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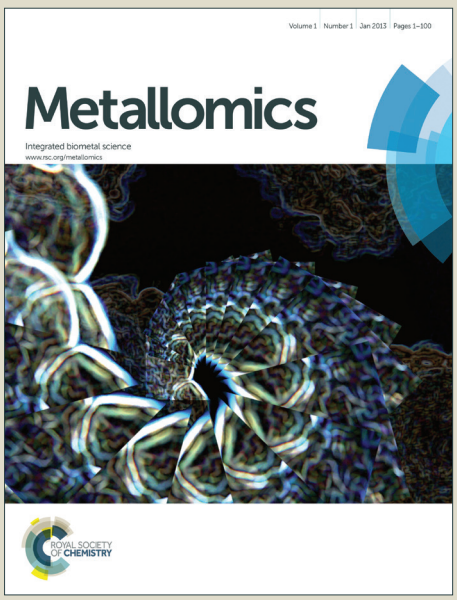

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Selenopeptides and Elemental Selenium in *Thunbergia alata* after Exposed to Selenite:
Quantification Method for Elemental Selenium

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Abstract

Three months old *Thunbergia alata* were exposed for 13 days to 10 μM selenite to determine the biotransformation of selenite in their roots. Selenium in formic acid extracts (80 ± 3 %) was present as selenopeptides with Se-S bonds and selenium-PC complexes (Selenocysteinyl-2-3-dihydroxypropionyl-glutathione, seleno-phytochelatin2, seleno-di-glutathione). An analytical method using HPLC-ICPMS to detect and quantify elemental selenium in roots of *T. alata* plants using sodium sulfite to quantitatively transform elemental selenium to selenosulfate was also developed. Elemental selenium was determined as 18 ± 4 % of the total selenium in the roots which was equivalent to the selenium not extracted by formic acid. The results are in an agreement with the XAS measurements of the exposed roots which showed no occurrence of selenite or selenate but a mixture of selenocysteine and elemental selenium.

Keywords: speciation, sulfite, selenosulfate, elemental selenium, selenite, selenate, selenocysteine, selenomethionine, anion exchange and reversed phase chromatography, XAS, ICP-MS, ESI-MS and *Thunbergia alata*.

Introduction

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Plants are the main source of selenium in human and livestock diets and, as such, knowledge of uptake, transformation, toxicity and translocation of selenium in plant tissues requires the use of reliable speciation techniques. Selenomethionine, selenocysteine and Se-methyl selenocysteine are well known selenium metabolites in crops and accumulator plants[1-3] while the presence of elemental selenium in plants has been recently proposed as a tolerance mechanism.[4,5] The identification and quantification of elemental selenium in plants, however, has never been shown due to the lack of appropriate analytical methods. Knowledge of selenium metabolites in plants is important for evaluation of genetic modification of plants for enhanced Se accumulation, to ensure that appropriate Se species are produced for nutritional purposes or therapeutic treatments.

The coupling of a high performance liquid chromatograph (HPLC) with an inductively coupled plasma mass spectrometer (ICP-MS) in parallel with a molecular specific detector (electrospray ionization, ESI-MS) provides a means of analyzing selenium speciation in plants. The main challenges with this technique are the extraction of the selenium species in identifiable and quantifiable amounts while preserving the integrity of the native species. Extraction procedures that are applicable for soluble non-protein bound components of plant tissues are not appropriate for insoluble and/ or protein bound components. The water soluble fraction including inorganic and non-protein species can be extracted with mild solvents such as water/methanol and acid solutions while maintaining species integrity[6-8] but often give extraction efficiencies below 50%.[6-8] Generally, the protein bound components require the use of enzymatic hydrolysis[7,9] to achieve extraction efficiencies of around 80%,[7,9] but this approach also destroys the Se proteins and only provides an indication of selenium moieties within proteins.

To preserve the native selenium species, and in particular the peptide bonds, Bluemlein *et al.* [10] used 1 % formic acid to extract selenium species from the roots of selenized *Thunbergia alata;* a model garden plant. Though the method has been used for arsenic speciation with high success in maintaining species integrity and detecting and quantifying metalloid-biomolecule complexes,[10-16] no information was provided regarding its extraction efficiency for selenium speciation.

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As previously mentioned, milder extraction methods for selenium in plants leave about half of the selenium species in the insoluble fraction. This insoluble fraction might be elemental selenium but to date there has not been a method specifically developed for its measurement in plants. The methods described in the literature for extraction of elemental selenium in biota are either not quantitative[17] or mobilise other selenium species and erroneously determined them as elemental selenium.[18-20]. Recently Loeschner et al.[21] demonstrated that sulfite extraction could be used to extract artificially formed nanoparticles of elemental selenium Se^{o}-NP from animal tissue by the formation of selenosulfate.

In this study, we investigated the selenium species in *Thunbergia alata* (Black-eyed Susan) exposed to low levels of selenite. *T. alata* was selected because the phytotoxicity to selenite is known from our previous study[10]. The aims of this study were to investigate whether *T. alata* can sequester selenium as elemental selenium at subtoxic levels and evaluate the use of sulphite to extract, selenium species and HPLC-ICPMS to separate and quantify elemental selenium in plants.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade or better. Deionised water (18 MΩ cm) was used throughout (Elga UK). Methanol (HPLC-grade) and hydrogen peroxide (32%) were sourced from Fisher Scientific UK. Sodium sulfite (hydrated), ammonia solution (28%), potassium sulphate (K_2SO_4) and sodium dihydrogen orthophosphate dihydrate and selenium powder supplied by BDH. L-glutathione, magnesium sulphate heptahydrate, calcium nitrate tetrahydrate, potassium nitrate and citric acid were purchased from Sigma Aldrich (UK). Sodium selenate, sodium selenite and sodium selenide were sourced from Alfa Aesar (Germany). Gallium used as internal standard was from High Purity Standards Charlston (USA). Sodium selenite (Se^{IV}), sodium selenate (Se^{VI}), L-selenocystine, methyl selenocysteine, and trimethyl selenonium iodide used for synchrotron experiments were from Sigma-Aldrich, Australia. DL-selenomethionine and γ-glutamyl selenocysteine were from PharmaSe, USA. Methyl selenomethionine was synthesised from DL-selenomethionine by

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acidification in aqueous formic acid and acetic acid with the addition of methyl iodide, methyl selenomethionine was precipitated by the addition of methanol to give up on drying a white powder.

Preparation of Standard Solutions

Ammonium citrate buffer (10 mM; pH 7) was prepared in 2 % v/v methanol from citric acid and ammonia solution. Sodium sulfite (1M) was prepared from sodium sulfite dissolved in ammonium citrate buffer. Stock solutions of glutathione, selenite, selenate, selenide, selenomethionine, and selenocystine were prepared by dissolving appropriate amount of corresponding compounds in deionised water. Working/diluted solutions (0.1 - 0.5 mM) of the different selenium standards were prepared from their stock solutions in the citrate buffer and kept in a refrigerator at 4^{o}C. Calibration standards for selenium determination were made by dilution of the selenite stock solution with 0.1 % v/v HNO_3.

Selenosulfate standard solutions from elemental selenium powder

A selenosulfate stock solution (10 mM), was prepared by adding 4.30 mg of selenium powder into a 15 mL plastic vial followed by 5.49 mL of the 1 M sodium sulfite solution. The vial was closed and placed in a water bath maintained at 90^{o}C for 3 hours with regular agitation at 15 min intervals to maintain a homogeneous solution. After 3 hours, the solution was allowed to cool and centrifuged for 10 minutes at 3,500 g. Aliquotes (1mL) were further centrifuged in 1.5 mL Eppendorf tubes for 5 min at 13,000 g. Diluted solutions of the selenosulfate stock (~500 µM Se) were prepared by pipetting 0.75 mL of the supernatant solutions into 15 mL vials and made up to 15 ml with ammonium citrate buffer. The prepared solutions were stored in a refrigerator at 4^{0}C prior to use and were stable for at least 3 hrs. Stability of standards were checked prior to use.

Selenosulfate standard solutions from selenite and glutathione

Sodium sulfite solution (100 mM) was prepared from the 1 M stock solution in ammonium citrate buffer (pH 7). Sodium selenosulfate was prepared by adding 0.75 mL of 10 mM sodium selenite to 3 mL of 13 mM GSH in a 15 mL via and shaking

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gently for about 1 minute after which 5 mL of 100 mM sodium sulfite was added. The prepared solutions were stored in a refrigerator at 4^{o}C prior to use.

Mixed selenium - sulphite species

A 0.75 mL aliquot of 10 mM stock solutions of each of selenite, selenate, selenide, selenomethionine and selenocystine, was added to 5 mL of 100 mM sodium sulfite. After mixing, the solutions were stored in a refrigerator at 4^{0}C prior to use.

Sample Preparation

Measurement of total selenium concentrations in plants

Lyophilised plant samples (n = 3) were weighed into a 50 mL plastic centrifuge tube (129 ± 50 mg), 2 mL of concentrated nitric acid was added and left to stand overnight at 25°C. Hydrogen peroxide (3 ml) and 250 µL of 20 µg g^{-1} of indium were added and the samples digested in a Mars 5 microwave oven (Matthews Inc, USA), using a 3 stage temperature program: Stage 1 ramped to 50^{o}C (1600 W), held for 5 min, stage 2 ramped to 75^{0}C (1600 W), held for 5 min and stage 3 ramped to 95^{o}C (1600 W) and held for 30 min. The heating was repeated until a colourless solution was obtained. Samples were cooled, diluted with deionised water to a final concentration of 2% (v/v) nitric acid. Selenium was then measured by high-resolution ICP-MS (Element 2, Thermo Fisher Scientific) at *m/z* 77. In order to correct for the effects of possible fluctuation in the plasma conditions or instrumental drift, and internal standard was added continuously before nebulisation. Gallium measured at *m/z* 69 was used in this study since no significant barium, a potential interferent, was present in the sample.

Quality control

For mass balances and total selenium measurements, the certified reference material (Rice Flour, NIST 1568a) was digested in quadruplicate along with 5 reagent blanks to gain information with regards to accuracy of the total selenium

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measurements. Measured selenium concentration in NIST 1568a was measured as 0.372 ± 0.001 µg g^{-}dry mass. (n=4) which is in agreement with the certified value of 0.38 ± 0.04 µg g^{-1} dry mass.. The limit of detection was calculated as 3 times standard deviation of the blank signal and gave 0.01 µg Se g^{-1} *d.m.* No spiking experiments were performed since the selenium spike would not have been incorporated into the sample matrix and the uncertainty as to what selenium species to use i.e. organoselenium species or elemental selenium.

Plants germination

Thunbergia alata (black-eyed Susan) was used as a model plant. Commercial seeds of *T. alata* were germinated in seed trays containing well-nourished, firmed and moistened compost/peat soil. Seeds (1 per hole) were placed about 6 mm deep into the compost and spaced about 5 cm apart. Germination took 2-3 weeks with the compost moistened regularly with water and maintained at about 70 % humidity while temperature was maintained between 18 and 25^{o}C. The trays were placed to the side of the greenhouse with adequate sunlight and artificial light was used during winter to maintain 12 hours of light per day. After 3 weeks and with the appearance of the first leaves, the plants were transplanted into 500 mL pots with the compost again moistened regularly with tap water and with modified Hoagland solution containing 0.6 mM KNO_3; 0.4 mM $Ca(NO_3)_2$; 0.1 mM $MgSO_4$; 0.4 mM $(NH_4)_2HPO_4$; 2 µM H_3BO_3; 0.36 µM $MnCl_2$; 0.06 µM $CuSO_4$; 0.04 µM $NaMoO_4$; 0.1 µM $ZnSO_4$; 20 µM FeNaEDTA; 0.4 mM MES pH 5 and pH adjusted to 6.0 ± 0.25 with KOH for fertilisation once in a week.

Plant exposure experiments

For each experiment, fully grown plants about 3 months old were used for exposure. The plant roots were freed from soil and washed with water before they were set in a hydroponic solution. The plants were nutrient starved for a week prior to exposure. For the exposure, an individual plant was placed into a 600 mL hydroponic plastic box. The hydroponic solution was made up of Hoagland solution supplemented with freshly prepared 10 µM selenite. Each box was covered with aluminium foil to prevent UV light but with provision of holes to allow air to enter. For each experiment, exposures were carried out in triplicate, maintained for 13 days with the hydroponic solutions changed every 72 hours. For the XAS experiments, plants were taken out of their hydroponic box after 12 days and with their roots wrapped in

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moistened tissue paper and transported live to the synchrotron site at Tsukuba, Japan where they were further exposed to 10 µM selenite for another 24 hours.

Prior to analysis, plant roots were separated from shoots and washed with tap water for about 5 min, rinsed in an ice-cold phosphate solution (10 mM KH_2PO_4) for about 5 – 10 min to desorb any adhered selenite from the roots followed by a final rinse in distilled water. Plant roots were blotted dry, ground under liquid nitrogen and divided into 2 sub-samples. One of the sub-samples was lyophilised in a Thermo Heto PowerDry LL3000 Freeze dryer at – 52^{o}C and selenium concentrations measured as described above. The other sub-sample was used for speciation analysis. Three replicates of each subsample were measured.

Preparation of extracts for HPLC-ICPMS/ESIMS – Formic acid extraction

Homogenised sub-samples were extracted with 1 % v/v formic acid using a modified method adapted from our previous studies.[16,17] Briefly, 1.0 ± 0.2 g of each sub-sample was extracted with 3 mL of 1 % v/v formic acid in an ice bath for 90 min. Extracted samples were centrifuged at 3,500 x g for 10 min and approximately1 mL of supernatant was syringed into a 1.5 mL Eppendorf vial and further centrifuged at 13,000 x g for 5 min and transferred into 1.8 ml HPLC vials for HPLC-ICP-MS and ESI-MS analysis. Analyses were performed within 4 hours of harvests and extraction.

Preparation of samples for X-ray Absorption Spectroscopy (XAS)

Sub-samples were prepared by grinding the roots in liquid nitrogen. The homogenised material was transferred frozen in cooled aluminium framed sample cells and sealed with polyimide (Kapton) tape. The samples were then immersed in liquid nitrogen and transferred into the cryostat for XAS measurement (at about 18 K).

Preparation of samples for HPLC-ICPMS measurement of elemental seleniumconcentrations

Standards of selenosulfate, selenite, selenate, selenocystine and selenomethionine standards were run alongside the root extracts from the exposed *T. alata* using the developed chromatographic method for elemental selenium in order to be able to establish whether any of these species were present in the root. For extraction, 1.5 ± 0.2 g of each sub-sample was extracted with 3 mL of the 1 M sulfite solution in

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citrate buffer for 3.5 hours, with regular mixing (2 min) on a vortex mixer at 30 min intervals. Preliminary experiments showed that a high excess of sulphite was required for complete conversion of elemental selenium to selnosulfate. Extracts were then centrifuged at 3,500 x g for 10 min after which 1.2 mL of each supernatant was syringed into a 1.5 mL Eppendorf vial and further centrifuged at 13,000 x g for 5 min and then transfered into 1.8 ml HPLC vials for HPLC-ICP-MS analysis. The residue of each extract was washed 3 times with distilled water and centrifuged at 3,500 x g. The washed residues were lyophilised and selenium concentrations measured as described above.

Speciation analysis

HPLC-ICP-MS/ESI-MS of hydrophilic selenium species

For the speciation experiments involving parallel HPLC-ICP-MS/ESI-MS, the instrumentation used consisted of an Agilent 1100 HPLC system coupled to a high-resolution ICP-MS (Element 2, Thermo Fisher Scientific) and a high-resolution ESI-MS (LTQ Orbitrap Discovery, Thermo Fisher Scientific). For the separation of the species, a Zorbax Eclipse XDB C-18 (4.6 mm x 150 mm, 5µm) column was used with a gradient program; a flow rate of 1 ml/min; injection volume of 100 µL, and the auto-sampler was held at 4^{o}C. Mobile phase A was 0.1% v/v aqueous formic acid and mobile phase B 0.1% v/v formic acid in methanol. The gradient was 100% A to 80 A in 20 min, held at 80% A for 10 min, to 100 % A in 5 min and held at 100 % A for a further 5 min to re-equilibrate the column. The eluate was split after the column; 80% to the ESI-MS and 20% to the ICP-MS. The ICP-MS was used in medium resolution mode for the measurement of selenium (*m/z* 77) and sulfur (*m/z* 32). In order to be able to correct for the effects of possible fluctuation in the plasma conditions and instrumental drift, here gallium (10 µg g^{-1}) was added post-column as an internal standard. Quantification (peak area vs. concentration) was performed using external calibration with sodium selenite and sulfate as calibrant. As methanol (carbon) in the mobile phase is known to change ICP-MS signal intensities for selenium,[15,22] quantification was performed by compensating for changes caused by the methanol gradient as described in Amayo et al.[23] . Briefly the calibration was determined during starting conditions of the mobile phase. Sulphate and selenite

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were added to the internal standard solution which was added post-column. During a blank run the selenium and sulphur response curves were recorded. The change in sensitivity with retention time was used tocorrect the sample response.
The ESI-MS was used in positive mode with an ESI-MS in high-resolution mode and mass range of 100 to 2000 m/z, resolution of 30,000, capillary voltage of 4.5 kV, 35 % collision energy.

X-ray absorption spectroscopy (XAS)

X-ray absorption spectroscopy experiments were performed at the Australian National Beam line Facility (BL20-B) at the Photon Factory, Tsukuba, Japan. BL20-B is equipped with a water cooled Si (111) monochromator which was calibrated using an elemental Se foil. The samples were mounted in a cryostat sample holder to hinder beam induced artefacts and analyzed at about 18K. XAS spectra were collected in fluorescence mode with a 36 element array detector (Canberra-Eurisys). Standards (sodium selenite, sodium selenate, selenomethionine, L-selenocystine, methyl selenocysteine, γ-glutamyl selenocysteine, methyl selenomethionine, trimethyl selenonium iodide) were prepared in aqueous solution (15 mM), injected through the polyimide tape into the measurement cells and frozen in liquid nitrogen before transferring into the cryostat. Each sample and the standards were measured in triplicate. The collected data spectra were reduced to an average using the AVERAGE 2.0 software. The spectra were analysed by linear combination fitting in k space ($Å^{-1}$) with the ATHENA software.[24]

Elemental selenium concentration measurement by HPLC-ICP-MS

For measurements, an Agilent Technologies (USA) HPLC 1100 equipped with an automatic degasser, a gradient pump, a thermostated auto-sampler tray and a thermostated column device, and a 7500c quadrupole ICP-qMS with an octopole-collision reaction cell system was used. A Hamilton, PRP - X100 anion exchange column was used with a mobile phase of 10 mM ammonium citrate in 2 % (v/v) methanol (pH 7), flow rate of 1 mL min^{-1}, injection volume of 100 µL with the auto-sampler held at 4°C. The citrate concentration in the mobile phase (10-50 mM) and pH (7-9) was investigated and 10 mM and pH 7 chosen so that selenosulfate was separated from all other selenium species without excessive peak broadening. Note

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selenosulphate could not be separated from the tail of the nearby sulphite peak. The ICP-MS was optimised in the oxygen mode for simultaneous measurement of selenium and sulfur at *m/z* 96 ($^{80}Se^{16}O$) and 50 ($^{34}S^{16}O$) respectively. Oxygen was used as the reaction gas as it gives better detection limits when sulfur is measured at *m/z* 48 ($^{32}S^{16}O$).[10,25] Measurements were carried out at *m/z* 50 with ^{34}S, however, because of problems with signals from the high concentration of sulfite (1 M) used for the Se^{o} extraction at *m/z* 48. For quantification (peak areas vs. concentration), calibration standards of sodium selenite and sodium sulfite in 10 mM ammonium citrate in 2 % (v/v) methanol (pH 7) were used.

RESULTS AND DISCUSSION

Total selenium concentrations and formic acid extraction efficiencies

The roots of the selenite exposed plants accumulated 3.2 – 4.1 µg Se g^{-1}, mean 3.5 ± 0.5 µg Se g^{-1}dry mass. (Table 1), and formic acid extracted 81 ± 3 % of the total selenium in the roots (Table 1).

Table 1: Total selenium and hydrophilic Se species concentrations in *Thunbergia alata* roots exposed to 10 µM selenite for 13 d. Quantities expressed in µg Se/g dry mass.

Sample ID	Totals [Se]	% Recovery	iSe[a]	UI[b]	Se^{II}-PC_2	Se-$(SG)_2$	SeCysDHP-GSH
Root 1	4.1	76.8	0.91	1.11	0.35	0.39	0.37
Root 2	3.3	81.0	1.29	0.81	0.31	-	0.27
Root 3	3.2	83.6	0.99	0.76	0.34	0.37	0.23
Mean	3.5 ± 0.5	81 ± 3	1.1 ± 0.2	0.89 ± 0.19	0.33 ± 0.02	0.38 ± 0.01	0.29 ± 0.07

[a]Inorganic selenium species, [b]Unidentified hydrophilic selenium species

HPLC-ICPMS/ESI-MS speciation of hydrophyllic selenium species

Exposing the *T. alata* plants to selenite produced at least 6 selenium species (Figure 1A). Three, out of the six species were identified using accurate mass of the $M+H^{+}$ by Orbitap-MS, and their fragmentation pattern using the MS/MS spectra data of the major elemental isotopes (Figure 1C and D). The species also showed the characteristic isotopic fingerprint of mono-selenium compounds on the HR-ESI-MS

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spectra. The use of the MS/MS spectra of the fragments confirmed the structure of Se species showing the characteristic fragments when the molecule fragments at the peptide bonds (Figure 1B, and Figures S1 and S2).

The three identified selenopeptides were; Selenocysteinyl-2-3-dihydroxypropionyl-glutathione (SeCysDHP-GSH) and seleno-diglutathione ($Se(GS)_2$) a widely proposed intermediate metabolite of selenium in plants,[26] but to the best of our knowledge, not previously identified in plants, while the third selenopeptide identified was seleno-phytochelatins 2 (Se^{II}-PC_2) which was first identified by Bluemlein *et al*..[10]

Figure 1 A-D

Full scan spectrum (HPLC-ESI-MS) of the extracts of the selenite exposed roots revealed the presence of a protonated selenopeptide $C_{16}H_{27}N_4O_{11}SSe^+$ (elution time 8.9 minutes, calculated [$M+H^+$] 563.0557, found 563.0548 Δm=1.6 ppm) (Figure 1A and C). The corresponding MS/MS fragmentation data (Figure 1B) and (Figure 1D) with signals at m/z 488 (indicating loss of glycine), m/z 434 (indicating loss of glutamic acid) and at m/z 256 (indicating the loss of glutathione) helped to identify the selenopeptide as selenocysteinyl-2-3-dihydroxypropionyl-glutathione conjugate with the structure shown below (Figure 1B and C).

This species (m/z 562) with a Se-S bond between a glutathione molecule and a selenocysteinyl residue has been previously identified in selenized yeast extracts,[27,28] but its structure could not be assigned because of the limitation of the methods used. The first elucidation of its structure in yeast extracts was made by Dernovics *et al.*[29] and later its isomer selenoglutathione-N-2,3-dihydroxypropionyl cysteine was identified by Preud'homme *et al*.[30] also in selenized yeast. The first Se-S conjugate of glutathione and selenocysteinyl residue to be identified in plants was selenocysteinyl-serine glutathione at m/z 561.[10] This species appears as the variant or modified version of the newly identified selenocysteinyl-2,3-dihydroxypropionyl-GSH with just one mass unit difference.

Another protonated selenopeptide; $C_{20}H_{33}N_6O_{12}S_2Se^+$, (elution time 12.9 minutes) (calculated [$M+H^+$] 693.0758, found 693.0744 Δm=2.0 ppm), again showing the characteristic isotopic fingerprint of a mono-selenium compound (Figure 1A and S1), was also present . The molecular structure of the species was assigned with the help

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of the MS/MS fragmentation data (Figure S1), with signals at m/z 564 (indicating loss of glutamic acid) and at m/z 435 (indicating additional loss of glutamic acid) and was consequently identified as seleno-diglutathione ($Se^{II}(GS)_2$).

The third species appeared as a protonated selenopeptide, $C_{18}H_{28}N_5O_{10}S_2Se^+$ (elution time 19.8 minutes) (calculated $[M+H^+]$ 618.0438, found 618.0432 Δm=1.0 ppm), also carrying with it the characteristic isotopic fingerprint of a mono-selenium compound (Figure 1A and S2). Its molecular structure was elucidated from the MS/MS fragmentation data (Figure S2) with signals at m/z 489 (indicating loss of glutamic acid) and at m/z 414 (indicating loss of glycine) and was consequently identified as phytochelatin selenide (Se^{II}-PC_2). Phytochelatins (PCs) are cysteine rich polypeptides that are formed by terrestrial plants in response to exposure to heavy metals and metalloids and they play a key role in detoxification and homeostasis in plants.[31,32] Induction of PC by selenium is rarely reported in plants except for those reported in yeast extracts[29,33] and in *Rauvolfia serpentine.* [34]

XAS speciation

The incomplete recovery for selenium by the formic acid extraction results in incomplete speciation information. Analysis of the selenium exposed roots was therefore undertaken using XAS. The XAS spectra of the roots were converted from energy to photoelectron momentum (k-space) and weighted by k^3. In Figure 2 it is evident that selenite and selenate were not present in the samples. The spectra of the root samples showed a structure similar to that of selenocysteine and elemental selenium. Linear combination fitting was performed using all the spectra of the standards reported in Figure 2. The best fit (R-factor 0.023) was obtained by a combination of selenocysteine (63%) and elemental selenium (37%). This is in agreement with the recovery by formic acid and the HPLC-ICP-MS/ESI-MS. It should also be considered that the scattering signal from lighter elements present in the organic molecules is less intense than that from adjacent Se atoms in elemental Se particles,[35,36] which are often found as a result of biological conversion of selenium to elemental selenium.[37]

Since the XAS data indicate that elemental selenium might account for the selenium not extracted by the formic acid extracts, a chromatographic method was developed to quantify the amount of elemental selenium in the plant roots.

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Figure 2

Elemental selenium (Se^0) concentration measurement

Elemental selenium is known to be sparingly soluble in water and hence it is difficult to extract using commonly employed solvents. Sodium sulfite is known to quantitatively dissolve elemental selenium[19] and specifically form a soluble compound, selenosulfate according to the equation below;[38,39]

$$Se^0_{(s)} + SO_3^{2-}{}_{(aq)} \rightleftharpoons SeSO_3^{2-}{}_{(aq)} \quad (1)$$

The reaction was expected to be stoichiometric and quantitative. This reaction is favoured at neutral to alkaline pH and published information indicates that this selenium species is stable in the presence of excess sulfite for at least 10 hours.[38-40] We have found that selenosulfate was stable for at least 3 hours. The chromatographic protocol used (Figure 3) gave a characteristic strong peak for selenium at 4.5 minutes and with a co-elution of sulfur indicating the presence of a compound containing both selenium and sulfur which was assumed to be the selenosulfate similar to the results of Loeschner et al.[21] who also used a sufite extraction for the quantification of Se^o nanoparticles. A series of $SeSO_3^{2-}$ standards were prepared by reacting Se^0 and sulfite (Table 2) and chromatographed. The concentrations of Se in the $SeSO_3^{2-}$ peaks were measured as 103 and 117 mg L^{-1} versus the theoretical Se concentrations of 126 and 140 mg L^{-1} respectively (82 % and 84 % recoveries). The Se/S molar ratios were determined to be 1.20 and 1.37 respectively for the solutions; slightly higher than the expected ratio of 1. The higher Se/S ratio was attributed to the error in S quantification due to the long tailing of sulfite which was added in 100 times excess as sulfite but considered as sufficient for identification purposes (Figure 3). The incomplete conversion (83 ± 1 %) using elemental selenium powder might be due to the purity of the selenium powder used and degree of crystallinity. The coarse nature of the powder might have led to aggregation of some particles which did not react with sulfite. Despite this, a complete dissolution of the powder was observed. The appearance of a single peak in the chromatogram, suggests that Se^o formed only one species with sulfite which was retained on the anion exchange column. The reproducibility of the retention

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time, the peak shape and peak areas of the chromatograms is a good indication of the reproducibility of the method.

Velinsky *et al*.[19] obtained a recovery of 105 ± 7 % for elemental selenium (red allotropic form) after dissolving the powder in 1 M sulfite for 1 hour at 60°C. They determined Se^0 by oxidising the resulting solution with nitric acid and analysing the solution for selenite using atomic absorption spectrometry (AAS). Chen *et al*.[17] also used Velinsky's method for dissolution of red powder of Se^o in sulfite but used a dissolution period of 8 hours and also obtained complete dissolution.

Figure 3 A-C

Table 2: Preparation of $SeSO_3^{2-}$: theoretical and experimental composition

Sample ID	*Experimental Concentration (mg L^{-1}) Se^0 as $SeSO_3^{2-}$*	*Theoretical Concentration (mg L^{-1}) Se^0*	*% Recovery Se^0 as $SeSO_3^{2-}$*
Se^0 powder A	103	126	82
Se^0 powder B	117	140	84
in-situ Se^o A#	142	149	95
in-situ Se^o B#	130	139	94

Notes: Experimental concentration is the concentration determined based on peak area vs. concentration calibration using sodium selenite and sulfate as calibrants. Theoretical concentration is the calculated concentration based on known amount of Se^0 dissolved in known volume of sulfite solution assuming complete dissolution and 100 % purity. # in-situ Seo A and *in-situ* Se^o B: details are in the text.

Though the selenosulfate obtained from elemental selenium powder gave a neat and reproducible peak from the chromatographic column, the powder contained particles that are likely to have properties (size and geometry) different from the particles of selenium likely to be encountered in biological systems. Therefore, conditions similar to those occurring physiologically in plants were used to simulate formation of Se^0. Reduction of selenite by GSH to Se^0 has been known since Ganther[41] published his work on this reaction in 1971. Debieux *et al.* [42] and Kessi and Hanselmann[37] independently suggested GSH as being responsible for microbial reduction of selenite to Se^0 and they both confirmed the nano-size of the formed Se^0. Kessi and

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Hanselmann[37] further confirmed that the Se^0 formed from biotic and abiotic reactions have similar size (nanometre), colour and spherical structure using transmission electron microscopy and energy dispersive x-ray analysis. We therefore prepared in-situ elemental selenium from glutathione reduction of selenite with the expected nanometre size to mimic what occurs in plants.

With the very high surface area of the *in-situ* Se^0, its reaction with sulfite to form selenosulfate would be expected to proceed faster than that between powder Se^0 and sulfite. Selenosulfate was therefore prepared from *in-situ* Se^0 produced from the reduction of selenite by GSH. The formation of *in-situ* Se^0 was based on the equation proposed by Painter[43] (equation 2).

$$4\ RSH + H_2SeO_3 \longrightarrow RS\text{-}Se\text{-}SR + RSSR + 3\ H_2O \quad (2)$$

and, in this study

$$4\ GSH + H_2SeO_3 \longrightarrow Se^{II}(GS)_2 + GSSG + 3\ H_2O \quad (3)$$

The reaction formed an intermediate compound, seleno(II)diglutathione $Se^{II}(GS)_2$ which is known to be stable in acidic solution and under condition of relatively high concentration of reactants and with excess of GSH.[41] This reaction was previously reported to proceed very rapidly within a minute.[37] At pH 7, seleno(II)diglutathione is expected to be rapidly converted into Se^0 and oxidised GSH (GSSG) according to equation 4 below. Addition of sulfite to $Se^{II}(GS)_2$ will raise the solution pH up to 7 and above.

$$Se^{II}(GS)_2 \xrightarrow[pH \geq 7]{} Se^0 + GSSG \quad (4)$$

In this study selenosulfate was produced from glutathione and selenite within 3 min. The chromatography of the solutions of formed selenosulfate gave a characteristic peak (peak 2) for selenium at 4.5 minutes (Figure S3) with the co-elution of sulphur. This observation confirmed the formation of selenosulfate from Se^0 as suggested by Loeschmer et al.[21] There was a small peak (peak 1) at the retention time of 2.3 min corresponding to selenate suggesting contamination either during the experiment or inherent in the selenite standard. The concentrations of Se^0 as $SeSO_3^{2-}$ were determined to be 142 and 130 mg L^{-1} as against the theoretical concentrations of 149 and 130 mg L^{-1} (Table 2). The conversions were calculated to be 95 and 94 %

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for solutions A and B respectively. And the Se/S molar ratios were determined to be 1.70 and 1.50 respectively. Again, the Se/S molar ratio is much higher with respect to the expected ratio of 1 and similar to that obtained in the experiment with black elemental Se^0 ($SeSO_3^{2-}$).

In order to confirm that sodium sulfite can only form selenosulfate with elemental selenium, other selenium species (selenite, selenate and selenide) were reacted with sulfite and the resulting solution subjected to the HPLC - ICP-MS. Selenide was included as it may be formed as an intermediate during the transformation of selenite to selenocysteine or selenomethionine. The chromatograms of the individual species (controls) were compared with the chromatograms of the reaction solutions (sulfite-selenium species). For selenite and selenate, the reaction solutions and the controls had the same chromatographic profiles and there were no peaks corresponding to selenosulfate (Figure S4 C-F). These observations suggested that neither selenite nor selenate formed selenosulfate with sulfite. A different behaviour was, however, observed with selenide (Figure S4 A-B). While there was no peak corresponding to selenide in both the control and the reaction solutions, there were peaks corresponding to selenite and selenate and an additional peak corresponding to selenosulfate in the reaction solutions. The selenide experiment clearly showed the instability of selenide as it was oxidised to the higher oxidation states. While the formation of small amounts of selenite was expected as selenide can be oxidised to selenite with some sulphite being reduced to sulfide, how selenate is produced is unclear. The peak corresponding to selenosulfate was probably formed from selenide that has been oxidised to elemental selenium. As previously indicated, selenide maybe formed as intermediate when selenite is biotransformed to SeCys or SeMet, but will occur most likely in minuscule concentration in the roots. Hence, the small conversion of selenide to selenosulfate should be considered as insignificant for the quantification of elemental selenium.

The study also investigated whether mixtures of sulfite with selenoamino acids will (i) form selenosulfate, (ii) oxidise selenoamino acids to higher oxidation states and (iii) cleave the C-Se bonds of selenoamino acids. Selenium standards and the reaction solutions gave the same chromatographic profiles which strongly indicated that the sulfite neither converted the selenoamino acids to selenosulfate nor broke their C-Se bonds (Figure S4 G-J).

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Detection and Quantification of Elemental Selenium in Plant roots

Since the extraction recovery for the hydrophilic inorganic and organic selenium species using formic acid extraction was 80 %, the remaining 20 % could either be lipid bound selenium or in the form of elemental selenium. Therefore having established a chromatographic method specific to detecting and quantifying elemental selenium, the study applied the developed method to plant extracts in order to test the hypothesis that plants can sequester elemental selenium in roots and form wholly or part of the 20% unextracted selenium in the formic acid extract.

The HPLC anion exchange chromatograms of the sulfite extracted roots revealed the presence of 5 selenium species (Figure 4). Although unknown selenium species elute close to where selenosulfate peak elutes, the selenosulfate peak is clearly separated and has the same peak shape and retention time as the selenosulfate standard. The observation of the characteristic strong peak of selenosulfate in the chromatograms of the root extracts is an indication of the presence of elemental selenium in the selenite exposed roots of *T. alata*. The concentrations of elemental selenium as selenosulfate were determined to be 63 ± 26 µg Se g^{-1} dry mass (n=3) while the sum of the species was 118 ± 29 µg Se g^{-1} dry mass (Table 4). The quantified elemental selenium from the chromatograms represents about 50 % of the extracted selenium species. The other selenium species were only present in small concentrations.

Taking the extraction efficiency with regards to the total selenium concentration into account, the measured elemental selenium represented about 18% of the selenium accumulated in the roots. The assignment of selenite and selenate to peaks 2 and 3 is only tentative and only selenosulfate is distinctively identified by its retention time and peak shape (Figure 4). The chromatographic recovery (i.e. sum of the species from the column as a percentage of the total selenium) from acid digest was quantitative and found to be 96 ± 17 % which is an indication of efficiency of the chromatographic conditions leading to quantitative elution of the species from the column. The reproducibility of selenosuulphate concentrations analysis in the three root samples is not as good as recorded for the synthesised elemental selenium solution presumable due to biological variation. Note that, because of the citrate

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concentration in the mobile phase, the presence of S from the tail of the sulphite peak and low sensitivity of S in the ESIMS system we were unable to further identify the selenosulfate in this peak by mass spectrometry.

Table 3: Total selenium (acid digest) and sum of selenium species concentrations in *Thunbergia alata* roots extract using sulfite extraction expressed in µg Se/g dry mass.

Sample ID	Total Se in residue (Acid digest)	Total Se in roots (Acid digest)	Total Se in extract [mass balance]	Extraction Efficiency [%]
Root 1	215	318	104	33
Root 2	258	434	176	41
Root 3	181	278	97.0	35
Mean	218 ± 39	344 ± 81	126 ± 44	36 ± 4

Notes: The total Se in extract by mass balance (total Se in roots – total Se in residue) is the difference between total selenium in roots (acid digest of whole roots) and total selenium in root residues (acid digest of the residue after digestion).

$$\% \text{ Extraction efficiency} = \frac{\text{Total extractable selenium (acid digest of extract by mass balance)}}{\text{Total selenium in roots (acid digest of whole roots)}}$$

The chromatographic recovery (sum of the species from the column as a percentage of the total selenium from acid digest) was quantitative and found to be 96.2 ± 16.7% (Table 4).

Table 4: Quantification results for elemental-Se in plant roots of *Thunbergia alata* and HPLC column recoveries expressed in µg Se/g dry mass (

Sample ID	Se^0 as $SeSO_3^{2-}$ (µg Se/g)	∑ Se (µg Se/g)	Se^0 [%] in the extract	Column recovery [%]	Se^0 in plant [%]
Root 1	63.0	119	52.9	115	19.8
Root 2	89.2	146	61.1	82.7	20.6
Root 3	36.4	88.4	41.2	91.1	13.1
Mean	63 ± 26	118 ± 29	52 ± 10	96 ± 17	18 ± 4

Notes: Peak 5 is the peak corresponding to selenosulfate, whilst ∑ Se is the summation of the concentration of the peaks determined from the peak area vs concentration from calibration using sodium selenite as calibrant.

$$\% \text{ Chromatographic recovery} = \frac{\text{Chromatographic sum of species (peak area vs concentration)}}{\text{Total extractable selenium (acid digest of extract by mass balance)}}$$

Conclusion

The suitability of the use of formic acid extraction for identification and quantification of seleno-peptides using the hyphenation of reversed phase HPLC with ICP-MS and ESI-MS in parallel was confirmed for identifying and quantifying selenium species in

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selenium enriched plants. Selenocysteinyl-2,3-dihydroxypropionyl-glutathione, $Se^{II}(GS)_2$ and Se^{II}-PC_2 were identified, however, elemental selenium cannot be quantified with this procedure. As predicted by XAS, selenium occurred in roots in its elemental form. The developed sulfite extraction procedure was able to be used to quantify Se^o by converting all Se^o to selenosulfate ($SeSO_3^{2-}$). The latter species cannot be formed by other stable selenium species expected in biological samples and can therefore be used for quantification of elemental selenium in plants. Here the challenged *T. alata* exposed to selenite accumulates selenium and converts approximately 20% of the selenium to Se^0. Whether the proportion of selenite biotransformation to elemental selenium is depending on the selenium exposure and time is the subject of a follow up study.

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Figures in the manuscript incl. Figure caption

Figure 1 A-D: HPLC-ICPMS/ESIMS of formic acid extract of plant roots with MS and MS/MS for selenocysteinyl-2,3-dihydroxypropionyl glutathione

Figure 2 A: Se XAS of standards and selenite exposed roots of *T. alata.*

Figure 3 A-C: Anion exchange ICPMS of elemental selenium standards

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Figure 4 A-B: Anion exchange ICPMS chromatograms of a sulfite extract of standards and selenite exposed roots of *T. alata*

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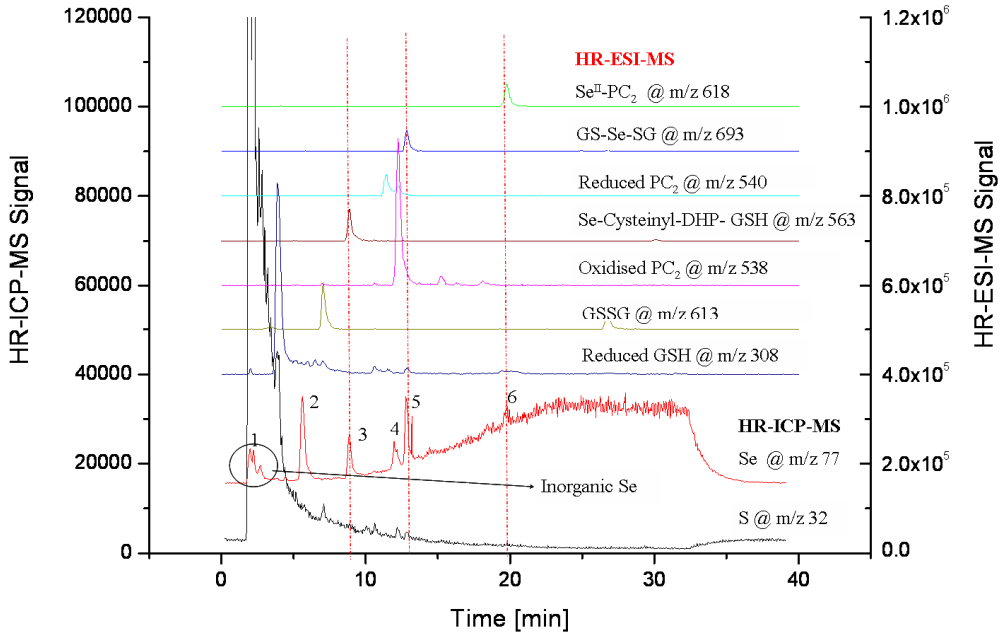

Figure 1A:HPLC-HR-ICP-MS/ESI-Orbitrap MS of formic acid extract of *Thunbergia alata* roots exposed to 10 µM selenite for 13 d, with overlaid ESI-MS (top) [M+H]+ for Se^{II}-PC2 @ m/z 618, $Se^{II}(GS)_2$ @ m/z 693, reduced PC2 @ m/z 540, Se-cysteinyl-2-3-dihydroxypropionyl-GSH @ m/z 563, oxidised PC2 @ 538, GSSG @ m/z 613, reduced GSH @ m/z 308, selenium and sulfur traces m/z 77 and 32 (ICPMS).

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B

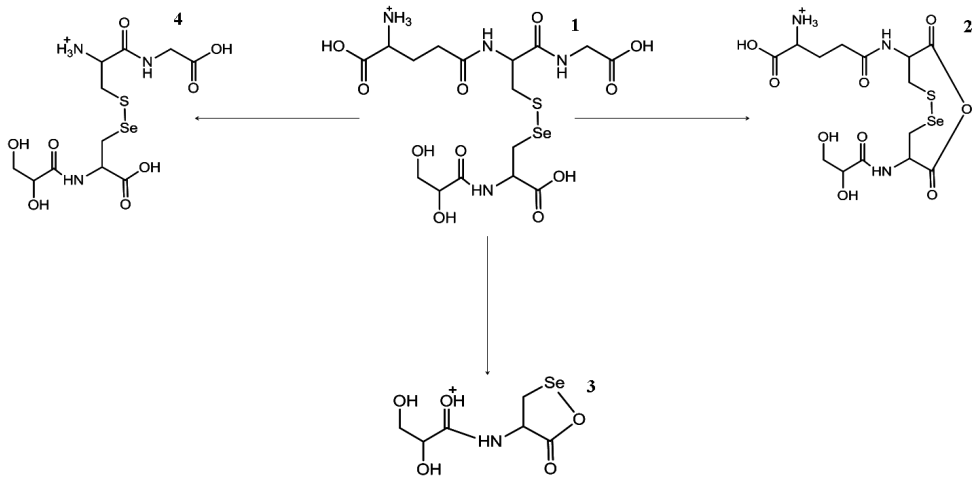

Figure 1B: Proposed structure [M + H]+ of selenocysteinyl-2,3-dihydroxypropionyl glutathione conjugate and its proposed fragmentation pathways from the MS2. For all accurate mass information (Table S1).

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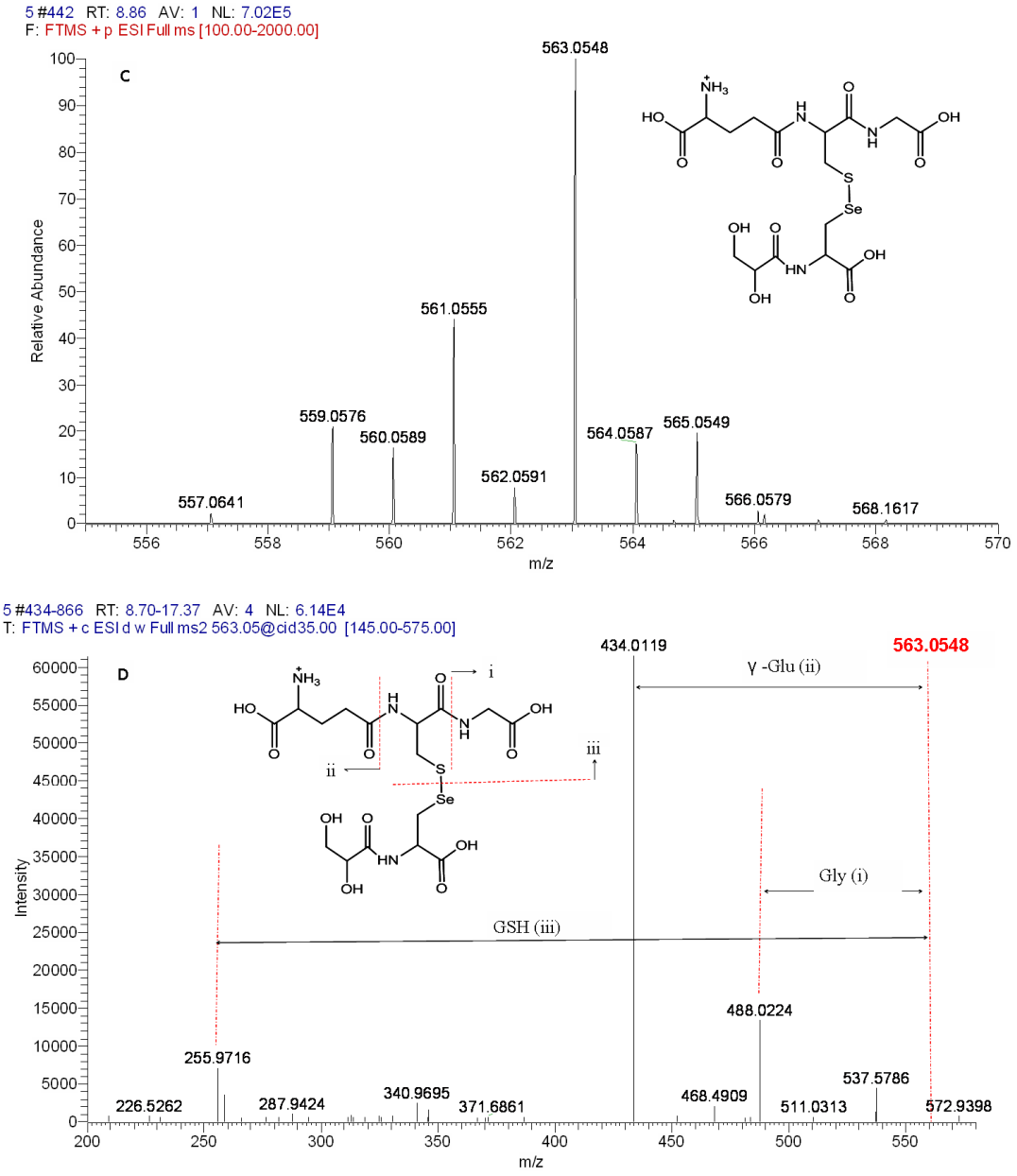

Figure 1C-D: C, ESI-Orbitrap MS of protonated selenocysteinyl-2-3-dihydroxypropionyl-GSH (ESI-MS m/z @563) showing the fingerprint of a mono-selenium isotopic compound for selenite exposed roots, D, MS2 of m/z 563→ (i) m/z 488= loss of glycine, (ii) m/z 434= loss of γ - glutamic acid, and (iii) m/z 256 corresponding to the loss of glutathione. For all accurate mass information (Table S1).

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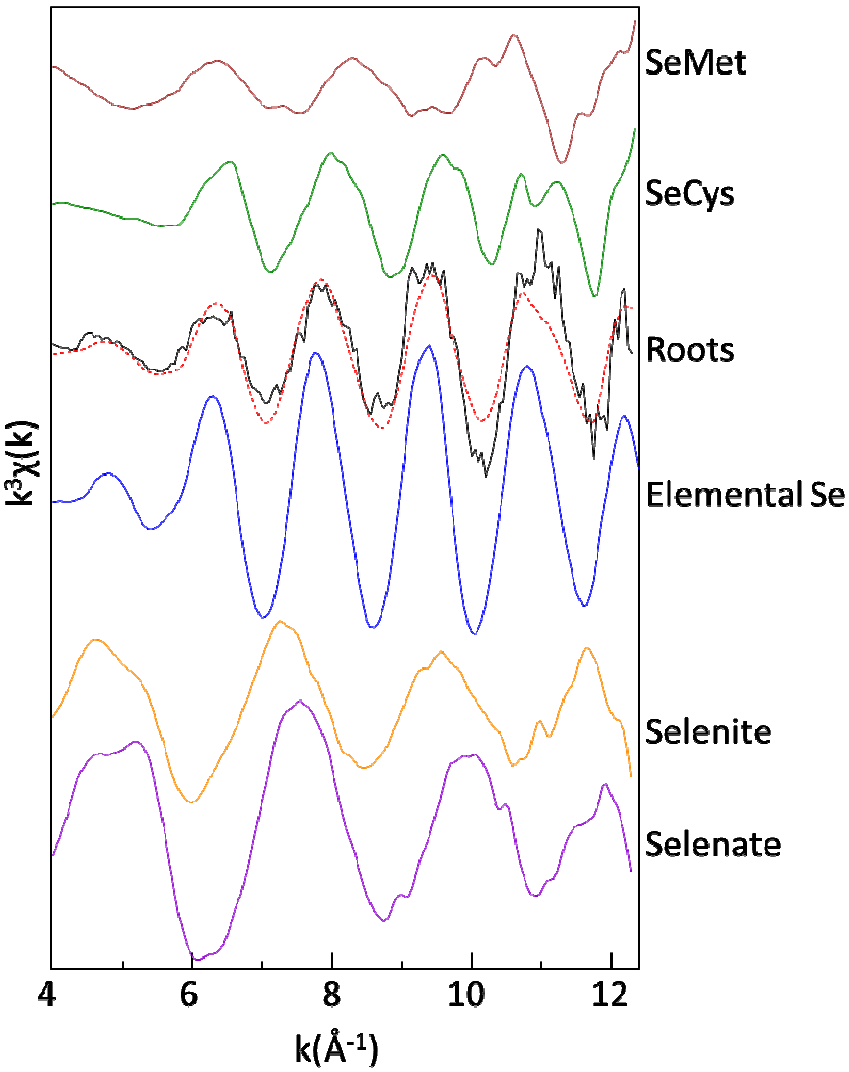

Figure 2: Se K-edge k^3-weighted XAS spectra for aqueous solutions of selected selenium model/standards (selenomethionine, selenocysteine, elemental selenium, selenite and selenate); fresh roots (black) and LCF fitting result (in red, broken line).

b

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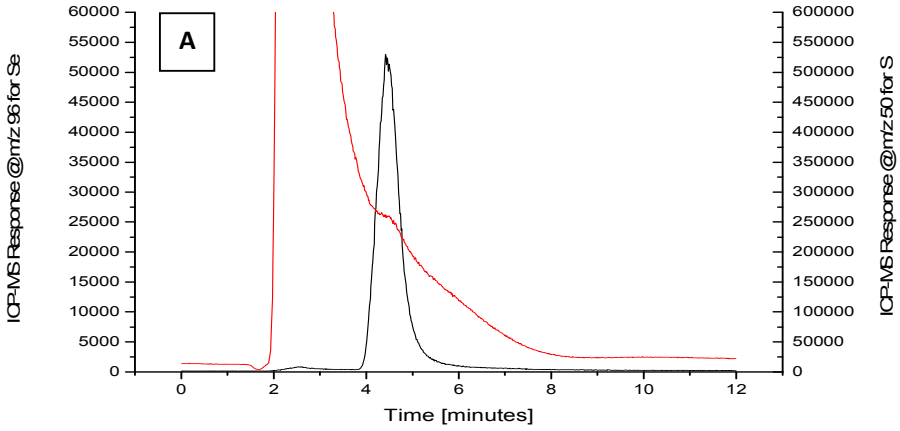

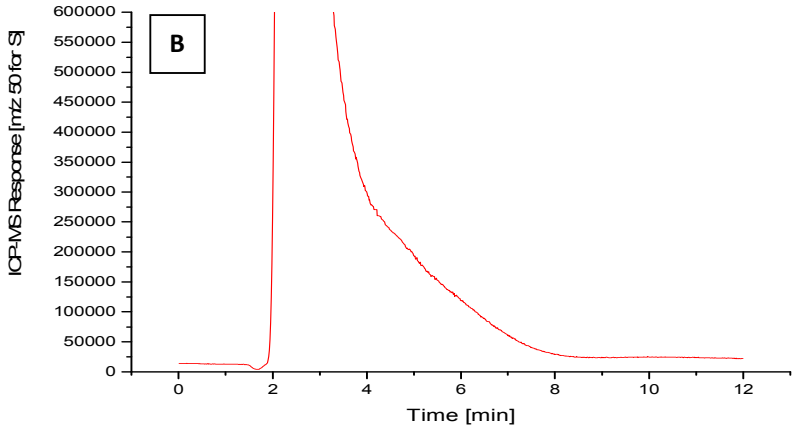

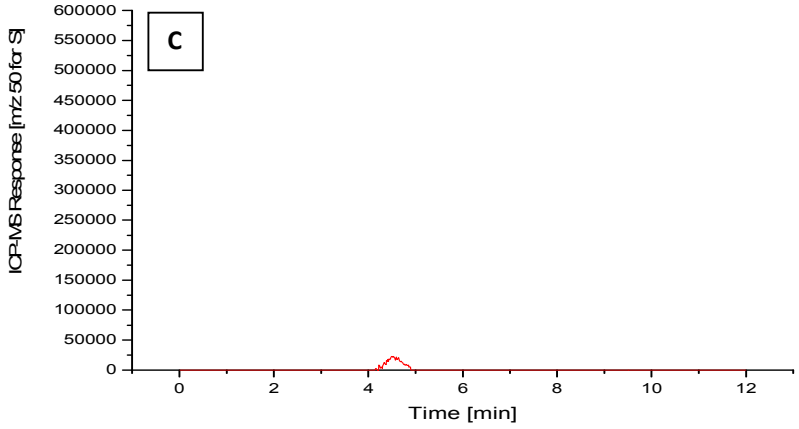

Figure 3: HPLC/ICP-MS chromatogram of solution of black elemental selenium powder in 1M sodium sulfite. A) Co-elution of selenium and sulfur @ 4.5 minutes indicating Se^0 eluted as selenosulfate. B) Extrapolated sulfur profile without the tiny peak, C) Sulfur peak residue after removing the extrapolated profile from the real sulfur profile in A.

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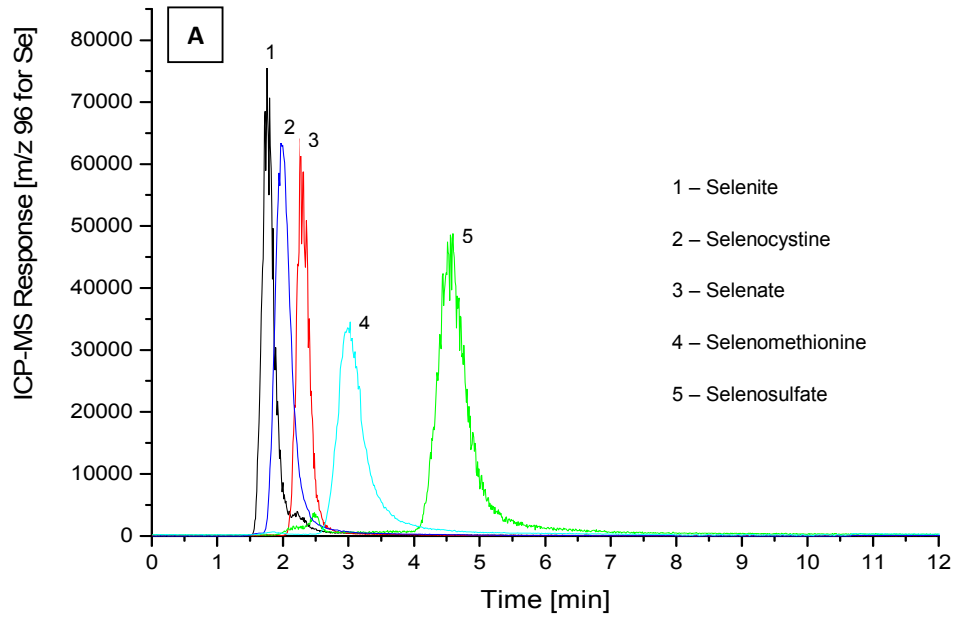

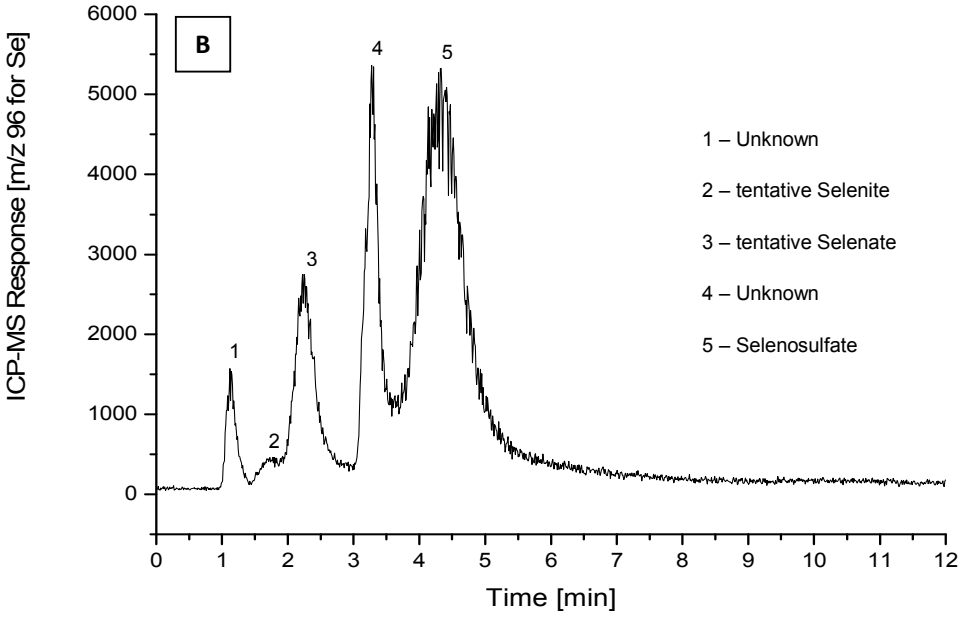

Figure 4: Separation of selenium species using anion exchange PRP100 with 10 mM ammonium citrate buffer, pH 7 and 2 % v/v methanol, 1 mL/min at 4 ^{o}C (A) selenium standards, (B) root extracts (sulfite extraction) showing elution of 5 different species labelled 1 – 5. Peaks 2 and 3 were tentative as selenite and selenate identified respectively but peaks 1and 4 did not match the retention time of any of the selenium standards used. Peak 5 is the strong peak characteristic of selenosulfate at 4.5 minute indicating the presence of elemental selenium in the plant roots.

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