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Tracking the metabolism of selenium compounds in *Spinacia oleracea* using multiple stable isotope tracers and HPLC-(ID)-ICP-MS

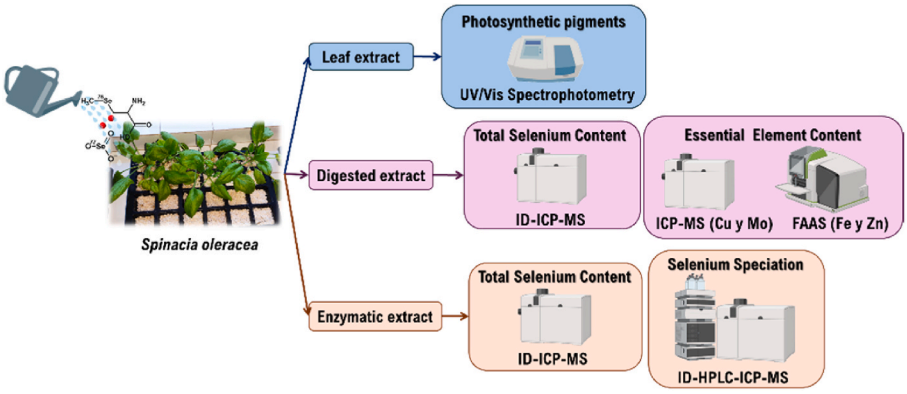
Gustavo Moreno-Martín [a,*], Marina Rodríguez-Marín [a], Silvia Queipo-Abad [b], Yolanda Madrid [a,**]

[a] Universidad Complutense de Madrid (ROR 0290gd045), Facultad de Ciencias Químicas, Ciudad Universitaria, 28040, Madrid, Spain
[b] Mass Spectrometry Unit, Scientific and Technical Services, Severo Ochoa Building, Campus of El Cristo, 33006, Oviedo, Spain

HIGHLIGHTS

- ^{76}SeMet, ^{77}Se(IV), Ch-SeNPs) reveal distinct metabolic behaviours in *S. oleracea*.
- ^{76}SeMet showed highest root translocation, while Ch-SeNPs stayed mostly in leaves.
- SeMet, Se(IV), and an unknown compound were the main species in *S. oleracea*.
- Isotopic tracers with HPLC-ICP-MS clarified selenium uptake and speciation paths.
- Selenium exposure increased chlorophyll *a*, Zn, and Fe content in spinach leaves.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Understanding the behaviour and metabolic transformations of different selenium species in plants requires conducting as many experiments as there are chemical forms to study. This approach does not allow for the evaluation of the coexistence between different selenium forms. Using multiple selenium isotopic tracers enables the simultaneous comparison of different selenium forms and their effects on plant metabolism in a single experiment. In this context, this study employed a mixture of two selenium isotopic tracers, ^{76}SeMet and ^{77}Se(IV), along with chitosan-modified selenium nanoparticles (Ch-SeNPs), to assess their metabolic transformations in *Spinacia oleracea* under foliar exposure.
Results: Selenium supplementation significantly increases chlorophyll *a* (800 ± 50 mg kg^{-1} DW) and total chlorophyll (1200 ± 80 mg kg^{-1} DW) content. ID-ICP-MS analysis showed that Ch-SeNPs and ^{76}SeMet were most readily incorporated into the aerial part (40 % and 31 % of total selenium), while ^{76}SeMet and ^{77}Se(IV) were rapidly transported to the roots (43 % and 36 %). ID-HPLC-ICP-MS revealed that SeMet was the predominant species in both the aerial part and root for ^{76}SeMet and ^{77}Se(IV). However, for Ch-SeNPs, SeMet and an unknown selenium compound were predominant in the aerial part, while Se(VI) and SeMet dominated in the roots. In the

* Corresponding author.
** Corresponding author.
E-mail addresses: gusmoren@ucm.es (G. Moreno-Martín), ymadrid@ucm.es (Y. Madrid).

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phloem sap analysis, the main species for selenium sources were SeMet for ^{76}SeMet, Se(IV) for ^{77}Se(IV) and unknown compound for Ch-SeNPs. Finally, selenium increased Zn and Fe levels in both plant parts, while reducing Mo concentration in the roots.

Significance: This study demonstrates the use of selenium isotope tracers to evaluate and compare the accumulation and metabolic transformation of different selenium forms in a single experiment. By combining post-column IDMS with HPLC-ICP-MS, all selenium species in spinach were quantified without the need for commercial standards. This innovative methodology offers a comprehensive approach for exploring selenium metabolic pathways and holds potential for advancing research in plant fertilization, particularly when paired with advanced mass spectrometry techniques.

1. Introduction

Selenium is a trace element of considerable importance in agriculture and nutrition, acting as an essential micronutrient for humans and animals due to its role in antioxidant defence and thyroid function [1]. Although selenium is not universally recognized as essential for plants, it has been shown to provide benefits at low concentrations, such as promoting growth, enhancing stress tolerance, and mitigating oxidative stress [2]. However, higher concentrations of selenium can lead to toxicity, posing challenges for both plant health and food safety. Plants acquire selenium primarily from the soil, where its availability and chemical forms, such as selenate, selenite, elemental selenium, and organic selenium compounds, are influenced by soil pH, redox potential, organic matter, and microbial communities [3,4]. Among the various chemical forms of selenium, selenate and selenite are the most bioavailable for plants, each following distinct uptake pathways. Selenate uptake occurs through sulfate transporters, while selenite is absorbed via phosphate transporters [5]. These differences in uptake mechanisms impact selenium accumulation and distribution within plant tissues, affecting its potential for toxicity or beneficial effects. Recently, selenium nanoparticles (SeNPs) have emerged as a novel form of selenium for agricultural use due to their lower toxicity, controlled release properties, and ability to be metabolized within the plant system with reduced environmental impact [6]. Studies suggest that SeNPs may enhance plant growth and stress resistance more effectively than traditional selenate or selenite applications, opening new avenues for sustainable selenium biofortification [7].

A powerful approach to understanding selenium metabolism in plants involves the use of selenium tracers, labelled with different isotopes, which allows for the simultaneous introduction and further evaluation of various selenium forms into plant growth media. This method not only enables the tracking of metabolites derived from distinct selenium precursors in a single experiment but also facilitates the comparison of key factors such as absorption rates, translocation, speciation, and potential competition mechanisms among different selenium species [8]. By maintaining identical growing and detection conditions for a single host plant, this technique provides a comprehensive and integrated view of selenium dynamics, offering insights into its metabolic processes and behaviour across different forms. However, while this isotopic tracer approach has been successfully applied in rats [9,10] and in environmental samples such as soils and sediments [11], its application in plants is still limited [8].

In the literature, the most widely used analytical technique to determine the total accumulated selenium content as well as the species formed by plant metabolism is ICP-MS [12,13]. The application of selenium tracers, labelled with highly enriched stable isotopes, significantly improves the information given by both ICP-MS and HPLC-ICP-MS by providing distinguishable signals for each selenium species, even at low concentrations. This strategy enhances the precision of detecting and quantifying both total selenium and the selenium species accumulated from each precursor using isotope dilution mass spectrometry (IDMS) in a single experiment [8,14]. Consequently, the integration of metabolic tracers, ICP-MS, HPLC-ICP-MS, and IDMS would provide a powerful approach for studying selenium dynamics in plants, offering valuable insights into selenium accumulation and transformation within plant systems.

In this study, spinach (*Spinacia oleracea*) was selected as model plant due to its ease of cultivation and the edibility of its aerial parts. A hydroponic cultivation system was established with simultaneous foliar exposure to three selenium species: two isotopically labelled (^{76}SeMet and ^{77}Se(IV)) and one with natural abundance (Ch-SeNPs) (triple spike). The main objectives of the study were: i) to assess the impact of selenium on photosynthetic pigments, ii) to quantify the accumulation of each selenium species added using ID-ICP-MS, iii) to determine the selenium species generated from each precursor through post-column ID-HPLC-ICP-MS and HPLC-ESI-MS/MS, and iv) to evaluate the effect of selenium on the incorporation of essential elements.

2. Instrumentation, materials and methods

2.1. Instrumentation

Extraction of chlorophyll pigments was carried out in a vertical oscillating shaker-incubator (Scientific Industries, CA, USA). Their quantification was performed using an HP Agilent 8453 UV/Visible spectrophotometer (Agilent Technologies, CA, USA).

A Sharp Freeze lyophilizer model 110 (aapptec, KY, USA) was used to lyophilize the sample.

For acid digestion of plant tissues, an analytical microwave oven (CEM, Matthews, USA) with EassyPrep vessels was employed.

Extraction of selenocompounds from samples was carried out at 37 °C in a digital control immersion thermostat model Digiterm 200 (J P. Selecta, Barcelona, Spain). Extracts were centrifuged in an Eppendorf 5804 F34-6-38 centrifuge (Fisher-Scientific, Madrid, Spain).

For chromatographic separations coupled to ICP-MS, a JASCO PU-2089 HPLC pump (Tokyo, Japan) was used, and injections were carried out using a model 7725i valve (Rheodyne, CA, USA) fitted with a 100 μL loop. Anion exchange column was performed on a Hamilton PRP-X100 column (250 × 4.1 mm, 10 μL) (Hamilton Company, Reno, Nevada, USA).

An Agilent 7700x ICP-MS instrument (Agilent Technologies, Tokyo, Japan) was used for the analysis of total selenium, selenium speciation, and Cu and Mo content. The operating conditions of ICP-MS are presented in Table 1.

For the confirmation of selenium species using an HPLC-ESI-MS/MS, a Phenomenex Kinetex EVO C18 column (Phenomenex, Madrid, Spain) was used. Analyses were conducted on an Agilent G6410B Triple Quadrupole LC/MS system (Agilent Technologies Inc., Santa Clara, CA, USA), equipped with an Agilent 1200 Series G1312 binary pump, a G1322A degasser, a G1329A standard autosampler with a temperature-controlled tray, and a G1316A column oven. A 5 μL sample volume was injected onto the column. The mobile phase consisted of 95 % solvent A (MilliQ water containing 0.1 % formic acid, Scharlab, Barcelona, Spain) and 5 % solvent B (acetonitrile with 0.1 % formic acid, Fisher Scientific, Fair Lawn, NJ, USA). Chromatographic separation was carried out under isocratic conditions at a flow rate of 0.5 mL min^{-1}. Detection was performed in positive electrospray ionization (ESI) mode using Multiple Reaction Monitoring (MRM), with fragmentor voltages ranging from 60

Table 1
ICP-MS operating conditions and data acquisition parameters.

Plasma parameters	
RF power (W)	1550
Plasma gas flow rate (L min^{-1})	15
Auxiliary gas flow rate (L min^{-1})	0.9
Carrier gas flow rate (L min^{-1})	1
Main chamber	Scott (double-pass or reversed-flow type)
Ion lens setting	Daily optimized for best sensitivity of 10 μg L^{-1} of Li, Y and Tl
Reaction parameters (only for selenium analysis)	
H_2 gas (mL min^{-1})	6.1
Octapole bias (V)	−16.9
QP bias (V)	−15.0
Data acquisition parameters (for total selenium. Cu and Mo determination)	
Nebulizer	Micromist
Monitored isotopes	^{65}Cu,^{76}Se,^{77}Se,^{78}Se,^{79}Br,^{80}Se,^{81}Br,^{82}Se,^{83}Kr,^{98}Mo
Points per peak	3
Acquisition time per point (s)	3
Replicates	5
Data acquisition parameters (for chromatographic analysis)	
Nebulizer	Multineb (Ingeniatrics, Sevilla, Spain)
Monitored isotopes	^{76}Se,^{77}Se,^{78}Se,^{79}Br,^{80}Se,^{81}Br,^{82}Se,^{83}Kr
Integration time (s)	0.1
Points per peak	1
Mobile phase	Ammonium citrate 10 mM, 2 % MeOH, pH 5.0
Elution mode	Isocratic
Flow rate (mL min^{-1})	1.0
Injection volume (μL)	100

to 110 V. The ion source parameters were as follows: gas temperature, 200 °C; drying gas flow, 10 L min^{-1}; nebulizer pressure, 35 psi; and capillary voltage, 200 V. A dwell time of 50 ms was applied for each transition. Data acquisition and processing were carried out using MassHunter Data Acquisition B.04.01 and MassHunter Qualitative Analysis B.07.00 software. The MRM transitions and collision energies (CE) for selenium species were optimized in a previous study [15].

The PerkinElmer FAAS model 1100 (PerkinElmer, USA) was used to determine the total Zn an Fe content. An air-acetylene mixture with a flow rate of 2.5/4.0 mL min^{-1}, respectively, was employed. Measurements were performed in continuously with a 0.2 nm band pass, lamp currents of 15 and 30 nm for Zn and Fe, respectively and wavelengths of 213.9 and 248.3 nm for Zn and Fe, respectively.

2.2. Standards, samples and reagents

Ultra-pure water (resistivity > 18.2 MΩ cm) was obtained from a Milli-Q water purification system (Millipore, Spain). Reagents of analytical purity grade were used for all experiments. $Ca(NO_3)_2{\cdot}4H_2O$, $CuSO_4{\cdot}5H_20$, FEDDHA, H_3BO_3, KH_2PO_4, KNO_3, $MgSO_4{\cdot}7H_2O$, $Na_2MoO_4{\cdot}2H_2O$ and $ZnSO_4{\cdot}7H_2O$ were obtained from Sigma-Aldrich (Steinheim, Germany) and used to prepare the one-tenth strength Hoagland's nutrient solution.

Acetone (Scharlab, Barcelona, Spain) was used for total chlorophyll and carotenoids extraction. 30 % (v/v) hydrogen peroxide (Dismadel, Madrid, Spain) and 65 % (w/v) nitric acid (Scharlab, Barcelona, Spain) were used for microwave acid digestion of the plant tissues.

Enriched ^{77}Se(IV) was obtained from Cambridge Isotope Laboratoires (Andover, MA, USA) as elemental powder and it was dissolved in a minimum volume of sub-boiled HNO_3 and diluted with ultra-pure water. Selenomethionine enriched in ^{76}Se ($^{76}SeMet$) was purchased from LGC Standards (LGC Group, UK) as powder and it was dissolved in 2 % (w/v) HCl (Scharlab, Barcelona, Spain). Chitosan-modified selenium nanoparticles (Ch-SeNPs) were synthesized and characterized according to a previously described procedure [16]. All these chemical forms of selenium were added to the one-tenth strength Hoagland's nutrient solution at a concentration of 1.5 mg Se L^{-1} each for foliar selenium supplementation of spinach.

Enriched ^{78}Se(IV) was obtained from LGC Standards (LGC Group, UK) as a solution of 10 μg mL^{-1} in 2 % HNO_3 and used as tracer to quantify the total selenium and selenium species content. The concentration of this solution was calculated by reversed IDMS.

Selenocystine ($SeCys_2$), selenomethylseleno-L-cysteine (SeMeSeCys), seleno-L-methionine (SeMet), selenite (Se(IV)) and selenate (Se(VI)) were purchased from Sigma-Aldrich (Steinheim, Germany).

To carry out enzymatic hydrolysis, protease type XIV from *Steptomyces griseus* and Base Trizma were supplied by Sigma-Aldrich (Steinheim, Germany). Tris-HCl solution was prepare with Base Trizma dissolved in water, and the pH adjusted to 7.5 with HCl.

Selenium species separation by anionic-exchange chromatography-ICP-MS was achieved using 10 mM citric acid (Sigma-Aldrich, Steinheim, Germany) in 2 % MeOH (Scharlab, Barcelona, Spain) as mobile phase. Selenium speciation by HPLC-ESI-MS/MS was performed by employing 0.1 % formic acid (Scharlab, Barcelona, Spain) and 5 % acetonitrile with 0.1 % formic acid (Fisher Scientific, Fair Lawn, NJ, USA) as mobile phase under gradient elution.

Nylon syringe filters (Dismadel, Madrid, Spain) with a pore size of 0.22 μm were used for filtration of extracts.

The spinach seeds (*Spinacea oleracea*) were obtained from a local supplier. The selenium enriched yeast Certified Reference Material (SELM-1) purchased from LGC Standards (LGC Group, UK) was used for validation of the methodology.

2.3. Methods

2.3.1. Plant growth

Seeds were germinated on filter paper moistened with ultra-pure water inside a polystyrene box during 20–25 days at 22 °C in darkness. After germination, 48 seeds, divided into two groups (24 control and 24 treated), were transferred to 24-compartment seed trays, each tray measuring 35.5 x 23 × 5 cm and each compartment 3.6 x 5 × 5 cm, filled with perlite as a substrate for hydroponic culture. Control and treated seeds were grown in separate seed trays to prevent cross-contamination between treatments. The trays were placed on 36.5 x 23.3 × 5.8 cm containers used to collect the excess Hoagland solution. Thirty-day-old plants of the treated group were exposed to Hoagland's nutrient solution enriched with 4.5 mg Se L^{-1} in form of $^{76}SeMet$, ^{77}Se(IV) and Ch-SeNPs (1.5 mg Se L^{-1} of each) by foliar spraying (Fig. 1a). To prevent the dripping of the solution from the leaves onto the lower nutrient solution, the perlite substrate was covered with plastic. In contrast, thirty-day-old plants of the control group were only exposed to Hoagland's nutrient solution by foliar spraying (Fig. 1b). The plants in each group were sprayed for 45 days and once a week in the evening (from 18:00 to 19:00 h) until the solution was evenly distributed over the surface of the leaves. With the aim of comparing results between groups, temperature was measured daily to keep this parameter under the same values ((22 ± 4) °C) in all the trays involved in the study. Plants growth took place between November–December 2023 and January–February 2024 under a 9/15 light cycle with a minimum solar radiation of 1.81 kWh m^{-2} and maximum of 3.72 kWh m^{-2}.

At the end of the growing period (45 days), plants from both groups were harvested, individually washed five times with ultrapure water, and then weighed and measured for root length. Phloem sap was collected by detopping plants 3–4 cm above the cotyledons. Stumps were allowed to bleed for 1 min, the first sap drops were discarded, the cut surface was wiped out with paper tissue, cleaned with ultra-pure water, and blotted dry. Then, phloem sap was collected for 10 min, using a micro-pipette, in Eppendorf tubes kept on ice. Finally, the roots and leaves were separated, lyophilized and finely ground with a pestle in an agata mortar for homogenization. Samples were stored at room temperature in high-density polyethylene (HDPE) tubes until analysis.

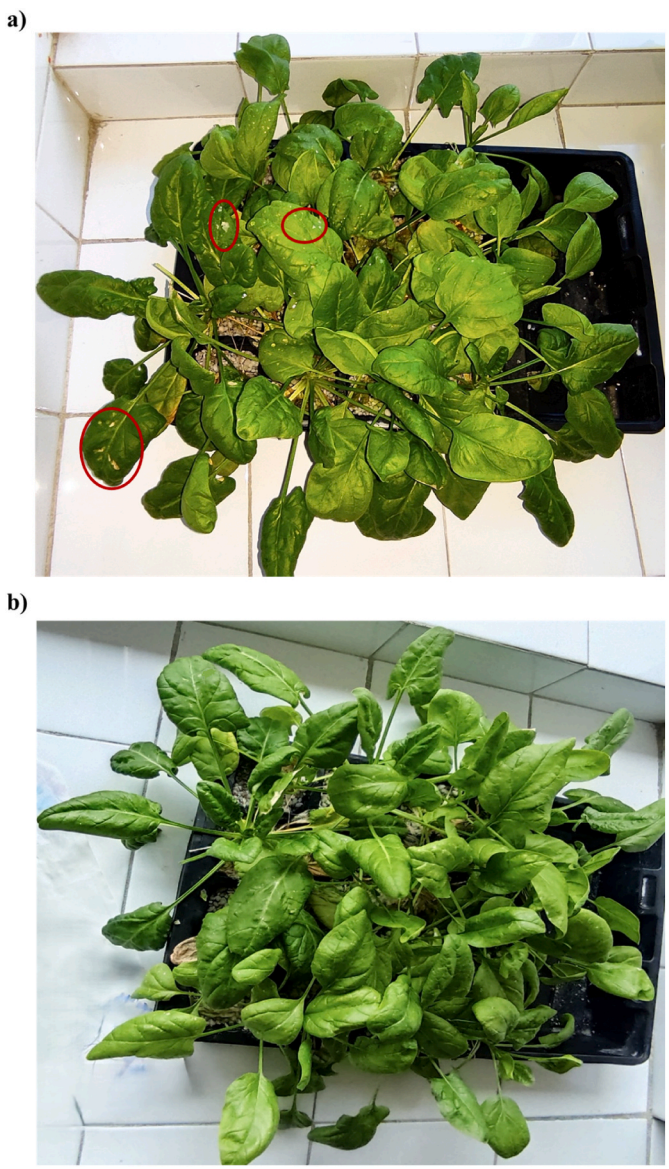

Fig. 1. *Spinacia oleracea* growth under (a) control and (b) selenium-treated conditions. Red circles indicate symptoms of chlorosis observed in leaves following foliar selenium application.

2.3.2. Determination of chlorophylls and carotenoids

Spectrophotometric analysis was conducted to quantify the chlorophyll *a*, chlorophyll *b*, total chlorophyll and total carotenoids in leaves with and without selenium foliar supplementation. These pigments were extracted from fresh leaf tissue by immersing 0.1000 g of the tissue in 5 mL of 80 % acetone into a 12 mL centrifuge tube. The samples were placed in a vertical oscillating shaker-incubator, where they were incubated in the dark at room temperature for 24 h at 25 rpm. After incubation, the samples were centrifuged at 9000 rpm for 5 min. The absorbance of the supernatants was recorded at 663.2 and 646.8 nm for chlorophyll *a*, chlorophyll *b* and total chlorophyll, and 470.0 nm for total carotenoids using an UV/Visible spectrophotometer. The pigment contents were estimated using the formulas described in the literature [17].

2.3.3. Total selenium determination by ID-ICP-MS

For aerial part, root and phloem sap sample amounts of 0.2500, 0.1000 and 0.0500 g, respectively, were weighed directly into EasyPrep tubes. An appropriate amount of the ^{78}Se(IV) spike and 2.5 mL of HNO_3 (65 % w/v) were added to perform a predigestion process, which was performed for 1 h at room temperature. Afterwards, 0.83 mL of H_2O_2 and 1.7 mL of H_2O were added to the predigested samples, and they were digested in an analytical microwave oven at maximum power of 1600 W. The digested procedure consisted of heating at 130 °C for 10 min, after a ramp of temperature of 15 °C min^{-1}, and finally 210 °C for 15 min after increasing the temperature to 25 °C min^{-1}. Finally, the acidified sample solutions were quantitatively transferred into 25.0 mL volumetric flasks, and diluted with ultra-pure water, filtered through 0.22 μm syringe Nylon filters and analyzed. The certified reference material, SELM-1, underwent the same procedure to validate the methodology.

The content of selenium in plant tissues derived from ^{76}SeMet, ^{77}Se (IV) and Ch-SeNPs enrichment was determined by ID-ICP-MS by measuring the ^{76}Se/^{78}Se (^{76}SeMet), ^{77}Se/^{78}Se (^{77}Se(IV)) and ^{80}Se/^{78}Se (Ch-SeNPs) selenium isotope ratio after correction of corresponding intensities to overcome the SeH^+ and BrH^+ interferences [18,19]. The proposed mathematical approach based on the use of isotope pattern deconvolution (IPD) [20] was then applied for the determination of SeMet, Se(IV) and Ch-SeNPs concentration. Thus, applying multiple linear regression the molar fractions of natural abundance and isotopically enriched compounds were calculated. Mass bias correction was performed using an internal correction based on the minimization of the square sum of the residuals of the multiple linear regression [21].

2.3.4. Enzymatic hydrolysis and quantification of selenium species by HPLC coupled to post-column ID-ICP-MS. Confirmation of selenium species identity HPLC-ESI-MS/MS

0.0500 g of protease XIV and 5.000 g of 30 mM Tris-HCl solution (pH 7.5) were added to 0.2500 g of aerial part and incubated for 48 h at 37 °C. The hydrolysed samples were then centrifuged (11000 rpm for 5 min) and filtered through 0.22 μm syringe Nylon filters. For root, an identical procedure was carried out, but sample and enzyme weights were halved compared to those used for aerial part. For phloem sap, an identical procedure was followed, but 0.2000 g of sample and 0.3000 g of 4 mg mL^{-1} of protease XIV solution prepared with 30 mM Tris-HCl solution (pH 7.5) were used.

To determine the percentage of selenium extracted during the enzymatic extraction, 1.0000 g of aerial part extracts, 0.5000 g of root extracts and 0.0500 g of phloem sap were mixed with an appropriate amount of the ^{78}Se(IV) standard solution prior to undergoing the enzymatic extraction described above. Finally, the extracts were diluted 1:200 with ultra-pure water, and selenium content was determined using ID-ICP-MS, following the procedure detailed in Section 2.3.3.

Chromatographic separation of selenium species from extracted samples was performed using an anion exchange column couple online to ICP-MS. The chromatographic conditions and instrumental parameters used for the separation are shown in Table 1. To quantify selenium species, post-column ID-HPLC-ICP-MS was employed. A^{78}Se(IV) standard solution of the appropriate concentration was continuously introduced at 0.85 mL min^{-1} through the second nebulizer inlet via the ICP-MS's peristaltic pump. To determine the concentration of selenium species separated by the column, the intensity chromatograms (counts s^{-1}) were converted into mass flow chromatograms (ng min^{-1}) [22]. After smoothing of the data using the Savitzky-Golay filter to reduce the noise, mathematical corrections were applied to correct for BrH^+ and SeH^+ interferences [19]. The ^{76}Se/^{78}Se (^{76}SeMet), ^{77}Se/^{78}Se (^{77}Se(IV)) and ^{80}Se/^{78}Se (Ch-SeNPs) isotope ratios were then calculated and further corrected for mass bias using an exponential model. Finally, the on-line isotope dilution equation was applied to each point of the chromatogram, obtaining a mass flow chromatogram. The amount of selenium in each fraction was calculated by integrating chromatographic peaks using the Origin 9.6.0.172 software (Microcal Software Inc., Northampton, MA, USA).

To confirm the identity of the selenium species previously detected by post-column ID-HPLC-ICP-MS, HPLC-ESI-MS/MS analyses were

performed under the conditions described in Section 2.1.

2.3.5. Effect of selenium application on essential metals

The effect of selenium application on the accumulation of essential metals (Cu, Fe, Mo and Zn) was evaluated using a microwave digestion process, as described in Section 2.3.3. The resulting acidified sample solutions were subsequently analyzed: Cu and Mo were determined by ICP-MS, while Fe and Zn were determined by FAAS. The operating conditions for ICP-MS are provided in Table 1, and those for FAAS are described in Section 2.1.

3. Results and discussion

3.1. Effect of foliar selenium treatment on growth parameters of Spinacia oleracea

Selenium foliar application may affect various morphological and physiological parameters, such as root length and biomass [23]. However, in the current study the root length of plants exposed to selenium ((8 ± 2) cm) did not show significant differences ($p > 0.05$) compared to control plants ((9 ± 2) cm). A similar result was observed for biomass, with selenium-treated plants showing a value of (1.3 ± 0.6) g and control plants a value of (1.9 ± 0.8) g. These findings suggest that foliar exposure to this concentration of selenium does not significantly affect the morphological and physiological parameters evaluated in *Spinacia oleracea*.

Although the present study involved simultaneous exposure to different chemical forms of selenium (SeMet, Se(IV), and Ch-SeNPs), the results obtained are consistent with those reported by Maršič et al. [24], who also observed no significant differences in root length and biomass when *Spinacia oleracea* was simultaneously exposed to 5 mg L^{-1} of Se (IV) and 5 mg L^{-1} of Se(VI), via foliar application. In contrast, another study performed with spinach, where Se(IV) was applied via the roots at concentrations ranging from 1 to 10 mg Se L^{-1}, reported a significant decrease in both plant growth parameters [23].

Consequently, growth parameters were not affected by selenium exposure; however, the appearance of fungi or chlorosis (Fig. 1a) was observed exclusively on some leaves of selenium-treated plants, indicating that this symptom was not widespread. This limited occurrence likely explains why no significant effects were detected in photosynthetic pigment content. On the other hand, the presence of these symptoms could be attributed to several factors. One possible explanation is the foliar application itself. Nevertheless, it is important to note that control plants also received a foliar treatment with a Hoagland's nutrient solution in the absence of selenium. A more likely explanation is that selenium incorporation may interfere with the availability or uptake of essential nutrients required for optimal spinach growth, thereby promoting fungal development or stress responses such as chlorosis [2].

3.2. Effect of selenium on chlorophylls and carotenoids

The results regarding chlorophyll and carotenoids concentrations are showed in Fig. 2. Chlorophyll *a* consistently appears in higher amounts than chlorophyll *b*, as it serves as the main pigment involved in photosynthesis. In contrast, chlorophyll *b* is primarily synthesized as a shade adaptation to enhance light absorption under low light conditions, and it is not essential for the photosynthetic process. In selenium-treated spinach, chlorophyll *a* level, and consequently the total chlorophyll content, increased significantly ($p < 0.05$) compared to the control, while chlorophyll *b* levels remained unchanged. Previous studies have shown that selenium can enhance the biosynthesis of photosynthetic pigments, contributing to chloroplast protection against oxidative stress and damage caused by reactive oxygen species (ROS) [25]. Additionally, several studies have indicated that selenium may regulate the photosynthetic antenna complex by increasing pigment levels, thereby safeguarding chlorophylls from oxidative damage [26].

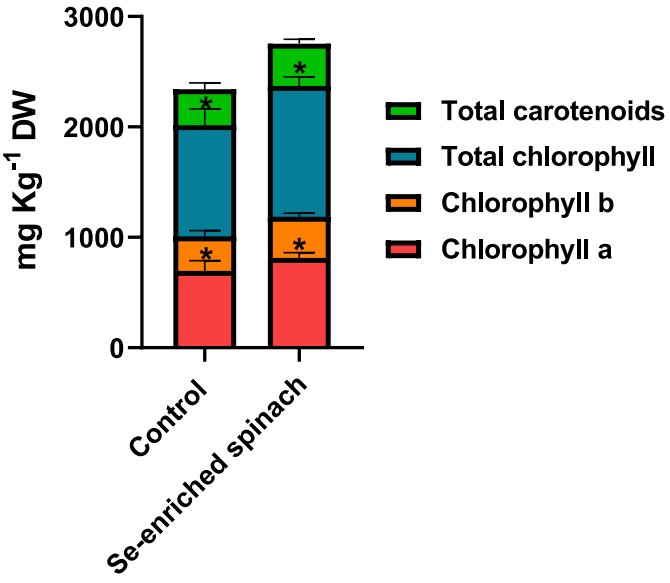

Fig. 2. Effect of selenium on chlorophyll *a*, chlorophyll *b*, total chlorophyll and total carotenoid contents in leaves of spinach plants. *in columns indicate a statistical difference within the same group according to Multi-way ANOVA (Sidak's multiple comparisons test) ($p < 0.05$).

Carotenoids, which function as non-enzymatic antioxidants is to mitigate ROS production in chloroplasts and peroxisomes, were not significantly affected by selenium treatment ($p > 0.05$), as their concentrations remained stable. The effect of selenium on photosynthetic pigments depends on the chemical form in which it is supplemented. For instance, Khan et al. [26] reported that selenite can enhance chlorophyll *b* production, while SeNPs promote overall chlorophyll synthesis. For their part, García-Tenesaca et al. [27] observed increases in chlorophyll *a*, chlorophyll *b*, and carotenoids in radish and pea, whereas these pigments decreased in alfalfa after hydroponic exposure to 20 μM selenium from a 1:1 M mixture of selenite and selenate. In our study, the joint effect of the different forms of selenium has altered the chlorophyll *a* content while maintaining the carotenoid levels in spinach. Therefore, these results suggest that the concentration of selenium applied to spinach did not induce stress in spinach as only the chlorophyll *a* content increased significantly. Furthermore, this may indicate that the synergistic effect of different forms of selenium modulates the photosynthetic antenna complex, enhancing chlorophyll levels as a protective mechanism.

3.3. Accumulation of ^{76}SeMet, ^{77}Se(IV) and Ch-SeNPs in Spinacia oleracea

Total selenium accumulated in aerial part and root of *S. oleracea* derived from the three selenium sources (^{76}SeMet, ^{77}Se(IV) and Ch-SeNPs) was analyzed by ID-ICP-MS and quantified by IPD. This method of quantification made it possible to determine how much of the total accumulated selenium corresponded to ^{76}SeMet, ^{77}Se(IV) and Ch-SeNPs, which had been added simultaneously to the plants by foliar application. Firstly, characterization of the tracers by inverse isotope dilution was carried out with the following results: for ^{77}Se(IV), showing a ^{77}Se abundance of (98.95 ± 0.02) %; for ^{76}SeMet with a ^{76}Se abundance of (98.95 ± 0.02) %, and for ^{78}Se(IV) with a ^{78}Se abundance of (98.1 ± 0.2) %. The isotope dilution methodology for total selenium determination was validated by analyzing the CRM SELM-1 certified for total selenium. No significant differences ($p > 0.05$) were found between the certified value ((2031 ± 70) mg Se kg^{-1} DW) and the experimental value obtained ((2026 ± 20) mg Se kg^{-1} DW), confirming the accuracy of the method. The results in Table 2 show that the selenium content was

Table 2
Total selenium and selenium species content in aerial part. roots and phloem sap of *Spinacia oleracea*.[a] In parentheses, the percentage represents the proportion of each chemical form of selenium in relation to the total selenium accumulated from each selenium source. [b] In parentheses, the percentages represent the proportion of each selenium species relative to the total extracted selenium content. DW (dry weight). nd (not detected).

	mg Se kg^{-1} (DW)			mg Se kg^{-1} (DW)							
	Total selenium[a]	Extracted Selenium after enzymatic hydrolysis	% recovery	SeMetO/SeCys[b]	SeMeSeCys[b]	Se(IV)[b]	SeMet[b]	Se(VI)[b]	Unknown A[b]	Unknown B[b]	Unknown C[b]
					Se-enriched aerial part						
^{76}SeMet	10.5 ± 0.2 (31.0 ± 0.8) %	10.3 ± 0.8	98 ± 7	0.27 ± 0.06 (2.5 ± 0.6) %	0.8 ± 0.1 (8 ± 1) %	nd	9.2 ± 0.7 (87 ± 6) %	nd	nd	nd	nd
^{77}Se(IV)	9.9 ± 0.2 (29.0 ± 0.5) %	8.4 ± 0.2	86 ± 2	0.4 ± 0.1 (5 ± 1) %	nd	0.6 ± 0.1 (7 ± 2) %	7.2 ± 0.4 (87 ± 4) %	0.15 ± 0.04 (1.7 ± 0.4) %	nd	nd	0.23 ± 0.02 (2.7 ± 0.2) %
Ch-SeNPs	13.59 ± 0.05 (40.0 ± 0.2) %	6.37 ± 0.05	48 ± 3	0.12 ± 0.01 (1.8 ± 0.1) %	nd	0.28 ± 0.03 (4.4 ± 0.4) %	1.75 ± 0.1 (27 ± 2) %	0.84 ± 0.09 (13 ± 1) %	nd	nd	2.6 ± 0.2 (40 ± 2) %
					Se-enriched roots						
^{76}SeMet	9.3 ± 0.2 (43.4 ± 0.9) %	8.9 ± 0.3	96 ± 3	0.17 ± 0.02 (1.9 ± 0.3) %	0.61 ± 0.04 (6.9 ± 0.5) %	nd	8.1 ± 0.2 (91 ± 2) %	nd	nd	nd	nd
^{77}Se(IV)	7.9 ± 0.01 (36.8 ± 0.6) %	6.72 ± 0.03	85.0 ± 0.4	0.22 ± 0.1 (3.3 ± 0.2) %	0.51 ± 0.03 (7.6 ± 0.4) %	0.20 ± 0.01 (2.98 ± 0.03) %	5.37 ± 0.02 (79.9 ± 0.3) %	0.42 ± 0.02 (6.2 ± 0.3) %	nd	nd	nd
Ch-SeNPs	4.3 ± 0.1 (19.8 ± 0.5) %	2.05 ± 0.02	47.8 ± 0.5	nd	nd	nd	0.75 ± 0.01 (36.56 ± 0.3) %	1.30 ± 0.02 (64 ± 1) %	nd	nd	nd
					Phloem sap						
^{76}SeMet	7.2 ± 0.2 (40 ± 1) %	6.9 ± 0.3	96 ± 5	0.56 ± 0.04 (8.1 ± 0.6) %	0.58 ± 0.05 (8.4 ± 0.7) %	nd	5.6 ± 0.2 (81 ± 3) %	nd	nd	nd	nd
^{77}Se(IV)	3.25 ± 0.06 (18.0 ± 0.3) %	2.8 ± 0.1	86 ± 3	0.17 ± 0.08 (6 ± 3) %	nd	1.00 ± 0.05 (36 ± 2) %	0.94 ± 0.03 (34 ± 1) %	nd	0.3 ± 0.1 (11 ± 4) %	nd	0.26 ± 0.01 (9.3 ± 0.4) %
Ch-SeNPs	7.58 ± 0.08 (42.0 ± 0.4) %	3.7 ± 0.2	48.8 ± 0.7	0.11 ± 0.02 (2.3 ± 0.5) %	nd	0.8 ± 0.2 (22 ± 5) %	0.37 ± 0.02 (10.0 ± 0.5) %	nd	1.15 ± 0.07 (31 ± 2) %	0.8 ± 0.2 (22 ± 5) %	0.28 ± 0.08 (8 ± 2) %

significantly higher in the aerial part than in the root, regardless the chemical form of selenium added, which is directly related to the foliar treatment applied. Also, these results reflect that all the chemical selenium forms added (^{76}SeMet, ^{77}Se(IV) and Ch-SeNPs) were capable of being transported by spinach from the aerial part to the root through the phloem sap. However, selenium accumulation in plants depends not only on the type of plant but also on the chemical form in which it is added [28].

Table 2 also reveals that the accumulation of these chemical forms varies both among the forms and between plant tissues. In the aerial parts, Ch-SeNPs ((13.60 ± 0.05) mg Se kg^{-1} DW) were the most efficiently incorporated form, followed by ^{76}SeMet ((10.5 ± 0.2) mg Se Kg^{-1}) and, lastly, ^{77}Se(IV) ((9.9 ± 0.2) mg Se kg^{-1} DW). Conversely, in the roots, the trend differed: SeMet was the most efficiently translocated selenium form, with an accumulation of (9.3 ± 0.2) mg Se kg^{-1} DW, followed by Se(IV) ((7.9 ± 0.1) mg Se kg^{-1} DW), while Ch-SeNPs showed the lowest accumulation ((4.3 ± 0.1) mg Se kg^{-1} DW). Significant differences ($p < 0.05$) were observed among the accumulation percentages of each of these species across the different plant tissues. Although all forms showed a decrease in concentration from leaves to root, this reduction was particularly pronounced for Ch-SeNPs, with root accumulation representing only half of the value found in the aerial parts. Similar results were reported by Li et al. [29] who applied selenite and SeNPs independently via foliar application to *Brassica chinensis*. When comparing total selenium concentrations, they observed that SeNPs accumulated predominantly in the aerial parts rather than in the roots, suggesting limited translocation to the root system. Moreover, if Ch-SeNPs remain in the leaves in nanoparticulate form, this could pose a food safety risk, since the leaves are the edible part of this plant. In contrast, selenite showed higher accumulation in the roots compared to SeNPs, suggesting a greater capacity for downward translocation. Likewise, a study by Wang et al. [30] which investigated the effects of five selenium species, including Se(IV) and SeMet, applied via foliar spraying to *Zea mays* (maize), reported that although Se(IV) accumulated more in the aerial parts than SeMet, the opposite trend was observed in the roots. These findings further support that selenium uptake and translocation are influenced by both the chemical form of selenium and the plant species involved. The ability of Se(IV) and SeMet to be incorporated by plants through either leaves or roots is well documented [5]. However, in the case of SeNPs, several additional factors (particle size, aggregation state, morphology, or the nature of the stabilizing agent) can significantly influence their uptake and mobility within plant tissues [31,32].

When evaluating the selenium content accumulated in the phloem sap, a higher accumulation of selenium originating from ^{76}SeMet and Ch-SeNPs was observed, similar to the values found in the aerial parts. However, in the case of ^{77}Se(IV), the percentages found were lower compared to both the aerial parts and the roots.

Although previous studies have examined plant responses to the co-application of two selenium species [27,33], there is a lack of evidence distinguishing the specific accumulation patterns of each individual form when three different selenium species are applied concurrently. In this study, where all three forms were applied simultaneously, potential competitive or synergistic interactions among them may have influenced their uptake and distribution.

3.4. Transformation of ^{76}SeMet, ^{77}Se(IV) and Ch-SeNPs in selenium species. Post-column ID-HPLC-ICP-MS and HPLC-ESI-MS/MS analysis

With the aim of evaluating the potential transformation of ^{76}SeMet, ^{77}Se(IV) and Ch-SeNPs by plant metabolism, the enzymatic extracts of root and aerial parts, after the proteolytic hydrolysis, were measured by post-column ID-HPLC-ICPMS to determine and quantify selenium species, as well as to identify the chemical form from which they originate. One of the most critical steps when performing selenium speciation is the extraction of different species. In this study, enzymatic hydrolysis

with protease XIV was chosen, as it does not degrade selenoamino acids [34]. In addition, enzymatic hydrolysis was performed at 37 °C for 48 h, as previous studies have shown improved extraction efficiency and better chromatographic profile of selenium species [15,35]. Total amount of selenium in the extracts was divided by the total amount of selenium in digested samples to evaluate the extraction efficiency of selenium species. The results in Table 2 indicate that the selected enzymatic protocol enabled quantitative extraction of selenium species with extraction efficiencies above 90 % for ^{76}SeMet in aerial part, root and phloem sap. For ^{77}Se(IV), extraction efficiencies were above 85 % in all tissues. In contrast, the lowest extraction efficiencies were observed for Ch-SeNPs, with values below 50 % in all tissues. These results suggest that spinach does not fully biotransform the nanoparticles into other selenium species, and that Ch-SeNPs would remain in their nanoparticulate form within the tissues. This incomplete biotransformation, as previously mentioned, could pose a potential food safety concern. Furthermore, these findings indicate that when *Spinacia oleracea* is fertilized with Ch-SeNPs via foliar application, the nanoparticles may largely persist in their original nanoparticulate form. This is consistent with previous studies reporting the presence of SeNPs in plant tissues following foliar application [36,37].

Chromatograms of the extracts of the aerial part (Fig. 3a) showed that ^{76}SeMet and ^{77}Se(IV) were mostly transformed into SeMet, a species identified by its retention time, while Ch-SeNPs were transformed into unknown compound C, Se(VI) and SeMet. In all chromatograms other minor Se-containing peaks were detected, as for instance the first selenium containing peak, eluting around 2 min that it could be identified either $SeCys_2$ or selenomethionine selenium oxide (SeMetO), since both species coelute when a PRP-X 100 column is used. However, giving the detection of SeMet, it is highly likely that this first peak corresponds to SeMetO, as oxidation of SeMet during the enzymatic hydrolysis process has been previously reported [38]. In the case of ^{76}SeMet, although it remained mostly unchanged, a minor amount of SeMetSeCys, was also detected. Interestingly ^{77}Se(IV) chromatogram presented a small Se-containing peak related detected as Se(IV) suggesting the almost complete transformation of Se(IV) to SeMet in the aerial part after foliar supplementation.

When analysing the root extracts (Fig. 3b), SeMet was once again the predominant species, except in the case of Ch-SeNPs, where Se(VI) was the major species followed by SeMet whereas the unknown compound C, previously detected in the aerial part was not found. This suggests that the downward translocation of Se species from the aerial part to the root does not significantly transform the Se species initially found in the leaves. Again, minor Se compounds as SeMetO, and SeMetSeCys were detected.

To further investigate the changes occurring during translocation, phloem sap was analyzed, as this tissue is responsible of transporting compounds from the leaves to the roots. The selenium species detected in the phloem sap (Fig. 3c) did not differ significantly from those identified in the aerial part extracts for ^{76}SeMet. SeMet remains unaltered from leaves to root via phloem sap. However, in the case of ^{77}Se (IV) and Ch-SeNPs, the formation of different unknown selenium compounds were detected: an unknown compound A was observed in both, and an unknown compound B and C was observed only in Ch-SeNPs. By comparing the chromatograms of the phloem sap and root corresponding to ^{77}Se(IV) and Ch-SeNPs, it seems to be that spinach metabolism transforms the unknown selenium species, present in both the aerial part and the phloem sap, in the root.

Selenium species detected in the aerial part, root, and phloem sap extracts were also quantified using post-column IDMS (Table 2). The column recovery percentage, based on the sum of the concentration of each selenium species found and the total extracted content, was higher than 95 % for ^{76}SeMet, ^{77}Se(IV), and Ch-SeNPs in all the extracts, except for Ch-SeNPs in aerial, whose percentage was 87 %. When comparing the percentage of SeMet derived from each selenium source between the aerial part and the root, an increase was observed when the selenium

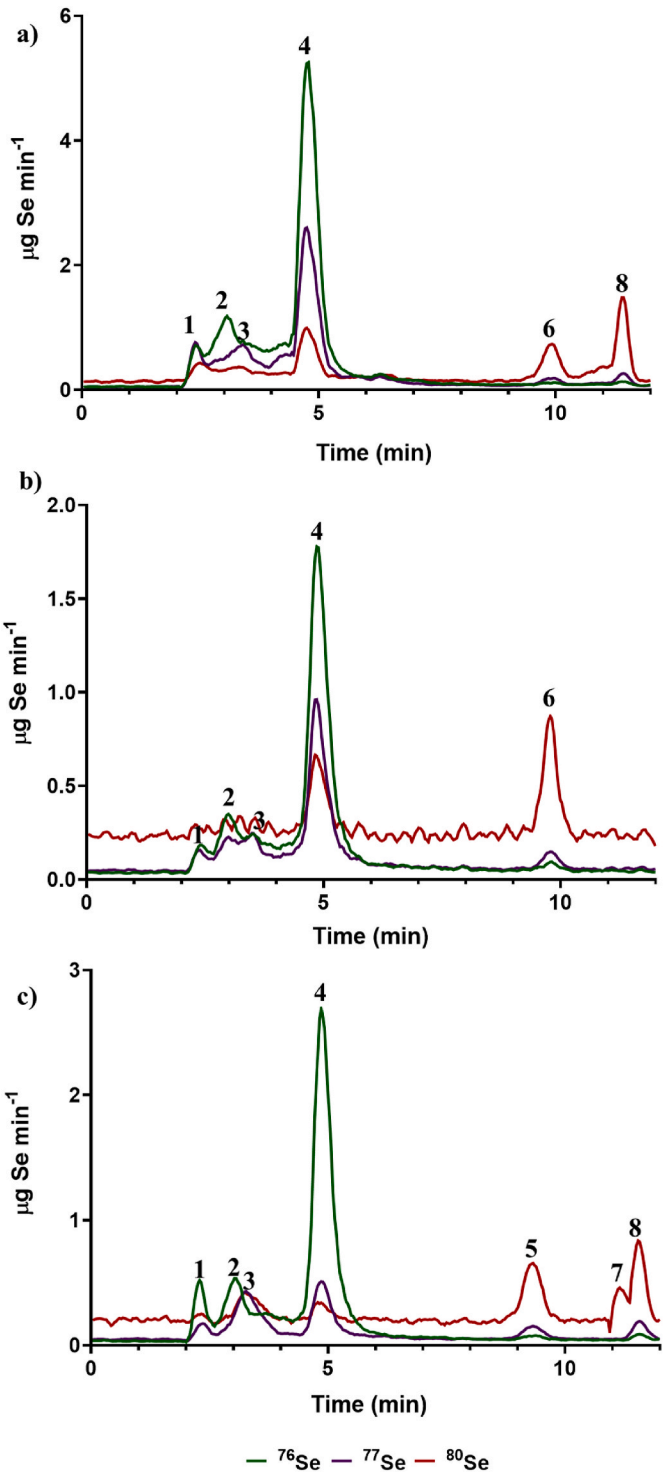

Fig. 3. Conventional mass flow chromatograms obtained from Se-enriched a) aerial part, b) root and c) phloem sap enzymatic extracts, considering the ^{76}Se/^{78}Se, ^{77}Se/^{78}Se and ^{80}Se/^{78}Se isotope ratios. Numbers above the chromatographic peaks identify different selenium compound (1: SeMetO/SeCys; 2: SeMetSeCys; 3: Se(IV); 4: SeMet; 5: Unknown A; 6: Se(VI); 7: Unknown B; 8: Unknown C).

source was either ^{76}SeMet or Ch-SeNPs. A similar trend was observed for Se(VI), whose percentage increased in the root regardless of whether it originated from ^{77}Se(IV) or Ch-SeNPs. In the case of unknown species, their variation across tissues could not be assessed due to the lack of commercial standards for identification by retention time or enrichment

studies. When evaluating these species in the phloem sap, it was observed that while SeMet content decreased across the different selenium sources, Se(IV) content was higher. Therefore, the results indicate that spinach is capable of metabolizing ^{76}SeMet, ^{77}Se(IV), and Ch-SeNPs into different selenium species, mainly SeMet.

This study, together with previous reports in the literature, reinforces the challenges associated with selenium speciation, particularly regarding the identification of individual species. The lack of commercially available standards continues to hinder the definitive identification of certain selenium compounds [39,40]. Nevertheless, the use of post-column IDMS proved to be a robust quantification strategy, allowing for the determination of selenium concentrations independently of species identification. Moreover, the application of isotopically labelled selenium forms enabled the simultaneous assessment of their accumulation and biotransformation within the plant system in a single experiment. This approach made it possible to quantify the amount of each selenium species present and, crucially, to trace their origin, thus providing valuable insight into the metabolic fate of different selenium inputs in spinach.

While HPLC-ICP-MS is highly sensitive for quantifying selenium species based on their elemental content, it does not provide structural information. Therefore, extracts from the aerial part, root, and phloem sap were also analyzed by HPLC-ESI-MS/MS using multiple reaction monitoring (MRM), which confirmed the presence of SeMet, SeMetO and SeMetSeCys. To identify the unknown selenium species, γ-glutamyl-SeMetSeCys and selenohomolanthionine were considered as potential candidates based on previous reports on selenium metabolism in plants [16,41,42]. However, none of the unknown peaks observed in the chromatograms matched the retention times or mass transitions of these reference compounds.

In view of the results and comparing the three chemical forms of selenium applied via foliar treatment to spinach, ^{76}SeMet remains as SeMet and ^{77}Se(IV) is mainly transformed to SeMet in leaves with no further transformation in roots. SeMet appeared with minor Se-containing peaks such as SeMetO and SeMetSeCys that it could be considered derived of SeMet or a precursor to the production of SeMet, respectively. The transformation of Ch-SeNPs is less clear with the presence of SeMet, Se(VI) and an unknown peak in leaves that disappeared in root.

Based on the biotransformation results, SeMet appears to be the most suitable source for foliar fertilization of *Spinacia oleracea*, as no inorganic selenium species were detected. Regarding Se(IV), the proportion of inorganic species in both roots and shoots was below 10 %, suggesting that this form could also be used effectively for spinach fertilization. However, in the case of Ch-SeNPs, although the proportion of inorganic species formed was the highest among the treatments, their persistence in nanoparticulate form within plant tissues raises concerns. This stability could limit their suitability as a selenium fertilizer for spinach.

To date, there are no studies in the literature that simultaneously use different isotopically labelled selenium forms to determine the species derived from each, as well as their respective quantities. García et al. [27] cultivated various microgreens simultaneously with Se(IV) and Se (VI). However, when identifying the selenium species, they were unable to determine the source of selenium for each species. Similarly, Di et al. [43] cultivated wheat with Se(IV) and Se(VI) in separate batches and assessed the resulting selenium species. In both cases, SeMet was the predominant species. A similar approach was employed by Freire et al. [44] who compared selenium species formed after foliar application of Se(IV) and SeNPs in independent wheat experiments. In this case, as in the present study, SeMet was again the predominant species.

3.5. Effect of selenium on essential trace elements

Previous studies have demonstrated that the application of different chemical forms of selenium can affect the absorption of essential macro- and micronutrients [45]. The concentrations of the essential elements in spinach exposed to selenium are shown in Fig. 4. Compared with the control, foliar application of ^{76}SeMet, ^{77}Se(IV) and Ch-SeNPs significantly increased ($p < 0.05$) the content of Zn and Fe in the aerial by 30 % and 11 %, respectively. A similar trend was observed in the root, where Zn and Fe content increased by 51 % and 8.2 %, respectively. This is a positive outcome, as Zn is a key component of several enzymes critical for crop growth and development, and Fe plays a vital role in chlorophyll pigment synthesis, and other metabolic processes [46]. Therefore, the increase in chlorophyll and total chlorophyll, as discussed in section 3.1, may therefore be associated with enhanced Fe availability. Likewise, the increase in Zn concentration in the aerial part may be associated with the chlorosis observed during spinach growth in the presence of selenium [47]. In contrast, the concentration of Mo, an essential element involved in nitrogen fixation [27], decreased significantly by 28 % in the root, while its concentration in the aerial part remained unchanged. Cu was the only essential element whose levels remained constant in both tissues. Overall, the content of all essential elements was higher in the root than in the aerial part, likely due to continuous exposure to Hoagland's nutrient solution via the root system. In a previous study involving *Raphanus sativus* and *Brassica juncea* exposed to Ch-SeNPs, Zn concentrations decreased in both species when the nanoparticles were added to the culture medium [16]. In contrast, other research has reported increased Zn and Cu content in *Brassica chinensis* grown with SeNPs [48]. Longchamp et al. [49] observed that in *Zea mays*, the accumulation of Zn, Cu, and Fe was strongly influenced by both selenium concentration and its inorganic form in the nutrient solution. Additionally, Garcia et el [27]. reported that co-application of selenite and selenate did not significantly alter Cu, Zn and Fe content in several plant species. These results are consistent with those reported by Piñero et al. [50] who observed similar effects in lettuce exposed to increasing concentrations of selenate.

The alteration of essential metal homeostasis, whether through enhancement or deficiency, can lead to synergistic or antagonistic interactions among nutrients. These changes in the balance of essential elements can also influence the antioxidant system, as several elements such as selenium, Cu, Fe, and Zn are components of various antioxidant enzymes. The distribution and uptake of these elements in response to selenium application are influenced by multiple factors, including the

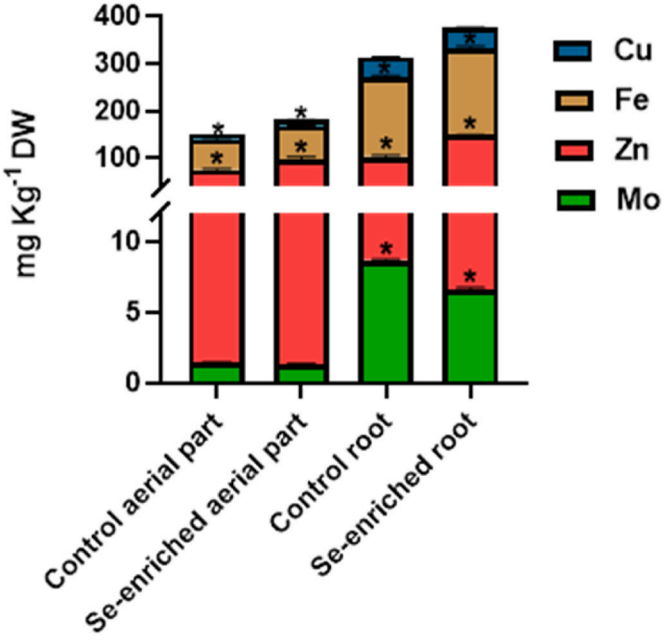

Fig. 4. Effect of selenium on macro- and micronutrients content in aerial part and root. *in columns indicate a statistical difference within the same group according to Multi-way ANOVA (Sidak's multiple comparisons test) ($p < 0.05$).

chemical form of selenium, the level of supplementation, the form of supplementation, and plant species-specific metabolic pathways. Consequently, modifications in one essential trace element may influence the concentration of others to maintain the balance of the redox system [16]. In this context, a key contribution of the present study is the simultaneous application of ^{76}SeMet, ^{77}Se(IV), and Ch-SeNPs to spinach, which may further influence this balance by altering the uptake of essential elements, potentially leading to synergistic effects. These findings highlight the relevance of considering the form and delivery method of selenium when aiming to optimize plant nutrition and stress resilience.

4. Conclusions

This study presents, for the first time, the application of triple stable selenium isotope tracers in *Spinacia oleracea* (exposed to ^{76}SeMet, ^{77}Se (IV), and Ch-SeNPs), encompassing the investigation of their effects on photosynthetic pigments, tracer distribution, speciation analysis, and their influence on the uptake of other essential nutrients.

Photosynthetic activity in spinach increased significantly, as evidenced by the elevated levels of chlorophyll *a*, and total chlorophyll following exposure to the different selenium sources. The use of isotopically enriched tracers demonstrated that ^{76}SeMet, ^{77}Se(IV), and Ch-SeNPs were all taken up by spinach plants, translocated, and biotransformed. The distribution of selenium among plant compartments and its metabolic fate depended on the chemical nature of the applied species. Ch-SeNPs accumulated predominantly in the aerial parts, while ^{76}SeMet and ^{77}Se(IV) showed a greater mobility as being also accumulated in an important extent in roots. Biotransformation studies confirmed the conversion of these selenium forms with a mainly in SeMet prevailing in both roots and shoots. However, it is worth noting the partial biotransformation of Ch-SeNPs by spinach plants, as these nanoparticles may remain relatively stable in their particulate form within plant tissues. Moreover, exposure to these selenium species led to a significant increase in Fe and Zn concentrations in both roots and aerial parts, which may be linked to the chlorosis observed. In contrast, Cu and Mo levels remained largely unchanged in both plant compartments, except for a significant decrease in Mo concentration in the roots.

In conclusion, this study demonstrates that the use of selenium isotope tracers enables the simultaneous evaluation and comparison of the accumulation and metabolic transformation of different selenium chemical forms within a single experiment. Additionally, the combination of post-column IDMS with HPLC-ICP-MS allowed for the quantification of all selenium species formed in spinach, even in the absence of commercial standards for their identification. This methodology thus offers a powerful approach for elucidating selenium metabolic pathways when complemented with molecular mass spectrometry techniques.

CRediT authorship contribution statement

Gustavo Moreno-Martín: Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Marina Rodríguez-Marín:** Methodology, Investigation, Formal analysis, Data curation. **Silvia Queipo-Abad:** Writing – review & editing, Writing – original draft, Data curation. **Yolanda Madrid:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

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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.

Data availability

Data will be made available on request.

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