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INVESTIGATION OF THE ROLE OF SELENIUM FORMS IN BIOLOGICAL
SYSTEMS (SOIL, PLANTS, AND ANIMALS)

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INVESTIGATION OF THE ROLE OF SELENIUM FORMS IN BIOLOGICAL
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LIST OF ABBREVIATIONS

AAS - Atomic Absorption Spectrometry

AdaBoost - Adaptive Boosting

AFS - Atomic Fluorescence Spectroscopy

ANOVA - Analysis of Variance

AsH₃ - Arsine

CNDs - Carbon Nanodots

DOF - Degrees of Freedom

EFSA - European Food Safety Authority

FTIR - Fourier-Transform Infrared Spectroscopy

GC–MS - Gas Chromatography-Mass Spectrometry

GEMAS - Geochemical Mapping of Agricultural Soils

GF-AAS - Graphite Furnace Atomic Absorption Spectrometry

GLS - Gas- Liquid Separator

H₂S - Hydrogen Sulfide

H₂Se - Hydrogen Selenide

HG-AFS - Hydride Generation Atomic Fluorescence Spectroscopy

HPLC - High-Performance Liquid Chromatography

ICP-MS - Inductively Coupled Plasma Mass Spectrometry

I₀/I - Fluorescence Intensity Ratio

LOD - Limit of Detection

LOQ - Limit of Quantification

ML - Machine Learning

NaBH₄ - Sodium Borohydride

NaOH - Sodium Hydroxide

NO - Nitric Oxide

OECD - Organisation for Economic Co-operation and Development

PCA - Principal Component Analysis

RF - Random Forest

R^2 - Coefficient of Determination

SD - Standard Deviation

Se - Selenium

Se⁰ - Elemental Selenium

SeNPs - Selenium Nanoparticles

SHAP - SHapley Additive exPlanations

SbH₃ - Stibine

TEM - Transmission Electron Microscopy

TOC - Total Organic Carbon

XRF - X-ray Fluorescence Spectroscopy

1. INTRODUCTION

Selenium (Se) is an essential trace element required for normal physiological functions in plants, animals, and humans. It plays a critical role in antioxidant defence, thyroid hormone metabolism, immune regulation, and cellular redox homeostasis through its incorporation into selenoproteins, most notably selenocysteine-containing enzymes. Despite its biological importance, selenium is characterised by a narrow margin between nutritional requirement and toxicity. Both selenium deficiency and excess intake are associated with adverse health effects, making accurate assessment, controlled application, and safety evaluation of selenium vital in environmental, food, and feed systems. One of the defining challenges in selenium research is its complex chemical behaviour. Selenium exists in multiple oxidation states and chemical forms, including inorganic species (selenite and selenate), organic forms (selenomethionine and selenocysteine), elemental selenium (Se^0), and nanoscale selenium. These species differ substantially in mobility, bioavailability, toxicity, and metabolic fate. As a result, total selenium concentration alone is often insufficient to describe selenium behaviour or biological relevance. Instead, selenium speciation, transformation pathways, and exposure context must be considered to obtain a meaningful understanding of its environmental and biological impact.

At the environmental scale, soil represents the primary reservoir of selenium and the starting point for its transfer into food chains. However, the distribution of selenium in soils is highly heterogeneous and controlled by a combination of geological, geochemical, and pedological factors. Large-scale, harmonised datasets are therefore essential for establishing reliable baseline concentrations and identifying regions of potential selenium deficiency or excess. While such datasets provide valuable context, they also highlight a fundamental limitation: bulk soil selenium data cannot resolve selenium speciation, reactivity, or bioavailability, which are critical for assessing environmental risk and nutritional relevance. This limitation creates a direct link between environmental assessment and the need for advanced analytical methods capable of targeting reactive and biologically relevant selenium species.

Among selenium intermediates, hydrogen selenide (H_2Se) occupies a central position in selenium biochemistry. H_2Se acts as a key metabolic intermediate linking inorganic selenium compounds to biologically active forms such as selenocysteine. It is also

increasingly recognised as a highly reactive redox species involved in cellular signalling and enzymatic regulation. Despite its importance, H_2Se remains poorly characterised due to its extreme chemical instability, volatility, and rapid transformation in aqueous and biological environments. Conventional selenium speciation techniques, including chromatographic and mass spectrometric methods, are generally optimised for stable selenium compounds and require complex sample preparation steps that preclude direct quantification of free H_2Se in aqueous systems. This analytical gap limits mechanistic understanding of selenium redox cycling and metabolism.

Beyond dissolved and volatile selenium species, elemental selenium (Se^0) has gained increasing attention due to its widespread use in nano-enabled food, feed, and biomedical applications. Compared to inorganic selenium salts, Se^0/SeNPs often exhibit improved bioavailability and reduced toxicity. However, their safety profiles depend strongly on dose, particle properties, exposure duration, and biological system. Reliable detection of Se^0 in real matrices and systematic evaluation of its biological effects are therefore essential for responsible application. Traditional analytical techniques for elemental selenium determination are often time-consuming, expensive, and laboratory-bound, limiting their suitability for rapid screening and routine monitoring.

In this context, fluorescent nanomaterials, particularly carbon nanodots (CNDs), have emerged as promising alternatives for selenium sensing. CNDs exhibit strong and tunable photoluminescence, high chemical stability, water solubility, and excellent biocompatibility. Importantly, they can be synthesised via green, low-cost routes using food-grade precursors, making them suitable for applications in environmental and food-related systems. Surface-functionalised CNDs enable selective interactions with target analytes, allowing the development of simple, rapid, and matrix-tolerant fluorescence-based sensing platforms for elemental selenium and related species.

Selenium chemistry also offers unique opportunities for hybrid sensing strategies. The strong chemical affinity between elemental selenium and mercury ions can be exploited to develop responsive sensing systems that transform selenium's reactivity into an analytical advantage. Such approaches are particularly relevant given the global concern surrounding mercury contamination and the need for accessible detection tools.

While analytical innovation is essential, understanding the biological implications of selenium exposure remains equally critical. Selenium toxicity is highly dose-dependent

and species-specific, and experimental evidence is scattered across diverse biological models with limited standardisation. Conventional toxicological assessment relies heavily on animal studies, which are resource-intensive and constrained by ethical considerations. As a result, there is growing interest in alternative biological models and data-driven approaches that can complement experimental studies. Invertebrate systems such as *Drosophila melanogaster* provide scalable, ethically acceptable platforms for preliminary toxicity screening. At the same time, machine learning offers powerful tools for integrating heterogeneous toxicity data and predicting biological outcomes across species.

Against this background, the present doctoral research adopts an integrated, multi-scale approach to selenium assessment. By combining environmental baseline analysis, advanced analytical method development, nano-enabled sensing strategies, biological evaluation, and machine learning-based toxicity prediction, this work aims to provide a coherent framework for understanding selenium behaviour from environmental occurrence to biological impact. Through this integrative strategy, the thesis seeks to contribute both fundamental insight and practical tools for the safer and more informed use of selenium in environmental monitoring, food and feed systems, and nano-enabled applications.

1.1 Research Gaps

- Environmental selenium assessment is limited by reliance on total concentration data.
- No validated method exists for quantitative H_2Se determination in aqueous systems.
- Rapid, selective detection of elemental selenium remains analytically challenging.
- Nano-enabled sensing strategies for selenium and mercury require further development.
- Nano-selenium toxicity data are fragmented and lack predictive integration.

Combined experimental and machine-learning validation frameworks are absent.

These gaps collectively justify an integrated, multi-scale research approach linking environmental data, analytical innovation, nanotechnology, biological assessment, and predictive modelling.

1.2 Objectives

The overarching objective of this doctoral research was to develop an integrated, multi-scale framework for understanding selenium behaviour by linking its environmental occurrence, chemical speciation, analytical accessibility, nano-enabled sensing, biological response, and predictive toxicology. To achieve this, the study combined geochemical assessment, analytical method development, nanomaterial-based sensing, in vivo experimentation, and machine-learning modelling. The specific objectives of the study were as follows:

- To assess the spatial distribution and variability of total selenium in European and Hungarian agricultural soils
- To develop and validate a modified hydride-generation atomic fluorescence spectroscopy (HG-AFS) method
- To develop a CND-based fluorescence sensing platform for the selective detection of elemental selenium (Se^0) and to construct a CND– Se^0 nanohybrid system for selective mercury (Hg^{2+}) detection,
- To compile and harmonise a cross-species *in vivo* nano-selenium toxicity database and to develop and evaluate machine-learning models for predicting nano-selenium toxicity.
- To experimentally validate the predictive toxicity framework using biological models of *Drosophila Melanogaster*

2. REVIEW OF LITERATURE

2.1 Selenium as a Trace Element: Chemical Nature and Biological Significance

Selenium (Se) is a chalcogen element located in Group 16 of the periodic table and is chemically related to sulfur and tellurium. Its unique redox flexibility allows it to exist in multiple oxidation states ($-II$, 0 , $+IV$, $+VI$), which underpins both its biological indispensability and its toxicological risk (Fordyce, 2013; Winkel et al., 2012). Selenium is incorporated into living systems primarily through its role in selenoproteins, where it is present as selenocysteine, an amino acid essential for redox regulation, antioxidant defence, thyroid hormone metabolism, and immune function (Hariharan & Dharmaraj, 2020; Rayman, 2012). Unlike many trace elements, selenium exhibits a particularly narrow margin between deficiency and toxicity. Insufficient intake has been linked to impaired immune response, cardiovascular disorders, thyroid dysfunction, and increased cancer susceptibility, whereas excessive exposure results in selenosis, characterised by neurological impairment, hair and nail loss, gastrointestinal distress, and metabolic disruption (Brigelius-Flohé & Flohé, 2020; Labunskyy et al., 2014). This duality has positioned selenium as both a micronutrient of public health importance and a contaminant of concern, necessitating precise control over its environmental distribution, chemical form, and biological exposure.

2.2 Environmental Occurrence and Soil-Based Selenium Distribution

2.2.1 Selenium in Soils and Agricultural Systems

Soils represent the primary environmental reservoir of selenium and the main gateway through which selenium enters terrestrial food chains (Ligowe et al., 2020; Rayman, 2012). Selenium concentration in soils varies widely depending on geological parent material, pedogenesis, climate, redox conditions, and land use practices. Agricultural soils are of particular relevance, as they regulate selenium uptake by crops and thus determine dietary selenium availability. Global studies have demonstrated extreme heterogeneity in soil selenium concentrations, ranging from selenium-deficient regions to selenium-enriched zones associated with seleniferous formations (Johnson et al., 2010). In Europe, this variability is influenced by glacial history, lithology, organic matter accumulation, and anthropogenic activities. As a result, Selenium deficiency remains a concern in several European regions despite the absence of overt contamination.

2.2.2 Large-Scale Geochemical Mapping and GEMAS

Harmonised geochemical mapping initiatives, such as the Geochemical Mapping of Agricultural Soils (GEMAS) project, have provided unprecedented continent-scale datasets for assessing total selenium concentrations in agricultural soils. GEMAS employs standardised sampling protocols, analytical techniques (including X-ray fluorescence spectroscopy), and quality control measures, enabling robust comparison across national boundaries (O'Connor, 2014a, 2014b).

These datasets are invaluable for establishing baseline selenium concentrations, identifying regional anomalies, and supporting environmental risk assessment. However, numerous studies based on GEMAS and similar surveys consistently report weak or inconsistent correlations between total selenium and individual soil parameters such as pH, organic carbon, or clay content.

2.2.3 Limitations of Total Selenium Data

While total selenium measurements are essential for baseline mapping, they fail to capture selenium's chemical speciation, mobility, and biological relevance. Selenium behaviour in soils is governed not only by concentration but also by redox transformations, adsorption–desorption processes, microbial activity, and interactions with iron oxides and organic matter. Consequently, total selenium alone cannot predict bioavailability or transformation pathways. This limitation highlights a critical conceptual gap: environmental selenium assessment must move beyond bulk concentration toward species-specific understanding (Schiavon et al., 2020).

2.3 Selenium Speciation and Redox Transformations

2.3.1 Inorganic, Organic, and Elemental Selenium

Selenium exists in environmental and biological systems in multiple chemical forms, broadly classified as inorganic (selenite, selenate), organic (selenomethionine, selenocysteine), and elemental selenium (Se^0) (Rayman, 2012). Each species exhibits distinct solubility, mobility, toxicity, and bioavailability (Bisht et al., 2022). In oxic soils, selenium commonly occurs as selenate or selenite, while reducing conditions promote conversion to Se^0 or metal selenides. Organic selenium species dominate in biological systems and food matrices. Elemental selenium, particularly in nano-scale form, has attracted increasing attention due to its reduced toxicity and controlled release behaviour (Benko et al., 2012; El-Ramady et al., 2014).

2.3.2 Hydrogen Selenide (H₂Se) as a Central Metabolic Intermediate

Hydrogen selenide (H₂Se) occupies a central position in selenium biochemistry. It is generated intracellularly through thiol-mediated reduction of selenite and serves as the direct precursor for selenocysteine biosynthesis. Beyond its metabolic role, H₂Se has been proposed as a highly reactive redox mediator with potential signalling properties analogous to hydrogen sulfide (H₂S) (Cupp-Sutton & Ashby, 2016; Kuganesan et al., 2019). However, H₂Se is extremely unstable. In aqueous environments, it rapidly dissociates ($\text{H}_2\text{Se} \leftrightarrow \text{HSe}^- \leftrightarrow \text{Se}^{2-}$), oxidises to elemental selenium, or reacts with thiols and disulfides to form transient selenosulfide species. Its volatility and short half-life severely limit direct experimental investigation (Hankins & Lukesh, 2024).

2.4 Analytical Challenges in Selenium Speciation

2.4.1 Conventional Selenium Analytical Techniques

Selenium determination is traditionally performed using atomic fluorescence spectrometry (AFS), atomic absorption spectrometry (AAS), and inductively coupled plasma mass spectrometry (ICP-MS) (Irizarry et al., 2001; Nakahara et al., 1980; Pedrero & Madrid, 2009). These techniques offer excellent sensitivity and selectivity but often require extensive sample preparation, including digestion, chemical reduction, hydride generation, and separation steps. Speciation analysis typically involves chromatographic separation coupled to ICP-MS or AFS detection (Y. Li et al., 2025; Sánchez-Rodas et al., 2010). While effective for stable selenium species, these methods are not suitable for highly reactive or volatile intermediates such as H₂Se (Moreno-Martín et al., 2026; Tutar et al., 2025).

2.4.2 Limitations in Hydrogen Selenide Detection

Current analytical strategies for H₂Se detection rely primarily on fluorescent probes designed for intracellular imaging. Although helpful in visualising relative changes, these probes do not provide quantitative information and are unsuitable for environmental or aqueous matrices (Kang et al., 2022; Tarze et al., 2007). No validated method exists for direct quantification of free H₂Se in solution. Hydride-generation atomic fluorescence spectroscopy (HG-AFS) theoretically offers an attractive platform due to its sensitivity and hydride specificity (Kong et al., 2016; Xin et al., 2020). However, standard HG-AFS systems suffer from hydride losses, signal instability, dead volume effects, and poor control of gas–liquid interactions, particularly for volatile selenium hydrides.

2.5 Elemental Selenium and Nano-Selenium

2.5.1 Elemental Selenium in Food and Feed Systems

Elemental selenium has emerged as a promising alternative to inorganic selenium supplements. In nano-scale form, SeNPs exhibit enhanced bioavailability, controlled release, and reduced toxicity compared to selenite or selenate. These properties have driven extensive research into their application in food fortification, animal nutrition, and biomedical therapy. Despite these advantages, SeNPs toxicity remains insufficiently characterised. Toxicity is influenced by particle size, surface chemistry, dose, exposure duration, and biological model, resulting in highly variable outcomes across studies (Benko et al., 2012; El-Ramady et al., 2014; Grudniak et al., 2025).

2.5.2 Analytical Gaps in Se⁰ Detection

Detecting elemental selenium in real matrices remains challenging. Traditional techniques require digestion or reduction steps that obscure selenium's original form. Rapid, selective, and matrix-tolerant detection methods for Se⁰ are scarce, particularly for screening applications (Gómez-Ariza et al., 1998; Muthu et al., 2025; Pedrero & Madrid, 2009).

2.6 Carbon Nanodots (CNDs) as Fluorescent Nanoprobes

Carbon nanodots (CNDs) are fluorescent carbon-based nanomaterials typically smaller than 10 nm, characterised by excitation-dependent photoluminescence, excellent aqueous solubility, chemical stability, and low toxicity (H.H. Nguyen et al., 2024; Nguyen, El-Ramady, et al., 2025). Their fluorescence originates from quantum confinement effects and surface-state emissions. CNDs can be synthesised via green, scalable routes using sugars and amino acids through Maillard reactions, producing nanomaterials rich in surface functional groups. These features make CNDs attractive for sensing applications in food, environmental, and biological systems. CND-based sensors have been widely reported for detecting pesticides, antibiotics, metal ions, and reactive species (Nguyen, Muthu, et al., 2025). However, their application for selective elemental selenium detection remains limited, and the mechanistic understanding of selenium–CND interactions is incomplete.

2.7 Selenium–Mercury Interactions and Nanohybrid Sensors

Mercury contamination poses severe environmental and health risks due to its neurotoxicity and bioaccumulation. Elemental selenium exhibits a strong affinity for mercury, forming stable Se–Hg complexes that reduce mercury bioavailability and

toxicity (Kumar et al., 2023; Ray et al., 2025). This chemical affinity has inspired the development of selenium-based mercury sensors. Combining selenium with fluorescent nanomaterials such as CNDs offers a promising strategy for selective, sensitive mercury detection (Bagheri et al., 2019; Dhamodharan et al., 2022; Hassanvand et al., 2021). However, systematic studies exploiting CND-Se⁰ nanohybrids for practical sensing applications remain scarce.

2.8 Biological Models for Selenium Toxicity Assessment

2.8.1 *In Vivo* Models and Limitations

Traditional selenium toxicity assessment relies on vertebrate animal models, which are costly, low-throughput, and ethically constrained. Reported toxicity thresholds vary widely due to differences in species, selenium form, dose, and exposure duration (Benko et al., 2012; Chen et al., 2023; Prokisch et al., 2012).

2.8.2 *Drosophila melanogaster* as an Alternative Model

Drosophila melanogaster offers a scalable, genetically well-characterised model for toxicity assessment. Its short life cycle, conserved metabolic pathways, and suitability for high-throughput screening make it attractive for preliminary evaluation of nano-selenium effects. However, its application to elemental selenium toxicity remains underexplored (Aleya et al., 2023; Buacheen et al., 2023).

2.9 Machine Learning in Nano-Selenium Toxicology

Computational toxicology, particularly machine learning (ML), provides powerful tools for integrating heterogeneous datasets and modelling nonlinear dose–response relationships (T. Wang et al., 2025; Zubrod et al., 2023). ML has been successfully applied in chemical toxicology and nanoparticle risk assessment, but remains underutilised in nano-selenium research. Existing studies often lack cross-species integration and experimental validation, limiting regulatory confidence. Interpretable ML models combined with biological validation are increasingly encouraged under Safe-by-Design and regulatory frameworks (Gasser et al., 2024; Liangruksa et al., 2025; Y. Zhou et al., 2023). In addition to the general background, previous research conducted within the research group has contributed significantly to the development of selenium analysis and nanomaterial-based sensing approaches. These studies provide an important foundation for the present work, particularly in the areas of analytical method development and nano-sensor applications. A more detailed discussion of these contributions is presented in Chapter 4, in direct relation to the results obtained.

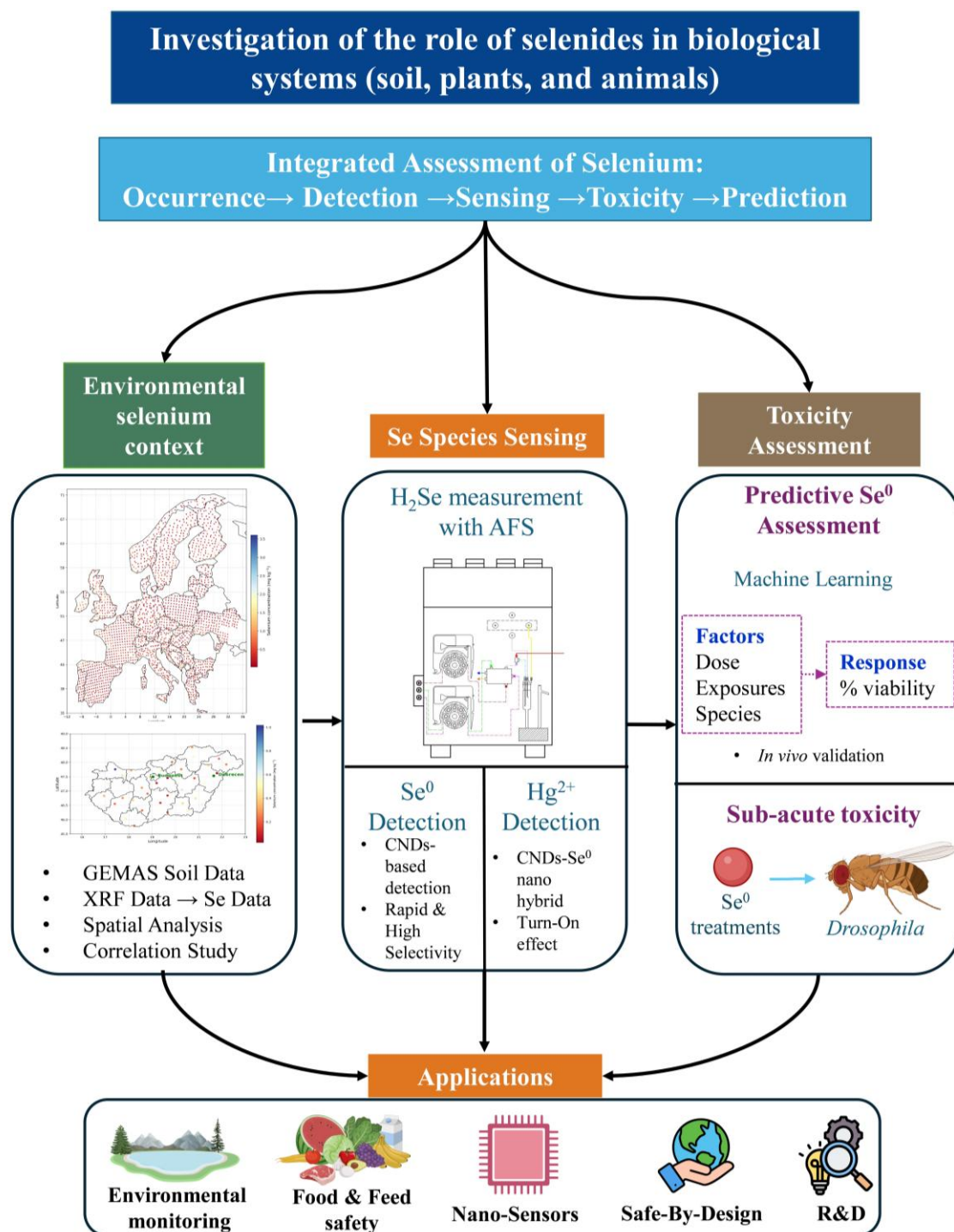


Figure 1. Illustration of the Integrated conceptual framework of the thesis objectives and their applications

3. MATERIALS AND METHODS

3.1 Chemicals and Reagents

Analytical-grade reagents were used throughout the experiment. Sodium selenite (Na_2SeO_3), sodium tetra borohydride (NaBH_4 , 98%, AR grade), sodium hydroxide (NaOH), hydrochloric acid (HCl , 37%), L-ascorbic acid, sodium chloride (NaCl), and ammonia solution (30%) were obtained from VWR International Ltd. (Lutterworth, Leics, UK) and Acros Organics (Belgium). Glycine and D-(+)-glucose, used as precursors for the synthesis of carbon nanodots, were purchased from Vital-Trend Ltd. (Budapest, Hungary). Certified single-element standard solutions of metal ions, including As^{3+} , Ca^{2+} , Cu^{2+} , K^+ , Pb^{2+} , Mg^{2+} , Mn^{2+} , Ni^{2+} , Na^+ , Zn^{2+} , and Hg^{2+} , were procured from Scharlab S.L. (Barcelona, Spain). Ultrapure water (resistivity $\geq 18.2 \text{ M}\Omega \text{ cm}$) was produced using a Milli-Q Advantage A10 purification system (Merck Millipore, Germany) and was used for all reagent preparation and experimental procedures.

3.2 Physical characterisation of Nanomaterials

Morphological characterisation was performed using a transmission electron microscope (TEM) - JEM-2000FXII (JEOL Ltd., Tokyo, Japan) and a Hitachi S-4300-CFE field emission SEM (Tokyo, Japan). For TEM sample preparation, a drop of the diluted, $0.22 \mu\text{m}$ -filtered sample solution was drop-cast onto a carbon-coated copper grid and left to dry at room temperature. For SEM analysis, samples were drop-cast onto carbon-coated stubs and then vacuum-dried. Elemental composition was assessed using a Bruker SDD-based EDX detector, which confirmed the presence of selenium with no detectable impurities. Raman spectroscopy was performed using the LabRAM HR Evolution Confocal Raman Microscope (Horiba Ltd., Kyoto, Japan) with a 532 nm excitation laser. The samples were placed on slides, and the Raman spectra were collected and recorded; Fluorescence spectroscopy, FP-8500 spectrofluorometer (JASCO, USA), using appropriate excitation and emission settings as described in subsequent sections (H.H. Nguyen et al., 2024).

3.3 Limit of Detection and Limit of Quantification

The limit of detection (LOD) and limit of quantification (LOQ) were determined in accordance with established analytical validation guidelines issued by AOAC (AOAC International, 2016) and ICH Q2(R1) (ICH, 1995). These parameters were calculated using Equations (1) and (2)

$$LOD = \frac{3.3\sigma}{S} \quad (eq. 1)$$

$$LOQ = \frac{10\sigma}{S} \quad (eq. 2)$$

where σ represents the standard deviation of ten blanks measured under identical conditions, and S is the slope of the calibration curve.

3.4 Assessment of Selenium Distribution in Agricultural Soils

3.4.1 Data Source and Study Area

Soil Se (Se) data were obtained from the Geochemical Mapping of Agricultural and Grazing Land Soil (GEMAS) project (O'Connor, 2014a, 2014b), a pan-European geochemical survey coordinated by EuroGeoSurveys and partnering national geological surveys. The GEMAS initiative systematically collected soil samples across 33 European countries (approximately 5.6 million km²) using harmonised field and laboratory protocols, enabling continental-scale geochemical comparison and baseline assessment of agricultural soils. Sampling targeted the agricultural topsoil (Ap horizon, 0–20 cm depth), reflecting the zone most critical for nutrient cycling, crop uptake, and agronomic relevance. The GEMAS dataset includes measurements for nearly 60 chemical elements and physicochemical parameters, analysed under strict quality assurance schemes. For the present study, Se concentrations measured by X-ray fluorescence spectroscopy (XRF) were extracted from the GEMAS database, representing total soil Se. A subset comprising all Hungarian sampling points was also generated to support national-scale comparative analysis with the broader European dataset.

3.4.2 Data Preprocessing and Quality Control

Before statistical analysis, all Se concentration values were standardised to mg kg⁻¹. Records lacking Se measurements or precise geographic coordinates were excluded. Consistency checks were performed via visual inspection and quantitative screening, including interquartile range (IQR) outlier assessment, to distinguish natural geochemical extremes from potential data errors. Outliers that were geochemically plausible were retained to preserve the integrity of natural soil variability. Final datasets comprised 2,113 European agricultural soil samples and 37 Hungarian samples. Quality control protocols followed standard practices in soil geochemistry, emphasising retention of real environmental variability while removing only clear artefacts. This approach mirrors quality assurance strategies recommended in continental geochemical mapping projects.

3.4.3 Descriptive Statistical Analysis

Descriptive statistical analysis was performed to characterise Se concentration levels and variability within European and Hungarian agricultural soils. The calculated statistical parameters included: Sample number (n), mean, median, minimum and maximum values, standard deviation, and interquartile range (IQR). Frequency distributions were examined using histograms to assess data dispersion and skewness. Comparative boxplots were constructed to evaluate differences in Se concentration ranges and variability between the European and Hungarian datasets

3.4.4 Correlation Analysis with Soil Parameters

To explore potential relationships between Se concentration and soil properties, Pearson (linear) and Spearman (rank-based) correlation analyses were conducted separately for the European and Hungarian datasets. Soil parameters evaluated included pH, soil organic carbon (SOC), and additional geochemical variables where data completeness and reliability permitted. Pearson correlation coefficients were used to assess linear relationships, while Spearman coefficients were applied to evaluate monotonic relationships and reduce sensitivity to non-normal data distributions. Correlation results were summarised using heatmaps, allowing comparison of association strength and direction between Se and selected soil parameters across spatial scales. Differences between Pearson and Spearman correlation patterns were used to identify potential non-linear or scale-dependent relationships.

3.5 Development of an AFS Method for Hydrogen Selenide (H₂Se) Determination

3.5.1 AFS Equipment configurations and their modifications

All H₂Se measurements and determinations were performed using a Millennium Excalibur 10.055 Atomic Fluorescence Spectrometer (PS Analytical, Orpington, UK) equipped with dual-channel peristaltic pumps, a standard gas-liquid separator (GLS), quartz atomizer, photomultiplier tube detector, a drying tube, and PSA Millennium software (version 2.23). To improve the stability and transfer efficiency of hydrogen selenide (H₂Se) into the atomizer, the original AFS flow path was reconfigured by installing a low dead-volume Teflon T-connector between the sample outlet and the gas-liquid separator (GLS) inlet. In this arrangement, the sample stream is acidified in-line by combining it with 3 mol L⁻¹ HCl upstream of the GLS, while a controlled external H₂ feed is introduced before the mixed stream enters the separator. This configuration supports a steady and reproducible flame that is less sensitive to fluctuations in the NaBH₄-HCl

reaction conditions. The reduced internal volume of the T-junction limits residence time and mixing turbulence, improves bubble dispersion, and shortens the hydride transport distance, thereby decreasing the likelihood of H₂Se degradation before atomization. The in-line neutralization primarily produces NaCl at concentrations well below its solubility limit; accordingly, no precipitation, blockage, or tubing fouling was observed during the experiments. Instrument optical and electronic settings (lamp current, slit width, and EHT voltage) were adjusted following the manufacturer's recommendations.

3.5.2 Method Optimisation

Method optimisation was carried out in a stepwise manner to achieve reliable hydride formation, efficient transport of hydrogen selenide (H₂Se), and consistent atomic fluorescence signal generation. The overall optimisation strategy and decision sequence are summarised schematically in **Figure 1**.

3.5.2.1 Instrumental optimisation: gas-liquid flow stability

Instrumental parameters governing gas-liquid interaction in the modified manifold were optimised using a full factorial (2²) experimental design. Two key instrumental variables were examined:

- Hydrogen gas flow rate: 0.5×10^{-6} and 1.0×10^{-6} m³ min⁻¹
- Reagent flow rate: 0.5 and 1.0 mL min⁻¹

These factors directly affect flame continuity, mixing behaviour at the T-junction, and the efficiency with which volatile H₂Se is transported to the gas-liquid separator (GLS). Flame behaviour was evaluated qualitatively based on continuity, absence of flickering, and atomizer noise, which are established indicators of hydride-system stability. In parallel, fluorescence peak height and integrated peak area (mAU) were recorded as quantitative performance metrics. Only parameter combinations that produced a stable and uninterrupted flame were selected for further chemical optimisation.

3.5.2.2 Optimisation of reagent matrix composition

Following instrumental optimisation, the chemical conditions governing hydride generation were refined to maximise H₂Se formation while avoiding excessive foaming or flow instability. Two reagent-related variables were investigated:

- NaBH₄ concentration: 10%, 40%, and 70% (coded as -1, 0, and +1)
- NaOH concentration: 2%, 8%, and 14%

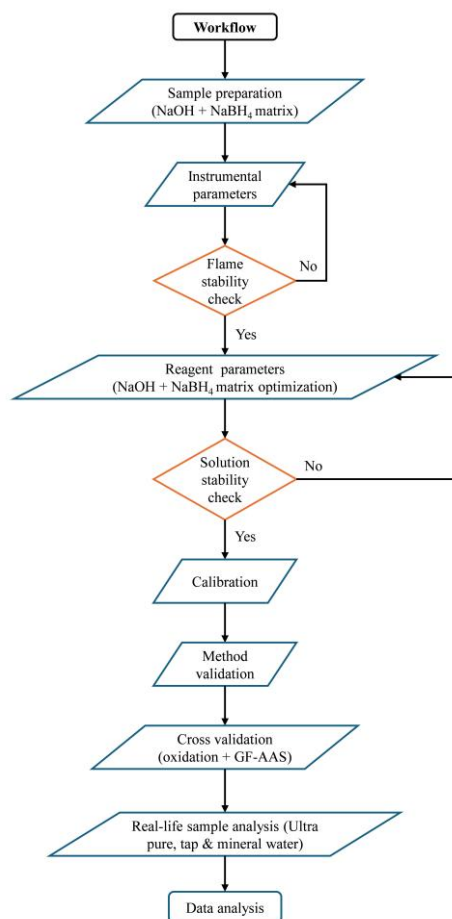


Figure 2. Workflow diagram for the development and validation of the optimized Atomic Fluorescence Spectroscopy (AFS) method

These reagents collectively regulate reduction kinetics, solution alkalinity, and hydride-formation efficiency within the in-line acidification region. Hydride flame behaviour was again qualitatively assessed by monitoring flame stability, flicker, and atomizer noise, whereas fluorescence peak height and integrated peak area (mAU) served as quantitative response variables.

Statistical evaluation of factor effects and interactions was performed using analysis of variance (ANOVA). Model adequacy was assessed through coefficients of determination (R^2 and adjusted R^2) and lack-of-fit analysis. Optimal operating conditions were selected based on combined statistical significance and experimental robustness

3.5.3 Method validation

Calibration of the AFS method was performed using seven Se standard solutions at concentrations of 5, 10, 50, 100, 250, 500, and 1000 $\mu\text{g L}^{-1}$. All calibration standards were prepared fresh by diluting the Se(IV) stock solution into the optimized reagent matrix.

Before measurement, each standard was combined with the optimized NaOH-NaBH₄ solution immediately before introduction into the system to maintain identical hydride-generation conditions for all concentration levels. Measurements were performed under the established optimized instrumental parameters, and each calibration point was analysed in triplicate. The calibration function was obtained by plotting fluorescence response (mAU) as a function of selenium concentration ($\mu\text{g L}^{-1}$), followed by linear regression analysis to evaluate linearity and sensitivity. LOD and LOQ were calculated based on Section 3.3. This approach ensures that both LOD and LOQ reflect the actual background variability of the optimized hydride-generation matrix.

3.5.4 Trapping and Recovery of H₂Se in different aqueous matrices

The applicability of the developed method and its tolerance toward matrix effects were assessed using three different aqueous media: ultrapure water, laboratory tap water, and commercial mineral water (ALDI, Debrecen, Hungary). Aqueous matrices were selected because H₂Se is generated exclusively via liquid-phase hydride reactions, and accurate quantification requires rapid stabilization of the volatile species in alkaline solution to prevent losses from volatilization, oxidation, or disproportionation. Hydrogen selenide was produced by reacting Se(IV) standards at concentrations of 5, 10, and 100 $\mu\text{g L}^{-1}$ with 1.4% NaBH₄ in the presence of 3 mol L⁻¹ HCl within a sealed hydride-generation vessel (Ueta et al., 2020). The generated H₂Se was transported by argon gas at a flow rate of $1.5 \times 10^{-6} \text{ m}^3 \text{ min}^{-1}$ for 10 minutes and subsequently absorbed into 0.1 mol L⁻¹ NaOH. To ensure matrix-matched trapping conditions, the alkaline absorbent solutions were prepared using the same water type as the corresponding test matrix. The trapped reduced selenium species were quantified by atomic fluorescence spectrometry. Method accuracy and matrix tolerance were evaluated by calculating recovery percentages and relative standard deviations (RSD%) from triplicate measurements. The inclusion of tap and mineral water matrices enabled evaluation of potential interferences from naturally occurring ions, such as Ca²⁺, Mg²⁺, HCO₃⁻, and Cl⁻, which may influence hydride generation efficiency, gas purging behaviour, or trapping kinetics.

3.5.5 Cross-Validation via GF-AAS

Graphite Furnace Atomic Absorption Spectrometry (GF-AAS) was used as an independent validation method for selenium recovery after trapping experiments, using a Shimadzu AA-7000 (Shimadzu, Japan) equipped with deuterated background correction and an autosampler. It is used to confirm the quantitative retention of selenium in the alkaline trapping solution following hydride generation. Because GF-AAS cannot

directly detect volatile H_2Se , the selenium species captured in the alkaline absorbent, which were present predominantly as reduced forms ($\text{HSe}^-/\text{Se}^{2-}$) in 0.1 mol L^{-1} NaOH, were chemically converted to Se(IV) before analysis, an oxidation state suitable for furnace atomization. Oxidation was achieved by acidifying each trapped solution to 2% (v/v) with concentrated nitric acid (69% HNO_3), a widely used procedure for the quantitative conversion of reduced selenium species before GF-AAS determination. This step ensured complete dissolution of selenium, minimized volatilization losses, and yielded a chemically homogeneous analyte suitable for calibration and measurement. 1% (w/v) nickel nitrate matrix modifier was added to enhance selenium thermal stability during the pyrolysis stage. Following the trapping step, the exact solutions were used to prepare calibration standards at concentrations of $1\text{--}150 \text{ }\mu\text{g L}^{-1}$ in 0.1 mol L^{-1} NaOH. After oxidation and addition of the matrix modifier, a $10 \text{ }\mu\text{L}$ aliquot was injected for each measurement. The complete furnace temperature program, including drying, pyrolysis, atomization, and cleaning steps, is provided in Annex 1. Because GF-AAS quantifies only oxidized selenium species and does not directly measure H_2Se , its application in this study was limited to verifying the efficiency of selenium capture and retention during the purge-and-trap process. Consequently, comparisons between GF-AAS and AFS results reflect the accuracy of the trapping step rather than hydride formation or transport processes within the furnace system.

3.6 Rapid detection of Se^0 and application to Hg^{2+} detection

3.6.1 Rapid detection of Se^0

3.6.1.1 Synthesising of Carbon nanodots (CNDs) and Se^0

CNDs were synthesized using a hydrothermal carbonization approach adapted from a previously reported method (H.H. Nguyen et al., 2024). Glycine was combined with glucose at an equal molar ratio (0.1 mmol L^{-1} each), and 20 mL of ultra-pure water was added. This ratio was selected based on prior studies to optimize carbonization and fluorescence yield by balancing the sugar (carbon source) and amino acid (nitrogen source) precursors. The mixture was heated at $120 \text{ }^\circ\text{C}$ for 12 hours, then cooled to room temperature. An equal volume of 40% cold ethanol was added, and the mixture was incubated at $-20 \text{ }^\circ\text{C}$ for 30 minutes. Subsequently, the mixture was centrifuged for 30 minutes at 5000 g , and the supernatant was collected. The supernatant was filtered through a $0.22 \text{ }\mu\text{m}$ filter and dried in a freeze dryer. The resulting powder served as carbon nanodots (CNDs). Se^0 was prepared following the method of Badger and Prokisch

(Badgar & Prokisch, 2021), where selenite was mixed with ascorbic acid in a 1:10 molar ratio in water. This green reduction reaction yields red Se^0 . All samples were filtered through a 0.22 μm filter before further analysis. CNDs were characterised using TEM, Raman, FTIR, and fluorescence spectroscopy performed using the procedure mentioned in Section 3.2

3.6.1.2 CNDs Stability Analysis

To assess the stability of CNDs, fluorescence spectra were collected over a pH range from 3 to 11, for 0 to 60 minutes, and at NaCl concentrations between 0.1 and 1.0 mol L^{-1} . An emission mode with a 5 nm emission bandwidth and a 2.5 nm excitation bandwidth was used. Measurements were taken using an excitation wavelength of 360 nm, an emission range of 300-700 nm, and a scan speed of 1000 nm per minute. All measurements were performed at $25 \pm 0.1^\circ\text{C}$.

3.6.1.3 Selectivity and Validation

To evaluate the selectivity of the synthesized CNDs for Se^0 , interference studies were performed with various potential interfering ions. A concentration of 100 mg L^{-1} was prepared for each metal ion solution, including Ca^{2+} , K^+ , Fe^{3+} , Mg^{2+} , Mn^{2+} , Ni^{2+} , Na^+ , and Zn^{2+} . Each metal ion was tested both with and without CNDs under identical conditions, and the response was recorded. I_0/I was calculated, where I_0 represents the intensity of the metal ion with CNDs, and I represents the intensity of the metal ion alone. Se^0 was detected in ultra-pure water, tap water from a laboratory faucet, and soft drink (7-Up) from a supermarket (Aldi, Hungary) as real-world samples. The samples were analyzed using the spiking method, with added Se^0 at concentrations of 316.5 $\mu\text{mol L}^{-1}$ and 633.3 $\mu\text{mol L}^{-1}$. Three tests were conducted, and the average results were reported.

3.6.2 Application of Selenium-based nanohybrids to detect Hg^{2+}

3.6.2.1 Synthesis of Nanohybrids (CND- Se^0)

CNDs were initially prepared according to the protocol described in Section 3.6.1.1. Later, 10 mg of dry CND powder, dissolved in 10 mL ultrapure water, was combined with 10 mL of 1 mM sodium selenite (Na_2SeO_3). To trigger the reduction of selenite to elemental selenium (Se^0), 1 mL of 0.1 mM ascorbic acid solution was added dropwise while stirring continuously at room temperature (Badgar & Prokisch, 2021). The CNDs served as both stabilizers and dispersants, thereby forming a stable CND- Se^0 nanohybrid suitable for fluorescence analysis. The solution was used immediately for all tests to maintain stability and reproducibility. Physical characterization of these nanohybrids was

done using TEM, Raman, and Fluorescence spectroscopy as mentioned in Section 3.2 (H.H. Nguyen et al., 2024; Muthu et al., 2025).

3.6.2.2 Stability Analysis

The stability of the CND-Se⁰ nanohybrid was evaluated under various environmental conditions, including pH (3–11), incubation time (0–60 minutes), and ionic strength (NaCl concentrations from 0.1 to 1.0 mol L⁻¹), as described in Section 3.6.1.2.

3.6.2.3 Selectivity and Validation

To assess the selectivity of the synthesized CND–Se⁰ nanohybrid toward Hg²⁺, potential interference from common metal ions was tested. Solutions of 100 mg L⁻¹ for As³⁺, Ca²⁺, Cu²⁺, K⁺, Pb²⁺, Mg²⁺, Mn²⁺, Ni²⁺, Na⁺, Zn²⁺, and Hg²⁺ were prepared. Each ion was examined both with and without the CND-Se⁰ probe under identical conditions. Fluorescence response was measured as the ratio I_0/I , where I_0 is the fluorescence intensity in the presence of the nanohybrid, and I is the fluorescence intensity in its absence. To validate the method on real samples, Hg²⁺ detection was performed in ultrapure water, tap water (from a laboratory faucet), and commercial mineral water (from Aldi, Debrecen, Hungary). The samples were spiked with Hg²⁺ at 1, 3, and 5 $\mu\text{mol L}^{-1}$, each analyzed in triplicate. The average recovery was calculated to evaluate accuracy and reproducibility, with the best results averaged across multiple runs.

3.7 Data-Driven and Biological Toxicity Assessment of Nano-Selenium

3.7.1 Data Acquisition and Database Construction

A systematic literature search was performed in PubMed to gather *in vivo* nano-selenium toxicity data. The search included terms such as “*nano selenium*”, “*selenium nanoparticles*”, “*toxicity*”, “*LD50*”, “*viability*”, “*in vivo*”, and “*animal model*”. Titles and abstracts were initially screened for relevance, followed by a full-text review based on criteria: (i) nano-selenium administered to vertebrate or invertebrate species; (ii) reporting at least one quantitative toxicological measure (e.g., viability, mortality rate, LD₅₀); and (iii) detailing key experimental parameters like dose, exposure duration, nanoparticle size, and synthesis method. Studies without *in vivo* experiments, nanoparticle characterization, or numerical outcomes were excluded. Ultimately, 86 experiments across eight biological models: mice, rats, quail, chicken, fish, *Drosophila*, *Lucilia sericata*, and *Spodoptera* were included. Data on each study were extracted and organized into a structured database capturing: (i) nanoparticle parameters (synthesis method: green or chemical; particle size in nm), (ii) dosage details (concentration in mg

Se kg⁻¹, exposure time in hours, frequency), (iii) biological descriptors (species and gender), and (iv) outcomes (viability in %). This dataset formed the foundation for all subsequent data processing, analysis, and machine learning modeling.

3.7.2 Data Cleaning and Preprocessing

Data preprocessing followed a structured workflow to ensure dataset consistency and readiness for analysis. Initial cleaning involved correcting typos, standardizing units (mg Se kg⁻¹ for dose, nm for particle size, and hours for exposure duration), and removing duplicate or overlapping dose–response entries. Nomenclature for species and nanoparticle synthesis methods was harmonized for consistency. Missing values in continuous variables were imputed using the median, which is robust against skewed distributions and outliers common in toxicological data. Categorical variables, like species, were encoded with integer labels (e.g., 1 = mice, 2 = rats, ... 8 = chickens), facilitating their use in tree-based regression models that are insensitive to label order. All continuous predictors were standardized with z-score scaling (eq.3),

$$X_{scaled} = \frac{X - \mu}{\sigma} \quad (eq.3)$$

Where X denotes the original variable, μ denotes the mean, and σ denotes the standard deviation, thereby preventing high-magnitude variables such as dose and exposure duration from biasing the model. The cleaned, imputed, and standardized dataset was then used for exploratory analysis and machine-learning workflows. This normalization process mitigated the influence of high-magnitude variables such as dose and exposure duration, preventing them from disproportionately affecting the optimization during model training. The resulting dataset, after cleaning, imputation, and standardization, provided a robust foundation for exploratory data analysis and subsequent machine learning procedures.

3.7.3 Exploratory Data Analysis (EDA)

Exploratory data analysis was conducted to characterize the dataset's structure and to inform feature selection for model development. Two complementary methods were used.

3.7.3.1 Principal Component Analysis (PCA)

PCA was conducted on the standardized continuous variables to reduce dimensionality and identify the most influential predictors of total variance. The PCA was performed using the scikit-learn library, and only the first two principal components were retained to assess variance and visually explore the multivariate structure.

3.7.3.2 Correlation analysis

Pearson correlation coefficients were calculated between all continuous variables to measure linear relationships. These analyses helped identify collinearity, evaluate the relative importance of exposure descriptors, and guide the selection of biologically meaningful predictors for modeling. Based on these exploratory analyses and mechanistic relevance, three features: dose (mg Se kg⁻¹), exposure duration (h), and animal model were chosen for machine-learning model development.

3.7.4 Machine Learning Workflow

All machine learning and statistical analyses were conducted using Python 3.11. The complete protocol was described in section 3.9.

3.7.4.1 Dataset split

The dataset was divided into training and testing sets using a 70:30 split, ensuring balanced representation of species across both sets. This division enabled an unbiased assessment of the model's generalization ability.

3.7.4.2 Model selection

A wide range of regression algorithms was evaluated to identify the optimal modeling approach for predicting nano selenium toxicity. The models tested included linear and regularized methods (Linear Regression, SGD Regressor), distance-based learning (KNN), kernel-based methods (SVR), tree-based ensemble models (Random Forest, Gradient Boosting, AdaBoost), and gradient-boosted decision tree techniques (XGBoost). All models were trained and evaluated within a consistent framework using R², RMSE, MAE, and 10-fold cross-validation. The models demonstrating the most potent combination of accuracy, stability, and generalization were chosen for detailed optimization and interpretability analysis with SHAP values. The top-performing model was then selected for experimental validation.

3.7.4.3 Training and evaluation metrics

Model performance was quantified using the following metrics:

a) Coefficient of determination (R²)

R² measures the proportion of variance in the observed viability explained by the model.

$$R^2 = 1 - \frac{\sum_{i=1}^n (y_i - \hat{y}_i)^2}{\sum_{i=1}^n (y_i - \bar{y})^2} \quad (eq. 4)$$

where y_i = actual values, $\hat{y}_i$ = predicted values, and $\bar{y}$ = mean of actual values.

b) Root Mean Squared Error (RMSE)

RMSE quantifies average prediction error and penalizes large deviations.

$$RSME = \sqrt{\frac{1}{n} \sum_{i=1}^n (y_i - \hat{y}_i)^2} \quad (eq. 5)$$

c) Mean Absolute Error (MAE),

MAE provides the average magnitude of prediction error.

$$MAE = \frac{1}{n} \sum_{i=1}^n |y_i - \hat{y}_i| \quad (eq. 6)$$

d) 10-fold cross-validation (K = 10).

Ten-fold cross-validation (CV) was employed to assess the model's robustness across various data partitions. The dataset was split into 10 folds; during each iteration, nine folds served for training while one was used for validation. The final CV score was calculated as

$$CV_{score} = \frac{1}{n} \sum_{k=1}^K Score_k \quad (eq. 7)$$

where, K=10 (folds), $Score_k$ refers to the performance metric (R^2 , RMSE, & MAE) from the k^{th} fold. This approach decreased overfitting risk and delivered a stable estimate of the model's generalizability.

3.7.5 Mechanism Analysis

SHAP (SHapley Additive exPlanations) analysis was used to quantify feature contributions and enhance the interpretability of the final machine-learning model. SHAP provides an additive contribution score for each feature in every prediction, allowing for the assessment of both overall and individual model behavior (Z. Li et al., 2022). Given that the chosen model was a tree-based ensemble, the SHAP Tree Explainer, which provides exact Shapley values for tree models, was used. SHAP values were calculated for all tests set observations to produce:

- Global feature importance rankings, derived from mean absolute SHAP values
- Beeswarm plots, showing sample-level variability in feature effects
- Summary profiles of contributions, illustrating how different factors influence prediction strength

This interpretability process facilitated a structured assessment of feature impact and verified that the model's decision logic was consistent with accepted toxicological principles. All SHAP analyses used the SHAP Python package (v0.44).

3.7.6 *In vivo* experimental Validation Using Quails

The *in vivo* feeding experiment with Japanese quail (*Coturnix japonica*) was part of collaborative study. The experimental design, animal handling, execution of the feeding trial, ethical approval, and initial data collection were carried out in the Animal House of the Institute of Animal Science, Biotechnology, and Nature Conservation at the University of Debrecen, Hungary, by Ms Aya Ferroudj under her approved research project. The aim of this experiment was limited to secondary data analysis, interpretation of biological outcomes (% viability), and integration of experimental results into the machine learning-based toxicity prediction framework developed in this thesis. This data was used solely for independent computational modelling and cross-species validation of nano-selenium toxicity predictions. It provided an external biological validation set to evaluate the predictive performance of the machine-learning models under conditions of nutritional and elevated selenium exposure. The details of the experimental design, ethical approval and procedure are provided in the Annex 2.

3.8 *Drosophila melanogaster* toxicity model

The *in vivo* nanotoxicity of Se⁰ was evaluated using a *Drosophila melanogaster* digestion-based exposure model, conducted at the Nutritional Genetics and Genomics Research lab in the Institute of Nutrition, University of Debrecen, Hungary. The w^{m4h} (white mottled 4) strain, obtained from the Bloomington *Drosophila* Stock Centre, was used for all experiments. The experimental design was based on the protocol reported by (Aleya et al., 2023) with minor modifications. Fly stocks were maintained as overlapping generations in culture bottles at 25 ± 1 °C and 60-70% relative humidity under complete darkness. All toxicity experiments were conducted under identical environmental conditions to ensure reproducibility. Experimental embryos were collected from population cages containing approximately 200 adult flies (equal numbers of males and females, 5 days old). Using a simple lab microscope, fifty synchronised embryos (0-2 h old) were selected and transferred into individual experimental vials containing control or Se⁰-supplemented media. Normal culture media were prepared by homogenising 70 g of commercial yeast paste in 1 L of distilled water, followed by the addition of 52 g and 30 g of wheat flour. Subsequently, 10 g of agar powder was added, and the mixture was boiled for more than 30 min until complete dissolution and homogeneity were achieved. The final volume of the medium was adjusted to 1 L. After boiling, the medium was cooled to approximately 50 °C in a water bath, and 1 g of Nipagin (ethyl 4-

hydroxybenzoate, 99%, A13172; Thermo Fisher Scientific) was incorporated as an antifungal agent. Elemental selenium (Se^0) was supplemented into the cooled media at five different concentrations: 0.1, 0.5, 1, 2, and 4 mg L^{-1} . Media without Se^0 supplementation served as the control. For each concentration, a minimum of five replicate vials were prepared. Embryo development was monitored daily for 20 days. The number of pupae formed and the number of adult flies emerged were recorded at 12-hour intervals and used as quantitative endpoints for toxicity assessment.

3.9 Statistical Analysis

Data preprocessing, statistical evaluation, and graphical visualization were performed using a combination of Python-based tools and dedicated statistical software. Environmental and analytical datasets were processed using Python (version 3.11) with the pandas and NumPy libraries. Graphical outputs, including histograms, boxplots, scatter plots, contour plots, surface plots, and correlation heatmaps, were generated using Matplotlib and Seaborn within a Jupyter Notebook environment. Additional data visualization and figure refinement were performed using Microsoft Excel, where appropriate. Statistical analyses for experimental and analytical validation studies were conducted using GraphPad Prism (version 9.0, GraphPad Software, USA) and IBM SPSS Statistics (version 26). Experimental results are reported as mean $\pm$ standard deviation (SD) based on triplicate measurements ($n = 3$) unless stated otherwise. Differences among multiple experimental groups were evaluated using analysis of variance (ANOVA). When significant effects were detected, Tukey's honestly significant difference (HSD) test was applied for post-hoc pairwise comparisons, with significance levels set at $p < 0.05$ or $p < 0.001$, as specified. In cases requiring classification of homogeneous subsets, Duncan's multiple range test was used. For factorial optimization experiments, ANOVA-based model evaluation was employed to assess the significance of main effects and interaction terms. Model adequacy was verified using F-tests, p-values, coefficients of determination (R^2 , adjusted R^2 , predicted R^2), and lack-of-fit analysis. Transmission electron microscopy (TEM) images and particle size distribution data were processed using ImageJ software (version 1.54D, National Institutes of Health, USA). Machine learning analyses were performed entirely in Python (version 3.11) using the scikit-learn library (version 1.4). NumPy and pandas were used for data handling, while SciPy and statsmodels supported statistical computations. Model performance was assessed using the coefficient of determination (R^2), root mean squared error (RMSE), mean absolute

error (MAE), and 10-fold cross-validation. All computational workflows were implemented within a reproducible analytical pipeline to ensure transparency and repeatability.

4. RESULTS AND DISCUSSIONS

4.1 Selenium Distribution in Agricultural Soils

4.1.1 Selenium Levels in European and Hungarian Agricultural Soils

Descriptive statistical evaluation of XRF-derived Se concentrations revealed that agricultural soils in Hungary exhibit Se levels comparable to those observed across Europe, with differences primarily related to variability rather than central tendency (Tóth et al., 2016). For the European dataset ($n = 2,113$), Se concentrations ranged from 0 to 3.836 mg kg^{-1} , a mean concentration of 0.405 mg kg^{-1} and a median of 0.354 mg kg^{-1} . The distribution showed moderate heterogeneity, reflected by a standard deviation of 0.293 mg kg^{-1} and an interquartile range (IQR) of 0.248 mg kg^{-1} , indicating substantial spatial variability at the continental scale. Hungarian agricultural soils ($n = 37$) exhibited Se concentrations between 0.025 and 1.017 mg kg^{-1} , with a mean of 0.355 mg kg^{-1} and a median of 0.327 mg kg^{-1} . Although the mean Se concentration in Hungary was slightly lower than the European average, the similarity of median values suggests comparable baseline Se levels (Zapletalová et al., 2025). The Hungarian dataset showed an IQR of 0.277 mg kg^{-1} , indicating a degree of internal variability consistent with European trends. Given the relatively small Hungarian subset ($n=37$), national-scale inference should be considered indicative rather than exhaustive. Overall, these findings demonstrate that Hungarian agricultural soils fall well within the broader European Se concentration range and do not exhibit evidence of extreme Se enrichment or depletion (Kovács et al., 2021).

4.1.2 Distribution Characteristics and Comparative Analysis

The distribution characteristics of Se (Se) concentrations in agricultural soils were evaluated using frequency distributions and comparative boxplot analysis (Figure 3). The European dataset (Figure 3a) exhibits a right-skewed distribution, with most Se concentrations concentrated between 0.2 and 0.5 mg kg^{-1} (Ander et al., 2013). A limited number of samples with elevated Se levels form an extended upper tail, contributing to the overall variability observed at the continental scale. In contrast, the Hungarian dataset (Figure 3b) displays a more compact distribution, with Se concentrations primarily confined to the $0.2\text{--}0.6 \text{ mg kg}^{-1}$ range. The fitted distribution curve indicates a clear central tendency and reduced influence of extreme values, reflecting the narrower concentration range and smaller sample size (Matys Grygar et al., 2022). Comparative boxplot analysis (Figure 3c) confirms that median Se concentrations are similar for Europe and Hungary, while variability differs markedly. The European dataset shows a broader upper range and a higher number of outliers, whereas the Hungarian dataset

exhibits a tighter interquartile range and fewer high-concentration values. Overall, these results indicate that Se concentrations in Hungarian agricultural soils are consistent with broader European patterns, differing mainly in the extent of variability rather than in central concentration levels (Manaljav et al., 2021).

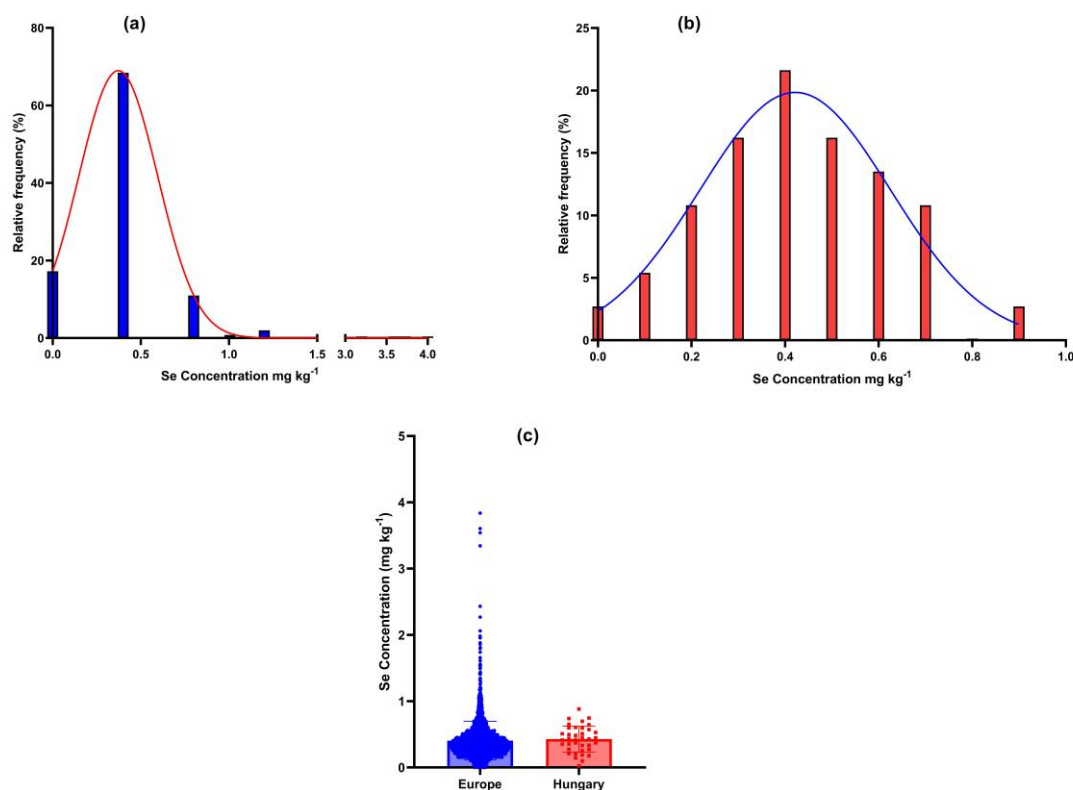


Figure 3. Distribution characteristics of Se (Se) concentrations in agricultural soils based on GEMAS XRF data: (a) Frequency distribution and fitted curve for European agricultural soils (n = 2,113); (b) Frequency distribution and fitted curve for Hungarian agricultural soils (n = 37); (c) Comparative boxplot illustrating median values, interquartile ranges, and outliers for Europe and Hungary.

4.1.3 Spatial Distribution of Selenium at European and National Scales

The spatial distribution of Se concentrations in agricultural soils across Europe and within Hungary is presented in Figure 4a and Figure 4b, respectively. At the European scale (Figure 4a), Se concentrations exhibit pronounced spatial heterogeneity, with low to moderate values dominating most agricultural regions (O'Connor, 2014a). Elevated Se concentrations occur periodically and are geographically localized rather than forming continuous regional patterns. No clear latitudinal or longitudinal gradient is evident across the continent, indicating that broad geographic trends do not govern Se distribution. Instead, the observed spatial variability suggests that regional and local geochemical

factors, including parent material composition, soil mineralogy, and soil-forming processes, primarily control Se occurrence (Saaltink et al., 2014). Large areas of Europe display uniformly low Se concentrations, while isolated zones exhibit comparatively higher values, contributing to the right-skewed distribution observed in the frequency analysis. Within Hungary (Figure 4b), Se concentrations also show spatial variability, although values remain within the broader European concentration range. Se levels vary among agricultural regions, with no distinct clustering indicative of widespread enrichment or depletion. The distribution around major reference locations, including Budapest and Debrecen, reflects Se concentrations comparable to surrounding areas, suggesting the absence of localized anomalies in these regions (Jones & Winkel, 2017). Overall, the spatial patterns observed at both continental and national scales highlight the patchy and site-specific nature of Se distribution in agricultural soils. These findings reinforce the importance of regional geological context in controlling total soil Se content and support the use of harmonized datasets, such as GEMAS, for baseline environmental assessment.

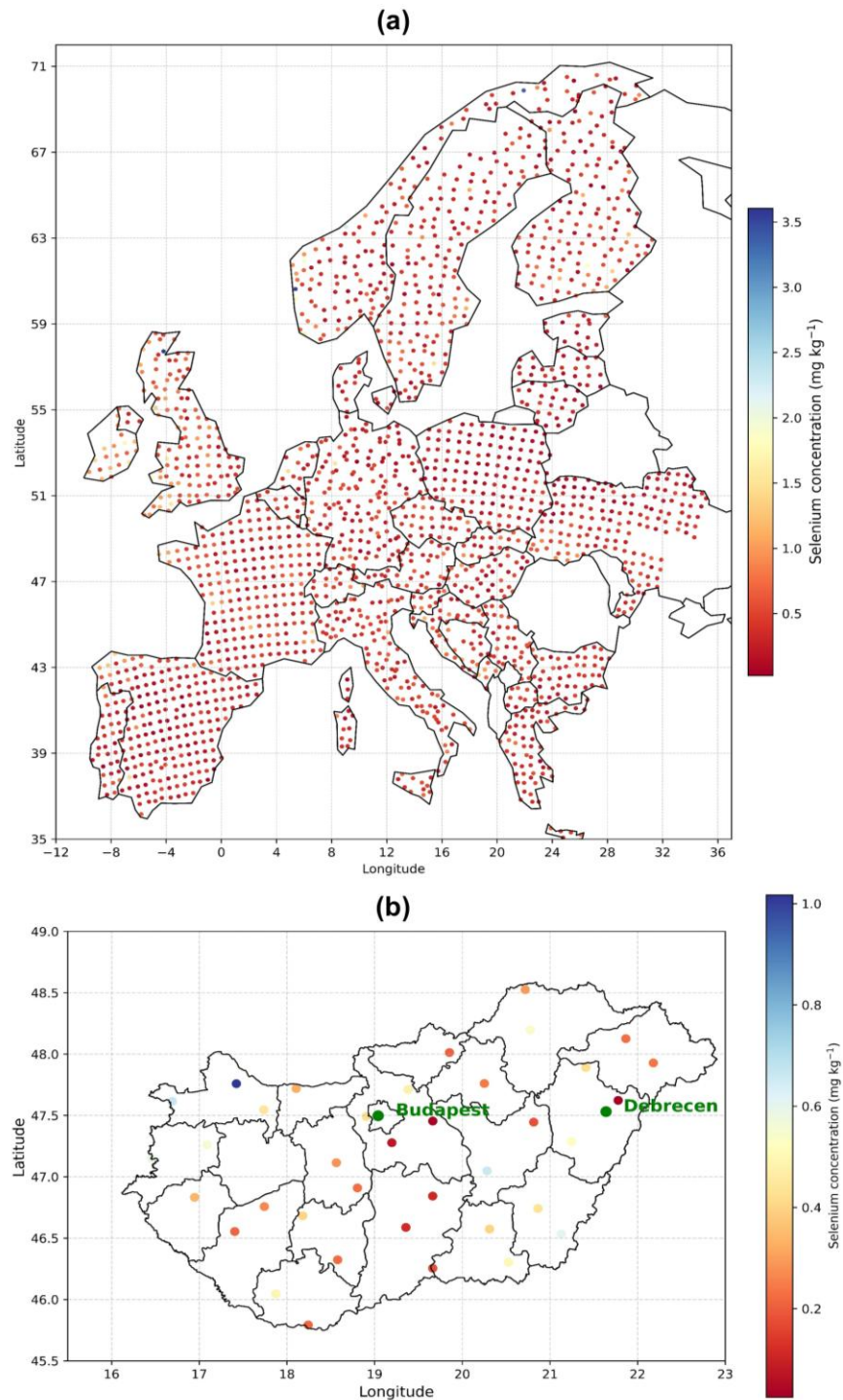


Figure 4. Spatial distribution of Selenium (Se) concentrations in agricultural soils based on GEMAS XRF data:(a) European-scale map showing heterogeneous Se distribution with localized areas of elevated concentration and no clear continental-scale gradient; (b) Hungarian-scale map illustrating spatial variability of Se concentrations within the national agricultural soil system, with values consistent with the broader European range.

4.1.4 Associations Between Selenium and Soil Geochemical Parameters

The relationships between Se concentration and selected soil parameters were evaluated using Pearson and Spearman correlation analyses at both the European and Hungarian scales (Figure 5). At the European level, Pearson correlation analysis (Figure 5a) revealed weak to moderate positive associations between Se and organic matter-related parameters, particularly total organic carbon (TOC) and loss on ignition (LOI) (Tóth et al., 2016). Moderate correlations were also observed between Se and selected elemental concentrations, while negative associations with silicon-related parameters suggest dilution effects in silica-rich soils. These relationships indicate that Se distribution in European agricultural soils is influenced by multiple geochemical factors rather than by a single dominant control (Tolu et al., 2014). Spearman correlation analysis of the European dataset (Figure 5b) revealed broadly consistent trends, with slightly strengthened monotonic relationships for several parameters. The similarity between Pearson and Spearman results suggests that most Se–non-linear effects do not strongly drive soil parameter relationships but instead reflect gradual, monotonic associations. (O'Connor, 2014a). In the Hungarian dataset, Pearson correlation analysis (Figure 5c) indicated generally stronger apparent correlations between Se and several elemental parameters. However, these elevated correlation coefficients should be interpreted with caution, given the small sample size, which increases sensitivity to individual data points and to co-variation among elements. The results primarily reflect shared geochemical or mineralogical associations rather than Se-specific processes (Novoselec et al., 2024). Spearman correlation analysis for Hungary (Figure 5d) showed slightly reduced but still consistent association patterns, confirming that the observed relationships are largely monotonic. Strong correlations among metallic elements (e.g., Zn, Pb, Al) were evident in both Pearson and Spearman analyses, reflecting familiar sources or soil matrix controls rather than direct interactions with Se. Overall, the correlation analysis demonstrates that complex, multivariate geochemical interactions govern Se behavior in agricultural soils. While organic matter and specific elemental associations appear to influence Se distribution, the absence of strong, universal correlations highlights the limitations of bulk geochemical parameters in predicting Se speciation or bioavailability (Jones & Winkel, 2017).

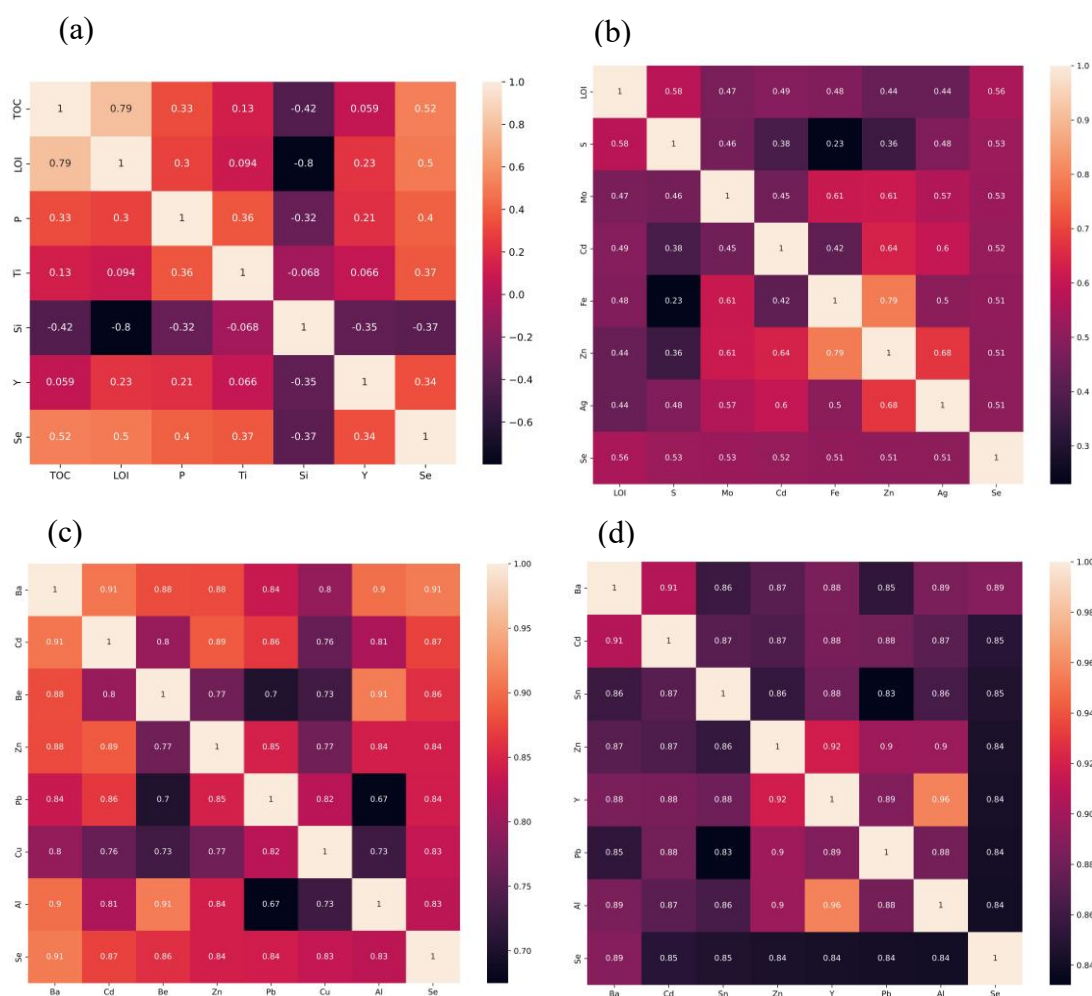


Figure 5. Pearson and Spearman correlation heatmaps illustrating relationships between Selenium (Se) and selected soil parameters based on GEMAS XRF data: (a) Pearson correlation matrix for European agricultural soils; (b) Spearman correlation matrix for European agricultural soils; (c) Pearson correlation matrix for Hungarian agricultural soils; (d) Spearman correlation matrix for Hungarian agricultural soils. Color intensity represents the strength and direction of the correlation coefficients

4.1.5 Interpretation and Methodological Considerations

The predominance of weak-to-moderate correlations highlights the multifactorial nature of Se behaviour in soils. Se distribution and retention are influenced by a complex combination of factors, including redox conditions, microbial activity, mineralogy, and organic matter composition, many of which cannot be fully captured using bulk geochemical parameters alone. Notably, the Se concentrations analysed in this study represent total soil Se as measured by XRF and do not provide information on Se speciation or bioavailability. Consequently, the results should not be interpreted as direct

indicators of Se uptake by plants or transfer into biological systems. Despite these limitations, the present analysis provides a robust and harmonised baseline assessment of Se distribution in agricultural soils at both continental and national scales.

While total selenium concentrations provide essential baseline information, they do not capture selenium speciation or transient intermediates relevant to biological and environmental transformations. Volatile hydrogen selenide (H_2Se) represents a critical but analytically challenging species in selenium redox cycling. Therefore, the following section focuses on the development and validation of a modified atomic fluorescence spectroscopy (AFS) method for sensitive and reproducible quantification of H_2Se . These baseline distributions define the environmental context in which selenium transformations occur and justify the need for complementary analytical methods capable of capturing reactive and volatile selenium species.

Although the GIS-based spatial analysis provides valuable insights into the regional distribution of selenium, the incorporation of advanced geostatistical approaches such as kriging or multivariate geochemical modelling could further improve spatial prediction and interpretation (Meng et al., 2013). However, the application of such methods requires high-resolution and spatially uniform datasets for reliable variogram construction and model validation (Xu & Zhang, 2023). Given the heterogeneity in sampling density within the GEMAS dataset, the present study focuses on descriptive spatial analysis (Hou et al., 2017). Future studies will aim to integrate geostatistical modelling to enhance predictive capability

4.2 Development of an Atomic Fluorescence Method for Hydrogen Selenide (H_2Se)

4.2.1 Instrumental Optimization for Hydride Generation and AFS Stability

The first stage of method development focused on improving hydride-transfer efficiency and stabilizing atomization in the modified AFS system. In the standard method, H_2 is usually produced in situ from the $\text{NaBH}_4\text{-HCl}$ reaction. It serves simultaneously as the reductant for hydride formation and the fuel gas for sustaining the quartz atomizer flame. However, the present workflow required selenium samples to be introduced into a strongly alkaline NaOH-NaBH_4 matrix, in which the conventional hydride-generation method is incompatible due to immediate hydrolysis, vigorous foaming, and unstable gas evolution. To overcome these limitations, an externally supplied hydrogen line was incorporated into the manifold to sustain the AFS flame and promote the controlled

reduction of selenium species to H_2Se following in-line acidification of the alkaline sample. A low dead volume Teflon T-joint was positioned between the sample outlet and the gas-liquid separator (GLS) to ensure rapid mixing and minimize hydride decomposition before separation.

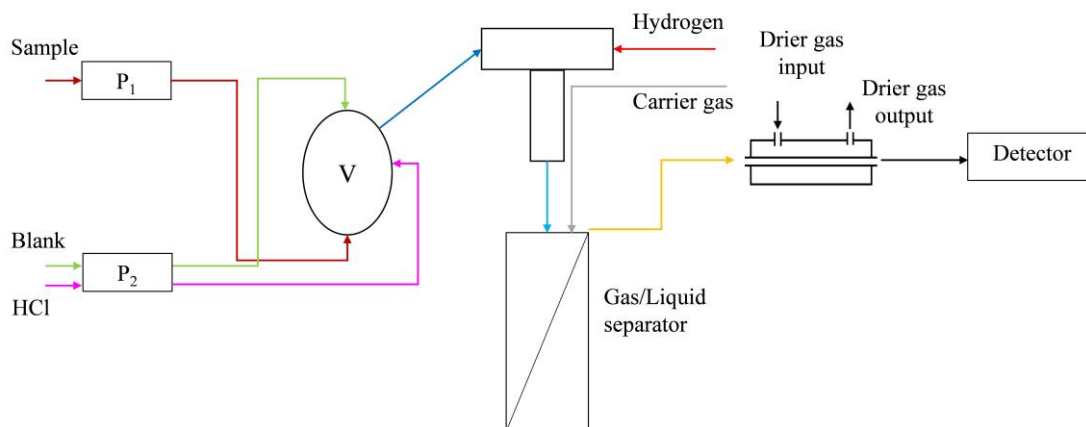


Figure 6. Modified flow injection system for Hydrogen selenide (H_2Se) detection. P_1 and P_2 : Peristaltic pumps; V: Valve system; Gas-liquid separator and drier modules are integrated before signal detection

In the modified configuration (Figure 6):

- Line 1 (dark maroon colored line): Sample solution in $0.1 \text{ mol L}^{-1} \text{ NaOH} + \text{NaBH}_4$
- Line 2 (green colored line): Blank ($0.1 \text{ mol L}^{-1} \text{ NaOH}$) for baseline correction
- Line 3 (magenta colored line): $3 \text{ mol L}^{-1} \text{ HCl}$ to acidify the sample immediately before reduction
- External H_2 line (red colored line): Introduced at the T-joint for controlled hydride formation

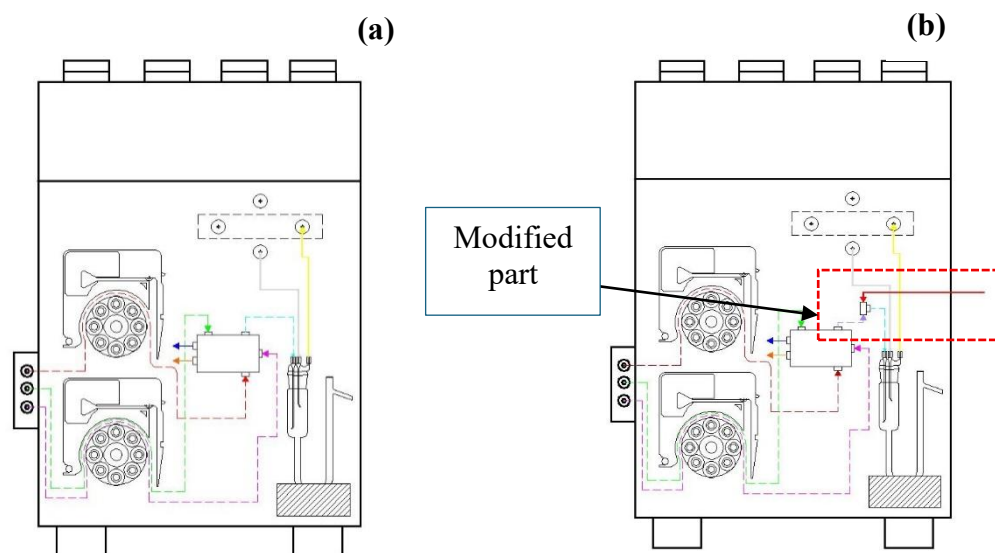


Figure 7. (a) 2D representation of the Atomic Fluorescence Spectroscopy (AFS) Instrument before modification and (b) after modification. Colored pathways indicate separate fluid and gas lines

Immediate acidification followed by H_2 exposure facilitates rapid conversion of $Se(IV)$ to volatile H_2Se while preventing premature decomposition during transit. In the conventional method, bubble coalescence, hydride breakdown, and back-pressure formation typically occur. Relocating the mixing point to a low-volume connector positioned close to the GLS significantly reduced these losses, producing smoother flow profiles, reduced flame flicker, lower baseline noise, and improved transport of freshly formed hydride. Figure 7a shows the 2D view of the AFS equipment before the modification, and Figure 7b represents the 2D view of the equipment after the modifications.

To identify optimal operational parameters, a two-factor full factorial design was applied, evaluating hydrogen flow rate (0.5×10^{-6} and $1.0 \times 10^{-6} \text{ m}^3 \text{ min}^{-1}$) and reagent flow rate (0.5 and 1.0 mL min^{-1}). Only the high-flow combination (Run E2) produced a stable flame, consistent hydride introduction, and reproducible signals. All other combinations resulted in intermittent atomization, insufficient hydride formation, or turbulence-driven loss of precision (Table 1 and Table 2). Overall, this externally assisted hydride-generation strategy addresses the key limitations noted in previous hydride-generation configurations, namely dead-volume-induced turbulence, insufficient reduction in alkaline matrices, and hydride decomposition during transport. The optimized configuration provides a mechanistically robust and reproducible platform tailored for the

trace-level detection of volatile H₂Se. Similar flame optimization strategies have been reported in hydride generation systems for arsenic and antimony detection (Rahman et al., 2000; Wietecha-Posłuszny et al., 2006), though their application to selenium, particularly in volatile hydride form, remains limited (Murillo et al., 2008). The current setup thus provides a tailored solution for trace H₂Se detection while maintaining AFS signal stability and operational simplicity.

Table 1. Instrumental parameters for factorial optimization

Factor name	Low value (-1)	High value (+1)
H ₂ gas flowrate (m ³ min ⁻¹)	0.5 × 10 ⁻⁶	1 × 10 ⁻⁶
Reagents flow rate (mL min ⁻¹)	0.5	1

Table 2. Experimental design matrix for instrumental optimization

Runs	H ₂ gas flowrate (m ³ min ⁻¹)	Reagents flow rate (mL min ⁻¹)	Flame stability
E ₁	-1	-1	Unstable
E ₂	1	1	Stable
E ₃	-1	1	Unstable
E ₄	1	-1	Unstable

4.2.2 Optimization of Reagent Composition

Following instrumental optimisation, the chemical composition of the hydride-generation system was systematically evaluated to maximise H₂Se formation and signal response. Although the modified AFS manifold employs externally supplied hydrogen to stabilise the atomizer flame and assist in-line reduction, the primary formation of H₂Se occurs inside the sealed hydride-generation reactor, where Se(IV) reacts with NaBH₄ under alkaline conditions. The efficiency of H₂Se generation depends critically on the concentration of both the reducing agent (NaBH₄) and the alkali stabilizer (NaOH), which control the reaction kinetics, pH, and stability of the intermediate species (Bax et al., 1986; Elsayed et al., 2000). Table 3 shows the parameters for reagent optimisation and the levels chosen for the optimisation. A two-factor, three-level full factorial design was employed to investigate the effects of NaBH₄ (10%, 40%, 70%) and NaOH (2%, 8%,

14%) on the fluorescence signal intensity (mAU). The experimental matrix and corresponding responses are shown in Table 4. All experiments were conducted under the previously optimized instrumental flow settings. The highest signal intensity (6300.49 mAU) was achieved at 40% NaBH₄ and 8% NaOH, indicating that this combination provides an optimal balance between reductive strength and solution stability. At higher NaBH₄ levels (70%), excessive hydrogen evolution occurred, leading to vigorous foaming and gas–liquid disturbance that impaired consistent hydride transfer to the atomizer (N. Wang & Tyson, 2014). Conversely, at lower NaBH₄ concentrations (10%), reductant availability was insufficient for complete conversion of Se(IV) to volatile H₂Se (Suzuki et al., 2012). The contour and surface plots (Figure 8 and Figure 9) clearly illustrate the strong positive influence of NaBH₄ concentration on signal intensity.

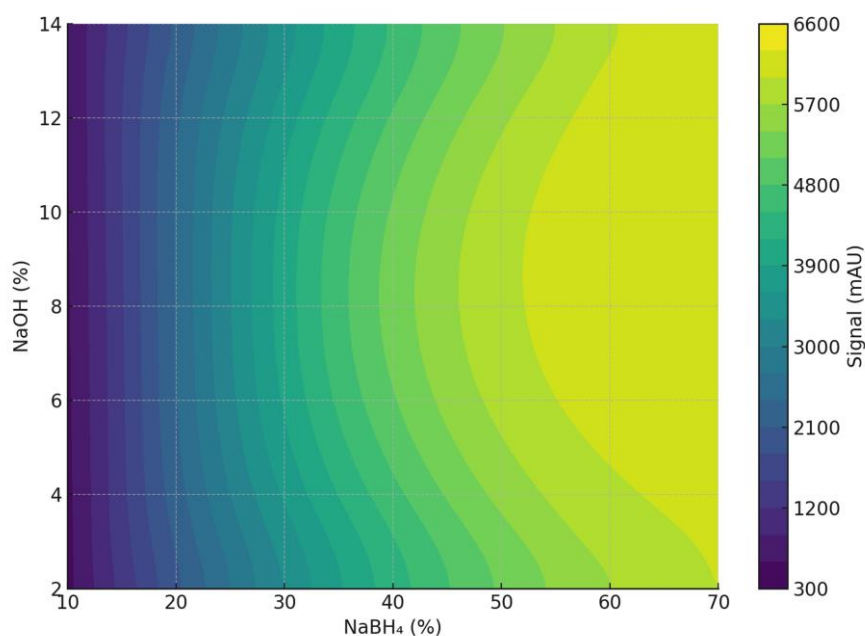


Figure 8. Contour plot showing the influence of Sodium borohydride (NaBH₄) and sodium hydroxide (NaOH) concentrations on signal intensity (mAU)

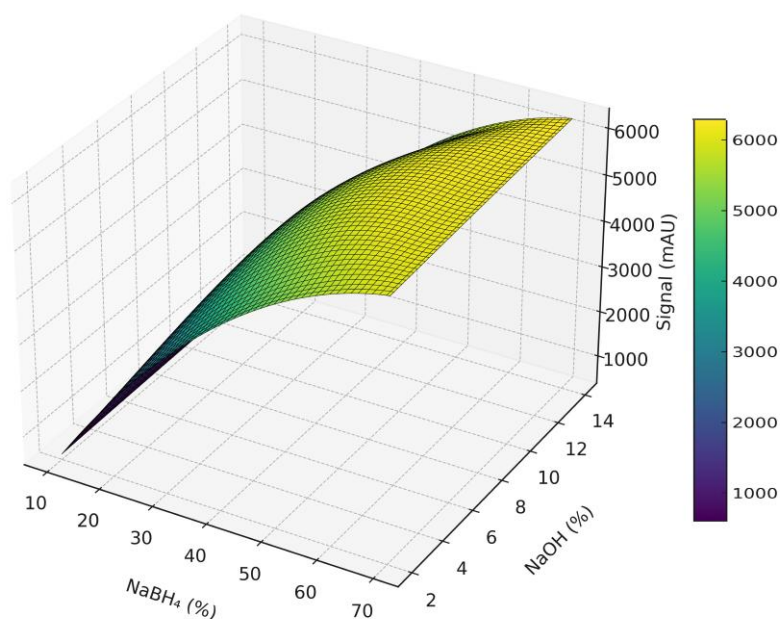


Figure 9. 3D surface plot representing the signal response (mAU) as a function of Sodium borohydride (NaBH₄) and sodium hydroxide (NaOH) concentrations

Statistical analysis using ANOVA revealed that NaBH₄ concentration had a significant influence on signal intensity ($p = 0.013$)(Table 5). In contrast, the effect of NaOH concentration and the interaction term were not statistically significant ($p > 0.05$). Although NaOH had a lesser impact on the magnitude of the signal, its role in stabilising NaBH₄, controlling the reaction pH, and preventing premature decomposition of intermediate hydride species remains critical. The model showed a good fit with an R^2 of 81.8% and a standard error of 1322.21 (Table 6). These results validate the selection of 40% NaBH₄ in 8% NaOH as the optimal reagent matrix for subsequent calibration and sample measurements. These findings are consistent with previous works, that moderate NaBH₄ concentrations enhanced signal strength in hydride generation AFS for arsenic detection, while excessive reductant led to reduced precision (Abdel-Lateef et al., 2013; Shraim et al., 1999). However, few studies have optimized this matrix specifically for H₂Se, underlining the novelty of the present approach in establishing conditions that maximize both signal intensity and stability for this volatile analyte (Kratzer & Dědina, 2007).

Table 3. Parameters for reagent optimization.

Factor name	Low value (-1)	Centre value (0)	High value (+1)
NaBH ₄ (%)	10	40	70
NaOH (%)	2	8	14

Table 4. Reagent experimental design matrix.

Runs	NaBH ₄ (%)	NaOH (%)	Response (signal, mAU)
E ₁	0	0	5220.00
E ₂	1	-1	6000.22
E ₃	0	0	5220.43
E ₄	1	1	6300.49
E ₅	0	0	5210.35
E ₆	-1	-1	500.49
E ₇	0	0	5230.31
E ₈	-1	1	600.43

Table 5. Coded Coefficients

Term	Effect	Coef	SE Coef	T- Value	P- Value	VIF
Constant		4285	467	9.17	0.001	
NaOH%	200	100	661	0.15	0.887	1
NaBH ₄ %	5600	2800	661	4.24	0.013	1
NaOH %*NaBH ₄ %	100	50	661	0.08	0.943	1

Table 6. Model Summary

S	R ²	R ² - (adj)	R ² -(pred)
1322.21	81.79%	68.13%	0.00%

4.2.3 Method Validation

4.2.3.1 Quantitative performance of the optimized method

The quantitative performance of the optimized method was evaluated by constructing a seven-point calibration curve using selenium standards at concentrations of 5, 10, 50, 100, 250, 500, and 1000 $\mu\text{g L}^{-1}$. Fluorescence intensity was plotted against concentration, and the curve showed excellent linearity across the tested range, with a regression coefficient of determination $R^2 = 0.9973$, indicating a strong correlation between signal and analyte concentration (Figure 10). The regression equation was derived as:

$$\text{Signal(mAU)} = 1.599 \times [\text{Se}] - 14.91 \quad (\text{eq. 9})$$

Where $[\text{Se}]$ is the selenium concentration in $\mu\text{g L}^{-1}$. The regression slope was statistically significant ($p < 0.0001$).

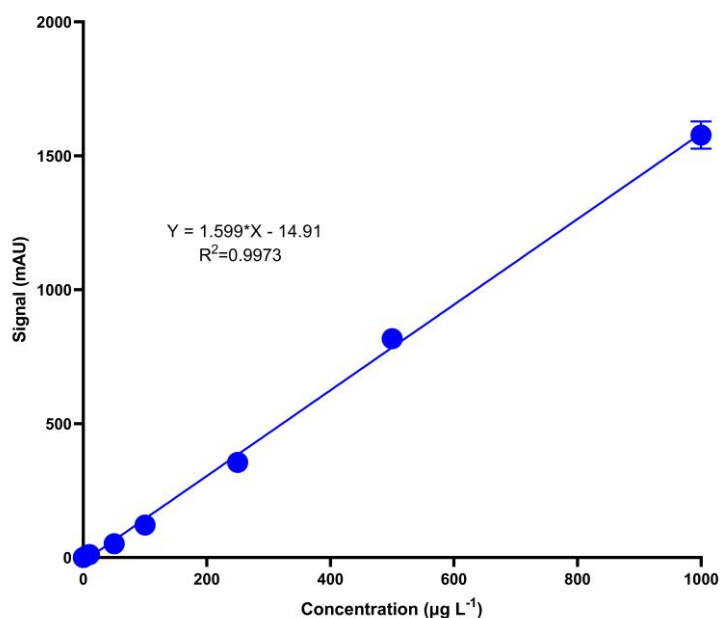


Figure 10. Calibration curve of selenium standards (0–1000 $\mu\text{g L}^{-1}$) measured using the optimized Atomic Fluorescence Spectroscopy (AFS) method

The limit of detection (LOD) and limit of quantification (LOQ) were calculated according to AOAC (AOAC International, 2016) and ICH Q2(R1) (ICH, 1995) guidelines using the standard deviation of the blank (σ) and the slope (S) of the calibration curve:

$$LOD = \frac{3.3\sigma}{S} = 1.4 \mu\text{g L}^{-1}$$

$$LOQ = \frac{10\sigma}{S} = 4.2 \mu\text{g L}^{-1}$$

These values demonstrate that the method is sufficiently sensitive for detecting trace levels of selenium in environmental and aqueous matrices, particularly in the form of volatile H_2Se . Compared to conventional approaches, the modified AFS system exhibits strong analytical performance. Previous studies utilizing hydride generation with atomic absorption spectroscopy (HG-AAS) or ICP-MS reported comparable or slightly lower LODs; however, these methods often require more complex instrumentation, pre-reduction steps, or inert gas shielding to stabilize reactive selenium intermediates (Haygarth et al., 1993; H. Li et al., 2012). The current method achieves comparable sensitivity with a simplified setup and minimal sample handling, offering a more accessible and cost-effective solution for selenium speciation. Repeatability across calibration levels was consistently $<5\%$ RSD, and signal stability was maintained over multiple runs, confirming the robustness of the modified system

4.2.3.2 Recovery and spiking of H_2Se

The accuracy and trapping efficiency of the developed AFS method were evaluated using spike and recovery experiments performed at two concentration levels (5 and 100 $\mu\text{g L}^{-1}$) across three aqueous matrices: ultrapure water, tap water, and commercial mineral water. All samples were prepared in the optimized NaOH- NaBH_4 matrix; the spiking experiments also evaluated the stability of hydride formation. In each case, Se(IV) standards were reduced with 1.4% NaBH_4 in the presence of 3 mol L^{-1} HCl under the optimized hydride-generation conditions, generating volatile H_2Se . The evolved gas was carried by argon into 0.1 mol L^{-1} NaOH, prepared using the corresponding matrix. The trapped selenium species were quantified using the modified AFS system. Figure 11 shows the schematic representation of the spiking experiment. Table 7 shows the recovery efficiency of the AFS method with various aqueous matrices with different spiked concentrations. Across all matrices and spike levels, recoveries ranged from 97.31% to 101.77%, with RSD values below 3.3% (Table 7).

Recoveries slightly exceeding 100% may be attributed to localized pH enhancement effects, improved bubble stabilization in the GLS, or minor spectral overlap, phenomena previously observed in hydride-based systems (Greda et al., 2015; Lampugnani et al., 2003). The consistency of recoveries across the tap and mineral water indicates that dissolved ions typically present in drinking water (e.g., Ca^{2+} , Mg^{2+} , HCO_3^- , Cl^-) do not significantly influence hydride formation or H_2Se capture. These results align with previous findings that variable recovery of volatile selenium species depends on reductant

concentration, reaction time, and carrier gas flow (Chatterjee et al., 2001; Suzuki et al., 2012). Importantly, no statistically significant differences were observed between matrices or spike levels (Tukey's HSD, $p > 0.05$), demonstrating the robustness of the method in diverse aqueous conditions. These findings position the developed AFS approach as a reliable tool for estimating the selenium hydride in metabolic pathways or other model studies that are looking to estimate the selenium speciation. Comparable recoveries (94–102%) have been reported for selenium in drinking water using hydride-generation ICP-MS, although those workflows required extensive pretreatment and inert gas shielding (Robberecht & Van Grieken, 1982).

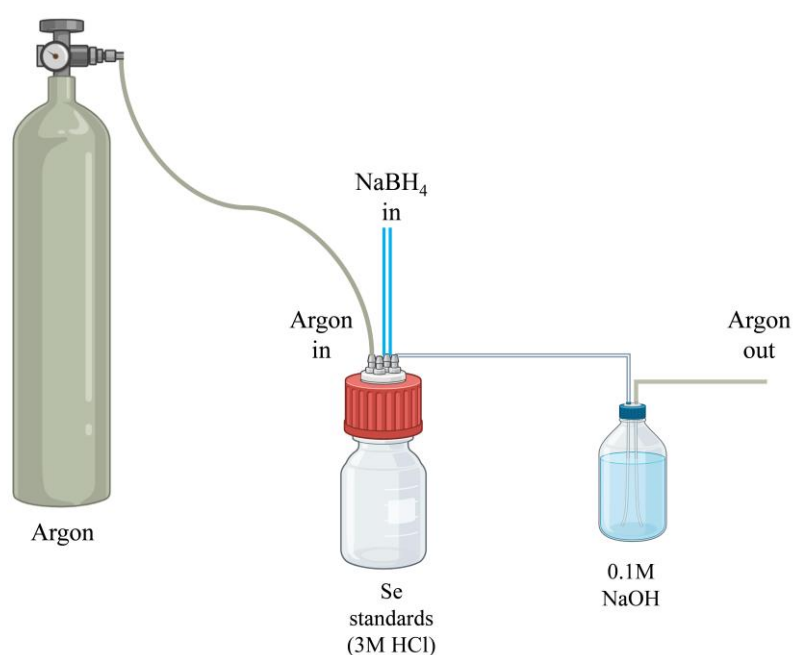


Figure 11. Schematic representation of the spiking experiment

Table 7. Recovery efficiency and precision of Hydrogen Selenide (H₂Se) detection in real samples

Sample	Spiked concentration ($\mu\text{g L}^{-1}$)	Recovery (%)	RSD (%)
Ultra-pure	100	101.77 $\pm$ 0.9 ^a	0.82
water	5	99.76 $\pm$ 1.6 ^a	2.48
Tap water	100	99.77 $\pm$ 1.70 ^a	1.71
	5	97.31 $\pm$ 2.10 ^a	2.15
Mineral	100	100.43 $\pm$ 0.91 ^a	0.90
water	5	101.01 $\pm$ 2.81 ^a	3.26

Data are presented as mean $\pm$ SD (n = 3). Superscript letters indicate statistically nonsignificant differences between groups based on Tukey's HSD test (p > 0.05).

4.2.3.3 Cross-validation Using GF-AAS

GF-AAS was used to confirm the total amount of selenium recovered in the NaOH trap after hydride generation, as GF-AAS is a well-established reference technique for trace elemental analysis. Trapped H₂Se is present as HSe⁻/Se²⁻, all solutions were oxidized to Se(IV) prior to GF-AAS detection. This oxidation step converts all trapped hydride species into the same oxidation state, ensuring matrix-matched quantification during furnace atomization. GF-AAS to serve as a validation tool, rather than a direct detector of volatile hydrides. As shown in Table 8, recovery values of 60.2 $\pm$ 0.34% for 5 $\mu\text{g L}^{-1}$ and 100.9 $\pm$ 0.30% for 100 $\mu\text{g L}^{-1}$ were obtained, with RSDs of 0.24% and 3.26%, respectively. The quantitative recovery at 100 $\mu\text{g L}^{-1}$ agrees closely with the AFS results, confirming the efficiency of the purge-and-trap process at moderate concentrations. In contrast, the lower recovery observed at 5 $\mu\text{g L}^{-1}$ reflects the known limitations of GF-AAS for selenium, including reduced atomization efficiency at trace levels (Kratzer & Dědina, 2007), higher susceptibility to background noise, and incomplete volatilization during the furnace drying/pyrolysis stages. Similar concentration-dependent losses have been documented in hydride-related selenium studies, particularly when operating near the method's sensitivity threshold (Ding & Sturgeon, 1996; Kratzer & Dědina, 2007). Tukey's HSD test indicated significant differences between spike levels (p < 0.001), with 100 $\mu\text{g L}^{-1}$ assigned to Group A and 5 $\mu\text{g L}^{-1}$ to Group B, demonstrating that GF-AAS performance declines at ultra-trace concentrations. In contrast, the modified AFS method provided consistent recoveries across all spike levels and matrices, confirming its superior

sensitivity and suitability for volatile selenium determination. Unlike GF-AAS, which detects selenium only after oxidation and high-temperature atomization, AFS responds directly to hydride-derived species, enabling improved detection limits, reduced sample handling, and greater analytical stability. These advantages position the modified AFS workflow as a more reliable and operationally efficient platform for trace-level H₂Se analysis, particularly in aqueous matrices where hydride behaviour plays a critical role (Heilier et al., 2005).

Table 8. Recovery efficiency and precision of selenium detection using Graphite Furnace Atomic Absorption Spectroscopy (GF-AAS)

Sample (µg L ⁻¹)	Recovery (%)	RSD (%)
5	60.2±0.34 ^b	0.24
100	100.9±0.30 ^a	3.26

Data are presented as mean ± SD (n = 3). Superscript letters indicate statistically significant differences between groups based on Tukey's HSD test (p < 0.001).

4.2.3.4 Comparison with existing H₂Se Detection methods

The developed AFS method achieves comparable or superior accuracy with a simplified and more cost-effective workflow, enabling broader adoption in routine monitoring applications. Table 9 compares this study with the previously reported methods, along with the LOD and their applications.

Table 9. Comparison of reported analytical methods for hydrogen selenide (H₂Se) detection, including detection limits, linear ranges, and application matrices.

Method	LOD ($\mu\text{g L}^{-1}$)	Linear range ($\mu\text{g L}^{-1}$)	Sample matrix used	Ref.
ESIPT-based ratio-metric fluorescent probe	16.2	0-7288	-	(Xin et al., 2020)
Fluorescent probe (Hcy- H ₂ Se)	55.1	0-8098	Cell imaging (HepG2 cells)	(Kong et al., 2017)
Near-infrared fluorescent	0.57	0-972	Cell imaging (HepG2 cells)	(Kong et al., 2016)
Activatable fluorescent probe	92.7	0-1215	Cell imaging (HepG2 cells)	(Tian et al., 2019)
Modified AFS	1.40	0-1000	water	This study

Methods based on fluorescent probes, such as ESIPT-based ratio metric sensors (Xin et al., 2020), Hcy-H₂Se conjugates (Kong et al., 2017), near-infrared fluorophores (Kong et al., 2016) and activatable probes (Tian et al., 2019) are primarily applied in biological contexts, especially for cell imaging (e.g., HepG2 cells), and offer high specificity but are generally not optimized for quantitative environmental analysis. In contrast, the modified atomic fluorescence spectroscopy (AFS) method developed in this study achieves a low limit of detection ($1.40 \mu\text{g L}^{-1}$) within a broad linear range ($0\text{--}1000 \mu\text{g L}^{-1}$) for aqueous samples, making it suitable for trace-level quantification in environmental and water quality applications. Overall, the method's compatibility with real sample matrices highlights its potential for ecological surveillance, water quality assessment, and regulatory compliance monitoring of volatile selenium species, particularly H₂Se.

While the modified HG-AFS method demonstrates good analytical performance under controlled conditions, its application to complex biological matrices may be influenced by matrix effects (Ueta et al., 2020). Components such as sulphur-containing species, proteins, and organic matter can interfere with hydride generation efficiency, potentially affecting sensitivity and selectivity (Sanchez-Rodas et al., 2013). Therefore, appropriate sample preparation strategies, including digestion, separation, or the use of masking agents, are necessary for reliable application in real biological systems (Ferreira et al.,

2022). Future work will focus on validating the method in complex matrices to enhance its practical applicability.

Although the developed AFS method provides robust quantification of volatile selenium species in aqueous systems, it is not directly applicable to solid-phase or particulate elemental selenium (Se^0), which is increasingly relevant in nano-enabled food, feed, and environmental applications. To address this analytical gap, carbon nanodot-based fluorescence sensing was explored as a complementary strategy for Se^0 detection.

4.3 Carbon Nanodots (CNDs) for Elemental Selenium (Se^0) Detection

4.3.1 Structural and Optical Characterisation of Synthesised CNDs

CNDs were prepared using the sample preparation protocol outlined in Section 3.3, and characterised by transmission electron microscopy (TEM), Fourier-transform infrared spectroscopy (FTIR), fluorescent spectroscopy, and Raman spectroscopy (Figure 12). TEM analysis showed that the CNDs are well-dispersed, spherical nanoparticles with a uniform morphology (Figure 12a). The particles lacked crystallinity, indicating their amorphous carbonaceous nature. The average particle diameter was calculated to be 3.90 ± 1.36 nm, which is consistent with previous reports (Chauhan & Chaudhary, 2021; Lamba et al., 2024; Pandey et al., 2020). Figure 12b shows the particle size distribution histogram, which confirms that most synthesised CNDs lie within the 2–5 nm range, with a sharp peak at around 3.5 nm. This narrow size distribution helps to ensure the CNDs have uniform optical properties and are readily dispersible in aqueous solutions. The photoluminescent behaviour of the CNDs was assessed using fluorescence spectroscopy. Three-dimensional spectra of CNDs (Figure 12c) show a characteristic excitation-dependent fluorescence pattern, with the highest intensity seen at an excitation wavelength of 360 nm and an emission maximum at around 430 nm. This attribute is due to multiple emissive traps or surface states introduced during the carbonisation process and surface functionalisation (D. Li et al., 2018, 2019; Wei et al., 2020). The two-dimensional spectra of the CNDs (Figure 12d) exhibit a clear red shift in the emission maximum as the excitation wavelength increases (from 320 to 440 nm), indicating a typical excitation-dependent emission profile. Such behaviour is often linked to heterogeneous energy states resulting from varying particle sizes, surface defects, or functional groups (Y.-P. Sun et al., 2006). The excitation-dependent fluorescence makes these CNDs ideal candidates for sensing applications, where tuneable photoluminescence is beneficial (Z. Guo et al., 2023).

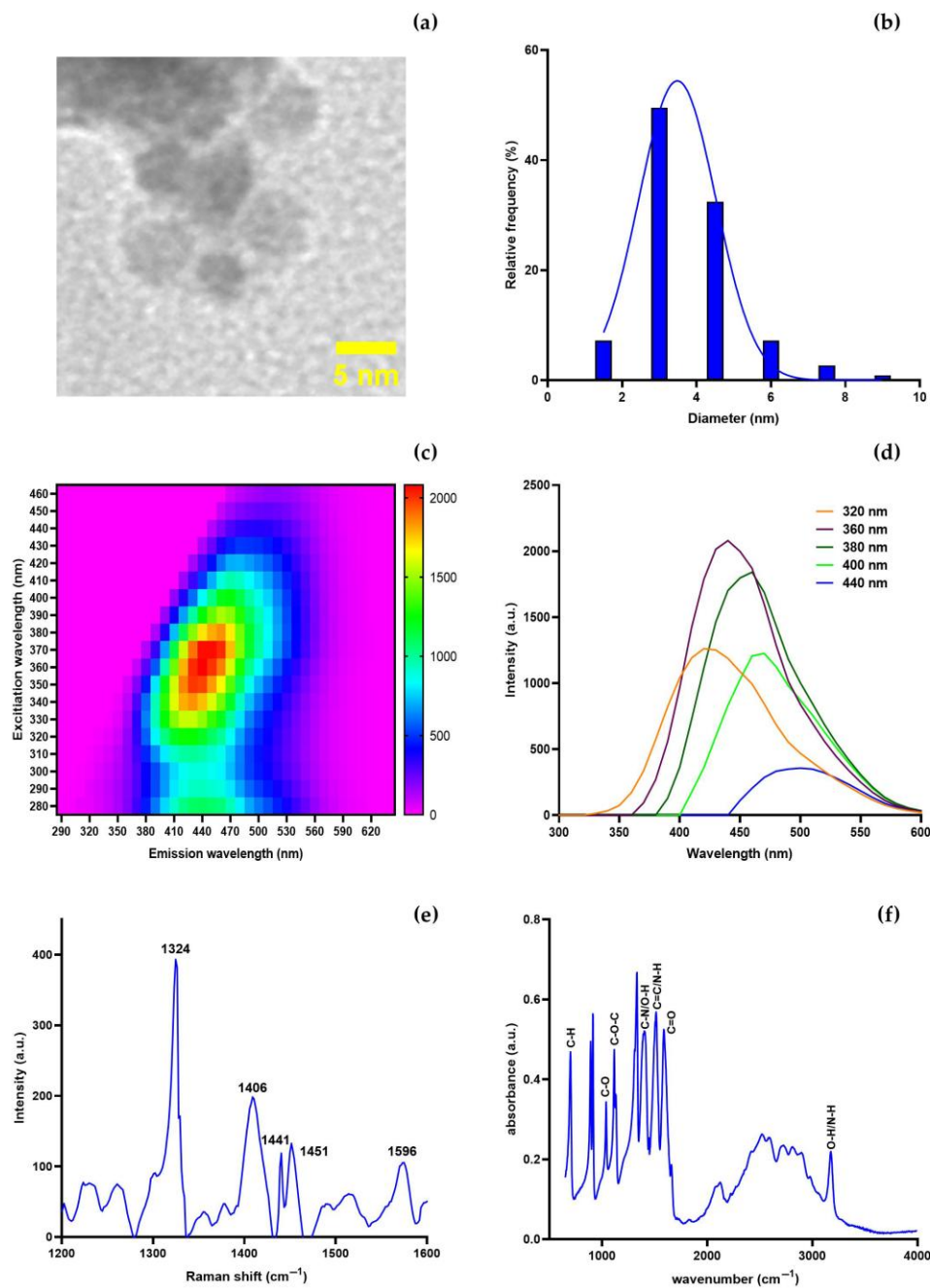


Figure 12. Characterisation of carbon nanodots (CNDs). (a) A transmission electron microscopy (TEM) image of CNDs at a scale of 5 nm. (b) A histogram with an average size of 3.90 ± 1.36 nm depicts the particle-size distribution. (c) CNDs' three-dimensional fluorescence spectra. (d) CNDs two-dimensional emission–excitation–intensity fluorescence spectrum. (e) CNDs Raman spectra. (f) FTIR spectra of CNDs.

Raman spectroscopy was conducted to investigate the carbon structure of the synthesised CNDs. As shown in Figure 12e, The following three significant peaks were observed: the D band at 1324 cm^{-1} , the F band at 1406 cm^{-1} , and the G band at 1596 cm^{-1} . The D band

corresponds to the breathing mode of sp^3 -hybridised carbon atoms and is indicative of structural defects or disorders in the graphitic framework. The bands at 1441 cm^{-1} and 1451 cm^{-1} are attributed to the vibrational modes of surface-bound functional groups, such as C–H bending ($-\text{CH}_2$, $-\text{CH}_3$) and C–N/C–O bonds, introduced during the Maillard reaction (Q. Wang et al., 2013). Usually, D bands situated at the $1330\text{--}1350\text{ cm}^{-1}$ range originate from defects and disorders within the carbon lattice and double resonant processes around the K point of the Brillouin Zone (BZ) boundary (Reich & Thomsen, 2004). The G band arises from the E_{2g} phonon of sp^2 -hybridised carbon atoms, reflecting the presence of graphitic domains (Ferrari & Robertson, 2000; Vidano & Fischbach, 1978). The intermediate F band, centred around 1406 cm^{-1} , is predominantly in the samples with high-intensity D bands. It has been attributed to additional structural disorder and surface functionalities such as amide or pyrrolic groups, likely introduced during the glycine-assisted synthesis (Graupner, 2007; Sanjuán et al., 2004). The intensity ratio of the D to G bands (I_D/I_G) suggests that the CNDs possess a defect-rich, amorphous carbon structure conducive to surface reactivity and photoluminescence.

Fourier-transform infrared (FTIR) spectroscopy was used to identify the functional groups present on the CND surfaces (Figure 12f). A broad absorption band centred at $\sim 3400\text{ cm}^{-1}$ corresponds to the O–H and N–H stretching vibrations, indicating the presence of hydroxyl and amine groups. Peaks at 2920 and 2850 cm^{-1} are attributed to asymmetric and symmetric C–H stretching vibrations from aliphatic chains. A distinct absorption peak at $\sim 1720\text{ cm}^{-1}$ is indicative of C=O stretching from carboxylic acid or amide groups. The C=C stretching or N–H bending vibrations are observed near $\sim 1600\text{ cm}^{-1}$, reflecting conjugated aromatic structures or peptide bonds. Peaks at $1400\text{--}1250\text{ cm}^{-1}$ correspond to C–N and C–O–C stretching, suggesting the presence of amines and ether linkages, while the bands below 1100 cm^{-1} are attributed to C–H bending modes of aromatic frameworks (Debes et al., 2024; Ji et al., 2019). These findings confirm that the CNDs are functionalized with a variety of hydrophilic and chemically reactive moieties, such as hydroxyl, carboxyl, amine, and ether groups, which improve aqueous dispersibility and facilitate interactions with target analytes such as Se^0 (X. Zhang et al., 2020).

4.3.2 Fluorescence stability of CNDs

Utilising the fluorescence of carbon nanodots (CNDs), making a nano-sensor detect Se^0 through CNDs is possible. The suggested sensing system comprises CNDs at an appropriate concentration (0.100 mmol L^{-1}) in an aqueous solution, where the CNDs

function as fluorometric reporters. Additionally, the fluorescence stability of CNDs is evaluated in terms of pH, salt, and time. To assess the ionic strength stability of the synthesised carbon nanodots (CNDs), their fluorescence behaviour was evaluated in aqueous solutions containing various concentrations of NaCl, ranging from 0.1 to 1.0 mol L⁻¹ (Figure 13a). Increasing the NaCl concentration resulted in a gradual decrease in fluorescence intensity at the maximum emission wavelength (~430 nm), suggesting that higher ionic strength leads to partial quenching of the CNDs emissions. Figure 13b shows a clearer picture of this trend, with statistically significant differences seen across the tested concentrations. At lower salt concentrations (0.1–0.3 mol L⁻¹), the fluorescence intensity stayed relatively stable. However, above 0.4 mol L⁻¹, a gradual decrease in emission intensity was observed, with fluorescence significantly reduced at concentrations of 0.6 mol L⁻¹. At 1.0 mol L⁻¹, the intensity dropped by over 30% compared to the control (0.1 mol L⁻¹), suggesting salt-induced quenching and possible aggregation or shielding of emissive sites (Dua et al., 2023; Ehtesabi et al., 2020).

The decrease in fluorescence intensity results from electrostatic screening and surface charge effects. At low ionic strengths, strong electrostatic repulsion keeps CNDs dispersed, maintaining their optical properties (Zeng et al., 2017). As ionic strength rises, the electrical double layer around the CNDs compresses, lowering zeta potential and causing colloidal instability and partial aggregation (L. Wang et al., 2017). This aggregation reduces the efficiency of radiative recombination because particles are closer together and may enable Förster resonance energy transfer (FRET) (J. Zhang et al., 2007). Moreover, Na⁺ ions shield negatively charged surface groups (–COOH, –OH), disrupting surface passivation and changing the emissive states. These factors lead to fluorescence quenching and demonstrate that CND's behaviour depends on ionic strength (Zeng et al., 2017). These findings indicate that the CNDs retain their photostability under mild ionic conditions, but excessive ionic strength may mask surface charges or interfere with surface passivation, resulting in fluorescence quenching. The results show that the CNDs have moderate ionic tolerance, making them suitable for sensing applications in biologically and environmentally relevant matrices that typically have salt contents below 0.5 mol L⁻¹ (Michenzi et al., 2024; Shen et al., 2021).

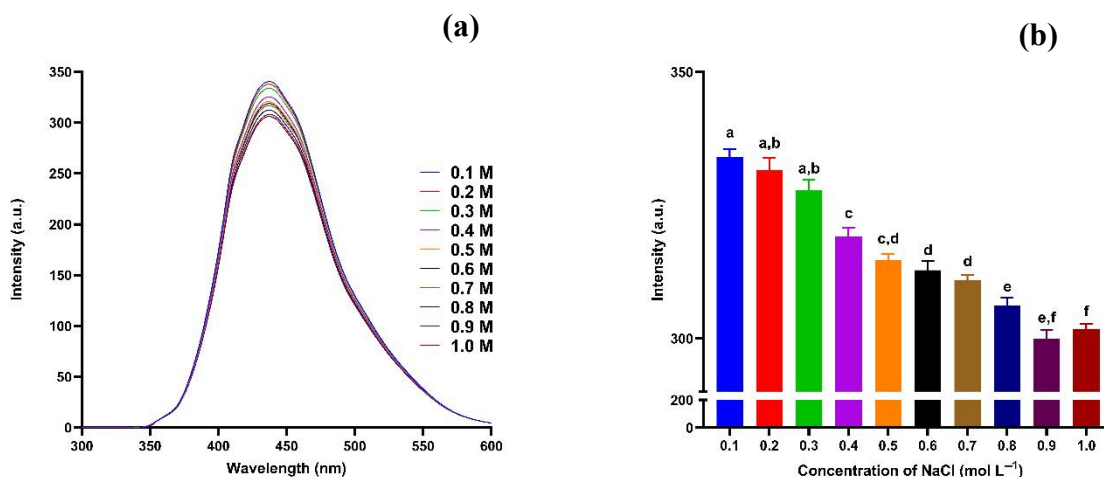


Figure 13. Stability of CNDs at NaCl 0.1–1.0 mol L⁻¹. **(a)** CNDs with varying concentrations of NaCl at a 360 nm excitation wavelength and an emission wavelength range of 300–700 nm. **(b)** CNDs with different concentrations of NaCl at a 360 nm excitation wavelength and a 430 nm emission wavelength. Values are represented as mean $\pm$ standard deviation, where $n = 3$, and different alphabetical letters show the level of significance, $p < 0.001$.

Further evaluation of the fluorescence stability of the CNDs was carried out over 60 min to assess their photostability in ambient aqueous conditions. The emission spectra recorded at different time intervals (Figure 14a) showed no significant shift in peak emission wavelength (~ 430 nm). Still, a gradual increase in fluorescence intensity was observed as the incubation time progressed. In Figure 14b, the fluorescence intensity increased steadily from 0 min to 45 min, with statistical analysis confirming notable differences among the various time points. At the initial time point (0 min), the lowest intensity was observed. The signal increased significantly within the first 30 min ($p < 0.001$), possibly due to improved surface hydration or the stabilisation of emissive traps on the CNDs surface (Dua et al., 2023). The observed increase in emission intensity may be attributed to environmental stabilisation or the structural relaxation of surface functional groups, both of which enhance radiative recombination. After 45 min the CNDs maintained high fluorescence intensity with no significant decline, indicating robust temporal stability under constant excitation (Y. Guo et al., 2016; Setianto et al., 2024). These findings support the high photostability and dispersion stability of the CNDs in aqueous media, a desirable feature for real-time sensing or long-term imaging applications. This result aligns with previous reports on CNDs exhibiting stable emission

behaviour under continuous observation and prolonged exposure in aqueous matrices (C. Li et al., 2023).

To check their environmental adaptability and sensing potential, the fluorescence properties of CNDs over a wide range of pH values, from 3 to 11, were evaluated. As shown in Figure 15a, the CNDs emitted strong fluorescence across all pH values, with the emission peak remaining consistent at around 430 nm, indicating that the emissive centres are structurally stable. However, significant differences in fluorescence intensity were observed (Figure 15b). The intensity was highest at pH 11, followed by pH 3, suggesting that the electronic environment or surface state is optimised under both acidic and basic conditions.

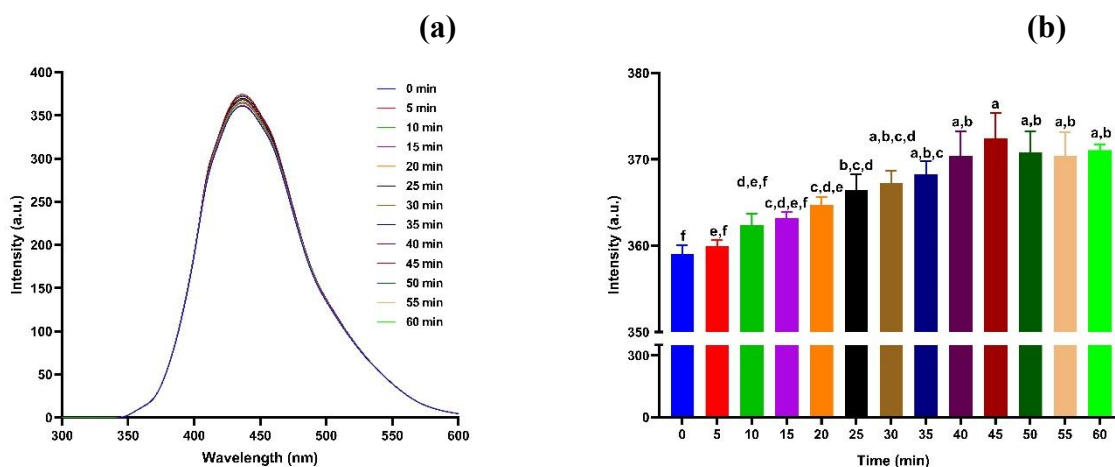


Figure 14. Stability of CNDs across different periods (0–60 min). (a) Stability of CNDs at different times (0–60 min) at 360 nm excitation wavelength and 300–700 nm emission wavelength. (b) Stability of CNDs at different times (0–60 min) at 360 nm excitation wavelength and 430 nm emission wavelength. All values are represented as mean $\pm$ standard deviation, where $n = 3$, and different alphabetical letters show level of significance, $p < 0.001$.

At neutral pH (7) the lowest fluorescence was recorded, indicating a substantial quenching effect. The initial increase in fluorescence at pH 3–4 may be due to the protonation of surface amine or hydroxyl groups, which reduces non-radiative pathways. Conversely, the decline around pH 6–7 suggests increased competition from protonated/deprotonated species, which may disrupt surface passivation or enhance dynamic quenching (Y.-P. Sun et al., 2006). The recovery of fluorescence at pH 10–11 could be due to deprotonation-induced electron delocalisation or aggregation-induced

emission effects in alkaline conditions (Fan et al., 2020). Overall, the results show that the CNDs exhibit pH-sensitive fluorescence with tuneable intensity but retain spectral stability, indicating suitability for biosensing or environmental applications where pH varies (Szapoczka et al., 2023). These findings are consistent with previous studies reporting that the photoluminescence of CNDs is modulated by the protonation–deprotonation equilibria of surface functional groups such as $-\text{COOH}$, $-\text{NH}_2$, and $-\text{OH}$ (Khan et al., 2024; Tammina & Rhim, 2023).

Based on the findings, the CNDs produced in this study demonstrated remarkable fluorescence stability, which is essential for ensuring accurate and reliable outcomes in Se^0 detection in feed, food, and beverage industries (Liang et al., 2021).

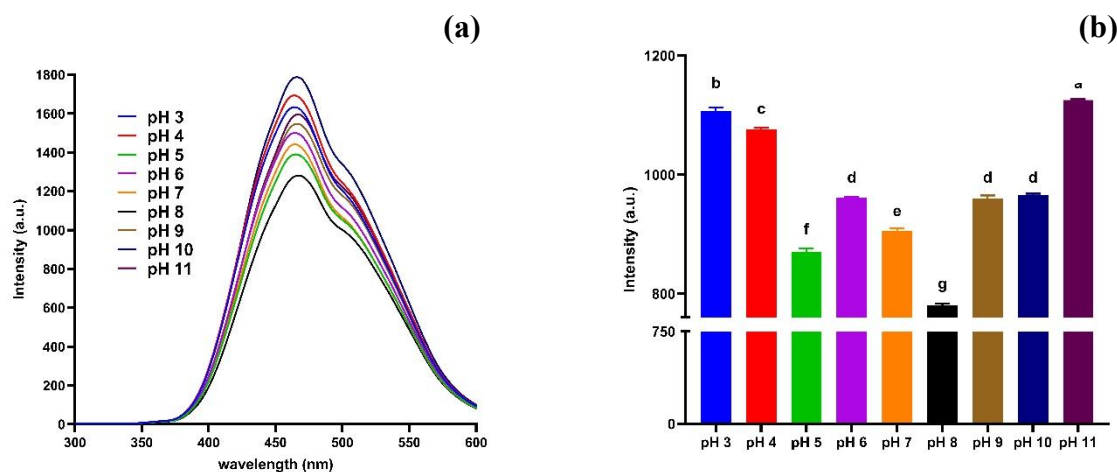


Figure 15. Stability of CND at different pH levels (3–11). (a) CNDs with different pH values (3–11) at 360 nm excitation wavelength and 300–700 nm emission wavelength. (b) CNDs with different pH values (3–11) at 360 nm excitation wavelength and 430 nm emission wavelength. Values are represented as mean $\pm$ standard deviation, where $n = 3$, and different alphabetical letters show level of significance $p < 0.001$.

4.3.3 Calibration, Sensitivity, and Selectivity towards Se^0

Standard curves were made by measuring the intensity of the fluorescence spectrum at a 360 nm excitation wavelength for various Se^0 concentrations. On the day of the measurement, a new standard curve was created. Over a ten-point calibration range that includes concentrations ranging from 0 to $12.665 \text{ mmol L}^{-1}$, the linear calibration line for Se^0 was established (Figure 16). The measurement has a repeatability of less than 0.1%. The method's determined detection limit was $0.131 \text{ mmol L}^{-1}$. The European Medicines Agency and AOAC International validation processes assessed this approach's precision,

reproducibility, daily variation, and linearity (AOAC International, 2016; ICH, 1995). The regression line, $Y = -1332 \cdot X + 18,179$, created the standard curve, producing an R^2 score of 0.9911. According to the findings the limit of detection (LOD) was $0.131 \text{ mmol L}^{-1}$ and the limit of quantitation (LOQ) was $0.397 \text{ mmol L}^{-1}$. The selectivity of the CNDs was evaluated by exposing them to a range of common metal ions, each at a concentration of 100 mg L^{-1} (Figure 17). The fluorescence response was expressed as the ratio I/I_0 (where I denotes the fluorescence intensity of the CNDs with the analyte and I_0 is the intensity in the absence of the analyte), which was recorded for Ca^{2+} , Fe^{2+} , K^+ , Mg^{2+} , Mn^{2+} , Ni^{2+} , Zn^{2+} , and Na^+ and compared against Se^0 . Among all the tested species, Se^0 had a significant decrease in fluorescence intensity, resulting in the lowest I/I_0 ratio (~ 0.34), indicating strong quenching and the high selectivity of CNDs for Se^0 . This response suggests a particular interaction between Se^0 and the surface functional groups of the CNDs, possibly involving charge-transfer or redox-based quenching mechanisms. In contrast, most metal ions exhibited negligible or even enhancing effects on fluorescence intensity (Devi et al., 2017). Notably, K^+ , Ni^{2+} , and Na^+ produced a statistically significant increase in I/I_0 , implying that these ions may stabilise the excited state of CNDs through electrostatic interactions (Dutta et al., 2021). Metal ions such as Ca^{2+} , Fe^{2+} , Mg^{2+} , and Zn^{2+} caused minor changes; these findings indicate that the CNDs fluorescence remains stable in the presence of physiologically or environmentally relevant concentrations of common metal ions, a desirable trait for selective sensing. These results suggest CNDs' high specificity towards Se^0 (XU et al., 2022).

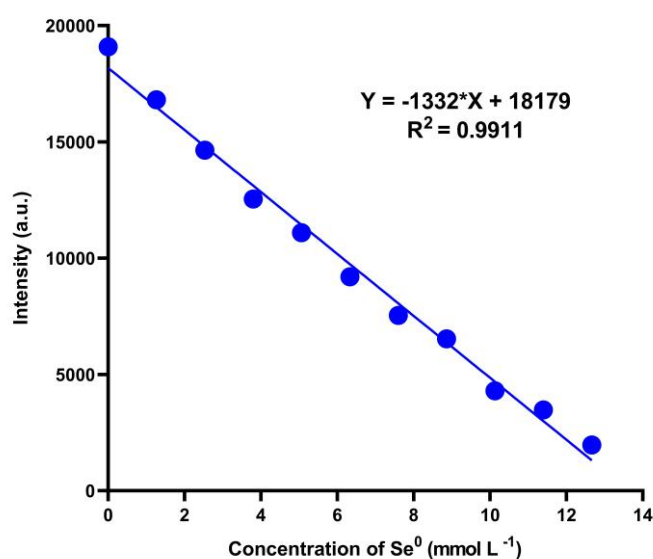


Figure 16. The standard linear calibration curve of Se^0 from 0 to $12.665 \text{ mmol L}^{-1}$, where $p < 0.0001$.

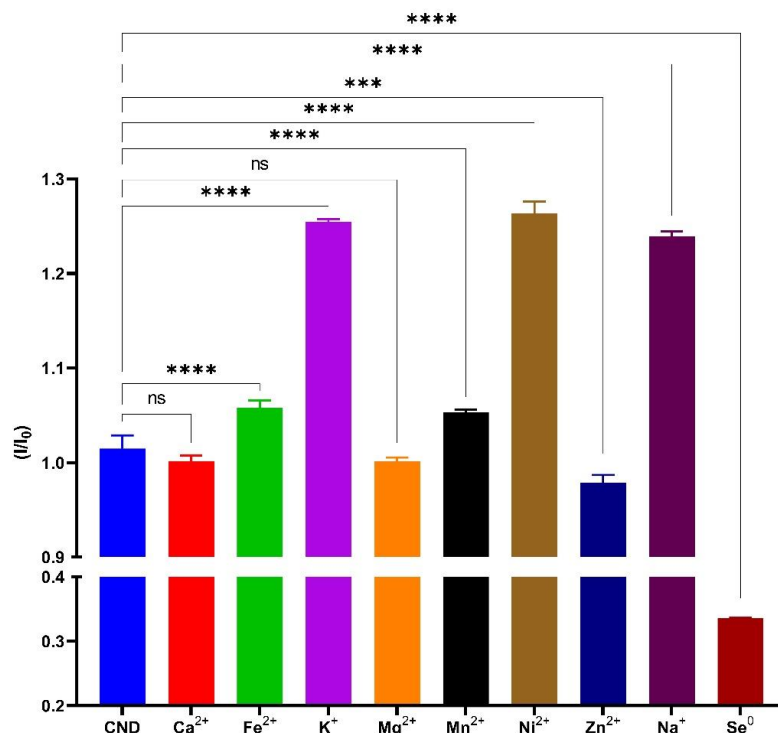


Figure 17. Selectivity of CNDs. Measures of 100 mg L⁻¹ of Ca²⁺, Fe²⁺, K⁺, Mg²⁺, Mn²⁺, Ni²⁺, Zn²⁺, and Na⁺, compared Se⁰. Data are expressed as mean ± SD (n = 3). Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test: **** = $p < 0.0001$ which indicates the level of significance; ns = not significant.

4.3.4 Application to Real Water and Beverage Matrices

To assess the practical applicability of the developed CND-based fluorescent sensor, spiking and recovery experiments were conducted using ultra-pure water, tap water, and a commercial soft drink matrix. Se⁰ was added at two concentrations (316.5 μmol L⁻¹ and 633.3 μmol L⁻¹), and the corresponding recovery percentages and relative standard deviations (RSDs) were recorded. As shown in Table 10. The CNDs have demonstrated excellent recovery, ranging from 98.60% to 108.1%, with low RSD values (0.2–3.4%) indicating high precision and reproducibility. Specifically, ultra-pure and tap water samples showed recoveries above 99%, while recoveries in soft drink matrices remained consistent with acceptable RSDs below 1.1%. The statistical groupings (a, b) suggest no significant deviation, which shows CND's stability across different sample types. The sample was represented as the mean of three replicates. A comparison with existing Se⁰ detection techniques is presented in Table 11. Comparison of previously available methods. Conventional methods such as hydride-generation AFS provide excellent

sensitivity (LOD-0.00152 $\mu\text{mol L}^{-1}$ for biological samples) (H. Sun et al., 2005) and colourimetric assays using AuNPs reach an LOD of 0.05 $\mu\text{mol L}^{-1}$ (Cao et al., 2017). Electrochemical-based sensors exhibit an LOD of 0.00152 $\mu\text{mol L}^{-1}$ and a limited range of 0.1267-0.6332 $\mu\text{mol L}^{-1}$. Our Maillard-synthesised CNDs probe achieves a lower LOD (0.131 mmol L^{-1}), a broader dynamic range, green synthesis, and robustness in beverage matrices. It is important to note that the current method targets elemental selenium (Se^0), which is not regulated in the same way as inorganic selenium species. The WHO guideline value for total selenium in drinking water is 0.127 $\mu\text{mol L}^{-1}$ (0.01 mg L^{-1}), applying to selenate and selenite (Vinceti et al., 2013). However, Se^0 concentrations in fortified or processed samples, such as Se^0 -enriched beverages or feed formulations, can be significantly higher. The linear detection range of 0 to 12.665 mmol L^{-1} used in this study accounts for such scenarios, demonstrating the method's reliability. With proper dilution or preconcentration, this platform can be adapted for detecting lower concentrations.

Table 10. The concentration of Se^0 in real samples.

Sample	Spiked Concentration of Se^0 ($\mu\text{mol L}^{-1}$)	Recovery (%)	RSD (%)
S1 Ultra-pure H_2O	316.5	103.8 ± 3.5 ^{a,b}	3.4
S2 Ultra-pure H_2O	633.3	98.60 ± 0.7 ^b	0.7
S3 Tap H_2O	316.5	102.9 ± 0.2 ^{a,b}	0.2
S4 Tap H_2O	633.3	108.1 ± 0.4 ^a	0.3
S5 soft drink	316.5	99.40 ± 0.1 ^b	1.1
S6 soft drink	633.3	100.7 ± 0.6 ^b	0.6

*Values represented as mean $\pm$ standard deviation (n=3). Where a represents $p < 0.001$. Superscripts a and b represent samples that show a statistically significant difference between the samples.

Table 11. Comparison of previously available methods.

Method	LOD ($\mu\text{mol L}^{-1}$)	Linear Range ($\mu\text{mol L}^{-1}$)	Green Synthesis	Sample Matrix	Ref.
Hydride generation (HG-AFS)	0.00152	>0.4052	No	Biological samples	(H. Sun et al., 2005)
Hydride generation with AuNPs and absorption with AgNPs	0.05	0.4–4.0	No	Biological and environmental sample	(Cao et al., 2017)
Electrochemical sensor with AuNPs	0.00152	0.1267– 0.6334	No	Seawater samples	(Segura et al., 2015)
Fluorescence quenching with CNDs	131	0–12,665	yes	Water, tap water, and soft drinks	This Study

4.3.5 Fluorescence Quenching Mechanism and Analytical Implications

In a hypothetical scenario of static quenching, if a non-fluorescent ground state complex developed between the fluorophore and quencher then the fluorescence lifetime ($\tau_0/\tau = 1$) would not change for static quenching (where τ_0 and τ denote the lifetime without and with the quencher, respectively), which is shown in Figure 18 (Y.-P. Sun et al., 2006; Wei et al., 2020). Accordingly, a dynamic quenching process is suggested by any change in the lifetime (Arimori et al., 2001). According to Mahajan et al. (Mahajan et al., 2015), the decrease in lifespan suggests that quenching is dynamic and that CNDs and Se^0 spontaneously interact through a radiative emission pathway in excited states. An excited state interaction with the excited nanoparticles is predicted when Se^0 molecules are close to surface-charged CNDs. This is explained by an electron or energy transfer process that causes quenching due to chelation. The creation of the complex at excited states, which transforms into fluorescence quenching by increasing Se^0 concentrations, was thus suggested by the decrease in the fluorescence lifetime of CNDs in the presence of Se^0 . These findings are consistent with earlier reports on carbon dot-based fluorescence

sensors, which have demonstrated robust performance in complex matrices with minimal interference (Su et al., 2020). The high recovery values and low variability further highlight the reliability of the proposed method for trace-level selenium determination in real-world samples. Such performance is crucial for environmental and food safety monitoring, where matrix effects often compromise analytical accuracy (Y. Wang & Hu, 2014).

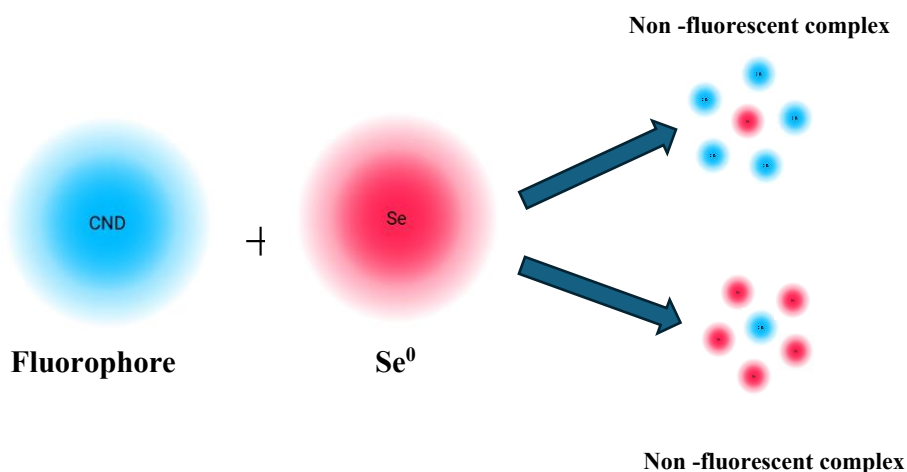


Figure 18. Mechanism of fluorescence quenching between CNDs and Se^0 .

Beyond direct Se^0 detection, the formation of a CND- Se^0 nanohybrid creates new opportunities for responsive sensing via fluorescence modulation. Given the strong chemical affinity between elemental selenium and mercury ions, the CND- Se^0 system was subsequently investigated as a selective “turn-on” probe for Hg^{2+} detection.

4.4 CND- Se^0 Nanohybrid System for Mercury (Hg^{2+}) Detection

4.4.1 Structural and Optical Characterization of CND- Se^0 Nanohybrids

CND- Se^0 was prepared using the sample preparation protocol outlined in sections 3.6.2.1 and 3.6.2.2. The morphological characteristics of the synthesized CND- Se^0 nanohybrid were examined by transmission electron microscopy (TEM). As shown in Fig. 1(a), the particles appeared as uniformly dispersed, dark, spherical structures with an average diameter of 3.12 ± 1.04 nm (Figure 19b), in agreement with previous observations for CNDs (Qi et al., 2021). The particle size remained well within the nanoscale range, with no signs of aggregation. A slight increase in average diameter compared to bare CNDs was also reported by (K. Yin et al., 2024), which may be attributed to the surface loading or interaction of elemental selenium. It is due to the marginal particle growth upon doping

CNDs with metallic or metalloid elements, such as sulfur or phosphorus, without significant alteration of core morphology (Kandasamy, 2019).

The fluorescence behavior of the nanohybrid was studied using 3D spectra and 2D analyses. The hybrid displayed strong excitation-dependent fluorescence with a maximum emission at 430 nm upon excitation at 360 nm (Figure 19c and Figure 19d), consistent with the typical optical signature of CNDs (Pan et al., 2015). However, compared to CNDs, a noticeable quenching of fluorescence intensity is observed in the CND-Se⁰ system. This reduction in emission intensity is likely due to nonradiative recombination introduced by the elemental selenium, which may act as an electron acceptor or trap state, suppressing fluorescence efficiency. Similar quenching phenomena have been reported in other CND-based hybrids where metal or metalloid dopants modify the surface energy level (Strauss et al., 2014).

Raman spectroscopy was employed to elucidate the structural integration of Se⁰ into the nanohybrid. As shown in Figure 19e, a distinct peak at 251 cm⁻¹ was observed, corresponding to the A₁ vibrational mode of amorphous red selenium (Lucovsky et al., 1967; Nagata et al., 1981). Although slightly shifted compared to the more commonly observed ~235 cm⁻¹ band for Se⁰, such shifts can arise due to quantum confinement effects, nanoscale interactions with carbon domains (Lorite et al., 2015). Additionally, the hybrid retained characteristic carbon peaks, including the D band at 1324 cm⁻¹ and the G band at 1562 cm⁻¹, are indicative of sp²-hybridized graphitic domains and structural disorder. The peak at 1451 cm⁻¹ represents the different vibrations of functional groups present on surfaces, which include C-O/C-N, -CH₃, and -CH₂ (Q. Wang et al., 2013). The co-existence of selenium and carbon signatures without spectral suppression confirms the formation of a stable CND-Se⁰ composite. Similar retention of spectral identity upon hybrid formation has been demonstrated in selenium-polymer nanoconjugates and other Se-functionalized nanocarbon systems (Lesnichaya et al., 2020; Rodrigues et al., 2008). Collectively, these results confirm the successful synthesis of a structurally and optically integrated CND-Se⁰ nanohybrid with nanoscale uniformity, preserved carbon structure, and retained or enhanced fluorescence, rendering it a promising candidate for Hg²⁺ sensing applications.

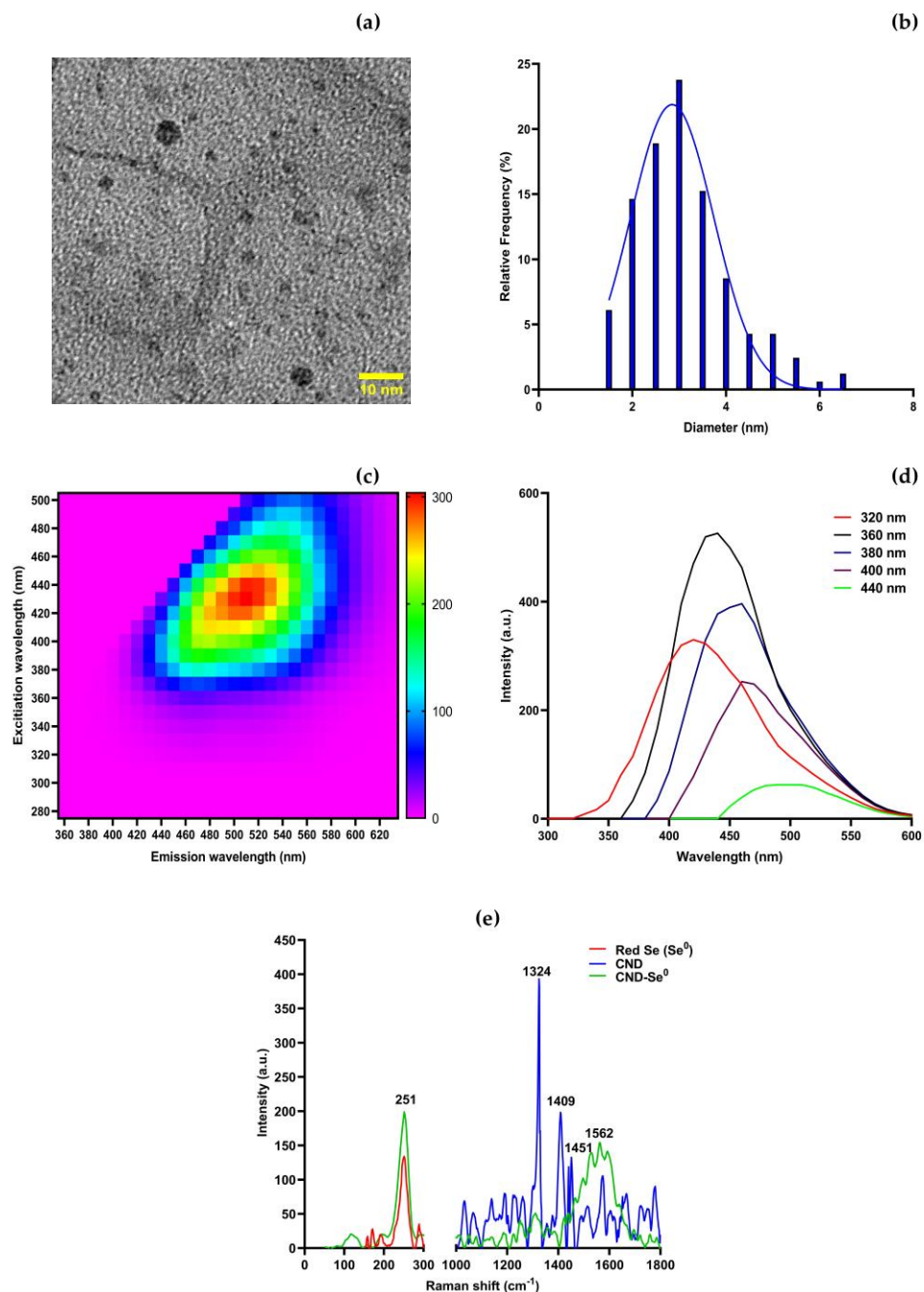


Figure 19. Characterization of CND-Se⁰ nanohybrid. (a) TEM image at 10 nm scale showing spherical CND-Se⁰ nanoparticles; (b) Histogram showing average size 3.12 ± 1.04 nm, (c) 3D fluorescence spectra, (d) 2D spectra with different excitation and emission wavelengths, and (e) Comparing the Raman spectra of CND-Se⁰ with red Se (Se⁰), & CND as controls.

4.4.2 Stability of the Nanohybrid System

The fluorescence response of the nanohybrid was monitored at increasing NaCl concentrations (0.1 to 1.0 mol L⁻¹). As shown in Figure 20a, The noticeable enhancement in fluorescence intensity was observed at higher salt concentrations, particularly at 1.0 mol L⁻¹. The corresponding bar plot Figure 20b reveals a statistically significant increase ($p < 0.001$) in emission at 1.0 mol L⁻¹, as denoted by distinct alphabetic grouping (Tukey's test). This unexpected enhancement may be attributed to salting-out effects, where ionic shielding reduces interparticle repulsion, promoting conformational stabilization and radiative transitions (Kewalramani et al., 2016). However, from 0.3 to 0.9 mol L⁻¹, the fluorescence intensity plateaued, suggesting that the probe remains functionally stable under moderate to high ionic strength conditions, which is essential for real-sample analysis (Becheru et al., 2018).

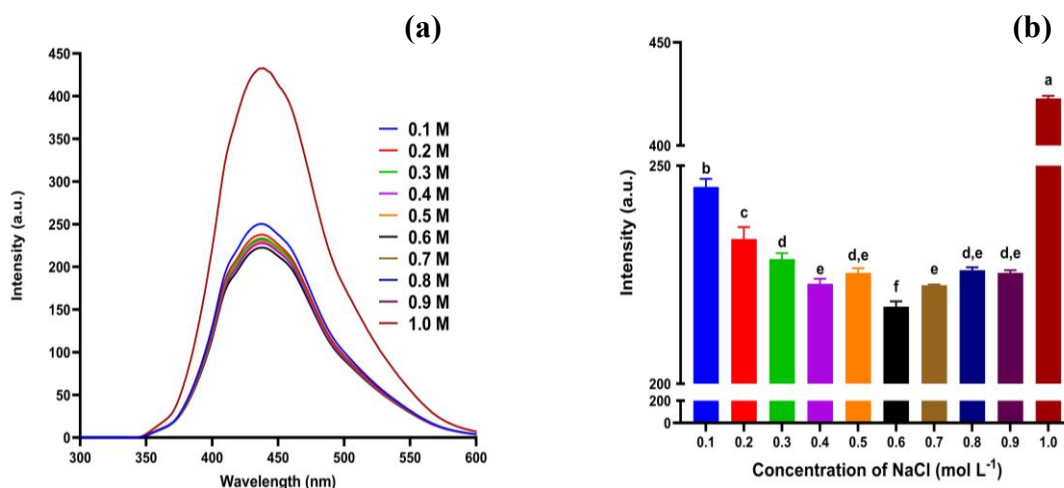


Figure 20. Stability of CND-Se⁰ at NaCl 0.1–1.0 mol L⁻¹. (a) CND-Se⁰ with varying concentrations of NaCl at 360 nm excitation wavelength and emission wavelength range of 300–700 nm. (b) CND-Se⁰ with different concentrations of NaCl at 360 nm excitation wavelength and 430 nm emission wavelength. Values are represented as Mean $\pm$ standard deviation, where $n = 3$, and different alphabetical letters show the level of significance, $p < 0.001$.

Time-based fluorescence measurements were conducted over 60 minutes to assess short-term photostability. As shown in Figure 21a, the fluorescence spectra remained nearly unchanged during the entire period. The fluorescence intensity increased over the first 30 minutes (Figure 21 b), after which no statistically significant change was observed. This temporal stability was confirmed by Tukey's test, where time points from 30 to 60

minutes shared the same superscript letter (a), indicating no significant difference ($p > 0.05$). This result confirms the nanohybrid's excellent temporal stability under ambient conditions. Similar kinetic stability has been reported for other selenium nanostructures stabilized by polymeric or carbon-based matrices, underscoring the role of CNDs as effective dispersing and protective agents (Bužková et al., 2025; Song et al., 2021).

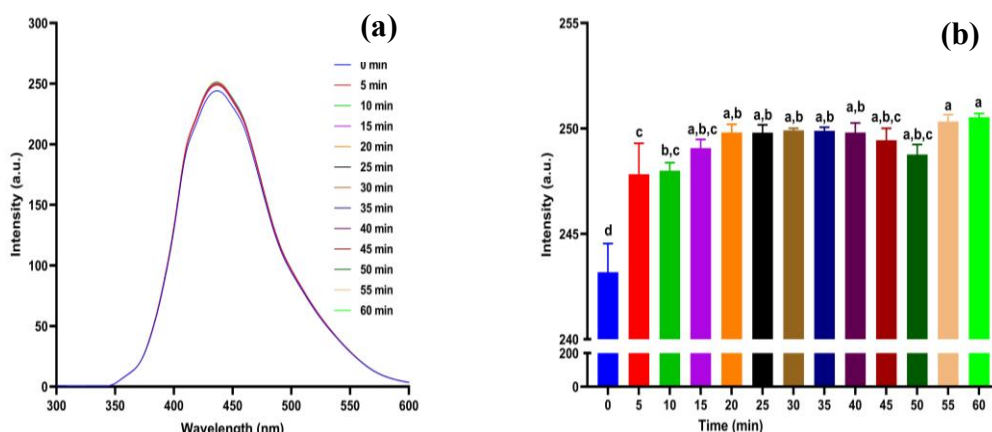


Figure 21. Stability of CND-Se⁰ across different periods (0-60 min). (a) stability of CND-Se⁰ at various time intervals (0-60 min) at 360 nm excitation wavelength and 300-700 nm emission wavelength. (b) stability of CND-Se⁰ at different time intervals (0-60 min) at 360 nm excitation wavelength and 430 nm emission wavelength. All values are represented as Mean $\pm$ standard deviation, where $n = 3$. Different superscript letters indicate statistically significant differences among time points based on Tukey's post hoc test ($p < 0.05$). Time points sharing the same alphabetical letter are not significantly different from each other.

The pH sensitivity of CND-Se⁰ was investigated over a wide range (pH 3-11). As illustrated in Figure 22a, maximum fluorescence emission was observed at acidic pH values (3-4), followed by a sharp decrease from pH 6 to 8. Beyond pH 9, a partial recovery in intensity occurred. Quantitative analysis (Figure 22b) confirms the highest emission at pH 3-4 (statistically indistinguishable, $p > 0.05$), while the lowest was at pH 7-8 ($p < 0.001$), likely due to structural disruption or protonation/deprotonation effects at near-neutral pH, altering surface functional groups and quenching emission (Dutta Choudhury et al., 2017; Ustun et al., 2023). Precipitation of Se⁰ was also observed at acidic and basic pH, which explains the high fluorescence intensities. In these pH ranges, Se⁰ tends to aggregate and grow from nanoscale to microscale particles, leading to a two-phase separation with solid Se⁰ particles deposited at the bottom of the solution (Liang et

al., 2021). Similar pH-responsive behavior has been reported for sulfur- and nitrogen-doped CNDs (Gong et al., 2019; Zhao et al., 2024). pH stability aligns with previous reports on pH-responsive graphene oxide (GO)-reinforced calcium alginate (CA) nanocomposite hydrogel beads, which exhibited minimal swelling in acidic environments and pronounced expansion at intestinal pH, enabling site-specific drug release in cancer therapy (Rasoulzadehzali et al., 2025).

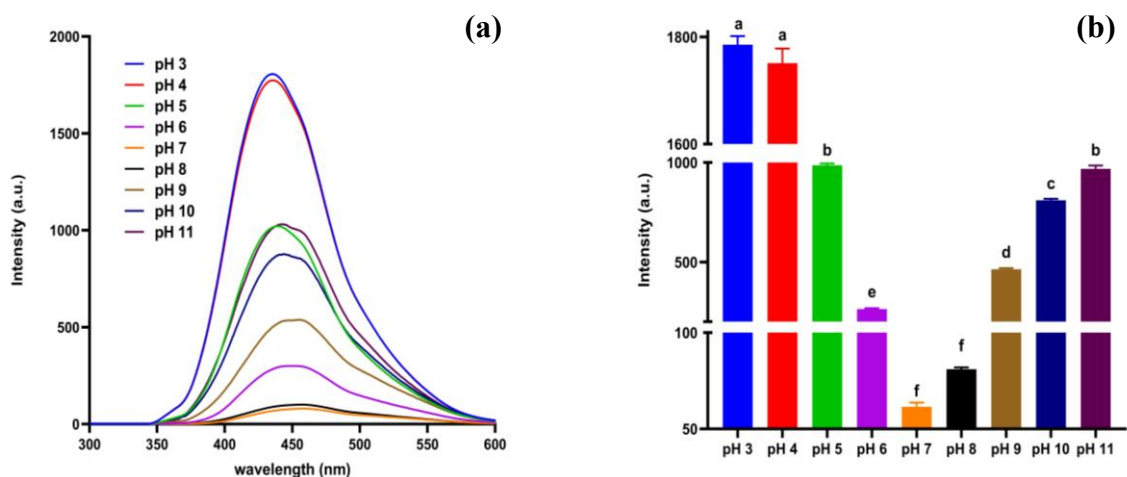


Figure 22. Stability of CND-Se⁰ at different pH levels (3–11). (a) CND-Se⁰ with different pH values (3–11) at 360 nm excitation wavelength and 300–700 nm emission wavelength. (b) CND-Se⁰ with different pH values (3–11) at 360 nm excitation wavelength and 430 nm emission wavelength; Values are represented as Mean $\pm$ standard deviation, where $n = 3$ and different alphabetical letters show the level of significance $p < 0.001$.

4.4.3 Fluorescence-Based Detection and Quantification of Hg²⁺

The sensitivity of the CND-Se⁰ probe toward Hg²⁺ ions was evaluated by measuring the relative fluorescence (equation 10), $\frac{I-I_0}{I_0}$ where I_0 represents the fluorescence intensity of the nanohybrid in the absence of Hg²⁺, and I is the intensity in its presence. As shown in Figure 23, the signal increased proportionally with rising Hg²⁺ concentrations ranging from 0.5 to 15 $\mu\text{mol L}^{-1}$. The resulting calibration plot showed excellent linearity, with a regression equation of:

$$\frac{I - I_0}{I_0} = 0.0066x + 0.0002 \quad (R^2 = 0.9919) \quad (\text{eq. 10})$$

The limit of detection (LOD) and limit of quantification (LOQ), calculated using the standard formulae (AOAC International, 2016; ICH, 1995) which are as mentioned below;

$$\text{LOD} = \frac{3.3\sigma}{S} = 8.7 \text{ nmol L}^{-1}$$

$$\text{LOQ} = \frac{10\sigma}{S} = 26.4 \text{ nmol L}^{-1}$$

where σ is the standard deviation of the six blank replicate measurements ($n=6$) and S is the slope of the calibration curve in the linear range ($0.5\text{-}15 \mu\text{mol L}^{-1}$)

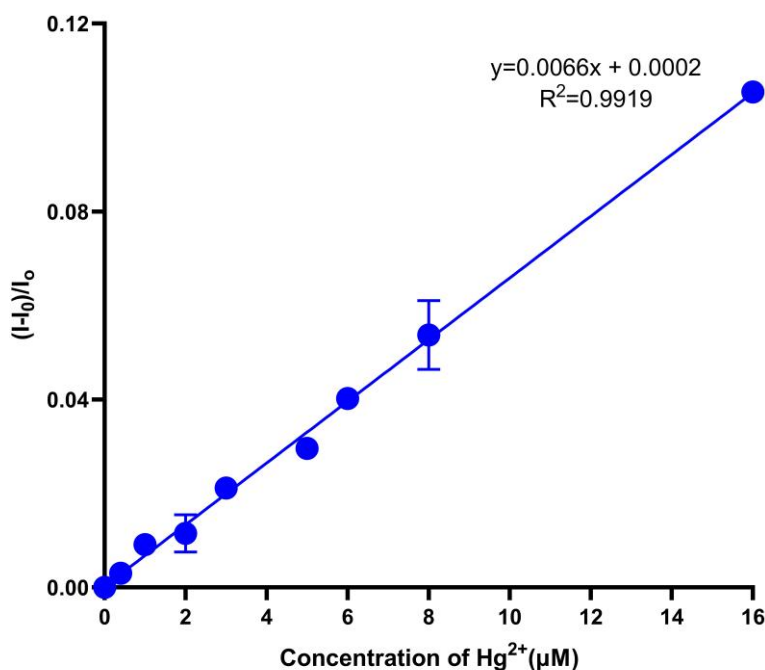


Figure 23. The standard linear calibration curve of Hg^{2+} from 0 to 15 $\mu\text{mol L}^{-1}$, where $p < 0.0001$.

These values underscore the high sensitivity of the proposed nanohybrid system, which is significantly lower than the WHO's standard value for inorganic mercury ($<6 \mu\text{g L}^{-1}$) in water systems. Unlike traditional fluorescence quenching approaches, the CND- Se^0 probe demonstrated an enhancement in fluorescence intensity upon interaction with Hg^{2+} , potentially attributed to mercury-induced modulation of surface passivation or electronic structure that alters nonradiative decay pathways (Afaneh & Schreckenbach, 2015). This distinct behaviour enhances detection contrast and minimizes false-positive effects caused by ambient interferents.

4.4.4 Selectivity and Real Sample Validation

The selectivity of the CND-Se⁰ nanohybrid sensor toward Hg²⁺ was evaluated in the presence of various potentially interfering metal ions, each tested at a concentration of 100 mg L⁻¹ under identical conditions. These included common mono-, di-, and trivalent cations such as As³⁺, Ca²⁺, Cu²⁺, Mg²⁺, K⁺, Na⁺, Mn²⁺, Ni²⁺, Pb²⁺, and Zn²⁺. The fluorescence response was expressed as the ratio I_0/I , where I_0 is the fluorescence intensity of the CND-Se⁰ probe in the absence of metal ions, and I represents the intensity in their presence.

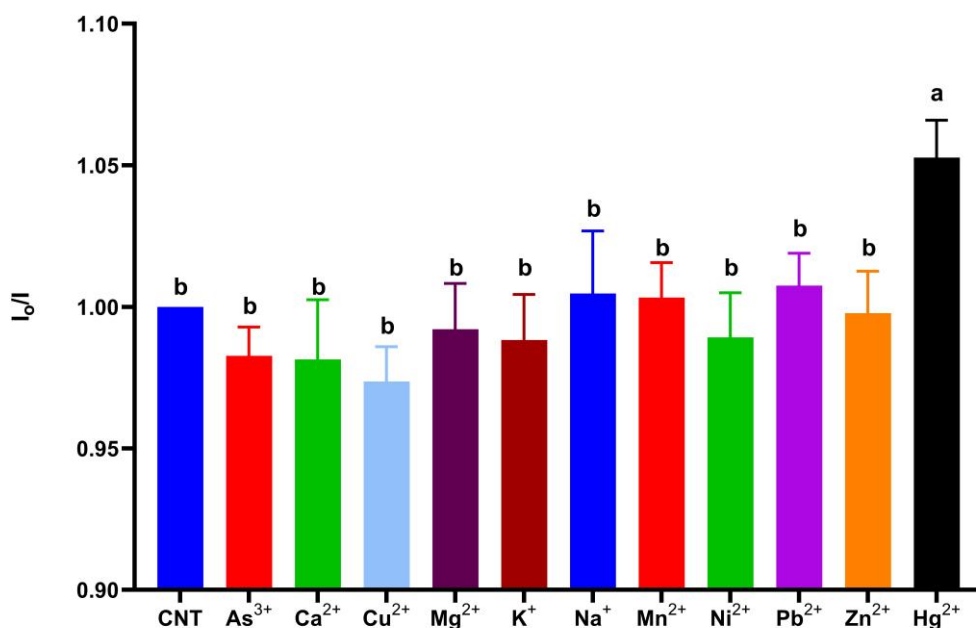


Figure 24. Selectivity of CND-Se⁰; 100 mg L⁻¹ of Ca²⁺, Fe²⁺, K⁺, Mg²⁺, Mn²⁺, Ni²⁺, Zn²⁺, and Na⁺, compared Hg²⁺. Data are expressed as mean $\pm$ SD (n = 3) and different alphabetical letters show the level of significance (p < 0.0001); CNT = control.

As shown in Figure 24, only Hg²⁺ produced a statistically significant fluorescence enhancement, while all other ions exhibited negligible effects on the probe's emission intensity. The selectivity pattern demonstrates the exceptional sensitivity of the CND-Se⁰ hybrid toward Hg²⁺, which may be attributed to the unique affinity of mercury ions for elemental selenium via soft-Lewis acid-base interactions (L. Zhou et al., 2012). This interaction likely induces a modulation of the surface state or electronic structure of the nanohybrid, promoting radiative recombination and increasing fluorescence emission (Dutta et al., 2021). The selectivity performance is crucial for practical applications, especially in environmental matrices that often contain high background concentrations

of alkali and alkaline earth metals. The ability of the probe to maintain high selectivity in the presence of these potentially interfering species highlights its robustness and analytical reliability (J. Yin et al., 2015). Similar selective interactions between selenium and mercury have been reported in selenium-based nanocomposites, reinforcing the probe's specificity in aqueous media (Tang et al., 2009).

To validate the analytical performance of the CND–Se⁰ fluorescent probe, recovery experiments were conducted using three real water matrices: mineral water (S1–S3), tap water (S4–S6), and ultra-pure water (S7–S9). Each sample was spiked with Hg²⁺ at concentrations of 1.0, 3.0, and 5.0 $\mu\text{mol L}^{-1}$, and the fluorescence response was recorded under optimized conditions. The recovery percentages and corresponding relative standard deviations (RSDs) are summarized in Table 12.

Table 12. The concentration of Hg²⁺ in real samples

Sample	Spiked Concentration of Hg ²⁺ ($\mu\text{mol L}^{-1}$)	Recovery (%)	RSD (%)
S1 Mineral H ₂ O	1.0	105.2±2.6 ^a	2.6
S2 Mineral H ₂ O	3.0	101.4±0.8 ^{b,c,d}	0.8
S3 Mineral H ₂ O	5.0	101.5±0.7 ^{b,c,d}	0.7
S4 Tap H ₂ O	1.0	105.1±3.5 ^{a,b}	3.3
S5 Tap H ₂ O	3.0	102.1±0.9 ^{a,b,c,d}	0.9
S6 Tap H ₂ O	5.0	102.1±0.5 ^{a,b,c,d}	0.5
S7 Ultra-pure H ₂ O	1.0	103.7±2.3 ^{a,b,c}	2.2
S8 Ultra-pure H ₂ O	3.0	99.92±0.9 ^d	0.9
S9 Ultra-pure H ₂ O	5.0	101.6±0.5 ^{a,b,c,d}	0.5

*Values represented as mean ± standard deviation. Different superscript letters indicate statistically significant differences among groups (Tukey's HSD test, $p < 0.001$). Comparisons were made across different samples and their spiked concentrations.

The results revealed satisfactory recoveries in the range of 99.92–105.2%, indicating the method's accuracy across different sample matrices. The acceptable analytical limits for trace analysis as outlined by AOAC and ICH guidelines (typically 80–120% depending on the matrix and concentration level) (AOAC International, 2016; ICH, 1995). RSD values were consistently low ($\leq 3.3\%$), demonstrating good repeatability and precision. Notably, the mineral water samples showed the highest recovery at the lowest spike level (105.2% ± 2.6), which may be attributed to minor matrix-induced enhancement effects (Henderson et al., 2009). Meanwhile, recoveries in ultra-pure water were slightly lower

but still within the acceptable range, affirming the method's applicability in both ionic-rich and low-conductivity matrices. The statistical significance among the recovery means was assessed using Tukey's multiple comparison test. Results denoted with different letters indicate statistically significant differences ($p < 0.001$). Although some variation was observed across samples, especially at lower spike concentrations, all recoveries remained within analytical tolerance limits, confirming the robustness of the method under real-world conditions.

4.4.5 Mechanism of the Fluorescence “Turn-On” Response

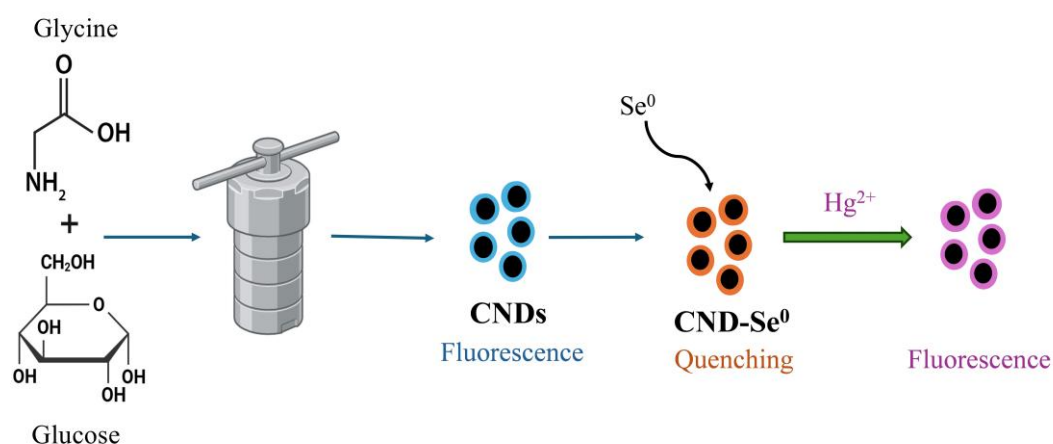


Figure 25. Mechanism of turn-on fluorescence quenching between CND- Se^0 & Hg^{2+} .

The underlying sensing mechanism of the CND- Se^0 nanohybrid towards Hg^{2+} is attributed to a reversible modulation of fluorescence through a quenching–recovery phenomenon, which has been illustrated in Figure 25 (Lan et al., 2014).

Carbon nanodots (CNDs), synthesized via the Maillard reaction route, exhibit strong intrinsic fluorescence due to abundant surface states and nitrogen-functional groups. Upon in situ formation of elemental selenium (Se^0) within the CND dispersion, a significant decrease in fluorescence intensity is observed (Arcudi et al., 2016). This quenching effect likely arises from energy transfer or charge transfer interactions between CNDs and Se^0 , which disrupt the radiative recombination of excited electrons. Similar quenching phenomena have been reported when electron-rich nanodots interact with redox-active species (Zu et al., 2017).

Interestingly, the introduction of Hg^{2+} ions into the CND- Se^0 system results in the partial recovery of fluorescence. This restoration may be due to a redox reaction between Hg^{2+} and Se^0 , forming Hg-Se^0 complexes or precipitates, thereby removing Se^0 from the CND surface and restoring the photoluminescent properties of the CNDs, which is also known

to be a “turn-on” effect (Ortiz Calderon et al., 2025). The high affinity between Hg^{2+} and Se^0 enables this selective interaction, leading to a measurable fluorescence enhancement. Such a mechanism supports the role of Se^0 as a modulating element, offering both quenching and responsive restoration capabilities, making the system highly suitable for selective Hg^{2+} sensing (Kumar & Singh, 2012; Tang et al., 2009). Given the green synthesis using food-grade precursors and the well-documented low toxicity of both glycine-derived carbon nanodots and elemental selenium, the nanohybrid holds potential for future biocompatible applications, although no direct toxicity or biocompatibility assays were performed in this study (Benko et al., 2012; Nguyen, El-Ramady, et al., 2025). The observed fluorescence modulation in the nanohybrid system aligns with prior studies demonstrating the tunability of carbon quantum dots’ emission properties when embedded in hybrid matrices. For instance, recent research incorporating halloysite Nanotubes with Malva extract into starch hyaluronic acid nanocomposite films reported enhanced CQD photoluminescence due to the synergistic interactions within the hybrid network (Abbaszadeh et al., 2025). This supports the hypothesis that Se^0 into the CNDs promotes favorable electronic interactions, contributing to the sensitive and selective fluorescence 'turn-on' response upon Hg^{2+} binding.

4.4.6 Comparative Evaluation with Existing Hg^{2+} Sensors

Table 13 compares the previously reported methods with this study on various factors. Such as synthesis type, LOD, biocompatibility, and its application.

Table 13. Comparison of previously available methods

Method	LOD (nmol L ⁻¹)	Linear Range (μmol L ⁻¹)	Green Synthesis	Sample Matrix	Ref.
Organo-selenium fluorescent probe	1	0-3.4	No	Cell (Macro phage)	(Tang et al., 2009)
Organo-selenium fluorescent probe	0.1	0.2–0.002	No	-	(Kumar & Singh, 2012)
Selenium-Based Fluorescence Chemosensor	73.5	0-10	No	-	(Rasin et al., 2022)
Selenium nanozyme	1.3	0.005-0.1	yes	water	(Shi et al., 2025)
CND-Se ⁰ Fluorescent probe	8.7	0-15	Yes	Water samples	This study

These findings highlight the potential of the CND–Se⁰ probe as a practical and reliable tool for on-site detection of Hg²⁺ in drinking and environmental water samples, without requiring extensive sample pretreatment or enrichment. The performance of the developed nano sensor was further evaluated in controlled aqueous samples, where satisfactory recovery and selectivity were observed. However, in complex food matrices, potential interferences such as light scattering, autofluorescence, and quenching by coexisting substances may affect the fluorescence response (Nguyen, El-Ramady, et al., 2025). Therefore, matrix-matched calibration or appropriate sample preparation is necessary for accurate quantification (Shanker et al., 2025). In terms of analytical performance, the detection limit of the developed nano sensor is comparable to those reported for CND-based fluorescent systems in the literature. Although some studies report lower detection limits under optimised conditions, these often involve more complex synthesis procedures or limited matrix applicability. The present approach

prioritises a balance between analytical performance, simplicity, and applicability in real samples (Laborda et al., 2016).

While nano-enabled sensing platforms highlight the functional advantages of elemental selenium at the nanoscale, their safe implementation requires a clear understanding of biological tolerance limits. Therefore, the final part of this chapter shifts from analytical performance to biological impact, integrating machine learning-based toxicity prediction with *in vivo* validation models. Because sensing platforms increasingly rely on nano-enabled selenium chemistry, evaluating biological tolerance and dose-dependent effects is essential to translate analytical capability into safe application.

4.5 Machine Learning Modelling of Nano-Selenium Toxicity

4.5.1 Dataset composition and variability

The curated dataset comprised 86 *in vivo* nano-selenium toxicity entries extracted from peer-reviewed studies, representing eight biological models: mice, rats, quail, chicken, fish, *Drosophila*, *Lucilia sericata*, and *Spodoptera* larvae. Nano-selenium doses ranged from 0.0375 to 198 mg kg⁻¹, encompassing environmentally relevant, nutritional, and supra-nutritional exposure levels reported in the literature. Exposure durations varied widely (18 to 6720 h), enabling the model to capture both acute and chronic toxicity patterns across taxa. Viability responses showed substantial inter-species variability. Vertebrate models such as quail and chicken generally maintained high survival rates at low to moderate doses, consistent with their higher tolerance to dietary selenium. In contrast, invertebrate models exhibited sharper declines in viability with increasing dose, reflecting differences in metabolic capacity and toxicokinetic handling of elemental selenium. Reported nanoparticle sizes (5–200 nm) demonstrated limited apparent influence compared with exposure intensity and duration, likely due to inconsistent reporting and methodological heterogeneity across studies. Overall, the diversity of species, dose ranges, and exposure conditions provided a broad toxicological landscape for model development, supporting robust training and enhancing the generalisability of machine-learning predictions across biological systems.

4.5.2 Exploratory Data Analysis and Feature Selection

4.5.2.1 Principal Component Analysis (PCA)

PCA was conducted to visualise multivariate structure and assess whether the predictors produced species-level clustering (Figure 26)(Jha et al., 2018). The first two principal

components explained approximately 50% of the total variance. PC1 was dominated by dose and exposure duration, reflecting their central roles in shaping toxicity responses, whereas PC2 captured inter-species variability. Distinct separation was observed between vertebrate models (mice, rats, quail, chicken) and invertebrate models (*Drosophila*, *Lucilia sericata*, *Spodoptera*), indicating fundamental differences in toxicokinetic and metabolic handling of nano-selenium. Quail entries formed a relatively compact cluster, consistent with their high tolerance to low and moderate SeNPs supplementation. In contrast, invertebrate groups displayed steeper dose-dependent shifts along PC1, reflecting higher sensitivity to increasing exposure levels (Benko et al., 2012). Overall, PCA confirmed that dose and exposure duration are major drivers of variance, while species identity significantly shapes toxicity outcomes (Zubrod et al., 2023). These patterns validated the inclusion of these predictors in the machine-learning framework and aligned with established biological expectations for selenium toxicodynamics (Rohn et al., 2018).

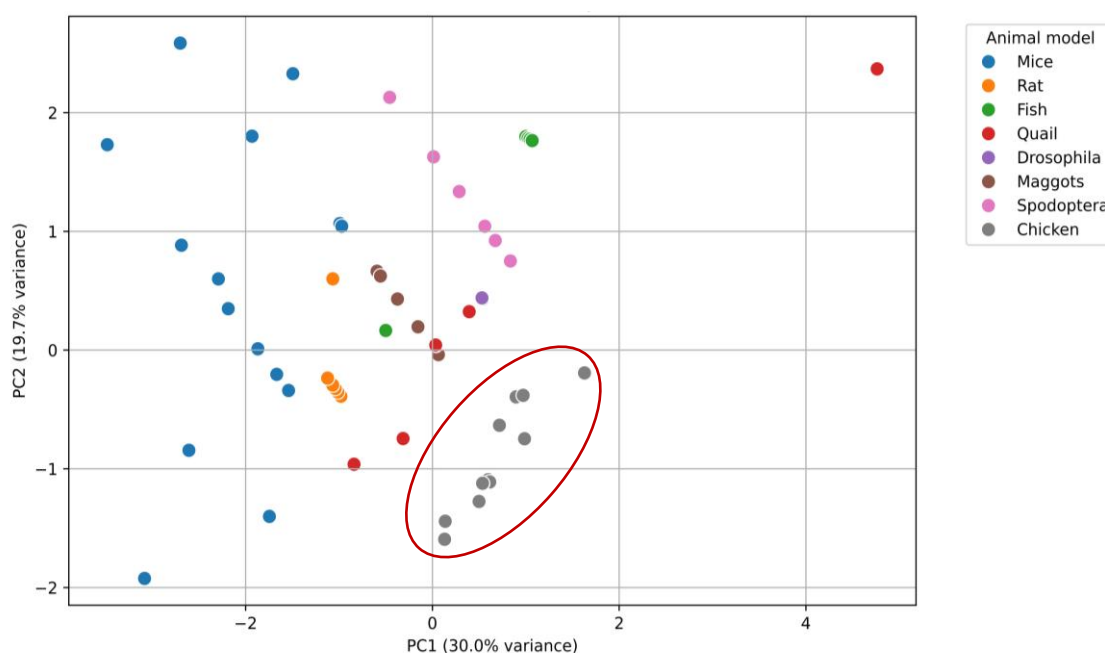


Figure 26. Principal Component Analysis (PCA) of the Nano-Selenium Toxicity Dataset, where PC1 and PC2 correspond to its total variance of ~50%.

4.5.2.2 Correlation Analysis

Pearson correlation coefficients were calculated to assess linear relationships among the continuous variables (Figure). Dose and viability exhibited a moderate negative correlation, reflecting reduced survival at higher concentrations and aligning with selenium's narrow therapeutic toxic range (Taylor & Maher, 2014). Exposure time

showed a weak positive correlation with viability, suggesting that longer low-dose exposures may allow physiological adaptation or steady-state metabolic handling. In contrast, particle size displayed no meaningful correlation with viability, likely due to inconsistent reporting across studies and the dominant influence of dose and exposure conditions in shaping toxicity outcomes (Mitjans et al., 2023; Warheit et al., 2007). Dose and exposure time were themselves only weakly correlated, indicating that the studies varied independently in their design parameters. Together with PCA findings and established toxicological relevance, these correlations supported the selection of dose (mg Se kg⁻¹), exposure duration (h), and species as the primary predictors for machine-learning modelling, representing exposure intensity, temporal accumulation, and organism-level sensitivity (Y. Zhou et al., 2023).

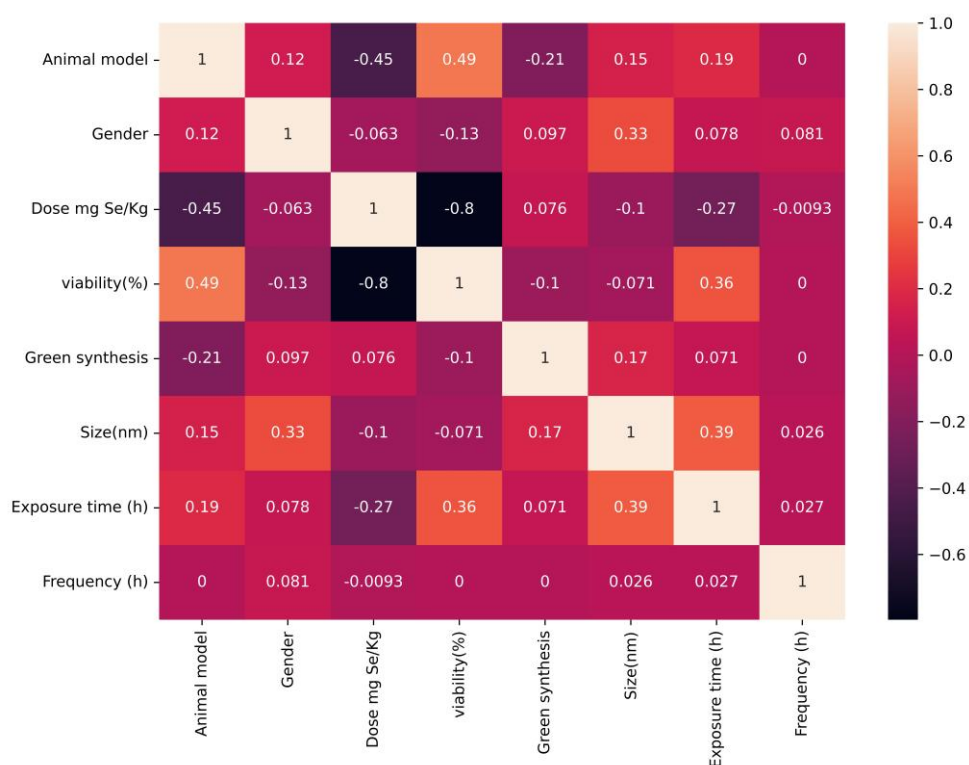


Figure 27. Pearson Correlation Matrix of Experimental Variables

4.5.3 Machine Learning: Model Screening and Comparative Performance Analysis

A comprehensive set of regression algorithms was evaluated to identify the most suitable approach for modelling nano-selenium toxicity across multiple species. Thirteen models representing linear, regularised, kernel-based, distance-based, and ensemble learning strategies were trained on the curated dataset using a consistent 70:30 train-test split. Model performance was assessed using R^2 , RMSE, MAE, and 10-fold cross-validation

(Table 14). Linear models, including ordinary least squares, Theil-Sen, and Huber regression, showed limited predictive capacity, reflected by low R^2 values and high error metrics, indicating an inability to capture the nonlinear dose–response interactions inherent to toxicological datasets (Balraadjsing et al., 2024). SVR similarly underperformed, suggesting that simple kernel boundaries could not resolve the heterogeneity present across species and exposure conditions (van den Berg et al., 2021). In contrast, ensemble tree-based methods demonstrated substantially superior performance. AdaBoost, Random Forest, Gradient Boosting, and XGBoost achieved high explanatory power on the training set ($R^2 > 0.95$) and competitive generalisation on the test set ($R^2 \sim 0.84$). Their comparatively lower RMSE and MAE values further indicated strong predictive alignment with reported viability outcomes (Gasser et al., 2024). Narrow cross-validation variance across these models highlighted robustness against data partitioning effects (Mei, 2025). Although XGBoost achieved the highest training accuracy, its reduced test-set performance and greater cross-validation variability indicated mild overfitting. To maintain methodological simplicity and interpretability, AdaBoost was selected as the final model. It achieved $R^2 = 0.971$ (train) and 0.848 (test) with low RMSE and MAE values, effectively capturing the nonlinear relationships among dose, exposure duration, and species. This model was therefore chosen for subsequent SHAP-based interpretability analysis and experimental validation in Japanese quails.

Table 14. Performance comparison of various machine learning models evaluated for predicting nano-selenium toxicity

Model	Trained Score			Test Score		
	R ²	RMSE	MAE	R ²	RMSE	MAE
AdaBoost Regressor	0.971	4.742	2.919	0.848	7.385	3.790
Random Forest Regressor	0.977	3.720	1.812	0.841	12.90	7.584
KNN Regressor	0.934	6.298	2.653	0.819	13.75	7.525
XGBoost Regressor	0.984	3.133	2.039	0.803	14.32	8.839
Gradient Boosting Regressor	0.999	0.787	0.581	0.838	12.99	7.529
CatBoost Regressor	0.905	7.560	4.965	0.771	15.45	10.84
LightGBM Regressor	0.744	12.45	7.343	0.614	20.08	14.67
SGD Regressor	0.673	14.06	10.24	0.619	19.93	13.10
Linear Regression	0.668	16.03	12.21	0.672	10.84	8.012
Huber Regressor	0.620	15.15	9.292	0.606	20.28	12.78
TheilSen Regression	0.400	21.54	13.56	0.574	12.36	7.830
SVR	0.110	23.20	14.08	-0.216	35.63	24.07

4.5.4 Selection and Validation of the AdaBoost Regressor Model

The AdaBoost Regressor was selected as the final predictive model due to its favourable balance of accuracy, generalisation capacity, and interpretability. Model robustness was further assessed using 10-fold cross-validation. The cross-validated mean R² was 0.828±0.099 with a corresponding RMSE of 9.920 ± 4.127 and MAE of 5.724 ± 2.523. The relatively narrow standard deviations across folds indicate stable predictive behaviour and minimal sensitivity to data partitioning, supporting the conclusion that the model did not overfit to specific subsets of the dataset (Merabet et al., 2025). These results demonstrate that AdaBoost provides reliable performance across heterogeneous multi-species toxicity data and is suitable for downstream interpretability analysis and experimental validation (Kiran et al., 2024).

4.5.5 Model Robustness: Residual Analysis and Error Distribution

Residual analysis was performed further to evaluate model reliability and bias (Figure 27). Most residuals were tightly clustered around zero, indicating good agreement

between predicted and observed viability values across most experimental entries. A limited number of samples exhibited larger positive or negative residuals, reflecting under- or overestimation by the model (Meneses et al., 2023). These deviations were primarily associated with high-dose invertebrate datasets (e.g., *Spodoptera* and *Lucilia sericata*), where abrupt, nonlinear declines in viability occur at elevated exposure levels (Benko et al., 2012). Despite these outliers, the overall residual distribution remained symmetrically centred near zero, and no systematic trend was observed across the predicted viability range. This pattern indicates the absence of systematic bias toward either low or high viability values (T. Wang et al., 2025).

Overall, the random and balanced distribution of residuals supports the robustness of the AdaBoost model and its suitability for general prediction of nano-selenium toxicity across heterogeneous biological systems.

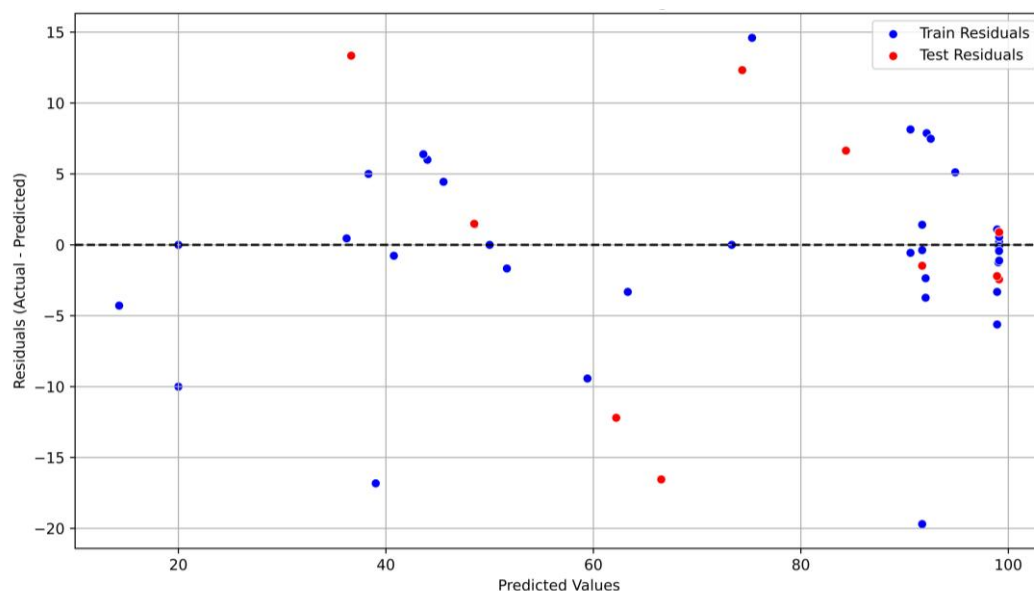


Figure 27. Residual plot representing the distribution of trained and tested residual errors across the samples

4.5.6 SHAP Analysis and Leave-One-Out Cross-Validation (LOOCV)

To interpret the prediction behaviour of the AdaBoost model and assess robustness across heterogeneous *in vivo* datasets, SHAP analysis and (LOOCV) were performed.

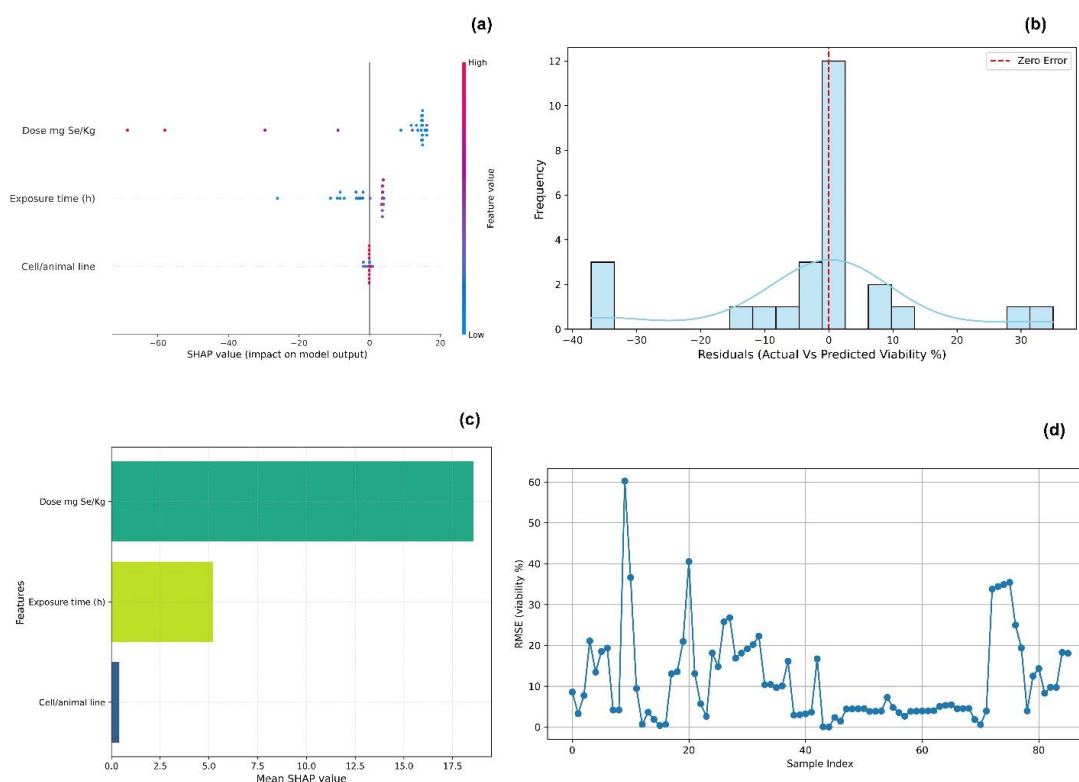


Figure 28. Model explainability and robustness assessment for the AdaBoost regressor. (a) SHAP beeswarm plot illustrating sample-level feature contributions to predicted viability. (b) Residual distribution highlighting model prediction errors and bias. (c) Global SHAP importance ranking showing dose as the dominant toxicity predictor, and (d) LOOCV RMSE profile across all samples, demonstrating model stability and identifying high-variance data points.

4.5.6.1 SHAP-based model interpretability

SHAP analysis revealed feature importance patterns consistent with classical dose–response toxicology (Figure 28). Global SHAP importance rankings (Figure 28c) identified dose (mg Se kg⁻¹) as the dominant predictor of viability, contributing substantially more to model output than any other variable. This finding aligns with the well-established dose-dependent nature of selenium toxicity across biological systems (Benko et al., 2012; Ferroudj, Muthu, et al., 2025; Ferroudj, Semsey, et al., 2025). Exposure duration (h) emerged as the second most influential feature, reflecting the importance of cumulative exposure effects in nano-selenium toxicity. The animal model code contributed comparatively less to overall predictions, indicating that the AdaBoost model generalised effectively across taxonomically diverse species. SHAP beeswarm plots (Figure 28a) further illustrated these trends, with higher doses associated with strongly negative SHAP values (reduced predicted viability). In contrast, low-dose and

short-duration exposures showed positive contributions (T. Wang et al., 2025). The dispersion of SHAP values across samples highlights nonlinear feature interactions, which were effectively captured by the ensemble model.

4.5.7 Error structure and model consistency

Residual distributions derived from SHAP analysis (Figure 28b) were centred near zero, indicating the absence of systematic prediction bias. A limited number of outliers were observed, primarily corresponding to high-dose exposure scenarios or studies with incomplete nanoparticle characterisation (Liangruksa et al., 2025). These deviations reflect inherent variability in published datasets rather than structural model deficiencies, supporting the model's resilience under heterogeneous data conditions.

4.5.8 LOOCV-based robustness assessment

LOOCV was applied to further evaluate generalisability by treating each observation as an independent test case. The resulting RMSE distribution (Figure 28d) showed that most samples exhibited low prediction errors, typically within $\pm 10\%$ viability deviation. Higher RMSE values were associated with extreme-dose exposures or datasets lacking detailed nanoparticle characterisation, underscoring the influence of data quality on predictive accuracy (Hataminia et al., 2019; Hataminia & Azinfar, 2025). Collectively, SHAP and LOOCV analyses demonstrate that the AdaBoost model combines strong interpretability with robust generalisation. The identification of dose and exposure duration as primary toxicity determinants is consistent with established toxicological principles, supporting the model's validity for guiding experimental design and identifying physiologically relevant exposure ranges.

To evaluate whether model predictions translate into real biological outcomes, independent *in vivo* validation was performed using Japanese quails as a nutritionally relevant vertebrate model.

4.6 Model experimental validation using Quails

To evaluate whether machine-learning-derived toxicity predictions translate to a real biological system, an independent *in vivo* validation experiment was conducted using Japanese quails (*Coturnix japonica*). This feeding trial provided an external test of model reliability under controlled conditions using well-characterised selenium nanoparticles.

4.6.1 Characterisation of Selenium Nanoparticles (SeNPs)

The synthesised SeNPs exhibited physicochemical characteristics consistent with previous reports on ascorbic acid-reduced Se⁰ (Badgar & Prokisch, 2021). SEM analysis

confirmed predominantly spherical particles with minimal aggregation (Figure 29a). The mean particle diameter was 20.05 ± 3.7 nm (Figure 29b), a size range associated with enhanced bioavailability while remaining below typical agglomeration thresholds. EDX spectra showed distinct selenium peaks without detectable impurities, confirming chemical purity (Figure 29c). Raman spectroscopy further verified structural identity through characteristic Se^0 vibrational bands at approximately 168, 190, and 250 cm^{-1} (Figure 29d) (Muthu et al., 2025). Collectively, these analyses confirmed that the SeNPs used for feeding trials were homogeneous, pure, and suitable for biological evaluation.

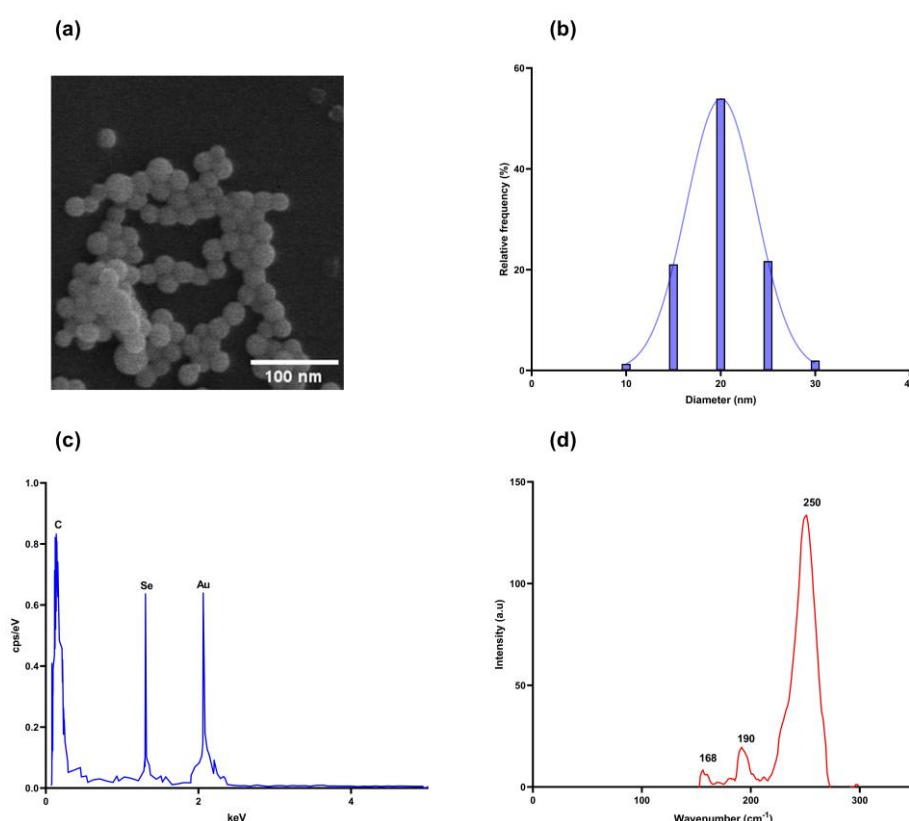


Figure 29. Characterisation of selenium nanoparticles (SeNPs) used for quail feeding. (a) Scanning Electron Microscopy image of SeNPs showing spherical morphology and low aggregation. (b) Particle size distribution histogram of SeNPs with an average particle size of 20.05 ± 3.7 nm, (c) EDX spectrum of SeNPs confirming elemental selenium and absence of impurities, and (d) Raman spectrum of SeNPs showing characteristic Se^0 vibrational bands at 168, 190, and 250 cm^{-1} .

4.6.2 *In Vivo* validation using Japanese Quails

Quails were fed diets supplemented with SeNPs at 0.05 and 0.5 mg Se kg^{-1} feed for 28 days, representing nutritionally relevant and moderately elevated exposure levels

commonly assessed in poultry studies (Al-Khalaifah et al., 2025; Ferroudj, Muthu, et al., 2025). SeNPs were uniformly incorporated into a balanced basal diet (Figure 30). Throughout the experimental period, no adverse effects, behavioural abnormalities, or reductions in feed intake were observed. Viability, defined as survival of clinically normal birds, remained 100% in both treatment groups (Figure 30), consistent with the reported low toxicity of SeNPs within their physiological tolerance range (Khalid et al., 2025).

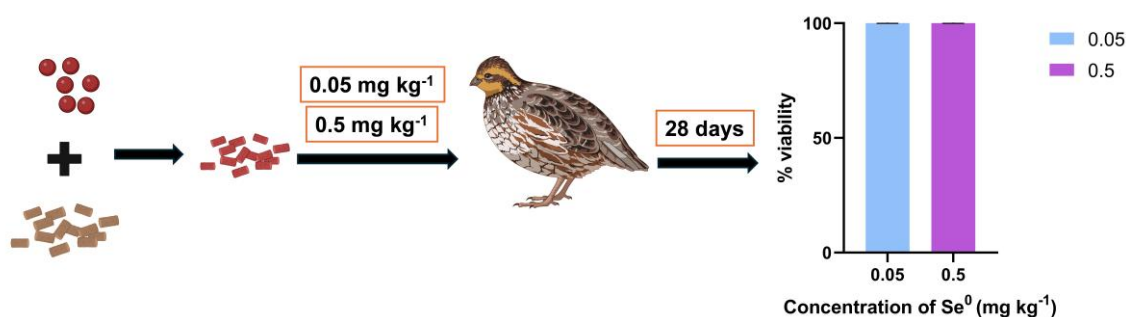


Figure 30. Schematic workflow and the viability plot of the quail validation experiment, where quails were fed SeNPs-supplemented diets of 0.5 and 0.05 mg Se kg⁻¹.

4.6.3 Comparison Between Experimental and Predicted Viability

The AdaBoost model predicted viabilities of 99.02% and 99.01% for the 0.05 and 0.5 mg Se kg⁻¹ treatments, respectively. The deviation between predicted and observed outcomes was less than 1%, corresponding to an overall prediction error of 0.988% (Figure 31). This high level of agreement indicates that dose-dependent patterns learned from heterogeneous, cross-species data successfully generalised to an independent biological system (Curtis et al., 2022). The results also confirm that the model captured nonlinear relationships between SeNPs dose and physiological tolerance, while remaining stable under real feeding conditions, where biological variability may influence outcomes (Selmani et al., 2020).

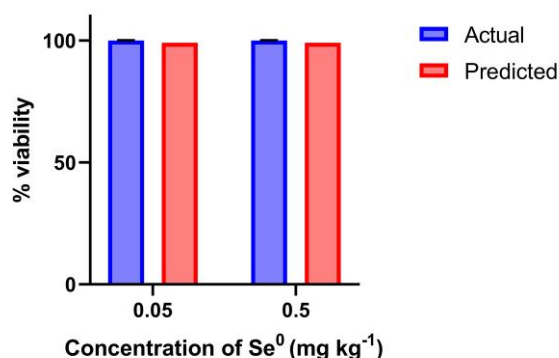


Figure 31. Comparison of actual vs predicted viability (%) of quails fed SeNPs-supplemented diets.

4.6.4 Toxicological Significance and Model Reliability

This experimental validation demonstrates that data-driven modelling approaches can complement *in vivo* studies by narrowing the range of doses requiring biological testing. The model accurately anticipated minimal toxicity at nutritionally relevant doses, consistent with prior evidence that nano-selenium exhibits lower toxicity than inorganic selenium forms (Bhattacharjee et al., 2019). By integrating nanoparticle characterisation, predictive modelling, and controlled biological validation, the study establishes a translational framework linking computational toxicology with practical feed safety assessment (Q. Zhang et al., 2018). These findings highlight the potential of machine-learning-assisted approaches to support “Safe by Design” nanoparticle implementation in animal nutrition and food systems, in line with emerging regulatory expectations (X. Zhang et al., 2025; Zouraris et al., 2025). Although the developed machine learning models demonstrate good predictive performance, the relatively small dataset ($n = 86$) may limit their generalizability. Cross-validation was employed to minimise overfitting and improve model reliability within the available data domain (Kuhn & Johnson, 2013). However, the predictive capability of the model is inherently dependent on the representativeness of the training dataset. Therefore, extrapolation of the model to other biological systems or experimental conditions should be performed with caution. Future work will focus on expanding the dataset and incorporating more diverse experimental conditions to enhance model robustness and external validity (Rajvanshi et al., 2024). To further resolve dose-dependent developmental effects and capture sub-lethal toxicity mechanisms, *Drosophila melanogaster* was employed as a complementary *in vivo* digestion-based model.

4.7 *In Vivo* Assessment of Elemental Selenium Toxicity in *Drosophila melanogaster*

4.7.1 Dose-Dependent Effects on Developmental Progression

The *in vivo* digestion exposure of *Drosophila melanogaster* to elemental selenium (Se^0) resulted in apparent, concentration-dependent effects on developmental progression and survival. The metamorphosis ratio (Figure 32) showed no statistically significant deviation from the control at low Se^0 concentrations (0.1 and 0.5 mg kg^{-1}), indicating that these exposure levels did not adversely affect larval-to-pupal transition. A statistically significant increase in metamorphosis efficiency was observed at 1 mg kg^{-1} , suggesting a stimulatory or hormetic response at moderate Se^0 concentrations. In contrast, higher concentrations (2 and 4 mg kg^{-1}) did not further enhance metamorphosis and showed increased variability, indicating the onset of dose-related stress (Miksanek et al., 2024). Such biphasic responses are consistent with selenium's well-documented dual biological role, acting as an essential micronutrient at low doses and a toxicant at elevated levels (Zheng et al., 2024). The absence of significant differences between the control and low-dose groups supports the biological tolerance of *Drosophila* to Se^0 within a defined exposure window.

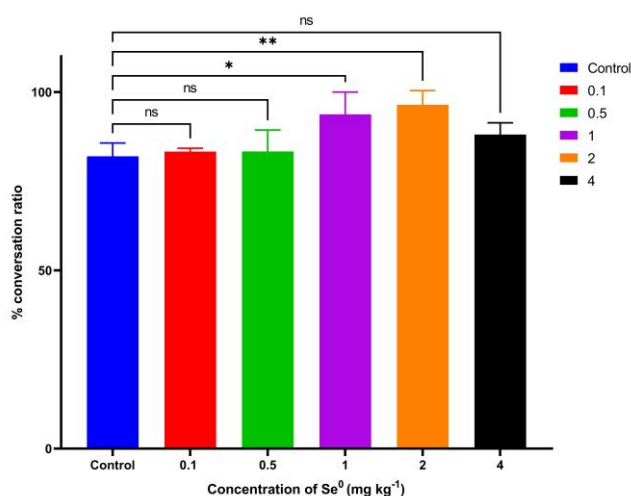


Figure 32. Effect of dietary elemental selenium (Se^0) concentration on metamorphosis ratio in *Drosophila melanogaster*. Bars represent mean $\pm$ standard deviation of the percentage of larvae successfully completing metamorphosis under control and Se^0 -supplemented conditions (0.1–4 mg kg^{-1}). Statistical significance relative to the control is indicated ($p < 0.05$; * $p < 0.01$; ns, not significant).

4.7.2 Pupal Viability and Developmental Kinetics

Pupal viability analysis revealed distinct dose- and time-dependent trends (Figure 33). Survival curves demonstrated that pupation onset was delayed at higher Se^0 concentrations, particularly at 2 and 4 mg kg^{-1} , indicating impaired developmental kinetics. This delay may result from the oxidative stress accumulation, disrupting energy balance and developmental timing (Mishra & Panda, 2021). Bar plot analysis (Figure 34) further confirmed that pupal viability was significantly enhanced at 1 mg kg^{-1} compared to the control. In comparison, no statistically significant reduction was observed at 0.1 and 0.5 mg kg^{-1} , reaffirming *Drosophila*'s tolerance to Se^0 within a specific threshold. This could be a hormetic effect that Se^0 can modulate redox systems, promoting cellular protection at sub-toxic levels while inducing damage at higher concentrations (Alaraby et al., 2024). The pupal stage, being metabolically intense and involving extensive tissue remodeling, appears particularly sensitive to oxidative imbalances. Excessive Se^0 may overwhelm antioxidant systems such as glutathione and thioredoxin pathways, leading to Cellular damage and developmental arrest (Mishra & Panda, 2021).

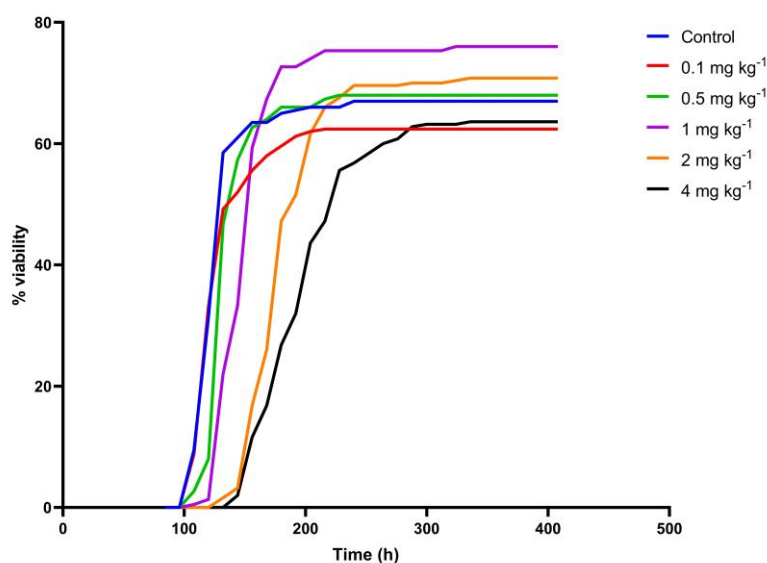


Figure 33. Time-dependent pupal viability of *D. melanogaster* exposed to different concentrations of elemental selenium (Se^0). Viability (%) was monitored over the developmental period under control and Se^0 -supplemented diets (0.1–4 mg kg^{-1}), illustrating dose-dependent effects on pupal development and survival.

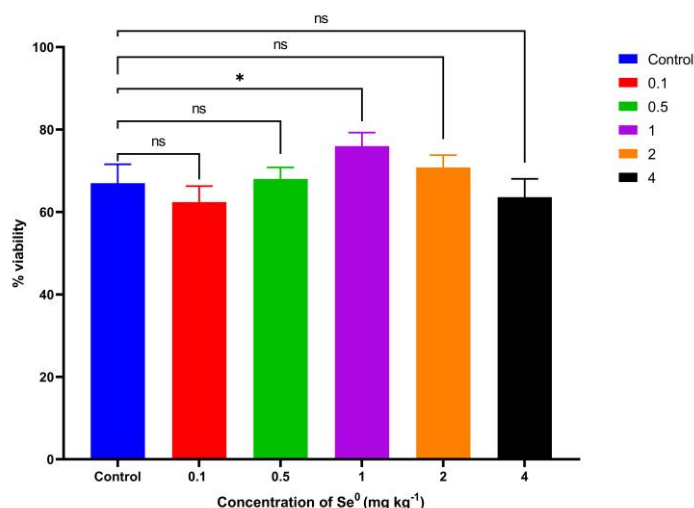


Figure 34. Pupal viability of *D. melanogaster* as a function of dietary elemental selenium (Se⁰) concentration. Bars represent mean $\pm$ standard deviation of pupal survival percentages after exposure to control and Se⁰-supplemented media. Statistical differences are indicated ($p < 0.05$; ns, not significant).

4.7.3 Adult Fly Viability and Survival Outcomes

Adult emergence and viability data showed a clear divergence between moderate and high Se⁰ exposure levels (Figure 35 and Figure 36). At 1 mg kg⁻¹, adult viability was significantly higher than the control, suggesting that moderate Se⁰ exposure during development may confer a survival advantage extending into adulthood (Miksanek et al., 2024). In contrast, exposure to 4 mg kg⁻¹ resulted in a pronounced reduction in adult viability, characterised by delayed emergence and lower final survival rates. The time-resolved viability curves indicate that high Se⁰ concentrations primarily affected early adult survival rather than causing immediate lethality, suggesting cumulative or delayed toxicity effects. This pattern is characteristic of sub-chronic toxicity, where physiological stress accumulates over developmental stages before manifesting in reduced adult fitness (Thomas & Janz, 2015).

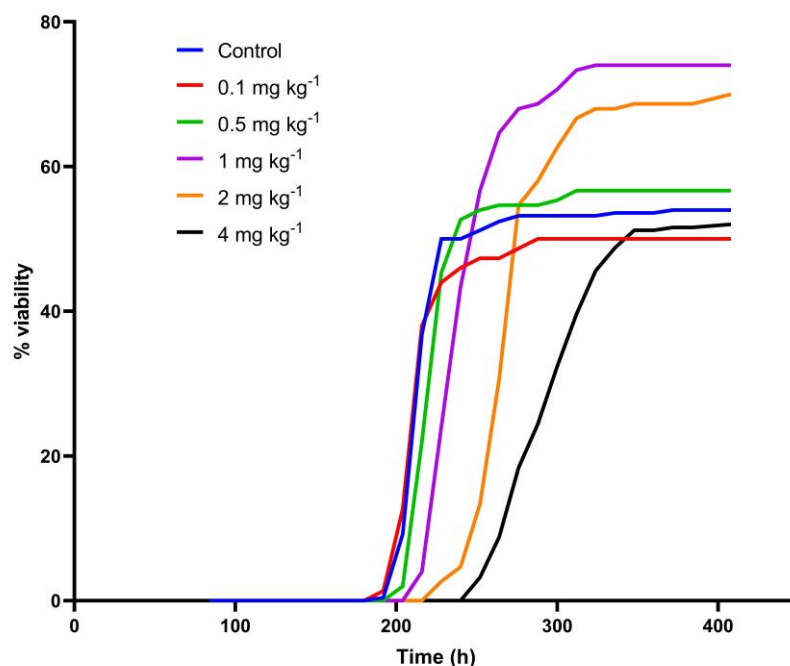


Figure 35. Time-resolved adult fly viability of *Drosophila melanogaster* following developmental exposure to elemental selenium (Se^0). Survival (%) of emerged adults was recorded over time under control and Se^0 -supplemented conditions, highlighting concentration-dependent survival dynamics.

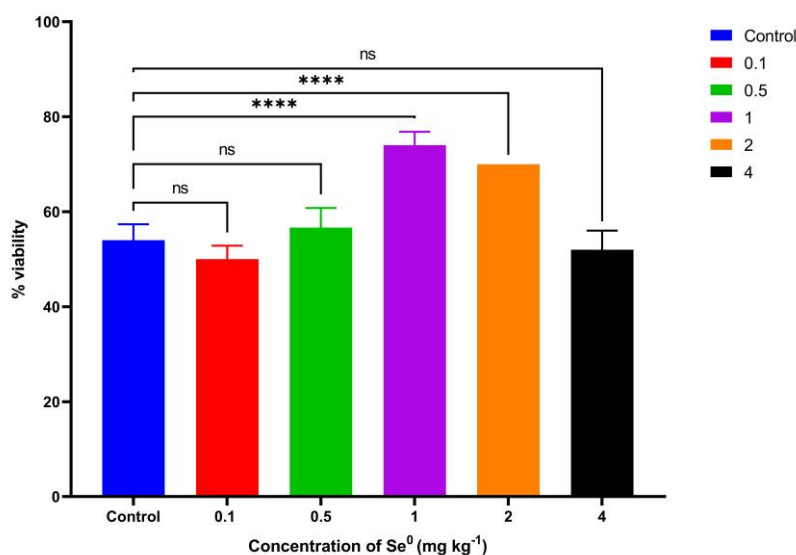


Figure 36. Adult fly viability of *Drosophila melanogaster* following dietary exposure to elemental selenium (Se^0). Bars represent mean $\pm$ standard deviation of adult survival percentages for control and Se^0 -treated groups (0.1–4 mg kg⁻¹). Statistical significance relative to the control is indicated (**** $p < 0.0001$; ns, not significant).

4.7.4 Toxicological Interpretation and Hormetic Behaviour

Overall, the results demonstrate a narrow but clearly defined threshold between beneficial and adverse Se⁰ exposure. Low to moderate concentrations support normal development and, in some cases, enhance survival, while higher doses induce developmental stress and reduced viability (Alaraby et al., 2016). These findings align with previous reports describing hormetic responses to selenium-based nanomaterials and reinforce the importance of dose optimisation in nano-selenium applications (Fairweather-Tait et al., 2011; Iavicoli et al., 2018).

The observed hormetic behaviour of Se⁰ can be explained by its dual role in biological systems. At low concentrations, selenium functions as an essential micronutrient and enhances antioxidant defence mechanisms through its incorporation into seleno-proteins such as glutathione peroxidase and thioredoxin reductase. These enzymes play a key role in mitigating oxidative stress (Lv et al., 2026). However, at higher concentrations, selenium can promote the generation of reactive oxygen species (ROS), leading to oxidative stress, cellular damage, and toxicity (Hariharan & Dharmaraj, 2020). This biphasic dose-response relationship is characteristic of hormesis, where low-dose stimulation and high-dose inhibition are observed (Banerjee et al., 2022). The consistency between metamorphosis ratios, pupal viability, and adult survival strengthens the robustness of the observed trends. It confirms *D. melanogaster* as a sensitive and reliable *in vivo* model for assessing nano-selenium toxicity (Zheng et al., 2024).

4.7.5 Significance of the Study

This study provides a comprehensive *in vivo* evaluation of elemental nano-selenium (Se⁰) toxicity using *D. melanogaster* as a digestion-based exposure model. By integrating developmental, pupal, and adult survival endpoints, the work offers a multi-stage perspective on nano-selenium toxicity that extends beyond single-time-point lethality assessments. The findings demonstrate that Se⁰ exhibits a dose-dependent dual effect, with moderate concentrations supporting developmental success and survival, while higher concentrations induce sub-chronic toxicity. This distinction is particularly relevant for food, feed, and nutraceutical applications of nano-selenium, where precise dosage control is critical due to selenium's narrow margin between nutritional benefit and toxicity. Notably, the results establish *D. melanogaster* as a scalable, ethically acceptable, and biologically informative *in vivo* platform for nano-selenium risk assessment.

The generated dataset also provides a strong experimental foundation for subsequent machine learning–based toxicity modelling and cross-species extrapolation, enhancing the translational relevance of the study. Collectively, this work contributes valuable experimental evidence toward the safe design, regulatory evaluation, and predictive modelling of nano-selenium in food-related and biomedical applications.

4.8 Integrated Discussion and Translational Significance

This study adopts a multi-scale, multi-methodological framework to investigate selenium (Se) behaviour across environmental, analytical, nanoscale, biological, and predictive domains. Rather than treating these components as isolated investigations, the results collectively demonstrate that selenium speciation, analytical accessibility, and biological impact are intrinsically interconnected. By integrating geochemical baseline assessment, species-specific analytical methods, nano-enabled sensing strategies, and biological validation supported by data-driven modelling, the work establishes a coherent and translational understanding of selenium behaviour from soil systems to biological outcomes.

At the environmental scale, the GEMAS-based assessment provides a harmonised baseline for total selenium concentrations in European and Hungarian agricultural soils. The results confirm that selenium distribution is highly heterogeneous and governed by complex, multivariate geochemical controls, with only weak-to-moderate correlations to bulk soil parameters. While such datasets are essential for continental-scale mapping and environmental benchmarking, the findings also highlight a fundamental limitation: total selenium concentrations alone cannot resolve selenium speciation, mobility, or bioavailability. This constraint provides a clear conceptual justification for the subsequent analytical focus on reactive and biologically relevant selenium species.

Volatile hydrogen selenide (H_2Se) represents a critical intermediate in selenium redox cycling and biological metabolism, yet its determination remains analytically challenging due to its instability and rapid transformation. The modified atomic fluorescence spectroscopy (AFS) method developed in this study directly addresses this gap by enabling stable, sensitive, and reproducible quantification of H_2Se in aqueous systems. By decoupling flame stability from reductant chemistry and minimising hydride residence time, the method improves analytical robustness while retaining a relatively simple instrumental configuration. The strong agreement between AFS and GF-AAS at moderate concentrations validates the efficiency of the purge-and-trap approach and confirms that

the developed workflow reliably captures volatile selenium species. In this context, the H_2Se method functions as a mechanistic bridge between total selenium occurrence and biologically active selenium forms.

While H_2Se analysis targets volatile and dissolved selenium species, elemental selenium (Se^0) is increasingly encountered in nano-enabled food, feed, and environmental applications, where it predominantly exists in solid or particulate form. To complement the AFS-based approach, carbon nanodot (CND)-based fluorescence sensing was employed to address the analytical gap associated with Se^0 detection. The synthesised CNDs exhibited stable, excitation-dependent photoluminescence and strong tolerance to variations in pH, ionic strength, and time, providing a robust optical platform for sensing applications. Their selective fluorescence quenching in the presence of Se^0 demonstrates that surface-mediated interactions between carbon nanomaterials and elemental selenium can be effectively harnessed for rapid, matrix-tolerant detection. This strategy extends selenium analysis beyond volatile species and highlights the utility of nanomaterial-based probes for monitoring particulate or solid-phase selenium.

Further integration of Se^0 into a CND- Se^0 nanohybrid enabled the development of a responsive “turn-on” fluorescence system for Hg^{2+} detection, exploiting the strong chemical affinity between mercury and selenium. The observed fluorescence recovery upon Hg^{2+} binding reflects a reversible modulation of electronic interactions within the nanohybrid rather than nonspecific ionic effects. This dual quenching–recovery behaviour illustrates how selenium’s inherent chemical reactivity can be strategically embedded within nanostructured sensing platforms, transforming an analytical limitation into a functional advantage. The successful application of this system to real water matrices confirms its practical relevance for environmental monitoring and contaminant screening.

Beyond analytical performance, the biological implications of nano-selenium exposure were addressed through a data-driven toxicity modelling framework. Machine learning analysis of curated *in vivo* datasets identified dose and exposure duration as the dominant predictors of nano-selenium toxicity across species, whereas nanoparticle size exerted a comparatively minor influence. SHAP-based interpretability confirmed that the model’s decision logic aligns with established toxicological principles, reinforcing confidence in its mechanistic relevance. Importantly, experimental validation in Japanese quails demonstrated close agreement between predicted and observed viability outcomes, providing independent biological confirmation of the model’s reliability.

The complementary *Drosophila melanogaster* digestion-based exposure model further revealed a narrow threshold between beneficial and adverse effects of Se⁰, characterised by hormetic responses at moderate concentrations and toxicity at higher doses. These findings reinforce selenium's dual biological role and emphasise the importance of precise dose control in nano-selenium applications. When interpreted alongside the machine learning results, the *in vivo* data support the concept that predictive modelling can effectively narrow biologically relevant exposure windows, thereby reducing the need for extensive animal testing while maintaining toxicological relevance.

Collectively, this integrated framework demonstrates that no single analytical or biological approach is sufficient to comprehensively characterise selenium behaviour. Meaningful assessment instead requires the convergence of geochemical baseline data, species-specific analytical methods, nanomaterial-enabled sensing, predictive modelling, and targeted biological validation. By linking these domains, the present study establishes a coherent pathway from environmental selenium occurrence to mechanistic understanding and risk-relevant biological outcomes. This integrative strategy provides a transferable blueprint for evaluating other redox-active trace elements in complex ecological, food, and biological systems.

5. CONCLUSIONS, RECOMMENDATIONS

This doctoral research presents an interdisciplinary investigation of selenium encompassing environmental assessment, analytical method development, nano-enabled sensing, biological evaluation, and data-driven modelling. By linking soil-level selenium occurrence with species-specific analytical techniques, nanoscale sensing platforms, and toxicity prediction, the study establishes a coherent framework for evaluating the behaviour, functionality, and safety of selenium, particularly in its elemental and nanoscale forms. Evaluation of GEMAS XRF data demonstrated that selenium concentrations in Hungarian agricultural soils are consistent with broader European baselines, with spatial variability controlled primarily by local geochemical factors rather than systematic enrichment or depletion. From an analytical perspective, a modified atomic fluorescence spectroscopy (AFS) method was developed for sensitive and reproducible determination of volatile hydrogen selenide (H_2Se) in aqueous systems. The externally assisted hydride-generation configuration enabled stable operation in alkaline matrices, low detection limits, and high recovery, with performance verified by graphite furnace atomic absorption spectrometry (GF-AAS). Complementary nano-enabled sensing strategies using green-synthesised carbon nanodots (CNDs) enabled selective fluorescence detection of elemental selenium (Se^0) in real water and beverage matrices. Integration into a CND– Se^0 nanohybrid further produced a selective “turn-on” fluorescent probe for mercury (Hg^{2+}), demonstrating the functional versatility of selenium-based nanomaterials. Biological relevance was addressed through a combination of machine-learning and experimental approaches. Predictive modelling identified dose and exposure duration as the primary determinants of toxicity, with strong agreement between predicted and observed viability in Japanese quails. *Drosophila melanogaster* experiments further confirmed a narrow threshold between beneficial and adverse effects, consistent with selenium’s hormetic behaviour.

Future research should focus on expanding selenium speciation analysis, validating nano-sensing platforms in complex biological matrices, refining toxicity models through additional material descriptors, and integrating predictive tools into “Safe-by-Design” strategies for selenium nanomaterials.

6. NEW SCIENTIFIC RESULTS

The outcomes of this doctoral research provide practical results applicable to environmental monitoring, analytical laboratories, food and feed safety evaluation, and nano-enabled risk assessment. The developed methods and frameworks are scalable, cost-effective, and compatible with real-world matrices.

1. Using harmonised GEMAS XRF data, total selenium concentrations in agricultural soils were systematically evaluated at continental and national scales. For European agricultural soils ($n=2113$), the mean selenium concentration was 0.405 mg kg^{-1} , with a median of 0.354 mg kg^{-1} . Spatial variability was moderate, indicating heterogeneous but stable background levels. For Hungarian agricultural soils ($n=37$), the mean selenium concentration was 0.355 mg kg^{-1} , with a median of 0.327 mg kg^{-1} . This indicates consistent internal variability with continental trends, despite the smaller dataset.
2. A novel, externally assisted hydride-generation atomic fluorescence spectroscopy (HG-AFS) system was developed and validated for the direct quantification of volatile hydrogen selenide (H_2Se). The method exhibited excellent linearity over $5\text{--}1000 \text{ } \mu\text{g L}^{-1}$ with $R^2 = 0.9973$. The limit of detection (LOD) was $1.40 \text{ } \mu\text{g L}^{-1}$, and the limit of quantification (LOQ) was $4.20 \text{ } \mu\text{g L}^{-1}$, with repeatability $<5\%$ RSD. Spike-recovery experiments in ultrapure, tap, and mineral water yielded recoveries of $97.3\text{--}101.8\%$ with $\text{RSD} < 3.3\%$, confirming matrix tolerance. Cross-validation using GF-AAS confirmed low quantitative recovery at $5 \text{ } \mu\text{g L}^{-1}$ concentrations while highlighting the superior sensitivity of the developed AFS method at ultra-trace levels.
3. A rapid method was developed with a carbon nanodot (CND) fluorescence platform for the selective detection of elemental selenium (Se^0) and application in aqueous matrices. A linear calibration was achieved over $0\text{--}12.665 \text{ mmol L}^{-1}$ of Se^0 with $R^2 = 0.9911$; The method sensitivity was quantified as $\text{LOD} = 0.131 \text{ mmol L}^{-1}$ and $\text{LOQ} = 0.397 \text{ mmol L}^{-1}$. Spiking and recovery in ultrapure water, tap water, and soft drink matrices at 316.5 and $633.3 \text{ } \mu\text{mol L}^{-1}$ yielded recoveries of $98.60\text{--}108.1\%$ with $\text{RSD} 0.2\text{--}3.4\%$, confirming robustness in real matrices.
4. A CND- Se^0 nanohybrid probe was developed with a “turn-on” response to Hg^{2+} . A linear calibration was achieved over $0.5\text{--}15 \text{ } \mu\text{mol L}^{-1}$ of Hg^{2+} with $R^2 = 0.9919$. The method achieved $\text{LOD} = 8.7 \text{ nmol L}^{-1}$ and $\text{LOQ} = 26.4 \text{ nmol L}^{-1}$, enabling trace-level

detection in drinking/environmental water contexts. Spiking and recovery validation in mineral, tap, and ultrapure water at 1, 3, and 5 $\mu\text{mol L}^{-1}$ achieved recoveries of 99.92–105.2% with $\text{RSD} \leq 3.3\%$, confirming accuracy and repeatability across ionic-rich and low-conductivity matrices.

5. A cross-species ML framework was developed to predict viability outcomes from nano-selenium exposure. A curated literature dataset of 86 in vivo entries spanning 8 biological models was compiled. After screening of 13 different models, AdaBoost Regressor had the most balanced performance and interpretability with: Train: $R^2 = 0.971$, Test: $R^2 = 0.848$, and validated with a k-fold validation method. Pearson analysis showed a moderate negative association between dose and viability, while particle size exhibited no meaningful correlation. External validation with quails at 0.05 and 0.5 mg Se kg^{-1} feed yielded observed viability of 100% in both groups, while AdaBoost predicted 99.02% and 99.01%, respectively.
6. A digestion-based *Drosophila melanogaster* model was applied to experimentally quantify developmental and survival responses to elemental selenium (Se^0), providing stage-resolved in vivo toxicity thresholds relevant for nano-selenium risk assessment. At low-dose Se^0 (0.1-0.5 mg kg^{-1}), there was no impairment of developmental progression. At moderate Se^0 exposure (1 mg kg^{-1}), a reproducible hormetic response was induced. At higher Se^0 doses (2-4 mg kg^{-1}), impaired developmental kinetics and reduced survival.

Together, these results establish an integrated experimental and computational framework that links selenium occurrence, analytical accessibility, nano-enabled sensing, biological responses, and toxicity prediction.

7. PRACTICAL RESULTS

The outcomes of this doctoral research provide several practical results that can be directly applied in environmental monitoring, analytical laboratories, food and feed safety evaluation, and nano-enabled risk assessment. The developed methods and frameworks are scalable, cost-effective, and compatible with real-world matrices.

The harmonised interpretation of GEMAS soil data provides a reliable baseline for evaluating selenium levels in European and Hungarian agricultural soils. This enables identification of selenium-deficient or marginal regions without reliance on speculative bioavailability indices. The modified atomic fluorescence spectroscopy (AFS) method developed for the determination of hydrogen selenide (H_2Se) provides a robust and accessible analytical tool for laboratories lacking advanced mass spectrometric instrumentation. The method is suitable for routine analysis of selenium transformation processes, validation of selenium trapping experiments, and laboratory-scale metabolic studies.

The carbon nanodot (CND)-based fluorescence platforms developed in this work enable rapid detection of elemental selenium and mercury ions without complex sample preparation. These features make the sensing systems suitable for preliminary screening, quality control, and fluorescence-based detection.

The machine-learning toxicity framework provides a practical decision-support tool for evaluating the safety of nano-selenium across species. The successful validation using Japanese quails confirms its utility for guiding experimental design and comparative risk assessment. The digestion-based *Drosophila melanogaster* model established in this study offers a rapid and ethically acceptable platform for preliminary nano-selenium toxicity screening.

Collectively, the practical outcomes of this doctoral research provide integrated tools for selenium assessment that span environmental evaluation, analytical measurement, nano sensing, toxicity prediction, and biological screening.

8. SUMMARY

Selenium is an essential trace element with a narrow margin between nutritional benefit and toxicity, making its accurate determination, controlled application, and biological evaluation critically important in environmental, food, and feed systems. The increasing use of elemental and nano-scale selenium has further intensified the need for reliable analytical tools, mechanistic understanding, and predictive safety approaches. This doctoral research addresses these challenges through an integrated framework combining environmental geochemistry, analytical method development, nano-enabled sensing, biological toxicity assessment, and data-driven modelling.

At the environmental level, selenium distribution in European and Hungarian agricultural soils was evaluated using harmonised GEMAS XRF data. To address analytical gaps, a modified atomic fluorescence spectroscopy (AFS) method was developed for the sensitive and reproducible determination of volatile hydrogen selenide (H_2Se).

The CND-based platform enabled selective and stable fluorescence detection of elemental selenium (Se^0) in real water and beverage matrices. Further integration into a CND- Se^0 nanohybrid yielded a selective “turn-on” fluorescent probe for mercury (Hg^{2+}).

The biological implications of nano-selenium exposure were evaluated using a combined machine-learning and experimental framework. Predictive modelling identified dose and exposure duration as the dominant drivers of toxicity across species, with strong agreement between predicted and observed viability in Japanese quails. Complementary *Drosophila melanogaster* experiments confirmed a narrow threshold between beneficial and adverse effects, consistent with selenium's hormetic behaviour.

Overall, this doctoral research demonstrates that meaningful evaluation of selenium requires the integration of environmental assessment, advanced analytical detection, nano-enabled sensing, biological validation, and predictive modelling. The methodologies and frameworks developed provide practical tools and scientific insight for safer and more informed use of selenium in environmental monitoring, food and feed systems, and nano-enabled applications.

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10. PUBLICATIONS IN THE FIELD OF RESEARCH



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Registry number: DEENK/5/2026.PL
Subject: PhD Publication List

Candidate: Arjun Muthu
Doctoral School: Doctoral School of Food Sciences
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List of publications related to the dissertation

Foreign language scientific articles in international journals (6)

1. **Muthu, A.**, Nguyen, H. H. D., Neji, C., Ferroudj, A., Prokisch, J., El-Ramady, H., Béni, Á.:
Determination of hydride-generated selenium in aqueous matrices by modified atomic
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Synthesised Carbon Nanodots Using the Maillard Reaction for the Rapid Detection of
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fluorescent "turn-on" system for selective detection of mercury (II) in aqueous solution.
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IF: 6

Foreign language abstracts (3)

33. Ferroudj, A., **Muthu, A.**, Sári, D., Seresné Törös, G., Béni, Á., Czeglédi, L., Knop, R., El-Ramady, H., Prokisch, J.: Impact of Red and Grey Nano-Selenium Supplementation on Growth and Feed Efficiency in Japanese quails.
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In: *Táplálkozástudományi Kutatások : XI. PhD konferencia programja és az előadások összefoglalói. Szerk.: Bíró Lajos, Gelencsér Éva, Lugasi Andrea, Rurik Imre, Simonné Sarkadi Livia, Magyar Táplálkozástudományi Társaság, Budapest, 16, 2023. ISBN: 9786155606144*

Total IF of journals (all publications): 112,4

Total IF of journals (publications related to the dissertation): 24,4

The Candidate's publication data submitted to the Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.



23 April, 2026

11. STATEMENTS

STATEMENT

I wrote this thesis in the framework of the University of Debrecen, Doctoral School of Food Sciences, for the purpose of obtaining a doctoral degree (Ph.D.) at the University of Debrecen.

Debrecen, 2026 January 10.



signature of the candidate

STATEMENT

I hereby certify that the doctoral candidate Arjun Muthu has carried out his work under my supervision within the framework of the above-mentioned Doctoral School between 2022-2026. The candidate has made a decisive contribution to the results of the thesis through his independent creative work, and the thesis is the candidate's independent work. I recommend that the thesis be accepted.

Debrecen, 2026 January 10.



signature of the supervisor

12. ANNEXES

Annex 1. GF-AAS instrumentation program

Ten μL of each oxidized sample was injected under the following operating conditions:

- Multiple Inject: Off
- Hot Inject: Off
- Modifier Injection: None
- Work head height: 8.5 mm
- Pre-last dry step: 2
- Post-last dry step: 3

Step	Temp ($^{\circ}\text{C}$)	Time (s)	Gas Flow (L min^{-1})	Gas Type	Read
1	85	5.0	3.0	Normal	No
2	95	40.0	3.0	Normal	No
3	120	10.0	3.0	Normal	No
4	1000	5.0	3.0	Normal	No
5	1000	1.0	3.0	Normal	No
6	1000	2.0	0.0	Normal	No
7	2600	0.8	0.0	Normal	Yes
8	2600	2.0	0.0	Normal	Yes
9	2600	2.0	3.0	Normal	No

Annex 2. Experimental in vivo validation using quails

a) Ethical Approval

All procedures involving animals adhered to EU Directive 2010/63/EU concerning the use of animals in scientific research. The Ethical Committee approved the experimental protocol for Animal Use at the University of Debrecen, Hungary (Protocol No. 5/2021/DEMAB). Every effort was taken to reduce stress and promote animal welfare.

b) Experimental Diet and Nutrient Composition

A basal diet formulated according to established nutrient guidelines for breeder Japanese quails (*Coturnix japonica*) was used. Ingredients included soybean meal, corn, wheat, sunflower oil, and a vitamin-mineral premix providing 0.042 mg/kg of background selenium from sodium selenite. SeNPs were synthesized using the method described in 3.6.1.1. (Muthu et al., 2025). The morphology of SeNPs was characterized using SEM, and elemental composition was assessed with a Bruker SDD-based EDX detector, confirming the presence of selenium without detectable impurities. Raman spectra were recorded to verify characteristic Se⁰ vibrational bands. Ingredients of the basal diet was mentioned below in the Table 2.1 These analyses confirmed the successful synthesis of chemically pure SeNPs. SeNPs were incorporated into the basal feed to produce three dietary treatments:

- C₀ (Control): Basal diet only (Total Se (0.042 mg kg⁻¹))
- LD: Basal diet + 0.05 mg kg⁻¹ SeNPs (Total Se = 0.092 mg kg⁻¹)
- HD: Basal diet + 0.5 mg kg⁻¹ SeNPs (Total Se = 0.542 mg kg⁻¹)

Table 2.1. Ingredient composition and calculated nutrient content of the basal quail diet.

Feed Ingredients	Inclusion Rate (%)
Soybean meal (46% CP)	34.88
Corn	30.37
Wheat	20.00
Sunflower oil	6.79
Limestone	5.64
MCP (monocalcium phosphate)	1.29
Salt	0.38
DL-Methionine	0.15
Vitamin-mineral premix ^a	0.50

Nutrient Content (%) or MJ/kg	Value
Metabolizable energy (MJ/kg)	12.13
Crude protein	20.0
Calcium	2.50
Available phosphorus	0.35
Sodium	0.15
Methionine	0.45
Methionine + cysteine	0.75
Lysine	1.08
Threonine	0.74
Leucine	1.59
Isoleucine	0.86
Arginine	1.33
Tryptophan	0.25

* Per kg premix: 1,000,000 IU vitamin A, 200,000 IU vitamin D₃, 4900 mg vitamin E, 200 mg vitamin K₃, 150 mg vitamin B₁, 500 mg vitamin B₂, 1200 mg Ca-d-pantothenate, 400 mg vitamin B₆, 2 mg vitamin B₁₂, 11 mg biotin, 2502 mg niacin, 60 mg folic acid, 300,000 mg choline chloride, 13,200 mg Zn, 1920 mg Cu, 9612 mg Fe, 13,200 mg Mn, 180 mg I, 42 mg Se, 12 mg Co

c) Experimental Design and Animal Management

Twenty adult male quails (11 weeks old) were used in a 28-day feeding trial. Birds were housed individually in wire cages to enable precise monitoring of feed intake and health status. Environmental conditions were kept at 25 ± 2 °C with a 16-hour light / 8-hour dark photoperiod. Feed was provided at approximately 18 g per bird per day, and water was available ad libitum. The birds were randomly assigned to three dietary treatments (C₀, LD, HD) using body-weight stratification to ensure uniform initial conditions (n = 5 per group). The selected doses represent a physiologically relevant supplementation level (0.05 mg/kg) and a higher exposure level (0.5 mg/kg) commonly used in toxicological studies. Birds were monitored daily for survival, overall health, and visible signs of toxicity (Ferroudj, Muthu, et al., 2025).

d) Outcome measurement

Viability (%) was assessed at the end of the trial according to:

$$\% \text{ viability} = \frac{\text{Number of surviving quails}}{\text{Total quails}} \times 100 \quad (\text{eq. 8})$$

One-way ANOVA was applied to compare viability among treatments (C0, LD, HD), and Tukey's HSD test was used for post-hoc comparisons when significance was detected ($p < 0.05$).

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