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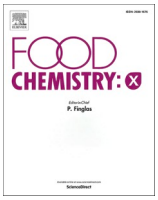

A screening for optimal selenium enrichment additives for selenium-enriched fish production: Application of a HPLC-ICP-MS method

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ABSTRACT

The production of selenium-enriched fish contributes to alleviating selenium deficiency for humans. In this study, selenium nanoparticles (SeNPs) comparable in bioavailability to selenomethionine (SeMet), increased SeMet content in *O. macrolepis* (*Onychostoma macrolepis*) muscle. Additionally, dietary SeNPs significantly enhanced selenocysteine ($SeCys_2$) and methylselenocysteine (MeSeCys) levels in *O. macrolepis* muscle. The effect of SeNPs on selenium speciation in grass carp muscle was consistent with *O. macrolepis* results. $SeCys_2$ and MeSeCys showed antioxidant capacity in HEK293T cells, indicating enhanced health benefits of Se-enriched fish produced using SeNPs. Furthermore, the addition of 0.3 mg/kg SeNPs significantly improved the flesh quality of *O. macrolepis* by reducing the content of crude fat and heavy metals, as well as increasing the levels of crude protein, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and the ratio of n-3/n-6 polyunsaturated fatty acids (PUFAs). Therefore, selenium-enriched fish produced from SeNPs is a good source for improving human dietary selenium intake.

1. Introduction

Selenium is an essential trace element in animals and the human body, primarily exerting its important functions through selenoproteins (Daniels, 1996). Approximately 1 in 7 individuals worldwide suffer from selenium deficiency (Tinggi, 2003). Selenium deficiency has been associated with various human disorders, including Kashin-Beck disease, Keshan disease and myxedematous endemic cretinism (Lobanov, Hatfield & Gladyshev, 2008). In China, over 70 % of the population is affected by selenium deficiency, with over 70 million people at risk of health problems associated with selenium deficiency (Gao et al., 2011). Consequently, addressing this problem is an urgent priority. Fish is an exceptional dietary source of selenium for humans due to its higher selenium concentration compared to many other foods. Moreover, selenium in fish predominantly exists in an organic form, which can be efficiently absorbed and retained by the human body (Wang et al., 2022). *O. macrolepis* (*Onychostoma macrolepis*) is highly popular in regions such as Shaanxi and Sichuan in China due to its delicious taste and high nutritional value (Sun et al., 2020). Grass carp (*Ctenopharyngodon idella*), with a global production exceeding 5.7 million tons, is currently the most highly produced fish species in the world (Li, Xue, Sun & Ji, 2023). Therefore, cultivating selenium-enriched fish could be a safe and effective solution to address human selenium deficiency. However, there is still a lack of research on how to produce selenium-enriched fish quickly and with high quality. Therefore, selecting a suitable selenium additive will be the focus of this study.

In the environment, selenium exists in various oxidation states and forms, including selenite and selenate (Na_2SeO_3, Na_2SeO_4), solid-state selenium in the form of nanoparticles (SeNPs), and biogenic selenium in the form of selenomethionine (SeMet) and others (Wadhwani, Shedbalkar, Singh & Chopade, 2016). The physiological effects and toxicity of selenium, as well as its metabolic pathways and bioavailability, are not solely determined by the intake level but are also significantly influenced by the chemical forms of the element (Weekley and Harris, 2013). Selenium in the form of selenate (SeO_4^{2-}) is considered more toxic than its selenite counterparts (SeO_3^{2-}) (Kumar and Prasad, 2021).

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Organic selenium has advantages over inorganic selenium, including high bioavailability, fewer side effects, and less environmental pollution (Schrauzer, 2000). SeMet is considered an effective replacement for inorganic selenium because it has a more significant deposition efficiency at the same level of addition (Zhang et al., 2021). Compared to inorganic forms, the high absorption rate and low toxicity of selenium in its nanoform have attracted attention in research (Bhattacharjee, Basu & Bhattacharya, 2019). SeNPs, widely used as a fish feed additive, enhance growth, antioxidant capacity, and immune function in grass carp at 0.3 mg/kg (Yu et al., 2020). Similar results have been reported in common carp and crucian carp, with optimal addition concentrations of 0.7 and 0.5 mg/kg, respectively (Saffari et al., 2017; Zhou, Wang, Gu & Li, 2009). Furthermore, SeNPs improve sperm motility and exhibit an antagonistic effect against heavy metals in fish (Khademzade et al., 2022; Saad et al., 2022; Yin, Wang, Huang & Zhang, 2021). Therefore, this study aims to select the most suitable selenium feed additives for fish from SeNPs, selenite, and SeMet.

When selecting selenium additives for producing selenium-enriched fish, it is crucial to consider not only the selenium deposition efficiency but also its impact on the selenium speciation in the flesh of selenium-enriched fish. This is an important indicator for assessing the value of selenium-enriched fish meat. Distinguishing between different forms of selenium in biological samples is a more challenging task than simply measuring the total selenium content. However, there is currently limited research on the analysis of selenium speciation in fish muscle. The presence of heavy metals in fish meat can impact human health. Consequently, the content of heavy metals in fish meat can be used as an indicator to evaluate the quality of fish meat (Łuczyńska et al., 2022; Manz et al., 2023). In addition, muscle proximate composition, and fatty acid composition are also indicators for assessing the nutritional value of fish meat (Li, Xue, Sun & Ji, 2023). Adding the appropriate form of selenium to achieve optimal levels in fish feed not only promotes fish health but also results in selenium-fortified aquaculture products. This enhances the functionality of an already nutritious product (Cotter, Craig & McLean, 2008).

Therefore, the objective of this study is threefold: (1) to evaluate the deposition efficiency and antioxidant capacity of different dietary selenium sources (selenite, SeMet, and SeNPs) and their impact on selenium species in *O. macrolepis*; (2) to further assess the potential health benefits of selenocysteine ($SeCys_2$) and methylselenocysteine (MeSeCys) to human cells (HEK293T); and (3) to investigate the influence of these selenium sources on the quality of *O. macrolepis* flesh, including its composition, levels of heavy metals, and fatty acid content. These findings will contribute to the selection of the most suitable selenium feed additive for producing selenium-enriched fish.

2. Materials and methods

2.1. Animals

All animal experiments were conducted in accordance with animal welfare and ethics regulations and approved by the Animal Care and Use Committee of Northwest A&F University.

2.2. Preparation of SeNPs

SeNPs were obtained from probiotic *Bacillus subtilis*, which was cultured in Luria-Bertani (LB) liquid medium containing 8 mM Na_2SeO_3, following the protocol described in our previous study (Zhu et al., 2023), and the microscopic morphology of the SeNPs was observed using scanning electron microscopy. To further confirm that the extracted nanoparticles were SeNPs, the XL30 ESEM (FEI, Hillsboro, OR, USA) equipped with an EDAX microanalytical system was used. Furthermore, the concentration of SeNPs was also measured by ICP-MS (Agilent 7900). The size distribution of dispersed SeNPs was determined using the PCCs technique (NANOPHOX 1 nm to 10,000 nm, Sympatec GmbH, Germany).

2.3. Experiment diets

Based on previous research, fish have shown a selenium requirement ranging from 0.1 to 0.9 mg/kg (Saffari et al., 2017; Zhou, Wang, Gu & Li, 2009; Cotter et al., 2008). Considering our previous work, we determined the optimal SeNPs requirement for grass carp is 0.3 mg/kg (Yu et al., 2020). In this experiment, selenium concentrations of 0.1, 0.3, and 0.9 mg/kg were employed. Diets for *O. macrolepis* included a basal diet (BD) (Table S1) and diets with SeNPs, selenite, and SeMet at three concentrations (0.1, 0.3, 0.9 mg/kg). Similar diets, including BD (Table S2) and those with 0.3 mg/kg SeNPs, selenite, and SeMet, were prepared for grass carp. Sodium selenite (purity 99 %) and SeMet (purity 98 %) were purchased from Sigma-Aldrich and Chuanqi Medical Co. Ltd, respectively. Ingredients and selenium sources were mixed, dried, and stored at −20 °C.

2.4. Experiment design and collection of samples

A total of 600 *O. macrolepis* (152.3 ± 4.7 g) were randomly distributed into 30 tanks (10 treatment groups, three replicates each), and 240 grass carp (255.24 ± 2.68 g) were placed in 12 tanks (4 treatment groups, three replicates each). Each 1000 L tank received flowing water throughout the 60-day period, and aeration maintained dissolved oxygen levels above 7 mg/L. Water temperature, pH, and oxygen were monitored regularly and kept at 19–23 °C, 8.0 ± 0.3, and 7.5 ± 0.1, respectively. After a two-week acclimation period, fish were fed three times daily (08:30, 12:30, 16:30) to satiation with experimental diets.

At the experiment's conclusion, fish were anesthetized with 0.1 g/L MS-222 (Sigma, St. Louis, MO, USA). From each tank (n = 12), four fish were randomly chosen for blood collection via the caudal vein, and the obtained serum was stored at − 80 °C after centrifugation (1,369.55 × g, 4 °C, 15 min). Left fillets from the dorsal fin to the head above the lateral line were swiftly excised, snap-frozen in liquid nitrogen, and stored at − 80 °C for total selenium and heavy metal analysis. For selenium species analysis, fillets were collected from six fish (2 per tank) randomly chosen from each treatment (n = 6). Muscle composition analysis involved four randomly selected fish from each tank (n = 12), with samples taken from the left dorsal fin to the lateral line of the head.

2.5. Serum antioxidant capacity analysis

We analyzed serum samples for total antioxidant capacity (*T*-AOC), superoxide dismutase (SOD) activity, malonaldehyde (MDA) content, Glutathione peroxidase (GPx), and catalase (CAT) using assay kits provided by Nanjing Jiancheng Bioengineering Institute in China. The experimental procedures were carried out in accordance with the manufacturer's instructions.

2.6. Total selenium content and selenium species analysis

The selenium content in fish muscle was detected using the Chinese national standard method (GB 5009.93–2017). Briefly, 0.2 g of dried muscle samples were placed in 50 mL digestion tubes containing 8 mL of high-grade pure nitric acid (HNO_3) (Beijing Institute of Chemical Reagents, China). After standing overnight at room temperature, the mixture was heated using a microwave digestion system (CEM-MARSX®, CEM Corporation, Matthews, NC, USA) with the following program: 5 min at 120 °C, 10 min at 150 °C, and 20 min at 190 °C. The liquid was then evaporated to approximately 1 mL using an electric evaporation block and transferred to a 25 mL volumetric flask. After adding ultrapure water to the flask to reach the mark, the solution was prepared for machine detection. Stock standard solutions of 1 μg/mL selenium were obtained from SPEX CertiPrep (Metuchen, USA).

Agilent 7900 ICP-MS was used to analyze the selenium species of

muscle. Muscle samples were frozen dried and ground into powder with liquid nitrogen. Next, 0.2 g of the powder was added to a 50 mL centrifuge tube with 10 mL of 100 mM Tris-HCl buffer (pH 7.4) (Solarbio Life Science, China), 0.02 g of protease type XIV (Solarbio Life Science, China), 0.02 g of Protease K (Solarbio Life Science, China), and 0.2 g of trypsin (Solarbio Life Science, China). The mixture was shaken at 37 °C for 24 h, sonicated for 1 h, and then centrifuged at 10,000 × g for 10 min. The supernatant was filtered through a 0.22 μm filter (Millipore, Bedford, MA, USA) and then processed with a 15 mL 10.0 k_D ultra centrifugal filter (Millipore, Bedford, MA, USA) before analysis by Agilent 7900 ICP-MS. Citric acid and sodium 1-hexanesulfonate used in the experiment were obtained from Aladdin Chemica (Shanghai, China).

2.7. Analyses of proximate compositions and heavy metals of O. macrolepis muscle

Following the methods described by AOAC (AOAC, 1995), we analyzed the proximate composition of diets and muscle. The sample was oven-drying at 105 °C until complete moisture evaporation, and the water content was determined, and the remaining sample was analyzed for crude protein content using the Kjeldahl method (N × 6.25), crude fat content by the Soxhlet extraction method, and crude ash content using a muffle furnace. The method described by Bosch et al. (2016) was used to measure the concentrations of copper (Cu), cadmium (Cd), and arsenic (As) in the muscle samples.

2.8. Analyses of fatty acid composition of O. macrolepis muscle

The muscle fatty acid composition was analyzed using the methods recommended by Gou et al. (2020). Initially, sample lipids were extracted using a mixture of chloroform–methanol (2:1, v/v). The lipid fraction was dissolved in 1 mL of normal hexane, and a 1-hour methyl esterification process with 1 mL of 0.4 M potassium hydroxide methanol. Upon adding 2 mL of double-distilled water, the resulting mixture was divided into two layers. The upper layer was separated, followed by GC analysis, and a single fatty acid methyl ester (FAME) was quantified by comparison to a recognized standard (47015-U, Sigma-Aldrich, Inc.).

2.9. Cell culture

HEK293T cells were obtained from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in a humidified atmosphere at 37 °C with 5 % CO_2 in uncoated plastic flasks (Corning, New York, USA) using DMEM/Glutamax supplemented with 10 % FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 % extra GlutaMAX (GIBCO BRL, New York, USA).

2.10. Assessment of the cell viability

To evaluate the effects of $SeCys_2$ and MeSeCys on human cells, we examined their impact on the antioxidant capacity of 293T cells at different concentrations. Initially, 293T cells were seeded in 96-well plates at a density of 1×10^4 cells/well. After cell adherence, when the confluence reached 40 %, the original culture medium was discarded, and the corresponding concentrations of $SeCys_2$, hydrogen peroxide (H_2O_2) or MeSeCys diluted in serum-free medium (similar to the aforementioned medium but without serum) were added. After 36 h, the culture medium was removed, and the cells were washed twice with PBS before adding 100 μL of CCK-8 solution. After a 2-hour incubation, the absorbance of each well was measured at a wavelength of 490 nm using a microplate reader. Finally, the absorbance values of each treatment group were compared to the control group to calculate cell viability.

2.11. Analysis of reactive oxygen species (ROS)

To evaluate the impact of $SeCys_2$ and MeSeCys on 293T cell antioxidant capacity, we employed the 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) kit (Beyotime, China) per the manufacturer's instructions. Post-treatment, cells were fixed with 4 % paraformaldehyde and incubated with DCFH-DA (1:1000) at 37 °C for 20 min. After three PBS washes, cell observation and imaging were performed using a Leica DM4000B microscope. Treated cells were collected, washed thrice with PBS, and ROS fluorescence intensity was quantified via flow cytometry. The results were analyzed for ROS fluorescence intensity using FlowJo software version 7.6.

2.12. Annexin V-FITC/PI staining

First, 293T cells were subcultured in 6-well plates. At 40 % cell density, the original medium was replaced with serum-free medium containing $SeCys_2$ or MeSeCys. After 36 h, cells were collected, washed thrice with PBS, and resuspended in 100 μL binding buffer. Then, 10 μL propidium iodide (PI) and 5 μL Annexin V-FITC were added. After a 15-minute incubation on ice in the dark, apoptotic cells were detected using flow cytometry, and results were analyzed with FlowJo software version 7.6.

2.13. Statistical analysis

The statistical analysis was carried out using the software SPSS 22.0 (SPSS, Inc., Chicago, IL, USA). For testing the normality and homogeneity of variance in all data, one-way ANOVA was utilized, whereas experimental data were subjected to Duncan's post-hoc test. The results were represented as the mean ± standard deviation (SD). Significance was considered at $P < 0.05$. The differences in Fig. 3A and B were compared by the two-sample two-tailed T-test method ($^{**}P < 0.01$, $^{*}P < 0.05$).

3. Results

3.1. Characterization of SeNPs

The SeNPs extracted from *Bacillus subtilis* were visualized using an XL30 ESEM with EDAX microanalysis (Fig. 1 A, B, and C). The nanoparticles exhibited a granular morphology with significant size non-uniformity. Elemental analysis indicated selenium as the primary constituent, along with silicon, oxygen, and platinum (Fig. 1 C). Silicon and platinum presence was attributed to the substrate and coating used during imaging. Oxygen might result from proteins on the SeNPs surface. The average particle size of SeNPs was 570.4 nm (Fig. 1D). Furthermore, our observations revealed the presence of selenate, selenite, SeMet, $SeCys_2$, and MeSeCys on the surface of SeNPs. Among these, $SeCys_2$ exhibited the highest content, followed by MeSeCys (Figure S1). Notably, an unidentified peak at approximately 5.6 min was also observed.

3.2. The impact of various selenium treatments on the bioavailability and selenium speciation in O. macrolepis muscle

Table S3 demonstrated reliable linear responses for total selenium and different selenium species. Average recoveries using the ICP-MS method for quantifying the total selenium content in muscle ranged from 97.2 % to 106.7 %. The measured selenium concentration of GBW10045a (0.062 ± 0.01 mg/kg) closely matched the known value (0.06 ± 0.01 mg/kg), demonstrating result accuracy. The selenium content in muscle increased from 0.191 ± 0.031 mg/kg in the selenite-0.1 group to 0.485 ± 0.041 mg/kg in the selenite-0.9 group, representing an increase of 28 % to 226 % compared to the control group. For SeNPs, muscle selenium content increased from 0.222 ± 0.007 mg/kg in

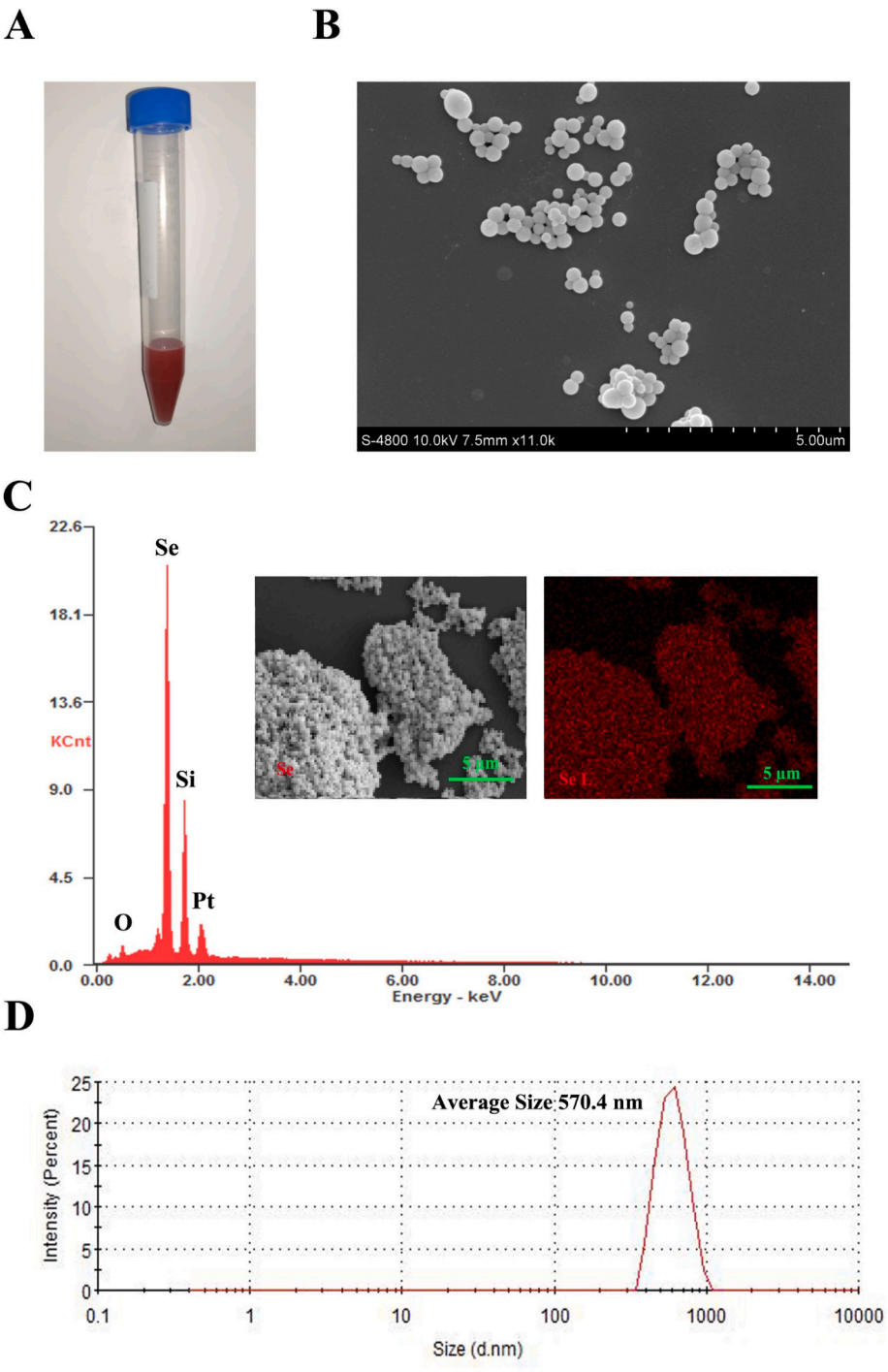

Fig. 1. Scanning electron microscopy analysis of SeNPs. SeNPs (A) were extracted from B. subtilis, which had been subjected to selenium enrichment. Scanning electron microscopy (B) was utilized to observe the ultrastructure of the SeNPs. SeNPs were further determined by an EDAX microanalytical system, the figure on the left shows the ultrastructure of SeNPs, and the red image on the right shows the location of selenium (C). The average particle size of SeNPs was detected by the PCCS technique (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the SeNPs-0.1 group to 0.664 ± 0.053 mg/kg in the SeNPs-0.9 group, representing an increase of 49 % to 346 % compared to the control group. For SeMet, muscle selenium content increased from 0.222 ± 0.013 mg/kg in the SeMet-0.1 group to 0.669 ± 0.047 mg/kg in the SeMet-0.9 group, representing an increase of 49 % to 349 % compared to the control group (Fig. 2A). These results suggest the deposition efficiency order of the three selenium sources was SeNPs ≈ SeMet > selenite.

To assess the effectiveness of our enzyme extraction method, a preliminary experiment was carried out to ascertain the efficiency of enzyme extraction at a concentration of 0.3 mg/kg (Table S4). The enzyme hydrolysis method proved suitable with an average extraction efficiency ranging from 91.16 % to 91.47 %, indicating its effectiveness. Fig. 2B-G revealed that as SeNPs, selenite, and SeMet feeding concentrations increased from 0.1 mg/kg to 0.9 mg/kg, the most pronounced changes in selenium species in *O. macrolepis* muscle were observed for SeMet, followed by $SeCys_2$ and MeSeCys, exhibiting dose-dependent effects. Compared to selenite, SeNPs and SeMet supplementation

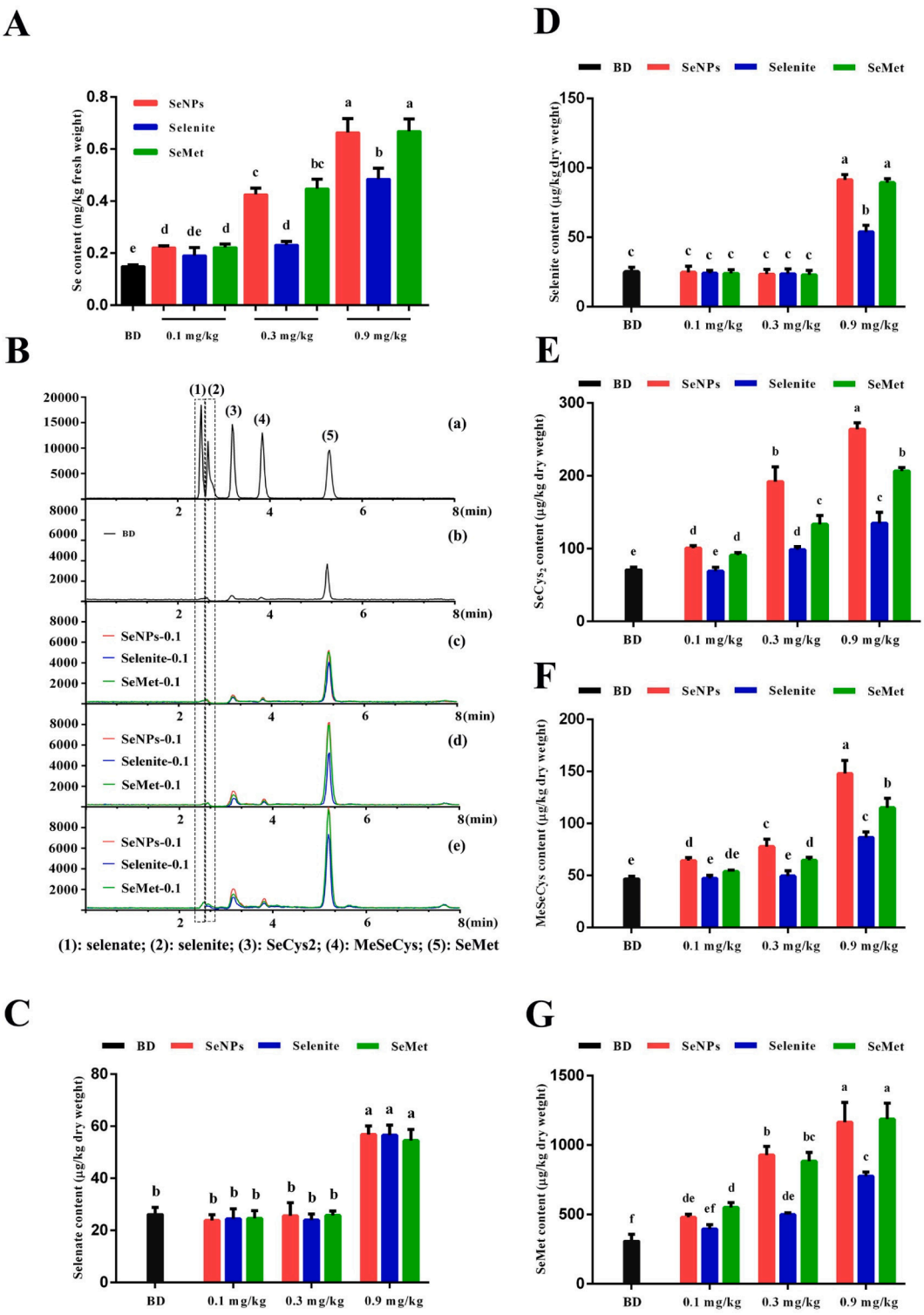

Fig. 2. Evaluate the bioavailability and speciation of selenium in the muscle tissue of *O. macrolepis* when subjected to different selenium treatments. The deposition efficiency of various selenium sources at different concentrations in the muscle tissue of *O. macrolepis* was investigated (A) (n = 9). Chromatograms of five standard selenocompounds (B: a), and muscle after feeding basal diet (BD) group (B: b), 0.1 mg/kg SeNPs, selenite and SeMet treatment (B: c), 0.3 mg/kg SeNPs, selenite and SeMet treatment (B: d), 0.9 mg/kg SeNPs, selenite and SeMet treatment (B: e). The levels of selenite (C), selenite (D), $SeCys_2$ (E), MeSeCys (F), and SeMet (G) in the muscle tissue of *O. macrolepis* were analyzed (n = 6). Different lower case letters between different groups denote statistically significant differences ($P < 0.05$). Data expressed as means values ± SD.

significantly increased SeMet content in the muscle ($P < 0.05$), with SeNPs showing a similar effect to SeMet. Interestingly, a more pronounced elevation of $SeCys_2$ and MeSeCys content in the muscle tissue was observed after SeNPs feeding compared to selenite and SeMet ($P < 0.05$). At selenium addition concentrations of 0.1 and 0.3 mg/kg, there were no significant changes in the levels of selenate and selenite in the muscle. However, at a selenium addition concentration of 0.9 mg/kg, all three selenium additives showed a significant increase in selenate and

selenite levels ($P < 0.05$). Regarding selenate, there was no significant difference in the promoting effect among the three selenium additives at the 0.9 mg/kg concentration. For selenite, feeding with 0.9 mg/kg of SeNPs and SeMet showed a stronger promoting effect compared to selenite ($P < 0.05$).

3.3. The health benefits (antioxidant capacity) of $SeCys_2$ and MeSeCys

Both SeNPs and SeMet exhibited higher bioavailability. However, selenium speciation analysis revealed that compared to SeMet, SeNPs significantly increased the levels of $SeCys_2$ and MeSeCys in the muscle tissue (Fig. 2B, E and F). To further investigate the impact of $SeCys_2$ and MeSeCys on human cells, we conducted *in vitro* studies using 293T cells. Results revealed that without 500 μM H_2O_2 supplementation, $SeCys_2$ and MeSeCys at concentrations from 0.01 to 10 μM did not enhance cell viability and, in some cases, even resulted in a decrease cell viability (10 μM $SeCys_2$) (Fig. 3A and B). However, when 500 μM H_2O_2 and $SeCys_2$/MeSeCys were co-administered, $SeCys_2$ at concentrations of 0.01 to 1 μM significantly improved cell viability of 293T cells ($P < 0.05$). Similarly, MeSeCys at concentrations of 0.01 to 10 μM significantly increased cell viability ($P < 0.05$). Under simultaneous exposure to 500 μM H_2O_2, both 1 μM $SeCys_2$ and MeSeCys maximally enhanced cell viability ($P < 0.05$), suggesting that 1 μM $SeCys_2$ and MeSeCys were the most suitable concentrations for supplementation. Flow cytometry analysis demonstrated that 500 μM H_2O_2 significantly induced cell apoptosis ($P < 0.05$), while 1 μM $SeCys_2$ and MeSeCys effectively inhibited H_2O_2-induced apoptosis in 293T cells (Fig. 3C and D) ($P < 0.05$). To further investigate related mechanisms, we assessed intracellular reactive oxygen species (ROS) levels. Results demonstrated that 1 uM $SeCys_2$ and MeSeCys significantly reduced intracellular ROS levels induced by 500 μM H_2O_2 (Fig. 3E-G) ($P < 0.05$). These findings suggest that $SeCys_2$ and MeSeCys protect against H_2O_2-induced cell damage by decreasing ROS levels in 293T cells, emphasizing the enhanced health benefits of selenium-enriched fish using SeNPs as a dietary supplement.

3.4. The impact of 0.3 mg/kg three different selenium sources on the antioxidant ability of *O. macrolepis*

The antioxidant capacity of *O. macrolepis* after feeding different selenium sources was detected in this study (Table S5). Feeding different selenium sources significantly improved the antioxidant capacity of *O. macrolepis*, particularly at a concentration of 0.3 mg/kg. Moreover, we found that among the three selenium sources, the SeNPs group showed the strongest antioxidant capacity in the 0.3 mg/kg group ($P < 0.05$).

3.5. The impact of 0.3 mg/kg three different selenium sources on heavy metal levels, proximate compositions, and fatty acid profiles in *O. macrolepis* muscle

The good linear responses for heavy metals are shown in Table S6. In Fig. 4A, a supplemented diet with 0.3 mg/kg of the three selenium sources significantly reduced As levels in *O. macrolepis* muscle (by approximately 16 % to 30 %; $P < 0.05$). SeNPs exhibited the most significant reduction (by approximately 30 %). Additionally, all three selenium sources significantly reduced Cd levels in *O. macrolepis* muscle (by approximately 20 %-31 %; $P < 0.05$), with SeNPs and selenite showing the most pronounced reduction effect (Fig. 4B) (by approximately 31 %; $P < 0.05$). Regarding Hg levels in *O. macrolepis* muscle, feeding with 0.3 mg/kg of SeNPs and selenite significantly reduced Hg content (by approximately 13 % − 19 %; $P < 0.05$), while SeMet had no effect on the Hg levels in the muscle (Fig. 4C). For Zn, Sr, and Pb levels in *O. macrolepis* muscle, feeding with the three selenium sources did not significantly reduce their content in the muscle (Fig. 4D-F).

For the crude composition of *O. macrolepis* muscle (Fig. 4G-J), SeNPs significantly reduced the crude lipid content in *O. macrolepis* muscle ($P < 0.05$), while the selenite and SeMet groups showed no significant changes ($P > 0.05$). Additionally, all three forms of selenium significantly increased the crude protein content in *O. macrolepis* muscle ($P < 0.05$), with no significant differences observed among the different selenium forms. Furthermore, feeding with the three forms of selenium did not significantly impact moisture and ash content in the *O. macrolepis* muscle.

As shown in Table 1, feeding *O. macrolepis* with the three selenium sources (SeNPs, selenite and SeMet) significantly increased C16:0 and C18:0 fatty acids, leading to an increase in ΣSFA (saturated fatty acids) ($P < 0.05$). Additionally, the decreased levels of C16:1n-7 and C18:1n-9 fatty acids resulted in a significant reduction in ΣMUFA (monounsaturated fatty acids) ($P < 0.05$). It is noteworthy that the decreased levels of C18:2n-6 and C18:3n-6 led to a significant decrease in ΣN-6 PUFA (polyunsaturated fatty acid) ($P < 0.05$), while the increased levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) resulted in a significant increase in ΣN-3 PUFA, which led to an increase of N-3/N-6 ΣPUFA ratio ($P < 0.05$).

3.6. The impact of feeding three different selenium sources on the speciation in the muscle of grass carp

To extend our investigation of selenium species impact on fish muscle, we chose grass carp, a globally significant species. Similar to *O. macrolepis*, significant changes in selenium species were observed in grass carp muscle, with SeMet showing the most pronounced changes, followed by $SeCys_2$ and MeSeCys (Fig. 5). Muscle levels of selenate and selenite remained unchanged ($P > 0.05$). Notably, SeNPs exhibited comparable promotion of SeMet as dietary SeMet, and a more pronounced increase in $SeCys_2$ and MeSeCys compared to selenite and SeMet ($P < 0.05$) (Fig. 5 A, D and E).

4. Discussion

4.1. Effects of three selenium sources and their supplementation levels on total selenium and selenium species content of *O. macrolepis*

Selenium as a feed additive can significantly increase the selenium content in fish muscle (Wang et al., 2022), which is consistent with our research findings (Fig. 2A). The optimal daily selenium requirement for humans is approximately 47–70 μg per day (Lin, 2014, Wang et al., 2022). Therefore, if consider the consumption of 100 g of *O. macrolepis* fillet per day (Lin, 2014), the selenium-enriched *O. macrolepis* fillets produced with the addition of 0.3 mg/kg of SeNPs and SeMet, as well as 0.9 mg/kg of SeNPs, selenite, and SeMet as additives, would meet the recommended daily intake of selenium for humans. However, due to the higher bioavailability of SeNPs and SeMet, the use of SeNPs and SeMet as feed additives may have greater application value in the production of selenium-enriched fish.

However, the quality assessment of selenium-enriched products should not solely rely on their total selenium content. The selenium species composition of selenium-enriched products is also an important indicator for evaluating their value (Zhang et al., 2020). The supplementation of various selenium sources at different levels in the feed predominantly influenced the concentrations of SeMet, followed by $SeCys_2$ and MeSeCys in *O. macrolepis* muscle (Fig. 2B-G). These differences in selenium species levels could be attributed to the metabolic pathways of selenium (Chen et al., 2022; Sarkar et al., 2015). The addition of SeMet and SeNPs demonstrated more pronounced effects on SeMet accumulation in *O. macrolepis* muscle (Fig. 2B and G). This may be attributed to the easier substitution of sulfur atoms in methionine by selenium in SeNPs and SeMet, resulting in higher bioavailability compared to selenite (Budisa et al., 1995). Regarding $SeCys_2$ levels in the muscle (Fig. 2B and E), the promoting effects of SeNPs and SeMet were significantly higher compared to selenite, with SeNPs exhibiting the strongest promotion effect. These findings can be attributed to the

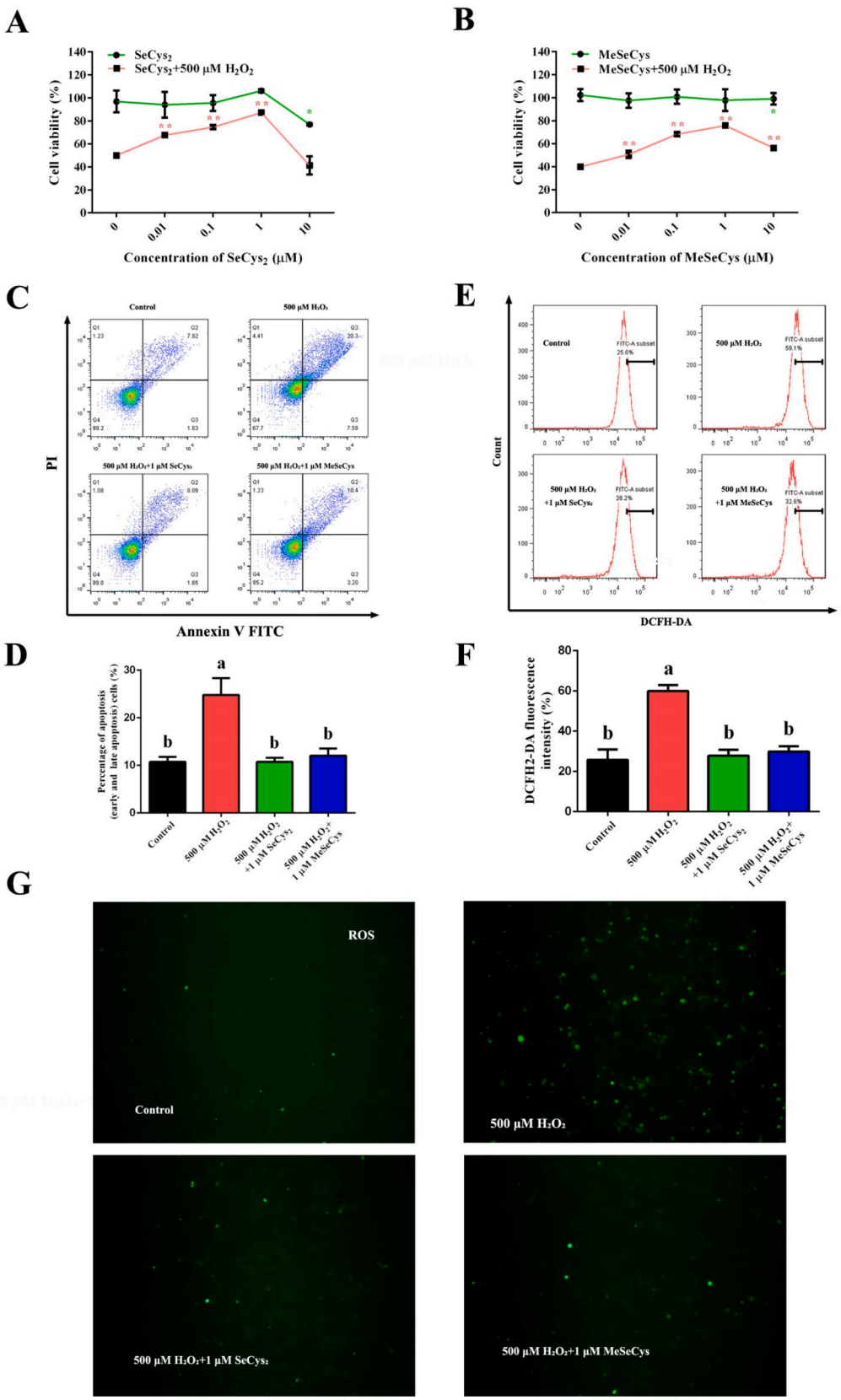

Fig. 3. The effects of $SeCys_2$ and MeSeCys on the viability parameters and apoptosis ratio in 293T cells under the condition of 500 μM hydrogen peroxide supplementation. The effects of different concentrations of $SeCys_2$ (A) and MeSeCys (B) on the viability of 293T cells under conditions with and without the addition of 500 μM H_2O_2. The impact of 1 μM $SeCys_2$ and MeSeCys on apoptosis in 293T cells under the condition of 500 μM H_2O_2 supplementation (C, D). The levels of ROS in 293T cells were measured using flow cytometry analysis (E, F). The fluorescence intensity of ROS in 293T cells was observed using fluorescence microscopy (G). Different lower case letters between different groups denote statistically significant differences ($P < 0.05$). Data expressed as means values ± SD. *$P < 0.05$, **$P < 0.01$.

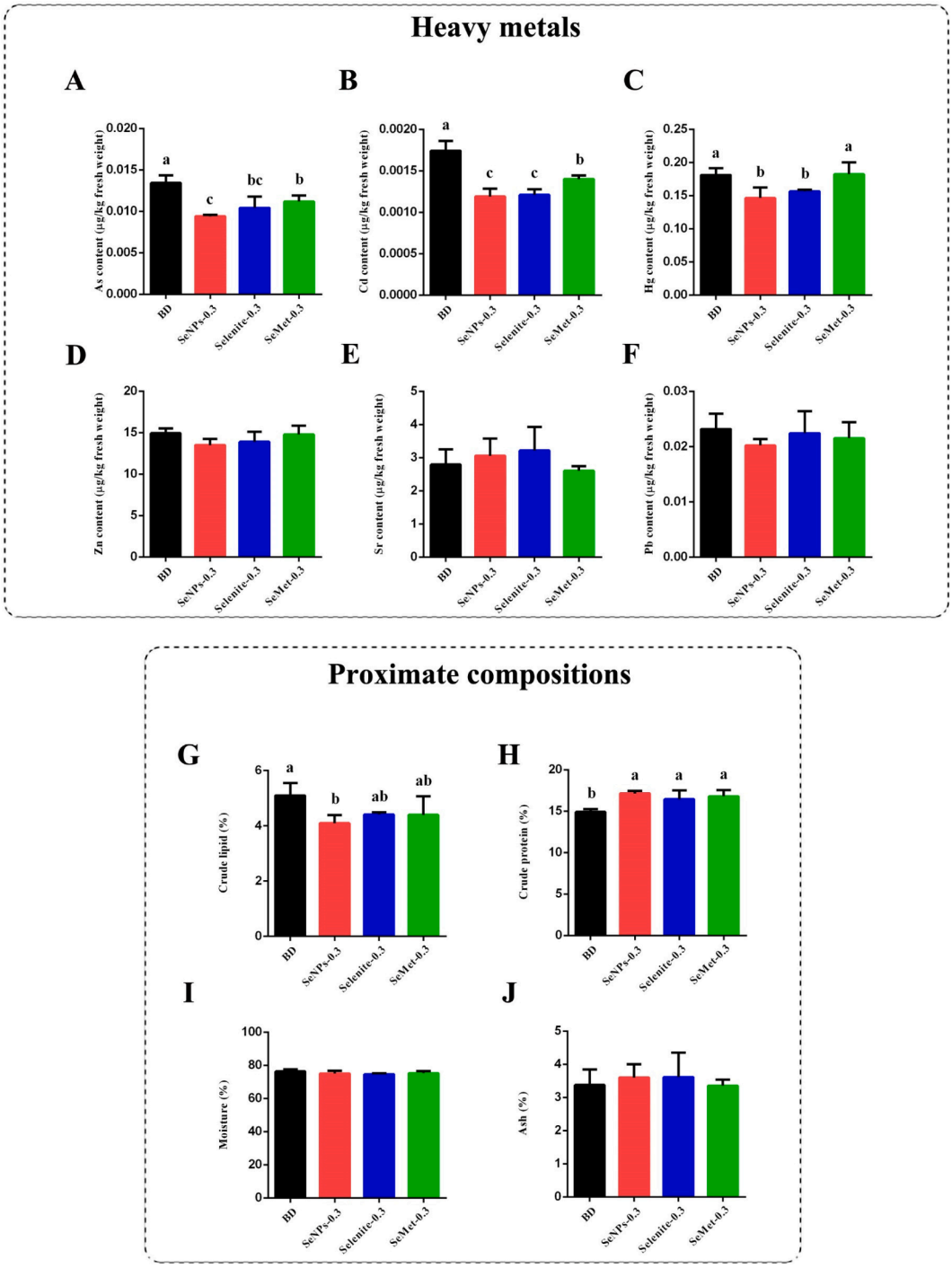

Fig. 4. Analysis of heavy metal element content and proximate composition in the muscle of *O. macrolepis* (n = 9). As (A), Cd (B), Hg (C), Zn (D), Sr (E) and Pb (F) contents in *O. macrolepis* muscle. The proportions of crude lipid (G), crude protein (H), moisture (I) and ash (G) in *O. macrolepis* muscle. Different lower case letters between different groups denote statistically significant differences ($P < 0.05$). Data expressed as means values ± SD.

direct conversion of SeMet into $SeCys_2$ or the synthesis of $SeCys_2$ via an intermediate metabolite (selenium hydride). In contrast, selenite can only undergo reduction to selenium hydride (dependent on the activity of thioredoxin reductase) before being converted into $SeCys_2$ (Kajander et al., 1991; Weekley et al., 2011). Feeding SeNPs leads to an increase in the levels of $SeCys_2$ in the muscle, which can be attributed to the more efficient internalization of SeNPs into cells, thereby facilitating their metabolism into $SeCys_2$ (Liu et al., 2020). Similarly, for MeSeCys (Fig. 2B and F), SeNPs exhibited the strongest promotion effect. We speculate that the increase in MeSeCys levels may also be related to the unique metabolic mechanism of SeNPs. Furthermore, we also speculate that the increase in $SeCys_2$ and MeSeCys levels may be associated with the presence of $SeCys_2$ and MeSeCys on the surface of SeNPs (Figure S1). Further research is needed to investigate the underlying mechanisms.

4.2. *The antioxidant capacity of $SeCys_2$ and MeSeCys in 293T cells*

MeSeCys is more prone to generate trimethylselenonium, eating MeSeCys-accumulators is better than Se-accumulators (Suzuki, Doi & Suzuki, 2006). MeSeCys are assumed to be the reactive selenium sources

Table 1
Fatty acid composition of *O. macrolepis* muscle (% total fatty acids) (n = 6).

	Groups			
Fatty Acids	BD	SeNPs-0.3	Selenite-0.3	SeMet-0.3
C14:0	1.42 ± 0.10^{a}	1.01 ± 0.01^{b}	1.05 ± 0.29^{b}	0.71 ± 0.08^{c}
C16:0	20.35 ± 0.04^{b}	21.92 ± 0.27^{a}	22.49 ± 0.58^{a}	22.61 ± 0.63^{a}
C18:0	10.29 ± 0.98^{c}	12.71 ± 0.08^{b}	14.28 ± 0.10^{a}	13.70 ± 0.19^{a}
ΣSFA	32.06 ± 0.94^{c}	35.64 ± 0.17^{b}	37.83 ± 0.47^{a}	37.02 ± 0.74^{a}
C16:1n-7	9.50 ± 0.53^{a}	8.72 ± 0.85^{ab}	8.13 ± 0.03^{b}	7.78 ± 0.20^{b}
C18:1n-9	24.79 ± 0.83^{a}	19.73 ± 0.45^{b}	20.50 ± 0.48^{b}	19.94 ± 0.30^{b}
ΣMUFA	34.29 ± 1.30^{a}	28.45 ± 0.63^{b}	28.62 ± 0.50^{b}	27.72 ± 0.36^{b}
C18:2n-6	11.66 ± 0.86^{a}	9.74 ± 0.19^{b}	9.12 ± 0.08^{b}	9.31 ± 0.34^{b}
C18:3n-6	1.49 ± 0.03^{a}	0.93 ± 0.07^{b}	0.68 ± 0.03^{c}	0.74 ± 0.05^{c}
C20:4n-6	3.51 ± 0.02^{b}	3.90 ± 0.12^{b}	3.91 ± 0.15^{b}	4.62 ± 0.38^{a}
ΣN-6PUFA	16.6 ± 0.90^{a}	14.57 ± 0.15^{b}	13.70 ± 0.09^{b}	14.67 ± 0.67^{b}
C18:3n-3	2.90 ± 0.09	2.70 ± 0.26	2.56 ± 0.04	2.51 ± 0.23
C20:5n-3 EPA	1.85 ± 0.07^{b}	2.43 ± 0.11^{a}	2.33 ± 0.19^{a}	2.24 ± 0.06^{a}
C22:5n-3	0.95 ± 0.00	0.92 ± 0.15	0.97 ± 0.06	0.83 ± 0.07
C22:6n-3 DHA	11.29 ± 0.94^{b}	15.29 ± 0.67^{a}	13.98 ± 0.05^{a}	15.02 ± 0.74^{a}
ΣN-3PUFA	16.99 ± 0.91^{c}	21.34 ± 0.66^{a}	19.84 ± 0.24^{b}	20.59 ± 0.53^{ab}
N-3/N-6ΣPUFA	1.02 ± 0.05^{b}	1.46 ± 0.03^{a}	1.45 ± 0.01^{a}	1.41 ± 0.06^{a}
Total	99.94	100.00	99.99	100.00

Abbreviations: saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA). Values are mean ± SD. (n = 6) with different superscripts in the same row are significantly different (P < 0.05) from each other.

for the anti-carcinogenic effect, foods rich in MeSeCys are considered to have better nutritional and health benefits (Brummell et al., 2011). Furthermore, compared to SeMet and selenite, MeSeCys exhibits the strongest ability to scavenge DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radicals (Sentkowska & Pyrzyńska, 2019). Our results also revealed that MeSeCys can reduce oxidative damage to the human body by scavenging ROS (Fig. 3). Research reports have shown that $SeCys_2$ exhibits anticancer and chemopreventive effects through different mechanisms, including reducing oxidative stress, inducing cell apoptosis, and enhancing chemotherapy activity. We also demonstrated that $SeCys_2$ can reduce oxidative damage to the human body by scavenging ROS (Fig. 3), further confirming the potential health benefits of selenium-enriched fish produced using SeNPs as an additive.

4.3. Nutritional response (proximate composition, heavy metal elements, and fatty acids) to different dietary selenium forms in O. macrolepis muscle

The appropriate addition of selenium effectively increases the selenium content in fish, while promoting fish health (antioxidant capacity), thus reducing production costs (Cotter, Craig & McLean, 2008). Notably, considering the antioxidant indicators of *O. macrolepis* (Table S5), a selenium supplementation concentration of 0.3 mg/kg is found to be the optimal choice. Hence, our research will concentrate on further exploring the impact of three selenium additives, administered at a concentration of 0.3 mg/kg, on the flesh quality of *O. macrolepis*. The levels of heavy metals, proximate composition, and fatty acids in fish muscle are important indicators for assessing its flesh quality (Li, Xue, Sun & Ji, 2023). Selenium's antagonistic effect on heavy metals is widely established, most studies have focused on the relationship between heavy metals and sodium selenite (Kumar et al., 2018; Yin et al., 2021). However, no comparative analysis of the effects of different selenium sources on heavy metal accumulation in fish muscle has been conducted so far. SeNPs had a greater advantage in reducing the levels of As, Cd, and Hg elements in *O. macrolepis* muscle (Fig. 4A-C). This could be attributed to the higher selenium concentrations accumulated in the muscle after feeding with SeNPs and SeMet. However, it could not be excluded that these differences in metabolic pathways of different selenium sources contributed to the observed outcomes.

The higher lipid content in artificially farmed fish leads to a decrease in the overall flesh quality and taste (Ergün, Soyutürk, Güroy & Merrifield, 2009). Supplementation of 0.3 mg/kg of SeNPs significantly reduces the lipid content in *O. macrolepis* muscle (Fig. 4G). This may be attributed to the regulation of SeNPs to lipid metabolism-related genes through the AMPK pathway (Liu et al., 2021). Abdel-Tawwab, Mousa & Abbass (2007) demonstrated that feeding organic selenium significantly increased the crude protein content in the muscle of African catfish, a phenomenon also observed in our results (Fig. 4H). However, it was found that dietary selenium supplementation did not have a significant effect on the crude protein content in the muscle of common carp and crucian carp (Saffari et al., 2017; Zhou, Wang, Gu & Li, 2009). This difference may be attributed to the variations in experimental species. Fish muscle contains a significant amount of PUFAs, particularly the n-3 series of PUFAs such as EPA and DHA (Tang et al., 2016). As a feed additive, SeNPs had a more pronounced effect in increasing the levels of EPA and DHA in fish muscle (Table 1). Moreover, a higher ratio of ∑n3/∑n6 PUFA has been associated with a reduced risk of aggressive prostate cancer and cardiovascular disease in humans (Sampels, 2015). SeNPs as a feed additive significantly increases the ratio of ∑n3/∑n6 PUFA. This further demonstrated the SeNPs was the most suitable additive for producing selenium-rich fish.

4.4. Effects of 0.3 mg/kg three selenium sources on selenium species of grass carp

To further investigate the influence of SeNPs on selenium species distribution in fish muscle, we conducted a study using grass carp and observed the occurrence of selenium species distribution similar to that found in *O. macrolepis*. This further demonstrated the widespread effectiveness of SeNPs in fish and highlighted the promising application prospects of SeNPs as a selenium-enriched additive in the production of healthier selenium-enriched fish meat.

5. Conclusion

In summary, our findings indicate that SeNPs exhibit comparable bioavailability to SeMet in *O. macrolepis*. Se-enriched fish produced with SeNPs offer humans a greater supply of healthy selenium species, including $SeCys_2$ and MeSeCys, in comparison to selenite and SeMet. These two forms of selenium have the significant ability to alleviate H_2O_2-induced apoptosis and cell viability decline of 293T cells, indicating selenium-enriched fish with higher health benefits for humans can be produced using SeNPs. Furthermore, the inclusion of SeNPs in the diet significantly enhanced the antioxidant capacity and flesh quality of *O. macrolepis*. Therefore, the production of Se-enriched fish with SeNPs as a supplementary source of selenium may be an efficient approach for increasing the human dietary intake of selenium in selenium-deficient areas.

6. Contributions

CZ conceived and designed the experiments. QL and YW performed the experiments. XW analyzed the data and wrote the paper with the help of YM. CZ provided technical support for the SeNPs preparation and characterization. HJ, FY and WD reviewed and edited the manuscript.

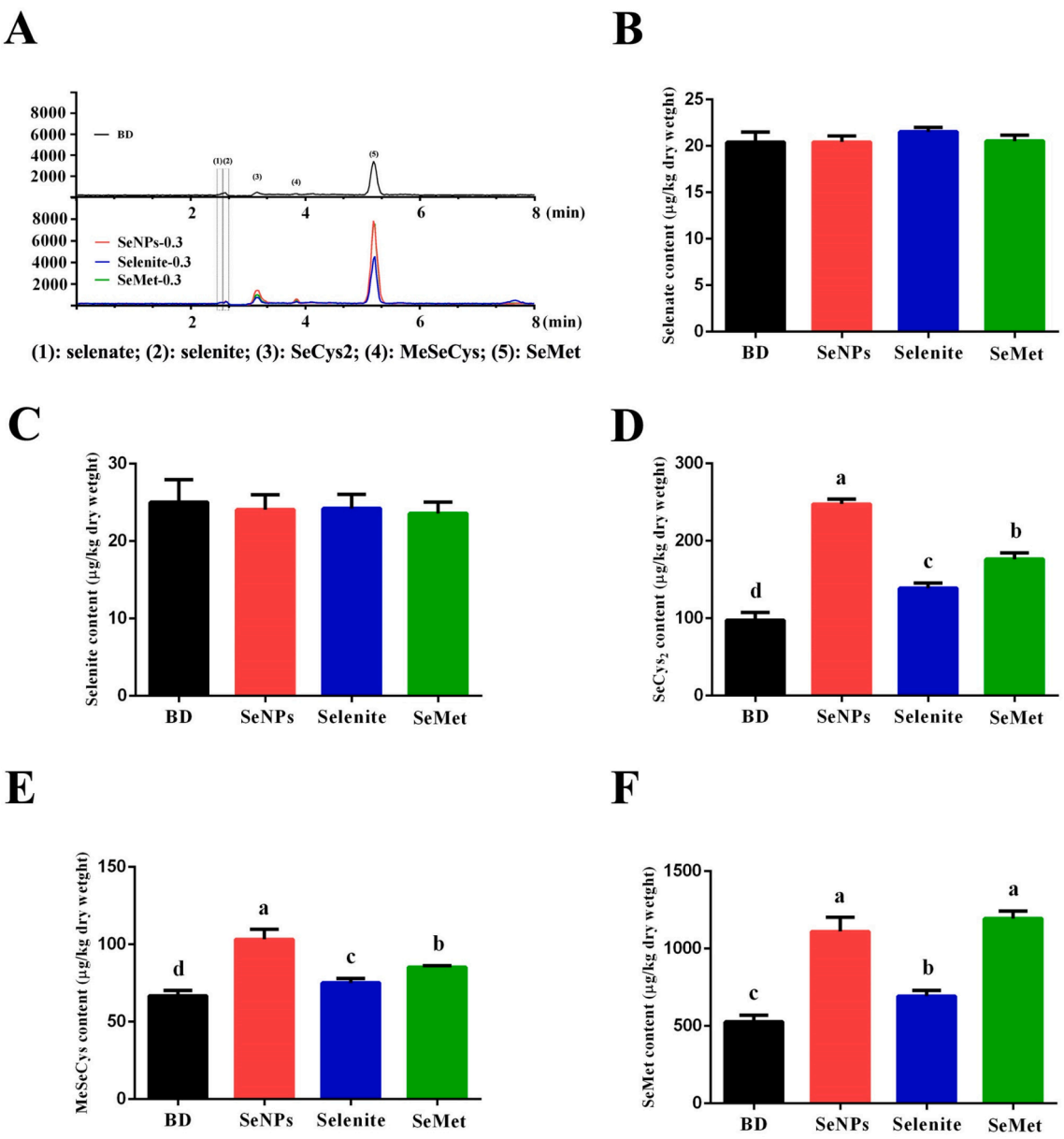

Fig. 5. Evaluate the speciation of selenium in the muscle tissue of grass carp after feeding 0.3 mg/kg three selenium sources. Chromatograms of grass carp after feeding basal diet (BD) group and 0.3 mg/kg SeNPs, selenite and SeMet treatment (A). The levels of selenite (B), selenite (C), $SeCys_2$ (D), MeSeCys (E), and SeMet (F) in the muscle tissue of grass carp were analyzed (n = 3). Different lower case letters between different groups denote statistically significant differences ($P < 0.05$). Data expressed as means values ± SD.

CRediT authorship contribution statement

Chao Zhu: Writing – review & editing, Writing – original draft, Resources, Methodology, Conceptualization. **Qimin Liu:** Validation, Supervision, Investigation, Formal analysis, Data curation. **Yang Wang:** Visualization, Validation, Supervision, Data curation. **Xiaolin Wang:** Validation, Software, Project administration, Formal analysis. **Yuxuan Ma:** Visualization, Validation, Supervision, Methodology. **Fangxia Yang:** Supervision, Project administration. **Wuzi Dong:** Supervision, Resources, Project administration, Funding acquisition. **Hong Ji:** Supervision, Resources.

Declaration of competing interest

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

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.101088.

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