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DOI: 10.2478/auoc-2025-0006

Ovidius University Annals of Chemistry Volume 36, Number 1, pp. 51 - 57, 2025

Ergothioneine-loaded chitosan nanoscale particles mitigate discoloration and lipid oxidation in frozen yellowfin tuna cubes

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Abstract. The present study was conducted to evaluate the effects of ergothioneine-loaded chitosan nanoscale particles (ECNP) on discoloration and lipid oxidation in yellowfin tuna cubes after 6 months of frozen storage. Tuna cubes were treated with ECNP, chitosan nanoscale particles (CNP), ergothioneine (EGT), or left untreated (control). Lipid oxidation was assessed through the total lipid hydroperoxide (HPO) and thiobarbituric acid reactive substances (TBARS), while myoglobin oxidation was determined based on metmyoglobin (metMb) concentration. Color stability was evaluated by L*, a*, b* values, and the redness index (RI = a*/b*). Results indicated that ECNP treatment significantly suppressed lipid and myoglobin oxidation compared to the control ($p < 0.05$). ECNP-treated samples presented the lowest metMb accumulation (43.85 ± 5.15%), with grade B (good) classification after 6 months of frozen storage. In contrast, the control group displayed the highest level of metMb (71.36 ± 2.95%), which belonged to grade D (unacceptable). CNP and EGT treatments also showed protective effects, but to a lesser extent than ECNP. The RI values strongly correlated with the oxidative indicators (HPO, TBARS, metMb), demonstrating its validity in reflecting the anti-discoloration effect of ECNP. These findings suggest that ECNP treatment can enhance the oxidative stability and color retention of frozen tuna cubes, and thus improving their overall quality during frozen storage.

***Keywords*:** biopolymer; natural antioxidant; sashimi; seafood preservation; *Thunnus albacares*.

1. Introduction

Tuna is a group of more than 48 species widely distributed across the Atlantic, Indian, and Pacific Oceans, and Mediterranean Sea, which makes it a world-wide significant resource. Among them, bluefin, bigeye, yellowfin, skipjack and albacore are the most common and commercially important of these species [1]. Tuna is highly favored for the characteristic bright red color of its muscle, which is a feature very few other fish possess. It is mostly due to high levels of ferrous myoglobin (Fe^{2+}) including deoxymyoglobin (deoxyMb) and oxymyoglobin (oxyMb). However, the bright red color of tuna meat is very susceptible to turning dark brown due to the formation of ferric myoglobin (Fe^{3+}) so-called metmyoglobin (metMb), an oxidation product of deoxyMb and oxyMb [2].

Previous researchers have found that the major problem encountered is metMb formation when tuna meat is frozen at -18 °C, a frequently used temperature for freezing meat [2, 3]. Although storing tuna meat at temperatures below -40 °C has been shown to significantly inhibit metMb formation [2-4], such low temperatures substantially increase preservation costs. So, the focus should be on finding a treatment for tuna meat that prevents metMb formation and retains its bright red color during frozen storage.

The color change in tuna meat in the course of storage is directly associated with oxidative reactions of lipids. Lipid hydroperoxides and free radicals are the major factors generated through lipid oxidation and these are capable of Mb oxidation triggering metMb formation. On the other hand, the metMb presence stimulates lipid oxidation by a positive feedback loop mechanism, which in turn, leads to the further creation of the oxidation products. It is this chain of reactions that constitutes the oxidative deterioration and the following tuna meat discoloration [5-8].

The counter to these oxidation reactions has been the use of antioxidants, which have been one of the most extensively researched and deployed [9,10]. With the increasing concern on food safety, there has been a significant shift in global food industry trends with the tendency of consumers to prefer products that have natural rather than artificial additives. Ergothioneine (EGT), a natural antioxidant that can be derived from some edible mushrooms, has the properties of maintaining the bright red color of tuna meat over time by its antioxidation mechanism, which is by suppressing the oxidation of myoglobin and lipids and thus minimizing metMb formation. The results of studies have revealed that minced tuna meat with an added 50 mg EGT/kg may be kept with its natural red color for even 7 days during ice storage [6, 11, 12]. However, the high price of EGT at effective doses, in particular, is a significant factor that hinders its widespread and practical use by industrial manufacturers.

One of the most researched and widely used biopolymers with antibacterial and antioxidant properties is chitosan, a natural biopolymer derived from the shells of crustaceans [13]. Antibacterial and

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antioxidative activities of chitosan depend on its molecular weight (MW) and degree of deacetylation (DD). Generally, chitosan with lower MW and higher DD has stronger antibacterial and antioxidant activities. Most recent research has shown that chitosan nanoscale particles (CNP) are more effective in preserving seafood compared to conventional chitosan. CNP tested by Ghorabi and Khodanazary [14] had proved to be more effective in preserving *Cynoglossus arel* than the conventional chitosan. Similarly, Binh et al. [15] reported that CNP in their experiment was more effective in inhibiting lipid oxidation in seasoned dried pangasius fillets than conventional chitosan of similar MW and DD.

CNP preparation, the ionic gelation technique with sodium tripolyphosphate (STPP) being the cross-linker, is the most widely used drug or bioactive compound encapsulation method in medicine, food, as well as cosmetic. It controls the release of bioactive compounds and particularly the release of drugs, which brings a gain in their stability, bioavailability, and efficacy [16-19].

Therefore, it would be a good idea to combine the EGT with CNP by the ionic gelation technique to create ergothioneine-loaded chitosan nanoscale particles (ECNP). The resulting products could have the added potential to not only improve the EGT antioxidant effect but also reduce the amount of EGT used for seafood preservation and thus lower the preservation costs, which seems more practical.

In order to develop a safe, effective, and economical preservation solution, the present study was carried out to evaluate the efficacy of ECNP in preventing discoloration and lipid oxidation in frozen yellowfin tuna cubes.

2. Experimental

2.1. Materials and chemicals

Yellowfin tuna (*Thunnus albacares*) was obtained from a seafood company in Khanh Hoa Province, Vietnam. The tuna loins were frozen at the company and then transported to the laboratory of Nha Trang University, where they were kept at -20 ± 2 °C with a freezer Sanaky VH-5699HY4K (Viet Nhat Electronic - Refrigeration Co., LTD, Vietnam) for testing in the current study.

Low molecular weight chitosan (viscosity ≤ 110 cPs, deacetylation degree ≥ 90.3%) was supplied by Vietnam Food Joint Stock Company (VNF), Vietnam. Ergothioneine (purity ≥ 98%) was purchased from Shaanxi Dideu Medichem Co. Ltd, Dideu Group, Shaanxi Province, China. Other chemicals used in this study were of analytical grade and were obtained from Sigma-Aldrich (USA) and Merck (Germany).

2.2. Preparation of ECNP and CNP

ECNP were prepared following the procedure described by Thinh [20], with slight modifications. In brief, chitosan was dissolved in 1% ascorbic acid solution to produce a 1 mg/mL chitosan solution. EGT was then dissolved and added to the chitosan solution at a concentration of 0.2 mg/mL. The mixture was stirred continuously at 750 rpm to ensure homogeneity. Subsequently, an STPP solution (1 mg/mL) was added dropwise to the mixture while stirring continuously at 750 rpm to achieve a chitosan-to-STPP ratio of 4/1 (w/w) in order to form the ECNP spontaneously. CNP were prepared using the same procedure without the addition of EGT. After 2 h of stabilization, the particle sizes of ECNP and CNP were determined by dynamic light scattering (DLS) using a Horiba SZ-100V2 spectrometer. The Z-average values of ECNP and CNP were 139.6 nm and 146.8 nm, respectively. The polydispersity index (PI) of ECNP and CNP was 0.319 and 0.308, respectively. The amount of EGT loaded in ECNP was 159.5 ± 3.4 µg/mg.

2.3. Treatment of tuna cubes

The yellowfin tuna cubes with a size of 20×20×20 mm were prepared from the tuna loins and randomly divided into four groups for different treatments: untreated (control), sprayed with ECNP at 100 mg/kg fish, sprayed with chitosan CNP at 100 mg/kg fish, and sprayed with EGT at a level equivalent to that in 100 mg ECNP/kg fish. After treatment, the tuna cubes were placed on Styrofoam trays (200 g per tray), vacuum-packaged in multilayer polyamide bags and stored at -20 ± 2 °C. Color ($L^*a^*b^*$), metMb, total lipid hydroperoxides (HPO), and thiobarbituric acid reactive substances (TBARS) of the tuna cubes were measured before and after 6 months of frozen storage. All treatments were performed in triplicate.

2.4. Measurement of color

The color ($L^*a^*b^*$) of tuna cubes was measured by a Konica Minolta CR-400/CR-40 (Japan). The redness index (RI) was calculated as a ratio of a^*/b^* [21].

2.5. Measurement of metmyoglobin (metMb)

The percentage of metMb was determined according to a procedure of Bito [2] with slight modifications. A 3 g portion of minced tuna meat was mixed with 10 mL of refrigerated phosphate buffer solution (0.04 M, pH = 6.8) and stirred with a Teflon-coated magnetic bar for 10 min. The mixture was centrifuged at 5000 rpm for 5 min, and the supernatant was filtered through Whatman filter paper No. 1. Absorption spectra of the supernatant were measured at 540 nm and 503 nm using a Biochrom Libra S50 UV/VIS spectrophotometer (Cambridge, UK). The percentage of metMb was calculated using the absorbance ratio at 540 nm and 503 nm, based on the regression equation derived from the data reported by Bito for tuna Mb [2].

$$\text{metMb (\%)} = 115.33 - 43.291 \times \frac{A_{540}}{A_{503}}$$

where: A_{540} is the absorbance at 540 nm and A_{503} is the absorbance at 503 nm.

2.6. Measurement of total lipid hydroperoxides (HPO)

HPO were determined according to a procedure of Shantha and Decker [22] with slight modifications. Lipids were extracted from 5 g of minced fish muscle using the Bligh and Dyer method [23]. The final extract was adjusted to 10 mL with chloroform/methanol (2:1). Subsequently, 50 µL of 30% ammonium thiocyanate and 50 µL of 2% ferrous chloride were added, vortexed, and incubated at room temperature for 5 min.

Absorbance of the reaction mixture was measured at 500 nm using the spectrophotometer. The results were expressed as nmol cumene hydroperoxide per gram of fish muscle based on a standard curve.

2.7. Measurement of thiobarbituric acid reactive substances (TBARS)

TBARS were determined according to a procedure of Uchiyama and Mihara [24]. Briefly, 0.5 g of minced fish meat was homogenized with 4.5 mL of 1.15% KCl solution. A 0.5 mL homogenate was mixed with 0.3 mL of 1% phosphoric acid and 1.0 mL of 0.6% thiobarbituric acid solution, then incubated at 95 °C for 45 min. After cooling, 4.0 mL *n*-butanol was added, vortexed, and centrifuged at 3000 rpm for 10 min. Absorbance of the reaction mixture was measured at 535 nm and 520 nm using the spectrophotometer. TBARS values were calculated from the difference in absorbance (A_{535} - A_{520}). Results were expressed as malondialdehyde (MDA) equivalents using a 1,1,3,3'-tetraethoxypropane standard curve.

2.8. Statistical analyses

Microsoft Excel 2013 was used for mean and standard deviation calculations and graph generation. R software version 4.2.2 was used for statistical analysis. Two-way ANOVA identified significant differences, and Tukey's Multiple Comparisons Test determined differences between samples at $p < 0.05$.

3. Results and discussion

3.1. Effect of ECNP treatment on lipid oxidation in tuna cubes during frozen storage

HPO and TBARS are two commonly used indicators for evaluating lipid oxidation levels in food in general and seafood in particular. The effect of ECNP treatment on the HPO content of tuna cubes before and after 6 months of frozen storage is shown in Figure 1.

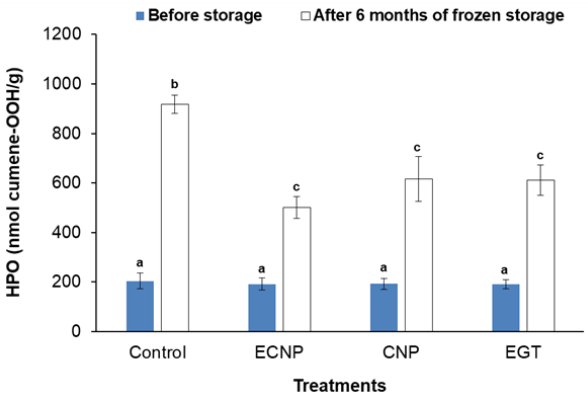

Figure 1. Changes in total lipid hydroperoxides (HPO) content of the yellowfin tuna cubes treated with ECNP, CNP, EGT, and control without treatment before and after 6 months of frozen storage. Data are presented as mean ± SD (n = 3). Columns labeled with different letters indicate significant differences ($p < 0.05$).

Changes in HPO content of fish muscle during the early stage of storage reflect the degree of lipid oxidation [10]. The HPO content of fresh fish muscle stored below 4 °C within one day after harvesting is typically less than 50 nmol/g [25]. Results from this study indicate that the initial HPO content of tuna cubes before storage was 204.37 ± 32.39 nmol/g (control), 191.91 ± 24.78 nmol/g (ECNP), 193.17 ± 22.31 nmol/g (CNP), and 191.64 ± 18.63 nmol/g (EGT). These values suggest that significant lipid oxidation had already occurred in the tuna cubes before storage. No significant difference ($p > 0.05$) was observed in the initial HPO content among the samples.

As shown in Figure 1, the HPO content of all tuna cube samples increased significantly ($p < 0.05$) after 6 months of frozen storage. However, the HPO contents were significantly lower ($p < 0.05$) in the ECNP, CNP, and EGT samples compared to the control. There was no significant difference ($p > 0.05$) in HPO content among samples treated with ECNP, CNP, and EGT.

TBARS are secondary products of lipid oxidation, and their contents in fish muscle during storage reflect the extent of lipid oxidation [25]. The effect of ECNP treatment on TBARS values in 6-month frozen storage of tuna cubes is shown in Figure 2.

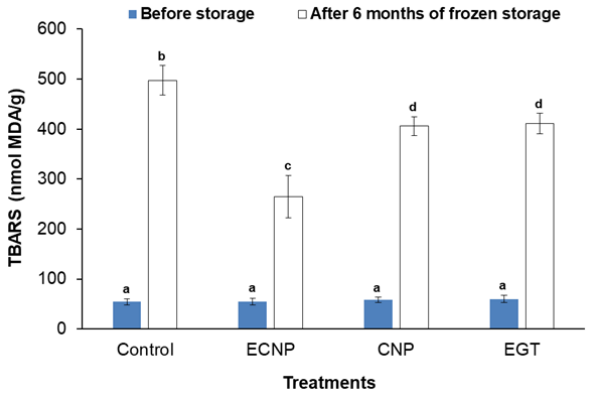

Figure 2. Changes in thiobarbituric acid reactive substances (TBARS) content of the yellowfin tuna cubes treated with ECNP, CNP, EGT, and control without treatment before and after 6 months of frozen storage. Data are presented as mean ± SD (n = 3). Columns labeled with different letters indicate significant differences ($p < 0.05$).

The results in Figure 2 indicate that the TBARS contents of tuna cubes in all treatments before storage were relatively low: control (54.19 ± 6.42 nmol MDA/g), ECNP (54.96 ± 6.67 nmol MDA/g), CNP (58.19 ± 5.38 nmol MDA/g), and EGT (59.90 ± 7.31 nmol MDA/g). No significant difference ($p > 0.05$) was observed in the TBARS contents among the samples before storage.

After 6 months of frozen storage, a substantial increase in TBARS contents was observed in all treatments, with significant differences among treatments ($p < 0.05$). The control group exhibited the highest TBARS contents (497.62 ± 29.56 nmol MDA/g), indicating severe lipid oxidation. This value far exceeds the threshold of 200 nmol MDA/g, which is associated with the development of rancid odors in fish, as reported by Ke et al. [26]. In contrast, the ECNP treatment significantly reduced TBARS accumulation (264.73 ± 42.29 nmol MDA/g), whereas the CNP and EGT treatments exhibited similar effects with TBARS contents of 405.71 ± 18.89 nmol MDA/g and 411.01 ± 21.00 nmol MDA/g, respectively. These results suggest that ECNP had the strongest

inhibitory effect on lipid oxidation in frozen tuna cubes, followed by CNP and EGT.

3.2. Effect of ECNP treatment on metMb concentration and RI of frozen tuna cubes

During low-temperature storage, the color of tuna meat gradually changes from red to dark brown due to the deoxyMb and oxyMb species being oxidized, leading to the formation of metMb [2-4]. The metMb value classification in the meat of yellowfin tuna as reported by Nurilmala et al. [21] is grade A (excellent) < 26%, grade B (good) < 46%, grade C (acceptable) < 52%, and grade D (unacceptable) > 52%.

The effect of ECNP treatment on metMb formation in tuna cubes after 6 months of frozen storage is shown in Figure 3.

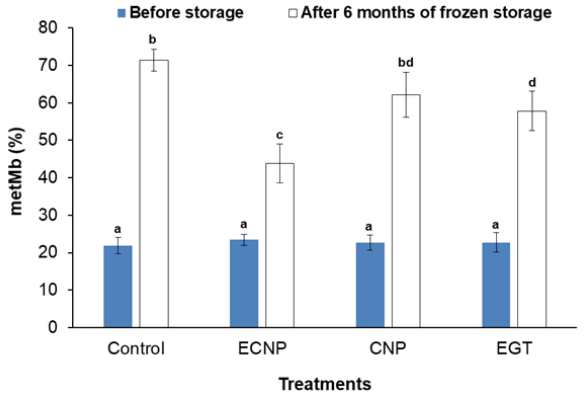

Figure 3. Changes in metmyoglobin (metMb) concentration of the yellowfin tuna cubes treated with ECNP, CNP, EGT, and control without treatment before and after 6 months of frozen storage. Data are presented as mean ± SD (n = 3). Columns labeled with different letters indicate significant differences ($p < 0.05$).

The results in Figure 3 indicate that the metMb concentration of tuna cubes in all treatments before storage showed no significant difference ($p > 0.05$) and were below 26%, corresponding to grade A. After 6 months of frozen storage, a considerable increase in metMb concentration of tuna cubes in all treatments was observed with significant variations among treatments ($p < 0.05$). The control group showed the highest metMb concentration (71.36 ± 2.95%), while those treated with CNP and EGT showed results of 62.16 ± 6.04% and 57.80 ± 5.24%, respectively; all these figures exceeded the 52% cutoff set by Nurilmala et al. [21] to classify as having substandard quality, thereby ranking them as grade D. However, the ECNP treatment showed the minimum formation of metMb (43.85 ± 5.15%), thereby retaining a grade B status. The results emphasize the greater efficacy of ECNP over CNP and EGT in preventing metMb formation, which indicates its prospects for maintaining color stability in tuna cubes during frozen storage. Inhibition of metMb formation by EGT in tuna meat and beef was previously demonstrated by Bao et al. [11]. Furthermore, the CNP may also contribute to suppressing metMb formation due to their antioxidant action that inhibits lipid oxidation by the elimination of lipid hydroperoxide and free radicals [27].

Nurilmala et al. [2] also reported a negative correlation between metMb content and the redness index (a^*/b^*) of yellowfin tuna meat. The redness index for yellowfin tuna is classified as follows: grade A (excellent) > 1.2, grade B (good) > 0.9, grade C (acceptable) ≥ 0.7, and grade D (unacceptable) < 0.7.

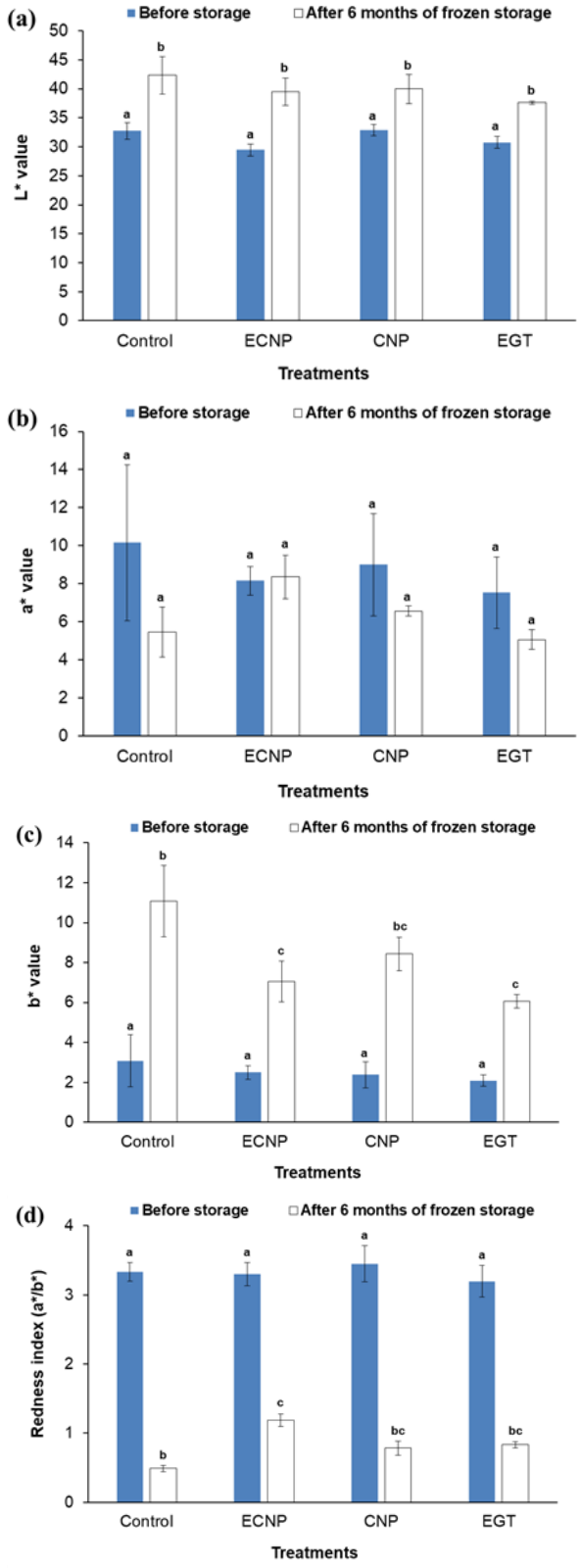

Figure 4. Changes in L^* value (a), a^* value (b), b^* value (c) and redness index (d) of the yellowfin tuna cubes treated with ECNP, CNP, EGT, and control without treatment before and after 6 months of frozen storage. Data are presented as mean ± SD (n = 3). Columns labeled with different letters indicate significant differences ($p < 0.05$).

The effect of ECNP treatment on changes in the redness index of tuna cubes after 6 months of frozen storage is shown in Figure 4.

The results indicate significant changes in the color values (L^*, a^*, b^*) and the redness index (a^*/b^*) of tuna cubes after 6 months of frozen storage. The L^* value (lightness) was higher ($p < 0.05$) in all treatments after storage, indicating loss of color intensity. The a^* value (red-green) decreased in all samples without differences among treatments, indicating an overall decrease in red color during storage. At the same time, the b^* value (blue-yellow) exhibited a tremendous increase, with the control treatment having the highest b^* value, followed by CNP, ECNP, and EGT treatments. This shift suggests a tendency toward a more yellowish hue during storage. After 6 months of frozen storage, the redness of all the tuna samples had visibly faded (Figure 5). The redness index, measured by the a^*/b^* ratio, is an important tuna quality indicator. The control group had the lowest redness index, falling below the grade D threshold (< 0.7), indicating an unacceptable quality. Meanwhile, the tuna cubes treated with ECNP held onto their red color the best, with a redness index of 1.19 ± 0.09, earning them a grade A rating. The CNP and EGT treatments also preserved the redness reasonably, with values of 0.79 ± 0.10 and 0.83 ± 0.05, respectively, both within the grade C range. These results suggest that ECNP is the most effective in preserving the redness index and overall color stability of frozen tuna cubes.

This finding aligns with the study by Nurilmala et al. [21], which reported a negative correlation between metMb concentration and the redness index in tuna meat.

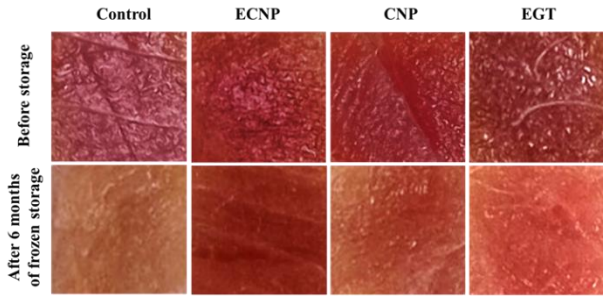

Figure 5. Changes in color of the yellowfin tuna cubes treated with ECNP, CNP, EGT, and the control without treatment before and after 6 months of frozen storage.

Previous studies reported that the change in color of tuna meat primarily results from the oxidation of lipids and Mb [6, 11]. The correlation matrix provides valuable insights into the relationships between lipid oxidation, metMb formation, and color parameters of yellowfin tuna cubes subjected to different treatments before and after 6 months of frozen storage in the present study, as shown in Figure 6.

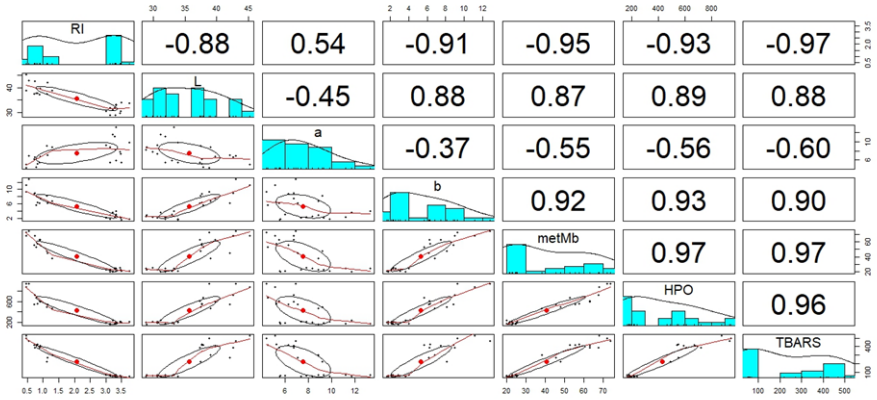

Figure 6. The correlation between the total lipid hydroperoxide (HPO) content, thiobarbituric acid reactive substances (TBARS) content, metmyoglobin (metMb) concentration, L^* value, a^* value, b^* value, and the redness index (RI) of all yellowfin tuna cubes treated with ECNP, CNP, EGT, and control without treatment before and after 6 months of frozen storage.

According to previous research, the main culprit causing discoloration of tuna muscle is lipid and myoglobin (Mb) oxidation because tuna muscle has a high content of unsaturated fatty acids and abundant Mb, which facilitates oxidation [6]. This is also consistent with what have been observed in practice, where tuna meat is more susceptible to discoloration than some other meats. In the present study, the authors measured HPO and TBARS contents and metMb concentration, which are indicators of Mb and lipid oxidation in tuna muscle. A strong negative correlation was observed between the redness index (RI) and lipid oxidation indicators-HPO content (r = -0.93) and TBARS content (r = -0.97). This suggests that as lipid oxidation progresses, the RI significantly declines, indicating deterioration in tuna color quality. Similarly, RI showed a highly negative correlation with metMb concentration (r = -0.95), reinforcing the role of oxidative processes in color degradation.

In contrast, RI exhibited a positive correlation with the a^* value (r = 0.54) but a strong negative correlation with the b^* value (r = -0.91) and L^* value (r = -0.88). This implies that a decrease in redness (a^*) and an increase in yellowness (b^*) and lightness (L^*) contribute to a lower redness index, reflecting quality deterioration. Additionally, lipid oxidation indicators (HPO and TBARS) were strongly positively correlated with metMb (r = 0.97), supporting the close link between lipid and myoglobin oxidation in frozen tuna storage.

These results confirm that lipid oxidation accelerates Mb oxidation, leading to increased metMb formation and a decline in the RI, which ultimately reduces the visual appeal and quality of frozen tuna cubes. Among the treatments, ECNP showed the most effective

preservation of RI, supporting its superior antioxidant potential in mitigating color deterioration.

4. Conclusions

The findings in this study show that the discoloration, metMb formation, and lipid oxidation in frozen tuna cubes were better inhibited by ECNP treatment than by CNP and EGT treatment. The treatment of ECNP enabled the tuna cubes to maintain higher redness index values and lower levels of lipid and myoglobin oxidation, thus better preserving color and chemical quality during 6 months of frozen storage. These results highlight the scope of ECNP as a potential natural antioxidant for the preservation of seafood products.

Conflict of interest

Authors have no conflict of interest to declare.

Acknowledgments

This research was funded by the Vietnam Ministry of Education and Training under project code B2023-TSN-11.

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Received: 02.04.2025
Received in revised form: 17.04.2025
Accepted: 21.04.2025