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Effect of freeze–thaw cycles on the physicochemical, water–holding properties, and histology of *Sardinella aurita*

Efecto de los ciclos de congelación y descongelación en las propiedades fisicoquímicas, capacidad de retención de agua y la histología de *Sardinella aurita*

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ABSTRACT

This study investigates the impact of freeze–thaw cycles on samples of *Sardinella aurita*, focusing on the examination of physicochemical properties, water–holding capacity, color changes, and histological alterations in fish meat. The present findings indicate significant variations in the studied parameters, i.e., pH, water activity, lightness (L*), redness (a*), yellowness (b*), protein solubility (mg·g^{-1}), moisture content (%), thawing loss (%), centrifugation loss (%), cooking loss (%), underscoring the importance of comprehending the consequences of freezing–thawing in the fishing and food processing industry. Initially, a statistically significant decrease in pH levels was observed (T0: 6.23 ± 0.1, T4: 6.19 ± 0.1), followed by a notable increase after the fifth freeze–thaw cycle (T5: 6.47 ± 0.1), possibly due to chemical and microbiological composition shifts. Water activity exhibited a gradual decrease (T0: 0.911 ± 0.009, T4: 0.899 ± 0.01), likely attributed to water loss during freezing–thawing (P<0.05). Colorimetry results demonstrated a significant decrease in brightness (L*) and a slight increase in yellow hue (b*) throughout the cycles, with values ranging from 63.51 (T0) to 33.64 (T5) for L* and from 26.74 (T0) to 17.28 (T5) for b*. These variations highlight notable and significant changes in the product's color over the freeze–thaw cycles (P<0.05). Histological analysis revealed structural changes, including muscle fiber dehydration. These observed changes hold implications for product quality and consumer perception. It is essential to recognize that various factors, such as fish size, seasonality, and environmental conditions influence these results. Further research is needed to delve deeper into these aspects. In essence, this study offers valuable insights for industry professionals, aiding them in making informed decisions regarding seafood products subjected to freezing–thawing cycles. This not only ensures product quality and safety but also helps prevent food fraud and provides consumers with high–quality products.

Key words: *Sardinella aurita*; histological changes; freeze–thaw cycles; water–holding

RESUMEN

Este estudio investiga el impacto de los ciclos de congelación y descongelación en muestras de Sardinella aurita, centrándose en el examen de propiedades fisicoquímicas, capacidad de retención de agua, cambios de color y alteraciones histológicas en la carne del pescado. Los hallazgos actuales indican variaciones significativas en estos parámetros [pH, actividad de agua, luminosidad (L*), rojez (a*), amarillez (b*), solubilidad de proteínas (mg·g^{-1}), contenido de humedad (%), pérdida de descongelación (%), pérdida por centrifugación (%), pérdida de cocción (%)]. Los resultados revelan alteraciones significativas en estos parámetros, destacando la importancia de comprender los efectos de la congelación y descongelación en la calidad del pescado dentro de la industria pesquera y de procesamiento de alimentos. Inicialmente, se observó una disminución estadísticamente significativa en los niveles de pH (T0: 6.23 ± 0.1, T4: 6.19 ± 0.1), seguida de un aumento notable después del quinto ciclo (T5: 6.47 ± 0.1), posiblemente debido a cambios en la composición química y microbiológica. La actividad del agua mostró una disminución gradual, probablemente atribuida a la pérdida de agua durante la congelación y descongelación (P<0,05). Los resultados de la colorimetría mostraron una disminución significativa en la luminosidad (L*) y un ligero aumento en el tono amarillo (b*) a lo largo de los ciclos, con valores que van desde 63.51 (T0) hasta 33.64 (T5) para L* y desde 26.74 (T0) hasta 17.28 (T5) para b*. Estas variaciones resaltan cambios notables y significativos en el color del producto a lo largo de los ciclos de congelación y descongelación (P<0,05) Estos cambios observados tienen implicaciones en la calidad del producto y la percepción del consumidor. Es importante reconocer que estos resultados están influenciados por diversos factores, como el tamaño del pescado, la estacionalidad y las condiciones ambientales. Se necesita investigación adicional para profundizar en estos aspectos. En resumen, este estudio proporciona información valiosa para los profesionales de la industria, ayudándolos a tomar decisiones informadas sobre productos de mar sometidos a ciclos de congelación y descongelación. Esto no solo garantiza la calidad y seguridad del producto, sino que también ayuda a prevenir el fraude alimentario y proporciona a los consumidores productos de alta calidad.

Palabras clave: *Sardinella aurita*; cambios histológicos; ciclos de congelación y descongelación; retención de agua

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INTRODUCTION

Consumer interests related to food safety and quality are paramount when selecting fish for purchase, and this holds true on a global scale [1, 2]. Consumers are increasingly vigilant when choosing fish, driven by concerns that the fish they buy may have undergone alterations. These alterations may involve opting for thawed fish rather than fresh ones [2, 3]. After death, fish undergo significant chemical and bacterial changes that render them more suitable for consumption, as highlighted in a study [4]. Furthermore, assessing the quality of frozen fish takes into account the condition of the fish before the freezing process [5, 6]. Certain fish species have a limited shelf life due to their low protein, fat, and moisture levels, as well as the presence of microorganisms [4, 7]. The shelf life of such fish has been extended through the development of methods for freezing perishable raw materials, enabling long-term storage. Freezing is one of the most common processes employed in the food industry today to preserve the quality of meat and other food products over time [8]. It leads to the deceleration of many biochemical reactions, resulting in alterations in food quality. Additionally, freezing reduces water activity, a critical aspect of the freezing process that inhibits microbial growth [9]. It also induces changes in muscle structure, impacting the overall sensory quality of fishery products [10, 11].

In 2018, It was reported that ice crystals formed in muscles during freezing cause irreversible structural damage to muscle fibers and myofibrils [12]. This damage is attributed to changes in muscle morphology and size due to the formation of ice crystals, which occur at varying rates depending on the freezing speed of the meat [13]. Freezing meat can impair its quality by denaturing proteins and aggregating them into larger masses. Both [14] and [15] found that this deterioration in quality persists even after thawing. This persistence is attributed to the formation of ice crystals during the freezing process, which can damage the structure of proteins, leading to their denaturation. Protein denaturation, in turn, alters their functional properties, affecting their ability to bind water, solubility, and three-dimensional structure. Notably, the influence of protein denaturation on the texture, flavor, and juiciness of meat persists even after the fish has been thawed. When assessing fish meat quality, it is essential to consider its fat content. When discussing the fat content of food, reference is made to the quantity of healthy fats it contains, a factor proven to benefit human health [16]. Fish meat loses quality when it oxidizes or is frozen and stored for extended periods [17]. The rate of meat spoilage is contingent on the release of fatty acids without interference from lipases responsible for breaking down fish proteins. [17] and [3] corroborated this fact. Moreover, the water content of meat affects its taste and texture after defrosting or refreezing. Freezing fish muscles alters their structure and composition, as indicated by histological analysis and chemical assessments.

By collecting data related to these aspects, the current study aims to demonstrate the effect of freezing on the muscles of Sardinella (*Sardinella aurita*). This introduction provides an overview of the primary factors that have led to the undertaking of this study on the impact of freezing on the quality of fish products in Algeria. It underscores the growing importance of food safety and product quality for consumers, as well as the challenges associated with preserving the freshness of fish products in a context where freezing has become commonplace.

MATERIALS AND METHODS

Sampling

Histological evaluation was carried out on a total of 30 fresh samples of *Sardinella aurita*, obtained from El-Kala fishing port, Algeria, with a weight of about 111.25 g and a length of 25 cm and brought refrigerated to the Laboratory of Histology and Histopathology of the University of Batna 1 within 12 hours of being caught. All samples were individually wrapped in polyethylene bags and randomly divided into 6 groups; the first group with zero freeze-thaw cycle (T0 (fresh), while the samples of the other five groups were subjected to freezing at -20°C for different days (d) (4 d, 7 d, 11 d, 15 d, and 20 d) separated by thawing at 4°C for 12 hours to obtain samples with different freeze-thaw cycles, i.e., T1 (one freeze-thaw cycle), T2 (two freeze-thaw cycles), T3 (three freeze-thaw cycles), T4 (four freeze-thaw cycles), T5 (five freeze-thaw cycles). The microstructure of meat tissues was observed after thawing.

Physicochemical properties

- **pH measurement**

The pH values were measured according to the technique described by Li *et al.* [18] and the samples were prepared by mixing 10 g of fish with 90 mL distilled water. The pH values were measured using an inoLab® digital pH meter (Xylem Analytics, WTW, Weilheim, Germany).

- **Water activity (Aw)**

Water activity (Aw) was measured with a Hygroscope Rotronic model BT-RS1.

- **Color**

A colorimeter (Konica Minolta CR-10, Japan) was used to evaluate the color changes due to freeze-thaw cycles. To ensure a comprehensive representation, as reported by Ali *et al.* [19], measurements included brightness (L*), redness (a*), and yellowness (b*) and they were recorded at three distinct locations on the surface of each of the six fish samples.

- **Protein solubility**

One gram of fish sample underwent homogenization using a Wiggins D-500 homogenizer (Straubenhardt, Germany) and treated as described by Choi *et al.* [20]. The homogenized sample was then mixed with 20 mL of ice-cold KI/potassium phosphate and left at a temperature of 4°C overnight. Afterward, the mixture underwent centrifugation (B. Braun Sigma 2K15 Centrifuge, Germany) (2,000 G) at 4°C for 20 min. Following this, a combination of 2 mL of the supernatant and 8 mL of a biuret solution was prepared and added. The absorbance of this solution was subsequently measured at a wavelength of 540 nm with a spectrophotometer (Shimadzu UV 120-01, Japan).

Water holding capacity

- **Moisture content (%)**

The water content of various commodities was largely assessed through oven drying method, and represent the difference between the initial weight of samples (10 g) and their subsequent weight after oven drying at 37°C for 3 d as reported by Avinee *et al.* [21].

To assess how well samples, retain water, we measured thawing loss, centrifugation loss, and cooking loss, with some minor changes to the methods reported by Zhu *et al.* [22]. Specifically, we modified the assessment of centrifugation loss by using 2 g samples instead of the 10 g mentioned by Zhu *et al.* [22]. Additionally, in the evaluation of cooking loss, we standardized the sample weight to 5 g.

- **Thawing loss (%)**

After the meat had thawed, the liquid in the package was poured out, and the samples of meat were wiped down with paper towels and weighed again. The freezing loss was measured as a percentage of the difference between the amount of weight lost before and after freezing.

- **Centrifugation loss (%)**

Two grams of fish samples, enclosed in filter paper, were loaded into a centrifuge tube and subjected to centrifugation at 210 G at 4°C for 10 min. The weight difference between the samples before and after centrifugation was used to calculate the centrifugation loss.

- **Cooking loss (%)**

To figure out how much food was lost during cooking, 5 g of samples were measured, wrapped in heat-resistant foil paper, and put in a water bath at 80°C for 30 min. The internal temperature was not measured, but based on an earlier study [23], it was estimated that it would take 30 min to reach the best internal temperature of 75 to 80°C. Samples are left out to dry and then weighed.

Histological and morphometric analysis

After preliminary microscopic examination of hematoxylin and eosin (H&E) stained sections, five hot spot areas of the samples were selected at low power magnification and measurements were made at 100X using a free software, ImageJ (version 1.52a). These measurements included average area of vacuoles, average area of myofibers containing vacuoles, and the ratio of total vacuole area to cell area were calculated to perform quantitative analysis of vacuoles with freeze–thaw cycles.

Statistical analysis

Statistical analysis was performed on the observed values by the application of variance (ANOVA) using SPSS software version 26 (IBM SPSS Statistics v26). Comparison of means was performed using the Tukey method. The difference is considered statistically significant when $P<0.05$ and considered nonsignificant when $P>0.05$.

RESULTS AND DISCUSSION

Physicochemical properties

The freezing–thawing cycles appear to have an effect ($P<0.05$) on the pH of *Sardinella aurita* samples (TABLE I). After several cycles, the pH showed a downward trend, followed by a significant increase in the fifth cycle. This may indicate changes in the chemical or microbiological composition of the fish due to the repeated freezing–thawing process.

The elevation in volatile basic components is what causes the pH to increase during storage. The latter acknowledges earlier research presented [18, 24]. The development of ice crystals at -20°C may give rise to the release of intracellular components, resulting in the highest pH value observed in sample T5. Similar findings have been reported by other authors [24, 25, 26]. pH values of fish can be influenced by various factors, including species, size, season, water composition, during location, stress levels during fishing, and muscle type [18].

The water activity values indicate a gradual decrease in water availability in sardinella samples over the freezing–thawing cycles (T0: 0.911 ± 0.009, T4: 0.899 ± 0.01), with some minor variations (TABLE I). This reduction may be attributed to water loss during the freezing–thawing process. Monitoring water activity is important as it can impact the microbiological stability and texture of food products. The variations in water activity are related to fluid migration and ice crystallization [27]. Significant differences ($P<0.001$) in water activity were observed in the abdominal and dorsal muscles of carp after freezing [1]. This study highlights a decrease in water activity after the first freezing, followed by an increase after the second freezing,

TABLE I
Changes in physiochemical and water–holding properties of *Sardinella aurita* subjected to multiple freeze–thaw cycles

Traits	T0	T1	T2	T3	T4	T5
Physicochemical properties						
pH	6.23 ± 0.10^{a}	6.17 ± 0.08^{a}	6.20 ± 0.10^{a}	6.15 ± 0.10^{a}	6.19 ± 0.10^{a}	6.47 ± 0.10^{b}
Water activity	0.911 ± 0.009^{a}	0.909 ± 0.005^{a}	0.903 ± 0.010^{a}	0.900 ± 0.010^{a}	0.899 ± 0.010^{a}	0.920 ± 0.010^{a}
L* value (lightness)	63.51 ± 11.01^{c}	58.77 ± 11.18^{bc}	45.61 ± 16.49^{abc}	42.48 ± 38.53^{abc}	38.53 ± 15.50^{ab}	33.64 ± 15.63^{c}
a* value (redness)	-9.27 ± 2.82^{a}	-8.17 ± 2.82^{ab}	-7.90 ± 2.70^{ab}	-6.80 ± 2.37^{abc}	-5.80 ± 2.76^{bc}	-5.05 ± 2.56^{c}
b* value (yellowness)	26.74 ± 3.06^{c}	25.48 ± 3.06^{bc}	21.36 ± 5.51^{abc}	20.00 ± 5.48^{abc}	18.94 ± 5.76^{ab}	17.28 ± 5.87^{a}
Protein solubility (mg/g)	15.99 ± 1.28^{a}	14.58 ± 0.43^{ab}	13.64 ± 0.84^{b}	12.7 ± 1.25^{bc}	11.23 ± 1.25^{c}	8.88 ± 0.80^{d}
Water–holding properties						
Moisture content (%)	78.68 ± 1.23^{b}	78.10 ± 1.97^{b}	76.90 ± 1.97^{b}	74.70 ± 0.60^{a}	74.08 ± 1.28^{a}	73.18 ± 1.34^{a}
Thawing loss (%)		0.78 ± 0.27^{a}	1.35 ± 0.49^{bc}	1.54 ± 0.69^{bc}	2.12 ± 0.67^{cd}	3.28 ± 1.04^{d}
Centrifugation loss (%)	8.64 ± 0.70^{a}	10.20 ± 3.09^{a}	15.84 ± 1.87^{b}	17.98 ± 1.59^{bc}	19.68 ± 1.77^{bc}	20.00 ± 1.58^{c}
Cooking loss (%)	13.72 ± 2.30^{a}	15.28 ± 0.40^{ab}	19.00 ± 20.4^{bc}	20.40 ± 1.70^{c}	21.20 ± 1.30^{c}	22.22 ± 1.69^{c}

Note: T0: fresh meat; T1: one freeze–thaw cycle; T2: two freeze–thaw cycles; T3: three freeze–thaw cycles, T4: four freeze–thaw cycles, T5: five freeze–thaw cycles. Data are given as mean values ± standard deviation (n = 5). Means within a row with different superscripts differ significantly ($P<0.05$)

regardless of the muscle type (dorsal or ventral). In contrast, our own results demonstrate a decrease in water activity over the first four cycles of freezing–thawing, followed by an increase in the fifth cycle. These variations could be attributed to specificities related to the types of fish studied and experimental conditions.

The colorimetry results reveal that sardinella samples subjected to freezing–thawing cycles undergo significant color changes (TABLE I). Brightness progressively decreases, indicating a darkening of the samples. The values of the (a*) component (redness) show significant variations, indicating a gradual increase from T0 to T5, while the yellow hue (b*) slightly increases. Uncertainty varies among samples, which may reflect measurement variability. These color changes can have significant implications for the quality and perception of the final product.

Proteins in sardinellas exhibit a gradual decrease in solubility following freezing–thaw cycles (TABLE I). This reduction in solubility is likely due to the effect of freezing and thawing on the protein's structure. The decrease in protein solubility may potentially affect the texture and quality of the final product. In a study conducted by Careche and his team [28] significant drops in the solubility of soluble proteins were observed in cod fillets at both -20°C and -30°C, with a more pronounced decrease at -20°C.

Water–holding capacity

The results show variability in the moisture content of sardinella samples subjected to freezing–thawing cycles. Initially, at T0 (fresh fish), the average moisture content was 78.68%, indicating a substantial presence of water in the samples. However, after the first two cycles (T1 and T2), while the moisture content remained relatively high, a slight non–significant reduction was observed compared to T0. The significant variation occurred from the third cycle (T3) onwards, where the moisture content started to decrease significantly, reaching 74.7% at T3 and 74.08% at T4. This downward trend continued with the fifth cycle (T5), where the moisture content reached 73.18%. (TABLE I). These variations may be influenced by freezing, thawing, and storage processes, as well as other environmental factors. According to other studies [29], the inability of muscle fibers to absorb water during thawing is the reason for the loss of water content after freezing. The melting of ice crystals positioned between muscle fibers is thought to be the source of this water. The effect of freeze–thaw cycles on *Sardinella aurita* water content is substantial ($P<0.05$).

The thawing loss, measured in percentage, progressively increases as the samples undergo more freezing–thawing cycles (TABLE I). At T1, the thawing loss is 0.78% with an uncertainty of ± 0.27%. This loss increases at T2, T3, T4, and reaches 3.28% at T5. This trend indicates that the samples lose water during the thawing process.

The centrifugation loss, also measured in percentage, significantly increases as the samples undergo freezing–thawing cycles (TABLE I). At T0, the centrifugation loss is 8.64% with an uncertainty of ± 0.7%. This loss increases at T1, T2, T3, T4, reaching 20% at T5. This trend indicates that the samples have progressively more difficulty retaining water during the process.

The cooking loss, also measured in percentage, significantly increases with freezing–thawing cycles (TABLE I). At T0, the cooking loss is 13.72% with an uncertainty of ± 2.3%. This loss increases at T1, T2, T3, T4, reaching 22.22% at T5. This trend indicates that the samples lose a significant amount of water during cooking, which can affect the quality of the product.

Histological analysis

Central and peripheral muscle fibers remained unchanged. No muscle bundles or muscle fibers appeared partially or completely fragmented (FIG. 1a). Instead, cross–sliced muscle fibers appeared polygonal in shape. Lyu *et al*. [30] found that fish muscle fibers in bundles have a polygonal shape and appear uniform when non–frozen. Strateva [1] noted that the carp muscle fibers lack torn bits when positioned uniformly. Dehydrated muscle fibers were observed with reduced morphological changes. Discarded muscle fibers with complete disruption of shape were also observed. Contracted muscle fibers were observed to be more abundant than fibers that maintained their integrity or completely disrupted in shape (FIG. 1b) When ice crystals form in the outside space, new osmotic pressure variations arise. Dehydration of muscle fibers can be seen via visual observation of reduced muscle volume. Strateva [1] determined that muscle fibers diminish in size due to osmotic processes and ice in the extracellular spaces. As a result of both of these impacts, a perpetual stream of water moves from the most internal region of muscle fibers to the outermost perimeters. Interstitial protein material in the form of a distinct basophilic granular substance is evident between muscle fibers (FIGS. 1c, 1d, 1e, 1f). A similar substance has been found in Carp and Merluccius fish [1, 31]. This protein–based substance is found in the intercellular space of these fish. Deformed muscle tissue merged into one figure as it was severely dehydrated and degenerated. This appeared to be the result of losing the ability to contact, which caused

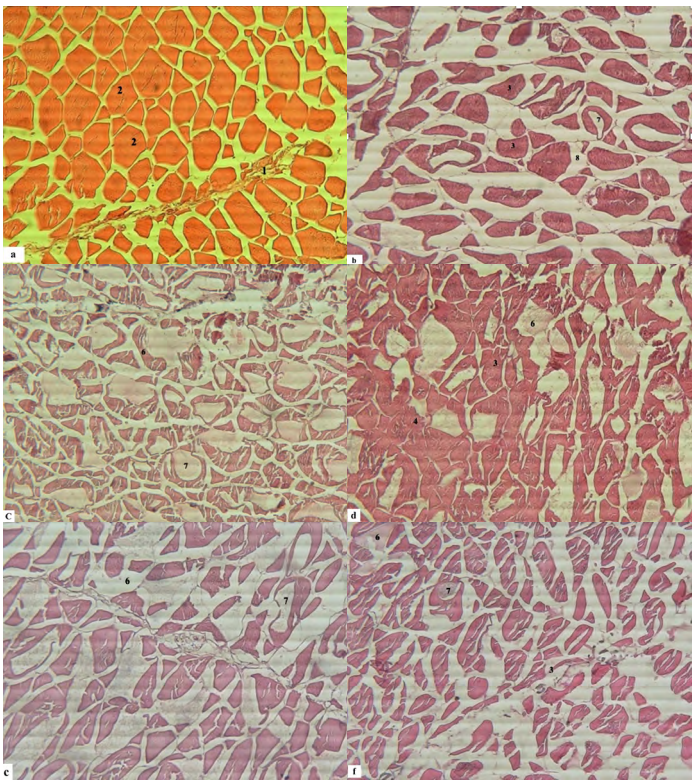

FIGURE 1. Muscle histology of *Sardinella aurita* subjected to different freeze–thaw cycles. (H&E, 100×). a: T0(fresh meat); b: T1(one freeze–thaw cycle); c: T2(two freeze–thaw cycles); d: T3(three freeze–thaw cycles); e: T4(four freeze–thaw cycles); f: T5(five freeze–thaw cycles). 1. perimysium internum; 2. skeletal muscle fibers with normal histostructure; 3. shrunken skeletal muscle fibers; 4. completely destructed skeletal muscle fibers; 5. skeletal muscle fibers with broken central and retained peripheral part; 6. interstitial protein material; 7. vacuoles; 8. Endomysium

the tissue to appear stiff (FIG. 1c). The size of cracks in the frozen material made it unlikely that crack patterns lined up with muscle fibers and bunched muscles. Kiani and Sun [9] found that large frozen crystals produced larger cracks. Additionally, the protein and vacuoles between the muscle fibers blurred their edges and contours. Freezing and thawing processes reduced significantly the amount of skeletal muscle tissue. Furthermore, the size and number of open spaces increased dramatically filling more areas (FIG. 1d, 1e, 1f)

Histomorphometric analysis

Quantitative analysis of ice crystal sizes (FIG. 2) and volumes was performed by calculating the ratio of the size or volume of a fish's ice crystals to the fish's cell surface area (FIG. 3). However, this was difficult for one part of the analysis; since some fish were thawed and some fibers were disrupted (T4 and T5). Calculating fish cell surface areas showed to be much easier than calculating ice crystal surface areas. Although there was significant difference (P<0.05) between first (603,63 μm^2) and fifth cycles (3954,18 μm^2), the trend remained the same a significant increase in cell area with the first cycle followed

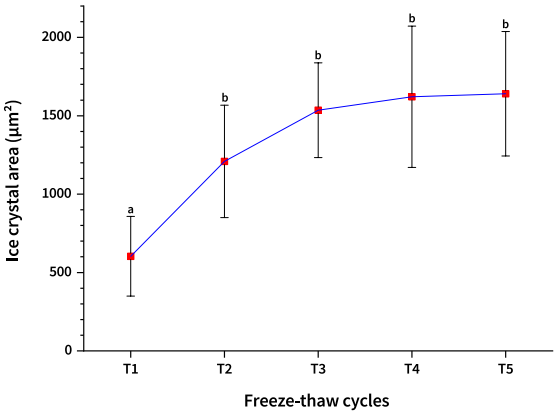

FIGURE 2. Mean area of ice crystals to different freeze–thaw cycles. T1: one freeze–thaw cycle; T2: two freeze–thaw cycles; T3: three freeze–thaw cycles, T4: four freeze–thaw cycles, T5: five freeze–thaw cycles. Different letters show a statistically significant difference (P<0.05)

by a larger increase in cell area with subsequent cycles. Furthermore, all samples demonstrated the same trend. When ice is stored frozen, its volume and size recrystallize. This process was well explained by Jiang *et al*. [32], during subsequent storage, small ice crystals will melt and grow into larger ones. This causes light melting of large crystals that can then grow again [33]. Ice damage to muscle fibers causes increased extracellular migration of water and thawing losses. Jiang *et al.* [32] stated that this led to increased protein denaturation when ice crystals form. Also, increased solute in the remaining water leads to further protein destruction, according to Kaale *et al.* [34].

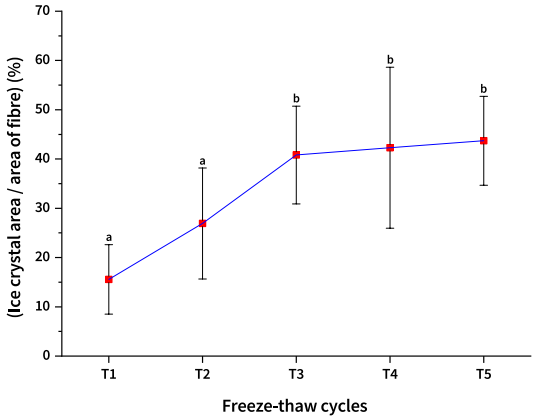

FIGURE 3. Ratio of the ice crystal surface to the cell surface. T1: one freeze–thaw cycle; T2: two freeze–thaw cycles; T3: three freeze–thaw cycles, T4: four freeze–thaw cycles, T5: five freeze–thaw cycles. Different letters show a statistically significant difference (P<0.05)

CONCLUSIONS

To summarize, this extensive investigation into the impact of freeze–thaw cycles on *Sardinella aurita* samples has uncovered noteworthy alterations in physicochemical characteristics, water retention, color, and histological structure of the fish. These findings emphasize the significance of comprehending how freezing–thawing affects fish quality, especially within the context of the fishing and food processing industry, with a view to thwarting food fraud. pH measurements indicate changes in pH after multiple cycles. Initially, a downward trend was observed, followed by a significant increase in the fifth cycle. Additionally, water activity values show a gradual decrease in water availability in sardine samples over the freezing–thawing cycles, with some minor variations. Furthermore, there is a color changes, that are not noticeable to most consumers. Additionally, histological changes such as muscle fiber desiccation can exert an influence on the fish's texture. It is important to acknowledge that these results may be influenced by various factors, such as the size of the fish, seasonal fluctuations, and environmental conditions. As a result, further investigations could explore these aspects to gain a more profound understanding of the underlying mechanisms. In essence, this study offers valuable insights for professionals in the fishing and food processing sector, enabling them to make informed decisions regarding the management of seafood products subjected to freezing–thawing cycles. Such measures not only assure product quality and food safety but also serve as a deterrent against food fraud for the benefit of consumers.

Conflict of interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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