ISSN: 2085-675X (Print)
ISSN: 2354-8770 (Electronic)

Jurnal Kefarmasian Indonesia
Available online at https://jkefarind.com/index.php/jki
Original Research Article
JKI

Effect of Storage Time and Temperature on Protein Content of White Anchovy (*Stolephorus tri*)

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ARTICLE INFO

Article history:
Received 26 February 2026
Revised 06 April 2026
Accepted 08 June 2026
Published online 30 June 2026

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Citation: Azizah A, Suhendi A. Effect of Storage Time and Temperature on Protein Content of White Anchovy (Stolephorus tri). Jurnal Kefarmasian Indonesia. 2026;16(1):112-121.

ABSTRACT

Anchovy (*Stolephorus tri*) is widely consumed in Indonesia as a source of protein, but its nutritional quality is strongly influenced by storage conditions. This study aimed to quantify changes in soluble protein content (mg/mL) during storage at room temperature (25–32 °C), refrigeration (2–8 °C), and freezing (–20 °C) over 60 days. Fresh anchovy samples (n = 3 per time point, 5 g each) were analyzed using the Lowry method, and data were evaluated with repeated-measures ANOVA followed by Tukey's post-hoc test ($p<0.05$). Results showed that protein content decreased significantly by 40.9% at –20 °C after 60 days ($p<0.01$), while refrigeration produced unstable fluctuations with a 74.9% increase at day 60 ($p<0.05$), and room temperature storage resulted in minor increases of up to 3.05%. Organoleptic evaluation indicated that freezer storage best preserved sensory quality compared to refrigeration and room temperature, although protein levels declined gradually. In conclusion, storage temperature and duration significantly affect protein content and sensory quality of anchovy, with freezing recommended as the most effective method to maintain quality during distribution, while confirmatory analyses such as SDS-PAGE, Bradford, or Kjeldahl are suggested to distinguish intact proteins from degradation products.

***Keywords*:** *Stolephorus tri;* Lowry assay; Frozen storage; Protein degradation

INTRODUCTION

Anchovy (*Stolephorus tri*) is one of the fish species widely found in Indonesia. According to traditional fisheries production data in 2023, anchovy reached a total production of 8,040.43 tons annually, indicating that this species is abundantly harvested from Indonesian maritime.[1] The nutritional composition of 100 g of fresh anchovy includes 77 kcal of energy, 16 g of protein, 1 g of fat, 500 mg of calcium, 0.01–0.17 mg of fluoride, 500 mg of phosphorus, 1 mg of iron, 47 IU of vitamin A, and 0.1 mg of vitamin B.[2]

The distribution of anchovy from fishermen to consumers is highly dependent on post-harvest handling, which determines the final product quality. In traditional markets, fish is often stored under open conditions or with block ice often fails to maintain optimal temperatures, accelerating spoilage due to microbial activity and proteolytic enzymes and reducing both sensory and nutritional quality of fish.[3] Temperature control is therefore critical, as deterioration occurs rapidly at room temperature but is slowed under refrigeration and freezing, with the

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ISSN: 2354-8770 (Electronic)

latter remaining the most reliable strategy for long-term preservation despite gradual protein loss.4,5,6

Storage temperature directly influences fish shelf life, with significant deterioration observed after only six hours at room temperature,[7] while chilled storage extends acceptability up to 14 days. However, storage at 2–5 °C still shortens shelf life to 5–9 days, and freezing at –30 °C to –35 °C can maintain quality for up to 12 months if stability is ensured [8]. Previous studies have shown that protein degradation occurs during both chilled and frozen storage, with differences in degradation patterns across species. These findings highlight the importance of evaluating protein stability in anchovy under varying storage conditions.[9,10]

Evaluation of protein degradation during storage can be performed using various analytical methods, including amino acid analysis (AAA) as a direct method, as well as indirect methods such as Dumas, Kjeldahl, Bradford, BCA, and Lowry assays. Among these, the Lowry method was selected in this study because of its high sensitivity at low protein concentrations and its ability to detect total protein changes in complex biological matrices. It also offers flexibility in measurement wavelengths (650–750 nm), lower variability compared to Coomassie-based methods, approximately 100-fold greater sensitivity than the Biuret method, and has been widely recognized for its accuracy and precision.[11,12,13]

This study aimed to quantify changes in total soluble protein content (mg/mL) of anchovy (*Stolephorus tri*) under different storage temperatures, including room temperature (25–32 °C), refrigeration (2–8 °C), and freezing (–20 °C) over 60 days. Protein content was analyzed using the Lowry method to evaluate the relationship between storage conditions and protein stability. It is hypothesized that lower storage temperatures significantly reduce protein degradation compared to room temperature storage, resulting in better preservation of protein content and sensory quality.

METHODS

Location and Time

The study was conducted at the Pharmaceutical Analysis Laboratory, Faculty of Pharmacy, Universitas Muhammadiyah Surakarta, from August 2025 to January 2026.

Instruments

The instruments used included a visible spectrophotometer (UV Mini 1280®, Shimadzu), sonicator bath (Branson® 2510), cuvettes (Hellma), glassware, refrigerator (Samsung), freezer (Sansio), ice box, and analytical balance (Semi-Micro EXP, Ohaus).

Materials

Materials used were anchovy (*Stolephorus tri*) obtained from Selokaton Fish Market, bovine serum albumin (pro analysis, Merck), Folin–Ciocalteu reagent (pro analysis, Merck), sodium carbonate (Na_2CO_3, pro analysis, Merck), sodium hydroxide (NaOH, pro analysis, Merck), copper sulfate ($CuSO_4$, pro analysis, Merck), and potassium sodium tartrate (pro analysis, Merck).

Sample Collection

Anchovy samples were collected from Selokaton Fish Market, Gondangrejo District, Karanganyar Regency, Central Java, on Wednesday, October 7, 2025, at 20:01. According to the vendor, fresh anchovy was caught in the afternoon and delivered to the market at approximately 17:00 on the same day with the addition of ice blocks and water to maintain freshness. Samples were weighed, placed in plastic bags, and transported using an ice box filled with ice. The ice box was placed in the motorcycle dashboard compartment and transported over approximately 10.5 km within about 30 minutes. A total of approximately several hundred individual fish (estimated 250–500 individuals) were collected and pooled prior to analysis. Samples were randomly assigned to different storage groups and labeled to minimize bias. Temperature during

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ISSN: 2354-8770 (Electronic)

transportation was not continuously monitored, which is acknowledged as a limitation of this study.

Sample Storage

Fish samples were divided into three groups based on storage conditions: room temperature (25 °C), refrigeration (2–8 °C), and freezing (–20 °C). Each group was further divided into 12 containers corresponding to four observation time points (0, 7, 30, and 60 days). Each container contained at least 5 g of anchovy, and three containers were used as replicates for each time point. This arrangement was intended to prevent cross-contamination and minimize fluctuations caused by repeated sampling or inconsistent storage. Each container represented a biological replicate (n = 3 per time point), and samples in each container consisted of pooled anchovy to reduce individual variability. Temperature stability in the refrigerator and freezer was monitored periodically; however, continuous temperature logging was not performed.

Preparation of Bovine Serum Albumin

A standard solution was prepared by accurately weighing 10 mg of bovine serum albumin and dissolving it in a 5 mL volumetric flask with distilled water.

Preparation of Lowry Reagent A

A mixture of 0.2 g potassium sodium tartrate and 10 g sodium carbonate was placed into a 100 mL volumetric flask, followed by addition of 50 mL of 1 N NaOH and dilution with distilled water to volume [14].

Preparation of Lowry Reagent B

A mixture of 0.2 g potassium sodium tartrate and 0.1 g copper sulfate was dissolved in 10 mL of 1 N NaOH and diluted with distilled water to 100 mL [14].

Preparation of Lowry Reagent C

Six milliliters of Folin–Ciocalteu reagent was diluted with distilled water to 100 mL [14].

Operating Time and Maximum Wavelength

A total of 750 µL of 0.2% BSA solution was mixed with 900 µL Lowry reagent A and 100 µL Lowry reagent B, then incubated at room temperature for 10 minutes. Subsequently, 3 mL of Lowry reagent C was added. Absorbance was measured every 2 minutes until stabilization to determine the operating time. Maximum wavelength was determined by scanning from 400 to 800 nm after stabilization.[14]

Preparation of Calibration Curve

Standard solutions (0.56, 0.34, 0.20, 0.12, and 0.07 mg/mL) were prepared. A volume of 500 µL of each concentration was mixed with 900 µL Lowry reagent A and 100 µL Lowry reagent B, incubated for 10 minutes, followed by addition of 3 mL Lowry reagent C. After operating time (38 minutes), absorbance was measured at maximum wavelength (749 nm,). Blank solution consisted of distilled water with Lowry reagents [14].

Sample Preparation

Anchovy samples were dried in an oven at 50 °C for approximately 8 hours [15] and weighed to determine moisture content. Samples were ground, and 100 mg was transferred into a 10 mL volumetric flask and dissolved with distilled water. The flask was placed in a sonicator bath for 10 minutes and incubated at 50 °C for 5 minutes. The solution was filtered using filter paper [14].

Accuracy

Accuracy was evaluated using the standard addition method at 0%, 80% (0.185 mg/mL), 100% (0.231 mg/mL), and 120% (0.278 mg/mL). Samples were prepared and analyzed following the same procedure as the calibration curve [13,14].

Precision

Precision was assessed by preparing sample solutions and analyzed with seven times replication on the same day

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ISSN: 2354-8770 (Electronic)

following the same procedure as the calibration curve [13,14].

Organoleptic Test

Organoleptic evaluation was conducted by several panelists using a simple descriptive assessment of color, odor, texture, and overall acceptability. The number of panelists and scoring system were limited, which is acknowledged as a limitation of this study[7].

Protein Determination

A total of 500 µL sample solution was mixed with 900 µL Lowry reagent A and 100 µL Lowry reagent B, incubated for 10 minutes, followed by addition of 3 mL Lowry reagent C. After operating time, absorbance was measured at maximum wavelength, and protein content was calculated using the calibration curve [14].

Data Analysis

Data of protein content were initially analyzed using the **Shapiro–Wilk normality test**. Differences among storage conditions and storage durations were evaluated using **Kruskal-Wallis test**, followed by **Tukey's post-hoc test** for multiple comparisons. Data are presented as **mean ± standard deviation (SD)**, with statistical significance set at **$p < 0.05$**. [16].

RESULTS AND DISCUSSION

Operating Time (OT) and Maximum Wavelength

Operating time (OT) refers to the time required for the color of the reacted solution to reach stability. Determination of OT is essential because measurements performed too early or too late may result in inaccurate absorbance values. During the incubation period leading to OT, two reaction stages occur until a stable color is formed, as illustrated in Figure 1. The first stage involves the formation of a complex between copper (II) ions (Cu^{2+}) and peptide bonds under alkaline conditions. The second stage involves the reduction of the Folin–Ciocalteu reagent (containing phosphomolybdic and phosphotungstic acids) by aromatic amino acid residues such as tyrosine and tryptophan. This reaction produces an intense blue color whose intensity is proportional to the protein concentration in the sample [17].

In this study, the operating time was obtained at 38 minutes with an absorbance value of 1.470. Furthermore, the maximum wavelength was determined using a visible spectrophotometer within the range of 400–800 nm, with an acceptable absorbance range of 0.1–2.0 A. Determination of the maximum wavelength was conducted to achieve optimal measurement sensitivity, thereby improving the accuracy of the results. The measurement showed an absorbance value of 0.971 at a wavelength of 749.0 nm.

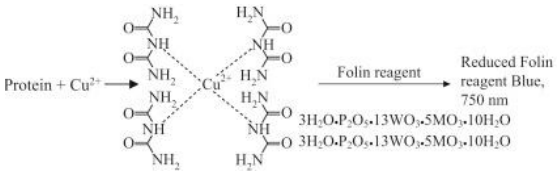

Figure 1. Reaction mechanism of the Lowry method showing complex formation between protein and Cu^{2+} followed by reduction of Folin–Ciocalteu reagent [18].

Determination of the Calibration Curve

The calibration curve was constructed by correlating the concentration of the Bovine Serum Albumin (BSA) standard solution as the x-axis (0.07–0.56 mg/mL) with the absorbance values as the y-axis. The result of calibration curve, demonstrate a linear relationship between concentration and absorbance, with a coefficient of determination (R^2) of 0.9918. The linear regression equation obtained from five concentration points was $y = 2.6617x + 0.5533$, as presented in Figure 2. The relatively high intercept (0.5533) suggests possible matrix interference or background absorbance, which may affect accuracy, as the Lowry method is known to be susceptible to interference from non-protein compounds present in complex biological samples.[19]

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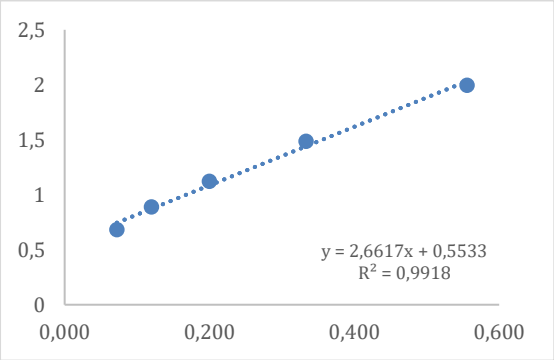

Figure 2 Calibration curve of BSA (bovine serum albumin) standard (0.07–0.56 mg/mL) versus absorbance using the Lowry method (λ = 749 nm)

Accuracy Parameter

Accuracy is a measure that indicates the closeness or agreement between the analytical result and the true value of the analyte. Accuracy is expressed as the percentage recovery (% recovery) of the added analyte. Accuracy can be determined using two approaches, namely the simulation method (spiked-placebo recovery) and the standard addition method [20]. In this study, the standard addition method was employed to evaluate accuracy with adding standard solution ranging from 80% to 120% on samples. The % recovery values obtained met the acceptance criteria of 98–102% according to AOAC International [21], with recovery results ranging from 98.10-101.13%, as presented in Table 1.

Table 1. Results of accuracy parameter determination of the Lowry method (n = 3)

Groups sample Spiked with (%)	Concentration (mg/ml)	RSD (%)	% Recovery	RSD (%)
0%	2.313 ±0.05	1.99	-	-
80%	2.495 ±0.05	1.86	98.10±0.32	0.32
100%	2.546 ±0.05	1.89	100.83±1.52	1.51
120%	2.594 ±0.05	1.83	101.13±0.58	0.57

Precision Parameter

Precision describes the closeness of agreement among independent test results obtained under specified conditions [21], which can be expressed in terms of repeatability or reproducibility [20]. In this study, precision was evaluated in terms of repeatability by assessing the consistency of measurement results when the method was applied repeatedly under the same conditions [22]. The acceptance criterion for precision is a relative standard deviation (RSD) or coefficient of variation (CV) value of ≤ 2% [20]. The precision test in this study yielded an RSD value of 1.975%, indicating that the method met the acceptance criteria for precision, as shown in Table 2.

Table 2. Results of precision parameter determination (repeatability)

Replicate	Absorbance	Concentration (mg/mg)
1.	0.924	0.203
2.	0.933	0.207
3.	0.930	0.205
4.	0.916	0.201
5.	0.957	0.212
6.	0.917	0.202
7.	0.952	0.210
MEAN (mg/mL)		**0.206**
SD		**0.004**
RSD (%)		**1.975**

Organoleptic Evaluation

The organoleptic evaluation of anchovy (*Stolephorus tri*) was conducted by observing color, odor, texture, and overall acceptability during storage at room temperature, refrigeration (2–8 °C), and freezing (–20 °C) on days 0, 7, 30, and 60, as presented in Table 3. On day 0, the samples exhibited fresh characteristics, indicated by a yellowish-white color, typical fresh fish odor, firm texture, and suitability for consumption. During storage, samples kept in the freezer remained relatively stable until day 60, as evidenced by the absence of significant changes in color, odor, and texture, indicating that they remained acceptable for consumption. In contrast, samples stored under refrigeration and at room temperature began to show quality deterioration from day 7, characterized by changes in color, odor, and progressively softer texture until day 60.

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ISSN: 2354-8770 (Electronic)

These findings indicate that freezer storage is the most effective method for maintaining the organoleptic quality of anchovy compared to refrigeration and room temperature storage. The decline in sample quality is attributed to oxidative processes [23] and microbial growth, including spoilage bacteria such as *Pseudomonas* and *Acinetobacter* [24], whose activity slows at low temperatures, particularly under freezing conditions. Rapid bacterial growth leads to spoilage characterized by a slimy surface, softened flesh, and off-odors caused by compounds such as indole, skatole, mercaptans, ammonia, hydrogen sulfide, and other volatile substances [25].

Table 3. Organoleptic observation results of samples from day 0 to day 60 (1= Freezer; 2=Refrigerator; 3= Room Temperature)

Day	Storage	Color	Odor	Texture	Slime	Acceptability
0	-	Yellowish white	fresh	Firm	×	✓
7	1	Yellowish white	fresh	Firm	×	✓
	2	Pale white slightly grayish	putrid odor	Slightly soft	✓	×
	3	Grayish black	strong putrid odor	Disintegrated	✓	×
30	1	Yellowish white slightly grayish	fresh	Firm	×	✓
	2	Gray	putrid odor	Soft and sticky	✓	×
	3	Dark brownish black	strong putrid odor	Disintegrated	✓	×
60	1	Yellowish white slightly grayish	fresh	Firm	×	✓
	2	Gray	strong putrid odor	Soft and sticky	✓	×
	3	Dark brownish black	strong putrid odor	Ikan disintegrated	✓	×

Protein Content Determination

Protein is a major component of fish whose level is strongly influenced by storage conditions. Therefore, accurate determination of protein content is essential to evaluate fish quality during storage. The Lowry method is one of the most sensitive techniques for measuring protein content in samples [26]. This method has a sensitivity range of approximately 0–0.1 mg, with accuracy partly depending on amino acid composition, and it may be affected by the presence of interfering substances such as acids, EDTA, DTT, phenol, and ammonium sulphate [11].

The results showed that protein content in samples stored in the freezer, refrigerator, and at room temperature changed over time, as presented in Table 4 and Figure 2. For samples stored in the freezer (–20 °C), protein content decreased by 1.84% on day 7, 31.84% on day 30, and 40.93% on day 60. These findings are consistent with previous studies reporting a significant decrease in protein content of *Saurida undosquamis* during frozen storage, with reductions of 3.82, 7.88, and 11.96% on days 7, 14, and 21, respectively [10].

Samples stored under refrigeration showed an increase in protein content of 20.69% on day 7, followed by a decrease of 7.66% on day 30, and the highest increase of 74.92% on day 60. These results differ from findings reported by Apituley and Tamaela, which showed a decrease in extractable protein during cold storage in both pelagic and demersal fish [9]. Storage at refrigeration temperature (around 4 °C) can lead to gradual degradation of structural proteins and may not sufficiently inhibit enzymatic activity and microbial growth, thereby contributing to instability in measured protein levels [27]. Similarly, anchovy samples stored at room temperature exhibited fluctuating increases in protein content, with increases of 25.39% on day 7, 0.85% on day 30, and 3.05% on day 60 compared to initial levels. Fresh fish stored at room temperature is known to undergo rapid quality deterioration due to continuous microbial growth and enzymatic activity [7].

During the early phase of storage, endogenous enzymes and spoilage bacteria may cause partial protein degradation into peptides and soluble protein fractions, which can lead to higher apparent protein values when measured using the Lowry method [28,29]. As storage progresses to around day 30, further degradation and protein oxidation may result in denaturation and reduced solubility, leading to a decrease in measured protein content. These processes are associated

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ISSN: 2354-8770 (Electronic)

with proteolysis, oxidation, and structural changes in myofibrillar proteins, which are key mechanisms of quality deterioration during chilled storage [30,31].

Table 4. Protein content of samples (n = 3)

Day	Concentration (mg/ml)		
	Freezer	Refrigerator	Room
0	2.797 ±0.18	2.797 ±0.18	2.797 ±0.18
7	2.745 ±0.22	3.375 ±0.06	3.507 ±0.11
30	1.906 ±0.47	2.582 ±0.08	2.820 ±0.04
60	1.652 ±0.25	4.892 ±0.41	2.882 ±0.32

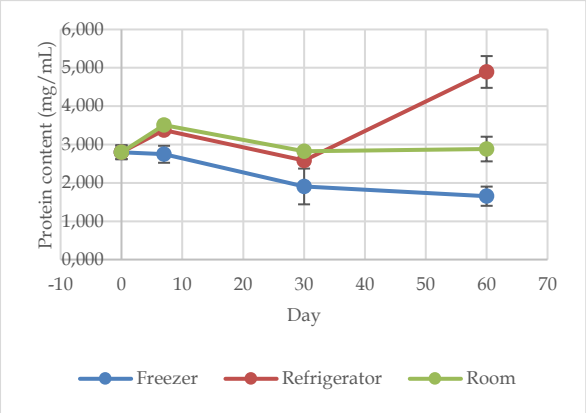

Figure 3. Graph of changes in sample protein levels over 60 days of storage

At longer storage periods, such as day 60, the apparent increase in measured protein may occur due to water redistribution, loss of other components such as lipids or moisture, and accumulation of soluble nitrogenous compounds that still react with the Lowry reagent, thereby increasing measured values. Structural changes and fragmentation of myofibrillar proteins may also produce smaller fragments that remain detectable, resulting in non-linear trends in protein content [32]. Additionally, the formation of carboxylic acids, such as octanoic and butanoic acids at temperatures between –3 °C and 6 °C, and pentanoic, 2-methylbutanoic, and 4-methylpentanoic acids at temperatures above 20 °C [33], can acidify the sample and potentially influence protein measurements, leading to overestimation [19,34]. The Lowry method cannot distinguish between intact proteins and degradation products; therefore, more specific methods may be required to accurately monitor protein changes during storage. Based on the literature, methods such as the Bradford Coomassie Blue assay may provide more specific assessment of protein changes [11,35]. Nevertheless, variability in results may also be influenced by experimental or handling errors during the study. The unexpected increase in protein content observed in refrigerated samples at day 60 may be attributed to the accumulation of soluble nitrogenous compounds, matrix interference in the Lowry assay, or potential experimental and calculation errors. Therefore, these results should be interpreted with caution.

CONCLUSION

Storage temperature and duration significantly influenced the protein content and organoleptic quality of anchovy (*Stolephorus tri*). Freezing (–20 °C) best preserved sensory characteristics, although soluble protein content decreased over time. In contrast, refrigeration and room temperature storage showed unstable protein values due to degradation processes and potential analytical limitations. Therefore, further confirmatory analyses are recommended to distinguish intact proteins from degradation products. Practically, freezing is suggested as the most effective method for maintaining anchovy quality during storage and distribution.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

ISSN: 2085-675X (Print)
ISSN: 2354-8770 (Electronic)

Acknowledgments

The author expresses gratitude to Allah SWT for His blessings that enabled the completion of this research and manuscript. The author sincerely thanks Dr. Andi Suhendi, S.Farm., Apt., M.Sc., as the research supervisor, for his guidance and valuable direction throughout the study. Appreciation is also extended to the Board of Examiners of the Faculty of Pharmacy, Universitas Muhammadiyah Surakarta, for their constructive feedback and suggestions. The author acknowledges the Laboratory of Food and Cosmetic Analysis, Faculty of Pharmacy, Universitas Muhammadiyah Surakarta, for providing research facilities. The author is also grateful to fellow students of the Pharmacy Study Program, class of 2022, for their technical assistance and support during the research, as well as to the author's family for their prayers, encouragement, and moral support.

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