Sains Malaysiana 53(10)(2024): 3369- 3381
*http://doi.org/10.17576/jsm-2024-5310-11*

# Surface Metabolite Amino Acids and Collagen Profiling in Refrigerated Tilapia Fish (*Oreochromis niloticus*): Implications for Identification and Quality Assessment

(Profil Metabolit Amino Asid dan Kolagen pada Ikan Tilapia (*Oreochromis niloticus*) yang Disimpan dalam Penyimpanan Sejuk: Implikasi untuk Pengenalpastian dan Penilaian Kualiti)

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*Received: 30 March 2024/Accepted: 13 August 2024*

ABSTRACT

Nile tilapia (*Oreochromis niloticus*) by-products, including the scales, skin, and mucus, are rich sources of protein with a balanced amino acid profile and high collagen content. Cold storage at 4 °C is commonly used to maintain the freshness of tilapia fish in retail settings. This study aims to investigate the changes in surface metabolites and collagen content in tilapia fish during cold storage at 4 °C. Fresh tilapia fish was stored at 4 °C for 7 days to monitor physicochemical and metabolite changes. Fish collagen content was extracted using acetic acid and pepsin, pH values were measured using a calibrated pH meter, protein content was determined via the Bradford method, and creatine and phenylalanine levels were assessed using High-Performance Liquid Chromatography (HPLC). Analysis was conducted on days 0, 3, and 7 of cold storage. Significant reductions ($p$<0.05) in collagen content from fish scales were observed on the 3rd day of storage, declining from 72.60 ± 12.40% to 30.17 ± 17.62%. pH levels of fish scales and mucus showed a slight alkaline shift, while the skin turned acidic due to bacterial and enzymatic activities. Protein content in the scales, skin, and mucus showed a substantial loss exceeding 50% after 7 days of cold storage. Changes in creatine and phenylalanine concentrations in the mucus further indicated a decline in tilapia fish freshness due to biochemical reactions post-mortem, compromising overall quality. In conclusion, the duration of cold storage significantly affects the composition of tilapia fish scales, skin, and mucus, with a 7-day storage period identified as a suitable freshness indicator in compliance with FDA guidelines permitting fresh fish to be stored at 4 °C for up to two days.

Keywords: Bradford; collagen extraction; creatine; phenylalanine; tilapia by-products

ABSTRAK

Sisa sampingan ikan tilapia Nil (*Oreochromis niloticus*), termasuk sisik, kulit dan lendir merupakan sumber protein yang kaya dengan profil asid amino yang seimbang dan kandungan kolagen yang tinggi. Penyimpanan sejuk pada suhu 4 °C biasanya digunakan untuk mengekalkan kesegaran ikan tilapia pada persekitaran runcit. Penyelidikan ini bertujuan untuk mengkaji perubahan metabolit permukaan dan kandungan kolagen dalam ikan tilapia semasa penyimpanan sejuk pada suhu 4 °C. Ikan tilapia segar disimpan pada suhu 4 °C selama 7 hari untuk memantau perubahan fiziko-kimia dan metabolit. Kandungan kolagen ikan diekstrak menggunakan asid asetik dan pepsin, nilai pH diukur menggunakan meter pH yang dikalibrasi, kandungan protein ditentukan melalui kaedah Bradford dan tahap kreatin serta fenilalanin dinilai menggunakan Kromatografi Cecair Prestasi Tinggi (HPLC). Analisis dijalankan pada hari 0, 3 dan 7 penyimpanan sejuk. Pengurangan ketara ($p$<0.05) dalam kandungan kolagen daripada sisik ikan diperhatikan pada hari ke-3 penyimpanan, menurun daripada 72.60 ± 12.40% kepada 30.17 ± 17.62%. Tahap pH sisik ikan dan lendir menunjukkan sedikit peralihan kepada alkali, manakala kulit menjadi berasid akibat aktiviti bakteria dan enzim. Kandungan protein dalam sisik, kulit dan lendir menunjukkan kehilangan ketara melebihi 50% selepas 7 hari penyimpanan sejuk. Perubahan dalam kepekatan kreatin dan fenilalanin dalam lendir menunjukkan penurunan kesegaran ikan tilapia akibat reaksi biokimia selepas kematian yang menjejaskan kualiti keseluruhan. Kesimpulannya, tempoh penyimpanan sejuk memberi kesan ketara terhadap komposisi sisik, kulit dan lendir ikan tilapia dengan tempoh penyimpanan 7 hari dikenal pasti sebagai petunjuk kesegaran yang sesuai selaras dengan garis panduan FDA yang membenarkan ikan segar disimpan pada suhu 4 °C sehingga dua hari.

Kata kunci: Bradford; fenilalanin; hasil sampingan tilapia; kreatin; pengekstrakan kolagen

## INTRODUCTION

Scientifically, the tilapia fish belongs to the *Cichlidae* family, specifically *Oreochromis niloticus*. It is a predatory fish that thrives in freshwater habitats and is native to Africa and the Middle East. Over time, it has been extensively bred worldwide. The Malaysian Fisheries Department has reported that tilapia is the second most commonly cultivated fish in Malaysia, accounting for over 80% of global tilapia aquaculture production (FAO 2019). According to Mili et al. (2022), this species is renowned for its ability to withstand harsh conditions, various aquaculture treatments, and resistance to diseases. Additionally, its high content of albumin, amino acids, and fatty acids makes it beneficial for human dietary needs (Agnes & Chrispin 2021). Figueiredo et al. (2023) states that Nile tilapia (*Oreochromis niloticus*) contains approximately 62.6% to 62.9% unsaturated fatty acids and 37.6% to 38.6% saturated fatty acids. High levels of unsaturated fatty acids are crucial for brain function and cell growth, although the structure is prone to instability due to carbon double bonds. Therefore, proper handling, including storage conditions and temperature, is essential to minimize the oxidation process.

According to Coppola et al. (2021), fish waste, which includes parts like skin, bones, fins, mucus, and scales, constitutes approximately 20% of the fish and can be a valuable source of collagen and amino acid metabolites. These components contain both organic and non-organic (mineral) elements (Chen et al. 2019; Sionkowska et al. 2017). Caruso et al. (2020) state that during the filleting process, tilapia heads, frames, skins, viscera, and scales account for about 70% of the co-products. Studies have shown that fish collagen has low antigenicity and shares the same genetic codes as collagen derived from mammals (Zhang et al. 2022). Previous research carried out by Faiqah, Ling and Zubairi (2018) explains that fish freshness is influenced by various amino acid metabolites, which can be categorized based on their roles and effects. Amino acid metabolites such as taurine, phenylalanine, tyrosine, and sarcosine significantly affect fish freshness. The level of creatine, which is correlated with the deterioration of muscular tissue, has been used as a diagnostic indicator of fish spoilage. Increased breakdown of phenylalanine and tyrosine, as well as the presence of taurine and sarcosine, also indicate spoiling. Fish freshness can be assessed by monitoring the levels of these amino acids, as well as other nitrogen-containing substances like histamine and uric acid.

However, the storage method of tilapia fish can have an impact on their overall quality and benefits. According to the US Food and Drug Administration, fish kept at 4 °C without any preservatives can only be stored for two days. Before storing in the chiller, the fish should be free of contaminants and should not be kept for more than seven days after being caught (Abusin & Emadi 2020). Immediately after the fish dies, a variety of quick physical, chemical, and organoleptic changes occur, eventually leading to spoilage, as claimed by Duarte et al. (2020). Freshly harvested fish deteriorates rapidly. Fish in tropical regions are susceptible to rigor mortis within 12 h of being caught. Rigor mortis refers to the condition where the fish's muscles become rigid and lose their suppleness (Tahiluddin et al. 2022). Additionally, fish will undergo further deterioration during storage due to enzymatic reactions such as lipase and endogenous enzymes, microbes, and the oxidation of proteins and fats (Eranda et al. 2024). As a result, the nutritional value and organoleptic properties of the fish will decline. Various by-products, including amines, sulfur compounds, aldehydes, ketones, esters, hypoxanthine, histamine, and cadaverine, will be produced. These by-products can cause changes in texture, increased mucus production, and undesirable odors, which will affect the fish's freshness and quality. Some of these by-products developed during cold storage can pose risks to consumer health (Adande et al. 2020; Zhang et al. 2020).

Fish quality monitoring techniques are essential for assessing fish quality throughout the processing, purchasing, and storage stages. These techniques help determine the freshness and overall quality of the fish. Traditional methods of measurement include the Quality Index Method (QIM), which involves sensory quality assessment based on the fish's skin, eyes, gills, and abdomen (Lauteri, Gianluigi & Luca 2023). Another traditional method is the Total Volatile Basic Nitrogen (TVB-N) method, which chemically measures bacterial development (Zhang et al. 2021). Physical characterization methods, such as the assessment of colour, texture, shape, size, volume, and weight using instruments like texture meters and colorimeters, are also commonly used. However, these traditional methods have limitations. They often rely on hazardous chemicals, lack quantitative assessment, and are time-consuming. To address these limitations, advanced scientific technologies have been developed to evaluate the physical, chemical, biological, and metabolic profile characteristics of fish. These technologies include pH-sensitive smart indicators, electronic noses, spectrophotometers, image analysers, and colorimeters (Franceschelli et al. 2021).

Considering the importance of fish freshness in evaluating food products, the study of amino acid metabolite content can help predict the extent of fish degradation during the cold storage period at 4 °C. This analysis can also aid in determining the overall quality of the product. In addition to focusing on the freshness and quality of the fish flesh during low-temperature storage, it is also possible to analyse amino acid metabolites in fish by-products such as scales, fins, skin, and mucus. This opens up opportunities for future product innovation and applications.

## MATERIALS AND METHODS

### MATERIALS AND CHEMICALS

A random fresh Nile tilapia fish (*Oreochromis niloticus*) as in Figure 1, weighing an average of 250 g, was randomly selected from a local market in Seri Kembangan, Selangor, Malaysia (3°01'49.6"N 101°41'55.4"E). The fish was placed in a clean polystyrene box filled with ice cubes to maintain a consistently low temperature (<0 °C) and promptly transported to the laboratory to preserve its freshness and quality. It was then stored in a refrigerator at 4 °C for 7 days. The chemicals used for collagen extraction, protein analysis and metabolites profiling were all analytical grade (Sigma-Aldrich™, Malaysia). Distilled water was obtained directly from a distiller unit.

### TILAPIA SCALES AND SKIN COLLAGEN EXTRACTION

A well-known method was used as the basis for the extraction of pepsin-soluble collagen (PSC) (Kaewdang et al. 2014). For sample pre-treatment, 1 gram of tilapia scales and skin was added to a centrifuge tube with a ratio of 1:10 (w/v) in a 0.1 M sodium hydroxide (NaOH) solution. The sample was immersed for two days at 4 °C to remove non-collagen proteins. The residual 0.1 M NaOH in the sample was then discarded by rinsing it with distilled water until the pH reached neutral. After that, the pre-treated sample was extracted with 0.5 M acetic acid at a ratio of 1:15 (w/v) at 4 °C for 24 h and then filtered using 125 mm filter paper. The supernatant was combined with 750 U/mg pepsin solution at 4 °C for 48 h to remove adipose tissue. The extract was then centrifuged for 30 minutes at 4 °C at 9,000 rpm and the supernatant was separated. Next, the supernatant was re-extracted with 0.5 M acetic acid containing 1.5% (w/w) pepsin for 12 h and centrifuged for 30 min at 4 °C at 9,000 rpm. The extracted supernatant was then mixed with sodium chloride (NaCl) until the final supernatant concentration for precipitation reached 0.7 M to facilitate salt precipitation. The precipitate was obtained by centrifuging the supernatant once again for 15 min at 2500 rpm. After dialysis (Beg Dialysis; Mw1200–1400, MD44-5M, MYM, USA) using distilled water at 4 °C, the material was lyophilized. The precipitate was then frozen and dried using a freeze dryer overnight at -80 °C. The collagen yield (%) was determined using Equation (1).

$$\text{Collagen yield (\%)} = \left(\frac{\text{Weight after extraction}}{\text{Initial weight of fish parts}}\right) \times 100 \quad (1)$$

### MUCUS COLLECTION

The technique described by Jurado et al. (2015) was slightly modified to obtain mucus samples from fish specimens. Mucus was collected by gently scraping the surface of the fish using a plastic spatula and transferring it into a centrifuge tube. The fish mucus was then centrifuged at 12,000 rpm for 10 min at 4 °C. The resulting supernatant was collected and kept at 20 °C until analysis of pH, protein, and metabolites.

FIGURE 1. Nile tilapia fish (*Oreochromis niloticus*) (Romana-Eguia, Eguia & Pakingking Jr. 2020)

### THE pH ANALYSIS

Approximately 1g of mucus sample was diluted with 10 mL of distilled water and homogenized. The diluted sample was then vortexed using a Velp Scientifica Vortex Mixture, F202A0173 ZX Classic (Usmate Velate (MB), Italy) for 10 min to ensure proper homogenization. The pH value was measured at 25 °C using a pH meter (MeterLabÒ PHM210, Japan). Prior to the analysis, the pH meter was calibrated with pH buffers of 4, 7, and 10. Readings were obtained in triplicate ($n$ = 3) for each sample (Masniyom, Benjakul & Visessanguan 2002).

### BRADFORD PROTEIN ANALYSIS

For fish scale and skin protein analysis sample preparation, approximately 30 mg of sample was combined with 2 mL of distilled water and mixed (Kaewdang et al. 2014). In contrast, the mucus sample was used directly without any dilution. A volume of 50 µL of the sample was then mixed with 1.5 mL of Bradford reagent (Biorad, United States). The absorbance of this mixture was measured at 595 nm using a spectrophotometer (Protein Assay Spectrophotometer, NanoPhotometer® NP80, Germany) after incubating it for 10 min at room temperature. A standard curve was generated using the Bradford protein (BSA) standard at concentrations of 50, 100, 150, and 200 µg/µL. For each sample, measurements were taken in triplicate ($n$ = 3).

### CREATINE AND PHENYLALANINE METABOLITES ANALYSIS

About 40 µL of fresh mucus was added to a 1.5 mL Eppendorf tube. Then, an equal volume of 5% (v/v) perchloric acid was added and thoroughly mixed. The supernatant was filtered using a PTFE syringe filter before being injected into the HPLC system for analysis. The analysis was performed using a XBridge C18 column (4.4 mm × 150 mm, 5 µm, Waters, Ireland) for isocratic elution of phenylalanine and creatine chromatography. The analysis was carried out at room temperature using a photodiode array detector. A 20 µL sample was injected and the analysis was run for 15 min using 5% (v/v) acetonitrile as the mobile phase at a flow rate of 1 mL/min and 40 °C. A standard curve was obtained using creatine and phenylalanine standards at concentrations of 20, 40, 60, 80, and 100 ppm. The sample concentrations were determined directly from the standard curve (Faiqah et al. 2017). Phenylalanine was detected at 2.7 min and creatine was detected at a retention time of 3.00 min.

### STATISTICAL ANALYSIS

All the data were analysed using SPSS 20 (IBM SPSS Statistics 20) for a two-way ANOVA. The Tukey test was used to statistically analyse the data at a 95% confidence interval ($p$<0.05) to determine significant results between the sample and cold storage duration. Each experiment was conducted with three readings ($n$ = 3). The data are expressed as mean ± standard deviation.

## RESULTS AND DISCUSSION

### COLLAGEN CONTENT

Collagen, which makes up around 30% of total animal protein, plays a crucial role in biomedical healing and meets human nutritional needs (Silvipriya et al. 2015). Fish collagen is a complex structural protein that supports the integrity of blood vessels, skin, ligaments, bones, joints, muscles, tendons, gums, eyes, nails, and hair. It does so by maintaining their strength and flexibility. Fish scales and skins can be used to produce collagen through advanced enzymatic digestion techniques employed in biotechnology (Jafari et al. 2020). A higher collagen value indicates better fish quality due to its significant benefits to human health and various industries. Table 1 presents the collagen content (%) obtained through acid extraction from both the scalp and skin of Tilapia (*Oreochromis niloticus*) fish during a 7-day cold storage period.

Cold storage is an economical technique often used to delay fish deterioration in the short term, which is very important for overall customer satisfaction. The results show a significant difference ($p$<0.05) in collagen yield from tilapia scalp on the 3$^{rd}$ day of cold storage compared to both the control (0$^{th}$ day) and the 7$^{th}$ day cold storage duration. This indicates that the duration of cold storage affects the collagen content in the tilapia scalp. The collagen yield during the 3$^{rd}$ day of cold storage, which is 30.17 ± 17.62%, was very low compared to other storage durations and shows signs of deterioration after three days. This result was consistent with Jiang et al. (2015), who observed a substantial reduction in the total collagen content of sea bass and grass carp (*Ctenopharyngodon idella*) after 3 days of cold storage. Several enzymes, particularly neutral and acidic proteases and collagenases, may cause autolytic alterations in fish, which in turn can lead to changes in collagen during cold storage (Walayat et al. 2023). However, during the 7$^{th}$ day of cold storage, the collagen content of the tilapia scalp gradually increased, indicating a rise in the ratio of nonhelical to helical groups. This increase was most likely caused by collagenase enzymes, such as collagenases, neutral proteinases, and acid proteinases, acting on the helical regions (Jiang et al. 2015).

In addition, the results also show a significant difference ($p$<0.05) in collagen yield between the scalp and skin parts of tilapia (*Oreochromis niloticus*) during the 7-day storage period. Specifically, the collagen content of

TABLE 1. Collagen yield (%, w/w) from tilapia (*Oreochromis niloticus*) fish scalp and skin during 0th, 3rd and 7th day cold storage

| Tilapia (*Oreochromis niloticus)* parts | Collagen yield (%, w/w) | | |
|---|---|---|---|
| | 0th day | 3rd day | 7th day |
| Scalp | $72.60 \pm 12.40^{Bb}$ | $30.17 \pm 17.62^{Aa}$ | $71.90 \pm 5.48^{Bb}$ |
| Skin | $45.37 \pm 20.44^{Aa}$ | $49.77 \pm 14.21^{Ba}$ | $46.97 \pm 12.39^{Aa}$ |
| Mucus | ND | ND | ND |

[a-b]Sample was significantly different ($p$<0.05) between cold storage day
[A-B]Sample was significantly different ($p$<0.05) between tilapia (*Oreochromis niloticus*) parts
ND: Not Detected

the tilapia scalp was higher than that of its skin. The collagen from the scalp reaches its highest concentration (72.60 ± 12.40%) on the 0th day of cold storage. Previous research has shown that fish scales are composed of highly organized collagen fibres with cross-linked areas, creating a bio-composition. However, separating collagen from scales is challenging due to the strong bond between collagen and hydroxyapatite in the scale matrix (Huang et al., 2016). Other studies have reported collagen contents in tilapia scales ranging from 1.48% to 12.3%, which may be attributed to variations in extraction methods, harvesting seasons, and breeding environments (Huang et al. 2016; Kittiphattanabawon et al. 2019).

Besides, the collagen content obtained from the tilapia scale in this research was also higher than previous research by several fish species reported by Sobanalakshmi and Brindha (2021) which is 58.87% in sardine, 41.10% in Japanese sea bass and 37.50% in red sea bream. This shows that tilapia scale has higher solubility in acid-pepsin solution during extraction than other fish types, thus highlighting its potential as a collagen source for food, cosmetic and pharmaceutical industries. In fact, Sulaiman and Sarbon (2020) also approved that acid and pepsin used during collagen extraction show an effective action in cleaving non-helical domains of collagen without cleaving the triple helix domain. Thus, the collagen from tilapia scales which is tightly linked to hydroxyapatite easily demineralizes to loosen the matrix of scale for better collagen solubility and yield (Huang et al. 2016). In addition, Huang et al. (2016) also claim that fish scale collagen generally has a less fishy flavour and smell than collagen from fish bones and skin because it contains a trace amount of lipids. This makes collagen from scales have a low oxidation level with better odour and flavour for better consumer preference.

Meanwhile, the collagen obtained from tilapia skin shows insignificant difference ($p$>0.05) across a 7-day cold storage duration, ranging from 45.37 ± 20.44% to 49.77 ± 14.21%. The collagen content extracted from the tilapia nil skin in this research is higher than that reported by Chen et al. (2016) and Li et al. (2018), who found only 27.20% and 20.03% collagen, respectively. This difference may be attributed to modifications in the extraction method used. The acetic acid solution, combined with the pepsin enzyme, aids in breaking collagen intermolecular cross-links at the telopeptide region of the collagen, making the collagen more soluble under acidic conditions (Zhang et al. 2020). However, when compared with other marine fish types such as salmon (25.95%), sturgeon (36.7%), and Giant groupers (~42.77%) (Hou & Chen 2023; Nilsuwan et al. 2022; Upasen et al. 2019), the tilapia skin in this research proved to be quite competitive. These differences are influenced by the species and environmental conditions of marine and freshwater fish. Furthermore, the high collagen content obtained from tilapia skin and scalp supports Avila, Rodriguez and Sánchez's (2018) statement that tilapia is a viable alternative source for replacing land-based mammal sources, such as pig skin or bovine tendon, in the production of industrial and commercial collagen. This is particularly important in contexts where religious barriers and disease transmission are concerns (Siahaan et al. 2022).

The presentation of collagen obtained from tilapia (*Oreochromis niloticus*) along the 7-day cold storage duration is shown in Figure 2. After 24 h of freeze-drying, it was observed that some collagen accumulated in a bulk shape due to the freeze-drying treatment. However, it is very brittle and easily crushed into a powder form. All the collagen obtained in this study was in the form of white powder. The visual appearance of the collagen powder obtained in this research supports the findings reported by Jafari et al. (2020), who also obtained white collagen powder with a small molecular size. The powder also increased in redness and yellowness due to the storage effect. When collagen is added to a product, this characteristic is very important as it will impact the final product's colour and texture.

pH PROFILES

The pH value is considered the simplest parameter that can be used to monitor fish quality during cold storage. According to the results shown in Table 2, the pH value of all tilapia parts, including fish scalp, skin, and mucus, was affected by the duration of cold storage. However, on the 3rd and 7th days of cold storage, there was a significant decrease in the pH value ($p<0.05$), making all samples slightly acidic. Based on Zhuang et al. (2020) and Yehia et al. (2022), the release of trichloroacetic acid (TCA)-soluble peptides and lactic acid during cold storage causes a decrease in pH at the beginning of storage. On the other hand, there was also a significant increase ($p<0.05$) in the pH value of tilapia skin on the 0th day and 3rd day of cold storage, making it slightly alkaline, except for the pH value of tilapia skin on the 0th and 7th day of cold storage, which showed a significant ($p<0.05$) acidic value of 6.50 ± 0.28 and 5.80 ± 0.49, respectively, compared to the other tilapia parts. Research conducted by Yehiya et al. (2022) also found similar trends in Mullet fish fillets, which became more acidic with an increase in cold storage time. The significant ($p<0.05$) increase in pH value of tilapia skin from the 0th to the 3rd day of cold storage was caused by the increase in total volatile basic nitrogen (TVBN) resulting from the decomposition of nitrogenous compounds by endogenous or microbial enzymes, which depends on the duration of storage (Shahrier et al. 2023). The increase in pH observed in this study can also be explained by the fermentation of carbohydrates into acid (Duarte et al. 2020).

In addition, the pH values of tilapia fish scalp, skin, and mucus during initial cold storage range from 6.50 ± 0.28 to 7.67 ± 0.19, which is slightly higher than the pH values reported by Chacon et al. (2020) and Yehiya et al. (2022). However, Yehiya et al. (2022) reported a pH value of 6.0 - 6.7 for Mullet fish species, while Chacon et al. (2020) reported a pH value of 6.20 - 6.70 for Tilapia *Oreochromis niloticus*. It should be noted that the fish used in this research was considered fresh on the 0th storage day and slowly lost its freshness over time. According to Admasu et al. (2023), the post-mortem pH of most fish species falls between 6.20 and 6.68, but this pH value can vary significantly depending on factors such as season, species, and other variables. Additionally, the nearly neutral collagen pH also indicates its suitability for application on human skin (Izzati, Zainol & Hanim 2017). The pH value is crucial for evaluating the glycolysis process as it reflects the major degradation and degeneration in fish, with post-mortem pH decreasing and then rising to 7 (Bernardo et al. 2020).

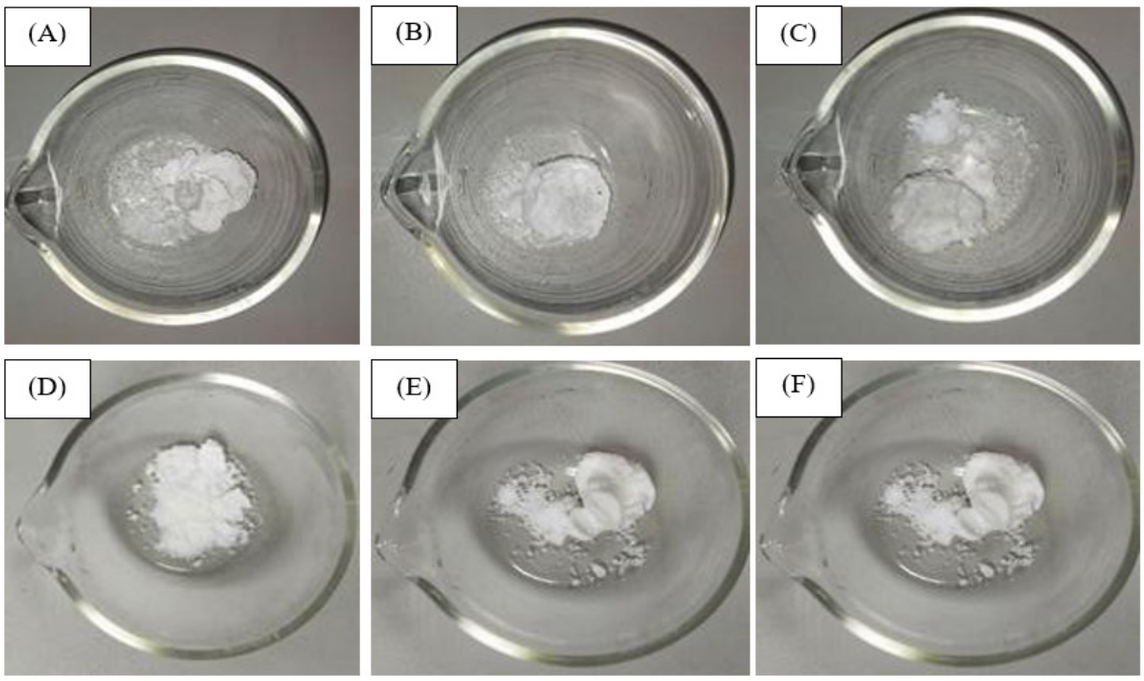



FIGURE 2. Collagen powder extracted from tilapia (*Oreochromis niloticus*) during 0th, 3rd and 7th day cold storage. A-C represent collagen powder from tilapia scalp, and D-F represent collagen powder from tilapia skin along a 7-day cold storage duration

TABLE 2. The pH profiles of tilapia (*Oreochromis niloticus)*'s scalp, skin, and mucus during 0th, 3rd and 7th day cold storage duration

| Tilapia (*Oreochromis niloticus)* parts | pH value | | |
|---|---|---|---|
| | 0th day | 3rd day | 7th day |
| Scalp | $7.64 \pm 0.26^{Bb}$ | $7.36 \pm 0.15^{Aa}$ | $7.46 \pm 0.02^{Ba}$ |
| Skin | $6.50 \pm 0.28^{Ab}$ | $7.61 \pm 1.12^{Bc}$ | $5.80 \pm 0.49^{Aa}$ |
| Mucus | $7.67 \pm 0.19^{Bb}$ | $7.37 \pm 0.06^{Aa}$ | $7.14 \pm 0.02^{Ba}$ |

[a-b]Sample was significantly different ($p$<0.05) between cold storage day
[A-B]Sample was significantly different ($p$<0.05) between tilapia (*Oreochromis niloticus*) parts

The trends of these pH value change during cold storage were affected by biochemical reactions such as lipid hydrolysis, autolysis, a proliferation of microorganisms and enzyme activities (Walayat et al. 2023). Moreover, Wang et al. (2021) also claimed that certain proteolytic bacteria increase the acidity of fish by producing acids as a by-product of the breakdown of carbohydrates. An increase in pH could also be a sign of the build-up of ammonia and other alkaline chemicals, which are mostly the result of microbial activity. This study discovered that the pH of all fish parts influences bacterial development, which in turn has a significant impact on the fish's freshness.

## PROTEIN CONTENT

Fish is widely recognized as an excellent source of protein for humans, and its high protein content indicates both nutritional value and quality. Tilapia processing co-products have been found to be potential sources of bioactive peptides due to their plentiful protein content. These co-product proteins are not only abundant but also cost-effective, making them suitable for use as bioactive peptide sources in functional food additives or nutritional supplements. The protein content of tilapia fish during cold storage duration was calculated using the standard curve for Bradford analysis, as shown in Figure 3 and summarized in Table 3.

Based on the results, it was observed that the protein content in all parts of tilapia during the 7th-day cold storage period is extremely low, ranging from 0.21 ± 0.02% to 43.07 ± 0.01%. The protein content in tilapia mucus was not detected during the 7th day of cold storage due to its very low concentration. However, the highest protein content was found in the skin and mucus of tilapia during the 3rd day cold storage period. Moreover, the protein content in tilapia skin showed a significant decrease ($p$<0.05) during the 7th day cold storage, while the protein content in tilapia mucus significantly increased from the 0th day storage period to the 3rd day storage period as a result of the isolation process after alkaline extraction.

Regarding the tilapia parts, there was a significant change in protein content ($p$<0.05) between the tilapia skin with scalp and mucus at the 0th and 3rd cold storage durations. Furthermore, at the end of the 7th-day cold storage duration, the protein content of the mucus was significantly lower ($p$<0.05) than that of the tilapia skin and scalp. The protein content of tilapia skin at the 0th cold storage duration was 28.79 ± 0.01, which aligns with the findings of Akter et al. (2016) and Li et al. (2018), who reported 21.31 ± 1.17% and 21.89 ± 0.08% tilapia skin protein content, respectively. In this study, the protein content of fresh tilapia skin was found to be similar to that of red snapper, parrotfish, and Pangasius, with values of 29.72%, 27.17%, and 26.73%, respectively (Nurilmala et al. 2022). The properties of fish can vary significantly among individuals based on age, sexual environment, and season. Meanwhile, the protein content of the tilapia scalp and mucus was observed to be very low, ranging from 0.21 ± 0.02 to 14.5 ± 0.01. The low protein content in this study was expected, as mucus typically contains low levels of protein, as noted by Green et al. (2019), who found that fish flesh usually contains 5 - 7% protein. However, the protein content in the mucus may vary depending on the specific species of tilapia.

These results demonstrate that protein from tilapia scalp and mucus is affected during 7-day cold storage. The efficiency of the extraction procedure, which uses acetic acid with pepsin, can influence protein yields. Therefore, further research should be conducted to optimize the extraction technique and enhance yields. Additionally, proteolytic enzymes can still be active at low temperatures, breaking down proteins. Microbial activity, including lactic acid bacteria, *Shewanella putrefaciens*, aerobic spoilers, and anaerobic rods, can also multiply at low temperatures and produce enzymes capable of degrading protein. Furthermore, oxygen is still present during cold storage and can react with proteins, causing them to break down.

When fish die, some proteins may lose their original structure and function due to aggregation, resulting in the formation of a precipitate and insoluble protein (Kontominas et al. 2021; Tavares et al. 2021; Walayat et al. 2023).

Protein and collagen contents significant correlation impacting the quality and functionality of tilapia. The protein content indicates its nutritional value, while collagen content as a vital structural protein affects the fish's tensile strength and flexibility (Silvipriya et al. 2015). In this study, the collagen yield in the scalp sharply decreases on the 3rd day (30.17 ± 17.62%), but it recovers by the 7th day (71.90 ± 5.48%). Similarly, the protein content in the scalp decreases noticeably over time. The skin shows fluctuating protein levels, reaching a peak on the 3rd day (43.07 ± 0.01%) and declining by the 7th day. These changes suggest that both collagen and protein contents were affected by cold storage, which impacts the fish's structural integrity and nutritional value.

## PHENYLALANINE AND CREATINE METABOLITES CONTENT

Phenylalanine and creatine are naturally occurring free amino acids and organic acids found in fish and fish products. These compounds are essential for maintaining the quality and determining the nutritional content of seafood. The amount of phenylalanine and creatine present greatly affects the sensory characteristics of tilapia fish (Faiqah et al. 2017). Figure 5 displays the HPLC chromatogram illustrating the phenylalanine and creatine levels in tilapia mucus on both the 0th and 7th day of cold storage. Additionally, Table 4 provides the concentrations of creatine and phenylalanine in tilapia mucus during the 7th day of cold storage. These values were obtained using the standard curve depicted in Figure 4.

It was observed that the peak of phenylalanine from tilapia fish mucus was detected at a retention time of 2.70 min on a chromatogram, based on a comparison with a

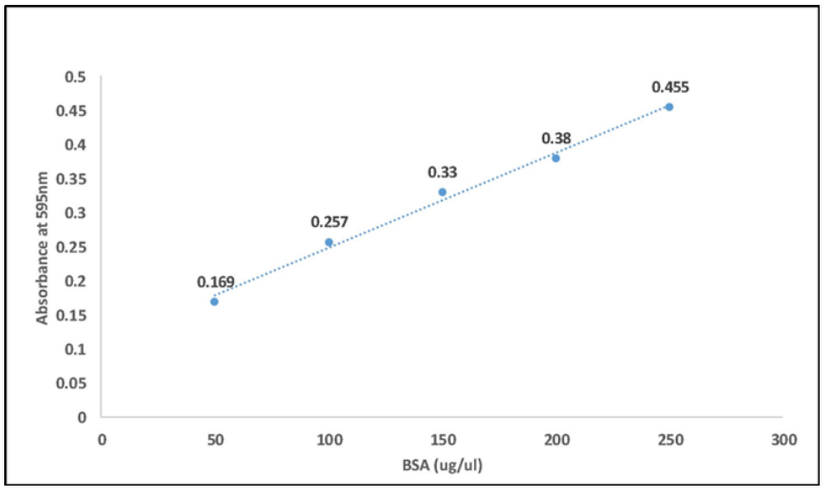
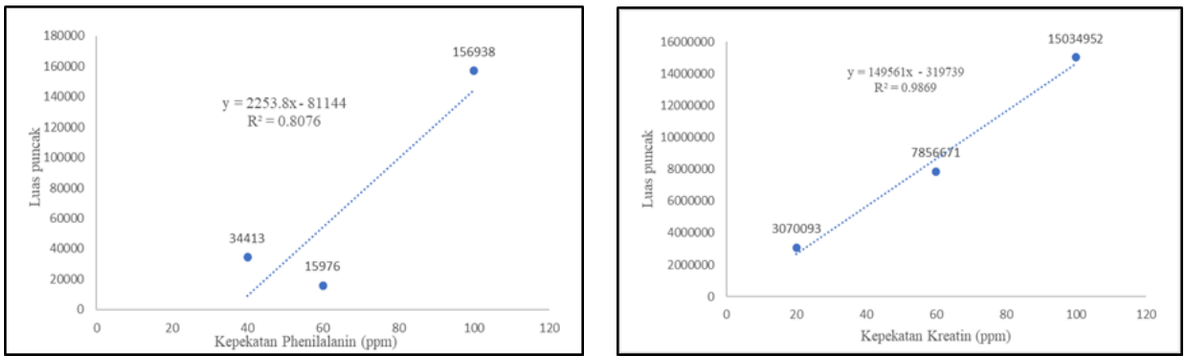



FIGURE 3. Standard curves for protein Bradford analysis at 50, 100, 150 and 200 µg/µL concentration (y = 0.0014x + 0.1097 (R2 = 0. 9923)

TABLE 3. Protein content of tilapia (*Oreochromis niloticus)*'s scalp, skin and mucus during 0th, 3rd and 7thday cold storage duration

| Tilapia (*Oreochromis niloticus)* parts | Protein content (%) (w/w) | | |
|---|---|---|---|
| | 0th day | 3rd day | 7th day |
| Scalp | $14.5 \pm 0.01^{Aa}$ | $7.36 \pm 0.30^{Aa}$ | $7.36 \pm 0.30^{Ba}$ |
| Skin | $28.79 \pm 0.01^{Bab}$ | $43.07 \pm 0.01^{Bb}$ | $14.5 \pm 0.01^{Ba}$ |
| Mucus | $0.21 \pm 0.02^{Aa}$ | $43.07 \pm 0.01^{Bb}$ | ND |

[a-b]Sample was significantly different ($p$<0.05) between cold storage day
[A-B]Sample was significantly different ($p$<0.05) between tilapia (*Oreochromis niloticus*) parts
ND: Not detected




a) b)

FIGURE 4. Metabolites standard curves for: a) Phenylalanine at 40, 60 and 100 ppm (y = 2253.8x - 81144 (R2 = 0.8076)); and b) Creatine at 20, 60 and 100 ppm (y = 149561x - 319739 (R2 = 0.9869))

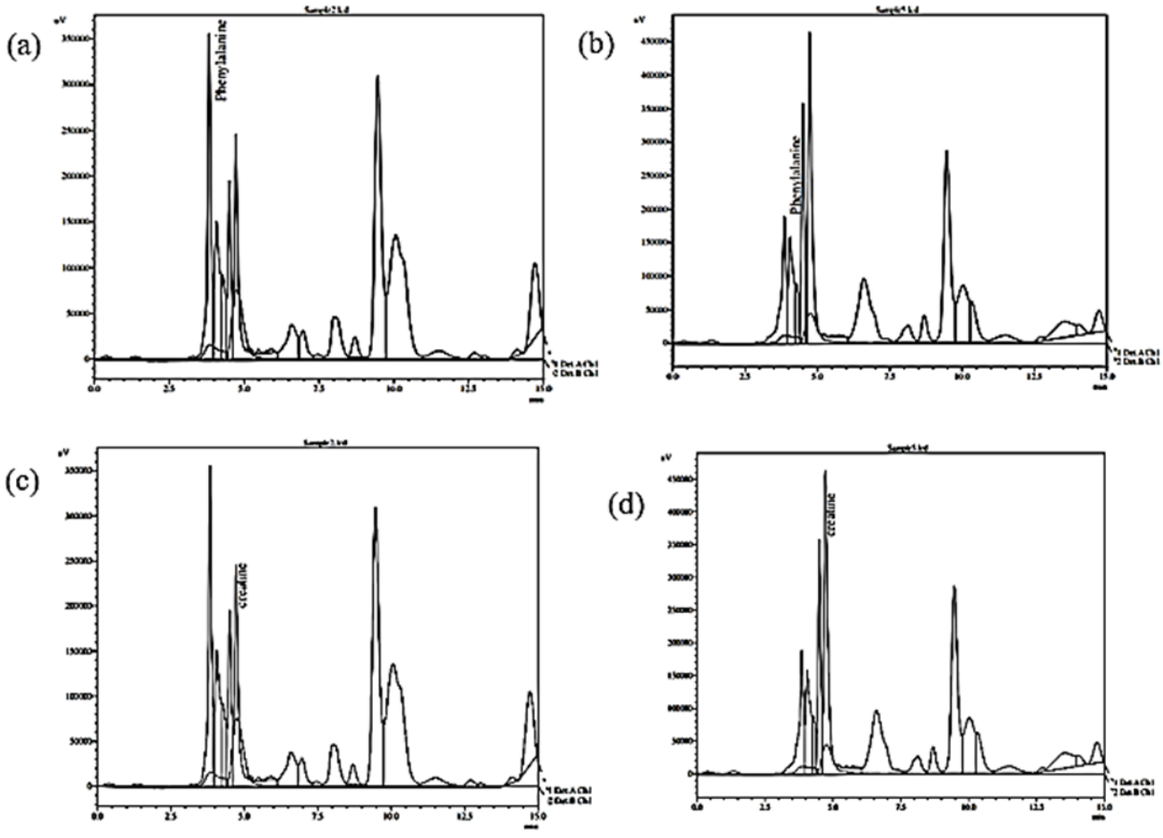



FIGURE 5. HPLC chromatogram for phenylalanine and creatine metabolites content from tilapia mucus during cold storage. (a) Phenylalanine on 0th day cold storage duration; (b) Phenylalanine on 7th day cold storage duration; (c) Creatine on 0th day cold storage duration; (d) Creatine on 7th day cold storage duration

TABLE 4. Creatine and phenylalanine metabolites concentration of tilapia (*Oreochromis niloticus)*'s scalp, skin and mucus during 7th-day cold storage duration

| Tilapia (*Oreochromis niloticus)* parts | Phenylalanine (ppm) | Creatine (ppm) |
|---|---|---|
| Scalp | ND | ND |
| Skin | ND | ND |
| Mucus | 102.67 | 105.65 |

ND: Not detected

standard chromatogram. The peak height and area of phenylalanine decreased from the 0th day to the 7th day of cold storage. This trend is similar to the findings of Faiqah et al. (2017) and Jiang et al. (2015) on tuna (*Euthynnus affinis*) mucus and grass carp fillets, respectively, during cold storage. The increase in *E. coli* bacteria, due to the fish's weakened defence system against microbes, was indicated by the production of phenylalanine. Therefore, phenylalanine greatly impacts fish welfare and health. It has been discovered to protect against oxidative stress, apoptosis, and damage to the tight junctions in fish gills. According to Hidalgo, León and Zamora (2016), the neutral, nonpolar, and hydrophobic amino acid phenylalanine breaks down complex components of fish tissue, such as proteins, during autolysis, creating nutrient-rich tissue that supports microbial growth. Through decarboxylation, it also produces biogenic amines. Fish mucus, as reported by Benhamed et al. (2014) and Tavares et al. (2021), contains phenylalanine, and the composition of mucus is affected by temperature and proteolytic enzymes. The decrease in phenylalanine also indicates impaired intestinal structural integrity (Feng et al. 2016). Therefore, the decrease of phenylalanine indicates a decrease in fish quality.

On the contrary, the creatine peak was detected at 3.00 min retention time on the chromatogram, with its peak height and area increasing from the 0th day to the 7th-day cold storage duration. According to Villasante et al. (2023) and Burns and Gatlin (2019), creatine is essential for fish physiology and may have advantages for muscle growth, performance, and energy use during osmoregulation. The increasing concentration of creatine might be due to the enzymatic activity of arginine-glycine amidinotransferase (AGAT) and guanidinoacetate N-methyltransferase (GAMT) in the kidney, as well as degradation and other biochemical processes (Post, Tsikas & Bakker 2019). However, it is predicted that the increment in creatine content during cold storage is temporary and will decrease during longer cold storage duration due to various bacterial and oxidation reactions after rigour mortis. During the post-rigor stage, the dead fish also releases N-compounds, including amino acids, uric acid, purines, methylamine, taurine, imidazoles, creatine, and creatinine (Faiqah & Ling 2021). Additionally, creatine can be found in blood, urine, brain, and muscle. After mortality, adenosine triphosphate (ATP) rapidly breaks down, resulting in the rigour mortis stage and the release of creatine compounds. When creatine phosphate and ATP are hydrolysed by phosphatase, creatine is categorized as a uremic toxin (Karim et al. 2019). Overall, these results show that both phenylalanine and creatine were affected by the duration of cold storage. According to Faiqah et al. (2017), decreasing the concentration of phenylalanine and creatine is a sign of protein denaturation and structural abnormalities, which leads to a decrease in fish quality.

The concentrations of creatine and phenylalanine were calculated using the standard curve equations. The equation for phenylalanine is $y = 2253.8x - 81144$, with an $R^2$ value of 0.8076. The equation for creatine is $y = 149561x + 319739$, with an $R^2$ value of 0.9869. In these equations, x represents the concentration of phenylalanine or creatine, while y represents the peak area of phenylalanine or creatine. The results indicate that there was insignificant difference ($p>0.05$) in the concentrations of phenylalanine and creatine during the 7th cold storage duration. However, when compared to the study by Faiqah et al. (2017) on mucus from tuna fish (~51 ppm), the phenylalanine concentration in mucus from tilapia (102.67 ppm) was higher ($p<0.05$). On the other hand, the creatine concentration in tilapia mucus (105.65 ppm) was lower than that in mucus from tuna fish (~4800 ppm) ($p<0.05$). This indicates that the composition of mucus varies among fish species and its natural habitat (freshwater and saltwater species) and can be influenced by the duration of cold storage.

## CONCLUSIONS

In conclusion, after seven days of refrigeration at 4 °C, discernible alterations were observed in the physicochemical properties and metabolite yield of tilapia (*Oreochromis niloticus*) scalp, skin, and mucus by-products. The significant decrease in collagen concentration (w/w) from 72.60 ± 12.40% w/w to 30.17 ± 17.62% in the scalp suggests potential degradation over time, highlighting the susceptibility of this tissue to storage conditions. Variations

in pH levels among different tissues between 5.80 ± 0.49 and 7.67 ± 0.19 indicate ongoing bacterial and enzymatic activities, underscoring the dynamic nature of freshness maintenance. The substantial reduction of protein content by over 50% during the 7-day storage period signals a notable protein deterioration, directly impacting the overall quality of the by-products. Furthermore, fluctuations in creatine and phenylalanine concentrations further compromise fish freshness and quality. These findings emphasize the critical importance of timely utilization and efficient storage methods for tilapia co-products to mitigate quality deterioration effectively.

## ACKNOWLEDGEMENTS

We express gratitude for the funding provided by Universiti Kebangsaan Malaysia (UKM) under grant ST-2023-043, which enabled us to conduct this study. Furthermore, we extend our appreciation to the Department of Food Sciences, Faculty of Science and Technology, UKM Bangi, for granting us access to their laboratory facilities. No data were used elsewhere to support this study and it was entirely a new set of data.

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