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Research Article

Physicochemical Characterization of Biofluid Metabolites from Liquid Residual of Tuna Fish *(Euthynnus affinis)* throughout Refrigerated Storage Condition

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The cold storage condition and use of chemical treatment to preserve the fish appearance sometimes cause difficulties to the consumers to estimate the freshness of fish in the market. However, during fish deterioration, some compound is released or formed due to microbial and biochemical process. Identification of released compound during fish spoilage is a crucial step in understanding the degree of spoilage. This study characterizes the physicochemical changes of metabolites biofluids from liquid residual of tuna fish *(Euthynnus affinis)* during refrigerated storage condition. Tuna fish were kept in ice at 0°C and stored in cold room (~4°C) for seven days in order to study the changes in fish freshness and loss of quality through the storage period. Liquid residual of fish was collected at 0, 1, 2, 3, 4, 5, 6, and 7 days of storage. LC-MS/MS analysis was carried out to determine the possible dominant compound which was later identified as creatine and phenylalanine. Quantification of creatine and phenylalanine using HPLC with UV detector found that creatine and phenylalanine increased significantly up to day 4 and day 5 upon storage time for creatine and phenylalanine, respectively ($p < 0.05$). The liquid residual pH increased from 6.5 on day 0 to 7.5 on day 7 ($p < 0.05$). Changes in chemical compounds were supported with physical analysis on gills colour of spoilage fish. L^* and a^* values decreased with storage time from 41.08 to 24.76 and 18.34 to 10.40, respectively, while b^* value increased from −3.80 to −0.46 ($p < 0.05$). The finding of biofluid derived compounds was found as useful and alternative indicators of fish freshness in later study on the development of optical biosensor.

1. Introduction

Fisheries sector is one of the most important food sources throughout the world. According to statistical data reported by Department of Fisheries, Ministry of Agriculture Malaysia, fish production increased from 2005 to 2014 with marine fish contributing the largest proportion of 1.74 million tons of fish in 2014. Tuna fish *(Euthynnus affinis)* (Figure 1) is categorized under marine (saltwater fish) that has a high market value. Tuna fish spread widely and almost consists of all coastal and offshore marine waters throughout the Indo-Pacific. *Euthynnus affinis* is the smallest species of tuna fish with an average length of about 50–150 cm or 200–500 grams/head. The chemical composition of a tuna fish as reported by Suzuki and Kume (1981) [1] is water (71%), protein (21.6%), fat (1.3%), minerals (1.2%), ash (1.45%), vitamin A (0.5%), and vitamin D (1.0%). Various types of dishes and fish product applications make demands of tuna fish increased by years. Hence, preserving fish freshness and quality is of high concern.

However, the smell, colour, taste, and physical form of the fish will change during storage before reaching customers. The microbiological and chemical process changes the protein and lipid fractions, leading to the formation of biogenic amines and hypoxanthine which are responsible for the deterioration of fish spoilage [2]. Briefly, once the fish

Figure 1: Lateral view of tuna fish *(Euthynnus affinis)* [14].

was caught and dead, the normal circulation system breaks down, and chemical signals leak into the muscle causing them to stiffen. This is called rigor-mortis process. The blood circulation stops and the supply of oxygen is prevented. The enzymes present in the muscle convert glycogen into lactic acid. Besides, fishing method, handling procedure, gutting methods, and storage conditions from the moment it is caught until it reaches the consumer affect the rapid spoilage [2]. A lot of study has previously reported on determination of fish freshness by physical observation on eyes, gills, and skin [2, 3], microbial counts [3, 4], presence of biogenic amines such as histamine, cadaverine, and putrescine [5–7], determination of trimethylamineoxide (TMAO) [8, 9], and lipid oxidation [10]. However, no study was found in determination of fish freshness by using biofluids from liquid residuals of fish. Biofluid is defined as biological fluid which is made by the body itself. It can be excreted through sweat and urine, secreted through bile, or developed as a result of a pathological process such as blood and serum [11].

The objective of this study was to evaluate the fish freshness according to the biofluid derived compounds. Compound concentration and its changes will be used as parameters for 7 days of storing periods. FDA (2016) [12] reported that refrigerated fish (4°C) could only last for two days. However, there are fishmongers and hypermarkets which sold fishes up to 10 days as reported by Prince (2013) [13]. Therefore, employment of biofluid derived compounds in evaluating the freshness of fish is needed to monitor and estimate its storage day at refrigerated temperature (4°C). Besides, the aim of this work is to identify dominant compounds from biofluids of *Euthynnus affinis* throughout refrigerated storage condition at 4°C for 7 days. Physical observation including pH and gills colour analysis were also studied in accordance with the changes in biofluid compound. Optical biosensor utilizing biofluid derived compounds as analytes could be developed for further studies. It is expected that study on the optical biosensor will be useful in monitoring fish quality and the application of the biosensor as kit could later be used in easy handling and it does not destroy the valuable fish muscle.

2. Materials and Methods

2.1. Chemicals. Phenylalanine, creatine, and cysteine were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Chromatographically pure acetonitrile (ACN) was obtained from Fisher Scientific (Pittsburgh, USA) and perchloric acid was obtained from Merck (New Jersey, USA).

2.2. Preparation of Fish Biofluids. Fresh caught tuna fish with average size of 250 g were supplied from local market (Seri Kembangan, Selangor, Malaysia). Upon arrival to the School of Chemical Sciences and Food Technology, Faculty of Science and Technology (UKM), the fishes were immediately transported to the laboratory in a polystyrene box filled with ice flakes. All fishes were stored in cold room (~4°C) for 7 days and subjected to experiments every 24 hours. The storage condition mimics the storage conditions applied by the fishmongers and hypermarket for fresh fish.

Biofluid samples were collected from fish specimens using the method described by Jurado et al. (2015) [15] with slight modifications. Briefly, the biofluid was collected by gently scrapping the skin surface of fish using plastic spatula. The liquid residual was centrifuged (12,000g, 4°C, 10 mins) and the supernatant was immediately stored at −20°C until further analysis. Control sample (fresh fish obtained after being caught before storage procedure) was presented as day 0 followed by 24 hours of storage (day 1), 48 hours of storage (day 2), 72 hours of storage (day 3), 96 hours of storage (day 4), 120 hours of storage (day 5), 144 hours of storage (day 6), and 168 hours of storage (day 7).

2.3. Fish Biofluid Analysis

2.3.1. pH Determination of Fish Biofluids. pH of liquid residual from fish was analyzed using a pH meter (Laquatwin, Horiba Scientific, Japan). Triplicates reading was taken for each sample.

2.3.2. Determination of Metabolites Using LC-MS/MS. Chromatography of crude biofluids (100 ppm) of fish was performed using UltiMate 3000 UHPL system (Dionex) with C18 column (3 × 150 mm, 3 μm particle size) (Thermo Scientific, Massachusetts, USA). Gradient elution was performed at 0.4 mL/min and 40°C using deionized water + 0.1% formic acid (A) and 100% ACN (B) with 22 mins total run time. The injection volume of sample was 1 μL. The gradient started at 5% B (0–3 min); 80% B (3–10 min); 80% B (10–15 min); and 5% B (15–22 min). High resolution mass spectrometry was carried out using a MicroTOF QIII Bruker Daltonic using an ESI positive ionization with the following settings: capillary voltage: 4500 V; nebulizer pressure: 1.2 bar; drying gas: 8 L/min at 200°C. The mass range was at 50–1000 m/z.

2.3.3. Quantification of Creatine and Phenylalanine upon Storage via HPLC. 40 μL biofluids were transferred to Eppendorf tube. An equal volume of 5% (V/V) perchloric acid was added and mixed thoroughly. The supernatant was filtered using PTFE syringe filter before it is injected into HPLC system for analysis. Chromatography of phenylalanine and creatine was carried out by isocratic elution on a XBridge C18 column (4.4 mm × 150 mm, 5 μm, Waters, Ireland) with photodiode array detector at room temperature. Isocratic elution was performed at 1 mL/min and 40°C using deionized water with 5% ACN for 15 mins total run time. The injection volume of sample was 20 μL.

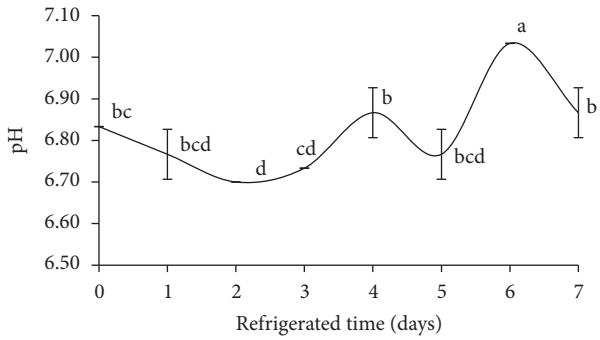


Figure 2: pH of biofluids of *Euthynnus affinis* upon storage at 4°C for seven days. Values are means ± SD ($n = 3$). Different superscript letters indicate significant differences ($p < 0.05$).

2.3.4. Gills Colour Determination. Gills colour was determined using a colour meter (Minolta, Osaka, Japan). Colours were expressed as CIELab coordinates. In this system, L^* represents the colour lightness on a 0–100 point scale from black to white; a^* is the position between red (+) and green (−); and b^* is the position between yellow (+) and blue (−). Triplicates reading was taken for each sample ($n = 3$).

2.4. Statistical Analyses. SPSS 20 was used in this study to analyze the data. One-way variance analysis was carried out using Duncan's test with confidence level as $p < 0.05$.

3. Results and Discussion

3.1. Biofluid pH. pH (Figure 2) of fish biofluid was between 6.5 and 7.5 and the trend showed increase in pH upon storage at 4°C although the pH values were inconsistent. Preliminary trial on a pH strip on biofluid showed similar results between pH 6 and pH 7. This result falls within the pH range obtained by Lillian (2007) [8] on cod fish in iced storage (pH 6.2–pH 7.1) after a few days of storage. The low pH (6.70–6.83) in the early iced storage (day 0 to day 2) indicated a good nutritional state of fish compared to day 4 to day 7 (pH 6.9–pH 7.1). The pH started to increase from day 2 to day 7 and indicated the production of alkaline bacterial metabolites in spoiling fish as well as increase in total volatile basic nitrogen (TVBN) [16]. However, it is quite difficult to use pH for fish grading due to inconsistent pH values, fish species, health status, catching method, and seasons [3, 8, 16].

3.2. Biofluid Metabolite Constituents. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) was performed to identify unknown dominant compound and its trend of changes by storage time for the preliminary study. Mass spectrometer works by atom ionization to give a positive ion, and the ions then accelerated to have the same kinetic energy before being deflected by a magnetic field according to the masses. The beam of ions passing through the machine was detected electrically [17].

Samples at day 1 and day 6 were used for peak comparison. LC-MS/MS chromatogram shows that there are two major peaks consistently changed during storage at retention times 1.7 and 3.9 on day 1 and day 6 (Figure 3), respectively. Peak at 0.3 mins was assumed as noise since it was also presented in the blank sample (data not shown). The m/z value and intensity of detecting compounds were then analyzed for fingerprinting identification using online Mass Bank. Mass Bank provides expected compounds having highest similarities based on the m/z (mass-to-charge ratio) value and intensity.

At retention time 1.7, Mass Bank showed similarities with creatine compound with a score of 93.66% and 89.37% similarities for sample on day 1 and day 6, respectively. The creatine compound decreased from day 1 to day 6. Meanwhile, at retention time 3.9, Mass Bank showed compound similarities with phenylalanine with a score of 93.89% and 91.69% for sample on day 1 and day 6, respectively. Relative current produced by ions of varying mass/charge ratio at day 1 was presented in Figures 4 and 5 for creatine and phenylalanine, respectively.

According to Wiegertjes and Flik (2004) [18], the entire surface of the fish is covered with mucus composed of mucopolysaccharides, steroids, bile salts, aliphatic acids, nucleotides, and carbon dioxide. After fish died, N-compounds chemical substances were also released comprised of amino acid, uric acid, purines, methylamine, taurine, imidazoles, creatine, and creatinine during postrigor stage [19]. Besides, the mucus mixed with blood and urinogenital or intestinal excretions once the fish died [15].

In fact, Karakuş et al. (2006) [20] mentioned that creatine level in blood serum and urine is an important factor in the evaluation of muscle damage. Creatine presents in muscle, brain, blood, and urine. In resting fish, most of the creatine is phosphorylated and supplies energy for muscular contraction. In the living organism, adenosine triphosphate (ATP) is formed by the reaction between adenosine diphosphate (ADP) and creatine phosphate, the latter being a reservoir of energy-rich phosphate in the muscle cell. After death, the ATP is rapidly degraded leading to rigor-mortis stage and at the same time released creatine compound [6]. During rigor mortis, the body of fish stiffens for a certain length of time after death [19]. Creatine is classified as uremic toxin after hydrolysis of creatine phosphate and ATP by phosphatase [21].

Phenylalanine is an amino acid, categorized as neutral, nonpolar, and hydrophobic nature due to benzyl side chain. In autolysis, the complex tissue components of fish such as proteins broke down into simple compounds by endogenous enzymes amino acids resulting in tissue softening. This nutrient-rich product becomes a medium for microbial growth. Decarboxylation of amino acid including phenylalanine produced phenylethylamine (biogenic amines) [19]. A lot of studies on biogenic amines such as histamine, tyramine, cadaverine, and putrescine as fish spoilage indicator have been recorded beforehand [22–26]. However, bacteria started to colonize in large extent on the skin surface once the fish died. Hence, phenylalanine which is naturally produced by Gram-negative bacteria *E. coli (Escherichia coli)* increased in quantity as *E. coli* increased [27–29]. In fact, Rahman et al. (2012) [30] have recorded the presence of phenylalanine in fish mucus. Moreover, Benhamed et al. (2014) [27] added

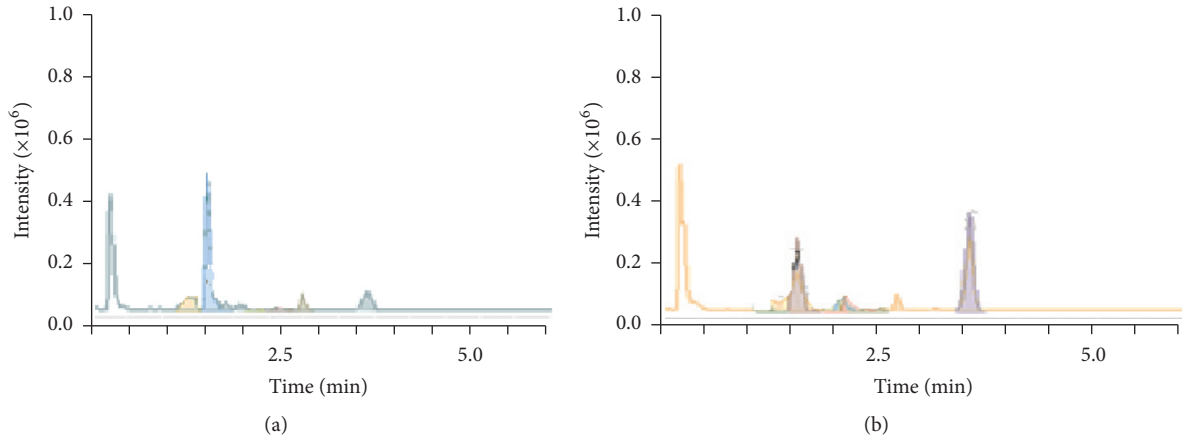
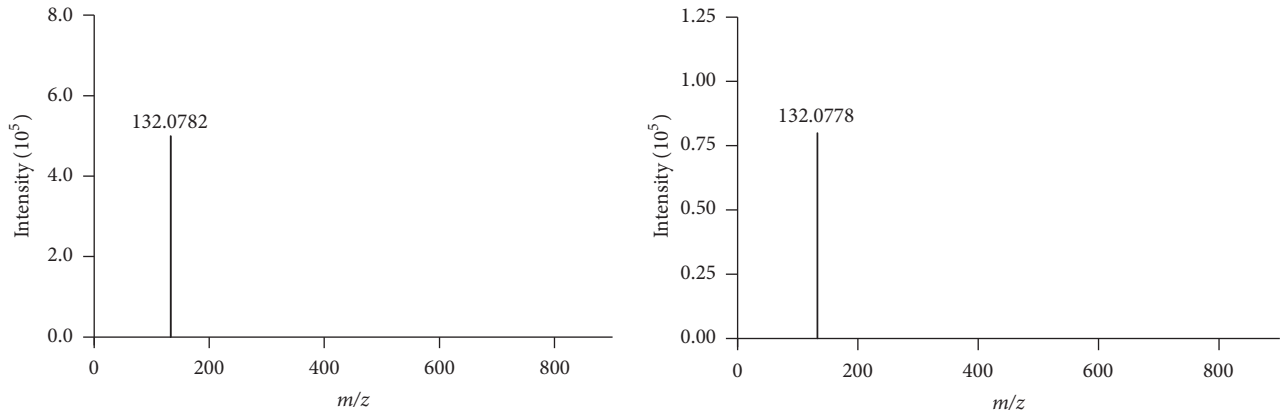


Figure 3: LC-MS/MS chromatogram of metabolites biofluid on day 1 (a) and day 6 (b).



#	m/z	Intensity
1	131.4489	615
2	132.0782	460159
3	132.2820	1195
4	132.3295	1118
5	132.4158	897
6	132.5129	983
7	132.6598	866
8	132.9274	623
9	133.0795	25459
10	263.1469	12228

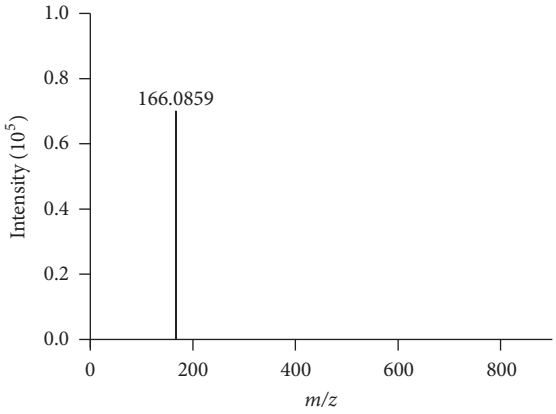
#	m/z	Intensity
1	114.0665	1503
2	115.0500	567
3	131.4576	149
4	132.0778	75597
5	132.2052	278
6	132.3301	212
7	132.4145	136
8	132.6633	136
9	133.0796	4332
10	134.0646	364

Figure 4: The m/z and intensity value of creatine on day 1.

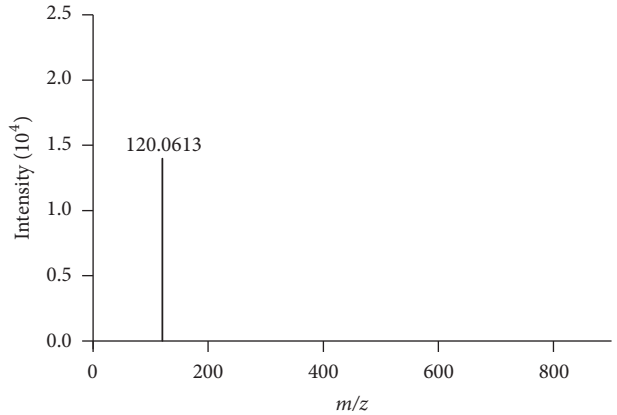
that temperature and proteolytic enzyme influence bacterial adhesion since they can change the mucus compositions.

3.3. Creatine and Phenylalanine Profiles. HPLC chromatogram profile of creatine and phenylalanine showed that the retention time of creatine and phenylalanine was 1.8 mins and 2.1 mins, respectively, with cysteine as internal standard (IS). The linear equation for creatine standard was $Y = 1.30^4X + 7.24^5$, $r^2 = 0.9916$, whereas linear equation for phenylalanine standard was $Y = 3.31^4X + 7.00^4$, $r^2 = 0.9997$, where X indicates the concentration of creatine or phenylalanine and Y indicates the peak area of creatine or phenylalanine. Figures 6 and 7 showed the effect of storage at 4°C on the concentration of creatine and phenylalanine of tuna fish, respectively.

Concentration of creatine increased from 4032.68 ppm (day 0) to 5650.85 ppm (day 4) and started to decrease at 5174.79 ppm (day 5) until 4631.28 ppm (day 7) before it remained unchanged until day 7 (3.31×10^3 ppm) (Figure 6). The trend was in agreement with preliminary testing using LC-MS/MS in which, at day 6, the creatine concentration was lower than the concentration at day 1. Physiological changes of fish after death involve prerigor stage, rigor-mortis stage, and postrigor stage. During prerigor stage, ATP was still high and the fish body was still soft before the ATP started to break down and the flesh became rigid at rigor-mortis stage. During postrigor stage, the proteolytic enzyme started to degrade the fish protein. Phosphatase hydrolyzed creatine phosphate and ATP producing creatine and phosphate. Once ATP decreased, creatine production may also be decreased



#	m/z	Intensity
1	166.0859	67271
2	167.0888	6611



#	m/z	Intensity
1	119.0688	187
2	119.5274	102
3	120.0813	12657
4	121.0884	776
5	121.5164	104
6	135.2362	129
7	149.0604	99
8	166.0766	118
9	167.0840	101
10	755.5137	120

Figure 5: The m/z and intensity value of phenylalanine on day 1.

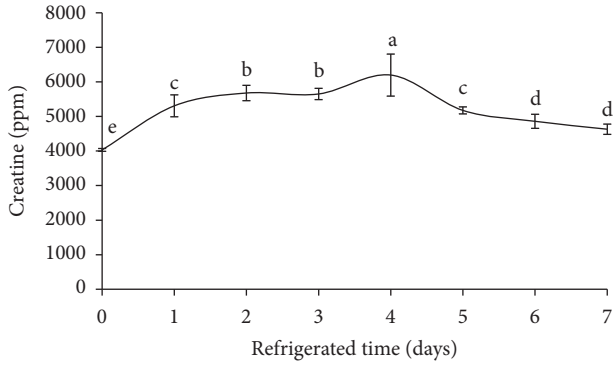


Figure 6: Creatine concentration of biofluids at 4°C for 7 days. Values are means ± SD ($n = 3$). Different superscript letters indicate significant differences ($p < 0.05$).

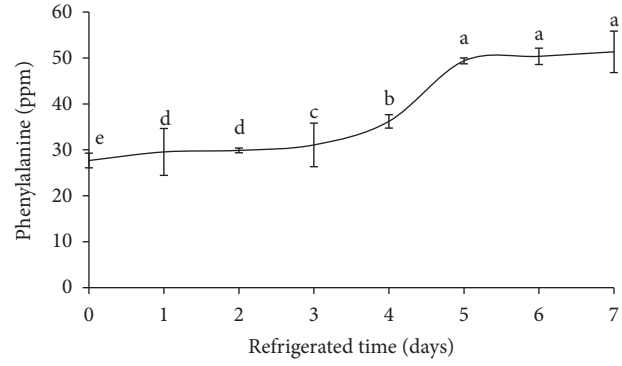


Figure 7: Phenylalanine concentration of biofluids at 4°C for 7 days. Values are means ± SD ($n = 3$). Different superscript letters indicate significant differences ($p < 0.05$).

as in Figure 6. However, study done by Huss (1988) [6] showed that creatine concentration unchanged when stored at 0°C for 20 days. Storage temperature tends to influence the releasing of biological fluid from fish body resulting from chemical and microbial activities [3]. This study used higher storage temperature (4°C) which may lead to the changes in creatine level. Besides, variations in glycogen content of different species contribute to different creatine content. Tuna fish contained the same amount of glycogen as that found in mammals, which contradicts most of the fish which has low amount of glycogen [6].

Figure 7 showed concentration of phenylalanine which increased from 27.68 ppm (day 0) to 49.36 ppm (day 5) and showed insignificant difference until day 7 ($p > 0.05$). The trend was similar with preliminary testing using LC-MS/MS in which, on day 6, the phenylalanine was higher than the concentration on day 1. Once the fish died, pepsin proteolyze denatured hemoglobin (substrate) in acidic condition into the trichloro ethanoic acid (TCA) soluble hydrolysis products, mainly tyrosine and phenylalanine. Besides, Gram-negative bacteria especially *E. coli* started to grow on the outer surface of spoilage fish after the body system stops functioning. Once the fish died, gills stop working as well as blood circulation. Small amount of microbes inside the body started to increase because defensive system against microbes stopped [6]. Increasing in phenylalanine from day 0 to day 5 indicated increasing of microbes producing phenylalanine especially *E. coli*.

Table 1: L^*, a^*, and b^* values of gills from *Euthynnus affinis* stored at 4°C for 7 days. Values are means ± SD ($n = 3$). Different superscript letters indicate significant differences ($p < 0.05$).

Day	L^*	a^*	b^*
0	41.08 ± 0.42^{a}	18.34 ± 0.55^{a}	-3.80 ± 0.12^{d}
1	37.65 ± 0.94^{b}	14.76 ± 0.28^{b}	-2.90 ± 0.56^{bcd}
2	36.30 ± 0.56^{b}	13.39 ± 0.43^{bc}	-2.95 ± 0.83^{cd}
3	30.58 ± 1.56^{c}	12.81 ± 1.76^{c}	-2.68 ± 0.07^{bcd}
4	27.36 ± 1.50^{d}	12.76 ± 0.27^{c}	-2.20 ± 0.11^{bc}
5	26.96 ± 0.31^{d}	12.08 ± 0.95^{c}	-1.43 ± 0.35^{ab}
6	25.91 ± 0.62^{de}	10.44 ± 1.09^{d}	-0.49 ± 1.40^{a}
7	24.76 ± 1.86^{e}	10.40 ± 0.49^{d}	-0.46 ± 1.34^{a}

3.4. Colour Properties of Fish Gills. Analysis on gills colour was done as a complementary result for biofluid analysis indicating fish spoilage. Table 1 shows colour changes on gills of *Euthynnus affinis* during 7 days of storage. The L^* value was decreased from 41.08 ± 0.42 to 24.76 ± 1.86 during iced storage indicating the changes on the gills which became darker and the red colour faded with respect to storage time. According to the quality index method (QIM) scheme, the gills colour changes from brightly red to rose colour, slightly pale, and green-yellowish upon fish spoilage. Similar trend on a^* value which represents the redness changes during storage of the fish gills was in fact decreased significantly throughout the storage time ($p < 0.05$). The b^* value increased significantly along the storage time which represents the yellowish colour formed during the storage. This result was most likely with the result obtained by Dowlati et al. (2013) [2] on the gills of gilthead sea bream (*Sparus aurata*) [10] where the gills colour changed from red to slightly pale and grey-yellowish during iced storage. Even though there were changes on the gills colour along the storage time, recently, some fish vendors are trying to apply chemicals such as formaldehyde for fish preservation [31]. The use of chemical treatment causes the fish apparently to look good and fresh making the fish freshness identification by naked eyes misleading. However, during storage, some liquid residual was secreted from the fish due to the protein hydrolysis which indicates the spoiling process is undergoing and this can represent how fresh the fish are.

4. Conclusion

Liquid residual of tuna fish *(Euthynnus affinis)* collected for seven days during iced storage at 4°C had shown presence of creatine and phenylalanine compounds derived from biofluids and microbial products, respectively, through LC-MS/MS analysis. Creatine and phenylalanine concentration increased through the storage period. Increase in pH upon storage indicated presence of microbes releasing basic compounds like sulphide. L^* and a^* values of fish gills decreased by times resulted in loss of gills redness colour. Concentration of creatine and phenylalanine determined during seven days of refrigerated incubation will be later used as limitation indicator for designing a biosensor kit for fish freshness application. FDA standard stated maximum 2 days of fresh fish in refrigerated temperature (4°C). Hence, the determined concentration of those metabolites in the biofluid could perhaps be used as an alternative indicator in the screening of fish spoilage.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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