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EFFECTS OF EXTRACTION METHODS AND STORAGE CONDITIONS ON HISTAMINE CONTENT IN FROZEN TUNA (*Thunnus albacares*)

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ABSTRACT

This study was undertaken to assess different extraction methods and the effects of times and temperatures on histamine produced in storage frozen tuna. The results showed that histamine in tuna samples could be efficiently extracted with perchloric acid 0.6 M and trichloroacetic acid 6%. The effective method for determining histamine concentration was benzoylation procedure. Tuna stored at 4°C resulted in higher histamine level (42.24 mg/kg) than tuna stored at 0°C (161.29 mg/kg) after 7 days of storage. Also, bacteria increased from 4.54 log_{10} cfu/g to 5.88 log_{10} cfu/g in the samples stored at 0°C, while for the samples stored at 4°C, an increase was shown dramatically in counts from 4.54 log_{10} cfu/g to 8.88 log_{10} cfu/g. These findings revealed that there was high correlation between storage conditions and histamine formation.

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1 INTRODUCTION

Currently histamine poisoning is one of the most common threats to consumers' health, its occurs after consuming seafood containing high levels of either histamine or other biogenic amines. This foodborne intoxication was originally called "scombroid poisoning" because primarily, it was associated with the consumption of fishes belonging to Scombridae and Scomberesocidae families such as tuna, mackerel, bonito, bluefish… These species adopted high levels of free histidine in their muscle that was decarboxylased to histamine. When conditions for the development of bacteria were available (for example improper temperature storage), decarboxylase enzymes produced by the bacteria started metabolizing histidine to histamine. Besides, other biogenic amines produced by bacteria might also increase the toxicity of histamine. The intoxication depends on the ability of individuals to metabolize normal dietary intakes of histamine. In some places of the world, histamine poisoning accounted for the largest population associated with fish and fish products (FAO, 2012). In Vietnam, histamine poisoning often occurs in cases of having tuna and mackerel.

Normally, after catching, fresh fish contained very low levels of histamine, but this amount increased with the decomposition of fish. Thus, histamine has also been used as an indicator to assess the quality of fish. US Food and Drug Administration (USFDA, 2012) have set the maximum histamine level at 50 mg/kg, above which it is considered as potential health hazard. In recent years, attention was paid to studying on the quality of seafood and its products through histamine assessment. Considering the importance of histamine in fish and fish products for legal, toxicological, and quality purposes, it is necessary to have effective analytical methods. For these reasons, the study was carried out to select the appropriate solvent for histamine

extraction, compare the effectiveness of benzoylation and evaluate the change of histamine contents in seafood over storage times and temperatures.

2 MATERIALS AND METHODS

2.1 Materials

Frozen tunas were purchased from local supermarkets in Can Tho City, Vietnam.

2.2 Methods

Histamine extraction procedures

Fresh tuna (*Thunnus albacares*) was preserved at 4°C for one week before analysis.

Extraction of histamine by 0.6 M perchloric acid (PCA): Extraction was carried out following the method of Ababouch *et al.* (1996). An amount of 10 g sample was homogenized with 20 mL 0.6 M PCA for 10 min. The homogenate was centrifuged at 7000 rpm for 10 min at 4°C, and the supernatant was made up to 50 mL with 0.6 M PCA, filtered and stored at 0°C until use.

Extraction of histamine using trichloroacetic acid 6%: Samples extraction was performed according to the method of Yung-Hsiang *et al.* (2001). An amount of 5 g sample was homogenized with 20 mL trichloroacetic acid (TCA) 6% for 10 min. The homogenates were centrifuged (10.000 g, 10 min, 4°C) and filtered. The filtrates were then placed in volumetric flasks, and trichloroacetic acid (TCA) was added to a final volume of 20 mL and stored at 0°C until use.

Extraction of histamine using methanol (99%): A modified method of Lin *et al.* (1976) was used. Sample (10 g) was homogenized with 50 mL methanol then transferred to a volumetric flask, which was then immersed in the water bath at 60°C for 15 min. The sample was cooled and then centrifuged at 7000 rpm for 10 min at 4°C. The supernatant was decanted, filtered and stored at 0°C until use.

Histamine determination by HPLC

Derivation of histamine by benzoyl chloride: The benzoyl derivatives of all samples were prepared according to a method of Hwang *et al.* (1997). One milliliter of NaOH 2 M and 10 µL of benzoyl chloride were added sequentially to 2 mL extracted solution. The mixture was vortex, and incubated at 70°C for 20 min. Benzoylation was stopped by cooling the test tubes on ice for 30 min, then the benzoyl products were extracted with 3 mL of diethylether and centrifugated at 3000 rpm, the supernatant was filtered through a 0.45 µm filter.

Derivation of histamine by dansyl chloride: Derivation was carried out following the method of Earola *et al.* (1993). A volume of 1 mL sample extract was alkalized by adding 200 µL of 2N NaOH. Then, 300 µl saturated sodium bicarbonate and 2 ml Dns-Cl were added to the alkalized extract. The reaction mixture was incubated at 40°C for 45 min, the residual Dns-Cl was removed by adding 100 µL ammonia, centrifugated for 30 min at 2500 rpm, the supernatant was filtered through a 0.45 µm filter.

Analysis of histamine on HPLC: Histamine in tested samples was analyzed on the reverse phase chromatography column Spherisorb 5 Si C18 pH-St, 250x4.6 mm (Phenomenex, Macclesfield, Cheshire, UK) using Shimadzu Prominence HPLC (Shimadzu, Kyoto, Japan) equipped with a SPD-M20A diode array detector (set at 254 nm) and two binary gradient pumps (Shimadzu LC-10AT), auto sampler (SIL 20AC), column oven (CTO-20AC), and a communication bus module (CBM-20A) with valve unit FCV-11AL. The elution solution was 95:5 (v/v) acetonitrile: water, with a flow rate of 1.0 mL/min.

All three histamine extraction methods and two methods of histamine determination were compared based on the yield of histamine (mg/kg).

Effects of time and temperature on histamine concentration and microbial flora in storage tuna samples

Fresh tuna (*Thunnus albacares*) was preserved at 4°C and 0°C. Samples were taken at different time points (0, 1, 2, 3, 4 and 7 days), and they all were stored at -20°C prior to analysis.

The total plate count (TPC) test of microorganism was done according to the method of Baranowski *et al.* (1990).

Statistical analysis

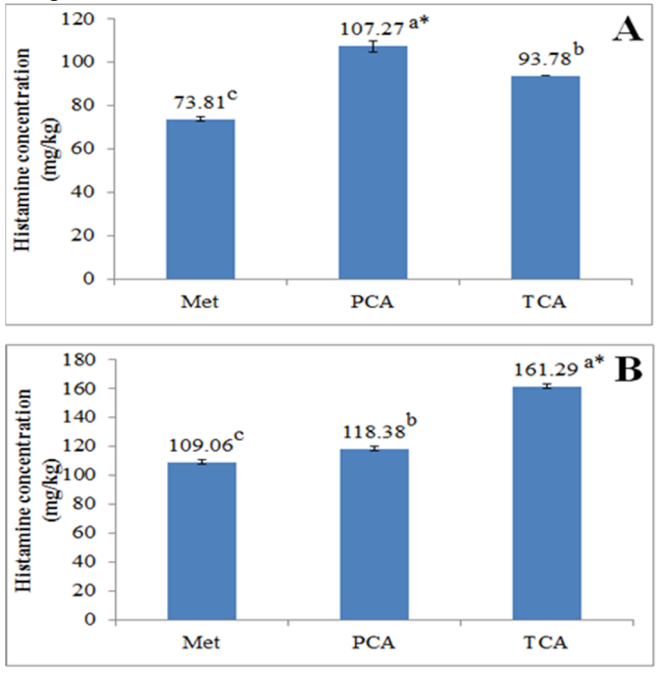
The experiment was conducted in triplicates. The results were reported as mean values ± standard deviation. The ANOVA test was used for data analysis. The differences between the mean values were considered significant when $p<0.05$. All data were analyzed using Statgraphic Centurion XV statistical package for windows (Statgraphic Centurion XV, Manugistics, Inc., Rockville, USA, 2009).

3 RESULTS AND DISCUSSION

3.1 Optimization of histamine extraction procedures

Complete and selective extraction of target analytes from complex food samples is of great

importance in food analysis. Solid samples are most frequently extracted with acidic solvents which act as deproteinisation agent during the liquid solid extraction. However, due to high content of fat in fish samples, extraction solvents that can remove fat or protein can be used for achieving high extraction efficiency. In this study, perchloric acid (0.6 M), trichloroacetic acid (6%), methanol (99%) were chosen as extraction solvents. Results revealed that different histamine concentrations were obtained from the fish samples with the three extraction solvents (Fig. 1).



* Mean that do not share a letter are significantly different.
A – Histamine was determined by using dansylation procedure.
B – Histamine was determined by using benzoylation procedure.

Fig. 1: Means of histamine concentrations in tuna samples extracted by methanol (Met), perchloric acid (PCA) and trichloroacetic acid (TCA)

As shown, for samples analysed by dansylation procedure (Fig. 1A) the highest histamine concentration was obtained with samples extracted by 0.6 M perchloric acid (107.27 mg/kg). In case of analysing by benzoylation procedure (Fig. 1B) the samples extracted by trichloroacetic acid 6% gave remarkable high histamine concentrations (161.29 mg/kg), which is statistically different in comparison with the samples tested in both histamine determination methods (dansylation and and benzoylation). These results coincide with those of Ben-Griggrey et al. (2001), who suggested perchloric acid and trichloroacetic acid are more effective than methanol, acetonitrile and acetone in extracting histamine from fish products. Ruiz-Capillas and Moral (2001) also reported that PCA and TCA are highly effective biogenic amine extractors for fish and fish products because of their affection on protein precipitation. Since most of the biogenic amines present in fish are in the complex forms, the histamine concentration was low when extracted by methanol (Shalaby, 1996). In this study, histamine concentration obtained from the tuna samples was quite high in the acidic fraction, suggesting that histamine in tuna is acid soluble. It appeared that perchloric acid and trichloroacetic acid provided good histamine peak shape on the chromatograms and effective separation, for this reason, they were chosen as optimum extraction solutions for the next following experiments. In addition, by dansylation procedure, perchloric acid 0.6 M was the appropriate acid to extract histamine from fish samples for analysis, while trichloroacetic acid 6% was a good solvent for histamine extraction used in benzoylation method.

3.2 Comparison of dansylation and benzoylation procedures for histamine determination from tuna

Amongst the available analytical techniques, HPLC is by far the most frequently used to separate and quantify histamine. Since histamine present in the food samples neither shows an adequate absorption, nor exhibits significant fluorescence, derivatisation has to be performed in order to in-

crease the sensitivity for a subsequent UV, VIS or fluorescence detection (Onal, 2007). In this work the amines were determined by HPLC using two procedures – dansylation and benzoylation – based on pre-column derivatisation. The chromatograms of dansyl chloride and benzoyl chloride derivatives are shown in Figure 2. As shown, histamine was well separated in the nearly 3 min retention time for benzoyl procedure, where as it was 5 min for dansyl procedure. The histamine levels determined from six tested samples revealed the significant differences in analysis efficency between the two procedures (Fig. 3). In general, the histamine content obtained by benzoylation procedure was higher than the one determined by dansylation procedure.

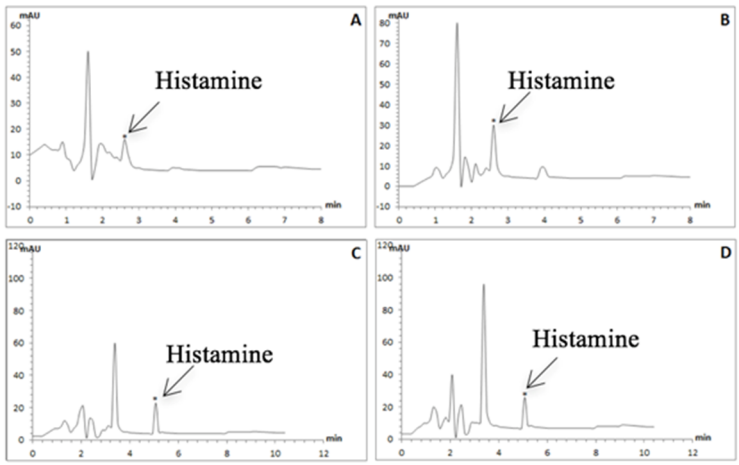


Fig. 2: Chromatograms of the histamine standard and samples. (A), (C) Histamine standard (1 µg/mL); (B), (D) tuna sample at day 7th of storage. (A) and (B): chromatography derived by benzoylation procedure; (C) and (D): chromatography derived by dansylation procedure

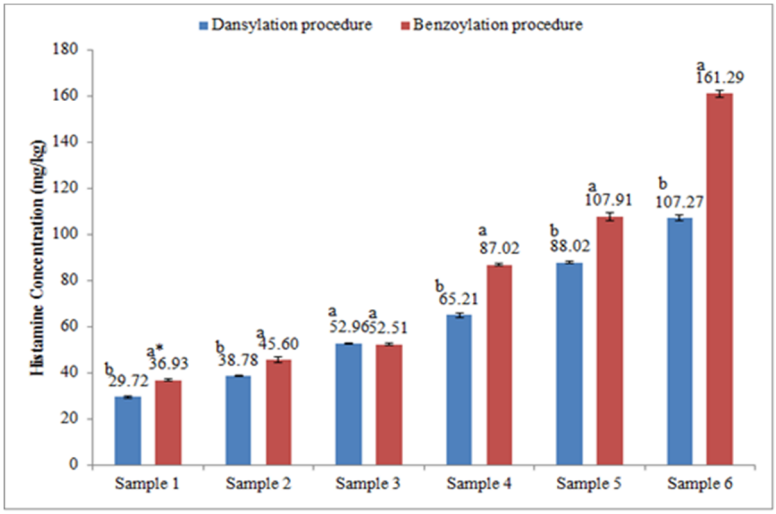


Fig. 3: Means of histamine concentrations in tuna samples determined by dansylation and benzoylation procedures

The results clearly suggested that benzoylation procedure was more accurate than the dansylation one for histamine quantification from tuna. This is related to the fact that benzoyl chloride forms stable compounds after reaction with both primary and secondary amino groups and the products are more stable than those formed using dansyl chloride. Moreover, the long derivatisation time is also an evident drawback of dansylation procedure, as it contributed to decreasing the stability of fluoresence compounds. Besides, ammonia has also to be used after the derivatisation to remove an excess of the dansyl reagent. Without this step, the by-products such as dansylamide (Dns-NH2), dansyl sulphonic acid (Dns-OH) and dansyl hydrazine (Dns-N2H3) most likely appeared on the chroma-

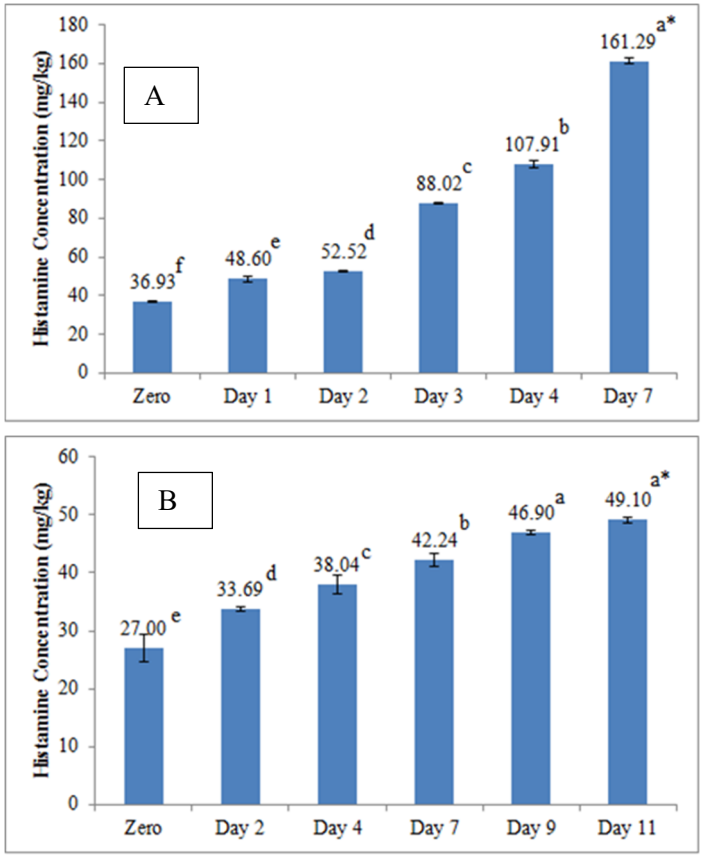
tograms (De Mey et al., 2011) and the excess of dansyl chloride may coelute with histamine. Unfortunately, an addition of 100 µL concentrated ammonia and incubation for 30 min at room temperature, as suggested by Earola *et al*. (1993), could not totally remove the excess of dansyl chloride. As a result, low amounts of the by-products can still be detected in the chromatograms and the peaks of histamine were obviously affected.

In contrast to the dansylation procedure, benzoylation is less frequently used for the determination of biogenic amines in fish and fish products. However, fast benzoylation gives an opportunity to reduce the analysis time. It was reported that the benzoyl derivatisation used to be performed at the temperatures ranging from room temperature to 70°C. At the room temperature, the derivatisation needs an overnight incubation, while at the increased temperatures the incubation time can range between 15 (Krause et al., 1995) and 30 min only (Chen et al., 2003). In most cases, an incubation period of 20 min at 70°C was recommended (Purohit et al., 2002). Based on the above mentioned advantages, the benzoylation procedure seemed to be the method of choice.

3.3 Changes in histamine content and microbial flora in tuna samples during storage time and temperature changes

Effect of time and temperature on histamine concentration during storage

The average histamine concentrations in tuna samples storaged at different temperatures during 7 days are presented in Figures 4. As shown, in the samples stored at 4°C, the increases of histamine varied from the lowest value 36.93 mg/kg to more than 161.29 mg/kg and there was a significant difference ($p<0.05$) in corelation to the storage time. A remakable increase in the histamine value was last until the day 7th, and it was above the FDA standard already at the 3rd day (>50 mg/kg). In tuna samples stored at 0°C, the results revealed a steady increase in histamine levels during seven days storage, however it was not as dramatic as at 4°C. The obtained results also showed a significant difference ($p<0.05$) between the mean concentrations of histamine during the observation period (7 days). However, histamine level does not exceed the safe limits (50 mg/kg) as it can be seen on the day of 11th when storage at 0°C (Fig. 4B).



* Mean that do not share a letter are significantly different

Fig. 4: Means of histamine concentrations in tuna samples stored at 4ºC (A) and 0ºC (B) by the time

According to the results, the storage temperature was one of the most important factor in histamine formation. Also, time and temperature have been reported as the key factors in controlling histamine development, these factors influenced the growths of histamine-producing bacteria and the formation of their histidine decarboxylase. The samples stored at 0°C showed little increase in histamine, while samples stored at 4°C presented toxic histamine levels. Similarly, Baranowski et al. (1990) showed the relationship between the formation of free amino acids and amines in mackerel stored at 0°C and 4°C. The contents of several amino acids decreased when fish were stored at 4°C with the resultant formation of phenylethylamine, tyramine, putrescine, histamine and cadaverine. However, no obvious changes were reported in fish stored at 0°C. Yamanaka et al. (1987) showed a similar result with little histamine formed in frozen mackerel, but high level of histamine were found at 8°C. Similarly, according to Jorgensen et al. (2000), low concentrations of histamine (80 mg/kg) in albacore loins stored at 0°C for 18 days were observed. Lopez-Sabater et al. (1996) reported that histamine content in large tuna fish pieces was 425 mg/kg after 6 days of storage at 8°C, but no histamine was detected after 12 days at 0°C.

Effect of time and temperature on microbial population during storage

Counts of total microorganism in tuna samples stored at 4°C relating to storage time were summarized in Figure 5. At the start of the incubation, total microbial count was low (4.54 log10 cfu/g). There was a steady increase over the period of storage to 8.88 log10 cfu/g. This count was comparable with the concentrations of histamine formed. As revealed by Suzuki et al. (1990), the number of bacteria were indirectly related to the histamine production because its ability to synthesize the enzyme histidine decarboxylase. Tuna samples stored at 0°C also showed increase in total aerobic bacteria, although the extent of this increase was not as dramatic as at 4°C. Apparently, the slow increase of bacteria at this temperature (Fig. 5B) correlated to the slow increase of histamine concentration in these samples.

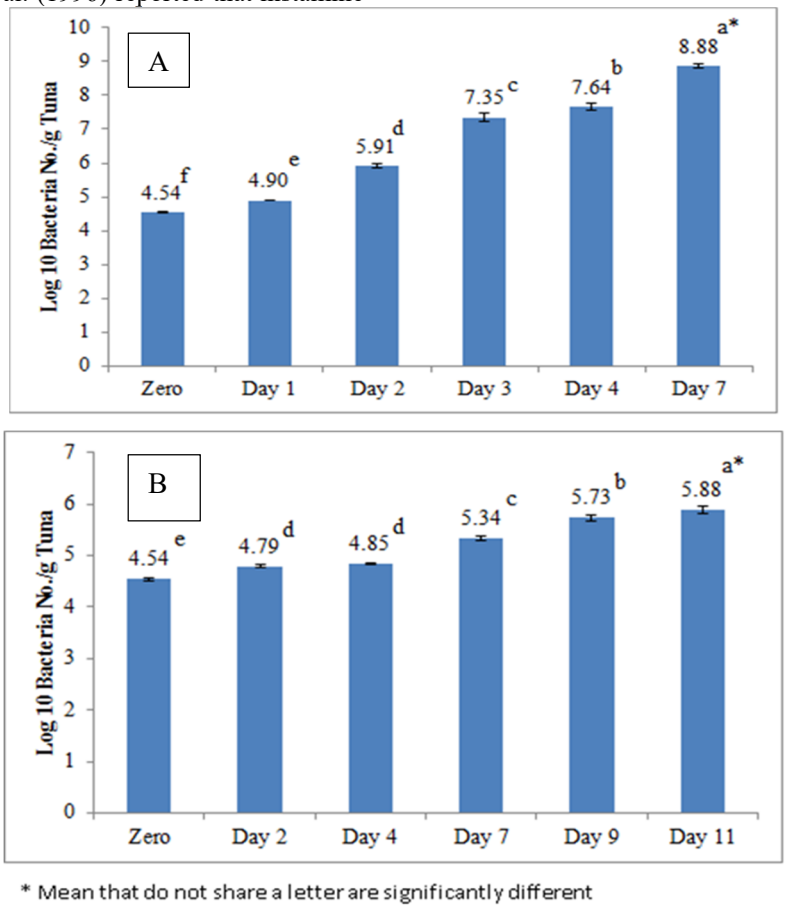


Fig. 5: Means of total viable counts in tuna samples stored at 4°C (A) and 0°C (B) during seven days storage

This study reveals that even at low temperatures, the longer the storage period the higher bacterial number and that exposed direct effect on the histamine value of the fish. Fish frozen at 0°C created an unfavourable conditions for the growth and survival of microorganisms, thereby reducing histamine formation, while keeping fish samples at 4°C temperature the proliferation of microorganisms occurred, thereby it caused histamine formation. The freezing process appeared to reduce the bacteria number or caused sublethal injuries to bacterial cells, as a result there was poor histamine production. According to Jorgensen et al. (2000), the growth of histamine producing bacteria can be monitored by low temperatures (2 - 4°C) but histamine formation can be controlled only by frozen storage. Baranowski et al. (1990) observed that the freezing period was an important factor for histamine formation during storage of mahi-mahi fish. Ben-Gigirey et al. (2000) also said that the survival of bacteria involving in biogenic amin development was poor at freezing temperatures. Recently, Dalgaard et al. (2006) evaluating the biogenic amin formation and microbial spoilage in fresh and chilled garfish, concluded that histamine formation depended strongly on previous freezing of fish, which probably killed freeze-sensitive bacteria like Photobacterium spp. or Vibrio spp. Staruszkiewicz and Rogers (2001) studied the changes of observed biogenic amines in mahi-mahi and tuna fish kept in freezer, and showed that histidine decarboxylase activity was retained in some frozen fish samples and could result in further increasing of histamine.

4 CONCLUSIONS

In sum up, the appropriate solvents for extraction of histamine from tuna fish were perchloric acid 0.6 M and trichloroacetic acid 6%. To determine histamine content in tuna fish the benzoylation procedure seemed to be the best method. Fish frozen at 0°C significantly reduced histamine concentration, it was better than keeping fish at 4°C. For good storage conditions, 0°C is recommended, since it will increase the shelf life of fish. The deterioration of fish samples was obvious from microbiological analyses, however, their control can be achieved at 0°C.

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