Advance Journal of Food Science and Technology 5(7): 822-828, 2013
ISSN: 2042-4868; e-ISSN: 2042-4876

Submitted: September 27, 2012 Accepted: November 11, 2012 Published: July 05, 2013

Effect of Different Temperatures on the Free Amino Acids, Physico-Chemical and Microbial Changes during Storage of Barramundi (*Lates calcarifer*) Fillets

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Abstract: The effects of storage days and temperature on free amino acids, TVB-N, pH and microbial changes in Barramundi (*Lates calcarifer*) fillets kept at 0°C and 8°C were investigated for 20 days. At the end of the storage, significant differences were observed ($p<0.05$) in the amino acids isoleucine, leucine, methionine, phenylalanine, valine, glutamic acid and glycine from the initial value of both storage temperatures. However, no significant difference ($p>0.05$) between two temperatures during the storage period were observed. Among two temperatures, the psychrophiles were initially 4.07 log CFU/g and exceeded the acceptable limit of 7 log CFU/g on the 12^{th} and 8^{th} day at 0°C and 8°C, respectively. Although, Total Plate Count (TPC) were initially 3.7 log CFU/g and exceeded the acceptable limit of 6 log CFU/g on the 12^{th} day in the both storage temperatures. Histamine Forming Bacteria (HFB) was significantly ($p<0.05$) lower in Barramundi fillets storage at 0°C compared to the 8°C. Significant differences ($p<0.05$) between the concentrations of Total Volatile Base-Nitrogen (TVB-N) in fillets kept at 0°C and 8°C were observed.

Keywords: Amino acids, shelf-life, Total Volatile Base-Nitrogen (TVB-N)

INTRODUCTION

Protein is the most important food component of fish. The basic units of proteins are amino acids which are produced when protein is hydrolyzed using acids, alkalis or enzymes. Peptide linkages into polymers resulting in peptides and proteins link the components amino acids (Liston, 1990). It is known that free amino acids play a very important role in bacterial spoilage and they are the major substrates responsible for rapid microbial spoilage in fish (Jay *et al*., 2005). This microbial spoilage leads to the most extensive deteriorative changes which eventually renders the fish unmarketable (Liston, 1990). The free amino acid profile is one of the characteristics which differ between fish species. There are always free amino acids present in the blood and flesh of fish, which are generated in the course of transportation, anabolism and catabolism (Ruiz-Capillas and Moral, 2004). The importance of free amino acids in the role of fish spoilage is illustrated by the fact that amino acids are major part of the substrates responsible for rapid microbial spoilage. As simple low molecular weight compounds, they provide the essential nutrients for the rapid growth of microbes after the death of a fish (Jay *et al*., 2005).

Microbial methods are generally used to indicate the presence or absence of microbes in a fixed quantity of product, or to measure the total numbers of organisms. One of these methods is the standard aerobic plate count which gives a measure of the degree of contamination (Wheaton and Lawson, 1985). Also, microbiological examinations may search for specific pathogens which may require the use of specialized media, e.g Niven's medium used for identification of histamine forming (Niven *et al*., 1981), psychrophilic bacteria (Arashisar *et al*., 2004) and anaerobic bacteria (Debevere and Boskou, 1996; Ravi Sankar *et al*., 2008).

The total Volatile Bases fraction (TVB) includes ammonia, monoethylamine, dimethylamine along with Trimethylamine (TMA). TVB is part of the NPN fraction of fish muscle as well. The residual by the subtraction of TMA from TVB is called Formalin-Bound Volatile Nitrogen (FBVN) (Debevere and Boskou, 1996). This fraction of TVB can increase slightly during storage due to some reactions of autolysis and deamination. TMA and TVB are considered responsible for unpleasant 'fishy' odor (Debevere and Boskou, 1996; Antoine *et al*., 2002). Huss (1994) and Antoine *et al*. (2002) stated that formation of volatile bases is one of the most characteristic features of fish spoilage. These volatile bases mainly include ammonia and lower amines such

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as TMA and Dimethylamine (DMA). The aims of this study was to assess the changes occurring in free amino acids, physico-chemical and microbial changes in Barramundi fillets during storage at refrigerated temperatures.

MATERIALS AND METHODS

Amino acids analysis: Extraction were carried out in triplicate by homogenizing Barramundi fillets with a Waring blender (Model 32BL79, USA) for 3 min and weighing 0.25 g and adding 15 mL HCl 6 N. After storage in oven overnight at 110°C and cooling, add 10 mL AABA (internal standard), filtrated and made up volume to 50 mL with deionised water. The supernatants were pooled and concentrated under vacuum and finally stored in the freezer at -20°C for 4 weeks. The free amino acid contents of the extracts were analyzed by an HPLC gradient system with precolumn Phenylisothiocyanate (PITC) derivatization (Rozan *et al*., 2000, 2001; Irvine and Davidson, 2003). Buffer A (0.1 M ammonium acetate, pH 6.5) and buffer B (0.1 M ammonium acetate containing acetonitrile and methanol, 44:46:10, v/v, pH 6.5) were used. For sample preparation, a 50-µL aliquot of extract was removed and dried under vacuum (37°C, 20 mmHg). Then 20 µL of a first coupling reagent [methanol, water, triethylamine (TEA) (2:2:1 v/v)] was added. After mixing, the sample was directly dried under vacuum during 10 min and was then reacted with 30 µL of PITC reagent [methanol, PITC, TEA, water (7:1:1:1 v/v)] at room temperature for 20 min before drying under vacuum to remove PITC. The derivatized samples were then redissolved in 500 µL of buffer A that is used as mobile phase for HPLC and filtered through a Millipore membrane (0.2 µm). A 20 µL sample was injected into an HPLC system (Waters model 991 equipped with a photodiode array detector) using a gradient system of buffer A (100-0 % after 50 min) and buffer B (0-100 % after 50 min) (Rozan *et al*., 2000, 2001; Irvine and Davidson, 2003). The operating temperature 43°C and a C18 reversed-phase column from Alltech (Alltima C18 5U, 250×4.6 mm) was used. The absorbance at 254 nm was used for calculations. The UV absorption spectrum was useful for the identification. A standard protein amino acid mixture (food hydrolysate A 9656, Sigma) was prepared as above (Rozan *et al*., 2000, 2001; Irvine and Davidson, 2003). Amino acid quantification was performed by internal standard method with AABA. AABA peak was used as reference peak during the chromatography. Each amino acid was calculated by:

mg a.a./g sample

$$= \frac{(\text{RFF}) \times (\text{area of a.a. in sample}) \times (\text{DF}\,(0.025 \text{ except Cystine was } 0.0125))}{(\text{area of AABA in sample}) \times (\text{weight of sample(g)})}$$

Relative response factor (RFF) = (Area of AABA/Area of a.a.) in standard.

The original concentration of amino acid standard was 250 pmoL (2.5 µmoL/mL).

The sum of the equation was multiplied with molecular weight of amino acid. Results were expressed as mg amino acids/g sample.

Microbiological analysis: Total Plate Count (TPC) and psychrophilic bacteria were done according to method of AOAC (2000). Twenty-five grams of Barramundi fillets were aseptically weighed and homogenized in stomacher bags (BAGMIXER® 400, Model P) with 225 mL sterile peptone water for 1 min. The homogenized sample was serially diluted using 9 mL peptone water. Further serial dilutions were made and 0.1 mL of each dilution was pipetted on to the surface of the plate count agar (Merck), in triplicates, after which they were incubated for 2 days at 30°C for TPC and 1 week at 8°C for psychrophilic bacteria. The HFB were carried out according to the procedure of Nivein's (Niven *et al*., 1981).

Total volatile base-nitrogen (TVB-N): The Total Volatile Base-Nitrogen (TVB-N) content of Barramundi fillet was determined according to the method of Goulas and Kontominas (2005) and expressed as mg TVB-N/100 g muscle.

pH determination: The fish fillet was homogenised in distilled water in the ratio of 1:10 (w/v) and the mixture was filtrated (Goulas and Kontominas, 2005). The pH of filtrate was measured using a digital pH-meter model DELTA 320 (METTLER TOLEDO, In Lab® Expert Probe) at room temperature.

Statistical analysis: All data collected were analyzed by Analysis of Variance (ANOVA) to determine the effect of temperatures and storage time on the parameters measured in the Barramundi fillets. The Tukey's test was used for mean comparison when a significant variation was found by the ANOVA test using SPSS software for windows (SPSS Inc. 2008).

RESULTS AND DISCUSSION

Amino acids profile: The changes in Free Amino Acids (FAA) in Barramundi fillets stored at 0°C and 8°C are as shown in Table 1. The FAA histidine, glutamine, lysine, phenylalanine, tyrosine, arginine are very important during fish spoilage, since these FAA can produced biogenic amine by decarboxylation (histamine, putrescine, cadaverine, phenylethylamine, tyramine and agmatine, respectively) which are important from the toxicity point of view and quality attribute of fish spoilage (Shalaby, 1996; Ruiz-Capillas and Moral, 2001b, 2002; Önal, 2007). However, in this

Table 1: Changes in free amino acids concentration[1] (mg/g sample) in Barramundi fillets at 0 °C and 8 °C

		Storage days					
Amino acids	Temperature(°C)	0	4	8	12	16	20
Histidine	0	2.69±0.16Da	2.06±0.09Ba	1.55±0.17Aa	2.61±0.13Ca	2.38±0.12BCa	2.48±0.05Ca
	8	2.69±0.16Ca	2.22±0.53Ba	1.46±0.11Aa	2.12±0.22ABa	2.38±0.05BCa	2.45±0.10BCa
Arginine	0	9.89±0.24Ca	7.47±0.25Ba	5.24±1.21Aa	10.41±0.41Ca	9.56±0.38Ca	10.26±0.26Cb
	8	9.89±0.24Aba	7.86±1.12Aa	7.96±1.58Aba	10.19±0.11Ba	9.91±0.29Aba	8.91±0.47Aba
Tyrosine	0	7.10±0.19Dea	4.78±0.17Ba	3.82±0.03Aa	7.52±0.27Ea	6.72±0.03Cda	6.62±0.10Ca
	8	7.10±0.19Ba	5.76±1.09Ba	4.06±0.07Ab	6.64±0.93Ba	7.29±0.15Bb	6.58±0.44Ba
Phenylalanine	0	8.82±1.08Ba	5.48±0.10Aa	4.71±0.14Aa	8.40±0.35Bb	7.68±0.07Ba	7.85±0.24Bb
	8	8.82±1.08Ba	6.92±1.92Aba	4.74±0.10Aa	7.30±0.16Ba	7.64±0.14Ba	6.78±0.22Aba
Lysine	0	56.20±1.84Da	39.16±4.40Ba	27.69±1.51Aa	48.91±0.83Ca	43.84±2.79Bca	44.55±1.43Bca
	8	56.20±1.84Ba	45.65±10.63Ba	30.69±0.93Aa	47.88±3.78Ba	49.42±0.68Bb	48.11±0.65Bb
Glutamine	0	19.32±0.59Ba	13.72±0.38Aa	12.06±0.57Aa	19.75±0.95Ba	20.67±1.62Ba	20.80±0.70Ba
	8	19.32±0.59BCa	15.84±2.81ABa	12.31±0.29Aa	19.45±1.63BCa	20.67±1.14Ca	19.64±1.17BCa
Aspartic acid	0	42.74±1.13Ba	30.28±2.26Aa	28.11±1.46Aa	49.43±1.30Ca	46.30±0.95Ca	48.73±1.03Cb
	8	42.74±1.13Ca	35.06±4.73Ba	29.58±1.17Aa	48.50±0.35Da	50.42±1.94Da	45.84±0.27Cda
Serine	0	7.98±0.47Ba	5.46±0.58Ab	4.64±0.43Ab	7.53±0.35Bb	7.73±0.02Bb	8.11±0.22Bb
	8	7.98±0.47Ca	2.03±0.51Aba	1.28±0.04Aa	2.09±0.28Ba	1.98±0.05Aba	2.40±0.04Ba
Glycine	0	9.93±0.16Ba	7.41±0.38Ab	7.13±1.01Ab	9.67±0.57Bb	9.59±0.26Bb	11.76±0.35Cb
	8	9.93±0.16Ca	2.57±0.48Ba	1.81±0.03Aa	2.96±0.41Ba	3.25±0.07Ba	2.93±0.19Ba
Threonine	0	9.36±0.23Da	7.83±0.33Ba	6.26±0.40Aa	9.07±0.08CDa	8.55±0.41Ca	9.77±0.26Da
	8	9.36±0.23Ba	9.14±0.89Ba	6.55±0.17Aa	9.61±0.44Ba	9.04±0.17Ba	8.95±0.36Ba
Alanine	0	12.40±0.54CDa	9.18±0.41Ba	7.68±0.72Aa	12.15±0.12CDa	11.57±0.05Ca	12.97±0.39Db
	8	12.40±0.54Ba	10.24±1.97Ba	8.10±0.21Aa	12.35±0.24Ba	12.13±0.34Ba	11.60±0.46Ba
Proline	0	6.49±0.02BCa	4.49±0.20Aa	3.92±0.55Aa	6.40±0.25BCa	5.83±0.28Ba	7.12±0.19Cb
	8	6.49±0.02Ba	5.12±1.16ABa	3.94±0.24Aa	6.34±0.52Ba	5.88±0.14Ba	5.71±0.28Ba
Valine	0	9.23±0.22CDa	6.486±0.16Ba	5.78±0.20Aa	9.43±0.13Da	8.70±0.37Ca	9.20±0.17CDa
	8	9.23±0.22Ba	7.60±1.48Ba	5.73±0.20Aa	9.28±0.46Ba	9.19±0.26Ba	8.61±0.45Ba
Methionine	0	5.59±0.16Ca	4.61±0.15Ba	3.33±0.06Aa	6.14±0.14Da	5.65±0.07Ca	5.54±0.14Ca
	8	5.59±0.16Ca	4.67±0.33Ba	3.27±0.31Aa	5.91±0.51Ca	5.83±0.20Ca	5.57±0.41Ca
Cystine	0	1.31±0.24a	ND	ND	ND	ND	ND
	8	1.31±0.24Ba	0.66±0.34A	0.62±0.085A	0.90±0.15AB	1.23403±0.06B	0.84±0.14AB
Isoleucine	0	8.51±0.29Aa	30.99±1.74Ca	23.02±0.49Ba	32.87±0.51CDa	32.48±1.07Ca	34.80±0.74Db
	8	8.51±0.29Aa	31.04±0.69Ca	24.21±1.07Ba	33.93±2.19Da	31.49±0.49CDa	31.64±0.51CDa
Leucine	0	19.01±1.19Ca	8.59±0.31Aa	7.40±0.38Aa	11.99±0.02Ba	11.66±0.26Ba	11.94±0.19Ba
	8	19.01±1.19Ca	7.61±1.70Aa	7.57±0.33Aa	12.01±0.12Ba	12.31±0.14Bb	11.45±0.55Ba

[1]: Means reported are the results of triplicate; [a,b-A-E]: Different lower case in the column and different upper case in the row (for the same amino acids) are significant difference ($p<0.05$).

study no clear relationship was observed between changes these FAA and the formation of biogenic amines. A significant increase ($p<0.05$) was observed in the biogenic amines histamine, putrescine, cadaverine, phenylethylamine, spermine, spermidine, tyramine and agmatine (data not shown) during 20 days of storage. This increase did not coincide with a decrease in the corresponding FAA since the initial concentration of FAA until 8 days decreased and enhanced to the same levels on 12 days after that were generally constant until the end of storage. This could possibly be because the degradation of this FAA was not due to decarboxylation but to deamination (Ruiz-Capillas and Moral, 2002). The release of free amino acids is due to the initial higher activity levels of cathepsins, calpains and aminopeptidases, which are capable of hydrolyzing a wide range of terminal amino acids (Alfaia *et al*., 2004). Thus, a reduction on the aminopeptidase activity together with degradation reactions may be mainly responsible for the decrease in the amounts of free amino acids observed between the 8 and 12 days. A similar profile was observed in hake (*Merluccius merluccius* L.) and bigeye tuna (*Thunnus obseus*) by Ruiz-Capillas and Moral (2002, 2004). A significant ($p<0.05$) increased between 3 to 4 times greater than initial value of isoleucine was observed in the end of storage. The similar observe was reported by Ruiz-Capillas and Moral (2002, 2004) who indicated in these hydrophobic FAA when the fish was stored in controlled atmosphere.

Microbial count: Figure 1a shows the total plate count, histamine-forming and psychrophilic bacteria in Barramundi samples stored at 0°C and 8°C. Higher counts were observed at 8 °C as compared to the counts at 0°C. At day 0, the initial aerobic colony counts were 3.47 log CFU/g. At day twentieth the counts were to 6.81 and 7.46 log CFU/g for samples stored at 0°C and 8°C, respectively. There were significant differences ($p<0.05$) in TPC of fish stored at 0°C and at 8°C on twentieth days. El-Marrakchi *et al*. (1990) also reported that the total plate count in sardines stored at ambient temperature exceeded the acceptable limit faster as compared to those kept on ice.

Initial psychrophilic bacteria of Barramundi fillets were 4.07 and log CFU/g indicating the good quality of Barramundi (Fig. 1b). Length of refrigerated storage (0°C and 8°C) had a significant ($p<0.05$) effect on APC which tended to increase as the storage time increased (Fig. 1). However, psychrophilic bacteria exceeded mesophilic count in all the samples after 4 days. Similar observations were made earlier in rainbow trout (Arashisar *et al*., 2004; Ravi Sankar *et al*., 2008). If 7 log CFU/g is taken as the APC and TPC limit of acceptability, therefore; the storage period of

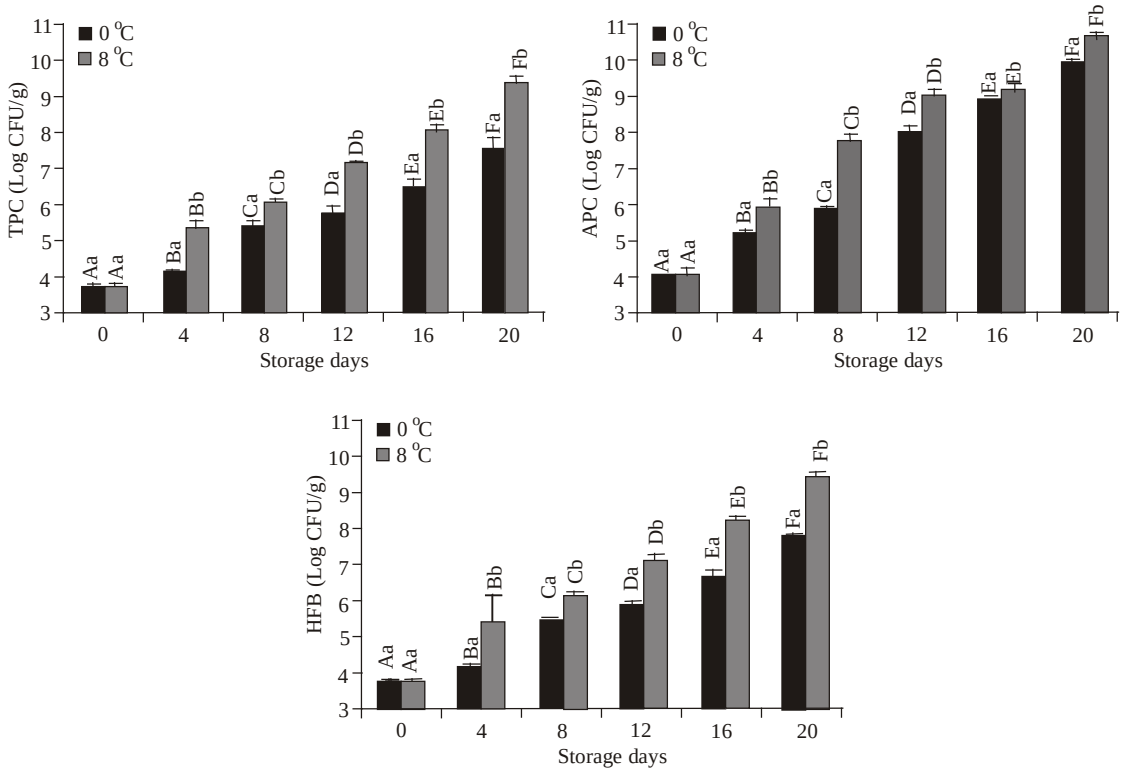

Fig. 1: Changes in TPC (a), APC (b) and HFB (c) of Barramundi fillets stored at 0°C and 8°C; TPC: total plate count, APC: psychrphilic bacteria, HFB: histamine forming bacteria; [a,b]: Means within groups with different lower case are significantly different ($p<0.05$); [A-F]: Means between groups with different upper case are significantly different ($p<0.05$)

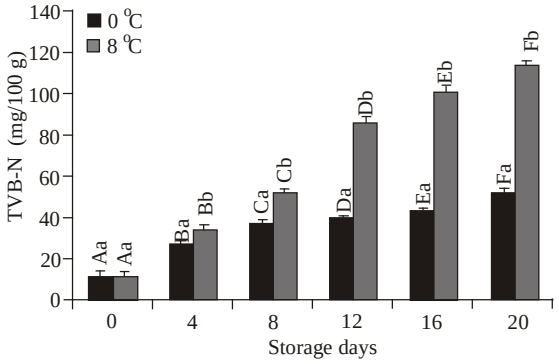

Fig. 2: Effect of temperature on TVB-N of Barramundi fillets stored at 0°C and 8°C; [a,b]: Means within groups with different lower case are significantly different ($p<0.05$); [A-F]: Means between groups with different upper case are significantly different ($p<0.05$)

Barramundi was approximately 12 and 8 days at 0°C and 8°C, respectively.

Figure 1c also shows the changes in the HFB of Barramundi fillets during storage at 0°C and 8°C. The HFB grew faster in Barramundi fillets stored at 8°C, as compared to at 0°C. In this study, although histamine, putrescine and cadaverine and other biogenic amines were not found at the beginning of the storage (data not shown). At the end of the storage, for samples kept at 0°C and 8°C, the HFB corresponded to 7.7 log CFU/g and 9.4 log CFU/g, respectively. Therefore, this indicates that HFB were naturally present at high levels in Barramundi.

Total Volatile Base-Nitrogen (TVB-N): The levels of total volatile basic nitrogen in the Barramundi fillets stored at two different temperatures studied are shown in Fig. 2. TVB-N levels in all samples increased throughout the day during on each day that sampling was done. Barramundi fillets stored at 0°C had lower levels of TVB-N, ranging from 12.10 mg N/100 g to 50.87 N/100 g for all 20 days compared with the range of 12.10 N/100 g to 112.47 mg N/100 g for samples stored at 8°C. The significant increases ($p<0.05$) in TVB-N levels during the 20 days may be due to handling practices, resulting in bacterial growth (Anderson, 2008). Hernández-Herrero *et al*. (1999) and Anderson (2008) reported a significant increase in TBV-N levels during the ripening of salted anchovies and attributed such increases to bacterial and enzymatic action. The 30-35 mg N/100 g by Connell (1995) suggested a TVB-N limit value of 35 mg N/100 g

Table 2: Two-way ANOVA results for effects of temperatures and storage days of all parameters evaluated for Barramundi fillets

		Two-way ANOVA		
Parameters		Day effect	Temperature effect	Interaction effect
pH		*	*	*
	p-value	0.000	0.000	0.000
	F-ratio	2861.09	2886.24	208.49
TVB-N		*	*	*
	p-value	0.000	0.000	0.000
	F-ratio	985.16	1985.18	251.71
TPC		*	*	*
	p-value	0.000	0.000	0.000
	F-ratio	1102.80	177.33	18.09
APC		*	*	*
	p-value	0.000	0.000	0.000
	F-ratio	2823.51	1045.45	84.81
HFB		*	*	*
	p-value	0.000	0.000	0.000
	F-ratio	1748.46	950.50	56.04
Glutamine		*	ns	ns
	p-value	0.000	0.719	0.364
	F-ratio	47.08	0.13	1.15
Histidine		*	ns	ns
	p-value	0.000	0.278	0.146
	F-ratio	34.31	1.23	1.82
Arginine		*	*	ns
	p-value	0.000	0.009	0.062
	F-ratio	42.70	8.07	2.46
Tyrosine		*	ns	*
	p-value	0.000	0.343	0.034
	F-ratio	49.18	0.94	2.91
Phenylalanine		*	ns	ns
	p-value	0.000	0.607	0.060
	F-ratio	23.51	0.27	2.48
Lysine		*	*	ns
	p-value	0.000	0.026	0.457
	F-ratio	34.72	5.63	0.97

TVB-N: Total volatile base nitrogen; TPC: Total plate count; APC: Aerobic psychrophilic count; HFB: Histamine forming bacteria; *: $p<0.05$ is significant difference; ns: not significant difference

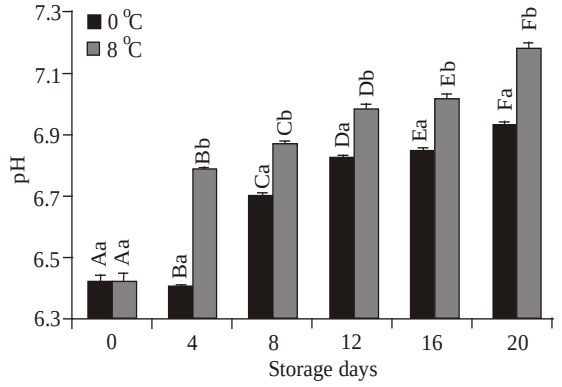

Fig. 3: Effect of temperature on pH values of Barramundi fillets stored at 0°C and 8°C; [a, b]: Means within groups with different lower case are significantly different ($p<0.05$); [A-F]: Means between groups with different upper case are significantly different ($p<0.05$)

muscle. Considering this TVB-N value as a limit of acceptability, Barramundi stored at 8°C would be expected to have storage period of 8 days, extended to 12 days when stored at 0°C.

pH: pH values for Barramundi stored at 0°C and 8°C increased progressively throughout the experiment and reached values of 6.92 and 7.17 after 20 days of storage, respectively (Table 2) (Fig. 3). For this species, the pH limit for consumption is about 7 (Huss, 1994). In this study, levels of pH 7 were exceeded after 12 days of storage at 8°C; on this day there were significant differences ($p<0.05$) in Barramundi. Increases in pH are related to the accumulation of basic substances, such as ammonia and TMA produced by microbial development muscle (Ruiz-Capillas and Moral, 2001a; Hebard *et al*., 1982). However, pH changes during refrigerated storage of fish differ according to the species of fish and other factors. Although this index is not very important, it can be used as a guide to the quality of fish (Huss, 1994; Ruiz-Capillas and Moral, 2001a).

Effect of temperature and storage day on the biogenic amines and amino acids: Results of two-way ANOVA (Table 2) indicated that temperatures and storage days did significantly ($p<0.05$) changed on the pH, TVB-N and microbial load of fish fillet. As shown in Table 2, the single and interaction effects of temperature did not significantly ($p>0.05$) affect

changes of amino acids glutamine, histidine and phenylalanine. However, the day of storage had significant ($p<0.05$) changed on those amino acids. Both independent variables had significant ($p<0.05$) effect on liberation of amino acids arginine and lysine, but their interaction did not significant ($p>0.05$) effect on their liberation. As illustrated by Montgomery (2001), the corresponding variable will be more significant ($p<0.05$) if the absolute F ratio became larger and P value became smaller.

CONCLUSION

The main and interaction effect of two independent variables (temperature and storage day) on microbial load and physicochemical properties of Barramundi fillets were study by two-way ANOVA. In general, this study showed that free amino acid and TVB-N formation were significantly ($p<0.05$) influenced by the main and interaction effects of independent variable. However, the single and interaction effects of temperature did not significantly ($p>0.05$) affect changes of amino acids glutamine, histidine and phenylalanine. The results of the present study also indicated that the physico-chemical and microbial property of Barramundi fillets increased after 12 and 8 days of storage at 0°C and 8°C, respectively.

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