1(3): 150-165 (2015) doi: 10.3153/JFHS15015

Journal of Food and Health Science

E-ISSN 2149-0473

ORIGINAL ARTICLE/ORİJİNAL ÇALIŞMA

FULL PAPER TAM MAKALE

EVALUATION OF BIOGENIC AMINE DEVELOPMENT OF ANCHOVY (*Engraulis encrasicolus*) MUSCLE COMPARED TO ITS QUALITY CHANGES AT DIFFERENT CHILLING CONDITIONS

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Received: 16.04.2015

Accepted: 02.06.2015

Published online: 12.06.2015

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Abstract:

This study investigates biogenic amine development (BA) *vs.* sensory, chemical and microbiological quality loss of anchovy (*Engraulis encrasicolus*) at three different chilled storage conditions which are commonly applied for fresh market and/storage prior to processing. The contents of BAs and sensory, chemical and microbiological quality parameters of anchovy were analysed daily during storage. Significant variations occurred ($p<0.05$) in the sensory, chemical and microbiological values among the storage groups. The highest shelf-life was found as 8 days for samples kept in chilled freshwater. Water-ice mix application resulted in lower TBA content indicating beneficial effect on its quality. Histamine results closely supported sensory values in terms of legally permitted levels set by US Food and Drug Administration (FDA). Histamine forming bacteria counts supported the formation of histamine in most groups while total bacteria counts were in agreement with sensory results in terms of acceptability. Moreover, some existing formulated indexes created from various BA levels were not found suitable to estimate spoilage degree of anchovy kept at these conditions. This study suggests that using ice and water-ice mix can improve shelf-life of anchovy stored at refrigerated temperatures in terms of food quality and safety. The results of this study can be used to guide seafood industry for testing fresh anchovy quality and safety at different chilled conditions.

Keywords:
Anchovy, Chilled storage, Chilled-freshwater, Sensory analysis, Histamine, Quality changes

Introduction

Anchovy is a pelagic species belonging to the *Engraulidae* family (Sahin and others 2008). It is one of the most significant captured species in the world fisheries consisting of about 10.4% of total captured marine fish in 2010 (FAO 2014). European anchovy (*Engraulis encrasicolus*) ranks the third amongst the common anchovy species with a production about 489.297 tons in 2012. It is ecologically and economically the most important fish for the Black Sea ecosystem (Sahin and others 2008; TUİK 2014).

Anchovy is a well liked and commonly consumed fish species of the world (FAO 2014). Although frozen storage is preferred for marketing of whole and/or headed and gutted anchovy, for longer distance and to extend shelf life to a long period up to a year, fresh market still exists, particularly in Turkey. Varying storage conditions are applied for anchovy prior to fresh market and processing in the world. Therefore, quality and safety in particular with histamine health risk of anchovy at these conditions are of interest. Its small size limits app-lication of mechanical pre-processing activities (i.e. heading, gutting and filleting). Therefore, manual processing is usually used for such applications resulting in longer handling and storage period of raw material before and during processing, even prior to freezing. Moreover, anchovy has a limited catching season with high volume production in a short period causing the most catch going to fish meal-oil factories. So, the majority of the world's anchovy catch is processed for fish meal-oil and the rest for non-human consumption (Eurofish 2012). Its small size makes it particularly susceptible to belly bursting, often produced by autolytical breakdown and the quality of processed anchovy closely depends on the initial quality of raw material (Soerensen and Motta 1989; Montaner and Zugarramurdi 1995; FAO 2014). For these reasons, effective storage applications to slow down spoilage is necessary to allow a longer processing and marketing period for this species.

Fish spoilage is mainly affected by bacterial activity, and other than sensory quality loss, some compounds, such as biogenic amines (BAs), can be formed by bacterial decarboxylation of precursor amino acids leading to food safety issues. Most reports have focused on histamine which is reported to cause scombrotoxin poisoning, closely linked to the consumption of fish kept at abused time/temperature conditions and species belonging to different families including *Engraulidae* due to high content of free histidine in their muscle (Lehane and Olley 2000; EU Directive 2005a). Other BAs, such as putrescine and cadaverine may enhance the toxicity effects of histamine. Several BAs are also known to associate with fish decomposition and the formation of cancerous nitrosamines (Lehane and Olley 2000; Pons-Sánchez-Cascado and others 2006a).

Previous studies demonstrated the advantage of refrigerated temperature or iced conditions over ambient storage of anchovy in terms of quality and BA formation for *E. encrasicolus* (Varlık and Heperkan 1990; Köse and Erdem 2004). However, short shelf-lives were obtained by either application. Moreover, dry fish surface is caused by cold air during refrigerated storage. Therefore, recent storage applications of fish prefer to use combination of ice in cold stores for fish preservation. The benefit of ice and/ice+saltwater application at cold stores was demonstrated by few studies mainly for different anchovy species from different seas (Soerensen and Motta 1989; Montaner and Zugarramurdi 1995; Köse and Erdem 2004; Pons-Sánchez-Cascado and others 2006a; Chotimarkorn 2011). However, no study exists on the quality changes of anchovy stored at the relevant conditions in comparison with its BA contents. Moreover, none of the mentioned studies covers the anchovy, *E. encrasicolus* caught from Black Sea. Therefore, this study aims to investigate the relationship between commonly used spoilage/quality parameters, and the levels of histamine and other BAs during storage of anchovy at different chilled temperatures.

Materials and Methods

Experimental design

Fresh anchovies were obtained directly from a fishing boat in the port of Rize, Turkey. The origin of fish was tracked as they were caught from the South-eastern Black Sea in January and kept in watertight expanded polystyrene boxes (EPS) in ice for 5h after catching and during transporting to the port. Three EPS boxes containing anchovies in ice, about 14-16kg each, were transferred to laboratory within 1h. Fish were immediately washed with tap water. Approximately 30 kg randomly chosen fish were divided into 3 groups weighing about 10 kg each (Fig. 1). The average length and

weight of fish were 11.89 ±1.46cm and 10.35 ±1.12g, respectively. Each group was kept in plastic containers (width: 62cm, length: 39cm, height: 33cm). Ice application on fish (for group IR) was carried out layer by layer. The containers were covered with plastic wrap (cling film). All samples were stored in a refrigerator at 4°C±1 (Arçelik, 8810NF, Turkey). The sample groups were;

(i) Control: Refrigerated fish without ice (CR): Storage in cold air

(ii) Iced storage in refrigerator (IR): Fish and ice ratio, (1:1 w/w)

(iii) Storage in chilled-freshwater (chilling supported with ice) at refrigerator (IFWR): Fish and freshwater-ice mix ratio, (1:1 w/w).

Sampling was carried out daily by randomly choosing anchovies until the products were spoilt based on sensory results. The ice was flake ice type obtained freshly from an ice maker (Hoshizaki, FM-80EE, Amsterdam, Netherlands). Ice and freshwater+ice mix were changed daily. The internal temperatures of fish in each group were measured on a daily basis using digital thermometer (Thermor PS100, Ontario, Canada). The refrigerator temperature was 4 ±1°C, fish temperatures were 4.1 ±0.7°C for CR, 1.8 ±0.6°C for IR, 1.2 ±0.4°C for IFWR.

Sensorial evaluation

Sensory analysis was carried out using eight experienced seafood quality assessors (panellists chosen from faculty staff) who judged the freshness of the samples using 10-point scale modified from Botta (1995) and Archer (2010). The panellists were comprised of 6 male and 2 females (5 academician and 3 administration staff). The subjects were qualified after passing the screening tests stated by Botta (1995). The panellists were chosen from previously trained 20 people who are regular anchovy consumers according to criteria given by Botta (1995).

Table 1 shows the sensory score sheets used to evaluate anchovy samples. This structured category scale is based on the traditional freshness quality grading system for whole iced and refrigerated anchovy. According to scale, 4 is limit for acceptable/unacceptable, <4: unacceptable. The results were presented as the means of data obtained from 8 panellists.

Triplicate samples (3-4 randomly selected fish) were taken at order intervals from each group for each day to evaluate their quality criteria at the sensory laboratory section. Fresh anchovy was also included in the evaluation starting from 2nd day to support the judgement of assessors as a reference sample (although the panellists were blind to its code). Different samples were simultaneously presented in plates coded. The panellists were expected to give the same score for the triplicated samples for the same group at the same sampling day. They were also expected to give the highest score for the blinded reference sample (freshly caught anchovy).

After the sensory analysis, the fish were used further for chemical analysis.

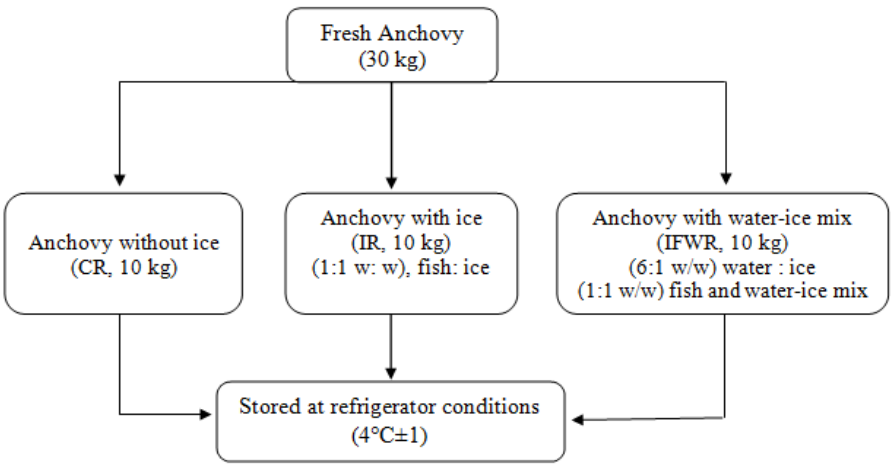


Figure 1. Experimental design

Table 1. Sensory score sheet used for fresh anchovy samples

Score	General Appearance					Odour	Texture
	Eyes	Gills	Skin	Flesh	Appearance of Abdomen (Belly Walls)		
10	Clear, transparent, Convex shape, Bright black and circular pupil	Bright red, clear (no slime)	Intact skin, Very bright colours, iridescence bluish violet, Transparent watery mucus	Velvety, translucent, glossy, fresh bright blood on fillet	Intact, firm	Seaweedy odour, peppery	Firm, stiff, smooth
9	Slightly convex, clear black pupil	Red, clear	Bright, slight iridescence, Clear mucus	Slight translucency, rosy, hue, bright blood on fillet.	No belly burst.	Slight loss of seaweedy odour, Oily	Loss of stiffness, still firm, smooth.
8	Flat, slightly convex, central opacity, dull black pupils	Red, slight brown, slight slime	Loss of brightness iridescence not bluish, Slightly cloudy mucus, slightly broken skin	Slight translucency, slight discoloration of belly flaps.	No belly burst or very slight belly burst.	Slightly seaweedy Oily	Less tense, slight softening, smooth.
7	Flat, slightly convex, central opacity, dull black pupils	Dull red, slight slime	Slightly dull, slightly broken skin, cloudy mucus	Slightly opaque, slightly brown, slight discoloration of belly flaps.	Slight belly burst.	Oily	Limp, slightly soft, slightly gritty.
6	Plane & flat eyes, slightly cloudy cornea, grey pupils	Brownish red, some slime	Dull, easy to break skin, cloudy mucus	Opaque, dull, brown, reddening on belly flaps. Pink	Slightly belly burst	Metallic, Neutral	Limp, slightly soft, slightly gritty
5	Flat, slightly sunken, slightly cloudy cornea, grey pupil, not so circular	Brownish red, discolouring, slime, bleached	Dull, Torn or damaged skin, cloudy mucus	Opaque, dull, brown, reddening on belly flaps. Pink	Definite belly burst.	Slightly rancid	Flaccid, soft (post rigor)
4	Concave, slightly sunken eyes, grey and distorted pupils, Cloudy cornea	Brownish red, Discolouring, slime, bleached	Dull, Torn or damaged skin, cloudy mucus	Brown, discoloured belly flaps and tail. Waxy, pink	Definite belly burst	rancid or sweet, acid, metallic	Flaccid, soft (post rigor)
3	Sunken eyes, grey pupils, cloudy cornea	Discoloured, Pink, bleached, brown slime, slats stick together	Dull colour, Torn and damaged skin, Plentiful (slippery) yellowish brownish mucus	Wax-like matt, dense, dark red colour (brownish colour)	Burst, very soft	Sour, stale blood	Flaccid, soft (post rigor)

Analysis of Chemical Quality parameters

The method of Lücke and Geidel (1935) was used to determine total volatile base-nitrogen (TVB-N) content as described by Goulas and Kontominas (2005). Thiobarbituric acid value (TBA) was estimated according to Smith and others (1992). The method of Boland and Paige (1971) was used for analyzing trimethylamine nitrogen (TMA-N).

Analysis of Biogenic Amines

Biogenic amines were analyzed using a high performance liquid chromatography (HPLC) method according to Köse and others (2012) and Koral and Köse (2012). HPLC equipment was Shimadzu Prominence LC-20 AT series (Japan) with autosampler (SIL20AC, Shimadzu, Japan), a Diode Array Detector (SPD-M20A, Shimadzu, Japan) and Intertsil column (GL Sciences, ODS-3, 5μm, 4.6×250mm). The HPLC method used was a modified method of Eerola and others (1993) and EU suggested methods (Malle and others 1996). To extract BAs, 10mL of 0.4M perchloric acid was added to 5g sample, and the mixture was homogenized using Ultra-turrax homogenizer (IKA T 25, Digital, Germany) in an ice bath and centrifuged (MPW 350R. MPW Med. Instruments, Warsaw, Poland) at 3,0009g at 4°C for 10min. The supernatant was collected, and the residue was extracted again with 10 mL of 0.4M perchloric acid solution. Both supernatants were combined and filtered through Whatman paper (No. 42). The final volume was adjusted to 25mL with 0.4M perchloric acid.

Each sample extract was mixed with 100μL of 2N sodium hydroxide and 150μL of saturated sodium bicarbonate. One millilitre of a dansyl chloride solution (10mg/mL) prepared in acetone was added to the mixture, mixed well and then incubated at 40°C for 45min and cooled down to room temperature in 10min. Then, the residual dansyl chloride was removed by the addition of 50μL 25% ammonia solution. After 30-min incubation at room temperature, the extract was adjusted to 5 mL with ammonium acetate: acetonitrile mixture (1:1 v/v) and mixed well with a vortex (Nuve NM 110, Ankara, Turkey). Extract was filtered through 0.45-μm-pore-size filters (Millipore Co., Bedford, MA, USA) and injected to HPLC. The gradient elution system was 0.1M ammonium acetate as solvent A and acetonitrile as solvent B. Gradient elution was initiated with 50% A and 50% B and terminated in 19 min with 90% B, run time 20 min. The system was equilibrated for 8 min before next run. The flow rate was 1.3mL/min), and 20μL sample was injected onto the column. Column temperature was 40°C, and amines were detected at 254nm.

The quality and biogenic amine index parameters were calculated as described by Koral and Köse (2012). All chemical analysis were carried out in triplicate sampling, and results were represented as means ±SD.

Bacterial analysis

Total aerobic viable bacteria counts (TVC) were enumerated using plate count agar according to Köse and others (2001). Twenty-five grams of samples was aseptically weighed into a sterile stomacher bag containing 225mL of sterile physiological saline (0.85%) and homogenised using a stomacher (Mayo, HG400V, Italy) for 4 min at the highest speed (4. step). Further decimal dilutions were prepared in physiological saline (0.85%). Total aerobic viable psychrotrophic and mesophilic microorganisms were counted using plate count agar incubated at 4°C for 8 days for psychrotrophic microorganisms and at 37°C for 48h for mesophilic microorganisms. Histamine-forming bacteria (HFB) were determined according to Yoshinaga and Frank (1982) using a modified Niven's medium (Niven and others 1981) by adjusting the pH to 6.5. Total HFB isolation agar contains 0.5% tryptone, 0.5% yeast extract, 2.35% L-histidine–HCl, 0.5% NaCl, 0.006% bromocresol purple, 0.1% $CaCO_3$ and 2% agar. Total mesophilic and psychrophilic HFB were determined using the same condition applied for TVB. Microbial counts were carried out in duplicate and expressed as log cfu/g. Triplicate samples were used for each type of analysis while each analysis was performed in duplicate. Counts of bacteria were expressed as log cfu/g. The results were presented as means of all counts ±SD.

Statistical analysis

All statistical analyses were performed in JMP software (Version 8, SAS Institute. Inc., Cary, NC, USA) (Sokal and Rohlf 1987). Analysis of variance (ANOVA) was used to compare the results within the groups as well as during storage period. When significant differences were found, comparisons among data were carried out by using Tukey test. A significant level of 95% ($p<0.05$) was used throughout analysis.

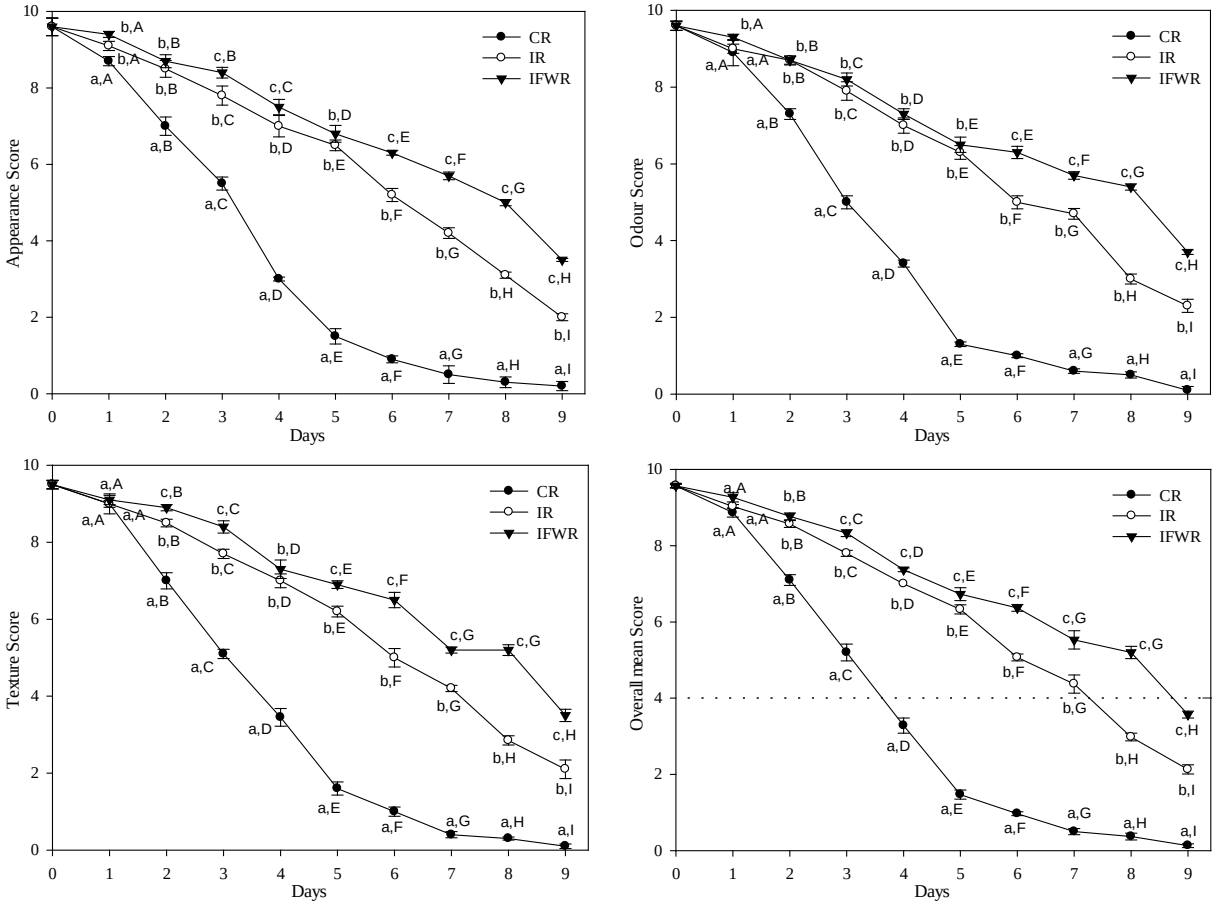
Other measurements

Moisture analysis was performed to calculate the weight gain of the samples. Moisture content was

determined by oven-drying of 5g of fish muscle at 105°C until a constant weight was obtained using the AOAC method (AOAC 1995, Method 985.14). Internal temperature of fish in each group was measured on a daily basis using a digital thermometer (Thermor PS100, Newmarket, Canada).

Results and Discussion

Figure 2 represents sensory results of all storage groups with the exception of reference sample. The panellists' scores for appearance, texture and odour (flavour) significantly decreased with time ($p<0.05$) for all groups. Significant changes occurred between the control group and others starting from the 1st day of storage ($p<0.05$) for each sensory parameter. Scores for appearance significantly varied between group containing ice (IR) and chilled freshwater with ice (IFWR) starting from 3rd day until the end of storage period ($p<0.05$). A similar situation occurred for overall sensory data. The variation in the scores of texture and odour were found significant amongst the groups within the same day throughout storage with some exceptions ($p<0.05$). According to overall sensory data, control group spoilt on the 4th day with a dull appearance and dryness on the surface. Using ice extended the sensory life for 4 days. Application of chilled freshwater using ice further improved the quality attributes for one more day. Therefore, the best quality attributes were observed for the group in chilled freshwater with 8 days of shelf-life. Different storage conditions were reported in literature for anchovy stored at different conditions including ambient and chilled conditions (Varlık and Heperkan 1990; Abbey 1998; Köse and Erdem 2004).



'4' limit for acceptability/unacceptability, Different lowercase letters represents statistical differences among groups ($p<0.05$).

Different uppercase letters represents statistical differences amongst different days within the same group during storage ($p<0.05$).

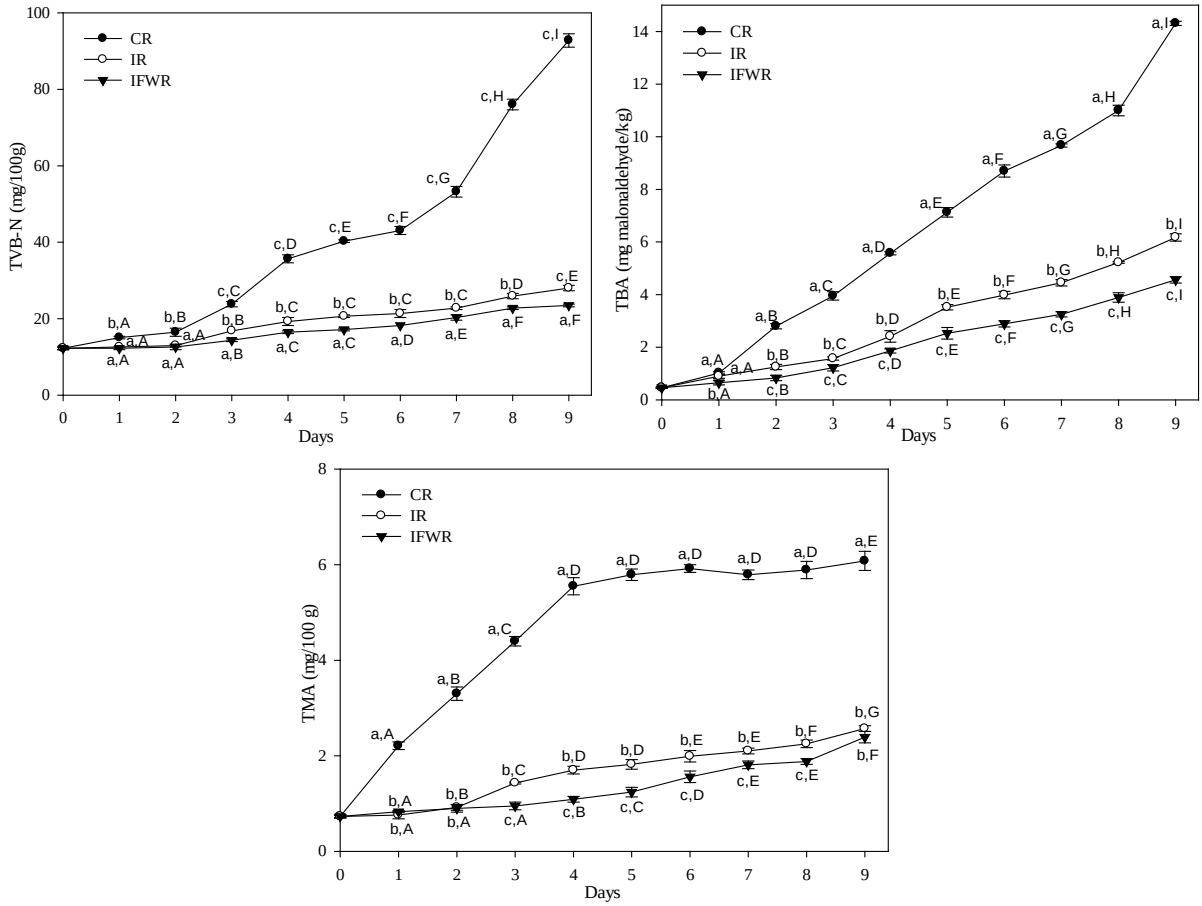
CR: Control, IR: Anchovy in ice, IFWR: Anchovy in water-ice (chilled-freshwater)

Figure 2. Sensory values of different anchovy storage groups

Figure 3 shows chemical quality changes of anchovy stored at three different conditions in refrigerator. The values of TVB-N increased significantly with increasing storage time for all groups ($p<0.05$). Significant differences were also observed between control and the experimental groups starting from the 1st day of storage, and between group containing ice and group kept in chilled freshwater starting from the 2nd day of storage period ($p<0.05$).

Varying levels of TVB-N have been suggested for different fish products to assess their freshness in literature (Connell 1990; Huss 1988). The European Union sets varying TVB-N limits as 25-35 mg/100 g for unprocessed fishery products which shall be regarded as unfit for human consumption where organoleptic assessment has raised doubts as to their freshness (EU Directive 2005b & 2008). However, anchovy is not included in EU regulation. Therefore, TVB-N levels can be used only in support of sensory values.

According to TVB-N values, the control group reached the spoilage limit on the 4th day which was also supported by the sensory results. The experimental groups (IR and IFWR) were within the acceptable level throughout the storage period. On the other hand, anchovy kept in chilled freshwater conditions had significantly lower TVB-N values than the group stored in ice ($p<0.05$). The TVB-N results also indicated that using ice and chilled freshwater at refrigerated temperature for anchovy storage have the advantage on the slowing down TVB-N development while prolonging the storage life. Similarly, Castañón and Barral (1990) demonstrated low TVB-N values for anchovy (*E. anchoita*) stored in ice kept at 0°C and chilled seawater. Our results were in accordance with the observation of Careche and others (2002) for the same species. These authors observed lower TVB-N values for anchovy stored in EPS boxes containing chilled freshwater with ice in comparison with the group kept in ice without water.



TVB-N:Total Volatile Basic Nitrogen, TBA: Thiobarbutiruc acid, TMA: Trimethyethylamine, Different lowercase letters represents statistical differences among groups ($p<0.05$). Different uppercase letters represents statistical differences amongst different days within the same group during storage ($p<0.05$). CR: Control, IR: Anchovy in ice, IFWR: Anchovy in water-ice (chilled-freshwater).

Figure 3. The changes of TVB-N, TBA, TMA during storage of different anchovy storage groups

Trimethylamine is a pungent volatile amine often associated with the typical "fishy" odour of spoiling seafood. Its presence in spoiling fish is due to the bacterial reduction of trimethylamine oxide which is naturally present in the living tissue of many marine fish species. Although TMA is believed to be generated by the action of spoilage bacteria, the correlation with bacterial numbers is often not very good (Huss 1995). A suggested acceptable level is reported as 12 mg/100 g (Goulas and Kontominos 2005). Initial TMA-N values were 0.7±0.0 mg/100g and significantly increased throughout storage period for all experimental groups with some exceptions (Fig. 3). Significant variations observed between control and other groups starting from the 1st day of storage, and between the experimental groups (i.e. IR and IFWR) starting from the 3rd day until the end of storage period ($p<0.05$). The lowest TMA-N values were found for the group in chilled freshwater while the highest was obtained for the control group. The values were within the acceptable levels for TMA throughout the storage. Therefore, TMA values did not support the sensory results for the control group.

TMA values obtained in this study were close to the findings reported by Careche and others (2002), Köse and Erdem (2004) and Pons-Sánchez-Cascado and others (2006b) for anchovy stored at similar chilling temperatures. However, higher TMA values were reported by Veciana-Nogues and others (1990), and Mol and others (2007) for this species stored in refrigerator. The differences might have caused due to different initial handling procedures prior to refrigeration. Higher TMA-N values were also obtained for different anchovy by Chotimarkorn (2011) and Rodtong and others (2005).

The TBA is a by-product of lipid oxidation and represents the degree of rancidity in products, is used to determine the quality of fish, particularly fatty fish. TBA value in good quality chilled fish is reported between 5 and 8 mg malonaldehyde/kg whereas levels of 8 mg malonaldehyde/kg flesh are generally regarded as the limit of acceptability for most species Schormüller (1969). The values of TBA increased significantly depending on time and significant variations were also observed between control and the other groups ($p<0.05$) (Fig. 3). The control group reached an unacceptable limit on the 6th day while others were within the levels of good quality. Therefore, TBA values supported histamine levels set by EU regulation. The lowest value was found for IFWR indicating the advantage of using iced freshwater at refrigerated storage. The results of Carache and others (2002) supported our findings. In our earlier study, TBA levels were unacceptable value on the 5th day at refrigerated storage (Köse and Erdem 2004).

Initial counts of mesophilic and psychrotrophic total viable bacteria, and total mesophilic and psychrotrophic histamine forming-bacteria (HFB) were 3.4 ±0.1, 2.9 ±0.1, 2.6 ±0.1 and 2.9 ±0.1 log cfu/g, respectively. The values significantly changed during storage ($p<0.05$) for all groups (Fig. 4). Bacteria growth was faster for the control group. It could be ascribed that application of ice and/or chilled-freshwater during storage can reduce the microbial growth in a great extent. Although there were significant differences within all groups for the bacteria counts ($p<0.05$), the differences were found lower between groups stored in ice and chilled freshwater with ice with the lowest counts representing samples kept in chilled water. Castañón and Barral (1990) also obtained lower bacteria counts for samples stored at chilled-seawater in comparison with the samples kept ice. Lower counts were attributed by the washing effect of water. This could also explain the low bacteria numbers in our results.

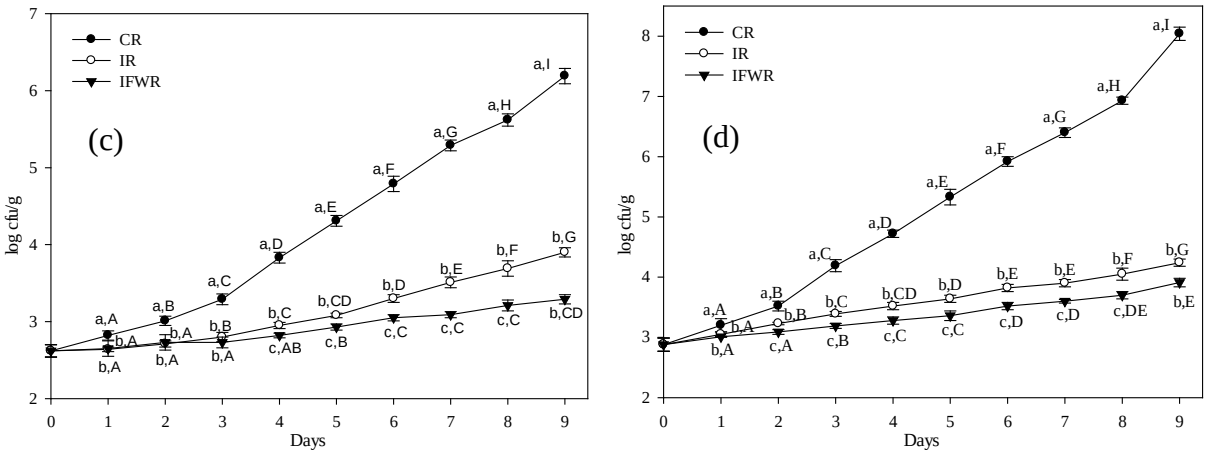
Initial bacteria counts can vary depending on season and region of fish caught, and handling conditions (Careche and others 2002). Therefore, previous authors reported varying levels in bacteria counts for anchovy caught at different seas (Castañón and Barral 1990; Ayala and others 2001; Careche and others 2002; Pons-Sánchez-Cascado and others 2006a; Chotimarkorn 2011) and some of them supported our findings. The recommended TVC limit for fresh fish consumption is reported between 6–7 log cfu/g (ICMSF 1992; Chotimarkorn 2011). TVC exceeded the recommended value on the 5th and 6th days for psychrotrophic bacteria and mesophiles, respectively for control group while the levels were below the suggested limit for the group stored in chilled freshwater throughout the storage. Mesophilic counts for the group stored in ice were below 6 log cfu/g on the 9th day of storage, the levels were unacceptable for psychrophiles on the 8th day. Sensory results supported microbial counts only for psychrotrophic bacteria for group kept in ice. For the other groups, samples were unacceptable depending on organoleptic judgment by the panellists despite the microbial counts were still below the suggested limits. Although Mol and others

(2007) obtained higher psychrotrophic and lower mesophilic bacteria growth for anchovy stored at refrigerated storage, their results indicated that estimation of the psychrotrophic microorganisms gives better results to the shelf-life estimation of chilled fish than mesophilic bacteria which also supported by our findings.

Both mesophilic and psychrotrophic TVC supported TVB-N and TBA values for IFWR group. However, none of the values correlated with TMA-N values in terms of rejection limits of TVC obtained for all groups. Chaouqy and Marrakchi (2005) obtained lower initial bacteria counts at ice storage of anchovy kept also at refrigerator followed by lower bacteria growth. Faster psychrotrophic bacteria growth at refrigerated storage was observed by Köse and Erdem (2004).

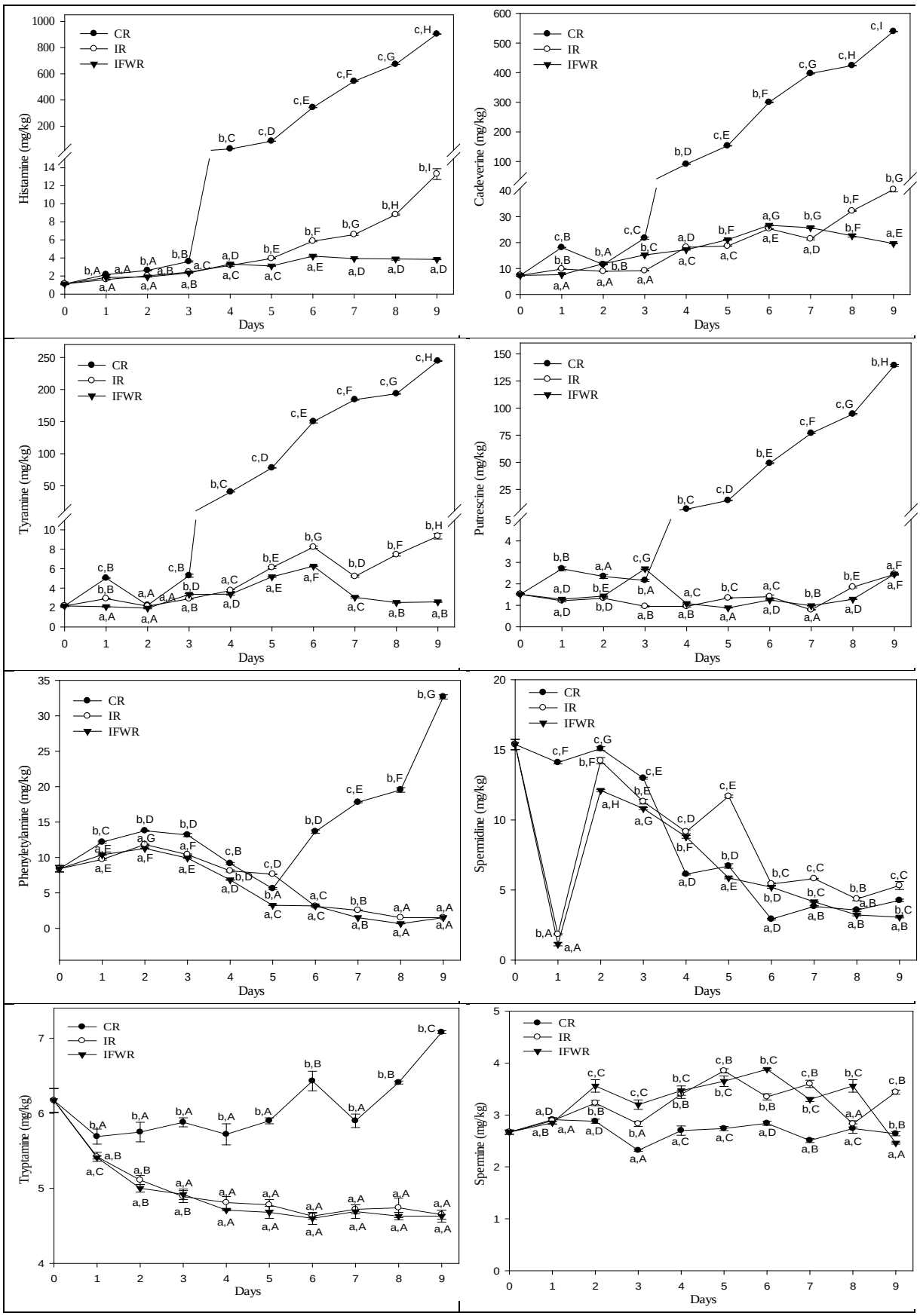
Various histamine-forming-bacteria (HFB) are reported including mainly members of the genera *Klebsiella, Morganella, Vibrio, Photobacterium* and others (Lehane and Olley 2000; Rodtong 2005; Köse 2010). Initial HFB in fish is important since previously formed histamine decarboxylases can continue to decarboxylase histidine to histamine even when histamine decarboxylase positive bacteria are no longer viable (Köse 2010). Initial HFB counts were 2.6±0.1 log cfu/g for mesophiles and 2.9±0.1 log cfu/g for psychrotrophic bacteria. The counts also increased significantly throughout the storage period for all experimental groups ($p<0.05$).

Figure 5 represents the results of biogenic amines (BAs). Significant variations occurred in the values of BAs depending on storage life and the groups with some exceptions ($p<0.05$). With the exception of spermidine and spermine, the levels of BAs significantly increased for control group throughout the storage period ($p<0.05$). Among BAs, only histamine is regulated for certain fish and fisheries products. The European Regulation permits up to 100 ppm for fresh and processed fish, and 200 ppm for fishery products which have undergone enzyme maturation treatment in brine (EU Directive 2005a) while FDA allows less than 50 ppm (FDA 2011). Anchovy is in the list of regulated fish species due to its high histidine content (Veciana-Nogués and others 1996). At the beginning of storage trial, histamine level was 1.1±0.1ppm and increased significantly throughout the storage ($p<0.05$). Histamine values reached an unacceptable level set by FDA on the 5th day as 85.4 ±0.6 ppm and by EU and 6th day as 342.9 ±1.5 ppm for control group. The values were below 14 ppm for the group kept in ice and <4 ppm for the group stored in chilled freshwater at the end of storage. These levels are well below the permitted levels set by various authorities.



Different lowercase letters represents statistical differences among groups ($p<0.05$). Different uppercase letters represents statistical differences amongst different days within the same group during storage ($p<0.05$). CR: Control, IR: Anchovy in ice, IFWR: Anchovy in water-ice. a) Total viable mesophiles b) Total viable psycrophiles c) Total mesophilic histamine forming bacteria d) Total psycrophilic histamine forming bacteria

Figure 4. Microbial changes of anchovy muscle during storage



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CR: Control, IR: Anchovy in ice, IFWR: Anchovy in water-ice (chilled-freshwater)

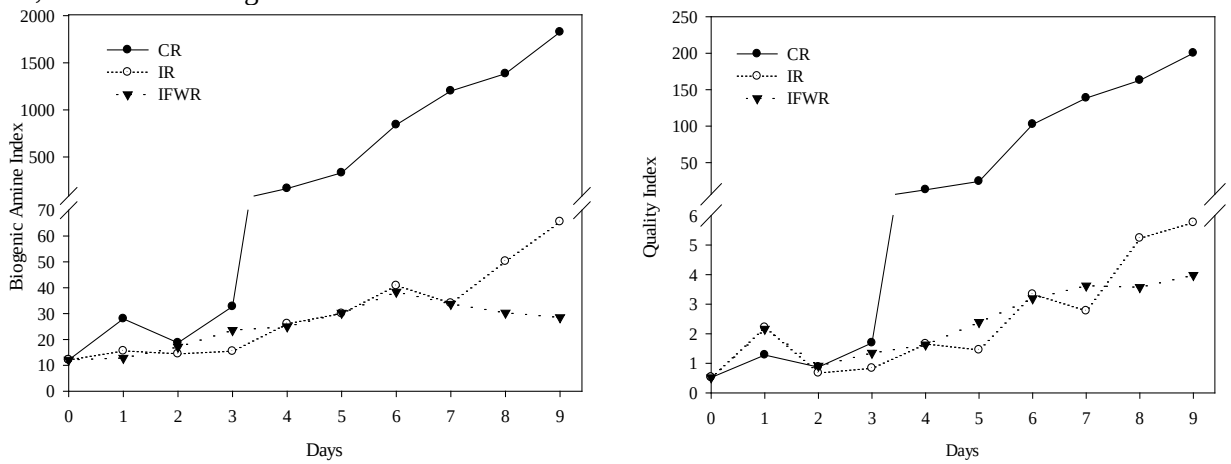
Figure 5. The changes in the levels of biogenic amines for anchovy storage groups

The results of Chaouqy and Marrakchi (2005), Rodtong and others (2005), and Pons-Sánchez-Cascado and others (2006a) also supported our findings in terms of low histamine values developed in anchovy kept in ice at a refrigerated room. However, Chotimarkorn (2011) found higher histamine levels at the same conditions for a different anchovy species (*S. heterolobus)* from Thailand indicating the different storage behaviour of different anchovy species. Unacceptable histamine values were reported within 1-3rd day of refrigerated storage by different studies (Veciana-Nogués and others 1990; Köse and Erdem 2004) indica-ting the benefit of combining refrigerated storage conditions for this species with ice and/or chilled water applications. Therefore, storing fish in ice or in chilled water while in refrigerated or chilled rooms is recommended to delay histamine formation prior to processing or fresh fish market.

Significant decrease in the levels of spermidine occurred for all groups ($p<0.05$). A similar trend also occurred for the contents of phenethylamine, tyramine and tryptamine for the experimental groups only, while fluctuations were observed in the values of cadaverine, putrescine and spermine for these groups throughout the storage period ($p<0.05$). The levels of these BAs were usually below 10 ppm at the end of storage period. Pons-Sánchez-Cascado and others (2006a), and Chotimarkorn (2011) found similar trends for the most BA levels in anchovy stored under similar iced storage conditions.

Some food migraines are related to BAs, particularly tyramine and phenylethylamine. Moreover, some BAs, mainly histamine, tyramine, putrescine, cadaverine and agmatine can be indicators of freshness or spoilage in fish: 100-800 ppm of tyramine and 30 ppm of phenylethylamine have been reported to be toxic doses in foods, respectively (Koral and Köse 2012). Our study showed that the levels of tyramine and phenylethylamine only reached toxic levels for control group on the 6th and 9th days, respectively. Therefore, the results show the advantage of using ice and chilled freshwater application during refrigerated storage to slow down various BA development in anchovy. Therefore, the benefit also applies to seafood safety by preventing histamine development in anchovy.

Biogenic amines are also suggested being used for the evaluation of fish spoilage and different indexes were reported by previous authors for various fish species (Mietz and Karmas 1977; Veciana-Nogues and others 1997). Pons-Sánchez-Cascado and others (2006a) studied the suitability of a BA index (BAI) to evaluate the freshness of anchovies stored in ice by comparison of different reported criteria. They suggested an acceptability limit of BAI for anchovy stored in ice as 15 ppm. In the present study, initial BAI of anchovy was 12.1 and quality index (QI) was 0.5 (Fig. 6). The values increased throughout storage. However, fluctuations in both BAI and QI values occurred for all sample groups. Decreasing levels for BAI seems to be affected by the decreasing levels of tyramine. Fluctuations in QI levels were also affected by the variations in BAs used in the calculations (Mietz and Karmas 1977; Veciana-Nogues and others 1997).Various other researchers obtained different levels of amine indexes in relation to spoilage for different fish species as revised by Al-Bulushi and others (2009).



CR: Control, IF: Anchovy in ice at refrigerator, IFWR: Anchovy in water and ice mixture at refrigerator

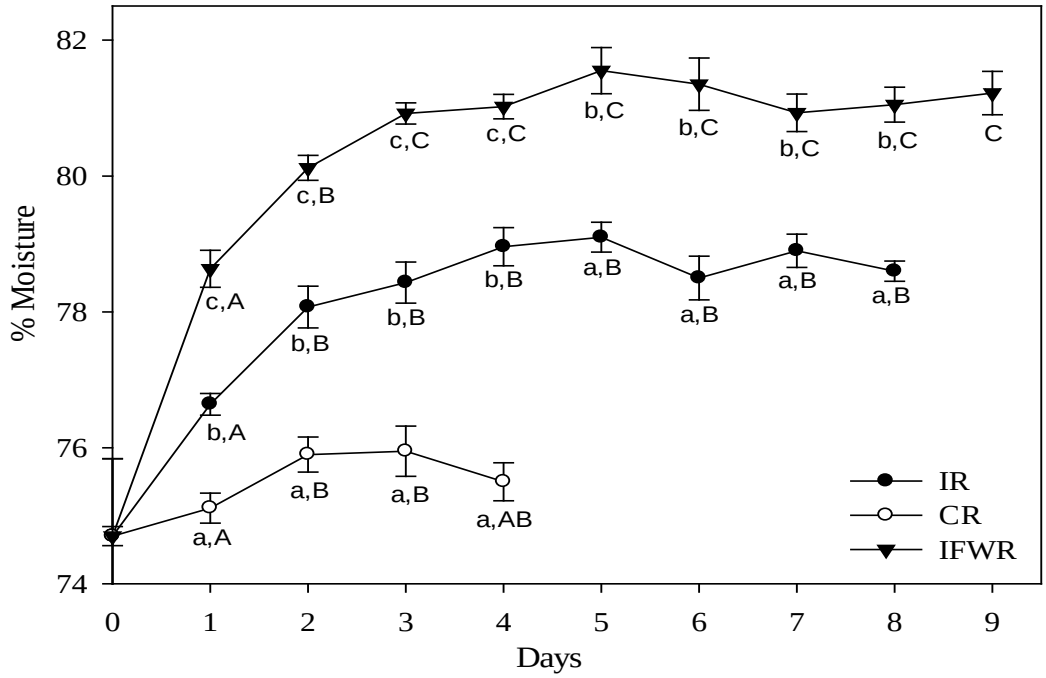
Figure 6. The results of BAI and QI for different anchovy storage groups

Sensory values closely supported histamine results since histamine levels were found below the regulated limits for the samples within the sensory shelf-life. Therefore, the results of the study seem to indicate that sensory evaluation of fresh anchovy might help to avoid histamine health risk which would arise from this species. However, it is known that histamine content can greatly vary in the final products depending on the initial bacteria load and type of bacteria which decarboxylase histamine. Thus, sensory values should be used with caution in the case of histamine health risk (Köse 2010). On the other hand, further studies with different anchovy batches may help to confirm our findings.

The levels of tyramine and phenylethylamine were also well below the toxic levels within the shelf-life of storage period for all groups. However, our results showed that the values for both QI and BAI do not correlate with sensory values. We also obtained a similar conclusion with bonito stored at various chilled conditions (Pons-Sánchez-Cascado and others 2005a). The reason might have caused by the possibility of non-spoilage bacteria involving in the formation of different BAs, which are used in the calculation. Therefore, the present study showed that using BAI and QI to estimate spoilage degree was not found suitable for anchovy samples stored at three different chilled conditions.

TVB-N values were also in support of histamine levels in terms of safety limits set by FDA. Al-Bulushi and others (2009) reported that mesophilic bacterial count of log 6–7 cfu/g has been associated with 50 ppm histamine. The suggested level only reached by the control group on the 6^{th} day. However, the results did not support histamine values since the level obtained was 342.9 ppm at the relevant storage time. An increase in histamine corresponded with the outgrowth of histamine-forming bacteria for the control group. The counts of mesophilic and psychrotrophic HFB were 4.3 ±0.1 and 5.3 ±0.1 log cfu/g, respectively for control group on the day when histamine exceeded the rejection level set by FDA. The counts were 4.8 ±0.1 and 5.9 ±0.1 log cfu/g, in the same order on the day when histamine exceeded the EU permitted value. However, the increasing rate for histamine formation was higher in comparison with growth of HFB for control group. The opposite situation was observed for other groups. The counts of psychrotrophic HFB were found higher in comparison with those of mesophiles indicating the effect of favourable chilled conditions supporting growth of psychrophilic bacteria.

Fish immersed in ice and water gains weight at first, and then slowly lose weight during subsequent storage. Weight gain depends on species and a number of other factors. A gain of 2 to 5% is accepted as normal for most species after a period of 1-2 weeks. The problem of water uptake is less critical with fatty fish (Graham and others 1992). Figure 7 represents the moisture contents of all groups demonstrating significant differences in moisture contents during storage period and also within the groups ($p<0.05$). Significant water uptake occurred at first for the samples stored in ice and chilled-freshwater mixture for the first 3 days ($p<0.05$) and then gradually stabilized during storage period. The water uptake seems to be higher for IFWR as 6.05% in comparison to IF as 3.7%. According to these results, the weight gain was within normal limits although a little over for the IFWR group. Control group had the lowest moisture contents during the storage period. However, this group also had significant water uptake during storage for the first 2 days ($p<0.05$) and then a gradual drop occurred which may be attributed to dryness caused by refrigerated air.



Different lowercase letters represents statistical differences among groups ($p<0.05$). Different uppercase letters represents statistical differences amongst different days within the same group during storage ($p<0.05$). CR: Control, IR: Anchovy in ice, IFWR: Anchovy in water-ice.

Figure 7. The changes in the levels of moisture contents for anchovy samples kept at various storage conditions.

In conclusion, this study demonstrates the quality changes of anchovy at different cold storage conditions in comparison with development of BAs in regarding to food safety. The results suggest that using ice and/or chilled-freshwater can improve the shelf-life of anchovy stored at refrigerated temperatures relating to food quality and safety. Good sensory quality was observed for fresh anchovy with the addition of ice and/or chilled-freshwater mixture during refrigerated storage. Chilled-freshwater with ice application increased the shelf-life for 8 days which is the highest shelf-life obtained for fresh anchovy. The worst sensory results represented anchovy without ice with a shelf-life less than four days. Storing fresh anchovy in chilled-freshwater also helped to improve its chemical quality and decrease biogenic amine development. Therefore, keeping fresh anchovy in ice and chilled freshwater during refrigerated storage is advised to delay histamine formation and retard quality loss prior to processing or fresh market. Histamine-forming bacteria counts supported histamine formation in most groups while total bacteria counts were in agreement with sensory results in terms of acceptability. Although sensory values supported histamine development for all storage conditions of anchovy, sensory criteria should be used in caution to avoid histamine health risk since histamine values also depends on initial bacterial load and types of HFB. Moreover, using BAI and QI parameters to estimate spoilage degree was not found suitable for anchovy samples stored at various chilled conditions.

Acknowledgements

This study was supported by Karadeniz Technical University Research Fund under the KTU-BAP project No.2006.117.001.4. We also acknowledge the help of Dr. George M. Hall, for correcting the English of this manuscript.

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