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Research article

Optimal level of added salt to minimize the risk of associated chemical hazards of small-sized anchovy (*Stolephorus indicus*) stored at ambient and refrigerated temperatures

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Abstract

Importance of the work: Practical protocol for post-harvest handling of small-sized (less than 12 cm) anchovy by means of chilling and salting is needed to minimize the risk of associated chemical hazards.

Objectives: The objective of this study was to establish the optimal level of added salt for anchovy (*Stolephorus indicus*) stored at different temperatures.

Materials & Methods: Changes in water phase salt (WPS) and quality of anchovy (7.99 ± 0.38 cm in length) salted at 2%, 4%, 6%, 8%, 10% and 12% w/w, were monitored during 5 hr post salting at ambient temperature (33 ± 3°C) and refrigerated temperature (4 ± 1°C).

Results: WPS levels of 10% or higher were achieved when the level of added salt was 8–12% w/w, while water activity values of 0.94 or lower were achieved when the level of added salt was 6–12% w/w for anchovy stored at both temperatures ($p < 0.05$). The time ranges practically required to reach 10% WPS during refrigerated storage of anchovy salted at 8–10% w/w and 12% w/w were 4 hr and 2 hr, respectively. After 5 hr of storage, salting anchovy at 8–12% w/w either stored at ambient or chilled temperature resulted in a histamine content below 2 mg/100 g and total volatile base nitrogen and trimethylamine contents below the limits of acceptability for human consumption.

Main finding: It could be concluded that the combination of a prescribed salt level of 8–12% w/w and refrigerated storage of anchovy being used as a raw material provided a high level of assurance that the microbial growth and chemical changes could be retarded.

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Introduction

Anchovies are a popular marine fish typically used as a raw material for production of dried salted products (Joint FAO/WHO Codex Alimentarius Commission, 2003; Siriskar et al., 2013; Chotimarkorn, 2014; Abraha et al., 2017) and fish sauce (Yongsawatdigul et al., 2004; Rodtong et al., 2005; Siringan et al., 2007; Joint FAO/WHO Codex Alimentarius Commission, 2018). In general, two potential chemical hazards of concern when using anchovy as a raw material are scombrotoxin (histamine) and botulinum toxin, where scombrotoxin is mostly associated with the consumption of certain fish prey that contain high levels of free histidine and in addition, when fish are subjected to temperature abuse during storage, bacterial decarboxylation of histidine leads to histamine formation (Magro et al., 2020; Visciano et al., 2020). Botulinum toxin is associated with the *Clostridium botulinum* bacterium that has long been reported to be distributed widely in marine environments such as mud, sand, seabed soil, sea water, intestinal tracts and gills of fish (Tanasugarn, 1979; Huss, 1980; Lalitha and Gopakumar, 2000).

Therefore, chilling is usually used to slow microbial growth and biochemical changes before an adequate salt treatment can be applied at the processing facility (Food and Agricultural Organization of the United Nations/World Health Organization, 2020). However, microbial growth and biochemical reactions start developing immediately after catching (Yu et al., 2018; Duarte et al., 2020), a process that is referred to as ripened or fermented fish (Zang et al., 2019). Similar to other fish caught, fresh anchovy usually are not processed within a short time post-harvest, resulting in non-compliant final products with reported histamine accumulation (Yongsawatdigul et al., 2004; Magro et al., 2020). In such cases, it was found that the changes in the biogenic amines during fish sauce fermentation were trivial, indicating that the raw material, rather than a fermentation process, was the major source of biogenic amine accumulation in the final product (Yongsawatdigul et al., 2004). Fish as raw materials are recommended to be chilled as soon as possible after being caught, either by being stored at 3°C or below or stored at 3–10°C in combination with salting to ensure the water phase salt (WPS), which is a concentration of salt at 10% or higher in the water portion of the fish flesh that controls microbial pathogens, scombrotoxin fish poisoning and decomposition (Food and Agricultural Organization of the United Nations /World Health Organization, 2020; U.S. Food and Drug Administration, 2020).

Anchovy post-harvest handling is mostly limited to refrigeration by either icing on board the fishing vessel (Yongsawatdigul et al., 2004; Rodtong et al., 2005; Czerner and Yeannes, 2010, 2013; Czerner et al., 2011; Chotimarkorn, 2014; Abraha et al., 2017; Rojas-De-Los-Santos et al., 2018) or icing at the landing site (Siriskar et al., 2013). However, post-harvest data are limited on the quality changes of refrigerated anchovy and thus inconclusive, especially when icing is conducted in combination with salting. A few studies on the effects of storage temperature revealed that anchovy (*S. heterolobus*) stored at 4°C with ice had a lower level of histamine than ones stored without ice (Chotimarkorn, 2014) and anchovy (*S. indicus*) was found with a high level of histamine when exposed to temperature abuse during storage (Yongsawatdigul et al., 2004; Rodtong et al., 2005). Apart from icing, salting is a process where the water content is reduced by the penetration of salt, resulting in ending or slowing down the activity of most of the spoilage bacteria (Turan et al., 2007; Czerner and Yeannes, 2010, 2013). Salting of anchovy could be done by either dry salting (Rodtong et al., 2005) or wet salting (brining) (Czerner and Yeannes, 2010, 2013; Rojas-De-Los-Santos et al., 2018). Rapid changes were observed in the salt and water contents during the first 5–10 hr of an extended time length post-brining of 50 hr (Czerner and Yeannes, 2010) and 60 hr (Czerner and Yeannes, 2013) for anchovy (*Engraulis anchoita*) that were affected by the cut type (Czerner and Yeannes, 2010), lipid content and salting temperature (Czerner and Yeannes, 2013). To date, despite some available information on the evolution of the salt and water contents post-brining of anchovy with a size length larger than 12 cm, such as *E. anchoita* (Czerner and Yeannes, 2010, 2013), this information is still lacking in smaller-sized (less than 12 cm) anchovy, such as *S. indicus,* in which no evisceration step is needed when being used as a raw material (Food and Agricultural Organization of the United Nations/World Health Organization, 2020).

Therefore, the present study monitored the WPS evolution and quality changes during 5 hr post-salting of anchovy (*S. indicus*) raw material to establish the optimal level of added salt to minimize the risk of associated chemical hazards of the anchovy. This information is needed to establish a practical protocol for post-harvest handling of anchovy (*S. indicus*) raw material by means of chilling and salting.

Materials and Methods

Chemical reagents

Ammonium thiocyanate, boric acid, bromocresol green, ferric alum, formaldehyde solution 35%, magnesium carbonate, methyl red, potassium carbonate, sodium chloride and trichloroacetic acid were obtained from Ajax Finechem (North Ryde, NSW, Australia). Histamine analytical standard, ion exchange resin (Dowex1-x8, 50–100 mesh), *O*-phthaldialdehyde (OPA) and silver nitrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol, nitric acid, hydrochloric acid, phosphoric acid and sodium hydroxide were obtained from VWR (Radnor, PA, USA). All chemicals used in this study were of analytical grade.

Sample preparation and experimental design

Anchovy (*S. indicus*) were captured in the Gulf of Thailand. Fish were iced immediately on board and transferred from Chonburi province to the laboratory facility of the Department of Fishery Products, Faculty of Fisheries, Kasetsart University within 2 hr. The average body size of the fish was approximately 7.99 ± 0.38 cm in length (Fig. 1). Therefore, no evisceration step was needed (Food and Agricultural Organization of the United Nations/World Health Organization, 2020). The protein, lipid and ash contents were determined following official methods of analysis numbers 981.10, 922.06 and 920.153, respectively (Association of Official Analytical Chemists International, 2016). Upon arrival, the fish were allocated into two lots. The first lot was left at ambient temperature (AT, 33 ± 3°C), the average tropical surrounding temperature at the time of the study, while the second lot was continually maintained at refrigerated temperature (RT, 4 ± 1°C). Each lot was divided equally into three replicates. Each fish was rubbed with dry salt (sodium chloride) at various concentrations: 0% w/w (weight by weight, the control), 2% w/w, 4% w/w, 6% w/w, 8% w/w, 10% w/w and 12% w/w. Fish samples were taken at each time interval to be analyzed for salt content, moisture content, water activity, pH, histamine, total volatile bases nitrogen (TVBN) and trimethylamine (TMA) contents.

Fig. 1 General appearance of fresh anchovy (*Stolephorus indicus*), scale in centimeters

Determination of salt content, moisture content and water phase salt

The salt content was determined following the official method of analysis number 937.09 (Association of Official Analytical Chemists International, 2012). In brief, each 2 g fish sample was added with 10 mL 0.1N silver nitrate and 20 mL concentrated nitric acid. The mixture was placed on a hot plate for 15 min and then left to cool before mixing gently with 50 mL distilled water and 2 mL ferric alum indicator. This mixture was titrated with 0.1 N ammonium thiocyanate until the color turned to a permanent brownish-red. The percentage of salt was calculated.

The moisture content was measured according to the official method of analysis number 931.04 (Association of Official Analytical Chemists International, 2016). In brief, each 5 g fish sample was placed in the oven at 105°C until the weight was constant.

WPS was defined as the concentration of salt in the water portion of the fish flesh and calculated using Equation 1:

$$\%WPS = [\%NaC1/(\%NaCl(1) + \%\ Moisture\ content)] \times 100 \quad (1)$$

Determination of pH and water activity

Each fish sample was 10-fold diluted with distilled water and homogenized using a disperser (T25 digital Ultra-Turrax®; IKA; Staufen, Germany). The pH of the sample was measured using a pH meter (MM-60R; TOA DKK; Tokyo, Japan) at room temperature. The water activity of the sample was measured using a Benchtop water activity meter (4TEV; AQUALAB; Pullman, WA, USA).

Determination of histamine content

The histamine content was determined according to the official method of analysis number 977.13 (Association of Official Analytical Chemists International, 2005). Briefly, each 5 g fish sample was homogenized with 20 mL methanol for 2 min and incubated in a water bath at 37°C for 15 min. The volume of the sample was adjusted to 50 mL with methanol and then filtered (Whatman No. 1). The filtrate was

loaded onto a column packed with Dowex1-x8. The eluents were derivatized using *O*-phthaldialdehyde. The intensity was measured using a fluorometer (FM109515; Barnstead Thermolyne Corporation; Dubuque, IA, USA) at excitation and emission wavelengths of 360 nm and 450 nm, respectively. The histamine contents in each sample was calculated from the standard curve prepared from a histamine analytical standard within the range 0.01–0.03 mg/100 g.

Determination of total volatile base nitrogen and trimethylamine contents

The TVBN and TMA contents were determined based on Conway microdiffusion assay (Conway and Byrne, 1933). In brief, each 2 g fish sample was mixed with 8 mL 4% trichloroacetic acid and incubated for 30 min at ambient temperature. Then, the mixture was filtered (Whatman No. 41). The filtrate was added with 10% formaldehyde to fix any ammonia present in the sample prior to TMA analysis. After saturated potassium carbonate was added into the outer ring of the Conway dish, the TVBN and TMA were released and diffused into boric acid solution in the inner ring after incubation for 60 min at 37°C. The sample was further titrated with 0.02N hydrochloric acid until the color changed from green to pink. The TVBN or TMA contents were calculated as milligrams per 100 g of fish sample.

Statistical analysis

Data were subjected to one-way analysis of variance using the SPSS software (Version 16; SPSS Inc.; Chicago, IL, USA). Comparison of means was done based on the Duncan′s multiple range test. Pearson′s correlation coefficient (r) was determined to express the relationship between WPS and other parameters consisting of moisture content, water activity, pH, histamine, TVBN and TMA. A test was considered significant at $p < 0.05$. Results were reported as the mean ± SD.

Results and Discussion

General quality characteristics of fresh fish

The general quality characteristics of fresh anchovy (*Stolephorus* sp.) are shown in Table 1. The proximate compositions of anchovy used in this study were consistent with the results reported for other fish species in the anchovy group, for example anchovies (*S. commersoni* and *S. indicus*) from the Mangalore coast of India (Siriskar et al., 2013) and anchovy (*E. anchoita*) from the coastal sector of Buenos Aires province (Massa et al., 2012). However, the lipid content (1.85 ± 0.04%) was clearly low compared with anchovy (*E. anchoita*) from the coastal sector of Buenos Aires province, Argentina (7.37 ± 1.93%; Massa et al., 2012), although it was in the range reported for *E. anchoita* (1.68–10.04%) in general (Czerner and Yeannes, 2013). The present results for moisture content were in the range reported for *E. anchoita* (72.34–79.56%; Czerner and Yeannes, 2013). Variation in fish proximate composition depends on several factors, such as age, sex, feed and season (Massa et al., 2012).

Water phase salt and water activity evolutions

Changes in the salt and moisture contents of the anchovy using different levels of salt and stored at two different temperatures are illustrated in Figs. 2 and 3, respectively. The salt contents increased rapidly in the beginning and gradually increased at a lower rate and then levelled off. In contrast, the moisture content decreased rapidly in the beginning and gradually decreased at a lower rate and then levelled off corresponding to the salt penetration profile. Generally, the salt penetration and moisture profiles from this study could be divided into three phases according to previous reports (Turan et al., 2007; Czerner and Yeannes, 2010, 2013). The first phase was a rapid salt penetration into the fish flesh and a removal of water during the first 120 min and 180 min post-salting for ambient storage samples and chilled storage samples, respectively. The second phase was a reduced salt penetration into the fish flesh and a removal of water during 120–180 min

Table 1 General quality characteristics of fresh anchovy (*Stolephorus indicus*)

Characteristic	Mean ± SD (n = 3)
Moisture content (%)	78.52±0.58
Protein (%)	16.81±0.11
Lipid (%)	1.85±0.04
Ash (%)	1.89±0.39
Salt (%)	0.19±0.09
Water activity	0.99±0.00
pH	6.56±0.01
Histamine (mg/100 g)	0.02±0.01
TVBN (mg/100 g)	20.16±1.34
TMA (mg/100 g)	1.12±0.07

and 180–240 min post-salting for ambient storage samples and chilled storage samples, respectively. The last phase was a ripening phase resulting from decomposition reactions such as lipolysis, lipid oxidation and proteolysis, during 180–300 min and 240–300 min post-salting for ambient storage samples and chilled storage samples, respectively. It was clear that the rate of salt uptake was more pronounced for a higher level of added salt and a higher storage temperature ($p < 0.05$). The salt penetration evolutions of anchovy used in this study (based on of dry salting) were completed quickly by 3 hr and 4 hr for ambient storage and chilled storage, respectively. These durations were much shorter than those reported in the previous studies. For examples, brining of anchovy (*E. anchoita*) with 26% sodium chloride solution, although having a similar profile of salt penetration evolution with the present study, the completion of the process took much longer (35–50 hr). Such a difference could be explained by differences in the size of the raw materials (7.99 ± 0.38 cm in the present study versus 13.6 ± 0.5 cm for *E. anchoita* in Czerner and Yeannes, 2010, 2013). The sizes of anchovies (family *Engraulidae*) were in the range of 5–10 cm for *S. heterolobus* (Chotimarkorn, 2014), 8 cm for *S. banganensis* and 14–16 cm for *E. anchoita* (Czerner and Yeannes, 2010, 2013; Massa et al., 2012) and 32 cm for *Setipinna brevifilis* (Rajan, 2018).

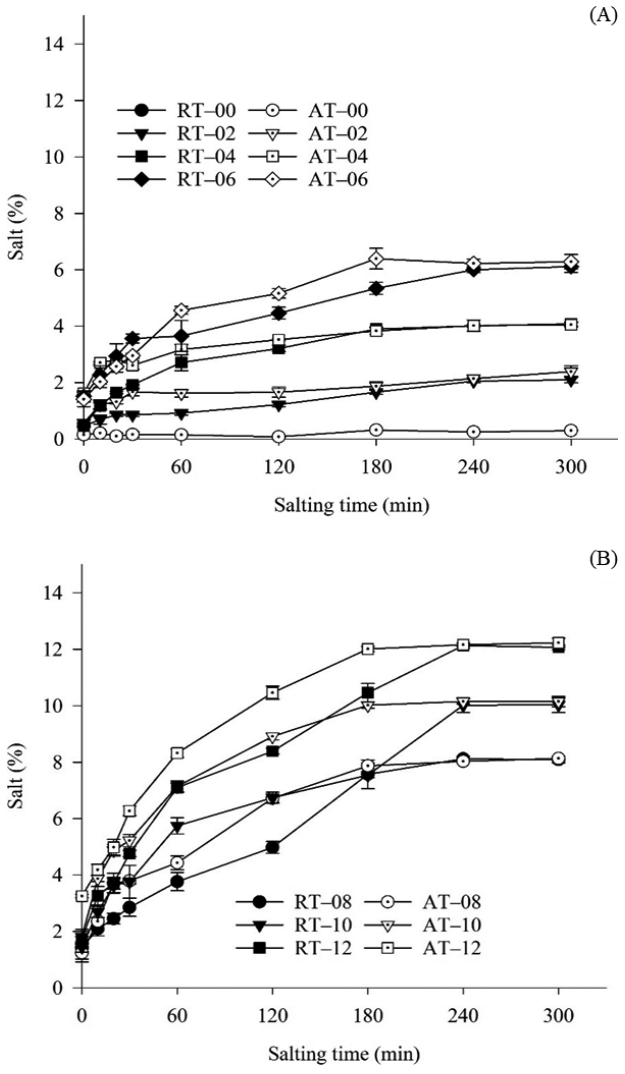

Fig. 2 Evolution of salt contents of anchovy stored at refrigerated temperature (RT, 4 ± 1°C) and ambient temperature (AT, 33 ± 3°C) in combination with salting at: (A) 0% (control), 2%, 4% and 6% w/w; (B) 8% w/w, 10% w/w and 12% w/w, where data are shown as mean ± SD, $n = 3$

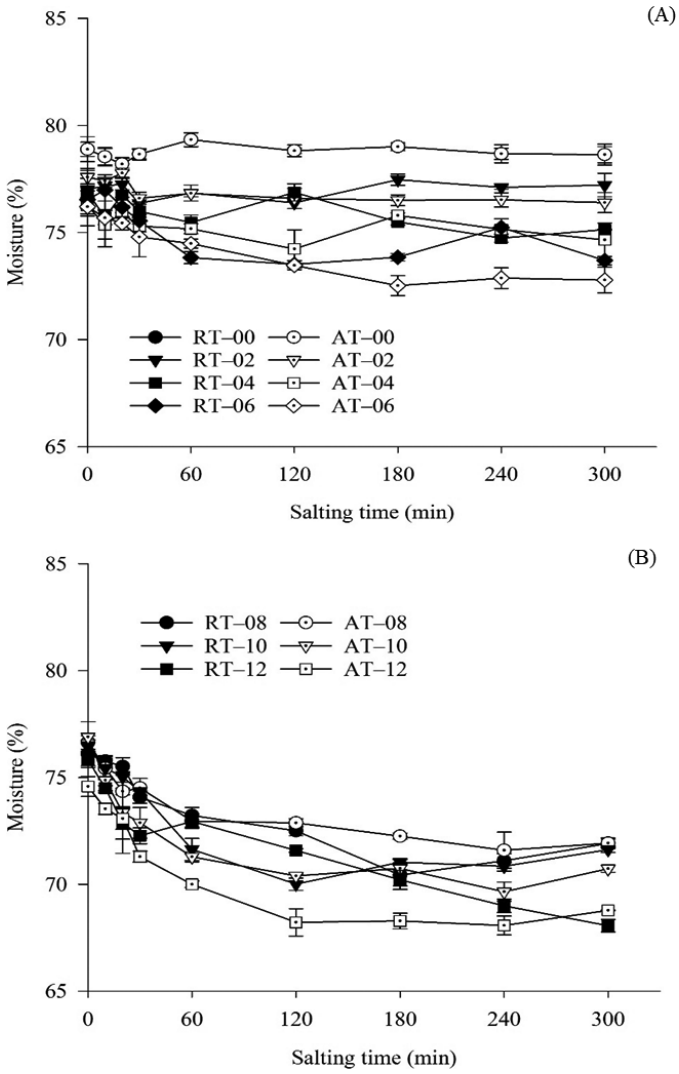

Fig. 3 Evolution of moisture content of anchovy stored at refrigerated temperature (RT, 4 ± 1°C) and ambient temperature (AT, 33 ± 3°C) in combination with salting at: (A) 0% (control), 2% w/w, 4% w/w and 6% w/w; (B), 8% w/w, 10% w/w and 12% w/w, where data are shown as mean ± SD, $n = 3$

The WPS content evolution patterns over a period of 5 hr were similar, with the salt content evolutions summarized in Table 2. It was clear that the WPS of the fish increased at a higher rate for a higher level of added salt than for a lower level of added salt. A level of WPS ≥ 10% is known to be able to control microbial pathogens, scombrotoxin fish poisoning and decomposition (Food and Agricultural Organization of the United Nations/World Health Organization, 2020; U.S. Food and Drug Administration, 2020). It has been reported that the growth of most bacteria is inhibited at WPS ≥ 10% (Rodtong et al., 2005), even if the growth of some halophilic bacteria is inhibited in media containing up to 15% salt (Turan et al., 2007). A level of WPS ≥ 10% was achieved by 4 hr (240 min) when the level of added salt was 8% w/w or higher for anchovy stored at either ambient or refrigerated temperature. Water and salt diffusions are known to be reduced at a refrigerated temperature (Czerner and Yeannes, 2013) as could be seen in the recent study for WSP ≥ 10% being attained earlier for the fish stored at ambient temperature compared with the refrigerated ones (120 min versus 240 min, respectively, with 10% salt and 60 min versus 120 min, respectively, with 12% salt).

The pattern of water activity reduction over a period of 5 hr was similar to the moisture content evolution, as summarized in Table 3. It was clear that the water activity of the fish decreased at a higher rate for a higher level of added salt than for a lower level of added salt. The relationship of water activity to WPS in NaCl/water solutions has been established for fish products, with the corresponding water activity value below 0.94 with 10% WPS (U.S. Food and Drug Administration, 2020). Salt penetration plays a key role in reducing water activity in fish flesh, with the mechanisms of action of fish salting that lower water activity being partial water removal from the flesh and partial replacement by salt (Turan et al., 2007; Czerner and Yeannes, 2010, 2013). Water activity values 0.94 or less were achieved when the level of added salt was 6% w/w or higher for anchovy stored at either ambient or refrigerated temperature. Similar to WPS, water activity 0.94 or less was accomplished earlier for the fish stored at ambient temperature compared with the refrigerated ones (120 min versus 180 min, respectively, with 6% salt, 60 min versus 120 min, respectively, with 8% salt and 20 min versus 30 min, respectively, with 10% salt, as the water and salt diffusions were known to be reduced at refrigerated temperature (Czerner and Yeannes, 2013). Other studies reported the growth of *C. botulinum* type E and non-proteolytic types B and F could be controlled by 5% WPS or higher or water activity of 0.97 or lower or both, and the growth of *C. botulinum* type A and proteolytic types B and F and other pathogens present in the finished product could be controlled by refrigeration (Lalitha and Gopakumar, 2005; Lalitha and Gopakumar, 2007).

Table 2 Evolution of water phase salt contents (mean ± SD) of anchovy stored at refrigerated temperature (RT, 4 ± 1 °C) and ambient temperature (AT, 33 ± 3°C) in combination with salting at 0% w/w (control), 2% w/w, 4% w/w, 6% w/w, 8% w/w, 10% w/w and 12% w/w

Sample	Salting time (min)								
	0	10	20	30	60	120	180	240	300
RT0%	0.19 ± 0.06^{fCD}	0.27 ± 0.06^{fBC}	0.13 ± 0.14^{gD}	0.20 ± 0.12^{hCD}	0.20 ± 0.02^{jCD}	0.10 ± 0.03^{lD}	0.41 ± 0.04^{iA}	0.33 ± 0.02^{hAB}	0.38 ± 0.02^{hAB}
RT2%	0.59 ± 0.01^{eF}	0.89 ± 0.21^{gE}	1.10 ± 0.06^{fD}	1.11 ± 0.06^{gD}	1.18 ± 0.09^{iD}	1.57 ± 0.08^{kC}	2.10 ± 0.08^{hB}	2.59 ± 0.07^{gA}	2.65 ± 0.15^{gA}
RT4%	0.59 ± 0.06^{eFG}	1.53 ± 0.12^{fF}	2.10 ± 0.07^{eE}	2.47 ± 0.05^{fD}	3.46 ± 0.36^{gC}	4.01 ± 0.14^{iB}	4.91 ± 0.09^{gA}	5.09 ± 0.07^{fA}	5.15 ± 0.08^{eA}
RT6%	1.94 ± 0.26^{bcdG}	2.86 ± 0.39^{eF}	3.72 ± 0.55^{cE}	4.50 ± 0.19^{dD}	4.71 ± 0.69^{eD}	5.72 ± 0.27^{gC}	6.74 ± 0.26^{fB}	7.39 ± 0.16^{eA}	7.65 ± 0.25^{dA}
RT8%	1.83 ± 0.50^{cdF}	2.69 ± 0.32^{eE}	3.14 ± 0.22^{dDE}	3.70 ± 0.40^{eDE}	4.88 ± 0.38^{eC}	6.43 ± 0.27^{fB}	9.71 ± 0.65^{dA}	10.25 ± 0.06^{dA}	10.12 ± 0.11^{cA}
RT10%	1.86 ± 0.24^{bcdF}	3.45 ± 0.26^{dE}	4.61 ± 0.31^{bD}	4.81 ± 0.71^{dD}	7.42 ± 0.33^{cC}	8.78 ± 0.20^{dB}	9.60 ± 0.11^{dB}	11.75 ± 1.09^{cA}	12.28 ± 0.29^{bA}
RT12%	2.25 ± 0.32^{bcH}	4.19 ± 0.41^{cG}	4.84 ± 0.47^{bF}	6.18 ± 0.17^{cE}	8.87 ± 0.18^{bD}	10.49 ± 0.12^{cC}	12.97 ± 0.31^{bB}	14.95 ± 0.13^{aA}	15.07 ± 0.10^{aA}
AT0%	0.19 ± 0.06^{fBC}	0.27 ± 0.06^{hAB}	0.13 ± 0.14^{gC}	0.20 ± 0.12^{hBC}	0.19 ± 0.02^{jBC}	0.10 ± 0.03^{lC}	0.40 ± 0.04^{iA}	0.32 ± 0.02^{hAB}	0.38 ± 0.02^{hA}
AT2%	0.73 ± 0.12^{eD}	1.55 ± 0.22^{fC}	1.67 ± 0.09^{eC}	2.14 ± 0.18^{fB}	2.07 ± 0.18^{hB}	2.12 ± 0.22^{jB}	2.39 ± 0.18^{hB}	2.72 ± 0.04^{gA}	3.03 ± 0.26^{fA}
AT4%	2.08 ± 0.04^{bcF}	3.45 ± 0.08^{dE}	3.58 ± 0.12^{cdE}	3.35 ± 0.19^{eE}	4.05 ± 0.24^{fD}	4.53 ± 0.05^{hC}	4.81 ± 0.14^{gB}	5.07 ± 0.24^{fAB}	5.15 ± 0.14^{eA}
AT6%	1.81 ± 0.31^{cdG}	2.61 ± 0.24^{eF}	3.30 ± 0.24^{cdE}	3.79 ± 0.06^{eD}	5.77 ± 0.17^{dC}	6.57 ± 0.19^{fB}	8.10 ± 0.47^{eA}	7.87 ± 0.21^{eA}	7.94 ± 0.29^{dA}
AT8%	1.59 ± 0.40^{dF}	3.03 ± 0.18^{dE}	4.75 ± 0.26^{bD}	4.84 ± 0.16^{dD}	5.72 ± 0.29^{dC}	8.44 ± 0.16^{eB}	9.82 ± 0.11^{dA}	10.09 ± 0.14^{dA}	10.15 ± 0.09^{cA}
AT10%	2.30 ± 0.34^{bG}	4.92 ± 0.16^{bF}	6.22 ± 0.15^{aE}	6.69 ± 0.31^{bD}	9.10 ± 0.19^{bC}	11.24 ± 0.13^{bB}	12.41 ± 0.16^{cA}	12.72 ± 0.10^{bA}	12.55 ± 0.18^{bA}
AT12%	4.18 ± 0.09^{aG}	5.38 ± 0.22^{aF}	6.38 ± 0.32^{aE}	8.08 ± 0.23^{aD}	10.62 ± 0.19^{aC}	13.29 ± 0.15^{aB}	14.96 ± 0.11^{aA}	15.16 ± 0.04^{aA}	15.09 ± 0.17^{aA}

Different lowercase superscripts indicate significant ($p < 0.05$) differences between means in the same column; different uppercase superscripts indicate significant ($p < 0.05$) differences between means in the same row

Table 3 Changes in water activity values (mean ± SD) of anchovy stored at refrigerated temperature (RT, 4 ± 1°C) and ambient temperature (AT, 33 ± 3°C) in combination with salting at 0% w/w (control), 2% w/w, 4% w/w, 6% w/w, 8% w/w, 10% w/w and 12% w/w

Sample	Salting time (min)							
	0	10	20	30	60	120	180	240	300
RT0%	0.99±0.00^{abAB}	0.99±0.00^{abAB}	0.99±0.00^{abAB}	0.99±0.00^{aA}	0.99±0.00^{aA}	0.99±0.00^{aA}	0.99±0.00a^{AB}	0.98±0.00^{bAB}	0.98±0.00^{aB}
RT2%	0.98±0.01^{abA}	0.98±0.01^{abcAB}	0.98±0.00^{abAB}	0.98±0.01^{bBC}	0.98±0.01^{bABC}	0.97±0.01^{bBC}	0.97±0.00^{cC}	0.97±0.01^{cBC}	0.97±0.00^{bC}
RT4%	0.99±0.00^{abA}	0.97±0.00^{deBC}	0.98±0.00^{bcB}	0.97±0.00^{bcBC}	0.96±0.00^{cdC}	0.96±0.00^{cdC}	0.97±0.00^{dC}	0.96±0.00^{dC}	0.96±0.00^{bcC}
RT6%	0.99±0.00^{abA}	0.98±0.00^{abcdA}	0.98±0.00^{bA}	0.97±0.00^{cB}	0.95±0.01^{efC}	0.95±0.01^{eD}	0.94±0.00^{eD}	0.94±0.00^{eD}	0.94±0.00^{dD}
RT8%	0.97±0.00^{dA}	0.97±0.01^{deA}	0.96±0.00^{deA}	0.95±0.00^{dB}	0.95±0.00^{fgB}	0.93±0.00^{ghCD}	0.93±0.00^{fC}	0.92±0.00^{fD}	0.92±0.00^{eD}
RT10%	0.98±0.00^{bcA}	0.97±0.01^{cdeB}	0.95±0.00^{fgC}	0.94±0.00^{eD}	0.93±0.01^{hE}	0.92±0.00^{hF}	0.90±0.00^{hG}	0.90±0.00^{gG}	0.90±0.00^{fG}
RT12%	0.98±0.01^{cdA}	0.96±0.01^{efB}	0.95±0.01^{fgC}	0.94±0.00^{eD}	0.92±0.01^{iE}	0.90±0.01^{iF}	0.89±0.00^{iG}	0.88±0.00^{hGH}	0.88±0.00^{gH}
AT0%	0.99±0.00^{aA}	0.99±0.00^{aA}	0.99±0.00^{aA}	0.99±0.00^{aA}	0.99±0.00^{abA}	0.99±0.00^{aA}	0.98±0.00^{bB}	0.99±0.00^{aA}	0.99±0.00^{aA}
AT2%	0.98±0.01^{abcA}	0.98±0.00^{abcdAB}	0.97±0.00^{dC}	0.97±0.00^{bBC}	0.97±0.00^{cC}	0.97±0.00^{bcC}	0.97±0.00^{cC}	0.97±0.00^{cBC}	0.97±0.00^{bcC}
AT4%	0.99±0.00^{aA}	0.97±0.00^{bcdB}	0.97±0.00^{cdC}	0.97±0.00^{bcC}	0.96±0.00^{deD}	0.96±0.00^{dD}	0.96±0.00^{dD}	0.96±0.00^{dD}	0.96±0.00^{cD}
AT6%	0.99±0.01^{abA}	0.97±0.01^{cdeB}	0.96±0.01^{deC}	0.95±0.00^{dD}	0.95±0.01^{fgDE}	0.94±0.00e^{fE}	0.94±0.01^{eDE}	0.94±0.00^{eE}	0.94±0.00^{dDE}
AT8%	0.98±0.00^{bcA}	0.97±0.00^{deB}	0.96±0.01^{efC}	0.95±0.00^{dD}	0.94±0.01^{gE}	0.93±0.01^{fgF}	0.92±0.00^{gG}	0.92±0.00^{fG}	0.92±0.00^{eG}
AT10%	0.98±0.01^{cdA}	0.96±0.01^{fB}	0.94±0.00^{hC}	0.93±0.00^{fD}	0.92±0.00^{hE}	0.90±0.00^{iF}	0.90±0.00^{hF}	0.90±0.00^{gF}	0.90±0.01^{fF}
AT12%	0.98±0.00^{bcA}	0.97±0.00^{deB}	0.95±0.01^{gC}	0.92±0.01^{gD}	0.90±0.00^{iE}	0.88±0.01^{jF}	0.88±0.01^{jF}	0.88±0.00^{hF}	0.88±0.00^{gF}

Different lowercase superscripts indicate significant ($p < 0.05$) differences between means in the same column; different uppercase superscripts indicate significant ($p < 0.05$) differences between means in the same row

Up to 5 hr storage, the pH of anchovy stored at chilled temperature and salted with 4% w/w or higher slightly decreased and remained steady (Fig. 4), which could have been due to lactic acid production postmortem (Chotimarkorn, 2014; Duarte et al., 2020). The pH of the control (0%) and of 2% w/w salted anchovy slightly increased due to the production of volatile compounds, indicating progressive accumulation of basic substances, such as ammonia and TMA (Dissaraphong et al., 2006; Duarte et al., 2020).

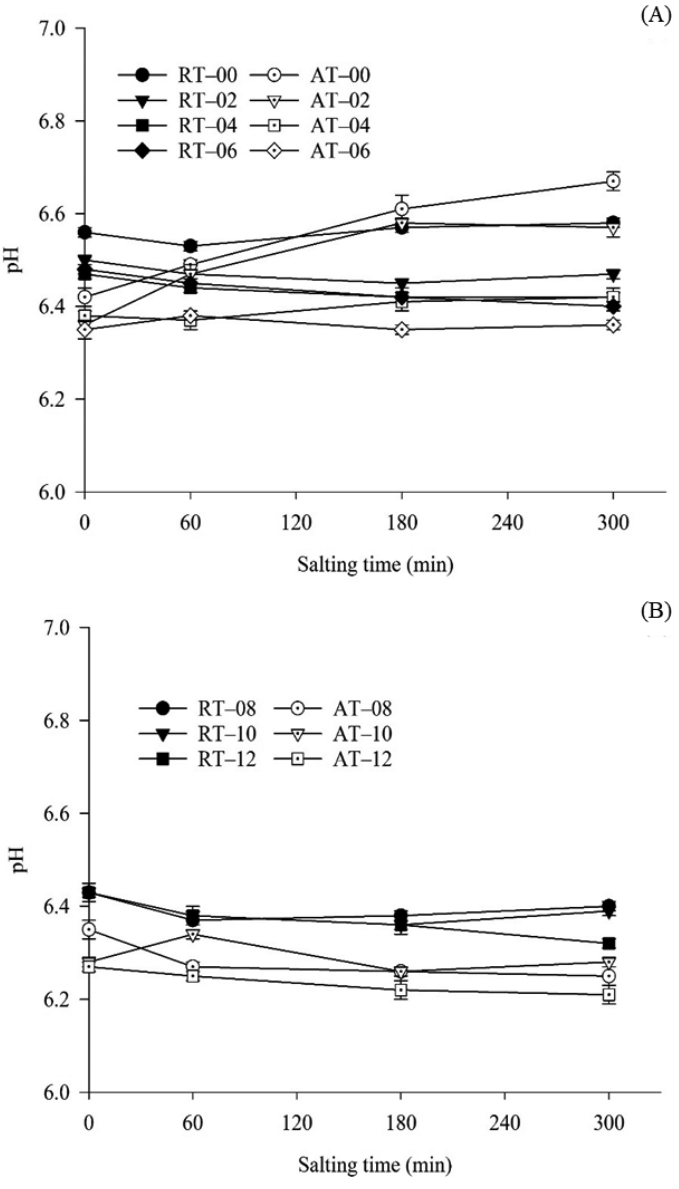

Fig. 4 Changes in pH values of anchovy stored at refrigerated temperature (RT, 4 ± 1°C) and ambient temperature (AT, 33 ± 3°C) in combination with salting at: (A) 0% (control), 2% w/w, 4% w/w and 6% w/w; (B), 8% w/w, 10% w/w and 12% w/w, where data are shown as mean ± SD, $n = 3$

Histamine accumulation

The histamine content of fresh anchovy stored in ice was 0.02 ± 0.01 mg/100 g (Table 1), which was in good agreement with another report that the fresh fish typically has histamine levels below 0.2 mg/100 g (Food and Agricultural Organization of the United Nations/World Health Organization, 2020). As shown in Fig. 5, the histamine contents of the control samples increased gradually to 0.21± 0.01 mg/100 g at refrigerated temperature, whereas it increased rapidly to 3.62 ± 0.07 mg/100 g at ambient temperature ($p < 0.05$). The histamine levels of all refrigerated samples were not higher than 0.21 ± 0.01 mg/100 g stored up to 5 hr post salting ($p \geq 0.05$), suggesting that storage temperature was the principal effect on accumulation of histamine (Rodtong et al., 2005; Chotimarkorn, 2014; Zou and Hou, 2017). On the other hand, the histamine levels of samples stored at ambient temperature were dependent on the added salt level ($p < 0.05$). The higher the level of salt added, the lower the histamine content accumulated, which was in good agreement with Hwang et al. (2012, 2020). Salting fish at 8% w/w or higher significantly delayed histamine formation to below 2 mg/100 g up to 5 hr storage ($p < 0.05$). The histamine contents of refrigerated fish were 1.90 mg/100 g after 15 d for *S. indicus*, 2.2 ± 0.19 mg/100 g for *S. heterolobus* after 7 d storage (Chotimarkorn, 2014), while it was reported rising to 19.0 mg/100 g at 15°C after 32 hr and to 25.4 mg/100 g at 35°C after 8 hr for *S. indicus* (Rodtong et al., 2005) and 4.9 ± 0.23 mg/100 g without ice after 7 d of storage for *S. heterolobus* (Chotimarkorn, 2014). Histamine formation in fish is affected by the free histidine content in the fish and the contamination load of histidine-decarboxylating bacteria that can grow over a broad range of temperatures, including refrigerated temperature (Zou and Hou, 2017; Magro et al., 2020; Visciano et al., 2020). The prescribed limits for histamine in fish are 5 mg/100 g as an indicator of decomposition and 20 mg/100 g as hazardous (U.S. Food and Drug Administration,

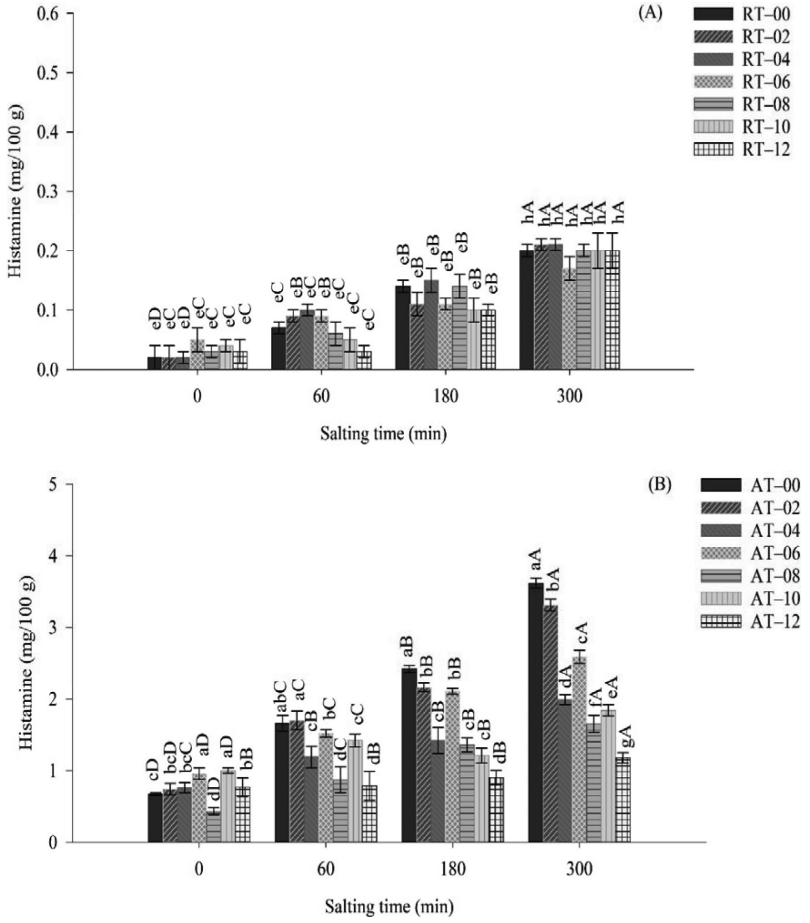

Fig. 5 Changes in histamine contents of anchovy salted at 0% (control), 2% w/w, 4% w/w, 6% w/w, 8% w/w, 10% w/w and 12% w/w and stored at: (A) refrigerated temperature (RT, 4 ± 1°C); (B) ambient temperature (AT, 33 ± 3°C), where data are shown as mean ± SD, $n = 3$, different lowercase letters on columns indicate significant differences between means within same salting time and different uppercase letters on columns indicate significant differences between means within same salt concentration ($p < 0.05$)

2020), and 10 mg/100 g as an indicator of decomposition and 20 mg/100 g as an indicator for poor handling and hygiene (Joint FAO/WHO Codex Alimentarius Commission, 2003). It was reported that 10% sodium chloride inhibited the growth of histamine-forming bacteria, such as *Morganella morganii*, *Proteus vulgaris* and *Enterobacter aerogenes*, isolated from Indian anchovy (*S. indicus*) (Rodtong et al., 2005).

Total volatile basic nitrogen and trimethylamine accumulations

The TVBN content of fresh anchovy stored in ice was 20.16 ± 1.34 mg/100 g (Table 1), which was in good agreement with another report of 18.43 ± 0.89 mg/100 g flesh for *E. anchoita* anchovy (Massa et al., 2012) and about 20 mg/100 g for *S. indicus* (Rodtong et al., 2005). Up to 5 hr storage, the TVBN contents of the control samples gradually increased to 26.50 ± 0.24 mg/100 g at refrigerated storage, whereas they increased up to 86.37 ± 2.01 mg/100 g at ambient temperature ($p < 0.05$), as seen in Fig. 6. Regardless of the percentage of added salt for the refrigerated samples, the TVBN content slightly increased within the range 16.93±0.78 mg/100 g to 30.71±0.42 mg/100 g during 5 hr of storage ($p < 0.05$). On the contrary, regardless of the percentage of added salt for ambient temperature storage samples, the TVBN content sharply increased within the range 25.36 ± 1.85 mg/100 g to 86.37 ± 2.01 mg/100 g during 5 hr of storage ($p < 0.05$). Notably, within only 60 min, the TVBN content was higher than the limiting level for fish with no salt (39.03 ± 1.49 mg /100 g), with 2% w/w salt (43.13 ± 0.44 mg /100 g) and 4% w/w salt (35.91 ± 1.81 mg /100 g) at ambient temperature ($p < 0.05$). However, the TVBN contents of the ambient temperature storage samples with 8–12% w/w added salt were in a range lower than 35 mg/100 g (25.36 ± 1.85 mg/100 g to 31.98 ± 1.19 mg/100 g) at 5 hr post storage. TVBN, mainly composed of trimethylamine, ammonia and dimethylamine and resulting from the degradation of proteins and non-protein nitrogenous compounds produced

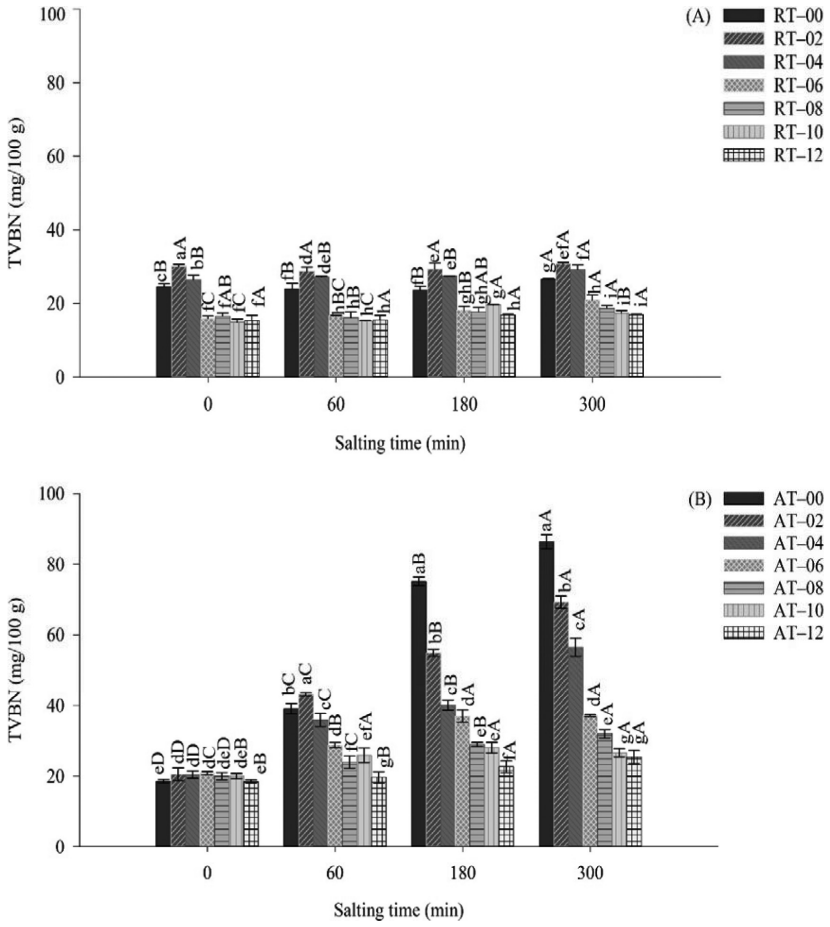

Fig. 6 Changes in total volatile bases nitrogen (TVBN) contents of anchovy salted at 0% w/w (control), 2% w/w, 4% w/w, 6% w/w, 8% w/w, 10% w/w and 12% w/w and stored at: (A) refrigerated temperature (RT, 4 ± 1°C); (B), ambient temperature (AT, 33 ± 3°C), where data are shown as mean ± SD, $n = 3$, different lowercase letters on columns indicate significant differences between means within same salting time and different uppercase letters on columns indicate significant differences between means within same salt concentration ($p < 0.05$)

by destructive activities of microorganisms, is commonly used as a good index to assess the freshness of fish during refrigerated storage (Sharifian et al., 2011; Massa et al., 2012; Chotimarkorn, 2014; Linn et al., 2021). A TVBN level of 35–40 mg/100 g in fish muscle is usually regarded as spoiled (Sharifian et al., 2011; Tavares et al., 2021; Linn et al., 2021). The increase in the TVBN contents of 0%, 2% and 4% w/w salt added fish samples, as shown in Fig. 6, were in accordance with the pH profiles, shown in Fig. 4. Fish stored at low temperature in combination with salting at 8% w/w or higher delayed the growth of bacteria, resulting in minimal changes in the TVBN content during the first 5 hr of storage.

The initial TMA content of the fresh fish samples was 1.12 ± 0.07 mg/100 g (Table 1), which was in good agreement with another report that the amount of TMA is typically less than 2 mg/100 g tissue in fresh fish (Summers et al., 2016). Up to 5 hr storage, the TMA contents of the control samples gradually increased to 1.40 ± 0.04 mg/100 g with refrigerated storage, whereas they increased up to 6.38 ± 0.13 mg/100 g at ambient temperature ($p < 0.05$), as shown in Fig. 7. Regardless of the percentage of added salt for the refrigerated samples, the TMA content slightly increased within the range 1.21 ± 0.16 mg/100 g to 1.57 ± 0.11 mg/100 g during 5 hr of storage ($p < 0.05$). On the contrary, regardless of the percentage of added salt for the ambient temperature storage samples, TMA increased within the range 2.52 ± 0.28 mg/100 g to 6.38 ± 0.13 mg/100 g during 5 hr of storage ($p < 0.05$). Notably, the accumulation of TMA during 5 hr storage of fish in the present study did not exceed the limit of acceptability for human consumption. TMA is rapidly accumulated in fish muscle under chilled storage; thus, it is considered as an indicator of fish freshness stored in ice (Chotimarkorn, 2014; Linn et al., 2021). The TMA results

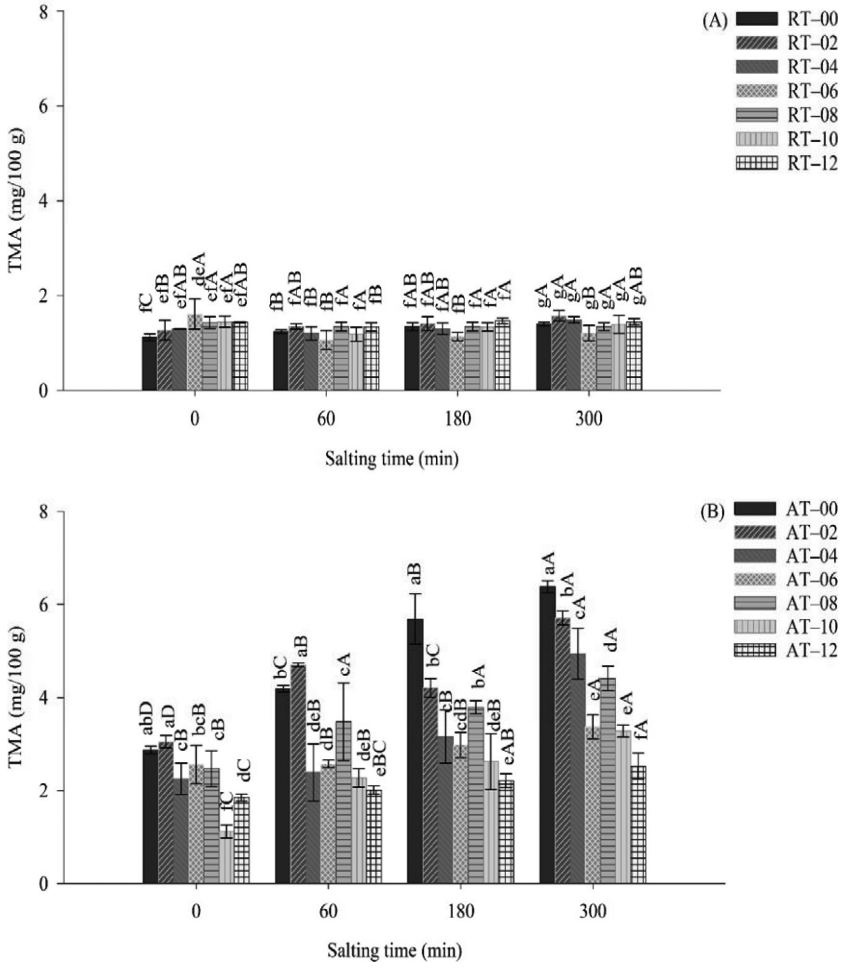

Fig. 7 Changes in trimethylamine (TMA) contents of anchovy salted at: (A) 0% w/w (control), 2% w/w, 4% w/w, 6% w/w, 8% w/w, 10% w/w and 12% w/w and stored at refrigerated temperature (RT, 4 ± 1°C; (B) ambient temperature (AT, 33 ± 3°C), where data are shown as mean ± SD, $n = 3$, different lowercase letters on columns indicate significant differences between means within same salting time and different uppercase letters on columns indicate significant differences between means within same salt concentration ($p < 0.05$)

from bacterial reduction of trimethylamine oxide that occurs naturally in marine fish to allow osmotic regulation (Duarte et al., 2020). A TMA level of 10–15 mg/100 g of fish muscle is usually regarded as the limit of acceptability for human consumption (Summers et al., 2016; Linn et al., 2021; Tavares et al., 2021).

Correlation between water phase salt and quality parameters

Pearson's correlation analysis was conducted to determine the relationship between WPS and the moisture content, water activity, pH, histamine, TVBN and TMA contents of anchovy treated with different levels of added salt under two different storage temperatures (refrigerated temperature, RT; and ambient temperature, AT) at different times post-salting (1 hr, 3 hr and 5 hr). As seen in Table 4, there was a positive correlation between WPS and salt concentration (correlation coefficient, r = 1, $p < 0.01$), since WPS is defined as the concentration of salt in the water portion of the fish flesh and calculated from the salt concentration and moisture content, regardless of the level of added salt, storage temperature and time post-salting. In general, negative correlations existed between WPS and moisture content (r, correlation coefficient ranged from -0.935 [RT-3 hr] to -0.995 [AT-5 hr]; $p < 0.01$), water activity (r ranged from -0.936 [RT-1 hr] to -0.986 [AT-5 hr]; $p < 0.01$) and pH (r ranged from -0.912 [RT-5 hr] to -0.955 [AT-3 hr]; $p < 0.01$). The results also revealed a negative correlation existed between WPS and the TVBN content (r ranged from -0.771 [RT-3 hr] to -0.957 [AT-5hr]; $p<0.01$), regardless of the level of added salt, storage temperature and time post-salting as well as negative correlations between the WPS of the ambient temperature storage samples and the histamine content (r ranged from -0.662 [AT-1hr]; $p < 0.05$ to -0.902 [AT-5hr]; $p < 0.01$) and TMA content (r ranged from -0.758 [AT-3hr] to -0.800 [AT-5 hr]; $p < 0.01$), regardless of the level of added salt and time post-salting.

These negative correlations between WPS and all the quality parameters studied could be explained by the fact that salting of fish resulted in water activity reduction and partial water removal from the flesh and partial replacement by salt (Turan et al., 2007; Czerner and Yeannes, 2010, 2013). The higher the salt added, the lower the quality parameters, such as aerobic plate count, total coliform, water activity, moisture content, TVBN and thiobarbituric acid accumulated in dried milkfish (Hwang et al., 2012). Negative correlations were noted between salt content and water activity (r = -0.92, $p < 0.05$) and salt content and histamine (r = -0.95,

Table 4 Pearson's correlation coefficients between water phase salt (WPS) and other parameters.

Time post-salting (hr)	Storage temperature	Salt content	Moisture content	Water activity	pH	Histamine content	TVBN content	TMA content
1	RT-WPS	1.000**	-0.980**	-0.936**	-0.854**	-0.348	-0.786**	-0.009
	AT-WPS	1.000**	-0.976**	-0.962**	-0.857**	-0.662*	-0.882**	-0.789**
3	RT-WPS	1.000**	-0.935**	-0.985**	-0.878**	-0.394	-0.771**	-0.128
	AT-WPS	1.000**	-0.992**	-0.983**	-0.955**	-0.821**	-0.923**	-0.758**
5	RT-WPS	1.000**	-0.987**	-0.977**	-0.912**	-0.118	-0.872**	-0.165
	AT-WPS	1.000**	-0.995**	-0.986**	-0.950**	-0.902**	-0.957**	-0.800**

RT = refrigerated temperature (4 ± 1°C); AT = ambient temperature (33 ± 3°C); TVBN = total volatile bases nitrogen; TMA = trimethylamine.
* = significance ($p < 0.05$); ** = highly significance ($p < 0.01$)

$p < 0.05$) of brined and dried milkfish products, regardless of the brine concentration (Hwang et al., 2020). Bacterial growth was mostly inhibited during ripening of salted anchovy (*E. encrasicholus*) according to Mohamed et al. (2016). Positive correlations existed between histamine and pH and water activity in all ripening temperatures studied in salted Atlantic bonito (*Sarda sarda*), while negative correlations were noted between histamine and ripening time, salt content and WPS (Ormanci and Colakoglu, 2017). However, no correlation existed between the WPS of iced storage samples and the histamine content, and the WPS of iced storage samples and the TMA content, which could be explained by bacterial growth inhibition by salting, as described by Turan et al. (2007), Czerner and Yeannes (2010, 2013), Hwang et al. (2012, 2020) and Mohamed et al. (2016), in combination with low temperature storage that allowed very low histamine and TMA accumulations during 5 hr of refrigerated storage. In addition, the relationship between WPS and water activity may be of practical use in estimating the water activity in salted fish when specific equipment is not available or to check individual measurements of water activity.

In conclusion, the highlight of this study was to establish the optimal level of added salt to minimize the risk of associated chemical hazards of small-sized anchovy (*S. indicus*) being used as a raw material, by using WPS as an indicator during 5 hr post salting. The results based on WPS evolutions revealed that salt penetration was completed during a short time range for small-sized anchovy (approximately 7.99 ± 0.38 cm in length) within 3 hr and 4 hr at ambient storage (33 ± 3°C) and chilled storage (4 ± 1°C), respectively, for all levels of added salt studied. WPS levels of 10% or higher were achieved only when the level of added salt was 8–12% w/w for anchovy stored at either ambient or refrigerated temperature ($p < 0.05$). This study also suggested that more attention should be paid to the time range practically required to reach 10% WPS for anchovy salted at 8–12% w/w, which was dependent upon the level of salt added. The time ranges during refrigerated storage practically required to reach 10% WPS for anchovy salted at 8–10% w/w and 12% w/w were 4 hr and 2 hr, respectively, ($p < 0.05$). Within the scope of the study, it could be concluded that the combination of prescribed salt level at 8–12% w/w and refrigerated storage provided a high level of assurance that the microbial growth and chemical changes would be retarded for anchovy being used as a raw material.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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