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Research article

Development of a novel Mohr's salt-based indicator for monitoring sea bass (*Lates calcarifer*) fillet spoilage in chilled storage

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Abstract

A novel Mohr's salt-based indicator was investigated with prospects for developing diagnostic packaging, as a chemical sensor for simultaneous observation of sea bass (*Lates calcarifer*) fillet putrefaction. In addition, the correlation was assessed between the microorganism enumeration and volatile compound quantification. This attached-to-package sensor consists of ammonium iron (II) sulfate hexahydrate ($[NH_4]_2Fe[SO_4]_2 \cdot 6H_2O$), termed Mohr's salt. Total volatile basic nitrogen (TVB-N) compounds such as ammonia, dimethylamine and trimethylamine were used as indicators of deteriorating metabolites since the extent of deterioration was associated with the quantity of heightened TVB-N throughout shelf life. Attributes of these sensing solutions were investigated, including their connection with NH_3. Then these indicator labels were prepared using solution casting. The appropriate formula consisted of Mohr's salt 0.1% weight per volume and acetic acid 0.05 % volume per volume (v/v) or 0.10 % v/v. The color changes (from colorless to dark green) for the total color difference of the Mohr's salt-based indicator corresponded well with the extent of TVB-N in the sea bass fillet. Experiments on fish fillets confirmed that the indicator signal was associated with the increased growth of microorganisms, which possibly allowed for concomitant observation of putrefaction.

Introduction

Consumer demand for either minimally processed or easily prepared "fresher" foods—in conjunction with both food business globalization and distribution logistics—pose key challenges for safety and quality of foods (Sonneveld, 2000). A decrease in the shelf life of food products due to microbial contamination and a rise in the risk of food-borne pathogens are motivations for an inventive way for monitoring microbial food deterioration and spoilage while strengthening food safety. As a legislative requirement, notably in

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the European Union, food traceability creates an entirely responsible food value chain (Aung and Chang, 2014). Thus, there is tremendous concern among food manufacturers, retailers, consumer rights organizations and food safety regulatory bodies to develop valid, cost-effective, speedy, trustable, non-invading and non-destructive techniques or devices to assess the simultaneous freshness of food products. An optional conception to reach this prerequisite is the development of smart packaging (SP) in the subclass of diagnostic packaging (DP) in relation to a spoilage indicator for sensing food freshness (Abreu et al., 2012; Suppakul, 2012; Janjarasskul and Suppakul, 2018). In addition to the invention of a food freshness indicator, the detection of chemical metabolites related to microbial agricultural and food deterioration and spoilage can provide an alternative to sensorial and microbiological evaluations which are generally costly and nonautomated (Dainty, 1996).

The conception for a visual indicator perceptive to metabolites indicating freshness and in turn indicating food spoilage status was first reported by Smolander et al. (2002). Nopwinyuwong et al. (2010) developed a pioneering, colorimetric blended, pH dye-based indicator label for monitoring spoilage of intermediate-moisture dessert. Subsequently, Rukchon et al. (2014) demonstrated a colorimetric indicator label for observing spoilage of skinless chicken breast. These studies proposed the prospect of creating energetic "best-before" dates which may result in crucial and engaging enhancements in the arena of quality assurance, thereupon, diminishing errors. The accentuated guarantee of product safety is an important consideration by consumers. Focusing on a fish spoilage indicator (FSI), as a member of DP as a subclass of SP (Suppakul, 2012; Janjarasskul and Suppakul, 2018), it can be defined as "a packaging system (or material) which exploits metabolites as "information" for observing fish freshness and spoilage status" (Suppakul, 2012). With rapidly decreasing protein content via enzymatic and bacteriologic activity, total volatile basic nitrogen (TVB-N) compounds such as ammonia (NH_3), dimethylamine (DMA) and trimethylamine (TMA) are responsible for the smell of spoiled fish and can be applied as metabolites to monitor fish spoilage status. Examples include indicators that are: pH dye-based (Pacquit et al., 2007), natural dye-based (Kuswandi et al., 2012b; Zhang et al., 2014), polymer-based (Kuswandi et al., 2012a; Li et al., 2014) and bio-based (Musso et al., 2017). These can be used to monitor either volatile amines or biogenic amines.

Fish fillet samples are stored at 4°C to maintain their freshness, quality and shelf life. Unfortunately, this condition cannot utterly retard the microbial growth. The total number of microorganisms, referred to as the total viable count (TVC), is a crucial index of fish quality in terms of freshness. It is arduous to apply published microbial counts in the public domain to delineate accurate spoilage thresholds as they differ depending on the fishing season, fishing maturity, geographical site and ultimately fish species (Pacquit et al., 2007). However, the greatest limitation of 6 log CFU/g is considered as the acceptable quality limit (AQL) for fish freshness (Jeon et al., 2002). *Pseudomonas* spp. represents one genus of specific spoilage organisms (SSOs) and it produces typical volatile compounds with unacceptable odor and flavor during chilled storage (Pacquit et al., 2008; Fernandes, 2009; Gram, 2009; Chun et al., 2014) and results in sensorial negation. The microbial enumeration of SSOs could be termed as the minimum spoilage level (MSL) and the extent of metabolites which are produced from SSOs could be used as a chemical spoilage index (CSI) (Dalgaard, 1993).

Volatile compounds can be detected by a coordination compound which consists of a transition metal as a central atom surrounded by molecules as ligands, leading to color change due to a ligand exchange reaction (Barrett et al., 2003; Atkins et al., 2010). Mohr's salt is a proposed coordination compound in the current research because it is a common laboratory reagent and is easy to find and cheap (Karami et al., 2013). Mohr's salt is a molecule of ammonium iron (II) sulphate dissolved in water; the iron (II) is surrounded by water molecules and an octahedral molecular geometry is obtained. The basic volatile compound can react with Mohr's salt to produce a color change from colorless to dark green due to the ligand reaction that is clearly visible to the naked eye (Cox, 2004; Housecroft and Sharpe, 2005). Therefore, the objectives of this research were to investigate the linkage between volatile compound quantification and microbial enumeration and to develop a Mohr's salt-based indicator for monitoring sea bass (*Lates calcarifer*) fillet spoilage in chilled storage.

Materials and Methods

Materials

Mohr's salt ($[NH_4]_2Fe[SO_4]_2 \cdot 6H_2O$) (Sigma-Aldrich; Singapore), acetic acid (J.T. Baker; USA), glycerol (Q-Rec; New Zealand), methylcellulose (Methocel™; Dow Chemical; USA) and de-ionized (DI) water were used to prepare the indicator film. Polypropylene adhesive tape (3M; Thailand) was used for coating and to protect the indicator film from dissolution. Plate count agar (PCA; Merck; Germany), *Pseudomonas* agar base (Merck; Germany) and potassium dihydrogen phosphate (KH_2PO_4) were used for microbiological analysis. Bromocresol green (BCG), methyl red (MR), boric acid, 95% ethanol, 4% trichloroacetic acid (TCA), saturated potassium carbonate (K_2CO_3) and 0.02 N hydrochloric acid (HCl) were used as reagents for evaluation of the TVB-N content. Visually transparent polyamide laminated with linear low-density polyethylene (PA/LLDPE, 80-µm grade) and LLDPE 50 µm grade films were supplied by Amcor Flexibles Bangkok Public Co. Ltd., Thailand.

Fish fillet spoilage study

Experimental setup

Descaled sea bass (average weight ranged 0.9−1.0 kg) were sampled from the fish market near Kasetsart University, Bangkok, Thailand. Descaled samples were washed with cooled tap water, filleted, packed in the low-density polyethylene (LDPE) zip lock bag and kept at 4°C before use. Filleted samples stored at 4°C were serially examined for microbiological and chemical changes during storage.

Microbiological analysis

Sea bass fillet samples (50 g) were aseptically prepared and transferred to a stomacher bag and homogenized in 450 mL of Butterfield's phosphate-buffered dilution water (pH 7.2) with a laboratory blender stomacher (stomacher 400 Type BA 7021; Seward; Germany) for 120 s at medium speed. Duplicate 1 mL samples of each decimal dilution were separately pipetted into marked Petri dishes (90 mm ×14 mm) and then pour-plated with 12−15 mL plate count agar and incubated at 35 ± 1°C for the aerobic plate count (Maturin and Peeler, 2001; Spencer and Ragout de Spencer, 2001). Each decimal dilution of 0.1 mL was duplicated and separately spread on the *Pseudomonas* agar base with selective isolation of *Pseudomonas* spp. and incubated at 30 ± 1°C for the *Pseudomonas* count (Pacquit et al., 2007). The colonies were counted after 48 ± 2 hr and determined as log colony-forming units (CFU)/g. All tests were performed in triplicate.

Quantification of total volatile basic nitrogen

Total volatile basic nitrogen (TVB-N) was quantified using Conway's microdiffusion method (Siang and Kim, 1992; Conway and Byrne, 1993). All tests were performed in triplicate. A dorsal fish sample (2 g) was extracted with 8 mL of 4% TCA weight per volume (w/v) and ground well before leaving for 30 min in ice and then passing through Whatman no.41 filter paper. The inner ring solution (1% boric acid comprising a mixture of 0.01 g of BCG and 0.02 g of MR in 10 mL of ethyl alcohol) was transferred to the inner ring of the Conway unit. A sample extract (1 mL) was transferred to the outer ring and then a saturated K_2CO_3 solution (1 mL) was added to activate the reaction. Blank (4% TCA) 2 mL was transferred to the outer ring instead of the sample for use as a reference color and then the reaction was activated using a saturated K_2CO_3 solution. The Conway unit was sealed and incubated at 35°C for 60 min. The concentration of TVB-N was determined by titrating the inner ring solution with 0.02 N HCl until its greenish color had changed to pink. The concentration of TVB-N was calculated using Equation 1:

$$\text{TVB-N content (mg N/100 g sample)} = \frac{(V_S-V_B)\times(N_{HCl}\bullet A_N)\times V_E\times 100}{W_S} \quad (1)$$

where V_S and V_B are the volumes of HCl for titration of the sample and blank, respectively, N_{HCl} is the concentration of HCl, A_N is the atomic mass of nitrogen, V_E is the volume of sample and W_S is the weight of the fish sample.

Indicator label fabrication

An amount (2 g) of methylcellulose and 1 g of glycerol as plasticizer were dissolved into 50 mL DI water, and then stirred at room temperature for 2 hr. Separately, 0.1 g of Mohr's salt was dissolved into 50 mL DI water, followed by adding 0.01, 0.05 and 0.1% volume per volume (v/v) of acetic acid as an oxidation retardant and then stirring for 3 min. Both solutions were well-mixed and stirring was continued for 1 hr before storing at 4°C. These three blended indicator solutions, labeled A1, A2 and A3 formulas, respectively, were achieved following degassing in an ultrasonic water bath (Model 275D; Crest Ultrasonics Corporation; USA) for 10 min. Then, each solution was poured onto separate 12.5 cm ×15 cm ×0.1 cm acrylic plates and left for 48 hr. Level adjustment was required to create a uniform thickness. When the indicator films had dried, they were cut into 2 cm × 3 cm strips, placed on 150 g paper and coated with polypropylene (PP) adhesive film to obtain the indicator label. These indicator labels were placed in polyethylene (PE) plastic bags and stored at -18°C and 4°C in a freezer and a refrigerator, respectively, to determine their color stability and stored at 4°C to investigate their color transition when exposed to ammonia vapor in the headspace of their solutions at various concentrations of 0.005, 0.01, 0.02, 0.04 or 0.08 M (Kuswandi et al., 2012a) prior to the fish spoilage trial.

Fish spoilage trial

Experimental setup

Sea bass fillet samples (100 g) were aseptically placed into sterilized 250 mL PE trays and then heat-sealed with PA/LLDPE film. Each type (A2 and A3 formulas) of indicator label was attached in each PE tray. All tests were performed in triplicate. The samples were kept at 4°C and periodically analyzed for product quality in terms of their TVB-N content during storage. Any color change of the colorimetric Mohr's salt-based indicator was measured instrumentally using a CR-400 chroma meter (Konica Minolta; Japan) with the L, a and b values used to describe the color of the indicator. The index describing the total color difference (TCD) was suggested by Hunt (1991) and is known also as delta *E* (Δ*E*) and was calculated using Equation 2:

$$\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2} \quad (2)$$

Additionally, a further approach was performed to permit the correlation of the response of the indicator label to the freshness of sea bass fillets during kept at 4 °C.

Statistical analysis

The data were analyzed using one-way analysis of variance in the SPSS software package (version 19; SPSS Inc.; USA). Significant differences among treatments were tested using Duncan's multiple range test at $p < 0.05$.

Results and Discussion

Microbiological and chemical changes in fish fillets

The initial TVC and *Pseudomonas* spp. count of fresh sea bass fillets were 4.38 log CFU/g and 1.97 log CFU/g, consecutively, whereas Paleologos et al. (2004) reported that *Pseudomonas* spp. was foremost in all cases for whole, gutted and filleted Mediterranean sea bass (*Dicentrarchus labrax*) over a complete storage time of 16 days, with a count of 4.6 log CFU/g at day 1 of refrigerated

conditions for their initial *Pseudomonas* counts. In the current study during storage at 4°C, the TVC and *Pseudomonas* spp. count gradually increased and reached the minimum spoilage level (6 log CFU/g) after 6.0 d and 6.74 d, respectively, (Fig. 1) with TVB-N levels of 22.84 mg N/100 g sample and 25.87 mg N/100 g sample, respectively (Fig. 2 and Fig. 3, respectively). The TVB-N contents, as a chemical spoilage index (CSI), was highly correlated with TVC (correlation coefficient, r = 0.98) and the *Pseudomonas* spp. count (r = 0.98), as a microbiological quality factor. This was consistent with Gui et al. (2014) who stated the TVB-N level in freshly caught fish is normally in the range 5−20 mg N/100 g sample.

The TVB-N level is another index that can be used to monitor fish quality. The last process of fish deterioration can involve an oxidation reaction of protein in the muscles, releasing volatile nitrogen compounds such as ammonia, DMA and TMA (Parlapani et al., 2015; Dehaut et al, 2016). Cheng et al. (2016) reported on the limitations on freshness of various fish species were enforced with no consolidated standard based on the TVB-N content in various countries and continents. National Standard of the People's Republic of China (2015) has the rejection limits for TVB-N in freshwater fish and marine fish at 20 mg N/100 g sample and 30 mg N/100 g sample, respectively. The acceptable limits for human consumption (cold-water fish in the European Union) are typically 30−35 mg N/100 g sample (European Union, 1995).

Color stability of indicator labels during chilled and frozen storage

During the ligand exchange reaction, Mohr's salt can be transformed to hexa-aqua iron (II) ions ($[Fe(H_2O)_6]^{2+}$) when it is dissolved in water as the water molecules which are surrounded by iron atoms can exchange with other molecules in the system causing variation in the oxidation number (Cox, 2004; Housecroft and Sharpe, 2005). In the current study, glycerol acted as a plasticizer and electron donor as shown in Equation 3; when the water receives electrons from glycerol, the hydroxide ion can be generated, as shown in Equation 4:

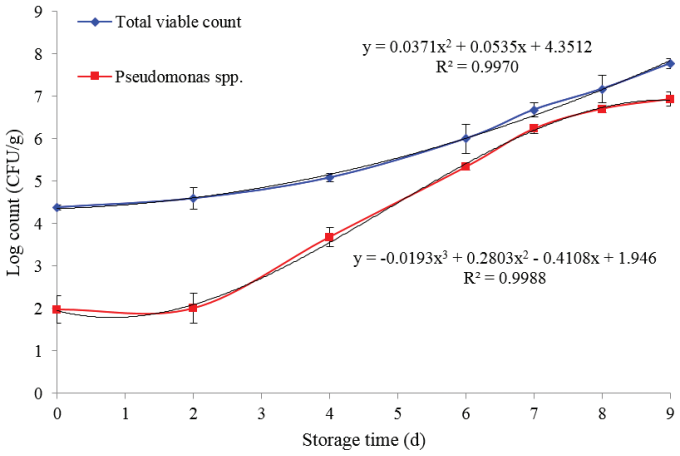

Fig. 1 Change in microbial counts of sea bass fillet kept at 4°C, where log count is in colony forming units (CFU) and error bars indicate ± SD

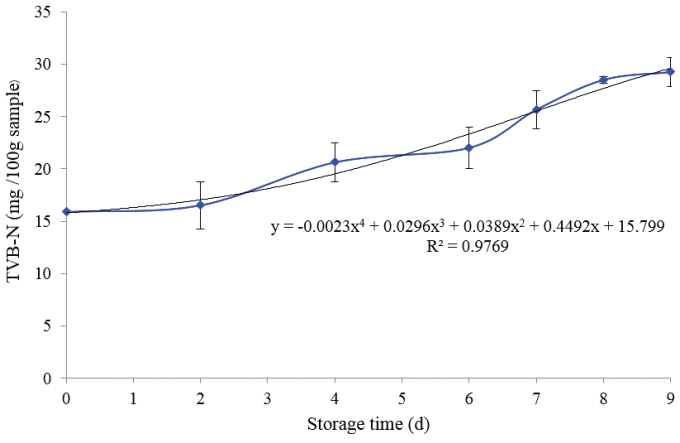

Fig. 2 Change in total volatile basic nitrogen (TVB-N) contents of sea bass fillet kept at 4°C, where error bars indicate ± SD

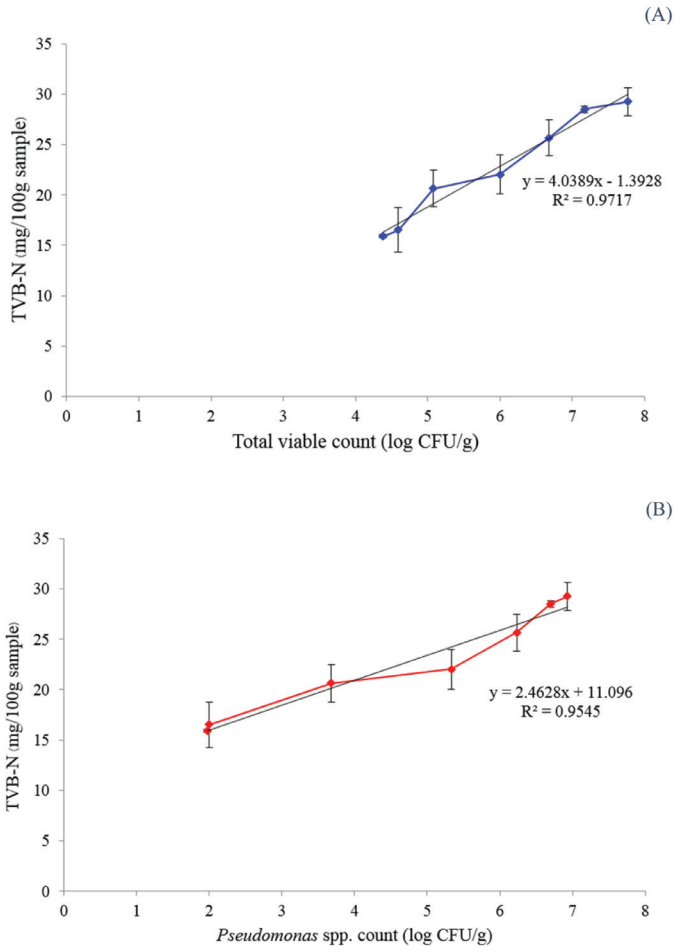

Fig. 3 Relationship between total volatile basic nitrogen (TVB-N) contents and microbial counts of sea bass fillet kept at 4°C: (A) total viable microorganism; (B) *Pseudomonas* spp., where error bars indicate ± SD

$$C_3H_8O_3 + 2OH^- \rightarrow C_3H_6O_3 + 2H_2O + 2e^- \quad (3)$$

$$\frac{1}{2}O_2 + H_2O + e^- \rightarrow 2OH^- \quad (4)$$

The hydroxide can react with Mohr's salt via a ligand exchange reaction as shown in Fig. 4 to yield green or yellow precipitate (Barrett, 2003). Prior to the usage of an indicator label, its color stability is necessary to prevent misleading consumers. As demonstrated in Table 1, the TCD (ΔE) of the indicator label decreased with increasing acetic acid concentration because the higher concentration, the more proton acid is formed. Nevertheless, there was strong color stability in the indicator label before its use was targeted, with TCD ≤ 1 set for this rigorous criterion. It was found that the TCD of the indicator labels stored at -18°C had lower values during the storage period.

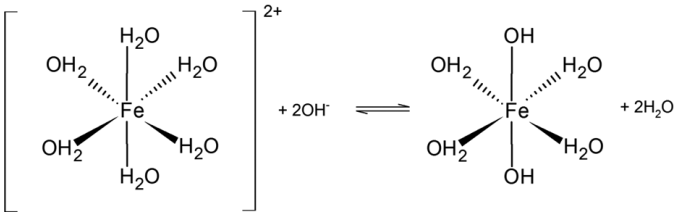

Fig. 4 Chemical reaction between Mohr's salt and hydroxide ion

Table 1 Total color difference of acetic acid-incorporated Mohr's salt-based indicator label under different storage conditions

-18°C			
Day	Concentration of acetic acid (% volume per volume)		
	0.01 (A1)	0.05 (A2)	0.10 (A3)
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
3	0.39 ± 0.01	0.32 ± 0.12	0.12 ± 0.03
5	0.42 ± 0.02	0.33 ± 0.03	0.25 ± 0.08
7	0.44 ± 0.03	0.38 ± 0.09	0.38 ± 0.12
9	0.50 ± 0.04	0.45 ± 0.04	0.42 ± 0.03
12	0.80 ± 0.16	0.46 ± 0.08	0.42 ± 0.05
4°C			
Day	Concentration of acetic acid (% volume per volume)		
	0.01 (A1)	0.05 (A2)	0.10 (A3)
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
3	0.43 ± 0.03	0.43 ± 0.09	0.21 ± 0.03
5	0.56 ± 0.08	0.57 ± 0.21	0.24 ± 0.07
7	0.76 ± 0.02	0.75 ± 0.23	0.40 ± 0.15
9	0.89 ± 0.05	0.81 ± 0.35	0.44 ± 0.11
12	1.03 ± 0.26	0.93 ± 0.39	0.58 ± 0.11

A1 = acetic acid 0.05% volume per volume (v/v); A2 = acetic acid 0.05% v/v; A3 = acetic acid 0.10% v/v

Therefore, the indicator labels with A2 and A3 formulas were screened for volatile sensitivity testing. Incorporation of acetic acid solution was used to retard the oxidation reaction, which in turn minimized the color change before use. In acid dissociation, the proton (H^+) combines with a hydroxide ion and makes a water molecule that forces the equilibrium to reverse (Chang, 2008), in turn, color stability of the indicator labels was achieved.

Color change of indicator labels during chilled conditions

The visible color changes of the colorimetric acetic acid-incorporated Mohr's salt-based indicator labels are illustrated in Fig. 5. The labels showed a manifest color transition from colorless to dark green when exposed to ammonia in the range 0–0.08 M. According to Table 2, the Mohr's salt-based indicator label with a lower concentration of acetic acid yielded greater TCD values when the ammonia concentration increased and vice versa because a lower acetic acid concentration contributed to less equilibrium reversal (Chang, 2008). The main purpose of applying these indicator labels to fish packaging is to be able to easily and responsibly inform on the degree of spoilage of the packaged fish fillets using a non-destructive approach during distribution and retail sale.

Color changes of indicator labels during fish spoilage trial

Fig. 6 illustrates the change in the TVB-N level of the sea bass fillet package with the Mohr's salt-based indicator label during the

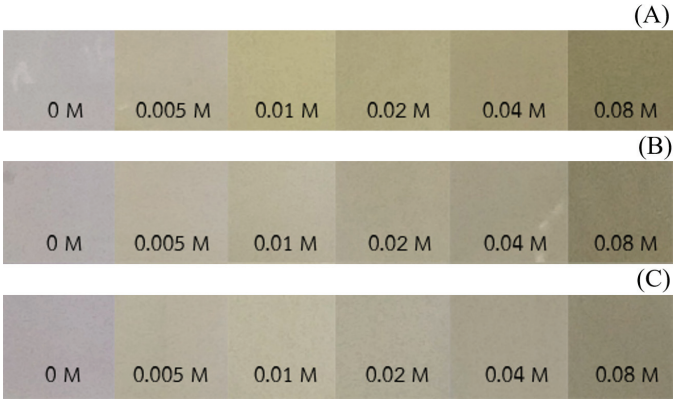

Fig. 5 Change in color of acetic acid-incorporated Mohr's salt-based indicator label kept at 4°C in response to ammonia at different concentrations: (A) A1, acetic acid 0.05% volume per volume (v/v); (B) A2, acetic acid 0.05% v/v; (C) A3, acetic acid 0.10% v/v

Table 2 Total color difference of acetic acid-incorporated Mohr's salt-based indicator label tested with various ammonia concentrations

Concentration of acetic acid	Concentration of ammonia (M)				
(% volume per volume)	0.005	0.01	0.02	0.04	0.08
0.01	8.43 ± 0.02^{aC}	8.82 ± 0.04^{bC}	9.12 ± 0.03^{cC}	9.39 ± 0.02^{dC}	10.63 ± 0.07^{eC}
0.05	6.22 ± 0.02^{aB}	7.34 ± 0.02^{bB}	8.82 ± 0.02^{cB}	9.11 ± 0.04^{dB}	9.86 ± 0.03^{eB}
0.1	5.07 ± 0.03^{aA}	6.42 ± 0.04^{bA}	6.93 ± 0.04^{cA}	8.46 ± 0.03^{dA}	9.10 ± 0.04^{eA}

Values (mean ± SD) in the same row with different lowercase superscripts are significantly ($p < 0.05$) different. Values (mean ± SD) in the same column with different uppercase superscripts are significantly ($p < 0.05$) different.

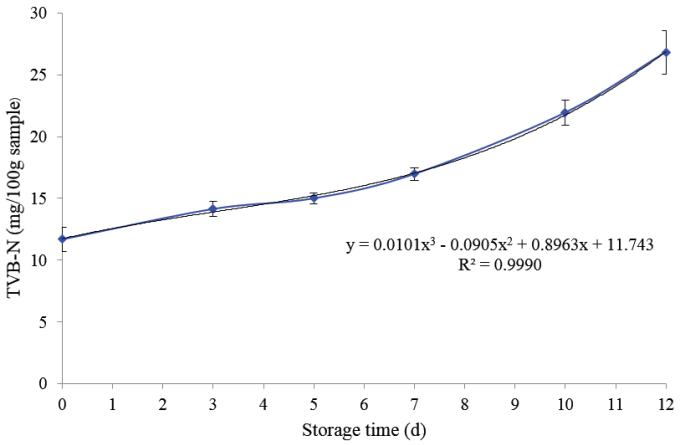

Fig. 6 Change in total volatile basic nitrogen (TVB-N) contents of sea bass fillet package with Mohr's salt-based indicator label stored at 4°C during fish spoilage trial, where error bars indicate ± SD

fish spoilage trial involving storage at 4°C. These findings showed that the MSL of sea bass fillet occurred on day 9, coinciding with a TVB-N level of 20 mg N/100 g sample during storage at 4°C. A rise in the TVB-N level was produced by the degradation of low molecular weight nitrogen-based compounds that released volatile nitrogen compounds including ammonia, DMA and TMA. These compounds can dissolute into water to form hydroxide ions as revealed in Equation 5−7, respectively. In addition, various amounts of biogenic amines (such as histamine, tyramine, putrescine and cadaverine) are normally generated during seafood decomposition, depending on the fish species (Lehane and Olley, 2000; Prester, 2011; Biji et al., 2016). The amines are derived from bacteria naturally found in putrefied fish in which decarboxylation of the free amino acids occurs (Halász et al., 1994; Önal, 2007).

$$NH_3 + O_2H \rightarrow NH_4^+ + OH^- \quad (5)$$

$$(CH_3)_2NH + H_2O \rightarrow (CH_3)_2NH_2^+ + OH^- \quad (6)$$

$$(CH_3)_3N + H_2O \rightarrow (CH_3)_3NH^2 + OH^- \quad (7)$$

Fig. 7 shows the color change in terms of the TCD of the Mohr's salt-based indicator label on sea bass fillet packaging stored at 4°C during the fish spoilage trial. While there was a a gradual rise in the TCD for both the A2 and A3 formulas, there was no difference during the onset of fish spoilage. After 7 d, there was a substantial increase in the TCD of both formulas, nearly reaching the stationary phase on days 10 and 12, respectively. The A2 formula had a higher slope than the A3 formula due to the former's lower acetic acid concentration. The relationship between the TVB-N content and TCD of the Mohr's salt-based indicator label on sea bass fillet packaging stored at 4°C during the fish spoilage trial is shown in Fig. 8. The TVB-N contents were positively correlated with the TCD of the A2 (r = 0.96) and A3 (r = 0.97) indicator labels. The basic volatile compounds which reacted with acetic acid-incorporated Mohr's salt resulted in a gradual change in color from colorless to an initial light brown at the periphery during 3 d of storage, followed by dark brown at the periphery and light brown in the center (the warning stage) during 5 d of storage, due to the diffusion process of these volatile compounds along the cross-section of label's thickness. Then, there was a rapid change to dark green (the expired stage) after 10−12 d of storage. Typically, TCD values greater than 5 can be easily perceived by the naked eye and TCD values greater than 12 signifies produces a very distinctive color space (Francis, 1983).

The results presented in this work indicated that exposure to spoilage metabolites in fish fillets could be determined using a non-invading colorimetric technique. The indicator signal was proved to be linked with the TVB-N contents from the chemical metabolites from microbial growth in the sea bass fillet samples, hence permitting the simultaneous indication of fish spoilage. This alternative colorimetric Mohr's salt-based spoilage indicator label assists in maintaining an useful shelf life for the food product by allowing an indicator of the freshness to be observed visibly next to the best before date, thus lessening the potential for mistakes by consumers in using expired products. A possible future research arena is the augmentation of this novel concept as an alternative spoilage indicator label to other food products.

Conflict of Interest

The authors declare that there is no conflict of interest.

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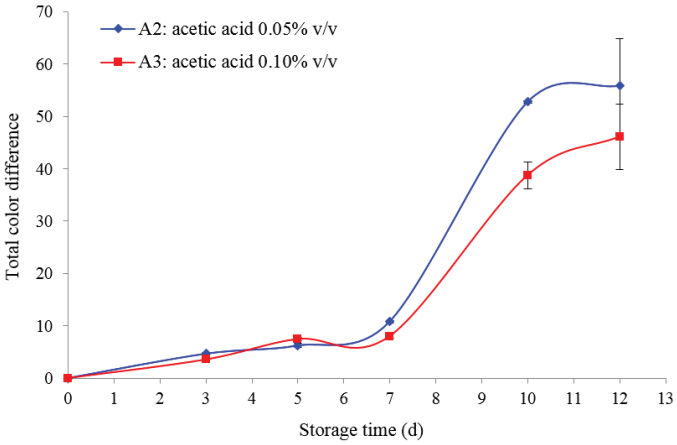

Fig. 7 Change in total color difference of Mohr's salt-based indicator label on sea bass fillet package kept at 4°C during fish spoilage trial, where error bars indicate ± SD

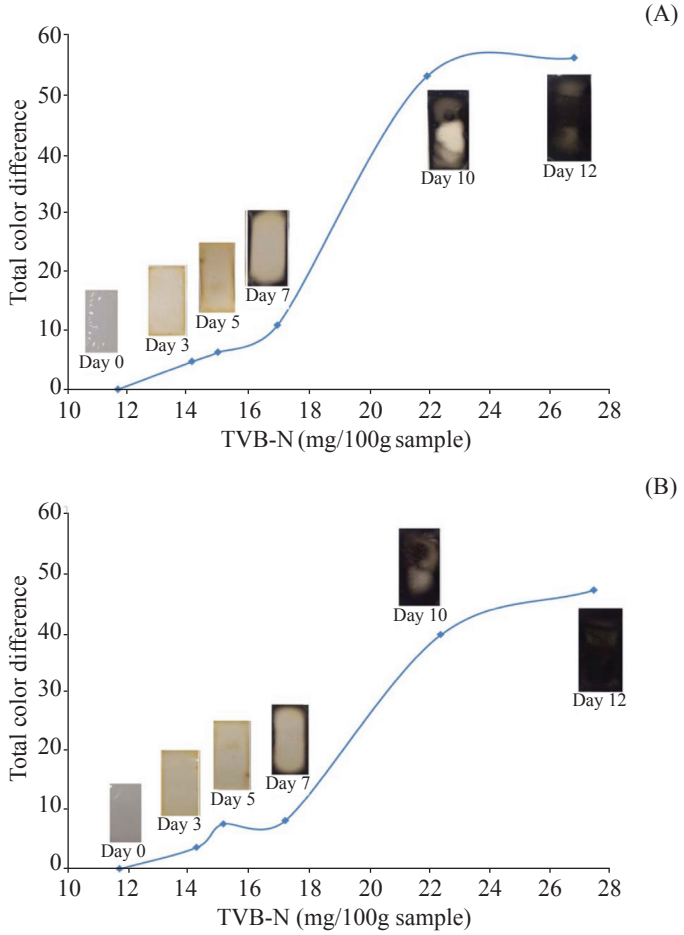

Fig. 8 Relationship between total volatile basic nitrogen (TVB-N) contents and total color difference of Mohr's salt-based indicator label on sea bass fillet package kept at 4°C during fish spoilage trial: (A) A2, acetic acid 0.05% volume per volume (v/v); (B) A3 acetic acid 0.10% v/v

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