CHAPTER VIII
NATURAL DYE-PLASTIC SENSOR FOR FISH FRESHNESS

8.1 Abstract

Presently, a number of people are apprehensive about the reliability of synthetic dyes as freshness sensors in food packaging. The natural dye extracted from Sappan wood (*Caesalpinia sappan* L.) has competency in shade sensitivity to acidic and alkaline atmosphere. To tap its potential, a colorimetric indicator for detecting fish freshness was developed based on ethylene vinyl acetate-sappan-dyed carboxymethyl cellulose (EVA-SAP-CMC) composite films by varying the amount of dye powder (1, 3, and 5 wt% of SAP-CMC). UV-Vis revealed that the more dye added, the higher absorbance level of the films observed. The color measurement was reported in the Hunter color system values (L*, a*, b*) and total color difference (ΔE). With respect to the amount of dye added to the composite, the film become darker as evidenced in lower lightness (L*). The redness (+a*) and yellowness (+b*) were higher. The color of the pH indicator film to various concentrations of standard ammonia was measured. After exposure to ammonia, the color gradually changed from orangish-red to orangish-pink at 0.1 mg/mL of ammonia (pH 9.25) and turned dark orangish-brown at 35 mg/mL (pH 11.31). The indicator responsiveness was found to correlate with higher ammonia concentration, thereby enabling the real-time monitoring of spoilage. Finally, the fish spoilage test was conducted via the total volatile basic nitrogen (TVBN) approach. The TVBN level increased during the storage time at room temperature. The TVBN level of 35 mg/100 g of fish sample was reached over 7 hours of storage time at room temperature implying that the fish was rarely acceptable for human consumption. The fish freshness was indicated by the relationship between TVBN values and the ΔE with greater than 2, from orangish-red to orange, during the fish starting lacking of freshness. The sensor film was able to detect the fish freshness. It could also be used for others.

Keywords: Carboxymethyl cellulose, EGMA, Intelligent film, Natural Dye, Sappan, Sensor Film

8.2 Introduction

A device to evaluate real-time freshness of food products has been developed. The globalization of the food business and the logistics of distribution from processing centers pose major challenges for food quality and safety to food industries, retailers, and consumers. They have fabricated the intelligent packaging based on the development of freshness indicator films. The films are useful for facilitating decision making to consumers because they provide a great deal of essential information on quality and safety, and especially warning possible problems to consumers (Rukchon *et al.*, 2011). In the case of fish products, both volatile and non-volatile amines are formed during the breakdown of their freshness. Total volatile basic nitrogen (TVBN) plays a crucial role in being a marker indicating fish freshness so it is a realiable method for assessing the quality of fish during storage (Rukchon *et al.*, 2011).

Recently, a great concern on the carcinogenicity and environmental pollution of man-made pigments has become an issue. Consequently, the use of natural dyes has captured the worldwide attention because of its eco-safety (Lee *et al.*, 2008). The natural dye extracted from Sappan wood (*Caesalpinia sappan* L.) is able to be shade sensitive to acidic and alkaline environments with thanks to its characteristic chromophore and auxochrome. Rattanapatiphan and Patanathabutr (2009) prepared an acid – alkaline indicator film based on polyvinyl alcohol (PVA) – sappan dye – aluminum silicate (Al_2O_3 – SiO_2) composites via the sol – gel technique, following by the film casting approach. The composite film showed visual change to orange and pink after contacting to acidic and alkaline conditions, respectively. They added that the as-prepared film was applicable for intelligent food packaging application (Rattanapatiphan, 2009). In addition to natural dyes, carboxymethyl cellulose (CMC) is a valuable and extensively used component in a number of foodstuffs, such as a stabilizer, binder, thickener, suspending and water-retaining agent (Hegedušі, 2000). The Food and Drug Administration (FDA) classifies the CMC as "GRAS" or "generally recognized as safe" (Elliot, 1974). In 2007, Dikobe and Luyt reported that the presence of poly (ethylene-co-glycidyl methacrylate) or EGMA in ethylene vinyl acetate (EVA) – cellulosic fiber composite showed better compatibility in comparison to that of uncompatibilized one due to the enhanced interaction between the hydrophilic fiber and the hydrophobic plastic matrix (Dikobe, 2007).

This work has focused on a colorimetric indicator film for detecting the fish spoilage based on natural dye and plastic. The sensor film was fabricated by melt-blending ethylene vinyl acetate (EVA) and sappan-dyed carboxymethyl cellulose (SAP-CMC) using poly (ethylene-co-glycidyl methacrylate) or EGMA as a compatibilizer. The absorption spectra of sensor films at various contents of dye powder were characterized by UV-Vis spectrophotometer. The response of the sensor films to standard ammonia was carried out as a model of fish spoilage. Furthermore, the determination of total volatile basic nitrogen (TVBN) and indicator films response to fresh fish tissue were performed. Finally, the direct and indirect leaching studies of sensor were also studied by UV-Vis spectrophotometric analysis.

8.3 Experimental Procedures

8.3.1 Materials

Commercial Ethylene Vinyl Acetate, EVA pellet under trade name Polene MV1055 (18% vinyl acetate) LOT 12080161, supplied by TPI POLENE Public Company (TPIPL). Dried sappan wood (*Caesalpinia sappan* Linn.) powder was purchased from a traditional drug store in Singburi, Thailand. Carboxymethyl cellulose, CMC was kindly provided by Division of Packaging Technology, School of Agro-Industry, Faculty of Agro-Industry, Chiang Mai University. Poly(ethylene-*co*-glycidylmethacrylate), EGMA was purchased from ALDRICH CAS NO. 26061-90-5. Ammonia 30 % (as NH_3) was purchased from Panreac CAS NO. 1336-21-6. The concentration of the ammonia concentration is 0.892 – 0.898. A fresh white perch (*Lates Calcarifer*) was freshly purchased from Samyan Market in Bangkok, Thailand. All of the materials were used as received.

8.3.2 Preparation of natural dye-carboxy methyl cellulose (SAP-CMC)

The dried sappan heartwood powder was extracted in a deionized water at the ratio of 1:4 (w/v). After that, the solid residue was filtered out. 200 g of carboxymethyl cellulose (CMC) was mixed with the dye extract at the ratio of

0.5:100 (w/v) and finally spraied dye at 150 °C to sappan dyed-carboxymethyl cellulose (SAP-CMC) powder.

8.3.3 Fabrication of pH Indicator films based on EVA-SAP-CMC

The Dyed-CMC powder and ethylene vinyl acetate pellet were dried in a vacuum oven at 60 °C for 24 hours prior to use. The ethylene vinyl acetate (EVA) were melt-mixed with 1, 3, and 5 wt% of the Dyed-CMCs using 5 phr of the ethylene glycidyl methacrylate (EGMA) as a reactive compatibilizer via the co-rotating twin screw extruder (Labtech types LTE-20-32 & LTE-20-40) with an L/D ratio of 40 and screw diameter of 20 mm. The temperature profiles of the barrel were 95 – 105 °C from the hopper to the die, and the screw speed was 30 rpm. Afterward, the extruded pellets were dried in a vacuum oven at 60 °C for 24 hours before being hot-compressed into thin film of ~150 μm in thickness. The composite was hot-compressed using compression molding machine (Labtech type LP 50) at 140 °C by pre-heating time of 15 min, compressing time of 5 min at pressure of 1500 psi., and cooling time of 5 min to thin film of sensor.

8.3.4 The Response of the Sensor Films to Standard Ammonia

25 ml of standard ammonia solutions in various concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 1, 5, 10, 15, 20, 25, 30, and 35 mg/mL) were pipetted into the polypropylene cup which was sealed to prevent the leakage of ammonia. Sensor films were faced-down inside the cup. The experiment was kept at room temperature for 6 hours. The color response of films at was measured by a color reader (HunterLab, ColorFlex EZ). Three different areas for each film were recorded. The result was recorded on Hunter color system, in which the outcome was expressed as the Hunter color L, a, b values and total color difference (TCD) or ΔE. The TCD value was calculated by following equation:

$$\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \qquad \text{(Eq. 8.1)}$$

Where, ΔL^* = The brightness difference between sample and target

Δa^* = The redness difference between sample and target

Δb^* = The yellowness difference between sample and target

The target color is (93.13, -0.96, 1.69) corresponding to (L, a, b) for white standard color in the Hunter system.

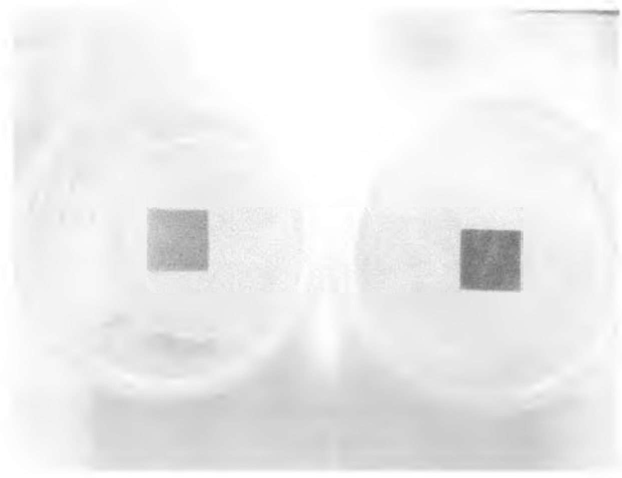


Figure 8.1 Experimental design for the response of the sensor films to different concentrations of standard ammonia (left) the original color of a sensor film and (right) the color change of sensor film at the ammonia concentration of 10 mg/mL after 6 hr of test time.

8.3.5 Determination of Total Volatile Basic Nitrogen (TVBN) and Indicator Films Response to Fresh Fish Tissue

The total volatile basic nitrogen released during the fish spoilage was measured following the procedure.

8.3.5.1 Sample Preparation

Grind the fish sample by homogenizer and 3 g of homogenized sample was placed into a centrifuge tube. Then, 12 ml of 4% trichloroacetic acid (TCA) solution was added to the centrifuge tube and the tube was sealed and shaken to make sure that it was properly mixed. After that, the sample was left at room temperature for 30 min with stirring from time to time. The sample mixture was filtrated using Whatman paper no.1.

8.3.5.2 Measuring of TVBN

Sealing agent (Vaseline) was first applied to the top edge of the Conway's unit. The inner ring solution (1% boric acid mixed with 1 ml of indicator) was pipette and placed into the inner ring of conway's unit. Then, 1 ml of filtered sample extract was pipette into the outer ring of conway's unit. 1 ml of saturated K_2CO_3 solution was pipette into the outer ring of conway's unit and placed on the opposite side of the sample solution. The Conway's unit was immediately shaken in order to mixed sample solution and K_2CO_3 solution without contaminating the inner ring of conway. The samples were kept at room temperature for 3 h. After the color of boric solution changed from pink to green, following the generation of volatile base, this sample was titrated with 0.01 N HCl containing in a micro-burette until the color changed back to pink.

8.3.5.3 Calculation

TVBN can be calculated by following equation:

$$\text{TVBN}\left(\text{mg}/100\ \text{g}\right) = \frac{\left(V_S - V_B\right) \times \left(N_{HC} \times A_N\right) \times \left[W_S \times \left(\frac{M}{100}\right) + V_E\right] \times 100}{W_S} \qquad \text{(Eq. 8.2)}$$

Where, Vs = Titration volume of 0.01 N HCl for sample extract (ml)

V_B = Titration volume of 0.01 N HCl for blank (ml)

N_{HCl} = Normality of HCl (= 0.01 N × factor of HCl)

A_N = Atomic weight of nitrogen (14.00)

W_S = Weight of tissue sample (g)

M = Percentage moisture of tissue sample (Assume 80%)

V_E = Volume of 4% TCA used in extraction

8.3.6 <u>The Response of the Sensor Films to Fresh Fish Storage</u>

A fresh white perch *(Oreochromis niloticus* L.) approximately 800 g was selected for the experiment. The indicator film was placed in a 6″ × 9″ of PPS20BEN film (from Chapter VI) with containing approximately 100 g of fresh fish. The indicator film was attached at the distance of 1/3 from the top border of the film. The cup was sealed to prevent the leakage of TVBN. The color change of indicator film after the sample started to deteriorate was observed at 3, 6, 9, and 12 hours during the storage.

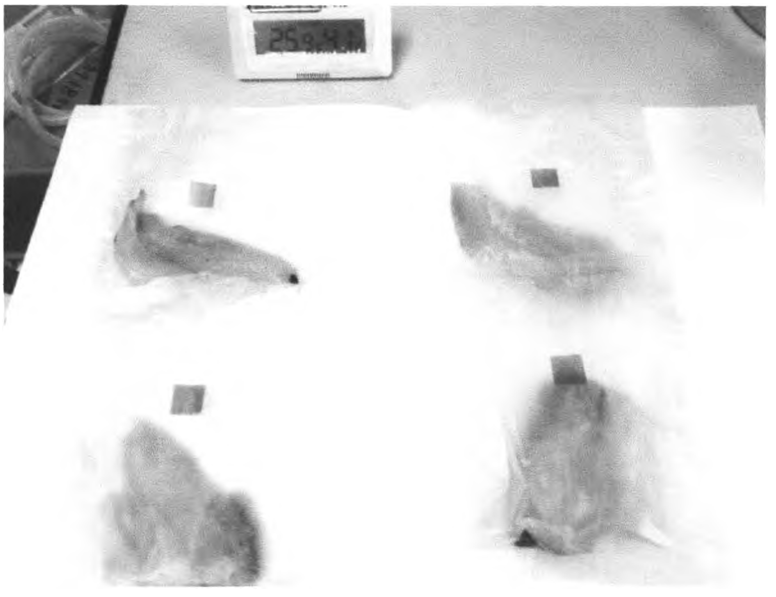


Figure 8.2 Experimental design for fish spoilage test.

8.3.6 Leaching studies

Indicator dye (SAP-CMC) was dissolved in water to prepare standard solutions of 1, 2, 5, 10, 20, and 100 ppm. The absorbance of standard solution was then investigated by the UV-Vis Spectrophotometer (Shimadzu Model UV-1800). The sensor films were cut into the rectangular shape with 2 x 2 cm and soaked in 30 mL of deionized water in a small container for approximately 24 hr as designed for " Direct leaching studies ". Another experiment was carried out by attaching sensor films on the lid of PP cup containing 30 mL of deionized water for 24 hr, designated as " Indirect leaching studies ". Afterward, the wavelength spectrum in range of 400 to 700 nm was carried out to detect the presence of pH dye. Each of the leaching studies was performed in 3 replication experiments.

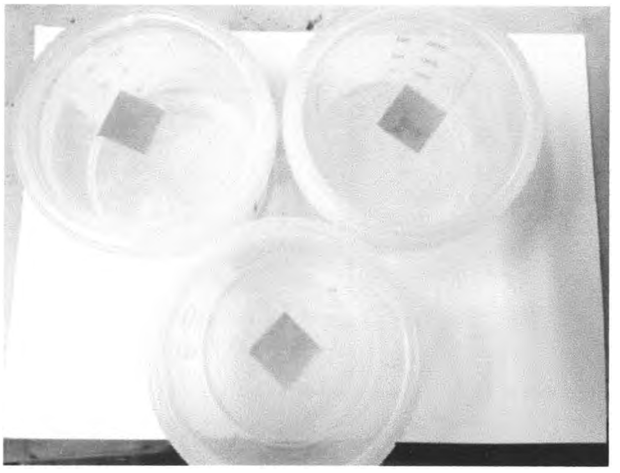


Figure 8.3 Experimental design set up for fish spoilage test designated as "Direct leaching study".

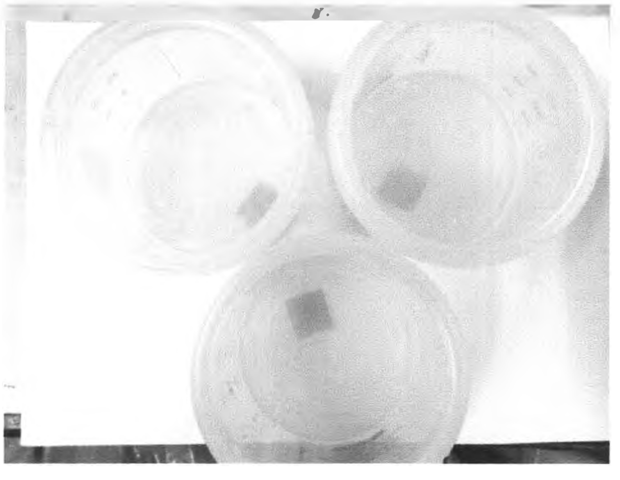


Figure 8.4 Experimental design set up for fish spoilage test designated as "Indirect leaching study".

8.3.7 Characterizations and Measurements

8.3.7.1 UV-Vis Measurement

For the optical characterization of the sappan extract, its absorption spectra were recorded on a UV/Vis spectrophotometer (Shimadzu UV-1800) while the absorbance spectra of films (2 cm x 2 cm) were collected on a UV/Vis spectrophotometer (Shimadzu UV-2550). The analyzed wavelength range was 400 - 700 nm with a resolution of 0.5 nm.

8.3.7.2 Color Reader

The color changes of the indicator film were measured through the HunterLab Model Colorflex with 45°/0° optical geometry and EasyMatch® QC software. The result expressed as Hunter system (L^*, a^*, *and* b^*) values and total color difference (ΔE).

8.4 Results and Discussion

8.4.1 Characterization of Sappan Wood Extract and EVA-SAP-CMC Films by the UV-Vis Spectroscopy

The Figure 8.5 shows the absorption wavelengths and absorbance of the extracted sappan wood. The maximum absorption wavelengths of the extract are observed at 448 and 539 nm in the UV-Vis spectrum. This indicates that the sappan dye is a multicolored material consisting the combination of two colors: yellow and red components according to Lee *et al.*'s work (2008).

The Figure 8.6 illustrates the UV-Vis spectra of sensor films at various contents of SAP-CMC (1, 3, and 5 wt%) compared to neat EVA film. There is no absorbance in the case of neat EVA films. On the other hand, the maximum absorbance spectra of EVA-SAP-CMC films are observed at around 450 and 550 nm, which are consistent with the two absorption bands of the sappan extract in the Figure 8.5. In addition, it is noticed that an increasing amount of SAP-CMC results in a greater absorbance level of the films. The highest absorbance is obtained from the film containing 5 wt% of SAP-CMC. As a result, the proportion of the light absorbed depends upon the concentration of dye molecules in the film.

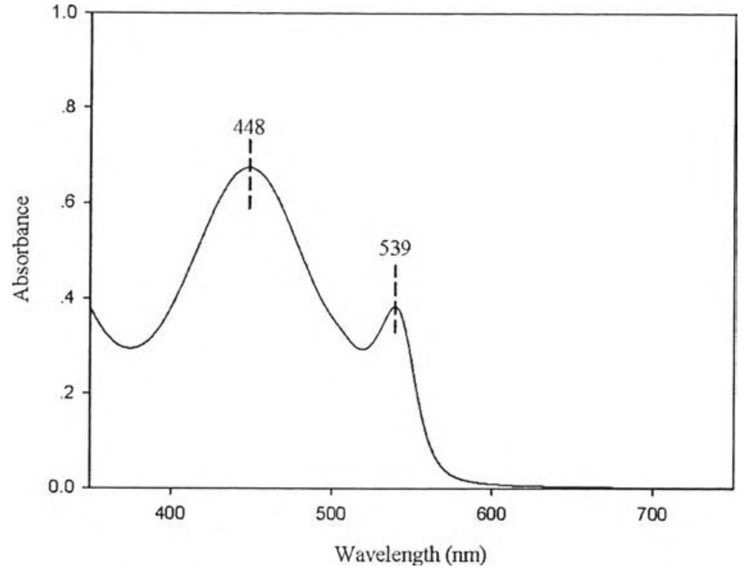


Figure 8.5 UV-Vis spectrum of sappan extract.

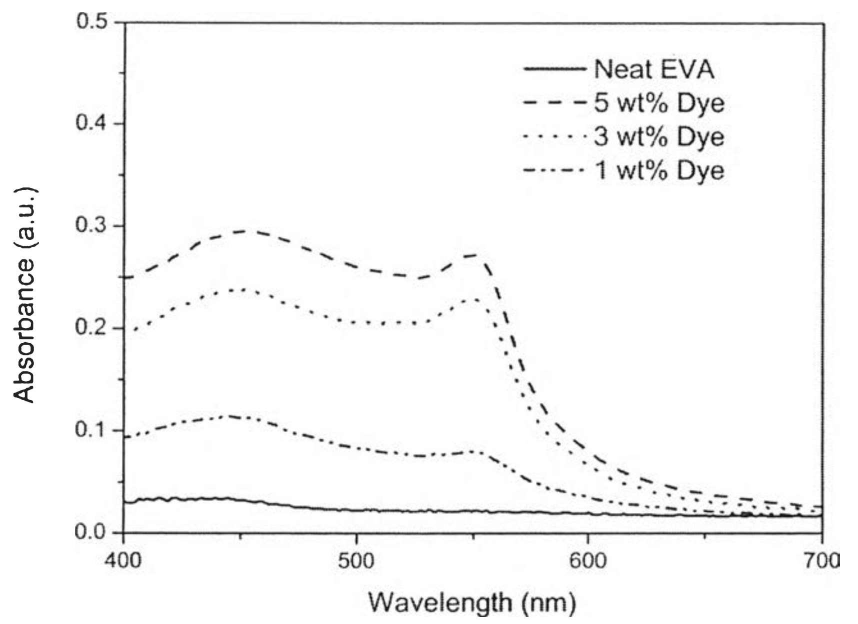


Figure 8.6 Absorption spectra of neat EVA and EVA-SAP-CMC films.

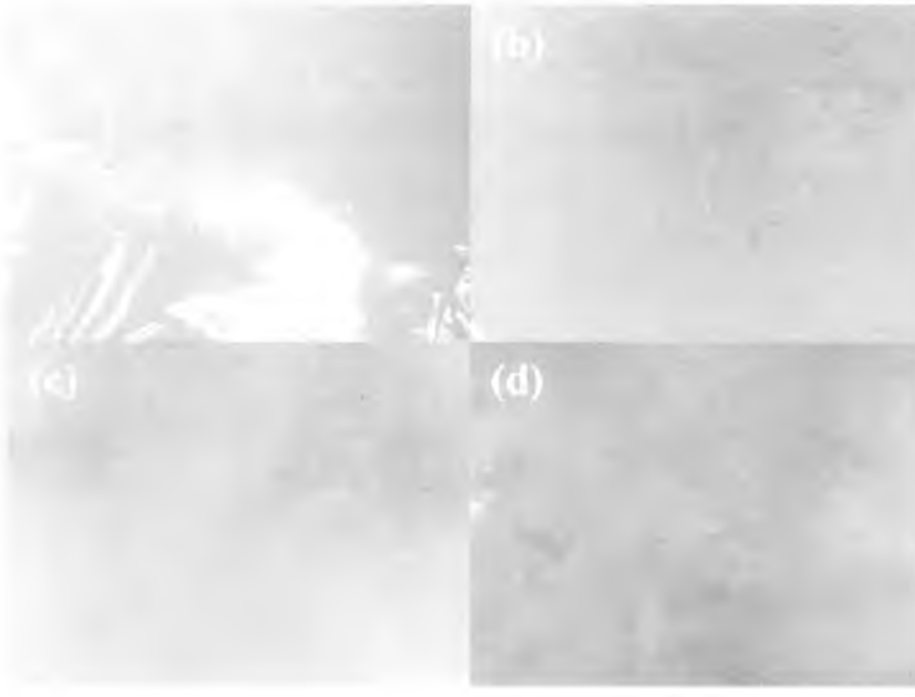


Figure 8.7 Photographs of EVA film and EVA composite films: (a) neat EVA, (b) EVA-1, (c) 3, and (d) 5 wt% of SAP-CMC films.

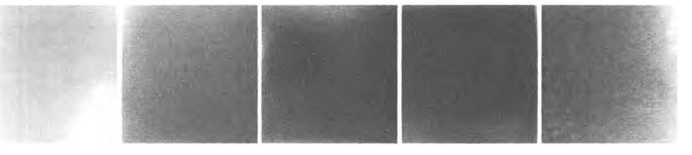
Table 8.1 Changes in Hunter color values of sappan-dyed EVA films

Sample	L*	a*	b*
EVA	15.93±0.11	-0.85±0.14	-2.27±0.26
EVA-1%dye	23.38±1.44	1.14±0.44	0.85±0.26
EVA-3%dye	21.68±0.90	4.02±0.45	2.14±0.28
EVA-5%dye	18.63±0.53	6.84±1.08	3.29±0.79

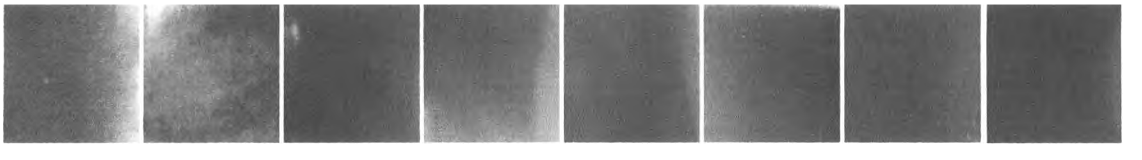
Moreover, the color measurement is reported in the Hunter color system values (L*, a*, b*) in the Table 8.1. With respect to the amount of dye added to the composite, the film becomes darker as evidenced in lower lightness (L*). The redness (+a*) and yellowness (+b*) are higher.

8.4.2 The Response of the Sensor Films to Standard Ammonia

Ammonia is one of the volatile compounds produced during the fish deterioration. Therefore, the study on sensor response to standard ammonia gas provides a representative model of the response to total volatile basic nitrogen (TVBN).

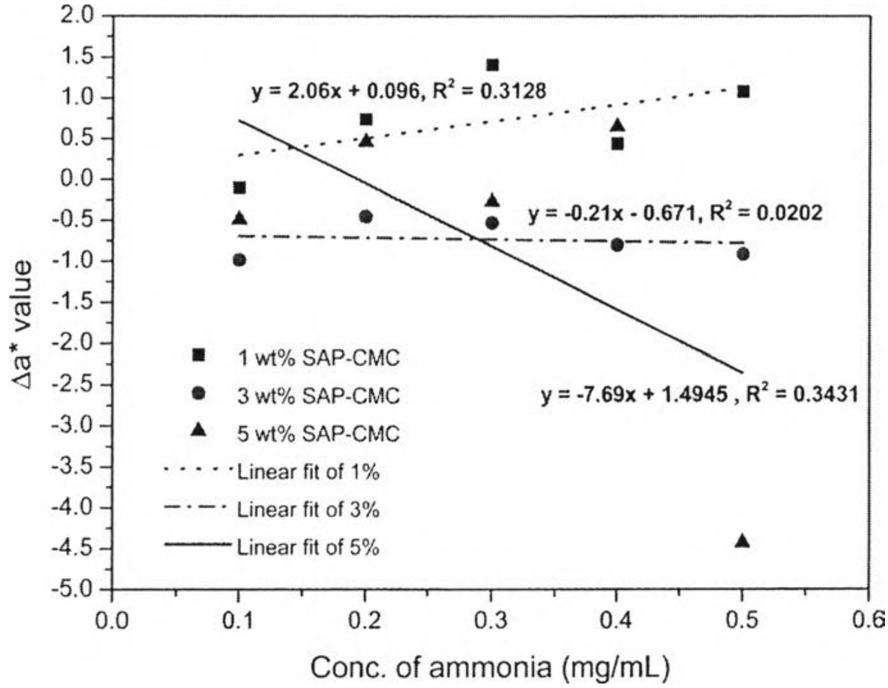


(a)

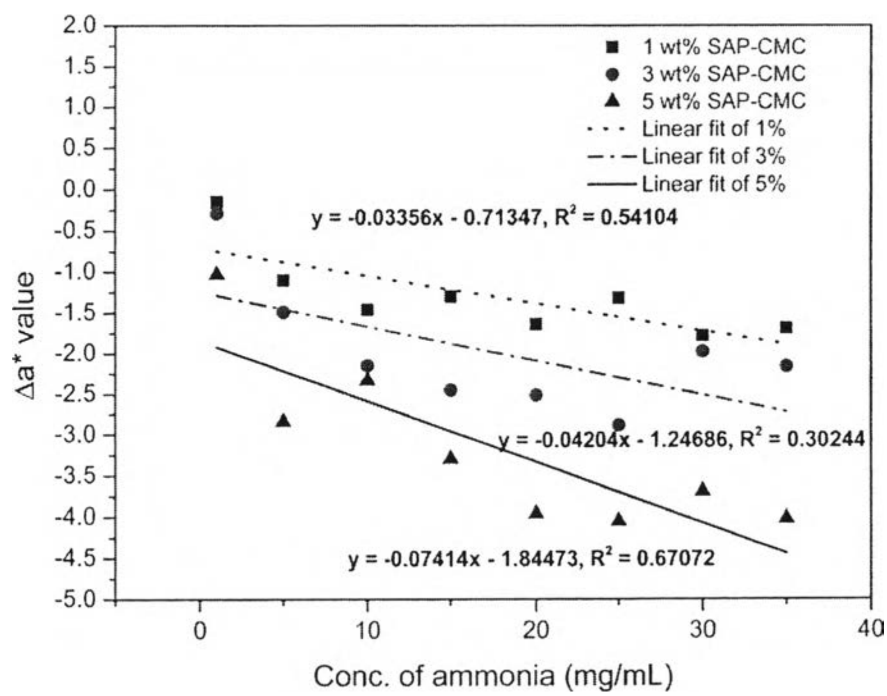


(b)

Figure 8.8 The photographs of EVA composites film with 5 wt% SAP - CMC after indirect contact with standard ammonia at (a) 0.1 – 1.0 mg/mL and (b) 1 – 35 mg/mL.



(a)



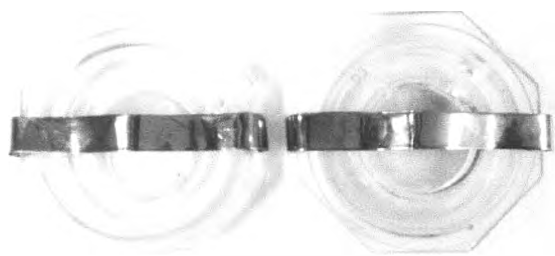
(b)

Figure 8.9 Changes in a* value of EVA-SAP-CMC films at 1, 3, and 5 wt% SAP-CMC after indirect contact at (a) 0.1 – 1.0 mg/mL and (b) 1 – 35 mg/mL.

After indirect contact at 1 – 35 mg/mL (pH 9.25 -11.31) of standard ammonia at room temperature, EVA-5 wt% SAP-CMC exhibits the highest change in a* or Δa* value (Figure 8.9 (a)). The changes of all sensor films provide the linear fitting trend indicating the good sensitivity to standard ammonia. The film at 5 wt% of SAP-CMC powder also manifests the greatest linearity corresponding to the highest R-squared value showing the visible change in color which can be detected by naked eyes. From the Figure 8.9 (b), its correlation between the change in a* value and ammonia concentration is reported as $y = -0.07414x - 1.84473$ where y is Δa* and x is concentration of ammonia in mg/mL, and R-square is equal to 0.67072. Therefore, the EVA – 5wt% SAP - CMC film can be applicable for detecting TVBN during the spoilage of fish.

8.4.3 Determination of Total Volatile Basic Nitrogen (TVBN) and Indicator Films Response to Fresh Fish Tissue

(a)



(b)

Figure 8.10 Photograph of (a) blank test (control) in comparison to the sample test at the outset of test and (b) after 3 hr of test time.

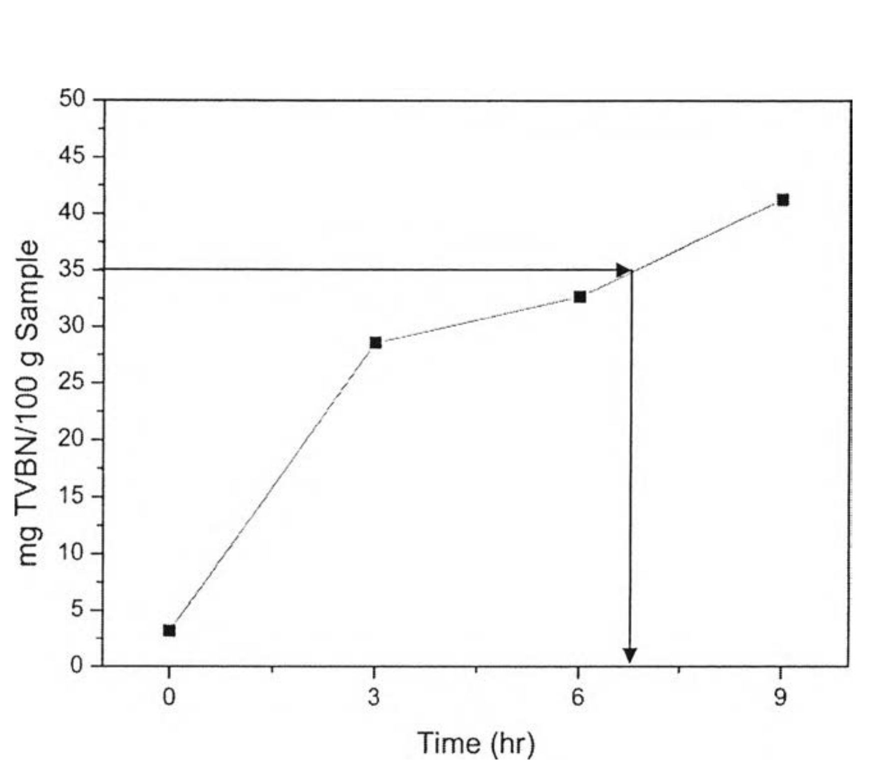


Figure 8.11 The TVBN level as a function of time.

Total volatile basic nitrogen (TVBN) in fish mainly consists of ammonia, trimethylamine (TMA), and dimethylamine (DMA). The TVBN is particularly detected in a fresh fish. The TVBN level increases with storage time as illustrated in the figure 7. The change in TVBN level relates to the microbial growth in a fish sample (Rukchon, 2011). Since fish starts deteriorating at a level of 35 mg TVBN/100 g, which is pertaining to spoiled fish meat, its quality is scarcely acceptable for human consumption (Seephueng, 2008). This level correlates to the graph (Figure 8.11) at around 7 hours of storage time at ambient temperature. As a consequence, an experiment on response test of the sensor film to TVBN during the fresh fish storage will be set up to monitor the sensing effect of the film.

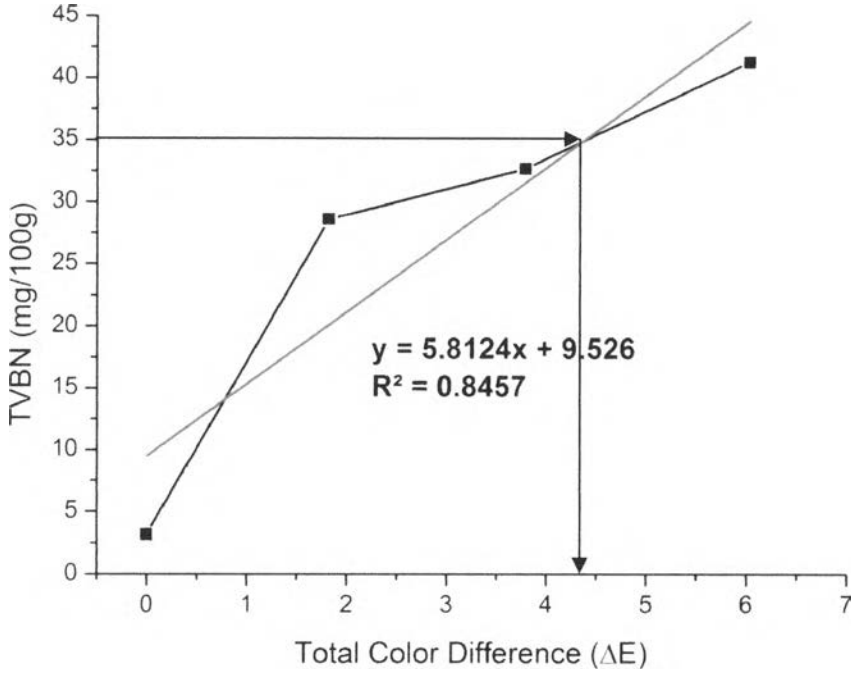


Figure 8.12 Correlation between the Total Color Difference (ΔE) and total volatile basic nitrogen (TVBN) value of the EVA – 5 wt% SAP – CMC film.

The sensitivity of the EVA-SAP-CMC film containing 5 wt% SAP-CMC obviously exhibits the linear increment of ΔE value as a function of TVBN value (Figure 8.12). For the value of total color difference (ΔE) of the sensor film, it was calculated according to the Eq. 8.1 as shown in section of experimental parts (8.3.4). Furthermore, the color change of the sensor film was compared to the original color of 5 wt% SAP-CMC in EVA-SAP-CMC films based on CIELAB system, of which the original values were designated as L* = 18.63, a* = 6.84, and b* = 3.29.

The correlation of total change in color and time is equal to y = 5.8124x + 9.526 where y is TVBN in mg/100g and x is ΔE value. The correlation of determination (R^2) of linear fitting of the data is 0.8457. It was found that the color of the sensor film was changed relating to 4.38 of ΔE because the large amount of TVBN, known as alkali volatiles, from the fish spoilage originating from microbial enzyme activity reacts with the proton in the dye structures causing the color change of the film (Rukchin, 2011). Evidently, the color change of the sensor appeared in orange rather than orangish pink as a result of lesser basic environment compared with standard ammonia. However, the visible change of sensor can be easily differentiated by naked eyes at the onset of spoilage with thanks to ΔE value greater than 2.

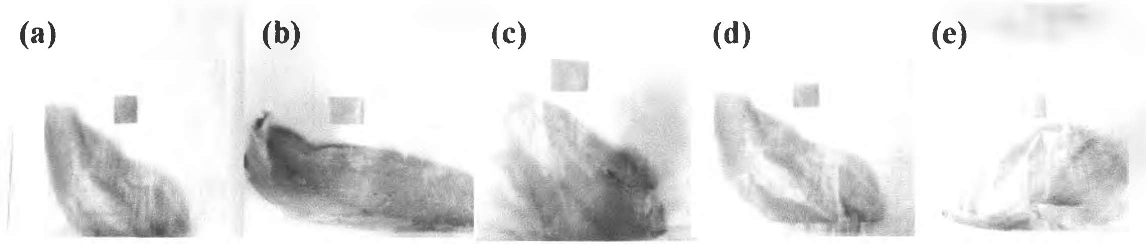


Figure 8.13 Response of sensor to fish spoilage test (a) at the onset of test, after test time of (b) 3 hr, (c) 6 hr, (d) 9 hr, and (e) 12 hr.

8.4.4 Leaching Studies

The leakage of the dye from the EVA-SAP-CMC 5% film with calibration curves was observed by UV-Vis spectrophotometer as shown in Figure 8.14 and Table 8.2, respectively. As the natural dye and carboxymethyl cellulose were water-soluble substance, the result demonstrated that there are certain amounts of Dye-CMC leaking away. After the sensor film was soaked in water and carried out to detect the presence of pH dye, the results showed that the concentration of dye leaking out in water was roughly 18.152 ± 0.934 ppm. In the application of intelligent packaging, the sensor film acts as a display within active films and only exposes to volatile gas during fish storage. The next experiment was carried out to study the indirect contact of sensor films. On the other hand, after indirect to water, the leaching of Dyed-CMC was around 0.937 ± 0.151 ppm, pointing out a very low amount of Dyed-CMC releasing in water compared to direct leaching measurment. Moreover, the water after leaching studies was as clear as the reference deionized water as evidently shown in the Figure 8.14 in both cases. The leakage of dye was not observed by naked eyes due to its clarity.

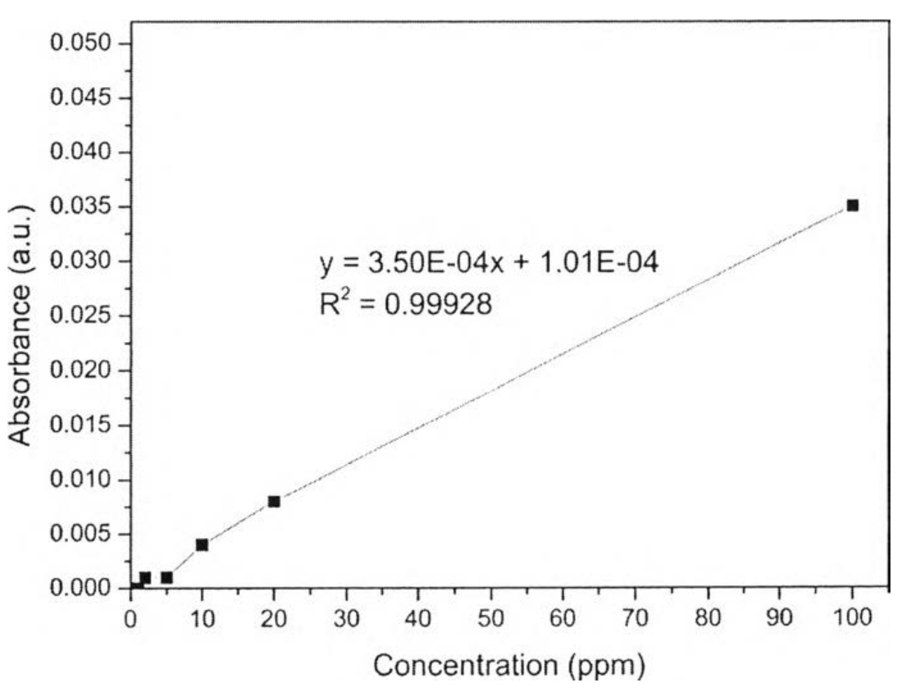


Figure 8.14 Standard curve of SAP-CMC solution.

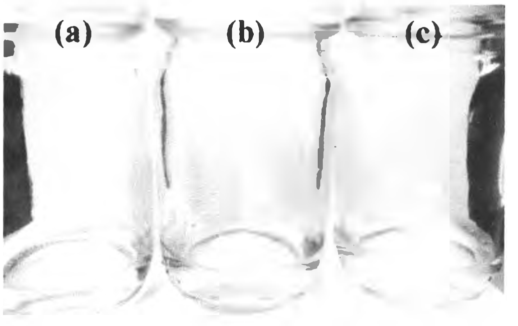


Figure 8.15 The deionized water after (a) indirect leaching study, (b) direct leaching study in comparison to (c) original deionized water.

Table 8.2 Concentrations of dye-cmc releasing from sensor films after direct immersion in water for 24 hours (A1 - A3) and indirect contact with water (B1 - B3) in three replication tests for each

Sample	Concentration (ppm)	Average Concentration (ppm)
A1	18.249	18.152 ± 0.934
A2	19.034	
A3	17.174	
B1	0.816	0.937 ± 0.151
B2	0.889	
B3	1.107	

8.5 Conclusions

Indicator films for detecting fish freshness were prepared based on EVA-SAP-CMC films using EGMA as a compatibilizer. The EVA-SAP-CMC containing 5 wt% of SAP-CMC provided the best sensitivity to detect the change in different concentrations of standard ammonia. The TVBN level of 35 mg/100 g of fish sample was accessed over 7 hours of storage time at room temperature implying that the fish was unacceptable for human consumption. The fish freshness was indicated by the correlation between TVBN values and the color changes in ΔE with value greater than 2, from orangish-red to orange, during the fish starting lacking of freshness. Therefore, the sensor is promising for being used as a pH-based indicator film in the smart packaging application, which benefits both consumers and fish industries.

8.6 Acknowledgements

This work was financially supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (FW0649A). The author is grateful for chemicals and the laboratory equipment support from Polymer Processing and Polymer Nanomaterials Research Unit, The Petroleum and Petrochemical College, Chulalongkorn University.

8.7 References

Dikobe, D. G., Luyt, A. S. (2007) Effect of Poly(Ethylene-*co*-Glycidyl Methacrylate) Compatibilizer Content on the Morphology and Physical Properties of Ethylene Vinyl Acetate–Wood Fiber Composites. Journal of Applied Polymer Science, 104, 3206–3213.

Elliot, J.H., Ganz, A.J. (1974) Some Rheological Properties of Sodium Carboxymethylcellulose Solutions and Gels. Rheologica Acta August/October, 13, 670-674.

Hegedušì, V. Herceg, Z. Rimac, S. (2000) Rheological Properties of CMC and Whey Model Solutions. Food Technology and Biotechnology, 38, 19–26.

Lee, D.-K. Cho, D.-H. Lee, J.-H. Shin, H.Y. (2008) Fabrication of Non-Toxic Natural Dye from Sappan Wood. Korean Journal of Chemical Engineering, 25, 354-358.

Rattanapatiphan, S. (2009) Investigation of Natural Dye-Aluminium-Silicate Polymer Composites for pH Indicators. M.Eng. Thesis, Department of Materials Science and Engineering, Silapakorn University, Nakhonpathom, Thailand.

Rukchon, C., Trevanish, S., Jinkarn, T., Suppakul, P. (2011) Volatile Compounds as Quality Indicators of Fresh Chicken and Possible Application in Intelligent Packaging. Online Proceeding of the 12th ASEAN FOOD CONFERENCE, 287-294. Bangkok, Thailand.

Seephueng, A. (2008) Smart Packaging for Fish Spoilage Indicator. M.S. Thesis, The Petroleum and Petrochemical College, Chulalongkorn University, Bangkok, Thailand.