STUDIES ON COLOR STABILITY OF BUTTERFLY PEA EXTRACT AND PREPARATION OF POLY(VINYL ALCOHOL) FILMS ENTRAPPED WITH BUTTERFLY PEA EXTRACT

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A SPECIAL RESEARCH PROJECT SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF ENGINEERING (CHEMICAL ENGINEERING)
FACULTY OF ENGINEERING
KING MONGKUT'S UNIVERSITY OF TECHNOLOGY THONBURI
2013

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A Special Research Project submitted in Partial Fulfilment
Of the Requirements for
The Degree of Master of Engineering (Chemical Engineering)
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Special Research Project Title	Studies on Color Stability of Butterfly Pea Extract and Preparation of Poly(vinyl alcohol) Films Entrapped with Butterfly Pea Extract
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Faculty	Engineering
Academic Year	2013

Abstract

This research aimed to develop a non-toxic, environmentally friendly seafood spoilage indicator in the form of label film by incorporating butterfly pea (BP) extract into a poly(vinyl alcohol) (PVA) matrix. The BP extract contains anthocyanins that respond through visible color change to volatile amines released during the seafood spoilage period. The dried BP extract was prepared by solid-liquid extraction using acidic ethanol as a solvent, followed by freeze-drying. The total anthocyanin content in the dried BP extract was equal to 44.17 mg/g as determined by the pH differential method. The stability study of anthocyanins revealed the decrease in the total anthocyanin content by 29.4% after 4 weeks of storage in darkness at 4 ˚C. The BP extract-PVA films were prepared via a solution casting technique. The amount of the dried BP extract added was varied between 0.2 and 1.6 wt% of the PVA matrix. By adjusting the pH of the casting solution to 5, the resultant films appeared blue. The color intensity of the films increased with increasing the BP extract content. The studies on color stability and mechanical properties revealed a small color loss of the BP extract-PVA films during storage at 4 ˚C and in darkness for 4 weeks as indicated by the slightly lower hue angle and chroma values, and the added BP extract did not significantly affect the tensile strength and elongation of the films. In addition, the BP extract-PVA films were tested for their ability to respond to NH_3 vapor as a model volatile amine. The films gradually changed from blue to green in response to the NH_3 vapor concentrations. The results indicate that the developed BP extract-PVA films have potential applications as seafood spoilage indicators.

Keywords: Seafood spoilage indicators / Volatile amines / Anthocyanins / Butterfly pea extract / Poly(vinyl alcohol)

หัวข้อโครงการศึกษาวิจัย	การศึกษาการเปลี่ยนแปลงสีของสารสกัดอัญชันและการเตรียมฟิล์มพอลิไวนิลแอลกอฮอล์ที่กักเก็บสารสกัดอัญชัน
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ผู้เขียน	นางสาวภิภัสรา สุขประสงค์
อาจารย์ที่ปรึกษา	ดร. นนทรี นิมิตศิริวัฒน์
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บทคัดย่อ

งานวิจัยนี้มีวัตถุประสงค์เพื่อพัฒนาตัวชี้วัดการเน่าเสียของอาหารทะเลที่เป็นมิตรต่อส่งแวดล้อมในรูปแบบฉลากฟิล์มโดยการเติมสารสกัดอัญชันลงในเมทริกซ์ของพอลิไวนิลแอลกอฮอล์ สารสกัดอัญชันประกอบไปด้วยสารให้สีในกลุ่มแอนโธไซยานิน ซึ่งสามารถเปลี่ยนสีเมื่อสัมผัสกับสารระเหยจำพวกเอมีนที่เกิดขึ้นจากกระบวนการเน่าเสียของอาหารทะเล สารสกัดอัญชันแห้งสามารถเตรียมโดยวิธีการสกัดด้วยสารละลายเอธานอลที่มีฤทธิ์เป็นกรดตามด้วยการทำแห้งแบบเยือกแข็ง สารสกัดอัญชันแห้งที่ได้มีปริมาณแอนโธไซยานิน 44.17 มิลลิกรัมต่อกรัม ซึ่งได้จากการวิเคราะห์ด้วยวิธี pH differential จากการศึกษาความเสถียรของแอนโธไซยานิน พบว่าปริมาณแอนโธไซยานินลดลงร้อยละ 29.4 เมื่อสารสกัดอัญชันแห้งถูกเก็บในที่มืด ที่อุณหภูมิ 4 องศาเซลเซียส เป็นเวลา 4 สัปดาห์ ฟิล์มพอลิไวนิลแอลกอฮอล์ที่กักเก็บสารสกัดอัญชันถูกเตรียมโดยวิธีการหล่อละลาย โดยปริมาณสารสกัดอัญชันแห้งที่เติมลงไป มีค่าผันแปรระหว่างร้อยละ 0.2 ถึง 1.6 โดยน้ำหนักของพอลิไวนิลแอลกอฮอล์ การปรับค่าความเป็นกรด-ด่างของสารละลายที่ใช้ในการเตรียมฟิล์มให้มีค่าเท่ากับ 5 ส่งผลให้ฟิล์มที่ได้มีสีน้ำเงิน และความเข้มของสีฟิล์มจะเพิ่มขึ้นเมื่อปริมาณสารสกัดอัญชันเพิ่มขึ้น จากการศึกษาความเสถียรของสีและคุณสมบัติเชิงกลของฟิล์มพอลิไวนิลแอลกอฮอล์ที่กักเก็บสารสกัดอัญชัน พบว่า สีของฟิล์มจางลงเล็กน้อยระหว่างการเก็บฟิล์มไว้ที่อุณหภูมิ 4 องศาเซลเซียส ในที่มืด เป็นเวลา 4 สัปดาห์ ซึ่งบ่งชี้โดยมุมของสีและความเข้มของสีที่มีค่าลดลง และการเติมสารสกัดอัญชันลงในฟิล์มพอลิไวนิลแอลกอฮอล์ส่งผลอย่างไม่มีนัยสำคัญต่อค่าความต้านทานแรงดึงและร้อยละการยืดตัวของฟิล์ม นอกจากนี้เมื่อนำฟิล์มพอลิไวนิลแอลกอฮอล์ที่กักเก็บสารสกัดอัญชันไปทดสอบการตอบสนองต่อไอแอมโมเนีย ซึ่งใช้เป็นตัวแทนของสารเอมีนที่ระเหยได้ พบว่าฟิล์มค่อยๆ

เปลี่ยนจากสีน้ำเงินเป็นสีเขียวเมื่อสัมผัสกับไอแอมโมเนียที่ความเข้มข้นต่างๆ จากผลการทดลองดังกล่าวสามารถสรุปได้ว่าฟิล์มพอลิไวนิลแอลกอฮอล์ที่กักเก็บสารสกัดอัญชันที่พัฒนาขึ้นนี้ มีศักยภาพในการนำไปประยุกต์ใช้เป็นตัวชี้วัดการเน่าเสียของอาหารทะเลได้

คำสำคัญ : ตัวชี้วัดการเน่าเสียของอาหารทะเล / สารเอมีนที่ระเหยได้ / แอนโธไซยานิน / สารสกัดอัญชัน / พอลิไวนิลแอลกอฮอล์

ACKNOWLEDGEMENTS

It is a pleasure to thank those who made this thesis complete. First of all, I would like to express my gratitude to my advisors, Dr. Nonsee Nimitsiriwat and Assoc. Prof. Dr. Jatuphorn Wootthikanokkhan for valuable supports, guidance and encouragements throughout the study period. I am very grateful to Dr. Boonnak Sukhummek, Dr. Saiwan Nawalertpanya, and Dr. Withawat Mingvanish for their encouraging comments, suggestions, and their time as members of the examination committee. Also, I would like to thank all laboratory member of PENTEC research group of School of Energy, Environmental and Materials, laboratory staff of Chemical Engineering Department and Food Engineering Department, King Mongkut's Univrsity of Technology Thonburi, for their helping suggestion and providing the laboratory equipment. Moreover, I would like to thank Chemical Engineering Practice School (ChEPS) staff, Mrs. Chadaporn Dammunee for her suggestion and information which was helpful to finish the master degree. Finally, I wish to thank my family and all friends for their assistance and encouragement.

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CHAPTER 1 INTRODUCTION

1.1 Background

Intelligent packaging is a new technology to monitor or inform the quality of the packaged food by containing some indicators that respond to environmental changes, for example, time-temperature indicators, gas or vapor indicators, ready-to-serve indicators, ripeness indicators and food spoilage indicators [1-5]. Intelligent packaging provides the customers confidence on the quality and safety of the food products they purchase, leading to a safer, easier, more interactive and more enjoyable life. Furthermore, it also helps the manufactures to identify supply chain inefficiencies, reduce costs and errors, improve product performance and ultimately increase profit. Food spoilage indicator is one of the intelligent packaging that has increasingly gained interest in the food industry as it increases the reliability of food safety and reduces the risk from consuming spoiled food.

Both seafood and seafood products are expensive and difficult to keep fresh. At high ambient temperatures, fresh seafood will start to spoil within 12 hr [6]. This spoilage can be caused by chemical, enzymatic or microbial activities. Microbial growth and metabolism are the major reasons, which produce volatile amines such as trimethylamine (TMA), dimethylamine (DMA) and ammonia (NH_3), and biogenic amines such as putrescine, histamine and cadaverine, along with other substances including organic acids, sulphides, alcohols, aldehydes and ketones [7-10]. These volatile amines, collectively known as total volatile basic nitrogen (TVB-N), are the characteristic substances responsible for the unpleasant fishy odor. The TVB-N level is therefore widely used to evaluate the seafood spoilage. In an enclosed package, the spoiled seafood causes an increase in the TVB-N level in the headspace.

Due to the fact that most food spoilage products cause the change in pH, food spoilage indicators are commonly developed by incorporating one or more halochromic compounds such as pH sensitive pigments into a polymer matrix [7, 11-13]. Although this technique is simple, it has a weak point that is the incorporated pH sensitive pigments could leach from the polymer matrix and mix with the food inside the package, leading to changes in taste and color. Moreover, most of common pH sensitive pigments are synthetic and generally toxic to human health if consumed continuously and in high quantity, which is a major concern in the development of food spoilage indicators. Although several techniques to prevent the pigment leaching have been developed, most of them involve complicated and expensive procedures [14]. Hence, natural pH sensitive pigments are suitable alternatives for detecting food spoilage. In addition to their low toxicity, natural pH sensitive pigments are obtained from sustainable and inexpensive resources. There are many groups of natural pH sensitive pigments such as carotenoids, anthocyanins and chlorophylls. Anthocyanins are natural substances, which their chemical forms depend on the pH of the medium in which they

are dissolved. Therefore, the color of anthocyanins changes in acid-base environments, for example, in strong acidic, weak acidic, neutral and basic environments their color is red, violet up to blue, green and greenish yellow, respectively [15]. Anthocyanins are considered as efficient replacement for synthetic pH sensitive pigments because of its bright, attractive color and water solubility. Considering the exhibited colors of anthocyanins in acid–base solutions compared to other natural pigments such as chlorophyll, anthocyanins would be a more effective pH indicator [16]. Examples of anthocyanin sources are butterfly pea flowers, red cabbages, and roselle red, dragon fruits, grapes etc.

Butterfly pea (BP) flowers are a richer source of anthocyanins compared to other fruits and vegetables [15]. Moreover, as being a local plant in Thailand, it is abundantly available and therefore suitable to be used as a raw material source for anthocyanins. Moreover, the BP extract is considered to have properties of anti-stress, anti-bacteria, anti-inflammations, and it is also proven to be a natural antioxidant that has hepatoprotective effects, indicating that it gives no negative impacts to human health even if it accidently leaches out. Poly(vinyl alcohol) (PVA) is biodegradable, water soluble and has excellent film forming properties. In addition, it is approved as an indirect food additive in products contacted with food. Therefore, it is suitable to be used as a polymer matrix for food spoilage indicators. This research aimed to develop a new type of non-toxic environmentally friendly seafood spoilage indicators in the form of label film, by incorporating the BP extract into a PVA matrix. Due to the fact that the BP extract is a natural substance that is sensitive to temperature, light and oxygen, the stability of anthocyanins in the dried BP extract under various storage conditions was investigated. The PVA films incorporated with the BP extract (BP extract-PVA films) were prepared and subsequently analyzed for the mechanical properties, color stability and ability to respond to ammonia (NH_3) vapor, a model volatile amine.

1.2 Objectives

1. To prepare the dried BP extract, and study the stability of anthocyanins in the dried BP extract
2. To prepare and characterize the PVA films incorporated with the BP extract
3. To investigate the potential of the prepared BP extract-PVA films as a pH sensitive film for qualitative detection of spoilage in seafood

1.3 Scopes of work

1. The dried BP extract was prepared by solid-liquid extraction technique using acidic ethanol as an extracting solvent and made to dry powder by freeze-drying technique.
2. The anthocyanin content in the dried BP extract was analyzed via the pH differential method, using UV-visible spectrophotometry.
3. The stability of anthocyanins in the dried BP extract was studied at 4 ˚C in darkness for 4 weeks.

4. The PVA films incorporated with different concentrations of the BP extract were prepared by solution casting technique.
5. The properties of the BP extract-PVA films that were analyzed were
   - Film thickness by using a digital vernier caliper.
   - Mechanical properties (ultimate tensile strength, ultimate %elongation and elastic modulus) by using a texture analyzer.
   - Color stability, at 4 ˚C in darkness for 4 weeks, by using a colorimeter.
6. The BP extract-PVA films were tested for ability to respond to NH_3 vapor, a model volatile amine, vaporized from NH_3 solution at different concentrations, at room temperature and at 4 ˚C.

CHAPTER 2 THEORY AND LITERATURE REVIEWS

This chapter presents theory and literature reviews regarding the BP extract and food spoilage indicators. The theory part mentions about food spoilage indicators, food spoilage indicators via pH change detection and polymer matrix. The literature reviews part presents the BP extract and the food spoilage indicators.

2.1 Theory

2.1.1 Food spoilage indicators

Food spoilage indicators are the interactive or responsive part of diagnostic packaging. The indicators may be defined as a substance that indicates the presence or absence of another substance by means of characteristic change, especially in color.

2.1.1.1 Fish spoilage mechanisms [6]

Food spoilage means the original nutritional value, texture, flavor of the food are damaged, the food becomes harmful to people and unsuitable to eat. For fresh fish and seafood, their spoilage results from 3 basic mechanisms, which are enzymatic autolysis, oxidation and microbial growth.

1) Autolytic enzymatic spoilage

Chemical and biological changes occur in dead fish due to enzymatic breakdown of major fish molecules. The autolytic enzymes reduce textural quality during early stages of degradation but do not produce the characteristic spoilage off-odors and off-flavors. The rate of degradation by enzymes proteolysis is reduced when the fish are kept at low temperature (0 ˚C) and pH in the acidic range (pH 5).

2) Oxidative spoilage

Lipid oxidation is a major cause of deterioration and spoilage for the pelagic fish species such as mackerel and herring with high oil/fat content stored in their flesh. There are 3 stages for lipid oxidation, which are initiation, propagation and termination. Initiation is a formation stage of lipid free radicals through catalysts such as heat, metal ions and irradiation. During the propagation stage, the peroxyl radicals react with other lipid molecule to form hydroperoxide and a new free radical. Termination occurs when a build up of these free radicals interact to form non-radical products. Oxidation typically involves the reaction of oxygen with the double bonds of fatty acids. Therefore, fish lipids, which consist of polyunsaturated fatty acids, are highly susceptible to oxidation.

3) Microbial spoilage

Microbial growth and metabolism is a major cause of fish spoilage, which produce amines, biogenic amines such as putrescine, histamine and cadaverine, organic acids, sulphides, alcohols, aldehydes and ketones. The compounds formed during spoilage

through microbial metabolism are listed in Table 2.1. Trimethylamine (TMA) is one of amine products from reduction of trimethylamine oxide (TMAO) by bacteria. The levels of TMA are universally used to determine microbial deterioration leading to fish spoilage.

Table 2.1 Bacterial spoilage compounds [6].

Specific spoilage bacteria	Spoilage compounds
Shewanella putrifaciens	TMA, H_2S, CH_3SH, $(CH_3)_2S$, HX
Photobacterium phosphoreum	TMA, HX
Pseudomonas spp.	Ketones, aldehydes, esters, sulphides
Vibrionacaea	TMA, H_2S
Aerobic spoilers	NH_3, acetic, butyric and propionic acid

TMA: Trimethylamine; H_2S: Hydrogen sulphide; CH_3SH: Methylmercarptan; $(CH_3)_2S$: Dimethylsulphide; HX: Hypoxanthine; NH_3: Ammonia

2.1.1.2 Types of food spoilage indicators [3]

Types of food spoilage indicators can be divided into 3 groups: freshness and microbial indicators, time-temperature indicators and gas concentration indicators. The first group is freshness and microbial indicators. The idea of freshness indicators is that they monitor the quality of the packed food by reacting in one way or another to changes taking place in the fresh food product as a result of microbiological growth and metabolism. Most of these concepts are based on a color change of the indicator tag due to the presence of microbial metabolites produced during spoilage. Freshness indicators are designed to respond to chemicals released by food as a result of spoilage; usually an oxidative process is effected by bacteria, yeasts and fungi, which break down food carbohydrates, proteins, and fats to a wide variety of low-molecular-weight molecules, such as CO_2, lactic and acetic acids, aldehydes, alcohols (e.g. ethanol), sulfur containing species (e.g. H_2S) and nitrogen containing molecules, such as ammonia and amines. For example, when proteins are bacterially decomposed, the products are amines that are related to the original amino acids that make up the protein. Thus, arginine is converted to putresceine, lysine, to cadaverine while histadine is converted to histamine. Putrescine, cadaverine and histamine are volatile amines, responsible for the smell of rotting protein, such as meat and seafood. By integrating the indicator into the food package, the freshness indicators can be realized as visible indicator tags going through a color change in the presence of the analyte [17].

The second type is the time-temperature indicators. There are 2 types of the time-temperature indicators: simple temperature indicators and time-temperature integrators (TTIs). Simple temperature indicators show whether products have been heated above or cooled below a reference temperature, warning consumers about the potential survival of pathogenic microorganisms and protein denaturation during freezing or defrosting processes. TTIs display a continuous temperature-dependent response of the food product. The response is made to chemical, enzymatic or microbiological changes that should be visible and irreversible, and is temperature dependent. TTIs provide an overall temperature history of the product during distribution.

The third type of food spoilage indicators is gas concentration indicators. Internal gas-level indicators are placed into the package to monitor the inside atmosphere. Most of these indicators induce a color change as a result of gas generated due to enzymatic and chemical reactions. In addition, the rapid label check can allow consumers to view the quality of the food inside the package by examining common redox dyes (e.g. methylene blue, which is used as a leak indicator).

2.1.2 Seafood spoilage indicators via pH change detection

There are several methods to evaluate the quality of fresh seafood and seafood products. According to literature review on freshness, quality and safety in seafood microbial, chemical, biochemical methods or other instrument are all appropriate methods for determination of seafood freshness. However, the most appropriate method must be convenient, fast-responding and inexpensive to determine the quality of seafood. During the seafood spoilage volatile basic amines such as TMA, DMA and NH_3 are generated, causing the pH change in the closed package. Seafood spoilage indicators via pH change detection are therefore considered as one of efficient methods for determining the seafood quality. Generally, seafood spoilage indicators contain one or more halochromic compounds or pH sensitive pigments that indicate the change in pH caused by the substances generated during seafood spoilage through a visible color change.

2.1.2.1 pH sensitive pigments

A pH sensitive pigment is a halochromic substance, which changes color with pH. When it is dissolved in water, it dissociates slightly and forms an ion. The undissociated form, so called acidic form, exhibits a different color from the ion, so called basic form or its conjugate base. The acid and basic forms can be expressed as the general formulas HIn and In^-, respectively. At equilibrium, the following equilibrium equation is established;

$$\underset{\substack{\text{Acid} \\ \text{(Color A)}}}{HIn\ (aq)} + H_2O\ (l) \leftrightarrow H_3O^+\ (aq) + \underset{\substack{\text{Conjugate base} \\ \text{(Color B)}}}{In^-\ (aq)}$$

At low pH values the concentration of H_3O^+ is high and so the equilibrium position lies to the left; the equilibrium solution has the color A. On the other hand, at high pH values, the concentration of H_3O^+ is low; the equilibrium position thus lies to the right and the equilibrium solution has color B. The color change of a pH sensitive pigment does not occur at specific H_3O^+ concentrations, but rather, occurs over a range of H_3O^+ concentrations. This rage is termed the color change interval, which is expressed as a pH range. Common synthetic pH sensitive pigments are shown in Table 2.2.

Table 2.2 Common synthetic pH sensitive pigments [18].

pH sensitive pigment	Color of acidic form	pH range	Color of basic form
Thymol Blue	red	1.2-2.8	yellow
Methyl orange	red	3.1-4.4	orange
Bromcresol green	yellow	4.0-5.6	blue
Methyl red	red	4.4-6.2	yellow
Bromcresol purple	yellow	5.2-6.8	purple
Phenol red	yellow	6.4-8.0	red
Cresol red	yellow	7.2-8.8	red
Thymol blue	yellow	8.0-9.6	blue
Phenolphthalein	colorless	8.0-10.0	red
Thymolphthalein	colorless	9.4-10.6	blue
Nile blue	blue	10.1-11.1	red
Nitramine	colorless	11.0-13.0	orange-brown
Trinitrobenzoic acid	colorless	12.0-13.4	orange-red

2.1.2.2 Natural pH sensitive pigments and their preparation [19]

In theory, any substance that undergoes a reversible chemical change when pH changes can be used as an acid-base indicator or pH sensitive pigment. Many pH sensitive pigments can be extract from plants. Many flowers, fruits and plants part contain a chemical compounds that can change color with pH such as alizarin, curcumin or tumeric yellow, anthocyanins, litmus, chlorophyll and logwood. Most flowers, fruits and plant parts that are red, blue or purple generally contain anthocyanins.

Anthocyanins

Anthocyanins are one of natural pigments that are found in various plant families and many edible plants; for example berries such as blackberries, grapes, blueberries etc., vegetable such as avocado, red onion, red cabbage etc., and some flowers such as butterfly pea, roselle red, canna indica etc. Other sources include oranges, olives, figs, sweet potatoes, mango, radishes and dragon fruits. They provide a variety of colors including orange, red, purple, green and yellow. Anthocyanins are water soluble glucosides of anthocyadinins. The basic chemical structure of them are shown in Figure 2.1; there are 6 groups of anthocyadinins comprised in fruits and vegetables (Table 2.3), which are delphinidin, cyanidin, pelargonidin, petunidin, peonidin and malvidin [20]. Anthocyanins change color depending on acid-base environment, for example, in strong acidic, weak acidic and basic environment their color is red, violet up to blue, green and greenish yellow, respectively [15]. Figure 2.2 shows the color and chemical form of cyadinin depending on the pH of solution.

Figure 2.1 The basic chemical structure of anthocyadinins.

Table 2.3 General groups of anthocyanidins in nature [20, 21].

Anthocyadinins	Substitution groups
Delphinidin	4,5,7,3′,4′,5′-OH
Cyanidin	4,5,7,3′,4′-OH : 5’-H
Pelagonidin	4,5,7,4′-OH: 3, 5-H
Petunidin	4,5,7,4′-OH; 3′-OMe
Peonidin	4,5,7,4′-OH; 3′-OMe, 5’-H
Malvidin	4,5,7,4′-OH; 3′,5′-OMe

Anthocyanins are valuable as kinds of important quality indicators in foods, and pharmaceutical and chemotaxonomic indicators in plants. Anthocyanins have characteristic physical and chemical properties that give unique colors and stability. They are highly reactive molecules and sensitive to degradation reactions. Oxygen, temperature, light, enzymes and pH are the factors that may affect to the chemistry, stability and color of anthocyanins. The color stability of anthocyanins is affected by temperature, oxygen and UV-light. Temperature and light can destroy the flavylium ion that causes loss of color. The use of anthocyanins is limited by these factors. Some loss of color during storage can be prevented by storing at low temperatures, in dark containers or under oxygen-free packaging.

Figure 2.2 The chemical forms of cyanidin depending on the pH of solution [22].

Butterfly pea flowers

Butterfly pea (*Clitoria ternatea L.*), common names including blue-pea and cordofan pea, is a plant species belonging to the Fabaceae family, and it widely grows in the tropical area including Southeast Asia. It is a perennial herbaceous plant. There are 2 types of butterfly pea, which are white and blue flowers. The blue color of butterfly pea flowers is come from anthocyanins, which are classified as ternatins. Ternatins are a group of delphinidin glucosides (Figure 2.3), a group of anthocyanins, which can be easily dissolved in water and give different colors according to the pH of solution. It is changed from blue in an alkaline solution to red in an acidic solution. Butterfly pea flower is one of natural sources that has a large amount of anthocyanins per dry weight when compared with other plants [15]. Butterfly pea flower is very useful as a companion crop, an ornamental plant, or animal feed. In Southeast Asia, an extract solution of butterfly pea is traditionally used in food colorants and hair dying.

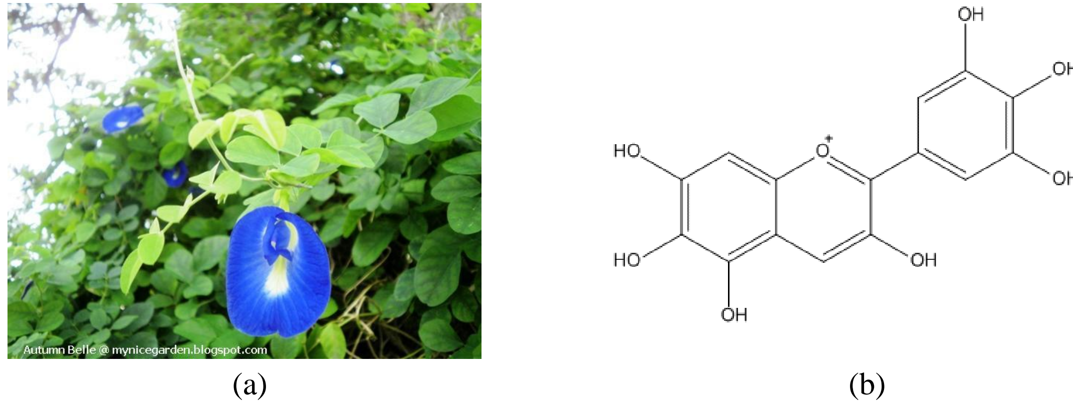

Figure 2.3 Butterfly pea flower (a) and the chemical structure of delphinidin (b).

Natural pigments extraction

Natural pigments are generally obtained by solid-liquid extraction of plant materials. The solid plant material is crushed or ground and extracted with an appropriate solvent, such as water, ethanol, or rubbing alcohol and acid solution. The solute is diffused from the solid into the solvent. Solid-liquid extraction is widely used in natural product, food and pharmaceutical industries.

Anthocyanin content [23]

Most studies on the quantitation and identification of anthocyanins have relied on expensive equipment, and/or difficult methods. These methods include paper chromatography, thin-layer chromatography, column chromatography, solid phase extraction, counter current chromatography, UV–Visible absorption spectroscopy (known as pH differential method), high performance liquid chromatography (HPLC), mass spectrometry (MS), and nuclear magnetic resonance (NMR) spectroscopy [24].

The pH differential method and HPLC are commonly used methods by researchers and the food industry for quantifying anthocyanins in a sample. The pH differential method is a rapid and easy procedure for the quantitation of monomeric anthocyanins, and it is used extensively by scientific and industry communities. In 2005, the pH differential method received first action approval from the Association of Analytical Communities (AOAC) as an international official method of analysis.

pH differential method

The pH differential method has been used extensively by the food technologists to assess the quality of fresh and processed fruits and vegetables. The method can be used for the determination of total monomeric anthocyanin content based on the structural change of the anthocyanin chromophore between pH 1.0 and 4.5 [21, 23]. Anthocyanin pigments undergo reversible structure transformations with a change in pH manifested by different absorbance spectra. For example, the flavylium cation form predominates at pH 1.0 and the hemiketal form at pH 4.5 (Figure 2.4). The pH differential method is

based on this reaction. The total anthocyanin content is calculated using the following equation.

$$\text{Anthocyanin content (mg/l)} = \frac{A \times M \times DF \times 1000}{\varepsilon \times 1}$$

Where A is the total absorbance = $[(A_{\lambda max} - A_{700})_{at\ pH\ 4.5} - (A_{\lambda max} - A_{700})_{at\ pH\ 1.0}]$, M is the molecular weight of anthocyanin, DF is the dilution factor (for example, if a 0.2 ml sample is diluted to 3 ml, DF = 15), ε is the molar absorptivity, and l is the path length (typically 1.0 cm).

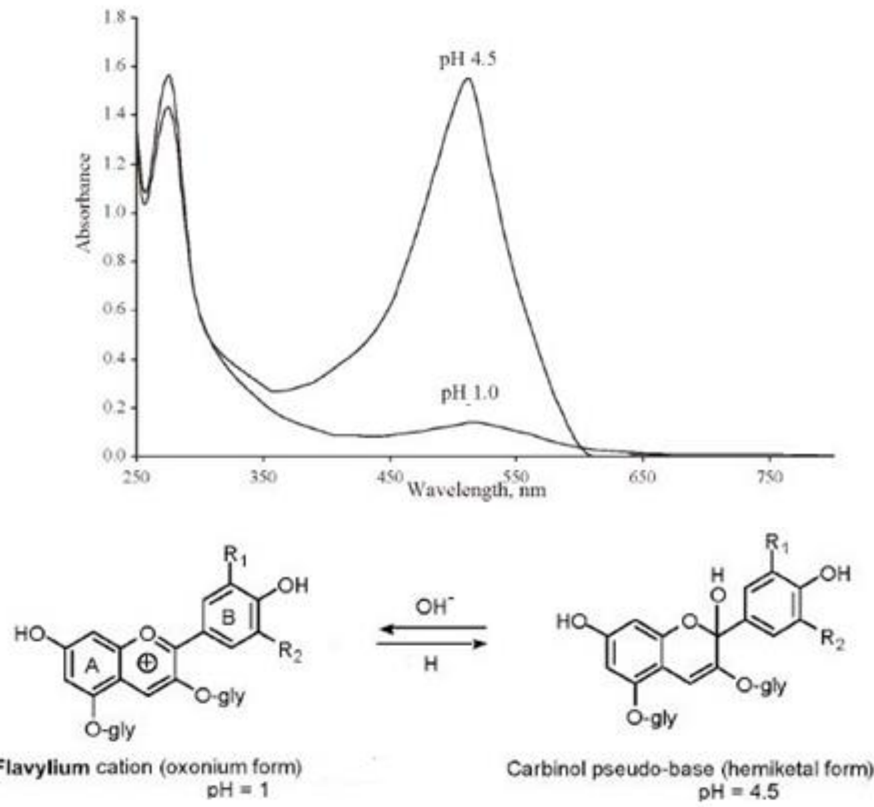

Figure 2.4 Spectral characteristics and molecular structures of an anthocyanin in oxonium form at pH 1.0 and hemiketal form at pH 4.5 [25].

2.1.3 Polymer matrix

2.1.3.1 Poly(vinyl alcohol) (PVA) [26]

Poly(vinyl alcohol) (PVA) is a polyhydroxyl polymer that is one of the largest-volume synthetic polymers. PVA is water-soluble, white solid, and is used in a wide range of industrial, commercial, medical and food applications including resins, lacquers, surgical threads and food-contact applications since 1930s. PVA is generally prepared from the hydrolysis or partial hydrolysis of poly(vinyl acetate), resulting in 2 types of PVA produced, which are fully hydrolyzed PVA and partially hydrolyzed PVA (Figure

2.5). General physical properties of PVA are shown in Table 2.4. These properties vary depending on molecular weight and % hydrolysis of PVA.

(a) (b)

Figure 2.5 The chemical structure of PVA; fully hydrolyzed (a) and partially hydrolyzed (b).

Table 2.4 General physical properties of PVA [26, 27].

Molecular weight	$(44.05)_n$ g/mole
Empirical formula	$(C_2H_4O)_n$
Physical appearance	Odorless, white to cream-colored granular powder
Melting point	Softens at about 200 ˚C with decomposition
Specific gravity	1.19-1.31 (water = 1)

The major use of PVA is in the textile industries as a sizing and finishing agents. PVA can be incorporated into a water-soluble fabric in the manufacture of degradable protective apparel, laundry bags for hospitals, rags, sponges, sheets and covers. PVA is also widely used in the manufacture of paper products. As with textile, PVA is applied as a sizing and coating agent. PVA is used as a thickening agent for latex paint and common household white glues or in other adhesive mixtures such as remoistenable labels and seals. Moreover, PVA is water-soluble, but relatively insoluble in organic solvents, and its solubility in aqueous solutions is adaptable to necessary applications.

PVA was evaluated for food use by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in June 2003, and it is also allowed by the US Food and Drug Administration (FDA) for use as an indirect food additive in products, which are contact with food. For example, PVA is approved as a diluent in color additive mixtures for coloring shell eggs. PVA has also been approved for use in packaging meat products and several medical applications such as transdermal patches, sustained release tablet formulations etc [26].

As an industrial and commercial product, PVA is valued for its solubility and biodegradability, which causes very low environmental impact. Several microorganisms ubiquitous to artificial and natural environments such as septic systems, landfills, compost and soil have been identified, which are able to degrade PVA through enzymatic processes. A combination of oxidase and hydrolase enzyme activities

degrades PVA into acetic acid [28, 29]. There are additional potential food uses for PVA currently under evaluation, including uses in confectionery products and high-moisture food products.

2.1.3.2 Film preparation

Plastic film is a thin continuous polymeric material. Plastic films are used to separate areas or volumes, to hold items, to act as barriers, or as printable surfaces. Plastic films are widely used in various applications including packaging, building construction, landscaping, electrical fabrication, photographic film, etc. Polymer films can be prepared from polymer solution via many methods, ranging from simple techniques for thick films to more complicate techniques for thin films, with either multilayers or single monolayers. The methods employed for film preparation can be divided into 2 groups based on the nature of preparation process, which are physical process (e.g. sputtering process, evaporation and resistive heating) and chemical process (e.g. chemical vapor deposition, spray pyrolysis and sol gel process) [30]. Solution casting is one of evaporation processes, which is the most simple technique for preparation of thick films. The solution casting technique consists of preparation of the solution, casting on the smooth, polished surface and removal of the solvent from the polymer. Since the solution casting technique is the simplest method used to create films. Therefore, it was selected as a method to prepare the BP extract-PVA films in this research.

2.2 Literature reviews

2.2.1 Solid-liquid extraction

Solid-liquid extraction has been the most common method in natural pigment sample preparation. Pigment compounds have been extracted from ground, dried, or freeze-dried flower and fruit samples or by macerating the fresh sample with the extracting solvent [31]. Most common solvents are aqueous mixtures with ethanol, methanol and acetone. The raw extracts produced contain also nonphenolic substances such as sugars, organic acids, proteins and pigments, which can interfere during antioxidant evaluation.

The extraction of anthocyanins from wine pomades [31] and from strawberries [32] has been studied using different solvents and acidifying conditions. The solvent used for anthocyanin extraction significantly affects the solid-liquid extraction. In 2011, Suppadit and co-workers [15] used 5 different solvents, distilled water (DW), ethanol (HE), acetic acid (AA), vinegar (V) and white liquor (WL) for anthocyanin extraction. 5 g of dried BP flowers was extracted with 50 ml of DW, HE, AA, V and WL, respectively at room temperature and were shaken at 180 rpm for 2 hr. The total anthocyanin content was measured by UV-Visible spectrophotometry and calculated by the equation of Fuleki and Francis (1968). DW gave the highest yield of anthocyanins followed by WL, HE, AA and V. Since DW has a stronger polarity than other solvents, hydrogen bonding can occur between the solvent and the anthocyanins having the molecular structure of oxonium salt; therefore, the DW could extract anthocyanins more

effectively than the other solvents. Although DW was the most effective solvent among the 5 solvents, the extract solution could not produce clearly definable colors when it had been tested with buffer solutions. In conclusion, the most appropriate solvent to extract anthocyanins from BP flowers were HE.

2.2.1.1 Stability of BP extract

The stability of anthocyanins is affected by temperature, oxygen and UV-light. Storage of anthocyanins in dark containers at low temperatures and in the absence of oxygen could reduce or prolong the degradation that leads to the color change or loss, which can be measured by UV-Visible spectroscopy. As reported by Kirca and co-workers (2007) [33] the color loss of the BP extract solution was more pronounced when it was stored for 4 weeks at 30 ˚C than at 4 ˚C. The color appearing after tested with different pH buffer solutions could not be separated clearly. The color loss was attributed to the conversion of the colored species, flavylium cations and quinoidal, to the colorless species, carbinol pseudobase and chalcone.

In 2010, Abdullah and co-workers [34] studied the color stability and structural transformation of BP extract in different pH solutions in the range of 0.05-12.0. The predominant forms of anthocyanins at pH <5, 5 to 7, 7 to 8 and 8 to 12 were the red flavylium cation, blue neutral quinonoidal bases, anionic quinonoidal bases, and chalcone, respectively. Red flavylium cation of anthocyanins in the BP extract was the most stable species on storage. The stability of the BP extract in different pH solutions were evaluated at various storage temperatures. The BP extract in solutions at pH 1.5, 3.0 and 6.0 were stable at 7 ˚C, at which the intensity of the color retained about 80-90% of its initial color on day 60 and remained stable for about a year. Therefore, the BP extract can be used as a colored ingredient for cold food products.

In an attempt to improve the color stability, microparticulated systems prepared by spray drying technique were developed [35]. The microparticulated systems can entrap chemicals such as colors in the microparticles, which may provide the protection against factors such as pH, light, heat and oxidation. For the aqueous BP extract, the color presented the most stable in pH 4 solution under darkness and the least stable in a pH 7 solution under UV light. The BP extract at pH 4.0 was selected for the preparation of microparticles due to its good stability. The gelatin microparticulated system showed better protection against photo-degradation compared to the HPMC microparticulated system and the aqueous color solution. Therefore, the polymer type affects the stability of the loaded BP extract and should be carefully selected.

2.2.2 Food spoilage indicators

At present, the developments and applications of food spoilage indicators are grown continuously. Food spoilage indicators can be found in several forms, e.g. chips, papers, and films. For fresh seafood and seafood product, their deterioration or spoilage can be determined by the levels of volatile amines generated from spoilage mechanisms. There are few seafood spoilage indicators that are commercially available; for example,

FreshTag™ (Cox Technologies, USA) is the chip indicator for the detection of volatile amines in packed seafood. This chip indicator will change its color to pink when it is exposed to the volatile amines in the headspace of package [36]. Another example is the product of LITMUS FQI [37], which can be used as an indicator to detect decomposition of seafood chain products. This indicator is in the form of colorimetric papers that respond to volatile amines generated by spoiled seafood. Likewise, the color of the paper indicator will change from yellow to blue when volatile amines have been exceeded limitation.

In addition, there are many research works reported on the development of seafood spoilage indicators. Immobilization of one or more pH sensitive pigments onto a solid substrate is one of techniques to prepare seafood spoilage indicators. There are 3 widely used methods for the immobilization of pH sensitive pigments; adsorption (or impregnation), covalent binding and entrapment. Although adsorption and entrapment are simple methods, the adsorbed or entrapped pigment can leach from the solid substrate and contaminates the food. The covalent binding method is a more complicated and time-consuming method; however, the leaching of the pH sensitive pigment can be avoided [14]. Khalil (2003) [38] developed an ammonia detection device in the form of film indicator, in which ammonia sensitive dyes were immobilized in or on a poly(tetrafluoroethylene) (PTFE) solid substrate. The PTFE solid substrate was in the form of a gas-permeable film. This film indicator can be used for detecting and measuring ammonia and volatile amines in both gas and liquid states. The sensitive dye was selected from the group consisting of bromophenol-blue, bromocreosol green, thymol blue, methyl crystal purple, chlorophenol, free-based porphyrins, and tetraphenylporphyrin (H_2TPP).

In 2004, Pacqult and co-workers [17] developed a colorimetric sensor for monitoring the fish spoilage in a packaging headspace. The sensor contained bromocresol green entrapped within a cellulose acetate matrix. Although this sensor was able to monitor volatile amines generated from spoiled fish, it had a serious problem about the leaking of bromocresol green from the cellulose acetate matrix. In 2007, they improved their sensor by adding tetraoctyl ammonium bromide salt to form an ion pair and create a liphophilic film to prevent the leaching of bromocresol green from the polymer matrix (cellulose acetate). The liphoplilic film could reduce the dye leaching by 82%, but this film acted as a barrier and prevented the sensor to be directly contacted with volatile amines. Therefore, the accuracy of the sensor was decreased [7].

For the covalent binding method, Liu and co-workers (2004) [14] studied a phenolphthalein immobilized cellulose membrane for an optical pH sensor. The procedure was started with phenolphthalein reacted with formaldehyde to produce a series of prepolymers of phenolphthalein-formaldehyde (PPF), which was then covalently immobilized to the diacetylcellulose membrane via hydroxymethyl groups. This sensor possessed several advantageous features including a large dynamic range

from pH 8 to 12.5 or even broader, rapid response time (2-30 s), easy of fabrication and a promising material for determination of high pH values.

Although the covalent binding method can prevent the leaching of the pH sensitive pigment, it is more complicated and time-consuming. Therefore, natural extracts that are sensitive to pH are of interest as alternative pH responsive substance for food spoilage indicators since they are non-toxic and unlikely cause health risks when they leach out and contaminate the food. There are several reports on the development of food spoilage indicators using natural extracts as pH responsive pigments. Santos and co-workers (2010) [16] developed biodegradable pH indicator films based on cassava starch plasticized with sucrose and inverted sugar and incorporated with grape and spinach extracts as sources of natural pH responsive pigments (anthocyanins and chlorophyll). The cassava films were homogeneous, transparent, colored with varying color intensity depending on quantity of the extracts. The mechanical properties and moisture barrier of the films were strongly influenced by the extract concentration. The films containing a higher concentration of the grape extract exhibited a greater color change at different pH values, suggesting that anthocyanins were more effective pH indicators than chlorophyll or the mixture of both extracts.

Kuswandi and co-workers (2012) [39] developed on-package sticker sensors for real-time monitoring of shrimp spoilage by using curcumin, the major yellow pigment extracted from turmeric, as a pH indicator source. The sensors were fabricated by absorption of curcumin onto bacterial cellulose membrane. The curcumin/bacterial cellulose membrane sensor worked based on the increase of pH as the spoilage volatile amines were produced gradually in the package headspace, and subsequently the color of the sensor would change from yellow to orange, and then to reddish orange. These sticker sensors were successfully used as on-package sensors for a visual detection of shrimp spoilage.

CHAPTER 3 EXPERIMENTAL PROCEDURES

3.1 Materials and chemicals

- Fresh butterfly pea (BP) flowers (*Clitoria ternatea L.*) (Pak Khlong Talat market, Thailand)
- Poly(vinyl alcohol) (PVA), fully hydrolyzed, Mw. approx. 60,000 g/mol, for synthesis (Merck, Germany)
- Ethanol (EtOH), 99.8%, analytical grade (Carlo Erbo, Germany)
- Ammonia (NH_3) solution, 30%, analytical grade (Carlo Erbo, Germany)
- Hydrochloric acid (HCl), fuming, 37%, analytical grade (Merck, Germany)
- Distilled water (H_2O)

3.2 Glassware and equipment

- Beakers
- Droppers
- Petri dishes
- Cylinders
- Burets
- Stirring rods
- Glass vials
- Funnels
- Pipettes
- Buchner funnels
- Dark bottles
- Glass plates (200x200x3 mm^3)
- Filter papers, Whatman No.42
- Micropipettes and pipet tips
- Nylon filter, 100 mesh
- Magnetic stir bars
- Zipper storage bags
- Hotplate stirrers (Arex, Velp Scientifica)
- Blender (HR2071, Philips)
- Analytical balance (ML802E, Mettler Toledo)
- Rotary evaporator (Laborota 4003, Heidolph)
- Vacuum oven (OV-11, Jeio Tech)
- Hot air oven (FD53, Binder)
- pH meter (S20 SevenEasy™ pH, Metter Toledo)
- Digital caliper (DC-04150, Digital micrometers)
- Colorimeter (Color QUEST XE, HunterLab)
- Texture analyser (TA.XTPlus, Stable micro system)
- UV-Visible spectrophotometer (Lambda-40, Perkin Elmer)

3.3 Experimental procedures

The experimental procedures are divided into 2 main parts: (1) preparation, characterization and stability analysis of the dried BP extract, and (2) preparation, characterization and analysis of the ability for detection of NH_3 vapor of the BP extract-PVA films. The experimental details of each part are described below.

3.3.1 Preparation, characterization and stability analysis of BP extract

3.3.1.1 Preparation of dried BP extract

1,500 g of fresh BP petals were cleaned twice by immersing them in 5,000 ml of tap water for 5 min. After the cleaned fresh BP petals were left to dry at room temperature for 6 hr, they were dried in a vacuum oven at 40 ˚C for 36 hr and then grounded to powder (<0.5 mm) by using a blender (speed level 3 for 3 min). Meanwhile, an acidic ethanol solution as an extracting solvent was prepared by adding 20 ml of 1.0 M HCl into 1,980 ml of 95 %v/v ethanol (95 %v/v ethanol was prepared by adding 96 ml of distilled water into 1,904 ml of 99.8 %v/v ethanol). 112.8 g of the dried BP powder was stirred in 1,128 ml of the acidic ethanol at 180 rpm at room temperature for 2 hr. The BP extraction solution was separated from the BP powder by filtration with a nylon filter. The extraction was repeated two times. The BP extract solutions from both extractions were combined and filtered through a filter paper. The acidic ethanol solvent was partially removed under reduced pressure with a rotary evaporator at 40 ˚C until the volume of the BP extract solution was reduced to approximately 50 ml. The concentrated BP extract solution was subjected to freeze-drying under vacuum at room temperature for 24 hr. After that, the dried BP extract was stored in a dark bottle at 4 ˚C until further used.

3.3.1.2 Characterization of dried BP extract

The anthocyanin content in the dried BP extract was determined by the pH differential method, which can be used for the determination of a total monomeric anthocyanin content based on the structural change of the anthocyanin chromophore between pH 1.0 and 4.5. Quartz cuvettes of 1 cm path-length were used and all measurements were carried out at room temperature. Absorbance readings were made against distilled water as a blank. The analysis procedure is as follows.

The BP extract solution was prepared by dissolving 0.5 g of the dried BP extract into 25 ml of distilled water. Then, 0.5 ml of stock solution was diluted by 8.5 ml of distilled water. The BP extract solution was filtered through a filter paper, and it should be analyzed immediately after preparation. Next, 2 dilutions of the BP extract solution, one with pH 1.0 HCl solution and the other one with pH 4.5 HCl solution, were prepared by adding 0.25 ml of the BP extract solution into 4.75 ml of each HCl solution. These acidic diluted solutions were left to equilibrate for 15 min. After that, the absorbance of each acidic diluted solution was measured by scanning the absorbance between 350 nm and 700 nm, with a UV-Visible spectrophotometer. All measurements should be made within 1 hr after sample preparation. The absorbance of the acidic diluted BP extract

solution (A) and the total anthocyanin content in the original BP extract solution were calculated as follows. The anthocyanin content was expressed in terms of total monomeric anthocyanins based on delphinidin-3-glucoside.

$$A = (A_{\lambda=543\ nm} - A_{\lambda=700\ nm})_{pH\ 4.5} - (A_{\lambda=543\ nm} - A_{\lambda=700\ nm})_{pH\ 1.0}$$

$$\text{Anthocyanin content (mg/l)} = \frac{(A \times MW \times DF \times 1000)}{\varepsilon \times 1}$$

Where MW is the molecular weight of delphinidin-3-gluciside (MW = 465.2)

DF is the dilution factor (for this experiment, DF = 20)

ε is the molar absorptivity of delphinidin-3-glucoside (ε = 29,000)

3.3.1.3 Stability analysis of dried BP extract

The effect of storage time on the stability of the dried BP extract was studied. The dried BP extract was kept in darkness and at 4 ˚C. The dried BP extract was analyzed every week for a period of 4 weeks to determine the total change in the anthocyanin content by the pH differential method.

3.3.2 Preparation, characterization and analysis of ability for detection of NH_3 vapor of BP extract-PVA films

3.3.2.1 Preparation of BP extract-PVA films

The BP extract-PVA films were prepared by a solution casting technique. 6.0 g of PVA was added into 60 ml of distilled water to obtain a 10 wt% PVA solution and left to hydrate overnight. The mixture was stirred and heated at 80 ˚C until PVA was completely dissolved (~2 hr) and then left to cool down to room temperature. After that, the PVA solution was adjusted pH to 5 by using 0.1 M HCl (< 1 ml). Meanwhile, an acidified 2 wt% BP extract solution was prepared by adding 0.5 g of the dried BP extract into 25 ml of a pH 5 HCl solution, and then filtered through a paper filter. The amount of the acidic BP extract solution that was added into the acidic PVA solution was varied from 0.6 to 4.8 ml (0.6, 1.2, 2.4, 3.6 and 4.8 ml in order to obtain the PVA films containing 0.2, 0.4, 0.8, 1.2 and 1.6 wt% BP extract). The BP extract-PVA solution was stirred continuously for 3 hr in order to obtain a uniformly colored PVA solution. The BP extract-PVA solution was poured into a glass plates (200x200x3 mm^3), and the surplus solution was wiped off. Then, the BP extract-PVA solution in a glass plate was allowed to slowly evaporate in a hot air oven at 40 ˚C for 24 hr. The BP extract-PVA film was peeled off and stored in a zipper storage bag, and kept in darkness at 4 ˚C until further used.

3.3.2.2 Characterization of BP extract-PVA films

Thickness

The thickness of the BP extract-PVA films was measured using a digital caliper at 9 positions to obtain an average thickness as shown in Figure 3.1.

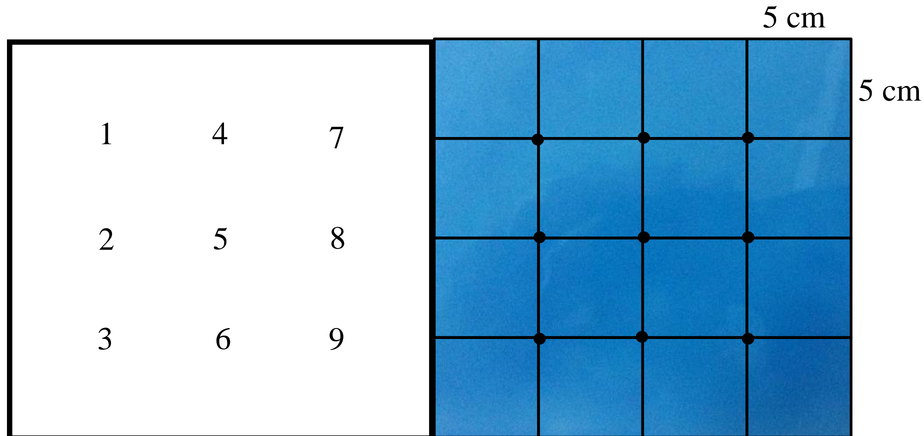

Figure 3.1 The positions of film thickness measurement.

Mechanical properties

The mechanical properties of the BP extract-PVA films – ultimate tensile strength, ultimate %elongation and elastic modulus, were examined by using a texture analyzer. The texture analyzer was used for measuring tensile properties by observations of the elongations by small loads. The BP extract-PVA films were tested according to ASTM D882-09 (the standard test method for tensile properties of thin plastic sheeting). Each film sample was cut into 4 strips of 2.5x10 cm^2 and left in a controlled condition at least 24 hr (at 25±2 ˚C and 50±5% relative humidity). Each strip was measured for thickness at 3 different points along the length. The displacement rate was controlled at 50 mm/min for all tests. The initial gauge length (grip separation) was set at 50.0 mm. The loads were measured using 500 kN load cell. Figure 3.2 shows the tensile test setup with a sample loaded. Neat PVA film was used as a reference for the analysis. The texture analyzer was calibrated by metal rod (100 g) before testing. The load versus extension was recorded.

Figure 3.2 Tensile test setup.

Color stability

The color stability of the BP extract-PVA films was studied by measuring the color change of the films that were kept in darkness and at 4 ˚C, for 4 weeks. The color of the BP extract-PVA films was measured weekly by colorimetric analysis, according to ASTM D1003-7 (the standard test method for haze and luminous transmittance of transparent plastics). The light source of a colorimeter was D65 illuminant and 10 ˚ of standard observer. The colorimeter was calibrated with black plate (L parameter = 0) before testing. Each film sample was cut into 2 pieces of 2.5x2.5 cm^2. The color measurement was repeated 4 times for each sample. The measured colors of the BP extract-PVA films before and after storage were expressed as the CIE Lab coordinates (L*, a* and b*). The total color difference (ΔE) was calculated by the equation below, according to ASTM D2244-09 (the standard practice for calculation of color tolerances and color differences from instrumentally measured color coordinates).

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

For identifying the direction of the color difference between 2 colors, the hue angle (h_{ab}) and chroma (C*) were calculated according to ASTM D2244-09.

$$h_{ab} = 180 - (180/\pi)\arctan(a^*/b^*) - 90\sin(b^*)$$

$$C^* = \sqrt{(a^*)^2 + (b^*)^2}$$

3.3.2.3 Analysis of ability for detection of NH_3 vapor of BP extract-PVA films

The ability to detect the presence of NH_3 vapor of the BP extract-PVA films was investigated. The response of the BP extract-PVA films to NH_3 vapor was measured in terms of their color change (the total color difference, ΔE), by colorimetric analysis according to ASTM D2244-09, as described in Section 3.3.2.2.

The BP extract-PVA films were tested with NH_3 vapor generated from 4 different concentrations of NH_3 solution, 0.01, 0.05, 0.1 and 1.0 M at 4 ˚C and room temperature. 10 ml of the NH_3 solution was added into a petri dish (9 cm diameter), and the BP extract-PVA films with different BP extract contents (2.5x2.5 cm^2) were attached inside the lid of the petri dish at 2 cm above the NH_3 solution, with transparent adhesive tape (Figure 3.3). Then, the petri dish was secured with paraffin film to prevent the NH_3 vapor leakage. The change in color of the BP extract-PVA films was monitored every 10 min for 1 hr. After that, the BP extract-PVA films were taken out to measure the color by a colorimeter immediately.

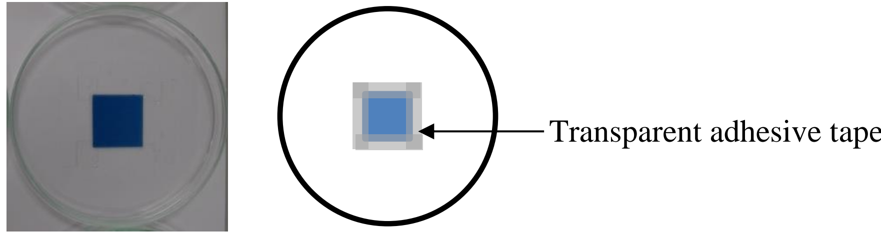

Figure 3.3 The experimental design for detecting NH_3 vapor.

CHAPTER 4 RESULTS AND DISCUSSION

The results and discussion in this chapter consist of 2 main parts, which are the works on the dried BP extract and the BP extract-PVA films. In the first part, the dried BP extract was prepared by solid-liquid extraction and then subjected to freeze-drying. The quality of the dried BP extract was analyzed by measuring the total anthocyanin content in the dried BP extract. As anthocyanins can be destroyed by temperature and light, the loss of the anthocyanin content during storage under controlled conditions was studied. In the second part, the BP extract-PVA films with different BP extract contents were prepared by solution casting technique. The physical and mechanical properties of the BP extract-PVA films were examined. Moreover, the ability of the BP extract-PVA films to detect NH_3 vapor, a model volatile amine, was also investigated.

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4.1 Dried BP extract

The dried BP extract was prepared from fresh BP petals through the procedure described in Chapter 3, and the results of each step are presented in Table 4.1. Briefly, 1,500 g of fresh BP petals was dried and grounded to 112.80 g of BP powder. After that, the BP powder was extracted twice by acidic ethanol solution as an extracting solvent. The freeze-drying of the concentrated BP extract solution resulted in 8.04 g of the dried BP extract, with the yield of 0.54 and 7.07 wt%, based on fresh BP petals and dried BP powder, respectively.

Table 4.1 The preparation results of the dried BP extract.

	Fresh BP petals	**Dried BP powder**	**Dried BP extract**
Weight (g)	1,500.00	112.80	8.04
Yield (%)	-	7.12 (on fresh BP petal basis)	0.54 (on fresh BP petal basis) 7.07 (on dried BP powder basis)

In general, extraction temperature and solvent significantly affect the final yield of the dried BP extract. High temperature can increase the extraction efficiency. However, anthocyanins are sensitive to heat, and can be easily converted to the colorless forms during a heating process [21]. Therefore, the extraction temperature should be lower than 50 ˚C for preventing the thermal degradation of anthocyanins. Acidic ethanol was selected as an appropriate solvent for extracting anthocyanins from the dried BP powder because it produced clearly definable colors for each range of pH [15]. Moreover, the stability of anthocyanins in the extract solution was influenced by the acidic solvent. At

low pH conditions, anthocyanins present as the red flavylium cation which is stable only in acidic conditions [40]. Increasing the pH results in decreasing the color intensity and the concentration of the flavylium cation as it is transformed into blue quinonoidal base [35]. The quinonoidal base is an unstable form, and immediately bond to water and form a colorless compound called carbinol pseudobase and chalcone [41]. Moreover, in most flowers, fruits and vegetables the anthocyanin pigments are located in cells near the surface. Extraction procedures have generally involved the use of acidic solvent which denature the membranes of cell tissue and simultaneously dissolve pigments [23].

Commonly used solvents for anthocyanins extraction are methanol, ethanol and water. According to the work of Korabathina and co-workers [42], methanol was a rapid, easy and efficient solvent for anthocyanins extraction, but it is toxic for use in contact with food as it can cause metabolic acidosis, neurologic sequelae, and even death, when ingested. On the other hand, water exhibited a slightly higher ability to extract anthocyanins from BP flowers when compared to ethanol. In this research, the BP extract was made to dry powder. The removal of ethanol solvent is easier than water because the boiling point of ethanol is lower. Therefore, acidic ethanol is the most appropriate solvent to extract anthocyanins from BP flowers.

4.1.1 Anthocyanin content in dried BP extract

Quantity and quality of the BP extract can be determined from the spectral characteristic of anthocyanins, which relies on the structural transformation of the anthocyanin chromophore varying with pH, by UV-Visible spectroscopy. The pH differential method is a rapid and easy procedure to measure the quantity of anthocyanins in the BP extract in terms of the total monomeric anthocyanin content, which is expressed as equivalent of delphinidin-3-glucoside since it is the most common anthocyanin pigment found in the BP flowers. However, natural materials normally contain a mixture of anthocyanins and the proportions of these anthocyanins vary naturally. The UV-Visible spectra of the dried BP extract solutions (0.0002 %w/v) at pH 1.0 and 4.5 are compared in Figure 4.1. Two absorbance peaks in the visible region were recorded at 543 nm for pH 1.0 and 632 nm for pH 4.5 as the maximum absorbance of delphinidin-3-glucoside, which were used to calculate the total monomeric anthocyanin content of the BP extract. At pH < 3.0, the red flavylium cation of anthocyanins in the BP extract was the predominant species and it absorbs visible light at 543 nm. At pH 3 to 5, the red flavynium cations were converted to blue neutral quinonoidal species, which absorbs the visible light at 632 nm. In addition, there was another strong absorbance peak was observed at 575 nm for pH 4.5, indicating that the BP flowers contain a mixture of anthocyanins.

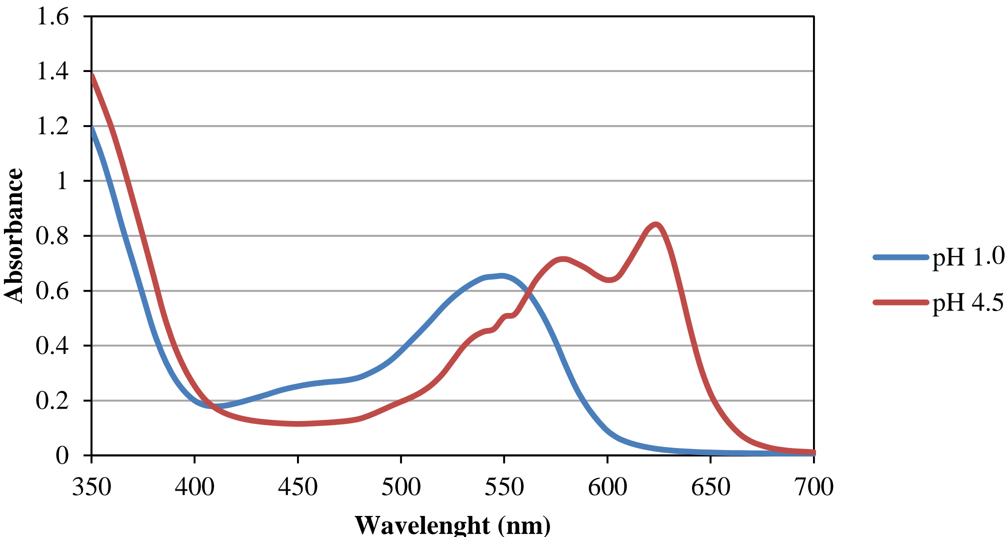

Figure 4.1 The UV-Visible spectra of the dried BP extract solutions (0.0002 %w/v) at pH 1.0 and 4.5.

As analyzed by the pH differential method, the total monomeric anthocyanin contents in the dried BP extract and dried BP powder were 44.17 and 3.24 mg/g, respectively (Table 4.2). The latter value was comparable to the extraction results reported by Suppadit and co-workers [15], in which acidic ethanol was also used as a solvent to extract anthocyanins from the dried BP powder. The reported total monomeric anthocyanin content was 3.03 mg/g of the dried BP powder.

Table 4.2 The total monomeric anthocyanin content in the BP samples.

Sample	Dried BP extract	Dried BP powder	Fresh BP petals
Anthocyanin content (mg/g)	44.17	3.24	0.24

4.1.2 Stability of anthocyanins in dried BP extract

In general, several factors are believed to affect the stability of anthocyanins in fruits, vegetables and their products during storage, which include pH, temperature, light, oxygen, metal ions, enzymes, and sugars. In this research, temperature and light were the controlled parameters. The dried BP extract was kept at 4 ˚C and in darkness for 4 weeks for studying the effect of storage time on its stability in terms of the change in the anthocyanin content. During the 4 weeks of storage, the dried BP extract was analyzed every week, and the results of the anthocyanin content in the dried BP extract are shown in Figure 4.2. In the first and second weeks, the anthocyanin content slightly decreased by only 0.5 and 1.3%, respectively, but it rapidly decreased by 16.3 and 29.4% after storage for 3 and 4 weeks, respectively. The significant decline in the anthocyanin content after 2 week could be due to the oxidation of anthocyanins caused by the presence of oxygen in the closed storage container, resulting in the structural change from colored species (e.g. flavylium cation) to colorless species (e.g. carbinol pseudobase and chalcone).

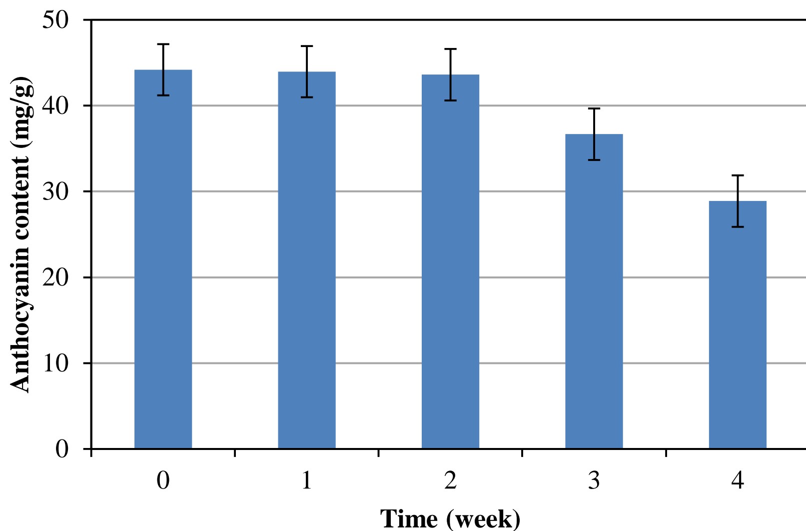

Figure 4.2 The anthocyanin content in the dried BP extract during storage for 4 weeks.

4.2 BP extract-PVA films

The BP extract-PVA films were prepared by a solution casting technique, and contained 5 different dried BP extract contents, which were 0.2, 0.4, 0.8, 1.2 and 1.6 wt% of PVA mass. By adjusting the pH of the casting solutions to 5, the films produced were transparent, blue color with varying color intensity depending on the amount of the BP extract added (Figure 4.3). From left to right, the color of the BP extract-PVA films can be referred as colorless, light blue, maya blue, denim, navy blue and prussian blue, respectively.

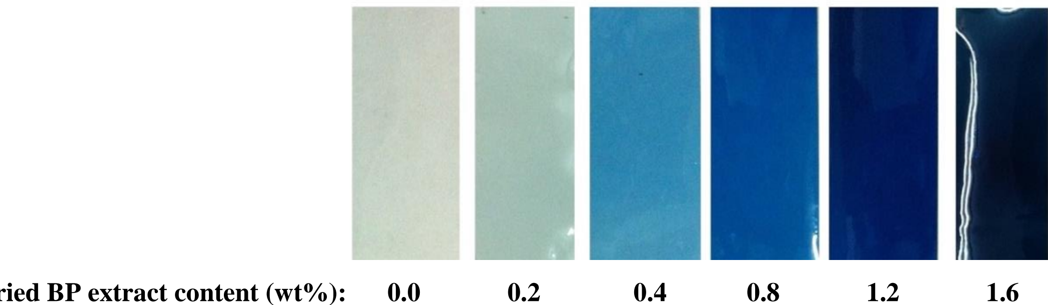

Figure 4.3 The BP extract-PVA films containing different dried BP extract contents.

At the lowest and highest dried BP extract contents (0.2 and 1.6 wt%), the blue color of the BP extract-PVA films were too pale and too dark, which were hard to observe the color change clearly by naked eyes. Therefore, the appropriate contents of the dried BP extract for producing the BP extract-PVA films in the further study were 0.4, 0.8 and 1.2 wt%.

4.2.1 Thicknesses of BP extract-PVA films

The thicknesses of the BP extract-PVA films were measured at 9 positions to obtain an average value. As seen in Figure 4.4, both of the neat PVA film and the BP extract-PVA films had uneven thickness throughout the whole film. The average thickness of the films varied from 198 to 214 μm. The deviation of the thickness of the BP extract-PVA films could be caused by the slightly inclined metal plates, on which the glass plates were placed in the hot air oven. However, the thickness of the films differed in a narrow range, suggesting that the amount of the BP extract that added into the PVA matrix did not influence the thickness of the BP extract-PVA films.

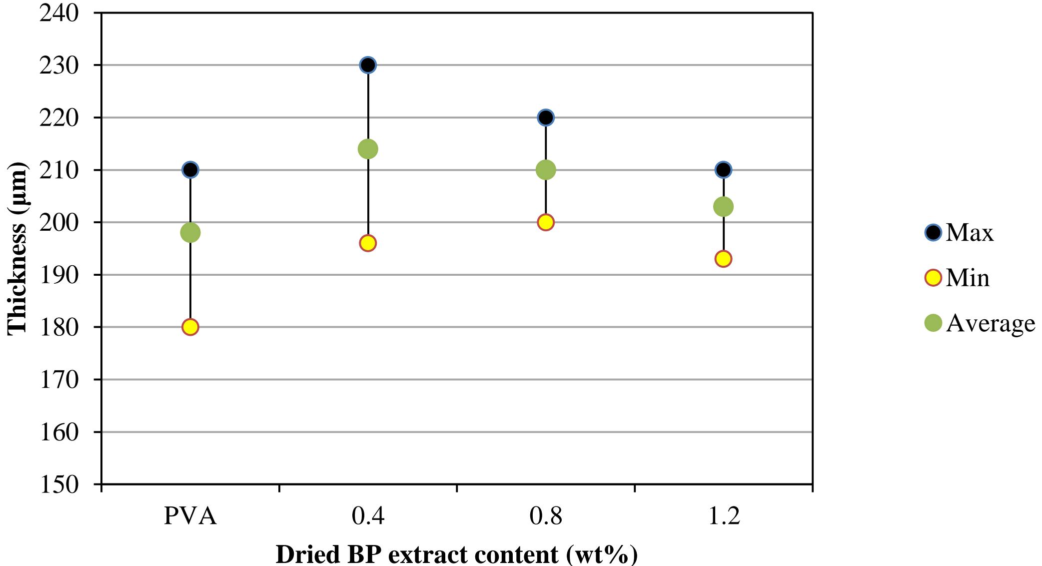

Figure 4.4 The thicknesses of the BP extract-PVA films containing different dried BP extract contents.

4.2.2 Mechanical properties of BP extract-PVA films

The mechanical properties of the BP extract-PVA films, ultimate tensile strength, ultimate %elongation and elastic modulus, were determined by tensile testing using a texture analyser (TA.XTPlus). The main product of a tensile test is a load versus elongation curve, which is then converted into a stress versus strain curve. The stress-strain curve relates the applied stress to the resulting strain and each material has its own unique stress-strain curve. Ultimate tensile strength is a measure of maximum force (tensile stress) required to pull a material to the point where it breaks. Ultimate elongation is the percentage increase in original length of a material as a result of tensile force being applied to the material to the point where it breaks. %Elongation represents for the ductility. High values of %elongation indicate that materials are very ductile. Low values indicate that materials are brittle and have low ductility. Elastic modulus defines the properties of a material as it undergoes stress, deforms, and then returns to its original shape after the stress is removed. It is a measure of the stiffness of a given

material. The elastic modulus can be calculated by dividing the tensile stress by the tensile strain.

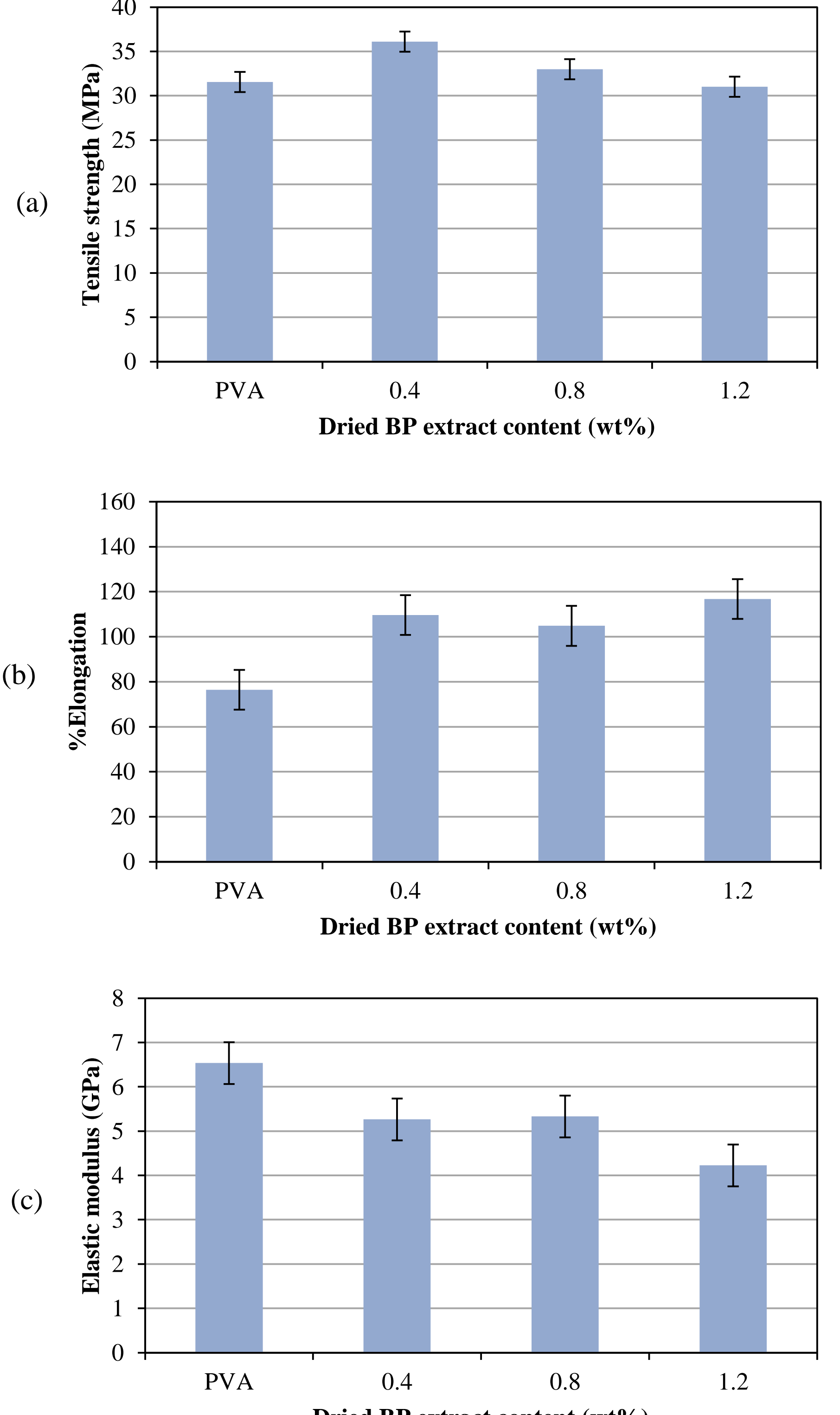

Figure 4.5 The mechanical properties of the BP extract-PVA films containing different dried BP extract contents: ultimate tensile strength (a), ultimate %elongation (b), and elastic modulus (c).

Figure 4.5 presents the mechanical properties of the BP extract-PVA films containing various BP extract contents. Same results were observed for the tensile strength, %elongation and elastic modulus, that is all the BP extract-PVA films exhibited comparable values, regardless of the different contents of the dried BP extract. In addition, a slight difference in the tensile strength between the neat PVA film and the BP extract-PVA films suggests that the presence of the BP extract in the PVA matrix did not have a significant effect on the film strength. However, the BP extract-PVA films exhibited higher %elongation but lower elastic modulus than the neat PVA film. The %elongation and elastic modulus of the BP extract-PVA films were in ranges of 104-116% and 4.1-5.3 GPa, respectively, whereas the neat PVA film had the elongation of 78% and the elastic modulus of 6.6 GPa.

4.2.3 Color stability of BP extract-PVA films

The BP extract-PVA films were kept in darkness and at 4 ˚C for 4 weeks for studying the effect of storage time on the color stability of the BP extract-PVA films, which was evaluated weekly in terms of the color change of the films. By using a colorimeter, the colors of the BP extract-PVA films were measured and expressed as the rectangular coordinates (L*, a* and b*), which were subsequently used to calculate the hue angle (h_{ab}), chroma (C*) and total color difference (ΔE). In the CIE Lab system, L* represents lightness, a* represents the red component (as positive values) and the green component (as negative values) and b* represents the yellow component (as positive value) and the blue component (as negative value). h_{ab} is the basic unit of color and can be interpreted, for example, as 0 ˚ or 360 ˚ is red, 90 ˚ is yellow, 180 ˚ is bluish green and 270 ˚ is blue. C* is the chromatic intensity; the color becomes more intense as the C* value increases. Figure 4.6 shows the relationships between the parameters L*, a* and b* and the BP extract content.

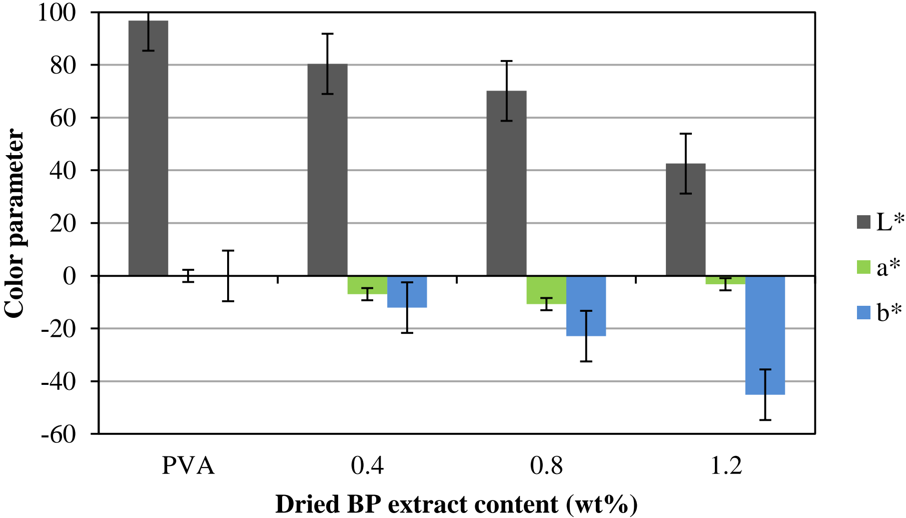

Figure 4.6 The color parameters (L*, a* and b*) of the BP extract-PVA films containing different dried BP extract contents.

As the BP extract-PVA films were blue color. The a* value that represents the red/green color was very small when compared with the L and b* values, and was not directly affected by the anthocyanin content in the BP extract-PVA films. On the other hand, the negative value of b*, which represents the blue color, became more negative with increasing the dried BP extract content as a result the BP extract-PVA films appeared more blue. Moreover, the L value relates to the color intensity, that is, it was found decreased with decreasing the b* value. The relationships between the parameters L*, a* and b* and the BP extract content were in agreement with the color of the BP extract-PVA films that were observed by naked eyes in Section 4.2, in which the blue color intensity of the films increased with increasing the content of the dried BP extract.

Figure 4.7 presents the hue angle (h_{ab}) and chroma (C*) values of the BP extract-PVA films during storage at 4 ˚C for 4 weeks. Both of the h_{ab} and C* values were found slightly decreased with storage time, suggesting that the color loss occurred during the storage. At the beginning, the BP extract-PVA films exhibited more dark blue color with the h_{ab} values in the range of 239 ˚-252 ˚, after 4 weeks of storage, the h_{ab} values of the films slightly decreased to 220 ˚-246 ˚, indicating that the films became more greenish. Similarly, the films exhibited a small decrease in the C* value. The decreases in the C* and h_{ab} values could be associated with the structural changes caused by oxidation of anthocyanins.

The total color difference (ΔE) is a useful property to measure the ability of human eyes to differentiate color differences without using equipment, that is, the color difference between 2 samples can be noticed when $\Delta E > 3.0$ [16]. As seen in Figure 4.8, the ΔE values of the BP extract-PVA films increased over storage time, and the $\Delta E > 3.0$ were observed after 4 weeks of storage. However, the color changes of the BP extract-PVA films were not obvious; therefore there should not be a significant effect on the film performance to detect the presence of NH_3 vapor.

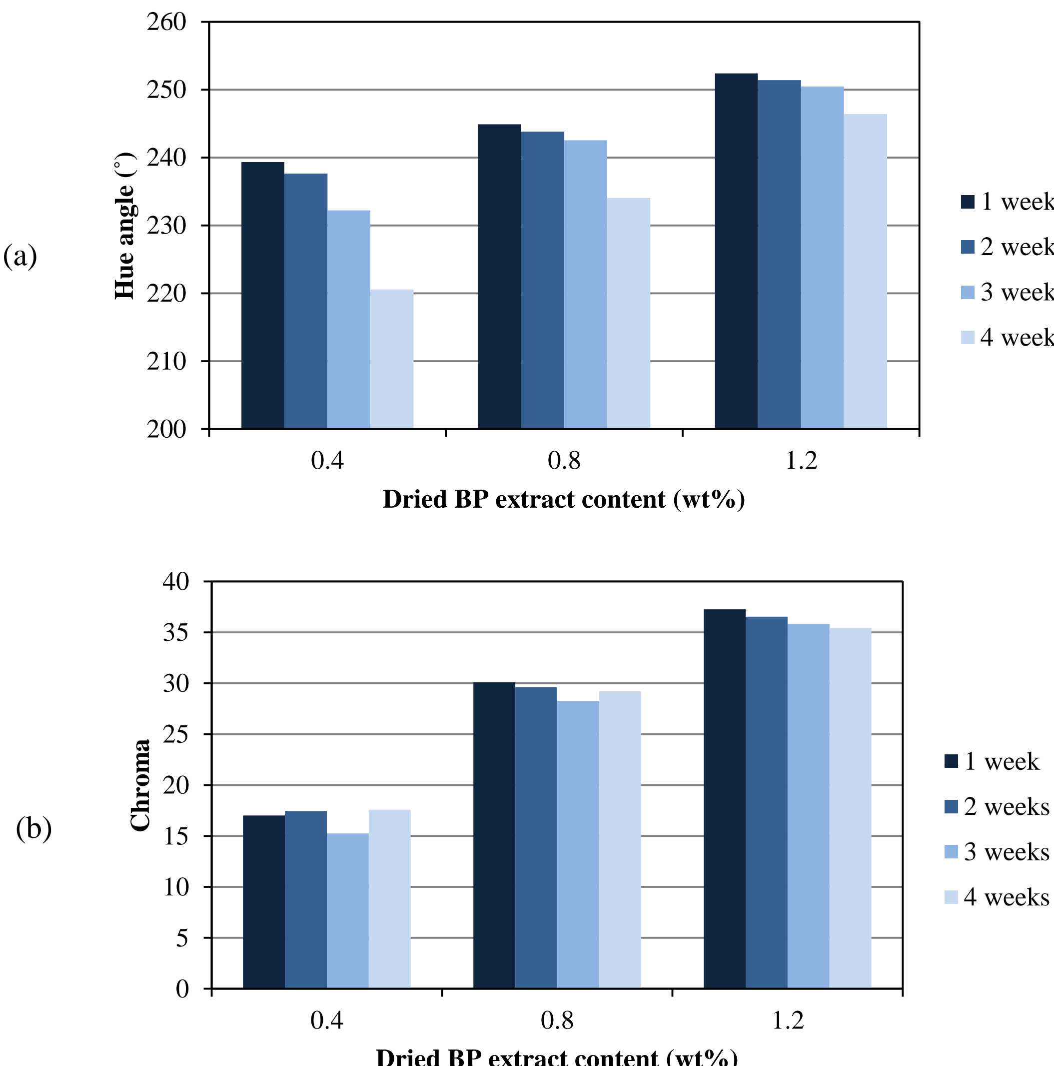

Figure 4.7 The hue angle (a) and chroma (b) values of the BP extract-PVA films containing different dried BP extract contents during storage at 4 ˚C for 4 weeks.

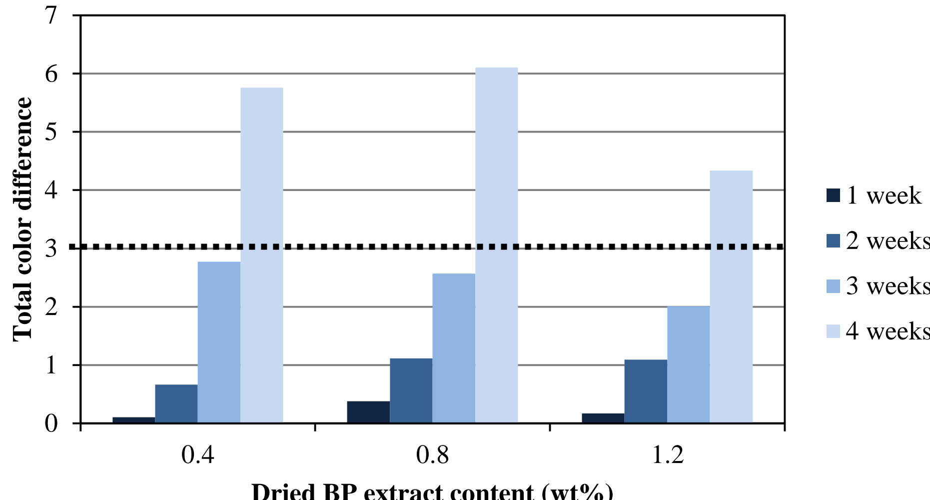

Figure 4.8 The total color difference of the BP extract-PVA films containing different dried BP extract contents during storage at 4 ˚C for 4 weeks.

4.2.4 Ability for detection of NH_3 vapor of BP extract-PVA films

The ability of the BP extract-PVA films to detect the presence of NH_3 vapor was investigated. The response of the BP extract-PVA films to NH_3 vapor in terms of their color change was evaluated by naked eyes, and also measured using a colorimeter in order to calculate the total color difference (ΔE). The NH_3 vapor was generated from the NH_3 solution at 4 different concentrations (0.01, 0.05, 0.1 and 1.0 M) at room temperature and 4 ˚C.

As the changes in color of the BP extract-PVA films when tested at 4 ˚C were not clearly seen because the inside of the containers were covered with condensed water vapor, only the color changes of the films toward various concentrations of the NH_3 solution at room temperature are presented here (Figure 4.9). The BP extract-PVA films gradually changed the color from blue (quinonoidal base as the main component) to green (a mixture of 3 components, which are quinonoidal base, carbinol pseudobase and chalcone) in respond to NH_3 vapor exposure over time, and their responses were faster at the higher concentrations of the NH_3 solution. The films became greenish blue within 5 min when tested with 1.0 M NH_3 solution whereas the films began to change color after 30 min when 0.01 M NH_3 solution was used. In addition, at all concentrations of the NH_3 solution, the color change was observed more clearly for the films with the higher BP extract contents.

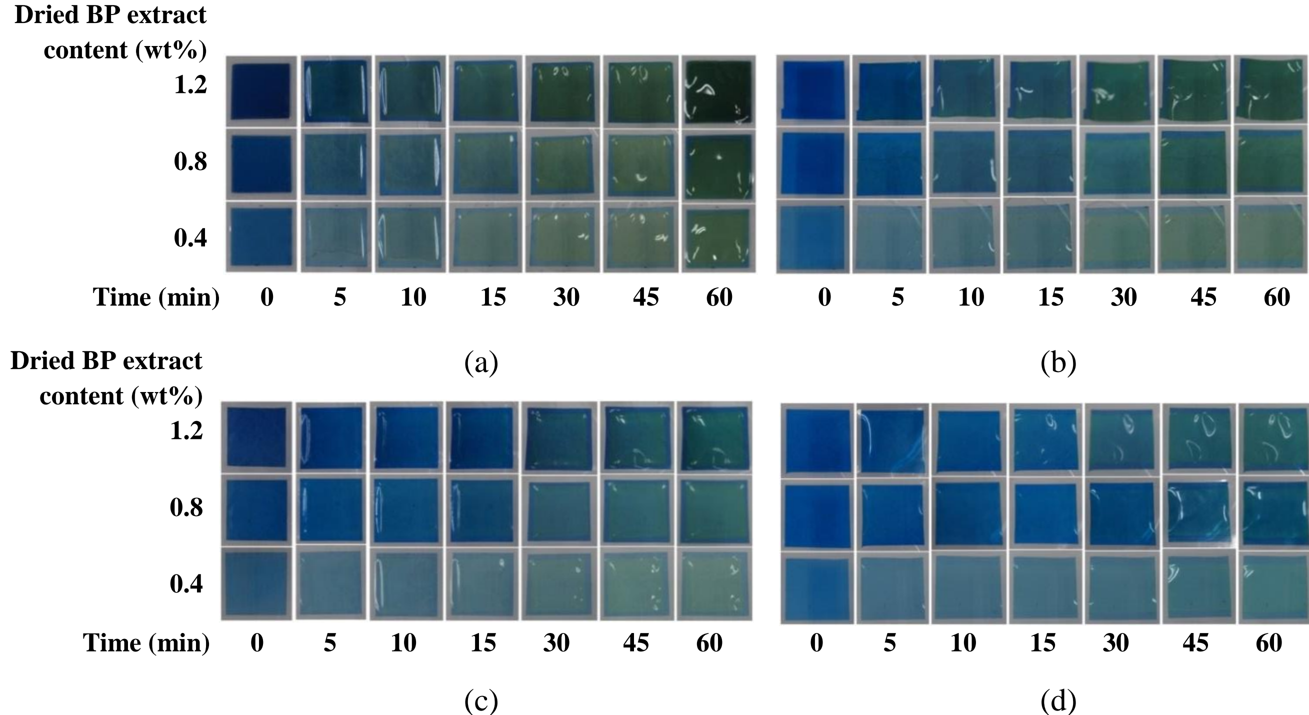

Figure 4.9 The color change of the BP extract-PVA films toward various concentrations of the NH_3 solution at room temperature: 1.0 M NH_3 solution (a), 0.1 M NH_3 solution (b), 0.05 M NH_3 solution (c) and 0.01 M NH_3 solution (d).

It is worth mentioning that the BP extract-PVA films were found to swell during the testing at both room temperature and 4 ˚C. The film swelling occurred within 5 min when tested at room temperature while the films began to swell after 15 min when tested at 4 ˚C. The swelling of the films can be explained by the fact that PVA is a hydrophilic polymer, which is sensitive to water and humidity. Accordingly, the films could swell when exposed to moisture that concurrently evaporates with the NH_3 vapor, and the film swelling is more pronounced at a higher temperature due to the higher water vaporization rate.

The color parameters of the BP extract-PVA films after tested with 4 concentrations of the NH_3 solution at room temperature and 4 ˚C are shown in Figure 4.10. After the BP extract-PVA films were exposed to the NH_3 vapor for 60 min, the L* value (indicates the lightness) of the films before and after tested were not much different. Since the final BP extract-PVA films exhibited green color instead of blue, the b* value became less negative while the a* value (indicate the green component) became more negative.

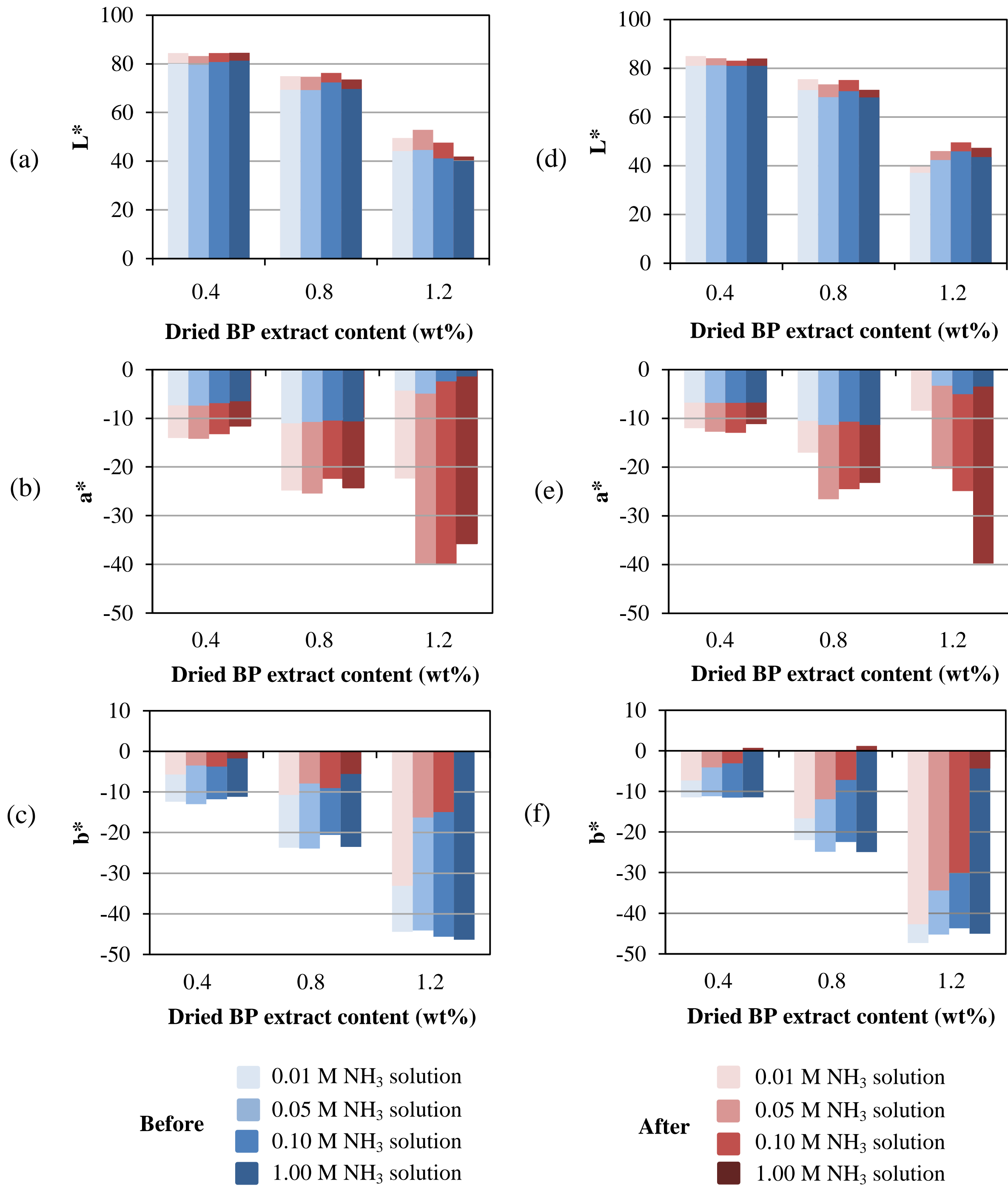

Figure 4.10 The color parameters (L*, a* and b*) of the BP extract-PVA films respond to the presence of the NH_3 vapor at room temperature and 4 ˚C: L* at room temperature (a), a* at room temperature (b), b* at room temperature (c), L* at 4 ˚C (d), a* at 4 ˚C (e) and b* at 4 ˚C (f).

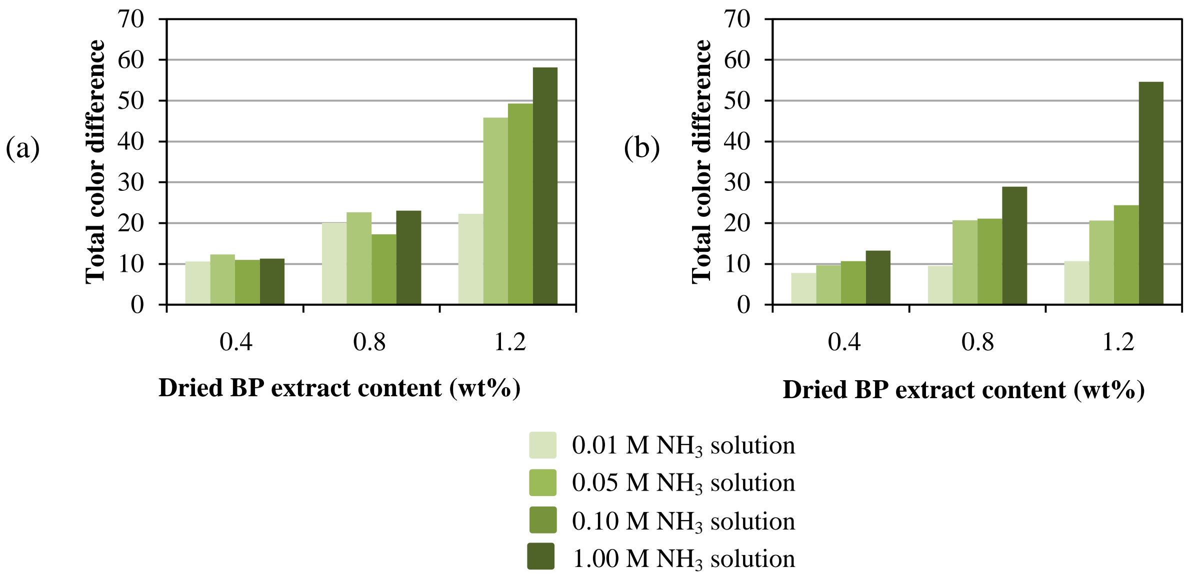

Figure 4.11 The total color difference of the BP extract-PVA films respond to the presence of the NH_3 vapor at room temperature (a) and 4 ˚C (b).

Figures 4.11 (a) and (b) illustrate the color difference (ΔE) between of the BP extract-PVA films before and after tested with different concentrations of the NH_3 solution at room temperature and 4 ˚C, respectively. The ΔE values of all BP extract-PVA films after the testing were in a range of 8 and 58 for which the ΔE value of the films tested at room temperature were greater than those of the films tested at 4 ˚C as the films exhibited more green color, which resulted from the higher vaporization rate of the NH_3 solution. The results of the color change of the BP extract-PVA films toward the NH_3 vapor exposure infer that the BP extract-PVA films should have an ability to respond to volatile amines that are generated from seafood spoilage, and therefore have potential applications as seafood spoilage indicators.

CHAPTER 5 CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

This research aimed to develop a non-toxic seafood spoilage indicator in the form of label film, by incorporating the BP extract into the PVA matrix. This BP extract-PVA film was expected to respond through visible color change to volatile amines released during seafood spoilage period.

The dried BP extract was prepared by the acidic ethanol extraction at room temperature for 2 hr, followed by freeze-drying. The yield of the dried BP extract based on fresh BP petals and dried BP powder were 0.54 and 7.07 wt%, respectively. The total anthocyanin content in the dried BP extract calculated by the pH differential method was 44.17 mg/g. The stability study of the dried BP extract revealed a slight degradation of anthocyanins over time; the total anthocyanin content in the dried BP extract decreased by 29.4% after storage for 4 weeks at 4 ˚C and in darkness.

The PVA films containing 0.2, 0.4, 0.8, 1.2 and 1.6 wt% of the dried BP extract were prepared via a solution casting technique. By adjusting the pH of the casting solution to 5, the resultant of the BP extract-PVA films appeared translucent blue, and the blue color of the films became darker as the BP extract content increased. The appropriate content of the dried BP extract were 0.4, 0.8 and 1.2 wt% because the blue color of the films containing 0.2 and 1.6 wt% of the dried BP extract were too pale and too dark, which were hard to observe the color change by naked eyes. The BP extract-PVA films had an average thickness of 198-214 μm and exhibited comparable tensile properties, regardless of the different dried BP extract contents.

The color stability of the BP extract-PVA films was investigated by storing the films in darkness at 4 ˚C for 4 weeks. A small color loss of the BP extract-PVA films was observed during storage as indicated by the slightly lower hue angle (h_{ab}) and chroma (C^*) values. The total color differences (ΔE) of the BP extract-PVA films after 4 weeks of storage were in the range of 4 to 6. However, the color change of the BP extract-PVA films during storage did not significantly affect the film performance to detect the presence of NH_3 vapor.

The BP extract-PVA films were tested for their ability to respond to NH_3 vapor as a model volatile amine. The films gradually changed from blue to green when exposed to NH_3 vapor over time, and their responses were faster at the higher concentrations of the NH_3 solution and the higher temperature. During tested with NH_3 vapor, the BP extract-PVA films were found to swell, which consequently lowered the film performance by reducing the color intensity. In addition, at all concentrations of the NH_3 solution, the color change was observed more clearly for the films with the higher BP extract contents. The obvious color change of the BP extract-PVA films when exposed to NH_3

vapor indicates that the developed BP extract-PVA films have potential applications as seafood spoilage indicators.

5.2 RECOMMENDATIONS

- Anthocyanins are a natural substance that can be degraded by several factors, including temperature, light and oxygen. The preparation of the BP extract should be carried out at low temperature as much as possible. In addition, the dried BP extract and the BP extract-PVA films should be kept at low temperature in dark containers and in the absence of oxygen in order to suppress the degradation of anthocyanins.
- The BP extract-PVA films should be tested with several types of seafood and seafood products in order to evaluate their accuracy to respond to spoilage components.
- As the BP extract-PVA films have the swelling problem when exposed to excess moisture, which leads to poor performance and appearance, the films should be modified by either physical or chemical means in order to minimize the swelling or the polymer matrix should be changed to other water-soluble polymers, which are less sensitive to moisture.

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APPENDIX A

Calculations of total anthocyanin content

Table A.1 Reported molar absorptivity of delphinidin.

Delphinidin (Dpd)	Solvent system	$\lambda_{vis\text{-}max}$ (nm)	Molar absorptivity (ε)
Dpd	0.1%HCl in ethanol	522.5	34,700
Dpd – 3 – glu	1%HCl in methanol	543	29,000
	10% ethanol, pH 1.5	520	23,700

Table A.2 Molecular weight of anthocyanidins, and anthocyanins commonly found in the nature.

Anthocyadinins	Pelargoni-din	Delphini-din	Cyanidin	Peonidin	Perunidin	Malvidin
Hex	180.2	180.2	180.2	180.2	180.2	180.2
Hex-H_2O^b	162.2	162.2	162.2	162.2	162.2	162.2
Acd + 1 hex	433.2	465.2	449.2	463.2	479.2	493.2
Acd + 2 hex	595.4	627.4	611.4	625.4	641.4	655.4
Acd + 3 hex	757.6	789.6	773.6	787.6	803.6	817.6
Pent	150.0	132.0	150.0	150.0	150.0	150.0
Pent - H_2O^b	132.0	435.0	132.0	132.0	132.0	132.0
Acd + 1 pent	403.0	597.2	419.0	433.0	449.0	463.0
Acd + 1 hex +1pent	565.2	164.2	581.2	595.2	611.2	625.2
Rhamnose	164.2	308.2	164.2	164.2	164.2	164.2
Rutinose	326.2	611.2	326.2	326.2	326.2	326.2
Rutinose - H_2O^b	308.2	773.4	308.2	308.2	308.2	308.2
Acd + rutinose	579.2	743.2	595.2	609.2	625.2	639.2
Acd+rutinose+1 hex	741.4	180.2	757.4	771.4	787.4	801.4
Acd+rutinose+1 pent	711.2	162.2	727.2	741.2	757.2	711.2

[a] Abbreviations : hex = hexose; pent = pentose; acd = anthocyanidin.

[b]-H_2O indicates a dehydrated sugar (water is lost upon forming a glycosidic bond).

Anthocyanin content

The BP extract solution was prepared by dissolving 0.5 g of the dried BP extract into 25 ml of distilled water as a stock solution. Then, 0.5 ml of the stock solution was diluted by 8.5 ml of distilled water. The final volume was then divided by the initial volume to obtain the dilution factor 1 (DF_1)

$$DF_1 = \frac{9}{0.5} = 18$$

2 dilutions of the BP extract solution, one with pH 1.0 HCl solution and the other one with pH 4.5 HCl solution were prepared by adding 0.25 ml of the BP extract solution into 4.75 ml of each HCl solution. The final volume was then divided by the initial volume to obtain the dilution factor 2 (DF_2).

For this research,

$$DF_2 = \frac{5}{0.25} = 20$$

The absorbance of the diluted sample (A) was calculated as follows:

$$A = (A_{\lambda vis\text{-}max} - A_{700})_{pH\ 4.5} - (A_{\lambda vis\text{-}max} - A_{700})_{pH\ 1.0}$$

The total anthocyanin content in the original sample solution was calculated using the following formula:

$$\text{Total anthocyanin content (mg/l)} = \frac{(A \times MW \times DF_2 \times 1000)}{\varepsilon \times 1}$$

Where MW is the molecular weight (Table A.2)
DF_2 is the dilution factor of sample solution ($DF_2 = 20$)
ε is the molar absorptivity (See Table A.1)

The total anthocyanin content in the dried BP extract was calculated using the following formula:

$$\text{Total anthocyanin content (mg/g)} = \frac{C \times 25}{1000} \times DF_1 \times \frac{1}{0.5}$$

Where C is the total anthocyanin content (mg/l)
DF_1 is the dilution factor of stock solution ($DF_1 = 18$)

Table A.3 The anthocyanin content in the dried BP extract after storage for 4 weeks.

Week	pH	Absorbance		A	Molecular weight	ε	Anthocyanin content	
		λ_{max}	at 700 nm		g/mole		mg/l	mg/g
0	1	0.738	0.007	0.153	465.2	26,900	49.09	44.17
	4.5	0.596	0.018					
1	1	0.652	0.007	0.151			48.45	43.95
	4.5	0.505	0.011					
2	1	0.399	0.003	0.127			40.75	43.60
	4.5	0.274	0.005					
3	1	0.42	-0.003	0.1			32.08	36.67
	4.5	0.324	0.001					
4	1	0.266	-0.002	0.09			28.87	28.87
	4.5	0.179	0.001					

APPENDIX B

Determination of thickness and mechanical properties of BP extract-PVA films

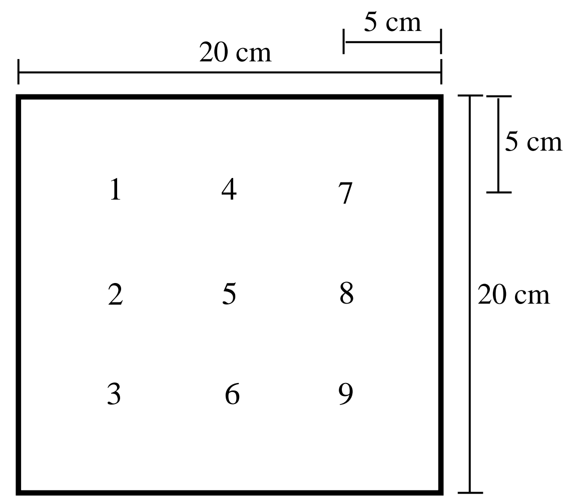

Figure B.1 The positions of film thickness measurement.

Table B.1 Thickness of the BP extract-PVA films.

Measurement point	Dried BP extract content (wt%)							
	0.0		0.4		0.8		1.2	
	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
1	188	179	228	190	218	220	203	210
2	185	195	215	192	212	200	199	205
3	209	200	189	205	220	195	198	190
4	182	210	227	230	200	205	208	194
5	208	210	229	228	214	208	193	188
6	210	225	219	209	220	210	210	180
7	199	182	230	212	205	240	203	174
8	203	190	205	208	201	238	206	180
9	180	230	180	215	203	225	202	170
Min	**180**	**179**	**196**	**190**	**200**	**195**	**193**	**170**
Max	**210**	**230**	**230**	**230**	**220**	**240**	**210**	**210**
Average	**198**	**202**	**214**	**210**	**210**	**216**	**203**	**188**

Table B.2 The mechanical properties of the BP extract-PVA films containing different dried BP extract contents: ultimate tensile strength, ultimate %elongation and elastic modulus.

Dried BP extract content (wt%)	**Film sample**	**Ultimate tensile strength**	**Ultimate %elongation**	**Elastic modulus**
		MPa	**-**	**GPa**
0.0	1	32.38	72.85	5.90
	2	34.20	81.52	6.59
	3	31.60	76.42	7.30
	4	27.99	74.88	6.53
	Average	**31.55**	**76.42**	**6.58**
0.4	1	39.43	106.86	5.20
	2	35.94	109.34	5.68
	3	32.96	112.74	5.07
	4	36.07	109.72	5.05
	Average	**36.11**	**109.65**	**5.25**
0.8	1	40.17	98.94	6.48
	2	35.24	113.58	5.58
	3	29.98	101.99	3.94
	4	23.58	103.96	5.35
	Average	**33.00**	**104.83**	**5.33**
1.2	1	33.07	119.36	4.43
	2	30.16	119.18	4.25
	3	29.06	111.79	3.99
	4	32.01	115.29	4.18
	Average	**31.03**	**116.77**	**4.23**

APPENDIX C

Colors measurement of BP extract-PVA films

Table C.1 The color parameters (L*, a* and b*) of the BP extract-PVA films.

Dried BP extract content (wt%)	Measurement repetition	L*	a*	b*
0.0	1	96.8	-0.02	-0.07
	2	97.2	-0.01	-0.08
	3	96.5	0	-0.07
	4	96.2	-0.03	-0.05
	Average	**96.68**	**-0.02**	**-0.068**
0.4	1	80.02	-7.33	-12.40
	2	79.60	-7.42	-13.01
	3	80.73	-6.91	-11.83
	4	81.28	-6.48	-11.21
	Average	**80.41**	**-7.04**	**-12.11**
0.8	1	69.28	-11.04	-23.74
	2	69.18	-10.74	-23.92
	3	72.40	-10.48	-20.55
	4	69.67	-10.62	-23.52
	Average	**70.13**	**-10.72**	**-22.93**
1.2	1	44.22	-4.30	-44.41
	2	44.63	-4.91	-44.10
	3	41.17	-2.42	-45.62
	4	40.20	-1.39	-46.36
	Average	**42.56**	**-3.26**	**-45.12**

Note: the L*, a* and b* values of the BP extract-PVA films were measured 4 times by rotating the film sample 90 ˚ to obtain an average parameter value.

Table C.2 The color parameters (L*, a* and b*), hue angle (h_{ab}), chroma (C*) and total color difference (ΔE) of the BP extract-PVA films after storage for 4 weeks at 4 ˚C.

Dried BP extract content (wt%)	Measurement repetition	0 week					1 week						2 weeks					
		L*	**a***	**b***	**h_{ab}**	**C***	**L***	**a***	**b***	**ΔE**	**h_{ab}**	**C***	**L***	**a***	**b***	**ΔE**	**h_{ab}**	**C***
0.4	1	78.2	-8.4	-14.2	239.6	16.5	78.4	-8.2	-14.4	0.2	240.2	16.5	78.1	-9.1	-14.4	0.8	237.7	17.0
	2	77.2	-9.0	-15.3	239.6	17.8	77.5	-9.3	-15.1	0.5	238.4	17.8	77.1	-9.5	-15.4	0.5	238.2	18.1
	3	77.8	-8.7	-14.6	239.1	17.0	77.6	-8.5	-14.4	0.3	239.4	16.8	77.8	-9.4	-14.5	0.7	237.0	17.3
	4	77.6	-8.5	-14.8	240.1	17.1	78.0	-8.5	-14.8	0.4	240.1	17.1	77.9	-9.0	-15.0	0.6	239.0	17.5
	Average	**77.7**	**-8.7**	**-14.7**	**239.4**	**17.1**	**77.8**	**-8.7**	**-14.6**	**0.3**	**239.3**	**17.0**	**77.7**	**-9.3**	**-14.8**	**0.7**	**237.6**	**17.5**
0.8	1	61.8	-12.9	-30.4	247.0	33.1	60.2	-13.0	-30.2	1.6	246.7	32.9	61.8	-13.7	-29.8	1.1	245.2	32.7
	2	66.0	-12.5	-26.8	244.9	29.6	66.8	-12.7	-26.2	0.9	244.7	29.6	66.2	-12.8	-28.6	0.9	243.8	28.9
	3	67.5	-12.1	-24.8	244.1	28.1	69.7	-12.6	-24.8	0.9	242.9	27.9	67.1	-12.7	-24.1	1.5	242.2	27.3
	4	64.8	-12.7	-28.0	245.5	30.2	63.4	-13.0	-28.1	0.3	244.9	30.2	66.4	-13.3	-23.9	1.1	243.7	29.7
	Average	**65.1**	**-12.5**	**-27.5**	**245.5**	**30.2**	**65.0**	**-12.8**	**-27.3**	**1.1**	**244.8**	**30.1**	**65.4**	**-13.1**	**-26.6**	**1.2**	**243.7**	**29.6**
1.2	1	57.9	-11.8	-34.4	251.5	36.6	56.9	-12.4	-34.6	0.4	250.9	36.6	56.8	-12.1	-33.8	1.0	250.4	35.9
	2	58.4	-11.9	-32.0	250.4	35.4	58.9	-11.9	-31.4	0.2	250.5	35.2	57.6	-12.6	-32.5	0.9	249.5	34.7
	3	52.8	-10.0	-38.6	255.5	39.9	52.7	-10.1	-38.7	0.5	255.4	40.0	51.5	-10.8	-37.9	1.4	254.0	39.2
	4	53.7	-10.3	-37.2	254.5	38.6	53.8	-10.8	-37.2	0.5	253.8	38.7	56.4	-11.2	-33.8	4.4	251.7	35.6
	Average	**55.8**	**-11.2**	**-35.5**	**252.6**	**37.2**	**55.7**	**-11.3**	**-35.5**	**0.4**	**252.3**	**37.3**	**55.5**	**-11.7**	**-34.6**	**1.1**	**251.3**	**36.6**

Note: the L*, a* and b* values of the BP extract-PVA films were measured 4 times by rotating the film sample 90 ˚ to obtain an average parameter value.

Table C.2 The color parameters (L^*, a^* and b^*), hue angle (h_{ab}), chroma (C^*) and total color difference (ΔE) of the BP extract-PVA films after storage for 4 weeks at 4 ˚C (Cont'd.).

Dried BP extract content (wt%)	Measurement repetition	3 weeks						4 weeks					
		L*	**a***	**b***	**ΔE**	**h_{ab}**	**C***	**L***	**a***	**b***	**ΔE**	**h_{ab}**	**C***
0.4	1	77.9	-9.1	-12.9	1.5	234.9	15.8	79.8	-13.3	-10.8	6.1	219.0	17.2
	2	78.3	-9.5	-13.0	2.6	233.8	16.1	78.0	-13.1	-12.4	5.1	223.5	18.0
	3	77.6	-9.4	-10.2	4.5	227.2	13.9	78.8	-13.6	-11.1	6.1	219.1	17.6
	4	79.0	-9.2	-12.5	2.8	233.6	15.5	77.4	-13.7	-11.6	6.1	220.3	18.0
	Average	**78.2**	**-9.3**	**-12.1**	**2.9**	**232.0**	**15.3**	**78.6**	**-13.4**	**-11.4**	**5.8**	**220.6**	**17.6**
0.8	1	65.3	-13.8	-27.4	5.0	242.9	30.4	62.7	-18.4	-26.2	7.0	234.9	32.0
	2	65.9	-12.0	-25.7	1.9	244.3	27.7	67.2	-16.0	-23.3	5.2	235.2	28.4
	3	65.8	-13.2	-23.2	2.9	240.4	26.7	68.5	-16.7	-21.5	6.2	231.9	27.3
	4	65.8	-12.7	-24.0	3.6	242.1	27.2	65.4	-17.8	-24.0	6.3	233.4	29.9
	Average	**65.7**	**-13.0**	**-25.1**	**3.3**	**242.5**	**28.3**	**66.0**	**-17.2**	**-23.7**	**6.1**	**234.0**	**29.2**
1.2	1	56.8	-12.8	-32.9	2.1	248.7	35.3	58.7	-15.9	-31.4	4.9	244.2	34.6
	2	58.3	-12.0	-31.1	2.2	248.9	33.3	59.3	-14.4	-30.5	3.8	244.8	33.7
	3	50.6	-11.1	-37.2	2.1	253.4	38.8	52.2	-13.7	-35.9	4.3	249.8	37.9
	4	56.0	-12.1	-34.2	4.2	250.5	36.3	53.8	-12.7	-31.7	6.0	248.2	34.1
	Average	**55.4**	**-12.0**	**-33.8**	**2.1**	**250.3**	**35.8**	**56.1**	**-14.2**	**-32.4**	**4.3**	**246.3**	**35.4**

Note: the L^*, a^* and b^* values of the BP extract-PVA films were measured 4 times by rotating the film sample 90 ˚ to obtain an average parameter value.

Table C.3 The color parameters (L*, a* and b*) and total color difference (ΔE) of the BP extract-PVA films before and after exposed to the NH_3 vapor generated from different concentrations of the NH_3 solution at room temperature.

Dried BP extract content (wt%)	NH_3 solution concentration (M)	Before NH_3 vapor exposure			After NH_3 vapor exposure			ΔE
		L*	a*	b*	L*	a*	b*	
0.4	0.01	80.02	-7.33	-12.40	84.45	-14.27	-5.71	10.61
	0.05	79.60	-7.42	-13.01	83.19	-14.40	-3.49	12.34
	0.10	80.73	-6.91	-11.83	84.42	-13.46	-3.79	11.01
	1.00	81.28	-6.48	-11.21	84.56	-11.72	-1.75	11.30
0.8	0.01	69.28	-11.04	-23.74	74.97	-25.22	-10.74	20.06
	0.05	69.18	-10.74	-23.92	74.70	-25.81	-7.91	22.67
	0.10	72.40	-10.48	-20.55	76.31	-22.74	-9.04	17.26
	1.00	69.67	-10.62	-23.52	73.63	-24.58	-5.59	23.07
1.2	0.01	44.22	-4.30	-44.41	49.51	-22.69	-33.09	22.23
	0.05	44.63	-4.91	-44.10	52.86	-40.38	-16.26	45.84
	0.10	41.17	-2.42	-45.62	47.65	-40.52	-14.98	49.32
	1.00	40.20	-1.39	-46.36	41.99	-36.18	0.15	58.11

Table C.4 The color parameters (L*, a* and b*) and total color difference (ΔE) of the BP extract-PVA films before and after exposed to the NH_3 vapor generated from different concentrations of the NH_3 solution at 4 ˚C.

Dried BP extract content (wt%)	NH_3 solution concentration(M)	Before NH_3 vapor exposure			After NH_3 vapor exposure			ΔE
		L*	a*	b*	L*	a*	b*	
0.4	0.01	80.99	-6.8	-11.45	84.94	-12.02	-7.31	7.75
	0.05	81.19	-6.85	-11.21	84.09	-12.76	-4.13	9.67
	0.10	80.93	-6.84	-11.54	83.1	-12.95	-3.07	10.67
	1.00	81.03	-6.75	-11.46	83.98	-11.17	0.71	13.28
0.8	0.01	71.06	-10.54	-22.00	75.52	-17.05	-16.66	9.53
	0.05	68.12	-11.35	-24.88	73.38	-26.61	-11.93	20.69
	0.10	70.62	-10.68	-22.44	75.17	-24.53	-7.2	21.09
	1.00	68.01	-11.38	-24.97	71.12	-23.28	1.2	28.92
1.2	0.01	37.12	0.65	-47.30	40.1	-8.46	-42.67	10.64
	0.05	42.28	-3.32	-45.26	46.06	-20.4	-34.37	20.61
	0.10	45.95	-5.06	-43.69	49.57	-24.95	-30.04	24.39
	1.00	43.56	-3.47	-45.05	47.3	-39.77	-4.39	54.63

CURRICULUM VITAE

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