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Graphical abstract
Highlights

- Colorimetric films were developed by using roselle anthocyanins, starch and PVA.
- Roselle anthocyanins improved the compatibility between starch and PVA.
- The colorimetric films had good color stabilities within 14 days at 4°C and 25°C.
- The colorimetric film with fewer roselle anthocyanins was more sensitive to NH₃.
- The colorimetric films can be used to monitor the fish freshness at 4°C.
Novel colorimetric films based on starch/polyvinyl alcohol incorporated with roselle anthocyanins for fish freshness monitoring

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Abstract

Novel colorimetric films were developed for real-time monitoring of fish freshness based on starch/polyvinyl alcohol (SPVA) incorporated with roselle (*Hibiseus sabdariffa* L.) anthocyanins (RACNs). Firstly, RACNs were extracted from roselle dehydrated calyces. Secondly, SPVA aqueous solution was obtained with a mass rate of 2:1 (starch/PVA). Thirdly, the colorimetric films were fabricated by immobilizing 30, 60 and 120 mg RACNs/100 g starch into SPVA matrix with casting/solvent evaporation method. FTIR spectra of the colorimetric films showed that RACNs were successfully immobilized into the SPVA matrix. X-ray diffraction spectra and SEM micrographs indicated that the crystallinity of PVA was reduced during the film-forming process and the compatibility between starch and PVA was improved, owing to the presence of RACNs. The incorporation of RACNs led to a decrease of water content and tensile strength and an increase of elongation at break of the colorimetric films compared with the SPVA film. The color stability test showed that the colorimetric films were stable at refrigeration temperature and room temperature up to 14 days with relative color changes below than 5%. The colorimetric films with lower content of RACNs were found more sensitive towards ammonia. An application trial was conducted to monitor the freshness of silver carp (*Hypophthalmichthys molitrix*) at refrigeration temperature. The colorimetric films presented visible color changes over time due to a variety of basic volatile amines known as total volatile basic nitrogen (TVB-N). Hence, these colorimetric films can be used to monitor the real-time fish freshness for intelligent packaging.

Keywords: Colorimetric film; Fish freshness; Roselle anthocyanins; Starch; Polyvinyl alcohol; Intelligent packaging.
1. Introduction

Fish is highly perishable due to enzymatic reaction and microbial contamination (Zhang, Sun, Xiao, Liu, & Zheng, 2016). Considering the food quality and safety, it is essential to evaluate the fish freshness during the supply chain. TVB-N has been widely regarded as an useful indicator of the fish freshness for a long period (Olafsdóttir, et al., 1997). It is comprised of ammonia (NH$_3$), trimethylamine (TMA) and dimethylamine (DMA) generated by the enzymatic decomposition of trimethylamine oxide (TMAO) (Byrne, Lau, & Diamond, 2002). A variety of approaches have been developed to determine the TVB-N level. Chemical methods such as Kjeldahl method can provide precise results, but they are generally time consuming and destructive to samples. Other rapid and non-destructive detection methods, such as FTIR spectroscopy, can also provide satisfactory results (Cai, Chen, Wan, & Zhao, 2011), whereas they need advanced instruments and highly skilled operators. Consequently, these methods are not suitable for consumers to evaluate the real-time freshness.

In the last decades, there was a rapidly growing interest in developing intelligent packaging systems for ‘on-package’ tracing the real-time food quality. Particularly, colorimetric indicators have received wide attentions because they can exhibit straightforward information by visible color changes. As regard to evaluating the fish freshness, Pacquit, et al. (2007) developed a colorimetric indicator by spin-coating bromocresol green onto a PET substrate. The color of the indicator gradually changed from yellow to green in response to the increasing TVB-N level at room temperature. Similarly, polyaniline-based colorimetric indicator has also been demonstrated to detect the fish spoilage (Kuswandi, et al., 2012). These colorimetric indicators fixed in the headspace of the packaged fish presented specific color changes upon reaction with the TVB-N in the form of gas sensors. In this way, these intelligent packaging systems had great potential to indicate the real-time fish freshness.

Recently, more researches have focused on the natural pigments as a source of color agents, because they are safer and more eco-friendly as compared to chemosynthetic dyes. Anthocyanins are natural water-soluble pigments that have wide
response ranges to pH variation. Several kinds of anthocyanins have been utilized to
fabricate the colorimetric indicators for sensing the food quality, such as anthocyanins
extracted from red cabbage (Pereira, de Arruda, & Stefani, 2015), grape skin (Ma &
Wang, 2016) and purple sweet potato (Choi, Lee, Lacroix, & Han, 2017). Zhang, Lu,
and Chen (2014) developed a pH sensing film by incorporating anthocyanins extracted
from Bauhinia blakeana Dunn with chitosan and the pH sensing film presented a
distinguishable color change from purple to green due to the basic volatile gases
generated from the fish, suggesting that the anthocyanins-based colorimetric films were
good candidates of gas sensors for monitoring fish freshness. When a constant amount
of fish samples was stored in a specific circumstance (e.g. temperature, packaging
volume), the concentration of the TVB-N in the headspace was definite after a specific
period of storage. Therefore, the extent of color change of the colorimetric film was
related to the content of the anthocyanins. However, the effect of the anthocyanins
content on color rendering properties of the colorimetric film has not been investigated
yet.

Roselle (Hibiseus sabdariffa L.) is an herbaceous plant, cultivated largely in
tropical and subtropical areas of both hemispheres (Sinela, et al., 2017). Its calyces
contain high amounts of anthocyanins up to 1.5 g/100 g on dry weight basis
(Degenhardt, Knapp, & Winterhalter, 2000). The biological activities of roselle
anthocyanin (RACNs), such as antioxidant activity (Tsai, McIntosh, Pearce, Camden,
& Jordan, 2002) and anti-hypertensive effect (Ajay, Chai, Mustafà, Gilani, & Mustafa,
2007) have been widely studied, while the potential use of RACNs for the development
of colorimetric indicators has not been explored. In order to immobilize the
anthocyanins, several natural polymers have been used for making different
colorimetric films, including chitosan (Zhang, et al., 2014), starch (Choi, et al., 2017,
Golasz, Silva, & Silva, 2013) and cellulose (Ma, et al., 2016). Among them, starch has
received greater attention because of its stability to heat, acid and base conditions.
However, pure starch film generally lacks the strength and processability, which can be
alternatively addressed by adding polyvinyl alcohol (PVA) (Sin, Rahman, Rahmat, &
Mokhtar, 2011). Since 1980s, starch/polyvinyl alcohol (SPVA) films have been studied
for packaging applications (Tang, Zou, Xiong, & Tang, 2008; Tang & Alavi, 2011). They have good transparency (Cano, Cháfer, Chiralt, & González-Martínez, 2015), which is beneficial for the development of colorimetric films. Furthermore, starch and PVA are both non-toxic, renewable and biodegradable (Lu, Xiao, & Xu, 2009; Rezaei, Nasirpour, & Fathi, 2015), which can eliminate the public concerns over food safety and environmental problems.

Therefore, in this study, we aimed to develop new colorimetric films by incorporating various content of RACNs into SPVA matrix through casting/solvent evaporation method. The microstructure of colorimetric films was studied by using X-ray diffractometer and SEM. The effect of the RACNs content on mechanical properties, color stability and sensitivity toward ammonia of the colorimetric films was investigated. Finally, the colorimetric films were employed to monitor the freshness of silver carp (Hypophthalmichthys molitrix) at refrigeration temperature (4°C).

2. Material and methods

2.1. Materials

Roselle dehydrated calyxes and live silver carp were obtained from the local market in Zhenjiang, China. Other materials including soluble starch, polyvinyl alcohol (MW: 1750 ± 50), ethyl alcohol (C₂H₆O), potassium chloride (KCl), sodium acetate (CH₃COONa·3H₂O), magnesium oxide (MgO), methyl red (C₁₅H₁₅N₃O₂), methylene blue (C₁₆H₁₆ClN₃S), boric acid (H₃BO₃), ammonia solution (NH₃·H₂O, 25%~27%), acetic acid (CH₃COOH) and hydrochloric acid (HCl) were all purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Plastic Petri dishes were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Extraction of anthocyanins from roselle dehydrated calyxes

The anthocyanins were extracted according to Chang, et al. (2012) with a slight modification. The roselle dehydrated calyxes were crushed and blended with 75% ethanol aqueous solution at a solid–liquid ratio of 1:10 for 2 h at 25°C. The extract was centrifuged at 8000 rpm for 20 min. The extraction procedure was repeated three times.
Ethanol in filtrate was removed with a rotary evaporator at 35°C in dark. Finally, the solution was freeze-dried under vacuum and the obtained anthocyanins extract powder was stored in sample vials at 4°C in the nitrogen atmosphere.

2.3. Determination of total anthocyanins content in extract powder

The anthocyanins content in extract powder was measured by pH differential method (Wang, Li, Chen, Xin, & Yuan, 2013) using a UV-Vis spectrophotometry (Agilent CARY 100, Varian Corporation, USA). The anthocyanins extract powder (20 mg) was dissolved in 10 mL distilled water, and 1 mL anthocyanins solution was dissolved in 9 mL of 0.025 M potassium chloride buffer (pH 1.0) and 9 mL of 0.4 M sodium acetate buffer (pH 4.5) respectively in separate test tubes. Absorbance of sample was measured at 520 and 700 nm. The anthocyanins content was expressed in mg/g.

2.4. Preparation of the colorimetric films

Firstly, 100 mL aqueous dispersion containing 2 g starch and 1 g PVA was heated at 100°C in a water bath and stirred with a magnetic stirrer until it was completely dissolved. Based on the calculated anthocyanins content (9.51 ± 0.41 mg/g) (refer to section 2.3), a certain amount of anthocyanins extract powder was then added to the cooled SPVA solution at 30, 60 and 120 mg RACNs/100 g starch, expressed as RACNs-30, RACNs-60 and RACNs-120, respectively. The mixtures were then homogenized (Ultra Turrax IKA T25 digital, Germany) at 8000 rpm for 5 min and degassed with a sonicator (Branson CPX5800H, USA) for 5 min at room temperature. Each film was prepared by casting 10 mL of the film-forming solution into a clean and smooth plastic Petri dish with a 53 mm diameter. The Petri dishes were placed on a level surface in an incubator at 35°C with 50% RH for 36 h. After that, the films were peeled from the Petri dishes and stored at 4°C with 75% RH for further use.

2.5. Spectral characteristic of RACNs

The color and spectra of RACNs solution at different pH (2-12) were recorded using a UV-Vis Spectrophotometer (Agilent CARY 100, Varian Corporation, USA) in
2.6. Characterization of the colorimetric films

2.6.1. FTIR spectroscopy

Fourier transform infrared (FTIR) spectra of the films were determined with a FTIR spectrometer (Perkine Elmer 16 PC spectrometer, Boston, USA). Spectra were recorded at the absorbance mode from 4000 to 650 cm$^{-1}$ at a resolution of 4 cm$^{-1}$ and the total number of scans was 32. OMNIC Spectra software (Thermo Scientific Co., USA) was used to configure the FTIR spectrometer for scanning and mathematical processing.

2.6.2. X-ray diffraction spectra

X-ray diffraction (XRD) spectra of films were measured using an X-ray diffractometer (D8 ADVANCE, Bruker, Germany) with a reference of target of Cu Ka radiation, voltage of 40 kV, current of 30 mA. The films were measured at an angle from 5° to 40° ($2\theta$) with steps of 2° ($2\theta$)/min.

2.6.3. Scanning electron microscopy (SEM)

The micrographs of the films were recorded by a field emission scanning electron microscopy (FE-SEM) (S-4800, Hitachi High-Technologies corporation, Japan). The films were first freeze-fractured by liquid nitrogen before measurement. Samples were attached to double-sided adhesive tape and mounted on the specimen holder, then sputtered and coated with gold under vacuum.

2.6.4. Thickness, water content and mechanical properties of the films

The thickness of the films was measured by a hand-held digital micrometer (Sanfeng Group Co., Ltd., Taiwan, China). The thickness was measured at 20 random positions on the films.

To determine the moisture content (MC), the films were dried to an equilibrium weight at 105°C in an oven. MC was calculated according to the following equation:
\[ MC(\%) = 100 \times \left( \frac{M_i - M_f}{M_i} \right) \]  
where \( M_i \) was the initial weight of films stored in 75% RH to moisture equilibrium (g) and \( M_f \) was the final weight of films dried at 105°C (g).

Tensile strength (TS) and elongation at break (EB) were measured with an Instron Universal Testing Machine (Model 4500, Instron Corporation, Canton, MA, USA) using a modified ASTM D882-00 (ASTM, 2000b) procedure. Each film was cut in rectangular strips with 40 mm length and 20 mm width. The initial grip separation and crosshead speed were set at 20 mm and 0.6 mm/s, respectively. Measurements represent an average of three samples. The TS and EB were calculated as the following equations:

\[ TS = \frac{F_{\text{max}}}{S} \]  
\[ EB = 100 \times \frac{\Delta l}{l_0} \]

where \( TS \) was the tensile strength (MPa); \( F_{\text{max}} \) was the maximum load (N); \( S \) was the initial cross-sectional area of the film sample (mm²); \( EB \) was the elongation at break; \( \Delta l \) was the extension of the films (mm) and \( l_0 \) was the initial length of the films (20 mm).

2.6.5. Color stability of the colorimetric films

The colorimetric films were stored in incubators at 4°C and 25°C with 75% RH under fluorescent lights. The images of the colorimetric films were captured every day for two weeks by an optical scanner (Scanjet G4050, HP) and analyzed by a user program in Matlab R2012a (Matworks Inc., Natick, MA, USA). The stability of the colorimetric films was defined as the relative color change (Xiaowei, et al., 2015):

\[ \Delta R = |R_0 - R_1| \]  
\[ \Delta G = |G_0 - G_1| \]  
\[ \Delta B = |B_0 - B_1| \]  
\[ S = \frac{\Delta R + \Delta G + \Delta B}{R_0 + G_0 + B_0} \times 100\% \]

where \( R_0, G_0, B_0 \) were the initial gray values of the red, green and blue, \( R_1, G_1, B_1 \) were the gray values of the red, green and blue after storage. \( S \) was the relative color change.
of $R$, $G$ and $B$ values.

2.6.6. Response of the colorimetric films to ammonia

Response of the colorimetric films toward volatile ammonia in term of their color changes was performed using absorbance measurements (Kuswandi, et al., 2012). The colorimetric films were cut into squares (10 × 10 mm) and hang up in an erlenmeyer flask (500 mL) at 1 cm above the ammonia solution (80 mL, 8 mM) for 24 min at 25°C. UV-vis spectra of the films in the range of 400-800 nm were recorded every 2 min by a hand-held fiber optical vis-NIR spectrometer (Ocean Optics Co., ltd, USA).

2.7. Application of the colorimetric films for monitoring fish freshness

2.7.1. Fish spoilage trial

Live silver carp was cut into strips after removing its innards, head, tail and feathers. Then, 20 g of silver carp was immediately transferred into covered Petri dishes with 90 mm diameters. The colorimetric films were placed in the headspace of the Petri dish. The Petri dishes were stored in a refrigerator at 4°C for 165 h. The color of the colorimetric films was recorded after every 15 h by using the optical scanner.

2.7.2. Determination of TVB-N

The TVB-N level of the fish sample was measured by a stream distillation method (Cai, et al., 2011). The 10 g portion was placed in a beaker, blended with 100 mL distilled water, then grounded by using a tissue homogenizer (A-88, Jintan medical instrument plant, China). The homogenate was filtered by using filter papers. Then, 5.0 mL filtrare was transferred to Kjeldahl distillation unit (ZLQ03, East China Glass Co. Ltd., China) with addition of 5 mL 1% magnesium oxide suspension (1 g/L). The distillate was collected in a flask containing 10 mL 2% aqueous solution of boric acid and 3 droplets of mixed indicator produced from dissolution of 0.2 g of methyl red and 0.1 g of methylene blue to 100 mL of ethanol. After that, the boric acid solution was titrated with a 0.01 M hydrochloric acid solution. TVB-N value was determined by the hydrochloric acid used during titration.
3. Results and discussion

3.1. Color and spectral properties of RACNs

Fig. 1a shows the color change of RACNs solutions in the pH range of 2 to 12. It can be seen that the color of RACNs solutions was pink at pH lower than 5, and changed gradually to purple at pH 6-7. When the solutions were basic, the color altered to blue and yellow at pH 8-9 and 10-12, respectively. The UV-vis spectra of RACNs solutions corresponding with color changes were recorded, as shown in Fig. 1b. When the pH value was lower than 4, the maximum absorption peak was obtained at 520 nm and the absorbance gradually decreased with the increase of pH value. As the pH increased from 5 to 8, the maximum absorption peak showed a bathochromic shift from 540 to 580 nm, accompanied with an increase of maximum absorbance. Furthermore, the absorbance at 580 nm decreased with the increase of pH in the range of 8-12.

The color variation and the corresponding peak shift was originated by their structure transformation (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). Different anthocyanins generally demonstrate different color response to pH variation (Garber, Odendaal, & Carlson, 2013). Herein, the color response of the crude RACNs extract solution was an integrated color response of several kind of anthocyanins, mainly including delphinidin-3-sambubioside and cyanidin-3-O-sambubioside (Grajeda-Iglesias, et al., 2016).

3.2. Characterization of the colorimetric films

3.2.1. FTIR analysis

Fig. 2 shows FTIR spectra of the starch, PVA, RACNs, SPVA film and the colorimetric films. In the spectrum of starch, the broad band at 3289 cm\(^{-1}\) and the peak at 1639 cm\(^{-1}\) were attributed to the stretching vibration and bending vibration of O-H, respectively. The peak at 2924 cm\(^{-1}\) corresponded to bending vibration of C-H. The characteristic peak occurred at 1639 cm\(^{-1}\) was the feature of tightly bound water present in starch. The bands from 761 to 1077 cm\(^{-1}\) corresponded to the C-O bond stretching
The spectrum for PVA showed the maximum absorption peak at 1086 cm\(^{-1}\) resulting from the C-O bond stretching and the peak at 1416 cm\(^{-1}\) corresponding to bending vibration of CH-CH\(_2\) certified the basic carbon skeleton of PVA \((\text{Pereira, et al., 2015})\). In terms of RACNs spectrum, the peaks between 2800-3300 cm\(^{-1}\), and the peaks at 1709 and 923 cm\(^{-1}\) were the three characteristic bands for the recognition of carboxyl group, assigned to the stretching vibration of O-H and C=O, and the out-of-plane bending vibration of -OH, respectively. The peak at 1596 cm\(^{-1}\) was related to the combinations and overtones of aromatic compounds \((\text{Pereira, et al., 2015})\). The anthocyanins spectrum showed the maximum absorption peak at 1023 cm\(^{-1}\), corresponding to aromatic ring C-H deformation. No significant difference between the spectra of colorimetric films could be observed. However, compared with the spectrum of SPVA film, a new peak around 1627 cm\(^{-1}\) which was related to the combinations and overtones of aromatic compounds appeared in the spectra of the colorimetric films, certifying that RACNs have been incorporated into the starch/PVA matrix.

**Fig. 2**

3.2.2. XRD analysis

For starch granules, there are usually A, B, and C-type by XRD spectra because of their different crystalline structures \((\text{Buléon, Colonna, Planchot, & Ball, 1998; Tian, Rickard, & Blanshard, 1991})\). A-type starch has strong diffraction peaks at about 15° and 23° and unresolved doublet at around 17° and 18°, while B-type starch possess the strongest diffraction peak at around 17°, some small peaks at around 15°, 20°, 22° and 24°, and a characteristic peak at about 5.6° \((\text{Guo, Liu, Lian, Li, & Wu, 2014})\). C-type starch is a mixture of A and B-type starch. As shown in Fig. 3, the starch showed the strongest diffraction peaks at 17.1°, and weak peaks at 15.1°, 19.8°, 22.2° and 24.1°. The weakest characteristic peak at 5.7° was also observed. The results indicated that the starch granules were B-type. In the diffractogram of PVA, the peaks at around 11.6°, 19.4°, 23.0° and the small hump at around 40.8° were associated to its crystalline structure \((\text{Sreekumar, Al-Harthy, & De, 2012})\).
As to the X-ray diffraction of SPVA film, no characteristic peak of starch granule appeared, which indicated that starch was well-dispersed in the SPVA film without crystalline structure, because the crystalline regions of starch granules were destroyed by heating and mechanical stirring during gelatinization. The broad peak in the range of 10-17° resulted from the amorphous state of starch in the SPVA film. However, there was a characteristic peak of PVA at 19.4°, indicating that there was partial crystal structure of PVA remained during the film-forming process. As regarded to the colorimetric films, the peak areas at 19.4° were smaller than that of SPVA film, and decreased with the increase of RACNs content. This indicated that there were fewer rearrangement of PVA molecules to crystallization. This phenomenon was probably due to the hydrogen bond formed between hydroxyl groups of anthocyanins and PVA. The dispersed phase of PVA molecules would be beneficial for the uniformity of films.

3.2.3. SEM micrographs analysis

The cross-section of the SPVA film and colorimetric films are shown in Fig. 4. It could be observed that the SPVA film showed a two-phase structure. The continuous phase on the left was the starch-rich phase, while the network-like phase on the right was the PVA-rich phase. This structure was due to a certain degree of immiscibility between starch and PVA, which was also observed in a previous research (Cano, Cháfer, Chiralt, & González-Martínez, 2015). However, when RACNs were incorporated into the SPVA film, the cross-section of colorimetric films became homogeneous without phase separation. This implied that RACNs had excellent compatibility with SPVA, and simultaneously improved the compatibility between starch and PVA. Generally, the compatibility of starch and PVA can be improved by adding plasticizers such as glycerol, sorbitol, poly(ethylene glycol) and monosaccharides (Jiang, et al., 2012). These plasticizers contain a number of hydroxyl groups that can form intermolecular hydrogen bonds with the hydroxyl groups of starch and PVA and thus reduce the intermolecular hydrogen bonds and entanglements between polymer chains (Jiang, et al., 2012). In this work, the enhanced compatibility
between starch and PVA may also be ascribed to the hydrogen bonds between the hydroxyl groups of RACNs, starch and PVA. Furthermore, the cross-sections of the colorimetric films became more compact as a result of an increasing content of RACNs, indicating the more intensive interactions.

### 3.2.4. Thickness, water content and mechanical properties of the films

Table 1 shows the thickness, water content and the mechanical properties of the SPVA film and the colorimetric films. The SPVA/RACNs-120 film had the highest thickness, and no significant difference was observed between the thicknesses of the SPVA film, SPVA/RACNs-30 film and SPVA/RACNs-60 film. The water content ($WC$) of SPVA/RACNs-30 film was close to the SPVA film. However, when the RACNs content was higher than 60 mg/100 g starch, the $WC$ of films significantly decreased with the increase of RACNs content. This was probably because the interaction between SPVA and RACNs could lower the availability of hydroxyl groups of SPVA, which would in turn limit the SPVA-water interactions (Wang, Dong, Men, Tong, & Zhou, 2013). The content of RACNs also had significant effect on the mechanical properties of the films. As can be seen, the tensile strength ($TS$) decreased with RACNs addition increasing from 30 to 120 mg/100 g starch, while the elongation at break ($EB$) increased with the increase of RACNs content. A similar behavior was found in SPVA films incorporated with hydroxyl group-riched glycerol (Yoon, Chough, & Park, 2006). The decrease of $TS$ could be due to that the intramolecular interaction of starch molecules and PVA molecules was interrupted by the RACNs molecules, whereas the increase of $EB$ was because the RACNs improved the compatibility of starch and PVA so that the films became more homogeneous, as shown in section 3.2.3, which resulted in enhanced extensibility.

### 3.2.5. Color stabilities of the colorimetric films

The self-stabilities of the colorimetric films were essential to their color
performance. Fig. 5 shows the relative color changes ($S$) of the colorimetric films. It can be seen that the colorimetric films stored at 4°C had small $S$ values which were lower than 1% within 14 days, showing that they had excellent color stabilities. There was no obvious difference between the $S$ values of the colorimetric films with different content of RACNs. By contrast, the colorimetric films had higher $S$ values when they were stored at 25°C. Obvious increase of $S$ values can be observed in the first day, which may be due to the moisture equilibrium process of the colorimetric films with the surrounding environment. After that, the $S$ values gradually increased with time, this was owing to that RACNs were partially oxidized by the oxygen. Furthermore, the $S$ values of the colorimetric films decreased with the increase of RACNs content, indicating that the colorimetric films with more RACNs possessed greater color stabilities. Nevertheless, the $S$ values were overall lower than 5%, implying that the colorimetric films had great color stabilities at 25°C as well.

Generally, the isolated anthocyanins are highly unstable and their stabilities are affected by several factors such as pH, storage temperature, chemical structure, light, oxygen, and their concentration (Castañeda-Ovando, et al., 2009). The color stability of RACNs solution stored at different temperatures has been studied by Sinela, et al. (2017) who found that the RACNs solution had great stability at 4°C. Apart from the storage temperature, in this work, the great color stabilities of the colorimetric films may be, on one hand, owing to the low water content of the colorimetric films that reduced the hydration of anthocyanins thus preserving the color (Lewis, Walker, & Lancaster, 1995). On the other hand, starch and PVA may protect the anthocyanins from being oxidized to some extent by entrapping the anthocyanins. Moreover, the RACNs incorporated into the colorimetric films were crude extract, in which the co-pigments such as sugar and phenolic acids could contribute to the color stability of anthocyanins (Sui, Bary, & Zhou, 2016).

3.2.6. Response of colorimetric films to ammonia vapor

In order to find out the response behavior of the colorimetric films towards the
volatile basic gas, the colorimetric films were exposed to the ammonia generated from
the 8 mM ammonia solution under 25°C for 24 min and UV-vis spectra were recorded.
The initial maximum absorption peak were all observed at 540 nm for these three
colorimetric films as shown in Fig. 6a, b and c, indicating a red shift compared with the
RACNs solution (520 nm). Similar red shift was also observed in chitosan films
containing bauhinia blakeana dunn anthocyanins (Zhang, et al., 2014). The absorption
peak at 540 nm decreased and another absorption peak at 640 nm gradually increased
with reaction time. These results indicated that the colorimetric films gradually
transferred to be more basic. The absorbance ratio at 640 nm versus 540 nm (A_{640}/A_{540})
represented the green color intensity compared to the red color intensity (Choi, et al.,
2017), which increased with time as shown in Fig. 6d. The calibration curves showed
that A_{640}/A_{540} increased exponentially with the reaction time as the following formulas,
where x was the reaction time and y was A_{640}/A_{540}:

\[ y = 0.1235e^{0.0644x}, R^2 = 0.992, \text{ for SPVA/RACNs-30 film}; \]  
\[ y = 0.0917e^{0.0611x}, R^2 = 0.9965, \text{ for SPVA/RACNs-60 film}; \]  
\[ y = 0.0505e^{0.0659x}, R^2 = 0.9819, \text{ for SPVA/RACNs-120 film}. \]  

The slope of calibration curve represented the rate of color variation from red to
green, a greater slope indicated a higher variation rate. At a certain reaction time, the
SPVA/RACNs-30 film had the highest color variation rate, followed by the
SPVA/RACNs-60 film and then the SPVA/RACNs-120 film. This result indicated that
the colorimetric film with fewer RACNs was more sensitive to NH₃. The color variation
mechanism of the colorimetric films was that the volatiled NH₃ firstly combined with
H₂O contained in the colorimetric film to form NH₃·H₂O which then hydrolyzed to
produce NH₄⁺ and OH⁻, the latter of which induced the color change of RACNs. The
higher color variation rate occored in the colorimetric films with fewer RACNs was
due to that the discolored RACNs took higher proportions of the total RACNs within
the same reaction time. It was generally expected that gas sensors could have fast
response to the analytes. Hence, the SPVA/RACNs-30 film that exhibited the highest
color variation rate would contribute to its application as a gas sensor.
3.3. Trials on monitoring the fish freshness

TVB-N level was used as the indicator to determine the fish freshness. As shown in Fig. 7a, the initial TVB-N value of the fresh fish was 6.61 mg/100 g, and then it increased up to 28.53 mg/100 g at 165 h. According to Chinese Standard (GB 2733-2015), the rejection limit of TVB-N level for silver carp is 20 mg/100 g. This implied that the fish sample could not be consumed almost after 135 h.

The color changes of the colorimetric films were shown in Fig. 7b. The SPVA/RACNs-30 film presented a purple color at the beginning, then green color at 90 h and finally yellow color after 135 h, while the SPVA/RACNs-60 film changed its color from initial pink to purple at 90 h and green after 150 h. As to the SPVA/RACNs-60 film, it showed a red color at first which turned to pink at 105 h and purple after 135 h. These color changes indicated that the colorimetric films became more basic due to the increasing TVB-N. However, it can be observed that the SPVA/RACNs-30 film exhibited the highest color change rate, followed by the SPVA/RACNs-60 film and then the SPVA/RACNs-120 film. This was in consist with the results found in 3.2.5 section in which the colorimetric films with lower content of RACNs had higher color change rates.

All of the colorimetric films displayed continuous color changes within the shelf life of fish (135 h), suggesting that they were capable to indicate the real-time fish freshness. Furthermore, it was well received for the colorimetric films to have rapid response to the TVB-N so that they could indicate the fish freshness in the early stages of storage. From this aspect, the SPVA/RACNs-30 film and SPVA/RACNs-60 film which presented earlier color variation were superior to the SPVA/RACNs-120 film, because the SPVA/RACNs-120 film did not show discriminative color changes until 75 h. However, it was worth mentioning that the colors displayed by the SPVA/RACNs-30 film were not deep enough to be easily seen by naked eyes. These results suggested that the colorimetric film with an appropriate content of anthocyanins would be favorable to its practical application for real-time monitoring the fish.
freshness. The colorimetric film with a high content of anthocyanins would take a long
time for its color shift, while the colorimetric film containing an extremely low content
of anthocyanins would present weak colors although it had rapid color changes.

439 4. Conclusions

Novel colorimetric films were successfully developed by incorporating 30, 60 and
120 mg RACNs/100 g starch with SPVA through casting/solvent evaporation method.
The FTIR spectra of the colorimetric films certified that RACNs were successfully
immobilized into the SPVA matrix. X-ray diffraction spectra and SEM micrographs
indicated that the crystallinity of PVA was reduced during the film-forming process
and the compatibility between starch and PVA was improved, owing to the presence of
RACNs. The incorporation of RACNs led to a decrease of water content and tensile
strength, and an increase of elongation at break. The color stability test showed that the
colorimetric films were stable within 14 days at 4°C and 25°C. The colorimetric film
with lower content of RACNs was more sensitive to ammonia. The results of the
application trial showed that the SPVA/RACNs-60 film were able to indicate the real-
time fish freshness by visible color changes. All the materials used to fabricate the
colorimetric films were nontoxic and biodegradable. Hence, the colorimetric films can
be used as safe and eco-friendly fish freshness indicators for intelligent packaging.
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Notes

The authors declare no competing financial interest.
Reference


Figure captions

Fig. 1. (a) Color and (b) UV-vis spectra of RACNs solution (0.12 mg/mL) at pH 2-12.

Fig. 2. FTIR spectra of (a) starch, (b) PVA, (c) RACNs, (d) SPVA film, (e) SPVA/RACNs-30 film, (f) SPVA/RACNs-60 film and (g) SPVA/RACNs-120 film.

Fig. 3. XRD spectra of starch, PVA, SPVA film, SPVA/RACNs-30 film, SPVA/RACNs-60 film and SPVA/RACNs-120 film.

Fig. 4. SEM micrographs of the cross sections of (a) SPVA film, (b) SPVA/RACNs-30 film, (c) SPVA/RACNs-60 film, and (d) SPVA/RACNs-120 film.

Fig. 5. The relative color change ($S$) of the colorimetric films stored at 4°C and 25°C for 14 d.

Fig. 6. UV-vis spectra of (a) SPVA/RACNs-30 film, (b) SPVA/RACNs-60 film and (c) SPVA/RACNs-120 film when exposed to ammonia generated from an 8 mM ammonia solution at 25°C for 24 min, and (d) the change of the absorbance ratio at 640 nm versus 540 nm ($A_{640}/A_{540}$).

Fig. 7. (a) The change of TVB-N level of stored silver carp within 165 h at 4°C and (b) the corresponding color changes of the colorimetric films.
Fig. 1

(a)

(b)
Fig. 3  revision  FOØHYD 3804

![Graph showing diffraction intensity (CPS) vs. 2θ (degree) for different samples: Starch, PVA, SPVA film, SPVA/RACNs-30 film, SPVA/RACNs-60 film, and SPVA/RACNs-120 film. Peaks are labeled with values such as 19.8, 22.1, 24.1, and 40.8.](image)
Fig. 4
Fig. 5
Fig. 6

(a) 

(b) 

(c) 

(d)
Fig. 7

(a) Graph showing the change in TVB-N (mg/100g) over time (h) for different films. The threshold of TVB-N is indicated.

(b) Circular diagrams showing the time (h) for the SPVA/RACNs-30, SPVA/RACNs-60, and SPVA/RACNs-120 films.
Table 1
Thickness, water content, tensile strength and elongation at break of the SPVA film and the colorimetric films.

<table>
<thead>
<tr>
<th>Films</th>
<th>Thickness (μm)</th>
<th>Water content (%)</th>
<th>Tensile strength (MPa)</th>
<th>Elongation at break (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPVA</td>
<td>88.06 ± 3.10(^b)</td>
<td>25.50 ± 0.98(^a)</td>
<td>48.97 ± 2.36(^a)</td>
<td>44.15 ± 2.42(^d)</td>
</tr>
<tr>
<td>SPVA/RACNs-30</td>
<td>88.40 ± 3.21(^b)</td>
<td>25.21 ± 1.47(^a)</td>
<td>48.21 ± 2.60(^a)</td>
<td>49.12 ± 2.09(^c)</td>
</tr>
<tr>
<td>SPVA/RACNs-60</td>
<td>89.25 ± 2.57(^b)</td>
<td>22.04 ± 1.33(^b)</td>
<td>45.17 ± 1.78(^b)</td>
<td>60.24 ± 3.18(^b)</td>
</tr>
<tr>
<td>SPVA/RACNs-120</td>
<td>93.89 ± 3.13(^a)</td>
<td>18.50 ± 0.98(^c)</td>
<td>41.85 ± 2.03(^c)</td>
<td>88.28 ± 3.51(^a)</td>
</tr>
</tbody>
</table>

Data were presented as mean ± standard deviation of three samples.

Data in the same column with different letter were significantly different (p < 0.05).