Plant-derived anthocyanin extract for qualitative test of food additives and preservatives

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Abstract. In this study, anthocyanin was extracted from purple cabbage and skin of vigna cylindrica skeels in aqueous solution at room temperature. The anthocyanin extracts indicated the pH-dependent color changes, envisaging the detection of the chemicals involving urea, nitrite, benzoate, formaldehyde and borax. The response time of the tests was quickly (either immediately or less than 5 min) and the solution colors were stable until 1 hour, offering the good sensitivities towards formaldehyde (detection limit of 250 ppm) and borax (detection limit of 10 ppm).

1. Introduction
In food production, the use of food additives in excess amounts and illegal food preservatives has been the biggest public health threat [1]. For instance, sodium nitrite has been widely used in order to enhance the color and extend the shelf-life of the processed meat, fish and some cheeses. The nitrite content permission is in the range of 60–150 ppm of processed products; in contrast, the daily nitrite consumption in higher amount can cause poisoning for humans, particularly methemoglobinemia [2]. Sodium benzoate was firstly authorized as the food additive by U.S. Food and Drug Administration (FDA) and listed as E211 in European food products. With the aim of inhibiting the growth of microorganisms (bacteria, mold, and insects), sodium benzoate has been commonly used in acidic foods and beverages. However, possible health problems caused by sodium benzoate involve inflammation, attention deficit hyperactivity disorder, appetite control, oxidative stress, allergies and the conversion to carcinogen [3]. Meanwhile, urea, formalin and borax are prohibited to use in food production due to their high toxicity and even acting as cancer agents [4, 5]. Formalin has been widely used in preservation of fruits, sea foods (fish, squid), tofu and wet noodles with the aim to keep the products fresh and make fruits attractive. Nevertheless, this substance causes kidney, liver and lung problems, even carcinogen. In fact, a study on mice exposed to 6–15 ppm formalin for 2 years exhibited the development of carcinogenic squamous-cell in nostril. Therefore, the control of limiting contents of these compounds in foods permits the consumers to make the safe food choices.

In general, those chemicals have been quantitatively analyzed by UV-vis spectrophotometry, chromatography (gas chromatography and high-performance liquid chromatography) or electrochemical methods [6–8]. Although the mentioned techniques can quantitatively analyze with the high precision and accuracy (even at very low concentrations), the analytical procedures involve
multi-steps with high-skilled requirements, restricting the out-of-lab applications with quick adapting. On the other hands, some qualitative tests based on the color observation of the reactions of chemicals and indicators have been developed: the purple for detection of formalin using chromotropic acid, the pink for detection of formalin using phenylhydrazine, the red for detection of borax using curcumin [9]. In general, the qualitative tests (like test kits) can determine the presence of some chemicals with the specific detection limits for a very short time, depending on the observed color and color intensity. Nevertheless, most indicators are commonly toxic, expensive and unavailable in living. Therefore, the natural indicators, mainly plant-derived extracts, have been currently developed as biosensors for detection of food additives and illegal food preservatives.

Anthocyanin is a natural coloring agent in flavonoid group, mainly found in fruits, flowers and vegetables. The basic structure of anthocyanin is anthocyanidin which can be bonded to either hydroxyl groups or methoxyl groups. The resonant structure of flavylum ion causes the changes in color or color intensity, depending on the pH. In fact, its color can be changed from red (acidic pH) to blue (neutral pH) and then yellow (basic pH). In general, the color of anthocyanin and its stability are dependent on its intrinsic factors (types of anthocyanidin, intermolecular bonds to counterparts) and environment (pH, light, temperature, oxygen, solvent) [10]. In this study, the anthocyanin extracts from purple cabbage and skin of vigna cylindrica skeels were obtained in aqueous solution at room temperature and then directly applied as biosensor to detect the presence of urea, nitrite, benzoate, formaldehyde and borax. The limit of detection (LOD) of positive-signal chemicals was also examined.

2. Experimental

2.1. Extraction of anthocyanin

Purple cabbage and skin of vigna cylindrica skeels were purchased at local markets in Ho Chi Minh City (Vietnam). The raw materials were washed by distilled water, dried at room temperature and then cut into small species. 10 g materials were soaked in the appropriate solvent at room temperature for 15–90 min. The solvent consisted of water and ethanol 96% with variable ratios of 100/0, 75/25, 50/50, 25/75 and 0/100 (v/v). The raw material-to-solvent ratios were performed from 1/1 to 1/12 (g/mL). The mixture was filtered, centrifuged with 3000 rpm for 15 min to remove the supernatant and then evaporated under vacuum to obtain the concentrated extract. The anthocyanin extracts were stored in dark glass bottles at 0–4 °C in refrigerator.

The anthocyanin content was determined following cyanidin-3-glucoside by the pH differential method (Equation 1) [11]. Anthocyanin mainly exists in oxonium form (orange to purple) with a maximum absorbance at 520 nm at pH 1 or chalcone (colorless) at pH 4.5.

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\text{Anthocyanin} \left( \frac{\text{mg}}{L} \right) = \frac{A \times MW \times DF \times 10^3}{\epsilon \times l}
\]  

(1)

Where: \( A = (A_{520\text{ nm}} - A_{700\text{ nm}})_{\text{pH 1}} - (A_{520\text{ nm}} - A_{700\text{ nm}})_{\text{pH 4.5}} \); \( MW = 449.2 \) g/mol for cyanidin-3-glucoside; \( DF = \) dilution factor; \( \epsilon = 26,900 \) \( \text{L}\times\text{mol}^{-1}\times\text{cm}^{-1} \) for cyanidin-3-glucoside and \( l = \) pathlength in cm.

2.2. Qualitative test of chemicals by anthocyanin extract

The anthocyanin extracted from both purple cabbage and skin of vigna cylindrica skeels was dissolved in water to ensure the concentrations of 100, 200 and 300 ppm. Besides, the solutions of urea, nitrite, benzoate, formaldehyde and borax were separately prepared with different concentrations from 10 to 10,000 ppm. The reactions between anthocyanin extract and chemicals were performed with a ratio of 1:1 (v/v) at room temperature. The color changes (if any) were immediately observed (or for the time less than 5 min) and being stable until 1 hour. The absorbance of solutions was measured by UV-vis spectrometry. Limit of detection of chemicals using anthocyanin extracts was tested for 10 times with 100% positive signals.
3. Results and Discussion

3.1. Extraction of anthocyanin

In this study, anthocyanin coming from purple cabbage and skin of vigna cylindrica skeels was extracted at room temperature by soaking method. The yield (mg anthocyanin / 10 g raw material) was used to evaluate the extraction efficiency. The extraction conditions were optimized involving solvent ratio (\(H_2O/Et = 100/0, 75/25, 50/50, 25/75\) and \(0/100\) mL/mL), material/solvent ratio (1/1 to 1/12 g/mL) and soaking time (15 to 90 min) as shown in Table 1.

Anthocyanin is sensitive to temperature and it is easily decomposed at high temperature. Practically, the extraction efficiency was unstable at higher than 60 °C although it increased following the temperature increase. With the aim to make a simple out-of-lab procedure, the extraction of anthocyanin was carried out at room temperature. Anthocyanin is water-soluble pigment thanks to polyphenolic groups, besides hydrophobic hydrocarbon chains. Normally, water was a good choice for the anthocyanin extraction from plants; however, water also dissolved other components than anthocyanin (like sugar, starch) to impede the target extraction [12]. Ethanol (Et) is more selective and the water/ethanol mixture (\(H_2O/Et\)) is the common solvent for such an extraction of polar organic compounds. In this term, \(H_2O/Et\) ratio of 50/50 (v/v) was the optimal solvent for both materials in order to obtain the high anthocyanin yield and save a large amount of solvent. In addition, the raw material-to-solvent ratio and time were also examined to the anthocyanin yield. As expected, the more the solvent volume was used, the higher anthocyanin yield was. However, at this ratio of 1/10 (for purple cabbage) or 1/6 (for skin of vigna cylindrica skeels), the anthocyanin dissolved in aqueous solvent reached equilibrium as proven by its unchanged concentration (mg/L). The results showed that the anthocyanin contents in the two materials are high enough to obtain the appropriate extracts for the further qualitative tests of some chemicals (see next section).

### Table 1. The optimal conditions for the extraction of anthocyanin.

| Entry | Material                          | Optimal conditions | Yield (mg/10 g) |
|-------|-----------------------------------|--------------------|-----------------|
| 1     | Purple cabbage                    | \(H_2O/Et = 50/50\) | 61.6            |
|       |                                   | Material/Solvent = 1/10 |                  |
|       |                                   | Time (min) = 45     |                  |
| 2     | Skin of vigna cylindrica skeels    | \(H_2O/Et = 50/50\) | 47.6            |
|       |                                   | Material/Solvent = 1/6 |                  |
|       |                                   | Time (min) = 60     |                  |

3.2. Qualitative test of chemicals by anthocyanin extract

Before testing the sensitivity of the anthocyanin extracts to chemicals, a study of pH-dependent anthocyanin extracts was performed by adjusting the pH in the corresponding aqueous solutions (from pH 1 to pH 14) as shown in Figure 1. Anthocyanin can be formed in the different structures depending on the pH of solution [10]. At acidic conditions (pH < 4), the predominant species of the flavylum ions attributed to red color. In the range of pH 4–6, the co-existence of four anthocyanin structures involving flavylum ion, anhydrous quinoidal base, colorless carbinol base and the pale yellow chalcone resulted in varying the color from red to purple and then blue. With an increase of pH to neutral solution (colorless carbinol base) and basic solution (yellow chalcone), the color changed from blue to green and then yellow. The pH-dependent anthocyanin extracts can be applied as a pH indicator.
Figure 1. The color of anthocyanin extracts at different pH conditions (from pH 1 to pH 14, left to right): (a) Purple cabbage and (b) Skin of vigna cylindrica skeels.

Taking into account the flexible structures of anthocyanin causing the color changes, the detection of some chemicals (urea: UR; sodium nitrite: SN; sodium benzoate: SB; formaldehyde: FM and borax: BR) using the anthocyanin extracts (AE) was performed by adding the 10,000 ppm chemicals into 300 ppm anthocyanin extract (1/1, v/v) (Figure 2). The color of the solution changed from violet (AE) to magenta (FM), eggplant (BR) and grape (SN) using AE extracted from purple cabbage; meanwhile, the color changes were observed from wine (AE) to rose (FM), raisin (BR) and mahogany (SB) using AE extracted from skin of vigna cylindrica skeels. Other chemicals have negative signals to the anthocyanin extracts. The further study focused on the effect of anthocyanin concentration towards the chemical sensitivity (Figure 3), indicating that a low AE concentration (< 100 ppm) gave a low sensitivity. Therefore, for AE extracted from both purple cabbage and skin of vigna cylindrica skeels, the AE solutions (higher than 100 ppm concentrations) permitted to detect the certain chemicals with relatively high selectivity.

Figure 2. Sensitivity of 300 ppm anthocyanin extracts to chemicals: (a) Purple cabbage and (b) Skin of vigna cylindrica skeels.

Figure 3. Sensitivity of anthocyanin extracts from purple cabbage to chemicals with different anthocyanin concentrations: (a) 300 ppm; (b) 200 ppm and (c) 100 ppm.
In order to understand mechanism of the color changes, the corresponding mixtures of anthocyanin extracts and chemicals were monitored by UV-vis spectrometry in the range of 450–650 nm wavelength (Figure 4). Obviously, there were the blue-shift to ~525 nm (FM) attributed to flavylium ion and the red-shift to ~575 nm (BR) associated to anionic quinonoid base, in comparison with the maximum absorbance at ~550 nm related to neutral quinonoid bases (AE) [13]. The forms of anthocyanin in aqueous solution are presented in Figure 5. In fact, the formalin solution has pH 3–4 favoring $A \rightarrow AH^+$ and the borax solution has pH 8.5–9.5 favoring $A \rightarrow A^-$. In addition, flavylium ions are week diacids; the electron-withdrawing pyrylium ring can react with nucleophiles (like nitrites) at the C4-position of flavylium structure forming the corresponding adducts [13]. It can be concluded that the color changes are based on not only the pH of solution but also the nucleophilic addition at C4 on flavylium ions.

**Figure 4.** UV-vis spectra of reaction mixtures between 200 ppm anthocyanin extracts from purple cabbage and chemicals.

**Figure 5.** The forms of anthocyanin in the extract solutions.

The limit of detection (LOD) of formaldehyde, borax, sodium nitrite and sodium benzoate using the anthocyanin extracted from purple cabbage and skin of vigna cylindrical skeels was examined for 10 times for each test, with 100% positive signals. The results summarized in Table 2 showed very good sensitivities of formaldehyde and borax for both anthocyanin extracts ($\geq$ 100 ppm anthocyanin concentrations), even at a very low borax concentration (10 ppm). In fact, the nature colors of AE
solutions are violet (purple cabbage) and wine (skin of vigna cylindrical skel's) interfered the detection of formaldehyde at low concentrations due to the red color of flavylium ions.

Table 2. Limit of detection (LOD) of chemicals using anthocyanin extracts.

| Entry | Chemicals (ppm) | Purple cabbage (ppm) | Skin of vigna cylindrica skel's (ppm) |
|-------|----------------|----------------------|---------------------------------------|
|       |                | 300      200  100 | 300      200  100                       |
| 1     | Formaldehyde   | Violet → Magenta | Wine → Rose                            |
|       |                | 250      250  250 | 500      500  500                       |
| 2     | Borax          | Violet → Eggplant | Wine → Raisin                          |
|       |                | 250      250  100 | 50       25  10                         |
| 3     | Sodium nitrite | Violet → Grape   | -                                    |
|       |                | 5,000    10,000 - | -                                    |
| 4     | Sodium benzoate| -        -       | Wine → Mahogany                       |
|       |                | -        -       | 500      500  500                       |

4. Conclusions
Anthocyanin extracted from purple cabbage and skin of vigna cylindrical skel's (in the range of 100–300 ppm anthocyanin concentrations) pointed out the high sensitivity towards formaldehyde (250 ppm LOD) and borax (10 ppm LOD). Meanwhile, the low sensitivities were observed towards sodium nitrite (5,000 ppm LOD) and sodium benzoate (500 ppm LOD). The response time of the tests was quickly (either immediately or less than 5 min) and the solution colors were stable until 1 hour. With the predominant features (such as environmental friendly, low cost, availability), the study of paper indicator application for chemical detection is currently underway.

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