Color Stability of Anthocyanins Copigmentation from Red Rice (*Oryza sativa L.*) Bran by Spectrophotometry UV-Vis

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Abstract. Red Rice (*Oryza sativa L.*) Bran is a waste that is often found in our environment. Pigment rice contains polar pigment anthocyanins (ACNs). Anthocyanins are a natural pigment that has low color stability and are easily degraded by environmental influences. In the present study, we have tried to increase the color stability of anthocyanins by copigmentation. Anthocyanins extraction was carried out using maceration techniques in 95% ethanol solvents. The extract of anthocyanins was characterized by spectrophotometer UV-Vis and Fourier transform infrared (FTIR). The results show that the spectrum UV-Vis and IR were obtained a characteristics peak at 470, 420, 340 nm and a hydroxyl group at 3400-3500 cm⁻¹, respectively. The various of Fe³⁺ and Al³⁺ as copigment and composition of anthocyanins to Fe³⁺ and Al³⁺ have been investigated. Anthocyanins stability was followed by measuring the loss of color by spectrophotometer UV-Vis, and the results show that Addition of Fe³⁺ and Al³⁺ could improve the anthocyanins stability.

1. Introduction

Rice bran (RB) is one of the by-products of rice milling and the main waste from the rice milling process. The rice milling waste is abundantly available in Indonesia which is known as one of the agricultural countries. However, the use of waste is still limited because it is considered to have low economic value. The utilization of the waste is currently only limited as animal feed. Recently researches regarding obtaining higher values from RB have been investigated. The previous studies have reported that the red rice bran has anthocyanins content as a red coloring pigment [1]. Anthocyanins are one of the many natural dyes in the red rice bran and are widely used as a natural dye. However, Munawarah et al (2015) reported that the anthocyanins as a natural dye are easily degraded by several factors such as pH, temperature, presence oxygen, light, and enzyme [2].

The color stability of anthocyanins can be increased through complex formation by copigmentation reaction. Copigmentation is an interaction between the structure of anthocyanin and other molecules such as metals (Al³⁺, Fe³⁺, Sn²⁺, Cu²⁺) and other organic molecules such as flavonoids (flavones, flavanone, and flavonol). There is a co-fermentation between anthocyanin and
other metal molecules that increase anthocyanin color and produce brighter colors and are protected from oxidation [3]. Fedenko et al (2017) have studied about copigmentation of anthocyanins. The results reported that the process could improve the color stability of anthocyanins through Me$^{3+}$-binding ability of anthocyanin structures [4]. This is because metals can stabilize the structure of anthocyanin pigments and can prevent the growth of microbes that can degrade the color. Some examples of suggested stabilization are intra-molecular promotional reactions of anthocyanins [5], ion formation between metal ions and anthocyanins [6] or decreased activity due to metal ions that inhibit flavylum ionic hydration [7]. In this study, we have tried to increase the color stability of anthocyanins through metal binding such as using Al$^{3+}$ and Fe$^{3+}$ as copigment. Copigmentation is known to be responsible for the color stability of anthocyanin and gives brighter and more stable colors than what would be expressed by an intact anthocyanin molecule through binding metal interaction.

2. Experimental
2.1 Materials
The materials are red rice bran, cyanidin 3-O-glucoside chloride as standard anthocyanin (Sigma Aldrich Singapore), ethanol (Merck), 1M NaOH, 1M HCl, 1% FeCl$_3$.6H$_2$O (Sigma Aldrich Singapore), 0.01% AlCl$_3$ (Sigma Aldrich Singapore) and distilled water. The specific equipments that are used including pH meter Jenway, thermometers, shakers, Shimadzu analytical balance ATX224, Spectroquant® Prove 600 spectrophotometer UV-Vis, and spectrophotometer FTIR Shimadzu IRAffinity-1S.

2.2 Procedure
2.2.1 Anthocyanin Extraction from Red Rice Bran
Anthocyanin extraction from bran rice bran was carried out by maceration with ethanol solvent [8]. Amount of 20 g of red rice bran was dissolved with 80 mL of ethanol solvent with the addition of 20 mL HCl 1M then allowed to stand for 24 hours in a dark room. Then filtered with Whatman Filter paper no 42 so that anthocyanin extract was obtained and then analyzed by Spectroquant® Prove 600 spectrophotometer UV-Vis and spectrophotometer FTIR Shimadzu IRAffinity-1S.

2.2.2 Complexation with Metals
The anthocyanin extract was added with 0.01% AlCl$_3$ and 0.01% FeCl$_3$.6H$_2$O with a concentration ratio of 1: 1, 1: 2, 1: 3, 1: 4, 1: 5 (w/w), respectively, and analyzed by UV-Vis and FTIR spectroscopy. The anthocyanin solution and metals AlCl$_3$ and FeCl$_3$ under optimum conditions were made in pH 1, 2, 3, 4, 5 then analyzed by Spectroquant® Prove 600 spectrophotometer UV-Vis and spectrophotometer FTIR Shimadzu IRAffinity-1S.

3. Results and Discussion
Anthocyanins are included in the flavonoid group of polyphenols. Generally, anthocyanins are unstable molecules as a natural dye. The color stability of anthocyanins depends on pH, temperature, presence oxygen and light. In this study, the extraction of natural pigment was carried out by the maceration process using 95% ethanol. Ethanol is a polar solvent so it can extract the anthocyanin from red rice bran. Anthocyanins have polar properties because they have hydroxyl groups, so the solvents used must be polar in accordance with the rules like dissolved like. The addition of acid to the solvent serves to denature the membrane of the bran so it dissolves the anthocyanin pigment and can be maximally extracted. The red rice bran and the results of extraction by maceration process are shown in Figure 1(b). The anthocyanin extract was characterized using a UV-VIS spectrophotometer at wavelengths of 350-700 nm. Spectra of anthocyanins characterization with UV-VIS as shown in Figure 1(a). Based on Figure 1(a), anthocyanins have a specific absorption at wavelengths 470, 420, and 340 nm. The presence of three absorption peaks obtained from sample extracts showed that the
presence of anthocyanin compounds. The results show the presence of an aromatic group which is at a wavelength of 340 nm (benzoyl groups) and 400-500 nm (cynnaoil groups) [9].

![UV-VIS spectra](image1.png)

**Figure 1.** (a) UV-VIS spectra of characterization of anthocyanin, (b) red rice bran and extracted anthocyanins

Samples were also characterized by infrared spectroscopy as shown in Figure 2. From the IR data, it was confirmed that the existence of aromatic groups from UV data was strengthened with IR spectrum which shows the aromatic C=C and C-H bonds at wave numbers 1639 cm\(^{-1}\) and 642 cm\(^{-1}\), respectively. In addition to aromatic groups from IR data, C-O bonds were also found which appeared at wave numbers 1046 cm\(^{-1}\). The hydroxy groups were detected in the wave number 3422 cm\(^{-1}\) and the absorption of C-H stretching appeared at wave numbers 2928 - 2977 cm\(^{-1}\). According to FTIR spectra and compared to standard anthocyanins (cyanidin 3-O-glucoside chloride), it is known that extracted anthocyanins have hydroxy groups and aromatic groups, so it can be concluded that the samples contain anthocyanin compounds.

![Infrared spectra](image2.png)

**Figure 2.** Infrared spectra of anthocyanin extract samples
As mentioned previously, copigmentation in this study was carried out by complexation reaction through metal binding using Al\(^{3+}\) and Fe\(^{3+}\). Metal binding with anthocyanins structures can improve the stability of anthocyanin through the chelation between hydroxyl groups and metals. Some suggested stabilization mechanisms are the promotion of intra-molecular reactions of anthocyanin, formation of ion pairs between metal ions and anthocyanin as illustrated in Figure 3 or decrease in water activity due to metal ions solvation which reduces the hydration of flavylum ions.

![Figure 3. Mechanism of the formation of anthocyanin complexes with metals [4]](image)

The effect of the ratio between anthocyanins to copigment (Al\(^{3+}\) and Fe\(^{3+}\)) was evaluated in this study. Anthocyanin and Al and Fe metal solutions were made by varying the volume anthocyanins to copigments (1:1, 1:2, 1:3, 1:4, and 1:5) respectively. The UV-Vis spectra is shown in Figure 4 and the result of anthocyanin copigmentation is shown in Table 1.

![Figure 4. UV-Vis spectra with copigment (a) Fe metals and (b) Al metals](image)

### Table 1. Maximum metal wavelength

| v/v     | \(\lambda_{\text{max}}\) | \% Fe copigmentation | \(\lambda_{\text{max}}\) | \% Al copigmentation |
|---------|----------------|---------------------|----------------|---------------------|
| 1:1     | 468           | 0.9657              | 466           | 0.8672              | 74.53               |
| 1:2     | 468           | 0.8672              | 465           | 0.5399              | 59.08               |
| 1:3     | 465           | 0.5627              | 464           | 0.67912             | 67.47               |
| 1:4     | 463           | 0.4275              | 464           | 0.3554              | 37.84               |
| 1:5     | 463           | 0.2902              | 463           | 0.2982              | 25.92               |

Based on Figure 4, the copigmentation is successfully formed both using metals Al\(^{3+}\) and Fe\(^{3+}\). Some kinds of literature have been reported that the copigmentation can be observed as a
bathochromic shift ($\Delta \lambda_{\text{max}}$) or a hyperchromic effect ($\Delta A$). In this study, we found that the copigmentation process observed as a hyperchromic effect as shown in Figure 4 (UV-Vis spectra). The maximum %copigmentation of the anthocyanins is obtained as in Table 1. The maximum %copigmentation was obtained in the ratio of 1:1 both Fe and Al metals with a percentage of 4.53% and 77.12%, respectively. In this copigmentation process, there was a blue or hyperchromic shift. Hyperchromic effects occur due to increased color intensity after copigmentation [7]. High metal concentrations can increase a solvent solvation capacity which causes a hydration reaction. Therefore, the process can significantly reduce the color of the solution so the optimum of ration ACNs to metal was found in the ratio 1:1.

Then, copigmented anthocyanins were tested for color stability in various pH (1-5). Based on Figure 5, the results show that copigmented anthocyanins are still stable in acidic solution at low pH. The maximum results show the anthocyanins stable at pH 2.0. At low pH, the flavylium cationic anthocyanin was very dominant and the most stable. In acidic solution, the anthocyanins with metal copigment exist as four main structure, namely quinonoidal base A, chalcone, AH$^+$ flavylium cation, and carbinol species. In addition, at low pH, the solubility of the metal will be higher so the metal can bind as well with the hydroxyl group of anthocyanin. Increasing the pH can decrease the color of anthocyanins because, at high pH, the dominant structure of anthocyanin will be in the form of carbinol [10,11]. The structure of carbinol has lost its conjugated bond and therefore does not absorb visible light. The comparative studies of increasing color stability of anthocyanins with different copigment are shown in Table 2.

![UV-Vis spectra with copigment](image)

**Figure 5.** UV-Vis spectra with copigment (a) Fe metals and (b) Al metals

**Table 2.** %copigmentation of anthocyanins with different copigment

| Copigments            | Rasio anthocyanins to copigments | %copigmentation | References |
|-----------------------|----------------------------------|-----------------|------------|
| Al$^{3+}$              | 1:1                              | 77.12           | Present study |
| Fe$^{3+}$              | 1:1                              | 74.54           | Present study |
| Ferrulic acid          | -                                | 59.4            | [10]       |
| Hydroxycinnamic acid   | -                                | 29.0            | [11]       |

Refer to Table 2, Generally intermolecular copigmentation using organic molecules as copigment showed that %copigmentation is more lower than metals as copigments. Sigurdson et al (2016) investigated the chelation of Me$^{3+}$ with anthocyanins at different pH (3-8). The results show that the
metal as copigments has a good interaction through covalent bonding with the functional groups in anthocyanins [12].

4. Conclusion
In this study, we summarize that anthocyanins from red rice bran have been successfully extracted and increased the color stability through copigmentation methods. Anthocyanin compounds can be complexed with metals $\text{Al}^{3+}$ and $\text{Fe}^{3+}$ through metal and covalent binding between the metals and functional groups on anthocyanins. The optimum ratio anthocyanins to copigment were found at 1:1 with %copigmentation of 77.12% and 74.54% at pH 2.0. The current results may be of use in improving the color stability of anthocyanins from red rice bran. However, the more study is still needed to improve the color stability in the other several factors such as temperature, presence oxygen, and light.

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