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# Identification of Anthocyanins Isolated from Black Bean Canning Wastewater by Macroporous Resin Using Optimized Conditions

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## ABSTRACT

Anthocyanins from black bean canning wastewater were isolated and purified on a laboratory scale by column chromatography, and then identified by high performance liquid chromatography electrospray tandem mass spectrometry. Dynamic adsorption and desorption were performed in glass columns packed with Sepabead Sp700 to optimize the purification process. Different temperatures during adsorption and desorption (25˚C and 35˚C) did not significantly affect the adsorption and desorption ratio. The adsorption ratio was significantly reduced when the flow rate increased from 1.5 mL/min to 2.5 mL/min. However, desorption ratio was not affected by flow rate (from 1.5 mL/min to 0.3 mL/min). Ethanol concentration (from 30% to 60%) was not a significant factor for desorption ratio. Four kinds of anthocyanins were identified in black bean canning wastewater. The major anthocyanins were delphinidin 3-glucoside, petunidin 3-glucoside and maldvidin 3-glucoside, with a small amount of petunidin 3, 5-diglucoside also present.

**Keywords:** Anthocyanin; Macroporous Resins; Purification

## 1. Introduction

Anthocyanins are water-soluble pigments responsible for the color of orange/red to violet/blue in many plants. They are one of the classes of flavonoids derived ultimately from phenylalanine [1]. The safety of artificial pigments has been questioned by some consumers and scientists. An association between ingestion of artificial food pigments and hyperactivity was suggested in a review of ten electronic databases for evaluating the effects of artificial food pigments [2].

Since the safety of artificial food pigments has been questioned, there has been a growing need for edible natural pigments like anthocyanin extracts [3]. Moreover, food and medicine containing anthocyanins may provide benefits like antioxidant activity [4], anticancer activity [5], and diabetes prevention [6]. Therefore, more and more anthocyanin-rich materials may be needed to meet with the increasing industry demands.

Black bean (*Phaseolus vulgaris*) has been considered to be a good anthocyanin source because of the high concentration of anthocyanins in its seed coat [7]. Since anthocyanins are water soluble, they may be dissolved into and lost in the wastewater during industrial canning processes, like washing and soaking. As a pigment, the anthocyanins in wastewater can absorb and prevent sunlight from entering water, which can inhibit the growth of certain bacteria that are able to degrade impurities in the water [8] and slow photosynthesis in aquatic plants [9]. To capture this anthocyanin resource and protect the environment, easy, low-cost, and effective technology should be designed to purify anthocyanins from black bean canning wastewater.

There is a long history of studying the anthocyanin components in black beans. The first four kinds of anthocyanins in the black beans were identified by Feenstra [10], including malvidin 3'-glucoside, petunidin 3'-glucoside, delphinidin 3'-glucoside, and delphinidin 3,5'-diglucoside. Since that time, more and more research about anthocyanins in different black bean cultivars has been reported. Delphindin 3'-glucoside and petunidin 3'-glucoside were the major anthocyanins found in the Korean cultivar [7], and delphindin 3'-glucoside in the Mexican cultivar [11]. Delphinidin 3'-glucoside, petunidin 3'-glucoside, and malvidin 3'-glucoside were the major anthocyanins in American commercial class black beans [12].

Although there is quite a bit of information for different anthocyanins in black beans, re-search on how to effectively collect and purify anthocyanins from black beans is limited.

Macroporous resins can be used for efficient purification. They are made of highly cross-linked nonpolar or slightly hydrophilic styrene-divinyl-benzene [SDVB; 12]. The advantages of macroporous resins include high adsorption capacities, long durability, easy regeneration, and low cost. One type of macroporous resins, SEPABEADS® Sp700, is a very good candidate for anthocyanin purification. It has a pore radius of about 90 Å and is designed for adsorbing or desorbing polyphenolic substances. Also, it has large surface area, which allows the resin to adsorb more chemical. Previous studies have already confirmed that Sp700 exhibited high adsorption and desorption capacities on anthocyanins from black bean canning wastewater.

The objective of this study was to determine the optimum conditions for using macroporous resin Sp700 for purification of anthocyanins from black bean canning wastewater, as affected by temperature, flow rate and ethanol concentration, on the anthocyanin profile.

## 2. Materials and Methods

Dry black beans were obtained from a local supermarket in Logan, UT, USA. The black bean canning byproduct was mimicked by soaking 1000 g of dry beans in 3000 mL distilled water for 24 hours at room temperature [13]. The wastewater was then drawn through Whatman filter paper (Qualitative 1) with a vacuum pump. Anthocyanin concentration of the wastewater was approximately 150 mg/L. The sample solution was kept in sterilized bottles at 4˚C (for immediate use) or at −20˚C (for storage).

Resins used in this study, Sepabeads® Sp700 (Resindion, Mistsubishi Chem Co., Chesapeake, VA, USA), are macroporous SDVB copolymer resins with no functional groups. The resins were activated according to manufacturer recommendations. Briefly, the resins were washed with distilled water, and then filtered through Whatman filter paper (Qualitative 1). They were then soaked overnight in a double resin volume of ethanol (95%, V/V), followed by rinsing with distilled water. Afterwards the resins were dried at 50˚C in a vacuum oven for 24 hours. Approximately 3 mL of the activated resin (0.7 g dry weight) was introduced into a glass column ($\phi$1.0 cm ×15 cm), and then washed with 6 ml of 95% ethanol and rinsed thoroughly with distilled water.

To analyze the relationship between the response function (anthocyanin effluent) and process variables and to optimize the adsorption process, the anthocyanin adsorption experiments on Sp700 were performed using a $2^2$ full factorial experiment design (**Table 1**). All columns were packed with 0.7 g of dry resin. The two independent variables studied were flow rate (2.5 mL/min or 1.5 mL/min) and temperature (25˚C or 35˚C). The anthocyanin content in the eluent was analyzed every 10 minutes until 600 mL of wastewater were passed through.

The adsorption ratio and amount adsorbed were described as follow:

Adsorption Ratio:

$$AR(\%) = \frac{(C_i - C_e)}{C_e} \times 100\% \quad (1)$$

Amount adsorbed:

$$Q_t = \frac{(C_i - C_t)V_i}{m} \quad (2)$$

where AR is the adsorption ratio (%), $C_i$, $C_t$ and $C_e$ (mg/L) are the concentrations of anthocyanins in liquid phase at the initial stage, at time t, and at equilibrium point, $Q_t$ (mg/g) is the quantity (mg) of anthocyanins on a unit amount (g dry weight) of adsorbent at time t, $V_i$ (L) is the volume of the solution, and m (g) is the mass of dry resins.

A $2^3$ full factorial experiment design (**Table 2**) was used to determine the relationship between the response function (anthocyanin yield) and process variables in the desorption process. The three independent variables studied were flow rate (0.3 mL/min or 1.5 mL/min), temperature (25˚C or 35˚C), and ethanol concentration (varying between 30 and 60% (v/v)). Ethanol was acidified with 0.1% HCL (v/v) to elute anthocyanins since acidified ethanol can facilitate anthocyanin solubilization and stabilization (3). Every 5 min, the anthocyanin content in the effluent was analyzed until the color of the resins was gone. During this length of time 50 mL of effluent was collected.

The extent of desorption was expressed as desorption ratio and desorption percentage, which were calculated as follows:

Desorption ratio:

**Table 1. Experimental values of the independent variables used for the $2^2$ full-factorial central composite design for adsorption process.**

| Code | Variables | |
|---|---|---|
| | *Temperature* (˚C) | *Flow rate* (mL/min) |
| **1** | 25 | 1.5 |
| **2** | 25 | 2.5 |
| **3** | 35 | 1.5 |
| **4** | 35 | 2.5 |

**Table 2. Experimental values of the independent variables used for the $2^3$ full-factorial central composite design for desorption process.**

| Code | Independent variables | | |
|---|---|---|---|
| | *Flow rate* (mL/min) | *Temperature* (˚C) | *Ethanol Concentration* (%) |
| **1** | 0.3 | 25 | 30 |
| **2** | 0.3 | 25 | 60 |
| **3** | 0.3 | 35 | 30 |
| **4** | 0.3 | 35 | 60 |
| **5** | 1.5 | 25 | 30 |
| **6** | 1.5 | 25 | 60 |
| **7** | 1.5 | 35 | 30 |
| **8** | 1.5 | 35 | 60 |

$$DR(\%) = \frac{C_d V_d}{(C_i - C_e)V_i} \times 100\% \quad (3)$$

Eluted Percentage:

$$EP(\%) = \frac{C_r V_r}{C_d V_d} \times 100\% \quad (4)$$

where DR is the desorption ratio (%), $C_d$ is the anthocyanin concentration in the desorption solution (mg/mL) and $V_d$ is the volume of the desorption solution (mL), EP is the eluted percentage (%), $C_r$ is the anthocyanin concentration in the desorption solution (mg/mL) from time range t to t + 5 minutes, $V_r$ is the volume of the desorption solution (mL) from time range t to t + 5 minutes, and $C_i$, $C_e$ and $V_i$ as described above.

An alternative method that involves measuring absorbance at different pH levels [14] was used with a UV-Vis spectrophotometer (Shimadzu UV-2100U, Shimadzu Corp., Tokyo, Japan) to calculate the anthocyanin concentration in the sample solution. The absorbance of the diluted sample was calculated as follows:

$$A = (A_{519} - A_{700})@\,pH_{1.0} - (A_{519} - A_{700})@\,pH_{4.5} \quad (5)$$

The monomeric anthocyanin pigment concentration in the original sample was calculated as follows:

$$\begin{aligned} &\text{Monomeric anthocyanin pigment}\,(\text{mg/L}) \\ &= (A \times MW \times DF \times 1000)/(? \times\ ) \end{aligned} \quad (6)$$

where *MW* is the molecular weight (449.2 Daltons), DF is the dilution factor (30), and £ is the molar absorptivity (26,900). The anthocyanin content was calculated as cyanidin-3-glucoside.

The effluent from desorption was collected and then concentrated to a small volume at 50˚C by a rotary evaporator until all the ethanol was evaporated from the solution. The concentrated anthocyanin was then dried to powder via freeze drying. Due to the limited sample size, powders from same desorption conditions were combined as one sample and then dissolved in water for subsequent pigment identification.

The anthocyanin solution was analyzed on an Agilent 1200 high performance liquid chromatography (HPLC) system equipped with Agilent 6130 LC-MS instrument (Agilent Technologies, Santa Clara, USA). An Agilent column (Zorbax SB-C18 column, 5 μm, 4.6 × 150 mm) was used at a flow rate of 1.0 mL/min at 25˚C. Mobile phase consisted of a combination of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient was varied linearly from 10% - 90% B (v/v) over 25 min. Diode array detector (DAD) was set at 210, 420 and 525 nm for real-time read-out and the UV/VIS spectra, from 190 to 650 nm, were continuously collected. Mass spectra were simultaneously acquired using electrospray ionization (ESI) in the positive and negative ionization modes (PI and NI) at low and high fragmentation voltages (70 V and 225 V, respectively) for the mass range of 100 - 1500 amu. Other parameters: Drying gas flow (13 L/min, 350˚C), nebulizer pressure (50 psi), PI (4000 capillary voltages), NI (3500 capillary voltages).

Statistical analysis was carried out according to full-factorial central composite design with three replicates for every group. It was performed using software package GraphPad Prism (GraphPad Software, San Diego, CA) and SAS 9.3 (SAS Institute Inc., Cary, NC). Proc GLM (general linear model) was conducted in SAS software, the significance of differences between groups was evaluated by a two-way analysis of variance (ANOVA), and differences were considered significant if $p < 0.05$.

## 3. Results and Discussion

In order to understand the effect of flow rate and temperature on the dynamic adsorption kinetics of Sp700, kinetic studies were performed based on a $2^2$ full factorial experimental design with a flow rate of 1.5 mL/min and 2.5 mL/min, and a temperature of 25˚C and 35˚C. Adsorption capacity increased with the amount of effluxent volume under different adsorption conditions (**Figure 1**).

During the first 200 mL, the adsorption capacities increased rapidly, slowing thereafter. At a flow rate of 1.5 mL/min, the system reached equilibrium when about 550 mL of wastewater was added. At a flow rate of 2.5 mL/min, the system reached equilibrium when about 600 mL of wastewater was added. The kinetic curves (**Figure 1**) at the same flow rate showed similar adsorption patterns for different temperatures, but the adsorption capacity increased more rapidly when flow rate was slower.

This may have been due to better particle diffusion in the solution.

Adsorption ratios were calculated as 42.10% ± 4.14% at 35˚C, 1.5 mL/min; 40.94% ± 5.80% at 25˚C, 1.5 mL/min; 34.96% ± 3.74% at 25˚C, 2.5 mL/min and 34.67% ± 3.38% at 35˚C, 2.5 mL/min. As shown in **Figure 2**, lower flow rate can provide higher adsorption ratio for both temperatures tested. A statistical analysis was performed on the adsorption ratio results, and the two main effects (flow rate and temperature) and their interaction effects were estimated. The test of statistical significance showed that only the effect of flow rate was significant ($p = 0.024$), which indicated that flow rate can significantly affect the adsorption ratio of anthocyanins from black bean wastewater on Sp700. This may be due to a longer contact time allowing the resins to adsorb more anthocyanins from the same amount of wastewater.

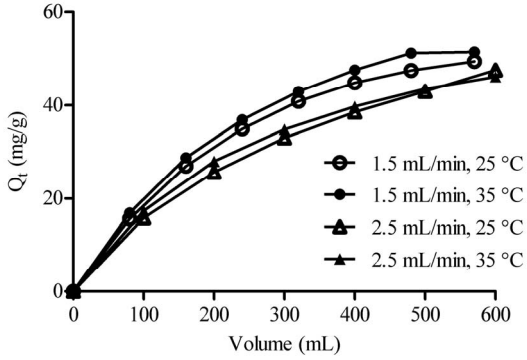



**Figure 1. Adsorption curves of black bean wastewater under different conditions. The four conditions were: 1.5 mL/min, 25˚C (○), 1.5 mL/min, 35˚C (●), 2.5 mL/min, 25˚C (△) and 2.5 mL/min, 35˚C (▲). $Q_t$ (mg/g) is the quantity (mg) of anthocyanins on a unit amount (g dry weight) of adsorbent at time t.**

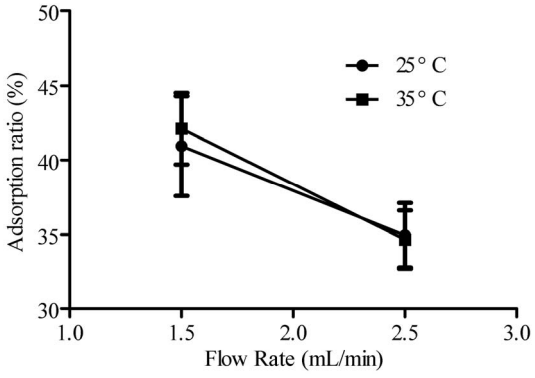



**Figure 2. Adsorption ratio of the anthocyanins using Sp700 at different flow rate (1.5 mL/min and 2.5 mL/min) with the temperature 25˚C (●) and 35˚C (▮). The curve shows the mean of three replicated tests and the error bars show standard deviations.**

Lower flow rate has also been shown to result in higher adsorption ratios for the phenolic compounds syringin, eleutheroside E, and isofraxidin from Radix Acanthopanax senticosus [Siberian ginseng; 15].

Temperature, on the other hand, did not significantly affect anthocyanin adsorption from black bean wastewater. This result is consistent with other adsorption studies, in which the adsorption ratio of anthocyanins from grape pomace extracts [16] and the adsorption capability of molinate from molinate water solution [17] using macroporous SDVB resins were not significantly influenced by temperature. This may be due to the adsorption process of anthocyanins on macroporous resins being controlled by a physical mechanism in the temperature range (25˚C to 35˚C) studied [18]. Apparently this temperature range did not significantly increase the relative mobility of the anthocyanins in the adsorption system.

### 3.1. Desorption with Different Temperature

The effect of temperature on the desorption process was investigated in this study (**Figure 3**). Two different temperatures (25˚C and 35˚C) were tested, and at the same flow rate and ethanol concentration, desorption curves showed similar patterns for different temperatures, which indicated that this desorption process is not significantly affected by temperature. Since the temperature from 25˚C to 35˚C, did not significantly affect the purification process, tight temperature control is not needed for the purification process within this temperature range.

### 3.2. Desorption with Different Acidified Ethanol Concentrations

The concentration of ethanol in the desorption solvent may affect anthocyanin desorption. As shown in **Table 3**, using the same temperature and flow rate, less ethanol volume was required to reach 80% recovery of anthocyanins when using the eluent with a higher concentration of ethanol. Anthocyanins were more easily eluted using solutions with a higher concentration of ethanol. This suggests that less volume of acidified eluent with a high concentration of ethanol could provide higher concentration of anthocyanin in the effluent. Additionally, effluents with lower concentrations of acidified ethanol are harder to concentrate because of the higher boiling point of water compared to that of ethanol [3]. Therefore, in order to simplify the concentration process when a higher anthocyanin yield is desired, a higher ethanol concentration in the eluent is preferred.

### 3.3. Desorption with Different Flow Rate

Flow rate is another factor that may affect the desorption

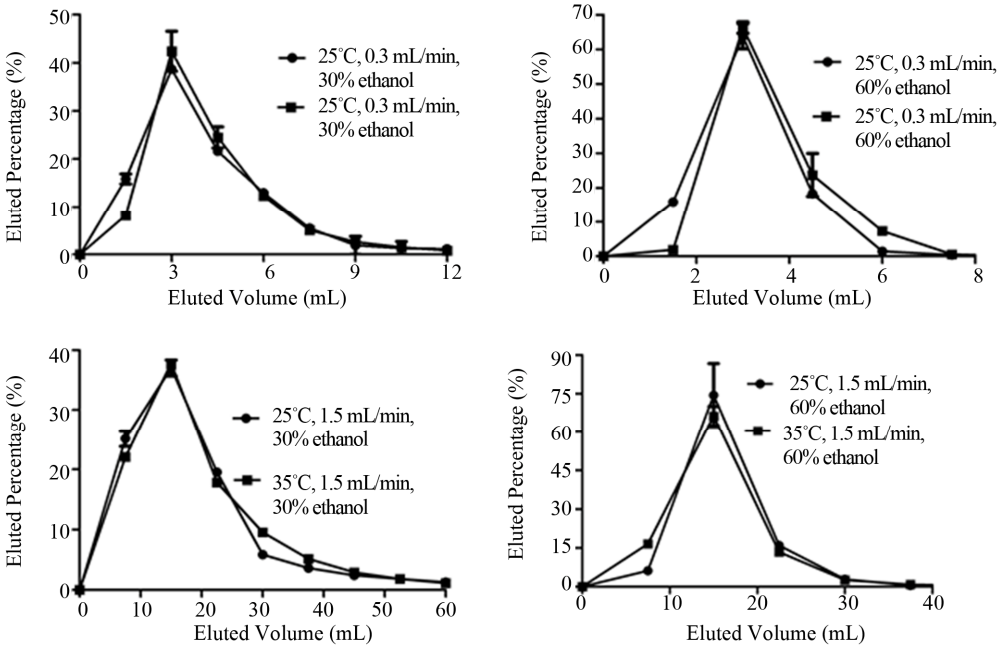



**Figure 3. Comparison of desorption parameters using different temperatures: 25˚C (●) and 35˚C (▌). The curve shows the mean of three replicated tests and the error bars show standard deviations.**

**Table 3. Ethanol volume and time used to reach 80% recovery of anthocyanins under different conditions.**

| Independent variables | | | | |
|---|---|---|---|---|
| *Flow rate* (mL/min) | *Temperature* (˚C) | *Ethanol concentration* (%) | *Ethanol volume* (mL)[a] | *Time* (min)[b] |
| 0.3 | 25 | 30 | 7.5 | 25 |
| | | 60 | 10.5 | 35 |
| | 35 | 30 | 7.5 | 25 |
| | | 60 | 12 | 40 |
| 1.5 | 25 | 30 | 45 | 30 |
| | | 60 | 60 | 40 |
| | 35 | 30 | 45 | 30 |
| | | 60 | 60 | 40 |

[a,b]Each value is the mean value of three replicated tests.

process. Two flow rates were investigated in this study; 0.3 mL/min and 1.5 mL/min. For desorptions at the same temperature and ethanol concentration, similar amounts of asorbed anthocyanin are recovered at both flow rates (**Table 3**). This indicates that at a flow rate of 0.3 mL/min, less desorption solvent was needed and the desorption process was shortened. Additionally, this introduces less water into the subsequent anthocyanin isolation process, saving time and energy. Therefore, 0.3 mL/min was the most efficient flow rate examined in this study.

## 3.4. Independent Variables and Desorption Ratio in the Desorption Process

A three-way factorial model was performed on the desorption ratio results, and the three main effects (ethanol concentration, flow rate, and temperature) and their interaction effect were estimated. These three effects did not significantly influence desorption ratio when enough eluent was used ($p > 0.05$). Since the independent variables did not significantly affect the desorption ratio, the most effective desorption condition should be determined according to other considerations, for example being more energy and resource efficient. Desorption conditions also affect the composition of the eluent and this may determine the desorption conditions for a given situation.

## 3.5. Identification of Anthocyanin

The anthocyanin extraction after resin purification was characterized by HPLC-MS at 520 nm (**Figure 4**). The chromatograms indicated the presence of 4 different kinds of anthocyanins with different [M]+ (molecular ion peak) values in black bean wastewater. The identification of anthocyanins was based on a comparison of their molecular weights with those in published papers. The characterizations of detected anthocyanins in black bean wastewater are presented in **Table 4**. It was found that petunidin 3, 5-diglucoside (peak 1; **Figure 4**), delphinidin 3-glucoside (peak 2; **Figure 4**), and petunidin 3-glucoside (peak 3; **Figure 4**) were the major anthocyanins

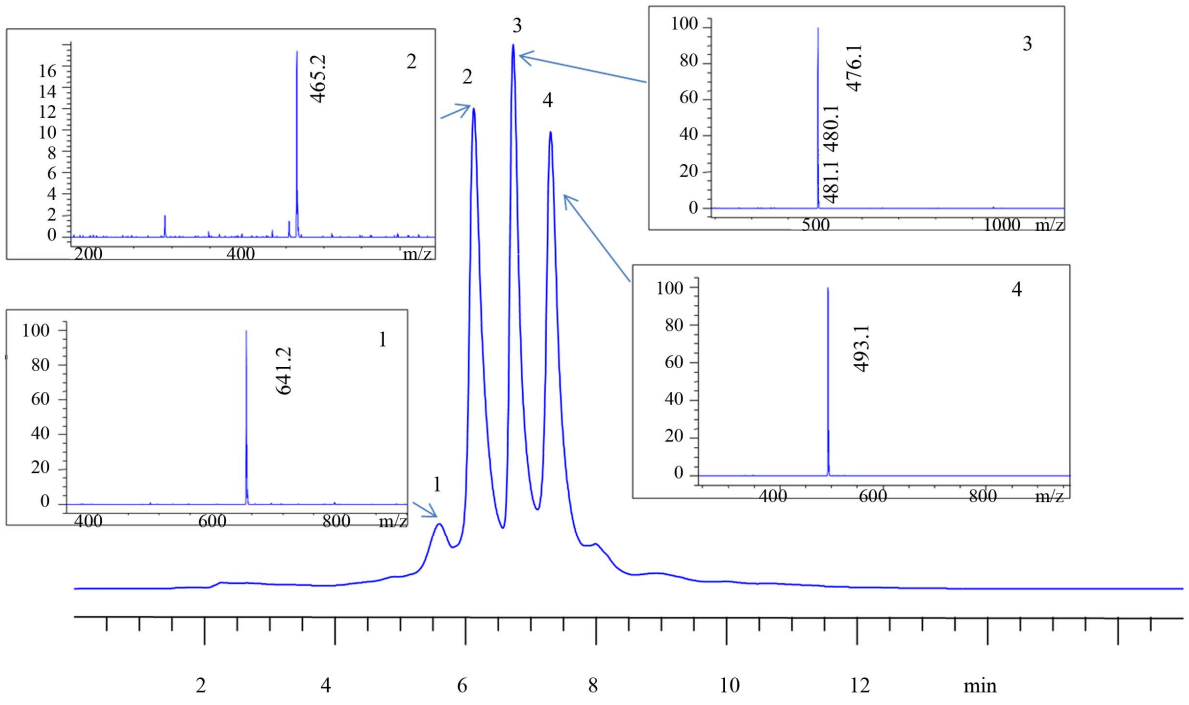
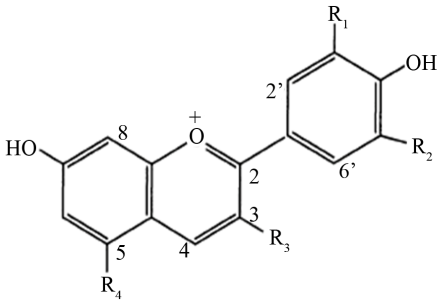



**Figure 4. HPLC profile of anthocyanin extraction. Mass spectrogram of compounds 1-4 are shown as HPLC-ESI/MS spectra.**

**Table 4. Characteristics of the anthocyanins found in black bean studied, related to their retention time ($t_R$), spectroscopic characteristics ($\lambda_{max}$), LC-MS data and chemical structures.**




| Peak | $t_R^a$ (min) | Anthocyanin | $\lambda_{max}$ (UV) | $\lambda_{max}$ (Vis) | LC-MS (m/z) | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 5.5 ± 0.1 | Petunidin 3,5-diglucoside | 218 | 525 | 641 | OH | OCH3 | O-$\beta$-D-glucose | O-$\beta$-D-glucose |
| 2 | 6.0 ± 0.1 | Delphinidin 3-glucoside | 235 | 525 | 465 | OCH3 | OCH3 | O-$\beta$-D-glucose | OH |
| 3 | 6.8 ± 0.1 | Petunidin 3-glucoside | 232 | 525 | 479 | OCH3 | OH | O-$\beta$-D-glucose | OH |
| 4 | 7.4 ± 0.1 | Maldivian 3-glucoside | 231 | 530 | 493 | OCH3 | OCH3 | O-$\beta$-D-glucose | OH |

[a]Each value is the mean value with standard error of three replicated tests.

in black bean wastewater extract [7,12,19], with a small amount of maldvidin 3-glucoside (peak 4; **Figure 4**).

The profile of the extract might be affected by the different conditions of desorption. All the powders from same desorption conditions were combined and tested as one sample in order to get big enough sample size. **Table 5** shows the percentages of the four anthocyanins identified and unidentified impurities in this study. Even though only one sample for each desorption condition was analyzed, qualitative and quantitative differences can be noticed among anthocyanin extractions from different desorption conditions. Lower flow rate, high temperature or high ethanol condition in desorption process may increase the numbers of the impurities.

**Table 5. Anthocyanin percentage and adsorbed impurities with different desorption conditions.**

| Method | Desorption Ratio (%)[a] | Anthocyanin percentage (%)[b] | | | | Impurities[c] |
|---|---|---|---|---|---|---|
| | | *Petunidin* 3,5-*diglucoside* | *Delphinidin* 3-*glucoside* | *Petunidin* 3-*glucoside* | *Maldvidin* 3-*glucoside* | |
| 0.3 mL/min 25˚C 30% ethanol | 76.5 ± 5.9 | 2.68 | 41.02 | 31.90 | 22.00 | 3 |
| 0.3 mL/min 25˚C 60% ethanol | 79.5 ± 6.5 | 3.46 | 36.09 | 29.96 | 28.50 | 4 |
| 0.3 mL/min 35˚C 30% ethanol | 76.9 ± 6.5 | 3.62 | 33.58 | 29.12 | 30.71 | 5 |
| 0.3 mL/min 35˚C 60% ethanol | 80.7 ± 3.5 | 3.37 | 27.07 | 28.59 | 38.50 | 3 |
| 1.5 mL/min 25˚C 30% ethanol | 82.7 ± 5.9 | 4.86 | 35.26 | 30.00 | 29.22 | 8 |
| 1.5 mL/min 25˚C 60% ethanol | 83.1 ± 9.3 | 4.70 | 37.50 | 28.60 | 27.04 | 6 |
| 1.5 mL/min 35˚C 30% ethanol | 83.0 ± 2.9 | 4.48 | 34.04 | 29.93 | 29.06 | 9 |
| 1.5 mL/min 35˚C 60% ethanol | 76.7 ± 0.2 | 5.55 | 33.24 | 30.24 | 29.42 | 8 |

[a]Each value is the mean value with standard error of three replicated tests. [b]Pigment fraction could absorb color in 520 nm. Each value is the mean value of three replicated tests; [c]Mean numbers of impurity peak can be detected by HPLC at 520 nm from three replicated testes.

## 4. Conclusion

The purification of anthocyanins from black bean canning wastewater as food colorants was examined. Temperature did not affect the purification process of anthocyanins from 25˚C to 35˚C. This is useful information for processors, because these results indicate that tight temperature control is not needed for the purification process when the purification process occurs around room temperature. Flow rate decrease from 2.5 mL/min to 1.5 mL/min can significantly increase the adsorption ratio. However, further flow rate decrease to 0.3 mL/min did not increase the desorption ratio. Acidified ethanol concentration (30% or 60% (v/v)) had no significant effect on the desorption ratio. The anthocyanins purified from black bean canning wastewater were then identified by HPLC-ESI/MS, and the major anthocyanins identified as delphinidin 3-glucoside, petunidin 3-glucoside, and maldvidin 3-glucoside. Qualitative and quantitative differences can be noticed among anthocyanin extractions from different desorption conditions.

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