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Isolation and Purification of Anthocyanins from Black Bean Wastewater Using Macroporous Resins

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ISOLATION AND PURIFICATION OF ANTHOCYANINS FROM BLACK BEAN WASTEWATER USING MACROPOROUS RESINS

by

Xiaoxi Wang

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ABSTRACT

Isolation and Purification of Anthocyanins from Black Bean Canning Wastewater Using Macroporous Resins

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Utah State University, 2013

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Isolation and purification of anthocyanins from black bean canning wastewater by column chromatography with macroporous resins were investigated in this study. Different adsorption materials and adsorption conditions were compared and the most effective material and adsorption conditions were selected to purify anthocyanins. Purified anthocyanins then were identified by high performance liquid chromatography electrospray tandem mass spectrometry. The most effective macroporous resin was selected by comparing the adsorption performance of five different types of macroporous resins (Diaion Hp20, Sepabeads Sp70, Sepabeads Sp207, Sepabeads Sp700, and Sepabeads Sp710). Equilibrium adsorption isotherms of five resins with wastewater were measured and analyzed using Langmuir and Freundlich isotherm models. Both Langmuir and Freundlich models could describe the adsorption process. The adsorption and desorption behaviors of anthocyanins were studied using a dynamic method on the five

types of resins, and Sp700 presented the highest adsorption capacity as well as desorption capacity, indicating that Sp700 is a good candidate for purification of anthocyanins from black bean canning wastewater. The most effective adsorption conditions were tested using Sp700. Dynamic adsorption and desorption were performed in glass columns packed with Sepabead Sp700 to optimize the purification process. Temperature during adsorption and desorption (25°C and 35°C) did not significantly affect the adsorption and desorption ratio. 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 1.5mL/min to 0.3mL/min). Ethanol concentration (from 30% to 60%) did not affect 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 in the final product.

(80 pages)

PUBLIC ABSTRACT

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Wastewater treatment is needed in almost all food factories. Removing color in wastewater is one of the most important tasks since colored wastewater could easily draw public attention. The color of wastewater is often due to the presence of anthocyanins, like the black color in the black bean canning wastewater resulting from anthoyanins in the black bean coat. Anthocyanins are colored water-soluble pigments that can provide attractive colors in foods. They are listed as natural colorants by the European Union and FDA. They have been used in beverages, fruit fillings, snacks, and dairy products. They also are antioxidants that may have health-promoting benefits such as increased visual acuity and lower cancer risk. They have received growing attention and increasingly been used in food and pharmaceutical products.

Our study aimed to investigate the effects of different adsorption materials and different adsorption conditions to purify anthocyanins from wastewater. Macroprous resin Sp700 was found to be an effective adsorption material. It has a good performance in both steps of the purification: adsorption and desorption. For adsorption, a low flow rate at room temperature should be used. For desorption, room temperature (25 °C), low flow rate (0.3 mL/min), and high ethanol concentration (60% acidified ethanol) was an effective desorption condition with low cost.

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Xiaoxi Wang

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LIST OF ABBREVIATIONS

a_L (L/mg)	Langmuir isotherm adsorption equilibrium constant
AR	Adsorption ratio
CCC	Countercurrent chromatography
Cd	Anthocyanins concentration in desorption solution (mg/mL)
Ce	Anthocyanin concentration at the adsorption equilibrium (mg/L)
Ci	Anthocyanins concentration at the initial stage (mg/L)
Ct	Anthlocyanins concentration at time t (mg/L)
Cy	Cyanidin
Dp	Delphinidin
DR	Desorption ratio
ESI	Electrospray ionization
EP	Eluted percentage
FDA	Food and Drug Administration
HPLC	High Performance Liquid Chromatography
KF (L/g)	An indication of the adsorption capacity of the adsorbent
K_L (L/g)	Langmuir isotherm adsorption equilibrium constant
MS	Mass Spectrometry
Mv	Malvidin
[M]+	Molecular ion peak
NI	Negative Ionization Mode
NMR	Nuclear Magnetic Resonance

ORAC	Oxygen Radical Absorbance Capacity
Pg	Pelargonidin
PI	Positive Ionization Mode
Pn	Peonidin
Pt	Petunidin
Q_e	Amount adsorbed at adsorption equilibrium
Q_{max} (mg/g)	The maximum amount adsorbed as the concentration of the adsorbate increases.
Q_t	Amount adsorbed at time t.
SDVB	Styrene-Divinylbenzene
TEAC	Trolox Equivalent Antioxidant Capacity
Vd	The volume of the desorption solution (mL).

CHAPTER 1

INTRODUCTION

Introduction

Phaseolus vulgaris is one of the most important industrial legumes, which can not only be used for human or animal feed but also has major importance in furnishing a variety of industrial, agricultural, food and pharmaceutical products (Black and others 2006) Kidney bean, which is one of the largest varieties of *Phaseolus vulgaris*, is relatively inexpensive and a good protein supply for animals and humans, especially important for growth and skeletal muscle nitrogen fractions (Marzo and others 2002). Even though it contains several phytochemical components that can limit its consumption, some processing techniques like soaping and cooking can be utilized to reduce its toxicity (Marzo and others 2002).

Canned beans have a long history of use and are sold in almost all supermarkets in the USA. Ironically, while the *Phaseolus vulgaris* itself provides health during its consumption, its resulting by-products (wastewater during bean canning) represent a serious environmental threat; it contains high amounts of sugar, starches and dark pigments, with a pollutional strength (Biochemical Oxygen Demand) approximately ten times greater as compared to domestic sewage (Lopez 1987). During the canning process of the beans, large amounts of water are required to soap, wash, blanch and cook the beans. This transfers part of the anthocyanin in the beans' coat into the industrial wastewater and gives the wastewater color.

Anthocyanins are the largest group of water-soluble pigments in *Phaseolus vulgaris*; the content and different types of anthocyanins in beans coats are responsible for the

color variation (red, black, brown, and white) of the beans (Choung and others 2003). Anthocyanin pigments, which are innocuous and easy to incorporate in aqueous media, are perfect as an alternative to substituting synthetic colorants which have toxic effects in humans (Castañeda-Ovando and others 2009). Takeoka and others (1997) reported that among the different colors of the beans, red and black pigments in the seed coats of the beans are an attractive source for natural food pigment in food industry. Wastewater from black or red bean canning containing anthocyanins is a potential resource to obtain natural pigments.

Also, currently research shows that anthocyanin not only can be used as food pigments, but also as active antioxidants in some food products and medicines (Goldstein 2010). Many kinds of chemical analyses, including the Folin-Ciocalteu method, the oxygen radical absorbance capacity (ORAC) assay and the Trolox equivalent antioxidant capacity (TEAC) assay were used to study the antioxidant capacity of anthocyanin (Prior and others 2005). Some researchers have also used electrochemical methods to study the antioxidant capacity of anthocyanins from Chilean red wine, grapes, and raspberries (Aguirre and others 2010). Thus, anthocyanins from canning wastewater have the potential to work not only as pigments, but also antioxidant products.

Even though there are several researches on extracting anthocyanins from some vegetables and fruits, and there are several methods on determining the antioxidant capacity of the anthocyanin, the research on extracting anthocyanin from food waste and using electrochemical methods to determine the products from the wastewater is still limited. Also, their genetic study and other related features such as anthocyanin composition and content are more limited in canning process wastewater, although

reusing food waste and reducing energy in waste management are becoming more important today. An understanding of the composition and contents of anthocyanins in canning process wastewater may aid in their further utilization as anthocyanin resource materials. The objective of this research was to characterize the colorant compounds present in the industrial wastewater from black bean canning and to investigate a potential industrial process for the extraction of anthocyanins for use as natural pigments.

Hypothesis

The extraction conditions, including resin materials, temperature, flow rate and eluent, which affect the purification of anthocyanins from wastewater using macroporous resin, may affect the purification effectiveness of the anthocyanins significantly.

Objectives

Objective 1: To investigate a potential industrial absorbent for the extraction of anthocyanins from black bean canning byproduct for use as a natural food colorant, and to test the suitability of the Langmuir and Freundlich adsorption models for describing the adsorption isotherms of anthocyanins on different kinds of macroporous resins.

Objective 2: To optimize pigment extraction process by comparing different resin extraction conditions in order to provide recommended guidelines for application.

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CHAPTER 2

LITERATURE REVIEW

Anthocyanins

The interest of the food industry in natural colorants to replace synthetic dyes has increased significantly over the last several years. Anthocyanins, which are colored water-soluble pigments that also are antioxidants and have health promoting benefits such as increased visual acuity and cognitive functions among humans, have received dramatically growing attention and increasingly been used in food and pharmaceutical products (Wallace and Giusti 2008). Another noteworthy property of anthocyanins is easy incorporation in aqueous media, which makes them suitable to be used as natural pigments (Alexandra Pazmiño-Durán and others 2001)

They are listed as natural colorants by the European Union (EU) legislation as product E163. Also being natural colorants, they are exempt from certification by the U.S. Food and Drug Administration (FDA), but their use is restricted (Mateus and Freitas 2009). They have been used in beverages, fruit fillings, snacks, dairy products (Choung and others 2003).

Anthocyanins are responsible for the colors displayed by many flowers, fruits, stems, and roots (Mateus and Freitas 2009). There are many sources suitable for extracting anthocyanins, including red beans, black beans, blood oranges, strawberries, raspberries, and grapes (Clifford 2000; Choung and others 2003; Aguirre and others 2010; Cao and others 2010; Cerezo and others 2010). Table 2.1 shows the average amount of anthocyanins in some foodstuffs.

Table 2.1. Average amount of anthocyanins in some foodstuffs

Anthocyanin source	Amount (mg/L[a])	Anthocyanin source	Amount (mg/L[a])
Blackberry	1150	Currant (black)	1300–4000
Blueberry	825–4200	Elderberry	2000–10000
Black bean	2100-2800	Red grapes	300–7500
Cherry	20–4500	Blood orange	2000
Chokeberry	5060–10000	Raspberry (black)	1700–4277
Cowberry	1000	Red bean	200-800

[a] mg/L or mg/kg
Summarized from Clifford (2000), Giusti and Wrolstad (2001).

From Table 2.1, we can see that black bean is a potential resource to extract anthocyanins. However, with new sources of natural colorants being in current demand by industry, food waste is being examined as a potential source to extract anthocaynins: for example, purple corn cob (*Zea mays*.), red grape pomace, and bract waste from harvesting bananas (Di Mauro and others 2000; Pazmino-Duran and others 2001; Giusti and Wrolstad 2001; Mantell and others 2002; Yang and Zhai 2010).

Red and black color pigments in the seed coats of kidney bean are an attractive potential for natural food colorants (Takeoka and others 1997). During canning of the beans, the process consumes large amounts of water to soap, wash, blanch and cook the beans, transferring part of the anthocyanin in the beans' coat into the industrial waste water (Brooks and Houpt 1973).

Therefore, due to the enormous potential of natural anthocyanins extracted from bean processing waste as natural pigments, there are an increasing number of reports found in the literature on analyzing anthocyanins in common beans, for example: purification and separation of anthocyanins from Korean cultivated kidney bean (Choung and others 2003), quantitative analysis of anthocyanins in common beans using chromatographic

methods such as high performance liquid chromatography (HPLC; Díaz and others 2010), antioxidant activity in common beans (Jiratantan and Liu 2004; Oomah and others 2010), and microwave-assisted extraction of anthocyanin from beans (Sutivisedsak and others 2010).

In this review, I will focus on bean anthocyanin chemistry and recent investigations about extraction, purification and identification of anthocyanins.

Bean Anthocyanin Chemistry

Anthocyanins are water-soluble vacuolar pigments that may appear as red, purple, or blue depending on pH. They are known as the anthocyanidins' glycoside form. The main anthocyanidin structure is an aromatic ring that contains oxygen, which is also bonded by a carbon-carbon bond to a third aromatic ring (Ananthaswamy and others 2004). There is a huge variety of anthocyanins spread in nature. Figure 2.1 shows the general structure of the anthocyanins and Table 2.2 shows the 6 anthocyanidins occurring most frequently in plants: pelargonidin (12%), cyanidin (50%), peonidin (12%), delphinidin (12%), petunidin (7%), and malvidin (7%; Kong 2003)

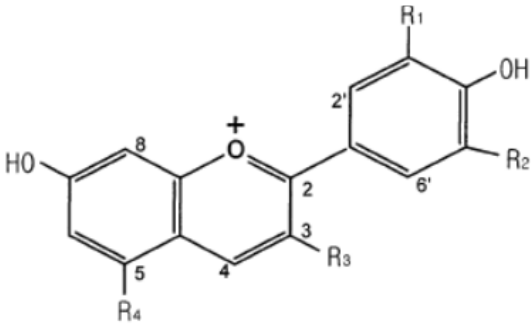

Figure 2.1 General anthocyanin structure

The sugars commonly linked to anthocyanidins are monosaccharides (glucose, galactose, rhamnose, and arabinose) and di- or tri-saccharides formed by combination of the 4 monosaccharides, which may also be acylated with a phenolic or aliphatic acid (Bureau and others 2009). The glycoside derivatives that are more widespread in nature are 3-monosides, 3-biosides, 3,5- and 3,7-diglucosides (Kong 2003). The huge variety of anthocyanins are formed by the different number of hydroxylated groups, the nature and number of the sugars bound to the anthocyanidin, the phenolic or aliphatic acid bound to the sugar in the molecule, and the different positions of these bonds. Several investigators (Feenstra 1960; Stanton and Francis 1966; Choung and others 2003) have identified different kinds of anthocyanins from diverse kidney bean varieties. Common anthocyanins in different bean cultivars are shown in Table 2.2. Distribution of anthocyanins between diverse bean varieties is shown in Table 2.3.

Table 2.2 Structural identification of anthocyanidins.

Anthocyanin	Abbreviations	R_1	R_2	λ_{max} (nm)*		colour
				R_3=H	R_3=gluc	
Delphinidin	Dp	OH	OH	546	541	Blue-red
Petunidin	Pt	OH	OCH_3	543	540	Blue-red
Malvidin	Mv	OCH_3	OCH_3	542	538	Blue-red
Cyanidin	Cy	OH	H	535	530	Orange-red
Peonidin	Pn	OCH_3	H	532	528	Orange-red
Pelargonidin	Pg	H	H	520	516	Orange-red

In methanol with 0.01% HCl (Giusti and Rolstad 2001; Castanedaovando and others 2009)

Table 2.3 Bean anthocyanins structure and cultivar

Anthocyanin	R_1	R_2	R_3	R_4	Cultivar
Cyaniding 3,5-diglucoside	OH	H	O-β-D-glucose	O-β-D-glucose	Korean red bean (KG98001),
delphinidin 3-glucoside	OH	OH	O-β-D-glucose	OH	Korean bean (KG98001, KG98287), Kurodanekinugasa black bean, Canadian Wonder bean, Yamashirokurosando black bean Black bean (US UI911)
cyanidin 3-glucoside	OH	H	O-β-D-glucose	OH	Korean red bean (KG98001)
petunidin 3-glucoside	OCH3	OH	O-β-D-glucose	OH	Korean black bean (KG98287), Black violet bean, Kurodanekinugasa black bean Black bean (US UI911) American commercial black bean
pelargonidin 3-glucoside	H	H	O-β-D-glucose	OH	Korean red bean (KG98001)
Malvidin 3-glucoside	OCH3	OCH3	O-β-D-glucose	OH	Kurodanekinugasa black bean Black bean (US UI911)
Malvidin 3,5-diglucoside	OCH3	OCH3	O-β-D-glucose	O-β-D-glucose	Kurodanekinugasa black bean American commercial black bean
delphinidin 3, 5-diglucoside	OH	OH	O-β-D-glucose	O-β-D-glucose	Black violet bean American commercial black bean
Cyanidin 3,5-diglucoside	OH	H	O-β-D-glucose	O-β-D-glucose	Canadian Wonder bean
Pelargonidin 3-glucoside	H	H	O-β-D-glucose	OH	Canadian Wonder bean
malvidin 3-galactoside	OCH3	OCH3	O-β-D-galactose	OH	American commercial black bean
petunidin 3-galactoside	OCH3	OH	O-β-D-galactose	OH	American commercial black bean
delphinidin 3-galactoside	OH	OH	O-β-D-galactose	OH	American commercial black bean
Pelargonidin 3,5-diglucoside	H	H	O-β-D-glucose	O-β-D-glucose	Canadian Wonder bean American commercial black bean

Chemical structures of anthocyanins in seed coats of different bean cultivar (Takeoka and others 1997; Choung and others 2003; Wu and Prior 2005)

Extraction and analysis of anthocyanins

Extraction is a very important step in the isolation, identification and use of anthocyanin compounds and there is no single and standard extraction method. Fruits, vegetables and herbs can be ground, dried, or lyophilized, and some fresh plants can be soaked with subsequent solvent extraction to extract phenolic compounds (Merken and Beecher 2000). These methodologies result in the co-extraction of non-phenolic substances such as sugars, organic acids, and proteins, requiring subsequent purification processes such as solid phase extraction (SPE; Castanedaovando and others 2009).

Chemical extraction

Anthocyanins are polar molecules, thus using solvents like aqueous mixtures of ethanol, methanol or acetone is better for extraction (Kahkonen and others 2001). Because anthocyanins are not stable in neutral or alkaline solutions, acidic aqueous solvents have been used as extraction solvents in order to disrupt cell membranes and at the same time dissolve the water-soluble pigments. The most common methods are those which use acidified methanol or ethanol as extractants. HCl (usually <1%) is chosen for acidulating the extraction solvent (Rodriguez-Saona and Wrolstad 2001; Amr and Al-Tamimi 2007). Ethyl acetate, methanol and aqueous mixtures (50%-90%,v/v), and ethanol and aqueous mixtures (10-90%) have been investigated (Ignat and others 2011).

Methanol is the most effective of these solvents. In anthocyanin extractions from grape pulp, extraction with methanol is 20% more effective than with ethanol, and 73% more effective than only water (Ignat and others 2011). It also has been found that acidified methanol resulted in significantly higher values for total anthocyanins than aqueous acetone, as the extraction with acidified methanol was twice as efficient as

aqueous acetone (Lee and others 2004). For food applications, although having a lower extraction capacity and being difficult to eliminate afterwards, ethanol is usually preferred due to its low toxicity (Mateus and Freitas 2009).

Aqueous acetone may not be an appropriate solvent for extracting anthocyanins from some plants like sorghum. Anthocyanin molecules can undergo significant structural modification in aqueous acetone through oxidative addition mediated by acetone and hence forming pyranoanthocyanins (Awika 2005).

When we use food waste as an anthocyanin resource, these solutions are used to extract solid food waste. For the aqueous food waste, these methods may not be appropriate. As mentioned, soaking has also been extensively used for anthocyanin extraction. In some food processes like canning beans, soaking is an important step which also results in a “natural” extraction process. It could be directly applied to raw material in the food industry, is easy to control the soaking conditions, and could extract a considerable amount of anthocyanins while maintaining the food product quality.

Physical extraction

In recent years, various novel extraction techniques have been developed as alternatives to conventional extraction which can take long extraction times. For example, ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid extraction and high hydrostatic pressure extraction, can be used to extract phytochemicals from plants.

Concentration

After extraction, rotary evaporator under vacuum at low temperature can be used in

the concentration process, in order to minimize anthocyanin degradation (Romero and Bakker 2000; Chaovanalikit and others 2012).

Purification

As mentioned, the extraction methods may not be selective for anthocyanins due to the co-extraction of non-phenolic substances such as sugars, organic acids and proteins. A subsequent purification processes is required. A wide variety of techniques have been examined, varying from extractions in solid phase (SPE) and liquid-liquid (LLE) to the use of sophisticated chromatographic techniques like countercurrent chromatography (CCC, Schwarz 2003; Wybraniec and others 2009), medium pressure liquid chromatography (MPLC) and HPLC (Díaz and others 2010). The most common method used for anthocaynin separation is HPLC with UV-Vis or photodiode array (PDA) detectors (Castanedaovando and others 2009).

Although some reports are available, research on the use of plants or fruits for anthocyanin extraction at an industrial plant scale are limited. This might be due to 3 limiting factors, often overlooked in scientific studies: The effectiveness of recovery and extraction; the marketability of resulting extracts; and the practical suitability for food and pharmaceutical products (Castanedaovando and others 2009).

Column chromatography

The components of a mixture can be separated based on their different relative mobilities using chromatographic methods. Figure 2.2 is a general column chromatography showing how 2 components, A and B, are resolved on a column by column chromatography. Column chromatography is an effective method for the extraction and purification of anthocyanins on a plant scale. Kammerer and others (2005)

evaluated ion exchange resin (Amberlite XAD 16HP resin) for recovery and concentration of anthocyanins from grape pomace extracts. The aqueous extract was prepared by adding water to fresh grapes skins (1:3 sample; water at 80°C). The crude extract was passed through the resin and eluted with methanol. The temperature of adsorption and desorption were 25°C and 50°C, respectively. Recovery rates reported ranged from 96% to 100%. The recovery rates on a pilot-plant scale were compared to bench-lab experiments and the results revealed no significant difference between the recoveries provided by both experiments.

In another study, macroporous resin was used to extract and purify the anthocyanins in 31 cultivars of mulberry, and a process for the industrial preparation of mulberry anthocyanins as a natural food colorant was studied. Of 6 resins tested, X-5 demonstrated the best adsorbent capability for mulberry anthocyanins. Residual mulberry fruit juice after extraction of pigment retained most of its nutrients, except for anthocyanins, and may provide a substrate for further processing (Liu and others 2004). This research showed that macroporous resins can be used for efficient purification. They are made of highly cross-linked nonpolar or slightly hydrophilic styrene-divinyl-benzene (SDVB). Figure 2.3 shows the chemical structure of SDVB and how SDVB forms macropores.

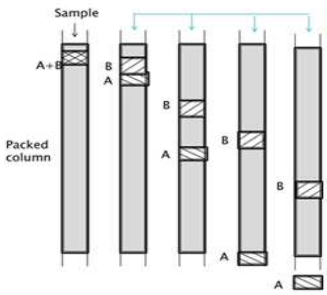

Figure 2. 2 General column elution chromatography showing how to separate a mixture of Components A and B

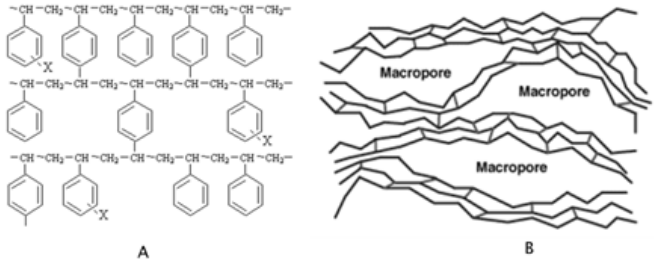

Figure 2.3 Chemical (A) and physical (B) structure of SDVB macroporous resin.

Adsorption models

An isotherm is usually used to describe an adsorption process on selected resins (Okeola and Odebunmi 2010). Adsorption isotherm is the equilibrium relationship between the concentration of solute in solution phase and on adsorbent phase at a specified temperature (Chabani and others 2007). The adsorbed molecules distribution between the liquid phase and the solid phase at the equilibrium state point can be indicated by the adsorption isotherm (Nwabanne and Igbokwe 2008). In order to obtain equilibrium adsorption isotherms of the adsorbent selected and predict the adsorption capacities, adsorption isotherms are usually tested on different types of resins. Two commonly used adsorption isotherm models are the Langmuir isotherm and the Freundlich isotherm.

The Langmuir isotherm assumes that each active site of the resins is independent and occupied only by one particle. It is a theoretical model suitable for the ideal conditions of monolayer adsorption (Scordino and others 2003; Liu and others 2007). Langmuir isotherm equation is described as follows:

$$Q_e = Q_{max}a_LCe/(1+a_LC_e)=K_LC_e/(1+a_LC_e)$$

where K_L (L/g) and a_L (L/mg) are the adsorption equilibrium constants. K_L/a_L is defined as Q_{max} (mg/g), which is the maximum amount adsorbed as the concentration of the adsorbate increases. C_e (mg/L) is the concentration of anthocyanins in liquid phase at equilibrium point, Q_e (mg/g) is the quantity (mg) of anthocyanins on a unit amount (g, dry weight) of adsorbent at the equilibrium point. This equation is usually applied using the following linear form:

$$1/Q_e=1/K_LC_e+1/Q_{max}$$

A dimensionless constant separation factor, R_L, has been defined (Hall and others 1966) to assess the validity of the Langmuir-type adsorption process given by:

$$R_L =1/(1+a_LC_i)$$

where, C_i is the initial sample solution concentration. The value of R_L indicates this type of the isotherm to be either unfavorable when $R_L>1$, linear if $R_L= 1$, favourable if $0<R_L<1$ or irreversible when $R_L = 0$.

The Freundlich isotherm, on the other hand, is an empirical model for non-ideal adsorption on heterogeneous surfaces. Freundlich isotherm is expressed as:

$$Q_e=K_F C_e^{b_F}$$

where K_F (L/g) provides an indication of the adsorption capacity of the adsorbent, and b_F (dimensionless) represents the adsorption intensity. C_e was described above. It can be rearranged to the linear form as follows:

$$\log Q_e= \log K_F +b_F*\log C_e$$

An Example of the Langmuir and Freundlich isotherm was shown in Figure 2.4. By comparing R^2 (coefficient of determination of the linear regression for both isotherms linear form), which model yields a better fit to the adsorption process could be

determined (Hameed and others 2007). However, Potgieter (1991) indicated that more than one isotherm can be applied to describe the adsorption process. Previous work (Liu and others 2007) has shown that both Langmuir and Freundlich models were suitable to describe the adsorption isotherms of purple potato anthocyanins on the 11 tested SDVB, methacrylic and acrylic resins when the R^2 for both isotherms were close.

Identification

The detection of anthocyanins can be achieved using UV-Vis spectroscopy (Giusti and Wrolstad 2001), spectrofluorometry (Mateus and Freitas 2009), mass spectrometry (FAB-MS, HPLC-MS, ESI-MS; Liu and others 2008; Huang and others 2009) or infrared spectroscopy (Dambergs and others 2006). The coupling of different analytical techniques improves the separation as well as the sensitivity of detection of anthocyanins. HPLC coupled to nuclear magnetic resonance (NMR) is one of the recent examples of such achievement (Valverde and Herve 2008; Veraderosso and others 2008; Lee and others 2009).

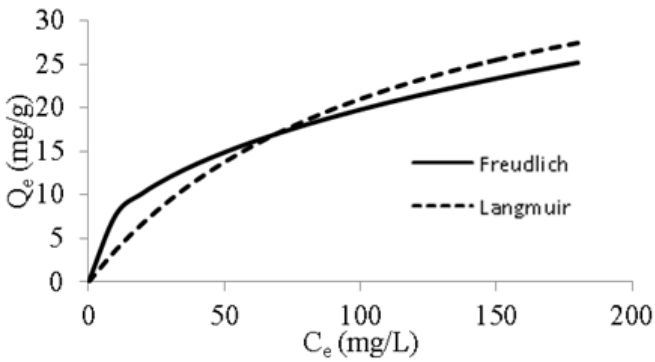

Figure 2.4 Example of the Langmuir (dashed line) and Freundlich (solid line) isotherms. Q_e is the amount adsorbed at adsorption equilibrium and C_e is the equilibrium concentration in the solution. The graph is for the values of the constants of K_L=0.4 and a_L=0.009 in Langmuir equation, K_F=3 and b_F=0.41 in Freundlich equation.

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CHAPTER 3

PURIFICATION OF ANTHOCYANINS FROM BLACK BEAN CANNING WASTEWATER WITH MACROPOROUS RESINS

Abstract:

This study investigated purification of anthocyanins from black bean canning wastewater by column chromatography with 5 types of macroporous resins (Diaion Hp20, Sepabeads Sp70, Sepabeads Sp207, Sepabeads Sp700, and Sepabeads Sp710). Byproduct of canned black beans was partially purified by filtration, in anticipation of higher performance during column chromatography. Equilibrium adsorption isotherms were measured and analyzed using Langmuir and Freundlich isotherm models. Both Langmuir and Freundlich models can describe the adsorption process of anthocyanins from black bean canning wastewater using the tested resins. The adsorption and desorption behaviors of anthocyanins were studied using a dynamic method on the 5 types of resins, and Sp700 presented the highest adsorption capacity as well as desorption capacity, indicating that Sp700 is better candidate in these 5 resins for purification of anthocyanins from black bean canning wastewater.

Introduction

Anthocyanins, a class of flavonoids derived from phenylalanine, are water-soluble pigments that can provide attractive colors ranging from orange/red to violet/blue (Tanaka and others 2008). The safety of artificial pigments has been questioned by some consumers and scientists, for example, an association between ingestion of artificial food pigments and hyperactivity was suggested in the review of 10 electronic databases for double-bind placebo-controlled trials evaluating the effects of artificial food pigments

(Schab and Trinh 2004). Due to this reason, there has been a growing need for edible natural pigments and anthocyanins are very good candidates (Giusti and Wrolstad 2003). Some research shows that anthocyanin extract and anthocyanin-rich mixtures of bioflavonoids can not only be used as food pigments, but may also provide various health benefits in some food products and medications.

Acquaviva and others (2003) proposed that anthocyanins are able to scavenge free radicals and exhibit antioxidant activity. Other researchers indicated that anthocyanins can provide anti-inflammatory activity (Rossi and others 2003), anticancer activity (Faria and others 2010) and may reduce the risk of diabetes (Wedick and others 2012). Taken together, more and more anthocyanins are and will be needed, and anthocyanin extraction methods that are more cost-effective and efficient can help meet this demand.

For a very long time, grape, elderberries, red cabbage, Roselle, and other materials including blood orange, black chokeberry and sweet potato were suggested to be the main sources of anthocyanins (Bridle and Timberlake 1997). However, more and more materials are needed to meet with the increasing demands. Black bean (*Phaseolus vulgaris*) is one kind of discovered anthocyanin source, and it has been considered to be a good candidate because of the high concentration of anthocyanins in the seed coat (Choung and others 2003). One study reported the concentration of anthocyanins in American common black bean could reach 44.5 mg/ 100g (Wu and others 2006). There are a variety of anthocyanins in black beans, including delphinidin 3′-glucoside, petunidin 3′-glucoside and malvidin 3′-glucoside (Wu and Prior 2005; Wu and others 2006). Large quantities of the anthocyanins in black beans are lost to the wastewater stream in the canning process. By absorbing and reflecting sunlight entering water,

anthocyanins in the effluent can cause problems like inhibiting the growth of bacteria which biologically degrade impurities in the water (Pierce 1994) and slowing down photosynthesis in aquatic plants (Slokar and Majcen Le Marechal 1998). However, new technology in processing may reduce these problems and bring commercially profitable production of anthocyanins from this wastewater.

Adsorption is an effective process for the treatment of contaminated wastewater, and it includes different kinds of adsorbents, such as activated carbon, macroporous resins, synthetic polymers, naturally occurring biopolymers and biomass (Soto and others 2011). Among all these adsorbents, macroporous resins are used because of their high adsorption capacity, which comes from their durable nonpolar or slightly hydrophilic styrene-divinylbenzene (SDVB, or acrylic polymers) structure, large surface area, and high degree of surface reactivity. Moreover, advantages like low-cost, easy regeneration and safety (FDA approved) of macroporous resins make these environmental friendly and long service life materials more suitable for food industry application. Past research has proposed that macroporous resins are good adsorbents for anthocyanins. Isolation and purification of anthocyanins from mulberry, purple-fleshed potato and blood oranges by column chromatography using macroporous resins was proved to be an efficient potential method (Liu and others 2004; Liu and others 2007; Cao and others 2010).

To achieve efficient large-scale separation of anthocyanins from canning bean wastewater, having predicable models of the adsorption process rather than empirical data is important. The objectives of this work were to investigate a potential industrial adsorbent for the extraction of anthocyanins from black bean canning byproduct for use as a natural food colorant, and to test the suitability of the Langmuir and Freundlich

adsorption models for describing the adsorption isotherms of anthocyanins on different kinds of macroporous resins.

Materials and Methods

Black bean canning wastewater

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 (Lopez 1987). The wastewater was then drawn through a Whatman filter paper (Qualitative 1) with a vacuum pump. The sample solution was kept in sterilized bottles at 4°C (for immediate use) or at -20°C (for storage).

Adsorbent resin treatment

All of the resins used in this study were macroporous SDVB copolymer resins, including Diaion® Hp 20, Sepabeads® Sp70, Sepabeads® Sp207, Sepabeads® Sp700, and Sepabeads® Sp710 (Resindion, Mitsubishi Chem, Co., Chesapeake, VA., U.S.A). They are characterized by a wide range of surface areas (590 ~ 1200 m^2/g) and pore radii (90 ~ 290 Å). Chemical and physical properties of the resins are shown in Table 3.1. Resins were activated prior to use, and the activation method was based on manufacturers' recommendations. Briefly, 0.6 g of dry resin was washed with distilled water and filtered using Whatman filter paper (Qualitative 1). Then they were soaked in 2 ml of 95% ethanol (V/V) overnight, followed by rinsing with distilled water. Afterwards the resins were dried at 50°C in a vacuum oven for 24 hours, then washed with 2 ml of 95% ethanol and rinsed thoroughly with distilled water to dispel the ethanol.

Stationary adsorption isotherms

Adsorption isotherms of black bean anthocyanins were developed for 5 different types of resins. For each type, 0.5 ~ 1.0 g (dry weight) of activated resin was mixed with 50 ml of the filtrated sample solution in a 25°C water bath. The solution was stirred gently every 5 minutes until the resins were saturated with anthocyanins. The resins were considered to be saturated with anthocyanins when anthocyanin content in the sample solution did not change.

The adsorption ratio (AR), the amount adsorbed in the resins (Q_t), and amount adsorbed at adsorption equilibrium (Q_e) were calculated using the relationships below (Fu and others 2005; Akar and others 2009):

Adsorption ratio (AR):

$$AR\ (\%)=100*(C_i-C_e)/C_e \quad (1)$$

Q_t value:

$$Q_t=(C_i-C_t)*V/m \quad (2)$$

Q_e value:

$$Q_e=(C_i-C_e)*V/m \quad (3)$$

Table 3.1 Chemical and physical properties of resins provided by manufacturer

Trade name	Particle size (μm)	Pore radius (Å)	Surface area (m^2/g)	Structure
Hp20	>250	290	590	SDVB[a]
Sp207	>250	210	600	SDVB
Sp70	>250	70	870	SDVB
Sp700	>250	90	1200	SDVB
Sp710	>250	90	900	SDVB

[a] styrene-divinyl-benzene structure (SDVB)

where Q_t (mg/g) is the quantity (mg) of anthocyanins based on a unit mass (g dry weight) of adsorbent at time t; Q_e (mg/g resin) is the adsorption capacity at adsorption equilibrium; C_i, C_t and Ce (mg/L) are the concentrations of anthocyanins in liquid phase at the initial stage, at time t and at equilibrium point; V (L) is the volume of the solution; and m (g) is the mass of dry resins.

Dynamic adsorption and desorption

Dynamic adsorption and desorption were performed after adsorption resin screening. Figure 3.1 is a schematic of the adsorption and desorption system. For each different resin type, about 0.6 g (dry weight) of the activated resin was introduced into a 10 ml disposable syringe. One adsorption run was defined as 100 ml of filtrated anthocyanin extraction (25°C) or effluent after being passed through the syringe once. Adsorption runs were repeated until the resins were saturated.

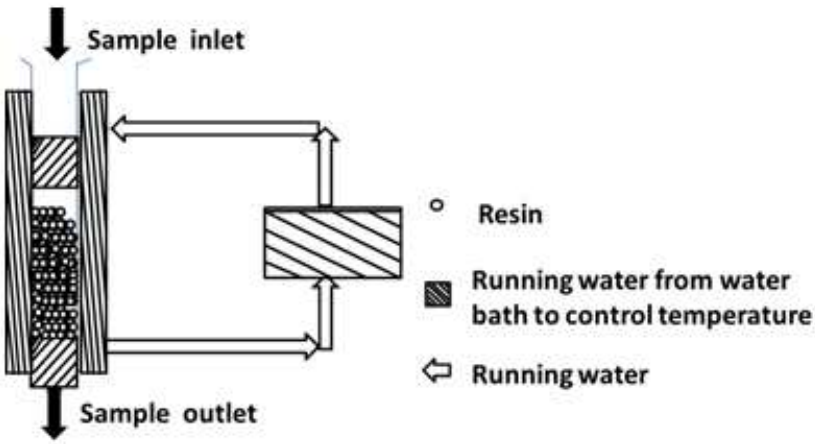

Figure 3.1 Schematic showing the dynamic adsorption and desorption system.

To select the best resin for extracting black bean anthocyanins, small aliquots were withdrawn from elution and the total anthocyanin content in the eluate was tested spectrophotometrically. The resins were considered to be saturated with anthocyanins when the anthocyanin content in the sample and in the eluent were equal. For desorption, 95% ethanol was used to elute the anthocyanins until the resin color of the resins did not change. All the effluent was collected and its anthocyanin content was analyzed.

Analysis of anthocyanin content

Anthocyanin content was determined by the pH differential method (Giusti and Wrolstad 2001) using a UV-Vis Spectrophotometer (Shimadzu UV-2100U, Shimadzu Corp., Tokyo, Japan). The absorbance values at 400nm and λ_{vis}-max (519nm) were measured, and the anthocyanin content was calculated as cyanidin-3-glucoside, using an extinction coefficient of 26900 L/cm/mg and molecular weight of 449. Table A.1 in Appendix A shows an example of different absorbances at different pH value and different wave length. Appendix A shows how to use these absorbances for sample calculation.

Analysis of pH, sugar and protein content

pH, sugar content , and protein content before and after filtration were also measured. pH was measured by a digital pH meter (Orion SA-700, Thermo Electron Corp., Beverly, MA). For sugar and protein measurements, all samples were freeze dried to solid phase and then dissolved with distilled water to a high concentration solution. Reducing sugar was measured by 3, 5-dinitrosalicylic acid reagent method (Miller 1959), and total sugar content was measured by phenol-sulphuric acid method (Xu and others 2005). Protein

content was determined according to the AOAC official Kjeldahl Method 973.48 (AOAC 2005). All analyses were performed at least in triplicate.

Statistical analysis

Statistical analysis 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 2-way analysis of variance (ANOVA), and differences were considered significant if $p < 0.05$. Values were presented as means ± SEM, unless otherwise indicated. (See Appendix B for detailed statistics).

Results and Discussion

Analysis of pH, sugar and protein content

In this research, the black bean wastewater was filtered with Whatman filter paper first, and the sugar and protein contents before and after filtration were determined (Table 3.2). The unfiltered extract contained about 3.24% protein and 6.35 mg/ml total sugar, which were reduced nearly 2-fold after filtration (1.90% protein and 3.3 mg/ml total sugar). However, only a relatively small proportion of the reducing sugar (about 1.92%) was removed. These results demonstrate that filtration using something similar to Whatman filters (Qualitative 1) can be used for filtration of canning wastewater to aid in extracting anthocyanins.

Table 3.2 Components in wastewater before and after normal filtration

	Protein (%)	Reducing sugar(g/L)	Total sugar(g/L)	pH
Before filtration	3.2±0.3 [a]	1.56±0.01	6.35±0.01 [a]	5.93±0.02 [a]
After filtration	1.9±0.2 [b]	1.53±0.01	3.30±0.03 [b]	6.04±0.01 [b]

Each value is the mean ± SD, n=3. Different letters within a column indicate significant difference at $p<0.05$.

Adsorption models

In order to screen the best resins for anthocyanin adsorption and predict the adsorption capacities, adsorption isotherms were performed on 5 types of resins. Adsorption isotherm is the equilibrium relationship between the concentration of solute in solution phase and on adsorbent phase at a specified temperature (Chabani and others 2007). The experimental isotherm data were fitted to two commonly used adsorption isotherm models, Langmuir isotherm and Freundlich isotherm.

The Langmuir isotherm assumes that each active site of the resins is independent and occupied only by one particle. It is a theoretical model suitable for the ideal conditions of monolayer adsorption (Scordino and others 2003; Liu and others 2007). Langmuir isotherm equation is described as follows:

$$Q_e = Q_{max}a_LCe/(1+a_LC_e)=K_LC_e/(1+a_LC_e) \quad (4)$$

where K_L (L/g) and a_L (L/mg) are the adsorption equilibrium constants. K_L/a_L is defined as Q_{max} (mg/g), which is the maximum amount adsorbed as the concentration of the adsorbate increases. This equation is usually applied using the following linear form:

$$1/Q_e=1/K_LC_e+1/Q_{max} \quad (5)$$

The Freundlich isotherm, on the other hand, is an empirical model for non-ideal adsorption on heterogeneous surfaces. Freundlich isotherm is expressed as:

$$Q_e=K_F{C_e}^{b_F} \quad (6)$$

where KF (L/g) provides an indication of the adsorption capacity of the adsorbent, and bF (dimensionless) represents the adsorption intensity. It can be rearranged to the linear form as follows:

$$\log Q_e= \log K_F +b_F*\log C_e \quad (7)$$

The isotherm parameters for these two models were calculated and are shown in Table 3.3. The experimental isotherm data for all the resins appeared well fitted to both Langmuri and Freundlich isotherm equations with respective regression coefficients (R^2 values) near unity. This suggested that either of these two models could provide a reasonable description of the adsorption process for all tested resins assuming the sample solution has a similar anthocyanin concentration with the black bean canning byproduct. The isotherm data can also be used to predict the adsorption capacity, and the resins with the highest amount absorbed (Q_e) at certain residual concentration (C_e) should be the most effective resin for anthocyanin adsorption. According to the constants shown in Table 3.3, the Q_e at different C_e were calculated and plotted in Figure 3.2. It showed that Sp700 had greater Q_e value than the other resins at residual concentrations ranging between 4.4-50.0 mg/L, suggesting that Sp700 is a more effective adsorbent for anthocyanins in black bean canning wastewater than other tested resins.

Table 3.3 Langmuir isotherm parameters and Freundlich isotherm parameters for the adsorption of anthocyanins on five types of resins (T = 25°C, pH=6.0)

Resins	N	Langmuir isotherm			Freundlich isotherm		
		Q_{max} (mg/g)	k_L	R^2	Kf (L/g)	B_f	R^2
HP20	4	41.32	0.24	0.9969	0.28	0.9969	0.9954
Sp70	4	37.59	0.53	0.9960	0.72	0.7929	0.9875
Sp207	4	104.17	0.38	0.9882	0.23	1.0992	0.9793
Sp700	4	188.68	0.47	0.9995	0.50	0.9504	0.9991
Sp710	4	52.63	0.50	0.9983	0.66	0.8424	0.9991

Each value is calculated from the experimental data.

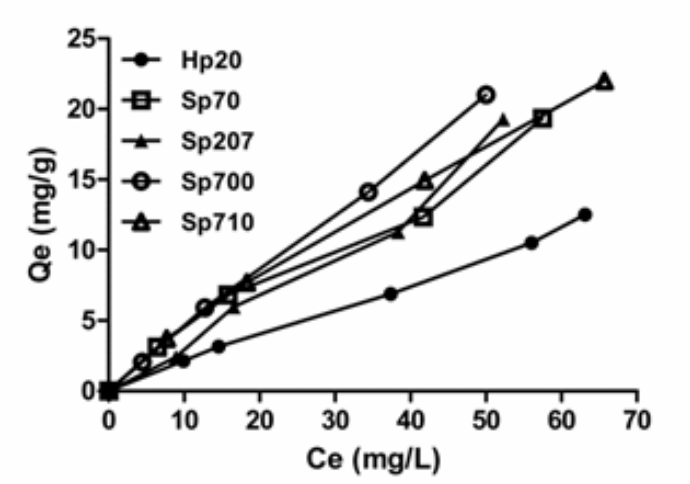

Figure 3.2 Equilibrium adsorption isotherms of anthocyanins on Hp20 (●), Sp70 (⊡), Sp207 (▲), Sp700 (○), and Sp710 (△) resins 25°C. Q_e (mg/g resin) is the adsorption capacity at adsorption equilibrium, C_e (mg/L) is the concentration of anthocyanin in liquid phase at equilibrium point. Each Value is the mean of three replicates.

Dynamic adsorption results

Dynamic adsorption studies were performed to study the adsorption behavior of anthoycanins on the 5 types of resins and in turn apply them in the further industrial chromatography process. Adsorption runs were repeated a number of times until the resins became saturated. The anthoycanins after each run by the resins was tested and calculated. The concentrations of anthoycanins were tested and Q_t at each cycle was calculated and plotted in Figure 3.3. For each resin, the anthocyanin amount adsorbed to the resin phase increased very quickly during the first 3 cycles, but thereafter they increased more slowly. Q_t had no significant change ($p > 0.05$) between the fourth and fifth run, which indicated that the resins were saturated after the fourth cycle. Among the five types of tested resins, Q_t varied significantly ($p < 0.05$) when the resins were saturated (Table 3.4).

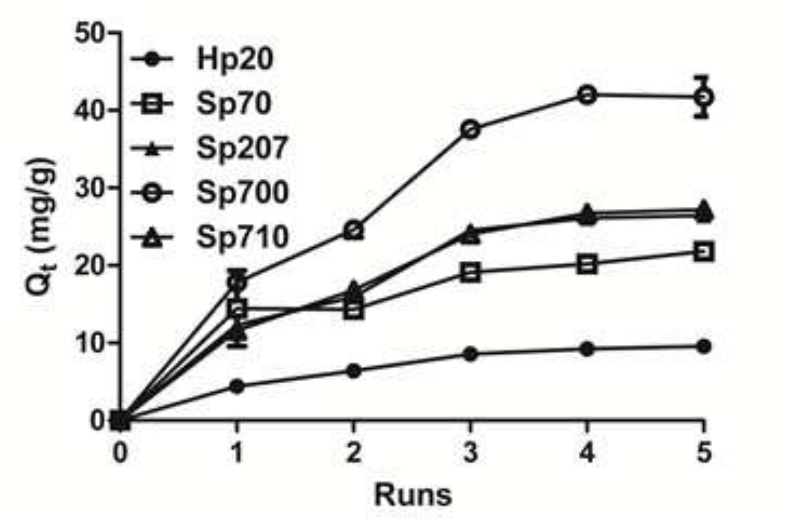

Figure 3.3 Changes in adsorption kinetics of black bean byproduct anthocyanins during repeated chromatography with Hp20 (●), Sp70 (⊡), Sp207 (▲), Sp700 (○), and Sp710 (△) resins at 25 °C. Q_t (mg/g resin) is the adsorption capacity at time t. The curve shows the mean of three replicated tests and the error bars show standard deviations.

Sp700 presented the highest adsorption capacity with an equilibrium concentration of 39±4 mg/g, and therefore, was the best material for adsorbing anthocyanins from black bean wastewater. Sp70, Sp207, and Sp710 had similar adsorption capacities ($p > 0.05$), and HP20 had the lowest capacity with an equilibrium concentration of 9.6±0.3 mg/g. These results are consistent with the screening results above. However, the dynamic adsorption results could compare the adsorption capacities of different resins more directly.

Chemical structures and adsorption capacity

The chemical structures of different types of resins determine their different adsorption capacities for anthocyanins. The adsorption of anthocyanins on macroporous resins is a physical action through van de Waals force and hydrophobicity. Surface area is one of the most important factors that can affect adsorption capacity (Li and others 2001).

Table 3.4 Comparison of dynamic adsorptive/desorptive capability for black bean anthocyanins on different resins.

Resins	Qt(mg)[e]	Ratio of desorption(%)[f]
Hp20	9.6±0.3[c]	4.3±0.2[d]
Sp70	21.9±0.1[b]	9.9±0.4[c]
Sp207	26±2[b]	12.4±0.1[b]
Sp700	39±4[a]	19±2[a]
Sp710	27.2±0.5[b]	12.7±0.2[b]

[a-d] Each value is the mean value with standard error of three replicated tests; mean values within a column followed by different letter superscripts are significantly different (p<0.05).
All columns were packed with 0.6 g dry weight, filtered anthocyanin concentration (135mg/L).
[e,f] See Table B.1 and Table B.2 in appendix B for Qt comparison statistical result details. See Table B.3 and B.4 in Appendix B for ratio of desorption comparison statistical result details.

In this research, the surface area and adsorption capacity for anthocyanins of Hp20, Sp70, Sp700, and Sp710 seem to be proportional, and therefore, larger surface area (1200 m^2/g) of Sp700 provides a better ability to adsorb anthocyanins than others. Another factor contributing to the adsorption ability is the pore structure, because the solute needs to migrate through the pores to the adsorbing surface. Therefore, the pore size should be big enough to accommodate anthocyanins. However, if the pores are too large, it will adsorb large molecules such as polysaccharides and proteins, and in turn prevent the binding capacities of anthocyanins. Sp700 has pore radius about 90 Å and is designed for adsorbing or desorbing polyphenolic substances. The suitable pore structure enhanced its purification capability. Special modifications on the resin surface can also affect the adsorption capacity. Sp207 is a modified brominated aromatic matrix resin in which bromine has been incorporated into the aromatic ring. The bromination of the aromatic ring provides an enhanced hydrophobicity and in turn increased binding force between anthocyanins and resin surface.

Desorption results

To be useful in anthocyanin purification, adsorbed anthocyanins should be easily desorbed under suitable conditions. Desorption ratio is defined as total anthocyanin content eluted over total anthocyanin adsorbed. Even though desorption method was not optimized and the ratio of desorption for all resins was not very high, the different resins' desorption capacities can still be compared. Sp700 had the highest desorption capacity with the desorption ratio of 19±2% (Table 3.4). Together with the fact that Sp700 also had the largest adsorption capacity, Sp700 was the most efficient macroporous resin for anthocyanin purification from black bean wastewater among all 5 types of tested resins in this research.

Conclusions

The purification of anthocyanins from black bean canning wastewater was investigated by column chromatography with five types of macroporous resins (Diaion Hp20, Sepabeads Sp70, Sepabeads Sp207, Sepabeads Sp700, and Sepabeads Sp710). The equilibrium isotherm data have been collected and fitted into Langmuir and Freundlich isotherm models. Both of the models appeared to provide a good fit for all the tested resins. Furthermore, dynamic adsorption and desorption were performed, and of the 5 resins, the maximum adsorption and desorption capacities were both observed on Sp700, suggesting Sp700 is the most efficient resin in this study for anthocyanins purification.

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CHAPTER 4

IDENTIFICATION OF ANTHOCYANINS ISOLATED FROM BLACK BEAN CANNING WASTERWATER BY MACROPOROUS RESIN USING OPTIMIZED CONDITIONS

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.

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 (Tanaka and others 2008). 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 (Schab and Trinh 2004). Since the safety of artificial food pigments has been questioned, there has been a growing need for edible natural pigments like anthocyanin extracts (Liu and others 2004). Moreover, food and medicine containing anthocyanins may provide benefits like antioxidant activity (Acquaviva and others 2003), anticancer activity (Faria and others 2010), and diabetes prevention (Wedick and others 2012). 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 (Choung and others 2003). 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 (Pierce 1994) and slow photosynthesis in aquatic plants (Slokar and Majcen 1998). 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 identifed by Feenstra (1960), 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 (Choung and others 2003), and delphindin 3′-glucoside in the Mexican cultivar (Aparicio-Fernadez and others 2005). Delphinidin 3′-glucoside, petunidin 3′-glucoside, and malvidin 3′-glucoside were the major anthocyanins in American commercial class black beans (Wu and Prior 2005a). Although there is quite a bit of information for different anthocyanins in black beans, research 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; Wu and Prior 2005b). 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 (Chapter 3).

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.

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 (Lopez 1987). 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), 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 (ϕ1.0 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 4.1). 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 was passed through.

Table 4.1 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		2.5
3	35	1.5
4		2.5

The adsorption ratio and amount adsorbed were described as follow:

Adsorption Ratio:

$$AR(\%)=(C_i-C_e)/C_e*100\% \quad (1)$$

Amount adsorbed:

$$Q_t=(C_i-C_t)V_i/m \quad (2)$$

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 4.2) was used to determine the relationship between the response function (anthocyanin yield) and process variables in the desorption process. The 3 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 (Liu and others 2004). 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.

Table 4.2 Experimental values of the independent variables used for the 2^3 full-factorial central composite design for desorption process.

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

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

Desorption ratio:

$$DR\ (\%) = C_d V_d/(C_i - C_e)V_i * 100\% \quad (3)$$

Eluted Percentage:

$$EP\ (\%) = C_r V_r/C_d V_d * 100\% \quad (4)$$

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 (Giusti and Wrolstad 2001) 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:

$$\text{Monomeric anthocyanin pigment (mg/L)} = (A \times MW \times DF \times 1000) / (£ \times 1) \quad (6)$$

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). 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 $p < 0.05$ (See appendix C for detailed statistics).

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 effluent volume under different adsorption conditions (Figure 4.1). 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 4.1) 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.

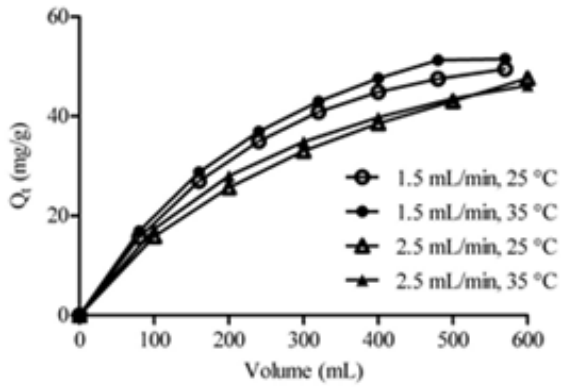


Figure 4.1 Adsorption curves of black bean wastewater under different conditions. The four conditions were:1.5 mL/min, 25°C (○), 1.5 mL/min, 35°C (●), 2.5 mL/min, 25°C (△) and 2.5 mL/min, 35°C (▲).Q_t (mg/g) is the quantity (mg) of anthocyanins on a unit amount (g dry weight) of adsorbent at time t.

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 4.2, 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 effect were estimated. The test of statistical significance showed that only the effect of flow rate was significant (p=0.024; See table C.1 and C.2 in Appendix C for statistical result details), 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. 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; Yang and others 2012).

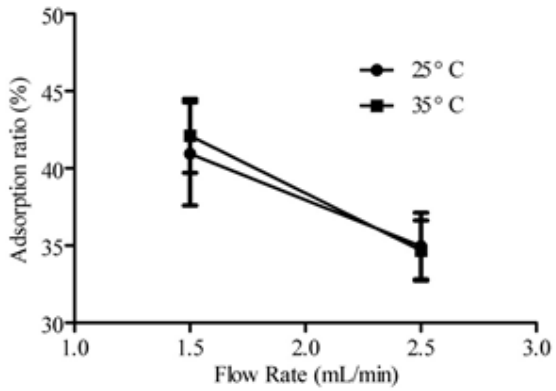


Figure 4.2 Adsorption ratio of the anthocyanins using Sp700 at different flow rate (1.5 mL/min and 2.5 mL/min) with the temperature 25°C (●) and 35°C (■).The curve shows the mean of three replicated tests and the error bars show standard deviations.

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 (Kammerer and others 2005) and the adsorption capability of molinate from molinate water solution (Silva and others 2004) 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 (Liu and others 2007). Apparently this temperature range did not significantly increase the relative mobility of the anthocyanins in the adsorption system.

Desorption with different temperature

The effect of temperature on the desorption process was investigated in this study (Figure 4.3). 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.

Desorption with different acidified ethanol concentrations

The concentration of ethanol in the desorption solvent may affect anthocyanin desorption. As shown in Table 4.3, 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.

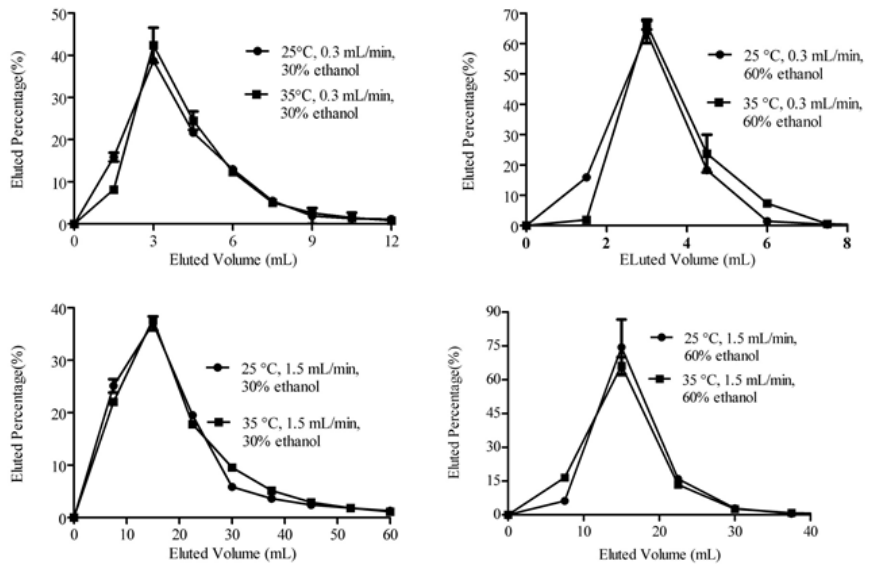


Figure 4.3 Comparison of desorption parameters using different temperatures: 25°C (●) and 35°C (■).The curve shows the mean of three replicated tests and the error bars show standard deviations.

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 (Liu and others 2004). Therefore, in order to simplify the concentration process when a higher anthocyanin yield is desired, a higher ethanol concentration in the eluent is preferred.

Desorption with different flow rate

Flow rate is another factor that may affect the desorption 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 4.3). 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, 0.3mL/min was the most efficient flow rate examined in this study.

Table 4.3 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.

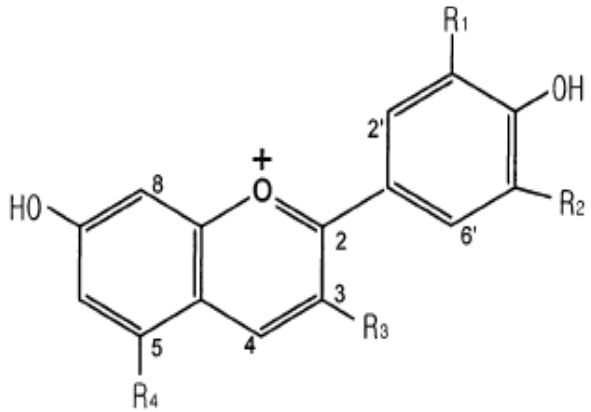
Independent variables and desorption ratio in the desorption process

A 3-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 ($p > 0.05$; See Table C.3 and C.4 in Appendix C for statistical result details). 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.

Identification of Anthocyanin

The anthocyanin extraction after resin purification was characterized by HPLC-MS at 520 nm (Figure 4.4). 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 4.4. It was found that petunidin 3, 5-diglucoside (peak 1; Figure 4.4), delphinidin 3-glucoside (peak 2; Figure 4.4), and petunidin 3-glucoside (peak 3; Figure 4.4) were the major anthocyanins in black bean wastewater extract (Choung and others 2003; Wu and Prior 2005a; Akond and others 2011), with a small amount of maldvidin 3-glucoside (peak 4; Figure 4.4).

Table 4.4 Characteristics of the anthocyanins found in black bean studied, related to their retention time (t_R), spectroscopic characteristics (λ_{max}), LC-MS data and chemical structures.



peak	t_R[a] (min)	anthocyanin	λ_{max} (UV)	λ_{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-β-D-glucose	O-β-D-glucose
2	6.0±0.1	Delphinidin 3-glucoside	235	525	465	OCH3	OCH3	O-β-D-glucose	OH
3	6.8±0.1	Petunidin 3-glucoside	232	525	479	OCH3	OH	O-β-D-glucose	OH
4	7.4±0.1	Maldivian 3-glucoside	231	530	493	OCH3	OCH3	O-β-D-glucose	OH

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

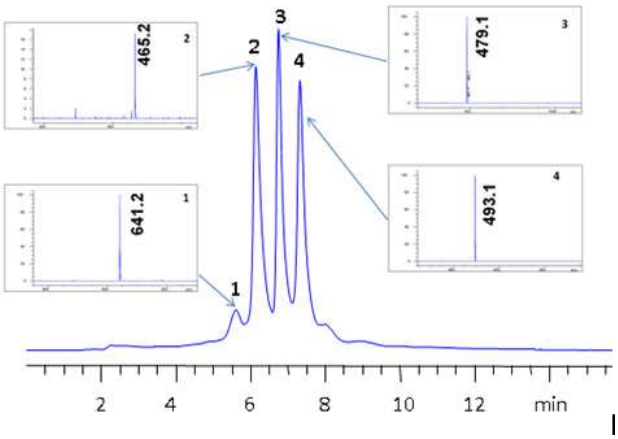


Figure 4. 4 HPLC profile of anthocyanin extraction. Mass spectrogram of compounds 1-4 are shown as HPLC-ESI/MS spectra.

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 4.5 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 4.5 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 520nm from three replicated testes.

Conclusions

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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CHAPTER 5

CONCULSIONS

The purification of anthocyanins from black bean canning wastewater was investigated by column chromatography with five types of macroporous resins (Diaion Hp20, Sepabeads Sp70, Sepabeads Sp207, Sepabeads Sp700 and Sepabeads Sp710). The equilibrium isotherm data were collected and fitted into Langmuir and Freundlich isotherm models. Both of the models appeared to provide a good fit for all the tested resins. Furthermore, dynamic adsorption and desorption were performed. Sp70, Sp207, and Sp710 had similar adsorption capacities during the stationary adsorption and dynamic adsorption. The adsorption and desorption capacity of Hp 20 was much lower than other resins, which indicated that Hp 20 is the least efficient resin of those examined in this study to purify anthocyanins from black bean canning wastewater. Of the five resins, the maximum adsorption and desorption capacities were both observed on Sp700, suggesting Sp700 is the most efficient resin examined in this study for purifying anthocyanins from black bean canning wastewater.

Then adsorption kinetic studies were investigated with Sp700 resin. The results showed that the best absorption condition is at 25°C and a flow rate of 1.5 mL/min in this study, though the desorption studies showed flow rate (0.3 mL/min or 1.5 mL/min), temperature (25°C or 35°C), and acidified ethanol concentration (30% or 60% (v/v)) had no significant effect on the desorption ratio. Temperature did not affect the purification process of anthocyanins, which indicated that tight temperature control is not needed for the purification process, as long as it takes place around room temperature. 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. Lower flow rate, high temperature or high ethanol condition in desorption process may increase the numbers of the impurities. The selected adsorption and desorption conditions may be applied to the industry-scale anthocyanin purification in the future.

APPENDICES

Appendix A Analysis of anthocyanin content

An alternative method that involved pH was used with a UV-Vis spectrophotometer to measure the anthocyanin concentration in the sample solution. Prepare two dilutions of the samples, one with 0.025 M potassium chloride buffer, pH 1.0 and the other with 0.4 M sodium acetate buffer, pH 4.5, diluting each by 30 times. Let these dilutions equilibrate for 15 min. Measure the absorbance of each dilution at 519 nm and at 700 nm, against a blank cell filled with distilled water.

The absorbance of the diluted sample was calculated as follows:

$$A = (A_{519} - A_{700}) \text{ @ pH }_{1.0} - (A_{519} - A_{700}) \text{ @ pH }_{4.5}$$

Table A.1 shows an example of the different absorbances of one sample at different pH values and wave lengths. Then the absorbance of the diluted sample was calculated as:

$$A = (0.304-0.003)-(0.004-0.003) = 0.300$$

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

$$\text{Monomeric anthocyanin pigment (mg/L)} = (A \times MW \times DF \times 1000)/ (£ \times 1)$$

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.

$$\text{Monomeric anthocyanin pigment (mg/L)} = (0.3*449.2*30)/26900 = 0.15 \text{ mg/L}$$

Table A.1 The absorbance at different pH values and different wave length of two diluted samples (example)

	A_{519}	A_{700}
pH $_{4.5}$	0.004	0.003
pH $_{1.0}$	0.304	0.003

Appendix B Detailed Statistics for Chapter III

Table B. 1 One –way ANOVA Model for adsorption performed by 5 different kinds of resins on extracting anthocyanins from black bean canning wastewater

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	1943.464500	485.866125	40.44	<.0001
Error	15	180.195500	12.013033		
Corrected Total	19	2123.660000			

Table B.2 REGWQ grouping for adsorption performed by 5 different kinds of resins on extracting anthocyanins from black bean canning wastewater

Means with the same letter
are not significantly different.

REGWQ Grouping	Mean	N	resin
A	38.620	4	Sp700
B	27.175	4	Sp710
B			
B	26.200	4	Sp207
B			
B	21.850	4	Sp70
C	9.550	4	Hp20

Table B.3 One-way ANOVA Model for desorption performed by 95% ethanol elute anthocyanins from 5 different kinds of resins.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	522.1600000	130.5400000	28.94	<.0001
Error	15	67.6680000	4.5112000		
Corrected Total	19	589.8280000			

Table B.4 REGWQ grouping for desorption performed by 95% ethanol elute anthocyanins from 5 different kinds of resins

Means with the same letter
are not significantly different.

REGWQ Grouping	Mean	N	resin
A	19.280	4	Sp700
B	12.700	4	Sp710
B			
B	12.400	4	Sp207
B			
B	9.900	4	Sp70
C	4.300	4	Hp20

Appendix C Detailed Statistics for Chapter IV

Table C.1 Two-way ANOVA Model for adsorption of anthocyanins performed by different temperature and flow rate with Sp700 resin.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Temperature	1	0.5808000	0.5808000	0.03	0.8657
Flow Rate	1	134.6700000	134.6700000	7.07	0.0289
Temperature*Flow Rate	1	1.5696333	1.5696333	0.08	0.7814

Table C.2 REGWQ grouping for adsorption of anthocyanins performed by different temperature and flow rate with Sp700 resin

Means with the same letter are not significantly different.			
REGWQ Grouping by hand	Mean	N	Number
A	42.10	3	35°C, 1.5mL/min
A	40.94	3	25°C, 1.5 mL/min
B	34.96	3	25°C, 2.5 mL/min
B	34.68	3	35°C, 2.5 mL/min

Table C.3 Two-way ANOVA Model for desorption of anthocyanins performed by different temperature, flow rate and ethanol concentration with Sp700 resin

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Temperature	1	0.55815000	0.55815000	0.01	0.9326
Flow Rate	1	46.87215000	46.87215000	0.62	0.4424
Tempearture*Flow Rate	1	6.12060000	6.12060000	0.08	0.7796
Ethanol	1	2.45760000	2.45760000	0.03	0.8591
Tempearture*Ethanol	1	26.67041667	26.67041667	0.35	0.5607
Flow Rate*Ethanol	1	48.11001667	48.11001667	0.64	0.4366
Temperature*Flow Rate*Ethanol	1	35.62406667	35.62406667	0.47	0.5021

Table C.4 REGWQ grouping for desorption of anthocyanins performed by different temperature, flow rate and ethanol concentration with Sp700 resin

Means with the same letter are not significantly different.

REGWQ Grouping by Hand	Mean	N	Number
A	83.1	3	1.5 mL/min 25°C 60% ethanol
A	83.0	3	1.5 mL/min 35°C 30% ethanol
A	82.7	3	1.5 mL/min 25°C 30% ethanol
A	80.7	3	0.3 mL/min 35°C 60% ethanol
A	79.5	3	0.3 mL/min 25°C 60% ethanol
A	76.9	3	0.3 mL/min 35°C 30% ethanol
A	76.7	3	1.5 mL/min 35°C 60% ethanol
A	76.5	3	0.3 mL/min 25°C 30% ethanol