LAPPEENRANTA UNIVERSITY OF TECHNOLOGY
LUT school of Engineering Science
Chemical and Process Engineering

Master's Thesis

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Recovery and purification of anthocyanins from purple-blue potato

Examiners: Professor Tuomo Sainio
D.Sc. (Tech) Jari Heinonen

ABSTRACT

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The aim of this work was to study techniques to extract and purify of anthocyanins from purple-blue potato. This topic was determined as a master's thesis and it was done in collaboration with the Food Chemistry and Food Development Department of University of Turku and Department of Chemical and Process Engineering at Lappeenranta University of Technology.

At first, purple-blue potatoes were pretreated in four types of boiled, raw, freeze-dried and dried boiled potato for extraction. They were mixed with aqueous acidified ethanol (ethanol:water:acetic acid 40%:53%:7% v/v) for conventional extraction. Boiled potato was selected as a best pretreated potato. Different ethanol concentration and extraction time were examined and the mixture of 80% in 24 h resulted in maximum anthocyanin content (132.23 mg/L). As conventional extraction method of anthocyanins was non-selective, some of impurities such as free sugars might accelerate anthocyanin degradation. Therefore, to obtain anthocyanins in purified form, adsorption as a promising selective method was used to recovery and isolate anthocyanins. It was carried out with six adsorbents. Among those, Amberlite XAD-7HP, a nonionic acrylic ester adsorbent, was found to have the best performance. In an adsorption column, flow rate of 3 mL/min was selected as the loading flow rate among four tested flow rates. Eluent volume and flow rate were 3 BV of aqueous acidified ethanol (75%, v/v) and 1 mL/min for desorption. The quantification of the total anthocyanin contents was performed by pH-differential method using UV-vis spectrophotometer. The resulting anthocyanin solution after purification was almost free from free sugars which were the major cause for degradation of anthocyanins. The average anthocyanin concentration in the purified and concentrated sample was obtained 1752.89 mg/L.

Foreword

First, I would like to thank God for giving me health and strength to conduct this project work. Then, I would like to thank Prof. Tuomo Sainio for allowing me to join his lab group and thank for his advice and support. I would also like to acknowledge D.Sc. Jari Heinonen for his valuable guidance, support and patience during my thesis. Without their comments, this thesis could not have been done.

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Lappeenranta, May 31, 2015

Hengameh Farahmandazad

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List of symbols and abbreviations

A	Absorbance	
q	Adsorption capacity	[mg of anthocyanin/g of adsorbent]
C	Concentration	[mg/l]
D	Desorption ratio	
DF	Dilution factor	
ε	Extinction coefficient	
b	Langmuir isotherm constant	
m	Mass	[g]
MW	Molecular weight	[g/mol]
V	Volume	[ml]

Subscript

a	Adsorbent
d	Desorption
e	Equilibrium
i	Initial
s	Saturation
λ	Wavelength

1. INTRODUCTION

Nowadays, there is a drastic attention to polyphenols due to their positive impacts on health by preventing cardiovascular, inflammatory and neurological diseases (Silva et al., 2007). Anthocyanins as a subsidiary of polyphenols have been under investigations in recent years, and sources of anthocyanin are utilized highly in pharmaceutical, food, and cosmetic industries. They obtained mostly from grape skin (Bleve et al., 2008, Corrales et al., 2009), blueberries (Buran et al., 2014, Wang et al., 2014), black rice (Kang et al., 2013), red cabbage (Chandrasekhar et al., 2012, Coutinho et al., 2004, Xavier et al., 2008), red radish (Patil et al., 2009), purple wheat (Hosseinian et al., 2008), eggplant peel (Todaro et al., 2009), purple corn (Yang and Zhai, 2010), and purple potato (Bridgers et al., 2010, Burgos et al., 2013, Fan et al., 2008a, Fan et al., 2008b, He et al., 2012, Lu et al., 2011, Motilla Elyana et al., 2011, Puertolas et al., 2013, Truong et al., 2010, Truong et al., 2012).

In order to use anthocyanins as the precious compounds, methods of recovery are required. By improvement in science and technology, advanced methods of extraction of valuable bio-compounds have been replacing the old-fashioned ones. New isolation and recovery techniques could effectively increase the purity and efficiency of the production. An increasing number of investigations found in the literatures on the methods of recovery of anthocyanins such as: development of separation techniques (Bleve et al., 2008, Buran et al., 2014, Corrales et al., 2009, Fan et al., 2008a, Galván D'Alessandro et al., 2013, Kang et al., 2013, Liu et al., 2004, Lu et al., 2011, Truong et al., 2012), applications (Giusti and Wrolstad, 2003), anthocyanin stability (Fan et al., 2008b, Lachman et al., 2012, Li et al., 2013, Moldovan et al., 2012), and analytical technique for identification of anthocyanins (Hosseinian et al., 2008, Lee et al., 2008).

The aim of this work was to study the methods for extraction and purification anthocyanins from purple-blue fleshed potato in order to test them in biological tests by the Food Chemistry and Food Development Department of University of Turku. Hence, the purified anthocyanin solution had to produce in a condition which made it to be edible. In this work, the most recent anthocyanin investigations are reviewed related to stability, extraction, purification, and identification methods. Then, it is focused on the potato which was studied in this project. This potato was cultivated in Finland with the name of "Synkeä Sakari". In the experimental part, the extraction was carried out by the conventional extraction technique. Due to the limitation in

using solvent, ethanol was selected as the most acceptable solvent in food industry. Moreover, to make an acidic environment in order to prevent the degradation of anthocyanins and have better extraction, acetic acid was preferred for food use. Different concentrations of aqueous acidified ethanol were tested as the extraction media. Adsorption method was selected for the recovery and isolation of anthocyanins. Six different adsorbents were tested. The concentration of the solution were done by rotary evaporator. The quantification of the total anthocyanin contents was performed by pH-differential method using UV-vis spectrophotometer. The analytical HPLC method showed that the high acylated anthocyanin glucosides are referred to mostly petunidin aglycons.

Another limitation in this study was that, anthocyanin pigment might degrade during the extraction, processing and storage. Therefore, it had influence on the final anthocyanin concentration. Moreover, to avoid the pigment degradation, dealcoholizationa and concentration of the anthocyanin extracts had to be done in the process with the temperature below 35 °C. Using rotary evaporator or membrane processes are the suitable options but they consume more time and in some cases more energy. In addition, information about anthocyanin structure, position, and the degree of acylation and glycosylation of anthocyanin were so important which could be detected by nuclear magnetic resonance (NMR) and analytical HPLC.

2. CHEMICAL AND PHYSICAL PROPERTIES OF ANTHOCYANINS

2.1. Anthocyanins as valuable compounds

Polyphenols are one group of the most favorable organic and natural chemical compounds due to their antioxidant activity and biological significance in plants. These components are known as secondary plant metabolites which means the chemicals produced by plants and divided into several classes according to the number of phenol rings that they contain and to the structural elements that bind these rings to each other. One of the main groups of polyphenols is flavonoids (Figure 1). They consist of two aromatic rings, which are joined together by a heterocyclic ring. Substitution patterns of heterocyclic ring are varied which cause to have different classes of flavonoids. Major flavonoids classes are anthocyanidins, flavones, isoflavones, flavanones, flavonols and flavanols. Anthocyanidins are the basic structure of anthocyanins. (Ignat et al., 2011)

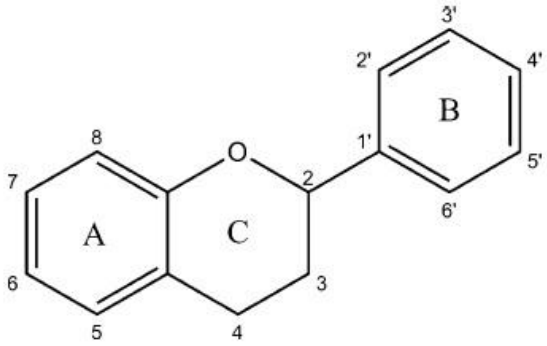

Figure 1. Basic structure of flavonoids (Ignat et al., 2011).

The terms anthocyanins originates from the Greek words "antho" meaning flower and "cyan" meaning blue. They are natural water soluble pigments which appear as red, blue and purple color in flowers, fruits and vegetables (Valls et al., 2009). Anthocyanins have great potential in food and pharmaceutical industries because they act as antioxidants by donating hydrogen to highly reactive radicals (Lapornik et al., 2005). They can increase the protection of the body against diseases such as cancer and they are in the class of antitumor compounds in medical science. The mechanism for enhancing the body protection by anthocyanins lies in the fact that they scavenge free radicals in the body and contribute to reduce the oxidative stress (Silva et al., 2007). They possess antimicrobial, antiviral and anti-inflammatory properties (Burgos et al.,

2013, Fan et al., 2008a). They can reduce risks of chronic diseases such as cancer, cardiovascular diseases, virus inhibition and Alzheimer's disease (Andersen and Markham, 2005). Another advantage of anthocyanins is anti-diabetic property. Eye function of anthocyanins can improve the human night vision, which was investigated by Ghosh and Konishi (2007).

Anthocyanins are also utilized in beverages as natural colors and in food industry as food colorants. Because anthocanins have high color power and they are natural colors, they have applications in cosmetic and paints industries (Andersen and Markham, 2005, Castañeda-Ovando et al., 2009). Moreover, they are practical natural colorants in coating products and as potential natural photosensitizers in solar cells (Anuar et al., 2013). Some natural sources of anthocyanins are mentioned in Table 1. Anthocyanin compositions differ from one source to another source and anthocyanin concentration can be changed in each cultivar batch due to cultivar condition or in different part of plants such as root, peel or leaves.

Table 1. Typical sources of anthocyanins.

Food Type	Food Items
Fresh Fruits	Strawberries, red onions, red grape, redcurrant, blue berries, black berries, raspberries, mangoes, plums, peaches, pomegranates, bananas
Beverages	Tea, coffee, herbal drinking, beer, wine
Fresh Vegetables	Purple potato, blue potato, red potato, broccoli, carrots, tomato, spinach, red cabbage, shallot, eggplant

2.2. Structure of anthocyanins

The basic structure of anthocyanins is anthocyanidins which consists of two aromatic hydrocarbon rings that are bound together by a heterocyclic ring containing oxygen (Figure 2). When the anthocyanidins are bound to a sugar moiety, they are known as anthocyanins. (Castañeda-Ovando et al., 2009) These sugars are monosaccharides such as glucose, galactose, rhamnose, fructose, and arabinose, or combination of three or four monosaccharides (Ignat et al., 2011).

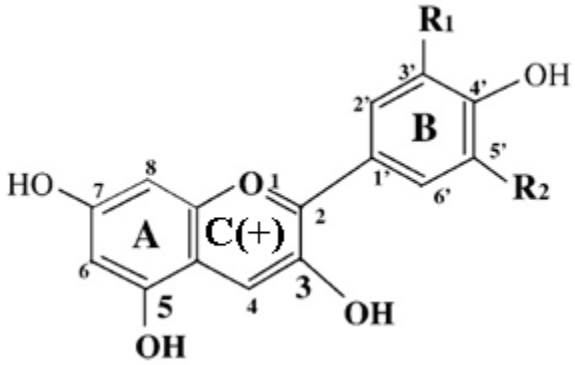

Figure 2. General anthocyanin structure. [A] aromatic hydrocarbon, [B] aromatic hydrocarbon, [C] heterocyclic ring (Giusti and Wrolstad, 2003).

Six anthocyanins found frequently in plants are presented in Table 2. They are different in the structure according to their aglycons, i.e., the chemical groups which are bound to the aromatic ring [B] of the anthocyanin and can have influence on the color of plants. If the sugars are removed from the anthocyanins by acid hydrolysis, the remaining molecule is an aglycone. (W. Pitakdantham, 2011)

Table 2. Six anthocyanins more commonly found in nature (Giusti and Wrolstad, 2003).

Aglycone	R_1	R_2	λ_{max} (nm) visible/color
Pelargonidin	H	H	494 nm/orange
Cyanidin	OH	H	506 nm/orange-red
Peonidin	OCH_3	H	506 nm/orange-red
Delphinidin	OH	OH	508 nm/red
Petunidin	OCH_3	OH	508 nm/red
Malvidin	OCH_3	OCH_3	510 nm/bluish-red

2.3. Stability of anthocyanins

There are several factors affecting on the stability of anthocyanins. These factors can affect chemically or physically the stability of anthocyanin by structural modifications with hydroxyl, methoxyl, glycosyl and acyl groups or by environmental factors such as pH, solvent or condition of pretreatment processing (Fan et al., 2008b). The isolated anthocyanins are highly instable and very susceptible to degradation. As it is shown in Figure 2, ring B substituent can have impact on the anthocyanin structure. Hence, anthocyanin stability is influenced by ring B substituent.

2.3.1. Acylation and glycosylation

The most significant parameters affecting the stability of anthocyanins are acylation and glycosylation.

Glycosylation means that the hydroxyl groups of the anthocyanin aglycone may be linked to sugar moieties such as glucose, rhamnose, xylose, galactose, arabinose and fructose through glycosidic bonds (Giusti and Wrolstad, 2003). Intramolecular H-bonding within the sugar moiety and between sugar moiety and anthocyanin molecule increase the molecular stability and therefore it prevents the high degradation rate of anthocyanin (Borkowski et al., 2005).

Acylation means that acyl groups are attached to glycosylated aglycones through ester bonds. Anthocyanin may be acylated with two types of acids, cinnamic, and aliphatic. Cinnamic acids contain an aromatic ring and include p-coumaric, caffeic, ferulic, gallic, and sinapic acids. Aliphatic acids contain straight chains, branched chains, or nonaromatic rings and include malonic, acetic, malic, succinic, and oxalic acids (Ignat et al., 2011). Acylated anthocyanins have been found to exhibit increased stability in food matrices as compared to non-acylated anthocyanins. Hence, the acylated anthocyanin extracts may be able to impart a higher level of chemoprevention than the non-acylated anthocyanin extracts, i.e., acylated anthocyanins can prevent or slow the development of cancer better than non-acylated anthocyanins. Therefore, they may be used widely in food and pharmaceutical industries. Most of fruits and berries contain very little or no acylated anthocyanins, while in potato almost all anthocyanins are acylated. Acylation makes the anthocyanins more favorable to use which means that more stable and have possible positive health effects.

As it is illustrated in Figure 3, in diacylated anthocyanins, anthocyanins are maintained between acylating groups and sugars by a sandwich type stacking although in monoacylated anthocyanin, only one side of anthocyanin can be protected by sugar and acylating group. Therefore, it can be attacked by other compounds and weak intramolecular effect might occur. The isolated anthocyanins are without any protection sides and other compounds can have direct effect on them and make them completely instable. (Giusti and Wrolstad, 2003)

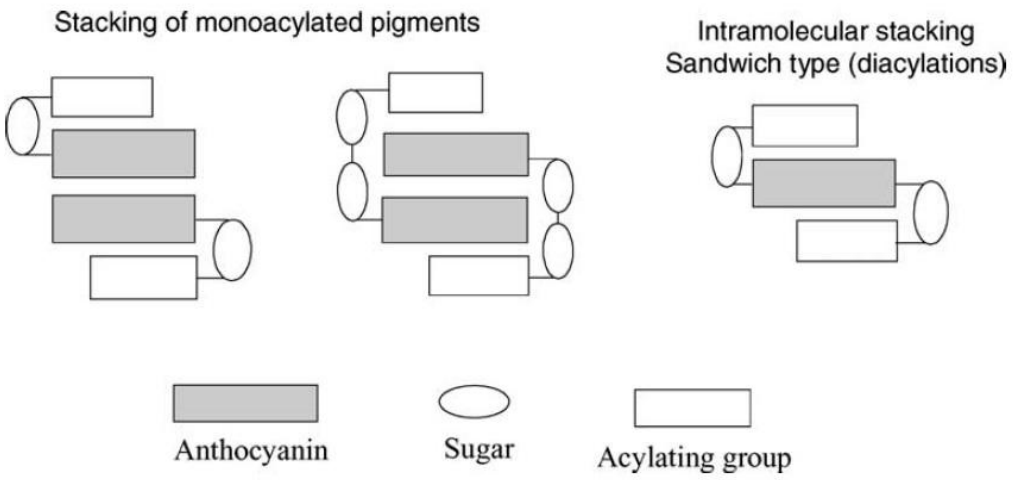

Figure 3. Stabilization mechanisms of acylated anthocyanins (Giusti and Wrolstad, 2003).

2.3.2. Influence of pH

pH has a significant impact on the anthocyanin molecules. This is seen as the change of color caused by changing chemical forms of anthocyanins. As shown in Figure 4, at pHs below 2, anthocyanins exist basically in cationic form (purple and red flavylium cation) and carrying oxygen by the positive charge. By increasing pH up to 4, the quinoidal blue species are predominant. A rapid proton transfer reaction occurs at oxygen and skeleton hydroxyl groups to make quinonoidal bases. Colorless species; a carbinol pseudobase and a yellowish chalcone exist at pH values between 5 and 6. The reason is due to the hydration reaction which occurs at C-2 and generate the colorless carbinol pseudo-base and further the light yellow chalcones. (Castañeda-Ovando et al., 2009, Kähkönen and Heinonen, 2003)

The degradation of anthocyanins occurs at basic condition. Therefore, to prevent the degradation of anthocyanins, by addition of a small amount of acids, we can have lower degradation rate of non-acylated anthocyanins. On the other hand, by high amount of acids, the hydrolysis reaction can occur and the acylated anthocyanins might be degraded. Therefore, sufficient amounts of acids to adjust pH around 3 provide a favorable condition for the formation of flavylium ions and the stabilization of anthocyanins (Li et al., 2013). Different acids such as HCl, ascorbic acid, citric acid and acetic acid are used for anthocyanin extraction to maintain pH in low acidic environment (Bridgers et al., 2010, Buran et al., 2014, Chandrasekhar et al., 2012, Fan et al., 2008a, Hillebrand et al., 2009, Kang et al., 2013, Puertolas et al., 2013, Truong et al., 2012).

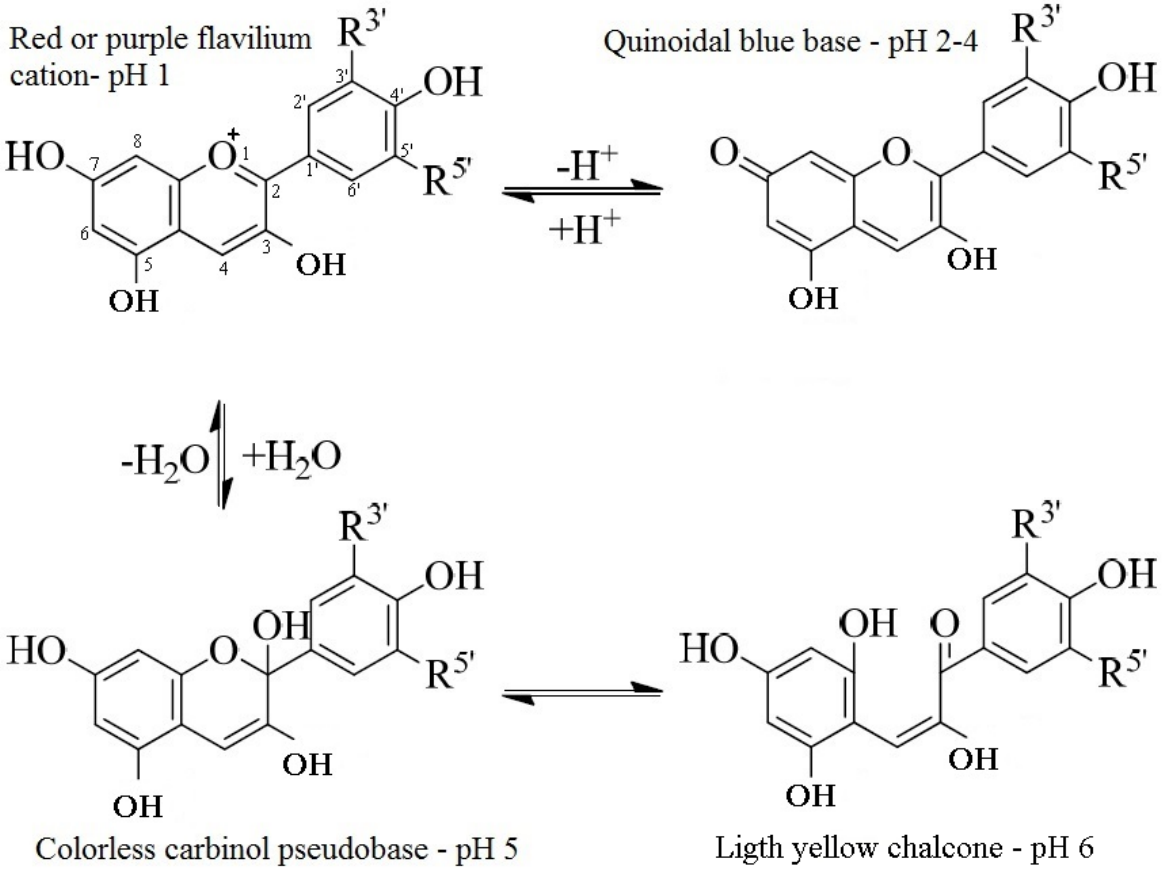

Figure 4. Effect of pH on the anthocyanin structure (Moldovan et al., 2012).

2.3.3. Solvent

Selection of suitable solvent is important for the extraction of anthocyanins. Suitable solvent is able to extract maximum amount of anthocyanins with minimum anthocyanin degradation rate and minimum solvent consumption. Also removal of the solvent after extraction and concentration of the extract should be considered. The easiest and economical way is better to use for removal of solvent from the extract. Moreover, some solvents are classified as hazardous ones for the food and pharmaceutical industries and, thus, cannot be utilized.

As anthocyanins are polar molecules, their solubility is high in the most common solvents such as aqueous methanol, ethanol, or acetone. Solvents with very high polarity such as water or little polarity such as hexane cannot extract anthocyanins efficiently. So an aqueous solvent can

improve the extraction efficiency. However, the number and linkage position of the sugar moieties can have impact on the solubility.

Aqueous acidified methanol and ethanol have been most commonly used in the extraction of anthocyanins (Bridgers et al., 2010, Chandrasekhar et al., 2012, Fan et al., 2008a, Kang et al., 2013, Puertolas et al., 2013, Truong et al., 2012). Methanol is not preferred for food use as it is toxic. In some cases, sulphur dioxide is used but it also causes health risks even at low concentrations. Compared to all these extracting media, ethanol is the most acceptable one for use in food industry (Bridgers et al., 2010, Burgos et al., 2013, Chandrasekhar et al., 2012, Kang et al., 2013, Lu et al., 2011, Patil et al., 2009, Truong et al., 2010, Truong et al., 2012).

2.4. Purple blue potato anthocyanins

The potatoes are different according to their cultivars and colors. Potatoes are found in various colors such as yellow, orange, red, blue, and purple. As it was discussed before, anthocyanins are responsible for the color in plants and vegetables. In comparison with other vegetables and plants, anthocyanins in purple potato have high stability due to acylated form. Therefore, extraction of anthocyanins from these potatoes has received more attention in recent years (Lu et al., 2011). The flesh or skin of potatoes can be entirely or partially pigmented.

The groups of anthocyanins in potato can be classified based on the peonidin/cyanidin ratio. First group is blue dominant group (cyanidin group) with a greater degree of blueness (peo/cya<1) and the second one is red dominant group (peonidin group) with a greater degree of redness (peo/cya>1) (Motilla Elyana et al., 2011).

3. ANALYSIS METHODS

pH-differential method and HPLC analysis are two common methods to quantify anthocyanins. According to the results studied by Hosseinian et al. (2008) and Lee et al. (2008), the measurement of total anthocyanin contents using HPLC and pH differential methods showed similar results about the total anthocyanin content and confirmed the precision of the methods. However, in pH-differential method, only the total anthocyanin content is obtained while in HPLC, the information of individual anthocyanins can also be obtained (Hosseinian et al., 2008, Lee et al., 2008).

3.1. pH-differential method

The first technique is pH-differential method which determines total monomeric anthocyanin content based on the structural changes of chemical forms of anthocyanins as a function of pH. At pH = 1, the colored oxonium form (red to purple) exists, while at pH = 4.5, the colorless hemiketal form is the major structural form. There is no absorbance in pH 4.5 buffer for monomeric anthocyanin and just polymeric and degraded anthocyanins will absorb at this pH. The difference in absorbance of the anthocyanin solutions between these two pH values permits an accurate and rapid determination of total monomeric anthocyanin content in the sample matrix, which is determined by UV- vis spectrophotometer. (Hosseinian et al., 2008)

The same amount of anthocyanin solution is mixed with 0.025 M potassium chloride buffer (pH 1.0) and 0.4 M sodium acetate buffer (pH 4.5), respectively and are equilibrated within 15-50 min. To determine the appropriate dilution factor, the solution is first diluted with pH 1.0 buffer until the absorbance at wavelength between 520 and 530 nm is within the linear range of the spectrophotometer. It is recommended to be between 0.2 and 1.4. This wavelength followed by 700 nm are two wavelengths at which the absorbances of these two diluted samples are measured. The final volume of the sample is divided by the initial volume to obtain the dilution factor. Due to correct for haze, the absorbance is measured also at 700 nm. The solution diluted by 0.4 M sodium acetate may become turbid so it is recommended to use millipore membrane filter with pore size of less than 1.2 μm which cannot absorb the anthocyanins. The following equations Eq. (1) and Eq. (2) are employed to calculate the total anthocyanin content as cyaniding-3-glucoside equivalents. (Lee et al., 2005)

$$anthocyanin\ concentration\ (mg/L) = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times L} \quad (1)$$

$$A = (A_{\lambda_{vis-max}} - A_{700})_{pH\ 1.0} - (A_{\lambda_{vis-max}} - A_{700})_{pH\ 4.5} \quad (2)$$

Molecular weight of anthocyanins (*MW*) and their extinction coefficient ($\mathcal{E}$) are considered as molecular weight and extinction coefficient of cyaniding-3-glucoside, because it is the most common anthocyanin in nature and they are valued 449.2 g/mol and 26900 L/cm mol. 10^3 is factor for conversion from g to mg. Quartz cuvette of 1 cm path (*L*) length is used for measurements. Absorbance (*A*) is obtained by UV-vis spectrophotometer and dilution factor (*DF*) are obtained as discussed before. (Lee et al., 2005)

3.2. Analytical HPLC

Another precise and reliable technique for analysis of anthocyanin is HPLC method. In this method, all the individual anthocyanins can be determined (Lapornik et al., 2005). In some cases, knowing the total monomeric anthocyanins cannot give us enough knowledge so pH-differential method cannot be sufficient for analysis. Therefore analytical HPLC coupled with photodiode array detection has been used for identification and quantification all individual anthocyanins. These information help to recognize the behavior of anthocyanins and can be followed to understand more and evaluate precisely.

Individual anthocyanins can be separated by their polarity and eluted at different times. Hence, external standards are needed (Lee et al., 2008). Due to a few commercially available anthocyanins, HPLC quantitation of anthocyanin is challenging. There are around 250 anthocyanins with the base of one of six aglycones glycosylated but they are various in sugar substitutes (Zhang et al., 2004).

Various factors such as selection of mobile phase and column type affect HPLC analyses of anthocyanins. The general procedure of the eluent selection for the mobile phase in the HPLC analyses of anthocyanins is that when anthocyanins and other phenolics are bound to the selected suitable column for analyses, other free sugars and polar compounds can be removed by acidified water. Hence, the strong acid is recommended to use to make the acidic condition in the pH range of 2-4. Moreover, the chemical structures and the colors of anthocyanins vary

according to pH. Hexane can be used to remove the residual water. The non-anthocyanin phenolics are eluted with diethyl ether followed by ethyl acetate. In some cases, after removal of non-anthocyanin phenolics with ethyl acetate, a mixture of water and acetonitrile is used to remove the more polar non-acylated pigments. The anthocyanin phenolic fraction is eluted with acidified methanol followed by acidified HPLC grade water. The appropriate column for the HPLC analyses has an excellent stability even under strongly acidic condition. Moreover, the selectivity separation of the column is one of the major factor because anthocyanins are slightly different in structure and hydrophobicity. Among the columns, a normal phase C18 or reversed phase (RP-C18) are the suitable columns for HPLC analyses of anthocyanins. (Khoddami et al., 2013)

Buran et al. (2014) measured anthocyanins of blueberries with an Agilent 1200 HPLC, A Zorbax SB-C18 column with binary mobile phase consisted of formic acid: water (5:95 v/v) and methanol with the detection wavelength of 520 nm for anthocyanin. Full scan mass spectra of anthocyanin can be recorded from m/z 350 to 650. In Liu et al. (2013) study, chromatographic separation has been performed with this column for purple sweet potato extract using a mixture of trifluoroacetic acid (0.1%) in water and acetonitrile. For quantification of mulberry anthocyanin, an Agilent 1200 HPLC with a Hypersil ODS column and solvents of (formic acid (3%) in water and methanol has been utilized (Wu et al., 2011). Blueberry anthocyanin chromatogram has been obtained by an Agilent 1100 HPLC system using a reversed phase Supelcosil-LC-18 column with mobile phase of formic anid (5%) in water and methanol (Grace et al., 2009). Anthocyanin analyses from grape pomace extract have been carried out with an Aqua C18 column(Kammerer et al., 2005).

4. EXTRACTION METHODS FOR ANTHOCYANIN EXTRACTION

In this chapter, different methods for extraction of anthocyanins are reviewed. Their advantages and disadvantages are mentioned briefly.

4.1. Conventional extraction

In conventional extraction, extractant solvent is used without any accelerator and energy sources such as the methods which are presented below. In this method, after choosing suitable solvent according to the nature of target molecule for extraction, extraction media is mixed with the solid materials to prepare the extract solution containing the target molecule. After passing the extraction time, solid-liquid separation techniques such as filtration and centrifugation are required to collect the supernatant. Solvent, extraction time, solid-liquid ratio, mixing time and extraction temperature are the factors which have great impacts on the extraction yield.

There are several studies under extraction of anthocyanins by solvent extraction. (Table 3). Extraction of anthocyanin from eggplant peel with acidified alcoholic solvent (ethanol:water:HCl;70:30:1, v/v/v) appeared more efficient than those without ethanol (Todaro et al., 2009). 60% ethanol (adjusted to pH 3) showed the optimum solvent for the extraction of anthocyanins from purple sweet potato (Lu et al., 2011).75% methanol and 80% methanol in acidified water were used for the extraction of anthocyanins from purple-fleshed sweet potato (Truong et al., 2010, Truong et al., 2012). 80% and 60% methanol were used efficiently for raw and boiled potato for anthocyanin extraction, respectively (Burgos et al., 2013). Maximum anthocyanin concentration from red cabbage was obtained as 387.6 mg/L and 372.6 mg/L for red radish at 50% acidified ethanol by comparing to other ethanol concentration (Chandrasekhar et al., 2012, Patil et al., 2009). As shown in Figure 5, the highest extraction of anthocyanins was 186.1mg/100 g fresh weight which was observed when 70% acidified methanol was used (Bridgers et al., 2010).

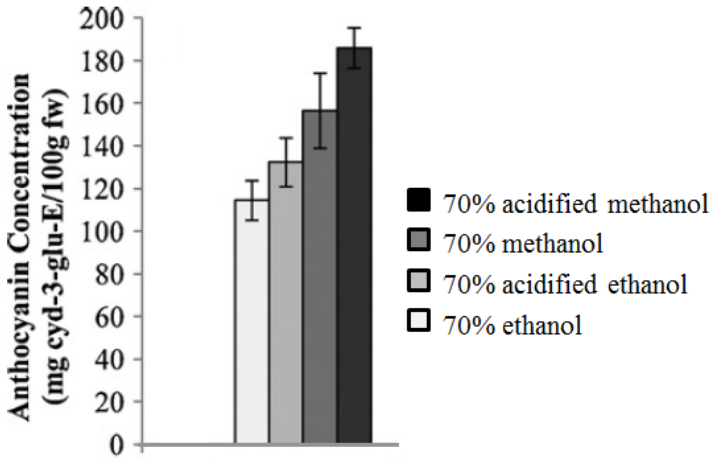

Figure 5. The anthocyanin concentrations of industrial purple-fleshed sweet potatoes obtained using different solvents (Bridgers et al., 2010).

The anthocyanin extraction from black rice, 70% methanol obtained higher anthocyanin concentration than 70% ethanol and it was more effective than water and 70% acetone (Figure 6). However ethanol has been used in the extraction of anthocyanins from black rice because of low toxicity application in food industries compared to methanol. (Kang et al., 2013)

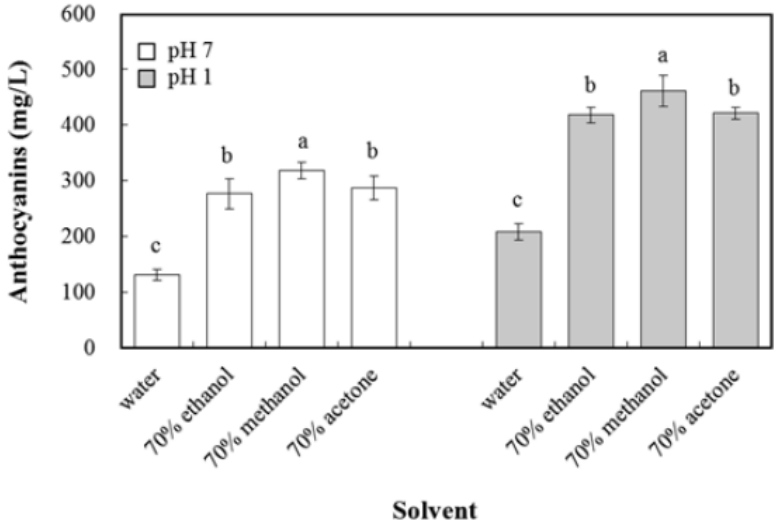

Figure 6. The anthocyanin concentrations of crude black rice obtained using different solvents at different pH values (Kang et al., 2013).

Table 3. Summary of experimental conditions for anthocyanin extraction.

Anthocyanin source	Solvent	Experimental condition	References
Eggplant peel	ethanol:water:HCl; 70:30:1 v/v/v	Solid-liquid ratio 1:2-1:80 (w/v) Extraction temperature:10-60 °C Extraction time: 0-60 min	(Todaro et al., 2009)
Purple sweet potato	60% ethanol (pH 3.0 with HCl)	Sliced and dried potato in 45 °C Solid-liquid 1:8 (w/v) Room temperature Extraction time: 24 h	(Lu et al., 2011)
Red cabbage leaves	ethanol:water:HCl; 50:49:1 v/v/v	Raw red cabbage leaves Solid-liquid ratio 1:2 (w/v)	(Chandrasekhar et al., 2012)
Red radish peel	ethanol:water:HCl; 50:49:1 v/v/v	Solid-liquid ratio 1:2 (w/v)	(Patil et al., 2009)
Industrial purple-fleshed sweet potato	Aqueous acidified methanol 70% (using acetic acid)	extraction temperature: 25,50, 80 °C solid loading: 3.3%, 17% w/v	(Bridgers et al., 2010)
Black rice	Aqueous acidified ethanol 70% (v/v) (pH 1 and 7 with HCl and NaOH)	Black rice flour Solid-liquid ratio 1:10 (w/v) Extraction temperature:25 °C Extraction time: 2 h	(Kang et al., 2013)
Red cabbage	Aqueous acidified solution (using acetic acid 10% v/v)	Extraction time: 24 h Solid-liquid ratio 1:4 (w/v)	(Xavier et al., 2008)

Different acids such as HCl, ascorbic acid, citric acid and acetic acid are used for anthocyanin extraction to maintain pH in low acidic environment (Bridgers et al., 2010, Buran et al., 2014, Chandrasekhar et al., 2012, Fan et al., 2008a, Hillebrand et al., 2009, Kang et al., 2013, Puertolas

et al., 2013, Truong et al., 2012). The effect of HCl concentration on the anthocyanin extraction from red cabbage showed that the degree of extraction of anthocyanins was higher in the case of acidified water when compared to water. Moreover, by increase in HCl concentration, the extraction of anthoycnains would be increased. However the high acid concentration is not preferable for food applications (Chandrasekhar et al., 2012, Patil et al., 2009). In the study on the acetic acid concentration, the highest yields of total anthocyanin were obtained at 5–10% acetic acid by Truong et al. (2012) and Truong et al. (2010) from purple-fleshed sweet potato and 7% of acetic acid was usually employed (Andersen and Markham, 2005). In the study on the extraction of anthocyanins from red cabbage by Xavier et al. (2008), the influence of acetic acid on anthocyanin concentration was shown that there was a non-linear relationship between the increase of extracted mass and acid concentration in which by slight increase of acetic acid, anthocyanin concentration was enhanced significantly.

Other factors such as cultivar, storage condition and type of pretreatment of the anthocyanin sources for extraction can have impact on the content of total anthocyanins. Moreover, free sugars can have influence on the anthocyanin extract. In the study of the cooking effect on the extracted anthocyanin content from five different cultivars of colored flesh potato by Lachman et al. (2012), cooked potato showed higher anthocyanin content in comparison with fresh uncooked tubers (Figure 7).

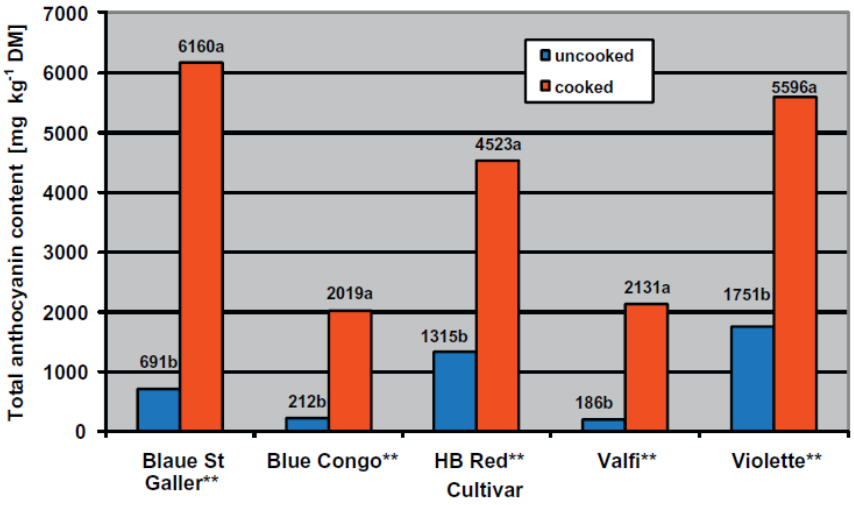

Figure 7. Comparison of cooked and uncooked different potato cultivars on the anthocyanin content (Lachman et al., 2012).

Moreover, influences of different cooking treatments on the total anthocyanin content were studied for colored flesh potato (Lachman et al., 2012). Cooking with steam, boiling in hot water, roasting in oven and using microwave energy were all the examined treatments by Lachman et al. (2012). According to Figure 8, boiling in water could enable the extraction of the highest amount of anthocyanins.

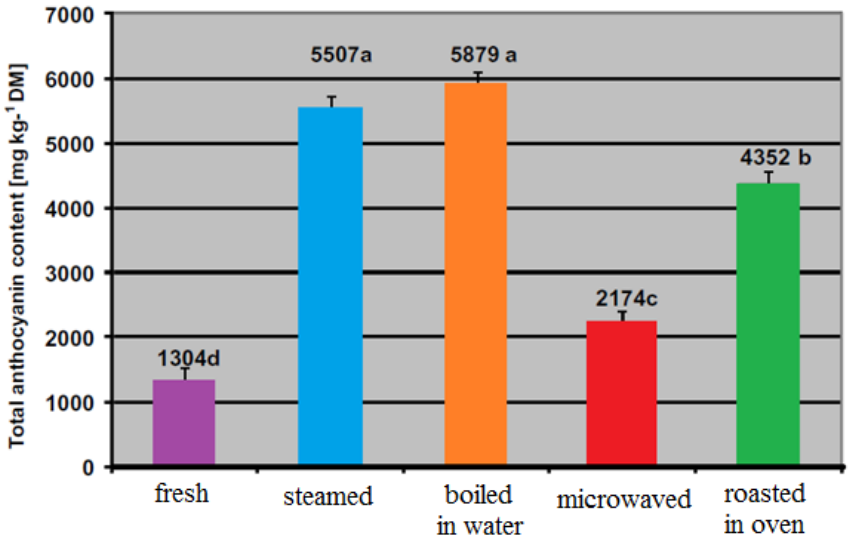

Figure 8. Effect of different cooking treatment on anthocyanin content (Lachman et al., 2012).

The impact of using heat and SO_2 during the extraction were examined on the anthocyanin content of blueberry during juice production by Lee et al. (2002). The blueberry juice processing were done in three different types: using heat, using SO_2, without any treatment. The results showed that the loss of anthocyanins were lower in case of using heat and SO_2 pretreatment. These enhancing methods can be considered by food industries which can maintain the nutritional value and intense color of anthocyanins from fruits during juice processing. (Lee et al., 2002)

Free sugars in the anthocyanin extract can cause the milliard reaction and form brown compounds in the extract. It can accelerate anthocyanin degradation during storage and shorten shelf life. It can be considered as the impurity to degrade and make worse the quality of anthocyanins. Therefore, removal of free sugars from anthocyanin extracts is significant. (Chandrasekhar et al., 2012, Jampani et al., 2014, Wu et al., 2011)

4.2. Supercritical fluid extraction

Supercritical fluid extraction is introduced as an environmentally beneficial alternative technique to the conventional organic solvent extraction. In this method, by slight change in pressure near the critical point, the solvent changes its properties and can diffuse better into the solid matrix. Lower solvent viscosity, lower solvent surface tension, and low solvent consumption are the advantages of this method. The possible degradation of anthocyanin during extraction can be decreased because of the absence of air and light during the extraction. Moreover, the filtration step can be eliminated after extraction. One of the most used supercritical fluids is supercritical carbon dioxide (CO_2). It has a relatively low critical temperature of 30 °C and a low critical pressure of 7.3 MPa. It is food grade, non-flammable and available with low cost and high purity. High cost for large scale industrial application of this extraction method can cause the limitation in use of this technique. (Andersen and Markham, 2005, Di Khanh, 2015)

In anthocyanin extraction, as anthocyanin is polar compound and CO_2 is relatively nonpolar solvent, therefore, a co-solvent such as ethanol is often added to CO_2. A yield of 85% of anthocyanin from grape skin was obtained using carbon dioxide as the supercritical fluid accompanying the co-solvent aqueous acidified ethanol. (Bleve et al., 2008) Optimization of supercritical fluid extraction was carried out by Ghafoor et al. (2010) and anthocyanin concentration of 1.176 mg/mL from grape peel was obtained at lower temperature by using CO_2 and 6% ethanol as modifier.

4.3. Accelerated solvent extraction

Accelerated solvent extraction is one technique similar to supercritical fluid extraction. It is low solvent consumption technique with low extraction time. However, there are some differences between these two methods. In the accelerated solvent extraction, the solvent does not reach the critical point and the extraction yield can be enhanced just by applying the pressure around 4-20 MPa and by using a temperature of 50-200 °C while keeping the solvent in liquid state at these applied temperature and pressure. Due to high temperature, the mass transfer becomes faster but degradation of anthocyanins might occur. (Silva et al., 2007, Wu et al., 2014)

Anthocyanin extraction from purple-fleshed sweet potato was carried out by Truong et al. (2012) using an accelerated solvent extractor by applying constant pressure of 10 MPa which could extract the highest anthocyanin concentration at temperature between 80-120 °C with aqueous acidified methanol. Total monomeric anthocyanin contents was reported 150 mg/100 g powder of purple-fleshed sweet potato.

In the investigation which was done byCardoso et al. (2013), pressurized liquid extraction could enhance the extraction yield of conventional solvent extraction for anthocyanin extraction. Aqueous acidified ethanol as a solvent at 100 bar and 80 °C obtained the best yield of anthocyanins. Also, they showed that pressurized liquid extraction was more efficient than supercritical CO_2 however addition of 5% (v/v) ethanol as the co-solvent to the CO_2 improved the anthocyanin extraction yield.

4.4. High-hydrostatic pressure extraction

Using high hydrostatic pressure can be classified to an environmentally friendly and energy efficient technique for extraction. It enhances the permeability of the solvent to the plant cell and increases mass transfer rate by the pressure which is generated by a hydraulic pump in combination with a pressure intensifier. (Corrales et al., 2008) Pressure intensity can affect the selective anthocyanin extraction.

In the study of the high hydrostatic pressure on the anthocyanin extraction from grape skins, ethanol was used as a solvent and the highest anthocyanin concentration of monoglucosides can be achieved at 200 MPa. By increasing pressure up to 600 MPa, more extraction of acylglucoside can be obtained (Corrales et al., 2009). Moreover, in the comparison between the techniques for anthocyanin extraction, Corrales et al. (2008) presented that acylated anthocyanin could be extracted better by high hydrostatic pressure whereas the monoglucosides had better extraction rate by pulsed electrical filed which is explained later.

4.5. Ultrasound-assisted extraction

Ultrasound-assisted extraction is rapid, simple, low cost, and efficient technique in which ultrasonic energy is used as the source of heating of solution. The technique can be applied to extract nonvolatile or semi-volatile organic compound. By ultrasonic energy, the cell incites to swell and sonication accelerates the penetration of the solvent to the solid matrices by

mechanical effects. Sound waves can vibrate the solid and liquid and cause the increase the contact surface area of the solid and liquid phase and enhance mass transfer. In some cases, it can be used as the pretreatment of the solid or instead of the conventional shaking and warming step for the better extraction to modify the conventional solvent extraction by decreasing the extraction temperature, reduction or elimination the use of organic solvents. (Andersen and Markham, 2005, Corrales et al., 2008, Di Khanh, 2015) The solid sample and solvent are put in the agitated glass contactor, which equipped by a generator of ultrasonic. The temperature of the extraction equipment is fixed by a circulation of water in external jacket. The solid particles are suspended in the solvent by agitation in the glass. After that the extracts are centrifuged in order to separate the extracts from solid particles.

In Burgos et al. (2013) study, different sonication times were tested during extraction with methanol as the solvent and it was found that 5 min sonication was enough for efficient extraction of anthocyanin form purple-fleshed potato. Complete extraction of anthocyanins from grape skins was also achieved using an ultrasonic bath at a frequency of 35 kHz for 30 min, followed by stirring at a temperature of 70 °C in a water bath for 2.5 h (Corrales et al., 2009). To compare the ultrasound assisted extraction operating at 22 kHz frequency and conventional extraction of anthocyanin from red raspberry, it was shown that the extraction yields were almost the same but the solvent consumption and extraction time were significantly decreased when sonication was applied (Chen et al., 2007). Ultrasound assisted extraction might have effect on the physical structure of the compounds because of the vibration and mechanical action but in the anthocyanin extraction from red raspberry, HPLC analysis indicated that there was not any impact on the anthocyanin composition and anthocyanin degradation(Chen et al., 2007).

4.6. Pulsed electrical field extraction

Pulsed electrical field extraction is a non-thermal technique which can increase the extraction rate by enhancing the mass transfer rate. By this technique, the tissue matrices and texture of the solid become softer by the stress caused by the electrical field and solvents can be diffuse better. (Corrales et al., 2008, Di Khanh, 2015)

The pulsed electrical field effects on the anthocyanin extraction yield from purple-fleshed potato has been investigated by Puertolas et al. (2013). 65.8 mg anthocyanin/100 g fresh weight was

obtained under the condition using pulsed electrical field for pretreatment of potato and using water as the solvent. The results was similar to the yield obtained from untreated potato using 96% ethanol as the solvent. In the extraction of anthocyanin from red grape by-product, the extraction yield was increased by 60% when pulsed electrical field was applied as a pretreatment for the conventional extraction (Wijngaard et al., 2012). Pulsed electrical field extraction enhance the anthocyanin extraction yields from grape by-products up to 10% compared to high hydro statistic pressure and up to 17% when just conventional extraction was used (Corrales et al., 2008).

Pulsed electrical field have also great influence on the extraction of monoglucosides which could enhance it remarkably. Acylatedglucoside anthocyanins seemed to be physically entrapped within the matrix, or form hydrogen bonds with cell wall polysaccharides and were consequently extracted in less proportion (Corrales et al., 2008). Considering the published results, this method can be modifier for the conventional extraction.

4.7. Microwave-assisted extraction

Microwave applicator can be considered as a source of energy to use for the extraction. This method of extraction is based on the heating of the water that exists inside of the plant cells or heating of the used solvent with microwave irradiation. Microwaves penetrate to cell wall and contact with the water inside and heat it. Therefore, the pressure inside of the cell increases up to when the cell wall cannot bear it and large amount of compounds release from the cell. It can decrease the extraction time compared to conventional extraction with less solvent consumption and high yield of extracted products. The used solvent in this technique is recommended to be polar solvent which is suitable for anthocyanin extraction and the particle size of the solid is better to be in the range of 0.01 to 2 mm. Solid sample and solvent are put in the reactor vessel which is equipped with a microwave generator. Temperature, microwave power and time of the extraction process should be controlled during the process. Filtration and centrifugation processes should apply to the extract after completion of the extraction. (Andersen and Markham, 2005, Di Khanh, 2015, Stalikas, 2007, Wijngaard et al., 2012)

Optimization of the microwave assisted anthocyanin extraction from purple corn was carried out by Yang and Zhai (2010): 185.1 mg anthocyanin/100 g was achieved with 95% aqueous

acidified ethanol as a solvent in 19 min and solid-liquid ratio of 1:20 by applying 555 W irradiation power. The same solvent with the ratio of 4:1 (mL/g) could extract 43.42 mg anthocyanin/100 g fresh red raspberries just in 12 min with the microwave power of 366 W(Sun et al., 2006). The extraction time was decreased from 5 h to 5 min by using microwave assisted extraction of anthocyanin from grape skins with 40% aqueous methanol (Liazid et al., 2011).

4.8. Summary

The most common technique to extract anthocyanins employ organic solvents. However methanol influences the yield of anthocyanin extraction, ethanol is preferred because it is the most acceptable solvent for food use with low health risk. It is difficult to place the extraction methods in order because all of them has their own advantages and disadvantages. Conventional extraction is simple and needs relatively simple and cheap apparatus but it requires large volume of organic solvents and in some cases, it consumes long extraction time. Supercritical fluid extraction, accelerated solvent extraction, high hydrostatic pressure extraction, ultrasound-assisted extraction, pulsed electrical field extraction, and microwave-assisted extraction can enhance the yield of anthocyanin extraction by shortening the extraction time and decreasing the solvent consumption but they requires their own apparatus and facilities. Therefore, the availability of the apparatus, solvent, sample complexity, and concentration of anthocyanin in the sample influence on the selection of the extraction method. Generally, ultrasound-assisted extraction is effective method for anthocyanin extraction with no or low anthocyanin degradation. In addition, pulsed electrical field extraction as a non-thermal technique without any temperature impact is recommended to increase the yield of anthocyanin extraction.

5. PURIFICATION METHODS FOR ANTHOCYANINS

Conventional extraction methods of anthocyanins from plant material are nonselective and some impurities are extracted with the desired molecules. Hence, after preparation of the extract, purification becomes one of the most significant stages in the recovery of anthocyanins with high purity. Choosing of the suitable method depends on the scale of the production, anthocyanin concentration in the extract, simplicity and availability of the needed equipment and facilities.

5.1. Adsorption

Adsorption is a selective process for the purification of the molecules from a complex mixture of molecules. As depicted in Figure 9, the adsorption is performed by contacting the solution with the porous solid materials which is named adsorbent. The solute molecules which are separated are called adsorbates. Adsorption is a surface phenomenon with high selectivity of separation and can purify the molecules based on the differences in affinity for the adsorbent. Adsorption strength depends on the molecular weight, polarity, size and shape of adsorbate. In this method, the adsorbates are bound to the adsorbent selectively. Thus, the release of these bound materials is required. This process is known as desorption. Compared to alternative technologies, adsorption is attractive for its relative simplicity of design, ease of operation and scale up, high capacity, ease of regeneration, and low cost. It is too common to use adsorption in biomolecule purification and biomedical analysis.

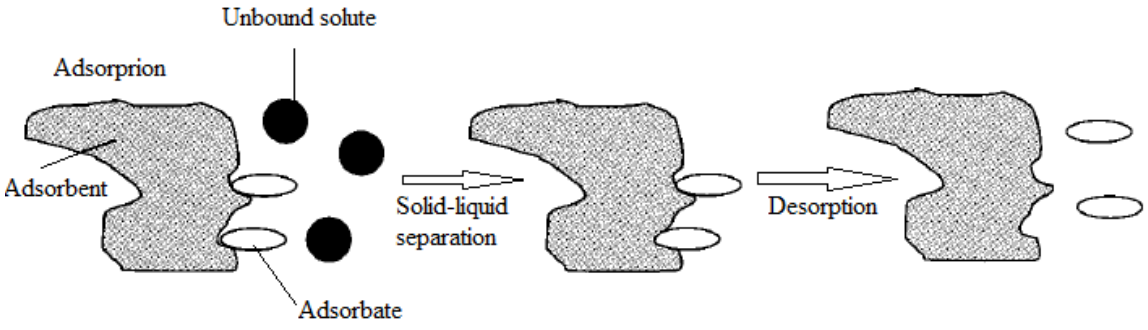

Figure 9. Adsorptive separation (Ghosh, 2006).

The equilibrium relationship between the solute concentration in the liquid phase and the solute on the adsorbent surface gives us the information which is presented by the mass of solute per

unit mass or volume of adsorbent. The following Eq. (3) is quantify the adsorption capacity (q_e) at equilibrium (Ghosh, 2006).

$$q_e = \frac{(C_i - C_e)V_i}{m_a} \quad (3)$$

Where C_i and C_e are the initial and equilibrium concentrations of the solute in the feed solution (mg/L) and V_i (L) and m_a (g) are initial sample volume and mass of adsorbent, respectively.

After reaching adsorption equilibrium, desorption of the adsorbates is done and desorption ratio (D) given by Eq. (4) is used to quantify the amount of solute which is desorbed from the adsorbents (Ghosh, 2006).

$$D = \frac{C_d V_d}{(C_i - C_e)V_i} \quad (4)$$

Where C_d and V_d are concentration of solute in eluted solution and volume of eluent.

5.1.1. Adsorbents

Adsorbents are solid materials which have large specific surface area that provides a place to keep large amount of adsorbed molecules. These are classified to natural and synthetic materials according to their nature. Adsorbents are also divided to different groups according to their types. Six major types of commercial solid adsorbents are activated alumina, silica gel, activated carbon, molecular-sieve carbon, molecular-sieve zeolites, and polymeric adsorbents.

Adsorbents are different in the surface area, particle porosity, and nature of hydrophobicity and hydrophilicity. Polymeric adsorbents are spherical beads with average diameter of 0.5 mm and pore diameter of around 10^{-4} mm. They are produced by polymerizing styrene and divinylbenzene for use in adsorbing nonpolar organics from aqueous solutions, and by polymerizing acrylic esters for adsorbing polar solutes.(Seader et al., 2010)

The molecules are bound to the adsorbents due to one or more of non-covalent interactions such as van der Waals forces, electrostatic interactions, hydrophobic interactions, hydrogen bonding, and partitioning. (Ghosh, 2006, Seader et al., 2010)

If electrostatic interactions occur, the adsorption is named ion-exchange. In this method, cation or anion exchangers can bind positively or negatively charged molecules, respectively. Ion exchangers are prepared by attaching charged groups onto insoluble support materials such as cellulose, acrylic polymers, and cross-linked dextran. The ligands for these support materials are charged groups. For cation exchangers, the ligands are carboxylic acid, carboxymethyl group, and sulphopropyl group. For anion exchangers, the ligands are diethylaminoethyl group, quaternary aminoethyl group, and quaternary ammonium. The elution of the adsorbate is done using a high concentration of salt solution such as sodium chloride. (Ghosh, 2006, Seader et al., 2010, Soto et al., 2011)

In affinity based adsorption, the adsorbates are bound to the adsorbent by hydrogen bonding, hydrophobic interaction, and van der Waals forces. By attaching the affinity ligand to an insoluble material such as cross-linked dextran, cross-linked cellulose, and synthetic resins, affinity adsorbents can be prepared. The separation of the attached molecules from the ligand as desorption part is done by extreme change of pH or high concentration of chaotropic salts, or organic solvents such as ethanol. (Ghosh, 2006, Seader et al., 2010)

Partitioning behavior of adsorbents can be carried out in the reverse phase adsorption which is suitable for the separation of nonpolar and low molecular weight molecules. In this type of adsorption, hydrophobic and low polarity substances such as aliphatic hydrocarbon chains are bound to the solid support material like silica. The target molecules in the solution of polar solvent is contacted with the adsorbent. The nonpolar molecules tend to bind to the hydrocarbon layer and are separated from the solution. For elution of these compound, nonpolar solvents are needed. (Ghosh, 2006)

One of the advantages of adsorption is the feasibility of regeneration of exhausted adsorbents. As it was discussed, physical adsorption which involves non-covalent interactions is reversible.

There are several methods for regeneration of adsorbents, which the common ones are explained as below. In some cases, combination of two or more methods may be used for regeneration.

- Thermal regeneration: In this method, heating is utilized to regenerate the adsorbents. Stream of hot gas or hot liquid can make this thermal processing. The temperature should

be set at which the adsorbed species are desorbed from bed and removed in the fluid stream. Flowing superheated steam, CO_2, and nitrogen are common for using in this technique. This method is suitable for strongly adsorbed species but it consumes long processing time and high energy. (Helfferich, 1985, Soto et al., 2011)

- Pressure swing: the regeneration of the bed is performed by reducing pressure at constant temperature and then purging. It is suitable for weekly adsorbed species and rapid technique. (Helfferich, 1985, Soto et al., 2011)
- Solvent regeneration: solvent regeneration is the most common regeneration method that after elution step, there is still small portion of adsorbates in resins which are not washed through elution step. Adsorbents are washed by high concentration of solvent and in some cases, then rinsed by deionized water to remove solvent. The temperature and pressure are constant and adsorbed species are displaced by a stream of solvent containing a competitively adsorbed species. In this technique, thermal aging of adsorbents are avoided and it is suitable for strongly adsorbed species. (Helfferich, 1985, Soto et al., 2011)

5.1.2. Adsorption isotherms

Understanding the behavior of an adsorption process and adsorbents are important. Adsorption equilibria gives the information about the relationship between the bound and free molecules to the adsorbents and how much those components can be accommodated by a solid adsorbents. If the adsorbate and adsorbent are contacted in sufficient time, an equilibrium will be established between the amount of adsorbate adsorbed and the amount of adsorbate in the solution. The equilibrium relationship is described by adsorption isotherms. Adsorption isotherm is typically defined by a curve relating the equilibrium concentration of a solute on the surface of an adsorbent to the concentration of the solute in the liquid phase. (Do Duong, 1998, Foo and Hameed, 2010)

Various fundamental theories and models predict this behavior. The Langmuir theory presents the most basic theory in adsorption (Do Duong, 1998). In Langmuir isotherms, it is assumed that the saturated and homogeneous monolayer distribution of the adsorbates is formed on the adsorbent surface. Adsorption of solutes by ion exchange and affinity based mechanisms often

follow Langmuir behavior. Langmuir isotherm is given as Eq. (5) as below and the curve in Figure 10 expresses the Langmuir behavior. (Foo and Hameed, 2010, Seader et al., 2010)

$$q_e = q_s \frac{bC_e}{1+bC_e} \quad (5)$$

Where q_e is the solid phase concentration of the adsorbed solute in equilibrium, q_s is saturation capacity, respectively, and b is the Langmuir isotherm constant.

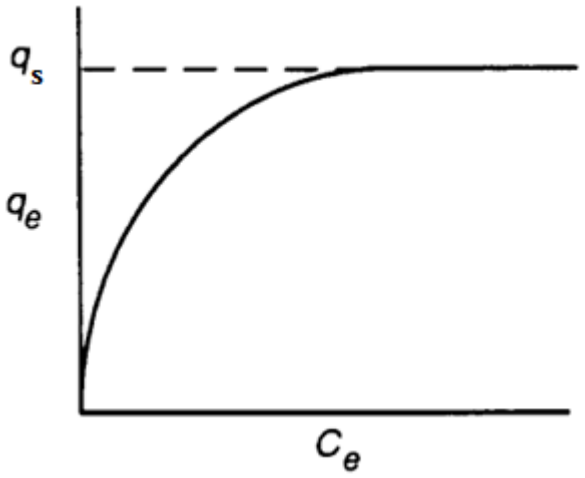

Figure 10. Langmuir isotherm model curve (Seader et al., 2010).

5.1.3. Packed bed adsorption

Adsorption can be done in a column into which the adsorbent is packed. This facilitates the reuse of the adsorbent. In adsorption step, the solution containing the target solutes is pumped through the column. Either the target solute or the impurities are adsorbed selectively to the adsorbent bed. The effluent stream from the packed bed is usually monitored for the target molecules as well as the impurities. After this stage, if the target solute adsorbed to the adsorbents, the impurities are usually washed away the column by a suitable wash liquid in the washing step. In the last step, the target molecule comes out of the column and are desorbed from the adsorbents by a suitable eluent. To have cyclic use of packed bed, it is washed again by wash liquid and then the feed is loaded again. (Ghosh, 2006)

The amount of pumped feed is important to enable the optimized use of the adsorption capacity of the packed bed. If it is pumped more, the target molecule will come out of the column with

the impurities to the effluent. If it is pumped less, the efficient capacity of the packed bed will not be utilized. Therefore, one point between these two points is required to optimize the usage of the capacity of the adsorbent. Breakthrough curve provides a simple way to define the best time as a breakthrough time to have the loading of the feed into the column (Figure 11). If the feed is pumped beyond the breakthrough time, the adsorbents will be saturated and the concentration of the target molecule in the effluent becomes equal to the feed solution (C_F). As a rule of thumb, the typical acceptable concentration of the target molecule in the effluent is 1% to 10% of the concentration in the feed (C''). Flow rate of the feed loading into the column is one of the significant parameters. Lower flow rates help the target molecule to have the time to adsorb to the resins better and increase the diffusivity. However, it increases the loading time which is not desired. Hence, optimization of the flow rate is one of the factor to have an efficient adsorption. It is also applied for the washing and elution steps. (Ghosh, 2006)

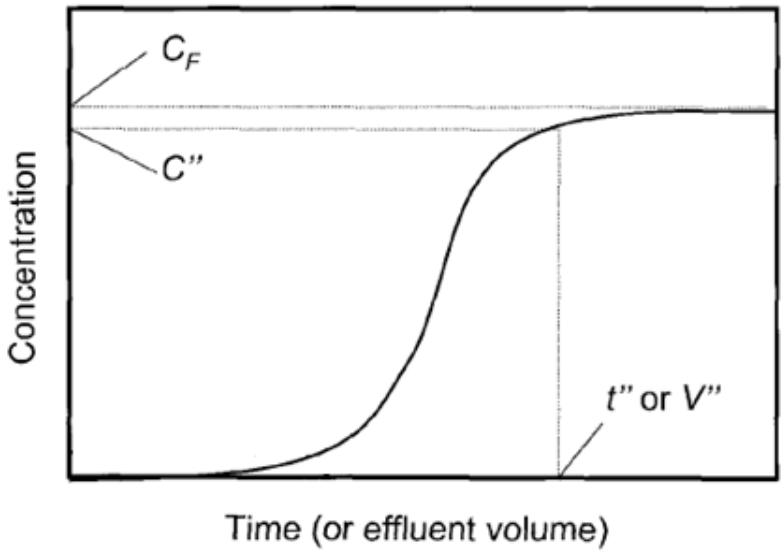

Figure 11. Breakthrough curve (Ghosh, 2006).

5.1.4. Anthocyanin purification by adsorption

Purification of anthocyanins from different sources by adsorption has been investigated quite much (Buran et al., 2014, Chandrasekhar et al., 2012, Jampani et al., 2014, Kammerer et al., 2005, Kang et al., 2013, Liu et al., 2004, Liu et al., 2007, Todaro et al., 2009). In this section, a review of different studies regarding the adsorptive purification of anthocyanins is given.

Chandrasekhar et al. (2012) studied the use of Silica gel, Amberlite IRC 80, Amberlite IR 120, DOWEX 50WX8, Amberlite XAD4 and Amberlite XAD7 for the purification of anthocyanins from red cabbage. Results in Figure 12 showed that Amberlite XAD-7HP as the nonionic acrylic ester adsorbent had the highest adsorption capacity (0.84 mg/mL of resin) and highest desorption ratio (92.85%). Therefore, XAD-7HP was selected as better adsorbents among others to purify anthocyanins. As free sugar was the degradation factor for anthocyanins (Chandrasekhar et al., 2012, Jampani et al., 2014, Wu et al., 2011), the free sugar concentration before and after purification were tested and it was decreased significantly from 224.2 µg/mL to 0.07 µg/mL after adsorption. Also 60% ethanol was used as a best eluent for desorption part. (Chandrasekhar et al., 2012)

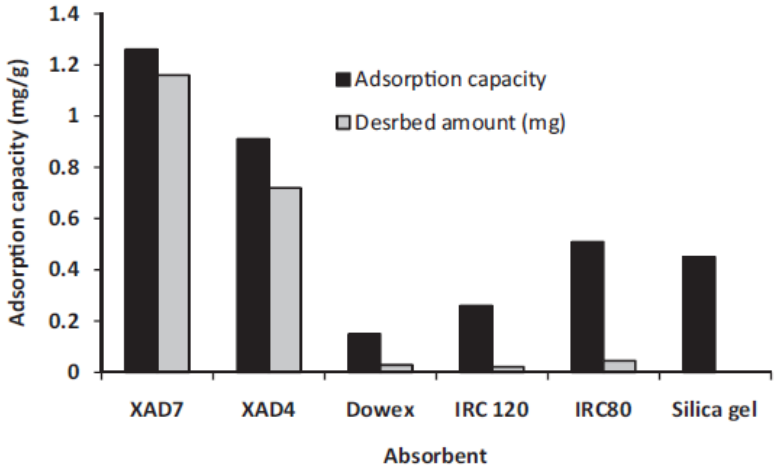

Figure 12. Comparison of different adsorbents for the adsorption of anthocyanins from red cabbage (Chandrasekhar et al., 2012).

The same adsorbents, Silica gel, Amberlite IRC 80, Amberlite IR 120, DOWEX 50WX8, Amberlite XAD4 and Amberlite XAD7, were examined for the purification of anthocyanins from jamun (Jampani et al., 2014). Also in this case, Amberlite XAD7HP had the highest capacity (1.07 mg/mL of adsorbent) and desorption ratio (87.62%) (Jampani et al., 2014).

The investigation for optimal extraction and purification of anthocyanins from black rice showed that among six examined adsorbents of XAD-4, XAD-7HP, HP20, HP2MG, SP70 AND SP207, the highest adsorption capacity of 0.406 mg/g and desorption ratio of 65% is for XAD-

7HP and aqueous acidified ethanol (60% v/v) could effectively elute anthocyanins (Kang et al., 2013).

XAD-7HP was also utilized for purification of anthocyanins from red cabbage juice by Coutinho et al. (2004) which recovered anthocyanins approximately two times more than Sephadex LH20. High purity anthocyanin mixtures were isolated by combination of two columns of XAD-7HP and Sephadex LH-20 with purity up to 68% which three pure monomeric anthocyanins of malvidin-3-O-glucoside, petunidin-3-O-glucoside, and delphinidin-3-O-glucoside were isolated (Wang et al., 2014). Amberlite XAD-7HP was successfully applied in a 36-litre-scale column by Kraemer-Schafhalter et al. (1998), after testing sixteen different adsorbents for the purification of anthocyanins.

Integration of extraction and recovery of anthocyanins from Aronia melanocarpa berries was shown as one of the simple and high productivity method in which liquid extract was circulated in a closed loop through a 1 L glass extractor and the packed adsorption column of XAD-7HP, which was resulted in 92% recovery of anthocyanins. Comparing the integrated extraction – adsorption process to normal extraction and adsorption processes, a clear positive impact of integration was observed, which process integration enhanced the extraction yields of anthocyanins of more than 25%. (Galván D'Alessandro et al., 2013)

Liu et al. (2007) carried out an investigation for recovery of anthocyanins from purple-fleshed potatoes. Investigation was carried out on 11 different adsorbents. Amberlite XAD-1180, XAD-4, XAD-1600, EXA-117, EXA-32, EXA-90, EXA-45, EXA-50, and EXA-118 were macroporous styrene-divinylbenzene (SDVB). EXA-31 and XAD-7HP were methacrylic and acrylic polymers, respectively. The highest adsorption capacity of anthocyanins was obtained with XAD-1600 (Figure 13). Moreover, membrane filtration was utilized to modify the purification before using adsorption which showed better purification to remove 90% of impurities. (Liu et al., 2007)

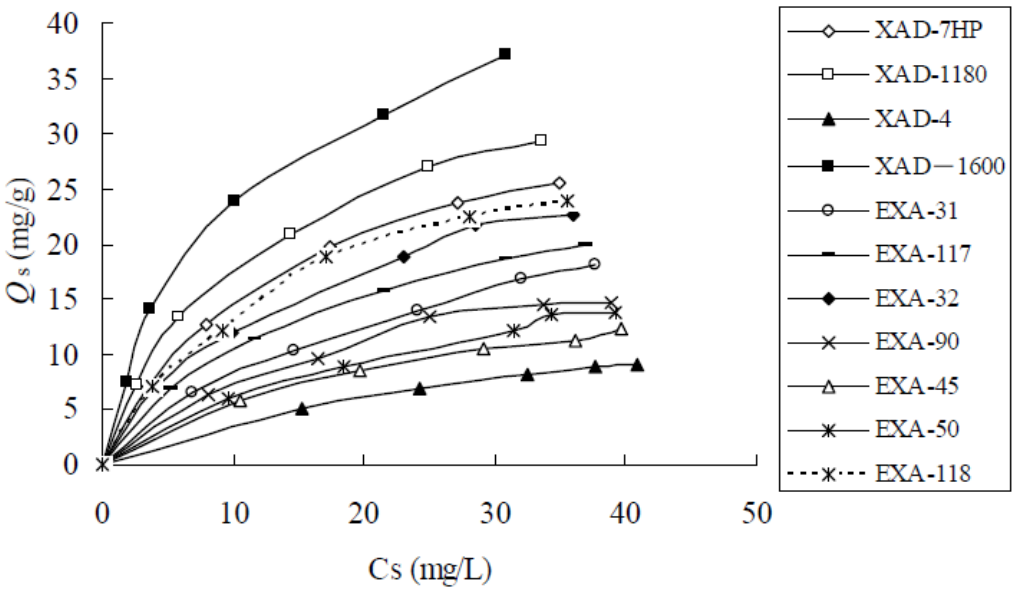

Figure 13. Equilibrium adsorption isotherms of anthocyanins from purple-fleshed potato on different adsorbents (Liu et al., 2007).

Extracting and purifying with macroporous resins was found to be an efficient method for the industrial production of mulberry anthocyanins as a food colorant. Among six resins tested, X-5, the cross-linked polystyrene copolymer with the pore radius of 290~300 Å and surface area of 500~600 m^2/g, demonstrated the best adsorbent capability for mulberry anthocyanins with adsorption capacity of 91mg/mL resin. (Liu et al., 2004)

Six commercial food-grade resins were tested by Di Mauro et al. (2002) to find the more suitable ones for adsorbing anthocyanins from pulp wash of pigmented oranges. Relite EXA-90 showed the best anthocyanin adsorption and anthocyanin recovery (Figure 14).

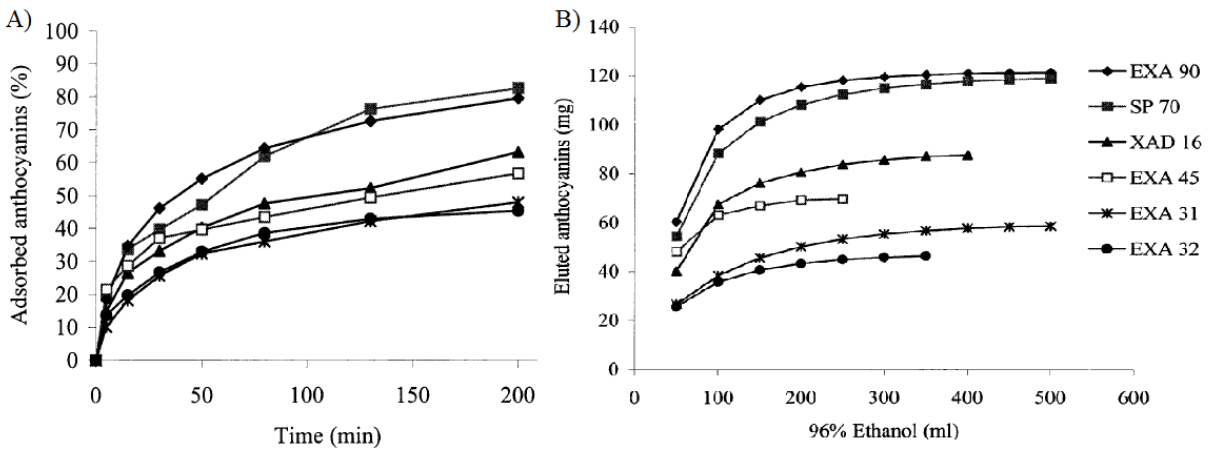

Figure 14. A) Kinetics of adsorption in batch system B) Elution of anthocyanins of pulp wash of pigmented oranges extract from different resins (Di Mauro et al., 2002).

5.2. Counter current chromatography (CCC)

Counter current chromatography is a highly reproducible process with high capacity for large scale production, however it is difficult. It is a liquid-liquid partition method with a high injection capacity which does not involve solid adsorbents. This technique is based on phase density difference and the procedures are mixing, settling and separation which can be done with gravity force or centrifugal force. The column is filled with the stationary phase and the mobile phase is then pumped in ascending mode (tail to head) or descending mode (head to tail) from another side of the column. One of the most used methods is "High Speed Counter Current Chromatography" (HSCCC) that the column consists of one or more coils of Teflon tubes which rotate around their own axis and centrifugal axis. Choosing the liquid phases for anthocyanin purification depend on the anthocyanin substituent. The elution of polymeric anthocyanins and diglucosides can be done by more hydrophobic solvents, but for monomeric anthocyanins, less hydrophobic ones are needed. (He et al., 2012, Valls et al., 2009)

In some studies, combination of adsorption and counter current chromatography were used. Two non-acylated and seven acylated anthocyanins from blue-fleshed potato were characterized by the aid of column XAD-7HP and high speed countercurrent chromatography and low speed rotary countercurrent chromatography (Hillebrand et al., 2009). Moreover, two anthocyanin compounds were separated by two steps of high speed countercurrent chromatography with the purity of 98.5% and 96.7%. After preparation of the extract, it was loaded on the HP2MGL, the

macroporous ion-exchanger column, and then separated by high speed countercurrent chromatography. This combination could increase the purity of the recovered anthocyanins (Lu et al., 2011).

5.3. Aqueous two-phase extraction

The aqueous two-phase extraction is considered as both extraction and purification method which comprises inorganic salts and short chain alcohols or hydrophobic organic solvents. Short process time, low cost and high extraction capacity are the advantages for this technique. In this method, a predetermined quantity of organic salt is dissolved in water. Then certain volumes of organic solvent is mixed with the material which contains anthocyanins (e.g., blueberry or purple potato). Then the mixture is added to the organic salt solution, and mixed well to form two phases. Anthocyanins in the top and bottom phases should be analyzed (Liu et al., 2013, Wu et al., 2014). After two phases become separated, removal of salts and solvents is required to have pure sample. A fair amount of salt could be precipitated by keeping the extract at 4 °C overnight and further eliminated by adding two volumes of 95% ethanol to allow more salt to precipitate. Then centrifugation and evaporation of alcohol are required (Wu et al., 2011).

The optimization of anthocyanin extraction from mulberry juice was carried out by Wu et al. (2011). The results showed that 30% ethanol and 20% of ammonium sulphate with the mulberry juice and 40% water could isolate the majority of anthocyanins and remove 90% of free sugars and impurities in comparison to mulberry juice in one step of extraction and purification(Wu et al., 2011). In the aqueous two-phase extraction of anthocyanins from purple sweet potato, the yield of 90.02% of anthocyanins was obtained under the condition of 25% ethanol and 22% ammonium sulphate with the solid-liquid ratio of 1:45. 85.1% of anthocyanin partitioned to the top phase enriched of ethanol solution (Liu et al., 2013). The recovery of 96.09% of anthocyanins from fruit residue was achieved using 30% ethanol as a short chain alcohol solvent and 19% ammonium sulfate as an inorganic salt combined with AB-8, the cross-linked polystyrene macroporous resin (Hua, 2013). Other inorganic salts such as dipotassium hydrogen phosphate and natrium carbonate were also examined by Hua (2013) and the results showed that ammonium sulphate was the best salt due to higher stability of anthocyanins in it due to pH of 4.3 than dipotassium hydrogen phosphate (pH 8.2) and natrium carbonate (pH 9.0). Therefore high pure anthocyanins can be achieved by this method in a single step.

5.4. Summary

To have purified anthocyanin solution, purification stage is required to recover the target molecules (anthocyanins) from the crude extract. However, counter current chromatography can characterize anthocyanins in high purity, it is difficult, high cost, and needs specific apparatus and instruments. It usually applies when isolation and characterization of individual anthocyanins is needed. Aqueous two-phase extraction is also a good method but removal of salts and solvents is required after purification which makes some drawbacks. Adsorption as a promising selective method for the purification has shown the feasibility for anthocyanin purification. The regeneration of used adsorbents makes it cost efficient. It can be scaled up to produce in high capacity. Considering the advantages and disadvantages of the mentioned purification methods, purifying with resin is found to be an efficient method for research and also industrial scale.

6. REMOVAL OF SOLVENT AND CONCENTRATION OF THE SOLUTION

In order to concentrate the extract and also the eluate samples, evaporation of solvent is required. The evaporation of alcohol as a solvent by a rotary evaporator under vacuum is an easy and common way of alcohol removal. The sample is put in the operator flask which is rotated in the water bath. Depending on the thermodynamic properties of the solvent to be evaporated, the combination of the bath temperature and a vacuum is applied to the solvent. The vapor of solvent is condensed by contacting to the cold water coil and flows into the receiving flask. The rotation and accelerates the evaporation process by increasing the surface area of the solvent.

Membrane process is considered as another technique to removal of alcohol and concentration. In Patil et al. (2009) investigation, an attempt has been made to remove alcohol from anthocyanin extract by a membrane process called membrane pertraction and further concentration anthocyanin by osmotic membrane distillation. In membrane pertraction, the anthocyanin extract containing alcohol is circulated on one surface of the hydrophobic membrane, while water is circulated on the other side of the membrane. Alcohol evaporates from the surface of the solution having higher partial pressure (high concentration of alcohol), diffuses in the form of vapor through the membrane and condenses on the surface of the other solution (pure water), which results in removing alcohol from the anthocyanin solution. In osmotic membrane distillation, calcium chloride dehydrate is utilized instead of water in the membrane to get the water by driving force and concentrate the anthocyanin solution. The advantages of this way of alcohol removal are that it perform in ambient temperature and atmospheric pressure. (Patil et al., 2009)

In order to overcome the drawbacks of the membrane processes such as low flux, limitation of achieving higher concentration, combination of different membranes were used (Patil and Raghavarao, 2007). When the combination of ultrafiltration, reverse osmotic and osmotic membrane distillation were utilized, the anthocyanin extract was concentrated from 400 mg/L to 7980 mg/L. It was concentrated more up to 8100 mg/L when just the combination of reverse osmotic and osmotic membrane distillation were applied (Patil and Raghavarao, 2007). In the study by Meng et al. (2006), multistep membrane process combining cross-flow microfiltration with a ceramic type membrane, reverse osmosis, and rotating evaporation was utilized. The extract was concentrated 9.4 times by reverse osmosis and then 5.4 times by rotating evaporation

which minimized time and energy consumption as compared to the traditional single evaporation process (Meng et al., 2006). In addition, in an investigation by Nayak and Rastogi (2010), it was indicated that the concentration of anthocyanin extract using forward osmosis had benefit over the thermal concentration in terms of higher stability when compared to thermally concentrated sample and it had the ability to scale up for large scale production.

7. EXPERIMENTAL

7.1. Materials

The potatoes used in this work as the anthocyanin source were a purple-blue potato with the name of "Synkeä Sakari". They are cultivated in Kokemäki in Finland. They were planted in May 2014 and harvested in September 2014. The harvesting was 132 days after planting and then they were stored in fridge at 4 °C. (Figure 15). They were prepared by Turku Univeristy. The anthocyanins in this potato are mostly petunidin aglycons: for example, petunidin-3-O-(4'''-p-coumaroylrutinoside) or petunidin -coumaroylrutinoside-glucoside. Due to high stability of acylated anthocyanin, the anthocyanins extracted from this potato are valuable.

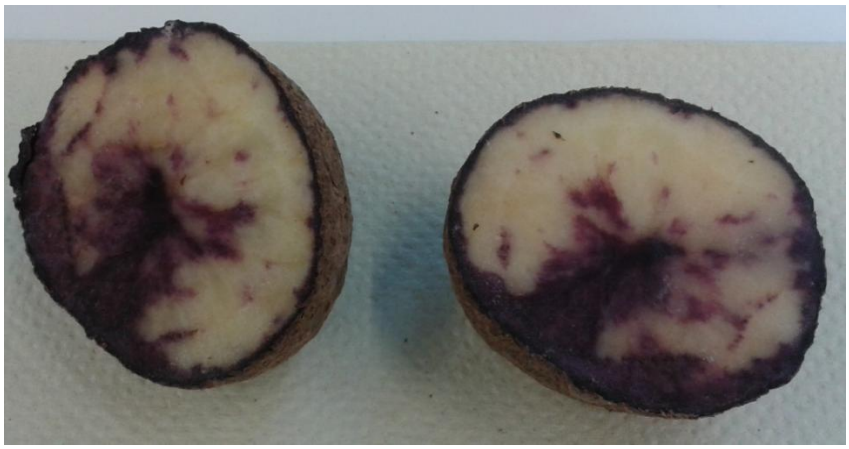

Figure 15. Cross section of partially pigmented Synkeä Sakari potato.

Analytical grade chemicals (ethanol, acetic acid, sodium chloride, potassium chloride and sodium acetate) were used in this work (Table 4). Purified water was used in all solutions.

Table 4. Information of analytical grade chemicals.

Chemicals	Purity	Provider
Ethanol	100%	VWR
Acetic acid	100%	Merck
Sodium chloride	99.7%	VWR
Potassium chloride	99.5%	Riedel-de Haën
Sodium acetate	99%	Riedel-de Haën

Six different adsorbents were used (Table 5). The resins were commercially available.

Table 5. Chemical and physical properties of the adsorbent used. Information provided by the manufacturers.

Trade name	Provider	Structure	Surface area(m^2/g)	Pore radius (Å)	Porosity (mL/g)	Density (g/mL)
XAD-4	Sigma-Aldrich	Styrene-divinylbenzene	725	50	0.98	1.02
XAD-7HP	Sigma-Aldrich	Acrylic ester	450	90	1.14	1.05
XAD-16N	Sigma-Aldrich	Styrene-divinylbenzene	900	100	1.82	1.02
MN-200	Purolite	Styrene-divinylbenzene	1000	-	1.00	1.04
Amberlyst 15 (H^+ form)	Sigma-Aldrich	Styrene-divinylbenzene	52	300	0.40	0.77
CS16GC (Na^+ form)	FINEX	Styrene-divinylbenzene	-	-	-	1.04

7.2. Pretreatment of potato

The potatoes were washed by tap water, maintained in room temperature to become dried after washing and then put in fridge at 4 °C. There were two kinds of potato, completely purple colored and partially purple colored. All the separation experiments were done with the partially purple colored potato batch due to its availability.

In order to evaluate the effect of thermal processing on the extraction of anthocyanins, three samples of boiled, freeze-dried, and dried boiled potatoes were prepared. Moreover, the raw sample was prepared for evaluation. In all cases, whole potato with the peel and flesh were used. Extraction yield were calculated for each samples to obtain more amount of extracted anthocyanins in comparison with lower amount of used fresh potato.

For boiled potato, whole potatoes were boiled in boiled water for 25 min and then smashed with mortar, and put in Ziploc plastic pack and placed in the freezer.

For freeze-dried potato, these were cut into 0.5 cm thick pieces. Then samples were placed in glass bottles and put into freezer. After three days, they were put into freeze-dryer (Christ Alpha 2-4 LSC Freeze Dryer) with -76 °C temperature and 1 bar pressure for four days. The freeze-dried samples were weighed and ground into powder using a mortar, placed in sample vials and kept in -20 °C storage until next part.

For dried boiled potato, whole potatoes were boiled in boiled water for 25 min and then smashed with mortar. Then, thin layer of smashed potato was put on the plate and into an oven at 110 °C temperature for 1 hour. After losing some part of free water, they were put in Ziploc plastic pack and placed in the freezer.

For raw potato, whole potatoes were cut into 0.5 cm thick pieces, put in Ziploc plastic and placed in the freezer. After 24 hours, they were shattered with a hammer and placed to the freezer again for further processing.

7.3. Extraction of anthocyanins

For the preparation of anthocyanin extract from ″Synkeä Sakari″ potato, conventional extraction was used. Extraction of the bioactive compound is influenced by various process parameters such as solvent composition, pH, temperature, extraction time, and solid-liquid ratio.

As the aim was to use the anthocyanins produced in this work in pharmaceutical and food industry, ethanol was chosen as the organic solvent for the anthocyanin extraction. In order to prevent the degradation of nonacylated anthocyanins and to maintain a low pH in the extraction of the desired and more stable form of anthocyanins (formation of flavylium ions), small amount of acid was needed to add to the extraction media. Among all acids which were used in different studies regarding anthocyanin extraction (Andersen and Markham, 2005, Truong et al., 2010, Truong et al., 2012, Xavier et al., 2008), acetic acid was selected as it is acceptable in food industry, and it is a weak acid which cannot hydrolyze acylated anthocyanins. As mentioned before, anthocyanins are polar molecules and their solubility are higher in polar solvent and, thus small amount of water was required for the extraction of hydrophilic anthocyanins. In order

to know the effect of ethanol concentration on the degree of extraction of anthocyanins from purple-blue potato, the concentration of ethanol was varied in range of 20%, 40% and 80% (v/v) in the extraction solvent. Amount of acid to maintain pH around 3 was kept 7% (v/v). pH measurements were made using a digital pH-meter (Consort C3010 Multi-parameter Analyser) calibrated with pH 1 and 7 buffers.

All the prepared potato samples were added to the extraction media with solid–liquid ratio (1:5) (wt/wt) and mixed for 15 min with magnetic stirrer bar. In order to determine the best extraction time, the samples were kept between 2 to 24 h in room temperature. In order to avoid the effect of anthocyanin degradation due to light, all the samples were put in the dark during extraction time. The anthocyanin extracts were filtered through a paper filter under vacuum to remove the coarse particles. Afterwards, all the extracts were centrifuged in a centrifuge (Heraeus Megafuge 1.0/1.0 R Centrifuge) at 4000 rpm for 20 min to remove all the suspended particles. The supernatant was collected and transferred into a dark bottles and kept in the freezer for further processing and anthocyanin quantification.

7.4. Removal of ethanol and concentration of the solution

The ethanol in the extract was evaporated using two methods. The first method was by using a rotary evaporator (Heidolph Hei-VAP Advantage Rotary Evaporator) at a temperature not exceeding 35°C and pressure around 60 mbar. The second method was evaporation of alcohol by normal heating with a heater plate. In this method, the temperature was kept around 60°C and the solution was stirred in order to distribute the heat evenly and avoid burning the solution. The concentrated extracts were put in the freezer at -18 °C for further processing.

7.5. Adsorption

Adsorption was selected as the purification process for the recovery of anthocyanin from purple-blue potato extract due to its advantages. By adsorption method, the purification procedure was done selectively based on differences in the molecules of the solution which leads to differences in affinity for the adsorbent. The regeneration of used adsorbents was the advantage of adsorption which made it cost efficient. Simplicity of design, scale up, and high capacity have made it favorable for this kind of separation. (Soto et al., 2011)

The adsorption process was carried out in the following manner. Six different adsorbents were examined to find out the best one for the anthocyanin recovery. The information about these adsorbents is given in Table 5.

Adsorbents are stable for years at room temperature but different salts such as sodium chloride or sodium carbonate salts are used to retard bacterial growth. Hence, prior to use the adsorbents, they needed to be washed. Preparation and activation of MN-200, XAD7HP, XAD-4 and XAD-16N was done by 5 BV of ethanol (20% v/v) and for pretreatment of amberlyst 15 and CS16GC, 5 BV of sodium chloride (1 M) was used. In order to make the preparation easy, they were put in open headed chromatography columns and washed by the related solvent with low flow rate. After that they were rinsed by abundant purified water to remove salts, impurities and solvents and followed by drying through centrifugation at 2600 rpm for 15 min to remove excess water.

To compare the adsorption capacity of the adsorbents, adsorption experiments were done in the batch mode at room temperature (23 °C). 4 g of each activated adsorbent was contacted with 16 g of centrifuged extract in a glass bottle while agitation on a vibratory shaker for 15 h. After reaching equilibrium, anthocyanin concentration of liquid phase was measured.

Desorption study was also performed on the adsorbents with higher adsorption capacity. The effluents were removed from the adsorbents and they were rinsed by 3 BV of deionized water. The adsorbents were centrifuged at 2600 rpm for 15 min to remove the excess water from the resins. Then the adsorbents were immersed in 16 mL of aqueous ethanol solution 50% in the glass bottles in batch mode under shaking for 1 hour. Anthocyanins were desorbed by the eluent. The elution samples were collected and transferred to the analyses.

7.5.1. Adsorption isotherms

The batch adsorption was used to determine the adsorption isotherms of the anthocyanins in order to predict the behavior of the adsorbents in dynamic systems. Feed solution and the adsorbent particles were brought into contact until equilibrium binding was achieved. 24 mL of concentrated extract with initial anthocyanin concentration of 134.32 was contacted to 24, 12, 7, 4, 3, 2.4, 1.4, 0.8 g of centrifuged adsorbents of XAD-7HP. Also 24 mL of concentrated extract with initial concentration of 219.61 mg/L was contacted to 1 and 0.4 g centrifuged

adsorbents of XAD-7HP. All the experiments were done in the glass bottles at 23 °C. The samples were on the vibratory shaker whole the night (15h). After reaching equilibrium, anthocyanin concentration was measured in the liquid phase to calculate adsorption capacity.

7.5.2. Adsorption in column

The adsorption of anthocyanins in a column was studied with Amberlite XAD-7HP adsorbent. The used equipment is shown in Figure 16 and the process diagram in Figure 17. The experimental setup consisted of scale, pump, column, water bath, fraction collector, and UV-vis spectrophotometer.

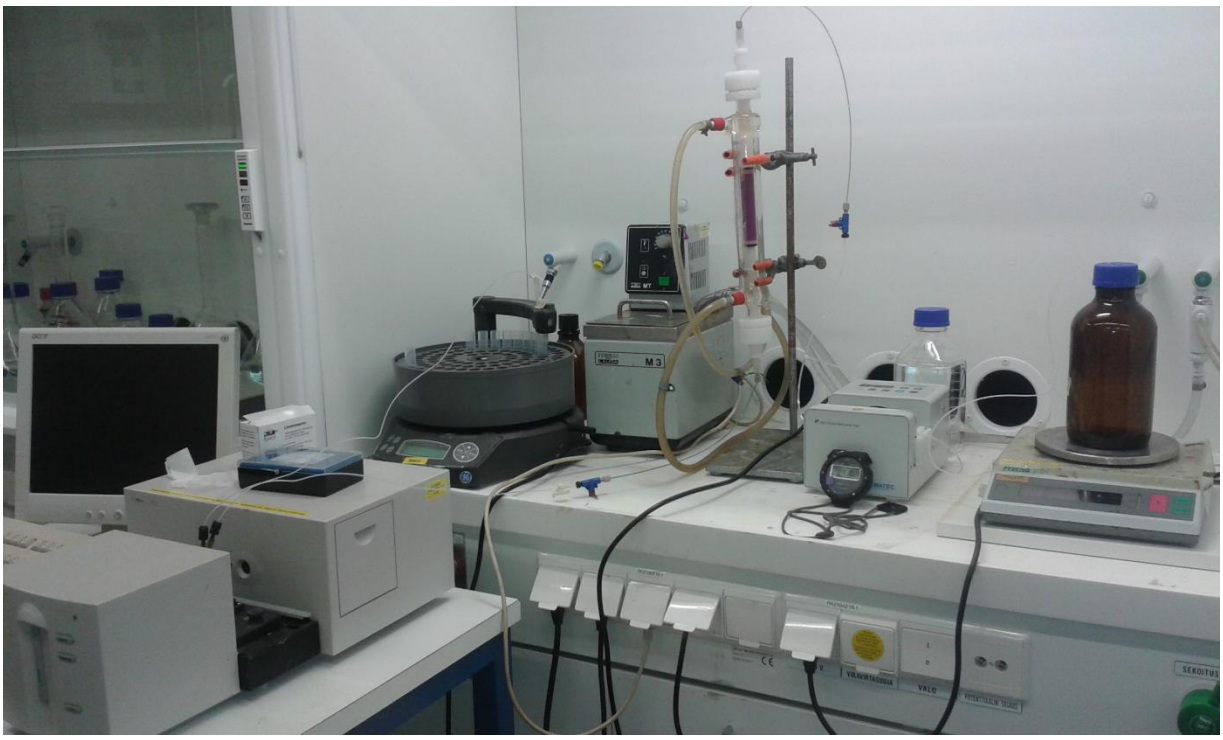

Figure 16. Adsorption column setup.

According to the block diagram of the adsorption process illustrated in Figure 17, the concentrated extract as the feed was pumped to the column from the top. Anthocyanins were adsorbed to the resins. Impurities and free sugars came out as the effluent by rinsing by deionized water. The absorbance of all the collected samples in each step were measured by online UV-vis spectrophotometer to follow the adsorption and desorption behavior.

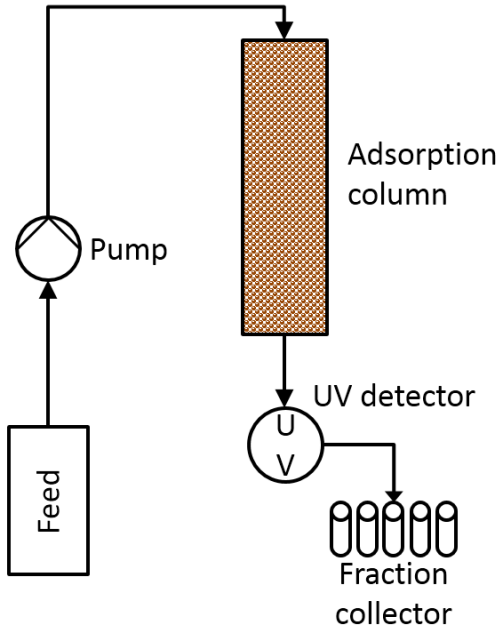

Figure 17. Process diagram of column setup.

Size of the adsorption column was 1.5 cm in diameter and 10 cm in height. Therefore, the bed volume (BV) of the column was calculated 17.66 mL (11.76 g of centrifuged XAD-7HP). The temperature was maintained constant at 23 °C by jacket of water around the column. Initial concentration of anthocyanins in the feed was 224.49 mg/L. Anthocyanin molecules were adsorbed by the resin, while free sugars and impurities were washed away with deionized water. The anthocyanins were then recovered from the resin by elution with aqueous acidified ethanol.

8. ANALYSIS

8.1. Quantification of anthocyanins

8.1.1. UV-vis spectrophotometer

A UV–vis spectrophotometer was used for the analysis and quantification of total anthocyanin contents. 0.025 M potassium chloride buffer and 0.4 M sodium acetate buffer were prepared in order to have the sample in pH 1.0 and 4.5 respectively. pH measurements were made using a digital pH-meter (Consort C3010 Multi-parameter Analyser) calibrated with pH 1, 4 and 7 buffers.

The wavelength for maximum absorbance for anthocyanin solution depends on the type of anthocyanins and anthocyanin sources differs. Therefore, it should be measured at first. This wavelength for maximum absorbance was measured for the "Synkeä Sakari" extract and the curve showed at 524 nm, the absorbance in the highest amount between 520-530 nm. Hence, all the samples were measured at 524 nm and 700 nm for both buffered solutions of pH 1.0 and 4.5. To determine the appropriate dilution factor for each sample, the extract was diluted with pH 1.0 buffer until absorbance at 524 nm was within the linear range of the spectrophotometer (between 0.2-1.4). The final volume of the sample was divided by the initial volume to obtain the dilution factor. Quartz cuvettes of 1 cm path length were used.

8.1.2. HPLC-DAD and UHPLC-MS analysis

Cyanidin 3-glucoside was used as external standard. A stock solution of 0.1 mg/ml in 5% Formic acid in methanol was prepared. Working standards were prepared in 5 % Formic acid in water. A seven point calibration curve between 0.001 to 0.099 mg/mL was used. R2=0.9986; y=3E-08x+0.0014.

The HPLC-DAD system consisted of a GT-154 vacuum degasser, two LC-10AT pumps, a SIL-10A autosampler, a CTO-10A column oven, and an SPD-M10AVP diode array detector linked to an SCL-M10AVP data handling station (Shimadzu Corporation, Kyoto, Japan). The system was operated using the LC solution Workstation software. HPLC separation was carried out on a Kinetex C18 column (100 ×4,60mm. 2.6 µm) and a guard column (AJO-8946). The chromatographic conditions were: flow rate: 1 mL/min; injection volume: 10 uL, mobile phase A: 5 % formic acid in water, B: acetonitrile; gradient: 0 min 5 % B, 2-4 min 10 % B, 10 min 40

% B, 13 min 90 % B, 18-23 min 5 % B. Anthocyanins were detected at 520 nm and quantified with the calibration curve of cyanidin-3-glucoside as an external standard.

In ultra-high performance liquid chromatography/mass spectrometry (UHPLC-MS), anthocyanins in samples were identified using an Acquity Ultra Performance LC (Waters, Milford, MA) interfaced to a Waters Quattro Premier quadruple mass spectrometer.

Analyses of anthocyanins was carried on a Kinetex C18 column (100 × 4.60mm, 2.6 µm) with a guard column (AJO-8946, Phenomenex). The mobile phases A and B were of LC-MS grade and had the same composition as previously mentioned. The gradient was: 0 min 5 % B, 4-44 min 11 % B, 44.01 min 12 % B, 50 min 14 % B, 65 min 17 % B, 69-72 min 80 % B, 76-80 min 5 % B. 0.33 ml/min was diverted to the MS. ESI-MS analysis for anthocyanins was performed in positive ion mode. The mass spectral conditions were: capillary voltage 3.25 kV, cone voltage 45 V, extractor voltage 2.50 V, source temperature 150 °C, desolvation temperature 400 °C, cone gas flow 47 L/Hr, desolvation gas flow 799 L/Hr. The MS analysis (full scan) was acquired at a range of m/z 250-1000.

8.2. Determination of free sugar and alcohol concentration

Purification of anthocyanin was done to have almost pure anthocyanin and remove the impurities. Free sugars as the major impurities and cause of anthocyanin degradation during storage were quantified for all the samples during extraction and purification (Chandrasekhar et al., 2012, Jampani et al., 2014, Wu et al., 2011). Glucose was chosen as the standard for the analysis of free sugars. HPLC analysis of glucose were performed under Shodex SP-0810 column.

In addition, after removal of ethanol, determination of ethanol concentration was needed for all extracts and elution samples. HPLC analysis of ethanol were also done under Shodex SP-0810 column.

The samples were filtered through 0.45 µm filters for HPLC analysis. The following solvent was water with the flow rate of 0.5 mL/min. The retention time for glucose and ethanol were 19 and 25 min.

9. RESULTS AND DISCUSSION

9.1. Effect of potato pretreatment method on extraction of anthocyanins

Four prepared samples were tested to see the effect of pretreatment on the extraction of anthocyanins. Three samples of the thermal processing and one raw sample were compared together. (Figure 18)

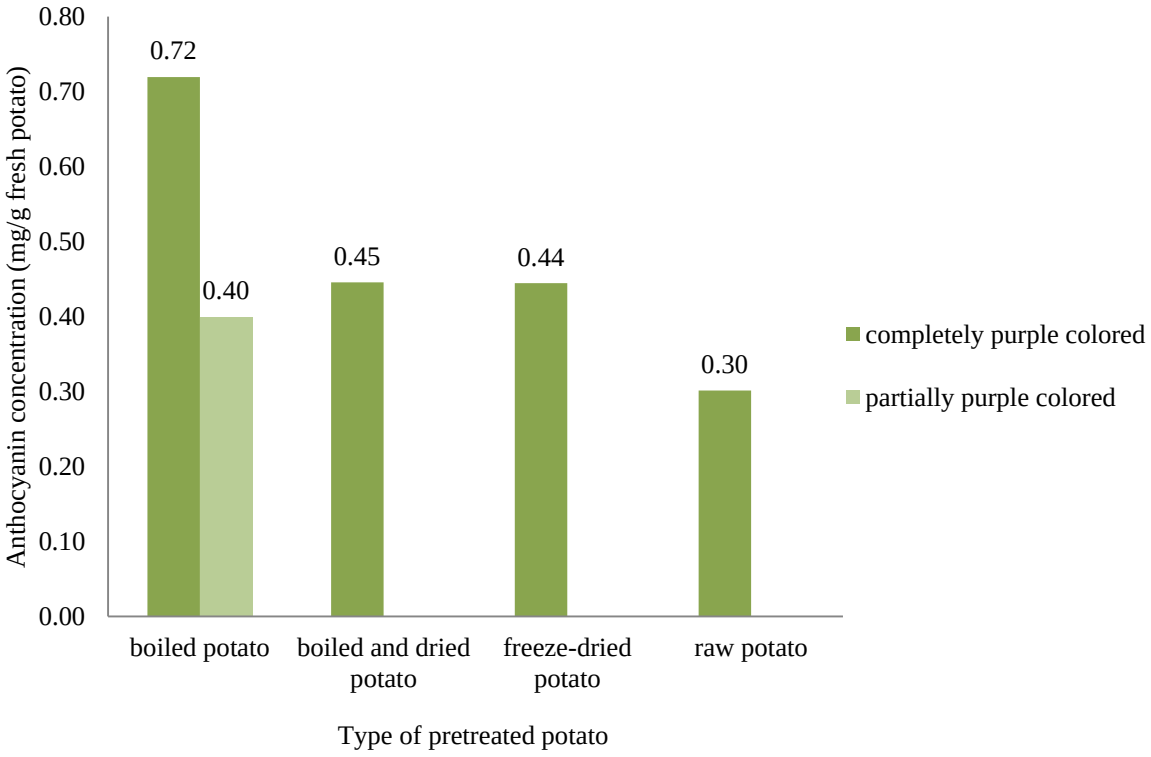

Figure 18. Effect of potato pretreatment on the total extractable anthocyanin contents. (1) Boiling potato in boiled water, whole unpeeled (2) Boiling potato in boiled water and dried at 110 °C oven, whole unpeeled (3) Freeze-drying at -76 °C and 1 bar, whole unpeeled (4) raw potato, whole unpeeled.

Boiling as the pretreatment method resulted in the highest amount of anthocyanins that could be extracted. The extracted anthocyanins in raw potato is relatively low in comparison with the thermal processed samples. Thermal processing affected the textural and rheological characteristic of potato. Thermal processing could be attribute to disruption of plant cell walls providing better extractability, breaking of chemical bonds of higher molecular weight

compound. Therefore, in these condition, anthocyanin could be released easier, and the extraction rate could be enhanced.

Boiling pretreatment were applied to both completely colored and partially colored potatoes. The completely purple colored batch contained higher amount of anthocynains (0.72 mg/g fresh potato) than the partially purple colored, which means that there is a direct relation between content of pigmented species and anthocyanin. Both kinds of potato were the same cultivar but some condition made them different in the anthocyanin contents.

The boiled potato was chosen as the pretreated sample for the further experiments.

9.2. Effect of time on the extraction of anthocyanins

To investigate the effect of extraction time, all other parameters were constant. Extraction parameters were ethanol:water:acetic acid, 40%:53%:7% (v/v) as extraction media with the solid-liquid ratio of 1:5 (wt:wt) and room temperature as the extraction temperature.

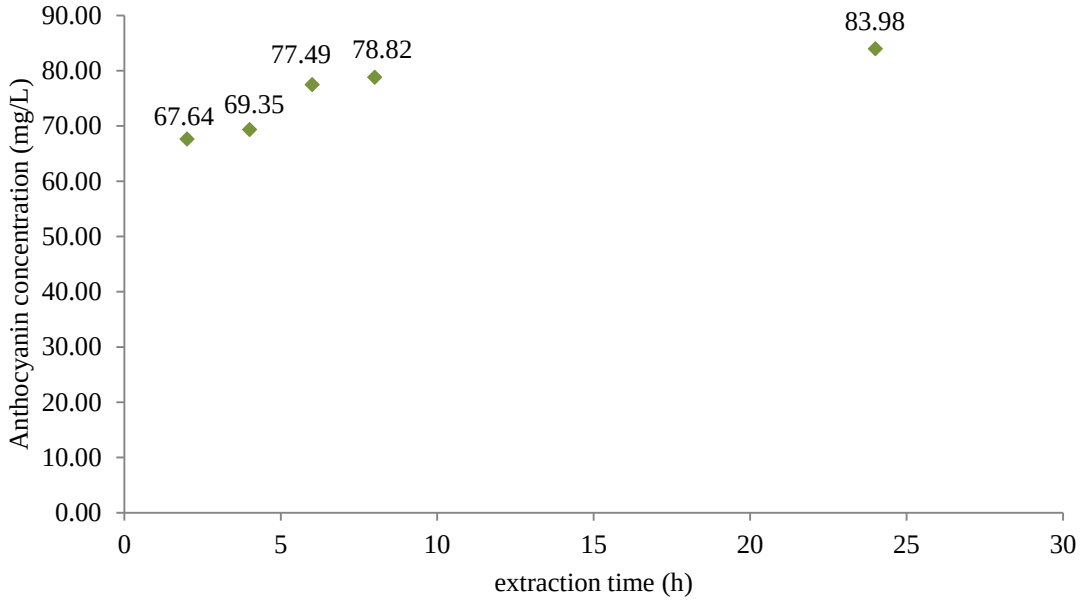

Figure 19. Effect of time on the anthocyanin concentration. ethanol:water:acetic acid, 40%:53%:7% (v/v), room temperature, solid-liquid ratio of 1:5 (wt:wt).

As it can be observed from Figure 19, longer extraction time increased the anthocyanin concentration in the extract. However the effect was not very strong, 24 h was chosen as the

extraction time for further processes in order to diffuse more solvent into the cell walls and help to get higher anthocyanin concentration.

9.3. Effect of ethanol concentration on the extraction of anthocyanins

In order to know the effect of ethanol concentration on the degree of extraction of anthocyanin from purple-blue potato, the concentration of ethanol were varied from 20% to 80%. Acetic acid with the concentration of 7% (v/v) was used in all the samples. The solid-liquid ratio was 1:5 (wt:wt). The prepared solution of boiled potato and extraction media were kept in room temperature for 24 h.

The results are shown in Figure 20. Higher ethanol concentration resulted in higher anthocyanin concentration in feed. By increasing ethanol concentration from 20% to 40%, anthocyanin concentration increased 3.9%, however, by increasing ethanol concentration from 40% to 80%, anthocyanin concentration enhanced 4.5%. The differences in anthocyanin concentration were not notable. 80% ethanol could extract the highest amount of anthocyanins among these three concentration but according to economical view in lower consumption of ethanol, 40% ethanol was chosen. Moreover, evaporation and removal of 40% ethanol was easier than 80%.

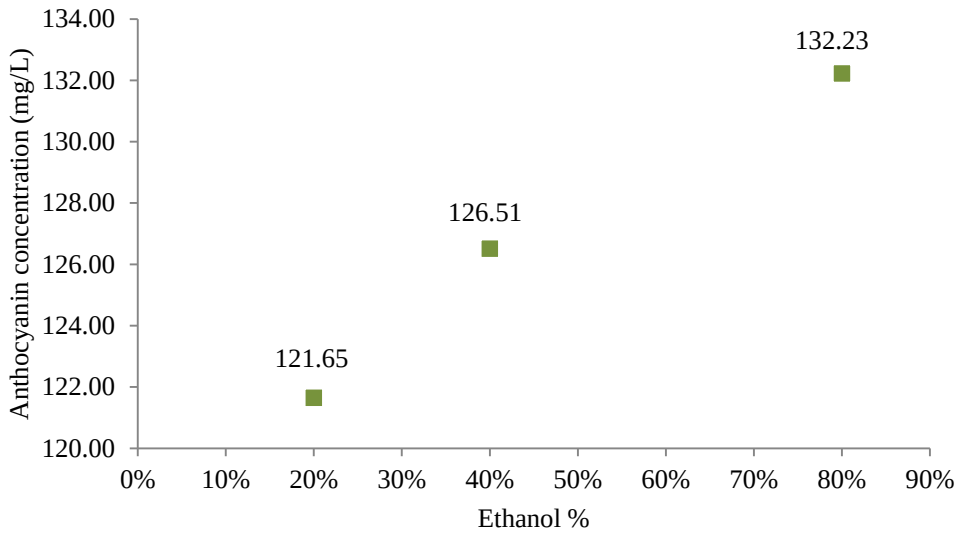

Figure 20. Effect of ethanol concentration on anthocyanin concentration. Acetic acid concentration of 7% (v/v), solid-liquid ratio 1:5 (wt:wt), room temperature, 24h.

9.4. Purification of anthocyanins

9.4.1. Selection of adsorbent

Six adsorbents were compared for the purification of anthocyanins from purple-blue potato by batch adsorption and desorption experiments. The adsorption capacities of six different adsorbents were calculated according to Eq. (3) and desorption ratio of four adsorbents with higher adsorption capacities were calculated by Eq. (4). The results are shown in Figure 21.

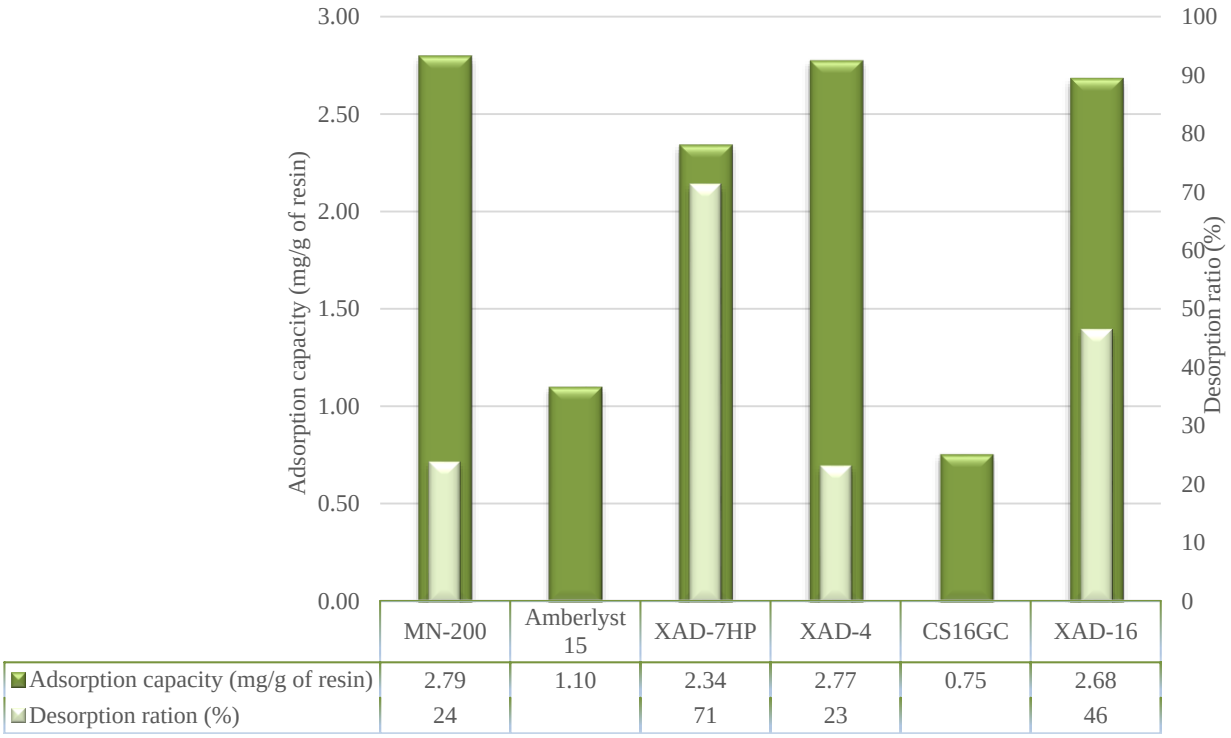

Figure 21. Adsorption capacity and desorption ratio of six adsorbents.

From the tested adsorbents, Amberlite XAD-7HP was selected as the best option for the purification due to several reasons which are expressed below:

- High adsorption capacity (2.34 mg/g of resin)
- High desorption ratio (71%)
- High surface area
- Similar in polarity to anthocyanin exhibited better adsorption ability
- Nonionic acrylic ester adsorbent
- Anthocyanin solution was free from free sugar (degradation cause) after purification

- Availability
- Low price

However, other adsorbent such as MN-200 showed high adsorption capacity, it was expensive and, thus, not economical to use. Also XAD-4 and XAD-16N had high adsorption capacities, but their desorption ratio were not so high in comparison to XAD-7HP with aqueous ethanol (50%). Moreover, XAD-4 and XAD-16N had hydrophobic polyaromatic chemical nature which were recommended to recovery of hydrophobic compounds while XAD-7HP with the acrylic ester structure was moderately polar and similar to anthocyanin in polarity with the dipole moment of 1.8.

9.4.2. Adsorption isotherms

By measuring anthocyanin concentration through pH-differential method, adsorption capacities were calculated by Eq. (3) for the isotherms samples and the results were exhibited in Figure 22.

The adsorption isotherm was performed for partial data and the adsorption isotherm curve by the "S" shape did not have the desired shape. This may be because of the forms of acylated and non-acylated anthocyanins. Non-acylated anthocyanins are less stable and they might be degraded during the processes. Also they were different in molecular size and molecular shape which could affect on the adsorption of anthocyanins to the resin. Moreover, due to the undesired polymerization of some anthocyanins during the processes, high molecular chain of anthocyanins prevent the diffusion into the adsorbents.

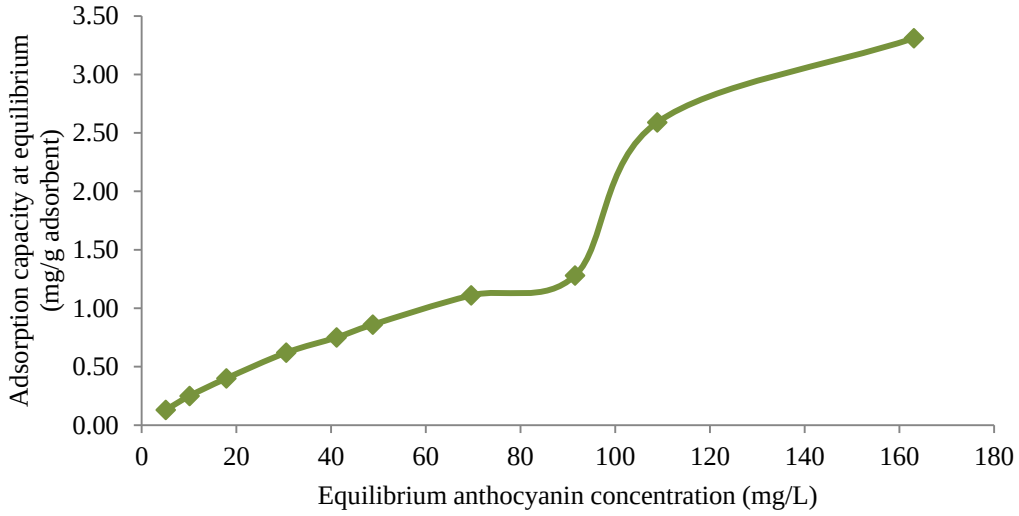

Figure 22. Adsorption isotherms of anthocyanin on Amberlite XAD-7HP.

9.4.3. Purification of anthocyanins in an adsorption column

9.4.3.1.Effect of flow rate on loading

Process parameters such as volume of feed and its flow rate were taken into consideration. Four different flow rates were examined to find out its effect on the loading of anthocyanin into the column of XAD-7HP. (Figure 23)

As it is observed from Figure23, there was no big differences among these four flow rates and anthocyanins began to elute from the column quickly after the void volume of the column. The reason for this behavior of the anthocyanins could that most of non-acylated anthocyanins and also polymerized anthocyanins might came out of the column followed by free sugars and impurities. Moreover, it might be needed to have longer column in height to let anthocyanin have enough time to adsorb to the resin. By having longer column, number of theoretical plates for mass transfer would be higher. Therefore, knowing the HPLC chromatogram of each effluent samples were required to understand the behavior of the anthocyanin in adsorption process.

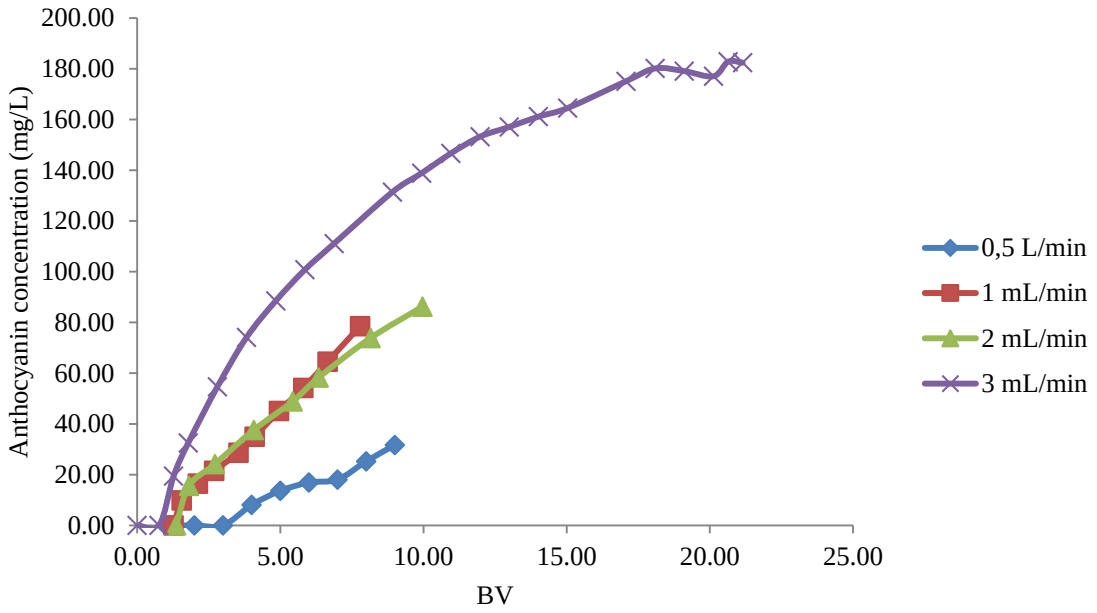

Figure 23. Effect of flow rate in the loading of anthocyanin on XAD-7HP. Temperature: 23°C.

The breakthrough curve was obtained at 3 mL/min flow rate. According to the color of the column during loading time, when 10 BV of feed with the flow rate of 3 mL/min was loaded on the resin, it became totally purple. Hence, 10 BV was selected as the processing volume with the flow rate of 3 mL/min.

The glucose concentration as the free sugar concentration was measured for the effluent samples in loading with 3 mL/min. As it is shown in Figure 24, the glucose concentration in the effluent samples are almost the same as feed samples. It can be resulted to the suitable selection of the adsorbent.

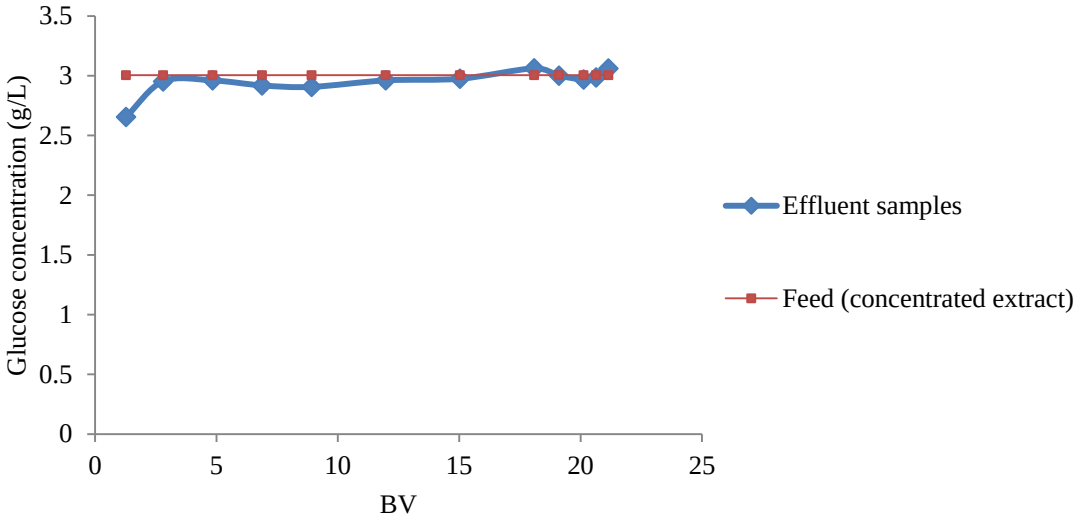

Figure 24. Glucose concentration of effluent samples in loading by flow rate of 3 mL/min.

9.4.4. Washing step

In order to remove the rest of those compounds that were not bound to the adsorbents in the loading step, washing step was needed. These compounds could become the impurities, free sugars and unbound anthocyanins. 3 BV of deionized water was pumped through the column with the flow rate of 1 mL/min after the loading of the feed was completed. Three samples of the outlet of column were collected after 1 BV, 2 BV, and 3 BV of water wash. According to the chromatograms which was shown in Figures 25, glucose with retention time of 19 min and unknown compounds with the retention time of 16 min were high when the first BV of water passed through the column. The amount of these decreased significantly in the second and third BV of water. In fact, after 3 BV of washing, there was no impurities in the column outlet. These results showed that 3 BV of deionized water could effectively washed almost impurities and was enough for washing step.

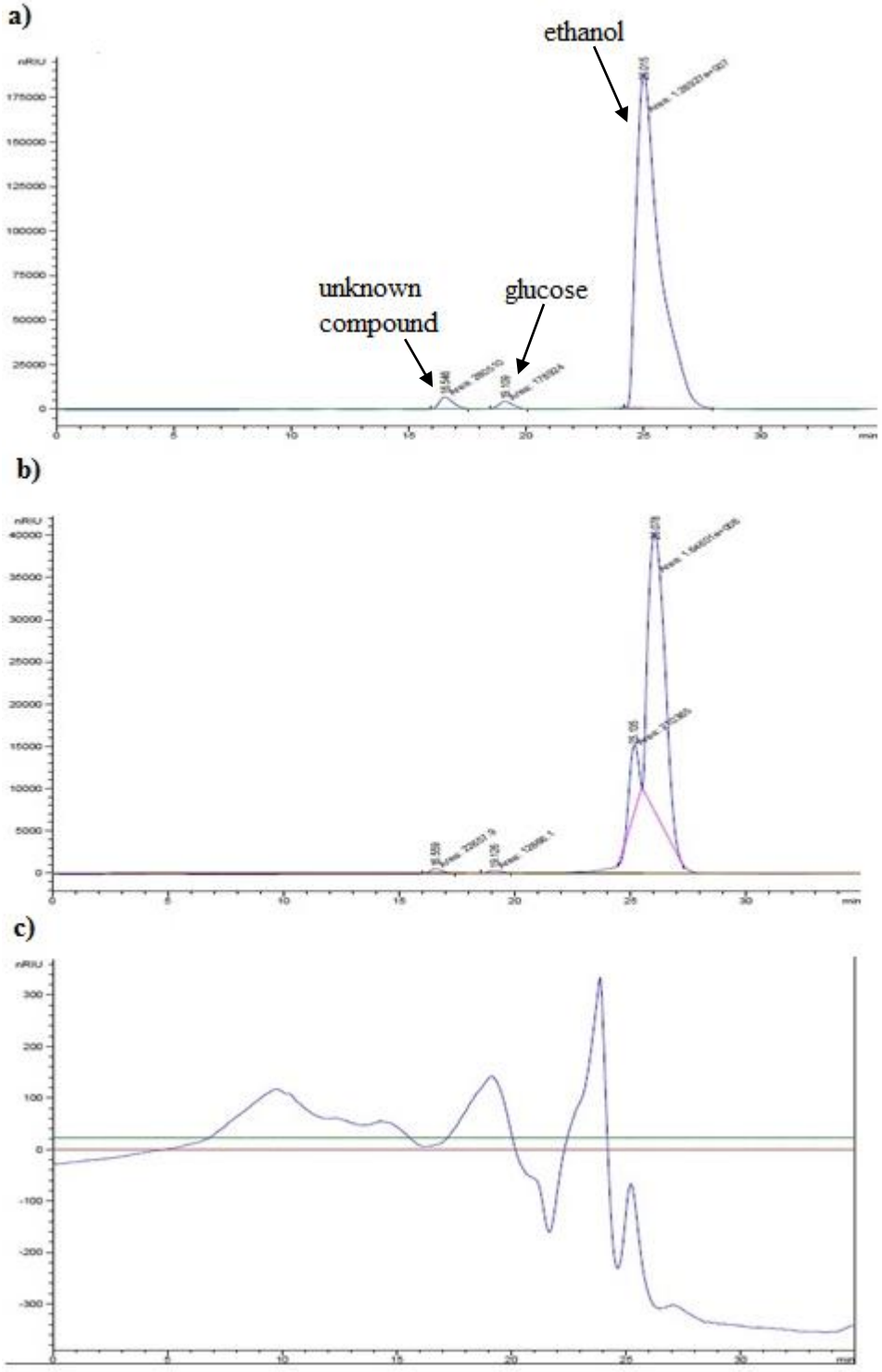

Figure 25. HPLC chromatogram of glucose in the washing samples: a) 1 b) 2 c) 3 BV of water.

9.4.5. Desorption of anthocyanins from the column

The effect of the eluent type and concentration on desorption of anthocyanins from the adsorption column was also investigated. In addition, the effect of eluent flow rate was also investigated.

9.4.5.1.Effect of ethanol concentration

Ethanol concentration is one of the most important factor for the elution of anthocyanins from the adsorbent. In order to know the influence of ethanol concentration on the elution of anthocyanins, studies were carried out with acidified aqueous ethanol with three different ethanol concentration (25%, 50% and 75%). The results are depicted in Figure 26. All the eluents were acidified form by acetic acid in order to maintain pH of eluent at 3.1 and prevented the anthocyanin degradation.

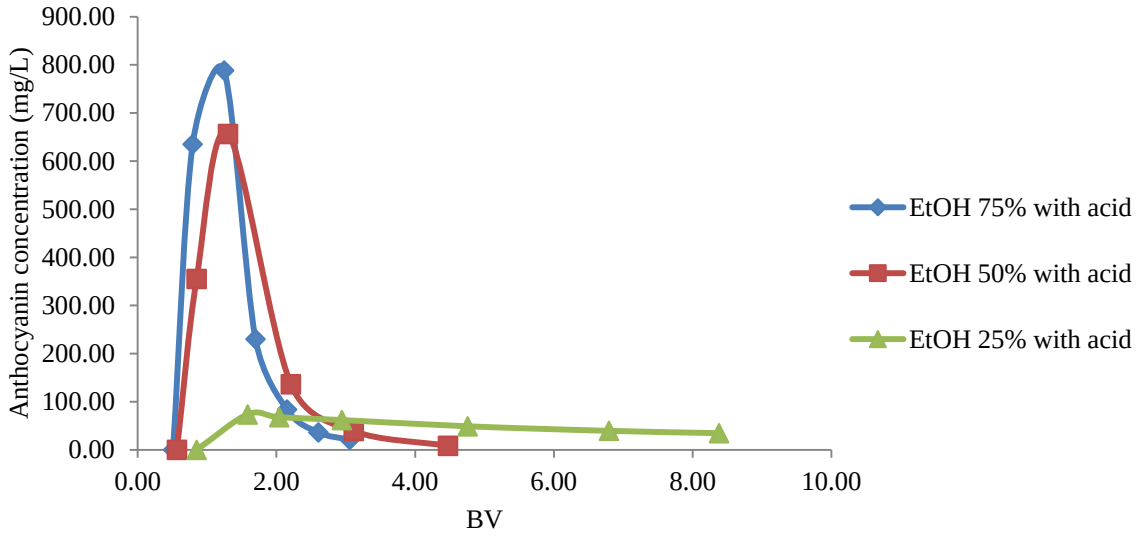

Figure 26. Effect of ethanol concentration in the elution of anthocyanin from XAD-7HP. Temperature: 23°C, flow rate: 1mL/min, Initial anthocyanin concentration in the feed**:** 224.49mg/L.

It can be seen from Figure 26 and Table 6 that acidified ethanol above 50% (v/v) could effectively elute anthocyanins from the adsorbents. 18.47 mg anthocyanins were adsorbed to the adsorbents, 50% and 75% ethanol could elute around 72% of anthocyanins from the column. Hence, for higher anthocyanin recovery, higher concentration of ethanol is recommended. There

was no big differences between ethanol concentration of 50% and 75% but in case of using ethanol concentration of 75%, 3 BV of eluent could elute the same of anthocyanin when 4.5 BV of eluent with 50% of ethanol concentration. The time and eluent consumption were 24 min and 24 mL less when ethanol concentration of 75% was used. Therefore, considering all these reasons, aqueous acidified ethanol with the concentration of 75% is most efficient of the studied eluents for desorption of anthocyanins.

Table 6. Effect of ethanol concentration on the elution of anthocyanins.

Ethanol concentration (%)	Eluted anthocyanin (mg)	Recovered anthocyanin (%)	Eluent consumption (BV)	Elution time (min)
25	6.49	35.16	8.34	147
50	13.25	71.37	4.47	78
75	13.34	72.19	3.06	54

9.4.5.2.Effect of acetic acid concentration in the eluent

Effect of acid can be seen in Figure 27. Aqueous acidified ethanol (75%) with 7% of acetic acid and aqueous ethanol (75%) were used to elute anthocyanin from XAD-7HP.

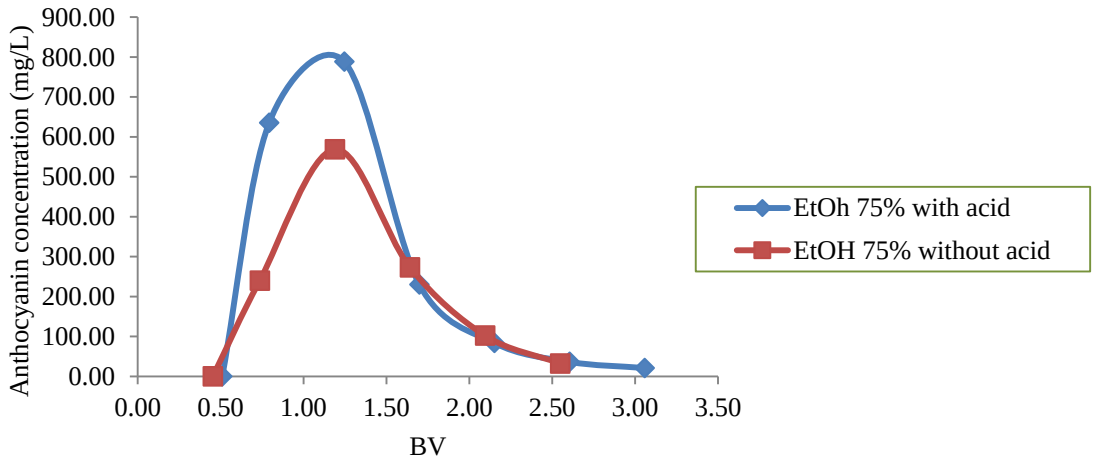

Figure 27. Effect of acid in the elution of anthocyanin from XAD-7HP. Temperature: 23°C, flow rate: 1mL/min, Initial anthocyanin concentration in the feed**:** 224.49mg/L.

It can be seen from Figure 27 and Table 7 that acidified ethanol could enhance the desorption of anthocyanins from the adsorbent. 3BV of aqueous acidified ethanol (75%) could elute 13.34 mg anthocyanins from 18.47 mg anthocyanins which was adsorbed to the adsorbents packed in the column. Aqueous acidified ethanol (75%) had the highest recovery of anthocyanins around 72% in comparison with aqueous ethanol (75%) which had the recovery of 50%. Moreover, elution without acid resulted in the change of red and purple color of the eluted anthocyanins to brownish color which is caused by the degradation of anthocyanins in high pH. Therefore, acidification of the alcoholic eluent leads to a better anthocyanin recovery.

Table 7. Recovery (%) of anthocyanin by aqueous acidified ethanol (75%) and aqueous ethanol (75%).

Eluent	Eluted anthocyanin (mg)	Recovery of anthocyanin (%)
Aqueous acidified ethanol (75%)	13.34	72.19
Aqueous ethanol (75%)	9.24	50.03

9.4.5.3.Effect of eluent flow rate

Volume of eluent and its flow rate were significant to standardize the dynamic desorption of anthocyanins. Hence, two different flow rates of eluent were tested to investigate the effect of flow rate on desorption of the anthocyanins. The dynamic desorption curve was obtained based on the volume of the eluent and anthocyanin concentration in eluate. Aqueous acidified ethanol (75%) was used for the elution of anthocyanins by maintaining the flow rates at 1 and 2 mL/min in order to compare these two flow rates and the results are shown in Figure 28.

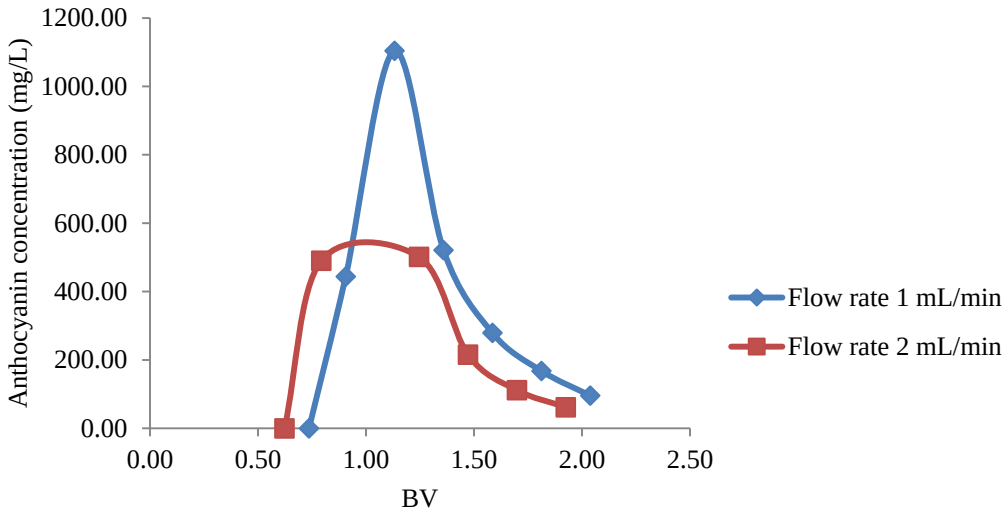

Figure 28. Effect of flow rate of eluent (aqueous acidified ethanol (75%)) in the elution of anthocyanin from XAD-7HP. Temperature: 23°C, pH: 3.1, Initial anthocyanin concentration in the feed**:** 188.20mg/L.

According to Figure 28 and Table 8, approximately 48 mL of aqueous acidified ethanol (75%) with flow rate of 1 mL/min could elute 10.65 mg of anthocyanins from 13.49 mg which was adsorbed to the resin but in case of using the eluent with 2 mL/min, the amount of consumed eluent became more. Hence, it can be understand that by flow rate of 1mL/min, the eluent could diffuse better in order to elute the anthocyanins.

Table 8. Effect of flow rate of eluent (aqueous acidified ethanol (75%)) in the elution of anthocyanins from XAD-7HP.

Flow rate (mL/min)	Eluted anthocyanin (mg)	Recovered anthocyanin (%)	Eluent consumption (mL)
1	10.65	78.95	48.03
2	7.46	55.28	84.06

9.4.6. HPLC analysis of anthocyanins

As it is shown in Figure 29, recovered and purified anthocyanin by the studied process in this project is exactly the same as the "Synkeä Sakari" anthocyanin which showed the success of the designed process for purification. Moreover, the chromatograms with two major peaks were

identified as the acylated anthocyanin; Petunidin -coumaroylrutinoside-glucoside (peak 5) and Peonidin-p-coumaroylrutinoside-glucoside (peak 8). The average anthocyanin concentration in the final sample of the recovered anthocyanin after concentration step was obtained as 1752.89 mg/L. The initial anthocyanin concentration in the concentrated extract before purification was 224.49 mg/L.

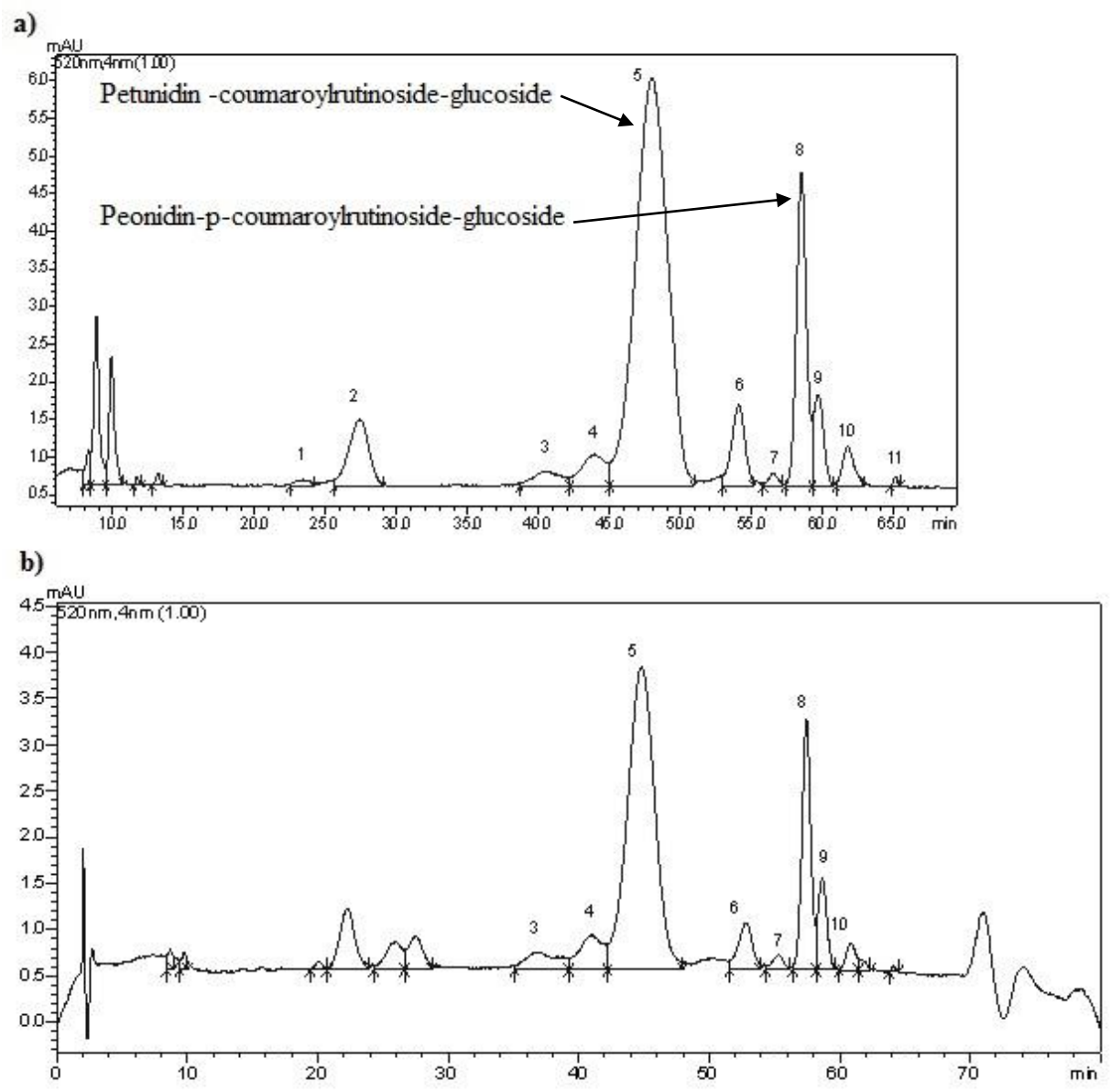

Figure 29. HPLC chromatogram of anthocyanin solution: a) Synkeä Sakari b) purified anthocyanin extracts by the method which was studied in this project.

Moreover, in order to identify the free sugar concentration, the HPLC chromatogram could determine the glucose concentration of the samples before and after purification (Figure 30).

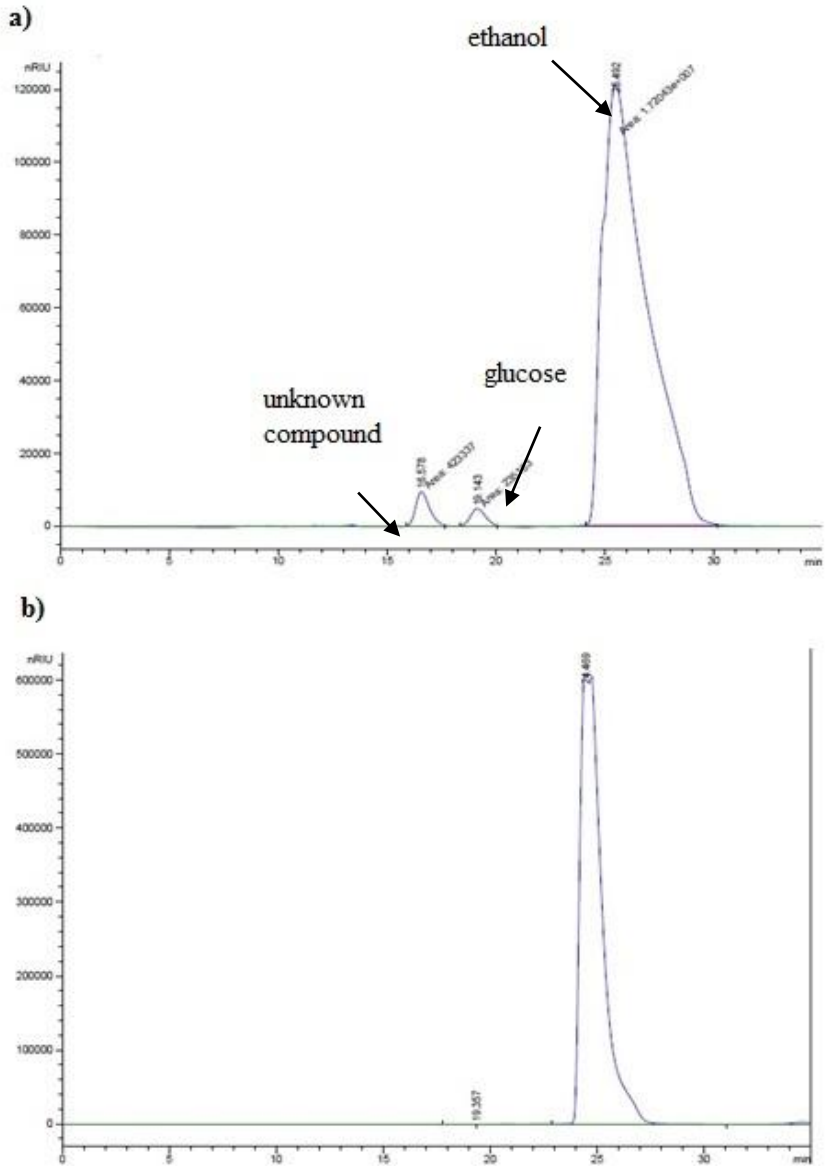

Figure 30. HPLC chromatogram of glucose in the anthocyanin extract a) before purification b) after purification by adsorption in XAD-7HP column.

As it can be observed from Figure 30 and Table 9, the concentration of glucose was decrease from an initial 1.35 g/L to 0.05 g/L after purification. In addition, the unknown compound with the retention time of 16 min was completely removed from the anthocyanin sample after

purification. Initial and final anthocyanin concentration in the concentrated extract and concentrated purified was 224.49 mg/L and 1752.89 mg/L.

Table 9. Glucose concentration before and after purification.

Retention time (min)	Component	Concentration before purification (g/L)	Concentration after purification (g/L)
19.143	Glucose	1.35	0.05

9.5. Process diagram

As the general review of the process, schematic of the process was illustrated in Figure 31.

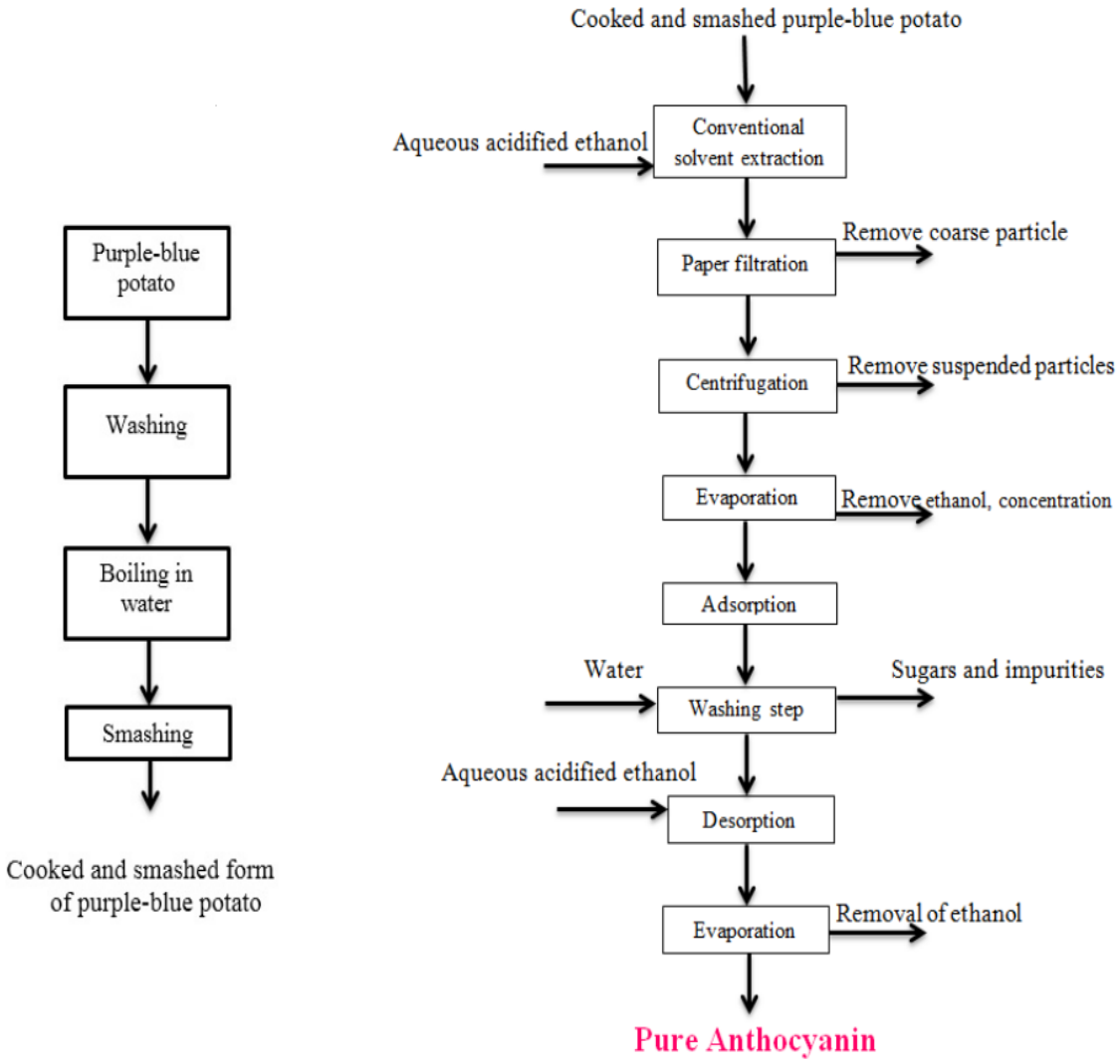

Figure 31. Schematic of the process of extraction and recovery of anthocyanins from purple-blue potato.

10. CONCLUSIONS

In this study, with the aim of the extraction and purification of anthocyanins from purple-blue fleshed potato, investigations and experiments were performed. The focus of recent investigations provided notable information regarding to anthocyanins and their recovery. Anthocyanins were investigated as valuable compounds for pharmaceutical and food industries. "Synkeä Sakari", the purple-blue fleshed potato which is cultivated in Finland was studied as the source of acylated anthocyanins. The extraction and purification methods, which cause to develop and facilitate the application of anthocyanins, were review.

The pretreatment of potato by four different studied conditions showed that boiled potato was the best pretreated potato. Among the different extraction methods, conventional extraction was selected. Aqueous acidified ethanol (ethanol:water:acetic acid 40%:53%:7% v/v) as the extraction media showed the better results with the extraction time of 24 h (anthocyanin content 126.51 mg/L).

The adsorption of anthocyanin from the extract was carried out and shown to be a very favorable process. The resin showing the best results regarding the adsorption process of anthocyanin was XAD-7HP, reaching adsorption capacity (2.34 mg/g) with the liquid phase concentration of anthocyanins of 176.48 mg/L, and desorption ratio (71%) of anthocyanins. Processing volume, flow rate and temperature were 10 BV, 3 mL/min, and 23 ± 1 °C, respectively. 3BV of deionized water completely washed free sugars and impurities from column. Eluent volume and flow rate were 3 BV of acidified aqueous ethanol (75%, v/v) and 1 mL/min for desorption, respectively.

Success of extraction and purification processes was proved by the HPLC chromatogram of recovered anthocyanins which was exactly the same as "Synkeä Sakari" anthocyanins with acylated anthocyanins. The average anthocyanin concentration in the final sample of the recovered anthocyanin was obtained as 1752.89 mg/L after concentration step. The initial anthocyanin concentration was 224.49 mg/L in the crude extract. The work was thus an important step in the direction of concentrating and selectively separating anthocyanin from purple-blue potato crude extracts.

Further studies considering the enhancing rate of extraction by using ultrasound, pulsed electrical field and microwave assisted extraction are suggested. Moreover, anthocyanin

analysis with analytical HPLC were required to understand the behavior of anthocyanins in each step so further works under HPLC analysis will give valuable information regarding to structure of anthocyanins and their behavior under different experimental condition. Testing longer column in order to have good breakthrough curve and also recirculating the feed to column to give time to anthocyanin to diffuse better into the adsorbents can be provide more investigations about recovery of anthocyanin by XAD-7HP.

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