

# Purification of Anthocyanin Extract from Roselle by Progressive Freezing: Effect of coolant temperature and stirring rate

Aisyah Nur Hanis Azhar<sup>2</sup>, Muhammad Athir Mohamed Anuar<sup>2</sup>, Siti Nor Adibah Mustapha<sup>2</sup> and Nurul Aini Amran<sup>1,2\*</sup>

<sup>1</sup>HICOE – Center for Biofuel and Biochemical Research, Institute of Self-Sustainable Building, Universiti Teknologi PETRONAS, 32610, Seri Iskandar, Perak, Malaysia.

<sup>2</sup>Chemical Engineering Department, Universiti Teknologi PETRONAS, 32610, Seri Iskandar, Perak, Malaysia.

**Abstract.** This study presents the process of progressive freezing as a potentially new and alternative approach for the purification of anthocyanin extract to replace the conventional methods of the aqueous two-phase system (ATPS) and adsorption. Specifically, anthocyanins extract was obtained from the roselle plant or also known as red hibiscus. It is essential to purify the anthocyanins extract before it can be used in various applications to remove the by-products of the extract i.e. free sugar, sugar alcohol, organic acids, amino acids, and proteins. Before the purification step, the anthocyanin was first extracted from the roselle flower through a maceration process. The total amount of anthocyanins in the extract was measured by a UV-VIS spectrophotometer. The extract was then concentrated via the progressive freezing process and the influence of cooling temperature and rate of stirring were analysed vis a vis the process performance, which was represented by the effective partition constant ( $K$ ) and concentration efficiency. The highest concentration efficiency of 55% and the smallest  $K$  value of 0.3 were found at the coolant temperature of -14 °C and 250 rpm rate of stirring. It was also found that lower coolant temperature and higher stirring rate produced a  $K$  value that was smaller and superior efficiency.

## 1 Introduction

Anthocyanins are pigments that are blue, red or purple which exist in abundance in flora such as berries and flowers [1]. The primary function of anthocyanins in the plants is to give colour. The examples of red flowers containing anthocyanins are red hibiscus, red roses, red pineapple sage, red clover and red blossom while blue flowers include cornflower, blue rosemary and blue chicory[2].

Roselle (*hibiscus subdariffa L.*) or also known as red hibiscus is a tropical plant and a native plant in West Africa. However, it can also be found abundantly in Malaysia. Chemical components that can be found in Roselle include anthocyanins, flavonoids and polyphenols. The presence of anthocyanins in Roselle made the flower appear to be in red. Dried petals of Roselle contain anthocyanins, delphinidin-3-mono glucosyde and cyaniding-3-monoglucosyde. Due to the presence of these chemicals, the petals are often used for beverages as well as food colouring agents [3].

The typical anthocyanins extract contains by-products like free sugar, sugar alcohol, organic acids, amino acids and proteins. These by-products may cause disturbance during the storage or downstream processing. Therefore, purification of anthocyanins

extract is needed to remove all the by-products. The conventional methods to purify the extracts include liquid-liquid separation process, adsorption [4], rotary evaporation and aqueous two-phase (ATP) system. These processes somehow require the use of chemical, expensive equipment and abundant energy. Additionally, ATP specifically requires further removal of the salts and solvents after the purification process which makes it more time consuming and costly [5, 6].

In this study, progressive freezing (PF) is introduced as an alternative method to purify anthocyanins. The general separation mechanism of the PF process is removing the solvent (water) through freezing which produces a layer of ice crystals the crystallizer's wall that results in highly concentrated liquid product [7]. The ice crystal layer can then be easily separated from the concentrated solution.

Theoretically, the crystals form in three stages. The solution in the first phase undergoes an initial cooling process that reduces the solution's temperature to the solvent's freezing point. Once the freezing point is reached and the equilibrium is altered at second stage, ice nucleation will start to occur. At this condition, fusion heat will be produced by ice nuclei formation. Finally, the third stage sees the stable ice crystal nuclei continuously growing till the actual set temperature is

\* Corresponding author: [nurul.amran@utp.edu.my](mailto:nurul.amran@utp.edu.my)

reached. Bulk temperature changes slowly and there is a continuous formation of solid ice crystals along with the increasing concentration of the mother liquor [8]. The main attraction of progressive freezing method is it offers a low-temperature separation process which could be extremely beneficial in retaining the heat-sensitive component in the solution, as compared to evaporation. As a result, the PF method has been applied in various industries, especially in liquid food processing such as fruit juice concentration, as well as dairy and coffee processing.

In this study, the PF process was applied to concentrate and purify the anthocyanins extract from roselle plant in a stirred tank crystallizer. The anthocyanins were first extracted through a maceration process before it was sent to the PF process. The impact of two operating parameters i.e. cooling temperature and rate of stirring on the resulting value of effective partition constant ( $K$ ) and concentration efficiency were investigated.

## 2 Methodology

### 2.1. Preparation of materials

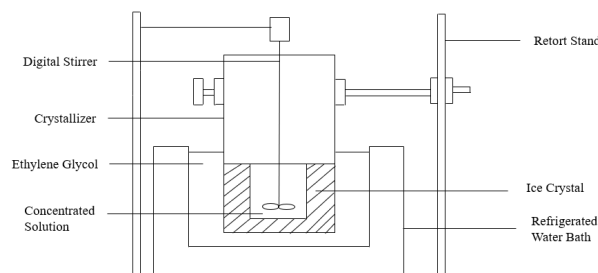
As the primary raw material, dried Roselle was purchased from a local supplier in Terengganu, Malaysia. The dried Roselle was cut into small pieces before grounded into fine particles using a blender. Then, a sieve was used to sieve the grounded Roselle into the desired particle size of 0.325 mm. After the desired particle size was obtained, the samples were kept in clearlinear low-density polyethylene (LLDPE) pouches and were stored in a cold environment until the extraction was carried out.

Ethylene glycol (Sigma Aldrich) was used as a coolant for the PF process. Approximately a 50:50 volume ratio of water and ethylene glycol is intermixed to obtain a favourable freezing temperature. By doing this, the freezing point can be obtained up to  $-27^{\circ}\text{C}$ . Besides, potassium chloride and sodium acetate (Sigma Aldrich) were used in the preparation of buffer solutions for the analysis of total anthocyanins content.

### 2.2 Anthocyanins extraction

The anthocyanin extraction was done using the maceration process. The solvent used in this process was water. A 500 mL volume of water was poured into a beaker containing 33.33 g of grounded Roselle (solvent to solid ratio of 15:1). A magnetic stirrer was then placed into the beaker. Then, the beaker was placed onto a hot plate with a temperature of  $40^{\circ}\text{C}$  and a 150 rpm rate of stirring. The solution was then heated for two hours. After two hours, the solution was left for cooling before being filtered using a filter paper. The filtered solution was the anthocyanins extract. The extract was stored in a refrigerator to be used in the next procedure.

### 2.3 Purification of anthocyanins extract via progressive freezing



**Fig. 1.** Schematic diagram for experimental setup for progressive freezing process.

Figure 1 shows a schematic diagram for the experimental setup of the progressive freezing process. As can be seen from the figure, ethylene glycol was put into the refrigerated water bath to act as a coolant to ensure that heat transfer occurred in very low temperature process [9]. Solution would be poured into the crystallizer where the ice crystal and concentrated solution would be formed. The digital stirrer provided the flow for the solution.

The refrigerated water bath was switched on and the temperature was set to the desired value ( $-10$  to  $-18^{\circ}\text{C}$ ). Once the desired temperature was achieved, 400 mL of Roselle extract was decanted into the crystallizer. The crystallizer was then immersed into the refrigerated water bath. At the same time, the digital stirrer was switched on with the desired stirring rate (100 to 300 rpm). The freezing process was conducted for 20 minutes.

After 20 minutes, the digital stirrer was turned off and the crystallizer was taken out from the water bath. The concentrate was then poured into a beaker and weighed. Readings of mass and volume for the concentrate were taken. A sample of 50 mL of the concentrated solution was stored in a vial and labelled for further use.

Next, the formed ice layer was left to melt before it was collected and placed into a beaker. Similar as before, the ice solution's mass and volume of were measured and recorded. A sample of 50 mL of the ice solution was stored in another vial and labelled for further use. The experimental procedures were repeated at different coolant temperatures.

As for the stirring rate parameter, the same procedure was repeated. The coolant temperature was set at desired value while the cooling time was set to be 20 minutes.

### 2.4 Analysis of total anthocyanins content

#### 2.4.1 Buffer solution (pH 1.0) preparation

A 1.86 g amount of potassium chloride, KCl and 500 mL of distilled water were put in a beaker and mixed. The

solution's pH was then measured using a pH meter and then adjusted until it reached the pH of 1.0 using hydrochloric acid, HCl. It was then moved to a 1 L volumetric flask and distilled water was used to dilute it to the mark [10].

#### 2.4.2 Preparation of pH 4.5 buffer solution

A 54.43 g amount of sodium acetate,  $\text{CH}_3\text{CO}_2\text{Na}\cdot 3\text{H}_2\text{O}$  and 500 mL of distilled water were put in a beaker and mixed. Then, the solution's pH was measured and adjusted using HCl to reach a pH of 4.5. Next, the solution was moved to a 1 L volumetric flask and distilled water was used to dilute it to the mark [10].

#### 2.4.3 Total anthocyanins content determination

A 1 mL volume of the roselle extract was put into a 10 mL volumetric flask. The extract was diluted with potassium chloride of pH 1.0 in the volumetric flask. Next, 1 mL of the sample was moved into another 10 mL volumetric flask. The extract was diluted with sodium acetate solution of pH 4.5 in the volumetric flask. Each diluted sample's absorbance was determined at a wavelength of 520 nm and 700 nm by a UV-Vis spectrophotometer [10].

The diluted sample's absorbance (A) was determined using Equation (1):

$$A = (A_{520} - A_{700})_{\text{pH}1.0} - (A_{520} - A_{700})_{\text{pH}4.5} \quad (1)$$

The anthocyanins concentration (AC) in each sample was determined using Equation (2):

$$AC \text{ (mg/L)} = (A \times MW \times DF \times 103) / \epsilon \quad (2)$$

Where  $A = (A_{520} - A_{700})_{\text{pH}1.0} - (A_{520} - A_{700})_{\text{pH}4.5}$ ; MW (molecular weight) = 287.24 g/mol; DF = dilution factor;  $\epsilon = 26900$  molar extinction coefficients, in  $\text{L} \times \text{mol}^{-1} \times \text{cm}^{-1}$ , for cyanidin; and  $10^3 =$  factor for conversion from g to mg [11].

#### 2.4.4 Effective constant determination

Effective partition constant (K) is the most essential variable in progressive freezing. The value of K is supposed to change in a range of 0 to 1. A value approaching 0 indicates a complete crystallization, while a value approaching 1 indicates no crystallization [7]. The determination of effective partition constant (K) was carried out using Equation (3):

$$K = 1 - [\log(C_o/C_L) / \log(V_L/V_o)] \quad (3)$$

Where  $V_L$  and  $V_o$  represent the volume of extract (mL) in concentrated and initial solutions respectively.  $C_L$  and  $C_o$  represent the anthocyanin concentration in concentrated and initial solutions, respectively [12].

#### 2.4.5 Determination of concentration efficiency

Concentration efficiency represents the interrelationship between the increases in the solution's concentration in the liquid phase corresponding to the solid phase's concentration as stated in Equation (4) [13]:

$$Eff = [(C_L - C_o) / C_L] \times 100 \quad (4)$$

Where Eff represents the concentration efficiency (100%),  $C_L$  is the anthocyanins concentration in the liquid phase after the freezing process and  $C_o$  is the initial solution's concentration.

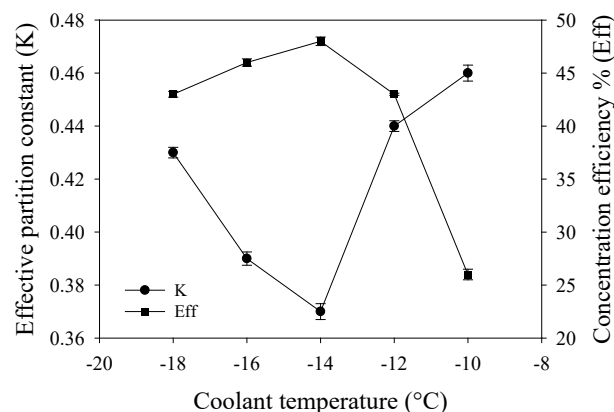
### 2.4 Statistical analysis

Analysis of variance (ANOVA) was carried out to detect any significant differences of error for experiments that were carried out at the studied range for coolant temperature and stirring rate. A *p*-value of  $< 0.05$  was specified to be statistically significant. The correlation coefficient ( $R^2$ ) was determined to verify the accuracy of the curves. Statistical analysis of data was performed with STATISTICA software Version 8.0 Statsoft Inc., USA.

## 3 Results and Discussion

### 3.1. Coolant temperature

In this experimental run, the varying parameter is set to be the coolant temperature. While the cooling time is set to be 20 minutes, the stirring rate is set to be 200 rpm. The range of coolant temperature is set to be within  $-10^\circ\text{C}$  until  $-18^\circ\text{C}$ .



**Fig.2.** Variations in effective partition constant and concentration efficiency at assorted coolant temperatures.

Fig. 2 shows the graph of the impact of cooling temperature to partition constant, K and concentration efficiency. The range of coolant temperature used is between  $-10^\circ\text{C}$  until  $-18^\circ\text{C}$ . The graph shows that K value decreases at temperatures of  $-10^\circ\text{C}$  until  $-14^\circ\text{C}$ , however as the temperature decrease, it begins to rise ( $p < 0.05$ ,  $R^2 = 0.96$ ). The value of K is the lowest (0.37) at  $-14^\circ\text{C}$  which also indicates the highest efficiency. A lower value of K also means that more ice crystal is

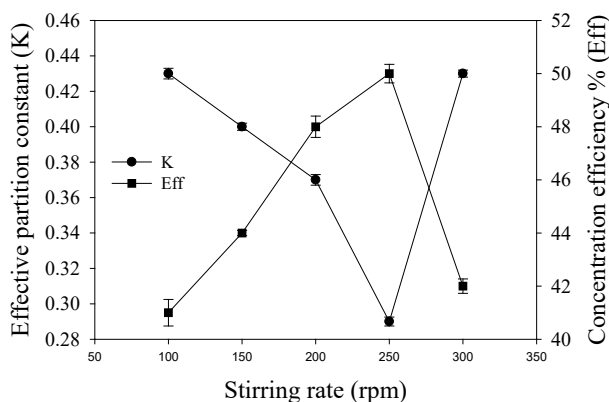
formed and the solutes remains in the liquid phase making the solution concentrated.

Coolant temperature significantly affects the final concentration of solute in progressive freezing [14]. The lower the temperature, the greater the ice formation. As the ice thickness increases, heat transfer efficiency between the coolant and solution will decrease. Coolant temperature also gives a significant impact on the growth of ice crystal [15]. The ice crystal's rate of growth rises along with the rise in the temperature difference between the entering solution and the surface temperature.

In this case, the entering solution is the Roselle extract while the surface temperature is the coolant temperature. Due to this, the ice crystal will trap more solids and later on reduces the purity of the ice. Also, it is possible for the Roselle extract to freeze at sufficiently low coolant temperature. This is because the freezing rate is faster and trapping more impurities in the ice crystal and ultimately decreasing the resultant ice purity. This explains the trend of the increase in the value of  $K$  and the decrease in efficiency at temperatures lower than  $-14\text{ }^{\circ}\text{C}$ .

### 3.2 Stirring rate

The varying parameter in this experimental is set to be the stirring rate. The coolant temperature is set to be constant at  $-14\text{ }^{\circ}\text{C}$  while the cooling time is set to be 20 minutes. The range of stirring rate used is within 100 rpm until 300 rpm.



**Fig. 3.** Variations in effective partition constant and concentration efficiency at assorted stirring rates.

Fig. 3 shows the graph of the impact of the stirring rate to partition constant,  $K$  and concentration efficiency. Stirring in this process is used to ensure that there is uniform distribution of flow along the freezing process. The range of stirring rate used is between 100 rpm to 300 rpm. The graph shows that the value of  $K$  decreased at stirring rate of 100 rpm until 250 rpm, however it increased at 300 rpm ( $p < 0.05$ ,  $R^2 = 0.98$ ).

The existence of shear force caused by high circulation flowrate has an impact on the concentration efficiency. The resultant shear force of the fluid flow can carry away the solute in the solution [16]. The high shear

force will remove the anthocyanins trapped between the dendritic structures of the ice layer and they will remain in the concentrated liquid, which results in a high concentration efficiency. It was found that higher flow rates will promote a slower solidification rate which later on resulted in less concentration captured by the ice crystal.

It is possible that the reason for the increase in value of  $K$  at stirring rate of 300 rpm is due to the stirring rate being too high. When the speed is too high, it is possible that it disturbs the formation of crystal by corroding the ice layer formed at the crystallizer's wall. The liquid phase's concentration is reduced because of the water from the ice layer enter the liquid solution causes the increase in water content.

## 4 Conclusions

Progressive freezing is introduced in this study as a method to purify anthocyanins extract from dried Roselle. The effect of cooling temperature towards the efficiency of the system was studied by varying the value of cooling temperature in the range of  $-10\text{ }^{\circ}\text{C}$  until  $-18\text{ }^{\circ}\text{C}$ . It was found that the lowest value of  $K$  obtained was 0.37 at  $-14\text{ }^{\circ}\text{C}$  which also indicates the highest efficiency. The effect of stirring rate towards the efficiency of the system was also studied by varying the stirring rate value in the range of 100 rpm to 300 rpm. The lowest value of  $K$  obtained was 0.29 at 250 rpm which also indicate sthe highest efficiency.

Financial support offered by Yayasan Universiti Teknologi PETRONAS Fundamental Research Grant (YUTP FRG) through cost centre of 015LC-042 is gratefully acknowledged. The support from the Chemical Engineering Department, UTP as well as the Ministry of Education Malaysia through the HICoE award to CBBR is duly acknowledged.

## References

1. H. E. Khoo, A. Azlan, S. T. Tang, S. M. Lim, Food Nutr. Res. **61**, 1 (2017)
2. T. L. Lin, H. H. Lin, C. C. Chen, M. C. Lin, M. C. Chou, C. J. Wang, Nutr. Res. **27**, 3 (2007)
3. B. Ben Amor, K. Allaf, Food Chem., **115**, 3 (2009)
4. J. Chandrasekhar, M. C. Madhusudhan, K. S. M. S. Raghavarao, Food Bioprod. Process. **90**, 4 (2012)
5. M. Iqbal, Y. Tao, S. Xie, Y. Zhu, D. Chen, X. Wang, L. Huang, D. Peng, A. Sattar, M. A. Hussan, S. Ahmed, Z. Yuan, Biol. Proced. Online **18**, 1 (2016)
6. H. Farahmandazad, Chemistry, (2015)
7. O. Miyawaki, L. Liu, and K. Nakamura, J. Food Sci. **63**, 5 (1998)
8. N. Yahya, Z. Y. Zakaria, N. Ali, and M. Jusoh, J. Teknol. **74**, 7 (2015)
9. N. Yahya, N. Ismail, Z. Y. Zakaria, N. Ngadi, R. A. Rahman, and M. Jusoh, Chem. Eng. Trans. **56** (2017)

10. AOAC, J. AOAC Int., 37.1.68, (2006)
11. L. Jungmin, R. Durst, and R. E. Wrolstad, AOAC 2005.02: Total Monomeric Anthocyanin Pigment Content of Fruit Juices, Beverages, Natural Colorants, and Wines- pH Differential Method. (2005)
12. S. Samsuri, N. A. Amran, L. J. Zheng, and M. M. M. Bakri, Mal. J. Fund. **13**, 4 (2017)
13. E. Hernández, M. Raventós, J. M. Auleda, and A. Ibarz, Innov. Food Sci. Emerg. Technol. **11**, (2010)
14. A. Ojeda, F. L. Moreno, R. Y. Ruiz, M. Blanco, M. Raventós, and E. Hernández, Chem. Eng. Commun. **204**, 8, (2017)
15. N. A. Amran and M. Jusoh, *Proceedings of the 2013 International Graduate Conference on Engineering Science and Humanity Universiti Teknologi Malaysia* (UTM, 2013).
16. M. Jusoh, Development of a Novel System for Progressive Freeze Concentration Process. Ph.D. thesis, Universiti Teknologi Malaysia (2010).