

## Extraction of Betacyanins from *Hylocereus polyrhizus* Peels Using Aqueous Two-Phase System

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### ABSTRACT

*Hylocereus polyrhizus*, known as red dragon fruit provides a betacyanin pigment source, which is not only concentrated in the flesh but also present in the peel with high content. Betacyanins are beautiful red-violet pigments used as food additives and stable in a wide pH range from 3 to 6. However, the stability of betacyanins is affected by heat, which causes discoloration. The aim of this work was to report the extraction of betacyanins from red dragon fruit peels by an aqueous two-phase system (ATPS) to overcome the drawback. By applying the aqueous two-phase extraction (ATPE) method on *Hylocereus polyrhizus* peels, the highest betacyanin yield of 85.07% was obtained in the optimal conditions of ammonium sulfate (12.75%, w/w); ethanol (30.00%, w/w); dragon fruit peel (8%, w/w), and pH 5.0 at 30 oC.

### KEYWORDS

Aqueous two-phase extraction;  
ATPE;  
*Hylocereus polyrhizus*;  
Betacyanin;  
Ethanol;  
Ammonium sulfate.

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## 1. Introduction

Dragon fruit, one among tropical fruits, belongs to *Hylocereus* (*H.*) genus, Cactaceae family. Based on the characteristics of their flesh and skin color, dragon fruits are classified into three types: red-skinned dragon fruit with red-skinned one with red flesh (*H. polyrhizus*), white flesh (*H. undatus*), and yellow-skinned one with white flesh (*H. megalanthus*) [1]. *H. polyrhizus* originated from the tropical and subtropical areas of Mexico and the United States. Up to now, it is grown in many tropical countries such as Australia, Israel, Cambodia, Vietnam, Philippines, China, Taiwan, Thailand, Japan, Nicaragua, Peru, Spain, Sri Lanka, and the United States [2].

Dragon fruit is known as a fruit rich in nutrients, especially vitamin B1/ B2/ B3, vitamin C, phosphorus, calcium, iron, and metabolites as antioxidants, including betalain, carotene, flavonoids, glucose, thiamin, niacin, pyridoxine, kobalamin, phenolic, polyphenol [3]. Betalain from red-flesh dragon fruit juice exhibited antioxidant capacity while non-betalanic phenolic compounds contribute only meagerly [4]. Based on the structural properties, betalains are generally classified into two groups: purple-red-colored betacyanins and yellow-colored betaxanthins [5].

Dragon fruit flesh is often used directly or processed into food products. Dragon fruit peel is considered a residual by-product of consumption and processing of the fruit and is often discarded. Interestingly, the presence of attractively colored betacyanins induced attempts to extract these pigments from the by-product [6]. The peel of red flesh dragon fruit (*H. polyrhizus*) removed during processing accounts for about 22% of the total weight of the fruit and can be reused to increase the value of raw materials. The dragon fruit peel becomes a potential raw material rich in betacyanins as a natural food colorant due to a high content (150.46 mg/100 g dried mass) in its peel [7].

Betacyanin is easily degraded by several factors such as metal, sulfur dioxide, exposure to light, high water activity, enzyme activity, pH, and high temperature [8]. Therefore, its applications in food are relatively limited. The researchers have focused mainly on discovering the stable conditions of betacyanin extraction. One of the modern techniques that can extract and purify betacyanin pigment is aqueous two-phase extraction (ATPE). This method is known to be a large-scale biological separation, which gives

high purity and efficiency, and maintains biological activities [9]. This technique has many advantages such as the ease of implementation, both selective extraction in a short time, and the ease of recovery. However, this technique has not been widely studied in Vietnam. In this work, we reported on the investigations of betacyanin extraction conditions using the ATPE method.

## 2. Material and method

### 2.1 Material and chemicals

#### 2.1.1 Sample preparation

*Hylocereus polyrhizus* was collected in the local market in Long An province, Vietnam. Preparation of *H. polyrhizus* peel paste was performed as follows. The collected peels were cleaned under the tap water flow and then pureed with a food processor (Philips HR3573/90, China). The paste sample was stored in a -18 °C freezer (Arctiko LTF 425, Denmark) for further work.

#### 2.1.2 Chemicals

The analytical grade chemicals were purchased from Xilong Scientific, China.

### 2.2 Methods

#### 2.2.1 Aqueous two-phase extraction

##### 2.2.1.1 Preparation of Aqueous two-phase system

Each experiment was carried out with a particular amount of each component. Therefore, in general, each aqueous two-phase system (ATPS) was prepared according to the following procedure: After dissolving a given mass of salt in a certain amount of distilled water, a pre-determined volume of absolute ethanol was added to the salt solution. The resulted mixture was vortexed and stayed stable until the phase separation equilibration was reached to obtain the expected ATPS.

##### 2.2.1.2 Phase-diagram construction

The phase diagram of a system composed of ethanol and salt was built by a turbidity titration method at room temperature described by Bensch et al. [10] with a slight adjustment described in our previous work [11].

##### 2.2.1.3 Preparation of betacyanin with ATPE

The pureed dragon fruit peel sample was added to a certain ATPS. The mixture was magnetically stirred well (IKA RET control-visc, Germany) at ambient temperature, the residue was discarded by filtration. The whole liquid was placed in the separatory funnel until the two phases were separated. The upper phase was an ethanol-rich solution containing betacyanin while the lower one was a salt-rich solution containing undesired material.

#### 2.2.2 Analytical procedure

##### 2.2.2.1 Determination of betacyanin content (C)

Betacyanin content (C, mg.L<sup>-1</sup>) was determined following Lim's method [12] by measuring the maximum absorbance value at 538 nm using UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan). The betacyanin content was determined by equation (1).

$$C = \frac{A \times (MW) \times (DF) \times 1000}{\epsilon \times L} \quad (1)$$

Here, A was the absorbance value at 538 nm, MW was the betacyanin molecular weight (550 g.mol<sup>-1</sup>); DF was the dilution factor;  $\epsilon$  was the betacyanin molar extinction coefficient at 538 nm ( $6.5 \times 10^4$  L.mol<sup>-1</sup>.cm<sup>-1</sup>) and L was the cuvette path length (1 cm).

##### 2.2.2.2 Determination of extraction efficiency

In order to optimize ATPE conditions for achieving the most extraction efficiency, the one-factor-at-a-time investigations were performed under various conditions of ammonium sulfate concentration (12.75-18.00%, w/w), ethanol concentration (23-30%, w/w), the ratio of sample and ATPS (5-10%, w/w), pH (3.5-6.0), and temperature (30-55°C). In each ATPE experiment, two phases were formed and then separated to determine phase volumes and betacyanin contents. The extraction efficiency was determined based on the parameters including phase volume ratio (R), betacyanin partition coefficient (K), and betacyanin recovery yield (Y).

- i) Determination of phase volume ratio (R)

The phase volume ratio (R) was defined as a ratio of the upper phase volume ( $V_T$ , mL) and the lower one ( $V_B$ , mL) and calculated by equation (2):

$$R = \frac{V_T}{V_B} \quad (2)$$

ii) Determination of partition coefficient (K)

The K value was calculated following (3)

$$K = \frac{C_T}{C_B} \quad (3)$$

Where  $C_T$ ,  $C_B$  ( $\text{mg}\cdot\text{L}^{-1}$ ) were betacyanin contents in the upper and the lower phases, respectively, at an equilibrium state.

iii) Estimation of betacyanin recovery yield (Y)

The betacyanin recovery yield in the upper phase (Y, %) was calculated by equation (4):

$$Y = \frac{C_T \times V_T}{C_T \times V_T + C_B \times V_B} \times 100 \quad (4)$$

### 3. Results and Discussion

#### 3.1 Determination of investigated parameters

##### 3.1.1 Selection of ATPS composition

The choice of ATPS composition was considered from two aspects: the alcohol composition (ethanol and methanol) and the phase-forming salt among commonly used salts. Since the research was targeted at food, methanol was limited. The degradation of betacyanin in the presence of di/trivalent metal cations ( $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Sn}^{2+}$ ,  $\text{Al}^{3+}$ ,  $\text{Cr}^{3+}$ ,  $\text{Cu}^{2+}$ ) was reported by Kuusi [13]. Therefore, the ATPSs in our work were carried out with monovalent metal salts such as  $\text{K}_2\text{HPO}_4$ ,  $\text{K}_2\text{CO}_3$ ,  $(\text{NH}_4)_2\text{SO}_4$ , and  $\text{NaH}_2\text{PO}_4$ .

To obtain the appropriate concentrations of phase composition in each ATPS, it is necessary to build a phase diagram for each salt. The phase diagrams (Fig.1) as binodal curves of four different ATPSs including  $\text{K}_2\text{HPO}_4$ /ethanol,  $\text{K}_2\text{CO}_3$ /ethanol,  $(\text{NH}_4)_2\text{SO}_4$ /ethanol, and  $\text{NaH}_2\text{PO}_4$ /ethanol were investigated by the turbidity titration method.

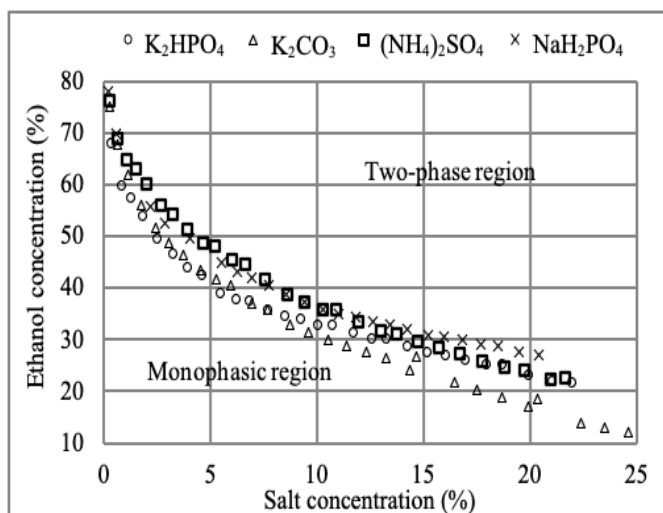
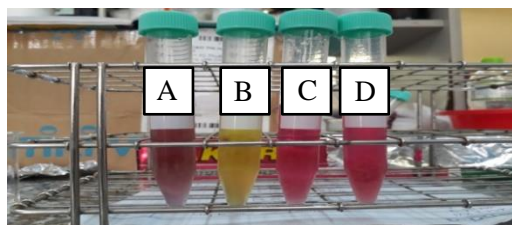


Fig. 1. The phase diagrams of the investigated ATPSs

The binodal curve of each salt separated the composition area of the ATPS into two regions. The phase composition in the above region formed the ATPS with two distinguished phases, and the one in the below region formed a homogenous solution or monophasic area. In the two-phase region, the upper phase was an ethanol-rich aqueous phase and the lower one was a salt-rich aqueous phase [14]. For further experiments, the concentration of phase composition in each ATPS was selected from the neighborhood above the binodal curve in the two-phase region.

The selection of phase-forming salt for the ATPE process resulted from the experimental results. Based on the phase diagram, the ATPSs for each salt were prepared with the dragon fruit peel sample added. The color changes in each experiment were given in Fig.2. The sample displayed a yellow color in  $K_2CO_3$  solution (pH 11.4) while the brown-purple color was obtained in  $K_2HPO_4$  solution (pH 7.2). The degradation of betacyanin in basic solutions has been mentioned by Elbe et al. [15]. In addition, the stability of betacyanin was observed in the solutions containing  $(NH_4)_2SO_4$  (pH 5.0) and  $NaH_2PO_4$  (pH 6.0) which were appropriate for the investigation of Casterllar [16]. Therefore, these salts have been used for further investigation.



**Fig. 2.** The color changes of betacyanins in ATPSs with different phase-forming salts (A– $K_2HPO_4$ ; B– $K_2CO_3$ ; C– $(NH_4)_2SO_4$ ; D– $NaH_2PO_4$ ).

**Table 1.** Effect of various salts in ATPSs on partition coefficient and betacyanin recovery yield

Ethanol/salt system	Salt (g)	Partition coefficient (K)	Recovery yield (Y, %)
$(NH_4)_2SO_4$	2.00	1.19	79.65±0.31
	2.40	1.25	79.39±1.08
	2.80	1.44	76.30±0.42
	3.20	1.22	63.22±0.31
	3.60	1.59	68.73±0.03
$NaH_2PO_4$	2.80	0.62	78.04±0.05
	3.20	0.66	75.41±0.18
	3.60	0.68	64.49±0.28
	4.00	0.68	61.56±0.44
	4.20	0.76	62.37±0.05

The ATPSs containing  $(NH_4)_2SO_4$  or  $NaH_2PO_4$  salt were prepared to evaluate the betacyanin recovery yield. The systems were composed of distilled water (6.0 mL), ethanol (6.0 mL), sample (2.0 g), and a certain amount of salt (see Table 1). As given in Table 1, it was found that the ethanol/ $(NH_4)_2SO_4$  system gave greater betacyanin partition coefficients ( $K > 1$ ) and greater betacyanin recovery yields (Y) compared with the ethanol/ $NaH_2PO_4$  system. The maximum recovery yield of 79.65% for betacyanin extraction demonstrated that the ethanol/ $(NH_4)_2SO_4$  system enhances the capacity of betacyanin extraction. Therefore, this system was selected to prepare the ATPSs for all subsequent extraction experiments.

### 3.1.2 Determination of parameter limits

The extreme phase separation points of the samples in the ATPSs were obtained from experimental results. The experiments were carried out with ATPSs selected along the binodal curve. The concentrations of ethanol and  $(NH_4)_2SO_4$  were calculated based on the phase diagram (Fig.1). The sample percentage of 10% was added to the ATPSs. The phase separation only occurred when the concentration of  $(NH_4)_2SO_4$  was in the range from 12.75% to 18.00% and the concentration of ethanol from 30% to 23%. The ATPSs outside this range cannot give a phase separation and salt was found to be precipitated in these systems. The increase in the concentration of ethanol in ATPS led to the precipitation of excessive ammonium sulfate [17]. This phase separation threshold, in which the concentration of  $(NH_4)_2SO_4$  from 12.75% to 18.00% and the one of ethanol from 23% to 30%, was applied for the further investigated experiments.

To obtain the best betacyanin extraction capacity, the appropriate ATPE condition needed to be established. Four extraction parameters were investigated using a one-factor-at-a-time approach, namely,

phase composition (phase-forming salt and phase-forming ethanol concentrations), sample concentration, pH, and temperature. The higher sample concentration resulted in a less invisible interface. Therefore, the sample concentrations in ATPSs were explored in the range of 5-10% (w/w) referenced from the research by Wu [17]. The pH range from 3.5 to 6.0 was considered due to the relatively stable red colour of betacyanin in the environment below pH 6 [18]. The high degradation rate of betacyanin above 60 °C has been demonstrated by Priatni [1], and the temperature was limited to the range of 30-55 °C.

### 3.2 Effects of the ATPS composition

#### 3.2.1 Effect of phase forming-salt concentration

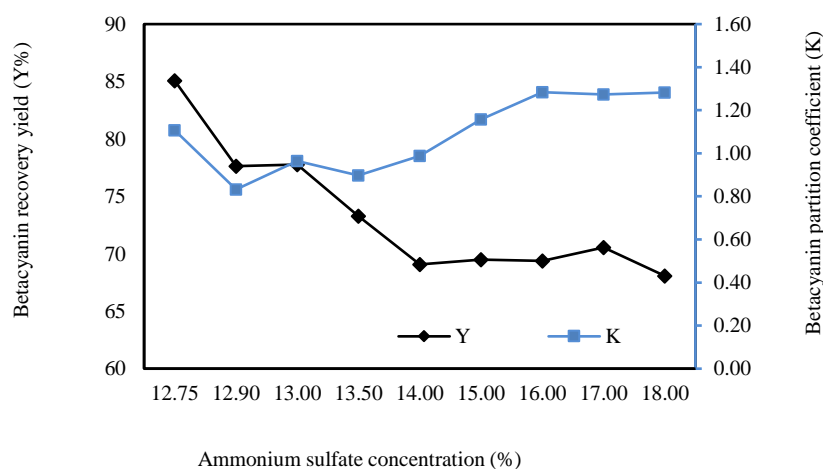
In order to investigate the effects of phase forming-salt concentration on the partition coefficient and the betacyanin recovery yield, the fixed ethanol concentration was 30% while the concentration of  $(\text{NH}_4)_2\text{SO}_4$  varied in the range from 12.75% to 18.00% (w/w), and 10% sample.

The effects of  $(\text{NH}_4)_2\text{SO}_4$  concentrations on the partition coefficient, the betacyanin recovery yield and the phase volume ratio were shown in Table 2 and Fig.3, respectively.

**Table 2.** Effects of  $(\text{NH}_4)_2\text{SO}_4$  concentration on phase volume ratio

$(\text{NH}_4)_2\text{SO}_4$ concentration (%)	Phase volume ratio (R)
12.75	5.15
12.90	4.17
13.00	3.63
13.50	3.06
14.00	2.26
15.00	1.97
16.00	1.77
17.00	1.88
18.00	1.66

The ATPSs composed of the constant ethanol and various ammonium sulfate concentrations from 12.75% (w/w) to 18.00% (w/w) resulted in a decrease in the phase volume ratio from 5.15 to 1.66 and an increase in the K value. The results can be due to the affinity between water and salt. Water moved from the upper phase to the lower one when the salt concentration increased, which resulted in a reduction of the R value. Betacyanins were partitioned in the ethanol-rich phase. As a result, the partition coefficient increased with increasing the salt concentration. The betacyanin recovery yield was found to be reduced from 85.07 to 68.08%. In other words, the increase in ammonium sulfate concentration cannot improve betacyanin extraction efficiency. The betacyanin recovery yield reached the maximum value of 85.07% at the concentration of ammonium sulfate of 12.75% (w/w). Therefore, a salt concentration of 12.75% was selected to investigate the ethanol concentration effect.



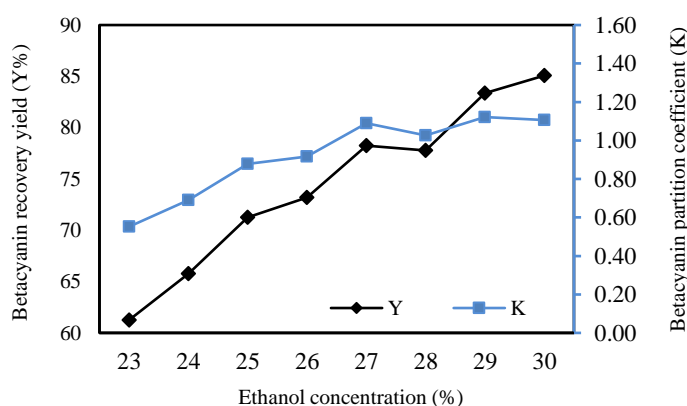
**Fig. 3.** Effect of  $(\text{NH}_4)_2\text{SO}_4$  concentration on partition coefficient and betacyanin recovery yield.

#### 3.2.2 Effect of phase forming-ethanol composition

The influences of phase-forming ethanol concentrations on the betacyanin extraction efficiency were investigated in the condition of a fixed  $(\text{NH}_4)_2\text{SO}_4$  concentration (12.75%) and the concentration of ethanol varying in the range from 23% to 30%, and sample (10%). The effects of ethanol compositions on the R, K values and the betacyanin recovery yield were given in Table 3 and Fig.4.

**Table 3.** Effects of ethanol concentration on phase volume ratio

Ethanol concentration (%)	Phase volume ratio (R)
23	2.86
24	2.78
25	2.83
26	2.98
27	3.30
28	3.41
29	4.46
30	5.15



**Fig. 4.** Effect of ethanol concentration on partition coefficient and betacyanin recovery yield.

When increasing the ethanol concentration in the ATPSs, the partition coefficient of betacyanin in the upper phase increased from 0.55 to 1.11, and the phase volume ratio also enhanced from 2.86 to 5.15. Betacyanin is dissolved well in ethanol. Therefore, the betacyanin concentration was enhanced by increasing the ethanol content. The betacyanin recovery efficiency increased gradually from 61.26% to 85.07% and reached the maximum value of 85.07% when the ethanol concentration was 30% (w/w). Finally, the ethanol concentration in the system was selected as 30% (w/w) to carry out the next survey.

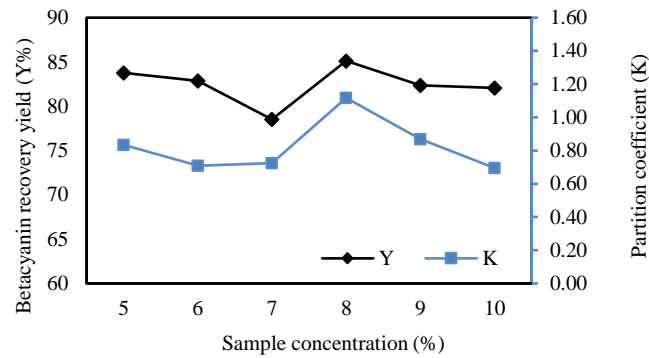
### 3.3 Effect of sample concentration

The effects of sample amount on the betacyanin extraction efficiency were investigated in the system composing of a fixed ethanol (30%) and  $(\text{NH}_4)_2\text{SO}_4$  (12.75%) concentrations, the pre-determined amount of sample (from 5-10%), and water was added to reach a final value of 100%. The experimental observations were displayed in Table 4 and Fig.5.

**Table 4.** Effect of sample concentration and ATPS on phase volume ratio

Sample concentration (%)	Phase volume ratio (R)
5	6.18
6	6.82
7	5.04
8	5.16
9	5.64
10	6.89

The phase volume ratio seems to be unchanged when increasing the sample concentration from 5% to 10%. The maximum partition coefficient and recovery yield of betacyanin were 1.12% and 85.12% at the sample concentration of 8%. The results can be attributed to the saturation of betacyanin in the organic phase at 8%. Therefore, a sample concentration of 8% (w/w) was chosen for the follow-up survey.



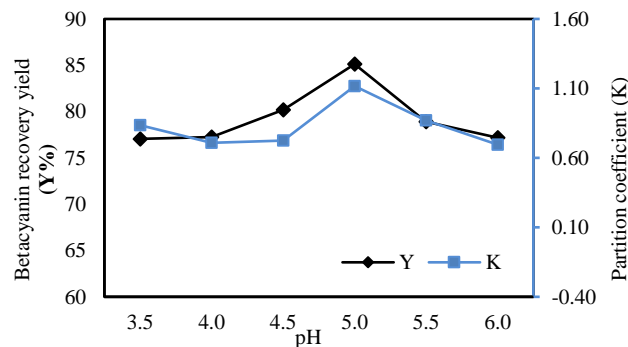
**Fig. 5.** Effect of sample concentration on partition coefficient and betacyanin recovery yield.

### 3.4 Effect of pH

The conditions of ethanol (30%),  $(\text{NH}_4)_2\text{SO}_4$  (12.75%), sample (8%), and water were utilized to investigate the effects of pH values on the betacyanin extraction efficiency in the ATPSs. The pH values were varied in the range from 3.5 to 6.0. It was noted that the pH values of ATPSs were adjusted by 0.1M HCl or 0.1M NaOH. The effects of pH values on the Y (%), R and K values were depicted in Table 5 and Fig.6.

**Table 5.** Effect of pH on phase volume ratio

pH	Phase volume ratio (R)
3.5	3.75
4.0	3.89
4.5	4.20
5.0	5.16
5.5	4.23
6.0	3.83



**Fig. 6.** Effect of pH value in the ATPSs on partition coefficient and betacyanin recovery yield.

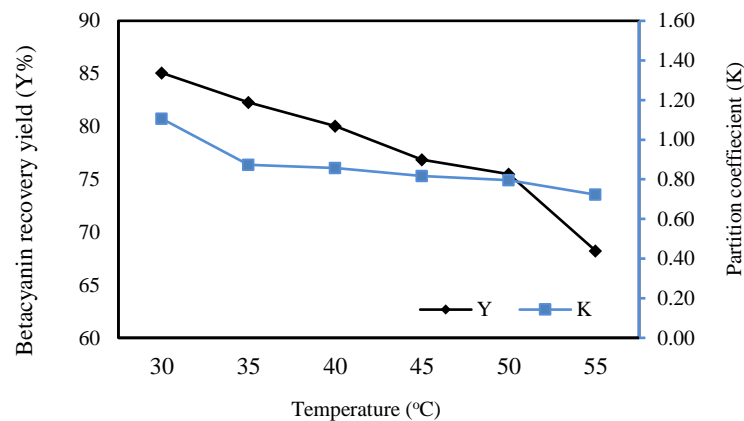
As shown in Table 5 and Fig.6, the phase volume ratio and the partition coefficient were not changed when varying pH. It was reported in the literature that betacyanin can be stable in the pH range from 4.0 to 6.0 [19]. The betacyanin recovery yield reached a maximum value of 85.12% at pH 5.0 and decreased at the pH values higher than 5.0. It can be concluded that betacyanin pigment is stable at the pH value of 5.0 approximately, which is consistent with the reported results [16]. The highest value ( $K=1.12$ ) of the partition coefficient was obtained at pH 5.0. Therefore, the pH of the system as 5.0 was chosen to carry out the next survey.

### 3.5 Effect of temperature

The betacyanin extract efficiency of ATPS was affected by temperature. The ATPS at pH 5.0 of ethanol (30%),  $(\text{NH}_4)_2\text{SO}_4$  (12.75%), and sample (8%) was used to investigate the effects of the temperature on the Y (%), K and R values. Temperature of the APTSs varied in the range from 30 to 55 °C. These values were given in Table 6 and Fig.7.

**Table 6.** Effect of temperature on phase volume ratio

Temperature (°C)	Phase volume ratio (R)
30	5.15
35	5.32
40	4.68
45	4.06
50	3.91
55	2.98



**Fig. 7.** Effect of temperature of ATPS on partition coefficient and betacyanin recovery yield.

When increasing the temperature from 30 to 55 °C, the partition coefficient and betacyanin recovery efficiency tended to decrease from 1.11 to 0.72 and from 85.07% to 68.24%, respectively. The results can be attributable to the betacyanin degradation at high temperatures and the degradation rate was accelerated with increasing temperature and heating time [20]. The maximum values of the betacyanin partition coefficient and the maximum betacyanin recovery efficiency were 1.11 and 85.07% at 30 °C. Therefore, the appropriate temperature for extraction was at 30 °C.

After investigating the effects of parameters on the betacyanin extraction efficiency by the ATPE method, the optimal ATPE conditions were established: the ATPS containing ammonium sulfate (12.75%, w/w), ethanol (30.00%, w/w), sample (8%, w/w), pH 5.0 at 30 °C. The highest betacyanin recovery yield was 85.07%, which corresponds to a betacyanin content of 6.083 mg/100 g of fresh peels. In Jamilah's report on *Hylocereus polyrhizus*, the betacyanin content was 150.46 mg/100 g of dried peel (the moisture 93%) when being diluted with McIlvaine buffer [7], corresponding to 10.53 mg/100 g of fresh peel. In our results, the betacyanin content was found to be less than the results reported by Jamilah. The observation can be originated from the remaining betacyanin in the lower phase. In 2015, Priatni investigated betacyanin extraction from *Hylocereus polyrhizus* at ambient temperature using the maceration method with methanol or water at pH 5.0. The betacyanin contents obtained from methanol and water extractions were 515.20 µg/100 g and 491.16 µg/100 g, respectively [1]. The betacyanin content obtained from our ATPE procedure was improved by 12-fold over the Priatni's result. It was noted that our experiments avoided using methanol, a highly toxic solvent. Therefore, ATPE should be considered as a potential method for betacyanin extraction.

#### 4. Conclusions

The betacyanin pigment from *Hylocereus polyrhizus* was extracted using the ATPE method under the appropriate conditions composing ammonium sulfate (12.75%, w/w); ethanol (30%, w/w); dragon fruit peel paste (8%, w/w), and pH 5.0 at 30 °C. The betacyanin recovery yield was achieved as 85.07% or 6.083 mg/100 g. ATPE can be an extraction method with high potential for application in the food industry due to its simple means of operation and the avoidance of toxic solvents.

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