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# Extraction and separation of anthocyanins from Kushui rose by ethanol-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> aqueous two-phase system†

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Simultaneous extraction of anthocyanins and removal of sugars from Kushui rose was performed using an ethanol-ammonium sulphate aqueous two-phase system (ATPS). The effects of different parameters, such as type of salt, concentrations of salt and ethanol, temperature and pH on the partition coefficient and recovery of anthocyanins in the top system and sugars in the bottom system were studied. Furthermore, an experimental design of a three-level three-factor Box–Behnken design response surface methodology (RSM) was used to obtain optimal extraction conditions. The maximum partition coefficient (5.64) and recovery (78%) of anthocyanins in the top system within the investigated range were obtained at 22% (w/w) concentration of ammonium sulphate, 25% (w/w) concentration of ethanol, pH 5 and 33.5 °C. During the discussion of the main factors, the maximum recovery of sugars reached 70.09%. The HPLC profile of anthocyanins obtained from the ATPS top phase was similar to that of anthocyanins extracted by ethanol, which indicated that the ethanol-ammonium sulphate ATPS was suitable for the extraction of anthocyanins. On the basis of the anthocyanin stability experiment, anthocyanins extracted from Kushui rose should be stored at low pH and temperature.

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## 1. Introduction

In recent years, the exploitation and application of natural pigments have caused widespread attention due to their apparent non-toxicity and safety compared with synthetic pigments.<sup>1</sup> Anthocyanins are water-soluble natural pigments belonging to flavonoids and are responsible for pink, red, violet and blue colors of flowers, vegetables and fruits. Anthocyanins have physiological functions such as antioxidant activity and free radical scavenging, which are important in the prevention of cancer, cardiovascular disease and neurological disease.<sup>2</sup> Therefore, finding good methods to separate anthocyanins effectively is of great significance.

The organic solvent extraction is commonly used for extraction of anthocyanins in plants and the most used solvents are mixtures of ethanol and methanol/acetone because anthocyanins are soluble in polar solvents.<sup>3</sup> However, some drawbacks of this method have emerged, such as low extraction rates, the presence of water-soluble impurities (such as sugars)

in the extract, and the consumption of large amounts of organic solvents. Other extraction methods with more advanced techniques have been reported, such as pressurized solvent extraction, subcritical water extraction and supercritical fluid extraction.<sup>4–6</sup> Limitations of these new methods are demanding more complex process conditions and higher cost in equipment.

As one of the separation methods, aqueous two-phase extraction (ATPE) has been proved to be a biocompatible and efficient technique for the downstream processing of biomolecules. Aqueous two-phase system (ATPS) not only has the function of easy processing, but also exhibits high performance in large-scale recycling of biological products. ATPS has been applied to the separation and purification of proteins,<sup>7,8</sup> enzymes,<sup>9</sup> antibiotics,<sup>10</sup> genetic materials<sup>11</sup> and low molecular weight products.<sup>12,13</sup> The hydrophilic alcohol-salt ATPS has advantages of low viscosity and low cost<sup>14</sup> and shows potential to achieve the desired purification and concentration of the product in a single step.<sup>15</sup> So far, the hydrophilic alcohol-salt ATPS has been applied in the Lithospermic acid B from *Salvia miltiorrhiza* Bunge,<sup>16</sup> lignans from seeds of *Schisandra*<sup>17</sup> and so on. Anthocyanins have been widely found in different kinds of roses<sup>18</sup> and Chinese Kushui rose dominates the domestic market share of more than 50% with high brand awareness. As far as we know, the paper on the hydrophilic alcohol-salt aqueous two-phase extraction of anthocyanins from rose has not been published.

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This study developed a simple aqueous two-phase system for the extraction and separation of anthocyanins from Kushui rose. The anthocyanins could be enriched in the top system while the sugars were enriched in the bottom system of the ATPS. The partition coefficient and recovery of the anthocyanins in this system were optimized. In order to obtain a higher extraction rate, the influences of process parameters, including the type and concentration of salt, the concentration of ethanol, temperature and pH were studied. The concentration of salt/ethanol and temperature were optimized using response surface methodology (RSM). In addition, HPLC analysis and the stability of anthocyanins from Kushui rose were also investigated.

## 2. Experimental

### 2.1. Chemicals and materials

Kushui rose was purchased from Gansu Longcuitang Nutrition Health Products Co., Ltd. (Gansu, China). Methanol (HPLC grade), phenol, sulfuric acid, ethanol, ammonium sulphate, sodium dihydrogen phosphate, potassium chloride, hydrochloric acid were supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Double distilled deionized water was used throughout the experiment and all the chemicals used in the experiment were of analytical grade.

### 2.2. Aqueous two-phase extraction

The Kushui rose petals were ground and sieved by 120 meshes, the powders were collected and subject to the experiment. A predetermined quality of ammonium sulphate solution was mixed with certain volumes of ethanol to obtain a final quality of 10.0 g. An ethanol-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> aqueous two-phase system was prepared. Then 0.1 g rose powders were added into the ATPS, the system was mixed up to form a homogeneous phase. Shaking the tube 2 min, complete phase separation was achieved by low-speed centrifugation at 3000 rpm for 10 min at 35 °C. After centrifugation the mixture was vortexed thoroughly for 2 h and held until the two phases were completely separated. The residues accumulated at the interface of two phases were discarded. The volumes of both top and bottom phases were noted. The anthocyanins and sugars concentrations in both the top and bottom phases were analyzed.

### 2.3. Estimation of anthocyanins and total sugars

The concentration of anthocyanins was determined by a dual-wavelength pH-differential method.<sup>19</sup> Two dilutions were prepared, one for pH 1.0 using potassium chloride buffer (0.03 M, 1.9 g KCl into 980 mL distilled water) and the other for pH 4.5 using sodium acetate buffer (0.4 M, 54.4 g CH<sub>3</sub>-COONa·3H<sub>2</sub>O in 960 mL distilled water). Samples under test were diluted 5 times with the buffer solutions. A UV-visible Spectrophotometer was used for the spectral measurements. With distilled water as blank, the absorbance of each sample was measured at 520 and 700 nm, respectively. The concentrations of Kushui rose anthocyanins were expressed as cyanidin-3-

glucoside (Cy-3-glc, molar extinction coefficient of 26 900) equivalents.

The phenol-sulphuric acid method, based on Dubois,<sup>20</sup> was carried out to determine the content of the total sugar in the phases. An aliquot of 1.0 mL of sample solution was mixed with 1.0 mL of phenol (5%, v/v) and 4.0 mL of sulphuric acid. The absorbance of the mixture, with the characteristic yellow-orange color, was measured at 490 nm. Dextrose was used as a standard for calibration.

The volume ratio (*R*) of top phase and bottom phase in the ATPS was obtained *via* eqn (1):

$$R = V_T/V_B \quad (1)$$

where *V<sub>T</sub>* and *V<sub>B</sub>* represent the volumes of top phase and bottom phase, respectively. The partition coefficient (*K*) of the anthocyanins/sugars was calculated using eqn (2):

$$K = C_T/C_B \quad (2)$$

where *C<sub>T</sub>* and *C<sub>B</sub>* are concentrations of the anthocyanins/sugars in the top and bottom phases, respectively.

The recovery rate *Y<sub>T</sub>* was the ratio of the anthocyanin amounts partitioned in the top phase to the total amount of anthocyanins, and it was calculated using eqn (3). The recovery rate *Y<sub>B</sub>* was the ratio of the sugar amount partitioned in the bottom phase to the total amount of sugars, and it was calculated using eqn (4):

$$Y_T = 100C_TV_T/(C_TV_T + C_BV_B) \quad (3)$$

$$Y_B = 100C_BV_B/(C_TV_T + C_BV_B) \quad (4)$$

### 2.4. Experimental design of RSM

In order to obtain the optimum extraction condition, the effect of ammonium sulphate on the differential partitioning of anthocyanins and sugars in ATPS (with fixed 25% (w/w) ethanol concentration at 25 °C, no pH adjustment) was investigated with different concentrations of ammonium sulphate (from 17% to 22%, w/w). Similarly, the ATPS with varied ethanol concentrations (from 21% to 25%, w/w), under other constant conditions (with fixed 22% (w/w) ammonium sulphate concentration at 25 °C, no pH adjustment), were studied to investigate the influence of ethanol concentration on the partition behaviors. Other parameters of the ATPS were also studied: the temperature was studied in the range of 15 °C to 55 °C, and the pH was tried in the range of 1–5. All the experiments were carried out in triplicate.

RSM was performed to optimize the ATPS conditions for the recovery of anthocyanins from Kushui rose. The independent variables tested were salt concentration (*A*), ethanol concentration (*B*) and temperature (*C*). A Box-Behnken design was selected for the optimization of the process at each of the two levels, with 17 runs including five replicates at the central point.



## 2.5. HPLC analysis

The HPLC system adopted in this work consisted of Agilent 1200 system (Thermo Fisher Scientific, America) equipped with a quaternary pump, surveyor plus detector. Chromatographic analysis was carried out on an Agilent TC-C18(2) column ( $4.6 \times 250$  mm,  $5 \mu\text{m}$ , Shanghai, China). Sample solution was filtrated through a syringe filter ( $0.22 \mu\text{m}$ ) and the injection volume was  $10 \mu\text{L}$ . The mobile phase consisted of water (solvent A) and 100% methanol (solvent B) at a flow rate of  $1.0 \text{ mL min}^{-1}$ . Type of isocratic elution was performed with 10% A and 90% B. The column temperature was set at  $35^\circ\text{C}$  and  $520 \text{ nm}$  was selected as the detection wavelength.

## 2.6. Stability analysis

The stability of anthocyanins from Kushui rose was evaluated at five different pHs (3.0, 4.0, 4.3, 5.0 and 6.0) by respectively fixing the temperature 70, 80 and  $90^\circ\text{C}$ . Anthocyanins concentrate ( $0.2 \text{ g}$ ) from Kushui rose was dissolved with  $50 \text{ mL}$  deionized water (pH 4.3) and four copies of  $50 \text{ mL}$  citrate-phosphate buffers (pH 3.0, 4.0, 5.0, 6.0) in the beakers from which aliquots of  $15 \text{ mL}$  anthocyanins solution were taken out and enclosed in five glass tubes in a thermostatic water bath (pre-heated to a given temperature which varied  $\pm 1^\circ\text{C}$ ), avoiding evaporation and shielded from light. At regular time intervals (0, 1, 3, 6, 9 and 11 h), samples were removed from the water bath and rapidly cooled by plunging into an ice water bath. The concentration of anthocyanins was measured immediately. Experiments were performed at triplicates.

# 3. Results and discussion

## 3.1. Effect of salt type

The anthocyanins are known to stably exist in the form of the flavylium cation at low pH.<sup>21</sup> The phase-forming salt selected should be acidic, ethanol- $\text{NaH}_2\text{PO}_4$  ATPS and ethanol- $(\text{NH}_4)_2\text{SO}_4$  ATPS were compared to discuss the partition coefficient and recovery rate of anthocyanins and sugars from Kushui rose.

In ethanol- $\text{NaH}_2\text{PO}_4$  ATPS and ethanol- $(\text{NH}_4)_2\text{SO}_4$  ATPS, the differential partition of anthocyanins and sugars was studied. The recovery value of anthocyanins is close to that in ethanol- $(\text{NH}_4)_2\text{SO}_4$ . The difference lies in the recoveries of sugars, which are 31.14% and 58.73% in the ethanol- $\text{NaH}_2\text{PO}_4$  ATPS and ethanol- $(\text{NH}_4)_2\text{SO}_4$  ATPS, respectively. Therefore, the ethanol- $(\text{NH}_4)_2\text{SO}_4$  ATPS was used for the extraction of anthocyanins and sugars from Kushui rose.

## 3.2. Effect of salt concentration

The influence of  $(\text{NH}_4)_2\text{SO}_4$  concentration on the partition coefficient and recovery rate of anthocyanins and sugars in the ATPS was shown in Fig. 1. In the case of 25% (w/w) ethanol, the results indicated that partition coefficient and recovery rate of anthocyanins increased with the increase of  $(\text{NH}_4)_2\text{SO}_4$  concentration in ATPS. When the concentration of  $(\text{NH}_4)_2\text{SO}_4$  increased from 17% to 22%, the partition coefficient of anthocyanins increased from 1.06 to 5.81 and recovery rate of

anthocyanins increased from 67.00% to 81.28%. It could be explained that the increase of  $(\text{NH}_4)_2\text{SO}_4$  concentration would lead to the increase of ethanol concentration in the upper phase and the increase of  $(\text{NH}_4)_2\text{SO}_4$  concentration in the bottom phase. On the one hand, the increase of ethanol concentration in the upper phase made anthocyanins favor partitioning towards the upper phase for that anthocyanins were freely soluble in ethanol. On the other hand, the increase of  $(\text{NH}_4)_2\text{SO}_4$  concentration in the bottom phase would enhance the salting-out effect.<sup>22</sup> The anthocyanins were readily dissolved in the ethanol-rich phase (the upper phase) due to the driving force produced by the strengthening of salting-out effect of the salt-rich phase (the bottom phase).

As the salt concentration increased, the partition coefficient of sugars in the ATPS slightly decreased from 0.85 to 0.56 while the recovery rate of sugars increased from 48.1% to 67.97%. This was because sugars were prone to be enriched in the salt-rich phase of ATPS in the presence of water-soluble small molecular organic solvents.<sup>23,24</sup> The recovery rate of sugar reached its maximum when  $(\text{NH}_4)_2\text{SO}_4$  concentration was 22%, therefore this concentration of  $(\text{NH}_4)_2\text{SO}_4$  was chosen for further discussion.

## 3.3. Effect of ethanol concentration

The influence of ethanol on the partition coefficient and recovery rate of anthocyanins and sugars in the ATPS was shown in Fig. 2. In the case of 22% (w/w)  $(\text{NH}_4)_2\text{SO}_4$ , the partition coefficient and recovery rate of anthocyanins increased with the increase of the ethanol concentration in the investigated ATPS. When the ethanol concentration increased from 21% (w/w) to 25% (w/w), the partition coefficient increased from 3.81 to 5.81 and recovery rate of anthocyanins increased from 64.91% to 81.28%.

Similar to the salt effect discussed above, the ethanol concentration in the upper phase would increase when increasing the total addition amount of ethanol. Accordingly,

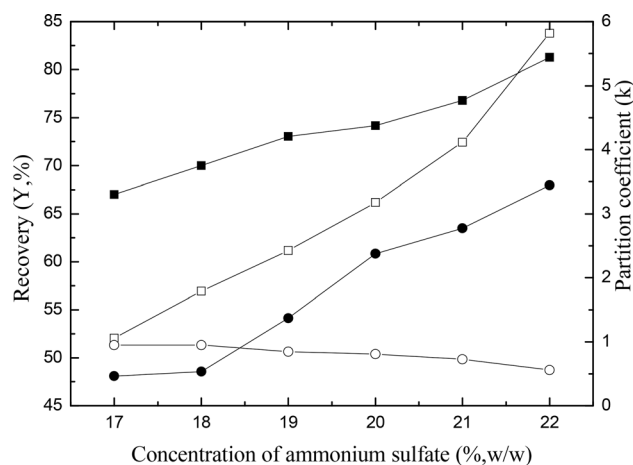


Fig. 1 Effect of the ammonium sulphate concentration on the partition coefficients and the recoveries of anthocyanins and sugars. The solid points represent recoveries, and the empty points are partition coefficients. Symbols: anthocyanins (■, □); sugars (●, ○).



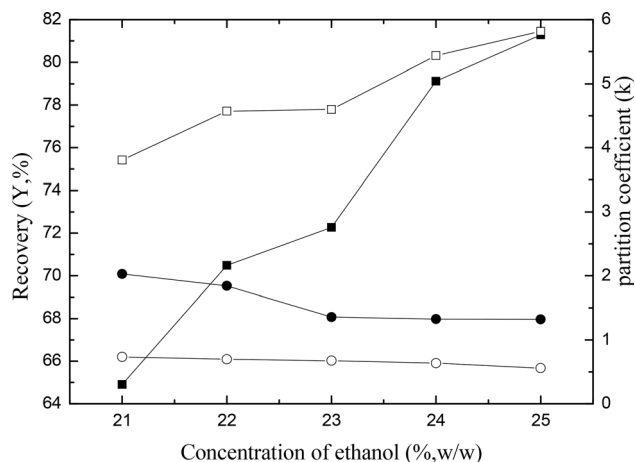


Fig. 2 Effect of the ethanol concentration on the partition coefficients and the recoveries of anthocyanins and sugars. The solid points represent recoveries, and the empty points are partition coefficients. Symbols: anthocyanins (■, □); sugars (●, ○).

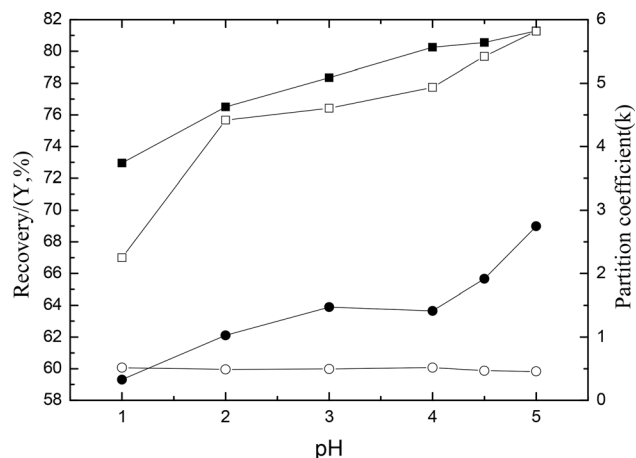


Fig. 4 Effect of pH on the partition coefficients and the recoveries of anthocyanins and sugars. The solid points represent recoveries, and the empty points are partition coefficients. Symbols: anthocyanins (■, □); sugars (●, ○).

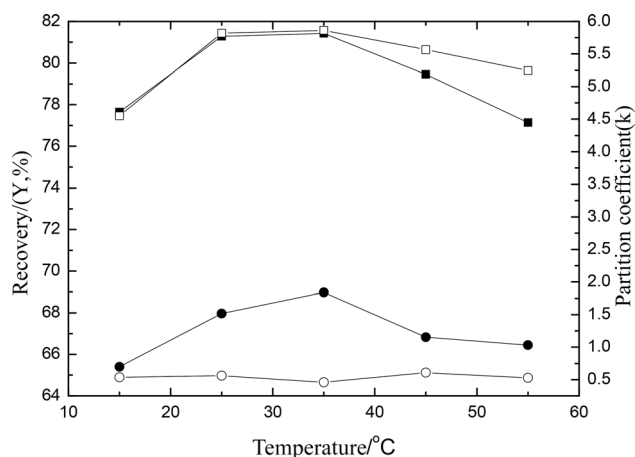


Fig. 3 Effect of the temperature on the partition coefficients and the recoveries of anthocyanins and sugars. The solid points represent recoveries, and the empty points are partition coefficients. Symbols: anthocyanins (■, □); sugars (●, ○).

the concentration of salt in the upper phase decreased but increased in the bottom phase.<sup>22</sup> Due to the strengthening of salting-out effect in the bottom phase and the increase of ethanol concentration in the upper phase, the anthocyanins were readily dissolved in the ethanol-rich phase. The contaminant sugars were also prone to distribute to the bottom phase due to the incompatibility between the water-soluble small molecular organic solvents and the water-soluble mono-saccharides or polysaccharides (such as glucose, glucan) as discussed above.<sup>23,24</sup> Both the partition coefficient of sugars (0.74 to 0.56) and the recovery rate of sugars (70.09% to 67.97%) decreased slightly as the ethanol concentration increased. Thereby, 25% ethanol was finally chosen for the following discussion.

### 3.4. Effect of temperature

Fig. 3 showed the influence of temperature on the partition coefficient and recovery rate of anthocyanins and sugars in the ATPS. It could be observed that the partition coefficient of anthocyanins increased from 4.55 to 5.81 and the recovery rate of anthocyanins increased with the system temperature from 15 °C to 35 °C. When the temperature increased from 35 °C to 55 °C, both the partition coefficient and recovery rate of anthocyanins decreased. The recovery rate of sugars reached maximum and the partition coefficient reached minimum at 35 °C. Increasing the temperature was beneficial to cell wall-broken, which could serve to extract anthocyanins and sugars. However, the anthocyanins would be subjected to a certain damage at high temperature.<sup>25–27</sup> This result indicated that the system temperature would influence the distribution behavior of anthocyanins and sugars in the experimental range and the best temperature was 35 °C.

### 3.5. Effect of pH

The influence of pH on the partition coefficient and recovery rate of anthocyanins and sugars in the ATPS was investigated and shown in Fig. 4. If the pH was high, the anthocyanins would degrade rapidly. Thus, the pH of the ATPS was tested in the range of 1–5. The data indicated that the recovery rate of anthocyanins increased (72.96% to 81.28%) and the partition coefficient of anthocyanins increased (2.25 to 5.82) with increasing pH (1 to 5). The partition coefficient of sugars decreased (0.52 to 0.46) slightly while the recovery rate of sugars increased (59.31% to 68.98%). Therefore, the optimal experimental conditions were obtained with a pH value of 5 within the pH range discussed in this experiment.

### 3.6. Modeling of the ATPS process from Kushui rose

Based on the discussion on the single factor experiment and the influence of various factors on the partition of anthocyanins,



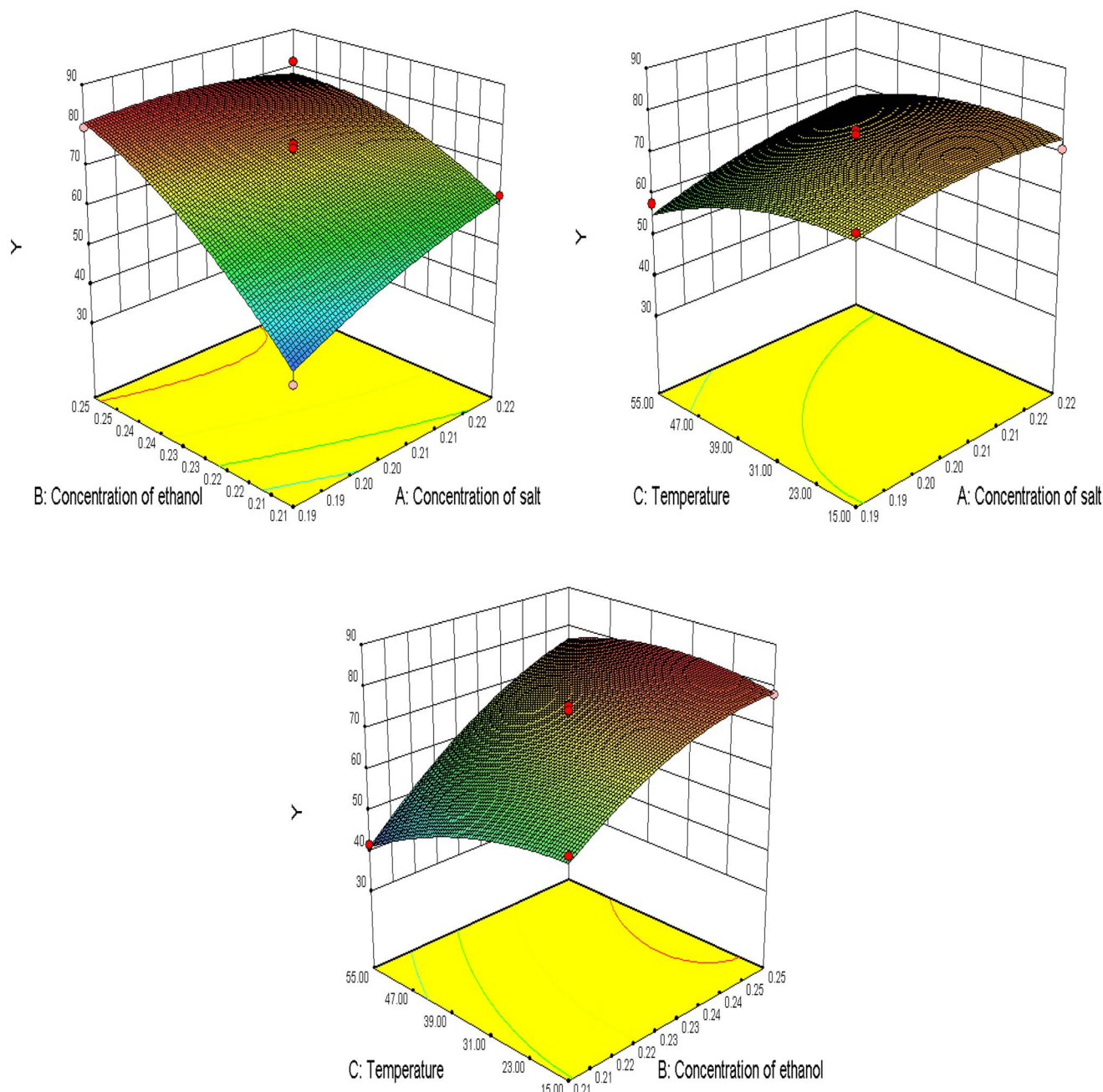


Fig. 5 Response surface plots for the yield of anthocyanins.

a Box–Behnken design was selected for the optimization of the process at each of the three levels, with 17 runs including five replicates at the central point. The recovery rate and partition coefficient of anthocyanins were identified as response value, and concentration of ammonium sulphate, concentration of ethanol and temperature were independent variables. And the experimental yields of three markers under various experimental conditions were presented in Table S1.†

The ANOVA result of response surface quadratic model for the recovery rate of anthocyanins are shown in Table S2,† the regression model established by partition coefficient is significant ( $P < 0.01$ ), and  $R^2$  of the model is 0.9707 which indicates

that the model shows satisfactory accuracy in fitting the experimental data. Lack of fit value  $P = 0.1412$  which indicates the lack of fit for the equation is not significant, and the model can be used to theoretical prediction for the partition coefficient of anthocyanins. In this case  $A$ ,  $B$ ,  $C$ ,  $AB$ ,  $BC$ ,  $B^2$ ,  $C^2$  are significant model terms.  $AC$  and  $A^2$  are not significant model terms.

The multiple regression analysis between recovery rate  $Y$  and independent variables was performed on the experimental data and the result was shown in eqn (5):

$$Y = 72.84 + 4.14A + 13.74B - 5.06C - 5.44AB + 2.16AC + 4.08BC - 2.32A^2 - 4.87B^2 - 4.03C^2 \quad (5)$$



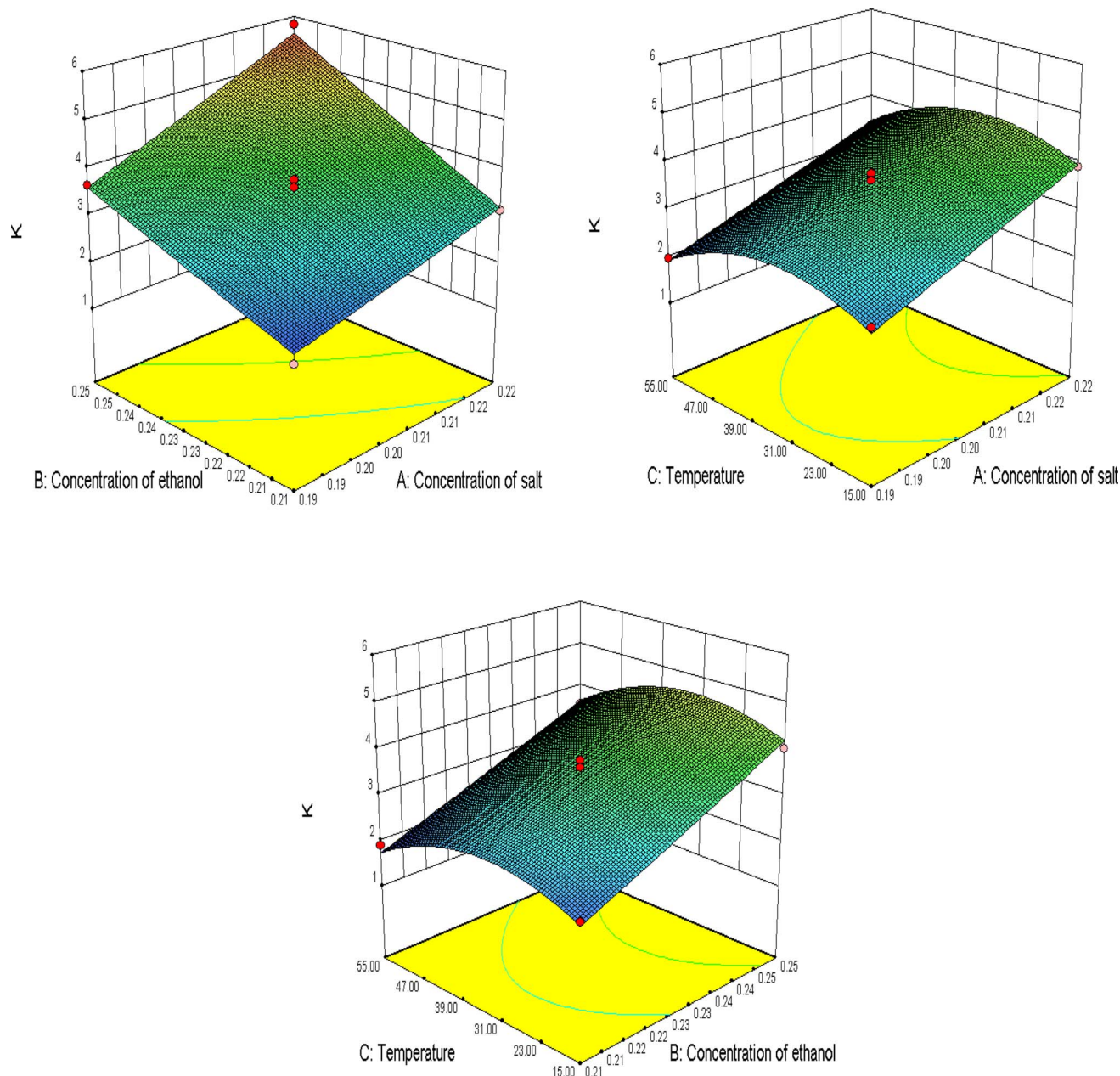


Fig. 6 Response surface plots for the partition coefficient of anthocyanins.

The 3D plots (Fig. 5) were constructed to predict the relationships between independent variables and dependent variables. It shows that the yield of anthocyanins increased with the increase in concentrations of salt and ethanol with the investigated range. The three factors influenced the partition coefficients are in the order: concentration of ethanol > concentration of salt > temperature.

The experimental values of the partition coefficients of anthocyanins were also presented in Table S3,<sup>†</sup> and multiple regression analysis between partition coefficient  $K$  and independent variables was performed on the experimental data and the result was shown in eqn (6):

$$K = 3.59 + 0.78A + 1.00B - 0.23C + 0.22AB - 0.013AC - 0.029BC + 0.01A^2 + 0.017B^2 - 0.68C^2 \quad (6)$$

The ANOVA result of response surface quadratic model for the partition coefficients of anthocyanins is shown in Table S3,<sup>†</sup> the regression model established by partition coefficients is significant ( $P < 0.01$ ), and  $R^2$  of the model is 0.9853 which indicates that the model shows satisfactory accuracy in fitting the experimental data. Lack of fit value  $P = 0.1412$  which indicates the lack of fit for the equation is not significant, and the model can be used to theoretical prediction for the partition coefficients of anthocyanins. In this case  $A$ ,  $B$ ,  $C$ ,  $AB$ ,  $C^2$  are



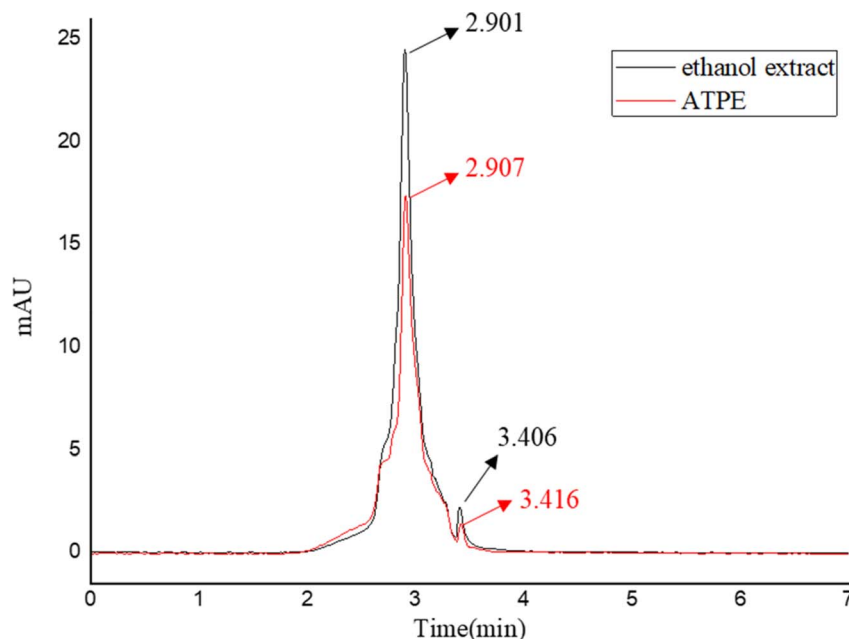


Fig. 7 HPLC chromatograms of ethanol extract and ATPE of anthocyanins from Kushui rose.

significant model terms.  $AC$ ,  $BC$ ,  $A^2$ ,  $B^2$  are not significant model terms.

3D plots (Fig. 6) were constructed to predict the relationship between independent variables and dependent variables. It can be seen that the partition coefficients of anthocyanins increase with the increase in concentrations of salt and ethanol within the investigated range. The three factors influencing the partition coefficient are in the order: concentration of ethanol > concentration of salt > temperature.

Due to the partition coefficient and the yield of anthocyanins are both important to the extraction of anthocyanins from Kushui rose in order to obtain the best extraction efficiency, the partition coefficient and the yield of anthocyanins are observed corresponding to its maximum value. The results from the software indicated that optimized condition for anthocyanins extraction occurred when the concentration of ammonium sulphate, concentration of ethanol, and the temperature were set at 22% (w/w), 25% (w/w) and 33.5 °C, respectively, giving a partition coefficient of anthocyanins, 5.64 and the yield of anthocyanins, 78%. Therefore, the optimization model of the BBD is able to predict the average partition coefficient and sublation yield of anthocyanins.

### 3.7. HPLC analysis

The HPLC results of the Kushui rose extraction obtained by ATPE and conventional extraction were shown in Fig. 7. Two peaks were found on each chromatogram. The HPLC profiles of anthocyanins extracted by the two methods were quite similar as well as the retention time of every peak labeled in Fig. 7, which confirmed that the anthocyanins extracted by ATPE and ethanol had no obvious change.

### 3.8. Kinetics of Kushui rose anthocyanins degradation

Previous studies have shown that thermal degradation of anthocyanins followed a first-order reaction which was expressed by the following equations:<sup>28,29</sup>

$$\ln(C_t/C_0) = -k \times t \quad (7)$$

$$t_{1/2} = -\ln 0.5/k \quad (8)$$

where  $C_0$  is the initial anthocyanin contents,  $C_t$  is the anthocyanin contents after time  $t$  (h) of heating at given temperature,  $k$  is the first order kinetic constant and  $t_{1/2}$  is the half-life of anthocyanins contents during heating.

The temperature-dependence degradation rate constant was represented by the Arrhenius equation:

$$\ln k = \ln k_0 - E_a/RT \quad (9)$$

where  $k_0$  is the frequency factor (per hour),  $E_a$  is the activation energy ( $\text{kJ mol}^{-1}$ ),  $R$  is the universal gas constant ( $8.314 \text{ J mol}^{-1} \text{ K}^{-1}$ ) and  $T$  is the absolute temperature (in Kel-vin, K).

It was clear from Fig. 8 that the thermal degradation of anthocyanins from Kushui rose followed first order reaction kinetics with respect to temperature. The kinetic parameters of Kushui rose anthocyanins degradation during heating (70, 80, 90 °C) at different pH levels are shown in Table 1. At the same pH levels, an increase of anthocyanins in the constant ( $k$ ) and a corresponding decline in the half-life values ( $t_{1/2}$ ) with increasing heating temperature were found, which suggested that anthocyanins from Kushui rose were unstable as the temperature increased. Anthocyanins were known to be more stable at lower pH, an increase in pH may result in reduction of pigment stability. Increasing the pH from 3.0 to 6.0 hastened



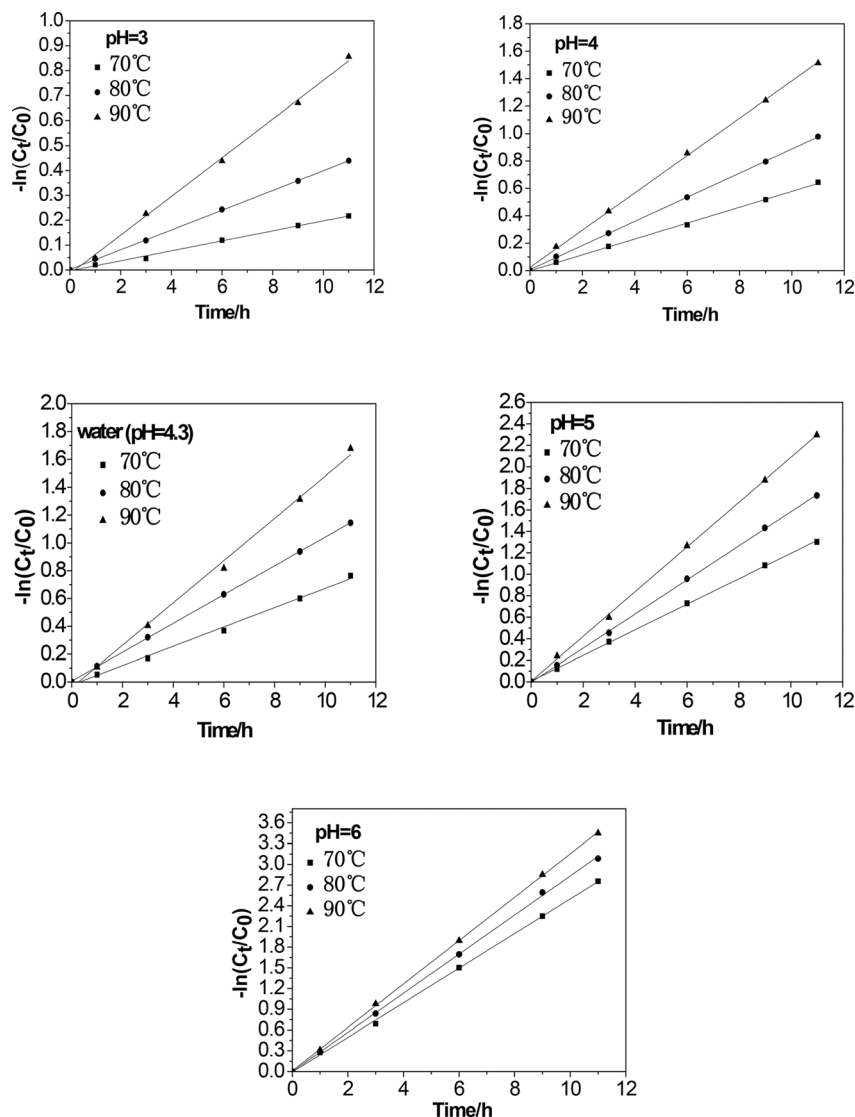


Fig. 8 Degradation of anthocyanins from Kushui rose during heating at different pH levels.

Table 1 Degradation kinetics of anthocyanins extracts

pH	$k$ ( $\text{h}^{-1}$ )			$t_{1/2}$ (h)			$E_a$ ( $\text{kJ mol}^{-1}$ )
	70 °C	80 °C	90 °C	70 °C	80 °C	90 °C	
3	0.02 $\pm 0.006$	0.040 $\pm 0.009$	0.078 $\pm 0.013$	34.6 $\pm 2.5$	17.5 $\pm 2.0$	8.9 $\pm 1.0$	70.40 $\pm 1.41$
4	0.058 $\pm 0.010$	0.088 $\pm 0.014$	0.136 $\pm 0.025$	12.0 $\pm 1.3$	7.9 $\pm 0.9$	5.1 $\pm 0.6$	44.33 $\pm 0.87$
4.3	0.069 $\pm 0.012$	0.104 $\pm 0.019$	0.152 $\pm 0.028$	10.0 $\pm 1.1$	6.7 $\pm 0.8$	4.6 $\pm 0.5$	40.78 $\pm 0.82$
5	0.119 $\pm 0.022$	0.159 $\pm 0.031$	0.208 $\pm 0.043$	5.8 $\pm 0.7$	4.4 $\pm 0.5$	3.3 $\pm 0.4$	29.10 $\pm 0.58$
6	0.251 $\pm 0.039$	0.283 $\pm 0.044$	0.314 $\pm 0.047$	2.8 $\pm 0.3$	2.4 $\pm 0.2$	2.2 $\pm 0.1$	11.73 $\pm 0.22$

the degradation of Kushui rose anthocyanins, which showed pH had a strong influence on the stability of Kushui rose anthocyanins (Table 1 and Fig. 8). With the increase of pH value, the

constant ( $k$ ) increased, while the half-life values ( $t_{1/2}$ ) declined, in other words, the degradation of Kushui rose anthocyanins accelerated as the pH increased at the same heating temperature. When it comes to the anthocyanin stability, reactions that altered their structures may occur because of the electronic deficiency of their flavylum nuclei. Anthocyanins stability increases with the number of methoxyl in the B ring and decreases as hydroxyls increase.<sup>30</sup> In a word, Kushui rose anthocyanins are unstable to high temperature or high pH conditions.

The  $t_{1/2}$  values of Kushui rose anthocyanins extracted by the ethanol-( $\text{NH}_4$ )<sub>2</sub>SO<sub>4</sub> ATPS were greater than those of black rice anthocyanins and blackberry anthocyanins reported at similar pH and similar temperature, which indicated that anthocyanins extracted from Kushui rose by ethanol-( $\text{NH}_4$ )<sub>2</sub>SO<sub>4</sub> ATPS were more stable.<sup>28,29</sup> It has been proven that the degradation rate of anthocyanins is related to the Maillard reaction of sugars to be degraded into furfural compounds.<sup>31,32</sup> The Maillard reaction





becomes strong at high temperatures, making the auxiliary color complexes of pigments unstable.<sup>32</sup> In this work, ethanol-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> ATPS could extract and separate anthocyanins by removing the majority of sugars, which reduced the Maillard reaction and improved the stability of anthocyanins extracted from Kushui rose by ethanol-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> ATPS during heating.

The calculated  $E_a$  values for Kushui rose anthocyanins concentrate in aqueous and buffer solutions (pH 3.0–5.0) were 70.40, 44.33, 40.78, 29.10, 11.73 kJ mol<sup>-1</sup> (Table 1). The  $E_a$  values decreased when pH changed from 3.0 to 5.0. The  $E_a$  values at pH 3.0 were highest which indicated that it was the most stable at pH 3.0. Consequently, higher  $E_a$  meant slower anthocyanins degradation, which also proved that Kushui rose anthocyanins in lower pH environment were more stable.

## 4. Conclusions

The present study has demonstrated the feasibility of using ATPS for the simultaneous extraction of anthocyanins and removal of sugars from Kushui rose. That the ethanol-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> ATPS has a unique advantage in terms of separation of anthocyanins and sugars needs to be particularly emphasized. The concentrations of salt and alcohol, temperature and pH discussed within investigated range produced effects on the partition behaviors of anthocyanins and sugars. Furthermore, optimal experiment was performed based on the BBD. The maximum partition coefficients (5.64) and recoveries (78%) of anthocyanins in the top system within investigated range were obtained at 22% (w/w) concentration of ammonium sulphate, 25% (w/w) concentration of ethanol, pH 5 and 33.5 °C. On the basis of HPLC analysis, the ethanol-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> ATPE was proved to be a suitable method for extraction of anthocyanins with structure unchanged. Finally, the thermal degradation of the Kushui rose anthocyanins corresponding to first-order kinetics implied that high temperature or pH should be avoided when storing or processing the anthocyanins extracted. In addition, anthocyanins extracted from Kushui rose were more stable than black rice anthocyanins and blackberry anthocyanins, so the superiority of separating anthocyanins and sugars by the ethanol-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> ATPE was further reflected through the thermal degradation experiment.

## Data availability

All data generated or analyzed in this study are included in this published article.

## Author contributions

Yuanyuan Li: conceptualization, methodology, formal analysis, data curation, writing – original draft, writing – review & editing. Tongyu Li: writing – review & editing. Hongxu An: investigation, validation, formal analysis. Xinyi Wang: formal analysis, data curation. Juan Han: investigation, resources, funding acquisition. Yun Wang: supervision, project administration, funding acquisition.

## Conflicts of interest

The authors declared that there are no conflicts of interest.

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