Aqueous two-phase system for the extraction of amygdalin from the debitterized water of apricot kernels

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ABSTRACT

Aqueous two-phase system (ATPS) is regarded as an effective and eco-friendly technique to extract some bioactive compounds. In order to promote the added value and reduce the discharge in the industrial processing of apricot kernels, the aqueous two-phase extraction conditions were optimized with the evaluation of the distribution coefficient (K), ratio of volume (R) and extraction yield (Y) to obtain a higher extraction of amygdalin from the debitterizing water concentrate (DWC) in this paper. The results indicate that the optimal conditions were as follows: 30% (w/w) ethanol and 20% (w/w) ammonium sulfate of the ATPS, at 35°C and pH of 7. Under these conditions, the highest extraction yield was 90.37% and the purity could be increased by more than two times. In a word, the ATPS can be considered as an effective method to recycle the amygdalin from the DWC in the apricot kernels processing.

1. Introduction

Apricot (Prunus armeniaca L.) is included in the genus prunus of the subfamily Prunoideae in the family Rosaceae, mainly distributed in the Central and West Asia, and Western China. Statistical Database shows that the global production of apricots has increased about 1.3 million tons during the last decade (FAOSTAT, 2016). Apricot kernels are the seeds of the apricot and contain rich carbohydrates, proteins, polyphenols flavonoids and amygdalin, etc. (Sharma, Gupta, Abrol, & Joshi, 2014), which has been reported to cause acute and sub-acute health problem due to its once higher intake (Alexander et al., 2006), and the main reason can be attributed to the toxic hydrogen cyanide (HCN) generated from the hydrolysis of amygdalin in the apricot kernels (Zhang, Song, Wang, Zhao, & Fan, 2016). In addition, the amygdalin greatly contributes to the bitterness of the apricot kernels, so it is essential to remove bitterness before consumption. There are several literatures about the removal of bitterness, i.e. the de-toxification or debitterizing, and the mostly employed conventional debitterizing method in industry is to immerse the apricot kernels with hot water (60–70°C) for 6–7 h till no bitterness tasted by sensory evaluation (Song, Zhang, Fan, & Zhang, 2017). However, the processing with longer maceration time and higher temperature of water may cause great amounts of water-soluble substances such as protein, amygdalin and phenolic compounds to be transferred to the debitterizing water, thus resulting in the great loss of nutritional compounds and the environmental pollution issues Zhang, Wei, Fan, & Shi, 2018. The amygdalin, the discharged compounds during the debitterizing processing of apricot kernels, is considered having perfect health promotions such as regulating the glucose level of serum (Hill & Backer, 2010), reducing the risk of some chronic symptoms and preventing certain forms of cancer (Guo, Wu, Sheng, Yang, & Tan, 2013). So it is very essential to think about its recycling from the debitterizing water and

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utilization in health food. It is noteworthy that the amygdalin itself is nontoxic, but the HCN with higher content might be toxic, which is one of the degraded products from amygdalin.

Aqueous two-phase system (ATPS) refers to mix two kinds of water-soluble substances, in a certain proportion, and is spontaneously separated into a mutually incompatible two-phase system (Madeira et al., 2013). Compared with the existing separation approaches, ATPS possesses many advantages including low cost, easy scale-up and continuous operation, and eco-friendly, which makes it an attractive method for the isolation of some bioactive compounds like proteins, macromolecules, enzymes, antibiotics, phenols, and amino acids (Babu, Rastogi, & Raghavarao, 2008; Du, Yu, & Wang, 2010; Han et al., 2011; Ketnawa, Rawdkuen, & Chaivut, 2010; Li et al., 2016; Wang, Han, Xu, Hu, & Yan, 2010). As a novel and eco-friendly extraction approach, it is reported that the efficiency of ATPS can be affected by many factors including the nature and concentration of the polymers, temperature, salt type and concentration, and the pH (Dembczynski, Bialas, & Jankowski, 2010; Raghavarao, Ranganathan, Srinivas, & Barhate, 2003; Sankaran et al., 2018). Moreover, the different compositions to be investigated can also influence the extraction rate. In a word, the parameters about the ATPS conditions should be optimized in order to achieve a higher yield of being isolated compounds.

However, to the best of our knowledge, there is no available literature about the recycling of the amygdalin with the ATPS from the discharged debitterizing water in the apricot kernels processing industry by now. In this paper, the main aim of the study was to investigate the factors of ATPS affecting the extraction yield of amygdalin to reduce the loss of nutritional compounds, the emission of polluted water and promote the added-value of processing for the apricot kernels.

2. Materials and methods

2.1. Materials

The debitterizing water concentrate (DWC) was kindly provided by the Shaanxi Tianshou almond food Co., Ltd (Shaanxi province, China), and its main properties were already described by our Lab (Zhang et al., 2018), and the suitable solution was diluted and prepared before use with the DWC.

2.1.1. Chemicals

Standard amygdalin, anhydrous ethanol, protocatechuic acid, chlorogenic acid were purchased from the Chengdu Pered Bio-Technology Co., Ltd. (Sichuan Province, China). Methanol (Chromatographic purity) was purchased from Fisher Scientific Co., Ltd. All the other reagents were of analytical grade. The solutions were all prepared with deionized water as the solvent.

2.2. Screening of inorganic salts for the ATPS composition

Aqueous two-phase systems were made by mixing the inorganic salt solutions with anhydrous ethanol to obtain a total system composition according to the phase diagrams. To be specific, absolute ethanol was added to the solutions contained with quantities of the ammonium sulphate (NH₄)₂SO₄, potassium hydrogen phosphate (K₂HPO₄), dipotassium hydrogen phosphate (KH₂PO₄), sodium carbonate (Na₂CO₃), sodium sulfate (Na₂SO₄), sodium chloride (NaCl), respectively (Chen, Dong, Yu, & Jiao, 2013; Wang, Jia, Liu, Li, & Liu, 2012). All the mixed solutions were shaken and stored at room temperature.

2.3. Diagram of the ATPS

Aqueous two phases system can be formed when the concentration reaches a certain proportion, and the phase diagram could be conducted by the turbidity method (Alhamouz & Ali, 2013). Taking the (NH₄)₂SO₄ solution of 50% (g/g) as an example, the anhydrous ethanol was slowly added into the (NH₄)₂SO₄ solution till the solution got turbidity, and the volume of added ethanol was recorded as mi. Followed by the addition of distilled water to the cloudy solution to make it clear again, then the added volume of water was recorded as mj. A series of cloudy points were achieved by continuously conducting the above operations, then the phase diagram of EtOH and (NH₄)₂SO₄ was depicted on the basis of all the cloudy points. The picture of the clear and cloudy point during the formation of ATPS was shown in the supporting file 1.

2.4. Determination of some parameters related to ATPS

All the systems were fixed at the total mass of 10 g for the solution, and the contents of (NH₄)₂SO₄ and EtOH were all expressed as the unit of g/100g solution. Specifically, a certain number of (NH₄)₂SO₄ and EtOH were added to the solution of DWC, and the total weight was supplemented by adding water to 10 g. The same composition was made with water replacing DWC solution as the control group. Then, the solutions were shaken and centrifuged for 20 min and kept stable in a water bath at 25°C for some time. Finally, the volumes of the two phases were read and the contents of amygdalin in each phase were also determined by HPLC. The extraction efficiency was evaluated by the parameters calculated by the following formulas:

The distribution ratio (R) of the volumes between the two phases was calculated and expressed as R:

\[ R = \frac{V_1}{V_2} \]

where \( V_1 \) and \( V_2 \) stand for the equilibrium volume in the top and bottom phase, respectively.

The distribution coefficient (K) stands for the amygdalin content distributed in the two phases and is given as:

\[ K = \frac{C_1}{C_2} \]

where \( C_1 \) and \( C_2 \) are equilibrium amygdalin concentrations in the top and bottom phase, respectively.

The extraction yield (\( Y(%) \)) of the amygdalin in the ATPS is given according to the equation:

\[ Y(\%) = \frac{C_1V_1}{(C_1V_1 + C_2V_2)} \times 100\% \]

2.5. Determination of amygdalin by HPLC

The content of amygdalin by the treated sample was analyzed by a modified method as previously described (Koo et al., 2005). The amygdalin content was determined by the Elite HPLC (Dalian
Elite Analytical Instrument Co. Ltd., China) equipped with a P230II binary pump, a Rheodyne injector (loop, 20 μL) and a UV230II detector (Elite). Chromatograms were recorded by the EC2006 software (Elite). Samples were separated on a column of TC-C18 (5.0 μm, 4.6 mm×250 mm, Agilent, USA). All the mobile phases were ultrasonically degassed for 25 min and filtered through a 0.45 μm membrane prior to use. An isocratic elution method was applied for the separation of amygdalin with the mobile phases of Methanol-H2O (28:72; v/v), 1.0 mL/min of flow rate, 35°C of column temperature. The detection wavelength was set at 214 nm, and the injection volume was 20 μL. Each sample was performed duplicates.

Figure 1. Flow diagram of the amygdalin extraction by aqueous two-phase system (ATPS).

Figura 1. Diagrama de flujo de la extracción de amigdalina por el sistema acuoso de dos fases (ATPS).

Figure 2. Phase diagrams of ATPS with EtOH-(NH4)2SO4, EtOH-K2HPO4 and EtOH-K2HPO4.

Figura 2. Diagramas de fase de ATPS con EtOH-(NH4)2SO4, EtOH-K2HPO4 y EtOH-K2HPO4.

Table 1. Distribution comparison of amygdalin in ATPS composed of EtOH-(NH4)2SO4, EtOH-K2HPO4 and EtOH-K2HPO4.

| Composition          | K value       | R value       | Y value (%) |
|----------------------|---------------|---------------|-------------|
| EtOH-(NH4)2SO4       | 3.76 ± 0.07a  | 1.83 ± 0.05a  | 89.43 ± 0.59a |
| EtOH-K2HPO4          | 3.89 ± 0.02b  | 0.91 ± 0.02b  | 75.96 ± 1.44b |
| EtOH-(NH4)2SO4       | 7.68 ± 0.31a  | 0.73 ± 0.06  | 84.16 ± 0.42b |

Note. Analyses were conducted in triplicate and results are listed as mean ± standard deviation (SD). Different superscripts in the same column mean the significant differences at p < 0.05.

Nota. Los análisis se realizaron por triplicado y los resultados figuran como la media ± desviación estándar (DE). Los diferentes superíndices en la misma columna indican diferencias significativas en p < 0.05.
2.6. Effect of the content of (NH$_4$)$_2$SO$_4$ and EtOH in ATPS on the extraction of amygdalin

Based on the above screened compounds for the ATPS, their appropriate contents were investigated in this section. To be specific, the content of (NH$_4$)$_2$SO$_4$ in the ATPS was fixed at a designated value, while the content of ethanol was changed within a certain range, then the parameters of R, K and Y were calculated to optimize the suitable content of ethanol for extracting the amygdalin. Regarding the screening of (NH$_4$)$_2$SO$_4$ content, the same procedure was conducted as that of the ethanol mentioned above.

2.7. Effect of pH on the extraction yield (Y) in ATPS

Generally, the different values of pH might influence the existing form, polarity and distribution of the compounds in the two phases. In the meantime, Chang also reported that the amygdalin was more stable when the pH range

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**Figure 3.** Effect of the EtOH and (NH$_4$)$_2$SO$_4$ concentration on the value of (A) R, (B) K, (C) Y, and (D) crystallization point.

**Figura 3.** Efecto de la concentración de EtOH y (NH$_4$)$_2$SO$_4$ en el valor de (A) R, (B) K, (C) Y y (D) el punto de cristalización.
was of 6.0–7.0 (Chang, 2013). Considering this situation, the pH range of ATPS was adjusted from the value of 5.0–8.0 with the KH$_2$PO$_4$-NaOH solution in order to increase the extraction of amygdalin and reduce the possible degradation.

2.8. Effect of temperature on the extraction yield ($Y$) in ATPS
A suitable amount of EtOH and (NH$_4$)$_2$SO$_4$ was added to the DWC solution and magnetically stirred for about 30 min within 25–65°C. The extraction yield ($Y$) of amygdalin was then investigated to explore the temperature’s influence.

2.9. Thermodynamic analysis about the extraction of amygdalin by ATPS
To investigate the extraction mechanism of amygdalin by the ATPS, the thermodynamic parameters such as the Gibbs energy, enthalpy and entropy were calculated according to the following equations during the extraction of amygdalin in the EtOH-(NH$_4$)$_2$SO$_4$ two-phase system at the given temperatures from 25°C to 65°C (Pei, Wang, Wu, Xuan, & Lu, 2009; Sé & Aznar, 2002).

\[
\Delta G = -RT \ln K \\
\Delta G = \Delta H - T\Delta S \\
\ln K = -\Delta H/RT + \Delta S/R
\]

2.10. The flowchart and purity of the extracted amygdalin by ATPS
The extraction of amygdalin from the DWC was conducted with the above optimized conditions of ATPS according to the following flowchart (Figure 1). Also, a certain weight of (NH$_4$)$_2$SO$_4$ and EtOH were added into the DWC solution to make the ATPS with their concentrations being as about 20% (g/g) and 30% (g/g), respectively. Then, the purity of the extracted amygdalin was calculated and expressed as $W$ by following the equation:

\[
W(\%) = \frac{A_0}{A_i} \times 100\%
\]

where $A_0$ and $A_i$ represent the peak area of amygdalin and all the peaks by HPLC, respectively.

2.11. Statistical analysis
The results were statistically analyzed by calculating the mean and standard deviation. Analysis of variance (ANOVA), multiple comparative analysis and paired T-test were conducted using the SPSS statistics software.

3. Results and discussion

3.1. The comparison of properties about ATPS formed with different inorganic salts
The formation properties of ATPS from different inorganic salts and ethanol were studied and the results illustrated that the systems with EtOH-(NH$_4$)$_2$SO$_4$, EtOH-K$_2$HPO$_4$ and EtOH-KH$_2$PO$_4$ were more stable and easier to be formed than that of the Na$_2$CO$_3$-EtOH. The ATPS formed with Na$_2$CO$_3$-EtOH was very unstable, and the salts were easily precipitated in the bottom of the container. Therefore, the ATPS formed with EtOH-(NH$_4$)$_2$SO$_4$, EtOH-K$_2$HPO$_4$ and EtOH-KH$_2$PO$_4$ were primarily screened for further study.

In order to further screen the suitable ATPS for the extraction of amygdalin, the phase diagrams of ATPS composed of EtOH-(NH$_4$)$_2$SO$_4$, EtOH-K$_2$HPO$_4$ and EtOH-KH$_2$PO$_4$ were conducted according to the cloudy point formed with the different contents of ethanol and inorganic salts. As shown in Figure 2, the phase is uniform, stable and no phase separation, whether the coordinates corresponding to the contents of ethanol and inorganic salts locate at the upper or the bottom of the cloudy-point curve. That is to say, the ATPS could be formed only with the coordinates on the curve. Figure 2 also shows that the content of EtOH becomes decreasing with the addition of K$_2$HPO$_4$, (NH$_4$)$_2$SO$_4$ or KH$_2$PO$_4$ into the system, and vice versa. Furthermore, the phase diagrams are different with the different inorganic salts to form the ATPS, i.e. the phase-forming ability of the inorganic salts in the ethanol is different.

The distribution coefficients of the amygdalin in the ATPS of EtOH-(NH$_4$)$_2$SO$_4$, EtOH-K$_2$HPO$_4$ and EtOH-K$_2$PO$_4$ were compared in Table 1. As can be seen, the maximum distribution coefficient ($K$) of amygdalin could be achieved in the ATPS of EtOH-(NH$_4$)$_2$SO$_4$, while the $K$ values in the ATPS of EtOH-KH$_2$PO$_4$ and EtOH-K$_2$PO$_4$ were lower and no significant difference exists between the ATPS formed with EtOH-KH$_2$PO$_4$ and EtOH-K$_2$HPO$_4$. For the value of $Y$, it is higher in the top phase of the EtOH-K$_2$HPO$_4$ system, while its higher $R$ value may cause the phase unsteady and be difficult to be adjusted.

Generally, the value of $Y$ in the ATPS could be increased by increasing the value of $R$ and $K$. For the value of $K$, it was very little selected due to its complexity influenced by many uncontrolled factors (Kim & Kim, 1986). On the contrary, the value of $R$ could be easily determined by the molecular polarity of and the capacity of associating with water between the upper and bottom phases. Moreover, the lower $R$ and higher $K$ values are beneficial to the extraction in the ATPS, so the EtOH-(NH$_4$)$_2$SO$_4$ was selected as the suitable composition to form the aqueous two-phase system in this study.

![Figure 4. Effect of the pH in ATPS on the extraction yield of amygdalin.](image-url)
3.2. Effect of the mass fraction of EtOH and (NH₄)₂SO₄ on the extraction of amygdalin in ATPS

The competitions between the EtOH, (NH₄)₂SO₄, and the water molecules determined the polarity of the upper and the lower phases, which consequently influenced the phase ratio of the two phases. As a result, the extraction of amygdalin in the concentrate was also affected in the ATPS. According to the preliminary experiment, the solution could be divided two phase by changing the EtOH content from 20% to 33% (w/w) and fixing the content of (NH₄)₂SO₄ at 18% (w/w). Meanwhile, the content of (NH₄)₂SO₄ was changed from the range of 14% to 22% with the content of EtOH fixed. Then, the values of K, R and Y were conducted to investigate the effect of the mass fraction of EtOH and (NH₄)₂SO₄ in ATPS on the extraction of amygdalin.

As shown in Figure 3(a), the value of R increased with the increasing of the EtOH concentration, while decreased with the increasing of the (NH₄)₂SO₄ concentration. Generally, the increase of EtOH concentration promotes the association between the water molecule and ethanol, causing the salting-out of (NH₄)₂SO₄ and the down phase decreasing, and finally the increasing of R value. Figure 3(b) shows that the K values demonstrated a similar increasing trend in the beginning and followed by a decreasing trend with the further increase of EtOH or (NH₄)₂SO₄, suggesting that the K values should be optimized in order to achieve a higher extraction efficiency. Figure 3(c) shows that the Y values also illustrated a similar increasing before dropping with the further increase of EtOH or (NH₄)₂SO₄ as did the K values. When the content of EtOH and (NH₄)₂SO₄ increased to a certain

![Figure 5](image-url)
range, the crystallization of (NH$_4$)$_2$SO$_4$ happened as shown in Figure 3(d), causing the solution system unstable and the extraction of amygdalin decreased. Considering all the above factors, the optimum mass fractions of EtOH and (NH$_4$)$_2$SO$_4$ were of 30% and 20%, respectively. According to this ratio to form the ATPS, the suitable values of K, R and Y could be achieved.

### 3.3. Effect of pH on the extraction of amygdalin in ATPS

As shown in Figure 4, the value of Y (extraction yield of amygdalin) firstly increased and followed by a decrease with the increase of pH from 5.0 to 8.0 in ATPS. Specifically, the Y value increased by changing the pH from 5 to 7, while it decreased from the pH of 7.0–8.0. Generally, the increasing of pH means the concentration increasing of hydroxyl ions, releasing a small amount of ammonia gas after binding with the ammonium ions and resulting in the instability of the ATPS. In addition, the configuration of the amygdalin isomer can be affected by the intermediate state isomerization, resulting in the re-equilibrium of the two isomers of D- and L- (Liu et al., 2016). Therefore, the pH 7.0 of the ATPS was chosen in this study.

### 3.4. Effect of the temperature on the extraction of amygdalin in ATPS

The extraction yield of amygdalin by ATPS had a slight rise with the increase of temperature from 25°C to 65°C (detailed information can be seen in the supporting file 2), but this increase was unnoticeable, which might be attributed to the chosen conditions when investigating the effect of temperature, i.e. the condition is farther to the critical point to form the ATPS. Generally, the temperature has effects on the formation of the phase diagram, the values of K, R, and the solubility of amygdalin in ATPS. Moreover, the nearer to the critical point of the phase diagram, the greater the effect of temperature on the R and K values. Considering the operability, the temperature was set at 35°C during the extraction of amygdalin by ATPS.

### 3.5. Thermodynamic analysis about the extraction of amygdalin by ATPS

As be seen in Figure 5(a), the fitting equation is $\ln K = -0.8719/T + 7.3936$. According to the equations about the Gibbs energy, enthalpy and entropy mentioned in the section of 2.9, the values of $\Delta H$ and $\Delta S$ were calculated as 15.563 and 0.061, respectively, when the temperatures were ranged from 25°C to 65°C. Since the values are all above zero, so the extraction of amygdalin could be identified as an endothermic entropy-driven process. Furthermore, the changes of $\Delta H$ and $\Delta S$ were relatively small, indicating that this process belongs to a mild extraction of the amygdalin. Due to all the values of $\Delta G$ lower than zero (Figure 5(b)), it suggests that the ATPS extraction of amygdalin was a spontaneous process. In the meantime, changes of the $\Delta G$ were observed, illustrating that the separation can spontaneously happen at room temperature. Therefore, the extraction of the amygdalin can be performed using the ATPS at room temperature.

### 3.6. Extraction of the amygdalin using the optimum ATPS

Figure 6 shows the chromatograms of amygdalin conducted by the HPLC before and after extraction from the DWC samples using ATPS, it can be seen that the purity of the sample about the amygdalin was greatly improved after the treatment of ATPS, since many peaks decreased or disappeared to a great extent except the peak of amygdalin. To be specific, the content of amygdalin was increased from 32.82% to 68.44%, and the extraction yield was up to 90.37%, which is significantly higher than those of the results of 48% and 71.2% obtained by the researchers of Li and Chen (2006) and

![Figure 6. Chromatograms of amygdalin by HPLC in untreated DWC sample (Bottle), standard amygdalin sample (Top) and treated sample with ATPS of the EtOH/(NH$_4$)$_2$SO$_4$ (Middle).](image)

**Figure 6.** Cromatogramas de amigdalina por HPLC en una muestra de DWC no tratada (botella), una muestra de amigdalina estándar (parte superior) y una muestra tratada mediante el ATPS de EtOH/(NH$_4$)$_2$SO$_4$ (centro).
Wang, Wang, and Zhan (2010) with conventional extraction methods, respectively. All these results suggest that the optimized ATPS composed of EtOH and (NH₄)₂SO₄ was suitable for the extraction of amygdalin, and can greatly increase the added value of apricot kernels and decrease the waste of resources.

4. Conclusions

The factors affecting the extraction efficiency of amygdalin using ATPS were investigated in this paper such as the composition of ATPS, the temperature and pH. The results suggest that the conditions were as follows: 30% (w/w) EtOH and 20% (w/w) (NH₄)₂SO₄ in ATPS, at the temperature of 35°C and pH value of 7. Under these conditions, the highest extraction yield was 90.37% and the purity of amygdalin compared with the untreated DWC sample increased more than two times. All these results indicate that as a novel extraction method, the ATPS could be used to recycle the amygdalin from the by-products (DWC) generated in the debitterizing of apricot kernels. However, the utilization of the recycled amygdalin should be further investigated in order to promote its practical application.

Disclosure statement

No potential conflict of interest was reported by the authors.

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