Aqueous Two-phase System for the Extraction of Flavonoids from Hawthorn (Crataegus pinnatifida Bge.) Leaves

Da-You SONG#, Juan-Juan MA#, Kun Li, Chuan-He ZHUa,* and Xiao-Cun ZHANGb,*

College of Food Science and Engineering, Shandong Agricultural University, Tai’an, China
achahzhu@sdau.edu.cn, bxczhang@sdau.edu.cn

#These authors contribute equally to this research
*Corresponding author

Keywords: Hawthorn Leaves, Flavonoids, Aqueous Two-Phase System.

Abstract. in the paper, the effect of aqueous two-phase system (ATPS) on the extraction of flavonoids from hawthorn (Crataegus pinnatifida Bge.) leaves was investigated. Based on the yield of flavonoids and partition coefficient (K), PEG 11.5% and (NH4)2SO4 14% was selected as the optimum solvent for extracting flavonoids. When the ratio of material to solvent was 1:35, the yield of flavonoids was 2.79%. The present study demonstrated that the aqueous two-phase system (ATPS) was an efficient method to extract flavonoids from hawthorn (Crataegus pinnatifida Bge.) leaves.

Introduction

Hawthorn (Crataegus pinnatifida Bge.) belongs to rosaceae plants and is cultivated in many parts of China [1]. According to the previous literature, leaves and fruits are widely used as medicinal and food materials in china for their pharmaceutical value and nutritive value [2, 3]. The pharmacological properties of them were related to the extracts such as flavonoids and polysaccharide, especially the flavonoids. And the content of flavonoids in hawthorn leaves is more than 20 times that of hawthorn fruits. Therefore, extracting flavonoids from hawthorn leaves has become a hot topic recently. The current studies obviously demonstrated that flavonoids have strong antioxidant activity, antimicrobial activity, anti-inflammatory activity and free radical scavenging activity which can prevent cancer and cardiovascular disease [3, 4]. The current studies showed the flavonoids have been used as functional food additives and materials of some medicine [5].

In recent years, many studies were reported about various methods of extracting flavonoids from plants. Such as the conventional method using water and ethanol has been widely used to extract flavonoids. Although the method is convenient and inexpensive, flavonoids cannot be extracted completely and the extracts are a complex mixture including tannins, polysaccharides and proteins [6, 7]. Therefore, the method is not conducive to the further purification. According to the present literatures, ATPS composed of short chain alcohols and inorganic salts has been used to extract nature compounds as a new method [8-9], which is based on the physicochemical properties of extract, such as solubility of the extracts. Compared with traditional extraction methods, the application of ATPS shows some advantages such as higher extraction yield, shorter extraction time lower cost and higher purity for target compounds in one step [10-11]. Besides, ATPS provides a suitable
environment to maintain biological activity of flavonoids. In this study, ATPS was used directly as a solvent to extract flavonoids from hawthorn leaves. The present aim of this study is to optimize the conditions of extracting flavonoids.

Materials and Methods

Plant Materials

Hawthorn leaves were collected from Laiwu city, Shandong province. The leaves were dried in the air and powdered using an electrical grinder and passed through a 60 mesh sieve. Then they were kept away from light until used.

Determination of Flavonoids

Flavonoids were measured at 510nm with colorimetric method by ultraviolet spectrophotometer (754N, APL, Shanghai). Rutin was chosen as the standard substance to make the standard curve. The concentration of flavonoids in hawthorn leaves was calculated based on the curve. The regression equation as follows:

\[ y = 9.0899x + 0.0159 \]

Where \( y \) is the absorbance of determined solution, and \( x \) is the concentration of rutin (mg/ml).

Partition of Flavonoids in ATPS

Predetermined quantities of alcohols and inorganic salt were added into the hawthorn leaves to extract flavonoids combined with ultrasound. After the separation of phases finished, the concentration of flavonoids in top phase and bottom phase was determined respectively. The partition coefficient (K) was calculated by the equation as follows:

\[ K = \frac{C_t}{C_b} \]

Where \( C_t \) and \( C_b \) were respectively equilibrium concentrations of flavonoids in the top phase and bottom Phases.

Selection of ATPS

The selection of ATPS was primary consideration in the study. A sample of 1.0000 g of powdered leaves was macerated in a glass flask with different ATPS which contained ethanol/\((\text{NH}_4)_2\text{SO}_4\), PEG/\((\text{NH}_4)_2\text{SO}_4\), propyl alcohol/\((\text{NH}_4)_2\text{SO}_4\) and ethanol/\(\text{K}_2\text{HPO}_4\). After the extraction finished, collecting the top and bottom phase respectively, and then analyzed flavonoids by colorimetric method with rutin as the standard. Taking into consideration the yield of flavonoids and K, the optimum ATPS by changing the concentration of alcohols and inorganic salt was selected. In generally, the polysaccharides transferred into the bottom salt-rich phase, while flavonoids were extracted into the top phase. The yield of flavonoids was calculated using the equation:

\[ \text{Yield of flavonoids (\%)} = 100\frac{C_tV_t}{M} \]

Where \( C_t \) and \( V_t \) were the measured concentration (mg/ml) and volume (ml) of the top phase, and \( M \) was the mass of leaves powder (g).

Statistical Analysis

All experiments were carried out in triplicate. Statistical analyses were conducted with a statistical analysis system (SAS 8.0 software).
Results and Discussion

Effects of ethanol/ (NH₄)₂SO₄ on the yield of flavonoids and K

The effects of different ethanol concentration on the distribution coefficient of flavonoids and the extraction yield were shown in Fig.1-A. The yield of flavonoids increased first and then decreased, with the increase of ethanol concentration, but the distribution coefficient K showed an increasing trend. According to its partition coefficient, the flavonoids were mainly distributed in the upper phase. With the increase of ethanol concentration, the amount of salt required to reduce the phase, the upper phase volume increases, resulting in the extraction rate of flavonoids increases. So, the concentration of ethanol was 44%. The effect of concentration of (NH₄)₂SO₄ on the on the distribution coefficient of flavonoids and the extraction yield were shown in Fig.1-B. With the increase of (NH₄)₂SO₄ mass fraction, the flavonoid yield and distribution coefficient increase first and then decrease. When the mass fraction of (NH₄)₂SO₄ was 18%, the yield of flavonoids was the highest, and the distribution coefficient of flavonoids was not significant (P> 0.05) in the range of 15% -18%. So 17% (NH₄)₂SO₄ was used to the following research.

![Figure 1: Effect of ethanol/ (NH₄)₂SO₄ ATP on the yield of flavonoids and K (A: effect of concentration of ethanol, B: effect of concentration of (NH₄)₂SO₄).](image)

Effects of PEG/ (NH₄)₂SO₄ on the yield of flavonoids and K

As can be seen from Fig. 2-A, according to the distribution coefficient, the flavonoids are basically distributed in the PEG phase. When the concentration of PEG increased, the flavonoid yield and its partition coefficient increased first and then decreased. After the concentration of PEG reached11.5%, the extraction rate of flavonoids reached the maximum. The partition coefficient reached a maximum at the concentration of 10%, which was not significantly different from that of 11.5% (P> 0.05). The 11.5% PEG was chosen as the concentration. From Fig 2-B, the yield and partition coefficient of flavonoids increased first and then decreased with the increase of the mass fraction of (NH₄)₂SO₄. The yield of flavonoids reached the maximum at the mass fraction of 14%, and the partition coefficient was 45, and the flavonoids almost all made into the PEG phase. So the mass fraction of (NH₄)₂SO₄ was 14%. 

![Figure 2: Effect of PEG/ (NH₄)₂SO₄ ATP on the yield of flavonoids and K.](image)
Effects of Propyl Alcohol/ (NH₄)₂SO₄ on The Yield of Flavonoids and K

From Fig.3-A, the yield of flavonoids and their partition coefficients increased first and then decreased with the increase of the mass fraction of (NH₄)₂SO₄. When the mass fraction of ammonium sulfate was 18%, the extraction rate of flavonoids and partition coefficient reached the maximum. Therefore, the final (NH₄)₂SO₄ mass fraction was selected as 18%. When the concentration of propyl alcohol was 60%, the extraction yield and partition coefficient were maximum (Fig.3-B). There are no significant differences (P>0.05) between 50% and 60%. But, the extraction rate reached the maximum value at 50% and the partition coefficient was smaller. The reason was as the concentration of propanol increases, the volume of the lower phase increases, and the yield and partition coefficient of flavonoids and polysaccharides increase. Taking the flavonoids as the main consideration, select the propanol concentration of 50%.

Effects of Ethanol/K₂HPO₄ on The Yield of Flavonoids and K

As can be seen from Fig.4-A, with the increase in the mass fraction of K₂HPO₄, the flavonoid yield was reduced. This may be due to the increase in the mass fraction of K₂HPO₄ will lead to salting out the phenomenon, so that the upper phase volume decreases, Change the ratio of phase, and then affect the distribution coefficient of flavonoids and extract. Therefore, the mass fraction of K₂HPO₄ was 20%. According to the distribution coefficient of flavonoids; the flavonoids were mainly distributed in the upper phase. As shown in Fig.4-B, The yield and partition coefficient of flavonoids increased with the increase of ethanol.
concentration, there was no significant difference in the concentration of ethanol in the range of 50%-60% (P>0.05). Overall, the ethanol concentration selected 50%.

![Figure 4](image)

Figure 4. Effect of ethanol/K$_2$HPO$_4$ ATP on the yield of flavonoids and K (A: effect of concentration of K$_2$HPO$_4$, B effect of concentration of ethanol).

Various ATPS, including ethanol/(NH$_4$)$_2$SO$_4$, PEG/(NH$_4$)$_2$SO$_4$, propyl alcohol/(NH$_4$)$_2$SO$_4$ and ethanol/K$_2$HPO$_4$ were studied for their effects on the yield of flavonoids and K with different concentrations of salt and concentration of alcohols. The results were respectively shown in Fig. 1-4. Taking into consideration of the yields of flavonoids and K, PEG/(NH$_4$)$_2$SO$_4$ was selected as the optimal ATPS. The partition of flavonoids in PEG/(NH$_4$)$_2$SO$_4$ was shown in figure 2-C and figure 2-D. It indicated that, with limits, the yield of flavonoids and K increased with the increasing concentrations of PEG and (NH$_4$)$_2$SO$_4$. So PEG (6000) 11.5% and (NH$_4$)$_2$SO$_4$ 14% were selected as the optimum ATPS for further study. Under the conditions of UA-ATPS, the yield of flavonoids was 2.802 ± 0.061 % and K was 33.35 ± 0.35.

**Effect of Ratio of Material to Solvent**

![Figure 5](image)

Figure 5. Effect of the ratio of material to solvent on the yield of flavonoids.

From the above experiments, ATPS with PEG 11.5 % and (NH$_4$)$_2$SO$_4$ 14 % was selected to extract flavonoids in this study. Fig. 5 indicated the influence of ratio of material on the yield of flavonoids. The data indicated that the yield of flavonoids increased with the increasing the ratio of material to solvent from 1:15-1:35, and there was a tendency of decrease of the extraction yield when the ratio exceeded 1:35. This can be explained that the ratio of material to solvent directly influenced the ultrasonic energy transfer process. If the ratio was too low, the solvent would be not enough for flavonoids to be dissolved. However, only small amount of heat was absorbed by the sample if the ratio was too high, so the yield of flavonoids decreased. Therefore, the ratio of material to solvent was selected as 1:35.
Conclusions

The present study has indicated the superiority and convenient of using ATPS for extracting flavonoids from hawthorn leaves. ATPS of PEG 11.5% and (NH₄)₂SO₄ 14% was the optimum solvent for extracting flavonoids. When the ratio of material to solvent was 1:35, the yield of flavonoids reached the maximum (2.79%). The method can not only save the extraction time, but also improve the extraction efficient. So the ATPS offers a theoretical basis for further extracting of flavonoids and represents a valuable alternative to some conventional solvent for extracting flavonoids from plants.

Acknowledgement

This work was supported by Fund Program: Shandong province science and technology plan project(J14LF11) and Funds of Shandong “Double Tops” Program (SYT2017XTTD04).

References

[1] Han Junyan, Tan Dehong, Liu Guangchun. Hawthorn-A Health Food, Applied Mechanics and Material Vol. 140 (2012) 350-354.

[2] Pengzhan Liu, Heikki Kallio, Baoru Yang. Phenolic Compounds in Hawthorn (Crataegus grayana) Fruits and Leaves and Changes during Fruit Ripening, Food Chemistry. 59(2011) 11141-11149.

[3] Jianli Liu, Jiangfeng Yuan, Zhiqi Zhang. Microwave-assisted extraction optimised with response surface methodology and antioxidant activity of polyphenols from hawthorn (Crataegus pinnatifida Bge.) fruit, Food Science and Technology. 45 (2010) 2400–2406.

[4] Adam Mekonnen Engida, Novy S. Kasim Extraction, Yeshitila Asteraye Tsigie. Extraction, identification and quantitative HPLC analysis of flavonoids from sarang semut (Myrmecodia pendaran), Industrial Crops and Products. 41 (2013) 392-396.

[5] Min-Jung Ko, Chan-Ick Cheigh, Myong-Soo Chung. Relationship analysis between flavonoids structure and subcritical water extraction (SWE), Food Chemistry. 143 (2014) 147–155.

[6] Mehmet Bilgin, Selin Sahin. Effects of geographical origin and extraction methods on total phenolic yield of olive tree (Olea europaea) leaves, Journal of the Taiwan Institute of Chemical Engineers. 44 (2013) 8-12.

[7] Yingchun Wu, Yun Wang, Wenli Zhang, Juan Han, Yan Liu, Yutao Hu, Liang Ni. Extraction and preliminary purification of anthocyanins from grape juice in aqueous two-phase system, Separation and Purification Technology. 124 (2014) 170–178.

[8] Guo Y.X., Han J, Zhang D.Y., Wang L.H., Zhou L.L. An ammonium sulfate/ethanol aqueous two-phase system combined with ultrasonication for the separation and purification of lithospermic acid B from Salvia miltiorrhiza Bunge, Ultrasronics Sonochemistry. 19 (2012) 719-724.
[9] Xiangyang Wu, Linghong Liang, Ye Zou, Ting Zhao, Jiangli Zhao, Fang Li, Liuqing Yang. Aqueous two-phase extraction, identification and antioxidant activity of anthocyanins from mulberry (Morus atropurpurea Roxb.), Food Chemistry. 129 (2011) 443-453.

[10] Y.X. Guo, J. Han, D.Y. zhang, L.H. Wang, L.L. Zhou. Aqueous two-phase system coupled with ultrasound for the extraction of lignans from seeds of Schisandra chinensis (turcz.) Baill, Ultrasonics Sonochemistry. 20 (2013) 125–132.

[11] Dongyang Zhang, Yuangang Zu, Yujie Fu, Wei Wang, Lin Zhang, Meng Luo, Fansong Mu, Xiaohui Yao, Minghui Duan. Aqueous two-phase extraction and enrichment of two main flavonoids from pigeon pea roots and the antioxidant activity, Separation and Purification Technology. 102 (2013) 26-33.