Chromatography
Separation Techniques

Hua et al., J Chromat Separation Techniq 2013, 4:2
DOI: 10.4172/2157-7064.1000167

Research Article Open Access

Extraction and Purification of Anthocyanins from the Fruit Residues of *Vaccinium uliginosum Linn*

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Abstract

An aqueous two-phase system was developed to extract anthocyanins present in the fruit residue during juice production from the fruit of *Vaccinium uliginosum Linn*. A maximum partition coefficient of 10.67 and a recovery of 96.09% for anthocyanins could be obtained using an extraction system consisted of 30% (w/w) ethanol and 19% ammonium sulfate. Compared with the traditional extraction using acidified ethanol, the novel aqueous two-phase extraction could not only yield a much higher concentration of anthocyanins, save more ethanol, energy, and time, but also decrease impurities in extract, e.g. proteins and sugars by 58% and 66%, respectively. AB-8 macroporous resin was applied to the purification of anthocyanins. A novel and simple separation technique for anthocyanins was developed by integrating aqueous two-phase extraction and macroporous resin column chromatography. This new technology might be a suitable for other bioactive natural products on industrial scale.

Keywords: Aqueous two-phase extraction; Ethanol/ammonium sulfate; Macroporous resin column chromatography; V*accinium uliginosum Linn*; Anthocyanin

Introduction

In the past decade anthocyanins have been found as health-promoting ingredients in many fruits and vegetables [1,2]. In such plants, the content of anthocyanins in *Vaccinium uliginosum Linn* (*V. uliginosum*) is the highest [3]. Anthocyanins are flavonoid pigments with a flavylium cation structure described as a C6-C3-C6 skeleton [1,4]. They play a vital role in the prevention of neuronal and cardiovascular illnesses, cancer and diabetes due to their antioxidant property [1,2]. In addition to being edible fruit, *V. uliginosum* fruits are mainly used to make fruit juice, producing lots of fruit residue as by-products. Anthocyanins, which are present mainly in the peel of *V. uliginosum*, constitute the most important pigments of the vascular plants [5]. They are harmless, easily dissolved in aqueous media, and therefore suitable natural water-soluble colorants.

Commercial recovery of natural pigments from plant materials is usually solvent extraction after homogenization. For examples, acidified solutions of methanol, ethanol, acetone, water, and a mixture of acetone/methanol/water are traditionally used to extract anthocyanins. From these methods, the extraction with acidified methanol is the most efficient [6-8]. Nevertheless, in food industry ethanol is preferred due to the toxicity of methanol. In addition, the extraction method needs large amount of organic solvents and much energy for recovering the solvent. Thus, there is a need for an efficient, economical and large-scale bioseparation method that can achieve high purity as well as high yield, while preserving the biological activity of these molecules. Aqueous two-phase extraction (ATPE) is likely to be developed as one extraction method to meet the above criteria.

ATPE used as a primary separation method has been widely applied for the recovery of biological products due to its easiness for scale-up, high capacity and yield, low cost and short processing time [9-12]. Furthermore, ATPE has the potential to achieve the desired purification and concentration of the product in a single step. In the separation of the pigments, polyethylene glycol (PEG)/ $(NH_4)_2SO_4$ system has been used to separate betalains from crude beetroot extract [13], and Mageste et al. studied ATPE of natural dye carmine with polyethylene oxide (PEO)/Li_2SO_4 system [14]. However, polymers are very expensive and not easy to recover. Recently, a novel aqueous two-phase system (ATPS) composed of short chain alcohol and salt has been used to extract natural compounds due to its low cost and easy recovery of solvent by evaporation. For example, ethanol/ $(NH_4)_2SO_4$ system has been used to separate piceid, resveratrol and emodin from *Polygonum cuspidatum* [15], and n-propanol/ phosphate system has been used for the purification of salvianolic B from crude extract of *Salvia miltiorrhiza* by counter-current chromatography (CCC) [16]. Encouraged by the previous results, ATPE is thought to be suitable for the extraction of natural pigments.

Generally, the purification of anthocyanin is carried out by HPLC or CCC [17,18]. However, these methods are too expensive to popularize. In recent years, macroporous adsorption resin is more and more used in the purification of pigments [19,20]. Especially, AB-8 resin is a kind of macroporous resin for the purification of flavonoid [21].

In this study, anthocyanins was extracted directly from *V. uliginosum* residue by ethanol/ammonium sulfate system and then purified by AB-8 macroporous resin. This method not only salvaged the wasted residue from *V. uliginosum* juice production, but also integrated extraction, clarification, concentration and purification into single steps without pretreatment to provide a simple method for the production of anthocyanins.

Materials and Methods

Chemicals and materials

V. uliginosum fruits were obtained from Blueberry Source of Dalian Science and Technology Co. Ltd (Dalian, China). AB-8 macroporous resin, whose particle size (>99%) was 0.3-1.25 mm, was bought from

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Received January 31, 2013; **Accepted** February 27, 2013; **Published** March 04, 2013

Citation: Hua Z, Yuesheng D, Ge X, Menglu L, Liya D, et al. (2013) Extraction and Purification of Anthocyanins from the Fruit Residues of *Vaccinium uliginosum Linn*. J Chromat Separation Techniq 4: 167. doi:10.4172/2157-7064.1000167

Tianjin Haiguang Chemical Co., Ltd (Tianjin, China). Prior to use, the resin was immersed with ethanol more than 12 h to remove the inert solvent, repeatedly washed with distilled water to no odour of ethanol, and then kept it in a beaker for later use. Ethanol and inorganic salts used were of analytical grade.

Preparation of fruit residue

V. uliginosum fruits (0.5 kg) were put into a JJ-2 Tissue Grinders (Shenzhen, China) and homogenized for 2 min at speed of 1200 rpm. After filtration, the solid residue was stored at -20°C.

Aqueous two-phase extraction

ATPSs were consisted of specific amount of ethanol, inorganic salt and water in a total amount of 20 g. Various systems were prepared in a range of ethanol (20%-40% (w/w)) and inorganic salt (10%-25% (w/w)) concentrations. Inorganic salt was first dissolved into water in 25-mL teat glass tubes. Through preliminary experiments, we found *V. uliginosum* residue hung between top phase and bottom phase. If *V. uliginosum* residue was too much, the solid phase was too thick to lower mass transfer. Therefore, in this study, the ratio of material and solvent was 1:20. 1.0 g *V. uliginosum* residue was then added and mixed by vortexing for 30 s, followed by addition of ethanol, and the whole mixture was then thoroughly mixed by vortexing for 30 s and then allowed to stand at room temperature for 0.5-1.0 h to enable phase separation. Alternatively, a specific amount of inorganic salt solution was added to 1.0 g of *V. uliginosum* residue by vortexing for 30 s, followed by addition of ethanol. The mixture was then treated as described above. After the two phases had separated, the volumes of the top and bottom phases were recorded, and anthocyanins were analyzed in the top and bottom phases, respectively. The extraction experiments were carried out in triplicate at room temperature. Furthermore, ATPE was scaled up on 3.0 kg scale in a 5.0-L beaker under optimized conditions. During experiments visual inspection of the residue subjected to ATPE showed a crimson top phase and a light purple bottom phase. The grey residue present in the extract was accumulated at the interface, which was discarded. The partition coefficient (K) of anthocyanins was defined as the ratio of the concentration of anthocyanins in the top phase to that in the bottom phase. The recovery (R) of anthocyanins was the percent of anthocyanins in the top phase to the total amount in ATPS.

The yield (Y) of anthocyanins was defined as the percent of the mass of anthocyanins in the extract to that obtained from acidified ethanol extraction.

Acidified ethanol extraction

Acidified ethanol extraction of anthocyanins was performed with 50% (w/w) ethanol at 50°C and pH 3.5 for 60 min [7].

Aqueous two-phase extraction of anthocyanins combined with column chromatography

100 g pretreated AB-8 macroporous resin was soaked by deionized water (pH=3.0-3.5) for 24 h to equilibrate, and then filtered.

After ATPE, the anthocyanin extract was diluted 5 times by water (pH=3-3.5) until ethanol elimination under vacuum at low temperature (37°C) on a rotary evaporator (Rotavapor RE-52A, Yarong, Shanghai, China). The diluted extract was added into a glass column (Φ1.5×40 cm) packed by 50.0 g pretreated AB-8 macroporous resin at a volume of 40 mL resin bed (BV). The anthocyanins adsorbed to the resins were washed with 1-2 BV water (pH 3-3.5), 1-2 BV 20% (v/v) ethanol solution (pH 3-3.5), 1-2 BV 30% (v/v) ethanol solution (pH 3-3.5), 1 BV 40% (v/v) ethanol solution (pH 3.0-3.5), and eluted from the column with 5 BV 60% (v/v) and 1-2 BV 80% (v/v) ethanol solution, respectively. The flow rate was controlled to 2.0 mL/min by metering pump. The effluents from the column were collected by 100-mL volumetric cylinders.

The desorption ratio (DR) was evaluated as follows:

$$DR(\%) = \frac{C_d V_d}{(C_0 - C_e)V_0} \times 100\% \quad (1)$$

where C_0 is the initial concentration of anthocyanin (mg/L); C_d is the desorption concentration of the solute in the desorption solution (mg/L); C_e is equilibrium concentration of anthocyanin (mg/L); V_d is volume of the eluent (L); V_0 is volume of initial anthocyanin solution (L).

Analytical procedures

Anthocyanins were directly determined by the pH-differential method as described by Wrolstad [22]. Samples were diluted with buffer and the absorbance was read at 520 and 700 nm using a Jasco V-560 UV/VIS spectrophotometer. Anthocyanin content was calculated as pelargonidin-3-glucoside, with molar absorbance of 22,400 M^{-1} cm^{-1} and molecular weight of 433.2 Da.

Total sugar concentration was determined by 3, 5-dinitrosalicylic acid colorimetry [23]. Protein concentration was determined by Coomassie Brilliant Blue method using BSA as standard protein [24].

Results and Discussion

Selection of aqueous two-phase system

Partition behavior and stability of anthocyanins were studied in ATPSs employing ethanol and different phase forming salts (dipotassium hydrogen phosphate, natrium carbonate and ammonium sulphate). Ammonium sulphate was selected as the best salt due to higher stability of anthocyanins in ammonium sulphate (pH 4.3) than dipotassium hydrogen phosphate (pH 8.2) and natrium carbonate (pH 9.0). The stability of anthocyanins was proved to be affected by the pH of the system: When pH of anthocyanin solution was above 8, the pyran ring in structure of anthocyanin was opened [4]. There was less than 33.5% anthocyanin in those systems comparing with the acidified ethanol extract.

The effects of ethanol and ammonium sulphate concentration on the partitioning of anthocyanins were determined on the basis of phase diagram of ATPS of ethanol/ $(NH_4)_2SO_4$ as shown in figure 1. Comparing with the previous phase diagram [25], the upper limit line of two phases was added in figure 1. As shown in figures 2 and 3, the partition coefficient and recovery of anthocyanins increased dramatically with increasing concentrations of $(NH_4)_2SO_4$ and ethanol, which indicated that anthocyanins tended to concentrate in the top phase. The partitioning behavior of anthocyanins was similar with another water soluble pigments-betalain in ATPS consisting of PEG and $(NH_4)_2SO_4$ [13]. Compared these ATPSs, the partition coefficient and recovery of anthocyanins in ATPS of 30% (w/w) ethanol and 19% (w/w) $(NH_4)_2SO_4$ were 10.67 and 96.09%, respectively. And the recovery was the highest. Thus this ATPS was chosen for the ATPE of anthocyanins in the next study.

Extraction of anthocyanins from *V. uliginosum* residue

To optimize ATPE, the effects of ATPE strategies on partition coefficient and recovery of anthocyanins were investigated (Figure 4). When $(NH_4)_2SO_4$ solution and ethanol were added to *V. uliginosum*

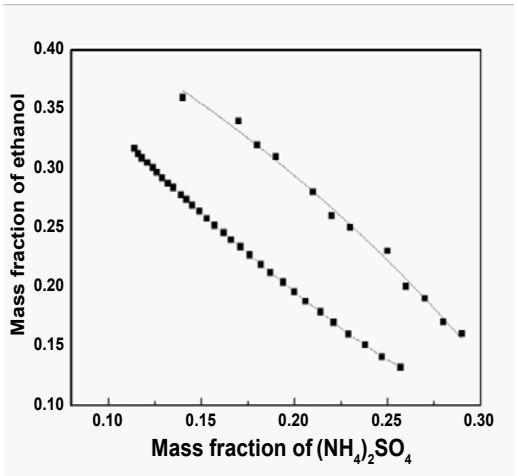

Figure 1: Phase diagram of aqueous two-phase system of ethanol/ ammonium sulfate.

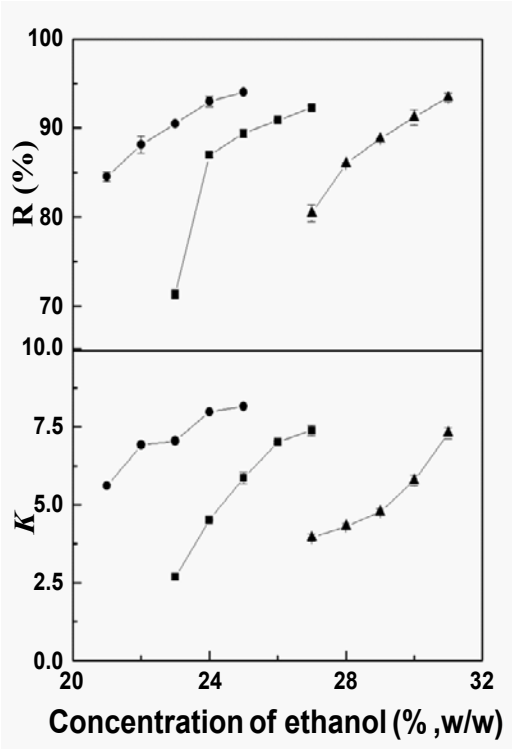

Figure 2: Effect of ethanol concentration on partition of anthocyanins. Concentrations of (NH4)2SO4 (w/w) were 16% (▲), 19% (■), 22% (●). Data are the means ± SDs from three different experiments.

residue without vortexing during addition, the highest partition coefficient of anthocyanins was obtained. The differences among the partition coefficients obtained from three different ATPE strategies were small; the highest value was 8.69 and the lowest value was 7.86, but the recovery and yield of anthocynins were highest when $(NH_4)_2SO_4$ solution was firstly added to the fruit residue, and the mixture was vortexed for 30 s before the addition of ethanol.

To further optimize ATPE, the effects of extraction time on partition coefficient, recovery and yield of anthocyanins were investigated. The

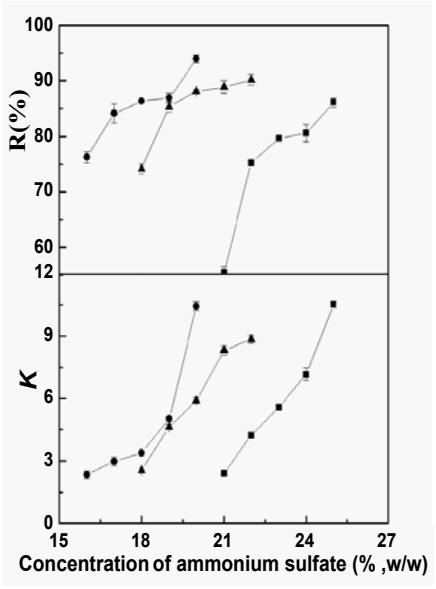

Figure 3: Effect of ammonium sulfate concentration on partition of anthocyanins. Concentrations of C2H5OH (w/w) were 20% (■), 24% (▲), 28% (●). Data are the means ± SDs from three different experiments.

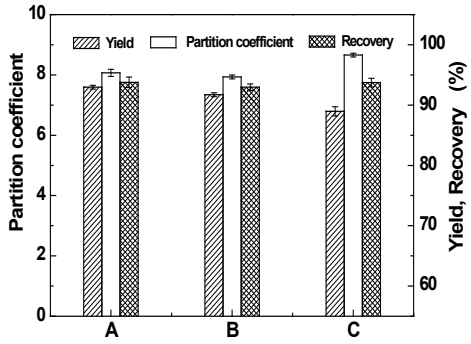

Figure 4: Effect of ATPE strategies on partition of anthocyanins. ① Addition of $(NH_4)_2SO_4$ solution; ② Addition of ethanol; ③ Vortexing for 5 s. The ATPE strategies were **A:** ①③②, **B:** ②③①, and **C:** ①②③. Data are the means ± SDs from three different experiments.

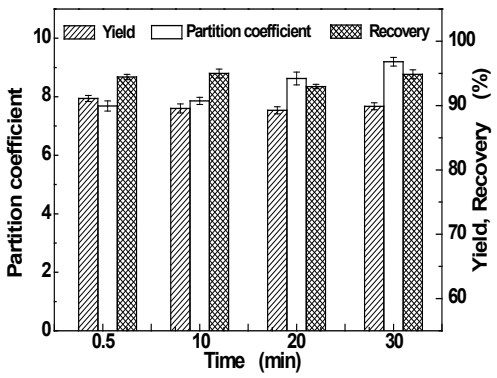

Figure 5: Effect of extraction time of $(NH_4)_2SO_4$ solution on partition of anthocyanins. Data are the means ± SDs from three different experiments.

Extraction methods	Temperature	Time	Volume of extract	Concentration of anthocyanins in extract	Proteins	Sugar
	(°C)	(h)	(mL)	(mg/L)	(mg)	(mg)
Acidified ethanol extraction	50	2	180	15.20 ± 1.36	16.86 ± 0.98	370.54 ± 10.38
Aqueous two-phase extraction	15-30	<1	33.5	80.34 ± 3.46	7.14 ± 0.32	127.60 ± 9.36

Data are the means ± SDs from three different experiments

Table 1: Comparison between acidified ethanol extraction and aqueous two-phase extraction.

Concentration of ethanol (%, w/w)	Volume of eluent (mL)	Content of anthocyanins (mg)	DR (%)
0	59	---	---
20	60	---	---
30	63	0.012	0.015
40	41	0.65	0.84
60	205	75.97	98.30
80	84	0.65	0.84

Table 2: Gradient desorption result of anthocyanins.

partition coefficient of anthocyanins increased as extraction time was increased (Figure 5). However, the recovery and yield of anthocyanins scarcely changed, indicating that the concentrations of ethanol and $(NH_4)_2SO_4$ were high enough to let anthocyanins dissolve from cells thoroughly. In order to obtain high recovery and improve extraction efficiency, after addition of $(NH_4)_2SO_4$ solution and vortexing thoroughly, ethanol should be added immediately.

Based on the above results, ATPEs of anthocyanins were carried out on different scales (20 g vs. 3 kg) under the optimized conditions. On the 20 g scale performed in glass tubes, partition coefficient, recovery and yield of anthocyanins were 10.67, 96.09% and 92.34%, respectively. On 3.0 kg scale ATPE of anthocyanins was performed from 150 g *V. uliginosum* residue in a 5-L beaker, partition coefficient, recovery and yield of anthocyanins were 8.89, 94.37% and 103.20%, respectively. No distinct difference in partition behavior was observed on different scales [26]. However, the yield of anthocyanins on 3.0 kg scale was higher than that on 20 g scale, it was attribute to the ratio of height to diameter of extractor. Because the smaller the ratio of height to diameter is; the thinner the solid phase between top and bottom phase in ATPS is, and the faster the mass transfer rate is. Therefore, the extraction in the beaker was performed more completely than in the tube. Moreover, on 3.0 kg scale, the yield of ATPS was also higher than that of acidified ethanol extraction. The experimental results showed that ATPS is more favorable for extraction of anthocyanins as it consumes less energy and is easy to scale up.

Comparison of different extraction methods for anthocyanins

Five different extraction methods for anthocyanins were compared. These methods were extraction in water at room temperature (pH=3.03), extraction in 19% (w/w) $(NH_4)_2SO_4$ solution at room temperature (pH=3.03), extraction in 50% ethanol solution at room temperature (pH=3.47), extraction in acidified 50% (w/w) ethanol at 50°C (pH=3.47) for 2 h (Heat reflux extraction by ethanol solution), and aqueous two-phase extraction with 30% (w/w) ethanol/19% (w/w) $(NH_4)_2SO_4$ system at room temperature, respectively. The yields of these methods were 7.56%, 42.78%, 51.86%, 100.00%, and 92.34%, respectively.

At room temperature, the yield from 19% (w/w) $(NH_4)_2SO_4$ solution or 50% (w/w) ethanol solution was much more than that of water, indicating that $(NH_4)_2SO_4$ and ethanol could facilitate the dissolution of anthocyanins from cells. The sum of yields from 19% (w/w) $(NH_4)_2SO_4$ solution and 50% (w/w) ethanol solution was a little higher than that of ATPE, because a small amount of anthocyanins remained in the bottom phase of ATPS. When a system composed of 30% (w/w) ethanol/ 19% (w/w) $(NH_4)_2SO_4$ was used, the yield of anthocyanins from the top phase was close to that obtained with heat reflux extraction by acidified ethanol at 50°C. On the contrary, ATPE don't require heating.

The yield of anthocyanins by aqueous two-phase extraction achieved 92.34% of that in acidified ethanol extration. Meantime, the concentration of anthocyanins in the top phase of ATPS increased about 4 times. Some researchers, including our lab, have reported that ATPE could be used remove protein impurities [26,27] and suger [12,27]. Similarly, in the top phase of our ATPS, the contents of proteins and sugar decreased by 58% and 66%, respectively (Table 1). Additionally, no heating requirement, short operation time and less ethanol consumption are the other import characteristics of the new application. ATPE thus integrated extraction, clarification, concentration and preliminary purification into a single step, and provides a simple and effective method for the separation of anthocyanins.

Column chromatography of anthocyanins on AB-8 resin

Column chromatography of anthocyanins on AB-8 resin was investigated by following ATPE of anthocyanins from *V. uliginosum* residue. Gradient elution was used to obtain anthocyanins from AB-8 resin as shown in table 2. Almost no product could be obtained when the column was eluted with ethanol at concentration of less than 30% (v/v). The yield of anthocyanins was 98.30% when the eluent containing 60% (v/v) ethanol. The ethanol in eluent was evaporate by a rotary evaporator under vacuum at 37°C, and then lyophilized to obtain anthocyanin product with a purity of 27.58%. CCC [18] or HSCCC [1] need higher equipment cost, and compared with them, the column chromatography with AB-8 resin was more suitable for the purification of anthocyanins.

Conclusions

An aqueous two-phase system composed of hydrophilic solvent and an inorganic salt, especially ethanol and ammonium sulfate, is suitable for the extraction of anthocyanins from *V. uliginosum* residue. The effect of ethanol and ammonium sulfate on the partition of anthocyanins was investigated to obtain the optimum condition for the extraction. The ATPS composed of 30% (w/w) ethanol/19% (w/w) ammonium sulfate was found to yield the best result. Meanwhile, the AB-8 resin fit for the purification of anthocyanins. Compared with conventional extraction based on acidified ethanol, an integrated process of ATPE combined with column chromatography has some advantages, such as short treatment time, lower ethanol consumption and no heating requirement to yield anthocyanins from the fruit residue of *V. uliginosum*. This new technology might be a suitable extraction method for other natural pigments and bioactive natural products on industrial scale.

Acknowledgments

This work was supported by the grant from Science and Technology Research of Liaoning Provincial Department (No.LS2010044). The authors thank Dr. Alan K Chang (Dalian University of Technology) for proof reading the manuscript.

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