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; Vaccinium 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 flavilium 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) / (NH4)2SO4 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)/Li2SO4 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/ (NH4)2SO4 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...
After filtration, the solid residue was stored at -20°C. Alternatively, a specific amount of inorganic salt solution was then allowed to stand at room temperature for 0.5-1.0 h to enable phase separation. The mixture was then thoroughly mixed by vortexing for 30 s and 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 out in triplicate at room temperature. During experiments visual inspection of the residue subjected to ATPE showed that the partitioning behavior of anthocyanins was similar with those systems comparing with the acidified ethanol extraction.

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=3.0-3.5) 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 3.3-3.5), 1-2 BV 20% (v/v) ethanol solution (pH 3.3-3.5), 1-2 BV 30% (v/v) ethanol solution (pH 3.3-3.5), 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 eluents from the column were collected by 100-ML volumetric cylinders.

The desorption ratio (DR) was evaluated as follows:

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

where \( C_d \) is the initial concentration of anthocyanin (mg/L); \( C_e \) is the desorption concentration of the solute in the desorption solution (mg/L); \( C_0 \) 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 acid colorimetry [23]. Protein concentration was determined by the 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 compared 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/ (NH₄)₂SO₄ 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 (NH₄)₂SO₄ 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 (NH₄)₂SO₄ [13]. Compared these ATPSs, the partition coefficient and recovery of anthocyanins in ATPS of 30% (w/w) ethanol and 19% (w/w) (NH₄)₂SO₄ 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 (NH₄)₂SO₄ solution and ethanol were added to V. uliginosum,
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 anthocyanins were highest when \((\text{NH}_4)_2\text{SO}_4\) 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 1: Phase diagram of aqueous two-phase system of ethanol/ammonium sulfate.

Figure 2: Effect of ethanol concentration on partition of anthocyanins. Concentrations of \(\text{C}_2\text{H}_5\text{OH} (\text{w/w})\) were 20% (▲), 24% (■), 28% (●). Data are the means ± SDs from three different experiments.

Figure 3: Effect of ammonium sulfate concentration on partition of anthocyanins. Concentrations of \((\text{NH}_4)_2\text{SO}_4\) (w/w) were 16% (▲), 19% (■), 22% (●). Data are the means ± SDs from three different experiments.

Figure 4: Effect of ATPE strategies on partition of anthocyanins. ① Addition of \((\text{NH}_4)_2\text{SO}_4\) 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 \((\text{NH}_4)_2\text{SO}_4\) solution on partition of anthocyanins. Data are the means ± SDs from three different experiments.
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| Extraction methods                  | Temperature (°C) | Time (h) | Volume of extract (mL) | Concentration of anthocyanins in extract (mg/mL) | Proteins (mg) | Sugar (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  |

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) (NH₄)₂SO₄ solution at room temperature, extraction in 50% ethanol solution at room temperature (pH=3.47), extraction in 19% (w/w) (NH₄)₂SO₄ 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) (NH₄)₂SO₄. 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) (NH₄)₂SO₄ solution at room temperature, extraction in 50% ethanol solution at room temperature (pH=3.47), extraction in 19% (w/w) (NH₄)₂SO₄ 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) (NH₄)₂SO₄ system at room temperature, respectively. The yields of these methods were 7.56%, 42.78%, 51.86%, 100.00%, and 92.34%, respectively.

| 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.

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. The ATPE method combined with column chromatography has some advantages, as short treatment time, lower ethanol consumption and no heating requirement to yield anthocyanins from the fruit residues of Vaccinium uliginosum Linn. New technology might be a suitable extraction method for other natural pigments and bioactive natural products on industrial scale.
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