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Extraction and preliminary purification of anthocyanins from grape juice in aqueous two-phase system

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ABSTRACT

Aqueous two-phase extraction (ATPE) was employed for the extraction and preliminary purification of anthocyanins in grape juice (GA). The influences of such factors as the type and concentration of salt, the concentration of alcohol and the quantity of grape juice, extraction time, system pH and temperature were investigated to obtain the optimal extraction conditions. They were listed as follow: sodium dihydrogen phosphate (NaH_2PO_4) concentration 28% (w/w), ethanol concentration 25% (w/w), the quantity of grape juice 1 mL, temperature 298.15 K, time 1 h and no pH adjustment. Under the optimal conditions, the relative and absolute recovery of GA in the top phase reached 99.35% and 99.26% in one step, respectively, while the sugar-removing rate reached 75.08%. The results of Multistage and scale-up extractions showed that it could remain over 90% GA in the top phase and remove more than 90% sugars after continuous extraction for two times. Scale-up extractions and the GA stability results showed the effectiveness and great potential of this method in processing mass grape juice.

1. Introduction

As a group of flavonoid compounds, anthocyanins are widely found in fruits, berries, as well as flowers and leaves. These water-soluble pigments providing attractive colors and beneficial health effects such as antioxidant, anti-inflammatory, anticancer, and anti-diabetic properties are a great interest in the food industry [1]. Artificial pigments had been favored over the past 100 years [2]. However, the recognition of artificial pigments can be harmful to humans have made the researchers turn to natural pigments as a response to the desire for a better and healthier diet. Grape juices, one of the products of grapes, are an important source of phytochemicals including anthocyanins. Anthocyanins present in grape juice are also reported to exhibit anti-microbial properties [3]. Previous studies have focused on the extraction, identification, anti-oxidant activities of anthocyanins from grape products [4–7]. Since the grape juices are relatively inexpensive and easily obtained, they can be a great potential source of anthocyanins.

Numerous isolation methods including conventional solvent extraction and modern techniques have been reported about the extraction of anthocyanins from plant materials. Time and solvent consuming, toxicity and low efficiency have limited the application of conventional solvent extraction. Moreover, extraction with heating over a long time could lead to the degradation of anthocyanins and decrease the antioxidant activity of the extracts [8]. Modern techniques like ultrasound assisted extraction (UAE) [9], microwave assisted extraction (MAE) [10] and high pressure CO_2 (HPCD) [11] require special equipments and energy consumption, which will inevitably increase the process risk and cost.

ATPE, as a powerful and valuable tool for the separation of bimolecular mixtures, has gained great attention over the last few decades. It has been widely applied as a first purification step in the separation of proteins [12,13], enzymes [14,15] and natural products [16–18]. The high water content of an aqueous two-phase system (ATPS) provides a mild and non-toxic environment, which is favorable for the biological activities. Meanwhile, the fast processing time, desirable extraction efficiency and low pollution burden have made ATPE become a very competitive technique compared to the other extraction methods. Compared with other systems ("polymer–polymer" and "polymer–salt"), ATPS consist of short chain hydrophilic organic solvents and salts are cheap, and in some way, they can be recycled and reused. This ATPS is both efficiency promoting and cost saving.

In the present work, an ATPS composed of ethanol and NaH_2PO_4 was applied for the extraction and preliminary purification of anthocyanins from grape juice by removing the majority of sugars. Single factor experiment was performed to obtain the optimal conditions. Multistage and scale-up extractions were carried out to remove more sugars and to explore the possibility of handling a

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large amount of grape juice, respectively. In addition, we also investigated the stability of GA in ethanol and salt solutions.

2. Materials and methods

2.1. Chemicals and materials

Grape juice was provided by Great Lake Co., Ltd. (Tianjin, China). Methanol (HPLC grade), Ethanol, hydrochloric acid and sulfuric acid were bought from Sinopharm Chemical Reagent Co., Ltd., (Shanghai, China). Potassium chloride, sodium acetate anhydrous, ammonium sulfate, sodium sulfite, sodium dihydrogen phosphate were purchased from NANJING CHEMICAL REAGENT CO., Ltd. (Nanjing, China). Phenol and trifluoroacetic acid (HPLC grade) were obtained from Aladdin Industrial Corporation (Shanghai, China). All of the above solvents and chemicals used are of analytical grade.

2.2. Selection of phase-forming salt

2.2.1. Phase diagrams

The phase diagrams of ethanol + $(NH_4)_2SO_4$/NaH_2PO_4 + H_2O systems were prepared by titration method [19,20]. A glass vessel (50 mL) was used to carry out the experiment. A $(NH_4)_2SO_4$ or NaH_2PO_4 solution of known concentration was titrated with ethanol until the mixture turned turbid. The compositions of the mixture were obtained by an analytical balance (FA1104B, Shanghai Yueping Instrument Co., Ltd., China), of which the precision is 0.0001 g. System temperature was controlled to within ±0.1 K by using a thermostat water bath (DF-101S, Yu Hua Instrument Co., Ltd., China).

2.2.2. Salt effect on the separation of GA

To choose an appropriate phase-forming salt, NaH_2PO_4 and $(NH_4)_2SO_4$ at 16%, 19%, 22%, 25%, 28% (w/w) were investigated by comparing the effect on the extraction of GA. An ATPS was prepared in a 10 mL centrifuge tube by adding appropriate amount of ethanol (2.5 g), salt, grape juice (1 mL) and distilled water with a total mass of 10 g. The compounds were mixed sufficiently and then the tube was settled in a water bath at 298.15 ± 0.1 K (DF-101S, Yu Hua Instrument Co., Ltd., China) for 1 h. After complete phase segregation, the volumes of the top and bottom phase were recorded. The GA and total sugars in each phase were measured.

2.3. Optimization of ATPE

Single factor experiment was carried out to investigate the effects of the added amount of salt, ethanol, raw material (grape juice), system temperature, extraction time and pH on the partitioning of GA and sugars in ATPS. The added amount of salt has already been studied in Section 2.2.2. ATPSs with ethanol ranging from 13% to 25%, under constant other conditions (at 298.15 K, 1 mL grape juice, 2.8 g salt, 1 h, no pH adjustment and a total mass of 10 g), were prepared to discuss the influence of ethanol concentration on the recoveries and partition coefficients of GA and total sugars. Similarly, the added amount of grape juice (0.6–1.4) mL, the temperature (288.15–328.15) K, the extraction time (10–120) min and the pH (2.0–4.0) were also investigated, respectively, to study their effects on the partitioning behaviors of GA and sugars in ATPE.

2.4. Stability of GA in ethanol and salt solutions

To evaluate the effect of ethanol on GA, systems with various ethanol concentrations (20%, 40% and 60%) were prepared by mixing 1 mL grape juice with appropriate amounts of ethanol and water to make the total weight equal to 10 g. Afterwards, the mixture was allowed to settled in the dark at room temperature. The preserving rate of GA of each system at 10, 30, 60 and 120 min, were analyzed, respectively.

Similarly, to investigate the effect of salt on GA, systems with various salt concentrations (20%, 30% and 40%) were prepared by mixing 1 mL grape juice with appropriate amounts of salt solution and water to make the total weight equal to 10 g. Afterwards, the mixture was allowed to settled in the dark at room temperature. The preserving rate of GA of each system at 10, 30, 60, 120 min, was analyzed respectively.

2.5. Multistage and scale-up extractions

2.5.1. Multistage extraction

Under the optimal conditions (2.8 g salt, 2.5 g alcohol, 1 mL grape juice, 298.15 K, 1 h, no pH adjustment with a total mass of 10 g), the first extraction was carried out. Then, the top phase of the first extraction was transferred to a fresh bottom phase that was the same composition and volume as the first extraction, the mixture was well blended and allowed for complete phase segregation for 1 h. Similarly, the third extraction was also carried out. The GA and total sugars partitioning in the each phase of each extraction were estimated [21].

2.5.2. Scale-up extraction

Scale-up assays were performed under such conditions as temperature (298.15 K), pH (no pH adjustment) and extraction time (1 h). The 0.1 kg and 1 kg scale extractions were studied, namely, the added amount of alcohol, salt and grape juice were increased 10 and 100 times, respectively. A secondary extraction was also carried out to investigate the possibility of further removal of the sugars. It should be pointed out that grape juice was last added into the system and the mixture should be stirred thoroughly and quickly. The GA and total sugars partitioning in each extraction were estimated.

2.6. HPLC analyses

HPLC analyses were carried out using Agilent 1200 system (Thermo Fisher Scientific, America) equipped with a quaternary pump, surveyor plus detector. Chromatographic analysis was performed on an Agilent TC-C18(2) column (4.6 × 250 mm, 5 μm, Shanghai, China). Sample solution was filtrated through a syringe filter (0.22 μm) and the injection volume was 20 μl. The mobile phase consisted of 0.05% trifluoroacetic acid in water (solvent A) and 100% methanol (solvent B) at a flow rate of 1.0 mL/min. Type of isocratic elution was performed with 60% A and 40% B. The column temperature was maintained at 35 °C and the detection wavelength was 520 nm".

2.7. Color and stability analyses

The anthocyanins extract was diluted with an appropriate amount of water. Colorant properties, such as color density, browning and degradation index were estimated before and after the extraction. Special measurements were carried out as described by previous reports, with minor modification [22,23].

Furthermore, storage stability and the stability of GA at different pH values were also investigated. Storage ability was determined at 5 and 25 °C. Colorant properties (color density, browning and degradation index) were estimated. To investigate the stability of GA at different pH values, citrate–phosphate buffer solutions at pH (2.0, 3.0, 4.0, 5.0, and 6.0) were prepared and then were colored with grape juice before and after ATPE. Color density, color intensity and degradation index were studied at 25 °C as described by previous literature, with minor modification [24].