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Extraction, Separation, and Purification of Blueberry Anthocyanin Using Ethyl Alcohol

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Abstract

Blueberry contains many substances that are important to the human body and can prevent cardiovascular diseases, protect the retina, and soften blood vessels. Anthocyanin, which is extracted from blueberry, can activate the retina, strengthen vision, reduce serum cholesterol, triglyceride and high-density lipoprotein, and protect cell nucleus tissues from radical oxidation; hence, blueberry is of importance to scientists from different countries. In this study, anthocyanin was extracted and separated from blueberry using ethyl alcohol to investigate the effects of factors, such as ethyl alcohol volume ratio on anthocyanin extraction and separation technologies. The extracting solution was then purified using the macroreticular resin purification method to investigate the effects of ethyl alcohol concentration and eluent dosage on anthocyanin extraction during purification. The research results demonstrated that 60 % ethyl alcohol volume fraction, 1 : 10 mass ratio of solid to liquid, and 60 °C ultrasonic temperature were the best conditions for anthocyanin extraction. The best purification conditions were 95 % ethyl alcohol, which had been acidized by 0.3 % hydrochloric acid and 70 ml of eluent. This work provides a reference for the application of ethyl alcohol in anthocyanin extraction.

Keywords

Blueberry, anthocyanin, extraction, separation, purification

1 Introduction

The blueberry, a blue fruit, belongs to *Ericaceae*, and it is favoured by people because of its sweet and sour taste, and a large amount of nutritional substances. It is rich in anthocyanin, which is important to the human body and can effectively protect retinal cells and prevent cardiovascular diseases.[1] Anthocyanin, as a water-soluble natural food colouring, can be dissolved by polar solvents, such as methyl alcohol and ethyl alcohol, as well as affected by elements, such as sunlight and temperature. Moreover, it has higher safety compared to the synthetic pigment.[2,3,4] Because of the antioxidant function of anthocyanin, which is 50 times that of vitamin E, it can induce proper crosslinking of collagen, eliminate free radicals, and protect the skin.[5] In addition, it can reduce serum cholesterol, triglyceride, and high-density lipoprotein, enhance low density lipoprotein, inhibit atherosclerosis, regulate blood fat, and prevent cardiovascular diseases and high blood pressure.[6] Blueberry anthocyanin has received much attention because it is edible and contains rich nutritional values.

J. Chen et al. investigated the protective effect and antioxidation mechanism of blueberry anthocyanin extractive *in vivo* and *in vitro* using acute CC14-induced mouse hepatic injury model, and found that blueberry anthocyanin extractive could effectively prevent acute hepatic injury.[7] *Y. Liu et al.* investigated the protective effect of blueberry anthocyanin on retinal pigment epithelium through establishing *in vitro* cell models of replicative senescence and photo-induced injury, and found that blueberry anthocyanin could inhibit the aging of retinal pigment epithelium and protect the cells from photoinduced injury.[8] Anthocyanin can promote the regeneration of rhodopsin and blood circulation, thereby preventing high myopia and improving vision.

J. Y. Gou et al. found that at least one of the miR156 targets, SPL9, could directly inhibit anthocyanin biosynthesis and reduce the accumulation of anthocyanin through destabilization of a MYB-bHLH-WD40 transcriptional activation complex.[9] Their research results revealed the direct connection between the transition to flowering and secondary metabolism, and offered a potential target for the processing of anthocyanin and flavonol content in plants. In the study of *A. Patras et al.*,[10] they mentioned some matters about anthocyanin degradation in thermal processing, and gave a summary of the mechanisms of anthocyanin degradation based on the current dynamics conclusions. The current anthocyanin extraction methods include solvent extraction method, ultrasonic extraction, *etc.* However, the former has low efficiency and the latter costs too much.

In this study, blueberry anthocyanin was extracted using ultrasonic-assisted solution, anthocyanin was purified with macroreticular resin purification, and the factors influencing the extraction, separation, and purification of anthocyanin were analysed, aiming to provide a reference for the extraction, separation, and purification of blueberry anthocyanin.

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2 Experimental

2.1 Materials

The used blueberries were picked from the blueberry planting base in Shijiazhuang, Hebei, China.

2.2 Reagents and equipment

Reagents included AB-8 macroporous resin, vanillic aldehyde (chemically pure), concentrated hydrochloric acid, methyl alcohol (analytically pure), distilled water, citric acid, sodium citrate, hydrochloric acid (36.0−38.0 %), ethyl alcohol (95 %) and ethyl acetate. Equipment included a beater, KQ5200DE numerical control ultrasonic cleaner, RE-52AA rotary evaporator, 722 visible spectrophotometer, and 80-1 medical centrifuge.

2.3 Blueberry anthocyanin extraction and purification techniques

There were three blueberry anthocyanin extraction techniques, *i.e.* solvent extraction, supercritical extraction, and ultrasonic-assisted solvent extraction. Ultrasonic wave can increase the dissolution of anthocyanin and promote the extraction and separation of anthocyanin through inducing a chemical combination between blueberry and solvent.[11] Hence, ultrasonic-assisted solvent extraction was adopted in this study.

There were also three anthocyanin purification techniques, *i.e.* resin purification, high-speed counter-current chromatography and high performance liquid chromatography. Resin purification was used in this study. The specific operating procedures were as follows.

2.4 Extraction and separation of blueberry anthocyanin

Twenty grams of blueberry were taken and made into fruit pulp using a beater. Ultrasonic extraction was performed using 0.3 % hydrochloric acid and 95 % ethyl alcohol at 40 °C for 30 min. After four extractions at a solid-liquid ratio of 1 g : 6 ml, extraction filtration was performed and the filtrate was collected. The red viscous fluid was then obtained after decompression at 40 °C.

2.5 Purification of blueberry anthocyanin

Firstly, AB-8 macroporous resin was immersed in ethyl alcohol for 24 hours. After it was fully swelled, it was washed until there was no turbidity. Then it was washed by deionised water until there was no smell of ethyl alcohol. Resin liquid was removed. Thirty millilitres of concentrated solution, 60 ml of distilled water and 60 ml of ethyl acetate were added for five extractions. The ethyl acetate was then removed by decompression at 40 °C. Sixteen grams of AB-8 macroporous resin, which had been preprocessed, were transferred to chromatographic column, and 30 ml of the concentrated solution obtained above was loaded. After sample loading, it was absorbed by AB-8 macroporous resin for three hours. The impurities, such as protein, were then washed away with 50 ml of distilled water. Anthocyanin, which adhered to the resin, was eluted with 140 ml of 0.3 % hydrochloric acid and 95 % ethyl alcohol. Finally, viscous fluid was obtained after decompression at 40 °C.

2.6 Calculation of anthocyanin content

Anthocyanin content:

$$\gamma = (A \cdot M \cdot F \cdot V)/(m \cdot \varepsilon \cdot l) \quad (1)$$

where γ stands for the mass concentration of anthocyanin, A is absorbance,[12] M is the molar mass of cyanidin 3-*O*-glucoside, 449.2 g mol^{-1}, F is the dilution ratio of blueberry fruit pulp, V is the volume of extracting solution, m is the mass of blueberry, ε is attenuation coefficient,[13] 29600 l mol^{-1} cm^{-1}, and l is the width of cuvette, 1 cm.

2.7 Calculation of anthocyanin purity

The mass of anthocyanin in the solution was calculated using Eq. (1). The purity of anthocyanin was then calculated using the following formula: purity = mass of anthocyanin in product/total mass of product.

3 Results and discussion

3.1 Analysis of investigation factors involved in extraction and separation of anthocyanin

3.1.1 Effects of volume fraction of ethyl alcohol

Twenty-four grams of blueberry were taken and made into fruit pulp. Ultrasonic extraction was then carried out at 40 °C by adding 40 %, 50 %, 60 %, 70 %, and 90 % ethyl alcohol, respectively, in a ratio of 1 : 6.6. After that, extraction filtration was performed and the filtrate was separated; the absorbance of anthocyanin was detected. The content of anthocyanin was calculated using Eq. (1). The experimental results are shown in Fig. 1.

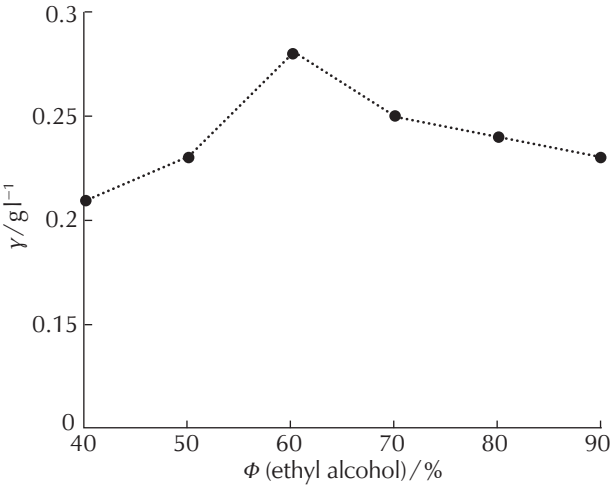

Fig. 1 – Effects of ethyl alcohol concentration on the mass concentration of anthocyanin

Fig. 1 shows that the content of anthocyanin increased with the increase in ethyl alcohol volume fraction (Φ) when ethyl alcohol volume fraction was between 40 % and 60 %, it reached the peak when it was 60 %, and decreased with the increase in ethyl alcohol volume fraction when it was between 60 % and 90 %. Ethyl alcohol with excessively high volume fraction damaged the internal structure of anthocyanin; hence, ethyl alcohol with a volume fraction of 60 % was the best in the extraction of blueberry anthocyanin. The difference in anthocyanin content using 60 % ethyl alcohol and ethyl alcohol with the other volume ratio had statistical significance ($p < 0.05$).

3.1.2 Effects of mass ratio of solid to liquid

Ethyl alcohol with a volume fraction of 60 % was used to extract blueberry anthocyanin at 40 °C for 30 min. The content of anthocyanin was detected when mass ratio of solid to liquid was 1 : 4, 1 : 6, 1 : 8, 1 : 10, 1 : 12, and 1 : 14, respectively.

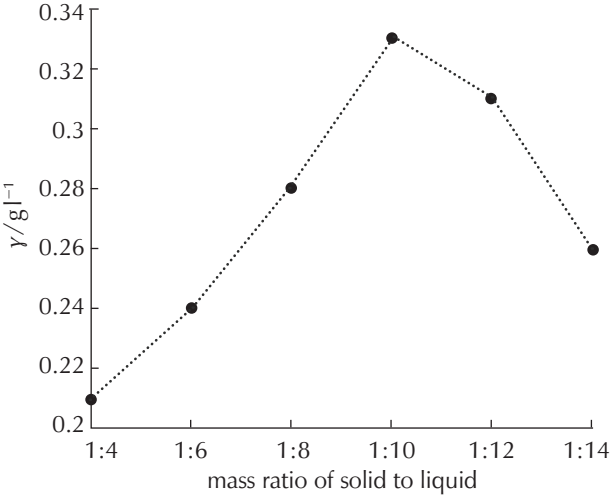

Fig. 2 – Effects of mass ratio of solid to liquid on the mass concentration of anthocyanin

As shown in Fig. 2, the content of anthocyanin increased with the decrease in mass ratio of solid to liquid when mass ratio of solid to liquid was between 1 : 4 and 1 : 10. When mass ratio of solid to liquid was 1 : 10, the content of anthocyanin extracted was the highest; when mass ratio of solid to liquid was lower than 1 : 10, the content of anthocyanin decreased with the decrease in mass ratio of solid to liquid. Therefore, the content of anthocyanin was the highest when mass ratio of solid to liquid was 1 : 10.

3.1.3 Effects of extraction temperature

Twenty-four grams of blueberry were taken and made into fruit pulp and evenly divided into 6 pieces. Ethyl alcohol at a volume fraction of 60 % was then added in a ratio of 1 : 6.6, and the anthocyanin was extracted by ultrasound at 15 °C, 30 °C, 45 °C, 60 %, 75 %, and 90 %, respectively, for 30 min. The absorbance of the concentrated solution, which was obtained by extraction filtration, was detected. The extraction content of anthocyanin was calculated using Eq. (1), and the results are shown in Fig. 3.

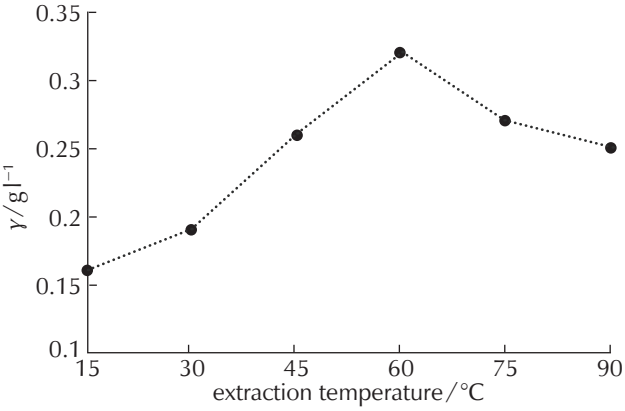

Fig. 3 – Relationship between extraction temperature and the mass concentration of anthocyanin

As shown in Fig. 3, the content of anthocyanin increased firstly, and then decreased with the increase in temperature. When the extraction temperature was 60 °C, the content of anthocyanin was the highest, and its difference with the content using the other temperatures had statistical significance ($p < 0.05$). Hence, it could be concluded that 60 °C was the best temperature for the extraction and separation of blueberry anthocyanin.

3.2 Analysis of investigation factors involved in anthocyanin purification

3.2.1 Effects of volume fraction of ethyl alcohol

As anthocyanin is a polar compound, regulating the volume fraction of ethyl alcohol can accelerate the dissociation and release of anthocyanin, greatly affecting the purification speed of anthocyanin in purification technique.

Thirty millilitres of concentrated solution, the impurities of which had been removed using ethyl acetate, were taken and loaded into a 16g AB-8 macroporous resin chromatographic column. The impurities, such as protein, were then washed away with 50 ml of distilled water; after that, 0.3 % hydrochloric acid and ethyl alcohol at volume fraction of 40 %, 50 %, 60 %, 70 %, 80 %, and 95 % were added to elute the volume fraction of ethyl alcohol.

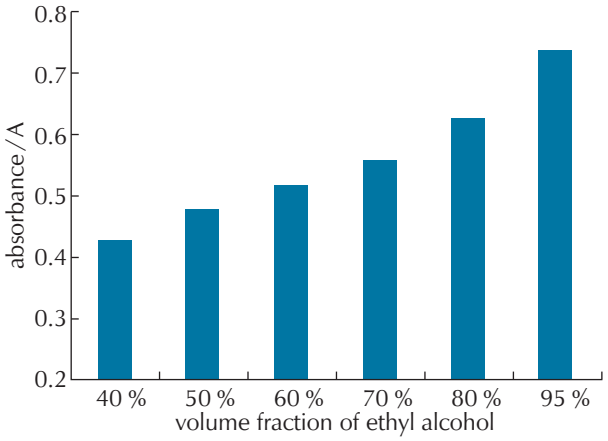

Fig. 4 – Effects of the volume fraction of ethyl alcohol on purification of anthocyanin

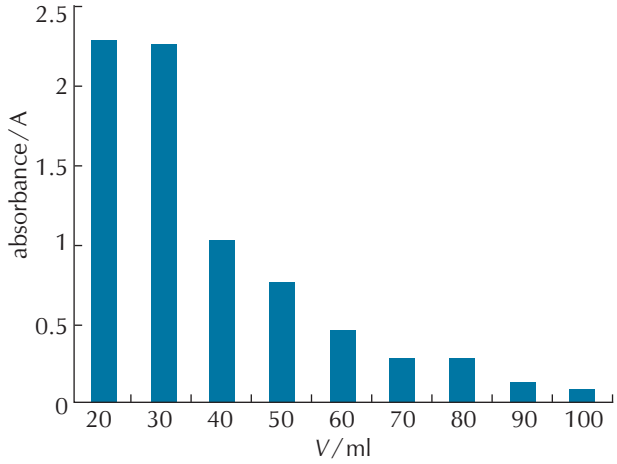

Fig. 5 – Effects of dose of eluent

As shown in Fig. 4, the absorbance and the content of anthocyanin increased with the increase of ethyl alcohol volume fraction. When the volume ratio of ethyl alcohol was 95 %, the absorbance was the highest, and the difference with that using ethyl alcohol with the other volume fractions had statistical significance ($p < 0.05$); the content of anthocyanin was also the highest and the elution efficiency was the best. Therefore, 95 % was the best volume fraction for ethyl alcohol in the test.

3.2.2 Effects of eluent dose

It was concluded from the above experiments that 95 % ethyl alcohol, which was acidized with 0.3 % hydrochloric acid, was the best eluent in purification of anthocyanin. Procedures of the test on the dose of eluent in purification of anthocyanin were as follows. Firstly, 30 ml of concentrated solution, the impurities of which had been removed by ethyl acetate, were taken and then loaded into a 16gAB-8 macroporous resin chromatographic column. After impurities were washed with 50 ml of distilled water, 95 % ethyl alcohol, which had been acidized with 0.3 % hydrochloric acid, was added to elute anthocyanin. Twenty millilitres of eluent were then added, and the absorbance tested and recorded. The dosage of eluent increased gradually, 10 ml each time. The results were recorded as follows.

As shown in Fig. 5, the absorbance decreased with the increase in eluent dose, rapidly decreased when the dose of eluent was between 20 ml and 70 ml, recovered after 70 ml, and finally decreased slowly. After comprehensive consideration, 70 ml was found to be the best dose.

In conclusion, the amount of blueberry anthocyanin extracted was the highest when the volume fraction of ethyl alcohol was 60 %, the mass ratio of solid to liquid was 1 : 10, and ultrasonic temperature was 60 °C. In the purification of anthocyanin, anthocyanin had the highest absorbance and purity when 70 ml of 95 % ethyl alcohol, which was acidized with 0.3 % hydrochloric acid, were used for elution.

4 Conclusion

In this study, blueberry anthocyanin was extracted and separated using ethyl alcohol as the solvent. The effects of ethyl alcohol volume fraction, mass ratio of solid to liquid, and extraction temperature on the content of blueberry anthocyanin were investigated through the experiments. Moreover, the extracted anthocyanin was purified by macroporous resin method. It was found that 95 % ethyl alcohol, which had been acidized with 0.3 % hydrochloric acid and 70 ml of eluent, achieved the best purification efficiency. This study provides some references for the extraction, separation, and purification of blueberry anthocyanin.

List of abbreviations and symbols

A – absorbance
F – dilution ratio of blueberry fruit pulp, $g\,ml^{-1}$
l – width of cuvette, cm
m – mass of blueberry, g
M – molar mass of cyanidin 3-*O*-glucoside, $g\,mol^{-1}$
V – volume of extracting solution, ml
γ – mass concentration
ε – attenuation coefficient, $l\,mol^{-1}\,cm^{-1}$
Φ – volume fraction, %

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SAŽETAK

Ekstrakcija, odvajanje i pročišćavanje antocijanina borovnice etanolom

Zhe Gao

Borovnica sadrži niz tvari koje su važne za ljudsko tijelo te mogu spriječiti kardiovaskularne bolesti, zaštititi mrežnicu i poboljšati stanje krvnih žila. Antocijanin, ekstrahiran iz borovnice, može aktivirati mrežnicu, ojačati vid, smanjiti serumski kolesterol, triglicerid i lipoprotein visoke gustoće te zaštititi tkivo stanica od radikalske oksidacije; dakle, borovnica je važna znanstvenicima diljem svijeta. Kako bi se istražili učinci čimbenika kao što je volumni omjer etilnog alkohola pri ekstrakciji antocijanina i tehnologija odvajanja, antocijanin je u ovoj studiji ekstrahiran i odvojen iz borovnice etanolom. Otopina za ekstrakciju je zatim pročišćena makroretikularnom smolom radi ispitivanja učinaka koncentracije etanola i doziranja eluenta na ekstrakciju antocijanina tijekom čišćenja. Rezultati istraživanja pokazali su da su volumni udjel etanola od 60 %, maseni omjer čvrste tvari i tekućine od 1 : 10 i ultrazvučna kupelj pri 60 °C najbolji uvjeti za ekstrakciju antocijanina. Najbolji uvjeti za pročišćavanje bili su 95 % etilnog alkohola koji je bio zakiseljen s 0,3 % klorovodične kiseline i 70 ml eluenta. Ovaj rad daje referenciju za primjenu etanola u ekstrakciji antocijanina.

Ključne riječi
Borovnica, antocijanin, ekstrakcija, odvajanje, pročišćavanje

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