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Extration, identification and stability ananlysis of anthocyanins from organic Guizhou blueberries in China

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Abstract

Guizhou Majiang organic blueberries from China are a prevalent fruit in the health industry and have high anthocyanin contents. This study used the ethanol extraction method, ultrasonic-assisted extraction, AB 8 macroporous resin adsorption purification, and the addition of 3% citric acid to extract anthocyanins, and through single-factor tests and orthogonal tests, the optimum extraction conditions of anthocyanins from blueberries was determined. The optimal extraction conditions were as follows: the extraction temperature was 40 °C, the ethanol volume fraction was 80%, the solid-liquid ratio was 1:10, the ultrasonic extraction time was 60 min, and the extraction amount was 2.56 mg/g. Anthocyanins were identified by HPLC, and the effects of storage conditions on their contents were investigated. K^+, Cu^{2+}, and Fe^{2+} had little influence on anthocyanin contents, while hydrogen peroxide had a highly destructive effect. With increasing illumination time, the retention rate of anthocyanins decreased gradually; the higher the temperature was, the stronger the damaging effect on anthocyanin contents, and the appropriate storage temperature was determined to be -4 °C or below. The absorbance was the highest when pH =2. Taken together, the results of our study revealed the optimal extraction process and appropriate preservation conditions of anthocyanins from organic Guizhou blueberries.

Keywords: blueberry anthocyanins; extraction; orthogonal test; HPLC; stability analysis.

Practical Application: Anthocyanins have strong antioxidant properties, and they are widely used in medicine and health care products. In this study, the optimal extraction conditions to achieve the highest extraction yield of anthocyanins from organic Guizhou blueberries were obtained. The appropriate conditions for the preservation of anthocyanins were also investigated. This study provides a data reference for the extraction and application of anthocyanins in blueberries.

1 Introduction

Blueberries are the health fruit of the 21st century, and they are gaining popularity as consumers demand healthier, more nutritious food products. Organic Majiang blueberries are a specialty of Guizhou Miao and Dong autonomous county and are one of China's national geographical indication products. In the first year of the 13th Five-year Plan, Guizhou Province proposed the "Great poverty alleviation", "Big Data", and "Great Health" as three big development strategies; the "great health" strategy covers pharmaceutical plant cultivation, drug development, health food, health tourism, health services, and other fields. Blueberries were rated as one of the five human health foods by the Food and Agriculture Organization of the United Nations, and Majiang County in Guizhou Province cultivate blueberries with advantageous geographical conditions because it is located in a humid subtropical monsoon climate zone with no cold winter sand hot summers, abundant rainfall, and an annual average temperature of 14~16 °C, so it is very beneficial to blueberry cultivation. Blueberries contain rich nutrients; in addition to protein, fat, carbohydrates, vitamin A, vitamin C, vitamin E, superoxide dismutase (SOD), Ca, P, Mg, Zn, Fe, Cu, etc., polyphenols, anthocyanins and phenolic acids are the main components of blueberry polyphenols and have good antioxidant activity and scavenging effects on reactive oxygen species in cells (Thomasset et al., 2009; Urias-Lugo et al., 2015). Blueberries have high anthocyanin contents and contain several anthocyanins (Hu et al., 2006; Mikulic-Petkovsek et al., 2012), such as delphinium, cornflower pigment, petunia pigment, peony anthocyanin, and mallow pigment (Chorfa etal., 2016). However, anthocyanins are particularly unstable in nature and combine with different monosaccharides, disaccharides, trisaccharides and other compounds to form anthocyanin compounds in plants. Therefore, anthocyanins in nature exist in the form of anthocyanin complexes.

Anthocyanins have a variety of biological functions, including antioxidant, antitumor, vision-protective, cardiovascular disease-preventive, anti-inflammatory, antiaging, immune-enhancing, estrogen-like effects (Diaconeasa et al., 2015), control metabolic syndrome and obesity (Yildiz et al.,2020). Therefore, it is particularly important and meaningful to explore a simple and safe method to extract anthocyanins from blueberries. At the same time, due to the instability of anthocyanins, it is necessary to explore their appropriate storage conditions. Ethanolacidified with 0.01% HCl was an effective extractant of anthocyanins, and ultrasound treatment may further improve the extraction of anthocyanins (Silva et al., 2017).

Therefore, in this work, the optimum extraction conditions of anthocyanins from Majiang blueberries were studied by orthogonal testing with the aid of ultrasonication and citric acid.

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The anthocyanins were identified by HPLC, and the stability of anthocyanins was analyzed. The aim of this study was to provide a basis for the extraction, preservation and application of blueberry anthocyanins.

2 Materials and methods

2.1 Materials

Fresh and organic samples of blueberry fruits of the Canlan variety were harvested in Majiang County in Guizhou (China). Ab-8 macroporous resin were purchased from Solarbio (USA). Petroleum ether was purchased from Nanjing Zhongchun Biotechnology Co., Ltd (China). A rotary evaporator was purchased from Shanghai Yarong Biochemical Instrument Factory (China). A vacuum freeze dryer was purchased from Shanghai Bilang Instrument Manufacturing Co., Ltd (China). An ultrasonic cleaner was purchased from Kunshan Shumei Ultrasonic Instrument Co., Ltd (China). A high-performance liquid chromatography (HPLC)instrument (1100 series) was purchased from Agilent (USA). Cyanidin 3-O-glucoside (CGE) was purchased from PhytoLab (GERMANY).

2.2 Extraction and purification of blueberry anthocyanins

Blueberry anthocyanins were extracted by using ethanol extraction, with the use Ab 8 macroporous resin for purification. Specifically, 200 g of blueberries was accurately weighed, combined with 3% citric acid (for extraction assistance and the prevention of anthocyanin oxidation) and ground into homogenate, according to a certain proportion of material to liquid. Then, a certain volume fraction of acid (hydrochloric acid content of 0.2%) was added, the mixture was subjected to room-temperature oscillation for 30 min, and then ultrasonication at a power of 100W was applied (this power can be achieved with an ordinary ultrasonic cleaning instrument in the laboratory and does not require special equipment, which is convenient for the popularization of this experimental method). Blueberry anthocyanins were extracted by ultrasonication for some time, and then filtration and petroleum ether extraction were implemented prior to AB 8 macroporous resin adsorption. The anthocyanins were eluted with 75%, the eluate was placed in a rotary evaporation apparatus at 45 °C to evaporate the ethanol, and the anthocyanin concentrate was obtained.

The AB 8 macroporous resin was pretreated by soaking the macroporous resin with distilled water for 24 h to achieve full swelling. A column was packed with the resin, which was then washed with 2 volumes of 95% ethanol and subsequently 2 volumes of deionized water to remove all ethanol. The column was then washed with an equal volume of a 4% hydrochloric acid solution and water until the pH was neutral.

2.3 Total anthocyanin content

The total anthocyanin content was measured by the pH differential (PD) method. In the determination of the anthocyanin content in blueberries, the PD method and HPLC method showed similar trends (Lee et al., 2016). In pH=1.0 and pH=4.5 buffer conditions, λvismax and 700 nm absorbance values were determined by ultraviolet spectrophotometry according to the Fuleki T formula to calculate the anthocyanin content:

$$Anthocyanin\ content\left(mg/L\right)=\frac{A\times MW\times DF\times 1000}{\varepsilon\times 1} \quad (1)$$

where A refers to (Absorbanceλ_{vismax}–Absorbance$\lambda_{700\ nm}$) pH 1.0 - (Absorbanceλ_{vismax}–Absorbance$\lambda_{700\ nm}$) pH 4.5, MW (449.2 g/mol) refers to the molecular weight of cya-3-glc, DF refers to the dilution ratio, and ε (26,900) refers to the molar absorptivity. According to the method by Giusti & Wrolstad (2001), λ_{vismax} =510 nm.

Through unit conversion of Formula 1, the total anthocyanin content (mg cyanidin-3-galactoside equivalent/g fresh weight) was estimated by Formula 2:

$$Anthocyanin\ content\left(mg/g\right)=\frac{Formula\left(1\right)\times V}{m} \quad (2)$$

where V refers to the extraction liquid volume and M refers to the mass of the blueberries.

2.4 Single-factor test

According to the above experimental procedure, under the condition of a fixed ultrasonic power of 100 W, the effects of four factors (the solid-liquid ratio, ethanol volume fraction, ultrasonic extraction time and extraction temperature) on anthocyanin extraction were investigated. The specific conditions were set as follows: the ultrasonic extraction temperature was set to 20 °C, 30 °C, 40 °C, 50 °C and 60 °C; the ultrasonic extraction time was set to 30 min, 40 min, 50 min, 60 min and 70 min; the ethanol volume fraction was set to 40%, 50%, 60%, 70% and 80%; and the solid-liquid ratio set to 1:4, 1:6, 1:8, 1:10 and 1:12.

2.5 Orthogonal test

Based on the results of the single-factor test, the orthogonal test with 4 factors and 3 levels was selected. Four influencing factors, namely, extraction temperature, extraction time, ethanol volume fraction and solid-liquid ratio, were named A, B, C and D, respectively. In combination with single-factor test results, the optimal three levels from each factor were combined, and a four-factor three-level orthogonal experiment was carried out.

2.6 Measurement of total anthocyanin contents by HPLC with CGE

A high-performance liquid chromatography (HPLC) system (Agilent Model 1100, USA) equipped with a C18 column (4.6 mm × 250 mm, 5.0 μm, Agilent, USA) was used. The method was performed as previously described (Wang et al., 2000), with appropriate adjustments. Gradient elution of anthocyanin extracts was conducted with an 8.5% aqueous methanol solution as eluent A, and 22.5:6.5:41.5 acetonitrile :

methanol : water. The flow rate and column temperature were maintained at 0.8 mL/min and 35 °C, respectively. The wavelength was set at 510 nm. Cyanidin 3-O-glucoside (CGE) was used as the standard.

2.7 Stability test

Metal ions on the stability of anthocyanins

Solutions of different concentrations of K^+, Cu^{2+}, and Fe^{2+} were used to dilute blueberry anthocyanin extracts to concentrations of 10 mmol/L and 20 mmol/L, respectively, placed in the dark, and the absorbance was measured at 510 nm every 24 h for 8 days.

Antioxidant effect on the stability of anthocyanins

In the pH = 3 blueberry anthocyanin extracts, different volumes of a H_2O_2 solution were added to achieve concentrations were 0.1 mL/100 mL, 0.5 mL/100 mL and 1.0 mL/100 mL and were placed in the dark. The absorbance was measured at 510 nm every 24 h for 8 days.

Effect of light on the stability of anthocyanins

Ten milliliters of blueberry anthocyanin extract was placed in transparent glass bottles with a cap, exposed to indoor lamplight illumination, sunlight (18~28 °C) and ultraviolet light, and the absorbance was measured every 2 h for 8 h.

Effect of temperature on the stability of anthocyanins

Five 30 mL brown bottles with caps, and 10 mL of blueberry anthocyanin extracts were added to each bottle at 4 °C, 20 °C, 40 °C, 60 °C and 80 °C thermostat water baths, and the absorbance was measured every 2 h for 8 h.

Effect of pH on the stability of anthocyanins

Eight 10 mL aliquots of blueberry anthocyanin extracts were taken, and the pH value was adjusted to 1, 2, 3, 4, 5, 6, 7, and 8 by disodium hydrogen phosphate and citric acid. The absorbance was measured at 510 nm every hour to calculate the retention rate of anthocyanins.

The retention rate was calculated by the measured absorbance value, and the Formula 3 is as follows.

$$Retention\ rate(\%) = \frac{Abosrbance\ after\ treatment}{Absorbance\ before\ treatment} \times 100\% \quad (3)$$

2.8 Statistical analysis

The data are expressed as the means ± standard deviations. SPSS software (version 20.0, SPSS Inc., USA) was used to analyze the significance of differences. Origin (version 9.0.0, Origin Lab, USA) was employed for statistical analysis and graphing. Orthogonal tests and analysis of variance were used. Statistical significance was taken at the $p<0.05$ level.

3 Results

3.1 Single-factor test results

Effects of extraction temperature

Five portions of 100 g of blueberry pulp were accurately weighed. According to the solid-liquid ratio of 1:6, the ethanol volume fraction was 60%, and the ultrasonic power was 100 W. Ultrasonication was performed at temperatures of 20 °C, 30 °C, 40 °C, 50 °C, and 60 °C for 60 min. After centrifugation, the supernatant was collected, and the absorbance values were measured at wavelengths of 510 nm and 700 nm.

As shown in Figure 1A, with other parameters being equal, the extraction amount of blueberry anthocyanins reached the highest when the extraction temperature reached 40 °C and then gradually decreased with the further increase in temperature. The reason may be that the anthocyanins were not completely dissolved when the temperature was low, and it can be completely dissolved when it reached approximately 40~50 °C. However, because anthocyanins are sensitive to heat, a further increase in temperature leads to anthocyanin decomposition. Therefore, 30~50 °C was selected as the factor level of the orthogonal test.

Effects of extraction time

Five portions of 100 g of blueberry pulp were accurately weighed. According to the solid-liquid ratio of 1:6, the ethanol volume fraction was 60%, the ultrasonic power was 100 W, and the temperature was 40 °C. Ultrasonication was performed at 30 min, 40 min, 50 min, 60 min, and 70 min. After centrifugation, the supernatant was collected, and the absorbance values were measured at wavelengths of 510 nm and 700 nm.

As shown in Figure 1B,with the other parameters held constant, the extraction amount of anthocyanins was the highest when the extraction time was 50~60 min, and the extraction amount decreased slightly as time increased, which indicated that the anthocyanins were completely extracted. Therefore, 50~70 min was selected as the factor level of the orthogonal test.

Effects of ethanol volume fraction

Five portions of 100 g of blueberry pulp were accurately weighed. The solid-liquid ratio was 1:6, the ultrasonic power was 100 W, the temperature was 40 °C, the ultrasonic time was 60 min, and the anthocyanins were extracted according to the ethanol volume fractions of 40%, 50%, 60%, 70% and 80%. After centrifugation, the supernatant was collected, and the absorbance values were measured at wavelengths of 510 nm and 700 nm.

As shown in Figure 1C, with the other parameters being held constant, the extraction amount of anthocyanins was the highest when the ethanol volume fraction was 70% and then decreased when the volume fraction increased. This result indicated that a suitable water content was helpful for anthocyanin extraction. Therefore, 60%~80% was selected as the volume fraction factor level of the orthogonal test.

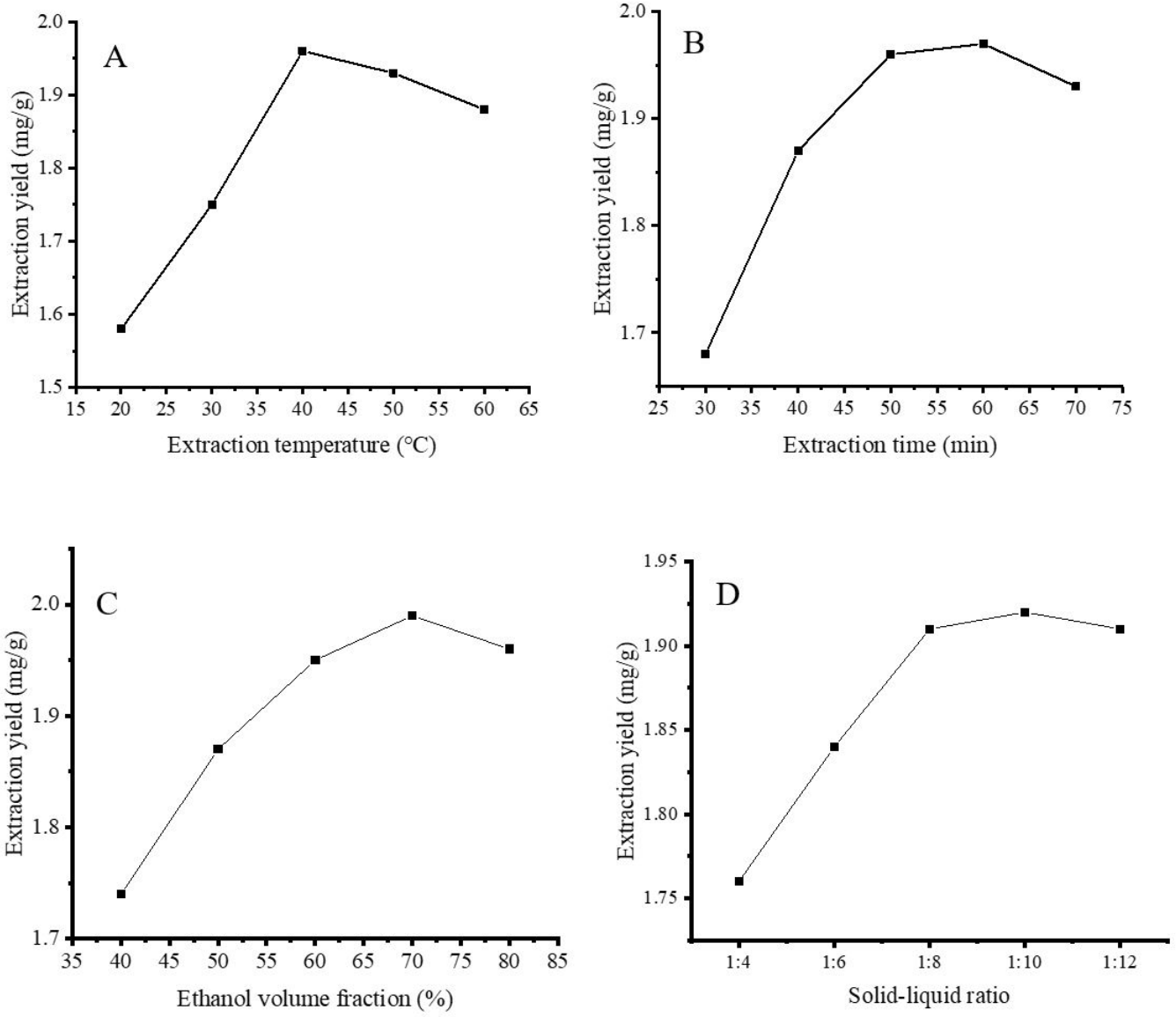

Figure 1. Effects of extraction temperature (A), extraction time (B), ethanol volume fraction (C) and solid-liquid ratio (D) on the extraction yield of anthocyanins.

Effects of solid-liquid ratio

Five portions of 100 g of blueberry pulp were accurately weighed. The ethanol volume fraction was 60%, the ultrasonic power was 100 W, the temperature was 40 °C, and the ultrasonic time was 60 min. Anthocyanins were extracted at solid-liquid ratios of 1:4, 1:6, 1:8, 1:10, and 1:12. After centrifugation, the supernatant was collected, and the absorbance values were measured at wavelengths of 510 nm and 700 nm.

As shown in Figure 1D, with other conditions unchanged, the extraction amount of anthocyanins reached the highest after the solid-liquid ratio reached 1:8 and then tended to plateau. Considering the subsequent concentration and drying of anthocyanin extract, 1:8~1:12 was selected as the factor level of the orthogonal test.

3.2 Orthogonal test results

Based on the results of the single-factor experiment, four factors were selected for the three-level orthogonal test: 30 °C, 40 °C and 50 °C were selected for extraction temperatures (A); 50, 60, and 70 min were selected for extraction times (B); 60%, 70%, and 80%were selected for ethanol volume fractions (C); and 1:8, 1:10, and 1:12 were selected for solid-liquid ratios (D). The orthogonal test results are shown in Tables 1 and 2.

The range of factor A was the largest, so the most significant influence on the extraction yield of blueberry anthocyanins was the temperature. The optimum process obtained by the orthogonal test was A2B2C3D1, but from the range analysis

Table 1. Orthogonal test results.

Test number	Factors				Anthocyanin content (mg/g)
	A (°C)	B (min)	C (%)	D	
1	30	50	60	1:8	1.63
2	30	60	70	1:10	1.72
3	30	70	80	1:12	1.81
4	40	50	70	1:12	2.31
5	40	60	80	1:8	2.48
6	40	70	60	1:10	2.40
7	50	50	80	1:10	2.13
8	50	60	60	1:12	1.98
9	50	70	70	1:8	1.92
K1	1.72	2.02	2.00	2.01	
K2	2.40	2.06	1.98	2.08	
K3	2.01	2.04	2.14	2.03	
R	0.68	0.04	0.16	0.07	
Primary and secondary order	A>C>D>B				
Optimal Level	A2B2C3D1				

results, it was concluded that the optimal process was A2B2C3D2; this inconsistency required validation of the method.

Anthocyanins were extracted according to the optimum range analysis process (40 °C, 60 min, 80%, 1:10), and the extraction amount was 2.56 mg/g, which was higher than that of the orthogonal test under the optimal process (2.48 mg/g). Therefore, the optimal process of A2B2C3D2 was finally chosen.

3.3 HPLC results

HPLC has been the most widely used tool for the identification of anthocyanins, in which the individual anthocyanins can be separated by their polarity (Lee et al., 2008). As shown in Figures 2 and 3, using cyanidin 3-O-glucoside (CGE) as the standard, CGE was isolated from blueberry anthocyanin extracts by HPLC.

3.4 The influence of different storage conditions on blueberry anthocyanin stability

The influence of metal ions

As shown in Figure 4, after adding K^+, Cu^{2+} and Fe^{2+} to the extracts, with increasing time, the blueberry anthocyanin retention rate gradually decreased but remained above 90%. The influence of 10 mmol/L K^+ and Cu^{2+} on the anthocyanin retention rate was relatively low, and when their concentrations increased to 20 mmol/L, there was no obvious decrease; however, Fe^{2+} addition at 10 mmol/L and 20 mmol/L showed notable and similar decreases in anthocyanin retention.

Table 2. Variance analysis of orthogonal test results.

Sources of variation	SS	f	F-ratio	F critical-values	Significance
A	0.691	2	345.500	19.000	*
B	0.002	2	1.000	19.000	
C	0.044	2	22.000	19.000	*
D	0.008	2	4.000	19.000	

Abbreviations and symbols: SS, deviation sum of squares; f, degree of freedom; *, $P<0.05$

The influence of hydrogen peroxide (H_2O_2)

As shown in Figure 5A, H_2O_2 had a great influence on anthocyanin stability when different volumes of hydrogen peroxide were added and incubated with the extracts for 24 hours, and the anthocyanin retention rates all decreased to approximately 5%.

The influence of light

As shown in Figure 5B, the influence of natural light and afluorescent lamp on the anthocyanin retention rate was similar; for 4 hours, the anthocyanin retention rate decreased to approximately 88% and then plateaued. However, after ultraviolet light irradiation for 2 hours, the retention rate fell to 86% and then plateaued.

The influence of temperature

As shown in Figure 5C, after storage at -4 °C, the anthocyanin retention rate after 8 h changed very little. At 20 °C, the anthocyanin retention rate was above 80%, and then, with increasing temperature and time, the anthocyanin retention rate gradually declined.

The influence of pH

As shown in Figure 5D, the absorbance value of blueberry anthocyanins decreased first and then increased in the pH range of 2-8. When pH=2, the retention rate reached its maximum, and when pH=5, it reached its minimum.

4 Discussion

Scholars have performed some studies on the extraction conditions of anthocyanins from blueberry, but some of the extraction amounts were not specific, or the extraction conditions required large volumes of solvent. In this study, the anthocyanins extraction amount from organic Guizhou blueberries was 2.56 mg/g by applying simple conditions and easily available instruments. Xu et al. (2016) used cellulose and pectinase as a raw material under the optimal extraction conditions of 37 °C, 2:1 (m/m), 75% (v/v) citrate ethanol and 6 h. The extraction rate of blueberry anthocyanins reached 25%, and the purity reached 49.6% after ethyl acetate extraction. In Duan et al.'s (2015) study, a microwave-assisted extraction was used to extract the anthocyanins from Chinese bayberry, the anthocyanin content was 2.95 ± 0.08 mg/g, the extraction time

was reduced to 15min; however the extraction temperature was 80 °C, anthocyanins may be affected. Xue et al. (2018) found that under microwave-assisted extraction conditions, the critical extraction temperature for optimum anthocyanin yield was 50.75 ± 0.88 °C, and the highest contents of pelargonidin, cyanidin, and delphinidin from blueberries were 1.02 μg/mL, 0.66 μg/mL, and 0.31 μg/mL, respectively. Hutabarat et al. (2019) used the Box–Behnken test to investigate the effect

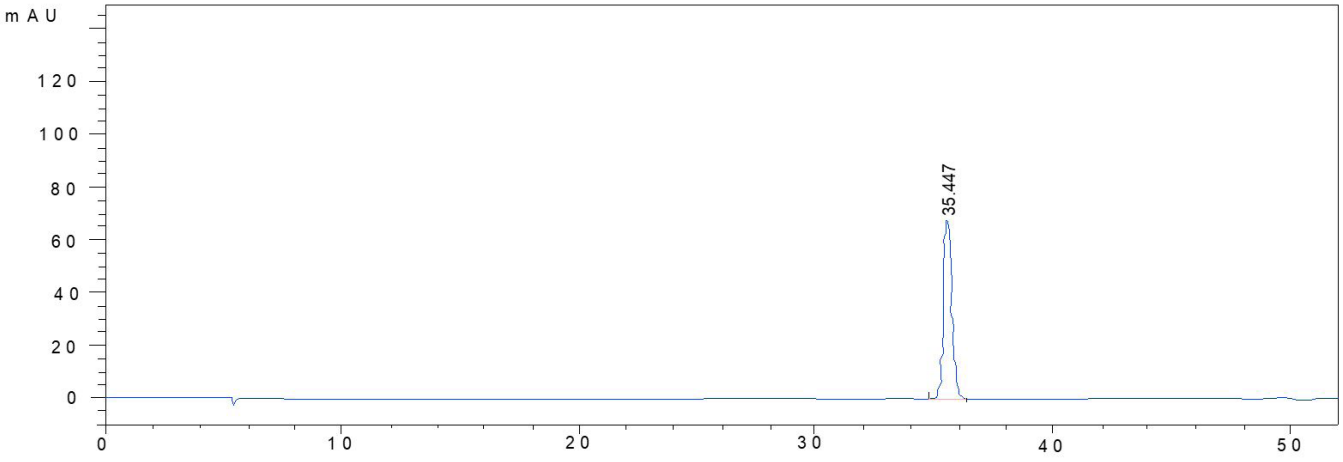

Figure 2. HPLC results of the CGE standard.

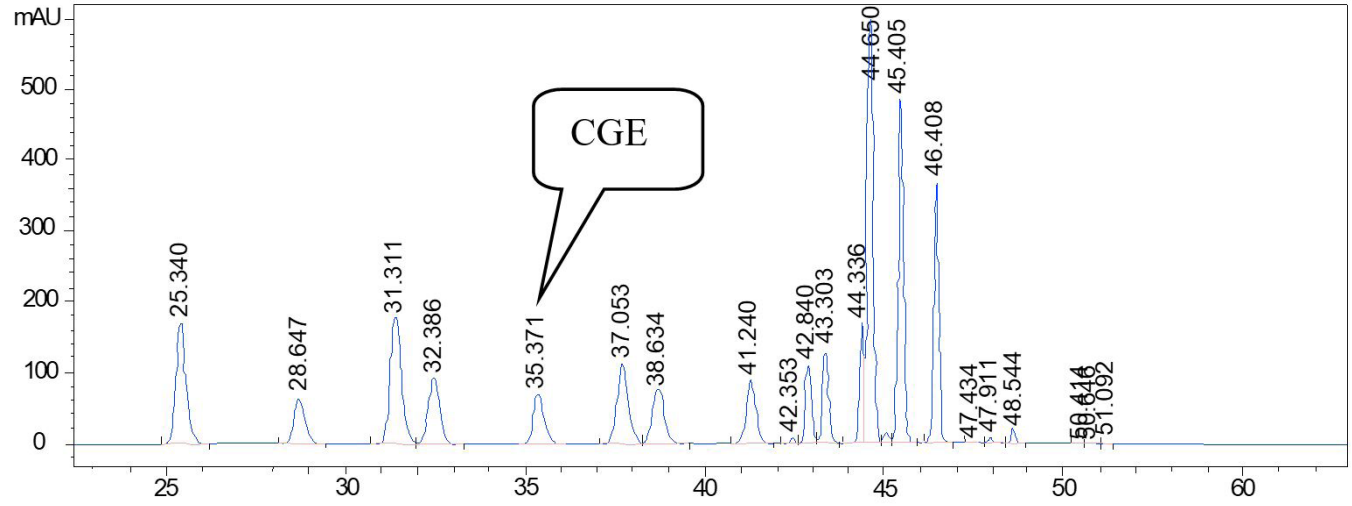

Figure 3. HPLC results of the sample.

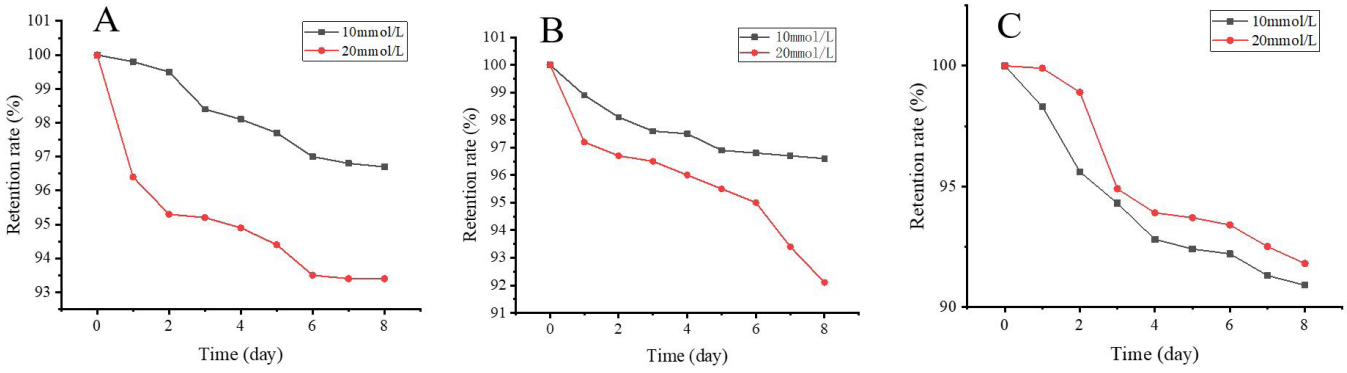

Figure 4. The influence of different concentrations of K^+(A), Cu^{2+}(B) and Fe^{2+}(C) on anthocyanin stability.

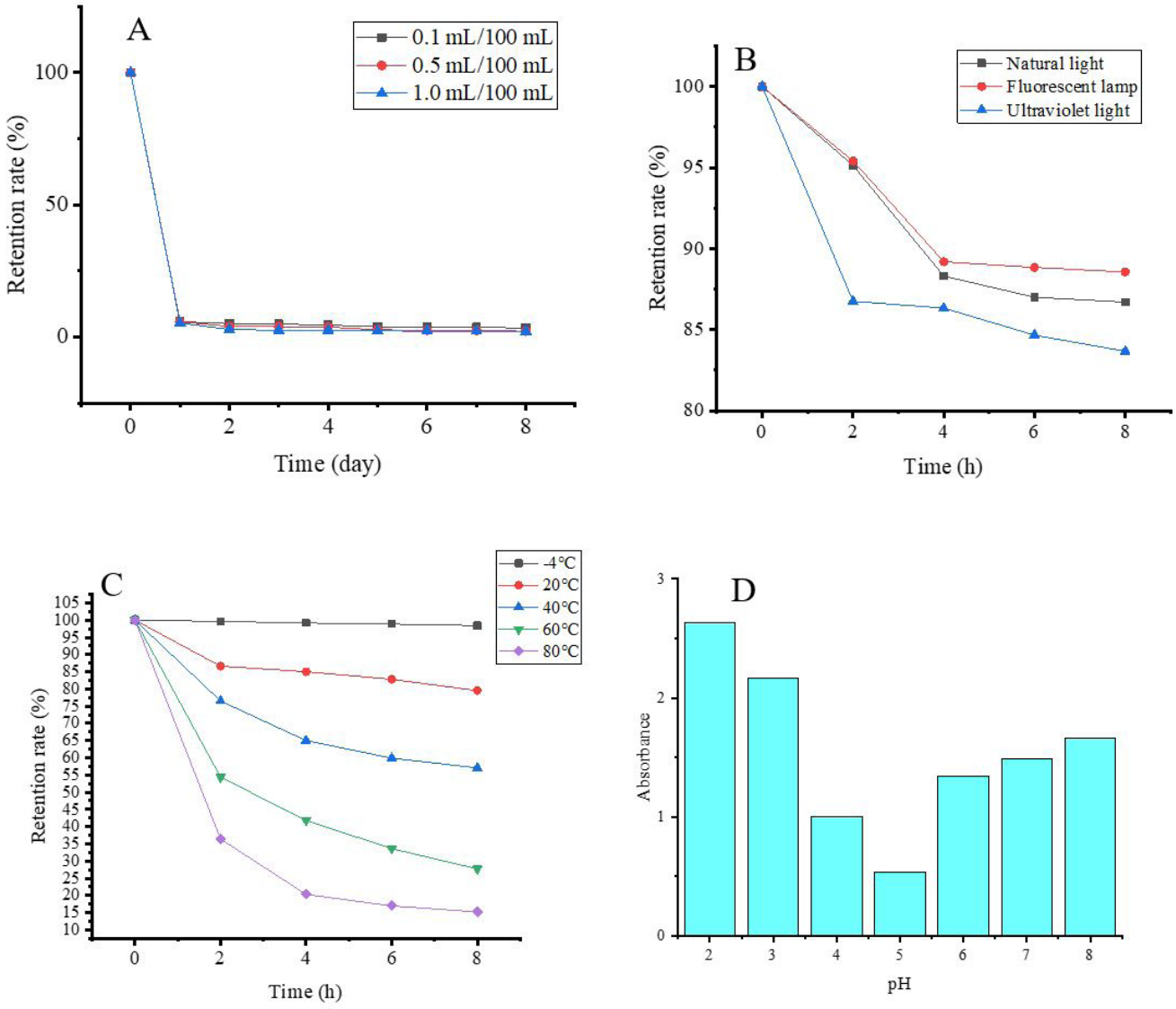

Figure 5. The influence of different volumes of H_2O_2(A), light conditions (B), temperature (C) and pH (D) on anthocyanin stability.

of extraction from rabbiteye blueberry fruits in Nanjing, the optimum conditions of the extraction were as follows: extraction time, 24 h; extraction temperature, 30 °C; extraction solvent, 72.50% ethanol containing 0.02% v/v hydrochloric acid; liquid-to-solid ratio, 20:1 v/w; and extraction yield, 16.21 ± 0.44 mg/g. The extraction amount was high, but the extraction time was long, and the efficiency did not seem to be high.

The stability of anthocyanins is often affected by light, temperature, pH, metal ions, oxygen and coexisting sugars (Riaz et al., 2012). The influence of temperature on the stability of anthocyanins is significant, and the increase in temperature leads to the thermal degradation of anthocyanins and the formation of brown products (Markakis, 2012). Light plays an important role in anthocyanin biosynthesis, but it also accelerates anthocyanin degradation. Amr & Al-Tamimi (2007) confirmed that light has a very adverse effect on the stability of anthocyanins during storage. High oxygen contents can lead to a decrease in anthocyanin contents because anthocyanins inhibit radical activity at high oxygen concentrations, causing depletion of the antioxidant pigments (Gustavo et al.,2009). Anthocyanins are sensitive to changes in pH. In this experiment, the color of blueberry anthocyanins changed with the change in pH. When the pH was 2-5, the solution turned red, and when the pH was 6-8, the color turned dark brown. This indicated that anthocyanins could maintain their original color under acidic conditions but cannot exist stably under alkaline conditions. When a given anthocyanin is dissolved in water, the flavonoid cations form a series of secondary structures based on different acid-base, hydration, and tautomerism reactions (Chandrapala et al., 2012), changing their colors.

5 Conclusions

In this study, the optimum extraction conditions of anthocyanins from organic Majiang blueberry from Guizhou were determined in terms of four parameters: solid-liquid ratio, ethanol volume fraction, ultrasonic extraction time and extraction temperature. The anthocyanin components were identified by HPLC, and the existence of CGE components was confirmed. The conditions of anthocyanin preservation were discussed, oxidationand temperature had the most serious influence on the stability of anthocyanins, followed by light and pH. Metal ions had little influence on the stability of anthocyanins.

Acknowledgements

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