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Full Length Article

Ultra-High Pressure Extraction of Anthocyanins from *Lonicera caerulea* and its Antioxidant Activity Compared with Ultrasound-assisted Extraction

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Abstract

The present study was aimed to determine the effect of ultrahigh pressure on the anthocyanin content and antioxidant activity of extracts from *Lonicera caerulea* berries. Response surface analysis predicted that the optimal conditions for ultrahigh-pressure extraction (UPE) included an extraction time of 7 min 43 sec, berry. Extraction buffer ratio of 1:14.70 (g:mL), and extraction pressure of 426 MPa showed that these conditions would yield a maximum anthocyanin content of 323.85 mg/100 g berries, which was significantly higher than those of extracts produced by ultrasound-assisted extraction and conventional solvent extraction ($P < 0.05$). In addition, radical scavenging assays indicated that the UPE extracts also exhibited higher antioxidant activity ($P < 0.05$), and HPLC-DAD-MS indicated that the UPE extracts had higher total anthocyanin contents and higher contents of two potent antioxidants, 5-methylpyranocyanidin-3-hexoside and cyanidin-3-hexoside-ethyl-catechin ($P < 0.05$). Therefore, current research indicated that UPE can be used as an effective extraction method for bioactive substances. © 2018 Friends Science Publishers

Keywords: Anthocyanins; Antioxidant activity; Ultrahigh-pressure extraction

Introduction

Haskap berries, which are the dark navy blue and long to oval to purple fruits produced by blue honeysuckle (*Lonicera caerulea*, Caprifoliaceae), are widely harvested in Russia, China, and Japan (Palíková *et al.*, 2009). Extracts of the berries contain high levels of phenolic compounds and anthocyanins (Roasto *et al.*, 2013), and research has shown that the anthocyanin content of *L. caerulea* berries can reach 22.73 mg cyanidin-3-glucoside equivalents/g dry weight (Celli *et al.*, 2015). Recently, anthocyanins, which are a type of polyphenol, have attracted increasing attention, owing to their antioxidant activity, since the consumption of antioxidants benefits human health by providing protection from DNA cleavage, inflammation, and cardiovascular and neurodegenerative diseases (Ramirez-Tortosa *et al.*, 2001; Matsumoto *et al.*, 2002; Lazze *et al.*, 2003; Dicko *et al.*, 2005; Rossi *et al.*, 2015).

However, different extraction techniques can affect the yield, quality, and composition of target compounds (Jing and Giusti, 2007; Rodríguez-Solana *et al.*, 2015). For example, ultrasound-assisted extraction (USE) has been used for the extraction *L. caerulea* anthocyanins (Zhang *et al.*, 2014; Celli *et al.*, 2015). Recent research on optimization of the ultrasound-assisted extraction of polyphenols compounds from mulberry (*Morusnigra*) pulp, the Optimum process conditions for anthocyanins extraction is the 76% methanol solution at pH 3, the extraction temperature is 48°C, the 70%ultrasound amplitude, a cycle of 0.7 s and an optimum ratio of liquid to raw material of 12:1.5. According to a previous study, thermal extraction can degrade anthocyanins, which subsequently reduces the antioxidant activity of the extract (Lapornik *et al.*, 2005), therefore the heat treatment of UPE.

In contrast, utilizing the high-pressure processing (100–600 MPa) to extract the bioactive ingredients from natural biological materials, known as ultrahigh-pressure extraction (UPE) or high hydrostatic pressure extraction, is emerging as a promising technology and can be performed at room temperature (Xi and Zhang, 2007; Corrales *et al.*, 2009). Recently, UPE has been successfully used to extract lycopene from tomato paste waste, anthocyanins in grape skins, ginsenosides in Korean panax ginseng powder, and main polyphenols and catechins in green tea (Corrales *et al.*, 2006; 2008; Margarita *et al.*, 2009) and studies have also shown that UPE can be used to shorten processing cycle, acquire higher

extraction rate, and preserve the bioactivity and structure of bioactive ingredients (Jun, 2006; Corrales *et al.*, 2008, 2009; Shin *et al.*, 2010).

The UPE represents a potential means for improving the process of extracting anthocyanins from *L. caerulea* berries; however, its application remains to be investigated. Therefore, the objectives of this research were to optimize the conditions for extracting *L. caerulea* anthocyanin s*via* UPE and to anthocyanin content, antioxidant activity, and anthocyanin composition of *L. caerulea* extracts obtained *via* USE and UPE.

Materials and Methods

Chemicals

In this study all of the chemical reagents used were of analytical and chromatography grade. With hydrochloric acid and ammonium acetate to measure total anthocyanin content; acetonitrile and formic acid were used for the analysis of anthocyanins; and 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,4,6-tripyridyl-s-triazine (TPTZ, 95% purity), and Trolox standard used for the DPPH, ABTS, and ferric reducing antioxidant power value analyses were bought from Dingguo Biological Technology Co., Ltd. (Shenyang, China).

Plant Material

Fully-ripe *L. caerulea* berries were mechanically harvested in Baishan City (Jilin Province, China), packed, quick-frozen, and shipped to Shenyang Agricultural University (Shenyang, Liaoning Province, China). The berries were kept at -80°C in an ultra-low temperature freezer (Revco ULT-1386-3V; Thermo Fisher Scientific, Waltham, MA, USA) until extraction.

Extractionof Anthocyanins

Ultrasound-assisted extraction (USE): The ultrasound-assisted extraction was performed according to a previously reported procedure (Celli *et al.*, 2015). Before extraction, 30 g *L. caerulea* berries was weighed, freeze-dried and grinded using a grinder (E710; Everyday Essentials, Gresham, OR, USA). Anthocyanins were extracted at the condition of ratio of raw material to liquid is 1:25 (g:mL), solvent 80% ethanol and 0.1% HCL, ultrasound bath temperature of 35°C, and extraction time 20 min. Mixtures of the berries and extraction solvent were centrifuged in a L535R refrigerated centrifuge at 4380 × *g* and 4°C for 15 min. The supernatants were collected, evaporated using a RE-52AA rotary evaporator, and freeze dried. The resulting powder was stored at -18°C in the dark until analysis.

Ultrahigh-pressure extraction (UPE): For this test, 30 g *L. caerulea* berries were weighed, freeze-dried, ground using a grinder mixed with acidified solvent of 80% ethanol and 0.1% HCl, poured into polyethylene terephthalate bottles, and then processed in a VX ultrahigh-pressure vessel (Systec GmbH, Linden, Germany). Afterward, mixtures of the berries and extraction solvent were centrifuged in L535R refrigerated centrifuge at 4380 × *g* at 4°C for 15 min, after which the supernatants were collected, evaporated using a RE-52AA rotary evaporator, and freeze dried. The resulting powder was stored at -18°C in the dark until analyzed.

Optimization of UPE conditions: Central composite design methods, with five levels and three-variables (15 experiments), was used to optimize UPE conditions (Chen *et al.*, 2015), and three levels of each variable were chosen for the present study. The independent variables (and their levels) included extraction pressure (300, 400 and 500 MPa), extraction time (6, 10 and 14 min), and solid: liquid ratio (1:10, 1:15 and 1:20, g:mL), and the predicted response of anthocyanin content (y) was calculated using a second-order polynomial model:

$$y = \beta_0 + \sum_{i=1}^{3}\beta_i x_i + \sum_{i=1}^{3}\beta_{ii} x_i^2 + \sum_{i}\sum_{j=i+1}\beta_{ij} x_i x_j \quad (1)$$

Where, β_0 is the offset term, β_i is the linear effect, β_{ii} is the squared effect, β_{ij} is the interplay, and X_i and X_j are independent variables. The response surface data were analyzed using the Box-Behnken program (Design Expert V8.0.6; State-East, Minneapolis, MN, USA), and the coefficients were analyzed by F-value. For determine the optimal conditions for extracting anthocyanins, an analysis of variance (ANOVA), regression analysis, and response surface analysis was accomplished.

Total Anthocyanin Content (TAC) Measurement

The TAC was measured using a slightly modified version of the pH differential method described previously (Giusti and Wrolstad, 2001). Briefly, the freeze-dried extract powders were prepared as aqueous solutions (1 mg/mL), 1 mL aliquots of the solution were mixed with 9 mL pH 1.0 and pH 4.5 buffer solution, respectively, and incubated at dark room temperature. Then, after 20 min, the absorbance of the solutions was determined at 510 nm and 700 nm wavelength, using distilled water as blank, in order to calculate the content of anthocyanins. The absorbance (A) of the sample was then count as follows:

$$\Delta A=(A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}, \quad (2)$$

The monomeric anthocyanin content of the samples was calculated as cyanidin-3-glucoside equivalents, on the basis of the following formula:

$$\text{Anthocyanin content (mg/L)} = (\Delta A \times Mw \times DF \times 1000\ \text{g/mg})/(\varepsilon \times L), \quad (3)$$

Where, Mw indicates the molecular weight of cyaniding-3-glucoside (449.2); the molar absorptivity, ε

indicates the molar absorptivity (26,900); *DF* indicates the dilution factor, and the value of 1000 is the factor for transform g to mg; *L* indicates cuvette path length (1 cm). The anthocyanin content (mg/L) was then converted to mg/100 g (cyaniding-3-glucoside content/100 g wet weight) using the wet weight of the berry sample.

Determination of Antioxidant Capacity

Sample preparation: The freeze-dried extract powders from the two extraction methods and VC (control group) were prepared as aqueous solutions (1 mg/mL) to determine antioxidant capacity.

DPPH radical scavenging activity assay: The DPPH assay measures reductions in the absorbance of DPPH after the introduction of antioxidant-containing samples, so that higher values indicate stronger antioxidant activity. In contrast, the ABTS assay measures reductions in the absorbance of $ABTS^+$ and compares the reduction to that achieved using Trolox (TEAC), so that higher values indicate stronger antioxidant activity. Meanwhile, the FRAP assay measures the reaction of antioxidants with TPTZ–Fe(III), which is converted to TPTZ–Fe(II) (Luo *et al.*, 2012) and quantified using the standard curve of $FeSO_4$,so that higher values indicate stronger ferric reducing power (*i.e*., antioxidant activity).The scaenging effects of the samples on the DPPH radical were monitored using a previously reported method (Zhou *et al.*, 2009). Briefly, 2 mL of the samples was added to 2 mL 0.2 m*M* DPPH, vortexed, then control to react at room temperature and dark environment for 30 min. The absorbance at 517 nm was determined by the spectrophotometer, and the inhibition ratio was calculated as follows:

$$\text{Scavenging ability (\%)} = \left(1 - \frac{A_0 - A_S}{A_0}\right) \times 100\% \quad (4)$$

Where, A_0 is the absorbance of control and A_s is the absorbance of the sample solution.

ABTS radical scavenging activity assay: The ABTS assay was conducted using a slightly modified version of a previously reported method (Zhang *et al.*, 2013). Briefly, ABTS radical cation ($ABTS^+$) solution was prepared by reacting ABTS stock solution with 2.45 m*M* potassium persulfate at room temperature in the dark environment for 12–16 h before use, and then the resulting $ABTS^+$ solution was add with 80% ethanol solution to adjust the absorbance to 0.70 ± 0.05 at 734 nm. Thenadded10μL of the individual samples or Trolox standard to 200 μL diluted $ABTS^+$ solution, absorbance at 734 nm was determined after 2–6 min at room temperature in the dark. Using Trolox as a standard substance, a calibrated solution with a concentration range of 0.15 to 1.5 m*M* was prepared and a standard curve was drawn. The results were presented as mmol/g Trolox equivalent antioxidant capacity (TEAC).

Ferric reducing antioxidant power (FRAP): FRAP was assayed base on the methods of the Beyotime Institute of Biotechnology (Luo *et al.*, 2012). Stock solutions consist of acetate buffer (300 Mm, pH 3.6), TPTZ solution (10 m*M* dissolved in 40 Mm HCl), TPTZ dilution, $FeSO_4$ solution (10 m*M* in deionized water), and 0.1 mL Trolox solution (10 m*M* in deionized water). A FRAP working solution was prepared freshly by mixing the TPTZ dilution, acetate buffer, and TPTZ solution in a ratio of 10:1:1 (v:v:v), respectively, and was warmed to 37°C before use. The samples or (5 μL) $FeSO_4$ solution (0.15–5.00 m*M*) (0.15–5.00 m*M*) were mixed with 180 μ*L* of the FRAP working solution and kept for 5 min at 37°C. The absorbance of the reaction mixture was then measured at 593 nm. A standard curve was prepared using $FeSO_4$ (0.15–5.00 m*M*) and was used to calculate the FRAP activity of the samples, in terms of $FeSO_4$ concentration.

Purification of Anthocyanins

The two samples from the different extraction procedures mentioned above were activated with 95% ethanol and then passed through an AB-8 Presin column (1.5 cm internal diameter, 30 cm length; Solarbio, Beijing, China) (Jordheim, 2007), followed by 0.1% HCl (v/v) in deionized water. Anthocyanins and phenols were adsorbed into the AB-8 macroporous resin, whereas the acids, sugars and other water-soluble matter were eliminated by washing the column with two times the column volume (120 mL) of 0.1% HCl (v/v) in deionized water. The anthocyanins were subsequently recovered by flushing the column with four times the column volume of ethanol containing 0.1% HCl (v/v). This acidified ethanol fraction was evaporated using an RE-52AA rotary evaporator at 35°C and then dissolved in 0.1% HCl (v/v) in deionized water. This purified anthocyanin solution was freeze dried and stored at -20°C until further analyses.

Identification of Anthocyanins

Sample preparation: A 10 mg of extract from the two extraction methods after Purification were weighed and diluted in 1 mL methanol, passed through a 0.45 μm filter and analyzed using HPLC-DAD-ESI-MS^2.

HPLC-DAD-EIS-MS^2analysis: Analysis of anthocyanins by HPLC system (Agilent1100; Agilent Technologies, Santa Clara, CA USA) equipped with a DAD detector (G4212B). A Dikma Platisil C18 column (4.6 mm × 250 mm, 5 μm) was used and operated at 25°C. The mobile phase included the use of aqueous 2% formic acid as solvent A, and acetonitrile as solvent B. The gradient profile consisted of 0 to 40% B for 45 min, 40 to 0% B for 2 min, isocratic 0% B for 5 min, then returned to initial conditions at 52 min, with a flow rate of 1.0 mL/min. Injection volumes were 30 μL, and anthocyanins were detected by their absorbance at 520 nm.

In-line MS^2 data was gathered using the MS (Agilent1100 series LC/MSD Trap SL version) that was provided with electrospray ionization. Analysis was used automatic secondary mass spectrometer scanning and data-dependent MS^2 scanning from 100 to 1200 m/z.

Control the capillary voltage in +3.0 kV. The atomizer gas is nitrogen, under 45 psi condition. Solvent gas was heated up to 350°C in 12 L/min flow delivery.

Statistical Analysis

All tests were repeated three times independently and the results of each analysis were presented as means ± SD. Data were analyzed by ANOVA and the significance of difference of different mean was measured on the basis of the Duncan's New Multiple Range test, with a significance level of $P < 0.05$. The statistical analyses were conducted using SPSS 18.0 (IBM Co., Armonk, NY, USA).

Results

Response Surface Analysis

ANOVA of the second-order response surface model indicated that the TAC of the UPE extracts (Y) was significantly affected by extraction time (X_1), solid: liquid ratio (X_2), and extraction pressure (X_3), as well as by the square of each variable (X_1^2, X_2^2, and X_3^2) and by the interaction between the materal:liquid ratio and extraction pressure (X_2X_3) ($P< 0.05$; Table 1). In addition, the quadratic polynomial equation that contained only the significant terms was a significant predictor of TAC ($F = 32.47$, $P< 0.05$):

$$Y = 24.35+3.65X_1 - 2.03X_2 +0.64X_3+0.43X_1X_2 – 0.57\ X_1X_3- 0.57\ X_1X_3 6.0.17\ X_2X_3 -2.86\ X_1^2 -6.08X_2^2- 5.12X_3^2 \quad (5)$$

Meanwhile, neither X_1X_2nor X_1X_3 had a significant effect on TAC, and the "Lack of Fit" F-value ($F = 32.47$) indicated that the Lack of Fit was not significant ($P = 0.067$) than in the pure error.

When extraction time was held constant at 10 min, the response surface analysis indicated that TAC increased with the solid: liquid ratio, until reaching a maximum at 1:15, and then decreased thereafter (Table 2 and Fig. 1A), because of that high solid: liquid ratio led to the greater loss of anthocyanins. TAC also increased with extraction pressure, reaching a maximum at 400 MPa, and decreased thereafter. On the basis of mass transfer theory, the rate of mass transfer = stress/drag of mass transfer. According to phase behavior theory, the solute will dissolve quicker under higher pressure. The pressure difference between the inside and the outside of the cell is tremendous under UPE conditions.

The maximum predictive value of TAC and the corresponding value of each variable were confirmed using Design-Expert8.0, which indicated that a maximum TAC of 25.64 mg/g could be achieved using an extraction time of 12.5 min, ratio of raw material to liquid of 1:21(g:mL), and extraction pressure 400 MPa. Using additional experimental trials found that optimal conditions yielded a mean TAC of 26.57 ±0.55 mg/g and that the 95% confidence interval (mean error of 3.62%) included the predicted maximum value, therefore validating the model.

Table 1: Analysis of variance (ANOVA) table for the second-order response surface model

Source	df	MS	F-value	p-value
Model	9	41.95	32.47	0.0007
X_1	1	106.65	82.55	0.0003
X_2	1	32.85	25.42	0.0040
X_3	1	3.25	2.52	0.1735
X_1X_2	1	0.72	0.56	0.4882
X_1X_3	1	1.29	1.00	0.3639
X_2X_3	1	0.12	0.09	0.7737
X_1^2	1	30.17	23.35	0.0047
X_2^2	1	136.42	105.58	0.0002
X_3^2	1	96.82	74.94	0.0003
Residual	5	1.29		
Lack of Fit	3	2.06	14.08	0.0670
Pure Error	2	0.15		
Cor Total	14			
S	1.14	R^2	0.9832	
Adj R^2	0.9529	Pred R^2	0.741	

Note: X_1 – extraction time; X_2 –materal: liquid ratio; X_3 – extraction pressure; S – standard error of the regression; R^2 – regression coefficient; df– degrees of freedom; MS – mean square

Extraction Yield

The UPE method yielded the highest TAC (26.57 ±0.55 mg/g), followed by the USE method (21.84±0.35 mg/g) ($P< 0.05$). According to the condition of the methods tested, UPE was the higher yielding and more efficient for extracting anthocyanins from *L. caerulea* berries.

Antioxidant Activity

In this study, DPPH, ABTS and FRAP measure were used to analyze the antioxidant activity of *L. caerulea* extracts. The DPPH radical-scavenging activity of the UPE extracts (91.54%) was significantly higher ($P< 0.05$) than that of the USE (88.17%) and VC (87.99%); whereas the DPPH radical scavenging activity of the USE extracts and VC were similar ($P> 0.05$; Fig. 2A). Similarly, mean TEAC value of the UPE extracts (0.365 m*M*) was significantly higher than those of the USE (0.339 m*M*) and VC (0.323 m*M*) extracts ($P< 0.05$; Fig. 2B). Moreover mean $FeSO_4$ value of the UPE extracts (0.843 m*M*) was significantly higher than those of the USE (0.751 m*M*) extraction and VC (0.619 m*M*), as well ($P < 0.05$; Fig. 2C).

Identification of Anthocyanins

In the present study, HPLC-ESI-MS was used in the identification and quantification of anthocyanins in *L. caerulea* extracts produced using two different extraction techniques. In the liquid phase, there were 14 peaks in the 520 nm condition; however, only 12 anthocyanins were identified. Based on the previous research results of our team, the composition and content of anthocyanins were dependent on the extraction method (Fig. 3 and Table 3). The anthocyanins content in the UPE and USE extracts were 161.98±0.69 and 148.86 ±0.59μg/mg, and cyanidin-3-glucoside (peak$_5$; *m/z* 449) was the most abundant of the 12 compounds,

Table 2: Effect of ultrahigh pressure extraction conditions on the total anthocyanin content of *L. caerulea* berry extracts, based on a central composite design for response surface analysis

Run[A]	Extraction conditions			Analytical results
	Extraction time (min)	Solid (g):liquid (mL) ratio	Extraction pressure (MPa)	Total anthocyanin content (mg/1 g berries)[B]
1	6	1:10	400	8.75 ±0.24
2	6	1:20	400	14.33 ±0.19
3	10	1:15	400	24.62 ±0.34
4	14	1:20	400	21.22 ±0.45
5	10	1:15	400	23.91 ±0.41
6	10	1:20	500	16.08 ±1.03
7	6	1:15	500	13.36 ±0.23
8	14	1:15	500	19.09 ±0.34
9	14	1:15	300	20.51 ±0.65
10	10	1:10	500	13.05 ±0.56
11	10	1:20	300	13.59 ±0.53
12	10	1:15	400	24.51 ±1.12
13	6	1:15	300	12.51±0.39
14	10	1:10	300	9.87 ±0.47
15	14	1:10	400	17.34 ±0.57

Note: A Run number was used for identification purposes only; B mg/L g indicates cyaniding3-glucoside content in 1 g *L.caerulea* berry dry weight;C Values indicate means ± SD (n = 3)

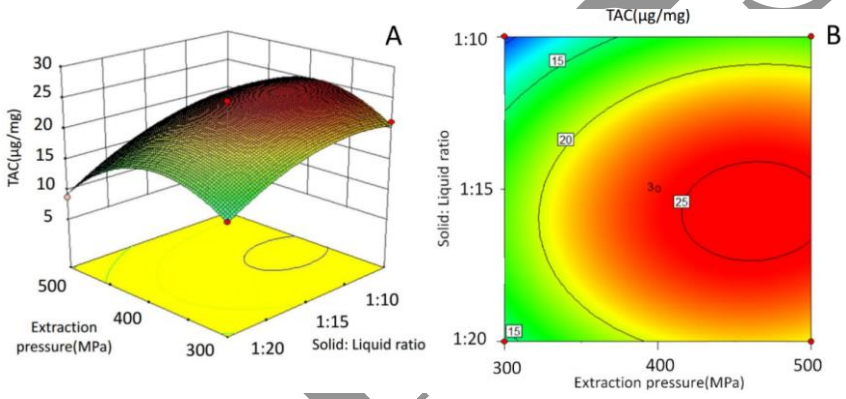

Fig. 1: Effect of extraction conditions on the total anthocyanin content (TAC) of *L. caerulea* berry extracts. (A) Response surface of TAC, as affected by extraction pressure (X2) and liquid: solid ratio (X3) from *L. caerulea*, using ultrahigh-pressure extraction (UPE) with a constant extraction time of 8 min. (B) Corresponding contour map

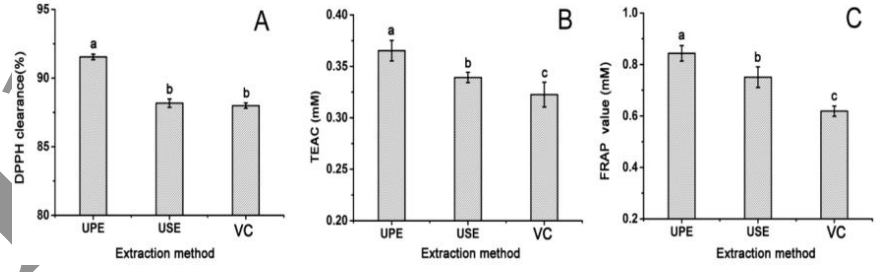

Fig. 2: Antioxidant capacity of *L. caerulea* extracts obtained using three different extraction methods. (A) DPPH radical scavenging ability assay. (B) ABTS radical scavenging activity assay. (C) Ferric reducing antioxidant power. Different lowercase letters indicate statistically significant differences at $P< 0.05$. VC, control group. USE, ultrasound-assisted extraction; UPE, ultrahigh-pressure

accounting for 78.56 and 77.64% of the total anthocyanins content in the UPE, USE extracts. From Table 3 the content of Cyanidin-3,5-dihexoside, Cyanidin-3-glucoside, Cyanidin-3-rutinoside, 5-Methylpyranocyanidin-3-hexoside, Cyanidin-3-hexoside-ethyl-catechin in UPE extract were higher than USE. This means that the retention rate of different anthocyanins was discrepant after ultrahigh pressure extraction: five above showed higher retention rate in 12 anthocyanins.

Table 3: Identities and peak areas of anthocyanins in the *L. caerulea* extracts

RT (min)	Tentative identification	Molecular mass (m/z)	Fragment mass (m/z)	Anthocyanins content (μg/mg)	
				USE	UPE
20.103	Cyanidin-3-hexoside-catechin	737	575, 287	2.05 ±0.24	1.25 ±0.76
21.521	Cyanidin-3,5-dihexoside	611	449, 287	6.54 ±0.33	7.61 ±0.24
23.310[A]	Delphinidin-3-glucoside	465	303	5.81 ±0.35	5.25 ±0.39
	Peonidin-3,5-dihexoside	625	463, 301		
25.612[B]	Cyanidin-3-glucoside	449	287	112.34 ±0.45	127.22 ±0.42
	Cyanidin-3-rutinoside	595	449, 287		
27.035	Dimer of cyanidin-hexoside	897	735, 573, 287	1.50 ±0.76	1.23 ±0.56
27.716	Pelargonidin-3-glucoside	433	271	3.78 ±0.54	3.32 ±0.72
28.664	Peonidin-3-glucoside	463	301	9.63 ±0.52	9.18 ±0.47
29.125	Peonidin-3-rutinoside	609	463, 301	1.07 ±0.69	1.00 ±0.87
30.423	5-Methylpyranocyanidin-3-hexoside	487	325	2.73 ±0.76	3.07 ±0.65
31.523	Cyanidin-3-hexoside-ethyl-catechin	765	603, 475, 313, 287	1.02 ±0.49	1.26 ±0.34
			Sum	148.86 ±0.59	161.98±0.69

Note: A. Delphinidin-3-glucoside coeluted with Peonidin-3,5-dihexoside; B. Cyanidin-3-glucoside coeluted with cyanidin-3-rutinoside; RT – retention time; USE – ultrasound-assisted extraction; UPE – ultrahigh pressure extraction

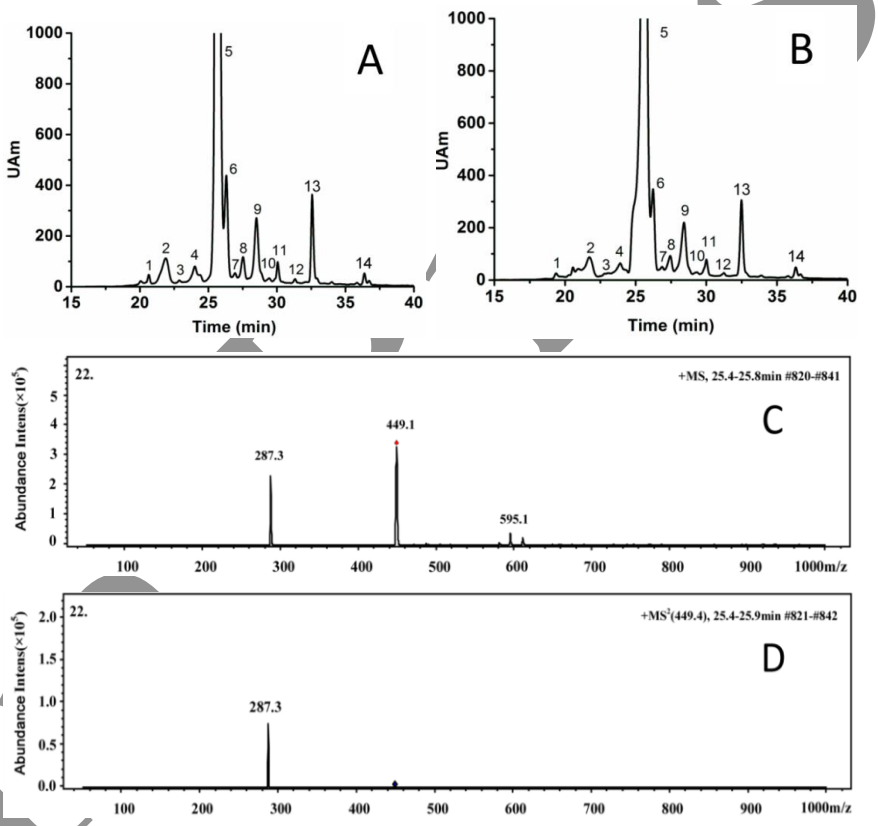

Fig. 3: The HPLC chromatograms (detected at 520 nm) of extracts obtained using different extraction methods and ESI-MS spectra of cyanidin-3-glucoside (MS = 449; MS2 = 287). (A) USE, ultrasound-assisted extraction; (B) UPE, ultrahigh-pressure extraction; (C) MS = 449; (D) MS2 = 287

Discussion

Under pressure difference, the matter will infiltrate very quickly through the broken membranes into cells, the mass transfer rate and the rate of dissolution of solute is very huge(Zhang *et al.*, 2004). However, in the present study, the volume of extracted bioactive substances did not increase with the increase in extraction pressure. Instead, a phenomenon was observed, as pressure increased. The TAC first increased and then decreased, the reason might be that the pressure is too high to reduce the flow of solvent (Zhang *et al.*, 2007).

Peak area is proportional to the content of anthocyanins and that anthocyanin content was positively correlated with

antioxidant capacity (Lee *et al.*, 2008). These results correspond to the result of total anthocyanins content and assays of radical scavenging activity. Furthermore, the differences in anthocyanin contents may have resulted from the binding of anthocyanins to proteins and polysaccharides (Kamachi *et al.*, 2005; Tang *et al.*, 2014), since ultrahigh pressure can affect the structure of proteins and polysaccharides (Tu *et al.*, 2008; Zhu *et al.*, 2016), which are then able to affect anthocyanins by combining with them. Therefore, it is suggested that the difference in the anthocyanin content occurred when ultrahigh pressure hindered the combination of anthocyanins with proteins, polysaccharides, and other biological macromolecules in *L. caerulea* berries and reduced the loss of anthocyanins in the extraction process.

Conclusion

The response surface was used to optimize the UPE conditions for extracting anthocyanins from *L. caerulea* berries. After optimization, the amount of extracted anthocyanins reached 26.57 ±0.55 mg/ g, which was significantly higher than the mean TAC of extracts obtained using USE Furthermore, all three radical scavenging assays (DPPH, ABTS, and FRAP) indicated the UPE extracts had greater antioxidant activity than the USE extracts ($P < 0.05$). In addition, HPLC-ESI-MS indicated that the composition and content of anthocyanins depended on the extraction method, that 12 anthocyanins were present in the *L. caerulea* extracts from all three extraction methods, and that the UPE extracts contained the highest levels of total anthocyanins. Compared with USE, UPE provided the higher yield and antioxidant activity of the *L. caerulea* extracts. In order to verify our conjecture and to provide a more theoretical basis for the application of ultrahigh pressure in berry processing, our team will continue to explore the effects of ultrahigh pressure on the combination of anthocyanins with biological macromolecules.

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