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Microwave-assisted extraction of anthocyanin from Chinese bayberry and its effects on anthocyanin stability

Wenkai DUAN[1,2], Shiping JIN[2], Guofu ZHAO[2], Peilong SUN[1]*

Abstract

Anthocyanins are present in high concentrations in Chinese bayberry, *Myrica rubra* Sieb. & Zucc. Herein, a microwave-assisted extraction was used to extract the anthocyanins from Chinese bayberry. The HPLC chromatogram of the extracts showed that the anthocyanin components were slightly hydrolysed during the extraction process. Further experiments confirmed that microwave irradiation slightly hydrolysed cyanidin-3-*O*-glucoside to cyanidin, but did not significantly influence the antioxidant activity of the extracts. Optimized extraction conditions for total anthocyanin content were a solid-to-liquid ratio, extraction temperature, and extraction time of 1:50, 80 °C, and 15 min, respectively. Under these conditions, the anthocyanin content was 2.95 ± 0.08 mg·g^{-1}, and the antioxidant activity yield was 279.96 ± 0.1 μmol.·g^{-1} Trolox equivalent on a dry weight basis. These results indicated that microwave-assisted extraction was a highly efficient extraction method with reduced processing time. However, under some extraction conditions it could damage the anthocyanins. These results provide an important guide for the application of microwave extraction.

Keywords: Chinese bayberry; anthocyanin extraction; microwave-assisted extraction; cyanidin-3-*O*-glucoside; anthocyanin stability.

Practical Application: This study showed that microwave irradiation would slightly hydrolysed cyanidin-3-*O*-glucoside to cyanidin, it provide an important guide for the application of microwave extraction.

1 Introduction

Chinese bayberry, *Myrica rubra* Sieb. & Zucc., is one of the six *Myrica* species native to China (Chen et al., 2004), and is noted for the attractive red colour of its fruits. Many pigments were extracted, identified, and characterized from different natural sources, especially red fruit extracts, for their safe use as colorants. Anthocyanins are among the most broadly distributed pigment groups in plants, and are present at high concentration in Chinese bayberry. The major anthocyanin present in bayberry fruits was identified as cyanidin-3-*O*-glucoside (C-3-G), which represents more than 95% of the total pigment content (Bao et al., 2005). Anthocyanins from Chinese bayberry can protect β-cells from oxidative-stress mediated injury (Zhang et al., 2011) and contribute to promoting good health and reducing the risk of chronic disease (Philpott et al., 2004).

Extraction is the first step in the commercial isolation of anthocyanins. Conventional extraction techniques for solid matrices include the well-known Soxhlet extraction, sonication, and blending. Although efficient extractions can be achieved using these simple techniques, they present major drawbacks. In particular, they feature long extraction times (especially for Soxhlet extraction), high solvent consumption, and low temperatures, and they are not yet readily automated (Heemken et al., 1997; Egizabal et al., 1998). Over the last decade, new techniques have emerged that will supersede traditional techniques. These include supercritical fluid, pressurized fluid, and microwave-assisted extraction (Schantz et al., 1997; Lopez-Avila et al., 1994).

Microwave-assisted extraction (MAE) has been demonstrated to be a fast and efficient unconventional extraction method that was developed for extracting analytes from solid matrixes, in particular, secondary metabolites from plant material (Kaufmann & Christen, 2002), such as saponins from ginseng (Kwon et al., 2003; Vongsangnak et al., 2004), glycyrrhizic acid from licorice root (Pan et al., 2000), anthocyanins in red raspberries (Sun et al., 2007), and alkaloids from the seeds of *Lupinus mutabilis* (Ganzler et al., 1990). Microwave energy is a non-ionizing radiation that results in molecular movement by migration of ions and rotation of molecules with permanent dipoles in liquids, without altering their molecular structures unless the temperature is too high (Kaufmann & Christen, 2002). Microwave extraction greatly reduces solvent consumption and extraction times and improves extraction efficiency (Eskilsson & Björklund, 2000).

The stability of anthocyanin is very important during the extraction, and is influenced by numerous factors, including temperature, pH, water activity, and light (Amr & Tamini, 2007). Recently, a study suggested that vitamin C was unstable under microwave conditions (Yuan et al., 2009), whereas some confirmed that microwaving preserved bioflavonoid content

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[1] *Laboratory of Food Energy, Zhejiang University of Technology, Hangzhou, PR, China*
[2] *Taizhou Vocational College of Science & Technology, Taizhou, PR, China*
*Corresponding author: sun_peilong@163.com

(Gorinstein et al., 2008; El-Adawy, 2002). Thus, the effect of microwave irradiation on the stability of anthocyanin needs to be further studied.

Herein, MAE was used to extract antioxidant compounds from Chinese bayberry. The aim of this work was to elucidate the optimal extraction conditions and evaluate whether it affects the structure or antioxidant activity of the extracted compounds.

2 Materials and methods

2.1 Chemicals and reagents

Cyanidin-3-*O*-glucoside standard was obtained from Extrasynthèse (Genay, France). 6-Hydroxy-2,5,7,8-tetramethyl-chroman-2- carboxylic acid (Trolox) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile (HPLC grade), formic acid, ethanol, and hydrochloric acid (analytical grade) were purchased from Shanghai Chemical Reagent Company (China). All solutions were prepared using distilled-deionized water.

2.2 Plant material

Myrica rubra Sieb. & Zucc. cv. Dongkui belongs to the genus *Myrica* in the family *Myricaceae*. Mature Chinese bayberry fruits were obtained from a fruit market in Taizhou, Zhejiang Province, China in June 2013. The samples were immediately dried in a lyophilizer (Labconco, Kansas City, USA), and then ground and sifted for homogenization before storing at −80 °C to avoid the degradation of the compounds.

2.3 Microwave-assisted extraction

The microwave-assisted extraction was performed using a microwave accelerated reaction system (MARSXpress, CEM Cooperation, Matthews, NC, USA) with a microwave power of 800 W, equipped with a digital timer and temperature controller.

The dried bayberry powder (0.50 g) was accurately weighed, placed in a tube, and mixed with an appropriate amount of extracting solvent (1% HCl in 95%ethanol). After thorough mixing, the tube containing the suspension was irradiated, using a predetermined extraction time and temperature, in the microwave device. After the microwave extraction, the sample was centrifuged at 8000 rpm for 10 min, and then the supernatant was collected and all samples were diluted to the same volume. All of the samples were filtered through a 0.45-μm syringe filter (Pall Life Sciences, Ann Arbor, MI, USA).

2.4 Determination of anthocyanin content

The total anthocyanin content of extracts was determined using a modified pH differential method described previously (Kim et al., 2003; Zhou et al., 2009). A general spectrophotometer (T6 New Century, Purkinje General Instrument Co., Beijing, China) was used to measure absorbance at 520 and 700 nm in buffers at pH 1.0 and 4.5. The absorbance measurements were converted into total milligrams of cyanidin-3-glucoside per gram dry weight of bayberry using the molar extinction coefficient, $\varepsilon = 26\,900\ L \cdot mol^{-1} \cdot cm^{-1}$, and absorbance of $A = [(A_{520} - A_{700})_{pH\ 1.0} - (A_{520} - A_{700})_{pH\ 4.5}]$. The data were reported as mean ± standard deviation for three replications.

2.5 HPLC-DAD-ESI-MS analysis

HPLC-DAD-ESI-MS analysis was performed using a Waters platform system, which was composed of a Micromass QUATTRO MICROTM API mass spectrometer, a Waters 600 pump system, and a Waters 2996 photodiode array detector (Waters Corp., Milford, MA, USA). Data were collected and processed on a personal computer running MassLynx software (Micromass, a division of Waters Corp., Beverly, MA, USA). Aliquots of bayberry extracts (20 μL) were resolved using a SUNFIRE C-18 column (250×4.6 mm, 5μm, Waters Corp., Milford, MA, USA), where solvent A was 0.05% (v/v) formic acid in water, and solvent B was 0.05% (v/v) formic acid in acetonitrile, and the flow rate was 0.7 $mL \cdot min^{-1}$. The injection volume was 20 μL, and the detection wavelengths was 520 nm. The elution system was: 0–5 min = 5% solvent B, 5–10 min = linear gradient from 5–10% of solvent B, 10–25 min = linear gradient from 10–90% of solvent B, and 25–50 min = 90% solvent B. Cyanidin-3-*O*-glucoside was used as a standard for quantitation of cyanidin-3-*O*-glucoside content, the ratio of cyanidin-3-*O*-glucoside to total anthocyanin was expressed to total anthocyanin content divided by the content of cyanidin-3-*O*-glucoside.

Mass spectra were obtained using electrospray ionization in positive ion mode. The following ion optics were used: capillary = 3.88 kV and cone = 60 V. The source block temperature was 90 °C and the desolvation temperature was 150 °C. Continuous mass spectra were recorded over m/z 50–800 with a scan time of 1 min and interscan delay of 0.15 s.

2.6 Evaluation of antioxidant activity using the DPPH method

The scavenging effects of the samples on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical were monitored using the previously reported method (Bao et al., 2005; Zhou et al., 2009). In summary, 0.1 mL of the diluted sample was added to 3.9 mL of 0.1 $mmol \cdot L^{-1}$ DPPH solution. It was vortexed and then allowed to react at ambient temperature for 30 min in the dark. The absorbance at 517 nm was measured using a spectrophotometer. A calibration curve was constructed for the decrease in absorbance based on trolox concentration, and 0.1 mL of 95% ethanol was used as a control. The inhibition ratio was calculated as follows:

Inhibition ratio (%) = $100 \times (A_o - A_s)/A_o$, where A_o and A_s represent the absorbance of the control and sample solutions, respectively. The antioxidant activity was expressed as μmol Trolox equivalent g^{-1} dry weight.

2.7 Experimental design and statistical analysis

A three-level three-factor Box–Behnken design was selected to evaluate the combined effect of three independent variables: solid to liquid ratio, extraction temperature, and time, which were coded as X_1, X_2, and X_3, respectively (Table 1).

In total, 15 experiments with three replicates at the centre were employed. The response surface methodology (RSM) was used to determine the optimum conditions for the extraction of anthocyanins from Chinese bayberry. The experimental design and statistical analysis were performed using Stat-Ease software (Design-Expert 7.0.10 Trial, Delaware, USA). The generalized second order polynomial model used for the response surface analysis was as follows:

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + b_{ii} \sum_{i=1}^{3} X_i^2 + \sum_{i=1}^{2} \sum_{m=i+1}^{3} b_{im} X_i X_m \quad (1)$$

where the response function, Y, was partitioned into linear, quadratic, and interactive components, b_0, b_i, b_{ii}, and b_{im} are defined as the constant, linear coefficient, quadratic coefficient, and cross-product coefficient, respectively, and X_i and X_m are the levels of the independent variables. Analysis of variance (ANOVA) tables were generated, and the effect and regression coefficients of the individual linear, quadratic, and interaction terms were determined.

3 Results and discussion

3.1 HPLC analysis of anthocyanins extracted from chinese bayberry

Anthocyanins were extracted from Chinese bayberry using a microwave-assisted method. The HPLC chromatogram of the anthocyanins is shown in Figure 1. Peak 1 displayed at *m*/*z* 287, corresponding to the molecular of cyanidin, was assumed to be cyanidin according to the reported by Zhao et al. (2013). Peak 2 has an m/z 449 corresponding to $[M]^+$, with a characteristic fragmentation $[M-162]^+$ at 287 corresponding to the cleavage of glucose, hence, it was assigned as cyanidin-3-*O*-glucoside. By comparing its HPLC retention time to a cyanidin-3-*O*-glucoside standard, peak 2 was further confirmed as cyanidin-3-*O*-glucoside. We suggest that cyanidin might be the hydrolysis product of cyanidin-3-*O*-glucose formed during the microwave-assisted extraction. Although it has been reported that malvidin-3-glucoside and malvidin-3,5-diglucoside might hydrolyze to the products of anthocyanone, hydroxycoumarins and dihydroxy phenylacetaldehyde under microwave treatment (Ramirez et al., 2015), this finding imply that there may be other anthocyanin degradation pathway under different conditions, such as cyanidin, delphinidin and

Table 1. Code and actual levels of three variables.

Independent variables	Units	Symbol	Code levels		
			−1	0	1
Solid to liquid ratio	1:X	X_1	30	40	50
T	[°C]	X_2	40	60	80
t	[min]	X_3	5	10	15

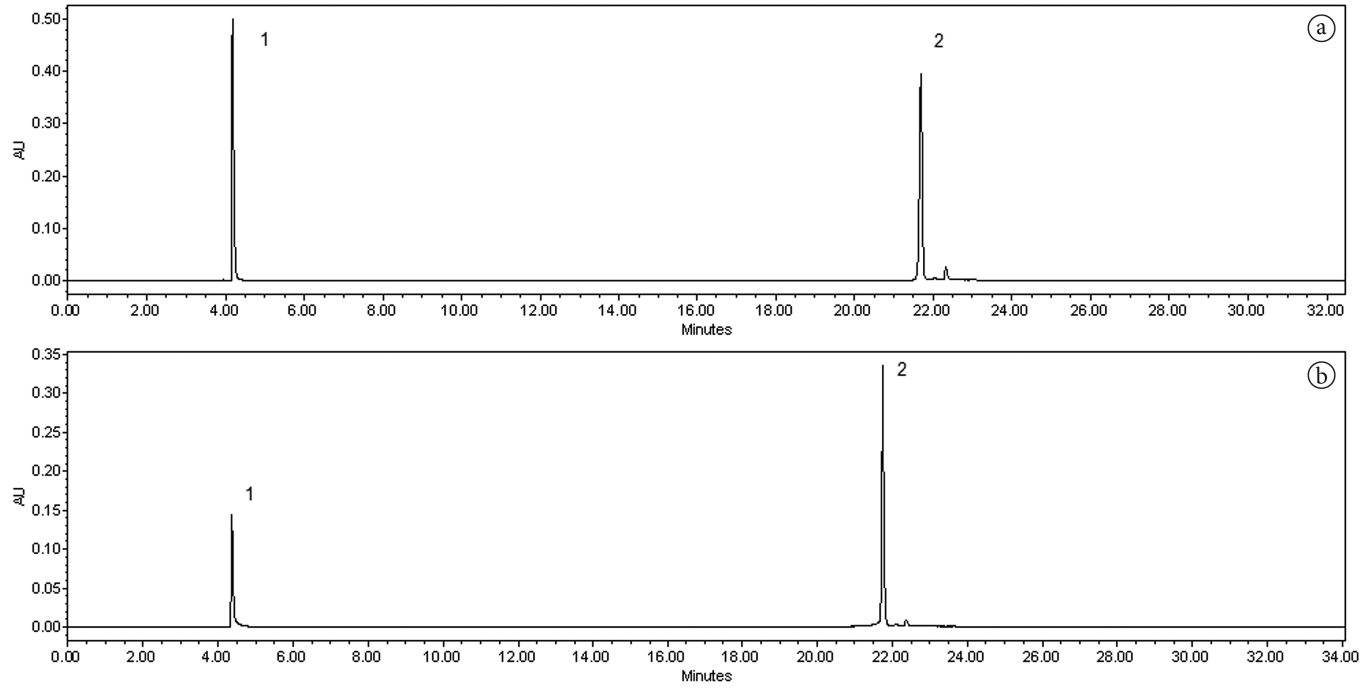

Figure 1. HPLC profile of the anthocyanin extracted from Chinese bayberry using a microwave-assisted method. 1 = Cyanidin; 2 = Cyanidin-3-*O*-glucoside (C-3-G). UV-Vis detection at 520 nm. (a) under 80 °C extract condition; (b) under 60 °C extract condition.

pelargonidin can be extracted from fresh floral and vegetative tissue by using 2M HCl leaching (Des Marais & Rausher, 2010).

3.2 Effects of microwave irradiation on Cyanidin-3-O-glucose hydrolysis

Cyanidin is an anthocyanin and is bound to glucose to form C-3-G, which is the main component of anthocyanins in the fruit of Chinese bayberry (Tanaka et al., 2008). Although it is well documented that there is no significant difference between the antioxidant activity of cyanidin and C-3-G (Kähkönen & Heinonen, 2003), we used a C-3-G standard to evaluate the effect of microwaves on the hydrolysis and release of cyanidin and its antioxidant activity. A two-factor and two-level experimental design was conducted to evaluate the influence of temperature and extraction time on the hydrolysis of C-3-G. The results are shown in Table 2. Two-way ANOVA results showed that increasing the temperature increased the total anthocyanin content, but decreased the ratio of C-3-G to total anthocyanin, whereas it had no significant effect on the antioxidant activity of the extracts. Increasing the extraction time did not increase the antioxidant activity of the extracts or the total anthocyanin content, but significantly decreased the ratio of C-3-G to total anthocyanin, especially when extracting at 80 °C.

3.3 Optimization of the microwave-assisted extraction conditions

A three-level three-factor Box–Behnken design was selected to evaluate the combined effect of three independent variables: solid to liquid ratio, extraction temperature, and time on the yield of anthocyanins extracted from Chinese bayberry using a microwave-assisted method. The C-3-G and total anthocyanin contents in the microwave-assisted extract were determined, and the ratio of C-3-G to total anthocyanin was calculated. The antioxidant activity of the microwave-assisted extracts was also determined. These results are listed in Table 3.

A positive correlation between the total anthocyanins and the antioxidant activity (R^2 = 0.8431, Figure 2) was observed, which agrees with previous findings (Kähkönen & Heinonen, 2003). Because C-3-G is hydrolysed into cyanidin in human bodies (Min et al., 2010), it is better to optimize the extraction

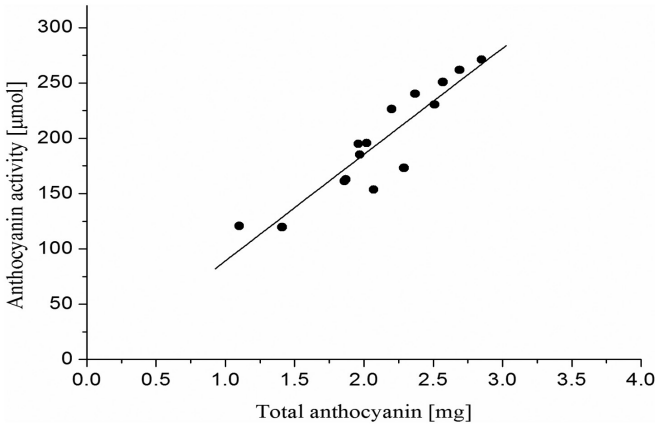

Figure 2. The correlation between total anthocyanin content and antioxidant activity (R^2 = 0.8431).

Table 2. Effect of microwave irradiation on the hydrolysis of C-3-G.

	40 °C		80 °C	
	5 min	15 min	5 min	15 min
DPPH [%]	60.73 ± 1.36	63.43 ± 3.20	64.17 ± 2.21	64.60 ± 0.96
Total anthocyanin [mg·L^{-1}]	26.33 ± 1.15	26.67 ± 1.15	28.67 ± 0.58	29.67 ± 1.15
C-3-G/total anthocyanin [%]	98.00 ± 0.61	97.00 ± 0.56	76.00 ± 0.92	48.00 ± 1.15

Table 3. Response surface design and experimental data[a].

Run	X_1	X_2	X_3	C-3-G yield [mg·g^{-1}]	Total anthocyanin yield [mg·g^{-1}]	C-3-G/total anthocyanin	Total antioxidant activity[b] [μmol·g^{-1}]
1	1	0	1	1.97	2.85	0.69	271.07
2	0	1	–1	1.83	2.51	0.73	230.56
3	1	–1	0	0.73	1.10	0.66	120.78
4	1	0	–1	1.30	1.86	0.70	161.26
5	–1	0	1	1.70	2.20	0.77	226.38
6	0	–1	–1	1.38	1.87	0.74	162.8
7	0	–1	1	1.39	2.07	0.67	153.56
8	–1	0	–1	1.60	2.29	0.70	173.14
9	–1	1	0	1.77	2.37	0.75	240.24
10	0	1	1	1.89	2.69	0.70	261.8
11	–1	–1	0	0.90	1.41	0.64	119.68
12	1	1	0	1.93	2.57	0.75	250.80
13	0	0	0	1.43	1.97	0.73	185.24
14	0	0	0	1.48	2.02	0.73	195.58
15	0	0	0	1.37	1.96	0.70	194.92

[a]Yields and activities are based on dry weight. X_1, X_2 and X_3 are defined in Table 1. [b]Antioxidant activity was expressed as μmol of Trolox equivalent per g of dry weight.

to target the total anthocyanin content if we are only concerned with their health benefits. However, the extraction conditions should be optimized to target the C-3-G content if purified C-3-G from Chinese bayberry is required.

The total anthocyanin content was optimized using an RSM analysis. The polynomial equation of the quadratic model was as follows:

$Y=1.98+0.014X_1+0.46X_2+0.16X_3+0.13X_1X_2+0.27X_1X_3-2.5\times10^{-3}X_2X_3-0.054X_1^2-0.067X_2^2+0.37X_3^2$ (F_{model} =7.32; $p < 0.05$; R^2 =0.9295), where Y, X_1, X_2, and X_3 are the total anthocyanin yield, solid to liquid ratio, extraction temperature, and extraction time, respectively.

The maximum total anthocyanin yield of 2.95 mg·g^{-1} on a dry weight basis was obtained when using the optimal extraction conditions: solid to liquid ratio, extraction temperature, and time of 1:50, 80 °C, and 15 min, respectively. The ANOVA statistics showed that the experimental data had correlation coefficient R^2 = 0.9295 with the calculated model.

To confirm these results, Chinese bayberry powder was extracted in triplicate using the above-mentioned conditions. The anthocyanin yield was 2.95 ± 0.08 mg·g^{-1} with an antioxidant activity yield of 279.96 ± 0.1 μmol·g^{-1} equivalent of Trolox on a dry weight basis.

The C-3-G content was also optimized using an RSM analysis. The polynomial equation of the quadratic model was as follows:

$Y=1.43+0.005X_1+0.43X_2+0.16X_3+0.083X_1X_2+0.14X_1X_3+0.11X_2X_3-0.088X_1^2-0.006X_2^2+0.30X_3^2$ (F_{model} =15.47; $p < 0.05$; R^2 =0.965), where Y, X_1, X_2, and X_3 are the yield of C-3-G, solid to liquid ratio, extraction temperature, and extraction time, respectively.

The maximum C-3-G yield of 2.01 mg·g^{-1} on a dry weight basis was obtained when using the optimal extraction conditions: solid to liquid ratio, extraction temperature, and time of 1:50, 75.8 °C, and 14.7 min, respectively. The ANOVA statistics showed that the experimental data had correlation coefficient R^2 = 0.956 with the calculated model.

The total anthocyanin and C-3-G contents extracted were affected by the three variables studied, but they were most greatly influenced by the extraction temperature. Increased temperature significantly increased the extraction of total anthocyanins and C-3-G (Figure 3), and was able to modify the equilibrium and mass transfer conditions of the solid-liquid extraction. Although higher temperatures had a positive effect on the extraction yields, the temperature cannot be increased indefinitely because of the thermal instability of anthocyanin compounds (Liazid et al., 2011).

The solid to liquid ratio also had an effect on the extraction of total anthocyanins and C-3-G. An increase in the solid to liquid ratio resulted in increased total anthocyanin and C-3-G extraction (Figure 3). At high extract concentrations, the solubility would have increased, and then an increase in the solid to liquid ratio resulted in a greater extraction of the total anthocyanin and C-3-G; however, values increased slowly at a high solid to liquid ratio because low extract concentrations would reduce the solubility.

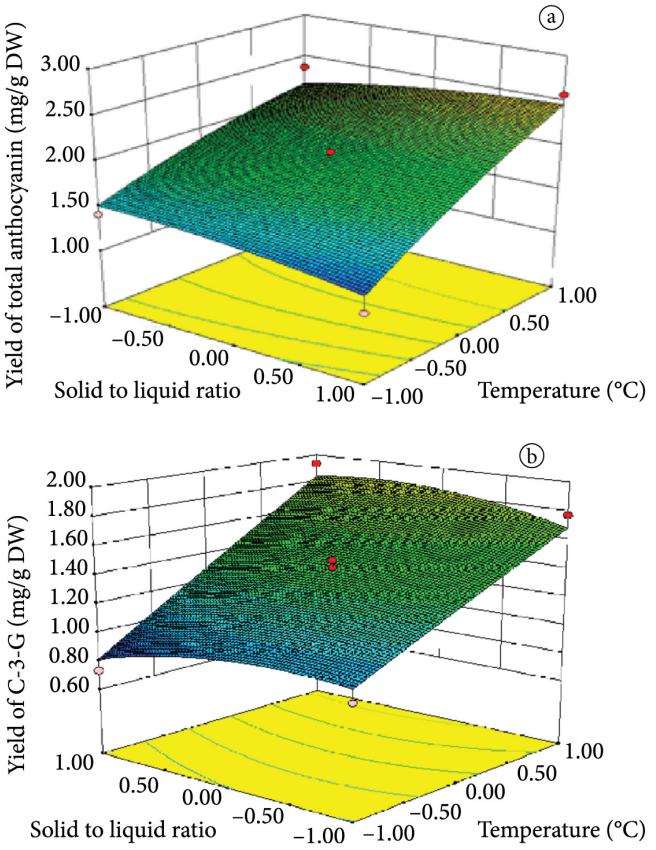

Figure 3. The response surfaces for the effects of temperature and solid to liquid ratio on the yield of total anthocyanin (a) and cyanidin-3-*O*-glucoside (b).

4 Conclusion

We first investigated the effect of microwave irradiation on the extracted components. HPLC-DAD-ESI-MS analysis of the extract implied that cyanidin-3-*O*-glucoside have been hydrolysed to cyanidin during the extraction process. To confirm whether microwave irradiation damaged the anthocyanins, a cyanidin-3-*O*-glucoside standard was used to evaluate the effects of microwave irradiation on the hydrolysis and release of cyanidin and its antioxidant activity. Our results showed that cyanidin-3-*O*-glucoside hydrolysed to cyanidin, but the antioxidant activity was not significantly changed during the extraction process featuring high temperature and long extraction times.

Finally, we used the RSM method to optimize the extraction conditions and a positive correlation was found between the total anthocyanin content and antioxidant activity, R^2=0.8431. The maximum total anthocyanin yield of 2.95 mg·g^{-1}, and an antioxidant activity yield of 279.96 ± 0.1 μmol·g^{-1} equivalent of Trolox on a dry weight basis were obtained under the optimal conditions where the solid to liquid ratio, extraction temperature, and extraction time were 1:50, 80 °C, and 15 min, respectively, whereas the maximum cyanidin-3-*O*-glucoside yield of 2.01 mg·g^{-1}

on a dry weight basis was obtained under conditions there the solid to liquid ratio, extraction temperature, and extraction time were 1:50, 75.8 °C, and 14.7 min, respectively.

These results provide an important guide for the application of microwave extraction when extracting anthocyanins from plants.

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