Comparison of microwave assisted extraction with hot reflux extraction in acquirement and degradation of anthocyanin from powdered blueberry

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Abstract: Acquirement and degradation kinetics of anthocyanin from powdered blueberry were studied to analyze the degradation reason and improve the yield of anthocyanin under microwave assisted extraction (MAE) conditions. Hot reflux extraction (HRE) method was employed as a reference with MAE to discriminate the influences of the microwave irradiation under the same extraction temperature at 30°C-70°C. Ethanol was selected as extraction solvent. Microwave volumetric heating improves the diffusion and solubility of anthocyanin within powdered blueberry particles in the pre-heating period. The kinetic coefficients, in terms of delivery kinetic and diffusion kinetic, are lower in MAE than that in HRE. Four anthocyanins including delphinidin, cyanidin, malvidin and pelaronidin were detected in the anthocyanin extracts from blueberry. Delphinidin, cyaniding, and pelargonidin contents significantly decrease, but malvidin was unchanged in MAE. Anthocyanin yield increases with elevating extraction temperatures, but anthocyanin degradation occurred at 41.2°C. The acquirement amount of anthocyanin is lower than its degradation amount when microwave extraction temperature is higher than 53.6°C. The self-aggregation of anthocyanin molecules and degradation results in the reduction of anthocyanin yield extracted from blueberry powder. In MAE, microwave volumetric heating results in the rapidly elevated temperature in pre-heating stage and generation of internal pressure to improve the acquirement of anthocyanin and the non-thermal effect from microwave field may weak the effect of temperature to decline the degradation of anthocyanin to monomer. The non-thermal microwave irradiation has pronounced effects compared to the effects of temperature on the structure changes of anthocyanin molecules at extraction temperature below 50°C. In HRE, convection mode of heat transfer in extraction system form the non-uniform distribution of temperature in the extraction vials to decrease the acquirement yield of anthocyanin from the blueberry powder. The research results provide valuable data to optimize extraction temperature for high extraction yield and bioactivity of anthocyanin in microwave extraction process.

Keywords: blueberry, microwave, anthocyanin, degradation, extraction, temperature, kinetic

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1 Introduction

Blueberries are well-known berry fruits containing abundant anthocyanin contents[1], which have high benefits for human health with respect to its anti-aging, free radical scavenging, and anti-inflammatory properties,

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as well as its ability to protect blood vessels from oxidative damage[2]. Extraction technology has been widely used to obtain the high-purity anthocyanin from blueberry fruits[3-5]. Improvement of the extraction efficiency and the yield of anthocyanin from blueberries is a concern issue in added-value processing field of berry industry. Obvious degradation of anthocyanin in conventional extraction method, for example, solid-liquid and heat-reflux, weakens the antioxidant activity of the extracts and reduces the yield due to the oxygen and temperature effects[6].

Microwave assisted extraction (MAE) is a novel technique that has many advantages including high extraction efficiency, low solvent consumption, high-purity extracts and shortened extraction time which make it well-suited for the extraction of bioactive compounds from plant materials[7,8], for example, polyphenols from *Myrtus communis* L. leaves[9], phenolic compounds from cherry laurel (*Prunus laurocerasus*) leaves[10], anthocyanin extracted from grape skins[11], raspberry[12], and antioxidants from sea buckthorn[13]. The mechanism of microwave volumetric heating involves the inherent ionic conduction and dipolar relaxation inside a dielectric material. Microwave irradiation induces the rapid elevating temperature of solvent to accelerate the diffusion of pure solvent into plant matrix[14], as well as the dissolution of the targeted compound into solvent[13]. Microwave energy penetration causes quick elevation of temperature to build the internal pressure inside the cell of plant material[15]. The high interior pressure may destroy the cell wall of plant material to easily release anthocyanins into solvents[16]. High temperature would cause the dehydration of cellulose, and reduce its mechanical strength in MAE, which promotes the solvent to penetrate into the cellular channels, and subsequently increase the extraction yield[17,18]. However, temperature may cause the degradation of anthocyanins because of its thermal susceptibility[19,20]. Cacace and Mazza[21,22] found that the extraction temperatures in range of 30°C-35°C has weak extraction kinetic to reduce the anthocyanin yield during solid-liquid extraction of milled berries. Temperatures in the range of 40°C-90°C would intensify the degradation kinetics of anthocyanin components, which results in the color changes from blood orange to black[23]. The degradation of anthocyanin caused by temperature in blueberries followed first order kinetic model[20]. Some explanations were proposed for the anthocyanin degradation caused by temperature. Abyari et al.[24] revealed that high temperature disrupts the ordered lattice structure of liquid water inside the material to release flavylium ions at the colorless hemiacetal form, which leads to the reduction in the extent of water hydrogen bonding and co-pigmentation complex. High temperatures might weaken the stabilization of co-pigmentation complexes[25], and low temperature benefits the exothermic co-pigmentation process because entropy drives the co-solvent phenomena[26]. Rein[25] attributed the degradation mechanism of anthocyanin to the *o*-dihydroxyphenol oxidase and laccase enzymes which produced colorless compounds by enzymatic oxidation or reaction. In addition, microwaves have the non-thermal effect other than dielectric heating of materials, which can improve extraction yield and reduces the degradation of anthocyanins[27].

Based on the results from above-mentioned studies, both acquirement and degradation of anthocyanin simultaneously occurs in the microwave extracting process. The determination of kinetic coefficients of degradation and extraction yield of anthocyanin would conduce to the elucidation of the extraction mechanism, improvement of anthocyanin yield and the optimization of parameters. High extraction yield and low degradation of anthocyanin are the desired results for anthocyanin extraction from blueberries in MAE. However, previous studies on the MAE mostly focus on either the extraction or degradation process. Existed research in the same microwave extraction system focus on the extraction characteristics and optimal parameters of anthocyanin from blueberry powder under MAE[28]. Limited research considered the relationship of anthocyanin extraction yield and bioactive degradation kinetics, as well as their interaction under microwave extraction process. The objectives of this study were as follows: 1) to determine the extraction and degradation kinetics of anthocyanin from the powdered blueberry under MAE;

2) to analyze the effects of microwave irradiation on the degradation of anthocyanin by means of the comparison of MAE with hot reflux extraction (HRE).

2 Materials and methods

2.1 Preparation of powdered blueberry samples

Fresh wild blueberry was collected from the Greater Khingan Mountains area in Northeastern of China. The uniform and ripened blueberries were picked out, and then soaked in purified water for 30 min. Fresh blueberry selected was cleaned then sealed in plastic bag at each package of 200g, and blueberry bags were placed into a cooling room of refrigerator (Haier, BC/BD-829HN, Qingdao, China) at 4°C mode in 1-2 d. The cooling stored fruits were taken out to get room temperature, then smashed by a fruit pulping machine (Philips-30, Zhuhai, China) to produce the blueberry purees. Then, the fruit purees were frozen in a refrigerator at temperature of –18°C for 24 h. The moisture content of fresh blueberry homogenate was 85%-90% (w.b.). If the fresh blueberry homogenate as solute is directly extracted in ethanol solvent, high moisture content in extraction system decreases the ethanol concentration to weak the dissolving capacity for the anthocyanin, and has the difficulty to purify the anthocyanin from extracts of blueberry homogenate. Moisture content of dehydrated blueberry was dehydrated by using a freeze-vacuum dryer. Although the freeze-vacuum drying process of blueberry puree is time-consuming, easy purification and high extraction yield of anthocyanin are the obvious advantages for the extraction of blueberry powder. The freeze drying was performed at –24°C, 500 Pa vacuum pressure, and 12°C heating temperature, on aluminum plates. Freeze drying operation was continuously run until the moisture content of the blueberry slices was below 0.5% (w.b.). The blueberry powders were prepared by pulverizing the dehydrated slices of blueberry. The dehydration berry powder was packed in a seal airtight plastic bag and stored in the refrigerator in –20°C for the later extraction experiments.

2.2 Microwave assisted extraction (MAE)

A multimode microwave extraction system (Ethos-1, Milestone Inc., USA) with microwave frequency of 2.45 GHz and maximum output power 1600 W was employed to conduct the MAE experiments. The extraction system consists of microwave cavity and control terminal. The microwave cavity is made of 18/8 stainless steel housing with multi-layer Teflon plasma coated. A microwave pyramid-shaped diffuser located above the microwave cavity evenly distributes microwaves throughout the cavity, preventing localized hot and cold spots. The rotating sample fixed frame inside the microwave cavity of extraction system holds 10 Teflon vials, each with a volume of 100 mL. The temperature and pressure of sample in extraction vial were monitored by using a fiber temperature sensor and a fiber pressure sensor inserted into a reference vial. The control terminal of the microwave extraction system embodying the Milestone Easy-Control software was operated via a compact terminal touch screen to set temperature and time for the extraction process. The extraction processes were carried out in a controlled environment. Based on the preliminary experimental results[28], extraction system were prepared as mass of lyophilized blueberry powder of 1 g solid to liquid ratio of 1:40 (g/mL) in ethanol concentration of 60%. Ethanol was selected as the solvent based on its high dielectric properties to absorb much more microwave energy and high solubility for the anthocyanin, as well as its non-toxicity in the environment[29]. The mixture of ethanol and blueberry powder was placed in closed vessels. The vessels were fixed at the chamber in the microwave extraction system. Extraction parameters were set at an external control palm. The pre-heating duration was 1 min to achieve the setting temperature.

Raising temperature improves diffusion of objective component and simultaneously intensities its degradation in microwave extraction system. From existed research results of microwave extraction of anthocyanin from blueberry powder[30], extraction temperature less than 30°C results in the low extraction efficient, which is unfit for the acquirement of anthocyanin; temperature higher than 70°C causes the drastic degradation of anthocyanin and the precipitous decline of anthocyanin content in extraction system. To determine the extraction and

degradation of kinetics of anthocyanin from powdered blueberry under extraction temperatures, the extraction temperatures were set at 30°C-70°C with 10°C intervals for MAE.

2.3 Hot reflux extraction (HRE)

HRE method was employed as a comparison to investigate the non-thermal effect from microwave irradiation during the anthocyanin extraction process. Dehydrated blueberry powder prepared and extraction conditions set under the HRE were the same as the MAE experiments in 2.2 section. For the Ethos 1 microwave extraction system, the extraction vials with modified Teflon material may be sealed by using the elastic safety helmet to prevent light and oxygen. An extraction vial used in MAE was employed in the HRE experiments. The extraction vial was placed in water bath (HZQ-C, Aohua Equipment Co., Changzhou, China) in 15 min to achieve the pre-setting temperature of 30°C, 40°C, 50°C, 60°C, 70°C, respectively, and then was poured the extracted mixture consisted of the solid to liquid ratio of 1:40 (g/mL), ethanol concentration of 60%.

The specific HRE experiments were conducted under the sealed vials to prevent light and oxygen to investigate the effect of temperature on the extraction of anthocyanin. The conditions of specific experiment were the same as that of MAE conditions in terms of temperature. All experiments were conducted three times and average data were reported.

2.4 Determination of anthocyanin contents

Anthocyanin content was measured by using a UV/Vis spectrometer (Lambda 35, Perkinelmer, Singapore) with a variable wavelength detector, according to the chemical analysis method[28]. A rotary evaporator (RE-52AA, Shanghai Yarong Biochemistry Instrument Factory, Shanghai, China) was employed to evaporate solvents from the extracts.

The highest absorbance of blueberry extract was achieved at 500 nm wavelengths in a UV/Vis spectrometer according to the preliminary experiments[28]. A standard curvilinear equation (determination coefficient R^2=0.9989) was fitted by plotting absorbance A and concentration C (mg/mL) as follows:

$$A = 1.934C - 0.0485 \quad (1)$$

The content of anthocyanin was expressed by Equation (2):

$$D = \frac{C \cdot V \cdot n}{W} \quad (2)$$

where, D is anthocyanin extraction rate (%); C is the concentration of anthocyanin, mg/mL; V is the constant volume, mL; n is the multiple of dilution (n=1.2), and W is the total content of anthocyanin, mg. The initial anthocyanin content in raw blueberry powder was measured triplicates, and the mean value with standard deviation was obtained as 73.008 ±0.015 mg/g.

2.5 Molecule structure analysis and types identification of anthocyanin

2.5.1 Acid hydrolysis of anthocyanin

Before the molecule structure analysis and types identification of anthocyanin extract from blueberry powder under MAE and HRE, it is necessary pre-treatment of acid hydrolysis of anthocyanin for the crude anthocyanin extracts to remove the glucosides attaching to anthocyanin. According to the existed method[31], 0.5 g anthocyanin sample and 7.5 mL ethanol at 98% concentration were uniformly mixed in a test tube. Six milliliter mixture was put into a 10 mL colorimeter tube, and 4 mL HCL with a concentration of 5 mol/L was added to formulate the analytical solution. After shaken, tube loaded analytical solution was placed into the water bath at 80°C for 1 h oscillating, then cooled to room temperature, followed by the centrifuged operation at rotation speed of 6000 r/min for 10 min. Supernatants were filtered through 0.45 μm membrane, and the filtrates were then for HPLC analysis.

2.5.2 Analysis of anthocyanins molecule structure using circular dichroism

Circular dichroism method[32] was employed to probe the conformational changes of anthocyanin molecules in a spectropolarimeter (J810, JASCO, Japan), which may investigate the degradation reason of anthocyanin in view of molecules structure under MAE and HRE. Crude anthocyanin extracted from blueberry powder was treated by a centrifugal separation operation at 4000 r/min for 5 min. Supernatant of anthocyanin was filtered by using 0.45 μm water films to collect filtrate. Macro-reticular resin was selected as chromatographic column filler to

purify the filtrate by sampling a volume of 30 mL at flow rate of 1 L/min, followed by elution using 40% methanol at flow rate of 2 mL/min. During the purification process, sampling operation was done every 3 min. The sample with higher absorbance reading was diluted to the concentration of 0.25 mg/mL. Purified anthocyanin samples were measured by using the spectropolarimeter at the absorbance wavelengths of 200-300 nm. Three measurements were completed for each sample and average value was reported.

2.5.3 Profiles of anthocyanin types using High Performance Liquid Chromatography (HPLC)

The determination of anthocyanin contents in section 2.4 is the total content of anthocyanin, as a mixture, in extract of blueberry. The anthocyanin mixture consists of different types, such as delphinidin and cyaniding. In order to discriminate the anthocyanin type in the extract mixture, profiles of anthocyanin compounds were measured by using HPLC. The profiles of anthocyanin types were determined by using HPLC method[33] with some modifications to investigate the anthocyanin changes under various extraction temperatures, and the differences of types in anthocyanin extracts between microwave extraction and heat reflux extraction. An HPLC system (HP 1100) equipped with the chromatographic column of Phenomenex Gemini C18 coupled with Chem Station software was used to measure the content of anthocyanin monomer. The mobile phase A containing 5% methane acid, and a mixture of 96% ethanol and 5% methane acid was used as mobile phase B. The gradient elutions were carried out as the following procedure: in 0-30 min, flowing mobile phase A from 90% to 10%; in 30-32 min, elution operation using 10% mobile phase A; in 33-38 min, elution operation using 90% mobile phase A; flow rate was set at 8 mL/min in room temperature, and inject volume was 20 μL. Anthocyanin monomers contents of extracts were measured at wavelength of 520 nm by using the ultraviolet with diode array detector. Standard samples were injected for identification and quantitative analysis.

Four anthocyanin standards solutions including delphinidin, cyaniding, malvidin and pelargonidin were prepared individually at six different concentrations. According to above mentioned HPLC measurement conditions, HPLC spectra of four anthocyanin standards were presented by peak area. The regression equations were fitted to represent the relationships of anthocyanin concentration with peak area of HPLC spectra as follows:

Pelargonidin: $Y=31.58x-152.4$ (3)

Cyanidin: $Y=34.08x-87.9$ (4)

Malvidin: $Y=55.11x-611.9$ (5)

Delphinidin: $Y=55.03x-152.1$ (6)

where, x is the concentration of the corresponding anthocyanin standard, and Y is the associated peak area of the HPLC spectra.

2.6 Extraction and degradation kinetics of anthocyanin mathematical models

Both extraction and degradation of bioactive compounds may simultaneously occur during microwave extraction of plant materials[16,34]. Thermal degradation of the anthocyanin in berry fruits follows the kinetic model of first-order reaction[20,23]. According to the change profiles of extraction (mentioned below in Figures 1 and 2) with the exponential trends, the detectable extraction of final anthocyanin substances derives from two transfer processes are involved in diffusion step from inside blueberry particles to surface, and diffusion step from the surface to solvent in the MAE and HRE. The diffusion processes are dependent upon the anthocyanin solubility in ethanol solvent in the MAE and HRE system. The anthocyanin content y_1 within blueberry particles is considered as the decreasing trend, which can be described by the exponential decay Equation (7).

$$y_1=a_1\exp(-b_1t)+c_1\exp(-d_1t) \quad (7)$$

where, a_1 is anthocyanin yields at equilibrium in the delivering stage of puree, %; b_1 is the transfer coefficients in the delivering step for puree, 1/min; c_1 is the anthocyanin yield at equilibrium in the diffusion stage for pomace, %; d_1 is the kinetic coefficients in the diffusion step for pomace, 1/min; t is the extraction time, min.

Acquirement amount is defined as total amount of anthocyanin in raw blueberry powder minus its residual amount in powder pomace extracted and degradation amount in extraction system. On the contrary to the decline process of anthocyanin content within extracted blueberry particles, the acquirement amount of

anthocyanin y_2 in extractant assumes the increasing trend expressed by Equation (8):

$$y_2=y_0+a_2[1-\exp(-b_2t)]+c_2[1-\exp(-d_2t)] \quad (8)$$

where, y_0 is the initial content of anthocyanin in solvent generated in rising temperature stage (in 1 min) to pre-set value, %; a_2 is anthocyanin yields at equilibrium in the delivering stage of extractant, %; b_2 is the transfer coefficients in the delivering step for extractant, 1/min; c_2 is the anthocyanin yield at equilibrium in the diffusion stage for extractant, %; d_2 is the kinetic coefficients in the diffusion step for extractant, 1/min; t is the same as in Equation (7).

From the viewpoints of mass transfer kinetics in Equations (7) and (8), the coefficients a, c (with lower superscript 1, 2) refer to the indications of internal resistance of material to the transfer process, named as resistance coefficient, and b, d (with lower superscript 1, 2) show the extraction capacity of the materials, named as kinetic coefficient[35]. Based on the change curves of extraction yield of anthocyanin under MAE and HRE (below mentioned), both resistance coefficient and kinetic coefficient was obtained by a regression fitting method.

Anthocyanin ratio R is defined as the ratio of anthocyanin content in blueberry extract system A_e to its initial content in raw blueberry powder A_i (with no unit) as expressed in Equation (9).

$$R=\frac{A_e}{A_0} \quad (9)$$

Anthocyanin degradation ratio Y is defined as the total anthocyanin content in raw blueberry extract (considered as 1) minus the acquirement yield (considered as fraction in Equation (9)) as expressed in Equation (10).

$$Y=(1-\frac{A_e}{A_i})\times 100\% \quad (10)$$

The degradation amount of anthocyanin were calculated according to the difference between the total anthocyanin contents Y_{tal} in raw materials and amount of anthocyanins from blueberry after microwave exacting by Equations (7) and (8), as expressed in Equation (11):

$$Y_{de}=Y_{tal}-(y_1+y_2) \quad (11)$$

where, Y_{de} is the degradation value of anthocyanin under the microwave extraction conditions. It was expected to determine the degradation kinetics of anthocyanin by means of the trace changes of Y_{de} in Equation (9) under MAE and HRE.

2.7 Statistical analysis

All experiments were conducted in triplicate, and analysis of variance (ANOVA) for each set of data was performed. The least significant difference (LSD) at $p<0.05$ was calculated by using Duncan's Multiple Range Test to analyze the significant differences in results using SAS software (8.0, SAS Institute Inc., NC, USA). Sigmaplot software (12.0, Systat Software Inc., U.S.) was employed to fit regression equations according to *SEE* and R^2 value. *SEE* is the abbreviation of the standard error of the estimate, which is a measure of the accuracy of predictions of regression. *SEE* is calculated by using Equation (12):

$$SEE=\sqrt{\frac{\sum(Y-Y')^2}{N}} \quad (12)$$

where, *SEE* is the standard error of the estimate; Y is an actual score; Y' is a predicted score, and N is the number of pairs of scores. In a regression line, the smaller the standard error of the estimate is, the more accurate the predictions are. In statistics, the coefficient of determination, denoted R^2, is a number that indicates the proportion of the variance in the dependent variable that is predictable from the independent variable. R^2 is calculated by using Equation (13):

$$R^2=1-\frac{S_r}{S_t} \quad (13)$$

where, S_r is the sum of squares of residuals, also called the residual sum of squares; S_t is the total sum of squares (proportional to the variance of the data). The closed to one indicates high credibility of regression equation.

3 Results and discussion

3.1 Kinetics of anthocyanin extraction and degradation at different MAE temperatures

Figure 1 indicated that anthocyanin acquirement amount positively depended on the extraction temperature in the range of 30°C-50°C. The elevating temperature dominantly promoted the diffusion rate of anthocyanin within the kernel of blueberry powder. At the extraction temperature of 60°C, the anthocyanin yields increased until the extraction time of 4 min, and then significantly

decreased until the end of extraction process. At the extraction temperature of 70°C, anthocyanin yield continually declined due to its degradation caused by high temperature.

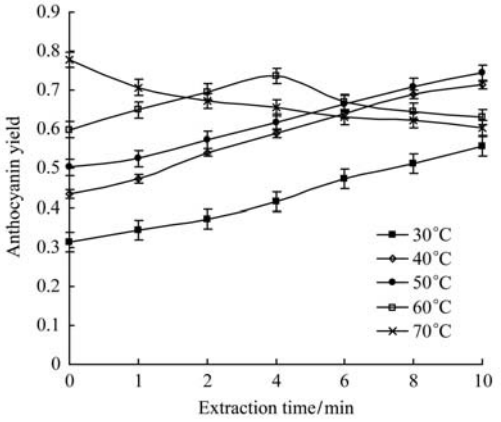


Figure 1 Effects of extraction temperatures on the yield of anthocyanin in extraction liquid in the MAE

From the view of degradation theory of bioactive compound, the degradation amount of anthocyanin as the function of extraction temperature may be presented by using Arrhenius equation (14) under MAE[28]:

$$M = M_0 \exp\left[-A\exp\left(\frac{-E_0}{RT}\right)t\right] \quad (14)$$

where, M is degradation amount of anthocyanin, g; M_0 is initial content of anthocyanin, g; E_0 is activation energy, kJ/mol; t is extraction time, min; R is gas constant, J/mol·K; T is temperature in extraction system, °C; A is constant. According to Equation (13), higher extraction temperature and longer time result in obvious significant degradation of anthocyanyin, and acquirement yield of anthocyanin decline during microwave extraction of blueberry powder.

The parameter's values of two terms of exponent Equation (8) were calculated by using a nonlinear fitting method with high determination coefficient (R^2>0.99) and low SEE value at a significant level p=0.001, as shows in Table 1. These results indicated that the initial anthocyanin value (y_0) derived from the rising temperature of microwave extraction increased with the extraction temperature except for at 70°C. In comparison of the kinetic coefficients b and d at different extraction temperatures, the temperature of 70°C had the highest mass transfer coefficient and lowest kinetic coefficient with a decreasing trend as shows in Table 1. It was attributed to the thermal degradation of anthocyanin due to the high microwave extraction temperature. Based on above results, the relatively high mass transfer and kinetic coefficients, and positive constants resulted in the highest anthocyanin yield at an extraction temperature of 60°C for 4 min. The degradation of anthocyanin also occurred in extractant diffusion stage in purees.

Table 1 Regression coefficients describing anthocyanin acquirement from blueberry powder under MAE conditions

Temperature/°C	y_0	a	b	c	d	R^2	SEE
30	0.315	0.5152	0.0269	0.5150	0.0269	0.9990	0.0051
40	0.4328	0.0904	0.3588	0.4429	0.0583	0.9939	0.0144
50	0.5009	0.2302	0.0284	0.3202	0.0870	0.9969	0.0089
60(1) *	0.5980	0.0898	0.3613	0.0932	0.3613	0.9970	0.0028
60(2) **	0.6266	0.9394	0.4637	–0.2361	0.4637	0.9985	0.0033
70	0	0.0955	1.1127	0.6823	0.012	0.9980	0.0038

Note: $y_2=y_0+a_2[1-\exp(-b_2t)]+c_2[1-\exp(-d_2t)]$. * and ** refer to at time period of 0-4 min and 4-10 min, respectively.

3.2 Kinetics of anthocyanin extraction and degradation at different hot reflux extraction (HRE) extraction temperatures

To distinguish the effect of microwave irradiation on the acquirement and degradation of anthocyanin, comparison between HRE and MAE process were conducted under the same extraction temperature. Figure 2 presents the change profiles of anthocyanin yield under five different extraction temperatures in HRE process.

The initial anthocyanin yield y_0 was obtained in a rising temperature period of 1 min (for all trails) from room temperature (25°C) to the set temperature levels in the range studied. The yields of anthocyanin increased with the extraction temperature from 30°C to 60°C. However, at extraction temperature of 70°C, the yield of anthocyanin increased to reach the highest yield with 65.07% in 2 min, and then decreased until the end of extraction.

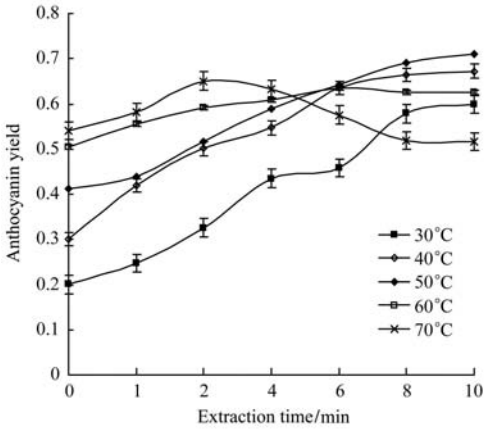


Figure 2 Changes of anthocyanin yield with extraction temperature in extraction liquid in the heat reflux extraction

As shows in Figure 1, the anthocyanin yield was 77.75% at 70°C for extraction time of 1 min, and 74.36% at 50°C at an extraction time of 10 min under MAE conditions. These yields were higher than that under the HRE conditions with 70.99% yield at 50°C in 10 min extraction time. The results indicated that MAE favorably improve anthocyanin yield from powdered blueberry, compared with HRE method under the same extraction temperature. This improvement was attributed to the dielectric effect of the electric-magnetic field on polar groups related to anthocyanin structure, and the destruction of cell walls in blueberry particles caused by microwave irradiation. In addition, the existence of light and oxygen in an open system of HRE impel the degradation process of anthocyanin in extracts. These factors resulted in higher degradation of anthocyanin in HRE than that in MAE under the same extraction temperatures.

Thermal and non-thermal effects of microwave irradiation on the anthocyanin extracted from blueberry may be revealed by means of the comparison experiments. Table 2 indicated that the yield of extracted anthocyanin from powdered blueberry under HRE follows the exponent equations with high R^2 and low *SEE*. Comparing to corresponding parameters for each extraction temperature in Table 1, the initial anthocyanin content (y_0) in extracts generated from pre-heating for a duration of 1 min were lower in the HRE process than that in the MAE process. The kinetic coefficients, in terms of delivery kinetic and diffusion kinetic, were higher in the HRE than that in MAE method. These results revealed that microwave volumetric heating mode improves the diffusion and solubility of anthocyanin within powdered blueberry particles, as well as the positive action of microwave irradiation on the anthocyanin yield in the pre-heating period (1 min duration).

Table 2 Regression coefficient describing anthocyanin acquirement from blueberry powder under HRE conditions

Temperature/°C	y_0	*a*	*b*	*c*	*d*	R^2	*SEE*	*p*
30	0.1953	0.6419	0.0654	0.1106	0.2680	0.9832	0.0348	0.0332
40	0.2998	0.0744	1.6088	0.3540	0.2000	0.9914	0.0223	0.0171
50	0.4017	0.1922	0.1589	0.2017	0.1589	0.9918	0.0186	0.0163
60	0.5041	0.0652	0.5629	0.0592	0.5629	0.986	0.0096	0.0279
70	0.535	0.5313	0.001			0.979	0.0001	
	0.183	0.5239	0.0484			0.9457	0.0205	0.0543

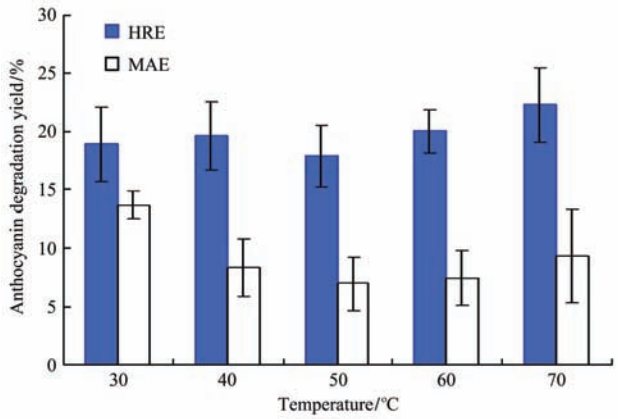
Note: $y=y_0+a[1-\exp(-bx)]+c[1-\exp(-dx)]$, when temperature is 30-60°C; $y=y_0+a[1-\exp(-bx)]$, when temperature is 70°C at time period of 0-2 min; $y=y_0+a\exp(-bx)$, when temperature is 70°C at time period of 2-10 min.

3.3 Analysis of microwave irradiation on the degradation of anthocyanin

3.3.1 Comparative analyses of anthocyanin degradation induced by MAE and HRE method

As shown in Figure 3, the amount of degraded anthocyanin extracted from powdered blueberry by the HRE was significantly higher than that by the MAE. In general, the process of microwave extracting anthocyanin from powdered blueberry was performed in the closed and pressurized vessels. This extraction process may protect anthocyanin from degradation of extracts due to shielding negative influences such as light and oxygen. Research results from study already indicated that microwave inducing pressure in extraction system achieves maximum level 0.068 bar at solid-liquid ratio of 1:30, 0.076 bar at ethanol concentration of 40%, 0.058 bar at microwave intensity of 100 W/g at extraction temperature of 50°C under MAE of anthocyanin from blueberry powder, which obviously accelerate the anthocyanin extraction in MAE under extraction

temperature of 50°C[15]. The pressure built in extraction system has the stronger swelling effect on the plant-material extracted to broken cluster formation and split cells, which promote the mass diffusion process, meanwhile the material organization mainly remained as the cluster formation under HRE[36,37]. Therefore, the amount of retained anthocyanin extracted from powdered blueberry in a closed MAE system is higher than in an open HRE system under the same extraction temperatures. As for the degradation trend of anthocyanin in extracts with the extraction temperature, a symmetric power equation was derived to describe the initial decrease followed by the increasing trend. The regression coefficients were well fitted with coefficients at $p<0.05$ levels and the data were shown in the table inlaid in Figure 3.



$y=y_0+abs(T_0-T)\wedge b$		
	HRE	MAE
a	0.0001	0.0005
b	3.0966	2.9880
T_0	41.2024	53.5711
y_0	18.7840	7.1181
R^2	0.8369	0.9978
SEE	1.3329	0.2539

Figure 3 Comparison of degradation amount of anthocyanin between heat reflux and microwave extracted method under different temperature in the extraction

Temperature higher than 50°C resulted in anthocyanin degradation in the extracts. The effect of extraction temperature on the anthocyanin degradation followed a symmetric power equation as shown in Figure 3. According to the power equation, the lowest anthocyanin degradation was at a temperature of 53.6°C. In HRE, no statistically significant differences were found in the degradation amounts of anthocyanin when the extraction temperature was lower than 50°C. The initial temperature of anthocyanin degradation was 41.2°C according to the critical temperature T_0 of power equation as shown in Figure 3. This critical temperature contributes to control degradation and improve yield of anthocyanin in the extraction process. As a result, the extraction temperature rising up to 70°C caused anthocyanin yield to decline compared to the lower temperatures (30°C-50°C).

The specific experiment results showed that the anthocyanin degradation percentage in the specific experiments are obtained as (16.23±1.62)% at 30°C, (16.92±0.85)% at 40°C, (14.77±2.11)% at 50°C, (17.77±1.66)% at 60°C, (19.78±0.96)% at 70°C. Comparing to Figure 3, anthochyanin degradation in closed HRE conditions are lower than that in open HRE, and are obvious higher than that in MAE. The results confirmed that the existence of light and oxygen in open HRE enhance the anthocyanin degradation. In both open and closed HRE system, temperature gradient of heat convection with relatively high temperature at outer location of vital results in the non-uniform temperature distribution. In MAE system, volumetric heating of microwave and microwave pyramid-shaped diffuser are located in ethanol result in uniform distribution. Non-uniform temperature in extraction system causes much more anthocyanin degradation than that in uniform one.

In comparison of MAE and HRE, prolonged exposure to oxygen resulted in greater degradation of anthocyanin in hot air heating[38]. High extraction efficiency of MAE was explained by greater destruction of bio-cellular structure and dissociation of membrane associated polyphenols[7], which were caused by microwaves and achievement of higher temperature levels in shorter period of time in case of microwave application. The variation of dielectric properties of extraction system improves its absorption capacity of microwave energy[39],

which leads to greater rate of release of compounds into the solvent.

3.3.2 Effects of MAE and HRE on the structure and composition of anthocyanin

To determine the effects of microwave irradiation on the components and chemical structure of anthocyanins from blueberry under MAE, HPLC and circular dichroism (CD) spectrum were employed for qualitative and quantitative analysis and the determination of the microstructure of components.

MAE and HRE methods were used to extract the anthocyanin from blueberry powder, respectively. Four anthocyanidins including delphinidin, cyanidin, malvidin and pelaronidin in the extractants were identified by using HPLC method. As shown in Table 3, microwave extraction methods caused delphinidin, cyaniding and pelargonidin contents significantly decreasing with elevating the extraction temperature due to thermal degradation (p<0.01), but malvidin was unchanged statistically in all extracts (p<0.01).

Table 4 Anthocyanins content of blueberry under different extraction temperatures using MAE and HRE methods (mg/g)

Extraction mode and temperature	Delphinidin	Cyanidin	Malvidin	Pelargonidin
MAE 40	1.1772±0.0118^{a}	1.0577±0.0098^{a}	0.2858±0.1566^{b}	2.1628±0.0192^{a}
MAE 50	1.1313±0.0444^{a}	1.0295±0.0552^{a}	0.2839±0.0035^{b}	2.0799±0.0758^{a}
MAE 60	1.1079±0.0246^{b}	0.8198±0.0252^{b}	0.3228±0.0118^{a}	2.0605±0.8433^{b}
MAE 70	1.0580±0.0163^{c}	0.7770±0.0189^{c}	0.3138±0.0157^{a}	1.9823±0.0343^{c}
HRE 40	1.0945±0.0103^{b}	0.7928±0.0203^{b}	0.3208±0.0108^{a}	2.0382±0.0207^{b}
HRE 50	1.1287±0.0450^{a}	0.7389±0.0136^{d}	—	1.9963±0.0791^{c}
HRE 60	1.1107±0.0304^{b}	0.7568±0.0175^{c}	—	1.9611±0.0725^{c}
HRE 70	1.0974±0.0210^{b}	0.7662±0.0119^{c}	—	1.9131±0.0378^{d}

Note: 1) All data in Table shown in average value ± SD, n=3, same letter behind data shown no significant difference (p<0.05); 2) No malvidin was detected in temperature of 50°C, 60°C and 70°C under heat reflux extraction; 3) MAE and HRE listed in 1st column represents microwave extraction method and heat reflux extraction method, respectively; 4) And the data behind letter represented extraction temperature.

For the anthocyanidin extracted by using the HRE method, the amount of cyanindin increased with the extraction temperature, meanwhile, pelargonidin contents significantly decreased, and deplhinidin contents did not statistically significantly change (p>0.05). Malvindin was detected only at a temperature of 40°C. A comparison analysis was performed for the changes of monomeric anthocyanins from two extraction methods under the same extraction temperature. Delphinidin content at a microwave temperature of 40°C was obviously higher than that in temperature of 40°C in HRE. However, there was an opposite difference occurred at the temperature of 70°C. No significant difference of delphinidin contents were found at the temperatures 50°C and 60°C under MAE and HRE. The cyanidin yield of MAE was higher than that extracted by the HRE method at the temperatures of 40°C, 50°C and 60°C. In these temperature ranges, pelargonidin yield from MAE was higher than that from HRE. CD may effectively detect conformational changes of molecular asymmetry of the anthocyanin during molecular association. In the conditions of extraction, π-electron groups of chromophor in anthocyanin molecules jump to produce UV-absorption due to thermal absorption causing the degradation of anthocyanin[40]. The generations of chromophoric groups enhance the intensity of CD absorption spectra.

According to the results from Figures 4a and 4b, two cotton peaks were found in the wavelength ranges of 200-240 nm and 260-280 nm, respectively, due to the existence of two chromophores of the aromatic rings-A and-B in the anthocyanin molecule structure. Relative weak negative cotton peaks of pure anthocyanin occur in wavelength ranges of 205-208 nm and 260-280 nm under extraction conditions in MAE and HRE mode as shown in Figures 4c and 4d. Positive cotton peaks at a wavelength of 220-240 nm, shown as extraordinarily large molecular ellipticities, indicated the self-aggregation of anthocyanins in extracts. As shown in Figure 4, the significantly differences were found in the CD spectra of anthocyanin structure in the extracts between MAE and HRE methods at the extraction temperatures of 40°C and 50°C. At 40°C, higher anthocyanin concentration in MAE caused the greater

self-aggregation of the flavylium cation of the four most common anthocyanindin 3-glucosides than that in HRE. However, the obvious intensity attenuation in CD band emerges at the extraction temperature of 50°C.

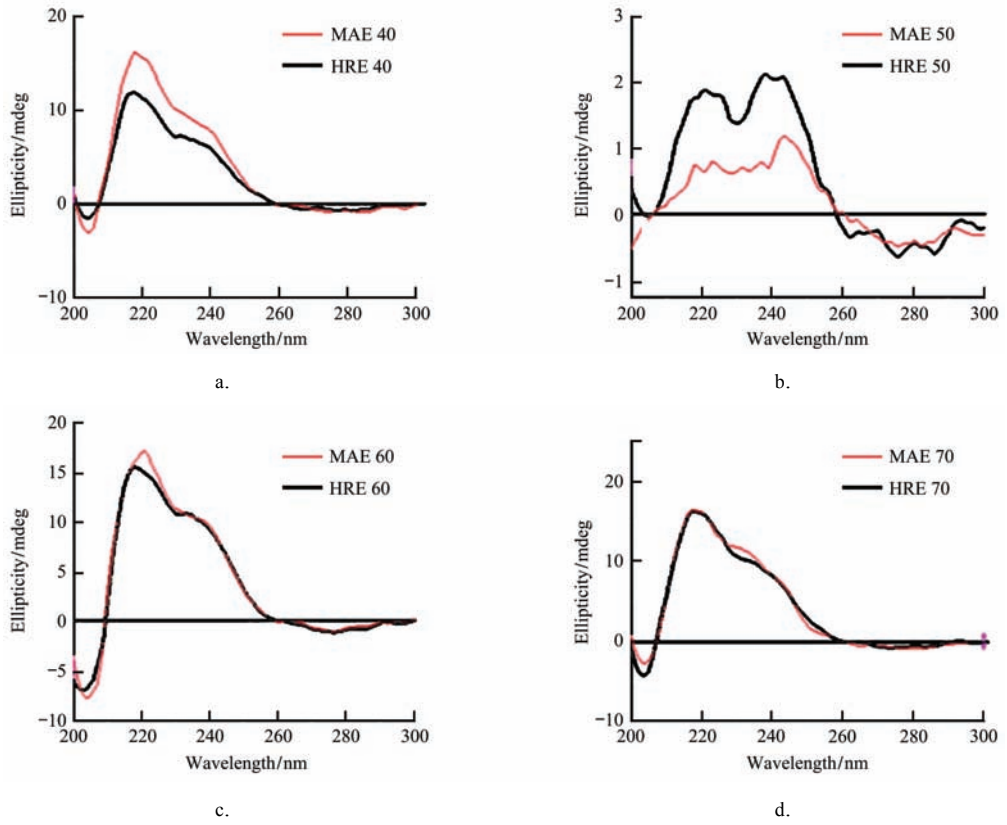


Figure 4 CD spectra of anthocyanin under different extraction methods and temperature conditions

3.3.3 Effect of microwave field on the anthocyanin and its derivatives

Anthocyanin is a natural flavonoid pigment with the basic structure of 2-benzofuranyl linked to the methoxyl group and hydroxyl. Anthocyanin, as a derivative from Anthocyanin, contains active polar groups of the benzoyl cation including in B-ring phenolic hydroxyl group with high antioxidant activity, B-4′-OH bond with strong active base[41]. Therefore, the solvation is comprised of ethanol and powdered blueberry irradiated by microwave field with non-equilibrium regime and enough free energy[42], which is positively associated with the solvent orientation polarization interaction energy, and negatively associated with the quantized solvent electronic polarization[43]. Therefore, the electromagnetic field at a microwave frequency of 2.45 GHz might exert strongly influence on the molecule of anthocyanin to cause the degradation of anthocyanin, which leads to some monomers of anthocyanin and anhydrobases. These monomers in neutral solutions have weak optical activity. The degradation results in the increase of methoxyl groups in the B-ring of anthocyanin nucleus to reduce the amount of their self-aggregation due to steric hindrance. The driving forces for self-aggregation are mainly hydrophobic interactions among the aromatic nuclei stacked parallel to each other, which are surrounded by the hydrophilic glucose moieties in a suitable spatial arrangement[44]. Electrostatic repulsion in a microwave field suppresses positive charge of the flavylium cation, agglomerating high order aggregates of anthocyanin by means of the hydration effect. At extraction temperatures 60°C and 70°C, the degree of aggregate formation of monomeric anthocyanin anhydrobases is higher than their hydration with decolorization. High extraction temperature would induce the self-aggregation of molecules and the degradation of anthocyanin. At extraction temperature of 40°C and 50°C, non-thermal effect of the microwave irradiation presented as an electrical magnetic field induces the movement of the charged ions and polar molecule to cause the self-aggregation of anthocyanin molecules. It was inferred that non-thermal of the

microwave irradiation has more significant effect on the changes of anthocyanin molecules than temperature at low microwave extraction temperature. With the extraction temperature increased up to 60°C, temperature plays a dominating role on the degradation of anthocyanin to monomer.

4 Conclusions

In MAE process, both acquirement and degradation of anthocyanin from powdered blueberry occur simultaneously with exponential function trends. Rising extraction temperature improves the diffusion of anthocyanin and aggravates its degradation, and the lowest anthocyanin degradation was at a temperature of 53.6°C. When extraction temperature is lower than 50°C, the non-thermal effect from microwave field plays the key role on the anthocyanin molecular structure to form self-aggregation; when extraction temperature is higher than 60°C, thermal effect from temperature dominates the degradation of anthocyanin to monomer.

MAE method has higher anthocyanin yield and lower its degradation in extraction system of blueberry powder than that in HRE under the same extraction conditions in term of temperature of 30°C-70°C. In MAE, the improvements of microwave on the acquirement of anthocyanin are as follows: (i) microwave irradiation could destroy the cell wall of blueberry particles to promote the mass diffusion and increase the anthocyanin yield to promote extraction kinetic at pre-heating stage; (ii) microwave volumetric heating results in the rapidly elevated temperature in the extraction system and generation of internal pressure to improve the acquirement of anthocyanin; (iii) the non-thermal effect from microwave field may weak the effect of temperature to decline the degradation of anthocyanin to monomer. In HRE, thermal convection modes form the higher temperature at surface area than that at center of extraction vials. The non-uniform distributions of temperature decrease the extraction yield of anthocyanin due to low interior temperature. The light and oxygen in the open system of reflux extraction also lead to the anthocyanin degradation during extraction process.

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