Carbohydrate Polymers 113 (2014) 314–324

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Ultrasound-assisted enzymatic extraction and antioxidant activity of polysaccharides from pumpkin (*Cucurbita moschata*)

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ARTICLE INFO

Article history:
Received 3 February 2014
Received in revised form 11 July 2014
Accepted 13 July 2014
Available online 21 July 2014

Keywords:
Polysaccharide
Cucurbita moschata
Ultrasound-assisted enzymatic extraction
Antioxidant activity
Response surface methodology

ABSTRACT

An efficient ultrasound-assisted enzymatic extraction (UAEE) of *Cucurbita moschata* polysaccharides (CMCP) was established and the CMCP antioxidant activities were studied. The UAEE operating parameters (extraction temperature, ultrasonic power, pH, and liquid-to-material ratio) were optimized using the central composite design (CCD) and the mass transfer kinetic study in UAEE procedure was used to select the optimal extraction time. Enzymolysis and ultrasonication that were simultaneously conducted was selected as the UAEE synergistic model and the optimum extraction conditions with a maximum polysaccharide yield of 4.33 ± 0.15% were as follows: extraction temperature, 51.5 °C; ultrasonic power, 440 W; pH, 5.0; liquid-to-material ratio, 5.70:1 mL/g; and extraction time, 20 min. Evaluation of the antioxidant activity in vitro suggested that CMCP has good potential as a natural antioxidant used in the food or medicine industry because of their high reducing power and positive radical scavenging activity for DPPH radical.

1. Introduction

The fruit of pumpkin (*Cucurbita moschata*) is one of the most important vegetables in the traditional agricultural system around the world (Qian, 2014), and it has received considerable attention in recent years because of its nutritional and health protective value. Several beneficial nutritive or medicinal properties of various *C. moschata* extracts have been reported, including antioxidant (Yang, Zhao, & Lv, 2007), antidiabetic (Jiang & Du, 2011), antiobesity (Choi et al., 2007), antihypercholesterolemic (Al-zuhair, Abd el-fattah, & Abd el latif, 1997), antibacterial (Park et al., 2010) and anti-prostate cancer activity (Gossell-Williams, Davis, & O'Connor, 2006). Macromolecular pumpkin polysaccharides, which are composed of galactose, glucose, arabinose, xylose, and glucuronic acid (Song et al., 2012), have chemical and pharmacological properties that play a nutritional important role in *C. moschata*. The polysaccharides from pumpkin fruit are demonstrated to increase the levels of serum insulin, reduce the blood glucose levels, improve tolerance of glucose, and thus can be developed as new antidiabetic agent (Zhang et al., 2013). It can also inhibit the H_2O_2-induced decrease of cell viability, lactate dehydrogenase leakage, and malondialdehyde formation, indicating that pumpkin polysaccharides have a cytoprotective effect and antioxidant activity (Yang et al., 2007).

The extraction technology used to obtain compounds with high aggregative value from natural plants is crucial for product quality. Application of different extraction methods mainly depends on their characteristics, including the complexity of materials, production cost, environmental effects and safety. Traditional extraction such as hydrothermal maceration has been applied to isolate polysaccharides from pumpkin (Huang, Chen, & Wang, 2011). The main shortcoming of conventional methods is the high-energy consumption with low extraction yield (Hromádková, Ebringerová, & Valachovič, 2002). Other techniques, which include the ultrasound-assisted extraction (UAE) and enzyme-assisted extraction (EAE) that are efficient, environmental friendly and capable of producing high quantities of polysaccharides, have become ideal alternatives to traditional techniques. Specifically, UAE can cause collapse of cavitation bubbles which generates sufficient energy to give rise to collisions between suspended plant particles as in the case of classical extraction by stirring (Chemat & Khan, 2011; Mason, Chemat, & Vinatoru, 2011). EAE uses enzyme preparations either alone or in mixtures that catalyze hydrolysis of the cytoderm and glycoproteins, and can enhance the release of bioactive substance by disrupting plant cells (Ptichkina, Markina, & Rumyantseva, 2008; You, Yin, & Zhao, 2013; Zhang, Jia, Liu, Wu, & Ran, 2011).

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http://dx.doi.org/10.1016/j.carbpol.2014.07.025
0144-8617/

Although EAE has been successfully used to extract pumpkin polysaccharides (Ptichkina et al., 2008; Qian, 2014), biotechnological application of enzymes is not currently used to its maximum potential within the food industry (Puri, Sharma, & Barrow, 2012). Demand for high performance extraction of *C. moschata* crude polysaccharides encourages the continuing search for better extraction techniques. In recent years, some researchers had confirmed that low-frequency ultrasonication improved the activity of cellulase preparations under certain ultrasonic intensity conditions (Aliyu & Hepher, 2000; Li et al., 2005; Nguyen & Le, 2013). Szabó and Csiszár (2013) indicated that enzymatic hydrolysis was always positive even though enzyme activity was severely reduced, and depended on ultrasonic parameters and advantageous effects of sonication that were used in the heterogeneous cellulose–cellulase reaction; these steps protected the enzyme from ultrasonication. Hence, ultrasound-assisted enzymatic extraction (UAEE), which uses the synergistic interaction of enzymatic hydrolysis and ultrasound, may meet the food industry's need for extraction of crude polysaccharides from pumpkin.

The UAEE has been used to isolate polysaccharides from *epimedium* leaves (Chen, Li, Liu, Yang, & Li, 2012), *Lentinus edodes* (Ke, 2014), corn silk (Chen et al., 2014), wheat bran (Wang, Sun, Liu, & Zhang, 2014) and *Lycium barbarum* (Liu et al., 2014). To date, however, there has been no report on ultrasound-assisted extraction combined with enzymatic treatment for polysaccharides from pumpkin and the synergistic extraction models between ultrasound, and enzymolysis in above mentioned studies exclude simultaneous operation. Therefore, in this work, the UAEE and antioxidant activity of *C. moschata* crude polysaccharides are investigated to determine an effective method for pumpkin polysaccharide extraction that has a higher yield and higher bioactivity. Various synergistic models of ultrasonication and enzymolysis are investigated in the UAEE process and multiple UAEE parameters, such as extraction temperature, ultrasonic power, pH, liquid-to-material ratio and extraction time, are optimized using response surface methodology (RSM) and simulated kinetic modeling. In addition, the antioxidant activity of crude extracts is evaluated by determining the 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging rate, hydroxyl radical scavenging rate and ferric reducing/antioxidant power (FRAP), compared with ascorbic acid as an antioxidant standard.

2. Materials and methods

2.1. Materials preparation

Fresh mature pumpkins (*C. moschata* Duch.) were purchased from a local supermarket (Qingdao, Shandong Province, China). The skin and seeds were peeled and the fruits were washed thoroughly with tap water to remove any impurities attached to the surface. The washed fruits were cut into small pieces (overall dimension: 1 cm × 1 cm × 1 cm) with a sharp knife and stored at 4 °C for subsequent experiments.

2.2. Reagents and equipment

Cellulase (40,000 U/g; the optimum temperature: 55–60 °C; pH: 4.8–5.2), pectase (30,000 U/g; the optimum temperature: 45–53 °C; pH: 3.5–4.5) and papain (200,000 U/g; the optimum temperature: 65 °C; pH: 5.0–7.0) were purchased from Lihua Enzyme Preparation Technology Co., Ltd. (Tianjin Municipality, China). DPPH and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). All other reagents used were of analytical grade.

Ultrasonic processing was performed in a thermostatic ultrasonic processor (SY-1000E, Beijing Hongxianglong Biotechnology Development Co., Ltd., Beijing, China), equipped with a powerful probe-shaped ultrasonic transducer (Φ15 mm) and a digital controlled low-frequency sonotrode (20 kHz), which can change ultrasonic power (0–900 W) during extraction processing. The processor was also equipped with an automatic heating/cooling system to control the medium temperature. In all of the following experiments, the ultrasonic time was set 2 s followed by cooling for 1 s.

2.3. Selection of synergistic models in UAEE procedure

Enzyme macromolecule-ultrasound interaction has a significant effect on the bioprocess efficiency (Mason, Paniwnyk, & Lorimer, 1996; Rokhina, Lens, & Virkutyte, 2009). In addition to the effects of substrate fragmentation and micro-mixing, modification of protein tertiary structure by ultrasound is another influencing factor that enhances enzyme activity in some cases when ultrasonication is applied to the enzymatic reactions as part of the preprocessing or simultaneous treatment (Basto, Tzanov, & Cavaco-Paulo, 2007; Nguyen & Le, 2013; Shah & Gupta, 2008). Thus, various synergistic models of ultrasonication and enzymolysis (C, U, E, U_E, E + U, E + U_E, U + E and U_E + E) were studied and an optimal operating mode was selected from following experiments: prepared pumpkin pieces (100 g) were well mixed and homogenized in a blender (ZA-70B, Tianjin, China) using an appropriate amount of distilled water and then placed in an ultrasonic processor inverted-cone vessel. Pumpkin pulp was prepared by maintaining a distilled water-to-pumpkin liquid-to-solid ratio of 6:1 mL/g. (I) C model (conventional water extraction): pumpkin pulp was incubated at 50 °C for 2 h without stirring extraction. (II) U mode (ultrasound-assisted extraction): pumpkin pulp was subjected to sonication using an output power of 500 W for 20 min at 50 °C. (III) E mode (enzymes-assisted extraction): pumpkin pulp was subjected to enzymatic hydrolysis using compound enzymes with optimal enzyme-to-material ratio at 50 °C for 2 h (cellulase, 100 mg/g; pectase, 140 mg/g; papain, 100 mg/g, according to our previous orthogonal testing), followed by immediate inactivation of enzymes with pasteurization at 90 °C for 30 s in a conical flask (Yang et al., 2010). (IV) U_E model (simultaneous processing): pumpkin pulp including the compound enzymes, was subjected to sonication with the ultrasonic power of 500 W for 20 min at 50 °C, followed by compound enzyme inactivation. (V) E + U model: pumpkin pulp was subjected to enzymatic hydrolysis using compound enzymes at 50 °C for 2 h followed by enzyme inactivation and sonicated with an output power of 500 W for 20 min at 50 °C. (VI) E + U_E model: pumpkin pulp was subjected to enzymatic hydrolysis with compound enzymes at 50 °C for 2 h, followed by sonication with an ultrasonic power of 500 W for 20 min at 50 °C; the enzyme activity was then terminated. (VII) U + E model: pumpkin pulp was subjected to sonication with an output power of 500 W for 20 min at 50 °C without enzymes, followed by enzymolysis at 50 °C for 2 h; the enzyme activity was then terminated. (VIII) U_E + E model: pumpkin pulp including the compound enzymes, was subjected to sonication with the ultrasonic power of 500 W for 20 min at 50 °C, followed by enzymolysis at 50 °C for 2 h; the enzyme activity was then terminated.

2.4. Determination of C. moschata crude polysaccharides (CMCP) extraction yield

After the extraction was complete, the extracts were centrifuged at 1000 × g for 15 min (Anting, Shanghai, China) and the supernatants were collected for analysis of the polysaccharide extraction yield. Total supernatant sugar content was measured using the phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, &

Table 1
Independent variables and their levels used in CCD.

Independent variables		Factor level					
		−2	−1	0	1	2	ΔX_i[a]
X_1	Extraction temperature (°C)	30	40	50	60	70	10
X_2	Ultrasonic power (W)	200	300	400	500	600	100
X_3	pH	2.5	3.5	4.5	5.5	6.5	1
X_4	Liquid-to-material ratio (mL/g)	2	4	6	8	10	2

[a] Step change values.

Smith, 1956) and the reducing sugar content of the supernatant was measured using the dinitrosalicylic acid method (Miller, 1959). D-Glucose was used as the standard in both methodologies. The percentage extraction yield of CMCP (Y) was calculated using Eq. (1):

$$Y(\%) = \frac{m_t - m_r}{\text{pieces weight}\,(100\,\text{g})} \times 100 \quad (1)$$

where m_t and m_r represent the total sugar content (g) and reducing sugar content (g), respectively.

2.5. Experimental design and statistical analysis

After selecting the synergistic UAEE model, a central composite design (CCD) was used to estimate the effect of independent variables (extraction temperature, X_1; ultrasonic power, X_2; pH, X_3; liquid-to-material ratio, X_4) on CMCP extraction yield. Based on single factor analysis, process variables with their ranges were selected and coded at five levels according to Eq. (2):

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad i = 1,\ 2,\ 3,\ 4 \quad (2)$$

where x_i is a coded value of the independent variable, X_i is the actual value of the independent variable, X_0 is the actual value of X_i on the center point, and ΔX_i is the value of the step change. The range of independent variables and their levels are represented in Table 1. In present study, the design consisted of 30 experimental points (16 factorial points, 8 axial points, and 6 center points).

All 30 experiments were performed to study the effect of independent variables on the response and the extraction conditions were optimized. The experiments were randomized to reduce error caused by external factors during the experimental process. The response values in each trial were analyzed using Design-Expert 8.0.5 (Stat-Ease Inc., Minneapolis, USA) and fitted to a second-order polynomial regression model expressing mathematical relationship between independent variables (X_1, X_2, X_3 and X_4) and response (Y):

$$Y = \beta_0 + \sum_{i=1}^{4}\beta_i X_i + \sum_{i=1}^{3}\sum_{j=i+1}^{4}\beta_{ij} X_i X_j + \sum_{i=1}^{4}\beta_{ii} X_i^2 \quad (3)$$

where, Y is the predicted response, X_i and X_j are input variables that influence the response variable Y, β_0 is the constant coefficient, β_i is the linear coefficient, β_{ij} is the interaction coefficient of the two factors, and β_{ii} is the quadratic coefficient.

2.6. Kinetics of the UAEE procedure

After each parameter was optimized using response surface design, the kinetic process of mass transfer was studied to describe different extraction phases. The effect of UAEE time variables (0–30 min) on CMCP extraction yield was studied using single factor experiment under the optimized extraction conditions derived from CCD. Experimental data was fitted to Weibull's model, which had been widely used to extract polysaccharides from botanic materials because of its simplicity and satisfactory fit to experimental data (Cheung, Siu, & Wu, 2013). The stabilized CMCP yield ($[CMCP]_\infty$) and extraction rate constant (k_e) was calculated using non-linear regression in Origin 8 (Microcal Origin, USA), according to Weibull Eq. (4) and the optimal extraction time was selected, which indicated that the integrated UAEE processing parameter optimization was complete.

$$[CMCP](\%) = [CMCP]_\infty (1 - e^{-(k_e t)^d}) \quad (4)$$

where d is the shape parameter. If $d = 1$, the Weibull equation reduces to a first-order equation and the curve is characterized by an exponential equation (Kitanović, Milenović, & Veljković, 2008).

2.7. Evaluation of antioxidant activity in vitro

After UAEE, solid crude polysaccharides from *C. moschata* to be used in antioxidant activity tests were prepared. The extract mixtures were centrifuged (1000 × g, 15 min) and then supernatants were filtered through a fast type filter paper (Xinxing, Hangzhou, China). Extracts obtained were concentrated using a rotatory evaporator (Yarong, Shanghai, China) at 50 °C under a vacuum and then deproteinized using the Sevag method (Staub, 1965). The polysaccharides in the remaining solution were precipitated using 95% (v/v) ethanol and incubated overnight at 4 °C. The precipitates were collected using suction filtration, washed sequentially using 95% (v/v) ethanol, acetone and ethanol, and lyophilized at −50 °C (Christ, Germany) to obtain crude polysaccharides. CMCP antioxidant activity was evaluated using the DPPH radical scavenging assay, hydroxyl radical scavenging assay, and FRAP assay.

2.7.1. DPPH scavenging assay

The CMCP scavenging activity on DPPH radicals was measured according to the procedure described by previous studies with some modifications (Tian et al., 2012). The reaction mixtures consisted of 2 mL DPPH (0.05 mM in ethanol) and 2 mL CMCP (2–20 mg/mL). The mixtures were incubated in darkness at 25 °C for 30 min, and the mixtures' absorbances were determined at 517 nm using ascorbic acid as a positive control. The DPPH radical scavenging rate was calculated using the following equation:

$$\text{Scavenging rate}\,(\%) = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100 \quad (5)$$

where A_0 is the absorbance of blank samples (2 mL ethanol and 2 mL DPPH solution), A_1 is the test group absorbance (2 mL samples solution and 2 mL DPPH solution) and A_2 is the control group absorbance (2 mL samples and 2 mL ethanol). The experimental values were fitted to a 4-parameter logistic model and the EC_{50} (mg/mL) corresponding to the response midway between estimates of the lower and upper plateaus was calculated using Origin 8 (Sebaugh, 2011).

2.7.2. Hydroxyl radical scavenging assay

The hydroxyl radicals scavenging assay was carried out using the method described by Chen et al. (2011) with some modifications. Reaction mixtures (8 mL) containing 2 mL CMCP (2–40 mg/mL),

0.6 mL 6 mM H_2O_2, 0.6 mL 6 mM $FeSO_4$, 0.6 mL 6 mM salicylic acid in Tris–HCl buffer solution (pH 7.4), were incubated at 37 °C for 30 min. The absorbance was observed at 510 nm using ascorbic acid as a positive control. The hydroxyl radicals scavenging rate was expressed as the following equation:

$$\text{Scavenging rate}\,(\%) = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100 \quad (6)$$

where A_0 is the absorbance of blank (distilled water instead of sample solution), A_1 is the sample absorbance, and A_2 is the absorbance of sample under identical conditions as A_1 (distilled water instead of salicylic acid solution). The EC_{50} value (mg/mL) was calculated using the logistic model, as in Section 2.7.1.

2.7.3. Determination of FRAP

The FRAP assay uses electron transfer reactions to measure the total antioxidant activity of natural compounds. The method used by Benzie and Strain (1996) was used in this experiment, with some modifications. CMCP aliquots (0.2 mL) were mixed with FRAP reagent (3.8 mL) made up of 0.3 M sodium acetate buffer (pH 3.6), 0.01 M TPTZ solution and 0.02 M ferric chloride solution (10:1:1, v/v/v). The reaction mixtures were incubated at 37 °C for 30 min. The increase in absorbance at 593 nm was measured. Ferrous sulfate solutions of known concentration from 0.1 mM to 1.0 mM were used for calibration. The ferric reducing ability of the samples was calculated from the following linear calibration curve equation (linearity range: 0.02–0.2 μmol, $R^2 = 0.9992$):

$$A_{593} = 4.185x - 0.0143 \quad (7)$$

where x is the amount of ferrous sulfate. The FRAP value of the samples was expressed as mmol $FeSO_4$ equivalents per gram of lyophilized CMCP and calculated as a linear regression slope using Origin 8 software.

2.8. Statistical analysis

All data were expressed as the mean ± standard deviation of three separate experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) and Duncan's multiple comparisons in SPSS (version 19.0, SPSS Inc.). The statistical significance was established when $p < 0.05$.

The UAEE model was also analyzed using the ANOVA and Design-Expert 8.0.5 (Stat-Ease Inc., Minneapolis, USA). The significance of all terms in the polynomial was statistically tested by computing the F-value at a probability (p) of 0.01 or 0.05. The quality of the response surface model was expressed by the determination coefficient (R^2), lack of fit, adjusted determination coefficient (Adj-R^2), predicted determination coefficient (Pre-R^2), coefficient of variation (C.V.) and adequate precision. The fitted polynomial equation was expressed as surface and contour plots to visualize the relationship between response and each factor (Wang, Zhang, Wang, & Wang, 2013).

3. Results and discussion

3.1. Selection of synergistic models

Independent enzymolysis and ultrasonication treatments were combined to investigate whether the EAE and UAE could work synergistically to increase CMCP extraction yields. Fig. 1 shows the CMCP extraction efficiency under the different synergistic modes. The ultrasonic or enzymatic treatments can significantly increase the extraction yield of pumpkin polysaccharides ($p < 0.05$) because EAE utilizes cellulase and pectinase to hydrolyze and degrade plant cell wall constituents to improve the release of intracellular contents (Karki, Maurer, Kim, & Jung, 2011) and UAE utilizes ultrasonic

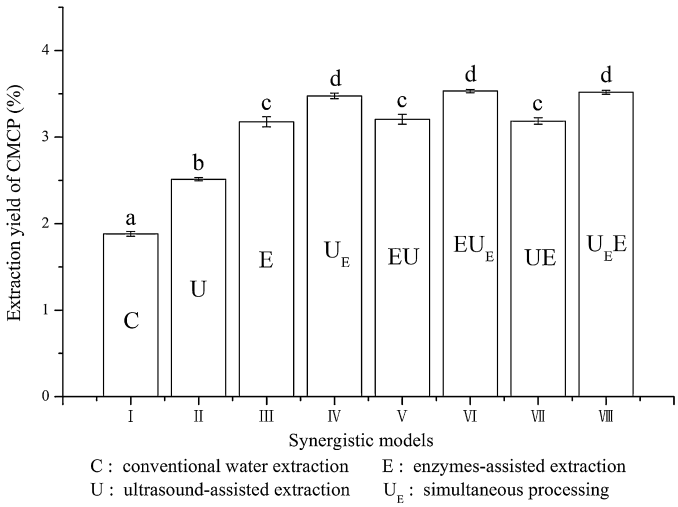

Fig. 1. Effect of different synergistic modes on extraction yield of CMCP. Values marked by the same letter are not significantly different ($p < 0.05$). CMCP: *Cucurbita moschata* crude polysaccharides.

cavitation, which is the major factor that enhances extraction (Chen et al., 2012). A significantly inferior yield using single sonication was found when model II and model III were compared, which indicates that enzymolysis makes a greater contribution to enhancing the CMCP extraction yield compared with ultrasonication ($p < 0.05$). This result is in agreement previous studies (You et al., 2013), which showed that EAE had the largest *Cornus officinalis* polysaccharides yield among the hot water extraction, EAE and UAE methods.

The effects of various synergistic models on CMCP extraction yield can be shown from the comparison among models IV, V, VI, VII and VIII. This comparison illustrates that the simple combination of two efficient treatments is not the best choice to improve the extraction efficiency. Polysaccharide transfer is significantly strengthened using both treatments simultaneously ($p < 0.05$). Capelo, Maduro, and Vilhena (2005) reported that there was an increased contact area between phases that was caused by cavitation, allowing a reduction of mass transfer limitations in the enzyme-substrate system. High intensity focused ultrasound causes a simple reduction in size, which allowed more substrate area to be in contact with the enzyme. Additionally, the enhanced reaction rates caused by ultrasonication have been attributed to an increase in collisions between the enzyme and the substrate. Moreover, comparing models IV, VI and VIII showed that the CMCP yield was not increased by excessive enzymatic hydrolysis ($p < 0.05$). Thus, the collaborative model IV, in which enzymolysis and ultrasonication are conducted simultaneously, was selected and used in subsequent experiments.

3.2. Effect of independent variables on extraction yield

3.2.1. Effect of the extraction temperature on CMCP yield

The effect of extraction temperature on CMCP extraction yield is shown in Fig. 2a. Different extraction temperatures were used while fixing the other extraction conditions as follows: ultrasonic power, 600 W; pH, 6.5; liquid-to-material ratio, 6:1 mL/g; extraction time, 20 min. A significant increase in CMCP yield was observed by increasing the extraction temperature from 20 °C to 50 °C because higher extraction temperatures can enhance enzyme catalyzing activities in favor of releasing the polysaccharides. The peak yield (4.08 ± 0.24%) was reached at an extraction temperature of 50 °C, and the yield significantly decreased with a further increase in the extraction temperature ($p < 0.05$). High temperatures decrease number of cavitation bubbles and weaken the impact of cavity

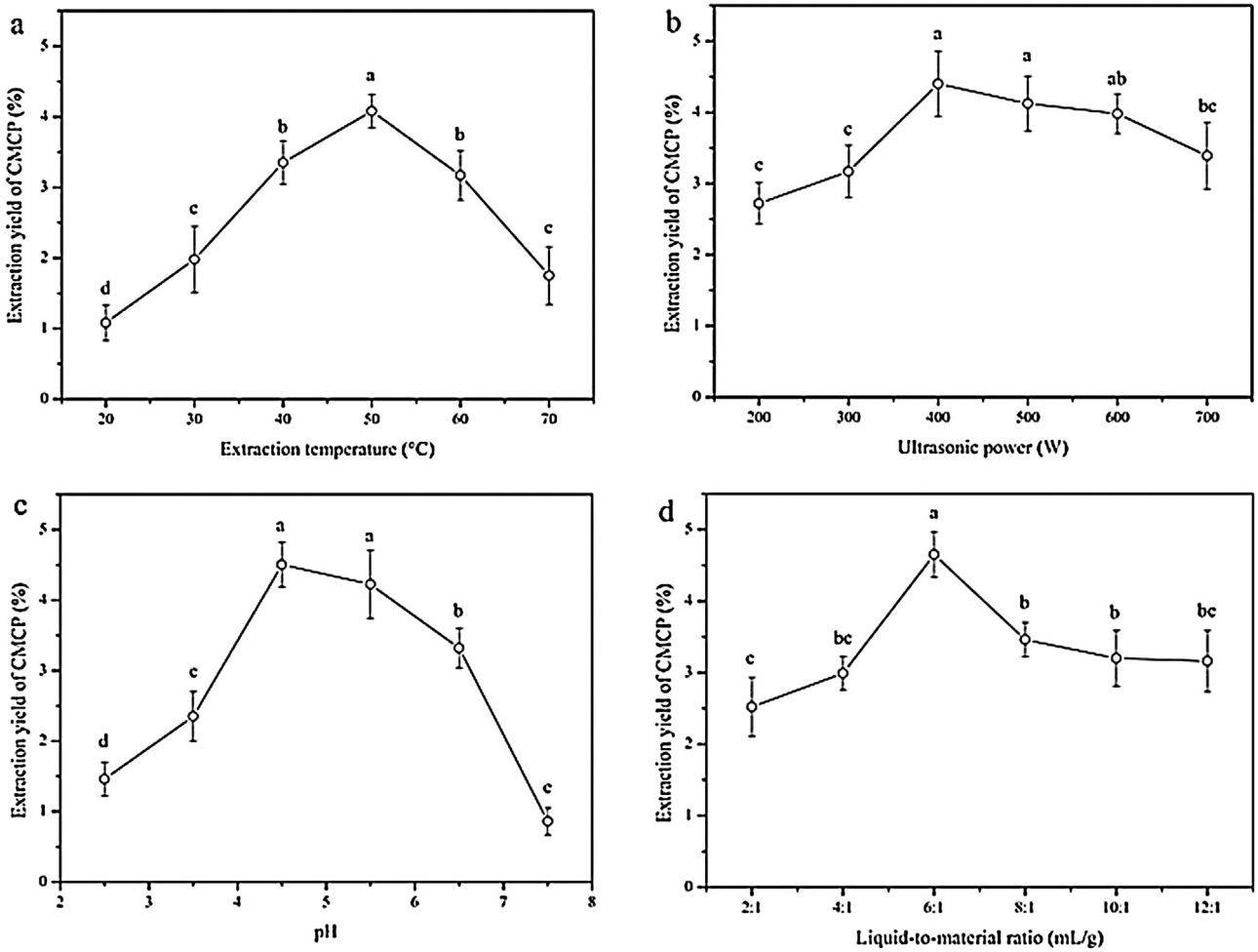

Fig. 2. Effect of dependent variables on the extraction yield of CMCP: (a) extraction temperature; (b) ultrasonic power (c) pH; (d) liquid-to-material ratio. CMCP: *Cucurbita moschata* crude polysaccharides.

collapse on homogenized samples, and the enzyme activity decreases (Palma & Barroso, 2002). The variable temperature range used in the RSM experiments was thus selected as 30 °C–70 °C.

3.2.2. Effect of the ultrasonic power on CMCP yield

To study the effect of power output on CMCP extraction yield, the UAEE process was implemented using different power parameters. The extraction temperature, pH, liquid-to-material ratio and extraction time were fixed at 50 °C, 6.5, 6:1 mL/g, and 20 min, respectively. As shown in Fig. 2b, increasing the ultrasonic power from 200 W to 400 W causes a continued improvement in yield because of an increase in the number of cavitation bubbles formed and enhanced mass transfer rates (Mason et al., 2011). Yield reached the maximum value (4.40 ± 0.46%) at 400 W. When the power was further increased, an insignificant reduction in yield was observed ($p < 0.05$). This may be because the powerful ultrasound may cause polysaccharide depolymerization, aggregation, and viscosity to decrease, which will result in a decrease in the extraction yield (Chen et al., 2012). No significant difference in extraction yield was found for a power between 700 W and 300 W. Therefore, the variable range of power used in the RSM experiments was selected as 200 W–600 W because higher energy consumption is derived from higher ultrasonic power.

3.2.3. Effect of the pH on CMCP yield

Fig. 2c shows the effect of pH on CMCP extraction yield while fixing the other extraction conditions as follows: extraction temperature, 50 °C; ultrasonic power, 400 W; liquid-to-material ratio, 6:1 mL/g; and extraction time, 20 min. Increasing pH values from 2.5 to 4.5 caused a significant increase in extraction yield, which reached a peak value (4.50 ± 0.32%) at pH 4.5. The extraction yield significantly decreased when the pH was further increased. A possible reason for this change may be that the appropriate pH of the complex enzymes is approximately 4.5, and the activity of the complex enzymes is decreased when the pH exceeds 4.5 because the enzyme spatial structure is changed. The variable pH range used in the RSM experiments was selected as 2.5–6.5 because the yield at pH 2.5 is significantly higher than that of pH 7.5 ($p < 0.05$).

3.2.4. Effect of the liquid-to-material ratio on CMCP yield

UAEE procedure is executed at different liquid-to-material ratios while fixing the other extraction parameters as follows: extraction temperature, 50 °C; ultrasonic power, 400 W; pH, 4.5; and extraction time, 20 min. As shown in Fig. 2d, extraction yield was affected by the liquid-to-material ratio indistinctively ($p < 0.05$). With an increased ratio from 2:1 mL/g to 6:1 mL/g, the CMCP yield increased and reached the maximum value (4.65 ± 0.32%) at 6:1 mL/g; the yield then decreased with further an increase to the liquid-to-material ratio. A liquid-to-material ratio that is too low or too high may not favor the movement of the plant cells to the active site of the enzyme and the polysaccharides to the medium under ultrasound treatment (Wang et al., 2014). No significant difference was observed between an extraction yield of 2:1 mL/g and 12:1 mL/g; thus, the variable range of

Table 2
Response surface central composite design and results for extraction yield of CMCP.

Run	X_1 (°C)	X_2 (W)	X_3	X_4 (mL/g)	Extraction yield of CMCP (%)	
					Experimental	Predicted
1	60(1)	500(1)	3.5(−1)	8:1(1)	3.78	3.80
2	50(0)	400(0)	4.5(0)	6:1(0)	4.28	4.41
3	40(−1)	300(−1)	5.5(1)	8:1(1)	3.55	3.59
4	60(1)	300(−1)	3.5(−1)	8:1(1)	3.62	3.70
5	40(−1)	300(−1)	3.5(−1)	4:1(−1)	3.79	3.86
6	50(0)	400(0)	4.5(0)	6:1(0)	4.47	4.41
7	50(0)	400(0)	4.5(0)	6:1(0)	4.41	4.41
8	70(2)	400(0)	4.5(0)	6:1(0)	3.95	3.95
9	50(0)	400(0)	6.5(2)	6:1(0)	4.22	4.20
10	60(1)	300(−1)	5.5(1)	4:1(−1)	4.04	4.00
11	40(−1)	500(1)	3.5(−1)	4:1(−1)	4.12	4.13
12	40(−1)	300(−1)	3.5(−1)	8:1(1)	3.65	3.53
13	50(0)	400(0)	2.5(−2)	6:1(0)	3.86	3.90
14	40(−1)	500(1)	5.5(1)	8:1(1)	3.85	3.93
15	30(−2)	400(0)	4.5(0)	6:1(0)	3.77	3.78
16	50(0)	400(0)	4.5(0)	6:1(0)	4.42	4.41
17	60(1)	300(−1)	5.5(1)	8:1(1)	3.99	4.01
18	60(1)	500(1)	3.5(−1)	4:1(−1)	3.97	3.88
19	60(1)	500(1)	5.5(1)	4:1(−1)	3.97	4.12
20	50(0)	200(−2)	4.5(0)	6:1(0)	3.81	3.79
21	40(−1)	300(−1)	5.5(1)	4:1(−1)	3.78	3.80
22	50(0)	400(0)	4.5(0)	2:1(−2)	3.77	3.80
23	50(0)	600(2)	4.5(0)	6:1(0)	4.16	4.20
24	50(0)	400(0)	4.5(0)	6:1(0)	4.42	4.41
25	40(−1)	500(1)	5.5(1)	4:1(−1)	4.25	4.12
26	40(−1)	500(1)	3.5(−1)	8:1(1)	3.86	3.84
27	60(1)	300(−1)	3.5(−1)	4:1(−1)	3.84	3.80
28	50(0)	400(0)	4.5(0)	10:1(2)	3.52	3.51
29	60(1)	500(1)	5.5(1)	8:1(1)	4.28	4.16
30	50(0)	400(0)	4.5(0)	6:1(0)	4.46	4.41

liquid-to-material ratio used in the RSM experiments was selected as 2:1 mL/g–10:1 mL/g because solvent consumption can increase production costs in the food industry.

3.3. Statistical analysis and model fitting

All 30 random sequential experiments under various extraction conditions are performed to study the reciprocal influence of independent variables (extraction temperature, ultrasonic power, pH, liquid-to-material ratio) on CMCP extraction yield and to optimize the operating parameters. The actual and predicted values according to the factorial design are presented in Table 2. Applying multiple regression analysis to the experimental data, the final quadratic equation obtained in terms of actual factors is given as below:

$$\begin{aligned} Y = {} & -2.99097 + 0.11391X_1 + 0.011234X_2 - 0.43434X_3 \\ & + 0.31855X_4 + 5.00625 \times 10^{-5}X_1X_2 + 6.50625 \times 10^{-3}X_1X_3 \\ & - 2.76562 \times 10^{-3}X_1X_4 + 9.68750 \times 10^{-5}X_2X_3 - 3.21875 \\ & \times 10^{-5}X_2X_4 + 0.014031X_3X_4 - 1.35552 \times 10^{-3}X_1^2 - 1.03927 \\ & \times 10^{-5}X_2^2 - 0.089802X_3^2 - 0.047388X_4^2 \end{aligned} \quad (8)$$

where, X_1, X_2, X_3 and X_4 are extraction temperature, ultrasonic power, pH and liquid-to-material ratio, respectively.

The ANOVA results for response surface quadratic model and multiple regression analysis are evaluated using the corresponding F and p values, and presented in Table 3. The F value is calculated to be 18.02, which suggests that the model is statistically significant. The model's coefficient of determination (R^2) is 0.9921, which indicates that more than 99.21% of the response variability is explained, supporting a good accuracy and ability of the established model within the range limits used (Li, Fabiano-Tixier, Tomao, Cravotto, & Chemat, 2013). The lack of fit relative to pure error at the 95% confidence level was not significant, and indicates that the model can be used to predict the polysaccharides extracted from pumpkin (Maran, Mekala, & Manikandan, 2013). In addition, Adj-R^2, Pre-R^2 and the coefficient of variation (C.V.) are calculated to check the model adequacy. Pre-R^2 is 0.7211, which is in reasonable agreement with the Adj-R^2 of 0.8915 (Adj-R^2 – Pre-R^2 < 0.2), and indicates a high degree of correlation between the observed and predicted data from the regression model (Erbay & Icier, 2009). The coefficient of variation (C.V.) expresses the standard deviation as a percentage of the mean, and was found to be 2.34% (<5.00%) for the CMCP extraction yield, which indicates that the model is reproducible (Samavati, 2013). Adequate precision measures the signal-to-noise ratio, which is useful when the value is less than 4. The ratio of 13.616, which is presented in Table 3, illustrates that the model is significant for the present UAEE process (Jin & Ning, 2013). Correlation analysis between predicted values and actual values can evaluate the suitability of the response surface model (Maran, Mekala, et al., 2013). A correlation coefficient of 0.971 is shown in Table 3, and indicates that a good positive correlation between the real data and the predicted data is obtained using CCD. Thus, the model can be used to predict the CMCP extraction yield under different extraction conditions during UAEE.

3.4. Effect of parameter interactions on CMCP extraction yield

To visualize the combined effects of two operational parameters on the extraction yield, the response was generated as a function of two independent variables: triaxial response surfaces and planar contour plots, and was determined using Design Expert software. Two variables were kept constant at their respective central test range values and the other two variables varied within their experimental ranges in order to understand their main and interactive effects on the dependent variables. Figs. 3 and 4 show the results of

Table 3
Analysis of variance for the extraction of polysaccharide yield.

Source	Coefficient estimate	Sum of squares	Degree of freedom	Standard error	Mean square	*F* value	*p* value
Model	4.41	2.21	14	0.038	0.16	18.02	<0.0001**
X_1	0.042	0.042	1	0.019	0.042	4.84	0.0439*
X_2	0.10	0.26	1	0.019	0.26	29.92	<0.0001**
X_3	0.074	0.13	1	0.019	0.13	15.14	0.0014**
X_4	−0.072	0.12	1	0.019	0.12	14.04	0.0019**
X_1X_2	−0.050	0.040	1	0.023	0.040	4.57	0.0493*
X_1X_3	0.065	0.068	1	0.023	0.068	7.73	0.0140*
X_1X_4	0.055	0.049	1	0.023	0.049	5.58	0.0321*
X_2X_3	0.009688	0.001502	1	0.023	0.001502	0.17	0.6848
X_2X_4	0.006438	0.0006631	1	0.023	0.0006631	0.076	0.7871
X_3X_4	0.028	0.013	1	0.023	0.013	1.44	0.2492
X_1^2	−0.14	0.50	1	0.018	0.50	57.49	<0.0001**
X_2^2	−0.10	0.30	1	0.018	0.30	33.79	<0.0001**
X_3^2	−0.090	0.22	1	0.018	0.22	25.23	0.0002**
X_4^2	−0.19	0.99	1	0.018	0.99	112.41	<0.0001**
Residual		0.13	15		0.008767		
Lack of fit		0.11	10		0.011	2.23	0.1940
Pure error		0.024	5		0.004809		
Cor total		2.34	29				
R^2	0.9439		Adep. precission	13.616			
Adj-R^2	0.8915		C.V.%	2.34			
Pred-R^2	0.7211		*r*	0.971			

* $0.01 \le p < 0.05$.
** $p < 0.01$

interactive influence of extraction temperature, ultrasonic power, pH and liquid-to-material ratio on CMCP extraction yield. This was also used to determine the optimum conditions.

Figs. 3a–c and 4a–c show the CMCP yield as a function of extraction temperature (X_1) and the other factors (X_2, X_3, X_4). The extraction yield first increased then decreased with the increase in extraction temperature from 40 °C to 60 °C, ultrasonic power from 300 W to 500 W, pH from 3.5 to 5.5, and liquid-to-material ratio from 4:1 mL/g to 8:1 mL/g.

The variance analysis results showed that the interactions containing extraction temperature (X_1) are significant, and that response value is sensitive to changes in temperature with power, pH, and the liquid-to-material ratio, similar to a previous study (Samavati, 2013). The possible interaction mechanism between

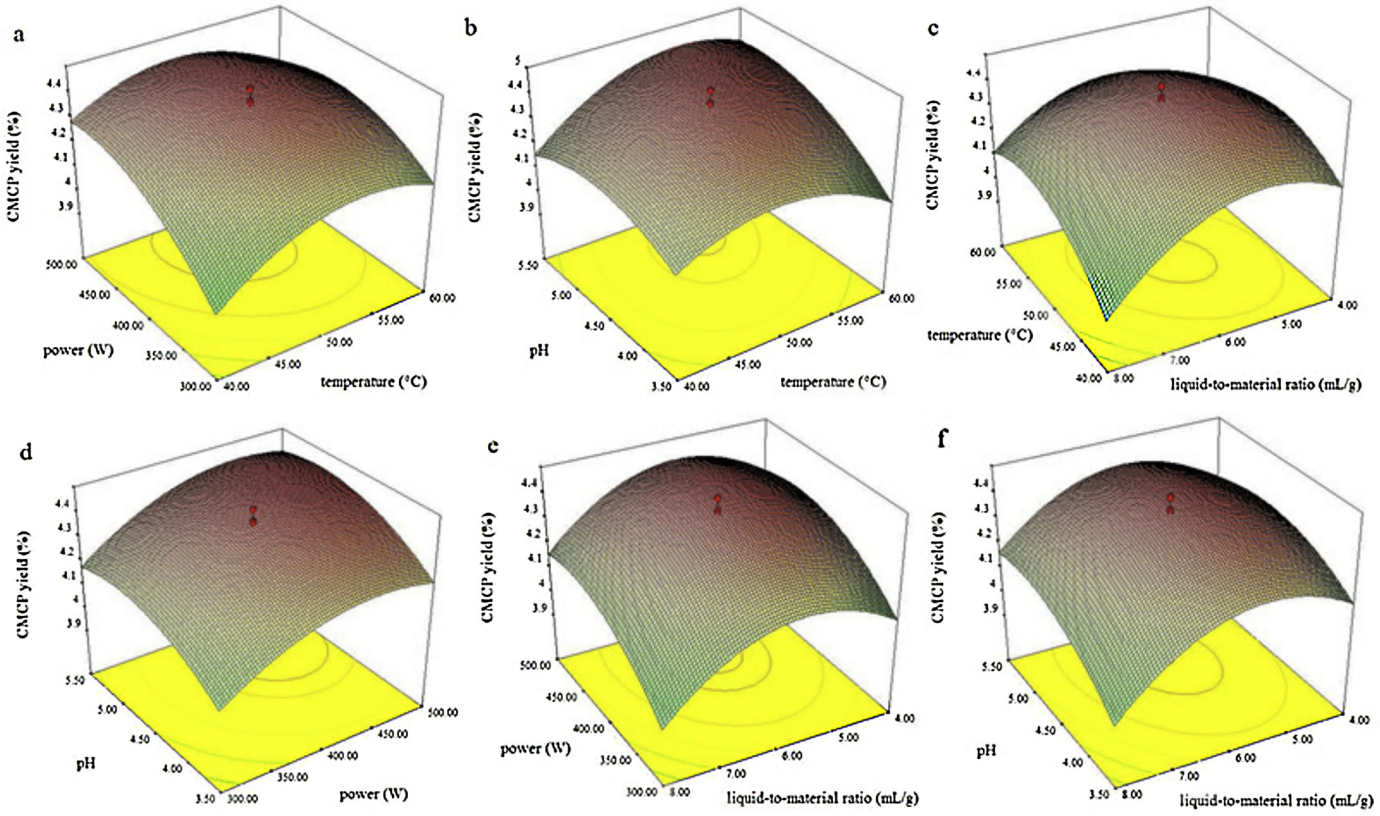

Fig. 3. Response surface showing the effects of four variables (ultrasonic power, pH, liquid-to-material ratio, temperature) on extraction yield of CMCP. CMCP: *Cucurbita moschata* crude polysaccharides.

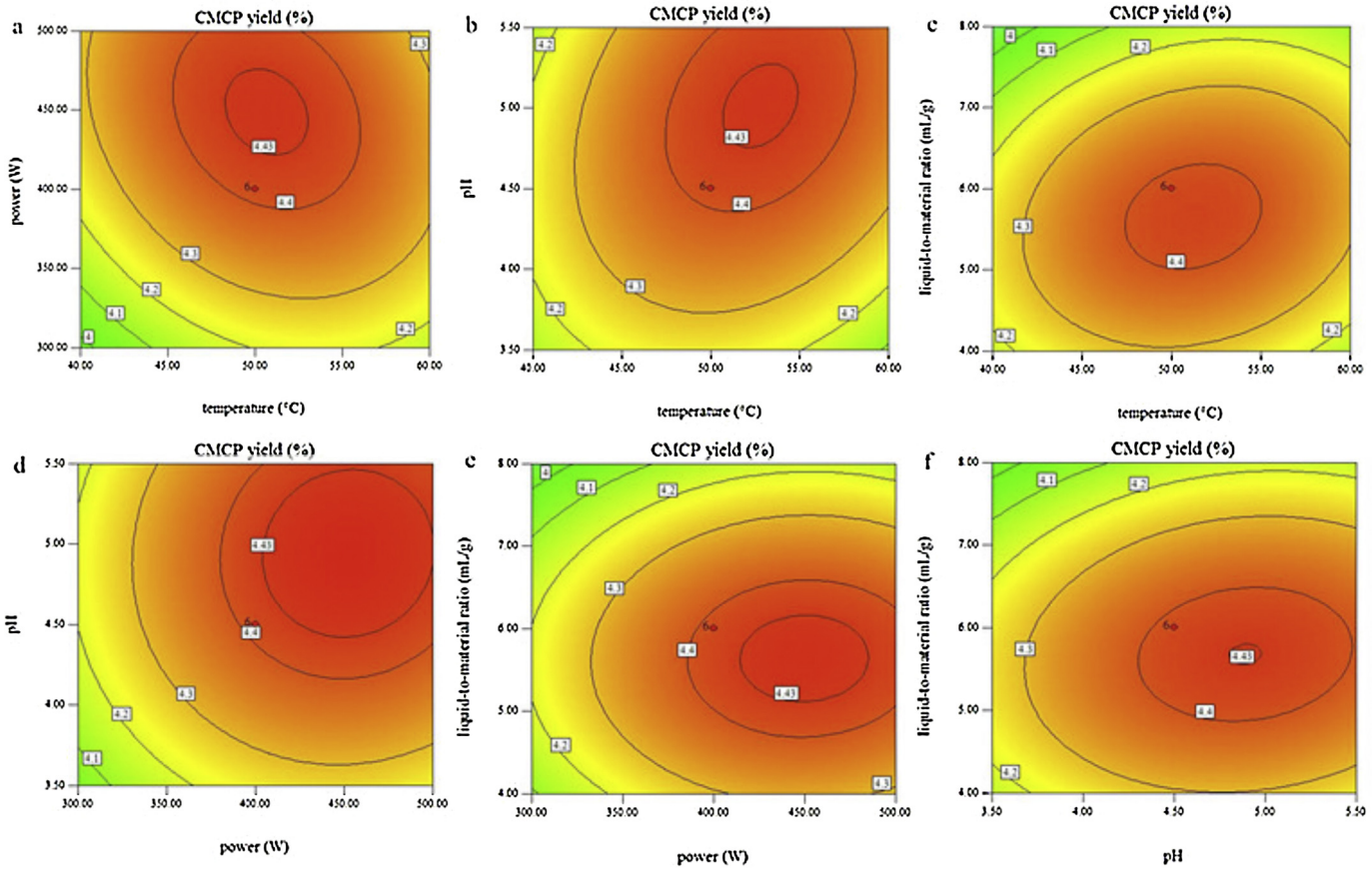

Fig. 4. Contour plots showing the effects of variables (ultrasonic power, pH, liquid-to-material ratio, temperature) on extraction yield of CMCP. CMCP: *Cucurbita moschata* crude polysaccharides.

temperature and power may be the change of cavitation threshold affected by changing temperature, which is responsible for acoustic cavitation and also results in the formation of a cavitational nucleus. The influence of relatively greater forces ruptures and erupts the formed cavitational nucleus and disrupts the cell tissues during extraction, which in turn enhances the mass transfer rate (Toma, Vinatoru, Paniwnyk, & Mason, 2001). In addition, better solvent solubility at higher temperatures may be the interaction mechanism between temperature and the liquid-to-material ratio, while surface tension and solvent viscosity decreased with increasing temperature, which will improve sample wetting and matrix penetration, respectively (Xie et al., 2010). In addition, the interactive effect of temperature and pH on extraction yield is the most significant interaction because both temperature and pH can affect the activity of complex enzymes.

As shown in Figs. 3d–f and 4d–f, the effects of ultrasonic power and pH, ultrasonic power and the liquid-to-material ratio, and pH and the liquid-to-material ratio are similar to that of temperature and power, pH, and the liquid-to-material ration. A CMCP yield of at least 4.43% was obtained from the planar contour plots in the range of extraction temperature from 47.38 °C to 55.02 °C, ultrasonic power from 403.93 W to 500 W, pH from 4.42 to 5.46, and liquid-to-material ratio from 5.01:1 mL/g to 6.30:1 mL/g.

3.5. Optimization of extraction conditions and verification of the predictive model

Parameter optimization based on an established mathematical model showed that the maximum predicted yield of CMCP is obtained according to Derringer's desired function methodology (Maran, Manikandan, Thirugnanasambandham, Nivetha, & Dinesh, 2013). The optimum extraction conditions are as follows: extraction temperature, 51.55 °C; ultrasonic power, 448.45 W; pH 4.98; and liquid-to-material ratio, 5.76:1 mL/g. Under these conditions, the predicted polysaccharide extraction yield is 4.46% with a desirability value of 0.993. The parameters were modified slightly in the verification experiment as follows: extraction temperature, 51.5 °C; ultrasonic power, 440 W; pH, 5.0; and liquid to material ratio, 5.70:1 mL/g. The experimental value of 4.39 ± 0.41% is obtained from three parallel experiments. The relative tolerance between the experimental value and predicted value is 1.57% < 5%, which confirms that the response model is adequately to reflects the expected optimization.

3.6. Kinetics of the UAEE process

CMCP extraction using distilled water is a solid-liquid extraction. Many theoretical and empirical kinetic models have been developed for solid–liquid extraction, and applied to the extraction of natural products from various sources such as food and medicinal plants (Cheung et al., 2013; Kitanović et al., 2008). After the enzyme is added to the extraction solvent, the enzyme can catalyze cell wall polysaccharide hydrolysis, while the ultrasound energy can enhance enzyme activity and accelerate the enzyme reaction rate; thus, the selected compounds can be transferred more quickly to the solvent and the extraction time can be reduced. In previous experiments, a fixed stationary extraction time of 20 min was selected for each experiment, but the mass transfer process during UAEE had not been studied. This may have caused unnecessary energy waste by prolonging the extraction time. A kinetic process

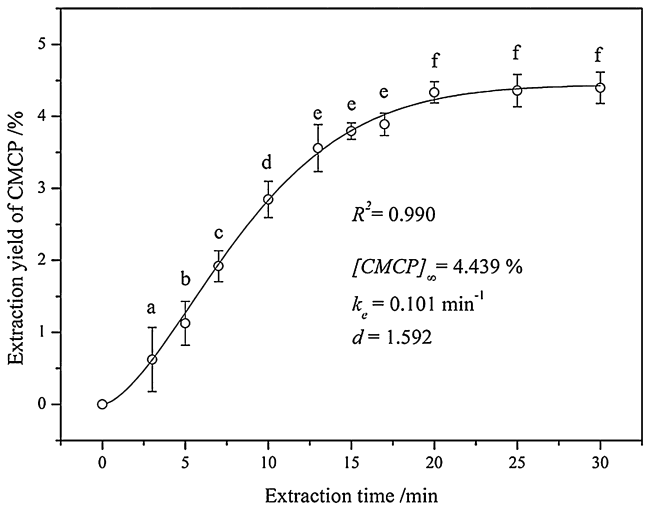

Fig. 5. Kinetics of UAEE process described by Weibull model including three parameters: stabilized extraction yield of CMCP ([CMCP]$_\infty$), extraction rate constant (k_e) and shape parameter (d). Values marked by the same letter are not significantly different ($p < 0.05$). UAEE: ultrasound-assisted enzymatic extraction; CMCP: *Cucurbita moschata* crude polysaccharides.

should be established and the extraction time should be optimized in the UAEE system. Fig. 5 shows the detailed CMCP extraction mass transfer process. An R^2 of 0.990 indicates a good fit to the model. Model parameters values are as follows: [CMCP]$_\infty$, 4.439%; k_e, 0.101 min^{-1}; and d, 1.592. The shape of simulated curve is the same as that observed from extracting various bioactive ingredients from plant materials (Kitanović et al., 2008; Perez, Carelli, & Crapiste, 2011; Zhang et al., 2012). Two extraction phases are clearly visible: (a) the rapid phase, occurring during the first 13 min; in this stage, a CMCP of 80.2% is rapidly transferred into solvent with an almost constant speed; and (b) the slow phase: further extraction progress is slow, and continues for about 17 min. A relatively slower diffusion occurred in this phase, which is most likely a result of exhaustion of the limited CMCP contained in the matrix. Thus, we conclude that the optimal extraction time is 20 min for CMCP, and this approximates the stabilized CMCP yield ($p < 0.05$). The maximum polysaccharide extraction yield in pumpkin is 4.33 ± 0.15% under the following optimized conditions: liquid-to-material ratio, 5.70:1 mL/g; ultrasonic power, 440 W; pH, 5.0; extraction temperature, 51.5 °C; and extraction time, 20 min.

3.7. CMCP antioxidant activity

3.7.1. DPPH scavenging activity

The DPPH radical is one of the few stable radical sources, and it is extensively used to determine antioxidant electron-donating and free radical-scavenging ability. DPPH is widely accepted as a tool for evaluating the free radical scavenging activities of natural compounds (Li, Huang, Lu, & Hou, 2011). DPPH shows a maximum absorption at 517 nm in ethanol. When DPPH encounters a proton-donating substance (antioxidants), the DPPH radical will

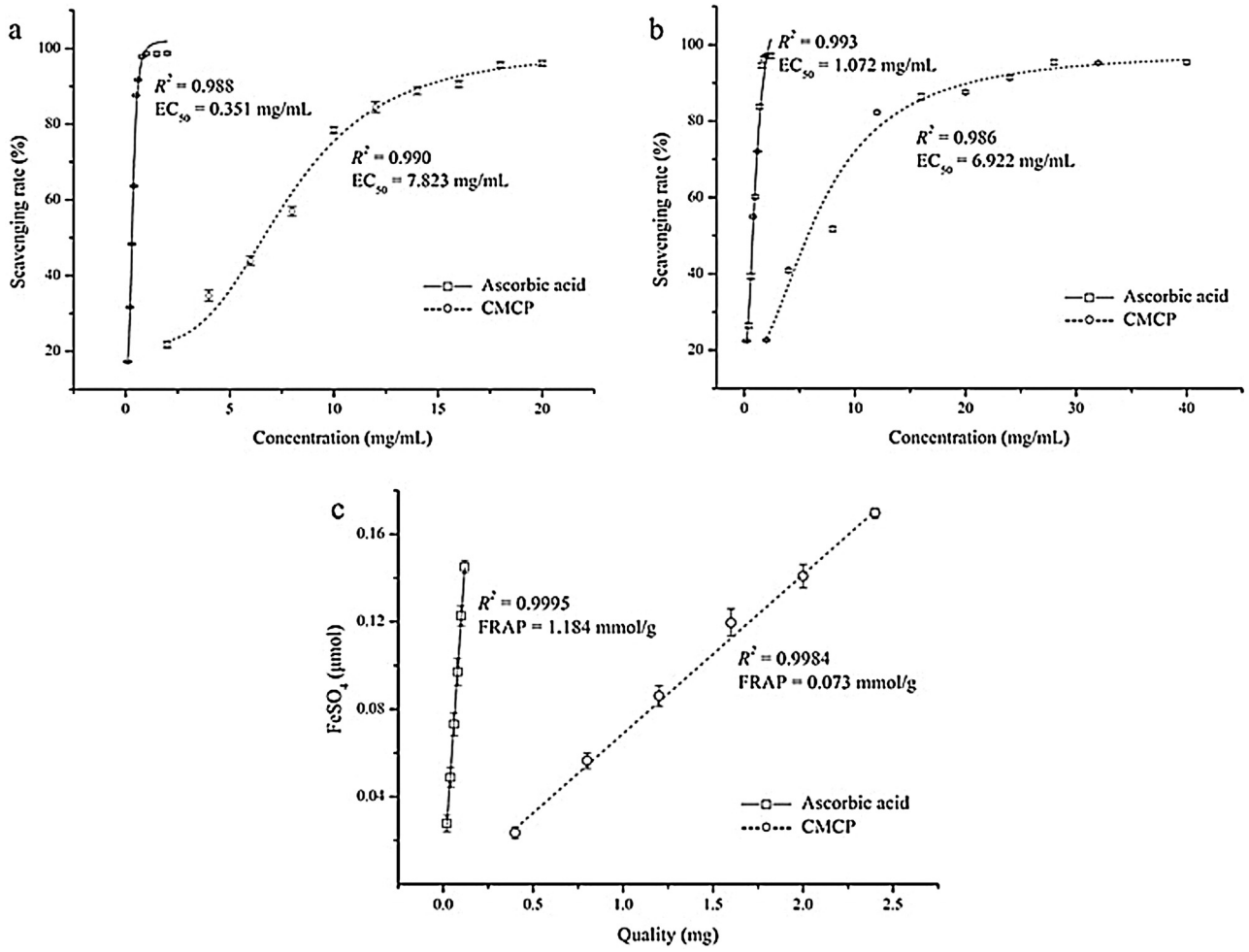

Fig. 6. Antioxidant activities of CMCP determined by DPPH• scavenging assay (a), hydroxyl radical scavenging assay (b) and FRAP assay (c). DPPH: 1,1-diphenyl-2-picrylhydrazyl; FRAP: ferric reducing antioxidant power; CMCP: *Cucurbita moschata* crude polysaccharides.

be scavenged and the absorbance at 517 nm is reduced with a noticeable color change from purple to yellow (Xu et al., 2009), which allows the DPPH radical scavenging effect to be measured. As shown in Fig. 6a, CMCP exhibits a concentration-dependent antiradical activity by inhibiting DPPH free radicals within the relatively low concentration range of 2–10 mg/mL. However, increased CMCP scavenging activity does not significantly increase within a relatively high concentration range of 10–20 mg/mL. The EC_{50} of ascorbic acid and CMCP is 0.351 mg/mL and 7.823 mg/mL respectively, which indicates that the DPPH scavenging activity of ascorbic acid is much higher than that of CMCP. The DPPH radical scavenging mechanism may occur through CMCP donating electrons or hydrogen atoms to DPPH. The differences of polysaccharides in molecular weight, monosaccharide composition, and conformation affect their hydrogen-donating abilities (Chen et al., 2011). In the present study, CMCP showed excellent DPPH radical scavenging ability compared with polysaccharides extracted from *Radix hedysari* (Wei, Cheng, Wei, & Zhang, 2012), which may result from its strong hydrogen-donating ability.

3.7.2. Hydroxyl radical scavenging activity

The hydroxyl radical is the most active free radical of the reactive oxygen species, and it attacks all biological molecules by setting off free radical chain reactions (Samavati & Manoochehrizade, 2013). Thus, removing hydroxyl radicals is important for protecting living systems. As shown in Fig. 6b, CMCP are capable of scavenging hydroxyl radicals in a dose-dependent manner within a relatively low concentration range of 2–12 mg/mL. However, increased CMCP scavenging activity does not significantly increase within a relatively high concentration range of 12–40 mg/mL. The EC_{50} of ascorbic acid and CMCP is 1.072 mg/mL and 6.922 mg/mL, respectively. These results indicate that the hydroxyl radicals scavenging activity of ascorbic acid is higher than that of CMCP. The possible antioxidant mechanism is that CMCP can combine with the radical ions, which are essential for the radical chain reaction, and the reaction is terminated (Shen et al., 2014). Different polysaccharide structures, which are affected by monosaccharide composition and molecular weight, have different chelating abilities. CMCP hydroxyl radical scavenging activity is relatively among reported cases (Chen et al., 2012; Samavati & Manoochehrizade, 2013; Wang et al., 2013).

3.7.3. Ferric reducing activity

The FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of TPTZ, and serves as an indicator of natural products' potential antioxidant activity. As shown in Fig. 6c, CMCP and ascorbic acid ferric ion reducing activities increase steadily as the concentration increases and a linear relationship between amount of $FeSO_4$ and sample quality will be displayed within a certain range. The CMCP and ascorbic acid FRAP values are 0.073 and 1.184 mmol $FeSO_4$/g, respectively. The results are similar to those of Košťálová et al. (2013), who indicate that the total antioxidant ability of crude polysaccharides DP1 from Styrian oil-pumpkin was 78.7 μmol $FeSO_4$/g. Compared with *Pteridium aquilinum* polysaccharide (Xu et al., 2009) and *Ampelopsis grossedentata* polysaccharide (Wang et al., 2011), CMCP has a higher antioxidant activity, and also has good potential as a natural antioxidant in the food or medicine industry.

4. Conclusion

This is the first report on the feasibility of UAEE used for CMCP extraction. The synergistic model of simultaneous enzymolysis and ultrasonication was selected in the UAEE process. Operating parameters were optimized using response surface design (CCD) and a kinetic study of mass transfer. The optimal experimental extraction yield of 4.33 ± 0.15% was obtained when the extraction temperature was 51.5 °C, ultrasonic power was 440 W, liquid-to-material ratio was 5.70:1 mL/g, pH was 5.0, and extraction time was 20 min. CMCP has a potential for application as a natural antioxidant because it shows a high reducing power and positive radical scavenging activity for DPPH radicals. The present study suggests an efficient and environmentally friendly extraction procedure that may be preferred by both consumers and producers, which could be used as the reference for the polysaccharide production from pumpkin (*C. moschata*).

Acknowledgments

Authors gratefully acknowledge the contribution of the Modern Vegetable Industry Technology System of Shandong Province (SDAIT-02-022-12), a project of Shandong province Higher Educational Science and Technology Program (J14LE11), Qingdao Applied Basic Research Project (14-2-4-71-jch), and Talents of Highlevel Scientific Research Foundation of Qingdao Agriculture University (631207). And authors are indebted to postgraduate in fruit and vegetable processing laboratory for providing help.

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