DOI: http://dx.doi.org/ 10.1590/1678-457X.13516

Optimization of ultrasound-assisted extraction of phenolic compounds, antioxidants and rosmarinic acid from perilla leaves using response surface methodology

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Abstract

Response surface methodology (RSM) was used to optimize ultrasound-assisted extraction (UAE) of functional components from perilla leaves. The factors investigated were ethanol concentration, extraction temperature, and extraction time. The results revealed that ethanol concentration had significant effects on all extraction parameters. Based on the RSM results, the optimal conditions were an ethanol concentration of 56%, a UAE temperature of 54 °C, and a UAE time of 55 min. Under these conditions, the experimental TPC (total phenolic content), RA (rosmarinic acid), FRAP (ferric reducing antioxidant power) and DPPH (1,1-diphenyl-2-picrylhydrazyl) values were 48.85 mg GAE/g DW (mg gallic acid equivalent /g of dry weight), 31.02 mg/g DW, 85.55 μmol Fe^{2+}/g DW and 73.35%, respectively. The experimental values were in agreement with those predicted by RSM models, confirming suitability of the model employed and the success of RSM for optimization of the extraction conditions.

Keywords: perilla leaves; ultrasound-assisted extraction; phenolic compounds; antioxidants; rosmarinic acid.

Practical Application: The study provides valuable information that ultrasound-assisted extraction is an environmentally-friendly, green process for the preparation of extracts from perilla leaves, and will help in the development of functional food resources.

1 Introduction

Perilla (*Perilla frutescens*), an aromatic vegetable, has been cultivated worldwide and used extensively for cooking and medicinal purposes in several Asian countries (Igarashi & Miyazaki, 2013; Li et al., 2015). The leaves of *P. frutescens* have been shown to have detoxicant, antitussive, antipyretic and antibiotic properties (Liu et al., 2013; Nakamura et al., 1998). Several studies have reported that extracts of perilla leaves have high antioxidant capacity, mostly due to the presence of polyphenolic compounds (Lee et al., 2013; Zhou et al., 2014). Phenolic compounds are well-known for their beneficial health properties, attributed to their antioxidant and antiradical activities (Tao et al., 2014; Krishnaswamy et al., 2013; Heim et al., 2002). Moreover, rosmarinic acid (RA) has been shown to be the main biologically active polyphenolic compound in perilla leaves (Liu et al., 2013). RA is a well-known hydroxycinnamic acid ester that has interesting biological properties beneficial to human health, including antioxidant, anti-inflammatory, anticancer, and anti-allergenic activities (Lamien-Meda et al., 2010; Tang et al., 2014), and preventing food spoilage (Petersen & Simmonds, 2003; Pérez-Tortosa et al., 2012). Recently, interest has increased considerably in naturally occurring antioxidants for use in foods or medicinal materials as replacements for synthetic antioxidants such as BHA (beta hydroxy acid) and BHT (butylated hydroxy toluene), whose use is restricted due to safety concerns (Ito et al., 2005; Zheng & Wang, 2001). An efficient extraction technique is therefore required to harvest the benefits of natural antioxidant phenolic compounds, including RA, present in perilla leaves.

Several methods are available for extraction of phenolic compounds from plants, including solvent extraction, ultrasound-assisted extraction, microwave-assisted extraction, and supercritical fluid extraction (Glisic et al., 2011; Hossain et al., 2012; Caxambu et al., 2016). Of these methods, ultrasound-assisted extraction (UAE) technology has gained increasing popularity due to its advantageous properties, including high extraction efficiency, good reproducibility, low solvent consumption, speed, low cost, environmental friendliness and easy scale up for industrial applications (Tao et al., 2014; Cravotto et al., 2008; Vinatoru, 2001; Vuong et al., 2014). The enhancement of extraction using ultrasound is mainly attributed to acoustic cavitations produced in the solvent (Ma et al., 2008; Velickovic et al., 2008), and ultrasound technology has been used to improve different food processes (Kiani & Sun, 2011; Kiani et al., 2012) in addition to extraction. However, the feasibility of using ultrasound for extraction of phenolic compounds from perilla leaves has not yet been explored.

Response surface methodology (RSM), which was first introduced by Box & Wilson (1951), is a collection of statistical

Received 23 May, 2016
Accepted 23 Sept., 2016
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and mathematical techniques that has been successfully used for developing, improving and optimizing processes (Liyana-Pathirana & Shahidi, 2005; Tabaraki & Nateghi, 2011). RSM can be used to evaluate the effects of multiple factors and their interactions with one or more response variables (Xu et al., 2013; Azmir et al., 2014). Different RSM methods such as Box-Behnken design (BBD), central composite design (CCD) and three-level full factorial design (TFFD) have been widely used. CCD has been shown to be a powerful design for RSM that is less time-consuming and more efficient than many other designs (Aybastier et al., 2013). It has been successfully used in the optimization of extraction of polyphenols from natural sources (Chen et al., 2015). It is therefore appropriate to study the use of CCD to optimize extraction of phenolic compounds from perilla leaves.

In the present study, ethanol concentration, extraction temperature and extraction time were optimized by RSM, employing a central composite design to maximize the extraction of phenolic compounds, antioxidant activities and RA from perilla leaves.

2 Materials and methods

2.1 Plant materials400W

P. frutescens leaves were collected in October 2014 from North University of China (Taiyuan, Shanxi Province, China). The leaves were air-dried at ambient temperature (~25 °C), milled with muti-function miller (CS-2000, Wuyi Haina Electric Company, Wuyi, Zhejiang, China), passed through a stainless steel sieve to approximately 250 μm and stored in closed desiccators at 4 °C until use.

2.2 Ultrasound-assisted extraction

Extractions were carried out in a tunable ultrasonic bath (TH-400BQG, Tianhua Ultrasonic Electronic Equipment Co., Jining, Shandong, China, 50 kHz, 400 W) using 1 g of the dried perilla powder and 20 mL of ethanol solution (ethanol was acidified with 0.1% aqueous HCl) for 3 times. The combined extracts were filtered and centrifuged at 12,000 *g* for 15 min. Solvent was removed on a rotary vacuum evaporator (SHZ-95B, Yuhua Ltd., Gongyi, Henan, China) at 40 °C. The residue was transferred into a glass vial and stored at −20 °C prior to analysis.

2.3 RSM design and statistical analysis

A CCD method with five levels and three variables was used to determine the optimal conditions for the UAE. Ethanol concentration (%, X_1), UAE temperature (°C, X_2), and UAE time (min, X_3) were taken as independent variables tested in a 20-run experiment. The independent variables were coded at five levels (−2, −1, 0, 1 and 2). Total phenolic content, RA content and antioxidant activity were selected as the responses of the design experiments (Y). A randomized experimental order was used to reduce the effect of unexplained variability on the observed response. Table 1 shows the run order, variable conditions, and the experimental and predicted values. A second degree polynomial equation derived from RSM was used as follows (Equation 1):

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \quad (1)$$

where Y is the response; b_0, b_1, b_2, b_3, b_{11}, b_{22}, … are the regression coefficients for intercept, linear, quadratic and interaction terms; X_1, X_2 and X_3 are the non-coded values for ethanol concentration, temperature and time, respectively.

Table 1. Central composite design of three variables with their observed responses using UAE.

Run	Ethanol concentration X_1 (%)	UAE temperature X_2 (°C)	UAE time X_3 (min)	TPC (mg GAE/g DW)	RA (mg/g DW)	FRAP (μmol Fe^{2+}/g DW)	DPPH (%)
1	30 (-1)	60 (1)	60 (1)	46.09	25.81	65.37	56.68
2	90 (2)	50 (0)	45 (0)	35.99	23.18	66.78	55.05
3	50 (0)	50 (0)	15 (-2)	47.00	29.34	67.61	68.40
4	30 (-1)	60 (1)	30 (-1)	43.76	21.84	62.65	59.61
5	30 (-1)	40 (-1)	60 (1)	43.21	21.55	50.03	41.21
6	50 (0)	70 (2)	45 (0)	45.80	31.14	84.61	70.22
7	50 (0)	30 (-2)	45 (0)	43.06	28.31	62.65	65.15
8	10 (-2)	50 (0)	45 (0)	32.65	16.55	35.63	28.18
9	50 (0)	50 (0)	45 (0)	49.06	32.23	85.31	70.62
10	70 (1)	60 (1)	60 (1)	45.35	31.74	76.46	71.50
11	50 (0)	50 (0)	45 (0)	47.59	31.89	80.08	76.06
12	70 (1)	40 (-1)	60 (1)	47.59	32.35	78.94	66.78
13	70 (1)	40 (-1)	30 (-1)	46.50	28.55	73.04	70.55
14	30 (-1)	40 (-1)	30 (-1)	40.85	18.14	38.63	30.94
15	50 (0)	50 (0)	45 (0)	49.13	29.20	83.22	71.76
16	50 (0)	50 (0)	45 (0)	47.44	31.98	86.10	76.02
17	50 (0)	50 (0)	75 (2)	46.77	30.28	84.73	76.38
18	50 (0)	50 (0)	45 (0)	47.34	28.19	81.97	68.44
19	50 (0)	50 (0)	45 (0)	49.21	28.12	79.00	67.09
20	70 (1)	60 (1)	30 (-1)	45.53	30.98	76.93	70.52

Data were analyzed by analysis of variance (ANOVA) to determine the lack of fit and the effects of linear, quadratic, and interaction variables on total phenolic content, RA content and antioxidant activity. Data analysis and RSM were performed with Design Expert software (Version 8; Stat-Ease, Inc., Minneapolis, MN, USA).

2.4 Analysis of total phenolic content

Total phenolic content (TPC) was determined by the Folin-Ciocalteu method (Dewanto et al., 2002). Extract (1.0 mL) was mixed with Folin-Ciocalteu reagent (0.5 mL), 7% (w/v) sodium carbonate solution (1.5 mL) and distilled water (7 mL). After 1 h at room temperature, absorbance was measured at 760 nm using a UV-visible spectrophotometer (UV9600, Bobang Co., Zhengzhou, China). Total phenolic content was expressed as mg of gallic acid equivalent (GAE) per g dry weight (DW) of perilla leaves.

2.5 Rosmarinic acid content

The rosmarinic acid (RA) content was measured using the ferrous sulfate method (Lopez-Amotdos et al., 1995). Extract (0.2 mL) was mixed with 0.1 M sodium acetate buffer (pH 6.0, 4.0 mL), 0.2 M ferrous sulfate solution (30 µL) and distilled water (0.77 mL). The solution was mixed thoroughly, allowed to stand for 5 min at room temperature, and then the absorbance was measured at 568 nm using a UV-visible spectrophotometer (UV9600, Bobang Co., China). RA content was expressed as mg per g DW of perilla leaves.

2.6 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to the method described by Benzie & Strain (1996). The FRAP reagent was freshly prepared by combining 300 mM sodium acetate buffer (pH 3.6), 10 mM TPTZ (tripyridyltriazine) in 40 mM HCl, and 20 mM ferric chloride solution in proportions of 10:1:1 (v/v), respectively. The sample (200 µL) was mixed with FRAP reagent (2.8 mL), warmed at 37 °C for 30 min, and then the absorbance at 593 nm was measured. The standard curve was constructed using $FeSO_4$ solution. The reducing power was expressed as µmol Fe (II) per g DW of perilla leaves.

2.7 DPPH radical-scavenging assay

DPPH radical-scavenging activity was determined according to the method reported by Choi et al. (2000) with some modification. Briefly, 0.1 mL of sample was mixed with a fresh 60 µM methanol solution of DPPH (2.9 mL). The mixture was shaken vigorously and incubated for 30 min at room temperature in the dark. The absorbance was measured at 517 nm using a UV-visible spectrophotometer (UV9600, Bobang Co., China). Results were expressed as percentage inhibition of the DPPH radical. Percentage inhibition of the DPPH radical was calculated as follows (Equation 2):

$$DPPH\ scavenging\ activity\ (\%) = \left[\left(OD_{control} - OD_{sample} \right) / OD_{control} \right] \times 100 \quad (2)$$

3 Results and discussion

3.1 Fitting the model

The coded and decoded values and the responses of each independent variable are listed in Table 1. Total phenolic content in perilla leaves extracts varied from 32.65-49.21 mg GAE/g DW. RA content ranged from 16.55-32.35 mg/g DW. FRAP and DPPH radical scavenging assays were used to determine the antioxidant activity of the extracts. As shown in Table 1, activity values varied from 35.63-86.10 µmol Fe^{2+}/g DW, and 28.18%-76.38% in the FRAP and DPPH assays, respectively. The relationships between independent variables and the responses are presented in Equations 3-6.

$$Y_1 = 48.55 + 1.11X_1 + 0.50X_2 + 0.32X_3 - 1.13X_1X_2 - 0.47X_1X_3 - 0.16X_2X_3 - 3.37X_1^2 - 0.84X_2^2 - 0.23X_3^2 \quad (3)$$

$$Y_2 = 30.78 + 3.10X_1 + 0.97X_2 + 0.86X_3 - 0.77X_1X_2 - 0.35X_1X_3 - 0.31X_2X_3 - 2.69X_1^2 - 0.23X_2^2 - 0.21X_3^2 \quad (4)$$

$$Y_3 = 81.59 + 9.44X_1 + 5.30X_2 + 3.36X_3 - 4.74X_1X_2 - 1.09X_1X_3 - 1.89X_2X_3 - 8.36X_1^2 - 2.76X_2^2 - 2.12X_3^2 \quad (5)$$

$$Y_4 = 70.78 + 9.04X_1 + 3.69X_2 + 1.28X_3 - 4.93X_1X_2 - 1.27X_1X_3 - 1.06X_2X_3 - 7.95X_1^2 - 1.44X_2^2 - 0.26X_3^2 \quad (6)$$

where Y_1: total phenolic content (mg GAE/g DW), Y_2: RA (mg/g DW), Y_3: FRAP (µmol Fe^{2+}/g DW), Y_4: DPPH (%), X_1: ethanol concentration (%), X_2: UAE temperature (°C) and X_3: UAE time (min).

In this study, a good model fit was obtained for TPC, RA, FRAP and DPPH, with R^2 values in Equations 3-6 of: 0.9517, 0.8496, 0.9448 and 0.8946, respectively, indicating good representation of the variability of the parameters by the models. According to Le Man et al. (2010), a model is adequate when $R^2 > 0.75$. In addition, the ANOVA showed that the quadratic polynomial model was highly significant with *P*-values ranging from 0.0041 to < 0.0001 (Table 2). The lack of fit statistics for all parameters, which measure the fitness of the model, were not significant ($P > 0.05$) and high *F*-values (6.2786–21.9148) further confirmed the reliability of the models within the studied range of process conditions.

3.2 Response surface analysis of TPC

For TPC, in addition to the linear and quadratic effects of ethanol concentration, the UAE temperature quadratic effects and the interaction of ethanol concentration and temperature were significant. Figure 1A shows the effect of ethanol concentration and UAE temperature on TPC at constant time (45 min). An increase in TPC was observed as the UAE temperature was increased to 51.83 °C, but decreased thereafter. Significant increases in TPC were observed at higher ethanol concentrations, but the trend was reversed as the ethanol concentration reached 51.94%. In general, the polarity of ethanol-water mixtures increases continuously as more water is added. A larger proportion of polar phenolic compounds may be extracted according to the "like dissolves like" principle (Tabaraki & Nateghi, 2011). However, the effect of UAE time on TPC was not significant

Table 2. Analysis of variance (ANOVA) for the quadratic polynomial mode.

Source	*P*-Value			
	TPC	RA	FRAP	DPPH
Model	< 0.0001[d]	0.0041[b]	< 0.0001[d]	0.0008[c]
X_1	0.0065[b]	0.0007[c]	< 0.0001[d]	0.0002[c]
X_2	0.1506	0.1650	0.0014[b]	0.0425[a]
X_3	0.3442	0.2096	0.0202[a]	0.4377
$X_1 X_2$	0.0338[a]	0.4192	0.0204[a]	0.0525
$X_1 X_3$	0.3261	0.7069	0.5395	0.5848
$X_2 X_3$	0.7299	0.7407	0.2983	0.6477
X_1^2	< 0.0001[d]	0.0004[c]	< 0.0001[d]	< 0.0001[d]
X_2^2	0.0086[b]	0.6626	0.0175[a]	0.2824
X_3^2	0.3993	0.6917	0.0536	0.8407
Lack of fit	0.1314	0.1785	0.0515	0.0576
CV	2.8765	9.3466	6.8589	10.0587
R^2	0.9517	0.8496	0.9448	0.8946
Adj.R^2	0.9083	0.7143	0.8951	0.7997
F-value	21.9148	6.2786	19.0158	9.4276

[a] Significant at $P \leq 0.05$. [b] Significant at $P \leq 0.01$. [c] Significant at $P \leq 0.001$. [d] Significant at $P \leq 0.0001$.

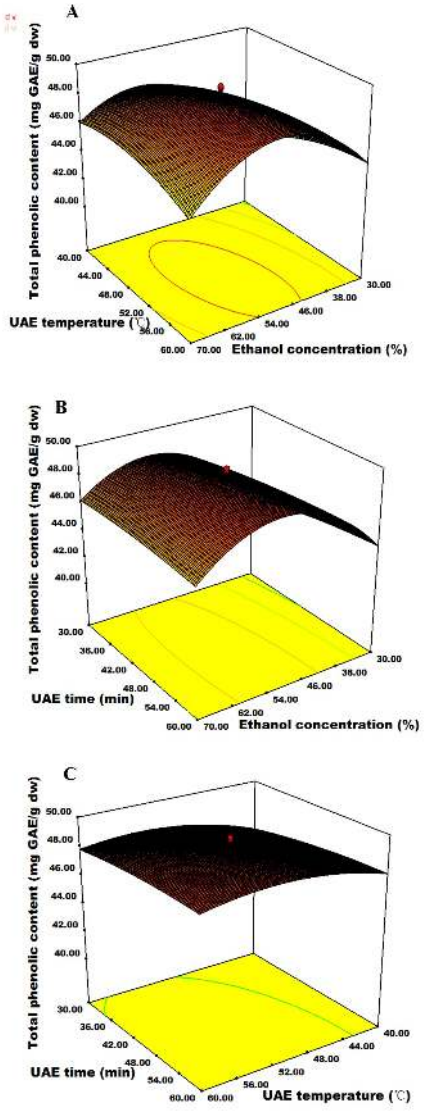

Figure 1. Response surface plots for the effect of (A) temperature and ethanol concentration (B) time and ethanol concentration and (C) time and temperature on the total phenolic content (TPC).

($P > 0.05$). TPC slowly increased with increasing extraction time until reaching a plateau at > 55.08 min (Figures 1B, 1C), indicating that prolonged sonication did not result in further improvements in extraction efficiency. Similar result was also obtained in perilla oil extraction (Li et al., 2015). Based on the linear and quadratic coefficients (Table 2), we concluded that the order of factors affecting TPC was ethanol concentration > UAE temperature > UAE time. Ultrasound-assisted extraction of phenolic compounds from other plants, such as rice bran, misai kucing, sugar beet molasses and sugarcane rinds, had been reported recently (Tabaraki & Nateghi, 2011; Ho et al., 2014; Chen et al., 2015; Feng et al., 2015).

3.3 Response surface analysis of RA content

Figure 2 shows the relationship between RA content and the three variables. The linear and quadratic effects of ethanol concentration were significant ($P < 0.001$), indicating that increasing the ethanol concentration favors extraction of RA only up to a certain value (58.31%). At higher ethanol concentrations, the RA content decreased. This behavior is clearly observed in Figures 2A and 2B. The effects of UAE temperature and UAE time on RA were not significant ($P > 0.05$), but had positive effects. RA content slowly increased with temperature and time, reaching a maximal value at 54.97 °C and 65.12 min (Figure 2C). It is likely that a higher extraction temperature improves target compound solubility, solvent diffusion rate and mass transfer (Hossain et al., 2011). Increased extraction time allows the solutes to be in contact with solvent for longer, facilitating higher diffusion of the target compounds (Ghafoor et al., 2009). Of the conditions generated by RSM, the highest RA content (31.54 mg/g) was observed at an ethanol concentration of 58.31%, UAE temperature of 54.97 °C and UAE time of 65.12 min.

3.4 Response surface analysis of antioxidant activities

All three factors had significant effects on the FRAP values of perilla leaves extracts. Ethanol concentration had the greatest effect, followed by temperature and time (Figure 3 and Table 2).

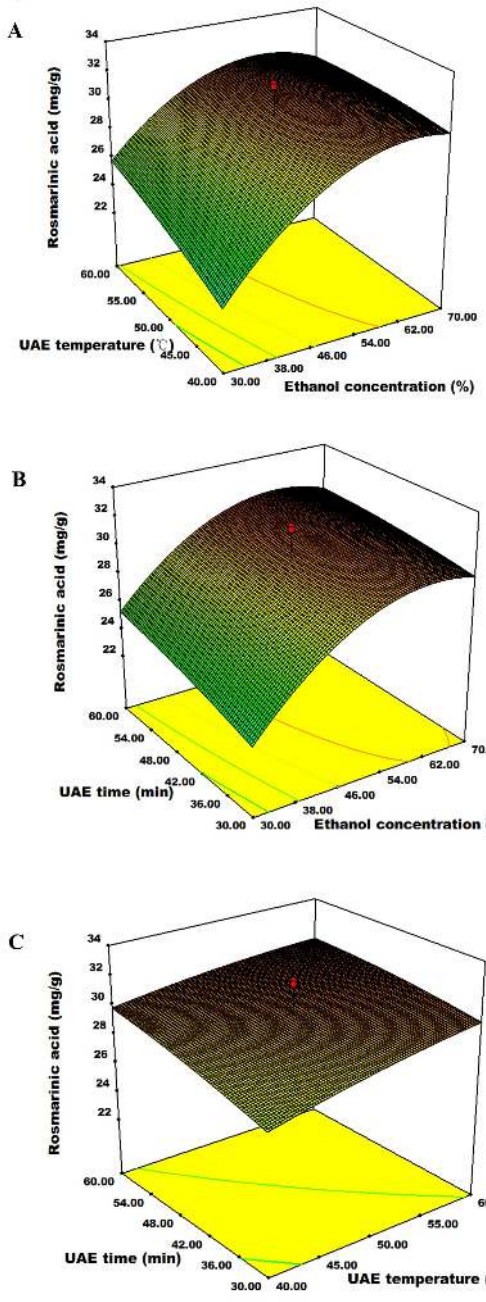

Figure 2. Response surface plots for the effect of (A) temperature and ethanol concentration (B) time and ethanol concentration and (C) time and temperature on the rosmarinic acid content (RA).

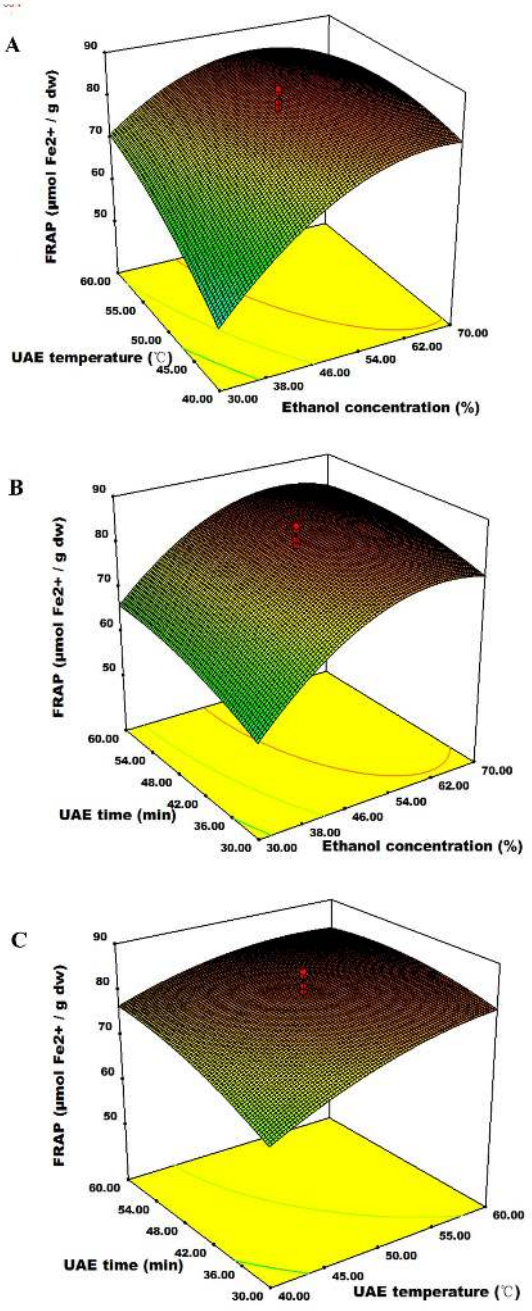

Figure 3. Response surface plots for the effect of (A) temperature and ethanol concentration (B) time and ethanol concentration and (C) time and temperature on the antioxidant activity assay (FRAP).

The interaction and quadratic effects of ethanol concentration and temperature were also significant. Figure 3A shows that FRAP values significantly increased with increasing ethanol concentration and UAE temperature, reaching a maximum at 58.12% and 54.44 °C. Significant increases in FRAP values were also observed as UAE time was increased to 52.31 min (Figures 3B, 3C). Under these optimal conditions, the highest FRAP value of 85.50 µmol Fe^{2+}/g DW was obtained (Table 3). These conditions increased the antioxidant activity as measured by FRAP by 86.80% compared with conventional solvent extraction (45.77 µmol Fe^{2+}/g DW).

The linear effects of ethanol concentration and UAE temperature on DPPH value were significant. Ethanol concentration also had a quadratic effect, indicating that ethanol concentration played the dominant role in antioxidant activity as measured by DPPH (Table 2). Figure 4A shows that DPPH values significantly increased as ethanol concentration and UAE temperature increased, reaching a maximum at 59.28% and 49.88 °C. As shown in the response surface plots for the effect of time on DPPH values (Figures 4B and 4C), at constant ethanol concentration and constant temperature, a longer extraction time had positive effects. Also, a high correlation between TPC, RA and antioxidant

Table 3. Predicted and experimental values of responses tested at optimal UAE conditions.

Response variables	Optimum UAE conditions			Optimum Value	
	Ethanol concentration (%)	Temperature (°C)	Time (min)	Predicted	Experimental[a]
TPC (mg GAE/g DW)	51.94	51.83	53.08	48.73	48.26
RA (mg/g DW)	58.31	54.97	65.12	31.54	31.75
FRAP (μmol Fe^{2+} / g DW)	58.12	54.44	52.31	85.50	85.94
DPPH (%)	59.28	49.88	65.28	73.72	73.46
Combination of TPC, RA, FRAP and DPPH	56.44	53.68	55.46	48.46	48.85
				31.37	31.02
				85.35	85.55
				73.66	73.35

[a] Mean of six determinations (n=6) from two replications.

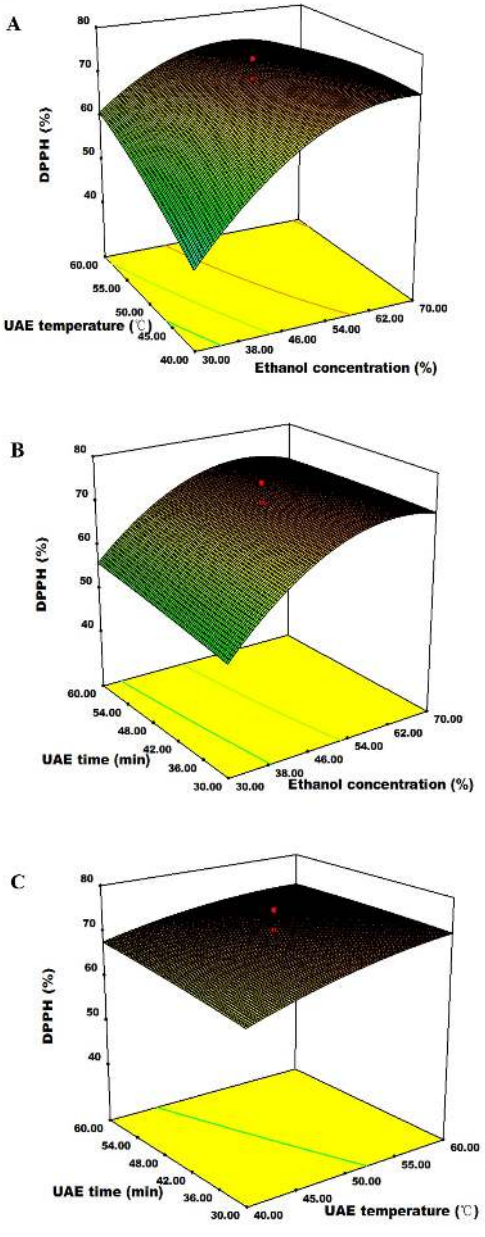

Figure 4. Response surface plots for the effect of (A) temperature and ethanol concentration (B) time and ethanol concentration and (C) time and temperature on the antiradical activity (DPPH).

activity of the extracts in the FRAP and DPPH assays were found. The Pearson's correlation coefficients (r) of TPC with FRAP and DPPH were both 0.780, and the coefficients (r) of RA with FRAP and DPPH were 0.913 and 0.933, respectively. These data suggest that RA, the main biologically active polyphenolic compound, might contribute significantly to the antioxidant capacity of perilla leaves extracts. The results are in agreement with the report by Liu et al. (2013).

3.5 Optimization and model validation

According to the second-order polynomial equation, the optimal conditions for all parameters combined were an ethanol concentration of 56.44%, UAE temperature of 53.68 °C, and UAE time of 55.46 min. Under these conditions, the predicted TPC, RA, FRAP and DPPH values were 48.46 mg GAE/g DW, 31.37 mg/g DW, 85.35 μmol Fe^{2+}/g DW and 73.66%, respectively. For convenience, these conditions were slightly modified to an ethanol concentration of 56%, a temperature of 54 °C, and an extraction time of 55 min. The experimental results were consistent with the predicted values and were found to be not significantly different at $P > 0.05$ using a paired t-test (Table 3). Therefore, the extraction conditions obtained by RSM may be considered accurate and reliable (Xu et al., 2013; Li et al., 2015).

4 Conclusion

In the present study, the effect of ethanol concentration, extraction temperature and time on ultrasound assisted extraction of polyphenols and antioxidants from perilla leaves were successfully evaluated by response surface methodology using a CCD method. The experimental values were in agreement with the predicted values. The optimal UAE conditions were an ethanol concentration of 56%, UAE temperature of 54 °C, and UAE time of 55 min. Under these conditions, the maximum TPC, RA, FRAP and DPPH values were 48.85 mg GAE/g DW, 31.02 mg/g DW, 85.55 μmol Fe^{2+}/g DW and 73.35%, respectively. The TPC, RA and antioxidant activities of the extracts from perilla leaves using UAE were strongly correlated. The study indicates that ultrasound-assisted extraction offers many advantages, including reduced solvent usage, lower temperature and shorter extraction time. Therefore, ultrasound-assisted extraction is an environmentally-friendly, green process for the preparation of extracts from perilla leaves that are rich in natural antioxidants that could replace synthetic antioxidants

(Chen et al., 2015; Tabaraki & Nateghi, 2011). There is clear potential for the utilization of polyphenols from perilla leaves in the pharmaceutical and food industries.

Acknowledgements

This work was supported by the Science and Technology Research Program of Shanxi Province in China (20150311009-4; 201603D321031).

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