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RESEARCH ARTICLE

Optimization of Microwave-assisted Extraction of Bioactive Alkaloid Compounds from Rhizoma Coptidis (*Coptis chinensis* Franch.)

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Abstract Microwave-assisted extraction (MAE) technique was employed and optimized for microwave power, irradiation time and solvent (ethanol) concentration using a central composite design and response surface methodology for the efficient extraction of bioactive alkaloid compounds from Rhizoma coptidis (*Coptis chinensis* Franch.). Alkaloid compounds were successfully isolated and quantified by high-performance liquid chromatography with ultraviolet detection. Maximum yields of alkaloid constitutes were predicted based on desirability option, and optimal conditions for MAE were microwave power of 180 W, irradiation time of 5 min, and ethanol concentration of 50%. Reliability of the method was confirmed by verification experiments performed under optimal conditions, and the experimental values (TAC of 333.94 mg BCE/g d.w., BC of 71.43 mg BCE/g d.w., and PC of 15.58 mg PCE/g d.w.) matched well with estimated values, suggesting that the estimated models were reliable and valid for MAE of alkaloids.

Keywords: alkaloids, berberine, microwave-assisted extraction, Rhizoma coptidis

Introduction

Rhizoma coptidis (*Coptis chinensis* Franch.), referred to Chuanhuanglian in China, is a traditional Chinese medicine that has been used to remove damp heat as well as treat dysentery and arrhythmia over 2,000 years (1). Bioactive alkaloids have been reported as main bioactive compounds in Rhizoma coptidis, and recent studies have discovered that alkaloid compounds from Rhizoma coptidis have pharmacological effects, including hepatoprotective (2), analgesic (3), anti-ulcer (4), anti-inflammatory (5), and broad antimicrobial effects (6,7). Eight types of alkaloids, including berberine, epiberberine, palmatine, coptisine, and jatrorrhizine, have been successfully isolated from Rhizoma coptidis (8), with berberine and palmatine accounting for 60% of the total alkaloid content (TAC) as main bioactive constituents (9).

Various extraction techniques have been applied to Rhizoma coptidis, including conventional solvent extraction (10), soxhlet extraction (11), high speed ultrasonic extraction (12), novel accelerated solvent extraction (13), supercritical fluid extraction (14), and pressurized liquid extraction (15). However, all of these techniques produce only low alkaloid yields, and some techniques required high pressure, substantial energy consumption or expensive devices. Microwave-assisted extraction (MAE) is a fast extraction approach based on microwave irradiation, which digests the outer walls of plant tissues, leading to a quick release of effective compounds into the extraction media. Compared with other extraction methods, MAE is faster, more convenient, safer, and more economically viable. Further, it is labor-efficient and requires less solvent consumption.

Thus, the present study aimed at employing a fast microwave-assisted method for the extraction of bioactive alkaloids from Rhizoma coptidis. It also investigated the effects of process variables, including microwave power, irradiation time, and ethanol concentration, on alkaloid extraction [TAC, berberine content (BC), and palmatine content (PC)]. Extraction process was simulated using a central composite design with five levels and three variables, and the best combination of process variables was determined

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using three dimensional surface plots in accordance with response surface methodology.

Materials and Methods

Chemicals Berberine chloride, palmatine chloride (purity >98%, w/w), and potassium dihydrogen phosphate were purchased from Sigma chemical Co., Ltd. (St. Louis, MO, USA). Ethanol (95%), acetonitrile (HPLC grade), and other relative solvents were purchased from Duksan Pure Chemical Company (Ansan, Korea).

Preparation of Rhizoma coptidis sample Dried Rhizoma coptidis, originally cultivated in Sichuan province in China, was purchased and authenticated under the guidance of the Pharmacopeia of China (2005 version). The Rhizoma coptidis sample was ground into powder form using an electric grinder and passed through a 40 mesh sieve with an aperture size of 250 μm. The powdered sample was then packed into a polyethylene zipper bag and stored in a freezer at −18°C during the experiment.

MAE process Microdigest (Soxwave 100; Prolabo, Fontenay, France) with a focused irradiation process under atmospheric pressure conditions was used for the MAE. The emission frequency of the extractor was 2,450 MHz and the microwave power was linear and adjustable between 60 W and 300 W with 30 W intervals. The extractor was equipped with one vessel that could access a 250 mL quartz tube and a cool water circulation system using a graham-type refrigerant column (400 mm length). Precisely, a 2.0 g sample was placed into the quartz tube and blended with 50 mL of the extraction solvent. Upon completion of the extraction, the extract tube was removed from the vessel and filtered using No. 1 filter paper under vacuum. The filter was transferred into a volumetric flask and the final volume was diluted up to 100 mL for quantitative analysis.

Determination of TAC The TAC of rhizoma coptidis extract was determined according to the method previously described by Xu *et al.* (16) using a 96-well ultraviolet-visible spectrophotometer. After scanning at maximal wavelength, 345 nm was selected as the detection wavelength with the smallest number of impure peaks. Thus, the TAC contained in rhizoma coptidis was measured by using 1 mL of adequately diluted sample (diluted 100 fold), and absorption values were recorded at 345 nm. Pure ethanol was used as a blank for setting zero, whereas berberine chloride was used as a reference for the calibration curve (R^2=0.9997). The results were expressed as mg of berberine chloride equivalent/g (mg BCE/g) of dry basis of rhizoma coptidis.

HPLC conditions Berberine and PCs were analyzed by using a high performance liquid chromatograph (Waters, Milford, MA, USA) equipped with an ultraviolet-visible detector (Jasco, Japan) according to the method described by Ye *et al*. (2). The separation process was carried out through a C18 reverse-phase column (A XTerra, 250 mm ×4.6 mm). The elute system consisted of acetonitrile (A) and 25 mmol/L of potassium dihydrogen phosphate in distilled water (B) at a mix ratio of 25:75 at a flow rate of 1.0 mL/min. Detection wavelength and retention time were set at 345 nm and 25 min, respectively.

Berberine chloride and palmatine chloride standard stock solutions were prepared by dissolving accurately weighted standards in 10 mL of pure ethanol, resulting in an initial concentration of 50 μg/mL for berberine chloride and 30 μg/mL for palmatine chloride. Standard working solutions used for calibration were prepared by diluting the above standard solutions to the desired concentrations with ethanol. Good linearity of the calibration curve for the berberine chloride and palmatine chloride standards was achieved with correlation coefficients of 1.0000 and 0.9970, respectively. Results were expressed as mg BCE/g and mg of palmatine equivalent/g (mg PCE/g) of dry basis of Rhizoma coptidis.

Experimental design Optimization of bioactive alkaloid contents from Rhizoma coptidis using MAE was conducted according to a central composite design (CCD) with three variables and five levels generated by the built-in package (ADX module) of the SAS system (version 9.3; SAS Institute, Cary, NC, USA). The CCD matrices consisted of six central points, eight factorial points, and another six axis points at a distance of ±2 from the center, resulting in 20 sets of experimental runs. Based on our primary experimental tests using single factor experiments, microwave power, irradiation time, and ethanol concentration were selected as independent variables with ranges of 60-300 W, 1-9 min, and 0-100%, respectively. The process variables Xi were coded as xi according to the equation below:

$$x_i = (X_i - \overline{X}_i)/\Delta X_i \quad (1)$$

where x_i is the coded level and X_i is the natural level of the independent variables; $\overline{X}_i$ is the mean of the natural level of the independent variables, and ΔX_i is the step change value. Coded and natural levels of the independent variables are shown in Table 1. TAC, BC, and PC were the dependent responses.

Experimental data were fitted into an empirical second order polynomial model using regression analysis and presented in the following equation:

Table 1. Central composite design (CCD) matrices with coded and natural variable levels, as well as experimental values for dependent responses of total alkaloid content (TAC), berberine content (BC), and palmatine content (PC)

Run	Coded variable level			Natural variable level			Dependent response		
	x_1 (W)	x_2 (min)	x_3 (%)	X_1 (W)	X_2 (min)	X_3 (%)	TAC (mg BCE/g)	BC (mg BCE/g d.w.)	PC (mg PCE/g d.w.)
1	-1	-1	-1	120	3	25	278.83	63.50	15.25
2	-1	-1	1	120	3	75	281.34	68.31	15.76
3	-1	1	-1	120	7	25	306.93	65.14	14.79
4	-1	1	1	120	7	75	308.91	71.58	15.60
5	1	-1	-1	240	3	25	291.58	63.79	15.16
6	1	-1	1	240	3	75	295.37	69.01	16.13
7	1	1	-1	240	7	25	320.75	70.37	14.29
8	1	1	1	240	7	75	324.12	72.38	15.15
9	-2	0	0	60	5	50	285.64	66.43	13.56
10	2	0	0	300	5	50	315.26	65.65	14.41
11	0	-2	0	180	1	50	271.13	61.52	16.38
12	0	2	0	180	9	50	328.12	70.44	15.53
13	0	0	-2	180	5	0	297.64	60.98	13.93
14	0	0	2	180	5	100	295.79	69.28	15.32
15	0	0	0	180	5	50	337.17	74.41	16.79
16	0	0	0	180	5	50	341.48	72.19	17.18
17	0	0	0	180	5	50	331.74	70.33	16.17
18	0	0	0	180	5	50	327.29	76.05	16.62
19	0	0	0	180	5	50	338.30	74.34	16.30
20	0	0	0	180	5	50	339.71	73.26	16.19

$$Y=\beta_0+\Sigma_{i=1}^{k}\beta_i x_i+\Sigma_{i=1}^{k}\beta_{ii}x_i^2+\Sigma_{1\le i\le j}^{k}\beta_{ij}x_i x_j+\varepsilon \quad (2)$$

where Y represents the independent responses, and β_0, β_i, β_{ii}, and β_{ij} represent the regression coefficients of the process variables for the intercept, linear, quadratic, and cross product terms, respectively, and ε represents error. Statistical significance of the coefficients in the regression equation was checked by analysis of variance (ANOVA). The fitness of the polynomial model equation to the responses was evaluated by the coefficient of *R*-squared along with the lack-of-fit using the *F*-test.

Statistical analysis All analytical experiments were performed in triplicate and expressed in mean values. Statistical analysis, including analysis of variance and estimation of coefficients, was carried out using SAS software (version 9.3; SAS Institute). Three dimensional surface and contour plots and desirability profiles were generated by Statistica 8.0 (Statsoft Inc., USA). Comparison of means was checked by Duncan's test in SAS system.

Results and Discussion

Modeling of MAE process for alkaloids from Rhizoma coptidis Yields of TAC, BC, and PC in Rhizoma coptidis extracts obtained from all 20 tests of CCD matrices are summarized in Table 1. The analytical values for BC and PC were in acceptable ranges as compared with narrow-bore high performance liquid chromatography (17). The fitness of models was analyzed and evaluated by ANOVA, and the results are summarized in Table 2. In general, the data fit well for all of the response models with high significance ($p<0.05$) and satisfactory multiple determination (R^2). Regression analysis was employed to fit the experimental data into second order polynomial models, and each coefficient was determined using the *t*-test. Three dimensional response surface and contour plots were constructed based on the fitted model in order to predict the relationships between independent and dependent variables.

Effects of process variables on TAC The TAC of Rhizoma coptidis extract obtained by MAE is presented in Table 1. ANOVA results in Table 2 revealed that the TAC model displayed good fitness with an R^2 value of 0.9600. The relationship between the process variables and TAC with only significant terms is shown in Eq. 3 below.

$$Y_{tac} = 334.64+7.19*x_1+14.22*x_2-9.53*x_1^2-9.74*x_2^2-10.46*x_3^2 \quad (3)$$

where, Y_{tac} is TAC, and x_1, x_2, and x_3 are coded values for the independent variables microwave power (W), irradiation

Table 2. Analysis of variance (ANOVA) results for second order polynomial model of responses of TAC, BC, PC

Source	Sum of squares	DF	Mean square	*F*-value	*p*-value
TAC (mg BCE/g)[1)]					
Model	9270.56	9	827.48	26.66	<.0001
(Linear)	4068.61	3	1356.20	35.10	<.0001
(Quadratic)	5200.32	3	1733.44	44.86	<.0001
(Cross product)	1.63	3	0.54	0.01	0.9976
Residue	386.39	10	38.64		
(Lack of fit)	242.00	5	48.40	1.68	0.2924
(Pure error)	144.39	5	28.88		
Total	9656.94	19			
BC (mg BCE/g)[2)]					
Model	341.79	9	37.98	13.22	0.0002
(Linear)	145.61	3	48.54	16.90	0.0003
(Quadratic)	190.67	3	63.56	22.13	<.0001
(Cross product)	5.51	3	1.84	0.64	0.6068
Residue	28.72	10	2.87		
(Lack of fit)	8.89	5	1.78	0.45	0.3365
(Pure error)	19.83	5	3.96		
Total	370.50	19			
PC (mg PCE/g) [3)]					
Model	17.05	9	1.89	15.61	<.0001
(Linear)	3.35	3	1.12	9.20	0.0032
(Quadratic)	13.47	3	4.49	37.00	<.0001
(Cross product)	0.23	3	0.08	0.62	0.6172
Residue	1.21	10	0.12		
(Lack of fit)	0.42	5	0.08	0.52	0.7513
(Pure error)	0.80	5	0.16		
Total	18.26	19			

[1)]The R squared value obtained in fit statistics for the response model of TAC was 0.9600.
[2)]The R squared value obtained in fit statistics for the response model of BC was 0.9225.
[3)]the R squared value obtained in fit statistics for the response model of PC was 0.9335.

Table 3. Estimates effect for significant coefficients of responses of TAC, BC, and PC

Term	Estimate	Std Err	*t*-value	*Pr*>*t*
TAC (mg BCE/g)				
X_1	7.19	1.55	4.63	0.0009
X_2	14.22	1.55	9.15	<.0001
X_1^2	-9.53	1.24	-7.69	<.0001
X_2^2	-9.74	1.24	-7.85	<.0001
X_3^2	-10.46	1.24	-8.44	<.0001
BC (mg BCE/g)				
X_2	2.04	0.42	4.82	0.0007
X_3	2.19	0.42	5.18	0.0004
X_1^2	-1.81	0.34	-5.37	0.0003
X_2^2	-1.83	0.34	-5.41	0.0003
X_3^2	-2.04	0.33	-6.04	0.0001
PC (mg PCE/g)				
X_2	-2.60	0.09	-2.99	0.0135
X_3	0.37	0.09	4.26	0.0017
X_1^2	-0.64	0.07	-9.21	<.0001
X_2^2	-0.15	0.07	-2.12	0.0596
X_3^2	-0.48	0.07	-6.91	<.0001

time (min), and ethanol concentration (%), respectively. Regression analysis (Table 3) showed that TAC was significantly (p<0.05) influenced by microwave power and irradiation time. Irradiation time and microwave power showed significant quadratic effects on TAC, as depicted in Fig. 1B. An increase in either irradiation time or microwave power led to enhancement of TAC, and maximal TAC was attained around 200 W and 6.5 min.

Effects of process variables on BC The BC of Rhizoma coptidis extract obtained by MAE is presented in Table 1. The calculated ANOVA data in Table 2 show that the BC model had significantly high linear and quadratic effects (p<0.05). The multiple coefficient of determination (R^2) was 0.9225 while the lack-of-fit was not significant (0.3365). Regression analysis was performed on the experimental data, and coefficients of the model were evaluated for their levels of significance. Equation 2 with only significant terms describes the relationship between microwave power, irradiation time, and ethanol concentration for MAE of BC.

$$Y_{BC}=73.50+2.04*x_2+2.17*x_3-1.81*x_1^2-1.83*x_2^2-2.04*x_3^2 \quad (4)$$

Estimated effects for each coefficient are listed in Table 3. The effects of irradiation time and ethanol concentration on BC were highly significant, as demonstrated in Fig. 2A. BC increased with an increase in either ethanol concentration or irradiation time, and the highest BC was achieved at around 6 min and a 60% ethanol concentration. Chen *et al.* (13) previously reported similar results in which maximal BC was obtained upon extraction with 60% aqueous ethanol. In addition, microwave power had no significant effect on BC, implying that the microwave irradiation in our tested range did not cause any decomposition of BC. Ong and Len (15) have reported that BC isolated from Rhizoma coptidis is not easily decomposed and can be extracted at temperatures as high as 120°C.

Effects of process variables on PC The PC of Rhizoma coptidis extract obtained by MAE is shown in Table 1. ANOVA results in Table 2 show that the fitted PC model had significantly high linear and quadratic effects (p<0.05). Moreover, insignificant lack-of-fit (p>0.05) as well as a high R-squared value (R^2=0.9325) revealed good fitness of the PC model to the data. Estimated effects from the

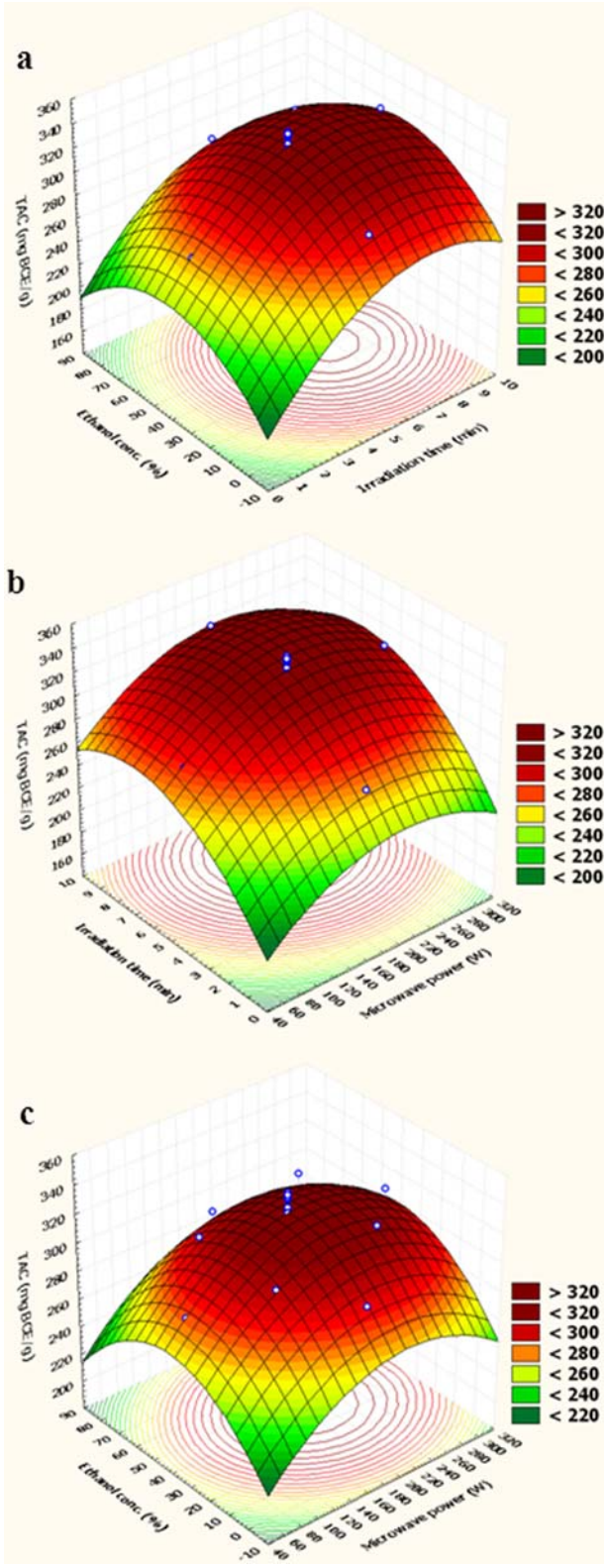

Fig. 1. Response surface plots for total alkaloid content (TAC) of Rhizoma coptidis as influenced by process variables under microwave-assisted extraction (MAE). (A) Ethanol concentration (%) and irradiation time (min). (B) Irradiation time (min) and microwave power (W). (C) Ethanol concentration (%) and microwave power (W)

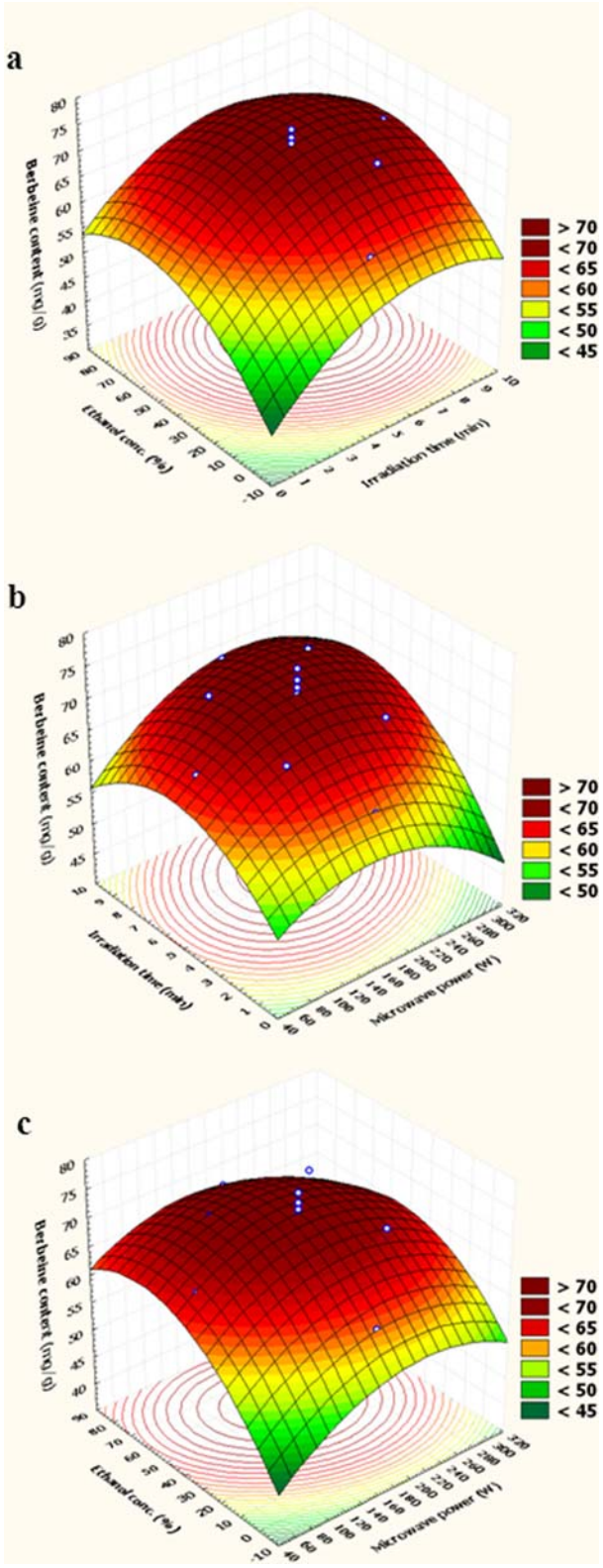

Fig. 2. Response surface plots for berberine content (BC) in Rhizoma coptidis as influenced by process variables under MAE. (A) Ethanol concentration (%) and irradiation time (min). (B) Irradiation time (min) and microwave power (W). (C) Ethanol concentration (%) and microwave power (W)

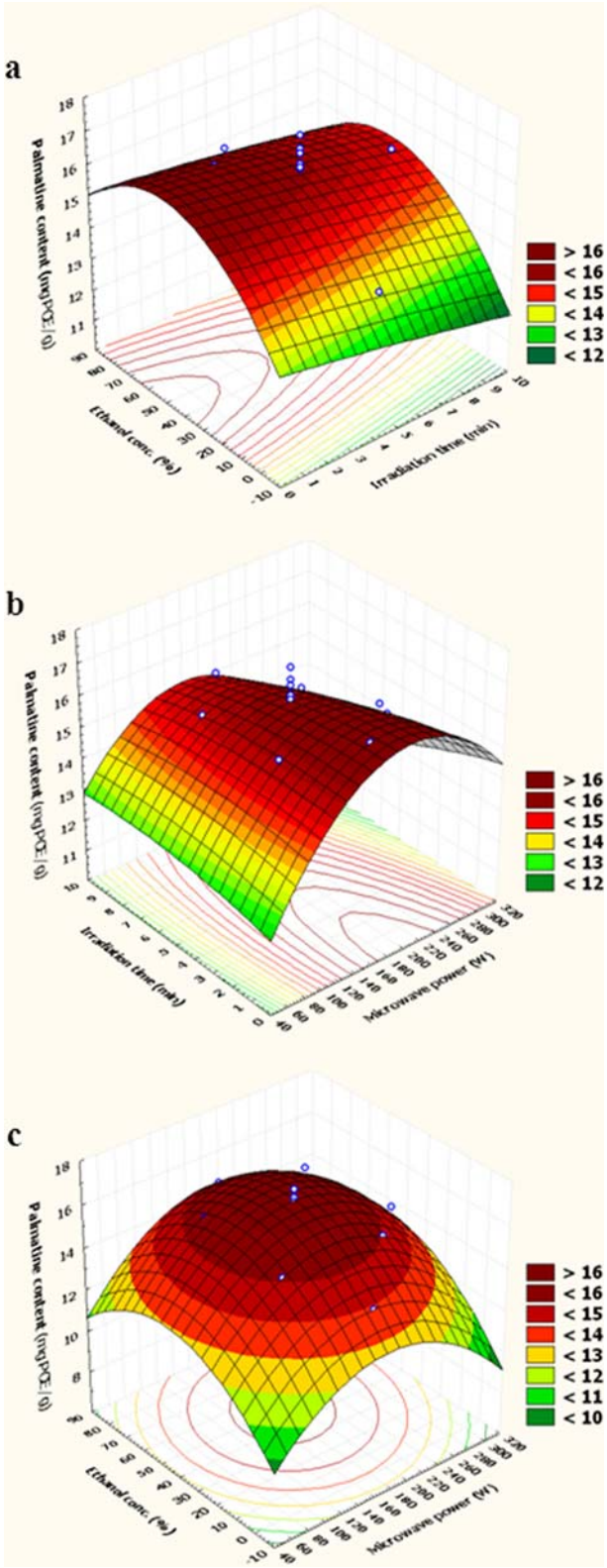

Fig. 3. Response surface plots for palmatine content (PC) in Rhizoma coptidis as influenced by process variables under MAE. (A) Ethanol concentration (%) and irradiation time (min). (B) Irradiation time (min) and microwave power (W). (C) Ethanol concentration (%) and microwave power (W)

regression analysis (Table 3) showed that the main extraction variables were irradiation time and ethanol concentration within a 5% significance level. The relationship between the extraction variables and PC is presented in Eq. 5.

$$Y_{PC} = 16.54 - 0.26 * x_2 - 0.37 * x_3 - 0.64 * x_1^2 - 0.15 * x_2^2 - 0.48 * x_3^2 \quad (5)$$

Good linear and quadratic effects of the fitted PC model were demonstrated in the response surface plot as shown in Fig. 3A-3C. Similar linear effects for irradiation time were observed in Fig. 3A, 3B, implying that irradiation time did not significantly influence PC. It has been reported that PC does not decrease below a temperature of 130°C (13). Thus, the microwave irradiation in our tested range did not cause any degradation of PC. Figure 3C presents the significant quadratic effects of ethanol concentration and microwave power, with maximal PC obtained at a microwave power of 190 W and ethanol concentration of 60%. These results were similar with those reported by Chen *et al.* (13).

Optimization of MAE process and model validation

The primary objective of MAE optimization was to determine the proper conditions to achieve maximal yields of all responses. Statistica 8.0 software was used for the optimization process with desirability option, and the profiles are shown in Fig. 4. The scale of the desirability function ranged between 0 (completely undesirable response) and 1 (fully desired response). To optimize the extraction of alkaloids from Rhizoma coptidis, all profiles for desirability were set to their maximums (in the right of Fig. 4). The predicted optimal microwave power, irradiation time, and ethanol concentration were 180 W, 5 min, and 50%, respectively. Estimated maximal yields for TAC, BC, and PC were 334.64 mg BCE/g d.w., 73.50 mg BCE/g d.w., and 16.54 mg PCE/g d.w., respectively, with a desirability value of 0.85. To confirm the validity of the estimated experimental values, verification experiments were performed under predicted optimal conditions and HPLC analysis was performed for alkaloid compounds in Rhizoma coptidis extracts. The experimental results were TAC of 333.94±5.21 mg BCE/g d.w., BC of 71.43±1.55 mg BCE/g d.w., and PC of 15.58±0.86 mg PCE/g d.w., which matched well with the estimated results and validated the RSM model with good correlation. Lee *et al.* (17) previously demonstrated ion-pair extraction of berberine and palmatine from Rhizoma coptidis. He obtained satisfactory yields of berberine (5.0%) and palmatine (2.1%), although the process involved expensive equipment and complex manipulation procedures. A comparison study

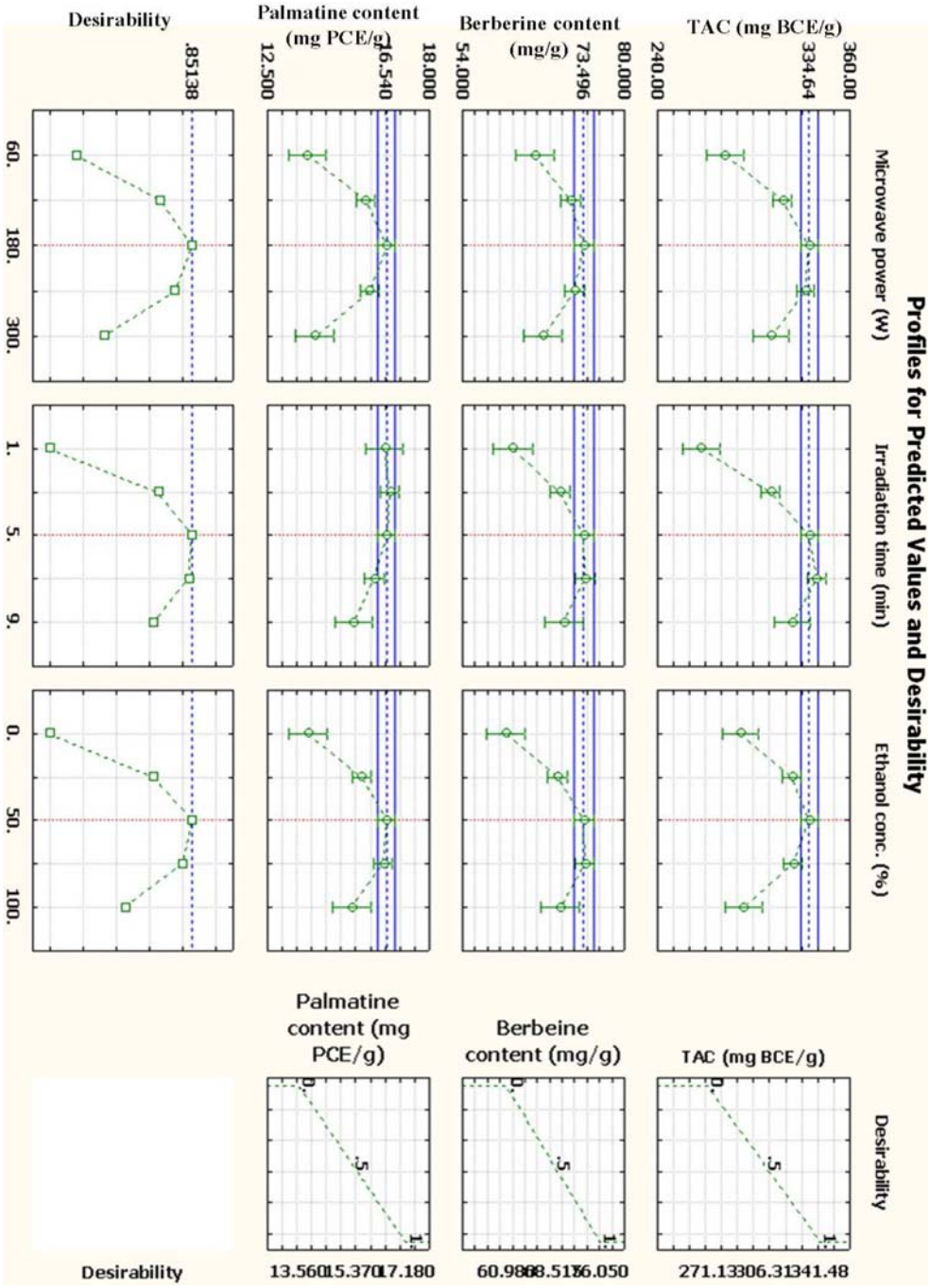

Fig. 4. Profiles for predicted values and desirability for maximal TAC, BC, and PC.

(18) that employed soxhlet and pressurized liquid for extraction of alkaloids from coptidis rhizome demonstrated BC of 7.1% by pressurized water extraction and 6.7% by soxhlet extraction. However, both techniques involved high pressure and temperature, which consume high amounts of energy and are dangerous. In this study, MAE was shown to be highly efficient in extracting a considerable amount of constituents in a very short time period, and the process did not involve any hazardous factors. In additional, alkaloids recovered by MAE were satisfactory and could be used for alkaloid extraction from other plant matrices.

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