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ResearchArticle

OPTIMIZATION OF ULTRASONIC-ASSISTED EXTRACTION PARAMETERS FOR ANTIOXIDANTS FROM *Curcuma longa* L.

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Abstract: In this study, ultrasonication was used to extract antioxidant compounds such as polyphenols, flavonoids, and curcuminoids from turmeric (*Curcuma longa* L.) The influences of time, ethanol concentration and temperature as three independent factors on the extraction of the total phenolic content were evaluated by the Folin-Ciocalteu method and the antioxidant capacities by the ABTS and chromium reducing antioxidant capacity (CHROMAC) methods. The central composite design (CCD) with a multi-response surface methodology (MRSM) was used for the statistical modeling of the response data followed by the regression and analysis of variance (ANOVA) to determine the significance of the model and the factors. The response predictions obtained at optimum extraction conditions of an extraction time of 64min, an ethanol concentration of 82% (v/v) and an extraction temperature of 32°C were 47.32mg GAE/g (for Folin-Ciocalteu), 29.15 (for ABTS) and 5.17mg TE/g (for CHROMAC). The predicted values obtained from the multi-response surface methodology agreed with the experimental values data 95% confidence level. These data indicate that the multi-response surface methodology is applicable for optimizing the ultrasonic-assisted extraction of antioxidant compounds from *C. longa*.

Keywords: *Curcuma longa* L., ultrasonic-assisted extraction, antioxidant capacity, phenolic compounds, multi-response surface methodology.

Özet: Bu çalışmada ultrasonikasyon, *Curcuma longa* L.'da bulunan polifenol, flavonoid ve kurkuminoid gibi antioksidan bileşiklerin ekstraksiyonu için kullanılmıştır. Zaman, etanol derişimi ve sıcaklık gibi 3 bağımsız değişkenin toplam fenolik madde (Folin yöntemi) ve antioksidan kapasite (ABTS ve CHROMAC yöntemi) üzerine etkisi değerlendirilmiştir. Merkezi kompozit dizayn ile çok yanıtlı yüzey analiz yöntemi, sonuçların istatistiksel modellenmesi, model ve faktörler arası etkileşimi belirlemek için regresyon ve ANOVA analizinde kullanılmıştır. Belirlenen optimum koşullarda (64dk ekstraksiyon zamanı, %82 (v/v) etanol derişimi ve 32˚C ekstraksiyon sıcaklığı) toplam fenolik madde miktarı 47,32mg GAE/g, antioksidan kapasite 29,15 (ABTS) ve 5,17 (for CHROMAC) mg TE/g bulunmuştur. Bu deneysel değerler ile tahmini değerler %95 güven aralığında birbiri ile uyumludur. Buna göre çok yanıtlı yüzey analiz tekniği (MRSM), *C. longa*'dan antioksidan bileşiklerin ultrasonik-destekli ekstraksiyon optimizasyonu için güvenle kullanılabilir.

Introduction

Curcuma longa L. (turmeric) distributed in East and South-East Asia (Xu *et al.* 2017)is generally used as a functional food and an herbal medicine (Péret-Almeida *et al.* 2005). The curcuminoids (curcumin-Cur, demethoxycurcumin-DMC, bisdemethoxycurcumin-BDMC) are natural and active phytochemicals in turmeric (Xu *et al.* 2017). A large number of studies reported that curcuminoids possess strong antioxidant (Martínez-Morúa *et al.* 2013), anti-inflammatory (Kant *et al.* 2014), antimicrobial (Kiamahalleh *et al.* 2016), and anticarcinogenic (Riela *et al.* 2014) properties and some other pharmaceutical activities (Lima *et al.* 2011, Mourtas *et al.* 2014).

Curcuminoids can be extracted from plants using a variety of methods. Non-conventional extraction techniques include ultrasound-assisted (Mandal *et al.* 2009), supercritical fluid, pressurized liquid and microwave extractions (Mandal *et al.* 2008). Among these techniques, ultrasound-assisted extraction (UAE) is widely used for the extraction of phytochemicals and is a simple, inexpensive, energy-saving, and efficient method when compared with other extraction techniques (Nasır *et al.* 2017). UAE was used for the extraction of antioxidant compounds from plants with higher yields (Şahin *et al.* 2013).

Different methods have been suggested for the extraction of phytochemicals from plants and the optimization of the extraction conditions due to the differences in the chemical and physical properties, concentrations, and matrix complexity related to the extractable phytochemical. The extraction solvent, pH, temperature, time, solid/liquid ratio, pressure, and particle

size are typical factors that contribute to the yield of extraction. Optimizing one factor at a time has been a generally used approach (Lai *et al*. 2014), but it is time-consuming and expensive and does not allow the analysis of possible interactions among the extraction variables (Matshediso *et al*. 2015). Therefore, a multi-response surface methodology (MRSM) is considered an efficient method for evaluating multiple individual parameters and their interactions (Baş & Boyacı 2007). The extraction of antioxidant compounds has been recently studied using different solvents such as dichloromethane, hexane, methanol, ethanol, acetone, dimethyl ether, diethyl ether, dimethyl sulfoxide, toluene, 2-propanol, and n-butanol. Due to the high solvent costs, the toxicity resulting from the solubility of the bioactive compounds in the solvent and low extraction efficiency, these solvents have been used regularly for the extraction of antioxidant compounds. Therefore, ethanol is one of the appropriate extraction solvents for antioxidant compounds due to its low cost, "green" characteristics, ease of access and safety for direct use in foods and pharmaceuticals (Xu & Bao 2014, Dailey & Vuong 2015).

The present study is focused on the application of UAE using a multi-response surface methodology to optimize the antioxidant extraction parameters for *C. longa*. Three extraction factors -the ethanol concentration, extraction temperature, and extraction time- were optimized by the MRSM. A three-variable and five-level, central composite design was used for simultaneously maximizing the antioxidant capacity and the total phenolic content.

Materials and Methods

Chemicals and reagents

Trolox, Folin–Ciocalteu reagent and ABTS were purchased from Sigma. Ethanol and methanol (HPLC grade), formic acid, phosphoric acid, sodium dihydrogen phosphate and HCl were purchased from Merck. Potassium dichromate, 1,5-diphenylcarbazide, sodium carbonate, potassium sodium tartrate, sodium hydroxide, copper(II) sulfate pentahydrate, and gallic acid in HPLC grade were obtained from Sigma.

Plant material

Dried *Curcuma longa* L. was purchased from a medicinal market in Bursa-Turkey and was stored at 4°C until extraction.

Ultrasound-Assisted Extraction (UAE) method

An ultrasonic cleaner (United model, Bursa, Turkey) was used for the extraction. The temperature was controlled using a resistance thermometer. *Curcuma longa* (0.5g) was placed in a vial, ethanol (30mL) was added, and the solution was placed in an ultrasonic cleaning bath. The operation was performed at 40 Hz ultrasonic wave frequency. The extraction parameters are given in Table 1.

Table 1. Range of coded and actual values for central composite design.

Factor	Level				
	-2	**-1**	**0**	**1**	**2**
Extraction time (min)	19	30	45	60	71
Ethanol concentration (%, v/v)	23	30	40	50	57
Extraction temperature (°C)	15	30	50	70	85

Antioxidant capacity

The antioxidant capacities of the extracts were determined with the chromium reducing antioxidant capacity (CHROMAC) (Işık *et al.* 2013) and ABTS (Re *et al.* 1999) methods with slight modifications (Şahin *et al.* 2013, Nasır *et al.* 2017). In the ABTS method, 3.8ml of ethanol was added to the *C. longa* sample (0.2ml). Then, 1ml of the ABTS solution (diluted with ethanol at a ratio of 1:10) was added. The absorbance of the sample was measured at 734nm against a blank sample after 6min by Varian Cary-50 UV/VIS spectrophotometer (Melbourne, Australia). The resulting antioxidant capacity was expressed as mg of Trolox equivalents (TE) per gram of sample. In the CHROMAC method, approximately 0.2ml *C. longa* sample, 0.3ml distilled water, 3.5ml phosphate buffer solution (pH 2.8) and 0.5ml $K_2Cr_2O_7$ (50mg l^{-1}) were added into a test tube. The $K_2Cr_2O_7$ solution was allowed to react with the sample. After incubation for 1min, approximately 0.5ml of 1,5-diphenylcarbazide (3.4×10^{-4}mol L^{-1}) was added and mixed thoroughly. The absorbance of the solution was measured at 540nm against a reagent blank after 50 min. The reagent blank at pH 1.2 was prepared with 0.1M citric acid and 6M HCl. A standard calibration curve was prepared using various concentrations of Trolox. The results were expressed as mg TE per gram of sample.

Total phenolic content

Total phenolic content was determined using the Folin-Ciocalteu method (Singleton *et al.* 1999) with slight modifications (Şahin *et al.* 2013). 50mL of Lowry A solution (2% aqueous Na_2CO_3 in 0.1M NaOH) was mixed with 1mL of Lowry B solution (0.5% $CuSO_4$ solution in 1% $NaKC_4H_4O_6$ aqueous solution) to produce Lowry C solution. Briefly, 1.8ml of distilled water and 2.5ml of Lowry C solution were added to *C. longa* sample (0.2ml), and the mixture was left for 10min. After 10min, Folin reagent (0.25ml) was added and the blue color of the mixture was allowed to stabilize under darkness for approximately 30min. The absorbance of the sample was measured at 750nm in triplicate. The total phenolic contents of *C. longa* extracts were expressed as mg gallic acid equivalent (GAE) per g of *C. longa*.

Response surface methodology

A central composite design (CCD) was selected for the optimal extraction conditions for the most successful RSM design (Aybastıer & Demir 2010). A three-factor, four-level central composite design experimental design matrix with factors such as the extraction time (min), ethanol concentration (%, v/v) and extraction temperature (°C) (Table 1) was formed, and the responses were

selected as the maximum antioxidant capacity and the total phenolic content. Twenty experiments were performed to optimize the parameters (Aybastıer & Demir 2010). Design Expert 7.0.0 software (Stat-Ease Inc., USA) was used for the statistical analysis.

Results

MRSM model

The MRSM was used to determine the extraction conditions that give the best results in terms of the antioxidant capacity and total phenolic content. The experimental and predicted total phenolic content and antioxidant capacity values are shown in Table 2. The extraction time, ethanol concentration and extraction temperature were studied with respect to the UAE of *C. longa*. The results indicate good correlations between the parameters because of a satisfactory *R*-squared value (R^2 =0.902) (Table 3). The *F*-value and *p*-values are indicated by larger corresponding coefficients. The x_1 (extraction time), x_2 (ethanol volume), x_3 (extraction temperature), x_1x_3, x_2x_3 and x_2^2 variables were the most significant factors ($p \leq 0.05$) (Table 4). However, x_1x_2, x_1^2 and x_2^2 had less effect (where $p > 0.05$) on the UAE in terms of the total phenolic content. The effects of the extraction factors on the total phenolic content were studied using response surface plots (Fig. 1). The MRSM of the data in Table 3 also showed good correlation (R^2 = 0.895 for ABTS and 0.826 for CHROMAC). The x_1, x_2, x_3, x_1x_3, x_2x_3, x_1^2, x_2^2 and x_3^2variables for ABTS and the x_1, x_2, x_3, x_2x_3, x_1^2 and x_3^2variables for CHROMAC were the most significant factors ($p \leq 0.05$) (Table 4) for the UAE of antioxidant compounds from *C. longa*. The $x_1x_2$variable for ABTS and x_1x_2, x_1x_3, x_2^2 for CHROMAC had minor effects on the antioxidant capacity derived from the UAE. The effects of the factors and antioxidant capacity values determined with the ABTS and CHROMAC methods were analyzed using response surface plots from the MRSM in Fig. 2.

Optimization of the extraction parameters

Validation tests were used to verify the reliability of the model by comparing the experimental and the predicted values for the MRSM. The optimum UAE conditions were presented in Table 5. An extraction time of 64min, an ethanol concentration of 82% (v/v), and an extraction temperature of 32ºC produced the maximum antioxidant capacities (29.15mg TE/g for ABTS and 5.17mg TE/g for CHROMAC) and the total phenolic content (47.32mg GAE/g) from *C. longa*. After comparing the predicted and experimental results, the RSM was more stable with good correlation (R^2>0.95) for *C. longa*.

Table 2. Central composite design of factors with experimental and predicted values.

Total phenolic content (mg GAE/g dried plant)			Antioxidant capacity (mg TE/g dried plant)			
			ABTS		CHROMAC	
Treatment	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1	42.32	43.68	7.63	5.33	2.16	1.33
2	18.34	17.95	5.56	5.21	0.16	0.17
3	44.21	47.70	19.92	22.36	3.51	4.36
4	42.93	42.36	11.05	10.81	2.55	2.47
5	46.55	48.12	9.49	9.84	3.62	3.74
6	42.74	42.79	5.43	7.49	2.62	3.26
7	41.49	43.68	4.81	5.33	1.09	1.33
8	38.54	43.68	6.33	5.33	0.67	1.33
9	46.35	43.68	2.86	5.33	1.51	1.33
10	46.77	46.01	12.91	10.58	4.09	3.34
11	32.46	32.23	14.57	15.04	2.93	2.90
12	47.37	43.68	6.63	5.33	1.25	1.33
13	36.57	38.62	5.46	8.58	0.94	1.78
14	46.45	41.98	13.67	10.64	3.97	3.06
15	46.00	43.68	3.70	5.33	1.31	1.33
16	45.34	46.86	13.05	13.19	1.62	1.72
17	20.78	24.47	6.65	7.53	0.18	0.39
18	45.80	43.37	20.76	20.66	2.10	1.96
19	33.46	30.98	10.64	9.91	1.38	1.22
20	41.00	39.98	19.56	16.90	3.45	2.76

Table 3. Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of extraction parameters.

Source	Folin (R^2 = 0.902)					ABTS (R^2 = 0.895)					CHROMAC (R^2 = 0.826)				
	DF	SS	MS	*F* value	*p* value	DF	SS	MS	*F* value	*p* value	DF	SS	MS	*F* value	*p* value
Model	9	1188.05	132.01	10.30	0.0006	9	519.61	57.73	9.55	0.0008	9	23.99	2.67	5.28	0.0079
Lack of fit	5	68.98	13.80	1.17	0.4354	5	43.45	8.69	2.55	0.1633	5	3.82	0.76	3.13	0.1178
Pure error	5	59.19	11.84			5	17.01	3.40			5	1.22	0.24		

Table 4. Second order polynomial equations and regression coefficients of the response variables (the extraction time; x_1; the ethanol concentration; x_2, the extraction temperature; x_3).

Responses	Second order polynomial equations
Total phenol content (mg GAE/g dried plant)	$y=43,68+2,13x_1+5,40x_2+4,67x_3-3,05x_1x_3-4,29x_2x_3-3,19x_2^2$
ABTS (mg TE/g dried plant)	$y=5,33+0,58x_1+3,75x_2-1,53x_3-1,96x_1x_3-2,29x_2x_3+1,42x_1^2+2,86x_2^2+2,46x_3^2$
CHROMAC (mg TE/g dried plant)	$y=1,33+0,45x_1+0,45x_2+0,25x_3-0,98x_2x_3+0,41x_1^2+0,69x_3^2$

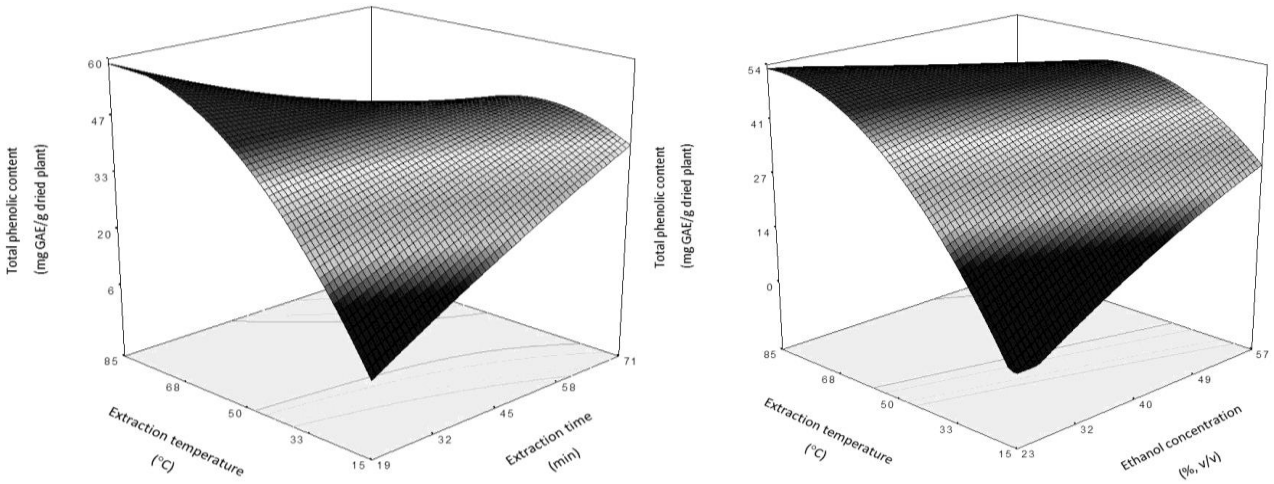

Fig. 1. Response surface plots of *Curcuma longa* showing the effects of (A) Extraction time and extraction temperature, (B) Ethanol concentration and extraction temperature on total phenolic content.

Discussion

Among the 15 runs (Table 3), experiment 12 (45min, 23% (v/v) of ethanol, 50ºC) produced the highest total phenolic content (47.37mg GAE/g), and experiment 10 (19min, 40% (v/v) of ethanol, 50ºC) produced the highest antioxidant capacity (4.09mg TE/g) for the CHROMAC method. For both the Folin-Ciocalteu and CHROMAC methods, experiment 2 produced the lowest values (18.34mg GAE/g and 0.16mg TE/g). For the ABTS method, experiment 18 produced the highest antioxidant capacity (20.76mg TE/g), but experiment 9 produced the lowest antioxidant capacity (2.86mg TE/g).

ANOVA revealed that the total phenolic content and antioxidant capacity values were described by quadratic polynomial models. The analysis showed that the large model *F*-values (10.30, 9.55 and 5.23 for Folin-Ciocalteu, ABTS and CHROMAC methods, respectively) and the lack of fit *p*-values (0.4354, 0.1633 and 0.1178 for Folin-Ciocalteu, ABTS and CHROMAC methods, respectively) were statistically significant at a 95% confidence level (Table 4). The larger *p*-values were statistically non-significant relative to the pure errors. The R^2 values were 0.902, 0.895 and 0.826 for the Folin-Ciocalteu, ABTS and CHROMAC methods, respectively. It was shown that the linear relationship between the extraction parameters and the responses were significant and the model was appropriate for optimization.

The three-dimensional images of the response surface methodology showed the interactions between the extraction parameters and the total phenolic content (Fig. 1). At higher extraction temperatures and time, the

highest total phenolic content was observed (Fig. 1a). The diffusion and solubility rates of the antioxidant compounds increased with a higher extraction temperature. When experiments 13 and 17 were compared (Table 3), the total phenolic content increased from 20.78 to 36.57mg GAE/g dried plant at a higher extraction temperature. Consequently, the effect of increasing the extraction temperature was significant for extracting antioxidant compounds in *C. longa.* Unfortunately, the extraction temperature was an important factor affecting the activity of the extracts due to both the degradation or loss of the antioxidant compounds (Yap *et al*. 2009, Dorta *et al*. 2012) and reactions with other components. However, the highest total phenolic content and antioxidant capacity values were obtained at 32°C by using the MRSM.

Ethanol was selected as the solvent for the UAE of antioxidant compounds from *C. longa.* A higher total phenolic content was observed at higher ethanol concentrations (Fig. 1b.). When experiments 6 and 8 are compared (Table 3), it appeared that the total phenolic content increased from 38.54 to 42.93mg GAE/g dried plant with increasing ethanol concentrations. It was reported that the higher ethanol concentration gave the highest extraction yield of the antioxidant compounds (Kwang *et al*. 2010). Therefore, the extraction yield of the antioxidant compounds was higher when the total phenolic content and antioxidant capacity of the extract was higher, as shown in our results.

It was clear that when the temperature was increased with a shorter extraction time, the antioxidant capacity was also increased for the ABTS method. The highest antioxidant capacity was observed at the shortest extraction time (19min) and the highest temperature (85°C). Fig. 2a-b shows the interactions between the ethanol concentration and extraction temperature on the antioxidant capacity for the ABTS and CHROMAC methods. The antioxidant capacity increased with increasing temperature at a low ethanol concentration. At lower ethanol concentrations and higher temperatures, the highest antioxidant capacity was observed. It was reported that the extraction yield of antioxidant compounds increased with increasing extraction time (Xu *et al.* 2015). Thus, an extraction time ranging from 19 to 71min was used in the present study, and the extraction yield of antioxidant compounds was affected by the extraction temperature. A high temperature may improve the solubility of antioxidant compounds (Liang *et al.* 2017), but there is an important risk of thermal degradation of antioxidant compounds. The solvent concentration is an important factor influencing extraction yield of antioxidant compounds and extraction yield of antioxidant compounds increased with increasing solvent concentration (volume) (Xu *et al.* 2015).With the antioxidant capacity as the reference value from Fig. 2, the optimum conditions obtained by the MRSM analysis were an extraction temperature of 85°C, an extraction time of 19min and an ethanol concentration of 23% (v/v). However, with the antioxidant capacity and the total phenolic content values as the reference value, the optimum conditions obtained by the MRSM analysis were an extraction temperature of 32°C, an extraction time of 64min and an ethanol concentration of 82% (v/v). The optimum conditions were obtained from the MRSM analysis to predict the maximum values for the antioxidant capacities and the total phenolic content. Multi-response surface methodology is the most suitable method for optimizing the ultrasonic-assisted extraction of antioxidant compounds from *C. longa* when compared a single response surface methodology. Because, multi-response surface methodology ensured the optimum extraction parameters with limit of time and solvent concentration and without no thermal degradation. Furthermore, the antioxidant capacity is dependent on the synergistic effect of the extracted antioxidant compounds. However, the synergistic effect will decrease when the total phenolic content increase (Thoo *et al.* 2010). Additionally, when compared with the available published data, similar results were found showing that the antioxidant capacity increased with increasing extraction time (Xu *et al.* 2015).

UAE was also used successfully for the extraction of antioxidant compounds from *C. longa* with yields higher than previously reported (Xu *et al.* 2015, 2017). The multi-response surface methodology was successful in optimizing the antioxidant compound content using UAE from *C. longa.*

Table 5. Optimum conditions, predicted and experimental values of responses.

Responses	Optimum Ultrasonic-assisted extraction conditions			Maximum values	
	Extraction time (min)	Ethanol concentration (%, v/v)	Extraction temperature (°C)	Predicted	Experimental
Total phenolic content (mg GAE/g dried plant)	64	82	32	47.63	47.32±0.02
ABTS (mg TE/g dried plant)				29.50	29.15±1.05
CHROMAC (mg TE/g dried plant)				5.13	5.17±0.08

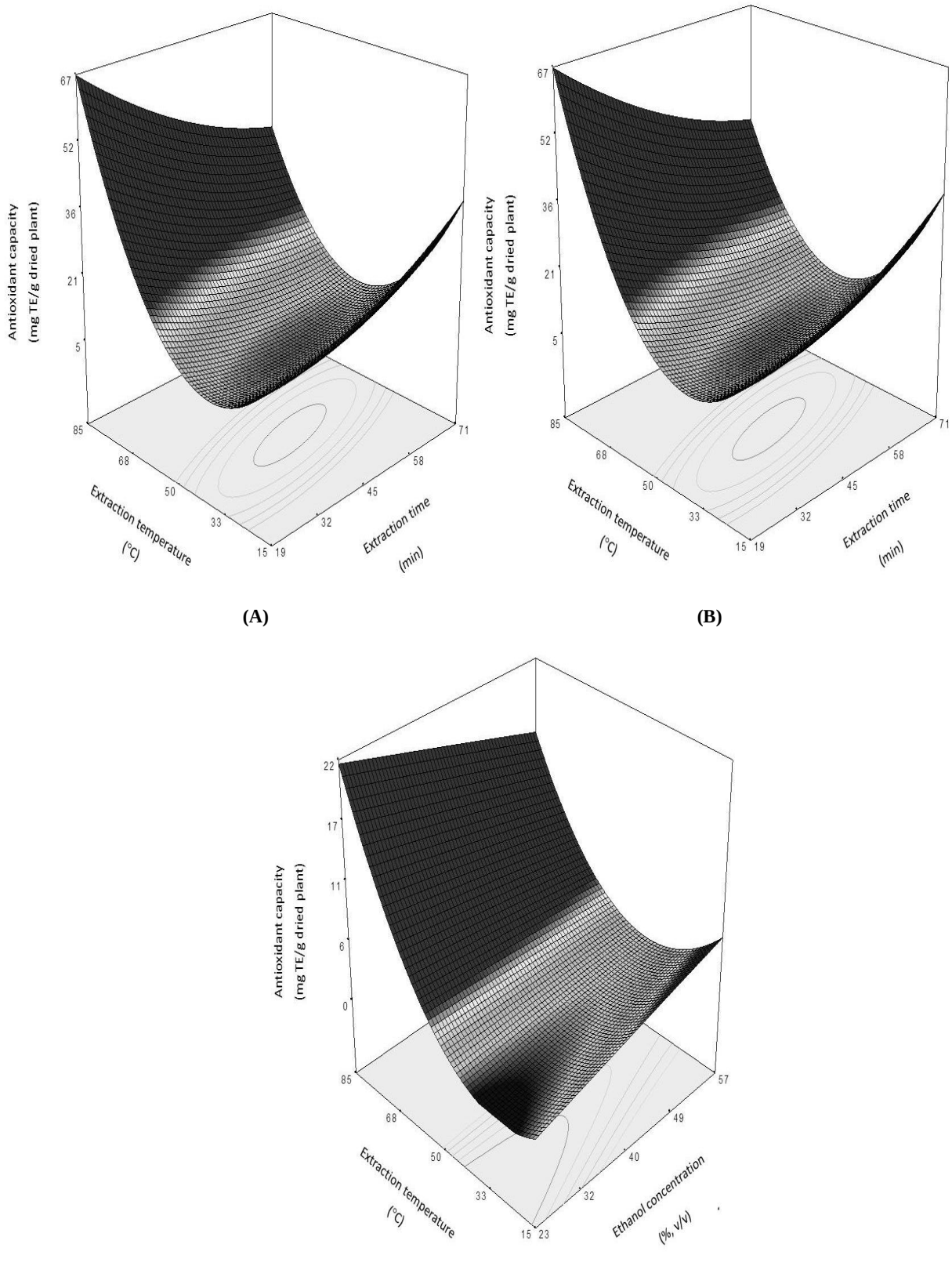

Fig. 2. Response surface plots of *Curcuma longa* showing the effects of (A) Extraction time and extraction temperature, (B) Ethanol concentration and extraction temperature on ABTS values, and (C) Ethanol concentration and extraction temperature on CHROMAC value.

The optimum conditions using UAE of antioxidants from *C. longa* are shown in Table 5.The study of Xu *et al.* (2017) with *C. longa* have demonstrated that phenolics such as curcuminoids are major contributors to antioxidant properties. Therefore, the total phenolic content and antioxidant capacity have been selected as the response of the MRSM model. All responses from each extraction factor were combined into a single set of optimum conditions. When the multi-response surface methodology was used, the total phenolic content of the extract increased with antioxidant capacity (Nasır *et al.* 2017). The present study is the first study about determination of the antioxidant capacity of *C. longa* by the CHROMAC method. It can be concluded that the MRSM is accurate and reliable within a 95% confidence interval to predict the results obtained with *C. longa*.

Conclusions

The multi-response surface methodology was used successfully for the optimization conditions of UAE of antioxidant compounds from *C. longa*. The CCD provided a powerful design for the optimization conditions using UAE. The extraction factors strongly influenced the extraction of the antioxidant compounds from *C. longa.* We conclude that *C. longa* is a good and reliable source of antioxidant compounds.

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