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Multi-response Optimization for Unfertilized Corn Silk Extraction Against Phytochemical Contents and Bio-activities

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Abstract

This study was designed to optimize ethanol extraction process of unfertilized corn silk (UCS) to maximize phytochemical contents and bioactivities. The response surface methodology (RSM) with central composite design (CCD) was employed to obtain the optimal extraction conditions. The influence of ethanol concentration, extraction temperature and extraction time on total polyphenol contents, total flavonoid contents, maysin contents, 2,2-diphenyl-1-picrylhydrazyl(DPPH) radical scavenging activities and tyrosinase inhibition were analyzed. For all dependable variables, the most significant factor was ethanol concentration followed by extraction temperature and extraction time. The following optimum conditions were determined by simultaneous optimization of several responses with the Derringer's desirability function using the numerical optimization function of the Design-Expert program: ethanol concentration 80.45%, extraction temperature 53.49°C, and extraction time 4.95 h. Under these conditions, the predicted values of total polyphenol contents, total flavonoid contents, maysin contents, DPPH radical scavenging activity and tyrosinase inhibition were 2758.74 μg GAE/g dried sample, 1520.81 μg QUE/g dried sample, 810.26 mg/100g dried sample, 56.86% and 43.49%, respectively, and the overall desirability (D) was 0.74.

Key words: unfertilized corn silk, ethanol extraction, phytochemical content, bioactivity, response surface methodology

Introduction

Corn silk (*Zea mays* L.) is a traditional herb, which has dried cut stigmata of maize female flowers that contains many bioactive compounds such as carbohydrates, proteins, vitamins, calcium, potassium, magnesium and sodium salts, volatile oils and steroids, saponins, tannins, alkaloids, flavonoids and other phenolic compounds with beneficial effects on human health (Ebrahimzadeh et al., 2008). Corn silk has been used for the treatment of several diseases with multiple pharmacological activities reported, such as antioxidant (Chen et al., 2014), antidiabetic (Guo et al., 2009; Pan et al., 2017), antitumor (Yang et al., 2014), immune enhancement (Kim et al., 2004), anti-fatigue (Hu et al., 2010), anti-obesity (Chaiittianan et al., 2016), and neuroprotective effects (Choi et al., 2014).

The operational conditions such as extracting solvent concentration, extraction time, extraction time, liquid to solid ratio, and the kind of extracting solvent for the extraction method play an important role in the conventional extraction process. Conventional solid-liquid extraction is widely used for phytochemical components, plus is safe, cheap, and easy to scale up (Mulinacci et al., 2011). Ethanol is a green solvent, environmentally friendly solvent, widely used in industry because of its low cost, easy availability, safety and biodegradability (Derrien et al., 2017).

The main advantage of response surface methodology (RSM) is reduced number of experimental trials needed to evaluate multiple variables and their interactions. Therefore, it is less laborious and time consuming than other approaches required optimizing a process (Derrien et al., 2017). Usually, it applies an experimental design such as central composite design (CCD) or Box-Behnken design (BBD) to fit a second order polynomial by a least square technique. An equation is used to describe how the test variables affect the response and determine the interrelationshipamong the variables (Liu et al., 2009).

In the present study, the Derringer's desirability function was used to improve phytochemical contents and bioactivities of extracts from unfertilized corn silk (UCS) by simultaneous optimization of five responses. The three independent variables considered were ethanol concentration, extraction temperature and extraction time, and were optimized according to the

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responses of the following dependent variables: total polyphenol contents, total flavonoid contents, maysin contents, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities, and tyrosinase inhibition.

Materials and methods

Materials

The UCS with 28.28±0.96 μm of mean diameter was obtained from Rural Development Administration (Jeonju, Korea) in 2016, and stored it at -70°C until use.

Extraction

The concentrations of ethanol used were varied from 30% to 90% (v/v), according to the experimental design. The reactor was heated at 20-60°C for 1-6 h in shaking incubator (HZQ-311, Neuronfit, Seoul, Korea) during the extraction process. The ratio of ethanol to UCS was 30 (v/w), and 1 L flask was used for extraction.

Experimental design

The optimization experiment was carried out using RSM with a CCD experimental design for extraction process from UCS. The CCD as an effective alternative to full factorial design enables to gather more data with lower number of experiments. The three independent variables were ethanol concentration (X_1), extraction temperature (X_2) and extraction time (X_3). In addition, the low, middle and high levels of each variable were designed as coded terms –1, 0, +1, respectively (Table 1). The experimental data were fitted to second-order poly nominal models to express the responses as a function of the independent variables according to following equation.

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i<j=1}^{3} b_{ij} X_i X_j \quad (1)$$

where, Y is the measured response variable, b_o is a constant, b_i, b_{ii}, and b_{ij} are the linear, quadratic, and interaction coefficients, respectively, and X_i and X_j are the levels of the independent variables.

Table 1. Factors and levels of experiment for ethanol extraction of unfertilized corn silk

Factors	Symbol	Code value		
		-1	0	1
Ethanol concentration (%)	X_1	30	60	90
Extraction temperature (°C)	X_2	20	40	60
Extraction time (h)	X_3	1.0	3.5	6.0

Total polyphenol contents

The total phenolic content of sample was analyzed by Folin-Ciocalteu colorimetric method with modification, using gallic acid as a standard (Liu et al., 2011). An aliquot (0.5 mL) of sample was transferred to the test tubes with 5 mL distilled water. After addition of Folin-Ciocalteu reagent (5 mL) and 10% aqueous Na_2CO_3 solution (2 mL), tubes were vortexed. After 60 min, the absorbance was recorded at 750 nm. The total phenolic content was determined using thestandard gallic calibration curve and expressed as microgram gallic acid equivalents per gram dry mass of sample (μg GAE/g dried sample).

Total flavonoid contents

The total phenolic content of sample was analyzed by Chew et al. (2009) with modification. The sample (0.5 mL) was placed in a volumetric flask. Ethanol (1.5 mL) was added followed by 10% aluminium nitrate (0.1 mL), 1 M potassium acetate (0.1 mL) and distilled water (2.8 mL). The solution was mixed and the absorbance was measured at 415 nm. Calculation were based on a standard curve obtained with quercetin. The total flavonoid content was expressed as micrograms of quercetin equivalents per gram of dry mass sample (μg QUE/g dried sample).

Maysin contents

Maysin contents were analyzed by HPLC; the operation conditions are described in Table 2. Standard maysin was obtained from Rural Development Administration, which was conformed by electrospray ionization-mass spectrometry (ESI-MS) using Micromass Electrospray Interface ZMD 4000 (Micromass, Manchester, UK). The maysin contents of the sample were measured by comparison retention time of standard maysin, and calculated based on the peak area of standard maysin (Kim et al., 2000).

Table 2. Instrument and analysis conditions for maysin

Instrument	YL9100 HPLC
Column	Agilent 5 TC-C18(2) 250 × 4.6 mm
Column temperature	30°C
Mobil phase	A - 10% ethanol + 0.1% phosphoric acid B - 90% ethanol + 0.1% phosphoric acid
Gradient condition	H_2O/ethanol linear gradient (from 20% ethanol to 80% ethanol for 35 min)
Flow rate	1.0 mL/min
Injection volume	20 micro liter
Detector wave length	340 nm

Antioxidant activity assay

A 0.2 mL of sample was added to 1 mL of 0.2 mM DPPH methanol solution, mixed, and allowed to stand for 30 min in the dark. Distilled water was used as a blank control. The percent inhibition of absorbance at 517 nm was calculated and plotted as a function of the concentration of standard for samples to determine the ascorbic acid equivalent antioxidant concentration (Sarepoua et al., 2013). The percentage of DPPH radical scavenging activity of sample was calculated according to the following equation.

$$\text{DPPH free radical scavenging activity (\% inhibition)} = (1 - A/B) \times 100 \quad (2)$$

Where, A is the absorbance of sample and B is the absorbance of control.

Tyrosinase inhibition

Tyrosinase inhibition of samples was analyzed using the method reported by Kwon et al. (2012). A 0.2 mL of sample was added to 2.3 mL of 0.2 M potassium phosphate buffer (pH 6.5) and 0.4 mL of 2 mM l-tyrosine solution. The solution was mixed and 0.1 mL mushroom tyrosinase (220 unit/mL) was added. After 20 min of reaction in 37°C water bath, the absorbance was recorded at 470 nm (A). The absorbance of 0.1 mL of distilled water instead of enzyme solution (B) and that of 0.2 mL of distilled water instead of the sample (C) were measured. Tyrosinase inhibition was then calculated according to the following equation.

$$\text{Tyrosinase inhibition (\%)} = (1 - (A - B)/C) \times 100 \quad (3)$$

Statistical analysis

Each experiment was performed in triplicate, and statistical analysis was performed using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA) software. Regression and variance analyses were carried out using RSREG procedure. Both carnonical and ridge analyses were conducted in addition to the Derringer's desirability function, known as an efficient method for optimization of multiple responses.

Results and Discussion

Fitting the response surface models

According to the CCD, 15 experiments were performed in triplicate and the obtained results are presented in Table 3. The experimental results of investigated responses (total polyphenol contents, total flavonoid contents, maysin contents, DPPH radical scavenging activity and tyrosinase inhibition) obtained under different ethanol concentrations, extraction temperatures and extraction times were fitted to a second order polynomial model (Eq. (1)), and multiple regression coefficients were generated for all responses, using a least square approach in Table 4. Their statistical significance based on determination coefficients and probability values for each investigated

Table 3. Central composite design for ethanol extracts from unfertilized corn silk and its dependent variables

Experiment number	X_1 [1)]	X_2 [2)]	X_3 [3)]	Total polyphenol contents (μg GAE/g dried sample)	Total flavonoid contents (μg QUE/g dried sample)	Maysin contents (mg/100g dried sample)	DPPH radical scavenging activity (%)	Tyrosinase inhibition (%)
1	0	-1	1	2420.2	1510.8	837.07	36.67	51.13
2	0	1	-1	2963.9	1687.5	853.02	42.69	24.71
3	0	0	0	2808.0	1655.3	868.72	41.77	39.63
4	0	-1	-1	2663.1	1604.3	854.74	40.53	37.43
5	-1	0	1	2623.2	1486.8	443.43	26.55	40.85
6	-1	-1	0	2141.2	1197.6	241.95	17.00	57.00
7	-1	0	-1	2224.5	1300.9	310.53	16.61	51.13
8	0	0	0	2799.0	1659.6	852.01	41.11	36.94
9	1	1	0	2576.1	1358.2	771.82	65.55	54.55
10	0	0	0	2822.5	1657.5	853.70	40.62	36.45
11	1	0	-1	2289.8	1155.2	826.42	65.77	60.67
12	1	0	1	2396.7	1270.7	814.94	65.73	59.20
13	1	-1	0	2088.6	1029.0	797.63	65.77	58.22
14	0	1	1	3043.6	1714.6	683.75	41.36	21.28
15	-1	1	0	2735.5	1575.0	482.52	31.08	28.13

[1)]Ethanol concentration (%)
[2)]Extraction temperature (°C)
[3)]Extraction time (h)

Table 4. Regression coefficients of second degree polynomials of five responses

Parameter	Estimate				
	Total polyphenol contents	Total flavonoid contents	Maysin contents	DPPH radical scavenging activity	Tyrosinase inhibition
Intercept	520.251	57.094	-1215.770	-14.501	108.866
X_1[1)]	57.873	44.744	46.208	0.601	-2.367
X_2[2)]	13.099	9.467	17.321	0.409	-0.047
X_3[3)]	32.782	9.918	70.283	2.866	1.898
X_1*X_1	-0.452	-0.385	-0.274	0.004	0.017
X_2*X_1	-0.045	-0.020	-0.111	-0.006	0.010
X_2*X_2	-0.044	-0.052	-0.095	0.000	-0.009
X_3*X_1	-0.973	-0.234	-0.481	-0.033	0.029
X_3*X_2	1.613	0.603	-0.758	0.013	-0.086
X_3*X_3	-3.117	-1.176	-2.052	-0.163	-0.044

[1)]Ethanol concentration (%)
[2)]Extraction temperature (^oC)
[3)]Extraction time (h)

Table 5. Determination coefficients and probability of five responses

	Regressions	Linear	Quadratic	Cross Product	Total regress
Total polyphenol contents	R^2	0.4255	0.4869	0.0399	0.9523
	Pr > F	<.0001	<.0001	0.006	<.0001
Total flavonoid contents	R^2	0.2900	0.6455	0.0079	0.9434
	Pr > F	<.0001	<.0001	0.4438	<.0001
Maysin contents	R^2	0.5698	0.3422	0.0435	0.9555
	Pr > F	<.0001	<.0001	0.0030	<.0001
DPPH radical scavenging activity	R^2	0.9555	0.0133	0.0198	0.9886
	Pr > F	<.0001	0.0013	0.0001	<.0001
Tyrosinase inhibition	R^2	0.4495	0.4082	0.1036	0.9613
	Pr > F	<.0001	<.0001	<.0001	<.0001

response are summarized in Table 5. For each terms in the models, a large determination coefficient or F value and a small Pr value would imply a more significant effect on the respective response variable (Yolmeh et al., 2014). According to statistically significant values of total regress (p<0.05) for all investigated responses, it was possible to conclude that applied mathematical model provides proper representation of experimental results. All of linear, quadratic and interactive coefficients were significant (p<0.05) for all responses except interactive coefficients of total flavonoid contents (p>0.05).

Effects of extraction conditions on total polyphenol contents

Corn silk is rich in phenolic compounds known to significantly benefit human heath, such as anthocyanins, p-coumaric acid, vanillic acid, proto-catechuic acid, derivatives of hesperidin and quercetin, and bound hydroxycinnamic acid forms composed of p-coumaric and ferulic acid (Ebrahimzadeh et al., 2008). The UCS used in this study had over 20 times higher maysin contents than commercial corn silk (unpublished data). The pharmacological effects of corn silk, such as anti-oxidant, anti-inflammatory or diuretic activity, are attributed to the presence of these phenolic compounds in it (Liu et al., 2011).

Accordingly, surface response plots of the model would allowfor visualizing the effect of the independent variables on the dependent variables by adjusting two of the factors simultaneously and keeping the third variable constant at the -1, 0, and 1 level. The contour plot for total polyphenol contents of extracts from UCS as functions of ethanol concentration, extraction temperature and extraction time (Fig. 1). In the case of total polyphenol contents, optimum range of ethanol concentration was around 60% (Fig. 1A-F). Increasing extraction temperature increased total polyphenol contents (Fig. 1A, B, C, G, H, I). Increasing extraction time just a little increased total polyphenol contents (Fig. 1G-I). The most significant factor for total polyphenol contents was ethanol

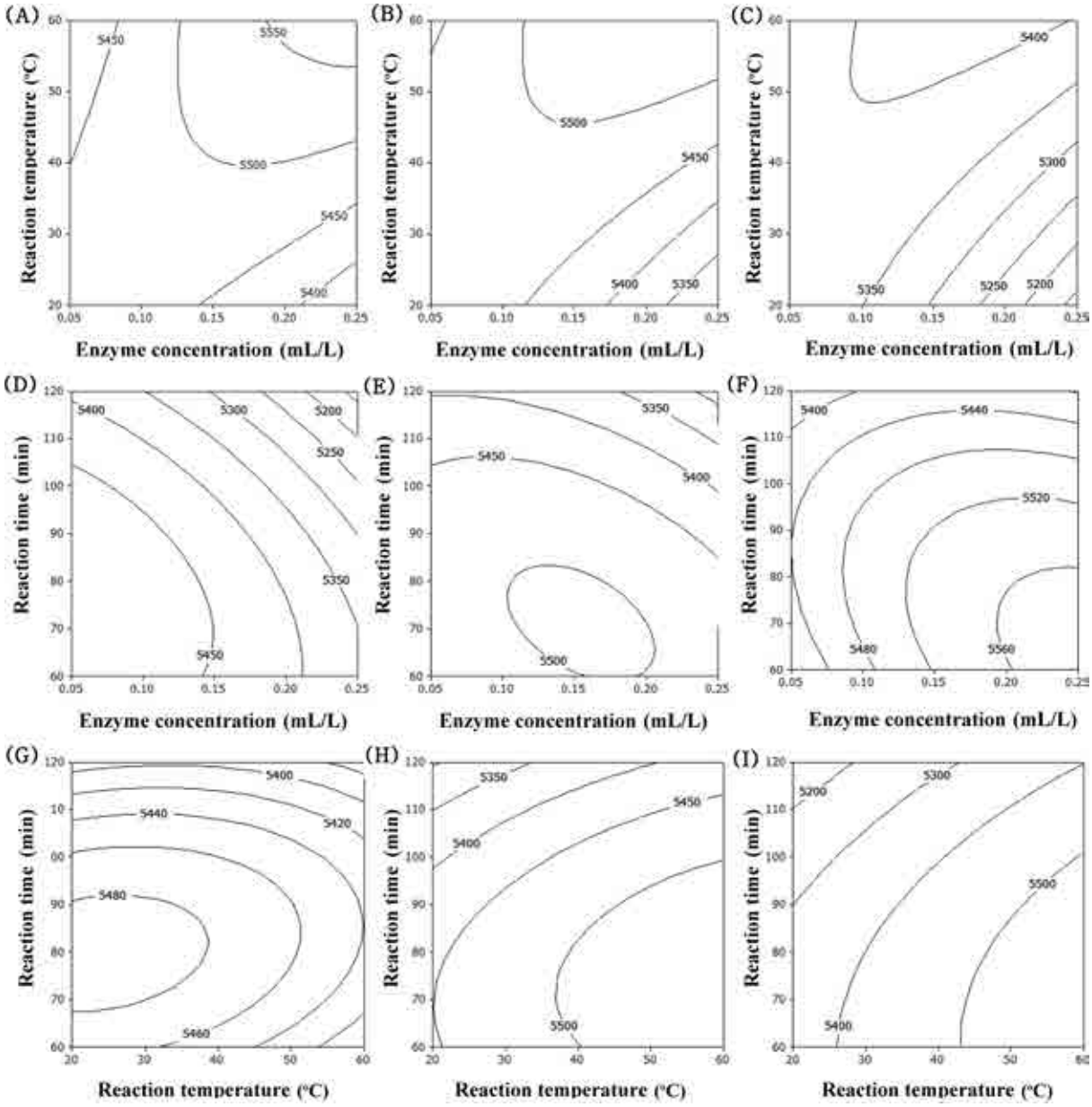

Fig. 1. Contour plot for total polyphenol contents (μg GAE/g dried sample) of ethanol extracts from unfertilized corn silk. Extraction time, A: 1 h, B: 3.5 h, C: 6 h; Extraction temperature, D: 20°C, E: 40°C, F: 60°C; Ethanol concentration, G: 30%, H: 60%, I: 90%).

Table 6. Analysis of variance for the effects of three variables on five responses

Factor		Total polyphenol contents	Total flavonoid contents	Maysin contents	DPPH radical scavenging activity	Tyrosinase inhibition
X_1[1)]	F value	54.41	65.98	105.95	424.79	77.71
	Pr > F	<.0001	<.0001	<.0001	<.0001	<.0001
X_2[2)]	F value	44.44	16.54	4.99	11.88	52.72
	Pr > F	<.0001	<.0001	0.0059	<.0001	<.0001
X_3[3)]	F value	5.28	1.53	2.06	3.69	4.97
	Pr > F	0.0045	0.2308	0.1244	0.0208	0.0060

[1)]Ethanol concentration (%)
[2)]Extraction temperature (°C)
[3)]Extraction time (h)

concentration as shown in Table 6. The second and third factors were extraction temperature and extraction time, respectively.

Effects of extraction conditions on total flavonoid contents

The flavonoid compounds of corn silk are major constituents which can scavenge the DPPH radical, due to the presence of the hydroxyl groups in their structure and their electron donating ability (Liu et al., 2011). The flavonoid compounds of corn silk were mainly composed of luteolin, formononetin and apigenin (Yu et al., 2008). The contour plot for the total flavonoid contents of extracts from UCS as functions of ethanol concentration, extraction temperature and extraction time is shown in Fig. 2. The optimal range of ethanol concentration was around 50-60% (Fig. 2A-F). As increased extraction temperature, total flavonoid contents was increased (Fig. 2A, B, C, G, H, I). Extraction time had not affected on total flavonoid contents (Fig. 2D-I). The most significant factor influencingthe total flavonoid contents was ethanol concentration as shown in Table 6, followed by extraction

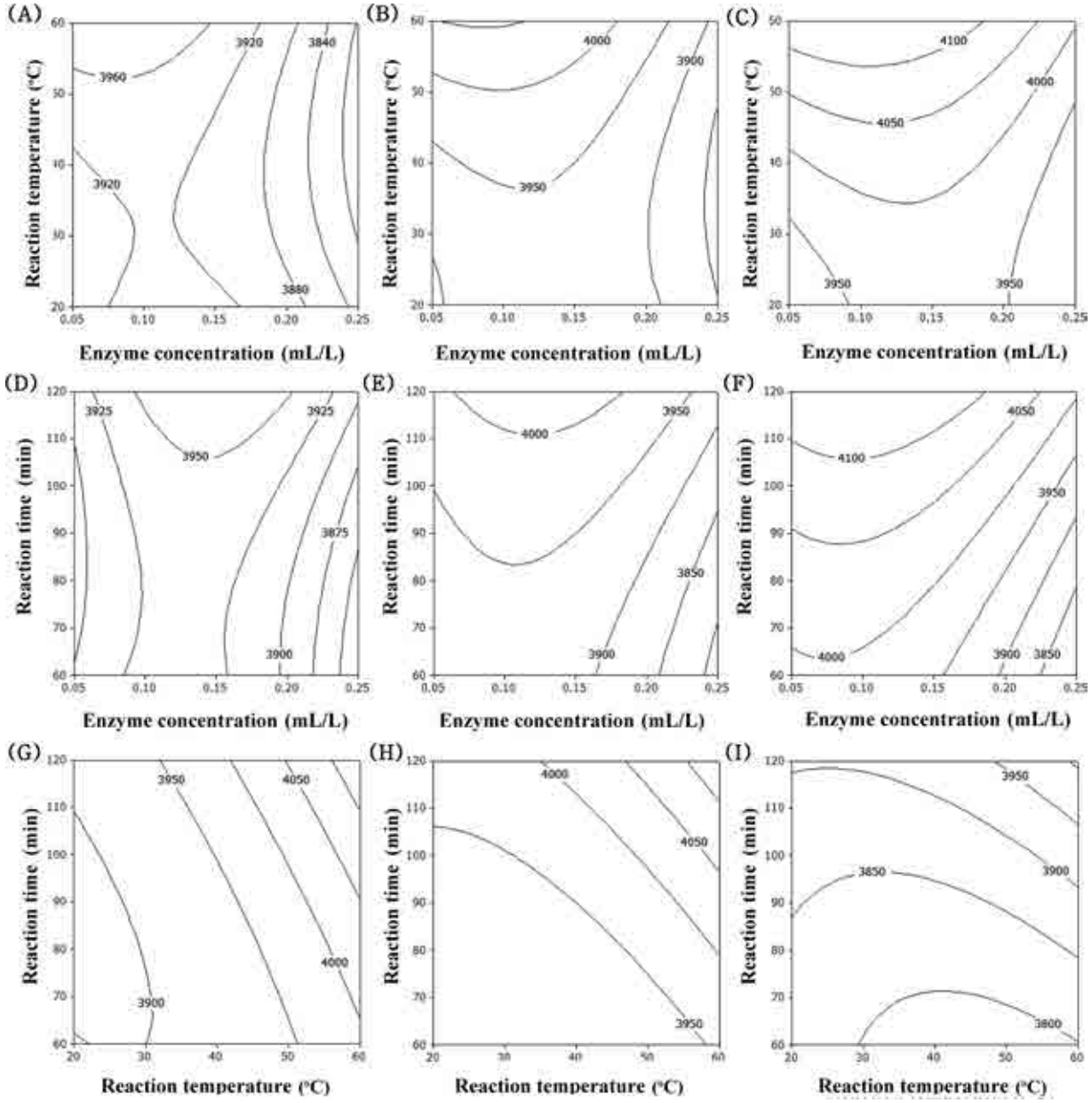

Fig. 2. Contour plot for total flavonoid contents (μg QUE/g dried sample) of ethanol extracts from unfertilized corn silk. Extraction time, A: 1 h, B: 3.5 h, C: 6 h; Extraction temperature, D: 20°C, E: 40°C, F: 60°C; Ethanol concentration, G: 30%, H: 60%, I: 90%.

temperature and extraction time. The effect of extraction time on total flavonoid contents did not show significant differences (p>0.05).

Effects of extraction conditions on maysin contents

The predominant phenolic compounds in corn silk extracts have been identified as maysin, apimaysin, 3'-methoxymaysin, isoorientin, and luteolin derivatives, among which maysin [rhamnosy-6-C-(4-ketofusyl)-5,7,3'4' tetrahydroxyflavone], a flavone glycoside containing a rhamnose residue, is the most abundant flavonoid (Lee et al., 2017). Maysin from purified corn silk acts anticancer (Lee et al., 2014), neroprotective (Choi et al., 2014), immunomodulating (Lee et al., 2014) and anti-obesity activities (Chaiittianan et al., 2016). The contour plot for maysin contents of extracts from UCS as functions of ethanol concentration, extraction temperature and extraction time is shown in Fig. 3. The optimal range of ethanol concentration was around 70-90% (Fig. 3A-F). Extraction temperature and extraction time had not affected on maysin contents is shown in Fig. 3. The most significant factor influencing the maysin content of extracts from UCS was the ethanol concentration, as shown in Table 6, followed by extraction

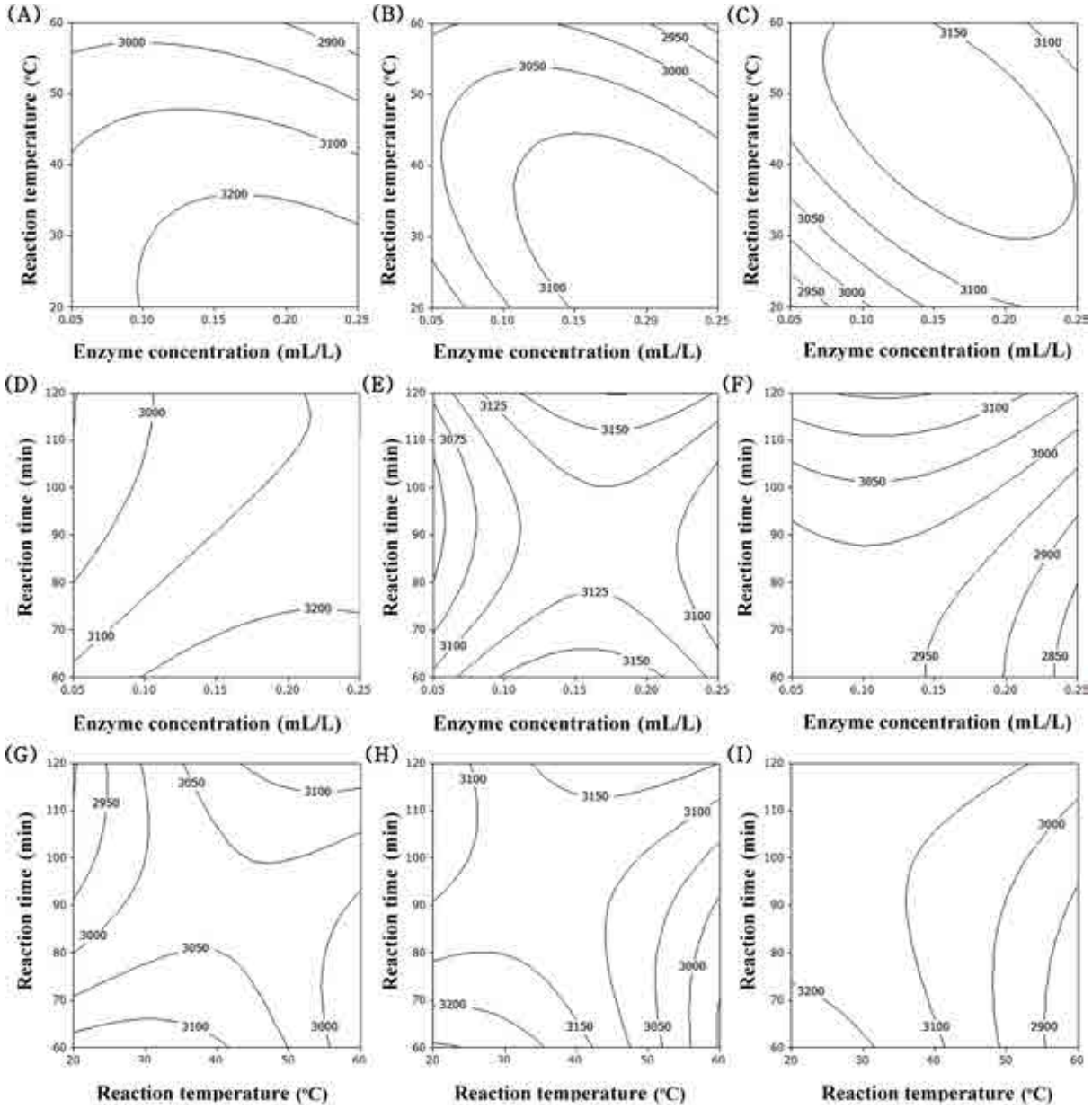

Fig. 3. Contour plot for maysin contents (mg/100g dried sample) of ethanol extracts from unfertilized corn silk. Extraction time, A: 1 h, B: 3.5 h, C: 6 h; Extraction temperature, D: 20°C, E: 40°C, F: 60°C; Ethanol concentration, G: 30%, H: 60%, I: 90%.

temperature and extraction time. Reaction time did not have a significant effect on maysin contents (p>0.05).

Effects of extraction conditions on DPPH radical scavenging activity

DPPH radical scavenging activity, based on the reduction of DPPH solution in the presence of a proton-donating substance, has been extensively employed to evaluate the radical scavenging ability of samples (Zeng et al., 2014). The DPPH radical scavenging activity of corn silk was shown to be related to the content of total polyphenol compounds (R=0.9415) and total flavonoids (R=0.9546) (Liu et al., 2011). The contour plot for DPPH radical scavenging activity of extracts from UCS as functions of ethanol concentration, extraction temperature and extraction time is shown in Fig. 4. Increasing ethanol concentration increased DPPH radical scavenging activity (Fig. 4A-F). With increased reaction temperature, DPPH radical scavenging activity was also increased (Fig. 4A, B, C, G, H). The most significant factor influencing DPPH radical scavenging activity of extracts from UCS was ethanol concentration, as shown in Table 6, followed by extraction temperature and extraction time.

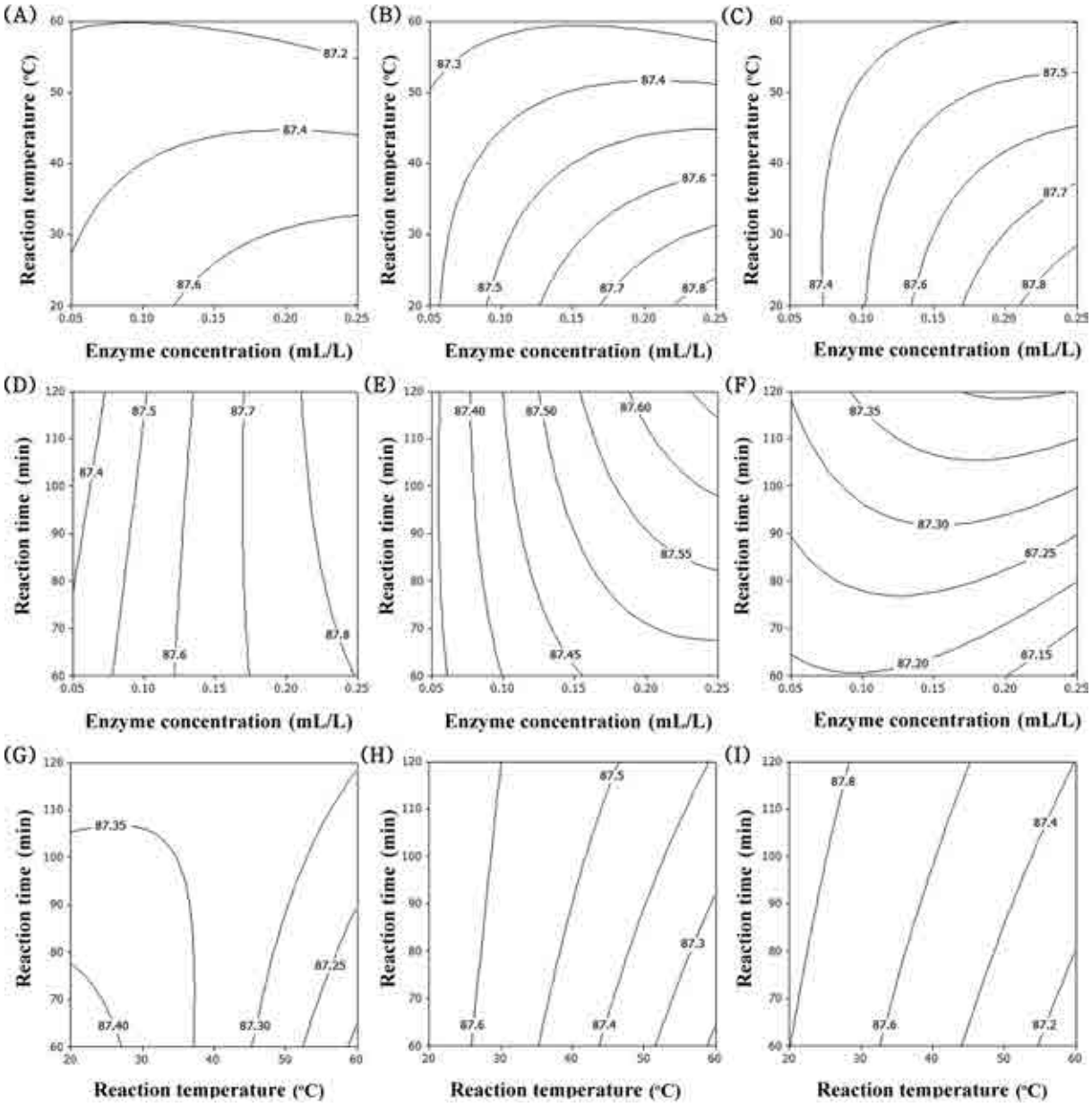

Fig. 4. Contour plot for DPPH radical scavenging activities (%) of ethanol extracts from unfertilized corn silk. Extraction time, A: 1 h, B: 3.5 h, C: 6 h; Extraction temperature, D: 20°C, E: 40°C, F: 60°C; Ethanol concentration, G: 30%, H: 60%, I: 90%.

Effects of extraction conditions on tyrosinase inhibition

There is an increased interest in finding natural tyrosinase inhibitors from herbs and applying them as skin care products, which have become potential sources of skin whiteners. Antioxidants are good inhibitors of tyrosinase activity and melanin production, and certain antioxidants have been applied as melanogenesis inhibitory agents (Yang et al., 2015). The contour plot for tyrosinase inhibition of extracts from UCS as functions of ethanol concentration, extraction temperature and extraction time is shown in Fig. 5. In the case of tyrosinase inhibition, optimum condition of ethanol concentration was 90% (Fig. 5A-F). As decreased reaction temperature and increased extraction time, DPPH radical scavenging activity was increased (Fig. 5). The most signifi- cant factor for tyrosinase inhibition was ethanol concentration, as shown in Table 6, followed by reaction temperature and traction time.

Multiple responses optimization

It is relatively simple to find the optimal conditions for a single response using RSM; however, in this study we sought to optimize five responses. Consequently, a Derringer's desirability function was utilized to optimize several responses

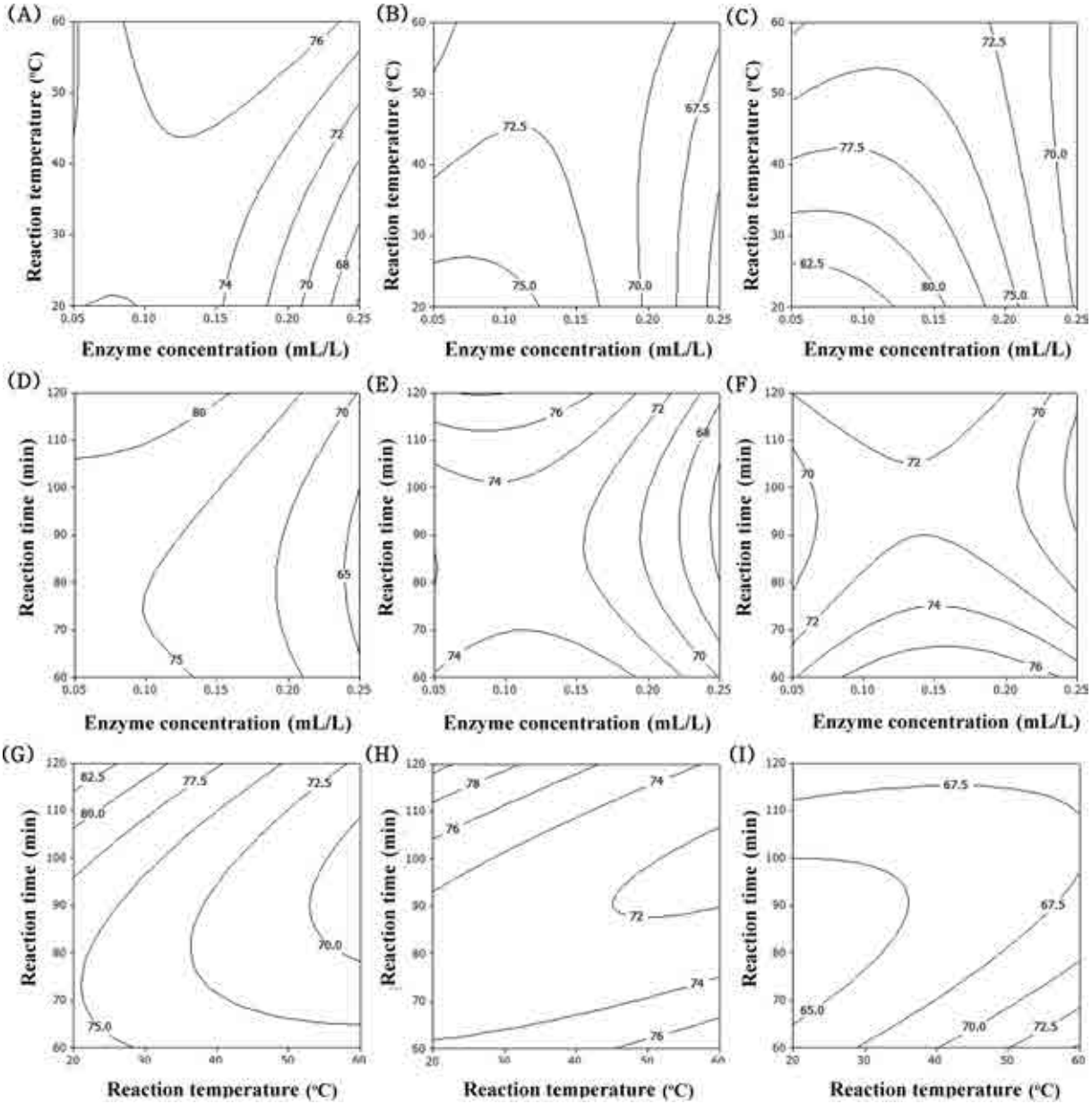

Fig. 5. Contour plot for tyrosinase inhibition (%) of ethanol extracts from unfertilized corn silk. Extraction time, A: 1 h, B: 3.5 h, C: 6 h; Extraction temperature, D: 20°C, E: 40°C, F: 60°C; Ethanol concentration, G: 30%, H: 60%, I: 90%.

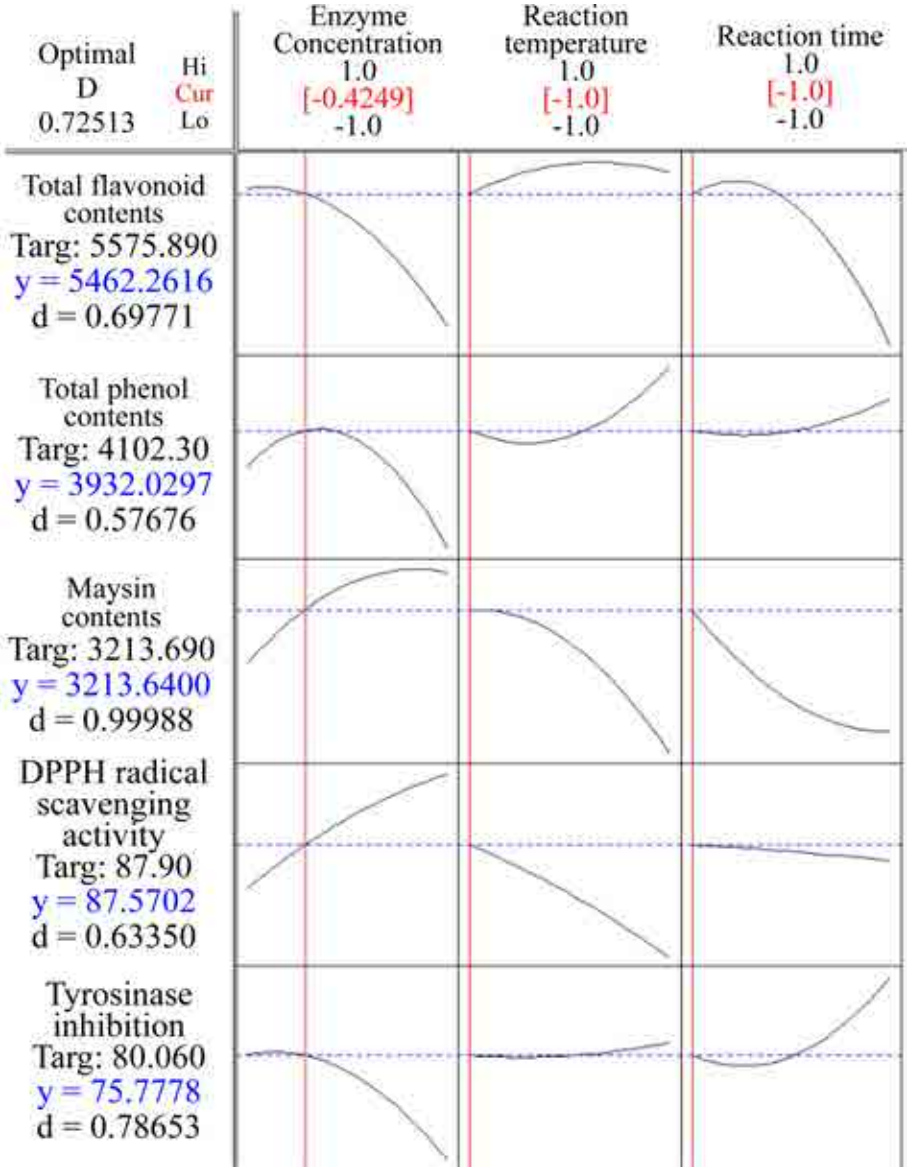

Fig. 6. Response optimization for multi-response surfaces of ethanol extracts from unfertilized corn silk.

simultaneously. The procedure involves constructing a function of each individual desirability $d_i$and then obtain an overall desirability function D. The function D is then used to maximize the ability of choosing the best conditions for the designated variable. The overall desirability function D is defined as the weighted geometric average of the individual desirability (d_i), according to following equation (Vera-Candioti et al., 2007).

$$D = (d_1 \times d_2 \times d_3 \times .... \times d_m)^{1/m} \quad (4)$$

where, d_1, d_2, d_3, ..., d_m correspond to the individual desirabilities function for each response.

Therefore, the optimal extraction process for improving the phytochemical contents and bioactivities from UCS were simultaneous optimized with the Derringer's desirability function using the numerical optimization function of the Design-Expert program. Response optimization for multi-responses of extracts from UCS are shown in Fig. 6. The optimum conditions given by the model were as follows: ethanol concentration 80.45%, extraction temperature 53.49°C, and extraction time 4.95 h, respectively. Under these conditions, the model predicted the values of total polyphenol contents, total flavonoid contents, maysin contents, DPPH radical scavenging activity and tyrosinase inhibition were 2758.74 μg GAE/g dried sample, 1520.81 μg QUE/g dried sample, 810.26 mg/100g dried sample, 56.86% and 43.49%, respectively, and the overall desirability

(D) was 0.74.

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