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Extraction of polyphenols from flowers of *Calendula officinalis*: modeling and optimization using response surface methodology

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- ✓ Response surface method.

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Abstract

Calendula officinalis flowers have been identified as a rich source of various phenolic compounds which are capable of combatting oxidative stress. Thus, effective extraction of phenolic compounds is important to efficiently utilize these flowers. The present study aims to make use of central composite design, to investigate the effects of extraction variables on the three response variables; total phenolic content, total anthocyanin content and antioxidant activity. Four independent variables including solid:liquid ratio, ethanol concentration, temperature and time were studied for the simultaneous optimization of response variables. The results showed that the polynomial models for all responses were significant ($p<0.05$) and did not show lack of fit and presented determination of coefficients above 95%. This indicates the suitability of the models for prediction purposes. The desirability function approach in the scale of 0-1 was used to determine the optimized conditions. The optimum process parameters generated were solid:liquid ratio 1:35, ethanol concentration 65.50%, extraction temperature 35°C and time 32 minutes. The experimental values obtained for the response variables under the generated optimum conditions confirmed the validity of the proposed second order polynomial model. The results demonstrated the application of feasible process parameters for the extraction of phenolics from Calendula officinalis flowers and effective utilization of these flowers in food as well as pharmaceutical industry.

1. Introduction

Calendula officinalis commonly known as Marigold is a medicinal aromatic herb belonging to the family Asteraceae. It is a native of central Europe and Mediterranean areas and is a commercially important plant source for many cosmetics as well as therapeutic products [1]. Despite the ornamental and culinary uses, various parts of this plant such as flowers and leaves have been used in folk medicine to treat ailments including skin disorders, eye inflammations, varicose veins and menstrual irregularities [2]. Among them, edible flowers of Marigolds have gained appreciable importance in pharmacological researches due to its disease healing drug-like properties attributed to its unique phyto-compositional diversity and associated bioactivities.

Scientifically validated pharmacological activities of Marigold flowers include anti-oxidant, anti-inflammatory, anti-viral, anti-hyperlipidemic, anti-diabetic, hepatoprotective and cardio-protective properties [3]. Previous investigations conducted by various authors have revealed that,

eminent therapeutic properties of Marigold flowers are associated with diverse groups of phenolics compounds such as phenolic acids (protocatechuic, vanillic, syringic acids), flavonoids (quercetin, isorhamnetin, isoquercitrin, rutin), carotenoids (flavoxanthin, zeaxanthin, lutein), steroids, tannins, quinines and coumarins [4,5] present in them. Thus, it becomes crucial to appropriately extract these phenolics compounds from Marigold flowers for their effective utilization in pharmaceutical and food industry. Diverse range of studies have been conducted in extracting various groups of phenolics from these flowers. For example, petroleum ether extract of Marigold flowers has been identified with the sitosterols, diesters of diols and erythrodiol [2], ethanol extracts have been reported with quercetin, rutin, calendoflavoside and neohesperodoside [6] and methanolic extracts were detected with violoxanthin, luteoxanthin and neoxanthin [7]. Thin layer chromatography analysis conducted by Ćetković et al., [8] using ethyl acetate, formic acid, acetic acid and water has reported that the ethyl acetate and n-butanol extracts of Marigold flowers contain coumaric, caffeic and chlorogenic acid, while water extract contains only chlorogenic acid.

Solid-liquid extraction is a widely employed technique to extract phenolics compounds from different plant matrixes. Extraction efficiency of phenolics in this technique is commonly a function of process parameters such as extraction temperature, pH, time, solvent composition, solid to solvent ratio, particle size, number of extraction cycles etc [9]. Thus in order to maximize the extraction efficiency and yield it is critical to appropriately select variables affecting the process and optimize the process parameters either empirically or statistically to quantify the phenolic compounds. Response surface methodology (RSM), is a mathematical and statistical approach, that has been successfully used to model and optimize biochemical and biotechnological processes related to food systems [10]. RSM estimates the effects of individual factors as well as multiple factors and their interactions on the responses, based on the fit of experimental data to the empirical models generated in relation to experimental design. This approach is beneficial over other traditional methods, due to its lower number of required experiments, a statistical basis to interpret the data, identification of the interactive effects between variables, less labor requirement and time-consuming than traditional optimization methods [11].The present study aims to apply RSM to simultaneously optimize the process parameters in order to maximize the yield of total phenolics and total anthocyanin content along with maximum antioxidant activity from Marigold flowers.

2. Methodology

2.1 Sample collection and preparation

Marigold flowers were collected from rural areas of Puttalam district, Sri Lanka. The morphologically perfect and completely bloomed flowers were manually cleaned and washed with distilled water to remove surface dirt. Then the samples were freeze-dried and powdered using a laboratory grinder. The powder obtained was stored at −18 °C until further analysis.

2.2 Extraction procedure

For the extraction, 1 g of sample was homogenized with ethanol (20 to 40 mL), at different concentrations (40% to 100%). The samples were then exposed to temperature in the range of 30 to 60°) for varying time period (30 to 60 minutes). The range of values for the process parameters were predetermined according to the experimental design shown in **Table 1**. The extracts were then filtered through Whatman filter paper # 1 and stored at -4°C until further analysis.

2.3 Experimental design and statistical analysis

The effects of the independent variables, (X_A: solid to liquid ration, X_B: ethanol concentration, X_C: extraction temperature and X_D: time) on the responses total phenolic content, total anthocyanin content and DPPH radical scavenging activity were investigated using a central composite design (CCD) consisting of 16 corner points, 7 center points and 8 axial points, with the total of 31 experiments. The experiments were conducted in random order, and the data were analysed by multiple regressions. The response function (Y) was partitioned into linear, quadratic and interactive components and the experimental data were fitted to the second-order polynomial model (**Eqn.1)**

$$\mathbf{Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_{ij}} \qquad \textbf{Eqn.1}$$

Y is the measured predicted response; β_0 is the intercept; βi, βii and βij are the model coefficients (linear, quadratic and interaction, respectively) and Xi and Xj are the values of the independent variables. The three-dimensional response surface plot was generated from the regression coefficients of the reduced polynomial equation. The plots were used to show the relationship between the response and the levels of each independent variable determine the optimum extraction conditions. The polynomial regression model was evaluated by analysis of variance (ANOVA). All statistical analyses were performed at a significance level of 5 % ($p \leq 0.05$) using Minitab software version 17.

2.4 Model verification

The optimum values of the selected independent variables were obtained by solving the regression equation and analysing the response surface plots. The predictive extraction model was verified by performing experiments using the optimized process parameters. The predicted values were compared to the experimental values to determine the validity and adequacy of the model.

2.5 Determination of Total Phenolic Content (TPC)

The TPC contents of the flower extracts were determined using the Folin-Ciocalteu method according to the method describe by Singleton *et al.,* [12] and with some modifications described in Janarny and Gunathilake [13]. Briefly, 100 µL of 0.5 N Folin–Ciocalteu reagent was mixed with 500 µL ethanolic extracts and the mixture was incubated at room temperature in dark for 15 minutes. Then 2500 µL of 7.5%sodium carbonate was added and further incubated for 2 hours in the dark. After incubation the absorbance of the mixture was measured at 760 nm using a UV/VIS spectrometer (840-210800 Thermo Fisher Scientific, USA). The concentration of total phenols was expressed as mg gallic acid equivalents (GAE) per g dry weight (DW) of flowers.

2.6 Determination of total anthocyanin content (TAC)

TAC of the flower extracts was determined based on the pH-differential method described in Janarny *et al.,* [14]. Accordingly, 500 µL of extract was mixed with 3500 µL potassium chloride buffer (0.025 M, pH 1) or 3500 µL of sodium acetate buffer (0.025 M, pH 4.5) separately and incubated for 15 min. After incubation, The absorbance of the resulting mixture was measured at 510 and 700 nm. The difference in the absorbance was calculated as follows: $A = [(A_{510} - A_{700})pH\ 1.0 - (A_{510} - A_{700})pH\ 4.5]$. The concentration of monomeric anthocyanin was calculated using the formula, absorbance × MW × dilution factor ×1000)/(ε × 1, where the molar absorptivity (ε) and molecular weights (MW) of cyanidin-3-glucoside was ε = 26900; MW = 449.2 respectively. Results were expressed as milligrams of cyanidin 3-glucoside equivalents (cy-3-glu) per gram of DW of flowers.

Table 1. Central composite design for process variables and corresponding response variables

Runs	Process variables				Response variables		
	Solid : Liquid ratio (X_A-W/V)	Ethanol concentration (X_B-%)	Temperature (X_C-°C)	Time (X_D-minutes)	TPC (mg GAE/g DW)	TAC (µg Cy-3-Glu/g DW)	DPPH (% scavenging /g DW
1	40	40	30	30	98.74	33.13	56.32
2	30	130	45	45	96.34	23.08	57.19
3	30	70	45	45	134.06	27.73	57.54
4	20	40	60	60	112.80	15.50	55.03
5	40	40	60	30	133.03	18.60	54.56
6	20	100	30	60	157.37	1.18	62.79
7	30	70	45	45	97.20	30.78	56.25
8	40	100	30	60	267.89	10.69	63.73
9	30	70	45	45	114.34	35.27	59.66
10	20	40	30	30	89.37	15.71	56.25
11	40	100	60	30	238.17	9.83	64.03
12	40	100	60	60	271.31	5.34	65.05
13	30	70	45	45	122.91	26.77	57.68
14	20	100	60	60	169.94	8.55	64.86
15	30	70	45	45	94.63	29.34	54.96
16	30	70	45	45	103.20	28.86	58.82
17	30	70	75	45	93.77	39.60	63.11
18	40	100	30	30	214.17	8.98	64.37
19	40	40	60	60	86.17	29.28	55.80
20	20	40	60	30	75.66	17.95	55.65
21	10	70	45	45	60.97	14.16	63.00
22	20	100	60	30	196.23	6.52	73.54
23	30	70	15	45	82.63	31.90	63.26
24	20	40	30	60	73.94	11.22	56.10
25	30	10	45	45	100.63	23.73	64.47
26	30	70	45	15	79.20	29.98	58.49
27	50	70	45	45	110.57	41.15	58.02
28	40	40	30	60	118.17	18.38	53.58
29	30	70	45	75	91.20	27.89	58.29
30	30	70	45	45	93.77	18.92	70.53
31	20	100	30	30	141.94	36.66	75.45

2.7 Determination of 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

As described by Kumari and Gunathilake., [15], 400 µL of flower extract was mixed with 3600 µL of ethanolic DPPH (100 µM) solution and incubated at 37°C for 30 minutes in the dark. After 30 min of the reaction, the absorbance of the remaining DPPH was measured at 517 nm against blank using a UV–Visible spectrophotometer. Percentage of DPPH radical scavenging was calculated using the formula;

% Scavenging = ($A_{control}$– A_{sample})/$A_{control}$ * 100

A_{sample} and $A_{control}$ denotes the absorbance of the samples and control respectively. Control was prepared by replacing the samples with the solvent.

3. Results and Discussion

3.1 Modelling using CCD

RSM is a widely used mathematical and statistical technique for modeling and analyzing a process in which the associated response is manipulated by different independent variables. CCD was used to determine the effect of four process parameters (Ethanol percentage, extraction time, temperature and solid to liquid ratio) on TPC, TAC and DPPH radical scavenging activity of Marigold flowers. The CCD consists of three types of points factorial points, a central point, and axial points which are at α distance from the central point. CCD is appropriate for progressive experimentation and provides acceptable range of information for testing lack-of-fit while not involving an unusually large number of experimental runs [16]. The levels for each independent variables were selected based on literature. The observed values of 31 experimental runs are presented in **Table 1**. The experimental data was analyzed by ANOVA and the significance of the regression coefficients were evaluated by their corresponding p-values **(Table 2)**. Modeling of the extraction parameters were carried out using second order polynomial equation. Fitting the model to the experimental data allowed estimation of the model coefficients. Some of them were not significant at a confidence level of 95% ($p > 0.05$) and were eliminated from the full model to build a reduced model with improved predictive capabilities.

In the current study, the ANOVA table revealed that all model responses (TPC, TAC and DPPH) were significant ($p<0.05$) while the lack of fit was insignificant for all model responses ($p>0.05$). The non-significant lack of fit indicates that the model term adequately explains the relationship between the process parameters and model responses. Also, it suggests that the models have a good predictive capacity and could be applied to predict the studied responses. Additionally, the visual interpretation of the interaction effects between process parameters towards the model responses is illustrated by the three-dimensional (3D) response surface plots.

3.2 Effect of process variables on TPC

Based on the experimental data a full model was generated and after the elimination of all insignificant extraction variables, the final reduced model generated for TPC by fitting the second order polynomial equation could be expressed as follows,

$$\textbf{TPC (mg GAE / g DW)} = 231 - 2.49\,X_B - 4.06\,X_A + 0.0089\,X_B^2 + 0.0504\,X_{AB} + 0.0113\,X_{BD} \quad \textbf{Eqn. 2}$$

From the outcomes of ANOVA, it was observed that the model p value was 0.028 and lack of fit was insignificant with the p value of 0.129, indicating that the proposed model is well fitted. Also the model displayed a good model prediction with $R^2 = 0.7594$ and Adj.$R^2 = 0.7320$. As suggested by the model, the variables ethanol concentration and solid:liquid ratio had a negative coefficient, indicating that the linear effects of these two variables negatively influence the yield of phenolics. Positive quadratic coefficient of ethanol concentration and positive interactive coefficients of solid to liquid ratio and ethanol concentration as well as interactive effects of ethanol concentration and extraction time concludes that positive changes in these variables can positively influence the TPC.

The TPC of the flower extracts were within the range of 60.97 to 271.31 mg GAE/g DW with the lowest and highest TPC observed in experimental runs 21 and 12 respectively. As illustrated in **Figure 1**, interactive effects of process variables on the yield of phenolics from Marigold flowers was

visualized using the response surface plots. As noted in **Figure 1 (a)**, at combinations of lower solid:liquid ratio and extraction times the observed yield was low and when gradually increasing the solid:liquid ratio the yield of phenolics have increased significantly ($p<0.05$) at lower extraction times. Though a slight increase in the yield was observed when increasing the extraction time to 60 minutes, it was not significant. The surface plots for the interactive effects of solid:liquid ratio and temperature **(Figure 1 d)** indicates that the yield has significantly increased when increasing the solid:liquid ratio whereas, increase in temperature at a fixed solid:liquid ratio slightly increased the yield. The above observations based on solid:liquid ratio could be explained using the mass transfer principle, which states that increasing solid:liquid ratio increases the concentration gradient facilitating more migration of phenolics from the flower matrix to the extraction solvent [17]. Also, when increasing the solid:liquid ratio it dilutes the concentration of the phenolics present in the surface and establishes more higher concentration gradient between the external and internal surface leading to higher leaching out rate [18]. This explains the observation where lower yield was observed at lower solid:liquid ratio. Migration of phenolics occur from the flower matrix to the solvent until the equilibrium is reached. Once the equilibrium is reached, yield of active components will not continue to increase [19] and this justifies the observation where lower extraction times lead to lower yield and high extraction times did not significantly increase the yield.

When considering the impact of ethanol concentration, despite the interactive effects of the other three variables, increasing ethanol percentage has increased the extractable content of phenolics from Marigold flowers. Under the fixed extraction parameters, type and composition of the extraction solvent plays an important role in the extraction efficacy of phenolics. Among the widely used organic solvents for extraction of phenolics from plant matrix, ethanol is highly recommended due to its broader spectrum of extraction compounds, low toxicity and acceptable for food-based applications [20]. Ethanol is capable of extracting phenolics and its glycosides as well as non-phenolic compounds from the matrix. Rather than the use of absolute ethanol, use of aqueous ethanol promotes the hydration of particles facilitating the penetration of the organic solvent into the matrix and, consequently, intensifying mass transfer by diffusion [21]. In the current study, increasing ethanol percentage up to 50% did not significantly increase the yield however further increase in the ethanol percentage increased the recovery of phenolics. As documented by previous studies TPC extracted from Sorghum shells and *Phaleria macrocarpa* fruits were maximized at 60% ethanol [22,23]. Interactive effects of temperature with ethanol concentration and solid:liquid ratio did not influence the TPC significantly, however increasing temperature more than 40°C significantly improved the TPC extracted from Marigold flowers. The findings of the present study were consistent with the reports of the previous investigations. Spigno et al, [24] has reported that phenolics extracted from grape marc was high at 60°C compared to the content extracted at 28°C.

3.3 Effect of process variables on TAC

The proposed model for the yield of anthocyanins from Marigold shown below had a model p value of 0.04 and insignificant lack of fit with p value of 0.11. The coefficient of determination of the predicted model is 0.81 indicating that it sufficiently explains the response. Based on the ANOVA studies, it is also apparent that the model for TAC is significant and implies that the experimental data well fits the model:

$$\text{TAC (µg cy-3-glu / g DW)} = 21.4 + 1.13\,X_B - 0.42\,X_C - 0.023\,X_A^2 + 0.009\,X_D^2 - 0.0118\,X_{AB} - 0.004\,X_{BC} \quad \text{Eqn.3}$$

Table 2. Regression coefficients and ANOVA results describing the effect of process variables on the total phenolic content, total anthocyanin content and DPPH radical scavenging activity of Marigold flowers and model adequacy

Factor	TPC	TAC	DPPH
Intercept	231	21.4	76.1
Linear		1.80	-0.54
X_A	-4.06		
X_B	-2.49	1.14	0.24
X_C	-0.71	-0.42	-0.58
X_D	-2.08	-0.22	-0.18
Quadratic			
X_A	0.04	-0.02	0.003
X_B	0.008	-0.003	0.004
X_C	0.02	-0.001	0.004
X_D	0.02	-0.009	-0.008
Cross product			
X_{AB}	0.05	-0.01	-0.003
X_{AC}	-0.02	0.003	0.001
X_{AD}	0.02	0.01	0.008
X_{BC}	0.009	-0.004	0.003
X_{BD}	0.01	-0.003	-0.002
X_{CD}	-0.02	0.01	0.002
R^2	0.76	0.80	0.73
Adjusted R^2	0.73	0.78	0.71
p value (model)	0.02	0.04	0.039
p value (Lack of fit)	0.12	0.11	0.42

As suggested by the model, linear effects of ethanol concentration and quadratic effects of extraction time influenced the yield positively whereas the linear effects of extraction temperature, quadratic effects of solid:liquid ratio and interactive effects of solid:liquid ratio and ethanol concentration as well as interactive effects of ethanol concentration and temperature influenced the TAC negatively. The yield of anthocyanins was observed within the range of 1.18 to 41.15 µg cy-3-glu/g DW. When considering the effect of solid:liquid ratio on the yield, it can be noticed that when varying the ratio from 1:20 to 1:40, the yield increased from 11.22 to 41.5 µg cy-3-glu/g DW and then gradually decreased upon further increase in solid:liquid ratio. A similar trend was observed with the other independent variables; ethanol concentration, temperature and time. Moderate concentrations of ethanol around 55% to 60% was suitable to obtain the maximum possible yield. When interacted with temperature, varying the temperature towards higher values at fixed ethanol concentration did not significantly increase the yield. Focusing on the interactive effects of ethanol concentration and time, mid values of the both the variables seemed to be the appropriate combination to effectively extract anthocyanins from Marigold flowers. Moving towards higher values of time as well as ethanol concentration synergistically reduced the TAC. This observation can be supported by the findings of Nour et al., [25], where 60% ethanol led to the extraction of significantly higher contents of anthocyanins from black currants compared to extremely higher ethanol percentages. As illustrated in **Figure 2 (f)**, lower temperature and lower extraction times was found to be suitable to maximize the TAC. Increasing extraction times at fixed temperature did not significantly increase the yield however prolonged extraction times lead to poor yield.

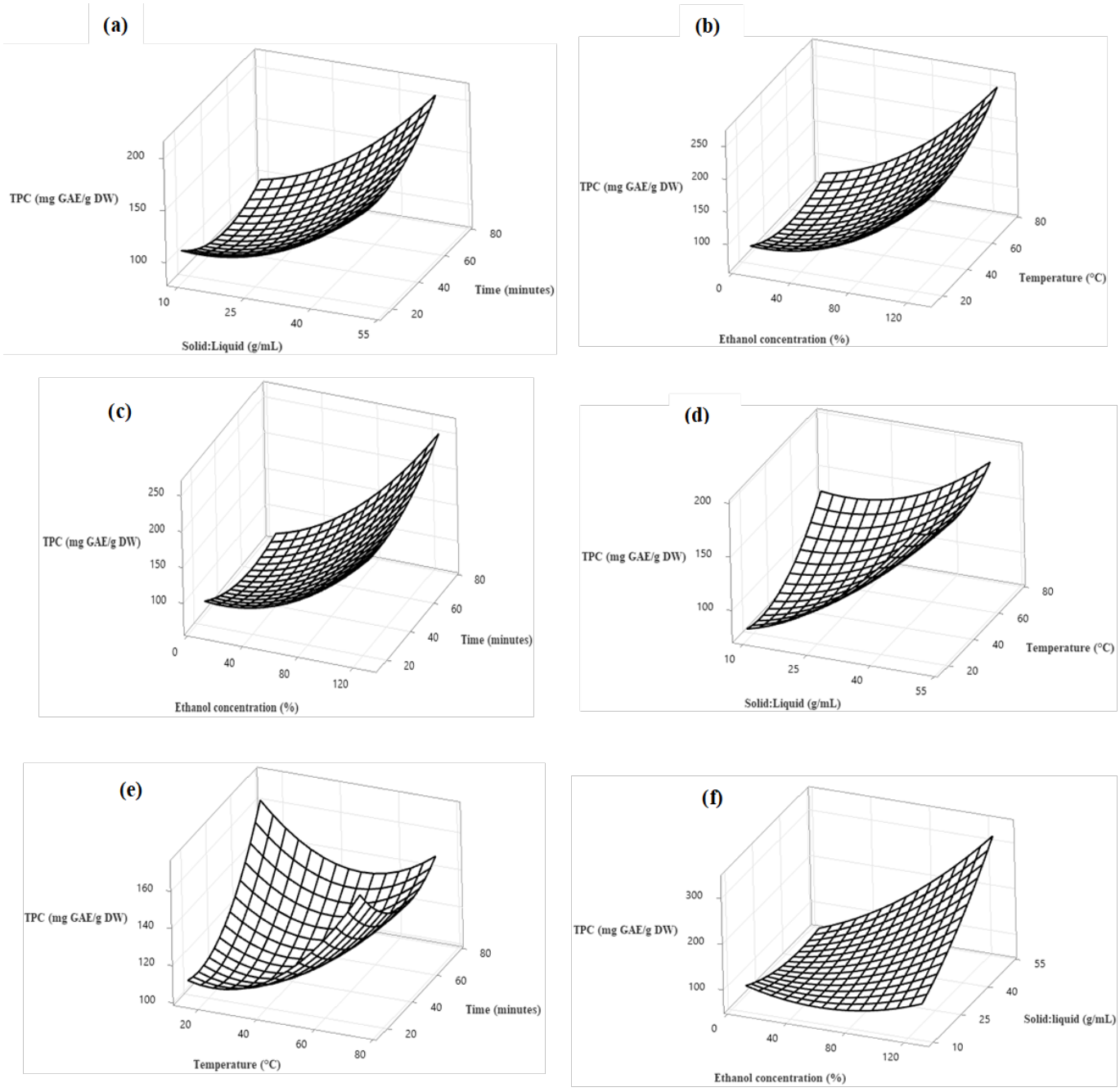

Figure 1. Response surface plots of phenolic extraction (mg GAE /g DW) extraction from *Calendula officinalis* flower as a function of solid to liquid ratio, ethanol concentration, extraction temperature and time. Values were kept constant as follows, Temperature 45°C, ethanol concentration 70%, Extraction time 45 minutes and solid to liquid ratio 1:30.

As noted here, temperature ranges between 45°C to 55°C was acceptable for the maximum extraction of anthocyanins from Marigold flowers. Temperature influences the extraction, since heat enhances the cell wall permeability, solubility of the compounds and the diffusion coefficient of the solvent. However, high temperatures (above 60° C) can degrade some anthocyanins and procyanidins [26]. Also, anthocyanins in high temperatures deteriorate and polymerize to form brown or colorless pigments. Due to the stability of polymeric anthocyanins in different pHs, they cannot be measured by pH differential method, and as a result, total measured anthocyanins could decrease [27].

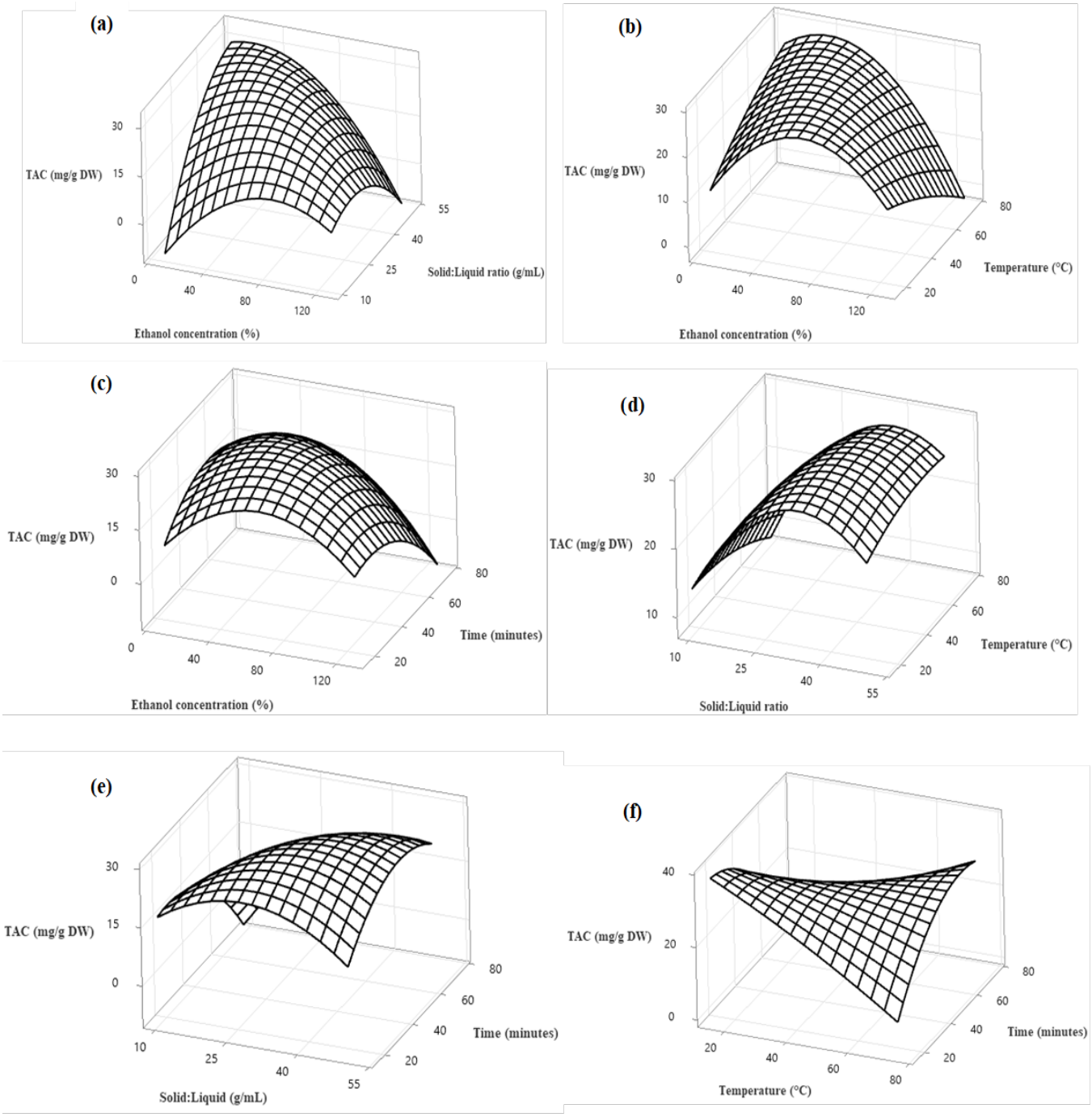

Figure 2. Response surface plots of anthocyanin (mg cyanidin-3-glucoside /g DW) extraction from *Calendula officinalis* flowers as a function of solid to liquid ratio, ethanol concentration, extraction temperature and time. Values were kept constant as follows, Temperature 45°C, ethanol concentration 70%, Extraction time 45 minutes and solid to liquid ratio 1:30.

3.4 Effect of process variables on DPPH radical scavenging activity

Antioxidant activity of the Marigold flower extracts was determined using the DPPH radical scavenging assay. The empirical reduced model obtained for the DPPH radical scavenging activity is shown below,

$$\textbf{DPPH scavenging activity (\%)} = 76.1 + 0.246\,X_B - 1.88\,X_D + 0.0045\,X_C^2 - 0.0014\,X_B^2 - 0.002\,X_{BD} - 0.008\,X_{AD} \quad \textbf{Eqn.4}$$

The obtained p values for the model and lack of fit were 0.039 and 0.42 respectively which confirm the model adequacies. The activity was observed within the range of 53.58% to 75.45% with the lowest and highest activities obtained in the experimental runs 22 and 31 respectively. The positive linear coefficient of ethanol concentration and quadratic coefficient of extraction temperature indicates that the radical scavenging activity is positively influenced by these two variables. The linear effects of extraction time, quadratic effects of ethanol concentration and interactive effects of ethanol concentration and time as well as interactive effects of solid:liquid ratio and extraction time negatively influences the radical scavenging activity.

Based on the response surface plot it was noted that increasing ethanol concentration increased the activity whereas prolonged extraction time decreased the activity. Considering the interactive effects, the lowest time and lower solid:liquid ratio combinations enhanced the activity. **Figure 3 (d)** suggests that at a fixed value of solid:liquid ratio, increasing the temperature increased the activity of the extracts. Combined effect of higher values of ethanol concentration and extraction temperature was observed to enhance the radical scavenging activity.

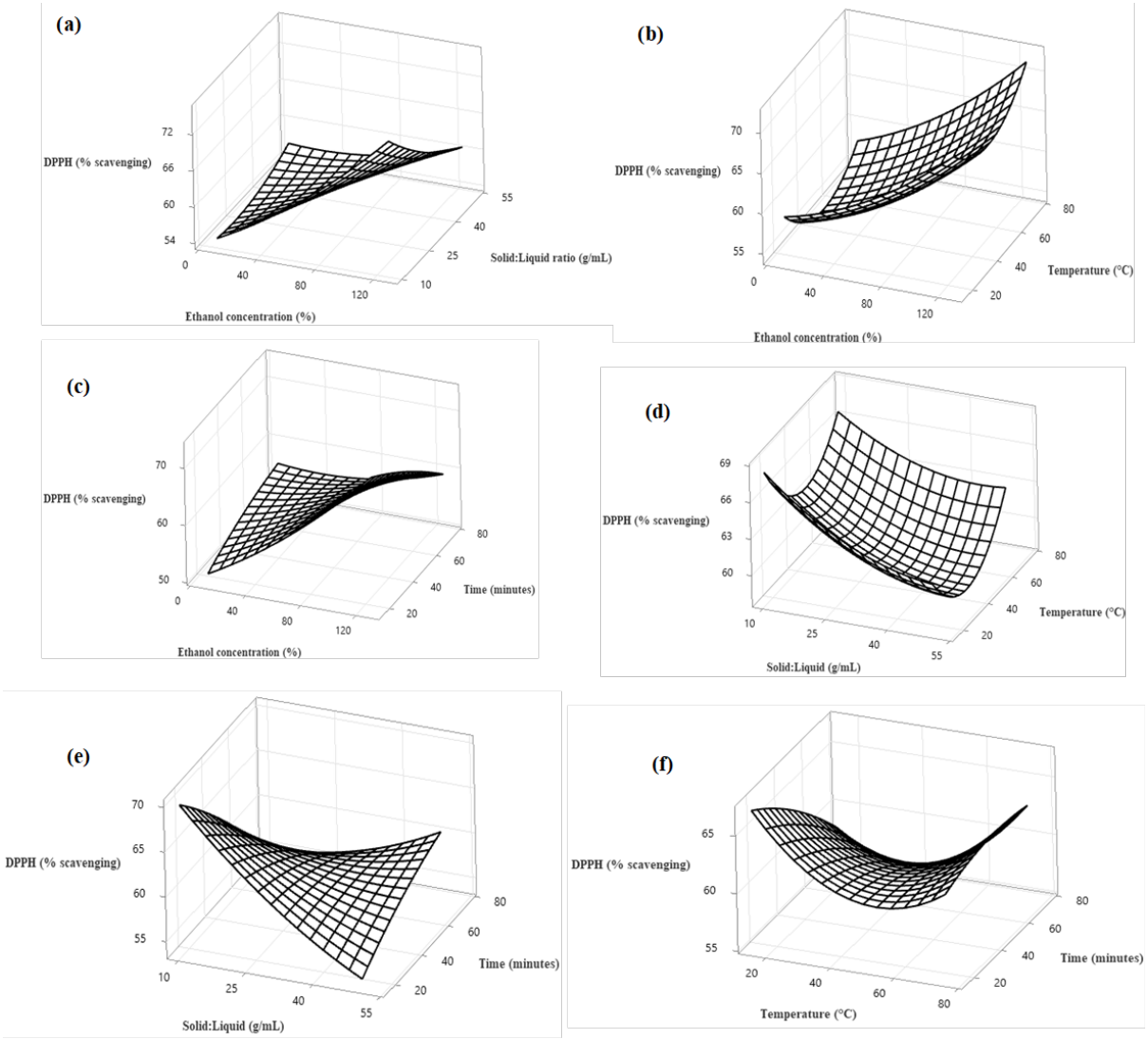

Figure 3. Response surface plots of DPPH radical scavenging activity (% scavenging) of *Calendula officinalis* flowers as a function of solid to liquid ratio, ethanol concentration, extraction temperature and time. Values were kept constant as follows, Temperature 45°C, ethanol concentration 70%, Extraction time 45 minutes and solid to liquid ratio 1:30.

3.5 Optimization of process variables and model validation

The software Minitab version 17 was used to simultaneously optimize the process parameters to maximize the response variables TPC, TAC and DPPH radical scavenging activity. Optimal conditions were obtained using the desirability function in the scale of 0-1. The generated optimum conditions were solid:liquid ratio 1:35, ethanol concentration 65.50%, extraction temperature 35°C and time 32 minutes. An experimental run was conducted with the identified optimum conditions and the experimental values for TPC, TAC and radical scavenging activity were compared with the predicted values to study the suitability of the response models. The obtained values are presented in **Table 3**. There was no statistically significant ($p>0.05$) difference between the predicted and experimental values at 95% confidence interval. The outcomes indicate the reliability of the parameters for the extraction of phenolics and anthocyanins with maximum antioxidant activity from Marigold flowers.

Table 3. Predicted and experimental values of responses under optimum conditions for simultaneous optimization of responses

Responses	Predicted values	Experimental values
TPC (mg GAE/ g DW)	198	197.32±1.89
TAC (µg cy-3-glucoside)	28.3	26.54±1.91
DPPH (% scavenging)	75.7	74.28±1.65

Experimental values are expressed as mean±standard deviation

Conclusion

The present study investigated the optimized conditions to maximize the recovery of phenolics and anthocyanins from Marigold flowers along with maximum DPPH radical scavenging activity. The second order polynomial models generated by RSM were adequate to optimize the process parameters for the extraction of antioxidant compounds based on the satisfactory ANOVA and descriptive statistics parameters. The optimum conditions obtained were: solid:liquid ratio 1:35, ethanol concentration 65.50%, extraction temperature 35°C and time 32 minutes. The findings of the current study is applicable, for the food industry as well as pharmaceutical industry, in order to develop industrial scale process in a cost effective and less labor intensive process for efficient extraction of antioxidant compounds.

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Compliance with Ethical Standards: This article does not contain any studies involving human or animal subjects.

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