

OPTIMIZATION OF PROCESS PARAMETERS FOR EXTRACTION OF POLYPHENOLIC COMPOUNDS FROM WILD CYCLAMEN (*Cyclamen purpurascens* Mill.) TUBERS

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Abstract

The aim of this study was to model the extraction of polyphenolic compounds from wild cyclamen (*Cyclamen purpurascens* Mill.) tubers using a central composite design (CCD). The extractions were performed under reflux using ethanol solutions (20-100%, v/v) at solid-to-liquid-ratio of 1:5-1:30 (m/v) for different extraction time (10-120 min). The extraction parameters were varied according to the CCD matrix in order to investigate their effect on the yield of total polyphenols. The obtained results were fitted using a second-order polynomial model. The analysis of variance was used to estimate the influence of extraction parameters and to determine the fitness of the model. After fitting the experimental data, the response was maximized to obtain the optimal conditions for the extraction of polyphenol compounds using a numerical optimization method. The experimental value of the yield obtained under optimal conditions was agreed with the value predicted using the proposed regression equation.

Key words: wild cyclamen, extraction, central composite design, modeling, total polyphenols

1. INTRODUCTION

Wild cyclamen (*Cyclamen purpurascens* Mill.) is a perennial herbaceous plant which grows from 5 to 15 cm (Fig. 1a). It belongs to the primrose family (Primulaceae). The leaves are kidney, heart shape, which mainly in the upper surface have silvery or white markings. The fragrant flowers are pink and grow during the period from June to September. They consist of 5 petals and the bottom of each is a dark pink colored part. This is a plant with the surface or underground tubers of 4-12 cm in diameter (Fig. 1b). Its habitats are the deciduous forests at the altitude between 250 and 1300 m (Oz *et al.*, 2013). Wild cyclamen grows in eastern France, Switzerland, northern Italy, southern Germany, Austria, Slovenia, Croatia, Bosnia and Herzegovina, Montenegro, Macedonia, Polish, Hungarian, Czech, Slovakia and Bulgaria. In Serbia there are many sites, particularly common in the vicinity of Bujanovac, Predejane, Ruple, Lebane, Vucje, Leskovac, Stalac, Niska banja, Jelasnica, Koritnik, Prizren, *etc.*



Figure 1. Wild cyclamen (a) and wild cyclamen tuber (b)

In folk medicine, the leaves and tubers of wild cyclamen are mainly used. The tubers in the form of a dry powder is used for hose cleaning and in the treatment of strong headache (migraine), anemia, nasal polyps, ulcers, to relieve menstrual problems, rheumatism, various swelling, *etc.* As a tincture, it has application in the ear infections. Spray (Nasodren[®]) prepared from the extract of wild cyclamen tubers is used in the treatment of acute rhinosinusitis, sinusitis, nose infections, as well as nasal polyps (Mullol, 2009; Pfaar *et al.*, 2012; Jurkiewicz *et al.*, 2016). This extract is rich in saponins, which act as a local

surfactant on the mucous membranes to promote the intranasal drainage of fluid from the sinuses through a physical mechanism (Passali *et al.*, 2015).

In the literature, there are data that the tubers extracts of various types of cyclamen (*e.g.*, *Cyclamen graecum* (Metin *et al.*, 2013), *Cyclamen persicum* Mill. (Jaradat *et al.*, 2015), *Cyclamen mirabile* (Sarikurkcü, 2011) possess a high content of antioxidant compounds. Recently, Savic *et al.* (2017) determined the content of total flavonoids and polyphenols in the ethanol extract of wild cyclamen tubers which is 2.60 mg rutin equivalent per gram dry extract and 1.27 mg gallic acid equivalent per gram dry extract, respectively.

The solvent extraction is a process, which widely employed for the phenolic extraction from various plant materials (Savić *et al.*, 2013). The extraction process depending on the various parameters, such as the type of solvent, pH, temperature, extraction time, solid-to-liquid ratio, particle size of the solid matrix, *etc.*, that affect the yield of defined bioactive compounds. The selection of optimal conditions is achieved using the standard optimization method one-variable-at-a-time (OVAT), in which a variable varies how to estimate its effect on the yield, while all other variables are constant, and *vice versa*. The main disadvantage of this method was obtaining a local optimum, which is in contrast to a global optimum.

In order to overcome this problem, the response surface methodology (RSM) has increased application in the optimization of extraction process in recent years (Savic *et al.*, 2015; Savic *et al.*, 2016). The RSM is a suitable mathematical approach for optimization of the extraction process. This method clearly defines the impact of independent variables. The optimization using RSM implies:

1. determination of the independent parameters and their levels,
2. choice of adequate experimental model, prediction and verification of equation model,
3. graphical representation of the model equations and determining the optimum conditions.

Unlike OVAT approach, the RSM simultaneously varies the level of extraction parameters. At the RSM method, the consumption of available resources is reduced due to smaller number of performed experimental runs.

Since the aforementioned results provide important information for the use of wild cyclamen tubers as a source of phytonutrients, the objective of research was to determine the optimal extraction conditions for polyphenolic compounds from wild cyclamen (*Cyclamen purpurascens* Mill.) using the central composite design (CCD) as one of RSM design. The main contribution of this paper was the potential application of the tuber extract enriched with polyphenolic compounds in the treatment of various human diseases.

2. MATERIAL AND METHODS

2.1. Reagents and chemicals

The standard of quercetin-3-O-rutinoside trihydrate (purity 97%) (Alfa Aesar Johnson Matthey Company-, Hays, United Kingdom), aluminum (III) chloride, potassium acetate, Folin-Ciocalteu reagent (AppliChem, Darmstadt, Germany), gallic acid (purity > 97.5%) (Sigma Aldrich, Steinheim, Germany), sodium carbonate, 96% (v/v) ethanol (MosLab doo, Serbia), absolute ethanol (Alkaloid, Macedonia) were used. All other reagents were analytical-grade (p.a.).

2.2. Plant material

Wild cyclamen (*Cyclamen purpurascens* Mill.) tubers are collected in the territory of Southeast Serbia (Rudarska cuka, Leskovac, 42°57'N and 21°58'E). The plant material was first grinded and dried in a dark place at room temperature up to 14.58% (w/w) moisture content. The moisture content in the plant material was less than 15% (m/m), which is in accordance with the recommendation of the World Health Organization (WHO, 2011).

2.3. The extraction process

The milled plant material was quantitatively transferred to the flask of 100 cm³ and treated with the different concentrations of ethanol solution. The total volume of all samples was adjusted at 50 cm³. The extractions were carried out under reflux in a water bath at the boiling temperature of the solvent. In order to optimize the process parameters, the extraction time, ethanol concentration and solid-to-liquid ratio were varied according to the matrix of CCD. The number of experiments necessary to model the extraction process was a total of 20. The solid matrix was separated from liquid phase by vacuum filtration. The liquid extract was evaporated to remove the residual solvent at a vacuum rotary evaporator at 50 °C, and after that dried and stored in a desiccator. Thus prepared extracts were further subjected to analyze the total polyphenol content (TPC).

2.4. Central composite design

In this study, the extraction time, ethanol concentration and solid-to-liquid ratio were analyzed as the independent variables. The extraction parameters were varied in order to investigate their effect on the yield of polyphenolic compounds. The independent variables were observed at 5 levels (- α , -1, 0, 1, + α). The value of coded level α was 1.68, so that the CCD corresponding to the rotatable design. The coded and uncoded values for five factor levels are given in Table 1.

Table 1. Uncoded and coded values of process parameters level

Process parameters	Uncoded	Coded	values of coded levels				
			-1.68	-1	0	+1	+1.68
extraction time, min	τ	X_1	10	32.3	65	97.7	120
ethanol concentration, (% v/v)	C_e	X_2	20	36.2	60	83.8	80
solid-to-liquid ratio, (m/v)	ω	X_3	1:5	1:10.1	1:17.5	1:24.9	1:30

The extraction of polyphenolic compounds was described using a second order polynomial equation, which can be represented by the general equation as follows (Eq. 1):

$$Y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_{11}x_1^2 + a_{22}x_2^2 + a_{33}x_3^2 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{23}x_2x_3 + \varepsilon \quad (1)$$

where are: x_1 , x_2 , x_3 - independent variables, a_0 - intercept, while a_1 , a_2 , a_3 , a_{11} , a_{22} , a_{33} , a_{12} , a_{13} , a_{23} - regression coefficients, Y -response, ε -residue.

The coefficients of polynomial model were determined using the least squares equation. Statistical analysis of experimental data was performed by commercial software Design Expert 7.0.0 (Stat Ease Inc., Minneapolis, USA). The regression coefficients of terms (linear, quadratic and interaction) included in the equation and their effects on the TPC were analyzed by analysis of variance (ANOVA). All terms of the equation were analyzed and verified statistically using F-test with the corresponding confidence level of 95% ($p < 0.05$). The adequacy of the developed models was also analyzed using the coefficient of determination (R^2), adjusted coefficient of determination (R^2_{adj}) and predicted coefficient of determination (R^2_{pre}). After fitting the data, the three-dimensional diagrams were constructed in order to estimate the relationship between the independent variables and their impact on the system response.

2.5. Determination of the total polyphenol content

The TPC in the extracts of wild cyclamen tube was determined according to the Folin-Ciocalteu method (Singleton *et al.*, 1999). The amount of total polyphenol was expressed as mg equivalents of gallic acid per gram dry extract (mg_{GAE}/g). The stock solution of gallic acid (1 mg cm⁻³) was prepared in 96% (v/v)

ethanol, from which a dilution series was made in the concentration range of 5-50 $\mu\text{g cm}^{-3}$. The reaction mixture is prepared by mixing 0.25 cm^3 of standard solution, 2.25 cm^3 of redistilled water, 0.25 cm^3 of Folin-Ciocalteu's reagent and 2.5 cm^3 of sodium carbonate solution (7%, m/m). The absorbance of samples was measured at 740 nm at room temperature after 30 min of incubation. The scans were performed on a Varian Cary-100 Conc UV-Vis spectrophotometer in the quartz cuvettes of 1×1 cm.

3. RESULTS AND DISCUSSION

3.1. Modeling of the extraction process

The composition of the extract and yield of bioactive compounds depends on the used extraction method, as well as the type and polarity of the solvent (Moller *et al.*, 1999). The extraction of polyphenol compounds are mainly performed using acetone, ethanol, methanol and combinations thereof, most frequently in the various proportions with water. In the literature, the most commonly used solvents are ethanol and methanol (Garcia-Salas *et al.*, 2010). According to the literature data, ethanol was used to extract the various types of plant materials, wherein the obtained extract had favorable characteristics in terms of the composition of pharmacologically important compounds (Savić *et al.*, 2013; Savić *et al.*, 2015; Savić *et al.*, 2016). The advantage of ethanol use in compared with other solvents is its non-toxicity and possible further application of the obtained extracts in the pharmaceutical and food industry. The yield of antioxidants with a polar character increases with increasing the polarity of solvents. The solvent polarity increases with increasing the water portion, so that the different ethanol concentrations should be considered as a suitable for the extraction of these compounds.

The optimization of extraction process was carried out in order to estimate the optimal conditions using the CCD. Since that is practically impossible to determine and control all extraction conditions, it was necessary to choose those that have the greatest influence on the extraction process. In this study, the extraction time, ethanol concentration and solid-to-liquid ratio were varied during extraction of polyphenol compounds. The matrix of CCD with three extraction parameters is presented in Table 2.

Table 2. The experimental matrix of CCD with three process parameters and the total polyphenol content

Std.	Run	t, min	Ce, % (v/v)	ω (m/v)	observed TPC	predicted TPC
					(mg _{GAE} /g)	
11	1	65.0	20.0	1:17.5	1.63	1.67
2	2	97.7	36.2	1:10.1	1.20	1.22
13	3	65.0	60.0	1:5.0	0.80	0.78
3	4	32.3	83.8	1:10.1	0.97	1.00
9	5	10.0	60.0	1:17.5	1.28	1.29
20	6	65.0	60.0	1:17.5	1.36	1.35
6	7	97.7	36.2	1:24.9	1.86	1.80
15	8	65.0	60.0	1:17.5	1.30	1.35
17	9	65.0	60.0	1:17.5	1.38	1.35
7	10	32.3	83.8	1:24.9	1.28	1.24
4	11	97.7	83.8	1:10.1	1.05	1.05
1	12	32.3	36.2	1:10.1	1.14	1.11
5	13	32.3	36.2	1:24.9	1.68	1.67

10	14	120.0	60.0	1:17.5	1.43	1.44
16	15	65.0	60.0	1:17.5	1.36	1.35
19	16	65.0	60.0	1:17.5	1.32	1.35
14	17	65.0	60.0	1:30.0	1.42	1.47
18	18	65.0	60.0	1:17.5	1.37	1.35
12	19	65.0	100.0	1:17.5	1.18	1.17
8	20	97.7	83.8	1:24.9	1.30	1.31

t – extraction time; **Ce** – ethanol concentration; **ω** – solid-to-liquid ratio; **TPC** – total polyphenol content; **GAE** – gallic acid equivalent, **std** - standard order.

In Table 2, the observed and predicted values of TPC are also given. The experimental runs are given in random order to avoid that the ordering of experimentation be associated with the level of one or several factors. The TPC in the wild cyclamen tuber extracts was varied in the range of 0.8 to 1.86 mg_{GAE}/g dry extract. These results indicate that the value of TPC was approximately 0.2% in the analyzed extracts.

In order to estimate the effect of independent variables (extraction time, ethanol concentration and solid-to-liquid ratio) on the dependent variable (TPC) and to investigate the suitability of the CCD in the modeling and optimization of the extraction process, a second order polynomial equation was used for fitting the experimental data. The model and their coefficients were obtained by statistical technique of nonlinear regression of experimental data, where the model gives the functional dependency between dependent and independent variables. The equation that described the extraction process consisted of linear and quadratic terms. The most acceptable model is determined by the maximum value of the correlation coefficient (*R*), *i.e.* the determination coefficient (*R*²), which is an indicator of agreement between the experimental and model predicted data and ideally is 1. In other words, a high value of *R*² indicates the adequate fitting of the experimental data. The regression coefficients of polynomial equation and coefficients of determination are given in Table 3. The value of *R*² for the second order polynomial model was found to be 0.9842. This value indicated that 1.58% of the variation in the TPC could not be explained using this regression model. Also, the value of predicted coefficient of determination (0.9010) was in reasonable agreement with the adjusted coefficient of determination (0.9699).

Table 3. The regression coefficient in the polynomial equation

Terms of polynomial equation	Regression coefficient	Standard error
Intercept	1.3479	0.0169
X ₁	0.0445	0.0112
X ₂	-0.1489	0.0112
X ₃	0.2051	0.0112
X ₁ X ₂	-0.0158	0.0146
X ₁ X ₃	0.0072	0.0146
X ₂ X ₃	-0.0784	0.0146
X ₁ ²	0.0069	0.0109
X ₂ ²	0.0246	0.0109
X ₃ ²	-0.0789	0.0109

R^2	0.9842
R^2_{adj}	0.9699
R^2_{pred}	0.9010

Frequently, the selected approximation model of the experimental data does not describe fully the investigated design space. Therefore, the detailed statistical analysis should be conducted in order to more accurately determine the justification of the chosen model. The results of ANOVA of the modeled response are presented in Table 4. The F-value (69.13) of regression model implied that the model was a significant, and there was only a 0.01% chance that this model value could occur due to noise. The lack of fit was not significant relative to the pure error (0.0047), because its value (2.63) was smaller than the critical value (5.05). This indicated that the model was adequate for prediction of the TPC in the wild cyclamen tuber extract. Based on the value of adequate precision, it can be evaluated the signal-to-noise ratio. The adequate precision is desirable to be greater than 4. An adequate precision of the proposed model was 34.9. This value indicated a low signal-to-noise ratio, so that the model could be used to navigate the design space.

The individual effects, the significance of defined factors and their interactions could be explained based on the p-value. When the p-value is lower than 0.05 for certain terms of polynomial equation, they can be considered statistically significant. The statistical significant terms are clearly indicated in Table 4. In other words, all linear terms, interaction between ethanol concentration and solid-to-liquid ratio, as well as the quadratic terms of ethanol concentration and solid-to-liquid ratio were statistical terms. In order to improve this regression model, the insignificant model terms could be excluded from the equation.

Table 4. Analysis of variance for response surface quadratic model

Source	Sum of squares	Degrees of freedom	Mean square	F-value	p-value Prob > F
Model	1.0630	9	0.1181	69.13	< 0.0001 ^a
X ₁	0.0271	1	0.0271	15.86	0.0026 ^a
X ₂	0.3029	1	0.3029	177.29	< 0.0001 ^a
X ₃	0.5743	1	0.5743	336.14	< 0.0001 ^a
X ₁ X ₂	0.0020	1	0.0020	1.17	0.3050
X ₁ X ₃	0.0004	1	0.0004	0.24	0.6328
X ₂ X ₃	0.0492	1	0.0492	28.79	0.0003 ^a
X ₁ ²	0.0007	1	0.0007	0.40	0.5391
X ₂ ²	0.0087	1	0.0087	5.11	0.0474 ^a
X ₃ ²	0.0897	1	0.0897	52.51	< 0.0001 ^a
Residual	0.0171	10	0.0017		
Lack of fit	0.0124	5	0.0025	2.63	0.1561
Pure error	0.0047	5	0.0009		
The total correction	1.0800	19			

^aStatistically significant at the confidence level of 95%.

Pareto chart of standardized effects for the proposed regression model is presented in Fig. 2. The length of the bars in the chart was proportional to the absolute magnitude of the estimated effects coefficients, while the referent line represented the minimal magnitude of statistically significant effects with respect to response. The effects are arranged in descending order of significance.

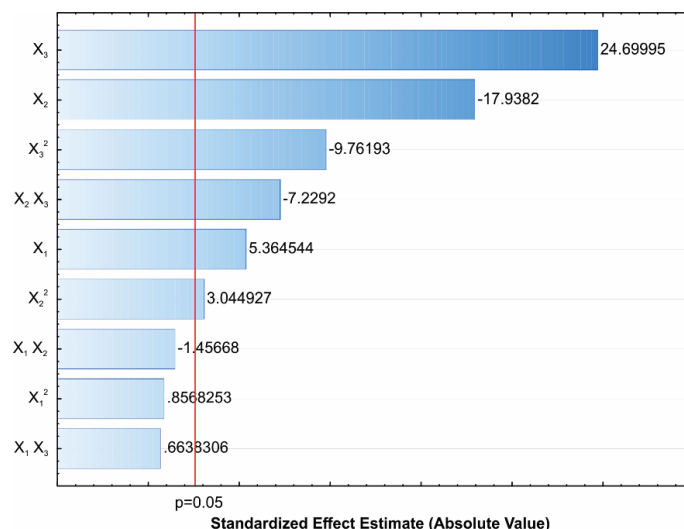


Figure 2. Pareto chart of the standardized effects

Based on the diagram, it can be noticed that the highest effect on the TPC had solid-to-liquid ratio. The second significant effect had ethanol concentration, while extraction time had the lowest effect in compared with other linear terms. Unlike the solid-to-liquid ratio and extraction time, the ethanol concentration had the negative effect on the TPC in the extracts. In other words, the TPC in the extracts will be increased by the use of ethanol solution with higher portion of water during extraction. Pareto chart clearly indicated that the interaction between ethanol concentration and solid-to-liquid ratio was the only significant interaction of polynomial equation. The quadratic effect of extraction time represented the insignificant term in the polynomial equation.

Normal probability plot of the standardized residuals (Fig. 3) was used to define whether the error term ε in the polynomial model was actually normally distributed. In this study, the functional dependency had the low scattering of the data, indicating that the residuals followed the normal distribution.

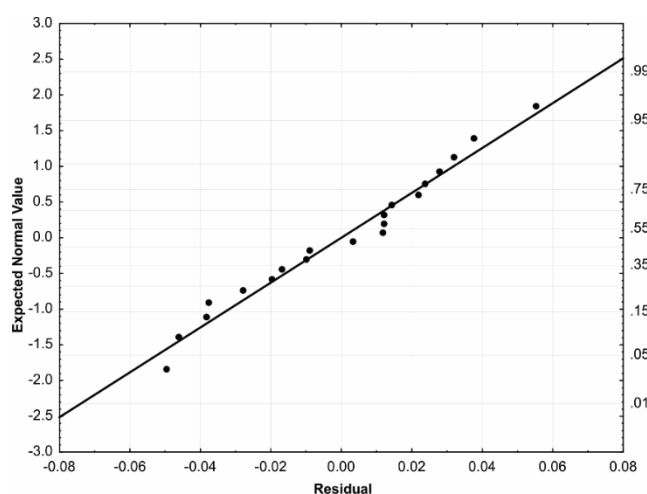


Figure 3. Normal probability plot of the standardized residuals

The three-dimensional diagrams that represent the effect of extraction parameters on the TPC are presented in Fig. 4. Based on these diagrams, it was possible to analyze the interaction between the extraction parameters, *i.e.* their effect on the TPC in the extract. In Fig. 4a, the effect of extraction time and ethanol concentration on the TPC at the solid-to-liquid ratio of 1:17.5 (m/v) is depicted. The impact of extraction time was significant at the higher portions of water in the ethanol solutions, while this effect was negligible at the higher ethanol concentrations. The statement obtained by analysis of Pareto chart that the effect of ethanol concentration was higher than the effect of extraction time was also confirmed by the analysis of three-dimensional diagrams. The impact of extraction time and solid-to-liquid ratio using ethanol concentration of 60% (v/v) is presented in Fig. 4b. The shape of response surface (Fig. 4b) indicated that the solid-to-liquid ratio had the strong effect than other defined factors. This effect was almost the same at the shorter and longer extraction times. It can be seen that the saturation of response surface was achieved at the solid-to-liquid ratio of 1:20 (w/v). The interaction between the ethanol concentration and solid-to-liquid ratio for extraction time of 65 min is depicted in Fig. 4c. The strong interaction between extraction parameters was only noticed in this case, and the conclusion was derived based on the shape of response surface. The area of the highest TPC was found for ethanol concentration up to 50% (v/v) and for solid to liquid ratio between 1:16 and 1:30 (m/v). The increase of ethanol concentration did not have the significant effect on the TPC at the lower solid-to-liquid ratios. This factor had a more pronounced impact at the solid-to-liquid ratio higher than 1:16 (m/v). As already mentioned at the Pareto chart analysis, the increase of ethanol concentration had a negative impact on the TPC in the extracts.

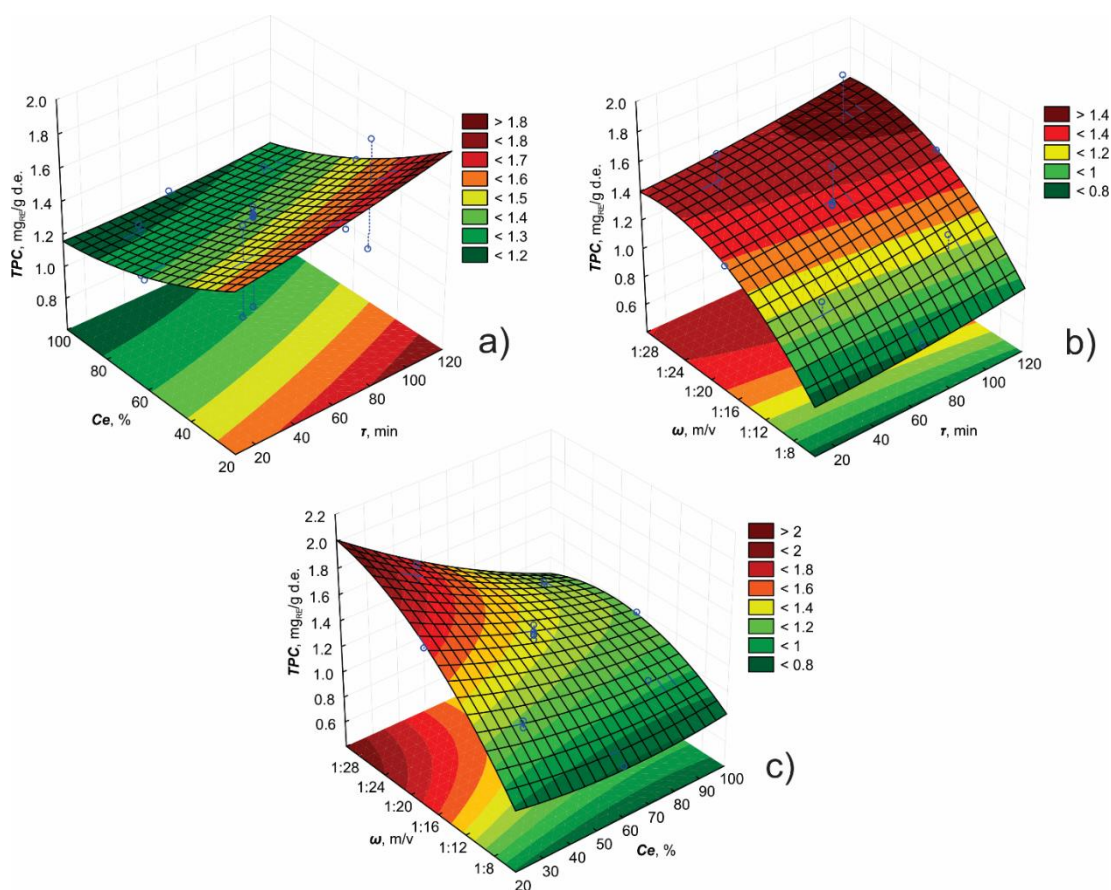


Figure 4. The effect of: a) extraction time and ethanol concentration at the solid-to-liquid ratio of 1:17.5 (m/v); b) extraction time and solid-to-liquid ratio using ethanol concentration of 60% (m/v); c) ethanol concentration and solid-to-liquid ratio for extraction time of 65 min on the total polyphenol content

3.2. Optimization of the extraction process

After modeling of the extraction of polyphenols from wild cyclamen tuber, it was also necessary to optimize this process. The optimization of the process was achieved using numerical optimization method. After processing the data, the optimal conditions were the extraction time of 97.7 min, ethanol concentration of 36.2% (v/v) and solid-to-liquid ratio of 1:25 (m/v). Under these conditions, the proposed regression model predicted the TPC of 1.77 mg_{GAE}/g dry extract. In order to confirm the predictability of the regression model, the extraction of polyphenols from wild cyclamen tuber was carried out at the suggested optimal conditions. After UV-Vis analysis of the obtained extracts using the standard procedure for determination of polyphenols, the TPC of 1.80 mg_{GAE}/g dry extract was determined. Based on this result, it can be also concluded that the polynomial model can successfully predict the yield of polyphenols. This conclusion has already derived because of the results obtained after ANOVA analysis.

4. CONCLUSION

In this study, the CCD with three factors at five levels was successfully employed to optimize and study the individual and interactive effects of the process variables. The effect of extraction time, ethanol concentration and solid-to-liquid ratio was monitored on the extraction yield of polyphenolic compounds from wild cyclamen tuber. The experimental data for the extraction yield was fitted using the second order polynomial mathematical model. This developed model for prediction of TPC had the high coefficient of determination (R^2) value (more than 0.98), indicating that 98.428% of the variation in the TPC could be explained using this regression model. Statistical and graphical analysis showed that the solid-to-liquid ratio was the most influential factor. The interaction effect of ethanol concentration and solid-to-liquid ratio was the most significant effect in compared with other interactions, which were statistically insignificant in the polynomial equation. Instead of all analyzed factors, the upper levels of ethanol concentration prevented a better extraction of polyphenols. The optimal conditions to obtain the maximum extraction yield of polyphenols were determined as follows: the extraction time of 97.7 min, ethanol concentration of 36.2% (v/v) and solid-to-liquid ratio of 1:25 (m/v). Under these conditions, the experimental value (1.80 mg_{GAE}/g dry extract) was agreed well with the predicted value (1.77 mg_{GAE}/g dry extract).

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