Modeling and Optimizing of Microwave-Assisted Extraction of Antioxidants and Phenolics from Wormwood (*Artemisia absinthium* **L.**) Using Response Surface Methodology

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ABSTRACT: In this study, modelling and optimizing microwave-assisted extraction (MAE) of antioxidants and phenolics from wormwood (*Artemisia absinthium* L.) was performed by using response surface methodology with face-centered composite design as factors of temperature, extraction time, solvent concentration, and solid-to-solvent ratio. The MAE process factors were optimized so that cupric reducing antioxidant capacity (CUPRAC) and total phenolic content (TPC) of the wormwood extract are maximized. All of the models calculated for the two responses (CUPRAC and TPC) were found significant ($p < 0.0001$) to show the relationship between the response and independent parameters. Extraction temperature was found as the most significant operational factor in MAE. Extraction time was found is the most insignificant parameter in MAE. The data obtained by the experimental model and the predicted by the model were found to be strongly accordance. It shows the suitability of the model and its success in optimization. The CUPRAC and TPC yields were obtained as 1.22 and 1.42 mmol TR/g-dried sample under the optimum operational conditions of MAE. According to the CUPRAC and TPC values, under the same operational conditions, MAE method was found to be approximately two times more efficient than classical heat extraction. As a result, the modeled methodology can be applied to the extraction of antioxidant and phenolics from the wormwood in the natural product industry.

Keywords: Wormwood, microwave-assisted extraction, cupric reducing antioxidant capacity, total phenolic content

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INTRODUCTION

Reactive free radicals can cause protein denaturation, lipid peroxidation, degradation of membrane fluid, oxidation of DNA, and change of platelet function in the human body (Fridovich, 1978; Kinsella et al., 1993). In this case, many serious health problems may occur such as inflammation, atherosclerosis, Alzheimer's, and cancer. Nowadays, many synthetic antioxidants are used to combat radicals that cause oxidative damage. However, recent research has shown that synthetic antioxidants can cause serious vital problems such as carcinogenesis and liver damage (Yuan et al., 2008). For these reasons, unlike synthetic antioxidants, the use of natural antioxidants to combat oxidative damage is becoming increasingly important (Kan et al., 2015).

Wormwood (*Artemisia absinthium* L.), a member of the Asteraceae family, is a species of plant with more than 200 species spreading from Europe to Asia (Singh et al., 2012; Şahin et al., 2013). Wormwood has traditionally been used as antipyretic, antimicrobial, antiseptic, diuretic, antifungal, and for treatment of stomach pains, leukemia, and sclerosis (Kordali et al., 2005; Lopes et al., 2008). Wormwood extracts have occasionally been used as a muscle relaxant or as a mild sedative (Canadanovic-Brunet et al., 2005). It is also an aromatic spice used as a sweetener in alcoholic and non-alcoholic beverages (Lachenmeier et al., 2006). Wormwood is also known as a good antioxidant source due to its phenolic, flavonoid, and other biologically active compounds (Hoffmann and Herrmann, 1982; Mahmoudi et al., 2009).

The development of new extraction methods for antioxidants has come to the forefront with the increase of interest in the natural plant-based antioxidant-rich diet for last years. Microwave-assisted extraction (MAE) is the process by which the solvent to be used to pass the analytes from the sample matrix to the appropriate solvent mixtures is heated by microwave energy (Eskilsson and Björklund, 2000). One of the critical advantages of this method is the rapid heating of the solvent mixture. It is possible to transfer mass at high temperatures by using closed vessel MAE systems. Other important advantages are the fact that it can be done in minutes rather than hours and using volumes about ten times lower than traditional methods (Kaufmann and Christen, 2002; Routray and Orsat, 2002). The MAE method also serves to the green analytical chemistry with features such as short time, low volume, and the possibility to study multiple samples at the same time. The MAE conditions are optimized by using factorial, central composite, and orthogonal array designs to perform an efficient extraction. The most studied parameters are solvent volume, process temperature, solvent type, process time, and amount of water in the solvent, respectively (Eskilsson and Björklund, 2000).

The MAE was used for antioxidants and phenolics extraction from the wormwood, and the effective parameters of the method were optimized using RSM in this study. In the optimization process, it is aimed to have the maximum cupric reducing antioxidant capacity (CUPRAC) and total phenolic content (TPC) of the wormwood extract. The optimized parameters are extraction temperature, extraction time, solid-to-solvent ratio, and solvent concentration, respectively. In addition, the effects of operational factors on CUPRAC and TPC were discussed. As a result, the optimized MAE method is presented as a simple, rapid, cost-effective, and efficient alternative to the extraction of antioxidants and phenolics from wormwood.

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MATERIALS AND METHODS

Materials

The Folin-Ciocalteau reagent, Neocuproine (Nc), ammonium acetate, copper(II) sulfate, and ethanol (EtOH) were purchased from Sigma-Aldrich; potassium sodium tartrate tetra hydrate, sodium carbonate, copper(II) chloride dihydrate, and sodium hydroxide were supplied from Merck. Wormwood was supplied from the medicinal plant garden belonging to Zeytinburnu Municipality in Istanbul (Turkey).

Microwave-assisted Extraction (MAE)

The air-dried raw wormwood sample was ground before MAE process. MAE process of wormwood was performed under various operational parameters such as temperature (50 - 100 °C), time (1 - 10 min), solvent concentration (20-80%, ethanol in water), and solid-to-solvent ratio (0.1 - 0.4 g/20 mL). This study was performed by using MAE system with closed vessels and the microwave power (0-1500 W) was adjusted automatically inside the oven by using the fiber optic temperature probe according to temperature. The extracts obtained as a result of each treatment were cooled, filtered, and stored at 4 °C until analysis.

CUPRAC Measurement

CUPRAC of wormwood extract was determined by using the CUPRAC method (Apak et al., 2004). CUPRAC method is based on the reduction of Cu(II)-Nc (chromogenic reagent) to Cu(I)–Nc by receiving an electron from antioxidants, and the absorbance changes was measured at 450 nm.

In this method, x ml of sample was added to 3 mL of reagent mixture (consisting of 1 mL of 10 mM $CuCl_2$, 7.5 mM Nc, and 1.0 M NH_4Ac), and distilled water is added to make the total volume is 4.1 mL. The absorbance was recorded at 450 nm after 30 min. The CUPRAC was expressed as trolox (TR) equivalent (mmol TR/g-dried sample (DS)).

TPC Measurement

The TPC of the wormwood extract was measured according to the Folin-Ciocalteau assay (Singleton et al., 1999). Reagents were prepared as follow: "Lowry A" solution consists of 2% Na_2CO_3 in 0.1 M NaOH; "Lowry B" solution consists of 0.5% $CuSO_4$ in 1% $NaKC_4H_4O_6$; "Lowry C" solution is a mixture of Lowry A solution (50 mL) and Lowry B solution (1 mL).

In this method, x mL of extract, 2.5 mL of Lowry C, and (1 - x) mL of distilled water were added to the test tube. After test tube was stand for 10 min, 0.25 mL of Folin reagent (three times diluted) was added to the mixture. Finally, the absorbance was recorded at 750 nm against a reagent blank after 30 min at room conditions. The TPC was expressed as TR equivalent (mmol TR/g-DS).

Statistical Analysis

Response surface methodology (RSM) with face-centered composite design (FCCD) (Design-Expert® Software Version 11) was used to analyze and optimize the experimental data. Four independent variables were chosen (X_1: extraction temperature, X_2: extraction time, X_3: solvent concentration (%, ethanol in water), and X_4: the solid-to-solvent ratio) in design study. The CUPRAC (Y_1) and TPC (Y_2) were selected as the dependent variable (two responses). Based on the experimental data, the independent variables and levels were determined as summarized in Table 1. These independent variables and their levels were entered into Design-Expert® Software, the experimental plan was obtained as presented in Table 2. It also shows the values of response calculated from experimental data.

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The quadratic model is fitted to the experimental data in this study. This model equation is shown in Eq. 1 (Stadler et al., 2002).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i\, x_i + \sum_{i=1}^{k} \beta_{ii}\, x_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij}\, x_i x_j + \varepsilon \tag{1}$$

In Eq. 1, β_0 is the constant regression coefficient, β_i, β_{ii}, β_{ij} are the interaction coefficients, k is the factor number, and ε is experimental error.

Table 1. The independent variables and levels with their units and symbols

Independent variables	Symbol of the variables	Units	Levels		
			-1	0	1
Temperature	X_1	°C	50	75	100
Time	X_2	min	1	5.5	10
Solvent concentration	X_3	%, v/v	20	50	80
Solid-to-solvent ratio	X_4	g/20 mL	0.1	0.25	0.4

RESULTS AND DISCUSSION

Table 2 summarizes the effects of independent variables on the CUPRAC and TPC of wormwood extract obtained by MAE on the experimental design. The CUPRAC values of extracts were found ranged from 0.374 to 1.193 mmol TR/g-DS. As for the TPC values changed between 0.436 and 1.402 mmol TR/g-DS.

Table 2. FCCD of the independent factors for the MAE and experimental results for the CUPRAC and TPC

Run No	Factors				CUPRAC (mmol TR/g-DS)	TPC (mmol TR/g-DS)
	X_1	X_2	X_3	X_4		
1	50	1	20	0.1	0.619	0.727
2	100	1	20	0.1	1.116	1.395
3	50	10	20	0.1	0.679	0.823
4	100	10	20	0.1	1.128	1.402
5	50	1	80	0.1	0.896	1.163
6	100	1	80	0.1	1.098	1.356
7	50	10	80	0.1	0.899	1.168
8	100	10	80	0.1	1.105	1.368
9	50	1	20	0.4	0.374	0.436
10	100	1	20	0.4	1.141	1.398
11	50	10	20	0.4	0.441	0.484
12	100	10	20	0.4	1.109	1.393
13	50	1	80	0.4	0.588	0.632
14	100	1	80	0.4	1.193	1.158
15	50	10	80	0.4	0.672	0.815
16	100	10	80	0.4	1.148	1.268
17	50	5.5	50	0.25	0.560	0.663
18	100	5.5	50	0.25	1.113	1.275
19	75	1	50	0.25	1.015	1.253
20	75	10	50	0.25	1.144	1.298
21	75	5.5	20	0.25	1.109	1.207
22	75	5.5	80	0.25	0.969	1.183
23	75	5.5	50	0.1	1.190	1.288
24	75	5.5	50	0.4	1.061	1.242
25	75	5.5	50	0.25	1.177	1.320
26	75	5.5	50	0.25	1.156	1.331
27	75	5.5	50	0.25	1.169	1.29
28	75	5.5	50	0.25	1.153	1.311
29	75	5.5	50	0.25	1.165	1.305
30	75	5.5	50	0.25	1.158	1.318

Modeling and Optimization of MAE Using RSM

Models calculated for the CUPRAC and TPC were found to be significant in terms of showing the relationship between response and independent factors (p<0.0001). Extraction temperature was the most significant operational factor on the CUPRAC and TPC responses. Table 3 shows the results of fitting quadratic models to the data. The results of the analysis of variance (ANOVA) shows that the contribution of the quadratic model was significant.

Table 3. The ANOVA for the quadratic equations for the CUPRAC and TPC

	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model (CUPRAC)	1.975	14	0.1249	27.31	< 0.0001
X_1	1.09	1	1.09	237.54	< 0.0001
X_2	0.0045	1	0.0045	0.9863	0.3364
X_3	0.0403	1	0.0403	8.81	0.0096
X_4	0.0559	1	0.0559	12.22	0.0033
X_1X_2	0.0046	1	0.0046	1.01	0.3307
X_1X_3	0.0497	1	0.0497	10.87	0.0049
X_1X_4	0.0844	1	0.0844	18.44	0.0006
X_2X_3	0.0002	1	0.0002	0.046	0.8331
X_2X_4	4.0×10^{-6}	1	4.0×10^{-6}	0.0009	0.9768
X_3X_4	0.0004	1	0.0004	0.0874	0.7715
X_1^2	0.1534	1	0.1534	33.52	0.0001
X_2^2	2.4×10^{-7}	1	2.4×10^{-7}	0.0001	0.9943
X_3^2	0.0043	1	0.0043	0.9430	0.3469
X_4^2	0.0054	1	0.0054	1.18	0.2941
Residual	0.0686	15	0.0046		
Lack of Fit	0.0682	10	0.0068	83.20	< 0.0001
Pure Error	0.0004	5	0.0001		
Cor Total	1.82	29			
Model (TPC)	2.39	14	0.0653	44.45	< 0.0001
X_1	1.45	1	1.45	377.19	< 0.0001
X_2	0.0139	1	0.0139	3.64	0.0758
X_3	0.0398	1	0.0398	10.37	0.0057
X_4	0.1930	1	0.1930	50.35	< 0.0001
X_1X_2	0.0027	1	0.0027	0.7053	0.4142
X_1X_3	0.1905	1	0.1905	49.7	< 0.0001
X_1X_4	0.0915	1	0.0915	23.87	0.0002
X_2X_3	0.0017	1	0.0017	0.4385	0.5179
X_2X_4	0.0029	1	0.0029	0.7606	0.3969
X_3X_4	0.0186	1	0.0186	4.86	0.0435
X_1^2	0.1763	1	0.1763	45.97	< 0.0001
X_2^2	0.0054	1	0.0054	1.41	0.2535
X_3^2	0.0031	1	0.0031	0.8196	0.3796
X_4^2	0.0032	1	0.0032	0.8362	0.3750
Residual	0.0575	15	0.0038		
Lack of Fit	0.0566	10	0.0057	29.79	0.0008
Pure Error	0.0009	5	0.0002		
Cor Total	2.44	29			

The quadratic models for the CUPRAC and TPC are summarized in Eqs. (2)–(3), respectively. The significance of each coefficient was obtained by using F-test and p-value from Table 3.

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$$CUPRAC = -1.77026 + 0.067929X_1 + 0.016546X_2 + 0.011557X_3 - 2.89086X_4 - 0.000151X_1X_2 - 0.000074X_1X_3 + 0.019367X_1X_4 - 0.000027X_2X_3 - 0.000741X_2X_4 + 0.001111X_3X_4 - 0.000389X_1^2 - 0.000015X_2^2 - 0.000045X_3^2 + 2.03080X_4^2 \qquad (2)$$

$$TPC = -2.05864 + 0.076805X_1 - 0.018756X_2 + 0.017827X_3 - 271538X_4 - 0.000116X_1X_2 - 0.000146X_1X_3 + 0.020167X_1X_4 + 0.000076X_2X_3 + 0.02X_2X_4 - 0.007583X_3X_4 - 0.000417X_1^2 + 0.002256X_2^2 - 0.000039X_3^2 + 1.56335X_4^2 \qquad (3)$$

The models obtained for the CUPRAC and TPC were found significant ($p > 0.05$). Furthermore, the predicted R^2 of 0.8470 and 0.9002 are in reasonable agreement with the adjusted R^2 of 0.9270 and 0.9545 (i.e. the differences are less than 0.2) for the CUPRAC and TPC respectively. It is desirable that the adequate precision value measuring the signal to noise ratio is greater than 4. Adequate precision ratio of the CUPRAC and TPC were found as 17.944 and 22.807, respectively. Independent variables of the MAE were optimized to have the maximum CUPRAC and TPC responses via Design-Expert program. The highest CUPRAC (1.22 mmol TR/g-DS) and TPC (1.42 mmol TR/g-DS) yields were obtained at the following conditions of $X_1 = 79$ °C, $X_2 = 7$ min, $X_3 = 49\%$ and, $X_4 = 0.13$ g/20 mL.

Effects of Operational Factors on MAE

The 3D response surface plots in Figures 1-3 were drawn to illustrate according to the Eqs. 2 to 4 to predict the interaction among the different factors and their corresponding effect on the CUPRAC and TPC responses. Figs. 1a and b show that the CUPRAC and TPC responses had a similar trend with respect to extraction temperature. The extraction temperature is one of the most critical parameters investigated for MAE as in all other extraction types. This fact confirms that our study has shown that temperature is the most effective factor in the extraction of antioxidants and phenolics from wormwood by MAE. In this study, the closed vessel system used for MAE. With the closed vessel system, extraction process can be applied beyond the boiling point of the solvent. The extraction efficiency is increased with the higher temperature due to the ease of dissolution of target compounds from the active sites in the sample matrix (Eskilsson and Björklund, 2000). In addition, due to temperature, the surface tension and viscosity of the solvent decreases, which cause sample wetting and increase matrix penetration, and consequently increase the solvent's solving capacity (Stadler et al., 2002; Chen et al., 2007). When Figs. 1a and b are examined, both the CUPRAC and TPC values increase with temperature. The temperature of 79 °C is determined as the optimum temperature. In the literature, it has been reported that structural decomposition of antioxidants and phenolics has begun after 80 °C (Wettasinghe and Shahidi, 1999; Spigno et al., 2007; Bener et al., 2013; Bener et al., 2016). As a result, it is not preferred to work at very high temperatures considering both efficiency decrease and economy and safety parameters.

The extraction times with MAE are considerably low compared to other techniques. In most of the studies in the literature, it was reported that the extraction efficiency increased over time, but after a certain period the increase was relatively lower (Mandal et al., 2007; Wang et al., 2008). The results of this study coincide with the literature. The extraction of the antioxidants and phenolics increased relatively with the extraction time and the optimum extraction time was determined as 7 min. It should be noted that an additional 3 min process was applied to the samples to reach the desired temperature in each extraction process. Solvents with a high dielectric constant (ethanol, methanol, etc.) have very high heating rates and, when mixed with water, their heating capacity increases. The use of long-term microwave energy in MAE processes where such solvent mixtures are applied can cause the analytes to

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warm up and decompose (Routray and Orsat, 2002). As a result, long-term MAE processes should be avoided due to both economic reasons and the risk of degradation of the analytes.
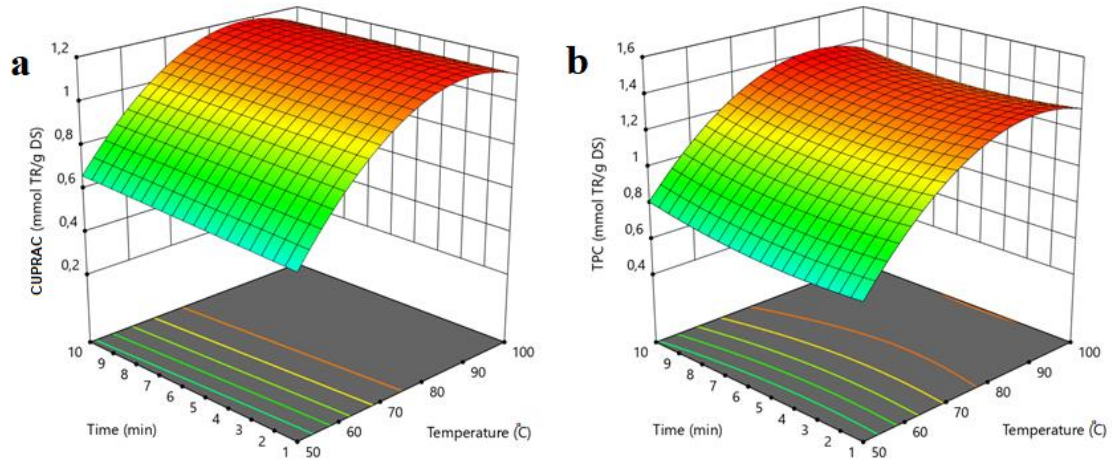

Figure 1. The 3D surface plot for the (a) CUPRAC and (b) TPC of the wormwood extract as a function of extraction time to extraction temperature

Figs. 2a and b show that the CUPRAC and TPC responses have similar trends with respect to solvent concentration. Polar solvents are popular solvent types for the extraction of polyphenols from plant samples. Methanol, ethanol, acetone, and their aqueous mixtures are the most suitable solvents for antioxidant extraction (Naczk and Shahidi, 2006; Do et al., 2014). In this study, the ethanol-water mixture which is ideal for human consumption was chosen as the solvent mixture and the ethanol concentration was determined for optimum analytes extraction. The amount of 49% ethanol in water was determined as the optimum solvent concentration for the extraction of antioxidants and phenolics. It is thought that water in this mixture plays a role in the plant blowing agent, and ethanol plays a role in the dissolution of the analytes by breaking the bonds between the analytes and the plant matrix (Lang and Wai, 2001; Wang and Weller, 2006).
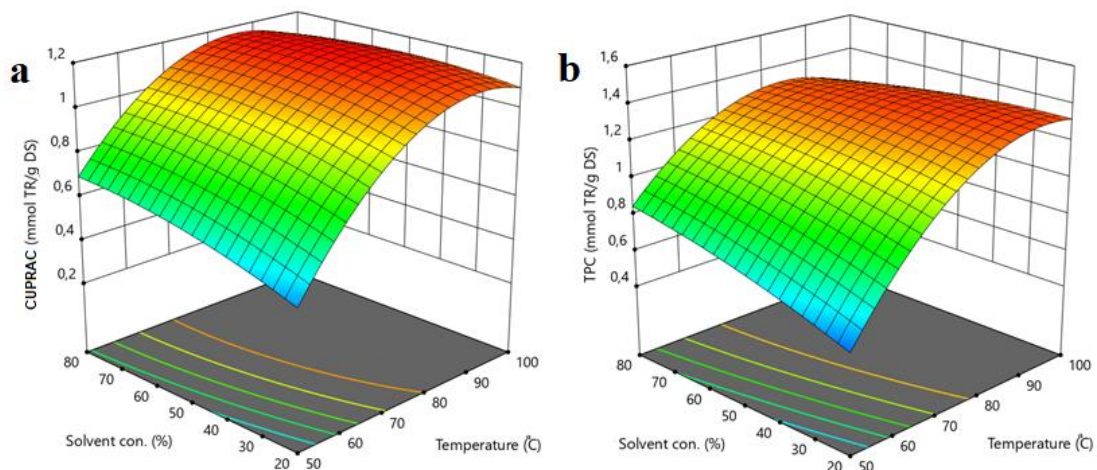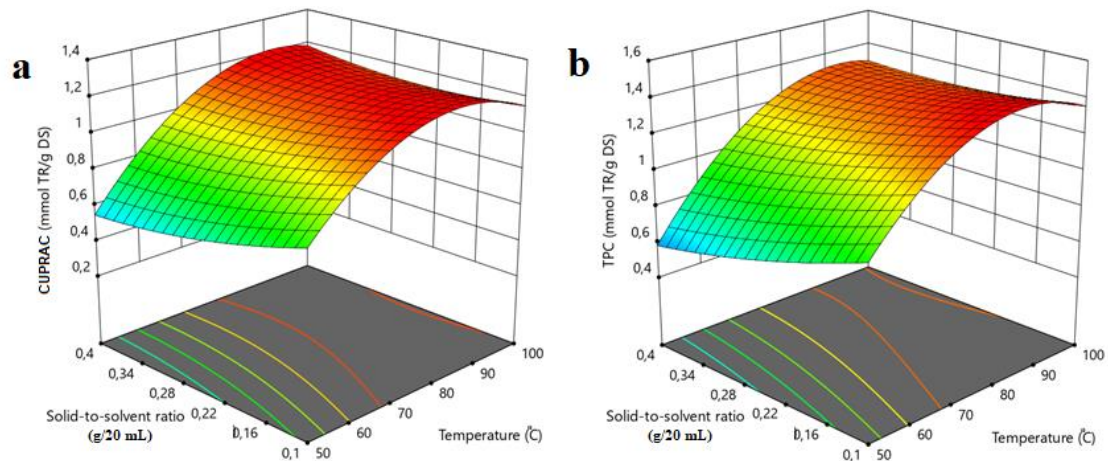

Figure 2. The 3D surface plot for the (a) CUPRAC and (b) TPC of the wormwood extract as a function of solvent concentration to extraction temperature

Figs. 3a and b show that the CUPRAC and TPC responses had a similar trend with respect to solid-to-solvent ratio. The reduction in the extraction efficiency after a given solid-to-solvent ratio is consistent with the principles of mass transfer because the decrease in the ratio of the solid in the solvent results

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causes to more effective and higher the pushing force (İlbay et al., 2014). According to the proposed model, the optimum solid-to-solvent ratio for extracting antioxidants and phenolics from wormwood with MAE was 0.13 g /20 mL. The use of solid-to-solvent ratio higher or lower than the optimum value may cause some problems. The use of excess mass per unit volume can significantly reduce the recovery of phenolics, while the use of excess solvent per unit of solid can be a disadvantage for both cost and environmental factors.

Figure 3. The 3D surface plot for the (a) CUPRAC and (b) TPC of the wormwood extract as a function of solid-to-solvent ratio to extraction temperature

Verification of Predictive Models

The relationship between the predicted (calculated with quadratic models) and the experimentally found the CUPRAC and TPC values of the wormwood extract prepared in different combinations of operational factors of MAE are shown in Figs. 4a and b. As a result, the significant correlation between the predicted and experimental values shows that the developed models reached the target.
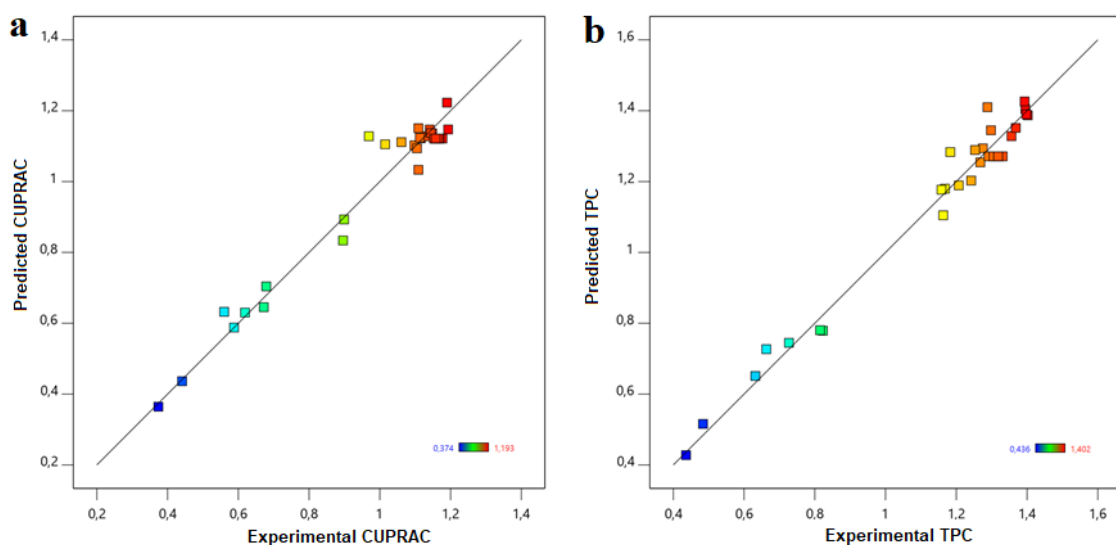

Figure 4. The correlation between the predicted *vs.* the experimental (a) CUPRAC and (b) TPC of the wormwood extracts

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Comparison of the CUPRAC and TPC of Wormwood Extract with Using MAE and Classical Heat Extraction (CHE)

The extract of wormwood plant was prepared by classical heat extraction under optimized MAE conditions. The CUPRAC values of the wormwood extracts obtained using MAE and CHE methods were found to be 1.22 and 0.53, respectively. In addition, the TPC values of the extracts obtained with MAE and CHE were found to be 1.42 and 0.68, respectively. As a result, the CUPRAC and TPC values of the wormwood extract obtained by the MAE method are approximately two times as high as the CHE.

CONCLUSION

The extraction of antioxidants and phenolics from wormwood were optimized and modeled using four different factors of MAE by RSM in this study. These factors are temperature, time, solvent concentration, and solid-to-solvent ratio. Models calculated for the CUPRAC and TPC were found to be significant in terms of showing the relationship between response and independent variables ($p<0.0001$). Extraction time was found as the most significant operational factor on the CUPRAC and TPC of wormwood extract; on the other hand, extraction time parameter is the most insignificant one. The predicted and experimental values were found close to each other. Folin method used in this study for the determination of TPC was used for determining the antioxidant capacity in many research. In this context, the TPC values determined in the study reflect the antioxidant capacity of the extract. The data obtained showed that the CUPRAC and TPC values showed the same response to the change of operational factors. On the other hand, the reason for TPC values are higher than CUPRAC is due to its ability to determine some phenolics which do not show antioxidant effect. As a result, the CUPRAC values are a better indicator than TPC to define the antioxidant property of the extract.

Finally, modelled and optimized MAE method in this study will be an important alternative method for the simple, cost-effective, fast, and high efficient antioxidants extraction from the wormwood in the food and pharmaceutical industry.

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