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Research Article/Araştırma Makalesi

Optimization of Antioxidant Activity and Phytochemical Properties of Dried Pepper Samples (*Capsicum annuum* L.) by Using Response Surface Methodology

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Abstract: The optimization of methanol extraction was performed to maximize antioxidant activity and phytochemical properties of dried pepper (*Capsicum annuum* L.) samples by using the surface response method (RSM) in this study. Box Behnken design model was used to investigate the influences of three independent variables, extraction temperature (25-60°C), extraction time (30-60 min), and dried pepper concentration (500-1000 mg/20 mL). The effect of the dried pepper concentration variable on all the responses was statistically significant, except reducing capacity data. DPPH radical scavenging, reducing capacity, and metal chelating activity increased with rising extraction temperature and dried pepper concentration variables. Total phenolic content (TPC) and total flavonoid content (TFC) yields were also observed to enhance with an increase in dried pepper concentration. TPC and TFC in samples ranged from 2.32 to 3.92 mg GAE/g and 0.063 to 0.210 mg RE/g. The highest antioxidant potential for all employed tests was recorded in 2 experimental runs (25°C, 45 min. and 1000 mg/20 mL). The coefficients of determination (R^2) for antioxidant analysis were determined as 0.9235-0.9238. These values for TPC and TFC were 0.9364-0.9925, respectively.

Keywords: Antioxidant, Box Behnken design, *Capsicum*, Phytochemical.

Yanıt Yüzey Metodolojisi Kullanılarak Kurutulmuş Biber Örneklerinin (*Capsicum annuum* L.) Antioksidan Aktivite ve Fitokimyasal Özelliklerinin Optimizasyonu

Öz: Bu çalışmada, kurutulmuş biber (*Capsicum annuum* L.) örneklerinin antioksidan aktivitelerini ve fitokimyasal özelliklerini arttırmak için yüzey yanıt metodu (YYM) kullanılarak metanol ekstraksiyonunun optimizasyon çalışmaları sürdürülmüştür. Üç bağımsız değişkenin (ekstraksiyon sıcaklığı (25-60°C); ekstraksiyon süresi (30-60 dakika) ve kurutulmuş biber konsantrasyonu (500-1000 mg/20 mL)) etkilerini araştırmak için Box Behnken tasarım modeli kullanılmıştır. Kurutulmuş biber konsantrasyonu değişkeninin tüm yanıtlar üzerindeki etkisi, indirgeme kapasitesi verileri hariç, istatistiksel olarak anlamlıdır. DPPH radikali giderme, indirgeme ve metal şelatlama aktivitesi, ekstraksiyon sıcaklığı ve kuru biber konsantrasyonu değişkenlerinin artması ile artmıştır. Kurutulmuş biber konsantrasyonunun artmasıyla total fenolik ve flavonoid içeriklerinin de arttığı gözlenmiştir. Örneklerdeki total fenolik ve flavonoid içerik 2.32-3.92 mg GAE/g ve 0.063-0.210 mg RE/g aralığındadır. Kullanılan tüm tasarımlar için en yüksek antioksidan potansiyeli 2. deneysel çalışmada (25°C, 45 dak. ve 1000 mg/20 mL) kaydedilmiştir. Antioksidan analizi için R^2 katsayısı 0.9235-0.9238 olarak belirlenmiştir. Total fenolik ve flavonoid içerik için ise bu değerler sırasıyla 0.9364-0.9925'dir.

Anahtar kelimeler: Antioksidan, Box Behnken tasarımı, *Capsicum*, Fitokimyasal.

1. Introduction

Capsicum genus belongs to the Solanaceae family and is native to Mexico but currently cultivated in Asia, Africa, and countries along the Mediterranean (Magied et al., 2014). This plant is consumed as a spice and seasoning for flavoring and coloring in Asian cuisines (Toontom et al., 2012). *Capsicum* genus consists of five main species *C. annuum, C. frutescens, C. chinense, C. baccatum,* and *C. pubescens* (Pino et al., 2006). *Capsicum annuum* L. is produced in Türkiye and especially in the Southeast Anatolia region (Oztekin & Yelmen, 2010).

It has various colors such as red, green, and yellow depending upon the ripening period (Magied et al., 2014). It is an important mineral source such as A, B, B1, C, E, K, and P vitamins and calcium, phosphorous, potassium, and iron, essential for developing cells (Keles, 2007; Darvishi et al, 2013; Ikuomola & Eniola, 2015). In addition, *Capsicum* species include carotenoids, tocopherols, flavonoids, phenolics, and capsaicinoids components that have antioxidant, antienflamatuar, antiallergic, antimicrobial, and anticarcinogen properties (Lee et al., 2005). Thus, it was widely used as a natural drug to treat gastric ulcers, alopecia, rheumatism, toothache, and diabetes in traditional medicine (Baldemir et al., 2015).

It becomes perishable within a few days of harvesting depending on weather conditions. Therefore, the alimentary characteristics of this fruit are preserved by drying (Fadhel et al., 2014).

Traditionally, fresh pepper is dried under the sun without any special treatment. Conventional procedures employed for sun dehydration of pepper take about 7-15 days (Toontom et al., 2012; Magied et al., 2014). This

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process is slow but conventional drying is a popular method for acquiring dry productions such as condiments, spice, and bell pepper in the southeast region of Türkiye, especially in Gaziantep and Kilis. Moreover, the consumption of dried pepper productions in these regions is higher than the other cities in Türkiye.

Therefore, pepper samples dried by conventional process from Gaziantep and Kilis local markets were purchased in the present study. Moreover, this study focuses on investigating the effects of temperature, time, and dried pepper concentration-independent variables on *in vitro* total phenolic, total flavonoid contents, and antioxidant activity by response surface method (RSM).

2. Material and Methods

2.1. Extraction process of dried pepper

Dried peppers were purchased from Gaziantep and Kilis local markets and then powdered by the grinder. Dried pepper powders were extracted by the Box-Behnken Experimental Design with two levels provided by the Response Surface Method (Box & Wilson, 1951). Extraction procedures were performed in a water bath by using different combinations of independent variables. Extraction conditions are shown in detail in Table 1. Extracts were obtained from 15 different experimental procedures and these were separately filtered by Wattman No. 4 filter paper. After centrifuging at 8500 rpm, the extracts were kept at 4°C for the following antioxidant and phytochemical analysis.

Table 1. The Box Behnken experimental design for antioxidant potential, total phenolic, and flavonoid contents of dried pepper extractions

	Temperature (°C)	Time (min)	Dried pepper oncentration (mg/20 mL)
Experimental no	X1	X2	X3
1	60.00	30.00	750.00
2	25.00	45.00	1000.00
3	60.00	60.00	750.00
4	42.50	30.00	1000.00
5	25.00	60.00	750.00
6	25.00	45.00	500.00
7	60.00	45.00	1000.00
8	42.50	45.00	750.00
9	25.00	30.00	750.00
10	42.50	45.00	750.00
11	42.50	30.00	500.00
12	42.50	60.00	500.00
13	60.00	45.00	500.00
14	42.50	45.00	750.00
15	42.50	60.00	1000.00

2.2. Antioxidant activities of dried pepper

2.2.1. DPPH radical scavenging capacity

Free radical scavenger method is based on turning colorless 1,1-diphenyl-2-picryl-hydrazyl (DPPH) reagent solution depending on the electron or proton-transfer ability of the samples (Blois, 1958). For this analysis, 100 µL of the extracts was added to 3.9 mL of DPPH reagent solution prepared in methanol (0.1 mM). This mixture was incubated at room temperature in the dark for 120 min. The absorbance of the mixture was spectrophotometrically measured at 517 nm (Biochrom, Libra S60, B) against methanol blank. DPPH scavenging activity expressed as % inhibition was calculated by the following equation. Butylated hydroxytoluene (BHT) at 200-1000 µg/mL concentrations were used as a standard antioxidant substance.

$$\text{Inhibition (\%)} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

2.2.2. Metal chelating activity

Fe^{2+} chelating activity of dried pepper samples was performed according to the method described by Dinis et al. (1994). This method is based upon the competition of metal-binding compounds in the extracts with ferrozine, a strong iron-chelating agent. The compounds having high metal ions binding capacity avoid the red Fe^{2+}/ferrozine complex formation. Briefly, 3.7 mL distilled water and 100 µL 2 mM $FeCl_2$ were added to 1 mL of the dried pepper extracts. After incubated at room temperature for 30 min, 200 µL of 5 mM ferrozine solution was added to the reaction and mixed for 10 min. The absorbance of the reaction mixture was read 562 nm (Dinis et al., 1994). The metal ions chelating capacity of the samples was evaluated by using EDTA (50-250 µg/mL) as a standard chelator agent. The chelating activity was expressed as % inhibition using the following equation:

$$\text{\% chelating activity} = (1-(A_{sample}/A_{control})) \times 100$$

2.2.3. Reducing Capacity Assay

In this assay, the reducing Fe^{3+} to Fe^{2+} capability of antioxidant substances in dried pepper extracts was tested (Oyaizu, 1986). The absorbance of the Prussian blue color formed by adding $FeCl_3$ in the reaction mixture was measured. A high absorbance value indicated a high reducing capacity of the samples. In brief, dried pepper extract (1 mL) was mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium $K_3Fe(CN)_6$. This reaction solution was incubated at 50°C for 20 min. To terminate the activity, 10% trichloroacetic acid (TCA) was added and the mixture was centrifuged at 2500 rpm for 10 min. The equal volume of distilled water and 0.5 ml $FeCl_3$ (0.1%) were added to 2.5 mL of supernatant. The absorbance of the reaction mixture was measured at 700 nm. A beta-hydroxy acid (BHA) was used as standard.

2.3. TPC and TFC of dried pepper

For the total phenolic content in all samples, Folin–Ciocalteu colorimetric method was performed. The total phenolic content was expressed as mg of gallic acid equivalents (mg GAE)/g of extract using a standard curve (Stankovic, 2011). The total flavonoid content of samples was determined using a method grounded by Sharm & Vig (2013). The results were calculated according to a standard curve prepared by using rutin and expressed as mg of rutin equivalent (mg RE)/g.

2.4. Box Behnken experiment design

The effects of the three independent variables: extraction temperature (X1: 25-60°C), extraction time (X2: 30-60 min.), and pepper concentration/20 mL (X3: 500-1000 mg/20

mL) on antioxidant activity and phytochemical properties were investigated by "Design Expert statistical software" (Design-Expert version 8.0.7). A two-level-three-factor Box-Behnken design (Box & Wilson, 1951) consisting of 15 randomized runs at 3 center points is shown in Table 1.

The compatibility of the design and the significant terms of each response were tested by using quadratic model variance analysis (ANOVA) at a 95% confidence level. Antioxidant activity (Y1), total phenolic (Y2), and flavonoid (Y3) content as the response was calculated by a second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{N} \beta_i \times X_i + \sum_{i=1}^{N} \beta_{ii} \times X_i^2 + \sum_{i=1}^{N-1} \sum_{j=i+1}^{N} \beta_{ij} \times X_{ij}$$

3. Results and Discussion

Optimization of extraction conditions was performed in 15 experimental runs to explain the effects of three independent variables on *in vitro* antioxidant activity and phytochemical properties of dried pepper samples. The adequacy of the model is determined by R^2 and the significance of the model (p-value). According to the results of the ANOVA, the coefficients of determination (R^2) were 0.9283, 0.9235, and 0.9238 for DPPH scavenging, reducing capacity, and metal chelating activity analysis. It shows that the experimental values agreed with those predicted of the model employed for antioxidant activity

The small p-values ($p<0.05$) in evaluated response per analysis indicate the significance of the performed model. P values for antioxidant activity tests ranged from 0.0213 to 0.0248. The low p-values indicate that independent variables have a more significant effect on antioxidant activity of dried pepper samples. The experimental and predicted responses for antioxidant activity tests are shown in Table 2.

As observed in polynomial equations of responses (Table 3), the most important variable on DPPH yields was the interaction effect between dried pepper concentration and extraction time (X2X3), followed by interaction between extraction temperature and time (X1X2) and dried pepper concentration (X3). Dried pepper concentration (X3), the first-order linear effect, and the interaction effect (X1X2 and X2X3) were significant ($p<0.05$). DPPH scavenging activity ranged from 36 to 85%. The maximum activity was recorded during experiment 2 (25°C, 45 min, and 1000 mg/20 mL) but experiment 11 (42.5°C, 30 min, and 500 mg/20 mL) resulted in the lowest activity.

The increase from 500 mg to 1000 mg of dried pepper concentration solved in 20 mL at the constant temperature (42.5°C) and extraction time (45 min) increased the DPPH radical scavenging activity of extracts. For the constant pepper concentration (750 mg/20 mL), DPPH activity increased with the rise in temperature. It was observed that an increase (from 30 to 60 min) in extraction time had accompanied a decrease in DPPH removal activity (Fig. 1).

According to the regression equations, dried pepper concentration (X3) had the largest influence on metal chelating activity followed by the-second order quadratic effects. The obtained responses for metal chelating activity were in the range of 4.47-26.01%. Three-dimensional (3D) response surface graphs are plotted for the metal chelating activity test in Figure 2. The highest metal chelating activity was calculated in 2 experimental runs (25°C, 45 min, and 1000 mg/20 mL). Figure 2a reveals that the increase to 42.5°C of extraction temperature decreased activity at constant dried pepper concentration (750 mg/20 mL). On the other hand, the chelating activity of samples was observed to increase with increasing to 60°C of extraction temperature and to reach the maximum level at 26.01% (predicted 25.53%). Variance analysis results presented that all of the tested interactions had no statistically significant effect on metal chelating activity.

As can be seen in Table 3, the highest effect on reducing power activity is the interaction between temperature and time. This interaction effect and the second-order term of dried pepper concentration ($X3^2$) had significant effects ($p<0.05$). However, the first-order linear, the other second-order quadratic and interaction effects were not found to be statistically significant ($p>0.05$). In the model, the other independent variables (X1 and X3), except extraction time (X2), showed a positive effect on the activity at a linear level for antioxidant activity analysis. It means that the antioxidant activity increases with the increasing extraction temperature and dried pepper concentration solved in methanol.

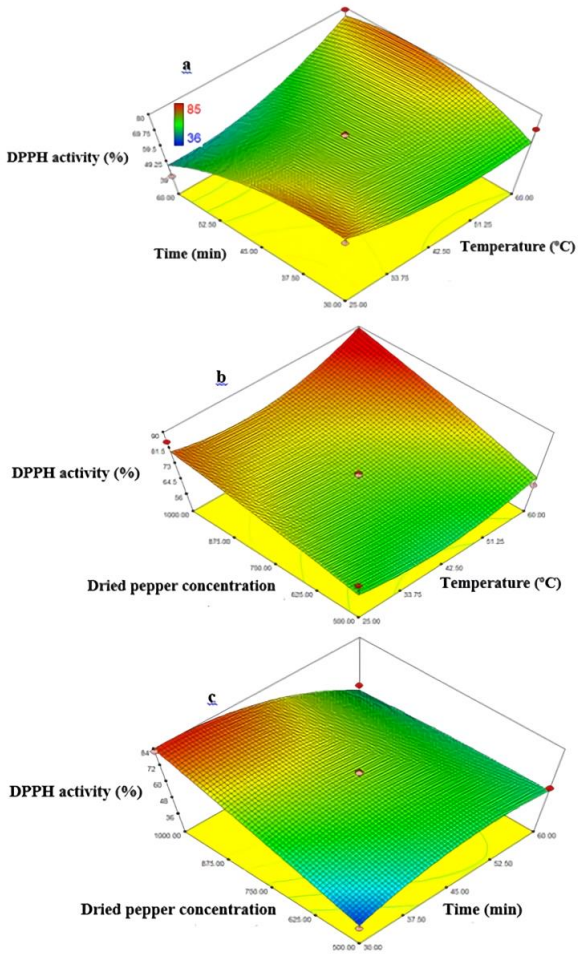

Figure 1. Response surface plots showing the effects of examined variables on the DPPH activity: (a) time/temperature interaction at constant dried pepper concentration (750 mg/20 mL) (b) dried pepper concentration/temperature interaction at constant time (45 min), (c) dried pepper concentration/time interaction at constant temperature (42.5°C)

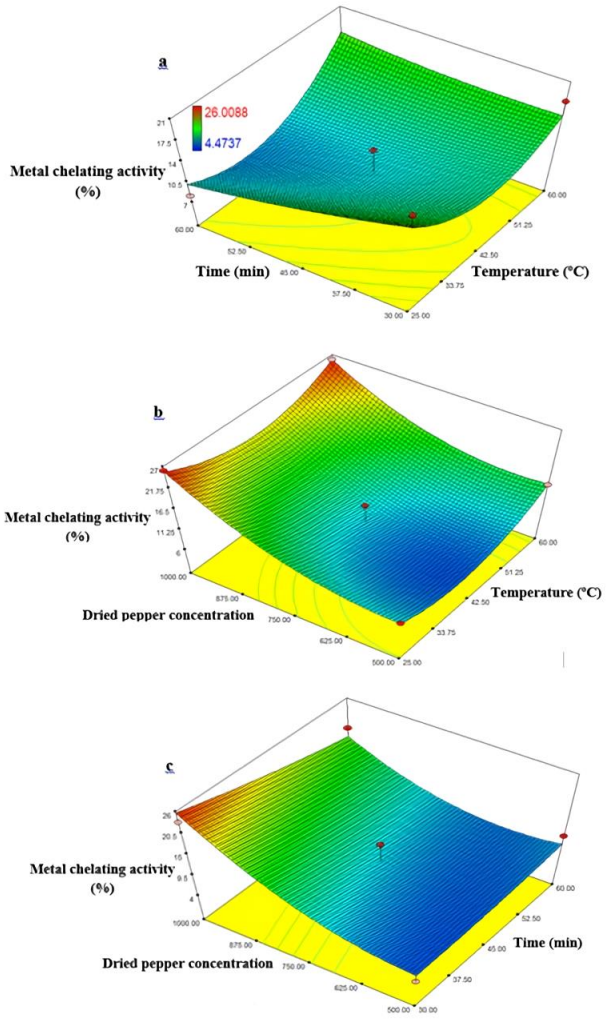

Figure 2. Response surface plots showing the effects of examined variables on the metal chelating activity: (a) time/temperature interaction at constant dried pepper concentration (750 mg/20 mL) (b) dried pepper concentration/temperature interaction at constant time (45 min), (c) dried pepper concentration/time interaction at constant temperature (42.5°C)

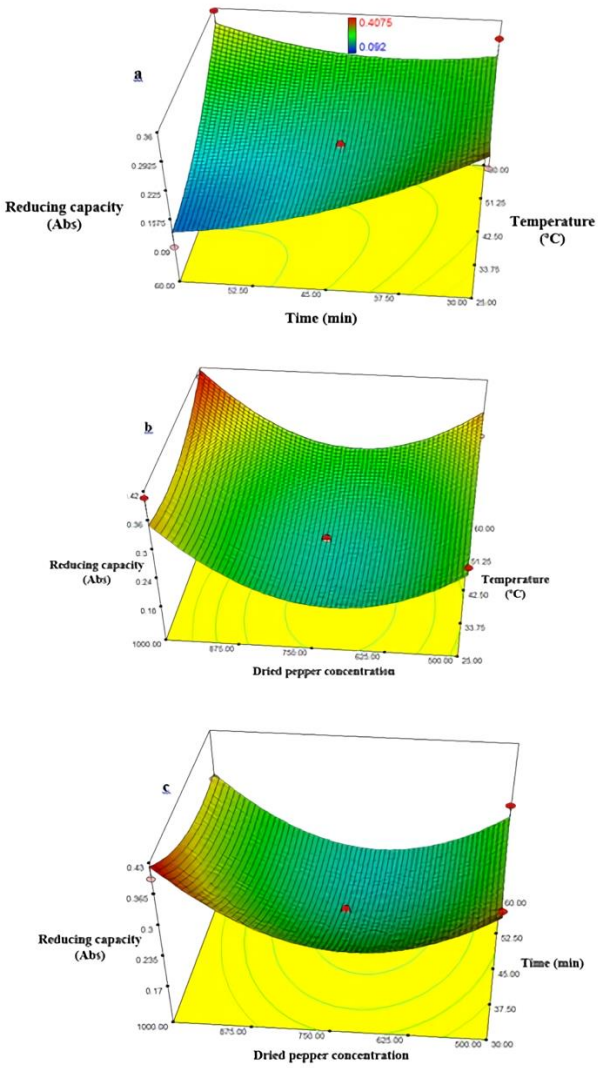

Figure 3. Response surface plots showing the effects of examined variables on the reducing capacity: (a) time/temperature interaction at constant dried pepper concentration (750 mg/20 mL), (b) dried pepper concentration/temperature interaction at the constant time (45 min), (c) dried pepper concentration/time interaction at constant temperature (42.5°C)

3D surface plots of the interactive influences of the independent variables on reducing power are shown in Figure 3. It can be seen that the increase in extraction temperature (from 25 to 60°C) did not affect reducing power at constant dried pepper concentration (750 mg/20 mL) and extraction time (45 min). Reducing capacity decreased when dried pepper concentration increased from 700 to 875 mg in 20 mL, reaching a maximum level by rising concentration (Fig. 3c).

The antioxidant activity increased with the rise of the temperature up to certain intervals and then decreased. It indicates that the high temperatures may increase the mobilization of active antioxidant compounds from the substrate up to a certain level. However, the possible loss of components can occur due to their decomposition at higher temperatures (Singh et al., 2012).

Wettasinghe & Shahidi (1999) state that high temperatures may mobilize certain antioxidants by promoting concurrent decomposition of antioxidants, already mobilized at a low temperature. It was also emphasized that the extraction rate of thermally stable antioxidants is higher than the rate of the decomposition of less soluble antioxidants. This proposition explains the high antioxidant activity observed in extracts prepared at high temperatures.

Table 3 shows the positive linear effect of dried pepper concentration on TPC and TFC yields. The experimental and predicted responses for TPC and TFC are shown in Table 4. This suggests that variations in dried pepper concentration have a significant impact on TPC and TFC in the extracts. P values of TPC and TFC analysis were 0.0161 and 0.0001, respectively. The coefficient of determination (R^2) was 0.9364 and 0.9925 for TPC and TFC respectively. While the effects of extraction time (X2) and dried pepper concentration (X3) on TPC content were found to be statistically significant, only the effect of dried pepper concentration on TFC content was found to be significant. TPC and TFC of dried pepper extracts ranged from 2.322 to 3.919 mg GAE/g and 0.063 to 0.210 mg RE/g. The maximum TPC was acquired under 42.5°C, 60 min, and 1000 mg/20 mL experimental conditions while the minimum was 42.5°C, 30 min. and 500 mg/20 mL. For TFC in extracts, the maximum content was recorded at 25°C, 45 min, and 1000 mg/20 mL while the minimum was 42.5°C,

60 min. and 500 mg/20 mL (predicted 0.061). The yield of total phenolic content increased between 25 and 42.5°C, then it decreased with the increase of temperature reaching 60°C (Fig. 4a). It was emphasized that as the dried pepper concentration parameter increased, TFC increased at a constant extraction temperature (42.5°C). The TF content in extracts was almost constant between 30 and 37 min, and then it decreased slightly with the increase of extraction time. Increasing the extraction temperature and dried pepper concentration enhanced the TFC yield (Fig. 5b).

Table 2. The experimental and predicted responses for antioxidant activity

Run	DPPH scavenging activity (%)		Reducing capacity (Abs)		Metal chelating activity (%)	
No	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1	71.00±0.004	64.13	0.29±0.003	0.26	17.98±0.001	15.28
2	85.00±0.005	80.00	0.40±0.011	0.35	26.01±0.007	25.53
3	79.00±0.007	76.13	0.35±0.026	0.33	15.13±0.013	17.09
4	82.00±0.001	84.13	0.40±0.006	0.42	23.42±0.037	25.86
5	40.00±0.006	46.88	0.09±0.016	0.12	6.05±0.051	8.75
6	64.00±0.003	59.25	0.30±0.008	0.29	9.87±0.019	9.61
7	83.00±0.005	87.75	0.40±0.002	0.41	25.83±0.008	26.09
8	67.00±0.003	67.67	0.20±0.015	0.19	8.11±0.077	9.65
9	75.00±0.004	77.88	0.32±0.005	0.34	20.34±0.043	18.38
10	68.00±0.002	67.67	0.19±0.009	0.19	7.89±0.013	9.65
11	36.00±0.001	37.88	0.37±0.001	0.36	4.47±0.053	6.70
12	56.00±0.001	53.88	0.30±0.008	0.28	10.53±0.024	8.09
13	62.00±0.006	67.00	0.30±0.003	0.35	13.82±0.005	14.29
14	68.00±0.002	67.67	0.19±0.001	0.19	12.94±0.012	9.65
15	51.00±0.003	49.13	0.33±0.013	0.34	18.88±0.001	16.65

Table 3. Second-order polynomial model equations for responses

Response	Second-order polynomial equations
DPPH removal activity	$Y=+67.67+3.87X_1-4.75X_2+10.38X_3+10.75X_1*X_2+0.000X_1*X_3-12.75X_2*X_3+7.92X_1^2-9.33X_2^2-2.08X_3^2$
Reducing capacity	$Y=+0.19+0.030X_1-0.038X_2+0.032X_3+0.072X_1*X_2+7.000*10^{-4}X_1*X_3+0.000X_2*X_3+0.036X_1^2+0.035X_2^2+0.12X_3^2$
Metal chelating activity	$Y=+9.65+1.06X_1-1.70X_2+6.93X_3+2.36X_1*X_2-1.03X_1*X_3-2.65X_2*X_3+5.14X_1^2+0.59X_2^2+4.09X_3^2$
Total phenolic content	$Y=+2.77-0.022X_1+0.18X_2+0.47X_3+0.028X_1*X_2-0.13X_1*X_3+0.040X_2*X_3-0.051X_1^2+0.22X_2^2+0.093X_3^2$
Total flavonoid content	$Y=+0.081+1.194*10^{-3}X_1-2.968*10^{-3}X_2+0.066X_3+3.725*10^{-4}X_1*X_2-1.933*10^{-3}X_1*X_3-3.282*10^{-3}X_2*X_3+0.010X_1^2-2.124*10^{-3}X_2^2+0.047X_3^2$

Table 4. The experimental and predicted responses for TPC and TFC

Run	TPC (mg GAE/g)		TFC (mg RE/g)	
No	Experimental	Predicted	Experimental	Predicted
1	2.84±0.033	2.71	0.090±0.060	0.093
2	3.42±0.023	3.43	0.210±0.020	0.210
3	3.13±0.042	3.12	0.090±0.050	0.088
4	3.36±0.025	3.33	0.200±0.043	0.200
5	2.97±0.007	3.11	0.089±0.010	0.085
6	2.40±0.017	2.24	0.064±0.080	0.070
7	2.97±0.006	3.13	0.210±0.050	0.200
8	2.88±0.029	2.77	0.077±0.010	0.081
9	2.80±0.050	2.81	0.090±0.060	0.092
10	2.73±0.079	2.77	0.088±0.140	0.081
11	2.32±0.028	2.47	0.068±0.029	0.060
12	2.72±0.031	2.75	0.063±0.030	0.061
13	2.47±0.061	2.45	0.072±0.070	0.076
14	2.70±0.022	2.77	0.078±0.035	0.081
15	3.92±0.079	3.77	0.180±0.015	0.190

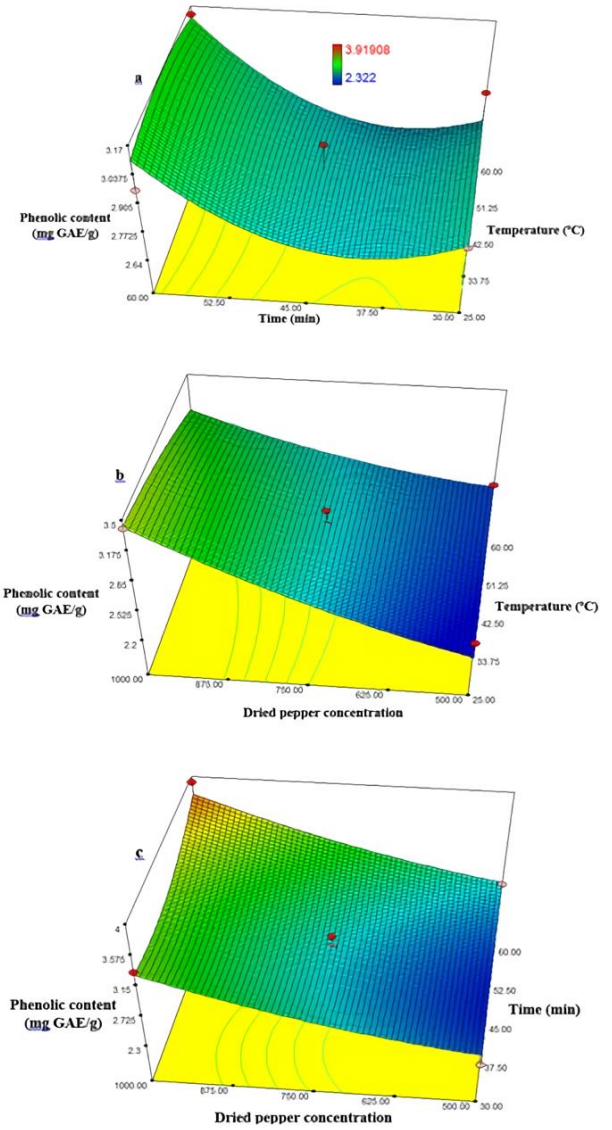

Figure 4. Response surface plots showing the effects of examined variables on the extraction yield of phenolic substances: (a) time/temperature interaction at constant dried pepper concentration (750 mg/20 mL), (b) dried pepper concentration/temperature interaction at the constant time (45 min), (c) dried pepper concentration/time interaction at constant temperature (42.5ºC)

Optimization studies related to *in vitro* phenolic substance extraction and antioxidant activity researches of *Capsicum annuum* L. have not been encountered in the literature. Our findings were discussed in light of the optimization experiments conducted with various plants. Singh et al. (2012) reported that high temperature increases the diffusion of phenolic and flavonoid from cells to extraction agents by speeding up molecule movements depending on the solvent polarity. Kumar et al. (2008) detected gradually enhance flavonoid contents in the range of 55-85°C with a 10°C temperature increase. He et al. (2005) stated that the temperature parameter shows a dual effect on the extraction process. Carciochi et al. (2015) recorded that high temperature enhances the solubility by accelerating diffusion of phenolic components but some phenolic substances can denature depending on the chemical and enzymatic degradation above certain temperature intervals. In another study, high temperature

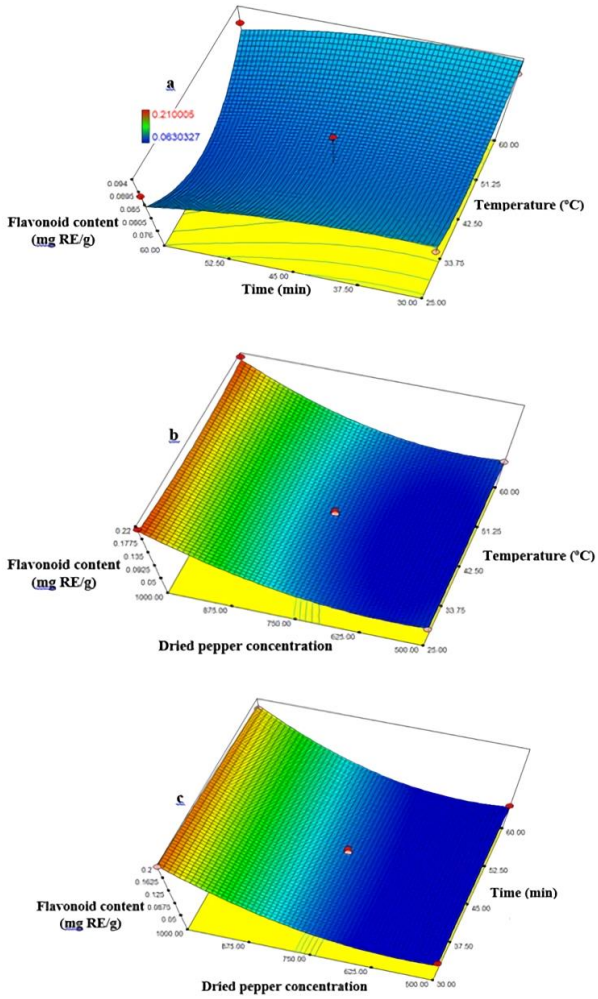

Figure 5. Response surface plots showing the effects of examined variables on the extraction yield of flavonoid substances: (a) time/temperature interaction at constant dried pepper concentration (750 mg/20 mL), (b) dried pepper concentration/temperature interaction at the constant time (45 min), (c) dried pepper concentration/time interaction at constant temperature (42.5ºC)

can increase extraction efficiency by decreasing solvent viscosity and surface tension (Yagcıoglu, 2015).

We revealed that phenolic content in methanol extract increased with a rise in the extraction time and the dried pepper concentration. Similar to our phenolic results, Bachir et al. (2014) notified that a long time of extraction under high temperature can result in the negative quadratic effect. This can be explained that mass transform increases with the increase of extraction time until maximum extraction efficiency is reached. It was also expressed that the increasing interaction time between matrix and solvent can cause a gradual dissolution from solid matrix to solvent.

An optimized procedure (response surface methodology) for the antioxidant activity and total phenolic and flavonoid contents of dried pepper extracts are described in this study. Our results showed that methanol extraction was effective in extracting total phenolic, flavonoid, and antioxidant components under various combinations of extraction conditions (temperature, duration, and dried pepper concentration).

The experimental responses agreed with predicted values for all responses. This proves the suitability of the model performed. The present paper is the first study that researches the optimization of methanol extraction of dried pepper (*Capsicum annuum* L.) samples by using the surface response method.

Ethics committee approval: Ethics committee approval is not required for this study.

Conflict of interest: The authors declare that there is no conflict of interest.

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