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THE EFFECTS OF TIME, TEMPERATURE, SOLVENT: SOLID RATIO AND SOLVENT COMPOSITION ON EXTRACTION OF TOTAL PHENOLIC COMPOUND FROM DRIED OLIVE (*Olea europaea* L.) LEAVES

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Abstract
The optimum extraction conditions for total phenolic compounds from dried olive leaves were determined by using response surface methodology. Central composite design was used to investigate the effects of four independent variables such as solvent composition (ethanol in water, 20 to 100%), extraction temperature (20 to 60 °C), extraction time (4-48 hours) and solvent/solid ratio (4 to 8). As a response total phenolic compound content of the extracts were chosen. The quadratic model is used for predicting the results and R^2 was found 0.8539. The olive leaves with the initial 43.94±0.25% moisture content were dried in vacuum oven at 60 °C to 2±0.07% moisture content before extraction. There are several solutions by choosing the target, in range, maximize or minimize the effective parameters in response surface methodology. In this study, recommended optimal conditions for the total phenolic compounds from olive leaves were found 43% ethanol in water (v/v), 50 °C, 15 hours and 7 times solvent/solid ratio. Under the chosen optimum conditions the corresponding predicted response value for total phenolic compounds was 4586.3 mg GAE/100 g dried leaves.

Keywords: Solvent extraction, total phenolics, olive leave, response surface methodology, optimization

KURUTULMUŞ ZEYTİN YAPRAĞINDAN (*Olea europaea* L.) TOPLAM FENOLOİK MADDE EKSTRAKSİYONU ÜZERİNE SÜRE, SICAKLIK, ÇÖZÜCÜ-KATI ORANI VE ÇÖZÜCÜ KOMPOZİSYONUNUN ETKİSİ

Özet
Kurutulmuş zeytin yaprağından toplam fenolik maddelerin ekstraksiyonu için optimum ekstraksiyon koşulları cevap yüzey yöntemi kullanılarak belirlenmiştir. Çözücü kompozisyonu (etanol-su, %20 - %100), ekstraksiyon sıcaklığı (20 - 60 °C), ekstraksiyon süresi (4 - 48 sa) ve çözücü katı oranı (4 - 8) gibi bağımsız dört farklı değişkenin etkisinin belirlenmesinde merkezi teşekküllü dizayn kullanılmıştır. Cevap olarak ekstraktaki toplam fenolik madde içeriği seçilmiştir. Sonuçlar ikinci dereceden denklem ile açıklanmış ve R^2 0.8539 olarak bulunmuştur. Ekstra çekleştirilmesi için parametrelerin minimize maksimize edilmesi veya aralığının seçilebilmesi nedeniyle çeşitli çözümler yapılabilmektedir. Bu çalışmada zeytin yaprağından toplam fenolik madde ekstraksiyonu için önerilen seçilmiş koşullar %43 etanol içeren su (h/h), 50 °C, 15 sa ve 7 kat çözgen/katı oranı olarak belirlenmiştir. Optimum koşullar uygulanarak belirlenen kurutulmuş zeytin yaprağının toplam fenolik madde içeriği ise 4586.3 mg GAE/100 g kuru yaprak olarak bulgulanmıştır.

Anahtar kelimeler: Çözgen ekstraksiyonu, toplam fenolikler, zeytin yaprağı, cevap yüzey yöntemi, optimizasyon

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INTRODUCTION

Epidemiological evidences have suggested that food phenolics may have protective effects against degenerative diseases (1). Many researches have been reported for most of the phenolic compounds from olives: they act as anti-oxidant, anti-inflammatory, anti-viral, anti-carcinogenic agents (2-4). Superheated liquid extraction, microwave or ultrasound assisted alcohol/water extractions were applied to the olive leaves and results were compared with the solvent extraction methods. It was determined that olive leaves contained in range of 14000-32000 mg/kg oleuropein, 488-737 mg/kg verbacoside, 976-1141 mg/kg apigenin-7-glucoside, 917-1079 mg/kg luteolin-7-glucoside (5-7). For the extraction of phenolics from olive leaves, methanol/water mixture (8, 9) or hexane is used mostly (10). Many factors such as solvent composition, extraction time, extraction temperature (11), solvent to solid ratio (12) and extraction pressure (13), among others, may significantly influence the extraction efficiency (14). In the literature no information was found about the optimization of phenolics extraction from olive leaves. Hence in this paper, it is aimed to define the optimum extraction conditions for total phenolic compounds from dried olive leaves by using response surface methodology (RSM) which allows the evaluation of different process variables such as solvent composition, temperature, time and solvent to solid ratio effects and their interactions on response variable as total phenolic compound content in the extract.

MATERIALS AND METHODS

Samples and reagents

Experiments were carried out on leaves of the Memecik cultivar of Olea europaea L., commonly cultivated in Ege University, Izmir, Turkey. Samples of fresh green leaves were collected at the end of olive morphology (March 2007). Leaves were selected randomly from around the tree and were dried directly in vacuum oven. Folin & Ciocalteau's phenol reagent and gallic acid were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Analytical grade ethanol was obtained from Riedel-de Haen (Seelze, Germany).

Total dry matter analysis

Total Dry Matter content of fresh and dried leaves were analysed by using a vacuum oven at 60-65 °C (15).

Preparation of samples

Fresh olive leaves were collected and dried at 60 °C vacuum oven and were ground in an hammer mill (Brook Cromption Controls, Wakefield England) at 8000 rpm and 0.03 mm diameter sieve to obtain a fine powder and stored in N^2 gas purged glass jars covered with aluminum foil at 0 °C until extraction.

Extraction procedure

Five grams of dried and milled leaves and different amount of extractant (ethanol–water mixture) were placed in a volumetric flask and subjected to stirring in Gerhard Thermoshake shaker (C. Gerhardt GmbH & Co. KG Königswinter, Germany) at different temperatures for different times. Extraction conditions were given in Table 1. After filtration, the extract was stored at -40 °C until total phenolic compound analysis.

Measurement of total phenols in the extract

The prepared extracts of 0.5 ml were used for total phenols determination. Colorimetric oxidation/reduction reaction was measured by UV spectrophotometry. The Folin Ciocalteau reagent was used as an oxidizing agent (16). 0.5 ml olive leave extract was diluted to 100 ml. The amount of 0.5 ml of diluted extract and 2.5 ml of Folin Ciocalteau reagent (diluted 10 times with water) was mixed together and within the time intervals 0.5 to 8 min, 2 ml of Na_2CO_3 (75 g/l) was added to that solution. The samples were incubated at 50 °C for 5 min and then cooled. For control sample 0.5 ml distilled water was used instead of extract. The absorbance was measured at 760 nm by Cary 50 UV-vis. Spectrophotometer. The results were expressed in gram gallic acid per liter of extract and converted to (g GA/100 g dried leaves).

Experimental Design

Optimization of phenolics from dried olive leaves in aqueous ethanol was carried out using RSM (20-22). Four-factor and a central composite design (CCD) consisting of thirty experimental runs was employed including six replicates at the center point.

Response Surface Methodology Analysis

Stat Ease Design Expert 7.0 software and Central Composite Design (CCD) was used for RSM analysis.

The effects of unexplained variability in the observed response due to extraneous factors were minimized by randomizing the order of experiments. The design variables were the solvent composition (X1 ,%, v/v, ethanol/water), extraction temperature (X2, °C), extraction time (X3, h) and solvent/solid ratio (X4) while response variable was total phenolic compound in dried leaves. Optimal conditions for the extraction of phenolic compounds from dried olive leaves on solvent composition, extraction temperature, time and solvent/solid ratio were obtained using the predictive equations of RSM. Table 1 shows the experimental design by RSM.

Table 1. Independent variables and their coded values used for optimization

Independent variable	Unit	Symbol	Coded level -alpha	+alpha
Solvent composition	% (v/v)	X_1	20	100
Temperature	°C	X_2	20	60
Time	h	X_3	4	48
Solvent/ solid	-	X_4	4	8

RESULTS AND DISCUSSION

Selection of lower, middle and upper levels of the design variables

Previous researchers used conventional method for bulk extraction of oleuropein and related biophenols from olive leaves. Conditions that were used as follows: ethanol/water (70/30, v/v), 40 °C for 48 h and 8 times solvent/solid ratio (5), ethanol/water (80/20, v/v), 40 °C for 24 h and 8 times solvent/solid ratio (6), ethanol/water (59/41, v/v), 40 °C for 24 h and 8 times solvent/solid ratio (7). There are other researches which the extractions were carried out for experimental determinations of total phenols with different combinations of solvents up to 100 ml total extract volume such as methanol/acetone/water (60/30/10, v/v/v), 25 ml of solvent, stirring at 700 rpm on a magnetic stirrer for 10 min (18) and methanol/water (80/20 v/v) extracts of phenolic compounds from olive fruits prior to HPLC analysis (19-21). Aqueous ethanol, yielded extracts of total phenolic compounds with higher total antioxidant activity compared to other aqueous solvents from whole wheat was considered as the most effective solvent (14). Ethanol is mostly food grade solvent according to methanol, acetone, hexane and diethyl ether etc. and consumption gives less hazardous to the human beings. Subsequently, the lower and upper levels of the solvent composition were selected based on these fundamentals and the ranges for optimization was chosen between 20-100 % ethanol in water for olive leaves. The mobilization of active compounds from the substrate may occur up to certain level followed by their possible loss due to decomposition at higher temperatures (14). Hence, the maximum extraction temperature was chosen 60 °C and minimum 20 °C which is close to the room temperature. Extraction time (4-48 h) and solvent/solid ratio (4-8) were chosen according to their usage possibility in industrial scales and previous researches' results and patent data. Under these circumstances, RSM has shown to be a powerful tool in optimizing experimental conditions to maximize various responses.

Fitting the models

Table 2 summarizes the data for TPC of olive leave extracts examined. The results of ANOVA for total phenolic compounds with corresponding coefficients of multiple determinations (R1) for olive leaves are shown in Table 3.

Fresh olive leaves containing 45.6±0.76 % moisture were dried in vacuum oven until its dry matter reaches to 98 %. Results were reported on dry weight basis for a more descriptive expression.

In general, proceeding with exploration and optimization of a fitted response surface may produce poor or misleading results unless the model exhibits an adequate fit (22). Total phenolic compound was found between 2062.2 and 5177.6 mg GAE/100 g dried leaves in the extracts. Makris et al. reported total phenolic compound content of olive leaves with 48.79 % moisture content was 2058±92 mg GAE/100 g sample (18). Proposed optimal conditions for the extraction of total phenolic compounds were found 43 % ethanol in water (v/v), 50 °C, 15 hours and 7 times solvent/solid ratio. Under the optimum conditions the corresponding predicted response value for total phenolic compounds was 4586.3 mg GAE/100 g dried leaves. During optimizing conditions, time range is targeted as minimized where the total phenolic content

Table 2. Four factor central composite design for RSM

Standard order	Run order	Factor 1 (A) Solvent composition (%) (v/v)	Factor 2 (B) Temperature (°C)	Factor 3 (C) Time (hours)	Factor 4 (D) Solvent/solid	Response: Totalphenolic compound (mg GAE/100 g dried leaves)
14	1	80	30	37	7	3735.90
20	2	60	60	26	6	3651.84
7	3	40	50	37	5	3623.40
21	4	60	40	4	6	3206.40
1	5	40	30	15	5	2062.20
13	6	40	30	37	7	3270.40
12	7	80	50	15	7	3932.60
4	8	80	50	15	5	2762.20
22	9	60	40	48	6	3378.36
5	10	40	30	37	5	2223.60
15	11	40	50	37	7	4537.96
29	12	60	40	26	6	3436.80
24	13	60	40	26	8	5177.60
11	14	40	50	15	7	4580.40
18	15	100	40	26	6	2305.92
2	16	80	30	15	5	3509.00
17	17	20	40	26	6	2275.68
8	18	80	50	37	5	3173.00
16	19	80	50	37	7	3338.58
9	20	40	30	15	7	3056.06
25	21	60	40	26	6	3698.40
19	22	60	20	26	6	3462.72
27	23	60	40	26	6	3158.88
23	24	60	40	26	4	2328.64
10	25	80	30	15	7	2953.86
28	26	60	40	26	6	3231.36
6	27	80	30	37	5	3168.50
3	28	40	50	15	5	3202.70
26	29	60	40	26	6	3313.56
30	30	60	40	26	6	3644.28

is maximized. Other variables were kept in range. As it was seen from Table 3, in 14th run order line, 40 % ethanol in water, 50 °C, 15 hours and 7 times solvent/solid ratio conditions, recovery of total phenolic compounds was 4580.4 mg GAE/100 g dry leaves. Both trials and optimization results gave similar values. The experiments also proved that the predicted values of total phenolic content for the model could be satisfactorily achieved. In Table 3, the quadratic model was found significant for olive leaves with satisfactory coefficient of determination (R^2) that is 0.8539 where the lack of fit value (0.0769) is not significant.

Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case B, D, AB, AD, A2 are significant model terms. As it was seen from Table 3, the mostly effective parameter is solvent/solid ratio (D). Extraction temperature (B) is another parameter which has significant effect on extraction. Liyana-Pathirana and Shahidi reported that the temperature and solvent composition were perhaps the most important factors that may significantly influence total antioxidant activity (14). With increasing temperature up to 60 °C, total phenolic compound extraction was increased. The effect of extraction time (C) is not found important in that model. Similar time effect was found in extraction of phenolic compounds from wheat as the extraction time had no significant effect (14). Solvent composition (A) has a greater effect than

time but not have significant effect on the model term while the interaction of solvent composition with temperature and solvent solid ratio were significant. Total phenolic compound content in the extract reached a maximum followed by a decrease with further increase in the proportion of the organic solvent in the extraction medium. Similar results were found in relation between total antioxidant activity of wheat and solvent composition (14). Temperature and solvent ratio effect which were found mostly significant on total phenolic compund extraction were shown in Figure 1.

Maximum total phenolic compund (5177.60 mg GAE/100 g dried leaves) gain was at 40 °C and 8 solvent ratio (Figure 1). In that combination, solvent composition was 60:40 ethanol:water(v/v) where the extraction time was 26 h. Proposed experimental design for total phenolic compounds from olive leaves (43 % ethanol in water (v/v), 50 °C, 15 hours and 7 times solvent/solid ratio) were found more suitable as its required time, ethanol concentration and amount of solvent is lower than other researchers' experiments. It is possible to change conditions as desired with the information of RSM results. It is very clear to introduce variables effect with this optimization procedure. The main significant factors on extraction of total phenolic compounds were found as extraction temperature and solvent solid ratio.

CONCLUSION

In solvent extraction procedures, several factors have effects on the extraction efficiency. There is lack of information in literature on determination of these variables effect involved in interactions

Table 3. The results of ANOVA for total phenolic compounds from dried olive leaves

Response	R					
ANOVA for Response Surface Quadratic Model						
Analysis of variance table [Partial sum of squares - Type III]						
	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	12211764	14	872268.9	6.26	0.0005	significant
A-A	7441.282	1	7441.282	0.05	0.8203	
B-B	1062419	1	1062419	7.63	0.0145	
C-C	143567.8	1	143567.8	1.03	0.3260	
D-D	5879689	1	5879689	42.22	< 0.0001	
AB	1558178	1	1558178	11.19	0.0044	
AC	61961.17	1	61961.17	0.44	0.5149	
AD	758937.2	1	758937.2	5.45	0.0339	
BC	78713.91	1	78713.91	0.57	0.4638	
BD	72269.57	1	72269.57	0.52	0.4824	
CD	2691.534	1	2691.534	0.02	0.8913	
A^2	1844954	1	1844954	13.25	0.0024	
B^2	89952.52	1	89952.52	0.65	0.4341	
C^2	2200.986	1	2200.986	0.02	0.9016	
D^2	309509.3	1	309509.3	2.22	0.1567	
Residual	2088899	15	139259.9			
Lack of Fit	1845935	10	184593,5	3.798787	0.0769	not significant
Pure Error	242963.8	5	48592.75			

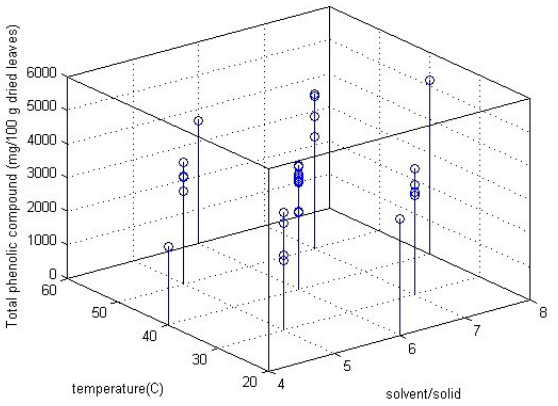

Figure 1. Total Phenolic compound (mg GAE/100 g dry leaves) data with different temperature and solvent/solid variables

during extraction processes. RSM is a powerful tool in determination of these factors effects. Experimental design for extractions which is not directly related to used equipment can be well defined with RSM. In this study effects of extraction temperature, solvent/solid ratio, solvent composition and time effects with their interactions was determined. The main significant factors on extraction of total phenolic compounds were found as extraction temperature and solvent solid ratio.

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