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Comparison between conventional and ultrasound-assisted extractions of natural antioxidants from walnut green husk

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Abstract−Agricultural industries produce substantial quantities of phenolic-rich by-products, which have gained much attention due to their antioxidant properties. Ultrasonic technology was applied for extraction of antioxidants from the walnut green husk using ethanol as a food grade solvent. Response surface methodology (RSM) was used to optimize experimental conditions. The responses were total phenolic content (TPC), ferric reducing antioxidant power (FRAP), scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and yield. TPC varied from 6.28 to 7.23 mg GA g^{-1} dry sample. FRAP and DPPH values varied from 0.33 to 0.46 mmol Fe^{2+} g^{-1} of dry sample and 33.98% to 56.31% inhibition, respectively. Extraction yields ranged from 33.04% to 38.72%. The optimal conditions were 60% ethanol-water mixture as solvent, temperature of 60 °C and extraction time of 30 min. Comparison of ultrasonic-assisted extraction (UAE) and conventional extraction was shown that TPC, FRAP, DPPH and yield obtained by UAE during 30 min were significantly higher than by conventional extraction during 16 hours. The extract can be used as substitute of synthetic antioxidants for food products, color and oxidative stabilization.

Keywords: Ultrasonic, Antioxidants, Walnut Green Husk, Response Surface Methodology

INTRODUCTION

The green husk, leaf, stem and bark of the walnut tree (*Juglandaceae*) have been widely used as folk medicine for treatment of cancer and dermatosis in Korea, Japan and China for a long time. The green walnut husk (*Juglans regia* L) is a traditional herbal medicine, which has long been used for clearing away heat, toxic materials, alleviating pain and treating skin disease [1]. Different works demonstrated the antioxidant content of walnut products, such as fruits [2,3], leaves [4], liqueurs [5] and husk [6-8].

Free radicals are any atom or molecule containing one or more unpaired electrons. For this reason they are highly reactive with a great variety of molecules and change their physical-chemical properties. Reactive oxygen species (ROS) are normally produced during cellular metabolism, and for this reason aerobic cells have antioxidant defense mechanisms which protect them from oxidative stress [9]. Excessive production of ROS can lead to accumulative damage in DNA, protein and lipids, which are involved in various disease processes, ranging from cancer to diabetes and neurodegenerative diseases [10]. Consequently, antioxidants have emerged as therapeutic agents for these diseases [11].

In recent decades an increasing tendency towards the use of natural substances instead of the synthetic ones has been observed. Natural antioxidants, such as phenolic compounds, are used as natural antioxidants. They are important, due to their benefits for human health, reduction of oxidative stress and inhibition of radicals. Their use as preserving food additives had increasing interest [12,13].

Several conventional extraction techniques have been reported for the extraction of target compounds from raw materials. However, they are usually associated with longer extraction times and higher temperatures but lower extraction efficiency. With the development of the "green chemistry" concept during the last few years, environment-friendly techniques are becoming more and more attractive. Recently, ultrasonic-assisted extraction (UAE) has been widely employed in the extraction of target compounds from different materials, owing to its facilitated mass transfer between immiscible phases through super agitation at low frequency [14]. It offers high reproducibility at shorter times, simplified manipulation, and lowered energy input, as well as solvent consumption [15]. By using conventional extraction under ultrasound irradiation (20-100 kHz), structural changes and degradation can be avoided. Thus, UAE may be an effective and advisable technique for the extraction of chemicals [16]. Many factors have been established to influence the extraction efficiency, such as extraction method, solvent type, solvent concentration and extraction temperature and time [17]. In many works, methanol comes up as a suitable extraction solvent to reach good yields of the phenolic compounds. However, environmentally benign and non-toxic food grade organic solvents like water and ethanol are recommended by the US Food and Drug Administration for extraction purposes [18].

Response surface methodology (RSM) has been used increasingly to optimize processing parameters because it allows more efficient and easier arrangement and interpretation of experiments compared to other methods [19]. The main advantage of RSM is the reduced number of experimental trials needed to evaluate multiple parameters and their interactions. Therefore, it is less laborious and time-consuming than other approaches required to optimize a process. Unlike the conventional empirical method, RSM can generate a mathematical model, and take into account the possible interrelationship among the test variables and minimize the number of experiments.

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The aim of this study was to optimize ultrasonic-assisted extraction variables such as temperature, time and concentration of ethanol as modifier for the maximum extraction of antioxidants from walnut green husk by response surface methodology. Finally, ultrasonic-assisted extraction (UAE) was compared with conventional extraction.

MATERIALS AND METHODS

1. Plant Materials

The walnuts were traditional Persian walnut (*Juglans regia* L), purchased from a local market in Shiraz in the 2010 season. Walnut green husks were handpicked from fruits and then rinsed with distilled water. The husks were dried in an oven (Memmert, GmbH+ Co. KG, DIN 40050, Germany) with air circulation at 40 °C and finely ground in a laboratory grinder (Pars Co., Iran). The ground sample was fractioned by a series of sieves (35, 60 and 80-mesh, Damavand Co., Iran) to obtain the particle size distribution. Samples were put in plastic bags and frozen at −20 °C until extraction.

2. Chemicals

2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ), Folin-Ciocalteu reagent and gallic acid were purchased from Merck. 1,1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich.

3. Extraction Procedure

The process of antioxidant extraction from walnut green husk by ultrasonic was performed in an ultrasonic bath S30H (Elmasonic, Germany) with maximum frequency of 60 KHz (280 W). Walnut green husk powder (0.5 g) was sonicated in the solvent (10 ml) for different times and temperatures. After the extraction, the walnut green husk extract was centrifuged at 2,000 rpm for 20 min. The extracts were concentrated by rotary evaporation at 40 °C under vacuum to dryness and the yield of extraction was determined.

4. Optimization of Solvent Type, Solvent-to-solid Ratio and Particle Size

Samples were extracted with ethanol, methanol, water and acetone, respectively, and kept for sonication at 45 °C for 30 min. The best solvent was selected according to the values of responses. The influence of the solvent-to-solid ratio on the extraction was investigated by considering four ratios: 20 : 1, 30 : 1, 40 : 1, 50 : 1; v : m. Walnut green husk (0.5, 0.33, 0.25 and 0.20 g) was sonicated in a fixed volume of 10 ml of ethanol solvent at 45 °C for 30 min. Samples with different particle sizes (35, 60, 80-mesh sieve) were sonicated for 45 °C for 30 min with ethanol (10 ml) and 40 : 1 solvent-to-solid ratio.

5. Total Phenolic Content (TPC)

Total phenol content in the obtained extracts was estimated by a colorimetric assay based on procedure described by Singleton and Rossi with some modifications [6]. Briefly, 1 ml of sample (or standard) was mixed with 1 ml of Folin-Ciocalteu's phenol reagent. After 3 min, 1 ml of sodium carbonate solution (7.5% w/v) was added to the mixture and adjusted to 10 ml with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm. Gallic acid was used for constructing the standard curve (0.1-2 mM). The results are expressed as mg of gallic acid equivalents/g of dry sample (GAEs).

6. DPPH Assay

The capacity to scavenge the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was monitored according to a previous method [20]. Various concentrations of sample extracts (0.3 ml) were mixed with 2.7 mL of methanolic solution containing DPPH radicals. The mixture was shaken vigorously and left to stand in the dark until stable absorption values were obtained (about 30 min). The reduction of the DPPH radical was measured by monitoring continuously the decrease of absorption at 517 nm. DPPH scavenging effect was calculated as percentage of DPPH discoloration using the equation:

$$\%\ \text{scavenging effect} = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100 \quad (1)$$

where A_S is the absorbance of the solution when the sample extract has been added and A_{DPPH} is the absorbance of the DPPH solution.

7. Ferric Reducing/Antioxidant Power (FRAP)

This procedure was conducted according to the modified method [21]. The working FRAP reagent was produced by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution and 20 mM $FeCl_3 \cdot 6H_2O$ in a 10 : 1 : 1 ratio prior to use and heated to 37 °C in a water bath. A total of 3 ml of FRAP reagent was added to a cell and a blank reading was taken at 593 nm, using the spectrophotometer. In total, 100 μL of sample or standard and 300 μL of distilled water were added to the cell. After addition of the sample to the FRAP reagent, a second reading at 593 nm was taken after 40 min of incubation at 37 °C in a water bath. The changes in absorbance after 40 min from the initial blank reading were compared with a standard curve. Standards of known Fe(II) concentrations were run using several concentrations ranging from 0.1 to 2.25 mM. A standard curve was then prepared by plotting the FRAP values of each standard versus its concentration. The final result was expressed as mmol Fe^{2+} g^{-1} sample.

8. Experimental Design

Three factors that can potentially affect extraction of antioxidants such as ethanol percentage (X_1, %), extraction temperature (X_2, °C) and extraction time (X_3, min) were chosen as key variables. The minimum and maximum levels (Table 1) given to each factor were chosen based on preliminary experiments, our experience and that of our previous works.

Box-Behnken design (BBD) with three independent variables was used for the optimization and to achieve maximal information from a minimal number of possible experiments. The dependent variables (response variables) were TPC (mg GAE g^{-1} sample), FRAP (mmol Fe^{2+} g^{-1} sample), DPPH free radical scavenging (%) and yield of extraction (%). Each variable was coded at three levels: −1, 0 and +1 (Table 1). The complete quadratic equation used is as follows:

$$Y = \beta_o + \sum_{i=1}^{3} \beta_i X_i + \sum_{i}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i-1}^{3} \beta_{ij} X_i X_j \quad (2)$$

where Y is the estimated response; β_o, β_i, β_{ii}, and β_{ij} are the regres-

Table 1. Coded and uncoded levels of the independent variables

Independent variables	Coded units	Coded levels		
		−1	0	1
Ethanol concentration (%)	X_1	45	55	65
Temperature (°C)	X_2	30	45	60
Time (min)	X_3	30	50	70

sion coefficients for intercept, linear, square, and interaction terms, respectively; and X_i and X_j are the independent variables.

All the analysis was done in triplicate and the experimental results were expressed as means±SD. Statistical analysis was performed using the Minitab 15.1 (Minitab Inc., State College, PA, USA) software and fitted to a second-order polynomial regression model containing the coefficient of linear, quadratic and interaction terms. An analysis of variance (ANOVA) with 95% confidence level was then carried out for each response variable to test the model significance and suitability. The significances of all terms in the polynomial was analyzed statistically by computing the *F*-value at a probability (p) of 0.01 or 0.05.

RESULTS AND DISCUSSION

1. Effect of Solvent Type, Solvent-to-solid Ratio and Particle Size on Extraction

Before the development of response surface models, effects of solvent type, solvent to solid ratio and particle size on TPC, FRAP value, DPPH scavenging activity and extraction yield were investigated. It is evident that the recovery of phenolic and antioxidant compounds from each fresh sample matrix is dependent on the extracting solvent. Methanol was better than ethanol, acetone and water in extraction of phenolics and antioxidants from walnut green husk (Fig. 1) under the same extraction conditions (20 : 1 (v : m), 45 °C and 30 min). However, environmentally benign and non-toxic food-grade organic solvents like water and ethanol are recommended by the US Food and Drug Administration for extraction purposes [18]. So ethanol was chosen as the extraction solvent for the next experiments.

The effect of the solvent-to-solid ratio on the extraction of polyphenols and antioxidants from walnut green husk was also studied with four ratios (20 : 1, 30 : 1, 40 : 1, 50 : 1; v : m) over a 30 min extraction period, with an ethanol solution, at 45 °C. A marked increase was observed at 40 : 1 solvent-to-solid ratio (Fig. 2). Consequently, a solvent-to-solid ratio of 40 : 1 (ml;g) was selected.

Walnut green husk powder was passed through three different standard-size sieves (pore sizes of 0.5, 0.25 and 0.18 mm). The results showed that smaller particle size increases the antioxidant extraction and yield over a 30 min extraction period with an ethanol solution at 45 °C and solvent-to-solid ratio 40 : 1 (Fig. 3). In general, smaller particle size was preferred for industrial processes to shorten

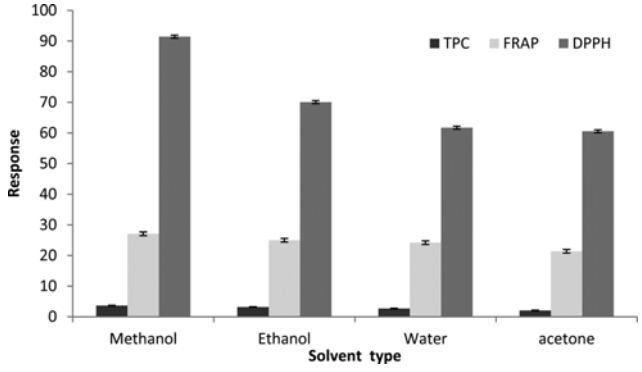

Fig. 1. Effect of solvent type on the extraction of total phenolics and antioxidants from walnut green husks. TPC (mg GA g^{-1}), FRAP (mmol Fe^{2+} g^{-1}) and DPPH (%).

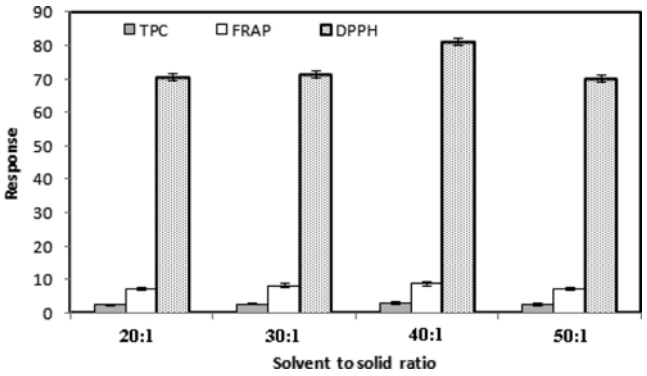

Fig. 2. Effect of the solvent-to-solid ratio on the responses (30 min extraction time and 45 °C). TPC (mg GA g^{-1}), FRAP (mmol Fe^{2+} g^{-1}) and DPPH (% inhibition).

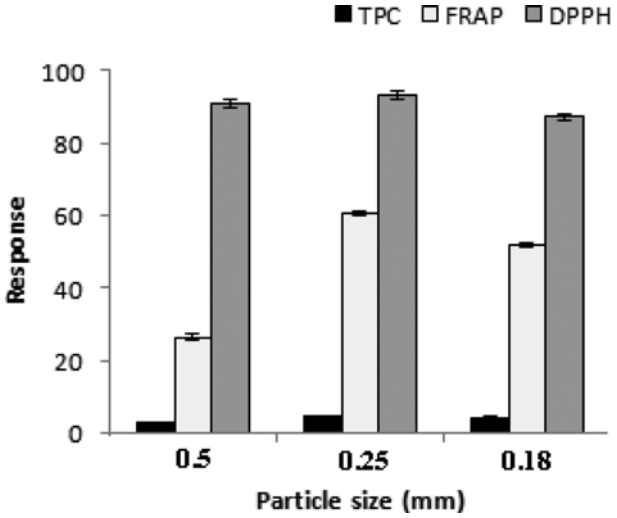

Fig. 3. Effect of particle size on the extraction of total phenolics and antioxidants from walnut green husks. TPC (mg GA g^{-1}), FRAP (mmol Fe^{2+} g^{-1}) and DPPH (%).

the extraction time even though the energy used for grinding needs to be considered. The increase of the extraction yield for the small particles is due to larger surface area per mass unit. In addition, the migration rate of the analyte through the pores of the solid matrix is also increased with the decrease in particle size. Particle size of 0.25 mm was used for next extraction experiments (Fig. 3).

2. Modeling of the Extraction Process from Walnut Green Husk

The responses (total phenolic content, antioxidant activities and yield) of each run of the experimental design are presented in Table 2. The coded and decoded values of independent variables for each experiment are also presented. Total phenolic content of walnut green husk extracts varied from 6.28 to 7.23 mg GA g^{-1} dry sample. FRAP and scavenging of DPPH radical assays were used to determine the antioxidant activity of the extracts. As shown in Table 2, activity values for FRAP and DPPH assays varied from 0.33 to 0.46 mmol Fe^{2+} g^{-1} of dry sample and 33.98% to 56.31% inhibition, respectively. Extraction yields ranged from 33.04% to 38.72%.

Regression coefficient and analysis of variance of the second-order polynomial models (Eq. (2)) for total phenolic content, antioxidant activity of walnut green husk extracts and yield are sum-

Table 2. Central composite design of three variables with their observed responses

Exp. no	X_1	X_2	X_3	E (%)	T (°C)	t (min)	TPC (mg GA g^{-1})	FRAP (mmol Fe^{2+} g^{-1})	DPPH (%)	Yield (%)
1	−1	−1	−1	45	30	30	6.82	0.36	41.80	34.88
2	−1	−1	1	45	30	70	6.88	0.41	33.98	35.84
3	−1	1	−1	45	60	30	6.28	0.40	56.31	36.80
4	−1	1	1	45	60	70	6.5	0.46	55.34	37.44
5	1	−1	−1	65	30	30	6.61	0.37	37.14	35.84
6	1	−1	1	65	30	70	6.43	0.38	51.12	33.12
7	1	1	−1	65	60	30	6.97	0.39	35.17	38.72
8	1	1	1	65	60	70	7.06	0.42	36.82	33.28
9	0	0	0	55	45	50	6.75	0.36	51.02	34.12
10	0	0	0	55	45	50	6.67	0.34	50.59	35.36
11	−1	0	0	45	45	50	6.37	0.38	43.34	33.04
12	1	0	0	65	45	50	6.73	0.33	38.88	33.48
13	0	−1	0	55	30	50	6.39	0.34	48.56	33.60
14	0	1	0	55	60	50	6.72	0.39	40.48	36.16
15	0	0	−1	55	45	30	7.23	0.35	49.86	36.96
16	0	0	1	55	45	70	7.01	0.42	55.06	35.04

Table 3. Regression coefficients of predicted polynomial models for the investigated responses

Coefficient	Response			
	TPC	FARP	DPPH	Yield
β_o	4.61^{*}	0.53	-218.92^{*}	−0.13
β_1	151.08×10^{-3}	-14.87×10^{-4}	$83.61\times10^{-1*}$	$1423.81\times10^{-3*}$
β_2	-2.28×10^{-3}	-13.77×10^{-4}	$39.39\times10^{-1*}$	-88.63×10^{-3}
β_3	$-91.68\times10^{-3**}$	-54.31×10^{-4}	-18.12×10^{-1}	-76.62×10^{-3}
β_{11}	$-1.83\times10^{-3*}$	0.38×10^{-4}	-0.68×10^{-1}	-10.33×10^{-3}
β_{22}	$-1.03\times10^{-3*}$	0.42×10^{-4}	-0.15×10^{-1}	2.61×10^{-3}
β_{33}	$0.96\times10^{-3**}$	$0.85\times10^{-4**}$	0.11×10^{-1}	$4.27\times10^{-3**}$
β_{12}	$1.59\times10^{-3**}$	-0.31×10^{-4}	$-0.43\times10^{-1**}$	-0.40×10^{-3}
β_{13}	-0.23×10^{-3}	-0.47×10^{-4}	0.15×10^{-1}	$-6.10\times10^{-3**}$
β_{23}	0.18×10^{-3}	0.13×10^{-4}	0.02×10^{-1}	-1.27×10^{-3}
Model	**	**	*	**
Linear	*	ns	*	ns
Quadratic	**	**	ns	*
Cross-product	**	ns	*	*
Lack of fit	0.372	0.610	0.044	0.735
R^2	0.94	0.94	0.85	0.93

ns, Not significant ($p>0.05$)
*Significant at $p\leq0.05$
**Significant at $p\leq0.01$

marized in Table 3. As shown, the regression parameters of the surface response analysis of the models, the linear, quadratic and interaction terms have significant effects ($p\leq0.01$ or $p\leq0.05$). These models were used for the construction of three-dimensional response surface plots to predict the relationship between independent and dependent variables.

3. Effect of Process Variables on Total Phenolic Compounds

Total phenolic content of walnut green husks extracts obtained by ultrasonic-assisted extraction is shown in Table 2. Regression analysis was performed on the experimental data and the coefficients of model (Eq. (2)) were evaluated for significance. The effect of ethanol concentration was highly significant on the extraction of phenolic compounds (Table 3). Eq. (3) shows the relationship between ethanol concentration, temperature and time for the extraction of total phenolic compounds:

$$\begin{aligned} Y_1\ (\text{mg GA/gdw}) = {} & 4.609 + 0.151\,E - 2.28\times10^{-3}\,T - 9.168\times10^{-2}\,t \\ & - 1.83\times10^{-3}\,E^2 - 1.03\times10^{-3}\,T^2 + 0.96\times10^{-3}\,t^2 \\ & + 1.59\times10^{-3}\,ET - 0.23\times10^{-3}\,Et + 0.18\times10^{-3}\,Tt \end{aligned} \quad (3)$$

where Y_1 represents total phenolic content in walnut green husk extract. E, T, and t are ethanol concentration (%), temperature (°C) and time (min), respectively.

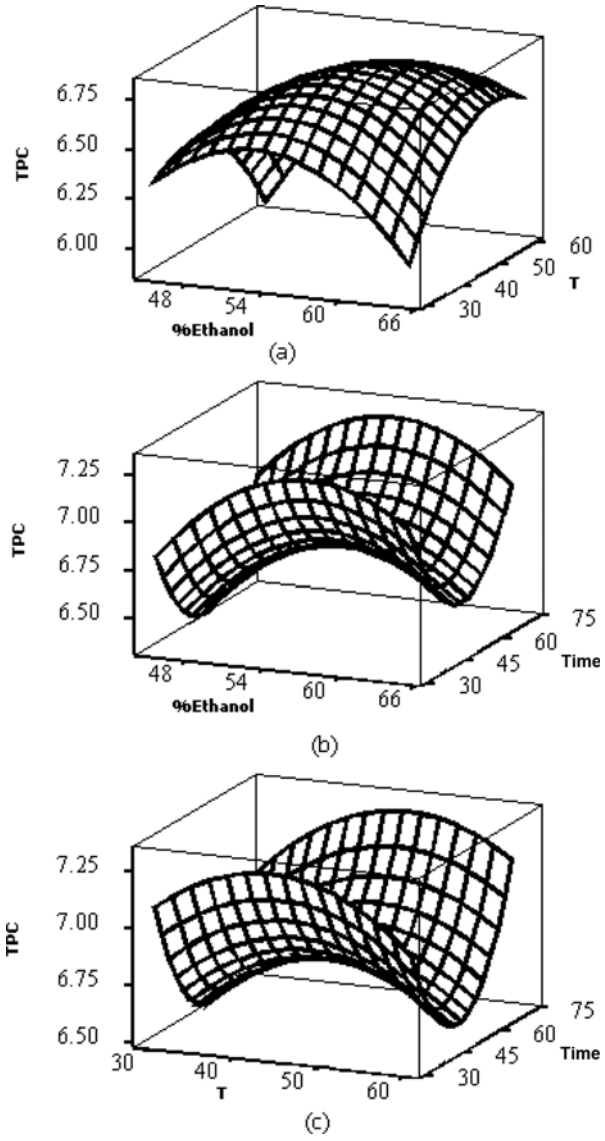

Fig. 4. Response surface plots for the effect of (a) EtOH/temperature (b) EtOH/time and (c) temperature/time on the total polyphenol content (TPC).

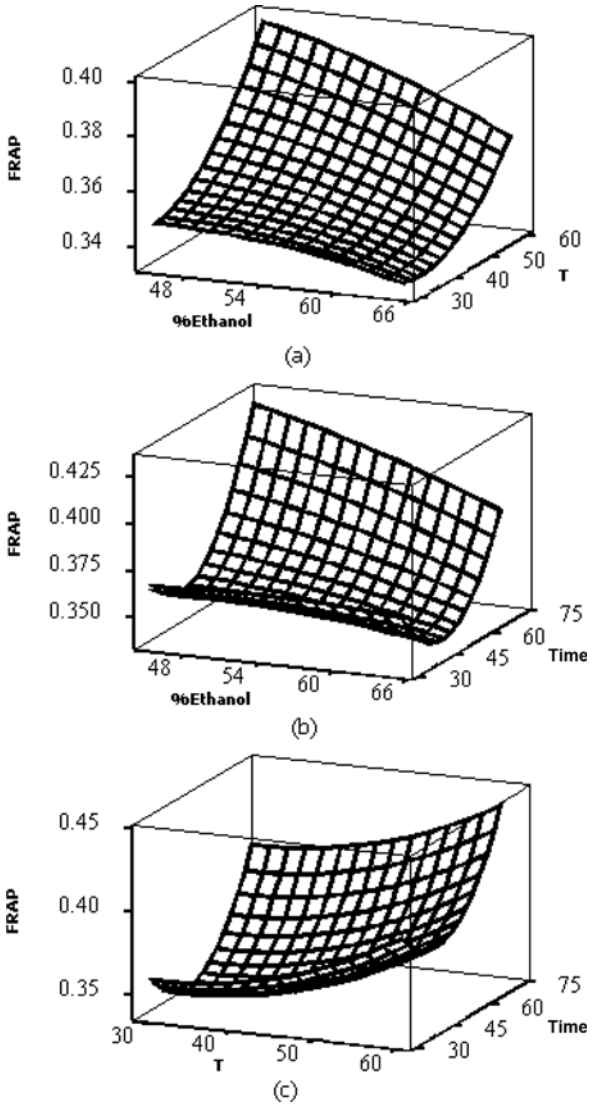

Fig. 5. Response surface plots for the effect of (a) EtOH/temperature (b) EtOH/time and (c) temperature/time on the antioxidant activity assay (FRAP).

The predicted model (Eq. (3)) can be visualized by the surface response plot. This graphical representation is an *n*-dimensional surface in the (n+1)-dimensional space. Usually, a two-dimensional representation of a three-dimensional plot can be drawn. Thus, if there are three or more variables, the plot visualization is possible only if one or more variables are set to a constant value. These surfaces were drawn by Minitab software. Figs. 4-7 illustrate the response surfaces for dependent variables (TPC, FRAP, DPPH and yield) in function of two factors when the third factor was kept constant at middle level (Table 1). The effect of ethanol concentration and extraction temperature on TPC at constant time appeared as a saddle shape (Fig. 4(a)). As shown, a higher amount of phenolics was yielded at ethanol concentration about 60% and temperature between 50-60 °C. TPC gradually increased with the increase of ethanol concentration and temperature, and achieved optimum value at about 60% and 50 °C, before it began to decrease. In general, the polarity of ethanol-water mixture would increase continuously with the addition of water to ethanol. More polar phenolic compounds may be extracted according to "like dissolves like" principle.

The effects of ethanol concentration and extraction time on TPC at fixed extraction temperature (45 °C) are shown in Fig. 4(b). Ethanol concentration demonstrated a pronounced influence on TPC in linear and quadratic manner (Table 3). At upper level of time, TPC increased with the increase of ethanol concentration up to 60% and further increase in ethanol concentration led to deceleration of polyphenol extraction.

The relationship of extraction temperature and time with TPC is shown in Fig. 4(c). Total phenolic content of walnut green husk extracts increased readily with increasing temperature up to 48 °C. Mild heating might soften the plant tissue, weaken the cell wall integrity, hydrolyze the bonds of bound phenolic compounds (phenol-protein or phenol-polysaccharide) as well as enhance phenolics solubility; thus more phenolics would distribute to the solvent.

4. Effect of Process Variables on Antioxidant Compounds

The analytical results of antioxidant activities (FRAP and DPPH)

of walnut green husk extracts are shown in Table 2. Each antioxidant assay (TPC, FRAP, DPPH …) only provides an estimate of antioxidant capacity that is subject to its conditions, reagents and different classes of antioxidants. Therefore, the use of different antioxidant assays help to identify variations in the response of the compounds extracted from the samples. These natural antioxidant compounds can be separated by HPLC from the extract. The relationship between the antioxidant activities of walnut green husk extracts and main variables is shown in Figs. 5 and 6. Antioxidant activities were affected by the quadratic term of time for FRAP and linear terms of ethanol concentration and temperature for DPPH. The models for antioxidant activities are represented in Eqs. (4) and (5):

$$Y_2\ (\text{mmol } Fe^{2+}/\text{gdw}) = 0.53 - 1.49\times10^{-3}\,E - 1.38\times10^{-3}\,T - 5.43\times10^{-3}\,t + 0.38\times10^{-4}\,E^2 + 0.42\times10^{-4}\,T^2 + 0.85\times10^{-4}\,t^2 - 0.31\times10^{-4}\,ET - 0.47\times10^{-4}\,Et - 0.13\times10^{-4}\,Tt \quad (4)$$

$$Y_3\ (\%\ \text{Inhibition}) = -218.92 + 8.36\,E + 3.94\,T - 1.81\,t - 0.68\times10^{-1}\,E^2 - 0.15\times10^{-1}\,T^2 + 0.11\times10^{-1}\,t^2 - 0.43\times10^{-1}\,ET + 0.15\times10^{-1}\,Et - 0.02\times10^{-1}\,Tt \quad (5)$$

where Y_2 and Y_3 represent ferric reducing antioxidant power (FRAP) and scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) of walnut green husk extracts.

The effects of ethanol concentration, temperature and time on FRAP and DPPH are shown in Figs. 5 and 6. As shown, the highest activities were obtained at ethanol concentration between 45-55% and extraction temperature between 50-60 °C. Antioxidant activity (DPPH) gradually increased with the increase of ethanol concentration and extraction temperature, and achieved optimum values before it began to decrease. As shown in response surface plots for the effect of time on FRAP (Figs. 5(b) and 5(c)) and DPPH values (Figs. 6(b) and 6(c)) at constant ethanol concentration and constant temperature, longer extraction time had a positive effect on the antioxidant activities of walnut green husk extracts.

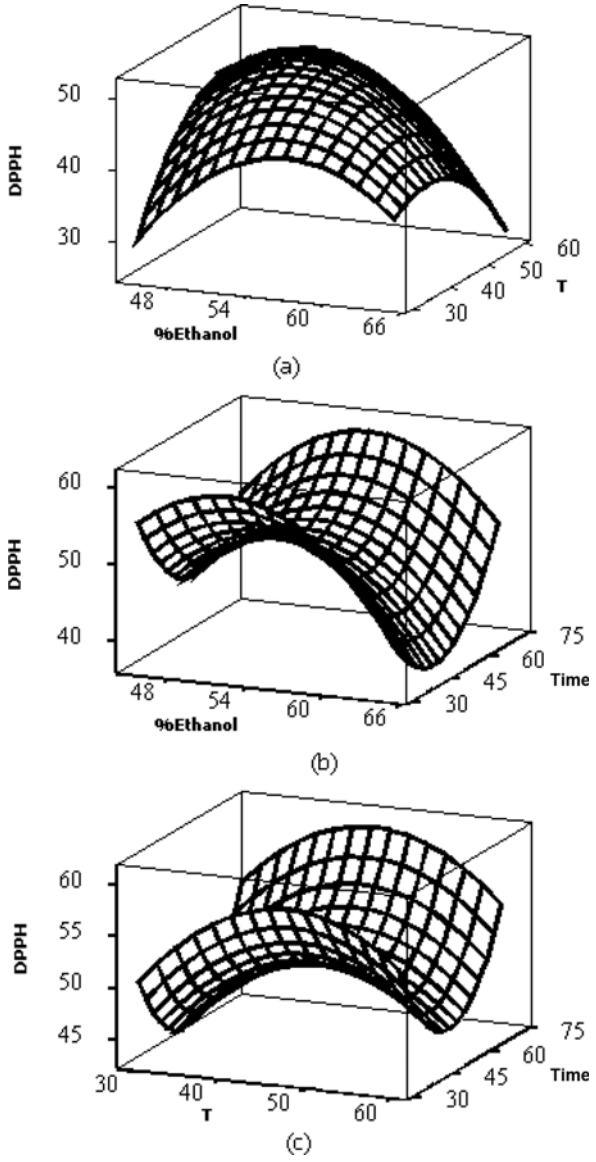

Fig. 6. Response surface plots for the effect of (a) EtOH/temperature (b) EtOH/time and (c) temperature/time on the antiradical activity (DPPH).

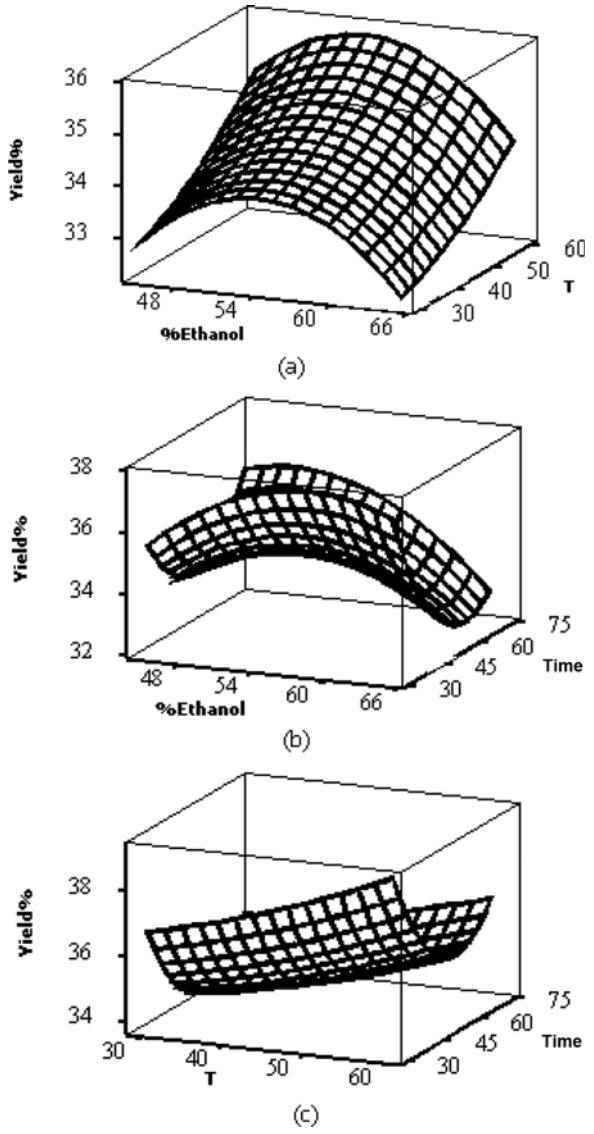

Fig. 7. Response surface plots for the effect of (a) EtOH/temperature (b) EtOH/time and (c) temperature/time on the yield (%).

Table 4. Estimated optimum conditions, predicted and experimental values of responses under these conditions

Response variables	Optimum UAE conditions			Maximum values	
	Ethanol (%)	T (C)	t (min)	Predicted	Actual*
TPC (mg GA g^{-1})	60	48	30	7.14	6.95±0.21
FRAP (mmol Fe^{2+} g^{-1})	45	60	70	0.47	0.45±0.04
DPPH (%)	46	60	30	58.10	56.32±2.61
Yield (%)	59	60	30	39.04	36.98±1.93

*Results are means±SD (n=3)

5. Effect of Process Variables on Extraction Yield

The yields of extractions are presented in Table 2. Solvent was removed from the extracts by evaporation under vacuum at 40 °C by a rotary evaporator. The regression analysis of the data showed that the extraction yield was significantly affected by the ethanol concentration and extraction time. The relationship of the extraction yield and process variables is depicted in Fig. 7. The optimal conditions of extraction yield were determined as follows: ethanol concentration 59% (v/v), extraction temperature 60 °C and extraction time 30 min. Eq. (6) shows the relationship between ethanol concentration, temperature, time and yield:

$$Y_4\,(\%\text{Yield}) = -0.13 + 1.42\,E - 8.86\times10^{-2}\,T - 7.66\times10^{-2}\,t - 1.03\times10^{-2}\,E^2 + 2.61\times10^{-3}\,T^2 - 4.27\times10^{-3}\,t^2 - 0.40\times10^{-3}\,ET - 6.10\times10^{-3}\,Et - 1.27\times10^{-3}\,Tt \quad (6)$$

where Y_4 is the extraction yield (%).

6. Optimal Conditions

Response surface optimization can be found depending on the three key variables. The optimal conditions obtained from the first derivatives of the second-order polynomial equations (Eqs. (3)-(6)). The derivatives were then equaled to zero and solved in an equation system. The optimum UAE conditions for the response variables from walnut green husk extract are presented in Table 4. The predictive ability of the models was examined by extractions at optimal conditions. For each condition, three experiments were done and the experimental values confirmed the validity and adequacy of the predicted models. The extract can be used as a substitute for synthetic antioxidants for food products, color and oxidative stabilization. Therefore, the highest yield is recommended for industrial applications. The optimal conditions for this aim were a solvent percentage of 60% ethanol, temperature of 60 °C and extraction time of 30 min.

7. Comparison of UAE vs. Conventional Extraction

The phenolic compounds extracted from walnut green husk by ultrasonic-assisted extraction and conventional extraction are shown in Table 5. The TPC, FRAP, DPPH and yield obtained by UAE during 30 min were significantly higher than by conventional extraction during 16 hours. The consumed energy was equal to the power multiplied by the exposure time. The energy consumption was (140 W ×0.5 h) and (500 W×16 h) for UAE and conventional methods, respectively. Although the same volumes of solvent were used in both extraction processes, the duration of the ultrasound-assisted process and consequently the energy input were reduced and better overall yields were obtained.

Table 5. Comparisons of ultrasonic-assisted and conventional extraction of antioxidants from walnut green husk (UAE conditions: 60% ethanol, 60 °C, 30 min; Conventional condition: 60% ethanol, 60 °C, 16 hour)

Response	UAE	Conventional
TPC (mg GA/g dw)	6.83	4.25
FRAP (mmol Fe^{2+}/g dw)	0.37	0.02
DPPH (%)	34.21	23.55
Yield (%)	37.52	14.31

CONCLUSIONS

The recent consumer interest in natural products drives the need for natural antioxidants to replace the synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). By-products of food processing industries assume significance because of their acceptability, nontoxicity and availability in large quantities. Walnut's green husk, a by-product of the walnut production, is a good source of phytochemicals with antioxidant and antimicrobial activities.

This study revealed application of ultrasonic technology for extraction of the polyphenols and antioxidants from walnut green husk. Response surface methodology (RSM) was used to simultaneously optimize the experimental variables such as ethanol concentration, temperature and time and evaluation of variable interactions. In the previous works, methanol came up as a suitable extraction solvent. However, environmentally benign and non-toxic food-grade organic solvents like water and ethanol were used for extraction purposes. Ultrasonic-assisted extraction (UAE) and conventional extraction methods were compared. The TPC, FRAP, DPPH and yield obtained by UAE during 30 min were significantly higher than by conventional extraction during 16 hours. Thus UAE permits higher extraction yields in shorter periods of time, thereby reducing the energy input.

REFERENCES

1. H. R. Li, Z. G. Yang, J. Wang and S. Kitanaka, *Nat. Prod. Res. Dev.*, **18**, 363 (2006).
2. J. A. Pereira, I. Oliveira, A. Sousa, I. C. F. R. Ferreira, A. Bento and L. Estevinho, *Food Chem. Toxicol.*, **46**, 2103 (2008).
3. L. Li, R. Tsao, R. Yang, C. M. Liu, H. H. Zhu and J. C. Young, *J. Agric. Food Chem.*, **54**, 8033 (2006).
4. J. A. Pereira, I. Oliveira, A. Sousa, P. Valentão, P. B. Andrade, I. C. F. R. Ferreira, F. Ferreres, A. Bento, R. Seabra and L. Estevinho,

J. Sci. Food Agric., **45**, 2287 (2007).
5. F. Stampar, A. Solar, M. Hudina, R. Veberic and M. Colaric, *Food Chem.*, **95**, 627 (2006).
6. I. Oliveira, S. I. Sousa, C. F. R. Ferreira, A. Bento, L. Estevinho and J. A. Pereira, *Food Chem. Toxicol.*, **46**, 2326 (2008).
7. J. Jakopic, R. Veberic and F. Stamper, *Acta Agr. Slov.*, **93**, 11 (2009).
8. A. Fernandez-Agullo, E. Pereira, M. S. Freire, P. Valentao, P. B. Andrade, J. González-Álvarez and J. A. Pereira, *Ind. Crop. Prod.*, **42**, 126 (2013).
9. T. Finkel and N. J. Holbrook, *Nature*, **408**, 239 (2000).
10. J. M. Mates, J. A. Segura, F. J. Alonso and J. Marquez, *Mini Rev. Med. Chem.*, **9**, 1202 (2009).
11. M. Valko, D. Leibfritz, J. Moncol, M. T. D. Cronin, M. Mazur and J. Telser, *Int. J. Biochem. Cell Biol.*, **39**, 44 (2007).
12. M. I. Bhanger, S. Iqbal, F. Anwar, M. Imran, M. Akhtar and M. Zia-ul-Haq, *Int. J. Food Sci. Technol.*, **43**, 779 (2008).
13. R. S. Farag, E. A. Mohamoud and A. M. Basuny, *Int. J. Food Sci. Technol.*, **42**, 107 (2007).
14. K. Vilkhu, R. Mawson, L. Simons and D. Bates, *Innov. Food Sci. Emerg.*, **9**, 161 (2008).
15. M. K. Khan, A. V. Maryline, A. S. Fabiano-Tixier, O. Dangles and F. Chemat, *Food Chem.*, **119**, 851 (2010).
16. F. Chemat, V. Tomao and M. Virot, *Ultrasound-assisted extraction in food analysis*, in: Semih Otles (Ed.), *Handbook of food analysis instruments*, CRC Press, USA, 85 (2008).
17. M. Pinelo, M. Rubilar, M. Jerez, J. Sineiro and M. J. Nunez, *J. Agric. Food Chem.*, **53**, 2111 (2005).
18. D. D. Bartnik, C. M. Mohler and M. Houlihan, US Patent, 20,060,088,627, April 27 (2006).
19. C. Liyana-Pathirana and F. Shahidi, *Food Chem.*, **93**, 47 (2005).
20. W. Brand-Williams, M. E. Cuvelier and C. Berset, *LWT - Food Sci. Technol.*, **28**, 25 (1995).
21. R. Pulido, L. Bravo and F. Saura-Calixto, *J. Agric. Food Chem.*, **48**, 3396 (2000).