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OPTIMIZED ULTRASONIC-ASSISTED EXTRACTION OF TOTAL PHENOLICS AND ANTIOXIDANT ACTIVITY OF DATE (PHOENIX DACTYLIFERA L.) USING RESPONSE SURFACE METHODOLOGY

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In the present study, we have optimized the ultrasonic-assisted extraction conditions of total phenolic compounds (TPC) and antioxidant activity of date fruit using response surface methodology (RSM). Three factors including methanol concentration (40-80%), sonication amplitude (50-100%) and extraction time (10-30 min) were investigated. The analysis of results with JMP software, using Box-Behnken model, indicated that 65% methanol, ultrasound amplitude of 84.50%, and of extraction time of 17.64 min were the optimal combination that maximized antioxidants extraction. Under the optimized conditions, the experimental values for both TPC and antioxidant activity were 246.46 mg EAG/100g and 26.48 mg EAG/100g, respectively. Predicted models were highly significant ($P < 0.01$) with high regression coefficients ($R^2 \geq 0.97$), while the lack of fit is insignificant. RSM was shown to be an adequate approach for modeling the total phenolic extraction and the antioxidant activity of date palm fruit.

Keywords: date, ultrasonic-assisted extraction, phenolics, antioxidant activity, response surface methodology

Introduction

Date palm fruits are an excellent source of carbohydrates, minerals and vitamins with a high dietary fibers content (Al-Shahib and Marshell, 2003). From immemorial time, date was considered as an important part of the daily diet in the hot arid regions of the world. They are also popular in other parts of the world due to their delicious taste, nutritional value and health benefits (Vayalil, 2002).

Cells of human body produce antioxidants in order to protect biomolecules including DNA, proteins and lipids, from oxidation, which is an inevitable phenomenon caused by free radicals produced by the organism. An overproduction of free radicals results in oxidative damage of biological molecules causing many human diseases and accelerated aging process. Hence, dietary antioxidants are necessary for a good health. Plant diet represents promising tools against the cellular damage and other human diseases caused by the free radicals (Fattahi and

Rahimi, 2016, Vasundhara *et al*., 2008). According to Menat (2006), fruits and vegetables phenolic compounds are considered as the most interesting group of natural antioxidants.

The extraction is a preliminary and essential step to take advantage of different natural resources. Extraction of phenolics using conventional methods have often disadvantages, such as the use large volume of solvent and a long time of extraction with less efficiency. Other modern extraction techniques such as microwave-assisted extraction, supercritical fluid extraction and ultrasound-assisted extraction have been developed. However, in comparison with these extraction methods, ultrasound equipment is simpler and economically cheaper (Awad *et al*., 2012). Ultrasound is one of the emerging technologies developed to minimize processing, maximize quality and ensure the safety of food product. This technique can dramatically enhance mass transport in various foods; it promotes homogenization and extraction of intracellular compounds (Jambrak, 2012).

The traditional optimization methods of extraction process consider variables individually. However, it is time consuming and dismisses influences that variables may exert on another one (Guldiken *et al*., 2015). Methodology of the experiment design has become an important tool in optimization procedures. It is a method for developing, improving and optimizing process; it can evaluate the effect of the variables and their interaction. Response surface methodology (RSM) based on the development of series of experimental tests is aimed to obtain the parameters involved in optimization. Conventionally, the methodology is to fix all parameters and varying one, while observing a response investigated. A number of experiments will increase according to the number of extraction factors to optimize.

There is no data reported about optimization of extraction conditions of phenolics date fruit using response surface methodology. Consequently, the aim of the current study is to optimize the ultrasound-assisted extraction conditions of total phenolics and antioxidant activity of date fruit using the response surface methodology. The investigated factors were methanol concentration, extraction time and sonication amplitude.

Materials and methods

Chemicals

Folin-Ciocalteu reagent was purchased from Biochem, Chemopharm (Montreal, Quebec), 1,1-diphenyl-2-picrylhydrazyl radical - from Sigma Aldrich (Sternhein, Germany), gallic acid - from Sigma Aldrich (St. Louise, MO, USA), sodium carbonate - from BiochemChemopharma (Georgia, USA) and methanol (99.7%) was purchased from Prolabo VWR (Fontenay- sous- bois, France).

Sample preparation

Tamdjohart mature cultivar harvested in M'zab oasis of Ghardaia department (Algeria) in November 2014 was used in the current investigation. It was characterized by its dark brown color. After harvest, the dates were transported to

the laboratory. Fruits with uniform size free of physical damage, insect injury and fungal infection, were selected.

Optimization of the extraction conditions

The initial step of the preliminary experiment was to select an appropriate extraction solvent for date antioxidants. Effect of solvent type (ethanol, methanol, acetone, and water), methanol concentration (20-100%), sample/methanol ratio (1/100, 2/100, and 3/100), extraction time (5-30 min), and sonication amplitude (25-100%) were determined. Three variables (solvent concentration, extraction time and sonication amplitude) were selected as significant. The obtained preliminary results were used in order to optimize the extraction conditions using RSM.

Preparation of date extract

An aliquot of date paste was weighed into a test tube and 20 mL of solvent were added. The extraction was carried out by an ultrasonicator equipped with a probe (Sonics vibracell. VCX 130 PB, USA) using a frequency of 20 KHz. Sonication was performed in an ice bath in order to keep the low temperature. Subsequently, the mixture was centrifuged at 500 rpm (NF 200, Nuve, Turkey) for 5 min and supernatant was used for further investigations.

Total phenolic contents

The method of Singleton and Rossi (1965) was adopted for determining the total phenolics. A quantity of 200µL of the extract was mixed with 750µL Folin-Ciocalteu reagent, and 400µL of sodium carbonate (7.5%) were added after 5 min. After 60 min, the absorbance was measured in a UV-vis spectrophotometer (UVline 9400, Secomam 30100, Ales France, EU) at 720nm. The total phenolic content was expressed as milligrams of gallic acid equivalent (GAE) per 100g of date fruit.

Antioxidant activity

The antiradical activity was evaluated according to Molyneux (2004). To 100 µl of extract was added one milliliter of a methanol solution of DPPH. After 30 min, the absorbance was measured at 515nm. The scavenging activity of date extracts was calculated using a calibration curve of gallic acid and expressed as mg GAE/100g of date fruit.

Experimental design

JMP software with Box-Behnken design was used to investigate and validate the parameters affecting the extraction of TPC and antioxidant activity. In this study, fifteen experiments were designed and carried out with different ranges of methanol concentration, sonication amplitude and extraction time (Table 1). Coded value 0 stands for center point, +1 for the maximal value and -1 for the minimal value.

Data analysis

In order to conduct the experimental design and statistical analysis, the response surface regression procedure of JMP 10 (statistical analysis system Inc., SAS)

software was used. Experimental data were fitted to a second order polynomial model and regression coefficient obtained presented in equation 1.

$$y = a_0 + \sum_{i=1}^{3} a_i x_i + \sum_{i=1}^{3} a_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j=1}^{3} a_{ij} x_i x_j \quad (i \neq j) \qquad (1)$$

where a_0, a_i, a_{ii}, a_{ij} are the regression coefficients for intercept, linear, quadratic, and interaction terms, respectively; x_i and x_j are the independent variables.

Fischer and Student's t-tests were performed for the determination of model equation and for determination of statistical significance of regression coefficient, respectively.

Model verification

A second-order equation was chosen to select optimal levels of the three variables, methanol concentration, sonication amplitude, and extraction time. Validity of models was confirmed by comparing the experimental and predicted values.

Results

Model Analysis

Preliminary study was conducted to determine the effect of five variables in wide range. Three variables (methanol concentration, extraction time and sonication amplitude) were selected as pertinent.

Table 1 shows the experimental design and obtained results according to the Box-Behnken model. It indicated that TPC was ranged from 215.47 to 246.61 mg GAE/100g, while the antioxidant activity varied from 19.26 to 26.54 mg GAE/100g. It indicated also the close agreement between experimental and predicted values.

Table 2 presents the influence of methanol concentration, extraction time and sonication amplitude as well as the interaction between these parameters on TPC and antioxidant activity.

The quadratic terms of methanol concentration and sonication amplitude, as well as interactions methanol concentration-sonication amplitude and extraction time-sonication amplitude influenced negatively both responses (TPC and antioxidant activity). However, the linear coefficient of extraction time (x_2) and interaction methanol concentration-extraction time were found to have no significant effect on phenolic extraction and antioxidant activity of date. Linear coefficient of sonication amplitude and quadratic term of extraction time influenced negatively TPC, while the linear coefficient of methanol concentration was insignificant. Linear coefficient of methanol concentration influenced positively the antioxidant activity, while the linear coefficient of amplitude and quadratic term of extraction time were found to have no significant effect.

Reduced second order regression model for total phenolic content (Y_{TPC}) and antioxidant activity (Y_{AA}) using significant terms for methanol concentration (x_1), extraction time (x_2) and sonication amplitude (x_3) were as following equations (Eq. 2 and Eq. 3):

$$Y_{TPC} = 245.82\text{-}3x_3 - 7.92x_1x_3 - 4.13x_2x_3 - 11.99x^2_1 - 3.51x^2_2 - 8x^2_3 \quad (2)$$

$$Y_{AA} = 26.32 + 0.76x_1 - 1.90x_1x_3 - 2.71x^2_1 - 2.14\,x^2_3 \quad (3)$$

P-values for both responses were 0.0018 and 0.0023, respectively, attesting a good model fits. In addition, other parameters are indispensable to ensure the performance of the models, which were the lack of fit and coefficient of correlation.

Table 1. Factors and levels for response surface methodology, Box-Behnken design matrix (in coded and uncoded level of three variables), experimental data and predicted values for three-level-three-factor response surface analysis.

Run	**Variable levels** [a]			**TPC** [b]		**Antioxidant activity** [b]	
	x_1	x_2	x_3	**Observed**	**Predicted**	**Observed**	**Predicted**
1	60 (0)	20 (0)	75 (0)	245.07	245.82	26.41	26.32
2	60 (0)	20 (0)	75 (0)	246.61	245.82	26.02	26.32
3	80 (+1)	10 (-1)	75 (0)	238.36	238.29	25.28	24.73
4	40 (–1)	30 (+1)	75 (0)	229.61	229.68	21.47	22.01
5	60 (0)	30 (0)	100 (+1)	227.33	225.31	21.42	20.87
6	60 (0)	20 (0)	75 (0)	245.78	245.82	26.54	26.32
7	80 (+1)	20 (0)	50 (-1)	234.41	232.46	24.44	24.43
8	40 (-1)	10 (–1)	75 (0)	220.33	217.73	19.88	19.38
9	60 (0)	10 (–1)	50 (0)	233.04	235.06	22.29	22.83
10	80 (+1)	20 (0)	100 (+1)	233.43	232.88	23.63	23.67
11	60 (0)	30 (+1)	50 (-1)	243.33	242.68	26.14	25.64
12	80 (+1)	30 (+1)	75 (0)	215.47	218.07	19.26	19.75
13	40 (-1)	20 (0)	50 (-1)	233.41	233.99	22.95	22.90
14	60 (0)	10 (-1)	100 (+1)	240.54	241.19	25.55	26.04
15	40 (–1)	20 (0)	100 (+1)	220.41	222.36	22.11	22.11

[a] *x_1, Solvent concentration (%); x_2, extraction Time (min); x_3, sonication amplitude (%)*

[b] *TPC and antioxidant activity were expressed in mg GAE per 100 g of date.*

ANOVA results of models were illustrated in Table 3. It indicated that the quadratic terms of the models were significant ($P < 0.01$) and lack of fits were not significant ($P \geq 0.05$) for both TPC and antioxidant activity; indicating that the models could be used to predict responses. The prob ≥ F value indicated the probability equals the proportion of the area under the curve of the F distribution that lies beyond the observed F-value (Zhang *et al*., 2014).

Data presented in Table 3 showed that the lack of fits of models were not significant. The coefficient of determination (R^2) was considered the best measure to ensure overall performance of model, for a good fit, R^2 should approach unity. Determination Coefficients for TPC and antioxidant activity responses were 0.974 and 0.986, respectively. The adjusted coefficients of determination (adjusted R^2),

that express the contribution of significant factors were 0.929 and 0.960 for TPC and antioxidant activity, respectively. The difference between the two coefficients was insignificant, implying that the insignificant factors didn't have a great influence on the adjustment of models.

Table 2. Regression coefficient, standard error, and Student's t-test results of response surface for TPC and antioxidant activity (mg GAE/100g).

Parameter	Estimate	Std Error	t Ratio	Prob>|t|
TPC				
Intercept	245.82	1.466	167.59	<0.0001*
x_1	2.251	0.898	2.51	0.0541
x_2	-1.885	0.898	-2.1	0.0899
x_3	-3.003	0.898	-3.34	0.0205*
x_1*x_2	3.105	1.270	2.44	0.0583
x_1*x_3	-7.917	1.270	-6.23	0.0016*
x_2*x_3	-4.125	1.270	-3.25	0.0228*
$x_1* x_1$	-11.998	1.322	-9.08	0.0003*
$x_2 *x_2$	-3.506	1.322	-2.65	0.0453*
$x_3* x_3$	-8.003	1.322	-6.05	0.0018*
Antioxidant activity				
Intercept	26.323	0.392	67.11	<0.0001*
x_1	0.775	0.240	3.23	0.0233*
x_2	-0.389	0.240	-1.62	0.1665
x_3	-0.589	0.240	-2.45	0.0579
x_1*x_2	0.008	0.340	0.02	0.9832
x_1*x_3	-1.903	0.340	-5.6	0.0025*
x_2*x_3	-1.995	0.340	-5.87	0.002*
$x_1* x_1$	-2.709	0.354	-7.66	0.0006*
$x_2 *x_2$	-0.332	0.354	-0.94	0.3913
$x_3* x_3$	-2.142	0.354	-6.06	0.0018*

*x_1, Solvent concentration; x_2, extraction time; x_3, sonication amplitude of; *P≤0.05.*

Discussion

Response surface analysis

The three-dimensional response surfaces of TPC and antioxidant activity as a function of methanol concentration, sonication amplitude and extraction time were given in Figures 1-3. It were plotted to evaluate the interaction of the variables and to determine the optimum level of each variable for maximum values of responses.

Table 3. ANOVA table for the effect of methanol concentration, time, and amplitude on TPC extraction and antioxidant activity (mg GAE/100g).

Source	DFa	Sum of Squares	F Ratio	Prob> F
TPC				
x1	1	40.54	6.28	0.054
x2	1	28.43	4.40	0.090
x3	1	72.18	11.18	0.021*
x1*x2	1	38.56	5.97	0.058
x1*x3	1	250.75	38.85	0.002*
x2*x3	1	68.06	10.54	0.023*
x1* x1	1	531.58	82.36	0.0003*
x2 *x2	1	45.39	7.03	0.045*
x3* x3	1	236.53	36.65	0.002*
Model	9	1232.44	21.21	0.0018*
Lack of fit	3	31.08	17.44	0.0547
Error	5	32.27		
Total model	14	1264.71		
R^2 = 0.97				
Adj. R^2 = 0.92				
Antioxidant activity				
x1	1	4.80	10.41	0.0233*
x2	1	1.21	2.62	0.1665
x3	1	2.77	6.01	0.0579
x1*x2	1	0.0002	0.0005	0.9832
x1*x3	1	14.48	31.37	0.0025*
x2*x3	1	15.92	34.49	0.002*
x1* x1	1	27.10	58.71	0.0006*
x2 *x2	1	0.41	0.88	0.3913
x3* x3	1	16.94	36.69	0.0018*
Model	9	80.17	19.29	0.0023*
Lack of fit	3	2.16	9.84	0.0937
Error	5	2.31		
Total model	14	82.48		
R2 = 0.98				
Adj. R^2 = 0.96				

*x_1, Solvent concentration; x_2, extraction time; x_3, sonication amplitude *P≤0.05; [a] Degrees of freedom.*

Figure 1 showed the effect of two parameters, solvent concentration and sonication amplitude on the TPC (Figure 1-A) and antioxidant activity (Figure 1-B). The

estimate coefficient for the quadratic term of sonication amplitude was higher in both TPC and antioxidant activity compared with the quadratic term of solvent concentration.

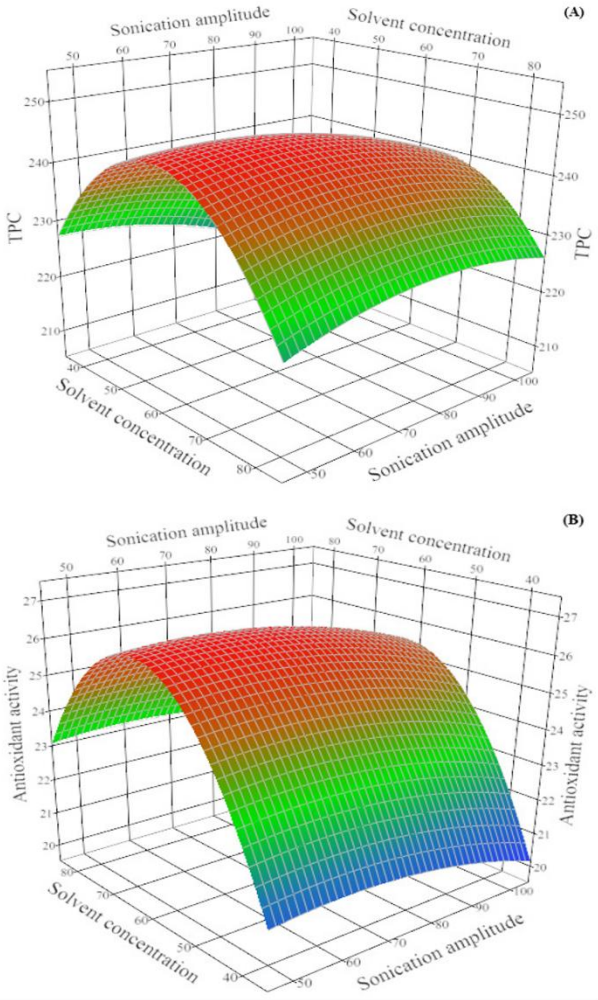

Figure 1. Response surface plots showing the effect of solvent concentration and sonication amplitude of (A) on TPC (mg EAG/100g), (B) on antioxidant activity (mg EAG/100g).

The sonication amplitude that gave the best extraction of phenolic compounds and antioxidant activity was of 84.5%. The quadratic effect of amplitude was significant on both TPC and antioxidant activity.

Ultrasound-assisted extraction had mechanical actions; they were aimed to release the intracellular constituents by rupture of the plant tissue and by diffusion in order to allow a better mass transfer and a better release of intracellular compounds. The origin of this destruction is the implosion of cavitation bubbles induced by

ultrasounds. This implosion produces shear forces and liquid jets directed to a solid surface in the case of a solid liquid extraction. These jets may have enough energy to cause physical damage to the cell walls (Chemat *et al*., 2008).

The results indicated that the extraction of TPC as well as the antioxidant activity of date palm fruit was significantly influenced by the extraction solvent, extraction, time and sonication amplitude. These results are similar to those obtained by Saci *et al*. (2018) for carob pulp.

At a certain sonication amplitude, the responses decreased. Although sonication was performed in an ice bath, the increase of the agitation rate at certain amplitude caused an increase in temperature around the probe. According to Suslick and Price (1999) implosion of cavitation bubbles generate local values of temperature and a high pressure which may induce the destruction of the thermo sensitive phenolic compounds. According to Veillet *et al*. (2009), the high temperature facilitates the generation of cavitation bubbles but they lose their ability to collapse, resulting in a decrease of responses**.**

Figure 2 shows the effect of two parameters - solvent concentration and extraction time - on TPC (Figure 2-A) and antioxidant activity (Figure 2-B). The quadratic term of methanol concentration significantly influenced the two models.

The interaction solvent concentration-extraction time was significant for both responses, the increase of this two parameters results the increase of both responses until a concentration of 65% and extraction time of 17. 64 min.

The methanol concentration of 65% gave the highest responses of 246.24 mg EAG/100g and 26.54 mg EAG/100g for both TPC and antioxidant activity, respectively. Methanol is the most polar solvent of the investigated organic solvents. The addition of water increased the polarity of the solvent; the results showed that both responses increased with increasing methanol concentration. However, with the methanol concentration of 80% (v/v), the responses decreased. When the extraction was done with this concentration, matrix didn't dissolve easily. The no dissolution can be explained by the low polarity of the solvent, which has prevented the total dissolution of sugars, hence the retention of glycosylated polyphenols.

According to the polarity principle "like dissolves like" (Zhang *et al*., 2007), methanol concentration which allowed to extract the maximum phenolic compound (65%) can be explained by the richness of date fruit in polyphenols median polarity.

Figure 3 showed the effect of extraction time and sonication amplitude on the TPC (Figure 3-A) and antioxidant activity (Figure 3-B). The estimated coefficient of quadratic term for extraction time was higher in both TPC and antioxidant activity compared with the quadratic term of sonication amplitude.

The interaction extraction time-sonication amplitude was significant for both responses, the increase of both parameters led to the increase of both responses until sonication amplitude of 84.5% and a time17.64 min.

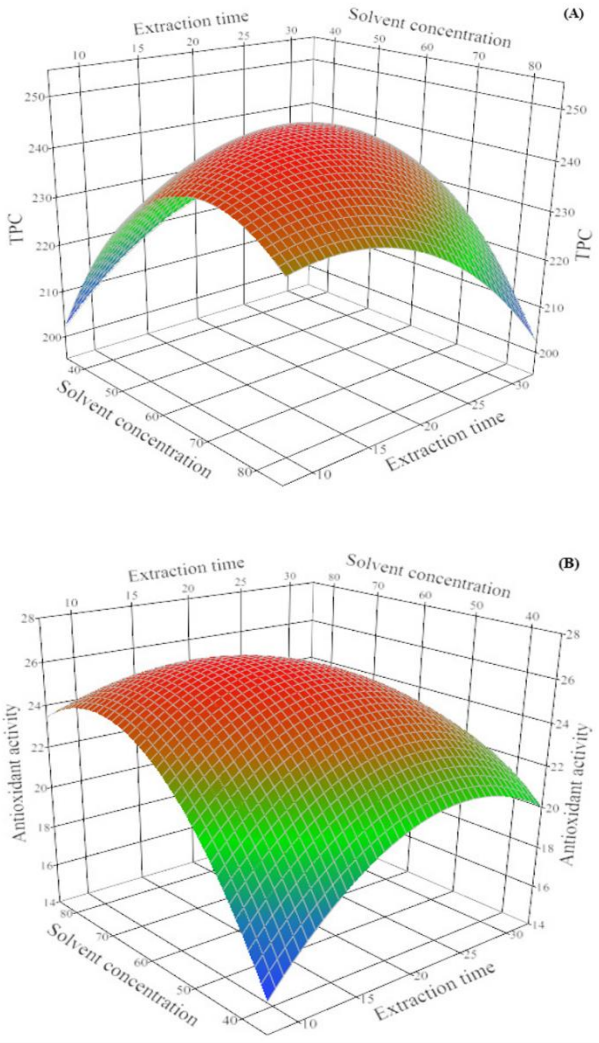

Figure 2. Response surface plots showing the effect of solvent concentration and extraction time**.** (A) On TPC (mg EAG/100g), (B) on antioxidant activity (mg EAG/100g).

Ultrasound assisted extraction greatly reduced the of antioxidant extraction time. In the case of dates, using maceration method during 48 hours, the concentration of TPC was 23.05 mg GAE/100g (Ghiaba *et al*., 2014), and using agitation method during 24h, the concentration of TPC was from 81 to 234mg GAE/100g DM (Singh *et al*., 2012). However, using ultrasound assisted extraction an extraction time of 17.64 min allowed to extract the maximum phenolic contents with the highest antioxidant activity.

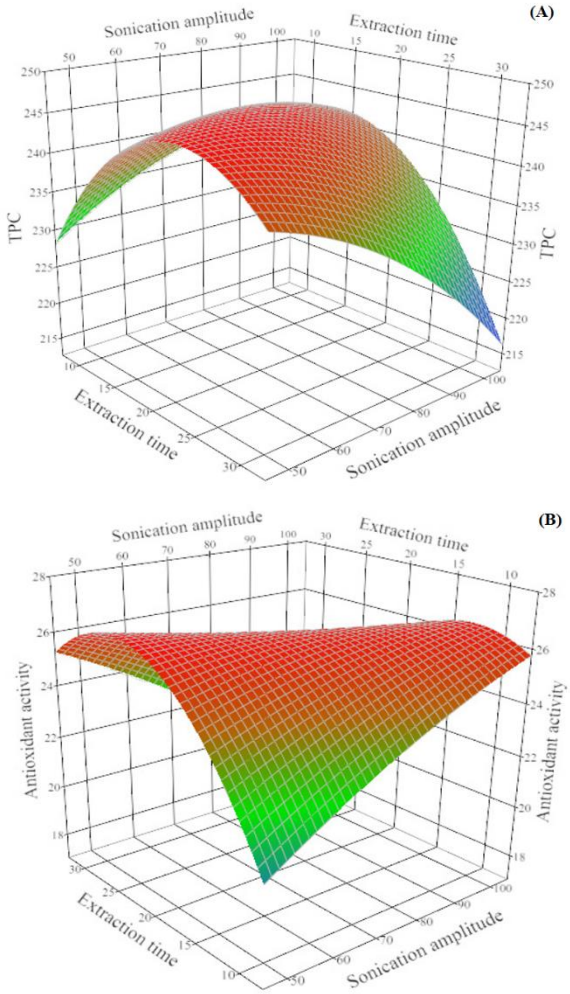

Figure 3. Response surface plots showing the effect of extraction time and sonication amplitude. (A) on TPC (mg EAG/100g), (B) on antioxidant activity (mg EAG/100g).

The quadratic effect of the extraction time was significant on both TPC and antioxidant activity. The generation of cavitations and implosion will lead to a better transfer of intracellular material. Consequently, the increase in extraction time will allow to produce more cavitations and chances that the micro-jets produced by this implosion to the matrix. In addition, it was stated that the increase of the extraction time means the increase of the contact solvent/sample time, which gave better responses (Ghafoor *et al.*, 2009).

The extraction time that gave the best performance in both responses was 17.64 min. This result agrees with those found by Lou *et al.* (2010). The present study confirmed that the responses increased during the first 20 minutes, as reported by Esclapez *et al.* (2011).

The decrease of responses with increase of extraction time could be explained by the degradation of the extracted compounds by the mechanical actions of ultrasound. The decrease could be also explained by the chemical effect of ultrasound; free radicals could be generated and react with extracted compounds (Veillet *et al*., 2009).

The interaction sonication amplitude-extraction time was significant for both responses, the increase of this two parameters result in the increase of both responses up to a concentration of 84.5% and a time of 17.64 min.

Determination and validation of optimal conditions

The RSM guided optimization demonstrated that the optimum conditions for maximizing TPC and antioxidant activity were methanol concentration of 65%, sonication amplitude of 84.5% and extraction time of 17.64 min. The optimal conditions predicted by the models were tested and the experimental values of the two responses were 246.63 and 27.00 mg EAG/100 g, respectively. The theoretical values were in the range of 246.43 and 26.46 00 mg EAG/100 g. It indicated that the results of the experimental and predicted were nearly similar. Therefore, the proposed models could be used to predict the response value.

Conclusions

In this study, ultrasound assisted extraction of total phenolics and antioxidant activity were optimized using RSM. The results indicated that the extraction of TPC as well as the antioxidant activity of date palm fruit were significantly influenced by the extraction solvent, extraction, time and sonication amplitude and the interaction between these parameters.

The experimental values were found to be in agreement with the predicted values and clearly indicated the suitability of the developed quadratic models. Therefore, it is suggested that the obtained models can be used to optimize the extraction of TPC and antioxidant activity from date palm fruit in used experimental conditions.

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