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ORIGINAL RESEARCH PAPER

OPTIMIZATION OF ULTRASOUND-ASSISTED EXTRACTION OF PHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITY FROM CAROB PULP (*CERATONIA SILIQUA* L.) BY USING RESPONSE SURFACE METHODOLOGY

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In this study, the ultrasonic-assisted extraction for phenolic compound extraction from the carob pulp (*Ceratonia siliqua* L.) was applied. The Box-Behnken design and response surface methodology (RSM) were used to study and optimize the effect of processing variables, acetone concentration (60-80%), extraction amplitude (30-100%) and extraction time (10-60 min) on the total phenolic content (TPC) and the antioxidant activity. The optimal values for the TPC yield and for the antioxidant activity were at 59.30% and 61.70% acetone concentration, 85.86% and 90.12% extraction amplitude, and 53.24 min and 56.82 min of extraction time, respectively. The experimental values were in accordance with those predicted by the model, which indicated the suitability of response surface methodology in optimizing the antioxidants extraction from carob pulp by ultrasonic-assisted extraction.

Keywords: optimization; carob pulp; phenolic compounds; antioxidant activity; ultrasonic-assisted extraction; response surface methodology

Introduction

Carob fruits are among the most important tree fruit crops in the Mediterranean countries and their production and consumption have increased considerably in recent years (FAO, 2014). The composition and quantification of polyphenols in carob fruit have been elucidated, as well as their antioxidant activity (Benchikh *et al.*, 2014; Benchikh *et al.*, 2016).

Phenolic compounds are a group of very diverse chemicals that includes simple phenols, benzoic and cinnamic acid derivatives and flavonoids (Moraes *et al.*, 2013). Many of these compounds have been shown to present useful traits that support their use as bioactive compounds for human health (Sanmukhani *et al.*, 2013; Vajic *et al.*, 2015).

Many methodologies have been used for the extraction of phenolic compounds from carob (Benchikh and Louaileche, 2014; Mulet *et al.*, 2015). Recently, new extraction techniques have been developed for the extraction of target compounds from different materials including ultrasound- and microwave-assisted, supercritical fluid and accelerated solvent extraction (Hammi *et al.*, 2015; Siddiqui and Aeri, 2017). Among these, ultrasound-assisted extraction (UAE) is one of the most rapid methods and efficient alternatives to conventional extraction techniques (Heydari Majd *et al.*, 2014). UAE has been applied to extract bioactive compounds from different materials owing to its high reproducibility, simplified manipulation, and significant reduction in solvent consumption (Liu *et al.*, 2013). In fact, the ultrasound induces a swelling of the cells, solvent uptake and an enlargement of the pores of the cell walls allowing higher diffusivity of the solvent across the cell walls. The ultrasound could even cause a breakdown of the cell walls and facilitates the washing out of the cell content (Talmaciu *et al.*, 2015). In recent studies, UAE has been applied to obtain bioactive compounds from different materials such *Tecomellaun dulata* Bark (Siddiqui and Aeri, 2017).

Many factors including solvent concentration, extraction amplitude and extraction time affect the efficiency of the extraction process. In order to optimize the operational conditions, the response surface methodology (RSM) has been widely used (Bachir bey *et al.*, 2014; Ilaiyaraja *et al.*, 2015; Siddiqui and Aeri, 2017). RSM is an effective statistical technique for modeling and optimizing the complex processes (Siddiqui and Aeri, 2017). Compared with the classical methods, as the one-factor-one method, the RSM is more efficient, requires fewer experiments, and provides interaction effects on the responses besides factor effects (Bernardo-Gil *et al.*, 2011).

In the present study, optimization of solvent concentration, ultrasound amplitude, and extraction time for extraction of total phenolic compounds from *Ceratonia siliqua* L. pulp by using UAE and evaluation of antioxidant activity of the extract was carried out according to RSM.

Materials and methods

Plant material

The carob mature fruits were randomly harvested from the same region (Bejaia, Algeria). The carob cloves were chosen for uniformity in shape and colour. The seeds were manually separated from carob, and the rest of the fruit (carob pulp) was oven-dried at 45°C for 5 days, then the dried pulp was ground with a crusher (IKA, A 11 basic, Staufen, Germany), and passed through a 500 µm sieve before analyses.

Chemicals and reagents

Folin–Ciocalteu reagent, methanol, and acetone were purchased from Biochem, Chemopharma (Montreal, Quebec), sodium carbonate anhydrous was from Sigma Chemical (Sigma–Aldrich GmbH, Switzerland), gallic acid was from Prolabo (Montreuil, France), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) was from Sigma Chemical (Sigma–Aldrich GmbH, Germany).

Ultrasound assisted extraction

Ultrasonic equipment (Sonics Vibra cell. VCX 130 PB, USA) was used for UAE with working frequency fixed at 20 kHz. The energy input was controlled by setting the amplitude of the Sonicator Probe. For extraction, 25 mg of samples were homogenized in 15 mL of acetone. The obtained suspension was exposed to acoustic waves for varying acetone concentration (40-80%), amplitude (30-100%), and extraction time (10-60 min) (Table 1). The temperature was fixed at 25°C. The extract was centrifuged at 1700 × g (Nüve NF 200, Ankara, Turkey) for 20 min and filtered.

Analytical methodology

Determination of total phenolic content

Total phenolic content was estimated using Folin–Ciocalteu method (Singleton and Rossi, 1965). A volume of 100 µL of the extracts was added to 1 mL of Folin-Ciocalteu solution (diluted 10-fold with distilled water). Subsequently, 800 µL of sodium carbonate (7.5%) were added. After 30 min of incubation in the dark at room temperature, the absorbance at 765 nm was measured (Uviline 9400 UV-visible spectrophotometer, Secomam, France). Total phenolic contents were expressed as mg gallic acid equivalent per 100 g dry matter (mg GAE/100g DM).

Antioxidant activity

Free radical scavenging activity of the extracts was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Brand-Williams *et al.*, 1995). A volume of 50 µL of extract was added to 1 mL of DPPH solution (60µM). The decrease in absorbance was determined at 517 nm, after incubation for 30 min in the dark. Gallic acid was used as a standard, and the antioxidant activity was expressed as mg gallic acid equivalents per 100 g dry matter (mg GAE/100g DM).

Statistical analysis

Experimental design

Response Surface Methodology (RSM) was used to investigate the optimal conditions for antioxidants extraction from carob pulp. A Box–Behnken design was applied to evaluate the effects of the solvent concentration (x_1, acetone/water %, v/v), ultrasound amplitude (x_2, %), and time (x_3, min) on total phenolic content and antioxidant activity. As indicated in Table 1, the three independent variables were analysed at 3 levels, coded -1, 0, and +1 for lowest, central, and highest value, respectively.

Table 1. Experimental values and coded levels of the independent variables used for the Box-Behnken design

Coded levels	Uncoded levels		
	Acetone concentration, %	Ultrasound amplitude, %	Extraction time, min
-1	40	30	10
0	60	65	35
+1	80	100	60

Data analysis

The experimental results of the response surface design were analyzed using JMP 10 (statistical analysis system Inc., SAS) software. All experiments were conducted in triplicate and the mean was reported. The complete design consisted of 15 experimental runs, including three replicates of the central point to evaluate the experimental error measurement (Table 2).

A second-degree polynomial regression model was used to correlate the relationship between independent variables and responses (TPC and antioxidant activity), and the second-degree polynomial model was as follows equation (1).

$$Y = \propto_0 + \sum_{i=1}^{3} \propto_i x_i + \sum_{i=1}^{3} \propto_{ii} x_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \propto_{ij} x_i x_j \quad (1)$$

Where Y was the response variables; α_0, α_i, α_{ii}, and α_{ij} are the intercept, linear, quadratic, and interactive coefficients, respectively; x_i and x_j are the levels of the independent variables ($i \neq j$).

Student's t-test permitted the checking of the statistical significance of the regression coefficient and Fisher's F-test determined the second-order model equation at a probability (p) of 0.001, 0.01 or 0.05. Model adequacy was evaluated using the lack of fit, the coefficient of determination (R^2) and the F-test value obtained from the analysis of variance (ANOVA). Regression analysis and three dimensional response surface plots were plotted to determine optimum conditions for TPC and antioxidant activity. The test of statistical significance was based on the total error criteria with a confidence level of 95.0%.

Table 2. Box-Behnken design and responses values for total phenolic compounds (TPC) extraction and antioxidant activity

Run	Variable levels [a]			TPC[b]		Antioxidant activity[b]	
	x_1	x_2	x_3	Observed	Predicted	Observed	Predicted
1	60(0)	65(0)	35(0)	28.41	28.68	20.31	20.52
2	60(0)	30(-1)	10(-1)	16.80	17.31	11.63	11.49
3	60(0)	65(0)	35(0)	28.72	28.68	20.35	20.52
4	60(0)	100(+1)	10(-1)	22.00	22.38	16.94	16.62
5	80(+1)	30(-1)	35(0)	16.30	16.40	12.85	12.93

Run	Variable levels [a]			TPC[b]		Antioxidant activity[b]	
	x_1	x_2	x_3	Observed	Predicted	Observed	Predicted
6	40(-1)	65(0)	10(-1)	20.04	19.76	14.78	15.18
7	60(0)	30(-1)	60(+1)	21.91	21.54	16.10	16.42
8	40(-1)	100(+1)	35(0)	24.69	24.59	19.40	19.32
9	80(+1)	100(+1)	35(0)	24.21	24.44	19.14	19.40
10	60(0)	65(0)	35(0)	28.92	28.68	20.89	20.52
11	60(0)	100(+1)	60(+1)	30.69	30.18	22.32	22.46
12	80(+1)	65(0)	10(-1)	18.96	18.36	13.40	13.46
13	80(+1)	65(0)	60(+1)	24.16	24.44	20.17	19.77
14	40(-1)	30(-1)	35(0)	19.15	18.92	14.88	14.62
15	40(-1)	65(0)	60(+1)	25.11	25.71	19.72	19.66

[a] x_1, Solvent concentration (%); x_2, Ultrasoundamplitude (%); x_3, Extraction time (min).

[b] TPC and antioxidant activity were expressed in mg GAE/g DM of carob pulp.

Results and discussion

Model fitting

A RSM approach was conducted in order to determine the correlative effect of the solvent concentration, amplitude and extraction time on both TPC and antioxidant activity. In accordance with the experimental design, the observed and predicted response values were indicated in Table 2. Overall, a close relationship between the experimental values and the predicted ones was observed attesting satisfactory of developed models. In addition, it was indicated that the TPC and the antioxidant activity ranged from 16.30 to 30.69 mg GAE/g, and from 11.63 to 22.32 mg GAE/g, respectively. The regression coefficients of the intercept, linear, quadratic and interaction terms of the models were calculated using the least squares technique and were displayed in Table 3.

Table 4 shows the results of fitting quadratic models of data. Analysis of variance (ANOVA) indicates that the contribution of quadratic effect was significant. The fitted quadratic models for TPC and antioxidant activity (AA) were given in Eqs. 2 and 3, respectively.

$$TPC = 28.6833 - 0.6700x_1 + 3.4288x_2 + 3.0088x_3 + 0.8950x_2x_3 - 4.1892x_1^2 - 3.4067x_2^2 - 2.4267x_3^2 \quad (2)$$

$$AA = 20.5167 - 0.4025x_1 + 2.7925x_2 + 2.6950x_3 - 1.8396x_1^2 - 2.1096x_2^2 - 1.6596x_3^2 \quad (3)$$

The fitness of model was evaluated by lack of fit, which was insignificant for TPC (0.101) and antioxidant activity (0.316), indicating the goodness of the both models.

P-values obtained for TPC and antioxidant activity indicated the suitability of models to accurately predict responses (Table 4). The quality of the fit model can be determined by the coefficient of determination (R^2) which was 0.993 and 0.994 for TPC and antioxidant activity, respectively.

The corresponding variables would be more significant if the absolute *F*-value becomes greater and the *P*-value becomes smaller. The *F*-value of the models was 82.66 in case of the TPC ($P< 0.0001$) and 93.29 for antioxidant activity ($P< 0.0001$). The *P*-value of the models was less than 0.0001, which indicates that the model was significant and could work well for the prediction of TPC and antioxidant activity of carob pulp.

Table 3. Regression coefficient, standard (Std.) error, and Student's t-test results of response surface for TPC and antioxidant activity

Parameter	Estimate	Std. error	*t* Ratio	Prob> |*t*|
TPC				
Intercept	28.6833	0.3593	79.8395	<0.0001*
x_1	-0.6700	0.2200	-3.0454	0.0286*
x_2	3.4288	0.2200	15.5851	<0.0001*
x_3	3.0088	0.2200	13.6760	<0.0001*
x_1-x_2	0.5925	0.3111	1.9043	0.1152
x_1-x_3	0.0325	0.3111	0.1045	0.9209
x_2-x_3	0.8950	0.3111	2.8766	0.0347*
x_1-x_1	-4.1892	0.3238	-12.9361	<0.0001*
x_2-x_2	-3.4067	0.3238	-10.5198	0.0001*
x_3-x_3	-2.4267	0.3238	-7.4935	0.0007*
DPPH assay				
Intercept	20.5167	0.2501	82,0309	<0.0001*
x_1	-0.4025	0.1532	-2,628	0.0466*
x_2	2.7925	0.1532	18,2326	<0.0001*
x_3	2.6950	0.1532	17,596	<0.0001*
x_1-x_2	0.4425	0.2166	2.0429	0.0965
x_1-x_3	0.4575	0.2166	2,1122	0.0884
x_2-x_3	0.2275	0.2166	1,0503	0.3417
x_1-x_1	-1.8396	0.2254	-8,1598	0.0004*
x_2-x_2	-2.1096	0.2254	-9,3574	0.0002*
x_3-x_3	-1.6596	0.2254	-7,3614	0.0007*

x_1, solvent concentration; x_2, ultrasound amplitude; x_3, extraction time

*Values statistically significant at $P < 0.05$.

The results showed that amplitude and extraction time had a positive linear effect on both TPC extraction and antioxidant activity as well as interaction term between amplitude and time for TPC. However, interaction terms between solvent concentration-amplitude and solvent concentration-time were found to have no effects.

Table 4. ANOVA table for the effect of acetone concentration, amplitude and time on TPC extraction and antioxidant activity (mg GAE/g DM)

Source	DF	Sum of squares	*F* value	*P* value
TPC				
x_1	1	3.5912	9.2746	0.0286*
x_2	1	94.0506	242.8941	<0.0001*
x_3	1	72.4206	187.0327	<0.0001*
x_1-x_2	1	1.4042	3.6265	0.1152
x_1-x_3	1	0.0042	0.0109	0.9209
x_2-x_3	1	3.2041	8.2749	0.0347*
x_1-x_1	1	64.7967	167.3434	<0.0001*
x_2-x_2	1	42.8506	110.6656	0.0001*
x_3-x_3	1	21.7429	56.1531	0.0007*
Model	9	288.0610	82.6604	<0.0001*
Error	5	1.9360		
Total	4	289.9970		
R^2	0.993			
Adj. R^2	0.981			
Lack of fit	3	1.8039	9.1064	0.101
DPPH				
x_1	1	1.2961	6.9063	0.0466*
x_2	1	62.3845	332.4275	<0.0001*
x_3	1	58.1042	309.6194	<0.0001*
x_1-x_2	1	0.7832	4.1736	0.0965
x_1-x_3	1	0.8372	4.4613	0.0884
x_2-x_3	1	0.2070	1.1032	0.3417
x_1-x_1	1	12.4950	66.5821	0.0004*
x_2-x_2	1	16.4320	87.5612	0.0002*
x_3-x_3	1	10.1694	54.1897	0.0007*
Model	9	157.5778	93.2982	<0.0001
Error	5	0.9383		
Total	14	158.5161		
R^2	0.994			
Adj. R^2	0.983			
Lack ok fit	3	0.7284	2.314	0.316

x_1, solvent concentration; x_2, amplitude; x_3, time; DF: Degree of freedom.
* Values statistically significant at $P < 0.05$.

Analysis of response surfaces

Interpretation of response surface plots

To illustrate the correlations between the independent and dependent variables considered, three-dimensional response surface plots were built on the basis of the model equations mentioned above. The type of plots showed the effects of two out of the three independent variables on the response factor, keeping the third one at level-coded zero (center value of the testing ranges). The profile of the response surface plots showed a strong interaction between the tested variables. From these three-dimensional profiles, it is easy to see the interaction effects between any two independent factors.

Response surface analysis of total phenolic contents

The effect results of extraction factors studied were shown in Figures 1 and 2. Each variable contributes significantly to affect the quantity of extractable phenolic compounds. Amongst them, the polarity of the solvent plays an important role which is due to its ability to extract these molecules by solubilisation. Since phenolic compounds have a wide spectrum of solubility; a combination of acetone and water may be desirable and effective for separation than any single solvents (Benchikh and Louaileche, 2014).

In our study, the influencing factors on TPC were determined by the significant coefficient of the second-order polynomial regression equation. The results indicated that the first-order linear effect was significant for acetone concentration (x_1), extraction amplitude (x_2) and extraction time (x_3); the second-order quadratic effect was significant for all the factors; the interactive effect was significant for ultrasound amplitude and time (x_2-x_3).

In our results, Figure 1A showed that when the extraction time was fixed at central levels, the phenolic compounds extraction increased slightly by increasing the acetone concentration and reached the maximum value at 59.30%. As shown in our previous study using the one-factor-one method for extraction of phenolic compounds from carob pulp, 70% acetone was the best extracting solvent (Benchikh and Louaileche, 2014), but in this work, 59.30% acetone was the optimum parameter which indicates that the combination of acetone with a bit more water was better than 70% acetone. The binary-solvent system was more efficient in the extraction of phenolic compounds from plant samples compared with a mono-solvent system (Chew *et al.*, 2011). These results can be explained by the principle of "like dissolves like"; any given solvent would only extract compounds that have a similar polarity (Zhang *et al.*, 2007). Furthermore, adding water to organic solvent can enhance the extraction efficiency of phytochemicals by causing the plant material to swell, allowing the solvent to penetrate more easily into the solid particles of the sample matrix (Gertenbach, 2001; Luthria, 2012). A linear increase of TPC was noticed with increasing amplitude, and a maximum of TPC was obtained with 85.86% amplitude, and thereafter, this content decreased. The ultrasound induces a swelling of cells, a solvent uptake and an enlargement of the pores of the cell walls allowing higher diffusivity of the solvent across the cell

walls. The ultrasound could even cause a breakdown of the cell walls and facilitate the washing out of the cell content (Talmaciu *et al.*, 2015).

Figure 1B showed the interaction between acetone concentration and extraction time on total phenolic extraction. An increase in the total phenolic contents was observed as increasing extraction time up to 53.24 min, but decreased thereafter. Also as shown in our previous study, 90 min was the best extraction time (Benchikh and Louaileche, 2014), but in the present results, we have gained 36.76 min because the model from Box-Behnken design takes into consideration the all the parameters in order to determine the optimum one. The decreasing of phenolic compounds after 53 min could be due to a phenolic compound oxidation occurring by prolonging exposure to environmental factors, such as light and oxygen (Chan *et al.*, 2009). The results obtained were explained by Fick's second law of diffusion, which predicts that after a certain time, there will be a final equilibrium between the solute concentration in the solid matrix (plant sample) and in the bulk solution (extraction solvent) (Silva *et al.*, 2007). Hence, excessive extraction time was not useful in extracting more phenolic compounds from carob pulps. Figure 1C reflects the effects of amplitude and extraction time on the ultrasonic-assisted extraction of TPC from carob pulp. The highest total phenolic content was observed at a higher amplitude and higher extraction time.

Response surface analysis of antioxidant activity

The relationship between the antioxidant activity and extraction factors was investigated by response surface plots in Figure 2. Figure 2A shows the effect of acetone concentration, amplitude and their mutual interaction on the antioxidant activity measured by DPPH radical scavenging assay. The concentration of acetone influenced significantly the antioxidant activity in first order linear ($p<0.05$) and second order quadratic ($p<0.05$). The regression coefficient for this factor was a negative value indicating that an increase in acetone concentration caused a decrease of the antioxidant activity. The highest antioxidant activity was observed at 61.70% of acetone concentration and 90.12% of amplitude extraction. The effect of extraction time, acetone concentration and their mutual interaction on the antioxidant activity was illustrated in Figure 2B. Generally, antioxidant activity was increased with increasing extraction time. An increase in the antioxidant activity was observed with the increasing of extraction time and the trend was reserved when the extraction time reached to approximately 56min, and then antioxidant activity began to decrease. As mentioned above, this decreasing of the antioxidant activity was probably due to the oxidation of the antioxidants. Figure 2C represents the effect of extraction amplitude, extraction time and their mutual interaction on the antioxidant activity. The highest antioxidant capacity was observed at higher extraction amplitude and higher extraction time.

Optimization of extraction parameters and validation of the model

After determining the optimum conditions and predicting the responses under these conditions, a new set of experiment was designed and conducted with the selected optimal conditions of the process parameters to predict and verify the accuracy of the mathematical model. The results were shown in Table 5.

The predicted results matched well with the experimental results obtained using optimum extraction conditions which validated the response surface methodology model with a significant correlation. As a result, the model from Box-Behnken design was considered to be accurate and reliable for predicting total phenolic content and antioxidant activity of carob pulp for ultrasonic assisted extraction.

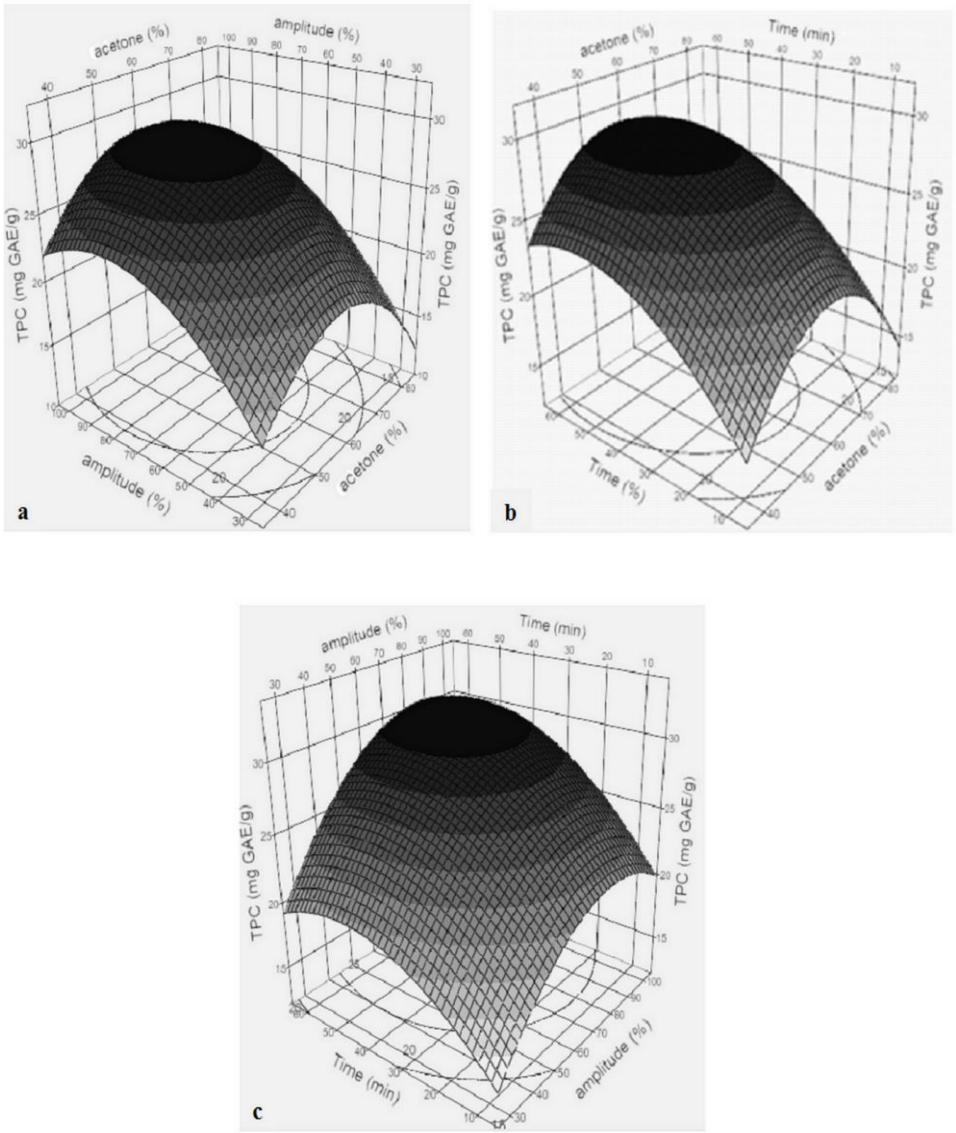

Figure 1. Response surface plots of the total phenolic content (TPC) of carob pulp as affected by (**a**) acetone concentration and extraction amplitude; (**b**) acetone concentration and extraction time; (**c**) extraction amplitude and extraction time following UAE

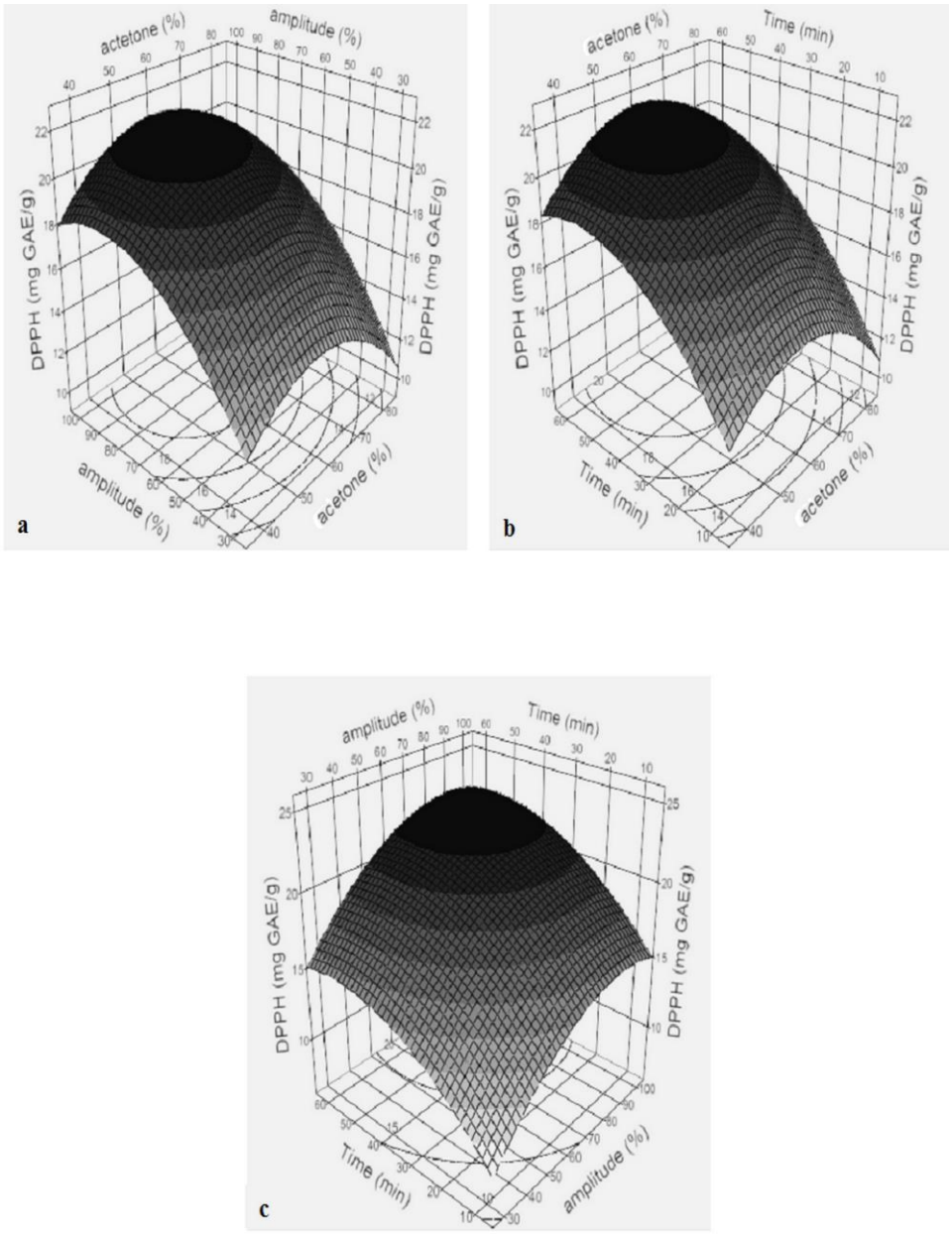

Figure 2. Response surface plots of the antioxidant activity of carob pulp as affected by (**a**) acetone concentration and extraction amplitude; (**b**) acetone concentration and extraction time; (**c**) extraction amplitude and extraction time following UAE

Table 5. Optimum conditions and predicting the responses under extraction conditions

Responses	Optimum UAE conditions			Maximum values	
	Acetone concentration, %	Extraction amplitude %	Extraction time, min	Experimental values	Predicted Values
TPC, mg GAE/g DM	59.30	85.86	53.24	30.75 ± 9.82	30.81
Antioxidant activity, mg GAE/g DM	61.70	90.12	56.82	22.96 ± 6.35	22.67

Conclusions

An ultrasonic-assisted extraction technique was applied for the extraction of phenolic compounds from carob pulp and optimized by response surface methodology. The high correlation of the models exhibited that the second-order polynomial model could be successfully used for optimizing the extraction parameters. The results showed that the extraction conditions including acetone concentration, extraction amplitude and extraction time markedly influenced the total phenolic content and antioxidant activity of carob pulp. The optimum ultrasonic assisted extraction conditions for TPC and antioxidant activity were respectively 59.30-61.70% of acetone concentration using extraction amplitude of 85.86–90.12% during 53.24–56.82min.

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