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Optimization of phenolic compound recovery and antioxidant activity from carob pulp using response surface methodology

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Abstract

The optimum extraction conditions for recovery of total phenolics and antioxidant activity of carob pulp were analysed using response surface methodology. The effects of acetone concentration (60-80%), extraction time (60-120 min), extraction temperature (50-90°C) and sample/solvent ratio (10-40 mg/10mL) were investigated. Second order polynomial models produced a satisfactory fitting of the experimental data with regard to total phenolic content (R^2 = 0.9949, P<0.0001) and antioxidant activity (R^2 = 0.9947, P<0.0001). Response surface analysis showed that the optimal extraction parameters that maximized antioxidant extraction were acetone concentration of 70.85%, extraction time of 101.50 min, temperature of 88.35°C and sample/solvent ratio of 16.65mg/10mL. The optimum values for total phenolic and antioxidant activity were 2557.96 mg GAE/100g and 2393.75mgGAE/100g, respectively. The experimental values agreed well with those predicted, indicating the suitability of the model and the success of response surface methodology in optimizing the extraction conditions.

Introduction

In many geographical regions, locally grown fruit and vegetables contribute substantially to local diet. The composition of such foods and their products is therefore a matter of considerable interest to food scientists. The carob tree (*Ceratonia siliqua* L.) has been grown since ancient times in most Mediterranean countries (Avallone *et al.*, 1997), usually in mild and dry places with poor soils (Bernardo-Gil *et al*., 2011). Carob is a drought resistant, perennial leguminous tree, with beanlike fruit.

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 (Fidan and Sapundzhieva, 2015). Some early reports indicated that carob pod extracts have several beneficial effects on health such as antioxidant properties in different in vitro test systems and cholesterol lowering activities in humans suffering from hypercholesterolemia (Zunft *et al*., 2001). These properties could presumably be attributed to the presence of phenolics.

Phenolic compounds, non nutrient but biologically active secondary plant metabolites which can act as antioxidants, are widely distributed in many foods of plant origin. There is increasing evidence that consumption of a variety of phenolic compounds present in foods may lower the risk of health disorders because of their antioxidant activity (Shahidi and Ambigaipalan, 2015). Phenolic content in carob pulp reported by literature is quite different, depending not only on the carob variety, geographical origin, weather conditions, harvesting and storage, but also on technological factors such as the extraction methodologies (Roseiro *et al.*, 2013).

Extraction is the first step in isolation of antioxidants from plant material and plays a crucial stage in quantification of these bioactive compounds. The extraction conditions may not be the same for different plant materials since they are influenced by several parameters, such as the chemical nature of the sample, extraction time (Chaalal *et al*., 2012), extraction temperature (Benchikh and Louaileche, 2014), the solvent used (Butsat and Siriamornpun, 2016), and sample/solvent ratio (Nugraha *et al.*, 2016). Furthermore, it is essential to choose an appropriate experimental design method that will evaluate effects of the major parameters involved in the treatment method and their probable interactions, through the minimum number of experiments (Bernardo-Gil *et al*., 2011). Response Surface Methodology (RSM) is an important tool in optimization of process. RSM is an empirical modeling approach for defining the relationship between various process parameters and responses with the various desired criteria and searching the significance of the process parameters on the coupled responses (Chamoli, 2015). RSM

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is an economical approach because a limited number of experiments are performed for assessing the interaction of independent parameters on the response. In conventional optimization, high number of experiments are necessary to perform the research leads to an increase in duration, and amounts of reagents and materials (Kirmizakis *et al.*, 2014).

The extraction of antioxidants from carob was performed using various solvents (100% acetone, 70% acetone, 70% methanol) (Avallone *et al*., 1997), extraction time (60, 90 and 120 min) (Benchikh and Louaileche, 2014) and temperature (20, 50 and 85°C) (Turhan *et al*., 2006). To the best of our knowledge, there are no investigations about the optimization of antioxidants extraction from carob pulp using RSM. Hence, this study aims to optimize the extraction of phenolic compounds from carob and to evaluate its antioxidant activity as a potential alternative to commercial antioxidants. For this, the optimization was carried out using RSM and four parameters were considered (solvent concentration, sample/solvent ratio, extraction time and temperature).

Material and Methods

Chemical reagents

Folin-Ciocalteu reagent, methanol and acetone were from Biochem, Chemopharma (Montreal, Quebec); sodium carbonate 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).

Plant material and sample preparation

The carob fruits were harvested in Bejaia department (Algeria). The fruits with uniform shape and colour were selected. The fruit pulp and seeds were manually separated, oven dried at 45°C for 5 days, ground with crusher (IKA, A 11 basic, Staufen, Germany) and passed through a 500-µm sieve.

Selection of appropriate extraction conditions

The initial step of the preliminary experiment was to select an appropriate extraction medium for carob pulp antioxidants. Effects of solvent nature (acetone, ethanol, methanol, and water), solvent concentration (40-100%), extraction temperature (25-90°C), and time (60-120 min), and sample/solvent ratio (15/10-75/10 mg/mL) were tested in our previous study (Benchikh and Louaileche, 2014).

Extraction procedure

Carob pulp sample was homogenized in 10 ml of extracting solvent; the mixture was shaken using a water bath shaker (WB 22, Memmert, Germany) at different temperature for different time (Table 1). The extract was centrifuged at 1700 g (Nüve NF 200, Ankara, Turkey) for 20 min and then filtered (Benchikh and Louaileche, 2014).

Quantification of total phenolic content (TPC)

Total phenolics were determined in all extracts, by using the Folin–Ciocalteu colorimetric method according to Singleton and Rossi (1965). Briefly, the extract (100 µL) was mixed with 1/10 (v/v) diluted Folin–Ciocalteu reagent (1 mL) and 7.5% sodium carbonate (800 µL). The mixture was incubated in the dark at room temperature for 30 min, and the absorbance was measured at 765 nm (Uvline 9400 spectrophotometer, Secomam, Alès, France). Total phenolic contents were expressed as mg gallic acid equivalents per 100 g dry matter (mg GAE/100g DM).

Antioxidant activity – DPPH method

DPPH assay is a rapid, simple and an inexpensive method to measure antioxidant capacity of extracts. It is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors. Radical scavenging activity of extracts was measured by a modified method of Brand-Williams *et al*. (1995). Each extract (50 µL) was added to 1mL 2,2-diphenyl-1-picryl-hydrazyl (DPPH) methanolic solution. The decolorizing process was measured at 517 nm after 30 min of reaction and compared to a blank control that contained the solvent and DPPH reagent. The antioxidant activity was expressed as mg gallic acid equivalents per 100 g dry matter (mg GAE/100g DM).

Experimental design

Box-Behnken (BB) design was used to determine the optimal conditions of antioxidant extraction from carob pulp. According to the principle of BB design, solvent concentration (x_1, acetone/water %, v/v), extraction time (x_2, min), temperature (x_3, °C) and sample/ solvent ratio (x_4, mg/10mL) were taken as independent variables tested in 27-run experiments. As indicated in Table 1, the four independent variables were prescribed into 3 levels, coded -1, 0, and +1 for low, intermediate and high value, respectively. Total phenolic content and antioxidant activity were taken as response of the design experiments.

Data analysis

The experimental results of the response surface design were analyzed using JMP 10 (statistical

Table 1. Box-Behnken design and response values for total phenolic compound and antioxidant activity

Variable levels [a]				TPC[b]		Antioxidant activity[b]	
x_1	x_2	x_3	x_4	Observed	Predicted	Observed	Predicted
80(+1)	60(-1)	70(0)	25(0)	1991.15	1946.54	1832.42	1818.28
70(0)	90(0)	70(0)	25(0)	2271.18	2284.93	2078.74	2090.58
70(0)	90(0)	90(+1)	40(+1)	2132.73	2111.75	1890.77	1871.05
70(0)	90(0)	90(+1)	10(-1)	2456.73	2507.62	2271.35	2330.12
70(0)	120(+1)	70(0)	40(+1)	1757.72	1768.58	1450.64	1476.78
80(+1)	90(0)	90(+1)	25(0)	2369.11	2380.29	2182.92	2215.19
70(0)	60(-1)	90(+1)	25(0)	2355.37	2343.82	2169.19	2153.68
60(-1)	120(+1)	70(0)	25(0)	2035.82	2064.25	1841.31	1881.59
70(0)	60(-1)	50(-1)	25(0)	1534.17	1571.87	1396.33	1401.72
70(0)	60(-1)	70(0)	10(-1)	2314.99	2303.28	2119.93	2093.01
80(+1)	90(0)	50(-1)	25(0)	1573.68	1581.70	1403.59	1427.86
70(0)	120(+1)	90(+1)	25(0)	2499.68	2479.01	2307.29	2276.55
60(-1)	60(-1)	70(0)	25(0)	2162.95	2158.42	1892.19	1934.22
60(-1)	90(0)	70(0)	10(-1)	2357.94	2343.70	2164.34	2131.04
80(+1)	120(+1)	70(0)	25(0)	2241.98	2230.33	2030.28	2014.39
60(-1)	90(0)	90(+1)	25(0)	2425.80	2416.93	2247.57	2222.52
70(0)	120(+1)	50(-1)	25(0)	1597.73	1626.31	1432.17	1422.33
70(0)	120(+1)	70(0)	10(-1)	2383.71	2348.17	2192.61	2182.66
80(+1)	90(0)	70(0)	40(+1)	1660.01	1691.28	1443.57	1451.51
70(0)	90(0)	70(0)	25(0)	2293.52	2284.93	2098.12	2090.58
70(0)	90(0)	70(0)	25(0)	2290.08	2284.93	2094.89	2090.58
70(0)	90(0)	50(-1)	10(-1)	1924.15	1928.95	1710.07	1755.93
60(-1)	90(0)	50(-1)	25(0)	1602.89	1590.87	1436.71	1403.66
80(+1)	90(0)	70(0)	10(-1)	2276.34	2282.14	2186.55	2152.10
60(-1)	90(0)	70(0)	40(+1)	1664.30	1675.53	1446.60	1455.70
70(0)	90(0)	50(-1)	40(+1)	1132.85	1065.78	0871.69	0839,06
70(0)	60(-1)	70(0)	40(+1)	1589.14	1623.84	1413.79	1422.96

x_1, solvent concentration (%); x_2, extraction time (min); x_3, extraction temperature (°C); x_4, sample/solvent (mg/10mL).
b TPC and antioxidant activity were expressed in mg GAE/100g DM of carob pulp.

analysis system Inc., SAS) software. All experiments were conducted in triplicate and the mean was reported. A second-degree polynomial regression model was used to correlate the relationship between independent variables and responses (total phenolic contents and antioxidant activity), and the second-degree polynomial model was as follows:

$$Y = \alpha_0 + \sum_{i=1}^{4} \alpha_i x_i + \sum_{i=1}^{4} \alpha_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \alpha_{ij} x_i x_j \quad (1)$$

where Y was the response variables. 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 ($i \neq j$). The statistical significance of the model equation and the model terms was evaluated via the Fisher's test. The quality of fit of the second-order polynomial model equation was expressed via the determination coefficient (R^2) and the adjusted R^2. The fitted polynomial equation was then expressed in the form of three dimensional surface plots, in order to illustrate the relationship between the responses and the experimental levels of each variable investigated in the current study.

Results and Discussion

Fitting the response surface model

The Box-Behnken design was employed to study effects and interactions among four individual parameters. The independent variables and the corresponding response values obtained in different experimental combination are listed in Table 1. The experimental values for phenolic content (TPC) and antioxidant activity (AA) were ranged from 1132.85 to 2499.68 mg GAE/100g DM and from 871.69 to 2307.69mg GAE/100g DM, respectively. Multiple regression analysis was used to analyze the experimental data and thus quadratic polynomial equations of the response surfaces obtained were shown in equations 2 and 3.

$$TPC = 2284.927 + 47.406x_2 + 406.163x_3 - 314.759x_4 + 94.490x_1x_2 + 116.825x_3x_4 - 98.923x_1^2 - 86.118x_2^2 - 193.558x_3^2 - 187.843x_4^2 \quad (2)$$

$$AA = 2090.883 + 35.871x_2 + 401.544x_3 - 343.983x_4 + 62.185x_1x_2 + 114.450x_3x_4 - 87.364x_1^2 - 91.101x_2^2 - 185.91x_3^2 - 205.631x_4^2 \quad (3)$$

As can be seen from Table 2, the three linear (x_2, x_3 and x_4), the four quadratic, and the interactions x_1-

Table 2. Regression coefficient, standard 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	2284.927	22.404	101.990	<0.0001*
x_1	-11.453	11.202	-1.020	0.3268
x_2	47.406	11.202	4.230	0.0012*
x_3	406.163	11.202	36.260	<0.0001*
x_4	-314.759	11.202	-28.100	<0.0001*
x_1-x_2	94.490	19.402	4.870	0.0004*
x_1-x_3	-6.870	19.402	-0.350	0.7294
x_2-x_3	20.188	19.402	1.040	0.3186
x_1-x_4	19.328	19.402	1.000	0.3388
x_2-x_4	24.965	19.402	1.290	0.2225
x_3-x_4	116.825	19.402	6.020	<0.0001*
x_1-x_1	-98.923	16.803	-5.890	<0.0001*
x_2-x_2	-86.118	16.803	-5.130	0.0003*
x_3-x_3	-193.558	16.803	-11.520	<0.0001*
x_4-x_4	-187.843	16.803	-11.180	<0.0001*
Antioxidant activity				
Intercept	2090.583	23.429	89.230	<0.0001*
x_1	4.218	11.714	0.360	0.7251
x_2	35.871	11.714	3.060	0.0099*
x_3	401.544	11.714	34.280	<0,0001*
x_4	-343.983	11.714	-29.360	<0.0001*
x_1-x_2	62.185	20.290	3.060	0.0098*
x_1-x_3	-7.883	20.290	-0.390	0.7045
x_2-x_3	25.565	20.290	1.260	0.2316
x_1-x_4	-6.310	20.290	-0.310	0.7611
x_2-x_4	-8.958	20.290	-0.440	0.6667
x_3-x_4	114.450	20.290	5.640	0.0001*
x_1-x_1	-87.364	17.571	-4.970	0.0003*
x_2-x_2	-91.101	17.571	-5.180	0.0002*
x_3-x_3	-185.914	17.571	-10.580	<0.0001*
x_4-x_4	-205.631	17.571	-11.700	<0.0001*

x_1, solvent concentration (%); x_2, extraction time (min); x_3, extraction temperature (°C); x_4, sample/solvent (mg/10mL).
* Values statistically significant at $P < 0.05$.

Table 3. ANOVA table for the effect of acetone concentration, time, temperature, and sample/solvent ratio on TPC extraction and antioxidant activity

Source	DF	sum of squares	*F* ratio	Prob>*F*
TPC (mg GAE/100g DM)				
x_1	1	1573.917	1.0453	0.3268
x_2	1	26967.756	17.9096	0.0012*
x_3	1	1979615.717	1314.684	<0.0001*
x_4	1	1188879.996	789.5479	<0.0001*
x_1-x_2	1	35713.440	23.7177	0.0004*
x_1-x_3	1	188.788	0.1254	0.7294
x_2-x_3	1	1630.141	1.0826	0.3186
x_1-x_4	1	1494.209	0.9923	0.3388
x_2-x_4	1	2493.005	1.6556	0.2225
x_3-x_4	1	54592.323	36.2553	<0.0001*
x_1-x_1	1	52190.632	34.6604	<0.0001*
x_2-x_2	1	39553.576	26.268	0.0003*
x_3-x_3	1	199811.558	132.697	<0.0001*
x_4-x_4	1	188186.460	124.9766	<0.0001*
Model	14	3584954.400	170.0576	<0.0001*
Error	12	18069.300		
Lack of fit	10	17779.903	12.2886	0.0775
Total	26	3603023.700		
R^2	0.9949			
Adj.R^2	0.9891			
Antioxidant activity (mg GAE/100g DM)				
x_1	1	213.448	0.1296	0.7251
x_2	1	15440.600	9.3767	0.0099*
x_3	1	1934852.613	1174.9890	<0.0001*
x_4	1	1419887.524	862.2632	<0.0001*
x_1-x_2	1	15467.897	9.3933	0.0098*
x_1-x_3	1	248.535	0.1509	0.7045
x_2-x_3	1	2614.277	1.5876	0.2316
x_1-x_4	1	159.264	0.0967	0.7611
x_2-x_4	1	320.947	0.1949	0.6667
x_3-x_4	1	52395.210	31.8183	0.0001*
x_1-x_1	1	40706.266	24.7199	0.0003*
x_2-x_2	1	44263.668	26.8803	0.0002*
x_3-x_3	1	184340.920	111.9458	<0.0001*
x_4-x_4	1	225515.792	136.9503	<0.0001*
Model	14	3750625.08	162.6903	<0.0001*
Error	12	19760.381		
Lak ok fit	10	19544.768	18.1295	0.0534
Total	26	3770385.47		
R^2	0.9947			
Adj.R^2	0.9886			

x_1, solvent concentration (%); x_2, extraction time (min); x_3, extraction temperature (°C); x_4, sample/solvent (mg/10mL).
* Values statistically significant at $P < 0.05$.

x_2 and x_3-x_4, parameters were significantly ($P < 0.05$) affected both the phenolic extraction and antioxidant activity, whereas x_1 and the four other interaction parameters (x_1-x_3, x_2-x_3, x_1-x_4 and x_2-x_4) were not significant.

The fitness of the model was evaluated by the lack of fit test, which was not-significant for TPC (0.0775) and antioxidant activity (0.0534), indicating the goodness of the two models. The ANOVA results of experiment model were shown in Table 3. The ANOVA analysis indicates a good model performance with the determination coefficient (R^2) values of 0.9949 and 0.9947 for TPC and antioxidant activity, respectively. This was an estimate of the fraction of overall variation in the data accounted by the model, and thus 99.4% of the variations in response could be explained by the fitted model. The F-value of the models was 170.05 in case of the TPC ($P<0.0001$) and 162.69 for antioxidant activity ($P<0.0001$). These results indicated that the two models could work well for the prediction of phenolic compound extraction and antioxidant activity from carob pulp.

The results showed that extraction temperature, sample/ solvent ratio, and extraction time were

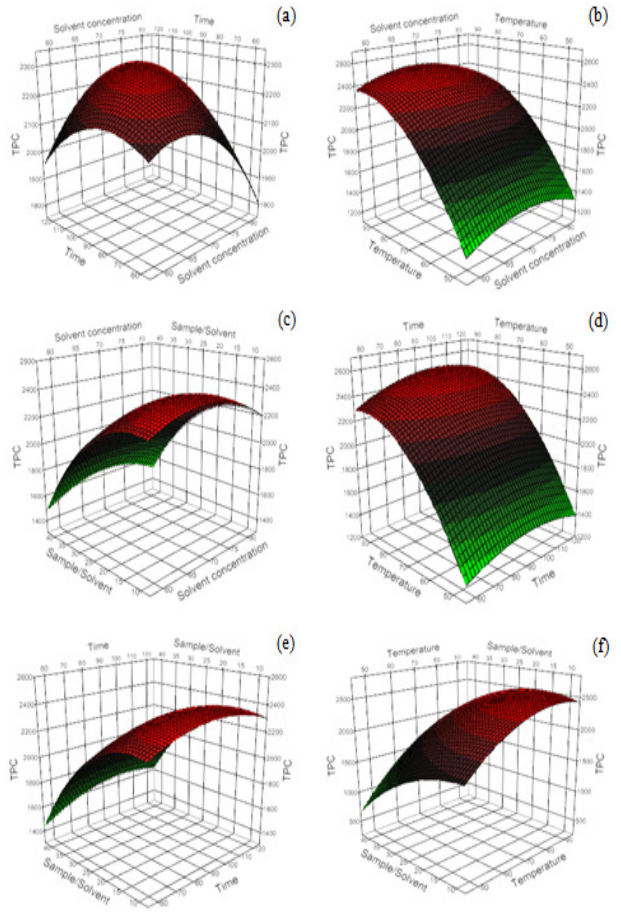

Figure 1. Response surface plots of total phenolic content of carob pulp as affected by solvent concentration and time (a), solvent concentration and temperature (b), solvent concentration and sample/ solvent ratio (c), temperature and time (d), time and sample/solvent ratio (e), and temperature and sample/solvent ratio (f)

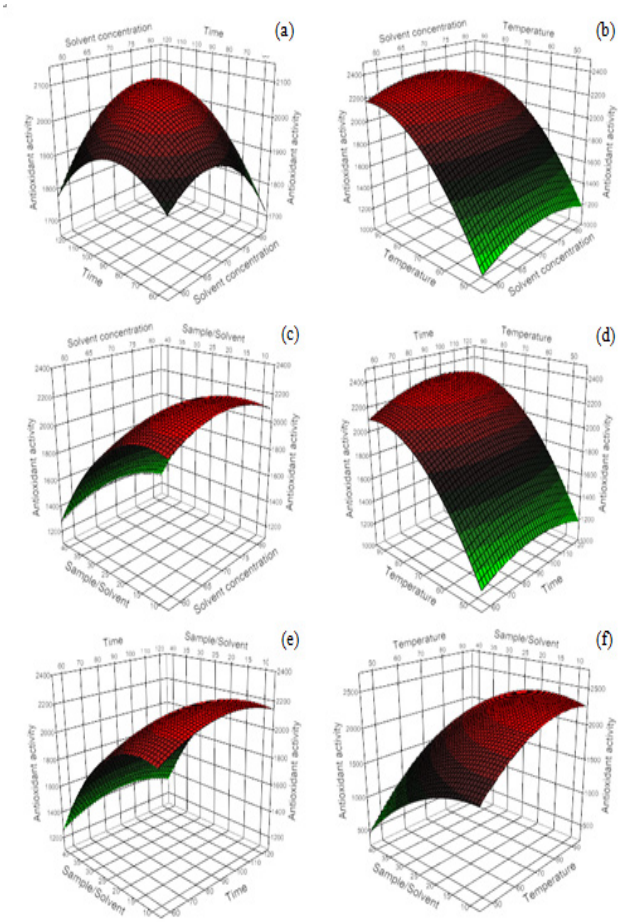

Figure 2. Response surface plots of antioxidant activity of carob pulp as affected by solvent concentration and time (a), solvent concentration and temperature (b), solvent concentration and sample/ solvent ratio (c), temperature and time (d), time and sample/ solvent ratio (e), and temperature and sample/solvent ratio (f)

the most significant factors, which affected the extraction of phenolics and antioxidant activity from carob pulp. The extraction time and temperature, and the interaction between solvent concentration-time and temperature-sample/solvent ratio were found to have positive influence on responses. The sample/ solvent ratio and the quadratic effects of the factors influenced negatively both phenolic compounds extraction and antioxidant activity. However, the solvent concentration and interaction terms between solvent concentration-temperature, solvent concentration-sample/solvent ratio, time-temperature and time-sample/solvent ratio were found to have no significant effect. The significance of coefficients was listed in Table 3. The P-value of the model is less than 0.0001, indicating that the model was significant and can be used to optimize antioxidants extraction.

Analysis of response surfaces

In order to determine the optimal levels of independent variables for the extraction of total phenolics from carob pulp, response surfaces were plotted. Each pair of variables within the experimental ranges was depicted in three dimensional surface plots, while the two other variables were kept constant at central level. Figures 1 (a, b and c) and 2 (a, b and c) are the three dimensional plots showing the effects of solvent concentration (x_1) with time (x_2), temperature (x_3) and sample/solvent ratio (x_4) on the phenolic extraction and antioxidant activity of carob pulp, respectively. The influence of solvent concentration was shown to be statistically insignificant. It can be observed that the two responses increased with increasing solvent concentration at first. However, the trend was reversed when the optimal was reached. This is due to the quadratic effect of acetone concentration, which showed a negative value, indicating that an increase in this parameter beyond a certain value tends to decrease the responses, while the interaction between solvent concentration and extraction time had a positive effect (Table 2).

In the current study, the total phenolic compound

extraction and antioxidant activity decreased when the acetone concentration was above 70.85%. Pure acetone was inefficient for the extraction of phenolics from carob pod. These results can be explained by the principle "like dissolves like"; any given solvent would easily extract compounds that have a similar polarity (Zhang *et al*., 2007).

Addition of small quantity of water to organic solvents creates a more polar medium which facilitates the extraction of phenolics. Our results are in agreement with results reported for other vegetal matrices; acetone/water 50% (v/v) and 61% were the most efficient solvent mixtures for phenolic extraction from mango seeds (Dorta *et al*., 2013) and dried dark figs (Bachir bey *et al*., 2014).

Changes in solvent concentration modify the physical properties of the solvent such as density, dynamic viscosity, and dielectric constant. Solubility of compounds would also be modified by changes in solvent concentration, and this may influence the extraction of phenolics (Cacace and Mazza, 2003).

Examination of the plots in Figures 1 and 2 revealed that extraction temperature, time, and sample/solvent ratio exert higher effects than acetone concentration. The extraction time was an important factor that influenced antioxidant extraction. The effects of time with each of the three other variables on total phenolic compound extraction and antioxidant activity of carob pulp are displayed in Figures 1 (a, d and e) and 2 (a, d and e), respectively. As shown, both phenolic content and antioxidant activity responses increased with increasing extraction time to achieve the maximum at 101.5 min. Beyond this value, a decrease in TPC, and, hence antioxidant activity was observed. These findings are in consonance with those reported in our previous study (Benchikh and Louaileche, 2014); the phenolic compound content of carob pulp extracts increased when extraction time was increased. Extended extraction time was expected to favour the extraction of phenolic compounds, since it takes time enough to solvent penetration into the plant tissue, dissolving the solute and subsequently diffusing out to the extraction medium (Viacava *et al*., 2015). However, according to Tay *et al.* (2013), long extraction time may cause increased exposure to light and oxygen which will eventually result in the oxidation of phenolic compounds. The optimal time for phenolic extraction depends upon the vegetal material investigated. It was of 93 min for TPC extraction from grape by-product (Rajha *et al*., 2014), whereas Gomez *et al*. (2014) found that 23 min was the optimum time for TPC extraction from avocado seeds.

Figures 1 (b, d and f) and 2 (b, d and f) show the effects of extraction temperature (x_3) with solvent concentration (x_1), time (x_2) and sample/ solvent ratio (x_4) on the phenolic extraction and antioxidant activity of carob pulp, respectively. In all cases, the extraction of phenolics was improved with temperature increase to reach the optimum at 88.35˚C. The estimate of the linear and the quadratic coefficients of extraction temperature was the highest in both responses compared with the other factors, which indicated that extraction temperature had the most critical role in phenolic extraction from carob pulp. This finding is also supported by ANOVA results (Table 3). With suitable temperature, more phenolic compounds would be extracted into the solvent, as mild heating might soften the plant tissue, weaken the cell wall integrity, hydrolyze the bonds of bound phenolic compounds (phenol-protein or phenol-polysaccharide) and enhance both the phenolic solubility (Tabaraki and Nateghi, 2011). However, beyond a certain temperature, some antioxidants like some phenolic compounds or vitamins can be denatured by chemical reactions. Other authors have also observed in different matrices an increase in phenolic extraction with increasing temperature up to 63 °C for avocado pits (Gomez *et al*., 2014) and 94 °C for grape pomace (Rajha *et al*., 2014).

The mass transfer from plant material to solvent was related to time and temperature. It can be observed from Figures 1(d) and 2 (d) that both the extraction time and temperature influenced phenolic extraction and antioxidant capacity of carob pulp. In this sense, the linear and the quadratic effects of temperature and time were showed clearly. It was noticed that both factors operate independently on phenolic extraction and antioxidant activity (Tables 2 and 3). Moreover, time coefficient was smaller than temperature coefficient, meaning that the time of extraction (x_2) is less influent for antioxidant extraction than the temperature.

Figures 1(c, e and f) and 2 (c, e and f) present the response surface showing the effects of sample/ solvent ratio with each of the three other variables on phenolic compound extraction and antioxidant activity, respectively. Three different ratios (sample/ solvent ratio, mg/10 mL; 10/10, 25/10 and 40/10) were used for TPC extraction and antioxidant activity. As shown, the increase in the sample leads to a gradual decrease in responses. Also, Table 2 indicated that sample/solvent ratio parameter had higher influence on the extraction of phenolics compared with solvent concentration and extraction time. The maximum phenolic compound concentration and the best antiradical capacity were obtained when a low sample/ solvent ratio was used. Consequently,

the sample/solvent ratio of 16.65mg/10mL was considered as suitable to obtain the highest extraction. Also, Benchikh and Louaileche (2014) reported that total phenolic compounds and antioxidant activity from carob pulp decreased when sample/solvent ratio was above 25mg/10 mL. This is consistent with mass transfer principles; the driving force during mass transfer is the concentration gradient between the liquid (extraction solvent) and the solid (sample), which is greater when a lower solid/solvent ratio is used (Al-Farsi and Lee, 2008). Therefore, further increase in solvent/sample ratio, a decline in total phenols extraction yield was observed (Wang *et al*., 2013). The reduction in extraction efficiency is due to poor solid-to-solvent interaction possibly due to caking of sample which decreases the solubility of phenolics in extraction solvent (Luthria, 2012). A high sample/solvent ratio produces incomplete extraction and the solvent becomes saturated before substrate exhaustion (Bachir bey *et al*., 2013).

Determination and experimental validation of the optimal conditions

Through the 3-D plots, the final step of the RSM after selecting the optimal conditions was to predict and to verify the accuracy of the mathematical model. Results of the optimal conditions to extract the highest amount of phenolics from carob pulp, and to obtain the extract with the best antioxidant activity, were acetone concentration (x_1) of 70.85%, extraction time (x_2) of 101.5 min, extraction temperature (x_3) of 88.35°C, and sample/solvent ratio (x_4) of 16.65mg/10 mL. Under these conditions, the experimental values were 2557.96 mg GAE/100g DM and 2393.75 mg GAE/100g DM for phenolics and antioxidant activity, respectively. These experimental results were in agreement with the predicted values for phenolics (2559.99 mg GAE/100g DM) and antioxidant activity (2385.06 mg GAE/100g DM), confirming the effectiveness of the response surface models to reflect the expected optimization. The phenolic content found in the current investigation is higher than that obtained by Avallone *et al*. (1997) using acetone 70% (17 mg GAE/g DM).

Conclusion

The current study confirmed the advantages of response surface methodology compared with the classical method. Response surface methodology was successfully implemented for optimization of total phenolics and antioxidant activity. The extraction temperature was found to be the most significant (positively) for phenolic extraction and antioxidant activity followed by the sample/solvent ratio (negatively). The experimental values were found to be in agreement with the predicted ones and clearly indicated the suitability of the developed quadratic models. These results confirm the predictability of the model for the extraction of TPC and antioxidant activity from carob pulp in the used experimental conditions. The optimized parameters could be exploited for the mass production of natural antioxidants for application in the cosmetic, pharmaceutical, and food industries.

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