CHEMISTRY & CHEMICAL TECHNOLOGY

Chem. Chem. Technol., 2020,
Vol. 14, No. 4, pp. 572–582

Chemical Technology

EXPLOITING RESPONSE SURFACE METHODOLOGY (RSM) AS A NOVEL APPROACH FOR THE OPTIMIZATION OF PHENOLICS AND ANTIOXIDANT ACTIVITY OF DATE PALM FRUIT

Soumaya Hachani[1, ✉], Sarah Boukhalkhal[1], Ziyad Ben Ahmed[1], Mohamed Harrat[1], Artur M.S. Silva[2], Mohamed Yousfi[1]

https://doi.org/10.23939/chcht14.04.572

Abstract. The Box-Behnken design was used to investigate the effect of three independent variables – time, temperature and solvent-to-solid ratio on the responses of total phenolics, total flavonoids, 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity and cupric ion reducing antioxidant capacity (CUPRAC) of date fruit methanolic extracts. Response surface analysis showed that the optimal ultrasound extraction parameters that maximized the responses were 30 min, 298 K and 74.4 ml/g. Under optimum conditions, UHPLC-DAD-MS/MS was used to tentatively characterize 11 phenolic compounds. The experimental values for the quantification of phenolic compounds and antioxidant activity are in accordance with the predicted values, indicating the suitability of the model and the success of response surface methodology in optimizing the ultrasound extraction conditions.

Keywords: date fruit, polyphenols, ultrasound extraction, response surface methodology, antioxidant activity, UHPLC-DAD-MS/MS.

1. Introduction

Date palm *Phoenix dactylifera* L. is one of the important fruit crops of the arid regions of the North Africa [1]. Millions of date cultivars are main income source and staple food and have played great role in the economy, society, and environment in the country they are cultivated [2]. Algeria, the largest country in Africa, is classified as the second producer of date fruits in Africa and the third one in the world after Iran and Egypt by an average production of 1,029,596 tons [3]. An outstanding diversity of date palms of this country attracted attention of several researchers from Algeria and other countries since the tree itself differs greatly from the neighboring trees of both Morocco and Tunisia [4]. Thus, recent researches focus on the genetic characterization of date palm, whereas other main goals focus on studying the chemical components of different fruit cultivars since they contain numerous active substances such as phenolic compounds, and frequent consumption of these fruits is associated with a lower risk of cancer, heart disease, hypertension, and stroke [5-9]. These studies showed that fresh and dried dates varied quantitatively and qualitatively in their phenolic content and antioxidant activity. This was due to the extraction type (maceration, soxhlet, under reflux, *etc*.) and parameters such as extraction time, solvent and temperature. Nowadays, ultrasound is widely applied for the extraction of different substances from the plant matrix due to the high efficiency, short extraction time and high yield of ultrasonic technology. Also, it reduced the consumption of solvents and energy. Ultrasound technology is based on ultrasonic waves that can penetrate to reach the plant cavitation and break out the cell walls leading to products releasement. Also, it improves viscosity and homogenization of the product [10].

Although ultrasound extraction can improve productivity and yield by maximizing the quality and minimizing the extraction processing, it can also modify the physical parameters and cause the degradation of major and minor target compounds. This was due to the critical time and temperature allied to the degradation of certain substances or the formation of free radicals [11, 12]. Therefore, the focus of this paper was to optimize the ultrasound extraction parameters that assure a good performance of ultrasound extraction when applied to extract phenolics from date fruit.

Extraction parameters are generally optimized using one-factor-at-a-time approaches. In this method, the interactions of other factors are ignored and therefore, it is difficult to find out the optimum conditions [13]. However, a mathematical modeling based on a statistical

[1] Laboratoire des Sciences Fondamentale, Université Amar Telidji, Laghouat, Algérie
[2] Department of Chemistry and QOPNA, University of Aveiro, 3810-193 Aveiro, Portugal
✉ *hachani.soumaya@gmail.com*
© Hachani S., Boukhalkhal S., Ben Ahmed Z., Harrat M., Silva A., Yousfi M., 2020

experimental protocol can overcome the one-factor-at-a-time design, which is called response surface methodology (RSM) [14]. RSM is a tool used by many researchers to maximize or minimize various independent variables in order to predict the optimal conditions and to attain the best system performance.

Thus, we fixed our objective on optimizing ultrasound extraction conditions: solvent-to-solid ratio, temperature, and time, in order to maximize both phenolic content and antioxidant activity of date fruit using RSM. This will be a basic study to promote industrial extraction of date antioxidants.

2. Experimental

2.1. Chemical Reagents

All reagents used in this experiment were obtained from Biochem, Sigma-Aldrich and Pearce.

2.2. Sample Collection

Date cultivar of *Phoenix dactylifera* L. namely *Takarmoust* is collected at "tamr stage" from Ain Salah region of Algeria at the end of 2015 harvest season. After cleaning the samples and removing the seeds, the edible part of date was dried at 313 K until the moisture content equaled to 17.49 %.

2.3. Selection of Appropriate Ultrasound Extraction Conditions

In order to achieve the best ultrasound extraction conditions, preliminary experiments were realized. Extractions were carried out in an ultrasonic bath (Ultrasonic Cleaner, Joident) at 40 kHz. Samples (1 g) of uniform size powder were used and placed in glass container (150 ml).

Extraction time. Starting with the extraction time, the samples were exhaustively extracted at different extraction time (5–50 min), at the extraction temperature of 298 K and the liquid-to-solid-ratio of 25:1 ml/g. After ultrasonic extraction, the extracts were filtered using Whatman filter paper (1:11 mm grade) and then evaporated to dryness using a rotary evaporator at 318 K. The dried residue was dissolved in 10 ml of methanol and kept at 277 K.

Extraction temperature. The ultrasound extraction process of different samples was carried out under extraction temperatures varying from 278 to 328 K for 35 min with the liquid-to-solid-ratio of 25:1 ml/g. The extracts were filtered using Whatman filter paper (1:11 mm grade) and then evaporated to dryness using a rotary evaporator at 318 K. The dried residue was dissolved in 10 ml of methanol and kept at 277 K.

Liquid-to-solid ratio. The ultrasound extraction was performed using the following parameters: T = = 308 K, τ = 35 min and methanol-to-dry material ratio of 10–110 ml/g. The extracts were filtered using Whatman filter paper (1:11 mm grade) and then evaporated to dryness using a rotary evaporator at 318 K. The dried residue was dissolved in 10 ml of methanol and kept at 277 K.

In each experiment, total phenolic content (TPC) were measured in order to find out the suitable parameters of time, temperature and liquid-to-solid ratio (Fig. 1). Based on the results of the preliminary experiments, the ranges of the three factors that varied in the experimental design were determined (Table 1).

2.4. Determination of Total Phenolics and Total Flavonoids in Palm Date Extract

Total phenolic content (TPC) was determined as follows: 100 µl of the sample was added to 500 µl of the aqueous solution of Folin-Ciocalteu reagent (10%). After 2 min incubation at room temperature, 2 ml of sodium carbonate (2%) were added and incubated for 30 min. Then the absorbance was measured at 760 nm using the Shimadzu 1601 visible spectrophotometer. TPC were expressed as mg of gallic acid equivalent (GAE)/g dry weight (DW) [15].

Total flavonoid content (TFC) was determined based on the formation of the complex flavonoids-aluminum, having an absorption maximum at 412 nm [16]. The reaction mixture was made up using 1 ml of the extract and 1 ml of aqueous aluminum chloride (2%). After 15 min of incubation, the absorbance of the mixture was determined spectrophotometrically. Quercetin was used for the calibration curve and the TFC were expressed as mg of quercetin equivalent (QE)/g DW.

2.5. Antioxidant Assay

DPPH radical scavenging activity. Radical scavenging activity was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH (2,2-diphenyl-1-picrylhydrazyl). 1 ml of date extract was added to 1 ml of methanolic DPPH (250 µM) and incubated for 30 min. The absorbance was measured at 517 nm [17]. The calibration curve of vitamin C was prepared, and the results were expressed in mg equivalent of ascorbic acid (EAA) pre-100 g of DW (mgEAA/100gDW).

CUPRAC (Cupric Ion Reducing Antioxidant Capacity) assay. 500 µl of $CuCl_2$ (0.01M), 500 µl neocuproine alcoholic solution (7.2 mM), 1 ml of NH_4 acetate buffer solution were added to a test tube and then

100 µl of extract were added and mixed well [18]. Absorbance against a reagent blank was measured at 456 nm after 30 min. The calibration curve of vitamin C was prepared, and the results were expressed in mgEAA/100gDW.

2.6. Experimental Design

The optimization using one-variable-at-a-time was widely applied in the extraction of organic substances, but its major drawback was neglecting the interactive effects among different variables. By varying just one factor and leaving the others constant the number of experiences will increase. As a consequence, the resulting response was not clearly described by the complete effects of the parameter [14].

For that, the response surface methodology (RSM) was used in this study. This method used multivariate statistic techniques based on mathematical and statistical equations. In this case, the resulting experimental data should fit polynomial equation and the influence of different variables on the behavior of response will be optimized to achieve the best system performance [14].

An experimental design called Box-Behnken design for quadratic response surfaces was used in order to evaluate the extraction parameters and optimize the conditions of date phenolics extraction. This design is more efficient and economical then three-level factorial designs (3^k designs), mainly for a large number of variables [14].

The chosen design includes three independent variables; time (X_1, min), temperature (X_2, K) and liquid-to-solid ratio (X_3, ml/g) at three factorial levels (-1, 0, +1) that lead to a total of 15 experimental runs with three replicates at the center points (0, 0, 0). In this study, the chosen responses were Y_{TPC} (response of TPC), Y_{TFC} (response of TFC), Y_{DPPH} (response of DPPH) and Y_{CUPRAC} (response of CUPRAC). These responses were influenced by the independent variables and these influences were represented by response surfaces, and in each test the optimal conditions, where the highest yield is marked, were chosen.

2.7. Data Analysis

All the experiments were carried out in triplicate, and the data presented are the mean values of these independent experiments. Statistical analysis of RSM was performed using Design-Expert (Design Expert software, Trial Version 10.0.7, Stat-Ease). The generalized second-order polynomial model used in the response surface analysis was presented by the following equation:

$$Y = a_0 + \sum_{i=1}^{3} a_i X_i + \sum_{i=1}^{3} a_{ii} X_i^2 + \sum_{i=1}^{3} a_{ij} X_i X_j \quad (i \neq j) \quad (1)$$

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

The responses obtained from each set of experimental design (Table 1) were subjected to further analysis using design expert software to plot response surface using 3-D response graphs. The analysis of variance tables (ANOVA, 95% confidence level) was generated, and the experimental data were refitted using the *p*-values by excluding the non-significant terms from the basic model [19]. The values of R^2, adjusted-R^2 of models were evaluated to check the model adequacies. The *F*-values in the ANOVA table and the lack-of-fit test were also used to check the significance of the model and to check the variability of the residues between the proposed model and the observations of repeated tests.

2.8. Verification of Model

In order to verify the validity of the statistical experimental design, additional confirmation experiments at the optimized conditions (time, temperature and liquid-to-solid ratio) were done.

Since the study objective was to optimize the extraction of phenolic compounds and maximize their antioxidant activities, the number of responses increased to four responses, and it was difficult to find the conditions that concurrently satisfy all the four responses. To overcome this problem, a multicriteria methodology was used. This methodology is widely used to find the optimal conditions between the total numbers of the considered responses [14]. The desirability function (*D*), also called Derringer function, was used, its values ranged from 0 to 1. The overall optimized conditions were calculated using design expert optimization tool and the maximum overall desirability (*D*) was defined by reducing the simultaneous optimization process to find out the appropriate levels of factors. After that, both experimental and predicted response values of the optimal conditions were compared.

2.9. UHPLC-DAD-ESI-MS/MS Analysis of Phenolic Compounds in Optimum Conditions

UHPLC-DAD-ESI-MS/MS apparatus (Ultimate 3000 'Dionex Co, USA). Diode array detector 3000 (Dionex Co, USA) was coupled to LTQ XL thermo ion trap mass spectrometry (thermo scientific, USA) equipped with an ESI source. The analysis was carried out on a C18 Hypersil Gold column (thermo scientific, USA) of 100 mm length, inner diameter 2.1 mm, particle diameter 1.9 µm, temperature 303 K.

The mobile phase for the separation of date extracts constituents was composed of acetonitril (A) and acidified water (0.1% formic acid, v/v, (B)), both degassed and filtered before use. The mobile phase gradient started with 5 % of (A) and 95 % of (B), reached 40 % of solvent (A) at 14.7 min; 100 % of (A) at 16.6 min and finally at 24 min it returned to the initial conditions. The flow rate was 0.2 ml/min and UV-Vis spectral data for all peaks were accumulated in the range 200–500 nm while the chromatographic profiles were recorded at 280 nm.

Control and data acquisition of MS were carried out with the Thermo Xcalibur Qual Browser data system (Thermo Scientific, USA). Nitrogen above 99% purity was used and the gas pressure was 520 kPa (75 psi). The instrument was operated in negative-ion mode with ESI needle voltage set at 5.00 kV and an ESI capillary temperature of 548 K. The full scan covered the mass range from m/z 100 to 2000. CID–MS/MS and MS^n experiments were simultaneously acquired for precursor ions using helium as the collision gas with collision energy of 25–35 arbitrary units.

3. Results and Discussion

3.1. Determination of the Experimental Ranges of Independent Variables

The investigated experimental field is defined by both the minimum and maximum limits of the experimental variables; time, temperature and liquid-to-solid ratio. The levels of each variable are different values giving codes -1, 0 and +1 of variable at which the experiments must be run out.

Ultrasound extraction time. The levels of the independent variable of time (X_1, min) were chosen after ranging the ultrasound time of experience from 5 to 50 min (Fig. 1a), while other factors were set as follows: liquid-to-solid-ratio was 25:1 ml/g, temperature was 298 K, and in each experiment the TPC was measured. Results show that the TPC increases by time and reaches the maximum level at 35 min. This could be explained by the fact that ultrasound time presents a positive effect on the TPC by giving a chance to full liquid penetration, releasement and dissolvent of different substances from the raw materials. After 35 min the decrease of TPC may be due to the longer extraction times that increase the phenolics oxidation. This oxidation is caused by the addition of some reducing agents to the solvent [20]. Furthermore, the influence of long exposure to ultrasound waves gives rise to the degradation of phenolic compounds. Many different studies have studied the effects of ultrasound on sonicated food products, their results show the decrease or degradation of compounds such as polyphenols, ascorbic acid, lycopene, isomers 15-cis-β-carotene, di-cis-β-carotene, and other compounds with C–O function after sonication. Moreover, the ultrasound extraction of orange juice lead to the formation of hydroxyl radical, which lead to the interaction between free radicals and ascorbic acid [12]. Based on the results, the levels of the independent variable of time (X_1) are 30, 35 and 40 min.

Ultrasound extraction temperature. Fig. 1b shows the effect of extraction temperature on the TPC. The extraction process was carried out at the temperatures ranging from 278 to 328 K, while other extraction parameters were set as follows: ultrasound extraction time 35 min and liquid-to-solid ratio 25:1 ml/g. Results indicated that the TPC increased significantly when the temperature increased from 278 to 308 K and reached the highest TPC value of 2.54 mgGAE/gDW. These results could be explained by the fact that higher adequate temperature enhances the solubility of the target substances in the solvent, insures better diffusion and improves good mass transfer [21]. Moreover, heating facilitates the liberation of polyphenols by the cleaving of the esterified and glycosylated bond or by the formation of Maillard reaction products, which are responsible for the intensification of polyphenols after heating [22]. However, applying temperature higher than 308 K decreases the TPC. These results are in good agreement with other previous researches that related the decreasing of TPC to the decomposition of phenolic compounds when they reach a higher critical temperature [23]. This critical ultrasound temperature can cleave chemical bounds between atoms and facilitate the destruction of phenolic molecules [22]. Based on the results, the levels of the independent variable of temperature (X_2) are 298, 308 and 318 K.

Liquid-to-solid ratio. Many studies indicated that liquid-to-solid ratio can affect the process of ultrasound extraction and should thus be taken into consideration [12]. In this work, different liquid-to-solid ratios were used (from 10:1 to 110:1 ml/g), while other parameters were set as follows: ultrasound extraction time 35 min and temperature 308 K. Results show that TPC increased with increasing the liquid-to-solid ratio from 10 to 60 ml/g.

This could be explained by the fact that larger ratio of methanol to plant material increases the concentration gradient between them leading to an increased diffusion rate of polyphenols from the extracted raw material into the solvent. However, after 60 ml/g, the TPC decreases (Fig. 1c). Similar previous researches have observed the same results indicating that the excessive dilution of the target material may not increase the TPC under the same investigated conditions [11]. In addition, higher liquid-to-solid ratio protracted the distance of diffusion towards the inner tissues causing a big loss during production [21]. Based on the results, the levels of the independent variable of liquid-to-solid ratio (X_3) are 40, 60, 80 ml/g.

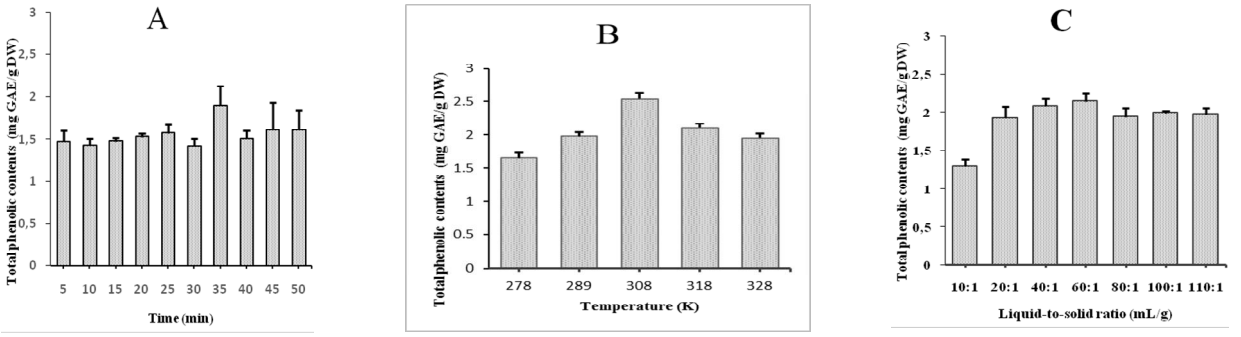

Fig. 1. Preliminary studies on extraction time (A); temperature (B) and liquid-to-solid ratio (C). Values represent mean (n = 3) ± standard deviation

Table 1

Box-Behnken design matrix (in coded and uncoded level of three variables) and the experimental data values for the responses used in response surface methodology (RSM)

Run	Time, min	Temperature, K	Liquid-to-solid ratio, ml/g	Total phenolic content [a] mgGAE/gDW	Total flavonoid content [b] mg QE/gDW	[c]DPPH [d] mgEAA/100gDW	[c]CUPRAC [d] mg EAA/100gDW
1	-1	-1	0	1.637	0.393	74.444	306.420
2	1	-1	0	1.694	0.322	71.719	313.873
3	-1	1	0	1.550	0.280	65.594	307.345
4	1	1	0	1.705	0.261	57.941	308.289
5	-1	0	-1	1.528	0.289	69.048	276.741
6	1	0	-1	1.395	0.270	58.364	280.929
7	-1	0	1	1.602	0.328	68.473	299.931
8	1	0	1	1.624	0.327	68.108	288.433
9	0	-1	-1	1.449	0.320	55.130	272.935
10	0	1	-1	1.473	0.319	65.867	271.040
11	0	-1	1	1.315	0.413	35.969	280.888
12	0	1	1	1.438	0.326	44.360	295.583
13	0	0	0	1.348	0.390	41.519	266.190
14	0	0	0	1.369	0.291	35.238	262.596
15	0	0	0	1.316	0.289	37.908	269.158
Uncoded level							
-1	30	298	40				
0	35	308	60				
+1	40	318	80				

Notes: [a] mg of gallic acid equivalent /100 g dry weight (DW); [b] mg of quercetin equivalent /100 gDW; [c] DPPH (2,2-diphenyl-1-picrylhydrazyl freeradical scavenging activity), CUPRAC (cupric ion reducing antioxidant capacity); [d] mg equivalent of ascorbic acid/100 gDW

3.2. Optimization of Extraction

According to the results of single factor experiments, the Box-Behnken design was created based on the following experimental domains: ultrasound extraction time of 35±5 min, the temperature of 308±10 K and liquid-to-solid ratio of 60±20 ml/g.

The mean experimental values of the TPC, TFC, DPPH and CUPRAC are shown in Table 1. The mean absolute percentage error (MAPE) was 3, 9, 12, and 1 % for TPC, TFC, DPPH, and CUPRAC, respectively, indicating the close agreement between experimental and predicted values. The TPC ranged from 1.31 (assay 11) to 1.71 mgGAE/gDW (assay 4). The highest value for TPC was corresponding to the experimental design with liquid-to-solid ratio of 60 ml/g for 40 min at 318 K.

The analysis of multiple regression of TPC values (Table 2) revealed that the model was significant (p = = 0.0003), the F-value was 17.59. If the p-value is low and the F-value is high, the model will indicate a more significant effect on the response variable. Moreover, the model did not present lack of fit (p = 0.1062), which

indicated the relevance of model to predict the variation with an exact way [24].

On top of that, the values of R^2, adjusted R^2 and predicted R^2 were 0.7456, 0.7032 and 0.6090, respectively. They indicate that the model is more accurate and fits the data [25]. The coefficient of variation (*CV*) is equal to 4.91 %, indicating the high degree of precision between the calculated and experimental result [26]. Withal, the adequate precision measures the signal to noise ratio and this ratio was 9.29. Usually a ratio greater than 4 is desirable and indicates an adequate signal. As a result, this model can be used to navigate the design surface.

Furthermore, the quadratic regression coefficients of both time (X_1) and temperature (X_2) were significant ($p < 0.05$). Thus, predicted model of TPC can be described by Eq. (2) in terms of coded values:

$$Y_{TPC} = 1.33 + 0.21X_1^2 + 0.093X_2^2 \quad (2)$$

The results suggested that the liquid-to-solid ratio had negligible effect on the TPC.

The TFC ranged from 0.26 (assay 4) to 0.41 mgQE/gDW (assay 11). The highest value for TFC was observed in the experimental design with 80 ml/g (liquid-to-solid ratio) for 35 min at 298 K (Table 1).

The analysis of multiple regression of TFC values revealed that the model was significant ($p = 0.0375$), the *F*-value is 5.37 and the model did not present lack of fit ($p > 0.05$) pointing out the relevance of model to predict the variation [24]. This model might explain just 29.22 % of all variance in data (adjusted $R^2 = 0.2377$) (Table 2). In addition, the coefficient of variation (*CV*) and the adequate precision values were 12.38 % and 4.50, respectively, which indicated that the polynomial model equation had quite good quality fit. Thus, this model can be used to navigate the design surface.

Only the linear regression coefficient of temperature (X_2) was negative and significant. Therefore, predicted model of TFC can be described by Eq. (3) in terms of coded values:

$$Y_{TFC} = 0.32 - 0.03X_2 \quad (3)$$

The results suggested that both time and the liquid-to-solid ratio had negligible effects on the TFC.

For DPPH test results, the extracts have remarkable antioxidant activity ranging from 35.24 to 74.44 mgEAA/100 gDW. Date extract obtained with run 1 (extraction for 30 min at 298 K with liquid-to-solid ratio equal to 60 ml/g) showed the best radical scavenging activity, while run 14 showed a relatively weaker free radical scavenging activity (Table 1).

As indicated in Table 2, the ANOVA analysis showed that the model was significant ($p = 0.0004$), with convenient performance ($R^2 = 0.6298$, adjusted $R^2 = 0.6014$) and there was no significance in the lack of fit ($p = 0.1025$) illustrating that the model could be used to predict the response. An adequate signal 6.66 was detected. The coefficient of variation (*CV*) was equal to 15.64 %.

The quadratic regression coefficient of time (X_1) was positive and significant. Therefore, a predicted model of DPPH antioxidant activity can be described by Eq. (4):

$$Y_{DPPH} = 45.14 + 21.57X_1^2 \quad (4)$$

The correlation between TPC and the DPPH test was good and positive ($R^2 = 0.826$). On the other hand, the correlation between TFC and the DPPH test was found weak and negative ($R^2 = -0.1404$), revealing that the DPPH antioxidant activity of dates extracts may be attributed to the presence of phenolic compounds (non-flavonoids) but not for the flavonoids [27].

The results of CUPRAC test are in the range from 262.60 (assay 14) to 313.87 mgEAA/100 gDW (assay 2). Moreover, the RSM analysis of CUPRAC values showed that the model was significant with *p*-value < 0.0001, did not present lack of fit ($p = 0.2818$) and could explain 92.73 % of calculated model (adjusted $R^2 = 0.9074$). In addition to that, the coefficient of variation (*CV*) equal to 1.84 %, demonstrates the high degree of precision between the calculated and experimental results [26], and the adequate precision is 16.06 as well.

The linear term of liquid-to-solid ratio (X_3) and the quadratic terms of both time (X_1) and temperature (X_2) were significant. Thus, the predicted CUPRAC response is written as follows:

$$Y_{CUPRAC} = 263.41 + 7.90X_3 + 25.02X_1^2 + 18.62X_2^2 \quad (5)$$

Table 2

Analysis of the response surface model

Response	R^2	Adjusted R^2	*F*-Value of model	*p*-Value of model	*p*-Value of lack of fit
Total phenolic content	0.7456	0.7032	17.59	0.0003	0.1062
Total flavonoid content	0.2922	0.2377	5.37	0.0375	0.8827
[a] DPPH	0.6298	0.6014	22.12	0.0004	0.1025
[b] CUPRAC	0.9273	0.9074	46.75	< 0.0001	0.2818

Note: [a] DPPH (2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity); [b] CUPRAC (cupric ion reducing antioxidant capacity)

The high correlation coefficient (R^2 = 0.811) between TPC and CUPRAC indicates that date fruit phenols have a reducing capacity and are able to reduce cupric ions [28]. On the contrary, flavonoids might not be the dominant components responsible for the reducing capacity since the correlation coefficient was weak and negative (R^2= –0.050).

3.3. Response Surface Analysis

For better visualization of the predicted models' equations, the response surfaces were plotted using design expert software. This graphical representation is a 2-dimensional surface in the (2 + 1)-dimensional space since the plot visualization is possible only if one variable is set to a constant value [14].

Figs. 2a1-a3 represents the 3-D plot response surfaces of TPC. Each figure showed the effects of two variables on TPC while the other one was kept in a constant level. Fig. 2a1 presents concave surface with minima equaled to 1.33 mgGAE/gDW when varying ultrasound extraction time and temperature. This plot shows that TPC increased with increasing of ultrasound time and temperature, this was due to the positive quadratic effect a_{11} and a_{22}. The influence of both liquid-to-solid ratio (X_3) and extraction time (X_1) was shown in Fig. 2a2. Due to insignificant effect of both a_3 and a_{33}, X_3 has no effect on the TPC. According to the response plot, TPC increased with increasing the ultrasound time to reach a maximum equal to 1.70 mgGAE/gDW. The same effect was found by varying liquid-to-solid ratio (X_3) and temperature (X_2). It can be seen that temperature has a positive relationship with TPC due to the significant positive effect of the quadratic term b_{22} (Fig. 2a3). Generally, long time exposure to high temperature had a positive effect on TPC since it enhances the ability to release more phenolic compounds from the vegetal matrix due to the formation of Maillard reaction products or the cleaving of some bounds such as esterified and glycosylated bonds [22].

Fig. 2b1 illustrates the evolution of the TFC according to extraction temperature and time, with the liquid-to-solid ratio kept constant. As can be seen from the plot, the TFC values increased only with decreasing extraction temperature. Thus, temperature has a strong negative significant effect, whereas ultrasound extraction time had no significant effect. These results are in good agreement with other studies which have shown that different dimers and oligomers of flavonoids form monomers by the hydrolysis of C-glycosides bonds. This reduces the TFC especially for molecules such as quercetin, kaempferol and isorhamnetin glucosides [22].

Fig. 2b2 illustrates the evolution of the TFC according to liquid-to-solid ratio and ultrasound extraction time. Time (X_1) has negligible effect on TFC and the variation of liquid-to-solid ratio could slightly increase TFC. This could be due to the influence of low fixed temperature (298 K) on the solvent viscosity. When the temperature was decreased, the viscosity of the solvent increased, thereby decreasing its ability to wet the powder and solubilize the solutes [29]. On the other hand, if the evolution of the TFC according to the variation of both liquid-to-solid ratio and ultrasound extraction time with a fixed temperature equal to 308 K, the slight increase of TFC value caused by the variation of liquid-to-solid ratio will disappear leading to the formation of flat plot that highlighted the negligible effect of both time and liquid-to-solid ratio on TFC.

The predicted response surface showing the influence of extraction temperature (X_2) and liquid-to-solid ratio (X_3) on TFC, with a constant ultrasonic time is represented in Fig. 2b3. The resulting plot shape is mainly due to the negligible effect of the liquid-to-solid ratio. Only the decrease of temperature could increase the TFC, highlighting the strong negative significant effect of the linear term b_2. Values under 308 K could enhance TFC, whereas temperature higher than 308 K decreases the TFC. This may be due to the degradation of flavonoids under high temperature. Higher temperature increases solvent vapor pressure and decreases surface tension. Moreover, under certain temperature of above 308 K, the reactions such as internal redox reaction or polymerization and hydrolysis reactions can occur and cause the degradation of flavonoids [11]. Therefore, an appropriate temperature must be achieved to overcome the problem of bioactive degradation.

Figs. 2c1-c3 represent the 3-D plot response surfaces of DPPH antioxidant activity. Fig 2c1 presents the effect of ultrasound time and temperature on the DPPH antioxidant activity where liquid-to-solid ratio was kept constant. According to the plot, only ultrasound time can affect the DPPH antioxidant activity. This was mainly due to the positive significant effect of the quadratic term b_{11}. The contrary was observed with the effect of temperature that has a negligible effect on the response.

The influences of the liquid-to-solid ratio (X_3) and extraction time (X_1) on DPPH antioxidant activity is shown in Fig. 2c2. The liquid-to-solid ratio (X_3) has negligible effect on DPPH antioxidant activity, whereas time affects the DPPH antioxidant activity. Increasing ultrasonic extraction time above 35 min enhances the DPPH activity indicating that substances responsible for this activity were highly extracted in this time interval. Fig. 2c3 presents the effect of temperature and liquid-to-solid ratio with time kept constant. It is clear that both variables do not affect the DPPH antioxidant activity.

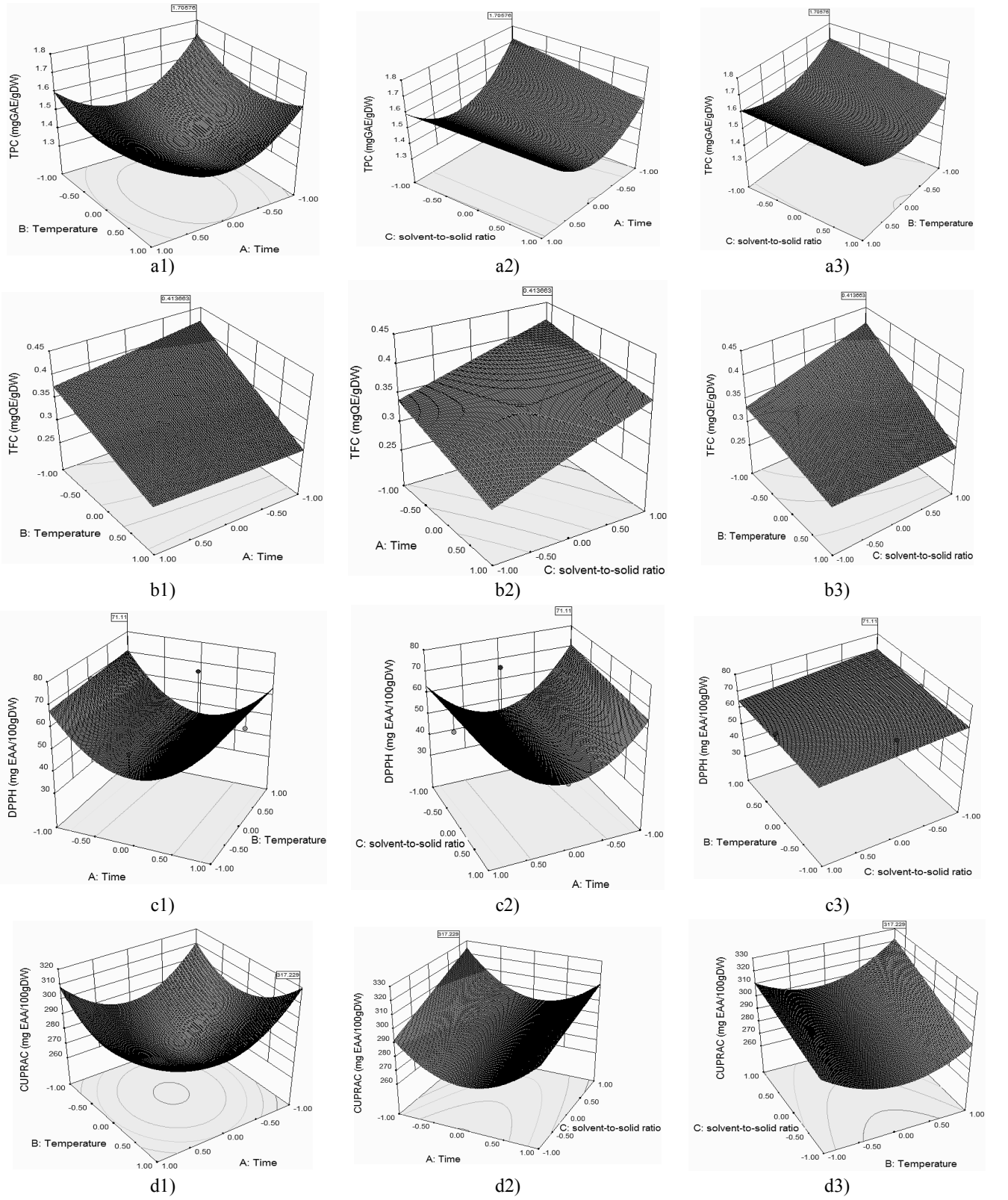

Fig. 2. Response surfaces for total phenolic content (a1-a3), total flavonoid content (b1-b3), DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity (c1-c3) and CUPRAC (cupric ion reducing antioxidant capacity) (d1-d3) in functions of temperature, time and liquid-to-solid ratio

Figs. 2d1-d3 represent the 3-D plot response surfaces of CUPRAC. Fig. 2d1 reflects the effect of the ultrasound extraction time and temperature. At 308 K the increase in the CUPRAC was observed with the decrease in ultrasound extraction time. Nevertheless, at 35 min, the increase in CUPRAC was observed with the increase of temperature.

Furthermore, as shown in Fig. 2d2, the increase in the liquid-to-solid ratio with the decrease of ultrasound extraction time significantly enhances the CUPRAC. This is mainly due to the linear and quadratic terms b_3 of liquid-to-solid ratio and b_{11} of ultrasound extraction time, respectively.

Generally, long-time exposure to ultrasound waves has a negative effect on some antioxidants since it can generate pro-oxidants such as $H^{\bullet}$ and $OH^{\bullet}$ radicals that reduces the CUPRAC. Other studies found that the content of some sensitive nutrients such as anthocyanin and ascorbic acid decreased by around 3.2 and 10 %, respectively, after the continuous ultrasound treatment.

The influence of liquid-to-solid ratio and temperature on the response of CUPRAC is shown in Fig. 2d3. The resulting shape is mainly due to the linear and quadratic terms b_3 and b_{22} of liquid-to-solid ratio and temperature. A significant increase in CUPRAC is due to the increased values of both liquid-to-solid ratio and temperature.

3.4. Verification of Predictive Models

The optimal conditions were predicted using design-expert prediction profiler in order to maximize the TPC, TFC, DPPH, and CUPRAC. This was done using multicriteria methodology based on the desirability function (D), also called Derringer function.

Results of maximized responses suggested that extraction for 30 min at 298 K with liquid-to-solid ratio equal to 74.4 ml/g is the best solution for this combination of variables. Using these optimal conditions, new extractions were submitted and results of TPC, TFC, DPPH, and CUPRAC were 1.634 ± 0.05 mgGAE/gDW, 0.309 ± ± 0.01 mgQE/gDW, 60.683 ± 3.06 mgEAA/100 gDW, and 293.342 ± 6.92 mgEAA/100 gDW, respectively, with p-value < 0.001.

These results are in agreement with the predicted values (TPC = 1.639 mgGAE/gDW, TFC = 0.354 mgQE/gDW, DPPH = 66.700 mg EAA/100gDW and CUPRAC = 314.417 mgEAA/100 gDW), where the mean absolute percentage error (MAPE) is equal to 8 %, emphasizing that the proposed model could be used to predict the response value.

3.5. Chromatographic Determination of Individual Phenolic Compounds

The characterization of phenolic compounds in optimum points (Table 3, Fig. 3) revealed that date fruit extract contains hydroxycinnamic acid derivatives such as caffeoyl glucoside-formic acid (peak 1) with molecular ion of peak = 387 ($C_{13}H_{23}O_{13}$) and base peak = 225, and their major MS^2 ion (100%) is at m/z 341 and 179, respectively, which indicates the loss of formic acid [5]. The major deprotonated ions (m/z 341 and 179) resulting from MS^2 show the base peaks in MS^3 = 179 with the loss of hexoside (162 Da) moiety ([M-H]-formic acid-hexoside)$^-$ and m/z = 143 with the loss of two molecules of water ([M-H]-formic acid-2×H_2O)$^-$ [5].

Compound 2 exhibited a deprotonated molecular ion $[M-H]^-$ = 225 and displayed the fragmented loss of −46 Da (formic acid adduct ion) in its MS^2 fragmentation plus 36 Da (-2×H_2O) in its MS^3 fragmentation. Based on this arguments compound 2 can be characterized as caffeic acid-formic acid [30].

Table 3

HPLC–ESI$^-$/MS^n analysis of phenolic compounds in optimum conditions

Peak	t_R, min	[M-H]$^-$, m/z	Major MS^2 ions	Major MS^3 ions	Tentative identification
1	2.62	225 387	179 341	143 179	Caffeoylglucoside-formic acid
2	3.24	225	179	143	Caffeic acid-formic acid
3	9.16	210	124	106	Unknown
4	9.59	335	179	135	Caffeoylshikimic acid
5	9.93	449	Nd	Nd	Ferulic acid-*o*-hexoside derivative
6	10.06	335	179	135	Caffeoylshikimic acid
7	10.85	163	119	87	*Para*-coumaric acid
8	11.57	609	301	179	Rutin
9	11.96	463	301	179	Quercetin 7-glucoside
10	12.08	435	Nd	Nd	Phloretin-O-hexoside (Phloridzin)
11	12.80	565	Nd	Nd	Caffeic acid-O-(sinapoyl-O-hexoside)
12	13.33	653	585	Nd	Unknown
13	15.85	285	285	285	Luteolin

Note: Nd means not detected

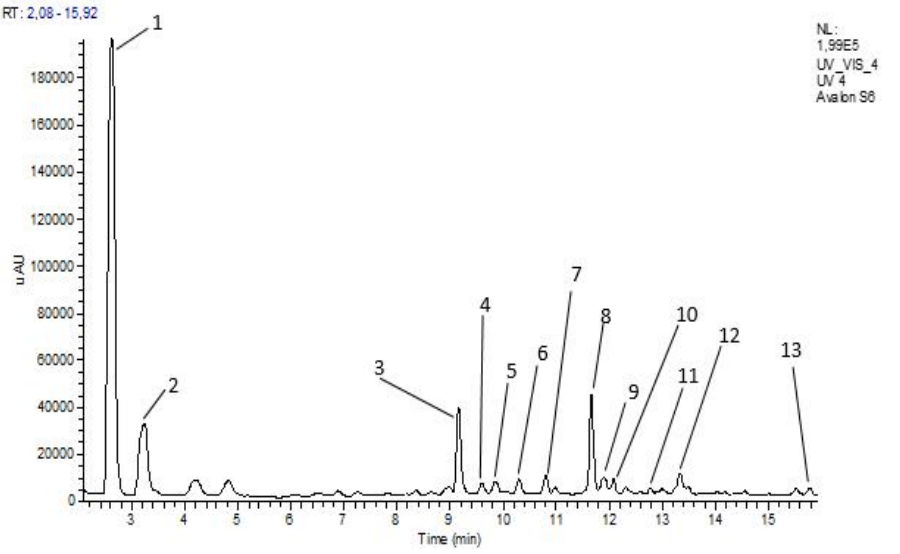

Fig. 3. UHPLC chromatogram of date fruit extract in optimum conditions (280 nm)

Compounds 4 and 6 with deprotonated molecular ion at m/z = 335 were characterized as caffeoyl-shikimic acid; compounds 5, 10 and 11 with molecular ions at m/z = 449, 435 and 565 are ferulic acid-*o*-hexoside derivative, phloretin-O-hexoside (Phloridzin) and caffeic acid-O-(sinapoyl-O-hexoside) [5]. The resulting compound contains three isomers which can be formed by the esterification of caffeic acid at three positions 3, 4 or 5 on shikimic acid [30]. These three isomers do not vary in their MS/MS spectra and their resulting ion peaks in MS^n are the same but their intensities were different. The MS^2 base peak at m/z = 179 is due to the neutral loss of CO_2 (44 Da) and the loss of cyclohexa-4-ene-1,2-diol (111 Da) by β-elimination ($[M-H]-44-C_9H_7O_4]^-$), MS^3 base peak at m/z = 135 results from the loss of 44 Da (loss of CO_2) [30]. In view of the above results, isomers were tentatively assigned as 3-, 4- and 5-caffeoyl shikimic acids.

The deprotonated *p*-coumaric acid (peak 7) showed a base peak at m/z = 163, the loss of 44 Da (loss of CO_2) was observed to give place to the MS^2 at m/z = 119 [31]. Thus, its identification was confirmed as being *p*-coumaric acid.

The analysis also revealed the presence of flavonoids – both rutin (compound 8) and quercetin 7-glucoside (compound 9). Their base peaks were 609 and 463, respectively. This was confirmed by the fragment signals at m/z = 301 and 179 corresponding to the loss of different glycosides moieties [30, 32]. Peak 13 was identified as luteolin, its MS spectra showed $[M-H]^-$ at m/z = 285 [33].

4. Conclusions

To conclude, the ultrasound extraction process was successfully applied to optimize the conditions for the extraction of antioxidant compounds from date fruit. Results of maximized responses revealed that extraction for 30 min at 298 K and with liquid-to-solid ratio of 74.4 ml/g is the best solution for this combination of variables. The model used for optimizing the TFC was not quadratic, only the linear regression coefficient of temperature influences the TFC. Under this optimal condition, the yield of TPC, TFC, DPPH, and CUPRAC increased significantly, indicating that the second-order polynomial model could be used to predict the response value.

In this study, UHPLC-DAD-MS/MS was used to separate and identify or tentatively characterize 11 compounds in the extract of date fruit at optimum point. The results indicate that date fruit could be considered as source of bioactive phenolic compounds. This study can be useful to promote industrial extraction of date antioxidants.

References

[1] Chao C., Krueger R.: HortSci., 2007, **42**, 1077. https://doi.org/10.21273/HORTSCI.42.5.1077
[2] Al-Alawi R., Al-Mashiqri J., Al-Nadabi J. *et al.*: Front. Plant Sci., 2017, **8**, 1. https://doi.org/10.3389/fpls.2017.00845
[3] http://www.fao.org/faostat/en/#data
[4] Moussouni S., Pintaud J., Vigouroux Y., Bouguedoura N.: PLoS One, 2017, **12**. https://doi.org/10.1371/journal.pone.0175232
[5] Hachani S., Hamia C., Boukhalkhal S. *et al.*: NFS J., 2018, **13**, 10. https://doi.org/10.1016/j.nfs.2018.10.001
[6] Djouab A., Benamara S., Gougam H. *et al.*: Emirates J. Food Agric., 2016, **28**, 601. https://doi.org/10.9755/ejfa.2015-12-1056
[7] Mansouri A., Embarek G., Kokkalou E., Kefalas P.: Food Chem., 2005, **89**, 411. https://doi.org/10.1016/j.foodchem.2004.02.051
[8] Zineb G., Boukouada M., Djeridane A. *et al.*: Med. J. Nutrition Metab., 2012, **5**, 119. https://doi.org/10.1007/s12349-011-0082-7

[9] Messaoudi R., Abbeddou S., Mansouri A. *et al*.: Int. J. Food Prop., 2013, **16**, 1037. https://doi.org/10.1080/10942912.2011.576355
[10] Benkerrou F., Bachir bey M., Amrane M., Louaileche H.: J. Food Meas. Charact., 2018, **12**, 1910. https://doi.org/10.1007/s11694-018-9805-5
[11] Bamba B., Shi J., Tranchant C. *et al*.: Molecules, 2018, **23**, 1685. https://doi.org/10.3390/molecules23071685
[12] Pingret D., Fabiano-Tixier A., Chemat F.: Food Control, 2013, **31**, 593. https://doi.org/10.1016/j.foodcont.2012.11.039
[13] Alberti A., Zielinski A., Zardo D. *et al*.: Food Chem., 2014, **149**, 151. https://doi.org/10.1016/j.foodchem.2013.10.086
[14] Bezerra M., Santelli R., Oliveira E. *et al*.: Talanta, 2008, **76**, 965. https://doi.org/10.1016/j.talanta.2008.05.019
[15] Singleton V., Rossi J.: Am. J. Enol. Viticult., 1965, **16**, 144.
[16] Lamaison J., Carnat A.: Pharm. Acta Helv., 1990, **65**, 315.
[17] Brand-Williams W., Cuvelier M., Berset C.: LWT-Food Sci. Technol., 1995, **28**, 25. https://doi.org/10.1016/S0023-6438(95)80008-5
[18] Apak R., Özyürek M., Güçlü K. *et al*.:Chapter 24 – The CUPRAC Methods of Antioxidant Measurement for Beverages [in:] Preedy V. (Ed.), Processing and Impact on Antioxidants in Beverages. Academic Press 2014, 235-244. https://doi.org/10.1016/B978-0-12-404738-9.00024-6
[19] Nandi P., Parsad R., Gupta V.: Open J. Stat., 2015, **5**, 430. https://doi.org/10.4236/ojs.2015.55045
[20] Mokrani A., Madani K.: Separ. Purif. Technol., 2016, **162**, 68. https://doi.org/10.1016/j.seppur.2016.01.043
[21] Zhao Z.-Y., Zhang Q., Li Y.-F. *et al*.: Carbohydr. Polym., 2015, **119**, 101. https://doi.org/10.1016/j.carbpol.2014.11.052
[22] Sharma K. *et al*.: J. Food Drug Anal., 2015, **23**, 243. https://doi.org/10.1016/j.jfda.2014.10.005
[23] Ben Ahmed Z. *et al*.: Anal. Methods, 2016, **8**, 6107. https://doi.org/10.1039/C6AY01739H
[24] Quanhong L., Caili F.: Food Chem., 2005, **92**, 701. https://doi.org/10.1016/j.foodchem.2004.08.042
[25] Yolmeh M., Habibi Najafi M., Farhoosh R.: Food Chem., 2014, **155**, 319. https://doi.org/10.1016/j.foodchem.2014.01.059
[26] Akalin M., Tekin K., Akyüz M., Karagöz S.: Ind. Crops Prod., 2015, **76**, 829. https://doi.org/10.1016/j.indcrop.2015.08.005
[27] Abbas F. *et al*.: Ind. Crops Prod., 2013, **44**, 634. https://doi.org/10.1016/j.indcrop.2012.09.008
[28] Cárdenas A., Gomez M., Frontana C.: Procedia Chem., 2014, **12**, 62. https://doi.org/10.1016/j.proche.2014.12.042
[29] Jentzer J., Alignan M., Vaca-Garcia C. *et al*.: Food Chem., 2015, **166**, 561. https://doi.org/10.1016/j.foodchem.2014.06.078
[30] Said R. *et al*.: Int. J. Mol. Sci., 2017, **18**, 512. https://doi.org/10.3390/ijms18030512
[31] Ibrahim R. *et al*.: Rev. Bras. Farmacogn., 2015, **25**, 134. https://doi.org/10.1016/j.bjp.2015.02.008
[32] Farag M., Mohsen M., Heinke R., Wessjohann L.: Food Res. Int., 2014, **64**, 218. https://doi.org/10.1016/j.foodres.2014.06.021
[33] Adetunji A., Duodu K., Taylor J.: Food Chem., 2015, **175**, 225. https://doi.org/10.1016/j.foodchem.2014.11.102

Received: January 28, 2019 / Revised: February 07, 2019 / Accepted: April 19, 2019

ВИКОРИСТАННЯ МЕТОДОЛОГІЇ ПОВЕРХНІ ВІДГУКУ ЯК НОВІТНЬОГО ПІДХОДУ ДО ОПТИМІЗАЦІЇ ВМІСТУ ФЕНОЛІВ ТА АНТИОКСИДАНТНОЇ АКТИВНОСТІ ПЛОДІВ ФІНІКОВОЇ ПАЛЬМИ

Анотація. *За допомогою факторного плану Бокса-Бенкена досліджено вплив трьох незалежних змінних – часу, температури та співвідношення розчинник-тверда речовина на очисну активність фенолів, флавоноїдів і 2,2-дифеніл-1-пірилгідразилу (DPPH) та зниження антиоксидантної активності йону міді (CUPRAC) метанольних екстрактів фінікових плодів. Аналіз поверхні відгуку показав, що оптимальні параметри ультразвукової екстракції, які максимізували відповіді, становили 30 хв, 298 K і 74,4 мл/г. За допомогою методу ультра-високоефективної рідинної хроматографії і тандемної мас-спектрометрії (UHPLC-DAD-MS/MS) за оптимальних умов визначено орієнтовні характеристики 11 фенольних сполук. Експериментально доведено, що кількість і антиоксидантна активність фенольних сполук відповідають прогнозованим значенням, що вказує на придатність моделі та успіх методології поверхні відгуку для оптимізації умов ультразвукової екстракції.*

Ключові слова: *плоди фінікової пальми, поліфеноли, ультразвукова екстракція, методологія поверхні відгуку, антиоксидантна активність, UHPLC-DAD-MS/MS.*