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Dahmoune Farid
Université de Béjaïa

Hocine Remini
Université de Béjaïa

Aoun Omar
Université de Béjaïa

Khodir Madani
Université de Béjaïa

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Optimization of Ultrasound-Assisted Extraction of Phenolic Compounds from *Citrus sinensis* L. Peels using Response Surface Methodology

Farid Dahmoune[a], Kamal Moussi[a], Hocine Remini[a], Amine Belbahi[a], Omar Aoun[a], Giorgia Spigno[b], Khodir Madani*[a]

[a]Laboratory of biomathematics, biochemistry, biophysics and scientometrics, Abderrahmane mira university of Bejaia, Bejaia 06000, Algeria
[b] Institute of Oenology and Food Engineering, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29122 Piacenza, Italy
madani28dz2002@yahoo.fr

The aim of this study was the optimization, by Response Surface Methodology (RSM), of Ultrasound Assisted Extraction (UAE) conditions for the recovery of phenolic compounds from C. sinensis peels with aqueous acetone at room temperature. A Central Composite Rotatable Design (CCD) was applied to determine the effects of extraction time (X1, 5-15 min), extraction amplitude (X2, 30-70%), and acetone concentration (X3, 30-70 %) on Total Phenolic Content (TPC expressed as Gallic Acid Equivalents GAE, according to the Folin's assay) of the extract. The independent variables were coded at five levels and their actual values were selected based on the results of single factor experiments. Results showed that acetone concentration and extraction amplitude were the most significant ($p<0.05$) factors affecting the TPC yield. The optimum extraction conditions were found to be 8.33 min extraction time, 65.94 % extraction amplitude and 75.79 % acetone concentration. Under the optimized conditions, a TPC yield of 13.57±0.71 mgGAE/g_{dw} was obtained which resulted very close to the predicted value of 14.16 mgGAE/g_{dw}.

1. Introduction

Extraction of natural phenolic compounds from agro-food by-products has attracted both academic and industrial interest (Dahmoune et al., 2013). Citrus is an important crop mainly used in food industries for fresh juice production. Peels, the main waste fraction of citrus fruits, represent roughly half of the fruit mass and have been widely studied because they contain numerous biologically active compounds including natural antioxidants such as phenolic acids and flavonoids (Dahmoune et al., 2013). Due to the worldwide diffusion of citrus crops, valorization of their by-products for the recovery of bioactive compounds with potential diversified applications (food, cosmetic, pharmaceutical and material sector) can represent an important source of additional income for the producers allowing, at the same time, the production at reduced costs of bioactive based products. Industrial large scale implementation of such recovery strategies requires development of cost-effective process. Emerging technologies based on ultrasounds assistance has been increasingly investigated for this purpose. This work demonstrated the possibility of exploiting ultrasound irradiation to reduce extraction time and solvent use to obtain good extraction yield thanks to a damaging effect on the vegetable tissue, preserving at the same time the antioxidant activity of the recovered compounds.

2. Materials and methods

2.1 Plant material

The fruit samples of *C. sinensis* were collected in the area of Oued Ghir (Bejaia, Algeria), washed with distilled water and peeled off with hands. Peels were dried in oven for a week at 40 °C. Dried peels were

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ground with an electrical grinder (IKA model A11Basic, Germany), the powder was passed through standard 125 μm sieve and only the fraction with particle size <125 μm was used. The powder was stored in airtight bags until use. The water activity (aw) was determined by HygroPalm AW and was 18 ± 0.2 %rh at 20.6 °C.

3. CCD assay

On the basis of the single-factor experimental results, critical influencing factors were confirmed, and then a CCD design with response surface methodology was conducted to design the experimental project (Yang et al., 2010). In the investigation, a 2^3 full factorial with augment design (CCD) was employed to fit a second order polynomial model which indicated 18 experiments to be required for this procedure. The general equation of the second degree polynomial equation is:

$$Y = B_0 + \sum_{i=1}^{k} B_i x_i + \sum_{i=1}^{k} B_{ii} x^2 + \sum_{i>j}^{k} B_{ij} x_i x_j + E \qquad (1)$$

Where Y represents the response function (in our case the TPC yield); B0 is a constant coefficient; Bi, Bii and Bij are the coefficients of the linear, quadratic and interactive terms, respectively, and xi and xj represent the coded independent variables. According to the analysis of variance, the regression coefficients of individual linear, quadratic and interaction terms were determined. In order to visualize the relationship between the response and experimental levels of each factor and to deduce the optimum conditions, the regression coefficients were used to generate 3D surface and contour plots from the fitted polynomial equation.

The factor levels were coded as −1 (low), 0 (central point or middle) and 1 (high), respectively. The variables were coded according to the following equation Eq(2):

$$x_i = \left(X_i - X_0\right) / \Delta X \qquad (2)$$

Where xi is the (dimensionless) coded value of the variable Xi; X0 is the value of X at the center point and ΔX is the step change.

4. Ultrasound Assisted Extraction

Extraction of natural products using ultrasound has been proposed to improve the efficiency and/or speed of this step. The pulses emitted by the ultrasound probe can often getting a higher yield because they promote breaking cellulose cell walls. An ultrasonic apparatus is used for UAE with working frequency fixed at 20 kHz. The energy input was controlled by setting the amplitude of the sonicator probe. For the extraction, one gram of fine powder was placed in a 250 mL amber glass bottle (Ø x H: 45 mm × 140 mm and cap size of 28 mm) containing water–acetone mixture; the obtained suspension was exposed to acoustic waves for varying time, acetone concentration and amplitude (Table 1). The temperature (27±2 °C) was controlled continuously by circulating external cold water and checking the temperature using a T-type thermocouple. After extraction, the extract was recovered by filtration in a Büchner funnel through Millipor 45 Micron filter paper, and collected in a volumetric flask. The extract was stored at 4 °C until use and analyzed for Total phenolic content determined according to the Folin–Ciocalteu assay (Jaramillo-Flores et al., 2003).

5. Results and discussion

In general, optimization of a process could be verified by either empirical or statistical methods; the former having limitations toward complete optimization. The traditional one-factor-at-a-time approach to process optimization is time consuming. Moreover, the interactions among various factors may be ignored hence the chance of approaching a true optimum is very unlikely.

According to the method of Central Composite designed experiment and the levels of independent variables were chosen based on the values obtained in the single factor experiment, extraction time (X1, min), extraction amplitude (X2, %) and acetone concentration (%, v/v) which have a great effect on the extraction rate of polyphenols, were selected as design variables in the RSM, the extraction rate of polyphenols (Y, mg GAE/g) was employed as a response value, and the three factors and five levels' RSM test were designed (Table 1).

Table 1 shows the experimental conditions and the results of extraction yield of TPC according to the factorial design.

Table 1: Central composite design with the observed responses for yield of total phenolic compounds of C. sinensis peels using UAE method

X1-Time	X2-Amplitude	X3-Solvent	TPC Yield (mgGAE/g)
5	30	30	10.60
15	30	30	12.18
5	70	30	12.51
15	70	30	12.37
5	30	70	13.40
15	30	70	13.79
5	70	70	13.63
15	70	70	13.37
10	50	50	13.16
10	50	50	13.82
10	50	50	13.85
10	50	50	13.05
17.05	50	50	12.71
2.95	50	50	12.69
10	78.2	50	14.16
10	21.8	50	13.06
10	50	78.2	14.37
10	50	21.8	11.87

The final mathematical models are expressed by Eq(3) represents TPC yields of *C. sinensis* (y) as a function of extraction time (X1), amplitude (X2) and acetone concentration (X3): $Y(TPC\ yield) = 10.44 + 0.02X_2 + 0.05X_3 - 0.003X_1X_2 - 0.0007X_2X_3 - 0.02X_1^2$ (1)

From Table 2, it can be seen that irradiation time (X1) did not influence the extraction yield (p>0.05) but its interaction with amplitude did it (p<0.05). The interaction amplitude and solvent was significant (p<0.05).The quadratic effects of amplitude and solvent were insignificant (p>0.05) while their linear effects were significant (p < 0.05) and the tendency was reversed for time effect. The recovery of TPC by using acoustic energy was found to be a function of the linear and interaction effect.

Table 2: Analysis of variance (ANOVA) for the experimental results obtained by using ultrasound assisted extraction.

Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	*p*-value Prob > F
Model	15.85	9	1.50	12.54	0.0008
X_1-Time	0.21	1	0.21	1.78	0.2186
X_2-Amplitude	1.00	1	1.00	8.35	0.0201
X_3-Solvent	8.44	1	8.44	70.52	0.0001
X_1X_2	0.70	1	0.70	1.79	0.0417
X_1X_3	0.21	1	0.21	5.48	0.2174
X_2X_3	0.66	1	1.66	14.62	0.0474
X_1^2	1.75	1	1.75	0.01	0.0050
X_2^2	0.001	1	0.001	4.42	0.9255
X_3^2	0.53	1	0.53	2.98	0.0686
Lack of Fit	0.58	5	0.12	0.46	0.7875
Pure Error	0.54	3	0.27		
Cor Total	17.38	17			

In fact, in ultrasound procedure, it must be underlined that these variables could be affecting the release of phenols from different matrix, which are able to modify equilibrium and mass transfer conditions in the solvent extraction and affect rupture of cell wall (Dahmoune et al., 2013). Table 2 shows the results of analysis of variance for the fitting model. Values of probability (P)>F less than 0.05 and 0.01 indicate that model terms are significant and highly significant respectively, and the values greater than 0.05 indicate that the model terms are not significant. From the Table 2, the ANOVA reveals that the model was highly significant for response surface model at p-value < 0.01, which means that the model represented the data satisfactorily. There is only a 0.01 % chance that Models F-value this large could occur because of noise. Furthermore, the values of pure error (0.54) are low which indicate good reproducibility of the data. The coefficients of determination (0.93) and adjusted coefficients (0.84) are close to 1, which revealed also, that there are excellent correlations between the predicted and experimental models. In addition, the small values of % CV than 10 (3.07) and higher values of adequate precision than 4 (12.75) for extraction yield give better reproducibility and adequate signal. In conclusion, these models can be used to navigate the design space.

5.1 Optimization of extraction conditions of TPC

The graphical representations of the regression Eq.1, called the response surfaces and the contour plots were obtained using JMP software version 7.0, and the results of extraction yield of TPC from *C. sinensis* affected by extraction time, amplitude and acetone concentration were presented in Figure. 1a and 1b. Response surface methodology plays a crucial role in identifying the optimum condition of the output variables efficiently, under which dependent variable could reach the maximum response. In the response surface plot (Figure 1a) and isoresponse curves (Figure 1b), the extraction yield of TPC was obtained along with two continuous variables, while the other two independent variables were fixed constant at their intermediate levels (center value of the testing ranges). The profile of the response surface and isoresponse curves shows a strong interaction between these tested variables. From these three-dimensional profiles, it is easy to see the interaction effects between any two independent factors.

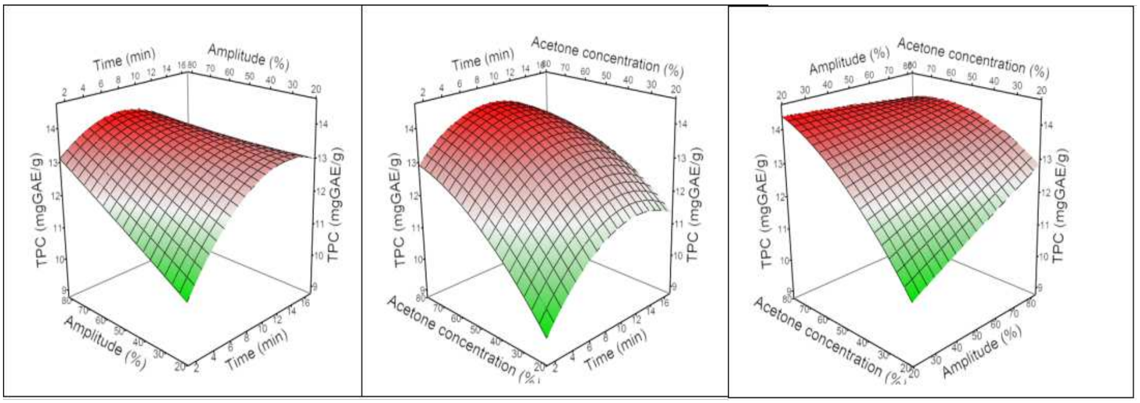

Figure 1a. Response surface analysis for the total phenolic yield from C. sinensis peels with ultrasound assisted extraction with respect to extraction time and amplitude; irradiation time and acetone percentage; amplitude and acetone concentration

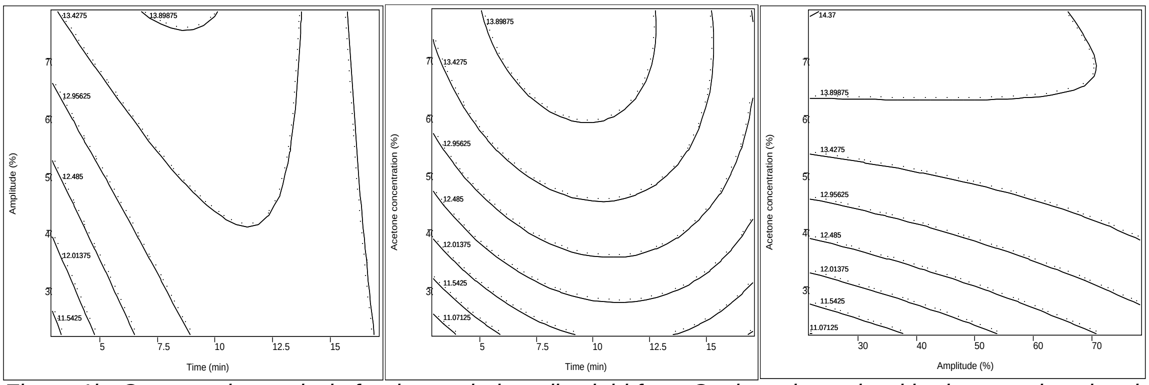

Figure 1b. Contour plot analysis for the total phenolic yield from C. sinensis peels with ultrasound assisted extraction with respect to extraction time and amplitude; irradiation time and acetone percentage; amplitude and acetone concentration.

Using the derived model Eq.1, the optimal UAE conditions for the TPC yield were listed on Figure 2 with a desirability of 0.9.

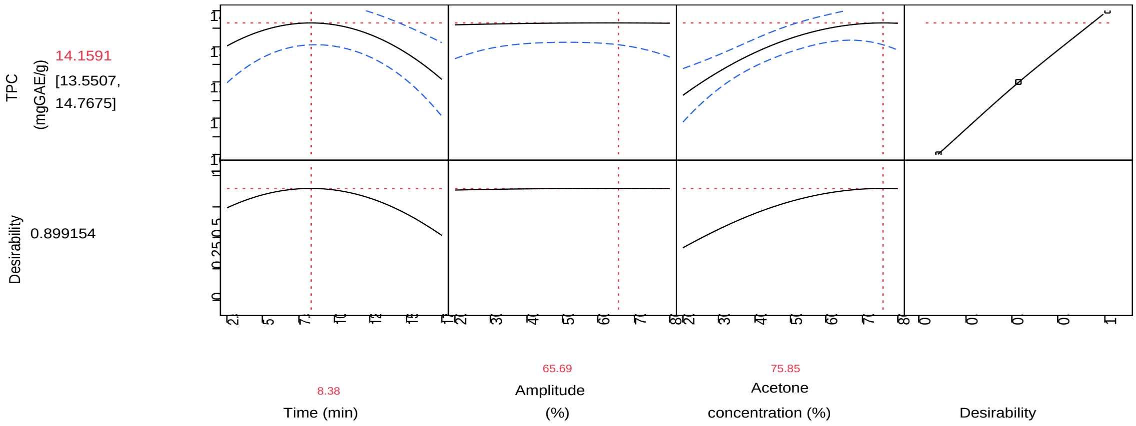

Figure 2: Prediction profiler for the optimal conditions obtained by CCD model

6. Validation of the developed models

Through these 3D plots and their respective contour plots, the final step of the RSM after selecting the optimum parameter combination is to predict and verify the improvement of the performance characteristics with the selected optimum parameters. In this work, after determining the optimum conditions and predicting the response 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 are shown in Table 3, the strong correlation between the actual (experimental) and predicted results confirm the effectiveness of the response surface models to reflect the expected optimization.

Table 3: Optimum conditions predicted and experimental value of response under those conditions

Optimum conditions			TPC Yield (mg GAE/g DW)	
Extraction time (min)	Ampitude (%)	Acetone Concentration (%)	Experimental	predicted
8.33	65.94	75.79 %	13.57±0.71	14.16

Furthermore, the adequacy of the model has been investigated also by the examination of residuals l. Analysis of residuals appears to be a very useful and remarkably simple tool in model building and model criticism. The residuals play an important role in judging model adequacy. The residuals from the TPC regression model are shown in Figure 3, which presents plot residuals (e) versus the predicted response (ý). A random pattern on these plots would indicate model adequacy (van Boekel, 1999)**.**

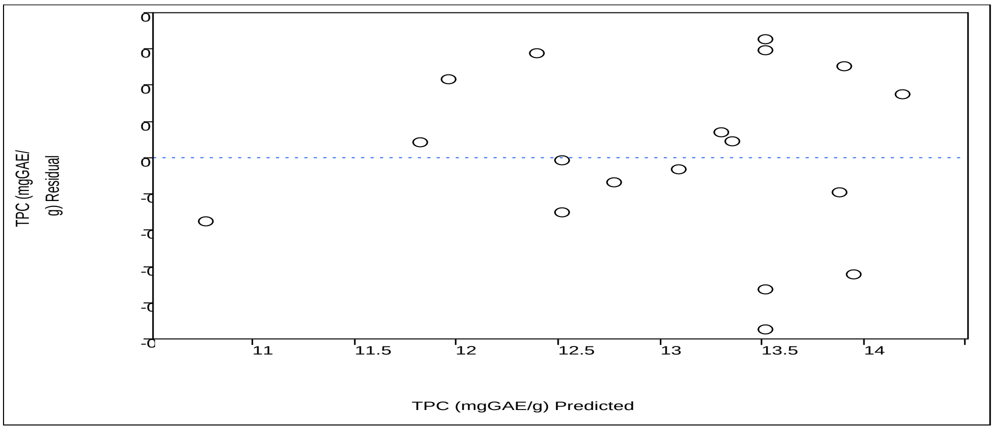

Figure 3: A residual plot for ultrasound assisted extraction method obtained by RSM

7. Conclusions

This present study indicates that *C. sinensis* L. peels can be considered as a good source of phytopharmaceutical interest compounds. UAE was optimized through a RSM based approach and the optimal conditions are 8.33 min as irradiation time, 65.94 % as amplitude and 75.79 % (v/v)aqueous acetone solvent. Under these conditions, a TPC yield of 13.57±0.71 mgGAE/g_{dw} was obtained which resulted very close to the predicted value of 14.16 mgGAE/g_{dw}.

Anyway, from the point of view of industrial application, this research could be the basis for further pilot-plant trials of UAE as a green extraction technology for the recovery of high-added value compounds from plant material. In addition, mathematical models are required to optimize and predict the process in order to modify the classical extraction methods". However, the standardized equations used in RSM have no methodological background. Consequently, the designed model can only be used within the experimental range and cannot be used for extrapolation.

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