Optimization of ultrasound and microwave-assisted extraction of phenolic compounds from olive leaves by response surface methodology

Hédia Hannachi[1*], Hasnia Benmoussa[2], Ezzedine Saadaoui[3], Ines Saanoun[4], Néjia Negri[4] and Walid Elfalleh[5]
1. Laboratoire de productivité végétale et contraintes environmentales (LR18ES04), Faculté des Sciences de Tunis, Université de Tunis El Manar, TUNISIA
2. Laboratoire d'Ingénierie des Procédés de l'Environnement, Faculté de Chimie, Université des Sciences et de la Technologie d'Oran-Mohamed BOUDIAF, Oran, 31000, ALGERIA
3. University of Carthage, National Institute of Research in Rural Engineering, Waters and Forests (INRGREF), LGVRF, Regional Station of Gabès, TUNISIA
4. Institut Supérieur des Sciences Appliquées et de Technologie de Gabès, Université de Gabès, TUNISIA
5. Laboratoire Energie, Eau, Environnement et Procèdes, (LEEEP), LR18ES35, Ecole Nationale d'Ingénieurs de Gabès, Université de Gabès, TUNISIA
*hannachi_hedia@yahoo.fr

Abstract
Plant secondary metabolites, phenolic compounds have been *extensively studied and are commonly used as antioxidants for a* wide range of applications. *The response surface methodology (RSM) based on a central composite design was applied to determine the optimum conditions for the ultrasound (UAE) and microwave-assisted extractions (MAE) of polyphenol and flavonoid components from olive leaves (Olea europaea L.). For UAE, two operational variables were evaluated: liquid/solid ratio (l/s ratio) (4, 12 and 20) and the extraction time (10, 25 and 40 min). For MAE, three process variables were evaluated: liquid/solid ratio (4, 12 and 20), the extraction time (1, 3 and 5 min) and the power (100, 200 and 300W). The total polyphenol content (TPC) and total flavonoid content (TFC) were significantly influenced by liquid/solid ratio. In fact, the RSM applied for UAE showed that 37.07 of the liquid/solid ratio and the time of 28.69 min were the optimum conditions.*

The RSM applied on MAE showed the optimal extraction conditions obtained by using liquid/solid ratio of 16.76, extraction time of 1.81 min and the power of 259.46 W. The MAE represented an interesting alternative protocol for rapid, economic and high eco-extraction using water as green solvent.

Keywords: Optimization, Response surface methodology, Microwave, Ultrasound, Polyphenols contents, Flavonoids contents**.**

Introduction

The *Olea europaea* L. Var. *europaea* comprising the cultivated olive trees or cultivars[9] is widely cultivated in the Mediterranean basin. The olive trees have been used widely in folk medicine and olive oil is commonly used in food and in cosmetic formulations[14]. The antioxidant properties of the olive extracts have recently been of great interest in both academia and food industries because of their radical scavenging potentials[13,16]. The phenols components extracted from olive leaves were used as an additive to improve the olive oil stability. However, the extraction of bioactive components varied according to various factors including the type of solvent, temperature, extraction time, number of extraction steps and the liquid/solid ratio[15].

The extraction parameters selected must avoid their chemical modification. Depending upon the stability and nature of the phenolic compounds, different solvent and extracting procedures are used[1, 25]. The traditional extraction methods of polyphenols are associated with long extraction time. However, the innovated extraction methods would be economic and rapid as the ultrasound assisted extraction (UAE) and microwave-assisted extractions (MAE).

In the classical optimization of extractions, only one factor is variable. This technique is expensive and failed to determine the interaction effects between variables affecting extraction process. Response surface methodology (RSM) is an efficient method to optimize experimental conditions and to evaluate the effects of multiple factors and their interactions on one or more response variables. RSM can effectively be used to find a combination of factor levels that produce an optimum response[17,24]. It has been used for optimization of various food processing methods such as extraction and fermentation[3]. The response surface methodology (RSM) as a collection of statistical and mathematical techniques is useful optimizing processes[18] as polyphenols extraction[12].

Hence, the aim of this research was to investigate the optimization of the polyphenols and flavonoids contents during UAE and MAE based on RSM approach using water as extraction solvent in order to optimize an eco-method of extraction for food industry or pharmacology uses.

Material and Methods

Plant material: The olive leaves were collected in February 2016 from the 'Chemlali' cultivar cultivated in Gabes, southern of Tunisia. The plant samples were identified by Dr. Ezzeddine Saadaoui (INRGREF, Gabes, Tunisia). They were dried and crushed into powder using a laboratory mill (type FW135).

Chemicals and reagents: Gallic acid, rutin and Folin-Ciocalteu's phenol reagent were purchased from Sigma Chemical Co. (USA). The water used in sampling was prepared with a Millipore Simplicity (Millipore S.A.S., Molsheim, France). Spectrophotometric measurements were performed on a T60 UV- Visible spectrophotometer.

Extraction methods: The UAE was applied using the ultrasonic bath (clean-120hd) under temperature of 40°C. Dried olive leaves were used to extract polyphenols and flavonoid components using the ultrasound extraction method under the variation of two operational factors: liquid/solid ratio (4, 12 and 20) and the extraction time (10, 25 and 40 min). The response variables were evaluated at three levels (13 experimental designs).

The MAE process was of olive leaves polyphenols and flavonoids under three operational factors: liquid/solid ratio (4, 12 and 20), extraction time (1, 3 and 5 min) and microwave powers (100, 200 and 300 W). The response variables were evaluated at three levels (20 experimental designs).

In each case, the olive leaves extracts was removed by centrifugation at 4000 rpm for 15 min. Then it was filtered with Whatmann paper filter. Thus, the leave extracts were obtained, kept in dark glass at 4°C for polyphenols and flavonoids analyses.

Total polyphenol content (TPC): TPC was estimated by the Folin-Ciocalteu method[10,19]. From each 0.1 ml of olive leaves extract, 0.5 ml of Folin- Ciocalteu (Prolabo) reagent was added. After 5 min, 4 ml of sodium carbonate (1 M) solution was added to the mixture solution. The tubes were laid for 90 min at room temperature in darkness. Absorbance was measured at 765 nm. Gallic acid was used to make a calibration curve. TPC was expressed as mg gallic acid equivalents per 100 g dry weight (mg GAE/100g DW).

Total flavonoid content (TFC): TFC were measured spectrophotometrically[6, 10, 11] based on the formation of a complex flavonoid–aluminium, having the maximum absorbance at 430 nm. Rutin was used to make a calibration curve. 1 ml of each olive leaves extract was mixed with 1 ml of $NaNO_2$ sodium nitrite 0.5 M and 150 µl $AlCl_3$ 0.3 M. After incubation at room temperature for 15 min, the absorbance of the reaction mixture was measured at 430 nm. Total flavonoid contents were expressed as rutin equivalents in mg per 100 g dry weight (mg RE/100 g DW).

Experimental design using RSM

Ultrasound-assisted extraction (UAE): The RSM was applied based on a central composite design (CCD) as a function of ultrasound-assisted extraction (UAE) using two factors: X_1 as the liquid/solid ratio (4, 12 and 20) and X_2 as the extraction time (10, 25 and 40 min). A total of 13 experiments were conducted in CCD, four factorial points, four axial points and five replicates of the central points (Table 1). The response variables were the total polyphenol (TPC) and total flavonoid (TFC) contents of olive leaves (Table 2).

The mathematical model corresponding to the central composite design is represented by second-order polynomials:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 + \varepsilon$$

where Y is the response variables (TPC and TFC), x_1 and x_2 are the factors respectively, the liquid/solid ratio and the time of extraction (min), β_0 is a constant fixing the response at the central point of the experiment, β_1 and β_2 are regression coefficients of linear effects and β_{11} β_{22} represent regression coefficients of quadratic effect, β_{12} is the interaction effect of the factors and ε is the pure error.

Microwave-assisted extraction (MAE): The RSM was applied based on CCD as a function of microwave-assisted extraction. Three factors were used: x_1 as the liquid/matter ratio (4, 12 and 20 ml/g), x_2 as the extraction time (1, 3 and 5 min) and x_3 as the microwave power (100, 200 and 300W). A total of 20 experiments were conducted in CCD, eight factorial points, six axial points and six replicates of the central points (Table 3). The response variables were the polyphenols (PC) and flavonoid (FC) contents of olive leaves (Table 4).

The mathematical model corresponding to the central composite design is represented by a second-order polynomials:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \varepsilon$$

where Y is the response variables, x_1, x_2, x_3 are the factors respectively, the liquid/solid ratio, the time of extraction (min) and the power (W), β_0 is a constant fixing the response at the central point of the experiment, β_1, β_2, β_3 represent regression coefficients of linear effects and β_{11}, β_{22}, β_{33} represent regression coefficients of quadratic effect, β_{12}, β_{13}, β_{23} are the interaction effects of the factors and ε is the pure error.

Statistical analysis: In RSM, natural factors are transformed into coded factors that have been defined as dimensionless with a mean zero and the standard deviation[18].

The polyphenols and the flavonoids compounds were obtained according to the recommended optimum conditions using RSM based on the CCD. The RSM predictive equations were used to determine optimal conditions for the highest extraction contents of polyphenols and flavonoids from olive leaves. Statistical tests were conducted to verify the accuracy of statistical experimental design. The results of experimental design were fitted by a second-order polynomial equation in order to correlate the response variables to the independent factors.

The software Design-Expert (version 10, Stat-Ease Inc., Minneapolis, USA) was used to assess the RSM-CCD results. The linear, quadratic and interaction effects of the investigated factors on the extraction of polyphenols and flavonoids were evaluated. Their significance was evaluated by analysis of variance (ANOVA).

Results and Discussion

Process optimization of ultrasound-assisted extraction (UAE): Two factors were used to optimize the UAE of polyphenols and flavonoids contents, the liquid/solid ratio (x_1) and the time of extraction (x_2). The TPC varied from 0.897 to 5.309 g GAE/100 g DW. The TFC varied from 0.042 to 0.239 g RE/100 g DW.

Results of model fitting were presented in table 5. A quadratic model was adequately fitted for the experimental data of the TPC and TFC extracted using UAE ($P < 0.0001$). The ANOVA demonstrated that the models are highly statistically significant. The higher model F-values (*F-Value* = 18.00 for TPC and 78.97 for TFC) indicated that the models were significant with low probability values ($p < 0.0001$ for TPC and TFC). ANOVA demonstrated that there was an insignificant lack of fit (*P-value* = 0.3236 and 0.9055 for TPC and TFC models respectively) that validates the proposed model. These results indicate that most of the variation in the TPC and TFC can be explained by the regression equation and indicated that the developed quadratic models were significant to predict TPC and TFC.

The coefficient of multiple determination (R^2= 0.989 and 0.9826 for TPC and TFC models, respectively) giving the proportion of the total variation in the response predicted by the model revealed that the second-order polynomial model sufficiently signified the experimental data. The factor with the largest effect on TPC and TFC extraction was the liquid/solid ratio (x_1) having a significant effect ($p < 0.05$). The predicted mathematical models obtained for TPC and TFC are:

$$TPC = 2.99 + 1.80x_1 + 0.071x_2 - 0.054x_1^2 - 0.18x_2^2 + 0.052x_1x_2 + 0.417$$

$$TFC = 0.15 + 0.097x_1 - 2.000.10^{-4}x_2 - 8.272.10^{-3}x_1^2 - 3.272.10^{-3}x_2^2 + 0.015$$

where x_1: liquid/matter ratio and x_2: time of extraction (min).

Figure 1 showed the 3D response surfaces for TPC and TFC respectively. The increase of liquid/solid ratio increased the TPC and TFC. UAE applied using liquid/solid ratio of 4 during 25 min showed a lower TPC and TFC respectively 0.897 GAE/100g DW and 0.042 RE/100 g DW. When liquid/solid ratio increases from 4 to 20, the TPC increased from 0.897 to 5.309 GAE/100g DW and the TFC increased from 0.042 to 0.239 RE/100 g DW which was explained by the fact that more phenolic component could permeate into the solvent under the high liquid/solid ratio[22]. It was reported that the extraction of phenol components was highly depending to the liquid/solid ratio[8,21] which was explained by the variation of the adsorption surface.

Using the liquid/solid ratio of 20, when the extraction time increases from 10 to 25 min, the TPC increased from 4.269 to 5.309 GAE/100g DW. Several factors affected the bioactive compounds extraction. It was reported that an extension of extraction time increases the extraction of polyphenols compounds[7].

The optimum conditions were determined to extract the higher yield for TPC and TFC obtained through UAE graphical and numerical optimizations using RSM. The highest TPC of 4.756 g GAE/100g DW and TFC of 0.241 g ER/100g DW were obtained by UAE for: a liquid/solid ratio of 37.07 and extraction time of 28.69 min

The UAE has been used to extract bioactive compounds from plants[12,23,26]. Compared with conventional extraction methods, the UAE of phenol components seemed to be more efficient and faster[5]. In fact, the effect of the cavitation improves the diffusion of the solvent into the plant material[27], therefore, the ultrasonic waves broke down the plant's wall to enhance the mass transport of the polyphenols and the flavonoids compounds into the green solvent (distilled water)[7].

On the other hand, the UAE reduces the use of the toxic solvent which encouraged the extraction of bioactive components used as the essential constituents in the food and pharmaceutical products.

Process optimization of microwave-assisted extraction (MAE): Three factors were used to optimize the MAE of polyphenols and flavonoids, the liquid/solid ratio (x_1), the time of extraction (x_2) and the power (x_3). The results of 20 experimental runs using CCD design represented in table 4 were showing the design, factors and response variables. The PC varied from 1.492 to 8.214 g GAE/100g DW). The FC varied from 0.042 to 0.343 g ER/100 g DM.

Results of model fitting are presented in table 6. A quadratic model was adequately fitted for the experimental data of the TPC and TFC extracted using MAE ($P < 0.0001$). The ANOVA demonstrated that there was an insignificant lack of fit (*P-value* = 0.49 and 4.83 for TPC and TFC models, respectively) that validates the proposed model. The coefficient of multiple determination (R^2= 0.9892 and 0.8576 for TPC and TFC models, respectively) revealed that the second-order polynomial model sufficiently signified the experimental data. The liquid/solid ratio (x1) showed a significant effect on TPC and TFC. The predicted mathematical models obtained for TPC an TFC are:

$$TPC = 4.74 + 0.03x_1 - 0.1x_2 + 3.22x_3 - 0.089x_1x_3 - 0.052x_2x_3 + 0.094x_1^2 + 0.082x_2^2 - 0.098x_3^2 + 0.389$$

$$TFC = 0.16 + 0.012x_1 - 0.03x_2 + 0.11x_3 - 0.014x_1x_2 + 8.296x_1x_3 + 0.015x_2x_3 + 0.094x_1^2 + 0.082x_2^2 - 0.098x_3^2 + 0.389$$

where x_1: liquid/matter ratio, x_2: time of extraction and X_3: the power.

Figure 2 showed the 3D response surfaces for TPC and TFC, respectively for MAE. The liquid/solid ratio increased the TPC and TFC. Indeed, the increase of liquid/solid ratio from 4 to 12 (for 3 min of extraction under 200 W) increased the TPC and TFC from 4.491 to 5.133 GAE g/100 g DW and from 0.0151 to 0.225 ER/100g DW respectively. This would be explained by the increase of the adsorption surface. The TPC has a lower value of 1.492 g GAE/100g DW when we used a liquid/solid ratio of 4, extraction time of 1 min and power of 100 W. The highest PC (8.270 g GAE/100g DW) and TFC (0.343 ER/100 g DW) were obtained for a liquid/solid ratio of 20, an extraction time of 1 min and a microwave power of 300 W.

To determine the optimum conditions to extract the highest levels of polyphenols and flavonoïds obtained through MAE graphical and numerical optimizations were done using RSM. The highest TPC of 8.196 g GAE/100g DW and TFC of 0.350 g ER/100g DW were obtained by MAE for: a liquid/solid ratio of 16.76, extraction time of 1.81 min and power of 259.46 W.

MAE is a potential alternative to conventional method of extraction that has been recently applied to extract the bioactive compounds from various plants, mainly due to the significant savings in processing time of extraction, decrease or eliminate the use of organic solvent, lower cost and less energy consumption[4,29].

It has been reported that a liquid/solid ratio of 20 was sufficient for extraction of polyphenols[21]. However, in this study, after the MSR used for UAE and MAE showed that a ratio of 37.07 and 16.76 respectively showed the highest polyphenols and flavanoids extractions. This would be explained by the effectiveness of the method of extraction applied.

Comparison of extraction methods: MSR was conducted for optimization of the TPC and the TFC by using tow extraction methods, UAE and MAE. The optimal extraction conditions of UAE were liquid/solid ratio of 37.07 and extraction time of 28.69 min to obtain the TPC of 4.756 g GAE/100g DW and TFC of 0.241 g ER/100g DW. The optimal conditions of MAE were: liquid/solid ratio of 16.76, time extraction of 1.81 min and power of 259.46 W to obtain the highest level of TPC (8.196 g EAG/100g DW) and the highest level of TFC (0.349 g ER/100g DW).

The MAE would be more efficient, faster to obtain high TPC and TFC compared to the levels obtained through UAE. These results showed that the extraction process seems to be the primary factor for the variation of the TPC and TFC.

Table 1
Experimental domain of central composite design of ultrasound assisted extraction

Factors	Units	Codes	Coded levels		
			-1	0	+1
Liquid/solid ratio	-	x_1	4	12	20
Time	min	x_2	10	25	40

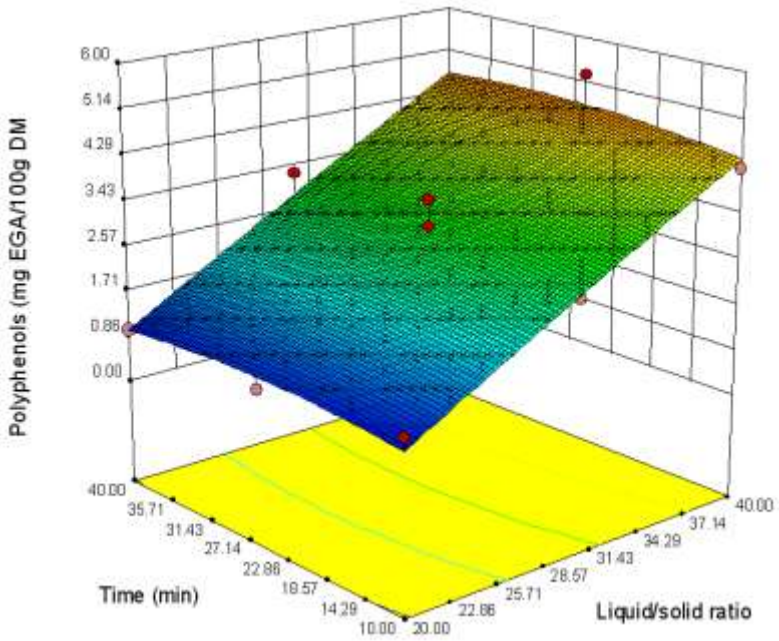
Table 2
Experimental matrix, factors and response variables based on central composite design of ultrasound assisted extraction

Experimental runs	Coded factors		Response variables	
	x_1	x_2 (min)	TPC (g EAG/100g DM)	TFC (g ER/100g DM)
1	0	0	2.691	0.144
2	0	0	2.687	0.143
3	-1	1	0.978	0.048
4	1	1	4.276	0.239
5	1	-1	4.269	0.239
6	0	-1	2.668	0.146
7	0	0	2.573	0.145
8	-1	-1	1.180	0.048
9	1	0	5.309	0.239
10	0	1	3.292	0.145
11	0	0	3.079	0.167
12	-1	0	0.897	0.042
13	0	0	3.585	0.174

x_1: liquid/matter ratio; x_2: Time of extraction (min); TPC: total polyphenol content; TFC: total flavonoid content; DM: dried matter

Table 3
Experimental domain of central composite design of microwave assisted extraction

Factors	Units	Codes	Coded levels		
			-1	0	+1
Liquid/solid ratio	-	X_1	4	12	20
Time	min	X_2	1	3	5
Power	W	X_3	100	200	300



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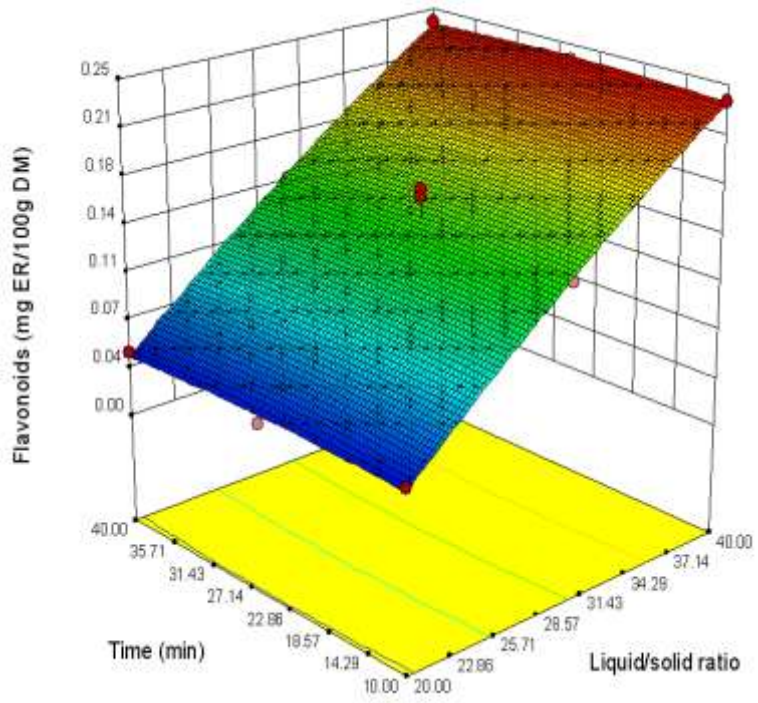

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Figure 1: Three-dimensional response surfaces plots for total polyphenol (a) and total flavonoid contents (b) as a function of liquid/solid ratio and time of extraction (min) using ultrasound assisted extraction

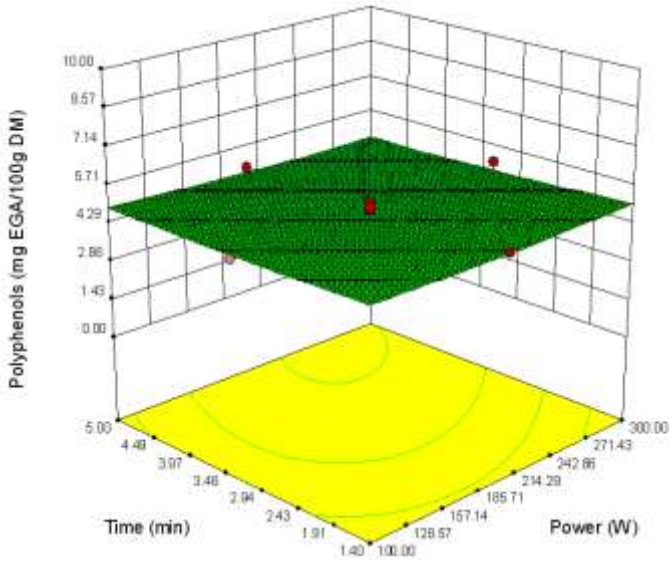
Table 4
Experimental matrix, factors and response variables based on the central composite design of microwave assisted extraction

Expérimental runs	Coded factors			Response variables	
	x_1	x_2 (min)	x_3 (W)	TPC (g EAG/100g DM)	TFC (g ER/100g DM)
1	0	0	0	4.491	0.151
2	-1	0	0	4.729	0.148
3	0	0	0	5.133	0.225
4	1	-1	-1	1.560	0.071
5	-1	1	-1	1.492	0.052
6	0	0	0	4.672	0.167
7	1	-1	1	8.270	0.343
8	-1	-1	1	8.127	0.254
9	1	1	-1	1.519	0.053
10	0	0	0	4.900	0.145
11	0	1	0	4.900	0.147
12	-1	1	1	8.214	0.266
13	0	-1	0	4.943	0.218
14	0	0	-1	1.560	0.042
15	1	0	0	5.138	0.156
16	0	0	0	4.843	0.168
17	0	0	0	4.016	0.157
18	1	1	1	7.493	0.268
19	-1	-1	-1	1.703	0.048
20	0	0	1	7.921	0.272

x_1 : the liquid/solid ratio ; x_2 : time of extraction (min); X_3 : microwave power (W); TPC : total polyphenol content ; TFC : total flavonoid content

Table 5
Analysis of variance of response surface quadratic model and the coefficient of determination of the model (R^2) for polyphenols and flavonoids contents obtained through ultrasound assisted extraction

Source	Sum of square	DF	Mean square	F-value	p-value
PC (g EAG/100g DM)					
Model	19.61	5	3.92	18.00	0.0007
x_1	19.44	1	19.44	89.18	< 0.0001
x_2	0.031	1	0.031	0.14	0.7188
x_1x_2	0.011	1	0.011	0.050	0.8302
x_1^2	8.10^{-3}	1	8.10^{-3}	0.037	0.8535
x_2^2	0.086	1	0.086	0.40	0.5491
Residual	1.53	7	0.22		
Lack of fit	0.83	3	0.28	1.59	0.3236
Total	21.14	12			
Pur error	0.69	4	0.17		
R^2	0.9278				
FC (g ER/100g DM)					
Model	0.056	5	0.011	78.97	< 0.0001
X_1	0.056	1	0.056	392.59	< 0.0001
X_2	$2.4.10^{-7}$	1	$2.400.10^{-7}$	$1.685.10^{-3}$	0.9684
X_1X_2	0.00	1	0.00	0.00	1.000
X_1^2	$1.890.10^{-4}$	1	$1.890.10^{-4}$	1.33	0.2871
X_2^2	$2.958.10^{-5}$	1	$2.958.10^{-5}$	0.21	0.6624
Residual	$9.969.10^{-4}$	7	$1.424.10^{-4}$		
Lack of fit	$1.180.10^{-4}$	3	$3.932.10^{-5}$	0.18	0.9055
Total	0.057	12			
Pur error	$8.790.10^{-4}$	4	$2.197.10^{-4}$		
R^2	0.9826				



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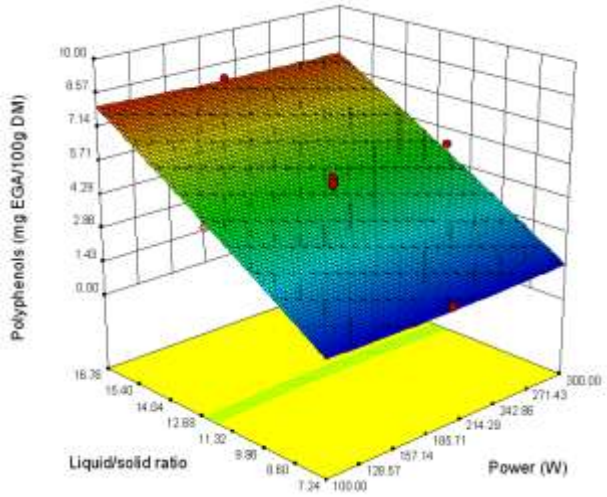

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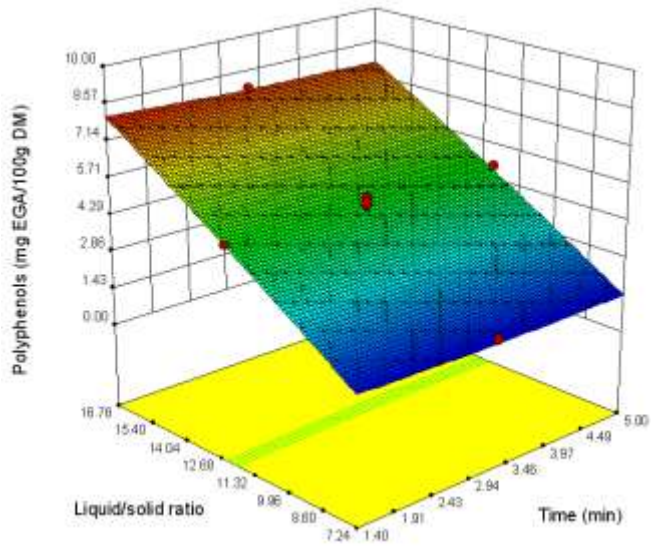

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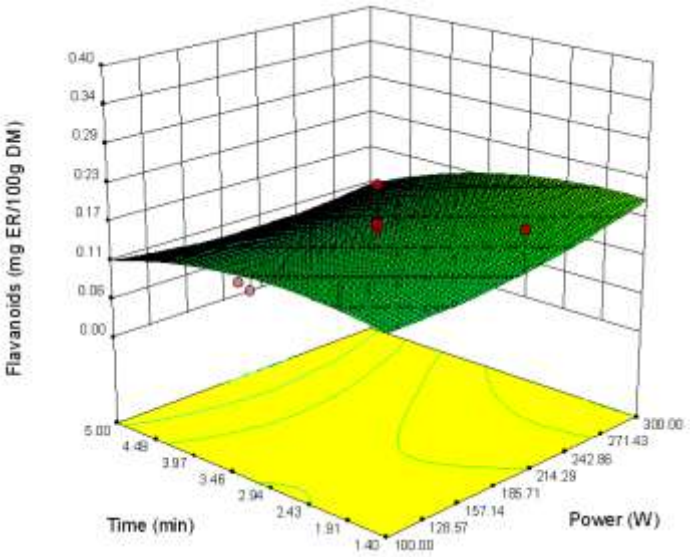
Figure 2: Three-dimensional response surfaces plots for total polyphenol content as a function of liquid/solid ratio, time of extraction (min) and power (W) using microwave assisted extraction



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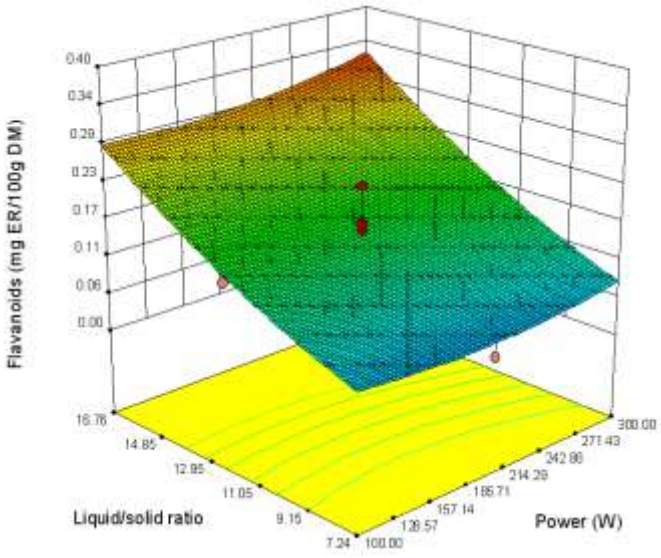

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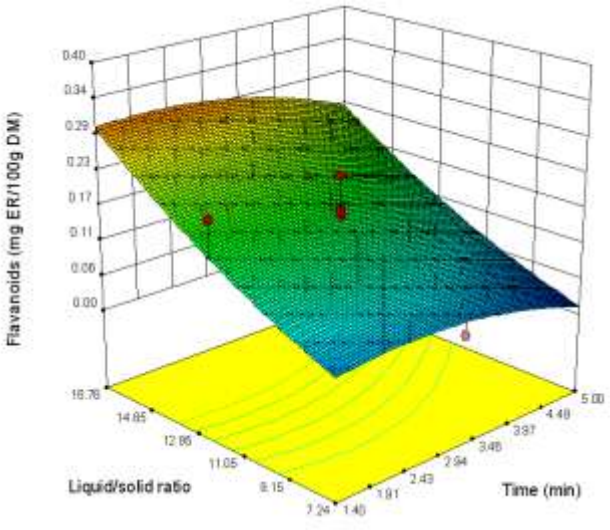

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Figure 3: Three-dimensional response surfaces plots for total flavonoid content as a function of liquid/solid ratio, time of extraction (min) and power (W) using microwave assisted extraction

Table 6
Analysis of variance of response surface quadratic model and the coefficient of determination of the model (R^2) for polyphenols and flavonoids contents obtained through microwave assisted extraction

Source	Sum of square	DF	Mean square	F-value	p-value
PC (g EAG/100g DM)					
Model	104.03	9	11.56	102.09	< 0.0001
X_1	103.74	1	103.74	916.26	< 0.0001
X_2	0.10	1	0.10	0.89	0.3678
X_3	$9.267.10^{-3}$	1	$9.267.10^{-3}$	0.082	0.7806
X_1X_2	0.022	1	0.022	0.19	0.6694
X_1X_3	0.025	1	0.025	0.22	0.6509
X_2X_3	0.064	1	0.064	0.56	0.4707
X_1^2	0.027	1	0.027	0.23	0.6385
X_2^2	0.019	1	0.019	0.17	0.6931
X_3^2	0.024	1	0.024	0.22	0.6520
Residual	1.13	10	0.11		
Lack of fit	0.37	5	0.075	0.49	0.7712
Total	105.16	19			
Pure error	0.76	5	0.15		
R^2	0.9892				
FC (g ER/100g DM)					
Model	0.14	9	0.016	6.69	0.0032
X_1	0.13	1	0.13	53.69	< 0.0001
X_2	$8.782.10^{-3}$	1	$8.782.10^{-3}$	3.65	0.0852
X_3	$1.536.10^{-3}$	1	$1.536.10^{-3}$	0.64	0.4430
X_1X_2	$3.142.10^{-4}$	1	$3.142.10^{-4}$	0.13	0.7254
X_1X_3	$5.506.10^{-4}$	1	$5.506.10^{-4}$	0.23	0.6427
X_2X_3	$1.512.10^{-3}$	1	$1.512.10^{-3}$	0.63	0.4464
X_1^2	$1.148.10^{-3}$	1	$1.148.10^{-3}$	0.48	0.5056
X_2^2	$2.043.10^{-3}$	1	$2.043.10^{-3}$	0.85	0.3786
X_3^2	$6.563.10^{-4}$	1	$6.563.10^{-4}$	0.27	0.6129
Residual	0.024	10	$2.407.10^{-3}$		
Lack of fit	0.020	5	$3.988.10^{-3}$	4.83	0.0544
Total	0.17	19			
Pure error	$4.128.\ 10^{-3}$	5	$8.256.10^{-4}$		
R^2	0.8576				

Compared to the conventional extraction methods, MAE and UAE were more efficient with a shorter extraction time and lower cost[2,20,28].

Conclusion

The RSM was applied to optimize the total polyphenol and flavonoid contents from olive leaves through two methods of extraction (UAE and MAE). The second order polynomial model could be used to optimize extraction of phenolic compounds from olive leaves for maximizing the total polyphenol and flavonoids content.

Results showed that the MAE technique seems to be faster, useful and efficient method to obtain the highest yield of polyphenols and flavonoids contents from the *Olea europaea* leaves by using the green solvent as the water which is considered as non-toxic and economic compared to the organic solvents.

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