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ORIGINAL RESEARCH PAPER

THE OPTIMIZATION OF DIRECT ENRICHMENT OF SOYBEAN OIL WITH MYRTLE SEED BIOACTIVE COMPOUNDS USING ULTRASONIC ASSISTED EXTRACTION

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Abstract

This study aimed to optimize Ultrasound-Assisted Extraction (UAE) parameters to enrich soybean oil with *Myrtus communis* L. (myrtle) seeds powder. The effect of the temperature and sonication time on antioxidant contents, namely total phenolics, total carotenoids, total chlorophylls and also on the antioxidant capacity was studied. The resulted optimized conditions were a temperature of 30 °C during 32.5 min and through their use, the experimental values for total phenolics, carotenoids, and chlorophylls were 103.09 ± 7.22, 1.96 ± 0.90 and, 1.88 ± 3.97 mg/kg oil, respectively. These values were very close to the predicted ones, which were 94.45 ± 10.13, 1.52 ± 0.12, and 1.57 ± 0.84 mg/kg oil, respectively. The results of the antioxidant capacity of the oil extracts indicated that the enriched oil using UAE appeared to be more potent than the control. Hence, myrtle seeds presented high amounts of bioactive compounds with antioxidant activity and were appropriate for soybean oil enrichment by UAE.

Keywords: myrtle seeds, ultrasound-assisted extraction, soybean oil, bioactive compounds, antioxidant activity

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Introduction

The limited oxidative stability of edible oils, induced by lipid oxidation, is one of the foremost constraints for the food industry, causing a decrease in nutritional and sensory properties and shelf life of food products including oil (Fadda *et al.*, 2022). Soybean oil has become the second most important vegetable oil in the global market ranking with a production of 62.25 million tons in 2021/2022 (USDA, 2022). In addition, this oil is the most famous employed in the food industry (Zhou *et al.*, 2020) and is an excellent source of essential fatty acids and fat-soluble vitamins, which are substantial compounds of the human diet (Viana da Silva *et al.*, 2021).

The main fatty acids found in soybean oil are unsaturated and mainly represented by oleic, linoleic, and linolenic acids. The occurrence of double bonds in these fatty acids, can provide various abilities to engender chemical and structural modifications essential to their functionality (Naeli *et al.*, 2017). At the same time, they are susceptible to oxidation when conserved at low temperature, and when employed for frying and cooking at high temperature (Blasi and Cossignani, 2020). So, the use of synthetic antioxidants namely butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tertiary butyl hydroquinone (TBHQ) is necessary (Farahmandfar *et al.*, 2018).

The extraction of natural antioxidants from vegetables is very advantageous when they are used as an alternative to synthetic ones, which are being constricted owing to their probable health hazards and typically undesirable effects, and their safeness has been queried for so long (González de Peredo *et al.*, 2018). Furthermore, several researchers pointed out that antioxidants from natural origins are more suitable for application in frying and also cooking as an effective alternative to inhibit lipid peroxidation (Viana da Silva *et al.*, 2021). Therefore, the enriched edible oils with natural antioxidants namely from herbs, fruits, seeds, and processing by-products have gained increasing interest in the last years for the food industry (Fadda *et al.*, 2022). For instance, extracts of marjoram, thyme, oregano, and rosemary were effective in increasing the shelf life of sunflower oil and can be recommended as a potent source of natural antioxidants to replace synthetic ones. The relevance of natural compounds is not only linked to their biological effect but also to the request of consumers, besides their economic influence, given that most of them can be extracted from under-usable food by-products (Lourenço *et al.*, 2019). In this context, the *Myrtus communis* L. seeds by-products were used to prevent the oxidation of soybean oil. Myrtle seeds extract exhibits many beneficial health effects such as neuro-protective, antidiarrheal, antimicrobial, antioxidant and antiulcer (Giampieri *et al.*, 2020) activities, that revealed its richness in beneficial active molecules such as phenolic compounds (González de Peredo *et al.*, 2018). Among these compounds found in seeds are flavonols including myricetin and quercetin derivatives, anthocyanins, and some hydroxybenzoic acid derivatives. The myrtle fruits were also found to be a rich source of carotenoids (*β*-carotene and lycopene) (D'Urso *et al.*, 2017). Carotenoids occur naturally in oils but most of them are lost during the bleaching operation. Consequently, the addition of myrtle seed can increase the carotenoid content in refined oil. Chlorophylls are also widely extracted

for industrial applications from various plants including *Myrtus communis* L. (Cvitković *et al.*, 2021). These compounds are widely used as a stable, non-toxic, physiologically harmless colorant for edible oils (Jinasena *et al.*, 2016). Hence, *Myrtus communis* is an edible plant that can be applied to improve and preserve food products prone to oxidation such as vegetable oils.

Ultrasound-assisted extraction (UAE) of bioactive compounds directly from plant powder into the oil can be performed. It was a better alternative method which has been successfully employed in several food matrices e.g. fruits, vegetables, and edible oils. This technique offers several advantages, namely the use of organic solvents is avoided or minimized; low temperature and extraction time are required, leading to less energy consumption, high extraction yields and preservation of the extract quality (Zia *et al.*, 2020; Kumar *et al.*, 2021).

This present work aimed to optimize for the first time the enrichment of edible oil (soybean oil) with bioactive substances from *Myrtus communis* L. by-products (fruit seeds) using ultrasonic-assisted extraction (UAE). Two parameters of UAE which are time and temperature were optimized by response surface methodology. Then, the effect of this enrichment on soybean oil composition (phenolic compounds, carotenoids, and chlorophylls) and its antioxidant activity was studied.

Materials and methods

Chemicals and standards

Gallic acid, ferric chloride ($FeCl_3 \cdot 6H_2O$), trichloroacetic acid, potassium ferricyanide ($C_6N_6FeK_3$), and sodium dihydrogen phosphate (NaH_2PO_4) were purchased from Biochem-Chemopharma (Loire, France). Sodium carbonate (Na_2CO_3), disodium hydrogen phosphate (Na_2HPO_4), Folin–Ciocalteau's phenol reagent, and aluminum chloride ($AlCl_3$) were obtained from Prolabo (Loire, France), and 1,-diphenyl-2-picrylhydrazil (DPPH) from Sigma Aldrich (St. Louis, MO, USA). All used solvents were of analytical grade.

Plant material and sample preparation

The harvesting of *Myrtus communis* L. samples was realized at optimal fruit maturity from the Addekar region (Bejaia, North East of Algeria); the fruits were washed with distilled water to remove any adhering soil and dust. The samples were dried with microwave drying – ultrasound assisted pretreatment method (4 mL of water were needed for 1g of seed sample and placed in an ultrasonic bath for 90 min, and treated with microwave at 500 W). The dried fruits were peeled manually and the seeds were recuperated and ground with an electrical grinder (IKA model A11 Basic, Staufen, Germany). The powder was stored in airtight bags until use.

Extraction procedures

Preliminary study and preparation of enriched soybean oil

A preliminary study was performed to determine the appropriate level of temperature, extraction time, and powder particle size for soybean oil enrichment by dried myrtle seeds. So, different macerations assisted by ultrasound process were

carried out as follows: myrtle seeds powder (10 g) was added to 100 mL of soybean oil. The mixture was sonicated at 20 KHz in an ultrasound water bath for 15, 30, 45, and 60 min at different temperatures (15, 25, 35, and 45 °C), and two powder particle sizes were used (125, and 250 µm). The mixture was filtered to remove traces of powder before analyses.

Extraction of phenolic compounds from enriched oil

The extraction of phenolics from oil enriched by myrtle seeds powder was achieved using 5 mL of hexane and 5 mL of methanol/water (6/4, *v/v*) which were added to 2.5 g of oil. Then the mixture was vortexed for 2 min and centrifuged at 3500 rpm for 10 min. The polar fraction was recovered while the apolar one was depleted. An additional extraction was done and the obtained fractions were combined and stored at 4 °C (Kalantzakis and Blekas, 2006).

Phytochemical analysis

Determination of total phenolics content

The Folin-Ciocalteu's assay followed by spectrophotometric measurement with absorbance monitored at 760 nm (Georgé *et al.*, 2005) was utilized to estimate the total phenolics content (TPC). The data were interpolated in a gallic acid calibration curve and expressed as mg of Gallic Acid Equivalent per gram of Dry Weight sample (mg GAE/g DW).

Determination of carotenoids and chlorophylls content

A sample of 7.5 g of oil was dissolved in cyclohexane (up to 25 mL). The maximum absorbance at 470 and 670 nm gives information on the carotenoids and chlorophylls, respectively (Mínguez-Mosquera *et al.*, 1991). Their quantification was assessed by adopting the following equations:

$$\text{Carotenoids (mg/Kg)} = \frac{A_{470} - 10^{-6}}{2000 - 100 \times T} \quad (1)$$

$$\text{Chlorophylls (mg/Kg)} = \frac{A_{670} - 10^{-6}}{613 - 100 \times T} \quad (2)$$

where A: absorbance; T: the thickness of the vessel (1 cm); 2000 and 613 are the values of the specific extinction coefficients used that correspond to lutein (the major component of carotenoids) and pheophytin (the major component of chlorophylls), respectively.

Antioxidant assays

For the DPPH$^{\bullet}$ radical scavenging assay, 3 mL of DPPH solution (60 µM) was mixed with 1 mL of the extract. After incubation for 20 min at 37 °C in the dark, the absorbance was measured at 515 nm. The inhibition rate of the extracts was calculated according to equation 3 (Dudonne *et al.*, 2009).

For the reducing power assay, 1 mL of extract was mixed with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% (*m/v*) potassium ferricyanide [$K_3[Fe(CN)_6]$], followed by incubation in a water bath at 50 °C for 20 min. After

that, 2.5 mL of 10% (*m/v*) trichloroacetic acid was added. Finally, 1 mL of this mixture was added to 5 mL of distilled water and 1 mL of $FeCl_3 \cdot 6H_2O$ at 0.1% (*m/v*). The intensity of the blue-green color was measured at 700 nm (Zou *et al.*, 2004). α-tocopherol and BHA were used as positive controls.

$$\text{Inhibition \%} = (A_{\text{control}} - A_{\text{extract}})/A_{\text{control}} \times 100 \quad (3)$$

where A_{control} is the absorbance of the DPPH solution. A_{sample} is the absorbance of DPPH in the presence of the extract sample.

Response surface methodology

To determine the effect of temperature and irradiation time on the extraction yield of bioactive compounds in enriched oil, RSM was applied with a Box–Behnken Design (BBD). The coded levels and their natural values were selected based on preliminary study and the variables were shown in table 1 which involved 30 experiments. The selected optimization parameters were total phenolics (Y_1), chlorophylls (Y_2), and carotenoids (Y_3) concentrations. To determine the validity of the model, the experimental values of TPC, chlorophyll, and carotenoid pigments were compared to the predicted values.

Results and discussion

Preliminary study

The effect of the temperature was firstly assessed at a fixed time and powder diameter factors while the temperature was varied from 15 to 45 °C. Table 1 illustrated the results of temperature effects on total phenolic, chlorophyll, and carotenoid contents. The TPC increased significantly ($p < 0.05$) with increasing temperature in the extraction medium from 15 to 35 °C, giving the highest yield of 73.97 ± 1.50 mg GAE /kg of oil. However, the TPC yield began slightly to decrease after 35 °C and reached TPC value of 61.93 ± 0.89 mg GAE /kg of oil at 45 °C. This result is in agreement with the observations of Bouaoudia-Madi *et al.* (2019) who have reported that reduced recoveries of TPC of myrtle could be due to the thermal degradation of the phytochemicals mainly anthocyanins at a higher temperature.

As shown in table 1, the recovery of carotenoids increased significantly from 1.54 ± 0.01 to 2.59 ± 0.06 mg/kg oil by increasing temperature from 15 to 35 °C, where the optimal was attained. These results were noticed also by Song *et al.* (2018) who studied the degradation of carotenoids in dehydrated pumpkins as affected by different temperatures. These authors demonstrated that higher carotenoid degradation in N_2-packaged dehydrated pumpkins stored at 40 °C occurred than that stored at a lower temperature. In addition, the found results were in agreement with the ones of Ordóñez-Santos and Martínez-Girón (2020) who investigated the thermal degradation kinetics of carotenoids in tomato juice.

Regarding the content of chlorophylls (Table 1), the maximum content (3.9 ± 0.01 mg/kg oil) was recorded at a temperature of 45 °C, which was 4 times higher than the control (0.55 ± 0.06 mg/kg). Sintra *et al.* (2021) studied the effect of temperatures ranged from 14.9 to 35.1 °C on the extraction of chlorophylls from *Anabaena cylindrica*. They showed that this independent variable has the highest influence on the extraction of chlorophylls with a maximum extraction yield observed for temperatures ranging between 24.0 and 30.0 ± 0.5 °C. These results were also in agreement with that found by Jinasena *et al.*(2016) who studied the extraction and degradation of chlorophylls from *Alternanthera sessilis*. They revealed that chlorophyll extraction improves with the increase in temperature up to 50 °C. The degradation of chlorophyll may become prominent at high temperatures and prolong extraction. According to Sarkar *et al.* (2020) temperature is a very critical factor for any extraction procedure since elevated temperature improves the speed of mass transfer of the cellular constituents to the solvent. Conversely, pigments such as chlorophylls and carotenoids are prone to denaturation at very high temperatures.

Table 1. Effect of temperature on total phenolics, carotenoids, and chlorophylls content of soybean oil enriched with bioactive substances from *Myrtus communis* L. using ultrasound-assisted method.

Temperature (°C)	**TPC** (mg GAE /kg of oil)	**Carotenoids** (mg/kg oil)	**Chlorophyll** (mg/kg oil)
Control	15.80 ± 1.52^{c}	0.48 ± 0.01	0.55± 0.06^{d}
15	49.62 ± 2.22^{b}	1.54 ± 0.01^{d}	1.50 ± 0.11^{c}
25	73.58 ± 4.49^{a}	1.8 ± 0.21^{c}	1.58 ± 0.18^{e}
35	73.97 ± 1.50^{a}	2.59 ± 0.06^{a}	3.85 ± 0.24^{a}
45	61.92 ± 0.89^{ab}	2.14 ± 0.01^{b}	3.93 ± 0.01^{b}

Each value in the table is the mean ± standard deviation (n = 3). Values sharing different letters within a column are significantly different ($p < 0.05$). Results are ranked in ascending order; a > b.

The effect of irradiation time was determined at 35 °C using a powder with a diameter of 250 µm. Then, the mixture was sonicated at times varied from 15 to 60 min. The effect of irradiation time on the recovery of TPC, carotenoids and chlorophylls was depicted in Table 2. The amount of TPC increased significantly ($p < 0.05$) and gave a maximum of 73.34 ± 4.40 mg GAE/kg of oil at 30 min (Table 2). Then, it decreased approximately by 40% at longer time irradiation (60 min). The same tendency was observed by Brahmi *et al.* (2022) who proved that TPC resulted from ultrasound assisted extraction was notable through the former 30 min and thereafter diminished progressively.

As shown in table 2, the amount of carotenoids increased with increasing extraction time. However, the maximum amount of carotenoids content (1.90 ± 0.01 mg/kg of oil) was obtained at 30 min. Several other investigations have also established that extraction time determined an increase in carotenoid content, while the rising yield

was ultimately decreased before the yield attained a maximum and then decreased with additionally extraction time (60 min). A diminution in carotenoid content after the maximum time may be due to the degradation of the dissolved carotenoids by light, heat and oxygen, while the equilibrium concentration was achieved (Chuyen *et al.*, 2017).

Optimal chlorophyll content (1.07 ±0.03 mg/kg of oil) was obtained at 45 min (Table 2). In the same sense, by varying the extraction time from 11.4 to 68.6 min, the optimized time for the efficiently recovering of chlorophylls from *Anabaena cylindrica* was 45 min (Sintra *et al.*, 2021).

Table 2. Effect of extraction time on total phenolics, carotenoids, and chlorophylls content of soybean oil enriched with bioactive substances from *Myrtus communis* L. using ultrasound-assisted method.

Extraction time (min)	**TPC** (mg GAE /kg of oil)	**Carotenoids** (mg/kg oil)	**Chlorophyll** (mg/kg oil)
Control	11.85 ± 0.04	0.096 ± 0.002^{e}	0.54 ± 0.03^{d}
15	34.66 ± 0.04^{b}	1.47 ± 0.04^{d}	1.10 ± 0.05c
30	73.34 ± 4.40^{a}	1.90 ± 0.01^{c}	0.75 ± 0.02^{b}
45	65.18 ± 4.19^{a}	0.48 ± 0.15^{b}	1.07 ± 0.03^{b}
60	43.85 ± 1.67^{b}	2.21 ± 0.09^{a}	1.27 ± 0.02^{a}

Each value in the table is the mean ± standard deviation (n = 3). Values sharing different letters within a column are significantly different ($p < 0.05$). Results are ranked in ascending order; a > b.

After fixing time and temperature, the powder granulometry of 250 and 125 µm were studied to determine the best powder size for oil enrichment. The results showed clearly that powder granulometry influences the migration of phenolic compounds, carotenoids, and chlorophylls from vegetable matrix to oil. It was observed that the diameter powder of 125 µm allows better oil enrichment; *i.e.*37.44 and 35% higher than enrichment using a powder diameter of 250 µm, for TPC, carotenoids, and chlorophylls, respectively. The results (Table 3) indicated that the small diameter powder allows better extraction; this may be due to the increase in the contact surface between the powder and the extraction solvent (Brahmi *et al.*, 2021).

Table 3. Effect of diameter powder on total phenolics, carotenoids, and chlorophylls content of soybean oil enriched with bioactive substances from *Myrtus communis* L. using ultrasound-assisted method.

Powder diameter (µm)	**TPC** (mg GAE/kg oil)	**Carotenoid** (mg/kg of oil)	**Chlorophyll** (mg/Kg of oil)
125	101.62 ± 6.28^{a}	2.597 ± 0.36^{a}	3.858 ± 0.21^{a}
250	73.92 ± 1.70^{b}	0.596 ± 0.07^{b}	2.854 ± 0.17^{b}

Each value in the table is the mean ± standard deviation (n = 3). Values sharing different letters within a column are significantly different ($p < 0.05$). Results are ranked in ascending order; a > b.

Mousavi *et al.* (2020) have fractionated fennel seeds on superfine powders (100–180 µm, 180–315 µm, 315–500 µm, > 500 µm and unsieved superfine powder). They proved that the best extraction of polyphenols was obtained in the 100–180 µm fraction. Furthermore, the phenolic content of grape seeds was also impacted by fluctuations in the sample particle size. A higher amount of TP was obtained with a particle ≤ 250 µm (Brahmi *et al.*, 2021).

Optimization of ultrasound enrichment conditions

Modeling and fitting the model using response surface methodology (RSM)

The quantity of TPC, carotenoids, and chlorophylls in the enriched refined oil was influenced by the studied variables such as temperature and treatment time. So, the effect of temperature (X_1) and sonication time (X_2) on the oil enrichment procedure was evaluated by RSM. The experimental design and corresponding response data for the total phenolic, carotenoid and chlorophyll contents from enrichment-refined oil was presented in Table 4.

Table 4. Central composite design with the observed responses and predicted values for the yield of Total Phenolic Compounds (TPC), carotenoids, and chlorophylls of of soybean oil enriched with bioactive substances from *Myrtus communis* L. using ultrasound-assisted method.

Run	**X_1:** Temperature (°C)	**X_2:** Sonication time (min)	**TPC** (mg GAE/kg oil)	**Carotenoid** (mg/kg of oil)	**Chlorophyll** (mg/Kg of oil)
1	45	15.0	99.85	2.46	11.94
2	30	32.5	110.01	1.36	1.09
3	30	50.0	33.09	2.07	2.46
4	30	32.5	91.23	1.67	1.53
5	30	32.5	91.26	1.50	2.76
6	30	15.0	27.65	1.03	1.99
7	30	32.5	80.96	1.57	1.72
8	15	50.0	111.41	2.60	2.76
9	15	32.5	142.42	1.95	0.58
10	45	50.0	14.81	2.95	8.31
11	45	32.5	123.85	2.58	7.90
12	15	15.0	28.54	1.00	0.63

The regression coefficients of the intercept, linear, quadratic, and interaction terms of the model were illustrated in Table 5.

Table 5. Estimated regression coefficients for the quadratic polynomial models and the analysis of variance (ANOVA) for the experimental results of total phenolics, carotenoids, and chlorophylls contentofsoybean oil enriched with bioactive substances from *Myrtus communis* L. using ultrasound-assisted method.

Parameters	Estimated coefficients	DF[a]	Sum of squares	Standard error	F ratio[b]	Prob > F
Results of phenolic contents						
Model		5	19696.15		47.85	<0.0001
Intercept	94.45			4.14		<0.0001
Linear						
X_1-Temperature	-7.31		320.53	-10.16	3.89	0.0959
X_2-Time	0. 54		1.78		0.02	0.8878
Quadratic						
X_1^2	36. 53	1	3557.08	5.56	43.22	0.0006
X_2^2	– 66.24	1	11700.26	5.56	142.14	<0.001
Interaction X_1-X_2	- 41.97	1	7047.71	4.54	85.62	<0.0001
Lack of fit		3	53.95		0.13	0.9408
Pure error		3	439.92			
R^2					0.98	
Adjusted R^2					0.96	
RMSE	9.0727					
Cor Total[c]		11	20190.26			
Results of carotenoids contents						
Model	5		4.51	72.12	<0.0001	
Intercept	1.53	1	0.05			0.0001
Linear						
X_1-Temperature	0.41	1	0.05	0.99	77.26	0.0001
X_2-Time	0. 52	1	0.04	1.63	127.14	<0.0001
Quadratic						
X_1^2	0.72	1	0.69	1.39	108.01	<0.0001
X_2^2	0.006	1	0.69	0.0001	0.008	0.9312
Interaction X_1-X_2	- 0.27	1	0.056	0.31	23.98	0.0027
Lack of fit		3	0.027	0.51	0.7009	
Pure error		3	0.05			
R^2				0.98		
Adjusted R^2				0.97		
RMSE	0.11					
Cor Total[c]		11		4.5799	< 0.0001	
Results of chlorophyll contents						
Model	5	144.17		50.86	<0.0001	
Intercept	1. 58			0.34		0.0038
Linear	4.03	1	97.44	0.30	171.88	<0.0001

X_1-Temperature	-0.17	1	0.17	0.30	0.31	0.5967
X_2-Time						
Quadratic						
X_1^2	3.07	1	25.21	0.46	44.47	0.0006
X_2^2	1.06	1	3.005	0.46	5.28	0.0612
Interaction						
X_1-X_2	-1.44	1	8.29	0.37	14.63	0.0087
Lack of fit		3	1.99		1.26	0.4259
Pure error		3	1.49			
R^2					0.98	
Adjusted R^2					0.96	
RMSE	0.7529					
Cor Total[c]		11	147.59			

[a]Degree of freedom; [b]the model mean square to error mean square ratio; [c]orrected total. DF. degree of freedom; F Ratio. Freedom ratio; Prob. Probability.

The quadratic terms of sonication time and the interactive effect of time and temperature parameters X_1X_2 affected the phenolic extraction ($p < 0.001$). Regarding carotenoids content, it was significantly affected by the linear temperature (X_1) and time (X_2) parameters and by the quadratic terms of temperature ($p \leq 0.0001$), followed by the interaction of time and temperature parameters X_1X_2 ($p = 0.0027$) while the quadratic term of time (X^2_2) showed no significant effect.

Chlorophylls content was affected by the linear and quadratic parameters of temperature at $p < 0.0001$ and $p = 0.0006$, respectively, followed by the interactive effect of time and temperature (X_1X_2) ($p < 0.0087$) than by the quadratic term of irradiation time X_2^2 at $p < 0.0612$.

The following second-order polynomial equations show the predicted models:

$$Y_1 = 94.44625 - 7.3X_1 + 0.540X_2 - 41.9775X_1X_2 + 36.52625X_1^2 - 66.23875X_2^2 \quad (4)$$

$$Y_2 = 1.53125 + 0.406667X_1 + 0.5216667X_2 - 0.2775X_1X_2 + 0.72125X_1^2 + 0.00625X_2^2 \quad (5)$$

$$Y_3 = 1.5716667 + 4.0304757X_1 - 0.171667X_2 - 1.44\ X_1X_2 + 3.0375X_1^2 + 1.06X_2^2 \quad (6)$$

where Y_1, Y_2, and Y_3 were total phenolic, carotenoid, and chlorophyll contents, respectively; X_1 is the temperature variable (°C) and X_2 is the sonication time (min).

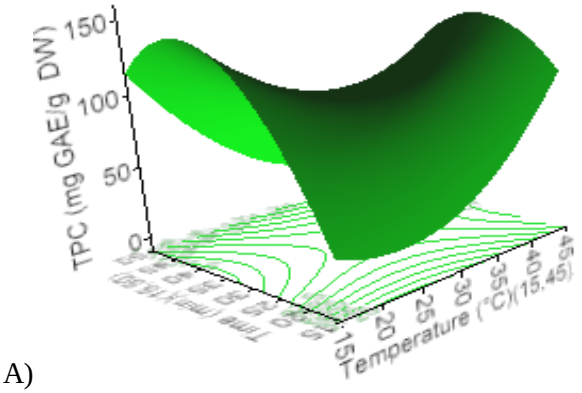
The model was highly significant at p-value < 0.0001. The coefficients of determination (R^2) and the adjusted coefficients of determination (R^2_{adj}) of TPC, carotenoid, and chlorophyll contents were close to 1. For TP content, the sample variations of 97.56% for the UAE efficiency on the oil enrichment were attributed to the independent variables which explain a positive degree of correlation between

experimental and predicted values of the TPC yield. For the carotenoids and chlorophylls, the variations were about 98.31% and, 97.69%, which explain a positive degree of correlations between experimental and predicted values. The *p*-values for lack-of-fit in the ANOVA table are not significant relative to pure error ($p > 0.05$) confirming the validity of the models.

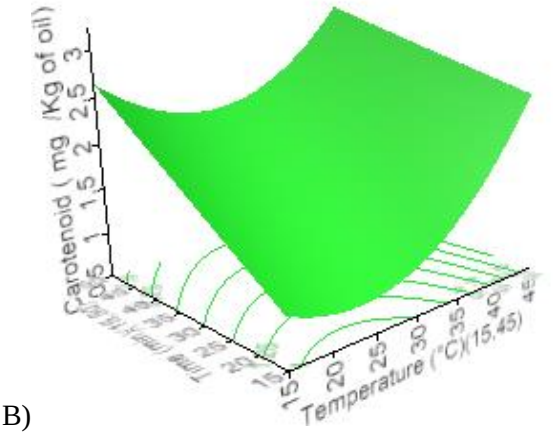
Response Surface Analysis (RSA)

According to Figure 1, the extraction efficiency in terms of TP, carotenoids, and chlorophyll concentrations increase with increasing temperature and time. Figure 1A shows the interactions between temperature and time on the recovery of TPC. The increase of temperature from 15 to 45 °C and from 15 to 50 min for a time resulted in a rapid enhancement of TPC with a maximum of 142.40 ± 0.71 mg/Kg of oil at 15 °C and 38 min. Our results were compatible with those obtained by Bouaoudia-Madi *et al.*(2019) who used UAE to extract phenolics from myrtle pericarp. The same tendency was recorded in the ultrasound-assisted enrichment of virgin olive oil with phenolic compounds from olive leaves (increase from 342.5 ± 1.5 to 414.2 ± 3.2 mg/kg of oil). These results show the efficiency of the ultrasounds that improved the hydration and fragmentation process and hence facilitates the mass transfer of bioactive compounds to the extraction solvent (Achat *et al.*, 2012).

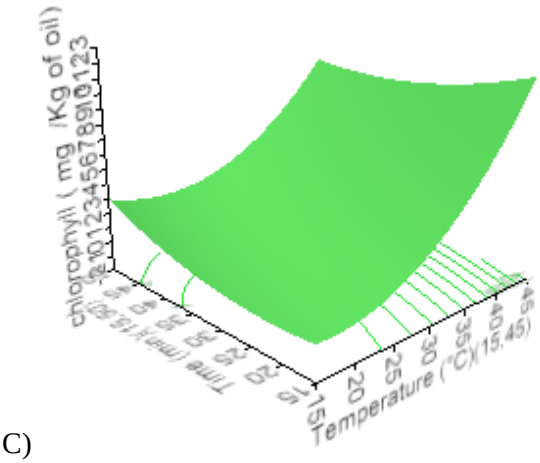
Carotenoid content increases by raising the temperature and sonication time with maximum extraction recovery of carotenoids (2.95 ±0.04 mg/kg of oil) achieved at temperature and extraction time of 45 °C and 50 min, respectively (Figure 1B). However, the maximum chlorophyll yield (11.94 ± 0.32 mg/kg of oil) was obtained under the conditions of 45 °C and 15 min (Figure 1C). The current results were in agreement with those of Chemat *et al.* (2012), that showed the effect of ultrasound time on carotenoid content in the enrichment of edible oil with sea buckthorn by-products. Goula *et al.* (2017) also noticed that an increase in the ultrasound extraction time, from 10 to 30 min, caused an increase in the amount of carotenoids extracted from pomegranate wastes into the vegetable oils. In addition, the chlorophyll yield increased rapidly using UAE with the increase of the sonication time (Kong *et al.*, 2014). However, the amounts of chlorophylls and carotenoids were highest in samples subjected to 8 minutes sonication treatment whereas they were lowest in olive oils subjected to ultrasound for 12 minutes (Aydar, 2018). This difference is probably due to the difference between the samples and the oils used, on which the effectiveness of the method adopted depends. Hence, the virgin olive oil was flavored with the seeds of green anise by three procedures including ultrasonic assisted maceration. The authors discovered that an enrichment in polyphenols estimated at 35% was found in the case of ultrasonic flavored oil, while an increase in the content of carotenoids and chlorophylls (67% and 21%, respectively) was recorded using classic maceration (Moustakime *et al.*, 2021).



A)



B)



C)

Figure 1. Response surface analysis for the TPC (A), carotenoid (B), and chlorophyll (C) contents of soybean oil enriched with bioactive substances from *Myrtus communis* L. using ultrasound-assisted method with respect to temperature and irradiation time.

Validation and verification of the predictive model

RSM method was applied for modeling and optimizing the ultrasound-assisted enrichment of refined soybean oil by *Myrtus communis* L. fruit seeds. According to the results of the response surface and prediction by these built models, the obtained values corresponding to optimal conditions were a temperature of 30 °C and a time of 32.5 min for the three studied responses. Using these optimized conditions, the experimental values for TP, carotenoid and, chlorophyll contents were 103.09 ± 7.22, 1.96 ± 0.90, and 1.88 ± 3.97 mg/kg of oil, respectively; which are very close to the predicted values (94.45 ± 10.13, 1.53± 0.12 and 1.57 ± 0.84 mg/kg oil for TP, carotenoid and chlorophyll amounts, respectively) with no significant difference ($p > 0.05$). This great correlation between experimental and the predicted values showed that the response surface modeling models could be applied effectively to predict the enrichment of refined oil with polyphenols, carotenoids, and chlorophylls from myrtle seeds powder.

Comparison between enriched and non-enriched soybean oils

The highest phenolic (103.09 ± 7.22 mg GAE/kg of oil), carotenoid (1.96± 0.90 mg/kg oil), and chlorophyll (1.88 ± 3.97 mg /kg oil) yields were obtained in enriched soybean oil using UAE under the optimal conditions which are a temperature of 30°C and a time of 32.5 min, compared to non-enriched oil (control) (Table 6).

Table 6. Comparison between soybean oil enriched with bioactive substances from *Myrtus communis* L. using ultrasound-assisted method and the control oil.

	Enriched soybean oil	**Non-enriched soybean oil**
TPC (mg GAE/ Kg oil)	103.09 ± 7.22^{a}	15.80 ± 1.52^{b}
Carotenoids (mg/Kg)	1.96 ± 0.90^{a}	0.48 ± 0.003^{b}
Chlorophyll (mg/Kg)	1.88 ± 3.97^{a}	0.55 ± 0.66^{b}
DPPH scavenging assay (%)	78.00 ± 0.15^{a}	26.00 ± 0.02^{b}
Iron reducing power (Absorbances at 700 nm)	0.12 ± 0.01^{a}	0.08 ± 0.01^{b}

Each value in the table is the mean ± standard deviation (n = 3). Values in the same line sharing different letters are significantly different ($p < 0.05$). Results are ranked in ascending order; a > b.

The total phenolics estimated in the studied enriched oil were superior to those determined by Sousa *et al.*(2021) who enriched sunflower oil with *Pelvetia canaliculata* and *Crithmum maritimum* using the UAE method. This can be attributed to the low solubility of the phenolic compounds found in these plants, which depends on the extraction method, their degree of polymerization, the interaction with other constituents, as well as the type of effect of the solvent used. The chlorophyll content was also higher than that recorded in sunflower oil supplemented by *Crithmum maritimum* L. (0.03 ± 0.01 mg /kg oil) (Sousa *et al.*, 2021). Nevertheless, the carotenoid content of the oils increased significantly by increasing the incorporation of dried sea buckthorn by-products (Corbu *et al.*, 2020).

Similarly, sunflower oil supplemented by *Pelvetia canaliculata* using UAE had the best chlorophyll content (25.84 to 72.11 mg/kg oils) (Sousa *et al.*, 2021). These results are also similar to those found by Goula *et al.* (2017) who enriched sunflower oil with pomegranate wastes using UAE.

The antioxidant activity of enriched oil was determined using DPPH and reducing power tests. The results of the anti-free radical capacity of the oils (enriched and control) extracts, indicated that enriched refined oil using UAE extraction appeared to be more potent in scavenging the $DPPH^{\bullet}$ radical (78.00 ± 0.15%) than the non-enriched (control) oil (26.00 ± 0.02%), which correlates positively with its higher concentration of antioxidants. Moreover, in reducing power assay, the results showed that the enriched refined oil has higher iron-reducing effect than the control. The higher reduction power explains the higher UAE yield of phenolic compounds in enriched oil.

The enriched oil by UAE had a new composition of phenolic compounds from myrtle extract with better antioxidant properties. Effectively, the concentration of total polyphenols in myrtle seeds was comprised between 25.25 and 147.56 mg GAE/g (Wannes and Marzouk, 2016; Jabri *et al.*, 2017) which were mostly extracted with UAE in soybean oil.

The antioxidant activity of myrtle could be mainly due to the presence of galloyl derivatives compounds. According to UHPLC-DAD-ESI-MSn analysis of UAE myrtle pericarp extract, myricetin-O-galloyl-hexoside, myricetin 3-(6”-O-galloyl galactoside) and flavonols, particularly myricetin-O-hexoside and myricetin-O-deoxyhexoside were the prevalent phenolic components (Bouaoudia-Madi *et al.*, 2019). Moreover, carotenoid and chlorophyll contents, besides their participation in the coloring of fruits, vegetables, and oils, are bioactive compounds that have antioxidant activity. Therefore, Fadda *et al.*(2022) stated in their review study that carotenoids extracted from tomato peel by-products stabilized refined olive and sunflower oil against oxidation occurring during long storage periods. Ultrasound-assisted extraction is a new process that permits the enrichment of soybean oil by myrtle seeds by-products, without any intermediate steps and without using any other solvent. Its effectiveness is due to the phenomenon of cavitation which accelerates the release of bioactive plant components by rupture of the cell wall and intensification of mass transfer (Kumar *et al.*, 2021).

Conclusions

This work presents the potential of an ultrasound-assisted enrichment of vegetable oil (soybean oil) with myrtle seeds using the RSM methodology. According to the result of the response surface and prediction by this built model, the optimal conditions that give the higher total phenolic, carotenoid, and chlorophyll contents were 30 °C for temperature, 32.5 min enrichment time using a power of 100 W, and frequency of ultrasounds bath of 20 kHz. The ultrasound-assisted process greatly facilitates the enrichment of soybean-refined oil in bioactive compounds (phenolic, carotenoids, and chlorophylls) since these constituents contained in the myrtle seeds were rapidly extracted into the refined oil. This made it possible to have the best

enriched oil with significantly higher total phenolic, carotenoid, and chlorophyll contents compared with non-enriched oil. In addition, the antioxidant activity correlated positively with bioactive compounds content with a higher DPPH$^{\bullet}$ scavenging effect and iron-reducing power. This novel research showed that UAE, as a green extraction technology, was effective by increasing the antioxidant activity of soybean oil through its enrichment with bioactive compounds of myrtle.

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Conflicts of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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