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ORIGINAL ARTICLE

Application of Microwave-Assisted Method for Lutein Extraction from Pistachio Waste

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K E Y W O R D S

Lutein extraction;
MAE method;
Pistachio hull;
RSM

A B S T R A C T

Lutein is a xanthophyll family of carotenoids, found in flowers, vegetables, and fruits either in esterified or non-esterified fatty acid form. It is mainly administered in pharmacological products, dietary additives, the food industry, and animal feeding industries. This study was conducted on the 'Fandoghi' variety from the Markazi province for pistachio hull lutein extraction and quantification. This study aimed to assess the lutein in pistachio hull and optimize its extraction protocol by new extraction methods with emphasis on microwave-assisted method (MAE). The powder from dried pistachio hulls obtained from fresh raw un-hulled pistachios was applied for further analysis. An experimental design based on the central composite design was applied for the extraction using the MAE method and extraction optimization. The lutein contents were quantitatively analyzed using a validated LC-MS/MS method. According to the free form of lutein, Ethyl acetate was applied as an extraction solvent with the MAE method followed by the setting up of the extraction time, temperature, and solvent/sample ratio as variables. Under optimal experimental conditions corresponding to 5 min extraction time at 40°C, and 30 mg ml^{-1} of the solvent/sample ratio, the amount of lutein obtained from dried pistachio hull was 3.86 mg 100 g^{-1}. The MAE method is a green, time-saving, and cost-effective method for lutein extraction from pistachio hull that can be suggested for lutein extraction from other plant materials and it can be applied in industrial scale.

Introduction

Carotenoids are tetraterpene pigments predominantly retrieved from yellow-orange fruits, green-leaf vegetables, and marigold flowers, which play a crucial role in photoprotection and photosynthesis (Walsh *et al.*, 2015). Over 1100 fat-soluble pigments of carotenoids confer the color of plants and antioxidant activity, which are found in all photosynthetic plants such as leaves, flowers, fruits, and roots (Hajare *et al.*, 2013). They are increasingly gaining attraction for marketing all over the world due to their antioxidant properties, biological, and pharmacological properties, in which there is about a 5.7% increase in annual marketing rates and lutein, a main family of carotenoids, approximately takes 23% of global marketing (McWilliams, 2018; Cheniany *et al.*, 2013). The current commercial lutein fatty acid ester is sourced from the flower of the *Tagetes* genus, and half of its weight consists of esterified fatty acid

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and must be removed using saponification to obtain pure lutein (Zhao *et al.*, 2022); hence, the microalgae takes over the lutein source due to higher lutein content and biomass productively (Leong and Chang, 2023). However, the main drawback in microalgae is that different cultivation conditions depend upon particular strains (Wolf *et al.*, 2021). So, other sources are considering obtaining higher amounts of lutein with less and inexpensive procedures.

Lutein is a xanthophyll family of carotenoids found in flowers, vegetables, and fruits either in esterified or non-esterified fatty acid form. It is mainly administered in pharmacological products, dietary additives, food, as well as animal feeding industries (Lin *et al.*, 2015). It is also applied in medicine with its antioxidant properties, mitigating some life-threatening diseases such as cancers, age-related macular degeneration (AMD), and cardiovascular disease (Ochoa Becerra *et al.*, 2020). Lutein is also applied as a natural food colorant thanks to its intense yellow color. So, to obtain the highest amount of lutein with higher antioxidant activity, accompanied by bio-accessibility and bio-availability, some elements should be considered during processing, such as processing level, food matrix composition, interaction with other components, absorption rate, and delivery system (Ochoa Becerra *et al.*, 2020; Jahanbani *et al.*, 2021).

Pistachio nuts (*Pistacia vera* L., family Anacardiaceous) are largely cultivated in Iran, China, Syria, Greece, Italy, Turkey, Tunisia, and the USA (Nazoori *et al*., 2022). It is wrapped with green hulls and shells consisting of about 45% agricultural byproducts because their value is neglected, it causes a problem for the environment, or they are applied as animal feed (Hamed *et al.*, 2020). So, to tackle this problem, recently, researchers focused on the bioactive component of pistachio hull, and they found that these green by-products are a source of fatty acid, carotenoids, chlorophyll, tocopherol, and phytosterol with antioxidant and anti-inflammatory properties (Hamed *et al.*, 2020). An increasing number of clinical studies revealed the beneficial effect of pistachio on human health due to being rich in vitamin K, lutein, potassium, dietary fiber, proteins, phenolic acids, and xanthophyll carotenoids (Roozban *et al*., 2006; Nazoori *et al*., 2024; Sharifkhah *et al*., 2020). It has been confirmed that Iran and the USA account for the largest countries in the production and cultivation of pistachio and produce over 70% of world pistachio. So, they produced a huge amount of by-products annually (Toghiani *et al.*, 2023; Sharifkhah *et al*., 2020). According to various studies, pistachio hull consists of quercetin-3-O-rutinoside, catechin, 4-hydroxybenzoic acid, isorhamnetin-3-O-glucoside, naringin, isorhamnetin-7-O-glucoside, eriodictyol-7-O-glucoside (Sadeghinejad *et al.*, 2019), and protocatechuic acid; Also, lutein accounts as a dominant bioactive product in pistachio hull (Mandalari *et al.*, 2022).

Microwave energy has a considerable efficiency for the rapid heating of material, so the application of microwave-assisted technology (MAE) would be a great option for extracting chemicals from plant material, which is a mild but rapid technique with a high recovery (Low *et al.*, 2022). The principle of this procedure is based on the direct emission of microwave energy on molecules by dipole rotation and ionic conditions. While microwave contributes to the electromagnetic migration of ions, the liquid phase of the material resists the electromagnetic fallow, resulting in breakage of solid materials (Hu *et al.*, 2021). These properties outweigh other conventional extractions of lipids and lutein from plants. According to various studies, the MAE method successfully extracted the lutein and lipids from dried microalgae (Low *et al.*, 2022).

As mentioned before, Iran accounts as the main source of pistachio in the world. To the best of our knowledge there isn't any data regarding the administration of pistachio hull as an agricultural by-product in food industries, health, and pharmaceutical fields, and taking into consideration that pistachio hull is rich in lutein content, in the current study, we the

investigated 'Fandoghi' variety from Markazi province for pistachio hull lutein extraction and quantification. Our study aimed to assess the lutein in the pistachio hull's content and optimize its extraction protocol by new extraction methods with emphasis on the microwave method. Lutein extraction would increase the nutrient value of this natural by-product and have economic benefits and positive environmental effects.

Materials and Methods

Chemicals and Plant Material

The pistachio hull was obtained from the "Fandoghi" variety harvested from the Markazi province of Saveh City. A standard lutein with above 95% (UV) purity was purchased from Extrasynthese (Lyon-France). Isopropanol, Ethyl acetate, Acetonitrile (ULC/MS- CC/SFC grade), and Formic acid 99% (ULC/MS- CC/SFC grade) were purchased from Biosolve (Dieuze, France). Other chemical materials applied in the current study were purchased from Sigma-Aldrich (St. Louis, MO, USA). The fresh raw un-hulled pistachios ('Round' or 'Fandoghi' variety) were harvested in September 2020 from Saveh City (Markazi province, Iran) following the dehulling 24 h after harvesting and were dried at 62 ˚C for 4h in a convection drier. Then, dried hulls were finely powdered with an electric blender and stored at -26 ˚C until use. All the experimental procedure was performed at the Department of Food Science and Technology, Science and Research Branch, Islamic Azad University, Tehran, Iran.

Experimental Design for MAE and Analysis of the Effect of Parameters using Response Surface Methodology (RSM)

The experimental design was carried out using Design-Expert software (Version 8.0.7.1, stat Ease, Inc., USA) to check the parameters leading to optimum lutein extraction condition with higher quality, time-- saving, and cost-effective experiments, as well as calculating the minimum level of experimental procedures for microwave extraction. Therefore, the RSM method based on the central composite design (CCD) was applied to identify the effect of three independent variables, including time (min), solvent-to-solid ratio (ml g^{-1}), and temperature (C°) on lutein extraction using the MAE method which is represented in table 1. Also, the elements and the consequence of various variables on the response were evaluated individually. Consequently, the three most significant variables that affected the microwave extraction method were selected, remaining variables were retained, and others were kept sustained. The ranges of given variables are indexed in Table 2.

Table 1. Experimental parameter ranges for the MAE method

Parameters	Ranges	Units
Tme	1-9	Min
Solvent/ solid ration	20-40	ml gl^{-1}
Temperature	20-60	C^{o}

Furthermore, the actual and coded levels of these variables are represented in Table 2 using the regression equation:

$$xi = \frac{(x_i - x_{i0})}{\Delta x_i}$$

Xi= independent variable coded value

X_i= independent variable real value

X_{i0}= independent variable real value on the center point

Δx_i= in interval, I (1,2,3)(Roriz *et al.*, 2009).

In the following, the quadric model equation was applied to explain the mathematical relation between the response function and with independent variable using this equation:

$$Y = \beta_0 \sum_{i-1}^{K} \beta_i x_i + \sum_{i-1}^{K} \beta_{ii} x_i^2 + \sum_{i-1}^{K-1} \sum_{j-2}^{K} \beta_{ij} x_i x_j + \varepsilon$$

Y= predicted response

$\beta_{i=}$ linear coefficient

$\beta_{ii=}$ interaction of coefficient

X_i .X_j= coded factors (Aghaie *et al.*, 2009)

Table 2. Ranges and designed levels of the variables.

Variable	Factor code	Level and range (coded)			
		-1	+1	-alpha	+alpha
Time	A	3	7	1	9
Solvent to solid ratio	B	25	35	20	40
Temperature	C	30	50	20	60

Furthermore, the graphical analysis was obtained from RSM to ascertain the desirable compound of variables. The preciosity of the model in fitting the polynomial equation was assessed using multiple correlation coefficients, R^2, and adjusted R^2.

Selection of optimal extract solvent for pistachio hull lutein

According to Grace *et al*., which indicated that the lutein in the pistachio hull is free from(Grace *et al.*, 2016), and due to the non-polarity of lutein, the different alcoholic solvents were applied to obtain the proper one for optimal extraction. Thus, two gr of dried pistachio hull were dissolved in 10 ml of ethyl acetate, isopropanol, and hexane following heated at 40˚C for 4 h using magnetic stirring and then passed through a 0.45 µm microporous membrane to remove any impurities. The drying procedure was performed using a rotary evaporator and a gentle stream of nitrogen. Accordingly, 3 mg of oleoresin was dissolved in 1 ml methanol, and 100 µl was applied for LC-MS/MS analysis. Finally, the most efficient solvent for further analysis was selected based on the maximum amount of extracted lutein.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Instrumentation

The chemical component pistachio hull, was analyzed to identify the lutein content using Liquid Chromatography- tandem Mass Spectrometry (LS-MS/MS) using Agilent 1290 Infinity series LC system from Agilent (Paolo Alto, CA, USA), and detection of mass was performed with a 6500 QTrap® triple quadrupole linear ion trap mass spectrometer equipped with electrospray ionization from AB Sciex (Darmstadt, Germany). The chromatographic separation was carried out using a Kinetex® C18 column (50 × 2.1 mm, 7 µm) (Phenomenex company, Brechbühler, Switzerland).

Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile heating at 40˚C. Gradient elution was prepared at a 500 µL min^{-1} flow rate according to the following: 0–0.4 min 15% B, 0.4–10 min from 15% to 95% B, 10–12 min 95% B, 12–12.1 min 15% B and 15% B until 16 min to equilibrate the column. The positive model detection was applied for lutein detection monitoring with two main transitions of lutein (551.4>90.9 and 551.4>104.9). The optimized de-clustering potential (DP) was 151 V for both transitions, and the optimized collision energy (CE) and cell exit potential (CXP) were respectively (121, 93 V) and (14, 16 V). Values of QTrap parameters are as follows: curtain gas =40 psi, collision gas =high, Ion Spray voltage =4500 kV, temperature =450 ˚C, ion source gas 1 =60 psi, ion source gas 2 =60 psi.

Experimental design for micro-wave assistant extraction of lutein

There are various techniques for extracting carotenoids, including lutein, which consists of liquid extraction, supercritical fluid, microwave-assisted

methods, and ultrasonic-wave-assisted methods (Hsu *et al.*, 2011; Liu *et al.*, 2011; Kang *et al.*, 2016). MAE technique applies microwave energy, which is electromagnetic radiation, to heat the solvent and break down the chemical by disrupting hydrogen bonds and penetrating solvent into the matrix and better solvation of the analyte (Kaufmann and Christen, 2002). Administration of the MAE technique outweighs other procedures in terms of using microwave radiation to rapid increment in the evaporation of raw material, less energy consumption, and easier post-extraction procedure due to a lower amount of solvent for extraction (Reddy *et al.*, 2020). There are two types of instruments predominantly applied in the MAE technique: extraction using a closed vessel with fixed and controlled pressure and temperature and an open-focused vessel system using atmosphere pressure. A closed Vessel system is highly recommended for digestion, mineralization using acid, and vigorous conditions for extraction with enhanced extraction speed and better efficacy(Kaufmann and Christen, 2002).

The Micro synth wave microwave (Sorisole (BG). Italy) was applied to extract lutein from pistachio hull dried powder based on closed vessel systems. We used the different range of solvent/ solid ratio, as well as different time and temperature obtained from Eskilsson *et al*.'s review article, which was as follow: time ranged from 3-9 min, solvent/ solid ratio ranged from 25-40 ml g^{-1}, and temperature ranged from 30-60ºC (Eskilsson and Björklund, 2000). Then, the level of experimental procedure using MAE was obtained using RSM as described earlier.

Statistical analysis

The effect of variables and their interaction were determined using Design-Expert software. Comparison of experimental design set as well as determination of significant model using RSM method and non-significant values were obtained using one-way ANOVA test and significance of experimental analysis was verified while $P < 0.05$. All experiments were carried out in triplicate, and quantitative data were expressed as mean ± standard deviation (SD. $p < 0.01$) was considered a statistically significant value. Finally, the 3D model of interaction between variables was indicated using response surface three-dimensional (3D) plots.

Results

Selection of optimal extract solvent for pistachio hull lutein

Considering the free form of the lutein, which was confirmed by Grace *et al*., we applied three different polar solvents, including ethyl acetate (polar), isopropanol (high polar), and hexane (non-polar), to 2 gr of dried pistachio hull and heated at 40˚C for 4 h while stirring; the impurities were removed using a 0.45 µm microporous membrane. The obtained supernatant was dried using a rotary evaporator. Then a gentle stream of nitrogen to obtain the oleoresin for LC-MS/MS procedure. 3 mg of obtained oleoresin was dissolved in 1 ml methanol in which 100 µl of solution was injected into the spectrometry. According to LC-MS/MS analysis, the amount of lutein extracted by ethyl acetate, isopropanol, and hexane were 3.58, 1.82, and 1.66 (mg $1000gr^{-1}$ dried pistachio hull), respectively, indicating that ethyl acetate accounts for as the most efficient solvent to have a high yield of lutein. Thus, ethyl acetate solvent was selected for lutein extraction by ultrasound-assisted method.

Microwave-assisted method

Microwave treatment optimization of lutein extraction using ethyl acetate was carried out using the RSM method. Our independent variables were extraction temperature, solvent-to-solid ratio, and time to define the CCD matrix. Seventeen defined experimental levels were then assayed by the LC-MS/MS method to identify the most efficient conditions for lutein extraction. The list of experimental levels and the obtained results are

represented in Table 3.

Microwave-assisted method optimization using RSM

To obtain an ideal condition for MAE extraction, we applied a central composite design (CCD) to evaluate the consequence of applying each variable to the microwave extraction as a retaliation. Table 3 shows the experiments' arrangement, the actual amounts of response (microwave extraction), and the designed levels of the variables investigated in the current study. The independent variables selected were A: Time, B: Solvent to solid ratio, and C: Temperature of microwave extraction, respectively; the microwave extraction was obtained in the range of 2.26 to 3.86 mg lutein per 100 gr dried pistachio hull. A total of 17 experiments were conducted separately to get the experimental response of microwave extraction.

Table 3. Factors in actual form

mg lutein per 100 gr dried pistachio	C*** (ºC)	B** (ml g^{-1})	A* (min)
2.5	30	25	3
3.33	50	35	7
3.86	40	30	5
3.4	50	25	7
	40	30	5
3.06	50	35	3
3.15	40	30	9
3.08	0	25	7
3.15	40	40	5
3.3	30	35	3
3. 08	60	30	5
2.26	40	30	1
2.61	40	20	5
2.82	20	30	5
3.26	30	35	7
2.62	50	25	3
3.77	40	30	5

*: Time; **: Solvent to solid ratio; ***: Temperature

A quadratic polynomial model fitted the response function. The obtained equation is demonstrated in both actual and coded forms in low equations.

Conditions for microwave extraction= +3.79+0.2106A+0.1519B+0.0494C-0.1413AB+0.0637AC-0.0762BC-0.2744A^2-0.2307B^2-0.2132C^2

The final equation in terms of actual factors:

Conditions for microwave extraction=-14.58071+1.08757A+0.7155650B+0.205272C-0.014125AB+0.003188AC-0.001525BC-0.068601A^2-0.009226B^2-0.002132C^2

Where A, B, and C, are Time, Solvent to solid ratio, and Temperature, respectively. The equation in terms of actual factors can be used to predict the response for given levels of each factor. Here, the

levels should be specified in the original units for each factor. This equation should not be used to determine the relative impact of each factor because the coefficients are scaled to accommodate the units of each factor, and the intercept is not at the center of the design space.

The analysis of variance (ANOVA) is a statistical calculation to determine the significance of the designed model and regression coefficient. The capability of rejection probability (Model p-value), R^2, adjusted R^2, predicted R^2, and adequate precision are being analyzed. The result of the ANOVA test on the fitted model is indexed in Table 3. The valid model is defined based on the obtained P-Value that must be lower than 0.05, and the lower p-value would be the best corresponding fitted model. As shown in Table 3, four independent variables have a significant impact on the response, although time has a foreground effect on the MAE method (Table 4). Furthermore, a significant interaction between time and temperature has been observed in the MAE method, although interaction between time and the solvent solid ration (AB), solvent to solid ration and temperature, along with adjusted R^2 in time (A^2), solvent to solid ratio (B^2), and temperature (C^2) are significantly related to optimized MAE extraction outcome. Additionally, the F-value of 137.43 is obtained with 95% confidence, indicating the acceptable scoring among experimental procedure and proposed model. There is only a 0.01% chance that an F-value this large could occur due to noise indicating a lack of significant response confirming the preciosity of the designed model with minimum regression errors.

Table 4. Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of extraction parameters in MAE

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3.28	9	0.3646	137.43	< 0.0001	significant
A-Time	0.7098	1	0.7098	267.56	< 0.0001	
B-Solvent to solid ratio	0.3691	1	0.3691	139.11	< 0.0001	
C-Temperature	0.0390	1	0.0390	14.70	0.0064	
AB*	0.1596	1	0.1596	60.17	0.0001	
AC**	0.0325	1	0.0325	12.26	0.0100	
BC***	0.0465	1	0.0465	17.53	0.0041	
A²	1.46	1	1.46	549.73	< 0.0001	
B²	1.03	1	1.03	388.41	< 0.0001	
C²	0.8800	1	0.8800	331.70	< 0.0001	
Residual	0.0186	7	0.0027			
Lack of Fit	0.0132	5	0.0026	0.9756	0.5764	not significant
Pure Error	0.0054	2	0.0027			
Cor Total	3.30	16				

***** Time and Solvent-to-solid ratio; ******Time and Temperature; ******* Solvent-to-solid ratio and Temperature

Validation of the models for MAE

The coefficient of determination (R^2), adjusted R^2, and predicted R^2 of the model were found at 0.99, 0.98, and 0.96, respectively. These values show a good agreement between the model and actual results. Another criterion to ensure the model's validity was the prediction error of the model. For this purpose, the model's prediction error for the experimental result was calculated for the case with a maximum

microwave extraction. The maximum microwave extraction was obtained at 5 min, Solvent to solid ratio of 30 ml g^{-1}, and Temperature of 40 **ºC**. For this case, the experimental and predicted microwave extraction was 3.86 and 3.79 mg. The prediction error was 0.034, less than 5%, and confirmed the model's validity in predicting and fitting the experimental data. The graph of the predicted and actual values of microwave extraction is shown in Fig. 1. The predicted values were found to be quite close to the experimental values, which confirmed the acceptable accuracy and validation of the model developed for establishing a correlation between the process variables and the microwave extraction. Additionally, the ratio of the distributed diagram of the predicted to the Distributed Diagram of the actual was about a 45-degree line, indicating the proper assessment of the RSM model. According to Fig. 1 amount of obtained predicted and actual data is the 45-degree line, and the suggested regression to estimate data could successfully fit and predict data with high precision.

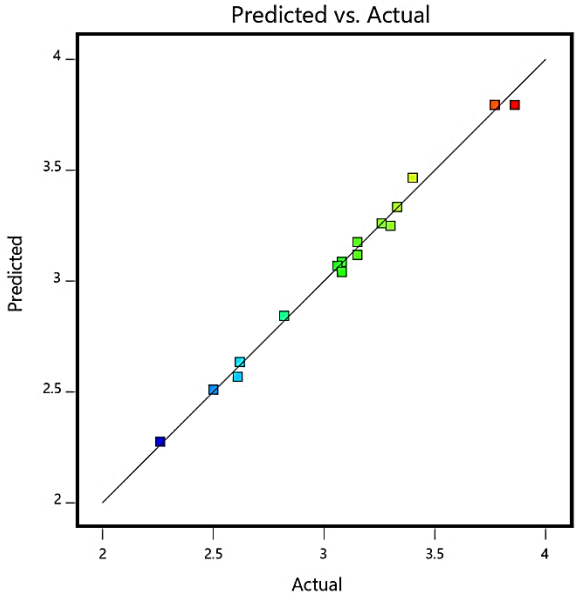

Fig. 1. Comparison between model-predicted and actual values of size for RSM mode

Effect of process parameters on the microwave extraction

operational conditions

The superlative values of the independent variables and their effect on the response were obtained using the regression equation and evaluating the response surfaces and contour plots. The synergistic effect is indexed with a positive sign, whereas the antagonistic effect is indexed with a negative sign. Considering the sign of the coefficients in Equations 4 and 5, it is clear that the microwave extraction increased with an increment in the time, solvent-to-solid ratio, and temperature. The two-dimensional contours or three-dimensional (3D) response surface plots indicate the association between the variable and the MAE method. Fig.2 shows the response surface 3D diagram presenting the effect of interaction between time and solvent to solid ratio (AB), time and temperature (AC), and solvent to solid ratio and temperature (BC), which have a significant effect in improving the microwave extraction.

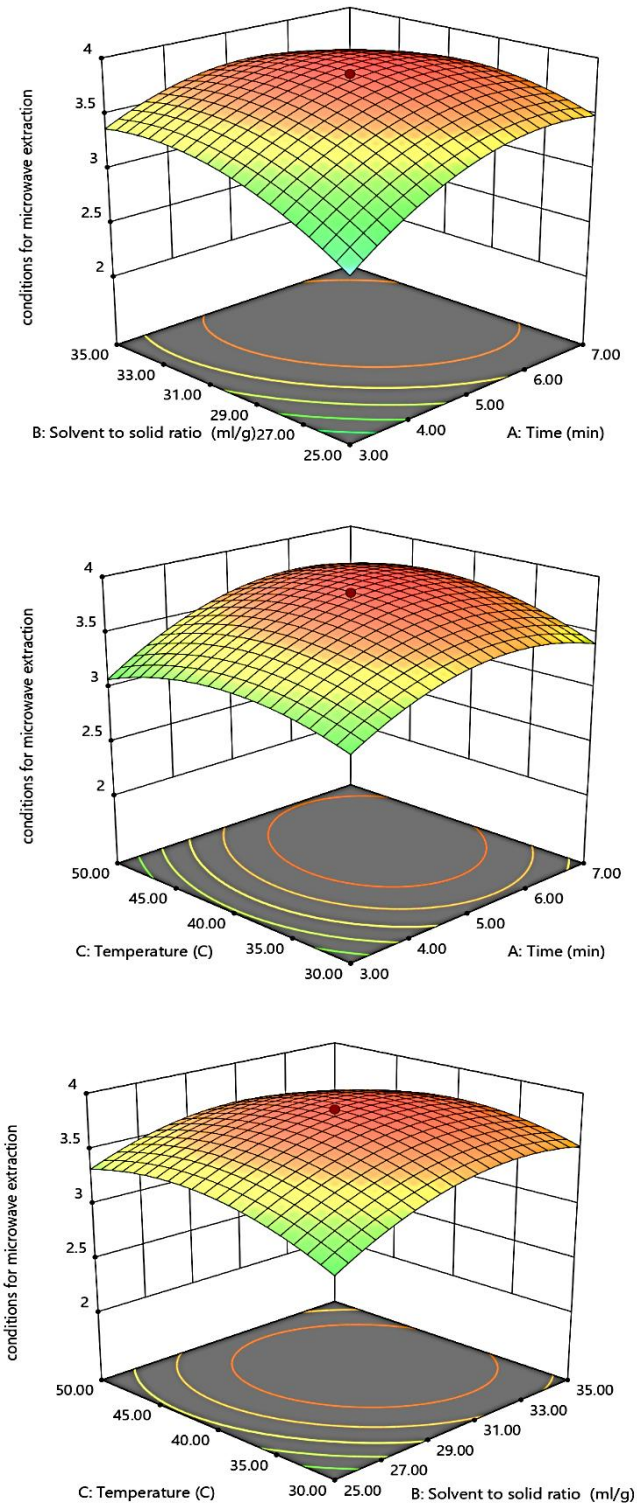

Fig. 2. response surface 3D diagram for the effect of interaction between Time and Solvent to solid ratio (AB), Time and Temperature (AC), and Solvent to solid ratio and Temperature (BC)

According to ANOVA test results and results obtained from equations microwave extraction and Conditions for microwave extraction, the maximum microwave extraction was obtained using the increase in the time, solvent to solid ratio, and temperature.

Optimization and model validation

The model optimization criteria obtained from the

software is shown in Table 5 shows, which, according to the data the optimum value for the microwave extraction as a response is obtained to gain the maximum microwave extraction efficiency. The suitable function approach was one of the most frequently applied in multi-response optimization techniques in practice and which originally had been developed by Harrington (Yeong *et al.*, 2009). The desirability lies between 0 and 1, representing the closeness of a response to its ideal value. Generally, total desirability is defined as the geometric mean of individual desirability (Raissi, 2009). The optimum conditions were regarding the maximum microwave extraction. The optimum extraction was obtained at 3.79 mg under experimental conditions corresponding to 5 min, 30 ml g^{-1}, and 40 ˚C for time, solvent to solid ratio, and temperature, respectively. The experimental response to the optimized microwave extraction condition was 3.86 ± 0.42 nm. While the predicted error is lower than 5%, the designed model is considered a valid model for MAE extraction. Furthermore, all synthetic model from statistical analysis is by experimental results, and the average error is ±1.01%. MAE extraction was carried out using the obtained condition and prediction error less than <0.05, authenticated the validity of the model.

Table 5. The optimized conditions listed.

Number	A* (min)	B**(ml g^{-1})	C*** (ºC)	Extract (mg)	Desirability	
1	5.00	30.00	40.00	3.795	1.000	Selected
2	3.00	35.00	30.00	3.250	1.000	
3	7.00	35.00	50.00	3.335	1.000	

*: Time; **: Solvent to solid ratio; ***: Temperature

Discussion

Lutein accounts for a xanthophyl family of carotenoids which is widely applied in medicine, food industries, and pharmaceutical products. Lutein supplements have gained much interest from specialists because of their several advantages for human health, as well as pharmaceutical and nutraceutical potentials (Wang *et al.*, 2006; Boonnoun *et al.*, 2012). Lutein is presented either in the form of lutein fatty acid esters or free from which only free from of lutein can be up-taken by humans (Khachik *et al.*, 1986; Khachik, 2001).

The Pistachio hull is a good lutein source, so we applied the Iranian pistachio hull to evaluate the amount of lutein and find the optimum condition for extraction of lutein using MAE. According to the data obtained from a recent paper about the extraction of lutein from pistachio hull by Grace *et al*., we found that lutein is free from and non-esterified (Grace *et al.*, 2016).

The current study revealed that using MAE, ethyl acetate is the best solvent for lutein extraction from pistachio hull. The optimum condition for lutein extraction using MAE is applying 30 ml g^{-1} solvent to solid ration and heating at 40ºC for 5 min.

Caretonid extraction, including lutein, is commonly performed using organic solvents, including isopropanol, diethyl ether, methylene chloride, and hexane (Kumar Kashyap *et al.*, 2022). Hence, most organic solvents are non-renewable, toxic, inflammable, and hazardous. So, researchers administered Hexane as an organic solvent due to low polarity, higher stability, easy removal by evaporation, and appropriate boiling point. However, it is restricted by European Directives and Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH), owing to petrochemical properties of Hexane and other green solvents which were eco-friendly and non-toxic, were introduced for chemical extraction (Kumar Kashyap *et al.*, 2022). Li. *et al.* evaluate the five different solvents, including acetonitrile, ethanol, water, methanol, ethyl acetate, and heptane, to isolate the polar component of lutein

and zeaxanthin using the shake-Flask experiment. The Kemin test indicated that the 5:3:1 volume of n-heptane/ ethanol/ water was the best option for purification using Centrifugal partition choreography (CPC) with a lower settling time, greater separation, and polarity range (Li and Engelberth, 2018). Another study by Boonnoun *et al*. revealed that Hexane: ethyl acetate with a 70:30 volume ratio was the best solvent for lutein separation from marigold flowers using choreography, which they could obtain free lutein with 97.1% purity (Boonnoun *et al.*, 2012). Similarly, the current study confirmed that ethyl acetate is considered the best solvent for lutein extraction from pistachio hull using MAE (Boonnoun *et al.*, 2012).

Microwave-assisted method for lutein extraction

Microwave-Assisted Method Optimization using RSM

Response surface methods are based on surface placement, which detects the maximum, minimum, local, and ridge lines to find the most relevant response and the relation between different conditions, resulting in optimal extraction operation with higher yield. It is used to fit a model and help optimize the effective variables with a minimum number of experiments, and analyze the interaction between the variables. Furthermore, this approach lends itself to analyzing problems in which a response of interest is influenced by several variables and operating conditions (Azargohar and Dalai, 2005; Zahangir *et al.*, 2007). There are two major experimental designing methods, including Box-Behnken design (BBD) and central composite design (CCD), in which the BBD is a spherical design, whereas CCD is a fractional design(Alev Yüksel, 2018). Herein, we apply the CCD designing method because of the highest accuracy and building a second-order quadratic model simpler than three three-level fractional experiments (Bhattacharya, 2021).

Application of traditional extraction approaches such as Soxhlet and maceration are time-consuming and require a large amount of solvent. Currently, some mechanical procedures, including autoclave, high-pressure homogenization, as well as ultrasonic application, pulse field electrophoresis, and microwave-assisted methods, are administered mostly in biological extraction that disturb the cell wall of plants (Low *et al.*, 2022). Low *et al*. revealed that using an optimized microwave-assisted binary solvent for extraction of lutein from microalgae renders the maximum amount of lutein with high quality, better antioxidant activity, with minimum time and energy consumption (Low *et al.*, 2022). Fu *et al*. applied the microwave method using 28% W W^{-1} ethanol, 20% W/W ammonium sulfate, 0.45 U g^{-1} hydrolysis enzyme, and with a microwave power of 270W at 45ºC temperature for 150 min to extract lutein from marigold flower. Based on their experimental condition, they could obtain 7.32 mg g^{-1} lutein (Fu *et al.*, 2018). The current study applied the MAE method for lutein extraction by setting the temperature at 40ºC, 30 ml g^{-1} solvent/ solid ratio, giving the microwave energy for 5 min, and 3.58 mg $1000gr^{-1}$ lutein was obtained from dried pistachio hull.

Effect of parameters on lutein extraction using MAE

Effect of solvent on amount of lutein using MAE

The selection of the proper solvent is a basic step for the optimal extraction process, and in terms of using a Microwave, the solvent should absorb waves emitted from the energy source. Also, the solvent should be compatible with the analytical method. Based on the microwave mechanism, application of a single solvent leads to higher energy absorption (Eskilsson and Björklund, 2000). We applied ethyl acetate as a cheap solvent to extract free lutein from the pistachio hull. Regarding the phenolic compound, ethanol as a polar solvent would be the best choice for the extraction of flavonoids (Georgiopoulou *et al.*, 2023)

Effect of Solvent Volume on the Amount of Lutein using MAE

The proper amount of solvent for sample extraction ranged between 10-30 ml. The volume of solvent is an important factor for efficient extraction and immersing the whole sample in solvent, especially while the hydrocarbons are being extracted from the sedimented sample. A total of 10-30 ml solvent is preferred for 1-5 g solid. According to Fig. 2, the proper solvent-to-solid ratio for higher extraction of lutein using MAE was 30mg ml^{-1}, and the 3.79mgr of lutein was recovered. Furthermore, the solid-to-solvent ratio shouldn't exceed 30-40% (w v^{-1}). While administration of a higher volume of solvent would increase the recovery rate in conventional methods, the higher solvent in the case of MAE has a negative effect on recovery(Eskilsson and Björklund, 2000).

Effect of temperature on the amount of lutein using MAE

Temperature accounts for an important factor for increased recovery applying all extraction techniques. As indicated in Fig. 2, the optimum temperature for lutein extraction using MAE is 40ºC. Occasionally, in the MAE technique, the given temperature is above the boiling point of the solvent, which leads to the increased desorption of analytes from active sites of the matrix. Also, higher temperatures render the higher solubility of solvent and decrease the viscosity and surface tension contributing to penetration of microwave energy to the solid and elevated level of extraction sufficiency (Eskilsson and Björklund, 2000). But herein, we applied a moderate temperature and obtained an optimum amount of lutein, which could prohibit the probability of chemical degradation. Applying a higher temperature contributes to a lower extraction rate.

Effect of time on the amount of lutein using MAE

Microwave energy needs a short time to penetrate the chemicals, so extraction time using MAE is very short compared to other conventional methods. The less extraction time of under 3 min showed a proper extraction efficiency, which prohibits the chance of chemical degradation or alternation of amino acid, which may occur application of microwave energy for a longer time, especially for thermolabile components (Eskilsson and Björklund, 2000). As is observed in Fig. 2, the optimal time for extraction of lutein using a microwave was 5 min to obtain the 3.7 mg lutein. Increment of microwave emission time contributes to the chemical degradation of lutein, and a lower amount of lutein is obtained.

Problem regarding using the MAE technique

On the right side of the microwave is the temperature sensor illustrating the temperature of the solution. At the first level, the temperature went up dramatically and even passed the desired temp. Then went down instantly While the independent variables were set as time: 5 min. solvent/ solid ration: 30mg gr^{-1}. Temperature: 40 min. To solve this problem, in the second run, we increased the ratio to 40ml g^{-1}, and found that the temperature sensor couldn't detect the temperature due to the lower amount of solvent. Thus, according to the data observed, we concluded that for the proper function of the temperature sensor, the amount of solvent should be more than 40ml g^{-1}, or the height of the balloon in the microwave should be heightened to prevent temperature increment during MAE. Temperature is being set according to the pressure in the vessels. The maximum pressure delivered to the vessels ranges from 600-1000W, indicating setting up the proper temperature to avoid excessive heating and degradation of solids (Kaufmann and Christen, 2002).

Another issue regarding MAE was inner temperature and reaching out the given temperature, which lasted about 30 sec. It should be mentioned that microwave uses the highest energy to reach the desired temperature in a given time, contributing to an increment of temperature higher than the desired one.

So, we increase the extraction time to 6 min to overcome this bias. It should be considered that the lower the temperature, the more bias and error would be observed, and the optimum temperature for microwave would be between 50-60°C (Talebpour *et al.*, 2009). In the current study, we find the optimum temperature at 40ºC, using 30 ml of solvent, and timing at 5 min with the highest lutein yield (3.86 mg per 100gr dried pistachio hall).

Conclusions

Currently, the administration of natural products in pharmaceutical marketing, medical applications, and food industries has gained attraction. Lutein, as one of the carotenoids, has an antioxidant property that is mostly applied in medicine and food substances. The agricultural by-products could be problematic for the environment. Iran accounts as a main source of pistachio in which the by-products of harvested pistachio are mostly applied as farm animal feed. Here, we investigated the importance of pistachio hull in terms of lutein's richness. So, this is the first experimental study on the lutein content of the Iranian pistachio hull. Using RSM as a time-saving and cost-effective way to develop the optimized extraction condition using microwave-assisted experiments. Our results showed that ethyl acetate is the most efficient solvent to obtain a high lutein yield from the pistachio hull. Our study also found the optimum Microwave-assisted method conditions for lutein extraction using ethyl acetate. Using the optimum conditions in this method effectively decreased the processing time, leading to a high-quality product. Therefore, the microwave-assisted method is suitable for lutein extraction from pistachio hull and can be scaled to industrial processes for lutein extraction and use in the pharmaceutical, cosmetic, and food industries.

Conflict of interest

All authors declared that they have no conflict of interest

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