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RESEARCH PAPER

Optimization and Economic Evaluation of Ultrasound Extraction of Lutein from *Chlorella vulgaris*

Aree Deenu, Srisuwan Naruenartwongsakul, and Sang Moo Kim

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Abstract The main carotenoid in *Chlorella vulgaris* is lutein. The ultrasound alone or together with enzymatic pretreatment for the extraction of lutein from *C. vulgaris* was optimized using response surface methodology (RSM) to improve the extraction process. The optimal ultrasound extraction condition was: ultrasound frequency, 35 kHz; ultrasound intensity, 56.58 W/cm^2; extraction temperature, 37.7°C; extraction time, 5 h; and ratio of solvent to solid, 31 mL/g, where the lutein recovery was 3.16 ± 0.03 mg/g wet *C. vulgaris*. The optimal enzymatic pretreatment was: reaction time, 2 h; enzyme concentration, 1.23% (v/w); pH, 4.5, and temperature 50°C. The optimal ultrasound extraction with enzymatic pretreatment was: ultrasound frequency, 35 kHz; ultrasound intensity, 56.58 W/cm^2; extraction temperature, 37.7°C; extraction time, 162 min; and ratio of solvent to solid, 35.6 mL/g wet *C. vulgaris*, where the extraction yield of lutein was 3.36 ± 0.10 mg/g wet *C. vulgaris.* This was much higher than for ultrasound treatment alone. The surface areas of microalga cells treated by ultrasound with/without enzymatic pretreatment increased significantly, which might contribute to the increase in lutein yield. There were no significant differences in structure, color, and antioxidant activity of lutein between the ultrasound and conventional methods. The highest cost of the crude and lutein was obtained by the ultrasound with enzymatic pretreatment due to the complex process and liquid waste in the enzymatic pretreatment process, but the ultrasound treatment alone was the lowest. Therefore, ultrasound extraction is the most economical method for the extraction of microalgal lutein.

Keywords: *Chlorella vulgaris*, economic evaluation, lutein, microalgae, optimization, ultrasound

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1. Introduction

Lutein, a carotenoid belonging to the xanthophylls family, is an essential component of the macular pigment in the eye retina. The low level intake of lutein results in the risk of age-related macular degeneration (AMD) and cataracts [1]. Moreover, lutein is recommended to prevent some types of cancer and cardiovascular diseases. Lutein is also a food colorant. Sales of lutein as a feed additive in the USA amount to about $150 million per year. The product of lutein from plant, especially marigold, is limited due to its very low concentration, 0.03% of dry weight. Therefore, several microalgae with higher lutein content, such as *Muriellopsis* sp. and *Chlorella* sp., have been used as a lutein source [2].

Chlorella vulgaris is a unicellular microalga that contains many bioactive compounds, such as protein, vitamins, polysaccharides, chlorophyll, and carotenoids. Microalgal cell walls are known to consist of multiple layers [3]. The cell wall of *C. vulgaris* was composed of two major constituents, hemicelluloses (25%) and alkali-insoluble rigid wall (65%) [4]. Many researchers have reported the health benefits of *C. vulgaris*, such as immune response, hampering cataract and atherosclerotic development [5-8]. The main carotenoid in *Chlorella* sp. is lutein, which is not only a food supplement but also an important natural food colorant.

The extraction is a very important stage for the production

of microalgal lutein. The recovery of this component is commonly performed through solvent extraction in which the concentration of solvent, time, and temperature are important parameters. Ultrasound, supercritical fluid, pressurized liquid, and microwave extractions are novel extraction techniques. Among these, ultrasound extraction is inexpensive and economical. It is a simple and efficient alternative to conventional extraction techniques due to its low instrumental requirements. The enhancement in the extraction yield by ultrasound is due to the effect of acoustic cavitations produced in the solvent by the passage of an ultrasound wave [9].

Ultrasound extraction has been applied to extract the components from plant and animal materials, such as oil, protein, polyphenolics, and pigments. The yield of carotenoids extracted from *Dunaliella salina* by ultrasound was higher than that by supercritical fluid extraction [10]. The yield of lutein from *C. vulgaris* by ultrasound was the highest when compared with maceration, soxhlet extraction, and pressurized liquid extraction [11].

In this study, ultrasound extraction with/without enzymatic pretreatment was optimized by response surface methodology (RSM) to obtain the optimal conditions for the extraction of lutein from *C. vulgaris.* Moreover, the effect of ultrasound on the morphology of the microalgal cell, the degradation and the antioxidant activities of lutein were also determined. Furthermore, the economic feasibility was evaluated to compare the extraction methods in terms of cost of manufacturing (COM).

2. Materials and Methods

2.1. Materials

Chlorella vulgaris purchased from Dae-sang Company (Incheon, Korea) was stored at -80°C until used. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), viscozyme, and standard lutein (purity minimum of 99%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The other chemicals used were of analytical grade.

2.2. Extraction

2.2.1. Ultrasound extraction

Ultrasound extraction was performed in a penetration 4-chanel ultrasonic generator (Flexonic-500, Mirae Ultrasound Technology, Bucheon, Korea) with fixed frequency of 35 kHz and intensity of 56.58 W/cm^2. One gram of *C. vulgaris* with 90% ethanol was put in a glass flask, and then the flask was held in the ultrasonic tank to extract at different times, temperatures and ratios of solvent to solid. Optimization of the ultrasound extraction condition was accomplished by employing the response surface methodology (RSM) with a Box-Behnken design (BBD). Three different factors (extraction temperature, extraction time and ratio of solvent to solid) were employed at three equidistant levels (-1, 0, and +1) (Table 1). The extract was filtered through Whatman No. 2 paper and finally stored at 4°C in the dark for further analysis.

Table 1. The experimental design for the ultrasound extraction of lutein from *C. vulgaris* (n = 3)

Run	Extraction conditions			Lutein (mg/g)	
	X_1, temperature (°C)	X_2, time (h)	X_3, solvent to solid ratio (mL/g)	Actual value	Predicted value
1	30	5	30	2.97 ± 0.07	2.97
2	30	5	30	2.98 ± 0.05	2.97
3	40	2	30	1.94 ± 0.05	1.96
4	30	5	30	2.89 ± 0.12	2.97
5	30	8	10	2.01 ± 0.07	1.98
6	30	5	30	3.06 ± 0.05	2.97
7	20	5	10	1.93 ± 0.05	1.97
8	40	8	30	2.95 ± 0.08	2.93
9	40	5	50	3.12 ± 0.04	3.07
10	20	5	50	2.29 ± 0.002	2.25
11	30	8	50	2.45 ± 0.07	2.52
12	20	2	30	1.40 ± 0.03	1.42
13	30	5	30	2.95 ± 0.06	2.97
14	30	2	10	1.38 ± 0.003	1.32
15	30	2	50	1.56 ± 0.01	1.58
16	20	8	30	2.08 ± 0.06	2.06
17	40	5	10	2.51 ± 0.14	2.55

2.2.2. Ultrasound extraction with enzymatic pretreatment

2.2.2.1. Enzymatic pretreatment

Enzymatic pretreatment of *C. vulgaris* was performed by incubating one gram of microalga in a 30 mL of 0.1 M acetate buffer (pH 4.5), then incubated at 50°C. The optimization of the enzymatic pretreatment condition was determined by response surface methodology (RSM) with a central composite design (CCD). Two independent variables chosen were time (h, A_1) and enzyme concentration {%(v/w), A_2}. The lutein yield was determined as the response variable (Y). The ranges of time and enzyme concentration were 0.6 ~ 3.0 h and 0.4 ~ 2.0% (v/w), respectively. One gram of enzymatic pretreated microalga with 31 mL of 90% ethanol was sonicated at 35 kHz, 56.58 W/cm^2 and 37.7°C for 2 h. The filtered extract was used for further analysis.

2.2.2.2. Ultrasound extraction after enzymatic pretreatment

Fresh *C. vulgaris* (one gram) was treated with 1.23% (v/w) viscozyme at the optimal conditions (pH 4.5, 50°C, 2 h) obtained from the previous experiment and then sonicated with various volumes of 90% ethanol at fixed frequency, intensity, and temperature (35 kHz, 56.58 W/cm^2, and 37.7°C). The ultrasound extraction after enzymatic pretreatment was optimized by response surface methodology (RSM), in which extraction time (1 ~ 4 h, B_1) and ratio of solvent to solid (10 ~ 50 mL/g, B_2) were designed according to central composite design (CCD). The extract was filtered through Whatman No. 2 paper and then stored at 4°C in the absence of light for further analysis.

2.3. Purification of lutein

The *C. vulgaris* extract (400 mL) was saponified by adding 6% KOH (w/v) (24 g) at 50°C for 30 min. The mixture was then evaporated by rotary evaporator (RA 10, IKA, Tokyo, Japan), and the dry solid obtained was dissolved in distilled water (400 mL) and partitioned with ethyl acetate (400 mL). The ethyl acetate fraction (400 mL) was evaporated by rotary evaporator (RA 10, IKA, Tokyo, Japan) and redissolved in 5 mL of acetone. The acetone solution (2 mL) was loaded on a silica gel column (2.0 × 60.0 cm), and then eluted with 150 mL of hexane, followed by 400 mL of hexane-acetone (7:3 v/v). The purified compound fraction was collected based on spectral characteristic of lutein using a spectrophotometer (V-530 UV/VIS, Jasco, Tokyo, Japan). The lutein fraction (150 mL) was evaporated and dried by nitrogen (JSVO-60T, JSR, Victoria, Australia), and stored at -20°C.

2.4. Identification and quantification of the purified compound

The purified compound was identified by LC-MS (HP-1100 MSD, Agilent Technologies, Santa Clara, CA). Twenty μL of the purified compound was injected to C_{18} column (5 μM, 150 mm × 46 i.d., Waters, Milford, MA). The mobile phase was a mixture of solvent A (methanol: ammonium acetate 0.1 N; 7:3) and solvent B (methanol) at 0.9 mL/min according to a step gradient, lasting 35 min, starting from 25% B, changing to 50% in one minute, rising up to 100% B at 10 min, and then keeping constant until the end of the analysis. The tentative identification was based on UV-vis spectral characteristics and compared to standard and data available in the literature.

The concentration of lutein was determined by measuring the absorbance of the samples at 445 nm (V-530 UV/VIS, Jasco, Tokyo, Japan). The calibration curve was constructed at a range of 0.02 ~ 3.36 μg/mL for the quantification of lutein in the extract.

2.5. Microalgal cell morphology

Microalgal cell morphology was studied on the dried cell obtained by each extraction processes (ultrasound extraction, ultrasound extraction with enzymatic pretreatment, and conventional extraction). The dried cell was fixed on a metal stub with a conductive tape and coated with gold. The surface characteristics were observed and recorded by Inspect™ Scanning Electron Microscope (Inspec F, FEI, Hillsboro, Oregon, USA) at the range of 30 ~ 200 kV accelerating voltage. The samples were placed in a preparation chamber, and the morphology of the samples was magnified and digitally recorded.

2.6. Microalgal surface area

The analysis for the specific surface area of the microalgal cell was determined by Nitrogen (N_2) adsorption isotherm using an automatic surface area analyzer (Macsorb HM-1200 series, Mountech, Tokyo, Japan). The values of the specific surface area were evaluated by the Brunauer-Emmett-Teller (BET) multilayers model [12]. The sample (0.3 g) was transferred in to the sample tube. Then the whole cryogen system was preheated at 350°C with isothermal jackets and degassed under vacuum. After the degassing process, the sample tube was immersed in liquid nitrogen and the temperature was kept at -196°C during the entire experiment. Nitrogen was adopted as the gas for the free space calculation. The equilibrium time between the two consecutive nitrogen gases was set at 60 sec.

2.7. Color measurement

The color of the purified lutein was measured by a spectrophotometer (CM-3500d, Minolta, Osaka, Japan). One milligram of the purified lutein obtained by ultrasound or conventional extraction was dissolved in 5 mL of acetone. The color parameters were measured using CIE

Lab color measurements.

2.8. Determination of antioxidant activities

2.8.1. DPPH radical scavenging assay

The DPPH assay was determined according to the method of Duan *et al*. [13]. 0.5 mL of the sample at different concentrations was mixed with 0.5 mL of 0.16 mM DPPH in methanol and then incubated at 37°C for 30 min in the dark. The absorbance was immediately measured at 517 nm. The percentage of DPPH radical scavenging activity was calculated using the following equation:

$$\%Inhibition = [(A_{control} - A_{sample})/A_{control}] \times 100 \quad (1)$$

2.8.2. ABTS radical scavenging activity

ABTS radical scavenging activity of lutein was determined according to the method of Re *et al*. [14] with some modifications. The radical $ABTS^{\cdot+}$ was produced by reacting 7 mM ABTS and 2.45 mM potassium persulfate buffer (1:1) and kept for 16 h in a dark at room temperature. After 16 h, ethanol was used to adjust the absorbance of the radical $ABTS^{\cdot+}$ to 0.70 ± 0.05 at 734 nm. 1.9 mL of the radical $ABTS^{\cdot+}$ was added to 50 μL of the sample at different concentrations. The mixture was stood at room temperature for 6 min, and then the absorbance was measured against the blank at 734 nm. Scavenging activity was calculated using the above equation (1).

2.8.3. Hydrogen peroxide radical scavenging activity

Hydrogen peroxide radical scavenging activity was determined according to the method of Muller [15]. The reaction mixture consisted of 20 μL of 2 mM H_2O_2, 100 μL of 0.1 M phosphate buffer (pH 5.0), and 100 μL of sample solution, and then incubated at 37°C for 5 min. Then, 30 μL of 1.25 mM ABTS and 30 μL peroxidase (1 unit/mL) were added to the reaction mixture and incubated at 37°C for 10 min. The absorbance was measured at 405 nm. The percentage of hydrogen peroxide radical scavenging activity was calculated by equation (1) as above.

2.8.4. Reducing power

The reducing power was measured according to the method of Chou *et al*. [16]. The sample at different concentrations (0.2, 0.4, 0.6, 0.8, and 1 mg/mL) in ethanol (0.25 mL) was added to 0.25 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 0.25 mL of 1% potassium ferricyanide. Then, the mixture was incubated at 50°C for 20 min. The 0.25 mL of 10% trichloroacetic acid was added to the mixture to stop the reaction, and then the mixture was centrifuged at 12,000 g for 10 min. The supernatant (0.4 mL) was mixed with 0.4 mL of ethanol and 80 μL of 0.1% ferric chloride solution to allow for standing for 10 min, and the absorbance was measured at 700 nm against a blank. A higher absorbance defines a higher reducing power.

2.9. Economic evaluation

The cost of manufacturing (COM) was estimated for the lutein extracts obtained by ultrasound extraction alone, ultrasound extraction with enzymatic pretreatment and conventional extraction according to the method of Turton *et al*. [17].

The cost of manufacturing (COM) consisted of the terms of fixed cost of investment (FCI), cost of operational labor (COL), cost of utilities (CUT), cost of waste treatment (CWT) and cost of raw material (CRM). The equation proposed by Turton *et al*. [17] was:

$$COM = 0.304FCI + 2.73COL + 1.23(CUT + CWT + CRM) \quad (2)$$

The conventional extraction in this study is the agitated bed extraction. Fifteen grams of *C. vulgaris* was immersed in 500 mL of 90% ethanol in an agitated tank. The equipment used comprised extractors, solution tank, evaporator, pump, condenser, and recycle solvent tank. For the ultrasound extraction, it was assumed that ultrasonic transducers were bonded to the tank walls [18].

The procedure of the scale-up assumed that the industrial scale unit had the same performance as the laboratory scale unit [19]. The process was designed to operate 7,920 h per year with continuous 24 h per day shifts. The extractor capacities at different volumes of 0.01, 0.05 and 0.4 m^3 were investigated.

2.10. Statistical analysis

The optimization experiments were performed by response surface methodology (RSM) according to Design Expert 7.0 (State-Ease, Inc., Minneapolis, MN, USA). All experiments were conducted in triplicate. Data were expressed as means ±SD and analyzed with Duncan's multiple comparison test ($p \leq 0.05$) using the SPSS software version 11.0 (SPSS Inc., Chicago, IL. USA).

3. Results and Discussion

3.1. Ultrasound extraction

In order to optimize the ultrasound process for the extraction of lutein from *C.vulgaris* by the response surface method (Table 1). The regression coefficients of the

Table 2. Regression coefficient estimation and analysis of variance of the regression model for the ultrasound extraction of lutein

Independent variable	Coefficient	SS[a]	DF[b]	MS[c]	*F*-Value	*p*-Value
Model		5.865	9	0.652	137.767	< 0.0001*
X_1	0.352	0.993	1	0.993	209.966	< 0.0001*
X_2	0.401	1.286	1	1.286	271.923	< 0.0001*
X_3	0.199	0.316	1	0.316	66.726	< 0.0001*
X_1X_2	0.083	0.028	1	0.028	5.868	0.0459*
X_1X_3	0.061	0.015	1	0.015	3.156	0.1189
X_2X_3	0.068	0.018	1	0.018	3.871	0.0898
X_1^2	-0.132	0.073	1	0.073	15.406	0.0057*
X_2^2	-0.743	2.326	1	2.326	491.810	< 0.0001*
X_3^2	-0.375	0.592	1	0.592	125.102	< 0.0001*
Residual		0.033	7	0.005		
Lack of fit		0.018	3	0.006	1.501	0.3427
Total		5.898	16			
Coefficient of variation		2.891				
R^2		0.994				
Adjusted R^2		0.987				
Adequate precision		33.285				

*Significant at the $p < 0.05$ level.
[a]SS: sum of squares.
[b]DF: degrees of freedom.
[c]MS: mean squares.

intercept, linear, quadratic and interaction terms of the model were calculated using the least square technique (Table 2). It was evident that the linear (X_1, X_2, X_3), quadratic (X_1^2, X_2^2, X_3^2) and one interaction (X_1X_2) parameters were found to be significant ($p < 0.05$). The *p*-value of the regression model indicated that the effect of the extraction time was the major contributing factor to lutein recovery. The mathematical model representing the yield of lutein as a function of the independent variables within the region under investigation was expressed as follows;

$$Y = 2.97 + 0.35X_1 + 0.40X_2 + 0.20X_3 - 0.13X_1^2 - 0.74X_2^2 - 0.37X_3^2 + 0.08X_1X_2 + 0.06X_1X_3 + 0.07X_2X_3 \quad (3)$$

The analysis of variance (ANOVA) for the optimization of ultrasound extraction is also shown in Table 2. The *F*-test suggested that model had a high *F*-value ($F = 6137.767$) and a very low *p*-value ($p < 0.0001$), indicating that the fitness of this model was most significant. The *p*-value of the lack of fit was 0.3427, which implied an insignificant difference relative to the pure error and a good fitness of model. The coefficient of variation (C.V.) less than 5% (C.V. = 2.891) indicated good precision and reliability of the experiments performed. Furthermore, the determination coefficient (R^2) of the model was 0.994, which indicated 99.4% of the variability in the response could be explained by this model, which reasonably agreed with the adjusted R^2 value of 0.987. The adequate precision value (33.285) that measures the signal-to-noise ratio suggested an adequate signal. Those values indicated a satisfactory fitness of the quadratic model.

The three-dimensional response surface curve was plotted to explain the interaction of three independent variables and to determine the optimum condition (Figs. 1A, 1B, and 1C). The effect of the extraction temperature and time on the yield of lutein at a fixed solvent to solid ratio of 30 mL/g is shown in Fig. 1A. At a fixed time, the lutein yield increased slightly as the temperature increased. When temperature was fixed, the lutein yield increased until a certain amount of (approximately 5 h), and then decreased. It could be explained that the chemical decomposition of bioactive compound present in the extract may occur as the extraction time prolongs, resulting in a decrease in the extraction yield [20]. The effect of temperature and ratio of solvent to solid on the extraction of lutein from *C. vulgaris* at a constant time of 5 is shown in Fig. 1B. At a fixed solvent to solid ratio, the lutein yield increased as extraction temperature increased. The lutein content increased rapidly with the increase of extraction time at a fixed ratio of solvent to solid, while at a fixed extraction time, the increasing ratio of solvent to solid resulted in the increase of lutein (Fig. 1C). Increasing the ratio of solvent to solid and extraction time would not further increase the lutein

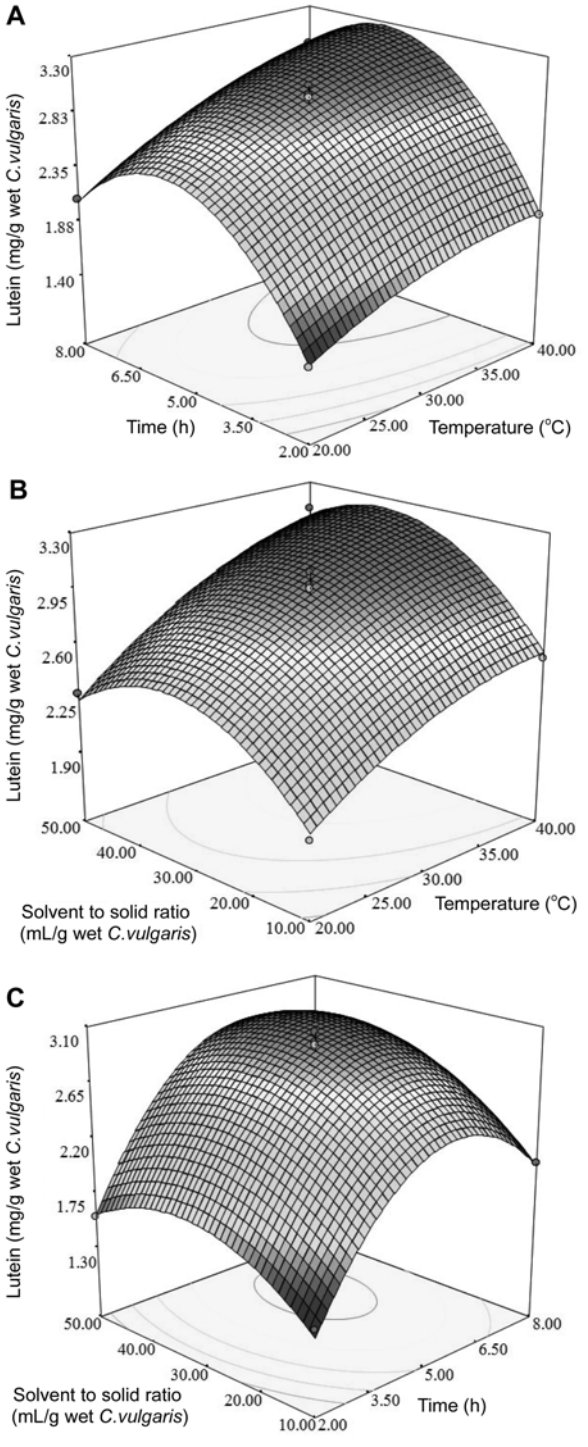

Fig. 1. The yield of lutein from *Chlorella vulgaris* by ultrasound extraction as a function of (A) temperature and time, (B) temperature and solvent to solid ratio, and (C) time and solvent to solid ratio.

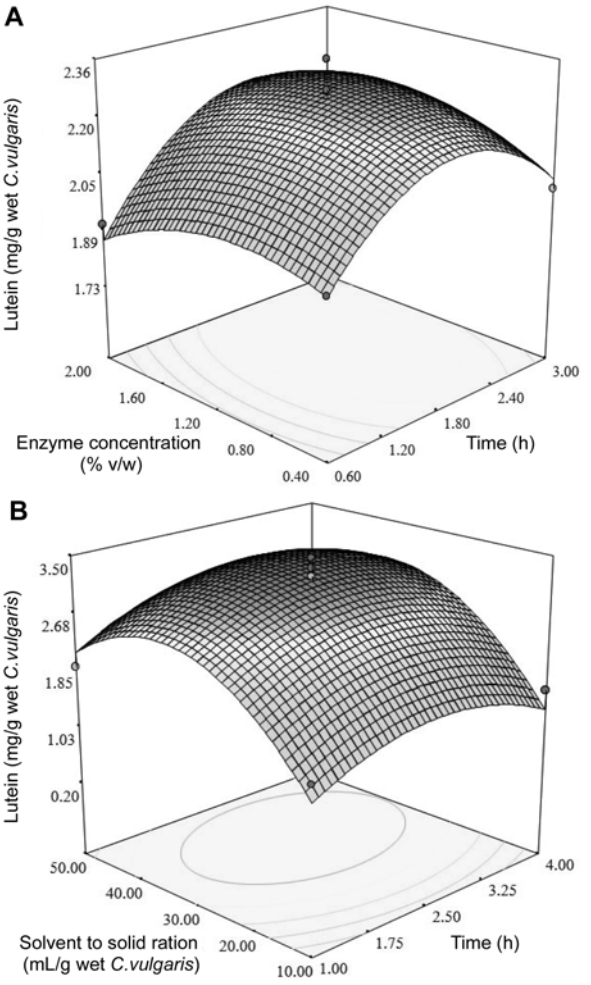

Fig. 2. The yield of lutein from *Chlorella vulgaris* by ultrasound extraction with enzymatic pretreatment as a function of (A) enzyme reaction time and enzyme concentration and (B) extraction time and ratio of solvent to solid.

yield [20]. This result was similar to those previously reported by Rodrigues *et al*. [21], Zhang *et al*. [22] and Zou *et al*. [23]. The optimal levels of the three variables determined by RSM were extraction time, 5 h; extraction temperature, 37.7ºC and ratio of solvent to solid, 31 mL/g, corresponding to the maximum lutein yield of 3.16 ± 0.03 mg/g.

3.2. Ultrasound extraction with enzymatic pretreatment

3.2.1. Optimization of enzymatic pretreatment

The effect of reaction time and enzyme concentration on the enzymatic pretreatment for the ultrasound extraction of lutein was investigated by response surface methodology (RSM) at the two-variable and five-level central composite design (CCD). The yield of lutein by the ultrasound extraction ranged from 1.74 to 2.36 mg/g (data not shown).

The following regression equation, an empirical relationship between the yield of lutein and the test variable in coded unit, as given in Equation (4), was obtained with the application of response surface methodology (RSM).

$$Y = 2.27 + 0.08A_1 - 0.002A_2 - 0.19A_1^2 - 0.08A_2^2 + 0.04A_1A_2 \quad (4)$$

where Y (lutein yield), mg/g; A_1 (time), h; A_2 (enzyme concentration), % (v/w)

Based on the ANOVA, the quadratic regression model was significant (p = 0.0003) with a very high F-value (23.555). The determination coefficient (R^2 = 0.944) and adjusted determination coefficient (Adj R^2 = 0.904) were satisfactory to confirm the significance of the model. Thus, it can be concluded that the quadratic model was statistically sound.

The suitability of the model equation for predicting the optimum response values was tested using the selected optimal conditions. Additional experiments using the predicted optimum conditions for enzymatic pretreatment were conducted at a reaction time of 2 h and enzyme concentration of 1.23% (v/w). The predicted maximum response was 2.28 mg/g, where as that of the experiment was 3.19 ± 0.11 mg/g (n = 4). The analysis confirmed that the response model was adequate for reflecting the expected optimization. Hence, the model of equation (4) was satisfactory and accurate.

The optimal condition of enzymatic pretreatment was: enzyme incubation time, 2 h; enzyme concentration, 1.23% (v/w); pH, 4.5; and temperature, 50°C.

3.2.2. Optimization of ultrasound extraction after enzymatic pretreatment

Preliminary experiments were performed to determine the main factors and the ranges of each factor for RSM. Extraction time (B_1) and ratio of solvent to solid (B_2) were the most significant variables. The extraction temperature of 37.7°C resulted in the highest yield of lutein. Therefore, the extraction temperature was fixed at 37.7°C for further experiment. The predicted lutein yield can be obtained by the following second-order polynomial equation:

$$Y = 3.22 + 0.11B_1 + 0.52B_2 - 0.49B_1^2 - 0.95B_2^2 + 0.07B_1B_2 \quad (R^2 = 0.9460,\ p = 0.00003) \quad (5)$$

The optimal condition for the extraction of the lutein by ultrasound with enzymatic pretreatment was: enzyme incubation time, 2 h; enzyme concentration, 1.23% (v/w); ultrasound extraction time, 2.60 h; ratio of solvent to solid, 35.61 mL/g, in which the predicted maximum lutein yield was 3.30 mg/g. The experimental value of lutein was 3.36 ± 0.10 mg/g, which did not differ from the predicted value. Therefore, the quadratic response model was satisfactory for the optimization.

The results of ultrasound extraction with/without enzymatic pretreatment were compared to that of the conventional method. The yields of lutein by ultrasound extraction with/without enzymatic pretreatment and conventional extraction were 3.36, 3.16, and 1.75 mg/g wet weight of *C. vulgaris*, respectively. Lutein was more effectively extracted by ultrasound extraction with/without enzymatic pretreatment than by the conventional method. The yields of lutein from *C. vulgaris* by soxhlet, pressurized liquid, supercritical CO_2, and supercritical CO_2 with 7.5% ethanol were 3.42, 3.78, 0.50, and 3.00 mg/g dry weight of *C. vulgaris*, in which the pressurized liquid method was the most efficient [11,24]. The yields of the lutein were 3.78 [11] and 9.22 [25] mg/g dry weight of *C. vulgaris* by ultrasound extraction, in which the dried *C. vulgaris* was used as a raw material. The different cultivations of microalgae caused the different lutein productions in microalgae [26]. Freeze-drying or lyophilization has been widely used for drying microalgae in research laboratories; however, freeze-drying is too expensive for use in a large-scale commercial recovery of microalgal products [27]. The yield of lutein extracted by ultrasound extraction at the optimal condition in this study was 12.38 mg/g dry weight of *C. vulgaris* (data not shown). Therefore, the ultrasound extraction with/without enzymatic pretreatment was an effective extraction method and can become an alternative method to extract the bioactive compound. Furthermore, the microalgae could become a potential source to make lutein production, because of the higher lutein content [28].

3.3. The Disruption of microalgal cell

The surface areas of microalgal cells treated at the optimal extraction condition of ultrasound with/without enzymatic pretreatment were determined (Table 3). The surface area of microalga cell treated by ultrasound with/without enzymatic pretreatment and conventional extraction was 26.561, 11.923 and 6.760 m^2/g, respectively. The surface significantly area values demonstrated that the ultrasound with/without enzymatic pretreatment leads to increase the porosity of microalgal cell and enlarge the amount of nitrogen entrapment [28]. Thus, the microalgal cell wall was destroyed effectively by ultrasound with/without enzymatic pretreatment, based on increasing the of surface area of the microalgal cell [28,29].

Table 3. Surface areas of *Chlorella vulgaris* cells by different extraction methods

Sample	Specific surface area (m^2/g)
C. vulgaris	3.831
Conventional extraction	6.760
Ultrasound extraction	11.923
Ultrasound extraction with enzymatic pretreatment	26.561

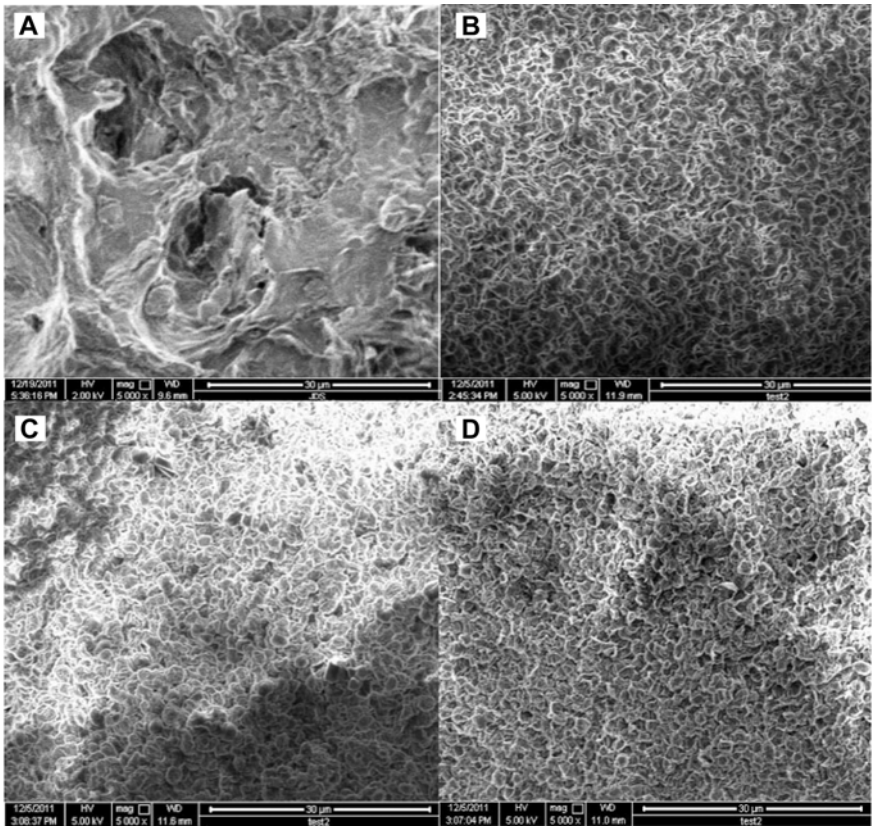

Fig. 3. Scanning electron microscope images of *Chlorella vulgaris*. (A) Control; (B) conventional extraction; (C) ultrasound extraction; and (D) ultrasound extraction with enzymatic pretreatment.

The microalgal cell morphology was also determined by scanning electron microscope. The images of *C. vulgaris* treated by ultrasound with/without enzymatic pretreatment and conventional extraction are shown in Fig. 3. The microstructure of the microalgal cell by ultrasound (Fig. 3C) had more interspaces and holes than the microalgal cell by the conventional method (Fig. 3B).The disruption of the microalgal cell by ultrasound extraction is shown in Fig. 3C. This was the same as the results of Li *et al*. [30] and Zhao *et al*. [28], in which ultrasound treatment disrupted tissues and cell walls. Moreover, the physical disruption of *C. vulgaris* cells by ultrasound, together with enzymatic pretreatment, was the most efficient method to destroy microalgal cell wall (Fig. 3D). Enzymatic pretreatment has recently been shown to be another alternative method, which opens up cell walls through biodegradation and releases bioactive compounds from microalga [31]. The cell walls were damaged by the application of ultrasound and enzymatic pretreatment, which resulted in the greater penetration of solvent into the sample matrix, increased the contact surface area between the solid and liquid phase, and as a result, the solute quickly diffused from the solid phase to the solvent [32]. Hence, ultrasound treatment is much more efficient and rapid for the extraction of the bioactive compound. Therefore, the sonication played an important role in breaking up the microalgal cell walls to enhance the extraction yield [33].

3.4. Structure and color of lutein

The quantity of trans-lutein in the purified lutein extract by ultrasound and conventional extraction methods were 0.86 ± 0.03 and 0.85 ± 0.02%, respectively. There were no significant differences in cis-lutein quantities between the purified luteins by ultrasound and conventional extraction. The UV spectra of the purified lutein extract by ultrasound extraction (Fig. 4) showed the wavelengths of maximum absorption and spectral fine structural values (%III/II). These agreed with standard lutein and data in the literature [34,35]. The mass spectra of the purified lutein by ultrasound extraction showed the molecular ion at m/z 568.4 $[M]^+$, a protonated molecule at m/z 569.5 [M+H]+, and the abundant fragment ion at m/z 551.4 $[M+H-H_2O]^+$ formed by the elimination of water from the protonated molecule (Fig. 4). The purified compound was identified as all-trans-lutein, based on the UV and mass spectra characteristics and confirmed by coelution with the lutein standard.

The measurement of color is a nondestructive and very

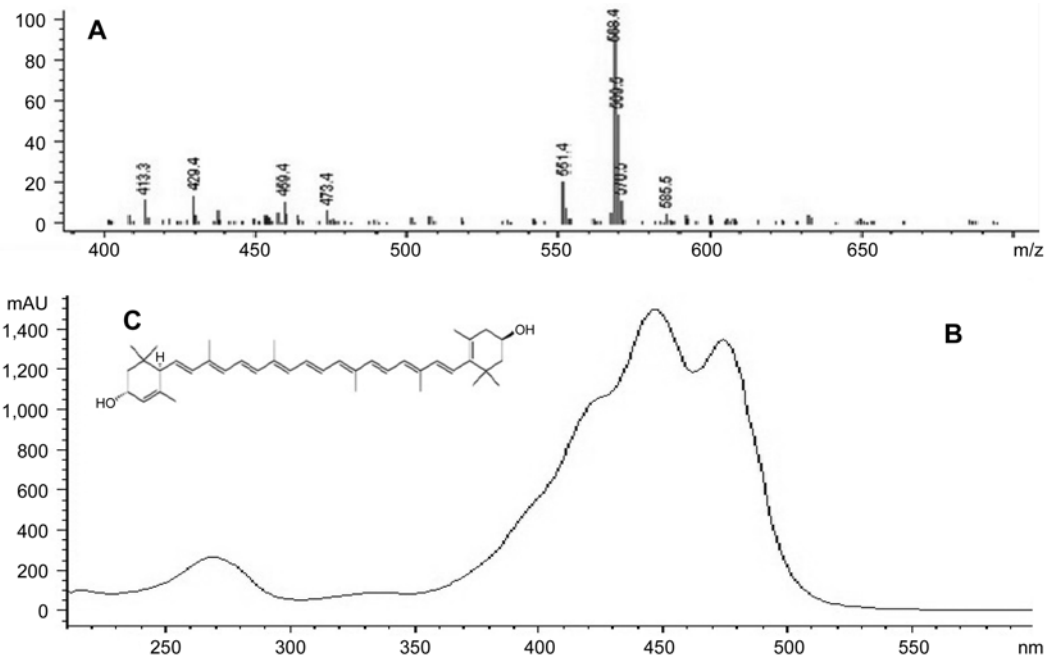

Fig. 4. Mass spectrum (A), UV spectra (B), and structure (C) of the purified microalgal luein by ultrasound extraction.

rapid technique in a few seconds. This is a very useful tool for the quality control of carotenoids in industry [36]. The values of color parameters of the purified lutein by ultrasound extraction were: lightness (L*), 93.92 ± 0.04; redness (a*), -6.17 ± 0.02; yellowness (b*), 37.39 ± 0.02; chroma (C*), 37.90 ± 0.02; and hue angle (h^o); 99.37 ± 0.03. These values are not significantly different from those of standard lutein and similar to the result of Maléndez-Martínez *et al*. [37]. Normally, the higher lightness value of the sample indicates that the lower amount of pigment is retained in the sample, while the higher content of pigment is represented by higher chroma values [38]. The terms of color coordinates of carotenoids depends on the chemical structure of carotenoids [37]. Hence, it was concluded that ultrasound extraction did not influence the chemical structure of lutein.

3.5. Antioxidant activity

It is well known that the evaluation of the antioxidant capacities on a selected antioxidant requires more test systems [39]. The DPPH free radical is a stable free radical that has been widely used as a tool for estimating the free radical-scavenging activity of antioxidant [40]. The ABTS is widely used in food analysis because it is not subject to pH variations and is useful to analyze both hydrophilic and lipophilic compounds [41]. Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to a cell, because it can raise the hydroxyl radical in the cells. Thus the removal of H_2O_2 is very important for antioxidant defense in cell or food systems [42]. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant [43]. In the DPPH, ABTS and hydrogen peroxide radical scavenging activity, and reducing power, the IC_{50} values of the purified lutein were 112.57, 184.25, 36.19, and 505.44 μg/mL, respectively. Meanwhile, the concentration of lutein showed a good linear relationship with the clearance rate of DPPH, ABTS and hydrogen peroxide radical as well as the reducing power in a dose-dependent manner (Fig. 5). Therefore, the *C.vulgaris* lutein by ultrasound extraction had significant antioxidant activities in a dose-dependent manner.

The lutein scavenged DPPH radical by 33.18% at 50 μg/mL was higher than 30.02% of yellow marigold flowers (*Tagetespatula L.*) [44]. The IC_{50} values of *C. vulgaris* lutein (112.57 μg/mL) against DPPH was lower than that of Thai gag (3.57 mg/mL), but it was higher than that of *Tagetuserecta L.* and water spinach (IC_{50} = 35 and 7.54 μg/mL, respectively) [45,46]. The IC_{50} value of lutein (184.25 μg/mL) against ABTS radical was lower than 344 μg/mL of red marigold flowers lutein (*Tagetespatula L.*) [43]. Moreover, the microalgal lutein was absorbed more efficiently by the human digestive system and its antioxidant activity was not significantly different from the other plant lutein sources [27].

3.6. Economic evaluation

The costs of manufacturing (COM) for the production of lutein from *C. vulgaris* by ultrasound extraction, the

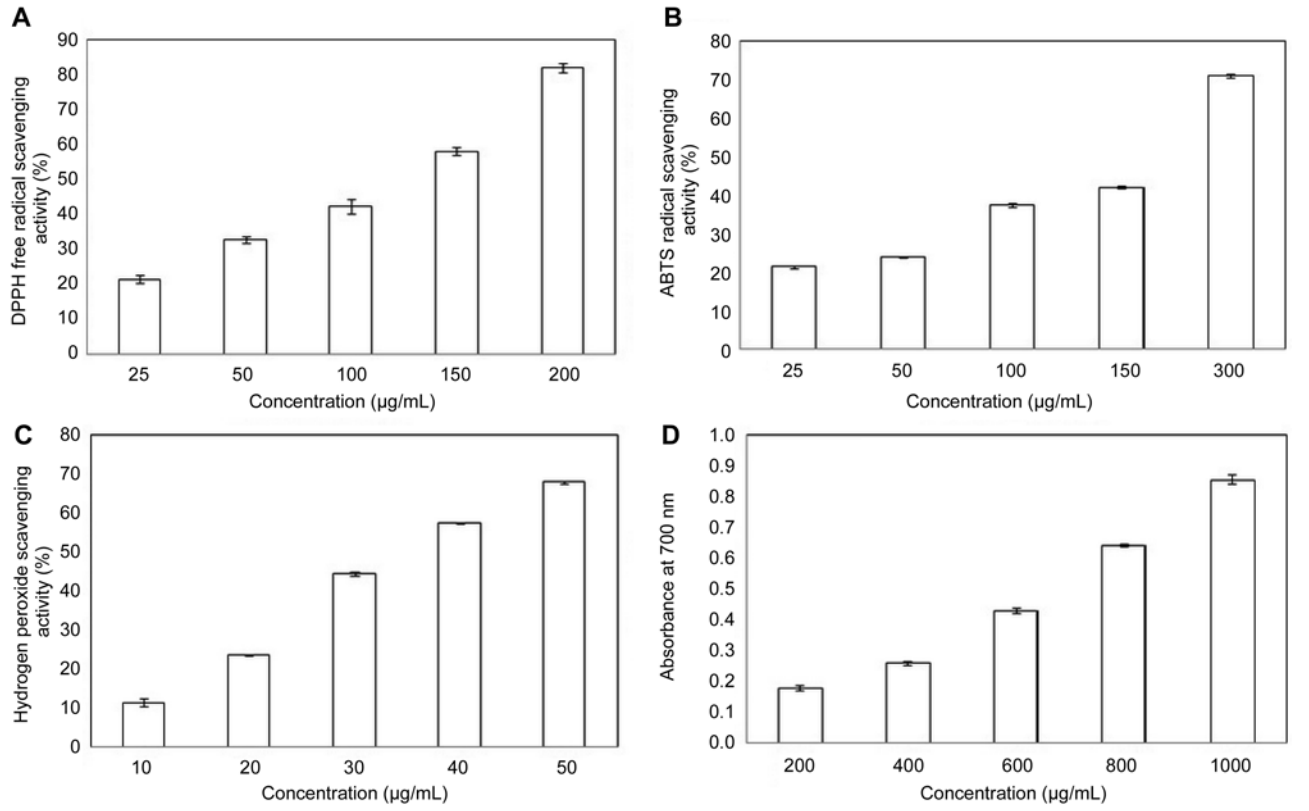

Fig. 5. Antioxidant activities of lutein at different concentrations by ultrasound extraction: (A), DPPH; (B), ABTS; (C), hydrogen peroxide; and (D), reducing power.

Table 4. Estimation of the cost of manufacturing (COM) for the crude and purified lutein extracts for ultrasound extraction, ultrasound extraction with enzymatic pretreatment, and conventional extraction

Method	Extractor capacity (m^3)	Crude extract (kg/kg)	COM for crude extract (US$/kg)	Content of lutein (g/kg)	COM for lutein (US$/g)	FCI (%)	CRM (%)	COL (%)	CUL (%)	CWT (%)
Ultrasound	0.05	0.20	232.56	3.16	14.72	22.95	13.07	32.68	0.80	0
	0.10		120.79		7.64	22.33	25.16	31.46	1.53	0
	0.40		36.31		2.30	19.42	83.69	26.17	3.71	0
Ultrasound with enzymatic pretreatment	0.05	0.47	265.10	3.36	37.08	5.79	8.43	12.63	14.60	28.82
	0.10		217.12		30.37	3.60	10.29	7.71	17.83	35.19
	0.40		151.67		21.22	1.38	14.73	2.76	9.74	50.37
Conventional*	0.05	0.15	512.90	1.75	44.22	22.20	4.50	31.61	1.15	0
	0.10		274.45		23.66	22.44	9.00	31.61	2.31	0
	0.40		94.23		8.12	23.46	35.98	31.61	7.54	0

*Stirring 15 g *Chlorella vulgaris* in 500 mL of 90% ethanol at room temperature for 8 h.

ultrasound extraction with enzymatic pretreatment and conventional extraction were evaluated.

The COM of the crude and purified lutein extracted at various extractor capacities by each extraction method are shown in Table 4. The ultrasound extraction with enzymatic pretreatment resulted in the highest COM for the crude and purified lutein, owing to the cost of liquid waste (buffer) treatment. The waste from the extraction process by the ultrasound and conventional method was the exhausted solid produced from biological material. This can be incorporated into the soil. Thus, there was no additional cost for the waste treatment. Moreover, the ultrasound extraction with enzymatic pretreatment had more equipment and material in the enzymatic pretreatment step, which increased CRM, FCI, CUT, and COL. The COM of the crude lutein extract ranged from 265.10 to 151.67 US$/kg,

while that of the purified lutein ranged from 37.08 to 21.22 US$/g. In contrast, the lowest COM for the crude and the purified lutein (36.31 and 2.30 US$/g) were found in the ultrasound extraction, because ultrasound extraction resulted in higher yield of the crude and purified lutein than the conventional extraction together with the decreasing of the extraction time and ratio of solvent to solid.

Although the highest yields of the crude and purified lutein were obtained from the ultrasound extraction with enzymatic pretreatment, their COMs were not attractive compared to those of three different extraction methods, due to the increasing of FCI and CWT. The cost of equipment and raw materials, such as enzyme, also increased. Thus, the ultrasound extraction with enzymatic pretreatment increased COM above that of the other extraction methods.

Normally, the COM decreased as the extractor capacity increased, which is advantageous for a large industrial scale. The fixed cost of investment (FCI) and cost of operational labor (COL) decreased as the extractor capacity increased (Table 4). The cost of raw materials (CRM) and the cost of utilities (CUT) increased due to the higher demand for the extractor capacity (Table 4). The CRM and CUT present a small portion of the COM, while the COL is the main portion following by FCI.

Therefore, the ultrasound extraction was not a time consuming process and loweredcost [47,48]. Furthermore, the ultrasound extraction is an easier and more comfortable process to scale-up in industry and can improve the efficiency of lutein extraction [18,49].

4. Conclusion

Ultrasound extraction was applied for the extraction of lutein from *Chlorella vulgaris* with an increase in the yield of extraction. The response surface method was chosen to get the optimal conditions of ultrasound extraction with/without enzymatic pretreatment. The ultrasound extraction with enzymatic pretreatment resulted in the highest lutein yield, followed by ultrasound and conventional extraction in order. In comparison with the conventional extraction, ultrasound extraction is a more efficient and rapid method to extract the lutein from *C. vulgaris*, due to the strong disruption of the cell wall under ultrasonic acoustic cavitation. The ultrasound extraction had no effect on the chemical structure, color and antioxidant activity of lutein. The cost of manufacturing of the ultrasound with enzymatic pretreatment was highest, whereas that the ultrasound extraction was the lowest. Thus, it is suggested that ultrasound extraction is the most economical method for the extraction of lutein.

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