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Optimization of Pressurized Liquid Extraction of Carotenoids and Chlorophylls from *Chlorella vulgaris*

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Pressurized liquid extraction (PLE) was applied to the extraction of carotenoids and chlorophylls from the green microalga *Chlorella vulgaris*. Four extraction techniques such as maceration (MAC), Soxhlet extraction (SOX), ultrasound assisted extraction (UAE), and PLE were compared, and both the extraction temperature (50, 105, and 160 °C) and the extraction time (8, 19, and 30 min), which are the two main factors for PLE, were optimized with a central composite design to obtain the highest extraction efficiency. The extraction solvent (90% ethanol/water) could adequately extract the functional components from *C. vulgaris*. PLE showed higher extraction efficiencies than MAC, SOX, and UAE. Temperature was the key parameter having the strongest influence on the extraction of carotenoids and chlorophylls from chlorella. In addition, high heat treatment (>110 °C) by PLE minimized the formation of pheophorbide a, a harmful chlorophyll derivative. These results indicate that PLE may be a useful extraction method for the simultaneous extraction of carotenoids and chlorophylls from *C. vulgaris*.

KEYWORDS: Pressurized liquid extraction; carotenoids; chlorophylls; *Chlorella vulgaris*; optimization

INTRODUCTION

Chlorella is a green microalga that contains very high concentrations of chlorophyll and produces proteins, vitamins, and many carotenoids, such as lutein, β-carotene, and astaxanthin (*1*). Numerous clinical studies have shown that chlorella ingestion has health benefits (*2−4*). Chlorella is especially noted for its protective role on bacteria, antiproliferative effects on cancer cells, and anti-inflammatory activity (*5*, *6*). These beneficial effects may be due to its antioxidant properties, primarily through carotenoids and chlorophylls.

Lutein, the main carotenoid from chlorella, is not only an important natural food colorant and additive but also an effective stimulant of the immune response, hampering cataract and atherosclerotic development (*7*). The minor chlorella carotenoids, β-carotene and astaxanthin, may reduce the risk of cardiovascular diseases and delay chronic disease (*8*). In addition, chlorophylls and their derivatives such as pheophorbides and pheophytins, which are abundant in chlorella, have been extensively studied for their biological activities (*9−13*).

The extraction techniques for these types of compounds are important for the growing nutraceutical industry. Maceration, sonication, and Soxhlet methods have previously been examined for carotenoid and chlorophyll extraction from several microalgae (*14*, *15*). These traditional extraction techniques have some drawbacks, such as the use of large quantities of toxic organic solvents, long extraction times, low selectivity and/or low extraction yields, and exposure of the extracts to excessive heat, light, and oxygen. As an alternative of traditional extraction methods, pressurized liquid extraction (PLE) has recently been used in the extraction of biologically active constituents (*16−18*). This method utilizes conventional solvents at elevated temperatures and pressures, and is well established for environmentally clean extraction. Since PLE is automated, uses less solvent in a shorter period of time, and maintains the sample in an oxygen- and light-free environment, PLE has the potential to be a powerful tool in the industry.

Although there have been many reports on the extraction of carotenoids and chlorophylls from microalgae, these studies have mainly focused on the application of supercritical fluid extraction, another environment-friendly technique (*19*, *20*). Only a few references can be found concerning the use of PLE to extract these compounds from microalgae, and PLE has not been applied to the chlorella species (*21−24*). Therefore, this study was designed to demonstrate the effects of PLE on the extraction of carotenoids and chlorophylls from *C. vulgaris*, a strain we used previously (*5*). The change of chemical compositions by PLE was investigated and characterized. To obtain the maximum extraction yield of lutein, β-carotene, and chlorophylls a and b, key factors such as the extraction solvents, temperature, and time were examined, and central composite design (CCD) was used to optimize PLE.

MATERIALS AND METHODS

Samples and Chemicals. *C. vulgaris* (KMCC C-024) was obtained from the Korea Marine Microalgae Culture Center (Busan, Korea), and it was cultured by Aquanet Co. (Tongyoung, Korea). The biomass was

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washed twice in distilled water and harvested by centrifugation. Chlorella cells were collected, freeze-dried in a vacuum freezer dryer (Ilshin Lab, Korea), and stored under vacuum in darkness until extraction. High performance liquid chromatography (HPLC)-grade acetone, *tert*-butyl methyl ether, ethanol, hexane, methanol, and water were purchased from Fisher Scientific (Springfield, NJ, USA). Standard lutein (98.2%) was from Chromadex Inc. (Santa Ana, CA, USA). β-Carotene and chlorophylls a and b were from Sigma-Aldrich (St. Louis, MO, USA). Both chlorophyll derivatives, pheophorbide a, and pheophytin a, were purchased from Wako Chemicals (Osaka, Japan). All other chemicals were of analytical grade.

Extraction. Several extraction methods were compared for the extraction of *C. vulgaris*. Maceration, the conventional extraction method, was performed with various solvents to select the optimum extraction solvent. A 0.5-g sample of freeze-dried chlorella powder was macerated in 50 mL of acetone, hexane, water, and various concentrations of ethanol (50−100%) at room temperature for 6 h. Soxhlet extraction was performed with 0.5 g of chlorella powder and 100 mL of 90% ethanol for 2 h. Ultrasound-assisted extraction was performed in an ultrasonic cleaning bath (model RK 158s, Bandelin, Germany). A 0.5-g sample of chlorella powder was sonicated with 50 mL of 90% ethanol for 2 h. The resulting extracts were filtered through Whatman No. 1 filter paper into a 50 or 100 mL volumetric flask. The extract was brought to the correct volume with the appropriate solvent and refiltered through a 0.2 μm membrane filter for HPLC analysis.

Pressurized liquid extraction was performed with 0.5 g of chlorella powder using a Dionex ASE 200 accelerated solvent extractor (Sunnyvale, CA, USA) equipped with 33 mL stainless steel extraction cells and 60 mL collection vials. The extraction procedure was as follows: (i) the sample was loaded in the cell; (ii) the cell was filled with solvent to a pressure of 1500 psi; (iii) heat was applied for the initial heat-up time; (iv) static extraction with all system valves closed was performed; (v) the cell was rinsed with 60% of the cell volume with extraction solvent; (vi) the solvent was purged from the cell with N_2 gas for 120 s; and (vii) the system was depressurized. The extractions were collected into glass collection vials. The extract was transferred to a 50-mL volumetric flask, which was brought up to its volume with methanol and filtered prior to injection into the HPLC system.

Experimental Design. Response surface methodology was used to study the effect of temperature and time on the extraction of carotenoids and chlorophylls from *C. vulgaris*. The response variables selected were the extraction yield of lutein (Y_1), β-carotene (Y_2), chlorophyll a (Y_3), and chlorophyll b (Y_4). The yields were expressed in mg of compound per g of chlorella dry weight. To determine the optimum conditions, we used a CCD with a total of 13 randomized chromatographic runs, including four cube points, five center points in the cube, and four axial points with an α value of 1.4142 (**Table 1**). The quadratic model proposed for each response variable (Y_i) was:

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{1,1} X_1^2 + \beta_{2,2} X_2^2 + \beta_{1,2} X_1 X_2 \quad (1)$$

Table 1. Central Composite Design with 13 Runs for All Possible Combination of Factors Levels

	coded variables		natural variables	
run order	X_1	X_2	temperature (°C)	time (min)
1	0	0	105	19
2	1	1	160	30
3	0	0	105	19
4	0	1.414	105	34.6
5	0	0	105	19
6	1.414	0	182.8	19
7	0	0	105	19
8	0	0	105	19
9	−1.414	0	27.2	19
10	1	−1	160	8
11	0	−1.414	105	3.4
12	−1	1	50	30
13	−1	−1	50	8

where β_0 is the intercept, β_1 and β_2 are the linear coefficients, $\beta_{1,1}$ and $\beta_{2,2}$ are the squared coefficients, $\beta_{1,2}$ is the interaction coefficient, and X_1 and X_2 are the coded levels of variables temperature and time, respectively.

Model parameters were estimated by multiple linear regression using the statistical package MINITAB for Windows, Release 14 (Birmingham, UK). This program allows both the creation and analysis of experimental designs. The analytical terms not significantly different from zero at $P \leq 0.05$ were rejected from the model, which was then refitted by multiple linear regression. From the new fitted model, the conditions that maximized the yield response variables (optimum conditions) were calculated. Surface plots were developed using the fitted second-order equations obtained.

HPLC Analysis. All extracts were analyzed in an Agilent Series 1200 liquid chromatographer equipped with a G1379B vacuum degasser, G1312A binary pump, G1329A auto sampler, G1316A column oven, and a G1315B DAD detector connected to Agilent ChemStation software. A YMC carotenoid column (3 μm particle size, 150 mm × 4.6 mm, Waters, Milford, MA, USA) was used to analyze the carotenoids and chlorophylls. The most suitable mobile phase system comprised methanol (100%) (A) and *tert*-butyl methyl ether (B) with the following conditions: isocratic at 0% B for 7 min followed by a linear gradient from 0 to 5% B in 1 min; isocratic at 5% B for 12 min, a linear increase to 35% B in 1 min; and isocratic at 35% B for 19 min followed by a linear decrease to 0% B in 4 min. The column was equilibrated for 10 min at the starting conditions before each injection. A flow rate of 1.0 mL/min at 30 °C with an injection volume of 20 μL was used. Simultaneous detection was performed at 445 nm for carotenoids (lutein and β-carotene) and 660 nm for chlorophylls.

Identification and Quantification. The carotenoids, chlorophylls, and their derivatives were identified by comparing retention times and absorption spectra of unknown peaks with reference standards and cochromatography with added standards. In addition, quadruple mass spectrometry (MS) with APCI (Varian, Palo Alto, CA, USA) in positive mode was used for detection. Mass spectra were acquired over the *m*/*z* 500−1000 scan range using a 0.1 unit step size with a drying gas flow of 4 L/min, nebulizing gas flow of 50 psi, vaporizer temperature of 550 °C, and dry gas temperature of 350 °C.

For quantitative analysis, the standard curves of lutein, β-carotene, chlorophyll a and b, pheophorbide a, and pheophytin a were calibrated using the linear least-squares regression equation derived from the peak area. The concentrations of the six major compounds were calculated according to the regression parameters derived from each standard curve.

RESULTS AND DISCUSSION

Choice of the Extraction Solvent. Hexane, acetone, ethanol and water, with dielectric constants of 1.9, 21.0, 24.3, and 78.5 at 25 °C and 1 bar, respectively, were tested to evaluate the influence of solvent polarity on carotenoid and chlorophyll extraction from the microalga. **Figure 1** shows the yield of each solvent using the

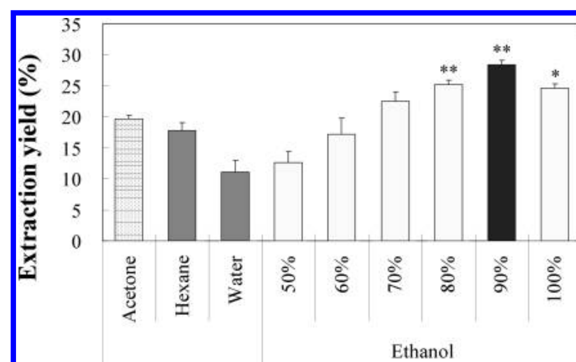

Figure 1. Solvent selection for the optimized extraction of carotenoids and chlorophylls from *C. vulgaris*. The extraction yield obtained from the dry extract weight/total sample weight is expressed as a percent. Values marked with an asterisk are significantly different from extraction with acetone (as the traditional method). ** $P < 0.005$ and * $P < 0.01$ using Student's *t*-test with $n = 3$.

Table 2. Comparison of the Chemical Composition of Extracts from *C. vulgaris* Using Four Different Extraction Methods[a]

	carotenoids (mg/g sample)		chlorophylls (mg/g sample)			
extraction method[b]	lutein	β-carotene	chlorophyll a	chlorophyll b	pheophorbide a	pheophytin a
MAC[c]	2.97 ± 0.31	0.08 ± 0.01	4.26 ± 0.53	2.58 ± 0.09	0.85 ± 0.09	2.31 ± 0.17
SOX[d]	3.42 ± 0.11	0.26 ± 0.09	3.32 ± 0.30	3.45 ± 0.28	** 5.15 ± 0.59	* 3.90 ± 0.16
UAE[e]	* 3.83 ± 0.28	0.10 ± 0.02	5.12 ± 0.29	* 3.71 ± 0.41	* 2.15 ± 0.71	2.64 ± 0.94
PLE 160[f]	** 3.78 ± 0.19	** 0.50 ± 0.25	*** 9.63 ± 0.65	*** 5.77 ± 0.68	*** 0.01 ± 0.00	** 5.64 ± 1.07

[a] Values marked with an asterisk are significantly different from MAC as the control method. *** $P < 0.001$, ** $P < 0.005$, * $P < 0.01$ using Student's t-test with $n = 3$. [b] All extractions used 90% ethanol as the extraction solvent. All components were quantified using the corresponding standard. [c] MAC was performed at room temperature for 6 h. [d] SOX was performed using hydrodistillation for 2 h. [e] UAE was performed at room temperature for 2 h. [f] PLE 160 was performed at 160 °C for 30 min.

maceration method. The highest extraction yield (~30%) was obtained with 90% ethanol, while those of hexane and water were comparatively low. Thus, the ethanol/water mixture, which had mild solubility compared to that of both hexane and acetone that are apt to accept the best solvents to extract hydrophobic carotenoids, was the most effective extraction solvent of the four tested. On the basis of these results, 90% ethanol was chosen as the extraction solvent.

Ethanol provides high yields of medium antioxidant activities when used for the extraction from microalgae such as *Spirulina planensis* and *Dunaliella salina* (*21*, *22*). This behavior can be explained by the high hydrophilic content of microalgae: 100 g of dry chlorella cells reportedly contain ~63 g of protein (*1*). The high extraction efficiency of 90% ethanol identified here might have been due to the high proportion of water-soluble contents in *C. vulgaris*.

Comparison of Extraction Methods. The results for PLE at 160 °C (PLE 160) were compared with those for maceration (MAC), Soxhlet (SOX), and ultrasound assisted extraction (UAE) using 90% ethanol as the extraction solvent (**Table 2**). Lutein, β-carotene, chlorophylls a and b, and pheophytin a were more effectively extracted by PLE than by other extraction methods. Although the efficiencies of UAE and PLE 160 for lutein extraction were similar, with total yields of 3.83 and 3.78 mg/g sample, respectively, PLE 160 was less time-consuming. The maximum yields of chlorophyll a and b using PLE 160 were approximately 15 mg/g. This result shows that PLE is a very effective extraction method for chlorophyll production from chlorella, compared with MAC (7 mg/g), SOX (7 mg/g), and UAE (9 mg/g). These extraction efficiencies of PLE on carotenoids and chlorophylls could be match the supercritical fluid extraction of those compounds from the same microalga (*19*, *28*).

Pheophorbide a, the chlorophyll derivative formed from chlorophyll a by endogenous chlorophyllase, was not detected in PLE 160, likely because the high operation temperature deactivated the chlorophyllase. In contrast, the compound was found in MAC, SOX, and UAE, which used mild heat processing (20−80 °C). Pheophorbide a can cause dermatitis in human and animal skins (*25*), leading the Environment Health Bureau, Ministry of Health and Welfare, Japan, to establish a safe limit (< 1.6 mg/g) for the total pheophorbide a content in processed chlorella (*26*). In SOX and UAE, the contents of pheophorbide a (5.15 and 2.15 mg/g) were beyond the standard limit, which might produce toxic symptoms. This result indicates that chlorophyllase was actually activated by mild heat treatment, accelerating the conversion of chlorophylls or chlorophyllides to pheophorbides.

Pheophorbide a formation increased in a time-dependent manner when PLE was performed at 60 or 85 °C (**Figure 2**), consistent with a previous study (*27*). The low level of pheophorbide a in PLE 110 or PLE 135 can be explained by the high temperature treatment, which may have deactivated chlorophyllase.

Optimization of PLE Conditions. The values of the independent process variables (X_1 and X_2) were investigated, and the contents

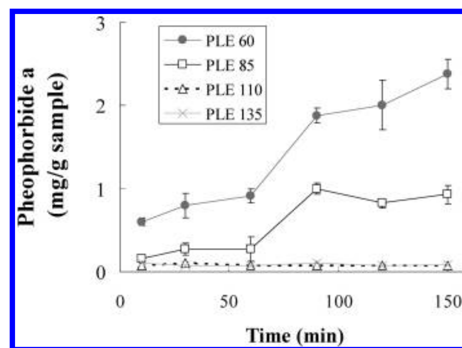

Figure 2. Effects of PLE on pheophorbide a extraction. *C. vulgaris* was extracted using PLE at different temperatures (60, 85, 110, and 135 °C) for 150 min. Data are expressed as the mean ± SD of three replicates.

Table 3. Values of 4 Responses for the PLE Experiment with 13 Runs

	responses (mg/g sample)			
run order	lutein (Y_1)	β-carotene (Y_2)	chlorophyll a (Y_3)	chlorophyll b (Y_4)
1	3.06	0.77	8.30	5.92
2	3.78	0.50	10.95	6.09
3	2.80	0.67	8.20	5.65
4	3.28	0.77	7.69	5.61
5	2.97	0.65	8.46	5.44
6	2.90	0.46	10.21	5.72
7	2.78	0.66	8.38	5.07
8	3.14	0.73	8.49	5.29
9	0.58	0.27	0.32	1.12
10	3.08	0.53	10.73	6.76
11	2.88	0.56	8.18	5.42
12	1.04	0.35	2.21	2.32
13	0.70	0.27	2.91	1.16

of the two carotenoids and two chlorophylls are shown in **Table 3**. The experiments were performed in a random order to avoid systematic errors. Experimental values of the four major compounds in PLE were analyzed by multiple regression to fit the second-order regression equation. Four second-order equations for the contents of lutein (Y_1), β-carotene (Y_2), chlorophyll a (Y_3), and chlorophyll b (Y_4) were generated as follows:

$$Y_1 = -1.843 + 0.066X_1 + 0.018X_2 - 0.00022{X_1}^2 \quad (2)$$

$$Y_2 = 0.994 + 0.121X_1 + 0.063X_2 - 0.274{X_1}^2 - 0.056{X_2}^2 \quad (3)$$

$$Y_3 = 5.421 + 1.985X_1 + 0.097X_2 - 1.114{X_1}^2 - 0.456X_1X_2 \quad (4)$$

$$Y_4 = 8.367 + 3.819X_1 - 0.147X_2 - 1.526{X_1}^2 - 0.191{X_2}^2 \quad (5)$$

X_1 and X_2 correspond to the coded values of the two independent variables of temperature and time. The statistical significance of

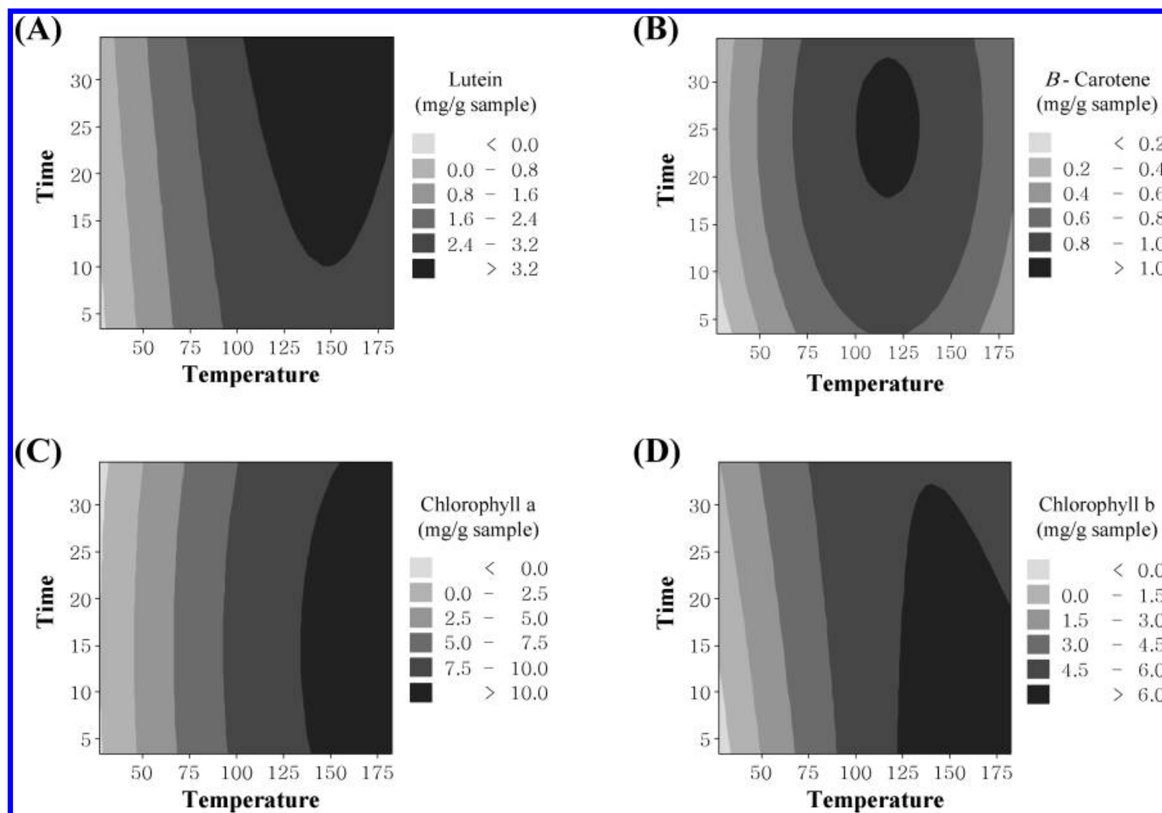

Figure 3. Surface maps of the four compounds from *C. vulgaris* as a function of extraction temperature and time. Contour plots for lutein (**A**), β-carotene (**B**), chlorophyll a (**C**), and chlorophyll b (**D**) were drawn according to the fitted mathematical regression models.

the second-order equations was determined using analysis of variance (ANOVA), and quality of fit of the regression equations was checked using the coefficient of determination (R^2). The R^2 values for lutein, β-carotene, chlorophyll a, and chlorophyll b were 0.968, 0.886, 0.958, and 0.992, respectively. All estimated models, except β-carotene, were adequate to describe the data.

Following model validation, graphs of the surface responses were drawn, revealing that temperature had an obvious effect on the extraction efficiency for all compounds (**Figure 3**). The extraction efficiency was slowly enhanced with increasing extraction time, but the trend was not apparent. Chlorophylls a and b had maximum extraction efficiencies at high temperatures of 150−160 °C. As mentioned above, severe heat treatment degraded the chlorophylls to Mg^{2+}-free chlorophyll derivatives, while using PLE at even 160 °C increased the extraction yield of chlorophylls (*25*, *29*, *30*). These inconsistent results may be due to the balance between the extraction efficiency and degradation speed of the major compounds. In PLE from *C. vulgaris*, the improvement of extraction efficiency by the high heat of the process was more decisive than chemical destruction by the degrading reaction. Chlorella accumulates large quantities of chlorophylls only in a chloroplast enclosed by a thick cell wall, which can hinder carotenoid and chlorophyll extraction (*1*). The enormous energy from the high temperature of PLE diminished the protective barrier in chlorella extraction. Temperature affects solvent viscosity and solubility but may also promote the isomerization and decompose of labile target chemicals (*16*). In our study, β-carotene was the most temperature-sensitive compound, with decreasing extraction yields in the temperature range of 120−160 °C. **Table 4** shows the optimum extraction temperature and time as determined by the response optimizer in the MINITAB program for each compound. The predicted and experimental values are also presented. In almost all cases, the maximum predicted values agreed well with the experimental values, indicating the quality of the fitted models. The optimum condition of time for lutein was 34.6 min, which is the maximum analyzed in the experimental design. The result indicates that our experiment design was not enough to optimize the PLE condition for lutein and that greater times must be studied in further studies.

Table 4. Predicted and Experimental Values for the Optimum Setting

	optimum conditions			
responses	temperature (°C)	time (min)	predicted values (mg/g)	experimental values (mg/g)
lutein	148.2	34.6	3.65	3.70
β-carotene	116.8	25.1	0.75	0.67
chlorophyll a	173.4	14.7	10.79	10.83
chlorophyll b	169.9	3.4	6.84	6.81

In conclusion, we analyzed the effects of PLE on the extraction of bioactive carotenoids and chlorophylls from *C. vulgaris*, and the PLE conditions for four main compounds were optimized with CCD. From the results, PLE showed an advantage for the extraction of oxygen- and light-sensitive carotenoids and chlorophylls. In addition, the formation of pheophorbide a, which can cause serious food-poisoning, was readily decreased by PLE.

ABBREVIATIONS USED

PLE, pressurized liquid extraction; CCD, central composite design; HPLC, high performance liquid chromatography; MS, mass spectrometry; APCI, atmospheric pressure chemical ionization; MAC, maceration; SOX, Soxhlet; UAE, ultrasound assisted extraction.

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