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Pressurized liquid method for fucoxanthin extraction from *Eisenia bicyclis* (Kjellman) Setchell

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Optimization of extraction conditions for fucoxanthin from brown algae *Eisenia bicyclis* was investigated through a pressurized liquid extraction (PLE) method and statistical experimental design. The process was optimized by Plackett–Burman design at first step to screen the most important variables in the extraction of fucoxanthin, and subsequently central composite design was applied to attain the optimum conditions of the selected factors for fucoxanthin extraction. Two factors, temperature and ethanol concentration, significantly influenced the extraction efficiency of fucoxanthin at 95% level ($P<0.05$). The maximum predicted value of fucoxanthin extraction was 0.42 mg/g at 110°C and 90% ethanol. The validation of the model was verified by triplicate experiments under the optimal conditions. The results demonstrated that the statistical strategy was successfully applied for optimization of PLE method for fucoxanthin extraction and that PLE can be a powerful method to extract fucoxanthin from *E. bicyclis*.

[**Key words:** Carotenoid; Fucoxanthin; Optimization; Pressured liquid extraction; Response surface methodology]

Fucoxanthin as a member of the carotenoid family occurs abundantly in edible brown algae, and contributes over 10% of the estimated total production of carotenoid in nature (1). This compound plays a role of light harvesting and energy transferring, thus it can help marine brown alga survive in shallow coastal waters by offering efficient photosynthesis for acclimatization in their environment (2). There have been a number of reported biological functions of fucoxanthin including anticancer, antihypertensive, anti-inflammatory, antioxidant and anti-obesity effects (3–7). For the isolation of this valuable pigment, several studies have focused on the extraction and purification of fucoxanthin from seaweeds. An improved isolation procedure for crystalline fucoxanthin from brown algae, *Fucus serratus*, was investigated through partition and silica column chromatography (8). Wang et al. (9) demonstrated that dimethyl sulfoxide was a much more effective solvent than acetone, the most commonly used organic solvent, for the extraction of fucoxanthin from fresh *Laminaria japonica* fronds. Fucoxanthin isolated from *Undaria pinnatifida* by solvent fractionation and silica gel chromatography, was assessed for the cleavage products formed by autoxidation of fucoxanthin in liposomal suspension (10). For the commercial-scale preparation of fucoxanthin, the waste parts of cultured kombu (*L. japonica*) were used as a resource of fucoxanthin. The results revealed that heating increased fucoxanthin recovery, and an additional washing step with tap water reduced the salt concentration of the fucoxanthin extract (11). Recently, a new extraction approach using supercritical carbon dioxide with ethanol as the co-solvent has been performed with brown seaweed (*U. pinnatifica*) oil to optimize the best extraction conditions (12).

Pressurized liquid extraction (PLE) is a sample preparation technique that combines elevated temperature and pressure with liquid solvents to achieve fast and efficient extraction of the analytes from the solid matrix (13). This technique has been considered as a green extract method due to its decreased solvent use, short operating time, and light- and oxygen-free environment (14). These factors can be advantageous for fucoxanthin extraction from brown seaweeds, because plant carotenoids are particularly sensitive to degradation during extraction and this can lead to *cis–trans* isomerisation, oxidative cleavage and/or epoxidation of the carotenoid backbone (15). Therefore, in this study, optimization of a PLE method was investigated in the extraction of fucoxanthin from *Eisenia bicyclis* (Kjellman) Setchell. *E. bicyclis*, a common edible brown algae, belongs to Lamianariaceae and inhabits the middle pacific coast around the East Sea (16). While a number of researches were performed on the pharmaceutical activities with the extract and the isolated compounds of this alga (17–19), none have been reported on the optimization of fucoxanthin isolation from this alga. Response surface methodology (RSM), which is a widely used statistical optimization procedure used to explore the relationships between several explanatory variables and one or more response variables (20), was applied for the experimental design of the PLE procedure.

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MATERIALS AND METHODS

Chemicals and materials The fronds of *E. bicyclis* were harvested from the coast of Ulleung Island, East Sea, Korea in June 2009. The collected seaweed was washed with fresh water three times and then stored at −20°C until use. The solvents used for extraction and PLE were analytical grade and were purchased from Daejung (Gyonggi, Korea). All HPLC-grade solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). Pure fucoxanthin were isolated from *E. bicyclis* and used for the construction of calibration curve.

Plackett–Burman design Plackett–Burman design (PBD) was introduced as a first optimization step to determine important factors in fucoxanthin extraction by PLE as described in Ghanem et al. (21). The technique is based on the first order polynomial model:

$$Y = \beta_0 + \sum \beta_i X_i \quad (1)$$

where Y is the response (fucoxanthin extraction efficiency, FE), β_0 is the model intercept, β_i is the linear coefficient and X_i is the level of the independent variable. According to the parameters of PLE, six independent parameters in PLE procedure were used as variables: X_1 = temperature (°C), X_2 = concentration of ethanol solvent in water (%), X_3 = static time (min), X_4 = pressure (psi), X_5 = weight of sample (g) and X_6 = flush (%) were examined at two levels: −1 for low level and +1 for high level to investigate how the key ingredients significantly affect FE, as seen in Table 1. Design-Expert statistical experiment design software 7.0.0 (Sta-Ease Inc., Minneapolis, MN, USA) produced 12 experimental designs based on PBD from six variables. The significant effect of each variable on fucoxanthin extraction was screened at a 95% significant level ($P<0.05$) by Analysis of Variance (ANOVA). The design matrix is shown in Table 2.

Pressurized liquid extraction Pressurized liquid extraction was carried out using a fully automated ASE 200 system (Dionex, Sunnyvalue, CA, USA). Fresh *E. bicyclis* was cut into small pieces (approximately 2×2 mm) and loaded into the stainless steel cell (11 mL) with sea sand (particle size 30–50 mesh, Fisher Chemicals) above and below the sample to avoid any void spaces. The extraction cell was placed into the carrousel and submitted to the experimental conditions given in Table 2. The automatic extraction sequence began with the loading of the cell into the oven. When the cell was heated to the preset extraction temperature with continuous solvent flow, the cell was pressurized for a fixed time and then allowed to flow into the collection vial with a fixed flush volume. After the extraction, the sample was rinsed with a portion of fresh solvent under low pressure. The extraction solution was made up to 25 mL with extraction solvent and filtered through a 0.45 μm membrane filter (Agilent Technologies, USA) prior to injection into HPLC system for quantification.

Central composite design For the optimization of the significant variables of the PLE, a full second-order central composite design (CCD) was applied on the extraction of fucoxanthin. Based on the result of the PBD, two significant factors, temperature (X_1) and ethanol concentration (X_2), were selected and five levels of each variable were applied for a more exact experiment. Other parameters have no significant effect on results, thus, they were set by economic and time-saving rules as follows: 2 g of fresh *E. bicyclis*, 5 min of extraction static time, 1500 psi of pressure, and 40% flush volume of the extraction cell. A 2^2 CCD with five replicates at the center point leading to 13 experiments was employed to optimize the fucoxanthin extraction using Design-Expert statistical experiment design software as above. A second-order polynomial, quadratic model was used for the prediction of optimal point, and its equation was expressed as follows:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (2)$$

where Y is the response (FE); β_0, β_i, β_{ii} and β_{ij} represent the interception coefficient, the linear coefficient, the quadratic coefficients and the interactive regression coefficient, respectively and X_i and X_j are the level of independent variables (X_1 and X_2). The actual levels are coded at five levels as seen in Table 3. Three-dimensional plots of two factors versus FE and corresponding contour plots were performed by Design-Expert statistical experiment design software.

Chromatographic analysis and quantification of fucoxanthin All extracts were analyzed by Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA) consisting of a G1312A binary pump, a G1367B autosampler, a G1315D PDA detector, a G1316A column oven and Chemstation software. Reverse phase YMC carotenoid column [150×4.6 mm i.d., 3 μm particle size, Waters, Milford, MA, USA] was used to analyze the fucoxanthin. The mobile phase was methanol and *tert*-butyl methyl ether (TBME) with flow rate of 1 mL/min, and a gradient program was used as follows: TBME was increased from 0% to 35% in 20 min and then, decreased to 0% in 10 min. The chromatogram was recorded at 440 nm for fucoxanthin. The calibration curve was constructed at a range of 0.85–850 μg/mL for the quantification of fucoxanthin in the extract solution.

Table 1. Independent variables and their levels in the statistical analysis of Plackett–Burman design.

Variables	Parameters	Experimental values		F value	P value[a]
		Low level (−1)	High level (+1)		
X_1	Temperature (°C)	40	100	14.42	0.0127*
X_2	Ethanol concentration (%)	50	100	165.71	<0.0001**
X_3	Static time (min)	5	15	4.63	0.0841
X_4	Pressure (psi)	1000	2500	4.05	0.1003
X_5	Weight of sample (g)	2	4	3.52	0.1196
X_6	Flush volume (%)	40	100	0.38	0.5651

[a] *$P<0.05$, **$P<0.01$ by ANOVA analysis.

Table 2. Experimental design and the responses (FE, fucoxanthin extraction efficiency) determined from 12 experiment runs generated by Plackett–Burman design.

Run	Process parameters[a]						Responses[b] FE (mg/g)
	X_1	X_2	X_3	X_4	X_5	X_6	
1	40	50	5	2500	2	100	0.01 ± 0.002
2	40	50	5	1000	2	40	0.01 ± 0.003
3	40	100	15	2500	2	40	0.35 ± 0.131
4	100	100	5	1000	2	100	0.42 ± 0.231
5	100	50	15	2500	2	100	0.10 ± 0.042
6	100	100	15	1000	2	40	0.42 ± 0.109
7	40	100	15	1000	4	100	0.29 ± 0.110
8	40	50	15	1000	4	100	0.086 ± 0.109
9	40	100	5	2500	4	40	0.200 ± 0.012
10	100	100	5	2500	4	100	0.327 ± 0.100
11	100	50	5	1000	4	40	0.094 ± 0.053
12	100	50	15	2500	4	40	0.072 ± 0.024

[a] Parameters (X_1 … X_6) are defined in Table 1.
[b] Values are expressed as mean ± standard deviation of five times replicate experiments.

RESULTS AND DISCUSSION

Screening of significant factors in PLE affecting FE The principle of PLE is simple. The sample is extracted with an organic solvent at temperatures ranging from room temperature to 200°C and at a relative high pressure (from 500 to 3000 psi). Several parameters affecting the PLE process were selected as seen in Table 1. In the PLE process, raising the temperature improves the contact of the analytes with the solvent and enhances the extraction through increase of diffusion rate, solubility of analytes and mass transfer, and decrease of the viscosity and surface tension of the solvents (13). The nature of the extraction solvent also has a profound effect on PLE efficiency. Generally, mixtures of low- and high-polar solvents provide more efficient extractions of the analytes than single solvents. Not only temperature and extraction solvents, but also pressure, extraction time (static time), flush volume, flow rate, packing the extraction cell and sample weight may well influence PLE efficiency and their effects may be either independent or interactive (20). In this study, these

Table 3. The central composite experimental design with two independent variables and fucoxanthin extraction efficiency (FE).

Run	Independent variables[a]				FE (mg/g)	
	X_1	Code X_1	X_2	Code X_2	Experimental values[b]	Predicted values[c]
1	40	−2	70	0	0.226 ± 0.02	0.26
2	80	−1	55	−1	0.299 ± 0.14	0.26
3	80	−1	85	1	0.439 ± 0.03	0.41
4	120	0	100	2	0.444 ± 0.03	0.43
5	120	0	40	−2	0.048 ± 0.02	0.053
6	120	0	70	0	0.361 ± 0.04	0.37
7	120	0	70	0	0.405 ± 0.02	0.37
8	120	0	70	0	0.331 ± 0.07	0.37
9	120	0	70	0	0.385 ± 0.03	0.37
10	120	0	70	0	0.401 ± 0.02	0.37
11	160	1	55	−1	0.092 ± 0.03	0.13
12	160	1	85	1	0.296 ± 0.01	0.36
13	200	2	70	0	0.127 ± 0.01	0.081

[a] X_1 = temperature; X_2 = ethanol concentration.
[b] Values are expressed as mean ± standard deviation of triplicate experiments.
[c] Predicted values are calculated from a second-order polynomial equation obtained from the experimental values.