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Antioxidant activity of *Spirulina platensis* extracts by supercritical carbon dioxide extraction

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Abstract

Supercritical CO_2 extraction of antioxidants from *Spirulina platensis* was optimized using response surface methodology. About 10.26 g/kg of extracts from *S. platensis* could be obtained under the optimum conditions of 48 °C at 20 MPa over a 4 h period. The antioxidant activity of the extracts prepared under the optimized condition, determined by linoleic acid peroxidation inhibition method, was lower compared with BHT and Trolox, but significantly higher than α-tocopherol in 300 min and became similar to α-tocopherol after that. The components of the extracts were further analyzed, and the results showed that the extracts contained 85.1 g/kg of flavonoids, 77.8 g/kg of β-carotene, 113.2 g/kg of vitamin A and 3.4 g/kg of α-tocopherol, which may contribute greatly to their high antioxidant activity. The main fatty acids in the extracts were palmitic acid (35.32%), linolenic acid (21.66%) and linoleic acid (20.58%).

Keywords: *Spirulina platensis*; Supercritical carbon dioxide; Extraction; Antioxidant

1. Introduction

Microalga species are receiving increasing attention mainly for their bioactive components such as polyunsaturated fatty acids, β-carotene and other pigments (antioxidants) (Bhat & Madyastha, 2000; Cohen & Vonshak, 1991; Madhava et al., 2000; Mahajan & Kamat, 1995), sulphated polysaccharides (anti-virals) and sterols (anti-microbials) (Otles & Pire, 2001; Xue et al., 2002). *Spirulina platensis* is a high quality health food with high levels of protein, vitamins, minerals, polyunsaturated fatty acids, zeaxanthin and myxoxanthophyll and has been reported to have pharmaceutical potential (Li, Guo, & Li, 2003; Morist, Montesinos, Cusido, & Godia, 2001).

The recovery of lipophilic bioactive components from *S. platensis* has posed a problem due to the presence of residual organic solvents and instability of the extracts. Supercritical carbon dioxide extraction (SC-CO_2) is an attractive alternative to conventional liquid extraction due to its mild environment in the process and no residue of harmful solvents. Furthermore, SC-CO_2 extraction has been proposed for antioxidants from rosemary leaves, sage and herbaceous matrices. The antioxidant activity of extracts prepared by SC-CO_2 was significantly higher compared to the conventional means. Therefore, supercritical fluid extraction may serve as a very promising process in future (King, 2000).

In this paper, response surface methodology was adopted to optimize temperature, pressure and time for supercritical CO_2 extraction and to obtain a available regression equation. Meanwhile, antioxidant activity of the extracts under optimized condition was determined by inhibition of peroxidation of linoleic acid. Furthermore, the components of the extracts which may contribute greatly to the antioxidant activity were analyzed. Thus, this work is expected to find natural antioxidants with great activity from *S. platensis*.

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2. Materials and methods

2.1. Materials

Spirulina platensis was provided by Jiangsu Academic of Agricultural Sciences (Nanjing, China). Butylated hydroxytoluene (BHT) was from Nanjing Chemical Industry (Nanjing, China); Linoleic acid, 2,2′-azobis (2-amidinopropane) hydrochloride (AAPH) was obtained from Wako Chemical Industries Ltd. (Osaka, Japan); Rutin, vitamin A, β-carotene, α-tocopherol-6-hydroxy-2,5,7,8-tetramethlchroman-2-carboxylicacid (Trolox) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA).

2.2. Optimization of process

The process of extraction was optimized with Box-Behnken design for a higher yield of antioxidants from *S. platensis* (Bosque-Sendra, Pescarolo, Cuadros-Rodrıguez, Garcıa-Campaña, & Almansa-López, 2001; Box & Behnken, 1960). The factors and levels investigated in the study were shown in Table 1. Experimental design, data analysis and quadratic model building were conducted using the software Design Expert (Version 6.0.5, Stat-Ease Inc., Minneapolis, MN, USA).

2.3. Extraction of antioxidants

Supercritical fluid extraction was performed on a Hua'an supercritical fluid extractor (Hua'an Co., Ltd. Nantong, China) with the extractor volume of 1 L. The schematic flow diagram of supercritical fluid extractor is shown in Fig. 1. The major parts of the apparatus are a high-pressure extractor and two separator flasks. The flow rate of CO_2, the extraction temperature and pressure were monitored by the dial plate on the front panel, and the extraction time was set by the timer. Liquid CO_2 was supplied from a gas cylinder. Before it passed into the extraction vessel filled with the samples by a pump, the liquid CO_2 was pressurized to the desired pressure and heated to the specified temperature to reach the supercritical state. Absolute ethanol acting as the co-solvent was added in a vaporizer and mixed with the sample in the extractor by another pump. The system was stabilized for 2 h under the desired conditions prior to the beginning of the experiment. The extracts extracted under the optimal conditions were collected for analysis of antioxidant activity and components.

Table 1
Experimental levels and codes of the factors used in Box-Behnken design

Factors	Symbols	Coded levels		
		−1	0	1
Temperature (°C)	A	32	40	48
Pressure (MPa)	B	20	30	40
Time (h)	C	2	3	4

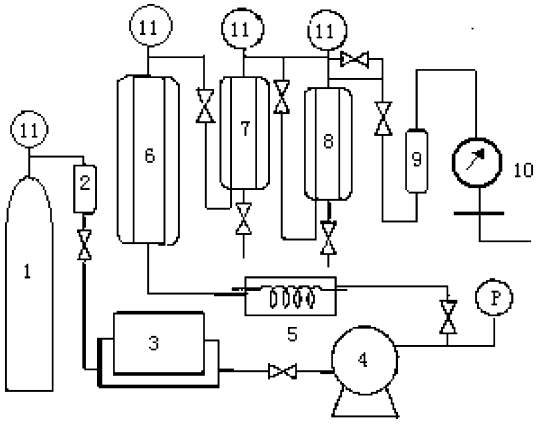

Fig. 1. The experimental equipment of supercritical fluid extraction: 1, gas cylinder; 2, filter; 3, cooler; 4, compressor; 5, pre-heater; 6, extractor; 7, 8, separator; 9, flow meter; 10, pressure cumulative flow meter; 11, pressure indicator.

2.4. Determination of antioxidant activity

The antioxidant activity of the extracts from *S. platensis* was assayed by a linoleic acid system (Huang, Mendis, & Kim, 2005; Takeshi, Mizuho, Reiji, Hachiro, & Nobutaka, 2001). One millilitre of five milligrams extracts/ml absolute ethanol, 4 ml of 0.05 M sodium phosphate buffer (pH 7.0), One millilitre of anhydrous ethanol and 2 ml distilled water were mixed with 2 ml of 2.5% (v/v) linoleic acid in ethanol. The preoxidation was initiated by the addition of 0.4 ml of 0.1 M AAPH and carried out at 37 °C in the dark. The degree of oxidation was measured by reading the absorbance at 500 nm after colouring with $FeCl_2$ and ammonium thiocyanate (Kikuzaki & Nakatani, 1993). BHT and α-tocopherol (1 mg/ml) in ethanol and the same concentration of Trolox in deionized water were used as references. The control was absolute ethanol.

2.5. Analysis of components of extracts

2.5.1. Determination of flavonoids content

The flavonoids content in the extracts from *S. platensis* was measured by the $AlCl_3$ method based on the formation of a complex flavonoid-aluminium (Djeridane et al., 2006). The extracts were diluted with absolute ethanol to an appropriate concentration. Then 1 ml of diluted sample was mixed with 1 ml of 2% (w/v) methanolic solution of aluminum chloride. After incubation at room temperature for 15 min, the absorbance of the reaction mixture was read at 430 nm with a spectrophotometer. Rutin was used as a standard.

2.5.2. Quantification of β-carotene, vitamin A and α-tocopherol

β-Carotene in the extracts was assayed by HPLC system (Shimadzu Co., Kyoto, Japan), equipped with DGU-14 A Degasser, LC-10AT quaternary pump, and SPD-10AV UV–Visible detector at 450 nm. Separations were per-

formed on a Zorbax SB-C18 column, 150 mm × 4.6 mm, 5 μm particle size (Agilent Technologies, Wilmington, DE, USA) (Zhang & Omaye, 2001). The mobile phase consisted of acetonitrile/methylene chloride 7:3 (v/v) and ran at a flow rate of 1.0 ml/min. Synthetic *all-trans* β-carotene was used as a standard.

Vitamin A and α-tocopherol in the extracts were determined simultaneously by HPLC analysis after saponification and extraction (Zhang & Omaye, 2001). The mobile phase remained at 100% methanol, after a gradient from 90% methanol/water (v/v) to 100% methanol in 10 min at 1.0 ml/min. Vitamin A and α-tocopherol were detected at 325 and 292 nm, respectively.

2.5.3. Analysis of fatty acid profile

The fatty acid profiles in the extracts were analyzed by GC–MS (Hartvigsen, Hansen, Lund, Bukhave, & Hølmer, 2000). In order to obtain the FAME, the sample were brought to a temperature of 40 °C, and dehydrated by filtering with Na_2SO_4. One millilitre of the oil was added to 1 ml of a 10% (v/v) solution of concentrated H_2SO_4 in MeOH, then heated at 110 °C for 2 h in closed vials. After cooling, the FAME phase was separated from the acidic solution. Injection was carried out at 250 °C. The oven temperature was programmed at 180 °C for 1 min, and then rose to 230 °C at 10 °C/min. The final temperature was maintained for 3 min. Then the compounds were identified by mass spectrometric analysis. Spectra of the compounds were compared with those in the US National Institute of Standards and Technology (NIST) library.

2.6. Statistics analysis

All computations were performed by SAS (version 8.0). The data were presented as means ± standard deviations of three determinations. Analysis of variance followed by Student's *t*-test was used to see the differences amongst various groups for each concentration. Multiple comparisons of means were done by LSD (least significant difference) test. A probability value of <0.05 was considered significant.

3. Results and discussion

3.1. Optimization of process

The Box-Behnken design matrix of the factors was given in Table 2 along with the experimental and predicted values of yield of the extracts from *S. platensis*. By applying multiple regression analysis methods, regression equation could be obtained and given as:

$$Y = 7.02900 - 0.26063A - 0.011275B - 1.14775C + 0.0032266A^2 + 0.00059000B^2 + 0.13400C^2 - 0.00065625AB + 0.011563AC - 0.0010000BC$$

where Y was the predicted yield of the extracts from *S. platensis*.

Table 2
Box-Behnken design matrix along with the experimental and predicted values of the yield of extracts

Std.	Temperature (°C)	Pressure (MPa)	Time (h)	Yield (g/kg)	
				Experimental	Predicted
1	−1	−1	0	4.00	3.96
2	1	−1	0	6.68	6.98
3	−1	1	0	4.28	3.99
4	1	1	0	4.89	4.93
5	−1	0	−1	4.23	4.79
6	1	0	−1	4.66	4.89
7	−1	0	1	4.90	4.67
8	1	0	1	9.09	8.53
9	0	−1	−1	4.26	3.74
10	0	1	−1	3.23	2.97
11	0	−1	1	5.48	5.74
12	0	1	1	3.97	4.49
13	0	0	0	2.33	2.31
14	0	0	0	2.06	2.31
15	0	0	0	1.98	2.31
16	0	0	0	2.50	2.31
17	0	0	0	2.69	2.31

The analysis of variance (ANOVA) was reported in Table 3. *F*-value and probability value indicated that the model was actually significant. The fit of the model was checked by the coefficient of determination (R^2), which was 0.9628, pointing out that less than 4% of the total variations could not be explained by the model. The value of R (0.9814) indicated good agreement between the experimental and predicted values of yield (Pujari & Chandra, 2000). The value of lack-of-fit was not significant ($P = 0.0580$), indicating that the model equation was adequate for predicting the yield under any combination of values of the variables (Rastogi & Rashmi, 1999).

The fitted response surfaces for the yield were generated using the Design Expert program (see Fig. 2). From these three-dimensional plots and corresponding contour plots, it is evident that the interactions of extraction temperature and pressure, extraction temperature and time were very significant, but the interaction of extraction pressure and time was not significant, as evaluated by statistical analysis with software Design Expert. From the plots, it was also easily seen that the yield of antioxidant was sensitive even to the minor alterations of extraction temperature and time, but was insensitive to the extraction pressure. The yield of antioxidants decreased with

Table 3
Analysis of variance (ANOVA) for the fitted quadratic polynomial model

Source	Sum of squares	d.f.	Mean square	*F*-value	Probability $(P) > F$
Model	50.16	9	5.57	20.13	0.0003
Lack-of-fit	1.59	3	0.53	6.00	0.0580
Pure error	0.35	4	0.088		
Corrected total	52.16	16			

$R = 0.9812$, $R^2 = 0.9628$, $R^2_{adj} = 0.9150$.

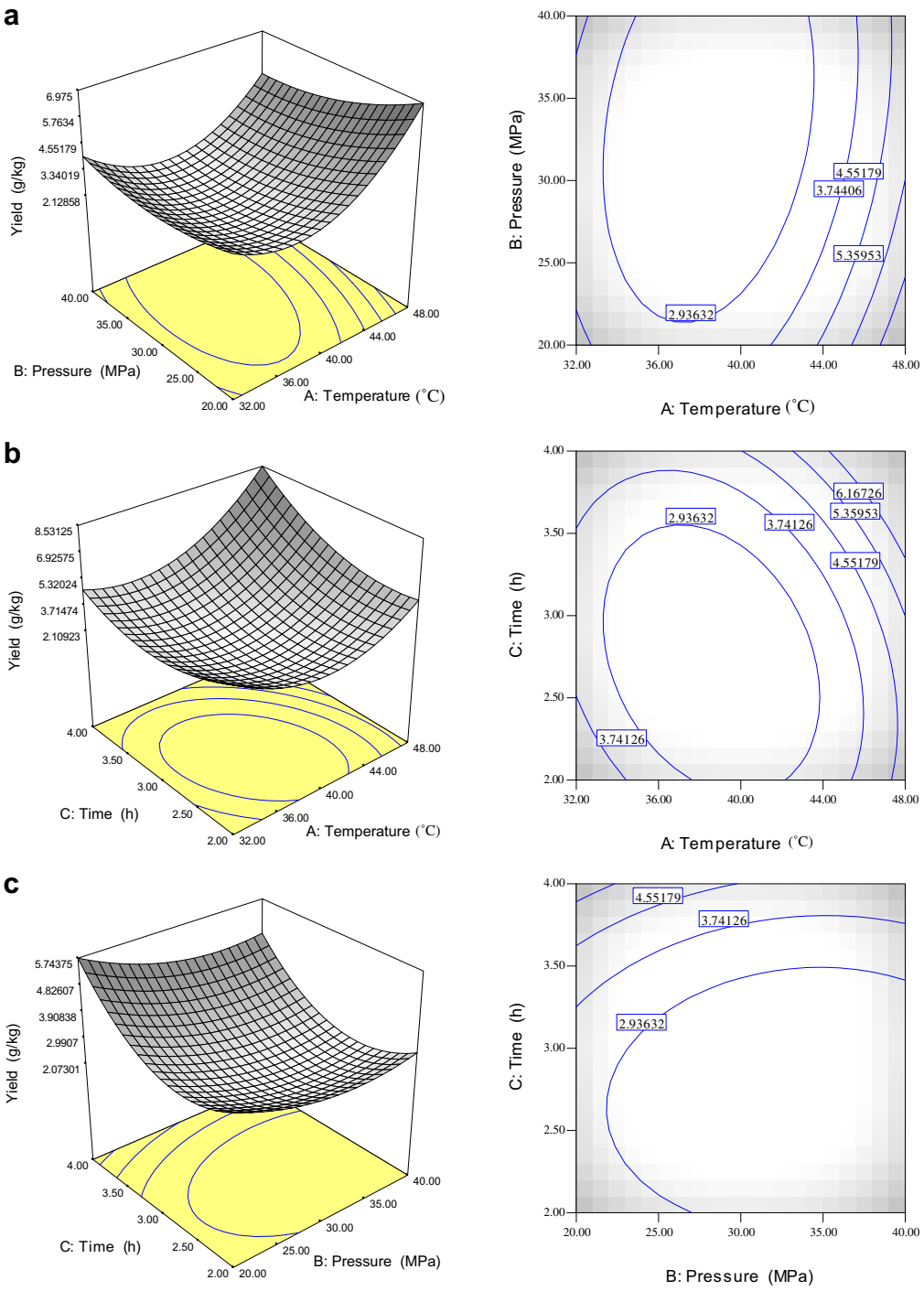

Fig. 2. Fitted response surface and corresponding contour: (a) yield vs. extraction temperature and pressure at fixed time of 3 h; (b) yield vs. extraction temperature and time at fixed pressure of 30 MPa; (c) yield vs. extraction pressure and time at fixed temperature of 40 °C.

increasing temperature up to 37 °C, and then increased sharply and reached maximum yield at 48 °C within the tested range (Fig. 2a and b). The yield increased with increasing time, and reached a maximum at 4 h within the tested range (Fig. 2b and c).

By solving the inverse matrix with software Design Expert, the optimum levels of the tested factors were 48 °C, 20 MPa and over 4 h. Under these conditions, the maximum predicted yield of the extracts from *S. platensis* was about 10.26 g/kg.

The adequacy of the model was further validated by verification experiments. A total of 10 verification experiments were carried out under different combination of process parameters (within the tested area) generated by software Design Expert. The correlation coefficient (R) between the experimental and predicted values was 0.9882, which

indicated that the experimental values were in good agreement with the predicted values, and also suggested that the model was satisfactory and accurate.

3.2. Antioxidant activity of extracts

The antioxidant activity of the extracts from *S. platensis* was assayed by inhibiting peroxidation of linoleic acid method, and results are shown in Fig. 3. High absorbance was an indication of high degree of peroxidation of linoleic acid and low antioxidant activity of antioxidants. It was clear that the extracts and antioxidants showed high inhibition activity towards peroxidation of linoleic acid. The activity of extracts from *S. platensis* was lower compared with that of BHT and Trolox, but was significantly higher than that of α-tocopherol in 300 min and became similar after that. The antioxidant activity of extracts from *S. platensis* and the reference antioxidants decreased in the following order: Trolox > BHT > extracts ⩾ α-tocopherol.

However, statistic analysis indicated no significant difference of antioxidant activity between Trolox and BHT. And this antioxidant pattern was quite similar to the results of BHT > Trolox > α-tocopherol reported by Dubuisson, Gülçin and Hu (Dubuisson et al., 2001; Gülçin, Mshvildadze, Gepdiremen, & Elias, 2006; Hu, Xu, Chen, & Yang, 2004). As for the reference antioxidants, several literatures indicated another different order of BHT > α-tocopherol > Trolox (Castro, Rogero, Junqueira, & Carrapeiro, 2006; Gülçin, 2006a; Gülçin, 2006b). The difference is of great interest and need to be further investigated.

3.3. Components of the extracts

The fatty acid profile was analyzed by GC–MS, palmitic acid (35.32%), linolenic acid (21.66%) and linoleic acid (20.58%) were the main components present (Table 4). Moreover, the components of the extracts, which may contribute greatly to the antioxidant activity, were analyzed tentatively. The result showed that 85.1 g/kg of flavonoids, 77.8 g/kg of β-carotene, 113.2 g/kg of vitamin A, 3.4 g/kg of α-tocopherol were contained in the extracts from *S. platensis* (Table 5).

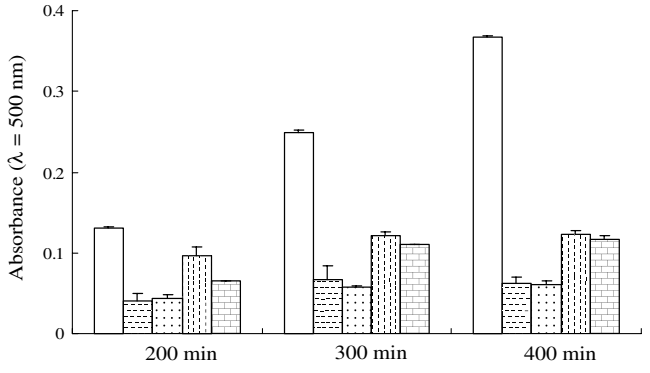

Fig. 3. Antioxidant activities assessed by inhibiting peroxidation of linoleic acid method. □, control; ▨, BHT; ▨, trolox; ▨, α-tocopherol; ▨, extracts from *Spirulina platensis*.

Table 4
Fatty acid profile and relative content of extracts from *Spirulina platensis*

Retaining time for GC–MS (min)	Component	Molecular formula	Similitude Index[a]	Relative content (%)
4.13	2,6-Diisopropyl phenol	$C_{12}H_{18}O$	884	1.71
4.97	Pentadecanoic acid	$C_{15}H_{30}O_2$	660	1.16
6.75	Palmitic acid	$C_{16}H_{32}O_2$	947	35.52
6.87	_[b]	_[b]	_[b]	1.57
7.06	Zoomaric acid	$C_{16}H_{30}O_2$	880	1.74
7.15	Zoomaric acid	$C_{16}H_{30}O_2$	880	5.96
9.05	_[b]	_[b]	_[b]	0.83
9.84	Stearic acid	$C_{18}H_{36}O_2$	858	2.02
10.21	Oleic acid	$C_{18}H_{34}O_2$	890	2.81
10.32	Oleic acid	$C_{18}H_{34}O_2$	867	2.70
10.65	Linoleic acid	$C_{18}H_{32}O_2$	798	1.28
10.99	Linoleic acid	$C_{18}H_{32}O_2$	935	18.40
11.50	Linolenic acid	$C_{18}H_{30}O_2$	913	21.66
12.39	Linoleic acid	$C_{18}H_{32}O_2$	778	0.90
12.86	_[b]	_[b]	_[b]	0.69
16.88	_[b]	_[b]	_[b]	1.06

[a] The data was produced by GC software. The higher the Similitude Index (0–1000), the more accurate the result was.
[b] Compound not searched in NIST library.

At the present time, several compounds, which may be attributed to the high antioxidant activity of extracts, have been identified, including some phenolic compound, carotenoids, phycobiliproteins, chlorophyll and some chlorophyll degradation products (Bhat & Madyastha, 2001; Jaime et al., 2005; Mendiola et al., 2005; Pinero Estrada, Bermejo Bescos, & Villar del Fresno, 2001). In this paper, we have tentatively analyzed only four compounds in the extracts, which included flavonoids, β-carotene, vitamin A and α-tocopherol. Flavonoids in the extracts from *S. platensis* were the first time reported. Additional work is therefore necessary to fractionate and identify the extracts further to elicit a better understanding of how each chemical fraction contributes to the overall antioxidant activity and whether the mixture of extracts contributes to a synergistic antioxidant activity.

Table 5
The components that may contribute to the antioxidant activity of the extracts from *Spirulina platensis*

Component	Content (g/kg)
Flavonoids	85.1 ± 7.3
β-Carotene	77.8 ± 6.8
Vitamin A	113.2 ± 2.7
α-Tocopherol	3.4 ± 0.3

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