



Original Article

Enhancement of antioxidant activity of C-phycoerythrin of *Spirulina* powder treated with supercritical fluid carbon dioxideMonchai Dejsungkranont,^a Ho-Hsien Chen,^b Sarote Sirisansaneeyakul^{a, c, *}^a Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok, Thailand^b Department of Food Science, National Pingtung University of Science and Technology, Pingtung, Taiwan^c Center for Advanced Studies in Tropical Natural Resources, National Research University-Kasetsart University, Bangkok, Thailand

ARTICLE INFO

Article history:

Received 29 July 2016

Accepted 16 June 2017

Available online 27 December 2017

Keywords:

Antioxidant activity

C-phycoerythrin

Spirulina sp.

Supercritical fluid carbon dioxide pretreatment

Taguchi method

ABSTRACT

The functionality and activity of proteins can be modified by supercritical fluid CO₂ (SCFCO₂). The objectives of this study were to investigate the possibility of enhanced antioxidant activity of C-phycoerythrin (C-PC) proteins from light-harvested *Spirulina maxima* powder using the SCFCO₂ pretreatment and to optimize the SCFCO₂ pretreatment conditions enhancing the antioxidant activity of C-PC. The Taguchi method was used to determine the optimum conditions for the SCFCO₂ pretreatment. The experimental factors were the pretreatment temperature, pressure, pretreatment mode (static, dynamic and conjugated) and duration. The optimal conditions of SCFCO₂ pretreatment were: 60 °C, 24.13 MPa and 60 min in static batch mode. Using these pretreatment conditions, the maximum antioxidant activity of C-PC from the treated residual biomass was 410.1 μmole trolox/mg, which was 1.7-fold higher than the untreated biomass (control). The factor that most affected the antioxidant activity of C-PC was temperature (59%). A high pretreatment temperature could damage C-PC, but promoted antioxidant activity. Of note is that this work was the first to explore SCFCO₂ treatment enhancing the antioxidant activity of C-PC in *Spirulina* sp. powder.

Copyright © 2017, Kasetsart University. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

C-phycoerythrin (C-PC) is a blue pigment and the most commercially promising substance found in *Spirulina* sp (Liang, et al., 2004; Iyer et al., 2007). It is a water-soluble phycobiliprotein (PBP) and photosynthetic accessory pigment (Eriksen, 2008). Its main function is to collect and transfer light energy for the chlorophyll when the chlorophyll experiences poor absorption and transfer (Bennett and Bogorad, 1973; Zilinskas and Greenwald, 1986; Grossman et al., 1994). C-PC absorbs light at a wavelength of approximately 620 nm and emits light (or fluoresces) at approximately 640 nm (Bennett and Bogorad, 1973). C-PC serves to store nitrogen and is selectively degraded when the cells are starved of nitrogen (Grossman et al., 2001; Sloth et al., 2006). C-PC has been mainly used as a nutritional ingredient, a fluorescent marker or as a natural dye in foods or cosmetics and is also known to be antioxidant, anti-inflammatory, antiplatelet, anti-cancer, antifungal and antiviral in

nature. It also has nephroprotective and hepatoprotective properties (Eriksen, 2008). Moreover, phycobiliproteins have yet to be reviewed in the literature (Manirafasha et al., 2016; Stadnichuk and Tropin, 2017). Among its numerous bioactivities, the antioxidant function of C-PC may have the most value. In fact, it can protect the living cell from oxidative stress by delaying or inhibiting lipid oxidation (Chadwick et al., 2003). Evidence taken from medical treatments has revealed that the intake of antioxidant food constituents can maintain a balance between the antioxidant system and reactive oxygen species (ROS) production (Chadwick et al., 2003; Samaranayaka and Li-Chan, 2011). Thus, the human body can fight various diseases such as atherosclerosis, alzheimer, cancer, diabetes mellitus, rheumatoid arthritis, inflammatory diseases and the aging process (Wu et al., 2005; Durackova, 2010).

Supercritical fluid CO₂ (SCFCO₂) is an interesting process to separate non polar compounds from materials. It is environmentally benign due to its non-toxic and non-flammable nature (Wimmer and Zarevúcka, 2010). Furthermore, it is inexpensive because the CO₂ used is a byproduct of industrial processes, substantially available, odorless, tasteless and can be removed from products easily (Fidder, 1999). It has low critical temperature and

* Corresponding author. Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Chatuchak, Bangkok 10900, Thailand.

E-mail address: sarote.s@ku.ac.th (S. Sirisansaneeyakul).

pressure (31.0 °C and 7.36 MPa, respectively) which can preserve thermally unstable substances (Wimmer and Zarevúcka, 2010; Mallikarjun et al., 2014). The solvent power can be tuned by the manipulation of the temperature and pressure (Crampon et al., 2013). Currently, SCFCO₂ is a valuable tool which is widely used for the large-scale extraction of natural compounds and pharmaceutical products (Mendes et al., 2003). Bioactive compounds have been successfully extracted from *Spirulina* sp. and other microalgae using SCFCO₂ include γ -GLA (Mendes et al., 2006), β -carotene (Careri et al., 2001), chlorophylls (Tong et al., 2011), vitamin E (Mendiola et al., 2008), phenolic compounds (Hsueh et al., 2012), antioxidant compounds (Mendiola et al., 2007; Qiuhui et al., 2007; Wang et al., 2007; Millao and Uquiche, 2016), phycocyanin (Deniz et al., 2016) and antimicrobial compounds (Mendiola et al., 2007; Mallikarjun et al., 2014). Several studies have demonstrated the successful use of SCFCO₂ pretreatment to improve the properties and quality of raw materials such as lignocellulosic biomass for ethanol and biofuel production (Serna et al., 2016) and whey protein for producing higher quality products in the food industry (Zhong and Jin, 2008). Other studies have achieved the inactivation of microorganisms and enzymes using SCFCO₂ (Balaban et al., 1991; Kim et al., 2007). In addition, the activities of enzymes (lipase and α -amylase) were increased after SCFCO₂ treatment (Giessauf and Gamse, 2000; Yan et al., 2001). These studies provide evidence of the potential for CFCO₂ pretreatment to modify the antioxidant activity of C-PC for various biochemical applications.

Generally, protein is damaged by higher temperature, especially above 70 °C (Opstvedt et al., 1984). Also, the higher pressures cause conformational changes and protein denaturation due to deprotonation of charged groups and the destruction of salt bridges and hydrophobic bonds (Barbosa-Cánovas et al., 1998). It has been reported that the stability and activity of proteins under the SCFCO₂ process depend on protein species, pressure and temperature (Wimmer and Zarevúcka, 2010). The three-dimensional structure of C-PC and other proteins changes under extreme conditions, causing their denaturation and loss of activity as minor structural changes induce an alternative active protein state with altered activity, specificity and stability (Wimmer and Zarevúcka, 2010; Deniz et al., 2016). None of the published work in this field has reported on SCFCO₂ pretreatment being used to improve the bioactivity or functionality of *Spirulina* powder. There has been only one report of using SCFCO₂ extraction to separate and purify the active component and remove the stench from *Spirulina* powder (Qiuhui, 1999). To date, there has been no published record of enhanced antioxidant activity of bioactive compounds in *Spirulina* sp. powder or other microalgae. However, there have been abundant reports of using SCFCO₂ extraction to obtain the highest yield and antioxidant activity of the extract (Mendiola et al., 2007; Qiuhui et al., 2007; Wang et al., 2007; Millao and Uquiche, 2016). Thus, it is an interesting target to study the general effect of SCFCO₂ pretreatment on the resultant C-PC and the antioxidant of *Spirulina* powder. A conceptual schematic diagram of the economical production of C-PC from *Spirulina* sp. through SCFCO₂ pretreatment is shown in Fig. 1. After SCFCO₂ pretreatment, the extracts (lipophilic products) can be used as healthy products, while the residual biomass (I) can be used for the extraction of C-PC. The residual biomass (II) from the C-PC extraction can then be used as healthy products using the biorefinery process. Therefore, the production of C-PC using SCFCO₂ pretreatment generates no waste and provides a variety of valuable products.

The objectives of the present study were to investigate the antioxidant activity of C-PC from the residual biomass of *S. maxima* powder after SCFCO₂ pretreatment and to determine the optimal conditions of SCFCO₂ pretreatment enhancing the antioxidant activity of C-PC identified using statistically designed experiments

based on the Taguchi method. This method has proven useful for optimization in many areas of biotechnology (Sirisansaneeyakul et al., 2007, 2011; Dejsungkranont et al., 2017). The biochemical and other bioactive compounds of the biomass residual were also investigated.

Materials and methods

Sample preparation

Spirulina maxima IFRPD1183 was obtained from Ratchaburi Electricity Generating Holding Public Company Ltd., Ratchaburi, Thailand. The algal biomass was freeze dried and ground into powder using a grinder (D3V-10; Yu Chi Machinery; Changhua, Taiwan). The *S. maxima* powder was sifted through a 140-mesh sieve. Then, the *S. maxima* powder was subjected to SCFCO₂.

Supercritical carbon dioxide extraction equipment

A batchwise SCFCO₂ extraction system is shown in Fig. 2. Fifteen grams of *S. maxima* powder were loaded into a 100 mL extractor vessel (UK-SFE-100-20; Ivorist; New Taipei, Taiwan). The pretreatment was conducted in three modes (static mode, dynamic mode and conjugated mode) at various pressures, temperatures and durations (Table 1). All runs were carried out in the separate modes. For the static mode, an extraction was kept at stable pressure and temperature for a desired duration before being released normally. In the dynamic mode, an extraction was kept with an inlet valve open to allow fresh SCFCO₂ to enter the autoclave and an outlet valve kept the system pressure equal with the open level. This was carried out until the end of the process. The conjugated mode involved a combination of the static and dynamic modes. The experiments were carried out in duplicate. The golden extracts from the *S. maxima* powder were collected in an empty volumetric flask sealed with rubber septum at low temperature. After each pretreatment, ethanol was used to flush the tubes and valves and added to the extracts, with the ethanol then separated using a rotary evaporator (Rotovapor R-210, type B-49; Buchi; Switzerland). Acetone was added into the extracts for subsequent analysis. The yield of extracts was calculated using Equation (1) from Huang et al. (2011):

$$Y_{\text{algal extract}}(\%) = \frac{C_{\text{extraction}}(\text{g})}{W_{\text{samples}}(\text{g})} \times 100\% \quad (1)$$

where, $Y_{\text{algal extract}}(\%)$ is the yield ratio of algal extract per unit mass of dry sample, $C_{\text{extraction}}(\text{g})$ is the mass of algal extract obtained from the supercritical fluid extraction, and W_{sample} is the weight of the freeze-dried *A. maxima* powder.

At the end of each run, the residual biomass after SCFCO₂ pretreatment or the treated biomass were analyzed for color, moisture, protein, lipid, carbohydrate, phenolic compounds, chlorophyll a (Chl-a), carotenoids and the C-PC contents compared with *S. maxima* powder (control) or the untreated biomass.

Experimental design

The experiment on the SCFCO₂ pretreatment process was carried out using the Taguchi method (Taguchi, 1990; Roy, 2001) and analysis of variance (ANOVA). The Taguchi method uses orthogonal arrays to reduce the factor numbers of the experimental sets and facilitates the identification of the influence of individual factors on performance and the optimum levels of factor values using a few well-defined experimental datasets (Sirisansaneeyakul et al., 2007). Four factors (A–D; Table 1), were optimized at three

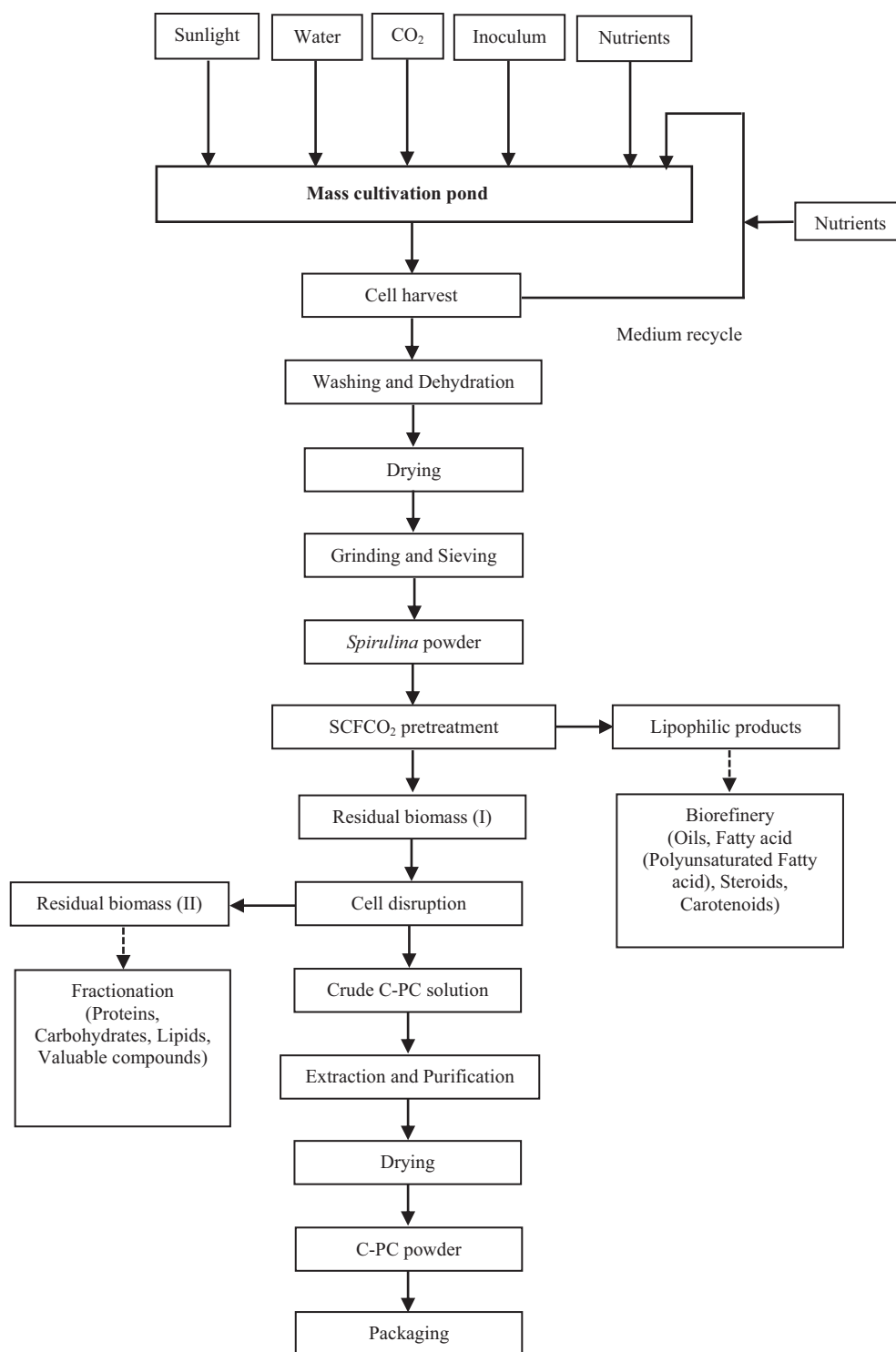


Fig. 1. A conceptual schematic diagram of the economical production of C-phycocyanin (C-PC) from *Spirulina* sp using supercritical fluid CO₂ (SCFCO₂) pretreatment.

levels. The factors were: A (temperature, 40 °C, 50 °C and 60 °C); B (pressure, 24.13 MPa, 31.03 MPa and 37.92 MPa); C (pretreatment mode, static mode, dynamic mode and conjugated mode); and D (duration, 60 min, 90 min and 120 min). Orthogonal arrays, L₉ (3⁴), were selected and are shown in Table 1. The Qualitek-4 software (Nutek Inc.; Bloomfield Hills, MI, USA) was used to identify the trial experimental profiles shown in Table 1. The experimental data (y_i) were converted to a signal-to-noise (S/N) ratio (Roy, 2001). The S/N

is a ratio of the average and standard deviation calculated from the experimental data. A high S/N ratio indicates that the signal is higher than the random effects of the noise factors. In generally, the noise is usually caused from the factors that cannot be controlled and completely eliminated. There are three the criterion of quality characteristics to be optimized: 1) the-smaller-the-better; 2) the nominally smaller-the-better; and 3) the larger-the-better (Yang et al., 2007). The objective in the current study was to find the

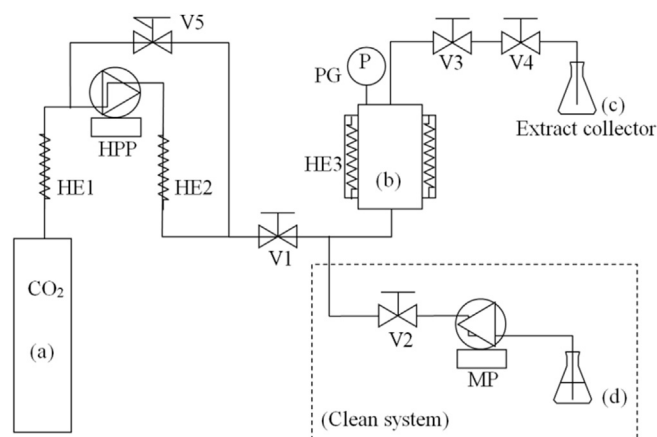


Fig. 2. Schematic diagram of the supercritical fluid CO₂ pretreatment extraction equipment, where (a) = CO₂ tank, (b) = extract autoclave, (c) = extract collector, (d) = cleaning collector, HE1–3 = heat exchangers, HPP = high-pressure pump, MP = liquid pump, PG = pressure gauges, V1–3 = exhaust valves, V4 = needle valve, V5 = back-pressure valve.

optimum SCFCO₂ pretreatment conditions to enhance the antioxidant activity of the C-PC of *S. maxima* powder. The antioxidant activity of the C-PC of *S. maxima* powder was treated as having larger-the-better performance characteristics. The higher-the-better ratio was calculated using Equation (2):

$$S/N = -\log_{10} \left(\frac{\sum \left(\frac{1}{y_i} \right)}{n} \right) \quad (2)$$

where y_i is the combination variable in experiment i for a certain combination of controlled factor levels and n is the number of trials (Roy, 2001). The expected values of observations (Y_{opt}), that is, the antioxidant activity of C-PC, were calculated using Equation (3):

$$Y_{opt} = \bar{T} + \sum (\bar{F}_i - \bar{T}) \quad (3)$$

where $\bar{T}$ and $\bar{F}_i$ are the grand averages of the S/N ratios and the factor averages at each factor level, respectively. The main effect was the difference between the maximum and minimum values of the factor averages at each factor level, while the percent main effect of each factor was calculated as the percentage of its main effect divided by the sum of the main effects of all the factors.

Table 1
Factor levels in the experimental design for optimization of the orthogonal arrays.

Experiment number	Factor							
	A (Temperature)	B (Pressure)	C (Mode)	D (Time)	Temperature (°C)	Pressure (MPa)	Mode	Time (min)
1	1	1	1	1	40	24.13	Static	60
2	1	2	2	2	40	31.03	Conjugated	90 ^a
3	1	3	3	3	40	37.92	Dynamic	120
4	2	1	2	3	50	24.13	Conjugated	120 ^b
5	2	2	3	1	50	31.03	Dynamic	60
6	2	3	1	2	50	37.92	Static	90
7	3	1	3	2	60	24.13	Dynamic	90
8	3	2	1	3	60	31.03	Static	120
9	3	3	2	1	60	37.92	Conjugated	60 ^c

^a Supercritical fluid CO₂ (SCFCO₂) pretreatment was conducted in static mode at 31.03 MPa at 40 °C for 45 min. Thereafter, it was switched to dynamic mode and continuous SCFCO₂ was supplied for 45 min.

^b SCFCO₂ pretreatment was conducted in static mode at 24.13 MPa at 50 °C for 60 min. Thereafter, it was switched to dynamic mode and continuous SCFCO₂ was supplied for 60 min.

^c SCFCO₂ pretreatment was conducted in static mode at 37.92 MPa and 60 °C for 30 min. Thereafter, it was switched to dynamic extraction mode and continuous SCFCO₂ was supplied for 30 min.

Taguchi's statistical optimization has been discussed previously (Sirisansaneeyakul et al., 2007).

Analytical methods

The color of the *S. maxima* powder (control) and the residual biomass were measured using an SP60 Series sphere spectrophotometer (X-rite Incorporated; Grand Rapids, MI, USA). The results were reported in terms of L , a and b , where L refers to the brightness of the samples (black (0) to white (100)). A negative a value ($-a$) indicates a green color, while a positive a value ($+a$) indicates a red color. A positive b value ($+b$) indicates a yellow color, while a negative b value ($-b$) indicates a blue color.

The average moisture content of the control and the residual biomass were evaluated using an infrared moisture determination balance (FD-610; Kett; Tokyo, Japan). The crude protein, total lipids and the total carbohydrate content of the control and residual biomass were determined using the Kjeldahl method (Association of Official Analytical Chemists, 2000), Soxhlet extraction method (Association of Official Analytical Chemists, 2000) and phenol-sulfuric acid method (Dubois et al., 1956), respectively. The concentrations of total phenolic compounds of the control, residual biomass and extract were estimated using the Folin-Ciocalteu colorimetric method (Wolfe et al., 2003).

The extraction of chlorophyll *a* (Chl-*a*) in control and residual biomass were followed the method from El-Baky et al. (2008). The content of Chl-*a* was estimated according to Lichtenthaler and Wellburn (1985). The amount of total carotenoids in control and residual biomass were determined by in accordance with the method of the Association of Official Analytical Chemists (2000). The extraction of C-PC in control and residual biomass were carried out using the modified method from Chaiklahan et al. (2012). The C-PC concentrations in the extract were determined spectrophotometrically using the equations of Bennett and Bogorad (1973). The C-PC purity in the *S. maxima* powder and residual biomass was calculated using Equation (4), the modified Equation of Deniz et al. (2016):

$$\text{C-PC Purity (\%)} = \frac{\text{C-PC in the biomass}}{\text{Total protein in the biomass}} \times 100\% \quad (4)$$

Antioxidant activity assay

The ability of crude C-PC extracted from *S. maxima* powder (control), residual biomass and extracts on free radical scavenging activity were estimated using the 2,2-diphenyl-2-picrylhydrazyl method (Alvarez Suarez et al., 2011). Trolox was used as the

reference for the antioxidant calibration curve. The antioxidant activities were expressed in terms of micromoles of trolox per milligram of C-PC ($\mu\text{mole trolox/mg}$) and micromoles of trolox per gram of extract ($\mu\text{mole trolox/g}$).

Results and discussion

Effect of SCFCO₂ pretreatment on biomass and its antioxidant activity

The extract from *S. maxima* was clearly golden in color. Apart from the treated biomass (discussed later), the yield of extract was highest in experiment 3 (0.72%) (Table 2). On the other hand, the lowest yield (0.27%) was found as confirmation under the optimal conditions, which maximized the antioxidant activity of C-PC in the treated biomass. The extract yields obtained after SCFCO₂ treatment were reasonable under those conditions for 60 min (experiments 1, 5 and 9), except for experiment 3 (120 min). The extraction rate highest and lowest from experiment 5 (4.54 mg/g/h) and experiment 8 (1.58 mg/g/h), respectively (Table 2). The highest value of total phenolic compounds was observed in experiment 7 (11.40 mg gallic acid/g), and it was likely that those experiments carried out at 60 °C (experiments 7, 8 and 9), gave favorable amounts of total phenolic compounds (Table 2). The antioxidant activity for experiments 1–9 were in the range 44.18–118.62 $\mu\text{mole trolox/g}$. The maximized extraction yield occurred under the conditions of 40 °C, 37.92 MPa, dynamic mode and 120 min (experiment 3), but had by far the lowest antioxidant activity (Table 2). The conditions optimized for highest antioxidant activity of C-PC in the treated biomass resulted in 227.43 $\mu\text{mole trolox/g}$ (Table 2), which was higher than the antioxidant activity found in the extracts (experiments 1–9) by approximately 1.92–5.15-fold.

Although the extraction yields by SCFCO₂ were very low (in the range 0.27–0.72%), the study focused on maximizing the antioxidant activity of C-PC and the profound composition of the treated biomass. However, the higher pressure (37.92 MPa) and the longer pretreatment time (120 min) could promote the extraction yield, as

shown clearly with experiment 3 (1.6–2.7-folds higher than the other experiments) (Table 2). Higher pressure was favorable to solvent power, the solubility of non-polar components and the extraction rate. These results agreed well with previous reports conducted under higher pressure with *S. platensis* and *Chlorella pyrenoidosa*, resulting in increases in the extraction yield and antioxidant activity for the extract (Qiuhi, 1999; Qiuhi et al., 2007). The components of the extract responding to the antioxidant activity might be the phenolic compounds, flavonoids, carotenoids, vitamin A, α -tocopherol, chlorophyll and degradation products of chlorophyll (Mallikarjun et al., 2014).

Effect of SCFCO₂ pretreatment on color of *S. maxima* powder

The *S. maxima* powder (treated biomass) was a homogeneous dark green without any colored contaminants. Compared to the control, except for the brightness (L), the green ($-a$) and the yellow ($+b$) of the residual biomass (experiments 1–9) were slightly lower (Table 2).

Effect of SCFCO₂ pretreatment on biochemical compositions of *S. maxima* powder

The biochemical compositions of the *S. maxima* powder (control) and treated biomass after SCFCO₂ are shown in Table 2. The control contained initially moisture content (7.95% of dry weight), crude protein (61.98% of dry weight), total lipids (3.12% of dry weight) and total carbohydrate (14.86% of dry weight) values which were similar to the other experiments. These values were also in a similar range to other *Spirulina* strains (Cohen, 1997; Belay, 2008). After SCFCO₂ pretreatment, the treated biomass had a higher moisture content than the control (Table 2). The results for crude proteins and total lipids in the *S. maxima* powder were consistent with the other reports (Qiuhi, 1999). The crude protein had not significantly changed from the initial value (60.37–62.26% of dry weight) (Table 2), while the total lipids reduced 7.7–31% from the initial value (2.15–2.88% of dry weight) caused by the removal of

Table 2

Effect of supercritical fluid CO₂ (SCFCO₂) pretreatment enhancing the antioxidant activity of C-phycoyanin (C-PC) in *S. maxima* powder, on color, biochemical composition, and pigments content of *S. maxima* powder (control) and treated biomass under various conditions.

	Experiment ^a										Optimum ^b
	Control	1	2	3	4	5	6	7	8	9	
Antioxidant activity C-PC											
Antioxidant activity ($\mu\text{mole trolox/mg}$)	241.67	259.61	231.05	227.27	246.18	235.43	267.14	287.14	309.92	288.67	410.07
Antioxidant activity (signal-to-noise ratio)	–	48.280	47.221	47.046	47.815	47.333	48.253	49.158	49.814	49.138	–
Crude extract after SCFCO ₂ treatment											
Yield of extracts (%)	–	0.35	0.42	0.72	0.43	0.45	0.42	0.37	0.32	0.36	0.27
Rate of extraction (mg/g/h)	–	3.50	2.79	3.59	2.15	4.54	2.78	2.45	1.58	3.64	2.74
Total phenolic compound (mg gallic acid/g)	–	7.72	11.10	9.03	10.26	10.79	6.44	11.40	10.45	10.69	8.47
Antioxidant activity ($\mu\text{mole trolox/g}$)	–	92.45	44.18	44.32	77.27	74.00	79.67	109.64	79.77	118.62	227.43
Color											
L ^a	10.06	9.71	9.97	10.04	10.10	10.26	9.31	10.30	9.29	10.25	10.25
a ^a	–4.74	–4.30	–4.51	–4.24	–4.46	–4.58	–3.69	–4.20	–3.53	–3.97	–4.19
b ^a	6.07	4.83	4.90	4.90	5.05	5.13	4.98	5.64	5.37	5.72	5.63
Biochemical composition (% of dry weight)											
Moisture content	7.95	9.00	8.65	8.88	7.60	8.80	9.25	7.75	10.70	9.20	9.25
Crude protein	61.98	61.17	61.24	61.86	61.77	62.26	61.14	62.04	60.37	60.62	60.73
Total lipid	3.12	2.52	2.88	2.57	2.15	2.64	2.36	2.56	2.20	2.62	2.38
Total carbohydrate	14.86	17.00	15.01	16.21	16.27	15.82	17.23	16.12	15.38	15.37	16.51
Total phenolic compound	0.21	0.23	0.27	0.25	0.24	0.28	0.21	0.24	0.25	0.27	0.22
Pigments content											
Chl-a (mg/g)	9.95	9.21	9.23	8.68	8.44	8.60	8.18	8.88	8.58	9.54	9.11
Total carotenoids (mg/g)	3.17	2.97	3.02	3.05	3.15	3.12	2.61	3.10	2.78	3.09	2.94
C-PC (mg/g)	84.55	93.53	92.87	90.55	89.54	86.79	83.04	76.70	73.88	79.04	83.92
C-PC purity (%)	13.64	15.30	15.16	14.64	14.50	13.94	13.58	12.36	12.24	13.04	13.82

^a See Table 1 for factor levels in the experimental design for optimization.

^b The measured data obtained under optimized conditions.

CO₂-soluble lipophilic substances and some neutral lipids that were extracted (Table 2). Qiuhui (1999) reported that the lipid content in *Spirulina* powder extracted using SCFCO₂ reduced 2.32% from its initial value. Tibbetts et al. (2015) found that *Nannochloropsis granulata* powder after SCFCO₂ extraction at 35 MPa pressure for 270 min at 70 °C and 90 °C, the crude lipid content reduced around 10.0% from its initial value. The components of interest, not solubilized in CO₂, could be extracted using a safe, polar modifier such as ethanol to increase the solvent power, (Crampon et al., 2011). The total carbohydrate increased 1–16% from its initial value (2.15–2.88% of dry weight) and was also consistent with the other studies. Tibbetts et al. (2015) reported that the carbohydrate contents in the treated biomass of *N. granulata* increased 11.27% and 4.56% from initial value after SCFCO₂ extraction at 70 °C and 90 °C, respectively. The amounts of total phenolic compounds were only slightly changed in the control and the treated biomass (Table 2).

Effect of SCFCO₂ pretreatment on pigments content of *S. maxima* powder

Spirulina sp. is known to have C-PC and chlorophyll as the main photosynthetic pigments, while carotenoids are found in considerable amounts (Cohen, 1997). Chlorophyll a and its derivatives are high molecular weight molecules and not soluble in CO₂. On the other hand, the carotenoids are liposoluble pigments important for photosynthesis that have a high potential antioxidant role, while the C-PC is a protein with a strong antioxidant role and is not soluble in CO₂. Therefore, it could remain in the residual biomass after SCFCO₂ pretreatment. In the present work, the contents of Chl-a, total carotenoids and C-PC in the *S. maxima* powder (control) were 9.95 mg/g, 3.17 mg/g and 84.55 mg/g, respectively (Table 2), which were consistent with those reported by the Siam Algae Company (12 mg/g, 4.77 mg/g and 162 mg/g, respectively) (Shimamatsu, 2004). The differences might depend on the strains, culturing and harvesting conditions and also the methodology of analysis. The highest Chl-a was observed with experiment 9. Clearly, experiments 4, 5, 6 and 8 produced lower Chl-a amounts due to being conducted under higher temperatures (50 °C and 60 °C) (Table 2). The carotenoids content was similar to the Chl-a; it was reduced markedly after SCFCO₂ pretreatment (Table 2). In particular, experiment 6 had the lowest Chl-a and carotenoids content in the residual biomass after pretreatment due to the temperature and SCFCO₂ extraction, respectively.

Clearly, temperature strongly influenced the content of C-PC. Therefore, the content of C-PC and C-PC purity markedly decreased with increased temperature (experiments 6, 7, 8 and 9 and the confirmation under optimized conditions). Contradictorily, these were increased with decreased temperature (experiments 1, 2, 3, 4 and 5). The highest level of purity of C-PC and C-PC of the treated biomass were obtained with experiment no. 1 (93.53 mg/g and 15.30%, respectively) at 40 °C. Therefore, the pretreatment conducted at 60 °C (experiment 8) provided the lowest purity level of C-PC and C-PC purity (73.88 mg/g and 12.24%, respectively).

Effect of SCFCO₂ pretreatment on C-PC antioxidant activity

The experimental results (Table 3) were processed using the Qualitek-4 software with the higher-the-better attribute to establish the optimum conditions of SCFCO₂ pretreatment enhancing the antioxidant activity of C-PC in the *S. maxima* powder and identifying the individual factors that influenced the antioxidant activity of C-PC. ANOVA was used to analyze the results of the experiment and to determine how much variation was contributed by each factor. A statistical analysis of the percentages of main effect of the factors on the antioxidant activity of C-PC is shown in Table 3. Temperature

Table 3
Analysis of main effect and analysis of variance^a of factors.^b

Level	Factor			
	A (Temperature)	B (Pressure)	C (Mode)	D (Time)
1	47.52	48.42	48.78	48.25
2	47.80	48.12	48.06	48.21
3	49.37	48.15	47.85	48.23
Minimum value	47.52	48.12	47.85	48.21
Maximum value	49.37	48.42	48.78	48.25
% Main effect	59.32	9.43	29.97	1.28

Analysis of variance					
Factor	Degrees of freedom	Sum of squares	Variance	F-ratio	Percent P (%)
A	2	5.98	2.99	1685.27	78.71
B	2	0.16	0.08	45.73	2.09
C	2	1.45	0.72	408.09	19.02
D	2	0.00		Pooled	
Error	0	0.00			0.18
Total	8	7.60			100.00

Expected values of observations = $Y_{opt} = \bar{T} + (\bar{A3} - \bar{T}) + (\bar{B1} - \bar{T}) + (\bar{C1} - \bar{T}) + (\bar{D1} - \bar{T})$					
---	--	--	--	--	--

^a Analysis of variance was performed using the signal-to-noise ratio (Table 2).

^b See Table 1 for an explanation of the factors A–D.

(factor A) was the main effect to enhance the antioxidant activity of C-PC (59.32%), while the pretreatment time (factor D) had the least effect (1.28%). ANOVA was used to determine the contributions of individual factors to variation in and revealed that temperature had the highest impact on the antioxidant activity of C-PC (78.71%) of the residual biomass (Table 3). The results were consistent with the percentages of the main effect. The antioxidant activity of C-PC was most strongly influenced by temperature (factor A) as also shown in Table 3. In contrast, the impacts of the pretreatment time and pressure were relatively minor. The equation resulting from the Taguchi method could be used to estimate the expected values (Y_{opt}) of antioxidant activity of C-PC in the residual biomass under various conditions (Table 3). As the principal criterion, the S/N ratio is very helpful in identifying the optimal conditions in fermentation processes (Sirisansaneeyakul et al., 2007). Fig. 3 shows the optimal conditions of SCFCO₂ pretreatment resulted from the highest S/N ratio (A3B1C1D1) at 60 °C, 24.13 MPa and a pretreatment time 60 min under a static batch mode.

Under the above specified optimal conditions, the expected antioxidant activity of C-PC was 320.53 μmole trolox/mg. The measured data under the optimized conditions are summarized in Table 2, which indicate that the measured data were 1.28 higher

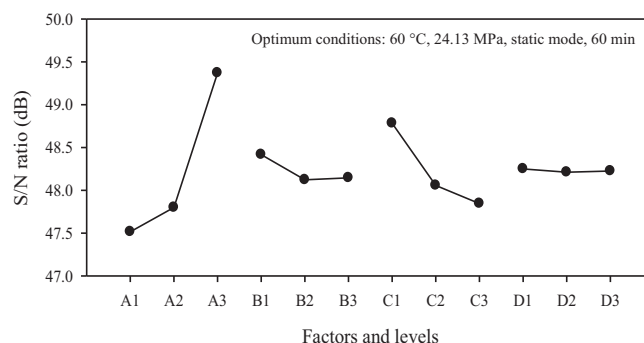


Fig. 3. Signal-to-noise (S/N) ratio for various factors and levels (A–D, Table 1) to maximize antioxidant activity of C-phycoerythrin from *S. maxima* powder after supercritical fluid CO₂ (SCFCO₂) pretreatment, where factors are A (temperature), A1 = 40 °C, A2 = 50 °C, A3 = 60 °C; B (pressure), B1 = 24.13 MPa, B2 = 31.03 MPa, B3 = 37.92 MPa; C (pretreatment mode), C1 = static mode, C2 = dynamic mode, C3 = conjugated mode; D (duration), D1 = 60 min, D2 = 90 min, D3 = 120 min.

than the expected. The maximum antioxidant activity of C-PC was 410.07 $\mu\text{mole trolox/mg}$, which increased 70% or was enhanced 1.7-fold higher than the control (241.67 $\mu\text{mole trolox/mg}$). Also, the S/N ratios calculated using equation (2) from the average antioxidant activity of C-PC are shown in Table 2; these ratios indicate that the antioxidant activities of C-PC from experiments 7–9 were approximately 1.2-fold, 1.3-fold and 1.2-fold higher than the control, obtained at 60 °C (Table 1). The lowest antioxidant activities of C-PC were obtained at lower temperatures (40 °C and 50 °C) with experiments 2, 3 and 5. Therefore, the temperature was more important than the pretreatment time in maximizing the antioxidant activity of C-PC.

Advantageously, the antioxidant activity of C-PC in *S. maxima* powder was increased by the suitable SCFCO₂ pretreatment conditions. This finding is the first report using the SCFCO₂ pretreatment for enhancement of the antioxidant activity of C-PC. Since C-PC is a protein, properties and degradation depend on the aggregation state of the protein, which is influenced by light, temperature, pH and protein concentration (Sarada et al., 1999). Generally, the protein was damaged by higher temperature (above 70 °C). Thermal denaturation of protein results in the formation of disulfide bonds between amino acids, liberating polypeptides and free amino acids (Opstvedt et al., 1984). These phenomena cause Coulombic repulsion and thermal vibration, leading to modification of the protein structure and under extreme conditions, the three-dimensional structure of proteins can be changed, resulting in their denaturation and loss of activity (Volkin and Middaugh, 1992). On the other hand, under mild conditions, the protein structure can have minor changes that cause alternative biological activity, specificity and stability (Volkin and Middaugh, 1992; Wimmer and Zarevúcka, 2010; Deniz et al., 2016). There was no clear enhanced antioxidant activity of C-PC in the *S. maxima* powder after the SCFCO₂ pretreatment at high temperature. The C-PC content decreased at higher temperature, but its three-dimensional structure was found to have had minor changes, which might have resulted in C-PC having better antioxidant activity. This are probably the most logical reasons why the antioxidant activity can increase with less C-PC.

The present work succeeded in using the Taguchi method to qualify *S. maxima* powder with the SCFCO₂ pretreatment, regarding proteins, lipids and carbohydrates. Clearly, the SCFCO₂ pretreatment had an influence on the pigment contents. The antioxidant activity of C-PC in *S. maxima* powder increased due to the SCFCO₂ pretreatment. This is the first report to show that SCFCO₂ pretreatment can enhance the C-PC antioxidant activity of the treated biomass. The yield of the crude extracts in this study was very low (0.27–0.72%) because the focus was on the antioxidant activity of C-PC and the composition of the treated biomass. However, the extraction yield could be increased with increased pressure and modifier concentration. The optimal conditions (60 °C, 24.13 MPa, 60 min, a static batch mode) of the SCFCO₂ pretreatment enhanced the antioxidant activity of C-PC in the *S. maxima* powder. The maximal antioxidant activity of C-PC was 410.07 $\mu\text{mole trolox/mg}$ and 227.43 $\mu\text{mole trolox/g}$, found in the treated biomass and the crude extract of the *S. maxima* powder, respectively. The factor that most affected the antioxidant activity of C-PC was temperature. The SCFCO₂ pretreatment had a minor effect on the appearance of the *S. maxima* powder. The Chl-a and carotenoids contents were markedly reduced after pretreatment using SCFCO₂. Although the C-PC content was markedly decreased at higher temperature, it promoted antioxidant activity.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

This work was supported by: the Ministry of Science and Technology, Thailand; the Department of Biotechnology, Kasetsart University, Bangkok, Thailand; the Center for Advanced Studies in Tropical Natural Resources, Kasetsart University Institute for Advanced Studies (KUIAS); the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission; and the Department of Food Science, National Pingtung University of Science and Technology, Pingtung, Taiwan.

References

- Alvarez Suarez, J.M., Tulipani, S., Diaz, D., et al., 2011. Antioxidant and antimicrobial capacity of several monofloral Cuban honeys and their correlation with color, polyphenol content and other chemical compounds. *Food Chem. Toxicol.* 48, 2490–2499.
- Association of Official Analytical Chemists, 2000. 967.04: Official Method of Analysis of the Association of the Analytical Chemists. Arlington, VA, USA.
- Balaban, M.O., Arreola, A.G., Marshall, M., Peplow, A., Wei, C.I., Cornell, J., 1991. Inactivation of pectinesterase in orange juice by supercritical carbon dioxide. *J. Food Sci.* 56, 743–746.
- Barbosa-Cánovas, G.V., Pothakamury, U., Palou, E., Swanson, B., 1998. High hydrostatic pressure food processing, pp. 9–52. In: Barbosa-Cánovas, G.V., Pothakamury, U., Palou, E., Swanson, B. (Eds.), *Nonthermal Preservation of Foods*. Marcel Dekker Inc, New York, NY, USA.
- Belay, A., 2008. *Spirulina (Arthrospira): production and quality assurance*. In: Gershwil, E., Belay, A. (Eds.), *Spirulina in Human Nutrition and Health*. CRC Press, Taylor and Francis Group, Boca Raton, FL, USA, pp. 1–26.
- Bennett, A., Bogorad, L., 1973. Complimentary chromatic adaptation in a filamentous blue-green alga. *J. Cell Biol.* 58, 419–435.
- Careri, M., Furlattini, L., Mangia, A., Musci, M., Anklam, E., Theobald, A., Von Holst, C., 2001. Supercritical fluid extraction for liquid chromatographic determination of carotenoids in *Spirulina pacifica* algae: a chemometric approach. *J. Chromatogr. A* 912, 61–71.
- Chadwick, R., Henson, S., Koenen, G., Liakopoulos, M., Midden, C., Moseley, B., Palou, A., Rechtemer, G., et al., 2003. *Functional Foods*. Springer, New York, NY, USA.
- Chaiklahan, R., Chirasuwan, N., Bunnaq, B., 2012. Stability of phycocyanin extracted from *Spirulina* sp.: influence of temperature, pH and preservatives. *Process Biochem.* 47, 659–664.
- Cohen, Z., 1997. The chemicals of *Spirulina*. In: Vonshak, A. (Ed.), *Spirulina platensis (Arthrospira): Physiology, Cell-biology and Biotechnology*. Taylor and Francis, London, UK, pp. 175–204.
- Crampon, C., Boutin, O., Badens, E., 2011. Supercritical carbon dioxide extraction of molecules of interest from microalgae and seaweeds. *Ind. Eng. Chem. Res.* 50, 8941–8953.
- Crampon, C., Mouahid, A., Toudji, S.A.A., Lépine, O., Badens, E., 2013. Influence of pretreatment on supercritical CO₂ extraction from *Nannochloropsis oculata*. *J. Supercrit. Fluids* 79, 337–344.
- Dejsungkranont, M., Chisti, Y., Sirisansaneeyakul, S., 2017. Optimization of production of C-phycocyanin and extracellular polymeric substances by *Arthrospira* sp. *Bioproc. Biosyst. Eng.* 40, 1173–1188.
- Deniz, I., Ozen, M.O., Yesil-Celiktas, O., 2016. Supercritical fluid extraction of phycocyanin and investigation of cytotoxicity on human lung cancer cells. *J. Supercrit. Fluids* 108, 13–18.
- Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28, 350–356.
- Durackova, Z., 2010. Some current insights into oxidative stress. *Physiol. Res.* 59, 459–469.
- El-Baky, H.H.H., El Baz, F.K., El-Baroty, G.S., 2008. Characterization of nutraceutical compounds in blue green alga *Spirulina maxima*. *J. Med. Plants Res.* 2, 292–300.
- Eriksen, N., 2008. Production of phycocyanin – a pigment with applications in biology, biotechnology, foods and medicine. *Appl. Microbiol. Biotechnol.* 80, 1–14.
- Fidder, W., 1999. Supercritical fluid extraction of organochlorine pesticides in eggs. *J. Agric. Food Chem.* 47, 206–211.
- Giessauf, A., Gamse, T.A., 2000. Simple process for increasing the specific activity of porcine pancreatic lipase by supercritical carbon dioxide treatment. *J. Mol. Catal. B Enzym.* 9, 57–64.
- Grossman, A.R., Bhaya, D., He, Q., 2001. Tracking the light environment by cyanobacteria and the dynamic nature of light harvesting. *J. Biol. Chem.* 276, 11449–11452.
- Grossman, A.R., Schaefer, M.R., Chiang, G.G., Collier, J.L., 1994. Phycobilisome and phycobiliprotein structure. In: Bryant, D.A. (Ed.), *The Molecular Biology of Cyanobacteria*. Kluwer Academic Publishers, Dordrecht, the Netherlands, pp. 139–216.
- Hsueh, Y.C., Yu, Z.R., Wang, B.J., 2012. A continuous preparation method of *Spirulina platensis* phenolic compounds and polysaccharides using supercritical carbon dioxide technology. *Hohhot* 524–527, 2229–2237.

- Huang, C.C., Chung, C.C., Wang, H.Y., Law, C.L., Chen, H.H., 2011. Formation of 6-Shogaol of Ginger oil under different drying conditions. *Dry. Technol.* 29, 1884–1889.
- Iyer, U.M., Dhruv, S.A., Mani, I.U., 2007. *Spirulina* and its therapeutic implications as a food product. In: Gershwin, E., Belay, A. (Eds.), *Spirulina in Human Nutrition and Health*. CRC Press, Taylor and Francis Group, Boca Raton, FL, USA, pp. 51–70.
- Kim, S.R., Rhee, M.S., Kim, B.C., Lee, H., Kim, K.H., 2007. Modeling of the inactivation of *Salmonella typhimurium* by supercritical carbon dioxide in physiological saline and phosphate-buffered saline. *J. Microbiol. Meth.* 70, 132–141.
- Liang, S., Liu, X., Chen, F., Chen, Z., 2004. Current microalgal health food R & D activities in China. *Hydrobiologia* 512, 45–48.
- Lichtenthaler, H.K., Wellburn, A.R., 1985. Determination of total carotenoids and chlorophyll a and b of leaf extract in different solvents. *Biochem. Soc. Trans.* 11, 591–592.
- Mallikarjun, G.K.G., Udaya, S.K., Sarada, R., Ravishanka, G.A., 2014. Supercritical CO₂ extraction of functional compounds from *Spirulina* and their biological activity. *J. Food Sci. Technol.* 52, 3627–3633.
- Manirafasha, E., Ndikubwimana, T., Zeng, X., Lu, Y., Jing, K., 2016. Phycobiliprotein: potential microalgae derived pharmaceutical and biological reagent. *Biochem. Eng. J.* 109, 282–296.
- Mendes, R.L., Nobre, B.P., Cardoso, M.T., Pereira, A.P., Palavra, A.F., 2003. Supercritical carbon dioxide extraction of compounds with pharmaceutical importance from microalgae. *Inorg. Chim. Acta* 356, 328–334.
- Mendes, R.L., Reis, A.D., Palavra, A.F., 2006. Supercritical CO₂ extraction of γ -linolenic acid and other lipids from *Arthrospira (Spirulina) maxima*: comparison with organic solvent extraction. *Food Chem.* 99, 57–63.
- Mendiola, J.A., Jaime, L., Santoyo, S., Reglero, G., Cifuentes, A., Ibañez, E., Señoráns, F.J., 2007. Screening of functional compounds in supercritical fluid extracts from *Spirulina platensis*. *Food Chem.* 102, 1357–1367.
- Mendiola, J.A., García-Martínez, D., Rupérez, F.J., et al., 2008. Enrichment of vitamin E from *Spirulina platensis* microalga by SFE. *J. Supercrit. Fluids* 43, 484–489.
- Millao, S., Uquiche, E., 2016. Antioxidant activity of supercritical extracts from *Nannochloropsis gaditana*: correlation with its content of carotenoids and tocopherols. *J. Supercrit. Fluids* 111, 143–150.
- Opstvedt, J., Miller, R., Hardy, R.W., Spinelli, J., 1984. Heat induced changes in disulphide bonds in fish protein and their effect on protein and amino acid digestibility in rainbow trout. *J. Agric. Food Chem.* 32, 929–935.
- Qiu, H., 1999. Supercritical carbon dioxide extraction of *Spirulina platensis* component and removing the stench. *J. Agric. Food Chem.* 47, 2705–2706.
- Qiu, H., Pan, B., Xu, J., Sheng, J., Shi, Y., 2007. Effects of supercritical carbon dioxide extraction conditions on yields and antioxidant activity of *Chlorella pyrenoidosa* extracts. *J. Food Eng.* 80, 997–1001.
- Roy, R.K., 2001. Design of Experiments Using the Taguchi Approach. John Wiley & Sons, London, UK.
- Sarada, R., Pillai, M.G., Ravishankar, G.A., 1999. Phycocyanin from *Spirulina* sp: influence of processing of biomass on phycocyanin yield, analysis of efficacy of extraction methods and stability studies on phycocyanin. *Process Biochem.* 34, 795–801.
- Samaranayaka, A.G.P., Li-Chan, E.C.Y., 2011. Food-derived peptidic antioxidants: a review of their production, assessment, and potential applications. *J. Func Foods* 3, 229–254.
- Serna, L.V.D., Alzate, C.E.O., Alzate, C.A.C., 2016. Supercritical fluids as a green technology for the pretreatment of lignocellulosic biomass. *Bioresour. Technol.* 199, 113–120.
- Shimamatsu, H., 2004. Mass production of *Spirulina*, an edible microalga. *Hydrobiologia* 512, 39–44.
- Sirisansaneeyakul, S., Luangpipat, T., Vanichsriratanana, W., Srinophakun, T., Chen, H.H., Chisti, Y., 2007. Optimization of lactic acid production by immobilized *Lactococcus lactis* IO–1. *J. Ind. Microbiol. Biotechnol.* 34, 381–391.
- Sirisansaneeyakul, S., Singhasuwan, S., Choorit, W., Phoopat, N., Garcia, J.L., Chisti, Y., 2011. Photoautotrophic production of lipids by some *Chlorella* strains. *Mar. Biotechnol.* 13, 928–941.
- Sloth, J.K., Wiebe, M.G., Eriksen, N.T., 2006. Accumulation of phycocyanin in heterotrophic and mixotrophic cultures of the acidophilic red alga *Galdieria sulphuraria*. *Enzym. Microb. Technol.* 38, 168–175.
- Stadnichuk, I.N., Tropin, I.V., 2017. Phycobiliproteins: structure, functions and biotechnological applications. *Appl. Biochem. Microbiol.* 53, 1–10.
- Taguchi, G., 1990. Introduction to Quality Engineering. Asian Productivity Organization, Japan, Tokyo.
- Tibbetts, S.M., Björnsson, W.J., McGinn, P.J., 2015. Biochemical composition and amino acid profiles of *Nannochloropsis granulata* algal biomass before and after supercritical fluid CO₂ extraction at two processing temperatures. *Anim. Feed Sci. Technol.* 204, 62–71.
- Tong, Y., Gao, L., Xiao, G., Pan, X., 2011. Supercritical CO₂ extraction of chlorophyll a from *Spirulina platensis* with a static modifier. *Chem. Eng. Technol.* 34, 241–248.
- Volkin, D.B., Middaugh, C.R., 1992. Stability of Protein Pharmaceuticals. In: Ahem, T.J., Manning, M.C. (Eds.), *Chemical and Physical Pathways of Protein Degradation*. Plenum Press, New York, NY, USA, pp. 109–134.
- Wang, L., Pan, B., Sheng, J., Xu, J., Hu, Q., 2007. Antioxidant activity of *Spirulina platensis* extracts by supercritical carbon dioxide extraction. *Food Chem.* 105, 36–41.
- Wimmer, Z., Zarevúcka, M., 2010. A review on the effects of supercritical carbon dioxide on enzyme activity. *Int. J. Mol. Sci.* 11, 233–253.
- Wolfe, K., Wu, X., Liu, R.K., 2003. Antioxidant activity of apple peels. *J. Agric. Food Chem.* 51, 609–614.
- Wu, L.C., Ho, J.A., Shieh, M.C., Lu, I.W., 2005. Antioxidant and antiproliferative activities of *Spirulina* and *Chlorella* water extracts. *J. Agric. Food Chem.* 53, 4207–4212.
- Yan, Y., Noritomi, H., Nagahama, K., 2001. A rise in the hydrolysis activity of *Candida rugosa* lipase caused by pressurized treatment with supercritical carbon dioxide. *Kobunshi Ronbunshu* 58, 674–678.
- Yang, K., Teo, E.C., Fuss, F.K., 2007. Application of Taguchi method in optimization of cervical ring cage. *J. Biomech.* 40, 3251–3256.
- Zhong, Q., Jin, M., 2008. Enhanced functionalities of whey proteins treated with supercritical carbon dioxide. *J. Dairy Sci.* 91, 490–499.
- Zilinskas, B.A., Greenwald, L.S., 1986. Phycobilisome structure and function. *Photosynth. Res.* 10, 7–35.