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Optimization of a Continuous Preparation Method of *Arthrospira platensis* γ-linolenic Acid by Supercritical Carbon Dioxide Technology using Response Surface Methodology

(Pengoptimuman Kaedah Penyediaan Berterusan *Arthrospira platensis* γ Asid Linolenik oleh Teknologi Supergenting Karbon Dioksida yang menggunakan Kaedah Gerak Balas Permukaan)

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ABSTRACT

γ-linolenic acid is an essential omega-6 unsaturated fatty acid made in the human body from linoleic acid. It can be metabolized to various important eicosanoids and it is also a precursor of prostaglandin E and several other active substances that are associated with anti-inflammatory properties. Arthrospira platensis *is known to contain relatively large quantities of γ-linolenic acid. The aim of this study was to investigate the optimal parameters under a continuous preparation method of γ-linolenic acid from* A. platensis *using supercritical carbon dioxide technology. A Box-Behnken experimental design and response surface methodology were used to optimize combinations among pressure (10, 20 and 30 MPa), temperature (40, 50 and 60°C) and flow rate of* A. platensis *extract liquor (1, 2 and 3 mL/min) for yield of γ-linolenic acid. The results showed that the extraction of γ-linolenic acid from* A. platensis *was optimized at a temperature of 60°C, a pressure of 30 MPa and a flow rate of 3 mL/min. These parameters could be used as a basis for facilitating future scale-up industrial applications.*

Keywords: Arthrospira platensis; Spirulina platensis; *supercritical carbon dioxide fractionation; γ-linolenic acid*

ABSTRAK

Asid linolenik γ adalah asid lemak tak tepu penting omega-6 yang dihasilkan dalam tubuh manusia daripada asid linolik. Ia boleh dimetabolismakan untuk pelbagai eicosanoid penting dan ia juga merupakan pelopor prostaglandin E dan beberapa bahan aktif lain yang berkaitan dengan sifat anti-radang. Arthrospira platensis *diketahui mengandungi kuantiti asid Linolenik γ yang agak besar. Kajian ini bertujuan untuk mengkaji parameter optimum dengan kaedah penyediaan berterusan asid Linolenik γ daripada* A. platensis *menggunakan teknologi supergenting karbon dioksida. Satu reka bentuk eksperimen Box-Behnken dan kaedah gerak balas permukaan digunakan untuk mengoptimumkan gabungan antara tekanan (10, 20 dan 30 MPa), suhu (40, 50 dan 60°C) serta kadar aliran cecair ekstrak* A. platensis *(1, 2 dan 3 mL/min) untuk hasil asid Linolenik γ. Keputusan menunjukkan pengekstrakan asid linolenik γ daripada* A. platensis *dioptimumkan pada suhu 60°C, tekanan pada 30 MPa dan kadar aliran 3 mL/min. Parameter ini boleh digunakan sebagai asas bagi tujuan memberi kemudahan kepada aplikasi industri pada masa hadapan.*

Kata kunci: Arthrospira platensis*; Asid linolenik γ; pemeringkatan supergenting karbon dioksida*; Spirulina platensis

INTRODUCTION

γ-linolenic acid (GLA, 18: 3), an omega-6 polyunsaturated fatty acid, is a metabolite of linoleic acid and the first intermediate in the conversion of linoleic acid to arachidonic acid (Tapiero et al. 2002; Ward & Singh 2005). It is also a precursor of prostaglandin E and several other active substances that are associated with anti-inflammatory properties. Evening primrose oil, which is rich in GLA, has been evaluated for the treatment of various disorders such as atopic eczema (Morse et al. 1989), diabetic neuropathy (Keen et al. 1993), hypercholesterolemia (Ishikawa et al. 1989), premenstrual syndrome (Puolakka et al. 1985), rheumatoid arthritis (Zurier et al. 1996) and schizophrenia (Vaddadi 1992).

Traditionally, plants are the main source for GLA production. However, alternative sources among microbes such as microalgae have been explored. The cyanobacterium, *Arthrospira platensis* (formerly known as *Spirulina platensis*), is known to contain large quantities of GLA of up to 30% of fatty acids and 1.4% of dry cell weight (Cohen et al. 1993; Sajilata et al. 2008a). Animal studies have demonstrated the consumption of *A. platensis* was safe and non-toxic (Chamorro et al. 1997; Salazar et al. 1998). More recent safety assessment also showed that cultivated *S. platensis* have minimal cytotoxicity and did not contain algal toxins such as microcystins (Yang et al. 2011). Furthermore, *A. platensis* has received the assignment of a Class A safety rating by the Dietary

Supplements Information Expert Committee of the United States Pharmacopeial Convention in 2011 (Marles et al. 2011).

Supercritical fluid extraction with carbon dioxide ($SC\text{-}CO_2$) could be considered as a promising technique in producing solvent-free extracts of *A. platensis*. $SC\text{-}CO_2$ is non-toxic, non-flammable, non-explosive, cost-efficient and readily available technology as an alternative to the traditional solvent extraction methods. A maximum recovery of 45% GLA from *A. platensis* by non-continuous $SC\text{-}CO_2$ with ethanol as a co-solvent at 350 bars and 60°C has been reported (Mendes et al. 2006). In addition, the constituents of extracts could be different depending on the extraction methods. For example, the supercritical extract of propolis contains more diterpens and less flavonoids than ethanol extracts (Matsuno et al. 1977). Therefore, the aimed of this study were to demonstrate a continuous preparation method with $SC\text{-}CO_2$ methods and to establish optimal parameters for GLA from *A. platensis*.

Materials and Methods

Materials

Dried powder of *A. platensis* (Biotechnology Research Center, Far East University, Tainan, Taiwan.) was extracted with 95% of ethanol at a ratio of 1:10 (w/v) for 24 h to produce *A. platensis* ethanol extract (E).

Palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), GLA (C18:3), potassium hydroxide, and boron trifluoride-methanol-complex solution (BF_3 13-15%) reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium chloride and sodium sulfate anhydrous reagents were obtained from SHOWA Co. (Tokyo, Japan). Methanol, n-hexane and hydrogen chloride were obtained from Tedia Co. (Fairfield, Ohio, USA).

γ-LINOLENIC ACID FRACTIONATION WITH SUPERCRITICAL CARBON DIOXIDE

The Box-Behnken experimental design and response surface methodology (RSM) were used to optimize the combinations among three levels of pressure (10, 20 and 30 MPa), three levels of temperature (40, 50 and 60°C) and three levels of flow rate (1, 2 and 3 mL/min) for yield of GLA. The Box-Behnken design is a rotatable second-order designs based on three-level incomplete factorial designs. With three factors, the design was constructed as three blocks of four experiments consisting of a full two-factor factorial design with the level of the third factor set at zero, a total of 13 experimental conditions (Table 1) (Began et al. 2000; Box & Benhnken 1960).

Both extracts (E) and $SC\text{-}CO_2$ were continuously fed countercurrently into a continuous supercritical fluid system equipped with a 500 mL cylinder (316L-50DF4-500; Swagelok Co., Solon, OH, USA) packed with glass balls (3 mm, Kimble, Vineland, NY, USA) and three separation vessels in series at 15, 10 and 5 MPa and 60°C to yield residual (R) (material insoluble in $SC\text{-}CO_2$) and three fractions (fraction 1, F1; fraction 2, F2; and fraction 3, F3, respectively) (material soluble in $SC\text{-}CO_2$). The extraction and fractionation liquid collected were frozen under -20°C until use.

The generalized model for prediction of the response variable (GLA content) as a function of independent parameters is given $GLA = C_1 \times \text{pressure} + C_2 \times \text{temperature} + C_3 \times \text{flow rate of liquid extract}$, where C_1, C_2, and C_3 are the linear regression coefficients.

DETERMINING CONCENTRATION OF Γ-LINOLENIC ACID

The samples for fatty acid analysis were first methyl esterified to form high-volatility fatty acid methyl esters (FAMEs). The amount of each fatty acid involved was analyzed and calculated using gas chromatography (Thermo Trace GC Ultra, Thermo Fisher Scientific, Waltham, MA, USA). DB-WAX (60 m × 0.25 mm, 0.25 μm) was utilized in chromatographic column under gas chromatograph analytic condition. Temperature settings for flame ionization detector (FID) and injector were 250°C and 225°C, respectively. The initial temperature of drying oven was set at 100°C for 4 min with heating rate increased by 2°C/min until the temperature reached up to 220°C for 40 min. The flow rate of gas nitrogen in travelling phase was set at 1.0 mL/min and the quantity of injecting sample was 1 μL (Sajilata et al. 2008b).

Statistical Analysis

The responses under the 13 different combinations as defined by the design (Table 1) were analyzed using analysis of variance (ANOVA) to estimate the statistical parameters. Correlations between GLA and pressure, temperature and flow rate of liquid extract were evaluated using Pearson product-moment correlation coefficient. All statistical procedures were conducted using SPSS Statistics for Windows, Version 17.0 (SPSS Inc., Chicago, USA).

Results and Discussion

The cyanobacterium *A. platensis* was submitted to a continuous preparation method using $SC\text{-}CO_2$ technology. Prior to the $SC\text{-}CO_2$ fractionation, dried powder of *A. platensis* was extracted with ethanol rather than other organic solvents such as hexane and acetone. It has been shown that the highest yield in lipids and GLA was obtained with ethanol because of the hydrogen bonding and iconic forces between the lipids and proteins in the cell membranes could be counterbalanced by ethanol (Mendes et al. 2005).

The results of the response surface model fitted in the form of ANOVA are shown in Figure 1. The ANOVA of the quadratic regression model demonstrated that the model was significant with a low probability value ($p<0.01$). The goodness of fit (R^2) of the model was 0.91.

TABLE 1. Box-Behnken design matrix for response surface methodology studies of pressure, temperature and flow rate for γ-linolenic acid extraction

Run No.	Pressure (MPa)	Temperature (°C)	Flow rate (mL/min)	Normalized pressure	Normalized temperature	Normalized flow rate
1	20	50	2	0	0	0
2	30	40	2	1	-1	0
3	10	60	2	-1	1	0
4	10	40	1	-1	-1	-1
5	20	40	3	0	-1	1
6	20	50	1	0	0	-1
7	10	50	3	-1	0	1
8	10	60	1	-1	1	-1
9	20	60	3	0	1	1
10	20	40	2	0	-1	0
11	30	50	1	1	0	-1
12	30	50	2	1	0	0
13	30	60	3	1	1	1

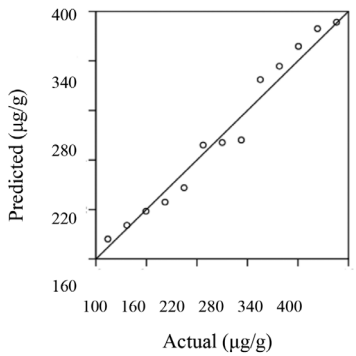

FIGURE 1. Response surface model predicted vs. experimental data of γ-linolenic acid

The effects of pressure, temperature and sample flow rate on GLA content are shown in Figure 2. Under the condition of low temperature of 40°C, a higher amount of GLA compounds could be obtained with higher pressure. When the pressure was increased from 10 to 30 MPa, the yield increased from 300 to 350 μg/g dry weight at 3 mL/min flow rate. In addition, the production of GLA was increased with the diverse flow rates along with the increased pressure. Optimal operation was under the pressure of 30 MPa, flow rate of sample 3 mL/min and temperature 60°C, GLA increased from 380 μg/g dry weight at 60°C to 340 μg/g dry weight at 40°C. Therefore, the purification effect of GLA would be the best with a higher temperature of 60°C and a high pressure of 30 MPa and a high flow rate of 3 mL/min.

In the present work, although the main objective was to increase the GLA content using SC-CO_2, it is also important to investigate the optimal conditions for GLA yield because of the consideration of scale-up process in the future. Figure 3 is the three dimensional plot showing predicted response surface of GLA content as a function of pressure, temperature and sample flow rate. The experimental design chosen for this study was an orthogonal design for three independent variables at three levels: pressure, temperature and flow rate of liquid extract. The regressed equation is shown as (1).

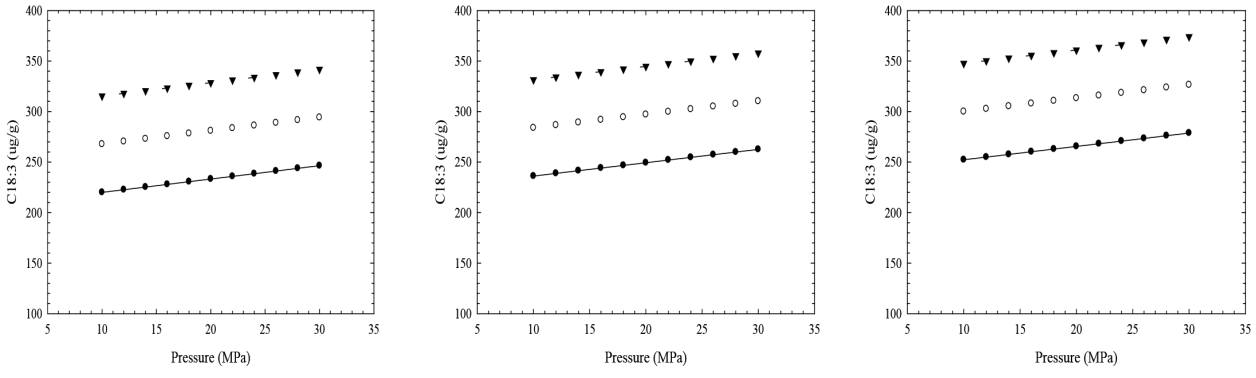

FIGURE 2. Predicted γ-linolenic acid in *A. platensis* extract prepared at various combinations of pressure and sample flow rate at 40°C (a), 50°C (b) and 60°C (c) (●: flow rate: 1 mL/min; ○: flow rate: 2 mL/min; ▼: flow rate: 3 mL/min)

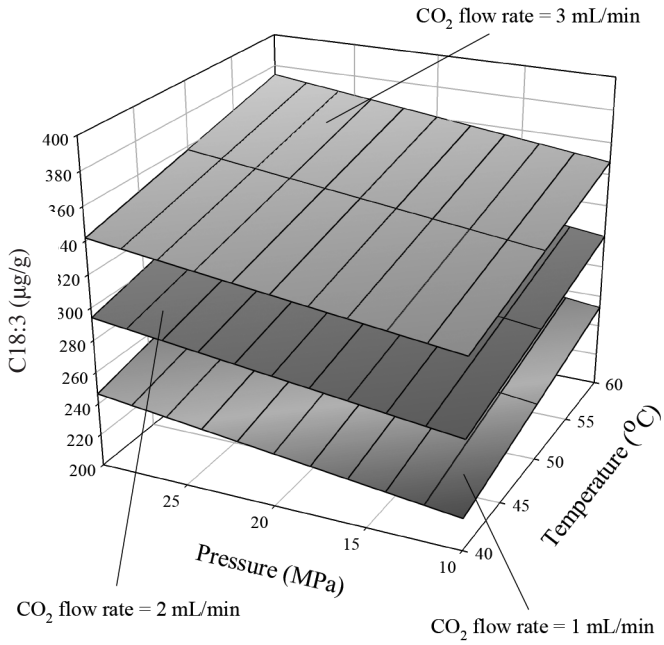

FIGURE 3. Response surface plot of predicted γ-linolenic acid compound as a function of pressure, temperature, and sample flow rate

$$\text{GLA} = C_1 \times \text{pressure} + C_2 \times \text{temperature} + C_3 \times \text{flow rate of liquid extract} \quad (1)$$

The regression coefficients of the response function with the statistical analysis were $C_1 = 1.302$, $C_2 = 4.767$, $C_3 = 16.135$ with a $R^2 = 0.91$. Strong correlations were observed between GLA and pressure (Pearson product-moment correlation coefficient, r=0.90, p<0.01), GLA and temperature (r=0.95, p<0.01) and GLA and flow rate of liquid extract (r=0.90, p<0.01). The higher GLA with increase in pressure might be the result of an increase in the density of carbon dioxide. The increase in temperature could increase the vapor pressure of the extractable compounds, thus, could provide a higher extraction yield of GLA. Similar observation was reported by Mendiola et al. (2008), in which a higher extraction pressure and temperature by SC-CO_2 significantly produced a higher concentration of vitamin E in *A. platensis* extracts.

Table 2 shows the GLA yield of extract and various fractions under optimal operating condition of the continuous preparation method. The concentration of GLA was increased from 109.2 µg/g in the extract to 146.1 and 136.4 µg/g in the F1 and F2 fraction, respectively, indicating that SC-CO_2 fractionation could increase the GLA yield. Figure 4 shows the bar diagram comparing the values of different fatty acids identified in the fractions fractioned at the optimal parameters compared to fatty acids found in *A. platensis* extract. After the SC-CO_2 fractionation, the concentrations of all the fatty acids in the sum of the fraction (F1 + F2 + F3) were higher than that of the extract. The results showed that continuous SC-CO_2 fractionation were able to increase the concentration of fatty acids in *A. platensis*.

CONCLUSION

In this study, we demonstrated the use of SC-CO_2 in supercritical flow fractionation to continuously prepare GLA from *A. platensis*. The continuous sample processing has the potential in applications for scale-up industrial processes comparing with the conventional supercritical extraction method. In addition, findings from this study

TABLE 2. γ-linolenic acid compound of extract and various fractions in a continuous supercritical fluid system operated at optimal condition

	Extract	Residual	Fraction		
			F1	F2	F3
Pressure (MPa)	–	30	15	10	5
Yield (wt. %)	–	21	25	36	18
γ-linolenic acid (µg/g)	109.2	57.5	146.1	100.2	136.4

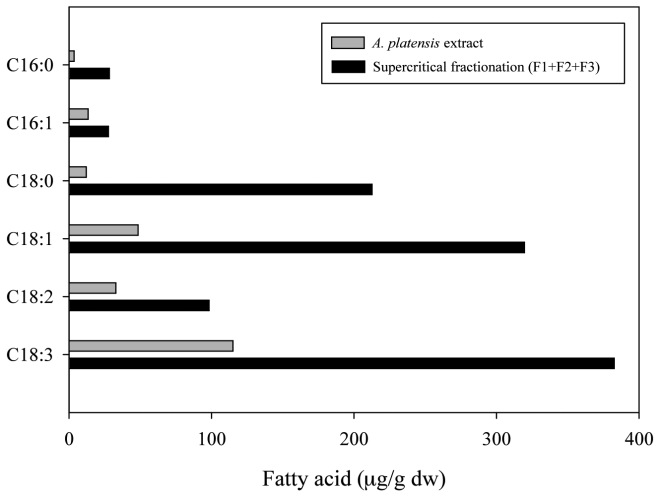

FIGURE 4. Bar diagram corresponding to the fatty acid profile (fatty acid μg/g dry weight) of *A. platensis* extract (E) and fractions in a continuous supercritical fluid system operated at optimal condition

showed that the extraction of GLA from *A. platensis* was optimized at 60°C at a pressure of 30 MPa and a flow rate of 3 mL/min.

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