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Supercritical CO_2 extraction and response surface optimization of ginkgolic acids from ginkgo biloba exopleura

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Abstract−Supercritical (Sc)-CO_2 extraction was adopted to extract ginkgolic (G.) acids from ginkgo biloba exopleura. Response surface optimization was employed to maximize extraction recovery of G. acids from ginkgo biloba exopleura. The effects of pressure, temperature, CO_2 mass flow rate, dosage of entrainer and extraction static-dynamic time on the yield of G. acids were investigated in detail, and the central composite design was used to maximize the extraction recovery of G. acids. The amounts of G. acids were analyzed by HPLC with the mixture of methanol and acetic acid solution as the mobile phase. The optimal process parameters for sc-CO_2 extraction were determined to be: 31.3 MPa extraction pressure, 46.1 °C extraction temperature and 11.1 g min^{-1} CO_2 flow rate, 30 mL ethanol entrainer, 1 h extraction static time and 2 h dynamic time. Under the conditions of optical extraction process, the average G. acids extraction rate was 74 mg g^{-1}.

Keywords: Supercritical (Sc)-CO_2 Extraction, Ginkgolic (G.) Acids, Ginkgo Biloba Exopleura, Response Surface Optimization

INTRODUCTION

Ginkgo biloba is one of China's peculiar tree species, which is also deemed to be living fossil on account of its existence over millions years. It is classified as a national secondary protection plant. In China, the resource of ginkgo biloba accounts for 70% of the total world. Many researchers investigated the effective components of ginkgo biloba, such as flavonoids and terpene, which are two kinds of the most widely used dietary supplements in the world [1]. As a traditional Chinese medicine, ginkgo biloba has been used for treating cerebral dysfunction associated with brain aging, neurodegenerative dementia [2] and resistance against many kinds of insects and bacteria [3]. Nevertheless, Ginkgo biloba exopleura has been usually discarded or stacked in farmlands and rivers near the ginkgo gardens, and the toxicant of abandoned ginkgo biloba exopleura, which could contaminate the environment, would inevitably do harm to the ecology of soil and the fish in the river [4]. As G. acids are are useful components in G. bilola exopleura, the application of ginkgo biloba exopleura has attracted much more attention than ever before.

G. acids are the major active ingredients in ginkgo biloba exopleura, and have been reported to have the activities of antimicrobial [5] and antitumor [6]. G.acids, which mainly consist of hydroginkgolic acid, hydroginkgolinic acid and bilobol, are derivatives of salicylic acid, where the C-6 position is substituted by a long chain (13-17) alkyl or alkenyl group and side chain's double bond (0-2) [7,8]; the structure of G. acids is presented in Fig. 1.

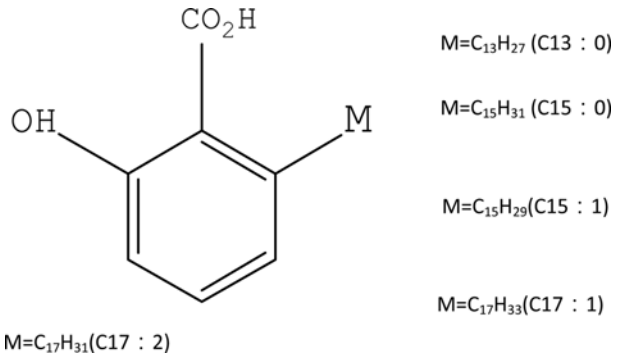

Fig. 1. Chemical structure of G. acids.

The technique of supercritical fluid extraction (especially sc-CO_2) has been widely used in the pharmaceutical industry, because the process has environmental compatibility, low critical temperature (304.1 K), and moderate operating critical pressure (7.38 MPa) [9, 10]. Nevertheless, there are no investigations reported concerning the extraction of G. acids using sc-CO_2 extraction method. G. acids are of relatively high polarity, so it would be inefficient for the direct extraction using nonpolar CO_2. Therefore, polar solvents should be added to increase the polarity of sc-CO_2, which could greatly improve the extraction efficiency [11]. Ethanol is an ideal modifier because of its non-toxicity and moderate polarity.

One-variable-at-a-time experiments could not reflect actual changes in the process and ignore mutual effect between factors. Response surface methodology (RSM) has been demonstrated to be a powerful tool for testing the impacts and interplay between each factor, which can fit a polynomial model to design the optimal extracting conditions. RSM has been successfully employed to model and optimize supercritical CO_2 extraction of essential oil and diosgenin from tribulus terrestris [12], chlorophyll a from spirulina [13], onion

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oil from onions [14], oils from cherry seed [15], walnut [16], apricot kernel [17], and hazelnut [18]. In this paper, Program Design Expert 8.0.5 was adopted to analyze the experiment. The research aims to maximize the extraction of G. acids from ginkgo biloba exopleura. The effects of independent extraction parameters such as pressure, temperature, and the CO_2 mass flow rate were investigated by RSM. Sc-CO_2 extraction efficiency was compared to conventional organic solvents extraction.

MATERIALS AND METHODS

1. Materials and Reagents

The ginkgo biloba exopleura was obtained from Southeast University. It was air dried, then ground by a disintegrator to get 40-60 mesh samples. CO_2 (purity 99.9%) was supplied by the Gas Company of NO.55 Institute. Methanol (analytical and HPLC grade), ethanol (analytical grade), acetic acid (analytical and HPLC grade, petroleum ether (boiling temperature ranges from 60-90 °C) were obtained from Nanjing Chemical Reagent Co. Ltd. A standard sample of G. acids (purity ≥90%) was purchased from Aladdin Chemistry Co. Ltd. Deionized water was obtained from Southeast University.

2. Experimental Apparatus and Procedure

A schematic drawing of the sc-CO_2 extraction pilot plant (SFE-100-2-base) is presented in Fig. 2. The apparatus, which was purchased from Thar Technologies Inc. (Pennsylvania, USA), consisted of a syringe pump, an extraction vessel (160 mm×28.6 mm internal diameter), an automated back pressure regulator, and a separation vessel. The electronic balance apparatus (JA2003N) was purchased from Shanghai Precision & Scientific Instrument Co. (Shanghai, P. R. China) and the HPLC Model LC-20AT was from Shimadzu Corp. (Kyoto, Japan).

3. Supercritical CO_2 Extraction

Dried ginkgo biloba exopleura powder (10.00±0.01 g) was mixed with a certain volume of entrainer and placed in the center of the cylindrical extractor. First, CO_2 entered into the cooling bath and was maintained at 0°C to facilitate a constant feeding rate of the pump; the required temperature was set by heat exchange, and the predetermined pressure was adjusted by automated back-pressure regulator (ABPR). Then, the needle valve was closed and the interactions of sc-CO_2, modifier, and biloba exopleur powder occurred under the required pressure and temperature. Finally, the needle valve was turned on; CO_2 flowed through the extractor and then was depressurized to atmospheric pressure. G. acids and the entrainer were collected in the separation vessel. The modifier was removed by vacuum distillation; the isolated extract was diluted by mobile phase for HPLC analysis. The effects of independent variables were investigated in the range of 10-50 MPa, 25-65 °C, 6-14 g min^{-1} CO_2 flow rate. Optimal conditions to provide the maximum amount of G. acids were used for further experimentation to examine the effect of dosage of entrainer and the extraction dynamic time.

4. Experimental Design

A three-variable, five-level central composite design (CCD) [19, 20] was applied to optimize the extraction conditions in order to obtain high G. acids recovery from the dried ginkgo biloba exopleura powder. The studied parameters were extraction pressure (A, MPa), temperature (B, °C), and flow rate of CO_2 (C, g min^{-1}). The experimental design was based on the CCD consisting of three variables; the adopted three-factors and five-levels are shown in Table 1. The levels of the independent parameters were based on the individual factor consequence. Repeated experiments were performed at all designed points except at the central point (0.0.0) where six replications were executed to allow for estimations of pure error. A second-order polynomial Eq. (1) was used to express the yield of G. acid:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum\sum \beta_{ij} X_i X_j \quad (1)$$

where Y represents the yield of the G. acids, β_0 is a constant, β_i, β_{ii},

Table 1. Levels of independent variables established according to the CCD

Independent variables	Independent variables level				
	−1.68	−1	0	1	1.68
Extraction pressure, A [MPa]	13.2	20	30	40	46.8
Extraction temperature, B [°C]	28.2	35	45	55	61.8
Flow rate of CO_2, C [g min^{-1}]	6.6	8	10	12	13.4

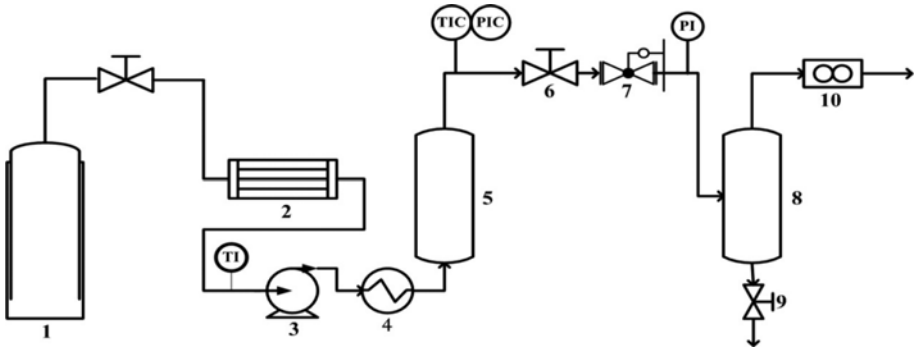

Fig. 2. The equipment of supercritical CO_2 extraction.

1. CO_2 cylinder with siphon
2. CO_2 cooler
3. High pressure pump
4. Heat exchanger
5. Extraction vessel
6. Needle valve
7. Automated back-pressure valve
8. Separation vessel
9. Collection valve
10. CO_2 flow meter

and β_{ij} are the linear, quadratic, and interactive coefficients, X_i and X_j are the levels of the independent variables.

5. Ginkgolic Acids Quantification Analysis

G. acids sample and standard G. acids were analyzed by an HPLC apparatus equipped with an Inertsil ODS-SP (4.6 mm×150 mm, 5 μm) chromatography column. The column was eluted by using the mobile phase of MeOH-4%HAc (90 : 10, v : v) at a flow rate of 0.6 mL min^{-1} and an operation temperature of 35 °C. The solution injection volume was 20 μL and detection was performed by an SPD-20A UV/VIS detector at 310 nm. The concentration of G. acids in the extract was calculated from peak area measurements and the extraction yield of G. acids by Eq. (2):

$$Y = \frac{C \times n \times V \times 10^{-3}}{m} \quad (2)$$

where C is the concentration of G. acids in the extract (mg L^{-1}), V is the liquid of the extract (mL), n is the dilution ratio of the extract, and m is the mass of dried ginkgo biloba exopleura (g).

6. Soxhlet Extraction

Traditional Soxhlet extraction was also carried out by the standard method [21]; 10.00±0.01 g dried ginkgo biloba exopleura powder was extracted in 50 mL petroleum ether (60-90 °C) at 70 °C for 6 h (two times, each time 3 hours). The extraction G. acids were diluted to be analyzed by HPLC.

RESULTS AND DISCUSSION

1. Effect of Static-dynamic Extraction Time

The effect of the static-dynamic extraction time on the yield of G. acids was investigated in the range from 0.5 to 1.5 h and 0.5 to 3 h (static-dynamic time: 0.5-0.5, 0.5-1.2, 0.5-1.9, 0.5-2.6; 1-1.5, 1-2.0, 1-2.5; 1.5-1.8, 1.5-2, 1.5-2.2; unit: h). The experimental conditions were as follows: 25 mL ethanol entrainer, 35 MPa extraction pressure, 40 °C extraction temperature and 10 g min^{-1} CO_2 flow rate. The results are presented in Fig. 4.

As can be seen in Fig. 4, the extraction yield increased when the static time was prolonged to 1 h and the dynamic time extended to 2 h. The increasing static time strengthened the intumescence of the gingko biloba exopleura cell. When the static time was further prolonged, the yield of G. acids did not increase. However, when the dynamic time was further extended, the process costs dramatically increased. Therefore, the optimum static-dynamic time was 1-2 h.

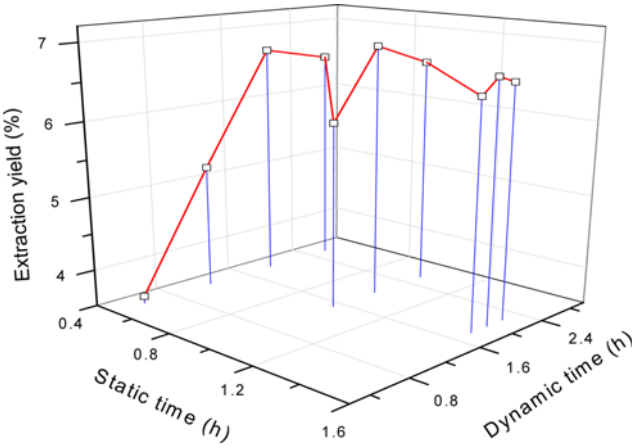

Fig. 4. Effect of static-dynamic extraction time on the extraction yield of G. acids. Experimental conditions: 25 mL ethanol entrainer, 35 MPa extraction pressure, 40 °C extraction temperature and 10 g min^{-1} CO_2 flow rate.

2. Effect of Entrainer Volume

The effect of the entrainer volume was investigated in the range from 10 to 40 mL. The experimental conditions were as flows: 1-2 h static-dynamic time, 35 MPa extraction pressure, 40 °C extraction temperature and 10 g min^{-1} CO_2 flow rate. The results are presented in Fig. 5.

Fig. 5 shows that the G. acids extraction yield gradually increased with entrainer volume up to 30 mL and then decreased with larger entrainer volume. The polarity and dissolving capacity of sc-CO_2 improved with the increase of the entrainer volume, finally, reinforcing the extraction yield of G. acids. However, with further increase of the amount of entrainer, its adverse influence of decreasing the sc-CO_2 density became the dominant factor. The dissolv-

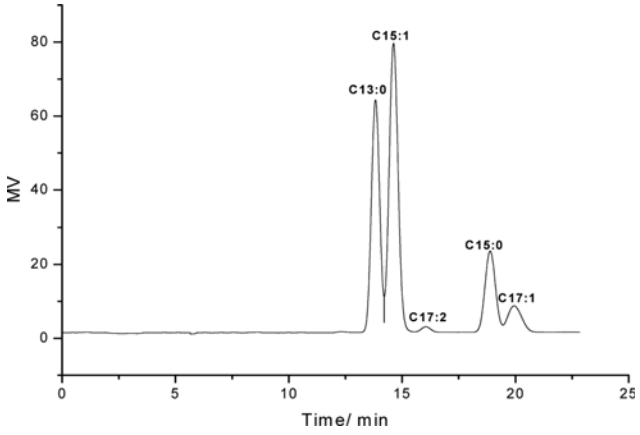

Fig. 3. HPLC fingerprints of the standard G. acids.

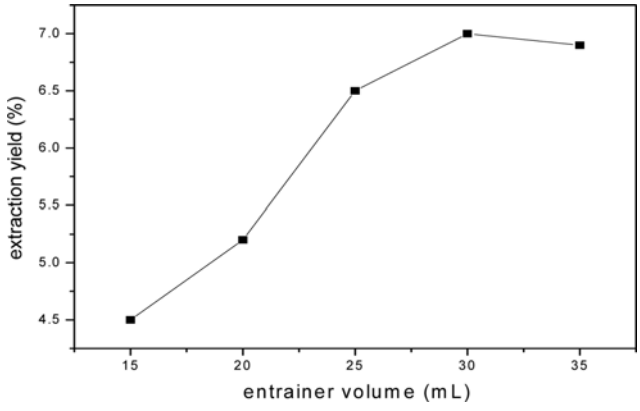

Fig. 5. Effect of entrainer volume on the extraction yield of G. acids. Experimental conditions: 1-2 h static-dynamic time, 35 MPa extraction pressure, 40 °C extraction temperature and 10 g min^{-1} CO_2 flow rate.

ing capacity of sc-CO_2 decreased with lower density. Consequently, 30 mL was chosen as the optimum modifier volume.

3. Model Fitting

The conditions for sc-SO_2 extraction of G. acids were optimized using different variable combinations according to the CCD. Table 2 presents the experimental design and the corresponding response data for the G. acids recovery. The experimental data were used to calculate the coefficients of the second-order polynomial equation (Eq. (3)); the analysis of variance for the response surface quadratic model is presented in Table 3.

$$\begin{aligned}\text{Yield} = &-24.163+0.319 * A+T0.854 * B+1.259 * C \\ &-3.25E\text{-}004 * AB+7.50E\text{-}003 * AC+3.125E\text{-}003 * BC \\ &-6.677E\text{-}003A^2-9.753E\text{-}003 * B^2-0.074 * C^2\end{aligned} \quad (3)$$

For any of analysis model, a small p-value would hint at more significant effects on the respective response variables. From the statistical analysis, the linear terms of temperature, flow rate of CO_2 and all of the quadratic terms affected the yield highly significantly ($p<0.01$); the interaction of pressure and flow rate of CO_2 had significant effect ($0.01<p<0.05$), and the linear pressure, interactions of pressure and temperature, temperature and flow rate of CO_2 were found to be insignificant ($p>0.05$).

The analysis of variance (ANOVA) proved the suitability of the fitted models. The linear regression coefficient R^2 was computed as 98.2%, indicating the model could adequately represent the real relationship between the parameters chosen. Moreover, the lack-of-fit was insignificant at p-value>0.05, demonstrating the adequacy of the selected quadratic model. The stable experimental data (C.V. %= 2.27) of less than 5% indicated that the model was reproducible [22].

4. Response Surface Analysis Data

Maintaining one constant and two independent variables plotted their three-dimensional response surface and contour plots to study the interactions between function of select factors. The yield of G. acids on the effect of extraction pressure and temperature with the flow rate of CO_2 maintained at 10 g min^{-1} is presented in Fig. 6. Fig. 7 illustrates the effect of extraction pressure and CO_2 flow rate on the G. acids recovery at a fixed extraction temperature of 45 °C, and Fig. 8 shows the effect of extraction temperature and CO_2 flow rate on the G. acids yield with the extraction pressure maintained at 30 MPa.

Table 2. Experiment design by CCD and the results of RSM

No.	A	B	C	Yield/%	
				Actual value	Predicted value
1	−1	−1	−1	5.27	5.15
2	1	−1	−1	4.81	4.94
3	−1	1	−1	5.24	5.30
4	1	1	−1	5.36	5.23
5	−1	−1	1	5.16	5.33
6	1	−1	1	5.75	5.72
7	−1	1	1	5.83	5.73
8	1	1	1	6.10	6.26
9	−1.68	0	0	5.36	5.37
10	1.68	0	0	5.70	5.64
11	0	−1.68	0	4.42	4.35
12	0	1.68	0	4.9	4.93
13	0	0	−1.68	6.00	6.05
14	0	0	1.68	7.17	7.07
15	0	0	0	7.32	7.39
16	0	0	0	7.48	7.39
17	0	0	0	7.52	7.39
18	0	0	0	7.38	7.39
19	0	0	0	7.41	7.39
20	0	0	0	7.25	7.39

Table 3. Analysis of variance for response surface quadratic model

Source	Sum of squares	Degree of freedom	Mean square	F-value	P-value
Model	20.72	9	2.30	121.50	<0.0001
A	0.087	1	0.087	4.61	0.0574
B	0.40	1	0.40	21.29	0.0010
C	1.25	1	1.25	65.82	<0.0001
AB	8.450E-003	1	8.450E-003	0.45	0.5194
AC	0.18	1	0.18	9.50	0.0116
BC	0.031	1	0.031	1.65	0.2218
A^2	6.43	1	6.43	339.02	<0.0001
B^2	13.71	1	13.71	723.31	<0.0001
C^2	1.25	1	1.25	66.05	<0.0001
Residual	0.19	10	0.19		
Lack of fit	0.14	5	0.028	2.80	0.1418
Pure error	0.050	5	9.987E-003		
Cor total	20.91	19			
R^2	0.982				
Adj R^2	0.966				
C.V. %	2.27				

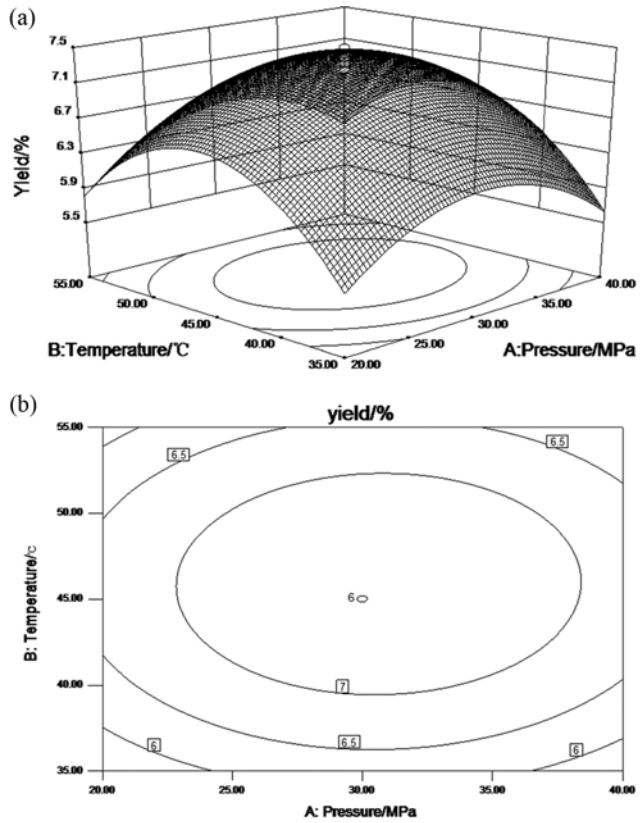

Fig. 6. Three-dimensional plot (a) and corresponding contour plot (b) of the effect of extraction pressure (A) and extraction temperature (B) on the extraction yield (Y) of G. acids with the flow rate of CO_2 maintained at 10 g min^{-1}.

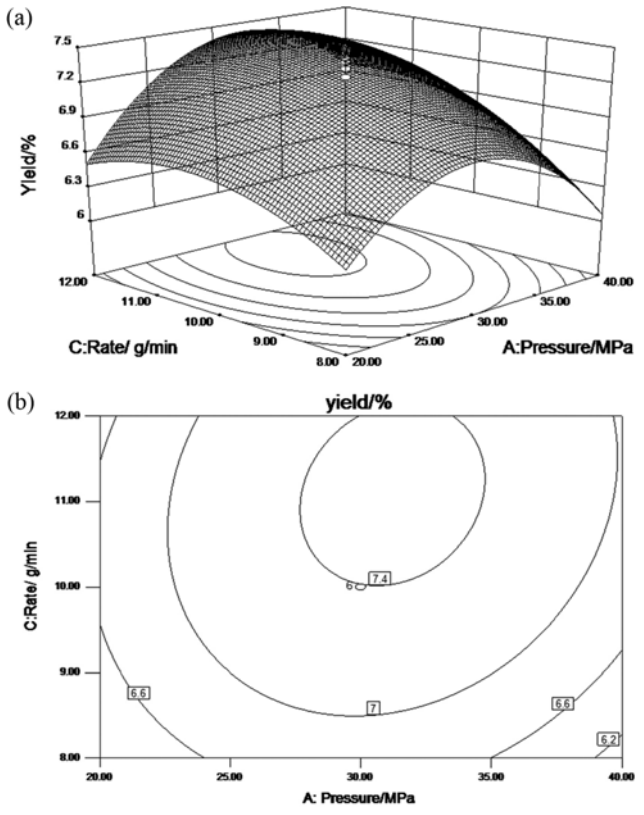

Fig. 7. Three-dimensional plot (a) and corresponding contour plot (b) of the effect of extraction pressure (A) and the flow rate of CO_2 (C) on the extraction yield (Y) of G. acids with the extraction temperature maintained at 45 °C.

The effect of extraction pressure on the G. acids yield was owing to a twin impact. The extraction yield of the G. acids increased with an extraction pressure less than 30 MPa. However, when the pressure was above 30 MPa, the extraction yield began to decrease. This result accords with those of other researchers' results for the sc-CO_2 extraction of natural products [23]. G. acids solubility depended on a complex interaction between sc-CO_2 density and solute vapor pressure. At a constant temperature, the density of the solvent increased with an increase in pressure, but the solute vapor pressure decreased with the increase of pressure. When the pressure increased to 30 MPa, the density of sc-CO_2 was dominant in this extraction process, resulting in favorable G. acids extraction lower than 30 MPa of pressure. Nevertheless, when the pressure was increased more than 30 MPa, the vapor pressure of the solute overcame the relatively small change of solvent density, resulting in a disadvantageous extraction at higher pressure.

Analogously, the temperature also showed a dual effect on the G. acids extraction. It was found that temperature could significantly increase the extraction yield, while a further increase above 45 °C led to a lower recovery. It can be explained that increasing the temperature could enhance the mass transfer between supercritical fluid and powders of ginkgo biloba exopleura, but at the same time, it will also reduce the density of the sc-CO_2. In front of the temperature not to exceed 45 °C, the mass transfer effect was a leading factor, while the density of sc-CO_2 was more significant in the ranges of 45-62 °C. The two factors interacted oppositely to make the influence of the extraction temperature on the yield of G. acids.

Based on the principle of dissolution in the similar material structure, the influence of CO_2 flow rate had a dramatic effect on the G. acids yield. When the amount of entrainer was fixed at 30 mL, an increase in the sc-CO_2 rate improved the solubility of G. acids. Fig. 7 and Fig. 8 show the optimal CO_2 flow rate was 10 g min^{-1}, and the polarity of G. acids was very near to this point of solvent.

From the RSM experimental results, the optimal process parameters for sc-CO_2 extraction could be predicted: 31.3 MPa extraction pressure, 46.1 °C extraction temperature and 11.1 g min^{-1} CO_2 flow rate. The theoretical maximum extraction yield of G. acids obtained 7.49%, which approximated the verification experimental results of 7.40%. Thus, the optimum process parameters for sc-CO_2 extraction obtained from RSM were credible and helpful for industrialized applications.

5. Comparison of Sc-CO_2 Extraction with Conventional Solvent Extraction

Conventional solvent extraction was investigated by petroleum ether (60-90 °C) at 70 °C for 6 h (two times, each time 3 h). The sc-CO_2 extraction under the optimum condition obtained from the RSM was compared with conventional solvent extraction experiment. The results are shown in Table 4. It is evident that the extraction yield of G. acids with sc-CO_2 was higher than obtained from conventional solvent extraction, which indicated that sc-CO_2 extraction

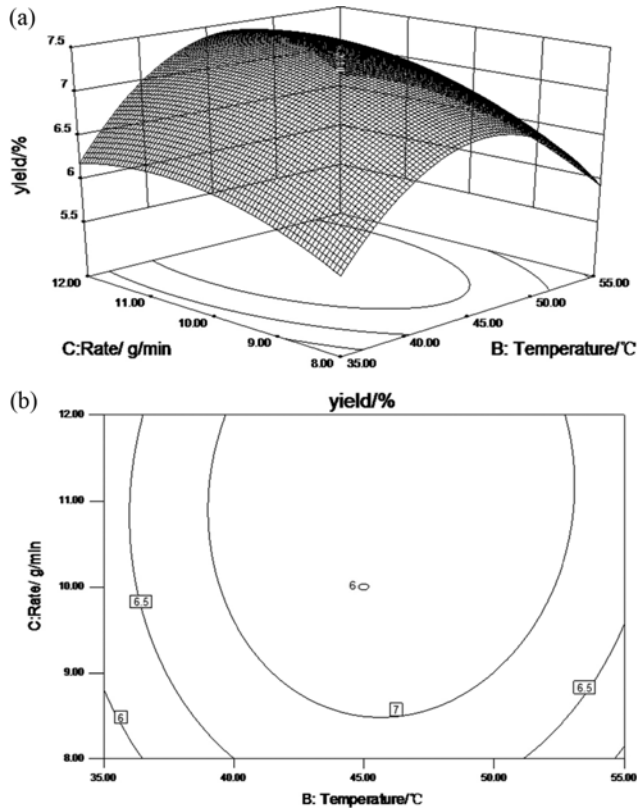

Fig. 8. Three-dimensional plot (a) and corresponding contour plot (b) of the effect of extraction temperature (B) and the flow rate of CO_2 (C) on the extraction yield (Y) of G. acids with the extraction pressure maintained at 30 MPa.

Table 4. Comparison of the extraction yield of G. acids between sc-CO_2 and conventional extraction

Extraction method	Extraction time [min]	Extraction yield/%
Sc-CO_2	180	7.4±0.1
Conventional solvent extraction	300	4.8±0.2

was more effective than the conventional solvent extraction.

CONCLUSIONS

The response surface optimization indicated that the CCD model could be used to optimize G. acids yield by sc-CO_2. From the single factor curve and three-dimensional response plots, the optical process parameters were as below: 31.3 MPa extraction pressure, 46.1 °C extraction temperature and 11.1 g min^{-1} CO_2 flow rate, 30 mL Ethanol entrainer, 1 h static time 2 h dynamic time. The experimental values carried out under the optical conditions were in good accordance with the predicted values.

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