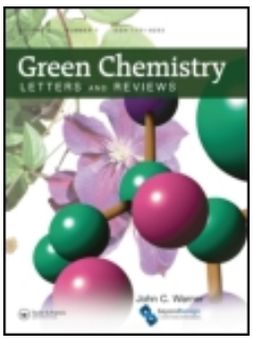

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RESEARCH LETTER

Optimization of supercritical fluid extraction of geniposidic acid from plantain seeds using response surface methodology

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The process parameters of supercritical CO_2 ($SCCO_2$) plus modifer for the extraction of geniposidic acid from plantain seeds were studied using a Box–Behnken design. The effects of independent variables, that is, ethanol concentration (0–70%, ethanol:water, v/v), extraction pressure (10–30 MPa), and temperature (50–80°C) on the yield of geniposidic acid were evaluated. Results indicated that the data could be well fitted to a second-order polynomial model. The effects of ethanol concentration and temperature, as well as the interaction between ethanol concentration and temperature were significant ($p < 0.05$). The yield (8.9 mg/g) of modified $SCCO_2$ extraction at optimal conditions was compared with that obtained by Soxhlet extraction or ultrasound assisted extraction.

Keywords: geniposidic acid; supercritical fluid extraction; Box–Behnken design; optimization; plantain seeds

1. Introduction

Plantain seeds come from *Plantago asiatica* L. or *Plantago* depressa Willd., a perennial herb growing in the woods and lowlands of China, Japan, and Korea. The seeds have been utilized in Chinese medicine for a long time due to their effects on inducing diuresis, treating stranguria, clearing heat from the liver for improving vision, and removing heat from the lungs to clear phlegm (*1*). Geniposidic acid (Figure 1) is an iridoid glucoside, found in a variety of plants such as *Eucommia ulmoides*, *Gardenia jasminoides* and *Plantago*. It has been reported that geniposidic acid has different pharmacological actions, such as antitumor and radioprotection (*2*), alleviating GalN/LPS-induced liver injury (*3*), and anti-inflammatory action (*4*).

Conventionally, the extraction of bioactive component can be optimized using "one at a time" variation of treatment variables. This method assumes that the various treatment parameters do not interact and that the response variable is a function of only the single varied parameter. However, the response obtained from an extraction process could be the result from the interactive influences of the different variables. When a combination of several independent variables and their interactions affects desired responses, response surface methodology (RSM) can be used as an effective tool for optimizing the process. RSM is a statistical method. This method uses quantitative data from an appropriate experimental design to evaluate the response of the statistically designed combinations, to estimate the coefficients by fitting it in a mathematical model that fits best the experimental conditions, to predict the response of the fitted model, to check the adequacy of the mode, and to search optimum condition of factors. As an experimental design, it may minimize assay numbers and time to keep the experimental cost at a minimum level with the possibility of revealing optimum information in studied experimental domain, and it has been applied in various experiments (*5*, *6*).

Due to the applications of geniposidic acid in pharmaceutics and the quality control of herbal medicines, it is interesting to study the extraction and analysis of the component from different plants. Some works dealing with the extraction and analysis of geniposidic acid from different plants have been carried out, such as high-pressure liquid chromatography (HPLC) analysis of water extraction from the leaves of *Plantago asiatica* (*7*), isolation antioxidants using HSCCC (*8*), comparison of methods for extraction of geniposidic acid from *Eucommia ulmoides* (*9*) and UPLC-MS analysis of the extract of *Eucommia ulmodies* (*10*). However, there is no report on the extraction of geniposidic acid from plantain seeds using supercritical fluid extraction (SFE). According to the requirement of Chinese pharmacopeia on the

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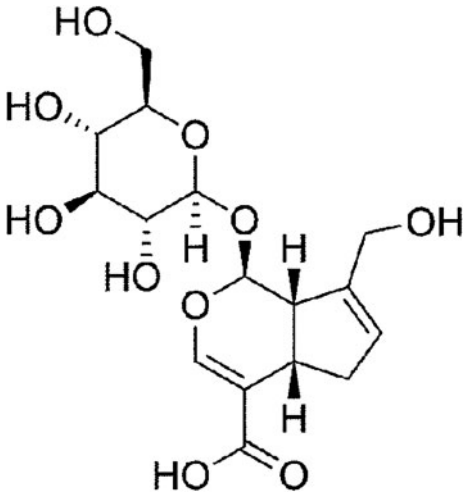

Figure 1. Structure of geniposidic acid.

content of geniposidic acid higher than 0.5% in plantain seeds (*1*), it is also necessary to evaluate the quality plantain seeds. SFE is regarded as a sustainable green technology because it does not use chemical solvents with drastic environmental impacts. The technology has been applied to extract bioactive compounds from different materials (*11*, *12*). Therefore, the main objective of the present work was to develop a SFE process, to optimize process parameters, and to investigate the effects of the three variables at three levels on the geniposidic acid yield using RSM in SFE.

2. Results and discussion

2.1. Model fitting

The effect of temperature, pressure, and modifier (ethanol concentration) on geniposidic acid yield obtained using supercritical CO_2 ($SCCO_2$) extraction was investigated using a Box–Behnken statistical model. They are presented in Table 1. The results were analyzed by using analysis of variance (ANOVA; Table 2). Judged by the model ($p < 0.0001$), coefficient of determination ($R^2 = 0.9955$) and the lack of fit ($p = 0.591$) show that the Box–Behnken model developed to predict geniposidic acid yield (Y) is considered adequate. ANOVA shows a highly significant effect on the yield for ethanol concentration and temperature ($p < 0.01$), and the interaction between ethanol concentration and temperature ($p < 0.05$).

The quadratic model from the Box–Behnken design can be used to generate a response surface image for the main interaction among the three independent factors. Fitting a regression surface to the experimental results, the following equation was obtained, applicable to predict the achievable extraction yield (Y) as a function of the studied process variables:

$$\begin{aligned} Y(\mathrm{mg/g}) = {} & 6.8 - 2.91X_1 + 0.16X_2 + 0.42X_3 \\ & - 2.03X_1^2 + 0.04X_2^2 + 0.46X_3^2 \\ & - 0.07X_1X_2 - 0.29X_1X_3 + 0.07X_2X_3 \end{aligned} \quad (1)$$

Table 1. Box–Behnken design matrix of independent variables and their corresponding geniposidic acid yields obtained from plantain seed.

	Uncoded (coded) variables			Responses
Trail no.	X_1 Ethanol concentration (ethanol:water,%, v/v)	X_2 Pressure (MPa)	X_3 Temperature (°C)	Geniposidic acid Yield (mg/g)[a]
1	0 (−1)	20 (0)	50 (−1)	7.45
2	35 (0)	10 (−1)	80 (1)	7.38
3	70 (1)	30 (1)	65 (0)	2.05
4	35 (0)	20 (0)	65 (0)	6.86
5	70 (1)	20 (0)	50 (−1)	2.01
6	35 (0)	30 (1)	50 (−1)	7.07
7	70 (1)	20 (0)	80 (1)	2.41
8	70 (1)	10 (−1)	65 (0)	1.93
9	35 (0)	20 (0)	65 (0)	7.18
10	35 (0)	30 (1)	80 (1)	7.88
11	35 (0)	20 (0)	65 (0)	6.71
12	0 (−1)	10 (−1)	65 (0)	7.41
13	35 (0)	20 (0)	65 (0)	6.74
14	35 (0)	10 (−1)	50 (−1)	6.85
15	0 (−1)	30 (1)	65 (0)	7.82
16	35 (0)	20 (0)	65 (0)	6.49
17	0 (−1)	20 (0)	80 (1)	9.03

[a]Results are mean of three determinations.

Table 2. ANOVA for the experimental results of the Box–Behnken design (quadratic model).

Source	Sum of squares	Degrees of freedom	*F* value	*P* value	Coefficient of determination (R^2)
Model	87.8	9	173.57	<0.0001	0.9955
X_1	67.92	1	1208.39	<0.0001	
X_2	0.20	1	3.47	0.1046	
X_3	1.38	1	24.51	0.0017	
X_1X_2	0.021	1	0.37	0.5601	
X_1X_3	0.35	1	6.19	0.0417	
X_2X_3	0.02	1	0.35	0.5734	
X_1^2	17.38	1	309.24	<0.0001	
X_2^2	6.16×10^{-3}	1	0.11	0.7503	
X_3^2	0.89	1	15.9	0.0053	
Residual	0.39	7			
Lack of fit	0.14	3	0.72	0.5910	
Pure error	0.26	4			
Cor. total	88.19	16			

where Y is the response (geniposidic acid yield), and X_1, X_2, and X_3 are the coded values of factors, ethanol concentration, pressure, and temperature, respectively. In Equation (1), the minus sign in front of the coefficient of ethanol concentration indicates that the factor reduce geniposidic acid yield. However, the plus sign in front of the coefficients of pressure and temperature has an increase effect on the yield.

Correlation graph (Figure 2) shows that high correlation exists between the experimental and predicted values. Each point is close to the regression line, which indicates the good fit of model. The optimal extraction condition was ethanol concentration 8%, pressure 30 MPa, and temperature 80°C with the predicted yield of 9.26 mg/g.

2.2. *Influence of factors*

Some factors, such as modifier, pressure, and temperature, can impact the yield of polar compounds in $SCCO_2$ extraction. The combined effects of ethanol concentration, pressure, and temperature on geniposidic acid yield are illustrated in Figures 3a–c, respectively. These graphs can be used for visually predicting future responses and for determining factor values that optimize the response function.

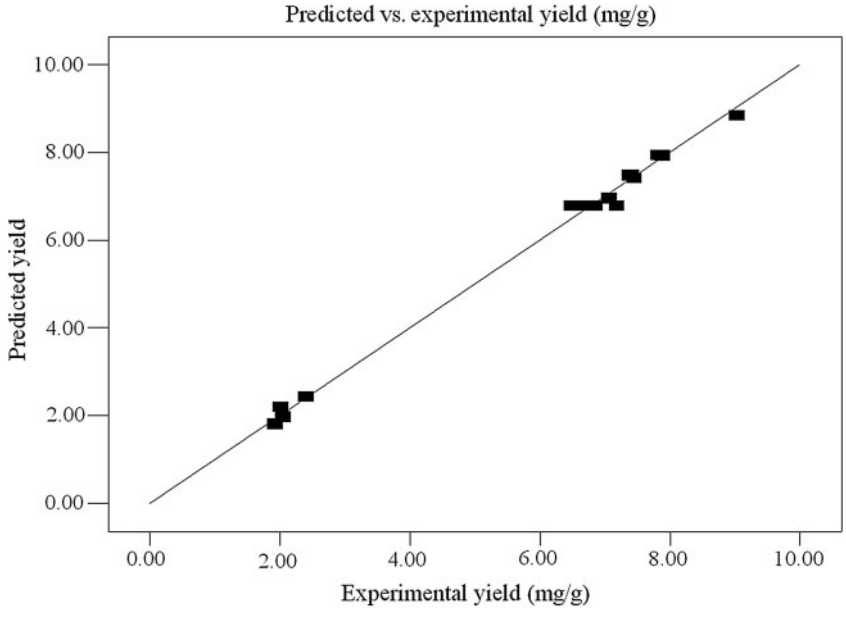

Figure 2. Correlation graph between the predicted and experimental yield.

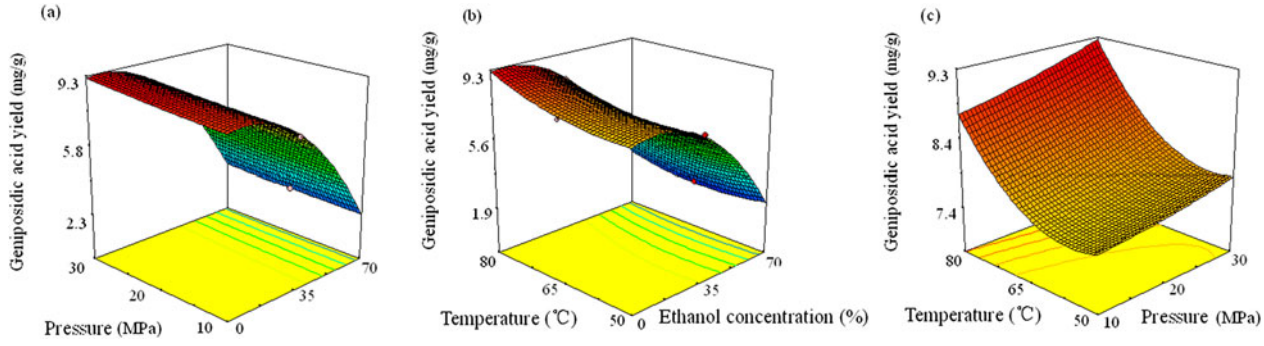

Figure 3. Response surface plots of geniposidic acid showing (a) the effect of pressure and ethanol concentration at constant temperature (80°C), (b) the effect of temperature and ethanol concentration at constant pressure (30 MPa), (c) the effect of temperature and pressure at constant ethanol concentration (8%, ethanol:water, v/v).

When considering the effect of different combination of two factors on extraction yield, 3D response surface plots can be used to provide a better understanding of the interaction between any two factors while the other factor (third factor) is held at constant optimum values.

For extraction of polar components with $SCCO_2$, it is necessary to add a small amount of polar modifier in CO_2 in order to increase the polarity of fluid, whose advantages include the improvement of extraction efficiency and the reduction of extraction time. Figure 3a shows the interaction effect of ethanol concentration and pressure ($X_1 \times X_2$) on geniposidic acid yield while temperature is kept constant at an optimum value of 80°C. It can be observed that the yield is influenced obviously by ethanol concentration. The yield increased from 2.4 mg/g to 9.2 mg/g when ethanol concentration decreased from 70% to 8%, and afterward the change of yield was small as the ethanol concentration further reduced. This response surface plot indicates that the highest yield (9.2 mg/g) is attained at ethanol concentration of 8% and pressure of 30 MPa. Any change in the value of pressure did not have significant interaction with increasing ethanol concentration. Higher yield was attained at higher pressure and lower ethanol concentration. Also, it is seen from Equation (1) that the coefficient (absolute value) of ethanol concentration term (X_1) is larger than that of pressure (X_2) term and $X_1 \times X_2$, which indicates that the ethanol concentration has a dominant effect over the pressure. The effect of pressure was not significant ($p > 0.05$). The effect of ethanol concentration could be explained by the fact of a similar polar solvent dissolving a similar polar solute. Geniposidic acid is an iridoid glucoside with high solubility in water, due to containing five hydroxyl groups and one carboxyl group in molecular structure. The decrease of the concentration of ethanol in water will increase solvent polarity. Higher yield can be attained when the polarity of the fluid matches with the polarity of the compound. It is believed that the solubility of geniposidic acid increases at a given concentration range of ethanol/water, which results in the increase in the extraction recovery.

The interaction effect of ethanol concentration and temperature ($X_1 \times X_3$) is displayed in Figure 3b. Lower ethanol concentration and higher temperatures favored the extraction. Higher yield was achieved at ethanol concentration within the range of 0–20% and temperature higher than 70°C while pressure was kept constant at an optimum value of 30 MPa. There was a strong interaction between ethanol concentration and temperature ($X_1 \times X_3$), and this interaction was highly effective on the geniposidic acid yield ($p < 0.05$). The effect of temperature on the yield could come from two ways. One is the increase of solute vapor pressure with temperature rise, causing an increase of solubility, and another is the decrease of solvent density with temperature rise, resulting in a decrease of solubility. The improvement of yield is dependent on which effect is more important. If the effect of vapor pressure were predominant, the solubility of solute in the supercritical phase would increase at higher temperatures, producing higher yield. On the contrary, if the effect of density were overwhelming, the solubility of solute would decrease at higher temperatures. In this study, higher temperature was favor of geniposidic acid extraction, which means that vapor pressure plays a major role in the effect of temperature (*14*). Temperature had significant effect on the yield ($p < 0.05$).

The effects of pressure and temperature are shown in Figure 3c. Higher temperature and pressure enhanced the extraction. The highest yield occurred at 30 MPa and 80°C, while lower yield (<7.5 mg/g) appeared within the range of 10–15 MPa and 50–60°C. The phenomenon of higher yield obtained at higher

pressure could be attributed to the increase of fluid density with elevating pressure, causing an increase of solubility. However, the effect of pressure was not significant ($p > 0.05$). For $X_2 \times X_3$ interaction, the coefficient of X_2X_3 term in Equation (2) is 0.07, which indicates that the interaction effect of X_2X_3 is small.

2.3. Validation of the model

In order to validate the adequacy of the model equation (Equation 1), a verification experiment was carried out under the optimal conditions (ethanol concentration 8%, pressure 30 MPa and temperature 80°C). Under the optimal conditions, the model predicted a maximum yield of 9.26 mg/g and a mean experimental value was 8.90 ± 0.63 mg/g ($n = 3$), which demonstrated the validation of the RSM model to be in good agreement with the predicted yield (Table 3).

2.4. Comparison of the yield of geniposidic acid obtained with three methods

Chinese pharmacopeia recommends to extracting geniposidic acid using 60% methanol as solvent (1). It was reported that geniposidic acid content in the tested 28 seed samples of 12 *Plantago* species was from 0.05 to 10.04 mg/g with 60% methanol in ultrasonic bath (*15*). Table 3 compares the results for extraction of geniposidic acid from the powdered plantain seeds using $SCCO_2$ + modifier under optimized conditions, Soxhlet extraction, and ultrasound-assisted extraction using several solvents. The yields obtained with SFE under the optimal conditions, Soxhlet extraction (60% methanol as solvent), and ultrasound-assisted extraction (60% methanol as solvent) were 8.9 mg/g, 9.24 mg/g, and 9.20 mg/g, respectively. Although there is a small difference in geniposidic acid yields, considering from environmental effect that ethanol is class 2 and ethanol is class 3 solvents (*16*), 8% ethanol modified $SCCO_2$ extraction is effective for exhaustive extraction of the component with lower environmental hazards.

3. Conclusion

This work studied the process parameters of SFE of geniposidic acid from the powdered plantain seeds. An RSM with varying ethanol concentration, pressures, and temperatures in $SCCO_2$ extractions indicated that the independent variables (ethanol concentration and temperature) significantly ($p < 0.01$) influenced the extraction yield of geniposidic acid. A synergetic effect of ethanol concentration and temperature was observed and it also had no significant effect ($p > 0.05$) with change of pressure. In general, high yield of geniposidic acid was attained in the low ethanol concentration region where extraction temperature was high. The model predicts the highest geniposidic acid yield (9.26 mg/g), which is similar with the yields obtained by Soxhlet extraction and ultrasound assisted extraction using 60% methanol as solvent. The SFE with subsequent HPLC analysis can offer an effective method for the quality evaluation of plantain seeds.

4. Experimental

4.1. Materials

The seeds of *Plantago asiatica* L., voucher no. YHM05614, came from Yinzhou Herbal Medicine Ltd (Ningbo, China). The seeds were ground into powder using an herbal pulverizer (FW 100, Tianjin Taisite Instrument Co. Ltd, Tianjin, China) and the powder was sieved through a 250 μm filter for extraction later. Geniposidic acid standard were purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China). CO_2 (99.5% purity) was from Fangxin Gas Ltd. (Ningbo, China). Methanol

Table 3. Comparison of the yield obtained by SFE, Soxhlet extraction, and ultrasound-assisted extraction.

Extraction method	Condition	Geniposidic acid yields (mg/g) (mean ± SD, $n = 3$)
SFE	Pressure, 30 MPa; temperature, 80°C; flow rate, 0.4 mL/min 8% ethanol and 2 L/min CO_2; dynamic extraction time, 1 hr	8.90 ± 0.63
Ultrasound	Extraction solvent, water; extraction time, 50 min; temperature, 45°C	7.26 ± 0.36
	Extraction solvent, 8% ethanol; extraction time, 50 min; temperature, 45°C	8.83 ± 0.47
	Extraction solvent, 35% ethanol; extraction time, 50 min; temperature, 45°C.	8.62 ± 0.09
	Extraction solvent, 60% methanol; extraction time, 50 min; temperature, 45°C	9.20 ± 0.56
Soxhlet	Extraction solvent, 95% ethanol; extraction time 6 hr	6.25 ± 0.58
	Extraction solvent, 8% ethanol; extraction time 6 hr	8.50 ± 0.67
	Extraction solvent, 60 methanol; extraction time 6 hr	9.24 ± 1.02

of HPLC grade was purchased from Tianjin Shield Company (Tianjin, China). Ethanol, methanol and acetic acid were analytical grade and were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Celite (Chemical grade) was from Fengcheng Chemical Ltd. (Shanghai, China).

4.2. Supercritical fluid extraction

A supercritical fluid extractor Spe-ed SFE-2 (Applied Separation, USA) was used, which operates with two pumps, a master pump for delivery of CO_2 and a second pump (Knauer pump, model K-501, Berlin, Germany) for the addition co-solvent. Each time an accurately weighed quantity of grounded sample (about 0.5 g) was placed in a 10 mL of extraction vessel (60 × 15 mm, i.d.) sandwiched with celite forming a fixed bed in the vessel. Before the extraction was started, the extraction vessel was preheated in the oven for 10 min. The extraction conditions were as follows: extraction time, static extraction for 5 min and then dynamic extraction up to 60 min; temperature, from 50 to 80°C; pressure, from 10 to 30 MPa, flow-rate of carbon dioxide (gaseous state), 2 L/min; flow-rate of co-solvent, 0.4 mL/min (correspond to 8% co-solvent). Collection is at room temperature and atmospheric pressure. The extracts are collected in glass vials (30 mL containing 4 mL of 35% ethanol, ethanol:water, v/v). The extracts were quantitatively transferred to a 25 mL volumetric flask and made up to the mark with 35% ethanol. This solution was quantitatively analyzed with HPLC.

4.3. Soxhlet extraction

A known quantity of grounded sample (0.5 g) was accurately weighed into a thimble and was extracted in a 50 mL of extractor with 50 mL of different concentration of methanol or ethanol at a syphon rate of 1 cycle/15 min for 7 h. The extracts were transferred to a 50 mL volumetric flask and made up to the mark with methanol.

4.4. Ultrasound assisted extraction

A known quantity of grounded sample (0.3 g) was accurately weighed into a 25 mL flask with stopper containing 20 mL of water or various concentration of methanol or ethanol (methanol or ethanol:water, v/v) and was extracted for 50 min with an ultrasonics processor SB-3200D with 40 KHz and 120 W (Ningbo Scientz Biological Technology Co. Ltd., China). The extracts were centrifuged (TDL-50B, Shanghai Anting Scientific Instrument Factory, China) at 4000 rpm for 5 min and the solution was ready for quantitative analysis.

4.5. HPLC analysis

Geniposidic acid contents were quantified by a HPLC (Hitachi, Japan) equipped with a Hitachi pump (model L-2130) and an ultraviolet-visible detector (model L-2400). The detection wavelength was set at 240 nm. An Inertsil C8-3 (4.6 × 250 mm, 5 μm) column (GL Sciences Inc., Japan) was used for the separation and quantification of geniposidic acid from the extracts. The mobile phase was methanol:water: acetic acid (20: 80: 1, v/v/v) with a flow rate of 1 mL/min. All extracts were filtered through a 0.45 μm membrane filter before injecting into the HPLC system. A 20 μL injection volume was used for all analysis. A series of geniposidic acid standards in the range of 5.1–112.7 μg/mL were prepared in 35% ethanol (ethanol:water, v/v) with a linear calibration curve ($y = 27045\times-44514$, $R^2 = 0.9995$, $n = 7$). The extraction yield of geniposidic acid was calculated according to the calibration curve. The peak of geniposidic acid in the extract was identified by comparing the retention time with that obtained with a pure geniposidic acid standard solution. A typical chromatogram of extract is shown in Figure 4 and the peak of geniposidic acid appears at a retention time of approximately 8 min.

4.6. Experimental design

Experimental design is a planned approach for determining cause and effect relationships of factors having influence on a system or model. In classical experimental design, it may be designed to investigate one factor at a time so that all other independent variables are held constant. However, it is impossible to establish the effects of interactions between the factors. Factorial experiment design was an optimization approach that allows for varying levels of the factors simultaneously rather than one at a time and studies interactions between the factors. In this study, a Box–Behnken design was used, which fits mathematically the experimental domain studies in the theoretical design through a response function to optimize process parameters. It has been confirmed by different researchers that modifier, temperature, pressure, and time are important factors that can impact the extraction yield of polar compounds in $SCCO_2$ extraction (*13*, *15*). However, the factor of time was not regarded as an independent variable for evaluation in this work in that >95% of geniposidic acid could be extracted within 1 hr based on the recovery of 2 hours. As a result, the time was fixed at 1 hr.

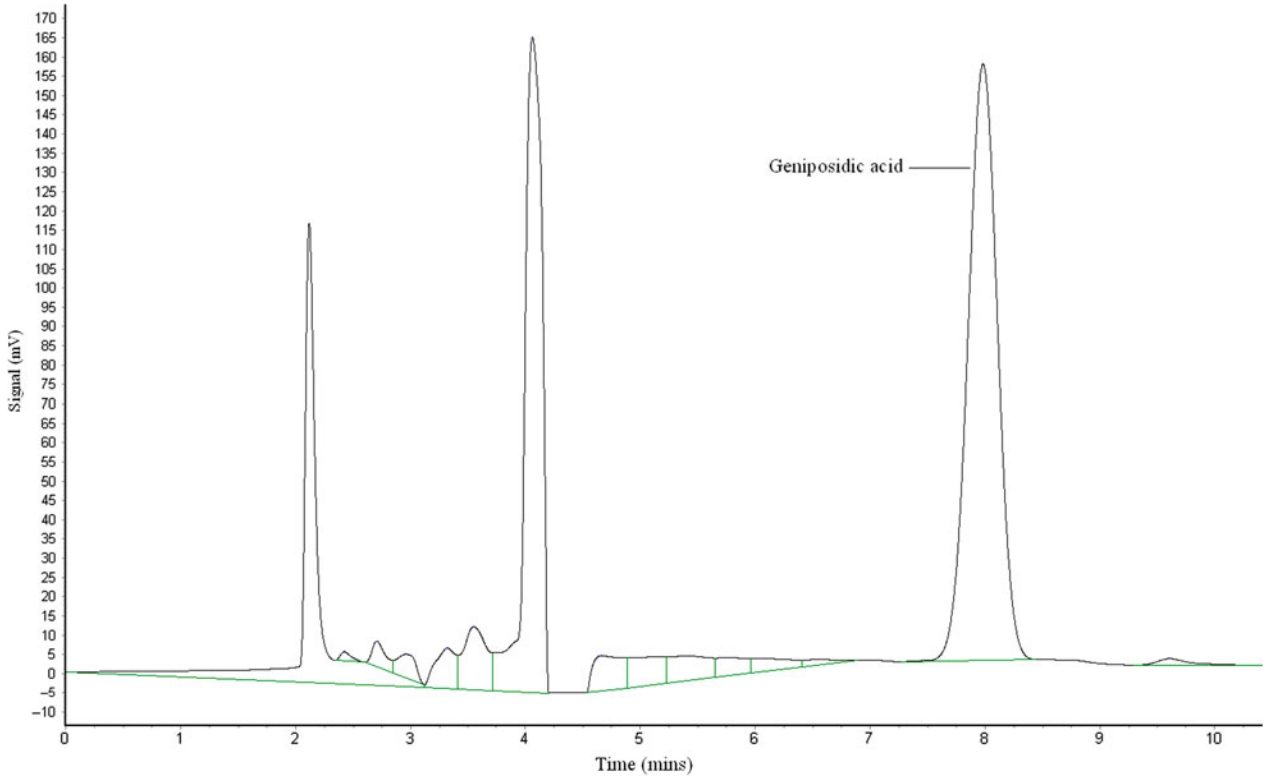

Figure 4. The chromatogram of extract obtained by $SCCO_2$ + 8% ethanol (ethanol:water, v/v) at 30 MPa and 80°C for 1 hr extraction.

Compared with commonly used polar solvent methanol, ethanol has more safety belonging to class 3 solvent (*16*). Thus, different concentration of ethanol was adopted. In our preliminary tests, it was found that there was no geniposidic acid to be extracted when using pure $SCCO_2$ as solvent and the yield of geniposidic acid was lower than 2.0 mg/g when higher ethanol concentration (>70% ethanol) was used as modifier. Thus, ethanol concentration was set between the range of 0% and 70% (ethanol:water, v/v). For the determination of pressure, it is observed that the yield attained under 30 or 40 MPa was similar when same ethanol concentration and temperature were applied. So pressure from 10 to 30 MPa was selected. Temperature was range between 50°C and 80°C so that the fluid was in a supercritical or near-critical state. Additionally, too high temperature could lead to damage the seals of extraction vessel. The design consists of a three-factored (n = 3) factorial design with three levels, which are based on the results of preliminary experiments. The factors, i.e. ethanol concentration in water (X_1), pressure (X_2), and temperature (X_3), and their levels are shown in Table 4. The matrix for the Box–Behnken design optimization experiment is summarized in Table 1. The experimental plan consisted of 17 trials with replicated five times at the central point of the design for experimental error determination. All the experimental units (run) were replicated three times.

A full second-order polynomial model of the design is given in Equation (2) and was used to evaluate the yield (response value, Y) as a function of the corresponding factors (X) and their interactions:

$$Y = \beta_0 + \sum_{i=0}^{3} \beta_i X_i + \sum_{i=0}^{3} \beta_{ii} X_i^2 + \sum_{i=0}^{3} \sum_{j=1}^{3} \beta_{ij} X_i X_j \quad (2)$$

where Y is the predicted response value; X_i and X_j are independent factors that influence the response Y; β_0 is the offset term; β_i is the ith linear coefficient; β_{ii} is the ith quadratic coefficient; and β_{ij} is the ijth interaction coefficient.

Table 4. Variables and experimental design levels for response surface.

Variables	Coded symbols	Levels		
		−1	0	1
Ethanol concentration (ethanol: water, v/v,%)	X_1	0	35	70
Pressure (MPa)	X_2	10	20	30
Temperature (°C)	X_3	50	65	80

All statistical analyses of the experimental data were carried out using Design Expert software (Stat-Ease Inc., Minneapolis, MN, USA). Design Expert software was also used to generate the main effect plots and response surface plots of factors.

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