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ORIGINAL RESEARCH PAPER

Extraction process and method validation for bioactive compounds from *Citrus reticulata* cv. Chachiensis: Application of response surface methodology and HPLC–DAD

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ABSTRACT

Citrus reticulata cv. Chachiensis, a traditional Chinese herb, has extensive medicinal and edible effects. 3',4',5,6,7,8-Hexamethoxyflavone (HM) and 5,6,7,8,4'-pentamethoxyflavone (PM) are main bioactive compounds in Chachiensis, which have been reported to possess various biological properties. In this study, supercritical CO₂ extraction (SCE) and high-speed countercurrent chromatography (HSCCC) were utilized to prepare HM and PM from Chachiensis. The contents of target compounds were determined by a high-performance liquid chromatography method with diode-array detection (HPLC–DAD), which was validated using the following parameters: linearity, sensitivity, repeatability, stability, precision and accuracy. The SCE conditions were optimized using response surface methodology with central composite design. Obtained optimum conditions were temperature of 37.9 °C, pressure of 26.3 MPa, and modifier volume of 81.0 mL. Under above conditions, the recoveries of target compounds were 92.52 ± 0.83 and 96.36 ± 0.43%, respectively. The most appropriate solvent system for HSCCC was selected as *n*-hexane/ethyl acetate/methanol/water (1:0.8:1:1.2, v/v). The HSCCC fractions were detected by HPLC–DAD, liquid chromatography-mass spectrometry (LC–MS) and nuclear magnetic resonance spectroscopy (¹H NMR and ¹³C NMR). The results indicated that this method was successfully applied to obtain HM and PM with high purities and high recoveries from Chachiensis.

KEYWORDS

HPLC–DAD, HSCCC, method validation, extraction

INTRODUCTION

Incidences of chronic diseases such as diabetes, obesity, heart disease, and hypertension have increased worldwide, especially in developed areas, drawing more attention and prompting people to focus on a balanced diet and healthy lifestyle [1]. It is reported that plants contain a variety of bioactive ingredients beneficial to human health, such as vitamins, flavonoid and volatile oils etc. Because of the advantages of low toxicity and nature, the acceptance of plant-medicine has been improved gradually. Many plants are used in diets and prescriptions. For instance, ethnic medicine with long-term application experience is easier to be accepted and used. As predicted by previous article, plant-based medicines and foods are growing at 6–8% per year and have great potential for development worldwide [2].

Citrus reticulata cv. Chachiensis is belonging to the Rutaceae family. Large quantities of Chachiensis are consumed in China and Southeast Asia yearly for its extensive activities,

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Fig. 1. The chemical structures of HM (I) and PM (II)

including antiviral [3], antioxidant [4], anti-inflammatory [5], anti-asthmatic [6] and hypolipidemic effects [7]. In addition to being used as medicine, Chachiensis is commonly employed in food therapy and medicated diets due to its high edible value. For example, Gan'pu Tea, a popular Chinese tea, made from Chachiensis peels and Pu'er tea, functions to moisten the lungs, strengthen the stomach and modulate the gut microbiota [8]. These beneficial effects are based on its secondary metabolites, such as volatile oils, flavonoids and amino acids, etc. 3',4',5,6,7,8-Hexamethoxyflavone (HM) and 5,6,7,8,4'-pentamethoxyflavone (PM) (Fig. 1), two of the major bioactive flavonoids in Chachiensis, have been found to possess broad biological activities.

Hence, the recovery and isolation of HM and PM from Chachiensis make perfect sense. There have been some attempts. Ada C. Gallo-Molina et al. described a process using microporous resin and silica gel column chromatography, which includes four steps by eluting with 43% ethanol in water, 90% ethanol in water, petroleum ether–ethyl acetate (20:1 to 1:3) and recrystallization [5]. This approach requires multiple steps and large amounts of organic solvents, results in low extraction efficiency.

Supercritical CO₂ extraction (SCE) is an eco-friendly technology and typically applied in chemical, food and pharmaceutical industries. SCE can be conducted under moderate operating conditions that help to protect thermally unstable components [9–11], making it especially suitable for the extraction of HM and PM. The most common supercritical fluid is CO₂, which is non-toxic, safe, reusable and readily available. Pure CO₂ with low polarity is a suitable extraction medium for nonpolar components [12, 13]. With the addition of modifier, solvent properties of CO₂ can be changed, which is useful for the extraction of moderately polar and polar components [14, 15].

Jiaojiao Xu et al. provided a way to prepare HM and PM from Chachiensis using SCE and silica gel column chromatography, including SCE process for 2 hours and several elution steps with petroleum ether, petroleum ether-acetone gradients (99:1 → 60:40, v/v), methanol, cyclohexane-ethyl acetate (300:1 → 1:1, v/v) and recrystallization [3]. Although advanced SCE was used in Xu's method, the extraction conditions need to be further investigated and optimized. Besides, the subsequent separation process was too complex and solvents consuming.

The high-speed countercurrent chromatography (HSCCC), a sort of liquid-partition chromatographic technique, allows both forward and reversed-phase operation and reduces sample adsorption in the system. The

separation of compounds in HSCCC depends on the rapid mixing and demixing of samples in two-phase solvent systems, which offers the following advantages: high sample-loading capacity, simple technology and complete recoveries of the target components [16, 17]. HSCCC has been broadly implemented to purify components with high quantity and quality from natural products. However, there is no reported study on the separation of the individual compound from Chachiensis by HSCCC.

This work aims to develop a novel method to prepare HM and PM from Chachiensis, evaluate the extraction parameters and their influence on the yields, and develop an analytical method for the determination of the target compounds. For this purpose, SCE and HSCCC processes were consecutively utilized, while a response surface methodology based on central composite design (RSM-CCD) program was carried out for the maximum yields. In our previous work, six kinds of solvents (methanol, anhydrous, ethanol, 95% ethanol, ethyl acetate and petroleum ether) were screened, ethyl acetate exhibited the best extraction effects of HM and PM [18]. Therefore, ethyl acetate was served as modifier in this study. To our knowledge, the purification of HM and PM from Chachiensis using consecutive application of ethyl acetate-modified SCE and HSCCC are reported for the first time. In addition, a high-performance liquid chromatography method with diode-array-detector (HPLC-DAD) was developed and validated according to the International Conference on Harmonization (ICH) guidelines to detect HM and PM in SCE extracts and HSCCC fractions.

EXPERIMENTS

Reagents

The peels of Chachiensis were provided by Changye Co., Ltd. (Xinhui, China). Carbon dioxide was obtained from Yigas Gases (purity >99.99%, Guangzhou, China). Ethyl acetate and formic acid were of analytical-grade (Damao Chemical, Tianjin, China). HPLC-grade acetonitrile (Anqua Global International Inc., Cleveland, USA) was used for the detection of samples. HM and PM reference substances were made by Must Biotechnology (purity >99%, Chengdu, China). Chloroform-d was purchased from Cambridge Isotope Laboratories (Cambridge, USA).

SCE procedure

SCE was carried out on an SCE apparatus (HA122-50-01, Hua'an Technology, Jiangsu, China). The peels of Chachiensis (100 g) was smashed, sieved (40 mesh) and placed into the extractor of the SCE apparatus. Supercritical CO₂ was delivered by a high-pressure pump with the constant flow rate of 20 L/h. Two separators (1 L each) were connected in series and allowed the periodic discharge of extracts during the extraction process. Pressure-regulating valves allowed different conditions to be implemented during separation. The pressures of separators I and II were maintained independently at 8 and 6 MPa with temperatures of 45 and 60 °C,

Table 1. The levels of independent variables in the RSM design

Pressure (MPa)	Temperature (°C)	Modifier (mL)	Coded variables
15	18	33	−1.68
19	25	50	−1
25	35	75	0
31	45	100	1
35	52	117	1.68

respectively. Dynamic extraction was carried out for 60 min with ethyl acetate as modifier. Each SCE extract was collected and evaporative dried in a rotational vacuum concentrator (N-1200A, Eyela, Tokyo, Japan). Then dried extract was weighed, dissolved by methanol, and filtered through 0.22-μm membrane for HPLC analysis.

A method for determining the recoveries of HM and PM was developed in this study. Chachiensis powder (0.5 g) was added into ethyl acetate (50 mL) and ultrasonic extracted for 30 min three times in an ultrasonic instrument (Scientz Biotechnology, Zhejiang, China) to achieve complete extraction. The extracts were evaporative dried to remove the solvent and dissolved with methanol for HPLC analysis. The recoveries of HM and PM were calculated as a ratio between the contents of target compounds in SCE extract and that in Chachiensis powder.

RSM-CCD experiments

Many factors affect the efficiency of SCE. The effects of the parameters on the response could not be depicted

Table 2. Central composite design and experimental results for the recoveries of HM and PM

Run	Independent variables			Recovery (%)	
	Pressure (MPa)	Temperature (°C)	Modifier (mL)	HM	PM
1	19 (−1)	45 (1)	50 (−1)	38.89	38.93
2	25 (0)	35 (0)	117 (1.68)	62.38	61.49
3	25 (0)	35 (0)	75 (0)	86.02	90.62
4	25 (0)	35 (0)	33 (−1.68)	33.46	36.53
5	19 (−1)	25 (−1)	50 (−1)	53.66	55.39
6	25 (0)	18 (−1.68)	75 (0)	52.42	53.81
7	25 (0)	52 (1.68)	75 (0)	60.57	56.43
8	25 (0)	35 (0)	75 (0)	91.18	96.36
9	31 (1)	25 (−1)	50 (−1)	45.17	46.83
10	31 (1)	45 (1)	100 (1)	81.50	79.76
11	19 (−1)	45 (1)	100 (1)	54.71	57.89
12	25 (0)	35 (0)	75 (0)	91.96	95.39
13	25 (0)	35 (0)	75 (0)	90.64	95.59
14	25 (0)	35 (0)	75 (0)	92.37	95.23
15	25 (0)	35 (0)	75 (0)	91.24	96.09
16	15 (−1.68)	35 (0)	75 (0)	59.52	60.89
17	35 (1.68)	35 (0)	75 (0)	71.30	72.17
18	31 (1)	25 (−1)	100 (1)	46.58	47.06
19	31 (1)	45 (1)	50 (−1)	54.48	56.62
20	19 (−1)	25 (−1)	100 (1)	57.81	58.60

Table 3. Results of HPLC method validation

Item	Values	
	HM	PM
Regression equation ($y = ax + b$) ^a	$y = 40384x + 6293.1$	$y = 21615x + 7501.9$
Linear range (μg/mL)	6.40–213.40	5.63–180.00
Correlation coefficient (r)	0.9999	0.9998
LOD (μg/mL)	0.064	0.057
LOQ (μg/mL)	0.129	0.071
Recovery ($n = 9$)	100.59%	99.29%
Repeatability (RSD%)	0.48%	0.30%
Intraday precision (RSD%)	0.37%	1.20%
Interday precision (RSD%)	1.80%	1.08%
Stability (RSD%)	1.60%	1.18%

^a Where y and x denote the peak area and corresponding concentration (μg/mL), respectively, and a and b denote the slope and intercept of the regression line, respectively.

completely using a traditional single-factor experiment, which does not account for the interactive effects among the studied variables [19, 20]. RSM is a multivariate analytical technique that could better describe the behavior of the data, make statistical predictions, and establish the optimal operational conditions [11, 21].

Table 4. Partition coefficients (K) of HM and PM in different solvent systems

No.	Solvent system (v/v)	Partition coefficient (K)	
		HM	PM
1	<i>n</i> -Hexane-ethyl acetate-methanol-water (1:0.8:0.8:1.2)	1.51	3.53
2	<i>n</i> -Hexane-ethyl acetate-methanol-water (1:0.8:1:1.2)	0.78	1.86
3	<i>n</i> -Hexane-ethyl acetate-methanol-water (1:0.8:0.8:1.5)	2.74	8.18
4	<i>n</i> -Hexane-ethyl acetate-methanol-water (1:1.2:1:1.5)	2.05	4.99
5	<i>n</i> -Hexane-ethyl acetate-methanol-water (1:1:1:1.5)	1.97	5.05
6	<i>n</i> -Hexane-ethyl acetate-methanol-water (1:0.8:1:1.5)	1.59	4.53
7	Petroleum ether-ethyl acetate-methanol-water (1:0.8:0.7:0.8)	1.12	2.56
8	Petroleum ether-ethyl acetate-methanol-water (1:0.8:0.6:0.9)	1.94	4.81
9	Petroleum ether-ethyl acetate-methanol-water (1:0.8:0.7:1)	1.15	5.60

Table 5. ANOVA of RSM quadratic model for HM recovery

Variance Sources	Sum of Squares	dF	Mean square	F-value	P-value (prob. > F)
Model	7,191.88	9	799.10	103.63	<0.0001
A (Pressure)	132.04	1	132.04	17.12	0.0020
B (Temperature)	117.50	1	117.50	15.24	0.0029
C (Modifier)	689.51	1	689.51	89.41	<0.0001
AB	481.96	1	481.96	62.50	<0.0001
AC	8.91	1	8.91	1.16	0.3076
BC	173.79	1	173.79	22.54	0.0008
A ²	1,164.35	1	1,164.35	150.99	<0.0001
B ²	2,124.47	1	2,124.47	275.50	<0.0001
C ²	3,317.91	1	3,317.91	430.26	<0.0001
Residual	77.11	10	7.71		
Lack of fit	50.43	5	10.09	1.89	0.2508
Pure error	26.68	5	5.34		
Cor total	7,268.99	19			
R ²	0.9894				
Adj-R ²	0.9798				
Pre-R ²	0.9368				
Adeq. pre.	27.986				
CV%	4.22				

Table 6. ANOVA of RSM quadratic model for PM recovery

Variance sources	Sum of squares	dF	Mean square	F-value	P-value (prob. > F)
Model	8,077.49	9	897.50	145.65	<0.0001
A (Pressure)	108.11	1	108.11	17.55	0.0019
B (Temperature)	64.79	1	64.79	10.51	0.0088
C (Modifier)	560.77	1	560.77	91.00	<0.0001
AB	445.03	1	445.03	72.22	<0.0001
AC	0.18	1	0.18	0.029	0.8675
BC	186.90	1	186.90	30.33	0.0003
A ²	1,414.28	1	1,414.28	229.52	<0.0001
B ²	2,800.70	1	2,800.70	454.51	<0.0001
C ²	3,735.40	1	3,735.40	606.20	<0.0001
Residual	61.62	10	6.16		
Lack of fit	38.98	5	7.80	1.72	0.2828
Pure error	22.64	5	4.53		
Cor total	8,139.11	19			
R ²	0.9924				
Adj-R ²	0.9856				
Pre-R ²	0.9584				
Adeq. pre.	33.688				
CV%	3.67				

RSM-CCD allows the screening of a broad range of parameters. In addition to evaluating the role of each individual factor, RSM-CCD provides information on the cumulative effect of the variables while helping to reduce the cost of the analysis.

In this work, RSM-CCD was utilized to screen and optimize different factors affecting the SCE of HM and PM. The variables with five levels including pressure (X_1 , MPa), temperature (X_2 , °C) and modifier volume (X_3 , mL) were studied (Table 1). Analysis of variance was conducted for quadratic regression equation based on the F value at 95% confidence intervals by Design-Expert V8.0.6. The CCD matrix was composed of 20 experimental points (Table 2).

HPLC-DAD analysis

HM and PM were quantitatively analyzed using a liquid chromatography system (LC-20AB, Shimadzu, Tokyo, Japan). The sample injection volume was set at 10 μ L using a SIL-20A autosampler. Acetonitrile and 0.1% formic acid in water were mixed proportionally to serve as mobile phase. The following gradient elution was programmed: 0–20 min, 40–41.5% acetonitrile; 20–26 min, 41.5–60% acetonitrile; 26–36 min, isocratic with 60% acetonitrile. The flow rate was 1.0 mL/min. The diluted extracts were injected into the system and monitored simultaneously at 334 and 323 nm by an SPD-M20A detector. The operating temperature was maintained at constant 35°C using a CTO-20A column oven.

Fig. 2. Response surface (a) and contour plots (b) for the effects of pressure and modifier volume on HM recovery while the temperature at zero level

Method validation

Standard solutions were prepared by dissolving reference substances (HM and PM) at six different concentrations and analyzed by HPLC with injection volumes of 10 μ L. The limits of detection (LOD) and quantitation (LOQ) were measured according to signal/noise ratios ($S/N = 3:1, 10:1$). The intra-day (within 24 h) and inter-day (on three consecutive days) precisions were evaluated by analyzing standard solutions. The calibration graph was made with the peak-area (y) vs the corresponding concentration (x). The accuracy of method was tested by the recovery procedure. Standard solutions of HM were added to the sample solutions ($n = 9$) at three different concentrations (85.36, 106.70 and 128.04 μ g/mL). Similarly, standard solutions of PM were added to the sample solutions ($n = 9$) at three different levels (82.16, 102.70 and 123.24 μ g/mL). Six different samples were detected to verify the repeatability. To validate the stability, the sample solutions were evaluated at 0, 2, 4, 8, 12 and 24 h after preparation, respectively. Table 3 showed the results of HPLC method validation.

Solvent systems of HSCCC

HSCCC separation is strongly influenced by the partition coefficient (K value), which is one of the most significant

Fig. 3. Response surface (a) and contour plots (b) for the effects of pressure and modifier volume on PM recovery while the temperature at zero level

parameters in determining peak resolution. To select an effective solvent system for HSCCC, K value should be in the range of $0.5 \leq K \leq 2.0$. K values (<0.5) generally result in lower stationary phase retention, leading to poor separation effect. While K value greater than 2 commonly lead to inordinately long elution time and excessive band broadening [22].

K value was determined referring to previous article [23]. The solvent system (10 mL) was mixed and stood until divided into two layers. The SCE extract (2 mg) was placed into a tube which contained the upper and lower phases (each of 2 mL). After fully shaken and equilibration, the upper and lower phases (each of 1 mL) were put into two test tubes and vacuum dried. Then the dried samples were dissolved for HPLC analysis. K value was obtained by calculating the ratio of peak areas of compounds in two phases. A series of two-phase solvents were evaluated by HPLC (Table 4).

HSCCC procedure

Separation of HM and PM was achieved using an HSCCC instrument (Counter Current Technology, Jiangsu, China) with an LC3000 detector and a CF80 pump. An optimal solvent system was prepared for separating sample. After equilibrated overnight, two phases were transferred to different bottles, and degassed by suction filtration before use. Two phases were

Fig. 4. Response surface (a) and contour plots (b) for the effects of pressure and temperature on HM recovery while modifier volume at zero level

injected into the column to establish hydrodynamic equilibrium in head-to-tail mode. The solvent (20 mL) consisted of equivalent upper and lower phases was used for dissolving SCE extract (200 mg). The rotate speed and operation temperature were 800 rpm and 25 °C. SCE extracts were injected into HSCCC system at 2 mL/min and monitored at 334 nm.

Identification

^1H NMR, ^{13}C NMR spectroscopy and liquid chromatography-mass spectrometry (LC-MS) were performed to identify structures of compounds. The data were recorded on an Advance III400 NMR Spectrometer (Bruker, Germany) and liquid chromatography-mass spectrometry (Thermo Scientific, Cambridge, USA).

RESULTS AND DISCUSSION

Validation of analytical method

Two calibration curves both had good linearity ($r > 0.9998$). The linear range was 6.40–213.40 and 5.63–180.00 $\mu\text{g/mL}$, respectively. Triplicate HPLC analyses of each sample indicate that average recovery of HM was 100.59% and that of PM was 99.29%. Relative standard deviation (RSD) calculated for validating accuracy was less than 2.00%. The LOD

Fig. 5. Response surface (a) and contour plots (b) for the effects of pressure and temperature on PM recovery while modifier volume at zero level

and LOQ of HM were 0.064 and 0.129 $\mu\text{g/mL}$, and that of PM were 0.057 and 0.071, respectively. Repeatability was validated as RSD varied from 0.30% to 0.48%. The sample solutions could be stable within 24 h. The intra-day and inter-day precisions were both qualified (RSD < 2.00%).

Optimization of SCE conditions

Three key parameters and their ranges were designated and further evaluated by RSM-CCD to maximize the extraction rates of HM and PM. The design matrix showing all the independent variables and corresponding response data for 20 RSM-CCD runs was displayed in Table 2.

The following second-order polynomial equation was formulated to explain the relationship between parameters (A , B , C , corresponding to the extraction pressure, temperature, and modifier volume) and response variable (recovery, R):

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

where X_i and X_j represent different independent variables. β_0 is the intercept and β_i , β_{ii} and β_{ij} are the linear, quadratic and cross product regression coefficients, respectively [24]. The optimal conditions were defined as those that maximized the recoveries of HM and PM.

Fig. 6. Response surface (a) and contour plots (b) for the effects of modifier volume and temperature on HM recovery while pressure at zero level

Tables 5 and 6 showed the significance of regression coefficients by P -value (terms with $P < 0.001$, $0.001 \leq P < 0.05$ and $P \geq 0.05$ are highly significant, significant and insignificant, respectively) [25]. The models of HM and PM were both highly significant ($P < 0.001$) and statistical equation was suggested for this optimization. Five model terms, C , AB , A^2 , B^2 and C^2 had highly significant effects on the recoveries of HM and PM ($P < 0.001$). A , B , and BC were significant ($P < 0.05$). The interaction parameter AC was not significant ($P > 0.05$). The results revealed that modifier volume had a stronger effect on the extraction rates of the target compounds compared to extraction pressure and temperature.

HM recovery (R) as a dependent variable was shown in Eq. (2):

$$R = 90.58 + 3.11A + 2.93B + 7.11C + 7.76AB + 1.06AC + 4.66BC - 8.99A^2 - 12.14B^2 - 15.17C^2 \quad (2)$$

PM recovery (R) as a dependent variable was shown in Eq. (3):

$$R = 94.86 + 2.81A + 2.18B + 6.41C + 7.46AB + 0.15AC + 4.83BC - 9.91A^2 - 13.94B^2 - 16.10C^2 \quad (3)$$

The regression coefficient R^2 of HM and PM (0.9894 and

Fig. 7. Response surface (a) and contour plots (b) for the effects of modifier volume and temperature on PM recovery while pressure at zero level

0.9924, respectively) and the non-significance of lack of fit ($P > 0.05$) proved high accuracy of the model. The adjusted R^2 and the predicted R^2 for HM were 0.9798 and 0.9368, and that for PM were 0.9856 and 0.9584, which illustrated that the model highly agreed with experimental value. The model exhibited good precision as CV% of 4.22 and 3.67% with adequate precision of 27.986 and 33.688, respectively [26].

Determination of optimum conditions

The optimum SCE conditions were: extraction pressure, 26.3 MPa; extraction temperature, 37.9 °C; modifier volume, 81.0 mL. The CO_2 flow rate and extraction time were set at 20 L/h and 60 min. The model was validated under the optimum conditions. About 2.362 g of SFE extracts were obtained from Chachiensis powder (100 g). The predicted recoveries of HM and PM were 92.37 and 96.07%, and the experimental value were $92.52 \pm 0.83\%$ and $96.36 \pm 0.43\%$ ($n = 3$), respectively. The results indicated the sufficient validity and accuracy of the quadratic model according to Adnan [27].

Three-dimensional response surfaces

Three-dimensional response surfaces were generated to show the mutual effects of three factors on the recoveries of HM and PM (Figs. 2–7). The plots were made by sketching

Fig. 8. Interaction plots of independent variables on HM recovery by RSM

Fig. 9. Interaction plots of independent variables on PM recovery by RSM

Fig. 10. Chromatogram of consecutive HSCCC separation

the recover versus two factors while the other factors at constant levels.

Effect of pressure

When the pressure increased, a bidirectional effect happened. As shown in Figs. 2–4, the HM recovery improved with the pressure growing from 15.0 MPa to 26.2 MPa, but higher pressure caused lower recovery. Similarly, The PM recovery increased with pressure from approximately 15.0–26.8 MPa, which was attributed to the increase in the density of supercritical CO₂ and the solubility of the solute with increasing pressure. However, when the pressure increased over 26.8 MPa, PM recovery began to decrease due to the decrease in the mass transfer speed and diffusion coefficient. Xiaojian Lv et al. reported that the recoveries of HM and PM increased as pressure increased until 28 MPa and then decreased in the extraction of *Citri Reticulate* Pericarpium by UAE [28]. The

effect of pressure on the extraction of the target compounds are similar to the results of this study.

Effect of temperature

Generally, temperature has a dual effect on extraction efficiency. Increasing temperature led to the increase in mass transfer speed and diffusion coefficient, and therefore improved the recovery. But further growing temperature caused the decrease in the density and dissolving power of supercritical CO₂. As shown in Figs. 3–6, HM recovery increased with the temperature within 18–37 °C. However, further growing in temperature caused the recovery to decrease. Temperature had also two opposite effects on the SCE of PM from *Chachensis*. The solubility and recovery gradually enhanced with temperature growing from 18 to 33 °C, and the phenomenon of gradual elevation was no longer exist at higher levels of temperature.

Effect of modifier

As shown in Figs. 1, 2, 5, and 6, the recoveries of HM and PM increased with the increasing ethyl acetate volume due to the increasing polarity of the supercritical solvent. However, the recovery decreased when the volume of ethyl acetate exceeded a certain value as a result of the excessive polarity of the system. Ethyl acetate was employed as the modifier in this study because it was thought to increase the solubility of the solutes by improving the polarity of supercritical CO₂ via hydrogen bonding. The polar modifier molecules compete with the solutes for active binding sites and disrupt the matrix structure, thus accelerating the desorption process [29].

Fig. 11. HPLC chromatograms of various samples: (A) SCE extract of Chachiensis; (B) HSCCC fraction corresponding to HM; (C) HSCCC fraction corresponding to PM

The interaction effects of different factors by RSM were displayed in Figs. 8 and 9. The interaction term between temperature and pressure was highly significant ($P < 0.001$), the interaction term between temperature and modifier was significant ($P < 0.05$), and other terms were insignificant ($P > 0.05$).

HSCCC separation results

Stationary phase retention is an important factor in HSCCC separation because it determines the peak resolution [30]. The flow rate and rotation speed largely affect stationary phase retention. The effects of the flow rate (1.0, 1.5, 2.0, 2.5,

3.0 mL/min) and rotation speed (600, 700, 800, 900, 1,000 rpm) on sample separation were screened by single-factor design. Finally, 2.0 mL/min and 800 rpm were selected. Stationary phase retention of 71.43% indicated that designed conditions were suitable for purification of the target compounds according to Engels [31].

Based on *K* values of HM and PM (0.78 and 1.86, respectively), n-hexane/ethyl acetate/methanol/water (1:0.8:1:1.2, v/v) was chosen as optimal solvent system. As showed by the HSCCC chromatogram in Fig. 10, the shaded parts of peak A and B (corresponding to HM and PM, respectively) were collected for HPLC analysis. Under the optimized conditions, HM (25 mg) and PM (28 mg) were obtained from (200 mg) with purities >98%, respectively (Fig. 11). The recoveries of HM and PM were 2.95 mg/g dry peel and 3.31 mg/g dry peel, respectively. Xiaojian Lv et al. extracted HM and PM from *Citri Reticulate* Pericarpium by SCE without modifier, and the total recoveries was 1.89 mg/g dry peel [28]. Manqin Fu et al. isolated and purified HM and PM by silica gel column, Sephadex LH-20, and ODS column chromatography, the recoveries of HM and PM were 0.006 mg/g dry peel and 0.005 mg/g dry peel, respectively [32]. The column chromatography method requires multiple steps and the sample loss is serious. In this study, ethyl acetate was used as modifier in SCE, which significantly increased the recoveries of the target compounds. Besides, HSCCC allows bidirectional elution, which help to reduce sample adsorption in the system and achieve effective recovery.

Identification of target compounds

Compound (I): 3',4',5,6,7,8-Hexamethoxyflavone (HM), $C_{21}H_{22}O_8$, yellowish-white powder; EI-MS, *m/z* 403.05 $[M+H]^+$. 1H NMR(500 MHz, $CDCl_3$): δ (ppm): 7.40 (1H, d, *J*=2.1 Hz, H-2'), 6.98 (1H, d, *J*=8.5 Hz, H-5'), 7.56 (1H, dd, *J*= 2.1, 8.5 Hz, H-6'), 6.61 (1H, s, H-3), 4.10, 4.06, 3.91, 3.93, 3.92 (6 s, OMe at C-5, -6, -7, -8, -3',-4'); ^{13}C NMR (125 MHz, $CDCl_3$): δ (ppm): 176.36 (s, C-4), 160.03 (s, C-2), 150.88 (s, C-4'), 150.40 (s, C-7), 148.24 (s, C-3'), 147.38 (s, C-9), 146.69 (s, C-8), 143.05 (s, C-5), 136.97 (s, C-6), 122.95 (s, C-1'), 118.60 (d, C-6'), 113.81 (s, C-10), 110.17 (d, C-5'), 107.48 (d, C-2'), 105.83 (d, C-3), 62.10, 61.90, 61.91, 61.50, 55.90, 55.82 (6 q, OMe at C-5, -6, -7, -8, -3',-4'). The NMR signals were in line with the data reported previously in the literatures [32].

Compound (II): 5,6,7,8,4'-Pentamethoxyflavone (PM), $C_{20}H_{20}O_7$, yellow powder; EI-MS, *m/z* 373.05 $[M+H]^+$. 1H NMR (500 MHz, $CDCl_3$): δ (ppm): 7.87 (2H, d, *J* = 8.0 Hz, H-2',6'), 7.02 (2H, d, *J* = 8.0 Hz, H-3',5'), 6.60 (1H, s, H-3), 4.11, 4.03, 3.88 (each 3H, s, OMe), 3.95 (6H, s, 2×OMe); ^{13}C NMR (125 MHz, $CDCl_3$): δ (ppm): 177.38 (C-4), 162.29 (C-4'), 161.20 (C-2), 151.38 (C-7), 148.39 (C-5), 147.74 (C-9), 144.07 (C-6), 138.08 (C-8), 127.72 (C-2', 6'), 123.83 (C-1'), 114.89 (C-10), 114.52 (C-3', 5'), 106.70 (C-3), 62.23, 61.99, 61.78, 61.61, 55.47 (5×OMe). The NMR signals were consistent with the data reported previously in the literatures [5, 33, 34].

CONCLUSION

HM and PM have good bioactivities but are found in low amounts in plants. It is difficult but of great significance to establish a rapid method for isolating them with high yields and high purities. For the first time, a two-step method combining SCE- CO_2 with HSCCC was conducted consecutively to prepare HM and PM from *Chachiensis*. Besides, SCE- CO_2 process was designed, modeling and optimized using RSM-CCD. The quadratic model obtained had good accuracy and effectively represented the experimental data. The SCE- CO_2 could realize the almost complete extraction of HM and PM from *Chachiensis*. Depending on the characteristic of two-way elution, HSCCC could mitigate the irreversible adsorption of samples in the system and reduce the loss in the separation process as much as possible. This sequential method had broad application prospects for purifying active compounds with lower content in natural products and can be applied in pharmaceutical and food industries.

Conflict of interest: The authors declare that they have no conflict of interest.

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