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Accelerated solvent extraction of monacolin K from red yeast rice and purification by high-speed counter-current chromatography

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ABSTRACT

Monacolin K from red yeast rice was extracted by accelerated solvent extraction (ASE). The effects of various extraction parameters including extraction temperature, static extraction time and cycle index on yield were investigated using a DIONEX ASE 300 system to select the optimal conditions by an orthogonal test design L_9 $(3)^3$. The optimum extraction conditions were determined as follows: extraction temperature 120 °C, static extraction time 7 min, and cycle index 3. Under the optimal conditions, the yield of ASE extract and monacolin K was 5.35% and 9.26 mg/g of dry red yeast rice, respectively. A separation and purification method of monacolin K was then established using high-speed counter-current chromatography (HSCCC) with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (8:2:5:5, v/v/v/v). From 300 mg of crude extract, 51.2 mg of monacolin K was obtained with the purity of 98.7%. The chemical structure of isolated compound was identified by UV, ESI-MS and ^{1}H NMR.

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1. Introduction

Red yeast rice is described as a fermented product of rice on which red yeast (*Monascus purpureus*) has been grown, which is a traditional food widely consumed in some Asian countries, especially in China, Japan and Korea. It has attracted attention because of its biologically active metabolite production [1], and its food and medicinal value could date back to more than a thousand years [2]. Red yeast rice is effective in decreasing blood pressure [3], reducing plasma cholesterol levels [4], and existing anti-inflammation activity [5]. The major active compound of red yeast rice is considered to be monacolin K (Fig. 1), which is an important secondary metabolite produced by *M. purpureus* in liquid fermentation [6]. Previous study discovered that monacolin K is a cholesterol-lowering agent and is able to reduce the risk of colon cancer [7]. Monacolin K decreases the amount of cholesterol by inhibition of 3-hydroxy-3-methyl glutaryl-coenzyme A reductase, which is a regulatory and rate-limiting enzyme of cholesterol biosynthesis [8], therefore, it also helps to decrease blood pressure [3,9].

The extraction and purification of monacolin K from red yeast rice by some conventional methods are usually tedious, time consuming, requiring multiple chromatographic steps on silica gel [10,11]. In recent years, new extraction and purification techniques have been introduced in order to reduce the amount of solvent required, reduce operation time, reduce the cost of sample preparation and improve the precision of analysis. Accelerated solvent extraction (ASE) and high-speed counter-current chromatography (HSCCC) are two of these new technologies. The former uses organic solvents at high pressures and temperatures above the boiling point as extraction solvent [12], which requires only small volumes of solvents, meets the requirements of environmentally friendly procedures being also very selective, allows faster extractions than classical methods, and it has been used for extraction of various compounds from natural products [13–15]. The later, a form of liquid–liquid partition chromatography technique that uses no solid support matrix, which eliminates the irreversible adsorptive loss of samples onto the solid support matrix as used in the conventional chromatographic column [16], this method has been successfully applied to separate and purify various natural products [17–20]. However, no report has been published on the use of ASE to extract and HSCCC to separate and purify monacolin K from natural products. We herein report an efficient method for the extraction, separation and purification of monacolin K from red yeast rice.

2. Experimental

2.1. Reagents and materials

Organic solvents including *n*-hexane, ethyl acetate, and methanol were all of analytical grade (Guangcheng Chemical Factory, Tianjin, China). Acetonitrile used for HPLC analysis was

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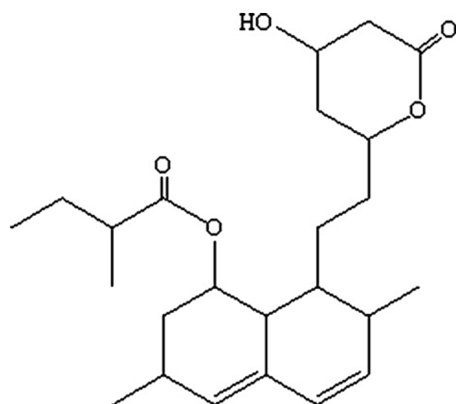

Fig. 1. Chemical structure of monacolin K from red yeast rice.

of chromatographic grade (Yuwang Chemical Factory, Yucheng, China). Reverse osmosis Milli-Q water (Millipore, USA) was used for all solutions and dilutions.

Red yeast rice was purchased from Huzhou Llissy Biology Technology Co., Ltd., Zhejiang, China.

2.2. Apparatus

Accelerated solvent extraction was performed on a DIONEX ASE 300 system (Dionex Corp., Sunnyvale, CA, USA) with 34-mL stainless steel cell.

The preparative HSCCC was carried out using a Model TBE-300A (Tauto Biotech Co. Ltd., Shanghai, China) equipped with three multilayer coil separation column connected in series (1.6 mm i.d., total volume of 260 mL) and a 20-mL sample loop. The revolution radius was 5 cm, and the β values (a geometrical parameter) of the multilayer coil range from 0.5 at internal terminal to 0.8 at the external terminal. The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1000 rpm. A Model HX 1050 constant-temperature circulating implement (Beijing Boyikang Lab Instrument Co., Ltd., Beijing, China) was used to control the separation temperature. A Model TBP5002 constant-flow pump (Tauto Biotech Co. Ltd., Shanghai, China) was used to pump the two-phase solvent system. Continuous monitoring of the effluent was achieved with a Model 8823A-UV Monitor (Beijing Institute of New Technology Application, Beijing, China) at 254 nm. A 3057-11 portable recorder (Yokogawa, Sichuan Instrument Factory, Chongqing, China) was used to draw the chromatogram.

HPLC was carried out on a Waters Empower system (Milford, MA, USA) including a model 600 pump, a model 600 system controller, a model 600 multisolvent delivery system, a model 996 diode-array detector (DAD), a sample injector with a 20-μl loop and an Empower workstation.

2.3. Optimization of ASE conditions

In order to determine the suitable extraction conditions in a wide range with a minimum number of trials by ASE, an orthogonal test design L_9 $(3)^3$ was employed where extraction temperature, static extraction time and cycle index were considered to be three major factors for effective extraction. Combinations of the three different levels of each factor were listed in Table 1. The instrument settings were as follows: 1500 psi, pre-heat 1 min, purge 60 s and flush volume 60%. In each test, 10.0 g of red yeast rice power was placed into the 34-mL stainless steel cell and extracted using ethyl acetate as extracting solvent. The crude extract was analyzed and the content of the target compound was determined by HPLC. Then, the extract was collected and evaporated to dryness at 60 °C under reduced pressure, and weighted.

2.4. Preparation of the crude extract

After the ASE conditions were optimized, the extraction was performed using the DIONEX ASE 300 system under the optimal conditions. The crude extract obtained was evaporated to dryness under reduced pressure at 60 °C, and it was used for subsequent HSCCC separation and purification.

2.5. Selection of two-phase solvent system

The partition ratio (K) values of target components were determined according to the literature [21,22] by HPLC analysis as follows: 2 mL of each phase of the equilibrated two-phase solvent system was added to approximately 1 mg of crude extract placed in a 10-mL test tube. Then the test tube was stoppered and shaken vigorously for 1 min to thoroughly equilibrate the sample between the two phases. Equal volumes (200 μl) of the upper and the lower phases were evaporated to dryness separately. The residues were diluted with methanol to 1 mL and analyzed by HPLC. The K value was defined as the peak area of the compound in the upper phase divided by their peak area in the lower phase.

2.6. Preparation of two-phase solvent system and sample solution

The selected two-phase solvent system utilized in the present study was thoroughly equilibrated in a separation funnel by repeated vigorous shaking at room temperature. The two phases were separated and degassed by sonication shortly before use. The sample solution was prepared by dissolving the crude extract in the mixture solution of upper phase and lower phase (1:1, v/v) of the solvent system.

2.7. HSCCC separation procedure

In each HSCCC separation, the multiplayer coiled column was first entirely filled with the upper phase (stationary phase), then the apparatus was rotated at a speed of 850 rpm, while the lower

Table 1
L_9 $(3)^3$ orthogonal test design.

Test no.	A (extraction temperature, °C)		B (static extraction time, min)		C (cycle index)	
1	A_1	80	B_1	5	C_1	1
2	A_1	80	B_2	7	C_2	2
3	A_1	80	B_3	9	C_3	3
4	A_2	100	B_1	5	C_2	2
5	A_2	100	B_2	7	C_3	3
6	A_2	100	B_3	9	C_1	1
7	A_3	120	B_1	5	C_3	3
8	A_3	120	B_2	7	C_1	1
9	A_3	120	B_3	9	C_2	2

Table 2
Analysis of L_9 $(3)^3$ test results.

Test no.	A (1)	B (2)	C (3)	Yield[a] (%)	Yield[b] (mg/g)
1	A_1	B_1	C_1	3.61	7.25
2	A_1	B_2	C_2	3.74	7.81
3	A_1	B_3	C_3	4.01	7.83
4	A_2	B_1	C_2	4.52	8.33
5	A_2	B_2	C_3	4.59	8.47
6	A_2	B_3	C_1	4.48	8.29
7	A_3	B_1	C_3	5.16	9.11
8	A_3	B_2	C_1	4.79	9.08
9	A_3	B_3	C_2	5.27	9.20

[a] Extraction yield (%) = (the amount of extract/sample mass) × 100.
[b] Extraction yield (mg/g) = the amount of monacolin K in extract/sample mass.

phase (mobile phase) was pumped into the head end of the column at a flow rate of 2.0 mL/min. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting from the tail outlet, the sample solution was injected through the injection valve. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm and the chromatogram was recorded. Each peak fraction was collected according to the elution profile and analyzed by HPLC. After completing the separation, retention of the stationary phase was measured by collecting the column contents into a graduated cylinder by forcing them out of the column with pressurized nitrogen gas.

2.8. Analyses and identification of HSCCC fractions

The crude extract and each purified fraction from the preparative HSCCC separation were analyzed by HPLC on a Symmetry Shield™ (Waters, Milford, MA, USA) RP-C_{18} column (250 mm × 4.6 mm, i.d., 5 μm) with the column temperature at 25 °C. The mobile phase, a solution of acetonitrile and 0.1% H_3PO_4 (70:30, v/v), was set at a flow rate of 1.0 mL/min. The effluent was monitored at 200–700 nm. The identification of HSCCC peak fractions was carried out by electrospray ionization mass spectrometry (ESI-MS) on an Agilent 1100/MS-G1946 (Agilent, California, USA) and by ^1H NMR spectra on a Varian-600 NMR spectrometer (Varian, Palo Alto, USA) with chloroform ($CDCl_3$) as solvent.

3. Results and discussion

3.1. Optimization of ASE conditions

According to the properties of monacolin K and consideration of previous studies on extraction [8,23], ethyl acetate was selected as extracting solvent. To obtain an efficient extraction, the extraction conditions of the target compound were optimized by ASE firstly. Since various parameters potentially affect the extraction process, the optimization of the experimental conditions is a critical step in the development of an ASE method. In fact, extraction temperature, static extraction time and cycle index are generally considered as the most important factors of ASE. All selected factors were examined using an orthogonal test design L_9 $(3)^3$. The total evaluation index was used for analysis by statistical method. The results of the orthogonal test and extreme difference analysis are presented in Tables 2 and 3. The analysis of variance was performed by statistical software SPSS 13.0 and the result is listed in Table 4.

As seen from the results of Table 3, we can find that the influence to the mean extraction yield of the compound decreases in the order: A > C > B according to the *R* values. The maximum yield of monacolin K was obtained when extraction temperature, static extraction time and cycle index were $A_3B_2C_3$ (120 °C, 7 min and 3). Table 4 indicates the yield of monacolin K significantly increased as the extraction temperature increased (Fig. 2), static extraction

Table 3
Analysis of L_9 $(3)^3$ test results.

	Monacolin K yield (mg/g)		
	A	B	C
K_1	22.89[a]	24.69	24.62
K_2	25.09	25.36	25.34
K_3	27.39	25.32	25.41
k_1	7.63[b]	8.23	8.21
k_2	8.36	8.45	8.45
k_3	9.13	8.44	8.47
R	1.50[c]	0.22	0.26
Optimal level	A_3	B_2	C_3

[a] $K_i^A = \sum$ extraction yield at A_i.
[b] $k_i^A = K_i^A/3$.
[c] $R_i^A = \max\{k_i^A\} - \min\{k_i^A\}$.

Table 4
Analysis of variance.

Source	Sum of squares	Degree of freedom	Mean square	*F* value	Significance
A	3.376	2	1.688	162.113	0.006 (<0.05)
B	0.094	2	0.047	4.522	0.181 (>0.05)
C	0.127	2	0.064	6.123	0.140 (>0.05)

time and cycle index have less significant influence on the yield of monacolin K. According to the *R* values and the result of analysis of variance, we can find the extraction temperature was the most important determinant of the yield.

Under the optimal extraction conditions of ASE, 535 mg crude extract was obtained from 10.0 g sample. Fig. 3A shows HPLC chromatogram of the ASE extract.

3.2. Selecting the suitable solvent system

Based on the physicochemical properties of monacolin K, a number of two-phase solvent systems composed of *n*-hexane, ethyl acetate, methanol and water were tested by changing the volume ratio of the solvent to obtain the optimum condition that could gave suitable *K* values. The solvent system of *n*-hexane–ethyl acetate–methanol–water was selected because it provides a broad range of hydrophobicity by modifying the volume ratio of the four solvents [16].

Table 5 shows the *K* values of two-phase systems composed of *n*-hexane–ethyl acetate–methanol–water with different volume ratios, the volume ratios of 7:3:5:5, 8:2:5:5 and 5:5:6:4 could be used to separate the crude extract. After trying all the above solvent systems, it was confirmed that *n*-hexane–ethyl acetate–methanol–water (8:2:5:5, v/v/v/v) was best to separate and purify the target compound. Fig. 4 shows the separation of HSCCC using this solvent system.

Table 5
Partition coefficients (*K*) of monacolin K.

Solvent systems	*K*
n-Hexane–ethyl acetate–methanol–water	
5:5:5:5	3.45
6:4:5:5	2.60
7:3:5:5	1.43
8:2:5:5	1.14
5:5:6:4	0.92
5:5:7:3	0.39

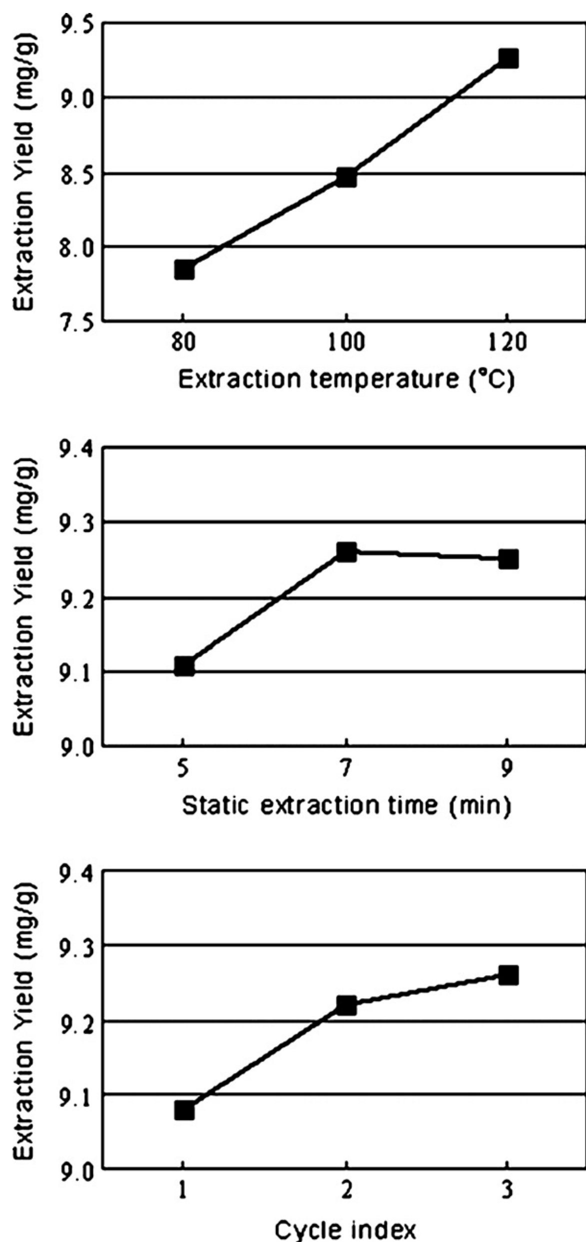

Fig. 2. Effects of extraction temperature, static extraction time and cycle index on yield of monacolin K.

3.3. Purification of monacolin K by HSCCC

The crude extract was purified by preparative HSCCC with *n*-hexane–ethyl acetate–methanol–water (8:2:5:5, v/v/v/v) as the solvent system. The retention of the stationary phase was 65.4%, and the separation time was about 180 min in each separation run. The absorbance was measured at 254 nm to draw an elution curve (Fig. 4) and the HSCCC fractions were analyzed by HPLC. All collected fractions were combined into different pooled fractions. Fig. 3B shows the HPLC analysis of the combined fractions. After only one step of separation by HSCCC, 51.2 mg of monacolin K (98.7% purity) was obtained from 300 mg of ASE extract.

3.4. Identification of monacolin K

Compound I (Fig. 4): Colorless needles, UV (λ_{max}^{MeOH}) absorption: 230, 237 and 246 nm. Positive ESI-MS, *m/z* 405 $[M+H]^+$. ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.99 (1H, d, *J* = 9.6 Hz, H-5), 5.79 (1H, dd, *J* = 7.8, 8.4 Hz, H-6), 5.53 (1H, brs, H-4), 5.39 (1H, brs, H-1), 4.61 (1H, m, H-5′), 4.34 (1H, m, H-3′), 2.72 (1H, dd, *J* = 4.8, 18 Hz, H_{ax}-2′), 2.62 (1H, d, *J* = 18 Hz, H_{eq}-2′), 2.43 (2H, m, H-3), 2.38 (1H, m, H-7), 2.36 (1H, m, H-2″), 2.27 (1H, d, *J* = 12 Hz, H-8a), 1.96 (1H, m, H_{eq}-4′), 1.93 (2H, m, H-2), 1.87 (1H, m, H-6′), 1.70 (1H, m, H-8), 1.65 (1H, m, H_{ex}-4′), 1.63 (1H, m, H-3″), 1.46 (1H, m, H-7′), 1.42 (1H, m, H-3″), 1.38 (1H, m, H-7′), 1.28 (1H, m, H-6′), 1.11 (3H, d, *J* = 6.6 Hz, H-2″-CH_3), 1.08 (3H, d, *J* = 7.8 Hz, H-3-CH_3), 0.88 (3H, d, *J* = 6.6 Hz, H-7-CH_3), 0.87 (3H, t, *J* = 7.8 Hz, H-4″). The spectral data was found to be in accordance with the data reported in reference [24] for monacolin K.

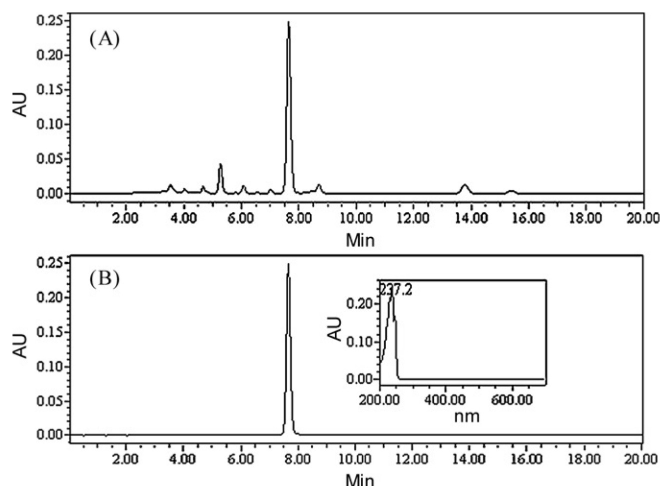

Fig. 3. (A) HPLC chromatogram of the extract from ASE and (B) HPLC chromatogram and UV spectrum of the monacolin K separated and purified by HSCCC. Experimental conditions: a Symmetry Shield^{TM} RP-C_{18} column (250 mm × 4.6 mm, i.d., 5 μm); column temperature: 25 °C; mobile phase: acetonitrile and 0.1% H_3PO_4 (70:30, v/v); flow rate: 1.0 mL/min; detection: 237 nm; injection volume: 5 μl.

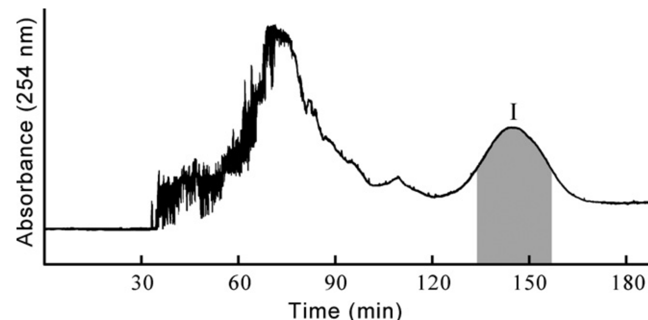

Fig. 4. Chromatogram of the crude extract by preparative HSCCC. Solvent system: *n*-hexane–ethyl acetate–methanol–water (8:2:5:5, v/v/v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 2.0 mL/min; revolution speed: 850 rpm; detection: 254 nm; sample size: 300 mg; retention of stationary phase: 65.4%. Peak I: monacolin K.

4. Conclusion

Monacolin K from the traditional Chinese fermented product of red yeast rice was extracted by the accelerated solvent extraction technique. Under the optimal conditions, an extraction temperature of 120 °C, a static extraction time of 7 min and a cycle index of 3, the yield of monacolin K was 9.26 mg/g of dry red yeast rice. From the crude ASE extract, monacolin K was obtained with 98.7% purity by HSCCC with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (8:2:5:5, v/v/v/v) in one step of separation. The results of the present study demonstrated that the combination of ASE with HSCCC is an efficient method for the extraction, separation and purification of monacolin K from red rice yeast.

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