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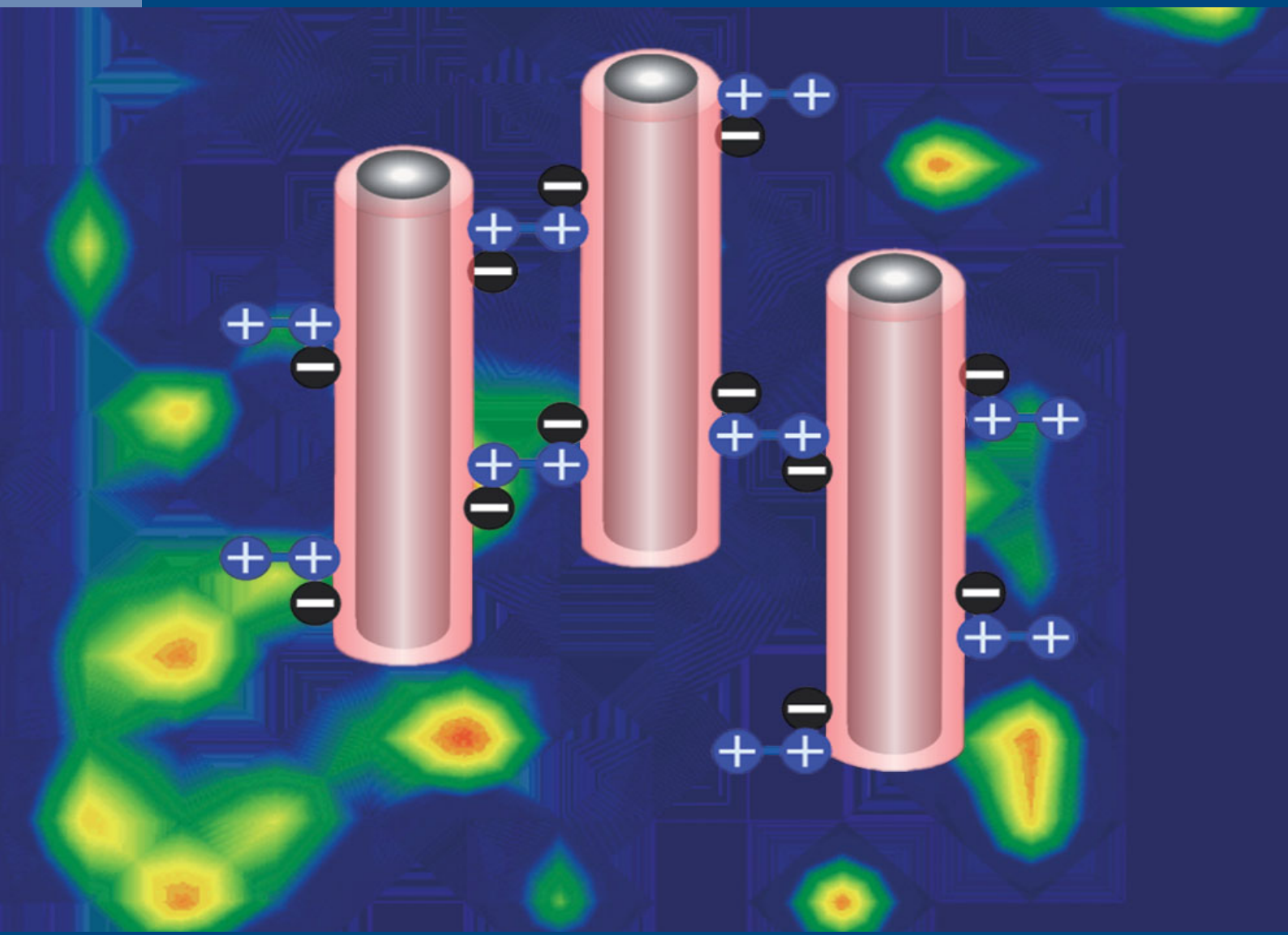

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Research Article

Quick method for separating target compounds from the bark of Maqian (*Zanthoxylum myriacanthum* var. *pubescens*) by high-performance countercurrent chromatography

Choosing a suitable solvent system for a countercurrent chromatography separation presents a challenge for many researchers. In this study, we introduce a quick method of separating a target compound from the bark of *Zanthoxylum myriacanthum* var. *pubescens* by countercurrent chromatography. This method relies on the thin-layer chromatography based generally useful estimation of solvent systems. This paper will present how to quickly choose a suitable solvent system with a thin-layer chromatography based generally useful estimation of solvent systems working chart. *O*-Methyltembamide (**1**) was enriched by countercurrent chromatography using *n*-hexane/ethyl acetate/methanol/water (6:4:6:4) as the solvent system. Further purification was achieved by high-performance liquid chromatography with purities of 98.2% from *Z. myriacanthum* var. *pubescens bark*.

Keywords: High-performance countercurrent chromatography / *O*-Methyltembamide / *Zanthoxylum myriacanthum* var. *pubescens*
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1 Introduction

In the modernization of traditional Chinese medicine, the key issue of Dai medicine research s still to clarify the pharmacodynamic material basis. Study of Dai medicine is seriously lagging behind the traditional Chinese medicine in this regard. The use of Dai prescription is basically limited in the Dai hospital. Therefore, the research of Dai medicine needs to be advanced by modern means. New methods for separating bioactive constituents at trace level must be developed to break through the restriction of the traditional research, and increase the possibility of finding new drug candidates.

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Abbreviations: **"GUESS" method**, the generally useful estimation of solvent systems method; **HEMWat**, hexane/ethyl acetate/methanol/water systems; **HPCCC**, high-performance countercurrent chromatography; **HSCCC**, high-speed countercurrent chromatography

High-speed countercurrent chromatography (HSCCC) is an advanced liquid–liquid chromatographic separation technique. HSCCC has been widely used for the preparative separation of various natural products because of its technical merits, such as shorter separation time, wider range of selection of solvent systems, and quantitative material recovery [1]. Many compounds were separated and purified with one step by HSCCC [2–4].

Choosing a suitable solvent system is a critical operation in CCC. Some CCC practitioners such as Ito have given guidance to beginners by outlining the steps to choosing a solvent system [5]. Another solvent system selection procedure has been described by Pauli: a generally useful estimation of solvent systems (GUESS) method [6]. This method employs TLC to identify a suitable solvent system with minimal labor. Up to now, the descriptions of the TLC-based GUESS method have been rare in the literature. However, an updated look at GUESS method has recently appeared [7].

The aim of this work was to expand on the TLC-based GUESS method by utilizing the TLC-based GUESS working chart, and by applying the method to separating *O*-methyltembamide (**1**) from bark of *Zanthoxylum myriacanthum* var. *pubescens*.

Maqian (*Z. myriacanthum* var. *pubescens* Huang (Huang)) is a valuable traditional Dai herb medicine native to the

southern Yunnan province of China. Dai people use Maqian bark as a home remedy for enteritis, pediatric hepatitis, and colitis. Maqian is used to create a stew flavored sauce for cooking in the Dai people's life. This herb is also used as a Dai herb medicine with detoxification, swelling reduction, and pain relief effects [8]. The fruits of Maqian are widely used as food flavorings in China [9]. Its branch, leaflet, and fruits have a distinctive pepper-like aroma [10]. Studies have shown that roots and stem bark of this plant genus are rich in triterpenoids, lignans [11], and alkaloids [12,13]. Extracts of leaves, stems, and bark have antitumor [14] and anti-inflammatory properties [15,16].

2 Materials and methods

2.1 General experimental procedures

Optical rotations were taken on a Jasco DIP-370 digital polarimeter. 1D-NMR and 2D-NMR spectra were obtained on a Bruker-DRX-600 spectrometer with chemical shifts recorded in δ (ppm) using tetramethylsilane as the internal standard. The coupling constants (*J*) were given in Hertz. Mass spectra were measured on a Waters XEVO-TQD spectrometer or an Agilent G6230 TOF Mass spectrometer. Fractions were monitored by TLC. Spots were visualized by heating silica gel plates sprayed with 10% aqueous H_2SO_4.

2.2 Plant material

The bark of Maqian (*Z. myriacanthum* var. *pubescens*) was collected from Mengwang township, Jinghong municipality, Xishuangbanna Dai Autonomous Prefecture, China in August 2014, and identified by Mrs. Chun-fen Xiao from Xishuangbanna Tropical Botanical Garden, Chinese Academy of Science. A voucher specimen (no.152673) for Maqian was deposited in the herbarium (HITBC).

2.3 Extraction and isolation

The samples were air dried and powdered by a laboratory mill. The powdered Maqian bark (18.0 kg) was extracted three times with 90% methanol in a hot water bath to produce the crude extract (2499 g). The water suspension of the condensed crude extract was successively extracted with petroleum ether, ethyl acetate and *n*-butanol for four to five times with each solvent. Following the solvent removal, the petroleum ether (15 g), ethyl acetate (473 g), and *n*-butanol (975 g) extracts were obtained. The ethyl acetate extract residue was loaded onto silica gel (200–300 mesh) and eluted with a chloroform (C)/methanol (M) gradient (100% C – 1:1) to produce seven fractions (1–7) based on TLC analysis. The sample (600 mg) from Fraction 2 (65 g) was prepared for high-performance countercurrent chromatography (HPCCC) work. Two compounds were both enriched by CCC. Compound **1** (67.5 mg) was separated by HPLC with purities 98.2% and identified by ESI-HRMS and NMR.

2.4 TLC-based GUESS method

The method represents a rapid and practical method to link CS *K* values and TLC retention factors (Rf values). A hexane/ethyl acetate/methanol/water system (HEMWat) was chosen. Based on previous studies, compounds with Rf values between 0.29 and 0.71 (optimal 0.5) will have *K* values between 0.4 and 2.5 (optimal = 1) and be eluted in the sweet spot of an HSCCC run [17]. So the first step, the target compound is chosen. Then the TLC-based GUESS method working chart (Supporting Information Fig. S1) is followed and the sample developed on silica gel TLC plates.

2.5 CCC separation procedure

The CCC was performed on a Spectrum HPCCC instrument (Dynamic Extractions, Berkshire, UK), which is connected to a quaternary gradient pump (KNAUER, Berlin, Germany). The fraction collector was a Shanghaihuxi automatic sampling instrument BS-100N (Shanghai, China). The isolation was carried out in RP mode using hexane/ethyl acetate/methanol/water (6:4:6:4) at 38°C. The semipreparative columns (total 300 mL) were filled with the upper phase as the stationary phase. The mobile phase (lower phase) was pumped in to the system using a flow rate of 15 mL/min while the bobbins were rotating at 1600 rpm until the instrument was equilibrated in 5 min. Six hundred milligrams of the sample (dissolved in 6 mL of upper phase/lower phase, 1:1, v/v) was injected after filtration. The elution run time was 20 min and the extrusion time was 25 min at the same flow rate. The fractions were collected per minute. Fractions were analyzed by TLC and similar fractions were combined. The solvent was evaporated with an EYELA N-1100 Series evaporator (Shanghai, China) to obtain two compounds.

2.6 HPLC conditions

A semipreparative Waters HPLC C_{18} column (10 μm, 10 × 250 mm) with an Extend C_{18}-guard column was used. The separation was achieved by using gradient elution and the mobile phase consisting of A (MeCN) and B (water) at a flow rate of 1 mL/min. The gradient was used as follows: 0–25 min (15–95% A), 25–35 min (95% A), 35–35.05 min (95–15% A), 35.05–45 min (15% A). The injection volume was 200 μL and the UV detector. The column temperature was set at 25°C. The purified compounds were identified by ESI-HRMS and NMR.

3 Results and discussion

The choice of an appropriate solvent system for CCC is a critical step in the purification of natural products. TLC-based GUESS method exhibited high efficiency to choose a suitable solvent system for CCC separation. A TLC-based "GUESS" method working chart (Supporting Information Fig. S1) was built for the first time. The working chart may be very useful for the isolation of phytomedicines and cosmeceuticals.

3.1 Proceeding TLC-based GUESS method working chart

3.1.1 Choosing suitable solvent system family for target compound

Generally, there are three useful solvent system families for laboratory technician. The laboratory technician can choose solvent system family according the polarity of the target compound. The three CCC solvent system families: hexane/ethyl acetate/methanol/water, chloroform/methanol/water, and ethyl acetate/butyl alcohol/water are recommended as the HEMWat, chloroform/methanol/water systems (ChMWat), and ethyl acetate/*n*-butyl alcohol/water systems (EBuWat) methods of solvent system selection. The HEMWat method was designed to provide a systematic process of choosing a CCC solvent system for separating a wide range of organic compounds of low and medium polarity. The ChMWat method was designed for the separation a wide range of organic compounds of medium and high polarity. The EBuWat method was designed for the separation a wide range of organic compounds of high polarity [6]. As the sample for HPCCC work was prepared from ethyl acetate extract, the HEMWat method was chosen for the work.

3.1.2 Making target compound near Rf value near 0.5

Confirm target compound on silica gel TLC plates. According the Table 1 of "GUESS" method, modify the target compound Rf value near 0.5 (Supporting Information Fig. S2).

3.1.3 Optimizing solvent systems

The solvent system of *n*-hexane/ethyl acetate/methanol/water (6:4:6:4) for CCC was chosen according the Table 1 of "GUESS" method [6]. First, one mixture of TLC solvents with *n*-hexane/EtOAc (5:5) was tested, the Rf value of target compound was above 0.5. Another mixture TLC solvents with *n*-hexane/EtOAc (6:4) to make Rf value of target compound near 0.5. Second, different ratios of two solvent systems including *n*-hexane/ethyl acetate/methanol/water (6:4:5:5) and *n*-hexane/ethyl acetate/methanol/water (6:4:6:4) in the Table 1 were tested. The upper phase of both of solvent systems could make the Rf value of the target compound near 0.5. Generally, there are two ways to select the optimized solvent system. One way is using the CCC to compare the performance of the two solvent systems; another way is to test the settling times of the two solvent systems. Finally, *n*-hexane/ethyl acetate/methanol/water (6:4:6:4) was chosen for CCC by comparing settling times of two solvent systems.

Table 1. Equivalence of HEMWat and SSE solvent systems

HEMWat	nHex	EtOAc	MeOH	Water	SSE	nHex	EtOAc
−7	9	1	9	1	1	9	1
−6	8	2	8	2	2	8	2
−5	7	3	7	3	3	7	3
−4	7	3	6	4	3	7	3
−3	6	4	6	4	4	6	4
−2	7	3	5	5	3	7	3
−1		4	5	5	4	6	4
0	5	5	5	5	5	5	5
+1	4	6	5	5	6	4	6
+2	3	7	5	5	7	3	7
+3	4	6	4	6	6	4	6
+4	3	7	4	6	7	3	7
+5	3	7	3	7	7	3	7
+6	2	8	2	8	8	2	8
+7	1	9	1	9	9	1	9
+8	0	10	0	10	10	0	10

3.2 Enrichment of target compound by HPCCC

According the TLC-based "GUESS" method and previous studies, compounds with Rf values between 0.29 and 0.71 (optimal 0.5) will have *K* values between 0.4 and 2.5 (optimal = 1) and be eluted in the sweet spot of an HSCCC run. With the chosen ratios of solvents, the target compound would come out after one column volume elution, as its *K*-value may be close to one (Supporting Information Figs. S3 and S4) [18]. The target compound and another compound were detected at Fraction 25 from CCC where the elution volume equals to one column volume.

3.3 Purification of the target compound

O-Methyltembamide (**1**) was purified by HPLC (Supporting Information Fig. S5) with purities 98.2% and identified by ESI-HRMS and NMR spectroscopy. It was obtained as 67.5 mg of white amorphous powder. The molecular formula was determined to be $C_{17}H_{19}NO_3$ on the basis of the $[M+Na]^+$ peak in the HR-ESI-MS spectrum at *m/z* 308.1258. The base peak in the EI-MS spectra corresponds to a $C_9H_{11}O_2^+$ fragment that is characteristic of this structure. An incomplete ^1H NMR spectrum was published in 1973 [19]. The ^{13}C NMR spectrum is in agreement with the one reported in the literature (Table 2) [20].

Table 2. ^{1}H and ^{13}C NMR spectral data of compounds **1** (600 MHz, in MD_3OD, δ values)

	1	
	$^{1}H_a$	^{13}C
1	—	132.78
2, 6	7.286, d, $J = 8.4$	129.34
3, 5	6.930, d, $J = 8.4$	115.13
4	—	161.25
7	4.394, dd, $J = 6.0, 5.9$	83.06
8	3.500–3.600, m	47.48
9	6.65, s	—
10	—	170.47
1′	—	135.85
2′, 6′	7.775, dd, $J = 7.2, 0.6$	128.42
3′, 5′	7.439, dd, $J = 7.8, 7.8$	129.67
4′	7.516, dd, $J = 7.2, 7.2$	132.91
4-OMe	3.785, s	55.85
7-OMe	3.222, s	57.09

a) *J* (Hz).
d, doublet; m, multiplet; s, singlet.

4 Concluding remarks

The TLC-based GUESS method was employed to choose a suitable solvent system for CCC separation of *O*-methyltembamide from the ethyl acetate extract of Maqian bark. In this case, the target compound was identified in the TLC of a complex fraction before enrichment by CCC and purification by HPLC. The only biological activity of *O*-methyltembamide that has been studied to date is the in vivo antispasmotic activity [19]. Therefore, more tests need to be undertaken to discover its potential bioactivity.

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The authors have declared no conflict of interest.

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