Rapid and efficient purification of chrysophanol in *Rheum Palmatum LINN* by supercritical fluid extraction coupled with preparative liquid chromatography in tandem

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ABSTRACT

Chrysophanol has high pharmaceutical values. However, it was difficult to use the traditional extraction method to extract high-concentration chrysophanol. Therefore, the purpose of this study is to purify and separate chrysophanol in traditional herb, *Rheum Palmatum LINN*, by using supercritical fluid extraction (SFE) and preparative high-performance liquid chromatography (P-HPLC) for rapid and large-scale isolation. The method is efficient for selective extraction of chrysophanol from the herbs, which have complex compositions. The extraction efficiency of chrysophanol with SFE is $25\times$ higher than that of boiled water extraction under the same extraction time. The optimal conditions for SFE were 210 atm and 85 °C for 30 min; for P-HPLC, a C18 column was used with a gradient elution of methanol and 1% acetic acid at a flow rate of 10 mL/min. According to ^{1}H NMR and LC–MS analyses, the purity of the isolated chrysophanol was as high as 99%. The recovery for chrysophanol in *Rheum* after SPE/PHPLC processing was in the range of 88–91.5%. Compared with other extraction and purification methods, the sequential system (SFE/P-HPLC) achieved the highest amount of extracted chrysophanol from *Rheum Palmatum LINN* (0.38 mg/g) and the shortest run time (3 h). Hence, this rapid and environmentally friendly method can separate compounds based on polarity with high efficiencies and, coupled with P-HPLC, it may be applicable in the large-scale production of foods and medicines in the future.

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1. Introduction

Rheum Palmatum LINN (*Rheum*), a well-known Chinese herbal medicine, has been used for thousands of years in China. It is a traditional purgative containing considerable amounts of hydrophilic glycosides, such as sennoside A, sennoside B, and emodin-6-monoglucoside [1,2]. Anthraquinones of *Rheum* have been used extensively since ancient times because of their therapeutic and biological properties, which include antioxidant [3–5], antifungal [6], antimicrobial [7,8], larvicidal [9], and anticancer activities [10–12]. Chrysophanol (1,8-dihydroxy-3-methylanthracenedione, Fig. 1) is an anthraquinone and has been identified as a metabolite responsible for antimicrobial activity, shortening of blood coagulation time, and anticancer action [13–16]. It is usually detected in the root of the plant *Rheum*. Anthraquinones are often used as a quality criterion of drug efficacy for medicinal herbs; therefore, numerous extraction and purification methods have been established for the isolation of anthraquinone derivatives, including aqueous methanol or acetonitrile extraction, high-speed countercurrent chromatography (HSCCC), and electrophoresis chromatography [17,18]. MS and UV have been used for the detection and quantification of anthraquinone derivatives [19,20]. Most studies on *Rheum* have focused on its hydrophilic components [21], whereas few studies have focused on its hydrophobic constituents. Hydrophilic glycosides are traditionally extracted with hot water or alcohol [22]. However, traditional extraction methods, such as stewing in hot water or alcohol immersion, cannot be used for efficient extraction of hydrophobic compounds (i.e., chrysophanol).

Supercritical carbon dioxide extraction is a promising technology that is a potentially more efficient alternative to conventional solvent extraction for bioactive components, such as for food and pharmaceutical applications, because it provides higher selectivity, shorter extraction times, and does not use toxic organic solvents. In the SFE process, by varying either pressure or temperature,

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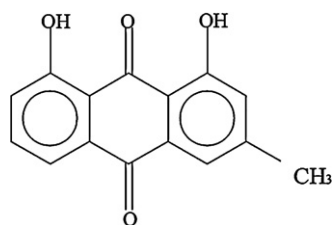
Fig. 1. Chrysophanol (1,8-dihydroxy-3-methylanthracenedione) structure, ($C_{15}H_{10}O_4$), molecular weight: 254.23.

supercritical fluid densities can be altered. In addition, extraction time and CO_2 flow rate can affect the efficiency of Sc-CO_2 extraction. Thus, a substance can be extracted selectively at a specific range of densities. SFE can also proceed at low temperatures to prevent the decomposition of thermally sensitive compounds [23–25]. In addition, Sc-CO_2 exhibits strong solvating power of a liquid and the fast exchange kinetics of a gas. When extraction is complete, liquid carbon dioxide can be removed by reducing the pressure and allowing the gas to evaporate. Carbon dioxide is chemically inert and is the solvent of choice for SFE in the manufacture of natural products, foods, flavoring, and medicine [26–30]. Therefore, in this study, we developed a system that combines supercritical CO_2 extraction (SFE) and preparative high-performance liquid chromatography (P-HPLC) in tandem to purify chrysophanol in large quantities and in a short period.

2. Experimental

2.1. Materials

The plant material of *Rheum Palmatum LINN* was collected from Sichuan, located in southwest China. Chrysophanol and the internal standard 1,8-dihydroxyanthraquinone were purchased from Sigma–Aldrich, USA. A known mass of solute was dissolved in ethanol to prepare different concentrations in 2, 5, 10, 15, 20, and 25 mg/L for the calibration curve. All chemicals and solvents were of analytical and HPLC grades (Sigma–Aldrich, USA).

2.2. Apparatus

The SPE/P-HPLC system was developed for the rapid and large-scale isolation of chrysophanol from the herbs. A schematic diagram of the tandem system is shown in Fig. 2. Supercritical fluid extraction was performed on an ISCO syringe pump (model
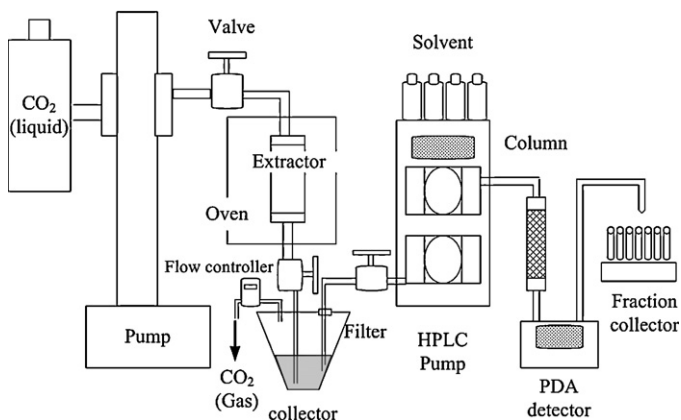
260D, Isco, Lincoln, NE, USA) equipped with a 50-mL stainless steel extraction vessel with two 0.45-μm filters, each positioned at the entrance and exit of the vessel. At the exit of the extraction vessel, 30 cm of stainless steel tubing (316 SS, 1/16-in. OD, 0.030-in. ID) was used as the pressure restrictor for the exit of CO_2. The entire extraction vessel and tubing were placed in a thermostatic oven. The extracts were extracted by supercritical CO_2 under proper conditions, and the released CO_2 was passed through a solvent to trap the target components in a 100-mL funnel-shaped collector with a stir bar at the bottom. The collector was connected with 2-mL sample loops of P-HPLC and was performed using a Prep-Star SD-1 pump linked to a Pro-Star 335 diode-array spectrophotometer (Hewlett-Packard, USA). Analytes were separated on a preparative C18 column (250 mm × 19 mm ID with a particle size of 5 μm, Waters, USA).

2.3. Procedure for the SPE/P-HPLC

All herbal samples were sun-dried, ground, and sieved, which resulted in a powder with particle sizes between 0.3 and 0.85 mm. A 5-g sample was placed in a stainless steel extraction vessel (50 mL) and was then extracted using the SFE procedure with Sc-CO_2. Optimal extraction conditions were achieved by varying the experimental parameters sequentially. The static extraction time, extraction pressure, and temperature were considered. The extraction conditions were altered as follows: temperatures of 40, 50, 60, 70, and 80 °C; pressures of 90, 120, 150, 180, 210, and 240 atm; and extraction times of 5, 10, 20, 30, 40, 50 and 60 min. After the appropriate extraction conditions were determined, the extracts were collected in 25 mL of ethanol at a CO_2 flow rate of 50 mL/L. Subsequently, 5 mL of the extractant in ethanol was injected into preparative P-HPLC for the separation and purification of chrysophanol from the hydrophobic fraction. A preparative C18 column was used under a gradient elution identical to the gradient used with the analytical column (Pursuit[TM] 250 mm × 4.6 mm ID with a particle size of 5 μm). The mobile phase was methanol and acetic acid (1%) in gradient mode as follows: 0–3 min: 50% methanol; 3–5 min: 50–10% methanol; 5–35 min: 10–85% methanol; and 35–40 min: 85% methanol. The flow rates through the analytical column and the preparative column were 1 mL/min and 10 mL/min, respectively.

2.4. Recovery

For percent-recovery experiments, 1 g samples of homogenized *Rheum* powder were spiked with 0.5, 1, and 2 mg of the chrysophanol standard reagent. These spiked samples were applied on the SFE/p-HPLC system, as described in Section 2.3. To calculate the recovery, the 1 g of *Rheum* was used as a blank sample. The recovery was calculated as follows:

$$\text{Recovery}(\%) = \frac{\text{chrysophanol in spiked sample} - \text{blank sample}}{\text{Amount of added chrysophanol}}$$

2.5. Characterization of chrysophanol

Chrysophanol identification was achieved by comparing the retention time and the wavelength of UV at 254 nm. Chrysophanol isolated from *Rheum* was identified by ^{1}H NMR and ESI-MS (Instrumentation Center, National Tsing Hua University).

3. Results and discussion

The experiments were performed to develop an SFE/P-HPLC tandem process for rapid obtainment of chrysophanol from *Rheum Palmatum LINN* and with a large yield. To determine the optimum

Fig. 2. Schematic diagram of the tandem system of supercritical fluid extraction and P-HPLC.
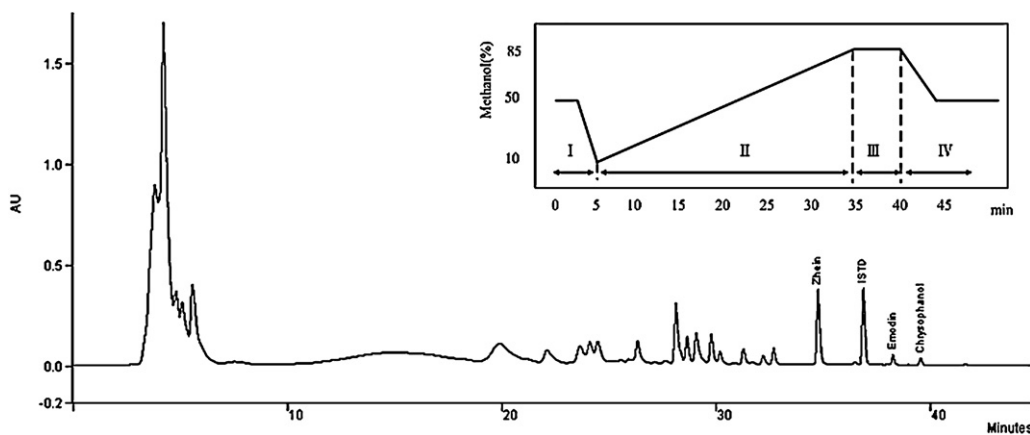
Fig. 3. Chromatogram of *Rheum* extracted by water. The last four compounds are rhein, ISTD, emodin and chrysophanol (ISTD refers to the internal standard, 1,8-dihydroxyanthraquinone). The inset figure shows the gradient programming of the mobile phase. The mobile phases were composed of methanol (solvent A) and 1% acetic acid (solvent B) and were eluted in gradient mode.

conditions for chrysophanol extraction from *Rheum*, several parameters were investigated (i.e., extraction temperature, pressure, and time).

### 3.1. Validation of HPLC conditions

Chinese herbal extracts contain numerous constituents; therefore, in this study, elution modes were employed to analyze crude extracts from *Rheum Palmatum LINN* by HPLC. A C18 column was used to analyze anthraquinone derivatives, which are weakly polar. Thus, *Rheum* analysis was achieved with a mobile phase composed of methanol and 1% acetic acid in a 50/50 ratio (v/v) for the first 3 min to elute polar compounds. Subsequently, the mobile phase was in gradient mode from 10% to 85% methanol for 35 min, with an increasing rate of 2.5%/min. As shown in Fig. 3, most compounds appeared in the high-polarity region, whereas a few others were found in regions of low and middle polarity. Chrysophanol, the analyte, was observed in the region of low polarity, and its retention time was 39.3 min. This analysis displayed the polarity distribution of the compounds in *Rheum* and isolated several components successfully, including *rhein*, *emodin*, and *chrysophanol*.

3.2. Comparison of supercritical and water extraction methods

Chrysophanol in *Rheum* is difficult to extract using traditional methodologies. As shown in Fig. 4, boiling-water extraction can be used to extract the majority of hydrophilic compounds in *Rheum*, but few hydrophobic substances such as chrysophanol are also obtained using this method. Fig. 4(b) shows the chromatogram for the extraction of 1 g of *Rheum* by boiling water for 20 min at 100 °C. Supercritical CO_2, however, can be used to extract the majority of hydrophobic substances, chrysophanol, without any hydrophobic compounds in *Rheum*. Fig. 4(c) shows the chromatogram for the extraction of 1 g of *Rheum* for 20 min at 60 °C and 150 atm with supercritical CO_2. In addition, the chrysophanol extraction efficiency of Sc-CO_2 is 25× higher than that of boiling water for the same extraction time. The results indicate that the selectivity of supercritical CO_2 extraction can enhance the purification and isolation of chrysophanol.

3.3. Optimization of supercritical CO_2 extraction conditions

Preliminary experiments were performed to determine the efficiency of the Sc-CO_2 for extraction purposes. The optimized procedure yielded the results shown in Fig. 5. The temperature within
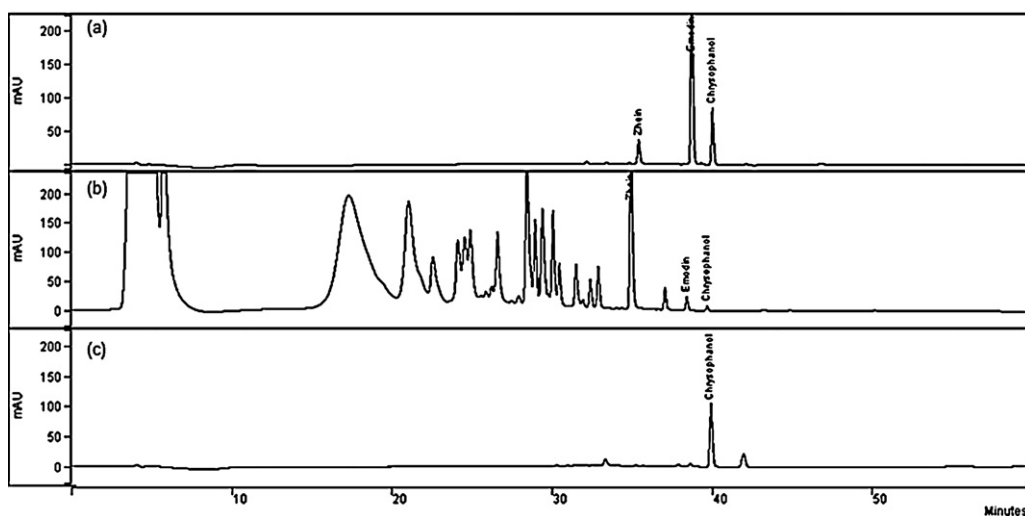
Fig. 4. Chromatogram of (a) 100 mg/L of standard rhein, emodin and chrysophanol, (b) water extracts of *Rheum* after 20 min at 100 °C and (c) supercritical-CO_2 extracts of *Rheum* after 20 min at 50 °C and 150 atm.
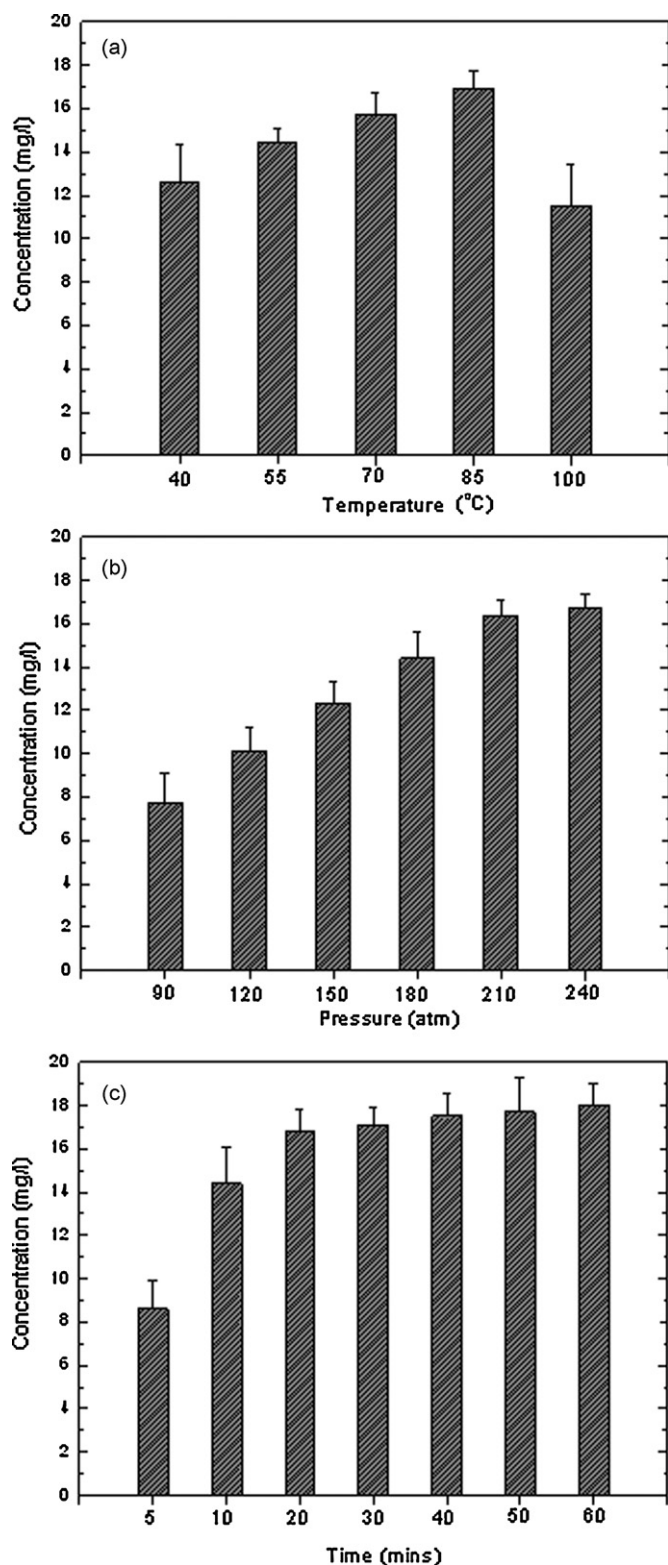
Fig. 5. Extraction efficiency of chrysophanol in supercritical CO$_2$ at (a) different extraction pressures, (b) different extraction temperatures and (c) different extraction times. Data are means from triplicate experiments ± SD.

the vessel was set at 40, 55, 70, 85, and 100 °C to observe the impact of temperature on the extraction efficiency at a constant pressure of 210 atm for 1 h. At this pressure, a higher temperature leads to a smaller supercritical fluid density, and the density of supercritical CO$_2$ is 840, 745, 630, 550, and 490 g/L, respectively [31]. Because the

density of Sc-CO$_2$ decreases with temperature, solubility tends to decrease with temperature as well. However, an increase in temperature may raise the solute vapor pressure, thereby increasing the concentration of the solute in supercritical CO$_2$. Therefore, in this case, an increase in solute concentration has a more significant effect on extraction efficiency than a decrease in solvent density. As shown in Fig. 5(a), a higher temperature leads to a larger amount of chrysophanol extracted from the mixture. However, when the extraction temperature was set above 85 °C, the extraction rate dropped significantly because of a decrease in the density of the supercritical CO$_2$. To investigate the effect of pressure on extraction efficiency, the pressure in the tank was adjusted to 90, 120, 150, 180, 210, and 240 atm at 85 °C for 1 h. The effect of pressure on the extraction efficiency is shown in Fig. 5(b). The results indicate that a higher pressure tended to extract more chrysophanol and that the extraction rate was smoother when the pressure was greater than 210 atm. At the optimum extraction temperature (85 °C) and pressure (210 atm), different extraction times were considered, as shown in Fig. 5(c). The results show that when extraction time exceeded 30 min, the extraction rate became smoother. Thus, in this extraction experiment, the optimum extraction condition was 30 min, 85 °C, and 210 atm.

3.4. Recovery of chrysophanol in SFE/P-HPLC system

Under the optimum extraction condition, recoveries were obtained by triplicating the sample at 3 concentration levels for chrysophanol. According to the results, the recoveries for chrysophanol in *Rheum* after sample processing were in the range of 88–91.5%, as shown in Table 2. The high-percent recovery of chrysophanol is attributed to the high permeability of supercritical CO$_2$. Supercritical fluids do not exhibit surface tension, and chrysophanol can therefore be extracted easily inside the *Rheum*.

3.5. Identification and purity determination

After supercritical CO$_2$ extraction, preparative HPLC was used to separate and purify chrysophanol from the hydrophobic fraction. A preparative C18 column was used under a gradient elution identical to the gradient used with the analytical column. The purity of chrysophanol isolated from *Rheum* is shown in Fig. 6(a); the chrysophanol was identified and its purity was determined using ^{1}H NMR and ESI-MS (Finnigan LCQ).

The ^{1}H NMR data of chrysophanol are as follows: ^{1}H NMR (400 MHz, CDCl$_3$): 12.14 (1H, s, C1–OH), 12.03 (1H, s, C8–OH), 7.80 (1H, d, J = 7.2 Hz, C5–H), 7.67–7.63 (2H, m, C6–H, C4–H), 7.27 (1H, d, J = 8.4 Hz, C7–H), 7.08 (1H, s, C2–H), 2.44 (3H, s, –CH$_3$). The ^{1}H NMR data were compared to those found in the literature [32], and the results indicated that chrysophanol had been isolated.

The ESI-MS spectrum of chrysophanol (shown in Fig. 6(b)) exhibits one peak at 252.9, which indicates [M–H]$^-$. The electronic delocalization of anthraquinone can stabilize anionic species (the phenolate) under electrospray ionization; thus, a negative-ion scan mode was used for chrysophanol identification. According to the results of the ^{1}H NMR and ESI-MS analyses, the structure of chrysophanol did not change during the extraction process, and the purity of the obtained analyte was greater than 99%.

3.6. Comparison of different extraction and purification methods

The study results were compared with published research results correlated with the amounts of extraction and purification of chrysophanol, and the published results include those obtained by using direct solvent extraction/HSCCC, reflux extraction/packing column and SFE/P-HPLC, as shown in Table 1 [18,33]. The solvent
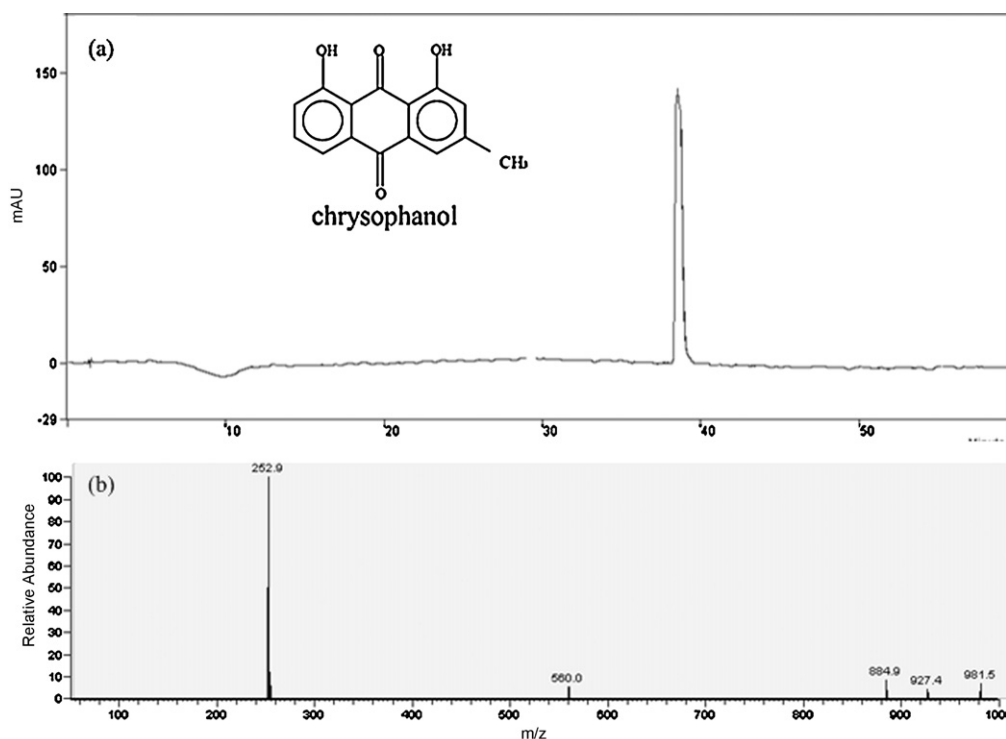
Fig. 6. (a) The purity of chrysophanol isolated from Rheum. (b) Mass spectrum of chrysophanol analyzed with ESI-MS (Finnigan LCQ).

Table 1
Recovery of chrysophanol using SFE/P-HPLC.

| Compound	Amount of chrysophanol present in Rheum (mg/g)	Amount of standard added (mg)	Amount of chrysophanol found in spiked sample (mg)	Recovery (%)
	0.38	0.5	0.82 ± 0.04	88
Chrysophanol	0.38	1	1.34 ± 0.03	96
	0.38	2	2.21 ± 0.06	91.50

Table 2
Comparison of isolating efficiency of chrysophanol using various extraction and purification methods.

Name of herbs	Extraction and purification method	Separation procedure	Extraction rate of chrysophanol from crude sample	Content ratio of chrysophanol in crude extracts	Total time of isolating chrysophanol	
Rheum officinale Baill.	Solvent Extraction	50 g crude sample was extracted by H_2SO_4/benzene (v/v = 1:5), 5% NaOH and aether	0.20 mg/g	8.4%	28 h	[a]
	HSCCC	HSCCC was performed as follows: aether was used as stationary phase, and 1% NaH_2PO_4 and 1% NaOH as mobile phase in gradient elution mode				
Rheum Palmatum LINN.	Refluxing extraction	200 g crude sample was extracted by hot chloroform; undesired compounds were removed by soda solution	0.26 mg/g	20%	120 h	[b]
	Packing column	Chrysophanol was separated by silica-gel packing column and crystallized in ethanol				
Rheum Palmatum LINN.	Supercritical fluid extraction	5 g crude sample was extracted by supercritical CO_2 at 210 atm at 85 °C	0.38 mg/g	77%	3 h	[c]
	Preparative-HPLC	Chrysophanol was separated by preparative C18 column				

[a] Renmin Liu, Journal of Chromatography A, 2004.
[b] Huawei Liao, China Pharmacy, 2006.
[c] This study.
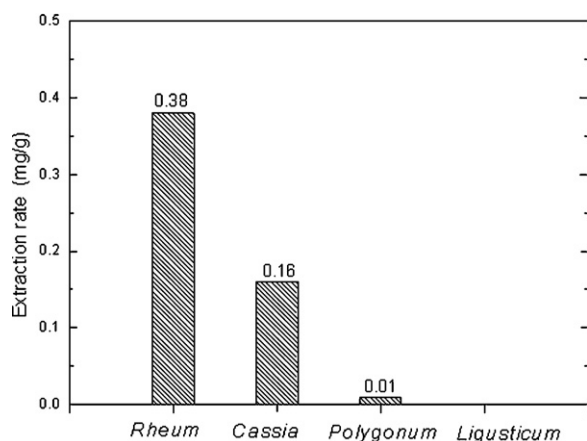
Fig. 7. Traditional Chinese herbal medicines that contain chrysophanol, such as *Rheum*, *Cassia*, *Polygonum* and *Ligusticum*. Data are means from triplicate experiments.

extraction/HSCCC method for preparative separation and purification of chrysophanol from the *Rheum* officinale Baill was developed using pH-gradient elution. The powder (50 g) was extracted with several solvents, and the sample solution was subsequently performed by the HSCCC system in gradient elution mode. The reflux extraction/packing column method for preparative separation and purification of chrysophanol from the *Rheum Palmatum Linn* was developed using solvent extraction and column chromatography. The 200-g crude sample was extracted by hot chloroform, and other undesired compounds were then removed by soda solution before separation of physcion and chrysophanol by using silica-gel packing column. Finally, the pure chrysophanol was obtained by several crystallizations in ethanol. Compared with other methods, the highest yield of chrysophanol (i.e., 0.38 mg/g) was achieved using the SFE/preparative-HPLC method developed in this study, followed by the reflux extraction/packing column (0.26 mg/g) and the solvent extraction/HSCCC (0.2 mg/g). The highest yield of chrysophanol obtained using the SFE/preparative-HPLC method was a result of the hydrophobic selectivity of Sc-CO$_2$ and the sample weights in different orders of magnitude for the three methods. The hydrophobic selectivity of Sc-CO$_2$ extraction resulted in the highest content of chrysophanol in crude extracts (77%), compared to other extraction methods. The others were only 8.4% chrysophanol (solvent extraction) and 20% chrysophanol (reflux extraction) in the crude extracts. Therefore, the SFE method can preconcentrate the target compound in the crude extracts, which can improve extraction efficiency greatly and shorten extraction time. As shown in Table 2, the sequential tandem SFE/P-HPLC system could massively extract and isolate chrysophanol in a short time. Hence, SFE/P-HPLC is an excellent extraction and purification method for chrysophanol because of its high extraction efficiency, short extraction time, and small volume requirement for an organic solvent.

HPLC, the system can massively extract and isolate chrysophanol in a very short time. Hence, SFE/Preparative HPLC was an excellent extraction and purification method for chrysophanol due to high extraction efficiency, short extraction time and extremely few organic solvent requirement.

3.7. Content of chrysophanol in different herbs

The SFE/P-HPLC system was also tested to isolate chrysophanol in other herbs containing chrysophanol, such as *Cassia tora* L., *Polygoni*, *Polygonum*, and *Ligusticum* [34–36], based on the optimization of SFE. The results are displayed in Fig. 7. A comparison of the herbs showed that *Rheum* contained the highest chrysophanol content

at 0.38 mg/g, whereas *Cassia* contained a chrysophanol content of 0.16 mg/g, and the other herbs contained little to no chrysophanol. This work is a good example of an SFE/P-HPLC system being applicable for the isolation of non-polar compounds and perhaps being more effective than water- and ethanol-based methods.

4. Conclusions

We have succeeded in developing a novel method to separate the bioactive ingredient chrysophanol from *Rheum Palmatum LINN* by using supercritical fluid extraction and P-HPLC in tandem. This route is rapid and efficient; in addition, the yield of chrysophanol is high in comparison with traditional procedures. In the future, we can use this green route to produce chrysophanol in large quantities for the production of safer medicines.

Acknowledgments

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