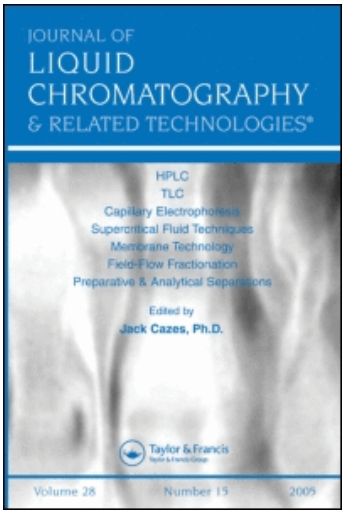

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Abstract: Preparative high speed countercurrent chromatography (HSCCC) has been successfully used for the isolation and purification of isorhamnetin from *Stigma maydis*. This was achieved in two stages: the first separation was performed with a two phase solvent system composed of n-hexane-ethyl acetate-methanol-water (HEMW) at a volume ratio of 5:5:5:5, yielding isorhamnetin at 65.6%, which is followed by the second run using a two phase solvent system composed of HEMW 5:5:6:4, v/v. From 700 mg of the crude extract, 11.8 mg of isorhamnetin was obtained at a high purity of 98%. The final identification was performed by MS, ^{1}H-NMR and ^{13}C-NMR spectra.

Keywords: High speed countercurrent chromatography, Isorhamnetin, Preparative isolation, *Stigma maydis*

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INTRODUCTION

Stigma maydis is the capital of *Zea mays* L., containing saponin, alkaloid, and flavones, which can be used in the treatment of diuresis.[1] Flavones and similar structural compounds have been recently shown to inhibit blood glucose and aspergillus activity.[2]

However, the separation of these active compounds from natural sources using conventional support based chromatography can have the problem of irreversible adsorption to the support. High speed countercurrent chromatography (HSCCC), being a support free liquid-liquid partition chromatographic technique, eliminates such adsorption problems,[3] and has been widely used in preparative separation of natural products.[4–6] Some researches have focused on the separation of flavone from *Stigma maydis* by macroporous resin.[78] The present paper describes the successful preparative separation and purification of Isorhamnetin from the crude extract of *Stigma maydis*, which is achieved by a two stage high speed countercurrent chromatography process with two phase solvent systems composed of n-hexane-ethyl acetate-methanol-water at volume ratios of 5:5:5:5 and 5:5:6:4.

EXPERIMENTAL

Materials

All organic solvents used for HSCCC were of analytical grade and purchased from Beijing Chemical Factory (Beijing, China). Methanol used for HPLC analysis was of chromatographic grade and purchased from Tianjin Huaxi Special Reagent Factory (Tianjin, China).

Stigma maydis was gifted from Enkang Factory (Hebei province, China).

Preparation of Sample

About 100 g of dried *Stigma maydis* were ground, and 7.5 g amount of the dried powder was extracted twice by refluxing for 3 hour with 800 mL of 80% ethanol solution, and then extracted with ethyl acetate, concentrated to dryness under reduced pressure and yielding 3 g of a crude sample that contained isorhamnetin.

Preparation of Two Phase Solvent System and Sample Solutions

The solvent systems utilized in the phase system optimization study were prepared by mixing n-hexane-ethyl acetate-methanol-water (5:5:5:5 or

5:5:6:4, v/v), or ethyl acetate-methanol-water (10:1:10, v/v). The mixtures were equilibrated in a separating funnel at room temperature; the two phases were separated shortly before use.

The sample solutions were prepared by dissolving the crude extract in the lower phase at the appropriate concentrations.

The Choice of Two Phase System

Three two phase systems including ethyl acetate-methanol-water (10:1:10 v/v), n-hexane-ethyl acetate-methanol-water (5:5:6:4, v/v), and n-hexane-ethyl acetate-methanol-water (5:5:5:5, v/v) have been used.

Each HSCCC peak has been determined by HPLC.

Two Stage Preparative HSCCC

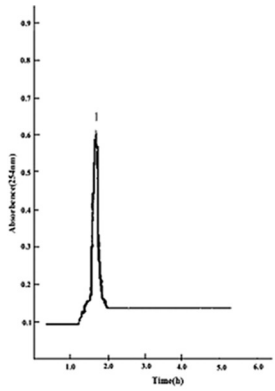
Two stage preparative HSCCC was performed on the Model GS 10AB HSCCC instrument as follows: the multiplayer coiled column was first entirely filled with the upper phase of the selected two phase solvent system composed of n-hexane-ethyl acetate-methanol-water with volume ratios of 5:5:5:5. The lower phase was then pumped into the head end of the inlet column at the selected flow rate, while the apparatus was running at a revolution speed of 800 rpm. After hydrodynamic equilibrium was established, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution at the selected concentration was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm. Each peak fraction was collected according to the chromatogram.

For the second preparative HSCCC stage, the peak 3 from the first stage was concentrated to dryness under reduced pressure, then injected again onto the HSCCC and using the upper phase of n-hexane-ethyl acetate-methanol-water (5:5:6:4, v/v) as the stationary phase and the upper phase as the mobile phase at a flow rate of 2.0 mL/min.

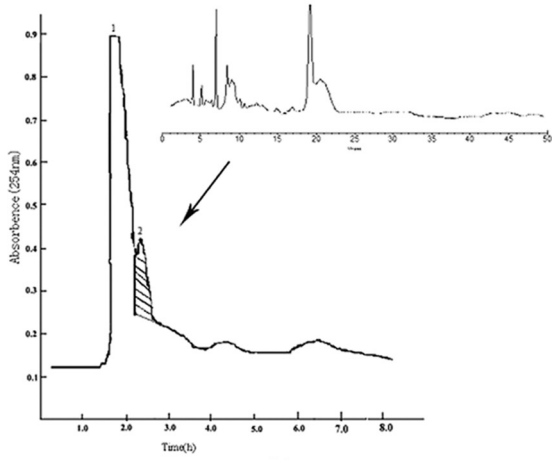
HPLC Analyses and Identification of HSCCC Peak Fractions

The crude extract of *Stigma maydis* and HSCCC peak fractions were each analyzed by HPLC using a YMC-PACK ODS column (150×4.6 mm) at room temperature. The mobile phase, composed of methanol and 0.05% H_3PO_4 aqueous solution (50:50, v/v), was isocratically eluted at a flow rate of 1.0 mL/min and the effluent monitored at 254 nm and 360 nm by a DAD detector.

Identification of the target compound (Isorhamnetin) was based on EI-MS, ^{1}H-NMR, and ^{13}C-NMR spectra.



(a)



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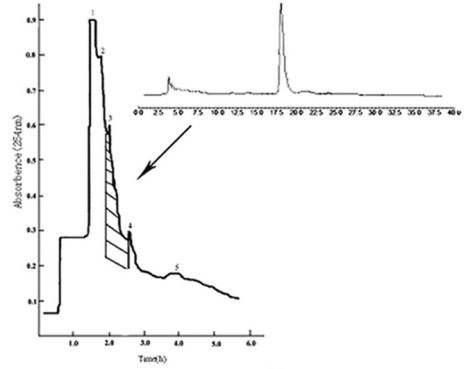

(c)

RESULTS AND DISCUSSION

The chromatogram was obtained by the solvent system ethyl acetate-methanol-water (10:1:10) and is shown in Figure 1a. An impurity peak was eluted at the solvent front while the target compound was retained in the column. Figure 1b was obtained using a less polar solvent system composed of n-hexane-ethyl acetate-methanol-water (5:5:6:4, v/v). Although the main peak contained isorhamnetin, HPLC analysis showed a large amount of impurity. The solvent system of n-hexane-ethyl acetate-methanol-water (5:5:5:5, v/v) gave the best result as shown in Figure 1c.

Figure 2 shows the HPLC analysis of the crude sample, in which the crude extract contained about 2.25% of isorhamnetin based on the external standard curve. In order to shorten the separation time, a two stage HSCCC process was developed: quick separation of isorhamnetin from *Stigma maydis* was achieved using a HEMW (5:5:5:5, v/v) solvent system where acceptable rapid fractionation was achieved in two hours, yielding 19.7 mg of isorhamnetin at 65.6% purity based on the external standard curve. Then, the portion (shaded) of the main peak containing isorhamnetin was subjected to the second HSCCC separation using the HEMW (5:5:6:4, v/v) two phase solvent system. As shown in Figure 3, two peaks were resolved and the second peak (shaded) contained 11.8 mg of isorhamnetin at a high purity of 98% based on the external standard curve.

The isorhamnetin separation with HEMW (5:5:5:5, v/v) is better than that with HEMW (5:5:6:4, v/v) at the first preparative HSCCC stage. The reason may be that the crude extract contains a large amount of water soluble materials, which should be removed using a two phase solvent system containing less methanol. After these polar impurities

Figure 1. Chromatograms of the crude extract from *stigma maydis* by HSCCC. (a) Solvent system: ethyl acetate-methanol-water (10:1:10, v/v), sample: 700 mg dissolved in 10 mL lower phase of solvent system. (b) Solvent system: n-hexane-ethyl acetate-methanol-water (5:5:6:4, v/v), sample: 800 mg dissolved in 10 mL lower phase of solvent system. (c) Solvent system: n-hexane-ethyl acetate-methanol-water (5:5:5:5, v/v), sample: 700 mg dissolved in 10 mL lower phase of solvent system. Peak 3: Isorhamnetin at the purity 65.64%. Stationary phase: upper organic phase, mobile phase: lower aqueous phase; flow rate: 2.0 mL/min; revolution speed: 800 rpm. HPLC conditions: YMC-PACK ODS column (150×4.6 mm) at column temperature of room temperature. The mobile phase, composed of methanol and water including 0.05% H_3PO_4 (50:50, v/v), was isocratically eluted at a flow rate of 1.0 mL/min and the effluent monitored at 254 nm and 360 nm by a DAD detector.

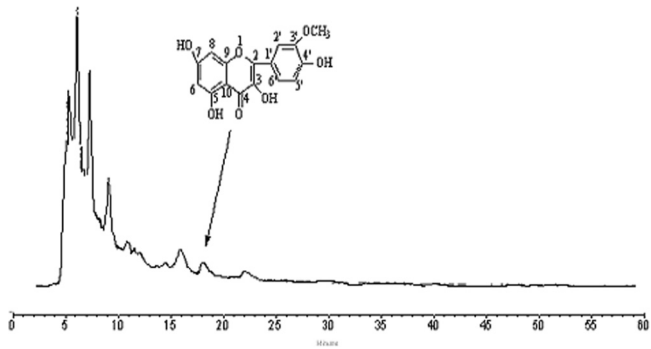

Figure 2. HPLC analysis of the crude extract from *Stigma maydis* with the chemical structure of isorhamnetin. HPLC conditions are the same as those in Figure 1.

have been removed, better separation of isorhamnetin from the rest of lipophilic material is achieved by increasing the methanol concentration in the second solvent system.

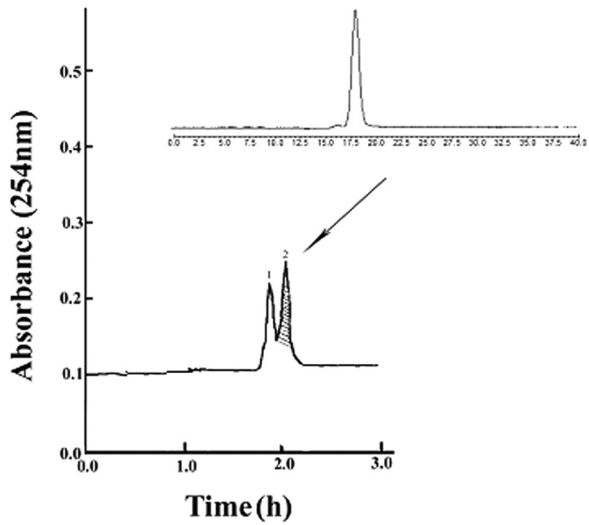

Figure 3. Chromatogram of the second stage HSCCC. Sample: a shaded portion of the peak in Figure 1c obtained by the first HSCCC run; solvent system: n-hexane-ethyl acetate-methanol-water (5:5:6:4 v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow rate: 2.0 mL/min; revolution speed: 800 rpm. HPLC conditions are the same as those in Figure 1. Peak 2: Isorhamnetin at the purity 98%.

The structural identification of isorhamnetin was carried out by EI-MS, ^{1}H-NMR, and ^{13}C-NMR Spectra as follows: the EI-MS: m/z 316, 301, 287, 245, 153,142, 128, 108, 69. It showed the molecular ion at m/z 316, which is in agreement with the molecular formula $C_{16}H_{12}O_7$ of isorhamnetin.

Isorhamnetin: ^{1}H-NMR (500 MHz, DMSO) δ ppm: 3.84 (3H, s, OCH_3), 6.20 (1H, d, 6-H), 6.48 (1H, d, 8-H), 6.94 (1H, d, 5′-H), 7.69 (1H, m, 6′-H), 7.75 (1H, d, 2′-H), 9.40 (1H, s, OH), 9.73 (1H, s, OH), 10.76 (1H, s, OH), 12.46 (1H, s, OH).

Isorhamnetin: ^{13}C-NMR (500 MHz, DMSO) δ ppm: 148.61 (2-C), 135.62 (3-C), 175.72 (4-C), 156.05 (5-C), 98.11 (6-C), 163.71 (7-C), 93.53 (8-C), 160.32 (9-C), 102.91 (10-C), 121.84 (1′-C), 111.56 (2′-C), 146.43 (3′-C), 147.21 (4′-C), 115.33 (5′-C), 121.64 (6′-C), 55.63 (OCH_3).

CONCLUSIONS

The results of our studies demonstrate successful preparative separation of isorhamnetin from *Stigma maydis* using two-step HSCCC operation. A 700 mg of the crude extract was first separated with a two phase solvent system composed of n-hexane-ethyl acetate-methanol-water at a volume ratio of 5:5:5:5 to improve the purity of isorhamnetin from 2.3% to 65.7%. This was followed by the second run using a slightly less polar solvent system of n-hexane-ethyl acetate-methanol-water at a volume ratio of 5:5:6:4 to remove the rest of the impurities, yielding 11.8 mg of isorhamnetin at a high purity of 98%.

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