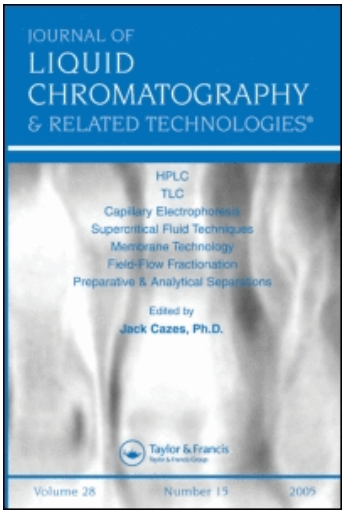

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Preparative Isolation and Purification of Anthraquinones from *Cassia Seed* by High-Speed Countercurrent Chromatography

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To cite this Article Xie, Y. , Liang, Y. , Chen, H. -W. , Zhang, T. -Y. and Ito, Y.(2007) 'Preparative Isolation and Purification of Anthraquinones from *Cassia Seed* by High-Speed Countercurrent Chromatography', Journal of Liquid Chromatography & Related Technologies, 30: 9, 1475 — 1488

To link to this Article: DOI: 10.1080/10826070701277117

URL: http://dx.doi.org/10.1080/10826070701277117

Journal of Liquid Chromatography & Related Technologies®, 30: 1475–1488, 2007

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070701277117

Preparative Isolation and Purification of Anthraquinones from *Cassia Seed* by High-Speed Countercurrent Chromatography

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Abstract: A high-speed countercurrent chromatography (HSCCC) technique in a semi-preparative scale has been applied to separate and purify anthraquinones from the extract of *Cassia seeds*. A high efficiency of HSCCC separation was achieved on a two-phase solvent system of *n*-hexane–ethyl acetate–methanol–water (4:1:3:2, v/v/v/v) by eluting the lower mobile phase at a flow rate of 1.5 mL/min under a revolution speed of 750 rpm. A total of five well separated peaks were obtained in the HSCCC chromatogram, and their purities were determined by HPLC-UV absorption spectrometry. These peaks were characterized by ESI-MS^n and the data compared with the reference standards. Five peaks were identified as 1,2,6-trihydroxy-7, 8-dimethoxy-3-methylanthraquinone (7 mg), 1,2,6,8-tetrahydroxy-7-methoxy-3-methyl-anthraquinone (4 mg), 2-hydroxy-1,6,7,8-teramethoxy-3-methylanthraquinone (9 mg), 6-dihydroxy-1,7,8-trimethoxy-3-methylanthraquinone (2 mg), and 1,2-dihydroxy-6,7,8-tri-methoxy-3-methylanthraquinone (3 mg) from 100 mg of the sample. The purities of obtained fractions were 98, 95, 96, 95, and 96%, respectively. HSCCC, thus, provides

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a cost effective alternative to preparative scale HPLC for the semi-preparative-scale separation and purification of anthraquinones from *Cassia seeds.* With appropriate modifications, the technique can also be applicable to other herbs in general.

Keywords: High-speed countercurrent chromatography (HSCC), *Cassia seed*, Anthraquinones, HPLC

INTRODUCTION

Cassia seed is a well known traditional Chinese medicinal plant. Seeds of the plant, named *Juemingzi* in Chinese, have been widely used in traditional Chinese medicine for the treatments of red and tearing eyes, headache, and dizziness, etc. The chemical constituents of the seed have been investigated thoroughly and a number of anthraquinones were isolated recently.[1] It was reported that the seed of *Cassia tora* L. contained a variety of bioactive anthraquinones (including chrysophanol, emodin, rhein, etc.), flavonoids, polysaccharides, inorganic elements (including Zn, Cu, Mn, Fe, Mg, Ca, Na, etc.), all of which were mainly responsible for its various pharmacological effects. The seed extract was also reported for its hypotensive activity. In addition, many medicinal properties such as antimicrobial, antihepatotoxic, and antimutagenic activities have been attributed to this plant.[2] In Chinese medicine, it was highly valued for the treatment of hyperlipidemia. Consequently, extracting the main functional components from *cassia seeds* is very valuable and essential to understand its medical functions.[3]

High-speed countercurrent chromatography (HSCCC) is a form of liquid–liquid partition chromatography,[4] where solute separation is based on partitioning between the two immiscible liquid phases: the mobile phase and the support-free liquid stationary phase. Without any solid matrix, the stationary phase is retained in the column by the aid of a centrifugal force field hence; the method eliminates irreversible adsorption of samples onto the solid support. Therefore, it is considered as a suitable alternative for separation of phenolic compounds such as flavonoids and hydroxyanthraquinones.[5–7] As an advanced separation technique, the method has been widely used for separation of active components from traditional Chinese herbs and other natural products in recent years. Since the past decade, successful applications of HSCCC have been reported for the purification of alkaloids,[8–10] hydroxyanthraquinones,[11,12] flavonoids,[13,14] saponins,[15] and so on. Besides a much larger separation capacity compared to HPLC, HSCCC allows the direct application of crude extracts and an excellent recovery of the analytes.

The aim of this work is to investigate the preparative separation and purification of anthraquinones, whose chemical structures are given in Figures 3–7, from the crude extract of *cassia seeds* by HSCCC.

EXPERIMENTAL

Apparatus

An HSCCC instrument, Model GS10A-2 (Beijing Institute of New Technology Application, Beijing, China) equipped with a polytetrafluoroethylene multilayer coil of 110 m long and 1.6 mm I.D., with a total capacity of 230 mL was employed for the present study. The β values of the preparative column were varied from 0.5 at the internal to 0.7 at the external ($\beta = r/R$, where r is the rotation radius or the distance from the coil to the holder shaft, and R is the revolution radius or the distances between the holder axis and central axis of the centrifuge). The rotation speed was adjustable from 0 to 1000 rpm, and 850 rpm was used in this work. The system was also equipped with one NS-1007 constant flow pump, a Model 8823A-UV monitor operating at 254 nm, a Yokogawa 3057 recorder, and a manual injection valve with a 2 mL (for the analytical HSCCC) or 20 mL sample loop (for the preparative HSCCC).

Reagents

Hexane, methanol, and ethyl acetate were of an analytical grade and purchased from Guangzhou Chemical Co., Ltd. (Guangzhou, China). Methanol used for HPLC analysis was of chromatographic grade and was purchased from Guangzhou Dikma Co., Ltd. (Guangzhou, China). *Cassia seeds* were purchased from Guangzhou Traditional Chinese Medicine Co., Ltd. (Guangzhou, China).

Preparation of Two-Phase Solvent System

The HSCCC experiments were performed with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (4:1:3:2, v/v/v/v). After thoroughly equilibrating the mixtures in a separation funnel at room temperature, two phases were separated shortly before use. The upper organic phase was used as stationary phase, and the lower aqueous phase as mobile phase.

Preparation of Sample

A 10 g amount of *cassia seeds* was extracted with 300 mL of 70% ethanol using reflux extraction for 3 h at 70°C in a water bath. The extracted solution was evaporated in reduced pressure to dryness, yielding 3 g crude extracts of *cassia seeds*. It was then stored in a refrigerator at 4°C for further purification by HSCCC.

HSCCC Procedure

HSCCC was performed as follows. The multilayer coiled column was first entirely filled with the upper phase as a stationary phase. The lower aqueous mobile phase was then pumped into the head end of the column inlet at a flow rate of 1.5 mL/min, while the apparatus was run at a revolution speed of 750 rpm. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (100 mg dissolved in 2 mL mixture consisting of equal volumes of each phase of the solvent system) 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 (further determined by HPLC and ESI-MS). After the separation was completed, retention of the stationary phase was measured by collecting the column contents by forcing them out of the column with pressurized nitrogen gas.

HPLC and ESI-MS Analysis and Identification of HSCCC Peak Fractions

The peak fractions from HSCCC were analyzed by HPLC, a Waters system (Series 600 liquid chromatograph) equipped with a UV detector (Waters 2487) and a chromatographic data system (HS). The analyses were performed with an analytical column Phenomenex ODS3 (250 mm × 4.6 mm, 5 μm). The mobile phase composed of methanol-acetic acid (70:30, v/v) was eluted at a flow rate of 0.8 mL/min and the effluent was monitored by a UV detector at 254 nm.

Identification of HSCCC peak fractions was carried out by ESI-MS using a Finnigan LCQ Deca ion trap mass XP MAX spectrometer equipped with an electrospray ionization source (Thermo Finnigan, San Jose, CA, USA).

RESULTS AND DISCUSSION

Selection of HSCCC Solvent System

Since HSCCC has no solid support, a proper amount of retention of the stationary phase in the column is a prerequisite for high peak resolution. Ideally, more than 50% of the stationary phase should be retained. The retention volume is highly correlated with the separation time of the two phases in a test tube, which should not exceed 20 s after vigorous shaking. Furthermore, partition coefficient (K) values between 0.5 and 1.0 are required for an efficient resolution in a short elution time.[16] In this study, retention of the stationary phase was in the range of 70–85%. The retention

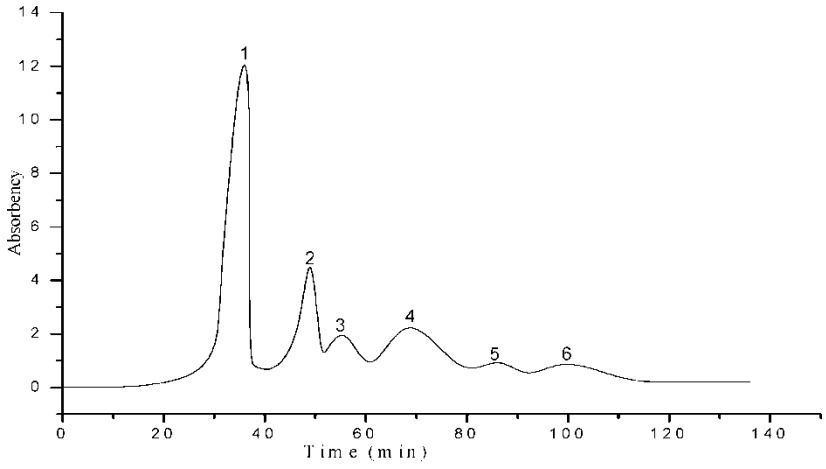

Figure 1. HSCCC chromatograms of the 70% ethanol crude extract of cassia seed. Peak 1: unknown mixed compounds. Experimental conditions: solvent system: n-hexane–ethyl acetate–methanol–water (4:1:3:2); coil volume: 233 mL; rotation speed: 750 rpm; flow rate: 1.5 mL/min; sample: 100 mg extract dissolved in 2 mL mixture solution of lower phase and upper phase (1:1, v/v); retention of the stationary phase: 79%.

of the stationary phase relative to the total column volume was determined from the volume of the stationary phase collected during the separation.

In a HSCCC experiment, selection of the two-phase solvent system is the first and critical step where a suitable solvent system can provide ideal partition coefficient values (K) for the target compounds. In the present study, HPLC was used to choose suitable solvent systems for HSCCC. To a certain extent, HPLC provided useful information for the solvent selection process from an enormous number of possible combinations. The selection of the two-phase solvent system for the target compounds is the most important step in HSCCC where searching for a suitable two-phase solvent system may be estimated as 90% of the entire work in HSCCC.[17]

In this work, search for a suitable solvent systems was carried out according to the method[17] previously reported as follows: If the sample is an extract of plant material, the search may start at any point according to the polarity of the solvent used for the extraction, e.g., if the sample is an ethyl acetate extract (relatively hydrophobic solvent), the search may start at hexane–ethyl acetate–methanol–water (1:1:1:1), whereas if the sample is a methanol extract (polar solvent), the search may start at 1-butanol–water. Since the sample of *cassia seeds* was extracted with 70% ethanol (moderately polar solvent), the search was started with the two-phase solvent system composed of hexane–ethyl acetate–methanol–water at a volume ratio of 3:5:3:5, which has a moderate degree of polarity, and the partition coefficient was adjusted by slightly modifying the solvent volume ratio.

The search was repeated for several trials, and finally hexane–ethyl acetate–methanol–water (4:1:3:2) was selected as a most suitable two-phase solvent system. The selected solvent system satisfies the following requirements: the analyte(s) was stable and has a high solubility in the system; the solvent system forms two phases with an acceptable volume ratio to avoid wastage.

Figure 1 shows the preparative HSCCC separation of 100 mg of the crude sample using n-hexane–ethyl acetate–methanol–water (4:1:3:2) as a two-phase solvent system. Five peaks were identified as 1,2,6-trihydroxy-7,8-dimethoxy-3-methylanthraquinone (7 mg), 1,2,6,8-tetrahydroxy-7-methoxy-3-methylanthraquinone (4 mg), 2-hydroxy-1,6,7,8-teramethoxy-3-methylanthraquinone (9 mg), 6-dihydroxy-1,7,8-trimethoxy-3-methylanthraquinone (2 mg), and 1,2-dihydroxy-6,7,8-trimethoxy-3-methylanthraquinone (3 mg). Purities of these obtained fractions were 98, 95, 96, 95, and 96%, respectively. HPLC analysis of each peak fraction of this preparative HSCCC revealed that five relatively pure anthraquinones could be obtained from the crude extract in a one step separation. The HPLC chromatograms of anthraquinones as purified from the preparative HSCCC are shown in Figure 2.

Identification of Separated Fractions by HSCCC

The identification of the structures of these five compounds by ESI-MS^n analyses are shown in Figures 3–7, with the negative mode analyses as follows:

HSCCC peak 2 in Figure 1: negative ESI-MS, m/z 329 (M-H).
HSCCC peak 3 in Figure 1: negative ESI-MS, m/z 315 (M-H).
HSCCC peak 4 in Figure 1: negative ESI-MS, m/z 357 (M-H).
HSCCC peak 5 in Figure 1: negative ESI-MS, m/z 343 (M-H).
HSCCC peak 6 in Figure 1: negative ESI-MS, m/z 343 (M-H).

The ESI-MS mass spectra of compound A (HSCCC peak 2 in Figure 1) in the negative mode gave m/z 329 as the deprotonated molecular ion [M-H], which confirmed the molecular mass as 330, the same as that for 1,2,6-trihydroxy-7,8-dimethoxy-3-methyl anthraquinone. Further experiments in MS^2 of the m/z 329 ion ([M-H]) produced a main fragment at m/z 314. The ion at m/z 314 is considered to be the loss of the methyl [M-H-15] from the parent ion m/z 329. The MS^3 spectrum of the ion at m/z 314 yielded one ion at m/z 299 by losing a methyl unit. Possible fragmentation pathways of anthraquinone are illustrated in Figure 3 (D).

The ESI–MS mass spectra of compound B (HSCCC peak 3 in Figure 1) in the negative mode gave m/z 315 as the deprotonated molecular ion [M-H], which confirmed the molecular mass as 316, the same as that for 1,2,6,8-tetrahydroxy-7-methoxy-3-methyl anthraquinone. Further experiments in MS^2 of the m/z 315 ion ([M-H]) produced a main fragment at m/z 300. The ion at m/z 300 is considered to be the loss of the methyl [M-H-15]

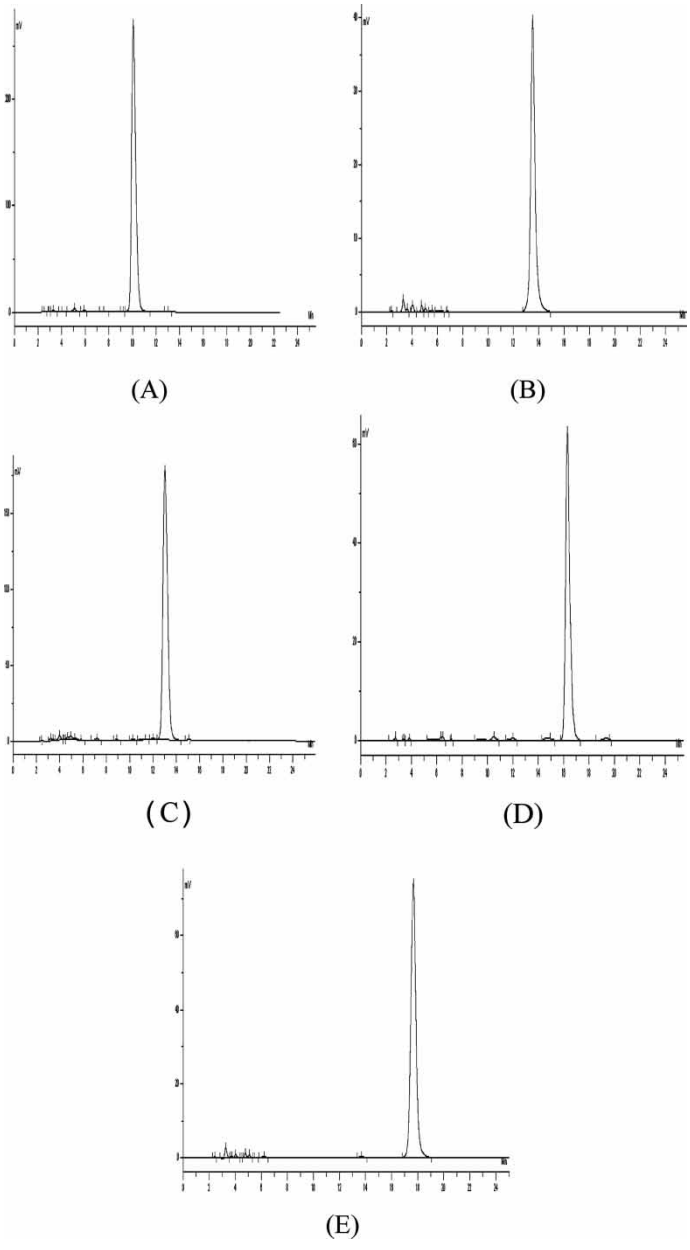

Figure 2. Results of HPLC analyses of the crude sample of *Cassia seed* and its HSCCC fractions. Column: PhenomenexODS3 (250 mm × 4.6 mm) at room temperature; mobile phase: methanol–0.4% Acetic acid (70:30,v/v); flow-rate: 0.8 mL/min. (A) HSCCC fraction from peak 2 (Figure 1); (B) HSCCC fraction from peak 3 (Figure 1); (C) HSCCC fraction from peak 4 (Figure 1); (D) HSCCC fraction from peak 5 (Figure 1); (E) HSCCC fraction from peak 6 (Figure 1).

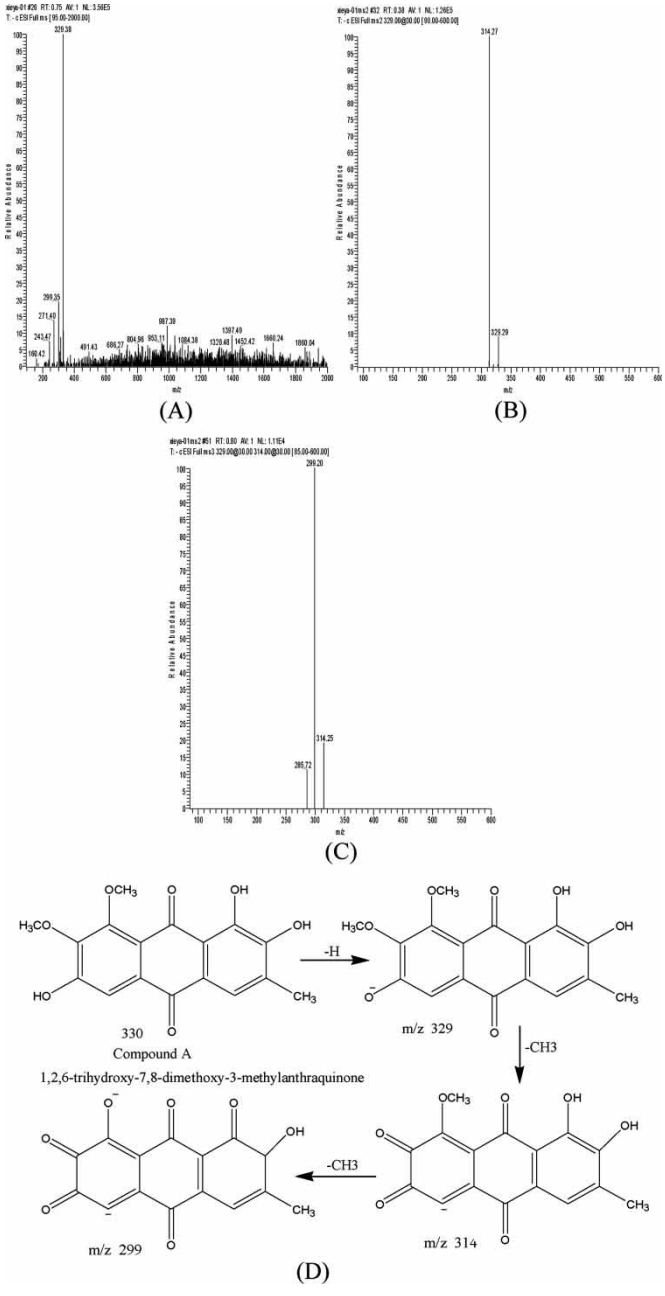

Figure 3. ESI-MS^n mass spectra of compound A (HSCCC peak 2 in Figure 1): (A) ESI-MS spectrum of the $[M\text{-}H]^-$ ion of compound A; (B) MS^2 on product ion m/z 329; (C) MS^3 on product ion m/z 314; (D) Proposed fragmentation mechanisms of $[M\text{-}H]^-$ ion of compound A.

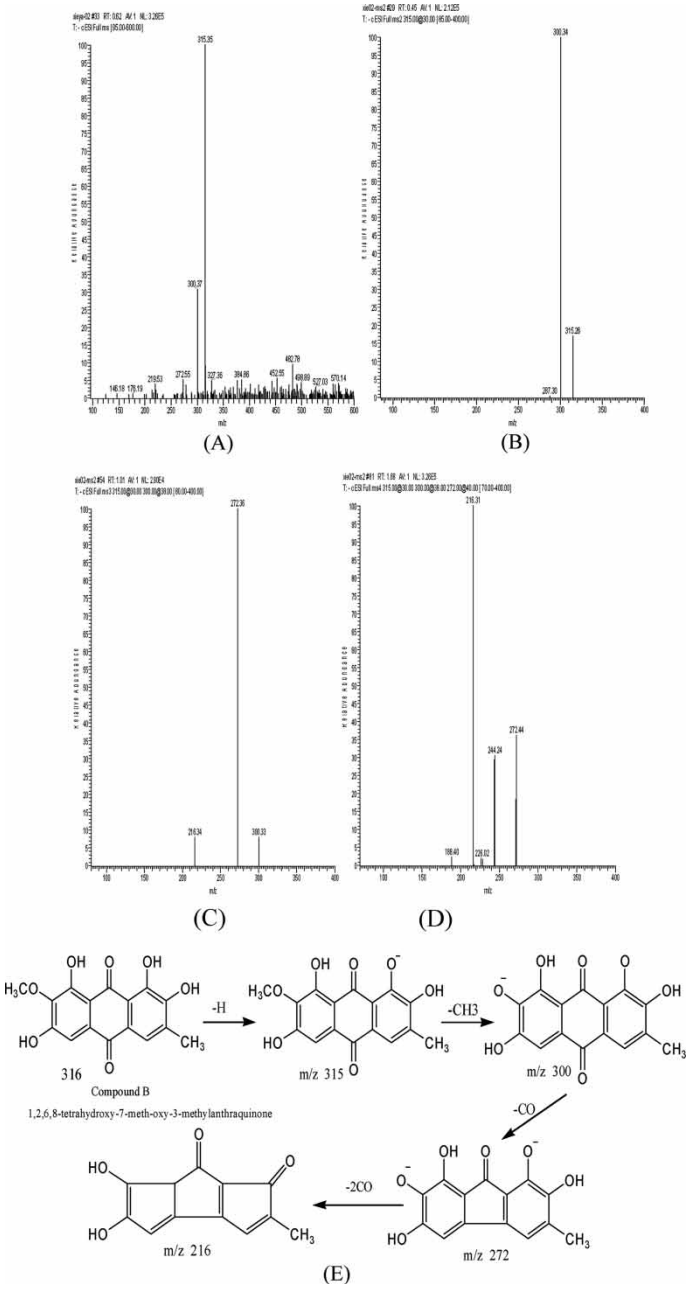

Figure 4. ESI-MS^n mass spectra of compound B (HSCCC peak 3 in Figure 1): (A) ESI-MS spectrum of the $[M\text{-}H]^-$ ion of compound B; (B) MS^2 on product ion m/z 315; (C) MS^3 on product ion m/z 300; (D) MS^4 on product ion m/z 272; (E) Scheme proposed fragmentation mechanisms of $[M\text{-}H]^-$ ion of compound B.

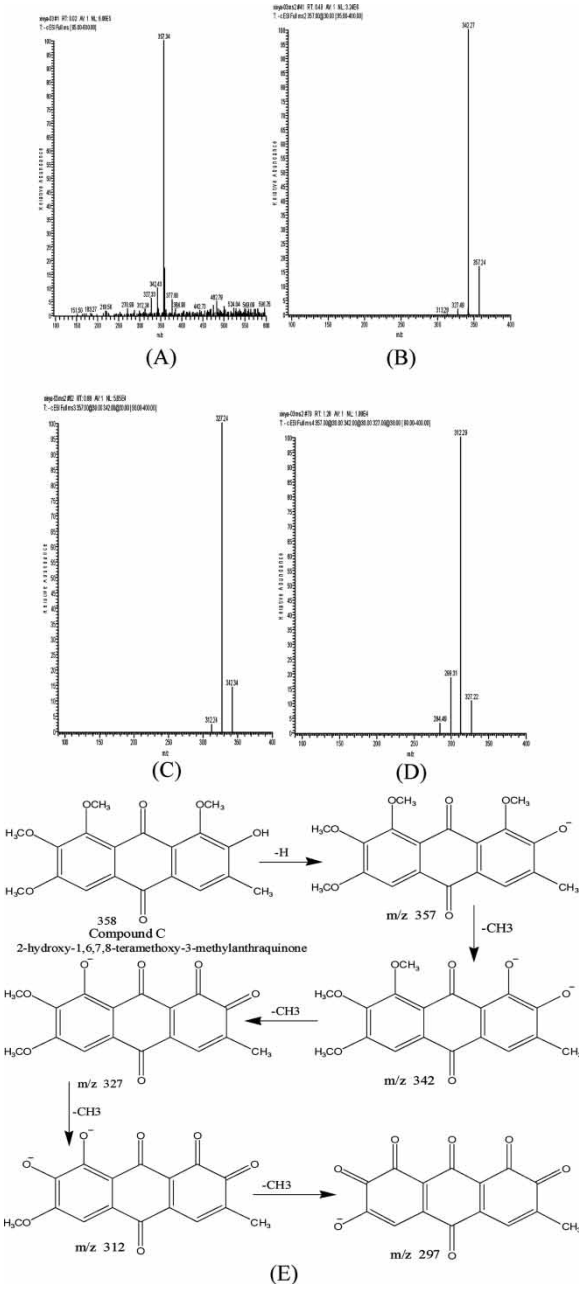

Figure 5. ESI-MS^n mass spectra of compound C (HSCCC peak 4 in Figure 1): (A) ESI-MS spectrum of the $[M\text{-}H]^-$ ion of compound C; (B) MS^2 on product ion m/z 357; (C) MS^3 on product ion m/z 342; (D) MS^4 on product ion m/z 327; (E) Scheme proposed fragmentation mechanisms of $[M\text{-}H]^-$ ion of compound C.

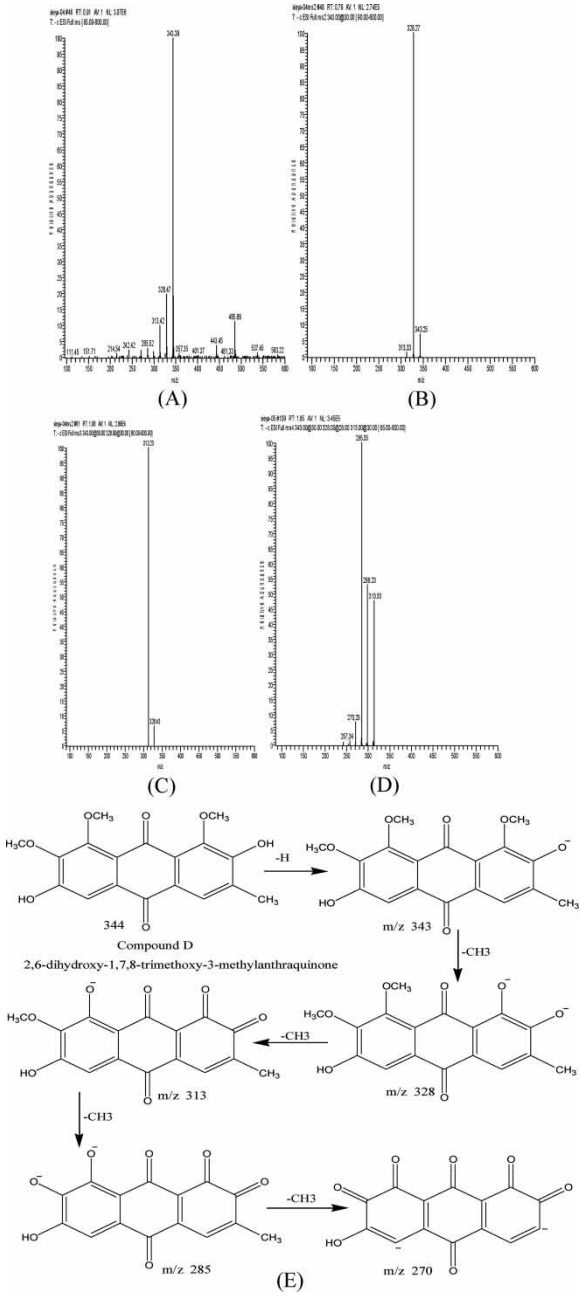

Figure 6. ESI-MS^n mass spectra of compound D (HSCCC peak 5 in Figure 1): (A) ESI-MS spectrum of the $[M\text{-}H]^-$ ion of compound D; (B) MS^2 on product ion m/z 343; (C) MS^3 on product ion m/z 328; (D) MS^4 on product ion m/z 313; (E) Scheme proposed fragmentation mechanisms of $[M\text{-}H]^-$ ion of compound D.

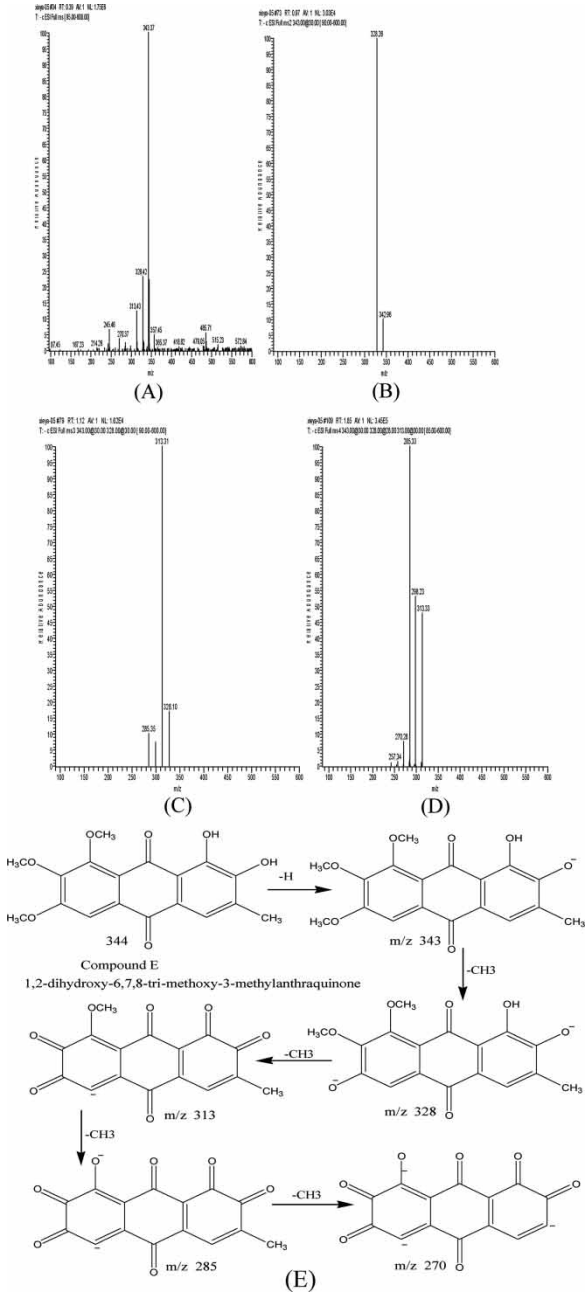

Figure 7. ESI-MS^n mass spectra of compound E (HSCCC peak 6 in Figure 1): (A) ESI-MS spectrum of the $[M\text{-}H]^-$ ion of compound D; (B) MS^2 on product ion m/z 343; (C) MS^3 on product ion m/z 328; (D) MS^4 on product ion m/z 313; (E) Scheme proposed fragmentation mechanisms of $[M\text{-}H]^-$ ion of compound E.

from the parent ion m/z 315. To our knowledge, the MS^3 spectrum of the ion at m/z 300 yielded one ion at m/z 272 by losing a CO unit [*M*-H-28] from the parent ion m/z 300. The MS^4 spectrum of the ion at m/z 272 yielded one ion at m/z 216 by losing two CO units. Possible fragmentation pathways of anthraquinone are illustrated in Figure 4 (E). The possible fragmentation pathways of compound C, D, E (HSCCC peak 3, 4, 5 in Figure1) are also illustrated in Figures 5–7.

For compound D (HSCCC peak 5 in Figure 1), the ESI–MS data provided m/z 343 as the deprotonated molecular ion [*M*-H], which indicates that it has the same molecular mass of 343 as compound E (HSCCC peak 6 in Figure 1) The MS^2 spectrum of the ion at m/z 328 and MS^3 spectra of the ion at m/z 313 were all the same as those of compound E, suggesting that these two compounds are isomers with similar structures.

Data collected from the ESI-MS^n experiment provided much more detailed structural information about these five compounds than the MS methods reported in the literature.[18] The analysis of these five compounds with ESI-MS^n showed that the mass spectrogram of them was almost identical to the literature[18] values of anthraquinones in *cassia seeds*.

CONCLUSION

An HSCCC technique has been developed and successfully applied to the separation and purification of five anthraquinones from crude cassia seed extracts. The results demonstrate the effectiveness of HSCCC as a semi-preparative separation technique for the isolation and purification of cassia seeds. A total of five major peaks were observed in the HSCCC chromatogram. The peaks were individually collected and their identities studied by ESI-MS analysis.

The HSCCC fractions from the extract were positively identified as anthraquinones of *cassia seeds* based on MS^n fragmentation patterns. However, further confirmation is needed for these peaks because of the lack of standards. In a typical run, tens of milligrams of samples can be separated with high efficiency to yield tens of milligrams of purified materials with over 95% purity. HSCCC thus, provides a cost effective alternative to preparative scale HPLC for the semi-preparative scale separation and purification of bioactive components in herbal extracts. In our present study, HSCCC has been successfully developed for the preparative separation of anthraquinones from the crude extract of *cassia* seeds. Furthermore, it demonstrates the wide applicability of the technique in the separation and purification of bioactive components from herbal extracts with diverse chemical compositions and properties.

ACKNOWLEDGMENT

This work is supported by the Bureau Science and Technology of Guangzhou, P. R. China.

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Received January 2, 2007
Accepted January 30, 2007
Manuscript 6025