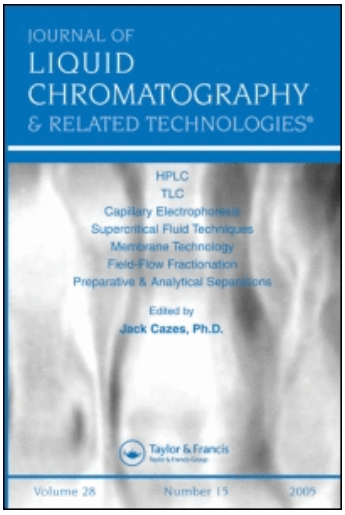

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Analysis of Flavonoids in the Extracts from the Seeds of *Oroxylum indicum* Using High Speed Countercurrent Chromatography/Mass Spectrometry

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To cite this Article Chen, L. J. , Song, H. , Du, Q. Z. , Li, Jianrong and Ito, Y.(2005) 'Analysis of Flavonoids in the Extracts from the Seeds of *Oroxylum indicum* Using High Speed Countercurrent Chromatography/Mass Spectrometry', Journal of Liquid Chromatography & Related Technologies, 28: 10, 1549 — 1555
To link to this Article: DOI: 10.1081/JLC-200058350
URL: http://dx.doi.org/10.1081/JLC-200058350

Journal of Liquid Chromatography & Related Technologies®, 28: 1549–1555, 2005

ISSN 1082-6076 print/1520-572X online
DOI: 10.1081/JLC-200058350

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Abstract: The HSCCC was successfully interfaced with a mass spectrometer possessing APCI- and ESI-MS mode for the first time. The coupling system gave an excellent separation of ethyl acetate extract from the seeds of *O. indicum* using a solvent system composed of hexane-ethyl acetate-methanol-0.2% formic acid (1:1.2:1:1); the online data of APCI-MS and APCI-MS-MS provided useful information for the identification of the compounds.

Keywords: Flavonoids, *Oroxylum indicum*, HSCCC-MS

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INTRODUCTION

During the preceding decade, high speed countercurrent chromatography (HSCCC) has been used mostly for preparative separation of natural products. HSCCC coupling to a mass spectrometer was not widely used due to the following reasons: 1) HSCCC-MS will result in a large amount of stationary phase being lost and a high baseline noise because high backpressure will arise after interfacing the two instruments; 2) The solvent system usually used in HSCCC would not give good sensitivity if no modified solution was added to help ionization of target components; 3) Most CCC instruments were of preparative scale and, therefore, unsuitable for lower flow rates required by a mass spectrometer. However, the benefits offered by coupling a mass spectrometer with many analytical instruments play a very important role in the identification and determination analyses in a complex sample matrix.

Mass spectrometry is a rapid detection method that lends itself well to many types of chromatography because of its high sensitivity and small amount of sample used. It is important to interface HSCCC with mass spectrometry because it combines the advantage of HSCCC with the lower detection limit and identification capability of MS. Recently, considerable effort has been made to develop analytical HSCCC for interfacing mass spectrometry. CCC-TSP-MS has been successfully applied mainly to the separation of natural products including the analyses of alkaloids, triterponic acids, and ligans.[1–3] However, an additional pump has to be connected between HSCCC and a mass spectrometer in order to decrease the backpressure.

Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are two atmospheric pressure ionization sources. Both of them are soft ionization techniques. Such sources ionize compounds at atmospheric pressure and then transfer the ions into the mass spectrometer. With the development of the CCC technique to analytical scale, ESI and APCI mass spectrometers can directly interface HSCCC.[4,5] In this study, an analytical HSCCC instrument interfaced directly with ESI and APCI mass spectrometry was explored for the isolation and identification of the flavonoids of extracts from the seeds of *O. indicum* and soybeans.

EXPERIMENTAL

Solvent and Reagents

HPLC grade solvents used in this study included hexane, ethyl acetate, methanol, and water. All these solvents were purchased from Fisher Chemicals (Shanghai, China). Analytical grade formic acid and acetic acid were also from the same supplier. All solvents were degassed prior to use. Standard baicalein-7-O-glucoside was isolated by our lab[6] and the purity

checked by UV and NMR. Baicalein and chrysin were purchased from the Sigma Company.

Instruments

The analytical HSCCC used in the present studies was made by our lab (West China University of Medical Sciences, Huaxi Medical School, Sichuan University. P.R. China). This HSCCC is a prototype J-type coil planet centrifuge high speed analytical instrument. It was assembled with two identical bobbins; each with 0.8 mm bore tubing connected in series, to provide a total coil volume of 25 mL with a value of 0.80. The instrument is designed to rotate at a speed up to 1500 rpm. In the present study, the speed up to 1200 rpm was used.

The mass spectrometer used in this study was a LCQ Ion Trap Mass Spectrometer (Finnigan MAT, San Jose, CA, USA). This instrument can perform MS and MSn and provide ESI and APCI spectra.

Sample Preparation

Seeds of *O. indicum* (50 g) were refluxed for 5 hours in 90% methanol. The extracts were then filtered and evaporated. The residue was redissolved in 200 mL H_2O and extracted three times with ethyl acetate. Final evaporation yielded 6.5 g of a yellow powder.

HSCCC Separation Method

A biphasic mixture of hexane-ethyl acetate-methanol-0.2% formic acid was prepared in different ratios and purged for 30 minutes with nitrogen to remove any dissolved gases. First, the coil was filled with the upper organic phase of the biphasic mixture. The coils were rotated in a forward direction at a speed of 1200 rpm and the lower aqueous phase was pumped into the coil from head to tail at a flow rate of 1.0 mL/min. When the two layers were observed, the equilibration point was determined when no more stationary phase was eluted (hydrodynamic equilibration). The retention volume of the system could then be calculated by subtracting the volume of the stationary phase eluted at the end of the equilibration process and dead volume from the total volume. The extra volume was calculated according to the Wood plot.[7]

HSCCC Interfacing with the Mass Spectrometer

Our analytical HSCCC can be interfaced directly with ESI/MS or APCI/MS without additional pumps between the mass spectrometer. When ESI/MS is interfaced with HSCCC, a split of flow rate is required for ESI ionization. Figure 1 shows the HSCCC interfacing with a mass spectrometer.

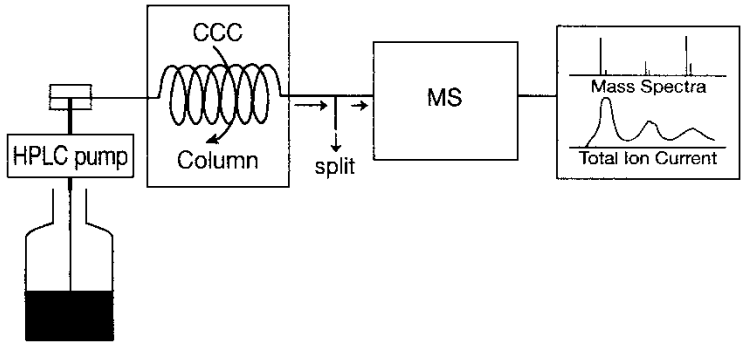

Figure 1. The HSCCC interface with mass spectrometry.

RESULTS AND DISCUSSION

Negative HSCCC-ESI-MS for the Separation of Flavonoids from the Ethyl Acetate Extract of the Seeds of *O. indicum*.

The seeds of *O. indicum* are known as the crude drug 'Mu Hu Die' in China, and it has been used as an analgesic, antitussive, and anti-inflammatory agent for the treatment of coughs, bronchitis, and other diseases. The flavonoids in the seeds of *O. indicum* are possibly bioactive components. Figure 2 shows the separation results by HSCCC-ESI-MS for the separation of flavonoids from

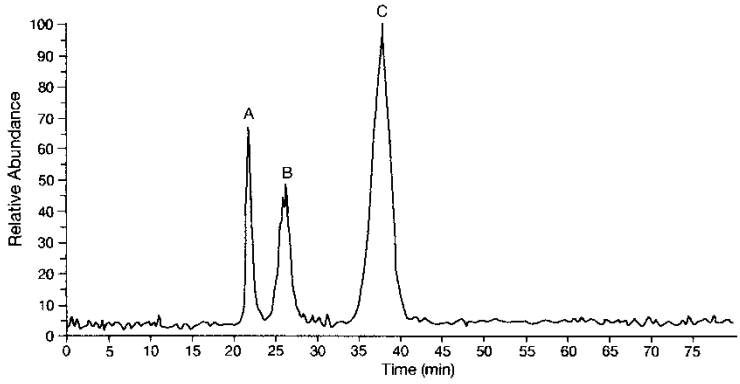

Figure 2. TIC chromatogram of the ethyl acetate extract of the seeds of *O. indicum* by negative HSCCC-ESI-MS. HSCCC conditions: Solvent system, Hexane-Ethyl acetate-Mathanol-0.2% formic acid (1.2:1:1, v/v); Upper phase, Stationary phase; Lower phase, Mobile phase; Stationary retention (%) $S_F = 65.41$; Mass Spectrometry conditions, Capillary temperature is 210°C; Capillary voltage 10.0 V; Sheath gas flow rate, 60 arbitrary and tube lens offset 5.0 V.

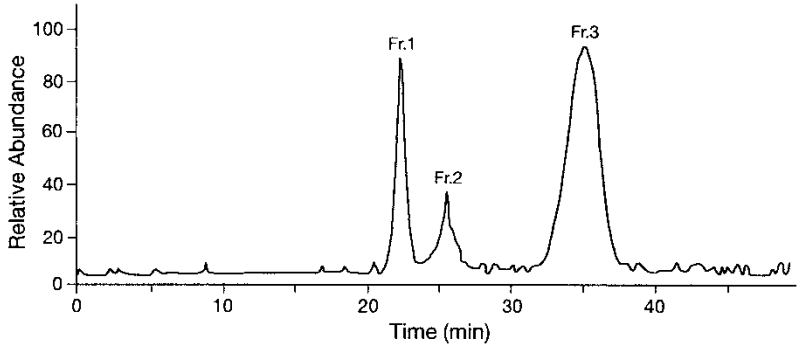

Figure 3. TIC chromatogram of the extract of the seeds of *O. indicum* by HSCCC-APCI-MS separation. Fr1, baicalein-7-O-glucoside; Fr 2, baicalein; Fr3, chrysin. HSCCC conditions: Solvent system, hexane-ethyl acetate-methanol-0.2% formic acid(1:1.2:1:1, v/v); Upper phase, Stationary phase; Lower phase, Mobile phase; Stationary retention (%), $S_F = 67.08$; Speed 1200 rpm; Flow rate, 1.0 mL/min. Mass spectrometer conditions: Vaporizer temperature, 500°C; Sheath gas flow rate, 90 arbitrary; Discharge current, 5.0; Capillary temperature, 170.

the extract. Here, the negative MS mode was adopted since it is better than the positive mode demonstrated by our experiment. A split of flow rate has to be used to provide the lower flow rates required by the mass spectrometer. In this case, an accurate flow rate into the mass spectrometer cannot be guaranteed. Therefore, a lower flow rate may be better for obtaining a reproductive chromatogram, but the analytical time has to be lengthened. To overcome these problems, we tried the HSCCC-APCI-MS to allow a higher flow rate.

HSCCC-APCI-MS for the Separation of Flavonoids from the Ethyl Acetate Extract of the Seeds of *O. indicum*

Figure 3 shows the TIC chromatogram of HSCCC-APCI-MS separating the ethyl acetate extract from the seeds of *O. indicum.* Three peak fractions,

Table 1. HSCCC-MSn spectra (m/z value) of fractions I,II, and III

Fraction	$[M\text{-}H]^-$	MSn data of compounds
I	431	$431\underline{MS^2}311\underline{MS^3}269\underline{MS^4}$ 251,241,225,197,169,141
II	269	$269\underline{MS^2}$251,241,225,197,169,141
III	253	$253\underline{MS^2}$ 235, 151,145,127,105

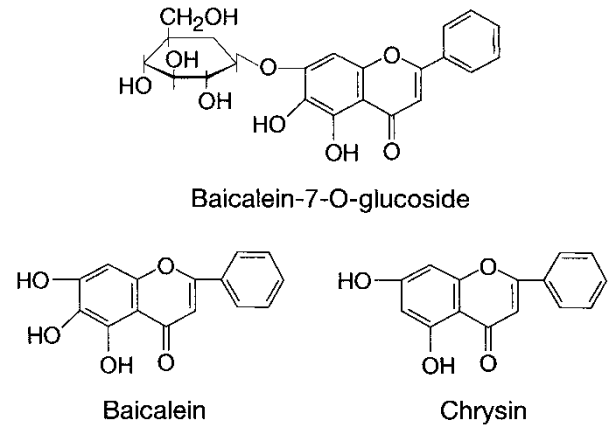

Figure 4. Structures of flavonoids in the seeds of *O. indicum*.

Fr1, Fr2, and Fr3 give online MS data $[M\text{-}H]^-$ of 431, 269, and 253 (m/z), respectively. Advanced data of MS-MS(MS^2), MS^3, and MS^4 are obtained online. These MS and MS^n data (Table 1) are consistent with those of the standard baicalein-7-O-glucoside, baicalein, and chrysin obtained in the same conditions. Thus, Fr1, Fr2, and Fr3 can be identified as baicalein-7-O-glucoside, baicalein, and chrysin (Fig. 4).

CONCLUSIONS AND DISCUSSION

The HSCCC was successfully interfaced with a mass spectrometer possessing APCI- and ESI-MS mode for the first time. The coupling system gave an excellent separation of ethyl acetate extract from the seeds of *O. indicum*, and the online data of APCI-MS and APCI-MS-MS provided useful information for the identification of the compounds. The system can play an important role for the analysis of natural products as a complement of HPLC.

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Received January 4, 2005
Accepted February 12, 2005
Manuscript 6568