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High-speed counter-current chromatography coupled online to high performance liquid chromatography–diode array detector–mass spectrometry for purification, analysis and identification of target compounds from natural products

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ABSTRACT

A challenge in coupling high-speed counter-current chromatography (HSCCC) online with high performance liquid chromatography (HPLC) for purity analysis was their time incompatibility. Consequently, HSCCC–HPLC was conducted by either controlling HPLC analysis time and HSCCC flow rate or using stop-and-go scheme. For natural products containing compounds with a wide range of polarities, the former would optimize experimental conditions, while the latter required more time. Here, a novel HSCCC–HPLC–diode array detector–mass spectrometry (HSCCC–HPLC–DAD–MS) was developed for undisrupted purification, analysis and identification of multi-compounds from natural products. Two six-port injection valves and a six-port switching valve were used as interface for collecting key HSCCC effluents alternatively for HPLC–DAD–MS analysis and identification. The ethyl acetate extract of *Malus doumeri* was performed on the hyphenated system to verify its efficacy. Five main flavonoids, 3-hydroxyphloridzin (**1**), phloridzin (**2**), 4′,6′-dihydroxyhydrochalcone-2′-*O*-β-D-glucopyranoside (**3**, first found in *M. doumeri*), phloretin (**4**), and chrysin (**5**), were purified with purities over 99% by extrusion elution and/or stepwise elution mode in two-step HSCCC, and 25 mM ammonium acetate solution was selected instead of water to depress emulsification in the first HSCCC. The online system shortened manipulation time largely compared with off-line analysis procedure and stop-and-go scheme. The results indicated that the present method could serve as a simple, rapid and effective way to achieve target compounds with high purity from natural products.

1. Introduction

Natural products play a highly significant role in drug discovery and development. From 1981 to 2010, about 50% of all the marketed-new chemical entities were of direct natural origins or underlying synthetic design principles [1]. As is known, isolation of pure compounds from natural products is the key step for lead discovery and drug screening. However, natural products are complex matrices, and the conventional fractionation steps are usually laborious, time-consuming and result in loss of some interesting compounds because of the dilution and decomposition [2].

Chromatography is a key technique to resolve complex natural products into single compound for structural identification and pharmacological testing [3,4]. Notably, hyphenated high-performance liquid chromatography (HPLC) [e.g. HPLC–diode array detector (DAD), HPLC–tandem mass spectrometry (MS^2), and HPLC–nuclear magnetic resonance (NMR)] [5,6] and two-dimensional HPLC (2D HPLC) [7–10] have been widely used for comprehensive analysis of natural products because of their large peak capacity, effective separation, high resolution and detailed structural information. Despite these achievements, the structural elucidation and biological evaluation of interesting compounds, especially for minor compounds, is still a challenging work. The chance of obtaining structural and biological information may be increased when compounds are efficiently purified from complex matrices.

High-speed counter-current chromatography (HSCCC), a unique liquid–liquid partition chromatography method based on

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partitioning of compounds between two immiscible liquid phases with a support-free matrix, no irreversible adsorption, low risk of sample denaturation, total sample recovery and large load capacity, is an optimal choice to purify compounds from complex matrix, and HSCCC using different modes (e.g. stepwise elution, extrusion elution) could isolate compounds with a wide range of polarities [11–13]. However, the relatively lower theoretical plates of HSCCC and the all-pervading existence of compounds with close polarities in natural products incidentally result in insufficient peak resolution, and then several compounds are commonly co-eluted in one fraction [13,14]. Thus, HSCCC fractions must be analyzed by HPLC to directly evaluate their compositions/purities. Off-line procedures need multiple manual steps and lengthy manipulation/analysis time. To tackle this challenge, different interfaces have been adapted to direct hyphenation of HSCCC with HPLC for online monitoring [15]. Similar to 2D HPLC, the second dimensional HPLC procedure requires a certain period of time to collect, load and analyze the HSCCC effluents, and then equilibrate the column to its initial conditions, therefore, the possible incompatibility between HSCCC and HPLC in terms of manipulation time should be considered. Zhou and coworkers firstly interconnected HPLC with HSCCC via a T-splitter and a six-port switching valve to online analyze hyperosides from *H. perforatum* [16] and xanthones from *A. asphodeloides* [17] by a fast HPLC. After that, Wu et al. developed HSCCC–HPLC with a six-port switching valve for preparative separation and analysis of flavonoids from alfalfa by stop-and-go scheme [18], or one target compound, arctiin, from *A. lappa* [19]. Recently, preparative HPLC was online coupled with HSCCC through a six-port switching valve to purify overlapped compounds in first HSCCC separation [20], in which, the flow rate of HSCCC was controlled to ensure that the elution time of each HSCCC fraction was corresponded to the isolation time of preparative HPLC. These previous developments are efficient for HPLC online analysis/preparation of HSCCC fractions. However, for natural products with a wide range of polarities, elution time of HSCCC fraction (difficult to be controlled) and HPLC analysis time (relatively long) were sometimes incompatible, and at this time, the conditions for HSCCC purification and HPLC analysis should be optimized to avoid overlapping between the HPLC analysis of HSCCC effluent n and $n+1$, while stop-and-go scheme lasts for extended time. Perhaps owing to the aforementioned limitations, HSCCC–HPLC has not been widely applied for simultaneous and comprehensive purification and analysis of target compounds from complex natural products.

In the present work, a novel online HSCCC–HPLC–DAD–MS system was developed for the simultaneous purification, analysis and identification of target compounds from natural products. To assess the applicability of this new hyphenated system, we applied it to the online harvesting main flavonoids from *Malus doumeri*.

2. Experimental

2.1. Chemicals and reagents

All solvents used for extraction and separation were of analytical grade and obtained from Chemical Reagent Factory of Hunan Normal University (Hunan, China). Acetonitrile and formic acid used for HPLC were of chromatographic grade (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China). Ultrapure water (18.2 MΩ) was purified and filtered using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Leaves of *M. doumeri* was collected from Jianghua, Hunan province of China, in August 2014. The plant material was authenticated by Prof. Zhaoming Xie, Research Institute of Chinese Medicine, Hunan Academy of Chinese Medicine, Changsha, China. A voucher specimen (No. MD201408) was deposited at the Research Institute of Chinese Medicine, Hunan Academy of Chinese Medicine, Changsha, Hunan, China.

2.2. Apparatus

Preparative HSCCC was performed using a model TBE-300B HSCCC (Shanghai Tauto Biotechnique Co. Ltd., Shanghai, China). The apparatus consisted of an upright coil type-J planet centrifuge with three multilayered coils connected in series (diameter of tube, 1.6 mm, total capacity 260 mL) and a 20 mL manual sample loop. The rotation speed was adjustable, ranging from 0 to 1000 rpm. The HSCCC system was equipped with a TBP-1002 pump, a TBD-2000 UV detector, an HX-1050 constant temperature regulator (Beijing Boyikang Lab Implement Co. Ltd., Beijing, China), and a WH V4.0 workstation (Shanghai Wuhao Information Technology Co. Ltd., Shanghai, China). In the separation process, the temperature of separation columns was maintained at 25 °C, and the effluents were achieved at 280 nm.

An Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA), equipped with an online vacuum degasser, a Quat-Pump, a manual injection valve with a 20 μL sample loop, a thermostated column compartment, a diode assay detector (DAD), and an Agilent ChemStation, has been selected to analysis of samples. Chromatographic separation was performed on a SunFire™ C_{18} (250 mm × 4.6 mm i.d., 5 μm, Waters, MA, USA) column in tandem with a Phenomenex C_{18} guard cartridge (4.0 mm × 3.0 mm, Phenomenex, Torrance, CA). The mobile phase consisted of A (0.4% acetic acid in water) and B (0.4% acetic acid in acetonitrile) was programmed as follows: 0–5 min, 20% B; 5–15 min, 20–30% B; 15–20 min, 30% B; 20–30 min, 30–45% B; 30–45 min, 45–90% B. The flow rate was 0.8 mL/min while the column temperature was set at 25 °C. Spectra were recorded from 190 to 400 nm (peak width 0.2 min and data rate 1.25 s^{-1}) while the chromatogram was acquired at 280 nm.

Agilent 6530 Accurate-Mass Q-TOF LC/MS system (Agilent Technologies, Santa Clara, CA) equipped with an electrospray ionization (ESI) interface was coupled in parallel by splitting the mobile phase 1:3 using an adjustable high-pressure stream splitter (Valco Instrument Company, Houston, TX, USA). MS data were acquired across the range m/z 100–1000 in positive and negative ion modes. The operating conditions were as follows: nitrogen as dry gas with temperature at 325 °C and flow rate at 5.0 L/min, and sheath gas with temperature at 400 °C and flow rate at 12 L/min; pressure of nebulizer, 55 psi; capillary voltage, 3500 V; skimmer, 65 V; OCT 1 RF Vpp, 750 V; fragmentor voltage, 130 V. The mass axis was calibrated using the mixture provided by the manufacturer mover the m/z 70–3200 range.

2.3. Preparation of M. doumeri extract

The dried and pulverized leaves of *M. doumeri* (1000 g) were decocted by 75% ethanol (10 L) at 85 °C for three times (each for 3 h). The combined filtrates were concentrated to dryness under vacuum by rotary evaporation at 60 °C to afford crude extract (106.9 g). A mass of 105 g of crude extract was then suspended in water and submitted to liquid–liquid fraction using petroleum ether and ethyl acetate with increasing polarities. The dried ethyl acetate fraction (22.9 g) was stored at 4 °C for further experiments.

2.4. HSCCC separation

2.4.1. Selection of two-phase solvent system

Suitable solvent systems were selected according to the partition coefficient (K) of each target compound. A series of solvent systems composed of ethyl acetate–n-butanol–25 mM ammonium acetate solution, ethyl acetate–n-butanol–methanol–25 mM