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Magnetic nanoparticles and high-speed countercurrent chromatography coupled in-line and using the same solvent system for separation of quercetin-3-O-rutinoside, luteoloside and astragalin from a *Mikania micrantha* extract☆

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ABSTRACT

A new in-line method of magnetic nanoparticles (MNPs) coupled with high-speed countercurrent chromatography (HSCCC) using a same solvent system during the whole separation process was established to achieve the rapid separation of flavonoids from *Mikania micrantha*. The adsorption and desorption capacities of five different MNPs for flavonoid standards and *Mikania micrantha* crude extract were compared and the most suitable magnetic nanoparticle Fe_3O_4@SiO_2@DIH@EMIMLpro was selected as the in-line MNP column. An in-line separation system was established by combining this MNP column with HSCCC through a six-way valve. The comparison between two solvent systems n-hexane-ethyl acetate-methanol-water (3:5:3:5, v/v) and ethyl acetate-methanol-water (25:1:25, v/v) showed that the latter solvent system was more suitable for simultaneously in-line separating three flavonoids quercetin-3-O-rutinoside, luteoloside and astragalin from *Mikania micrantha*. The purities of these three compounds with the ethyl acetate-methanol-water solvent system were 95.13%, 98.54% and 98.19% respectively. Results showed the established in-line separation system of MNP-HSCCC was efficient, recyclable and served to isolate potential flavonoids with similar polarities from natural complex mixtures. The in-line combination of magnetic nanoparticles with high-speed countercurrent chromatography eluting with the same solvent system during the whole separation process was established for the first time.

1. Introduction

The invasive plant *Mikania micrantha* contains flavonoids [1], which have considerable antioxidant properties, antibacterial bioactivities and antioxygenation [2]. Thus, separation and enrichment of the useful flavonoid components from *Mikania micrantha* is an essential step to derive a benefit from the invasive plant.

New separation techniques of flavonoids continue to be of great interest since these compounds have significant effectiveness in coronary heart disease, senile dementia and cerebral ischemia and are widely used in herbal medicines and health foods. Though methods of macroporous resin [3], aqueous two-phase extraction [4], solid-phase extraction (SPE) [5], magnetic solid phase extraction (MSPE) [6,7], preparative high performance liquid chromatography (HPLC) [8] and high-speed countercurrent chromatography (HSCCC) [9,10] were widely used to separate flavonoids, most of these methods were non-coupled fashion. Even though some references reported that the two methods mentioned above were combined during flavonoid separation, two solvent systems were used [3,11]. Jiang et al. reported a two-dimensional HSCCC strategy by which five flavonoids quercetin, 3-O-rutinoside-3′-O-β-glucopyranoside, kaempferol, rutin and kaempferol-3-rutinoside were successfully separated from tartary buckwheat grains with two solvent systems of n-hexane-ethyl acetate-methanol-water 3:5:3:5 (v/v) and ethyl acetate-n-butanol-water 7:3:10 (v/v) [12]. The above methods were done as two or more steps during separation process of complex natural products. So, an in-line coupling system was set up for one-step separation. The advantages of in-line mode are time saving, high separation efficiency and less organic solvent consuming, which would give a promising environmental friendly chromatography. An integrated in-line HSCCC-SPE-HSCCC system was applied to isolate organic

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compounds from *Ligusticum chuanxiong* rhizomes [13]. Chen et al. obtained polar polyphenols from tea extract using two phase HSCCC coupled with HPLC [14]. Lu et al. reported a two-dimensional countercurrent chromatographic method (CCC) for preparative isolation and purification of three prenylflavonoids from *Artocarpus altilisis* with a pair of two-phase solvent systems composed of n-hexane-ethyl acetate-methanol-water (5:5:7:3 and 5:5:6.5:3.5, v/v). With a pair of two-phase solvent systems composed of n-hexane–ethyl acetate–methanol–water (1:5:1:5 and 3:5:3:5, v/v), the two-dimensional CCC system was successfully used for isolation and purification of oridonin and ponicidin from *R. rubescens* [15,16]. These reported methods used different solvent systems that were not further used in the next process. Thusthese methods were time-consuming and complicated due to solvent removal after first pretreatment. So in-line separation system with the same solvent system is a better choice to solve the aforementioned problems during separation process.

In the present work, we established the device of magnetic nanoparticles (MNP) in-line coil combined with HSCCC through a six-way valve to achieve in-line separation and enrichment of flavonoids quercetin-3-O-rutinoside, luteoloside and astragalin from *Mikania micrantha.* The established in-line separation system of MNP-HSCCC was highly effective, recyclable and using the same solvent system during the whole separation process. Moreover, this strategy would be helpful for developing the systematic in-line separation technology of natural products.

2. Experimental

2.1. Equipment

The in-line MNP coil was performed in 1.6 mm I.D., 3.73 m long PTFE tubing with a total capacity of 7.5 mL. The tubing was filled with Fe_3O_4@SiO_2@DIH@EMIMLpro (200 mg). The preparative HSCCC instrument (Beijing Institute of New Technology Application, Beijing, China) was used with a Model GS10AB multilayer coil planet centrifuge equipped with a PTFE (polytetrafluoroethylene) multilayer coil of 110 m × 1.6 mm I.D. with a total capacity of 230 mL. The β values of the coil values varied from 0.5 at the internal terminal to 0.75 at the external terminal. Although the revolution speed of the apparatus could be regulated with a speed controller in a range between 0 and 1000 rpm, an optimum speed of 800 rpm was used in the present study.

The solvent system was pumped into the column by a Model NS-1007 constant flow pump (Beijing Institute of New Technology Application). The sample solution of the in-line MNP coil was pumped by the NE-4000 Double Syringe Pump (New Era Pump Systems Inc., New York, USA). The continuous monitoring of the effluent was achieved with a Model 8823A-UV detector (Beijing Institute of New Technology Application) operating at 254 nm. A manual sample injection valve with a 10 mL loop was used to introduce the sample into the column. A three-way valve (Beijing DIKMA Technology Company Limited) was used for injecting sample to the MNP coil. A six-way valve(Tanjin Keqi High&New Technology Corporation)was used for combining this MNP coil with HSCCC. Two portable recorders (Yokogama Model 3057, Sichuan Instrument Factory, Chongqing, China) were used to plot the chromatograms. An RE-52A rotary evaporator (Shanghai Yarong Biochemistry Instrument Factory, Shanghai, China) was used to concentrate fractions.

The analytical HPLC equipment used was a Shimadzu LC-20AVP system equipped with two LC-20AT solvent pumps, a SPD-M20AVP UV–vis photodiode array detector (DAD) system, a Model 7725 injection valve with a 20 μL loop and a Shimadzu SIL-20A autosampler, a SCL-20AVP system controller, and a Class-VP-LC work station (Shimadzu, Kyoto, Japan).

The identification of separated compounds was carried out with quadrupole time-of-flight mass spectrometry (Q-TOF-MS) (Waters Q-TOF Micro, USA), ^1H and ^{13}C nuclear magnetic resonance spectrometry (^1H NMR and ^{13}C NMR) (Bruker Biospin Corporation, USA).

2.2. Materials and reagents

Mikania micrantha plants harvested in 2014 September were supplied by the Chinese Academy of Agricultural Sciences. All standards were purchased from National Institute for the Control of Pharmaceutical & Biological Products (Beijing, China). All organic solvents used for crude sample preparation and HSCCC were of analytical grade and purchased from Beijing Chemical Factory (Beijing, China). Methanol used for HPLC analysis was of chromatography grade and purchased from Beijing Chemical Factory (Beijing, China).

Other chemicals were of analytical grade commercially available (Tianjin Damao Chemical Reagent Factory, China), including ferric chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), tetraethyl orthosilicate (TEOS), γ-aminopropyltriethoxysilane (KH550), succinic acid, urea, 1,2-propylene glycol (PG), concentrated hydrochloric acid (12 mol/L HCl), ammonium hydroxide ($NH_3{\cdot}H_2O$), and dimethyl sulfoxide (DMSO). 1, 6-Diisocyanatohexane (DIH) was purchased from J&K Scientific Ltd (Beijing, China). The chiral ionic liquid 1-ethyl-3-methyl-imidazolium L-proline (EMIMLpro) was obtained from Shanghai Cheng Jie Chemical Co., LTD. The circular magnets were purchased from Beijing Kehuajingwei Scientific Company Limited.

2.3. Synthesis of MNPs

The Fe_3O_4 and Fe_3O_4@SiO_2 MNPs were synthesized by a solvothermal reaction according to a previous study [17]. Briefly, a mixture consisting of 0.81 g (3 mmol) $FeCl_3{\cdot}6H_2O$, 1.8 g (30 mmol) urea, and 0.12 g (1 mmol) succinic acid was completely dissolved in 30 mL of propylene glycol by ultrasonication. Then the solution was transferred to a Teflon-lined stainless steel autoclave, sealed and heated at 200 °C for 8 h. After the completion of reaction, the synthetic MNPs were put in a breakerflask, and then dispersed in ethanol or water under vigorous shaking or ultrasonic vibration for 5 min. Then, the MNPs were rapidly isolated from the dispersed solution with the presence of an external magnetic field (magnets under the bottom of breakerflask), and the supernatant was poured out. The MNPs were washed 5 times using ethanol or water with the help of a magnet, and kept in ethanol.

The above produced Fe_3O_4 MNPs were suspended in 1 mol/L HCl aqueous solution (50 mL) and ultrasonicated for 10 min. After washing three times with deionized water, the magnetically collected Fe_3O_4 MNPs were then dispersed in the 200 mL mixture of ethanol and deionized water at the ratio of (4:1, v/v), along with an addition of 5 mL of concentrated ammonia. The suspension was treated with ultrasonication for 30 min, followed by the dropwise addition of 100 μL of TEOS diluted in 20 mL of ethanol. That mixture was mechanically stirred for 4 h. The pH adjusted to 7 using 1 mol/L HCl to terminate the reaction and the product Fe_3O_4@SiO_2 MNPs were collected with the help of magnet, washed with ethanol and water several times, and finally kept in ethanol.

The above obtained Fe_3O_4@SiO_2 MNPs of 400 mg were dispersed in 150 mL of anhydrous toluene by sonication, and then excessive KH550 (4 mL), 1 mL triethylamine as catalyst were added. Then the mixture was stirred at 130 °C for 24 h under nitrogen. The resultant magnetic nanoparticles were separated from the solvent by magnet, washed 5 times with methanol and then the product Fe_3O_4@SiO_2@KH550 was dried under vacuum at 70 °C for 8 h.