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Preparation, characterization and application of molecularly imprinted monolithic column for hesperetin

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ABSTRACT

The molecularly imprinted solid-phase extraction (MISPE) monolithic column coupled with high-performance liquid chromatography (HPLC) was firstly developed for the extraction of hesperetin in the flesh of *Citrus reticulata cv. Chachiensis*, which is a traditional Chinese medicine (TCM). The molecularly imprinted polymers (MIPs) have been prepared by a thermal polymerization method using hesperetin as the template, acrylamide (AM) as functional monomer and ethylene glycol dimethacrylamide (EGDMA) as cross-linker in the mixed porogen of methanol, toluene and dodecanol. The prepared MIPs were characterized in detail by SEM and FTIR. The results confirmed the uniform and open structure of network skeleton with large flow-through pores. The influence of synthesis conditions on the specific recognition properties of hesperetin MIPs were also investigated systematically. The results showed that high adsorption capacity and good selectivity of MIPs were achieved when using non-imprinted polymer monolith (NIP) and structure similarly compound rutin as references. Furthermore, several parameters of the MISPE method have been optimized, and then it was successfully applied to the extraction of hesperetin from the flesh of *Citrus reticulata cv. Chachiensis*. Good gathering and impurity removing ability of prepared MIP were demonstrated. The MISPE method was proven to be a potentially competitive technique for separation and cleanup of hesperetin in complex TCM with satisfied recovery (90.8 ± 3.2%) and good precision (RSD = 6.48%).

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1. Introduction

Hesperetin, a flavonoid occurs naturally in citrus fruits [1,2], exerts a variety of pharmacological effects main including anti-inflammatory, antioxidant and anti-rheumatic [3]. The dried pericarp of *Citrus reticulata Blanco* which contains hesperetin has been widely used as important medicinal herb in China, Korea and Japan for a long time [4,5], and the clinical application include relieving cough, eliminating phlegm and alleviating stomachache [3]. Although the pericarp has been widely used as traditional Chinese medicine (TCM), the flesh of *Citrus reticulata Blanco* was usually thrown away as useless. Little research has been reported about the hesperetin in the flesh of *Citrus reticulata Blanco*. Due to the complexity of natural matrices, the extraction and purification methods are essential for the utilization of hesperetin from *Citrus reticulata Blanco*.

The conventional extraction methods of active components from natural products are usually based on the liquid–liquid extraction, solid-phase extraction and matrix solid phase dispersion. These methods are lack of selectivity and lead to the co-extraction of many compounds which have similar physicochemical characteristics. Over the last decades, molecularly imprinted solid-phase extraction (MISPE) has received increasing attention due to the advantages of low cost, storage stability, high mechanical strength, robustness, and the resistance to a wide range of pH, solvents and temperatures [6,7]. The molecularly imprinted polymers (MIPs) are synthetic receptors with a predetermined selectivity toward specified analyte. The recognition sites were generated with a "memory" for the shape, size and functional group positions of the template molecule. They were able to specifically rebind target molecule and offer significantly higher affinity and selectivity, avoiding the co-elution of interfering species [8–10]. Owing to these reported high selectivity, affinity and simplicity, MIPs have been applied as selective sorbent material for the cleanup and preconcentration of several molecules such as nucleosides, analgesics, pesticides, carbohydrates, and steroids [11–15]. However, neither the synthesis of hesperetin MIPs nor the application of MISPE method in

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the extraction of hesperetin from the flesh of *Citrus reticulata cv. Chachiensis* has yet been reported. Therefore, based on the expectation that MISPE could offer advanced specificity and affinity, MIPs were introduced to extract hesperetin directly from the flesh of *Citrus reticulata cv. Chachiensis*.

In this study, non-covalent imprinted polymers using hesperetin as template molecule were synthesized adopting acrylamide (AM) as functional monomers, and ethylene glycol dimethacrylate (EGDMA) as cross-linker in the mixed porogen of methanol, toluene and dodecanol. High-pressure liquid chromatography (HPLC) was used to evaluate the adsorption and selectivity properties of the MIPs. Finally, the proposed MISPE procedure was firstly developed and it was proven to be applicable for the selective extraction and cleanup of hesperetin from the flesh of *Citrus reticulata cv. Chachiensis*.

2. Experimental

2.1. Materials and regents

Hesperetin was supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ethylene glycol dimethacrylate and acrylamide were purchased from Aladdin Reagents (Shanghai, China). Azobisisobutyronitrile (AIBN), toluene, dodecanol and acetic acid were purchased from Tianjin Kermel Chemical Reagent Co. Ltd. (Tianjin, China). Methanol and acetonitrile were of chromatographic grade (Darmstadt, Germany). Water was prepared using an ultrapure water system (Millipore Corporation, Bedford, MA, USA). All solutions used for HPLC were filtered through a 0.22 μm filter before use.

2.2. Instruments

The chromatographic analysis was carried out on an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a G1328B manual injector (with a 20 μL loop). A reversed phase Diamonsil-C_{18} column (250 mm × 4.6 mm i.d., 5 μm) provided by Dikma Technologies Incorporation (Beijing, China) was used for the separation of analytes. The solid-phase extraction apparatus (Zhizhen Biological Technology Co., Ltd, Guangzhou, China) was used to control the volume and flow speed of solvents. A JSM-6330 Feld emission scanning electron microscope and an AUW220D electronic balance were gained from Japan Electronics Co. Ltd (Qingdao, China) and Shimadzu (Guangzhou, China). FT-IR spectra were obtained via a Bruker Tensor - 37 Fourier Transform Near Infrared spectrometer (Bruker Optik GmbH, Ettlingen, Germany).

2.3. Preparation of MIPs and NIPs

In order to prepare the hesperetin-imprinted polymer monolith column, the template molecule hesperetin (0.025 mmol) and the functional monomer acrylamide (0.05 mmol) were dissolved in 1.0 mL of mixed porogenic solvents (toluene:dodecanol:methanol, 1:2:2, v/v). 2.0 mL of syringe sealed at the tip end was filled with the above mixture, and then the mixture was ultrasonicated for 20 min. After that, cross-linker EGDMA (188 μL) and initiator AIBN (10 mg) were added and ultrasonicated for 20 min. To remove oxygen, the mixed solution was purged with nitrogen gas for 10 min, and then the syringe was sealed with silicon rubber at the other end. Subsequently, polymerization was performed at 60 °C for 24 h. After polymerization, the silicon rubber was removed and the sealed end of the syringe was cut off. Finally, the resultant MIP monolith was set on the digital control solid-phase extraction apparatus and washed with methanol–acetic acid (80:20, v/v) to remove the template molecule. Non-imprinted polymer monolith (NIP) was also prepared simultaneously in an identical manner to MIPs, although in the absence of the template molecules.

2.4. Sample preparation

Citrus reticulata cv. Chachiensis was collected from Xinhui district of Guangdong Province (Guangdong, China). The flesh of *Citrus reticulata cv. Chachiensis* was dried under the temperature of 60 °C for 12 h and then smashed to powder by pulverizer. The powder (5.0 g) was placed into a 250 mL of round-bottom flask, followed by 100 mL of methanol. Subsequently the flask was marinated into a water bath at 70 °C for 90 min. And then reduced pressure distillation was used to concentrate the volume of extracts to 50 mL.

2.5. MIP and NIP-SPE procedures

The prepared MIP or NIP was installed on the digital control solid-phase extraction apparatus. Firstly, the MIP/NIP was conditioned with 2.0 mL methanol and 1.0 mL of 0.1% ammonia water respectively. Secondly, 1.0 mL sample solution was loaded on the MIP/NIP at a flow rate of 1.0 mL/min, and then the MIP/NIP was washed with 4 mL of 5% methanol in water (v/v) at a flow rate of 0.1 mL/min to remove the impurities in sample. Finally, the elution of analytes was conducted by using 6 mL methanol–acetic acid (80:20, v/v) solution at the flow rate of 0.1 mL/min. The collected loading solution, washing solution and elution solution were evaporated to dryness under nitrogen and the residues were redissolved in 1 mL of methanol before HPLC analysis.

3. Results and discussion

3.1. Preparation and evaluation of hesperetin-imprinted monolithic column

3.1.1. Optimization of synthesis conditions

The imprinting effect of MIPs has close relationships with the conditions of polymerization, and a number of factors require careful consideration such as the nature of functional monomer, porogenic solvent and cross-linker, the amount of cross-linker and the molar ratio of template molecule to functional monomer. Among these factors, it is crucial to choose a suitable functional monomer which can strongly interact with template and form specific "donor–receptor" or "antibody–antigen" complexes prior to polymerization [16,17]. As can be seen in Fig. 1, the hesperetin molecule contains three hydroxyl groups and they can act as the hydrogen-bond donor sites. Therefore, acrylamide (AM) with the carbonyl group was chosen as the functional monomer because it could form a stable complementary complex with hesperetin, and this is crucial for the subsequent affinity and selectivity of the MIPs based on the non-covalent molecular imprinting technique.

The selection of porogenic solvents was also significant in preparing MIP monolithic column, because the strength of non-covalent interactions is highly determined by the porogen, and the porogenic solvents also influence polymer morphology in terms of specific surface area and pore size [18–20]. In the selection of right porogenic mixture, some properties must be considered. First, all the components including template molecule, initiator, functional monomer and cross-linker could be dissolved in the porogenic solvents and brought into one phase. Otherwise, the undissolved floccule would result in unfavorable MIPs with inhomogeneous pores and reduced internal surface area [21]. Second, the porogenic solvents should both generated large pores and small pores for providing adequate flow-through properties and large surface area of the resulting polymers [22]. Finally, the polarity of porogenic solvents should be relatively low for reducing the interferences during complex formation between the template molecule

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