Journal of Chromatography A, 1483 (2017) 20–29

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Extraction, isolation, and aromatase inhibitory evaluation of low-polar ginsenosides from *Panax ginseng* leaves

Yuchi Zhang[a], Jianxu Zhang[b], Chunming Liu[a,*], Min Yu[c,*], Sainan Li[a]

[a] Central Laboratory, Changchun Normal University, No. 677 North Changji Road, Erdao District, Changchun 130032, China
[b] Department of Rehabilitation, Second Branch of the First Hospital of Jilin University, Erdao District, Changchun 130031, China
[c] Department of Nephrology, Affiliated Hospital, Academy of Military Medical Sciences, No. 8 East Avenue, Fengtai District, Beijing 100071, China

ARTICLE INFO

Article history:
Received 30 September 2016
Received in revised form 5 December 2016
Accepted 22 December 2016
Available online 23 December 2016

Keywords:
Accelerated solvent extraction
Centrifugal partition chromatography
Aromatase inhibitor
Mathematics
Panax ginseng leaves

ABSTRACT

A hyphenated accelerated solvent extraction (ASE) technique was elaborately coupled with centrifugal partition chromatography (CPC), ultra-high-performance liquid chromatography (UHPLC), and photodiode array detector (PDA). This approach was applied to obtain low-polar ginsenoside fractions from the leaves of *Panax ginseng*. The CPC fractions were isolated and analyzed using the hyphenated technique, and followed by testing and evaluation of their aromatase inhibitory effects. Subsequently, the aromatase inhibition rates of the compositions in the CPC fractions were calculated using a multivariable linear regression model. A biphasic ethyl acetate/*n*-butanol/ethanol/water solvent system with respective volume ratios of 10:2:2:8 was used for the ASE and CPC separation of 200 g of leaves of *P. ginseng* raw material. The (lower) aqueous phase of the abovementioned solvent system was used as the extraction solvent. The ginsenosides were subjected to ASE, and the extraction solution was pumped into the sample loop and then directly into the CPC column. The CPC fractions were collected and monitored by an online UHPLC/PDA system at 5-min intervals. The aromatase inhibitory activities of CPC fractions were analyzed by a fluorescence method, with mathematical calculations indicating that the inhibition rates of ginsenosides Rk_1, Rg_5, Rs_5, 20R-Rg_3, and Rs_4 exceeded 50.00%; indicating that the aforementioned chemical compounds have potential for further development. The results were validated by comparison with authentic standards, indicating that the method used in this research was accurate and advantageous for matrix analysis.

1. Introduction

The extraction, fractionation, and screening of the bioactive chemical constituents of medicinal herbs are a challenging field in natural drug development [1]. The conventional process for active chemical component screening is mature and effective, with a wide application range. However, the extraction, filtration, concentration, separation, fraction analysis, and bioactivity screening processes are performed independently. Following extraction, the extracted solution should be filtered, concentrated, dried, and re-dissolved in appropriate solvents prior to separation. After separation, the chromatographic eluents still need to undergo concentration, drying, and other processes for analysis [2,3]. To expedite these non-automatic and non-industrial production processes, integrated techniques [4] and automated extraction and separation processes [5,6] have been pursued for the extraction and isolation of natural products. Herein, we establish a new hyphenated strategy of accelerated solvent extraction (ASE), coupled online with centrifugal partition chromatography (CPC) and ultra-high-performance liquid chromatography/photodiode array detector (UHPLC/PDA) for the separation and analysis of fractions from medicinal herbs.

Panax ginseng C.A. Meyer, belonging to the *Panax* family, is one of the most famous medicinal herbs in eastern Asia [7]. Being a valuable medicinal herb, the roots of *P. ginseng* is highly priced. The chemical composition of ginseng leaves is similar to that of ginseng roots [8]; therefore, in some cases, the ginsenosides from ginseng leaves can be used as substitutes for the ones from *P. ginseng* roots. The chemical nature of the ginsenosides obtained is dependent on the processing temperature of ginseng roots and leaves [9,10]. Ginsenosides Rb_1, Rb_2, Rc, Rg_2, *etc.* are the major extract constituents at normal temperature (<100 °C), while less polar ginsenosides such as Rg_3, Rg_6, F_4, Rs_5, Rs_4, Rg_5, and Rk_1 are the unique extract constituents at higher temperatures (>120 °C) [11,12]. Several reports

* Corresponding authors.
E-mail addresses: chem_lab@sina.cn (C. Liu), papaym@163.com (M. Yu).

http://dx.doi.org/10.1016/j.chroma.2016.12.068
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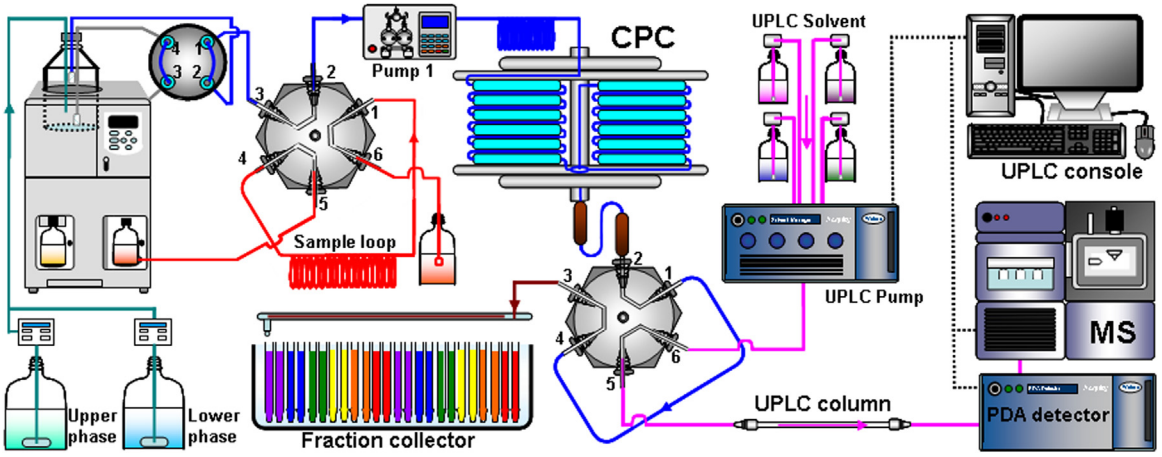

Fig. 1. Diagram of the instrumental setup enabling hyphenation of ASE, CPC, and UHPLC/PDA/MS.

have shown that low-polar ginsenosides exhibit potent biological activities, such as radical scavenging, vasodilating, neuroprotective, and anti-tumor ones [13,14], with the anti-tumor activity of low-polar ginsenosides exceeding that of their polar counterparts [15].

The activity screening of high-temperature *P. ginseng* leaves extracts showed their strong aromatase inhibitory activity. Therefore, these extracts were investigated to analyze the aromatase inhibitory activities of their constituent monomeric compounds, using the ASE/CPC/UHPLC/PDA technique for compound extraction and separation. However, the leaves of *P. ginseng* contain more than 20 compounds, making their complete separation challenging. Furthermore, using chromatography to separate natural compounds makes it difficult to obtain compound fractions with purities above 98% [16–18], complicating the accurate activity evaluation of each compound. Moreover, the separation of all compounds in the high-temperature extracts of *P. ginseng* leaves would waste a lot of manpower, material resources, and time. To solve this problem, mathematical analysis was employed as the method of choice, with chromatographic fractions and their activities as its object. The fraction chemical compositions and activities were fitted with functional relations, and the activity of each compound in the fraction was calculated.

The aims and achievements of this article are a) to establish a new "hyphenated" strategy of ASE coupled online with CPC and UHPLC/PDA for the high-temperature extraction, online separation, and analysis of fractions (low-polar ginsenosides) from *P. ginseng* leaves; and b) to calculate the activities of individual compounds by analyzing the composition and activities of CPC fractions. The method established in this study is advantageous for the activity evaluation of complex compounds, modifying the conventional usage of CPC, and can be used for the analysis and screening of active components from medicinal herbs.

2. Experimental

2.1. Reagents and raw materials

n-Butanol, ethyl acetate, and methanol were of analytical grade (Beijing Chemicals, Beijing, China). Water was purified using a Milli-Q water purification system (Millipore, Boston, USA). Acetonitrile was of HPLC grade (Fisher Scientific, Shanghai, China). Leaves of *Panax ginseng* were purchased from the Changchun YuZhenTang Medicinal Store and identified by Prof. Yuchi Zhang (Changchun Normal University, Changchun, China). Aromatase was purchased from Yuanye (Shanghai, China), and phosphate buffered saline was purchased from Bueke (Switzerland).

2.2. Apparatus

An Accelerated Solvent Extraction 150 System (Dionex, Sunnyvale, CA, USA) with a 100-mL stainless steel ASE vessel was used. Centrifugal partition chromatography was performed on an SIC CPC-240 high-performance centrifugal partition chromatography system (System Instruments Co., Ltd., Japan) modified in our laboratory. The CPC column featured a stacked circular partition disk rotor containing 2136 partition cells with a total internal volume of ~240 mL. The column was connected to the injector and the detector *via* two high-pressure rotary seals. A four-port valve integrated into the CPC apparatus allowed operation of the column in either ascending or descending mode, depending on the relative density of the mobile and stationary phases. UHPLC analysis was conducted using a Waters Acquity H class instrument coupled with a Waters diode array detector (Milford, USA). UHPLC analysis was conducted using a Thermo Scientific LCQ Fleet^{TM} mass spectrometer with an electrospray ionization (ESI) source (Massachusetts, USA).

2.3. Online ASE/CPC/UHPLC/PDA

A schematic diagram of the online ASE/CPC/UHPLC/PDA system is shown in Fig. 1. Samples were ground in a high-speed disintegrator (Model SF-2000, Chinese Traditional Medicine Machine Works, Shanghai, China) to obtain a fine powder (particle size: 0.6 mm). The dry powder (10.0 g) was placed into the 100-mL ASE vessel, and the chemical reagent pumps were set at a certain flow rate to pump the reagents into the mixer and degasser. Subsequently, the mixed solvent separated into two layers in an ASE solvent bottle, and the four-port valve was turned to link ports 1/2 and 3/4, while the left six-port valve was turned to link ports 1/6, 2/3, and 3/4. The lower phase in the solvent bottle was then poured into the ASE vessel to extract the raw materials using the following extraction parameters: extraction time, 5 min; number of extractions, one; extraction temperature, 130 °C. During the extraction, the upper phase in the ASE solvent bottle was pumped into the CPC column by pump 1, and column rotation was started (1100 rpm, 10 min). The four-port valve was then turned to link ports 1/4 and 2/3, while the lower phase in the solvent bottle was pumped into the CPC column at a flow rate of 2.0 mL/min. After ASE extraction, the extraction solution was flowed into the sample loop *via* the left six-port valve (ports 1/6, 2/3, and 3/4 were still linked), which was then turned to link ports 1/2, 3/4, and 5/6, and the extraction solution in the