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Preparative separation of the polar part from the rhizomes of *Anemarrhena asphodeloides* using a hydrophilic C18 stationary phase

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ABSTRACT

The goal of this study was to develop a method that utilized a hydrophilic C18 stationary phase in the preparative high performance liquid chromatography to isolate the polar part from the rhizomes of *Anemarrhena asphodeloides*. The results showed that an initial mobile phase of pure water for the separation could greatly increase the retention and solubility of the polar compounds at the preparative scale. Introducing polar groups on the surface of the hydrophilic C18 column together with the use of optimized mobile phase compositions improved the column separation selectivity for polar compounds. Eleven previously undescribed compounds in *Anemarrhena asphodeloides* were obtained, indicating that the method developed in this study would facilitate the purification and separation of the polar part of traditional Chinese medicines.

1. Introduction

Anemarrhena asphodeloides (A. asphodeloides), as a traditional Chinese medicine, has been commonly used for therapeutic purposes for thousands of years. Over 100 compounds have been isolated and identified from *A. asphodeloides*, including steroidal saponins, xanthones, phenylpropanoids, alkaloids, organic acid and others [1–3]. Among them, most are from the category of the saponins [4–6]. Of note, there are only a few studies that focus on the separation and identification of compounds from the polar part of *A. asphodeloides* due to the lack of proper separation materials and methods. In order to further explore the compositions, it is important to develop the new separation material-based methods for the purification of the polar part in *A. asphodeloides*.

Separation of the polar part of traditional Chinese medicines (TCMs) remains a challenge due to their poor retention on traditional C18 stationary phases. Normal phase liquid chromatography (NPLC) and hydrophilic interaction liquid chromatography (HILIC) could resolve the retention problem [7–9]. However, those polar compounds have poor solubility in the mobile phase with high level of organic solvent, which is typically used as the initial mobile phase in the NPLC and HILIC-based methods [8,10]. This defect becomes more obvious in the preparative chromatography. Development of hydrophilic C18 stationary phase is another feasible way to overcome this contradictory. So far various types of hydrophilic C18 stationary phases have been synthesized and studied, including the polar-endcapped, polar-embedded and polar-copolymerized stationary phases [11–13]. With the introduction of additional polar groups, hydrophilic C18 stationary phase could be potentially used in the highly aqueous mobile phase, which would greatly improve the retention of the polar compounds [14–16]. In addition, those polar groups also play a role in the retention of the polar compounds together with C18, providing with different separation selectivity [17,18].

Recently, we have synthesized a hydrophilic C18 stationary phase, C18HCE, based on the approach of horizontal polymerization of silica surface [19]. As demonstrated, the C18HCE column can be used under the condition of 100% aqueous mobile phase, suggesting that it may have a great potential for the separation of the polar part of TCMs. Therefore, we employed this hydrophilic C18 stationary phase to develop an HPLC-based method for the separation of the compounds from a polar part of *A. asphodeloides*. It should be noted that the selectivity of those stationary phases was influenced significantly by the types of bonded polar groups and the compositions of mobile phase. The retention of the polar compounds on hydrophilic C18 stationary phase would be improved dramatically when pure water was used as the initial mobile phase. Eleven compounds with high purity were isolated

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and identified from *A. asphodeloides* for the first time, demonstrating an alternative method to isolate the compounds from the polar part of TCMs.

2. Experimental

2.1. Apparatus

The analytical experiments were performed on an Alliance high performance liquid chromatography (HPLC) system (Waters, Milford, MA, USA) consisting of a Waters 2695 pump and a Waters 2998 UV detector. Data acquisition and processing were conducted using Waters Empower 3 software.

The pretreatment was performed on a dynamic axial compression (DAC) HB50 purification system (Hanbon, Jiangsu, China). A Waters Purification system was used for further purification, which consisted of a Waters 2545 binary gradient module, a Waters 2767 sample manager and a 2489 UV/Vis detector. Data were recorded by MassLynx 4.1 software (Waters, Milford, MA, USA).

The HRMS analysis was performed on Agilent 1290 Infinity LC/6530 Q-TOF MS (Agilent, USA). ^{1}H NMR spectra and ^{13}C NMR spectra were measured on a BRUKER AVANCE III-500 spectrometer (Bruker, Germany) (^{1}H NMR at 500 MHz; ^{13}C NMR at 125 MHz) with DMSO-d_6 as the solvent.

2.2. Chemicals and materials

Preparative grade acetonitrile and methanol were purchased from Fulltime special solvent Co., Ltd (Anhui, China). HPLC grade acetonitrile and trifluoroacetic acid were purchased from J & K (Beijing, China). The water used in this study was purified by Milli-Q water purification system (Millipore, Bedford, MA, USA).

The rhizomes of *A. asphodeloides* were purchased from Lei Yun Shang Pharmaceutical store (Shanghai, China). Activated carbon was purchased from Shanghai Activated Carbon Co., Ltd (Shanghai, China). 3-chloropropyl trichlorosilane ligand bonded phase (CP) (60 μm, 100 Å) was home-made based on previous reports [20]. C18 stationary phase (60 μm, 100 Å) was purchased from Acchrom Technologies Co., Ltd (Beijing, China).

Unitary C18 column (4.6 × 250 mm, i.d., 5 μm), XAqua C18 column (4.6 × 250 mm, i.d., 5 μm) were purchased from Acchrom Technologies Co., Ltd (Beijing, China). C18HCE columns (4.6 × 250 mm, i.d., 5 μm, 10 × 250 mm, i.d., 5 μm, 20 × 250 mm, i.d., 10 μm,) were prepared in our laboratory.

2.3. Preparation of a polar part

The *A. asphodeloides* powder (17 kg) was extracted twice with ethanol for 1 h. Filtrate of both times was gathered and 0.4% activated carbon (g mL^{-1}) was added for decolorization. The solution was refluxed for 0.5 h and filtered after cooling to room temperature. The discolored filtrate was concentrated by rotary evaporation at 55 °C under reduced pressure. The residue was dissolved in 5 L of water for solid phase extraction (SPE) with the cartridge packing a reversed-phase (RP) material named CP; successively eluted using water and ethanol with three column volumes each time. The ethanol eluate was concentrated by vacuum distillation, which yielded 420 g of residue (called crude extract below). 125 g of crude extract were dissolved in 2.5 L of methanol for further isolation using a preparative DAC column packed with 300 g C18 stationary phase (50 × 250 mm, i.d., 60 μm) with a gradient elution. The polar part (12.8 g) was eluted within five minutes for further separation.

2.4. Chromatographic conditions

Initially, the rough separation of the crude extract was performed on a preparative C18 column (50 × 250 mm, i.d., 60 μm). The mobile phases were water (A) and acetonitrile (B). The gradient condition was 0–30 min, 10–90%B; 30–32 min, 90–90%B. The flow rate was 70 mL min^{-1} and the detective wavelength was 203 nm.

Subsequently, a preparative C18HCE column (20 × 250 mm, i.d., 10 μm) was used to fractionate the polar part. The mobile phase A was 0.01% trifluoroacetic acid aqueous solution, and the mobile phase B was 0.01% trifluoroacetic acid acetonitrile. The gradient elution program was 0–30 min, 10–15%B and the flow rate was 19 mL min^{-1}. Two fractions were collected and marked as P1 and P2.

Compound isolation was performed on a semi-preparative C18HCE column (10 × 250 mm, i.d., 5 μm). The mobile phases were 0.01% trifluoroacetic acid aqueous solution (A), 0.01% trifluoroacetic acid methanol (B) and 0.01% trifluoroacetic acid acetonitrile (C). The gradient elution and injection volume were optimized respectively.

3. Results and discussion

3.1. Preparation of a polar part of A. asphodeloides

The extract of *A. asphodeloides* contains numerous compounds with the polarity and molecular weight varying in a wide range. Thus, it is necessary to pretreat the sample to remove the pigment and polysaccharide, in the forms of active carbon decolorization and simple SPE. To simplify the sample, a conventional C18 column was used for preliminary separation. It was worthy to mention that the C18 material (60 μm, 100 Å) was packed into a DAC column, which was applied in industrial level HPLC separation rather than SPE process. This method provided online detection, higher column efficiency and better separation repeatability relative to SPE. As shown in Fig. 1A, a part that was rapidly eluted before 5 min on C18 column was collected. Herein, the polar part of *A. asphodeloides* in this study refers to the peak that contains the compounds with small molecular weight and is eluted early on traditional C18 column.

The polar part was re-fractionated on a preparative hydrophilic C18 stationary phase (C18HCE, 20 × 250 mm, i.d., 10 μm) using a mild elution condition. Two fractions, P1 and P2, were collected (Fig. 1B). To achieve a better separation, P1 was selected as a representative fraction and was further explored in column screening and separation conditions optimization.

3.2. Column screening for separation of the polar part

As displayed in Fig. 1B, a deformed peak P1 was observed, which was speculated to contain several compounds. In order to characterize the content of this peak, two hydrophilic C18 columns (XAqua C18 and C18HCE) (Fig. 2) were screened for further separation. For comparison, a conventional C18 column (Unitary C18) was subjected to a same condition. The chromatograms for three different columns were shown in Fig. 3. Consistent with the above experimental results, the sample was eluted nearly at the dead time and only three peaks were observed on Unitary C18 column (Fig. 3A). Although the retention time of the peak P1 was just slightly increased on two hydrophilic C18 columns, seven and ten peaks could be distinguished on C18HCE column and XAqua C18 column respectively (Fig. 3B and C). The results revealed that introducing the polar groups on the surface of C18 silica benefited the separation of those polar compounds. The two hydrophilic C18 columns were further investigated for the separation of the peak P1 under a weaker elution condition. To this end, 100% aqueous solution was chosen as an initial mobile phase. As shown in Fig. 4A and B, the retention time of those polar compounds from the peak P1 increased significantly on both columns with improved peak shape and resolution. Noticeably, the retention behaviors of those compounds were dramatically different between these two hydrophilic C18 columns, suggesting a close relationship between the kinds of polar groups and selectivity [21]. Intuitively, C18HCE provided preferable separation as