Separation of vitexin-4"-O-glucoside and vitexin-2"-O-rhamnoside from hawthorn leaves extracts using macroporous resins

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1. Introduction

Hawthorn (Crataegus) leaves are a well-known traditional Chinese medicine and are commonly used to treat blood stasis, memory loss, chest distress, palpitations, dizziness and tinnitus [1]. Phytochemical studies reveal that flavonoids are the major active compounds of hawthorn leaves. Modern pharmacological studies demonstrate that hawthorn leaf flavonoids exhibit many types of biological activities such as cardiovascular regulation [2], antioxidant activity [3], inhibitory effects on α-glucosidase [4], and regulation effects on glucose and lipid metabolism [5,6].

Earlier phytochemical studies on hawthorn leaves resulted in the isolation of approximately 30 flavonoids including vitexin-4"-O-glucoside (VOG), vitexin-2"-O-rhamnoside (VOR), vitexin, quercetin, rutin and hyperoside [7,8]. It has been noted that two C-glycosylflavonoids, VOG and VOR, are major constituents extracted from hawthorn leaves. These two compounds are often selected as markers in the quality control of hawthorn leaf flavonoids because of their high contents and physiological activities [8,9].

It has been reported that VOG and VOR can protect the heart against anoxia/reoxygenation injury [10], inhibit the effects of α-glucosidase [4] and significantly alleviate oxidative stress and apoptosis in human adipose-derived stem cells (hADSCs) caused by H2O2–induced injury [11]. In addition, VOG has been proven to possess the antioxidant activity to protect ECV304 cells from TBHP [12]. Other studies have also suggested that VOR can inhibit DNA synthesis in MCR-7 human breast cancer cells [13] and has a protective effect on injured cardiac myocytes and endothelial cells [14,15]. Hence, it is necessary to develop an effective purification technology to obtain VOG and VOR with high purity for pharmaceutical application.

The conventional method to purify flavonoid compounds from plant materials is by solid–liquid extraction followed by liquid–liquid extraction and various subsequent column chromatography methods (such as silica gel, polyamide, Sephadex LH-20, etc.) [16]. However, these methods are not appropriate
for large-scale preparation because of their several shortcomings, including low recoveries, organic solvent wastage, high cost, time-consuming procedure and environmental pollution. Recently, macroporous resins have been considered promising adsorption materials because of their large surface area, high stability, low operational cost, reduced solvent consumption and easy regeneration [16,17]. Resins have been widely used to purify many types of secondary metabolites such as flavonoids, glycosides, saponins, alkaloids, and lignans [18–24]. In our previous study, macroporous resins have been successfully applied in large-scale separation of chlorogenic acid from Helianthus tuberosus leaves [25].

Recently, macroporous resins as an efficient adsorbent received great progress in the separation of C-glycosylflavonoids, including orientin-2′-O-galactopyranoside and orientin from trollflowers [26], vicenin-2, isoschaftoside and schaftoside from Abrus mollis [16], vitexin and isovitexin from pigeonpea [27]. Hence, it is of interest to determine whether the macroporous resins can exhibit good performance for the separation of VOG and VOR in hawthorn leaves. This has stimulated the present study, which employed macroporous resins for preparative separation of VOG and VOR from hawthorn leaves. The VOG and VOR adsorption and desorption properties of macroporous resins with different chemical and physical properties were investigated in detail. In addition, the variables for the dynamic adsorption and desorption of the selected resin were optimized.

Fig. 1. Adsorption/desorption capacities and desorption ratios of VOG (A) and VOR (B) on different resins; adsorption kinetics curves for VOG and VOR on HPD-400 resin at 25 °C (C); adsorption isotherms for VOG (D) and VOR (E) on HPD-400 resin at different temperatures (25 °C, 35 °C and 45 °C); effects of the pH value on the VOG and VOR adsorption capacities of the HPD-400 resin (F).
2. Materials and methods

2.1. Samples and reagents

VOG and VOR standards (98% purity) were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). The standard was dissolved in 50% methanol to obtain a stock solution. The hawthorn leaf extracts were obtained from Shanxi Pure Source Bio-Tech Co., Ltd. (Xian, China). Distilled water was prepared using a Milli-Q water purification system (Millipore, Boston, USA). Analytical-grade ethanol, acetic acid and HPLC-grade acetonitrile were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Adsorbents

Macroporous resins (HPD-200A, HPD-700, HPD-450, HPD-400 and HPD-600) were purchased from Cangzhou Bonchem Co., Ltd. (Hebei, China), and ADS-21 and ADS-7 were purchased from Nankai Hecheng S &T Co., Ltd. (Tianjin, China). The physical properties of the resins are summarized in Table 1. The resins were first soaked in ethanol for 24 h and subsequently washed with distilled water. Then, the adsorbent beads were treated with 5% (w/v) NaOH solution for 4 h and washed with distilled water. Finally, the resins were soaked in 5% (w/v) HCl solution for 4 h and washed with distilled water until neutral. Prior to use, the pretreated resins were soaked in ethanol and thoroughly washed with distilled water.

2.3. Preparation of sample solutions

The hawthorn leaves extracts were dissolved in distilled water to obtain solutions at different concentrations.

2.4. Static adsorption and desorption tests

2.4.1. Screening of adsorption resins

The static adsorption tests on macroporous resins were investigated as follows. The pretreated hydrated resins (2.00 g) were placed into 100 mL conical flasks with stoppers, and 50.0 mL of sample aqueous solution was added. Then, all flasks were successively shaken at 120 rpm in a constant-temperature shaker (25 °C) for 12 h. Before and after the adsorption, the VOG and VOR concentrations were determined using HPLC.

After attaining the adsorption equilibrium, the resins were washed with distilled water (50.0 mL); then, 50.0 mL of 70% ethanol (v/v) solution was added for desorption. All flasks were shaken at 120 rpm for 12 h at 25 °C. After desorption, the concentrations of VOG and VOR were analyzed using HPLC. The preliminary selection of resins was based on their adsorption/desorption capacities and desorption ratios, which are calculated according to the following equations:

\[
Q_v = \frac{(C_0 - C_e)V_0}{W}
\]

Table 1
Physical properties of the test macroporous resins.

<table>
<thead>
<tr>
<th>Name</th>
<th>Polarity</th>
<th>Surface area (m²/g)</th>
<th>Average pore diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPD-200A</td>
<td>Non-polar</td>
<td>700–750</td>
<td>8.5–9.0</td>
</tr>
<tr>
<td>HPD-700</td>
<td>Non-polar</td>
<td>650–700</td>
<td>8.5–9.0</td>
</tr>
<tr>
<td>HPD-450</td>
<td>Middle-polar</td>
<td>500–550</td>
<td>9.0–11</td>
</tr>
<tr>
<td>HPD-400</td>
<td>Middle-polar</td>
<td>500–550</td>
<td>7.5–8.0</td>
</tr>
<tr>
<td>HPD-600</td>
<td>Polar</td>
<td>550–600</td>
<td>8.0</td>
</tr>
<tr>
<td>ADS-21</td>
<td>Polar</td>
<td>80.0–100</td>
<td>15–20</td>
</tr>
<tr>
<td>ADS-7</td>
<td>Strong-polar</td>
<td>≥100</td>
<td>25–30</td>
</tr>
</tbody>
</table>

Fig. 2. Effects of the initial concentration of the sample solution on the adsorption capacities and adsorption ratios of VOG (A) and VOR (B) on HPD-400 resin; dynamic leakage curves of VOG and VOR in the column with the HPD-400 resin (C); dynamic desorption curves of VOG and VOR in the column with the HPD-400 resin (D) (the column packed with saturated HPD-400 resin was gradually flushed with water (5 BV), 20% (10 BV), 30% (5 BV), 40% (5 BV) and 100% (5 BV) ethanol in water (v/v)).
Desorption capacity

\[ Q_d = \frac{C_d V_d}{W} \]  

(2)

Desorption ratio (%)

\[ D = \left[ \frac{C_d V_d}{(C_0 - C_e)V_0} \right] \times 100\% \]  

(3)

where \( Q_d \) is the adsorption capacity (mg/g); \( Q_d \) is the desorption capacity (mg/g); \( D \) is the desorption ratio (%); \( C_0 \) and \( C_e \) are the initial and equilibrium concentrations of VOG or VOR in the solutions (mg/mL), respectively; \( C_d \) is the concentration of VOG or VOR in the desorption solutions (mg/mL); \( V_0 \) and \( V_d \) are the volumes of the initial sample and desorption solutions (mL), respectively; and \( W \) is the weight of dry resin (g).

2.4.2. Adsorption kinetics

The adsorption kinetics of VOG and VOR on the selected HPD-400 resin were studied according to the method described in Section 2.4.1. The VOG and VOR concentrations were analyzed using HPLC at certain time intervals until equilibrium.

2.4.3. Adsorption isotherms

The adsorption isotherms on HPD-400 resin were performed by contacting the aqueous sample (50.0 mL) at different initial concentrations with a pre-weighted amount of hydrated resins (2.00 g) in a series of 100 mL conical flasks with stoppers. The flasks were shaken at 25, 35 and 45 °C (120 rpm) for 12 h. The initial and equilibrium concentrations of VOG and VOR were determined using HPLC.

In addition, the adsorption properties of the selected resin were evaluated under different initial pH values (2.0–7.0) of the sample solutions.

2.5. Dynamic adsorption and desorption tests

The separation properties of the selected resin were evaluated using dynamic adsorption and desorption tests. First, 15.0 g (wet weight) of HPD-400 resins were wet-packed into a glass column (12 mm × 350 mm), with a bed volume (BV) of 22.0 mL. The adsorption process was performed by pumping the sample solution through the pretreated glass column at a rate of 2 BV/h. After adsorption equilibration, the adsorbed column was washed with distilled water and subsequently desorbed with gradient ethanol-water solutions at a rate of 2 BV/h. The VOG and VOR concentrations in each eluent were analyzed using HPLC. Then, the eluent was concentrated to dryness under vacuum to calculate the product purity.

Several variables were studied, including the initial concentration of sample solution, feeding volume and different proportions of ethanol-aqueous solutions for desorption.

2.6. HPLC analysis of VOG and VOR

The VOG and VOR concentrations were analyzed with an Agilent 1200 series HPLC system (Palo Alto, CA, USA), which consisted of 1200 ChemStation software, a G1311A quaternary pump, a G1329A autosampler, a G1316A column oven and a G1314A variable-wavelength DAD detector. Analysis was performed on an Agilent Zorbax Extend C18 column (4.6 × 250 mm, 5 μm) with an Agilent

Fig. 3. Chromatograms of the sample before (A) and after (B) purification on the column packed with HPD-400 resin.
Zorbax Stable Bond C18 guard cartridge (12.5 × 4.6 mm I.D., 5 μm). Water with 1% acetic acid (A) and acetonitrile (B) was used as the mobile phase. The solvent gradient program was set as follows: 0–15 min, 15% B; The gradient was ascended to 100% B for an additional 15 min to clean up the column, followed by reequilibration of the column for 15 min with 15% B before the next run. The column temperature was maintained at 30 °C. The flow rate was 1.0 mL/min, the injection volume was 5 μL, and the detection wavelength was 350 nm. The regression equations for VOG and VOR are $A = 8.122C + 7.209 (R^2 = 0.9997)$ and $A = 7.816C - 8.812 (R^2 = 0.9988)$, respectively, where $A$ is the peak area and $C$ is the concentration (μg/mL).

3. Results and discussion

3.1. Adsorption and desorption capacities, desorption ratio of the resins

Seven macroporous resins ranging from non-polar to highly polar were applied to purify VOG and VOR, and their adsorption/desorption capacities and desorption ratios are shown in Fig. 1A and B. It can be observed that the HPD-400, HPD-200A, HPD-450, HPD-600, HPD-700 resins have considerably higher VOG and VOR adsorption capacities than other resins. Both VOG and VOR are composed of non-polar flavone aglycone and polar glucoside groups and are easily adsorbed by HPD-400 and HPD-450 resins with moderate polarity. The non-polar HPD-700 and HPD-200A resins also show better adsorption and desorption capacities because of their high surface areas. Among the polar resins, the HPD-600 resin with higher surface area has better adsorption capacity than the ADS-7 and ADS-21 resins. In addition, the HPD-400 resin has the highest VOG and VOR adsorption and desorption capacities, and its desorption ratio is only slightly lower than that of the HPD-200A resin. Hence, the HPD-400 resin was selected as a suitable resin to further study the adsorption behaviors towards VOG and VOR.

3.2. Adsorption kinetics

The adsorption kinetics curves for VOG and VOR on HPD-400 resin were obtained at 25 °C and are shown in Fig. 1C. The VOG and VOR adsorption capacities rapidly increased with contact time in the first 3 h and subsequently slowly increased. The adsorption equilibrium for two compounds was observed at approximately 4 h. Hence, 4 h is sufficient to establish the adsorption equilibrium for VOG and VOR.

3.3. Adsorption isotherms

The equilibrium adsorption isotherms were studied by adding 50.0 mL of aqueous solution with different initial concentrations (0.0710–0.365 mg/mL and 0.260–1.37 mg/mL for VOG and VOR, respectively) to the HPD-400 resin at 25, 35 and 45 °C. As shown in Fig. 1D and E, the VOG and VOR adsorption capacities of the HPD-400 resin dramatically increased at lower initial concentrations and subsequently attained adsorption equilibrium at 0.299 and 1.13 mg/mL, respectively. In addition, the temperature distinctively affects the adsorption. High adsorption capacity was observed at low temperature, which indicates that the adsorption process is an exothermic chemical process.

In general, information about the affinity between solutes and adsorbents can be obtained from the equilibrium adsorption isotherms. The following Langmuir and Freundlich equations are commonly used to reveal the linearity fitting and describe how the solutes interact with the resin during the adsorption process.

Langmuir equation:

$$Q_e = \frac{Q_{\text{max}}K_C}{1 + KCT}$$

(4)

where $Q_e$ (mg/g) and $C_t$ (mg/mL) are identical to those in Eq. (1); $Q_{\text{max}}$ (mg/g) is the maximum adsorption capacity; and $K$ (mg/mL) is the adsorption constant.

Freundlich equation:

$$Q_e = K_fC_t^{1/n}$$

(5)

where $K_f$ is the Freundlich constant, which indicates the adsorption capacity; $1/n$ is an empirical constant related to the adsorption affinity of the adsorbent for the adsorbate.

The Langmuir and Freundlich parameters at different temperatures (25, 35 and 45 °C) are listed in Table 2. The correlation coefficients (0.9632–0.9988) of both Langmuir and Freundlich equations for VOG and VOR adsorbed on HPD-400 resin are notably high, which suggests that the models are suitable for describing the tested adsorption system in the studied concentration range. The adsorption is commonly considered likely to occur when $1/n$ is between 0.1 and 0.5 in the Freundlich equation [28]. In Table 2, all values of $1/n$ are in the range of 0.1891–0.2384, which demonstrates that VOG and VOR can be easily adsorbed by the HPD-400 resin. Hence, the HPD-400 resin is suitable for the purification of the two compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Initial content in extracts (%)</th>
<th>Content in product (%)</th>
<th>Recovery yield (%)</th>
<th>RSD for recovery yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VOG</td>
<td>0.720</td>
<td>6.08</td>
<td>79.1</td>
<td>1.41</td>
</tr>
<tr>
<td>VOR</td>
<td>2.63</td>
<td>22.2</td>
<td>81.2</td>
<td>1.51</td>
</tr>
</tbody>
</table>

Table 2

Langmuir and Freundlich adsorption parameters of VOG and VOR on HPD-400 resin at different temperatures.

<table>
<thead>
<tr>
<th>Temperature(°C)</th>
<th>Langmuir equation</th>
<th>Freundlich equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>VOG</td>
<td>$C/Q_e = 0.0645C_e + 0.00007$</td>
<td>$Q_e = 22.8C_e + 0.2336$</td>
</tr>
<tr>
<td>25</td>
<td>0.9932</td>
<td>0.9948</td>
</tr>
<tr>
<td>35</td>
<td>0.9959</td>
<td>0.9932</td>
</tr>
<tr>
<td>45</td>
<td>0.9962</td>
<td>0.9958</td>
</tr>
<tr>
<td>VOR</td>
<td>$C/Q_e = 0.0171C_e + 0.00007$</td>
<td>$Q_e = 62.9C_e + 0.2384$</td>
</tr>
<tr>
<td>25</td>
<td>0.9938</td>
<td>0.9911</td>
</tr>
<tr>
<td>35</td>
<td>0.9988</td>
<td>0.9632</td>
</tr>
<tr>
<td>45</td>
<td>0.9971</td>
<td>0.9828</td>
</tr>
</tbody>
</table>
3.4. Effect of the sample solution pH value

The initial pH value of the sample solution has an important effect on the adsorption capacity of the resin. The pH value can affect the adsorption affinity by determining the extent of ionization of the sample molecules. As shown in Fig. 2F, for VOG and VOR, the HPD-400 resin showed higher adsorption capacities at pH 5.0 than at other pH values. The results suggest that hydrogen bonding may play an important role in the adsorption process on the HPD-400 resin. The phenolic hydroxyl groups of VOG and VOR can dissociate to form H+ and corresponding anions at higher pH values. Then, the ionization process decreases the hydrogen bonding interactions and consequently reduces the adsorption interaction between VOG or VOR and the HPD-400 resin. Therefore, the pH value of the sample solution was adjusted to 5.0 for all subsequent experiments.

3.5. Effect of the sample concentration

The initial concentration of the sample solution has an important effect on the affinity of VOG and VOR to the resins. In general, the adsorption capacity of the resin increases with the concentration of the sample, whereas the adsorption rate decreases. The effect of the sample concentration on the dynamic adsorption capacity of the HPD-400 resin was evaluated by loading different concentrations of hawthorn leaf extract onto six resin columns. As shown in Fig. 2A and B, for VOG and VOR, the adsorption capacities rapidly increased with increasing concentration and reached the saturation plateau when the initial concentration of hawthorn leaf extract was 60.0 mg/mL. In contrast, the adsorption rate significantly decreased at higher concentrations. Thus, 60.0 mg of extracts per 1 mL of solution was selected as the initial concentration of the sample solution for subsequent experiments.

3.6. Dynamic breakthrough curves of the HPD-400 resin

The breakthrough curves of the HPD-400 resin were provided based on the volume of effluent liquid and the concentrations of VOG and VOR at a flow rate of 2 BV/h. In general, it is defined that the adsorption process reaches the leakage point when the concentration in the effluent is 10% of the original concentration. As shown in Fig. 2C, VOG and VOR have identical leakage points because of their similar structure and polarity. Before 8 BV, both VOG and VOR in the extract solutions were almost completely adsorbed by the HPD-400 resin. Then, the concentrations of VOG and VOR in the effluent liquid rapidly increased until it reached a steady plateau at 14 BV. A sample loading of 7 BV (154 mL) was selected for the dynamic adsorption.

3.7. Dynamic desorption of the HPD-400 resin

Ethanol is the most common desorption solution because it is easily recovered and lacks toxicity. The ethanol concentration is one of the most important factors in the dynamic desorption process of adsorbates from the resins. Herein, different concentrations of ethanol solution were used for the dynamic desorption of VOG and VOR at a flow rate of 2 BV/h. After the desorption, the fully saturated column was washed with distilled water (5 BV) and subsequently flushed with 20% (10 BV), 30% (5 BV), 40% (5 BV) and 100% (5 BV) ethanol in water (v/v). As shown in Fig. 2D, for both VOG and VOR, 20% ethanol (v/v) was the best eluent. When the ethanol concentration was 30%, more impurities were desorbed. Hence, 10 BV of 20% ethanol was selected as the desorption solution for high yield.

The VOG and VOR concentrations in each eluent were analyzed using HPLC. As shown in Table 3, after one run treatment with the HPD-400 resin, the VOG and VOR contents in the product were increased 8.44-fold and 8.43-fold from 0.720% and 2.63% to 6.08% and 22.2%, respectively, and the recovery yields were 79.1% and 81.2%, respectively.

The HPLC chromatograms (Fig. 3) of the samples before and after the treatment with HPD-400 show that some impurities in the hawthorn leaf extracts were removed and that the relative peak areas of VOG and VOR significantly increased.

4. Conclusion

An effective method to purify VOG and VOR from hawthorn leaves using macroporous resin has been developed in the present study. Seven macroporous resins were tested, and the HPD-400 resin shows the best adsorption and desorption capacities for VOG and VOR. The adsorption equilibrium experimental data of two compounds on HPD-400 resin at different temperatures (25, 35, and 45 °C) were well fitted to the Langmuir and Freundlich isotherms. After one treatment on the column packed with HPD-400 resin under optimal conditions, the VOG and VOR contents increased from 0.720% and 2.63% in the extracts to 6.08% and 22.2% in the product, respectively. The recovery yields of VOG and VOR were 79.1% and 81.2%, respectively. Compared to the conventional method, the adsorption-desorption method is more advantageous because of its low cost, high efficiency, reduced solvent consumption, harmlessness to the environment, etc. Therefore, the developed method in this study is suitable for large-scale purification of the flavonoid C-glycosides VOG and VOR from the leaves of hawthorn and other herbal plants.

Competing interests

The authors declare that they have no competing interests. This article does not contain any studies with human or animal subjects.

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