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Preparative separation of four individual flavonoids in *Scutellaria barbata* D. Don based on high selectivity polymeric adsorbents with different polarities

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ABSTRACT

The individual flavonoid component, scutellarin, scutellarein, luteolin and apigenin, in *Scutellaria barbata* plant was isolated based on the macro porous adsorbent with high adsorption selectivity. These adsorbents were synthesized based on the copolymerization of methyl acrylate and divinylbenzene (MA-co-DVB). So the polarity and the adsorption affinity of these adsorbents can be adjusted through changing MA content in the adsorbents. And then the ability of the adsorbent with different MA contents for isolation of these four individual flavonoid was also investigated. Adsorbents M2 and M4, with MA content of 25% and 45%, respectively, demonstrated the best separation ability. Complete separation of the four flavone compounds was achieved in a continuous process based on combination of adsorbents with different polarities (M2 and M4). Gradient elution using adsorbent M4 separated the four flavonoids into three fractions, which were determined to contain scutellarin, scutellarein and a mixture of luteolin and apigenin. The latter was separated completely by adsorbent M2 subsequently. All four compounds were obtained at high resolution and high recovery yield (96.7%, 94.1%, 95.8% and 93.8%, respectively), suggesting the efficiency of sequentially combined columns with different segregation patterns.

1. Introduction

Scutellaria barbata D. Don (Lamiaceae; SB), a perennial herb found in southern China, has been used as an anti-inflammatory and antitumor agent in traditional Chinese medicine [1–3]. The main biologically active components in SB are flavonoids, among which scutellarin, scutellarein, luteolin and apigenin have been studied mostly [4–7]. Although the four flavonoids are similar in chemical structure (Fig. 1), each of them shows different pharmacological activities according to recent studies. For example, scutellarin and scutellarein have definite efficacies on the cardiovascular system of human body [8,9]. Scutellarin has often been applied in prevention of neurodegenerative disorders [10] and inhibition of HIV-1 virus replication [11]. Scutellarein has already been proved to be an anticancer agent [12], and luteolin and apigenin have also been used in the treatment of various cancers [13–16]. However, large-scale separation of each individual flavone component from SB plant extracts is challenging, therefore it is of paramount importance to develop efficient separation methods to support the biological studies and clinical usage of these flavonoids.

Column chromatography is a well-developed and high resolution technology for separation of compound with similar structure, that includes silica gel chromatography, preparative chromatography, and high-speed counter-current chromatography [17–20]. However, due to their separation mechanism, these methods are of some obvious limitations, such as low processing capacity, large solvent consumption, and harsh operating conditions (for example, the operation must be done with high pressure and the packing size must be quite uniform). These limitations have restricted the application of the techniques mentioned above to large-scale industrial preparation.

In recent years, macroporous polymeric adsorbents have been widely used to extract and purify bioactive components [21–23], and to separate different components [24–26] of natural products. As one of the important packing material of column chromatography, polymeric adsorbent is of obvious advantage, such as higher processing capacity, lower operation expense, environmentally friendly and suitable for large-scale production with easy operation. But polymeric adsorbent is limited from the separation of the compounds with very similar structure, because of the low adsorption selectivity and unitary hydrophobic adsorption mechanism of the resin material, which usually contains only hydrophobic groups. Introducing the amide groups to the adsorbents could enhance the adsorption selectivity towards flavone compounds, presumably through the introduction of synergistic effect of

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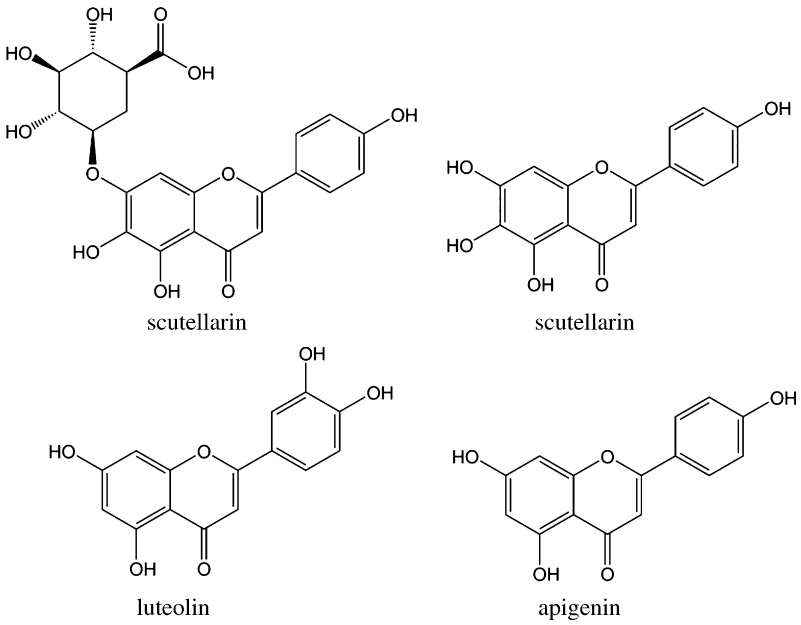

Fig. 1. Chemical structure of scutellarin, scutellarein, luteolin and apigenin.

hydrogen bonding [27,28]. However, the adsorbent with amide group failed to separate each individual flavonoids based on the hydrogen bonding interaction. This is because that every flavone compound has the same phenolic hydroxyl group and can produce hydrogen bonding affinity with amide group in the adsorbent. Actually, we got the same result as that referred in the literature [28]. Although we can extract the active principle of flavonoids with high purity from *S. barbata* plant based on the adsorbent with amide functional group, the every flavone compound could not be isolated. In the previous studies, our group demonstrated that it was possible to separate two kinds of structurally similar molecules according to their different molecular polarities. The main consideration is how to adjust the synergistic effect of the hydrophobic and dipole interactions between adsorbates and adsorbents [29].

In this paper, we sought to apply the similar principle to a more challenging and complicated case, complete separation of the four flavonoids from SB during a continuous operation technology. The polarity of these four kinds of flavonoids is different owing to the difference in the amount or location of the substituent in four flavonoids (Fig. 1). Therefore, a series of methyl acrylate-co-divinylbenzene polymeric adsorbent (MA-*co*-DVB) were synthesized. With varying amount of MA, the adsorbent exhibited the different binding strength to every flavone molecular due to the different polarity of the adsorbent. Meanwhile, the ability to separate the four flavonoids in SB based on the synthesized adsorbent was evaluated, and the effects of the adsorbents' polarity and operating conditions of the separation processes were also investigated. More important in this paper, in order to achieve the complete separation of every flavone compound only through a continuous operation course, it should be important to optimize the operation technology and combine the multiple adsorbents ingeniously.

It was considered that in above-mentioned chromatographic separation, the resolution could be improved by enhancing the separation factor greatly between the adsorbate and the adsorbent without increasing the theoretical plate number, a common way to enhance efficiency of chromatography. Our method is also featured by the ability to be performed under atmospheric conditions, making it suitable for large-scale industrial preparations.

2. Experimental

2.1. Materials

Methyl acrylate (MA, AR) and divinylbenzene (DVB, AR, 80% of purity) were obtained from Chemical Plant of Nankai University (Tianjin, China). 2,2′-Azobisisobutyronitrile (AIBN), toluene, liquid paraffin, phosphoric acid, sodium chloride and ethanol of analytical grade were purchased from Tianjin Chemical Co. (Tianjin, China). *S. barbata* (collected in Anhui province, China) was purchased from Anshun Pharmacy (Tianjin, China).

The standards of scutellarin, scutellarein, luteolin and apigenin were obtained from J&K Chemical Ltd. (Beijing, China). Methanol of HPLC grade was purchased from Tianjin Concord Co. Ltd. (Tianjin, China). Deionized water was purified by a Milli-Q Water Purification system (Millipore, MA, USA).

2.2. Synthesis of the adsorbents with different MA contents

The synthesis of the adsorbent was performed in a three-necked, round-bottomed flask fitted with mechanical stirrer, reflux condenser and thermometer. The spherical adsorbent was prepared based on the method of suspension polymerization. The organic phase consisted of monomer (MA and DVB, 50%, w/w), porogenic agent (toluene, 16.7%, w/w, and liquid paraffin, 33%, w/w) and AIBN (0.5%, w/w) as an initiator. The aqueous phase consisted of polyvinyl alcohol (1%, w/w) and NaCl (5%, w/w). The detailed synthesis process was referred in Ref. [29].

The ratio between MA and DVB was changed in the synthesis process to obtain polymeric adsorbents with different hydrophobic affinities. The adsorbents were named M1–M5 corresponding to 20%, 25%, 35%, 45%, 50% MA content in weight of total monomers, respectively.

2.3. Determination of the adsorbents' physical parameters

2.3.1. Determination of pore structure

Pore structure parameters of the adsorbents synthesized were measured using an automatic surface area analyzer (Autosorb-1-MP, Quantachrome Instruments, Boynton Beach, FL, USA) based on

Table 1
Physical properties of polymeric adsorbents.

Adsorbents	MA content (%, w/w)	Particle diameter (mm)	Surface area (m^2/g)	Average pore diameter (nm)	Moisture content (%, w/w)
M1	20		223	15.1	61.2
M2	25		210	15.3	62.4
M2	35	0.2–0.3	191	14.1	66.3
M3	45		172	14.6	71.8
M4	50		161	13.1	73.5

the BET nitrogen adsorption method.

2.3.2. Determination the moisture proportion imbedded in the adsorbent bead

The hydrated adsorbents disposed of deionized water were weighed accurately and then dried in an oven at 110 °C until constant weight. The following equation was used to calculate the moisture content imbedded in the adsorbent.

$$\alpha = \frac{W_{wet} - W_{dry}}{W_{wet}} \times 100\% \quad (1)$$

where α is the moisture content imbedded in the adsorbent (%), W_{wet} is the weight of the hydrated adsorbent (g), W_{dry} is the weight of the dry adsorbent (g).

2.4. HPLC analysis of the four flavone compounds

The HPLC analysis was carried out on a Waters high performance liquid chromatography system (Waters, Milford, MA, USA), which consisted of a Waters 510 HPLC pump and a Waters 486 UV detector. The analysis was performed on Kromasil C18 reverse phase column (200 mm × 4.6 mm I.D., 5 μm) at room temperature. The detection wavelength was 335 nm, the flow rate was 0.8 mL/min, and the injection volume was 10 μL. The mobile phase consisted of 0.3% phosphoric acid aqueous solution (eluent A) and methanol (eluent B) with the following gradient program: 35% eluent B in 20 min; 35% to 55% eluent B from 25 min to 35 min, and 55% eluent B from 35 min to 45 min. All of the tested samples were filtered through a 0.45 μm membrane filter prior to inject into the HPLC system. The working calibration curve based on the standard solutions of scutellarin, scutellarein, luteolin and apigenin showed good linearity over the range of 10–600 μg/mL.

2.5. Preparation of SB sample solutions

One hundred grams of dried *S. barbata* was pulverized to a suitable size and extracted twice with 800 mL of 70% ethanol aqueous solution in Soxhlet Extractor at 60 °C for 2 h each time. And the extract solution was filtered and concentrated under reduced pressure in a water bath at 40 °C. Finally, the residue fraction was dissolved in 700 mL water, filtered to give the SB sample solution used in the following experiments. The concentration of scutellarin, scutellarein, luteolin and apigenin in the SB sample solution was found to be 487.4, 105.2, 25.6, and 21.8 μg/mL, respectively, analyzed by HPLC.

2.6. Separation procedure

The total separation procedure consisted of dynamic adsorption and gradient desorption.

Dynamic adsorption tests were carried out as follows: the hydrated test adsorbent was packed into a glass column (diameter 10 mm) with a bed volume (BV) of 30 mL. Then 30 mL of SB sample solution flowed through the adsorbent column at rate of 0.5 BV/h, and then the adsorbent column was rinsed by 6 BV of deionized water.

Dynamic desorption tests exploiting the gradient elution method were performed as follows: the adsorbate-laden column was desorbed by 20%, 40%, 50%, 60%, and 80% (v/v) aqueous ethanol solutions in succession at rate of 1 BV/h, and the volume of each solution was 2 BV. The concentration of scutellarin, scutellarein, luteolin and apigenin in each eluent solution was monitored by HPLC analysis.

2.7. Optimization of operating conditions and acquirement of products

As the main operating condition, the maximal processing capacity and the optimal eluent amount based on the selected adsorbent with the best separation ability was validated.

The fractions of the gradient eluent were, respectively, collected, concentrated, and dried under vacuum, to give the products. The products derived from the different gradient eluents were named as product A, B, C, and D, in which the major flavonoid was scutellarin, scutellarein, luteolin and apigenin, respectively. The products were then analyzed by HPLC. The following equation was used to calculate the recovery of the individual flavonoid.

$$R = \frac{m}{M} \times 100\% \quad (2)$$

where R is the recovery rate in percentage, M is the weight of the individual flavonoid loaded onto the selected adsorbent, and m is the weight of the individual flavonoid after column chromatography.

The dynamic adsorption and desorption tests were done in triplicates under the optimal separation conditions.

3. Results and discussion

3.1. Characterization of resins

The physical properties of resins synthesized in Section 2.3 are summarized in Table 1. It was found that the moisture content of the adsorbents increased with the decrease of DVB content in the adsorbent, indicating the decrease of the hydrophobicity of the adsorbents.

3.2. Influence of MA content on the separation of SB flavonoids by adsorbents

After the dynamic adsorption as described in Section 2.6, each adsorbent loaded with the flavone compounds was desorbed under the gradient elution test and the desorption curves of adsorbents M1–M5 are shown in Fig. 2.

As shown in Fig. 2, for all synthesized adsorbents, the adsorption affinity towards scutellarin was relatively weaker than that towards the other three kinds of flavonoids. After being eluted by 40% aqueous ethanol solution, almost all the scutellarin loaded on the adsorbents was desorbed while the other three flavonoids were not. But at the same time, when MA content of the adsorbents was increased to 50% (adsorbent M5, as shown in Fig. 2E), the adsorption affinity dropped as 50% aqueous ethanol solution

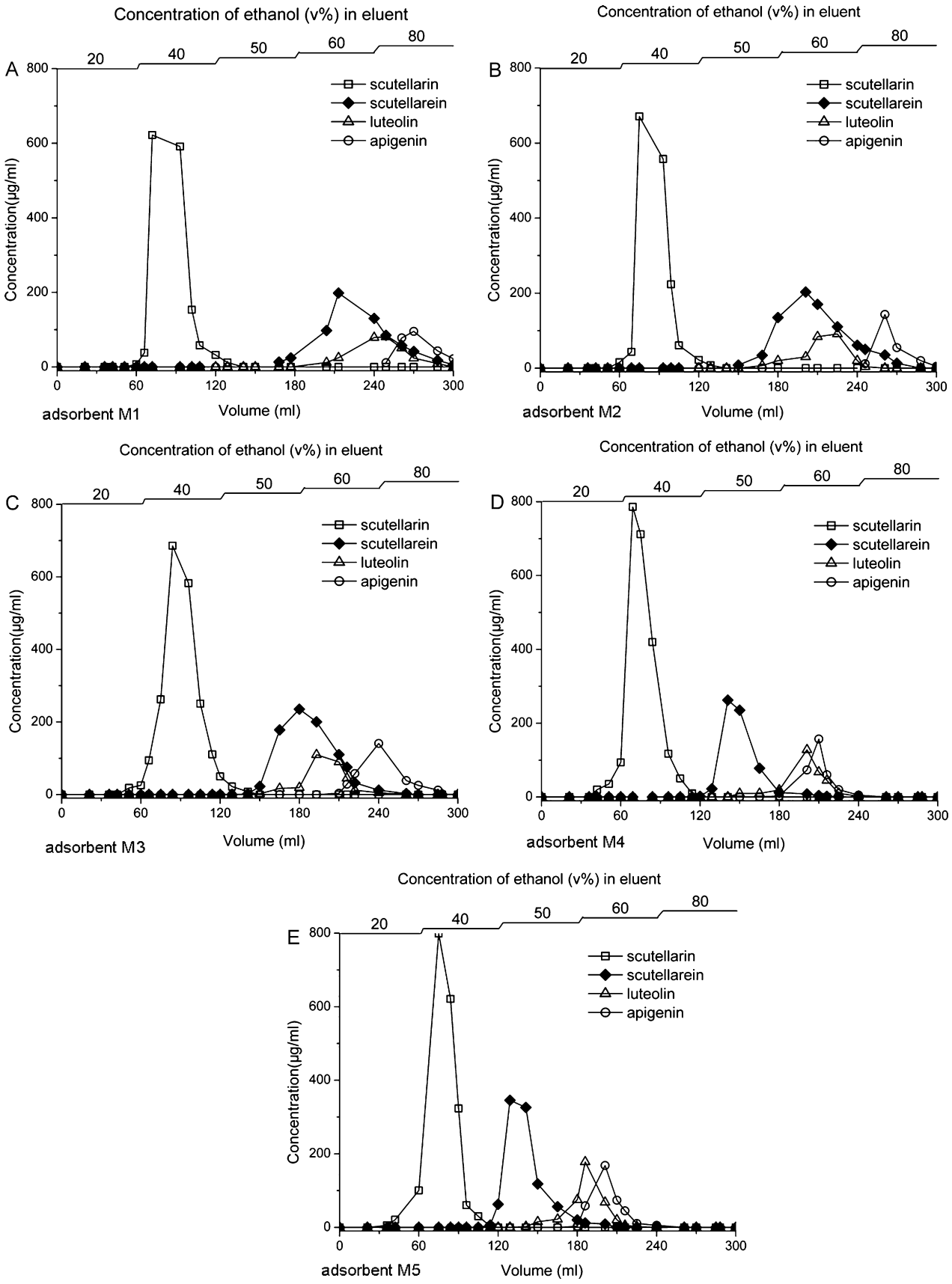

Fig. 2. Desorption curves of the gradient elution of SB flavonoids tests with adsorbents M1–M5.

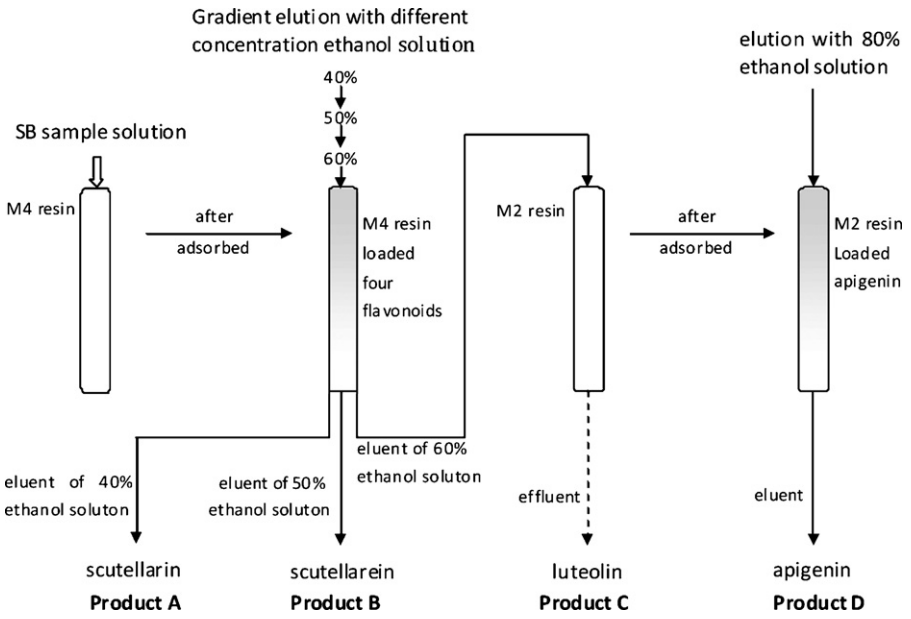

Fig. 3. Separation process of SB flavonoids.

could desorb all of scutellarein and also part of luteolin and apigenin. At lower MA content (adsorbent M4, as shown in Fig. 2D), the adsorption affinity was increased so that 50% ethanol solution could no longer desorb luteolin and apigenin. In comparison, 60% aqueous ethanol solution could elute both of them simultaneously due to the still weak adsorption affinity in adsorbent M4. As MA content of the adsorbent kept decreasing, the adsorption affinity was increased gradually as expected (as shown in Fig. 2C, B, and A). And adsorbent M2 could separate luteolin and apigenin based on the gradient elution with 60% and 80% aqueous ethanol solution, respectively (as shown in Fig. 2B). Unfortunately, although luteolin and apigenin had been separated by adsorbent M2, scutellarein was still inevitably mixed with luteolin in 60% aqueous ethanol desorption solution. This is because that 50% aqueous ethanol solution could hardly elute scutellarein completely owing to the excessively strong adsorption affinity of adsorbent M2.

According to the above analysis, it was concluded that the four flavonoids in SB plant could be separated completely only through a continuous operation, which was based on the ingenious design and reasonable combination of adsorbent M2 and M4. The whole separating process of SB flavonoids is illustrated in Fig. 3

To be specific, adsorbent M4 showed proper polarity and was the optimum adsorbent to separate the four flavonoids in SB plant into three fractions, scutellarin, scutellarein and a mixture of luteolin and apigenin which were eluted by 40%, 50% and 60% aqueous ethanol solution, respectively. Afterwards, the resulting eluent of 60% ethanol solution from adsorbent M4 would be fed on the column of adsorbent M2 directly. Only apigenin could be adsorbed and luteolin was passed through the adsorbent column directly.

In addition, from Fig. 3, it can be seen that the adsorption solutions for adsorbent M4 and M2 were different, hence the volumn of adsorption solution for M4 and M2 were different. In order to ensure a reasonable proportion between the amount of adsorbent M4 and M2 in one continuous operating process, the processing capacity of M4 and M2 for their different adsorption solutions should be investigated carefully. Thus, further studies on the adsorption and desorption properties of adsorbents M4 and M2 were carried out.

3.3. Operating conditions of adsorbent M4 and separation of scutellarin and scutellarein

The adsorption solution for adsorbent M4 was the SB sample solution prepared as described in Section 2.4. The breakthrough curve on adsorbent M4 (1 BV = 30 mL) was obtained based on the volume of the effluent solution and the concentration of the four flavonoids herein. The results are shown in Fig. 4.

Because the affinity between each of the four flavonoids and adsorbent M4 had considerable differences (Fig. 4), the appearance of breakthrough point for each flavone component was also quite different from each other. Adsorption of scutellarin and scutellarein reached equilibrium when the process volume of the sample solution was 60 mL (approximately equal to 2 BV), but at this point, luteolin and apigenin were still adsorbed by the adsorbent due to their stronger affinity to the adsorbent. Therefore, the optimum process volume of the SB sample solution for adsorbent M4 was determined as 60 mL (2 BV).

After adsorption with adsorbent M4, 40%, 50% and 60% aqueous ethanol solutions were used, respectively, in the gradient elution of

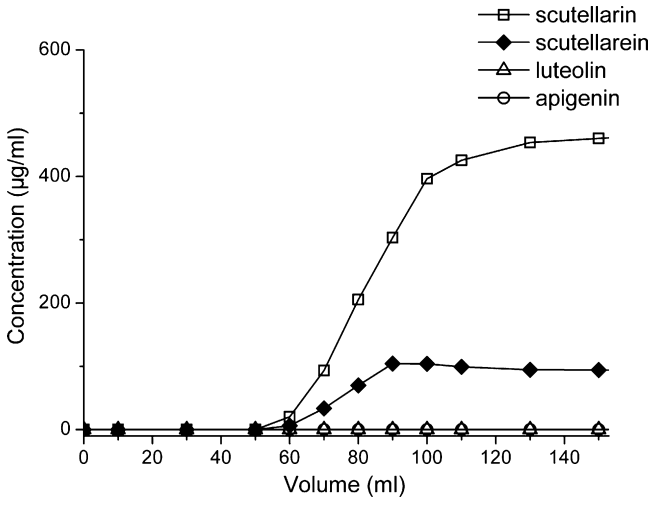

Fig. 4. Breakthrough curves of SB flavonoids on adsorbent M4.

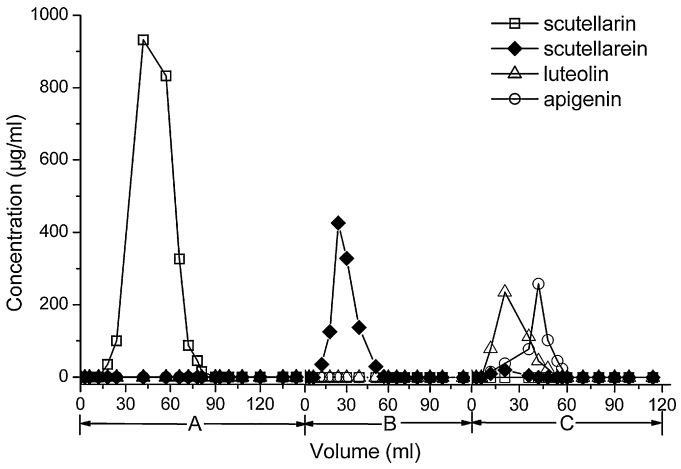

Fig. 5. Dynamic desorption curves of SB flavonoids on adsorbent M4. The column packed with adsorbent M4 was gradually flushed with 40% (A), 50% (B), 60% (C) ethanol solutions (v/v) of different volumes.

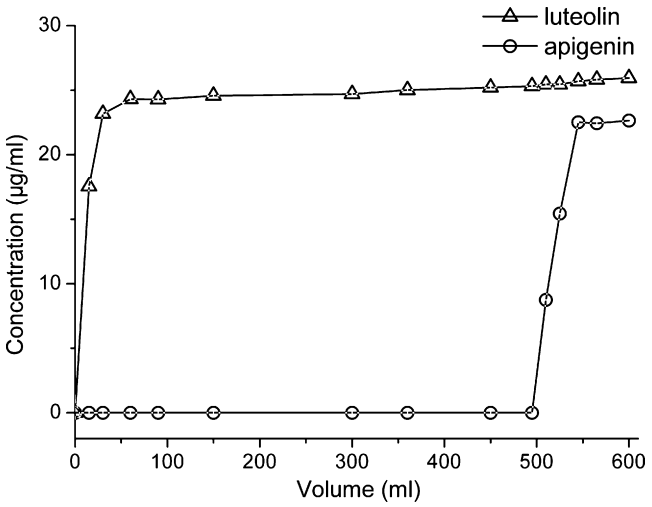

Fig. 6. Breakthrough curves of luteolin and apigenin on adsorbent M2.

scutellarin, scutellarein, luteolin and apigenin at the rate of 1 BV/h. The desorption curve shown in Fig. 5 was based on the volume of the eluent and the concentration of flavonoids.

Scutellarin was firstly eluted by 90 mL (3 BV) of 40% aqueous ethanol solution, and then scutellarein was eluted by 60 mL (2 BV) of 50% aqueous ethanol solution. Finally, the eluent of 60 mL (2 BV) 60% aqueous ethanol solution was used to obtain a mixture of luteolin and apigenin.

3.4. Operating conditions of adsorbent M2 and separation of luteolin and apigenin

According to the separation process illustrated in Fig. 3, the adsorption solution for adsorbent M2 should be the eluent of 60% ethanol solution from adsorbent M4. The breakthrough curves on adsorbent M2 (1 BV = 30 mL) were obtained for luteolin and apigenin based on the volume of the effluent solution and the concentration of two flavonoids (Fig. 6).

As expected, luteolin was not adsorbed onto adsorbent M2 completely. The product of luteolin was obtained by collecting the effluent that passed through adsorbent M2. Adsorption of apigenin reached equilibrium when the process volume was 510 mL (approximately equal to 17 BV). Thus, the optimum proportion between the amount of adsorbent M2 and M4 could be obtained by a simple conversion as follows: for 8.5 BV of adsorbent M4 used, it should produce 17 BV eluent of 60% ethanol solution, which should be the adsorption solution for 1 BV adsorbent M2. Therefore, the optimum M4-to-M2 proportion should be 8.5:1 (v/v).

The dynamic desorption curve on adsorbent M2 was obtained for apigenin based on the volume of the eluent solution and the concentration of apigenin. As shown in Fig. 7, 120 mL (4 BV) of 80% aqueous ethanol solution was able to completely desorb apigenin from adsorbent M2.

3.5. Validation of the separation process of SB flavonoids

In brief, the optimized process for the preparative separation of SB flavonoids was carried out as follows:

Firstly, 200 mL of the SB sample solution was fed onto 100 mL of adsorbent M4 (1 BV = 100 mL) at a flow rate of 0.5 BV/h, followed by the gradient elution at a flow rate of 1 BV/h. The 3 BV eluent of 40% ethanol aqueous solution gave product A, the 2 BV eluent of 50% ethanol aqueous solution gave product B, then 2 BV (200 mL) of

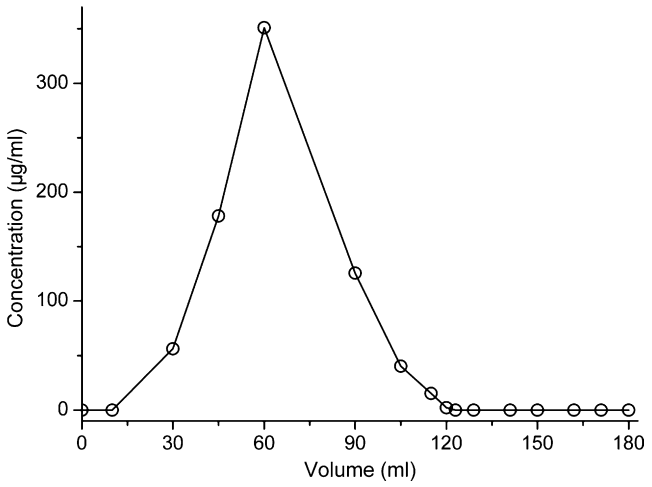

Fig. 7. Dynamic desorption curve of apigenin on adsorbent M2.

Table 2
Results of the separation of SB flavonoids by adsorbents M2 and M4.

	Initial sample	Product A		Product B		Product C		Product D		Recovery	
	W (mg)	W_{ave}[a] (mg)	SD[b]	W_{ave} (mg)	SD	W_{ave} (mg)	SD	W_{ave} (mg)	SD	R_{ave}[c] (%)	SD
Scutellarin	97.48	94.26	0.12	0	0	0	0	0	0	96.7	1.2
Scutellarein	21.04	0	0	18.91	0.08	0.8	0.03	0	0	94.1	1.8
Luteolin	5.12	0	0	0.1	0.02	4.61	0.05	0.21	0.03	95.8	2.2
Apigenin	4.36	0	0	0	0	0	0	4.09	0.08	93.8	1.1

[a] W_{ave} is the average weight of flavonoid in product of four tests.
[b] SD is standard deviation.
[c] R_{ave} is the average recovery of flavonoid of four tests.

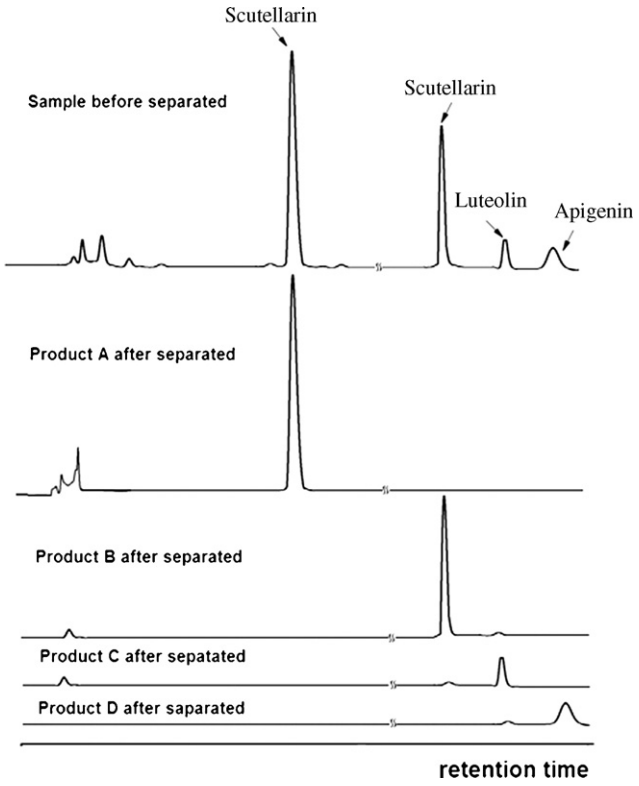

Fig. 8. HPLC schematic profile of products before and after separated.

the eluent of 60% ethanol aqueous solution from adsorbent M4 was directly fed onto 12 mL of adsorbent M2 (1 BV = 12 mL). The effluent from adsorbent M2 gave product C. Then 4 BV eluent of 80% ethanol aqueous solution gave product D.

The contents of the individual flavonoid compound in product A–D and their recovery rates are listed in Table 2. HPLC profile of products before and after separated showed the perfect effect (Fig. 8). Each of the flavonoids was obtained at high resolution and high recovery after passing through M4 and M2 columns sequentially (Table 2).

In summary, the four flavonoids in SB were successfully separated by the combined use of two columns each packed with MA-co-DVB copolymeric adsorbents with different MA contents. Coupling two columns different in polarity allowed effective separation of four compounds with closely related chemical structures, which would be challenging to separate using traditional chromatography.

4. Conclusion

In the present study, the method for adjusting polarity between polymeric adsorbent and adsorbates was developed to be suitable for the preparative separation of the four individual flavonoids in *S. barbata* D. Don. Polymeric adsorbents with different MA contents were synthesized according to the different polarity of the four flavonoids. The influence of polarity of adsorbents on the adsorption and desorption of the four flavonoids was studied. It was found that the affinity strength between the adsorbents and the four flavonoids was dependent on the polarity of the adsorbent. Through the optimal process based on the reasonable combination of adsorbents M2 and M4, the four flavonoids were separated successfully in one continuous operating process. Each flavonoid was obtained with high resolution and high recovery. In each final product, there is mainly one kind of flavonoid to be detected and the recovery of each individual flavonoid was 96.7%, 94.1%, 95.8% and 93.8%, respectively.

The results in this paper certainly show another successful application of macroporous resin-based chromatography as a very effective method to separate compounds, as it provides the simplicity of varying polarity of adsorbents, and also flexibility of combining adsorbents of different polarities to achieve optimal separation of compounds that are otherwise challenging to be isolated.

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