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Research Article

Preparation of two flavonoid glycosides with unique structures from barley seedlings by membrane separation technology and preparative high-performance liquid chromatography

Barley seedlings are rich in flavones that can have positive effects on people with antihypoxia and antifatigue. Lutonarin and saponarin are two major flavonoid glycosides that have unique structures in barley seedlings. This study presents a new approach for the preparation of lutonarin and saponarin from barely seedlings by membrane separation technology and preparative high-performance liquid chromatography. Preparative conditions of these two flavonoid glycosides by membrane separation technology were studied using response surface methodology. Under the optimized conditions, the total contents of these two flavonoid glycosides amounts to 17.0%.

Keywords: Barley seedlings / Lutonarin / Membrane separation technology / Response surface methodology / Saponarin
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1 Introduction

Barley (*Hordeum vulgare*), a well-known local crop of Qinghai-Tibet Plateau, is traditionally used as a staple in the Southwest China provinces of Xizang, Sichuan, Qinghai, and Yunnan [1]. Previous studies on barley primarily focused on the nutrition, physiology, and plant aspects of this crop [2–7]. However, with increased public interest in health-promoting compounds, the bioactive compounds in barley seedlings have captured people's attention. Recent research on the flavonoids in barley seedlings has shown that they are suitable to be used in healthcare products due to their positive effects on antihypoxia and antifatigue [8, 9]. Both of these conditions are of great significance for people living on the plateau. Thus, an aim of this paper is to develop a method for the preparation of barley with high flavonoid content.

Membrane separation technology provides a useful approach to extract, concentrate, separate, or fractionate different compounds [10]. Over the years, many industries have come to accept cross-flow filtration, including microfiltration, ultrafiltration, nanofiltration, and reverse osmosis, as standard technologies for clarification or concentration. A membrane is defined as a material that forms a thin wall capable of selectively resisting the transfer of different constituents of a fluid and, thus, effecting a separation of the constituents. Membrane separation technology has been applied in many fields, such as chemical engineering and biotechnology, as a new type of separation and purification technology, [11–14], but has been applied less in the field of traditional Chinese medicine pharmacy. In this paper, preparative conditions of barley flavonoids with high content by UF membrane separation technology were studied by response surface methodology.

Lutonarin and saponarin are two major flavonoid glycosides with unique structures in barley seedlings. As seen in Fig 1, there was a glucose taking a C–C connection with nuclear parent, which is different from the common C–O connection. With the increase in research applications of lutonarin and saponarin, the demands for highly pure amounts of these compounds are rapidly increasing. In our previous study, a separation method was established by high-speed counter-current chromatography [15], which took a lot of time to select the two-phase solvent system. Besides, it took about 6 h for one run. Thus, the other aim of this paper is to develop an efficient method for the separation of lutonarin and saponarin.

Preparative HPLC is a robust, versatile, and fairly rapid technique that provides advantages of high efficiency, high resolution, and good repeatability that are not found in other chromatographic techniques [16]. It takes advantage of high-performance separation, online detection, and automatic control to realize the efficient separation of target compounds

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Abbreviations: ANOVA, analysis of variance; **DAD**, diode array detection

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Colour Online: See the article online to view Fig. 2 in colour.

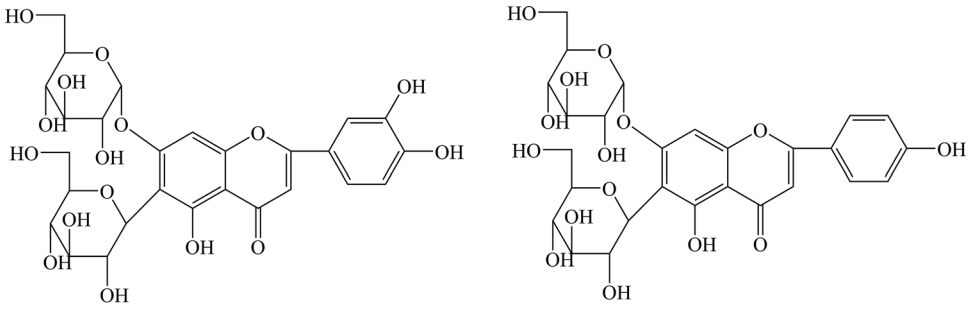

Figure 1. Chemical structures of lutonarin (1) and saponarin (2).

[17]. The various modes available to date, e.g., normal phase, RP, size exclusion, and ion exchange, can be used to purify most classes of natural products [18–20]. In addition, prep-HPLC systems which were "state-of-the-art" some 10 years ago are now within the reach of most research groups for that the relative cost of prep-HPLC systems has fallen due to increased competition, with the arrival of numerous column and equipment manufacturers. More and more studies have been reported on the separation of natural products by prep-HPLC [21–24]. In this paper, an efficient method for the separation of lutonarin and saponarin was successful by prep-HPLC.

2 Materials and methods

2.1 Apparatus

HPLC analysis was performed on an Agilent 1200 system (Agilent Technologies, USA). The Agilent 1200 system was equipped with a G1354A solvent delivery unit, a G1315B UV-vis photodiode array detector, a G1316A column thermostat, a G1313A autosampler, an Agilent Eslipse-XDB C_{18} (250 × 4.6 mm, 5 μm) analytical column, and an Agilent HPLC work station.

The membrane separation process was conducted on a miniature multifunctional membrane separation equipment (HEFEI YUWANG MEMBRANE ENGINEERING, China). The ultrafiltration membrane was purchased from GE Healthcare.

The prep-HPLC experiment was conducted on a LC-8A chromatography system (Shimadzu, Japan) equipped with a LC-8A solvent delivery unit, a SPD-20A UV-vis detector, a CBM-20A communication module and a FRC-10A Fraction collector.

2.2 Reagents and materials

All the chemical reagents used were of analytical grade and were purchased from Yuwang Chemical (Shandong, China). Deionized water was used throughout the experiment. Reference standards of lutonarin and saponarin were produced in our preliminary study.

Barley seedlings were collected in Huangzhong County, Qinghai Province, China, in June 2013. The species was identified by Professor Yuhu Shen (Northwest Institute of Plateau Biology, Chinese Academy of Sciences). Voucher specimens were deposited in the archives of the Northwest Institute of Plateau Biology (HNWP), Chinese Academy of Sciences (QTPMB-0286185).

2.3 Preparation of sample and standard solutions

Dried barley seedlings (20 kg) were ground into powder and underwent three extractions using 30% ethanol at 85°C. The solid/liquid ratio was 1:20 for the three extractions. The extraction time for the three extractions was 2, 2, and 1 h, respectively. All the filtrates were combined and vacuum-dried to produce 4 kg of crude sample.

A standard solution of lutonarin was prepared by dissolving 5.77 mg lutonarin in 50 mL of 30% ethanol. A standard solution of saponarin was prepared by dissolving 3.05 mg saponarin in 50 mL of 30% ethanol. All the solutions were stored at 4°C until use.

2.4 HPLC analysis

An Agilent Eclipse-XDB C_{18} column (250 × 4.6 mm, 5 μm) was used throughout the experiment. The mobile phase was composed of water (solvent A) and methanol (solvent B). A gradient elution program was performed as follows: 0 min, 20% B; 30 min, 60% B. The flow rate was 1.0 mL/min, the column temperature was 25°C, and the detection wavelength was 270 nm.

2.5 Method validation

Method validation addressed the evaluation of variation of retention times and peak areas for analytes, building of calibration curves, LOD, accuracy, and precision. Linearity was measured at six injection volume (4, 6, 8, 10, 12, 14 μL) for each of the analytes. Calibration curves were constructed by plotting peak area versus injected amount (μg) in the range of 0.4616–1.6156 and 0.2440–0.8540 μg, respectively. LOD

Table 1. Uncoded and coded levels of the independent variables of membrane separation process

Level	Factor		
	X_1 Volume (L)	X_2 Membrane pore size (Dalton)	X_3 Separation pressure (Mpa)
−1	2.0	5000	4
0	4.0	20 000	6
1	6.0	35 000	8

was calculated at S/N = 3. The method repeatability was investigated by six injections of 10 μL standard solution. The precision was expressed as the percentage of the RSD%. The accuracy of the analytical method was determined by spiking with a known amount of standard into serum samples.

2.6 Membrane separation process

100 g of the crude sample was dissolved in deionized water and then introduced to the membrane separation equipment. The solution was pumped across the membrane under the selected pressure and membrane pore size; smaller molecules pass through the pores while larger molecules were retained. The solution passing through the membrane was then concentrated by a 300 Da membrane. Finally, the retained solution was vacuum-dried to produce crude barley flavonoids.

2.6.1 Single factor experimental design

The effects of the volume of deionized water used to dissolve the sample (2, 3, 4, 5, 6, and 7 L, respectively), membrane pore size (5000, 10 000, 20 000, 30 000, 40 000, and 50 000 Da, respectively) and separation pressure (3, 4, 5, 6, 7, and 8 MPa, respectively) on the total content of lutonarin and saponarin were studied by a single factor design as follows: one factor was changed while the other factors were kept constant in each experiment.

2.6.2 Optimization of membrane separation process

Response surface methodology was used to optimize the preparative conditions of lutonarin and saponarin by membrane separation technology from barley seedlings. A Box–Behnken design with three independent variables was employed [25]. The variables used were as follows: separation pressure (X_1), the volume of deionized water used to dissolve the sample (X_2), and membrane pore size (X_3). The total content of lutonarin and saponarin in crude barley flavonoids was selected as the response value. The uncoded and coded values for the three variables can be observed in Table 1. The complete design consisted of 17 experiments including 12 factorial experiments and five replicates at the center point (Table 2) [25]. The experiments were carried out at random to minimize the effect of unexplained variability in the observed responses due to systematic errors.

Table 2. Box–Bohnken design matrix and experimental response

No	X_1	X_2	X_3	Y
1	2	20 000	4	13.11
2	6	20 000	8	13.57
3	2	20 000	8	13.57
4	6	20 000	4	15.43
5	6	35 000	6	11.43
6	4	35 000	8	10.71
7	4	20 000	6	17.39
8	4	20 000	6	18.21
9	4	20 000	6	17.86
10	2	5000	6	12.86
11	4	5000	8	13.36
12	6	5000	6	15.60
13	4	20 000	6	16.57
14	4	35 000	4	11.79
15	2	35 000	6	13.57
16	4	5000	4	12.21
17	4	20 000	6	17.02

The least square multiple regression methodology was used to enquire the relationship between the independent and dependent variables. The multiple regression equation was used to fit the second-order polynomial equation based on the experimental data as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1 X_1 + \beta_{22} X_2 X_2 + \beta_{33} X_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

where Y represents the predicted response, β_0, is the model intercept, β_1, β_2, β_3, β_{11}, β_{22}, β_{33} and β_{12}, β_{13}, β_{23} are linear quadratic and interaction coefficients respectively, and X_1, X_2, and X_3 are the independents. The models were compared based on the coefficient of determination (R^2), adjusted coefficient of determination (R^2-adj), and the predicted coefficient of determination (R^2-pred). The coefficient of determination (R^2) is defined as the regression of the sum of squares proportional to the total sum of squares, which illustrates the adequacy of a model. R^2 ranges from 0 to 1. An R^2 value close to 1 suggests the model has high accuracy. The highly adjusted and predicted coefficients of determination also illustrate whether or not the model adequately fits the data [26]. After selecting the most accurate model, an analysis of variance test was used to investigate the statistical significance of the regression coefficients by conducting Fisher's F-test at 95% confidence level. The interactive effects of the factors were observed using surface plots derived from the chosen model.

Finally, the entire process was optimized. The aim of the optimization was to maximize the responses with the same weight ($w = 1$), and the credibility of the optimum conditions was diagnosed through the desirability values of

the responses that range from 0 to 1. The closer the values of desirability are to 1, the more desirable and credible the optimal conditions are.

2.7 Prep-HPLC separation

The prep-HPLC experiment was conducted on an LC-8A chromatography system (Shimadzu, Japan) equipped with an LC-8A solvent delivery unit, an SPD-20A UV-vis detector, a CBM-20A communication module and a FRC-10A Fraction collector.

A 200 mg of the crude barley flavonoids were dissolved in 10 mL 30% ethanol and introduced to the prep-HPLC system. Further separation was performed on a SinoChrom ODS-AP column (300 × 30 mm i.d., 10 μm) column. The mobile phase was composed of water (solvent A) and methanol (solvent B). A gradient elution program was performed as follows: 0–20 min, 40% B; 20–30 min, 40–60% B. The flow rate was 15 mL/min and injection volume was 10 mL. The effluent was monitored at 270 nm and peak fractions were collected according to the elution profile.

2.8 Identification of prep-HPLC peak fractions

Identification of the prep-HPLC peak fractions was carried out by comparing the retention times with reference standards.

3 Results and discussions

3.1 Analytical method validation

The HPLC with diode array detection (DAD) method validation was evaluated as described in Method validation section. The linear regression equation, correlation coefficients, LOD, and reproducibility of retention time and peak area are given in Table 3. This method yielded a correlation coefficients of >0.9998, indicating excellent linearity, and with DAD detection offering the low LOD of 4.2 and 3.8 ng, respectively. The accuracies were determined by analyzing the percentage recovery and calculated as follows: recovery (%) = 100 $(a-b)/c$, where a was the measured concentration obtained from the extracted serum samples which were spiked standard; b was the concentration of analyte in the matrix and c was the added known concentration to the matrix. The analyses were repeated three times, and the experimental accuracy obtained was 98.24 and 102.16%, respectively (Table 3). The inter- and intra-day variabilities were investigated to evaluate the precision of the proposed method and expressed as RSD%. The intraday assay variabilities were 2.46 and 2.62%, respectively (Table 3), while interday assay variabilities were 3.72 and 3.63%, respectively (Table 3). These results demonstrated the suitability of the proposed method for the determination of the target analytes in terms of sensitivity, accuracy, and precision. The total content of lutonarin and saponarin in barley seedlings was 1.8 and 8.7% in the crude extract.

3.2 Single-factor experiments

3.2.1 Effect of the volume of deionized water used to dissolve the sample on the total content of lutonarin and saponarin

The different volumes of deionized water used to dissolve the samples were 2, 3, 4, 5, 6, and 7 L, respectively. This was used to investigate the influence that the volume of deionized water had on the total content of lutonarin and saponarin with a membrane pore size of 10 000 Da and a separation pressure of 6 MPa. The results indicated that the total content of lutonarin and saponarin increased as the volume of deionized water increased until it reached its peak value at approximately 4 L. There was no increase in content as the volume of deionized water rose past 4 L. Therefore, 2–6 L was considered to be optimal volume range of deionized water in this experiment.

3.2.2 Effect of membrane pore size on the total content of lutonarin and saponarin

Different membrane pore size was set at 5000, 10 000, 20 000, 30 000, 40 000, and 50 000 Da, respectively, to investigate the influence of membrane pore size on the total content of lutonarin and saponarin 4 L of deionized water and a separation pressure of 6 MPa. The results indicated that the total content of lutonarin and saponarin increased as the membrane pore size increased, but subsequently decreased and reached a peak value at about 20 000 Da. Therefore, 5000–35 000 Da

Table 3. Linear regression equation, *r*, LOD, reproducibility of retention time and peak area, accuracy and intra- and inter-day precision

Analytes	Regression equation	*r*	LOD (ng)	Repeatability		Accuracy		Precision	
				RSD (%) ($n = 6$)		% ($n = 3$)		RSD (%) ($n = 6$)	
				Retention time	Peak area	Mean	RSD	Intraday	Interday
Lutonarin	$y = 2053.7x + 1.4$	0.9999	4.2	0.86	1.18	98.24	2.33	2.46	3.72
Saponarin	1827.5*x*–0.93	0.9998	3.8	0.79	1.24	102.16	2.12	2.62	3.63

was considered to be optimal range for membrane pore size in this experiment.

3.2.3 Effect of separation pressure on the total content of lutonarin and saponarin

The different separation pressures were set at 3, 4, 5, 6, 7, and 8 MPa, respectively, to investigate the influence of separation pressure on the total content of lutonarin and saponarin in 4 L of deionized and a membrane pore size of 10 000 Da. The results indicated that the total content of lutonarin and saponarin increased by increasing the separation pressure but subsequently decreased and reached the peak value at about 6 MPa. Therefore, 4–8 MPa was considered to be the optimal range for separation pressure in this experiment.

3.3 Optimization of membrane separation process

According to the created design, 17 experiments were performed in duplicate and the obtained results are depicted in Table 2.

The R^2, R^2-adj, and R^2-predicted values were 94.0, 92.06, and 85.48, respectively, and showed that the full quadratic models were more efficient than the other models for obtaining the total lutonarin and saponarin contents.

Table 4. The analysis of variance

Term	*F*	*P*
Model	29.13	< 0.0001
X_1	3.15	0.1191
X_2	15.81	0.0053
X_3	0.65	0.4450
$X_1 \times_2$	17.71	0.0040
$X_1 \times_3$	3.97	0.0864
$X_2 \times_3$	3.64	0.0981
X_1^2	14.34	0.0068
X_2^2	110.59	< 0.0001
X_3^2	73.05	< 0.0001
Lack of fit	0.51	0.6941

The model is

$$Y = 18.61 + 0.35X_1 - 0.79X_2 - 0.16X_3 - 1.18X_1X_2 - 0.56X_1X_3 - 0.53X_2X_3 - 1.03{X_1}^2 - 2.87{X_2}^2 - 2.33{X_3}^2 \quad (2)$$

Analysis of variance (ANOVA) was used to evaluate the significance of the model and the results are shown in Table 4. For each term in the model, a large F-value and a small *P*-value implies a more significant effect on their respective response

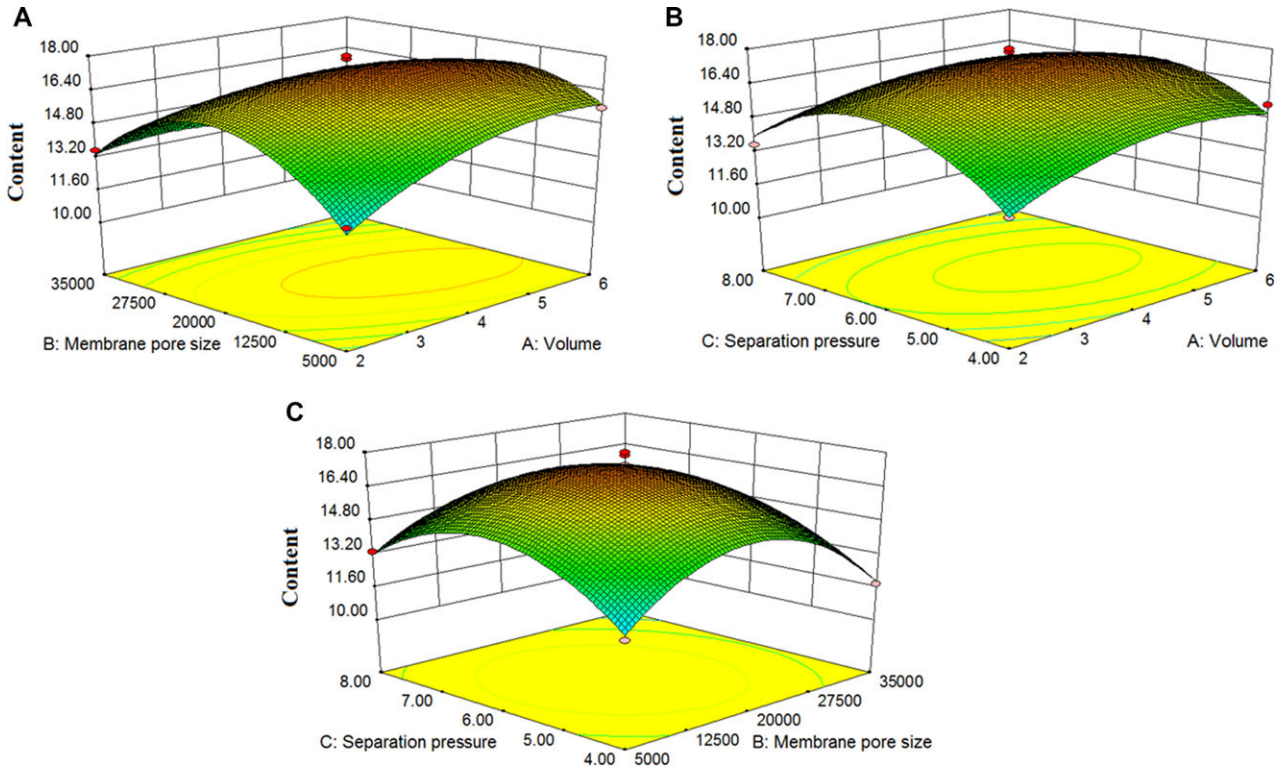

Figure 2. Response surface showing the interactive effect of the variables on the total content of lutonarin and saponarin: (A) membrane pore size vs. the volume of deionized water used to dissolve; (B) separation pressure vs. the volume of deionized water used to dissolve; (C) membrane pore size versus separation pressure.

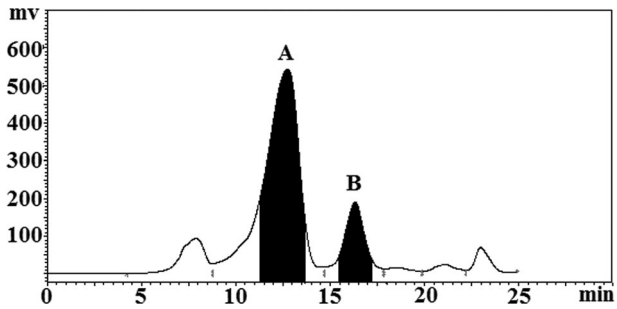

Figure 3. Prep-HPLC chromatogram of the target compounds. Conditions: column, SinoChrom ODS-AP column (300 × 30 mm i.d., 10 μm); mobile phase: H_2O (solvent A) and CH_3OH (solvent B), 0–20 min, 40% B, 20–30 min, 40–60% B; flow rate, 15 mL/min; detection wavelength, 270 nm.

variable [27]. Therefore, the linear term X_2, the interactive term $X_1 \times_2$, and the quadric terms X_1^2, X_2^2, and X_3^2 showed a significant effect ($P < 0.05$) on the total content of lutonarin and saponarin. However, the linear terms X_1 and X_3, and the interactive terms $X_1 \times_3$ and $X_2 \times_3$, did not show a significant effect ($P > 0.05$).

The fitness of the model was investigated through lack-of-fit test ($P > 0.05$), which indicated suitability of models to accurately predict the variation [27].

The 3D plots are the graphic representations of the regression models and provide a method to visualize the relationship between responses and experimental levels of each variable and the type of interactions between two test variables. The relationship between independent and dependent variables was illustrated as a 3D representation of the response surfaces for the total lutonarin and saponarin content (Fig. 2). Two variables were depicted in one 3D surface plot, while the other variable was kept at level zero.

Figure 2A shows the interaction between the membrane pore size and the volume of deionized water used to dissolve the sample. Initially, the total content of lutonarin and saponarin increased by increasing the membrane pore size but subsequently decreased. This was due to the transmission of other large molecules when the membrane pore size exceeded 20 000 Da. These results were consistent with the single-factor experiment.

Figure 2B depicts the interaction between separation pressure and the volume of deionized water used to dissolve the sample. Initially, the total lutonarin and saponarin content increased with increasing separation pressure, but eventually began to decrease again. The concentration polarization gel layer model can be used to illustrate this phenomenon. When the separation pressure was low, the membrane filtration resistance and viscosity remain nearly unchanged, and the total content of lutonarin and saponarin increased by increasing the separation pressure. When the separation pressure exceeded 6 MPa, the total content of lutonarin and saponarin decreased for the gel layer resistance caused by membrane pollution. These results were also consistent with the single factor experiment.

The separation pressure of 5.9 MPa, 4.6 L of water, and a membrane pore size of 17 101 Da were found as the optimal conditions for the membrane separation process. The predicted total content of lutonarin and saponarin was 17.4%, with desirability values equal to 1. According to the actual conditions, the parameters were set as follows: separation pressure 5.9 MPa, 4.6 L of deionized water to dissolve the sample, and a membrane pore size of 20 000 Da. Under the optimal conditions, the experiment

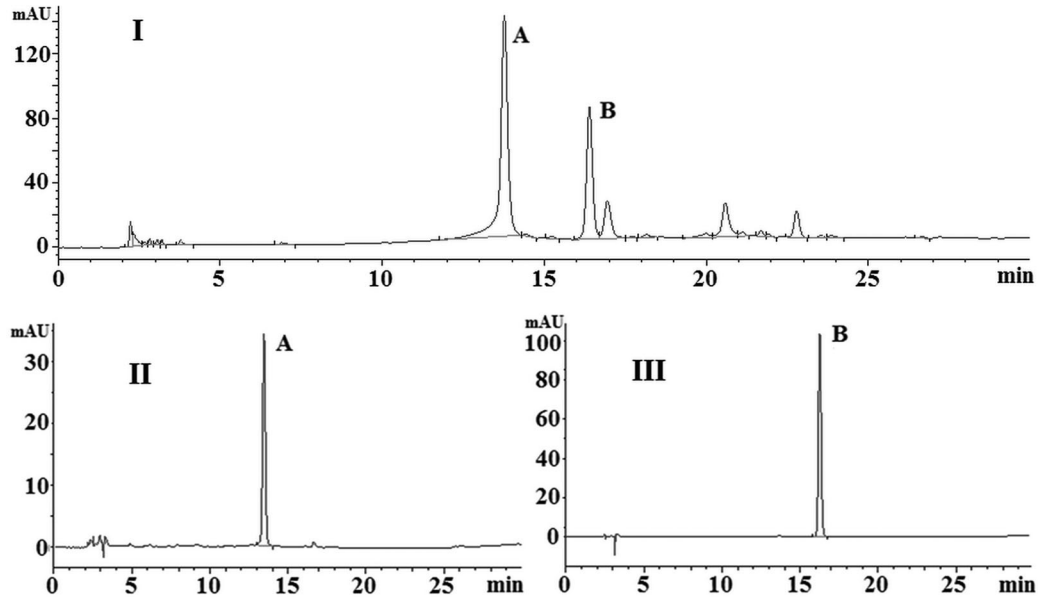

Figure 4. HPLC chromatograms of the sample after membrane separation (I) and the target compounds purified by Prep-HPLC (II and III). Conditions: column: Agilent Eslipse-XDB C_{18} (250 × 4.6 mm, 5 μm); mobile phase: H_2O (solvent A) and CH_3OH (solvent B), 0–30 min, 20–60%B.; flow rate: 1.0 mL/min; column temperature: 25°C; and detection wavelength: 270 nm.

was conducted in triplicate. As a result, 36.4 g of crude barley flavonoids were obtained, with the total content of lutonarin and saponarin amounting to 17.0%. The recovery rate of the total lutonarin and saponarin content following the membrane separation process was 68.8%. This demonstrated that the response surface methodology, with an appropriate experimental design, can effectively be applied to the optimization of the membrane separation process. The results showed that membrane separation technique could be a good choice for the preparation of barley flavonoids with high content.

3.4 Prep-HPLC separation

In this work, the mobile phase, flow rate, and sample-loading amount were optimized. With increases in organic solvent content, the flow rate, and the sample-loading amount, the purification efficiency of the compounds decreased. Finally, water (solvent A) and methanol (solvent B) were used as the mobile phase. A gradient elution program was performed as follows: 0–20 min, 40% B; 20–30 min, 40–60% B, 15 mL/min. The sample injection volume for each run was 10 mL and contained 200 mg sample. Figure 3 shows the prep-HPLC chromatogram, which yielded 20 mg of fraction A and 12 mg of fraction B. HPLC analysis showed that the purities were 95.8 and 97.2%, respectively (Fig. 4). The results showed that the prep-HPLC was more efficient than the previously developed high-speed counter-current chromatography method.

3.5 Structural identification

Fractions A and B were identified as lutonarin and saponarin by comparison of the retention times with reference standards. These two flavonoid glycosides were known compounds [15].

4 Conclusions

In our paper, a novel method for the preparation of lutonarin and saponarin from barley seedlings by membrane separation technology and prep-HPLC was established. The results demonstrated that membrane separation technology coupled with prep-HPLC could be a powerful technique for separation of bioactive compounds from natural products.

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The authors have declared no conflict of interest.

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