Study on the Separation of Triterpene Saponins from *Panax notoginseng* by Off-Line Two-Dimensional Supercritical Fluid Chromatography–Ultrahigh-Performance Liquid Chromatography

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The off-line two-dimensional supercritical fluid chromatography (SFC)–ultrahigh-performance liquid chromatography (UHPLC) was selected to separate the triterpene saponins from *Panax notoginseng*. The separation by SFC was performed on an Atlantis® HILIC silica column. Methanol was selected as a modifier, and the most time-saving gradient was developed. The decrease of the column temperature and the increase of the back pressure could shorten the retention time but had no effect on the separation selectivity. Then, the back pressure, column temperature, and flow rate were set as 131 bar, 45 °C, and 4.0 mL min^{-1}, respectively. The retention behavior of the saponins from *P. notoginseng* was different between SFC and reversed-phase liquid chromatography (RPLC), which facilitated to construct an off-line SFC/RPLC–mass spectrometry (MS) system. In first dimension, a total of eight fractions were collected under SFC and further analyzed by RPLC–MS in second dimension. The result indicated that the retention behavior of triterpene saponins was mainly controlled by the hydrogen bonding interactions which were affected by the number and types of sugars, as well as the aglycone in the structure of triterpene saponins. Thus, the presence of "clustering effect" under SFC was observed, namely, one SFC peak always contained several saponins with same number of sugars and similar structure of aglycone. The clustering effect of triterpene saponins promised SFC to be used as first dimension to complete the preliminary crude separation in the two-dimensional liquid chromatography.

Keywords: Supercritical fluid chromatography, reversed-phase liquid chromatography, mass spectrometry, *Panax notoginseng*, saponins

Introduction

Panax notoginseng is a famous Chinese medicine, which has been used to treat cardiovascular diseases, inflammation, different body pains, trauma, internal and external bleeding due to injury, etc. [1–3]. Chemical and pharmacological studies on *P. notoginseng* have demonstrated that its major bioactive constituents are triterpene saponins [4–6]. Triterpene saponin is an important class of compounds, which is consisted of one or more sugars, linked to a triterpene type of aglycone with four- or five-member ring structures [7]. There were several studies in analyzing triterpene saponins from various plants using high-performance liquid chromatography (HPLC) [8, 9]. For example, reversed-phase liquid chromatography (RPLC) has been the most common method for the content determination of the triterpene saponins in *P. notoginseng* [10–13]. HPLC coupled tandem mass spectrometry (MS/MS) is an effective method for rapidly characterizing the saponins in *P. notoginseng* [14]. In recent years, the hydrophilic interaction liquid chromatography (HILIC) also showed the advantages at the analysis of triterpene saponins, e.g., the isomeric saponins were separated using methanol as a weak eluent under HILIC based on a Click XIon zwitterionic stationary phase by Guo et al. [15]. Although advanced techniques have been used in the separation of saponins, challenges still remain due to the diversity and similarity of the triterpene saponins, as well as the complexity of their plant origins. There is an urgent need to expand the analytical method for further research of the triterpene saponins in *P. notoginseng*.

Recently, SFC has gained increasingly attention. The supercritical fluid (SF) is used as mobile phase in SFC, which is the most significant difference between SFC and other chromatographic technologies. Compared with gas and liquid, the SF has more advantages. The diffusion property of the SF is higher than that of liquid; therefore, SFC has lower mass transfer resistance between stationary phase and mobile phase than HPLC. The viscosity of SF is close to that of gas, and the density is similar to that of certain liquid, which facilitates lower pressure drop at relatively high flow rate [16, 17]. In addition, the densities and solvating power of SF could be sharply changed with a slight change in pressure near the critical point; thus, the pressure is an important parameter to improve the separation. In summary, SFC can offer high-throughput and high-resolution analysis [18]. CO_2 is the most frequently used SF and methanol, and ethanol and isopropanol are usual polar organic modifiers.

Nowadays, packed columns are widely accepted in SFC, which are more useful for routine separations than earlier open tubular capillary columns. Moreover, particles for stationary phases could be smaller than 5 μm in SFC, while column pressure is not an obstacle. Difficulties in back pressure regulation, flow rate stability, sample injection, and automation have been resolved. Owing to the low polarity of CO_2, SFC is effective for the analysis of hydrophobic compounds such as carotenoids, tocopherols, triterpenoids, and lipids and so on [19–24]. In recent years, with the improvement of the SFC, the applications can be extended to the study of peptides, nucleobases, and other hydrophilic analytes by the incorporation of various polar organic modifiers [25–28]. In the analysis of saponins by SFC, the most widely used application was the analysis and purification of diastereomeric saponins [29]. Agrawal et al. developed the SFC method for standardization of isolation of bacoside A_3 and bacoside II in *Bacopa monnieri* extract [30]. However, studies on separation of triterpene saponins from *P. notoginseng* using SFC were rarely reported.

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Two-dimensional (2-D) chromatography has become an effective method for the separation of complex samples [31]. In a previous study, the retention behavior of 46 solutes of varying molecular properties was studied using RPLC, SFC, gas–liquid chromatography (GLC), and micellar electrokinetic capillary chromatography (MECC) modes, respectively. The orthogonality of different 2-D systems combined with these separation modes was evaluated, and the result proved that different separation mechanism could improve separation selectivity and peak capacity [32]. Our previous study about the analysis of saponins in *P. notoginseng* adopted a comprehensive HILIC–RPLC with mass spectrometry detection, and 224 saponins were successfully found [33]. The preparative RPLC–HILIC was also used to purify the saponins from the leaves of the *P. notoginseng* [34]. Sandra et al. developed an automated off-line SFC–SFC system using octadecyl silica gel (ODS) and silver-loaded stationary phases for the characterization of triglycerides in vegetable oils [35]. In summary, 2-D chromatography developed with two separation modes would lead to orthogonality separation due to different mechanism.

The current work was to explore the application of an SFC system for the separation of the triterpene saponins from the *P. notoginseng*. Firstly, a HILIC silica column was selected to separate the triterpene saponins under SFC, and SFC conditions such as modifier, gradient condition, back pressure, column temperature, and flow rate were optimized. Secondly, an off-line 2-D SFC/RPLC–ultraviolet (UV)–MS method was developed and evaluated. Moreover, the retention behavior of saponins under SFC could be studied clearly, and the relationship between the structure of saponins and retention behavior under SFC could be proved.

Materials and Methods

Apparatus. SFC separation was carried out using the Waters ACQUITY UltraPerformance Convergence Chromatography™ system (ACQUITY UPC^2 system). It includes a binary solvent delivery pump, an autosampler, a column manager, a photodiode array (PDA) detector, and a back pressure regulator (BPR). The Waters ACQUITY UPLC H-Class system is equipped with a quaternary solvent manager, an autosampler, a column manager, and a PDA detector. Data acquisition and control of the SFC and ultrahigh-performance liquid chromatography (UHPLC) system were performed using the Waters Empower™ Pro 3 Software. UPLC–MS was carried out using the Waters ACQUITY UPLC-I-Class/Xevo G2-S QTOF system. The data was processed using Masslynx (4.1).

Reagents. CO_2 (food grade) was provided by Zhenxin Gaisi (Shanghai, China). Methanol and acetonitrile of HPLC grade were obtained from J&K Chemical (Beijing, China). Ethanol (HPLC grade) was purchased from Tedia (Fairfield, USA). The neutral alumina (Al_2O_3) was produced by the Sinopharm Chemical Reagent Co., Ltd. The C18 solid-phase extraction (SPE) cartridge was purchased from Acchrom (Beijing, China). The Ginsenoside Rg1 was purchased from Dalian Meilun Biotech Co., Ltd. (China).

Sample Preparation. Ten grams of sample (stems of *P. notoginseng*) was ground into powder and decocted in 100 mL water for 120 min. The decoction was filtrated out, and the residue was decocted in another 100 mL water for 90 min. These two decoctions were combined and concentrated, and then methanol was added until the concentration of methanol reached 75%. After standing for 24 h in refrigerator at 4 °C, the supernatant was concentrated to 10 mL. Two grams of neutral alumina was packed into a 6 mL SPE cartridge and washed with water in conditioning step and methanol in equilibration step. One milliliter of sample solution was loaded on Al_2O_3 SPE and eluted successively with 10 mL methanol and 10 mL water. The methanol elution was evaporated to dryness and then dissolved in 1 mL water. It was rechromatographed over a C18 SPE, eluting successively with 10 mL 90% water (10% methanol) and 10 mL methanol. The portion eluted with methanol was found to contain the saponins of interest, and it was evaporated to dryness with nitrogen gas and redissolved in 1 mL methanol.

Chromatographic Condition. The separation of the sample under SFC was operated on the column Atlantis® HILIC Silica (150 mm × 4.6 mm i.d., 5 μm, Waters), the detection wavelength was 205 nm, and the mobile phases were CO_2 (A) and methanol (B). The gradient program was as follows: 0–1 min, 22% B; 1–3 min, 22–32% B; 3–10 min, 32% B.

Pressure effect: The flow rate was 4.0 mL min^{-1}, the column temperature was 45 °C, and the bark pressure ranged from 110, 131, 172 to 207 bar.

Temperature effect: The flow rate was 4.0 mL min^{-1}, the back pressure was 131 bar, and the temperature was 40, 45, 50, and 55 °C.

Flow rate effect: The column temperature was 45 °C, the back pressure was 131 bar, and the flow rate changed from 2.0, 3.0, 3.5 to 4.0 mL min^{-1}.

Optimized conditions: The back pressure was 131 bar, the column temperature was 45 °C and the flow rate was 4.0 mL min^{-1}.

The fractions from the SFC were reanalyzed under RPLC. The column was Agilent Poroshell EC-C18 (3.0 × 50 mm i.d., 2.7 μm, Agilent); the flow rate was 0.6 mL min^{-1} with the mobile phase H_2O (A) and acetonitrile (B); the column temperature was 30 °C; and the detection wavelength was 205 nm. The gradient program was as follows: 0–10 min, 20–60% B; 10–15 min, 60–95% B. The condition of MS was set as follows: nitrogen was used as the desolvation gas at 850 L h^{-1}; source temperature and desolvation temperature were 120 °C and 500 °C; sampling cone and source offset were 40 V and 60 V; and capillary voltage was 2.50 kV in the positive mode and 2.0 kV in the negative mode. The mass spectrometer was scanned from *m*/*z* 350 Da to 1700 Da in full scan mode. MS data was processed using a Masslynx (v 4.1).

Results and Discussion

Optimizing the Conditions of SFC. The structure of triterpene saponins consists of a triterpene type of aglycone and one or more sugar moieties. Thus, the polar stationary phase was used in this study. The preliminary experiment showed that saponins had good retention on the silica column under SFC mode with CO_2 as the mobile phase and methanol as a modifier. Later in this study, an off-line 2-D SFC–RPLC system would be developed. In order to guarantee the concentration of the first fractions, the larger sampling amount was needed. Also, the report has proved that current ultrahigh-performance supercritical fluid chromatography (UHPSFC) instruments are more ready to function properly with 4.6 mm i.d. columns [36]. Therefore, the larger dimension of the silica column (150 × 4.6 mm i.d.) was selected. A series of optimization experiments were performed.

First, the effects of three modifiers were investigated, including acetonitrile, methanol, and ethanol. The triterpene saponins could not be eluted when using acetonitrile as a modifier, while the other two modifiers could ensure the elution of the peaks. Separation with methanol showed more information than ethanol at the same condition. In addition, methanol had smaller viscosity and better solubility. Thus, methanol was selected as a modifier for further separation. Furthermore, three elution gradients were performed by adjusting the ratio of methanol in mobile phase and the most time-saving gradient was used without losing the separation selectivity.

Pressure and temperature are the important factors for the separation under SFC. The effect of the back pressure (110 bar, 131 bar, 172 bar, and 207 bar) on the retention behavior of the triterpene saponins was studied (Figure 1a). As the back pressure was increasing, the fluid density and solvating power of the supercritical CO_2 increased. Then, the retention time of all the analytes decreased, but the separation selectivity remained unchanged. The lower back pressure was more easily to stabilize in the subsequent flow split; thus, the conventional condition (131 bar) was selected. The effect of column temperature (varying between 40 and 55 °C) was also investigated, and the retention time of triterpene saponins increased with the temperature increasing (Figure 1b). It could be attributed to the decrease in density of the supercritical CO_2, which resulted in a decrease in the elution power of the mobile phase. The column temperature had slight effect on the separation. In view of the stability of the triterpene saponins, the column temperature was set as 45 °C.

SFC facilitates lower pressure drop at relatively high flow rate. The four flow rates, 2.0, 3.0, 3.5, and 4.0 mL min^{-1}, were investigated (Figure 1c). The retention time of the triterpene saponins shortened from 11.0 min to 7.0 min by improving the flow rate from 2.0 mL min^{-1} to 4.0 mL min^{-1} without any change in resolution. Therefore, the flow rate was set as 4.0 mL min^{-1}. Based on the result, it showed that the chromatographic parameters could affect the retention time of the saponins but not the separation selectivity under SFC.

Construction of the Off-line 2-D Chromatography. After optimization, the triterpene saponins were well separated within 7 min by SFC (Figure 2a), and the retention time of the main saponins was distributed in the range of 2 min to 4 min. The separation of the triterpene saponins was also performed using UHPLC under reversed phase mode. Good and fast separation of the saponins was achieved under UHPLC (Figure 2b). The different selectivity of SFC and UHPLC was observed (Figure 2). It was promising for the construction of 2D-LC-system-based SFC and RPLC for the separation of triterpene saponins samples. SFC was selected as the first dimension, and the fractions were collected every 0.3 min from 2.0 min to 4.4 min. These fractions were named according to the time order as a, b, c, d, e, f, g, and h, which was further analyzed by RPLC–UV–MS.

Eight fractions were further reanalyzed under RPLC (Figure 3). After identification by MS, the saponins were numbered with Arabic numbers. A total of 17 saponins in the 8 fractions were detected under RPLC. The fraction c contained one main peak under SFC (Figure 2a), which comprised compounds 3, 4, and 5 under RPLC (Figure 3c), and the fraction e contained 6 saponin peaks under RPLC. Not only the coeluted peaks under SFC were separated under RPLC but also the elute sequence changed. For example, the fraction d was eluted before the fraction e under SFC (Figure 2a). However, the retention times of compounds 6 and 7 in the fraction d were longer than the compounds in fraction e (Figures 3d and e) under RPLC. As the retention mechanisms were different between the SFC and RPLC, it could be an indication of the good orthogonality between the two modes.

Studying the Retention Behavior of the Triterpene Saponins under SFC. The off-line SFC/RPLC–MS was set to study the retention behavior of the saponins under SFC. The MS was combined to identify the chemical structure of the compounds, which was referred to the former work of the author [33]. The fragments $[M + H]^+$ and $[M + FA]^-$ were used to calculate the molecular weight, and the fragments in the positive mode were used to identify the type of aglycone (Figure 4) and sugars. The structure information of 17 compounds was shown in Table 1 after removing the repeated data. The compound 17 was determined by comparison with the standard compound as Ginsenoside Rg1. The identification of the other compounds was based on matching the molecular ions and the fragment ions with data reported in the literature [14]. Due to the diversity and the similarity of the saponins in *P. notoginseng*, the structure was difficult to determine. The possible structures of the saponins were shown in Table 1.

According to the result, it could be concluded that the retention behavior of the triterpene saponins under SFC were affected by the number of the sugar moiety. The triterpene saponins with one sugar residue had shorter retention time than that with two sugar residues. The effect of the sugar moiety's type on retention was also observed. Compounds 14–17 in fractions f or g all had two sugar residues including hexose, 6-deoxyhexoses, and pentoses (Table 1). Compound 17 that had two hexose residues exhibited stronger retention than compounds

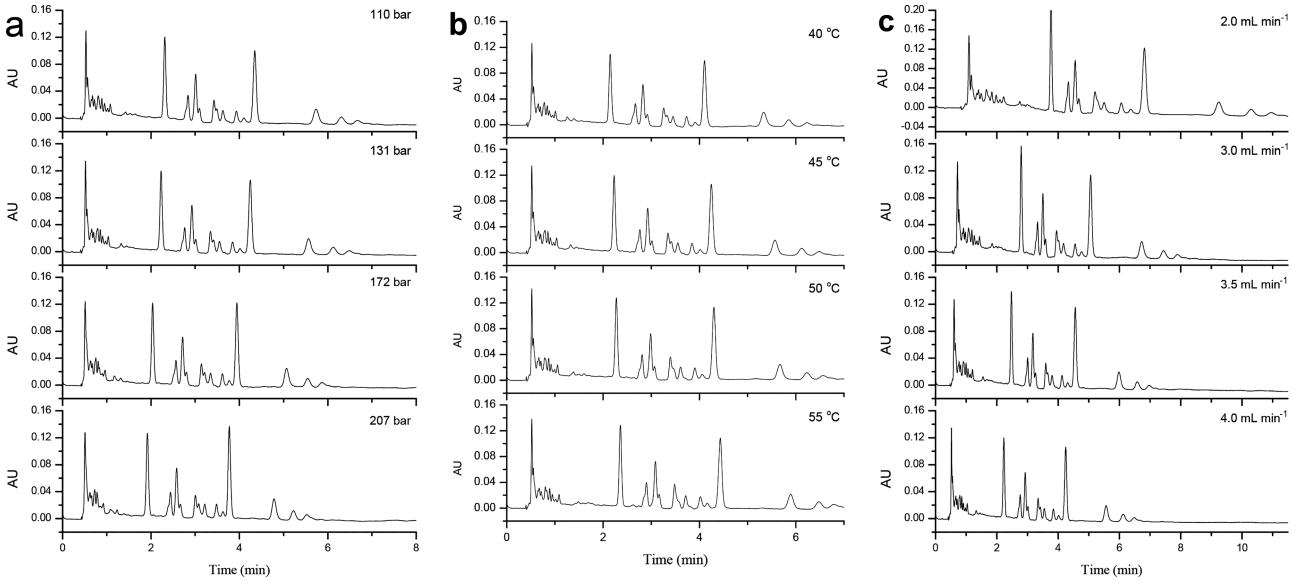

Figure 1. Chromatograms of triterpene saponins from *Panax notoginseng* with different back pressure (a), column temperature (b), and flow rate (c) under SFC. The column: Atlantis® HILIC Silica (150 mm × 4.6 mm, 5 μm, Waters); mobile phase: supercritical CO_2 (A) and MeOH (B); gradient: 0–1 min, 22% B–22% B; 1–3 min, 22% B–32% B; 3–10 min, 32% B–32% B; flow rate: 4.0 mL min^{-1}; column temperature: 45 °C; ABPR: 131 bar; wavelength: 205 nm

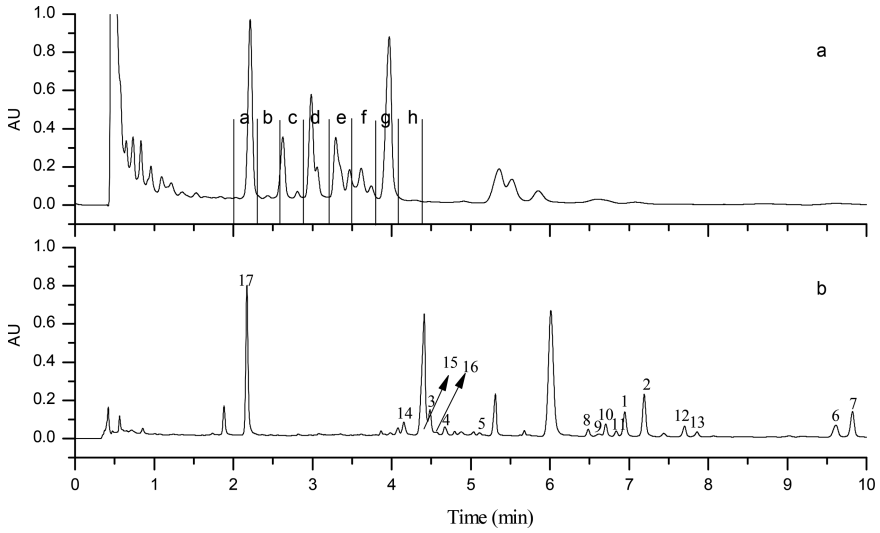

Figure 2. Fast analysis of triterpene saponins from *Panax notoginseng* under SFC (a) and UHPLC (b). The condition of SFC: the column was Atlantis® HILIC silica (150 mm × 4.6 mm, 5 μm, Waters), the mobile phase was supercritical CO_2 (A) and MeOH (B), the flow rate was 4.0 mL min^{-1}, the column temperature was 45 °C, the back pressure was 131 bar, and the gradient was 0–1 min, 22% B–22% B; 1–3 min, 22% B–32% B; 3–10 min, 32% B–32% B. Wavelength: 205 nm. The condition of UHPLC: the column was EC-C18 (50 mm × 3.0 mm, 2.7 μm, Agilent), the mobile phase was H_2O (A) and ACN (B), the flow rate was 0.6 mL min^{-1}, the column temperature was 30 °C, the wavelength was 205 nm, and the gradient was 0–10 min, 20% B–60% B; 10–15 min, 60% B–95% B.

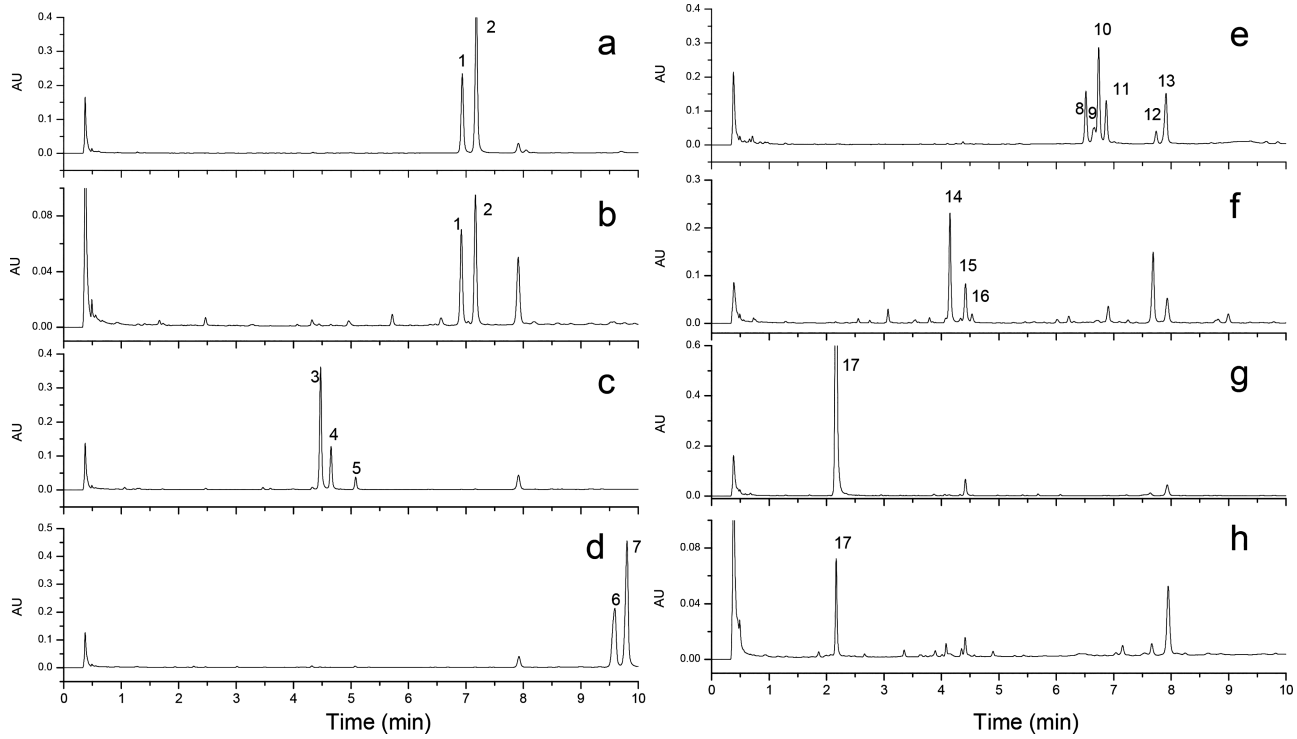

Figure 3. The analytical chromatograms of separation fractions a–h from SFC under RPLC. The column was EC-C18 (50 mm × 3.0 mm, 2.7 μm, Agilent). The mobile phase was composed of H_2O (A) and ACN (B). The linear gradient was 0–10 min, 20% B–60% B; 10–15 min, 60% B–95% B. The column temperature was 30 °C. Flow rate: 0.6 mL min^{-1}. The wavelength was 205 nm

14–16 which had one hexose residue and one pentose or 6-deoxylhexose residue. Therefore, separation of compound 17 from other compounds by SFC was successfully achieved. Unfortunately, the number of the hydroxyls of 6-deoxyhexoses was the same as the pentose's, and then compounds 14–16 were not separated by SFC, which were coeluted in fraction f. In addition, the retention behavior of the triterpene saponins under SFC was also affected by the aglycone. The compounds in fractions a and c contained the same kind of sugar moiety. The aglycone of the saponins in fraction a was I-2/III-1/III-2 type, while the aglycone in fraction c was I-4/II-1 type. The difference in retention behavior was attributed to that the aglycone in fraction a had one less hydroxyl than that in fraction c (Figure 4).

The silica column belonging to the polar stationary phase provided considerable hydrogen bonding interactions, which mainly controlled the retention behaviors of triterpene saponins under SFC [37]. The hydrogen bonding interactions were affected by the number of hydroxyls of triterpene saponins. Therefore, the aglycone and sugar moiety containing different number of hydroxyl groups determined the retention behavior of triterpene saponins. The presence of "clustering effect" under SFC was observed, namely, one SFC peak always

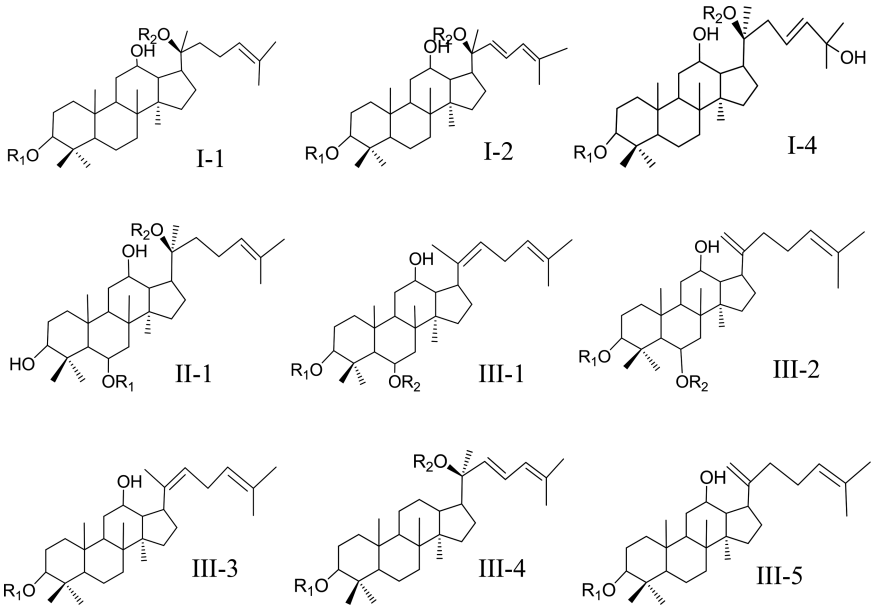

Figure 4. The aglycone skeletons of saponins in *Panax notoginseng*

Table 1. The MS fragmentation information of the triterpene saponins from *Panax notoginseng*

No.	Fraction no.	$t_{R(SFC)}$ (min)	$t_{R(RPLC)}$ (min)	$[M + H]^+$ *m/z*	$[M + FA]^-$ *m/z*	$[Aglycone + H]^+$ *m/z*	Fragment ions of the aglycone *m/z*	Aglycone	Sugar residues	Possible structure
1[a]	a/b	2.0–2.3	6.94	621.4368	665.4274	459	441, 423, 405	I-2/III-1/III-2	162	Ginsenoside Rh4/Rk3
2[a]	a/b	2.3–2.6	7.18	621.4374	665.4277	459	441, 423, 405	I-2/III-1/III-2	162	
3[a]	c	2.6–2.9	4.47	639.4455	683.4395	477	441, 423, 405	I-4/II-1	162	Ginsenoside F1/20S-Ginsenoside Rh1/20R-Ginsenoside Rh1
4[a]	c	2.6–2.9	4.65	639.4464	683.4417	477	441, 423, 405	I-4/II-1	162	
5[a]	c	2.6–2.9	5.08	639.4465	683.4396	477	441, 423, 405	I-4/II-1	162	
6[a]	d	2.9–3.2	9.59	767.4951	811.4862	443	443, 425, 407	III-3/III-4/III-5	162 + 162	Ginsenoside Rg5/Rk1
7[a]	d	2.9–3.2	9.80	767.4952	811.4869	443	443, 425, 407	III-3/III-4/III-5	162 + 162	
8[b]	e	3.2–3.5	6.52	753.4768	797.4724	459	441, 423, 405	I-2/III-1/III-2	162 + 132	Unidentified
9[a]	e	3.2–3.5	6.66	767.4954	811.4883	459	441, 423, 405	I-2/III-1/III-2	162 + 146	Ginsenoside Rg6/F4
10[b]	e	3.2–3.5	6.74	753.4804	797.4729	459	441, 423, 405	I-2/III-1/III-2	162 + 132	Unidentified
11[a]	e	3.2–3.5	6.87	767.4943	811.4883	459	441, 423, 405	I-2/III-1/III-2	162 + 146	Ginsenoside Rg6/F4
12[a]	e	3.2–3.5	7.74	785.5118	829.4980	461	443, 425, 407	I-1	162 + 162	20S-Ginsenoside
13[a]	e	3.5–3.8	7.91	785.5076	829.4992	461	443, 425, 407	I-1	162 + 162	Rg3/20R-Ginsenoside Rg3
14	f	3.5–3.8	4.15	771.4893	815.4839	477	441, 423, 405	I-4/II-1	162 + 132	Notoginsenoside R2/Unidentified
15[b]	f	3.5–3.8	4.42	771.4817	815.4850	477	441, 423, 405	I-4/II-1	162 + 132	
16	f	3.5–3.8	4.53	785.5133	829.4987	477	441, 423, 405	I-4/II-1	162 + 146	Ginsenoside Rg2
17	g/h	4.1–4.4	2.17	801.5013	845.4957	477	441, 423, 405	I-4/II-1	162 + 162	Ginsenoside Rg1

The most common sugar residues presented in *Panax notoginseng* are hexose (glucose), 6-deoxyhexoses (rhamnose), and pentoses (arabinose, xylose) with the molecular weight as 162, 146, and 132. Xyl and Ara cannot be differentiated.

[a]The molecular weight of the compounds was the same, and the structure was similar which cannot be distinguished.

[b]The unidentified compounds that were not found in the literature.

contained several saponins with same number of sugars and similar structure of aglycone.

The clustering effect of triterpene saponins promised SFC to be used as first dimension to complete the preliminary crude separation in the 2-D liquid chromatography.

Conclusion

In this work, the off-line 2-D SFC/RPLC–MS was selected to separate the triterpene saponins from *P. notoginseng*. The SFC condition was optimized at first. The Atlantis® HILIC Silica (150 mm × 4.6 mm i.d., 5 μm, Waters) was chosen due to the outstanding separation and appropriate size. Later, methanol was selected as a modifier. Then, the most time-saving gradient was developed. The back pressure, column temperature, and flow rate were optimized as 131 bar, 45 °C, and 4 mL min^{-1}, respectively. As the retention mechanisms were different between the SFC and RPLC, it could be an indication of good orthogonality between the two modes. Eight SFC fractions were collected in first dimension and then were analyzed by RPLC–MS in second dimension. The orthogonality between the SFC and RPLC could be used to set 2-D liquid chromatography to separate as many triterpene saponins as possible from *P. notoginseng*. The off-line SFC–RPLC was used to study the retention behavior of triterpene saponins under SFC. It could be concluded that the retention behavior of triterpene saponins was affected by two aspects: the number of the sugar moiety and the structure of the aglycone. The more sugar residues and more hydroxyls in the aglycone, the longer retention time of the triterpene saponins presented. The separation of saponins showed the clustering effect under SFC, which promised SFC to be used as first dimension to complete the crude separation of triterpene saponins in the 2-D liquid chromatography.

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