Fast separation of triterpenoid saponins using supercritical fluid chromatography coupled with single quadrupole mass spectrometry

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A R T I C L E   I N F O

A B S T R A C T

Triterpenoid saponins (TSs) are the most important components of some traditional Chinese medicines (TCMs) and have exhibited valuable pharmacological properties. In this study, a rapid and efficient method was developed for the separation of kudinosides, stauntosides and ginsenosides using supercritical fluid chromatography coupled with single quadrupole mass spectrometry (SFC-MS). The separation conditions for the selected TSs were carefully optimized after the initial screening of eight stationary phases. The best compromise for all compounds in terms of chromatographic performance and MS sensitivity was obtained when water (5–10%) and formic acid (0.05%) were added to the supercritical carbon dioxide/MeOH mobile phase. Beside the composition of the mobile phase, the nature of the make-up solvent for interfacing SFC with MS was also evaluated. Compared to reversed phase liquid chromatography, the SFC approach showed higher resolution and shorter running time. The developed SFC-MS methods were successfully applied to the separation and identification of TSs present in *Ilex latifolia Thunb.*, *Panax quinquefolius L.* and *Panax ginseng C.A. Meyer*. These results suggest that this SFC-MS approach could be employed as a useful tool for the quality assessment of natural products containing TSs as active components.

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1. Introduction

Triterpenoid saponins (TSs) is a major class of chemical compounds which exist in plants, such as kudinosides in *Ilex latifolia Thunb* [1], stauntosides in *Stauntonia chinensis DC* [2]. and ginsenosides in *Panax quinquefolius L.* and *Panax ginseng C.A. Meyer* [3]. Their pharmacological activities, including anti-inflammatory [4], hypoglycemic [5], immunological adjuvant [6], and anti-endotoxin [7] properties, have been well studied. Various methods including UV spectrophotometry [8] and chromatography [9] coupled with different kinds of detectors have been reported for the determination of TSs in traditional Chinese medicines (TCMs). Among these methods, liquid chromatography (LC) coupled with various detection modes, such as UV detector [8], evaporative light scattering detector (ELSD) [9] and mass spectrometry (MS) [10] were the most widely used. However, all of these methods cannot be considered as fast approaches because the LC separation of TSs often takes up to 20–45 min [9,11] or even over 60 min [10], which limits its appli-

cation in high-throughput analysis. Therefore, it is highly desirable to develop a rapid and efficient separation method for the analysis of TSs.

Supercritical fluid chromatography (SFC), considered as a green separation technique, is a potential alternative to LC for the analysis of TSs. By using supercritical fluids with low viscosity and high diffusivity, such as supercritical carbon dioxide ($scCO_2$), as mobile phase, SFC exhibits some interesting features [12], such as high separation efficiency, high flow-rates and thus reduced analysis times. Beside the successful achievements in chiral separations, SFC has also showed great potential in the separation and isolation of active components in TCMs and natural products, such as lipids [13], vitamins [14], ginkgolides [15], triterpenoids [16], regioisomeric spirostanol saponin diastereomers [17]. However, very few applications have been focused on the separation of TSs using SFC [18,19]. Agrawal et al. reported the determination of two TSs present in Bacopa monnieri L. extract, i.e. bacoside A3 and bacopaside II, using SFC coupled with diode array detector (SFC-DAD) on a Finepak SIL-5C-18 column [18]. However, due to the weak or no UV absorbance of most TSs [18], low sensitivity remains a major problem for the SFC-DAD approach. Samimi et al. reported the isolation of ginsenosides from North American ginseng using SFC-ELSD on a moderately polar cyanopropyl packed column. Although SFC-ELSD

is a sensitive and simple detection system [19], structural information about TSs could not be obtained simultaneously. In addition, in both studies only a limited number of TSs were separated, which did not demonstrate the universality of the SFC approach for TSs analysis. Compared to DAD or ELSD detection, MS is a very useful tool for providing structural information and improving detection sensitivity. Recently, LC coupled with various types of MS has been successfully applied to the analysis of TSs [20–23]. However, to the best of our knowledge, the application of SFC-MS to the analysis of TSs has not yet been reported.

In the present study, rapid and efficient SFC-MS methods were developed for the first time for the separation of both TSs standards (kudinosides, stauntosides and ginsenosides) and TSs from natural product extracts. The separation conditions, including temperature, pressure, the mobile phase modifier and additives and the column type, were systematically optimized. The selected conditions were then applied to the analysis of TSs present in TCMs extracts, including *Ilex latifolia Thunb.*, *P. quinquefolius L.* and *P. ginseng C.A. Meyer*. Moreover, a comprehensive comparison between LC–MS and SFC-MS with respect to selectivity and running time was carried out using a mixture of TSs as test sample.

2. Experimental

2.1. Chemicals and materials

Food grade liquid carbon dioxide (99.5% purity) for SFC separations was supplied by Yinglai Gas Company (Guangzhou, China). Formic acid (FA) and ammonium acetate (AA) were purchased from Aladdin Chemicals (Shanghai, China). HPLC grade acetonitrile (ACN), methanol (MeOH), ethanol (EtOH) and isopropanol (IPA) were all obtained from Merck (Shanghai, China). The distilled water was filtered through 0.22 μm membrane before use.

All tested kudinosides, including Ilekudinoside G, kudinosides A, C, E, F, G and O, latifolosides H and Q, were isolated from *Ilex latifolia Thunb.* according to [1]. The reference compounds of stauntosides (stauntosides H, I, X, akebia saponin D, yemuoside YM10, and yemuoside YM14) and ginsenosides (ginsenosides Rb1, Rb2, Rb3, Rc, Rd, Re, Rf, Rg1, F2, compound K (CK), notoginsenoside K (NK)) were all kindly gifted by Dr. Hao Gao from Jinan University. The structures of these TSs (Fig. 1) were confirmed by spectral data (UV, IR, MS and NMR). Their purity was found to be higher than 98% by LC-MS analysis, so that they could be used as reference standards. Five batches of dried leaves of *Ilex latifolia Thunb.* were collected from Guangdong, Guangxi, Hainan, Hubei and Zhejiang provinces of China, respectively. Samples of *P. quinquefolius L.* and *Panax ginseng C.A. Meyer* were purchased from a local drugstore in Guangzhou (China).

2.2. Instrumentation

Both SFC-MS and LC–MS experiments were performed on a 1260 Infinity Hybrid SFC/UHPLC analytical system (Agilent Technologies, Santa Clara, CA, USA) coupled with a Agilent 6130 single quadrupole mass spectrometry detector (Agilent Technologies). The 1260 Infinity Hybrid SFC/UHPLC analytical system consisted of an Infinity SFC binary pump, an Aurora A5 Fusion Module, a degasser, an autosampler with 5 μL loops, a DAD detector, a column oven, a make-up flow pump and a 2-position/10-port valve. Alternating between SFC and LC modes is accomplished by switching the 2-position/10-port valve. Additionally, a make-up flow was introduced prior to the back-pressure regulator (BPR) through an Agilent zero dead volume T-connector. Agilent OpenLab ChemStation Edition C.01.05 was used to control the SFC/MS instrument. All chromatograms were converted into .txt files and then redrawn using Microcal Ori-

gin 8.5. Sonication extraction was performed using an ultrasonic water bath (Kun Shan, Jiangsu, China).

The following columns were used in this research: ZORBAX SB-C18 column (150 mm × 4.6 mm, 5 μm) and ZORBAX RX-SIL column (150 mm × 4.6 mm, 5 μm) were obtained from Agilent Technologies; X Amide column (150 mm × 4.6 mm, 5 μm) was purchased from Acchrom Technologies (Beijing, China). Venusil NP column (250 mm × 4.6 mm, 5 μm), Venusil PFP column (250 mm × 4.6 mm, 5 μm), Venusil ASB Phenyl column (250 mm × 4.6 mm, 5 μm), Venusil Imidazolyl column (250 mm × 4.6 mm, 5 μm) and Venusil HILIC column (250 mm × 4.6 mm, 5 μm) were all generously donated by Bonna–Agela Technologies (Tianjin, China).

2.3. Sample preparation

The stock solutions of each reference standard were prepared in MeOH at a concentration of 1 mg mL^{-1} and stored at –20 °C. Dry raw materials including the dried leaves of *Ilex latifolia Thunb.* and the roots of *P. quinquefolius L.* and *Panax ginseng C.A. Meyer* were first ground into powder with an electric grinder. An amount of 0.25 g accurately weighed ground powder was transferred to a 50 mL conical flask with stopper, and 25 mL of MeOH was added. After ultrasonication at room temperature for 30 min, MeOH was added to compensate for the weight lost during the extraction. The solution was then centrifuged at 3000 × g for 10 min, and the supernatant was stored at 4 °C before use. All sample solutions were filtered through 0.22 μm membrane before injection.

2.4. Chromatographic and mass spectrometric conditions

The SFC separation of TSs was carried out using gradient elution mode at a flow rate of 3 mL min^{-1}. ScCO$_2$ and the organic modifier (MeOH, EtOH, ACN and IPA) were used as mobile phase components A and B, respectively. Additives (water and FA) were added to the organic modifier in appropriate amounts. The full loop injection mode was employed to inject 5 μL sample solution. MeOH was selected as needle wash solvent. Both BPR and temperature were optimized in order to obtain satisfactory separation. A make-up solvent made of MeOH containing different concentrations of AA was delivered at 0.3 mL min^{-1}. After a systematic optimization, different gradient elution programs were chosen for kudinosides, stauntosides and ginsenosides, respectively (Table 1). The selected temperature and backpressure for all experiments were 20 °C and 160 bar, respectively. The MS conditions were tuned in positive ESI mode for SFC separations as follows: nitrogen and air were used as curtain gas and nebulizer gas, respectively; capillary voltage, 3.5 KV; nebulizer gas flow rate, 11 L min^{-1}; nebulizer pressure, 35 psi; dry gas temperature, 300 °C. The analyses were performed in selected ion monitoring (SIM) mode using the precursor ions ([M + Na]$^+$) (Supplementary information Table S-1).

The LC–MS separation of TSs was performed on a ZORBAX SB-C18 column using gradient elution. Water (containing 0.5% FA (v/v)) and ACN were used as mobile phase components A and B, respectively. The elution gradient was as follows: 0 min/25% B, 6 min/25% B, 12 min/30% B, 20 min/40% B, 20.1 min/25% B, 25 min/25% B. The injection volume, flow rate and column temperature were 5 μL, 1.0 mL min^{-1} and 30 °C, respectively. The MS was operated in negative ESI mode as follows: capillary voltage, 3.5 KV; nebulizer gas flow rate, 11 L min^{-1}; nebulizer pressure, 35 psi; dry gas temperature, 300 °C. The analyses were performed in SIM mode using the precursor ions ([M + HCOO]$^-$) (Supplementary information Table S-2).
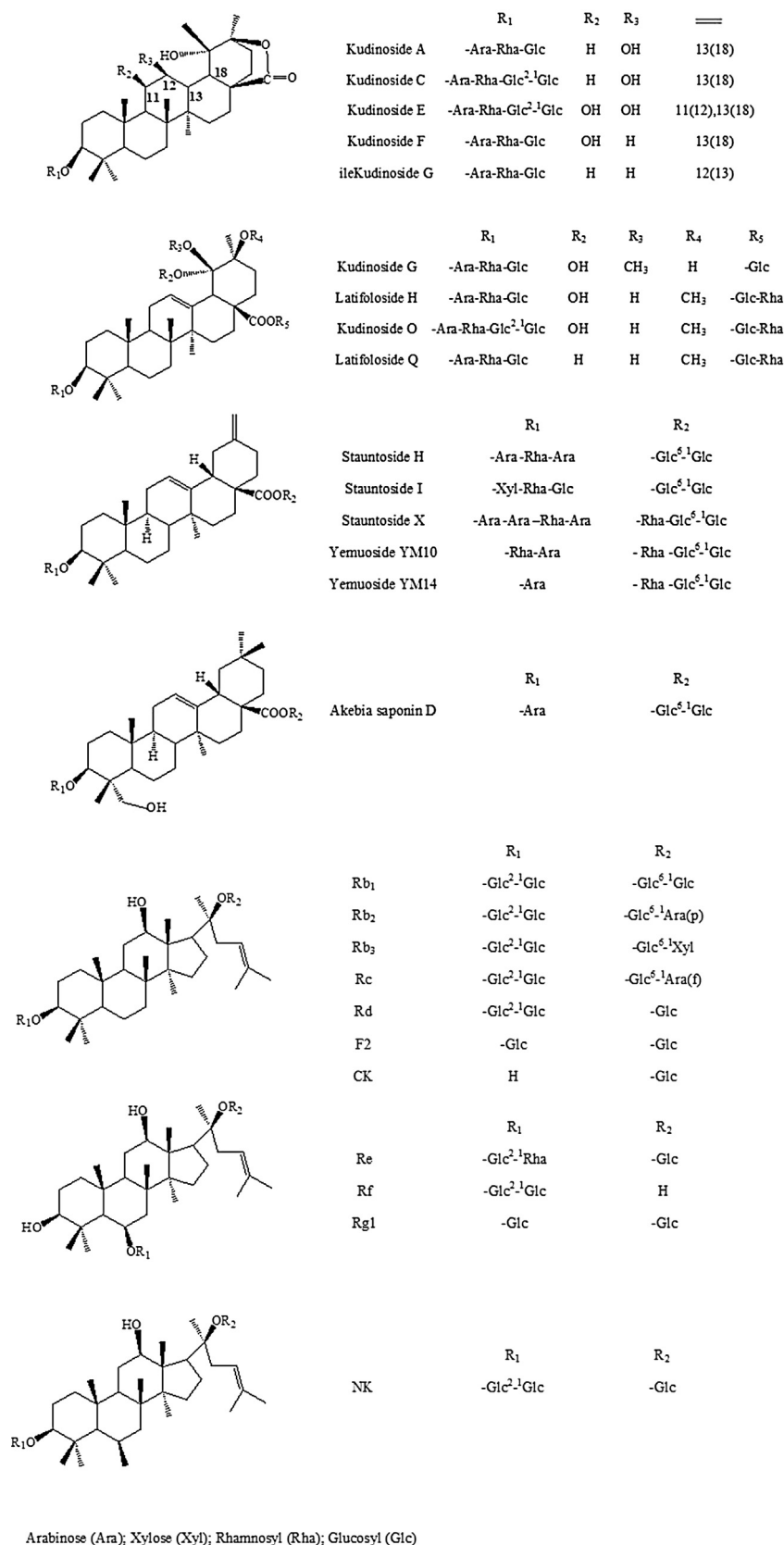
	R_1	R_2	R_3	$=\!=$
Kudinoside A	-Ara-Rha-Glc	H	OH	13(18)
Kudinoside C	-Ara-Rha-Glc$^{2-1}$Glc	H	OH	13(18)
Kudinoside E	-Ara-Rha-Glc$^{2-1}$Glc	OH	OH	11(12),13(18)
Kudinoside F	-Ara-Rha-Glc	OH	H	13(18)
ileKudinoside G	-Ara-Rha-Glc	H	H	12(13)

	R_1	R_2	R_3	R_4	R_5
Kudinoside G	-Ara-Rha-Glc	OH	CH$_3$	H	-Glc
Latifoloside H	-Ara-Rha-Glc	OH	H	CH$_3$	-Glc-Rha
Kudinoside O	-Ara-Rha-Glc$^{2-1}$Glc	OH	H	CH$_3$	-Glc-Rha
Latifoloside Q	-Ara-Rha-Glc	H	H	CH$_3$	-Glc-Rha

	R_1	R_2
Stauntoside H	-Ara-Rha-Ara	-Glc$^{6-1}$Glc
Stauntoside I	-Xyl-Rha-Glc	-Glc$^{6-1}$Glc
Stauntoside X	-Ara-Ara–Rha-Ara	-Rha-Glc$^{6-1}$Glc
Yemuoside YM10	-Rha-Ara	-Rha -Glc$^{6-1}$Glc
Yemuoside YM14	-Ara	-Rha -Glc$^{6-1}$Glc

	R_1	R_2
Akebia saponin D	-Ara	-Glc$^{6-1}$Glc

	R_1	R_2
Rb$_1$	-Glc$^{2-1}$Glc	-Glc$^{6-1}$Glc
Rb$_2$	-Glc$^{2-1}$Glc	-Glc$^{6-1}$Ara(p)
Rb$_3$	-Glc$^{2-1}$Glc	-Glc$^{6-1}$Xyl
Rc	-Glc$^{2-1}$Glc	-Glc$^{6-1}$Ara(f)
Rd	-Glc$^{2-1}$Glc	-Glc
F2	-Glc	-Glc
CK	H	-Glc

	R_1	R_2
Re	-Glc$^{2-1}$Rha	-Glc
Rf	-Glc$^{2-1}$Glc	H
Rg1	-Glc	-Glc

	R_1	R_2
NK	-Glc$^{2-1}$Glc	-Glc

Arabinose (Ara); Xylose (Xyl); Rhamnosyl (Rha); Glucosyl (Glc)

Fig 1. Structural formulas of TSs.

3. Results and discussion

3.1. Optimization of the SFC-MS separation of TSs

In order to select the most suitable column for the SFC separation of TSs, eight commercially available columns (ZORBAX SB-C18; ZORBAX RX-SIL; X Amide; Venusil NP; Venusil PFP; Venusil ASB Phenyl; Venusil Imidazolyl; Venusil HILIC) representing different polarities and surface chemistries were screened in this study. For

Table 1
Selected elution gradient programs for the three different classes of TS standards.

No.	TSs	Elution gradient
1	kudinosides	0 min/30% B-1, 4 min/35% B-1, 8 min/45% B-1, 10 min/50% B-1, 10.1 min/30% B-1, 15 min/30% B-1.
2	stauntosides	0 min/29% B-1, 5 min/29% B-1, 9 min/35% B-1, 15 min/40% B-1, 15.1 min/30% B-1, 20 min/30% B-1.
3	ginsenosides	0 min/20% B-2, 5 min/20% B-2, 7 min/37% B-2, 9 min/48% B-2, 12 min/55% B-2, 12.1 min/20% B-2, 17 min/20% B-2.

B-1: MeOH containing 0.05% (v/v) formic acid and 10% (v/v) water; B-2: MeOH containing 0.05% (v/v) formic acid and 5% (v/v) water.

example, the ZORBAX RX-SIL phase can establish different types of polar interactions with the solutes such as dipole-induced dipole interactions and dipole–dipole interactions [24]. The more polar Venusil HILIC and X Amide phases could provide even stronger hydrophilic interactions as well as hydrogen bonding interactions [25]. The Venusil ASB phenyl and Venusil NP phases could provide π–π interactions through the phenyl rings, whereas the Venusil Imidazolyl phase could give rise to strong ion-exchange interactions [26]. Besides, the Venusil PFP phase could involve multiple retention mechanisms such as hydrophobic, π–π, dipole–dipole and H-bonding interactions as well as shape selectivity [27]. The nine kudinoside standards (Fig. 1) were selected as test analytes and the gradient elution program shown in Table 1—Program 1 was used for all eight columns, the mobile phase component B being made of MeOH without additives in this case.

Reversed phase C18 columns have been commonly used for the separation of TSs in LC mode, while all nine kudinosides showed no retention at all on a ZORBAX SB-C18 column in SFC mode (Supplementary information Fig. S-1-A). This could be attributed to the polar characteristics of TSs. Therefore, the ZORBAX SB-C18 phase was not a suitable choice for the separation of TSs in SFC mode although Agrawal et al. separated bacoside A3 and bacopaside II on a C18 column [18]. A slightly increased retention for few kudinosides was observed on Venusil ASB phenyl and Venusil Imidazolyl phases. Although the retention of all nine kudinosides clearly increased on both Venusil PFP and Venusil NP phases, co-elution remained a challenge for further optimization. Notably,

these four columns exhibited different selectivities for kudinosides, which could be evidenced by the elution order of the nine standards (Supplementary information Fig. S-1-B-E). On the other hand, the two most polar phases among those tested, i.e. Venusil HILIC and X Amide columns, led to an extremely strong retention for kudinosides. Only kudinoside F and kudinoside A could be eluted from them but they were not separated (Supplementary information Fig. S-1-F-G). Among the eight columns tested, the ZORBAX RX-SIL phase exhibited the best separation performance for the nine kudinoside standards (Supplementary information Fig. S-1-H). All analytes were baseline or partially separated within 11 min. Therefore, the ZORBAX RX-SIL column was chosen for further optimization even if the peak shapes were still not perfect at this point.

Organic modifiers, such as MeOH, EtOH, ACN and IPA [28], are often added to scCO$_2$ in order to avoid the precipitation of the analytes within the column [29], reduce retention times and improve separation efficiency. Due to the low solubility of the polar kudinosides in the non-polar scCO$_2$, a relatively polar solvent had to be added to the mobile phase and the influence of this modifier on the SFC separation performance was studied using these four typical organic solvents. Under the selected gradient program for kudinosides (Table 1-Program 1), unsatisfactory separation or extremely strong retention of kudinosides was observed when ACN, EtOH or IPA (Supplementary information Fig. S-2) was used as modifiers, instead of MeOH and without additives. It was found that compared to these solvents, the use of MeOH as mobile phase component B was able to significantly improve resolution and reduce both the
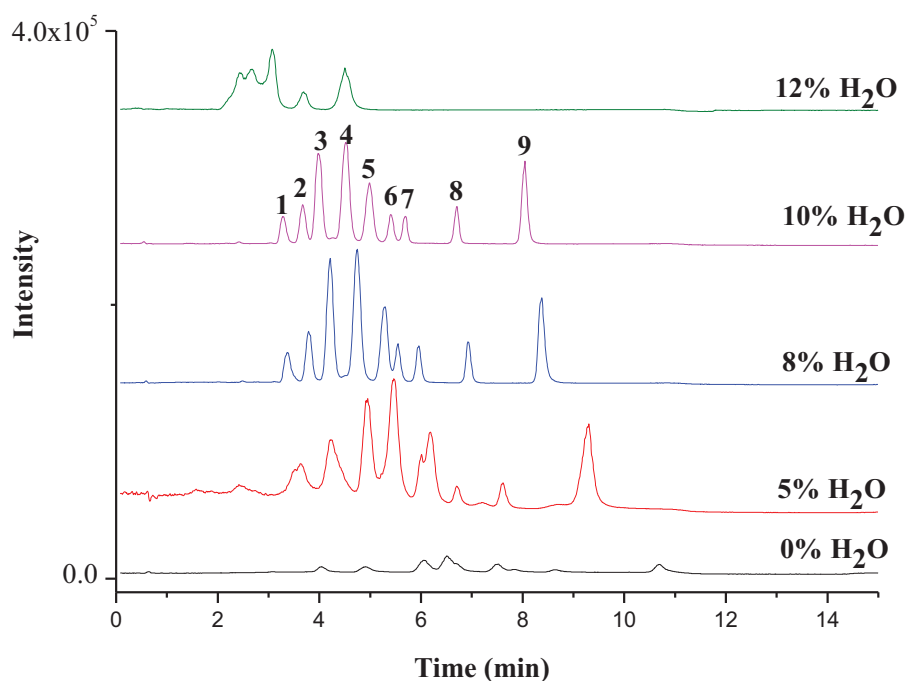

Fig. 2. Effect of water content in the mobile phase. Experimental conditions: column: ZORBAX RX-SIL column (150 mm × 4.6 mm, 5 μm); mobile phase components: (A) scCO$_2$; (B) MeOH containing 0-12% (v/v) water; gradient, 0 min/30% B, 4 min/35% B, 8 min/45% B, 10 min/50% B, 10.1 min/30% B, 15 min/30% B; column temperature: 25 °C; backpressure: 160 bar; injection volume: 5 μL; flow rate: 3 mL min^{-1}; detection mode: SIM using the precursor ions ([M + Na]$^+$) in ESI$^+$; compounds: 1. Kudinoside F, 2. Kudinoside A, 3. Ilekudinoside G, 4. Kudinoside E, 5. Kudinoside C, 6. Kudinoside G, 7. Latifoloside Q, 8. Latifoloside H, 9. Kudinoside O.

peak tailing and retention of TSs. Therefore, MeOH was chosen as modifier for further experiments.

In order to further improve resolution and peak shape, low concentrations of additives, such as water, acids, bases or salts, are often used in SFC [12]. Water is an interesting additive in SFC, due to its positive effect on peak shape and reproducibility of retention times [29]. The benefit of the addition of water to the mobile phase on the separation of the highly polar kudinosides was investigated by varying its proportion in mobile phase component B from 0 to 12% (v/v). As can be seen in Fig. 2, by increasing the water content in mobile phase component B from 0% to 10%, the resolution was clearly improved, while the analysis time was reduced consistently. Fig. 2 also shows that the highest detection sensitivity was obtained with a water content of 8%. Water in contact with $scCO_2$ becomes acidic due to the formation and dissociation of carbonic acid [30], and its ability to function both as a hydrogen bond acceptor and a hydrogen bond donor has been recognized to enhance its role as an additive in SFC [31]. However, it is worth noting that both resolution and peak shape were found to worsen with a further increase of the water content to 12%. This may be due to the pH change after adding water [31]. Finally, a water content of 10% in mobile phase component B was selected for further experiments.

Previous studies showed that the addition of FA could improve the MS response in LC mode [1]. Therefore, the effect of FA concentration (ranging from 0 to 0.2% (v/v)) in mobile phase component B was investigated in SFC mode. It was observed that the addition of FA could significantly increase the MS response of the nine standards (Fig. 3). However, the overall resolution also decreased with increasing percentage of FA in mobile phase component B. Kudinosides G, O and latifolosides Q, H could not be baseline separated after adding 0.1–0.2% (v/v) FA. As the best compromise between MS signal intensity and overall resolution, an FA concentration of 0.05% (v/v) in mobile phase component B, for which the highest MS responses were obtained, was selected for the analysis of kudinosides.

Ionization in SFC-MS is difficult because of the high flow rate of the mobile phase [15]. Thus, a make-up solution of AA in MeOH was used for interfacing SFC with MS at a flow rate of 0.3 mL min^{-1} in order to obtain satisfactory MS responses for kudinosides. As shown in the diagram from Fig. 4, the effect of the AA concentration (0–20 mM) in the make-up solution on detection sensitivity was evaluated. The MS responses of kudinosides were improved when a make-up solution containing 5 or 10 mM AA was used while a significant loss of signal intensity was observed when the AA concentration reached 20 mM. At this higher AA concentration, the ionization of kudinosides might be somewhat decreased. Finally, 10 mM was selected as the optimal AA concentration in the make-up solution for the MS analysis of TSs.

The backpressure and temperature can affect fluid density, and thus the retention of the analytes [32]. Therefore, the effect of backpressure (from 140 to 200 bar) and temperature (from 20 to 35 °C) was also investigated in order to further improve the separation performance. The effects of column backpressure on the SFC separation were studied by monitoring the retention times. As shown in Fig. S3, the variations in backpressure have very little effect on retention over the investigated range. This might be explained by the relatively high proportion of organic modifier added to $scCO_2$, which lowers the mobile phase compressibility [12]. As can be seen from Fig. 5, the decrease in temperature from 35 to 20 °C can significantly increase the resolution of the critical peak pair comprising kudinosides C and G from 0.98 to 2.02 without increasing the running time. Therefore, a temperature of 20 °C and a backpressure of 160 bar were selected as the optimal conditions for the separation of kudinosides.

Finally, the optimal SFC separation conditions for kudinosides were obtained on the RX-SIL column at 160 bar and 20 °C. MeOH
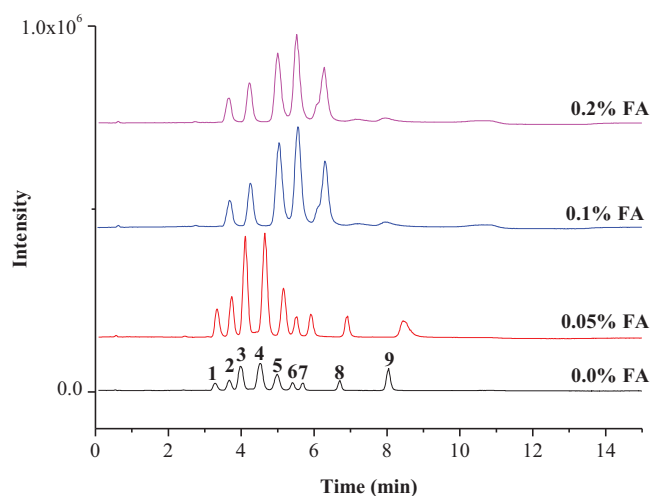

Fig. 3. Effect of the addition of formic acid to the mobile phase. Experimental conditions: mobile phase: (A) $scCO_2$, (B) MeOH (containing 10% (v/v) water) and 0-0.2% (v/v) FA; gradient: 0 min/30% B, 4 min/35% B, 8 min/45% B, 10 min/50% B, 10.1 min/30% B, 15 min/30% B; other experimental conditions as in Fig. 2.

containing 0.05% (v/v) formic acid and 10% (v/v) water was used as mobile phase component B (30-50%) in the gradient elution program 1 (Table 1).

3.2. Comparison of SFC and LC methods

Previously, we have developed a LC–MS method for the separation of TSs [1]. For the purpose of comparison, the same standard mixture of kudinosides was separated using both SFC and LC modes. A C18 column was used for the LC separation under the optimized conditions described in [1]. As shown in Fig. 6, under the selected conditions, all nine kudinoside standards can be baseline separated in SFC mode within 10 min, while it takes approximately 20 min to achieve an acceptable separation in terms of overall resolution under LC conditions. As expected, the elution order in LC mode is totally opposite to that in SFC mode due to different retention mechanisms. These results demonstrate the complementarity of the two separation modes and the usefulness of SFC as an alternative approach for tuning selectivity when LC cannot provide a satisfactory separation.

3.3. Separation of other TSs

The applicability of the SFC approach to other classes of TSs, such as stauntosides and ginsenosides, was also evaluated. Similar processes as those employed for optimizing the separation conditions of kudinosides were conducted using six stauntosides and 11 ginsenosides as test samples. It was found that the optimal separation conditions for both stauntosides and ginsenosides were similar to those selected for kudinosides except for the proportion of water added to the organic modifier in the case of ginsenosides. For the latter compounds, the best SFC separation performance was obtained when 5% water was added to MeOH (Supporting information Fig. S4). Under these optimal conditions (Table 1 - programs 2 and 3), both test mixtures of stauntosides (Fig. 7) and ginsenosides (Fig. 8) could also be well separated within 10 min. Compared with Samimi's research [19], more ginsenosides were separated, and higher selectivity was observed for ginsenosides Rb_2, Rc and Rb_1 in the present study.
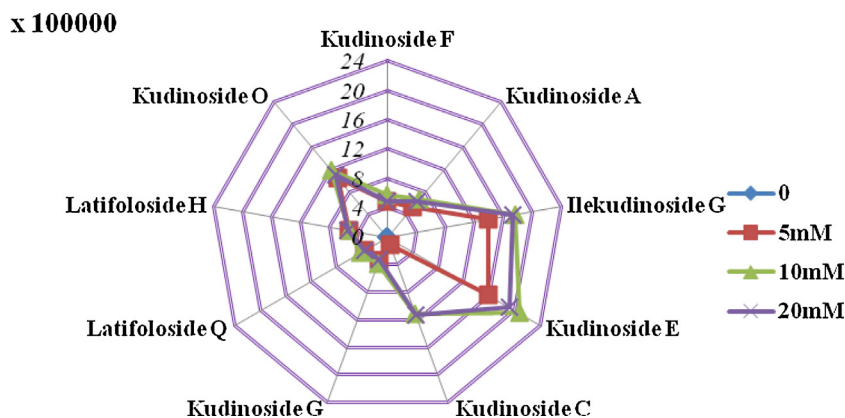
Fig. 4. Effect of the ammonium acetate concentration in the make-up solution on MS response. Experimental conditions: mobile phase: (A) $scCO_2$, (B) MeOH (containing 10% (v/v) water and 0.05% (v/v) FA); gradient, 0 min/30% B, 4 min/35% B, 8 min/45% B, 10 min/50% B, 10.1 min/30% B, 15 min/30% B; other experimental conditions as in Fig. 2.
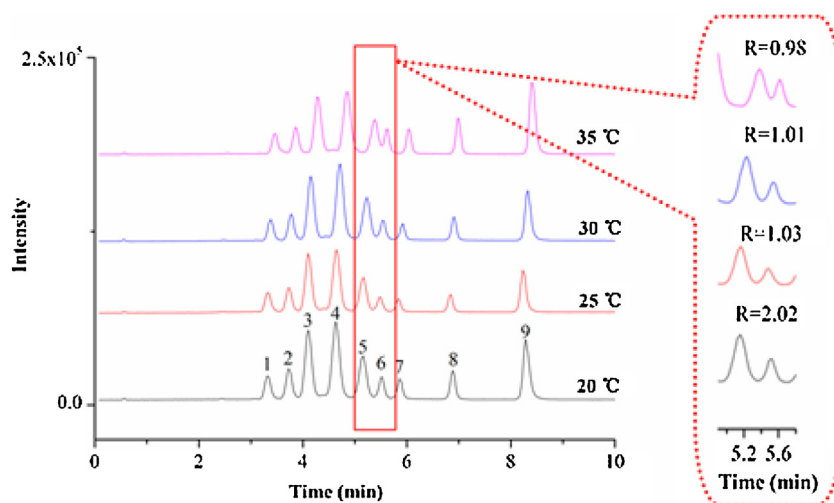

Fig. 5. Effect of temperature on resolution. Experimental conditions: make-up solution, MeOH (containing 10 mM AA); flow rate of make-up solvent, 0.3 mL min^{-1}; backpressure, 160 bar; other experimental conditions as in Fig. 3.
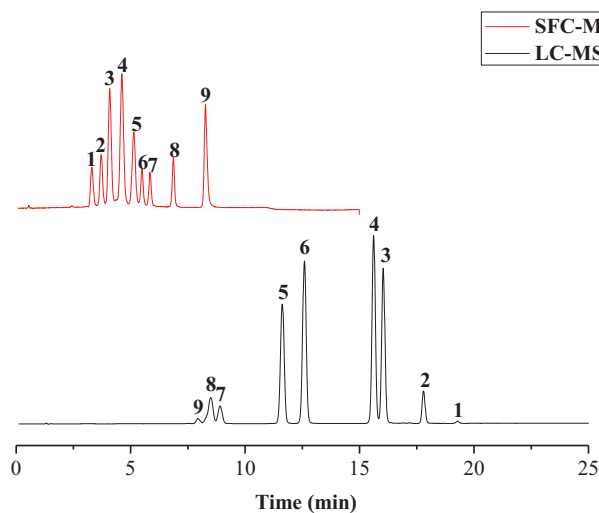
Fig. 6. Separation of the nine kudinosides in both SFC and LC modes. Experimental conditions: (LC–MS): column: ZORBAX SB-C18 column (150 mm × 4.6 mm, 5 μm); mobile phase: (A) H_2O containing 0.5% FA (v/v), (B) ACN; gradient: 0 min/25% B, 6 min/25% B, 12 min/30% B, 20 min/40% B, 20.1 min/25% B, 25 min/25% B; flow rate: 1.0 mL min^{-1}; injection volume: 5 μL; column temperature: 30 °C; detection mode: SIM using the precursor ions ([M + HCOO]$^-$) in ESI$^-$ (SFC-MS): column temperature: 20 °C; backpressure: 160 bar; other experimental conditions (SFC) as in Fig. 5.
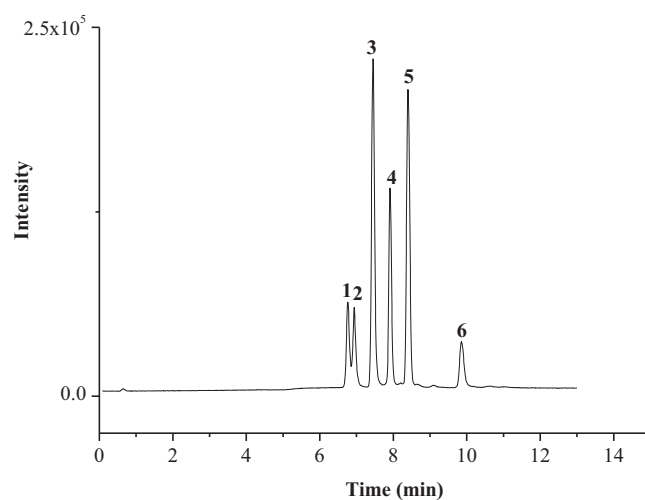

Fig. 7. Total ion chromatograms of the six stauntsoide standards. Experimental conditions: mobile phase: (A) $scCO_2$, (B) MeOH (containing 10% (v/v) water and 0.05% (v/v) FA); gradient: 0 min/29% B, 5 min/29% B, 9 min/35% B, 15 min/40% B, 15.1 min/30% B, 20 min/30% B; column temperature, 20 °C; compounds: 1. Yemuoside YM$_{14}$, 2. Akebia saponin D, 3. Yemuoside YM$_{10}$, 4. Stauntoside I, 5. Stauntoside H, 6. Stauntoside X; other experimental conditions as in Fig. 5.
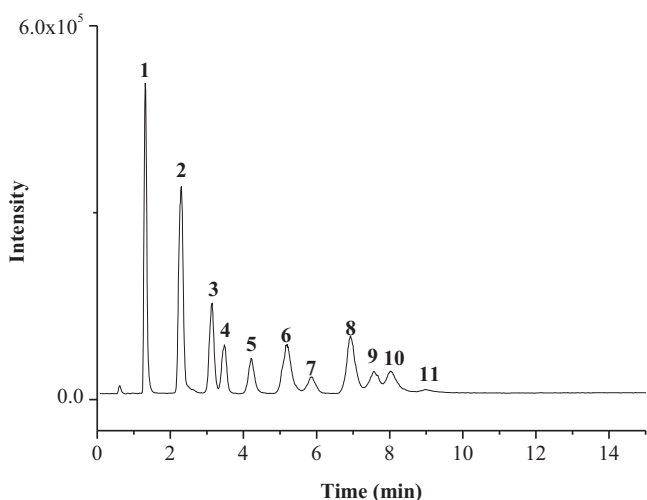
Fig. 8. Total ion chromatograms of the 11 ginsenoside standards. Experimental conditions: mobile phase: (A) $scCO_2$, (B) MeOH (containing 5% (v/v) water and 0.05% (v/v) FA); gradient, 0 min/20% B, 5 min/20% B, 7 min/37% B, 9 min/48% B, 12 min/55% B, 12.1 min/20% B, 17 min/20% B; compounds: 1. CK, 2. F2, 3. Rf, 4. Rg_1, 5. Rd, 6. NK, 7. Re, 8. Rc, 9. Rb_2, 10. Rb_3, 11. Rb_1; other experimental conditions as in Fig. 6.

3.4. Separation of TSs present in natural products

In order to further evaluate the applicability of the SFC-MS approach for the analysis of TSs, a series of natural products containing TSs were tested using the developed SFC-MS methods. Kudinosides were found to be a class of active components in *Ilex latifolia Thunb* [1]. A previous study [1] showed that the type and concentration of kudinosides are slightly different in the dried leaves of *Ilex latifolia Thunb.* collected from different origins, production processes, storage conditions, collection time, etc. In this research, 5 batches of dried leaves of *Ilex latifolia Thunb.*, collected from Guangdong, Guangxi, Hainan, Hubei and Zhejiang provinces of China, were analyzed by means of SFC-MS. Both the nine standards and the kudinosides present in *Ilex latifolia Thunb.* were examined under the selected SFC-MS conditions. As shown in Fig. 9 A–E, kudinosides present in *Ilex latifolia Thunb.* samples could be identified by MS and by comparison with the retention times of the standards. Interestingly, the amount of kudinoside G (peak 6) present in samples from Hainan (C) and Zhejiang (E) provinces was clearly lower than that in other samples, while the amounts of the other kudinosides were almost the same in five batches of samples. These results indicate that the SFC-MS approach could be a potential fast tool for the identification and quality control of *Ilex latifolia Thunb.* samples.

Moreover, the 11 ginsenoside standards and the ginsenosides present in *P. quinquefolius L.* and *Panax ginseng C.A. Meyer* were also examined under the selected SFC-MS conditions. It is well known that ginsenosides present in *P. quinquefolius L.* and *Panax ginseng C.A. Meyer* extracts are the main components responsible for their many pharmacological effects [33]. A SFC-ELSD method was reported earlier for the separation of ginsenosides in ginseng extracts [19]. Nevertheless, six of these ginsenosides were not separated. As shown in Fig. 10, several ginsenosides can be identified in *P. quinquefolius L.* (F2; Rf; Rg_1; Rd; NK; Re; Rc; Rb_2; Rb_1) and *Panax ginseng C.A. Meyer* (CK; Rf; Rg_1; Rd; Re; Rc; Rb_3; Rb_1). It was found that the amounts of ginsenosides Rg_1 (peak 4), Rc (peak 8) and Rb_2 (peak 9) present in the *P. quinquefolius L.* extract are much lower, which is consistent with literature [19,34]. CK is the metabolite of ginsenosides Rb_1, Rb_2, and Rc [35], and normally its concentration is too low to be detected in real samples. However, it was detected in the *Panax ginseng C.A. Meyer* extract using SFC-MS. In addition, a significant difference in the amounts of gingenosides Rf (peak 3),
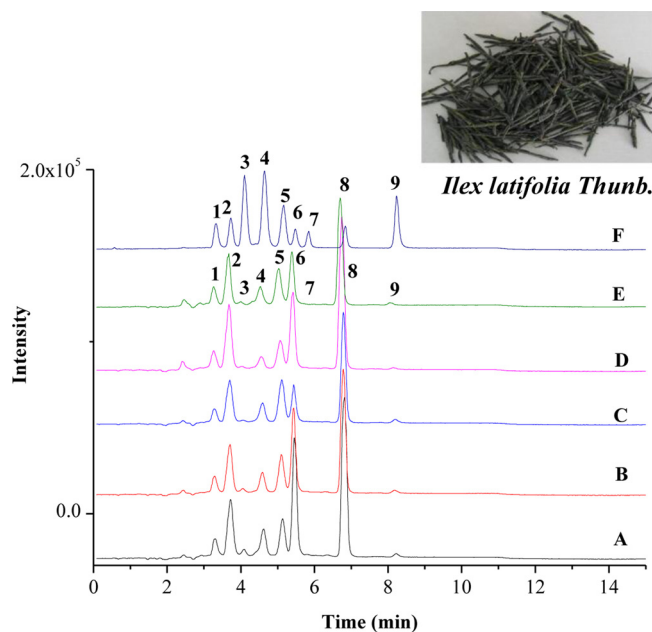

Fig. 9. Total ion chromatograms of the nine kudinosides standards and the extracts of *Ilex latifolia Thunb.* Experimental conditions: *Ilex latifolia Thunb.* samples collected from different regions: A. Guangdong, B. Guangxi, C. Hainan, D. Hubei, E. Zhejiang, F. Nine kudinoside standards; other SFC experimental conditions as in Fig. 6.
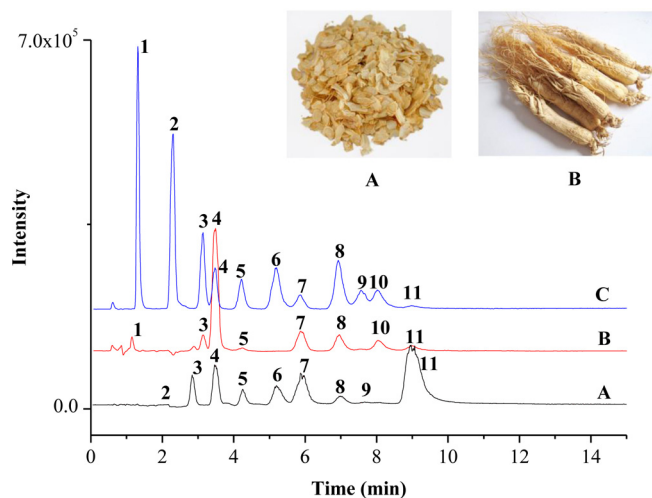

Fig. 10. Total ion chromatograms of the 11 ginsenoside standards (C) and the extracts of *Panax quinquefolius L.* (A), *Panax ginseng C.A. Meyer* (B). Experimental conditions as in Fig. 8.

Rd (peak 5) and RB_1 (peak 11) in *P. quinquefolius L.* and *Panax ginseng C.A. Meyer* extracts could also be observed using the SFC-MS approach. These observations indicate the usefulness of the SFC-MS approach for distinguishing different ginseng species.

4. Conclusions

In this work, rapid and efficient SFC-MS methods were developed for the separation and identification of kudinosides, stauntosides and ginsenosides. The separation conditions were carefully optimized. Under the selected SFC-MS conditions, all kudinosides, stauntosides and ginsenosides tested were separated on a RX-SIL column within 10 min using gradient elution. Compared to reversed phase liquid chromatography, the SFC approach provided higher overall resolution and shorter running time for the separation of TSs. Meanwhile, the developed methods were suc-

cessfully employed for the analysis of TSs present in the extracts of *Ilex latifolia Thunb.*, *P. quinquefolius* L. and *Panax ginseng C.A. Meyer*. In conclusion, the SFC-MS approach shows great potential for the qualitative analysis of TSs and could be used in the future as a quality control method for assessing the authenticity of medicinal products.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2015.12.056.

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