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Qualitative and quantitative analysis of steroidal saponins in crude extracts from *Paris polyphylla* var. *yunnanensis* and *P. polyphylla* var. *chinensis* by high performance liquid chromatography coupled with mass spectrometry

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ABSTRACT

High performance liquid chromatography coupled with electrospray ionization multi-stage tandem mass spectrometry (HPLC–ESI-MS^n) and triple quadrupole mass spectrometric detection (HPLC–ESI-MS/MS), respectively, had been employed for the simultaneous identification and quantification of steroidal saponins in the rhizomes of *Paris polyphylla* var. *yunnanensis* and *P. polyphylla* var. *chinensis*, which are the qualified plants of "Chonglou" in Chinese. The HPLC experiments were performed by means of a reversed-phase C-18 column and a binary mobile phase system consisting of 0.1% aqueous formic acid and acetonitrile under gradient elution conditions. The characteristic fragmentation patterns of diosgenin- and pennogenin-type steroidal saponins were investigated using ESI-MS^n in negative ion mode. The MS^n data of the $[M-H]^-$ ions provided structural information on the sugar sequence of the oligosaccharide chains and the aglycones of steroidal saponins. As a result, ten and seven saponins were determined in *P. polyphylla* var. *yunnanensis* and *P. polyphylla* var. *chinensis*, respectively, including four unknown compounds. One unknown compound was tentatively identified as diosgenin-3-*O*-α-L-rhamnopyranosyl(1 → 4) [α-L-rhamnopyranosyl(1 → 2)]-β-D-glucopyranoside and the aglycones of the other three new compounds were reported from Chonglou for the first time. The developed HPLC–ESI-MS/MS method was validated and found to be satisfactorily linear, selective and robust. The limits of detection (LODs) and quantitation (LOQs) ranged, respectively, from 0.5 to 10 ng/mL and 2 to 34 ng/mL depending on six various compounds. The intra- and inter-day precisions of the method were evaluated and were less than 5.0%. Recoveries ranged from 92% to 104% for all compounds. The established quality evaluation method was successfully used for simultaneous quantification of six predominant steroidal saponins in the rhizomes of these two *Paris* species.

1. Introduction

Rhizoma Paridis ("Chonglou" in Chinese), the dried rhizomes of both *Paris polyphylla* Smith var. *yunnanensis* (Franch.) Hand. -Mazz. and *P. polyphylla* Smith var. *chinensis* (Franch.) Hara, is a famous and precious traditional Chinese medicine and well documented in the Chinese Pharmacopoeia [1]. It is one of the major components of many well-known prepared Chinese medicines like Yunnan Baiyao Powder, Gongxuening Capsules and Jidesheng Sheyao Tablet, and has frequently played an important role in clinics for immunity adjustment and treating fractures, parotitis, tumours, analgesia and bleeding [2]. A lot of steroidal saponins have been isolated and identified from Chonglou and other species of the genus *Paris* [2], and they were considered as the major effective constituents for significant biological activities, such as antitumour [3,4], antifungal [5], inhibitory activities against abnormal uterine bleeding [6,7], chronotropic effects on spontaneous beating of myocardial cells [8], and protective effects on ethanol- or indomethacin-induced gastric mucosal lesions in rats [9]. The structures of these saponins were mainly classified into two types, pennogenin (**PNA**–**PNE**) and diosgenin (**DSF**–**DSK**), according to the differences of steroidal aglycones (Fig. 1).

The isolation and identification of these steroidal saponins by conventional procedures are tedious and time-consuming for their unfavorable inherent features, such as high polarities, poor volatilities and low contents in plant materials [10]. Therefore, there is a

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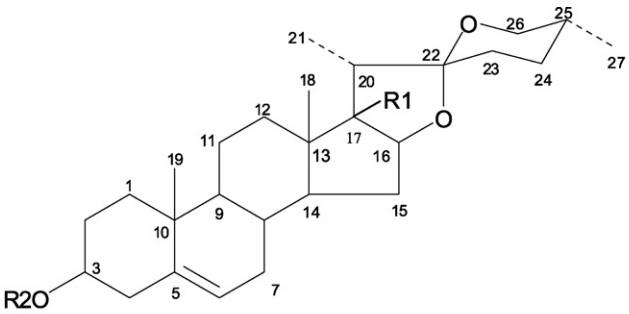

Standards	Aglycone Type	R1	R2	Molecular mass
PNA	Pennogenin	-OH	Rha (1→4)- Rha (1→4) [Rha (1→2)] -Glc-	1030
PNB	Pennogenin	-OH	Rha (1→2)- [Glc (1→3)] -Glc-	900
PNC	Pennogenin	-OH	Rha (1→2)- [Ara (1→4)] -Glc-	870
PND	Pennogenin	-OH	Rha (1→2)-Glc-	738
PNE	Pennogenin	-OH	Ara(1→4)-Glc-	724
DSF	Diosgenin	-H	Rha (1→4)- Rha (1→4) [Rha (1→2)] -Glc-	1014
DSG	Diosgenin	-H	Rha (1→2)- [Glc (1→3)] -Glc-	884
DSH	Diosgenin	-H	Rha (1→2)- [Ara (1→4)] -Glc-	854
DSI	Diosgenin	-H	Rha (1→2)-Glc-	722
DSJ	Diosgenin	-H	Ara(1→4)-Glc-	708
DSK	Diosgenin	-H	Glc-	576

Abbreviations: Glc: β-D-glucopyranosyl; Rha: α-L-rhamnopyranosyl; Ara: α-L-arabinofuranosyl

Fig. 1. Structures of eleven steroidal saponins used in this study.

great need for a sensitive, selective and practical method to structurally analyze steroidal saponins in complex mixture. With its superior performances in sensitivity and selectivity, the coupling of mass spectrometry with high performance liquid chromatography (HPLC–MS) has opened up the door to chemical research and quality control of traditional Chinese medicine. Especially, electrospray ionization mass spectrometry (ESI-MS) is a soft ionization technique that forms mainly molecular ion peaks, and the data from multi-stage tandem mass spectrometry (MS^n) with collision induced dissociation (CID) reactions on the molecular ions can provide more rich structural information [11–13]. The combinatorial HPLC–ESI-MS^n technique, which complements the excellent separating ability of liquid chromatography with the power of ESI-MS^n, is widely employed in characterization and quantification of saponins [14,15], differentiation of some specific saponin isomers [16,17], and investigations on the fragmentation behavior of saponins, such as saponin molecular species, aglycones, the saccharide sequence [18,19]. Therefore, it has been applied not only for separation and elucidation of the known steroidal saponins [20–22], but also for identification of unknown compounds in many botanical extracts [23,24]. Moreover, it is worthwhile to mention that the introduction of triple quadrupole mass spectrometric detection (MS/MS) has also led to a significant improvement in detectability and selectivity by employing multiple reactions monitoring (MRM) data acquisition [25,26].

In our previous study, the steroidal saponins in extracts from the rhizomes of *Paris* species and prepared Chinese medicines had been detected by HPLC–ESI-MS [27], whereas there were no methods available for elucidation of the structural characteristics by HPLC–ESI-MS^n and quantitative analysis of the steroidal saponins in Chonglou by LC–MS/MS. In this study, the fragmentation behavior, corresponding mechanisms and quantitative analysis of the steroidal saponins in methanol extracts, prepared from the dried rhizomes of *P. polyphylla* var. *yunnanensis* and *P. polyphylla* var. *chinensis*, had been investigated by HPLC–ESI-MS^n and HPLC–ESI-MS/MS, respectively. The interpretation of their fragmentation pathways and major diagnostic fragment ions had been accessed to the differentiation of the two types of steroidal saponins, i.e. diosgenin- and pennogenin-type. As a result, six saponins were unambiguously determined in both *P. polyphylla* var. *yunnanensis* and *P. polyphylla* var. *chinensis* by comparison with reference compounds while the structure of one compound was elucidated and the aglycones of other three new constituents were firstly reported from *P. polyphylla* var. *yunnanensis*. In addition, a simple and effective analytical method using HPLC–ESI-MS/MS had been established for the simultaneous quantification of six predominant steroidal saponins in the rhizomes of these two *Paris* species. The presented strategy herein allows a rapid, selective and sensitive analysis of saponin distribution and composition in crude extracts of Chonglou.

2. Experimental

2.1. Samples, chemicals and reagents

The dried rhizomes of *P. polyphylla* var. *yunnanensis* (YCDI0316) and *P. polyphylla* var. *chinensis* (YCHU0203) were collected from Yunnan and Anhui Provinces in China, respectively. The voucher specimens were authenticated by Prof. Qiang Wang and deposited in the Department of Chinese Materia Medica Analysis, China Pharmaceutical University. Eleven steroidal saponins were isolated from *P. polyphylla* var. *yunnanensis* in our laboratories and their structures were determined by MS, ^{1}H- and ^{13}C-NMR by comparison with the data in references [2]. The purities of these chemical standards were determined to be more than 98% by HPLC-ELSD analysis based on a peak area normalization method, and these compounds were very stable in methanol solution. Their structures were showed in Fig. 1. The solvents acetonitrile was HPLC grade from Honeywell (USA), formic acid and the other reagents were analytical grade from Nanjing Chemical Factory (Nanjing, China). Water was purified using a Milli-Q water purification system (Millipore, USA).

2.2. Sample preparation

The dried rhizomes of *P. polyphylla* var. *yunnanensis* and *P. polyphylla* var. *chinensis* were ground into powder, respectively. An aliquot (0.5 g) of the powder was accurately weighted and ultrasonicated for 30 min with 50 mL methanol. The extract was filtered and concentrated to dryness *in vacuo* at 40 °C and the residue was dissolved with methanol in a 10 mL flask for qualitative analysis. For quantitative analysis, 0.5 g of the powder was weighted and ultrasonicated for 60 min with 50 mL methanol. The extract was filtered, and residue was ultrasonicated again according to the above condition. The combined solution was concentrated to dryness *in vacuo* at 40 °C and the residue was dissolved in a 10 mL flask with methanol. All resultant solution was filtered through a 0.45 μm nylon filter before qualitative and quantitative analysis. The resultant filtrants were stored at −4 °C.

2.3. HPLC–ESI-MS^n analysis

An Agilent MSD SL Trap mass spectrometer was connected to an Agilent Technologies Series 1100 HPLC system via an ESI interface, consisting of an automatic sampler injector, a binary pump, a continuous vacuum degasser and a column heater-cooler (Agilent Technologies, USA). All the operations, the acquiring and analysis of data were controlled by MSD Trap Control Version 4.2 software (Agilent Technologies, USA).

The chromatographic separation was performed on a Kromasil RP-C18 (4.6 mm × 150 mm, 5 μm) column at a temperature of 30 °C. The mobile phase consisted of water containing 0.1% formic acid (A) and acetonitrile (B). All solvents were filtered through a 0.45 μm nylon filter prior to use. The mobile phase flow rate was kept constantly at 1 mL/min. The outflows were divided into two shares, and only one share (about 0.3 mL/min) entered MS detector. A gradient program was used according to the following profile: 0–3 min, 5–26% B; 3–9 min 26% B; 9–10 min 26–30% B; 10–15 min, 30–44% B; 15–35 min, 44–55% B; 35–40 min, 55–60% B; 40–45 min, 60–100% B. The sample injection volume was 10 μL.

The MS conditions were as follows: collision energy (Ampl), 0.6–1.0 V; collision gas, He; HV capillary voltage, 3.5 kV; nebulizer/drying gas N_2, 10 L/min; temperature, 350 °C; pressure of nebulizer gas, 40 psi; HV voltage, 3.5 kV; flow rate, 1.0 mL/min and scan range, *m*/*z* 400–1600 in negative ion mode.

2.4. HPLC–ESI-MS/MS analysis

For quantitative determination of the six steroidal saponins, all experiments were carried out in the negative ion mode by using an Accela High Speed LC System (Thermo Fisher Scientific, USA) equipped with a quaternary pump with on-line degasser, an autosampler and a TSQ Quantum Access MAX triple stage quadrupole mass spectrometer detector with an ESI interface. The raw data were acquired and processed by Xcalibur 2.0 software (Thermo Fisher Scientific, USA).

The chromatographic separation was achieved at 25 °C on an Eclipse Plus RP-C18 HPLC column (2.1 mm × 150 mm, 3.5 μm particle sizes, Agilent Technologies, USA). Mobile phases A and B were water containing 0.1% formic acid and acetonitrile, respectively. Gradient elution was as follows: 0–4 min, 40–52% B; 4–8 min, 52–58% B; 8–8.1 min, 58–100% B; 8.1–10.9 min, 100% B; 10.9–11 min, 100–40% B; 11–14 min, 40% B. The flow rate was 0.4 mL/min and the sample injection volume was 5 μL.

The operating conditions for the ESI interface were as follows: negative ionization mode; spray voltage, 4 kV; capillary temperature, 270 °C; nitrogen was used as the sheath gas (15 psi) and auxiliary gas (10 psi); skimmer offset was set as 35 V and argon was used as the collision gas at a pressure of approximately 1.0 mTorr (1 Torr = 133.3 Pa).

Quantitative analysis of **PNA**, **PNC**, **DSF**, **DSG**, and **DSH** was performed using MRM and its conditions were optimized for each saponin during infusion. The collision energy was 25 eV for **PNA** and **DSF**, 20 eV for **DSG**, and 15 eV for **PNC** and **DSH**. The **DSI** was analyzed in selective ion monitoring (SIM) mode by monitoring deprotonated molecular ions $[M-H]^-$. The retention times, MRM transitions selected for the analysis of six steroidal saponins in ESI negative mode were shown in Table 2.

3. Results and discussion

3.1. Structural characterization of the reference compounds by ESI-MS^n

A steroidal saponin molecule consists of an aglycone and several sugar moieties. Structural analysis of saponins is a difficult task owing to various linkage possibilities of the saccharides and diverse types of the monosaccharides. In order to determine details of the linkages of the sugar moieties of saponins, eleven known steroidal saponins previously isolated were investigated by ESI-MS^n, including pennogenin- (**PNA**–**PNE**) and diosgenin-type (**DSF**–**DSK**) aglycone skeletons. As shown in Fig. 1, the distinguishing difference between these two type saponins is the substituted position of hydroxyl, pennogenin at C-3, -17 and diosgenin at C-3. The ESI negative ion mode was more simple, stable and easy to interpret than the positive ion mode, and the diagnostic ions in negative ion mode could be obviously observed to distinguish two types of steroidal saponins. Thus, the negative ion mode was further conducted for comments and discussions concerning fragmentation characteristics. We used compounds **PNC** and **DSH** to discuss in detail the fragmentation pathways in the successive ESI-MS^n experiments that were performed for the stepwise elucidation of the molecular structure. Similar fragmentation patterns were observed in the spectra of the other reference compounds (Table 1).

The ESI-MS^n spectrum of **PNC** in negative ion mode was shown in Fig. 2a. ESI-MS analysis of **PNC** yielded a predominant $[M-H]^-$ ion at *m*/*z* 869. The $[M+Cl]^-$ ion at *m*/*z* 905 were observed at the same time. The 36 Da mass differences between the two dominating ions provided a straightforward means of determining the molecular weight of **PNC**, in this case 870 Da. The quasi-molecular ion $[M-H]^-$ was selected to carry out an MS^2 experiment. In the MS^2 spectrum of the parent ion at *m*/*z* 869, a fragment ion at *m*/*z* 737

Table 1
HPLC–ESI-MS^n data of steroidal saponins from Chonglou.

Standards	MW	MS	ESI-MS^n data		*P. polyphylla* var. *yunnanensis*	*P. polyphylla* var. *chinensis*
			MS^2	MS^3		
PNA	1030	1065 $[M+Cl]^-$	1029 $[M-H]^-$ 883 $[M-H-Rha]^-$ 737 $[M-H-Rha-Rha]^-$	883 $[M-H-Rha]^-$ 737 $[M-H-Rha-Rha]^-$ 591 $[M-H-Rha-Rha-Rha]^-$	+	+
PNB	900	899 $[M-H]^-$ 935 $[M+Cl]^-$	737 $[M-H-Glc]^-$	591 $[M-H-Glc-Rha]^-$		
PNC	870	905 $[M+Cl]^-$	869 $[M-H]^-$ 737 $[M-H-Ara]^-$	591 $[M-H-Ara-Rha]^-$	+	+
PND	738	737 $[M-H]^-$ 773 $[M+Cl]^-$	591 $[M-H-Rha]^-$			+
PNE	724	723 $[M-H]^-$	591 $[M-H-Ara]^-$			
DSF	1014	1013 $[M-H]^-$ 1049 $[M+Cl]^-$	867 $[M-H-Rha]^-$ 721 $[M-H-Rha-Rha]^-$	575 $[M-H-Rha-Rha-Rha]^-$	+	+
DSG	884	883 $[M-H]^-$ 919 $[M+Cl]^-$	721 $[M-H-Glc]^-$	575 $[M-H-Glc-Rha]^-$	+	
DSH	854	889 $[M+Cl]^-$	853 $[M-H]^-$ 721 $[M-H-Ara]^-$	721 $[M-H-Ara]^-$ 575 $[M-H-Ara-Rha]^-$	+	+
DSI	722	721 $[M-H]^-$ 757 $[M+Cl]^-$	575 $[M-H-Rha]^-$		+	+
DSJ	708	707 $[M-H]^-$	575 $[M-H-Ara]^-$			
DSK	576	575 $[M-H]^-$				
Compound of peak 5	868	813 $[M+HCOO]^-$ 903 $[M+Cl]^-$	867 $[M-H]^-$	721 $[M-H-Rha]^-$ 575 $[M-H-2Rha]^-$	+	+
Compound **1**-**1**	1194	1193 $[M-H]^-$ 1229 $[M+Cl]^-$	1047 $[M-H-Rha]^-$ 1029 $[M-H-Rha-H_2O]^-$ 901 $[M-H-2Rha]^-$ 883 $[M-H-2Rha-H_2O]^-$ 755 $[M-H-3Rha]^-$ 737 $[M-H-3Rha-H_2O]^-$ 721 $[M-H-2Rha-Glc-H_2O]^-$ 575 $[M-H-3Rha-Glc-H_2O]^-$ 413 $[M-H-3Rha-2Glc-H_2O]^-$	901 $[M-H-2Rha]^-$ 885 $[M-H-Rha-Glc]^-$ 755 $[M-H-3Rha]^-$ 737 $[M-H-3Rha-H_2O]^-$ 593 $[M-H-3Rha-Glc]^-$ 575 $[M-H-3Rha-Glc-H_2O]^-$ 431 $[M-H-3Rha-2Glc]^-$	+	
Compound **1**-**2**	1064	1063 $[M-H]^-$	917 $[M-H-Rha]^-$ 901 $[M-H-Glc]^-$ 755 $[M-H-Rha-Glc]^-$ 575 $[M-H-Rha-2Glc-H_2O]^-$ 413 $[M-H-Rha-3Glc-H_2O]^-$	755 $[M-H-Rha-Glc]^-$ 739 $[M-H-2Glc]^-$	+	
Compound **1**-**3**	1034	1033 $[M-H]^-$	901 $[M-H-Ara]^-$ 887 $[M-H-Rha]^-$ 755 $[M-H-Ara-Rha]^-$ 737 $[M-H-Ara-Rha-H_2O]^-$ 593 $[M-H-Ara-Rha-Glc]^-$ 575 $[M-H-Ara-Rha-Glc-H_2O]^-$	737 $[M-H-Ara-Rha-H_2O]^-$ 755 $[M-H-Ara-Rha]^-$ 593 $[M-H-Ara-Rha-Glc]^-$ 575 $[M-H-Ara-Rha-Glc-H_2O]^-$ 431 $[M-H-Ara-Rha-2Glc]^-$ 413 $[M-H-Ara-Rha-2Glc-H_2O]^-$	+	

Notes: + detected.

was produced by the loss of a pentose unit (Ara 132 Da), and it was considered to be $[M-Ara-H]^-$. An MS^3 main fragment ion at *m*/*z* 591 derived from the MS^2 precursor ion at *m*/*z* 737 was observed, and determined to be $[M-Ara-Rha-H]^-$, due to the elimination of a deoxyhexose unit (Rha 146 Da). The fragment ion at *m*/*z* 591 was $[pennogenin+Glc]^-$. Fig. 2b presented the ESI-MS^n spectrum of **DSH** in negative ion mode. The molecular weight of **DSH** was confirmed to be 854 Da according to the deprotonated molecular ion $[M-H]^-$ at *m*/*z* 853 and the adduct ion $[M+Cl]^-$ at *m*/*z* 889 in the ESI-MS spectrum. The MS^2 main fragment ion at *m*/*z* 721 from the parent ion at *m*/*z* 853 could be explained by ejecting a pentose unit (Ara 132 Da). The MS^2 precursor ion at *m*/*z* 721 yielded the fragment ion at *m*/*z* 575 in the MS^3 spectrum and the mass difference between the two fragment ions was 146 Da, which suggested that the fragment ion at *m*/*z* 575 was formed by the loss of a deoxyhexose unit (Rha), i. e. $[M-Ara-Rha-H]^-$. And the ion at *m*/*z* 575 was also $[diosgenin+Glc]^-$.

The proposed fragmentation pathway for the deprotonated molecular ion $[M-H]^-$ of **PNC** and **DSH** was assigned to the characteristic cleavage of glycosidic bonds of steroidal saponins, and the detailed structural information about the monosaccharide sequence was directly provided by the fragmentation patterns.

3.2. Chromatographic and fragmentation behavior of steroidal saponins in negative ion mode with HPLC–ESI-MS^n

In our previous HPLC–ESI-MS analysis, the chromatographic behavior of eleven steroidal saponins was confirmed to comply with the two rules: the retention times of all pennogenin-type saponins (**PNA**–**PNE**) were shorter than those of all diosgenin-type saponins (**DSF**–**DSK**); the retention times of the two types saponins increased with the numbers of monosaccharides decreased, which gave a very helpful clue to the fully structural elucidation of unknown steroidal saponins [27].

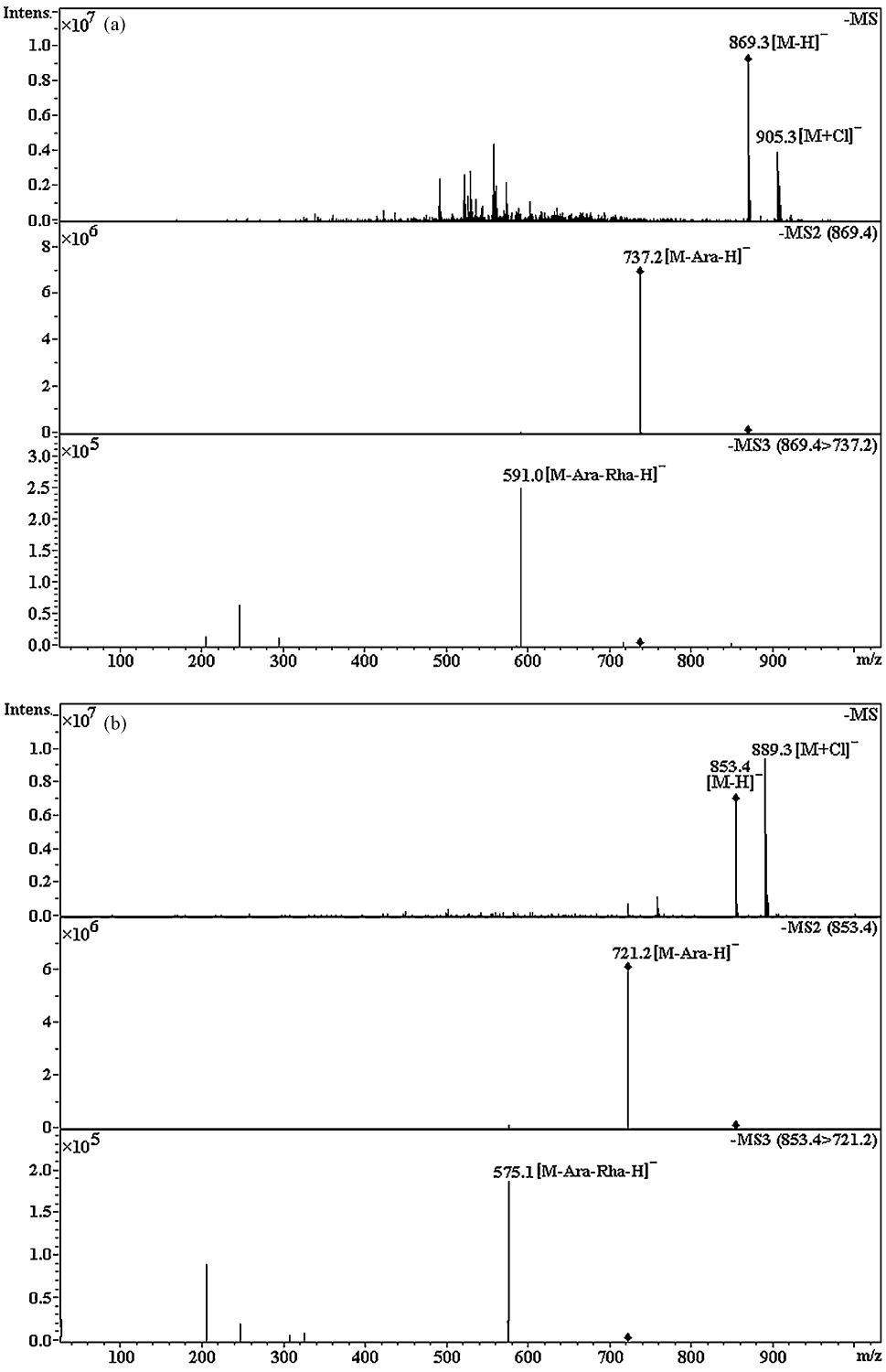

Fig. 2. (a) Negative ESI-MS^n spectra of **PNC**; (b) Negative ESI-MS^n spectra of **DSH**.

The fragmentation patterns of the eleven compounds were summarized in Table 1. The two type spirostanol saponins had obviously the similar ESI-MS^n fragmentation patterns with the variations in major product ions due to different aglycones, pennogenin with a mass of 429 Da and diosgenin with a mass of 413 Da. The fragment ions at m/z 591 [pennogenin+Glc]$^-$ and 575 [diosgenin+Glc]$^-$ could be observed in the MS^n data of these two type saponins, respectively, so they were considered as diagnostic ions in order to distinguish the pennogenin- and diosgenin-type steroidal saponins in negative ion mode. A mass difference of 16 Da between these two ions was attributed to the substituted hydroxy group at C-17 in pennogenin. As we know from the literature [2], a unique structural characteristic of steroidal saponins isolated from Chonglou was deduced from a sugar linkage mode, indicating that most of inner sugar moieties were glucose connected to the aglycone at C-3 and the normal outer sugar units were glucose, rhamnose or arabinose attached to C-2′, C-3′ and C-4′ of glucose. The fragmentation patterns could be concluded from the characterization of the sugar moieties in **PNB**, **PNC**, **DSG** and **DSH** that the monosaccharide cleavage (Glc or Ara) attached to C-3′ or C-4′ of glucose connected to

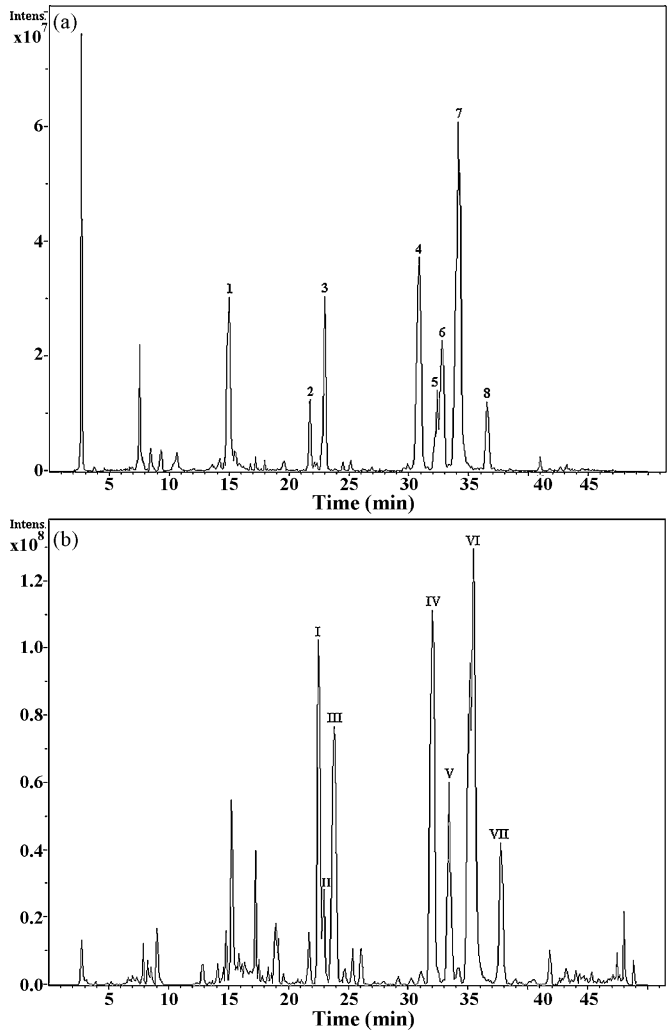

Fig. 3. HPLC–ESI-MS^n total ion current (TIC) chromatogram of methanol extracts from *P. polyphylla* var. *yunnanensis* (a) and *P. polyphylla* var. *chinensis* (b).

the aglycone occurred much more easily than the monosaccharide cleavage (Rha) attached to C-2′ of glucose.

3.3. Identification and characterization of steroidal saponins in extracts from Chonglou

Fig. 3 showed the HPLC–ESI-MS^n chromatograms of the methanol extracts from Chonglou in negative ion mode. Eight major peaks (**1**–**8**) were simultaneously detected in the total ion current (TIC) chromatogram of the extracts from *P. polyphylla* var. *yunnanensis*. By comparing their retention times (t_R), *m*/*z* values for $[M-H]^-$ ions and CID fragment ions with those of the eleven reference compounds, peaks **2**, **3**, **4**, **6**, **7** and **8** were unambiguously identified as **PNA**, **PNC**, **DSF**, **DSG**, **DSH** and **DSI**, respectively. Seven major peaks (**I**–**VII**) were simultaneously detected in extracts from *P. polyphylla* var. *chinensis*, and peaks **I**–**IV**, **VI** and **VII** were confirmed to be **PNA**, **PNC**, **PND**, **DSF**, **DSH** and **DSI**, respectively. The *m*/*z* values for $[M-H]^-$ ions and the CID fragment ions of each peak were listed in Table 1.

3.4. Analysis of unknown steroidal saponins in extracts from Chonglou

For unknown peaks, the structures were tentatively established according to the general fragmentation rules and chromatographic behavior of steroidal saponins summarized above. In the TIC chromatograms of Chonglou, there were three unknown peaks, i.e. peaks **1**, **5** from *P. polyphylla* var. *yunnanensis* and peak **V** from *P. polyphylla* var. *chinensis*. The retention times and molecular weights of them were absolutely different to those of the authentic standards, but their major fragmentations were extremely similar to those of steroidal saponins.

The extraction ion current (EIC) chromatogram of peak **5** was shown in Fig. 4a. In the ESI-MS^n spectrum (Fig. 4b), adduct ions $[M+HCOO]^-$ and $[M+Cl]^-$ were at *m*/*z* 913 and 903, respectively, and the low abundant deprotonated molecular ion $[M-H]^-$ was at *m*/*z* 867, with the molecular weight assigned as 868 Da. During the fragmentation of the $[M+HCOO]^-$ ion at *m*/*z* 913, the product ion at *m*/*z* 867 was observed with the separate mass difference of 46 Da, implying the loss of a $[HCOO]^-$ ion. In the MS^3 spectrum, the fragment ions at *m*/*z* 721 and 575 were produced from the MS^2 fragment ion at *m*/*z* 867 through the loss of a deoxyhexose (146 Da) and two deoxyhexoses (146 + 146 Da), respectively. The above analysis suggested that peak **5** might have the similar structure as that of **DSF**, expect for the absence of one deoxyhexose. Thus, peak **5** was a diosgenin-type saponin deduced from the diagnostic ion at *m*/*z* 575 appeared in the MS^3 spectrum, including three monosaccharides (two deoxyhexoses and one hexose). On the other hand, the retention time of peak **5** was far beyond that of **DSF** (four monosaccharides) and near to that of **DSG** (three monosaccharides) in the HPLC analysis under the same chromatographic condition, implying that peak **5** differed from **DSF** and **DSG** in the sugar moieties, which was in agreement with the chromatographic behavior of diosgenin-type saponins. On the basis of the above structural analysis and reference literature [2,28], the structure of peak **5** was tentatively deduced as diosgenin-3-*O*-α-L-rhamnopyranosyl(1 → 4)[α-L-rhamnopyranosyl(1 → 2)]-β-D-glucopyranoside and displayed in Fig. 4c. The similar deductive reasoning was applied to peak **V**, indicating that it shared the common structure as that of peak **5**.

The TIC and EIC chromatograms of peak **1** were shown in Fig. 5a. Three unknown saponins with the molecular weights of 1194 Da (**1**-**1**), 1064 Da (**1**-**2**) and 1034 Da (**1**-**3**) were simultaneously detected, but it was very difficult to complete baseline separation in the chromatographic condition 2.3. To obtain more detailed structural information of the related constituents, ESI-MS^n spectra were further acquired in negative ion mode (Table 1).

In Fig. 5b, deprotonated molecular ion $[M-H]^-$ at *m*/*z* 1193 and adduct ion $[M+Cl]^-$ at *m*/*z* 1229 were observed, which confirmed the molecular mass of compound **1**-**1** to be 1194 Da. One main MS^2 fragment ion at *m*/*z* 1047 and three middling abundant ions at *m*/*z* 901, 883, 755 were observed in the CID of the parent ion at *m*/*z* 1193. The fragment ions at *m*/*z* 1047, 901 and 755 were in agreement with the losses of a deoxyhexose, two deoxyhexoses and three deoxyhexoses. Simultaneously, five low abundant ions at *m*/*z* 1029, 737, 721, 575 and 413 were also observed in the MS^2 spectrum. The elimination of H_2O was found in the fragment ion at *m*/*z* 1029, due to the loss of 164 Da (water molecular and a deoxyhexose) from the parent ion at *m*/*z* 1193. The ion at *m*/*z* 1029 would be further dissociated to form the ion at *m*/*z* 883, owing to the loss of a deoxyhexose unit, followed by the separate losses of a deoxyhexose and a hexose to produce the ions at *m*/*z* 737 and 721. The ion at *m*/*z* 575 was formed by the elimination of a hexose from the ion at *m*/*z* 737. The MS^2 fragment ion at *m*/*z* 1047 yielded two main fragment ions at *m*/*z* 901 and 755 in the MS^3 spectrum, corresponding to the loss of a deoxyhexose and two deoxyhexoses, respectively. At the same time, five low abundant ions at *m*/*z* 885, 737, 593, 575 and 431 were also observed in the MS^3 spectrum. The fragment ions at *m*/*z* 593 and 431 accorded with the separate losses of 454 Da (two deoxyhexoses and one hexose) and 616 Da (two deoxyhexoses and two hexoses) from the precursor ion at *m*/*z* 1047. The mass difference

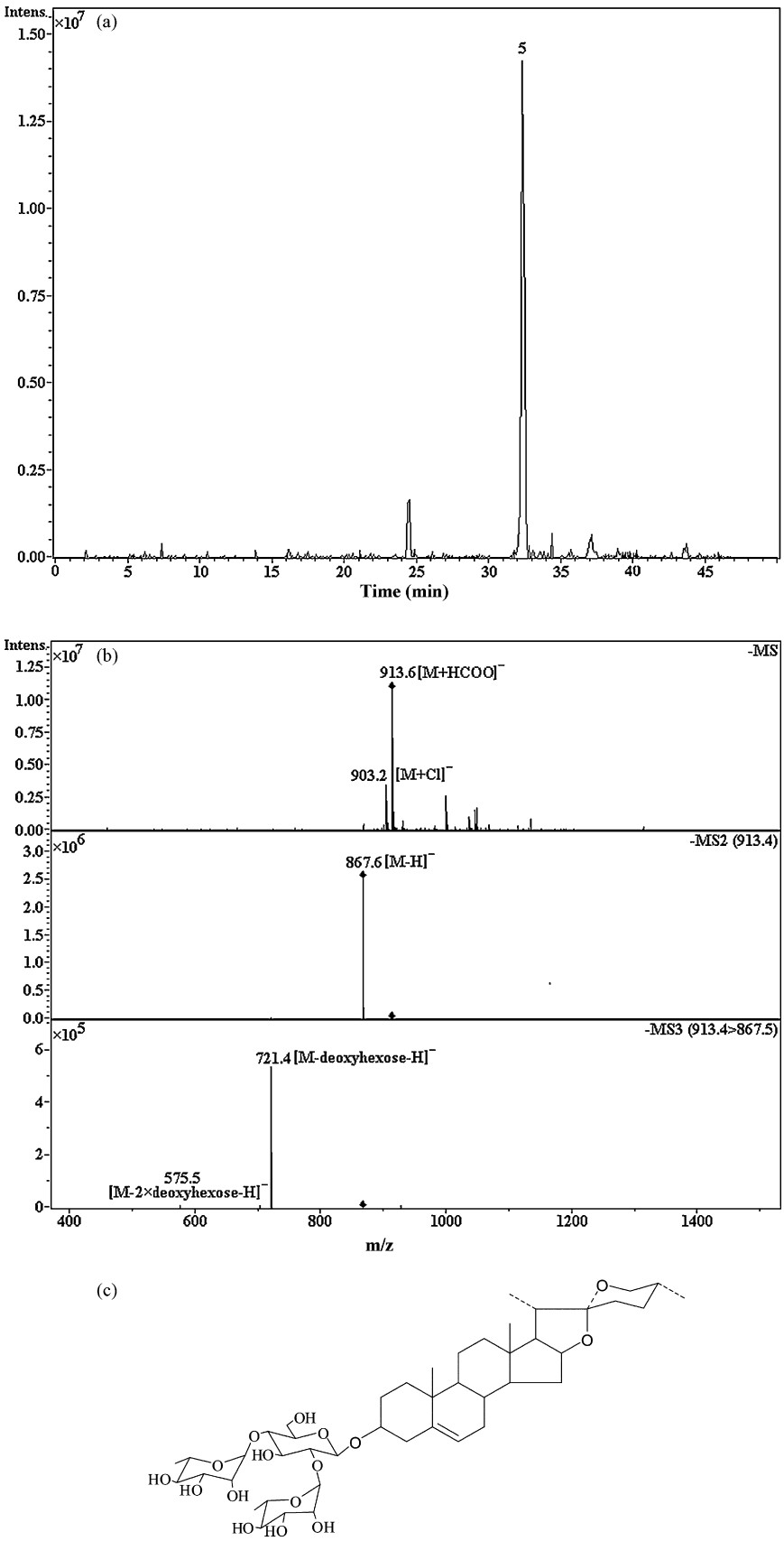

Fig. 4. (a) Extraction ion current (EIC) chromatogram of *m*/*z* 913; (b) ESI-MS^n spectrum of peak **5**; (c) The proposed structure of peak **5**.

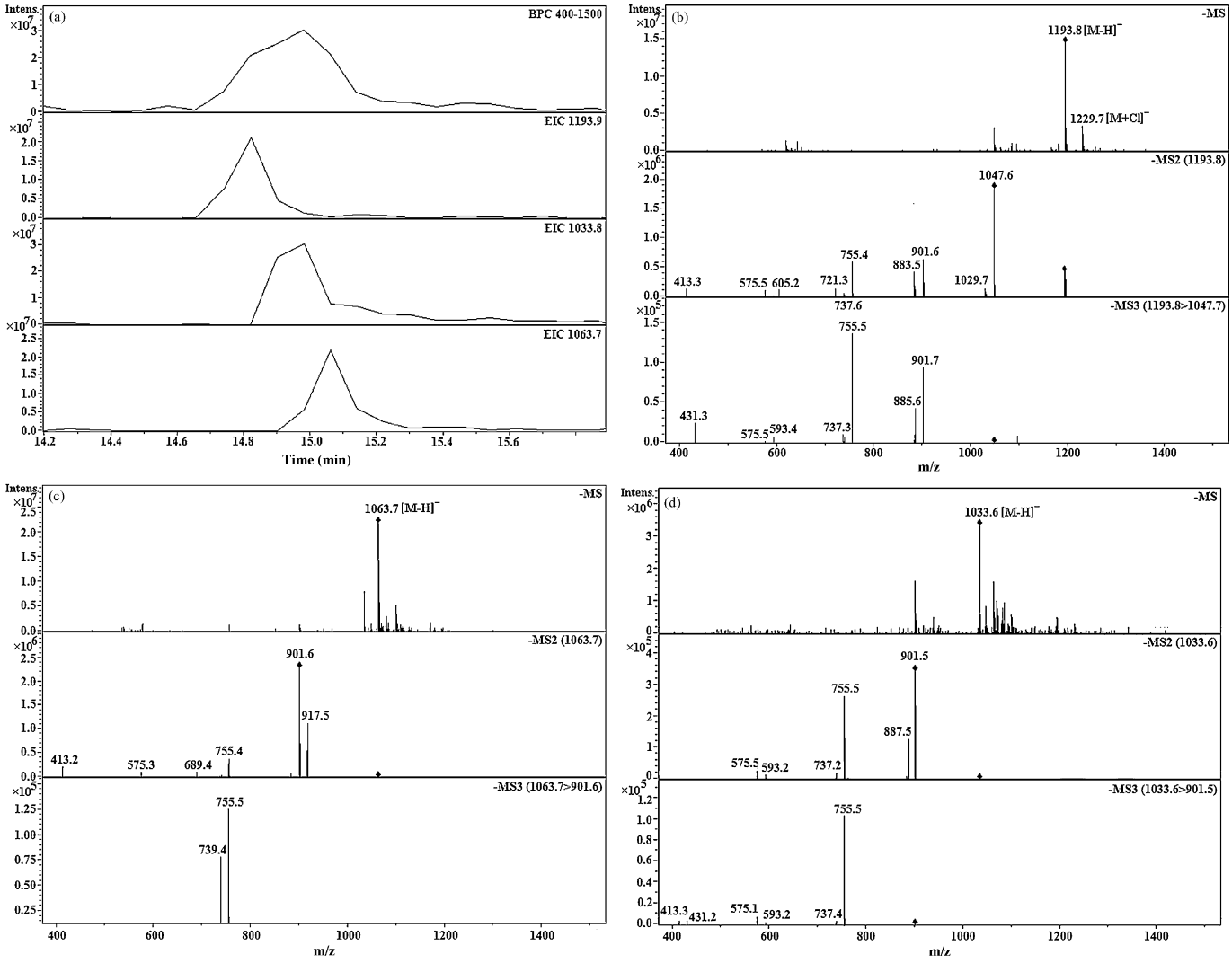

Fig. 5. (a) TIC chromatogram of peak **1** and EIC chromatograms of *m*/*z* 1193, 1063 and1033; (b) MS^n spectrum of the ion at *m*/*z* 1193 (compound **1**-**1**); (c) MS^n spectrum of the ion at *m*/*z* 1063 (compound **1**-**2**); (d) MS^n spectrum of the ion at *m*/*z* 1033 (compound **1**-**3**).

between the two fragment ions at *m*/*z* 413 in the MS^2 spectrum and 431 in the MS^3 spectrum was 18 Da, which suggested that the fragment ion at *m*/*z* 431 could lose one water molecule to produce the ion at *m*/*z* 413. The above analysis suggested that compound **1**-**1** contained five monosaccharides (three deoxyhexoses and two hexoses) and the molecular weight of its aglycone was 432 Da.

The deprotonated molecular ion $[M-H]^-$ of compound **1**-**2** was observed at *m*/*z* 1063 (Fig. 5c), which indicated its molecular weight to be 1064 Da. It was noteworthy that the $[M-H]^-$ ion at *m*/*z* 1063 underwent the separate losses of a deoxyhexose and a hexose to generate the two main fragment ions at *m*/*z* 917 and 901. Because the other product ions at *m*/*z* 755, 575 and 413 in the MS^2 spectrum were identical to those of **1**-**1**, they might share the similar tetrasaccharide chain. In the ESI-MS^3 spectrum, the product ions at *m*/*z* 755 and 739 were yielded by the separate losses of a deoxyhexose and a hexose from the MS^2 fragment ion at *m*/*z* 901. Based on the above structural information, it could be inferred that compound **1**-**2** tended to have four monosaccharides, three hexoses and one deoxyhexose.

The molecular weight of compound **1**-**3** was confirmed to be 1034 Da, according to the deprotonated molecular ion $[M-H]^-$ at *m*/*z* 1033 (Fig. 5d). The $[M-H]^-$ ion (*m*/*z* 1033) eliminated 132 Da and 278 Da in the MS^2 experiment assigned as a pentose and a pentose with a deoxyhexose unit to yield the fragment ions at *m*/*z* 901 and 755, respectively. Four low abundant fragment ions at *m*/*z* 887, 737, 593 and 575 were simultaneously observed. The loss of a deoxyhexose from the ion at *m*/*z* 1033 generated the fragment ion at *m*/*z* 887. In the MS^3 experiment of the MS^2 ion at *m*/*z* 901, one main product ion at *m*/*z* 755 and five low abundant ions at *m*/*z* 737, 593, 575, 431, 413 were yielded, which were also present in the MS^n spectrum of compound **1**-**1**. The structural information showed that **1**-**3** shared the major fragmentation patterns with **1**-**1**, and four monosaccharides (two hexoses, one deoxyhexose and one pentose) were identified in the structure of compound **1**-**3**.

The monosaccharides in the saccharide chains of steroidal saponins reported in *P. polyphylla* var. *yunnanensis* and *P. polyphylla* var. *chinensis* [2] were usually glucose (hexose), rhamnose (deoxyhexose) and arabinose (pentose). So the sugar moieties of compound **1**-**1** (three rhamnoses and two glucoses), compound **1**-**2** (three glucoses and one rhamnose) and compound **1**-**3** (two glucoses, one rhamnose and one arabinose) were proposed from the fragmentation patterns and reference literatures. The molecular weight of the aglycone of these three compounds was 432 Da, which differed from that of pennogenin (430 Da) or diosgenin (414 Da). The mass difference of 2 Da between pennogenin and the aglycone implied that the three compounds could be considered as furostane-type steroidal saponins (open F ring) and the molecular formula of their aglycone was $C_{27}H_{44}O_4$. From

Table 2
Statistical analysis of regression equations, correlation coefficients (R^2), linearity ranges, limits of detection (LODs), limits of quantitation (LOQs), retention times (t_R) and MRM transitions in the determination of the six steroid saponins employed by HPLC–MS/MS.

Marker compound	Regression equation[a]	R^2	Linear range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	t_R (min)	MRM transitions
PNA	$Y = 39{,}223.3X + 2{,}219.5$	0.9981	4340–86.8	10	34	3.03	1029 > 737
PNC	$Y = 552{,}067.7X + 95{,}073.9$	0.9984	2990–11.9	1	5	3.44	869 > 737
DSF	$Y = 51{,}086.5X + 26{,}987.0$	0.9925	7850–314	6	25	5.45	1013 > 721
DSG	$Y = 142{,}537.3X + 1{,}812.1$	0.9999	3740–29.9	5	20	6.05	883 > 721
DSH	$Y = 745{,}324.5X + 8{,}742.9$	0.9994	1012–20.2	2	8	6.37	853 > 721
DSI	$Y = 3{,}021{,}035.5X + 22{,}599.2$	0.9992	684–5.5	0.5	2	7.24	721

[a] Y, peak area count and X, concentration of standard (μg/mL).

Table 3
Repeatability, intra-day and inter-day precisions, recoveries tests and contents of the six steroid saponins in the rhizomes of *P. polyphylla* var. *yunnanensis* and *P. polyphylla* var. *chinensis*.

Compound	Repeatebility ($n = 6$, RSD, %)	Precision (RSD, %)		Recovery (%) ($n = 5$)		Content (mg/g, mean ± SD, $n = 3$)	
		Intra-day ($n = 6$)	Inter-day ($n = 12$)	Average	RSD	*P. polyphylla* var. *yunnanensis*	*P. polyphylla* var. *chinensis*
PNA	4.0	2.7	3.5	104	4.4	1.13 ± 0.007	0.81 ± 0.02
PNC	5.8	2.2	4.0	97	3.5	0.80 ± 0.01	0.78 ± 0.003
DSF	3.7	1.3	3.4	102	2.8	5.84 ± 0.1	0.017 ± 0.0005
DSG	3.8	2.8	3.1	93	3.7	3.09 ± 0.1	0.011 ± 0.0004
DSH	3.2	1.5	4.1	97	3.4	8.25 ± 0.1	0.0078 ± 0.0004
DSI	2.5	1.1	3.4	92	4.3	0.51 ± 0.002	0.00094 ± 3E−05

the above data, the molecular formulae of compounds **1-1**, **1-2** and **1-3** were tentatively deduced as $C_{57}H_{94}O_{26}$, $C_{51}H_{84}O_{23}$ and $C_{50}H_{82}O_{22}$, respectively. To our knowledge from literatures, there was no report on isolation and structural elucidation of the three compounds from *P. polyphylla* var. *yunnanensis*. They could be new compounds on the structural constitution through searching SciFinder database. Therefore, further isolation and NMR elucidation would be very helpful to confirm the presence of them in *P. polyphylla* var. *yunnanensis*.

3.5. Quantitative analysis of steroidal saponins and method validation

Six steroidal saponins including compounds **PNA**, **PNC**, **DSF**, **DSG**, **DSH**, and **DSI**, were selected for quantitative analysis and method validation by HPLC–ESI-MS/MS. All calibration curves were plotted based on linear regression analysis of the integrated peak areas (Y) versus the quantity (X, μg/mL) of the six analytes in the standard solution at a series of concentrations. The regression equa-

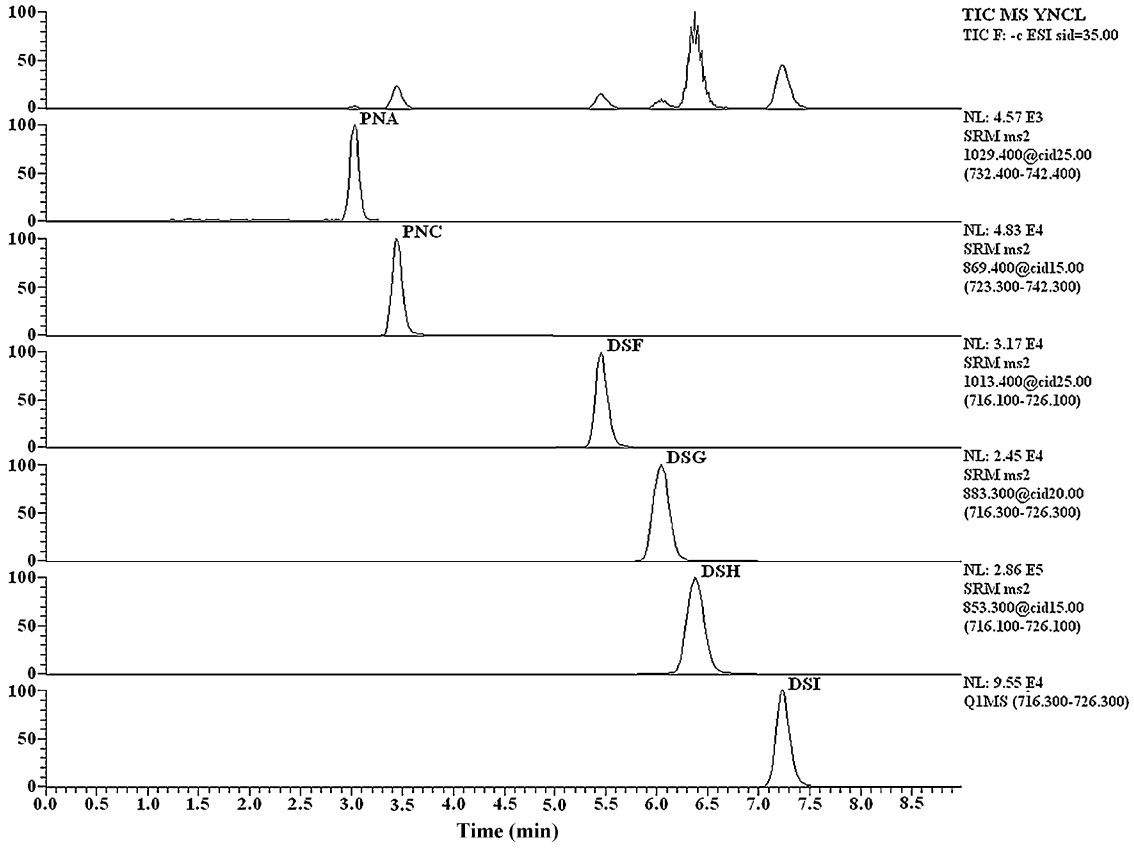

Fig. 6. HPLC–ESI-MS/MS chromatogram of six steroidal saponins in methanol extracts from *P. polyphylla* var. *yunnanensis*.

tions, correlation coefficients (R^2) and linear ranges for the six target analytes were shown in Table 2. The results indicated good linearity between the peak areas and compound concentrations over a relatively wide concentration range. The limits of detection (LODs) and quantitation (LOQs) were determined at signal-to-noise ratios (S/N) of 3 and 10, respectively. The LODs ranged from 0.5 ng/mL for **DSI** to 10 ng/mL for **PNA**, showing high sensitivity under these chromatographic conditions (Table 2).

Validation studies of the chromatographic method proved that this established method had good precision, repeatability and accuracy. The precision of the proposed method in terms of the peak areas for six replicate injections was determined in intra-day experiments. For inter-day precision, measurements of six times a day on two consecutive days were conducted. All of the measurements precision was expressed as relative standard deviation (RSD). As shown in Table 3, both intra-day and inter-day RSD of the six investigated components were less than 5.0%. Six independently prepared sample solutions of *P. polyphylla* var. *yunnanensis* with the same amount were analyzed and the variations within six measurements were calculated for evaluation of repeatability. The RSD of the repeatability test were less than 5.8% for all determination of the six marker compounds (Table 3). Recoveries were performed for evaluation of accuracy by the standard addition method. Five portions of *P. polyphylla* var. *yunnanensis* were spiked with mixed known contents of six steroidal saponins. Then the samples were pretreated as described in Sections 2.1 and 2.4, and the results were summarized in Table 3. The mean recoveries of the six steroidal saponins were 92–104%, with RSD values ranging from 2.8% to 4.4% ($n = 5$), indicating that the proposed method has an adequate degree of accuracy for the simultaneous determination of the six target constituents in the samples.

The newly established method had been applied to the simultaneous determination of the six target constituents in the rhizomes of *P. polyphylla* var. *yunnanensis* and *P. polyphylla* var. *chinensis*. Data of the quantitative analyses were expressed as means ± SD ($n = 3$, Table 3). Fig. 6 showed HPLC–ESI-MS/MS chromatogram of *P. polyphylla* var. *yunnanensis*.

4. Conclusion

In the present study, HPLC–ESI-MS^n had been established as a powerful tool for characterizing some steroidal saponins in two *Paris* species. The fragmentation behavior in negative ion ESI-MS^n spectra provided rich structural information on glycosidic bond cleavage, which could be beneficial to identify the types and sequences of sugar moieties attached to aglycone and to distinguish different aglycones (pennogenin or diosgenin). The proposed fragmentation rules made the identification of six known steroidal saponins possible. Most important, the structures of one spirostanol and three new furostanol steroidal saponins were tentatively elucidated based on their ESI-MS^n spectra from the extract of *P. polyphylla* var. *yunnanensis*, and furostanol aglycone was reported for the first time. Like other mass spectrometric methods, the established analytical method still has some limitations in differentiating geometrically isomeric compounds and it is essential to further investigations for a definite identification of these unknown saponins. However, HPLC–ESI-MS^n still provide a sensitive, selective, rapid and guided method for phytochemical research and quality control of steroidal saponins in Chonglou, as well as for the development of new medicinal resources from other *Paris* species.

In addition, an HPLC–ESI-MS/MS method for simultaneous quantitative analysis of six steroidal saponins in Chonglou was firstly developed, which could offer quite good linearity, accuracy and precision. All results demonstrated that this method could provide a reliable, sensitive and simple methodology in the quality evaluation of the widely consumed Chonglou, and new medicinal sources exploration in the future.

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