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Qualitative and quantitative determination of major saponins in *Paris* and *Trillium* by HPLC-ELSD and HPLC–MS/MS

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ABSTRACT

High-performance liquid chromatographic (HPLC) with evaporative light scattering detection (ELSD) and HPLC with electrospray ionization multistage tandem mass spectrometry (HPLC–ESI-MS^n) were used to identify and quantify steroid saponins in *Paris* and *Trillium* plants. The content of the known saponins such as Paris I, II, III, V, VI, VII, H, gracillin and protodioscin in *Paris* and *Trillium* plants was determined simultaneously using the developed HPLC-ELSD method. Furthermore, other 12 steroid saponins were identified by HPLC–ESI(+/−)-MS^n detection. In the end, a developed analytical procedure was proved to be a reliable and rapid method for the quality control of *Paris* and *Trillium* plants. In addition, the alternative resources for *Paris yunnanensis* used as a traditional Chinese medicine were discovered according to the hierarchical clustering analysis of the saponin fraction of these plants.

1. Introduction

The rhizoma of *Paris yunnanensis* (*Rhizoma Paridis*) is used as a traditional Chinese medicine (TCM) in China for a long time. The *Rhizoma Paridis* saponins (RPS) including diosgenyl, pennogenyl and protodioscin saponins [1–3], as the active ingredient, play an important role in the treatment of tumor, hemostasis, antibacterial action and inflammation counteraction, bearing some analogy to Gongxuening and Yunnanbaiyao in efficacy. But up to now, the source of *Rhizoma Paridis* becomes smaller and smaller, which needs us to discover alternate resources for it.

Its kindred plants belonging to *Paris* and *Trillium* mainly contain steroid saponins. Therefore, we chose nine typical saponins which showed strong antitumor effect before [2,4–16] and some derived saponins for qualitative and quantitative comparison of major saponins in *Paris* and *Trillium*.

As we know, a number of constituents and different possibilities of sugar chain composition and attachment cause great natural diversity of saponin structures. To identify these saponins, many methods such as TLC, HPLC, LC/MS and specific ELISA tests were selected [17]. It included further progress in the application of ELSD for saponin profiling and quantification, which was a universal detector for compounds of medium and low volatility, thus it "sees" everything including the interfering sample constituents. Up to now, ELSD has made an important progress towards successfully measuring platycosides [18], dioscin, protodioscin, gracillin [19], and C21 steroidal saponins in *Radix Cynanchi Atrati* [20]. It has also been reported in the literature for some species of the genus *Paris*, commercially available *Rhizoma Paridis* samples and prepared Chinese medicines [21,22].

In this study, a simple, accurate and reliable analytical method for the simultaneous determination of nine saponins was developed by HPLC-ELSD. The validated method was successfully applied to the quantitative analysis of important medicinal compounds which were classified as three types according to their aglycones (Fig. 1 and Table 1).

Meanwhile, some progress in the development of new applications especially for HPLC–ESI-MS^n has also been performed. This method plays an important role in the identification of natural products, particularly of saponins [23,24]. Highest sensitivity, relatively short analysis time, considerable structural information, low amount of sample and on line identification of separated saponins should be recommended as their competitive advantages. In our previous research, we identified 23 steroid saponins from

Abbreviations: Ara, α-L-arabinofuranosyl; Glc, β-D-glucopyranosyl; Rha, α-L-rhamnopyranosyl; PA, *Paris axialis*; PB, *Paris bashanensis*; PF, *Paris fargesii*; PM, *Paris mairei*; PT, *Paris thibetica*; PV, *Paris verticillata*; PY, *Paris yunnanensis*; Tt, *Trillium tschonoskii*.

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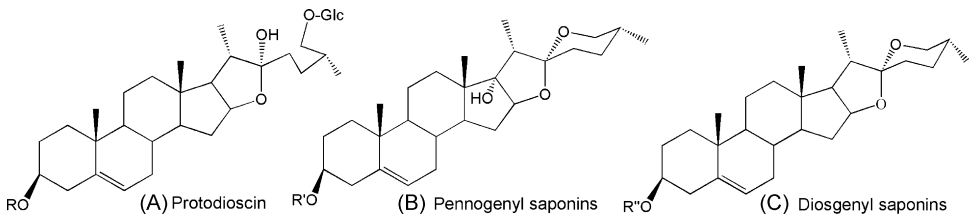

Fig. 1. Chemical structures of pennogenyl steroid saponins (A), protodioscin (B), and diosgenyl steroid saponins (C).

P. yunnanensis and deduced their fragmentation pathways [25]. In the present paper, we used LC–MS/MS to identify the common steroid saponins and compared their ingredient variability in *Paris* and *Trillium* plants. In the end, with the cluster analysis, we wanted to discover alternate resources for *Rhizoma Paridis*.

2. Experimental

2.1. Samples, standards and reagents

The dried rhizomes of *P. yunnanensis* (PY200805 and PY200605), *Paris fargesii* (PF200805), *Paris mairei* (PM200805), *Paris thibetica* (PT200805) and *Paris axialis* (PA200805) were purchased from Lijiang (Yunnan Province, China); *Paris bashanensis* (PB200805) was purchased from Xingshan (Hubei Province, China). *Paris verticillata* (PV200805) was purchased from Changchun (Jilin Province, China). *Trillium tschonoskii* (TT200805) was purchased from Sichuan, China. They had been identified by Dr. Gao, and voucher specimens were deposited at the School of Pharmaceutical Science and Technology at Tianjin University.

Reference standards of Paris I, Paris II, Paris VI and Paris VII were purchased from the National Institute for the Control of Pharmaceutical and Biological Products, China. Their batches were 111590-200402, 111591-200402, 111592-200402 and 111593-200402, respectively. Paris III and protodioscin were purchased from Phytomarker Ltd., Tianjin, China. Paris H was purchased from Kobeyuan Biomedical Technology Company, Beijing, China. Gracillin was purchased from Jianfeng Technology Company, Tianjin, China. Paris H was isolated and purified in our laboratory and confirmed by ESI-MS and ^{1}H, ^{13}C NMR (purity >98%, HPLC). The purities of these standards were determined to be more than 98% by normalization of the peak areas detected by HPLC, and were very stable in methanol solution for 1 month.

HPLC-grade methanol and acetonitrile were purchased from Tedia (USA). Water was purified using a Milli-Q water purification system (Millipore, France). The other reagents were commercially available and of analytical purity. All solvents and samples were filtered through 0.22 μm filter (Xinjinghua Co., Shanghai, China) before injecting into HPLC.

2.2. Sample preparation for HPLC-ELSD analysis

Every kind of dried roots was powdered to a homogeneous size by a mill, sieved through a No. 40 mesh, and further dried at 40 °C in the oven for 2 h. The powder samples accurately weighed (0.5 g) were added to a round-bottomed flask containing 50 mL of 80% ethanol and the mixture was heated under reflux for 2 times, 1.5 h for each time. The ethanol solution was filtered and evaporated with a rotary evaporator (Shensheng Co., Shanghai, China), and then made up to exactly 10 mL with 80% ethanol using a volumetric flask. Every sample solution was injected in triplicate, and the contents of the analytes were determined from the corresponding calibration curves.

2.2.1. Analysis of parissaponins by HPLC-ELSD

HPLC on parissaponins were carried out on an Agilent 1100 liquid chromatograph system (Agilent Technologies, USA), equipped with a quaternary pump, an online degasser, and a column temperature controller, coupled with an ELSD (Alltech Associates, USA) as the detector. The analytical column temperature was kept at 35 °C. The samples were separated with a Kromasil RP-C_{18} column (4.6 mm × 250 mm, 5 μm, AKZO NOBEL, Sweden) using water (A) and acetonitrile (B) under gradient conditions (0–5 min, linear gradient 33–36% B; 5–10 min, linear gradient 36–39% B; 10–12 min, linear gradient 39–45% B; 12–13 min, linear gradient 45–47% B; 13–18 min, linear gradient 47–50% B; 18–20 min, isocratic 50% B; 20–23 min, linear gradient 50–43% B; 23–42 min, isocratic 43% B; 42–45 min, linear gradient 43–55% B; 45–50 min, linear gradient 55–90% B) as the mobile phase at a flow rate of 1 mL/min within 50 min. The injection volume was 20 μL. The drift tube temperature for ELSD was set at 100 °C, and the nebulizing gas flow rate was 2.7 L/min. Peaks were assigned by comparing their retention time with that of each reference compound eluted in this mobile phase and by spiking samples with reference compounds.

2.2.2. Calibrations

Each of parissaponins were accurately weighed, dissolved in methanol and diluted with methanol to an appropriate concentration. A mixed solution of nine standard saponins, containing 254 μg/mL of protodioscin, 268 μg/mL of Paris I, 142 μg/mL of Paris II, 128 μg/mL of Paris III, 228 μg/mL of gracillin, 202 μg/mL of Paris V, 198 μg/mL of Paris VI, 190 μg/mL of Paris VII and 198 μg/mL of Paris H, was prepared in methanol and stored in the refrigerator at 4 °C until required for analysis.

Calibration curves were plotted by the peak area versus concentration of each analyte. The linear range was evaluated by linear regression analysis calculated by the least square regres-

Table 1
Structures of standard samples in *Paris* and *Trillium* plants.

Carbohydrate side chain in C_3 of aglycons	R	R′	R″
-3-*O*-Rha(1 → 4)-Rha(1 → 4)-[Rha(1 → 2)]-Glc		Paris VII (2)	Paris II (5)
-3-*O*-Ara (1 → 4)-[Rha(1 → 2)]-Glc		Paris H (3)	Paris I (8)
3-*O*-Rha (1 → 2)-Glc		Paris VI (4)	Paris V (9)
-3-*O*-Rha(1 → 4)-[Rha(1 → 2)]-Glc	Protodioscin (1)		Paris III (6)
-3-*O*-Rha(1 → 2)-[Glc (1 → 3)]-Glc			Gracillin (7)

Ara, α-L-arabinofuranosyl; Glc, β-D-glucopyranosyl; Rha, α-L-rhamnopyranosyl.

Table 2
Linearity of calibration curve for nine saponins.

Marker compound	t_R [a] (min)	Regression equation[b]	R^2	Linear range (ng)	LOD[c] (ng)	LOQ[d] (ng)
Protodioscin	6.55	$Y = 1.4585x - 6.3849$	0.9939	508–5080	188.2	300.25
Paris VII	17.1	$Y = 1.5458x - 5.7127$	0.9981	360–3600	203.4	350.6
Paris H	18.3	$Y = 1.5442x - 5.9097$	0.9993	198–3960	118.8	190.6
Paris VI	19.3	$Y = 1.5353x - 5.6664$	0.9991	198–3960	120.6	189.0
Paris II	32.2	$Y = 1.2371x - 4.4340$	0.9959	284–2840	140.0	224.0
Paris III	39.5	$Y = 1.2117x - 4.1598$	0.9986	256–2560	132.8	250.4
Gracillin	41.0	$Y = 1.5373x - 7.1186$	0.9965	456–4560	228.2	400.2
Paris I	46.3	$Y = 1.4464x - 5.6280$	0.9983	536–5360	209.2	384.2
Paris V	49.0	$Y = 1.6252x - 6.0401$	0.9991	202–4040	121.3	189.3

[a] t_R, retention time.
[b] Y, logarithmic value of peak area; x, logarithmic value of amount injected (ng).
[c] LOD, limit of detection, $S/N = 3$.
[d] LOQ, limit of quantification, $S/N = 10$.

sion method. Limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined on the basis of response and slope of each regression equation at a signal-to-noise ratio (S/N) of 3 and 10, respectively.

2.2.3. Validation

The intra-day and inter-day precision was determined by analyzing calibration samples during a single day and on three different days, respectively. The intra-day variation was determined by analyzing the six replicates on the same day and the inter-day variation was determined on three consecutive days. The relative standard deviation (RSD) was taken as a measure of precision.

The recovery test was used to evaluate the accuracy of this method. Accurate amounts of nine standards were added to accurately weighted 0.25 g preparations of *P. yunnanensis*. The above-prepared samples ($n = 6$) were extracted and analyzed as described in Section 2.2. The average recoveries were determined by the formula: recovery (%) = (observed amount − original amount)/spiked amount × 100, and RSD (%) = (SD/mean) × 100.

2.3. Sample preparation for HPLC–MS analysis

Every dried, crushed root (100 g) of *Paris* and *Trillium* plants were extracted with 80% ethanol (0.4 L) three times for 1.5 h under reflux. The combined 80% ethanol extracts were concentrated without ethanol and then suspended in distilled water (200 mL) and extracted with n-butanol (250 mL, six times) to obtain the n-butanol fraction. Then these fractions were evaporated with a rotary evaporator, transferred with methanol, dried at 40 °C in the thermostat-controlled water-bath, and further dried at 40 °C in the oven for 2 h. The powder samples accurately weighed (12 mg) which were only small parts of the n-butanol fractions were added to 5 mL methanol using a volumetric flask and an aliquot of 20 μL of the filtrate was injected into HPLC–MS for analysis.

2.3.1. Analysis of steroid saponins by positive and negative ion ESI-MS/MS

Samples were analyzed using an Agilent 1200 HPLC–MS system containing of a surveyor autosampling system, interfaced to a 6310 ion-trap mass spectrometer via an electrospray ion source. Source settings used for the ionization of steroidal saponins were: nebulizer gas pressure of 30.00 psi; dry gas flow rate of 8.00 L/min; electrospray voltage of the ion source of 3000 V; capillary temperature of 350 °C; capillary exit of −158.5 V; skimmer of 40 V. Nitrogen (>99.99%) and He (>99.99%) were used as sheath and damping gas, respectively.

2.3.2. Analysis of crude extract by LC–ESI-MS/MS

The chromatographic separation of the crude extract was performed on the same column and mobile phase which is described in Section 2.2.1. The HPLC system (Agilent technologies 1200 series Diode Array detector) with an ion-trap ESI-mass spectrometer was interfaced to MS described in Section 2.3.1 and the samples were injected into the column using an autosampler. The total ion chromatogram was obtained using a LC–ESI-MS/MS at 200–1500 m/z in the negative or the positive ion mode. The fragment ions were obtained using collision energy of 35% for both MS^2 and MS^3 experiments. Analyses were conducted at ambient temperature and the data were operated on the Xcalibur software.

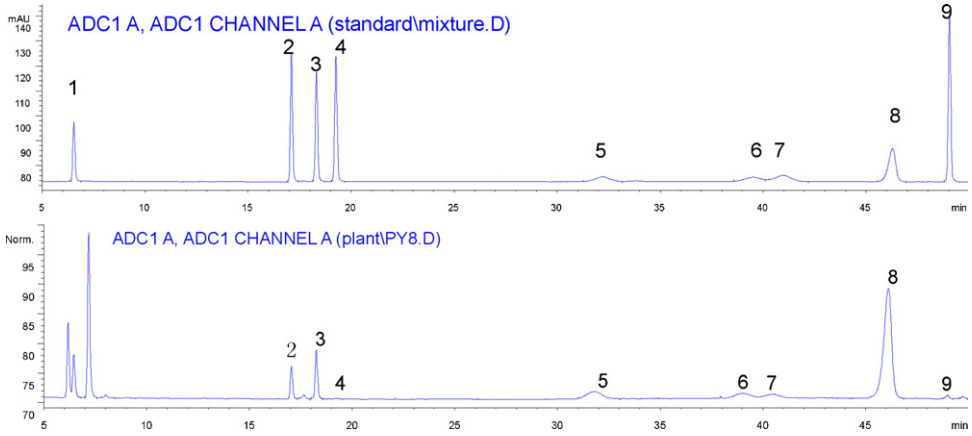

Fig. 2. Representative HPLC chromatograms of mixed standards. Column: Kromasil C_{18} (4.6 mm × 250 mm, 5 μm); detector: ELSD; drift tube temperature: 100 °C; nitrogen flow rate: 2.7 bar. (A) Mixed standards. (B) Extract of *Paris yunnanensis*. The numbers indicated each parissaponin in Table 1.

2.4. Character coding and data analysis

Based on the research above, we selected 20 steroidal saponins detected by LC–MS from these 9 samples as evaluative factors.

It was encoded as binary variable whenever the saponin was present or absent in plants. If the plant owned this saponin, the character state and coding of the plant was encoded 1, while not, it was encoded 0.

SPSS15.0 for Windows (SPSS Inc.) was used to analyze these data and hierarchical clustering: within-groups linkage as clustering method, Squared Euclidean distance as rescaled distance measure.

3. Results and discussion

3.1. Analysis of parissaponins by HPLC-ELSD

Optimizations of extraction [26] and chromatographic conditions were investigated systematically. Because the ingredients in the sample could not be separated with isocratic HPLC elution, gradient elution was carried out. Optimized chromatographic conditions were achieved after several trials. The results showed that a linear gradient elution of acetonitrile:water system, which was better than methanol:water or methanol:acetonitrile:water system, gave the best resolution during chromatographic separation (Fig. 2). Unlike the UV–vis detector, the ELSD system is universal and does not require a chromophore in the analyte so that any molecule present in the injected sample, assuming the concentration is appropriate, will be detected.

With these proposed chromatographic conditions, a strong response was obtained with ELSD showing good retention features and baseline shape. The mixed standards were identified by comparing the retention time of the reference standards. For determination of the bioactive markers, a calibration curve for each marker was constructed and tested thrice for linearity. For calibration, the log–log plots for the peak area versus concentration were drawn to obtain linearity, because the peak area varies exponentially with the mass of analyte [27].

As shown in Table 2, all calibration curves showed good linear regressions ($R^2 \geq 0.99$). LOD and LOQ for each compound were also shown in Table 2.

As demonstrated in Table 3, the results of precision and accuracy showed good reproducibility for the quantification of nine saponins in *Paris* and *Trillium* plants with intra- and inter-day variations of less than 1.80% and 1.94%, respectively. In addition, the related compounds showed the overall recoveries ranging from 95.47% to 103.12% with RSD ranging from 1.46% to 4.01%. These results demonstrated that this HPLC-ELSD method was precise, accurate and sufficiently sensitive for the quantitative determination of nine major saponins in *Paris* and *Trillium* plants.

Table 3
Intra-day and inter-day precisions, recovery tests of the nine steroid saponins.

Compound	Precision (RSD, %)		Recovery (%) ($n = 6$)	
	Intra-day ($n = 6$)	Inter-day ($n = 6$)	Average	RSD
Protodioscin	1.62	1.82	98.32	2.63
Paris VII	1.44	1.67	98.15	2.03
Paris H	1.46	1.49	95.47	3.98
Paris VI	1.43	1.68	102.42	2.23
Paris II	1.28	1.38	98.38	1.46
Paris III	1.80	1.94	103.12	4.01
Gracillin	1.57	1.83	98.49	3.48
Paris I	1.68	1.73	101.42	2.87
Paris V	1.60	1.91	102.58	2.01

Recovery (%) = (Observed amount − Original amount)/spiked amount × 100.
RSD (%) = (SD/mean) × 100.

3.2. Content and distribution of the known saponins identified by HPLC-ELSD

The method was subsequently applied to a simultaneous determination of nine bioactive markers in *Paris* and *Trillium* plants. The assay results are shown in Table 4. Due to the different growth environment of the medical plant, there were remarkable differences between the samples, in terms of concentration of the nine bioactive markers.

It is known that LC/MS spectrum can certify the existence of known saponins, as well as confirm the reliability of ELSD detection. Among these plants, the relative peak of protodioscin was obvious in ELSD detector, but disappeared in the next LC–MS analysis. Of course, the reason may be that protodioscin was too polar and may remain in the aqueous phase. However, there was no record for the existence of protodioscin in *Paris* and *Trillium*. Thus there was no proof for the existence of protodioscin in these plants.

Meanwhile, PY with different batches showed different content of eight saponins, indicating that the fresh plant may contain higher percentage of saponins. In addition, compared with PA, PF and Tt, PY owned more diosgenyl saponins which showed strong antitumor effects, rationalizing our previous conclusion that PY extract had an antitumor role [1].

Among these plants, the content of Paris VI with two saccharide groups exhibited trace amount, while Paris VII and II both with four glycons displayed almost in every plants. It indicated that less than two saccharide groups including aglycones easily combined with glycons in these plants.

All in all, we can quickly identify these kinds of plants and estimate their fresh level by this HPLC-ELSD analysis.

3.3. Identification and distribution of other saponins by ESI-MS and MS/MS

Although there were many kinds of saponins in *Paris* and *Trillium* plants, some non-saponin components of the analyte may overlap

Table 4
The mean contents of nine saponins in *Paris* and *Trillium* plants (mg/g, $n = 3$).

Plant	Protodioscin	Paris VII	Paris H	Paris VI	Paris II	Paris III	Gracillin	Paris I	Paris V
Paris axialis	–[a]	0.289	2.892	8.393	1.023	0.102	Tr	3.723	Tr
Paris bashanensis	–	0.700	0.890	–	0.210	–	Tr	0.380	0.082
Paris fargesii	–	15.590	9.253	19.520	0.338	0.128	0.129	4.354	0.149
Paris mairei	–	1.212	–	–	Tr	–	–	–	–
Paris thibetica	–	0.773	0.544	–	0.729	–	0.102	1.550	0.100
Paris verticillata	–	0.518	–	–	0.289	0.182	–	–	–
Paris yunnanensis 6[b]	–	0.498	0.584	0.154	0.406	0.072	0.160	0.329	–
Paris yunnanensis 8	–	2.086	3.044	0.128	4.504	2.664	3.258	15.270	0.529
Trillium tschonoskii	–	4.440	–	11.770	Tr	Tr	Tr	0.100	0.370

[a] "-" not detected; Tr, trace (it cannot be determined by ELSD, but can be identified by ESI-MS).
[b] *Paris yunnanensis* 6 means *Paris yunnanensis* was purchased in 2006, while *Paris yunnanensis* 8 means it was purchased in 2008.

Table 5
HPLC–ESI(+/−)-MS^n data of analyses and distribution of steroid saponins.

t_R (min)	Mass (m/z)	ESI(+/−)MS^n data			Possible name or structure	Distribution
		MS^1	MS^2	MS^3		
5.0	1194	**1177**[M+H−H_2O]$^+$	869[1177−Glc−Rha]$^+$; **723**[1177−Glc−2Rha]$^+$	577[723−Rha]$^+$; 415[723−Rha−Glc]$^+$	Dichotomin	PA; PM PT; PY
		1193[M−H]$^-$;	**1047**[1193−Rha]$^-$; 901[1193−−2Rha]$^-$; 575[1193−3Rha−Glc−H_2O]$^-$	755[1047−2Rha]$^-$; 593[1047−2Rha−Glc]$^-$		
6.1	1196	1179[M+H−H_2O]$^+$; **1017**[M+H−H_2O−Glc]$^+$	885[1017−Ara]$^+$; 855[1017−Glc]$^+$	723[855−Ara]$^+$; 577[**855**−Rha−Ara]$^+$; 415[855−Rha−Ara−Glc]$^+$	Protosaponin 3Glc–Rha–Ara	PY
		1195[M−H]$^-$; **1033**[M−H−Glc]$^-$	**901**[1033−Ara]$^-$; 755[1033−Ara−Rha]$^-$	755[901Rha]$^-$; 575[901−Rha−Glc−H_2O]$^-$		
6.3	1208	**1177**[M+H−MeOH]$^+$; 885[1177−2Rha]$^+$	**869**[1177−Rha−Glc]$^+$; 723[1177−2Rha−Glc]$^+$	577[869−2Rha]$^+$; 415[869−Glc−2Rha]$^+$	Methyldichotomin	PA; PB; PF; PM PT; PV; PY
		1207[M−H]$^-$	**1061**[1207−Rha]$^-$; 915[1207−2Rha]$^-$	915[1061−Rha]$^-$; 769[1061−2Rha]$^-$		
6.6	1062	**1031**[M+H−MeOH]$^+$	**869**[1031−Glc]$^+$	725[869−144]$^+$; 561[869−Glc−Rha]$^+$	Methyl protodioscin	PM; PY
		1061[M−H]$^-$	**915**[1061−Rha]$^-$; 769[1061−2Rha]$^-$	769[915−Rha]$^-$; 575[915−Rha−Glc−MeOH]$^-$		
6.9	1194	1163[M−MeOH+H]$^+$; **1017**[1163−Rha]$^+$	**855**[1017−Glc]$^+$	723[855−Ara]$^+$; 577[855−Ara−Rha]$^+$	Methylprotosaponin 2Glc–2Rha–Ara	PT; PY
		1193[M−H]$^-$; 1061[M−Ara−H]$^-$; **1015**[1193−MeOH−Rha]$^-$	**883**[1015−Ara]$^-$; 737[1015−Ara−Rha]$^-$	737[883−Rha]$^-$; 575[883−Glc−Rha]$^-$		
7.3	1176	1177[M+H]$^+$; **885**[M+H−2Rha]$^+$	**723**[885−Glc]$^+$; 577[885−Glc−Rha]$^+$	579[723−144]$^+$	Diosgenin 2Glc–3Rha	PA; PB; PF; PM PT; PV; PY
	1176	**1175**[M−H]$^-$	**1029**[1175−Rha]$^-$; 883[1175−2Rha]$^-$	737[1029−2Rha]$^-$		
7.5	1048	**1017**[M−MeOH+H]$^+$; 885[1017−Ara]$^+$	855[1017−Glc]$^+$; **723**[1017−Glc−Ara]$^+$	577[723−Rha]$^+$; 415[723−Rha−Glc]$^+$	PolyPhyllin H	PA; PB; PF; PM PT; PV; PY
		1047[M−H]$^-$	**915**[1047−Ara]$^-$; 769[915−Rha]$^-$	769[915−Rha]$^-$; 589[915−Rha−Glc−H_2O]$^-$		
7.7	1078	**1047**[M+H−MeOH]$^+$	**885**[1047−Glc]$^+$; 723[1047−2Glc]$^+$	577[885−Glc−Rha]$^+$	Methylprotogracillin	PY; Tt
		1077[M−H]$^-$; 753[M−H−2Glc]$^-$	**915**[1077−Glc]$^-$; 769[1077−Glc−Rha]$^-$	769[915−Rha]$^-$; 591[915−2Rha−MeOH]$^-$		
8.1	1016	**1017**[M+H]$^+$; 885[M−Ara+H]$^+$	**855**[1017−Glc]$^+$; 723[1017−Glc−Ara]$^+$	579[855−Ara−144]$^+$; 415[855−Ara −Rha−Glc]$^+$	Diosgenin 2Glc–Rha–Ara	PA; PB; PF; PM PT; PV; PY
		1015[M−H]$^-$	**883**[1015−Ara]$^-$; 737[1015−Ara−Rha]$^-$	737[883−Rha]$^-$ 575[883−Glc−Rha]$^-$		
18.1	884	**885**[M+H]$^+$	**723**[885−Glc]$^+$; 577[885−Glc−Rha]$^+$	579[723−144]$^+$	Pennogenin Glc–2Rha	PT; PV; PY; Tt
	884	**883**[M−H]$^-$	**721**[883−Glc]$^-$	575[721−Rha]$^-$		
18.4	900	**923**[M+Na]$^+$	**777**[923−Rha]$^+$	615[777−Glc]$^+$	Pennogenin	PT; PY
	900	**899**[M−H]$^-$	**737**[899−Glc]$^-$	591[737−Rha]$^-$	2Glc–Rha	
34.0	986	**1009**[M+Na]$^+$; 863[M+Na−Rha]$^+$	**863**[1009−Rha]$^+$; 731[1009−Rha−Ara]$^+$	731[863−Ara]$^+$; 599[863−2Ara]$^+$	Diosgenin 2Ara–Rha–Glc	PA; PF; PT; PY
	986	**985**[M−H]$^-$; 839[M−H−Rha]$^-$	**721**[985−2Ara]$^-$	575[721−Rha]$^-$		

t_R, retention time. Boldface indicates the molecular ion of the steroidal saponins.
Shading represents ESI(+)-MS data.

with saponins, making determination difficult. To overcome these problems and identify common saponins in these plants, several trials were performed. In the end, we applied liquid–liquid extraction. It was especially suitable for lipophilic compounds. Saponins as bipolaritic compounds were mainly extracted by n-butanol. Meanwhile, LC–MS was selective and sensitive enough to carry out the study of saponins. Generally, ion sensitivities for saponins were greater in the negative ion mode, while more structural information was obtained in the positive ion mode. Therefore, both modes have been used for saponins bioanalysis.

Under the LC–MS described above, the MS of 20 steroidal saponins from *Paris* and *Trillium* plants were acquired in the positive and negative ion modes and the base peak chromatograms of these saponins were obtained. Table 5 presents their retention times (t_R), mass and ESI(+/−)-MS^n fragmentation ions and distribution. In the MS, the steroid saponins exhibited quasi-molecular ions [M+H]$^+$, [M−H]$^-$, or whilst in the MS^n spectra fragment ions were formed by successive or simultaneous loss of saccharide groups, H_2O or CH_3OH. The detailed fragmentation pathways have been discussed before [25].

In this analysis, PY owns the most kinds of saponins. Methyldichotomin, polyphyllin H, diosgenin 2Glc–3Rha and diosgenin 2Glc–Rha–Ara almost exist in every kinds of plants. Meanwhile, dichotomin, methyldichotomin and diosgenin 2Glc–3Rha displayed similar positive ions. Through the comparison of the negative ions at the responsive retention time, we identified these saponins in Table 5. The analogous deductive method was also used in identification of polyphyllin H and diosgenin 2Glc–Rha–Ara.

3.4. Hierarchical cluster analysis

Base on the research above, we selected 20 steroidal saponins detected by LC–MS from these 9 samples as evaluative factors. It was noticeable that the samples were clustered in different

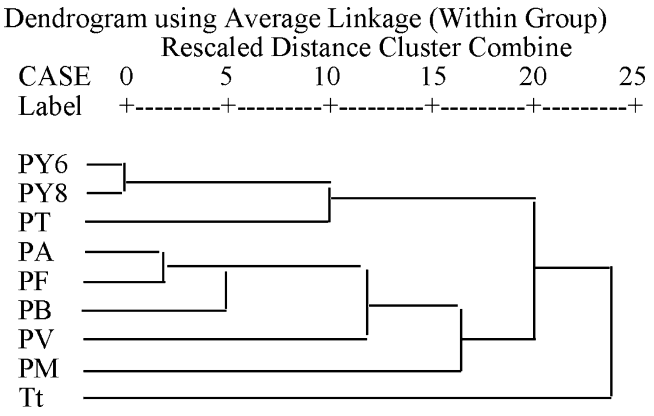

Fig. 3. Dendrogram of 9 samples from different *Paris* and *Trillium* plants scored for 20 steroid saponins (Tables 1 and 5). Using 'within-group' as average linkage clustering, nine plants were classified into five groups.

domains, which represented the "similarities" and "differences" (Fig. 3) by the score plots derived from 20 saponins indicated in Table 5.

Clustering by within-group linkage (Fig. 3) showed that PT shared similar 20 kinds of saponin components with PY, indicating an alternate resource for PY may exist. In addition, PA grouped robustly with PF, and PB was clustered with PA and PF. PV followed by PM was further to PB in the cluster. Tt was farthest to others for it had fewest kinds of saponins separated.

4. Conclusions

The analytical method described in this paper is the first combination of HPLC-ELSD and HPLC–ESI-MS/MS methods for the quantitative determination and identification of common saponins in *Paris* and *Trillium* plants. The method of HPLC-ELSD is accurate and precise and is successfully used to analyze the crude extract of *Paris* and *Trillium* plants. Under ESI-MS/MS conditions, LC/MS spectrum certifies the existences of known saponins, as well as confirms the reliability of ELSD detective results. The fragmentation patterns of $[M-H]^-$ ions exclusively show signals corresponding to cleavage of the glycosidic bonds, thus allowing a rapid identification of saponins in the crude extract of *Paris* and *Trillium* plants. The results demonstrate that the proposed method can be readily utilized for quality control of *Paris* and *Trillium* plants. And *Paris thibetica* maybe act as an alternate resource for *Rhizoma Paridis* in future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.08.033.

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