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Simultaneous quantification of six major active saponins of *Panax notoginseng* by high-performance liquid chromatography-UV method

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Abstract

A simple, sensitive and specific high-performance liquid chromatography-UV (HPLC-UV) method has been developed for the first time to simultaneously quantify the six major active saponins of *Panax notoginseng*, namely notoginsenoside R_1, ginsenoside Rg_1, Rb_1, Rg_2, Rh_1 and Rd. Astragaloside IV is used as the internal standard. This HPLC assay was performed on a reversed-phase C_{18} column with gradient elution of acetonitrile and 0.01% formic acid in 30 min. The method provided good reproducibility and sensitivity for the quantification of six saponins with overall intra- and inter-day precision and accuracy of less than 4.0% and higher than 90%, respectively. This assay is successfully applied to the determination of the six saponins in 23 notoginseng samples. The results indicated that the developed HPLC assay can be readily utilized as a quality control method for *P. notoginseng*.

Keywords: High-performance liquid chromatography; *Panax notoginseng*; Ginsenosides; Notoginsenoside

1. Introduction

Panax notoginseng(Burk) F. H. (Araliaceae) (Sanchi) is one of the most commonly used Chinese herbal drugs. It was highly prized in China for its therapeutic effects to treat cardiovascular diseases, inflammation, different body pains, trauma, internal and external bleeding due to injury, etc. Various chemical and pharmacological studies on Sanchi had demonstrated that the major bioactive constituents presented in this TCM herb were dammarane type saponins [1–6]. Recent studies showed that the pharmacological effects of these saponins included attenuation of cisplatin-induced nephrotoxicity [7], protection of the injured brain [8], anti-inflammation due to the reduction of the level of the intracellular free calcium concentration in neutrophils [9]. Currently, the roots of *P. notoginseng* are used to treat coronary heart disease, cardiac angina, apoplexy and atherosclerosis in clinics. The saponins isolated there from include 20(s)-protopanaxadiol type and 20(s)-protopanaxatrial type saponins. The 20(s)-protopanaxadiol type saponins (such as Rb_1, Rd) showed effective tranquilizing effects to central nervous system and the 20(s)-protopanaxatrial type saponins (such as Rg_1, Rg_2, Rh_1) possessed the properties of exciting central nervous system, anti-fatigue and hermolysis [10]. Therefore, the quality control of *P. notoginseng* was focused mainly on the determination of these major saponins.

In the past 20 years, many attempts had been made to assay the active saponins in *P. notoginseng* by various methods including capillary supercritical fluid chromatography [11], thin layer chromatography [12–14], gas chromatography [15,16], high-performance liquid chromatography [17–22], etc. However, most of these methods were insufficient to quantify the major active saponins, since the resolutions were limited to less than five saponins with external standard method, which made the reproducibility and extraction yield become critical for the quantification.

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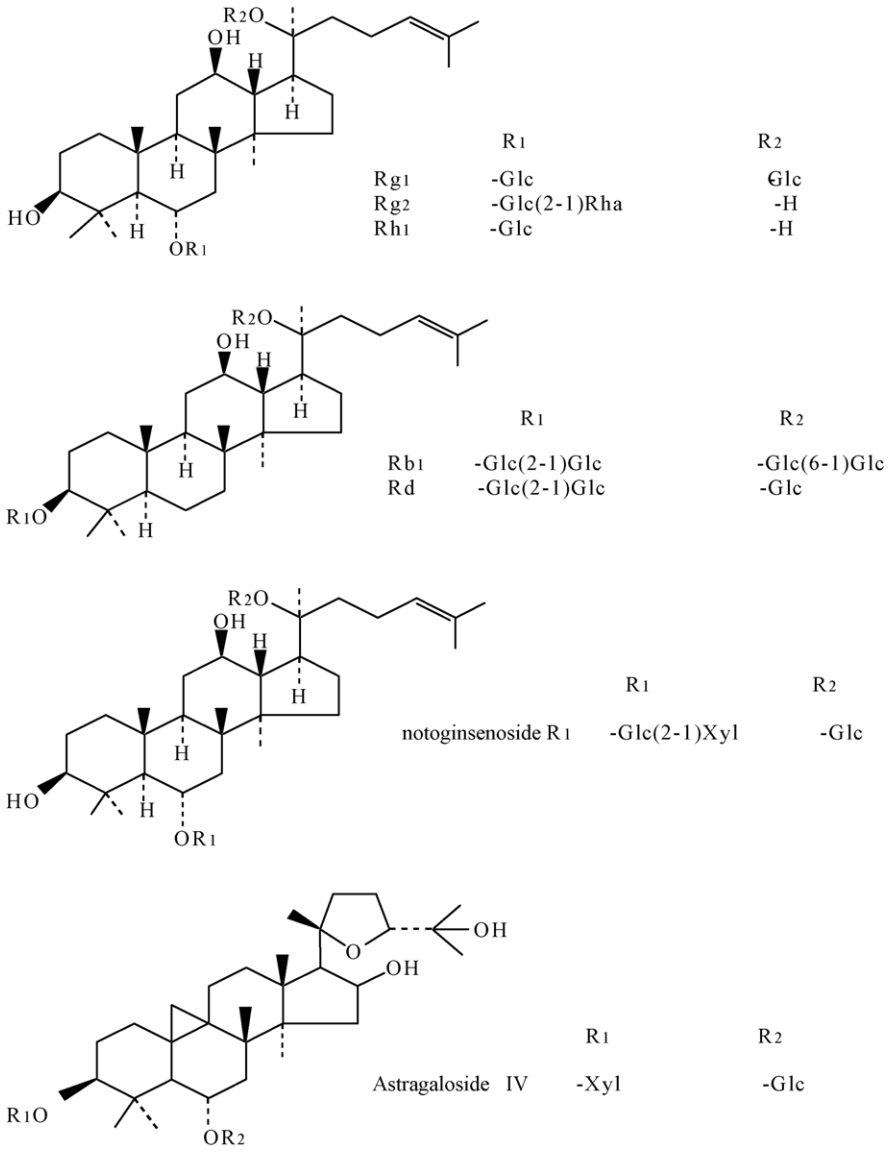

Fig. 1. The structures of notoginsenoside R_1, ginsenoside Rg_1, ginsenoside Rg_2, ginsenoside Rh_1, ginsenoside Rd and astragaloside IV.

Zhou et al. developed an internal standard method for simultaneous determination of notoginsenoside R_1, ginsenoside Rg_1, Re and Rb_1 by using Asahipak NH_2 P-50 column [23], in addition theophylline was used as the internal standard but its UV absorbance was not in accordance with saponins of Sanchi since it is an alkaloid.

We described here the development of a direct and rapid method for determining six saponins (shown in Fig. 1) in notoginseng crude drugs within 30 min. Astragaloside IV was chosen as the internal standard, which was structurally close to Sanchi saponins and almost has the same UV absorbance as that of Sanchi saponins. The developed method was successfully applied to the quantification of six major constituents in 23 notoginseng samples.

2. Experimental

2.1. Chemicals and materials

Twenty-three Sanchi samples were obtained locally from different provinces (see Table 4). HPLC grade acetonitrile was purchased from Merck Company (Merck, Darmstadt, Germany). Deionized water was purified by Milli-Q system (Millipore, Bedford, MA, USA). Formic acid for analysis was of analytic grade from Beijing Reagent Company (Beijing, PR China).

Ginsenoside Rg_1, Rb_1 and astragaloside IV were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). Notoginsenoside R_1, ginsenoside Rg_2, Rb_1, Rd, Rh_1 were

kindly presented by Professor Yingjie Chen, College of Pharmacy, Shengyang Pharmaceutical University, PR China.

2.2. Apparatus

An Angilent 1100 liquid chromatography system, equipped with a quaternary solvent delivery system, an autosampler and UV detector, was used. The column configuration consisted of an Angilent Zorbax SB-C_{18} reserved-phase column (5 μm, 250 mm × 4.6 mm) and an Angilent Zorbax SB-C_{18} guard column (5 μm, 20 mm × 4 mm). UV absorption was measured at 203 nm.

2.3. Chromatographic conditions

Gradient elution was employed using solvent A (acetonitrile) and solvent B (0.001% aqueous formic acid, v/v) at 35 °C; the gradient program used as follows: initial 0–8 min, linear change from A–B (25:75, v/v) to A–B (26:74, v/v); 8–10 min, linear change to A–B (33:67, v/v); 10–30 min, linear change to A–B (45:55, v/v). The flow rate was kept at 0.8 ml/min and the sample injection volume was 5 μl.

2.4. Calibration curves

Eighty percent of aqueous methanol stock solution containing notoginsenoside R_1, ginsenoside Rg_1, Rb_1, Rd, Rg_2 and Rh_1 was prepared and diluted to appropriate concentration ranges for the establishment of calibration curves. The concentration of the internal standard, astragaloside IV, was 500 μg/ml for all analytes. Each calibration curve was performed with six different concentrations in triplicate. The peak area ratio (analyte/internal standard) for each analyte was determined. Consequently, calibration curves were constructed.

2.5. Precision

The measurements of intra- and inter-day variability were utilized to determine the precision of the developed assay method. Two different concentration solutions (low and middle) of six standards with internal standard (500 μg/ml for the two solutions) were prepared. The quantity of each analyte was obtained from corresponding calibration curve. The relative standard deviation (R.S.D.) was taken as a measure of precision; and the inter-day reproducibility was examined on four separate days.

2.6. Limits of detection

The standard solutions were diluted with 80% aqueous methanol to provide appropriate concentrations. The limit of detection for each analyte was determined when the ratio of the testing peak signal-to-noise was 4.

2.7. Accuracy

Known quantities of six analytes were added to 5 ml of 80% aqueous methanol solution with 1 ml internal standard solution (2.5 mg/ml) and powder of *P. notoginseng* (30 mg). The resultant samples were extracted and analyzed as described in Section 2.8. The quantity of each analyte was subsequently obtained from the corresponding calibration curve.

2.8. Analysis of the six saponins in P. notoginseng

To the dried powders of Sanchi samples (40 mesh, 30 mg or so), 5.0 ml of 80% aqueous methanol solution with 1 ml internal standard solution (2.5 mg/ml) was added and extracted in an ultrasonic bath for 60 min. The resultant mixture was filtered, then the aliquots of the mixture were filtered through syringe filter (0.45 μm) before HPLC analysis. The contents of the analytes were determined from the corresponding calibration curves.

3. Results and discussion

3.1. Extraction method development

In order to obtain quantitative extraction, variables involved in the procedure such as solvent and extraction time were optimized. Pure and aqueous methanol solutions were tried as the extraction solvent. The best solvent was found to be 80% aqueous methanol that allowed complete extraction of all the saponins in high yield. Ultrasonic extraction was compared with refluxing. It was found that ultrasonic extraction was simpler and more effective for extraction of saponins with little impurity. Hence the ultrasonic bath extraction was chosen as a preferred method. The influence of the extraction time on the efficiency of extraction was also investigated, in which powdered samples were extracted with 80% aqueous methanol for 30, 60, 90 and 120 min, respectively. The results suggested that the highest amount of saponins were obtained with the extraction time of 60 min. After extraction, the residue was further extracted with 80% aqueous methanol for additional 60 min, and almost no saponins were detected by HPLC. Therefore, later samples were extracted for 60 min.

3.2. HPLC separation optimization

The selection of the HPLC conditions was guided by the requirement for obtaining chromatograms with better resolution of adjacent peaks within a short time especially when large amount of samples were analyzed. Since the saponins in Sanchi are non-chromophoric, mixture of water and acetonitrile was therefore the best choice for the separation. Different ratios of water and acetonitrile were compared but no satisfied separation was reached. Hence phosphorate buffer, formic acid or acetic acid was added to optimize the separation. It was found that formic acid could improve the separation.

Table 1
Calibration curves of six saponins in *P. notoginseng*

Analyte	Retention time (min)	Standard curve	r^2	Test range (μg/ml)	Limit of detection (μg/ml)
Notoginsenoside R_1	8.8	$Y = 0.0076x - 0.0119$	0.999	15.6–156	0.23
Ginsenoside Rg_1	11.2	$Y = 0.0087x - 0.0187$	0.997	40.2–402	0.22
Ginsenoside Rb_1	20.5	$Y = 0.0061x + 0.0368$	0.997	31.2–312	0.23
Ginsenoside Rg_2	22.9	$Y = 0.0095x - 0.0011$	0.999	3.2–32	0.22
Ginsenoside Rh_1	23.9	$Y = 0.0053x + 0.0016$	0.998	2.0–20	0.26
Ginsenoside Rd	28.4	$Y = 0.0063x + 0.0021$	0.998	15.4–154	0.24

Y: peak area ratio (analyte/internal standard); *x*: concentration of analyte (μg/ml); limit of detection: $S/N = 4$.

Finally, gradient elution program was practiced so as to ensure that each run of analysis was completed within 30 min. According to the Chinese Pharmacopoeia, 203 nm was suitable for the detection of ginsenosides, therefore, it was chosen as the detection wavelength.

3.3. Choice of internal standard

Blank controls were generally unavailable for the study of herbal materials, and calibrations were normally constructed without using the internal standard method. Therefore, reproducibility and extraction yield become critical for the quantification of the chemical constituents in herbs. Various methods reported to determine the contents of saponins in Sanchi were external standard method. Zhou et al. [23] used theophylline as the internal standard to analyse the saponins in *P. notoginseng* while the deficiency is that theophylline is an alkaloid with different absorption properties. Therefore, several compounds (β-sitosterol, oleanolic acid and astragaloside IV) for their suitability as the internal standards were compared in our experiment. It was found that astragaloside IV was a suitable internal standard in the present study. Since it was structurally close to ginsenosides and had a relatively strong UV absorption at 203 nm like Sanchi saponins. Secondly it had the most appropriate retention time when compared with other compounds examined and it was well resolved from the Sanchi saponins with a baseline separation.

3.4. Linearity, precision and accuracy

Under the chromatographic conditions used in this study, all six calibration curves exhibited good linear regressions as shown in Table 1, and the limits of detection (LOD) were in the range of 0.22–0.26 μg/ml for ginsenoside Rg_1, Rb_1, Rd, Rg_2, Rh_1 and notoginsenoside R_1. The results in Table 2 demonstrated that the developed analytical method was reproducible with good accuracy and sensitivity for all analytes

Table 2
Intra- and inter-day variability for the assay of six saponins in *P. notoginseng*

Concentration (μg/ml)	Intra-day ($n = 5$)			Inter-day ($n = 3$)		
	Found	R.S.D.[a] (%)	Accuracy[b] (%)	Found	R.S.D. (%)	Accuracy (%)
Notoginsenoside R_1						
39.0	37.49 ± 0.07	0.2	96.4	38.03 ± 0.97	2.6	97.5
93.6	96.03 ± 2.97	3.1	102.6	97.56 ± 1.21	1.2	104.2
Ginsenoside Rg_1						
100.5	96.97 ± 1.16	1.2	96.5	98.82 ± 4.1	4.1	98.3
241.2	247.38 ± 4.95	2.0	102.6	251.97 ± 3.5	1.4	104.5
Ginsenoside Rb_1						
78.0	79.82 ± 1.76	2.2	102.3	75.36 ± 3.0	4.0	96.6
187.2	187.71 ± 4.13	2.2	100.3	190.61 ± 2.7	1.4	101.8
Ginsenoside Rg_2						
5.5	5.52 ± 0.14	2.5	100.4	5.62 ± 0.24	4.3	102.2
13.5	13.13 ± 0.41	3.1	97.3	12.63 ± 0.44	3.5	93.6
Ginsenoside Rh_1						
5.0	5.01 ± 0.19	3.7	100.2	5.26 ± 0.08	1.5	105.2
12.5	13.00 ± 0.17	1.3	104.0	13.00 ± 0.25	1.9	104.0
Ginsenoside Rd						
38.5	37.75 ± 0.72	1.9	98.1	37.15 ± 1.46	3.9	96.4
92.4	94.37 ± 1.98	2.1	102.1	93.70 ± 1.01	1.1	101.4

[a] R.S.D. (%) (relative standard deviation) = (S.D./mean) × 100.
[b] Accuracy (%) = [1 − (nominal concentration − mean of measured concentration)/nominal concentration] × 100.

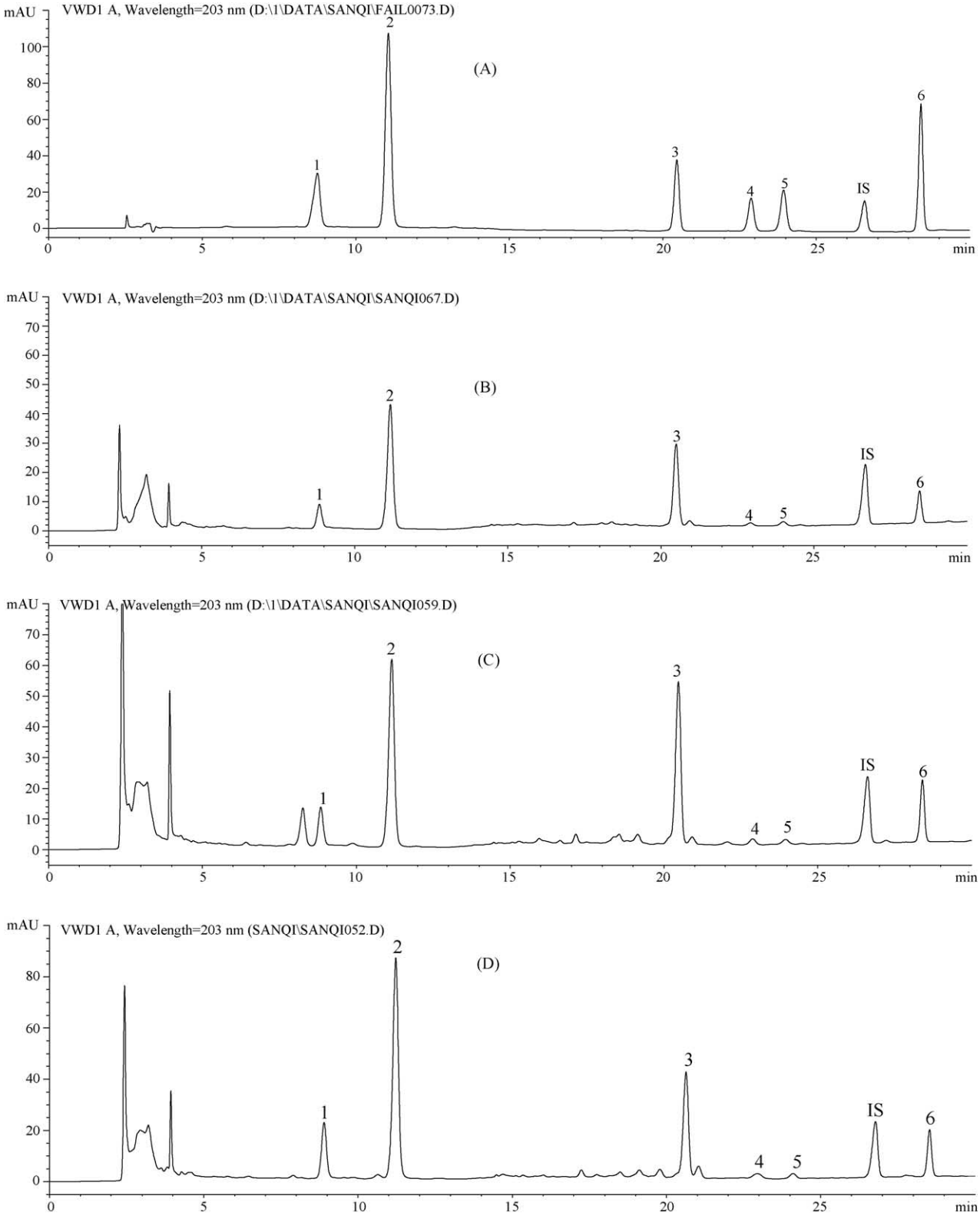

Fig. 2. Representative HPLC chromatograms of (A) standard solution at high concentration; (B) *P. notoginseng* (Wenshan,Yunnan, China, 60 heads); (C) *P. notoginseng* (Haerbing, Heilongjiang, China, 80 heads); (D) *P. notoginseng* (Changsha, Hunan, China, 60 heads). I.S., internal standard; 1, notoginsenoside R_1; 2, ginsenoside Rg_1; 3, ginsenoside Rb_1; 4, ginsenoside Rg_2; 5, ginsnoside Rh_1; 6, ginsenoside Rd.

Table 3
Recoveries of the six saponins in *P. notoginseng*

Saponin spiked (μg/ml)	Found (μg/ml)	R.S.D.[a] (%)	Recovery[b] (%)
Notoginsenoside R_1			
15.6	15.85 ± 0.02	0.1	101.6
124.8	119.85 ± 1.56	1.3	96.0
Ginsenoside Rg_1			
40.2	39.00 ± 0.63	1.6	96.9
321.6	338.92 ± 5.42	1.6	105.4
Ginsenoside Rb_1			
31.2	30.54 ± 1.01	3.3	97.8
249.6	236.75 ± 7.34	3.1	94.9
Ginsenoside Rg_2			
5.4	5.10 ± 0.05	1.0	94.4
20.2	20.85 ± 0.50	2.4	103.2
Ginsenoside Rh_1			
5.0	4.59 ± 0.08	1.7	91.8
15.0	13.85 ± 0.14	1.0	92.3
Ginsenoside Rd			
15.6	16.17 ± 0.27	1.7	103.6
123.2	115.75 ± 1.16	1.0	93.9

[a] R.S.D. (%) = (S.D./mean) × 100.
[b] Recovery (%) = [1 − (spiked concentration − mean of measured concentration)]/spiked concentration] × 100.

examined. The overall intra- and inter-day variations were less than 5.0% for all six analytes. The recovery assays of the saponins were carried out by adding the standards to the crude drug powders, and the results were shown in Table 3, from which it is clear that the recoveries for all the six saponins determined were in the range of 90–105%.

3.5. Results analysis

This newly developed HPLC-UV assay method was subsequently applied to a simultaneous determination of the major saponins in different notoginseng samples. Representative chromatograms of the extracts of these notoginseng samples were shown in Fig. 2. Their contents were summarized in Table 4. The results demonstrated a successful application of this HPLC-UV assay for the quantification of the major active saponins in different notoginseng samples. According to traditional Chinese custom, Sanchi was divided into different quantity types by its weight and color, such as 30 heads, 60 heads, etc. As a common sense, it was considered that the bigger the size, the better the quality. However, the results showed that different head numbers of notoginseng species contained different type and quantity of saponins. For the six saponins examined, there was no absolute linkage between the content of saponins and the head quantities of Sanchi. For example the so-called high quality Sanchi (30 heads) had a relatively low content of Rg_1, Rb_1 and notoginsenoside R_1. While the lower quality Sanchi (80 heads) contained a relatively high amount of notoginsenoside R_1 and ginsenoside Rg_2. Further studies on the relationships between the quality of Sanchi and its head quantities are currently under the investigation in the group.

Table 4
Contents of saponins in different *P. notoginseng* samples

No.	Quantity[a] (heads)	Collected place	Content (%)[b] ($n = 2$)					
			Notoginsenoside R_1	Ginsenoside Rg_1	Ginsenoside Rb_1	Ginsenoside Rg_2	Ginsenoside Rh_1	Ginsenoside Rd
1	60	Xian, Shaanxi	1.02 ± 0.01	3.46 ± 0.04	2.97 ± 0.08	0.08 ± 0.00	0.06 ± 0.00	0.66 ± 0.02
2	20	Suzhou, Jiangsu	1.53 ± 0.05	3.51 ± 0.09	2.56 ± 0.13	0.11 ± 0.00	0.04 ± 0.00	0.27 ± 0.01
3	60		0.91 ± 0.02	3.24 ± 0.08	2.16 ± 0.11	0.07 ± 0.00	0.03 ± 0.00	0.45 ± 0.02
4	20	Changsha, Hunan	1.25 ± 0.02	3.33 ± 0.05	2.46 ± 0.11	0.13 ± 0.01	nd[c]	0.43 ± 0.02
5	40		0.56 ± 0.02	2.15 ± 0.05	3.43 ± 0.15	0.16 ± 0.00	nd	0.64 ± 0.03
6	60		1.84 ± 0.09	4.00 ± 0.07	3.11 ± 0.17	0.15 ± 0.00	0.04 ± 0.00	0.66 ± 0.03
7	80		1.10 ± 0.02	3.61 ± 0.04	2.80 ± 0.08	0.08 ± 0.00	0.03 ± 0.00	0.43 ± 0.02
8	60	Shanghai	1.12 ± 0.03	3.57 ± 0.06	3.52 ± 0.17	0.13 ± 0.00	0.04 ± 0.00	0.87 ± 0.03
9	30	Nanjing, Jiangsu	0.76 ± 0.02	4.87 ± 0.20	3.28 ± 0.11	0.21 ± 0.00	0.09 ± 0.03	0.67 ± 0.04
10	40		1.19 ± 0.05	3.85 ± 0.21	2.10 ± 0.09	0.15 ± 0.01	0.06 ± 0.00	0.45 ± 0.02
11	60		1.57 ± 0.03	5.00 ± 0.10	2.98 ± 0.15	0.31 ± 0.01	0.10 ± 0.00	0.86 ± 0.04
12	40	Haerbing, Heilongjiang	0.77 ± 0.03	3.25 ± 0.07	2.74 ± 0.05	0.05 ± 0.00	0.08 ± 0.00	0.47 ± 0.01
13	60		1.37 ± 0.04	2.78 ± 0.08	2.79 ± 0.09	0.11 ± 0.00	0.08 ± 0.00	0.39 ± 0.00
14	80		1.15 ± 0.04	4.05 ± 0.05	4.10 ± 0.21	0.21 ± 0.00	0.05 ± 0.02	1.11 ± 0.00
15	40	Xinjie Village, Wenshan	0.65 ± 0.01	3.63 ± 0.11	2.94 ± 0.14	0.10 ± 0.00	0.07 ± 0.00	0.63 ± 0.02
16	60	Leshichong, Wenshan	2.47 ± 0.12	3.08 ± 0.04	2.46 ± 0.14	0.13 ± 0.00	nd	0.48 ± 0.02
17	60	Feibai hamlet, Wenshan	2.44 ± 0.04	3.98 ± 0.16	4.20 ± 0.21	0.18 ± 0.01	0.04 ± 0.00	0.94 ± 0.04
18	60	Santaihua hamlet, Wenshan	1.58 ± 0.05	2.81 ± 0.12	3.30 ± 0.09	0.07 ± 0.00	0.05 ± 0.00	0.72 ± 0.04
19	60	Debai hamlet, Wenshan	1.13 ± 0.06	3.95 ± 0.13	4.51 ± 0.05	0.15 ± 0.00	0.05 ± 0.00	0.68 ± 0.02
20	60	Laojunshan, Wenshan	0.82 ± 0.03	3.27 ± 0.16	3.22 ± 0.04	0.14 ± 0.00	0.04 ± 0.01	0.71 ± 0.02
21	60	Midi hamlet, Pingbian	1.36 ± 0.07	3.82 ± 0.11	3.36 ± 0.08	0.10 ± 0.00	0.05 ± 0.00	0.65 ± 0.02
22	60	Xinjie village, Wenshan	1.73 ± 0.09	2.81 ± 0.08	3.29 ± 0.07	0.13 ± 0.00	0.03 ± 0.00	0.79 ± 0.04
23	60	Xinjie village, Wenshan	1.55 ± 0.03	2.54 ± 0.13	2.34 ± 0.11	0.12 ± 0.00	0.03 ± 0.00	0.42 ± 0.02

[a] Quantity (heads): the head numbers of Sanchi per 500 g.
[b] Content = mean ± S.D. ($n = 2$).
[c] nd: not detected.

4. Conclusion

This is the first report on the simultaneous determination of six major saponins in *P. notoginseng* with internal standard method, which was proved to be simple, rapid and accurate. This HPLC assay can be readily utilized as a suitable quality control method for the determination of the major biologically active constituents in Sanchi, the most commonly used anti-inflammatory traditional Chinese medicine in China.

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