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Short communication

Simultaneous determination of nine saponins from *Panax notoginseng* using HPLC and pressurized liquid extraction

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Abstract

A HPLC and pressurized liquid extraction (PLE) method was developed for simultaneous determination of nine saponins, including notoginsenoside R1, ginsenoside Rg1, Re, Rf, Rb1, Rc, Rb2, Rb3 and Rd in *Panax notoginseng*. The analysis was performed on C_{18} column with water–acetonitrile gradient elution and the investigated saponins were authenticated by comparing retention time and mass spectra with their reference compounds. Several methods including PLE, ultrasonication, soxhlet extraction and immersion were used for sample preparation and their extraction efficiency was compared. The results showed that PLE has the highest extraction efficiency and repeatability, which would be valuable on standardization of sample preparation for quality control of Chinese medicines. The developed HPLC and PLE is an effective approach for simultaneously quantitative determination of sapoinins in *P. notoginseng,* which could be used for quality control of *P. notoginseng* and its preparations.

Keywords: Pressurized liquid extraction; *Panax notoginseng*; Notoginsenoside; Ginsenoside

1. Introduction

Ginseng, also named as Asian ginseng (*Panax ginseng*) and American ginseng (*Panax quinquefolius*) are medicinal plants known worldwide for their tonics. The root of *Panax notoginseng*, called Sanqi or Tanqi in China, is a less popular medical material which is largely cultivated in Yunnan and Guangxi provinces of China. *P. notoginseng* is well known for its efficacy in promoting blood circulation, removing blood stasis, inducing blood clotting, relieving swelling, and alleviating pain [1–3]. It has been used as a creditable drug for the treatment of haemoptysis, haemostatic and haematoma in China for more than 400 years [1,3]. It is also contained in several famous Chinese traditional formula, such as "*Yunnan Baiyao*" which is used for treatment of injury induced trauma and bleeding, and "*Pian Zi Huang*" used to treat hepatitis. Current pharmacological studies revealed that *P. notoginseng* and its saponins possess anticarcinogenic [4,5] and hepatoprotective [6] properties, as well as protective effects on cardiovascular and cerebrovascular diseases [7–9].

The pharmacological properties of *P. notoginseng* are generally attributed to its triterpene glycosides. To date, over 50 different saponins have been isolated and characterized from *P. notoginseng* including ginsenosides, notoginsenosides and gypenosides. These neutral saponins are mainly drammarane triterpenes with 20(*S*)-protopanaxadiol or 20(*S*)-protopanaxatriol aglycon moieties [1]. Among these saponins, ginsenoside Rg1, Re, Rf, Rb1, Rc, Rd and notoginsenoside R1 are considered to be the major components in *P. notoginseng* [3]. As the major constituents of *P. notoginseng* (i.e. damaranetype saponins) are very similar to those of Ginseng, the methods used to determine the ginsenosides in Asian and American ginseng could be suitable for the analysis of *P. notoginseng*. Thin layer chromatography was initially used for separation and analysis of ginsenosides. However, the method suffers from low resolution and repeatability [10–12]. In recent years, HPLC with UV [13–15], ELSD [16] or MS [17,18] detection have been extensively used for analysis of ginsenosides in *P. notoginseng*. Unfortunately,

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less than six saponins were quantitatively determined in those reports.

Sample preparation has been reported to be the bottleneck of the most analytical procedures, as it is one of the least evolved parts of the whole method. During the past few years, one of the most promising sample preparation techniques is pressurized liquid extraction (PLE; Dionex trade name ASE for accelerated solvent extraction) which has been widely applied in environmental [19–21], food [22,23] and pharmaceutical fields [24]. Compare to conventional extraction methods, PLE technique has the advantages of short extraction time, less solvent consumption, high extraction efficiency and automatic operating capabilities [19–25]. Generally, ultrasonication [13,15–17], hot reflux [26] and soxhlet extraction [14] have been employed for extraction of saponins from *P. notoginseng*. However, the extraction efficiency and repeatability of these methods are far from satisfaction.

In this paper, a HPLC and PLE were developed for simultaneous determination of nine saponins in *P. notoginseng*. The different methods including PLE, ultrasonication, soxhlet extraction and immersion were also compared for extraction of saponins from *P. notoginseng*.

2. Materials and methods

2.1. Materials and chemicals

P. notoginseng was obtained from Wenshan, Yunan province, China. The botanical origin of material was identified and the voucher specimens of *P. notoginseng* were deposited at the Institute of Chinese Medical Sciences, University of Macau, Macao, China.

Ginsenoside Rb2, Rb3, Rc, Rd, Re and Rf (Fig. 1) were purchased from Chromadex Company (Santa Anna, CA, USA) and ginsenoside Rb1 and Rg1 were supplied by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Notoginsenoside R1 was kindly offered by Kunming Institute of Botany (Kunming, China). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA). Methanol and acetonitrile for HPLC were purchased from Merck (Darmstadt, Germany).

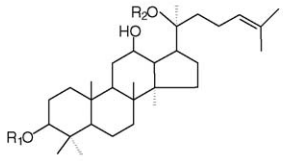
2.2. Sample preparation

2.2.1. Pressurized liquid extraction

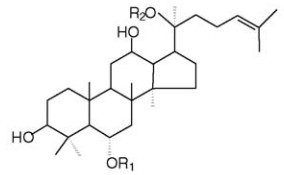
PLE were performed on a Dionex ASE 200 system (Dionex Corp., Sunnyvale, CA, USA) under optimized conditions. In brief, dried powder of *P. notoginseng* (0.5 g) were placed into an 11 ml stainless steel extraction cell. The optimized conditions were: particle size, 0.3–0.45 mm; solvent, methanol; temperature, 150 °C; pressure, 6.895×10^3 MPa; static time, 15 min; and one static cycle and one extraction times [27]. PLE extract was transferred into a 50 ml volumetric flask which was brought up to its volume with the same solvent and filtered through a 0.45 μm Econofilter (Agilent Technologies) prior to injection into the HPLC system.

2.2.2. Ultrasonic extraction

A portion of the dried sample powder (0.5 g) was placed into a 50 ml centrifuge tube containing 30 ml methanol and the capped tube was shaken for 30 s before ultrasonication for 2 h at room temperature (25 °C) in an ultrasonic bath (Transsonic T700/H, Lab-Line instrument, Inc., USA). After centrifuge (Thermo Inc., MA, USA) at 2000 rpm for 5 min, the supernatant was transferred into a 50 ml volumetric flask which was brought up to its volume with methanol, and filtered through a 0.45 μm filter prior to HPLC analysis.



20S-Protopanaxadiol (R_1=R_2=H)



20S-Protopanaxatriol (R_1=R_2=H)

Saponin	R1	R2	M.W.
Rb1	-glc(2-1)glc	-glc(6-1)glc	1108
Rb2	-glc(2-1)glc	-glc(6-1)arap	1078
Rb3	-glc(2-1)glc	-glc(6-1)xyl	1078
Rc	-glc(2-1)glc	-glc(6-1)araf	1078
Rd	-glc(2-1)glc	-glc	946

Saponin	R_1	R_2	M.W.
Re	-glc(2-1)rha	-glc	946
Rg1	-glc	-glc	800
Rf	-glc(2-1)glc	-H	800
R1	-glc(2-1)xyl	-glc	932

Glc= β-D-glucose
Arap= α-L-arabinose (pyranose)
Araf= α-L-arabinose (furanose)
Xyl= β-D-xylose
Rha= α-L-rhamnose
M.W. = Molecular Weight

Fig. 1. Chemical structures and molecular weights of investigated saponins from *Panax notoginseng*.

2.2.3. Soxhlet extraction

Dried powder (0.5 g) was placed in cellulose extraction thimbles, and was extracted in micro-Soxhlet extractors with 60 ml of methanol for 6 h. After cooling, partial methanol was removed in vacuo and the residue was transferred into a 50 ml volumetric flask which was brought up to its volume with methanol. The final solution was filtered through a 0.45 μm filter before analysis.

2.2.4. Immersion

The powder (0.5 g) was placed into a 50 ml centrifuge tube containing 30 ml methanol and statically extracted in capped tube at room temperature for 48 h. Sample tubes were then centrifuged at 2000 rpm for 5 min. The supernatant was transferred into a 50 ml volumetric flask which was brought up to its volume with methanol. The final solution was filtered through a 0.45 μm filter before HPLC analysis.

2.3. HPLC analysis

Analysis were performed on a Agilent 1100 liquid chromatograph system (Palo Alto, CA, USA), equipped with vacuum degasser, quaternary gradient pump, autosampler and DAD, connected to a Agilent ChemStation software. A Zorbax ODS C_{18} column (4.6 mm × 250 mm, 5 μm) and a Zorbax ODS C_{18} guard column (4.6 mm × 12.5 mm, 5 μm) were used. A binary gradient elution system consisted of water (A) and acetonitrile (B) and separation was achieved using the following gradient program: 0–30 min, 18–19% B; 30–35 min, 19–35% B; 35–60 min, 35–55% B; and finally, reconditioning the column with 18% B isocratic for 10 min. The flow-rate was 1.5 ml/min and the system operated at 40 °C. The detection wavelength was set at 203 nm.

2.4. HPLC–ESI-MS conditions

An Agilent 1100 Series LC/MSD Trap system (Palo Alto, CA, USA) were applied for authentication of the peaks. The same column, elution program and flow-rate of HPLC were used for LC–ESI-MS analysis. The mobile phases were 8 mmol/l aqueous ammonium acetate (A) and acetonitrile (B), the column temperature was set at 25 °C and the injection volume was 10 μl. About 32% of flow was introduced into MS after split. The MS conditions were optimized in order to achieve maximum signal. ESI-MS conditions: negative-ion mode; source voltage, 3.5 kV; dry gas N_2, 7 l/min; temperature, 325 °C; pressure of nebulizer, 3.62 kPa. The ESI-MS/MS was set with fragment amplification 1.5 V and isolation width four. Scan range of both ESI-MS and ESI-MS/MS was fixed at *m/z* 200–1400 U.

2.5. Calibration curves

Aqueous methanol stock solution containing ginsenoside Rg1, Re, Rf, Rb1, Rc, Rb2, Rd and notoginsenoside R1 was prepared and diluted to appropriate concentration for the establishment of calibration curves. So did ginsenoside Rb3 independently. Seven concentrations of the nine analytes were injected in triplicate, and then the calibration curves were constructed by plotting the peak areas versus the concentrations of each analyte.

2.6. Precision and accuracy

Intra- and inter-day variations were chosen to determine the precision of the developed assay. The relative standard deviation (R.S.D.) was taken as a measure of precision. Intra- and inter-day repeatability was determined on five times within one day and five separate days, respectively.

The recovery was determined by performing consecutive pressurized liquid extraction on the same sample under the optimized PLE conditions until no investigated compounds were detected by HPLC analysis. The recovery was calculated based on the total amount of individual investigated components and the average recoveries were counted by the formula: recovery (%) = first amount found/total amount of individual investigated components × 100%.

3. Result and discussion

3.1. Optimization of HPLC conditions

Several previous studies showed that ginsenoside Rg1 and Re could not be well-separated [28–31]. Therefore, different mobile phases, including acetonitrile 0.05% aqueous phosphoric acid and acetonitrile–phosphate buffer (pH 5.8), were tested in order to obtain good resolution, but failed. At last, ginsenoside Rg1 and Re were completely separated by using reasonable elution program of water and acetonitrile, though the peaks were broadened (Fig. 2). Unfortunately, ginsinoside Rb3, rich in the leaf of *P. notoginseng*, could not be separated with its isomer, ginsinoside Rb2, after careful trials. Therefore, the total amount of ginsenosides Rb2 and Rb3 was calculated as ginsenoside Rb2.

3.2. Identification of investigated compounds in P. notoginseng

Total ion chromatograms (TIC) of nine investigated saponins and PLE extract of *P. notoginseng* were shown in Fig. 3. As some saponins have same molecular weight, MS^2 information was also used for confirmation of the analytes. The nine investigated saponins were identified by comparing the retention time, MS and MS^2 spectra with those of their reference compounds and the fragmentation pathway of the saponins showed a successive deglycon process (Table 1).

3.3. Validation of the developed method

The linearity, regression and precision of nine saponins were performed using the developed HPLC method. The high correlation coefficient ($r^2 > 0.9997$) values indicated good correlations between investigated compounds concentrations and their peak areas within the test ranges. Both intra-day and inter-day repeatability (R.S.D.) of nine peaks area detected for the investigated components were less than 3.10% (Table 2).

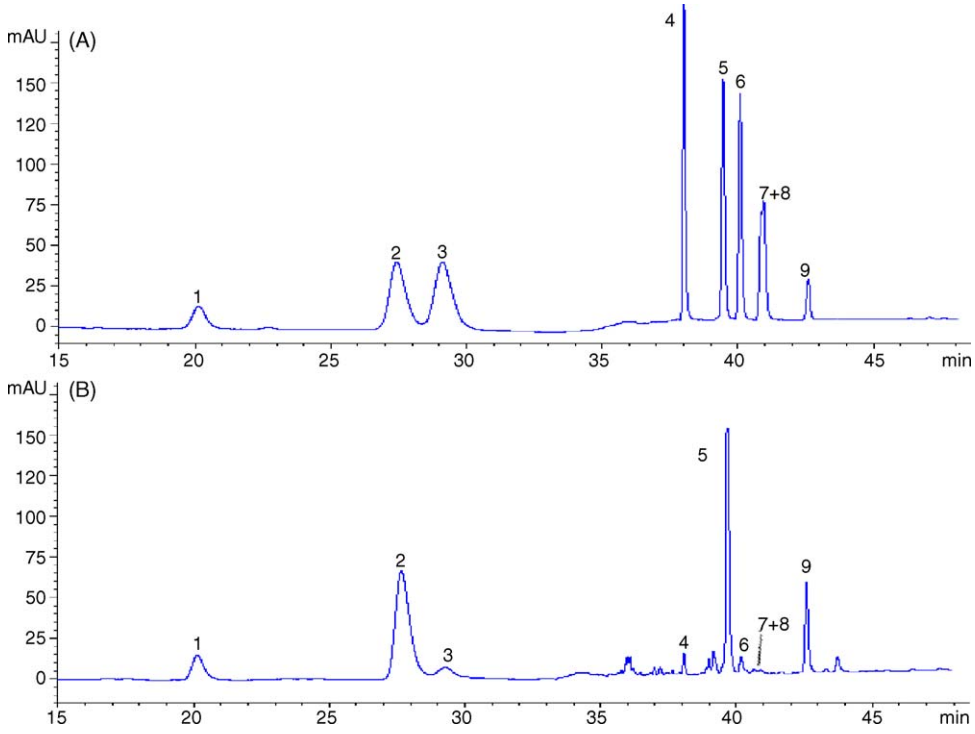


Fig. 2. HPLC profiles of (A) nine investigated saponins and (B) PLE extract of *Panax notoginseng*. 1, Notoginsenoside R1, 2–9, ginsenoside Rg1, Re, Rf, Rb1, Rc, Rb2, Rb3 and Rd, repectively.

The PLE recovery was calculated based on the total amount of individual investigated components. The result showed that the recovery of ginsenoside Rg1, Re, Rf, Rb1, Rc, Rb2, Rd and notoginsenoside R1 was 95.62, 97.32, 96.71, 93.96, 97.81, 97.44, 95.01 and 96.32% at the first time extraction, respectively.

3.4. Quantitation of nine saponins in P. notoginseng

The nine investigated saponins in four extracts of *P. notoginseng* were determined and the data were shown in Table 3. Based on the results, we can see that PLE has distinct advan-

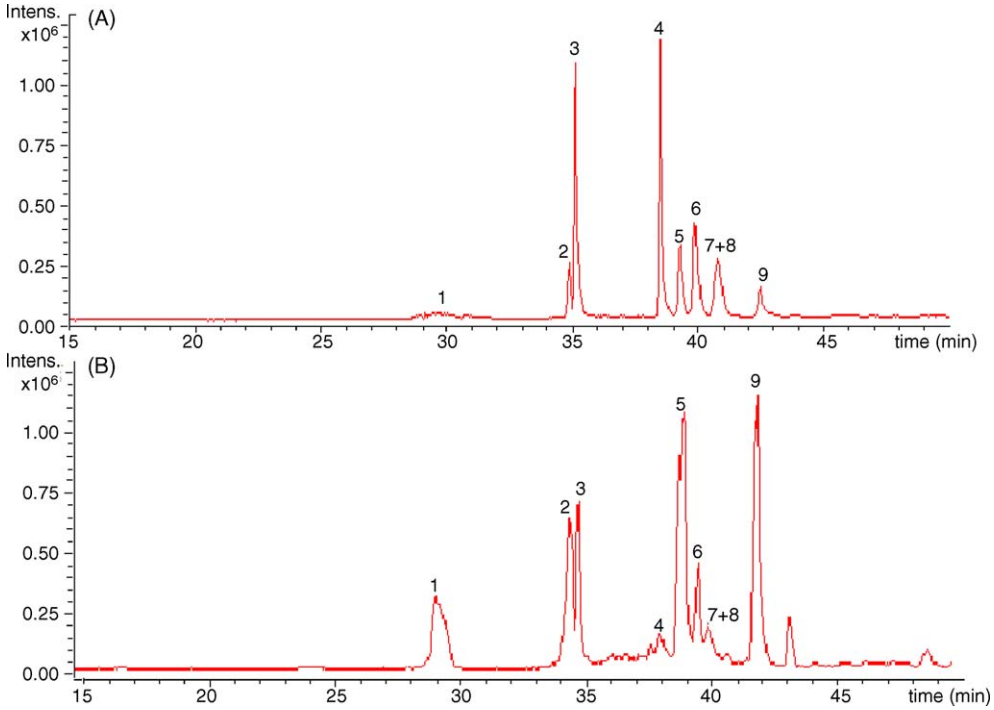


Fig. 3. Total ion chromatograms (TIC) of (A) nine investigated saponins and (B) PLE extract of *Panax notoginseng*. 1, Notoginsenoside R1, 2–9, ginsenoside Rg1, Re, Rf, Rb1, Rc, Rb2, Rb3 and Rd, repectively.

Table 1
MS and MS^2 data of investigated saponins from *Panax notoginseng*

Peak	Identification	RT (min)	MS (*m/z*)	MS^2 fragment ion (*m/z*)
1	R1	29.6	932	799(100)[M-H-Xyl]$^-$; 769(32)[M-H-Glc]$^-$; 637(10)[M-H-Xyl-Glc]$^-$; 475(1)Agl
2	Rg1	34.9	800	637(100)[M-H-Glc]$^-$; 475(10)Agl
3	Re	35.1	946	799(45)[M-H-Rha]$^-$; 783(100)[M-H-Glc]$^-$; 765(20)[M-H-Glc-H_2O]$^-$; 637(63)[M-H-Glc-Rha]$^-$; 619(21)[M-H-Glc-Rha-H_2O]$^-$; 475(20)Agl
4	Rf	38.5	800	637(100)[M-H-Glc]$^-$; 475(45)Agl
5	Rb1	39.2	1108	945(100)[M-H-Glc]$^-$; 783(36)[M-H-2Glc]$^-$; 621(7)[M-H-3Glc]$^-$; 459(1)Agl
6	Rc	39.9	1078	945(100)[M-H-Araf]$^-$; 915(12)[M-H-Glc]$^-$; 783(35)[M-H-Araf-Glc]$^-$; 621(4)[M-H-Araf-2Glc]$^-$; 459(1)Agl
7	Rb2	40.6	1078	945(100)[M-H-Arap]$^-$; 915(27)[M-H-Glc]$^-$; 783(49)[M-H-Arap-Glc]$^-$; 621(4)[M-H-Arap-2Glc]$^-$; 459(1)Agl
8	Rb3	40.8	1078	945(100)[M-H-Xyl]$^-$; 915(29)[M-H-Glc]$^-$; 783(48)[M-H-Xyl-Glc]$^-$; 621(4)[M-H-Xyl-2Glc]$^-$; 459(1)Agl
9	Rd	42.4	946	783(100)[M-H-Glc]$^-$; 621(10)[M-H-2Glc]$^-$; 459(3)Agl

Note: relative abundance of ion is shown in parentheses; Xyl, xylose; Glc, glucose; Rha, rhamnose; Agl, aglycone; Araf, arabinose; Arap, arabinose.

Table 2
Linear regression and precision data of investigated saponins from *Panax notoginseng*

Analyte	Linear regression data				Precision, R.S.D. (%)	
	Linear range (μg)	Slope	Intercept	r^2 ($n=7$)	Intra-day ($n=5$)	Inter-day ($n=5$)
R1	0.270–4.320	187.9	0	0.9999	0.61	1.54
Rg1	0.200–10.000	340.8	−6.275	1.0000	0.30	0.55
Re	0.022–2.240	306.9	−9.172	0.9997	0.76	1.58
Rf	0.101–2.020	182.1	−0.178	0.9999	0.81	1.34
Rb1	0.200–10.000	229.5	0.542	1.0000	0.42	0.68
Rc	0.050–0.515	295.9	−1.045	0.9999	0.73	2.50
Rb2	0.060–0.290	190.5	−0.122	0.9999	0.45	1.95
Rb3	0.080–0.380	234.8	−2.012	0.9998	0.34	1.48
Rd	0.027–1.070	279.9	−0.446	1.0000	0.49	3.10

tages over other extraction methods such as Soxhelt extraction, immersion and ultrasonication. First, the extraction efficiency of PLE was the highest among the four extraction methods. Secondly, the repeatability of PLE was the best (the lowest R.S.D.) due to high automation which could reduce human handling errors. Finally, the extraction time and solvent consumption of PLE were the least. In summary, the different data would be obtained when different sample preparation methods were employed. That is why the standardization of sample preparation for quantitative analysis should be emphasized and PLE is the most promising sample preparation technique.

Table 3
The content and repeatability data of investigated saponins from *Panax notoginseng* using different sample preparation methods

Saponin	PSE		Ultrasonication		Soxhlet		Immersion	
	(%)[a]	R.S.D.[b] (%)	(%)	R.S.D. (%)	(%)	R.S.D. (%)	(%)	R.S.D. (%)
R1	0.81	2.61	0.63	4.60	0.75	4.82	0.69	5.82
Rg1	2.37	0.92	1.79	1.90	2.20	2.96	1.88	8.46
Re	0.28	0.87	0.22	3.04	0.26	4.69	0.22	7.74
Rf	0.23	3.70	0.21	4.42	0.20	3.64	0.21	23.67
Rb1	2.74	0.82	2.08	4.95	2.70	3.76	2.24	7.62
Rc	0.20	3.02	0.19	2.71	0.20	8.89	0.18	17.09
Rb2 + Rb3[c]	0.11	2.67	0.13	13.90	0.10	4.73	0.08	7.28
Rd	0.62	0.14	0.52	1.22	0.58	7.73	0.50	8.99
Sum/mean	7.36[d]	1.84[e]	5.77	4.59	6.99	5.15	6.00	10.33

[a] Mean value of samples ($n=3$).
[b] Relative standard deviation.
[c] Calculated as Rb2.
[d] Total amount of the nine saponins.
[e] Mean of R.S.D. for nine saponins.

4. Conclusion

The developed HPLC and PLE method for simultaneous determination of ginsenoside Rg1, Re, Rf, Rb1, Rc, Rb2, Rb3, Rd and notoginsenoside R1 from *P. notoginseng,* has good precision, repeatability, recovery and less time-consuming. The results indicated that PLE could be beneficial to the standardization of sample preparation for quality control of Chinese medicine.

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