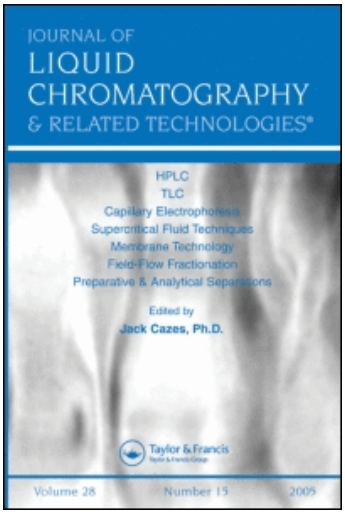

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Abstract: A simple and sensitive high performance liquid chromatography with diode array detection (HPLC-DAD) method was established and validated to simultaneously quantify seven major saponins, i.e., notoginsenoside R_1, ginsenoside Re, Rg_1, Rb_1, Rh_1, Rg_2 and Rd, in "Compound Danshen Dropping Pills" (DSDP), the best sold traditional Chinese medicine (TCM). The method involved the solid-phase extraction (SPE) and chromatographic separation on a reversed-phase Agilent Zorbax SB-C_{18} column. The mobile phase consisted of 0.01% acetic acid in water and 0.01% acetic acid in acetonitrile for gradient elution. The detection wavelength was 203 nm. Linearity ($R^2 > 0.994$), intra- (R.S.D $< 2.05\%$) and inter-day (R.S.D $< 3.24\%$) precision, and limit of quantification (0.011–0.054 mg/mL) were determined, and the recoveries of selected compounds were in the range of 90.06%–103.08% with RSD less than 4.3%. Subsequently, the method was employed to analyze commercial DSDP samples and frauds produced with inferior *Radix Notoginseng*. The results clearly demonstrated that the quality of the DSDP samples was closely related to plant materials and could be assessed by the proposed analytical method in conjunction with a chemometrics approach.

Keywords: Saponins, HPLC, Quality control, Compound Danshen Dropping Pill, *Radix Notoginseng*, Traditional Chinese medicine

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INTRODUCTION

Compound Danshen Dropping Pill (DSDP) is one of the best sold traditional Chinese medicines in China because of its wide range of therapeutic properties[1,2] towards cardiovascular diseases, including atherosclerosis and coronary heart disease. Saponins, known as ginsenoside and notoginsenoside,[3–6] and depsides are proven to be the major bioactive components contributing to pharmacological activity and clinical indications,[1] and they are from two composition plants, *Radix Notoginseng* and *Salvia Miltiorrhiza*, respectively. According to Chinese pharmacopoeia,[1] only Danshensu, a depside from *Salvia Miltiorrhiza*, has been evaluated. To reflect the quality of products more accurately, more bioactive constituents are required to be determined, especially an assay of DSDP samples reflecting another composition plant, *Radix Notoginseng*, is urgently needed.

Saponins of *Radix Notoginseng* have been analyzed by several approaches such as high performance liquid chromatography with UV detector (HPLC-UV),[7–10] HPLC with evaporative light-scattering detection (HPLC-ELSD),[11] and near infrared spectroscopy (NIR).[12] To the best of our knowledge, only subter-six saponins,[7,8] were quantified by HPLC in previous works, and the remarkable ginsenosides Re and Rg_1 were seldom determined simultaneously for limited resolution.[9] As we all know, TCM products are produced from crude materials using complex processes, not simply the extracts of medicinal materials. In past years, although much attention had been paid to quantify the active saponins in crude extracts of *Radix Notoginseng*, study on TCM products produced with *Radix Notoginseng* was rarely reported.

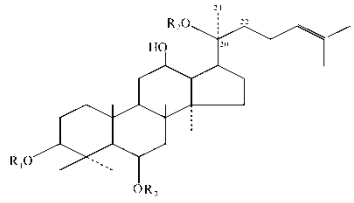
As is well known, the electrospray ionization technique is a soft ionization approach to obtaining a screening of molecular weight. It had been utilized to characterize herbal extracts, such as tomato,[13] ginkgo,[14] Panax ginseng,[15,16] and Chinese medicine Si-Wu-Tang.[16] The results demonstrated that it was especially suitable to identify thermolabile complex mixtures. Meanwhile, high performance liquid chromatography still holds the authoritative position on quantitative analysis. Thus, a reliable method for the qualitative and quantitative analysis of DSDP samples by HPLC-ESI-MS is highly desirable.

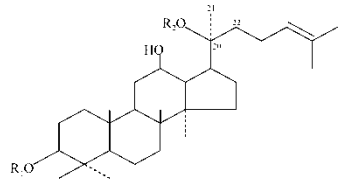
This paper aims to develop, for the first time, a specific HPLC-DAD method to simultaneously quantify the seven major active saponins (shown in Fig. 1) of DSDP samples. HPLC-ESI-MS was taken for purpose of identification and verification for the specificity of the method. The developed method would be applied to the determination of seven saponins in DSDP samples.

EXPERIMENTAL

Reagents and Materials

Acetonitrile and methanol were HPLC grade from Tedia (Fairfield, USA). Acetic acid and phosphoric acid were A.R. grade from Hangzhou Reagent





Ginsenoside Re R_1– H R_2– Glc R_3– O-Glc-Rham
Ginsenoside Rg_1 R_1= H R_2= Glc R_3= O-Glc
Ginsenoside Rg_2 R_1= H R_2= H R_3= O-Glc-Rham
Ginsenoside Rh_1 R_1= H R_2= H R_3= O-Glc
Notoginsenoside R_1 R_1= H R_2= Glc R_3= O-Glc-Xyl
Glc= β -D-glucose; Rham= α -L-rhamnose; Xyl= β -D-xylose

Ginsenoside Rb_1 R_1–Glc-Glc R_2– Glc-Glc
Ginsenoside Rd R_1=Glc-Glc R_2= Glc

Figure 1. Structures of the studied saponins in DSDP.

Company (Hangzhou, China). Ammonia solution (25% extra pure) was purchased from Shanghai Reagent Company (Shanghai, China). Water was purified by a Milli-Q academic water purification system (Milford, MA, USA). Nylon film, 0.45 μm, (Shanghai Institute of Pharmaceutical Industry, Shanghai, China) and Supelco C_{18} column (3 mL) (500 mg, Supelco Park Bellefonte, USA) were also used for pretreatment of DSDP samples.

All of the *DSDP* samples were supplied by Tasly Pharmaceutical Co., Ltd (Tianjin, China). The fauds were produced from inappropriate *Radix Notoginseng*, and the eligible samples were produced from *Radix Notoginseng* planted in the GAP farms. Meanwhile, all of the crude materials were identified by the research institute of Tasly Pharmaceutical Co., Ltd (Tianjin, China).

Reference compounds, notoginsenoside R_1, ginsenoside Re, Rg_1, Rb_1, Rh_1, Rg_2, and Rd were purchased from the National institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

HPLC-DAD-ESI-MS System

An Agilent 1100 series HPLC system (Agilent Company, USA) was equipped with quaternary pump, vacuum degasser, autosampler, diode array detector, column heater cooler, and ChemStation system. An Agilent 1100 series MSD (Agilent Company, USA) hyphenated with the HPLC system described above was also used. The column used was a Zorbax SB-C_{18} column (4.6 × 250 mm, 5 μm, Agilent Company, USA) coupled with Agilent C_{18} precolumn (4 × 5 mm).

HPLC-DAD-ESI-MS Operation Conditions

The linear gradient elution of solvent A (CH_3COOH: H_2O = 0.01:100) and solvent B (CH_3COOH: CH_3CN = 0.01:100) was used at a flow rate of 0.8 mL/min. The gradient program is presented in Table 1. Column temperature was set at 30°C and the effluent was monitored at 203 nm.

The MS spectra were acquired in negative ion mode for detection of saponins. N_2 was used as both drying gas with a flow rate of 12 L/min and as nebulizing gas with a pressure of 60 psi. The nebulizer temperature was set at 350°C and the capillary voltage was set at 3500 V. The mass spectra were recorded in the range of 400–1500 amu.

Sample Preparation

A DSDP sample (1.0000 g) was dissolved in 10 mL 4% ammonia solution with an ultrasonic process, in an ultrasonic bath for 15 min. The solution was filtrated through 0.45 μm nylon film. Then, 5 mL filtrate was applied on a Supelco C_{18} column (3 mL) and successively eluted with 15 mL 20%methanol-4% ammonia solution to entirely elute off the phenolic compounds. Finally, the saponins was eluted with 5 mL methanol. The centrifuged methanol elution was collected for HPLC analysis. The sample injection was 20 μL.

Preparation of Standard Solutions

The standard stock solutions were prepared by accurately weighing each compound and then dissolving with methanol. Three composite stock solutions were prepared: composite solution 1 consisted of ginsenoside Rg_1 and Rb_1, each at a concentration of 1 mg/mL. Composite solution 2 consisted of notoginsenoside R_1, ginsenoside Re, Rh_1, and Rd, each at a concentration of 0.5 mg/mL. Composite solution 3 consisted of ginsenoside Rg_2

Table 1. Solvent gradient grogram of HPLC analysis

Time (min)	A (% V/V)	B (% V/V)
0 min	80	20
40 min	80	20
55 min	65	35
65 min	65	38
80 min	57	43

Solvent A (CH_3COOH: H_2O = 0.01:100), solvent B (CH_3COOH: CH_3CN = 0.01:100).

at a concentration of 0.5 mg/mL. Calibration standards and daily calibration plots were prepared either from the mixture of stock solutions or from dilutions. Three injections were performed for each dilution.

Another solution, containing notoginsenoside R_1, ginsenoside Re, Rg_1, Rb_1, Rh_1, Rg_2, and Rd, was prepared in methanol as a fortified sample. The solution consisted of notoginsenoside R_1, ginsenoside Re, Rg_1, Rb_1, Rh_1, Rg_2, and Rd at a concentration of 0.07 mg/mL, 0.25 mg/mL, 0.04 mg/mL, 0.48 mg/mL, 0.03 mg/mL, 0.17 mg/mL, and 0.09 mg/mL, respectively.

Method Validation

Linearity

To carry out this study, six solutions with different concentrations were prepared from each reference saponin. Each dilution was injected in triplicate. The calibration curve was obtained by plotting the chromatography peak area against the concentration. The regression equation was calculated in the form of $Y = AX + B$, where Y and X were values of peak area and sample amount, respectively.

Precision

The instrument precision was obtained by analyzing a sample six times, consecutively. The repeatability was evaluated by the intra- and inter-day assays. Six replicate samples were prepared and analyzed in a single day (intra-day variation), and three replicate samples were prepared and analyzed during the same day, for four consecutive days (inter-day variation). The intra- and inter-day variation (RSD) of seven saponins in the samples was calculated. The limit of quantification (LOQ, signal/noise = 10) was also validated.

Recovery

The recoveries of the saponins were determined by the standard addition method. The standards were spiked into the samples (DSDP) in the form of solution and then processed as described above.

RESULTS AND DISCUSSION

Development of SPE and HPLC Analysis

Ratios of acetonitrile/water with acetic or phosphoric acid in mobile phase, column temperature, etc., were investigated for the best separation.

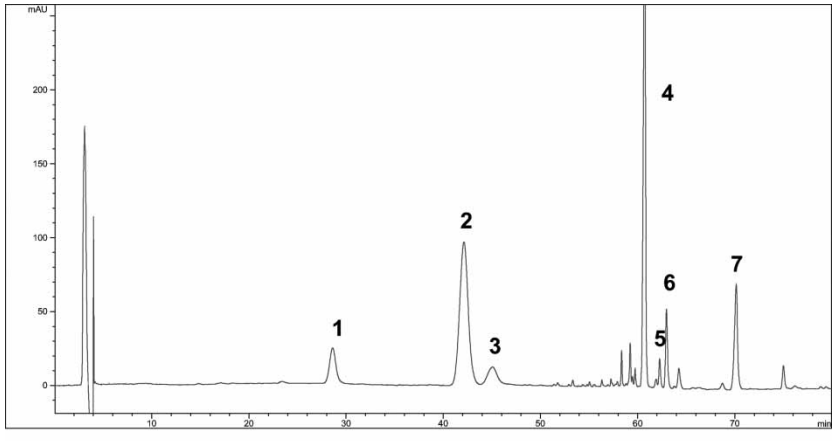
As shown in a typical chromatogram of Notoginseng saponins from DSDP at 203 nm (Fig. 2), baseline resolution was obtained for all the analytes.

As depsides, which were the major active compounds contained in DSDP, could best represent at 280 nm and also have the profile at 203 nm, a pretreatment method, based upon the solid-phase extract (SPE) technique, was used to remove depsides. After the pilot experiment, each step of SPE was optimized. The filtrate (5 mL) was applied on a C_{18} column (500 mg, Supelco, Park Bellefonte, USA) and successively washed with 15 mL 20%methanol-4% ammonia solution to entirely elute the phenolic compounds off. Finally, the cartridge was eluted with 5 mL methanol.

After SPE, the chromatogram (Fig. 3) at 280 nm showed the absolute absence of depsides and proved that there would also be none of the depsides peaks which appeared at 203 nm in the chromatogram (Fig. 2). It was concluded, that the developed SPE method was selective and the assay for Notoginseng saponins had no interference from depsides.

Identification of Saponins

Due to the complexity of the chemical components, usually several peaks were eluted close together. Thus, UV detection could not provide enough specificity and an MS detector was applied for further identification. Prior to the HPLC/ESI-MS analysis, studies on the fragmentation behavior of authentic chemicals of ginsenoside in DSDP were performed by Zhang et al.



1: Notoginsenoside R_1, 2: Ginsenoside Rg_1, 3: Ginsenoside Re, 4: Ginsenoside Rb_1, 5: Ginsenoside Rg_2, 6: Ginsenoside Rh_1, 7:Ginsenoside Rd.

Figure 2. A typical chromatogram of DSDP sample after SPE, at 203 nm.

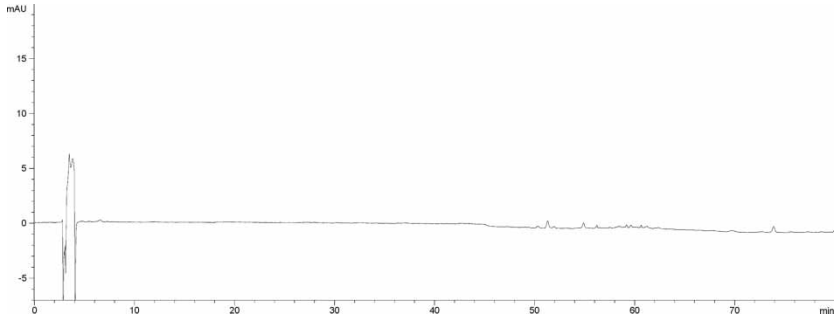


Figure 3. A representative chromatogram of DSDP sample after SPE, at 280 nm.

in our laboratory.[17] The MS spectra were detected in negative ion mode and their TIC chromatogram was shown in Fig. 4. In the MS spectra, most of the saponins in DSDP exhibited their quasi-molecular ions $[M-H]^-$ and adducted ions [M + CH3COOH-H]- in the negative ion mode. For each peak, the MS spectra exhibited several peaks at different m/z, respectively. The identification results are listed in Table 2. The m/z of main compounds in the sample was 931, 859, 945, 1107, 697, 783, 945, and it was consistent with the m/z of $[M-H]^-$ ion for notoginsenoside R_1, ginsenoside Re, Rb_1, Rg_2, and Rd (931, 945, 1107, 783, 945) and the $[M + CH3COOH-H]^-$ ion for ginsenoside Rg_1, Rh_1 (859, 697). The peaks were identified in another way by comparing their retention times with those of the standards. ESI-MS data were in good agreement with those obtained by HPLC analysis.

Based on the m/z value, UV spectra, literature data, and comparison with standards compounds, main peaks were unambiguously identified as notoginsenoside R_1, ginsenoside Re, Rg_1, Rb_1, Rh_1, Rg_2, and Rd.

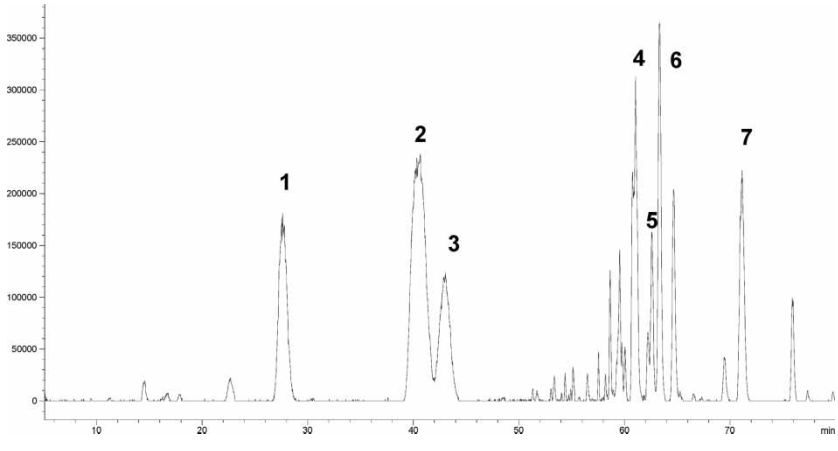


Figure 4. MS-TIC chromatogram of DSDP sample in negative ion mode.

Table 2. HPLC-DAD-ESI-MS identification

Peak	Compound	[M] (m/z)	$[M-H]^-$ (m/z)	$[M+HAC-H]^-$ (m/z)	[M + HAC] (m/z)	$[M+H]^+$ (m/z)	$[M+H]^+$ (m/z)
1	Notoginsenoside R_1	932	931	991			
2	Ginsenoside Rg_1		799	859	860		
3	Ginsenoside Re	946	945	1005			
4	Ginsenoside Rb_1	1108	1107				
5	Ginsenoside Rg_2	784	783	843	844		
6	Ginsenoside Rh_1			697	698		
7	Ginsenoside Rd	946	945				

Table 3. HPLC-UV linearity of seven saponins in DSDP

Compound	Slope (*A*)	Intercept (*B*)	R^2 (n = 5)	Linear range (mg/mL)	LOQ (mg/mL)
Notoginsenoside R_1	341.8	−0.4786	0.9942	0.18–2.8	0.054
Ginsenoside Rg_1	608.34	88.958	0.9991	1.52–12.2	0.043
Ginsenoside Re	174.7	178.18	0.9963	0.42–3.3	0.050
Ginsenoside Rb_1	348.9	0.9995	0.9999	0.62–15.4	0.011
Ginsenoside Rg_2	664.96	−6.5538	0.9999	0.04–0.9	0.032
Ginsenoside Rh_1	549.57	−3.1837	0.9999	0.18–3.63	0.024
Ginsenoside Rd	359.78	1.2978	0.9999	0.20–5.04	0.022

Method Validation

The validation of the method, including sensitivity, linearity, repeatability, and recovery were examined as described above. The results were listed in Tables 3–5.

For all of the quantitative components, good linearity with $R^2 > 0.994$ was achieved. Limits of quantification for each compound were listed in

Table 4. Repeatability of the method

Compound	Intra-day RSD (%)	Inter-day RSD (%)
Notoginsenoside R_1	1.79	2.70
Ginsenoside Rg_1	1.94	2.24
Ginsenoside Re	1.99	3.24
Ginsenoside Rb_1	1.67	0.84
Ginsenoside Rg_2	2.05	1.83
Ginsenoside Rh_1	1.19	2.60
Ginsenoside Rd	1.92	3.08

Table 5. Recoveries of seven saponins in DSDP

Compound	Actual added (mg)	Amount determined (mg)	Recovery (%)	RSD (%)
Notoginsenoside R_1	0.090948	0.092421	101.62	3.31
Ginsenoside Rg_1	0.496618	0.511914	103.08	2.36
Ginsenoside Re	0.002996	0.002698	90.06	4.24
Ginsenoside Rb_1	0.607763	0.547838	90.14	1.21
Ginsenoside Rg_2	0.033161	0.031185	94.04	1.49
Ginsenoside Rh_1	0.209853	0.190211	90.64	0.55
Ginsenoside Rd	0.102836	0.099720	96.97	1.64

Table 6. Contents of saponins (mg/g DW) of DSDP with different populations of *Radix Notoginseng*

No.	R_1	Rg_1	Re	Rb_1	Rg_2	Rh_1	Rd
1	0.409592	2.257894	0.316295	1.746339	0.050336	0.206389	0.300929
2	0.173957	1.131961	0.131042	0.743837	0.028753	0.105991	0.12265
3	0.140523	0.910958	0.102997	0.643193	0.019434	0.090559	0.089263
4	0.135332	0.89221	0.153974	0.440072	0.024253	0.098027	0.060181
5	0.154631	0.876766	0.128837	1.278397	0.097237	0.70977	0.227218
6	0.52586	2.585875	0.270233	2.488908	0.116233	0.657565	0.444547
7	1.164295	5.07662	0.47335	5.297968	0.105224	0.271037	1.386097
8	0.800353	3.647363	0.384076	3.74984	0.077	0.185827	0.947145
9	0.81099	2.227669	0.380162	3.836152	0.0771	0.185905	0.967664
10	1.158422	5.14028	0.468571	5.581988	0.108419	0.277261	1.455428
11	0.136798	0.631621	0.051926	0.534418	0.017391	0.057561	0.111236
12	0.35189	1.91587	0.172276	1.692575	0.059122	0.339524	0.267125
13	0.418866	2.285974	0.204935	2.085349	0.076356	0.464096	0.35797
14	0.463042	2.333086	0.213649	2.096557	0.078453	0.426602	0.346613
15	0.387066	1.955453	0.199281	1.692074	0.068644	0.395788	0.272384
16	0.427342	2.09212	0.199749	1.818035	0.061512	0.352033	0.288178
17	0.363931	1.952472	0.227468	1.791131	0.075095	0.368673	0.273969
18	0.442328	2.381419	0.290748	2.096	0.077044	0.467629	0.368267
19	0.472334	2.381165	0.262788	2.231834	0.096051	0.465714	0.3516
20	0.392443	1.977434	0.208701	1.778493	0.081727	0.411868	0.269515
21	0.431525	2.132034	0.245234	1.909045	0.076504	0.373783	0.288661

Table 3. The RSD ranged between 0.19–2.05% for intra-day assays and 0.84–3.24% for inter-day assays. The average recoveries of notoginsenoside R_1, ginsenoside Rg_1, Re, Rb_1, Rh_1, Rg_2, and Rd were 90.06%–103.08% with RSD less than 4.3%.

Analysis of DSDP Samples

Samples labeled from 1 to 11 are frauds and samples labeled from 12 to 21 are eligible samples, which were produced from materials grown in implantation bases. From the results from the HPLC analysis and curves calibration, the contents of saponins in DSDP samples were calculated, as listed in Table 6.

To better understand the difference between the commercial samples and frauds, a well-known chemometrics approach, Principal Components Analysis (PCA), was further employed in this study. Here, we produced a data matrix X in which each row refers to a sample and each column refers to the content of 7 compounds of the tested samples. The score plots derived from the first two principal components (PCs) were shown in Fig. 5, where each sample was represented as a marker. It was noticeable that samples 12–21 were as a cluster and the others scattered. This result agreed very well with the contents of saponins in Table 6. Members of cluster one were commercial employed *Radix Notoginseng* from Luoshang, one of the GAP farms, while others were produced with material collected from the market even without any knowledge of grades and sources. Therefore, quality of the *DSDP* samples was closely related to plant materials, and could be assessed by the presented analytical method in conjunction with the chemometrics approach.

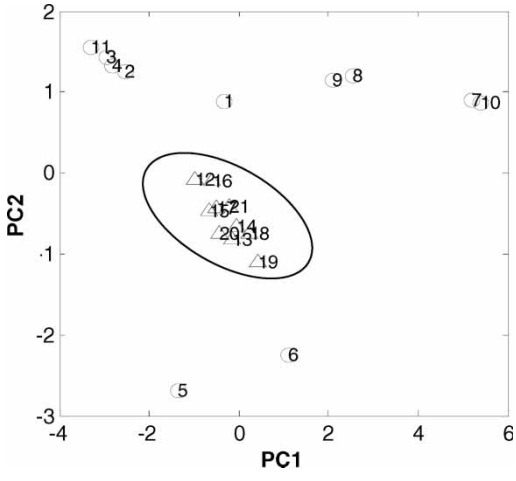


Figure 5. Score plots of the first two PCs of commercial DSDP samples and frauds. Δ, commercial samples; ○, frauds produced from inferior *Radix Notoginseng*.

CONCLUSIONS

The present study proposed an HPLC-DAD-ESI-MS method for simultaneous quantification of seven saponins including notoginsenoside R_1, ginsenoside Re, Rg_1, Rb_1, Rh_1, Rg_2, and Rd in the *Radix Notoginseng* preparation, DSDP. The method presented acceptable linearity ($R^2 > 0.994$), precision (with intra-day variation $<2.05\%$ and inter-day variation $<3.24\%$) and recoveries (between 90.06%–103.08%). This method had been successfully employed to differentiate DSDP produced with well formed *Radix Notoginseng* from unqualified ones.

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