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**RESEARCH NOTE**

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# Determination of 11 Ginsenosides in Black Ginseng Developed from *Panax ginseng* by High Performance Liquid Chromatography

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**Abstract** A high performance liquid chromatography (HPLC) method has been developed for determination of 11 ginsenosides in black ginseng (BG, white ginseng that is subjected to 9 cycles of 95°C for 3 hr). After eluted by gradient elution of water-acetonitrile without buffer in 70 min, 11 ginsenosides in BG were identified. The proposed method provided good linearity ($R^2$>0.9995), accuracy (92.2-106.6%), and intra- and interday precision (RSD<2.6%). In addition, ginsenosides compositions in white, red, and black ginsengs were investigated using this method, respectively. Interestingly, in BG, the content of ginsenoside $Rg_3$ which does not existed in white ginseng was 7.51 mg/g, approximately 20 times than that in red ginseng.

**Keywords:** black ginseng, ginsenoside, high performance liquid chromatography

## Introduction

*Panax ginseng* C. A. Meyer has been used as an herbal remedy in eastern Asia for over 2000 years. As we all known, the major pharmacologically active components in ginseng are a class of dammarene type triterpene saponins, more commonly known as ginsenosides (1,2). Today, ginsenosides have been extensively studied as one of the crucial active ingredients in ginseng (3,4). Among these ginsenosides, $Rg_3$ has reported to exhibit neuroprotective (5), antinociceptive (6), hematopoietic (7), anti-carcinogenic, and antimetastatic effects (8,9), etc. Nowadays, some commercial products of ginsenoside $Rg_3$ appeared in the market. For example, 'Shen-Yi Capsule' and 'Li-Li Capsule' have already proved a good market as a new anticancer drug and an antifatigue, respectively (10). Although ginsenoside $Rg_3$ has many pharmacological activities and wide marketing foreground, the content of ginsenoside $Rg_3$ is very low in normal commercial red ginseng (RG, fresh ginseng steamed at 95-100°C for 2-3 hr) and white ginseng (WG, fresh ginseng dehydrated by sun light) products. Fortunately, ginsenoside $Rg_3$ markedly increased by heat-activated *P. ginseng* root (11,12). Therefore, a new ginseng product containing a large amount of ginsenoside $Rg_3$, namely black ginseng (BG), was produced from 4-year WG by 9 cycles of steam treatment (95°C for 3 hr). As a multi-steamed ginseng, BG has more potent various biological activities such as anti-tumor activity, inhibition of obesity, neuroprotective and immuno-stimulating activities than WG and RG (13-15).

Up to now, numerous studies about ginseng have been mainly focused on WG and RG products, but few scientific researches on BG products have been reported. It has become a pressing issue to establish an authoritative quality control standard for evaluating BG. In this study, we aimed to establish an efficient high performance liquid chromatography (HPLC) method for the quantification of 11 individual ginsenosides in BG. In addition, ginsenosides composition in WG, RG, and BG were respectively investigated using this proposed HPLC method, and the results suggested that BG might have a great potential for neuroprotection due to a high content of ginsenoside $Rg_3$.

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## Materials and Methods

**Materials** All 4-year white and red ginsengs (*Panax ginseng* C.A. Meyer) were purchased from the Korean largest ginseng market in Geumsan (Chungnam, Korea). Black ginsengs were manufactured by 9-time repeated steaming the 4-year white ginseng at 95°C for 3 hr in pottery apparatus and followed by drying at 60°C for 18 hr. Standard ginsenosides $Rg_1$, Re, Rd, Rf, $Rh_1$, $Rb_1$, $Rg_2$, Rc, $Rb_2$, Rd, and 20(S)- and 20(R)- $Rg_3$ were purchased from the Hongjiu Biotech Co., Ltd. (Jilin, China). Solid-phase extraction (SPE) of Sep-Pak $C_{18}$ cartridges were from Waters (Milford, MA, USA). All solvents used in chromatography were HPLC grade; other chemicals were of reagent grade.

**Sample preparation** The ginsengs were ground with pulverizer, and extracted by ultrasonication (250 W) 3 times using 80% aqueous methanol at 50°C. Three extracts were combined, and the solvent was evaporated to dryness by rotary evaporator in vacuum and dried under $N_2$. The residue was dissolved in 2.0 mL water. This solution (0.5 mL) was loaded onto a preconditioned SPE Sep-Pak $C_{18}$ cartridge column, and then eluted sequentially by 0, 20, and 90% aqueous methanol (MeOH) (10 mL). The fraction of 90% MeOH was blown up under $N_2$, then dissolved in 1 mL 50% MeOH, filtered through syringe filter (0.45-μm) and subjected to HPLC analysis.

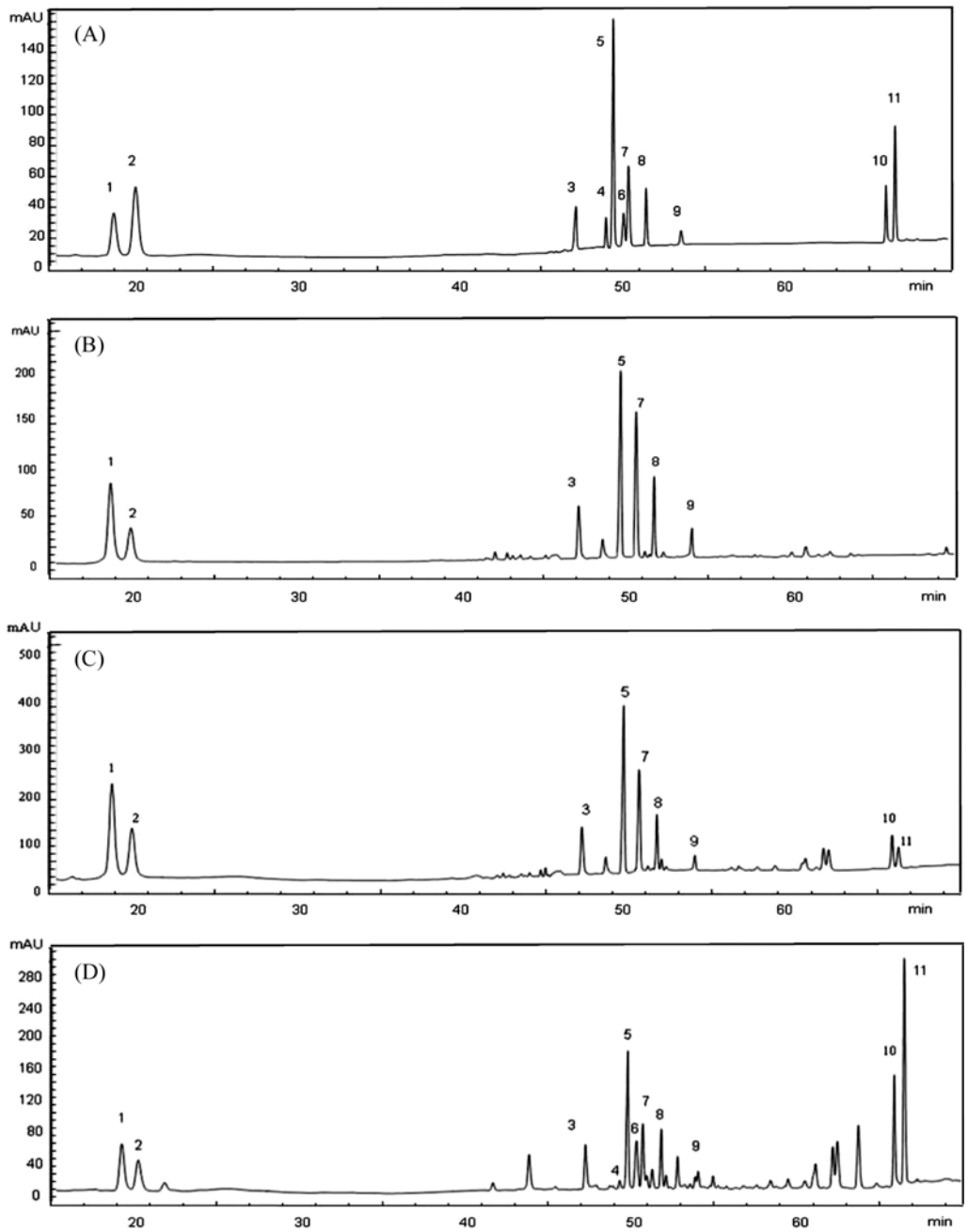


**Fig. 1. HPLC-UV chromatograms of mixed standards (A) and extracts of white (B), red (C), and black ginsengs (D).** Peaks: 1, $Rg_1$; 2, Re; 3, Rf; 4, $Rh_1$; 5, $Rb_1$; 6, $Rg_2$; 7, Rc; 8, $Rb_2$; 9, Rd; 10-11, 20(S)- and 20(R)-$Rg_3$.

**Analysis by HPLC** HPLC analysis was carried out by Agilent 1100 series HPLC system (Palo Alto, CA, USA), which was equipped with vacuum degasser, quaternary gradient pump, and diode-array detector. The separation was performed on the Discovery $C_{18}$ column (5 μm, 250 ×4.6 mm) at 30°C. The binary gradient elution system consisted of water (A) and acetonitrile (B) and separation was achieved using the following gradient program: 0-30 min, isocratic 20% B; 30-60 min, 20-45% B; 60-70 min, 45-62% B. The detection wavelength was set at 203 nm and the flow rate was set at 1.0 mL/min. All injections were 15 μL in volume. The stock solution of mixed standards was prepared and diluted to appropriate concentration for calibration. Six concentrations of the 11 saponins solution were injected in triplicate, and then the calibration curves were constructed by plotting the peak areas against the concentration of each analyte.

## Results and Discussion

**Optimization of HPLC-UV conditions** In traditional method of ginsenosides extraction from ginseng, diethyl ether and water-saturated *n*-butanol were used to remove nonpoar components and polysaccharids, respectively. However, it could not obtain ideal separation and stable baseline on chromatogram (16). SPE Sep-Pak $C_{18}$ cartridge

**Table 1. Intra- and interday variability for determination of 11 ginsenosides in black ginseng**

| Analytes | Concentration (μg/mL) | Intraday ($n$=6) | | | Interday ($n$=5) | | |
|---|---|---|---|---|---|---|---|
| | | Found (μg/mL) | RSD[1] (%) | Accuracy[2] (%) | Found (μg/mL) | RSD (%) | Accuracy (%) |
| $Rg_1$ | 209.5 | 210.5 | 1.3 | 100.5 | 212.8 | 1.3 | 101.6 |
| | 104.8 | 105.8 | 1.2 | 101.9 | 109.0 | 2.6 | 104.0 |
| Re | 327.4 | 326.2 | 1.5 | 99.6 | 318.5 | 1.6 | 97.3 |
| | 163.7 | 163.0 | 1.1 | 99.6 | 158.2 | 2.4 | 96.6 |
| Rf | 133.1 | 135.1 | 0.8 | 101.5 | 138.8 | 2.4 | 104.3 |
| | 66.5 | 65.3 | 2.6 | 98.2 | 63.5 | 2.7 | 95.5 |
| $Rh_1$ | 71.2 | 71.8 | 1.6 | 100.8 | 73.3 | 1.9 | 102.9 |
| | 35.6 | 35.7 | 1.9 | 100.3 | 37.0 | 2.1 | 103.9 |
| $Rb_1$ | 379.7 | 381.6 | 2.6 | 100.5 | 382.7 | 1.5 | 100.8 |
| | 189.8 | 187.1 | 1.2 | 98.6 | 183.7 | 2.2 | 96.8 |
| $Rg_2$ | 69.9 | 71.12 | 1.2 | 101.7 | 71.6 | 1.6 | 102.4 |
| | 34.9 | 34.6 | 2.4 | 99.1 | 33.5 | 2.4 | 96.0 |
| Rc | 252.3 | 252.5 | 0.7 | 100.1 | 260.6 | 2.2 | 103.3 |
| | 121.2 | 122.1 | 1.2 | 100.7 | 124.7 | 1.5 | 102.9 |
| $Rb_2$ | 208.5 | 208.8 | 0.7 | 100.1 | 217.1 | 2.3 | 104.1 |
| | 104.3 | 102.9 | 0.9 | 98.7 | 99.3 | 2.5 | 95.2 |
| Rd | 62.8 | 63.6 | 1.2 | 101.3 | 63.5 | 0.8 | 101.1 |
| | 31.4 | 31.1 | 2.3 | 99.0 | 31.1 | 2.2 | 99.0 |
| 20(S)-$Rg_3$ | 157.9 | 156.7 | 0.7 | 99.2 | 161.7 | 2.9 | 102.4 |
| | 79.0 | 79.5 | 1.7 | 100.6 | 78.6 | 0.7 | 99.5 |
| 20(R)-$Rg_3$ | 353.7 | 352.2 | 0.7 | 99.6 | 340.9 | 2.1 | 96.4 |
| | 176.8 | 177.7 | 0.8 | 100.5 | 182.4 | 2.7 | 103.2 |

[1]RSD (%)=(SD/mean)× 100
[2]Accuracy (%)=[1−(nominal concentration−mean of measured concentration)/nominal concentration]× 100.

used for sample preparation was proved to be a useful method, and could be alternative to traditional method due to its advantage such as using less amounts of organic solvent and the better efficient for ginsenosides purification. Usually, poor separation of ginsenosides $Rg_1$ and Re has been reported as a common problem in some cases (17,18). In this study, ginsenosides $Rg_1$ and Re were well separated from each other on stable baseline since the composition of mobile phase was programmed in the ratio of 20% acentonitrile to 80% water up to 30 min during the gradient elution.

**Method validation** ***Linearity***: Linearity of reference standards was studied for ginsenosides $Rg_1$, Re, Rd, Rf, $Rh_1$, $Rb_1$, $Rg_2$, Rc, $Rb_2$, Rd, and 20(S)- and 20(R)- $Rg_3$, respectively, and expressed in terms of correlation coefficient ($R^2$). The correction coefficient ($R^2$) was found to be better than 0.9995 for all of the reference ginsenoside standards in the range of 0.5-1.0 mg/mL (data not shown).

***Detection limits***: The limits of detection (LODs), determined according to signal/noise=3, were less than 3.9 μg/mL on the Discovery $C_{18}$ column (data not shown).

***Precision and accuracy***: Intra- and interday variations were chosen to determine the precision of the method. In the intraday variability test, the standard solutions were analyzed in triplicate for 6 times within 1 day, while in the interday variability test, the samples were examined in triplicate for 5 consecutive days. The overall intra- and interday variations of the 11 saponins were less than 2.9% (RSD) (Table 1).

Recovery was used to evaluate the accuracy of the method. The recovery experiments were performed by adding the spiked standard solutions into the 100 mg of BG powder, which were processed according to the section 'sample preparation'. The quantity of each analyte was subsequently obtained from the corresponding calibration curve. The developed method had fine accuracy with the overall recovery of 92.2-106.6% for the analytes (Table 2).

**Comparison Ginsenosides in white, red, and black ginsengs** The content of each ginsenoside in WG, RG, and BG are shown in Table 3. In BG, interestingly, ginsenoside $Rg_3$ (7.51 mg/g) which was not detected in WG comprised 51.89% of total 11 ginsenosides (14.32 mg/g) and was 20 times higher than that (0.37 mg/g) in RG. The sum content of the other 7 ginsenosides ($Rg_1$, Re, Rf, $Rb_1$, Rc, $Rb_2$, and Rd) was 6.26 mg/g, showing 43.72% of total gensenoside. Whereas, the sum of ginsenosides content ($Rg_1$, Re, Rf, $Rb_1$, Rc, $Rb_2$, and Rd) in WG and RG was 11.89 and 18.66 mg/g, respectively. In addition, the content of ginsenosides $Rg_1$ plus $Rb_1$ which were responsible for the learning and memory function of brain was 3.99 mg/g in BG, and was lower than that in WG (5.50 mg/g) or RG (10.41 mg/g). The contents of total protopanaxdiol (PPD) and protopanaxtriol (PPT) were also investigated. Among 11 ginsenosides, $Rb_1$, Rc, Rb, Rd, 20(S)- and 20(R)- $Rg_3$ belong to the type of PPD, and $Rg_1$, $Rg_2$, Re, Rf, and $Rh_1$ belong to the type of PPT. In BG, the content of PPD was higher than that (7.17±0.10 mg/g) in WG, but lower than that (13.12±0.13 mg/) in RG. The

**Table 2. Recoveries for the assay of 11 ginsenosides in black ginseng**

| Analytes | Original (μg) | Spiked (μg) | Found (μg) | RSD[1] (%) | Recovery[2] (%) |
|---|---|---|---|---|---|
| $Rg_1$ | 139.0 | 243.0 | 372.7 | 1.12 | 96.2 |
| | | 121.5 | 266.0 | 1.58 | 104.5 |
| Re | 70.0 | 102.0 | 166.0 | 1.63 | 94.1 |
| | | 51.0 | 123.6 | 0.35 | 105.1 |
| Rf | 45.0 | 60.0 | 108.9 | 3.16 | 106.5 |
| | | 30.0 | 73.2 | 1.30 | 94.0 |
| $Rh_1$ | 18.0 | 48.0 | 63.1 | 1.21 | 94.0 |
| | | 24.0 | 42.8 | 0.86 | 103.3 |
| $Rb_1$ | 251.0 | 310.0 | 547.2 | 1.36 | 95.5 |
| | | 155.0 | 412.5 | 1.02 | 104.2 |
| $Rg_2$ | 28.0 | 65.0 | 95.3 | 1.26 | 103.5 |
| | | 32.5 | 58.6 | 1.04 | 94.2 |
| Rc | 53.0 | 94.0 | 142.0 | 1.55 | 94.7 |
| | | 47.0 | 95.1 | 1.86 | 96.0 |
| $Rb_2$ | 39.0 | 78.0 | 122.2 | 1.73 | 106.6 |
| | | 39.0 | 75.3 | 1.00 | 93.1 |
| Rd | 20.0 | 59.0 | 81.7 | 0.58 | 104.6 |
| | | 29.5 | 47.2 | 0.55 | 92.2 |
| 20(S)-$Rg_3$ | 226.0 | 630.0 | 837.7 | 0.38 | 97.1 |
| | | 315.0 | 550.8 | 0.77 | 103.1 |
| 20(R)-$Rg_3$ | 499.0 | 910.0 | 1,378.1 | 0.31 | 96.6 |
| | | 455.0 | 964.3 | 0.82 | 102.3 |

[1]RSD (%)=(SD/mean)×100
[2]Recovery (%)=[(amount found−original amount)/amount spiked]×100.

**Table 3. Comparison of ginsenosides content in Korean white, red, and black ginsengs[1]**

| | White ginseng | Red ginseng | Black ginseng |
|---|---|---|---|
| G-$Rg_1$ | 2.21±0.04[a] | 3.23±0.05[b] | 1.45±0.07[c] |
| G-Re | 1.26±0.04[a] | 1.58±0.06[b] | 0.62±0.03[c] |
| G-Rf | 1.24±0.05[a] | 1.11±0.08[b] | 0.41±0.02[c] |
| G-$Rh_1$ | ND[a] | ND[a] | 0.22±0.02[b] |
| G-$Rb_1$ | 3.29±0.05[a] | 7.18±0.04[b] | 2.54±0.09[c] |
| G-$Rg_2$ | ND[a] | ND[a] | 0.33±0.02[b] |
| G-Rc | 1.70±0.07[a] | 2.65±0.10[b] | 0.56±0.02[c] |
| G-$Rb_2$ | 1.37±0.05[a] | 2.00±0.06[b] | 0.43±0.03[c] |
| G-Rd | 0.81±0.04[a] | 0.97±0.06[b] | 0.23±0.01[c] |
| 20(S)-$Rg_3$ | ND[a] | 0.25±0.03[b] | 2.32±0.07[c] |
| 20(R)-$Rg_3$ | ND[a] | 0.12±0.03[b] | 5.19±0.15[c] |
| PPD[2] | 7.17±0.10[a] | 13.12±0.13[b] | 10.53±0.06[c] |
| PPT[3] | 4.71±0.07[a] | 5.91±0.08[b] | 3.02±0.72[c] |
| Total | 11.89±0.04[a] | 19.03±0.05[b] | 14.32±0.02[c] |

[1]Values were expressed as the means±SD ($n$=3); values within the same raw not sharing a superscript letter are significantly different ($p$<0.05); ND, not detected.
[2]Protopanaxdiol type ginsenosides: $Rb_1$+Rc+$Rb_2$+Rd+$Rg_3$.
[3]Protopanaxtriol type ginsenosides: $Rg_1$+$Rg_2$+Re+Rf+$Rh_1$.

content of PPT (3.02±0.72 mg/g) in BG was lower than that in WG or RG. The ratio of PPD and PPT in BG, approximately 3.5 : 1, was higher than that in white (1.7:1) or red ginsengs (2.2:1). Previous pharmacological study has shown that $Rg_3$ possessed neuroprotective activities since ginsenoside $Rg_3$ could significantly reduced lipid peroxides and scavenged free radicals in cerebral ischemia-induced rat (5). Based on these results, BG might have strong medical value, especially for neuroprotective ability due to a high content of ginsenoside $Rg_3$.

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