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Simultaneous quantification of 19 ginsenosides in black ginseng developed from *Panax ginseng* by HPLC–ELSD

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ABSTRACT

A high-performance liquid chromatographic method with evaporative light scattering detection (HPLC–ELSD) has been developed to identify and quantify 19 ginsenosides (Rg_1, Re, Rf, Rb_1, Rc, Rb_2, Rd, F_4, Rg_6, Rk_3, Rh_4, 20(*S*)-, 20(*R*)-Rg_3, 20(*S*)-, 20(*R*)-Rs_3, Rk_1, Rg_5, Rs_4, and Rs_5) in black ginseng (BG, Korean white ginseng that was subjected to nine cycles of steam treatment). Ultrasonication is employed for sample preparation, and the analysis is achieved on a Discovery C_{18} column using gradient elution of CH_3CN–H_2O–CH_3COOH without buffer in 40 min. The method was validated by linearity ($r^2 \geq 0.9994$), precision (92.0–107.5%), intra- and inter-day accuracy (R.S.D. < 3.21%), and limit of detection (LOD ≤ 93 ng). The quantification method was applied to analyze the composition of ginsenosides in Korean white, red, and black ginsengs. During the preparatory process of BG, ginsenosides transform into constituents of low polarity by hydrolysis, isomerization, and dehydration at C-20, and hydrolysis also occurs at C-3 or C-6. The validated HPLC method is expected to provide the basis for the quality assessment of ginseng products.

1. Introduction

Ginseng (*Panax ginseng* C.A. Meyer, Araliaceae) has been traditionally used as an expensive and precious medicine in Asian countries for more than 2000 years. Ginsenosides, the ginseng saponins, are the main effective components responsible for their antidiabetic, anti-inflammatory, and antitumor activities [1–9]. To date, more than 30 different ginsenosides have been isolated and characterized, and shown to have different pharmacological effects. Based on their aglycone moieties, ginsenosides can be classified into two categories: 20(*S*)-protopanaxadiol (ginsenosides Rb_1, Rb_2, Rc, Rd, Rg_3, and Rs_3) group and 20(*S*)-protopanaxatriol (ginsenosides Re, Rg_1, and Rf) group (Fig. 1).

Ginseng is one of the most popular herbal medicines used as a dietary supplement in recent years. There are a variety of commercial ginseng products, including white ginseng (WG), red ginseng (RG), and black ginseng (BG). WG is produced from fresh ginseng by dehydration by sunlight, while RG is manufactured by steaming fresh ginseng at 95–100 °C for 2–3 h [10]. BG is developed from WG by nine cycles of steaming at 98 °C for 3 h [11,12]. BG contains some new ginsenosides (Rg_3, Rg_5, F_4, Rg_6, Rk_3, Rs_3, Rs_4, etc.) which are not present in WG, and exhibits more potent biological activities than WG and RG [12,13]. Recently, there have been several reports showing that these unique compounds have potent biological activities, such as anticancer, neuroprotective, and anti-inflammatory activities [14–20]. Therefore, the analysis of ginsenosides in BG is of great significance to promote the understanding regarding the components that are responsible for BG's special pharmacological effects.

High-performance liquid chromatography (HPLC) has been extensively used for the analysis of ginsenosides in the last two decades. However, the saponins in *P. ginseng* products show poor UV absorptivity and low-wavelength range (198–205 nm) is required for the detection, which greatly increases the baseline noise and lowers the sensitivity of the detection [16,21,22]. In comparison with UV detection, evaporative light scattering detection (ELSD) is a universal, nonspecific method, in which signal intensity is related to the concentration of the solute in the effluent but not its optical characteristics. Therefore, ELSD can provide a stable baseline even with steep gradients [23], which has been successfully applied for quantitative determination of ginsenosides in various ginseng products [24–26]. However, current HPLC–ELSD methods for ginsenosides analysis in various ginseng products suffer from long analysis time of more than 50 min [25–28]. In addition, the number of determined ginsenosides was still insufficient to identify all ginsenosides in the steamed ginseng products. Although Fuzzati et al. [26] reported a HPLC–ELSD method detecting as many as 25 ginsenosides in Asian ginseng (*P. ginseng*) extracts within 60 min,

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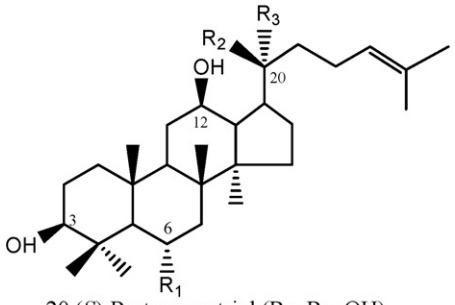
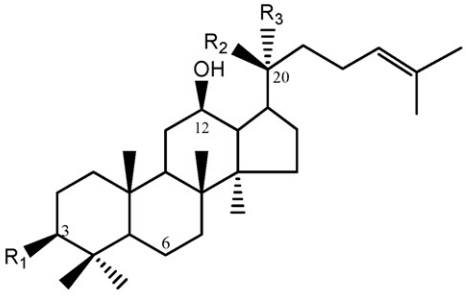


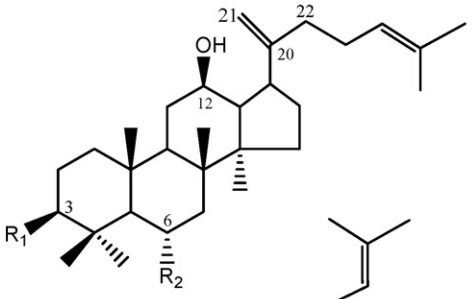
20 (*S*)-Protopanxatriol (R_1=R_2=OH)

Ginsenoside	R_1	R_2	R_3
Rg_1	-Oglc	-Oglc	$-CH_3$
Re	-Oglc(2-1)rha	-Oglc	$-CH_3$
Rf	-Oglc(2-1)glc	-OH	$-CH_3$

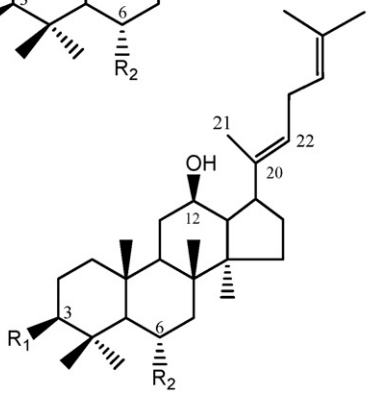


20 (*S*)-Protopanxadiol (R_1=R_2=OH)

Ginsenoside	R_1	R_2	R_3
Rb_1	-Oglc(2-1)glc	-Oglc(6-1)glc	$-CH_3$
Rc	-Oglc(2-1)glc	-Oglc(6-1)araf	$-CH_3$
Rb_2	-Oglc(2-1)glc	-Oglc(6-1)arap	$-CH_3$
Rd	-Oglc(2-1)glc	-Oglc	$-CH_3$
20(*S*)-Rg_3	-Oglc(2-1)glc	-OH	$-CH_3$
20(*R*)-Rg_3	-Oglc(2-1)glc	$-CH_3$	-OH
20(*S*)-Rs_3	-Oglc(2-1)glc-Ac	-OH	$-CH_3$
20(*R*)-Rs_3	-Oglc(2-1)glc-Ac	$-CH_3$	-OH



Ginsenoside	R_1	R_2
Rk_1	-Oglc(2-1)glc	-OH
Rg_6	-OH	-Oglc(2-1)rha
Rk_3	-OH	-Oglc
Rs_5	-Oglc(2-1)glc-Ac	-OH



Ginsenoside	R_1	R_2
Rg_5	-Oglc(2-1)glc	-OH
F_4	-OH	-Oglc(2-1)rha
Rh_4	-OH	-Oglc
Rs_4	-Oglc(2-1)glc-Ac	-OH

Fig. 1. Structure of the major ginsenosides in black ginseng developed from 4-year *Panax ginseng*. Glc, β-D-glucose; Rha, α-L-rhamnose; Arap, α-L-arabinose (pyranose); Araf, α-L-arabinose (furanose); Ac, 6′-O-acetyl.

this method was not appropriate for the steamed ginseng products since only polar ginsenosides were considered for analysis and the thermally unstable malonyl-ginsenosides definitely did not exist in the steamed ginseng products. What is more, most HPLC–ELSD methods focused on the quantitative analysis of polar ginsenosides (Rg_1, Re, Rf, Rb_1, Rc, Rb_2, Rb_3, Rd, etc.) only and paid less attention to the simultaneous quantification of multiple low polarity ginsenosides such as ginsenosides Rg_6, Rk_3, Rh_4, Rg_3, and Rs_4, the unique compounds in the heat-activated ginseng products [25–29]. Up to now, most reports concerning quality control of commercial ginseng products are limited on WG and RG, few papers on BG products have been published. It has become an important task to establish an authoritative quality control standard for evaluating the BG products.

In this study, a HPLC–ELSD method was developed for the simultaneous determination of 19 individual saponins including 12 less polar ginsenosides F_4, Rg_6, Rk_3, Rh_4, 20(*S*)-, 20(*R*)-Rg_3, 20(*S*)-, 20(*R*)-Rs_3, Rk_1, Rg_5, Rs_4, and Rs_5, which are the unique compounds in the steamed ginseng. This newly developed quantitative method could be applied for the quality control of several types of commercial ginseng products, such as WG, RG, and BG.

2. Experimental

2.1. Materials and equipment

Nineteen ginsenosides Rg_1, Re, Rf, Rb_1, Rc, Rb_2, Rd, F_4, Rg_6, Rk_3, Rh_4, 20(*S*)-, 20(*R*)-Rg_3, 20(*S*)-, 20(*R*)-Rs_3, Rk_1, Rg_5, Rs_4, and Rs_5 standards (Fig. 1) were purchased from the Hongjiu Biotech Co., Ltd. (Jilin, China). The purity of all these standards was over 98% as indicated by the manufacturer. Solid-phase extraction (SPE) of Sep-Pak C_{18} cartridges (6 cm^3, 1 g) were from Waters (Milford, MA, USA).

HPLC-grade acetonitrile was purchased from Merck Co. (Merck, Darmstadt, Germany). Deionized water was purified by Milli-Q system (Millipore, Bedford, MA, USA). Other chemicals were of reagent grade.

All 4-year Korean white and red ginsengs (*P. ginseng* C.A. Meyer) were purchased from the largest Korean ginseng market of Geumsan county (Chungcheong Province, South Korea). BG was manufactured by nine-time repeated steaming WG at 98 °C for 3 h in pottery apparatus and drying at 60 °C for 18 h. Three types of the above-mentioned ginsengs were pulverized into fine powder with a pulverizer (Hanil, Seoul, Korea).

2.2. Sample preparation

Based on the method of Shi et al. [30] with modification, 1 g dried ginseng powder was extracted three times with 50 ml of 70% ethanol aqueous solution at 75 °C by ultrasonication (60 kHz, heat power 330 W; JAC Ultrasonica 2010, KOPO, Korea) for 60 min. After filtration using filter paper (ADVANTEC, Dublin, CA, USA), the solvent was removed using an evaporator (EYELA N-N, Tokyo, Japan), and the residue was dissolved in 20 ml of distilled water. 1 ml of the aqueous sample solution was applied to a SPE Sep-Pak C_{18} cartridge previously conditioned with 5 ml of methanol and equilibrated with 10 ml water, and then eluted sequentially by 0, 20, and 90% aqueous methanol (MeOH) (10 ml). The 90% MeOH fraction was dried under a stream of nitrogen at 50 °C. The residue was dissolved in 1 ml of solvent (MeOH:H_2O = 1:1, v/v), and then filtered through a 0.45 μm polytetrafluoroethylene (PTFE) syringe filter (Waters, Milford, MA, USA) and it was ready for HPLC analysis.

2.3. Chromatographic conditions

HPLC analysis was carried out on an Agilent 1100 series HPLC system (Palo Alto, CA, USA), equipped with vacuum degasser, quaternary gradient pump, and autosampler, evaporative light scattering detector (Shimazu-model ELSD-LT). A Discovery C_{18} column (250 mm × 4.6 mm, i.d., 5 μm; Sigma–Aldrich, St. Louis, MO, USA) was used for all separations at a column temperature of 40 °C. The binary gradient elution system consisted of acetonitrile:water:5% acetic acid aqueous solution (10:85:5, v/v/v) (A) and acetonitrile:water (80:20, v/v) (B). The separation was achieved using the following gradient program: 0 min (0% B), 0–10 min (30% B), 10–25 min (50% B), 25–40 min (100% B), 40–50 min (100% B), 50–53 min (0% B), and 53–60 min (0% B). The flow-rate was set at 1.5 ml/min and the sample injection volume was 10 μl. ELSD was set to a probe temperature of 60 °C, and the nebulizer for nitrogen gas was adjusted to 1.8 l/min. A Bruker Esquire LC (Billerica, MA, USA) ion-trap mass spectrometer with electrospray ionization (ESI) was used in HPLC–MS method. ESI-MS conditions of HPLC–MS analysis were as follows: negative ion mode, drying gas N_2, 8 l/min, temperature 320 °C, pressure of nebulizer 12 psi, octapole voltage 2.35 V, ion-trap voltage 32.2 V, scan range 400–1400 u. ESI-MS–MS conditions were as follows: negative ion mode, separation width 0.9, fragment amplification 1.5, scan range 200–1300 u.

2.4. Validation of the HPLC method

2.4.1. Calibration curves, limits of detection

Due to the distinct variation in contents of saponins in the BG, the two methanol stock solutions of standards, one containing ginsenosides Rg_1, Re, Rb_1, Rd, Rg_6, Rh_4, 20(*S*)-ginsenoside Rg_3; 20(*S*)-Rs_3, Rk_1, and Rs_5; the other containing ginsenosides Rf, Rc, Rb_2, F_4, Rk_3, 20(*R*)-Rg_3, 20(*R*)-Rs_3, Rg_5, and Rs_5, were prepared and diluted with 20% (v/v) methanol aqueous solution to appropriate concentration for the establishment of calibration curves. Six concentrations of the 19 saponins solution were injected in triplicate, and then the calibration curves were constructed by plotting the peak areas against the concentration of each analyte. Each aliquot (10 μl) from WG, RG, and BG extracts were injected into HPLC, and the content of each ginsenoside was calculated using the calibration curves.

The limits of detection (LOD) and limit of quantification (LOQ) under present chromatographic conditions were determined on the basis of response at a signal-to-noise ratio (S/N) of 3 or 10, respectively.

2.4.2. Precision and accuracy

Intra- and inter-day variations were chosen to determine the precision of the HPLC method. 1 g of black ginseng powder was extracted and analyzed as described in Sections 2.2 and 2.3. The intra-day precision was performed by triplicate extraction and analysis on a single day. The inter-day precision was carried out on 5 different days. Variations were expressed by the relative standard deviations (R.S.D.). The recovery test was used to evaluate the accuracy of this quantification method. Accurate amounts of 19 ginsenosides were added to approximately 1 g of black ginseng powder and then extracted and analyzed as described in Sections 2.2 and 2.3. The average recoveries were calculated by the following formula: recovery (%) = 100 × (amount found − original amount)/amount spiked, with R.S.D. (%) = (S.D./mean) × 100%.

2.5. Analysis of finished ginseng products

1 g of each prepared white, red, and black ginseng powders was extracted and analyzed as described in Sections 2.2 and 2.3. Red ginseng powder was prepared from the red ginsengs produced by two different companies, (A) and (B). Black ginseng powder was prepared from three different batches of black ginsengs manufactured by nine-time repeated steaming WG at 98 °C for 3 h and drying at 60 °C for 18 h. The amounts of the 19 ginsenosides in each ginseng sample were determined simultaneously by HPLC.

3. Results and discussion

3.1. Optimization of extraction conditions

In order to obtain quantitative extraction of the investigated saponins, variables involved in the procedure such as solvent and extraction time were optimized. Ultrasonic extraction was compared with refluxing. The results showed that ultrasonic extraction was simpler and more effective for extraction of saponins. Hence ultrasonication was chosen as the preferred method. An orthogonal experiment was employed to optimize the ultrasonication extraction conditions. It involved the following experimental factors and corresponding levels: solvent volume (20, 50, 80 ml), ethanol concentration (0, 70, 100%, v/v), extraction repetitions (2, 3 or 4 times) and extraction time (30, 60 or 90 min). The optimal condition for extraction of RG and BG powders could be obtained by intuitionistic analysis of the experimental results of the orthogonal design $L_9(3^4)$. So the relative sum area of the identified characteristic peaks was used as a criterion for the selection of the optimal sonication conditions. According to statistic analysis theory, ethanol concentration was the most important factor in the extract conditions of ginseng powder, and 70% was the best concentration for extraction of the investigated saponins. Extraction repetition was the minimal influencing factor. The results suggested that three times extraction of the saponins was complete. The optimal condition for the extraction of ginseng powders was selected and presented in details in Section 2.2.

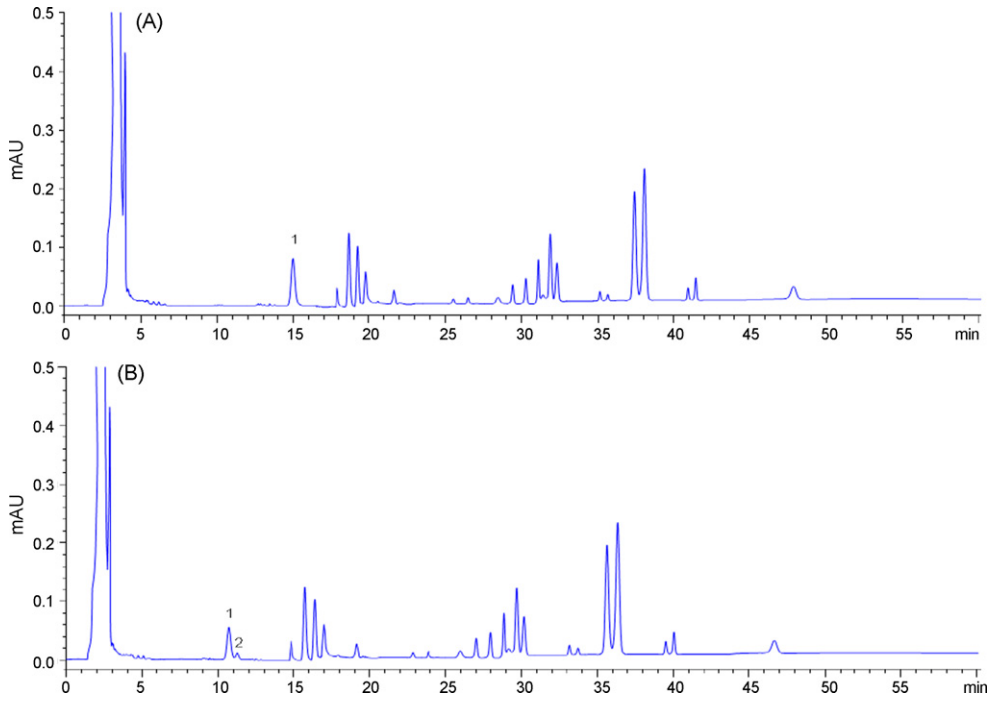


Fig. 2. HPLC profiles of black ginseng at the flow-rate of (A) 1.0 ml/min and (B) 1.5 ml/min. HPLC was performed on a Discovery C_{18} column (250 mm × 4.6 mm, i.d., 5 μm) at column temperature of 40 °C. The mobile phase consisted of acetonitrile:water:5% acetic acid aqueous solution (10:85:5, v/v/v) (A) and acetonitrile:water (80:20, v/v) (B) using the following gradient program: 0 min (0% B), 0–10 min (30% B), 10–25 min (50% B), 25–40 min (100% B), 40–50 min (100% B), 50–53 min (0% B), and 53–60 min (0% B). The sample injection volume was 10 μl. ELSD was set to a probe temperature of 60 °C, and the nebulizer for nitrogen gas was adjusted to 1.8 l/min. (1) Ginsenoside Rg_1 and (2) ginsenoside Re.

3.2. Optimization of HPLC–ELSD conditions

Previous studies reported a LC determination of ginsenosides in the processed ginseng using an amino column [14,29]. However, we found that epimers and geometric isomers did not separate on this. Ginsenosides 20(*S*)-Rg_3 did not separate from its 20(*R*) epimer, and 20(*S*)-Rs_3 from 20(*R*)-Rs_3; geometric isomers of ginsenoside Rk_1 and Rg_5, Rg_6 and F_4, Rs_5 and Rs_4, and Rk_3 and Rh_4 were not separated either. Our efforts to separate these isomers by modifying the solvent system, column temperature and flow-rate were unsuccessful. As an alternative, the use of a reversed-phase separation was attempted.

Subsequently, several binary and ternary combinations of methanol, acetonitrile, and water with ammonium acetate buffer or acetic acid were evaluated for use as the mobile phase in order to improve the resolution and sensitivity on a C_{18}-bonded silica column. After trying several types of solvent systems, gradients and varying duration, an optimum solvent system (CH_3CN:H_2O:CH_3COOH) in gradient mode was found optimal as described in Section 2.3. In addition, the important parameters that affect the sensitivity of ELSD, including the flow-rate of nebulizer gas (pressure) and drift tube temperature, were evaluated at different drift tube temperatures from 30 to 100 °C and the flow-rate from 1.2 to 2.5 l/min by the injection of ginsenoside Rg_1. Finally, the drift

Table 1
Calibration curves and LODs for 19 ginsenosides.

Ginsenoside	RT (min)	Calibration curve[a]	Correlation coefficient (r^2)	Test range (mg/ml)	LOD[b] (ng)	LOQ[c] (ng)
Rg_1	10.78	$y = 6129.5x - 170.95$	0.9995	0.050–1.000	88	166
Re	11.24	$y = 4012.8x - 810.66$	0.9996	0.050–1.000	65	110
Rf	14.91	$y = 4812.4x - 51.43$	0.9995	0.050–1.000	32	45
Rb_1	15.87	$y = 8028.9x - 820.69$	0.9999	0.050–1.000	93	186
Rc	16.47	$y = 3573.0x - 45.45$	0.9997	0.050–1.000	20	160
Rb_2	17.14	$y = 4441.2x - 84.12$	0.9998	0.050–1.000	27	81
Rd	19.24	$y = 6814.4x - 215.89$	0.9998	0.050–1.000	82	136
Rg_6	26.02	$y = 5347.1x - 726.34$	0.9994	0.010–0.080	28	52
F_4	26.96	$y = 4169.5x - 342.12$	0.9995	0.010–0.080	36	102
Rk_3	27.93	$y = 5218.5x - 145.23$	0.9997	0.010–0.080	29	87
Rh_4	28.94	$y = 4786.9x - 85.39$	0.9995	0.010–0.080	32	120
20(*S*)-Rg_3	29.86	$y = 3494.4x - 11.72$	0.9996	0.050–0.500	26	62
20(*R*)-Rg_3	30.24	$y = 3598.7x - 19.35$	0.9997	0.050–0.500	28	84
20(*S*)-Rs_3	33.24	$y = 3625.7x - 428.42$	0.9994	0.010–0.080	21	72
20(*R*)-Rs_3	33.87	$y = 3987.5x - 645.25$	0.9996	0.010–0.080	27	81
Rk_1	35.54	$y = 5176.4x - 315.62$	0.9994	0.010–0.080	42	117
Rg_5	36.32	$y = 4732.8x - 204.43$	0.9995	0.010–0.080	37	124
Rs_5	39.57	$y = 4367.4x - 216.54$	0.9998	0.010–0.080	21	37
Rs_4	40.12	$y = 4048.7x - 115.34$	0.9994	0.010–0.080	18	72

[a] y = peak area and x = concentration (mg/ml).
[b] Limit of detection (S/N = 3).
[c] Limit of quantification (S/N = 10).

tube under temperature of 60 °C and the flow-rate of 1.8 l/min were selected for detecting the analytes by comparing peak area values. These optimized parameters allow a complete solvent evaporation and produce negligible baseline noise.

Owing to the high polarity of ginsenosides, much water was used in the mobile phase for elution. On the other hand, the flow-rate of HPLC was set at 1.5 ml/min, with which was necessary for separating ginsenosides Rg_1 and Re satisfactorily (Fig. 2). Compared to the HPLC method described by the others [25–28], only 40 min was necessary for the simultaneous quantification of 19 individual saponins and a solvent system (CH_3CN:H_2O:CH_3COOH) was successful in separating 20(*S*) and 20(*R*) epimers of ginsenosides Rg_3 and Rs_3 on a C_{18}-bonded silica column and geometric isomers at C-20 position, i.e. ginsenosides Rg_6 and F_4, Rk_3 and Rh_4, Rs_5 and Rs_4 were also clearly separated through this system (Fig. 3D). Peaks were identified by careful studies of their retention times, their MS and MS–MS spectra, and by comparison with literature data [26,31–33].

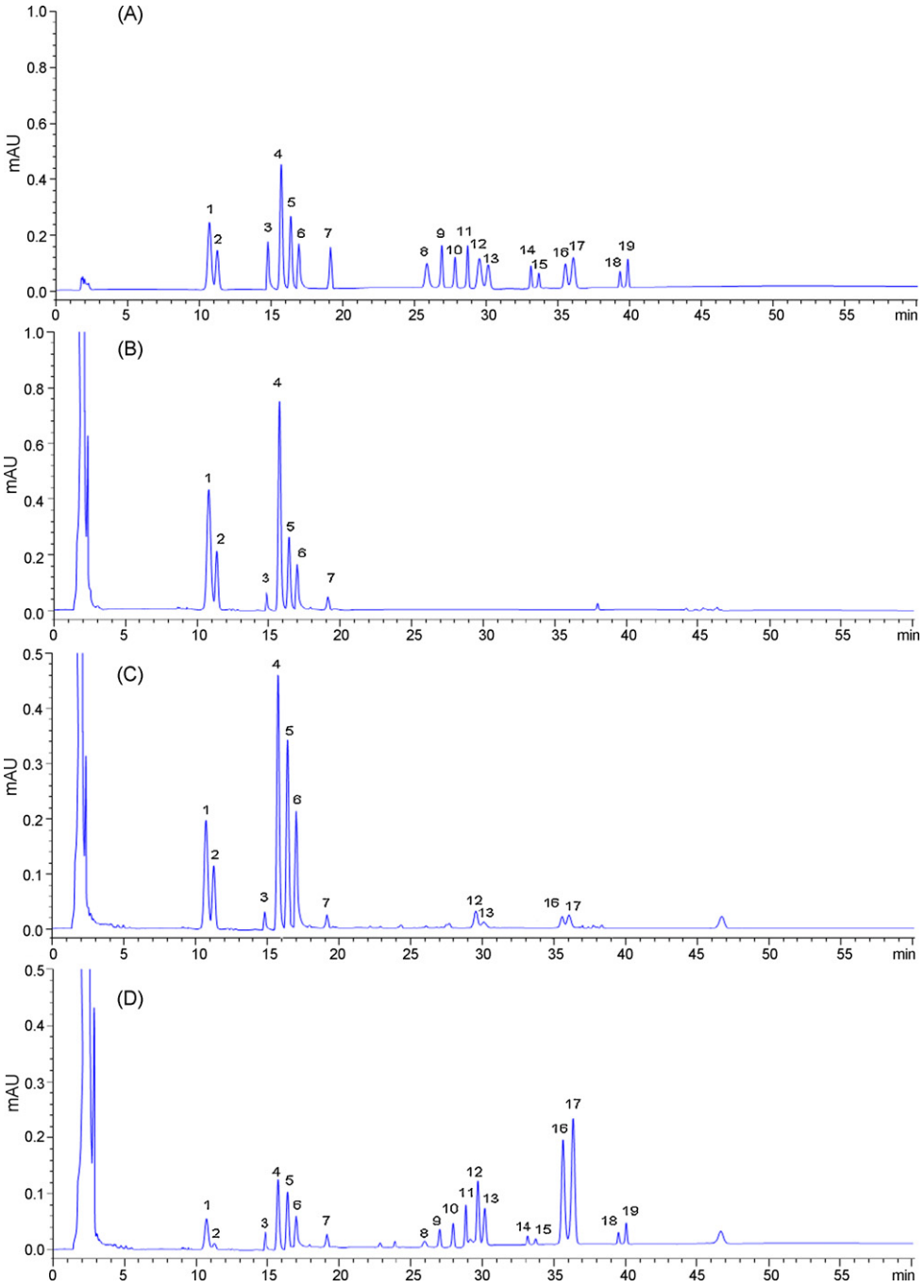


Fig. 3. HPLC–ELSD chromatograms of mixed standards (A) and ginsenosides in Korean white ginseng (B), red ginseng (C), and black ginseng (D). HPLC was performed on a Discovery C_{18} column (250 mm × 4.6 mm, i.d., 5 μm) at column temperature of 40 °C. The mobile phase consisted of acetonitrile:water:5% acetic acid aqueous solution (10:85:5, v/v/v) (A) and acetonitrile:water (80:20, v/v) (B) using the following gradient program: 0 min (0% B), 0–10 min (30% B), 10–25 min (50% B), 25–40 min (100% B), 40–50 min (100% B), 50–53 min (0% B), and 53–60 min (0% B). The flow-rate was at 1.5 ml/min and the sample injection volume was 10 μl. ELSD was set to a probe temperature of 60 °C, and the nebulizer for nitrogen gas was adjusted to 1.8 l/min. Peaks: 1, Rg_1; 2, Re; 3, Rf; 4, Rb_1; 5, Rc; 6, Rb_2; 7, Rd; 8, Rg_6; 9, F_4; 10, Rk_3; 11, Rh_4; 12 and 13, 20(*S*)- and 20(*R*)-Rg_3; 14 and 15, 20(*S*)- and 20(*R*)-Rs_3; 16, Rk_1; 17, Rg_5; 18, Rs_5; 19, Rs_4.

Table 2
Intra- and inter-day variations of HPLC–ELSD method for determination of 19 ginsenosides.

Analytes	Intra-day ($n=3$)		Inter-day ($n=5$)	
	Content (mg/g)	R.S.D.[a] (%)	Content (mg/g)	R.S.D. (%)
Rg_1	1.68 ± 0.08	2.62	1.57 ± 0.05	2.32
Re	0.66 ± 0.02	1.14	0.62 ± 0.03	1.86
Rf	0.44 ± 0.03	1.45	0.48 ± 0.04	1.58
Rb_1	2.66 ± 0.07	2.14	2.43 ± 0.05	1.84
Rc	1.54 ± 0.06	1.86	1.58 ± 0.04	1.45
Rb_2	0.52 ± 0.04	1.32	0.49 ± 0.05	1.74
Rd	0.48 ± 0.02	0.72	0.47 ± 0.06	1.69
Rg_6	0.19 ± 0.02	0.52	0.23 ± 0.04	1.38
F_4	0.46 ± 0.04	1.23	0.50 ± 0.04	1.27
Rk_3	0.66 ± 0.03	1.36	0.62 ± 0.05	1.62
Rh_4	0.82 ± 0.05	1.42	0.78 ± 0.03	1.02
20(*S*)-Rg_3	2.84 ± 0.18	2.44	2.80 ± 0.12	1.73
20(*R*)-Rg_3	2.20 ± 0.09	1.87	2.12 ± 0.05	1.21
20(*S*)-Rs_3	0.16 ± 0.05	1.15	0.14 ± 0.06	1.46
20(*R*)-Rs_3	0.14 ± 0.02	0.87	0.16 ± 0.05	2.13
Rk_1	2.38 ± 0.23	2.37	2.24 ± 0.13	1.78
Rg_5	3.08 ± 0.14	2.76	3.01 ± 0.24	3.21
Rs_5	0.24 ± 0.02	1.67	0.28 ± 0.04	2.25
Rs_4	0.50 ± 0.04	1.29	0.47 ± 0.06	1.87

[a] R.S.D. (%) = 100 × S.D./mean.

3.3. Method validation

The linearity, regression, and linear ranges of 19 ginsenosides were performed using the developed HPLC–ELSD method. The correlation coefficient ($r^2 \geq 0.9994$) values indicated the appropriate correlations between concentrations of investigated compounds and their peak areas within the test ranges. The LODs were less than 93 ng and the LOQs ranged from 37 to 186 ng on the Discovery C_{18} column (Table 1). As shown in Table 2, the HPLC–ELSD method showed good reproducibility for the quantification of the 19 ginsenosides, with intra- and inter-day variations of the 19 ginsenosides less than 2.76 and 3.21%, respectively. The developed method also had good accuracy with the overall recovery of 92.0–107.5%, with the R.S.D. ranging from 0.72 to 3.16% (Table 3). These results indicated that the HPLC–ELSD method is precise, accurate, and sensitive for quantitative determination of 19 ginsenosides in ginseng samples.

For the stability test, 19 ginsenoside standards were dissolved in 20% methanol aqueous solution, and black ginseng powder was extracted with 70% (v/v) ethanol aqueous. The stability of the ginsenosides in these solutions at room temperature was evaluated. The analyses were performed by injecting the stability solutions every 12 h within 5 days, and the analytes were found to be rather stable within 5 days (R.S.D. < 3.32%).

3.4. Analysis of ginsenosides in WG, RG, and BG

Three types of ginseng samples (WG, RG, and BG) were analyzed with the newly developed quantification method. The HPLC chromatograms of the various ginseng extracts are shown in Fig. 3, and the contents of 19 ginsenosides in the ginseng products are presented in Table 4.

Fig. 3B and C shows the HPLC chromatograms for WG and RG, respectively. Ginsenosides Rg_1, Re, Rf, Rb_1, Rc, Rb_2, and Rd were found in both WG and RG, but only the RG contained ginsenosides Rk_1, Rg_5, 20(*S*)-, 20(*R*)-Rg_3. These results are consistent with previous reports of the generation of new types of ginsenosides under conditions of high temperature and pressure, including Rk_1, Rg_5, Rg_3. Because antitumor activity of ginsenoside Rg_3 has been reported, many studies have been performed recently using RG [6,34]. RG was found to contain more ginsenosides than white ginseng (Table 4).

Table 3
Accuracy of HPLC–ELSD method for the determination of 19 ginsenosides.

Analytes	Original (mg)	Spiked (mg)	Found (mg)	Recovery[a] (%)	R.S.D. (%)
Rg_1	1.66	3.00	4.54	96.0	2.18
		1.50	3.20	102.7	1.67
Re	0.69	1.00	1.66	97.0	1.23
		0.50	1.20	102.0	0.75
Rf	0.47	1.40	1.90	102.1	3.16
		0.70	1.19	102.9	1.37
Rb_1	2.64	5.00	7.52	97.6	2.36
		2.50	5.22	103.2	1.43
Rc	1.57	2.00	3.64	103.5	1.75
		1.00	2.59	102.0	2.16
Rb_2	0.52	1.20	1.74	101.7	2.73
		0.60	1.15	105.0	1.25
Rd	0.49	1.40	1.93	102.9	0.88
		0.70	1.17	97.1	0.75
Rg_6	0.18	0.60	0.79	101.7	1.24
		0.30	0.46	93.3	1.69
F_4	0.47	0.80	1.29	107.5	2.21
		0.40	0.90	92.0	0.86
Rk_3	0.62	1.20	1.84	101.7	2.64
		0.60	1.20	96.7	0.73
Rh_4	0.83	1.60	2.41	98.8	1.72
		0.80	1.65	102.5	1.24
20(*S*)-Rg_3	2.84	5.00	7.90	101.2	2.48
		2.50	5.32	99.2	0.79
20(*R*)-Rg_3	2.15	3.00	5.17	100.7	0.61
		1.50	3.69	102.7	0.84
20(*S*)-Rs_3	0.20	0.70	0.93	104.3	2.21
		0.35	0.53	94.3	1.36
20(*R*)-Rs_3	0.16	0.70	0.84	97.1	2.15
		0.35	0.49	94.3	1.20
Rk_1	2.32	3.00	5.42	103.3	1.74
		1.50	3.78	97.3	0.73
Rg_5	3.14	5.00	8.20	101.2	1.96
		2.50	5.60	98.4	2.40
Rs_5	0.23	0.50	0.75	104.0	1.38
		0.25	0.47	96.0	0.87
Rs_4	0.47	1.50	2.00	102.0	1.49
		0.75	1.20	97.3	0.72

[a] Recovery (%) = 100 × (amount found − original amount)/amount spiked; R.S.D. (%) = 100 × S.D./mean.

Fig. 3C and D are the chromatograms from RG and BG. Ginsenosides Rg_6, F_4, Rk_3, Rh_4, 20(*S*)-, 20(*R*)-Rs_3, Rs_5, and Rs_4 were observed in BG but were not detected in RG. In addition, the BG contained a substantially higher amount of ginsenosides Rg_3, Rk_1, and Rg_5 than RG. Ginsenosides Rh_4 and Rk_3 can be formed by deglucosylation of Rg_6 and F_4 at C-6, while ginsenosides Rg_3 can be formed by eliminating the glycosyl residue at C-20 of many protopanaxdiol ginsenosides (Rb_1, Rc, Rd, etc.) [35]. Ginsenosides Rk_1 and Rg_5, which have side chains that are quite different from ginsenosides Rb_1, Rc, and Rb_2, have reduced polar properties (Fig. 1), but the glycosylation patterns are similar to Rg_3. These observations indicated that Rk_1 and Rg_5 were formed from Rg_3 under high temperature and pressure, but the pathway of formation has not been defined. Previous pharmacological study has shown ginsenoside 20(*S*)-Rg_3 could provide good neuroprotection against cerebral ischemia-induced injury [36]. Thus, BG might have strong medical value, especially for neuroprotective ability due to high contents of ginsenosides 20(*S*)-Rg_3.

Table 4
Comparison of ginsenosides content (mg/g) in Korea white, red, and black ginsengs[a].

Ginsenoside	White ginseng	Red ginseng		Black ginseng		
		A[b]	B[c]	1[d]	2[e]	3[f]
Rg_1	2.20 ± 0.08	3.28 ± 0.07	2.78 ± 0.05	1.64 ± 0.03	1.58 ± 0.02	1.61 ± 0.04
Re	1.34 ± 0.05	1.56 ± 0.03	2.03 ± 0.04	0.68 ± 0.03	0.64 ± 0.02	0.65 ± 0.01
Rf	1.19 ± 0.04	1.09 ± 0.02	1.17 ± 0.01	0.48 ± 0.02	0.52 ± 0.01	0.53 ± 0.02
Rb_1	3.23 ± 0.02	7.08 ± 0.12	7.26 ± 0.14	2.62 ± 0.03	2.59 ± 0.01	2.63 ± 0.03
Rc	1.72 ± 0.06	2.67 ± 0.09	3.14 ± 0.07	1.58 ± 0.04	1.60 ± 0.03	1.62 ± 0.02
Rb_2	1.32 ± 0.05	2.12 ± 0.07	2.83 ± 0.06	0.56 ± 0.02	0.64 ± 0.01	0.63 ± 0.03
Rd	0.78 ± 0.02	0.92 ± 0.04	0.98 ± 0.03	0.47 ± 0.01	0.44 ± 0.02	0.46 ± 0.01
Rg_6	ND[g]	ND	ND	0.17 ± 0.02	0.15 ± 0.03	0.19 ± 0.02
F_4	ND	ND	ND	0.49 ± 0.02	0.52 ± 0.04	0.47 ± 0.02
Rk_3	ND	ND	ND	0.64 ± 0.03	0.63 ± 0.04	0.65 ± 0.02
Rh_4	ND	ND	ND	0.86 ± 0.04	0.82 ± 0.06	0.87 ± 0.03
20(*S*)-Rg_3	ND	0.22 ± 0.02	0.32 ± 0.01	2.87 ± 0.12	2.98 ± 0.09	2.85 ± 0.08
20(*R*)-Rg_3	ND	0.15 ± 0.03	0.27 ± 0.02	2.17 ± 0.08	2.37 ± 0.06	2.24 ± 0.09
20(*S*)-Rs_3	ND	ND	ND	0.18 ± 0.02	0.20 ± 0.01	0.22 ± 0.03
20(*R*)-Rs_3	ND	ND	ND	0.12 ± 0.01	0.14 ± 0.03	0.15 ± 0.04
Rk_1	ND	0.17 ± 0.04	0.20 ± 0.05	2.34 ± 0.21	2.38 ± 0.15	2.32 ± 0.17
Rg_5	ND	0.23 ± 0.02	0.27 ± 0.01	3.12 ± 0.17	3.24 ± 0.13	3.19 ± 0.16
Rs_5	ND	ND	ND	0.22 ± 0.03	0.25 ± 0.02	0.26 ± 0.04
Rs_4	ND	ND	ND	0.48 ± 0.02	0.52 ± 0.03	0.54 ± 0.02
PPD[h]	7.05 ± 0.06	13.16 ± 0.08	14.8 ± 0.12	10.67 ± 0.08	10.96 ± 0.06	10.80 ± 0.10
PPT[i]	4.73 ± 0.10	5.93 ± 0.06	5.98 ± 0.08	2.80 ± 0.07	2.74 ± 0.04	2.79 ± 0.06
Total	11.78 ± 0.17	19.49 ± 0.11	21.25 ± 0.13	21.79 ± 0.13	22.21 ± 0.15	22.54 ± 0.17

[a] All values were expressed as the means ± S.D. ($n = 3$).
[b] Produced by company A.
[c] Produced by company B.
[d] The first batch of the manufactured black ginseng.
[e] The second batch of the manufactured black ginseng.
[f] The third batch of the manufactured black ginseng.
[g] Not detected.
[h] Protopanaxdiol type ginsenosides: $Rb_1 + Rc + Rb_2 + Rd + Rg_3 + Rs_3$.
[i] Protopanaxtriol type ginsenosides: $Rg_1 + Re + Rf$.

Table 4 shows the amounts of 19 ginsenosides in both red ginsengs manufactured by two different companies and three different batches of black ginsengs developed from WG by nine cycles of steaming at 98 °C for 3 h. There were no differences in the number of ginsenosides between the two red ginseng products, but the total quantity of ginsenosides was somewhat different. Similar results were also found among three different batches of the manufactured black ginsengs.

From these results, it can be concluded that substantial differences exist between the different types of ginseng products such as WG, RG, and BG. These results strongly suggested that guidelines and quality control for commercial ginseng products are required.

4. Conclusions

In this study, HPLC–ELSD conditions were optimized for the quantitative and qualitative determination of 19 ginsenosides in white ginseng, red ginseng, and black ginseng (BG, white ginseng that is subjected to nine cycles of 98 °C for 3 h). The developed method can be used to analyze less polar ginsenosides Rg_6, F_4, Rk_3, Rh_4, 20(*S*)-, 20(*R*)-Rg_3, 20(*S*)-, 20(*R*)-Rs_3, Rk_1, Rg_5, Rs_5, and Rs_4, which are the unique compounds of steamed *P. ginseng*. The quantification method is rapid, accurate, and precise, and it can simultaneously determine the amounts of 19 ginsenosides in various ginseng products (WG, RG, and BG). These results are definitely helpful to control the quality of BG products and provide a scientific basis for the search for the components that are responsible for BG's pharmacological effects.

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