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Steaming-Induced Chemical Transformations and Holistic Quality Assessment of Red Ginseng Derived from *Panax ginseng* by Means of HPLC-ESI-MS/MS^n-Based Multicomponent Quantification Fingerprint

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ABSTRACT: The purpose of this study is to evaluate the steaming-induced chemical transformation of red ginseng manufactured from fresh ginseng by means of simultaneous quantitative and qualitative analyses with a combinative high-performance liquid chromatography−electrospray tandem mass spectrometry (HPLC-ESI-MS/MS^n) technique. Thirty-six ginsenosides were identified in red ginseng and white ginseng by comparing the mass spectrum and/or matching the empirical molecular formula with that of known published compounds, and 11 of them were determined to be newly generated during the red ginseng preparatory process. The mechanisms involved were further deduced to be hydrolysis, dehydration, isomerization, and decarboxylation at C-20, and hydrolysis also occurs at C-3 or C-6 of the original ginsenosides through the mimic process of steaming and heating in laboratory. The multicomponent quantification fingerprint of ginseng was also established by HPLC-UV method, and the contents of 12 ginsenosides in red and white ginsengs from different sources were determined simultaneously. The ratio of the total content of determined malonyl ginsenosides to the corresponding neutral ginsenosides ($T_{m\text{-}PPD}/T_{PPD}$) in white ginseng ranged from 0.46 to 0.62 and from 0 to 0.19 in red ginseng. The validated method is expected to provide an effective approach to standardize the processing procedures of ginseng products and regulate the usage of ginseng in Traditional Chinese Medical prescription.

KEYWORDS: *red ginseng, ginsenosides, multicomponent quantification fingerprint, HPLC-ESI-MS/MS^n, chemical transformation, steaming*

INTRODUCTION

Radix ginseng, the root of *Panax ginseng* C.A. Meyer (Araliaceae), has been traditionally used as an elixir medicine in traditional oriental medicine for several thousands of years in Asia,[1] and it is one of the most popular herbal medicines used as a dietary supplement in recent years. The main bioactive components of ginseng are triterpene saponins, termed ginsenosides, which are considered to be the main bioactive components responsible for a variety of pharmacological effects such as antidiabetic, anti-inflammatory, and antitumor activities. On the basis of their aglycone moieties, ginsenosides can be classified into two main categories: most are dammarane type triterpene saponins with 20 (*S*)-protopanaxadiol (ginsenosides Rb_1, Rb_2, Rc, Rd, Rg_3, and Rs_3) and 20 (*S*)-protopanaxatriol (ginsenosides Re, Rg_1, and Rf) as the aglycone; ginsenoside R_0 is the only oleanane type saponin identified in Radix ginseng (as shown in Figure 2).[2−5]

In Asia, two types of commercial ginseng products manufactured by two different processing methods after harvest for different purposes are available in the herbal medicine market, including garden ginseng (also named white ginseng, WG) and red ginseng (RG). WG is produced from peeled fresh ginseng by dehydration in sunlight, which is usually used to "supply *qi* and promote the production of body fluids" as well as enhance physical fitness and disease resistance; RG, which is manufactured by steaming the nonpeeled fresh ginseng at 95−100 °C for 2−3 h and then drying, is used for "boosting *yang*" and replenishing vital essence for its "warming effect".[6] Recently, increasing reports have shown various pharmacological effects of red ginseng and its constituents, such as anti-inflammatory, antioxidative, and anticancer effects.[7−9] In some cases, the bioactivity of red ginseng was compared with that of white ginseng, and it seemed the red one showed better potential than the white one.[10,11] The chemical profiles differ considerably between white and red ginseng and have been comprehensively reviewed.[11−16] During steaming or heating, the polar ginseng saponins decreased, and less polar ginseng saponins (Rg_3, Rg_5, Rg_6, Rh_1, Rh_2, Rk_1, and Rs_3, etc.) increased.[17,18] It has been reported that the polar ginsenosides exhibited poor absolute bioavailability following oral administration in vivo, which may be metabolized via a deglucosylation pathway in the stomach and/or liver to generate the less polar ginsenosides, such as ginsenoside Rg_3 and a ginsenoside named compound K.[19,20] These less polar ginsenosides exhibited much more potent anticancer, antidiabetic, and anti-inflammatory bioactivities.[21−24] Because of its special bioactivities compared with WG, RG was widely used in

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some Traditional Chinese medicine (TCM) formulas instead of WG for the treatment of cardiovascular diseases or cancer, such as *Shenmai* injection, *Shengmai* injection, and *Delisheng* injection. Therefore, exploring characteristic chemical components as chemical markers to discriminate WG and RG is important for quality evaluation, formulation, and standardizing the processing procedures, as well as the effective and safe usage of ginseng. In the current standards of Chinese Pharmacopoeia, three ginsenosides, Rg_1, Re, and Rb_1, which are major components of both WG and RG, are stilled used as chemical markers for quality control of WG and RG.[25]

During the past few years, with multiginsenosides as chemical markers, the holistic chemical profiling and discrimination methods of various *Panax* herbs, such as *P. ginseng* (Asian ginseng, WG and RG), *P. quinquefolius* (American ginseng), and *P. notoginseng*, have been developed by many modern hyphenated techniques including HPLC-ELSD, HPLC-UV, HPLC-ESI-MS/MS^n, and UPLC-(Q) TOF-MS (/MS), together with chemometrics methods such as PCA and OPLS-DA.[26−33] Ginsenoside Rg_3 and ginsenoside 20(*R*)-Rh_1 were detected as characteristic components of RG, whereas malonyl ginsenoside Rb_1/isomer and malonyl ginsenoside Rg_1/isomer were found to be characteristic components of WG. However, these studies did not systematically compare the holistic chemical profiles between Chinese commercial WG and RG by simultaneous qualitative and quantitative analyses. The conclusion may be partial and one-sided, which is not reasonable enough to put forward more suitable marker components for distinguishing these two types of ginseng.

In this paper, a HPLC-ESI-MS/MS^n-based multicomponent quantification fingerprinting approach was developed to evaluate the holistic qualities of commercial RG and WG from herbal markets in China. An approach to screen and identify the main constituents in RG and WG by combining the accurate mass measurement of LC/TOF-MS to generate empirical formulas and the complementary fragmentation data for structure confirmation provided by LC/ion trap MS^n would be described.[34] The contents of 12 ginsenosides in different sources of commercial RG and WG would be determined simultaneously. Through the mimic process of steaming and heating in the laboratory, chemical conversions and the possible mechanisms involved in the RG preparatory process were also investigated. The validated method is expected to explore characteristic chemical components as chemical markers to ensure the consistent quality of RG and standardize the processing procedures for RG.

MATERIALS AND METHODS

Chemicals. Reference standards of ginsenosides Rg_1, Re, and Rb_1 were obtained from the National Institutes for Food and Drug control (Beijing, China). Another five ginsenosides, Ro, Rf, Rb_2, Rc, and Rd, were obtained from Sichuan Victory Biopharmaceuticals Co. Ltd. (Chengdu, China). All standards were of biochemical reagent grade and at least 98% pure as confirmed by HPLC. Acetonitrile (HPLC grade) was purchased from Fisher (USA); potassium dihydrogen phosphate (A.R. grade) was obtained from Sino-pharm Co. (Beijing, China). Ultrapure water (18.2 MΩ) was prepared with a Milli-Q water purification system (Millipore, France). Other reagents were of analytical grade.

Herbal Materials. Ten batches of commercial RG samples (CRG-001−010) and two batches of commercial WG samples (CWG-001−004) were purchased from various pharmacies in Jilin, Liaoning, Beijing, and Sichuan provinces of China. All samples were morphologically authenticated as the root of *P. ginseng* C.A. Meyer by Dr. Ying-ni Pan (Shenyang Pharmaceutical University, China). Two batches mimicking RG sample (SRG-001−002) were manufactured by steaming of WG (CWG-002) at 98 °C for 3 or 1 h in a pottery apparatus and drying at 60 °C for 12 h. Other heated WG samples (HWG-001−005) were manufactured by heating of WG in an oven at 98 °C for 1−5 h. Voucher specimens were deposited into the Department of Chemistry, Tsinghua University (as shown in Table 4).

Sample and Reference Solutions Preparation. The roots of ginseng samples were pulverized, and approximately 0.2 g of ground sample was accurately weighed and ultrasonically extracted (44 kHz, 250 W) with 5 mL of 70% aqueous methanol for 30 min at room temperature. The extracted solutions were then filtered through a 0.2 μm PTFE syringe filter, and 20 μL of each filtrate was subjected to HPLC-ESI-MS/MS^n analysis. The eight reference compounds, ginsenosides Rg_1, Re, Ro, Rf, Rb_2, Rc, Rb_1, and Rd, were dissolved with methanol to obtained stock solutions at approximately 1.0 mg/mL, and they were stored at 4 °C. Specific amounts of these eight reference compound stock solutions were mixed and diluted with methanol to obtain a series of standard reference compound mixture solutions. The solutions were filtered using a 0.2 μm PTFE syringe filter, and 20 μL of each filtrate was subjected to HPLC analysis.

HPLC Analysis. HPLC-DAD analysis was performed with an Agilent 1200 series HPLC-DAD system (Agilent Series 1200, Palo Alto, CA, USA), equipped with a binary solvent delivery system, autosampler, and diode array detector, scanning from 200 to 400 nm; the wavelength was then selected and fixed at 203 nm for qualitative and quantitative analysis, considering the wavelength of maximum absorption of ginsenosides. The chromatography was performed on an Agilent TC-C_{18} analytical column (250 mm × 4.6 mm i.d., 5 μm, AgilentCorp, USA) at 25 °C. The mobile phase consisted of (A) 2 mM potassium dihydrogen phosphate buffer solution at pH 6.0 and (B) acetonitrile with gradient elution (0−32 min, 21−23% B; 32−70 min, 23−38% B; 70−80 min, 38% B). Re-equilibration duration was 10 min between individual runs. The flow rate was kept at 1.0 mL min^{-1}, and 20 μL of standard and sample solution was injected in each run.

Mass Spectrometry. The HPLC system was also connected to an Agilent 1100 LC/MSD trap (Agilent Corp., Santa Clara, CA, USA) equipped with an electrospray interface to provide sufficient fragmentation data. The chromatography separation was also carried out on an Agilent TC-C_{18} analytical column. The mobile phase was changed because the phosphoric acid buffer salt solution was not allowed to be used in the mass spectrum analysis, which consisted of (A) water and (B) acetonitrile with gradient elution (0−30 min, 23−26% B; 30−52 min, 26−41% B; 52−62 min, 41 −61% B; 62−72 min, 61−63% B; 72−80 min, 63−100% B). The flow rate was kept at 1.0 mL min^{-1}, and one-third of the eluent was introduced into the MS system with a split valve. The ion trap MS analysis was carried out in negative mode using the following operation parameters: capillary voltage, 3500 V (ESI$^-$) or 4000 V (ESI$^+$); skimmer voltage, 40 V; capillary exit voltage, 137 V; nebulizer pressure, 30 psig; drying gas, 8 L/min; gas temperature, 325 °C; target mass, *m*/*z* 622; compound stability, 100%; trap drive level, 60%; threshold, 50000 (ESI$^+$) and 10000 (ESI$^-$); ion charged control (ICC), on; target, 10000; accumulation time, 200 ms. An amplitude voltage of 0.6 V was typically used for fragmentation in the ion trap auto-MS^3 experiments. Data were processed by Agilent Chemstation Rev. A. 09.01 software (Agilent, Palo Alto, CA, USA).

HPLC/TOF-MS Analysis. The HPLC system was coupled to an Agilent 1200 LC/MSD TOF (Agilent Corp, Waldbronn, Germany) equipped with an electrospray interface. The electrospray source includes dual nebulizers, one nebulizer for the LC eluent and the other for the internal reference solution. The reference nebulizer, along with the LC/MSD TOF's automated calibrant delivery system (CDS), provides continuous introduction of reference mass standards into the ion source for automated mass calibration. Accurate mass measurements were obtained with this CDS, and thus enhanced accuracy was achieved. The HPLC conditions for the LC/TOF-MS analysis were the same as the ion trap MS analysis method. TOF-MS analysis was also performed in negative (ESI$^-$) ion mode under the following

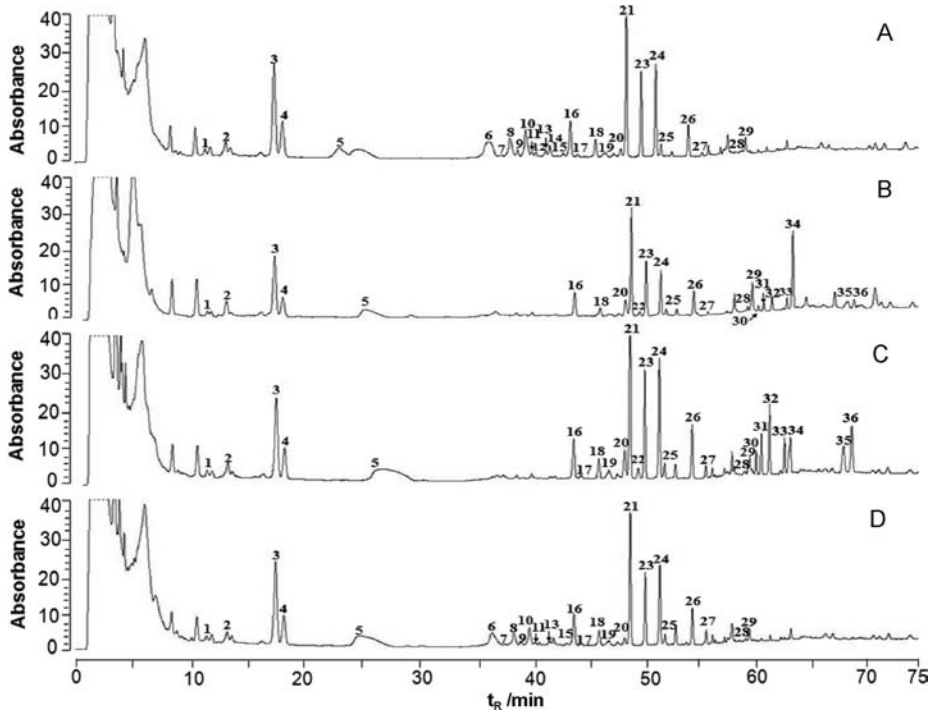

Figure 1. HPLC profiles of white ginseng (CWG-002, A), red ginseng (CRG-003, B), steamed white ginseng (SRG-001, C), and heated white ginseng (HWG-001, D) at 203 nm. See Table 1 for the peak numbers, and see Materials and Methods Mass Spectrometry for qualitative analysis conditions.

operation parameters: capillary voltage, 3500 V (ESI^-) or 4000 V (ESI^+); drying gas, 8.0 L min^{-1}; nebulizer pressure, 40 psig; gas temperature, 325 °C; fragmentor voltage, 175 V (ESI^+) and 190 V (ESI^-); skimmer voltage, 60 V; octopole dcl, 37.5 V (ESI^+) and −38.0 (ESI^-); octopole RF, 250 V. The full-scan carried out by LC/MSD TOF was recorded across the mass range m/z 50−1500. The reference solution was used as a continuous calibration using the following reference masses: m/z 121.0509 and 922.0098. Analyst QS software (Applied Biosystems, Framingham, MA, USA) was used to process the accurate mass data. Exact masses corresponding to particular elemental compositions were also calculated by the formula calculator in this software. Daily instrument tuning was carried out using the tuning solution (G1969-85000, Agilent Corp., USA) to ensure no more than 5 ppm mass error prior to running samples.

RESULTS AND DISCUSSION

Optimization of the Chromatography Conditions. Column types, mobile phase compositions, gradient elution procedure, flow rate of the mobile phase, and column temperature were optimized (data not shown), respectively, to achieve good separation of as many peaks as possible within a short analysis time. The applicability of the established analysis method by different HPLC instrument systems has been verified. A stationary baseline and satisfactory resolution of all quantified components are preconditions for accurate quantification, and the phosphoric acid buffer solution and acetonitrile were applied as mobile phase system for the quantitative analysis. The phosphoric acid buffer salt solution was not allowed to be used in the mass spectrum analysis, so another HPLC condition was proposed for further MS analysis to compare the chemical composition of different ginseng products. Representative HPLC chromatograms of commercial WG (CWG-002) and RG (CRG-003), together with the heated white ginseng (HWG-001) and steamed white ginseng (SRG-001) under the qualitative analysis condition are shown in Figure 1.

Identity Assignment and Confirmation of the Detected Ginsenosides in RG and WG. Under the present chromatographic and MS conditions, a total of 36 major ginsenosides were identified from the decoctions of WG and RG (Figure 2), 13 of which [Re, Rg_1, Rf, Rg_2, Rb_1, Ro, Rc, Rb_2, Rb_3, Rd, Rh_1, Rh_2 and 20(*R*)-Rg_3] were confirmed by comparing the mass spectra and retention times with those of reference compounds, whereas the others were tentatively assigned by matching the empirical molecular formulas with those of the published known ginsenosides and/or further confirmed by elucidating the low-energy CID fragment ions, in particular for the isomeric ginsenosides. In addition, the chromatographic behaviors of some ginsenosides in the literature were considered to be complementary data for the identity confirmation of isomers. The details of identified ginsenosides are summarized in Table 1. As shown in Table 1, the mass accuracy for all molecular ions, quasi-molecular ions, and fragment ions was <30 ppm, indicating that the empirical molecular formula well matched the putative deprotonated ions, quasi-molecular ions, and fragment ions.[26,27]

Seventy percent aqueous methanol extracts of commercial WG (CWG-002) and RG (CRG-003), together with the heated white ginseng (HWG-001) and steamed white ginseng (SRG-001), were compared by the established HPLC-ESI-MS/MS^n-based chemical profiling method. As demonstrated in Table 1, there was a significant difference between the chemical profiles of 70% aqueous methanol extracts from commercial WG and RG. Peaks 6−15, which were identified as malonyl ginsenosides, were detected both in the commercial WG sample (CWG-002) and in the heated WG sample (HWG-005), which was manufactured by heating the WG in an oven at 98 °C for 5 h. However, they were not detected in either the commercial

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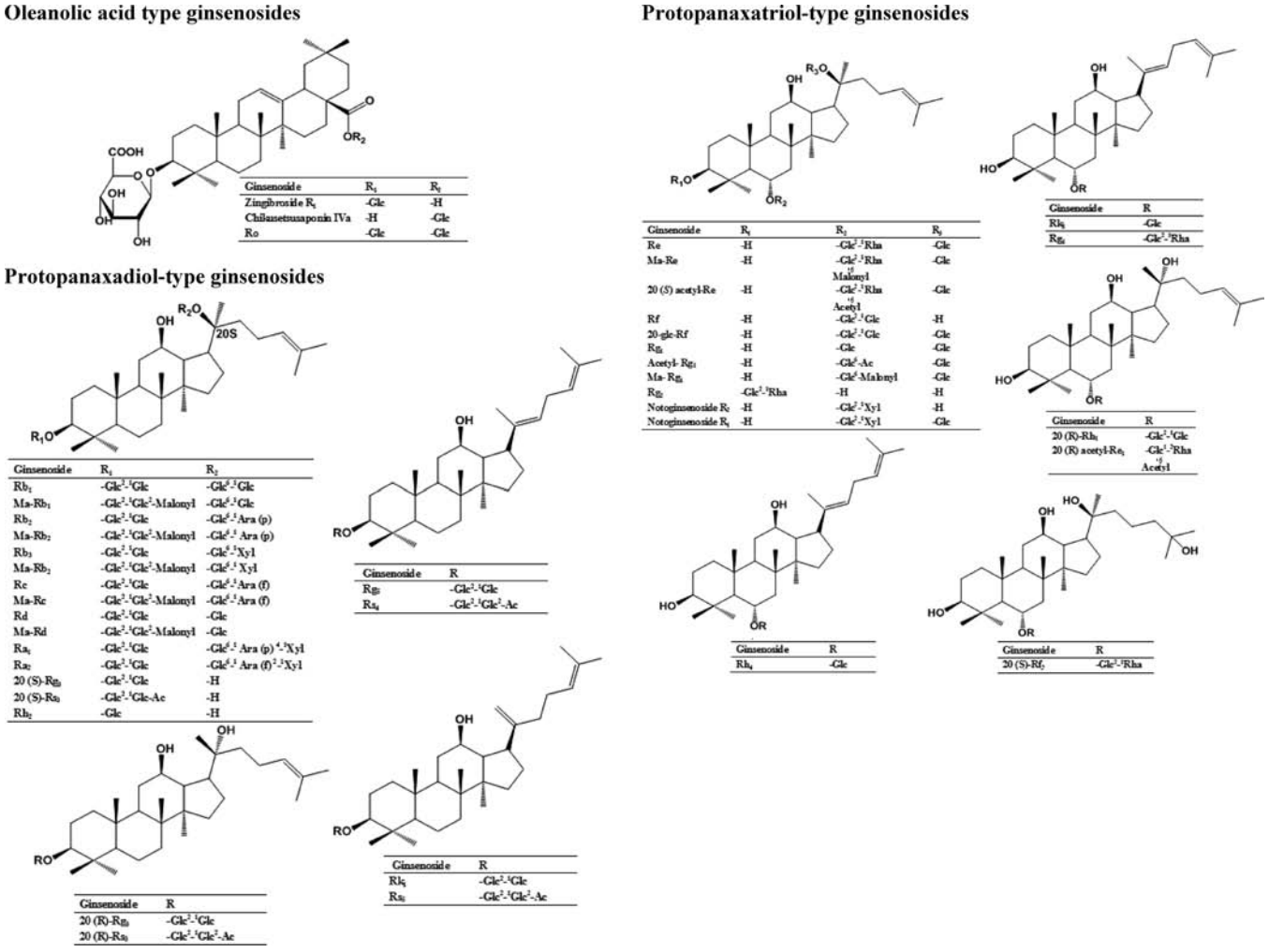

Figure 2. Structures of components identified in white and red gingseng.

RG or the steamed WG (SRG-001) sample, which was manufactured by steaming the WG at 98 °C for 3 h in a pottery apparatus and drying at 60 °C for 12 h. This result supported the previous findings that the content of malonyl ginsenosides decrease during steaming WG to RG, the mechanisms of which were deduced to be enzyme-involved hydrolysis of malonyl ginsenosides into their corresponding neutral ginsenosides as described.[26,27] Peaks 22, 30, 31, 32, 33, 34, 35, and 36 were detected only in the commercial RG sample (CRG-003) and were identified as the less polar ginsenosides Rh_1, 20(*R*) acetyl-Re, Rk_3, Rh_4, 20(*S*)-Rg_3, 20(*R*/*S*)-Rs_3, Rk_1, and Rg_5, respectively. The mechanisms involved were deduced to be the loss of a glycosyl moiety at C-20-OH of dammarane type ginsenosides through hydrolysis to generate the stereoisomers.[17,26,27] Due to unavailable reference standards, the identifications of isomers 20(*R*) acetyl-Re, Rk_3, Rh_4, 20(*S*)-Rg_3, 20(*R*/*S*)-Rs_3, Rk_1, and Rg_5 were conducted on the basis of the chromatographic behaviors reported in the literature.[35,36] The comparison of chemical compositions in the mimicked sample processed by steaming (SRG-001) and heating (HWG-001) in laboratory showed that malonyl ginsenosides characteristic to WG can be detected in the heated WG sample (HWG-001) and that the less polar ginsenosides which are characteristic to RG were not detectable. Malonyl ginsenosides were found in traces in the steamed WG sample (SRG-001). The above results suggested that the hydrolysis reaction would happen only in the presence of water vapor. Taken together, the malonyl ginsenosides characteristic to WG as well as the less polar ginsenosides characteristic to RG can be explored as chemical markers to ensure the consistent quality of RG and standardize the processing procedures for RG.

Validation of Quantitative Analytical Method. The HPLC method was validated by defining the linearity, limits of quantification and detection, identification and quantification of the analytes, repeatability, precision, stability, and recovery. All calibration curves were plotted on the basis of linear regression analysis of the integrated peak areas (Y) versus concentrations (X, μg) of the eight marker constituents in the standard solution at seven different concentrations. The regression equations, correlation coefficients, and linear ranges for the analysis of the eight marker constituents are shown in Table 2. The limit of detection (LOD) value was calculated as the amount of the injected sample that gave a single-to-noise ratio of 3 (S/N = 3). The LOD values of the method for the seven components are also listed in Table 2.

The precision of the HPLC method was determined for intra- and interday variations. An aliquot of powdered red ginseng sample was weighed and extracted in methanol. The intraday variability was performed six times on the same extract prepared on a single day. The interday reproducibility was determined by analyzing the samples on three separate days. The validation studies showed overall intra- and interday

Table 1. Components Identified from White and Red Ginseng

				[M − H]$^-$			fragment ions of [M − H]$^-$		
peak	identity	t_R (min)	molecular formula	measd mass	calcd mass	mass accuracy (ppm)	MS (m/z)	MS^2 − MS^3 (m/z)	sample items
1	20-glc-Rf	12.401	$C_{48}H_{81}O_{19}$	961.5445	961.5372	7.59	961.5 [M − H]$^-$	799.3 [M − H − Glc]$^-$ 619.3 [M − H − 2Glc − H_2O]$^-$	WG, RG, SWG, HWG
2[a]	notoginsenoside-R_1	14.12	$C_{47}H_{79}O_{18}$	931.529	931.5266	2.5316	931.5 [M − H]$^-$	799.4 [M − H − Xyl]$^-$ 637.4 [M − H − Xyl − Glc]$^-$ 475.1 [M − H − Xyl − 2Glc]$^-$	WG, RG, SWG, HWG
3[a]	Rg_1	17.974	$C_{42}H_{72}O_{14}$	799.5016	799.4843	17.2172	799.4 [M − H]$^-$	859.5 [M + Cl]$^-$, 799.4 [M − H]$^-$ 637.3 [M − H − Glc]$^-$	WG, RG, SWG, HWG
4[a]	Re	18.734	$C_{48}H_{82}O_{18}$	945.5506	945.5422	8.78	945.6 [M − H]$^-$	783.4 [M − H − Glc]$^-$ 637.3 [M − H − Glc − Rha]$^-$ 475.2 [M − H − 2Glc − Rha]$^-$	WG, RG, SWG, HWG
5[a]	Ro	25.918	$C_{49}H_{80}O_{18}$	955.5104	955.5266	7.8053	955 [M − H]$^-$	955.6 [M − H]$^-$ 793.4 [M − H-Glc]$^-$ 613.1 [M − H − Glc − Glu]$^-$	WG, RG, SWG, HWG
6	Ma-Rb_1	37.697	$C_{57}H_{94}O_{26}$	1193.6118	1193.5955	13.6562	1193.5 [M − H]$^-$	1149.6033 [M − H − CO_2]$^-$	WG, HWG
7	chikusetsusaponin IVa	38.499	$C_{42}H_{66}O_{14}$	793.4686	793.4373	19.2799	793.4 [M − H]$^-$	793.4 [M − H]$^-$ 631.3 [M − H − Glc]$^-$	WG, RG, SWG, HWG
8	Ma-Rb_2	39.506	$C_{56}H_{92}O_{25}$	1163.5998	1163.5849	12.7667	1163.4 [M − H]$^-$	1119.9 [M − H − CO_2]$^-$	WG, HWG
9	Ma-Rb_1 isomer	40.031	$C_{57}H_{94}O_{26}$	1193.6118	1193.5955	13.6562	1193.5 [M − H]$^-$	1149.6033 [M − H − CO_2]$^-$	WG, HWG
10	Ma-Rc	40.804	$C_{56}H_{92}O_{25}$	1163.5998	1163.5849	12.7667	1163.4 [M − H]$^-$	1119.9 [M − H − CO_2]$^-$	WG, HWG
11	Ma-Rb_3	41.365	$C_{56}H_{92}O_{25}$	1163.5769	1163.5849	−6.8753	1163.4 [M − H]$^-$	1119.9 [M − H − CO_2]$^-$	WG, HWG
12	Ma-Rc isomer	41.744	$C_{56}H_{92}O_{25}$	1163.5769	1163.5849	−6.8753	1163.5 [M − H]$^-$	1119.9 [M − H − CO_2]$^-$	WG, HWG
13	Ma-Rb_3 isomer	42.577	$C_{56}H_{92}O_{25}$	1163.5819	1163.5849	2.5782	1163.4 [M − H]$^-$	1119.9 [M − H − CO_2]$^-$	WG, HWG
14	Ma-Rd	42.939	$C_{51}H_{84}O_{21}$	987.5826	987.5528	10.1175	1031.2 [M − H]$^-$	987.8 [M − H − CO_2]$^-$	WG, HWG
15	Ma-Re	44.169	$C_{51}H_{84}O_{21}$	1031.5767	1031.5426	32.973	1031.0 [M − H]$^-$	987.8 [M − H−CO_2]$^-$	WG, HWG
16[a]	Rf	44.766	$C_{42}H_{72}O_{14}$	799.5016	799.5148	−16.629	799.5 [M − H]$^-$	799.4 [M − H]$^-$ 637.3 [M − H-Glc]$^-$	WG, RG, SWG, HWG

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Table 1. continued

peak	identity	t_R (min)	molecular formula	[M − H]⁻ measd mass	[M − H]⁻ calcd mass	[M − H]⁻ mass accuracy (ppm)	fragment ions of [M − H]⁻ MS (m/z)	fragment ions of [M − H]⁻ $MS^2 - MS^3$ (m/z)	sample items
								475.1 $[M - H - 2Glc]^-$	
17	Ra_3	46.671	$C_{59}H_{100}O_{27}$	1239.6515	1239.6373	11.3949	1239.8 $[M - H]^-$	1239.8 $[M - H]^-$ 1107.7 $[M - H - Xyl]^-$ 945.4 $[M - H - Xyl - Glc]^-$ 783.4 $[M - H - Xyl - 2Glc]^-$ 622.3 $[M - H - Xyl - 3Glc]^-$	WG, RG, SWG, HWG
18	notoginsenoside R_2	47.028	$C_{41}H_{70}O_{13}$	769.4935	769.4738	25.5777	769.7 $[M - H]^-$	769.5 $[M - H]^-$ 805.4 $[M + Cl]^-$ 637.2 $[M - H - Xyl]^-$ 475.1 $[M - H - Xyl - Glc]^-$	WG, RG, SWG, HWG
19	zingibroside R_1	47.987	$C_{42}H_{66}O_{40}$	793.4466	793.4373	11.7211	793.4 $[M - H]^-$		WG, RG, SWG, HWG
20	20(R/S)-Rg_2	49.29	$C_{42}H_{72}O_{13}$	783.513	783.4894	30	783.5 $[M - H]^-$		WG, RG, SWG, HWG
21[a]	Rb_1	49.869	$C_{54}H_{92}O_{23}$	1107.6165	1107.5951	19.3068	1107.7 $[M - H]^-$	945.6 $[M - H - (Glc - H_2O)]^-$ 783.4 $[M - H - 2(Glc - H_2O)]^-$	WG, RG, SWG, HWG
22[a]	Rh_1	50.453	$C_{36}H_{62}O_9$	637.4508	637.4315	30.2768	637.3 $[M - H]^-$	697.3 $[M + AcO]^-$ 637.3 $[M - H]^-$ 475.1 $[M - H - Glc]^-$	RG, SWG
23[a]	Rc	51.117	$C_{53}H_{90}O_{22}$	1077.6209	1077.5845	33.7311	1077.8 $[M - H]^-$	945.6 $[M - H - Ara\ (f) - H_2O]^-$ 783.5 $[M - H - Ara\ (f) - H_2O - (Glc - H_2O)]^-$ 621.2 $[M - H - Ara\ (f) - H_2O - 2(Glc\text{-}H_2O)]^-$	WG, RG, SWG, HWG
24[a]	Rb_2	52.438	$C_{53}H_{90}O_{22}$	1077.5833	1077.5845	−1.1605	1077.8 $[M - H]^-$	945.6 $[M - H - (Ara(P) - H_2O)]^-$ 915.5 $[M - H - (Glc - H_2O)]^-$ 783.4 $[M - H - (Ara(P) - H_2O) - (Glc - H_2O)]^-$	WG, RG, SWG, HWG
25[a]	Rb_3	52.769	$C_{53}H_{90}O_{22}$	1077.585	1077.5845	0.4	1077.5 $[M - H]^-$	945.5 $[M - H - (Xyl - H_2O)]^-$ 915.5 $[M - H - (Glc - H_2O)]^-$ 783.4 $[M - H - (Xyl - H_2O) - (Glc - H_2O)]^-$	WG, RG, SWG, HWG
26[a]	Rd	55.327	$C_{48}H_{82}O_{18}$	945.5822	945.5422	42.2049	799.5 $[M - H]^-$	783.5 $[M - H - Glc]^-$ 621.4 $[M - H - 2Glc]^-$	WG, RG, SWG, HWG
27	Rs_1/Rs_2	56.539	$C_{55}H_{91}O_{23}$	1119.6285	1119.5951	29.8322			RG, SWG

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Table 1. continued

peak	identity	t_R (min)	molecular formula	$[M - H]^-$ measd mass	$[M - H]^-$ calcd mass	$[M - H]^-$ mass accuracy (ppm)	fragment ions of $[M - H]^-$ MS (m/z)	fragment ions of $[M - H]^-$ $MS^2 - MS^3$ (m/z)	sample items
28	acetyl-Rg_1/isomer	60.043	$C_{44}H_{74}O_{14}$	825.5282	825.5	34.1201			RG, SWG
29	Rg_6	60.447	$C_{42}H_{70}O_{12}$	765.4879	765.4789	11.7573	765.4 $[M - H]^-$		RG, SWG
30	20(*R*) acetyl-Re	61.044	$C_{44}H_{74}O_{14}$	825.5365	825.5	44.1738			RG, SWG
31	Rk_3	61.472	$C_{36}H_{60}O_8$	619.4476	619.4209	42.9503	619.3 $[M - H]^-$	679.3 $[M + AcO]^-$ 619.3 $[M - H]^-$	RG, SWG
32	Rh_4	62.232	$C_{36}H_{60}O_8$	619.448	619.4209	43.5961	619.3 $[M - H]^-$	679.3 $[M + AcO]^-$ 619.3 $[M - H]^-$	RG, SWG
33[a]	20(*S*)-Rg_3	63.517	$C_{42}H_{72}O_{13}$	783.5248	783.4894	45.0934	783.4 $[M - H]^-$	783.4 $[M - H]^-$ 621.3 $[M - H - Glc]^-$ 459.1 $[M - H - 2Glc]^-$	RG, SWG
34	20(*R*/*S*)-Rs_3	64.041	$C_{44}H_{74}O_{14}$	825.5485	825.5	58.7089	825.4 $[M - H]^-$	825.4 $[M - H]^-$ 861.4 $[M + Cl]^-$ 783.4 $[M - H - Ac]^-$ 621.3 $[M - H - Ac - Glc]^-$	RG, SWG
35	Rk_1	68.825	$C_{42}H_{70}O_{12}$	765.5139	765.4789	45.7164	765.4 $[M - H]^-$	765.4 $[M - H]^-$ 603.2 $[M - H - Glc]^-$	RG, SWG
36	Rg_5	69.548	$C_{42}H_{70}O_{12}$	765.5139	765.4789	44.8965	765.4 $[M - H]^-$	765.4 $[M - H]^-$ 603.2 $[M - H - Glc]^-$	RG, SWG

[a]Identified with reference standard.

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Table 2. Regression Equation, Linear Range, and LOD of the Developed Method

ginsenoside	regression eq[a]	correlation factor (r)	linearity range (μg)	LLOQ[b] (ng)	LOD[b] (ng)
Rg_1	$Y = 7.478X - 71.87$	0.9995	0.550−22.2	92	185
Re	$Y = 6.143X - 3.260$	0.9995	0.364−14.6	91	182
Ro	$Y = 4.218X + 0.0676$	1.000	0.505−20.2	92	184
Rf	$Y = 7.777X - 41.66$	0.9996	0.276−11.1	69	138
Rb_1	$Y = 3.983X - 36.64$	0.9995	0.436−17.5	109	218
Rc	$Y = 5.578X - 36.55$	0.9996	0.364−14.6	91	182
Rb_2	$Y = 6.145X - 34.80$	0.9998	0.352−12.7	88	176
Rd	$Y = 6.342X - 42.16$	0.9997	0.358−14.3	89	179

[a] Y and X are, respectively, the peak areas and concentrations (μg mL $^{-1}$) of the analytes. [b] The LLOQ was defined as the concentration foe which the signal-to-noise ratio was 10, and the LOD was defined as the concentration for which the signal-to-noise ratio was 3.

variations (RSD) of less than 1.17 and 1.48%, respectively. For the stability test, retention times and peak areas of seven compounds in sample solutions were analyzed at 0, 4, 8, 12, 16, and 24 h. RSD values of the peak area of seven compounds were <3.0%, respectively. The percentage difference between amounts determined and spiked was considered to be a measure of accuracy. Known amounts (low, medium, and high) of the eight standard references were spiked into samples and then prepared as test solutions. The determination was performed in triplicate, and the average recoveries and relative standard deviation (RSD) were calculated. The developed method had good accuracy with the overall recovery of 96.5−104.6%, with the RSD ranging from 0.95 to 3.80% (Table 3). These results indicated that the HPLC-UV method is precise, accurate, and sensitive for the quantitative determination of 12 ginsenosides in RG samples.

Constituent Analysis of Samples. Chemical profiling and quantification of the 12 ginsenosides from RG and WG using the HPLC-UV method were carried out (Figure 3). Because of the absence of less polar ginsenosides characteristic to RG, we mainly paid attention to the amounts of the 12 representative components including 3 protopanaxtriol ginsenosides (Rg_1, Re, and Rf), an oleanolic acid type ginsenoside (Ro), 4 protopanaxdiol ginsenosides (Rb_1, Rc, Rb_2, and Rd), and 4 malonylated protopanaxdiol ginsenosides (m-Rb_1, m-Rc, m-Rb_2, and m-Rd) that are characteristic to WG. The concentrations of ginsenosides Rg_1, Re, Rf, Rb_2, Rc, Ro, Rb_1, and Rd were calculated precisely according to the respective calibration curves. However, four malonyl ginsenosides (m-Rb_1, m-Rb_2, m-Rc, and m-Rd) without reference substances were relatively determined by quantitative analysis of multicomponents by a single marker approach.[37] In theory, if the structures of compounds were similar, the relative correction factors (RCF) of components were around 1,[38] so the RCFs of four malonyl ginsenosides without authentic samples to corresponding demalonylated compounds were determined approximately as 1. Therefore, the concentrations of m-Rb_1, m-Rb_2, m-Rc, and m-Rd were calculated relatively on the basis of the calibration curves of Rb_1, Rb_2, Rc, and Rd, respectively. The structures of these four compounds were confirmed by accurate molecular weight measured by LC/TOF-MS to generate empirical formulas.

Table 4 shows the amounts of 12 main ginsenosides in commercial RG samples and WG samples. There were no differences in the variety of ginsenosides among the four WG products, but the total quantities of ginsenosides were somewhat different. Similar results were also found among 10 different batches of RGs. As shown in Figure 4, the average content of 12 determined ginsenosides in WG (av = 4.14 ± 1.49%, n = 4) was much higher than that in RG (av = 1.75 ± 0.71%, n = 10), and the four malonylated protopanaxdiol ginsenosides (m-Rb_1, m-Rc, m-Rb_2, and m-Rd) are characteristic components of WG, which were not detected at all or detected in only small or trace amounts in RG. We have proposed that the malonyl ginsenosides may be esterolyzed and generate the corresponding neutral ginsenosides during previous processing of RG, and the amount of malonyl ginsenosides may be directly bound with the processing procedures.[17] The determined result of the two batches of RGs developed from WG by steaming at 98 °C for 3 h (sample SRG-001) and 1 h (sample SRG-002) demonstrated the rationality of this thesis quantificationally. The total amount of

Table 3. Accuracy of HPLC-UV Method for the Determination of Eight Ginsenosides

analyte	original (mg)	spiked (mg)	found (mg)	recovery (%)	RSD (%)
Rg_1	0.40	0.32	0.72	99.5	3.40
		0.40	0.82	104.1	0.95
		0.48	0.90	104.6	1.05
Re	0.12	0.096	0.21	98.9	3.35
		0.12	0.24	96.5	1.59
		0.144	0.26	100.3	3.52
Ro	0.44	0.35	0.79	99.1	1.08
		0.44	0.88	100.2	1.76
		0.53	0.98	103.1	3.28
Rf	0.080	0.064	0.14	100.8	3.48
		0.080	0.16	98.9	2.66
		0.096	0.17	96.6	1.81
Rb_1	0.56	0.45	1.01	99.7	3.61
		0.56	1.12	100.8	2.48
		0.67	1.22	99.0	1.68
Rc	0.21	0.17	0.38	100.5	3.80
		0.21	0.42	100.3	3.77
		0.25	0.46	100.0	3.15
Rb_2	0.16	0.13	0.29	100.9	2.93
		0.16	0.32	102.6	3.03
		0.19	0.35	101.2	2.51
Rd	0.080	0.064	0.14	101.0	2.41
		0.080	0.16	102.9	1.24
		0.096	0.18	103.2	1.70

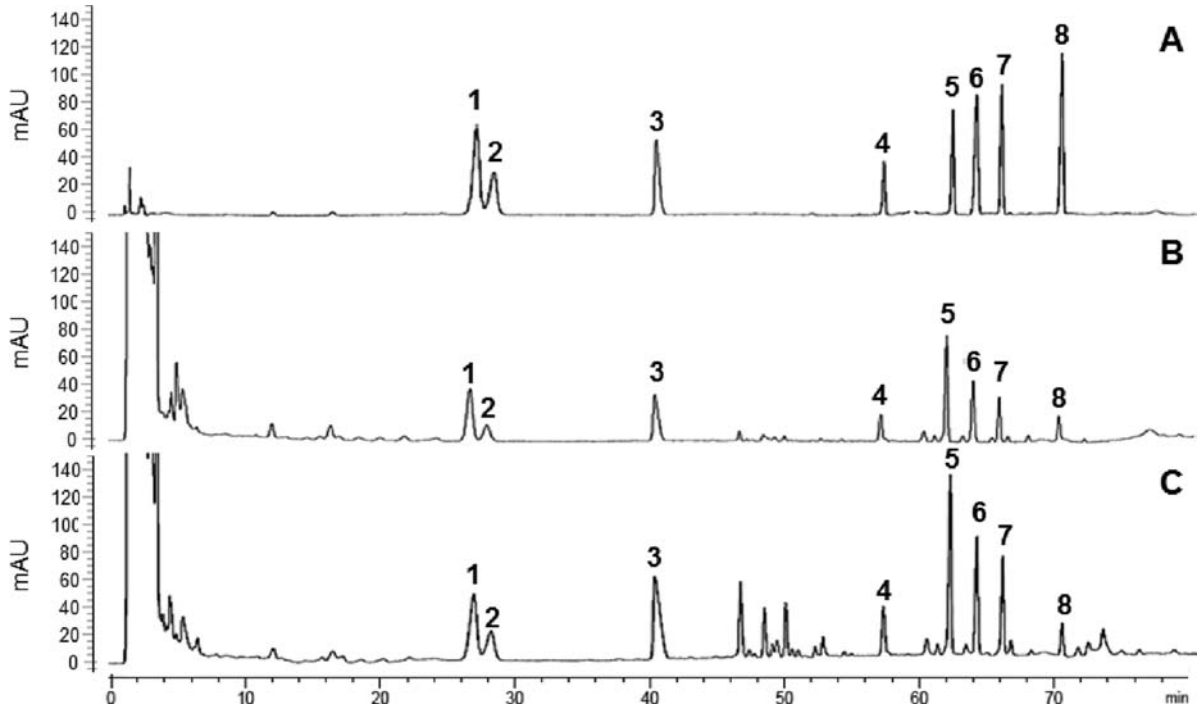

Figure 3. Multicomponent quantitative fingerprint chromatograms of reference standards (A), red ginseng (CRG-003, B), and white ginseng (CWG-002, C) at 203 nm. See Materials and Methods HPLC Analysis for quantitative analysis conditions. Peaks: 1, ginsenoside Rg_1; 2, ginsenoside Re; 3, ginsenoside Ro; 4, ginsenoside Rf; 5, ginsenoside Rb_1; 6, ginsenoside Rc; 7, ginsenoside Rb_2; 8, ginsenoside Rd.

malonyl ginsenosides in SRG-002, which was steamed for 1 h, was higher than the 3 h steamed one (SRG-001), which indicated that the content of malonyl ginsenosides is related to the steaming time. Traditionally, RG is prepared from fresh ginseng by steaming and drying. However, the temperature and time of steaming and drying have not been specified, which could explain why the malonyl ginsenosides characteristic to WG were detected in some commercial RG samples (e.g., CRG-001), indicating that sample CRG-001 was not properly processed. Although some scholars have realized that malonyl ginsenosides were effective chemical markers to discriminate WG from RG, which can be used in the quality control of processing procedures,[39,40] it is still not clear whether the content of malonyl ginsenosides is relatedonly to the processing temperature and heating time and whether the presence of water vapor is an essential condition for the manufacture of RG. To figure out the critical control index in the processing procedure of RG and clarify the above-mentioned questions, five heated WG samples (HWG-001−005) were manufactured by heating of WG in an oven at 98 °C for 1−5 h, and the contents of 12 ginsenosides were compared. As shown in Table 4, along with the increase of heating time, there is only a slight decrease in the content of the 12 determined marker components, as well as the content ratio of malonyl ginsenosides to the corresponding neutral ginsenosides. The peak origins from malonyl ginsenosides did not disappear in the process of heating, similar to steaming, which proved that the traditional process technology of RG cannot be replaced by heating at a high temperature without water vapor.

It is well-known that because of the different growth years, localities, cultivation practices, harvest times, storage conditions, and processing methods, the content of ginsenosides may vary greatly. For the same varieties of ginseng products, the content ratio of corresponding components should be relatively stable and seems to be more reasonable for the holistic quality control of ginseng products. In this study, the ratio of the total content of determined malonyl ginsenosides to the corresponding neutral ginsenosides ($T_{m\text{-}PPD}/T_{PPD}$) in WG ranged from 0.46 to 0.62, which is range from 0 to 0.19 in RG. All aforementioned results suggested that the content ratio of malonyl ginsenosides to the corresponding neutral ginsenosides in RG and WG may become important assessment criteria for quality control of ginseng products. These results also provided an effective approach to standardize the processing procedures of ginseng products and regulate the usage of ginseng in Traditional Chinese Medical prescription.

In summary, a HPLC-ESI-MS/MS^n-based multicomponent fingerprinting approach was developed to evaluate the holistic qualities of commercial RG and WG from herbal markets in China in this paper. Thirty-six components in RG and WG would be screened and identified by combining the accurate mass measurement of LC/TOF-MS to generate empirical formulas and the complementary fragmentation data for structure confirmation provided by LC/ion trap MS^n. HPLC-UV conditions were optimized for the quantitative and qualitative determination of 12 ginsenosides in white ginseng and red ginseng, The quantification method is rapid, accurate, and precise, and it can simultaneously determine the amounts of 12 ginsenosides in various ginseng products (WG and RG). Character markers for discriminating RG from WG have been proposed. There are still some limitations in the present study, such as the HPLC analysis time was too long to realize high-throughput detection, and the developed method cannot be used to quantitatively determine the content of less polar ginsenosides Rg_6, F_4, Rk_3, Rh_4, 20(*S*)-, and 20(*R*)-Rg_3, which are unique compounds of RG. The relationship between the chemical profiles of different ginseng products and the bioactivity also needs further investigation. However, these

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Table 4. Comparison of Ginsenosides Content (Percent) in Different Ginseng Products[a]

			protopanaxtriol type				oleanane type	protopanaxdiol type ginsenoside												
batch	sample item	collection region/ date	Rg_1	Re	Rf	T_{PPT}	Ro	m-Rb_1	m-Rc	m-Rb_2	m-Rd	Rb_1	Rc	Rb_2	Rd	T_{PPD}	$T_{m\text{-}PPD}$	$T_{m\text{-}PPD}/T_{PPD}$	T_G	T_{PPD}/T_{PPT}
CWG-001	WG	Jilin/20110305	0.13	0.12	0.06	0.30	0.43	0.53	0.06	0.06	tr	0.40	0.13	0.12	0.04	1.34	0.65	0.48	2.07	4.47
CWG-002	WG	Jilin/20080512	0.34	0.21	0.13	0.68	0.71	0.89	0.14	0.15	0.06	0.72	0.34	0.28	0.08	2.66	1.24	0.46	4.05	3.91
CWG-003	WG	Tongrentang/2005	0.36	0.34	0.11	0.81	0.90	1.68	0.23	0.14	0.10	0.70	0.22	0.33	0.05	3.45	2.15	0.62	5.16	4.26
CWG-004	WG	Jilin/2005	0.42	0.29	0.13	0.84	1.03	1.36	0.18	0.12	0.09	0.97	0.33	0.30	0.07	3.42	1.75	0.51	5.29	4.07
av_{CWG}			**0.31**	**0.24**	**0.11**	**0.66**	**0.77**	**1.12**	**0.15**	**0.12**	**0.08**	**0.70**	**0.26**	**0.26**	**0.06**	**2.72**	**1.45**	**0.52**	**4.14**	**4.18**
± SD (n = 4)			**0.13**	**0.10**	**0.03**	**0.25**	**0.26**	**0.51**	**0.07**	**0.04**	**0.02**	**0.23**	**0.10**	**0.09**	**0.02**	**0.99**	**0.65**	**0.07**	**1.49**	**0.24**
CRG-001	RG	Jilin/20080513	0.39	0.19	0.10	0.68	0.60	0.28	0.07	0.04	tr	0.86	0.41	0.26	0.15	2.07	0.40	0.19	3.35	3.03
CRG-002	RG	Fusong/20100912	0.15	0.08	0.04	0.28	0.27	tr	nd	nd	nd	0.29	0.14	0.10	0.04	0.57	nd	—	1.11	2.02
CRG-003	RG	Fusong/20101103	0.23	0.10	0.07	0.41	0.30	tr	nd	nd	nd	0.41	0.19	0.12	0.06	0.78	nd	—	1.49	1.94
CRG-004	RG	Dunhua/20101012	0.15	0.08	0.05	0.27	0.20	tr	nd	nd	nd	0.34	0.11	0.08	0.05	0.58	nd	—	1.05	2.14
CRG-005	RG	Antu/20101031	0.19	0.16	0.08	0.43	0.35	0.17	nd	nd	nd	0.47	0.26	0.19	0.09	1.18	0.17	0.14	1.96	2.72
CRG-006	RG	Fusong/20100912	0.21	0.08	0.07	0.36	0.29	tr	nd	nd	nd	0.39	0.19	0.12	0.06	0.75	nd	—	1.40	2.07
CRG-007	RG	Fusong/20090312	0.19	0.10	0.07	0.36	0.26	tr	nd	nd	nd	0.44	0.19	0.12	0.06	0.82	nd	—	1.44	2.26
CRG-008	RG	Fusong/20110123	0.16	0.08	0.06	0.30	0.28	tr	nd	nd	nd	0.37	0.16	0.10	0.05	0.68	nd	—	1.26	2.29
CRG-009	RG	Ji'an/20101012	0.25	0.13	0.08	0.47	0.39	0.15	nd	nd	nd	0.54	0.21	0.15	0.07	1.12	0.15	0.13	1.97	2.40
CRG-010	RG	Yanji/20100823	0.21	0.22	0.09	0.52	0.39	0.22	nd	nd	nd	0.65	0.33	0.20	0.12	1.53	0.22	0.14	2.45	2.93
av_{CRG}			**0.21**	**0.12**	**0.07**	**0.41**	**0.33**	**0.21**	**0.07**	**0.04**	**—**	**0.48**	**0.22**	**0.14**	**0.08**	**1.01**	**0.23**	**0.15**	**1.75**	**2.38**
± SD (n = 10)			**0.07**	**0.05**	**0.02**	**0.13**	**0.11**	**0.06**	**—**	**—**	**—**	**0.17**	**0.09**	**0.06**	**0.04**	**0.48**	**0.11**	**0.03**	**0.71**	**0.39**
SRG-001[b]		steam/20120112	0.33	0.19	0.15	0.67	0.92	tr	tr	tr	0.11	1.06	0.46	0.41	0.16	2.20	0.11	0.05	3.79	3.29
SRG-002[b]		steam/20120112	0.35	0.22	0.13	0.70	0.85	0.33	0.07	tr	0.13	0.95	0.43	0.39	0.13	2.44	0.54	0.22	3.98	3.49
HWG-001[c]		heated/20120112	0.35	0.27	0.19	0.80	0.67	0.72	0.12	0.13	0.19	0.74	0.38	0.35	0.10	2.73	1.15	0.42	4.20	3.39
HWG-002[c]		heated/20120112	0.32	0.23	0.16	0.72	0.61	0.76	0.12	0.12	0.16	0.68	0.37	0.31	0.09	2.61	1.16	0.45	3.94	3.65
HWG-003[c]		heated/20120112	0.34	0.24	0.17	0.75	0.68	0.79	0.13	0.14	0.18	0.72	0.37	0.33	0.09	2.75	1.24	0.45	4.18	3.66
HWG-004[c]		heated/20120112	0.32	0.23	0.18	0.73	0.59	0.70	0.12	0.12	0.16	0.67	0.34	0.32	0.09	2.51	1.10	0.44	3.83	3.46
HWG-005[c]		heated/20120112	0.36	0.26	0.16	0.78	0.69	0.61	0.10	0.11	0.17	0.69	0.33	0.32	0.10	2.41	0.98	0.40	3.88	3.10

[a]All samples were morphologically authenticated as white ginseng (WG) and red ginseng (RG) according to the current standard of Chinese Pharmacopoeia. T_G, T_{PPT}, T_{PPD}, and $T_{m\text{-}PPD}$ represent the sum quantities of 12 ginsenosides, protopanaxtriol type ginsenosides, protopanaxdiol type ginsenosides, and malonylated protopanaxdiol type ginsenosides, respectively. nd, not detectable (<LOD). tr, trace (>LOD and < minimum of the linear range), as described under Validation of Quantitative Analytical Method. [b]SRG-001 and SRG-002 were manufactured by steaming of WG (CWG-0002) at 98 °C for 3 h or 1 h in a pottery apparatus and drying at 60 °C for 12 h. [c]The heated WG samples (HWG-001−005) were manufactured by heating of WG in an oven at 98 °C for 1−5 h, respectively.

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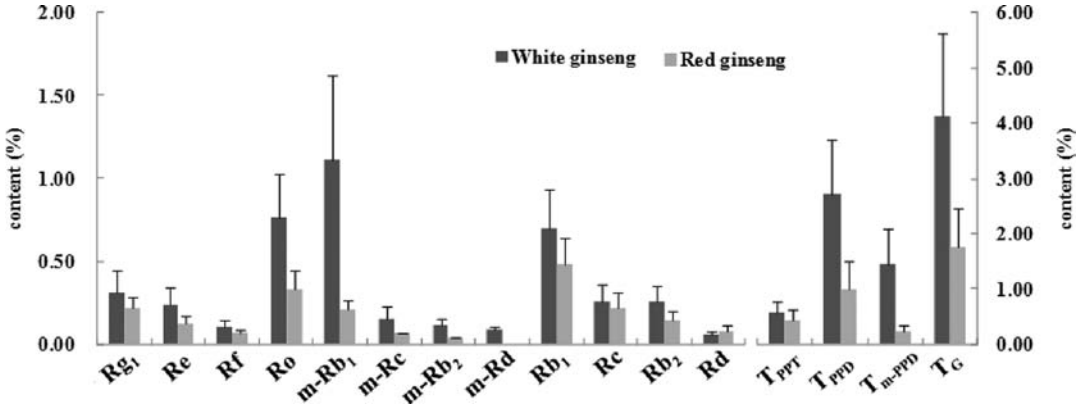

Figure 4. Comparison of contents of 12 ginsenosides in white and red ginseng. T_G, T_{PPT}, T_{PPD}, and $T_{m\text{-}PPD}$ represent the sum quantities of 12 ginsenosides, protopanaxtriol type ginsenosides, protopanaxdiol type ginsenosides, and malonylated protopanaxdiol type ginsenosides, respectively.

results are definitely helpful for quality evaluation, formulation, and standardizing the processing procedures, as well as the effective and safe usage of ginseng, and also provide a scientific basis for the search for the components that are responsible for red ginseng's pharmacological effects.

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Notes

The authors declare no competing financial interest.

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