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Research Article

Ginsenosides in Commercial Ginseng Products Analyzed by Liquid Chromatography-Tandem Mass Spectrometry

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Although the LC/MS-MS method has been developed to analyze ginsenosides in ginseng roots, this method has not yet been utilized to quantify ginsenosides in ginseng products. In this paper, an internal standard method by LC/MS-MS was developed to analyze the ginsenosides content in the commercial ginseng products such as the ginseng capsules. We compare the standard addition method and the internal standard methods for the quantification of several ginsenosides (e.g., Rb1, Rb2, Rb3, Rc, Rd, Re, Rg1, and Rg3) in five commercial ginseng capsules. The results showed that the internal standard method is as good as the standard addition method and is a less tedious approach.

1. Introduction

Asian ginseng, called Panax ginseng C. A. Meyer [1], is widely available as health food products in the form of powders, capsules, tablets, softgel, teas, or liquid extracts. Chemically, ginseng contains different ginsenosides, which are triterpenoidic saponins, with the general structural formula shown in Figure 1 [2]. They are believed to be the active ingredients responsible for the therapeutic effects of ginseng. Ginsenosides can be classified into 20S-protopanaxadiol-type (PPD) or 20S-protopanaxatriol-type (PPT), as listed in Table 1 [3]. In general, for PPD-type ginsenosides, the R3 group in Figure 1 is hydrogen, whereas in PPT-type ginsenosides, the R3 group is either an OH or O-glycosylated by various sugars. These sugar substituents are usually hexoses (glucose and rhamnose) or pentoses (arabinose and xylose) [4]. Because of the structural complexity and low abundance of these ginsenosides, very selective and sensitive analytical methods are needed to differentiate between various ginsenosides.

On the commercial side, the total sale of ginseng dietary supplements in the United States was US$ 12 million in 2005 [5], with a steady annual growth of 3-4% till 2010 [6], indicating that the ginseng products are widely used. Therefore, it is important to determine the contents of various ginsenosides in commercial ginseng products. It is because different ginsenosides are responsible for various health-promoting effects [7]. These biological effects range from antiaging effect and improvement of mental/physical ability to modulation of the immune system [8]. A number of laboratory studies have observed strong therapeutic effects of ginsenosides against cardiovascular diseases (e.g., Re), diabetes (e.g., Re), obesity (e.g., Rg1 and Rg3), cancer (e.g., Rd, Rg3, and Rh2), and estrogen deficiency (e.g., Rb1 and Rg1) [9–14].

The analysis of ginsenosides in ginseng root is well documented, but there is a paucity of information on ginsenosides levels in commercial ginseng products [15]. Typical analytical techniques for ginsenoside analysis include thin-layer chromatography, liquid chromatography with either ultraviolet or fluorescence detection, and gas chromatography-mass spectrometry [16]. Recently, liquid chromatography-mass spectrometry (LC/MS) with electrospray ionization (ESI) has been used to confirm the composition of ginseng products [17]. Liquid chromatography coupled with tandem mass spectrometry has also been employed for ginsenoside quantitation in ginseng roots [18–21] and in blood plasma

Table 1: Classification of ginsenosides in terms of their aglycones PPD or PPT.

Ginsenoside	MW (Da)	Molecular formula	R1	R2	R3
PPD	460.39	$C_{30}H_{52}O_3$	H	H	H
Rb1	1109.29	$C_{54}H_{92}O_{23}$	Glc^2-Glc	Glc^6-Glc	H
Rb2	1079.27	$C_{53}H_{90}O_{22}$	Glc^2-Glc	Glc^6-Ara(p)	H
Rb3	1079.27	$C_{53}H_{90}O_{22}$	Glc^2-Glc	Glc^6-Xyl	H
Rc	1079.27	$C_{53}H_{90}O_{22}$	Glc^2-Glc	Glc^6-Ara(f)	H
Rd	947.15	$C_{48}H_{82}O_{18}$	Glc^2-Glc	Glc	H
Rg3	785.01	$C_{42}H_{72}O_{13}$	Glc^2-Glc	H	H
PPT	476.38	$C_{30}H_{52}O_4$	H	H	OH
Re	947.15	$C_{48}H_{82}O_{18}$	H	Glc	O-Glc^2-Rha
Rg1	801.03	$C_{42}H_{72}O_{14}$	H	Glc	O-Glc

Glc: β-D-glucose; Ara(p): α-L-arabinose(pyranose); Ara(f): α-L-arabinose-(furanose); xyl: xylose; Rha: α-L-rhamnose.

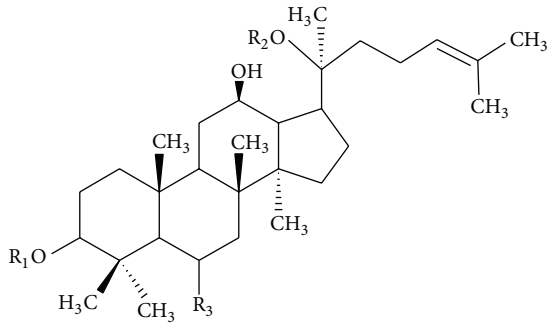

Figure 1: Structure of ginsenosides.

[22–24], but none of these studies were performed on ginseng products.

In this paper, the LC/MS-MS method was developed to analyze the ginsenosides content in the commercial ginseng products such as the ginseng capsules. To achieve higher selectivity and sensitivity needed for this study, LC-MS/MS with multiple reaction monitoring (MRM) was used. We compare the standard addition method and the internal standard methods for the quantification of several ginsenosides (e.g., Rb1, Rb2, Rb3, Rc, Rd, Re, Rg1, and Rg3). The results showed that the internal standard method, which is a less tedious approach, is as good as the standard addition method.

2. Material and Methods

2.1. Chemicals and Materials. Five brands of ginseng capsules, coded as GC-C, GC-O, GC-H, GC-U, and GC-W, were purchased from Nutrition House (Burnaby, BC), IDA Pharm (Coquitlam, BC), and Ginseng Enterprise Ltd. (Vancouver, BC). Four samples were made from Asian or Korean ginseng (Panax ginseng C.A. Meyer), whereas only GC-U was made from American ginseng (Panax quinquefolius).

Ethanol was obtained from Commercial Alcohols Inc. (Boucherville, QC). Deionized water was obtained from a Milli-Q system (Millipore, Bedford, MA). HPLC-grade acetonitrile was obtained from Sigma-Aldrich (St. Louis, MO) and acetic acid was obtained from EM Science (Gibbstown, NJ). Pure standards of ginsenosides Rb1, Rb2, Rb3, Rc, Rd, Re, Rg1, and Rg3 were purchased from Tauto Biotech (Shanghai, China). Digitoxin, which was used as the internal standard (IS), was purchased from Sigma-Aldrich.

2.2. Ginsenoside Extraction. For each sample, 1 g of each ginseng product was put into a test tube, and 10 mL of 90% ethanol was added. After sonication in an ultrasonic bath (Branson 5510) for 30 min, each test tube was centrifuged for 5 min. at 4000 rpm (Eppendorf 5810). The supernatant was pipetted out and put in a collection tube. The residues were reextracted with an additional 10 mL of 90% ethanol and the above process was repeated a second time. The supernatants obtained in these 2 subsequent extractions were combined into the same collection tube. This process was also repeated for a third time to get another fraction of supernatant. The need of several extraction steps was evaluated by analyzing the ginsenoside content after one, two, three, and four extractions. The solvent in the collection tube was evaporated using a Genevac EZ-2 evaporator until there was less than 1 mL of liquid left. Then, the liquid sample was made up to a final volume of 2 mL using 50% aqueous acetonitrile. This sample was diluted by 1/2000 times by dissolving 1 μL in 2 mL of 50% aqueous acetonitrile. Prior to LC-MS/MS analysis, 20 μL of digitoxin (100 μg/mL) was added into the diluted sample and mixed well.

2.3. HPLC Conditions. An Agilent 1100 HPLC system equipped with a LC pump, column compartment, degasser, UV-detector, and an autosampler was used with a Waters reversed phase Pico Tag C18 Column (3.9 mm × 300 mm, 4 μm). The injection volume was 10 μL for each sample. A solvent gradient was used for the separation (Table 2), with solvent A being water containing 0.02% acetic acid and solvent B being acetonitrile containing 0.02% acetic acid. The flow rate was 0.375 mL/min, and the total run time was 79 min. These are the HPLC conditions (long column and solvent gradient) employed to enhance the separation of ginsenosides, especially for the closely eluted Rb2 and Rb3.

Table 2: Solvent gradient used for HPLC analysis. A is 0.02% acetic acid in water, and B is 0.02% acetic acid in acetonitrile.

Time (min)	% A	% B
0.0	95	5
0.6	70	30
30.0	67	33
60.0	5	95
70.0	95	5

2.4. MS/MS Conditions. A Micromass Quattro Ultima mass spectrometer (Waters) was used with the HPLC system using MassLynx 4.0. All data generated was analyzed using QuanLynx. Negative electrospray (ESI) mode was used to ionize the samples as it has been reported that the ESI^- ion mode was more sensitive than the ESI^+ ion mode [15]. The MS was operated in MRM mode using argon as the collision gas. Table 3 lists the parameters used to quantify the ions. The capillary voltage was 3.0 kV. The source temperature and the desolvation temperature were set at 120°C and 400°C, respectively. The desolvation gas flow and the cone gas flow were 600 l/h and 25 l/h, respectively. The dwell time for each ion for the MRM experiments was set at 0.05 s and the interscan delay was 0.01 s.

2.5. Standard Solutions. Nine standard solutions (i.e., eight ginsenosides Rb1, Rb2, Rb3, Rc, Rd, Re, Rg1, and Rg3 plus the internal standard, digitoxin) were prepared for MS experiments. Each solution contained 0.5 mg/mL ginsenoside in 50% aqueous acetonitrile.

Internal Standard Method. Six calibration solutions with the concentrations of 0.125, 0.25, 0.625, 1.25, 2.50, and 6.25 μg/mL of each of the eight ginsenosides were prepared. Each 2 mL of calibration solution was spiked with 20 μL of digitoxin (100 μg/mL). After LC-MS/MS analysis, a calibration curve of the peak area ratio (i.e., area of sample peak divided by area of digitoxin peak) versus concentration was plotted for each of the 8 ginsenosides and the concentrations of Rb1, Rb2, Rb3, Rc, Rd, Re, Rg1, and Rg3 in the extracted ginseng samples were determined. Each sample was analyzed in triplicate.

Standard Addition Method. For each ginseng sample, 4 individual aliquots of 2 mL were spiked with 2, 5, 10, and 20 μL of standard mixture (containing 0.625 mg/mL of each of the 8 standards and 1 μg/mL of digitoxin), respectively. After LC-MS/MS analysis, a family of calibration curves (for 8 ginsenosides), of the peak area ratio versus added concentration was plotted for each sample. Then, the concentrations of Rb1, Rb2, Rb3, Rc, Rd, Re, Rg1, and Rg3 in the extracted ginseng samples were determined by extrapolation on the standard addition calibration curves.

3. Results and Discussion

3.1. Development of Extraction Method. To evaluate the volume of 90% ethanol needed for extraction, two ginseng samples GC-O and GC-U were randomly chosen. The supernatants after 1, 2, 3, and 4 extraction steps were analyzed (Figure 2). The comparison was made by using the sum of all ginsenosides as analyzed with LC-MS/MS.

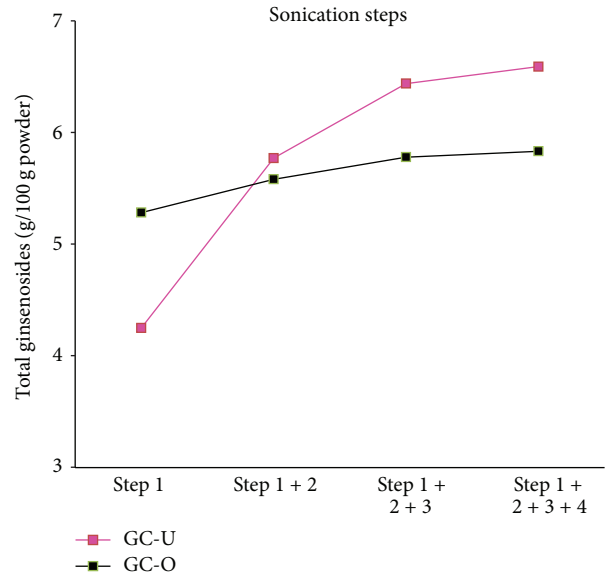

Figure 2: Total ginsenosides in GC-U and GC-O after one, two, three, and four sonication steps.

It was observed that the ginsenoside content increased from the first to the third step of extraction. The fourth step did not result in more ginsenosides being extracted. Therefore, it was concluded that three steps were sufficient for the extraction of ginsenosides.

3.2. Qualitative Analysis of Ginsenosides in Ginseng Capsules. Ginsenosides extracted from different commercial ginseng capsules were analyzed by LC-MS/MS. The separation of the ginsenoside standards is shown in Figure 3 with their retention times (in min.) in parentheses: Rb1 (39.59), Rb2 (51.33), Rb3 (54.10), Rc (44.99), Rd (66.82), Re (12.33), Rg1 (12.65), and Rg3 (68.29). A sample chromatogram of sample GC-U is shown in Figure 4. The presence of various ginsenosides in GC-C, GC-O, GC-H, GC-W, and GC-U was determined by comparing their retention times to those of the standards (Table 4). Rb1, Rc, Rd, Re, Rg1, and Rg3 were detected in all analyzed ginseng products (GC-C, GC-O, GC-H, GC-W, and GC-U). Rb3 could be detected in all except GC-U, whereas Rb2 could only be detected in GC-W.

3.3. Quantitation of Ginsenosides in Ginseng Capsules. In order to improve accuracy of ginsenoside quantitation, the internal standard method was used. The results were compared with those obtained by the more accurate, though more tedious, standard addition method [25]. The comparison is accomplished using Student's t test at 95% confidence level. Since no stable isotope-labelled ginsenosides are commercially available, digitoxin was used as the internal standard instead.

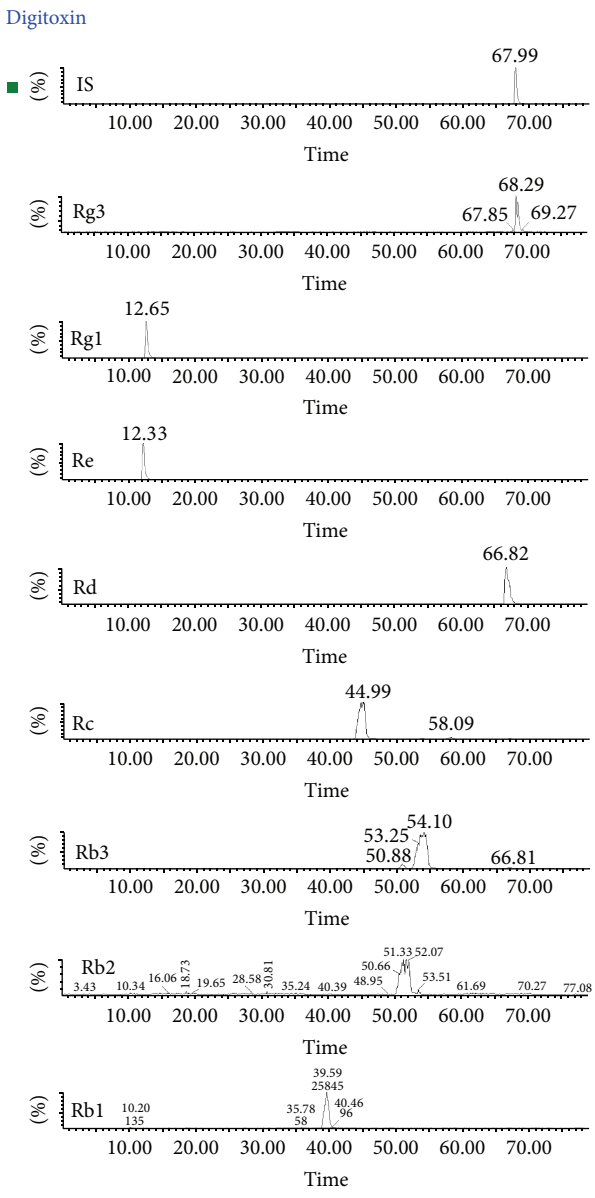

Figure 3: Typical MRM results of internal standard (IS) and ginsenoside standards Rb1, Rb2, Rc, Rd, Re, Rg1, and Rg3 at 0.5 mg/mL. For LC-MS/MS conditions, see text.

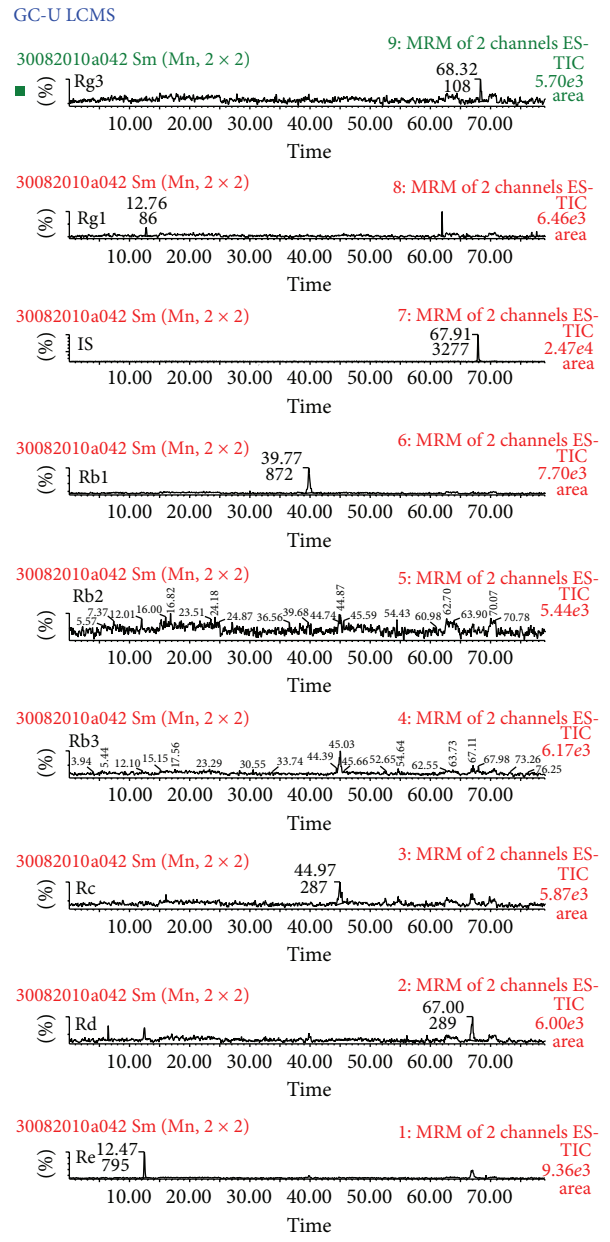

Figure 4: MRM results of the ginsenosides detected in ginseng product GC-U. For LC-MS/MS conditions, see text.

3.3.1. Internal Standard Method. The area ratios of the ginsenosides peaks and the digitoxin peaks (internal standard) were calculated. Calibration curves were constructed and then analyzed using linear regression, with the regression equations of the eight analytes listed in Table 5.

From the calibration curves, the amounts of the 8 ginsenosides of all commercial ginseng products (with standard deviations) were expressed as g/100 g powder, see Table 6. These values were correlated with the mass of the original ginseng powders.

In sample GC-C, relatively low amounts of Rb1, Rb3, Rc, Rd, Re, and Rg1 were found. However, the Rg3 content is high (2.0 ± 0.1%), which has been associated with steamed ginseng, but not raw ginseng [16]. Among the 5 ginseng products, GC-C is found to contain the highest Rg3 level, which is consistent with the company's claim of high Rg3 content. In GC-O, the amounts of Re and Rg1 are very high, which have been determined to be 1.9 ± 0.3% and 1.1 ± 0.1%, respectively. Rb1, Rb3, Rc, and Rd in GC-O are low but in comparable levels to GC-C. In GC-H, almost the same low amount of every ginsenoside (except Rb2) has been detected, with Rg1 having the highest percentage of 0.3 ± 0.1%. GC-W is special because of the presence of all eight analyzed ginsenosides; Re (0.7 ± 0.1%) and Rg1 (0.7 ± 0.1%) are high and Rb3 is the lowest. In GC-U, Rb1 and Re are high in content. Here, only a small amount of the ginsenosides Rc, Rd, Rg1, and Rg3 is detected,

Table 3: Parameters used for multiple reaction monitoring (MRM) of ginsenosides and internal standard.

Ginsenoside	Precursor ions (Da)	Product ions (Da)	Cone voltage (V)	Collision energy (eV)
Rb1	1108.29	221.2	95	60
		323.0	95	50
Rb2	1078.27	450.5	95	45
		766.6	95	45
Rb3	1078.27	292.8	90	50
		946.2	90	50
Rc	1078.27	784.1	95	50
		947.4	95	45
Rd	946.15	622.5	90	40
		784.5	90	30
Re	946.15	638.6	95	40
		784.5	95	40
Rg1	800.00	476.2	100	40
		638.3	100	25
Rg3	784.00	460.4	95	40
		622.5	95	35
Digitoxin	763.60	373.3	90	55
		503.3	90	30

Table 4: Presence (+) or absence (−) of ginsenosides in different commercial ginseng products analyzed by LC-MS/MS.

Ginsenoside	Name of the sample				
	GC-C	GC-O	GC-H	GC-W	GC-U
Rb1	+	+	+	+	+
Rb2	−	−	−	+	−
Rb3	+	+	+	+	−
Rc	+	+	+	+	+
Rd	+	+	+	+	+
Re	+	+	+	+	+
Rg1	+	+	+	+	+
Rg3	+	+	+	+	+

Table 5: Regression equations and correlation coefficients of ginsenoside calibrations.

Rb1	$y = 201.250x + 0.029$	$R^2 = 0.999$
Rb2	$y = 32.168x + 0.025$	$R^2 = 0.999$
Rb3	$y = 150.416x + 0.019$	$R^2 = 0.999$
Rc	$y = 448.943x - 0.018$	$R^2 = 0.999$
Rd	$y = 214.853x + 0.037$	$R^2 = 0.999$
Re	$y = 138.681x - 0.004$	$R^2 = 0.999$
Rg1	$y = 48.114x + 0.005$	$R^2 = 0.999$
Rg3	$y = 98.683x + 0.032$	$R^2 = 0.992$

whereas Rb2 and Rb3 are undetectable. As we know, GC-U is not Asian ginseng (P. ginseng), and this is confirmed from its high Rb1/Rg1 value because it has been reported that Rb1/Rg1 values of P. quinquefolius are higher than that of P. ginseng [16].

3.3.2. Standard Addition Method. The standard addition method has been widely used to reduce quantitation errors due to matrix effects. Although it was a labour intensive procedure, this method was still employed to confirm the results obtained by the internal standard method. The results (with standard deviations) were tabulated in Table 7. In GC-C, Rg3 has been found to have the highest content (2.3 ± 0.2%). In GC-O the amount of Re, which has been calculated to 1.3 ± 0.3%, is very high, whereas Rb1, Rb3, Rc, Rd, Rg1, and Rg3 show comparable low levels like GC-C. In GC-H, except Rg3 (0.7 ± 0.2%), all the analyzed ginsenosides have a percentage lower than 0.5%. GC-W has shown a similar amount of all tested ginsenosides, with Re (0.8 ± 0.2%) having the highest analyzed ginsenoside. In GC-U, Rb1 (1.4 ± 0.1%) and Re (1.1 ± 0.3%) are high in content. However, only a small amount of ginsenosides Rc and Rg1 is detected.

The detection of each ginsenoside using the two analytical methods is compared and shown in Figure 5. For instance, in GC-C, Rg3 is found to have 2.3 ± 0.2% by the standard addition method, which is similar to 2.0 ± 0.4% found by the internal standard method. In addition, total ginsenosides analyzed by internal standard method are found to be higher in GC-O (4.2 ± 0.5%), GC-C (3.1 ± 0.4%), and GC-W (2.5 ± 0.4%) than in GC-U (1.9 ± 0.3%) and GC-H (1.4 ± 0.2%). The results of the standard addition method show total ginsenosides of 4.5 ± 0.8% (GC-U), 4.2 ± 0.7% (GC-C), 4.1±0.9% (GC-W), 3.6±1.0% (GC-O), and 1.8±0.5% (GC-H). Even though there are variations between the two methods, the ginsenoside contents of the different commercial ginseng products are in the same range. The findings in the standard addition method are consistent with those in the internal standard method. In particular, the quantitations of Rb2, Rb3, Rc, Re, and Rg1 are not significantly different using either method, based on Student's t-test.

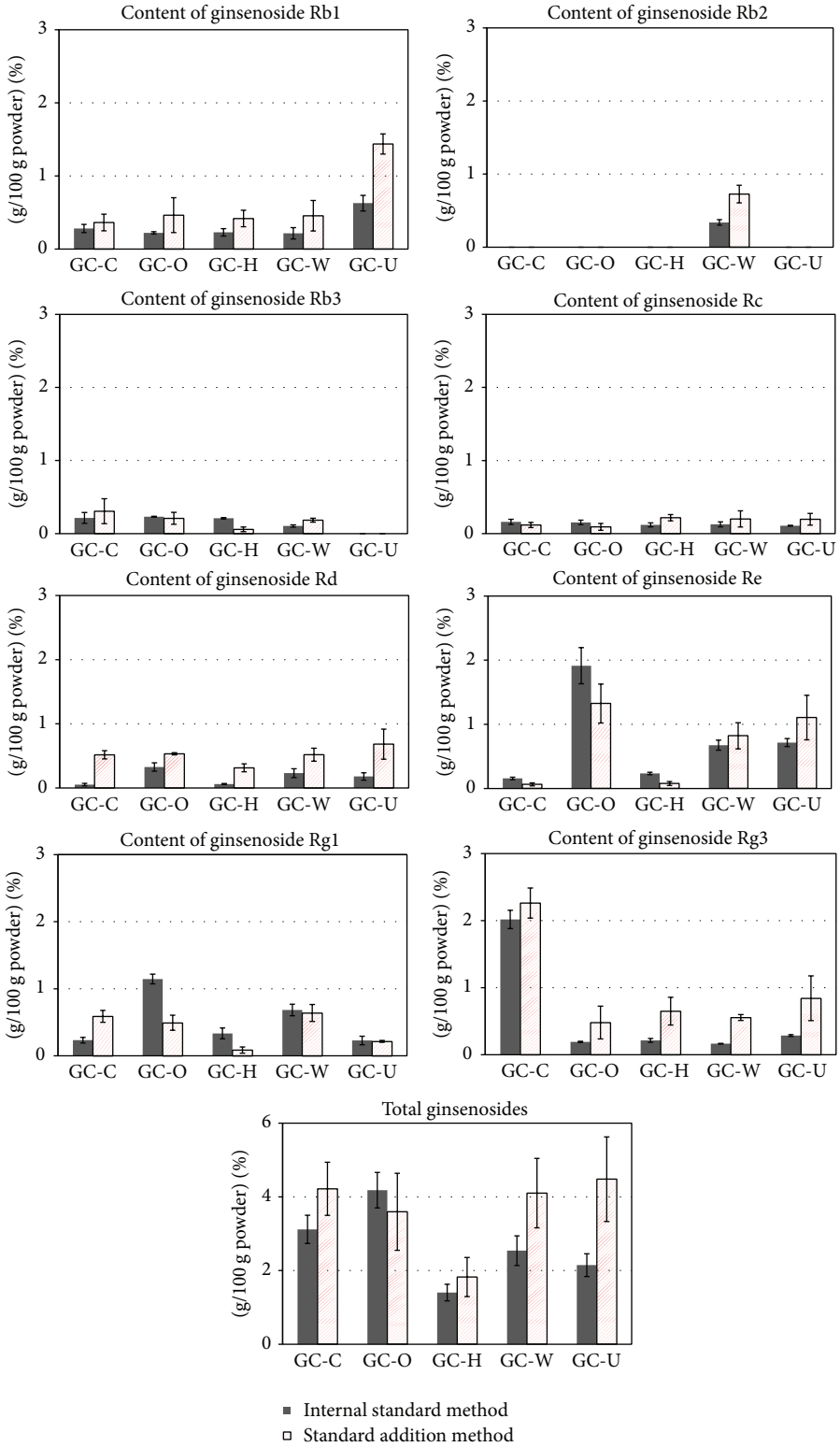

Figure 5: Content of ginsenosides Rb1, Rb2, Rb3, Rc, Rd, Re, Rg1 and Rg3 and total amount of ginsenosides in commercial ginseng products GC-C, GC-O, GC-H, GC-W, and GC-U, as determined by internal standard and standard addition calibration methods.

Table 6: Ginsenoside analysis using the internal standard calibration method. Percentage (g/100 g powder) and standard deviation ($n = 3$) of the ginsenosides Rb1, Rb2, Rb3, Rc, Rd, Re, Rg1, and Rg3 in different commercial ginseng products.

Name	Name of the sample				
	GC-C	GC-O	GC-H	GC-W	GC-U
Rb1	0.3 ± 0.1	0.22 ± 0.02	0.2 ± 0.1	0.2 ± 0.1	0.6 ± 0.1
Rb2	n.d.	n.d.	n.d.	0.34 ± 0.04	n.d.
Rb3	0.2 ± 0.1	0.23 ± 0.01	0.21 ± 0.01	0.10 ± 0.02	n.d.
Rc	0.16 ± 0.04	0.15 ± 0.03	0.12 ± 0.03	0.13 ± 0.03	0.11 ± 0.01
Rd	0.05 ± 0.02	0.3 ± 0.1	0.06 ± 0.01	0.2 ± 0.1	0.2 ± 0.1
Re	0.16 ± 0.02	1.9 ± 0.3	0.23 ± 0.02	0.7 ± 0.1	0.7 ± 0.1
Rg1	0.23 ± 0.04	1.1 ± 0.1	0.3 ± 0.1	0.7 ± 0.1	0.2 ± 0.1
Rg3	2.0 ± 0.1	0.19 ± 0.01	0.21 ± 0.03	0.17 ± 0.01	0.30 ± 0.01

n.d.: not detected.

Table 7: Ginsenoside analysis using the standard addition calibration method. Percentage (g/100 g powder) and standard deviation ($n = 3$) of the ginsenosides Rb1, Rb2, Rb3, Rc, Rd, Re, Rg1, and Rg3 in different commercial ginseng products.

Name	Sample				
	GC-C	GC-O	GC-H	GC-W	GC-U
Rb1	0.4 ± 0.1	0.5 ± 0.2	0.4 ± 0.1	0.5 ± 0.2	1.4 ± 0.1
Rb2	n.d.	n.d.	n.d.	0.7 ± 0.1	n.d.
Rb3	0.3 ± 0.2	0.2 ± 0.1	0.06 ± 0.03	0.18 ± 0.03	n.d.
Rc	0.12 ± 0.03	0.09 ± 0.05	0.22 ± 0.04	0.2 ± 0.1	0.2 ± 0.1
Rd	0.5 ± 0.1	0.53 ± 0.02	0.3 ± 0.1	0.5 ± 0.1	0.7 ± 0.2
Re	0.06 ± 0.02	1.3 ± 0.3	0.08 ± 0.03	0.8 ± 0.2	1.1 ± 0.3
Rg1	0.6 ± 0.1	0.5 ± 0.1	0.09 ± 0.05	0.6 ± 0.1	0.22 ± 0.01
Rg3	2.3 ± 0.2	0.5 ± 0.2	0.7 ± 0.2	0.56 ± 0.04	0.8 ± 0.3

n.d.: not detected.

The reason for using the standard addition method is that it will reduce the problem of the sample matrix effect, most likely due to the ionic suppression phenomenon [26]. But we know that the internal standard method is less tedious than the standard addition method. In order to match the results obtained from the standard addition method, we have chosen a proper sample dilution (1/2000) in the internal standard method.

In terms of total ginsenosides, there is 4% found in GC-O which is consistent with the company's claim of 4%. However, our finding of total ginsenosides (4%) in GC-C is much lower than the company's claim of 20%. It is disappointing to find a low amount of ginsenoside (2%) in GC-H (claim of 10%), especially because it is the most expensive ginseng product among the five. The total ginsenoside of 2–4% found in GC-U is also lower than the company's claim of 6%.

4. Conclusion

The HPLC-MS/MS method was employed to analyze the ginsenoside contents in various commercial ginseng products. GC-C was found to have the highest amount of Rg3, and GC-O was found to have the highest amount of Re. GC-O was found to have the highest total ginsenoside amount of 4%, consistent with the claimed amount by the manufacturer. Without any stable isotope-labeled ginsenosides, both the internal standard method and the standard addition method were used for quantitation, showing comparable results. This indicates that matrix effects due to ion suppression have not affected the analysis of ginsenosides by internal standard method, at the chosen dilution level of the sample.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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