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Saponins Composition in American Ginseng Leaf and Berry Assayed by High-Performance Liquid Chromatography

CHONG-ZHI WANG,[†,‡] JI AN WU,[†,‡] ERYN MCENTEE,[†,‡] AND CHUN-SU YUAN*,[†,‡,§]

Tang Center for Herbal Medicine Research, Department of Anesthesia and Critical Care, and Committee on Clinical Pharmacology and Pharmacogenomics, The University of Chicago, Chicago, Illnois 60637

The root of American ginseng is a commonly used herbal medicine in the United States. However, the compositions of American ginseng leaves and berries are not clear to date. In this study, we improved a method for the analysis of 12 ginsenosides based on solid phase extraction and high-performance liquid chromatography−ultraviolet. Good resolution was obtained for all tested ginsenosides: Rb1, Rb2, Rb3, Rc, Rd, Re, Rg1, Rg2, 20(R)-Rg2, Rg3, Rh1, and Rh2. Ginsenosides Rh1, Rg2, and 20(R)-Rg2 were easily separated with this column. The modified gradient elution program resulted in satisfactory linearity and precision. Solid phase extraction made the analysis accurate and efficient. Other investigators recently observed that ginsenoside Rb3 is a potent neuroprotective compound; it can promote learning and memory. In this report, we found that the major ginsenoside in American ginseng leaves and berries was ginsenoside Rb3, while Rb3 only had limited amounts in the root of American ginseng and other species of the *Panax* genus. Ginsenoside Rb3 was quantified as 4.71% in American ginseng leaves and 5.35% in berries, suggesting that American ginseng leaves and berries are new sources of ginsenoside Rb3.

KEYWORDS: ***Panax quinqueforlius*** **L.; American ginseng; leaf and berry; saponins; ginsenoside Rb3; high-performance liquid chromatography**

INTRODUCTION

American ginseng (*Panax quinquefolius* L.) is an important species in the family Araliaceae. Another species in this family, *Panax ginseng* C. A. Meyer (Asian ginseng), has been used for centuries in many Oriental countries as a panacea that promotes longevity (*1*). Several studies have demonstrated the beneficial effects of Asian ginseng. However, relatively few studies have focused on American ginseng, which is a popular herb in the United States for patients suffering from nervous system and cardiovascular diseases (*2*, *3*). American ginseng is also a dietary supplement (*4*, *5*); its extract has been included as a component in many supplement formulations (*6*, *7*).

It is generally believed that the bioactive constituents of American ginseng extract are dammarane saponins, commonly referred to as ginsenosides. Many analytical approaches have been developed to separate and assay ginsenosides in ginseng extracts. Among these methods, high-performance liquid chromatography (HPLC) is the most widely used (*8*). Fluorescence is one of the most sensitive detector methods in HPLC assay, but ginsenosides have to be derivatized before analysis (*9*). For the direct determination of ginsenosides, evaporative light scattering detectors (*10*, *11*) and mass spectrometry detectors (*12*, *13*) have been employed. Because HPLC-ultraviolet (UV) is the most commonly used method, most phytochemical laboratories are equipped with HPLC accompanied by UV detectors. Several assays for the determination of ginsenosides using HPLC-UV have been carried out (*14*−*17*), but only up to six ginsenosides were determined in previous studies (*9*, *15*−*17*). Furthermore, no trace ginsenosides were assayed in many of these methods. Although some trace saponins, such as ginsenoside Rg2, Rh1, and Rh2, were tested, the chromatographic conditions contained a long column (250 mm) and utilized an elution program of more than 130 min (*14*). Because of these limitations, it is necessary to improve the chromatographic conditions for analysis of ginsenosides, especially trace amounts of ginsenosides.

Ginsenoside Rb1, Re, and Rg1 have been studied extensively because of the high content of these saponins in the *Panax* genus (*18*, *19*). Pharmacological studies of ginsenoside Rb3 showed its strong neuroprotective effect, which may contribute to improve learning and memory (*20*, *21*). However, because of its low content in ginseng, studies on ginsenoside Rb3 were largely neglected in the past. Ginsenoside Rg2 was found to inhibit nicotinic acetylcholine receptor-mediated Na^+ influx and channel activity, indicating its effect on the human nervous system (*22*); ginsenosides Rg3, Rh1, and Rh2 showed anticancer effects (*23*, *24*). However, ginsenosides Rb3, Rg2, Rg3, Rh1,

* To whom correspondence should be addressed. Tel: 773-702-1916. Fax: 773-834-0601. E-mail: CYuan@dacc.uchicago.edu.
[†] Tang Center for Herbal Medicine Research.
[‡] Department of Anesthesia and Critical Care.
[§] Committee on Clinical Pharmacology and Pharmacogenomics.

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and Rh2 have not been well-analyzed using HPLC-UV methods because they appear only as trace components in the roots of different species of the *Panax* genus. In the present study, we established a simple HPLC-UV method to assay 12 ginsenosides using a reverse phase 150 mm × 4.6 mm i.d. column and gradient elution program. The assay process took less than 95 min. This method was applied to analyze the trace ginsenosides in American ginseng leaves and berries, and the data were compared to those from the roots and rootlets. We observed very high contents of ginsenoside Rb3 in American ginseng leaves and berries, providing important information for new sources of ginsenoside Rb3.

MATERIALS AND METHODS

Plant Material. Dried American ginseng (*P. quinquefolius* L.) roots, rootlets, leaves, and fresh American ginseng berries were obtained from Roland Ginseng, LLC (Wausau, WI). The seeds of the berries were removed, and the pulp of the berries was lyophilized to obtain dried American ginseng berries. All samples were taken during 2003 and stored at −20 °C until analysis.

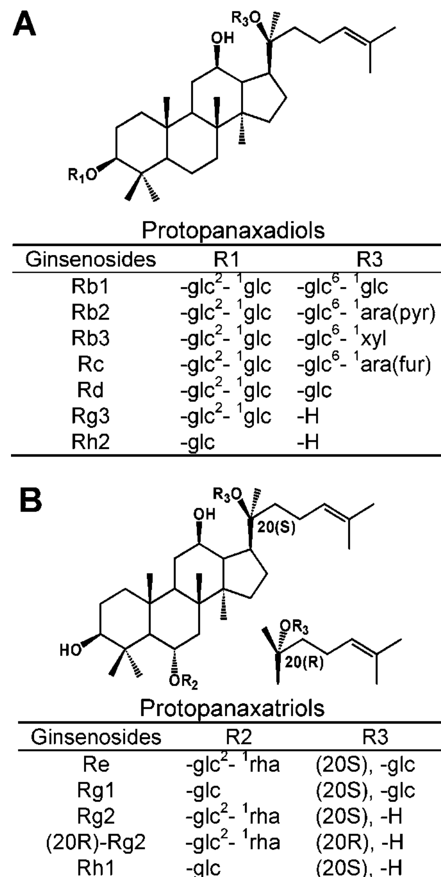
Reagents and Standards. HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Norcross, GA). Milli Q water was supplied by a water purification system (US Filter, Palm Desert, CA). Standards of ginsenosides Rb_1, Rb_2, Rc, Rd, Re, and Rg_1 were purchased from Indofine Chemical Co. (Somerville, NJ); ginsenosides Rb3, Rg2, 20(R)-Rg2, Rg3, Rh1, and Rh2 were purchased from Delta Information Center for Natural Organic Compounds (Xuancheng, AH, China). All standards were of biochemical reagent grade and at least 95% pure as confirmed by HPLC.

Sample Preparation. The preparation process for the sample was as follows: Dried American ginseng roots, rootlets, leaves, and berries were crushed into powder before 0.5 g of the sample was extracted with methanol in a Soxhlet extractor for 8 h. The extract was condensed, transferred into a 25 mL volumetric flask, and diluted to the desired volume with methanol. One or 2.0 mL of the solution was purified by solid-phase extraction (SPE). A Sep-Pak C8 Vac 3 cm^3 500 mg cartridge (Waters, Milford, MA) was employed. The cartridge was placed on a SPE vacuum manifold, cleaned with 2 mL of methanol, and then preconditioned with 4 mL of water. The methanol extract solution was dried by spraying with nitrogen in a 50 °C water bath. The residue was dissolved with water, and then, the solution was applied to the SPE cartridge, washed with 5 mL of water, and dried with air. The ginsenosides were then slowly eluted from the cartridge with 2 mL of methanol into a glass vial; the elution was evaporated to 1 mL with a gentle stream of nitrogen and stored at 4 °C until HPLC analysis.

Analytical Methods. The HPLC system was a Waters 2960 instrument, with a quaternary pump, automatic injector, a photodiode array detector (model 996), and Waters Millennium[32] software for peak identification and integration. The separation was carried out on a Prodigy ODS(2) column (150 mm × 4.6 mm i.d.) (Phenomenex, Torrance, CA) with a guard column (Phenomenex Prodigy, 3.0 mm × 4.0 mm i.d.). Another column used for comparison was the Gemini C18 column (250 mm × 4.6 mm i.d.) (Phenomenex). For HPLC analysis, a 20 μL sample was injected into the columns and eluted at room temperature with a constant flow rate of 1.0 mL/min. Acetonitrile (solvent A) and water (solvent B) were used. Gradient elution started with 20% solvent A and 80% solvent B, changed to 20.3% A for 25 min, then was changed to 26.8% A for 3 min and held for 26 min; changed to 35.6% A for 20 min; changed to 50% A for 11 min; changed to 68% A for 10 min; changed to 95% A for 1 min and was held for 3 min; changed to 20% A for 3 min and was held for 8 min. The detection wavelength was set to 202 nm. All solutions were filtered through Millex 0.2 μm nylon membrane syringe filters (Millipore Co., Bedford, MA) before use.

RESULTS AND DISCUSSION

The chemical structures of 12 ginsenosides are shown in **Figure 1**. We developed an analytical method that determined



Protopanaxadiols

Ginsenosides	R1	R3
Rb1	-glc^{2}- ^{1}glc	-glc^{6}- ^{1}glc
Rb2	-glc^{2}- ^{1}glc	-glc^{6}- ^{1}ara(pyr)
Rb3	-glc^{2}- ^{1}glc	-glc^{6}- ^{1}xyl
Rc	-glc^{2}- ^{1}glc	-glc^{6}- ^{1}ara(fur)
Rd	-glc^{2}- ^{1}glc	-glc
Rg3	-glc^{2}- ^{1}glc	-H
Rh2	-glc	-H

Protopanaxatriols

Ginsenosides	R2	R3
Re	-glc^{2}- ^{1}rha	(20S), -glc
Rg1	-glc	(20S), -glc
Rg2	-glc^{2}- ^{1}rha	(20S), -H
(20R)-Rg2	-glc^{2}- ^{1}rha	(20R), -H
Rh1	-glc	(20S), -H

Figure 1. Chemical structure of 12 ginsenosides. (**A**) Protopanaxadiols type and (**B**) protopanaxatriols type. Abbreviations for carbohydrates are as follows: Glc, β-D-glucose; Ara(pyr), α-L-arabinose(pyranose); Ara(fur), α-L-arabinose(furanose); Xyl, β-D-xylose; and Rha, α-L-rhamnose.

these 12 ginsenosides simultaneously using a 150 mm × 4.6 mm i.d. reverse phase column. To date, there have been few reports on the separation of ginenosides using a reverse phase column 150 mm long, and only seven ginsenosides have been analyzed with such a column (*12*). To increase the resolution of separation, buffers are usually used in these elution systems (*17*). However, because salts are in the eluent, the buffer cannot be stored in the column for a long time, and a complex, time-consuming process has to be conducted each time the assay is complete. To avoid buffer, we used acetonitrile and water, two simple, easily prepared eluents that are safe for the column.

To increase the load capacity and extend the pH stability, many chromatographic companies are developing new HPLC columns. The Phenomenex Gemini C18 column is one such column. Using this type of analytical column, many of the 12 selected ginsenosides can be well-separated, with the exception of ginsenosides Rc, Rh1, Rg2, and 20(R)-Rg2. After modifying the chromatographic condition carefully, the separate resolution of the four ginsenosides was unacceptable (**Figure 2a**). Thus, the Phenomenex Gemini C18 column cannot be used to separate these four ginsenosides using the elution system of acetonitrile and water.

Another analytical column, the Phenomenex Prodigy column, was employed to assay the ginsenosides. This 150 mm × 4.6 mm i.d. ODS(2) column has different characteristics than the Gemini column. Because the retention times of ginsenosides Rh1, Rg2, and 20(R)-Rg2 are between those of ginsenosides Rb1 and Rc, the former could not be separated by the Gemini column. Because the retention times of Rh1, Rg2, and 20(R)-Rg2 preceded those of ginsenoside Rb1, we thought separation

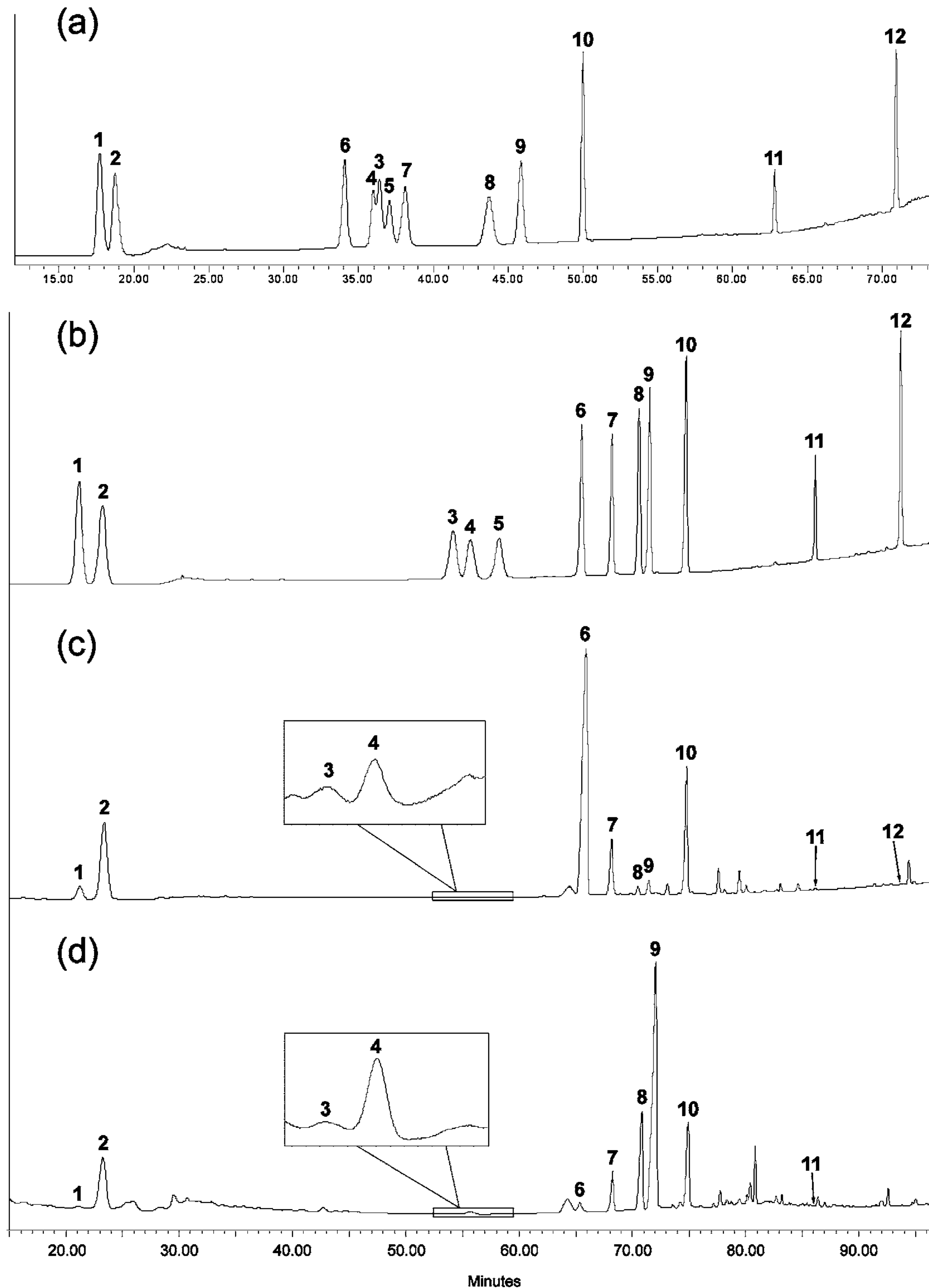


Figure 2. HPLC-UV chromatograms for the determination of ginsenosides in American ginseng samples: (**a**) mixed standard solution separated with Gemini C18 column and (**b**–**d**) analyses using a Prodigy ODS(2) column. Analytical samples: (**b**) mixed standard solution, (**c**) American ginseng root, and (**d**) American ginseng berry. Ginsenoside peaks: 1, Rg1; 2, Re; 3, Rh1; 4, Rg2; 5, 20(R)-Rg2; 6, Rb1; 7, Rc; 8, Rb2; 9, Rb3; 10, Rd; 11, Rg3; and 12, Rh2.

was possible with the Prodigy column. The eluent composition was changed to separate these three ginsenosides. When the gradient of elution was kept at 27.5% acetonitrile, ginsenosides Rh1 and Rg2 could not be separated. However, when the gradient was changed to 26% acetonitrile, the three ginsenosides could be well-separated at 35 min, but the peaks were too broad. With 26.8% acetonitrile, conditions for separation were better. **Figure 2b** shows the HPLC chromatogram of ginsenoside standards using the modified program, which is described in the Materials and Methods. This result demonstrates the applicability of the proposed method to the separation of 12 ginsenosides.

From pharmaceutical studies, it is evident that ginsenosides Rh1, Rh2, and Rg2 are becoming more and more important (*22*−*24*). However, in analytical reports, ginsenosides Rg2 and Rh1 were difficult to separate from ginsenoside 20(R)-Rg2 in HPLC. To simultaneously assay ginsenoside Rh2, a longer column (250 mm × 4.6 mm i.d.) and more time for elution

Table 1. Retention Time, Linear Regression, and Linear Range of 12 Ginsenosides from Our HPLC-UV Method

compound	retention time (min)	regression equations $Y = bX + a$	correlation coefficiency (R^2)	linear range (μg/mL)
Rg1	21.2	$Y = 390.2X + 964.9$	0.9998	2.8–200
Re	23.2	$Y = 327.9X - 5218.4$	0.9998	2.8–997
Rh1	54.3	$Y = 492.3X + 3255$	0.9999	1.3–94
Rg2	55.7	$Y = 348.3X + 1835$	0.9999	1.5–104
20R-Rg2	58.2	$Y = 377.8X + 250.6$	1.0000	1.3–90
Rb1	65.5	$Y = 280.4X + 7452$	0.9999	2.8–993
Rc	68.1	$Y = 232.2X + 4250.7$	0.9999	2.8–197
Rb2	70.6	$Y = 270.0X + 1854.5$	1.0000	1.4–94
Rb3	71.7	$Y = 259.6X + 1695.3$	0.9999	2.8–990
Rd	74.8	$Y = 325.2X + 6345.9$	0.9999	2.8–199
Rg3	86.1	$Y = 416.2X + 9029.3$	0.9999	2.7–189
Rh2	93.7	$Y = 461.0X + 2623.2$	0.9999	1.3–94

Table 2. Ginsenoside Content (Average ± Standard Deviation; Percentage) in Different Parts of American Ginseng[a]

ginsenosides	American ginseng samples			
	root	rootlet	leaf	berry
Rg1	0.157 ± 0.005	0.199 ± 0.007	0.672 ± 0.026	0.044 ± 0.005
Re	1.196 ± 0.024	1.677 ± 0.038	1.778 ± 0.048	1.663 ± 0.054
Rh1	0.003 ± 0.001	0.005 ± 0.001	0.016 ± 0.002	0.004 ± 0.001
Rg2	0.013 ± 0.001	0.022 ± 0.003	0.044 ± 0.002	0.073 ± 0.002
20(R)-Rg2				
Rb1	3.419 ± 0.052	4.600 ± 0.138	0.102 ± 0.007	0.175 ± 0.011
Rc	0.522 ± 0.013	1.595 ± 0.014	0.384 ± 0.018	0.680 ± 0.019
Rb2	0.060 ± 0.003	0.217 ± 0.006	1.194 ± 0.039	1.416 ± 0.026
Rb3	0.112 ± 0.010	0.345 ± 0.021	4.707 ± 0.106	5.348 ± 0.086
Rd	0.871 ± 0.031	2.034 ± 0.023	3.701 ± 0.129	0.847 ± 0.038
Rg3	0.004 ± 0.001	0.013 ± 0.001	0.022 ± 0.001	0.003 ± 0.001
Rh2	0.002 ± 0.001			
total	6.36	10.71	12.62	10.25

[a] $n = 3$ determinations; blank cell, not detected.

were necessary (*14*). In the present study, a gradient elution program enabled the separation of ginsenosides Rg2, 20(R)-Rg2, and Rh1. Also, the separation time of the 12 ginsenosides, including ginsenoside Rh2, was shortened to less than 95 min. These data indicate that the separation ability of 150 mm × 4.6 mm C18 analytical column can be extended to complex herbal constituents.

The linearity of the method was assayed by analyzing standard solutions in the range of 3−1000 μg/mL for ginsenosides Rb1, Rb3, and Re; 3−200 μg/mL for ginsenosides Rc, Rd, Rg1, and Rg3; and 1.5−100 μg/mL for ginsenosides Rb2, Rg2, Rh1, Rh2, and 20(R)-Rg2. Correlation coefficients (R^2) were ≥0.9998 (**Table 1**). These linear ranges are well-suited to amounts usually found in American ginseng (*11*, *25*). Recovery experiments were conducted to determine the accuracy of the SPE for the quantification of the 12 ginsenosides. The recovered ginsenosides of an American ginseng root extract were spiked at three levels of 0.01, 0.05, and 0.1 mg/mL for 12 ginsenosides and were between 89.5 and 97.2% ($n = 5$). The relative standard deviations (RSD) are less than 9.0% for any of these 12 ginsenosides.

Because the compositions of herbal medicine extracts are very complex, sample preparation is a key procedure in HPLC assay (*26*, *27*). The American ginseng samples were extracted with Soxhlet extractors using methanol. The ginseng extract solution was injected directly for the HPLC assay in some reports (*11*, *17*). However, because many hydrophilic compounds such as saccharides, phenolic acids, and water soluble pigments can interfere with the analysis, liquid−liquid extraction is the normal method to purify extracts before HPLC analysis (*10*, *28*). This method is time-consuming and not easy to manipulate. SPE is another widely used sample purification technique in pharmaceutical research (*29*). Although American ginseng roots and rootlet extracts can be assayed without purification, they cannot be determined in American ginseng leaves and berries before purification because of the many other constituents influencing the determination of ginsenosides. After treatment with a Waters Sep-Pak C8 solid extraction column, the extracts were purified, and the accuracy of the analysis was guaranteed.

American ginseng contains a variety of saponins, mainly dammarane saponins, which have been proven to be active constituents for the treatment of many diseases. Some trace saponins show special pharmacological activities. In this study, an analysis of 12 ginsenosides, including six trace ginsenosides [ginenosides Rb3, Rg2, 20(R)-Rg2, Rg3, Rh1, and Rh2] (*30*, *31*), in American ginseng samples gave a good indication of the distribution of trace ginsenosides in the different parts of the American ginseng plant. The HPLC-UV chromatogram of the American ginseng berry was much different from the chromatogram of the root (**Figure 2c**,**d**). The results of the analysis, shown in **Table 2**, indicate that the content of ginsenosides is diverse among the different parts of the American ginseng plant.

In the United States, only the main roots of American ginseng are commercially available, while the rootlets, leaves, and berries of the plant are considered as byproducts. In this study, as compared with the rootlets, leaves, and berries, we found that the total ginsenoside content in the main root was relatively lower. Thus, the byproducts are good sources of total ginsenosides.

As for the individual ginsenosides, ginsenoside Re is present in similar amounts in the different parts of American ginseng. The ginsenoside Rb1 content, which is the main constituent of the roots and rootlets, is low in the leaves and berries. In contrast with Rb1, the ginsenoside Rb2 content is lower in the roots and rootlets but higher in the leaves and berries. In the 12 assayed ginsenosides, 20(R)-Rg2 was not detected in any parts of American ginseng. This result is similar with other reports (*8*, *11*). Not only is the total saponin content different between the underground and the aboveground parts of American ginseng, but also the proportions of individual ginsenosides are different.

An important result of this study was the determination of the content of ginsenoside Rb3 in different samples. Although some HPLC assays have determined the ginsenoside content of American ginseng leaves and berries, only ginsenosides Rb1, Rb2, Rc, Rd, Re, and Rg1 had been determined (*25*, *32*, *33*). Focusing on the analysis of trace saponins, the assay data from the present study showed that the content of ginsenoside Rb3 was much lower in American ginseng roots and rootlets and significantly higher in the leaves and berries. This single saponin, ginsenoside Rb3, makes up 52% of the total ginsenosides in American ginseng berries (**Table 2**).

Ginsenoside Rb3 enhances neurite outgrowth in human neuroblastoma cells (*20*). In one study of neuroprotective effect of ginseng saponins, ginsenoside Rb3 was recognized as the most potent in promoting learning and memory (*21*). Another study found that a new dammarane glycoside derived from ginsenoside Rb3 showed a cytotoxic effect (*34*). Although the content of ginsenoside Rb3 in the roots of Asian ginseng and American ginseng is low, our study indicates that American ginseng leaves and berries are good sources of ginsenoside Rb3.

Our laboratory previously reported some pharmacological effects of American ginseng leaf and berry extracts (*33*, *35*, *36*). We also tested the effect on central nervous system activity and brain protection of ginsenoside Rb_1 (*37*). Because the main constituent of American ginseng leaves and berries is ginsenoside Rb3, in the future, we will focus our studies on the neuroprotective effect of ginseng leaf and berry extracts.

In this study, a simple HPLC-UV method was developed for the simultaneous determination of 12 ginsenosides in different parts of the American ginseng plant. The separation of selected ginsenosides, especially ginsenosides Rg2, 20(R)-Rg2, and Rh1, with a 150 mm × 4.6 mm i.d. C18 analytical column was successful. This result extends the potential of short analytical columns for the separation of complex botanical constituents. Twelve ginsenosides were separated within less than 95 min. The linear range of regression equations was extended, suited to different American ginseng sample assays. SPE guaranteed the accuracy and efficiency of the analysis. The method was successfully used to determine ginsenosides in American ginseng leaves, berries, roots, and rootlets. This is the first report indicating that the content of ginsenoside Rb3 was very high in American ginseng leaves and berries; thus, the leaves and berries could be new sources of ginsenoside Rb3. In addition, our analytical method has potential usage of other phytochemical laboratories for searching new trace ginsenosides.

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