# Determination of Polyacetylenes and Ginsenosides in *Panax* Species Using High Performance Liquid Chromatography

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**A new HPLC method was developed to separate and identify three polyacetylenes (panaxynol, panaxydol and 1,8-heptadecadiene-4,6-diyne-3,10-diol) found in *Panax* species. The mobile phase was a linear gradient of 2 : 1 : 3 to 2 : 1 : 1 (v/v/v) methanol/acetonitrile/water in 40 min. HPLC analysis was performed at a flow rate of 1.5 ml/min with UV detection at 254 nm. The contents of the polyacetylenes and ginsenosides in *Panax* ginseng (white ginseng and red ginseng), *P. quinquefolium*, *P. japonicus*, and *P. noteginseng* were determined using these methods. The species containing the highest polyacetylene content (0.080%) was *P. quinquefolium* cultivated in Nagano, Japan. Meanwhile, the species with the highest ginsenoside content (9.176%) was *P. noteginseng* cultivated in Yunnan, China.**

**Key words** polyacetylene; ginsenoside; *Panax* species; HPLC; validation

*Panax* species have been traditionally used as medicinal herbs since ancient times. In the Japanese Pharmacopoeia XIV, *Panax ginseng* (white[1] and red ginseng[2]), and *P. japonicus*[3] are listed, and are called "Ninjin," "Kojin" and "Chikusetsu-ninjin," respectively. The roots of *Panax* species are known to contain various dammarane saponins, including ginsenosides $Rb_1$, $Rb_2$, Rc, Rd, Re and $Rg_1$.

Recently, more than 10 polyacetylenes were isolated from white ginseng and *P. quinquefolium*,[4—8] some of which exhibit anti-inflammatory properties,[9] anti-platelet action,[10] inhibition of lipoxygenase,[11] cytotoxic activity against leukemia cells,[12] inhibition of 15-hydroxyprostaglandin dehydrogenase,[13] and inhibition of aggregation, release reaction, and thromboxane formation.[14] Despite the usefulness of the polyacetylene compounds in *Panax* plants, only one quantification method by GC has been reported.[15] Therefore, we developed and validated an HPLC method for determination of three polyacetylenes (panaxynol, panaxydol and 1,8-heptadecadiene-4,6-diyne-3,10-diol) and six ginsenosides $Rb_1$, $Rb_2$, Rc, Rd, Re and $Rg_1$.

## Experimental

**Herb Materials** Fifteen samples (white ginseng (W1—W8), red ginseng (R1—R3), *P. japonicus* (J1—J2) and *P. noteginseng* (N1—N2)) were collected from a Japanese market (supplied from Tochimoto Tenkaido Co., Ltd.). Lot No. of W4, W5, W6, W7, W8, R1, R2, R3, N1 and N2 were 0003G086-2, 0003G087, 9912C086, 9912C087, 9912C088, 0102034-1, 0102034-8, 0008C034, 0110C297 and 0110C298, respectively (Lot No. of W1, W2, W3, W4, J1 and J2 were unknown). Two samples of *P. quinquefolium* (Q1—Q2) were collected from Nagano Agricultural Technique and Training Center in Japan.

**Polyacetylenes Reference Standards** Powdered roots of *P. ginseng* (750 g) were extracted with *n*-hexane (3×1000 ml). The solvent was evaporated under reduced pressure. The concentrated extract was applied to a silica gel column (4 cm i.d.×20 cm, *n*-hexane/EtOAc, 19 : 1 to 4 : 1) to give 71.3 mg of panaxynol, and fractions 10—15. Fractions 10—11 were evaporated under reduced pressure to dryness, dissolved in benzene/EtOAc (5 : 1) and applied to a silica gel column (2 cm i.d.×20 cm, benzene/EtOAc, 5 : 1) to give 163 mg of panaxydol. Fractions 12—15 also evaporated under reduced pressure to dryness and applied to a silica gel column (1 cm i.d.×20 cm, benzene/EtOAc, 4 : 1) to give 14.7 mg of 1,8-heptadecadiene-4,6-diyne-3,10-diol. Identity and purity of the compounds were confirmed by chromatographic method (TLC and HPLC) and by a comparison with published spectrum data ($^1$H- and $^{13}$C-NMR, and CIMS).[4—7]

**Ginsenoside Reference Standards** Ginsenosides $Rb_1$, Rc, Rd, Re and $Rg_1$ were purchased from Waco Pure Chemicals Industries, Ltd., and ginsenoside Rb2 was purchased from Toray Co., Ltd.

**Reagents for Analysis** Acetonitrile and methanol of HPLC grade, and phosphoric acid and naphthalene of analytical reagent grade were purchased from Waco Pure Chemicals Industries, Ltd. Solutions were prepared in puri-

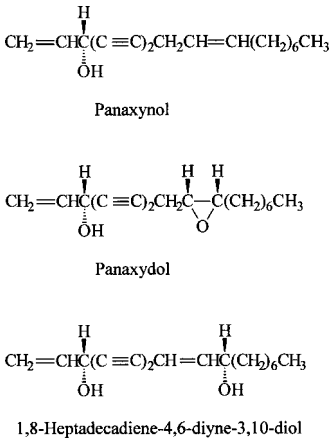
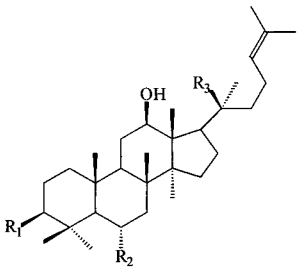



Fig. 1. Chemical Structures of Polyacetylenes




| Name | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| $Rb_1$ | O-Glc$^{2\text{-}1}$Glu | H | O-Glc$^{6\text{-}1}$Glc |
| $Rb_2$ | O-Glc$^{2\text{-}1}$Glu | H | O-Glc$^{6\text{-}1}$Ara(p) |
| Rc | O-Glc$^{2\text{-}1}$Glc | H | O-Glc$^{6\text{-}1}$Ara(f) |
| Rd | O-Glc$^{2\text{-}1}$Glc | H | O-Glc |
| Re | OH | O-Glc$^{2\text{-}1}$Rha | O-Glc |
| $Rg_1$ | OH | O-Glc | O-Glc |

Fig. 2. Chemical Structures of Ginsenosides

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fied water (filtrated through a 0.45 $\mu$m filter), which was prepared in our laboratory.

**Sample Preparation for Polyacetylene Quantification** The finely powdered sample (about 1 g) was extracted three times with 20 ml methanol using a sonicator for 30 min at 40 °C. After centrifugation, the supernatant was concentrated under reduced pressure. The residue was dissolved in 5.00 $\mu$g/ml methanolic naphthalene. The solution was transferred into a 10-ml volumetric flask and adjusted to final volume with 5.00 $\mu$g/ml methanolic naphthalene solution.

**Validation of Polyacetylene Quantification** Precision: Six samples from the same batch were extracted and processed in accordance with the sample preparation procedures for polyacetylene quantitative analysis. Recovery: Samples in triplicate were spiked with know amounts of the polyacetylenes (50 $\mu$g each), and then extracted. Linearity: Calibration curves for HPLC were created with six standard solutions in a concentration range of 0.1 to 100 $\mu$g/ml. Qualification and detection limits: The limits were calculated according to analytical method validation procedures described in JP XIV.[16]

**HPLC Conditions for Polyacetylene Quantification** For HPLC, the column used was LiChrosorb RP-18 (4.6 mm i.d.×250 mm) with a gradient mobile phase of 2 : 1 : 3 to 2 : 1 : 1 acetonitrile/methanol/water in 40 min. The flow rate was 1.5 ml/min, column temperature was 45 °C, and the injection volume was 20 $\mu$l. Detection was accomplished by monitoring at 254 nm. Retention times ($t_R$) were 19.6 min for 1,8-heptadecadiene-4,6-diyne-3,10-diol, 22.7 min for panaxydol and 36.1 min for panaxynol.

**Sample Preparation of Ginsenoside Quantification** The finely powdered sample (about 1 g) was extracted three times with 20 ml 80% methanol using a sonicator for 60 min at 40 °C. After centrifugation, the supernatant was concentrated under reduced pressure. The residue was dissolved in 80% methanol. The solution was transferred into a 10-ml volumetric flask and adjusted to final volume with 80% methanol. A calibration curve containing three data points using external standard solutions was obtained.

**Validation of Ginsenoside Quantification** Precision: Six samples from the same batch were extracted and processed in accordance with sample preparation procedures for ginsenoside quantification analysis (RSD≦4.5%). Recovery: Triplicate samples were spiked with know amounts of the ginsenosides (500 $\mu$g each), and then extracted (97.7—102.9%). Linearity: Calibration curves for HPLC were created with six standard solutions in a concentration range 10 to 5000 $\mu$g/ml ($r^2$≧0.998). Quantification and detection limits: The limits were calculated according to analytical method validation procedures described in JP XIV.[16] These limits were shown in Table 3.

**HPLC Conditions for Ginsenoside Quantification** For HPLC, the column used was LiChrosorb RP-18 (4.6 mm i.d.×250 mm) with a mobile phase of 31 : 69 acetonitrile/0.5% phosphoric acid for the ginsenoside Rb group ($Rb_1$, $Rb_2$, Rc and Rd) and 20 : 80 acetonitrile/0.5% phosphoric acid for the ginsenoside Rg group (Re and $Rg_1$). The flow rate was 1.0 ml/min, column temperature was 40 °C, and the injection volume was 10 $\mu$l. Detection was accomplished by monitoring at 203 nm. Retention times ($t_R$) were 16.8 min for $Rb_1$, 26.7 min for $Rb_2$, 21.1 min for Rc, 43.4 min for Rd, 28.8 min for Re and 26.5 min for $Rg_1$.

## Results and Discussion

**Validation of Polyacetylene Quantification** The chromatogram of *Panax* extract is shown in Fig. 3. The three polyacetylenes were separated and identified in same run, with retention times 19.6 min for 1,8-heptadecadiene-4,6-diyne-3,10-diol, 22.7 min for panaxynol, and 36.1 min for panaxydol. Elution of all three compounds within 40 min was possible after carefully assessing the performance of several mobile phases. An isocratic mobile phase was inadequate because of the low polarity of panaxynol. Therefore, a linear gradient mobile phase system was necessary. First, combination of simple organic solvents (methanol, acetonitrile or ethanol) and water were investigated. However, 1,8-heptadecadiene-4,6-diyne-3,10-diol and panaxydol could not be separated under those conditions. Therefore, combinations of mixed organic solvents and water were investigated. A good separating condition could be found using the combination 2 : 1 methanol/acetonitrile as an effective organic solvent phase, and programmed flow rate of 50 : 50 to 75 : 25 organic solvent/water in 40 min in a linear gradient. Naphthalene, with a retention time of 11.9 min, was chosen as the internal standard (Fig. 3).

The validation results (precision, recovery, linearity, and quantification and detection limits) are shown in Table 1. The validation study demonstrated the suitability of this method for polyacetylene quantification. Precision was indicated by relative standard deviation (RSD), which was not more than 4.7% for each compound. The recovery rates ranged from 92.5% to 101.6% indicated that the sample treatment did not result in loss of the drug.

**Validation of Ginsenoside Quantification** Over 30 types of saponins are known to be present in *Panax* species. In our work, commercially available ginsenoside saponins ($Rb_1$, $Rb_2$, Rc, Rd, Re and $Rg_1$) were quantified. HPLC conditions were modified per the method reported by Yamaguchi

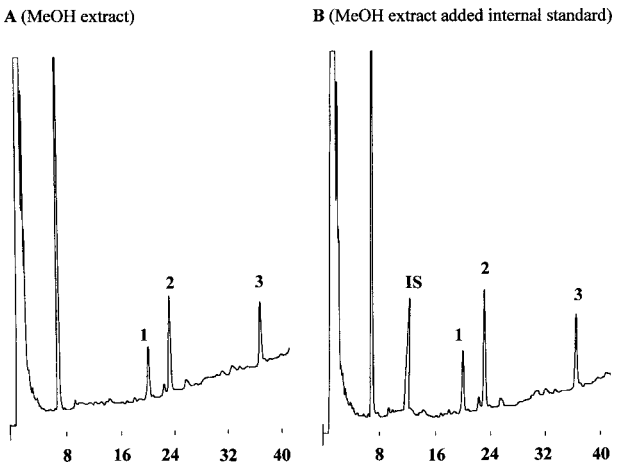


Fig. 3. HPLC Chromatograms of Methanolic *P. ginseng* Extract

Column: LiChrosorb RP-18 (7 $\mu$m, 4.6 mm i.d.×250 mm); Mobile phase: $CH_3CN$–$CH_3OH$–$H_2O$ (Liner gradient, 2 : 1 : 3→2 : 1 : 1, 40 min); flow rate: 1.5 ml/min; detection: 254 nm; Injection volume: 20 $\mu$l; Column temperature: 45 °C. IS: naphthalene (internal standard), 1: 1,8-heptadecadiene-4,6-diyne-3,10-diol, 2: panaxydol, 3: panaxynol.

Table 1. Validation Results of HPLC Method for Quantification of Three Polyacetylenes

| | | Panaxynol | Panaxydol | 1,8-Heptadecadiene-4,6-diyne-3,10-diol |
|---|---|---|---|---|
| Precision[a] | RSD % | 4.7 | 1.7 | 4.5 |
| Recovery[a] | Mean % (RSD %) | 92.5 (4.7) | 101.6 (2.9) | 95.2 (2.1) |
| Linearity[b] | Correlation coefficient $r^2$ | 0.997 | 0.996 | 0.998 |
| Quantification limit | Concentration $\mu$g/ml (in the drug %) | 1.83 (0.002) | 1.53 (0.002) | 1.55 (0.002) |
| Detection limit | Concentration $\mu$g/ml (in the drug %) | 0.56 (0.001) | 0.51(0.001) | 0.51 (0.001) |

*a*) Six injections of one sample. *b*) Concentration range (in the drug): 0.1—100 $\mu$g/ml (1—1000 $\mu$g/g).

*et al.*[17] Chromatograms of *Panax* extracts are shown in Fig. 4. Assay validation of precision, recovery, linearity, and quantification and detection limits was conducted in a manner similar to that used for the polyacetylenes. The detection limits of these ginsenosides were 0.033—0.056 $\mu$g (calculated into $\mu$g unit) in this study. The detection limits of these ginsenosides were reported to be 0.3 $\mu$g by Soldati *et al.*[18] and 0.09—0.20 $\mu$g by Samukawa *et al.*[19] Therefore, our method was excellent with regard to detection ability of ginsenosides.

**Quantifications of Polyacetylenes and Ginsenosides in *Panax* Species** Our validated methods were used successfully to analysis polyacetylenes and ginsenosides in several different *Panax* species. Polyacetylene content is shown in Table 2. Panaxydol was the dominant compound and was found in all of the samples. Three polyacetylenes were detected in white ginseng, red ginseng, *P. quinquefolium* and *P. noteginseng*; however, panaxynol was not detected in *P. japonicus*. Total polyacetylene content of white ginseng (W1—W8) was 0.020—0.073%, red ginseng (R1—R3) contained 0.019—0.055%, *P. quinquefolium* (Q1—Q2) 0.067—0.080%, *P. japonicus* (J1—J2) 0.004—0.006%, and *P. noteginseng* (N1—N2) 0.045—0.056%. Red ginseng is manufactured by steaming raw ginseng root. Polyacetylenes are considered unstable under conditions of high temperature and humidity. However, the polyacetylene content of red ginseng was similar to that of white ginseng, it suggested that polyacetylene compounds do not decompose during the steaming process.

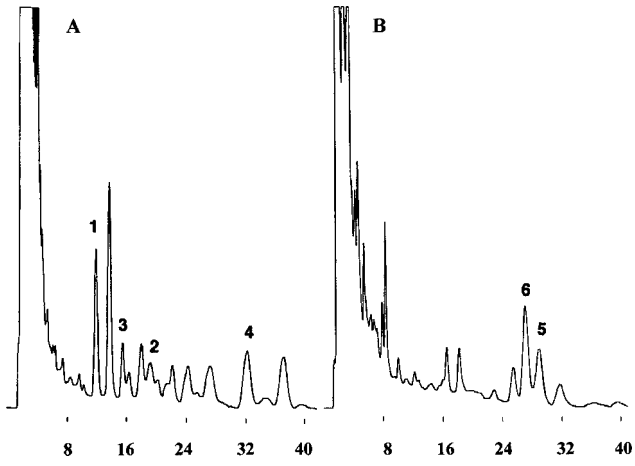


Fig. 4. HPLC Chromatograms of 80% Methanolic *P. ginseng* Extract

Column: LiChrosorb RP-18 (7 $\mu$m, 4.6 mm i.d.×250 mm); Mobile phase: $CH_3CN$–0.5% $H_3PO_4$ (A: 31 : 69, B: 20 : 80); flow rate: 1 ml/min; detection: 203 nm; Injection volume: 10 $\mu$l; Column temperature: 40 °C. 1: ginsenoside $Rb_1$, 2: ginsenoside $Rb_2$, 3: ginsenoside Rc, 4: ginsenoside Rd, 5: ginsenoside Re, 6: ginsenoside $Rg_1$.

Ginsenoside content of the samples is shown in Table 3. Total ginsenoside content of white ginseng (W1—W8) was 1.072—3.029%, red ginseng (R1—R3) contained 1.588—2.811%, *P. quinquefolium* (Q1—Q2) 5.582—6.024%, *P. japonicus* (J1—J2) 0.230—0.345%, and *P. noteginseng* (N1—N2) 8.661—9.276%. Six ginsenosides were quantified in white ginseng, red ginseng, and *P. quinquefolium*. However, *P. japonicus* did not contain ginsenosides $Rb_2$, Rc and Rd. The ginsenoside content of *P. noteginseng* was much higher than that found in other *Panax* species, although ginsenosides $Rb_2$ and Rc were not detected. In *Panax quinquefolium* and *P. noteginseng*, the polyacetylene contents were high as well as ginsenoside contents. In contrast, the ginsenoside content of *P. japonicus* was as low as its polyacetylene content. From these qualification results, the correlation of the polyacetylenes with the ginsenosides contents was not found. Therefore, in the case *Panax* plants are used for not only the ginsenosides but also the polyacetylenes bioactivities, it is possible to evaluate *Panax* plants quality by using these HPLC methods.

In conclusion, we developed a validated HPLC method useful for determination of three polyacetylenes and six ginsenosides in *Panax* species, which will be helpful for quality control of the *Panax* plants.

Table 2. Polyacetylene Contents (w/w %) of Roots of *Panax* Plants

| Sample No. | Source (region) | Panaxynol | Panaxydol | 1,8-Heptadecadiene-4,6-diyne-3,10-diol | Total |
|---|---|---|---|---|---|
| W1 | Japan (Nagano) | 0.002 (7.3) | 0.017 (0.9) | 0.001 (5.0) | 0.020 (1.7) |
| W2 | Korea | 0.008 (1.7) | 0.018 (3.2) | 0.001 (3.7) | 0.027 (2.9) |
| W3 | Korea | 0.006 (1.3) | 0.022 (3.9) | <QL | 0.028 (3.0) |
| W4 | China (Jilin) | 0.007 (6.1) | 0.016 (2.1) | 0.002 (3.1) | 0.025 (3.4) |
| W5 | China (Jilin) | 0.007 (4.8) | 0.063 (3.4) | 0.003 (3.8) | 0.073 (4.0) |
| W6 | China (Heilongjiang) | 0.010 (3.2) | 0.011 (3.2) | 0.005 (4.8) | 0.026 (3.4) |
| W7 | China (Heilongjiang) | 0.004 (4.1) | 0.014 (3.8) | 0.002 (3.5) | 0.020 (3.8) |
| W8 | China (Heilongjiang) | 0.009 (2.7) | 0.052 (3.6) | 0.003 (2.7) | 0.064 (3.0) |
| R1 | Japan (Nagano) | 0.015 (1.2) | 0.037 (4.2) | 0.003 (3.8) | 0.055 (4.0) |
| R2 | Japan (Nagano) | 0.003 (6.3) | 0.016 (3.2) | <QL | 0.019 (2.8) |
| R3 | China (Jilin) | 0.008 (3.9) | 0.030 (2.7) | 0.003 (4.1) | 0.041 (3.8) |
| Q1 | Japan (Nagano) | 0.015 (2.0) | 0.049 (1.2) | 0.003 (4.9) | 0.067 (3.1) |
| Q2 | Japan (Nagano) | 0.022 (2.6) | 0.053 (4.8) | 0.005 (1.9) | 0.080 (2.3) |
| J1 | Japan (Yamagata) | ND | 0.004 (5.2) | <QL | 0.004 (4.3) |
| J2 | Japan (Miyazaki) | ND | 0.004 (2.8) | 0.002 (4.0) | 0.006 (3.1) |
| N1 | China (Yunnan) | 0.010 (5.7) | 0.041 (4.4) | 0.005 (1.3) | 0.056 (2.1) |
| N2 | China (Yunnan) | 0.006 (4.0) | 0.036 (5.7) | 0.003 (2.5) | 0.045 (4.4) |

Percentage of compound in the drug (Relative Standard Deviation %) for $n=3$. <QL: under quantification limit, ND: not detected. W1—W8: White ginseng; R1—R3: Red ginseng; Q1—Q2: *P. quinquefolium*; J1—J2: *P. japonicus*; N1—N2: *P. noteginseng*.

Table 3. Ginsenoside Contents (w/w %) of Roots of *Panax* Plants

| Sample No. | Source (region) | Ginsenoside | | | | | | Total |
|---|---|---|---|---|---|---|---|---|
| | | $Rb_1$ | $Rb_2$ | Rc | Rd | Re | $Rg_1$ | |
| W1 | Japan (Nagano) | 0.451 (1.4) | 0.219 (3.6) | 0.422 (4.5) | 0.049 (3.1) | 0.160 (1.8) | 0.368 (4.2) | 1.669 (1.3) |
| W2 | Korea | 0.235 (5.7) | 0.073 (4.7) | 0.270 (4.1) | 0.041 (5.9) | 0.165 (2.6) | 0.288 (5.7) | 1.072 (3.2) |
| W3 | Korea | 0.548 (4.3) | 0.266 (4.3) | 0.278 (2.3) | 0.090 (4.6) | 0.261 (3.3) | 0.318 (1.9) | 1.761 (2.2) |
| W4 | China (Jilin) | 0.614 (4.0) | 0.421 (4.0) | 0.678 (3.8) | 0.085 (3.3) | 0.355 (2.6) | 0.483 (1.1) | 2.636 (1.6) |
| W5 | China (Jilin) | 0.358 (4.6) | 0.189 (4.1) | 0.840 (2.6) | 0.071 (3.5) | 0.418 (3.1) | 0.342 (3.0) | 2.218 (3.3) |
| W6 | China (Heilongjiang) | 0.735 (3.5) | 0.329 (4.5) | 0.865 (1.0) | 0.121 (4.8) | 0.390 (3.0) | 0.589 (3.1) | 3.029 (3.6) |
| W7 | China (Heilongjiang) | 0.861 (2.9) | 0.231 (4.0) | 0.838 (2.8) | 0.072 (2.7) | 0.303 (3.0) | 0.438 (3.3) | 2.743 (3.0) |
| W8 | China (Heilongjiang) | 0.425 (3.7) | 0.161 (3.9) | 0.769 (3.6) | 0.046 (3.6) | 0.245 (3.2) | 0.334 (4.8) | 1.980 (3.7) |
| R1 | Japan (Nagano) | 0.913 (2.9) | 0.240 (3.6) | 0.737 (4.8) | 0.064 (2.9) | 0.276 (4.2) | 0.336 (3.0) | 2.566 (3.3) |
| R2 | Japan (Nagano) | 0.547 (2.7) | 0.109 (4.1) | 0.451 (3.1) | 0.086 (3.2) | 0.114 (4.7) | 0.281 (1.3) | 1.588 (2.9) |
| R3 | China (Jilin) | 1.053 (3.2) | 0.373 (1.4) | 0.833 (4.2) | 0.089 (3.5) | 0.233 (2.2) | 0.230 (1.5) | 2.811 (2.3) |
| Q1 | Japan (Nagano) | 2.269 (3.6) | 0.219 (3.5) | 0.355 (3.6) | 0.319 (2.6) | 2.123 (4.2) | 0.297 (4.2) | 5.582 (3.6) |
| Q2 | Japan (Nagano) | 2.561 (5.1) | 0.194 (0.7) | 0.468 (1.3) | 0.435 (4.8) | 2.070 (1.6) | 0.296 (1.5) | 6.024 (2.8) |
| J1 | Japan (Yamagata) | 0.152 (3.2) | ND | ND | ND | 0.061 (2.9) | 0.017 (3.2) | 0.230 (2.7) |
| J2 | Japan (Miyazaki) | 0.277 (2.2) | ND | ND | ND | 0.063 (4.8) | 0.014 (5.0) | 0.354 (2.9) |
| N1 | China (Yunnan) | 3.814 (3.5) | ND | ND | 0.511 (4.0) | 0.372 (3.4) | 3.964 (2.8) | 8.661 (3.5) |
| N2 | China (Yunnan) | 3.861 (5.0) | ND | ND | 0.631 (0.9) | 0.414 (4.5) | 4.270 (4.0) | 9.176 (3.7) |

Percentage of compound in the drug (Relative Standard Deviation %) for $n=3$. ND: not detected. Qualification (detection) limits % of ginsenosides $Rb_1$, $Rb_2$, Rc, Rd, Re and $Rg_1$ in the drug are 0.010 (0.003), 0.015 (0.005), 0.010 (0.004), 0.014 (0.005), 0.013 (0.006) and 0.013 (0.006), respectively. W1—W8: White ginseng; R1—R3: Red ginseng; Q1—Q2: *P. quinquefolium*; J1—J2: *P. japonicus*; N1—N2: *P. noteginseng*.

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