Simultaneous Determination of 25 Ginsenosides by UPLC-HRMS via Quantitative Analysis of Multicomponents by Single Marker

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A method using UPLC-HRMS has been developed for a rapid, simultaneous qualitative and quantitative analysis of twenty-five ginsenosides. Chromatographic separation was achieved on a C18 analytical column with an elution gradient comprising 0.1% aqueous formate/acetonitrile as the mobile phase. HRMS detection acquired full mass data for quantification and fullms-ddms2 (i.e., data-dependent scan mode) yielded product ion spectra for identification. Furthermore, quantitative analysis of multiginsenosides by single marker (QAMS) was developed and validated using a relative correction factor. Under optimal conditions, we could simultaneously separate eight groups of isomers of the 25 ginsenosides. Good linearity was observed over the validated concentration range for each analyte ($r^2 > 0.9924$), showing excellent sensitivity (LODs, 0.003–0.349 ng/mL) and lower limit quantification (LOQs, 0.015–1.163 ng/mL). The LC-MS external standard method (ESM) and QAMS were compared and successfully applied to analyze the ginsenoside content from Panax ginseng roots. Overall, our UPLC-HRMS/QAMS approach provides high precision, stability, and reproducibility and can be used for high-throughput analysis of complex ginsenosides and quantitative analysis of multiple components and quality control of traditional Chinese medicines (TCM).

1. Introduction

Ginseng (Panax ginseng C.A. Meyer) has been used as an herbal medication for centuries. Ginsenosides, the major pharmacologically active ingredient in ginseng, have a wide range of pharmacological and therapeutic properties, e.g., improving brain function, preventing cancer, enhancing immune responses, and exhibiting antiaging, antiobesity, and antidiabetic effects [1, 2]. Up to now, about 300 ginsenosides have been detected from ginseng [3]. However, it has remained problematic to quantify all ginsenosides, difficult to distinguish among ginsenosides with the same molecular weight, and hard to acquire sufficient numbers of ginsenoside standards for quantification of their chemical and physical properties.

Several methods have been developed to assess ginsenoside content in ginseng. Among these approaches, high-performance liquid chromatography (HPLC) and HPLC-MS are by far the most employed analytical methods [4–8]. However, HPLC-UV has always shown considerable baseline noise and relatively poor sensitivity due to the weak UV absorption of ginsenosides. Moreover, ginsenosides that have been reported were not well separated, leading to inaccurate quantitation. HPLC-MS is a powerful tool for determining ginsenosides in diverse ginseng extracts [9, 10]. For instance, thirteen ginsenosides were simultaneously and quantitatively determined by HPLC-MS [9]. Among the various LC approaches, ultra-performance LC (UPLC) has provided better separations over short periods of time and increased sample throughput and sensitivity [11]. Among different MS analyzers, Orbitrap has provided much better accuracy, precision, and greater mass resolution [12].

In our present study, we report a novel UPLC-Orbitrap-HRMS approach that has been validated for rapid,
simultaneous qualitative/quantitative analysis of twenty-five ginsenosides. UPLC and HRMS methods were optimized to obtain excellent peak separations and maximum signal in the MS detector. Quantitative analysis of complex ginsenosides is difficult in the absence of reference standards. Therefore, a quantitative analysis of the multiginsenoside by single marker (QAMS) was developed and validated using a relative correction factor. The LC-MS external standard method (ESM) and QAMS were compared and successfully applied to analyzing ginsenoside content from Panax ginseng root. The present work has provided a sensitive and accurate approach for elucidating ginsenoside constituents that can also be used for multicomponent quality control of TCMs.

2. Materials and Methods

2.1. Chemicals and Reagents. HPLC-grade acetonitrile and methanol were purchased for Fisher Scientific (Waltham, MA); ultrapure water (18.25 MΩ cm) was prepared by a Milli-Q water system (Millipore, Bedford, USA). Reference ginsenosides, including ginsenosides Rg1, Re, Rf, Rb1, Rg2, Rc, Rh1, Rb2, Rb3, F1, Rd, GXVII, nFe, CO, nFd, F2, G75, Rg3, PPT, Mc, CY, CMx, CK, Rh2, and PPD, were isolated or transformed from ginseng and purified by a series of chromatography procedures in our laboratory. The purity of the twenty-five standards was >95%.

2.2. Liquid Chromatography. Ultra-performance liquid chromatography (UPLC) was performed using an UPLC system ( Dionex Ultimate 3000, Thermo Scientific, USA) equipped with a binary gradient pump, an autosampler, and a thermostatic column compartment. Chromatographic separation was achieved on a HyperSil GOLD C18 column (2.1 mm × 50 mm, 1.9 μm) at a flow rate of 0.35 mL/min. The gradient elution system consisted of water/0.1% formic acid (A) and acetonitrile (B) using the following gradient program: 0–4 min, 23–30% B; 4–8 min, 30% B; 8–8.5 min, 30–35% B; 8.5–12 min, 35% B; 12–12.5 min, 35–39% B; 12.5–16 min, 39% B; 16–17.5 min, 39–41% B; 17.5–20 min, 41–80% B; 20–21 min, 80–95% B; 21–21.5 min, 95–23% B; 21.5–25 min, 23% B. Column temperature was set at 30°C. The temperature of the autosampler was set at 15°C, and the injection volume was 5 μL.

2.3. Mass Spectrometry. The UPLC system coupled to a Q-Orbitrap HRMS (Thermo-Fisher, USA) with ESI operating in the negative ion mode and using full mass and fullsms-ddms2 scan types. The UPLC-Q-Orbitrap-HRMS acquired full MS data for quantification over the scan range of m/z 100–1500. The MS resolution was set at 70,000 FWHM, and the AGC target and the maximum inject time were set at 1.0 e6 and 100 ms, respectively. The fullsms-ddms2 (i.e., data-dependent scan mode) provided product ion spectra for identification using the mass inclusion list. The data-dependent- (dd-1) ms2 resolution was set at 17500 FWHM, and the AGC target was set at 2.0 e6 with a maximum injection time of 100 ms. The top 5 most intense ions were selected to perform MS/MS acquisition. Precursor ions were filtered by the quadrupole, which operates at an isolation window of m/z 4 Da. The normalized collision energy (NCE) of each ginsenoside was optimized by injecting a working mix standard solution at a concentration of 1 μg/mL. UPLC-HRMS data were controlled using Thermo Xcalibur 4.3 software (Thermo Finnigan, San Jose, CA, USA). In addition, the spray voltage was set at 2.8 kV, the capillary temperature was 320°C, and the S-lens RF level was fixed at 50 to obtain the best experimental conditions. Nitrogen was used as the sheath gas with a flow rate set at 40, and the aux gas flow rate set at 10. The aux gas heater temp was 300°C to achieve the highest signal intensity.

2.4. Preparation of Calibration Standards. The standard stock solutions of ginsenoside Rg1, Re, Rf, Rb1, Rg2, Rc, Rh1, Rb2, Rb3, F1, Rd, GXVII, nFe, CO, nFd, F2, G75, Rg3, PPT, Mc, CY, CMx, CK, Rh2 and PPD were prepared by dissolution in methanol at a final concentration of 1.0 mg/mL. Appropriate aliquots of the 25 stock solutions were then mixed to prepare a final standard solution. The stock solution was diluted with methanol to achieve serial working solutions.

2.5. Ginseng Sample Solutions. Freeze-dried ginseng root powder (purchased from Ningbo Gianon Biotech Co., Ltd.) was dissolved in 1 mL methanol and filtered through a 0.22 μm filter for further UPLC-Q-Orbitrap-MS analysis.

2.6. Method Validation

2.6.1. Selectivity. The chromatogram of a blank methanol sample was compared with a mixed ginsenoside standard solution and sample solution to investigate whether the target analytes had the same retention time with other unrelated components present in the sample.

2.6.2. Calibration Curve and Sensitivity. The mixed solution of 25 kinds of single standards with concentrations of 0.001, 0.002, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, and 1.0 μg/mL for mass spectrometer detector was injected in sextuplicate (n = 6). A standard regression line was drawn with the standard sample concentration (x) and peak area (y). The areas under analyte peaks of standards and noise around corresponding analyte peaks were measured by injection samples in sextuplicate (n = 6). The limit of detection (LOD) had a signal-to-noise ratio of 3 (S/N = 3), and the limit of quantitation (LOQ) had a signal-to-noise ratio of 10 (S/N = 10).

2.6.3. Precision and Accuracy. 0.2 μg/mL of mixed ginsenoside standard solutions was prepared and injected six consecutive times (n = 6). The precision obtained by calculating the relative standard deviation (RSD) (set to be less than ±15%) and the accuracy were expressed as a relative error (RE) between measured and targeted values required to be within ±15%.
2.6.4. Stability. To determine sample stability, solutions were prepared for sequential injections at 0, 2, 6, 12, 24, and 48 h \((n = 6)\). Specific peaks over accurate mass ranges were identified, and the area under each ginsenoside peak was recorded. The stability was expressed as RSD%.

2.6.5. Reproducibility. The reproducibility of the method was assessed by preparing 0.2 ug/mL of mixed ginsenoside standard solutions in six replicates \((n = 6)\). After injection, the exact peak with accurate mass range area of each ginsenoside was recorded, and the RSD% was calculated.

2.7. Calculation of Relative Response Factors of Ginsenosides. When a single reference was used to determine multiple components in samples, the concentration of each analyte \((Cx)\) was calculated as the ratio between the peak area of the analyte in the sample solution \((Ax)\) and the peak area of a chosen reference analyte in a standard solution as a unit concentration \((As/Cs)\) and then calibrated by the relative response factor \((RCF)\) of each analyte \((Fx)\). The formula is as follows:

\[
Cx = \frac{Ax}{As/Cs} \times Fx. \tag{1}
\]

The relative response factor \((Fx)\) for each ginsenoside was calculated as the ratio of the peak area in a unit concentration between standards \((As/Cs)\) and the analyte \((Ax/Cx)\):

\[
Fx = \frac{As/Cs}{Ax/Cx}. \tag{2}
\]

It is worth mentioning that the final value of RCF is the average value of multiple RCFs determined using a series of concentrations of the internal reference sample.

For comparison of this new QAMS approach with an ESM, the standard method difference (SMD) was calculated according to the following equations:

\[
SMD = \frac{C_{ESM} - C_{QAMS}}{C_{ESM}} \times 100%, \tag{3}
\]

where \(C_{ESM}\) and \(C_{QAMS}\) are the concentrations of analytes assayed by the external standard and QAMS methods, respectively.

3. Results and Discussion

3.1. Optimization of UPLC Conditions. The separation of mixtures of ginsenosides by chromatography is very difficult. This is especially true for the simultaneous separation of eight groups of isomers \((Rg1/Rf, Re/Rd/\text{GXVII}, Rh1/F1, Rc/Rb2/Rb3, nFe/CO/nFd, Rg2/G75/Rg3/F2, Mc/CY/CMx, and CK/Rh2)\). The use of an appropriate chromatographic column is a key factor for separating ginsenosides. Therefore, two chromatographic columns, including HyperSil GOLD C18 \((2.1 \text{ mm} \times 100 \text{ mm, 1.9 }\mu \text{m})\) and HyperSil GOLD C18 \((2.1 \text{ mm} \times 50 \text{ mm, 1.9 }\mu \text{m})\) columns, were employed and compared as shown in Figure S1 in the Supplementary Materials for comprehensive image analysis. Our results demonstrate that the HyperSil GOLD C18 \((2.1 \text{ mm} \times 50 \text{ mm, 1.9 }\mu \text{m})\) column not only exhibited better resolution and higher peak capacity but also could separate ginsenoside isomers Rg3 and G75. Therefore, the HyperSil GOLD C18 \((2.1 \text{ mm} \times 50 \text{ mm, 1.9 }\mu \text{m})\) column was chosen for this study.

To improve sensitivity and resolution, weak acid has usually been added to the water phase \([13, 14]\). Using acetonitrile as the organic phase, the effect of formic acid as an additive to the aqueous phase was investigated using the HyperSil GOLD C18 \((2.1 \text{ mm} \times 50 \text{ mm, 1.9 }\mu \text{m})\) column. Our results show that the addition of 0.1% aqueous formate/acetonitrile enhanced the intensity of mass signals and improved peak shape, thus providing the optimal choice (Figure S2 in the Supplementary Materials).

The effects of varying flow rate from 0.2 mL/min to 0.35 mL/min were assessed by observing the resolution of each analyte (Figure S3 in the Supplementary Materials). Increasing the flow rate significantly shortened analysis time and improved resolution. At an optimal flow rate of 0.35 mL/min, all isomeric compounds were effectively separated over a short period of time, except for ginsenosides Rg3 and G75. Finally, column temperatures of 30°C, 35°C, and 40°C were investigated. As shown in Figure S4 in Supplementary Materials, increasing the column temperature slightly influenced the retention time and resolution of each ginsenoside. In general, a temperature of 30°C was optimal due to a shorter retention time and better resolution for ginsenosides Rg3 and G75.

Overall, optimal chromatograms for the 25 analytes were obtained within 25 min and showed stable baselines and high resolutions.

3.2. Optimization of ESI-HRMS Conditions. In this study, ESI positive and negative modes were compared. All of the investigated ginsenosides exhibited strong \([M-H]^−\) ions in the negative ion mode but comparatively low-abundance \([M+H]^+\) ions and \([M+Na]^+\) ions in the positive ion mode. Furthermore, the negative ion mode provided more direct structural information, consistent with previous studies \([15, 16]\). In order to rapidly acquire MS/MS fragmentation data for the identification of chemical constituents, the fullms-ddms2 data acquisition mode was used to simultaneously collect the information from both precursor ion and their related fragment ions in a single run. Mass parameters of the spray voltage \((2.0–4.0 \text{kV})\), the capillary temperature \((250°C–350°C)\), and S-lens RF level \((30–70)\) were optimized by infusing standard solutions to achieve maximum responses of precursor ions. Moreover, the sheath gas flow rate \((20–40)\), aux gas flow rate \((5–10)\), and aux gas heater temperature \((250°C–350°C)\) were also optimized manually to achieve the greatest signal intensity. Furthermore, to make optimization easier, the stepped normalized collision energies \((NCE)\) of \((10 \text{ V, 15 V, and 20 V})\), \((10 \text{ V, 20 V, and 35 V})\), and \((20 \text{ V, 30 V, and 40 V})\) were used to obtain representative product ion spectra for each compound.
3.3. Method Validation. To assess the selectivity of this method, chromatograms of a blank methanol sample, ginsenoside standards, and the ginseng sample itself are shown in Figure 1. All ginsenoside isomers were adequately separated on the column with elution times of 1.46 and 4.08 min for Rg1 and Rf (m/z 799), 1.46, 9.25, and 10.07 min for Re, Rd, and GXVII (m/z 945), 5.02 and 6.73 min for Rh1 and F1 (m/z 637), 6.09, 7.13, and 7.59 min for Rc, Rb2, and Rb3 (m/z 1077), 10.8, 11.61, and 12.01 min for nFe, CO, and nFd (m/z 915), 5.08, 13.68, 15.63, and 15.89 min for Rg2, F2, G75, and Rg3 (m/z 783), 17.29, 18.02, and 18.46 min for Mc, CY, and CMx (m/z 753), 19.31 and 19.6 min for CK and Rh2 (m/z 621), 14.75 min for PPT, and 20.99 min for PPD. There was no interference peak near the corresponding retention time of each target peak, which shows that this method has reasonably good specificity.

In addition, characteristics of calibration curves, including the range of linearity, the square of correlation coefficient ($r^2$), limit of quantification (LOQ), and limit of detection (LOD) of each ginsenoside, are listed in Table 1. All compounds showed excellent linearity over a relatively wide concentration range, with correlation coefficients ($r^2$) of all calibration curves ranging from 0.9924 to 0.9998. The range of LOQs was within the range of 0.015–1.163 ng/mL. The range of LODs fell within the range of 0.003–0.349 ng/mL. In the stability test, analytes did not significantly degrade after storage of test solutions at room temperature for 48 h (RSD $\leq 3.77\%$). Additionally, variation in RSD reproducibility was less than 3.86%. In terms of precision, RSDs ranged from 0.52% to 2.37%, and accuracy (RE%) ranged from 0.06% to 3.92%. These results are shown in Table 2, with all values being within an acceptable range.

3.4. Qualitative Analysis of Ginsenosides. Twenty-five authentic standards and ginsenoside mixture from Panax ginseng roots analyzed by UPLC-HRMS in negative ion mode.
Ginsenoside root were determined using UPLC-Orbitrap-HRMS under optimized conditions. Typical total ion chromatograms are shown in Figure 1. In the present study, the use of the full ms-ddms² acquisition mode with scans ranging from 100 to 1500 m/z in negative ion mode combined with MS/MS fragmentation for the top 5 ions was proposed to aid in

Table 1: Calibration curves, limit of quantification (LOQ), and limit of detection (LOD) of UPLC-MS methods for determination of 25 ginsenosides.

| Ginsenoside | RT   | Calibration curve               | Test range (ug/mL) | \(r^2\) | LOQ (ng/mL) | LOD (ng/mL) |
|------------|------|---------------------------------|--------------------|-------|-------------|-------------|
| Rg1        | 1.46 | \( Y = 64984X + 980278 \)      | 0.02-2             | 0.9990 | 0.106       | 0.032       |
| Re         | 1.46 | \( Y = 41225.1X + 688583 \)    | 0.02-2             | 0.9959 | 0.164       | 0.049       |
| Rf         | 4.08 | \( Y = 42146.9X - 44017.1 \)   | 0.01-0.5           | 0.9994 | 0.116       | 0.035       |
| Rh1        | 5.02 | \( Y = 238413X - 153145 \)     | 0.002-0.2          | 0.9994 | 0.025       | 0.007       |
| Rh2        | 5.44 | \( Y = 6240.9X + 85602.4 \)    | 0.02-2             | 0.9664 | 1.163       | 0.349       |
| Rg2        | 5.08 | \( Y = 52298.5X - 612776 \)    | 0.05-1             | 0.9924 | 0.012       | 0.003       |
| Rc         | 6.09 | \( Y = 22275X - 24796.1 \)     | 0.01-1             | 0.9991 | 0.182       | 0.055       |
| F1         | 6.73 | \( Y = 292355X - 175557 \)     | 0.002-0.2          | 0.9995 | 0.029       | 0.009       |
| Rb2        | 7.13 | \( Y = 20778.7X - 36514.3 \)   | 0.01-1             | 0.9994 | 0.313       | 0.094       |
| Rb3        | 7.59 | \( Y = 20695.1X + 48835.8 \)   | 0.05-2             | 0.9994 | 0.513       | 0.154       |
| Rd         | 9.25 | \( Y = 61029.8X - 558838 \)    | 0.1-2              | 0.9899 | 0.015       | 0.005       |
| G17        | 10.07| \( Y = 85248.5X - 57819.7 \)   | 0.002-0.2          | 0.9988 | 0.217       | 0.065       |
| nFe        | 10.8 | \( Y = 29919.9X - 7299.02 \)   | 0.002-0.2          | 0.9996 | 0.192       | 0.058       |
| CO         | 11.61| \( Y = 31396.7X - 13591.7 \)   | 0.001-0.2          | 0.9990 | 0.217       | 0.065       |
| nFd        | 12.01| \( Y = 20835.8X - 25094.8 \)   | 0.01-0.2           | 0.9959 | 0.833       | 0.250       |
| F2         | 13.68| \( Y = 212189X - 115446 \)     | 0.002-0.2          | 0.9998 | 0.029       | 0.009       |
| G75        | 15.63| \( Y = 231218X - 99660.6 \)    | 0.001-0.2          | 0.9991 | 0.042       | 0.013       |
| Rh3        | 15.89| \( Y = 149379X - 82845.1 \)    | 0.002-0.2          | 0.9993 | 0.154       | 0.046       |
| PPT        | 14.75| \( Y = 27249.2X - 50845.3 \)   | 0.002-0.05         | 0.9984 | 0.150       | 0.045       |
| Mc         | 17.29| \( Y = 58309.3X - 14125.5 \)   | 0.001-0.2          | 0.9995 | 0.294       | 0.088       |
| CY         | 18.02| \( Y = 41233.6X + 36194 \)    | 0.001-0.2          | 0.9993 | 0.175       | 0.053       |
| CMx        | 18.46| \( Y = 80902.7X - 22662.5 \)   | 0.002-0.2          | 0.9990 | 0.189       | 0.057       |
| CK         | 19.31| \( Y = 140955X + 96724.4 \)    | 0.001-0.2          | 0.9998 | 0.018       | 0.005       |
| Rh2        | 19.6 | \( Y = 316346X - 188172 \)     | 0.002-0.2          | 0.9996 | 0.016       | 0.005       |
| PPD        | 20.99| \( Y = 3099.89X - 484321 \)    | 0.002-0.2          | 0.9990 | 0.535       | 0.160       |

Table 2: Instrument precision, accuracy, sample stability, and repeatability of the assay (n = 6).

| Ginsenoside | Precision (RSD%) | Accuracy (RE%) | Stability (RSD%) | Repeatability (RSD%) |
|------------|------------------|----------------|------------------|---------------------|
| Rg1        | 0.88             | 0.06           | 1.05             | 0.92                |
| Re         | 0.97             | 0.87           | 1.23             | 1.26                |
| Rf         | 0.78             | 0.11           | 0.84             | 1.10                |
| Rh1        | 0.63             | 0.25           | 0.68             | 1.41                |
| Rb1        | 0.93             | 1.06           | 1.89             | 1.24                |
| Rg2        | 0.52             | 3.92           | 1.71             | 1.45                |
| Rc         | 1.76             | 2.13           | 1.56             | 1.58                |
| F1         | 0.91             | 0.93           | 0.95             | 1.03                |
| Rb2        | 1.38             | 2.24           | 1.77             | 1.14                |
| Rb3        | 1.55             | 1.07           | 2.71             | 2.00                |
| Rd         | 1.60             | 1.64           | 0.70             | 0.78                |
| GXVII      | 0.79             | 0.31           | 2.14             | 2.01                |
| nFe        | 1.93             | 0.22           | 3.77             | 3.31                |
| CO         | 0.97             | 0.11           | 1.54             | 2.48                |
| nFd        | 1.55             | 0.22           | 2.15             | 1.74                |
| F2         | 0.74             | 1.71           | 0.65             | 1.34                |
| G75        | 1.16             | 2.60           | 1.06             | 3.40                |
| Rh3        | 1.85             | 3.65           | 0.71             | 1.58                |
| PPT        | 1.63             | 0.36           | 1.32             | 3.86                |
| Mc         | 1.10             | 0.73           | 1.84             | 2.15                |
| CY         | 1.02             | 0.19           | 2.87             | 1.86                |
| CMx        | 0.67             | 0.17           | 1.36             | 1.39                |
| CK         | 0.85             | 2.07           | 1.76             | 1.79                |
| Rh2        | 1.42             | 2.25           | 1.13             | 1.17                |
| PPD        | 2.37             | 0.94           | 1.08             | 1.23                |

RSD, relative standard deviation, RE, relative error.
| No. | Identification  | Formula          | $T_R$ (min) | $[M+COOH^-]$ (m/z) | $[M^-]$ (m/z) | $[M+COOH^-]-[M-H]^-$ (m/z) | $[M-H-Ara(Glc)]^-$ (m/z) |
|-----|-----------------|------------------|-------------|--------------------|---------------|-----------------------------|---------------------------|
| 1   | 20-Glc-ginsenoside Rf | C48H82O19        | 1.05        | 1007.5447          | 961.5384      | 799.4883[M-H-Glc]^- 637.4325[M-H-2Glc]- 475.3785[M-H-3Glc]- | 799.4872[M-H-Xyl]-637.4320[M-H-Xyl-Glc]- 475.3792[M-H-Xyl-2Glc]- |
| 2   | Notoginsenoside R1 (nR1) | C47H80O18        | 1.17        | 977.5344           | 931.5288      | 799.4872[M-H-Xyl]-637.4320[M-H-Xyl-Glc]- 475.3792[M-H-Xyl-2Glc]- |
| 3   | Ginsenoside Rg1 | C42H72O14        | 1.42        | 845.4910           | 799.4869      | 637.4324[M-H-Glc]- 475.3803[M-H-2Glc]- |
| 4   | Ginsenoside Re | C48H82O18        | 1.46        | 991.5502           | 945.5451      | 637.4338[M-H-Glc-Rha]- 475.3784[M-H-2Glc- Rha]- |
| 5   | Ginsenoside Rf | C42H72O14        | 4.05        | 845.4898           | 799.4855      | 637.4332[M-H-Glc]- 475.3791[M-H-2Glc]- |
| 6   | Ginsenoside Ra3 | C59H100O27       | 4.33        | 1285.6436          | 1239.6372     | 1107.6023[M-H-Xyl]-945.5419[M-H-Xyl-Glc]- 783.4863[M-H-Xyl-2Glc]- |
| 7   | Ginsenoside F3/F5 | C41H70O13        | 4.48        | 815.4810           | 769.4754      | 637.4333[M-H-Ara]- 475.3789[M-H-Ara-Glc]- |
| 8   | Ginsenoside Rh1 | C36H62O9         | 4.96        | 683.4381           | 637.4322      | 475.3778[M-H-Glc]- |
| 9   | Ginsenoside Rg2 | C42H72O13        | 5.03        | 829.4963           | 783.4898      | 637.4306[M-H-Rha]- 475.3807[M-H-Rha-Glc]- |
| 10  | Ginsenoside Ra2 | C58H98O26        | 5.23        | 1255.6328          | 1209.6276     | 1107.6023[M-H-Xyl]-945.5419[M-H-Xyl-Glc]- 783.4863[M-H-Xyl-2Glc]- |
| 11  | Ginsenoside Rb1 | C54H92O23        | 5.40        | 1153.6014          | 1107.5956     | 945.5421[M-H-Glc]- 783.4899[M-H-2Glc]- |
| 12  | Ginsenoside Rc | C53H90O22        | 6.06        | 1123.5912          | 1077.5854     | 647.5374[M-H-3Glc]- 459.3847[M-H-Ara(Glc)-3Glc]- |
| 13  | Ginsenoside Ra2-isomer | C58H98O26      | 6.27        | 1255.6329          | 1209.6266     | 1077.5870[M-H-Xyl]-945.5418[M-H-Xyl-Ara(Glc)-3Glc]- |
| 14  | Ginsenoside Ro | C48H76O19        | 6.44        | -                  | 955.4915      | 475.3782[M-H-Glc]- |
| 15  | Ginsenoside F1 | C36H62O9         | 6.64        | 683.4385           | 637.4288      | 459.3840[M-H-2Glc- GlcA]- |
| 16  | Ginsenoside Ra1 | C58H98O26        | 6.68        | 1255.6334          | 1209.6271     | 1077.5790[M-H-Xyl]-783.4831[M-H-Xyl-Ara(Glc)-p-Glc]- |
| 17  | Ginsenoside Rb2 | C53H90O22        | 7.09        | 1123.5912          | 1077.5859     | 945.5415[M-H-Ara(Glc)-p]-783.4908[M-H-Ara(Glc)-2Glc]- |
| 18  | Ginsenoside Rb3 | C53H90O22        | 7.48        | 1123.5916          | 1077.5838     | 621.4372[M-H-Xyl-2Glc]- 459.3855[M-H-Xyl-3Glc]- |
| 19  | Ginsenoside Ra1-isomer | C58H98O26    | 8.03        | 1255.6338          | 1209.6284     | 1077.5868[M-H-Xyl]-945.5466[M-H-Xyl-Ara(Glc)-p]-783.4907[M-H-Xyl-Ara(p-Glc)-2Glc]- |
| 20  | Chikusetsusaponin Iva (CS-Iva) | C42H66O14     | 8.91        | -                  | 793.4395      | 631.3858[M-H-Glc]- 455.3555[M-H-GlcA]- |
| 21  | Ginsenoside Rd | C48H82O18        | 9.21        | 991.5497           | 945.5438      | 783.4911[M-H-Glc]-621.4377[M-H-2Glc]- 459.3850[M-H-3Glc]- |
| 22  | Gypenoside XVII | C48H82O18        | 10.01       | 991.5506           | 945.5432      | 783.4890[M-H-Glc]-621.4405[M-H-2Glc]- |
| 23  | Notoginsenoside Fe (nFe) | C47H80O17      | 10.74       | 961.5374           | 915.5348      | 783.4901[M-H-Ara(Glc)-p]-621.4399[M-H-Ara(Glc)-2Glc]- 459.3845[M-H-Ara(Glc)-3Glc]- |
| 24  | Compound O (CO) | C47H80O17       | 11.17       | 961.5401           | 915.5327      | 783.4915[M-H-Ara(Glc)-p]-621.4379[M-H-Ara(Glc)-2Glc]- 459.3847[M-H-Ara(Glc)-3Glc]- |
| 25  | NotoginsenosideFd (nFe) | C47H80O17     | 11.54       | 961.5401           | 915.5330      | 783.4908[M-H-Xyl]-621.4370[M-H-Xyl-Glc]- |
| 26  | Ginsenoside Rg6 | C42H70O12        | 12.09       | 811.4872           | 765.4798      | 619.4219[M-H-Rha]- |
| 27  | Ginsenoside Rk3 | C36H60O8         | 12.51       | 665.4287           | 619.3349      | 619.4219[M-H-Rha]- 457.3643[M-H-Rha-Glc]- |
| 28  | Ginsenoside F4 | C42H70O12        | 12.90       | 811.4871           | 765.4786      | 619.4219[M-H-Rha]- 457.3643[M-H-Rha-Glc]- |
| 29  | Ginsenoside Rh4 | C36H60O8         | 13.24       | 665.4284           | 619.3344      | 619.4219[M-H-Rha]- 457.3643[M-H-Rha-Glc]- |
| 30  | Ginsenoside Rg2 | C42H72O13        | 13.62       | 829.4974           | 783.4861      | 621.4400[M-H-Glc]-459.3816[M-H-2Glc]- |

**Table 3:** Characterization of compounds in *Panax ginseng* roots using UPLC-HRMS.
the structural identification of the components. The formula data, retention time, and experimental molecular mass and MS/MS fragment information are shown in Table 3. This provided abundant information that can be used as the basis for identifying constituents from Panax ginseng roots. Here, a total of 43 ginsenosides were identified. Among the 43 ginsenosides found, 25 ginsenosides (including peaks 1, 2, 3, 4, 5, 7, 8, 9, and 15 and twenty-three PPDs ginsenosides such as compounds 6, 10, 11, 12, 13, 16, 17, 18, 19, 21, 22, 23, 24, 25, 30, 33, 34, 35, 36, 37, 38, 39, and 42. As an example, peak 11 (RT = 5.40), identified as ginsenoside Rb1, produced [protopanaxadiol-H]− at m/z 459.3840 in the MS/MS spectrum by successive loss of four Glc-H₂O (162 Da) groups. As another example, peak 4 (RT = 1.46), identified as ginsenoside Re, produced [protopanaxatriol-H]− at m/z 475.3784 via successive elimination of one Rha, one Glc, and two Glc, respectively. Moreover, the OAs displayed an aglycone ion m/z 455 corresponding to [oleanolic acid-H]−. There are three OAs (e.g., compounds 14, 20, and 32). Peak 20 was identified as Chikusetsusaponin Iva by comparing retention times and ion fragments with those previously reported [22]. This peak displayed a [M−H]− ion at m/z 793.4395. In the full-scan-ddms² experiment, two major fragment ions at m/z 631.3858 and 455.3555 were observed, indicating that the structure of saponins contains one glucose and one glucuronate group. The fragment ion at m/z 455.3555 indicated the presence of an oleanolic acid aglycon moiety that lost all linked glycosidic units. These results are shown in Figure 2.

3.5. Quantitative Analysis of Ginsenosides by QAMS

3.5.1. Relative Response Factors of Ginsenosides. Selecting a proper internal standard is vital for the accuracy of multi-components using QAMS. Using Rb2 as an internal reference (1.00), the relative response factors (RCF) against ginsenoside Rb2 for all of the ginsenoside standards for the m/z signals of the exact peaks with accurate mass ranges in the full scan mode were established by using five different concentrations. The average RCF of each compound, calculated using Equation (2), can be seen in Table 4. It should be noted that the RSDs of the RCFs of all ginsenosides were less than 5.08%, suggesting that the RCFs obtained on the same instrument at different concentrations have good reproducibility.

| No. | Identification | Formula | Tᵣ (min) | [M + COOH]− (m/z) | [M-H]− (m/z) | MS² fragment ions (m/z) |
|-----|----------------|---------|----------|------------------|--------------|------------------------|
| 32  | Glycoside D3a  | C42H66O14 | 14.70    | -                | 793.4392     | 613.3735[M-H-Glc-H₂O]− 455.3517[M-H-Glc-GlcA]− |
| 33* | Gynenoside LXV (G75) | C42H72O13 | 15.51    | 829.4975        | 783.4913     | 621.4393[M-H-Glc-459.3840|M-H-2Glc]− |
| 34* | Ginsenoside Rg3 | C42H72O13 | 15.79    | 829.4951        | 783.4912     | 621.4393[M-H-Glc-459.3845[M-H-2Glc]− |
| 35   | 20 (R)-Rg3  | C42H72O13 | 16.28    | 829.4974        | 783.4905     | 621.4382[M-H-Glc-459.3864[M-H-2Glc]− |
| 36* | Ginsenoside Mc | C41H70O12 | 17.31    | 799.4866        | 753.4793     | 621.4371[M-H-Ara (f)-Glc]− 459.3869[M-H-Ara (f)-Glc]− |
| 37* | Compound Y (CY) | C41H70O12 | 17.94    | 799.4866        | 753.4804     | 621.4374[M-H-Ara (p)]− 459.3866[M-H-Ara (p)-Glc]− |
| 38* | Compound Mx (CMx) | C41H70O12 | 18.42    | 793.4969        | 753.4770     | 621.4406[M-H-Xyl] 459.3858[M-H-Xyl-Glc]− |
| 39* | Compound K (CK) | C36H62O8 | 19.30    | 667.4473        | 621.4392     | 459.3828[M-H-Glc]− |
| 40  | Ginsenoside Rk1 | C42H70O12 | 19.46    | 811.4865        | 765.4803     | 603.4224[M-H-Glc]− |
| 41  | Ginsenoside Rg5 | C42H70O12 | 19.55    | 811.4869        | 765.4843     | 603.4230[M-H-Glc]− |
| 42* | Ginsenoside Rh2 | C53H90O22 | 19.59    | 667.4438        | 621.4372     | 459.3831[M-H-Glc]− |
| 43* | PPD | C30H52O3 | 20.99    | 505.3909        | —            | 459.3843 |

*Identified with a standard reference.
3.5.2. Reproducibility of RCFs and Relative Retention Times.

The effects of using various column temperatures (30°C, 35°C, and 40°C) and flow rates (0.25, 0.3, and 0.35 mL/min) on RCF were investigated in order to evaluate the reproducibility of the QAMS approach. Results are shown in Table 5. RSDs measured under different conditions were all less than 5.97%, indicating that the RCF calculated by the established QAMS method has good reproducibility. When the content of analytes is determined by QAMS, the correct localization of the peaks is crucial. The relative retention time (RTR) between measured components and the internal reference (Rb2) was calculated here using the ratio of \( t_{Rk} \) to \( t_{Rs} \). RSDs of RTR were calculated to evaluate the influence of column temperature (30°C, 35°C, and 40°C) and flow rate (0.25, 0.3, and 0.35 mL/min). Our results demonstrated that RSDs measured at different temperatures were less than 1.79%, and at different flow rates were less than 7.60%. This indicates that RTB is reproducible at different temperatures when the flow rate is constant. Thus, RTr can be used to accurately locate the chromatographic peak of each analyte.

3.5.3. Quantitative Measurement of Panax ginseng Roots.

In this study, a new UPLC-HRMS approach combined with the external standard method (ESM) and QAMS was developed to simultaneously determine 25 ginsenosides from Panax ginseng root, with calibration curve-based quantification provided in Table 6. The comparison of the two methods on the precision and accuracy is listed in Table S1 (in Supplementary Materials). The results indicated that the precision (RSD%) range of ESM and QAMS was 0.52%–2.37% and 0.45%–3.44%, respectively, and the accuracy (RE%) range was 0.06%–3.92% and 0.03%–5.01%, respectively. Both the ESM method and QAMS method had good precision and accuracy for the contents of 25 ginsenosides.

Figure 2: The MS/MS spectrum of ginsenoside-Rb1 (a), ginsenoside-Re (b), and ginsenoside-CS-Iva (c).
within the allowable error range. Using the ESM method, the amounts of Rg1, Re, Rf, Rh1, Rg2, and F1, which are all PPTs type, were calculated as 53.85, 151.28, 29.34, 1.39, 29.22, and 0.55 mg/g, respectively. The amounts of Rb1, Rc, Rb2, Rb3, Rd, GXVII, nFe, nFd, F2, G75, Rg3, Mc, CY, CMx, CK, and Rh2, which are PPDs type, were detected as 324.15, 29.34, 32.45, 3.42, 3.45, 3.48, and 3.86 mg/g, respectively.

### Table 4: Relative response factor (RCF) and relative standard deviation (RSD) values of 25 ginsenosides.

| Ginsenoside | Concentration (ng/mL) | Mean | RSD (%) |
|-------------|------------------------|------|---------|
| Rb2         | 10                     | 1.00 | —       |
| Rg1         | 20                     | 1.00 | —       |
| Rg1         | 50                     | 1.00 | —       |
| Rg1         | 100                    | 1.00 | —       |
| Rg1         | 200                    | 1.00 | —       |
| Rb2         | 0.32                   | 0.30 | 0.31    |
| Rb2         | 0.46                   | 0.47 | 0.48    |
| Rb2         | 0.48                   | 0.52 | 0.48    |
| Rb2         | 3.45                   | 3.25 | 3.05    |
| Rb2         | 3.50                   | 3.18 | 3.22    |
| Rb2         | 0.44                   | 0.43 | 0.46    |
| Rb2         | 0.09                   | 0.09 | 0.09    |
| Rb2         | 0.93                   | 0.94 | 0.94    |
| Rb2         | 0.07                   | 0.08 | 0.08    |
| Rb2         | 0.97                   | 0.97 | 0.98    |
| Rb2         | 0.36                   | 0.34 | 0.35    |
| Rb2         | 0.24                   | 0.24 | 0.23    |
| Rb2         | 0.62                   | 0.67 | 0.66    |
| Rb2         | 0.60                   | 0.63 | 0.66    |
| Rb2         | 1.05                   | 1.00 | 0.99    |
| Rb2         | 0.10                   | 0.10 | 0.10    |
| Rb2         | 0.09                   | 0.09 | 0.08    |
| Rb2         | 0.91                   | 0.86 | 0.67    |
| Rb2         | 0.08                   | 0.09 | 0.61    |
| Rb2         | 0.98                   | 1.06 | 0.00    |
| Rb2         | 0.32                   | 1.29 | 0.45    |
| Rb2         | 0.25                   | 1.41 | 0.41    |
| Rb2         | 0.67                   | 1.52 | 0.38    |
| Rb2         | 0.64                   | 1.64 | 0.71    |
| Rb2         | 1.06                   | 1.69 | 0.34    |
| Rb2         | 0.10                   | 0.97 | 1.92    |
| Rb2         | 0.09                   | 2.87 | 0.52    |
| Rb2         | 0.14                   | 2.05 | 1.49    |
| Rb2         | 0.34                   | 2.43 | 0.24    |
| Rb2         | 0.45                   | 2.53 | 0.40    |
| Rb2         | 0.26                   | 2.59 | 1.68    |
| Rb2         | 0.11                   | 2.69 | 1.14    |
| Rb2         | 0.07                   | 2.09 | 1.18    |
| Rb2         | 0.07                   | 2.90 | 1.43    |
| Rb2         | 0.67                   | 1.78 | 2.90    |

### Table 5: Effects of column temperature and flow rate upon relative response factor (RCF) and relative retention time (RT).

| Ginsenoside | RCF | RSD% | RT | RSD% |
|-------------|-----|------|----|------|
| Rg1         | 0.29| 3.45 | 0.21| 0.00 |
| Rg1         | 0.47| 2.44 | 0.21| 0.00 |
| Rg1         | 0.48| 1.21 | 0.56| 1.79 |
| Rg1         | 3.08| 3.83 | 0.77| 0.75 |
| Rg1         | 0.32| 4.45 | 0.70| 1.43 |
| Rg1         | 0.09| 3.94 | 0.70| 0.66 |
| Rg1         | 0.91| 3.34 | 0.86| 0.67 |
| Rg1         | 0.08| 0.00 | 0.95| 0.61 |
| Rg1         | 0.98| 3.11 | 1.06| 0.00 |
| Rg1         | 0.32| 3.13 | 1.29| 0.45 |
| Rg1         | 0.25| 4.00 | 1.41| 0.41 |
| Rg1         | 0.67| 1.49 | 1.52| 0.38 |
| Rg1         | 0.64| 2.40 | 1.64| 0.71 |
| Rg1         | 1.06| 1.44 | 1.69| 0.34 |
| Rg1         | 0.10| 5.97 | 1.92| 0.52 |
| Rg1         | 0.09| 3.27 | 2.19| 0.00 |
| Rg1         | 0.13| 4.56 | 2.21| 0.90 |
| Rg1         | 1.03| 2.03 | 2.05| 1.49 |
| Rg1         | 0.34| 2.94 | 2.43| 0.24 |
| Rg1         | 0.45| 4.44 | 2.53| 0.40 |
| Rg1         | 0.26| 3.85 | 2.59| 1.68 |
| Rg1         | 0.11| 5.41 | 2.69| 1.14 |
| Rg1         | 0.07| 5.50 | 2.72| 1.18 |
| Rg1         | 6.76| 1.78 | 2.90| 1.43 |

within the allowable error range. Using the ESM method, the amounts of Rg1, Re, Rf, Rh1, Rg2, and F1, which are all PPTs type, were calculated as 53.85, 151.28, 29.34, 1.39, 29.22, and 0.55 mg/g, respectively. The amounts of Rb1, Rc, Rb2, Rb3, Rd, GXVII, nFe, CO, nFd, F2, G75, Rg3, Mc, CY, CMx, CK, and Rh2, which are PPDs type, were detected as 324.15,
Table 6: Comparative experiment results of the external standard method (ESM) and quantitative analysis of multiginsenosides by single marker method (QAMS) (mg/g).

| No. | Ginsenoside | ESM   | QAMS   | SMD% |
|-----|-------------|-------|--------|------|
| 1   | Rg1         | 53.85 | 53.80  | 0.10 |
| 2   | Re          | 151.28| 143.83 | 4.92 |
| 3   | Rf          | 29.34 | 29.47  | 0.46 |
| 4   | Rb1         | 324.15| 316.05 | 2.50 |
| 5   | Rg2         | 29.22 | 30.21  | 3.38 |
| 6   | Rh1         | 1.39  | 1.35   | 2.54 |
| 7   | Rc          | 97.77 | 96.82  | 0.97 |
| 8   | F1          | 0.55  | 0.57   | 2.52 |
| 9   | Rh2         | 112.03| 112.03 | -    |
| 10  | Rh3         | 17.94 | 17.88  | 0.38 |
| 11  | Rd          | 97.66 | 98.03  | 0.38 |
| 12  | GXVII       | 2.56  | 2.54   | 0.91 |
| 13  | nFe         | 3.92  | 3.71   | 5.33 |
| 14  | CO          | 2.22  | 2.20   | 0.52 |
| 15  | nFd         | 1.75  | 1.76   | 0.50 |
| 16  | F2          | 1.25  | 1.24   | 0.71 |
| 17  | G75         | 0.11  | 0.10   | 5.45 |
| 18  | Rg3         | 2.95  | 2.86   | 3.05 |
| 19  | PPT         | 1.03  | 1.01   | 1.53 |
| 20  | Mc          | 0.91  | 0.88   | 3.69 |
| 21  | CY          | 2.42  | 2.42   | 0.21 |
| 22  | CMx         | 0.38  | 0.37   | 1.40 |
| 23  | CK          | 1.42  | 1.41   | 0.18 |
| 24  | Rh2         | 0.33  | 0.32   | 4.12 |
| 25  | PPD         | 0.47  | 0.46   | 2.09 |

97.77, 112.03, 17.94, 97.66, 2.56, 3.92, 2.22, 1.75, 1.25, 0.11, 2.95, 0.91, 2.42, 0.38, 1.42, and 0.33 mg/g, respectively. The aglycon of PPT and PPD was detected as 1.03 and 0.47 mg/g, respectively. Among them, Re, Rb1, Rc, Rh2, and Rd had the highest content in Panax ginseng root. Moreover, the content of F1, G75, PPT, Mc, CMx, Rh2, and PPD was generally low.

At present, QAMS, as an alternative approach for the quality control of TCMs, has attracted increasing interest in terms of quality assessment and control of complex multi-component systems [23, 24]. In this study, the content of 25 compounds was determined using the QAMS method. Using Rb2 as internal reference (1.00), RCFs of Rg1, Re, Rf, Rb1, Rg2, Rc, Rh1, Rc, F1, Rh3, Rd, GXVII, nFe, CO, nFd, F2, G75, Rg3, PPT, Mc, CY, CMx, CK, Rh2, and PPD were 0.31, 0.47, 0.49, 3.23, 0.43, 0.09, 0.92, 0.08, 0.98, 0.35, 0.24, 0.66, 0.63, 1.03, 0.10, 0.09, 0.14, 0.98, 0.34, 0.48, 0.25, 0.14, 0.07, and 6.71, respectively.

To validate the feasibility of using QAMS for quantitative analysis of ginsenosides, the standard method difference (SMD) was established between the QAMS and ESM. As shown in Table 6, there were no significant differences between results from QAMS and ESM methods according to the SMD% falling between 0.1% and 5.45%, illustrating that the use of QAMS in our approach is reliable and accurate for detecting the concentration of each component.

4. Conclusions

In this study, we report an innovative approach based on UPLC-HRMS combined with QAMS and ESM for the simultaneous determination of multiple ginsenosides. By optimizing detection conditions, we can acquire for each ginsenoside within 25 min a high-resolution UPLC chromatogram with excellent sensitivity and stability. The QAMS method established using relative response factors shows minimal difference (SMDs below 5.45%) compared to the external standard method, implying that the UPLC-MS-QAMS approach can be used as a replacement for the external standard method when standard substances are absent. For these 25 different ginsenosides, data reproducibility was very good at low concentrations for MS detection. Furthermore, our approach is simple to use, highly sensitive and accurate, and applicable for all kinds of ginseng samples and for quality control of ginseng containing products.

Data Availability

The data used to support findings in this study are included within the article.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supplementary Materials

The TIC of twenty-five ginsenoside standards by UPLC-HRMS with different kinds of the chromatographic column and different kinds of mobile phase and comparison of flow rates and column temperatures of ginsenoside detection in this article are available in the Supplementary Materials document. In other words, the comparison of the external standard method (ESM) and quantitative analysis of multiginsenosides by single marker method (QAMS) on the precision and accuracy is available in the Supplementary Materials document. (Supplementary Materials)

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