# Towards differentiation of *Panax vietnamensis* and *Panax vietnamensis* var. *fuscidiscus* by UPLC-QTOF-MS

Long Huynh Kim Vu[1], Duc Minh Nguyen[1], Long Ke Phan[2],
Yen Trang Hai Nguyen[1], Huy Truong Nguyen[1*]

[1]Ton Duc Thang University, Ho Chi Minh City, Vietnam
[2]Vietnam National Museum of Nature, Vietnam Academy of Science and Technology, Hanoi, Vietnam
[*]Corresponding author: nguyentruonghuy@tdtu.edu.vn

| ARTICLE INFO | ABSTRACT |
|---|---|

Vietnamese ginseng (*Panax vietnamensis* Ha *et* Grusvh., Araliaceae - PV) is a high-value medicinal plant of Vietnam. Recently, a new variety of this plant *P. vietnamensis* var *fuscidiscus* (PVF), so-called Lai Chau ginseng, was discovered and circulated on the Vietnam market along with PV. PV and PVF rhizomes and roots are preliminarily reported to possess similar saponin composition. However, PV is much more expensive than PVF due to the higher regard of the consumer, which consequently leads to the inattentive misuse or intentional adulteration of PV by PVF. In this study, a UPLC-ToF-MS method was developed to distinguish PV and PVF based on their saponin profiles. The UPLC qualitative result showed that the saponin constituent of PVF is more complex than that of PV. The significant differences in the chromatogram of PV and PVF extracts are the peaks eluted from 32 - 48 min. Vina-ginsenoside R2 (9), chikusetsusaponin L8 (10), and notoginsenoside R4 (12) are characterized for PVF, while notoginsenoside Fa (13), quinquenoside R1 (16), and pseudoginsenoside Rs1 (19) are specialized for PV. The preliminary quantitative determination result showed that the content of majonoside R2, ginsenoside Rg1, ginsenoside Rb1, and ginsenoside Rd in PV is higher than in PVF. In contrast, the content of majonoside R1 and vina-ginsenoside R2 in PVF is significantly higher than in PV. The developed method could be used as an effective method to distinguish PV and PVF.

## 1. Introduction

Vietnamese ginseng or Ngoc Linh ginseng (*Panax vietnamensis*, Ha *et* Grusvh., Araliaceae - PV) is Vietnam's endemic and precious medicinal plant. Before its discovery in 1973, PV was used as a "secret remedy" by Se Dang ethnic people living in Ngoc Linh Mountain for health enhancement and the treatment of various diseases. Over 50 years since its discovery, PV has attracted much attention from domestic and international scientists in terms of botany, chemistry, and biological activities. From PV rhizomes and roots, 52 compounds were isolated with a surprisingly high yield of Ocotillol-Type (OT) saponins, especially majonoside R2 (M-R2), with over 5% of the content (Nguyen et al., 1993, 1994a, 1994b). Many remarkable

biological activities of PV were revealed, including anti-stress, anti-depression, anti-tumor, antioxidant, hepato-, and nephroprotective effects (Konoshima et al., 1999; Nguyen et al., 1996; Nguyen, Matsumoto, Kasai, Yamasaki, & Watanabe, 1998; Nguyen et al., 2000; Vu et al., 2019).

In 2003, Zhu et al. reported a new variety of PV named it as Ye-Sanchi growing in Yunnan, China with the scientific name of *P. vietnamensis* var *fuscidiscus* (PVF) (Zhu, Fushimi, Cai, Chen, & Komatsu, 2003). In 2014, Phan et al. found PVF in Lai Chau Province, Vietnam, named it Lai Chau ginseng, and developed a genetic method to differentiate PV and PVF (Phan et al., 2014). Preliminary chemical study by HPLC-UV showed that PVF also shared the common saponins with those of PV, such as M-R2, ginsenoside Rg1 (G-Rg1), ginsenoside Rb1 (G-Rb1), ginsenoside Rc (G-Rc), ginsenoside Rd (G-Rd), and vina-ginsenoside R2 (V-R2) (Zhu, Zou, Cai, Meselhy, & Komatsu, 2004). However, due to the limitations of UV detection of OT saponins containing no chromophore in structure, the complete chemical profile of PVF was not established. Moreover, until now, there has been no report on a comparative study of the saponin composition of PV and PVF.

In terms of economic concerns, PV has better regard for consumers in the Vietnam market, which leads to a much higher price than PVF. However, it is hard to differentiate these two varieties due to their similar appearance. Until now, DNA sequencing has been the only reliable method to distinguish PV and PVF, which requires specific equipment and high-level technicians in biological experiments. Therefore, in this study, we aimed to use the UPLC-MS approach to elucidate the differences in the saponin composition between PV and PVF, which could be used to authenticate these two related varieties.

## 2. Object and research method

### 2.1. Objects

PV rhizomes and roots aged 06 years old (n = 15) were cultivated on Ngoc Linh Mountain and provided by Vietnamese Ginseng K5 Kontum Joint Stock Company (VINGIN JSC., Kon Tum City, Kon Tum Province). The PVF rhizomes and roots (n = 15) at the same age were harvested in Lai Chau Province and purchased from ThuKa company (Muong Te Ward, Lai Chau Province). The representative samples of each group (n = 3) were identified as PV or PVF variety at the Vietnam National Museum of Nature by Associate Professor Phan Ke Long using ITS-rDNA gene sequencing. The fresh rhizomes and roots were dried in an oven at under 60°C until dry and then ground to under 0.5mm fine powder. Specimens were deposited at the Faculty of Pharmacy, Ton Duc Thang University.

Reference standards (purity), including M-R1 (97.07%), M-R2 (98.86%), V-R2 (98.25%), G-Rg1 (96.43%), G-Rb1 (99.17%), G-Rb2 (97.31%), G-Rc (98.52%), and G-Rd (94.48%), were supplied by The Center of Pharmaceutical Science and Technology (SAPHARCEN), University of Medicine and Pharmacy Ho Chi Minh City.

### 2.2. Research method

#### 2.2.1. Sample preparation

An exact amount of 100mg of PV or PVF powder was placed into a 15mL test tube, and 10mL of 70% MeOH was added. The tubes were capped tightly and simultaneously extracted by sonication for 40min. The extract was filtered through a 0.22μm filter before being analyzed by UPLC-QTOF-MS.

*2.2.2. UPLC-QTOF-MS analysis*

The analysis was conducted in an Agilent Infinity 1290 system coupled with a 6456 QTOF detector. An EclipsePlus C18 RRHD column (2.1 × 50mm, 1.8μm) was used to separate the saponins in samples. The compounds were eluted by the mobile phase composed of acetonitrile (A) and water (B) containing 0.1% of formic acid in both channels with the gradient elution as follows: 0 - 23min: 17 - 18% A; 23 - 33min: 18 - 27% A; 33 - 47min: 27 - 28% A; 47 - 50min: 28 - 40% A; 50 - 55min: 40 - 95% A; 55 - 55.1min: 95 - 17% A; 55 - 60min: 17% A at the flowrate of 0.3 mL/min. An amount of 2μL of samples was injected into the UPLC system. As for the TOF-MS condition, the detection was carried out in negative mode with the scan range *m/z* 100 - 1,700Da. The gas temperature was kept at 300°C at the flow of 5 L/min. The compounds were ionized using the fragmentor of 350V with capillary voltage of 3,500V. The data was acquired by MassHunter software (ver. 10.0).

*2.2.3. Preliminary quantitative determination of saponin content of PV and PVF*

A series of standard mixtures of M-R1, M-R2, V-R2, G-Rg1, G-Rb1, G-Rc, and G-Rd was prepared in 70% MeOH with the concentration shown in Table 1. Regression equations established based on the natural logarithmic concentration of standards and their peak areas were used to determine the saponin content of PV and PVF rhizomes and roots.

**Table 1**

The concentrations of prepared standard mixtures used in the quantitative determination of saponins in PV and PVF by UPLC-TOF-MS

| No | Concentration (mg/mL) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **M-R1** | **G-Rg1** | **M-R2** | **V-R2** | **G-Rb1** | **G-Rc** | **G-Rd** |
| 1 | 1.000 | 1.070 | 1.010 | 1.010 | 1.080 | 1.045 | 1.080 |
| 2 | 0.500 | 0.535 | 0.505 | 0.505 | 0.540 | 0.523 | 0.540 |
| 3 | 0.250 | 0.268 | 0.253 | 0.253 | 0.270 | 0.261 | 0.270 |
| 4 | 0.125 | 0.134 | 0.126 | 0.126 | 0.135 | 0.131 | 0.135 |
| 5 | 0.063 | 0.067 | 0.063 | 0.063 | 0.068 | 0.065 | 0.068 |
| 6 | 0.031 | 0.033 | 0.032 | 0.032 | 0.034 | 0.033 | 0.034 |

*2.2.4. Data analysis*

The data were acquired by MassHunter Qualitative Analysis software version 10.0, and the comparison of the mean was carried out by One-way ANOVA using SPSS version 22.

**3. Result and discussion**

***3.1. Optimization of the analysis method***

The UPLC-TOF-MS condition was optimized in terms of MS detection parameters and gradient elution program. To improve the method's sensitivity, Figure 1 shows that the increase of fragmentor also increases the peak height, and the value of 350V leads to the highest signal intensity.
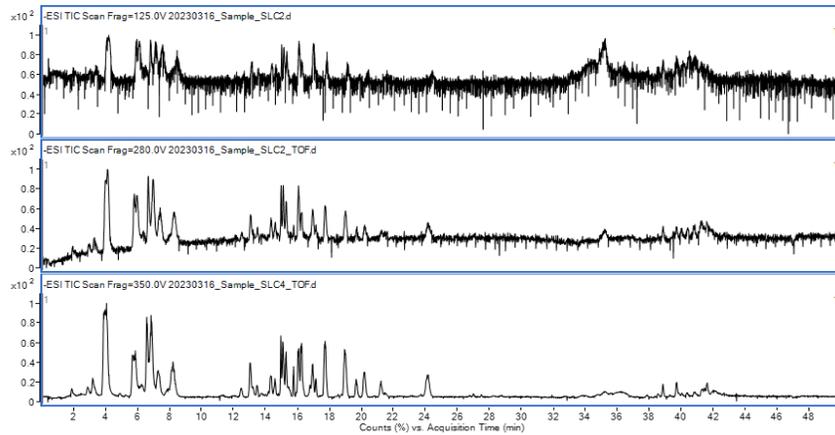
**Figure 1.** The chromatogram of PVF extract analyzed by UPLC-TOF at the fragmentor voltage of 125V (A), 280V (B), and 350V (C)

The gradient elution program was also optimized, including the gradient elution program and the addition of formic acid as the solvent modifier. The decrease in the initial acetonitrile concentration from 19% to 17% could result in the excellent separation of the early elute peaks such as majonoside R1 and notoginsenoside R1; ginsenoside Rg1, majonoside R2, and vina-ginsenoside R11 (Figure 2A-C). Notably, the separation of majonoside R2 and vina-ginsenoside R11 is essential due to the similar mass of these two compounds (*m/z* 785.47). The prolonged increase of acetonitrile content from 27 - 28% for 15min (33 - 47min) could separate the protopanaxadiol peaks' complex peak profile (Figure 2C-D). Although the MS detector could differentiate the peaks by mass value, the good separation of the peaks could visually express the difference in chemical composition in these two varieties.

Figure 2C showed that the mobile phase without formic acid could give a noisy baseline. Adding this mobile phase modifier agency could significantly reduce the background noise, as shown in Figure 2D, which exhibits the chromatogram at the optimized condition. The addition of formic acid may increase the ionization of the saponins leading to a higher peak intensity and thereby improving the sensitivity of the analysis.
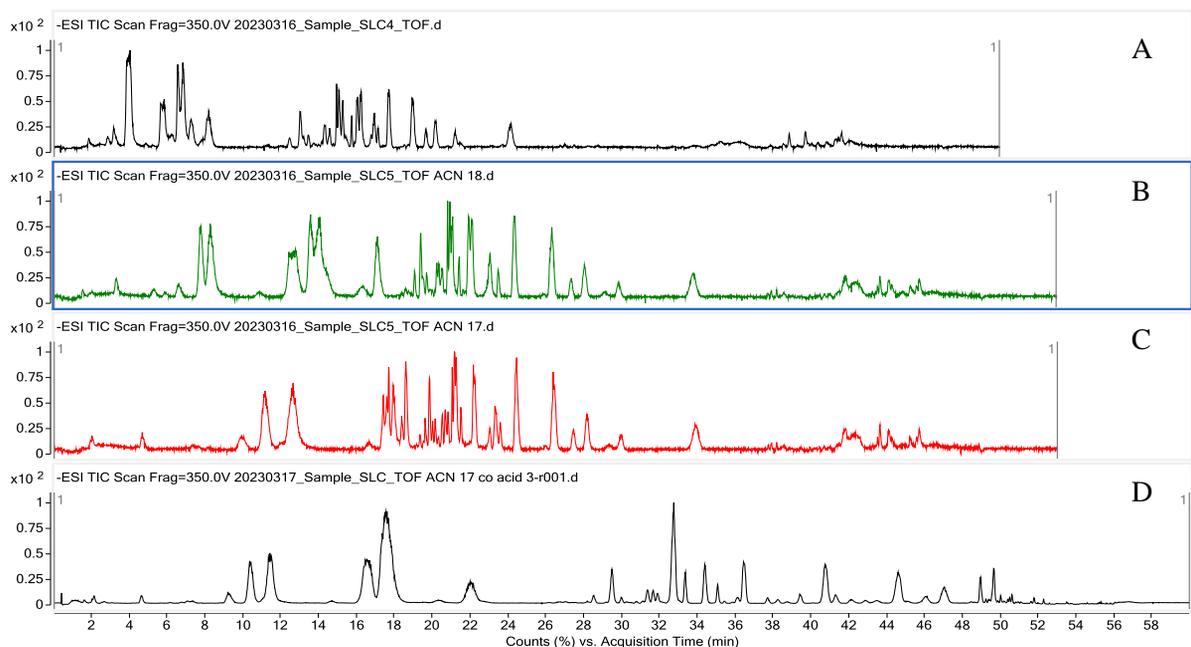


**Figure 2.** The mobile phase composition optimization's result of the LC-TOF-ESI-MS method

### *3.2. Qualitative differentiation of PV and PVF*
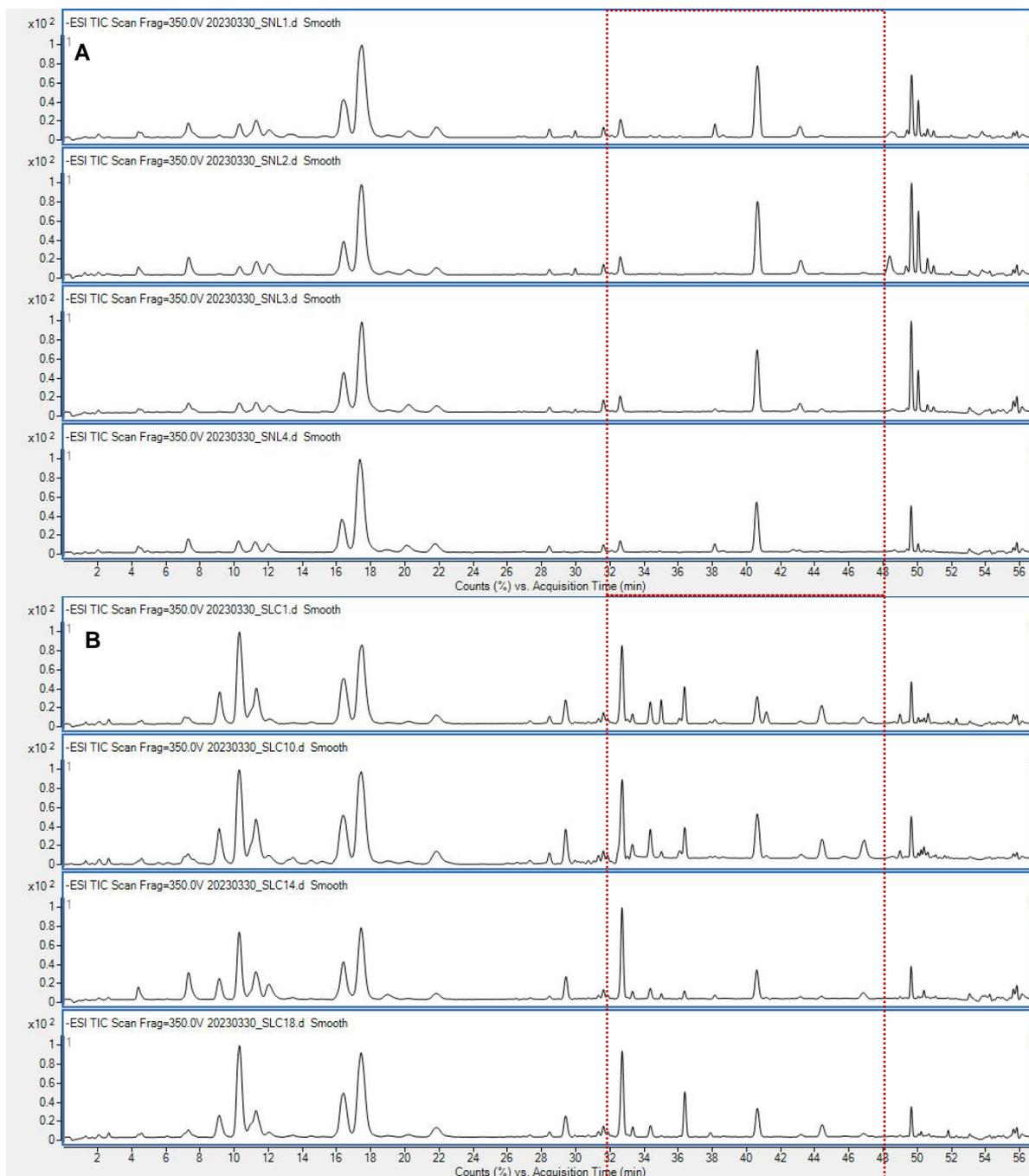


**Figure 3.** Representative chromatograms of PV (A) and PVF (B) extracts with distinct differences in eluted peaks of saponins from 32min to 48min

The representative UPLC-TOF-MS chromatograms of PV and PVF are shown in Figure 3. Visually, the two chromatograms share some similar peaks but also many differences. The representative chromatograms of PV and PVF extracts are shown in Figure 4. These two varieties of Vietnamese ginseng shared the common peaks of PPT and OT saponin such as M-R1 (2), notoginsenoside R1 (N-R1) (3), G-Rg1 (4), M-R2 (5), pseudoginsenoside F11 (6), V-R2 (9) as well as the two PPD-type saponins such as ginsenoside Rb1 and Rd. However, the peak abundance of M-R1 and V-R2, OT-type saponins, in PVF chromatogram is significantly higher than in PV. In addition, there are apparent differences in the peak profile of PV and PVF.

Regarding early eluted peaks, there is a peak of V-R4 (1) at the RT of 9.21 in the PVF chromatogram, while no peak of this compound is detected in the PV chromatogram. Moreover, the ratio of M-R1 (2) and N-R1(3) in PVF is much higher than in PV.

The peak profile of PVF eluted from 32 - 48min is much more complex than that of PV. In the PVF chromatogram, two unknown peaks [8: RT 29.38min, m/z 857.4891, and 11: RT 35min, m/z 1,371.6790] are absent in the PV chromatogram. The peak of V-R2 (9) is also much higher in PVF than PV. There are also high peaks of saponins, including chikusetsusaponin L8 (10, RT 34.33min), N-R4 (12, 36.37min), notoginsenoside Q (15, RT 41.15min), and notoginsenoside Fc (17, RT 44.37min) in PVF chromatogram, which is not found in PV chromatogram. In contrast, N-Fa (13, RT 37.16min), quinquenoside R1 (16, RT 43.13min), and pseudoginsenoside Rs1 (19, RT 50.05min) are present in PV chromatogram but not found in PVF chromatogram. In addition, the two PPD-type ginsenosides, such as G-Rb1 (14) and G-Rd (18), are present in both PV and PVF but their content in PV is significantly higher than in PVF.
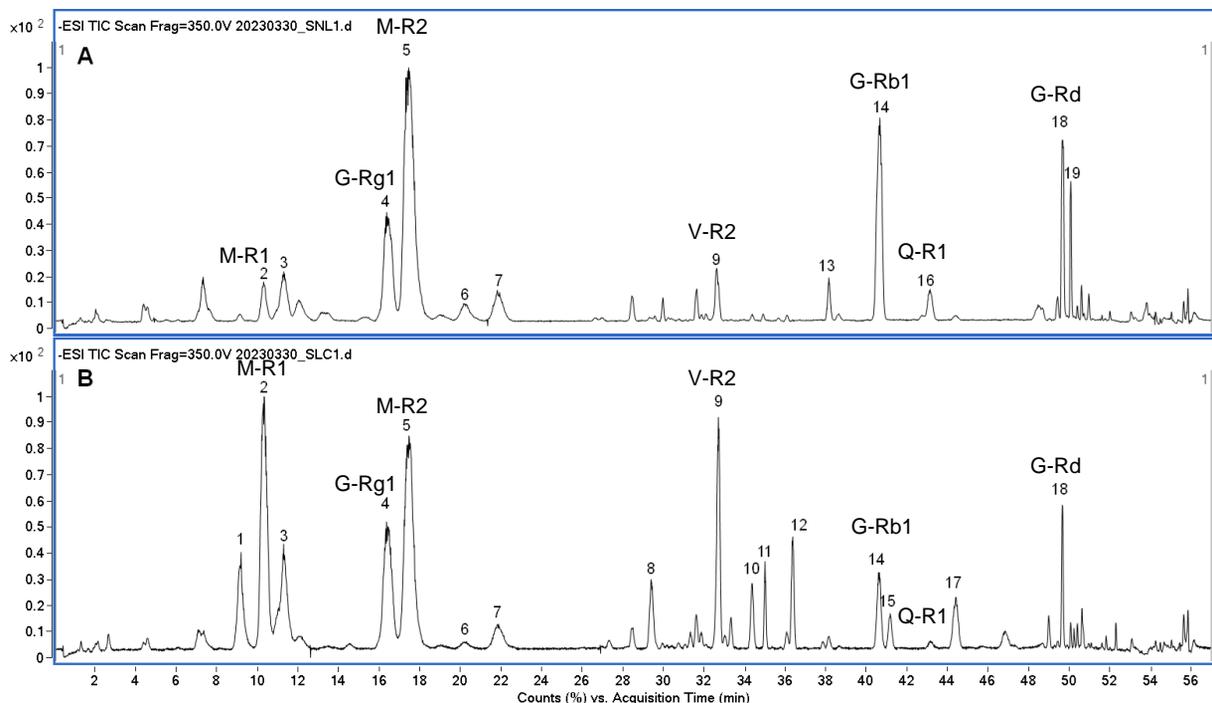


**Figure 4.** Peak identification of the chromatogram of PV extract (A) and PVF extract (B). The distinct differences are shown in saponin peaks eluted from 32min to 48min

**Table 2**

Compounds detected in PV and PVF extracts

| Peak No. | RT (min) | Compound | m/z | PV | PVF |
|----------|----------|----------|-----|-----|------|
| 1 | 9.21 | Vinaginsenoside R4 | 961.5371 ([M-H]⁻) | × | ✔ |
| 2 | 10.32 | Majonoside R1 | 815.4796 ([M-H]⁻) | ✔ | ✔ |
| 3 | 11.32 | Notoginsenoside R1 | 931.5269 ([M-H]⁻) | ✔ | ✔ |
| 4 | 16.39 | Ginsenoside Rg$_1$ | 799.4839 ([M-H]⁻) 845.4895 ([M+HCOO]⁻) | ✔ | ✔ |
| 5 | 17.44 | Majonoside R2 | 785.4689 ([M-H]⁻) | ✔ | ✔ |

| Peak No. | RT (min) | Compound | *m/z* | PV | PVF |
|---|---|---|---|---|---|
| 6 | 20.24 | Pseudoginsenoside F11 | 799.4842 ([M-H]⁻) | ✓ | ✓ |
| 7 | 21.89 | Vinaginsenoside R11 | 785.4691 ([M-H]⁻) | ✓ | ✓ |
| 8 | 29.38 | Unknown | 857.4891 ([M-H]⁻) | × | ✓ |
| 9 | 32.73 | Vinaginsenoside R2 | 827.4793 ([M-H]⁻) | ✓ | ✓ |
| 10 | 34.33 | Chikusetsusaponin L8 | 769.4739 ([M-H]⁻) | × | ✓ |
| 11 | 35.00 | Unknown | 1,371.6790 ([M-H]⁻) | × | ✓ |
| 12 | 36.37 | Notoginsenoside R4 | 1,239.6363 ([M-H]⁻) | × | ✓ |
| 13 | 37.16 | Notoginsenoside Fa | 1,239.6369 ([M-H]⁻) | ✓ | × |
| 14 | 40.63 | Ginsenoside Rb1 | 1,107.5951 ([M-H]⁻) | ✓ | ✓ |
| 15 | 41.15 | Notoginsenoside Q | 1,341.6702 ([M-H]⁻) | × | ✓ |
| 16 | 43.13 | Quinquenoside R1 | 1,149.6044 ([M-H]⁻) | ✓ | × |
| 17 | 44.37 | Notoginsenoside Fc | 1,209.6269 ([M-H]⁻) | × | ✓ |
| 18 | 49.65 | Ginsenoside Rd | 945.5415 ([M-H]⁻) | ✓ | ✓ |
| 19 | 50.05 | Pseudoginsenoside Rs1 | 987.5528 ([M-H]⁻) | ✓ | o |

### 3.3. Quantitative differentiation of PV and PVF

The regression equation, range, and correlation value of the compounds of interest determined by UPLC-TOF-MS are shown in Table 3. The $R^2$ value is above 0.985, which exhibits that this method is reliable for the preliminary quantitative determination of saponins in PV and PVF. Due to the non-linear regression of the method, the content calculation must be calculated using the regressive equation established by analyzing the range of six concentrations of standards. The equation was based on the area (y) and the natural logarithm of the concentration (x).
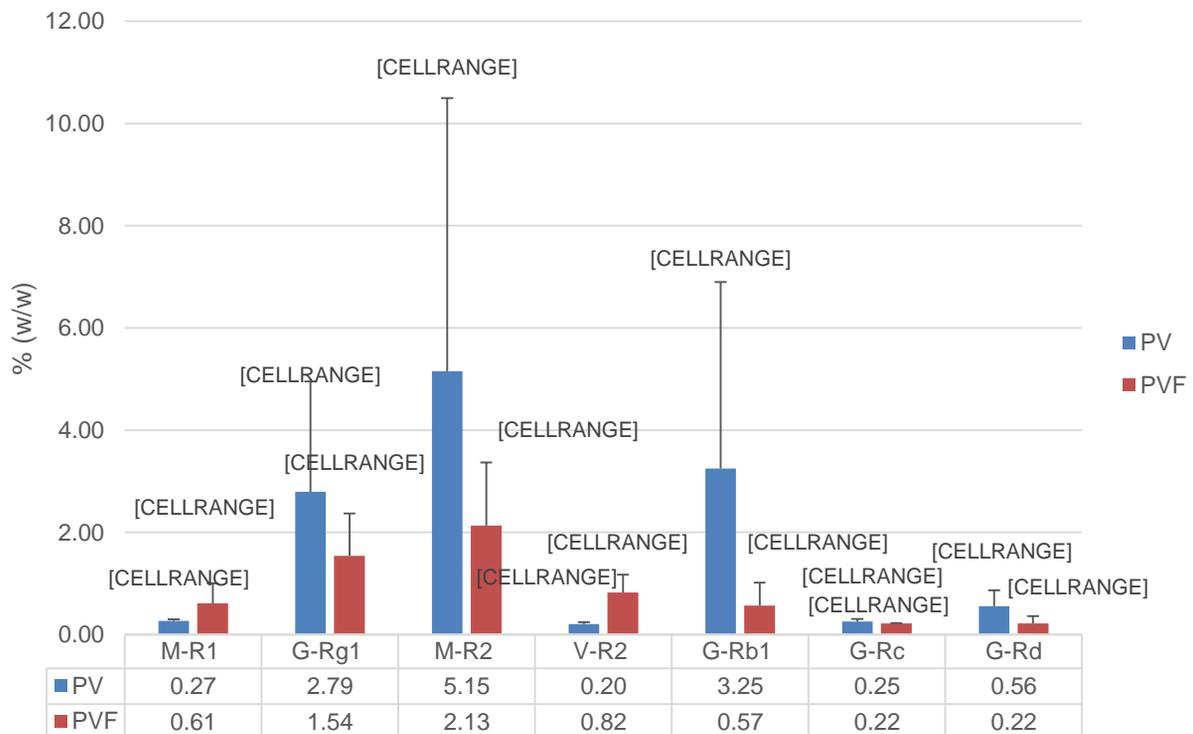
**Table 3**

Regression equation, range, and the correlation value of saponins determined by UPLC-TOF-MS

| Compounds | Regressive equation | Range (mg/mL) | $R^2$ |
|---|---|---|---|
| M-R1 | y = 794,335,060 × ln(x) + 3,057,142,849 | 0.50 - 0.031 | 0.9884 |
| G-Rg1 | y = 424,826,009 × ln(x) + 1,537,677,056 | 0.54 - 0.033 | 0.9852 |
| M-R2 | y = 820,669,938 × ln(x) + 3,156,025,831 | 0.51 - 0.032 | 0.9946 |
| V-R2 | y = 393,962,946 × ln(x) + 1,666,401,389 | 0.51 - 0.032 | 0.9929 |
| G-Rb1 | y = 691,864,195 × ln(x) + 2,769,480,918 | 0.54 - 0.033 | 0.9928 |
| G-Rc | y = 832,594,233 × ln(x) + 3,208,541,169 | 0.53 - 0.033 | 0.9909 |
| G-Rd | y = 171,994,379 × ln(x) + 886,309,110 | 0.54 - 0.034 | 0.9911 |

The content of 07 saponins in PV and PVF roots is summarized in Figure **5**. Overall, the content of four main saponins, G-Rg1, M-R2, G-Rb1, and G-Rd, in PV, is significantly higher than those in PVF. The content of G-Rg1, M-R2, and G-Rd in PV is twice as high as in PVF,

while the PV's G-Rb1 content is about 5.7 times higher than in PVF. These main saponins contribute to the high total saponin contents of PV (12.48%), two times higher than that of PVF (6.11%). However, the content of the two minor OT-type saponins in PVF, including M-R1 and V-R2, is two times and four times higher than in PV, successively. In detail, in PVF, the content of M-R1 is 0.67%, and that of V-R2 is 0.82%. However, the presence of these two saponins in PV is nearly at a trace level. This result is tightly correlative with the qualitative identification in 0. Therefore, M-R1 and V-R2 could be used as markers to differentiate PV and PVF.



| | M-R1 | G-Rg1 | M-R2 | V-R2 | G-Rb1 | G-Rc | G-Rd |
|---|---|---|---|---|---|---|---|
| PV | 0.27 | 2.79 | 5.15 | 0.20 | 3.25 | 0.25 | 0.56 |
| PVF | 0.61 | 1.54 | 2.13 | 0.82 | 0.57 | 0.22 | 0.22 |

**Figure 5.** Content of saponin in PV and PVF roots

*: $p < 0.05$; **: $p < 0.005$; ***: $p < 0.001$

## 4. Conclusion

By the developed UPLC-TOF-MS method, the saponin composition of PV and PVF could be qualitatively distinguished based on their saponin profiles, especially the saponin peaks eluted from 32 - 48 minutes. In addition, it was revealed that PVF has a more complex saponin composition but a lower content of the characteristic saponins, including G-Rg1, M-R2, G-Rb1, and G-Rd. On the contrary, PV has a higher saponin content and a simpler saponin profile. The developed method could help differentiate PV and PVF crude drugs on the market effectively to ensure the authentication of Vietnamese Ginseng and prevent the inattentive misuse or intentional adulteration of PV by PVF. Moreover, the established UPLC-QTOF-MS method could be extended for the analysis of finished products manufactured from the two sources of plants.

## References

Konoshima, T., Takasaki, M., Ichiishi, E., Murakami, T., Tokuda, H., Nishino, H., … Yamasaki, K. (1999). Cancer chemopreventive activity of majonoside-R2 from Vietnamese ginseng, *Panax vietnamensis. Cancer Letters, 147*(1), 11-16. doi:10.1016/S0304-3835(99)00257-8

Nguyen, H. T. T., Matsumoto, K., Kasai, R., Yamasaki, K., & Watanabe, H. (1998). In vitro antioxidant activity of Vietnamese ginseng saponin and its components. *Biological Pharmaceutical Bulletin, 21*(9), 978-981.

Nguyen, H. T. T., Matsumoto, K., Yamasaki, K., Nguyen, M. D., Nguyen, N. T., & Watanabe, H. (1996). Effects of Vietnamese ginseng on opioid agonist - and conditioned fear stress - induced antinociception. *Phytomedicine, 3*(1), 33-39. doi:10.1016/S0944-7113(96)80007-9

Nguyen, M. D., Nguyen, N. T., Kasai, R., Ito, A., Yamasaki, K., & Tanaka, O. (1993). Saponins from Vietnamese ginseng, Panax vietnamensis Ha et Grushv. Collected in central Vietnam. I. *Chemical and Pharmaceutical Bulletin, 41*(11), 2010-2014.

Nguyen, M. D., Nguyen, N. T., Kasai, R., Ito, A., Yamasaki, K., & Tanaka, O. (1994a). Saponins from Vietnamese Ginseng, Panax vietnamensis Ha et Grushv. Collected in central Vietnam. II. *Chemical and Pharmaceutical Bulletin, 42*(1), 115-122.

Nguyen, M. D., Nguyen, N. T., Kasai, R., Ito, A., Yamasaki, K., & Tanaka, O. (1994b). Saponins from Vietnamese ginseng, Panax vietnamensis Ha et Grushv. Collected in central Vietnam. III. *Chemical and Pharmaceutical Bulletin, 42*(3), 634-640.

Nguyen, T. D., Villard, P. H., Barlatier, A., Elsisi, A. E., Jouve, E., Nguyen, M. D., . . . Lacarelle, B. (2000). Panax vietnamensis protects mice against carbon tetrachloride-induced hepatotoxicity without any modification of CYP2E1 gene expression. *Planta Medica, 66*(08), 714-719.

Phan, L. K., Vu, D. D., Phan, L. K., Nguyen, S. G., Nguyen, T. T. P., Le, L. T. M., & Le, S. T. (2014). Phylogenetic relationships of the Panax samples collected in Lai Chau Province based on MATK and ITS-rDNA sequences. *Journal of Biotechnology, 12*(2), 327-337.

Vu, L. K. H., Le, V. T. H., Nguyen, H. T., Kim, H. M., Kang, K. S., Park, J. H., & Nguyen, M. D. (2019). Increase in protective effect of Panax vietnamensis by heat processing on cisplatin-induced kidney cell toxicity. *Molecules, 24*(24), Article 4627.

Zhu, S., Fushimi, H., Cai, S., Chen, H., & Komatsu, K. (2003). A new variety of the genus Panax from southern Yunnan, China and its nucleotide sequences of 18S ribosomal RNA gene and matK gene. *Journal of Japanese Botany, 78*(2), 86-94.

Zhu, S., Zou, K., Cai, S., Meselhy, M. R., & Komatsu, K (2004). Simultaneous determination of triterpene saponins in ginseng drugs by high-performance liquid chromatography. *Chemical and Pharmaceutical Bulletin, 52*(8), 995-998. doi:10.1248/cpb.52.995