## Original article

# Chemical Analysis of Xueshuantong Lyophilized Powder by LC-MS Profiling

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| ARTICLE INFO | ABSTRACT |
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| *Article history*<br><br>Received: July 7, 2014<br><br>Revised: September 1, 2014<br><br>Accepted: October 10, 2014<br><br>Available online:<br><br><br>DOI: | **Objective**   To elucidate the chemical basis of Xueshuantong (XST) Lyophilized Powder and primarily disclose the chemical difference between XST and *Panax notoginseng* roots. **Methods**   Liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC-ESI-MS$^n$) was used to profile the saponins in XST and *P. notoginseng*. Structural elucidation was based on combined analyses of the obtained negative and positive ESI-MS$^3$ data and comparing the retention behaviors. **Results**   The optimized LC-MS profiling approach enabled well resolution of major saponins. The negative mode ESI-MS$^3$ fragmentation gave diagnostic information on the nature (neutral loss 162 Da for Glc, 146 Da for Rha, and 132 Da for a pentose) and sequence (priority: terminal > inner) of sugars and sapogenins ($m/z$ 475 for protopanaxatriol; $m/z$ 459 for protopanaxadiol), while the intact glycosyl portion could be characterized by characteristic $Z_{0\alpha}^+$, $C_{n\alpha}^+$, and $C_{n\beta}^+$ ($n$ = 2 or 3) obtained in the positive mode. Ultimately, totally 30 saponins were characterized from XST. Compared with the roots of *P. notoginseng*, three malonyl-ginsenosides, ginsenoside Rd, and gyponoside XVII (or its isomer) were almost undetectable, and showed potential significance for their differentiation. **Conclusion**   The established LC-MS profiling approach is powerful for the chemical analysis of *P. notoginseng* and its preparations such as XST. |

## 1.   Introduction

The roots and rhizomes of *Panax notoginseng* (Burk) F. H. Chen are used as a well-known traditional Chinese medicine San-qi (or San-chi) in East Asian nations (Liu et al, 2014). *P. notoginseng* in clinic is widely prescribed due to its capacity of promoting blood circulation and hemostasis. Several subclasses of plant secondary metabolites, such as saponins, non-protein amino acids, polyacetylenes, phytosterols, flavonoids, etc, have been isolated from different parts of *P. notoginseng* (Wang et al, 2006). Of these, the saponins can modulate circulatory disorders and exert protective and

therapeutic activities against cardiovascular diseases. In addition, *L*-dencichine, a neuroexcitotoxic non-protein amino acid, is regarded as the major hemostatic component from *P. notoginseng*, which can enhance the hemostasis of activated platelets via AMPA receptors (Hang et al, 2014). Undoubtedly, most researches on *P. notoginseng*, in terms of the quality control, new chemicals discovery, pharmacological and pharmacokinetic studies, have focused on the involved saponins (Qi et al, 2011; Liu et al, 2014).

Xueshuantong (XST) is currently covering extensive clinical application for the treatment of cardio- and cerebrovascular diseases (Wang and Ding, 2007; Wang et al, 2014a). XST is prepared from Notoginseng Total Saponins (NTS) in the dosage forms of lyophilized powder and injection. There also have been documents reporting the therapeutic function of XST against nephrotic syndrome, senile chronic hepatitis B, diabetes mellitus, traumatic vitreous retinal hemorrhage, and paroxysmal epicophosis. XST mainly involves 20(*S*)- protopanaxatriol (PPT) type notoginsenoside $R_1$, ginsenosides Re and $Rg_1$, and 20(*S*)-protopanaxadiol type (PPD) ginsenosides $Rb_1$ and Rd. Tang and Wu (2008) developed a UPLC-based assay method to determine these five saponins in XST injections. The use of a 1.7-μm BEH $C_{18}$ column with acetonitrile-water as the mobile phase enabled well resolution of all analytes within 9.5 min. Wang et al (2013) compared the performance of HPLC and UPLC in the quantitative analysis of these five saponins in XST injection, and also demonstrated the higher efficiency of UPLC. All the available literature has focused on the major components. Aside from them, still there are minor or trace saponins in XST, a systematic study of which has not been witnessed.

Liquid chromatography coupled with mass spectrometry (LC-MS) has gained increasing application in the rapid screening of plant metabolites (Cheng et al; Wang et al, 2014b; Yang et al, 2009; Zhou et al, 2009). LC-MS based analytical approaches, when used for analyzing ginseng saponins, have been demonstrated with several merits: (1) providing good response in both negative and positive ionization modes with complementary structure information (Yang et al, 2012; 2013); (2) high sensitivity enabling quantitative analysis of saponins at trace level and pharmacokinetic studies (Guo et al, 2013; Qi et al, 2013); (3) high selectivity by selective ion monitoring or selective reaction monitoring; (4) high-accuracy mass measurement for unknown structures using a time-of-flight (TOF) or an Orbitrap mass analyzer (Peng et al, 2011; Zhang et al, 2012); (5) powerful differentiation of congeneric *Panax* Linn. species and different producing regions by combined use of fingerprint profiling and multivariate data analysis (Chen et al, 2011; Sun and Chen, 2011).

This study reports an LC-MS based qualitative method aimed to profile minor and trace saponins in XST and compare the chemical difference between XST and the roots of *P. notoginseng*. Results showed that this method was suitable and powerful for the on-line rapid profiling and chemical elucidation of *P. notoginseng* and its preparations.

## 2.  Materials and methods

### 2.1  Materials and reagents

Eight reference compounds (purity > 90% determined by HPLC), including notoginsenosides $R_1$, $R_2$, ginsenosides $Ra_3$, Re, $Rg_1$, $Rg_2$, $Rb_1$, and Rd, were isolated from the roots of *P. ginseng* by the authors (Yang et al, 2012). The analyzed XST Lyophilized Powder and NTS were friendly provided by Wuzhou Pharmaceutical Company (Guangxi, China). The drug materials of *P. notoginseng* roots were collected from Wenshan of Yunnan, China. The specimens for all analytes were deposited at the author's laboratory in Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

Methanol and acetonitrile of HPLC grade (J.T. Baker, Phillipsburg, NJ), ammonium acetate of ACS grade (Fluka, Netherland), and ultra-pure water prepared by a Milli-Q Water Purification System (Millipore, USA) were used in the mobile phase for chromatographic separation.

### 2.2  Sample preparation

For XST and NTS, an aliquot of 5.0 mg was accurately weighed and dissolved in 1 mL of 50% aqueous methanol with ultrasound assistance. The solution was further diluted quadruply (1.25 mg/mL) and filtered by a 0.22-μm membrane for LC-MS analysis. For *P. notoginseng*, 0.2 g of fine powder was soaked in 10 mL of 50% aqueous methanol and then ultrasonically extracted at 40 °C for 40 min. The extract (20 mg/mL) was filtered through a 0.22-μm membrane prior to LC-MS analysis.

### 2.3  HPLC-UV and LC-MS conditions

Chromatographic separation was performed on an Agilent 1100 series HPLC system (Germany) using a YMC-Pack ODS-A column (250 mm × 4.6 mm, 5 μm). With the view of well resolving major saponins, particularly for ginsenosides Re and $Rg_1$, a three-component mobile phase consisting of methanol (A), acetonitrile (B), and water containing 3 mmol/L ammonium acetate (C) was used following the elution program: 0 min: 12% (A), 35% (B), 53% (C); 10 min: 20% (A), 30% (B), 50% (C); 25 min: 32% (A), 18% (B), 50% (C); 40 min: 50% (A), 0 (B), 50% (C); 50 min: 75% (A), 0 (B), 25% (C); 52 min: 80% (A), 0 (B), 20% (C); 57 min: 80% (A), 0 (B), 20% (C); 59 min: 12% (A), 35% (B), 53% (C). The column was maintained at 35 °C. The UV detector was set at 203 nm. A flow rate of 0.8 mL/min was used.

A Finnigan LCQ Advantage Ion-trap Mass Spectrometer (Thermo Fisher, USA) hyphenated with the Agilent 1100 HPLC equipped with an ESI source was applied for chemical elucidation. The LC effluent after chromatographic separation was introduced into the ESI source in a splitting ratio of 5:1. Mass spectra were obtained in both negative and positive ion modes to offer sufficient fragment information. The collision gas and sheath/auxiliary gas were helium (He) and nitrogen ($N_2$) with ultra-high purity, respectively. A source

fragmentation of 30 V was applied to diminish adduct precursor ions in the negative mode. The source parameters such as spray voltage of 4.5 kV, capillary temperature of 320 °C, sheath gas of 45 arbitrary units, and auxiliary gas of 10 arbitrary units were adopted. Capillary voltage −22 V/15 V, tube lens offset −60 V/50 V were separately used in the negative/positive ion modes. The mass analyzer scanned over $m/z$ 400−1800 for MS and $m/z$ 150−1800 for data-dependent MS$^3$ acquisition. Collision energy 34% and 36% were used for the negative and positive MS$^3$ fragmentation. To obtain the MS$^3$ product ions information of coeluted components, the data dependent acquisition-dynamic exclusion (DDA-DE) was set using the following parameters: repeat time of 3; repeat duration of 0.5 min; exclusion list size of 25, and exclusion duration of 0.7 min. Date were performed using Xcalibur 2.0 software.

## 3. Results and discussion

Characterization of the chemicals in XST was based on the strategies: (1) unequivocal identification by comparing with reference standards in terms of the retention time ($t_R$, min) and ESI-MS$^2$ product ions; (2) tentative identification or characterization for those without reference by analyzing their negative/positive mode ESI-MS$^3$ information, comparing the retention behavior, and surveying the literature. As a result, a total of 30 saponins got identified or tentatively characterized, of which peaks **5** ($t_R$ 11.03 min), **8** ($t_R$ 12.89 min), **9** ($t_R$ 13.49 min), **14** ($t_R$ 26.94 min), **16** ($t_R$ 29.43 min), **24** ($t_R$ 34.31 min), **25** ($t_R$ 34.65 min), and **29** ($t_R$ 39.85 min) were unambiguously identified as noto-R$_1$, Re, Rg$_1$, noto-R$_2$, Rg$_2$, Ra$_3$, Rb$_1$, and Rd by comparing with the reference standards. Structural information, involving the retention time, negative and positive precursor ions ([M − H]$^-$/ [M + Na]$^+$), ESI-MS$^3$ product ions and the identity for these compounds are offered in Table 1. Nomenclature for fragments was consistent with that used in our previous study (Yang et al, 2012), and Xyl was used to represent all the pentose residues characterized by neutral loss of 132 Da in this study for the convenient depiction of the identity of characterized saponins.

**Table 1    Saponins characterized from XST Lyophilized Powder by comprehensive (±)-ESI-MS$^n$ analyses**

| No. | $t_R$ / min | [M − H]$^-$ / [M + Na]$^+$ | (−) ESI-MS$^3$ | (+) ESI-MS$^3$ | Identity |
|---|---|---|---|---|---|
| 1 | 8.35 | 961/985 | [961] 799, 637, 475 <br> [961 > 637] 475 | [985] 365 | PPT-20-GlcGlc-6-Glc |
| 2 | 8.89 | 961/985 | [961] 799, 781, 637, 475 <br> [961 > 799] 637 | [985] 805 <br> [985 > 805] 643 | PPT-20-Glc-6-GlcGlc |
| 3 | 10.16 | 1093/1117 | [1093] 961, 931 <br> [1093 > 931] 637 | [1117] 937, 365 <br> [1117 > 937] 497 | PPT-20-Glc-6-GlcGlcXyl |
| 4 | 10.35 | 961/ 985 | [961] 799, 637, 619, 475 <br> [961 > 799] 637, 475 | [985] 805 <br> [985 > 805] 365 | PPT-20-Glc-6-GlcGlc |
| 5 | 11.03 | 931/ 955 | [931] 799, 769, 637, 475 <br> [931 > 637] 475 | [955] 775 <br> [955 > 775] 643, 335 | noto-R$_1$ |
| 6 | 11.75 | 931/ 955 | [931] 799, 769, 637, 475 <br> [931 > 799] 637, 475 | [955] 775 <br> [955 > 775] 335 | isomer of noto-R$_1$ |
| 7 | 12.65 | 961/ 985 | [961] 799, 637, 475, 323 <br> [961 > 799] 637 | [985] 805 <br> [985 > 805] 365 | PPT-20-Glc-6-GlcGlc |
| 8 | 12.89 | 945/ 969 | [945] 799, 783, 637, 475 <br> [945 > 783] 637, 475 | [969] 789 <br> [969 > 789] 389 | Re |
| 9 | 13.49 | 799/ 823 | [799] 637,475 <br> [799 > 475] 391 | [823] 683, 643 | Rg$_1$ |
| 10 | 15.43 | 1123/1147 | [1123] 961, 799, 637, 475 <br> [1123 > 799] 475 | [1147] 805, 365 <br> [1147 > 805] 365 | PPT-20-GlcGlc-6-GlcGlc |
| 11 | 16.28 | 959/ 983 | [959] 797, 635, 473 <br> [959 > 797] 635 | [983] 803, 387 <br> [983 > 803] 365 | noto-G or its isomer |
| 12 | 23.07 | 961/ 985 | [961] 799, 475 <br> [961 > 799] 637, 475 | [985] 805 <br> [985 > 805] 365 | noto-E or its isomer |
| 13 | 26.59 | 799/ 823 | [799] 475, 323 | [823] 365 <br> [823 > 365] 305 | Rf or its isomer |
| 14 | 26.94 | 769/ 793 | [769] 637, 619, 475 <br> [769 > 637] 475 | Not detected | noto-R$_2$ |
| 15 | 28.16 | 1371/1395 | [1371] 1239, 1107, 945 <br> [1371 > 1107] 945, 621 | [1395] 921,497 <br> [1395 > 497] 437 | noto-T or its isomer |
| 16 | 29.43 | 783/ 807 | [783] 637, 619, 475 <br> [783 > 475] 457, 391 | Not detected | Rg$_2$ |
| 17 | 29.87 | 1269/1293 | [1269] 1107, 945, 621, 459 | [1293] 951, 527 | PPD-20-GlcGlc-3-GlcGlcGlc |

Continued Table 1

| No. | $t_R$ / min | $[M-H]^-$ / $[M+Na]^+$ | (−) ESI-MS$^3$ | (+) ESI-MS$^3$ | Identity |
|---|---|---|---|---|---|
| 18 | 30.37 | 637/− | [637] 569, 475 | Not detected | Rh1 |
| | | | [637 > 475] 457, 391 | | |
| 19 | 30.67 | 1239/1263 | [1239] 1107, 945, 783 | [1263] 789, 497 | noto-R$_4$ |
| | | | [1239 > 1107] 945, 783, 459 | [1263 > 497] 437, 365 | |
| 20 | 32.22 | 1371/1395 | [1371] 1239, 1107 | [1395] 921, 789, 497 | noto-T or its isomer |
| | | | [1371 > 1239] 1107, 945, 783 | [1395 > 497] 437, 467 | |
| 21 | 32.72 | 1239/1263 | [1239] 1107, 945 | [1263] 921 | isomer of noto-R$_4$ |
| | | | [1239 > 945] 783, 459 | [1263 > 921] 497 | |
| 22 | 32.85 | 1091/1115 | [1091] 929, 767 | [1115] 773, 365 | noto-I or its isomer |
| | | | [1091 > 929] 767, 605 | [1115 > 773] 365 | |
| 23 | 33.05 | 1105/1129 | [1105] 943, 781 | [1129] 787, 365 | 5,6-didehydro-Rb$_1$ or its isomer |
| | | | [1105 > 943] 781 | [1129 > 787] 365 | |
| 24 | 34.31 | 1239/1263 | [1239] 1107, 945, 783 | [1263] 789, 497 | Ra$_3$ |
| | | | [1239 > 1107] 783, 459 | [1263 > 497] 437, 407 | |
| 25 | 34.65 | 1107/1131 | [1107] 945, 783, 621 | [1131] 789, 365 | Rb$_1$ |
| | | | [1107 > 945] 783, 459 | [1131 > 365] 305 | |
| 26 | 35.78 | 1209/1233 | [1239] 1077, 945, 783 | Not detected | Ra$_1$ |
| | | | [1239 > 1077] 945, 783, 459 | | |
| 27 | 37.19 | 1077/1111 | [1077] 945, 915, 783, 621 | [1111] 789 | Rb$_2$ |
| | | | [1077 > 945] 783, 459 | [1111 > 789] 365 | |
| 28 | 37.62 | 1077/1111 | [1077] 945, 783, 621 | [1111] 789 | Rb$_3$ |
| | | | [1077 > 945] 783, 621, 459 | [1111 > 789] 365 | |
| 29 | 39.85 | 945/ 969 | [945] 621, 459 | [969] 789 | Rd |
| | | | [945 > 621] 459 | [969 > 789] 365 | |
| 30 | 41.98 | 945/ 969 | [945] 783, 621, 459 | [969] 365 | gyponoside XVII or its isomer |
| | | | [945 > 621] 459 | [969 > 365] 305, 275 | |

### 3.1 Interpretation for fragmentation of eight saponins compounds

Collision-induced dissociation (CID) of eight saponins was firstly studied, and the deduced common fragmentation pathways and diagnostic product ions were used to characterize other saponins present in XST Lyophilized Powder.

In general, these eight saponin molecules were ionized into $[M-H]^-$ and $[M+Na]^+$ precursors in the negative and positive modes, respectively. In the cases for representative notoginsenoside R$_1$ and ginsenoside Ra$_3$, their $[M-H]^-$ and $[M+Na]^+$ precursor ions were observed at $m/z$ 931/955 and 1239/1263, respectively (Figure 1). Therefore, a peak at the same $t_R$ with precursor mass difference 24 Da refers to the same component. CID of the $[M-H]^-$ precursors underwent successive neutral loss of sugars, and could yield sapogenin-related product ions. The elimination of Glc, Rha, and Xyl corresponded to neutral losses of 162, 146, and 132 Da. The product ions $m/z$ 475 (for noto-R$_1$, R$_2$, Re, Rg$_1$, and Rg$_2$) and 459 (for Ra$_3$, Rb$_1$, and Rd) were the deprotonated PPT and PPD molecules. Moreover, the terminal sugars were easy to be eliminated, as seen in the MS/MS fragmentation of noto-R$_1$ ($[M-H-Xyl]^-$, $m/z$ 799), noto-R$_2$ ($[M-H-Xyl]^-$, $m/z$ 637), Re ($[M-H-Rha]^-$, $m/z$ 799), Rg$_2$ ($[M-H-Rha]^-$, $m/z$ 637), and Ra$_3$ ($[M-H-Xyl]^-$, $m/z$ 1107). For the positive CID, the $[M+Na]^+$ precursors were prone to eliminate entire 20-glycosyl portion and produced the sodium-adduct remaining portion ($Z_{0\alpha}^+$) and 20-saccharide chains ($C_{n\alpha}^+$) product ions. When $Z_{0\alpha}^+$ was further dissociated, predominant $C_{n\beta}^+$ ($n$ = 2 or 3) was generated. Importantly, the transitions from $Z_{0\alpha}^+$ to $C_{n\beta}^+$ for PPT type and PPD type were 440 and 424 Da, respectively, assigned to [PPT-2H$_2$O] and [PPD-2H$_2$O], which can be diagnostic for indirect characterization of the sapogenins. On the other hand, the sugar number ratio of α-chain (C-20) and β-chain (C-6 for PPT and C-3 for PPD) would influence the intensity ratio of $Z_{0\alpha}^+$ and $C_{n\alpha}^+$ in the MS/MS spectra. For noto-R$_1$ (α-chain:β-chain 1:2), $Z_{0\alpha}^+$ at $m/z$ 775 was predominant, however, for Ra$_3$ (α-chain:β-chain 3:2), abundant $C_{3\alpha}^+$ at $m/z$ 497 was generated, in contrast to the weak $Z_{0\alpha}^+$ at $m/z$ 789 (Figure 1). Therefore, the relative intensity of $Z_{0\alpha}^+$ to $C_{n\alpha}^+$ could assist in characterizing the sugar distribution.

### 3.2 Chemical elucidation of XST Lyophilized Powder by LC-ESI-MS$^3$

The above deduced regular fragmentation behaviors together with the interpretation guideline we previously proposed were applied for characterizing unknown saponins in XST (Yang et al, 2012). In addition to Ra$_3$ (peak **24**, $t_R$ 34.31 min), its other two isomers **19** and **21** were found with $t_R$ of 30.67 and 32.72 min. The negative CID of these three saponins all generated product ions at $m/z$ 1107, 945, 783, and 459, which suggested the presence of four Glc, one pentose, and PPD sapogenin. In the positive mode ESI-MS$^3$ fragmentations, peak **19** produced the same $Z_{0\alpha}^+$ ($m/z$ 789),
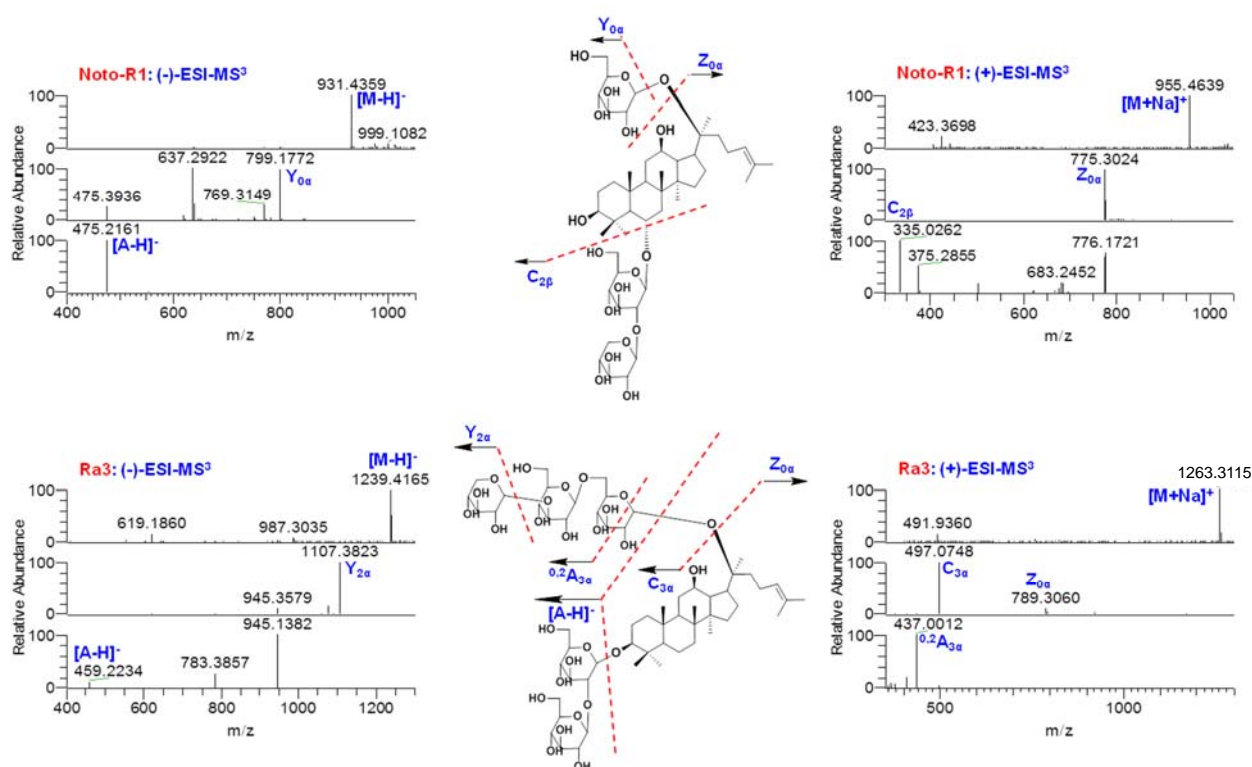
**Figure 1   Illustration for negative and positive mode ESI-MS³ fragmentations of representative notoginsenoside R₁ and ginsenoside Ra₃**

$C_{3\alpha}^+$ ($m/z$ 497), and $C_{2\beta}^+$ ($m/z$ 365) as those of Ra₃, based on which peak **19** was characterized as PPD-20-GlcGlcXyl-3-GlcGlc, possible to be notoginsenoside R₄ (Matsuura et al, 1983). However, $Z_{0\alpha}^+$ for peak **21** was found at $m/z$ 921 ([M + Na − GlcGlc − H₂O]⁺), which was further fragmented into $C_{3\beta}^+$ at $m/z$ 497, indicating the GlcGlc and GlcGlcXyl glycosyl portions glycosylated at C-20 and C-3, respectively. The mass difference calculated at 424 Da from $Z_{0\alpha}^+$ to $C_{3\beta}^+$ could also provide the evidence for confirming the PPD sapogenin of **21**. In consistency, peaks **19** and **21** possessing an additional pentose than Rb₁ (peak **25**, $t_R$ 34.65 min), they were both eluted from the column prior to Rb₁. The analysis of the CID spectral data and comparison of retention behavior aided in characterizing most of the saponins in XST.

Comparing the retention behaviors of unknown peaks with those of reference compounds could also assist in characterizing and differentiating sapogenin isomers. Peaks **1** ($t_R$ 8.35 min), **2** ($t_R$ 8.89 min), **4** ($t_R$ 10.35 min), and **12** ($t_R$ 23.07 min) were four isomeric compounds with the same molecular weight (MW) of 962 since they all possessed the deprotonated precursors $m/z$ 961 and sodium-adduct precursors $m/z$ 985. Their negative mode CID could generate product ions $m/z$ 799, 637, and 475, which indicated the presence of three Glc residues and the sapogenin with MW of 476. In contrast to Re (the trisaccharide glycoside of PPT, $t_R$ 12.89 min), peaks **1**, **2**, and **4** were all eluted ahead of Re, while peak **12** was eluted after Re. We therefore characterized the sapogenin of **12** as an isomer of PPT. Only by the negative CID information, the distribution of three sugars for these four isomers could not be characterized. Fortunately, the

positive mode ESI-MS³ fragmentation offered complementary evidence for characterizing the glycosyl portions. Abundant $Z_{0\alpha}^+$ at $m/z$ 805 and $C_{2\beta}^+$ at $m/z$ 365 were observed for **2**, **4**, and **12**, indicating the unique Glc and two Glc residues attached to C-20 and C-6 (or C-3), respectively. However, case is different for peak **1**. The [M + Na]⁺ precursors were dissociated into dominant product ion at $m/z$ 365, which informed a possible GlcGlc sugar chain glycosylated at C-20. According to these evidence and literature survey, peaks **1** was characterized as PPT-20-GlcGlc-6-Glc, peaks **2** and **4** as PPT-20-Glc-6-GlcGlc, and **12** as notoginsenoside E or its isomer (Yoshikawa et al, 1997).

Even when no diagnostic ions were generated to indicate the sapogenin, the positive mode ESI-MS³ could also give structural information for the tentative characterization. Of the identified saponins in XST, peaks **15** ($t_R$ 28.16 min) and **20** ($t_R$ 32.22 min) possessed the largest MW of 1372. Their deprotonated precursors ($m/z$ 1371) were fragmented into product ions $m/z$ 1239, 1107, 945, and 783, suggesting that they had an additional pentose in contrast to Ra₃. The sapogenin ion failed to be produced possibly due to the relative low collision energy (34%). The CID of [M + Na]⁺ precursors ($m/z$ 1395) both yielded weak $Z_{0\alpha}^+$ $m/z$ 921 and abundant $C_{3\alpha}^+$ $m/z$ 497. The MS/MS base peak $C_{3\alpha}^+$ informed a GlcGlcXyl glycosyl portion attached to C-20. The $Z_{0\alpha}^+$ ($m/z$ 921), the same as that for peak **21**, also suggested a saccharide chain comprising GlcGlcXyl glycosylated at C-3. However, these characterization results need verification by comparing with pure saponin compounds.

### 3.3 Primary comparison on saponins between XST and roots of P. notoginseng

Based on the characterization strategy, the chemicals in the roots of *P. notoginseng* were also investigated, and further primarily compared with those in XST. The holistic comparison among NTS, XST, and *P. notoginseng* is shown in Figure 2.

The chemical composition between NTS and XST was analogous. XST was prepared from NTS via certain preparation steps. However, the global profiling fingerprint of *P. notoginseng* roots displayed obvious differentiation from that of XST. By a comprehensive LC-MS analysis, five major differential components (**I−V** in Figure 2) were found and characterized. The negative and positive ESI-MS$^3$ spectra of **I**, **II**, and **III** are given in Figure 3. Peaks **I** ($t_R$ 22.69 min), **II** ($t_R$ 23.66 min), and **III** ($t_R$ 28.50 min) were three malonyl-substituted ginsenosides. In their negative MS spectra, $m/z$ 1149 (based peak), 1193, and 1215 were observed, corresponding to $[M − H − CO_2]^-$, $[M − H]^-$, and $[M − 2H + Na]^-$, respectively. The sodium adduct precursors $m/z$ 1217 ($[M + Na]^+$) were detected in the positive mode. The negative ESI-MS$^3$ spectra showed product ions at $m/z$ 1107 ($[M − H − 86\ Da]^-$), 1089, 945, 927, 783, 621, and 459, suggesting the PPD sapogenin glycosylated with four Glc and a malonyl group. Based on

the positive mode CID data, differential components **I** and **II** both generated $Z_{0\alpha}^+$ at $m/z$ 875 by neutral loss of 342 Da, indicating the presence of 20-GlcGlc. These $Z_{0\alpha}^+$ ions could be further cleaved into product ions $m/z$ 831 ($[Z_{0\alpha} − CO_2]^+$) and (or) 451 ($[malonyl − GlcGlc + H_2O + Na]^+$), which informed the composition of 3-glycosyl portion as malonyl-GlcGlc. Thus, **I** and **III** were tentatively identified as PPD-20-GlcGlc-3-GlcGlc-malonyl. For differential component **II**, an abundant $C_{2\alpha}^+$ at $m/z$ 451 as well as $Z_{0\alpha}^+$ at $m/z$ 789 was obtained by CID of the precursors ($m/z$ 1217). Based on these diagnostic product ions, we could primarily characterize **II** as PPD-20-GlcGlc-malonyl-3-GlcGlc. In addition, another two significant differential components (**IV** and **V**) were observed at $t_R$ of 39.85 and 41.98 min, of which the former was ginsenoside Rd by comparing with reference standard. Compound **V** was an isomer of Rd, whose negative MS/MS fragmentation generated the same product ions ($m/z$ 783, 765, 621, and 459) as those of Rd. Differently, CID of the positive precursors $m/z$ 969 yielded abundant $C_{2\alpha}^+$ at $m/z$ 365, rather than $Z_{0\alpha}^+$ $m/z$ 789, suggesting the presence of 20-GlcGlc. The $C_{2\alpha}^+$ ($m/z$ 365) could be further dissociated into $m/z$ 305 ($^{0,2}A_{2\alpha}^+$) and 275 ($^{0,3}A_{2\alpha}^+$), indicating these two glucoses were in 6→1 linkage (Liu et al, 2009). Ultimately, it was charac-terized as gyponoside XVII or its isomer (Wang et al, 2008).
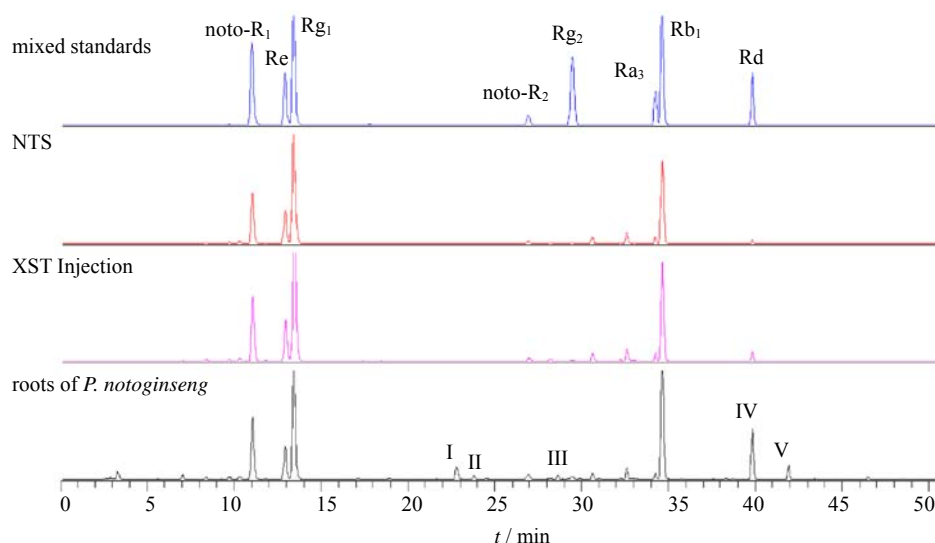


**Figure 2    Total ion current chromatograms of mixed standards, NTS, XST, and roots of *P. notogineng***

According to *Chinese Pharmacopoeia 2010*, the NTS are prepared from the ethanol extract of *P. notoginseng* by macroporous resin purification. The primary water eluate is discarded, and the subsequent 80% ethanol fraction is finally prepared into NTS. The malonyl-ginsenosides can be eluted by pure water due to their strong polarity, while partial ginsenoside Rd and gyponoside XVII (or its isomer) are not washed by 80% ethanol. This might be the reason for the chemical differentiation we discovered between NTS and XST.

## 4.    Conclusion

Despite the fact that XST has been extensively used in clinic, its chemical composition, in particular those minor and trace saponins, has not been fully elucidated. To this end, this study reports an LC-MS based method for comprehensively analyzing the saponins in XST Lyophilized Powder and primarily comparing the chemical difference between XST and the roots of *P. notoginseng*. The integrated analyses of the negative and positive mode ESI-MS$^3$ spectral data offered
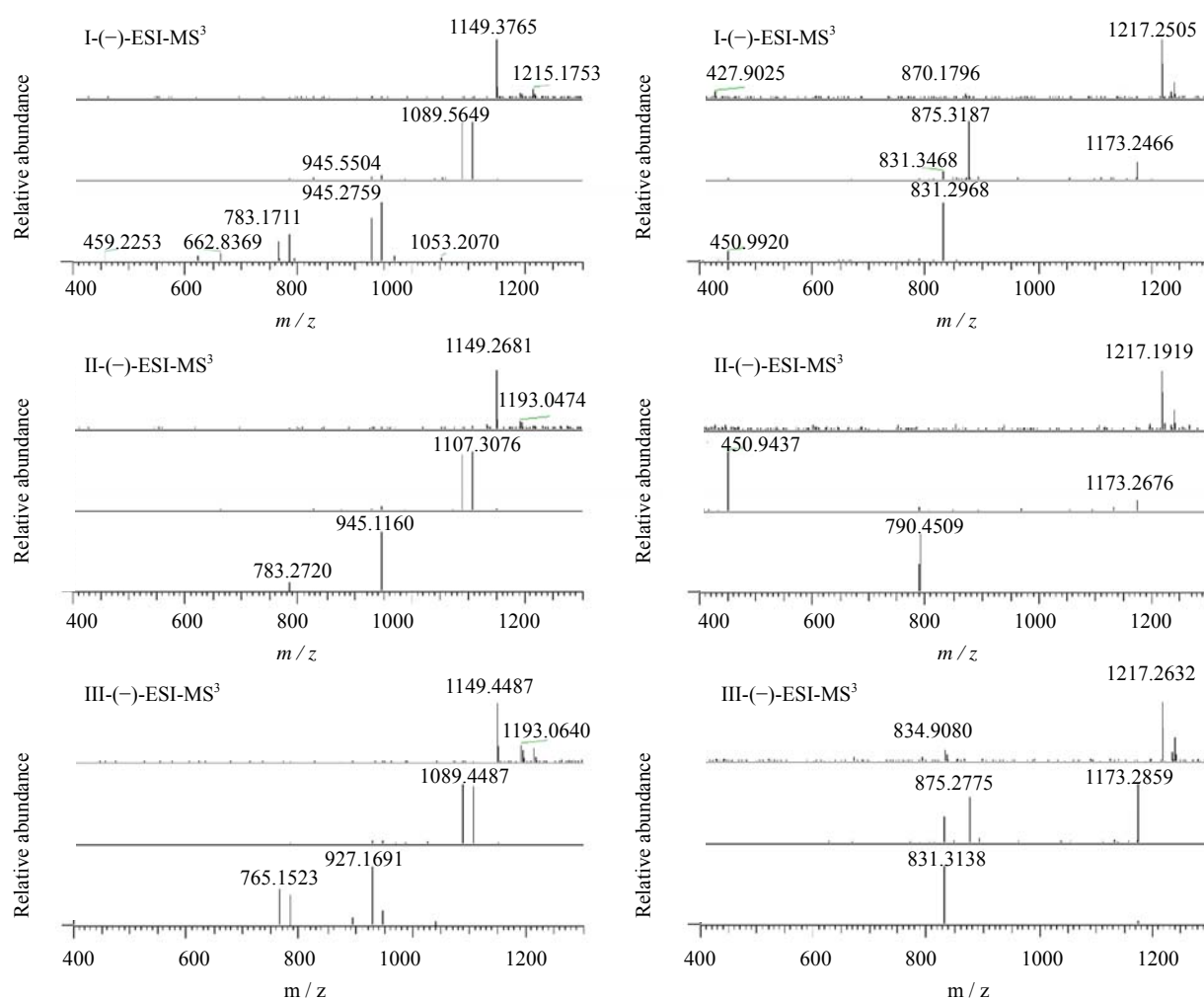
**Figure 3   (−)- and (+)-ESI-MS³ spectra of three significantly differential malonyl-ginsenosides between XST and *P. notoginseng* roots**

complementary structure information on the nature and sequence of sugars, sapogenins, and composition of glycosyl portion of the saponins. The obtained $Z_{0\alpha}^+$, $C_{n\alpha}^+$, and $C_{n\beta}^+$ could even be used to differentiate isomeric saponins with different sugar distribution. As a result, totally 30 saponins were identified or tentatively characterized from XST, involving the eight unambiguously identified by comparing with reference standards. In addition, five major differential components, comprising three malonyl-ginsenosides, Rd, and gyponoside XVII (or its isomer), were found, which were present or abundant in *P. notoginseng* rather than XST. The established LC-MS approach is proven as suitable and powerful for the qualitative analysis of the chemicals in *P. notoginseng*, NTS, and their preparations like XST.

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