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# Simultaneous Analysis of Eight Bioactive Steroidal Saponins in Gongxuening Capsules by HPLC–ELSD and HPLC–$MS^n$

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**Summary.** Rapid high-performance liquid chromatographic methods with evaporative light scattering detection (HPLC–ELSD) and electrospray ionization multistage mass spectrometry (HPLC–ESI–$MS^n$) have been established and validated for simultaneous qualitative and quantitative analysis of eight steroidal saponins in ten batches of Gongxuening capsule (GXN), a widely commercially available traditional Chinese preparation. The optimum chromatographic conditions entailed use of a Kromasil $C_{18}$ column with acetonitrile–water (30:70 to 62:38, *v*/*v*) as mobile phase at a flow rate of 1.0 mL $min^{-1}$. The drift tube temperature of the ELSD was 102°C and the nebulizing gas flow rate was 2.8 L $min^{-1}$. Separation was successfully achieved within 25 min. LC–ESI–$MS^n$ was used for unequivocal identification of the constituents of the samples by comparison with reference compounds. The assay was fully validated for precision, repeatability, accuracy, and stability, then successfully applied to quantification of the eight compounds in samples. The method could be effective for evaluation of the clinical safety and efficacy of GXN.

**Key Words:** column liquid chromatography, HPLC–ESI–$MS^n$, HPLC–ELSD, gongxuening capsule

## Introduction

Gongxuening capsule (GXN), a well-known traditional Chinese preparation, has been widely used for more than ten years and is commercially available in most cities in China. Classically, this patent medicine has been used to cure female patients suffering from metrorrhagia, menorrhagia, metrostaxis, functional uterine bleeding, and chronic pelvic inflammatory

diseases [1], and its therapeutic effectiveness and few side effects have been confirmed by long-standing clinical practice and modern pharmacological research [2–5]. To ensure the clinical efficacy and safety of this product, quality control is extremely important.

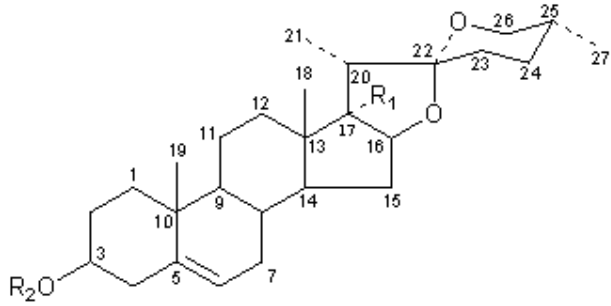
*Paris polyphylla* Smith var. *yunnanensis* (Franch.) Hand.-Mazz. (genus *Paris*, Trilliaceae) is the main raw material of GXN. Chemical and pharmacological investigations on the genus *Paris* have revealed that steroidal saponins, which are mainly divided into the two types pennogenin and diosgenin, depending on whether or not the structures contain a 17-hydroxyl group [6], are the major bioactive constituents with hemostasis, anti-inflammatory, analgesic, antithrombotic, and uterine contraction effects [6–12]. Accordingly, the two types of steroidal saponin should, together, be taken into account as marker compounds for chemical evaluation and standardization of GXN. Unfortunately, according to China Pharmacopeia, only one pennogenin saponin was selected as marker compound for quality control of GXN [1]. In previous studies, HPLC–UV was used for analysis of four pennogenin saponins in GXN [13]; HPLC–MS has also been used for qualitative analysis of this preparation [14]. Methods for the quality control of GXN by simultaneous quantification of both types of steroidal saponins are still not available, however.

In the work discussed in this paper HPLC–ELSD and HPLC–ESI–$MS^n$ methods has been developed for qualitative and quantitative analysis of eight major bioactive saponins in ten samples of GXN. This comprehensive and simple analysis was successfully applied to quality assessment of GXN for the first time. It is suitable for analysis of samples with the complexity of traditional Chinese medicines (TCM).

# Experimental

## Reagents and Materials

The saponin reference compounds **1–8** were isolated from the dried rhizomes of *P. vietnamensis* and *P. mairei* in our laboratory by column chromatography. Their chemical structures (*Fig. 1*) were elucidated by comparison of their physical and spectroscopic data (IR, UV, MS, $^1$H NMR, $^{13}$C NMR) with those reported in the literature [6, 15–20]. The purity of each saponin was determined to be greater than 98% by normalization of the peak areas detected by HPLC with ELSD. Ten batches of GXN samples (Batch no. 20041004 to 20070109), produced by Yunnan Baiyao Group (Kunming, China), were collected from 2004 to 2007 and stored at room temperature.




| standards | $R_1$ | $R_2$ |
|---|---|---|
| 1 | OH | Rha (1→4)- Rha (1→4)[Rha (1→2)]-Glc |
| 2 | OH | Rha (1→2)[Glc (1→3)]-Glc |
| 3 | OH | Rha (1→2)[Ara (1→4)]-Glc |
| 4 | OH | Rha (1→2)-Glc |
| 5 | H | Rha (1→4)- Rha (1→4)[Rha (1→2)]-Glc |
| 6 | H | Rha (1→2)[Glc (1→3)]-Glc |
| 7 | H | Rha (1→2)[Ara (1→4)]-Glc |
| 8 | H | Rha (1→2)-Glc |

*Fig. 1*. Structures of eight steroidal saponins (compounds **1–8**) used in this study. Glc: *β*-D-glucopyranosyl; Rha: *α*-L-rhamnopyranosyl; Ara: *α*-L-arabinofuranosyl

Acetonitrile was HPLC grade from Merck (Darmstadt, Germany) and redistilled water was obtained from a Millipore (Bedford, MA, USA) Milli-Q system. Other solvents from Nanjing Chemical Factory (Nanjing, China) were of analytical grade.

## Sample Preparation

An accurately weighed sample of GXN powder (0.5 g) was suspended in 100 mL methanol and ultrasonically extracted for 60 min, with methanol being added to compensate for the lost volume. The extracted solution was then centrifuged at 10,000 rpm for 15 min. Subsequently, 30 mL of the su-

pernatant was evaporated to dryness by rotary evaporation. The residue was dissolved in methanol and the solution was transferred to a 5-mL volumetric flask and diluted to volume with the same solvent. The solution was filtered through a membrane filter (0.45-μm), then 20 μL was injected for HPLC analysis.

## Preparation of Standard Solutions

Stock solutions of the eight standards (1.09, 1.08, 1.09, 1.00, 1.11, 1.09, 0.98, and 1.12 mg $mL^{-1}$ for compounds **1–8**, respectively) were prepared by dissolving accurately weighed amounts of the standards in methanol in 5-mL volumetric flasks. The stock solutions were further diluted with methanol to appropriate, different, concentration ranges. The solutions were filtered through a 0.45-μm membrane filter and 20 μL was injected for HPLC analysis. A mixed solution of the eight standard saponins was prepared in methanol, containing 272.5, 270, 272.5, 250, 277.5, 272.5, 245, and 280 μg $mL^{-1}$ of compounds **1–8**, respectively; this solution was used for method development. All solutions were stored under refrigeration at 4°C.

## HPLC Apparatus and Chromatographic Conditions

Liquid chromatography was performed with Agilent Technologies (USA) series 1100 equipment comprising a quaternary pump, an on-line degasser, a column temperature controller, and a injection valve with 20-μL sample loop, coupled with an Alltech Associates (USA) 2000 evaporative light-scattering detector and a Lanke Technologies (Tianjin, China) WYK-2 air-compressor. Compounds were separated on a 150 mm × 4.6 mm, 5-μm particle, Kromasil $C_{18}$ analytical column protected by a 12.5 mm × 4.6 mm, 5-μm particle, Agilent $C_{18}$ guard column. The mobile phase was a gradient prepared from acetonitrile (component A) and redistilled water (component B); the gradient program was: 0–5 min, 30%→42% A; 5–14 min, 42% A; 14–15 min, 42%→55% A; 15–22 min, 55%→62% A; and 22–25 min, 62%→100% A. The column was rinsed for 10 min with 100% A then equilibrated for another 10 min with 30% A before the next injection. The flow rate was 1 mL $min^{-1}$. The column temperature was maintained at 25°C. The drift tube temperature for ELSD was set at 102°C and the nebulizing gas flow rate was 2.8 L $min^{-1}$.

### LC–ESI–MS$^n$ System and Conditions

Mass spectrometry was used to confirm the chromatographic profile obtained by ELSD detection. MS analysis was performed with an Agilent Technologies LC–MSD Trap SL system equipped with an ESI interface. The chromatographic conditions were as described above, except the injection volume was 5 µL. By solvent splitting, 0.35 mL min$^{-1}$ of the column effluent was delivered into the ion source of the mass spectrometer. The ESI–MS spectra were acquired in the negative-ion mode, furnishing $[M + Cl]^-$ ions. The conditions were: drying gas (nitrogen) flow rate 9 L min$^{-1}$, temperature 350°C, pressure of nebulizer gas 40 psi, HV potential 3.5 kV, and scan range 400–1600 amu. All operations, acquisition, and analysis of data were controlled by Agilent Chemstation software.

## Results and Discussion

### Optimization of Sample Preparation

To achieve efficient and quantitative extraction, conditions involved in the procedure, for example extraction solvent, extraction method, and extraction time were optimized. Total peak area of the eight steroidal saponins was used as the marker for evaluation of extraction efficiency. Water, 20% (*v*/*v*) methanol, 40% methanol, 60% methanol, 80% methanol, and neat methanol were evaluated as extraction solvents. Marker size increased approximately twofold from water to 20% methanol then there was a steady rise of the marker from 20% to 80% methanol. The marker was barely different for use of 80% methanol or neat methanol. Therefore, neat methanol was the most suitable extraction solvent, considering time-saving and operational convenience. Heating under reflux and ultrasonic extraction were both evaluated using neat methanol as solvent. Because the yield of these two methods was the same, ultrasonic extraction was chosen as the preferred method because it is simple and rapid. The effect of extraction time was also investigated. When samples were extracted with methanol for 30, 60, and 90 min it was found that all eight compounds were almost completely extracted within 60 min.

## Optimization of Chromatographic Separation Conditions

The chromatographic conditions were investigated systematically. Because the ingredients in the sample could not be separated by isocratic elution, gradient elution was used. Different mobile phase compositions were also evaluated. Resolution of adjacent peaks was not satisfactory when the mobile phase contained methanol. When methanol was replaced with acetonitrile, all the analytes could be eluted with baseline separation in 25 min. Representative chromatograms obtained from the eight standards and a sample of GXN are shown in *Fig. 2*. *Fig. 2A* shows that the eight steroidal saponins were well separated and that the resolution between any two compounds was greater than 1.5. *Fig. 2B* shows that no other compounds in the sample interfered with analysis of the eight constituents.

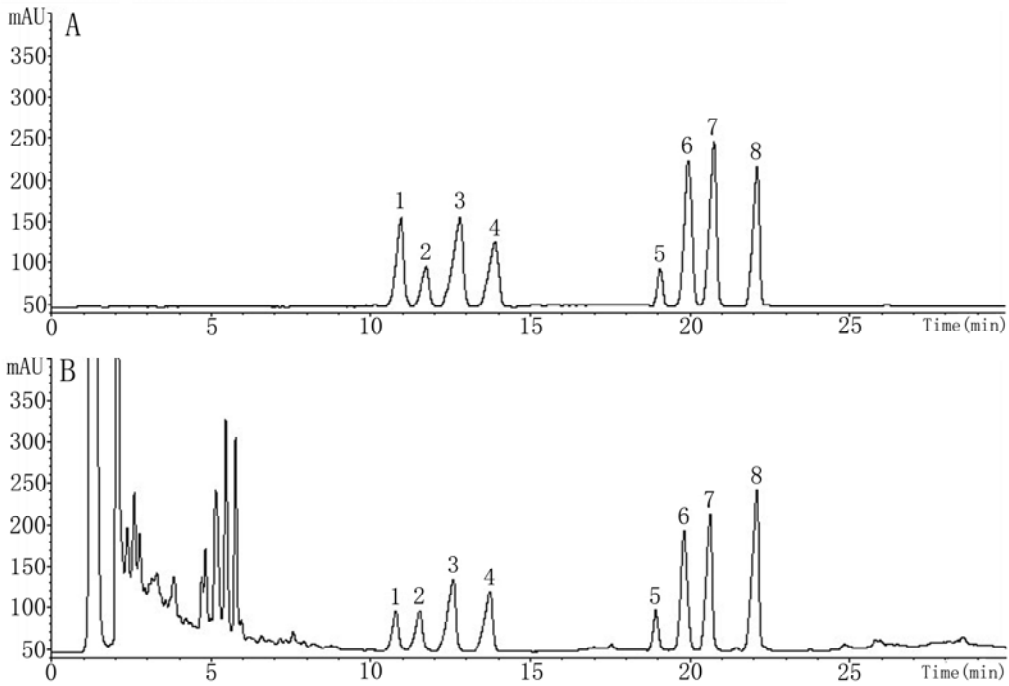



*Fig. 2*. Representative chromatograms obtained from mixed standards (A) and from a methanol extract of GXN (B)

## Optimization of ELSD Conditions

ELSD is universal and does not require a chromophore in the analyte, so any compound present in the injected sample, assuming an appropriate

concentration and molecular weight, will be detected. Good responses were obtained by ELSD of the steroidal saponins investigated. The operating conditions for ELSD, i.e. the nebulizing gas flow rate and the drift tube temperature were optimized to obtain the best signal-to-noise ratio ($S/N$). The flow rate of the gas and the temperature of the detector were evaluated systematically from 2.0 to 3.0 L $min^{-1}$ and 90 to 110°C, respectively. In general, large droplets form at low flow-rate of gas, which results in spikes and noisy signals. On the other hand, increasing the flow-rate leads to an obvious decrease of signal response. The optimum flow-rate in this work was 2.8 L $min^{-1}$. With regard to drift tube temperature, solvent evaporation is not complete at low temperatures but detector response decreases at high temperature. The optimum drift tube temperature was found to be 102°C.

## Identification of Steroidal Saponins

LC–ESI–$MS^n$ is a powerful analytical technique for analysis of TCM, furnishing not only the molecular weigh but also the fragments characteristic of compound structure. To identify the compounds of interest in the samples and to avoid possible errors arising from use of a single method of identification, the chromatographic peaks of samples were assigned by comparing both retention times and MS data with those obtained from reference compounds. Under the HPLC–ESI–$MS^n$ conditions described above, eight steroidal saponins from samples were detected in the negative ion mode. The retention times ($t_R$), and MS, $MS^2$, and $MS^3$ fragment ions of the analytes are listed in *Table I*. Studying of the mass spectra of these compounds revealed that all formed quasi-molecular ions $[M - H]^-$ and $[M + Cl]^-$. The $MS^2$ and $MS^3$ spectra of compounds **1** and **5** contained fragment ions formed by consecutive neutral loss of three molecules of rhamnose ($\Delta m/z$ = 146 Da). The fragment ions of compounds **2** and **6** were formed by consecutive loss of glucose ($\Delta m/z$ = 162 Da) and rhamnose as a neutral fragments. For compound **3** and **7** the fragment ions observed were attributed to consecutive neutral losses of one molecule of arabinose ($\Delta m/z$ = 132 Da) and rhamnose, in that order. For compounds **4** and **8** the fragment ions were produced by neutral loss of one molecule of rhamnose. These fragmentation patterns were highly consistent with their chemical structures (*Table I*; *Fig. 1*). With LC–ESI–$MS^n$ analysis, therefore, these active components of the samples can be unequivocally identified.

*Table I*. The main fragments of the eight steroidal saponins present in GXN determined by HPLC–ESI–MS$^n$

| Cmpd | $t_R$ (min) | ESI–MS | | ESI–MS$^n$ |
|---|---|---|---|---|
| | | $[M - H]^-$ | $[M + Cl]^-$ | |
| **1** | 11.6 | 1029 | 1065 | 883 $[M - H - Rha^a]^-$, 737 $[M - H - 2Rha]^-$, 591 $[M - H - 3Rha]^-$ |
| **2** | 12.3 | 899 | 935 | 737 $[M - H - Glc^b]^-$, 591 $[M - H - Glc - Rha]^-$ |
| **3** | 13.3 | 869 | 905 | 737 $[M - H - Ara^c]^-$, 591 $[M - H - Ara - Rha]^-$ |
| **4** | 14.5 | 737 | 773 | 591 $[M - H - Rha]^-$ |
| **5** | 19.4 | 1013 | 1049 | 867 $[M - H - Rha]^-$, 721 $[M - H - 2Rha]^-$, 575 $[M - H - 3Rha]^-$ |
| **6** | 20.4 | 883 | 919 | 721 $[M - H - Glc]^-$, 575 $[M - H - Glc - Rha]^-$ |
| **7** | 21.2 | 853 | 889 | 721 $[M - H - Ara]^-$, 575 $[M - H - Ara - Rha]^-$ |
| **8** | 22.6 | 721 | 757 | 575 $[M - H - Rha]^-$ |

[a] $\alpha$-L-rhamnopyranosyl
[b] $\beta$-D-glucopyranosyl
[c] $\alpha$-L-arabinofuranosyl

## Validation of the Method

### Linearity, and Limits of Detection and Quantification

The calibration plots for these components were assessed for at least six concentrations. Triplicate injections were performed at each concentration and the average value was used to establish the standard plots. For ELSD, response is usually a nonlinear function of the concentration of the sample injected, and the regression equations can be described as $Y = aX^b$, so the calibration curves should be plotted in double logarithmic coordinates [21]. Therefore, the regression equation was calculated in the form $y = ax + b$, where $y$ and $x$ were the values of the log-transformed peak area and the log-transformed amount of each reference compound (μg), respectively. Results of regression analysis and the correlation coefficients ($r^2$) obtained are shown in *Table II*. The high correlation coefficients ($r^2 \geq 0.9995$) were indicative of satisfactory linearity of relationships between the logarithms of peak area and compound amount injected in ELSD over a relatively wide concentration range.

The dilute solution of the eight reference compounds was further diluted with methanol to a series of concentrations for assessment of the limits

of detection (*LOD*) and quantification (*LOQ*). The *LOD* and *LOQ* under these chromatographic conditions were determined at signal-to-noise (*S*/*N*) ratios of 3 and 10, respectively. *LOD* and *LOQ* for each compound, also shown in *Table II*, ranged from 0.10 to 0.31 μg and 0.27 to 0.76 μg, respectively, indicative of high sensitivity under these chromatographic conditions.

## Precision, Repeatability and Stability

Precision was examined by performing intra-day and inter-day assay of a standard solution. Intra-assay precision was assessed at 4-h intervals on the same day whereas inter-assay precision was assessed over three days. The results are listed in *Table II*. The relative standard deviations (*RSD*) of peak areas were less than 5% for all eight analytes, indicative of the high precision of the HPLC instrument used.

The repeatability of the method was estimated by injecting six individual samples prepared with the same procedure. As showed in *Table II*, the repeatability, as *RSD*, ranged from 1.98% to 4.39%, indicative of the excellent repeatability of the sample-preparation procedure.

*Table II*. Calibration plots, regression data, and precision and repeatability data for the eight saponins

| Analyte | Linear regression | | | | | Precision (*RSD*, %) | | Repeatability |
|---|---|---|---|---|---|---|---|---|
| | Regression equation[a] | $r^2$ | Test range (μg) | *LOD* (μg) | *LOQ* (μg) | Intra-day ($n$ = 6) | Inter-day ($n$ = 3) | *RSD* (%) |
| **1** | $y = 1.8168x + 1.4271$ | 0.9998 | 0.44–7.63 | 0.10 | 0.27 | 0.77 | 1.21 | 1.98 |
| **2** | $y = 1.5283x + 1.5142$ | 0.9995 | 0.54–11.88 | 0.24 | 0.49 | 0.85 | 2.09 | 2.03 |
| **3** | $y = 1.7378x + 1.7356$ | 0.9996 | 0.54–9.81 | 0.22 | 0.43 | 1.73 | 3.20 | 4.14 |
| **4** | $y = 1.7847x + 1.4390$ | 0.9999 | 0.40–9.00 | 0.20 | 0.38 | 1.84 | 4.53 | 4.39 |
| **5** | $y = 2.1267x + 0.8874$ | 0.9998 | 1.11–16.65 | 0.31 | 0.76 | 0.86 | 2.62 | 3.31 |
| **6** | $y = 2.0109x + 1.4615$ | 0.9997 | 1.09–19.62 | 0.24 | 0.59 | 1.40 | 3.22 | 3.55 |
| **7** | $y = 2.0364x + 1.4702$ | 0.9995 | 0.98–14.70 | 0.21 | 0.52 | 0.87 | 1.95 | 2.03 |
| **8** | $y = 1.8727x + 1.5946$ | 0.9997 | 0.45–10.08 | 0.15 | 0.36 | 0.68 | 2.34 | 3.12 |

[a] $y$ and $x$ are, respectively, the logarithms of peak area and compound amount injected in HPLC–ELSD

Stability was studied with a sample solution (Batch no. 20060802) on two consecutive days ($n$ = 8). It was found that the analytes in the sample solution were stable for two days with *RSD* values from 1.7 to 3.4%.

## Accuracy

Recovery was studied to evaluate the accuracy of the method. Accurate amounts of the eight saponins were added to nine portions of GXN (each 0.05 g; Batch no. 20061002) which had been analyzed previously. The mixtures were then extracted and analyzed by means of the proposed procedure. Standard solutions were also prepared in three different concentrations (high, medium, and low) in the range of the calibration plots and triplicate fortified samples were prepared at each level. As is apparent from *Table III*, the accuracy of the method was good, with overall recovery >94.3% for all the analytes, suggesting the negligible loss of the eight compounds during the extraction process.

*Table III*. Recovery of the eight analytes ($n$ = 3)

| Analyte | Original (mg) | Spiked (mg) | Found (mg) | Recovery (%) | Average (%) | RSD (%) |
|---|---|---|---|---|---|---|
| 1 | 0.377 | 0.225 | 0.590 | 94.7 | 97.9 | 4.27 |
| | 0.377 | 0.375 | 0.762 | 102.7 | | |
| | 0.377 | 0.530 | 0.888 | 96.4 | | |
| 2 | 0.534 | 0.317 | 0.851 | 100.0 | 98.4 | 1.73 |
| | 0.534 | 0.535 | 1.051 | 96.6 | | |
| | 0.534 | 0.742 | 1.266 | 98.6 | | |
| 3 | 0.371 | 0.222 | 0.603 | 104.5 | 99.5 | 4.56 |
| | 0.371 | 0.374 | 0.739 | 98.4 | | |
| | 0.371 | 0.505 | 0.854 | 95.6 | | |
| 4 | 0.430 | 0.257 | 0.697 | 103.9 | 99.0 | 4.26 |
| | 0.430 | 0.426 | 0.844 | 97.2 | | |
| | 0.430 | 0.594 | 0.999 | 95.8 | | |
| 5 | 0.595 | 0.348 | 0.947 | 101.1 | 98.5 | 2.55 |
| | 0.595 | 0.597 | 1.169 | 96.1 | | |
| | 0.595 | 0.836 | 1.416 | 98.2 | | |
| 6 | 0.582 | 0.348 | 0.921 | 97.4 | 97.8 | 2.33 |
| | 0.582 | 0.597 | 1.180 | 100.2 | | |
| | 0.582 | 0.816 | 1.363 | 95.7 | | |
| 7 | 0.552 | 0.332 | 0.882 | 99.4 | 96.9 | 2.35 |
| | 0.552 | 0.553 | 1.077 | 94.9 | | |
| | 0.552 | 0.774 | 1.299 | 96.5 | | |
| 8 | 0.481 | 0.283 | 0.758 | 97.9 | 94.3 | 3.44 |
| | 0.481 | 0.485 | 0.924 | 91.3 | | |
| | 0.481 | 0.657 | 1.096 | 93.6 | | |

Recovery (%) = (amount found − original amount)/spiked amount × 100

The method is, therefore, precise, accurate and sensitive enough for simultaneous quantitative analysis of these active compounds in GXN.

## Application

The method has been successfully used for simultaneous analysis of the eight saponins in ten samples of GXN. The amounts ($n = 3$) of the eight marker compounds are listed in *Table IV*. From these results it was apparent that the total amounts of diosgenin type saponins (compounds **5–8**) was almost as high as that of the pennogenin type saponins (compounds **1–4**), indicating that both types of steroidal saponins should be used as chemical markers for quality control of GXN. In addition, levels of **1–8** in the individual sample varied substantially. It should be noticed that compounds **6** and **2** were the most abundant in most of the samples, each making up approximately 15% of the total saponin content. Compounds **1** and **3**, in contrast, accounted for a small percentage of total saponin content. It should also be mentioned that the concentrations of some compounds in different samples was also significantly different. The variability was most noticeable

*Table IV*. Amounts (mg $g^{-1}$) of the eight steroidal saponins in samples of GXN

| Batch no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| 20041004 | 6.42 | 12.21 | 8.48 | 10.50 | 8.63 | 9.85 | 9.32 | 10.85 |
| 20060506 | 6.77 | 10.61 | 7.30 | 8.57 | 9.57 | 10.29 | 8.84 | 9.56 |
| 20060604 | 7.65 | 11.86 | 7.25 | 7.13 | 10.80 | 14.84 | 11.32 | 7.61 |
| 20060802 | 8.11 | 11.70 | 8.19 | 8.24 | 13.82 | 13.09 | 11.19 | 8.93 |
| 20060901 | 6.98 | 11.92 | 7.71 | 8.49 | 9.41 | 12.01 | 9.80 | 9.13 |
| 20061002 | 7.54 | 10.67 | 7.42 | 8.60 | 11.89 | 11.65 | 11.04 | 9.62 |
| 20061102 | 7.24 | 10.48 | 7.62 | 8.85 | 10.40 | 9.75 | 9.45 | 9.77 |
| 20061109 | 7.15 | 11.17 | 8.10 | 9.87 | 9.88 | 8.39 | 8.10 | 10.36 |
| 20070102 | 6.72 | 11.14 | 7.28 | 9.38 | 9.79 | 11.18 | 8.67 | 11.74 |
| 20070109 | 6.76 | 11.24 | 7.38 | 8.85 | 8.95 | 11.41 | 8.96 | 10.08 |

for compound **6**, from 8.39 mg $g^{-1}$ (no. 20061109) to 14.84 mg $g^{-1}$ (no. 20060604), followed by Compound **5**, from 8.63 mg $g^{-1}$ (no. 20041004) to 13.82 mg $g^{-1}$ (no. 20060802). These differences suggest that each procedure involved in the manufacture of GXN should be standardized to ensure batch uniformity, because variations of these 'marker compounds' may affect the potency of GXN. These differences may, however, arise from incon-

sistency among batches of the herb plants, which are from different sources or harvested at different times. To overcome this problem, it has been suggested that different batches of the crude drug should be blended together to improve the dosage content uniformity of TCMs [22, 23], but the feasibility of its application to GXN needs further studies.

## Conclusion

HPLC–ELSD and HPLC–ESI–$MS^n$ have been successfully used for qualitative and quantitative analysis of eight compounds, four diosgenin saponins and four pennogenin saponins, in evaluation of samples of GXN. These new, validated methods are convenient, rapid, and reliable and could be used for effective and comprehensive monitoring of the quality of GXN. This approach of combined analysis is also believed to be of significant importance for clinical use and modernization of TCMs.

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