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Short communication

# An approach based on HPLC-fingerprint and chemometrics to quality consistency evaluation of Liuwei Dihuang Pills produced by different manufacturers

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ABSTRACT

Similarity analysis based on fingerprints has been commonly used in quality consistency evaluation of herbal medicines. However, very little efforts have been paid in interpreting of the differences in the fingerprints for the purpose of quality control. In this study, a facile and reproducible HPLC-fingerprint of Liuwei Dihuang Pills (LWPs) was developed. Both similarity analysis and Principal Component Analysis (PCA) were employed to evaluate quality consistencies of LWPs produced by five manufacturers. Contribution plots generated by PCA were performed to interpret differences in the chromatograms of samples from different groups and some peaks which importantly contribute to classification were separated and identified. Then, differences in these samples were verified by quantitative analysis and the way to improve quality consistency was discussed. TCM was usually complex in chemical constituents, it is difficult to identify and quantify all of them. Our study demonstrated that the combination of fingerprints of TCM and PCA not only offered a powerful way to quality consistency evaluation of TCM preparations, but also might simplify the process of quality control by quantitation of some important constituents.



## 1. Introduction

The curative effects of Traditional Chinese Medicine (TCM) are principally based on the synergic effect of their multi-targeting, multi-ingredient preparations, in contrast to modern pharmacology and drug development that often focus on a single chemical entity [1]. Therefore, the method employing a few markers or pharmacologically active constituents to assess the quality and authenticity of the complex preparations has a number of severe challenges [2]. Now, chromatographic fingerprint technique plays an important role in controlling the quality of TCM for the systemic characterization of compositions of samples and focusing on the identification and assessment of the stability of the components [3]. Both Food and Drug Administration (FDA) [4] and European Medicines Agency (EMEA) [5] clearly denoted that appropriate fingerprint chromatograms should be applied to assess the consistency of the botanical drugs. Chemometrics is the application of mathematical and statistical techniques to retrieve more information from data. Therefore, State Food and Drug Administration of China (SFDA) suggested that all of herbal chromatograms should be evaluated by their similarities, which derive from the correlative coefficient and/or cosine value of vectorial angle of the original data [6,7]. Currently, quality consistency evaluation of TCM products or herbal medicines based on similarities among chromatograms is extensively used. But, very little efforts have been paid in interpreting or understanding of the differences in the chromatograms for the purpose of quality control [8].

Liuwei Dihuang Pills (LWPs), one of the classical TCM prescriptions for "nourishing the kidney-yin", is widely used in preventing and curing many diseases, especially the disorder of immune and endocrine systems such as diabetes, dizziness, tinnitus, etc. [9]. The effectiveness of it has been well documented during long-term clinical practice [10]. LWPs consists of six Chinese herbs including *Radix rehmannia*, *Fructus corni*, *Rhizoma dioscoreae*, *Rhizoma alismatis*, *Cortex moutan radicis and Poria cocos*. There are more than twenty pharmaceutical manufactures that produce LWPs in China. According to the Chinese Pharmacopoeia, loganin and paeonal in LWPs are required to be qualified by two different HPLC systems for the purpose of quality control, which is laborious and time-consuming. Some researchers [11–13] developed HPLC-fingerprints and quantified contents of some bioactive compounds of LWPs. Yet, no reports have been concerned about consistency of it from different suppliers according to their global composition. Therefore, developing a suitable method for quality consistency evaluation of products from different manufactures based on fingerprint is quite necessary.

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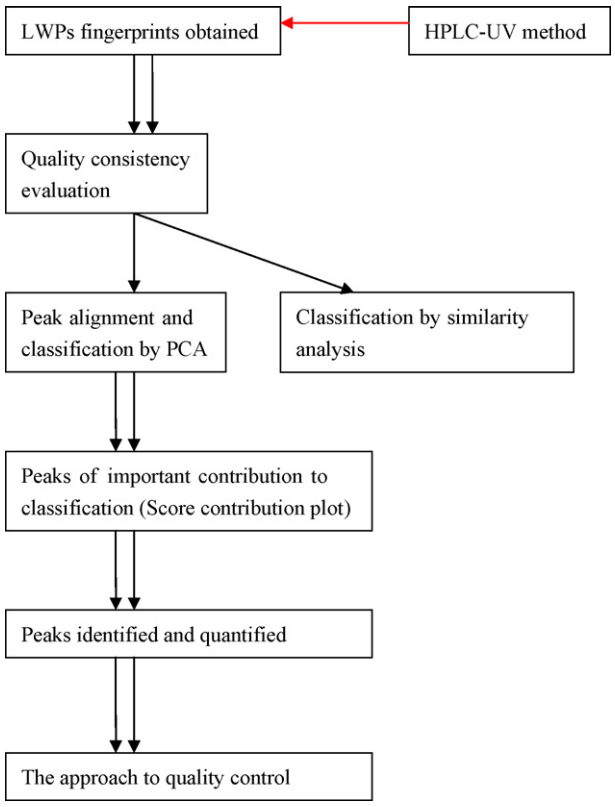


**Fig. 1.** The process to quality consistency evaluation of LWPs produced by different manufacturers for the purpose of quality control.

In the present study, a simple and facile HPLC-fingerprint of LWPs was developed. To assess the quality consistency of LWPs from five manufactures, classification of samples by similarity analysis and Principal Component Analysis (PCA) based on fingerprints was performed. Furthermore, contribution plots generated by PCA were employed to interpret differences in the chromatograms for the classification. With the help of semi-preparative HPLC and HPLC–MS, some peaks which importantly contribute to classification were identified and quantified by standard compounds. The process in this paper was shown in Fig. 1, it is equally applicable to other herbal medicines based on fingerprints in quality assessment for the purpose of quality control.

## 2. Experimental

### 2.1. Reagents and samples preparation

Samples of LWPs from five manufacturers were summarized in Table 1. Loganin was purchased from Sigma Chemical Co. (USA). Paeoniflorin was purchased from Zelang Medical Technology Co., Ltd (Nanjing, PR China). Paeonal was provided by Xi'an Acetan Bio-Tech Co., Ltd. (Xi'an, PR China). Aqueous methanol (50%, v/v) stock solutions containing standard compounds were prepared and diluted to appropriate concentrations for the construction of calibration curves for the quatitative analysis. Acetonitrile (HPLC grade) was purchased from Kermel Chemical Reagents Development Center (Tianjin, PR China). Water was prepared using aquapro water purification system (Taiwan, China). All other chemicals were of analytical grade and used without further purification.

Five grams of grinded powder of Liuwei Dihuang Pills were accurately weighted and extracted ultrasonically by 20.0 mL 50% (v/v) alcohol–water solution for 0.5 h. This extraction process was repeated once and the extracted solution was mixed together. Then, 1.5 mL of the mixed solution was centrifuged at 15,000 rpm for 10 min at 10 °C. Finally, the sample for HPLC injection was obtained by diluting 0.10 mL supernatant of solution with 0.90 mL 50% (v/v) methanol–water solution and kept at −20 °C until analysis.

### 2.2. HPLC instrumentation and chromatographic condition

HPLC–UV fingerprints were acquired on a Varian series ProStar HPLC system which was equipped with a ProStar 210 solvent delivery module and a photodiode array detector (DAD). System control and data-analysis were carried out using ProStar software (Version 6.3, Varian, USA). The chromatographic separation of samples was achieved by a reversed-phase HPLC column (Hypersil ODS C18, 250 mm × 4.6 mm, 5 μm particle size, Elite, Dalian, PR China) protected by a pre-column (Chromguard C18 column, Varian, USA). The column was maintained at 35 °C. Solvent A was distilled water–phosphoric acid (100:0.015, v/v), while solvent B was acetonitrile. The mobile phase flow rate was 1.0 mL $min^{-1}$ and the gradient elution was carried out as follows: 0–3 min, isocratic 5% B; 3–30 min, linear gradient 5–60% B; 30–33 min, linear gradient 60–70% B; 33–37 min, isocratic 70% B.

### 2.3. Similarities among fingerprints

The software "Similarity Evaluation System for Chromatographic Fingerprint of TCM" was published by Chinese Pharmacopoeia Commission (Version 2004A) and mainly applied in the similarity analysis of chromatographic and spectral patterns. In this study, the software was employed to synchronize and make quantitative comparison among different samples and the Mean Chromatographic Fingerprint could be provided by this software.

### 2.4. Peak alignment and data pretreatment for PCA

10 common peaks in the whole chromatogram as references were aligned according to a typical spectrum following the Computer Aided Similarity Evaluation System designed by Dr. Yi-Zeng Liang, and a new chromatographic fingerprint dataset (6000 × 44) for samples was reconstructed by cubic spline data interpolation technique (Fig. 2) [7,14,15].

The obtained data after peak alignment was a matrix for samples. The row of dataset represents the UV-intensity (variable) at one point in retention time, and the column represents different sample. The purpose of data normalization of each sample was to modify data derived from all samples and make them directly com-

**Table 1**
Some information of LWPs used in this work

| Samples name | Manufacturer | Batch number | Province in PR China | Loganin (mg/g) | Paeoniflorin (mg/g) | Paeonal (mg/g) |
|---|---|---|---|---|---|---|
| GLZ | Longzhong Pharmaceutical Co., Ltd | 050802 | Hubei | 1.10 ± 0.08 | 0.73 ± 0.02 | 2.13 ± 0.17 |
| QD | QingDa Pharmaceutical Co., Ltd | 070505 | Hubei | 2.12 ± 0.43 | 0.37 ± 0.04 | 1.22 ± 0.28 |
| GB | Lemon Pharmaceutical Co., Ltd | 06090402 | Shandong | 1.65 ± 0.21 | 0.74 ± 0.02 | 3.39 ± 0.09 |
| ZL | Zhenling Pharmaceutical Co., Ltd | 070105 | Jilin | 1.40 ± 0.12 | 0.66 ± 0.04 | 2.51 ± 0.11 |
| TRT | Tongrentang Group Co., Ltd | 070522 | Beijing | 1.47 ± 0.24 | 0.62 ± 0.03 | 2.76 ± 0.12 |

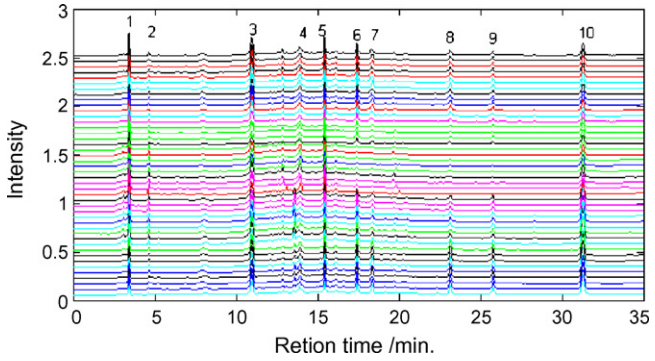


**Fig. 2.** HPLC–UV (238 nm) fingerprints of LWPs from five manufacturers after peak alignment.

parable with each other. Because LWPs were accurately weighted in this study, each variable on one column (sample) was normalized to the total sum of the variables as follows ($x_{i,new} = x_{i,old}/\Sigma(x_{i,old})$, where $x_i$ represented any variable in one column.

All routines for peak alignment and data pretreatment in this study were performed in the MATLAB 6.5 environment (The Math Works Inc.). PCA was performed by SIMCA-P software (version 10.0, Umeå, Sweden). Statistical analysis was performed by Origin 7.0 (OriginLab Corporation, USA).

### 2.5. Preparation of some peaks in fingerprint and HPLC–MS analysis

The instrument and separation condition for preparation of peaks in fingerprint were the same as described in Section 2.2, besides the semi-preparative column (Hypersil ODS C18, 250 mm × 10 mm, 5 μm particle size, Elite, Dalian, China) and the flow rate (1.8 mL min$^{-1}$).

The HPLC condition was the same as described above. The MS analysis was applied on a triple-quadrupoles mass spectrometry coupled with an ESI source (Waters Corporation, USA). Masslynx V4.1 software was used to control the HPLC–MS system. Full scan mass spectra were acquired by scanning MS from $m/z$ 80 to 800. The capillary voltage was 2.80 kV. The ion source temperature and the desolvation temperature were 100 °C and 250 °C, respectively. The desolvation gas flow (nitrogen) was 600 L h$^{-1}$.

## 3. Results and discussion

### 3.1. Optimization of the chromatographic system

Optimization of parameters in HPLC was done through investigating the influence of the mobile phase and detection wavelength. In this work, methanol or acetonitrile was chosen as the mobile phase. Considering the complex of the sample, a little amount of phosphoric acid was added to the mobile phase to reduce the ionization and lower the polarity of these compounds. Three concentrations of phosphoric acid (0.06%, 0.03% and 0.015%) with methanol or acetonitrile were investigated. The results showed that acetonitrile with 0.015% phosphoric acid had best peak shapes and baseline resolution. In order to obtain a sufficiently large number of detectable peaks on the HPLC chromatogram, the spectra of all peaks in the chromatogram were investigated with DAD and 238 nm was selected as detection wavelength. The optimal condition was presented in detail in Section 2.2.

Method precision was based on analysis of the sample solution for five times. The relative standard deviation (R.S.D.) values of 10 common peaks (Fig. 2) height and retention time were better than 4.0% and 1.5%, respectively. The repeatability was assessed by analyzing five independently prepared samples of LWPs. The R.S.D. values of 10 common peaks height and retention time were lower than 5.0% and 1.5%, respectively. The stability test was performed with a sample solution over 3 days in room temperature. The corresponding R.S.D. values of 10 common peaks height and retention time were less than 5.7% and 2.1%, respectively. The results indicated that the developed method was validated and applicable for sample analysis.

### 3.2. Quality consistency evaluation of LWPs from five manufacturers

#### 3.2.1. Classification by similarity analysis based on fingerprints

The relationship within a set of chromatographic fingerprints could be analyzed through comparison in terms of similarity of the objects with a certain reference, and correlation coefficients were the most commonly used standards for evaluation of similarity of the multivariate systems [15]. In this study, 44 samples from five manufacturers were investigated. The correlation coefficients of these fingerprints to their Mean Chromatographic Fingerprint were provided by the software "Similarity Evaluation System for Chromatographic Fingerprint of TCM" and each similarity value versus sample no. was plotted in Fig. 3. As revealed by the plot, it is obvious that the samples can be classified into two classes. Samples from GLZ and QD have relatively low similarity to Mean Chromatographic Fingerprint as a group (0.93–0.96) and the other as another group (0.97–1.0). The classification by these samples indicated that quality consistency of LWPs produced by different manufactures was different from factory to factory.

#### 3.2.2. Peak alignment and classification by PCA

PCA is a statistical approach to facilitate an understanding into the causes and effects behind the relationships of multivariate dataset, the core of this method is to generate new principal components (PCs) which are independent of the original variables but shows linear combinations of them, and simultaneously capture most features of the original data. Each PC consists of a set of values called "scores" defining the position of each sample in the new coordinate space, and the other set of values called "loadings" that give the relative contributions of each variable in calculating the scores. Recently, to achieve pattern recognition using PCA, more attention has been paid to data-analysis methods in entire chromatogram [8,15]. Using this method, the disadvan-

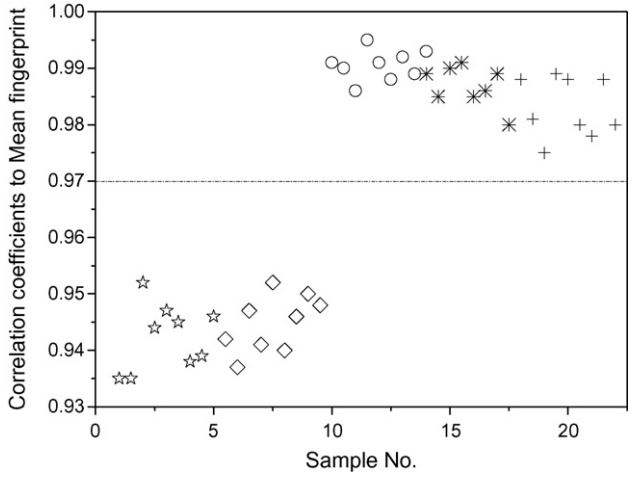


**Fig. 3.** The correlation coefficients of samples from five manufacturers to their Mean Chromatographic Fingerprint. GLZ (☆), GB (○), QD (◊), TRT (*) and ZL (+).

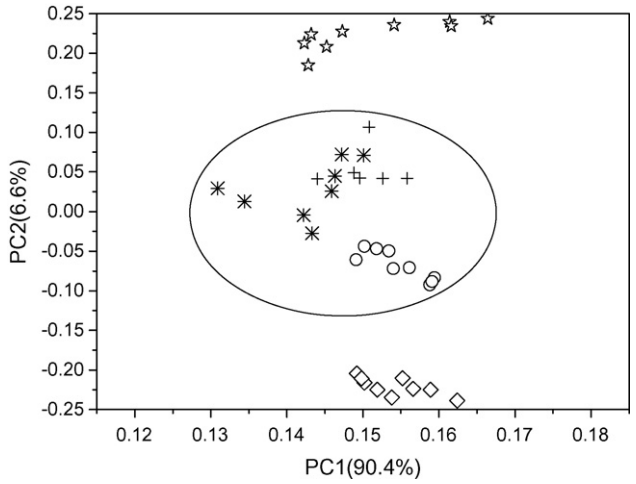


**Fig. 4.** Scores plot PC1 versus PC2 of PCA based on chromatographic fingerprints of samples from five manufacturers. GLZ (☆), GB (○), QD (◊), TRT (*) and ZL (+).

tages of peak detection and integration, and of the introduction of a subjective peak selection could be avoided. In addition, it interprets the chemometric-derived model more directly. However, a major problem with the direct use of chromatogram for comparison is the presence of retention time shift [8,16], which could be large in HPLC system. Several retention alignment algorithms have been proposed to solve this problem [7,15,17]. In the paper, retention time shifts in chromatographic fingerprints were aligned as described in Section 2.4. After peak alignment of fingerprints, a new chromatographic dataset was reconstructed and the entire chromatographic data were normalized as a column operation.

The results of PCA based chromatographic fingerprints of all samples were shown in Fig. 4. In the scores plot, samples were mapped in the space spanned by the first two principal components PC1 versus PC2, they could explain over 96% of the variability. It was obvious that all samples were classified into three classes. Samples produced by TRT, GB as well as ZL were almost in the same region and exhibited a distinct separation from ones by GLZ or QD. The result indicated that samples produced by these manufacturers resembled each other in chemical characteristic as a group, it was in accordance with the result by similarity analysis. But, samples which come from GLZ and QD were in two different groups, it indicated there were differences in chemical characteristics of samples from those two manufacturers even in a class by similarity analysis.

Both similarity analysis and PCA clearly indicated that quality consistencies of LWPs produced by five manufacturers were low among samples from GLZ and QD to other suppliers. Comparing with the similarity analysis, PCA could give a more visual and refined comparison of these chromatograms.

### 3.3. Differences in the chromatograms of samples from factory to factory

As a fuzzy approach, similarity analysis based on fingerprint techniques was extensively used alone or combined with PCA to access the quality consistency of herbal extracts or preparations [8,18,19]. However, very little efforts have been paid in interpreting or understanding of the differences in the chromatograms. In present study, to interpret the differences in the chromatograms of samples from QD or GLZ to the other group, we presumed samples from TRT, GB as well as ZL as the control group for their higher similarities to Mean Chromatogram as a group. Entire chromatographic data as mentioned above from control group and QD (or GLZ) were performed by PCA. Score contribution plots generated by SIMCA-P software were shown in Fig. 5. It is used to understand why an observation differs from the others in a scores plot and displays the difference between the average values of two groups. From Fig. 5, it clearly showed that peaks 5, 6, 10 (or peaks 1, 8) made important contribution to separate control group from QD (or GLZ).

### 3.4. Separation and identification of interesting peaks

Five interesting peaks which importantly contribute to classification were successfully isolated into discrete chromatographic peak respectively after semi-preparative HPLC. These fractions were then the subject of further analysis by HPLC–MS. In the ESI-MS experiment, the molecular weight of each peak and some fragments could be obtained. In our study, both ESI in positive and negative mode were used. Most of the $m/z$ data were $[M+H]^+$ or $[M+NH_4]^+$ in positive mode as well as $[M-H]^+$ in negative mode. In those ionization mode, saccharide group of glycoside was lost and the characteristic was indicated in $m/z$ data as $[161+NH_4]^+$or $[M-H-162]^-$ respectively, from which we can deduce whether the substance had glucopyranosyl unit. By comparing the retention time, UV and characteristic in ESI-MS spectrum of each peak separated by semi-preparative HPLC with the literatures [20,21], 3 peaks which made important contribution to separate control group from QD were unambiguously identified by standard substances. Some information about these peaks and structures of these constituents were shown in Table 2 and Fig. 6 respectively.

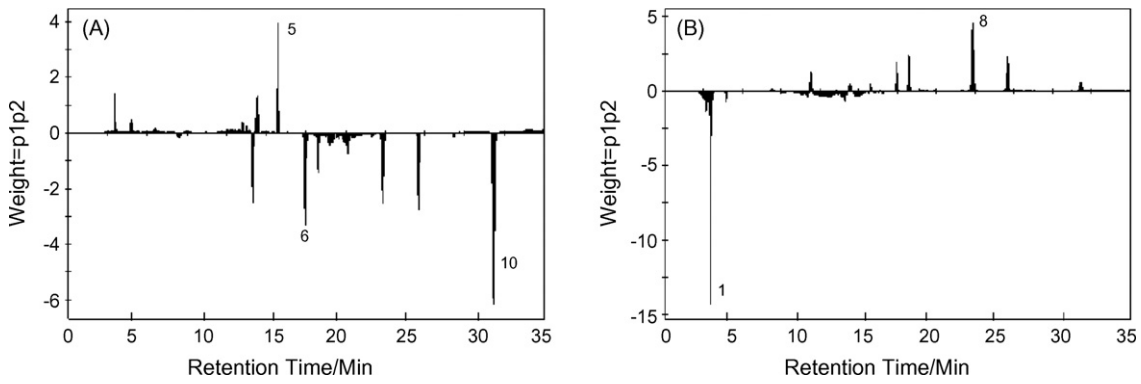


**Fig. 5.** Score contribution plot generated by PCA based on chromatographic fingerprints of samples from different manufacturers. (A): Samples from QD comparing with control group. (B): Samples from GLZ comparing with control group, peak 5, peak 6, peak 10, loganin, paeoniflorin and paeonal.

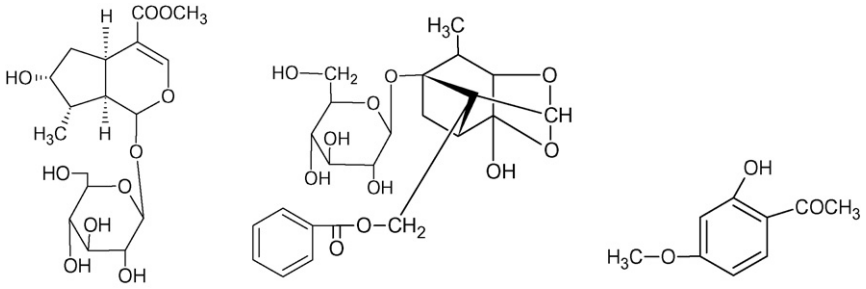


**Fig. 6.** The structure of compounds in fingerprint of LWPs which made important contribution to classification of samples from QD to control group.

**Table 2**
Peaks of important contribution to separate control group from QD (or GLZ)

| Peak number | Contents in control group | Contents in QD (GLZ) | Weight factor | UV ($\lambda_{max}$) | Molecular weight | ESI-MS main fragments | Identification |
|---|---|---|---|---|---|---|---|
| 5 | 1.55 ± 0.21 (mg/g, $n$ = 26) | 2.12 ± 0.43* (mg/g, $n$ = 9) | +3.8 | 238.2 | 390 | 408,229(+) 389,227(−) | Loganin |
| 6 | 0.68 ± 0.06 (mg/g, $n$ = 26) | 0.37 ± 0.04* (mg/g, $n$ = 9) | −2.9 | 232.2 | 480 | 498,179(+) 479,449(−) | Paeoniflorin |
| 10 | 2.89 ± 0.31 (mg/g, $n$ = 26) | 1.22 ± 0.28* (mg/g, $n$ = 9) | −5.4 | 276.4 | 166 | 184,152(+) 165,150(−) | Paeonal |
| 1 | 16.87 ± 0.72 (%, $n$ = 26) | 7.23 ± 0.55* (%, $n$ = 9) | −13.7 | 264.2 | – | – | unkown |
| 8 | 1.43 ± 0.11 (%, $n$ = 26) | 5.04 ± 0.29* (%, $n$ = 9) | +5.6 | 225.3 | 294 | 312,279(+) 293,120(−) | unidentifed |

The contents of peak 1 and peak 8 in samples from control and GLZ were expressed as relative peak area (%) generated by ProStar software. (+) in positive scan mode in HPLC–MS, (−) in negative scan mode in HPLC–MS.
* Denotes significant difference ($P$ < 0.05) from control group (one-way ANOVA).

### 3.5. Quantitative analysis to verify differences among samples

Three bioactive constituents of LWPs, namely loganin, paeoniflorin and paeonal, which made important contribution to separate control group from QD, were quantified by standard compounds. The recovery was preformed by adding a known amount of individual standards into a sample. The mixture was analyzed using the method mentioned above. Three replicates were performed for the test. The mean recoveries ($n$ = 3) of loganin, paeoniflorin and paeonal were 100.8 ± 2.6%, 99.2 ± 3.3%, and 99.9 ± 1.9%, respectively. At least six concentrations of the standard solution were analyzed, and then the calibration curves were constructed by plotting the peak heights versus the concentration of each analyte. Correlation coefficients for three compounds were over 0.999. The contents of these compounds in 44 samples from five manufacturers were shown in Table 1. It was obvious that samples from QD were different from others, the results were in agreement with similarity analysis and PCA. The contents of peaks 1 and 8 in samples from control group and GLZ were expressed as relative peak area (%) generated by ProStar software as these two peaks were unidentified (Table 2).

The results of quantitative analysis clearly indicated that the fluctuation of manufacturing quality when comparing control group with QD stemmed mainly from contents of loganin, paeoniflorin and paeonal for obvious differences of those in samples (Table 2), which have a statistical significance ($P$ < 0.05, one-way ANOVA). As both paeoniflorin and paeonal derived from *Cortex moutan*, as well as loganin from *Fructus corn,* sample quality consistency from QD could be improved by increasing the content of *Cortex moutan* and reducing *Fructus corni* properly in formula in the process of production. In addition, contents of peak 1and peak 8 were the main cause for samples produced by GLZ different from control group. Probing into which herbs these peaks derived from, which is an ongoing task in our group, we could optimize the formula for the purpose of quality control as QD.

## 4. Conclusion

The effective materials exerting efficacy in TCM are complicated, which may be compatible to or may antagonize with each other, and the difference in the effectiveness of the products from different suppliers lies in the variation of the chemical constituents and their relative contents. Thus, simultaneous determination of maximum number of compounds was commonly used strategy for quality control. However, it is difficult to identify and quantify all chemical constituents in TCM. Our study in the paper demonstrated the approach that fingerprints of TCM conjunction with PCA not only offered a powerful way to quality consistency evaluation of complex TCM system but also might optimize formula in the process of production by quantitative analysis of some important constituents which cause differences in fingerprints for the purpose of quality control.

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