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# Development of the fingerprints for the quality of the roots of *Salvia miltiorrhiza* and its related preparations by HPLC-DAD and LC–MS$^n$

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**Abstract**

High-performance liquid chromatographic (HPLC) fingerprints were developed for identification of both lipophilic and hydrophilic components of the roots of *Salvia miltiorrhiza* and four related preparations. These samples were separated with an Agilent Zorbax Extend $C_{18}$ reserved-phase column (5 μm, 250 mm × 4.6 mm) by linear gradient elution using water-phosphoric acid (100:0.026, v/v) and acetonitrile as mobile phase. The flow rate was 0.8 ml/min and the detector wavelength was set at 280 nm. Mean chromatograms and correlation coefficients of samples were calculated by the software "Similarity Evaluation System for Chromatographic Fingerprint of TCM". The correlation coefficients of Danshen and Fufang Danshen tablets (FDT) samples were in the range of 0.352–0.993 and 0.768–0.987, respectively. The correlation coefficients of Compound Danshen dripping pills (CDDP), Danshen injection (DSI) and Xiangdan injection (XDI) samples were higher than 0.928, 0.850 and 0.960, respectively. It was the first time to identify 34 peaks by comparing with standard compounds and using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS$^n$) technique. All results indicated that the developed fingerprint assay could be readily utilized as a quality control method for *S. miltiorrhiza* and its related preparations.
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*Keywords:* *Salvia miltiorrhiza*; Salvianolic acids; Tanshinones; Fingerprint; HPLC-DAD; LC–MS$^n$

## 1. Introduction

Herbal medicines and their derivative products are widely used as therapeutic products in many countries. Their worldwide use has increased in the last decade [1]. There may be hundreds of active components in these herbs and it is almost impossible to identify all these compounds. Therefore, controlling the quality of the herbal medicines and their derivatives is difficult. Recently, the chromatographic fingerprint technique was regarded as a useful method to control the quality of the herbal medicines and their derivatives because this technique emphasizes on the systemic characterization of compositions of samples and focuses on the identification and assessment of the stability of the components [2]. Accordingly, fingerprint technology was introduced and accepted by WHO and SFDA as a strategy for the quality evaluation of herbal medicines and their products [3,4]. The chromatographic methods include high-performance liquid chromatographic (HPLC), CE, GC, X-ray and TLC, while HPLC fingerprint analysis has been regarded as the first choice [5–8].

The roots of *Salvia miltiorrhiza* (Danshen), a popular traditional Chinese medicinal herb, has been used extensively for the treatment of coronary heart disease, cerebrovascular disease, hepatitis, hepatocirrhosis, chronic renal failure, dysmenorrhea and neuroasthenic insomnia [9–14]. There are two classes of major active compounds in Danshen, namely tanshinones which are lipophilic and salvianolic acids which are hydrophilic [15–21]. Both of them are considered to be biologically active [22–24]. At the same time, there are many traditional Chinese medicine preparations (TCMPs) containing Danshen, such as Fufang Danshen tablets (FDT), Compound Danshen dripping pills (CDDP), Danshen injection (DSI) and Xiangdan injection

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(XDI). These TCMPs are mainly used to treat coronary heart disease, heart-stroke, cerebrovascular diseases and cardiovascular diseases [25]. Danshen is widely distributed and used in China [26]; so, the composition of the various active compounds in Danshen varies significantly with the geographic location, climate conditions and other factors [27]. Due to the existence of such differences, the quality of Danshen and derived TCMPs also varied greatly [28].

Till now, there are few reports [29] on the fingerprints of Danshen and its related TCMPs. So, in the present paper, we aimed to establish HPLC fingerprints to investigate the quality of Danshen and its related TCMPs. The mean chromatograms and correlation coefficients of Danshen and its derived products were obtained by using the software "Similarity Evaluation System for Chromatographic Fingerprint of TCM" [30–32]. Then the relative retention time (RRT) and relative peak area (RPA) of "common peaks" were calculated at last, the corresponding quality was evaluated. At the same time, 34 compounds in the fingerprint chromatogram were identified by HPLC-DAD with reference standards and LC–MS$^n$ technique.

## 2. Experimental

### 2.1. Materials and reagents

The roots of *S. miltiorrhiza* and TCMPs samples were purchased from drugstores around China (see Table 1).

Vanillic acid and ferulic acid were purchased from the National Institute for Control of Biological and Pharmaceutical Products of China. Danshensu, procatechuic acid, procatechuic aldehyde, caffeic acid, salvianolic acid D, salvianolic acid E, rosmarimic acid, lithospermic acid, salvianolic acid B, salvianolic acid A, tanshindiol C, tanshindiol B, salvianolic acid C, tanshinone II-B, praewa tanshinone A, dihydrotanshinone I, methltanshinonate, cryptotanshinone, tanshinone I, dehydromiltirone and Tanshinone IIA were isolated from the roots of *S. miltiorrhiza* by author (Fig. 1). Their structures were unambiguously identified on the basis of their spectral data and their purities determined by HPLC were above 98%.

HPLC grade acetonitrile and methanol (E. Merck, Darmstadt, Germany) were used for the HPLC analysis. Deionized water was purified by Milli-Q system (Millipore, Bedford, MA, USA). Phosphoric acid and acetic acid were of analytical grade from Beijing Beihua Fine Chemicals Co. Ltd. (Beijing, China).

### 2.2. Instrumentation and chromatographic condition

#### 2.2.1. HPLC instrumentation and chromatographic condition

An Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) comprised a quaternary solvent delivery system, an on-line degasser, an auto-sampler, a column temperature controller and photodiode array detector coupled with an analytical workstation. The column configuration consisted of an Agilent Zorbax Extend $C_{18}$ reserved-phase column (5 μm, 250 mm × 4.6 mm) and an Agilent Zorbax Extend $C_{18}$ guard column (5 μm, 10 mm × 4.6 mm). The sample injection volume was 10 μl.

The detection wavelength was set at 280 nm, the flow rate was 0.8 ml/min and the column temperature was maintained at 20 °C. The mobile phase consisted of deionized water-phosphoric acid (A; 100:0.026, v/v) and acetonitrile (B). Initial condition was A–B (98:2, v/v), linearly changed to A–B (32:68, v/v) at 60 min. Over the next 20 min, the percentage of mobile-phase A increased linearly to 70%.

The software "Similarity Evaluation System for Chromatographic Fingerprint of TCM" published by GPC (Version 2004A), which was run on a Pentium III 850 (Intel) personal computer, was employed to generate the mean chromatogram, calculate the correlation coefficients and carry out the similarity analysis of the samples with the mean chromatogram.

#### 2.2.2. LC–MS$^n$ instrumentation and chromatographic condition

Except for replacing phosphoric acid (0.026%) by acetic acid (0.8%), the instrumentation and chromatographic condition of HPLC for LC–MS$^n$ were the same as that described in Section 2.2.1. The mass spectra were acquired using a Finnigan LCQ Advantage ion trap instrument with an ESI source (ThermoFinnigan, San Jose, CA, USA). Nitrogen ($N_2$) was used as the sheath and auxiliary gas and helium (He) was used as the collision gas. For the phenolic acids, the ionization mode was negative, and the interface and MSD parameters were as follows: sheath gas, 50 arbtitrary units; auxiliary gas, 10 units; spray voltage, 4.5 kV; capillary temperature, 340 °C; capillary voltage, −10 V; tube lens offset, −30 V. While the ionization mode was positive for the tanshinones, and the interface and MSD parameters were as follows: sheath gas, 50 arbitrary units; auxiliary gas, 10 units; spray voltage, 4.5 kV; capillary temperature, 320 °C; capillary voltage, 30 V; tube lens offset, 20 V.

#### 2.2.3. Software

The software "Similarity Evaluation System for Chromatographic Fingerprint of TCM" was published by GPC (Version 2004A) and mainly applied in the similarity analysis of chromatographic and spectral patterns. The mathematical bases for a certain software to analyze fingerprint of TCM are the chemical pattern recognition techniques such as principle component analysis (PCA), simple classification algorithm (SIMCA), nonlinear mapping (NLM), fuzzy information analysis, artificial neural net (ANN) and so on. The software used in this experiment was based on PCA and fuzzy information analysis, which were suitable for complicated system. This software could analyze the chromatograms of samples directly which made this method simple, rapid and accurate. In this study, the software was employed to synchronize and do quantitative comparison among different samples, as well as to compute and generate the mean chromatogram as a representative standard fingerprint chromatogram for a group of chromatograms. Then the correlation coefficients of samples with mean chromatogram could be provided by this software.

Table 1
The informations and correlation coefficients of samples

| No. | Batch number or harvest time[a] | Collection province | Correlation coefficient[b] | No. | Batch number or harvest time | Collection province | Correlation coefficient |
|---|---|---|---|---|---|---|---|
| 1[c,d] | 200107 | Shanxi | 0.962 | 40[c,e] | 200405 | Shanxi | 0.985 |
| 2[c,d] | 200203 | Henan | 0.991 | 41[c,e] | 200405 | Hebei | 0.993 |
| 3[c,d] | 200408 | Shanxi | 0.995 | 42[c,e] | 200306 | Hebei | 0.973 |
| 4[c,d] | 200302 | Henan | 0.996 | 43[c,e] | 200405 | Shandong | 0.864 |
| 5[c,d] | 200301 | Shanxi | 0.995 | 44[c,e] | 200403 | Sichuang | 0.361 |
| 6[c,d] | 200210 | Henan | 0.992 | 45[c,e] | 200402 | Ganshu | 0.352 |
| 7[c,d] | 200207 | Shanxi | 0.999 | 46[c,e] | 200401 | Shanxi | 0.991 |
| 8[c,d] | 200403 | Hebei | 0.974 | 47[f,e] | 030902 | Guangdong | 0.951 |
| 9[c,d] | 200409 | Hebei | 0.997 | 48[f,e] | 031207 | Beijing | 0.845 |
| 10[c,d] | 200405 | Shanxi | 0.997 | 49[f,e] | 040703 | Shenzheng | 0.768 |
| 11[c,d] | 200405 | Shandong | 0.998 | 50[f,e] | 9919 | Hebei | 0.947 |
| 12[c,d] | 200203 | Shanxi | 0.975 | 51[f,e] | 030203 | Sichuang | 0.955 |
| 13[f,d] | 040210 | Henan | 0.963 | 52[f,e] | 040237 | Shanghai | 0.818 |
| 14[f,d] | 03040111 | Liaoning | 0.952 | 53[f,e] | 4120369 | Beijing | 0.890 |
| 15[f,d] | 030207 | Zhejiang | 0.979 | 54[f,e] | 20040561 | Yunan | 0.974 |
| 16[f,d] | 20040561 | Yunnan | 0.974 | 55[f,e] | 040401 | Jiangxi | 0.914 |
| 17[f,d] | 020301 | Jiangxi | 0.925 | 56[f,e] | 030703 | Guangdong | 0.962 |
| 18[f,d] | 030901 | Guangdong | 0.931 | 57[f,e] | 040107 | Sichuang | 0.902 |
| 19[f,d] | 031211 | Shanghai | 0.961 | 58[f,e] | 0301900 | Guangdong | 0.920 |
| 20[f,d] | 040309 | Guangxi | 0.935 | 59[f,e] | 040427 | Beijing | 0.966 |
| 21[f,d] | 040403 | Jiangxi | 0.968 | 60[f,e] | 0405231 | Guangxi | 0.923 |
| 22[f,d] | 20040407 | Liaoning | 0.995 | 61[f,e] | 040301 | Guangdong | 0.847 |
| 23[f,d] | 0403006 | Jiangshu | 0.992 | 62[f,e] | 030902 | Guangdong | 0.987 |
| 24[f,d] | 20040604 | Xinjiang | 0.994 | 63[f,e] | 030311 | Guangxi | 0.950 |
| 25[f,d] | 0403006 | xianjiang | 0.989 | 64[f,e] | 030206 | Guangdong | 0.491 |
| 26[f,d] | 040201 | Shanghai | 0.995 | 65[f,e] | 0403006 | Xinjiang | 0.896 |
| 27[f,d] | 04051005 | Guangdong | 0.991 | 66[f,e] | 20040409 | Shanxi | 0.975 |
| 28[f,d] | 04011008 | Guangdong | 0.990 | 67[g] | 20030618 | Tianjing | 0.979 |
| 29[f,d] | 04041012 | Guangdong | 0.986 | 68[g] | 20031001 | Tianjing | 0.946 |
| 30[f,d] | 04021008 | Guangdong | 0.990 | 69[g] | 20040618 | Tianjing | 0.928 |
| 31[f,d] | 04031018 | Guangdong | 0.910 | 70[g] | 20040218 | Tianjing | 0.988 |
| 32[f,d] | 03111005 | Guangdong | 0.965 | 71[g] | 20040508 | Tianjing | 0.982 |
| 33[f,d] | 04081001 | Guangdong | 0.958 | 72[h] | 040828 | Guangdong | 0.980 |
| 34[f,d] | 03121020 | Guangdong | 0.941 | 73[h] | 040811 | Guangdong | 0.982 |
| 35[c,e] | 200305 | Liaoning | 0.776 | 74[h] | 040915 | Guangdong | 0.854 |
| 36[c,e] | 200401 | Yunan | 0.814 | 75[i] | 040710 | Shanghai | 0.967 |
| 37[c,e] | 200305 | Sichuang | 0.963 | 76[i] | 20040508 | Shangxi | 0.966 |
| 38[c,e] | 200303 | Shanxi | 0.708 | 77[i] | 040801 | Jiangshu | 0.980 |
| 39[c,e] | 200306 | Zhejiang | 0.959 | | | | |

[a] Batch number used in the FDT, harvest time used in Danshen.
[b] The similarity of Danshen and samples was calculated by the software "Similarity Evaluation System for Chromatographic Fingerprint of TCM" by comparing with mean chromatogram of Danshen and FDT.
[c] Danshen crud drug.
[d] The standard sample.
[e] The random sample.
[f] Fufang Danshen tablet.
[g] Compound Danshen dripping pills.
[h] Danshen injection.
[i] Xiangdan injection.

### 2.3. Sample preparation

The dried roots of *S. miltiorrhiza* were sheared to be about 1 cm in length and then homogenized. After the coating was removed by abrasive cloth, FDT and CDDP were ground in a mortar. Each solid sample (100 mesh, 0.300 g) was accurately weighed and extracted with 10 ml of 70% methanol in a refluxing bath for 60 min. One milliliter of either DSI or XDI liquid sample was diluted to 6 ml with deionized water. The solutions were filtered through a membrane filter (0.45 μm) and then injected into the HPLC.

### 2.4. Data analysis of chromatogram

The simulative mean chromatogram was calculated and generated by using the software "Similarity Evaluation System for Chromatographic Fingerprint of TCM". Then the correlation coefficients of all samples with mean chromatogram

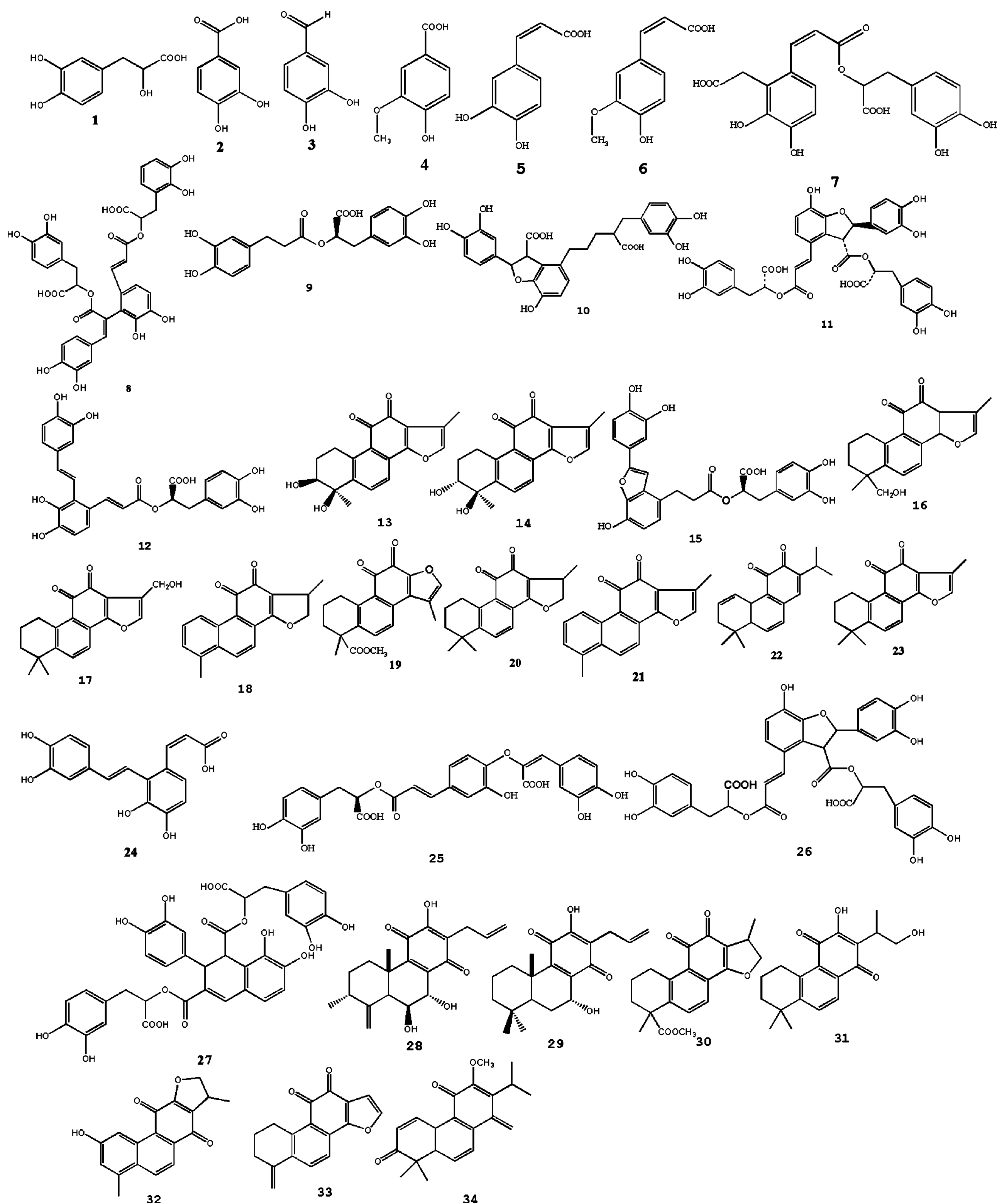

Fig. 1. The structures of compounds identified in Danshen: (1) danshensu; (2) procatechuic acid; (3) procatechualdehyde; (4) vanillic acid; (5) caffeic acid; (6) ferulic acid; (7) salvianolic acid D; (8) salvianolic acid E; (9) rosmarimic acid; (10) lithospermic acid; (11) salvianolic acid B; (12) salvianolic acid A; (13) tanshindiol C; (14) tanshindiol B; (15) salvianolic acid C; (16) tanshinone II-B; (17) praewa tanshinone A; (18) dihydrotanshinone I; (19) methltanshinonate; (20) cryototanshinone; (21) tanshinone I; (22) dehydromiltirone; (23) tnshinone II A; (24) salvianolic acid F; (25) salvianolic acid I; (26) isosalvianolic acid B; (27) salvianolic acid L; (28) royleanone-4; (29) 7a-hydroxyallyl-royleanone; (30) trijuganone; (31) neocryptotanshinone; (32) triiuganone A; (33) methylenetanshinone; (34) miltirone.

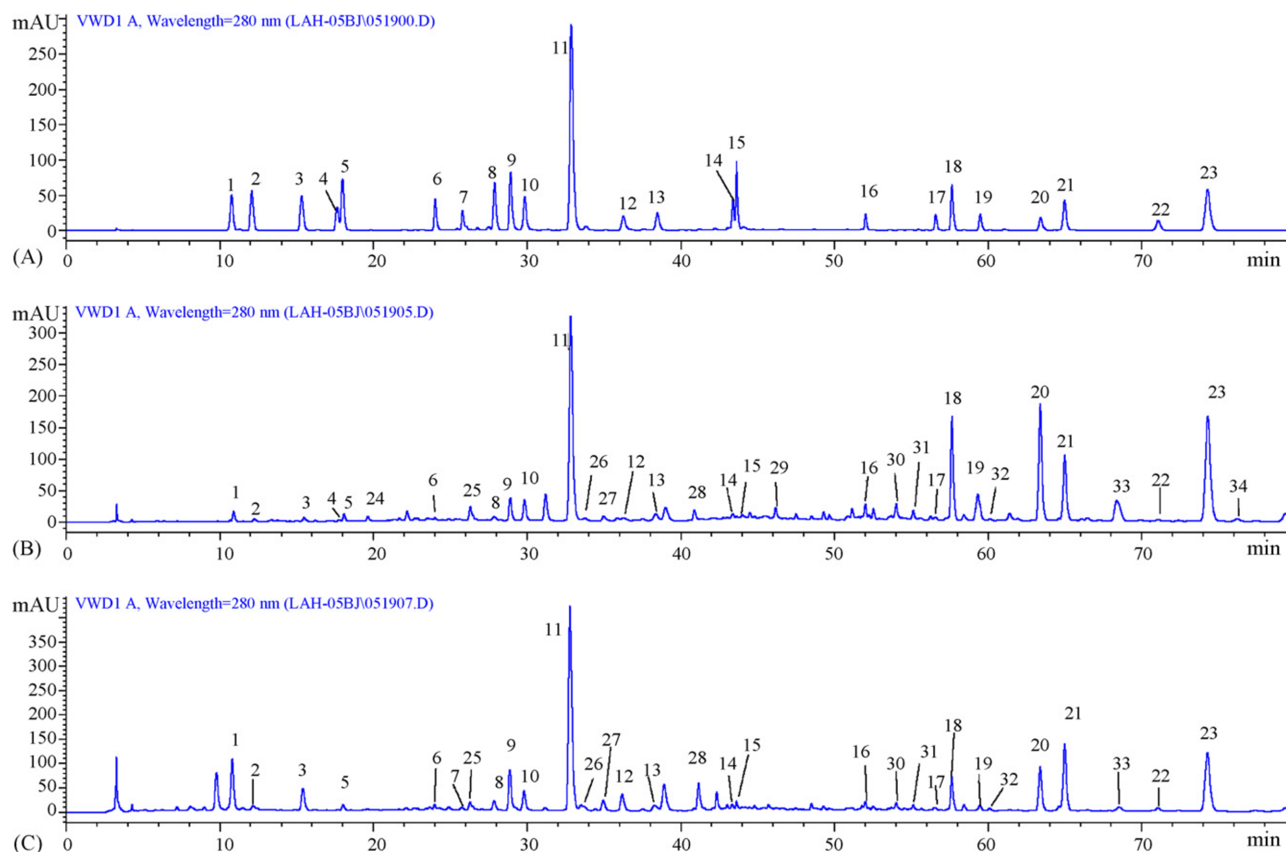


Fig. 2. The chromatograms of standard compounds (A). Danshen crude drug (B) and Fufang Danshen tablet (C). The compound name corresponding to each peak is given in Fig. 1.

were also calculated by this software based on the peak area.

There were 10 “common peaks” (peak nos. 1, 8, 9, 10, 11, 16, 18, 20, 21 and 23) existing in Danshen and FDT samples (Fig. 2). The RRT and RPA of each common peak were calculated, which could semi-quantitatively express the chemical properties in the chromatographic profile of samples. As to CDDP, DSI and XDI samples, the “common peaks” were not calculated for tanshinones were almost undetected in these samples.

## 3. Results and discussion

### 3.1. Extraction procedure

In this study, there were two classes of components in Danshen, and the polarity of them was drastically different. In order to obtain the most efficient extraction procedure, a series of factors including solvent, extraction method and extraction time were investigated.

The FDT powder (each 0.300 g 100 mesh) samples were extracted by ultransonication for 30 min with 10 ml water, 30% methanol, 50% methanol, 70% methanol, methanol, $CH_3OH$: $CHCl_3$ (2:1), $CH_3OH$: $CHCl_3$ (1:1), $CH_3OH$: $CHCl_3$ (1:2) and $CHCl_3$, respectively. The areas of the 10 “common peaks” were used as response to evaluate the extraction solvent, and the results indicated that 70% aqueous methanol was the most efficient solvent. After the solvent was ascertained, the extraction methods such as ultrasonication, soaking and refluxing were also investigated. Refluxing was found to be the most suitable extraction method. Finally, the extraction time was also compared. Danshen sample (0.300 g) was extracted with 10 ml 70% aqueous methanol for 30, 60, 90 and 120 min, respectively. The results signified that 60 min was the most suitable extraction time. Finally, the optimum extraction procedure was established as refluxing extraction by 10 ml 70% aqueous methanol for 60 min.

### 3.2. Optimization of HPLC systems

According to the literature [33], acetonitrile–water mobile phases were recommended to separate the constituents of Danshen. When the pH was adjusted to 2.5, the ionization of phenolic acids could be restrained to get better peak shapes. Four columns were screened with acetonitrile/0.026% phosphoric acid (B/A) as mobile phase. A Zorbax Extend $C_{18}$ was found to be better than BDS-Hypersil $C_{18}$, YMC-Pack ODS-A $C_{18}$ or Luna $C_{18}$. A mobile phase including phosphate was also examined. However, no improvement in resolution was observed. Additionally, it was beneficial for the robustness of the method if a relatively simple mobile phase was used. Thus, it was decided to use the chromatographic system consisting of the Zorbax Extend $C_{18}$ reserved-phase column and an acetonitrile/0.026% phosphoric

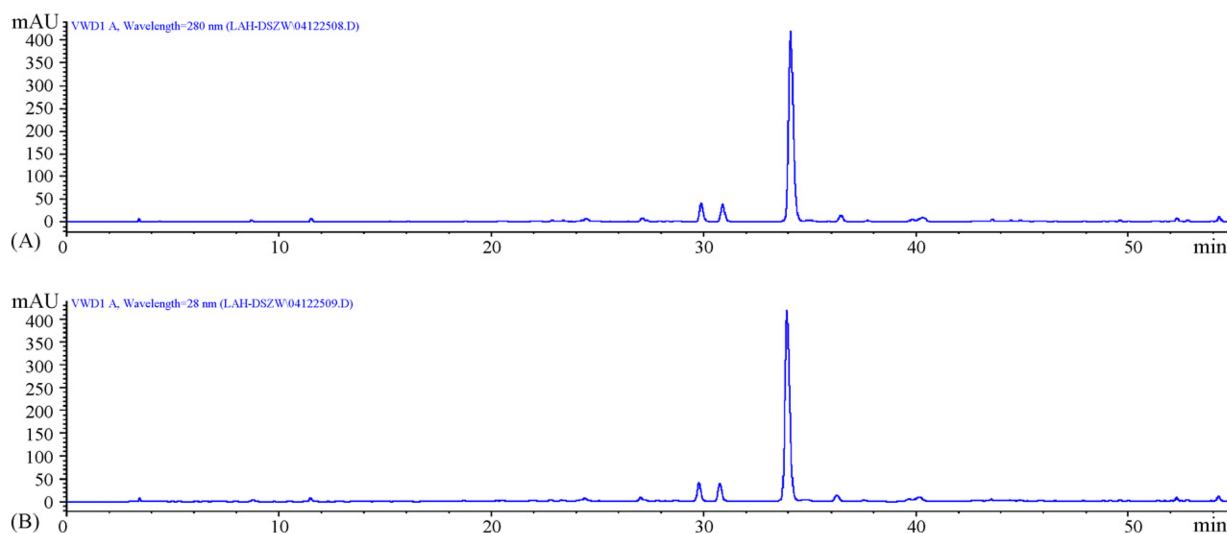


Fig. 3. (A) Chromatogram obtained when 0.026% phosphoric acid was used; (B) chromatogram obtained when 0.8° oacetic acid was used.

acid mobile phase combination, to proceed to the next optimization step.

After the suitable stationary and mobile phase were ascertained, other determinants of the chromatogram were investigated including column temperature, detection wavelength and injection volume. Column temperature is recognized as an important parameter influencing retention, selectivity, system pressure and column stability. In this experiment, 15 °C, 20 °C and 30 °C were screened and the results indicated that 20 °C was the best. The maximum absorbance of salvianolic acids and tanshinones was at 288 nm and 270 nm, respectively. Taking both classes of compounds into consideration, 280 nm were selected as the detection wavelength. Since phosphoric acid could reduce the ionization of phenolic acid groups and keep the baseline stable, three concentrations of phosphoric acid (0.052%, 0.026% and 0.013%) were investigated. Although both concentrations of phosphoric acids (0.052% and 0.026%) could reduce the ionization, the latter was selected for ensuring the robustness of fixed phase. Additionally, injection volume was also investigated, and 10 μl was found to be the most suitable volume.

According to the experiments, detection wavelength, column temperature, acid concentration and injection volume were set at 280 nm, 20 °C, 0.026% and 10 μl, respectively.

In LC–MS experiment, the phosphoric acid (0.026%) was replaced by acetic acid (0.8%) for the requirement of the instrument. In our study, the chromatograms in which 0.026% phosphoric acid or 0.8% acetic acid was used were compared, and found to be almost same (Fig. 3). So, the change of acids didn't influence the chromatogram.

### 3.3. Methodology validation

HPLC fingerprint determination is usually different from general assaying method. The authentication and identification of a drug and its products can be accurately performed using the chromatographic fingerprints, even if batches or concentrations varied among samples [34]. Considering these characteristics of fingerprints, the RRT and the RPA of 10 "common peaks" were used to evaluate the quality of the samples.

#### 3.3.1. Injection precision

The injection precision was determined by replicate injections of the same sample six times in one day. The relative standard deviations of RRT and RPA were lower than 2.49% and 4.21%, respectively.

#### 3.3.2. Repeatability

The repeatability was assessed by analyzing six independently prepared samples of Danshen. The RSDs of RRT and RPA were lower than 2.86% and 4.71%, respectively.

#### 3.3.3. Sample stability test

The sample stability test was determined with one sample during three days. In this period, the solution was stored at room temperature. The RSDs of RRT and RPA were lower than 4.45% and 4.37%, respectively. The results indicated that the sample remained stable for three days.

All results of injection precision, repeatability and stability test indicate that this method was adequate, valid and applicable.

### 3.4. Standardization of fingerprint

#### 3.4.1. Selection of standard samples

To gain the standardized fingerprint, the standard samples with good quality should be selected to establish the mean chromatogram. Twelve Danshen and 22 FDT samples (see Table 1) which met the requirement of the Pharmacopoeia of People's Republic of China (2005 Edition) were selected as the standard samples [9]. All standard samples were analyzed with the developed procedure. Due to the fact that there were almost no tanshinones in the CDDP, DSI and XDI samples, the standard samples of them were not selected.

Table 2
The RPA of 10 common peaks in Danshen and FDT random samples

| Sample no. | The RPA[a] of common peaks | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1[b] | 8 | 9 | 10 | 11 | 16 | 18 | 20 | 21 | 23 |
| Mean chromatogram of Danshen | 0.0132 | 0.0086 | 0.1165 | 0.0451 | 1.0000 | 0.0128 | 0.0798 | 0.1191 | 0.0988 | 0.2501 |
| 35 | 0.0180 | 0.1553 | 0.1253 | 0.1531 | 1.0000 | 0.0000 | 0.4865 | 0.7002 | 0.3080 | 0.8485 |
| 36 | 0.0171 | 0.0213 | 0.1295 | 0.0961 | 1.0000 | 0.0032 | 0.0108 | 0.0077 | 0.0181 | 0.0359 |
| 37 | 0.0329 | 0.0397 | 0.0996 | 0.0835 | 1.0000 | 0.0010 | 0.0214 | 0.0083 | 0.0114 | 0.0728 |
| 38 | 0.0053 | 0.0421 | 0.0902 | 0.1286 | 1.0000 | 0.0652 | 0.4449 | 0.7001 | 0.6593 | 1.5316 |
| 39 | 0.0049 | 0.0718 | 0.1832 | 0.1154 | 1.0000 | 0.0123 | 0.0614 | 0.0287 | 0.1380 | 0.2203 |
| 40 | 0.0175 | 0.0098 | 0.1402 | 0.0451 | 1.0000 | 0.0045 | 0.0365 | 0.0485 | 0.0477 | 0.1893 |
| 41 | 0.0004 | 0.0048 | 0.1191 | 0.0449 | 1.0000 | 0.0107 | 0.0732 | 0.1194 | 0.0611 | 0.1629 |
| 42 | 0.0243 | 0.0085 | 0.0886 | 0.0513 | 1.0000 | 0.0011 | 0.0164 | 0.0193 | 0.0292 | 0.0957 |
| 43 | 0.0013 | 0.0077 | 0.0759 | 0.0000 | 1.0000 | 0.0000 | 0.0000 | 0.3860 | 0.3264 | 0.8007 |
| 44 | 0.0355 | 0.0386 | 5.5905 | 0.0867 | 1.0000 | 0.0000 | 2.2676 | 3.5482 | 2.9654 | 11.1783 |
| 45 | 0.0141 | 0.0500 | 2.1312 | 0.1086 | 1.0000 | 0.0000 | 2.8753 | 5.2007 | 4.7636 | 13.7708 |
| 46 | 0.0031 | 0.0071 | 0.1097 | 0.0577 | 1.0000 | 0.0113 | 0.0502 | 0.0710 | 0.0808 | 0.1809 |
| 47 | 0.0751 | 0.0171 | 0.2244 | 0.0706 | 1.0000 | 0.0201 | 0.0973 | 0.1642 | 0.0074 | 0.4641 |
| Mean chromatogram of FDT | 0.164 | 0.0291 | 0.2575 | 0.1464 | 1.0000 | 0.0363 | 0.1498 | 0.2167 | 0.2821 | 0.4139 |
| 48 | 0.5822 | 0.0000 | 0.2033 | 0.1154 | 1.0000 | 0.0365 | 0.1048 | 0.2458 | 0.0379 | 0.5010 |
| 49 | 0.3309 | 0.0592 | 0.5054 | 0.1056 | 1.0000 | 0.0526 | 0.3042 | 0.3740 | 0.0260 | 1.3113 |
| 50 | 0.0811 | 0.0202 | 0.1264 | 0.1017 | 1.0000 | 0.0261 | 0.0601 | 0.0787 | 0.0124 | 0.2550 |
| 51 | 0.1234 | 0.0382 | 0.1818 | 0.0775 | 1.0000 | 0.0190 | 0.0478 | 0.1055 | 0.0066 | 0.5045 |
| 52 | 0.4676 | 0.0526 | 0.3143 | 0.1100 | 1.0000 | 0.1335 | 0.4297 | 0.8001 | 0.0862 | 1.4514 |
| 53 | 0.2104 | 0.0570 | 0.5239 | 0.0964 | 1.0000 | 0.1131 | 0.2605 | 0.2328 | 0.0519 | 0.9115 |
| 54 | 0.1151 | 0.0170 | 0.1315 | 0.0672 | 1.0000 | 0.0406 | 0.1435 | 0.2376 | 0.0205 | 0.4917 |
| 55 | 0.1910 | 0.0408 | 0.1465 | 0.1176 | 1.0000 | 0.1068 | 0.2353 | 0.4536 | 0.0204 | 0.9601 |
| 56 | 0.2369 | 0.0401 | 0.2003 | 0.1060 | 1.0000 | 0.0302 | 0.1193 | 0.1600 | 0.0118 | 0.3534 |
| 57 | 0.0419 | 0.0346 | 0.1038 | 0.0646 | 1.0000 | 0.0054 | 0.0373 | 0.0538 | 0.0016 | 0.2734 |
| 58 | 0.0465 | 0.0133 | 0.0829 | 0.0937 | 1.0000 | 0.0330 | 0.1261 | 0.2563 | 0.0134 | 0.1925 |
| 59 | 0.2390 | 0.0368 | 0.3035 | 0.0987 | 1.0000 | 0.0538 | 0.1237 | 0.1695 | 0.0177 | 0.5261 |
| 60 | 0.1340 | 0.0299 | 0.4775 | 0.0913 | 1.0000 | 0.0350 | 0.1263 | 0.2325 | 0.0057 | 0.7294 |
| 61 | 0.5985 | 0.0590 | 0.2445 | 0.1601 | 1.0000 | 0.0566 | 0.1567 | 0.2401 | 0.0294 | 0.5087 |
| 62 | 0.1920 | 0.0306 | 0.1267 | 0.1034 | 1.0000 | 0.0377 | 0.1057 | 0.1869 | 0.0093 | 0.4077 |
| 63 | 0.0754 | 0.0482 | 0.0921 | 0.0941 | 1.0000 | 0.0490 | 0.1977 | 0.3131 | 0.0275 | 0.6161 |
| 64 | 0.2673 | 0.1144 | 2.4427 | 0.1786 | 1.0000 | 1.4956 | 3.0987 | 4.9544 | 0.4450 | 19.0866 |
| 65 | 0.0582 | 0.0415 | 0.0990 | 0.0881 | 1.0000 | 0.0284 | 0.0694 | 0.1245 | 0.0130 | 0.1828 |
| 66 | 0.1504 | 0.0353 | 0.2618 | 0.0951 | 1.0000 | 0.0513 | 0.1582 | 0.1305 | 0.0174 | 0.3330 |

[a] RPA = the area of peak/ the area of reference (peak no. 11).
[b] Peak number.

### 3.4.2. *Selection of reference substance*

To calculate the RRT and RPA, a reference substance should be chosen [35]. There are two kinds of reference substances; one is an internal reference substance which belongs to common peaks, the other is an external reference substance which is added to the sample. In this study, the peak no. 11 (salvianolic acid B, Fig. 2) was chosen as the internal reference substance because this peak, which was present in the middle of the chromatogram with maximum content, existed in all chromatograms. The RRT and RPA of common peaks in the two standard chromatograms and random samples were calculated, and the data of RPA were shown in Table 2.

## 3.5. *Identification of constituents in fingerprint chromatograms*

In order to identify the chemical constituents in the chromatograms, both HPLC-DAD and LC–MS$^n$ techniques were adopted.

### 3.5.1. *HPLC analysis for compound identification*

The identification method in which standard compounds were used to compare by HPLC-DAD was accurate and fast. There were 23 peaks identified by comparing their retention time and ultraviolet absorption with standard compounds (Fig. 2). The areas of these 23 peaks accounted for 62% of all peaks' areas. Among the 23 standards, there were 13 phenolic acids and 10 tanshinones. The structures of these standard compounds were shown in Fig. 1.

### 3.5.2. *LC–MS$^n$ analysis for compound identification*

The standard methods to isolate different standard compounds from drugs are laborious and restricted. However, HPLC coupled with mass spectrometry (HPLC–MS) is a powerful approach to solve this problem. MS is a sensitive and selective detector and allows the detection of minor or even trace amounts of constituents from a microscale sample. Furthermore, MS provides abundant structural information and thus, facilitates the structural identification of unknown compounds. In this study,

Table 3
The on-line detected chromatogram and spectrometric data of these identified and deduced compounds

| Peak no. | $t_R$ (min) | Mass data | $\lambda_{max}$ (nm) | Identification |
|---|---|---|---|---|
| 1 | 10.77 | 197 $[M-H]^-$, 257 $[M-H+60]$ | 280, 288 | Danshensu |
| 2 | 12.08 | 153 $[M-H]^-$, 213 $[M-H+60]^-$ 307 $[2M-H]^-$ | 228, 260, 294 | Procatechuic acid |
| 3 | 15.33 | 137 $[M-H]^-$, 275 $[2M-H]^-$ | 222, 280, 310 | Procatechu aldehyde |
| 4 | 17.64 | 167 $[M-H]^-$ | 226, 260, 292 | Vanillic acid |
| 5 | 17.99 | 179 $[M-H]^-$, 135 $[M-H\text{-}44]^-$, 239 $[M-H+60]^-$, 359 $[2M-H]^-$ | 295(sh), 324 | Caffeic acid |
| 6 | 24.00 | 193 $[M-H]^-$ | 238, 293(sh), 322 | Ferulic acid |
| 7 | 25.78 | 417 $[M-H]^-$ | 248, 288(sh), 328 | Salvianolic acid D |
| 8 | 27.88 | 717 $[M-H]^-$, 519 $[M-H\text{-}198]^-$ | 238, 290(sh), 330 | Salvianolic acid E |
| 9 | 28.92 | 359 $[M-H]^-$, 419 $[M-H+60]^-$, 719 $[2M-H]^-$ | 224, 288(sh), 328 | Rosmarimic acid |
| 10 | 29.84 | 537 $[M-H]^-$, 493 $[M-H\text{-}44]^-$ | 232, 254, 290(sh), 310 | Lithospermic acid |
| 11 | 32.86 | 717 $[M-H]^-$, 519 $[M-H\text{-}198]^-$, 321 $[M-H\text{-}198\text{-}198]^-$ | 216, 234, 288, 210(sh) | Salvianolic acid B |
| 12 | 36.25 | 493 $[M-H^-$, 295 $[M-H\text{-}198]^-$ | 232, 288, 340(sh) | Salvianolic acid A |
| 13 | 38.47 | 313 $[M+H]^+$, 373 $[M+H+60]^+$, 295 $[M+H\text{-}18]^+$, 267 $[M+H\text{-}18\text{-}28]^+$ | 226, 254(sh), 270 | Tanshindiol C |
| 14 | 43.40 | 313 $[M+H]^+$, 373 $[M+H+60]^+$, 295 $[M+H\text{-}18]^+$, 267 $[M+H\text{-}18\text{-}28]^+$ | 226, 254(sh), 270 | Tanshindiol B |
| 15 | 43.64 | 491 $[M-H]^-$, 293 $[M-H\text{-}198]^-$ | 224, 288, 364 | Salvianolic acid C |
| 16 | 52.01 | 311 $[M+H]^+$, 293 $[M+H\text{-}18]^+$, 283 $[M+H\text{-}28]^+$ | 254(sh), 272 | Tanshinone II-B |
| 17 | 55.580 | 311 $[M+H]^+$, 293 $[M+H\text{-}18]^+$, 275 $[M+H\text{-}18\text{-}18]^+$ | 252(sh), 268 | Przewa tanshinone A |
| 18 | 57.64 | 279 $[M+H]^+$, 261 $[M+H\text{-}18]^+$, 233 $[M+H\text{-}18\text{-}28]^+$ | 240, 292, 334 | Dihydrotanshinone I |
| 19 | 59.49 | 339 $[M+H]^+$, 307 $[M+H\text{-}32]^+$, 289 $[M+H\text{-}32\text{-}18]^+$ | 270, 251, 222 | Methyltanshinonate |
| 20 | 63.40 | 297 $[M+H]^+$, 279 $[M+H\text{-}18]^+$, 251 $[M+H\text{-}18\text{-}28]^+$ | 218, 264, 299(sh), 360 | Cryptotanshinone |
| 21 | 64.97 | 277 $[M+H]^+$, 337 $[M+H+60]^+$, 455 $[2M+H]^+$, 249 $[M+H\text{-}28]^+$ | 246, 278(sh), 324 | Tanshinone I |
| 22 | 71.07 | 281 $[M+H]^+$, 461 $[2M+H]^+$, 266 $[M+H\text{-}15]^+$ | 224(sh), 277 | Diehydromiltirone |
| 23 | 74.28 | 295 $[M+H]^+$, 278 $[M+H\text{-}18]^+$, 249 $[M+H\text{-}18\text{-}28]^+$, 278 $[M+H\text{-}18\text{-}28\text{-}18]^+$ | 252(sh), 270, 354 | Tanshinone IIA |
| 24 | 19.64 | 313 $[M-H]^-$, 269 $[M-H\text{-}18]^-$, | 242(sh), 292(sh), 330 | Salvianolic acid F |
| 25 | 26.29 | 537 $[M-vH]^-$, 339 $[M-vH\text{-}198]^-$ | 254(sh), 288, 307(sh) | Salvianolic acid I |
| 26 | 33.76 | 717 $[M-H]^-$, 519 $[M-H\text{-}198]^-$ | 288, 235(sh) | Isosalvianolic acid B |
| 27 | 34.95 | 717 $[M-H]^-$, 519 $[M-H\text{-}198]^-$, 501 $[M-H\text{-}198\text{-}18]^-$ | 244(sh), 288, 328(sh) | Salvianolic acid L |
| 28 | 40.89 | 345 $[M+H]^+$,367 $[M+Na]^+$,327 $[M+H\text{-}18]^+$, 309 $[M+H\text{-}18\text{-}18]^+$ | 275, 345(sh) | Royleanone-4 |
| 29 | 46.18 | 353 $[M+Na]^+$, 331 $[M+H]^+$, 313 $[M+H\text{-}18]^+$ | 272, 320 | 7α-hydroxyallyl-royleanone |
| 30 | 54.01 | 341 $[M+H]^+$, 309 $[M+H\text{-}32]^+$, 281 $[M+H\text{-}32\text{-}18]^+$ | 274, 288, 335 | Trijuganone |
| 31 | 55.11 | 315 $[M+H]^+$, 295 $[M+H\text{-}18]^+$, 279 $[M+H\text{-}18\text{-}18]^+$ | 266, 308 | Neocryptotanshinone |
| 32 | 60.11 | 317 $[M+23]^+$, 295 $[M+H]^+$, 280 $[M+H\text{-}15]^+$ | 282 | Trijuganone A |
| 33 | 68.36 | 339 $[M+H+60]^+$, 301 $[M+23]^+$, 279 $[M+H]^+$, 261 $[M+H\text{-}28]^+$, 233 $[M+H\text{-}28\text{-}28]^+$ | 222, 288 | Methylenetanshinone |
| 34 | 76.22 | 283 $[M+H]^+$, 268 $[M+H\text{-}15]^+$, 240 $[M+H\text{-}1\text{-}28]^+$ | 224, 265(sh), 281 | Miltirone |

LC–MS$^n$ was adopted to identify the chemical constituents of Danshen.

ESI in both negative and positive mode was tried to detect salvanolic acids and tanshinones. The results indicated that negative mode was sensitive to salvianolic acids, while positive mode was sensitive to tanshinones. The negative and positive modes were respectively, adopted in two separate chromatograms because the salvianolic acids and tanshinones were interlaced in the chromatogram (Fig. 2).

In the ESI-MS$^n$ experiment, the molecular weight of each peak was obtained. Then using ultraviolet absorption, HPLC retention time and standard chromatogram [36–46], we identified the structures of the 23 standard compounds and deduced the possible structures of 11 peaks in the chromatogram (see Table 3 and Fig. 2). In these 34 compounds, there were 17 phenolic acids and 17 tanshinones.

When the ionization mode was negative, most of the $m/z$ data were $[M-H]^-$or $[2M-H]^-$. According to the structures (Fig. 1), it was found that most polyphenolic acids were composed of single phenolic acid such as danshensu or caffeic acid. Therefore, the fragments of $[M-H\text{-}198]^-$ and $[M-H\text{-}180]^-$ indicated the loss of danshensu and caffeic acid. While the fragment ion $[M-H\text{-}44]^-$ corresponding to the loss of "$CO_2$" indicated the existence of "–COOH" group in the structure. When the ionization mode was positive, most of the $m/z$ data were $[M+H]^+$ or $[M+Na]^+$. Most peaks of tanshinones had the fragments of $[M+H\text{-}18]^+$, $[M+H\text{-}28]^+$ and $[M+H\text{-}15]^+$ corresponding to the loss of "$H_2O$", "CO" and "$CH_3$", respectively. Furthermore, there were some fragments of $[M+H\text{-}32]^+$ which corresponded to the loss of "$CH_3OH$" and indicated the occurrence of "–$OCH_3$" group in the structure. Due to the use of acetic acid in the experiment, there were ions of $[M-H+60]^-$ or $[M+H+60]^+$ corresponding to $[M-H+CH_3COOH]^-$ or $[M+H+CH_3COOH]^+$, respectively.

### 3.6. Application

All samples were extracted and determined according to the method described in Section 2.3. In this study, there were 24 Danshen, 34 FDT, five CDDP, three DSI and three XDI samples analyzed by the developed method. From the results depicted in Fig. 4, it could be concluded that pro-

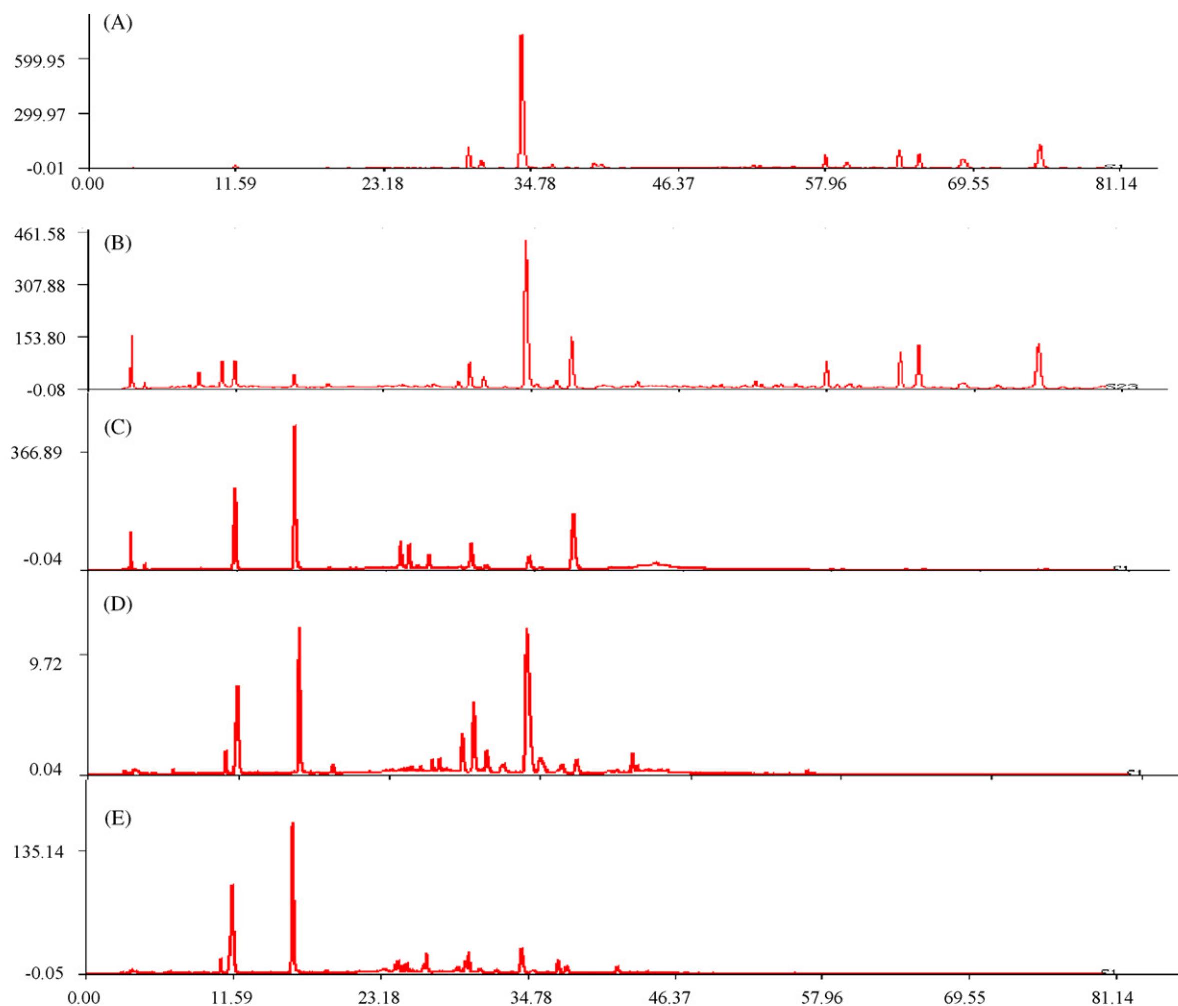


Fig. 4. The mean chromatograms of Danshen (A); FDT (B); CDDP (C); DSI (D); and XDI (E).

files of Danshen, FDT, CDDP, DSI and XDI were distinctly different.

By using the software "Similarity Evaluation System for Chromatographic Fingerprint of TCM", the mean chromatogram and correlation coefficients of the samples could be determined (Fig. 4 and Table 1). It was found that correlation coefficients of standard Danshen samples were higher than 0.960 and the correlation coefficients of random Danshen samples varied considerably in the range of 0.352–0.993. Similarly, it was also shown that the correlation coefficients of standard FDT samples were higher than 0.910 and the correlation coefficients of random FDT samples varied from 0.768 to 0.987 except for the correlation coefficient of no.64 sample which correlation coefficient was 0.491. The quality of five CDDP samples was stable because their correlation coefficients were higher than 0.92 (Table 1). As to DSI and XDI, the correlation coefficients of them were higher than 0.85 and 0.96, respectively. The high variation of correlation coefficients indicated the inconsistent quality of samples and the importance of fingerprint analysis.

There were 10 "common peaks" existing in the chromatogram of Danshen and FDT samples. The RRT and RPA of common peaks in the two standard chromatograms and random samples were calculated, and the data of RPA were shown in Table 2. The RPA of Danshen mean chromatogram and random samples were smaller than FDT. These results were consistent with the results of the profile of the chromatogram. The RPA of nos. 44, 45 and 64 samples was much higher than other Danshen samples and the results were in accordance with the correlation coefficients. The significant difference of RPA also showed the importance of the fingerprint analysis.

## 4. Conclusion

We developed an HPLC-DAD method for fingerprint analysis of Danshen and four related TCMPs. The mean chromatograms and correlation coefficients were obtained with standardized procedure. This was the first time to identify 34 compounds in Danshen by comparing with reference standards and using LC–MS$^n$. The results demonstrated that this developed method was feasible for comprehensive quality evaluation of Danshen and its related TCMPs.

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