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Evaluation of quality of *Salvia miltiorrhiza* Bunge from different provenances by HPLC-DAD fingerprint combined with Chemometrics Method

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Abstract. **Objective** To compare the difference of HPLC fingerprints of *Salvia miltiorrhiza* Bunge from different seed sources under the same ecological environment and harvest time, combined with chemometrics. **Methods** A DiamonsilC18 (250 × 4.6 mm, 5 μm) column was used with a 0.02% phosphoric acid-water (methanol: acetonitrile = 1:1) elution system. The detection wavelength was 270 nm, the flow rate was 1 ml / min and the column temperature was 30 °C. The HPLC fingerprints of *Salvia miltiorrhiza* Bunge from 9 different provenances were tested, and the similarity, cluster analysis and principal component analysis (PCA) of relative peak area were carried out. **Results** The HPLC fingerprints of *Salvia miltiorrhiza* Bunge from different provenances were established, and 27 common peaks were established, with the similarity of 0.989 and 0.999. All sample were divided into 3 groups by systematic cluster analysis, and the PCA score map and payload map were obtained by principal component analysis (PCA). The main chemical components which lead to the difference were selected by VIP value as peak 19, peak 18, peak 17 and peak 2. **Conclusion** There was no significant difference in the composition of *Salvia miltiorrhiza* Bunge, but there was a great difference in the content of components.

1. Introduction

Salvia miltiorrhiza Bunge is used as rhizome as medicine, It has functions such as activating blood circulation dredging stasis relieving pain cooling blood eliminating abscess and relieving heart disease etc [1]. Lipid soluble and water soluble components were active components of *Salvia miltiorrhiza* Bunge respectively. They were compounds containing Er Tie compounds and phenolic acids respectively [2]. Modern pharmacological studies indicate that improving microcirculation inhibiting platelet aggregation antioxidant activity increasing coronary flow rate and myocardial oxygen supply are essential components of salvianolic acid composition. Modern pharmacological studies indicate that salvianolic acids have functions such as improving microcirculation, inhibiting platelet aggregation, antioxidation, increasing coronary flow etc. . It were mainly active components of danshen stasis and blood circulation. There are many reports on determination of *Salvia miltiorrhiza* Bunge content and genetic differences among different provenances [4-7], but the sample chemical composition is complex only through one or several chemical components of *Salvia miltiorrhiza* Bunge quality evaluation is not



representative. But traditional Chinese medicine fingerprint is a kind of multi-index quality control mode which can comprehensively reflect the variety and quantity of chemical components which can reflect comprehensively and comprehensively the integral function of TCM components. Therefore, we can evaluate better the quality of Chinese medicine and its preparation [8]. Although there are many researches on fingerprint of *Salvia miltiorrhiza* Bunge [9 ~ 14], but mainly involved in fingerprint quality evaluation of the rhizome from different habitats, but no report on HPLC fingerprint of *Salvia miltiorrhiza* Bunge from different provenances; It was unable to clarify whether different provenances affected the quality of its. Therefore, in this paper, HPLC chromatographic analysis technique was used to test fingerprint of all sample, and fingerprint similarity evaluation, systematic cluster analysis and principal component analysis were performed. It also laid to explore the influence of provenances on quality of *Salvia miltiorrhiza* Bunge and provide reference for introducing cultivation and quality evaluation of *Salvia miltiorrhiza* Bunge.

2. Materials and methods

2.1 Instruments and reagents

All samples of *Salvia miltiorrhiza* Bunge were collected selected from import and research units selected from main producing areas of China and planted in Bijie Prefecture Guizhou Province respectively. After 1 year harvest their roots and rhizomes were harvested the sample of different provenances. Result is shown in Table 1. These samples were identified by Prof. Xiangpei Wang (Guizhou University of traditional Chinese Medicine) and stored in a cool, dry place. The voucher specimens were deposited at the Herbarium of Guizhou University of traditional Chinese Medicine. UPLC-grade methanol and acetonitrile were purchased from TEDIA (USA). Watsons distilled water were purchased from Watsons Food & Beverage Co., Ltd. Products (Guangzhou, China). Other chemicals (Tianjin Kemio Chemical Reagent Co., Ltd., China) were of analytical grade reagents.

Table.1 Source of *Salvia miltiorrhiza* Bunge

No.	Provenance	Place	Growth cycle(year)	similarity
S1	Henan Province	Guizhou City Guizhou Province	1	0.997
S2	Shaanxi Province	Guizhou City Guizhou Province	1	0.995
S3	Rizhao Shandong	Guizhou City Guizhou Province	1	0.998
S4	Shandong River Mr Ozawa	Guizhou City Guizhou Province	1	0.997
S5	Hebei Anguo Da Hong Pao	Guizhou City Guizhou Province	1	0.999
S6	Anhui Hao Zhou	Guizhou City Guizhou Province	1	0.994
S7	Puyang Henan Province	Guizhou City Guizhou Province	1	0.998
S8	Aerospace	Guizhou City Guizhou Province	1	0.998
S9	Sichuan No.1	Guizhou City Guizhou Province	1	0.999

2.2. Instrument and chromatographic conditions

Chromatographic analysis was performed on a Shimadzu LC-20AT performance liquid chromatograph (Japan), Equipped with binary solvent pump, vacuum degasser, column oven, and DAD detector. The chromatographic separations were carried out on DiamonsilC18 (250 × 4.6 mm, 5μm) column. The mobile phase consisted of 0.02% phosphoric acid-water (A) : (methanol: acetonitrile = 1:1) (B) with a gradient elution(B): 0~10min, 5%~20%; 10~15min, 20%~27.5%; 15~35min, 27.5%~50%; 35~40min, 50%~65%; 40~45min, 65%~75%; 45~60min, 75%~90%; 60~70min, 90%~90%. The flow rate was 1 mL/min, the sample injection volume was 10 μm, and the column temperature was 30 °C. The detection wavelength was 270 nm.

2.3. Preparation of samples

All the samples were milled into powder and oven-dried at 50 °C until it reached a constant weight. The sample powder is about 1.0 g, Precision weighing. A 50 mL volume of methanol was added to the sample and the solution was refluxed and extracted for 3 times for each 1.5 h, at a constant temperature of 60 °C, respectively. The extract was filtered through filter paper and evaporated, then diluted to volume with methanol in a 1 mL volumetric flask. After that, the sample solution was subsequently filtered through a 0.22 µm microporous membrane and injected into the HPLC system for analysis.

2.4. Data analysis

Data analysis was performed by professional software named Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine composed by Chinese Pharmacopoeia Committee (Version 2004 A), which was recommended by SFDA of China. Cluster analysis was carried out by SPSS 22.0 statistical software (IBM Inc., USA). The hierarchical clustering analysis (HCA) and principal components analysis (PCA) were performed on SIMCA 14.0 software from (Umetrics, Inc., Sweden).

3. Results

3.1. UPLC method validation

The test samples are determined by the method under "2.2", the retention time and peak area of each common peak were recorded, and peak 16 was selected as the reference peak. Precision stability repeatability relative retention time and relative peak area RSD of all samples were less than 3.0% indicating that methodology meets requirements.

3.2. Establishment of UPLC fingerprint

A total of *Salvia miltiorrhiza* Bunge samples were used to prepare the sample solution according to "2.3", and the chromatographic conditions under "2.2". It were used to detect and analyze by HPLC-DAD detector. The peak value, peak number and peak position of HPLC-DAD chromatogram were compared and analyzed the fingerprint of all sample. The relative retention time and area of the common peak of the sample were calculated. The average relative retention time of common peaks in all samples is 0.087, 0.095, 0.123, 0.191, 0.207, 0.394, 0.417, 0.454, 0.635, 0.682, 0.653, 0.855, 0.88, 0.917, 0.939, 1, 1.05, 1.07, 1.11, 1.14, 1.36, 1.41, 1.44, 1.61, 1.66, 1.70, 1.72. The results is shown in Fig. 1, 2. and Table. 2.

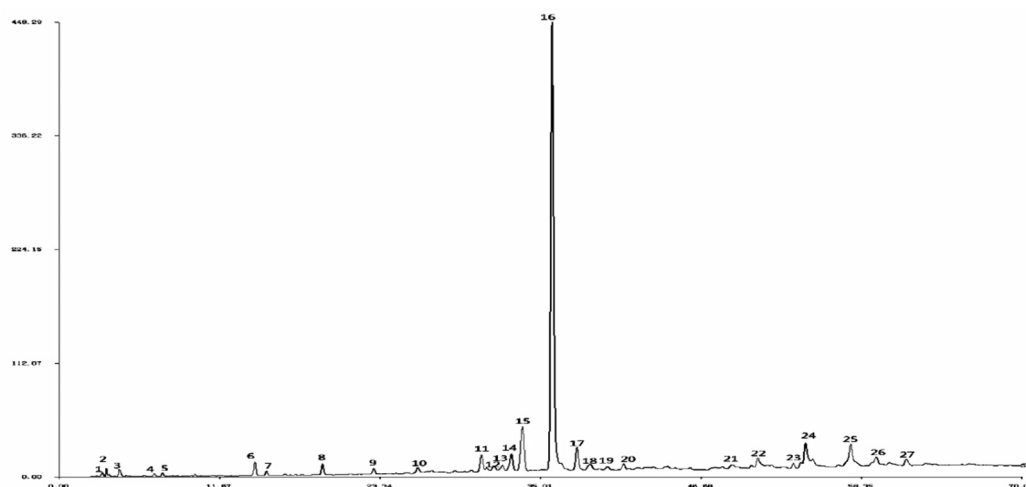
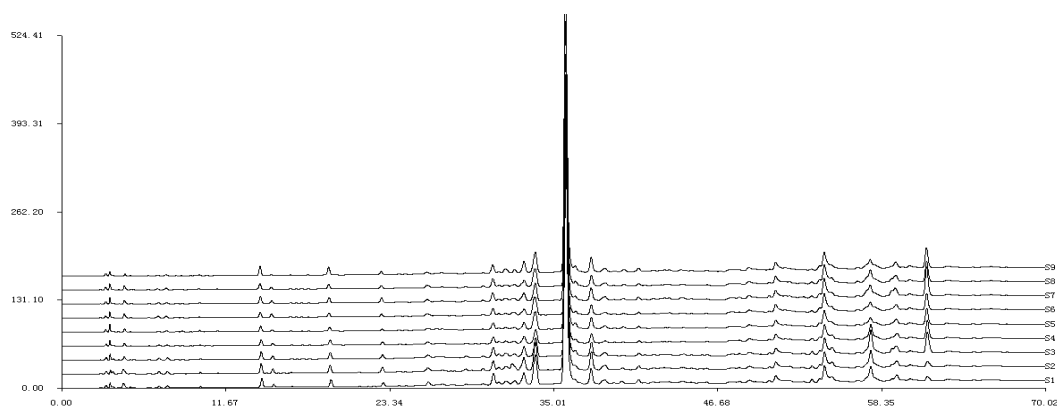


Fig. 1 Common pattern of HPLC fingerprint of *Salvia miltiorrhiza* Bunge

Fig. 2 Comparative of HPLC fingerprint of *Salvia miltiorrhiza* BungeTable. 2 Relative peak area of common peaks of HPLC fingerprint of *Salvia miltiorrhiza* Bunge

Common peak	S1	S2	S3	S4	S5	S6	S7	S8	S9
1	0.0061	0.0049	0.0053	0.0054	0.0063	0.008	0.0055	0.0079	0.0053
2	0.0066	0.0044	0.0074	0.0109	0.0153	0.0162	0.0103	0.0159	0.0086
3	0.0115	0.0107	0.0082	0.0083	0.0092	0.0179	0.0098	0.013	0.0035
4	0.0044	0.0055	0.0051	0.0048	0.0033	0.0096	0.0045	0.0043	0.0017
5	0.0049	0.0046	0.0055	0.0049	0.0055	0.009	0.0042	0.0066	0.0035
6	0.0202	0.0185	0.0189	0.0213	0.0184	0.024	0.0209	0.0208	0.0245
7	0.0069	0.0084	0.0083	0.0081	0.0049	0.0168	0.0073	0.0071	0.0024
8	0.002	0.0019	0.0017	0.0019	0.0013	0.0026	0.0016	0.002	0.001
9	0.0106	0.0124	0.011	0.0129	0.0072	0.0139	0.0153	0.0118	0.0102
10	0.0024	0.0022	0.0014	0.0015	0.0019	0.0022	0.0014	0.0024	0.0012
11	0.0126	0.0189	0.0179	0.0189	0.0143	0.0266	0.0163	0.0203	0.0103
12	0.0383	0.0288	0.0358	0.0363	0.0325	0.0435	0.032	0.0422	0.032
13	0.0207	0.0208	0.0226	0.0204	0.0148	0.0199	0.0176	0.0186	0.0232
14	0.047	0.0469	0.0429	0.0468	0.0356	0.0529	0.0408	0.0448	0.0473
15	0.114	0.091	0.0742	0.0834	0.0995	0.1516	0.0802	0.1152	0.0934
16	1	1	1	1	1	1	1	1	1
17	0.0472	0.0472	0.0466	0.0485	0.0519	0.0617	0.0513	0.0533	0.0553
18	0.02	0.0203	0.0204	0.0169	0.0213	0.0244	0.0244	0.0261	0.0217
19	0.0105	0.0067	0.0056	0.0059	0.011	0.0099	0.0101	0.0111	0.0131
20	0.0143	0.0122	0.0123	0.0114	0.0119	0.0166	0.0146	0.0142	0.0163
21	0.0178	0.0155	0.0196	0.0318	0.0256	0.0418	0.0265	0.0321	0.0235
22	0.0365	0.0311	0.0383	0.0648	0.0529	0.0882	0.0542	0.0664	0.0503
23	0.0133	0.0119	0.0127	0.0249	0.0167	0.0282	0.0179	0.0228	0.015
24	0.0924	0.0831	0.1285	0.1588	0.081	0.106	0.1034	0.1476	0.0972
25	0.0305	0.0272	0.041	0.0558	0.0456	0.0605	0.0457	0.0575	0.0499
26	0.0287	0.0242	0.0299	0.0521	0.0183	0.0577	0.0184	0.049	0.0361
27	0.0239	0.0241	0.0848	0.1126	0.0828	0.1176	0.1302	0.1129	0.0832

3.3. Fingerprint analysis

The chromatogram parameters of the *Salvia miltiorrhiza* Bunge were compared. Using peak16 (retention

time 35.8 min) as the reference peak, 27 common peaks were identified among the sample. Similarity results of *Salvia miltiorrhiza* Bunge from different provenances were shown in table 1.

3.4. Cluster Analysis (CA)

The relative peak area values of 27 common peaks in HPLC-DAD Chromatogram of the samples in the same plant were standardized to form 9×27 order original data matrix. The cluster analysis was carried out by SPSS 20.0 software, the intergroup connection method was used, and the Euclidean distance (Euclidean) was used as the measure of samples. According to the combination of correlation coefficients between the 9 samples, it can be divided into three groups. In the samples, S1, S2 was clustered into I, S6 into II, and S3, S4, S5, S7, S8, S9 into III. The results is shown in figure 7.

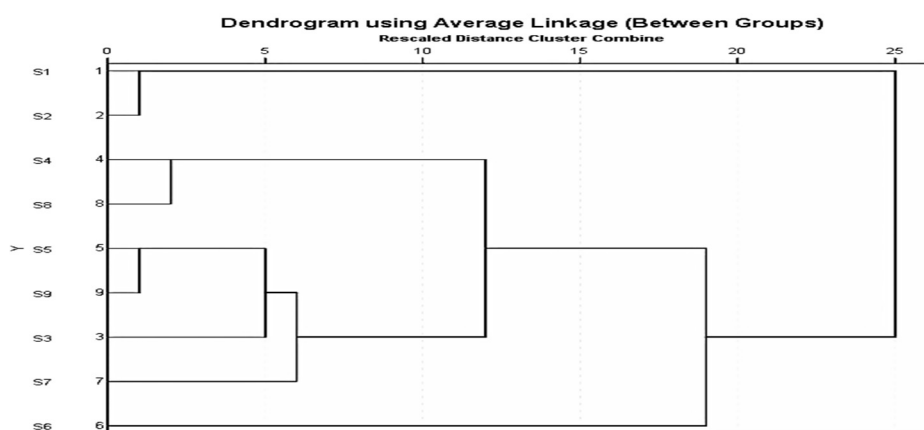


Fig. 3 Cluster analysis of relative peak area of common peaks of HPLC fingerprint of *Salvia miltiorrhiza* Bunge

3.5. Principal components analysis (PCA)

In order to evaluate and synthetically analyze the chemical similarity of the same, the relative peak areas of the samples were analyzed by principal component analysis (PCA). The relative peak area of the sample was introduced into SIMCA 14.0 software, three characteristic principal components (PC1, PC2) are obtained. The results is shown in figure 4. According to the variance contribution rate, the cumulative explanatory power parameter R2X and predictive power parameter Q2 of the model are 0.998, 0.991, respectively. The results show that the model has a good degree of discrimination and prediction. Therefore, the two principal component analysis can basically reflect the main characteristics of plant. Using principal component to establish coordinate system, the PCA score diagram and load diagram of the sample were obtained. Each point on the load graph represents a variable, and the farther away from the origin, the greater the contribution of the content of the component to the classification. The results is shown in figures 5 and 6. The results showed that there was a certain difference among the sample. According to the VIP value of the PLS-DA model, the main chemical components that lead to the differences are screened. The results is shown in figure 12. It is generally believed that variables with $VIP > 1$ play a key role in classification. it indicated that differences in species and quantity of chemical constituents of *Salvia miltiorrhiza* Bunge are distinguished obviously.

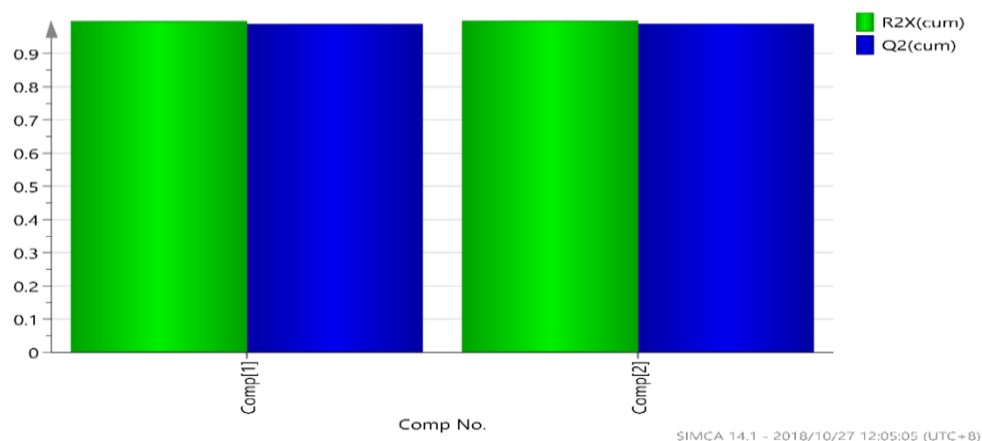


Fig. 4 Relative peak area of common peak of *Salvia miltiorrhiza* 1, 2 principal component variance cumulative contribution rate

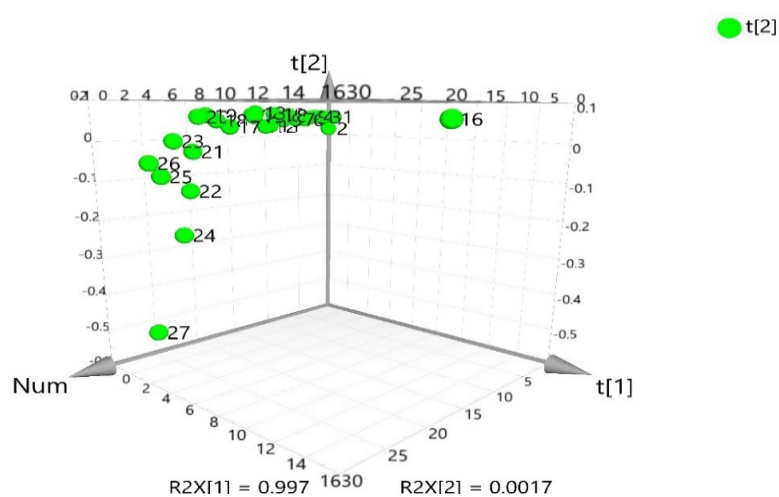


Fig. 5 Relative peak area principal component PCA 3D score diagram of common peak of *Salvia miltiorrhiza* Bunge

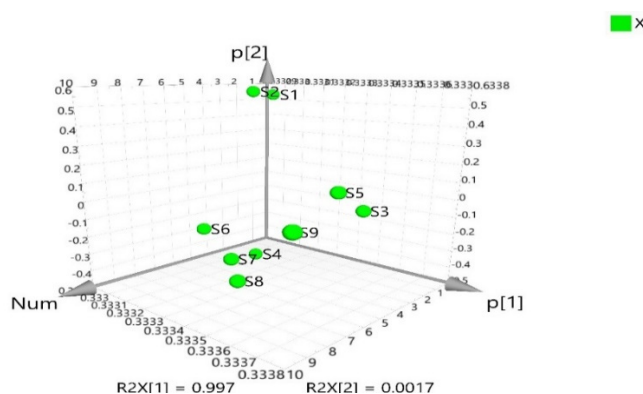


Fig. 6 Relative peak area principal component PCA load 3D diagram of common peak of *Salvia miltiorrhiza* Bunge

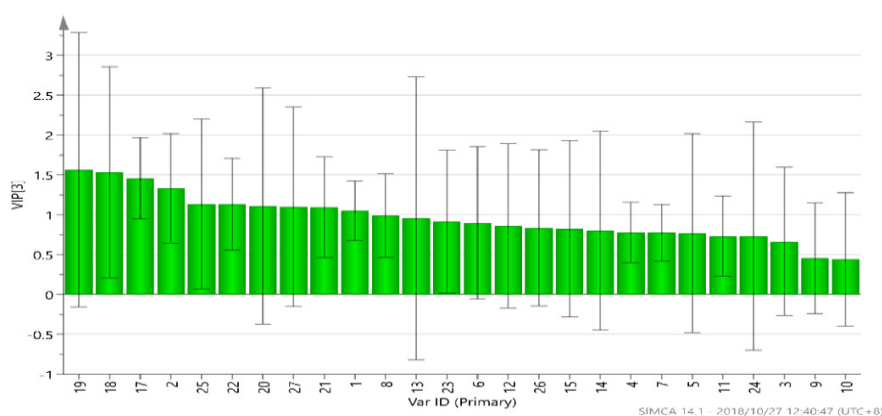


Fig. 7 Relative peak area of common peak of *Salvia miltiorrhiza* Bunge VIP

4. Discussions

During chromatographic optimization process, chromatographic conditions were reported in literatures [8-13], and tested by the samples. Results showed that chromatographic peaks were not high, information was less and baseline was unstable. HPLC fingerprint of *Salvia miltiorrhiza* Bunge could not be reached. Therefore, methanol-phosphoric acid, acetonitrile-phosphoric acid water, methanol and acetonitrile (1:1) - phosphoric acid water as mobile phase systems were reviewed. The separation degree of each peak is better. And baseline stability is beneficial to HPLC fingerprint analysis; The wavelength of 254 nm, 270 nm and 280 nm during chromatographic separation was investigated, the results showed that 270 nm was selected as detection wavelength. Column temperature was investigated at 25 °C, 30 °C and 35 °C during chromatographic separation process, it show that at 30 °C information is abundant, this paper is chosen as 30 °C column temperature.

This paper establishes fingerprint chromatogram analysis methods of *Salvia miltiorrhiza* HPLC from different provenances under same ecological environment and harvesting period, and compares similarity, cluster analysis and principal component analysis. The results showed that there was no obvious difference between the samples from different provenances indicating that there was little difference between *Salvia miltiorrhiza* Bunge species in Guizhou Bijie Prefecture. However, there were significant differences between each sample fingerprint spectra peak area, and through cluster analysis, the sample of *Salvia miltiorrhiza* planted from Guizhou Bijie were divided into 3 categories. Principal component analysis showed that there were significant differences among different provenances of *Salvia miltiorrhiza* Bunge in relative peak area indicating differences in contents of components in

Salvia miltiorrhiza Bge. It suggested that provenances had important effects on quality of *Salvia miltiorrhiza* Bge. Therefore, seed source selection is very important during cultivation process of *Salvia miltiorrhiza* Bge.

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