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Simultaneous Determination of Three Major Active Components in *Salvia miltiorrhiza* and its Relative Species by HPLC

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A high performance liquid chromatographic method was established for determination of three phenolic acids constituents (protocatechuic aldehyde, salvianolic acid B and salvianolic acid A) in 25 samples, including *Salvia miltiorrhiza* of different strains and its related species (*S. brevilabra*, *S. castana*, *S. cavaleriei*, *S. cavaleriei* var. *simpliciflia*, *S. digitaloides*, *S. paohsingensis*, *S. plebeia*, *S. prezwalskii*, *S. trijuga* and *S. yunnanensis*). The three components were successfully separated on a Shimadzu Shim-pack VP-ODS C_{18} reserved phase column (5 µm, 250 mm × 4.6 mm i.d.) by gradient elution using acetonitrile and 0.03 % (v/v) phosphoric acid as the mobile phase, the flow rate was 1 mL min^{-1} and the detection was set at 288 nm. The linearity was obtained over 0.648-99.06 µg mL^{-1} for protoctechuic aldehyde, 14.63-1786 µg mL^{-1} for salvianolic acid B and 2.561-334.14 µg mL^{-1} for salvianolic acid A. The average recovery rates of protoctechuic aldehyde, salvianolic acid B and salvianolic A were 95.17, 94.06 and 91.43 %, respectively. This method was successfully applied to the determination of three important phenolic acids constituents in 25 samples. The proposed method is simple, effective and suitable for quality control of *S. miltiorrhiza* and its relative species.

Key Words: *Salvia miltiorrhiza*, Salvianolic acid B, Salvianolic acid A, Protocatechuic aldehyde, HPLC.

INTRODUCTION

The dried root and rhizome of *Salvia miltiorrhiza* (Lamiaceae: *Salvia*), one of the popular traditional Chinese medicine (TCM), is officially listed in Chinese Pharmacopoeia under the name Danshen[1]. *S. miltiorrhiza* have been widely used for treatment of coronary heart disease, cerebrovascular disease, bone loss, hepatitis, hepatocirrhosis and chronic renal failure[2-6]. Although only *S. miltiorrhiza* was recorded in the Chinese Pharmacopoeia, the dried root of other *Salvia* species containing *S. brevilabra*, *S. castana*, *S. cavaleriei*, *S. cavaleriei* var. *simpliciflia*, *S. digitaloides*, *S. paohsingensis*, *S. plebeia*, *S. prezwalskii*, *S. trijuga* and *S. yunnanensis* that is frequently used as the substitute for *S. miltiorrhiza* in many regions of China[7]. To ensure their quantity and clinical efficiency, quality control of *S. miltiorrhiza* and its related species is greatly importance.

The chemical investigations have showed that there are two kinds of major active constituents in *S. miltiorrhiza*: water-soluble phenolic acids and lipophilic diterpene quinone. Due to the notable pharmacological activities of phenolic acids, the research attention has been focused on them in the past 30 years[8-10]. Protocatechuic aldehyde (Pro A), salvianolic acid A (Sal A) and salvianolic acid B (Sal B) are major water-soluble phenolic acids and important effective components of Danshen. Pharmacological tests have revealed that they have such biological activities as anti-oxidative effects, antiliver injury, anti-tumor, antiatherosclerosis, antiphlegmonosis and protecting myocardial damage[11-15] and salvianolic acid A was found to protect against focal cerebral ischemia and inhibit platelet aggregation by collagen-induced. The effects were better than other water-soluble compounds[16,17]. Up to now, there are some articles have been reported[18,19] about simultaneous determination of protocatechuic aldehyde, salvianolic acid B and other components on the *S. miltiorrhiza*, while could not focused on simultaneous determination of salvianolic acid A. Moreover, some related species products are similar in the effectiveness for the treatment of cardiovascular diseases by some clinical trials, sufficient quality control is still lack. Hence, quantitative analysis of protocatechuic aldehyde, salvianolic acid B and salvianolic acid A is significant for the quality control of *S. miltiorrhiza* and its related species. Fig. 1 shown the chemical structures of the above medicinal components.

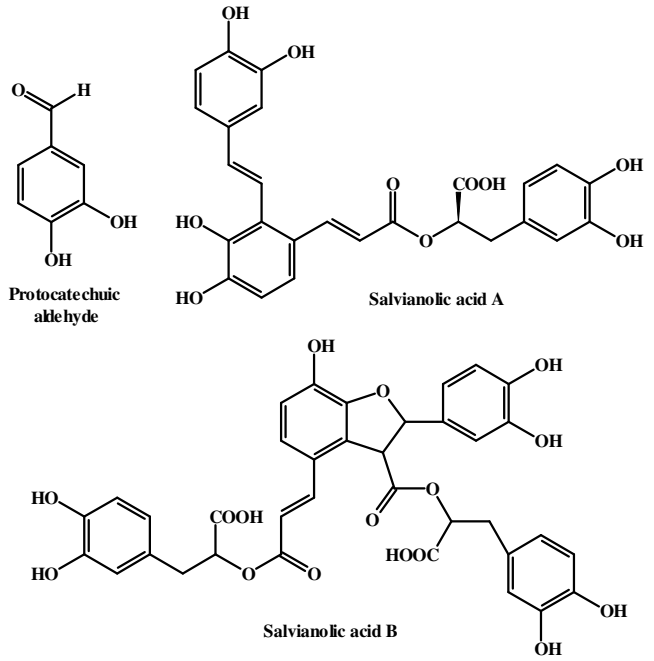

Fig. 1. Chemical structures of the standard substances

In the present study, a simple and sensitive HPLC method was established for simultaneous determination of protocatechuic aldehyde, salvianolic acid B and salvianolic acid A in the roots of *S. miltiorrhiza* and its related species. Then, the extraction solvents and extraction time have been investigated to find the simplest and most efficient sample preparation method for *S. miltiorrhiza* and its related species. The results are presented to show that these methods can aid in the assessment of quality control of raw herbal medicines and provide the basis for rational development and utilization of medicinal on *S. miltiorrhiza* and its related species.

EXPERIMENTAL

Twenty five samples of *Salvia* were collected from different provinces in China. The medicine plants and their names, locations, characteristics, medicinal parts are listed in Table-1.

Standards substances of protocatechuic aldehyde and salvianolic acid B were purchased from National Institute for Control of Pharmaceuticals and Biological Products (Beijing, China). Salvianolic acid A was bought from Chendu Mansite (Chendu, China). Acetonitrile (HPLC grade) were supplied by Fisher (Fair Lawn, NJ, USA). All the other reagents used in this research were analytical grade. Deionized water was purified in a RM-220 water purification system (Water purifier corp., Sichuan, China).

Chromatographic analysis was performed on a Shimadzu LC-20A HPLC system (Shimadzu Technologies, Japan) equipped with a dual-pump solvent delivery system, an online degasser, an auto-sampler, a column temperature controller and an ultraviolet detector coupled. Chromatographic separation was carried out on a Shimadzu Shim-pack VP-ODS C_{18} reserved phase column (5 μm, 250 mm × 4.6 mm i.d.) coupled with a Phenomenex C_{18} guard column (5 μm, 10 mm × 4.6 mm i.d.). The detection was performed at 288 nm, the flow rate was 1.0 mL min^{-1} and the column temperature was maintained at 25 ºC. The mobile phase was gradient elution which was consisted of solvent A (0.03 % aqueous phosphoric acid, v/v) and B (acetonitrile). The gradient programme was optimized and conducts as follows: 0-20 min, 8-23 % B; 20-35 min, 23-28.5 % B; 35-40 min, 28.5 % B; 40-45 min, 28.5-8 % B. An aliquot of 10 μL of the filtrate was injection into HPLC for analysis.

TABLE-1
STUDIED SAMPLES OF *S. miltiorrhiza* AND ITS RELATED SPECIES

No.	Name	Location	Characteristic	Medical parts
S1	*S. miltiorrhiza* 'Anhuibaiye'	Deyang, Sichuan	Culture	root
S2	*S. miltiorrhiza* 'Zupeixiaoye'	Deyang, Sichuan	Culture	root
S3	*S. miltiorrhiza* 'Sibeitixiaoye'	Deyang, Sichuan	Culture	root
S4	*S. miltiorrhiza* 'Zupeidaye'	Deyang, Sichuan	Culture	root
S5	*S. miltiorrhiza* 'Gaogandaye'	Deyang, Sichuan	Culture	root
S6	*S. miltiorrhiza* 'Aigandaye'	Deyang, Sichuan	Culture	root
S7	*S. miltiorrhiza*	Beijing	Culture	root
S8	*S. miltiorrhiza*	Zhenjiang, Jiangsu	Culture	root
S9	*S. miltiorrhiza*	Xi'an, Shaanxi	Culture	root
S10	*S. miltiorrhiza*	Shaoxing, Zhejiang	Culture	root
S11	*S. miltiorrhiza*	Bozhou, Anhui	Culture	root
S12	*S. miltiorrhiza*	Nanyang, Henan	Culture	root
S13	*S. miltiorrhiza*	Nanyang, Henan	Wild	root
S14	*S. miltiorrhiza*	Huanggang, Hubei	Wild	root
S15	*S. miltiorrhiza*	Taian, Shandong	Wild	root
S16	*S. brevilabra*	Ganzizhou, Sichuan	Wild	Whole plant
S17	*S. castanea*	Liangshanzhou, Sichuan	Wild	root
S18	*S.cavaleriei* var. *simplicifolia*	Leshan, Sichuan	Wild	Whole plant
S19	*S. cavaleriei*	Nanchuan, Chongqing	Wild	Whole plant
S20	*S. digitaloides*	Liangshanzhou, Sichuan	Wild	root
S21	*S. paohsingensis*	Ya'an, Sichuan	Wild	Whole plant
S22	*S. plebeia*	Ya'an, Sichuan	Wild	Whole plant
S23	*S. przewalskii*	Ganzizhou, Sichuan	Wild	root
S24	*S. trijuga*	Liangshanzhou, Sichuan	Wild	root
S25	*S. yunnanensis*	Kunming, Yunnan	Wild	root

Preparation of standard solutions: Standard stock solution of protocatechuic aldehyde, salvianolic acid B and salvianolic acid A were, respectively dissolved in 75 % methanol and stored at 4 ºC. Working standard solutions containing each of the compounds were prepared to appropriate concentration

range for the construction of calibration curves. The concentration of 16.48 μg mL^{-1} for protocatechuic aldehyde, 297.6 μg mL^{-1} for salvianolic acid B and 55.68 μg mL^{-1} for salvianolic acid A. The calibration curve of each compound was performed with at least six concentrations using the same HPLC condition as described in section 2.2.

Preparation of sample solutions: All the samples were milled into powder and over-dried at 50 ºC until the weight remained constant. 0.5 g powder of each dried samples were accurately weighed and extracted with deionized water in Soxhlet's apparatus extracting for 4 h. The extract solution was concentrated and metered volume to 25.00 mL with deionized water. For HPLC analysis, a moiety of solution was filtered through a 0.45 μm membrane filter and 10 μL of the filtrate was injected into the HPLC system.

RESULTS AND DISCUSSION

Selection of HPLC condition: A better HPLC condition was considerated with the need that the mark peaks have greatly baseline separation with adjacent peaks within a short analysis time as far as possible. In this study, detection wavelength, gradient program of mobile phase and column temperature were optimized, respectively.

The mobile phase (water-methanol and water-acetonitrile with different modifiers, including acetic acid and phosphoric acid) were compared. The binary mixtures of water-acetonitrile system showed more powerful separation ability for detected compounds than the water-methanol system. The 0.03 % (v/v) phosphoric acid was added to reduce the peak tailing and thus improve the peak shape. Protocatechuic aldehyde, salvianolic acid B and salvianolic acid A were difficult to be baseline resolved in isocratic elution profile, so gradient elution was employed in HPLC analysis. The ultimately selected mobile phase system was consisting of acetonitrile-0.03 % phosphoric acid aqueous, which provides lower pressure and greater baseline stability.

The most suitable flow rate were found to be at 1.0 mL min^{-1}. It was also suggested that separation were more suitable when column temperature were kept at 25 ºC and 288 nm were selected as the chromatograms detection wavelength.

Selection of the extraction procedure: In order to obtain the most efficient extraction procedure, a series of factors including extraction solvent, method and extraction time were investigated.

The dried root of sample 1 (0.3 g 80 mesh) was extracted by refluxing (1, 2, 3 and 4 h) and Soxhlet's apparatus (1, 2, 3 and 4 h) with deionized water, respectively. The contents of the three components were used as response to evaluate the extraction method and the results signified that Soxhlet's apparatus extracting for 4 h was the most suitable method (Fig. 2). After the extraction method and the extraction time were ascertained, the extraction solvents containing deionized water, methanol (10, 20, 30 and 40 %) and ethanol (10, 20, 30 and 40 %) were also investigated. The water was found to be the most suitable extraction solvent (Fig. 3). Finally, the optimum extraction procedure was established as Soxhlet's apparatus extraction by 100 mL deionized water for 4 h.

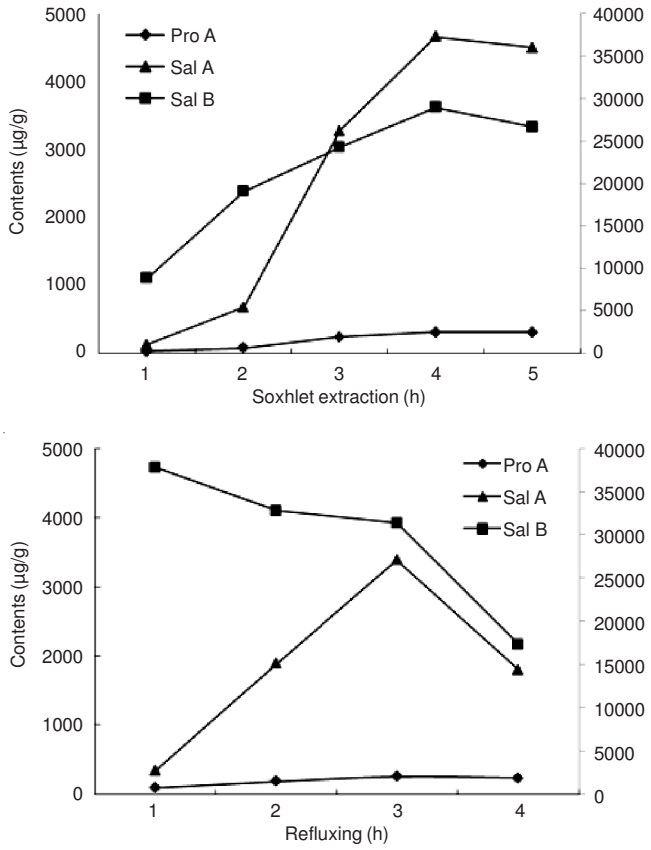

Fig. 2. Effect of different extracted method for water-soluble contents

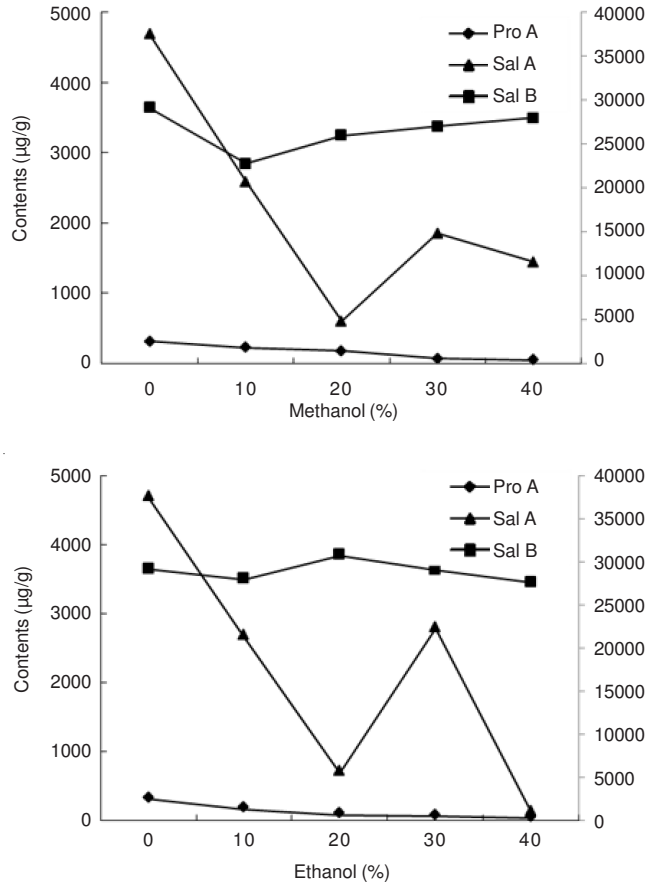

Fig. 3. Effect of different extractant on water-soluble contents

TABLE-2
CALIBRATION CURVE DATA AND LOD DATE FOR THREE COMPONENTS BY HPLC ANALYSIS

Analyte	Standard curve	R^2	Linear range (μg mL^{-1})	LOD (μg mL^{-1})
Protocatechuic aldehyde	y = 1.04624e – 005x + 0.149353	1.0000	0.648 -99.06	0.039
Salvianolic acid B	y = 8.81402e – 005x + 2.148873	1.0000	14.63 -1786	0.85
Salvianolic acid A	y = 6.18786e – 005x + 0.793650	0.9999	2.561 -334.14	0.13

y: Peak area; x: the concentration of each reference compound (μg L^{-1}); R^2: correlation coefficient of regression equations; LOD: limit of detection (S/N = 3).

Methodology validation

Calibration curves and the limit of detection: Under the carefully optimum chromatographic conditions, calibration graphs were obtained by injecting standard solution consisted of protocatechuic aldehyde, salvianolic acid B and salvianolic acid A at six different concentrations and each concentration performed in triplicate. The calibration graphs for each compounds was plotted based on linear regression analysis of the integrated peak areas (y) *versus* concentrations (x) of the standard phenolic acids. The regression equations and linear ranges for the analysis of the phenolic acids are shown in Table-2. The limit of detection value (LOD) was determined at signal-to-noise ratios (S/N) of 3. The LOD values of the method for three components are also given in Table-2.

Precision: The precision test was assessed by repetitive of the same sample for six times in one day. The RSD of relative retention time and relative peak area of protocatechuic aldehyde, salvianolic acid B and salvianolic acid A were not exceeding 1.9, 2.0 and 1.0 %, respectively (Table-3).

TABLE-3
VALIDATION DATA FOR HPLC QUANTITATIVE OF *S. miltiorrhiza* AND ITS RELATED SPECIES

Analyte	Precision RSD (%)	Repeatability RSD (%)	Recovery (%)	Stability RSD (%)
Pro A	1.9	0.62	95.17	0.89
Sal B	2.0	0.59	94.06	0.56
Sal A	1.0	1.0	91.43	2.2*

*Stability RSD (%) of Sal A within 12 h; Pro A = Protocatechuic aldehyde; Sal B = Salvianolic acid B; Sal A = Salvianolic acid A

Repeatability: Repeatability was checked by analyzing six independently prepared samples of *S. miltiorrhiza* using the same method. The RSD were 0.62 % for protocatechuic aldehyde, 0.59 % for salvianolic acid B and 1.0 % for salvianolic acid A were, respectively (Table-3). The results indicated that there is little variability in the instrumental response and thus showed good repeatability.

Recovery: In order to determined the accuracy of the developed method, recovery experiments were carried out by adding authentic standards to the samples and then extracted as the above procedure. For each level of concentration 3 replications were performed. The results are reported in Table-3. It can be seen that the recoveries were 95.17, 94.06 and 91.43 %, indicating that the proposed method has an adequate degree of accuracy.

Sample stability: Sample stability test was determined with one sample at 0, 2, 4, 6, 8, 12 and 24 h and the solution was stored at ordinary temperature. The RSD of protocatechuic aldehyde, salvianolic acid B and salvianolic acid A were below 0.89, 0.56 and 19 %, respectively. The results indicated that the protocatechuic aldehyde and salvianolic acid B remained stable for 24 h, but the salvianolic acid A was not robustness in 24 h. In this reason, the solution was assessed at 0, 2, 4, 6, 8 and 12 h at 4 ºC and the salvianolic acid A was found to be stable within 12 h (RSD < 2.2 %). The results were shown in Table-3.

Sample analysis: The established method has been applied to the simultaneous determination of protocatechuic aldehyde, salvianolic acid B and salvianolic acid A in the roots of *S. miltiorrhiza* and its related species. The HPLC profiles were showed in Fig. 4 and the results were summarized in Table-4.

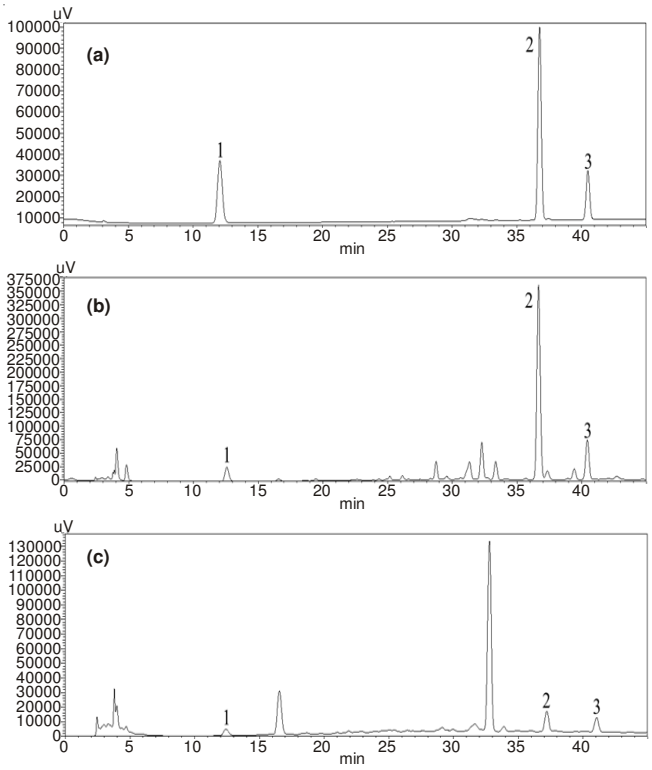

Fig. 4. Chromatograms of standards compounds (a), the sample of *S. miltiorrhiza* (b) and the sample of *S. paohsingensis* (c) (1-protocatechuic aldehyde; 2-salvianolic acid B; 3- salvianolic acid A)

As seen in Table-4, the concentration of three components were greatly difference, especially the content of salvianolic acid B. The contents range (mg g^{-1}) were 0.0873-0.4211 (protocatechuic aldehyde), 1.136-4.747 (salvianolic acid A), 5.131-31.40 (salvianolic acid B), respectively. The results showed that the samples from Sichuan province (S1, S4, S5, S6) and Henan province (S12) had a high content of salvianolic acid B (>25.00 mg g^{-1}) and Jiangsu (S8), Zhejiang (S9), Shaanxi province (S10) had a very lower content of salvianolic acid B (<8.00 mg g^{-1}). he variation of contents may be due to the different geographical location, the variations

TABLE-4
EFFECTIVE COMPONENTS IN SAMPLES (n = 3, UNIT mg g^{-1})

No.	Protocatechuic aldehyde	Salvianolic acid B	Salvianolic acid A
S1	0.2224	25.20	1.823
S2	0.1430	10.98	3.012
S3	0.2850	12.73	7.001
S4	0.4211	30.64	1.339
S5	0.3014	29.47	4.747
S6	0.2894	31.40	1.739
S7	0.2664	15.15	3.619
S8	0.1028	5.131	1.308
S9	0.1292	7.610	1.952
S10	0.1280	7.821	2.622
S11	0.2450	25.19	4.241
S12	0.1004	15.21	3.089
S13	0.0873	12.69	1.136
S14	0.2237	19.55	2.872
S15	0.1914	15.05	3.606
S16	0.04968	0.2553	0.3491
S17	0.1258	0.1361	–
S18	0.06187	3.241	0.9157
S19	0.06660	3.012	0.3286
S20	0.1366	1.253	–
S21	0.07632	1.796	0.6989
S22	0.02765	0.1600	0.3766
S23	0.1316	0.2788	–
S24	0.1260	6.066	4.215
S25	0.02972	1.009	0.3205

Note: Table-4 and Table-1 share the same serial No.

cultivation conditions, the effect of genuine quality and other factors.

S. brevilabra, *S. castanea*, *S. cavaleriei*, *S. cavaleriei* var. *simplicifolia*, *S. digitaloides*, *S. paohsingensis*, *S. plebeian*, *S. przewalskii*, *S. trijuga* and *S. yunnanensis* were reported for the first time on the simultaneous determination of three phenolic acids. All of them showed very low contents, even the salvianolic acid A were not detected in *S. brevilabra*, *S. cavaleriei* and *S. digitaloides*. The results indicated that these species were not suitable for being substitutes of *S. miltiorrhiza*. However, it should be noted that this study has only examined on the phenolic acids, further studies on the tanshinones constituents will be summarized in our next study.

Conclusion

In this paper, we developed an HPLC method for simultaneously quatitying the three phenolic acids of *S. miltiorrhiza* and its relative species. The results suggested that the validated HPLC method with high linearity, precision, accuracy and reliability. It could not only be considered as quality control of *S. miltiorrhiza* and its relative species, but also for other related botanical medicines.

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