Multi-component HPLC Fingerprinting of Radix Salviae Miltiorrhizae and Its LC-MS-MS Identification

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The multi-component fingerprinting method of Radix Salviae Miltiorrhizae (Root of *Salvia miltiorrhiza* BGE.), an important and popular medicinal herb in traditional Chinese medicine, was studied using reverse-phase HPLC and LC-MS-MS. Extract containing both the water-soluble phenolic compounds and nonpolar diterpenoid compounds known to be the herb's main bioactive components was prepared by a two-step extractive procedure. An HPLC fingerprinting method which can simultaneously separate these two types of compounds was established with gradient elution mode and photodiode array detection at 280 nm. Eighteen peaks in this HPLC fingerprint were structurally identified by employing LC-MS-MS techniques. The electrospray ionization (ESI) MS-MS spectra of most salvianolic acids displayed a characteristic behavior of loss of danshensu and caffeic acid moieties, while those of tanshinones showed a particular behavior of loss of H_2O which is quite different from the fragmentation pattern in electron ionization mass spectrometry (EI-MS). The HPLC fingerprints of 7 batches of crude drugs showed similar separation pattern and provided much chemical information of the pharmacologically-active compounds in the crude drugs, which is useful for the authentication and quality evaluation of this medicinal herb.

Key words *Salvia miltiorrhiza*; HPLC fingerprint; LC-MS-MS; salvianolic acid; tanshinone

Radix Salviae Miltiorrhizae (Root of *Salvia miltiorrhiza* BGE., commonly known as "Danshen" 丹参), is an important Chinese medicinal herb used to promote blood flow to overcome blood stasis and to cool the blood to resolve abscesses in traditional Chinese medicine.[1)] Because of the increase of cardiovascular diseases in modern society, it is now experiencing a particularly strong and rapid upsurge in usage.

In chemical studies over several decades, it has been found that there are a variety of diterpenoids, phenolics, flavonoids, triterpenoids and sterols in Radix Salviae Miltiorrhizae.[2)] In the early stages of bioactivity research on this herb, the diterpenoids attracted a great deal of attention from pharmacologists, since diterpenoid tanshinones are the major constituents of the nonpolar fraction of Radix Salviae Miltiorrhizae. Up to now, more than 30 diterpenoidal compounds have been chemically identified from this herb.[3)] They have been shown to possess a variety of biological activities including anti-ischemia, antioxidant and anti-tumor properties.[4,5)] Among them, tanshinone IIA was approved to be a drug which has been clinically used in China.

However, water-soluble phenolics are attracting more attention recently since water decoctions of Radix Salviae Miltiorrhizae are commonly used in clinics in China. In the water extract, the main chemicals are caffeic acid metabolites, such as caffeic acid monomer (*e.g.*, caffeic acid itself, danshensu and isoferulic acid), caffeic acid dimers (rosmarinic acid, salvianolic acids D and F), caffeic acid trimers [lithospermic acid (lithospermic acid A), salvianolic acids A, C, H, I, J and K] and caffeic acid tetramers [salvianolic acid B (lithospermic acid B) and salvianolic acid E] (Fig. 1).[6,7)] Pharmacological studies showed that the phenolics can protect the myocardium from ischemia-induced derangement, protect neural cells from injuries caused by anoxia, inhibit platelet aggregation, reduce hepatic fibrosis and depress the activities of HIV-1.[8—12)] Total salvianolic acids purified from Radix Salviae Miltiorrhizae have been clinically used for cardiovascular diseases in China.

Although Radix Salviae Miltiorrhizae itself and the preparations containing its whole extracts were proved to be highly effective for the treatment of some diseases by pharmacological studies and clinical studies in China,[1)] they have not been officially recognized internationally. One important reason for this is the lack of an effective method to evaluate the quality of the raw medicinal materials.

Usually, the methods of chemical testing and TLC identification are adopted for the authentication of traditional Chinese medicine in the Pharmacopoeia of the People's Republic of China.[13)] However, chemical testing based on the chemical group reaction is not a specific method for authenticating this herbal medicine. TLC identification is not powerful due to its relative weak ability of separation and reproducibility. In recent years, chromatographic fingerprinting has been shown to be an efficient method for evaluating herbal medicines since the chromatography has very powerful separation ability.[14,15)] Multiple components in traditional Chinese medicines can be simultaneously separated by using HPLC, gas chromatography (GC), high performance thin layer chromatography (HPTLC) and capillary electrophoresis (CE). Indeed, the use of HPLC coupled with a diode array detector (DAD) for the fingerprinting of Radix Salviae Miltiorrhizae and its preparations has been reported.[16—18)] However, these fingerprints only dealt with either the diterpenoidal compounds or the phenolic compounds in the crude drug. It is obvious that they are not sufficient for authenticating this herb since both the nonpolar and water-soluble compounds are pharmacologically-active components in Radix Salviae Miltiorrhizae. Therefore, a multi-component HPLC fingerprint method that can simultaneously analyze both water-soluble and nonpolar compounds including most of the pharmacologically-active components should be developed.

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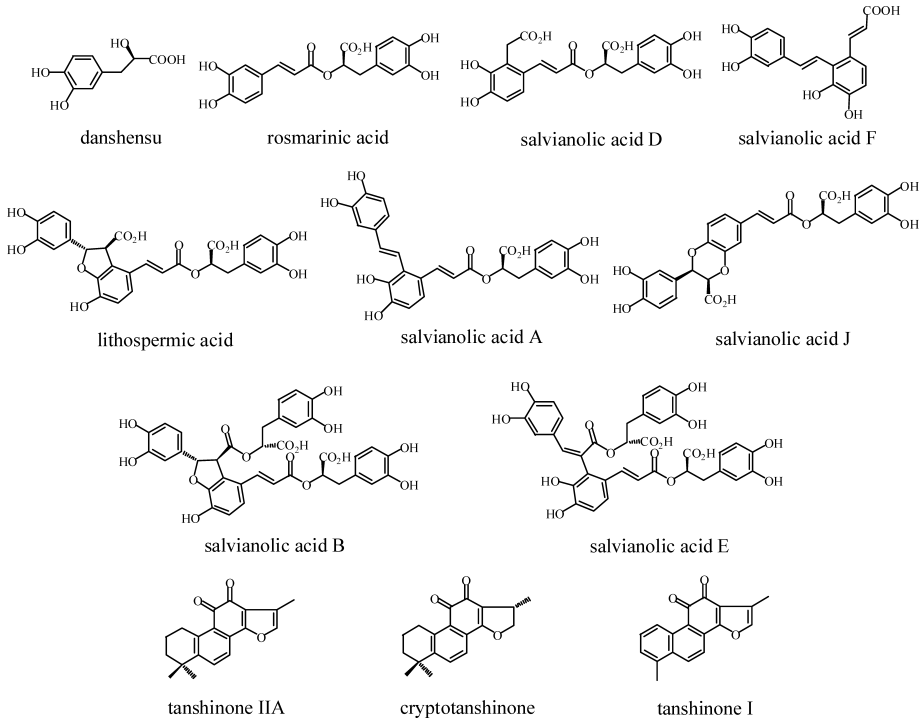

Fig. 1. The Structures of Phenolics and Tanshinones in Radix Salviae Miltiorrhizae

Liquid chromatography hyphenated with tandem mass spectrometry (LC-MS-MS), a relatively new technique rapidly growing in popularity, has been successfully applied to elucidate the structures of the active compounds in herbal extracts.[19] For example, non-volatile phenolic compounds in several plants have been analyzed using LC-MS and LC-MS-MS.[20—22] We believe that this technique could be successfully applied to identify the peaks in the HPLC fingerprint of Radix Salviae Miltiorrhizae.

In this paper, a multi-component (including water-soluble and lipid-soluble compounds) HPLC fingerprinting method of Radix Salviae Miltiorrhizae was developed and LC-MS-MS identification of the major constituents in the HPLC chromatogram was made. As an application, seven batches of Radix Salviae Miltiorrhizae samples collected from different regions of China were analyzed using the established methods.

Results and Discussion

Selection of Extraction Methods On the one hand, sonication was considered to be suitable for extracting the nonpolar components since tanshinone II_A and cryptotanshinone are not stable in hot solvents.[23] On the other hand, several simple phenolics, such as danshensu, protocatechuic aldehyde and caffeic acid were very difficult to extract out by methanol with sonication. On the basis of the above considerations, a two-step procedure, *i.e.*, extraction by methanol and then by boiled water, was designed as the extraction method for the sample preparation of HPLC fingerprint. Compared with the one-step extract method of decocting with water or sonicating with methanol commonly described in the literature, this two-step extraction process has two advantages. First, most of the bioactive components in the crude drug can be quantitatively extracted out. Second, most of the interference from macromolecules such as proteins, polysaccharides, can be easily removed from the test solution.

Table 1. Composition and Gradient of the Mobile Phase for HPLC Analysis

Time (min)	A (water/MeCN/formic acid, 90 : 10 : 0.4, v/v)/%	B (MeCN)/%
0	100	0
40	70	30
50	20	80
70	15	85

Optimization of HPLC Condition In the mobile phase A (Table 1), formic acid was added to depress the tailing of the peaks of phenolic compounds. The effect of the concentration of formic acid in mobile phase A on the separation of water-soluble compounds was investigated. It was found that the distortion of peaks of polyphenolics would occur at long intervals if the concentration of formic acid was less than 0.3%. Finally, the concentration of 0.4% formic acid was selected to ensure the reproducibility of the fingerprints of Radix Salviae Miltiorrhizae.

Linear gradient of constant slope was not suitable for the simultaneous separation of water-soluble and nonpolar compounds in Radix Salviae Miltiorrhizae due to the significant difference in polarity of these two types of components. In this fingerprint, a high-gradient slope was used in order to elute the tanshinones within a reasonable time, but it was necessary to selectively increase the resolution of phenolics and tanshinones within the gradient. Therefore, two sections of lower gradient slopes were built into the gradient so that it covered the parts of the chromatogram where increased resolution is desired. This three-slope gradient of the mobile phase could achieve maximum throughput and optimal resolution.

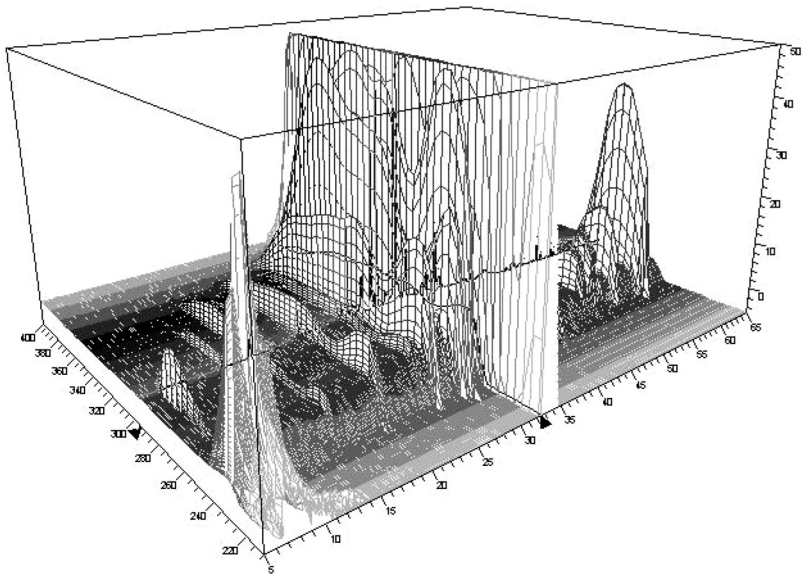

Fig. 2. HPLC-DAD Plot of Radix Salviae Miltiorrhizae for the Selection of Detection Wavelength

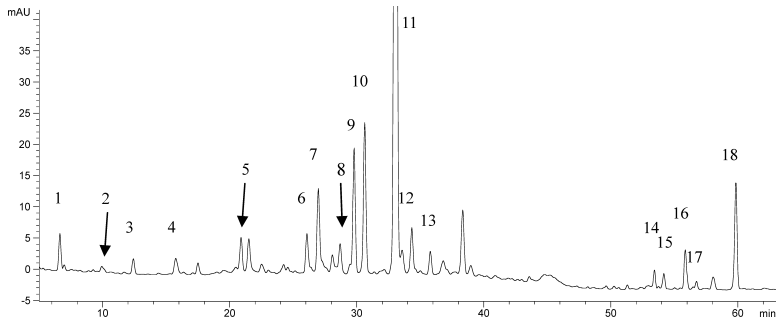

Fig. 3. Multi-component HPLC Fingerprint of Radix Salviae Miltiorrhizae

The peaks in chromatogram was identified by LC-MS-MS as following: 1. Danshensu; 2. protocatechuic acid; 3. protocatechualdehyde; 4. caffeic acid; 5. salvianolic acid F; 6. salvianolic acid D; 7. salvianolic acid J/isomer; 8. salvianolic acid E; 9. rosmarinic acid; 10. lithospermic acid; 11. salvianolic acid B; 12. salvianolic acid B/E/isomer; 13. salvianolic acid A; 14. dihydrotanshinone I; 15. tetrahydrotanshinone/isomer; 16. cryptotanshinone; 17. tanshinone I; 18. tanshinone IIA.

The wavelength for the detection of the components in Radix Salviae Miltiorrhizae was selected by using a DAD detector (Fig. 2). The maximum number and the height of peaks of the active compounds could be obtained and the baseline of chromatogram was stable at 280 nm. Therefore, 280 nm was chosen as the detection wavelength.

Identification of Water-Soluble and Nonpolar Compounds in Radix Salviae Miltiorrhizae by LC-MS-MS The multi-component HPLC fingerprint of authenticated herbal material was obtained according to the developed HPLC method described above (Fig. 3). Identification of the peaks in this fingerprint profile was carried out by using the LC-MS-MS technique. The reference standards rosmarinic acid and tanshinone IIA were analyzed by direct injection in order to optimize the electrospray ionization (ESI) MS-MS conditions. Tuning experiments showed that the negative ion mode is more sensitive than the positive ion mode for identifying water-soluble phenolic compounds, while the positive ion mode was found to be more suitable for the nonpolar tanshinones. The HPLC-ESI-MS-MS chromatogram exhibited good agreement with that obtained from HPLC-DAD analysis.

Eighteen characteristic peaks presented in fingerprint profile were tentatively identified by detailed studies of their MS-MS spectral data (Table 2) and by comparison with published data.[7—10,19,21] The identification of peaks 1—4, 9—11, 13, 14, 16—18 was further confirmed by comparing the their retention time values with those of the standards. Among these 18 peaks, peaks 1—13 correspond to water-soluble phenolic compounds and peaks 14—18 represent nonpolar diterpenoidal compounds. To our best of knowledge, this is the HPLC fingerprint of Radix Salviae Miltiorrhizae which exhibited the most comprehensive chemical information.

The ESI-MS-MS spectra of salvianolic acids displayed a characteristic fragmentation behavior. Except for salvianolic acid F (Mw 314), the MS-MS spectra of all salvianolic acids exhibited the fragment ions derived from neutral loss of one molecule of danshensu $[M-H-198]^-$ and one molecule of caffeic acid $[M-H-180]^-$ from the molecular ion. The fragment ion $[M-44-H]^-$ corresponding to the loss of CO_2 molecule is also present in the spectra of most of the phenolics including caffeic acid monomer and its metabolites due to the existence of –COOH group in all of these compounds. The abundance of this de-carboxyl fragment ion peak $[M-44-H]^-$ at *m/z* 493 in LC-ESI-MS-MS spectrum of peak 10 (lithospermic acid) is very high while the abundance of its

Table 2. HPLC-ESI-MS-MS Ions of Phenolics and Diterpeniods in Radix Salviae Miltiorrhizae

Peak	Identification	MS (m/z)[a]	MS-MS (m/z)[b]
1	Danshensu	197	197, 179, 137
2	Protocatechuic acid	153	153
3	Protocatechualdehyde	137	137
4	Caffeic acid	179, 135	179, 135
5	Salvianolic acid F	313, 269	313, 269, 159
6	Salvianolic acid D	417	417, 373
7	Salvianolic acid J/isomer	537, 296	537, 339, 493, 296
8	Salvianolic acid E	717, 519	717, 519, 537
9	Rosmarinic acid	359, 197, 161	359, 161, 197, 179
10	Lithospermic acid	493, 295	537, 493, 295, 383
11	Salvianolic acid B	717, 519	717, 519, 321, 537
12	Salvianolic acid B/E/isomer	717, 519	717, 519, 537
13	Salvianolic acid A	493	493, 296
14	Dihydrotanshinone I	279	279, 261, 233
15	Tetrahydrotanshinone/isomer	281, 263	281, 263, 235
16	Cryptotanshinone	297, 179, 251	297, 279, 269, 251, 234
17	Tanshinone I	277	277, 249, 259, 231
18	Tanshinone IIA	295, 277	295, 277, 249

a) Relative abundance of peaks are large than 10%. *b*) Derived from $[M-H]^-$.

quasi-molecular peak $[M-H]^-$ at m/z 537 was especially low and is easily to be ignored. These feature often lead to mistaking lithospermic acid for salvianolic acid A (Mw=494). In this paper, the existence of lithospermic acid and salvianolic acid in Radix Salviae Miltiorrhizae and their chromatographic character were unambiguous demonstrated. Examples of MS-MS spectra of salvianolic acid B and rosmarinic acid and the rules for the proposed fragmentation of salvianolic acid B are illustrated in Fig. 4.

The ESI-MS fragmentation of tanshinones mainly involved in the loss of a molecule of H_2O $[M+H-18]^+$ due to enolic rearrangement of a ketone group which did not occur in its electron ionization mass spectrometry (EI-MS) spectra. Further loss of CO from this fragment ion gave rise to $[M+H-46]^+$.

Fingerprinting of Representative Samples Seven samples of crude drug collected from the cultivation bases of Salvia Miltiorrhiza in different regions of China were analyzed according to the above methods. Their multi-component HPLC fingerprint profiles were shown in Fig. 5. The HPLC fingerprints of all samples, regardless of the origin, show a very similar pattern in terms of peak distribution and morphology. However, the relative height (comparing with the peak height of rosmarinic acid) of characteristic peaks exhibited somewhat difference. For example, the relative heights of tanshinones, especially tanshinone I, cryptotanshinone and dihydrotanshinone are lower in the samples from Zhongjiang, Sichuan Province than in the samples from the other provinces.

In a conclusion, two groups of pharmacologically-active compounds in Radix Salviae Miltiorrhizae were successfully simultaneously analyzed by using HPLC fingerprinting techniques. More than 20 peaks were observed in this multi-component HPLC profile, of which, 18 were structurally identified by LC-MS-MS analysis and by comparison of their retention time values with those of the standard compounds. Among the identified peaks, 13 are water-soluble phenolic compounds and the others are nonpolar diterpenoidal compounds. Several polyphenolic compounds such as lithospermic acid and salvianolic acid A were unambiguously confirmed and employed as characteristic markers of the fingerprint of Radix Salviae Miltiorrhizae for the first time. The fingerprint obtained by using our established techniques provides most comprehensive chemical information for the identification and assessment of Radix Salviae Miltiorrhizae. The multi-component HPLC fingerprints of 7 batches of representative crude drugs displayed a good repeatability in separation pattern which demonstrated that the fingerprint presented in this paper is a rapid, reliable and effective method suitable for the authentication and quality evaluation of Radix Salviae Miltiorrhizae.

Experimental

Standards and Samples Standards of danshensu, cryptotanshinone, tanshinone I, tanshinone II_A and dihydro-tanshinone (98%) were purchased from the National Institute for the Control of Pharmaceutical & Biological Products (Beijing, China). Caffeic acid, protocatechuic acid, protocatechuic aldehyde and rosmarinic acid (97%) were obtained from Sigma (St. Louis, MO, U.S.A.). Salvianolics acid A and B (98%) were purchased from Kunming Tongchi Pharmaceutical R&D Co. Ltd. (Kunming, China). Lithospermic acid was isolated in our laboratory, whose structure was confirmed by NMR and MS data. The samples of Radix Salviae Miltiorrhizae were collected from the cultivation base of *Salvia miltiorrhiza* in different regions of China and were air-dried according to the procedure described in China Pharmacopoeia. All of these samples were identified by one of the author (Z. Zhao) and were deposited in the Centre of Chinese Materia Medica, Hong Kong Baptist University.

Solvents and Reagents HPLC-grade acetonitrile (MeCN) and methanol (AR grade) were obtained from Labscan (Stillorgan, Ireland). Formic acid (GR grade) was purchased from Merck (Darmstadt, Germany), and the water used for HPLC was purified by a Milli-Q system (Millipore, Milford, MA, U.S.A.).

Sample Preparation Approximately 0.2 g of powdered Radix Salviae Miltiorrhizae and 5 ml of methanol were mixed in a 10 ml centrifugal tube. After sonication for 30 min in an Ultrasonic Cleaner (Branson, Danbury, CT, U.S.A.), the sample was centrifuged in a centrifuge (Eppendorf, Hamburg, Germany) for 5 min at about 2000 rpm. The supernatant was transferred into a 25 ml amber volumetric flask. The residue was re-extracted by 5 ml of methanol twice and the two supernatants were combined into the 25 ml volumetric flask. The residue was then transferred into a 50 ml round-bottom flask and refluxed with 10 ml water for 30 min. After centrifuged for 5 min at about 2000 rpm, the supernatant was combined with above methanol solution which was made up to 25 ml with water. The sample solution was filtered through a 0.2 μm Nylon filter (Iwaki Glass, Tokyo, Japan) into a HPLC amber sample vial for HPLC analysis.

The extracting procedure of crude drugs for LC-MS-MS identification was the same as that for HPLC fingerprint analysis except for the amount of sample increased to 1.0 g.

HPLC Method HPLC/DAD analysis was carried out on an Agilent Series 1100 (Agilent, Wilmington, Germany) equipped with G1312A Binary Pump, G 1329A automatic sample injector and G1315A Diode Array Detector. Separation was performed on a Alltima C18 column (4.6 mm×250 mm, 5 μm, Alltech, Illinois, U.S.A.) at ambient temperature with a sample injection volume of 20 μl and the wavelength was 280 nm. Solvent A (water/MeCN/formic acid, 90 : 10 : 0.4, v/v) and solvent B (MeCN) were used as mobile phase (1.0 ml/min). The gradient was presented in Table 1.

LC-MS-MS Identification The HPLC analytical conditions for the LC-MS-MS were the same as those used for the HPLC-DAD analysis, except for that the one-third of the eluant was introduced into the MS system by using split technique. Agilent 1100 HPLC/MSD Trap mass spectrometer (Agilent, Wilmington, Germany) equipped with an electrospray ionization source was used. An HPLC system coupled with DAD was controlled by an HPLC-MSD ChemStation software system.

Auto MS2 mode of Mass spectrometer was chosen to analyze the sample. Nitrogen was used as both dry gas and nebulizer gas, and dry temperature was at 300 °C. Other conditions are listed in Table 3.

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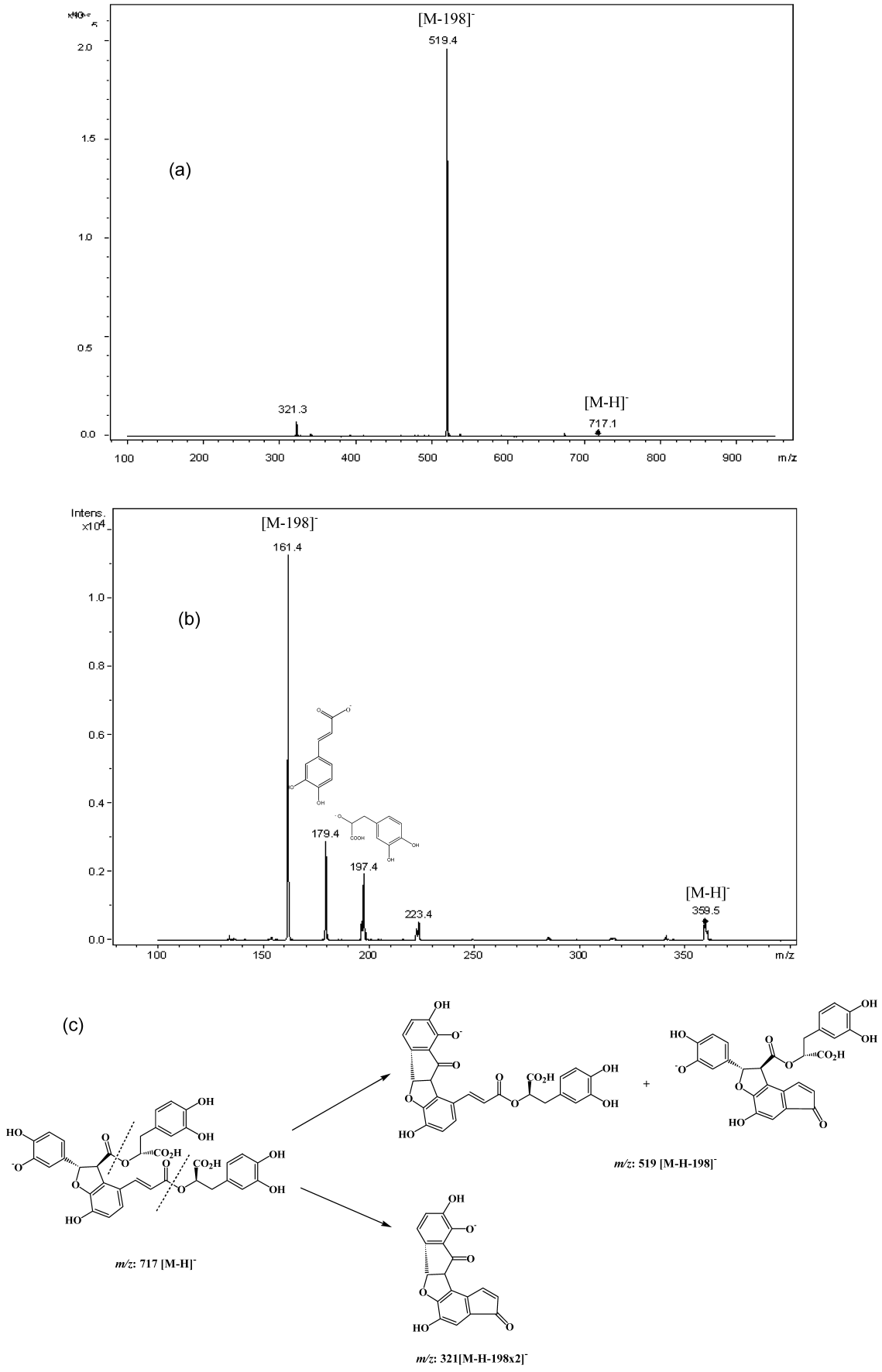

Fig. 4. The MS-MS Spectra of Polyphenolics (a) MS/MS Spectrum of *m/z* 717 of Salvianolic Acid B, (b) MS/MS Spectrum of *m/z* 359 of Rosmarinic Acid (c) ESI-MS-MS Fragmentation of Salvianolic Acid B

Table 3. ESI-MS Conditions for the Identification of Peaks in the Fingerprints of Radix Salviae Miltiorrhizae

Time range (min)	Scan mode	Scan range	Dry gas (l/min)	Neb. gas (psi)	Threshold	Fragmentation width (*m*/*z*)	Fragmentation amplitude (v)
0—45	Negative	100—1000	9	35	5000	20	1.50
45—70	Positive	100—800	8	30	10000	20	1.50

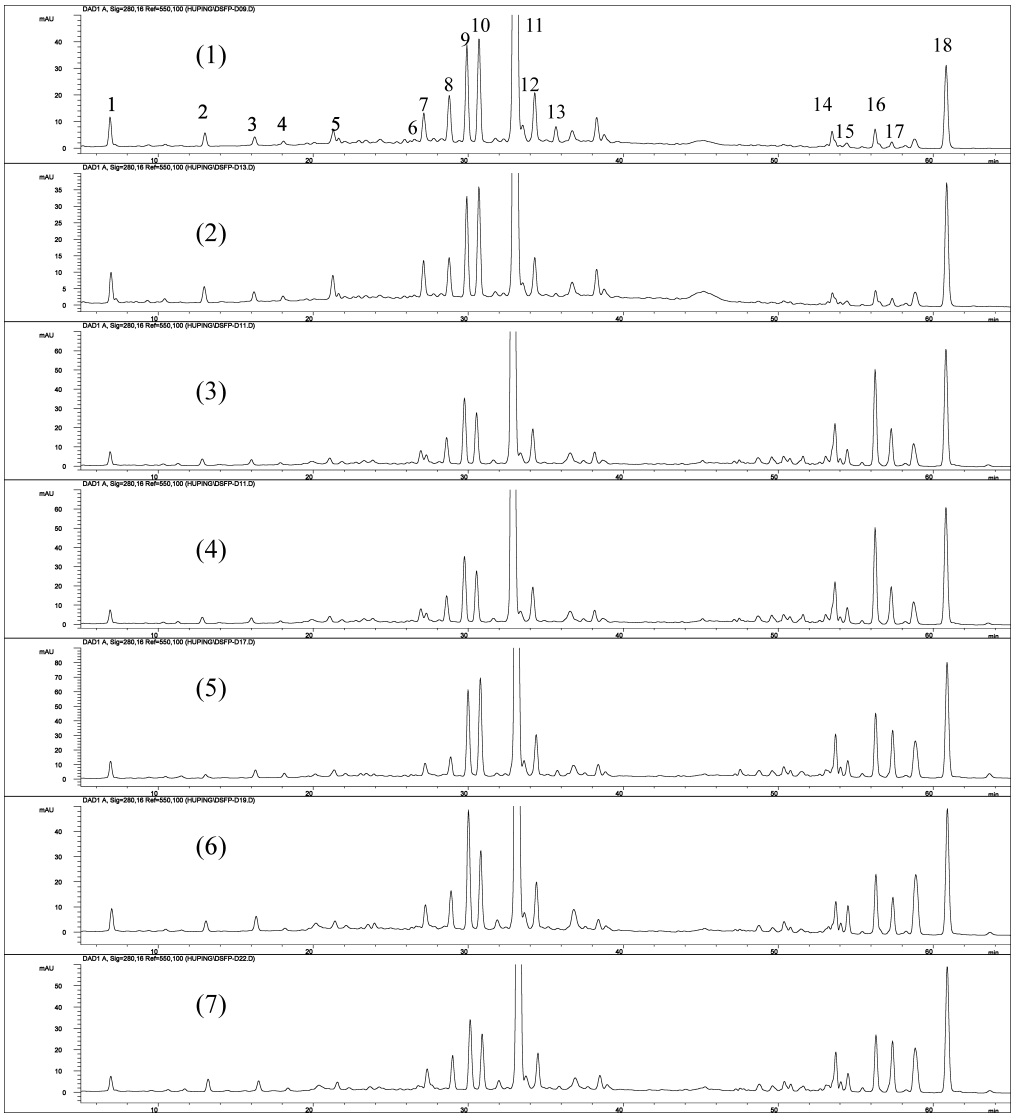

Fig. 5. Multi-component HPLC Fingerprint of Radix Salviae Miltiorrihizae from Different Regions of China (1) Zhongjiang-1, Sichuan Province; (2) Zhongjiang-2, Sichuan Province; (3) Yinan, Shandong Province; (4) Lushi, Henan Province; (5) Pingdingshan, Henan Province; (6) Luoyang, Henan Province; (7) Shangluo Shanxi Province

The peak numbers in the chromatogram represent the corresponding compounds as in Fig. 3.

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