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Rapid Differentiation of *Schisandra sphenanthera* and *Schisandra chinensis* by Matrix-assisted Laser Desorption/Ionization Mass Spectrometry

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Abstract

Schisandrae sphenanthera (southern magnoliavine fruit, Nan-Wuweizi) and *Schisandrae chinensis* (northern magnoliavine fruit, Bei-Wuweizi) belong to *Fructus Schisandrae* (Wuweizi), and are commonly used herbal medicines. The quality of *S. chinensis* is believed to be superior to that of *S. sphenanthera*. In this study, a matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)-based approach has been developed and demonstrated for rapid differentiation of *S. sphenanthera* and *S. chinensis* for the first time. The MALDI-MS spectra acquired with a brief extraction of the samples showed significantly different patterns for the two species. *S. sphenanthera* and *S. chinensis* could be unambiguously differentiated based on detection of the specific compounds (e.g., schisandrin at *m/z* 432 for *S. chinensis* and schisantherin A at *m/z* 575 for *S. sphenanthera*), intensity ratios of characteristic peaks (e.g., *m/z* 416 vs. *m/z* 415 and *m/z* 138 vs. *m/z* 136), and principal component analysis of the spectra. Direct analysis of the herbal powders by MALDI-MS could also allow differentiation of *S. sphenanthera* and *S. chinensis*. These approaches are rapid, simple, robust, and can be used for high throughput analysis of *S. sphenanthera* and *S. chinensis*.

Keywords: MALDI-MS; herbal medicines; differentiation; *Schisandra sphenanthera*; *Schisandra chinensis*

1. Introduction

Schisandra sphenanthera (southern magnoliavine fruit, Nan-Wuweizi) and *Schisandra chinensis* (northern magnoliavine fruit, Bei-Wuweizi) are the ripe fruits of *Fructus Schisandrae* (Chinese magnoliavine, Wuweizi). *S. sphenanthera* is mainly found in provinces in southern China, such as Shanxi, Shaanxi and Anhui, while *S. chinensis* is mainly distributed in provinces in northern China including Heilongjiang, Jilin and Liaoning. These two fruits can be used to replenish and promote production of body fluids and tonify the kidney to relieve mental strain [1]. Due to the therapeutic effects of these fruit, they are widely used in Korea, Japan and China. Major active ingredients in *Fructus Schisandrae* are lignans and volatile oils. *S. sphenanthera* and *S. chinensis*, however, have different chemical constituents and contents of bioactive components [1, 2] and *S. chinensis* is considered as better in quality [2, 3]. Therefore, it is necessary to develop approaches to differentiate these two fruits.

Thin layer chromatography (TLC) method is suggested by the Chinese Pharmacopoeia for differentiation between *S. chinensis* and *S. sphenanthera* [1]. Gao [4] reported that schisandrin, schisandrol B and schisandrin B were the major components detected in TLC analysis of *S. chinensis*, while schisantherin A and deoxyschisandrin were dominant in *S. sphenanthera*. Although the TLC method is relatively simple and convenient, it has poor resolution and sensitivity. High performance liquid chromatography (HPLC) coupled with an ultraviolet (UV) detector is suggested by the Chinese Pharmacopoeia as separation and quantitative determination method of markers in *S. chinensis* and *S. sphenanthera*. According to the Chinese Pharmacopoeia, Schisantherin A in *S. sphenanthera* should be no less than 0.2% while schisandrin in *S. chinensis* should be no less than 0.4% [1]. HPLC can be used to separate and quantitatively determine these markers, thus allowing the authentication of these two herbs [4-7]. In addition to TLC and HPLC, gas chromatography (GC) [8] and capillary electrophoresis

(CE) [9] were also used to characterize *Fructus Schisandrae*. With the couple of mass spectrometry (MS) detector, the sensitivity of quantification can be improved [8, 10, 11]. However, the above-mentioned methods usually require tedious sample pretreatment as well as component separation and are thus time-consuming. Development of rapid and effective methods [12-14] has been attempted. For example, a TLC-direct analysis in real time (DART)-MS method was developed for quantification of major dibenzocyclooctane lignans in schisandrae fructus [15], and Hu et al. presented a simple TLC-ESI-MS method which could successfully differentiate *S. chinensis* and *S. sphenanthera* [16].

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is a powerful technique for analysis of various compounds [17-20]. This technique is simple, rapid, tolerant of impurities and salts in the samples, and can be used for directly analyzing complex mixtures without chromatographic separation [21], which make it suitable for analysis of complex samples such as herbal medicines [22]. MALDI-MS has been applied for characterizing alkaloid profiling of Fuzi [23, 24], *Sinomenium acutum* [25], bioactive components in *Panax Ginseng* [26], *Angelica sinensis* and *Scutellaria barbata* [27]. Comparing to other techniques, MALDI-MS can allow loading hundreds of samples in one sample target and analysis of these samples within a very short time, and is very suitable for high-throughtput screening.

The current study for the first time introduces rapid and simple MALDI-MS-based approaches for differentiation between *S. sphenanthera* and *S. chinensis*. The samples were analyzed by MALDI-MS either after simple extraction of their powders or directly in their powder form. The results revealed that these two species could be differentiated based on the MALDI-MS spectra acquired.

2. Experimental

2.1 Materials

Dried *S. sphenanthera* and *S. chinensis* used in the study were purchased from authentic stores in Hong Kong and mainland China, and confirmed by experts in authentication of herbal medicines. Their identities are listed in Table S1. MALDI matrices α-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA), and 2,5-dihydroxylbenzoic acid (DHB) were purchased from Fluka (Pittsburgh, PA). HPLC grade solvents acetonitrile (ACN), methanol, ethanol, chloroform, and acetone were purchased from Tedia (Fairfield, OH). Trifluoroacetic acid (TFA) was purchased from International Laboratory U. S. A. (San Bruno, CA). Schisantherin A, schisandrin and schisandrol B standards were purchased from Tauto Biotech (Shanghai).

2.2 Sample preparations

2.2.1 Extraction method

Herb samples were ground into fine powder by a mortar grinder. A portion of 5 mg of the fine powder was weighed into a 1.5 mL eppendorf centrifuge tube, and 500 μL of the extraction solvent was added. The tube was put in an ultrasonic water bath and sonicated for 3 min. The extraction solution was then centrifuged at 13,000 rpm for 30 s and the resulting supernatant was used for analysis.

2.2.2 Powder method

A small piece of double-sided tape was first attached onto a spot of the target plate. Approximately 0.1 mg of herb powder was then transferred onto the tape surface with a spatula and pressed onto the tape surface until the herb powder was firmly adhered. Subsequently, 1

μL of CHCA matrix solution was spotted onto the top of the adhered herb powder, and then air-dried for analysis.

2.3 MALDI-MS

An aliquot of 10 μL of standard solution or extraction supernatant was mixed with 10 μL of 10 mg/mL CHCA matrix in 50/50 (v/v) ACN/H_2O with 0.1% TFA. An aliquot of 1 μL of the sample-matrix mixture was spotted onto the stainless steel target plate and air-dried. The target plate was then mounted onto a MALDI Micro-MX Time-of-Flight mass spectrometer (Waters, Milford, MA) for analysis. The laser of the MALDI source was a 337 nm pulse laser (Model 337Si-63, Spectra Physics, Mountain View, CA) operating at a pulse frequency of 10 Hz. The mass spectrometer was operated in positive and reflectron mode. The flight tube and reflectron voltage of the TOF mass analyzer were set at +12000 and –5200 V respectively. The extraction delay (Time Lag Focusing (TLF)) was set at 500 ns. For data acquisition, i.e., *m/z* 100 – 600, the "low-mass bias" mode was used for more sensitive detection of low mass ions. The laser energy and acceleration voltage were set at 220 A.U and 1.7 kV respectively. The mass peaks of the CHCA matrix were used for mass calibration in this mass range. Mass spectra were obtained by accumulation of 200 scans (10 laser shots per scan).

2.4 MALDI-MS/MS

MS/MS spectra were acquired by an Autoflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany) equipped with a Nd:YAG smart beam laser. Spectra were acquired from an average of 2000 laser shots. In the MALDI-MS reflector mode, ions generated by a pulsed UV laser beam were accelerated to a kinetic energy of 23.5 kV. A mass window of 0.5% was used for peak alignment. Peaks of interest were further analyzed on a separate platform using the LIFT function of the instrument. In the MALDI-MS/MS mode,

precursor ions were accelerated to 8 kV and selected in a time–ion-gate. The fragments were further accelerated by 19 kV in the LIFT cell and their masses were analyzed after the ion reflector passage. The mass spectrometer was calibrated using the mass peaks of the CHCA matrix and peptide standards.

2.5 Principal component analysis

Principal component analysis (PCA) was carried out using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). Normalized intensities (absolute intensity of the peak observed/total absolute intensity of all peaks observed in the mass spectrum) of those peaks with signal intensities higher than 5% were used for the analysis. The first (PC1) and the second principal components (PC2) were chosen to obtain a score plot.

3. Results and discussion

3.1 Optimization of experimental conditions

Different solvents, sample scale and extraction duration were examined in this study for optimal extraction efficiency. Moreover, the various matrices and sample preparation methods were compared as well.

3.1.1 Extraction solvent

Various solvents, namely 50% ACN, 50% MeOH, 100% H_2O, 100% ACN, 100% MeOH, 100% acetone and 100% chloroform, with the addition of 0.1% TFA were attempted as extraction solvents for the herb powders. There were no significant signals observed in the extraction with acetone and chloroform apart from the matrix peaks (Fig. S1). Spectra of similar patterns and with sample ions of significant intensities were observed for the extraction

with the other solvents. 50% ACN with 0.1% TFA was finally chosen for extraction since it is a common solvent system for the MALDI matrix.

3.1.2 Sonication duration and sample scale

The extraction efficiency of different sonication duration (0, 1, 3, 10, 20, 40 and 60 min) was examined as well. Sample peaks were significantly observed with 3 min sonication and there were no further improved with the extension of sonication (Fig. S2). Thus, 3 min sonication was chosen for the extraction. Sample scale was enlarged to test its effect on the extraction and the spectrum. 1 g of the herb powders was extracted with 5 mL of the extraction solvent, and the spectra obtained were similar to those obtained by extracting 5 mg of the herb powders with 500 μL extraction solvent.

3.1.3 Matrix selection

DHB, CHCA, SA and THAP, four commonly used MALDI matrices, were tested. CHCA was chosen since it produced more abundant sample peaks with very low-level matrix interferences. Most sample peaks observed with CHCA could also be detected when using SA, but the matrix interferences of SA were more significant. For DHB and THAP, fewer sample peaks and strong matrix peaks were observed (Data not shown).

According to the optimization, the final sample extraction conditions were as follows. 5 mg of powders of *S. sphenanthera* and *S. chinensis* were subjected to ultrasonic extraction for 3 min using 500 μL of ACN/H_2O (50/50, v/v) with 0.1% TFA as the extraction solvent. The extraction solution was then centrifuged at 13000 rpm for 30 s and the resulting supernatant was used for analysis.

3.1.4 Repeatability

Spectral repeatability of *Fructus Schisandrae* samples was examined in this study. First, the repeatability of the entire experimental methodology, including solvent extraction and MALDI-MS analysis, was tested by performing three independent experiments on the same herb sample. As showed in Fig. S3a, the mass spectra obtained in different experimental runs were highly similar. Second, herb samples of the same species but from different harvesting location were also analyzed. As showed in Fig. S3b, the mass spectra obtained for three samples from Liangning (Fushan), Jilin (Jian) and Heilongjiang (Yichun) had high similarity to each another, although the relative peaks intensities were slightly different. These data demonstrated a high degree of "inter-run' and 'inter-sample" repeatability of the present MALDI-MS fingerprinting method.

3.2 Differentiation between *S. sphenanthera* and *S. chinensis*

3.2.1 MALDI-MS spectra of *S. sphenanthera* and *S. chinensis*

The extracts were analyzed by MALDI-MS with CHCA as the matrix. No significant peaks were observed beyond 600 Da. Common peaks at *m/z* 136, 138, 184, 266, 399, 415, 416 and 520 were observed for both *S. sphenanthera* and *S. chinensis* and may act as the fingerprint of *Fructus Schisandrae* species (Fig. 1 & Table S2). Major bioactive components of *Fructus Schisandrae* including lignans, schisantherin A, schisandrol B, schisandrin B, schisandrin and deoxyschisandrin, which had been previously investigated by LC-ESI-MS [28]. Some peaks in the acquired MALDI-MS spectra could be assigned to lignans (Table 1) according to their masses and by comparison with the MALDI-MS spectra of the standards (Fig. S4). Peaks at *m/z* 432 and 575, confirmed to be schisandrin and schisantherin A by comparing with the MS/MS results with the standards (Figs. S5 & S6), were observed only for *S. chinensis and S. sphenanthera*, respectively (Fig. 1). These results were consistent with the literature [11] that

reported schisandrin as a marker of *S. chinensis* and schisantherin A as a marker of *S. sphenanthera*, indicating that the current approach could be effective for rapid identification of *S. sphenanthera* and *S. chinensis* based on the detection of these specific peaks. Other specific peaks observed included *m/z* 455, 534 and 553 for *S. sphenanthera* and *m/z* 287, 330, 346 and 368 for *S. chinensis*, respectively. These peaks could be used as fingerprints for identification of the two species.

3.2.2 Intensity ratios of peaks at *m/z* 416 and *m/z* 415 (I_{416}/I_{415})

Peak at *m/z* 415 in the spectra might arise from schisantherin A, schisandrin, schisantherin B or schisantherin *C* since all these compounds could give the fragment ion *m/z* 415. Similarly, peak at *m/z* 416 might be contributed by schisandrol B or deoxyschizandrin (Table 1). For *S. sphenanthera*, the intensity of *m/z* 416 was significantly higher than that of *m/z* 415, while for *S. chinensis*, the opposite result was obtained (Fig. 2a). This phenomenon was observed for all investigated *Fructus Schisandrae* samples except for N6, for which these two peaks were absent. I_{416}/I_{415}, the intensity ratio of these two peaks, was determined to be 2.19 ± 0.05 (n=27) for *S. sphenanthera*, and 0.39 ± 0.01(n=27) for *S. chinensis* with standard errors less than 2%, showing a significant difference between the two species. The logarithm of the I_{416}/I_{415} ratio clearly indicated positive and negative values for *S. sphenanthera* and *S. chinensis*, respectively, allowing easy and unambiguous differentiation between the two species (Fig. 2b).

3.2.3 Intensity ratios of peaks at *m/z* 138 and *m/z* 136 (I_{138}/I_{136})

Peaks of *m/z* 138 and 136 were another pair of peaks that showed a significant difference in the spectra for *S. sphenanthera* and *S. chinensis*. For *S. sphenanthera*, the peak at *m/z* 136 had a much higher intensity than *m/z* 138, while the opposite was observed for *S. chinensis* (Fig. 3a). This observation was highly reproducible in our study. With ten samples of each herbal

species from different sources and three independent experiments performed on each sample, the I_{138}/I_{136} ratios determined for *S. sphenanthera* and *S. chinensis* were 0.38 ± 0.02 (n=30) and 2.07 ± 0.01 (n=30) respectively. These data demonstrated that the I_{138}/I_{136} ratio could be a useful parameter for the differentiation between these two *Fructus Schisandrae* species. The logarithm of the I_{138}/I_{136} ratio gave negative and positive values for *S. sphenanthera* and *S. chinensis*, respectively, allowing easy and unambiguous differentiation between these two species (Fig. 3b).

3.2.4 Principal component analysis (PCA) results

PCA was employed for the differentiation between *S. sphenanthera* and *S. chinensis* in this study. Data of obtained from three independent measurements of each sample were averaged and input for PCA analysis. It could be found in the score plot (Fig. 4) that the samples of two species, *S. sphenanthera* and *S. chinensis*, closely clustered together respectively and located in different quadrants. The two-component PCA model cumulatively accounted for 76% of the variation (PC1 = 60% and PC2 = 16%). One of the *S. sphenanthera* samples, N6, was out of the circle and was found to have been subjected to different processing with the major compounds lost. This in turn demonstrated the reliability of the method for distinguishing the species.

3.3 Direct analysis of *Fructus Schisandrae* by the powder method

The patterns of the spectra for the two species obtained by the powder method (Fig. S7) were very similar to those obtained by the extraction method. Specific peak at *m/z* 575, corresponding to marker schisantherin A, was also observed only for *S. sphenanthera*, and *m/z* 432, corresponding to marker schisandrin, was detected only for *S. chinensis*. The I_{416}/I_{415} ratio obtained by this method for ten samples was 4.79 $\pm$ 0.72 for *S. sphenanthera* and 0.40 $\pm$ 0.02

for *S. chinensis.* The I_{138}/I_{136} ratio obtained by the powder method for the ten samples was 0.30 $\pm$ 0.20 for *S. sphenanthera* and 3.52 $\pm$ 1.00 for *S. chinensis.* Same as the extraction method, logarithm of the I_{416}/I_{415} ratio gave positive and negative values for *S. sphenanthera* and *S. chinensis*, respectively, and logarithm of the I_{138}/I_{136} ratio gave negative and positive values for *S. sphenanthera* and *S. chinensis*, respectively, allowing easy and unambiguous differentiation of these two herbal species. PCA was also applied to analyze the spectra obtained by the powder method. As shown in Fig. S8, the PCA results indicated that *S. sphenanthera* and *S. chinensis* could be differentiated based on the acquired MALDI-MS spectra. The two-component PCA model accounted for 67% of total variance of data obtained by the powder method (PC1 = 51% and PC2 = 16%). The PCA differentiation of the two species by the powder method was not as good as that by the extraction method, probably due to the poorer quality of the spectra obtained by the powder method. The *Fructus Schisandrae* samples investigated were wet and sticky. It was difficult to grind them into very fine powders to enable efficient extraction in a very short time and without sonication. This method can still be an alternate for preliminary screening.

4. Conclusions

MALDI-MS was successfully applied to analyze *S. sphenanthera* and *S. chinensis*. A brief extraction of a small amount of samples was sufficient to provide qualified spectra. *S. sphenanthera* and *S. chinensis* could be differentiated from each other based on the specific peaks (e.g., *m/z* 432 for *S. chinensis and m/z* 575 for *S. sphenanthera*), intensity ratios of characteristic peaks (e.g., *m/z* 416 vs. *m/z* 415 and *m/z* 138 vs. *m/z* 136), and PCA analysis of the spectra. Direct analysis of herbal powders by MALDI-MS was also attempted and the results demonstrated that *S. sphenanthera* and *S. chinensis* could be differentiated by this simpler and faster approach.

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Table 1. Summary of the peaks observed in the MALDI-MS spectra of the extracts from the *Fructus Schisandrae* samples[a].

Fructus Schisandrae	Peaks observed (*m/z*)	Proposed form
S. sphenanthera	399	[Schisandrol B+H-H_2O]$^+$
		[Schisantherin D+H-C_6H_5COOH]$^+$
	415	[Schisandrin +H-H_2O]$^+$
		[Schisantherin A+H-C_6H_5COOH]$^+$
		[Schisantherin B+H-C_4H_7COOH]$^+$
		[Schisantherin C+H-C_4H_7COOH]$^+$
	416	[Schisandrol B]$^{+\bullet}$
		[Deoxyschizandrin]$^{+\bullet}$
	455	Unknown
	520	[Schisantherin D]$^{+\bullet}$
	553	[Angeloylgomisin Q+Na]$^+$
		[Tigloylgomisin Q +Na]$^+$
	575	[Schisantherin A+K]$^+$
S.chinensis	399	[Schisantherin A+H-H_2O]$^+$
		[Schisantherin D+H-C_6H_5COOH]$^+$
	415	[Schisandrin+H-H_2O]$^+$
		[Schisantherin A+H-C_6H_5COOH]$^+$
		[Schisantherin B+H-C_4H_7COOH]$^+$
		[Schisantherin C+H-C_4H_7COOH]$^+$
	416	[Schisandrol B]$^{+\bullet}$
		[Deoxyschizandrin]$^{+\bullet}$
	432	[Schisandrin]$^{+\bullet}$
	520	[Schisantherin D]$^{+\bullet}$

[a] Only non-matrix peaks with relative abundances higher than 5% in the mass range of 110-600 Da were included.

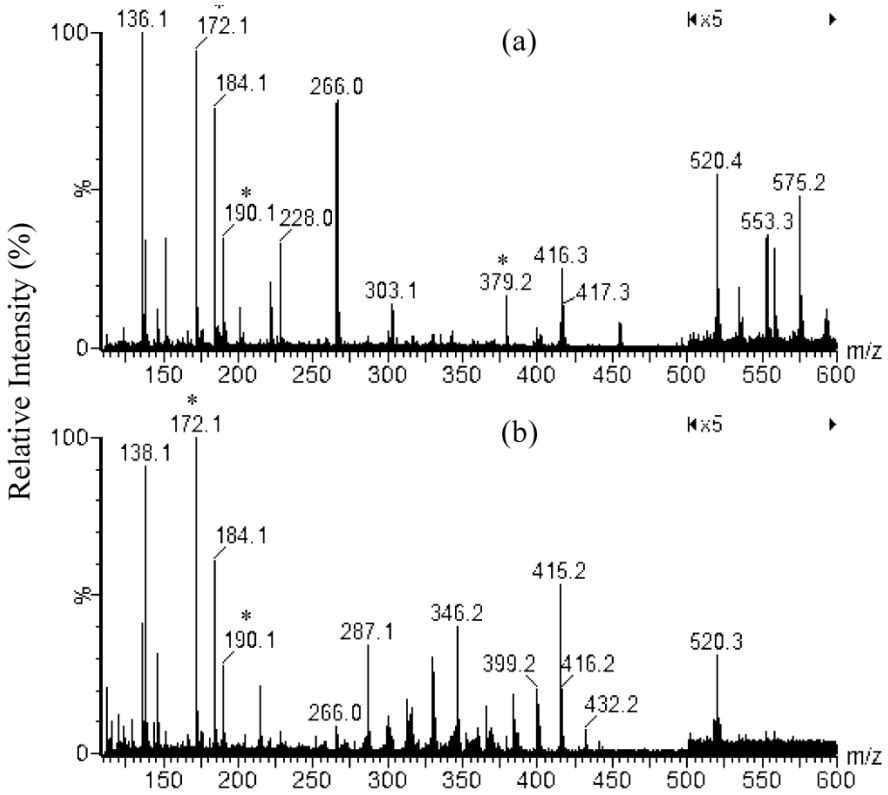

Figure 1. MALDI-MS spectra for extracts of *S. sphenanthera* (a) and *S. chinensis* (b) (peaks from the matrix are labelled with "*").

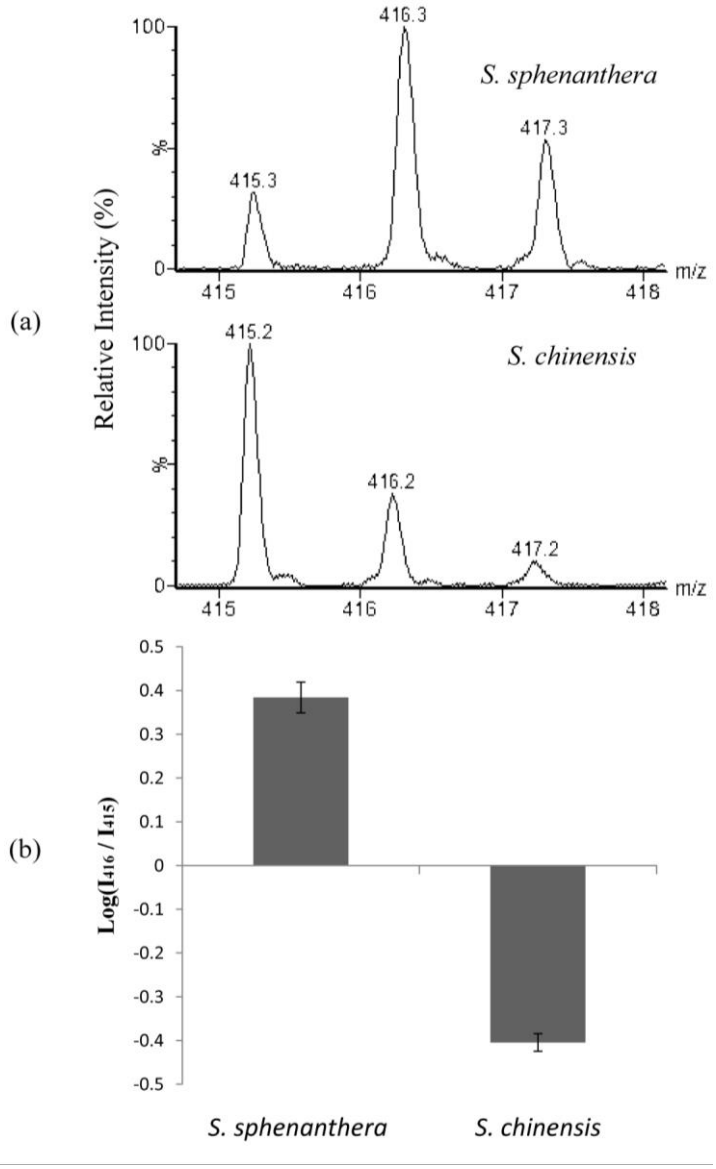

Figure 2. (a) MALDI-MS spectra for peaks at m/z 415 and m/z 416 for *S. sphenanthera* and *S. chinensis*. (b) Plot for logarithm of I_{416}/I_{415} against the *Fructus Schisandrae* sample.

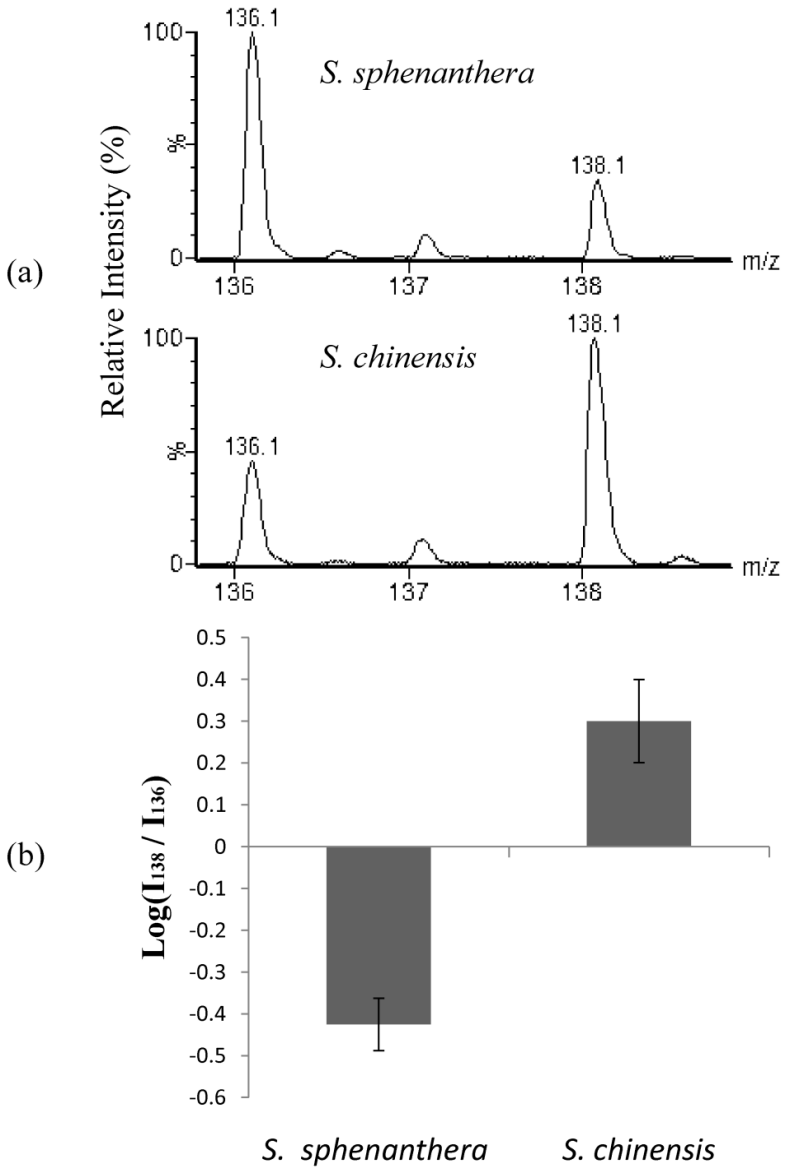

Figure 3. (a) MALDI-MS spectra at mass range of 136 -138 Da for *S. sphenanthera* and *S. chinensis*. (b) Plot for logarithm of I_{138}/I_{136} against the *Fructus Schisandrae* sample.

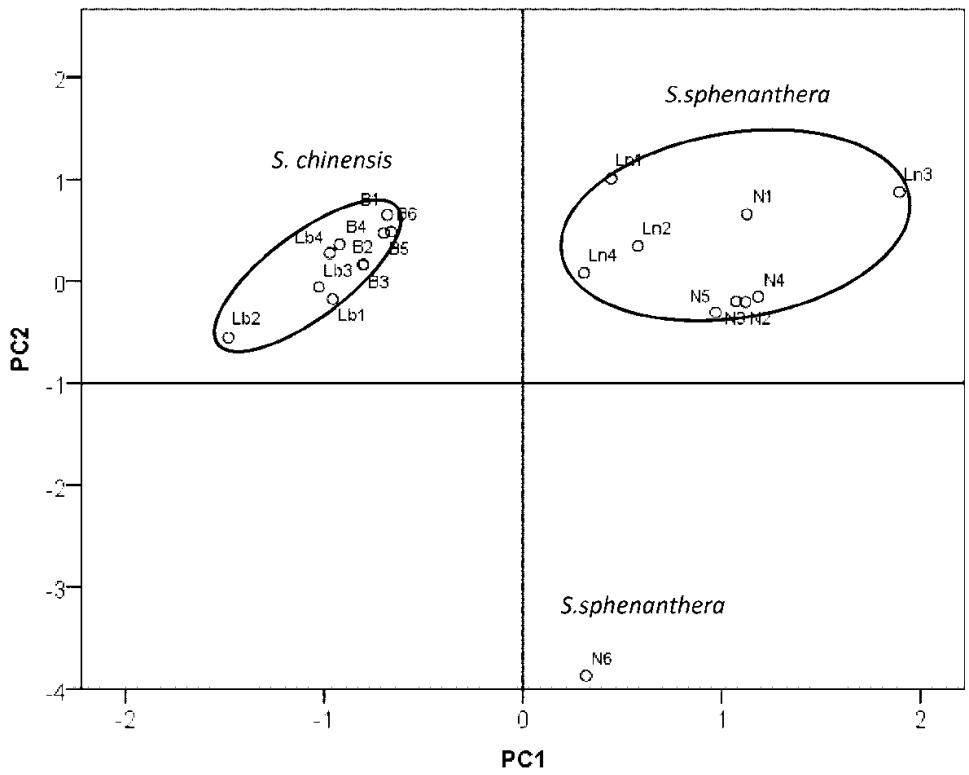

Figure 4. PCA score plot for *Fructus Schisandrae* samples with principal components 1 and 2.