RAPID DIFFERENTIATION OF HERBAL MEDICINES BY MATRIX-ASSISTED LASER DESORPTION/IONIZATION MASS SPECTROMETRY

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Rapid Differentiation of Herbal Medicines by Matrix-assisted Laser Desorption/Ionization Mass Spectrometry

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CERTIFICATE OF ORIGINALITY

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Abstract

A matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)-based approach has been developed and demonstrated for rapid differentiation of herbal medicines in this study. This approach typically involved a brief extraction to a small amount of herbal powder followed by MALDI-MS analysis. The MALDI-MS spectra obtained were characteristic of the herbal species and thus could act as the fingerprints of the species. This approach was used to differentiate between *Panax ginseng* (Asian ginseng) and *Panax quinquefolius* (American ginseng), between *Schisandrae sphenanthera* (southern magnoliavine fruit, Nan-Wuweizi) and *Schisandrae chinensis* (northern magnoliavine fruit, Bei-Wuweizi), and among *Angelica sinensis* (Chinese Danggui)*, Angelica acutiloba* (Japanese Danggui)*, Angelica gigas* (Korean Danggui) *and Angelica archangelica L. (Europe Danggui).* The results revealed that unambiguous differentiation could be achieved in each case, based on the mass patterns or principal component analysis of their respective MALDI spectra. Direct analysis of herbal powder or small pieces of raw samples by MALDI-MS, which avoids the extraction and/or homogenization procedures, was attempted and the acquired spectra also allowed differentiation of the herbal medicines. The approach developed in this study is simple, rapid, robust, and can be used for analysis of other herbal medicines.

Research Publications

Conference Papers

1. Lai, Y.H., So, P.K. and Yao, Z.P. Rapid Differentiation of Herbal Medicines by Matrix-assisted Laser Desorption/Ionization Mass Spectrometry, *HKSMS Mass Spectrometry Symposium 2012*, Tsim Sha Tsui, Hong Kong, June 2012.

2. Lai, Y.H., So, P.K. and Yao, Z.P. Rapid differentiation of *Schisandra sphenanthera* and *Schisandra chinensis* by MALDI-MS, *Proceedings of the 59th ASMS Conference on Mass Spectrometry and Allied Topics*, Denver, U.S.A, June 5 - 9, 2011.

3. Lai, Y.H., So, P.K. and Yao, Z.P. Rapid Differentiation of Herbal Medicines by MALDI-MS, *The Eighteenth Symposium on Chemistry Postgraduate Research in Hong Kong*, City University of Hong Kong, Hong Kong, May 2011.

Journal Publications

4. Lai, Y.H., So, P.K., Lo, S.C.L., Ng, W.Y., Poon, T.C.W. and Yao, Z.P. Rapid Differentiation of *Panax ginseng* and *Panax quinquefolius* by Matrix-assisted Laser Desorption/Ionization Mass Spectrometry, *Anal. Chim. Acta* **2012,** *753,* 73-81.

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List of Abbreviations

Full Form	Abbreviation
Acetonitrile	ACN
Angelica acutiloba	*A. acutiloba*
Angelica archangelica L.	*A. archangelica L.*
Angelica gigas	*A. gigas*
Angelica sinensis	*A.sinensis*
Automated peak alignment by beam search	Auto-PABS
Capillary electrophoresis	CE
Capillary zone electrophoresis	CZE
Capillary gel electrophoresis	CGE
Chemiluminescence detection	CL
Diode array detector	DAD
Electron ionization	EI
Electrospray ionization	ESI
European Medicines Agency	EMA
Evaporate light scattering detection	ELSD
Fingerprint analysis	FA
Flame ionization detector	FID
Fourier transform infrared spectroscopy	FTIR
Gas chromatography	GC

Herbal medicine	HM
High performance liquid chromatography	HPLC
High performance thin layer chromatography	HPTLC
Infra-red	IR
Ion-exchange chromatography	IEC
Mass spectrometer/ Mass spectrometry	MS
Mass to charge ratio	m/z
Matrix assisted laser desorption/ionization	MALDI
Micellar electrokinetic capillary chromatography	MEKC
Methanol	MeOH
Near IR	NIR
Nuclear magnetic resonance	NMR
Oil-assisted sample preparation	OASP
Pericarpium Citri Reticulatae	PCR
Pericarpium Citri Reticulatae Viride	PCRV
Principal component analysis	PCA
Quandrupole-ion-trap	QIT
Quadrupole time-of-flight	Q-TOF
Schisandrae chinensis	*S.chinensis*
Schisandrae sphenanthera	*S. sphenanthera*
Sinapinic acid	SA

Shuang-Huang-Lian	SHL
Surface desorption atmospheric pressure chemical ionization	DAPCI
Tandem mass spectrometry	MS/MS
Thermal conductivity detector	TCD
Thin layer chromatography	TLC
Time-of-flight	TOF
Traditional Chinese medicine	TCM
Transmission electron microscopy	TEM
Trifluoroacetic acid	TFA
Ultra high performance liquid chromatography	UHPLC
Ultraviolent	UV
World Health Organization	WHO
a-cyano-4-hydroxycinnamic acid	CHCA
2, 5-dihydroxybenzoic acid	DHB
2, 4, 6-trihydroxylacetophenone	THAP

Chapter 1: Introduction

1.1 Introduction of herbal medicines (HMs)

Herbal medicines (HMs) are widely used across the world as medicines and as dietary supplement. Many Asian countries such as China, Korea and Japan have made use of traditional HMs to treat diseases for thousands of years. According to a report of the World Health Organizations (WHO), 80% of the populations in some Asian and African countries depend on traditional medicines as their primary health care, and 70-80% of the populations in a lots of the developed countries have used some form of alternative or complementary medicines.[1] Unlike western medicine, HM consists of hundreds of complex phytochemicals. It is well known that the therapeutic effects of HMs are through synergic actions of their mass constituents.[2] Even for the same species of HMs, their therapeutic effects can vary due to many factors, such as cultivation site, harvesting time and post harvesting processing. Due to their high chemical complexity and variability in therapeutic effects, quality assurance and species authentication of HMs are of crucial importance.

Although HMs have been used worldwide, they have not been officially accepted in most countries.[3] This is usually because the safety and efficacy data of traditional medicines are unable to meet the requirements of existing health care policies and accepted research methodologies. The therapeutic mechanisms of many HMs are still unclear. Analysis of active components in extracts of HMs is an important key to explore the secret of their effectiveness. The major compound types in HMs include alkaloids, flavonoids, saponins, anthraquinones, terrpenoids, courmarins, lignans,

polysaccharides, polypeptides and proteins. Effective detection and rapid characterization of these components on a molecular basis play an important role in understanding the pharmacological basis of HMs.

1.2 Quality control of HMs

Mok and Chau categorized the approaches for authentication and quality control of HMs into the "component-based" and "pattern-based" approaches.[4] Both of these two approaches involve the use of various analytical methods among which chromatography is the most commonly used since it is very powerful for separation of complicated components of HMs. Thin layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) can be hyphenated to other instruments such as mass spectrometer (MS) and diode array detector (DAD) to provide additional spectral information. These hyphenated techniques are known as HPLC-DAD, HPLC-MS, GC-MS and CE-DAD. Hyphenated techniques allow for reduced instrumental interferences and retention time shift, and improved selectivity, chromatographic separation and precision in measurement.[4-6]

1.2.1 Single marker approach

Constituents (or groups of constituents) of HMs that can be used for authentication purposes are defined as chemical markers by the European Medicines Agency (EMA).[7] An ideal chemical marker should possess therapeutic effects of the concerned HMs.

However, this may not be the case because in most cases, we are not sure which components correspond with which therapeutic effects; and usually only a small number of chemical components are responsible for pharmacological actions of an HM.[7] One approach for the quality control of HMs is to use one of the active or standard ingredients as marker to assess the pharmacological quality of the HMs.[8] According to the Chinese Pharmacopoeia (2005), 282 chemical markers are used for the quality control of various traditional Chinese Medicines.[9]

This single marker approach was commonly used, but it has its drawbacks as some markers are not specific for one HM. For example, ferulic acid is present in many HMs, e.g., *Rhizoma Cimicifugae* and *Angelicae Sinensis*.[8] When the single marker method is used for the authentication of these herbs, it is difficult to get the correct identity of the examined herb. In addition, single component fails to account for the synergic actions of multiple components in HMs. Another component-based approach, the multi-component approach, is thus more desirable for the purpose of quality control.[2]

1.2.2 Multi-component approach

The multi-component approach is an extension of the single marker approach. The Chinese Pharmacopoeia (2005) suggests the use of multiple compounds instead of one single compound for the quality control of HMs. For example, ginsenosides Rg1, Re and Rb1 are used together to differentiate *Panax ginseng*, *Radix notoginseng* and *Panax quinquefolium L*. based on their different compositions in these three compounds.[9] Peng

et al.[10] used LC-DAD for the quality control of *Angelicae sinensis* from different regions of China by qualifying nine major components in the samples.[10] Chemometrics has also been employed in this approach for quality control. Song et al.[11] combined principal component analysis (PCA) with ultra high performance liquid chromatography-ultraviolet-quadrupole-time of flight (UHPLC-UV-Q-TOF-MS) for quality control of *Radix Salviae Miltiorrhizae.*[11] This method was found effective in the differentiation of raw materials, in-house prepared aqueous extracts of *Radix Salviae Miltiorrhizae* and the commercial products of the extract granules of *Radix Salviae Miltiorrhizae*.[11]

1.2.3 Pattern approach

The pattern-based approach can be used as an alternative method of the compound-oriented approach. It is also known as all-information based approach that uses the chromatogram or spectrum as a pattern or an image to characterize samples.[12] Fingerprint analysis (FA) is a good example of the pattern-based approach. It is employed to determine the authenticity and quality of complex HMs, and has been accepted by WHO for assessment of HMs.[3] Different HMs have different chemical fingerprints. Even for the same species of HMs, if collected from different sources or processed in different ways, they can have different fingerprints and thus can be distinguished. Among the various experimental techniques, chromatographic methods have been highly recommended for determining the fingerprints of HMs. [3, 13, 14] Chromatograms obtained by TLC, GC, HPLC and CE have been employed as fingerprints of HMs.

Cai et al. [15] found that the chromatographic profile of a herbal medicine depends not only on the preparation process, but also on the quality of the crude herb material, which varies with origin, harvest time and pretreatment process.[15] Xie[2] stated that chromatographic fingerprinting was not only an alternative analytical technique for identification, but also an approach for expression of various distribution patterns of chemical ingredients in HMs and preservation of such wholeness-target "database" for further multi-faceted studies.[2]

1.2.3.1 Thin layer chromatography

TLC is a simple, low cost and versatile method commonly used in the authentication of HMs according to various Pharmacopeia, such as Chinese Pharmacopeia,[9] American Herbal Pharmacopedia[16] and European Pharmacopedia.[17] TLC is widely used for fast screening of HMs and differentiation of closely related species.[18-21] It can analyze several samples simultaneously on a single plate. However, the use of TLC in quality control is limited by its poor resolution, low sensitivity and low accuracy. These can be improved by using high performance thin layer chromatography (HPTLC) with scanning and documentation software. [23]

1.2.3.2 Gas chromatography

Gas chromatography (GC) is capable of separating volatile compounds. Due to its high sensitivity, stability and efficiency, GC can be used to analyze volatile oils from HMs. With the use of capillary column, many volatile compounds can be separated in a short time. Common detectors used in GC include flame ionization detector (FID), thermal conductivity detector (TCD) and MS. When GC is combined with MS, reliable information for both qualitative and quantitative analysis of complex constituents can be obtained. The disadvantages of GC are its limitation on analysis of polar or non-volatile compounds. These compounds need to be derivatized before they can be analyzed by GC. Qiu et al. [22] used GC-time-of-flight (TOF)-MS and GC×GC-FID to study volatile oils in the rhizomes and radixes of *Notopterygium incisum Ting ex H.T. chang*, in order to differentiate the herbs that are grown in different geographic regions.[22] Qiu et al. [23] also studied volatile oils in *Radix ginseng* of different ages by using GC-TOF-MS.[23] Zuo et al. [24] analyzed anthraquinone in *Radix Polygoni multiflori* by using GC-MS and GC-FID after derivatization of anthraquinones.[24]

1.2.3.3 High performance liquid chromatography

HPLC is a popular method for the analysis of HMs because its analysis is not affected by the volatility or thermal stability of sample compounds. It can couple with various detectors for different applications, e.g., UV and DAD for UV absorbing compounds, evaporate light scattering detection (ELSD) and chemiluminescence detection (CL) for non-UV absorbing compounds, and mass spectrometer for identification of separated

compounds. With the use of DAD and MS, qualitative analysis of HMs becomes much easier due to the availability of UV spectra and MS spectra of individual peaks in chromatogram. The spectra can then be compared online with literature data and/or standard compounds, and analytes can thus be identified without isolation.

Li et al.[25] reported the use of HPLC-DAD for evaluating the quality of *Radix Polygalae* from different cultivation regions and harvest seasons in China, through simultaneous determination of nine phenols compounds.[25] They found that the samples cultivated in Shanxi had a higher concentration of phenols than those from other provinces. Within the same province, samples harvested in spring had a higher concentration of phenols than those harvested in other seasons. Peng et al.[10] used LC-DAD-MS to identify and quantify the major constituents of *Angelicae Sinensis* from different locations of China.[10] Qi et al.[26] used HPLC-DAD-ELSD and HPLC-electrospray ionization (ESI)-TOF-MS for the identification and quantification of major constituents in processed *Radix Astragali* products.[26] Apart from raw HMs, some commercial processed products of HMs can also be evaluated. Cao et al.[27] developed chromatographic fingerprints to assess the quality of Shuang-Huang-Lian (SHL) oral liquid manufactured in different pharmaceutical factories.[27]

1.2.3.4 Capillary electrophoresis (CE)

CE is a powerful analytical and separation technique due to its high resolution, minimum sample and solvent consumption, short analysis time and high separation efficiency.[28] Separation of analytes in CE is based on the charges and sizes of dissolved ions when they travel through an open tubular column under the influence of an electric field. Commonly used CE techniques include capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE) and micellar electrokinetic capillary chromatography (MEKC). Sun et al.[29] employed CE in the fingerprint analysis of *Flos Carthani* and found that the fingerprints of 10 batches of samples from Fengqiu had 29 "common peaks".[29] Fan et al.[30] created an integrated method to obtain MEKC fingerprints for the quality control of Sheung-Man-San oral liquid.[30]

1.2.4 Multi-pattern approach

Multi-pattern approaches have also been used for quality control of HMs. A combination of chromatographic fingerprints and biological activity profiles was employed[4] to discover bioactive components, evaluate therapeutic effects, correlate between chemical fingerprints and pharmacological indices, and assess the quality.[31-34] Wang et al.[31] studied the bioactive components and metabolites of Danggui Buxue decoction in rabbit plasma by the metabolite fingerprinting technique and LC-DAD-MS.[31] Wang et al.[32] also utilized abstract fingerprint and plasma fingerprint to find out that 25 chemical components in *Radix Astragali* had been absorbed by the rabbit body after the administration of oral solution of *Radix Astragali*.[32]

1.2.5 Chemometric methods for fingerprint analysis

The ingredients and the corresponding data of HMs can be very complicated. Data treatment including interpretation, prediction and extraction can be employed to simplify the analysis process. Chemometrics uses mathematical and statistical methods to retrieve more information from data and has been applied to compare data among herbs with hundreds or even thousands of chemical constituents.[12] The information obtained can be used to identify the origins of herbs, and to authenticate herbs. Yao et al.[35] used the method of automated peak alignment by beam search (Auto-PABS) to solve the problem of peak shift in chromatographic fingerprinting of HMs by piecewise shifting and linearly interpolating.[35] Yi et al. [36] used HPLC fingerprinting and multivariate statistical analysis for the quality control of *Pericarpium Citri Reticulatae* (PCR) and *Pericarpium Citri Reticulatae Viride* (PCRV).[36] They found that PCR and PCRV had similar properties because their chromatograms were similar in terms of correlation coefficient and absolute peak areas of characteristic compounds. [36]

1.2.6 MS-based approach

In addition to coupling with HPLC or GC for fingerprinting of HMs,[10, 24, 37-41] MS alone can also be used for the characterization of HMs. In this case, a mass spectrum is obtained for the HM sample, and the components or peaks of the sample are indicated by their masses (m/z values) in the spectrum. Mass is a more specific parameter when compared with other parameters that are involved in the fingerprinting of herbs, such as retention time in chromatography. Different mass spectrometric techniques have been

used for direct characterization of HMs without chromatographic separation. For example, Hung et al. [41] used matrix-assisted laser desorption/ionization mass spectrometry (MALDI–MS) to analyze the molecular weight and sequence of glucan in *Ganderma lucidum*.[41] Wang et al.[42] used ESI-MS combined with tandem MS to investigate protoberberine alkaloids in *Rhizoma Coptidis*.[42] Zhang et al.[43] developed surface desorption atmospheric pressure chemical ionization mass spectrometry (DAPCI-MS) for distinguishing Liuwei Dihuang Teapills of different sources without any sample pretreatment.[43] Among these techniques, MALDI-MS is the most commonly used one.

1.3 MALDI-MS for quality control of HMs

1.2.1 History of MALDI-MS

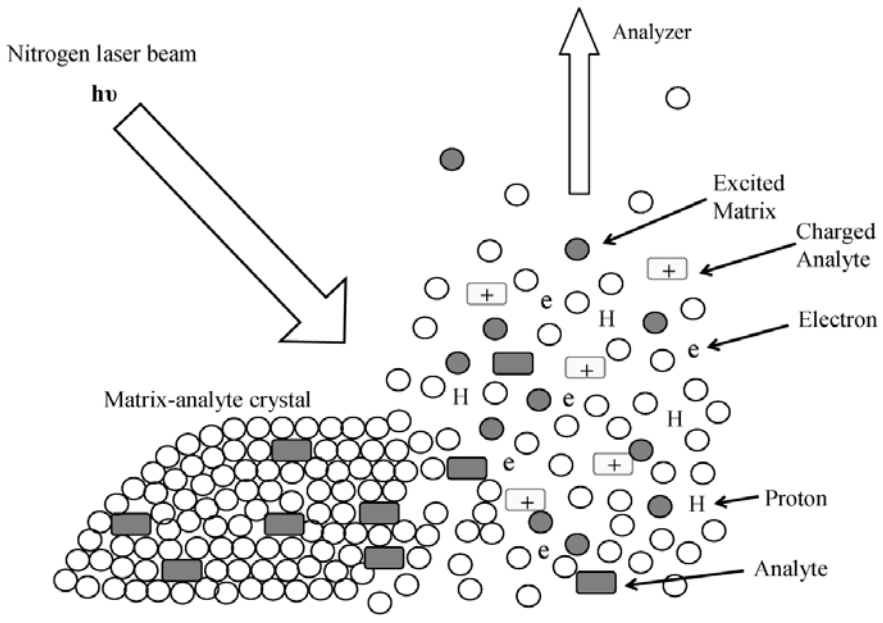
MS is a powerful analytical technique for analysis of various samples. A mass spectrometer includes three basic parts, ion source, mass analyzer and detector. Ion source is used to generate ions from the sample, and mass analyzer and detector are to separate and measure the m/z values of the ions and thus produce a mass spectrum. Before 1980, MS was usually applicable only for analysis of volatile and thermally stable compounds due to the lack of effective techniques to ionize and desorb non-volatile and thermally unstable analyte molecules.[44] Some ionization techniques, such as electron ionization (EI), require thermal evaporation of samples and are so energetic that can cause extensive fragmentation. In the late 1980s, two soft ionization techniques, namely ESI and MALDI were developed to allow ionization and desorption of large,

non-volatile and thermally labile compounds. Nowadays, MS has become a routine tool for analysis of biomolecules.[44, 45]

MALDI was first introduced by Karas and Hillenkamp in 1987.[46] They found that alanine could be ionized easier when mixed with tryptophan and irradiated with a pulsed laser of 266nm.[47] They extended their work to ionize larger, nonvolatile compounds with masses of up to 2843 Da.[47] Later, Koichi Tanaka et al.[48] reported the use of glycerol and metal powders for laser desorption/ionization of proteins and polymers with molecular weights of up to 25 kDa.[48] This success led to a Noble Prize in chemistry for Koichi Tanaka in 2002. Karas and Hillenkamp then further extended their method for analysis of biomolecules with masses larger than 10 kDa [49] and their method has become widely used since then.

1.3.2 Principle of MALDI

The MALDI method typically involves co-crystallization of analyte with a matrix, which is generally a low mass compound, such as α-cyano-4-hydroxycinnamic acid (CHCA) and 2, 5-dihydroxybenzoic acid (DHB), that can absorb the energy of laser usually at the 337 nm wavelength. Absorption of the laser energy causes the matrix to undergo a phase transition and assist desorption and ionization of the analyte. The ionized analyte is then analyzed by a mass analyzer, typically TOF. The mechanisms by which ions are formed in MALDI are still not fully understood.[45, 50] The most widely accepted model is that the analyte is ionized by proton transfer between matrix and analyte molecules in the solid phase before desorption or in the expanding plume.[51] Scheme 1.1 shows the MALDI process by which ions are formed.



Scheme 1.1 Schematic diagram of the MALDI ionization process.[46]

Time-of-flight (TOF) mass analyzer is commonly used in the analysis of large biomolecules, mainly due to its high sensitivity and wide m/z range for mass measurement. The pulsed operation mode of TOF also makes it suitable for coupling with MALDI. The TOF mass analyzer separates ions based on their differences in flight time when travelling through the flight tube. Before entering the flight tube, an ion with mass ***m*** and charge ***q*** (***q*** = ze) is accelerated by an electric field (V), its kinetic energy (E) and velocity v can be expressed by the following equation:

$$E = \frac{mv^2}{2} = qV = zeV \qquad [1.1]$$

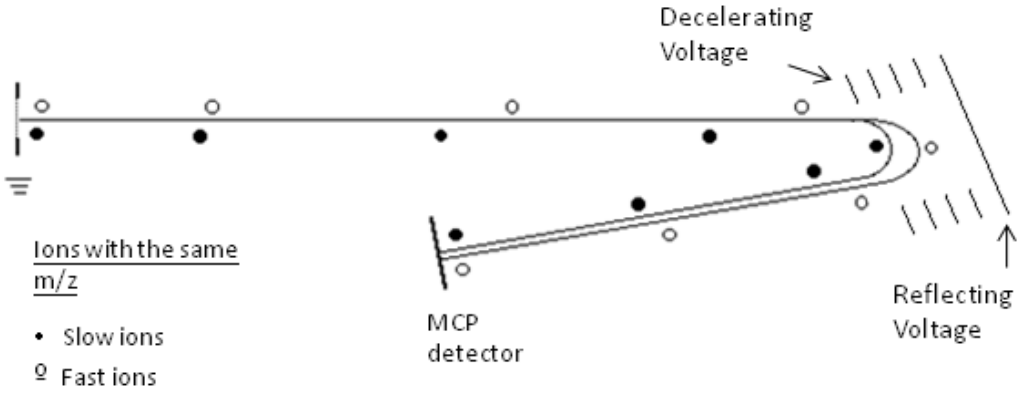
The time t needed for the ion to travel the flight path with length d is given by t = d/v. Thus, Eqn [1.1] becomes:

$$t^2 = \left(\frac{m}{z}\right)\left(\frac{d^2}{2Ve}\right) \qquad [1.2]$$

Since d and V are fixed, *m/z* can be calculated from a measurement of t. That is, the lower the m/z of an ion, the faster it reaches the detector, and vice versa.

Ions with the same m/z can have different kinetic energy, causing poor resolution in TOF mass measurement. Reflection TOF has thus been used to reduce such kinetic energy dispersion and increase the resolution. Reflection TOF uses a retarding electric field that acts as a mirror by deflecting the ions and sending them back through the flight tube (Scheme 1.2). The reflectron makes corrections on the energy dispersion of the ions with the same m/z value by allowing ions with more kinetic energy to penetrate

farther into the mirror and thus spend more time in it. Ions with less energy will travel shorter distance and spend less time. As a result, ions with different kinetic energy reach the detector at the same time.



Scheme 1.2 Schematic diagram showing the ion pathways in the reflectron-TOF analyzer.

1.3.3 MALDI matrix

Aromatic acids with a chromophore that strongly absorbs laser wavelength are the typical matrices used in MALDI. Table 1 is a list of the commonly used matrices for different substrates. CHCA, sinapinic acid (SA) and DHB are the most common matrices.

However, the use of some matrices causes a number of problems in MALDI-TOF MS analysis. Firstly, the analyte must be miscible and co-crystallized with the matrix in the solution-based method. The selection of proper matrix is a matter of trial and error. Secondly, the co-crystallization process does not produce any uniform mixture, and

good signals are only available from some spots ("sweet" spots). Thirdly, severe matrix interference in the low mass regions (< m/z 700), especially the adduct peaks from CHCA, makes the analysis of low mass compounds difficult. There are some strategies to solve the problems in the analysis of low mass compounds. These include the use of porous silicon,[52-55] nanostructured materials such as silicon nano-particles,[56] carbon nano-tubes,[57] and gold nano-particles,[58] high molecular weight matrices[59] and surfactant,[60] and derivatization of analytes. [61]

Table 1.1 Matrices and their typical substrates (taken from the Micromass MALDI micro MX Mass Spectrometer Operation Manual)

Matrix	Full name	Typical substrate
CHCA	α-cyano-4-hydroxy cinnamic acid	Peptides, polymers, intact bacteria
Sinapinic acid	3,5-dimethoxy-4-hydroxycinnamic acid	Proteins, peptides, polymers
DHB	2,5-dihydroxybenzoic acid	Sugars, peptides, nucleotides, polymers
CMBT	5-chloro-2-mercaptobenzothiazole	Proteins, peptides, intact bacteria
Dithranol	1,8-Dihydroxy-9(10H)-anthracenone	Synthetic polymers
THAP	2,4,6-trihydroxyacetophenone	Oligonucleotides
HABA	2-(4-hydroxyphenylazo)benzoic acid	Glycolipids, peptides, proteins
HPA	Hydroxypicolinic acid	Oligonucleotides, peptides, glycoproteins
IAA	β-indole acrylic acid	Polymethyl methacylates

1.3.4 Sample preparation methods for MALDI-MS

1.3.4.1 Solution-based MALDI

The most common protocol for MALDI sample preparation is called dried droplet method.[47, 62] The sample solution is mixed with a matrix solution, and a drop of the mixture is loaded onto a MALDI target plate and slowly dried in the ambient air or by a gentle stream of air. The major drawback of this method is the formation of inhomogeneous crystals, which leads to poor point-to-point signal reproducibility.[63]

To improve the resolution and mass accuracy of MALDI measurement, the fast evaporation method was introduced. In this method, matrix and sample solutions are loaded separately. The matrix solution is applied to the target plate first and the solvent is allowed to evaporate. One top of the matrix a drop of analyte solution is applied and allowed to dry.[64]

The double layer method[62] is also commonly used in the sample preparation of conventional MALDI. The matrix solution is first loaded onto the target plate to form a layer of very small and homogeneous crystals. A mixture of matrix and analyte solution was then loaded on the top of the crystal layer. Due to the increasing of the analyte-matrix ratio and improved isolation between the analyte molecules, this method provides higher sensitivity and excellent spot-to-spot reproducibility.[65]

1.3.4.2 Solvent-free method

Traditional solution-based techniques require good solubility of analytes in solvents and thus have limited applicability to insoluble compounds. Recently, an alternative preparation technique, called solvent-free sample preparation, has been developed.[66] No solvent is required in this method so the problems caused by solubility, miscibility, and segregation effects are avoided and the characterization of insoluble samples such as synthetic polymers becomes possible.[66-68] The sample preparation approaches of the solvent-free technique include grinding by mortar and pestle, ball-mill and vortexing. Analyte, matrix, and salt are added to a vessel for grinding, and the resulting powder mixture is then pressed on the MALDI target plate. In the case of the ball-mill or vortex methods, suitably sized balls are used to assist in the homogenization process.

1.2.4.3 Oil-assisted sample preparation (OASP)

Although solvent-free MALDI MS is successfully applied for analysis of insoluble compounds, it still has several drawbacks. Firstly, the sample preparation procedures are more complicated and time-consuming when compared to conventional MALDI MS analysis. Secondly, large amount of both analyte and matrix are required. Thirdly, contamination occurs during sample loading of the solid analyte-matrix mixture on the target plate. Fourthly, contamination of samples on the target plate and contamination to the ion source also occurs since no adhesive medium is present. Recently, the OASP method was developed for MALDI analysis of solid sample.[69] In this method, a drop of paraffin oil is used as the mixing and adhering media of solid sample and matrix. Using

this method, on-target sample preparation is allowed and the above problems can be avoided.

1.3.5 Characteristics of MALDI-MS

The development of MALDI made great contribution to the analysis of biomolecules such as peptides/proteins[62, 70-74], oligosaccharides[75] and synthetic polymers.[66, 76-80] MALDI-MS is a simple and rapid technique. It can directly analyze complex mixtures without the need of chromatographic separation.[45, 46] Compared with ESI, MALDI is more tolerant to salts and detergents. MALDI-MS spectra are predominant by singly charged ions instead of multiply charged ions as in ESI spectra.[45, 50] Because of these advantages, MALDI-MS profiling of proteolytic digests from a protein followed by database searching, i.e., peptide mass fingerprinting, has become a standard approach for protein identification in proteomics.[81] MALDI-MS is also an effective imaging technique for in situ analysis of proteins, peptides and metabolites from tissues or cell lines.[82-86]

1.3.6 HMs analysis by MALDI-MS

MALDI-MS not only can be applied for characterization of large biomolecules, but also can be used for analysis of small molecules, e.g., analysis of small molecules from HMs either following solution-based extraction[41, 87-94] or by direct analysis of sliced tissue of HMs.[95-97]

For the solution-based extraction method, homogenized HMs are extracted with solvents and the extracts, either directly or after some further purification, are mixed with matrix solutions and analyzed by MALDI-MS. Cai et al.[87, 98] used MALDI-quandrupole-ion-trap (QIT)-TOF MS to characterize phenolics such as hydrolysable tannins, flavonols and anthocyanins in *Rosa chinensis*, and identified some hydrolysable tannins that were not detected by LC-MS.[87] Chapagain et. al.[94] used MALDI-MS for determining the metabolites of saponins in *Balanites aegyptiaca*. Their results also revealed that compared to LC-MS, additional saponins could be detected by MALDI-MS.[94] Zhu et al.[93] developed a rapid method to identify the gallotannins components in the crude extract of Chinese galls by MALDI-QIT-TOF MS.[93] They suggested that their method could be applied for rapid evaluation and screening of hydrolysable tannins in medicinal plants.[93] Wang et al.[88] developed a high throughput and robust approach for qualitative profiling of alkaloids in *Aconitum carmichaeli Debx* by MALDI-MS.[88] The potential application of MALDI-MS in semi-quantitative measurement was confirmed in their study by comparison with results obtained from LC-MS.[88]

Chen et al.[89, 90] integrated ion-exchange chromatography (IEC) fractionation with reversed-phase LC-atmospheric pressure chemical ionization (APCI)-MS and MALDI-MS for isolation and identification of compounds in *Psoralea corylifolia* and Honeysuckle extract.[89, 90] More than 188 components in *P. corylifolia* extract and more than 117 components in Honeysuckle extract were detected with this integrated approach, and some of the components were preliminarily identified according to their UV spectra and MS spectra obtained by APCI-MS and MALDI-MS respectively.[89, 90]

Hung et al.[41] used MALDI-MS to determine molecular weights and sequences of glucans in *Ganoderma lucidum*.[41] Liu et al.[92] recently reported utilizing graphene or graphene oxide as MALDI matrix to identify the components in *Angelica sinensis* and *Scutellaria baicalenesis Georgi*.[92] Apart from analysis of single HM , analysis of formula medicines containing several HMs was also attempted by using MALDI-MS. Tao et al.[91] investigated the components from three herbs of Shengmaisan using Fourier transform infrared spectroscopy (FTIR), MALDI-MS and transmission electron microscopy (TEM).[91] They found that some components found in decoction from a single herb disappeared when different herbs were decocted together.[91]

Solution-based MALDI-MS analysis of HMs involves extraction using solvents, which can cause degradation or loss of active components, especially those components that are present at low levels in plant tissues.[95, 96] Direct analysis of HMs by MALDI-MS without solvent extraction is thus preferred. For direct MALDI-MS analysis, tissue that was sliced from raw HMs was placed on MALDI target plate and matrix solution was applied onto the tissue surface before it was introduced for MALDI-MS analysis. Wu et al.[95, 96] used this method to characterize alkaloids in *Aconitum Carmichaeli Debx* and in crude and processed *Strychnos nux-vomica* seeds.[95, 96] They found that this method could be valuable for discovery of new compounds and for quality control of medicinal herbs.[95, 96] Ng et al.[97] developed a MALDI-MS method for the spatial profiling and semi-quantitative measurement of alkaloids in *Sinomenium actum* from different regions.[97] They used PCA to analyze the characteristic metabolites in specific tissue regions, allowing unambiguous differentiation of samples from different geographic

locations.[97] Recently, Taira et al.[98] localized ginsenosides Rb1, Rb2 and Rf on *Panax ginseng* root by direct MALDI-MS analysis.[98]

Direct analysis of other plants, rather than HMs, by MALDI-MS has also been reported.[99-105]

MALDI-MS has become a more and more popular tool in analysis of HMs and plants. This can be explained by the 3S advantages of MS: speed, specificity and sensitivity.

1) Speed

   MALDI-MS spectrum can be obtained in less than 1 min for each sample, much faster than the chromatographic fingerprinting method. The chromatographic fingerprinting method requires separation of numerous chemical components in herbal extracts and thus takes a much longer time. Furthermore, re-equilibrating the analytical column is required between each sample run.[18, 90] On the other hand, high-throughput MALDI-MS allows analysis of multiple samples at the same time on one MALDI plate.

2) Specificity

   Mass in MALDI-MS spectra is obviously a more specific parameter than other parameters involved in fingerprinting of herbs, such as retention time in chromatography and various spectroscopic parameters involved in spectroscopy such as infra-red (IR) and near IR (NIR). Besides, retention time shift is always a

problem on peak specificity in the chromatographic fingerprinting approach, although chemometrics has been applied to correct the retention time shift in chromatographic analysis of HMs.[4] However, peak shift is usually not a significant problem in mass spectral profiling.[4]

3) Sensitivity

The sensitivity of MALDI-MS is usually comparatively much higher than that of chromatography. This high sensitivity allows the extraction to proceed in a fast and simple way, while typical chromatographic fingerprinting usually require harsher conditions (e.g. refluxing) and longer periods (e.g. 1h), and optimal solvent systems for extraction of herbs. [90]

1.4 Objectives of the present study

Chemical fingerprinting is recognized as an effective approach for quality control of HMs since it is compatible with the chemical complexity and holistic properties of HMs.[4, 106-109] In addition to chromatographic fingerprinting, alternative fingerprinting methods should be developed to obtain multi-dimension fingerprint data to increase the "coverage" of chemical information. In this study, MALDI-MS is applied for the quality control of HMs and MALDI profile of a HM is used as the fingerprint of the HM. The approach can thus be considered as a pattern approach. MALDI-MS is a fast and sensitive technique that can directly analyze complex mixtures without the need of chromatographic separation. As a result, HMs can be analyzed by MALDI-MS rapidly and efficiently, and a MALDI-MS-based database can be developed. Chemometrics will also be used for MALDI-MS fingerprinting in this study, and HMs including ginseng, *Fructus Schisandrae* (Chinese name: wuweizi) and *Radix Angelicae* (Chinese name: danggui) will be investigated.

1) *Panax ginseng* (Asian ginseng) and *Panax quinquefolius* (American ginseng) are two HMs commonly used for disease treatment and dietary supplement. They are closely related but have different pharmacological effects. The active ingredients of these two HMs are ginsenosides which are triterpene saponins. In this study, MALDI profile and chemometrics method will be used to differentiate these two species purchased from different traditional Chinese medicine (TCM) pharmacies. Details of the investigation will be discussed in Chapter 2.

2) *Fructus Schisandrae* (Wuweizi) can be classified into two types: *Schisandrae sphenanthera* (southern magnoliavine fruit, Nan-Wuweizi) and *Schisandrae chinensis (*northern magnoliavine fruit, Bei-Wuweizi). *S. sphenanthera* is mainly found in the southern provinces of China, such as Shanxi, while *S. chinensis* is distributed in the northern provinces of China such as Heilongjiang. These fruits mainly contain lignans and volatile oils. It is well known that the quality of *S. chinensis* is superior to that of *S. sphenanthera*. MALDI-MS spectra will be obtained for these two fruits and will be used to differentiate them. Chemometrics methods will also be used to differentiate fruits from different sources. Details of the studies will be discussed in Chapter 3.

3) *Angelica sinensis* (Chinese Danggui) is the roots of *Angelica,* which is one of the most common HMs in Hong Kong. This HM has several substitutes in the market, e.g., *Angelica acutiloba* (Japanese Danggui) and *Angelica gigas* (Korean Danggui). Ferulic acid and *Z-ligutilide* are the bioactive components of these HMs and are used as markers for quality assessment.[9] However, these two compounds are also present in other plants, so new authentication approach is desirable. In this project, *A. sinensis, A. acutiloba, A. gigas* and *Angelica archangelica L.* (European Danggui) will be analyzed and differentiated by their MALDI-MS spectra and the chemometrics method. Details will be provided in Chapter 4.

Chapter 2: Rapid Differentiation of *Panax ginseng* and *Panax quinquefolius* by Matrix-assisted Laser Desorption/Ionization Mass Spectrometry

2.1 Introduction

Panax ginseng (Asian ginseng) and *Panax quinquefolius* (American ginseng), two closely related herbal species both belonging to the *Panax* genus, are two of the most commonly used medicinal herbs, and their extracts have been widely used as dietary supplements.[110] *Panax ginseng* is "warm" in nature and known to replenish "heat" of the human body, whereas *Panax quinquefolius* is "cool" in nature and functions to remove excessive "heat" from the human body.[39, 111] Due to their significant differences in biological properties, differentiation of these two analogue herbal species is of paramount importance for proper medicinal formulation and food safety. The illegal mixing of *Panax ginseng* into *Panax quinquefolius* products has been reported, as the former is usually much cheaper than the latter.[111, 112] However, due to their similarity in morphology, and the fact that many of their commercial products are sold in the form of ground-powder, tablets or slices, differentiation between these two species based on traditional morphological inspection is difficult,[39, 111-114] and misidentification has been reported.[115] DNA analysis allows identification of herbal species, but is easily susceptible to DNA degradation and contamination during sample processing.[111, 116] Chemical analysis of these two herbs is also challenging because of the similarity in their chemical compositions.[112, 117] For these reasons, the developing of new methodologies to differentiate these two analogue herbal species has attracted considerable research interests. For instance, differentiation based on protein markers [118] and SNP markers from external transcribed spacer region of ribosomal DNA [111] has been reported.

Analysis of ginsenosides, triterpene saponins that are known to be the bioactive chemical components of *Panax* herbs, by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) is a common approach in the study and differentiation of *Panax* herbs.[110, 119] Mass spectrometry (MS) has been the method of choice for detecting ginsenosides in LC eluents because of its high sensitivity, specificity, and that sample derivatization is not required.[15, 39, 112, 114, 117, 120-131] In addition, the techniques of tandem mass spectrometry (MS/MS) and in-source dissociation allow fragmentation of sample ions, enabling differentiation of structural isomers based on the fragment ions generated.[15, 39, 117, 121, 124, 125, 127, 132-134]

In this study, we presented a MALDI-MS-based approach for differentiation between *Panax ginseng* and *Panax quinquefolius*. We demonstrated that *Panax ginseng* and *Panax quinquefolius* could be unambiguously differentiated by comparison of their MALDI-MS spectra thus obtained or by principal component analysis (PCA) of the spectra. The approach is simple, rapid and allows high throughput analysis of *Panax ginseng* and *Panax quinquefolius*.

2.2 Experimental

2.2.1 *Materials*

Samples of *Panax quinquefolius* from Canada, USA, and China, *Panax ginseng* from China and red ginseng from China and South Korea were purchased from local TCM pharmacies, and further confirmed by Ms. Dawn Tung Au. MALDI matrices α-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA), and 2,5-dihydroxylbenzoic acid (DHB) were purchased from Fluka. 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). Polyethylene glycol standards were purchased from Sigma. Sodium iodide was purchased from Panreac Química S.A (Barcelona, Spain). All chemicals were used without further purification.

2.2.2 Preparation of herb extracts.

Herbs were ground into fine powder with a motor grinder. A portion of 5 mg of ground herb powder was weighed into a 1.5 mL plastic eppendorf tube, and 500 μL of extraction solvent, 50/50 ACN/H_2O with 0.1% TFA, was added. The eppendorf tube with sample was then put in an ultrasound bath and sonicated for 2 min. All herb samples were sonicated together with the use of a vial stand. The extract solution was centrifuged at 13000 rpm for 30 s for sedimentation of herb residues.

2.2.3 MALDI-MS

An aliquot of 10 μL of extraction supernatant was mixed with 10 μL of 10 mg mL^{-1} CHCA matrix in 50/50 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 at high mass region, i.e., m/z 750 – 2000, the laser energy and acceleration voltage were set at 320 A.U and 2000 V, respectively. The mass spectrometer was calibrated with a PEG mixture (PEG600/PEG1000/PEG2000NaI = 1:1:3:1 (w/w)). For data acquisition at low mass region, 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).

2.2.4 MALDI-MS/MS

MS spectra (average of 2000 laser shots) of the precursor ions were acquired in reflection mode by using Ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany), which was equipped with a Nd:YAG smart beam laser. The

acceleration voltage was 19 kV. Voltage settings for the ion source 1, source 2, lens, reflector 1 and reflector 2 were 25.00 kV, 21.95 kV, 9.00 kV, 26.30 kV and 13.30 kV, respectively. A mixture of known amino acids was used for external calibration of the m/z scale. Tandem MS (average of 8000 laser shots) was performed in the LIFT mode. The precursor ions were accelerated at 8 kV, and selected with an optimized ion selection gating. The acceleration voltage of the LIFT cell was 19 kV. All mass spectra were analyzed with the Flex Analysis software (version 2.0) for peak detection. For the centroid algorithm, baseline subtraction and smoothing, default parameters were used.

2.2.5 Sample preparation for direct analysis of solid herbal materials

- *Powder method*

A small piece of double-sided tape was first attached onto a spot of the target plate. Approximately 0.1 mg 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.

- *Slice method*

The ginseng root sample was first cut into half from the middle of the root body with a stainless steel surgery knife. A thin slice of herbal material was then cut out from the interior of the root body. The thin slice obtained was then transferred and adhered to the surface of a double-sided tape pre-attached on the target plate. Afterward, 1 μL of CHCA matrix solution was spotted onto the slice surface, and then air-dried for analysis.

- *Sandpaper method*

A small piece of sandpaper (P1000-grit SiC abrasive paper) was first fixed onto a spot of the target plate with a double-sided tape. The ginseng root was cut into half, and the interior of the root body was directly rubbed on the sandpaper until a certain amount of herb powder was adhered onto the sandpaper surface. Subsequently, 1 μL of CHCA matrix solution was spotted onto the sandpaper deposited with herb powder, and then air-dried for analysis.

- *Solvent-free method*

5 mg of powder, 5 mg of CHCA and one small steel ball with a diameter of 2.5 mm were placed in a 500 uL eppendorf tube and vortexed for 1 min. The resulting mixture was pressed onto the target plate using a microspatula. A thin layer of the homogenized solid analyte-matrix mixture was obtained after extra solid mixture was removed with air flow.

- *Oil-assisted sample preparation (OASP) method*

A droplet of paraffin oil was spotted onto the target plate, then a minimal and transferable amount of herbal powder and approximately equal amount of solid matrix were transferred onto the oil-droplet, and mixed gently on-target with a micro-spatula until visual homogeneity was achieved.

2.2.6 LC-ESI-MS

LC-ESI-MS study was performed on an electrospray ionization quadrupole-time-of-flight (Q-TOF) mass spectrometer (Q-TOF2, Waters, Milford, MA) coupled to a liquid chromatography system (CapLC, Waters, Milford, MA). An aliquot of 30 μL of herb extract was transferred into a vial, and a 2 μL portion was injected into the C3 reversed phase HPLC column (Poroshell 300SB-C3 - 1x75mm - 5μ, Agilent Technology, USA). The mobile phase solvent system was composed of solvent A, H_2O with 0.5% formic acid, and solvent B, 90% ACN with 0.5% formic acid. Samples were first desalted by washing with 100% mobile phase solvent A at a flow rate of 40 μL/min for 3 min and then eluted with a 70 min gradient of 100% mobile phase solvent A to 100% mobile phase solvent B at a flow rate of 40 μL/min. The capillary voltage and cone voltage of the ESI source were set to 3KV and 30V, respectively. The mass spectrometer was scanned over a m/z range of 100 – 2000 m/z. Sodium iodide was used for calibration of the mass spectrometer.

2.2.7 Principal component analysis (PCA)

Principal component analysis (PCA) was carried out using the program of SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). Details of PCA are shown in *Section 2.3.3* in **Results and discussion**.

2.3 Results and discussion

2.3.1 Optimization of extraction condition

The solvent system used for extraction was 50/50 ACN/H_2O with 0.1% TFA, a commonly used solvent system for MALDI-MS. This solvent system was selected since quality mass spectra could be generated with high reproducibility at different positions within a sample spot (position-to-position reproducibility). Similar mass spectra could be obtained using other relatively polar solvents, e.g., methanol, and methanol-H_2O mixture, but the position-to-position reproducibility was less decent. (Fig. 2.1) The use of other relatively nonpolar solvents, e.g., acetone and chloroform, were also explored, but only weak ion signals of the sample peaks could be obtained, probably due to low extraction efficiency and/or that these solvents are less compatible with MALDI-MS.

A brief solvent extraction was found to be sufficient to produce desirable mass spectra. The extraction was simply performed by placing the eppendorf tube that contained the sample and solvents in an ultrasound bath and sonicating it for 2 min. (Fig. 2.2b and e) Further extending the sonication period to up to 30 minutes did not exert any obvious influence on the mass spectra. (Fig. 2.2c and f) The use of "harsher" sonication conditions, e.g., use of ultrasonic disintegrator (Soniprep 150, MSE Scientific Instrument, UK)) that is commonly applied for cell breakage in biological research, was also explored and found to generate similar mass spectra.

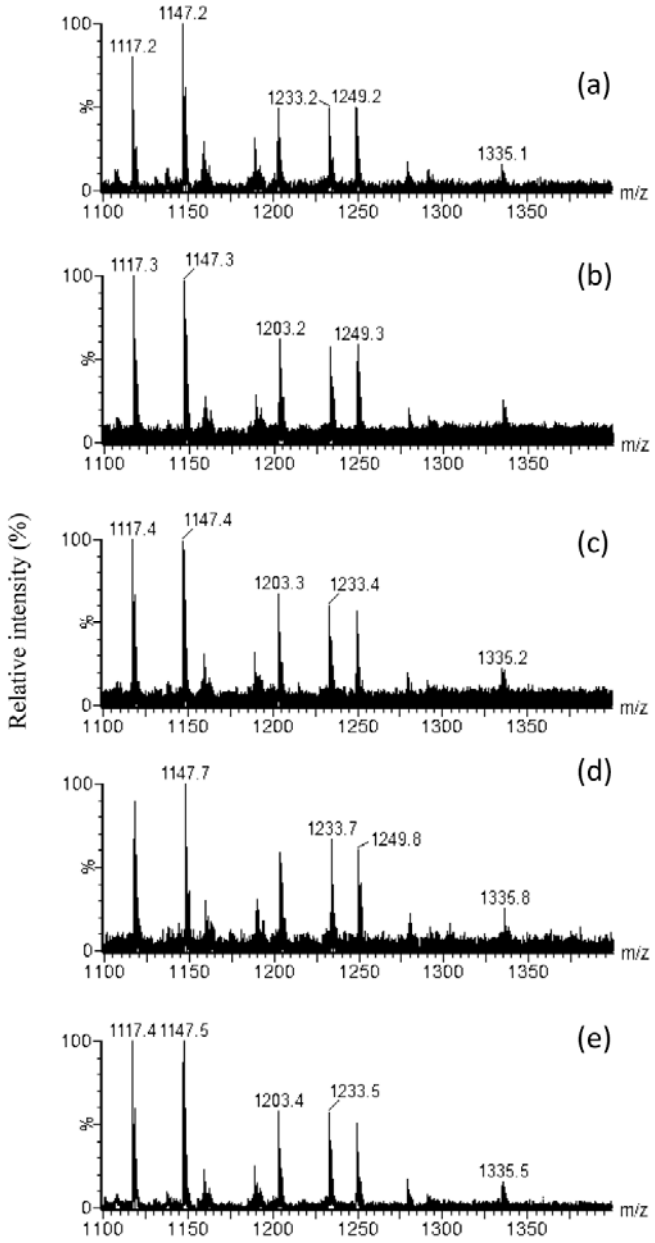


Figure 2.1. MALDI-MS spectra obtained for *Panax ginseng* in high mass region with different solvent extractions (a) 50% ACN, (b) 50% MeOH, (c) 100% H2O, (d) 100% ACN, (e) 100% MeOH. All the solvents contained 0.1% TFA. Mass peaks from CHCA matrix are labeled with "*".

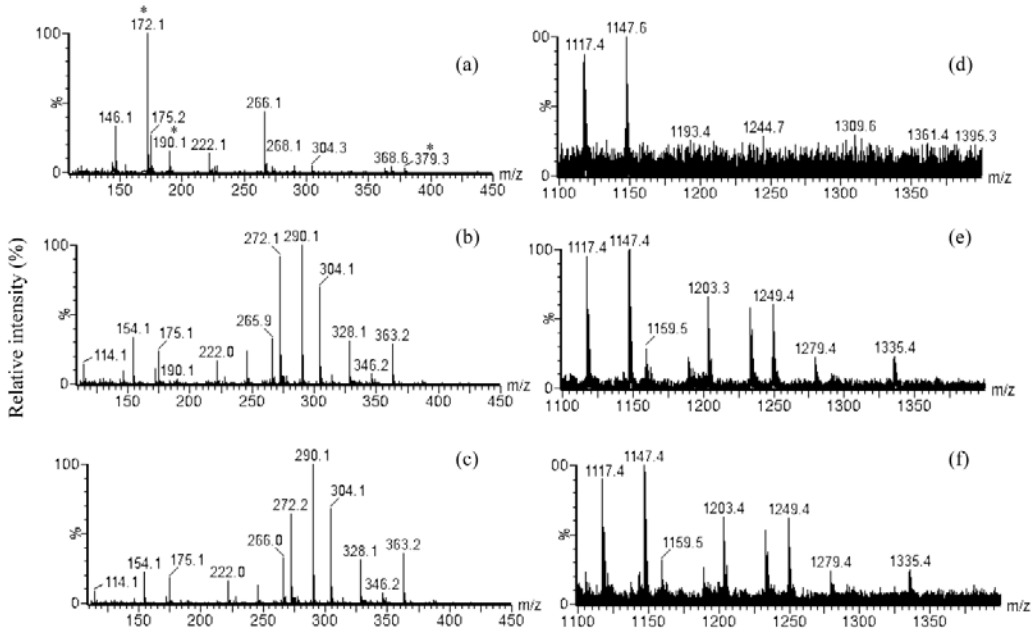


Figure 2.2. MALDI-MS spectra obtained for *Panax ginseng* with different extraction time (a) 0 min (b) 2 min, (c) 30 min for low mass region, (d) 0 min, (e) 2 min, (f) 30 min for high mass region. Mass peaks from CHCA matrix are labeled with "*".

Our experimental results showed that extraction of a small scale of sample, e.g., 5 mg herb powder, with 500 μL solvent was adequate to generate quality mass spectra. Attempts were also made to enlarge the sample scale to up to the range of 1 g herb powder extracted with 5 ml solvent, yet no significant differences in the mass spectra were observed. Homogenizing and sieving the herb powder to different powder size within 0.075 – 0.15 mm also did not exert any remarkable effect on the mass spectra.

The herb extract obtained was mixed with CHCA matrix for MALDI-MS analysis and quality spectra were obtained (Fig. 2.3). The use of other commonly used MALDI matrices, including SA and DHB, was also explored, but the quality of mass spectra acquired was less decent. For example, more significant matrix interference was observed when SA was used as the matrix, and fewer sample peaks could be observed using the DHB matrix (data not shown).

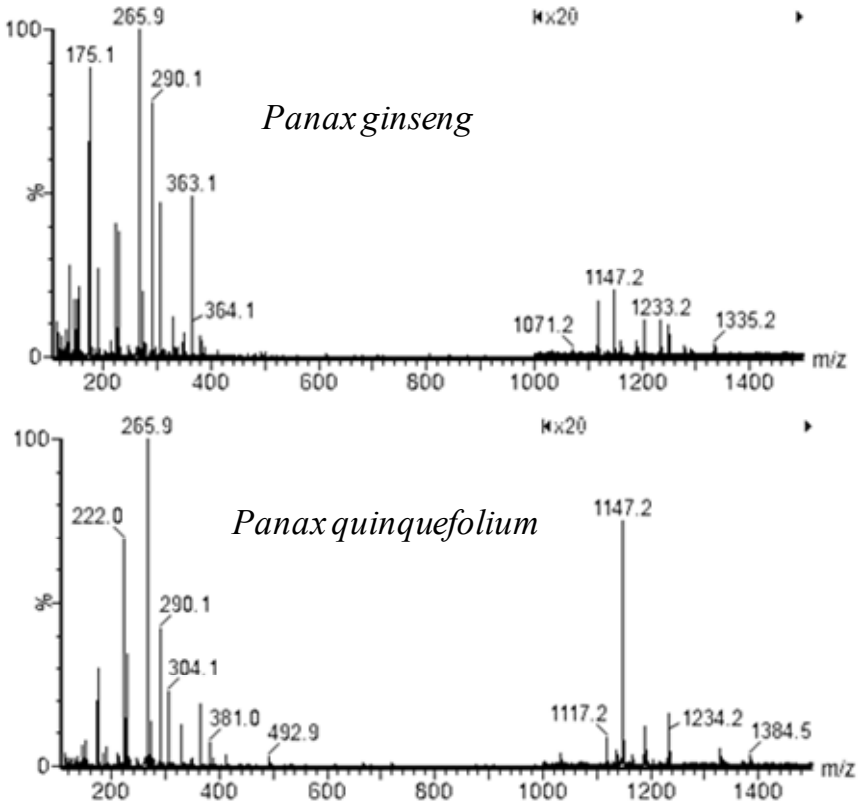


Figure 2.3. MALDI-MS spectra for *Panax ginseng* and *Panax quinquefolius* (Canada) obtained by analysis of the extracts from brief extractions and with CHCA as the matrix.

2.3.2 MALDI-MS spectra of Panax ginseng and *Panax quinquefolius*

Two typical spectra for *Panax ginseng* and *Panax quinquefolius* are shown in Fig. 2.3. No significant peaks were observed between 500 – 1000 Da. The spectrum can thus be divided into two regions. One is the high mass region, m/z 1000 – 1500, for observation of the ginsenoside pattern. The other is the low mass region, m/z 100 – 500, which might cover small molecules useful for differentiation. It was found that *Panax ginseng* and *Panax quinquefolius* had different patterns in these two mass regions, which allowed differentiation between the two species. In order to achieve optimal spectral quality, these two mass ranges were acquired separately with different instrumental parameters. The obtained spectra were then analyzed and compared for differentiation between *Panax ginseng* and *Panax quinquefolius*.

2.3.2.1 High mass region

For *Panax ginseng*, distinct mass peaks, identified as potassium adducts of different common ginsenosides including Rb_2/Rb_3/Rc (m/z 1117.5), Rb_1 (m/z 1147.5), pseudo-G-F_8/RS_1/RS_2 (m/z 1159.5), malonyl-Rb_2/Rb_3/Rc (m/z 1203.4), malonyl-Rb_1 (m/z 1233.4), Ra_1/Ra_2 (m/z 1249.4), Ra_3 (m/z 1279.5) and malonyl-Ra_1/Ra_2 (m/z 1335.5) based on their masses, literatures [120, 121, 130, 135] and MALDI-MS/MS spectra, could be observed (Fig. 2.4a and Table 2.1). The formation of potassium adducts was most likely due to the use of relatively polar extraction solvent that favored the extraction of alkali metal salts. More ginsenosides were detected herein than a previous MALDI-MS imaging study.[98]

The high mass region observed for *Panax quinquefolius* showed several remarkable differences compared with that of *Panax ginseng* (Fig. 2.4 and Table 2.1), thus allowing differentiation between the two *Panax* species. The mass spectra obtained for *Panax quinquefolius* from three major countries, Canada, the USA and China, were highly similar to one another (Fig. 2.5). Among the mass peaks of different malonyl-ginsenosides detected in *Panax ginseng*, only malonyl-Rb_1 (m/z 1233.4) could be observed, while others, including malonyl-Rb_2/Rb_3/Rc (m/z 1203.4) and malonyl-Ra_1/Ra_2 (m/z 1335.5), were absent in the mass spectra of *Panax quinquefolius* (Fig. 2.4 & Table 2.1). These observations could be correlated to previous findings that the levels of malonyl-Rb_2 and malonyl-Rc relative to malonyl-Rb_1 for *Panax quinquefolius* were lower than for *Panax ginseng* [126] and that only malonyl-Rb_1 but not the others could be detected in *Panax quinquefolius* when extracted with 50% ethanol. [131] Other remarkable differences are that the mass peaks corresponding to Ra_1/Ra_2 (m/z 1249.4), pseudo-G-F_8/Rs_1/Rs_2 (m/z 1159.5), and Ra_3 (m/z 1279.5) observed for *Panax ginseng* were not detected in *Panax quinquefolius*. These data are consistent with the literature that Ra_1, Ra_2, and Ra_3 could be detected in *Panax ginseng* [121], but have not been observed in *Panax quinquefolius* so far. [135] On the other hand, two mass peaks, m/z 1328.7 and 1384.8, were present only in *Panax quinquefolius*, but not in *Panax ginseng*. These two peaks could not be assigned to any known ginsenosides based on their masses and MS/MS studies, but were useful parameters for differentiation between the two *Panax* species. The identification of these two chemical components is under further investigation.

Table 2.1. Summary of the presence or absence of the major mass peaks in high mass region for *Panax ginseng* and *Panax quinquefolius*.

m/z	Identity	*Panax ginseng*	*Panax. quinquefolius*
1117	$[Rb_2/Rb_3/Rc + K]^+$	√	√
1147	$[Rb_1 + K]^+$	√	√
1159	$[\text{pseudo-G-}F_8/Rs_1/Rs_2 + K]^+$	√	×
1203	$[\text{malonyl-}Rb_2/Rb_3/Rc + K]^+$	√	×
1233	$[\text{malonyl-}Rb_1 + K]^+$	√	√
1249	$[Ra_1/Ra_2 + K]^+$	√	×
1279	$[Ra_3 + K]^+$	√	×
1335	$[\text{malonyl-}Ra_1/Ra_2 + K]^+$	√	×
1328	unknown	×	√
1384	unknown	×	√

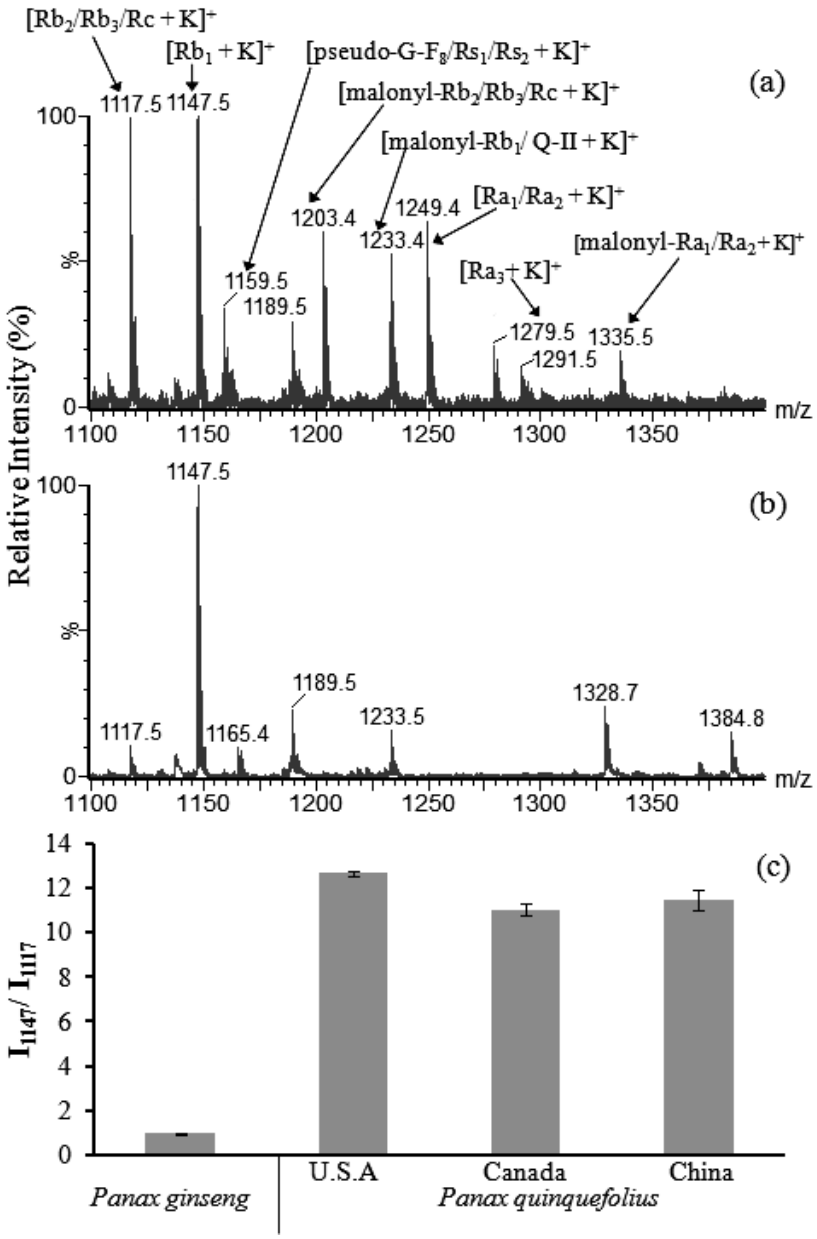


Figure 2.4. MALDI-MS spectra acquired in high mass region for *Panax ginseng* (a) and *Panax quinquefolius* (Canada) (b) respectively. (c) A chart showing the intensity ratio of m/z 1147 to m/z 1117 (I_{1147}/I_{1117}) in the MALDI-MS spectra obtained for *Panax ginseng* and *Panax quinquefolius.*

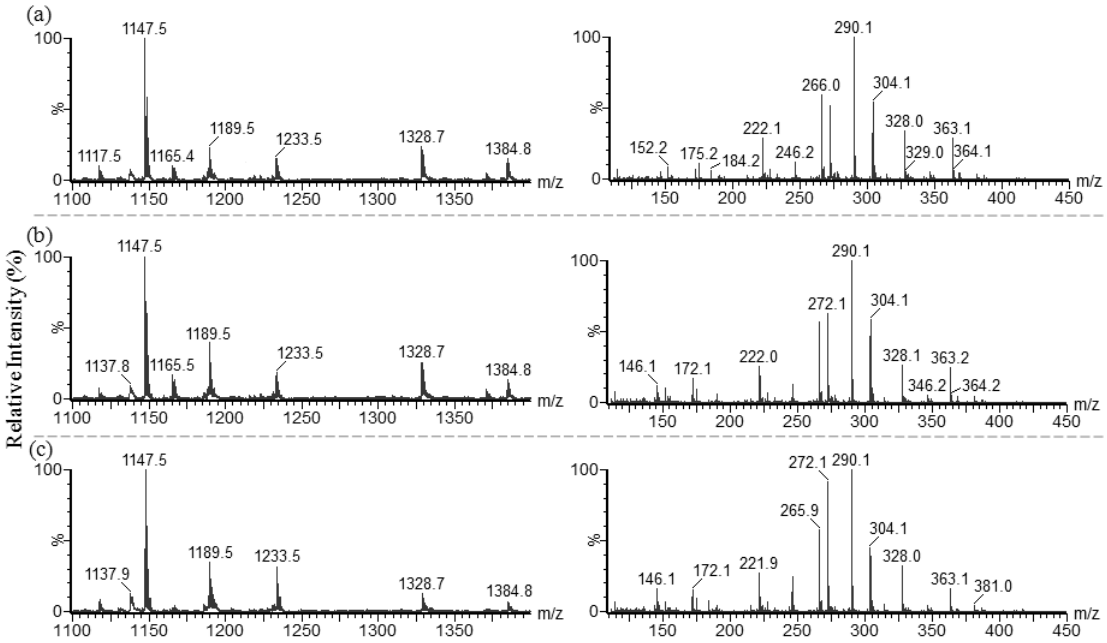


Figure 2.5. MALDI-MS spectra for *Panax quinquefolius* from Canada (a), USA (b), and China (c) acquired in the high (left column) and low (right column) mass regions.

The reproducibility of the mass spectra was tested in two aspects in this study. First, "inter-experiment" reproducibility, i.e. the reproducibility of the entire experimental methodology, including solvent extraction and MALDI-MS analysis, was tested by performing three independent experiments with the same herb sample. Second, "inter-sample" reproducibility, i.e. the reproducibility of the results obtained for the same species of herb samples but purchased from different local TCM pharmacies, was studied. As shown in Fig. 2.6, the mass spectra of the high mass region obtained from different experiments and different samples of the same herb species were very similar, indicating a high level of "inter-experiment" and "inter-sample" reproducibility of the

present MALDI-MS method even though the peaks at the high mass region were of lower intensities.

Another difference observed in the mass spectra between the two *Panax* species was the intensity ratio of the mass peaks at m/z 1147.5 and 1117.5 (I_{1147}/I_{1117}), which corresponded to Rb_1 and $Rb_2/Rb_3/Rc$, respectively (Fig. 2.4). For testing the "inter-experiment" and "inter-sample" reproducibility, seven samples of each herbal species purchased from different pharmacies were extracted and subjected to MALDI-MS analysis, and three independent experiments were performed on each sample. The I_{1147}/I_{1117} determined for *Panax quinquefolius* from Canada (3 samples), USA (3 samples) and China (1 sample) were 11.02 ± 0.26, 12.66 ± 0.11 and 11.45 ± 0.43 respectively, all three figures/values very close to one another. For *Panax ginseng* (7 samples from China), however, the I_{1147}/I_{1117} determined was 0.96 ± 0.06, which was significantly (~11 folds) smaller than that of *Panax quinquefolius* (Table 2.2). The standard errors obtained for the experimental data were as small as 2 – 9 %, indicating a high level of "inter-experiment" and "inter-sample" reproducibility. These data are consistent with the results of a previous quantitative study that the concentration of Rb_1 is similar to and higher than Rc and Rb_2 for *Panax ginseng* and *Panax quinquefolius*, respectively.[125]

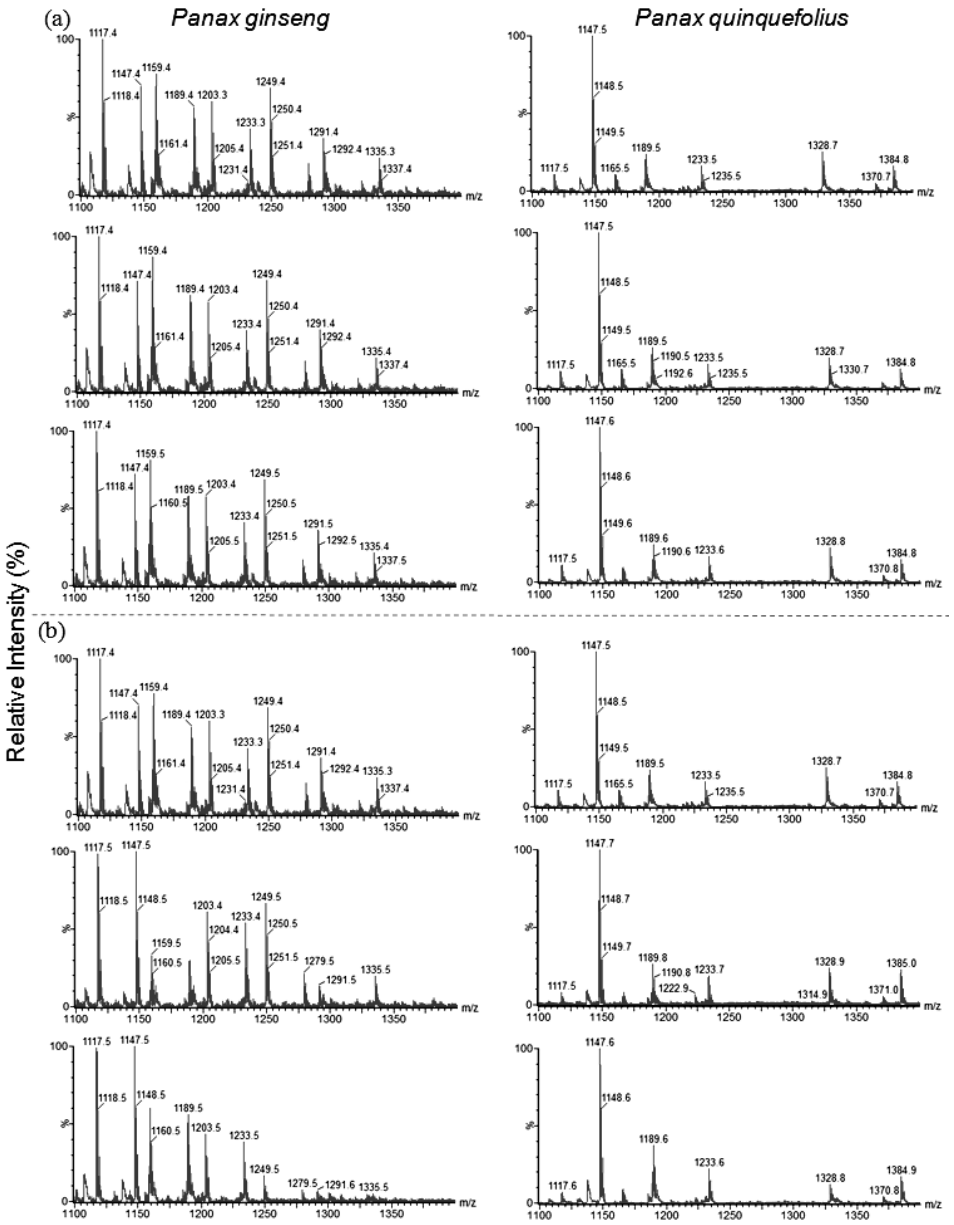


Figure 2.6. Test for reproducibility of the mass spectra obtained for *Panax ginseng* (left column) and *Panax quinquefolius* (Canada) (right column) acquired in the high mass region. (a) MALDI-MS spectra of the same herb samples obtained in three independent experiments. (b) MALDI-MS spectra obtained for the same species of herb samples purchased from three different pharmacies.

Table 2.2. Summary of the intensity ratio of m/z 1147 to m/z 1117 (I_{1147}/I_{1117}) and m/z 154 to m/z 152 (I_{154}/I_{152}) for *Panax ginseng* and *Panax quinquefolius*.

		Panax ginseng	*Panax quinquefolius*		
			U.S.A	Canada	China
I_{1147}/I_{1117}	solution-based method	0.96 ± 0.06	12.66 ± 0.11	11.02 ± 0.26	11.45 ± 0.43
	powder method	0.95 ± 0.02	6.71 ± 0.60	9.82 ± 0.60	6.26 ± 0.53
I_{154}/I_{152}	solution-based method	15.17 ± 3.73	0.41 ± 0.08	0.41 ± 0.07	0.49 ± 0.01
	powder method	10.92 ± 1.12	0.29 ± 0.03	0.37 ± 0.07	0.24 ± 0.02
	slice method	21.48 ± 1.85	0.38 ± 0.08	0.38 ± 0.10	0.18 ± 0.03
	sandpaper method	18.08 ± 5.11	0.27 ± 0.07	0.40 ± 0.19	0.19 ± 0.01

There is a notable feature in our MALDI-MS data, namely the absence of some of the commonly observed ginsenosides in LC-ESI-MS studies such as Rg_1, Re, Rd, Rf (only in *Panax ginseng*), and F_{11} (only in *Panax quinquefolius*).[39, 112, 114, 117, 120-131] To investigate if the non-detection of them was due to poor extraction efficiency or signal suppression, LC-ESI-MS analysis was performed on the same herbal extract, and the data are shown in Fig. 2.7. Strikingly, the common ginsenosides that could not be observed in MALDI-MS were detected in LC-ESI-MS. In particular, we noted that by investigating the fragmented ions generated by in-source dissociation, Rf was only detected in *Panax ginseng* and F_{11} was only observed in *Panax quinquefolius* in our LC-ESI-MS study (Fig. 2.7), results that were consistent with some previous studies.[39, 112, 114, 117, 135] In general, the characteristics of ginsenoside patterns observed in MALDI-MS were also present in LC-ESI-MS (data not shown). These experimental results indicated that the brief extraction used in this study was adequate for extracting most common

ginsenosides for MS analysis. The absence of some common ginsenosides in MALDI-MS was most likely due to signal suppression, although the detailed mechanism behind this requires further investigation.

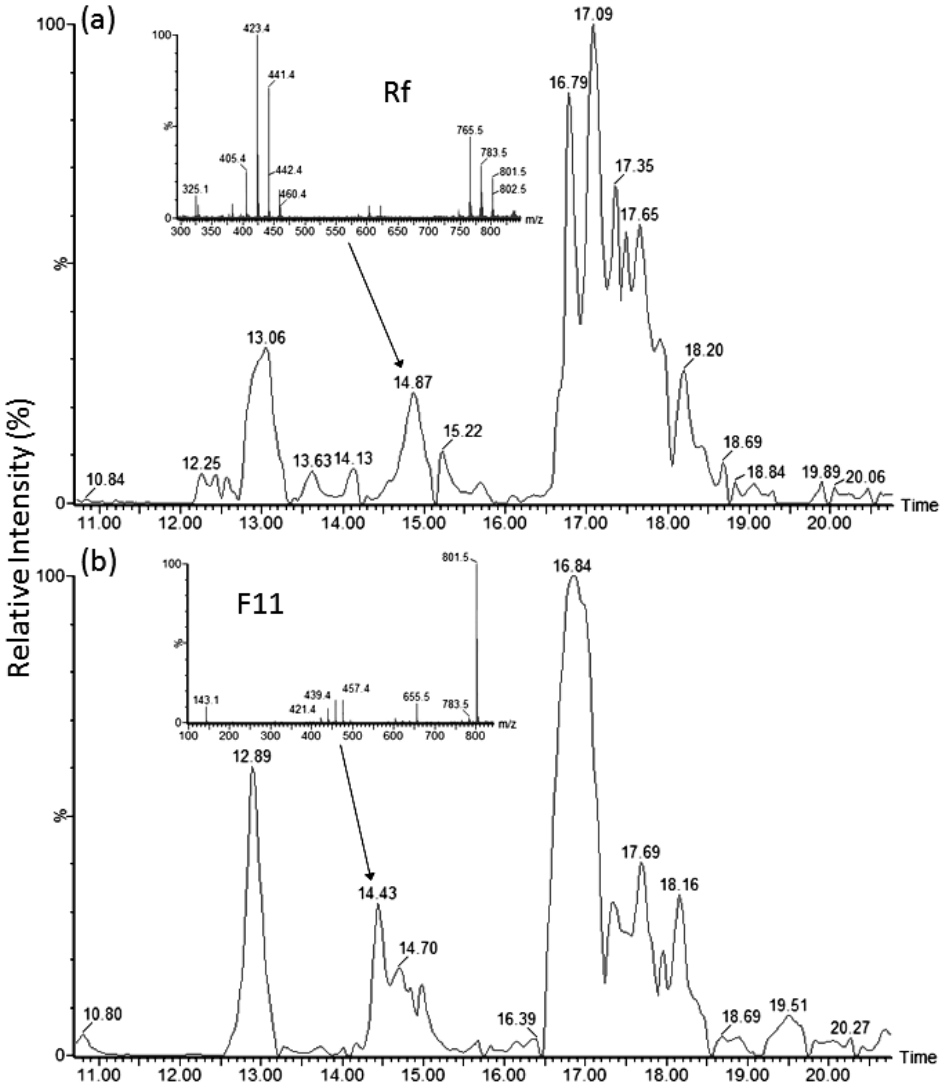


Figure 2.7. LC-ESI-MS total ion chromatogram obtained for *Panax ginseng* (a) and *Panax quinquefolius* (Canada) (b). ESI-MS spectra of Rf and F_{11} are shown in the embedded figures in (a) and (b), respectively.

2.3.2.2 Low mass region

Apart from the examination on ginsenoside pattern, we also investigated the low mass region of 100 – 500 Da, which might detect some small molecules useful for differentiation between the two *Panax* species. There were so far, to our knowledge, no mass spectrometric studies on *Panax* herbs focusing on this mass range of small molecules. The mass spectra that display the low mass region of the two *Panax* herbs are shown in Fig. 2.8. Matrix interference, a common problem in MALDI-MS analysis of small molecules, was insignificant in our study. The mass spectra obtained for *Panax ginseng* and *Panax quinquefolius* showed some common distinct mass peaks, e.g., m/z 266, 272, 290, 304, 328, and 363. The "inter-experiment" and "inter-sample" reproducibility of the mass spectra were indicated in Fig. 2.9, and the similarity of the mass spectra between *Panax quinquefolius* from the three major countries is shown in Fig. 2.5. When comparing the mass spectra obtained for other medicinal herbs, e.g., *Coptis chinensis* Franch, *Bupleuri Radix*, *Aconitum Carmichaeli* Debx, and *Schisandra chinensis*, these mass patterns were only observed for *Panax ginseng* and *Panax quinquefolius*, thus they might be applied for distinguishing the two *Panax* species from other medicinal herbs (Fig. 2.10).

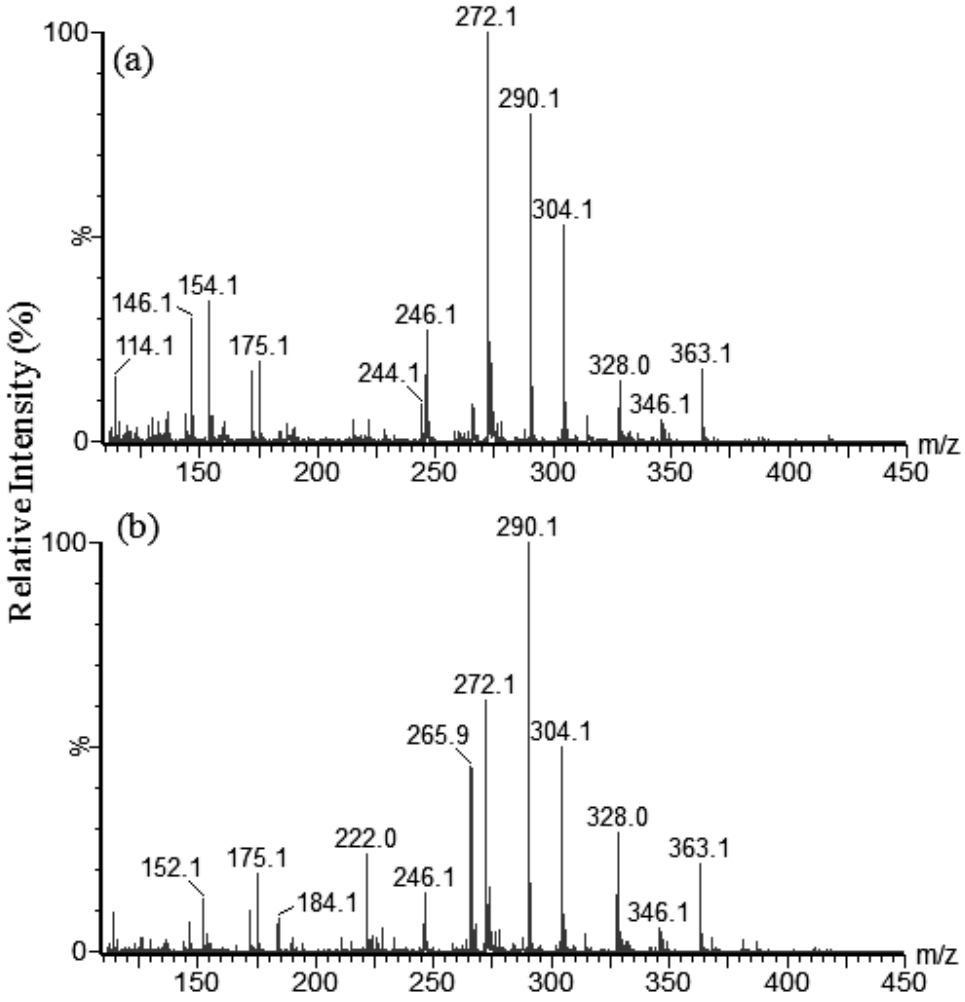


Figure 2.8. MALDI-MS spectra acquired in the low mass region for *Panax ginseng* (a) and *Panax quinquefolius* (Canada) (b) respectively.

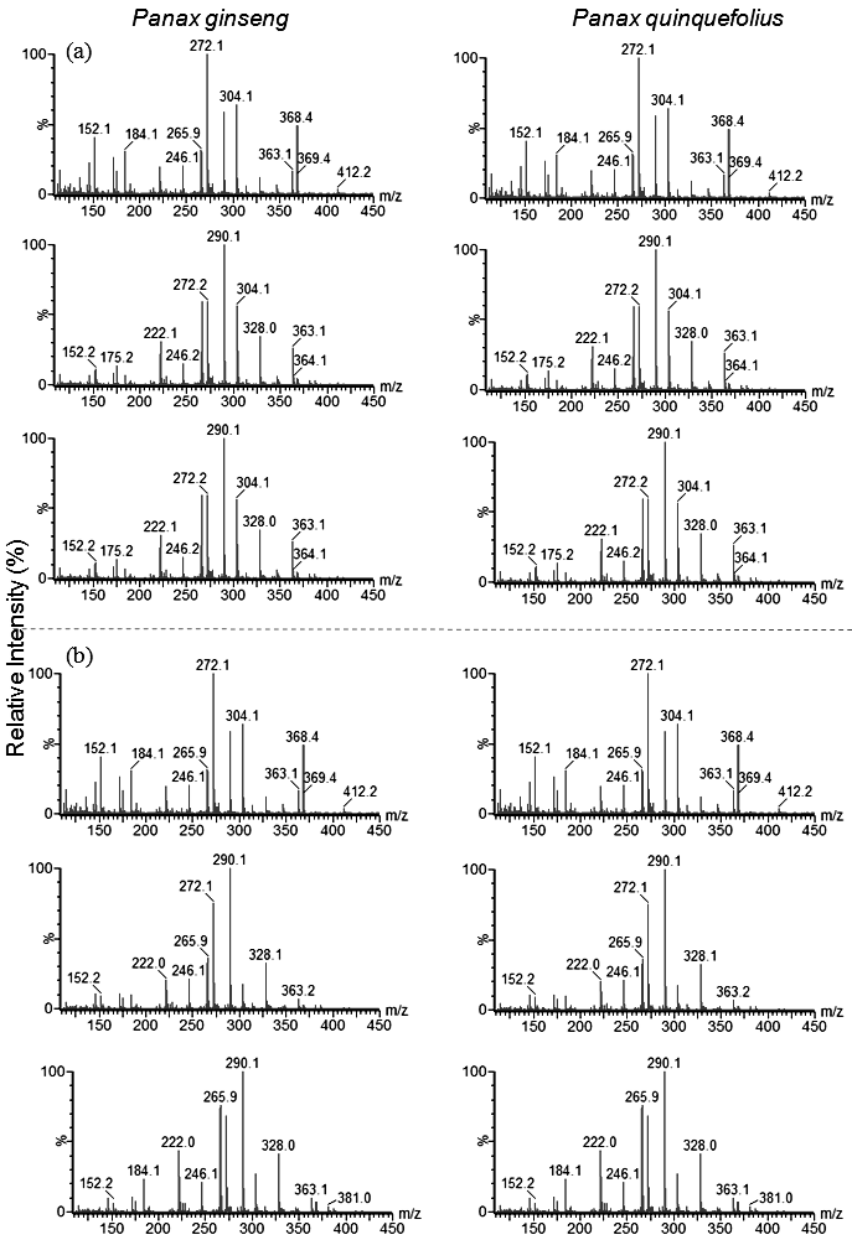


Figure 2.9. Test for reproducibility of the mass spectra obtained for *Panax ginseng* (left column) and *Panax quinquefolius* (Canada) (right column) acquired in the low mass region. (a) MALDI-MS spectra of the same herb samples obtained in three independent experiments. (b) MALDI-MS spectra obtained for the same species of herb samples purchased from three different pharmacies.

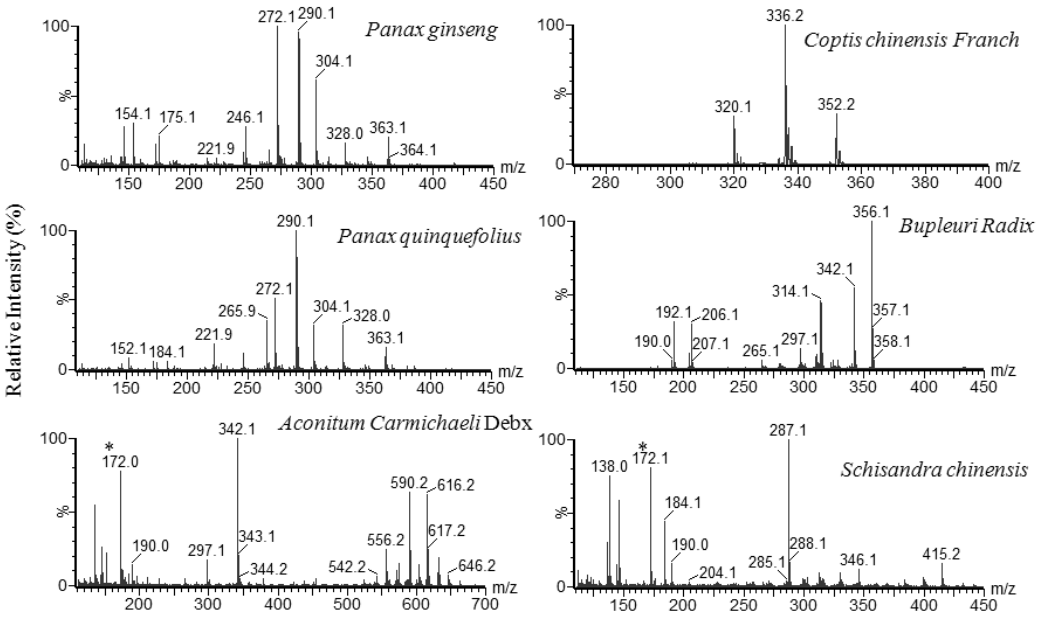


Figure 2.10. MALDI-MS spectra of different TCM herbs acquired in the low mass region. Mass peaks from CHCA matrix are labeled with "*".

Although the two *Panax* species exhibited similar mass patterns at the low mass region, detailed examination of the mass spectra showed an obvious difference. For *Panax ginseng*, the intensity of mass peak at m/z 154 was significantly higher than that at m/z 152, while for *Panax quinquefolium,* the opposite result was obtained (Fig. 2.11). For seven samples of *Panax ginseng* (three from Canada, three from the USA and one from China) and seven samples of *Panax quinquefolium* (all from China), the averaged I_{154}/I_{152} ratio calculated for *Panax quinquefolius* from Canada (0.41 ± 0.07), the USA (0.41 ± 0.08) and China (0.49 ± 0.01) were similar to one another and significantly (~ 30 folds) smaller than that for *Panax ginseng* (15.17 ± 3.73) (Table 2.2). Similar to the case for I_{1147}/I_{1117}, the low standard errors herein indicated the high level of the "inter-experiment" and "inter-sample" reproducibility and the reliability of using these values

to distinguish the two species. After logarithm of the I_{154}/I_{152} ratios, negative values and positive values were obtained for *Panax quinquefolium* and *Panax ginseng*, respectively, allowing easy and unambiguous differentiation between the two herbal species (Fig. 2.11c).

Attempts to identify these low mass components using MALDI-MS/MS were made. The identities of these two important markers were not completely determined yet due to the limited fragmentation observed in the spectra and the limited MS/MS reference spectra available for comparison. Potential compounds included (*Z*)-4-hydroxyphenylacetaldehyde-oxime for m/z 152, and pseudopelletierine and methyl 2,5-dimethyl-1H-pyrrole-3-carboxylate for m/z 154. MS/MS spectra of poor quality were obtained for other low mass marker ions. These low mass components could not be detected in LC-ESI-MS, restricting their identification using the ESI-based equipment. Further investigation in this respect is still going on.

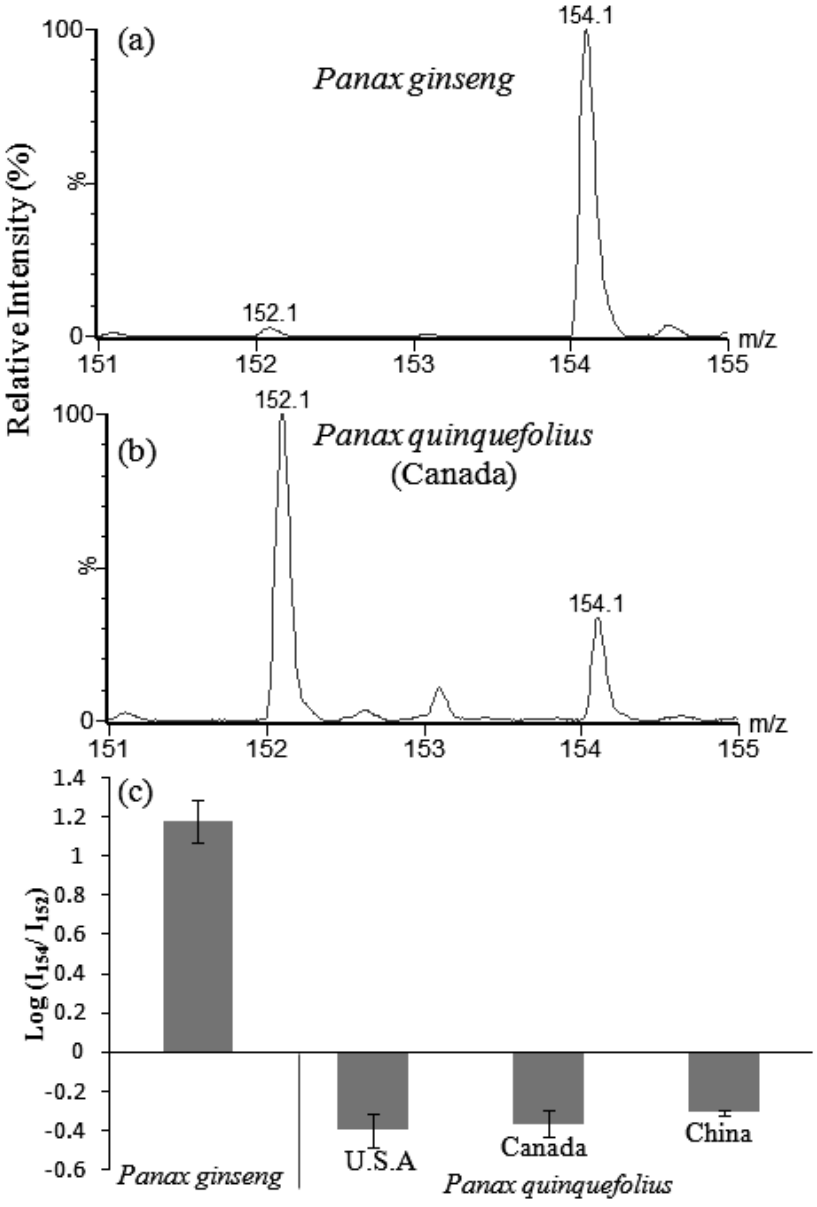


Figure 2.11. MALDI-MS spectra showing the relative abundance of the m/z 154 and m/z 152 components detected for *Panax ginseng* (a) and *Panax quinquefolius* (b) respectively. (c) A chart showing the logarithm of the intensity ratio of m/z 152 to m/z 154 (I_{154}/I_{152}).

2.3.3 Principal component analysis (PCA)

The relative ion intensities of mass peaks observed in the MALDI-MS spectra were applied for PCA for differentiation between *Panax ginseng* and *Panax quinquefolium*. The data obtained from three independent experiments on each sample were averaged and input for PCA analysis of the sample. The first (PC1) and second (PC2) principal components were chosen to generate a score plot (Fig. 2.12a), which showed that based on the obtained MALDI-MS data, *Panax ginseng* and *Panax quinquefolium* could be unambiguously differentiated. The two-component PCA model accounted for 74% of total variance of data (PC1 = 59% and PC2 = 15%).

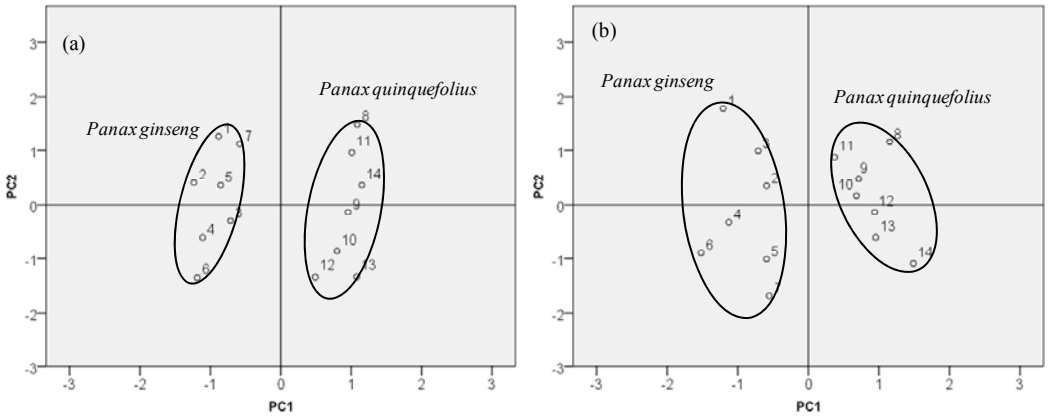


Figure 2.12. PCA plot of the MALDI-MS results obtained for *Panax ginseng* and *Panax quinquefolius* using the solution-based method (a) and powder method (b) respectively. Only the first and second principal components (PC1 and PC2) are shown. 1-7 are *Panax ginseng* samples from China; 8-10, 11-13 and 14 are *Panax quinquefolius* samples from Canada, the USA and China respectively.

2.3.4 Quantitative determination of ginsenosides

MALDI-MS is usually not recommended for quantitative measurement due to its poor signal reproducibility. In this study, measurement of intensity ratios of characteristic ions showed very good reproducibility, a result that inspired us to explore the application of measuring intensity ratios for quantitative determination of chemicals in the *Panax* species. As an example, I_{1147}/I_{1117} was measured for quantitation of ginsenoside Rb1 (m/z 1147) and standard addition method [136] was used. A set of *Panax quinquefolius* (Canada) extracts with ginsenoside Rb1 standard added in different concentrations was analyzed, and the I_{1147}/I_{1117} value obtained for each sample was plotted against the added Rb1 standard concentration. As shown in Fig. 2.13, a calibration line was obtained with $R^2 = 0.9875$. From the x-intercept, the concentration of Rb1 in the ginseng sample was determined to be 38.0 ± 1.7 mg/g, a value comparable to the concentration of 30.9 mg/g determined by LC/MS/MS for the same kind of ginseng.[124] The peak at m/z 1117 herein was actually used as an internal standard to improve the analysis. The good correlation coefficient and the comparable data demonstrated the applicability of our approach in quantitative measurement.

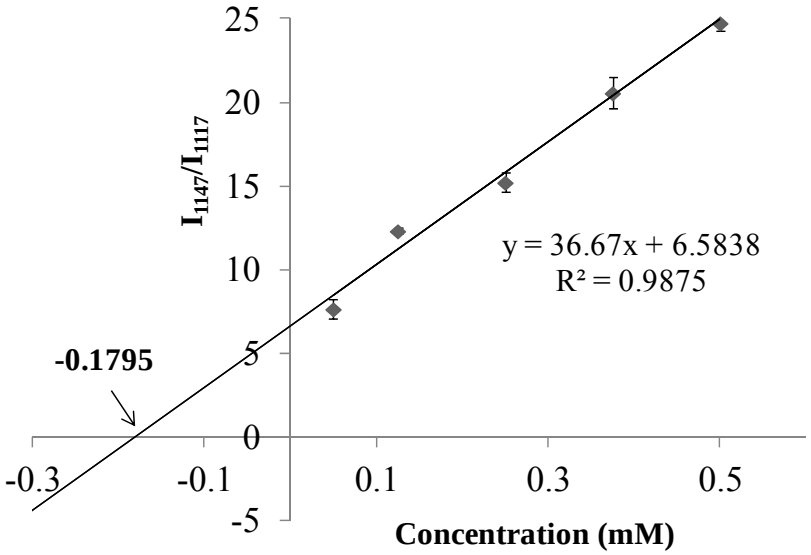


Figure 2.13. Plot of I_{1147}/I_{1117} against the added ginsenoside Rb1 standard concentration in the ginseng extract.

2.3.5. Direct analysis of solid herbal materials

The solution-based method introduced above requires homogenization and extraction procedures and is thus sample-consuming, time-consuming and laborious. In this study, we attempted direct analysis of ginseng powders to avoid the extraction step and direct analysis of raw ginseng samples to avoid both the homogenization and extraction steps.

For direct analysis of the powder samples (please refer to the powder method in this thesis), the obtained spectra showed mass patterns similar to those obtained by the solution-based method for both the low mass and high mass regions (Figs. 2.14a and d). The observed I_{154}/I_{152} and I_{1147}/I_{1117} values obtained by the powder method were generally smaller than those obtained by the solution-based method (Table 2.2).

However, the logarithms of I_{154}/I_{152} for *Panax ginseng* and *Panax quinquefolium* also produced positive and negative values respectively, and the I_{1147}/I_{1117} value for *Panax quinquefolium* was still remarkably larger (6 – 10 folds) than that obtained for *Panax ginseng* (Table 2.2), allowing clear differentiation between the two *Panax* species. PCA analysis also revealed that *Panax ginseng* and *Panax quinquefolium* could be unambiguously differentiated by the spectra obtained by direct MALDI-MS analysis of the powders (Fig. 2.12b). The two-component PCA model accounted for 70% of total variance of data (PC1 = 48% and PC2 = 22%).

Direct analysis of HM powders has previously been successfully attempted for *Aconitum carmichaeli* Debx.[88] Its mechanism for ion generation is, however, still unclear, and was investigated in this study. When the ginseng powder was analyzed using the solvent-free method[66, 68] and OASP method,[69] no sample signal was observed (data not shown), suggesting that the sample ions were not directly generated from the sample powders and matrix solvents played an important role in observation of the spectra. When the ginseng powder was soaked with the CHCA solution for a very short time (e.g., 30s) and the resulting extract was directly analyzed by MALDI-MS without further addition of matrix solution, a spectrum (Fig. 2.15a) similar to that obtained by direct analysis of the powder (Fig. 2.14a) was observed. No big changes in the spectra were observed with the extension of extraction time, indicating that extraction of the sample powder could be finished within a very short time, i.e., before the matrix solution became completely dried. Replacing the matrix solvent with 100% acetone with 0.1% TFA for direct analysis and extraction of the ginseng powder resulted in spectra

(Figs. 2.15b and c) different from those obtained using 50/50 ACN/H_2O with 0.1% TFA (Figs. 2.14a and 2.15a). But again, the spectrum obtained by direct analysis of ginseng powder (Fig. 2.14b) was similar to that obtained for the extract of the ginseng powder using the same matrix solution as the extraction solvent (Fig. 2.15c). These results revealed that extraction of chemicals from herb powders by the matrix solution during drying of the matrix solution accounted for the ion signals observed in the powder method.

Two other methods, the slice method and sandpaper method, were also attempted in this study. Note that the small piece of material analyzed in the slice method was directly taken from the raw sample, different from the thin tissue slices prepared using a cryotome in the previous studies.[95-97] In the sandpaper method, solid HM material was scratched against a piece of sandpaper adhered onto the MALDI plate to provide herb powders for MALDI-MS analysis. Sample preparation in both methods was thus very simple and required a very small amount of sample. The MALDI-MS spectra obtained from these two methods are shown in Figs. 2.14b and c. No ion signals for the various ginsenosides could be detected in the high mass region, a result probably due to the low abundances of ginsenosides in the samples and the small amount/small surface area of samples accessible for extraction. For the low mass region, the mass patterns observed for these methods were generally similar to those obtained by the solution-based method and powder method. Particularly, the logarithms of I_{154}/I_{152} obtained for *Panax ginseng* and *Panax quinquefolium* also produced positive and negative values respectively, allowing easy differentiation between the two *Panax* species (Table 2.2). These results

demonstrated that *Panax ginseng* and *Panax quinquefolium* could be rapidly differentiated by MALDI-MS without sample homogenization and solvent extraction.

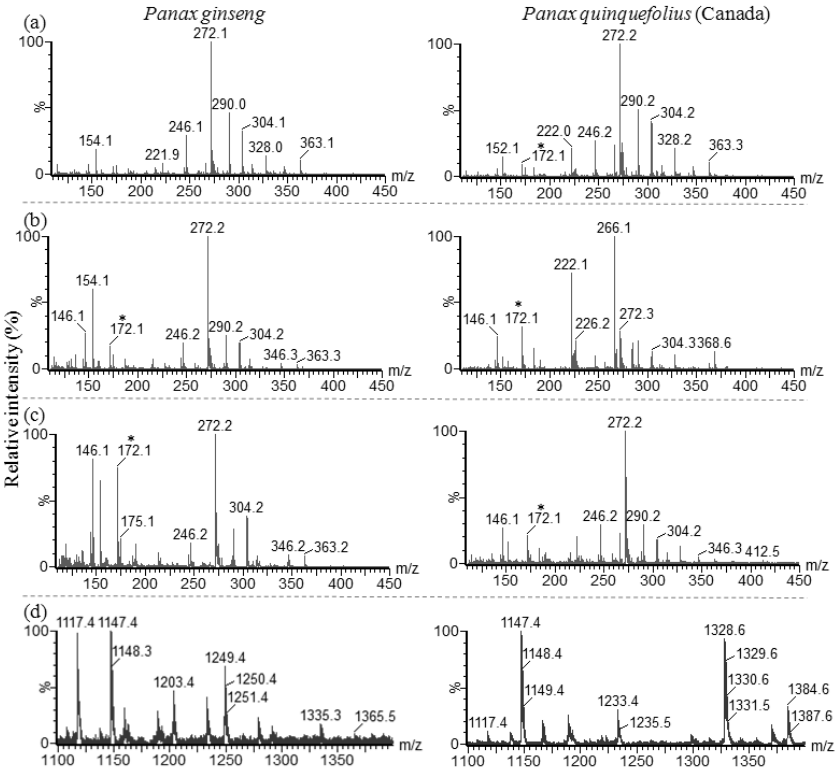


Figure 2.14. MALDI-MS spectra obtained for *Panax ginseng* (left column) and *Panax quinquefolius* (right column) using different solid sample preparation methods. (a) – (c): Mass spectra acquired in low mass region using the powder, slice, and sandpaper method, respectively. Mass peaks from CHCA matrix are labeled with "*". (d): Mass spectra acquired in high mass region using the powder method.

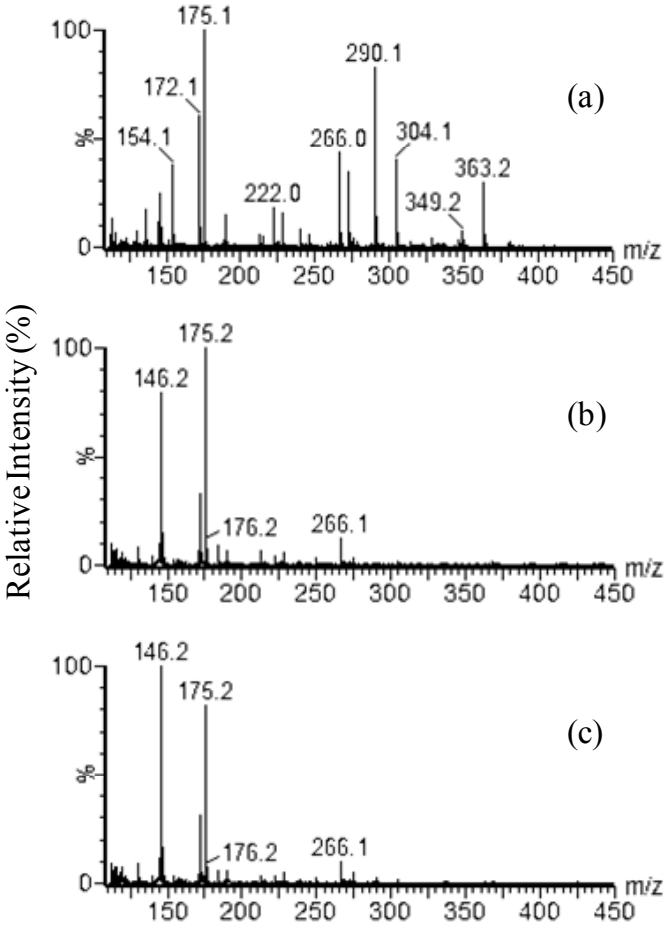


Figure 2.15. MALDI-MS spectra for *Panax ginseng* obtained by: (a) analysis of the extract of sample powder with matrix solution (10 mg/mL CHCA in 50/50 ACN/H_2O with 0.1% TFA) as the extraction solvent, (b) direct analysis of sample powder with CHCA (10 mg/mL in 100% acetone) as the matrix solution, and (c) analysis of the extract of sample powder with matrix solution (10 mg/mL CHCA in 100% acetone with 0.1% TFA) as the extraction solvent.

2.3.6 Analysis of red ginseng

Red ginseng is *Panax ginseng* that has been steamed [137, 138] and thus shows different color than the unheated *Panax ginseng* and *Panax quinquefolium* (usually white or yellow). Bulky red ginseng samples can be easily distinguished from *Panax ginseng* and *Panax quinquefolium* based on their colors. Extracts and powders of the red ginseng samples were tested using the solution-based method and powder method in this study. Two red ginseng samples, from China and South Korea respectively, were analyzed and it was found that they had very similar spectra. Compared to the spectrum of *Panax ginseng* (Fig. 2.4a), some malonyl-ginsenoside peaks, e.g., malonyl-Rb2/Rb3/Rc (m/z 1203.4), malonyl-Rb1 (m/z 1233.4) and malonyl-Ra1/Ra2 (m/z 1335.5), were absent in the MALDI spectrum of red ginseng (Fig. 2.16). This result was consistent with some previous conclusions that malonyl-ginsenosides are unstable and can be readily de-malonylated upon heating [139], and that red ginseng contains much lower levels of malonyl-ginsenosides than *Panax ginseng*.[126, 140]

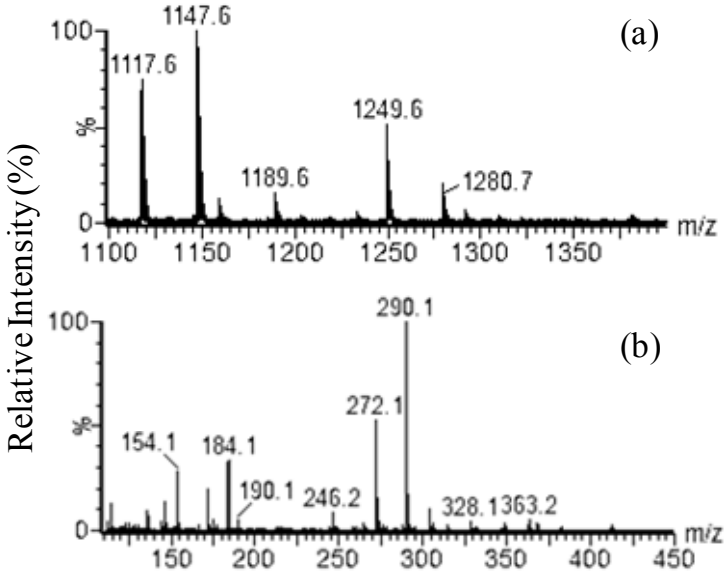


Figure 2.16. The high mass region (a) and low mass region (b) of the MALDI spectra for red ginseng (South Korea) obtained by the solution-based method.

The I_{1147}/I_{1117} ratios determined for the red ginseng samples from China and South Korea were 1.14 ± 0.02 and 1.18 ± 0.01 respectively using the solution-based method, and 1.29 ± 0.03 and 0.96 ± 0.02 respectively using the powder method. These ratios were very similar to those obtained from *Panax ginseng*, and much smaller than those of *Panax quinquefolius* (Table 2.2), suggesting that an I_{1147}/I_{1117} ratio around unity can be used as an indicator for both *Panax ginseng* and red ginseng. The I_{154}/I_{152} ratios determined for the red ginseng from China and South Korea were 6.79 ± 1.55 and 8.27 ± 0.29 respectively using the solution-based method, and 4.26 ± 0.02 and 4.48 ± 0.03 respectively using the powder method. These ratios were significantly lower than those obtained from *Panax ginseng* (Table 2.2), allowing differentiation between red ginseng and *Panax ginseng*. On the other hand, these ratios were significantly larger than those of *Panax quinquefolius* and the logarithms of I_{154}/I_{152} produced positive values for both

Panax ginseng and red ginseng, enabling unambiguous differentiation of them from *Panax quinquefolium* which had negative values for logarithms of I_{154}/I_{152}. PCA analysis also demonstrated that *Panax ginseng,* red ginseng and *Panax quinquefolium* could be unambiguously differentiated based on their mass spectra acquired using the solution-based method or the powder method (Fig. 2.17). The two-component PCA model accounted for 66% of total variance of data (PC1 = 50% and PC2 = 16%) for solution-based method and 63% of total variance of data (PC1 = 35% and PC2 = 28%) for the powder method.

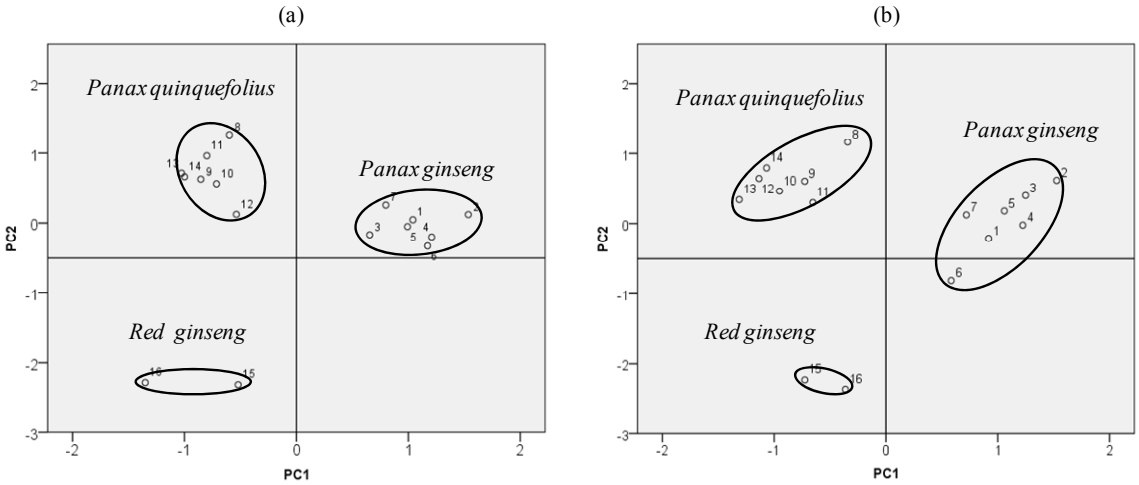


Figure 2.17. PCA analysis of the MALDI-MS results obtained for *Panax ginseng*, *Panax quinquefolius* and red ginseng using the solution-based method (a) and powder method (b). Only the first and second principal components (PC1 and PC2) are shown. 1-7 are *Panax ginseng* samples from China; 8-10, 11-13, and 14 are *Panax quinquefolius* samples from Canada, the USA and China respectively; 15 and 16 are red ginseng samples from South Korea and China respectively.

2.4 Conclusions

In this paper, we reported a MALDI-MS-based method for differentiation between two analogue herbs, *Panax ginseng* and *Panax quinquefolium*. From the acquired MALDI-MS spectra, different patterns of ginsenosides and small molecules were observed for *Panax ginseng* and *Panax quinquefolium*, allowing unambiguous differentiation between the two herbs species based on the specific ions, intensity ratios of characteristic ions or PCA analysis. The highly reproducible intensity ratios of characteristic ions enabled quantitative determination of herbal components, e.g., ginsenosides, by MALDI-MS. Red ginseng could also be distinguished from *Panax ginseng* and *Panax quinquefolium* by the method. Only a brief extraction and a small sample scale were required for acquisition of quality MS data for differentiation. In addition, differentiation could be achieved by direct analysis of solid herbal materials, bypassing the need of the solvent extraction and even the homogenization process.

As a comparison, LC-ESI-MS analysis provided a more complete profile of gensinosides for *Panax ginseng* and *Panax quinquefolium*, while MALDI-MS could not detect some of the ginsenosides presumably due to the ion suppression. However, MALDI-MS could detect some characteristic small molecules that could not be observed by LC-ESI-MS. MALDI-MS and LC-ESI-MS are thus complementary for analysis of *Panax ginseng* and *Panax quinquefolium*. Moreover, the MALDI-MS approach developed in this study is rapid, simple and robust. Further applications of this approach to other families of herbal medicines are being tested.

Chapter 3: Rapid Differentiation of *Schisandra sphenanthera* and *Schisandra chinensis* by Matrix-assisted Laser Desorption/Ionization Mass Spectrometry

3.1 Introduction

Both *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.[9] Due to the therapeutic effects of these fruits, they are widely used in Korea, Japan and China. The 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, [9, 141] and *S. chinensis* is considered as better in quality.[141, 142] Therefore, it is necessary to develop approaches for differentiation between these two fruits.

Thin layer chromatography (TLC) method is suggested by the Chinese Pharmacopoeia for differentiation between *S. chinensis* and *S. sphenanthera*.[9] Gao reported [143] 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 TLC method is relatively simple and convenient, it has poor resolution and sensitivity. In addition, it usually involves a tedious sample cleanup procedure and development using a solvent system. High performance liquid chromatography (HPLC) coupled with an ultraviolet (UV) detector is suggested by the

Chinese Pharmacopoeia as an alternative method for differentiation between *S. chinensis* and *S. sphenanthera*.[9] According to the Chinese Pharmacopoeia, [9] *S. sphenanthera* contains schisantherin A of no less than 0.12% while *S. chinensis* contains schisandrin of no less than 0.4%. HPLC can be used to separate and quantitatively determine these markers, thus allowing the authentication of these two herbs.[143-146] In addition to TLC and HPLC, gas chromatography (GC)[147] and capillary electrophoresis (CE)[148] were also used to characterize *Fructus Schisandrae*.

This chapter introduces a rapid and simple MALDI-MS-based approach for differentiation between *S. sphenanthera* and *S. chinensis*. *S. sphenanthera* and *S. chinensis* samples were analyzed by MALDI-MS either after a brief extraction of their powders or directly in their powder form or on their raw materials. The results revealed that these two species could be differentiated based on the MALDI-MS spectra acquired.

3.2 Experimental

3.2.1 Materials

Dried *S. sphenanthera* and *S. chinensis* from both local and mainland markets were used in the study. These samples were supplied by Prof. C. T. Che (School of Chinese Medicine, The Chinese University of Hong Kong) and Dr. K. W. Mok (Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University). Their identities are listed as Table 3.1 and 3.2:

Table 3.1. *S. sphenanthera* samples from different harvesting locations

	Original Sample ID	**Sample ID in this study**	**Production/ collection area**
Local samples	Lnwwzs 001	Ln1	Guizhou
	Lnwwzs 002	Ln2	Liaoning
	Lnwwzs 003	Ln3	Shaanxi
	Lnwwzs 004	Ln4	Henan
Mainland samples	Nwwzs 001	N1	Shanxi, Yunchun
	Nwwzs 002	N2	Shaanxi, shangluo
	Nwwzs 003	N3	Shanxi, Yongji
	Nwwzs 004	N4	Shanxi, Hengqu
	Nwwzs 005	N5	Hubei. Guangshui
	Nwwzs 006	N6	Henan, Lushi

Table 3.2. *S. chinensis* samples from different harvesting locations

	Orginal Sample ID	**Sample ID in this study**	**Production/ collection area**
Local samples	Lwwzs 001	Lb1	Heilongjiang
	Lwwzs 002	Lb2	Jilin
	Lwwzs 003	Lb3	Shaanxi
	Lwwzs 004	Lb4	Liaoning
Mainland samples	Wwzs 001	B1	Liaoning, Fushan
	Wwzs 002	B2	Liaoning, Benxi
	Wwzs 003	B3	Jilin, Fusong
	Wwzs 004	B4	Jilin, Jian
	Wwzs 005	B5	Heilongjiang, Yichun
	Wwzs 006	B6	Liaoning, Anshan

MALDI matrices α-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA), and 2,5-dihydroxylbenzoic acid (DHB) were purchased from Fluka. 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).

3.2.2 Preparation of herb extracts

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 uL 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 13000 rpm for 30 s and the resulting supernatant was used for analysis.

3.2.3 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 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).

3.2.4 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 23.5kV. 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 8kV and selected in a time–ion-gate. The fragments were further accelerated by 19kV 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.

3.2.5 Sample preparation for direct analysis of solid herbal materials

-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 firm

ly adhered. Subsequently, 1 μL of the CHCA matrix solution was spotted onto he top of the adhered herb powder, and then air-dried for analysis.

-*Slice method*

A thin slice of raw herb sample was cut and adhered to the surface of a double-sided tape pre-attached on the target plate. Afterward, 1 μL of CHCA matrix solution was spotted onto the slice surface, and then air-dried for analysis.

-*Sandpaper method*

A small piece of sandpaper (P1000-grit SiC abrasive paper) was first fixed onto a spot of the target plate using a double-sided tape. Raw herb sample was directly rubbed on the sandpaper until a certain amount of herb powder was adhered onto the sandpaper surface. Subsequently, 1 μL of CHCA matrix solution was spotted onto the sandpaper deposited with herb powder, and then air-dried for analysis.

3.2.6 Principal component analysis (PCA)

Principal component analysis (PCA) was carried out using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). The first (PC1) and the second principal components (PC2) were chosen to obtain a score plot. Details of PCA are shown in *Section 3.3.3.4* in **Results and discussion**.

3.3 Results and discussion

3.3.1 Optimization of extraction condition

Different solvents, sample scales and extraction durations were examined in this study for optimal extraction efficiency.

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, and the MALDI spectra were predominant with the matrix peaks (Fig. 3.1). 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 preparation of MALDI matrix.

The extraction efficiency of different sonication durations (0, 1, 3, 10, 20, 40 and 60 min) were examined. As shown in Fig. 3.2, sample peaks were significantly observed with 3 min sonication and were not further improved with extension of sonication. 3 min sonication was thus chosen for 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. The spectra obtained were similar to those obtained by extracting 5 mg of the herb powders with 500 μL extraction solvent.

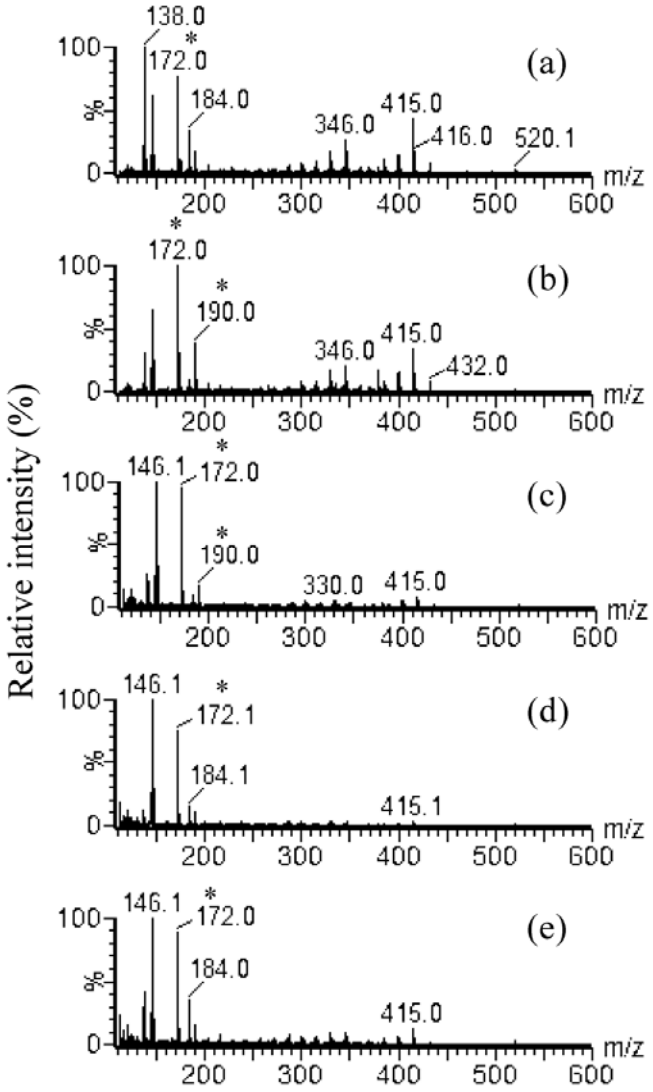


Figure 3.1. MALDI-MS spectra obtained for *S. sphenanthera* after extracted with 0.1% TFA in: (a) 50% ACN; (b) 50% MeOH; (c) 100% H_2O; (d) 100% ACN, and (e) 100% MeOH. Mass peaks from the CHCA matrix are labeled with "*".

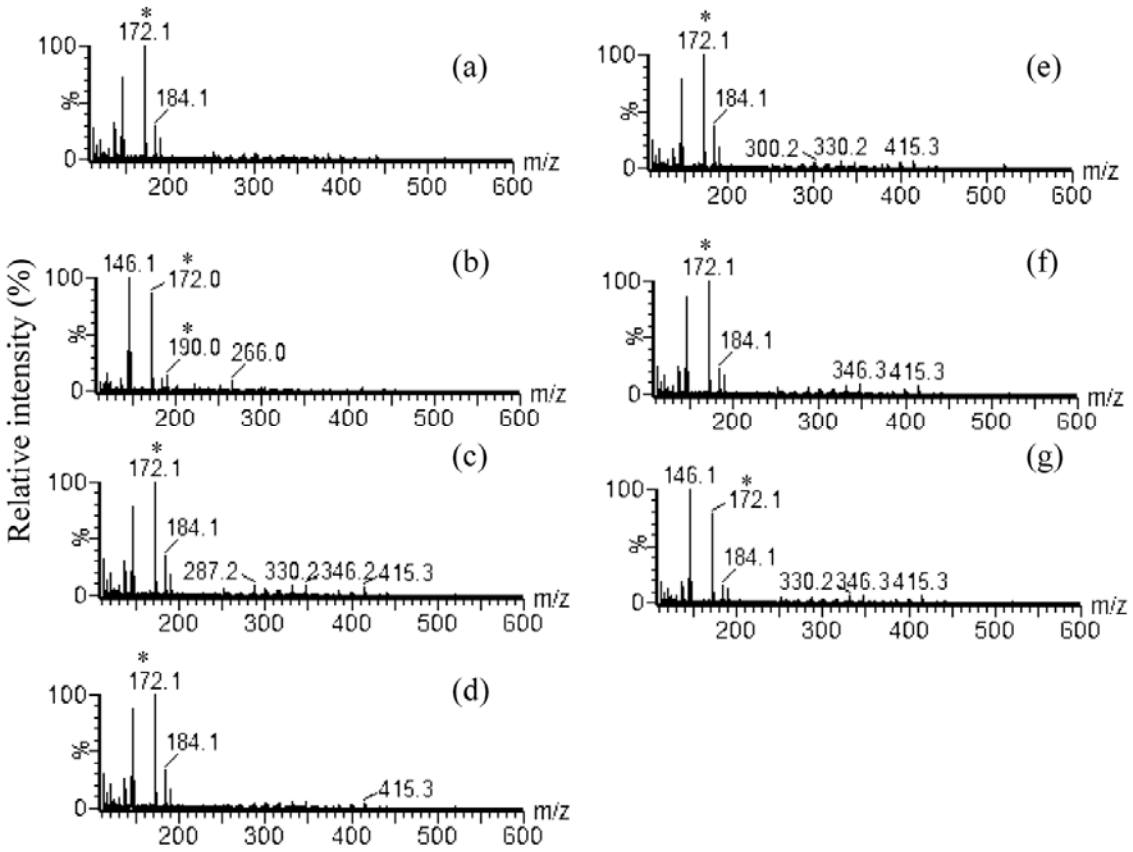


Figure 3.2. MALDI-MS spectra obtained for *S. sphenanthera* with different extraction time: (a) 0 min; (b) 1 min; (c) 3 min; (d) 10 min; (e) 20 min; (f) 40 min and (g) 60 min. Mass peaks from the CHCA matrix are labeled with "*".

DHB, CHCA, SA and THAP, four commonly used MALDI matrices, were tested. CHCA was chosen since it produced more sample peaks and had very low-level matrix interferences. Most sample peaks observed with CHCA could also be detected with SA, but the matrix interferences of the latter were more significant. For DHB and THAP, fewer sample peaks and strong matrix peaks were observed.

3.3.2 Differentiation between S. sphenanthera and S. chinensis

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

Powders of *S. sphenanthera* and *S. chinensis* were subjected to ultrasonic extraction for 3 min using 50/50 ACN/H_2O with 0.1% TFA as the extraction solvent. The extracts were analyzed by MALDI-MS with CHCA as the matrix. No significant peaks were observed beyond 600 Da. As shown in Fig. 3.3 and Table 3.3, common mass 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.

To our knowledge, this is the first time that MALDI-MS was used to study *Fructus Schisandrae*. Lignans, schisantherin A, schisandrol B, schisandrin B, schisandrin and deoxyschisandrin are the major bioactive components of *Fructus Schisandrae* and had been previously investigated by LC-ESI-MS.[142, 149-155] Some mass peaks in the acquired MALDI-MS spectra (Fig. 3.3) could be assigned to lignans (Table 3.4) according to their masses[151] and by comparison with the MALDI-MS spectra of the standards (Fig. 3.4). Mass peaks at m/z 432 and 575 were observed only for *S. chinensis* and *S. sphenanthera* respectively (Fig. 3.3). These two peaks were confirmed to be schisandrin and schisantherin A respectively by comparison of their MALDI-MS/MS spectra with those of the standards (Figs. 3.5 and 3.6). These results were consistent with the literature that reported schisandrin as a marker of *S. chinensis* and schisantherin A as a marker of *S. sphenanthera*,[141] indicating that our 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*. These peaks could be used as fingerprint for identification of the two species respectively.

Spectral reproducibility of the *Fructus Schisandrae* samples was examined in this study. First, the reproducibility 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 shown in Fig. 3.7a, the mass spectra obtained in different experimental runs were highly similar. Second, herb samples of the same species but from different harvesting location were analyzed. As shown in Fig. 3.7b, the mass spectra obtained for three samples from Liangning (Fushan), Jilin (Jian) and Heilongjiang (Yichun) were very similar to one another, although the relative intensities of their peaks were slightly different. These data demonstrated a high degree of "inter-run' and 'inter-sample" reproducibility of the present MALDI-MS fingerprinting method.

Table 3.3. Summary of the mass peaks observed in the MALDI spectra of the extracts from the *Fructus Schisandrae* samples.[a]

Fructus Schisandrae	Peaks observed
S. sphenanthera	136, 138, 184, 222, 266, 399, 415, 416, 455, 520, 534, 553 , 558, 575
S. chinensis	136, 138, 184, 222, 266, 287, 330, 346, 368, 399, 415, 416, 432, 520

[a] Only non-matrix peaks with relative abundances higher than 5% were included.

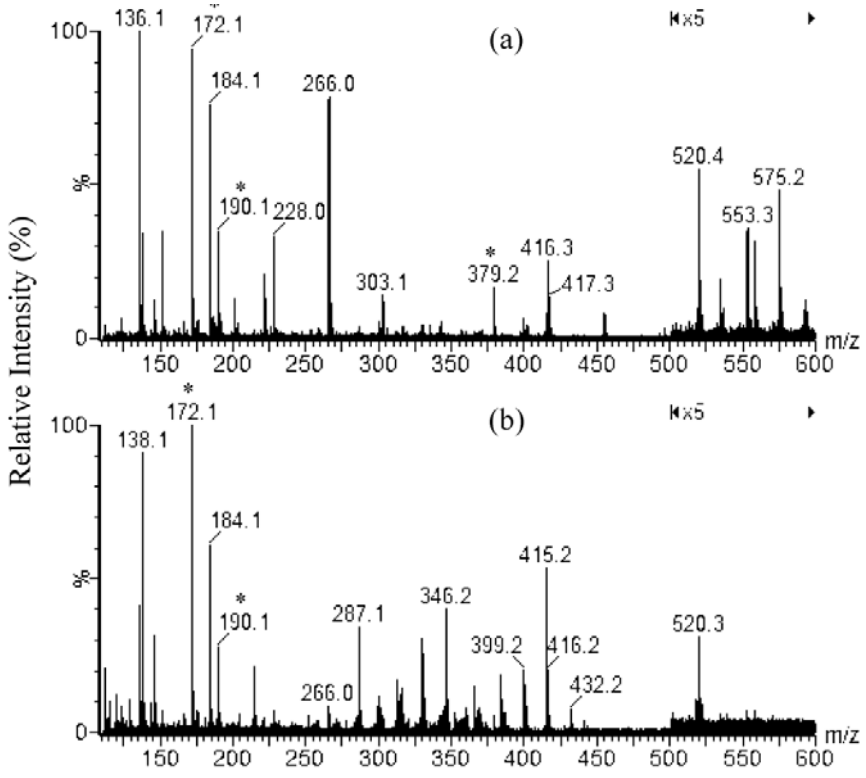


Figure 3.3. MALDI-MS spectra for extracts of *S. sphenanthera* (a) and *S. chinensis* (b). Mass peaks from the matrix are labeled with "*".

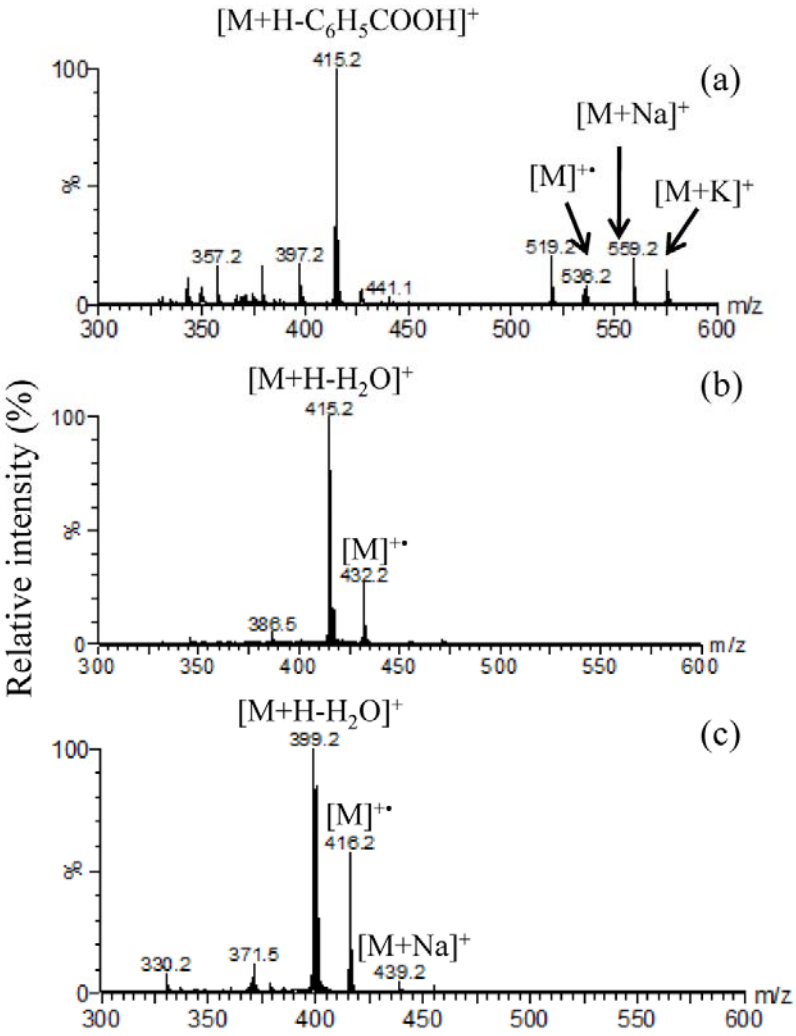


Figure 3.4. MALDI-MS spectra of schisantherin A (a), schisandrin (b) and schisandrol B (c).

Table 3.4. Summary of the mass peaks observed in the MALDI-MS spectra of the extracts from the *Fructus Schisandrae* samples that could be assigned to lignans.[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.

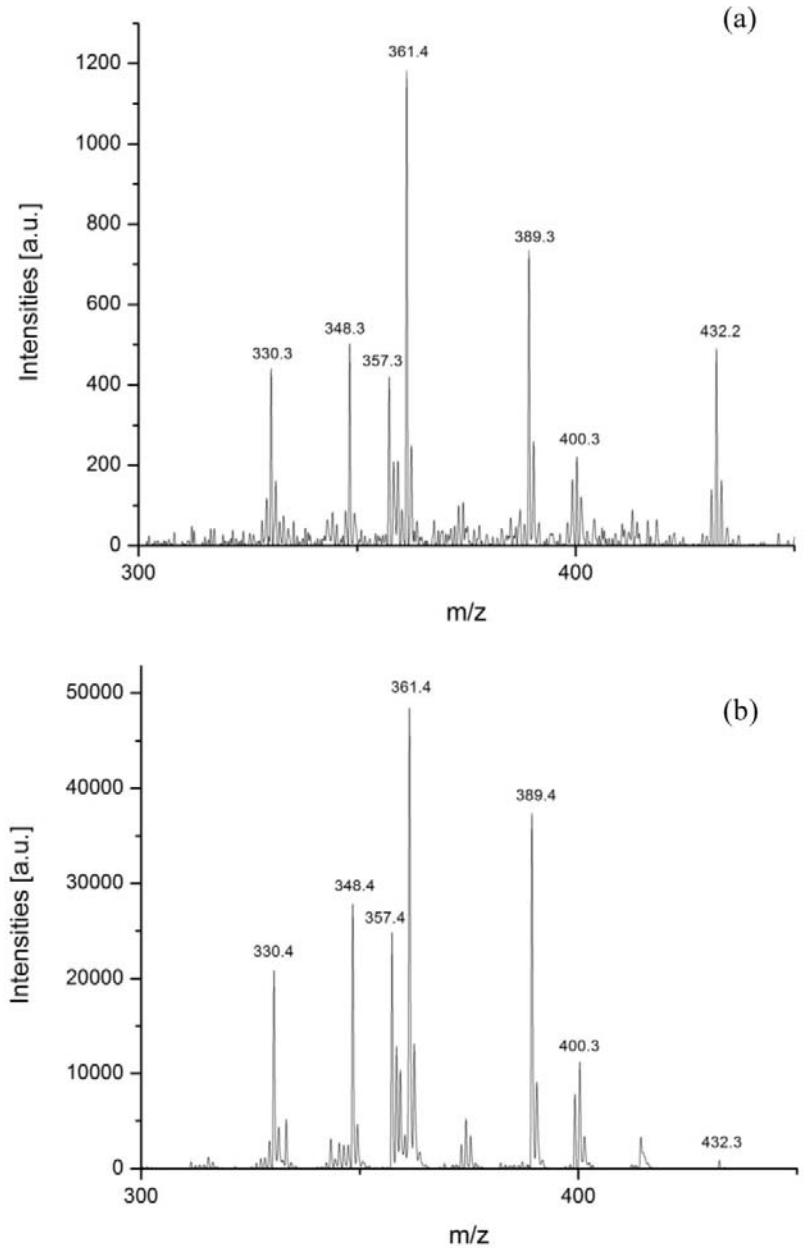


Figure 3.5. MALDI-MS/MS spectra of mass peaks at m/z 432 from *S. chinensis* (a) and schisandrin (b).

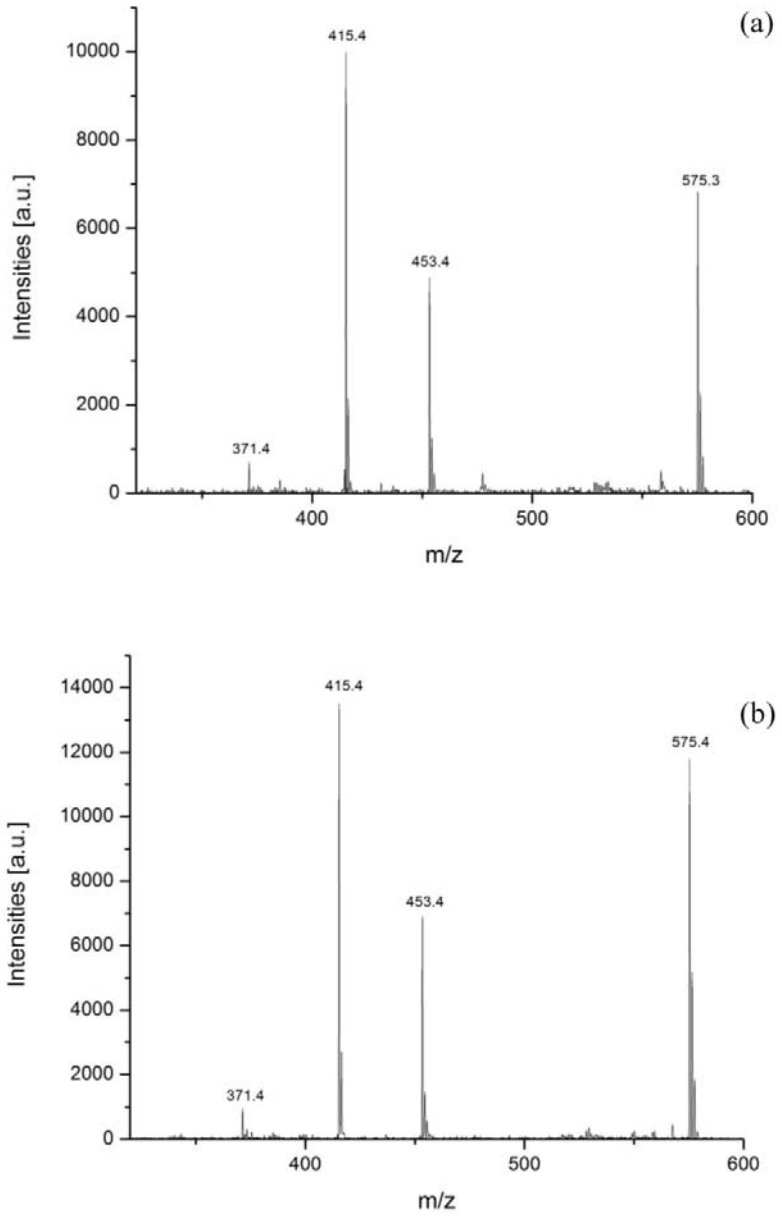


Figure 3.6. MALDI-MS/MS spectra of mass peaks at m/z 575 from *S. sphenanthera* (a) and schisantherin A (b).

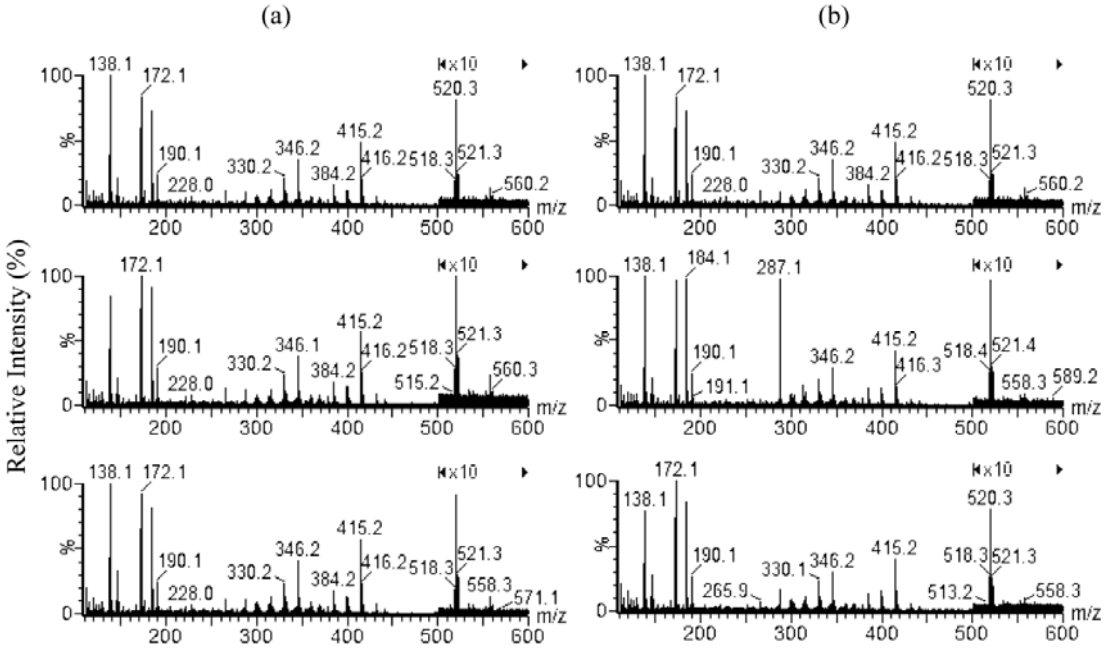


Figure 3.7. MALDI spectra for *S. chinensis*: (a) "inter-run" reproducibility and (b) "inter-sample" reproducibility.

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

The mass 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, the mass peak at m/z 416 might be contributed from schisandrol B or deoxyschizandrin (Table 3.2). 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. 3.8a). This phenomenon was observed for all investigated *Fructus Schisandrae* samples except for N6, for which these two mass peaks were absent. I_{416}/I_{415}, the intensity ratio of these two mass peaks, was determined to be 2.19 ± 0.05 for *S. sphenanthera*, and 0.39 ± 0.01 for *S. chinensis*, showing a significant difference

between the two species. The standard errors obtained for the experimental data were as small as 2 %, indicating a high level of “inter-run” and “inter-sample” reproducibility. After the logarithm of the I_{416}/I_{415} ratio was carried out, positive and negative values were obtained for *S. sphenanthera* and *S. chinensis* respectively, allowing unambiguous differentiation between the two herbal species (Fig. 3.8b).

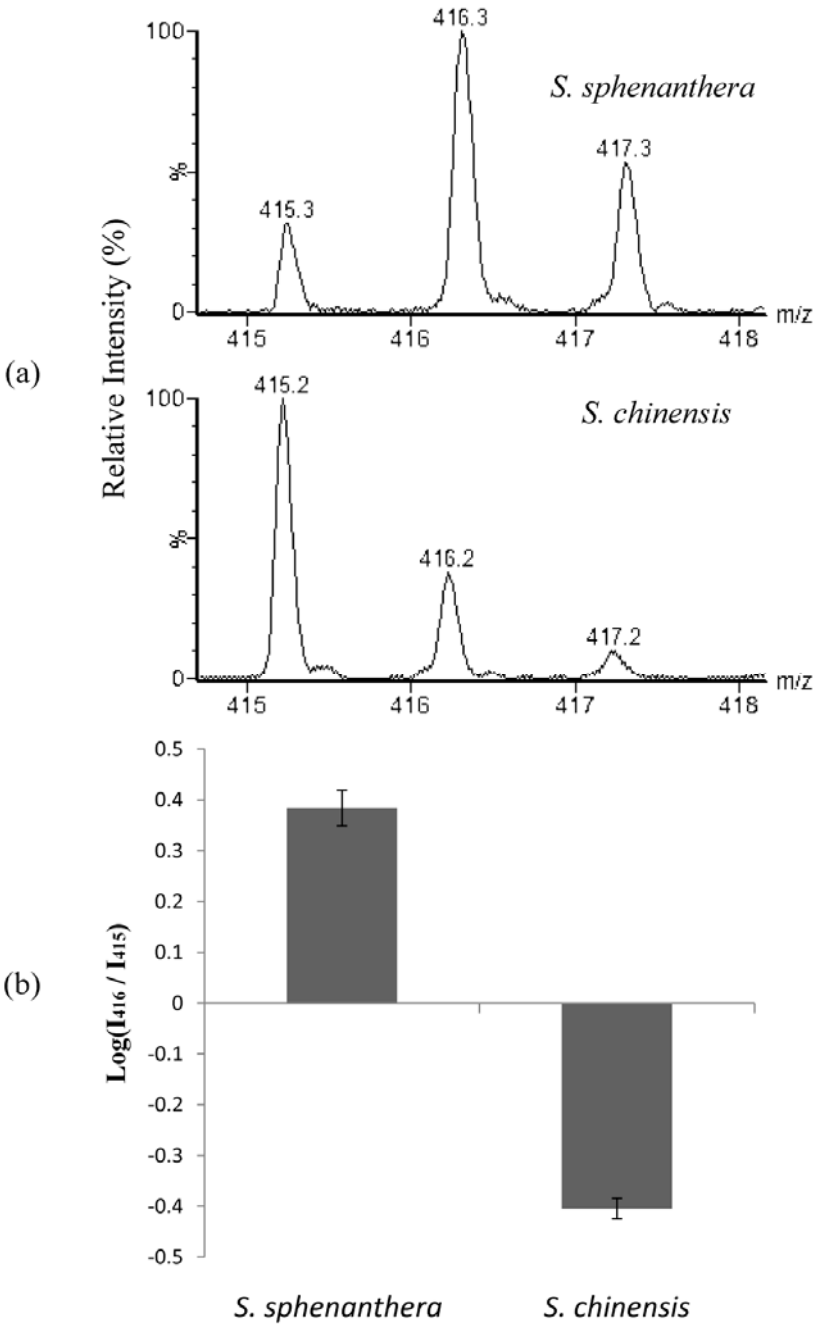


Figure 3.8. (a) MALDI-MS spectra for mass peaks at m/z 415 and m/z 416 for *S. sphenanthera* and *S. chinensis*. (b) Plot for logarithm of I_{416}/I_{415} against the *Fructus Schisandrae* sample.

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

Mass peaks of m/z 138 and 136 were another pair of peaks that showed significant difference in the spectra for *S. sphenanthera* and *S. chinensis*. For *S. sphenanthera*, the mass peak at m/z 136 had much higher intensity than m/z 138, while the opposite was observed for *S. chinensis* (Fig. 3.9a). 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 and 2.07 ± 0.01 respectively. These data demonstrated that the I_{138}/I_{136} ratio could be a useful parameter for 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 unambiguous differentiation between these two herbal species (Fig. 3.9b).

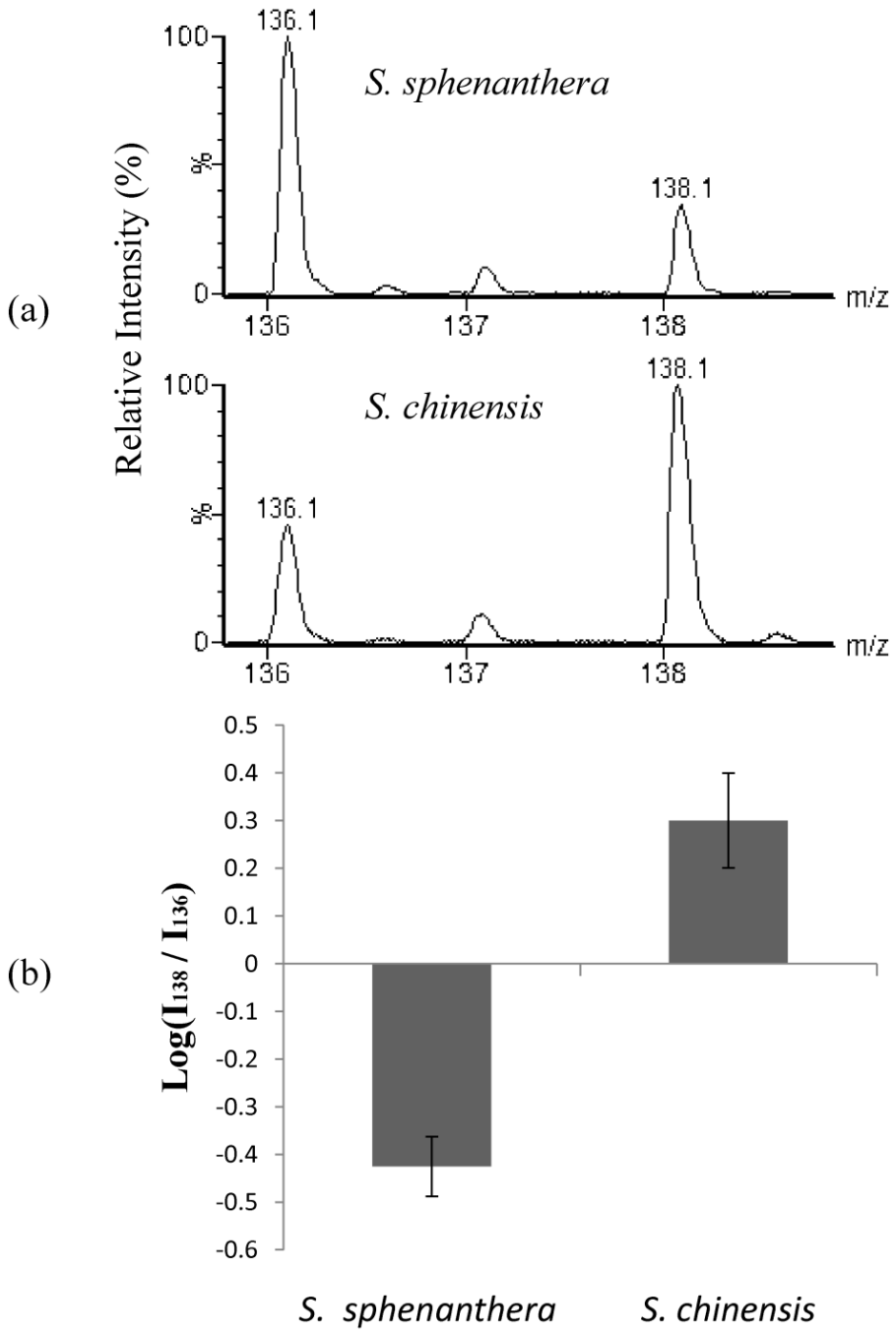


Figure 3.9. (a) MALDI-MS spectra at mass range of 136 -138 Da for *S. sphenanthera* and *S. chinensis*. (b) Plot for logarithm of I_{138}/I_{136} against the *Fructus Schisandrae* sample.

3.3.3.4. Principal component analysis (PCA)

PCA was also employed for differentiation between *S. sphenanthera* and *S. chinensis* in this study. The data obtained from three independent measurements of each sample were averaged and input for PCA analysis. *S. sphenanthera* and *S. chinensis* were clearly distinguishable in the PCA score plot (Fig. 3.10). 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 a different processing.

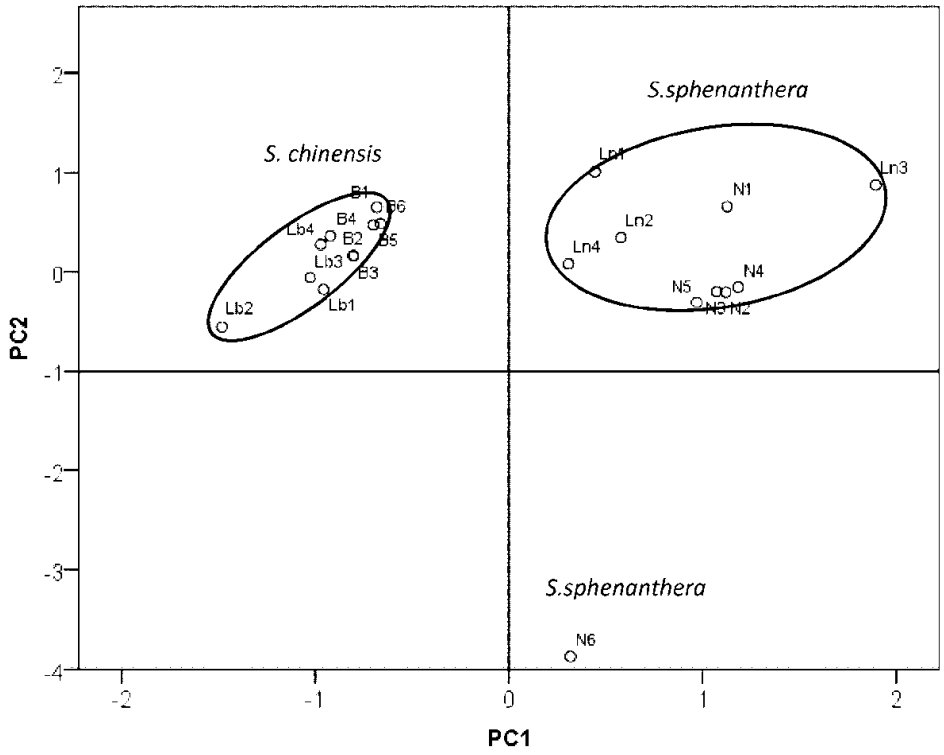


Figure 3.10. PCA score plot for *Fructus Schisandrae* samples with principal components 1 and 2.

3.3.3 Direct analysis of Fructus Schisandrae

3.3.3.1 Powder method

The patterns of the spectra for the two species obtained by the powder method were very similar to those obtained by the solution-based method (Fig. 3.11). Specific mass peak at 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 the powder method for the 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 solution-based 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. 3.12, 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 solution-based 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 to very fine powders to enable efficient extraction in very short time and without sonication.

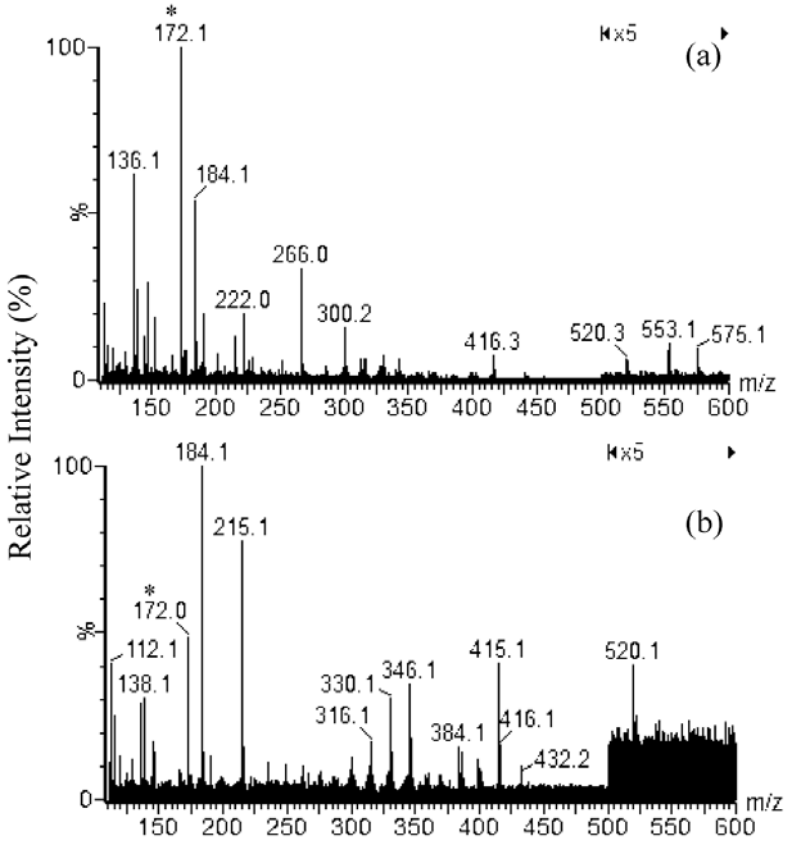


Figure 3.11. MALDI-MS spectra for *S. sphenanthera* (a) and *S. chinensis* (b) obtained by the powder method. Mass peaks from the matrix are labeled with "*".

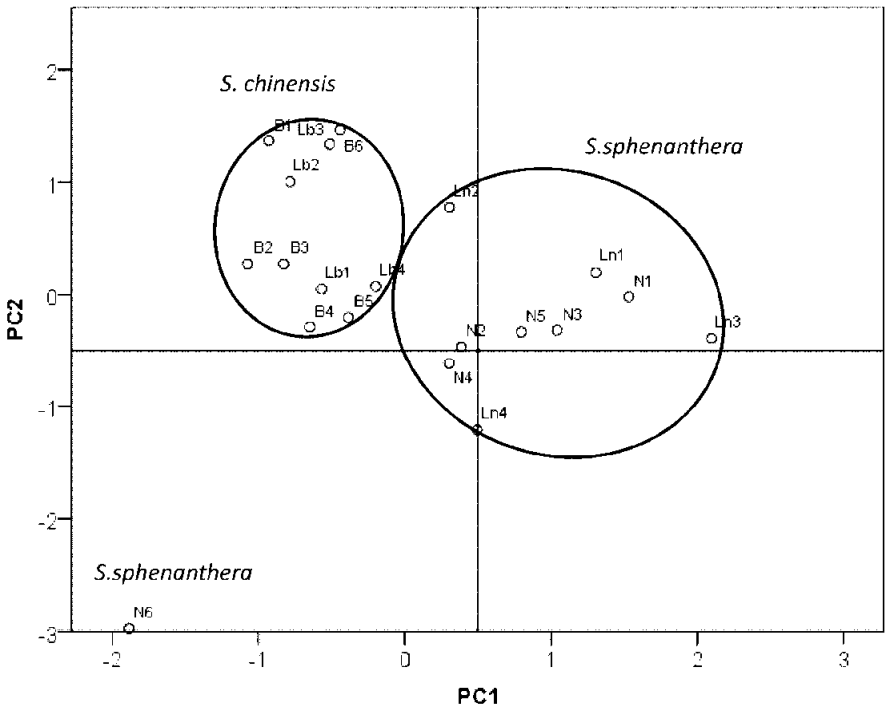


Figure 3.12. PCA score plot for different types of *Fructus Schisandrae* samples based on their MALDI-MS spectra obtained by the powder method.

3.3.3.2 Slice method and sandpaper method

Three samples were available in raw forms for *S. sphenanthera* and *S. chinensis* each. They were attempted to be analyzed by the slice method and sandpaper method with three independent experiments performed on each sample. Compared to the spectra obtained by the solution-based method, the MALDI-MS spectra obtained by these two methods generally had similar mass patterns but poorer quality and reproducibility due to the low extraction efficiency of the two methods. Specific mass peaks such as m/z 432 and 575 were not always observed for the corresponding species. For the slice

method, the I_{416}/I_{415} ratio obtained was 1.77± 0.30 for *S. sphenanthera* and 0.44 ± 0.14 for *S. chinensis,* and the I_{138}/I_{136} ratio obtained was 0.54 ± 0.19 for *S. sphenanthera* and 6.27 ± 0.87 for *S. chinensis,* indicating that the two species could be differentiated using the slice method, by comparing the I_{416}/I_{415} or I_{138}/I_{136} ratios, or simply by the positive or negative values of logarithm of I_{416}/I_{415} or logarithm of I_{138}/I_{136}. For the sandpaper method, mass peaks at m/z 416 and m/z 415 could not always be observed in the spectra. The I_{138}/I_{136} ratio obtained was 0.55 ± 0.22 for *S. sphenanthera* and 6.20 ± 2.07 for *S. chinensis*, indicating that the two species could be differentiated using the sandpaper method, by comparing the I_{138}/I_{136} ratios, or simply by the positive or negative values of logarithm of I_{138}/I_{136}.

3.4 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 quality spectra. *S. sphenanthera* and *S. chinensis* could be differentiated from each other based on the specific mass peaks (e.g., m/z 432 for *S. chinensis* and m/z 575 for *S. sphenanthera*), intensity ratios of characteristic mass 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 and raw herbal materials by MALDI-MS was also attempted and the results demonstrated that *S. sphenanthera* and *S. chinensis* could be differentiated by these simple and fast approaches.

Chapter 4: Differentiation of *Angelica* Roots by Matrix-assisted Laser Desorption/Ionization Mass Spectrometry

4.1 Introduction

Angelica Sinensis (Chinese Danggui) is a common traditional Chinese medicine (TCM) that has been used for thousands of years. Traditionally, this herbal medicine has been mainly used for prompting blood circulation system and treating gynecology-related health problems such as menstrual disorder.[156, 157] Scientific investigation indicated that this herbal medicine was effective in treatment of a wide range of physiological disorders or diseases, such as hypertension, chronic bronchitis and asthma.[156, 157] Due to its versatile functions, *A. Sinensis* has been extensively used worldwide.

Apart from *A. sinensis* that is grown in China, other analogous *Angelica* roots grown in different countries have also been widely used. In southern Asia, *Angelica acutiloba* (Japanese Danggui) and *Angelica gigas* (Korean Danggui), two *Angelica* roots grown in Japan and Korea respectively, are commonly used as substituent of *A. sinensis* because of the shortage of *A. sinensis*.[156, 158-160] *Angelica archangelica* (European Danggui), another analogous herb grown in Europe, is commonly used not only for healthcare, but also as spice or cake decorations in European countries.[158] These four common *Angelica roots* from different harvesting locations were, however, found to exhibit different pharmacological properties and efficacies.[157, 161] Therefore, differentiation of these herbal species is of great importance for proper medicinal formulation and food safety.

Different scientific approaches have been developed to distinguish *Angelica* roots from different harvesting countries. DNA analysis has been used to identify *Angelica*

roots.[161] However, this technique involved tedious procedures including extraction of DNA and PCR analysis and the DNA templates could be easily degraded and contaminated during the sample processing.[28] *Angelica* roots could also be distinguished based on their chemical compositions as determined by techniques such gas chromatography-mass spectrometry (GC-MS) and liquid chromatography (LC)-MS.[10, 156, 157, 162-173] Various chemical compounds, including Ferulic acid and phthalides such as Z-ligutilide, were believed to be the active components of *Angelica* roots and thus often targeted for assessing the quality of these herbs.[156, 157, 161] These approaches involved chromatographic separation, and were usually labor-intensive and time-consuming.

In this study, MALDI-MS was used to distinguish *Angelica* roots from different harvesting locations. The major goal of this study was to develop a simple and rapid method for differentiation of these herbal species. The herbal samples were analyzed by MALDI-MS either after a brief extraction or directly on the herbal powders. The patterns of the spectra obtained and principal component analysis (PCA) of the MALDI-MS data were used for differentiation of *Angelica* roots from different sources.

4.2 Experimental

4.2.1 Materials

Angelica root samples, as listed in Table 4.1, were provided in grounded powder forms by Dr. Leung, Kelvin S. Y. (Department of Chemistry, Hong Kong Baptist University).

Table 4.1. *Angelica* root samples from different harvesting locations.

Sample name	Type of *Angelica* root	Harvesting location
D1	*A. sinensis*	China, Sichuen
D2		
D3		
J1	*A. acutiloba*	Japan
J2		
J3		
E1	*A. archangelica L.*	Europe, Poland
E2		Europe, UK
E4		
K1	*A. gigas*	Korea, Seoul
K2		
K3		
K4		
K5		
K6		

MALDI matrices α-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA), and 2,5-dihydroxylbenzoic acid (DHB) were purchased from Fluka. 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).

4.2.2 Preparation of herb extracts

Herbal powder samples were further grounded into finer powder by a mortar grinder. 5 mg fine powder was weighed into a 1.5 mL eppendorf centrifuge tube, and 200 uL of 50/50 MeOH/H_2O (v/v) with 0.1% TFA was added. The samples were sonicated for 3 min in an ultrasonic water bath. All samples were sonicated together with the help of a vial stand. The samples were then centrifuged at 13000 rpm for 30 s.

4.2.3 MALDI-MS

An aliquot of 10 μL of extract supernatant was mixed with 10 μL of 10 mg mL^{-1} CHCA matrix in 50/50 ACN/H_2O (v/v) 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 modes. 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. A mass range of 100 – 600 Da was used for spectral acquisition with the use of "low-mass bias" mode. 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. All mass spectra were obtained by accumulation of 200 scans (10 laser shots per scan).

4.2.4 Direct analysis of herb powder of Angelica roots

A small piece of double-sided adhesive tape was attached onto a spot of the target plate. Approximately 0.1 mg 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

4.2.5 Principal component analysis (PCA)

Angelica roots from different harvesting locations were differentiated by PCA based on the percentage of relative ion intensities of mass peaks observed in MALDI-MS spectra. PCA was carried out using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA) program. The first and the second principal component were used to obtain the score plot. Details of PCA are shown in *Section 4.3.2.2* in **Results and discussion**.

4.3 Results and discussion

4.3.1 Optimization of extraction conditions

Various extraction conditions, including different extraction solvents, sample scales (amount of herb powder and volume of extraction solvent) and duration of sonication, were investigated for optimal extraction efficiency. Solvent systems of different polarities were used for extraction and the mass spectra obtained are shown in Fig 4.1. Relatively polar solvent systems such as 50/50 ACN/H_2O (v/v), 50/50 MeOH/H_2O (v/v), 100% H_2O, 100% ACN and 100% MeOH (all containing 0.1 % TFA) were found to produce most sample peaks with acceptable intensity (Fig.4.1a-e), indicating that desirable extraction efficiency could be achieved by using these solvent systems. The spectral patterns obtained for these relatively polar solvent systems were similar to each other. The solvent system of 100% MeOH with 0.1% TFA was finally chosen for extraction of *Angelica* roots since quality mass spectra could be generated with high reproducibility at different positions within a sample spot (position-to-position reproducibility). For relatively non-polar solvent systems, e.g., 100% acetone and 100% chloroform (both containing 0.1% TFA), fewer sample peaks were observed, implying that the extraction efficiency with non-polar solvents was unsatisfactory.

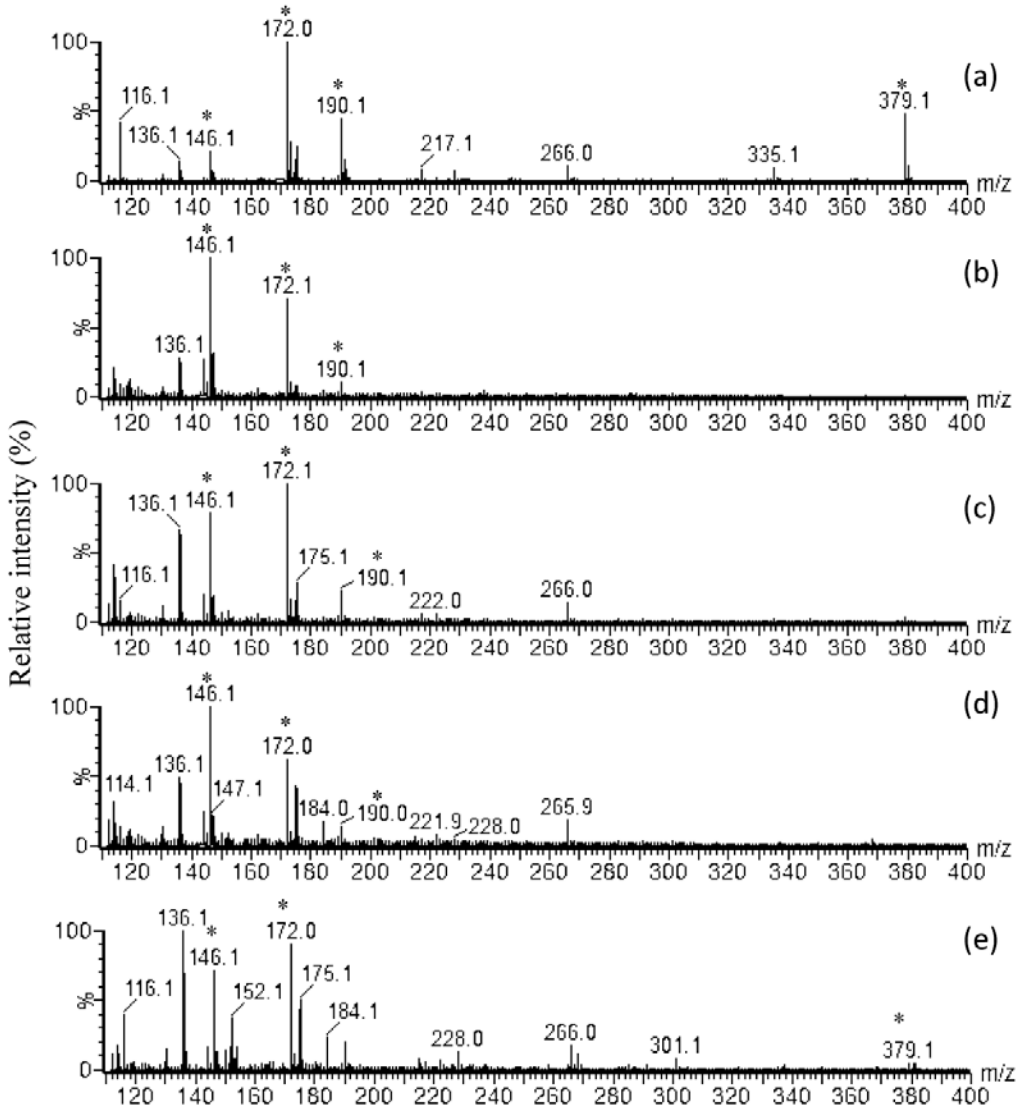


Figure 4.1. MALDI-MS spectra obtained for *A. acutiloba* with different extraction solvents: (a) 50% ACN, (b) 50% MeOH, (c) 100% H_2O, (d) 100% ACN and (e) 100% MeOH. All solvents contained 0.1% TFA. Mass peaks from the CHCA matrix are labeled with "*".

The effect of sample scale on the extraction was investigated. Our results showed that a sample scale of 5 mg herb powder extracted with 200 μL extraction solvent was adequate to produce desirable MALDI-MS spectra. Similar mass spectra were obtained by further enlarging the sample scale to up to 1 g herb powder extracted with 5 mL of extraction solvent (data not shown).

To test the effect of the ultrasonic extraction duration, different sonication durations (0, 1, 3, 10, 20, 40 and 60 min) were employed. With the sonication time of 0 or 1 min, the sample peaks observed were relatively weak, indicating that such short sonication durations was not enough to extract sufficient amount of chemicals for MALDI-MS analysis (Fig 4.2a and b). When the sonication duration was extended to 3 min, sample peaks with acceptable intensities could be observed (Fig 4.2c). No significant improvement in peak intensities and mass pattern was observed when the sonication duration was further extended to up to 60 min (Fig 4.2d and g). Therefore, the sonication time of 3 min was applied for solvent extraction in this study.

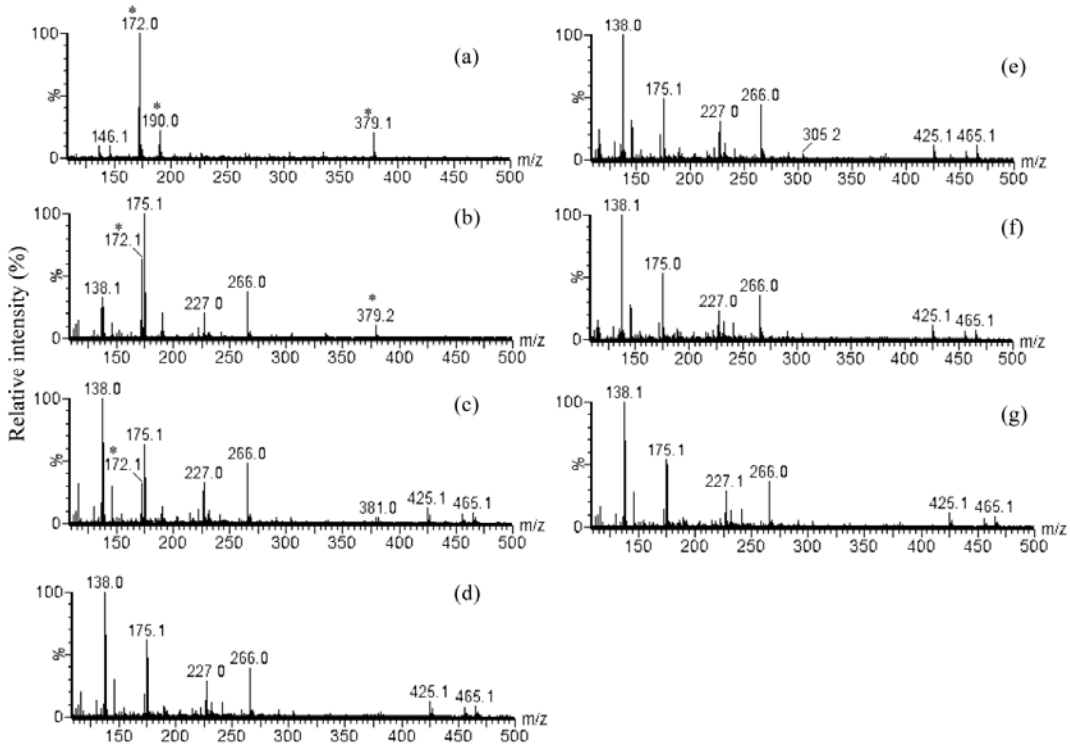


Figure 4.2. MALDI-MS spectra obtained for *A. archangelica L.* with different sonication time: (a) 0 min, (b) 1 min, (c) 3 min, (d) 10 min, (e) 20 min, (f) 40 min and (g) 60 min. Mass peaks from the CHCA matrix are labeled with "*".

DHB, CHCA, SA and THAP, four commonly used matrices, were compared in this study. CHCA was finally chosen since sample peaks of highest intensities were observed with this matrix.

4.3.2 Differentiation between Angelica roots

4.3.2.1 MALDI-MS spectra of Angelica roots from different harvesting countries

The mass spectra obtained for *Angelica* roots from different harvesting locations are shown in Fig.4.3 and the mass peaks observed are summarized in Table 4.2. In general, the mass peaks observed fell into the m/z range of 100-500 Da, beyond which no sample peak could be clearly observed (even when the "low-mass bias" function was deactivated). The CHCA matrix showed significant signals in the spectra *of A. sinensis* and *A. acutiloba*. The reported active chemical constituents of *Angelica* roots, e.g., ferulic acids and ligustilides,[156] were not observed in the spectra, probably due to the low abundances of these compounds and the ion suppression under the MALDI conditions. Some mass peaks, e.g., m/z 136 and 266, were commonly observed for the four species (Fig.4.3 and Table 4.2) and may be used as the indicators of *Angelica* roots. Each herb species was found to possess specific mass peaks that could act as the fingerprint of the species. Mass peaks at m/z 235, 294 and 484, mass peaks at m/z 217 and 301, mass peaks at m/z 227, 269, 305, 425, 455 and 465, and mass peaks at m/z 229, 247 and 329 were observed exclusively for *A. sinensis*, *A. acutiloba*, *A. archangelica L.* and *A. gigas* respectively (Fig. 4.3 and Table 4.2). Each species could thus be identified based on the observation of these specific peaks. According to the literatures, the mass peak at m/z 247 could be assigned to marmesin, decursinol, or 7-hydroxy-6-(2R-hydroxy-3-methylbut-3-enyl) coumarin, and the peak at m/z 229 could be resulted from loss of a water molecule from m/z 247.[172] The mass peak at 329 m/z could be assigned to decursin or dersinol angelate. [172, 173] Further investigation is needed for confirmation of these identities and for identification of other mass peaks.

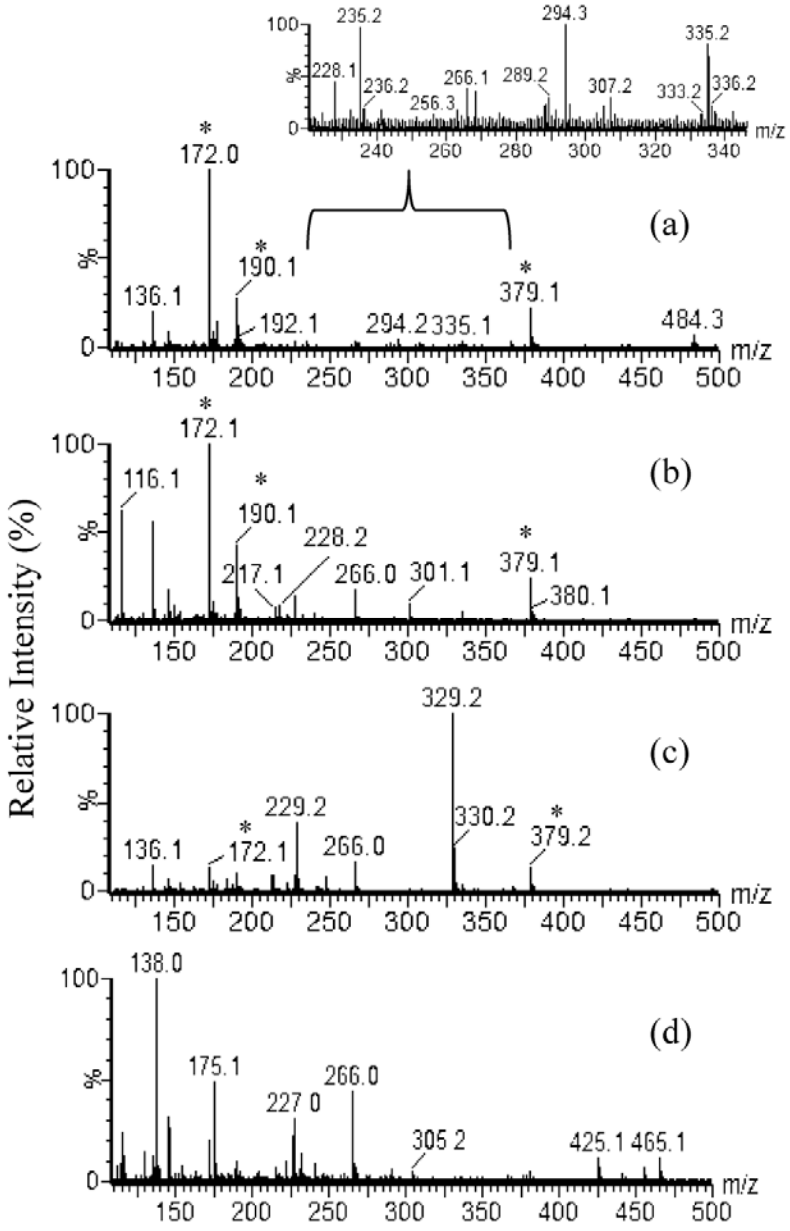


Figure 4.3. MALDI-MS spectra of *Angelica* roots from different harvesting locations: (a) *A. sinensis*, (b) *A.Acutiloba*, (c) *A. archangelica L.*and (d) *A.gigas.* Mass peaks from the CHCA matrix are labeled with "*".

Table 4.2. Summary of the mass peaks observed in the MALDI-MS spectra of *Angelica* roots investigated. [a]

Angelica roots	Peaks observed (m/z)
A. sinensis	136, 228, **235**, 266, **294**, 335, **484**
A. acutiloba	116, 136, **217**, 228, 266, **301**, 335
A. archangelica L.	116, 136, 138, **227**, 266, **269, 305**, 335, **425, 455, 465**
A. gigas	136, 184, **229, 247**, 266, **329**

[a]Specific mass peaks of the species are in bold form. Only non-matrix mass peaks with relative intensity higher than 5% were included.

Two aspects of reproducibility were examined in this study. First, the reproducibility of the entire experimental methodology, so called "inter-experiment' reproducibility, was tested by performing three independent experiments for the same herb sample. As shown in Fig. 4.4, taking *A. gigas* and *A. archangelica L.* as examples, the mass spectra obtained for different experimental runs were very similar. Second, reproducibility of the mass spectral results obtained for different herb samples from the same harvesting location was investigated. As shown in Fig. 4.5, different herb samples from the same harvesting country exhibited highly similar mass spectra. These data demonstrated the high degree of "inter-experiment' and 'inter-sample" reproducibility of the present MALDI-MS method.

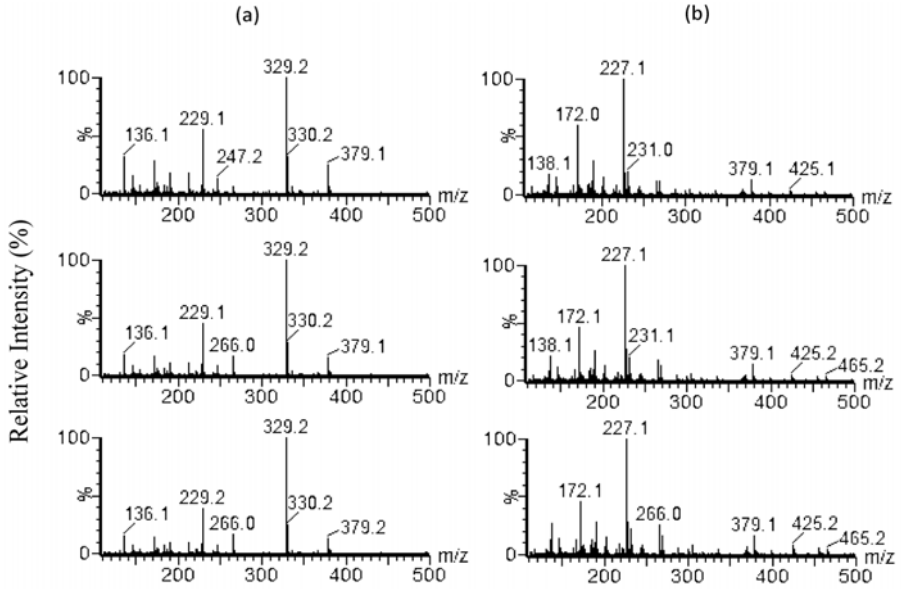


Figure 4.4. MALDI-MS spectra obtained for *A. gigas* (a) and *A. archangelica L.* (b) with three independent experiments for each.

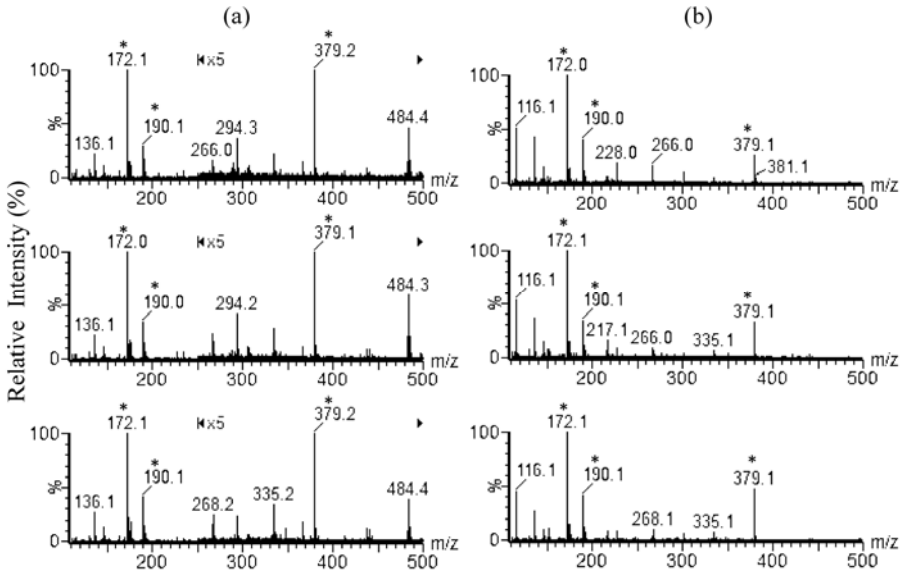


Figure 4.5. MALDI-MS spectra obtained for three different samples of *A. sinensis* (a) and *A. acutiloba* (b).

4.3.2.2 Principal component analysis (PCA)

PCA was performed to distinguish *Angelica* roots from different harvesting countries based on the MALDI-MS data obtained. The relative abundances of sample peaks observed were used for the analysis. The data obtained from three independent experiments were averaged and input for PCA analysis. As shown in Fig. 4.6, four independent clusters could be obtained for the *Angelica* roots from the four different harvesting locations, indicating that the MALDI-MS data obtained in the present study allowed unambiguous differentiation of these four herb species. The two-component PCA model cumulatively accounted for 72% of variation (PC1 = 49% and PC2 = 23%).

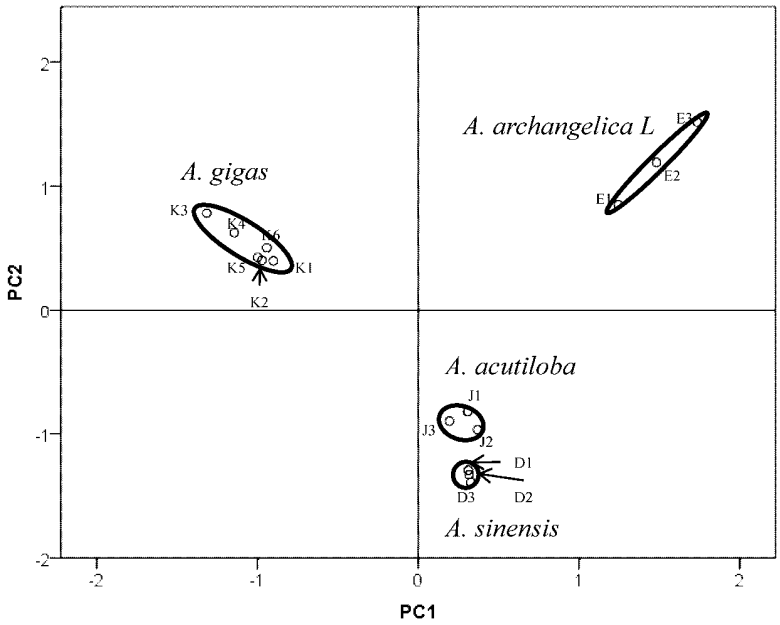


Figure 4.6. PCA analysis of the MALDI-MS data for *Angelica* roots from four different harvesting locations. Only the first and second principal components (PC1 and PC2) are shown.

4.3.3 Direct analysis of grounded powder of Angelica roots

The MALDI-MS spectra of *Angelica* roots obtained by the powder method are shown in Fig. 4.7. For *A. Sinensis* and *Angelica archangelica L.*, the MALDI-MS spectra obtained were dominated with a series of mass peaks with a mass interval of 12 Da in m/z range of 180-300 Da, which were most likely due to the formation of carbon clusters (Fig. 4.7a and b). The sample peaks observed in the solution-based method could not be detected for these two samples. Formation of carbon clusters during laser desorption/ionization or MALDI process is not uncommon, and was believed to be brought out by ionization of carbon materials.[174, 175] A possible reason is that since baking was involved in processing of the *Angelica* roots,[9] some portions of these two species might be burnt to generate carbon materials, which were ionized to form carbon cluster ions during the MALDI process.

For *A. Acutiloba*, a number of distinct peaks were observed in m/z range of 100-500 Da using the powder method, yet the mass pattern (Fig 4.7c) was significantly different from that observed in the solution-based method. For example, distinct mass peaks at m/z 156, 175 and 368 observed in the powder method were not observed in the solution-based method, while the sample peaks previously detected in the solution-base method generally could not be observed in the powder method. For *A. gigas*, the mass spectrum obtained by the powder method displayed a predominated peak at m/z 329 (corresponding to decursin or dersinol angelate), similar to that by the solution-based method (Fig 4.7d). However, other mass peaks observed in solution-based method and useful for differentiation, e.g., m/z 229 and 247, were not detected in the powder method.

The differences in the spectra obtained by the powder method and solution-based method could be due to the fact that for the powder method, the chemical components on the powder surfaces might be preferentially extracted and ionized and thus dominated in the mass spectrum, while for the solution-based method, chemical components inside the powders could also be extracted and detected. The results obtained for the four *Angelica* roots by the powder method also indicated that they might previously be subjected to different processing.

4.3 Conclusions

A simple and rapid MALDI-MS approach has been developed for differentiation of *Angelica sinensi* (Chinese Danggui)*, Angelica acutiloba* (Japanese Danggui), *Angelica gigas* (Korean Danggui) and *Angelica archangelica* (Europe Danggui), four *Angelica* roots from different harvesting countries. Only a brief ultrasound extraction and a small scale of samples were required to produce spectra that exhibited specific mass peaks for each herb species. PCA results indicated that these four *Angelica* roots could be unambiguously differentiated based on the MALDI-MS data. Differentiation by the powder method was attempted, and spectra obtained were very different from those by the solution-based method. Further investigated will be needed, in order to understand the mechanism of the difference, to identify the specific mass peaks, and to improve the spectral quality.

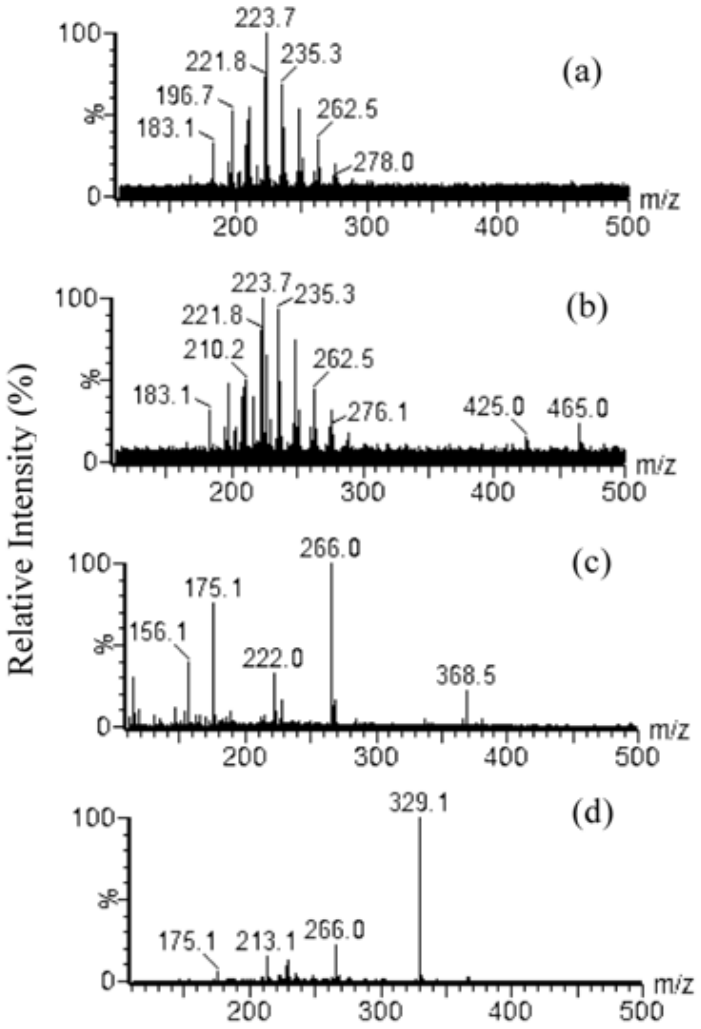


Figure 4.7. MALDI-MS spectra obtained for *Angelica* roots using the powder method. (a) *A. sinensis*, (b) *A. archangelica L.*, (c) *A. Acutiloba* and (d) *A.gigas.*

Chapter 5: Overall Conclusions

Herbal medicine (HM) has become more and more popular worldwide with increasing public concerns over the quality of HM products. Current approaches for the quality control of HMs are usually labor-intensive, time-consuming and material-consuming. In this study, we developed a MALDI-MS-based fingerprinting approach for the quality control of HMs, particularly for differentiation of different types of ginseng roots, *Fructus Schisandrae* and *Angelica* roots.

For differentiation between *Panax ginseng* and *Panax quinquefolium*, a brief ultrasound extraction and a small sample scale were sufficient for acquisition of quality MALDI-MS data. From the MALDI spectra obtained, different patterns of ginsenosides and small molecules were observed for *P. ginseng* and *P. quinquefolium*, allowing unambiguous differentiation between the two herbs species based on observation of the specific mass peaks or the intensity ratios of the characteristic mass peaks. The PCA score plot also showed that *P. ginseng* and *P. quinquefolium* could be clearly distinguished from each other. More rapid differentiation between *P. ginseng* and *P. quinquefolium* was achieved by direct analysis of the solid herb materials using the powder method, the slice method and the sandpaper method, all of which could be done without any extraction or homogenization procedures.

Similar approaches were employed for differentiation between *Schisandrae sphenanthera* and *Schisandrae chinensis*, and for differentiation among *Angelica sinensis, Angelica acutiloba, Angelica gigas* and *Angelica archangelica L.* The results

revealed that analogous species from different harvesting locations could be differentiated by their respective characteristic MALDI spectra and the PCA analysis of the spectra.

In brief, our results demonstrated that MALDI-MS could be a rapid and efficient approach for fingerprinting and differentiation of HMs. Even closely related species could be differentiated from each other based on their characteristic MALDI spectra and through PCA analysis. It is anticipated that this approach could be applied for other HMs and will become more and more popular in the quality control of HMs.

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