APPLICATIONS AND MECHANISTIC STUDIES OF DIRECT IONIZATION MASS SPECTROMETRY

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CERTIFICATE OF ORIGINALITY

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Wong Ho Yi

Abstract

Direct ionization mass spectrometry (DI-MS) is a one-step technique that could directly analyze raw solid samples, including Chinese herbal medicines (CHMs), within minutes. This technique has the advantages of simplicity, reliability and short analysis time, compared to conventional methods that typically involve homogenization, extraction, separation and centrifugation, which are labor intensive and time-consuming. The aims of this study are to develop DI-MS for rapid characterization of CHMs and to investigate its extraction and ionization mechanisms that were still unclear.

In this study, DI-MS has been developed for rapid characterization of popular and valuable CHMs, i.e. *Gastrodiae rhizoma* (known as *Tianma* in Chinese) and *Ganoderma* (known as *Lingzhi* in Chinese). Characteristic mass spectra of these CHMs could be generated by DI-MS directly from the raw herbal medicines with the application of a high voltage and solvents. Rapid authentication of *Gastrodiae rhizoma* could be achieved based on this method, as the acquired DI-MS spectra showed that the major active components of *Gastrodiae rhizoma* could be found only in genuine *Gastrodiae rhizoma* samples but not in counterfeit samples.

Similarly, rapid differentiation of the *Ganoderma* species that are officially stated in the Chinese pharmacopoeia from easily confused *Ganoderma* species could also be achieved. In addition, classification of wild and cultivated *Gastrodiae rhizoma* and *Ganoderma* and potential differentiation of *Gastrodiae rhizoma* and *Ganoderma* from different geographical locations could be accomplished based on their different intensity ratios of characteristic ions or principal component analysis (PCA) or hierarchical clustering analysis (HCA). Our experimental results confirmed that DI-MS is rapid, simple and reproducible, and can be further extended to the analysis of other herbal medicines.

Apart from the applications of DI-MS, the extraction and ionization mechanisms for DI-MS of CHMs were also investigated. CHMs with different classes of active ingredients were investigated with solvents of various properties, and the products generated at different extraction and ionization stages were collected, analyzed and compared, in order to understand the process and factors governing the signal responses of this direct analysis technique and to enhance the detection of active ingredients of CHMs. The experimental results revealed that the solvent loaded onto the surface of CHMs was crucial for the observation of desired ion signals as it served simultaneous extraction of compounds from the sample and ionization of

the extracted compounds in DI-MS. The detailed extraction and ionization mechanisms for DI-MS of CHMs were further investigated by comparing the physical properties of the analytes such as the surface activity, polarity and acidity of the analytes. The experimental results demonstrated that the extraction and ionization mechanisms for DI-MS of CHMs were related to the physical properties of the analytes, which are useful parameters for the prediction of DI-MS signal responses of analytes. These results allowed further insight into the extraction and ionization mechanisms of DI-MS in order to explore the ways to enhance the sensitivity and selectivity of DI-MS.

Research Publications

Journal Publications

1. **H.Y. Wong**, M.Y.M. Wong, B. Hu, P.K. So, C.O. Chan, D.K.W. Mok, Z.P. Yao, Rapid Differentiation of *Ganoderma* Species by Direct Ionization Mass Spectrometry. *Analytica Chimica Acta* 999 (**2018**) 99 - 106.

2. **H.Y. Wong**, B. Hu, P.K. So, C.O. Chan, D.K.W. Mok, G.Z. Xin, P. Li, Z.P. Yao, Rapid Authentication of *Gastrodiae Rhizoma* by Direct Ionization Mass Spectrometry. *Analytica Chimica Acta* 938 (**2016**) 90 - 97.

Conference Papers

3. **H.Y. Wong**, M.Y.M. Wong, B. Hu, P.K. So, Z.P. Yao, Investigation of the Extraction and Ionization Mechanism for Direct Ionization Mass Spectrometry of Traditional Chinese Medicines. *The 13th International Postgraduate Symposium on Chinese Medicine (IPSCM)*, Hong Kong Convention and Exhibition Center, Hong Kong, 17 August **2017**. **(Organizing Committee Member)**

4. **H.Y. Wong**, M.Y.M. Wong, B. Hu, P.K. So, Z.P. Yao, Investigation of the Extraction and Ionization Mechanism for Direct Ionization Mass Spectrometry of Chinese Herbal Medicines. *Hong Kong Society of Mass Spectrometry (HKSMS) Symposium 2017*, The Hong Kong Polytechnic University, Hong Kong, 24 June **2017**. **(Best Poster Award)**

5. **H.Y. Wong**, B. Hu, M.Y.M. Wong, P.K. So, Z.P. Yao, Investigation of the Extraction and Ionization Mechanism for Direct Ionization Mass Spectrometry of Chinese Herbal Medicines. *The 24th Symposium on Chemistry Postgraduate Research in Hong Kong*, City University of Hong Kong, Hong Kong, 6 May **2017**.

6. **H.Y. Wong**, B. Hu, P.K. So, C.O. Chan, D.K.W Mok, Z.P. Yao, Rapid Analysis of Chinese Herbal Medicines by Direct Ionization Mass Spectrometry. *The Sunney and Irene Chan Lecture in Chemical Biology 2016*, The Hong Kong Polytechnic University, Hong Kong, 3 October **2016**. **(Best Poster Award in Chemistry - First Prize)**

7. **H.Y. Wong**, B. Hu, P.K. So, C.O. Chan, D.K.W Mok, Z.P. Yao, Rapid Analysis of Chinese Herbal Medicines by Direct Ionization Mass Spectrometry. *The 12th International Postgraduate Symposium on Chinese Medicine (IPSCM)*, Hong Kong Convention and Exhibition Center, Hong Kong, 12 August **2016**.

8. **H.Y. Wong**, B. Hu, P.K. So, C.O. Chan, D.K.W Mok, Z.P. Yao, Rapid Analysis of Traditional Chinese Medicines by Direct Ionization Mass Spectrometry. *Hong Kong Society of Mass Spectrometry (HKSMS) Symposium 2016*, City University of Hong Kong, Hong Kong, 25 June **2016**.

9. **H.Y. Wong**, B. Hu, P.K. So, C.O. Chan, D.K.W Mok, Z.P. Yao, Rapid Analysis of Traditional Chinese Medicines by Direct Ionization Mass Spectrometry. *The 23rd Symposium on Chemistry Postgraduate Research in Hong Kong*, The Hong Kong Polytechnic University, Hong Kong, 19 April **2016**. **(Best Poster Presentation for Analytical and Environmental Chemistry)**

10. **H.Y. Wong**, B. Hu, P.K. So, C.O. Chan, D.K.W Mok, Z.P. Yao, Rapid Authentication of *Gastrodiae Rhizoma* by Direct Ionization Mass Spectrometry. *The Sunney and Irene Chan Lecture in Chemical Biology 2015*, The Hong Kong Polytechnic University, Hong Kong, 2 November **2015**

11. **H.Y. Wong**, B. Hu, P.K. So, C.O. Chan, D.K.W Mok, Z.P. Yao, Rapid Authentication of *Gastrodiae Rhizoma* by Direct Ionization Mass Spectrometry. *The 11th International Postgraduate Symposium on Chinese Medicine (IPSCM)*, Hong Kong Convention and Exhibition Center, Hong Kong, 14 August **2015**.

12. **H.Y. Wong**, B. Hu, P.K. So, C.O. Chan, D.K.W Mok, Z.P. Yao, Rapid Differentiation of *Ganoderma* Species by Direct Ionization Mass Spectrometry. *The 63rd American Society of Mass Spectrometry (ASMS) Conference on Mass Spectrometry and Allied Topics*, St Louis, Missouri, United States of America, 31 May – 4 June **2015**.

13. **H.Y. Wong**, B. Hu, P.K. So, C.O. Chan, D.K.W Mok, Z.P. Yao, Rapid Authentication of *Gastrodiae Rhizoma* by Direct Ionization Mass Spectrometry. *The 22nd Symposium on Chemistry Postgraduate Research in Hong Kong*, Hong Kong Baptist University, Hong Kong, 18 April **2015**.

14. **H.Y. Wong**, B. Hu, P.K. So, C.O. Chan, D.K.W Mok, Z.P. Yao, Direct Ionization Mass Spectrometry of *Ganoderma lucidum*, *Ganoderma sinense* and *Ganoderma duropora* Lloyd for Rapid Differentiation. *Hong Kong Society of Mass Spectrometry (HKSMS) Symposium 2014*, Courtyard Sha Tin, Hong Kong, 7 June **2014**.

15. **H.Y. Wong**, B. Hu, P.K. So, C.O. Chan, D.K.W Mok, Z.P. Yao, Rapid Differentiation of *Ganoderma lucidum*, *Ganoderma sinense* and *Ganoderma duropora* Lloyd by Direct Ionization Mass Spectrometry. *The 21st Symposium on Chemistry Postgraduate Research in Hong Kong*, The University of Hong Kong, Hong Kong, 12 April **2014**.

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List of Abbreviations

Full Form	Abbreviation
Acetonitrile	ACN
Aluminum	Al
Caffeoylquinic acid	CQA
Capillary Electrophoresis	CE
Chinese Herbal Medicines	CHMs
Chloroform	$CHCl_3$
Dalton	Da
Dichloromethane	DCM
Diode Array Detector	DAD
Direct Ionization	DI
Electron-capture Dissociation	ECD
Electrospray Ionization	ESI
Formic Acid	FA
Flame Ionization Detector	FID
Fourier Transform Infrared Spectroscopy	FTIR
Gas Chromatography	GC

Herbal Medicines	HMs
Hierarchical Clustering Analysis	HCA
High-performance Liquid Chromatography	HPLC
High Voltage	HV
Infrared	IR
Internal Standard	IS
Ion Mobility	IM
Liquid Chromatography	LC
Mass Spectrometer / Mass Spectrometry	MS
Mass-to-charge Ratio	*m/z*
Matrix-assisted Laser Desorption/Ionization	MALDI
Methanol	MeOH
Micellar Electrokinetic Chromatography	MEKC
Microchannel Plate	MCP
Near-infrared Spectroscopy	NIRS
Nuclear Magnetic Resonance	NMR
Polar Surface Area	PSA
Principal Component Analysis	PCA
Proton	H^+

Quadrupole Time-of-Flight	Q-TOF
Relative Standard Deviation	RSD
Selected Ion Chromatogram	SIC
Signal-to-noise Ratio	S/N
Solvent-Accessible Surface Area	SASA
Tandem Mass Spectrometry	MS/MS
Thermal Conductivity Detector	TCD
Thin Layer Chromatography	TLC
Time-of-flight	TOF
Total Ion Chromatogram	TIC
Traditional Chinese Medicines	TCMs
Ultra-Performance Liquid Chromatography	UPLC
Ultraviolet	UV
van der Waals Surface Area	vdWSA
World Health Organization	WHO

Chapter 1. Introduction

1.1. Introduction of Herbal Medicines

Herbal medicines are plant-derived materials or preparations that contain either raw or processed ingredients from one or more plants and can provide therapeutic or other health benefits to humans [1]. Many scientific studies have been conducted to indicate the therapeutic effects and health benefits of herbal medicines [2-4]. Owing to the efficacies and lower costs of herbal medicines, they have been used for thousands of years for elevating human health and treatments of diseases over the world. Also, public attention and acceptance of herbal medicines have been remarkably raised nowadays due to their less unfavourable influences on human body systems. The global herbal medicines and supplements market is estimated to reach US$107 billion by the year 2017 according to the analysis of Global Industry Analysts consulting [5]. The demands on herbal medicines increase greatly world-wide, leading to global concerns about the safety and quality of herbal medicines. It is a challenge to assure the safety and quality of herbal medicines, since unlike chemical drugs, thousands of chemical constituents may be present in one herb and can cause different effects on biological systems [6]. In general, combination of herbal medicines may be used in each medical treatment, causing the analysis to become much more difficult.

Moreover, multiple factors might influence the chemical constituents of herbal medicines, including growth conditions, harvesting period and species in nature [7]. Different species of the same herbal medicines might have remarkably different efficacy [8-11]. The increased global demands for herbal medicines and the profitability of trading herbal medicines have given rise to the problems of species confusion of herbal medicines and herbal medicine counterfeiting, which compromises the safety and efficacy in the use of herbal medicines [12]. Therefore, species differentiation and authentication of herbal medicines are important issues. Overall, quality control and assurance of herbal medicines are consequently essential to ensure the efficacy and safety of herbal medicines for human consumption.

Analytical techniques are essential to differentiate and authenticate herbal medicines. Among various analytical techniques, mass spectrometry (MS) is a powerful technique that allows rapid, accurate and precise qualification and quantification of analytes based on measuring the mass-to-charge ratios (*m/z*) of their ionized forms. This technique analyzes various analytes including both organic and inorganic analytes. Various mass spectrometric instrumentssuch as time-of-flight (TOF), triple quadrupole (QqQ), orbitrap, fourier-transform ion

cyclotron resonance (FTICR) and ion mobility (IM) mass spectrometers, have been invented to allow rapid and sensitive analysis [13-15]. Another powerful tool is the tandem mass spectrometry (MS/MS) which incorporates several stages of mass spectrometric selections and fragmentations between the stages. Diverse tandem mass spectrometric scanning modes, i.e precursor ion scan, neutral loss scan, product ion scan, selected reaction monitoring and mutiple reaction monitoring, enable a greater extent of specific and sensitive detection of analytes. As a result, numerous active ingradients of herbal medicines, e.g., parishins, ganoderic acids, ginsenosides, alkaloids, schisandrins, flavonoids, saponins, caffeoylquinic acids, kudinosides, latifolosides, can be successfully detected and quantified by mass spectrometry [16-21].

Although mass spectrometry is a robust and powerful analytical technique, complex sample matrix of herbal medicines is still a major challenge for MS analysis. Complex sample matrix of herbal medicines could cause the MS detection of targeted analytes to become less sensitive and less specific due to the undesired ion suppression effect from the sample matrix [22, 23]. Moreover, undesired ion peaks of sample matrix could interfere the resulting mass spectra of targeted analytes, leading to a high difficulty and complexity in interpreting the

mass spectra. To enhance the sensitivity and specificity of MS detection, comprehensive sample homogenization, extraction, separation and detection are needed, which could lower the sample complexity and ion suppression effect from the sample matrix. However, these steps are usually labor-intensive and time-consuming [24, 25]. Therefore, development of rapid, sensitive and specific mass spectrometric techniques for herbal medicines analysis is an important task.

Various rapid and direct mass spectrometric techniques for herbal medicines analysis have been developed recently, which usually are ambient ionization techniques that allow ionization of analytes of herbal medicines at atmospheric conditions with no or minimal sample preparation [26-28]. A wide range of methods based on electrospray ionization (ESI) with solid substrates have been developed and applied to facilitate analysis of herbal medicines by reducing the sample pretreatment steps and avoiding clogging problems in conventional capillary-based ESI [29, 30].

In this study, direct ionization mass spectrometry (DI-MS), which is a rapid and simple technique for direct analysis of herbal medicines at ambient conditions, is applied for rapid analysis of various herbal medicines. In addition, the mechanisms

of DI-MS are also investigated in this study, which allows a deeper insight into the extraction and ionization mechanisms of DI-MS and thus explore the ways to enhance the sensitivity and selectivity of DI-MS. Applications, mechanisms and prospects of DI-MS are discussed and summarized in this study.

1.1.1. Conventional analysis of herbal medicines

Conventional analysis of herbal medicines could be divided into two major aspects, i.e. physical analysis and molecular-level analysis [31, 32]. Physical analysis mainly involves the morphological method [33-36], taxonomic method [37-39] and microscopic method [40-44], in which the physical characteristics of herbal medicines including appearance, size, texture, color, smell, structural and cellular tissue features, etc., are comprehensively examined. In molecular-level analysis, various analytical techniques such as DNA molecular marker technology [45-51], fingerprint chromatography [52-58] and mass spectrometry [59-61] are commonly applied to analyze biological and chemical molecules of herbal medicines including small organic molecules, peptides, proteins, lipids, carbohydrates, DNA, etc. Fingerprint chromatography and mass spectrometry are usually used for assessing the quality of herbal medicine in the past decades as they can provide

more comprehensive information, both qualitative and quantitative. LC-MS and GC-MS are the most commonly used MS-based methods in conventional herbal medicine analysis. Relatively, LC-MS is usually adopted for herbal medicines analysis compared to GC-MS as most of the ingradients of herbal medicines are thermally labile and non-volatile compounds that are not suitable for GC-MS analysis without derivatization [62]. However, these method typically involves sample homogenization, extraction, separation of components using capillary electrophoresis (CE) [63, 64], micellar electrokinetic chromatography (MEKC) [65, 66], thin-layer chromatography (TLC) [67, 68], gas chromatography (GC) [69, 70] and liquid chromatography (LC) [71-74], and detection using techniques such as mass spectrometry (MS) [61, 75], near infrared (NIR) spectroscopy [76, 77], electrochemical detection (ECD) [78], diode array detection (DAD) [79, 80] and nuclear magnetic resonance (NMR) spectroscopy [81, 82], which are usually labor-intensive and time-consuming. Development of simple, rapid and high-throughput analytical methods for species differentiation of herbal medicines and authentication of herbal medicines is thus an important and necessary task for the increasing analytical demand of herbal medicines.

1.1.2. Direct ionization mass spectrometry (DI-MS) analysis of herbal medicines

In the past few years, new mass spectrometric techniques have been developed for facilitating analysis of raw samples including herbal medicines [83-88]. They are mainly ambient ionization mass spectrometric techniques that allow analysis of raw samples with little to no sample preparation [26, 89-92]. The first significant technique in the development of ambient ionization mass spectrometry was desorption electrospray ionization (DESI) invented by Cooks's research group in 2004 [26]. In the past thirteen years, various ambient ionization techniques have been subsequently developed, including direct analysis in real time (DART) developed by Cody's research group in 2005 [93], and various solid-substrate electrospray ionization techniques such as paper spray developed by Cooks's research group in 2010 [94], wooden-tip ESI in 2011 [95], direct ionization (DI) in 2012 [96], pipette-tip ESI [97] and ESI with aluminum foil in 2014 [98] invented by Yao's research group. In solid-substrate electrospray ionization techniques, solid substrates such as chromatography paper, wooden tips or aluminum foils are first cut into a small triangular shape with a sharp end. Samples, which could be in the form of solution, semi-solid or solid, are then loaded onto

the surfaces of solid substrates. By applying a high voltage and loading extraction solvents, the samples are extracted and ionized for MS detection. The solid-substrate electrospray ionization techniques allow simple and easy sampling for mass spectrometric analysis without the clogging problem which is usually encountered in conventional capillary-based ESI [29, 30]. Several review articles have systematically summarized the details of various ambient ionization techniques [90-92, 99].

Direct ionization mass spectrometry (DI-MS) [100] is a solid-substrate ESI-MS-based technique for direct analysis of solid raw samples [16, 100-103]. In this technique, a high voltage is applied to a small piece of raw sample cut in a sharp triangular shape and placed in front of the mass spectrometer inlet; with solvent loaded onto the sample surface, spray ionization could be induced at the tip of the sample to generate corresponding mass spectra. The analysis of one sample can be completed within minutes and no specialized device or pneumatic assistance is needed. It is a fast and efficient method for analysis of herbal medicines [100].

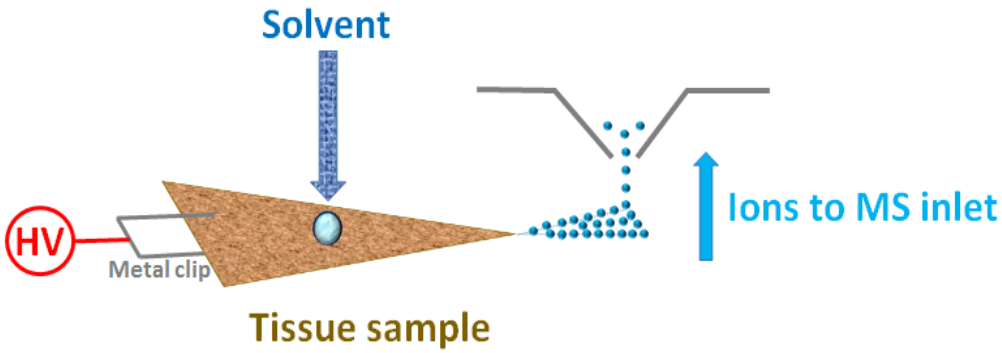


Figure 1.1. Schematic diagram of DI-MS analysis of tissue sample.

In a previous study, DI-MS was applied to differentiate two herbal species, i.e. Fructus Schisandrae Sphenantherae (FSS) and Fructus Schisandrae Chinensis (FSC), based on specific lignan patterns of the mass spectra [96]. DI-MS was also used for direct analysis of Coptis Chinensis Franch (CCF) in the same study, with various alkaloids preponderantly observed. Several similar direct analysis techniques, e.g., leaf spray [103] and tissue spray [104], were also developed. Wild and cultivated Panax quinquefolius, which have remarkably different efficacy and price, could be rapidly differentiated by tissue spray based on the chemical components observed in the mass spectra, including amino acids, oligosaccharides and ginsenosides [104]. Steviol glycosides from Stevia leaves could be successfully detected by leaf spray [105]. In addition, Japanese and Chinese star anises could also be rapidly differentiated by leaf spray based on different anisatin contents observed in the mass spectra [106]. Furthermore, an alternative type of

DI-MS, termed as field-induced direct ionization mass spectrometry (FI-DI-MS), was developed subsequently [107]. In FI-DI-MS, a high voltage is applied at the mass spectrometer inlet and sample is electrically grounded for mass spectrometric analysis. *In vivo* sampling and analysis are significantly favoured by FI-DI-MS as the high voltage is not directly connected to the samples. Chemical compositions of Changsu, which is a famous traditional Chinese medicine, could be analyzed with FI-DI-MS by detecting fresh venom secreted by a living toad upon stimulation. Real-time monitoring of chemical variation in alkaloids of a living plant, Catharanthusroseus, upon heat stimulation was also accomplished by FI-DI-MS [107]. Another study showed that major active ingredients of Coptis Chinensis Franch (CCF), Glycyrrhiza uralensis Fisch, Sophora flavescens and Radix Scutellariae could be successfully detected by FI-DI-MS [85]. Overall, various studies have demonstrated the advantages and robustness of DI-MS for rapid, reliable and high-throughput quality control of herbal medicines.

1.2. Objectives and Outline of This Thesis

Quality control and assurance of herbal medicines are important tasks to ensure the safety and efficacy of herbal medicines. MS is one of the most reliable, sensitive and specific techniques for the analysis of herbal medicines. However, sample pretreatment and chromatographic separation involved in conventional mass spectrometric analysis are usually laborious and time-consuming. The increased global demands for herbal medicines has given rise to the development of simple, rapid and reliable techniques for analysis of herbal medicines. Recently, direct ionization mass spectrometry (DI-MS), a simple and rapid sample preparation and ionization technique, has been developed to enable direct analysis of herbal medicines by mass spectrometry. In this study, DI-MS is applied for direct analysis of various Chinese herbal medicines (CHMs), and the extraction and ionization mechanisms for DI-MS of CHMs was also investigated in order to explore the ways to enhance the sensitivity and selectivity of DI-MS.

The research in this study includes two major parts. The first part discusses development of applications of DI-MS. The second part is about investigation of the extraction and ionization mechanisms of DI-MS. The related work in this thesis includes:

Chapter 1. Introduction

Chapter 2. Rapid Authentication of *Gastrodiae Rhizoma* by Direct Ionization Mass Spectrometry

Chapter 3. Rapid Differentiation of *Ganoderma Species* by Direct Ionization Mass Spectrometry

Chapter 4. Mechanistic Studies of Direct Ionization Mass Spectrometry

Chapter 5. Overall Conclusions and Prospects

Chapter 2. Rapid Authentication of *Gastrodiae Rhizoma* by Direct Ionization Mass Spectrometry

2.1. Introduction

Gastrodiae rhizoma (also known as *Tianma* in Chinese) is one of the most popular herbal medicines in China and it has been used as medicine for thousands of years. *Gastrodiae rhizoma* belongs to the Orchidaceae family. The most commonly used species of *Gastrodiae rhizoma* is called *Gastrodia elata* Bl. Tuber of *Gastrodiae rhizoma* is usually harvested from early winter to late spring, and is used as herbal medicine after washed and dried. *Gastrodiae rhizoma* is mainly found in Sichuan province and Shaanxi province of China [108], and is a valuable medicine widely used in China, Japan, and Korea. Studies have revealed that *Gastrodiae rhizoma* could produce pharmacological effects such as neuroprotection, memory improvement, anti-depressant activity, anti-oxidation, anti-inflammation and could be used for treatment of mental disorder, headache, convulsion, dizziness and paralysis [109-112]. Gastrodin (p-hydroxy-methylphenyl-β-D-glucopyranoside), parishin B / parishin C (bis-[4-(β-D-glucopyranosyloxy)benzyl] citrate) and parishin (tris-[4-(β-D-glucopyranosyloxy)benzyl] citrate) are the major active components of *Gastrodiae rhizoma* [113-116] that contribute to the pharmacological effects [117-123]. Gastrodin, also called gastrodia elata glycosides, is specified as marker for quality control of *Gastrodiae rhizoma* [120],

with the requirement that the level of gastrodin in *Gastrodiae rhizoma* should not be less than 0.20 % by weight [124]. Detection of gastrodin in *Gastrodiae rhizoma* is typically carried out by high-performance liquid chromatography (HPLC)-based methods [125]. On the other hand, *Gastrodiae rhizoma* is easily confused with two counterfeit species, *Cacalia davidii* (Franch.) Hand.-Mazz. [126] and *Canna edulis* Ker [127], which look very much like the genuine species (see Figure 2.1 for photos of genuine and counterfeit *Gastrodiae rhizoma*) but differ greatly in prices and pharmacological values. These two counterfeit species lack the active components of the genuine species and have no known or remarkable pharmacological activities [128-131]. It is difficult to distinguish between genuine and counterfeit species of *Gastrodiae rhizoma* visually because of their very similar appearances, and thus it is essential to establish an effective method for the authentication.

Figure 2.1. Photos of genuine and counterfeit *Gastrodiae rhizoma*.

Gastrodiae rhizoma is available in wild and cultivated types. Wild *Gastrodiae rhizoma* grows in natural environments, especially in mountains while cultivated *Gastrodiae rhizoma* is grown in farms and is the major source of *Gastrodiae rhizoma* in commercial markets. Pharmacological activities between wild and cultivated *Gastrodiae rhizoma* are different due to their different growing conditions such as soil, climate, and growth time [132]. Studies have revealed that wild *Gastrodiae rhizoma* has better pharmacological effects than the cultivated one [133-135]. Wild *Gastrodiae rhizoma* is thus more expensive than cultivated *Gastrodiae rhizoma* due to its better quality and limited availability. Moreover, herbal medicines originating from different geographical locations also differ in selling prices because of their different growth environments and physiological efficacies [136]. Therefore, it is worth developing a reliable, simple and rapid analytical method to differentiate the types (wild or cultivated) and discriminate the origins of *Gastrodiae rhizoma* in addition to authenticating the genuine species from counterfeit.

In the present study, DI-MS was applied and developed for rapid authentication of *Gastrodiae rhizoma*. We demonstrated that DI-MS could unambiguously distinguish between genuine and counterfeit *Gastrodiae rhizoma* samples based on their mass spectra. Meanwhile, *Gastrodiae rhizoma* of the wild type and cultivated type as well as of different geographical origins could be differentiated based on their different intensity ratios of characteristic ions or principal component analysis (PCA) of the spectra.

2.2. Experimental

2.2.1. Chemicals and materials

Genuine samples from different geographical origins and counterfeit samples (*Cacalia davidii* (Franch.) Hand.-Mazz. and *Canna edulis* Ker) of *Gastrodiae rhizoma* used in this study were provided by the local farmers or manufactures [137] or purchased from Chinese herbal medicine markets (see Table 2.1 for the sample information), and confirmed by Mr. Jifeng Zhao and Mr. Songyun Qin, who are experts in authentication of herbal medicines. All samples were sealed and stored in an electronic dry cabinet before analysis. Methanol and all the other solvents were of HPLC grade and purchased from Tedia (Fairfeild, OH, USA). Sodium iodide was purchased from Panreac Química (Barcelona, Spain). All chemicals were used directly without further purification.

Table 2.1. Sample list of the genuine and counterfeit *Gastrodiae rhizoma* analyzed in this study.

Sample ID	Species	Species name	Type	Geographical origin
1 - 3	Genuine	*Gastrodia elata* Bl. (Orchidaceae)	Wild	Sichuan, China
4	Genuine	*Gastrodia elata* Bl. (Orchidaceae)	Wild	Liaoning, China
5	Genuine	*Gastrodia elata* Bl. (Orchidaceae)	Wild	Heilongjiang, China
6	Genuine	*Gastrodia elata* Bl. (Orchidaceae)	Wild	Yunnan, China
7 - 9	Genuine	*Gastrodia elata* Bl. (Orchidaceae)	Cultivated	Gansu, China
10 - 17	Genuine	*Gastrodia elata* Bl. (Orchidaceae)	Cultivated	Hubei, China
18 - 23	Genuine	*Gastrodia elata* Bl. (Orchidaceae)	Cultivated	Henan, China
24 - 36	Genuine	*Gastrodia elata* Bl. (Orchidaceae)	Cultivated	Sichuan, China
37 - 43	Genuine	*Gastrodia elata* Bl. (Orchidaceae)	Cultivated	Shaanxi, China
44 - 50	Genuine	*Gastrodia elata* Bl. (Orchidaceae)	Cultivated	Yunnan, China
51	Counterfeit	*Cacalia davidii* (Franch.) Hand.-Mazz.	N.A.	Sichuan, China
52	Counterfeit	*Canna edulis* Ker	N.A.	Sichuan, China

2.2.2. Setup for DI-MS of Gastrodiae rhizome

The schematic diagram of experimental setup for DI-MS analysis of *Gastrodiae rhizoma* samples is shown in Figure 1.1. A small piece of raw tissue sample was cut into a sharp triangular shape (about 0.5 cm for the base width and about 1 cm for the height) and placed orthogonal to the mass spectrometer inlet by using a clip, with distances of 0.5 cm in vertical (y-coordinate) and 1 cm in horizontal (x-coordinate) from the sample tip to the MS inlet (see Figure 2.2 for photo of the experimental setup). The high voltage supply from the mass spectrometer was then connected to the clip. With the application of a high voltage (3.8 kV) and some solvents (15 μL) to the center of the tissue sample, spray ionization could be induced from the sharp end of the herbal medicine to generate mass spectra.

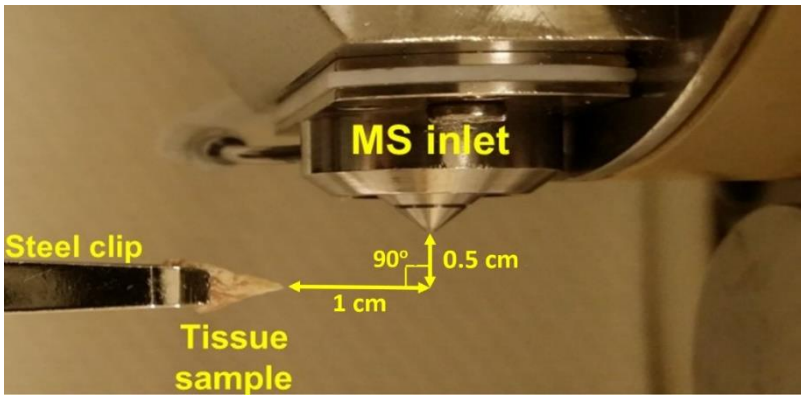


Figure 2.2. Photo of experimental setup for DI-MS analysis of *Gastrodiae rhizoma*.

2.2.3. Mass spectrometric measurements

Mass spectra were acquired on a QToF II mass spectrometer (Waters, Milford, MA) using positive ion mode. Data acquisition and instrumental control were conducted by using MassLynx 4.1 software. Instrumental parameters, including sample cone voltage, source temperature and voltage of microchannel plate (MCP) detector, were set at 30 V, 40 ºC and 2.1 kV, respectively. Capillary voltage applied to the samples was typically set at 3.8 kV, a voltage obtained after optimization. Mass spectra were acquired in a *m/z* range of 100 – 1100 Da with a scan time of 1 s and an interscan time of 0.1 s. Typically, data from the first minute was accumulated to generate the spectra. Sodium iodide was used for *m/z* calibration of the instrument before DI-MS analysis. Tandem mass spectrometry (MS/MS) experiments were performed for identification of the major active components of *Gastrodiae rhizoma*.

2.2.4. Principal component analysis (PCA)

Principal component analysis (PCA) is a statistical tool that converts a set of possibly correlated variables into a set of values of principal components by orthogonal transformation [138]. PCA was carried out using Umetrics SIMCA 13. For each DI-MS spectrum, the normalized intensities (absolute intensity of the peak observed / total absolute intensity of all peaks observed in the mass spectrum) of those monoisotopic peaks with signal intensities higher than 5% were input for the analysis.

2.3. Results and Discussion

2.3.1. Optimization for direct ionization mass spectrometry of Gastrodiae rhizome

Three important factors, i.e. solvent used for extraction and ionization of compounds from the sample, the high voltage used to induce spray ionization from the sample and the configuration of the experimental setup used for the DI-MS analysis, were optimized for DI-MS of *Gastrodiae rhizoma*.

In DI-MS, solvent loaded onto the surface of sample serves for extraction of compounds from the sample and ionization of the extracted compounds, and is thus crucial for the observation of desired ion signals. Solvents of different polarities and acidities, including ethyl acetate, dichloromethane, acetone, acetonitrile, ethanol, methanol, methanol with 0.1% formic acid, acetonitrile/water (1/1) with 0.1% formic acid, methanol/water (1/1) with 0.1% formic acid and water, were tested for DI-MS analysis of *Gastrodiae rhizoma*. The resulting mass spectra are shown in Figure 2.3, and the detectability of gastrodin, parishin B / parishin C and parishin, the three major active components of *Gastrodiae rhizoma,* and the

signal intensities of the DI-MS spectra for the genuine *Gastrodiae rhizoma* are summarized in Table 2.2. The spectra varied significantly with the solvents used. Detection of all the three major active components could be achieved with methanol, ethanol, acetonitrile or acetone as the solvent, but not with solvents of lower polarity i.e., dichloromethane and ethyl acetate, or of higher polarity, i.e., acetonitrile/water (1/1), methanol/water (1/1) and water, indicating that solvents of middle polarity were more suitable for the extraction and ionization of gastrodin, parishin B/C and parishin. Methanol with 0.1% formic acid was eventually chosen as the solvent for DI-MS analysis of *Gastrodiae rhizoma* because the corresponding mass spectrum allowed detection of all the three major active components with the highest signal intensity.

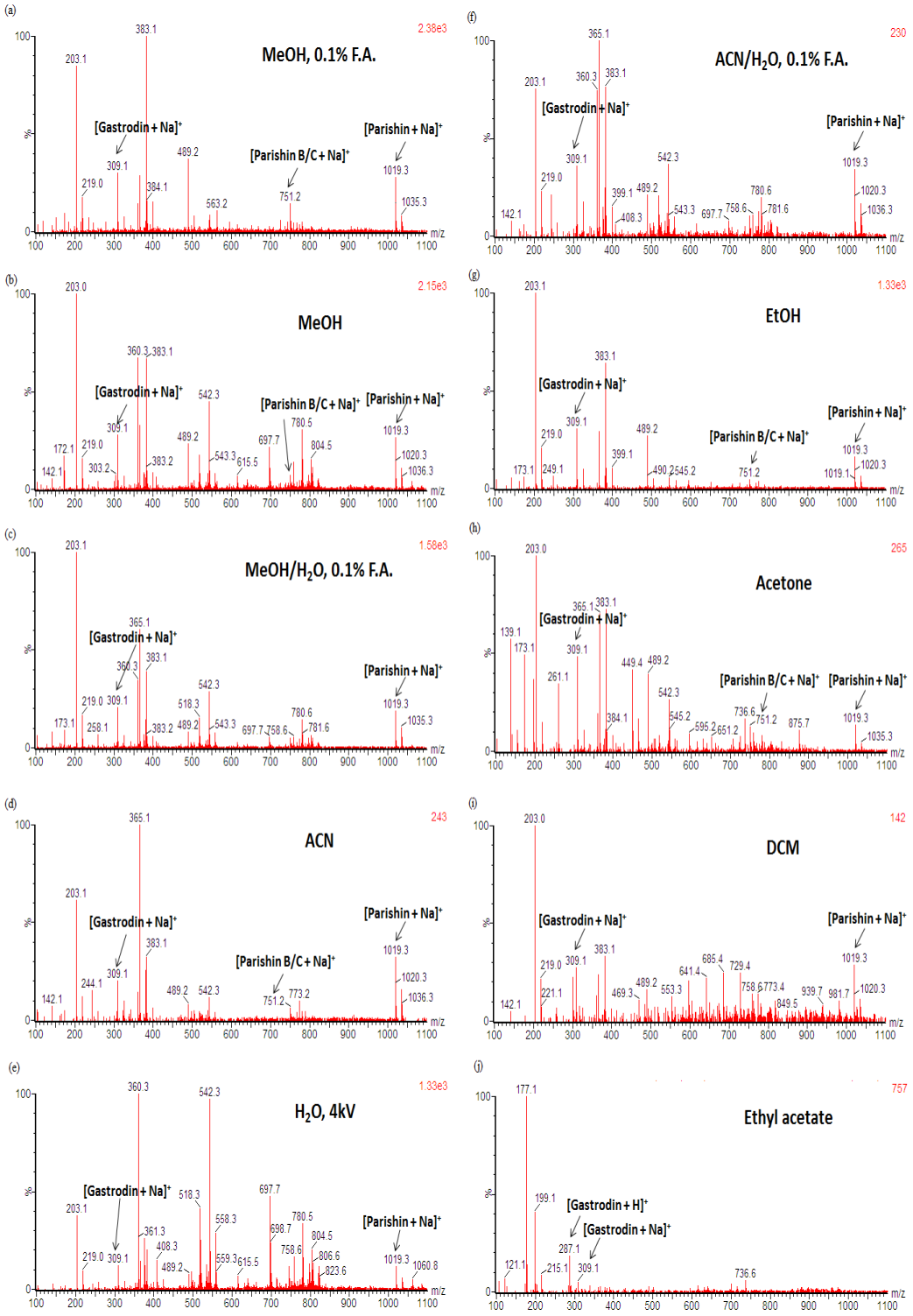


Figure 2.3. DI-MS spectra of *Gastrodiae rhizoma* with the application of different solvents: (a) methanol with 0.1% formic acid, (b) methanol, (c) methanol/water (1/1) with 0.1% formic acid, (d) acetonitrile, (e) water with capillary voltage of 4 kV, (f) acetonitrile/water (1/1) with 0.1% formic acid, (g) ethanol, (h) acetone, (i) dichloromethane, and (j) ethyl acetate.

Table 2.2. Summary of the detectability of the major active components and signals intensity of the DI-MS spectrum for genuine *Gastrodiae rhizoma* with the application of different solvents.

Solvent	**Major active components**			**Signal intensity of DI-MS spectrum**
	Gastrodin	Parishin B/C	Parishin	
Methanol with 0.1% formic acid	√	√	√	Strong
Methanol	√	√	√	Strong
Ethanol	√	√	√	Medium
Acetonitrile	√	√	√	Weak
Acetone	√	√	√	Weak
Water	√	x	√	Medium
Methanol/water (1/1) with 0.1% formic acid	√	x	√	Medium
Acetonitrile/water (1/1) with 0.1% formic acid	√	x	√	Weak
Dichloromethane	√	x	√	Weak
Ethyl acetate	√	x	x	Weak

Different voltages were tested for DI-MS analysis of *Gastrodiae rhizoma* and it was found that ion signals could be observed only when the applied voltage was higher than 3.1 kV. Increasing the voltage could enhance the intensity of ion signals. However, if the applied voltage was higher than 3.8 kV, undesirable electrical discharge could be easily induced on the sample tip. Therefore, a high voltage of 3.8 kV, which gave relatively intense and stable ion signals, was employed for the subsequent DI-MS analysis of *Gastrodiae rhizoma*.

Moreover, configuration of the experimental setup used for the DI-MS analysis, i.e. the distance between and the orientation of the tissue sample and MS inlet, was also optimized. When the tissue sample was getting close to the mass inlet, ion signals would be increased accordingly, but when the tissue sample was too close to the MS inlet, undesirable electrical discharge could be easily induced on the sample tip. A configuration with distances of 0.5 cm in y-coordinate and 1 cm in x-coordinate and an angle of 90^{o} between the tissue tip end and MS inlet (see Figure 2.2) was found to allow relatively stable and intense signals and thus used for the analysis.

2.3.2. DI-MS spectra of Gastrodiae rhizoma samples

Typical DI-MS spectra of wild and cultivated genuine *Gastrodiae rhizoma* are shown in Figure 2.4. Gastrodin, parishin B / parishin C and parishin, three active components used for characterization of *Gastrodiae rhizoma* [139], could be observed in the DI-MS spectra. These compounds were detected as potassium adduct ions at *m/z* 325, 767 and 1035, respectively in the spectra with the masses consistent with the literatures [108, 120, 140-143], and further confirmed with MS/MS in this study (see Figure 2.5 for the MS/MS structural confirmation). The CID of the ion at *m/z* 325 produced fragment ions at *m/z* 219 and 145, corresponding to the two moieties obtained by cleavage of the glycosidic linkage of the potassium adduct ion of gastrodin (Figure 2.5a). MS/MS spectrum of the ion at *m/z* 767 showed fragment ions at *m/z* 499, 393 and 231 (Figure 2.5b), which corresponded to loss of one 4-(β-D-glucopyranosyloxy)benzyl group ($C_{13}H_{16}O_6$, 268 Da), further loss of another 4-(β-D-glucopyranosyloxy)benzyl group, and loss of the benzyl alcohol (C_7H_6O, 106 Da) through the cleavage of the glycosidic linkage of 4-(β-D-glucopyranosyloxy)benzyl group of potassium adduct ion of bis-[4-(β-D-glucopyranosyloxy)benzyl] citrate, respectively (Figure 2.5b). Parishin B and parishin C are two isomers of bis-[4-(β-D-

glucopyranosyloxy)benzyl] citrate, which are co-existed in *Gastrodiae rhizoma* [139, 141-143], and are overlapped in the DI-MS spectrum. The MS/MS spectra of the ion at *m/z* 1035 gave fragment ions at *m/z* 767, 661, 499, 393 and 231, among which the ions at *m/z* 767, 499 and 231 were produced by loss of one, two and three 4-(β-D-glucopyranosyloxy)benzyl group(s) from potassium adduct ion of parishin, respectively, and the ions at *m/z* 661 and 393 were formed by loss of benzyl alcohol through the cleavage of the glycosidic linkage of 4-(β-D-glucopyranosyloxy)benzyl group of ions *m/z* 767 and 499, respectively (Figure 2.5c). In addition to potassium adduct ions, sodium adduct ions of the three major active components could also be observed in the DI-MS spectra of some *Gastrodiae rhizoma* samples that were mainly from Hubei and Shaanxi. These sodium adduct ions were also confirmed with MS/MS (Table 2.3). The MS/MS fragmentation patterns of these sodium adduct ions were similar to those of the corresponding potassium adduct ions, however, higher collision energy was required for the MS/MS fragmentation of the sodium adduct ions. The predominance of sodium or potassium adduct ions in the DI-MS spectra was believed to be related to the presence of alkali metal salts in the herbal samples and that the use of relatively polar solvent (methanol with 0.1% formic acid) favored the extraction of these salts. As shown in Figure 2.4, potassium adduct

ions of disaccharides and its dimer and trimer were also observed in the DI-MS spectra, and were confirmed with MS/MS (Table 2.3).

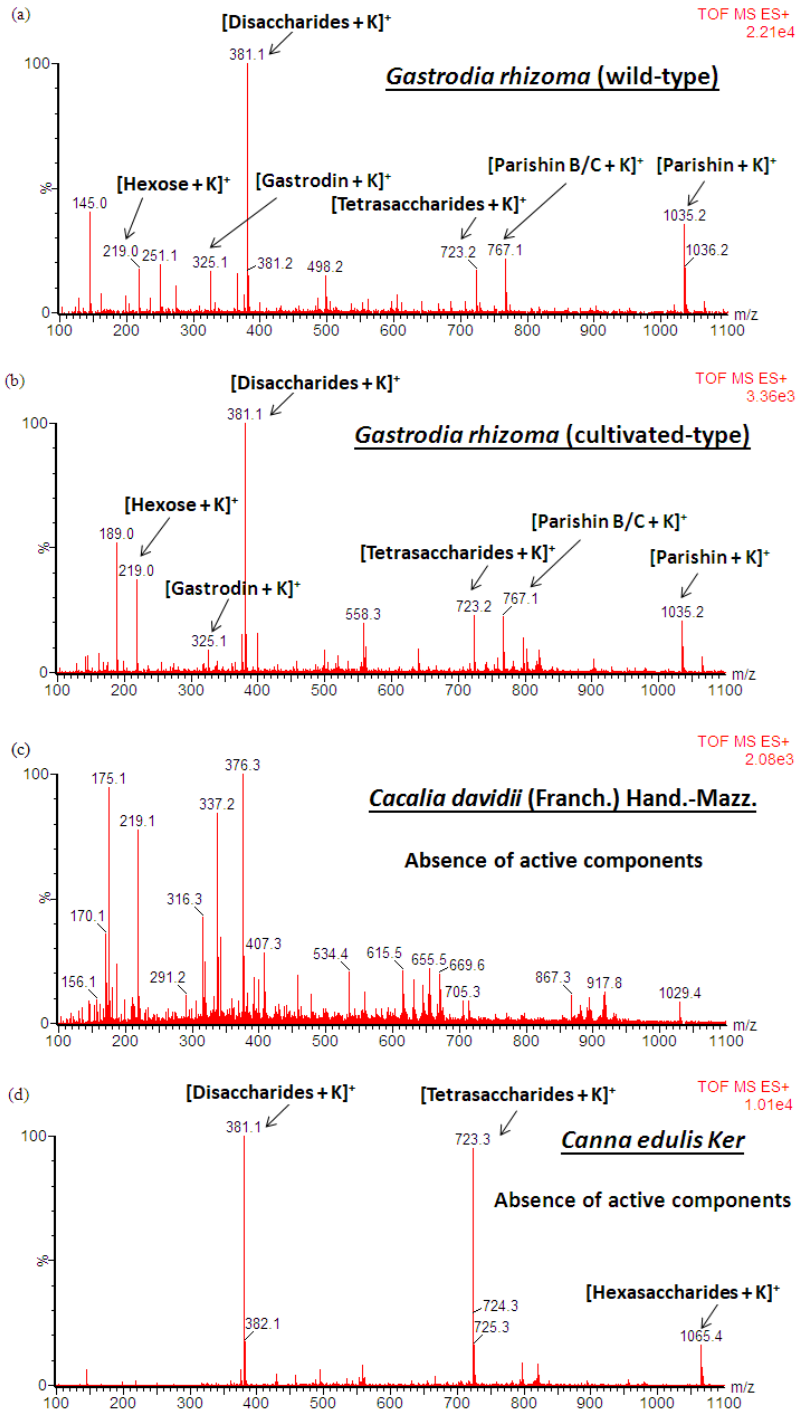


Figure 2.4. DI-MS spectra of genuine and counterfeit *Gastrodiae rhizoma*: (a) wild *Gastrodiae rhizoma*, (b) cultivated *Gastrodiae rhizoma*, (c) *Cacalia davidii* (Franch.) Hand.-Mazz. and (d) *Canna edulis* Ker.

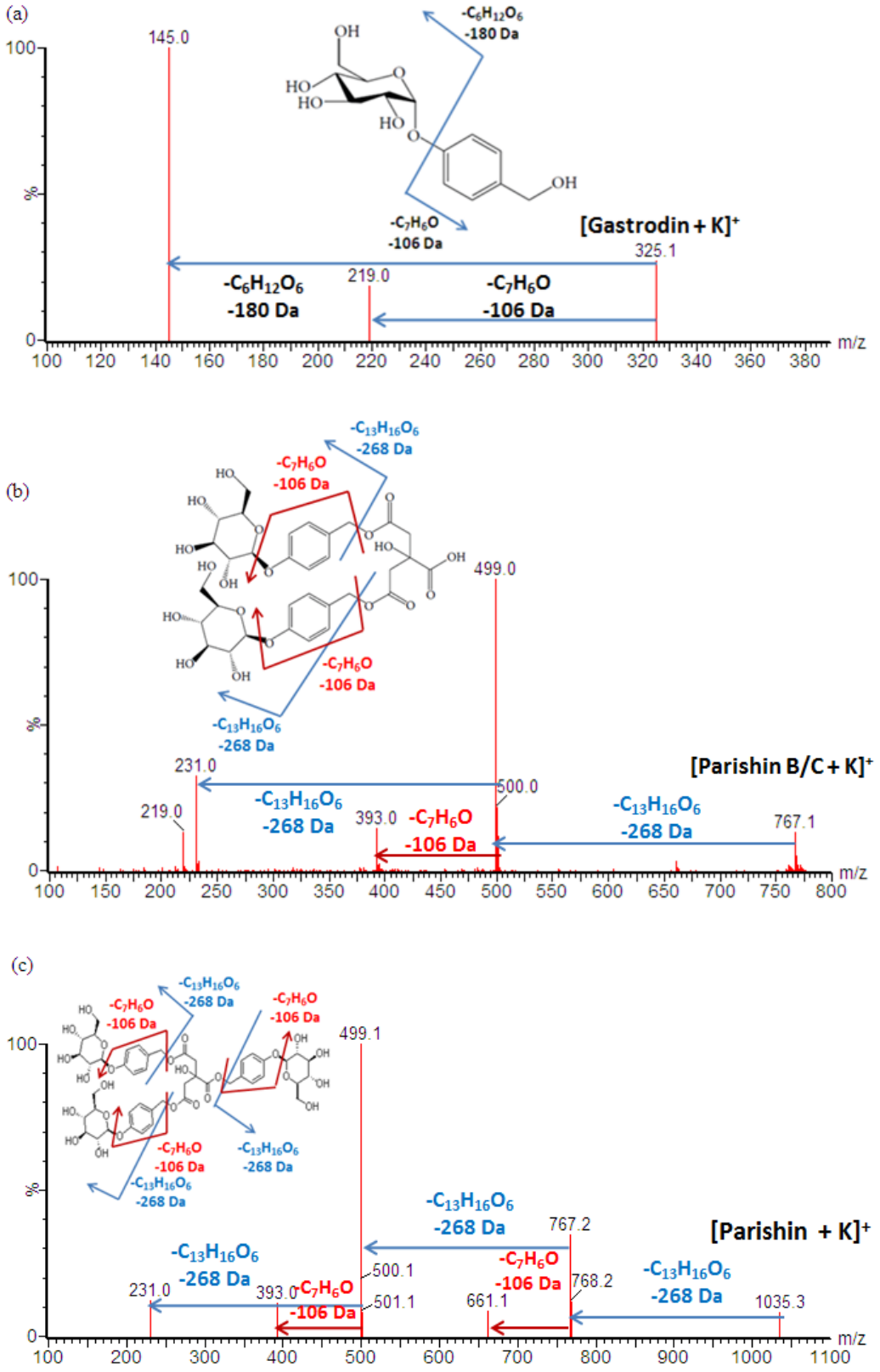
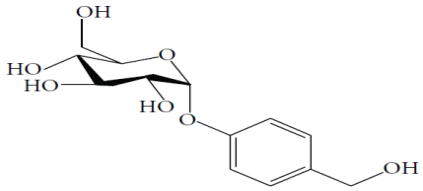
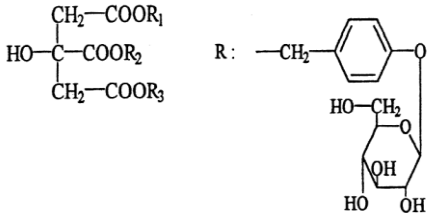
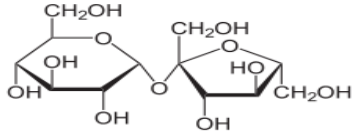
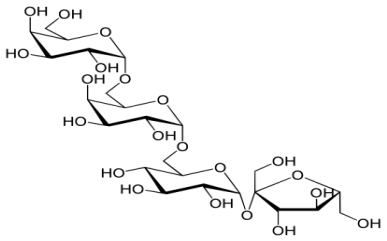
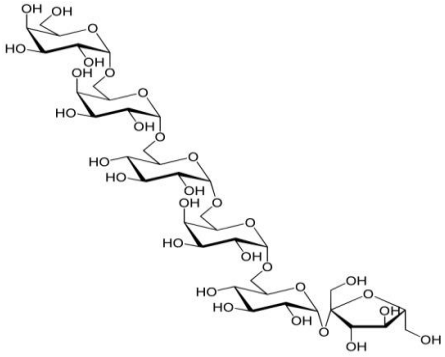


Figure 2.5. MS/MS spectra of precursor ions corresponding to (a) gastrodin, (b) parishin B/C and (c) parishin.

Table 2.3. MS/MS results of the compounds detected in the DI-MS spectra of *Gastrodiae rhizoma*.

Compound [M]	Structure	Molecular weight (Da)	MS/MS transition(s)	Optimum collision energy (eV)
Gastrodin		286	$[M + Na]^+$: 309 → 203, 129 $[M + K]^+$: 325 → 219, 145	$[M + Na]^+$: 49 $[M + K]^+$: 14
Parishin B/C		728	$[M + Na]^+$: 751 → 483, 377, 215 $[M + K]^+$: 767 → 499, 393, 231	$[M + Na]^+$: 70 $[M + K]^+$: 34
Parishin	1. Parishin B: R_1=R_2, R_3=H 2. Parishin C: R_1=R_3, R_2=H 3. Parishin: R_1=R_2=R_3=R	996	$[M + Na]^+$: 1019 → 751, 645, 483, 377, 215 $[M + K]^+$: 1035 → 767, 661, 499, 393, 231	$[M + Na]^+$: 88 $[M + K]^+$: 52
Disaccharides		342	$[M + K]^+$: 381 → 203	8

Tetrasaccharides		684	$[M + K]^+$: 723 → 381	9
Hexasaccharides		1026	$[M + K]^+$: 1065 → 723, 381	16

The reproducibility of the DI-MS spectra was tested by performing three independent experiments with different pieces of the same herb sample for method validation in this study. Taking sample 2 (wild type) and sample 27 (cultivated type) as examples, similar peaks were detected with similar signal intensities in the DI-MS spectra obtained from three independent experiments (see Figure 2.6), indicating a high level of reproducibility of the present DI-MS method.

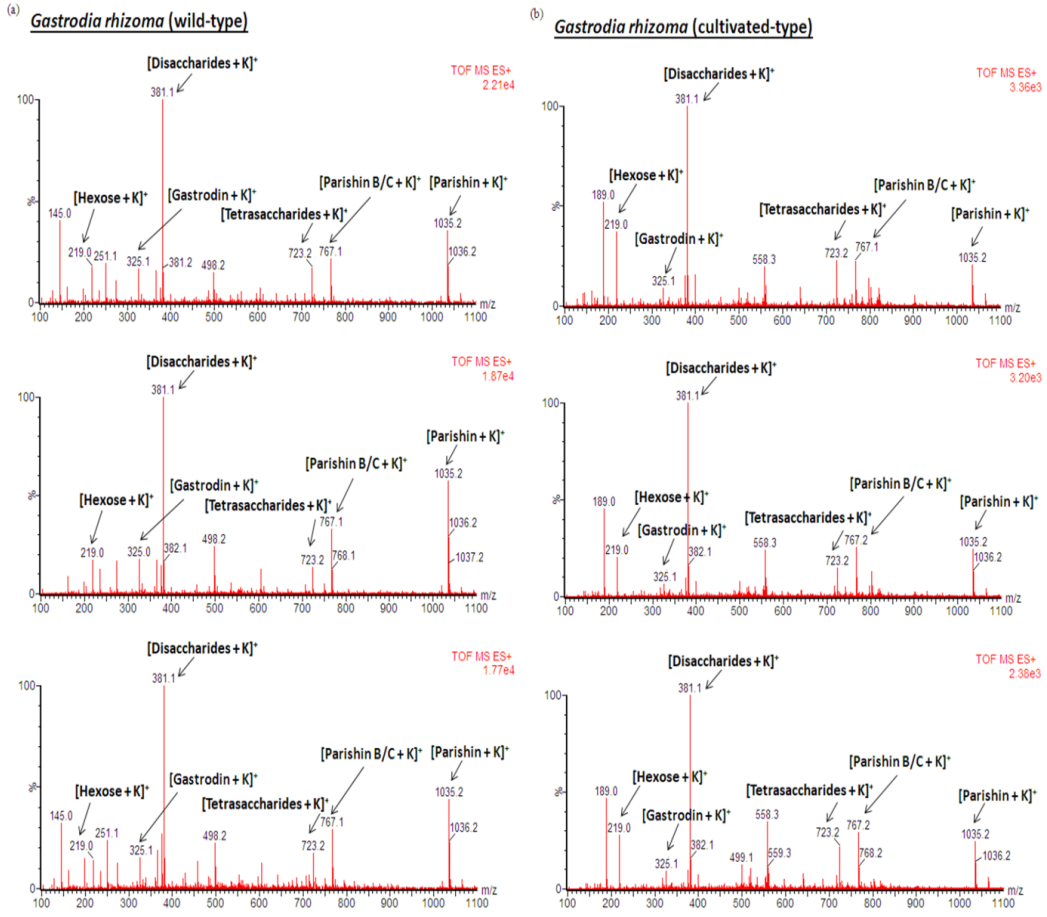


Figure 2.6. DI-MS spectra obtained by DI-MS analysis of different pieces of the same herb samples for (a) wild-type and (b) cultivated-type *Gastrodia rhizoma* in three independent experiments.

2.3.2.1. Wild and cultivated Gastrodiae rhizome

To quantitatively compare the contents of the active components in each sample, hexose, an endogeneous coumpound with intensities similar to those of gastrodin, parishin B/C and parishin, was chosen as the internal reference compound for the comparison. Similar to gastrodin, parishin B/C and parishin, hexose was observed as both sodium and potassium adduct ions in the DI-MS spectra. In this study, the relative abundances of both sodium and potassium adduct ions of each of these compounds were summated as the intensity of the compound for comparison. The intenstity ratios of each active component to hexose, i.e. $I_{Gastrodin}$ / I_{Hexose}, $I_{Parishin\ B/C}$ / I_{Hexose} and $I_{Parishin}$ / I_{Hexose}, for wild and cultivated *Gastrodiae rhizoma* are summarized in Table 2.4 and Figure 2.7. It could be found that the ratio of active components to hexose for the wild types were significantly higher than for the cultivated types. The ratio of total ion intensity of the three active components to the ion intensity of hexose ($I_{Gastrodin,\ Parishin\ B/C,\ Parishin}$ / I_{Hexose}) in the wild types were about four times as those in the cultivated types (Table 2.4), allowing unequivocal differentiation of the wild and cultivated *Gastrodiae rhizoma*. These results were consistent with previous results obtained by using conventional LC-MS [144, 145], and agreed with the general belief that wild-type *Gastrodiae rhizoma* has higher

contents of active components than the cultivated type and can have better pharmacological effects. The relative standard deviations obtained by three independent measurements for the above ratios were generally below 10 % (see Tables 2.4), demonstrating usefulness of the use of the endogenous compound as the internal reference compound and that good reproducibility could be achieved by the DI-MS method. Quantification has been always a problem for direct analysis techniques such as DI-MS [100, 146]. The above results indicated that by choosing a suitable endogenous compound as internal reference, relative quantification could be achieved with acceptable accuracy.

Table 2.4. $I_{Gastrodin}$ / I_{Hexose}, $I_{Parishin\ B/C}$ / I_{Hexose}, $I_{Parishin}$ / I_{Hexose} and $I_{Gastrodin,\ Parishin\ B/C,\ Parishin}$ / I_{Hexose} as observed in the DI-MS spectra of the *Gastrodiae rhizoma* samples.

Sample ID	Type	Geographical origin	$I_{Gastrodin}$ / I_{Hexose} (n=3)	$I_{Parishin\ B/C}$ / I_{Hexose} (n=3)	$I_{Parishin}$ / I_{Hexose} (n=3)	$I_{Gastrodin,\ Parishin\ B/C,\ Parishin}$ / I_{Hexose} (n=3)
1	Wild	Sichuan, China	1.128 ± 0.039	1.443 ± 0.046	1.159 ± 0.076	3.729 ± 0.098
2	Wild	Sichuan, China	1.043 ± 0.107	1.564 ± 0.177	2.416 ± 0.216	5.024 ± 0.297
3	Wild	Sichuan, China	1.050 ± 0.024	1.457 ± 0.137	2.090 ± 0.154	4.596 ± 0.246
4	Wild	Liaoning, China	1.492 ± 0.206	0.999 ± 0.129	1.817 ± 0.244	4.308 ± 0.155
5	Wild	Heilongjiang, China	0.967 ± 0.060	1.440 ± 0.079	2.551 ± 0.261	4.957 ± 0.386
6	Wild	Yunnan, China	0.480 ± 0.065	2.647 ± 0.253	3.032 ± 0.222	6.159 ± 0.293
7	Cultivated	Gansu, China	0.238 ± 0.063	0.444 ± 0.015	0.172 ± 0.010	0.854 ± 0.049
8	Cultivated	Gansu, China	0.301 ± 0.006	0.408 ± 0.037	0.121 ± 0.012	0.831 ± 0.054
9	Cultivated	Gansu, China	0.299 ± 0.029	0.465 ± 0.048	0.113 ± 0.010	0.877 ± 0.081
10	Cultivated	Hubei, China	0.351 ± 0.036	0.150 ± 0.028	0.343 ± 0.061	0.844 ± 0.057
11	Cultivated	Hubei, China	0.302 ± 0.006	0.131 ± 0.014	0.357 ± 0.057	0.790 ± 0.052
12	Cultivated	Hubei, China	0.330 ± 0.013	0.136 ± 0.002	0.346 ± 0.018	0.812 ± 0.023
13	Cultivated	Hubei, China	0.326 ± 0.043	0.297 ± 0.016	0.167 ± 0.008	0.789 ± 0.050
14	Cultivated	Hubei, China	0.217 ± 0.043	0.180 ± 0.042	0.271 ± 0.041	0.669 ± 0.040

15	Cultivated	Hubei, China	0.181 ± 0.035	0.150 ± 0.024	0.192 ± 0.022	0.522 ± 0.080
16	Cultivated	Hubei, China	0.185 ± 0.023	0.134 ± 0.008	0.200 ± 0.005	0.518 ± 0.026
17	Cultivated	Hubei, China	0.176 ± 0.025	0.153 ± 0.012	0.175 ± 0.022	0.505 ± 0.057
18	Cultivated	Henan, China	0.398 ± 0.009	0.042 ± 0.001	0.007 ± 0.001	0.447 ± 0.009
19	Cultivated	Henan, China	0.233 ± 0.012	0.333 ± 0.012	0.177 ± 0.015	0.743 ± 0.026
20	Cultivated	Henan, China	0.378 ± 0.009	0.183 ± 0.034	0.028 ± 0.002	0.589 ± 0.045
21	Cultivated	Henan, China	0.368 ± 0.017	0.035 ± 0.003	0.018 ± 0.002	0.421 ± 0.018
22	Cultivated	Henan, China	0.225 ± 0.017	0.212 ± 0.002	0.160 ± 0.002	0.598 ± 0.015
23	Cultivated	Henan, China	0.389 ± 0.028	0.240 ± 0.038	0.042 ± 0.008	0.671 ± 0.071
24	Cultivated	Sichuan, China	0.569 ± 0.022	0.547 ± 0.015	0.556 ± 0.014	1.671 ± 0.050
25	Cultivated	Sichuan, China	0.394 ± 0.021	0.161 ± 0.002	0.142 ± 0.003	0.697 ± 0.024
26	Cultivated	Sichuan, China	0.243 ± 0.003	0.606 ± 0.057	0.542 ± 0.061	1.390 ± 0.120
27	Cultivated	Sichuan, China	0.622 ± 0.011	0.256 ± 0.017	0.353 ± 0.082	1.231 ± 0.104
28	Cultivated	Sichuan, China	0.164 ± 0.009	0.127 ± 0.015	0.258 ± 0.027	0.549 ± 0.048
29	Cultivated	Sichuan, China	0.288 ± 0.016	0.790 ± 0.042	0.476 ± 0.050	1.554 ± 0.100
30	Cultivated	Sichuan, China	0.143 ± 0.017	0.252 ± 0.047	0.117 ± 0.021	0.512 ± 0.080
31	Cultivated	Sichuan, China	0.150 ± 0.015	0.210 ± 0.018	0.093 ± 0.005	0.453 ± 0.012
32	Cultivated	Sichuan, China	0.149 ± 0.008	0.204 ± 0.015	0.111 ± 0.014	0.464 ± 0.036
33	Cultivated	Sichuan, China	0.150 ± 0.013	0.223 ± 0.006	0.112 ± 0.018	0.485 ± 0.026

34	Cultivated	Sichuan, China	0.349 ± 0.012	0.186 ± 0.004	0.155 ± 0.007	0.689 ± 0.022
35	Cultivated	Sichuan, China	0.535 ± 0.033	0.260 ± 0.036	0.312 ± 0.024	1.108 ± 0.073
36	Cultivated	Sichuan, China	0.232 ± 0.006	0.509 ± 0.068	0.490 ± 0.036	1.232 ± 0.101
37	Cultivated	Shaanxi, China	0.409 ± 0.076	0.289 ± 0.009	0.121 ± 0.008	0.819 ± 0.081
38	Cultivated	Shaanxi, China	0.338 ± 0.008	0.270 ± 0.043	0.148 ± 0.025	0.756 ± 0.059
39	Cultivated	Shaanxi, China	0.380 ± 0.041	0.282 ± 0.055	0.212 ± 0.024	0.875 ± 0.098
40	Cultivated	Shaanxi, China	0.305 ± 0.042	0.334 ± 0.035	0.175 ± 0.007	0.814 ± 0.065
41	Cultivated	Shaanxi, China	0.234 ± 0.031	0.389 ± 0.068	0.203 ± 0.021	0.826 ± 0.077
42	Cultivated	Shaanxi, China	0.292 ± 0.008	0.285 ± 0.038	0.163 ± 0.021	0.740 ± 0.066
43	Cultivated	Shaanxi, China	0.385 ± 0.027	0.204 ± 0.035	0.189 ± 0.010	0.778 ± 0.067
44	Cultivated	Yunnan, China	0.188 ± 0.019	0.191 ± 0.032	0.310 ± 0.028	0.689 ± 0.071
45	Cultivated	Yunnan, China	0.243 ± 0.034	0.227 ± 0.027	0.251 ± 0.047	0.721 ± 0.021
46	Cultivated	Yunnan, China	0.228 ± 0.040	0.217 ± 0.038	0.233 ± 0.045	0.678 ± 0.032
47	Cultivated	Yunnan, China	0.202 ± 0.020	0.187 ± 0.027	0.364 ± 0.026	0.753 ± 0.027
48	Cultivated	Yunnan, China	0.243 ± 0.020	0.182 ± 0.004	0.420 ± 0.028	0.846 ± 0.041
49	Cultivated	Yunnan, China	0.240 ± 0.047	0.231 ± 0.025	0.241 ± 0.042	0.712 ± 0.030
50	Cultivated	Yunnan, China	0.268 ± 0.051	0.216 ± 0.037	0.220 ± 0.039	0.704 ± 0.048

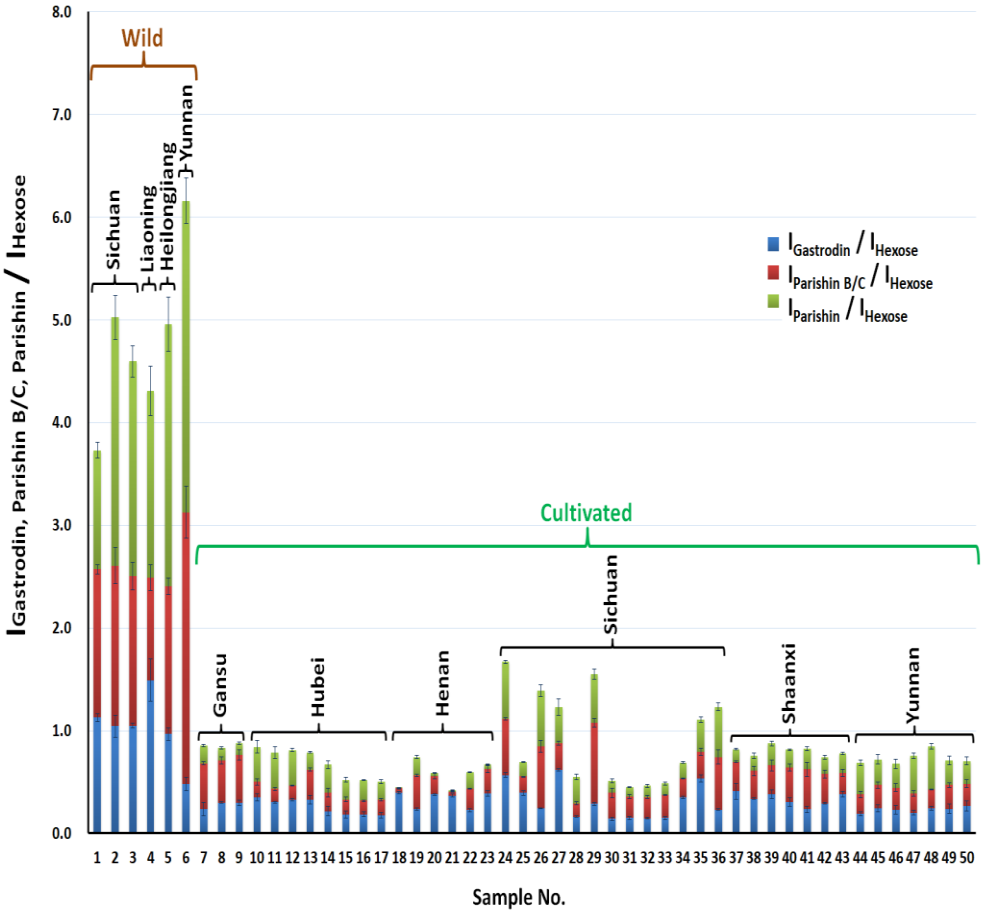


Figure 2.7. A chart showing the ratio of $I_{Gastrodin}$ / I_{Hexose}, $I_{Parishin\ B/C}$ / I_{Hexose}, $I_{Parishin}$ / I_{Hexose} and $I_{Gastrodin,\ Parishin\ B/C,\ Parishin}$ / I_{Hexose} for differentiation of wild-type and cultivated-type *Gastrodiae rhizoma* samples as observed in their DI-MS spectra.

To further compare the DI-MS spectra obtained from different *Gastrodiae rhizoma* samples, the normalized intensities of mass spectrometric peaks obtained from the mass spectra of each sample in three independent experiments were averaged and input for principal component analysis (PCA). A score plot (Figure 2.8) was generated from the first and second principal components based on the DI-MS data, and the two-component PCA model accounted for 78% of total variance of data.

From the PCA plot, it could be found that clusters of wild and cultivated *Gastrodiae rhizoma* samples were well separated in the PCA plot, allowing unequivocal differentiation between them.

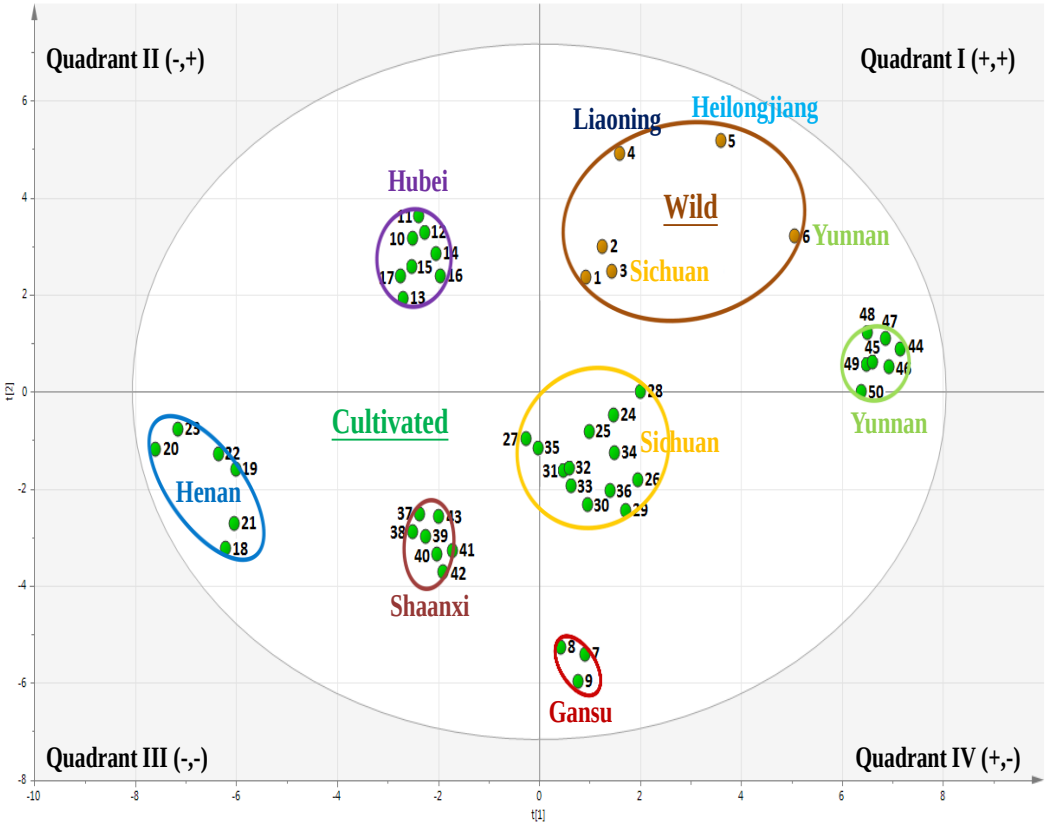


Figure 2.8. PCA plot for *Gastrodiae rhizoma* samples from different geographical origins based on their DI-MS data. Samples 1-6 are wild-type samples and samples 7-50 are cultivated-type samples from different provinces of China.

2.3.2.2. Gastrodiae rhizoma from different sources

It could be found in the PCA plot (Figure 2.8) that cultivated *Gastrodiae rhizoma* samples of the same geographical origin were closely clustered and located in the same circle region in the PCA plot. Although for some sources, there were only limited numbers of samples available for study, the PCA results clearly showed that samples from different origins could be differentiated. The variance of gastrodin, parishin B/C and parishin, the three active components of *Gastrodiae rhizoma*, dominated in quadrant III, IV and I of the PCA plot, respectively (see Figure 2.9 for PCA loading scatter plot showing the locations of the variance). The *Gastrodiae rhizoma* samples from Shaanxi and Henan were located in quadrant III of the PCA plot (Figure 2.8) since they had a higher level of gastrodin than parishin B/C and parishin (see Table 2.5). The *Gastrodiae rhizoma* samples from Gansu were located in quadrant IV of the PCA plot since they had a higher level of parishin B/C than gastrodin and parishin. The cultivated *Gastrodiae rhizoma* samples from Yunnan were located in quadrant I of the PCA plot since they had a higher level of parishin than gastrodin and parishin B/C. The cultivated *Gastrodiae rhizoma* samples from Sichuan had similar level of the three active components thus were located in the middle of the PCA plot. The cultivated *Gastrodiae*

rhizoma sample from Hubei were located in quadrant II of the PCA plot since it had similar level of gastrodin and parishin. On the other hand, as shown in Table 2.5, cultivated *Gastrodiae rhizoma* samples from different sources had very similar $I_{Gastrodin}$ / I_{Hexose}, while samples from Gansu and Sichuan exhibited significantly higher $I_{Parishin\ B/C}$ / I_{Hexose} than samples from Hubei, Henan, Shaanxi and Yunnan, and samples from Hubei, Sichuan and Yunnan showed significantly higher $I_{Parishin}$ / I_{Hexose} than samples from Gansu, Henan and Shaanxi. For $I_{Gastrodin,\ Parishin\ B/C,\ Parishin}$ / I_{Hexose}, the cultivated samples from Henan were the lowest one while the cultivated samples from Sichuan were the highest one.

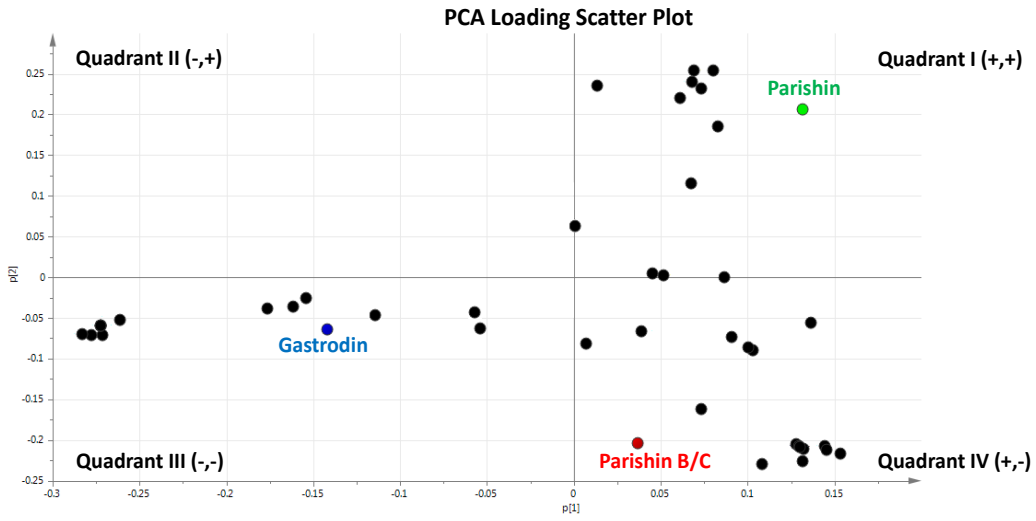


Figure 2.9. PCA loading scatter plot, showing the locations of the variance of gastrodin, parishin B/C and parishin, generated by PCA analysis of the *Gastrodiae rhizoma* samples from different geographical origins based on their DI-MS data.

Table 2.5. Mean values of $I_{Gastrodin}$ / I_{Hexose}, $I_{Parishin\ B/C}$ / I_{Hexose}, $I_{Parishin}$ / I_{Hexose} and $I_{Gastrodin,\ Parishin\ B/C,\ Parishin}$ / I_{Hexose} for *Gastrodiae rhizoma* samples from different sources.

Sample ID	Type	Geographical origin	Mean $I_{Gastrodin}$ / I_{Hexose}	Mean $I_{Parishin\ B/C}$ / I_{Hexose}	Mean $I_{Parishin}$ / I_{Hexose}	Mean $I_{Gastrodin,\ Parishin\ B/C,\ Parishin}$ / I_{Hexose}
1 - 3	Wild	Sichuan, China	1.074 ± 0.071	1.488 ± 0.128	1.888 ± 0.582	4.450 ± 0.605
4	Wild	Liaoning, China	1.492 ± 0.206	0.999 ± 0.129	1.818 ± 0.244	4.308 ± 0.155
5	Wild	Heilongjiang, China	0.967 ± 0.060	1.440 ± 0.079	2.551 ± 0.261	4.957 ± 0.386
6	Wild	Yunnan, China	0.480 ± 0.065	2.647 ± 0.253	3.032 ± 0.222	6.159 ± 0.293
7 - 9	Cultivated	Gansu, China	0.280 ± 0.046	0.439 ± 0.040	0.135 ± 0.029	0.854 ± 0.058
10 - 17	Cultivated	Hubei, China	0.259 ± 0.076	0.166 ± 0.056	0.256 ± 0.084	0.681± 0.146
18 - 23	Cultivated	Henan, China	0.332 ± 0.077	0.174 ± 0.111	0.072 ± 0.071	0.578 ± 0.121
24 - 36	Cultivated	Sichuan, China	0.307 ± 0.169	0.333 ± 0.204	0.286 ± 0.177	0.926 ± 0.461
37 - 43	Cultivated	Shaanxi, China	0.335 ± 0.067	0.293 ± 0.065	0.173 ± 0.034	0.801 ± 0.076
44 - 50	Cultivated	Yunnan, China	0.230 ± 0.039	0.207 ± 0.031	0.291 ± 0.079	0.729 ± 0.064

The wild *Gastrodiae rhizoma* samples from Sichuan, Liaoning, Heilongjiang and Yunnan were clustered differently in the PCA plot (Figure 2.8), indicating that the sources of wild *Gastrodiae rhizoma* samples might also be distinguished by the method. However, due to the difficulty in obtaining the wild *Gastrodiae rhizoma* samples, only 3 wild-type samples from Sichuan, and 1 wide-type sample each from Liaoning, Heilongjiang and Yunnan were investigated in this study. Data from more wild-type samples were needed to confirm the differentiation of sources for wild *Gastrodiae rhizoma*.

2.3.2.3. Genuine and counterfeit Gastrodiae rhizome

Two frequently found counterfeit *Gastrodiae rhizoma* species, *Cacalia davidii* (Franch.) Hand.-Mazz. [126] and *Canna edulis* Ker [127], were investigated using DI-MS and the obtained spectra were shown in Figure 2.4. Significantly different spectral patterns were observed with the two counterfeit *Gastrodiae rhizoma* samples, compared to those from the genuine samples. Particularly, characteristic peaks of the major active components of *Gastrodiae rhizoma*, i.e. gastrodin, parishin B/C and parishin, could not be detected with *Cacalia davidii* (Franch.) Hand.-Mazz. and *Canna edulis* Ker, (Figure 2.4), enabling rapid authentication of the herbal samples.

2.4. Conclusions

In this study, a DI-MS-based analytical method was developed for rapid authentication of *Gastrodiae rhizoma*. Genuine and counterfeit *Gastrodiae rhizoma* species could be unambiguously differentiated based on the detection of the major active components that present in *Gastrodiae rhizoma.* Differentiation between wild and cultivated *Gastrodiae rhizoma* could also be achieved based on the intensity ratio of characteristic ions and PCA analysis. Cultivated *Gastrodiae rhizoma* from different geographical origins could be distinguishable based on the DI-MS spectra and PCA analysis. The results also suggested that by using a suitable endogenous compound as the internal reference compound, DI-MS could be efficiently used for quantitative comparison of active ingredients in herbal samples. The overall method is simple, rapid, and can be further extended to analyze other herbal medicines.

Chapter 3. Rapid Differentiation of *Ganoderma* Species by Direct Ionization Mass Spectrometry

3.1. Introduction

Ganoderma (also known as *Lingzhi* in Chinese) is one of the most popular and valuable herbal medicines, and has been used as folk medicine in China for thousands of years [147]. As early as 200 A.D., *Ganoderma* was cited in the *Shen Nong's Herbal Classics*, which is widely considered as the oldest Chinese book on oriental herbal medicines agriculture and the foundation of traditional Chinese medicines. *Ganoderma* is used for enhancing "vital energy" of peoples and promoting "longevity" [148], and is a valuable medicinal mushroom widely used in countries such as China, Korea and Japan [149]. The global sale of products derived from *Ganoderma* exceeded 2.5 billion US dollars each year [150]. *Ganoderma* is a genus of polypore mushrooms that grow on wood, mostly on deciduous trees such as maple, chestnut and beech in subtropical regions [151]. *Ganoderma* belongs to the Ganodermataceae family of the Fungi kingdom, and there are approximately 80 *Ganoderma* species in the world while only about 20 species are used for medical purposes [152]. In China, only 2 *Ganoderma* species are officially described in Chinese Pharmacopoeia [153], i.e. *Ganoderma lucidum* (Leyss. ex Fr.) (*Chizhi* in Chinese) [154] and *Ganoderma sinense* (*Zizhi* in Chinese) [155], which are the most popularly used *Ganoderma* in the market and are

cultivated in many countries due to their health benefits and commercial values [156]. Owing to the increasing demand of *Ganoderma* and the limited supply and extremely high prices of wild *Ganoderma*, cultivation has become the major source of *Ganoderma* since early 1970s [157]. A variation of cultivated *Ganoderma* is called antler-shaped *Ganoderma ludicum*, which is grown by varying the cultivation conditions such as the carbon dioxide levels, light, humidity or pH, leading to an antler-shaped appearance of the herb [158]. Studies have revealed that *Ganoderma* exhibited therapeutic effects such as anti-inflammation [159], anti-tumor [159], anti-HIV [160], anti-hypertension [161], anti-bacteria [162], anti-diabetic [163], anti-aging [164], immune system-enhancing activities [162], hepatoprotective activities [165] and anti-hypercholesterolemic activities [166]. Ganoderic acids are the major active components of *Ganoderma* with structures closely related to tetracyclic triterpenoids [167].

However, some other *Ganoderma* species [168-170], such as *Ganoderma atrum*, *Ganoderma duropora* Lloyd and *Ganoderma applanatum*, which have appearances similar to *Ganoderma lucidum* and *Ganoderma sinense* (see Figure 3.1 for photos of various *Ganoderma* species) but few reported significant

pharmacological values [171], are commonly found as the adulterants to the official *Ganoderma* species, which are generally several times higher in prices in the market. Under the increased profitability of trading *Ganoderma* and the global demands for *Ganoderma,* the problem of species confusion of *Ganoderma* is getting serious. *Ganoderma* of different species, including the official and confused species of *Ganoderma,* have now flooded into the market, and it is difficult to distinguish them based on traditional morphological inspection. Particularly, most of the commercial *Ganoderma* products are in slice form, which makes accurate differentiation more difficult. Mislabeling and misuse of *Ganoderma* species would be a potential threat to product safety [172]. In the other hand, more than 70 % of *Ganoderma* are cultivated in the northern and middle areas in China, including Zhejiang, Hubei and Shandong provinces [171]. *Ganoderma* originating from different geographical locations is believed to have different quality and different curative effects, and thus differ in selling prices. However, it is not easy to determine the geographical origins with the existing analytical tools as well as visible inspection. Therefore, it is necessary to develop a reliable, simple and rapid analytical method to differentiate official species from confused species and wide type from cultivated type, and discriminate the origins of *Ganoderma*.

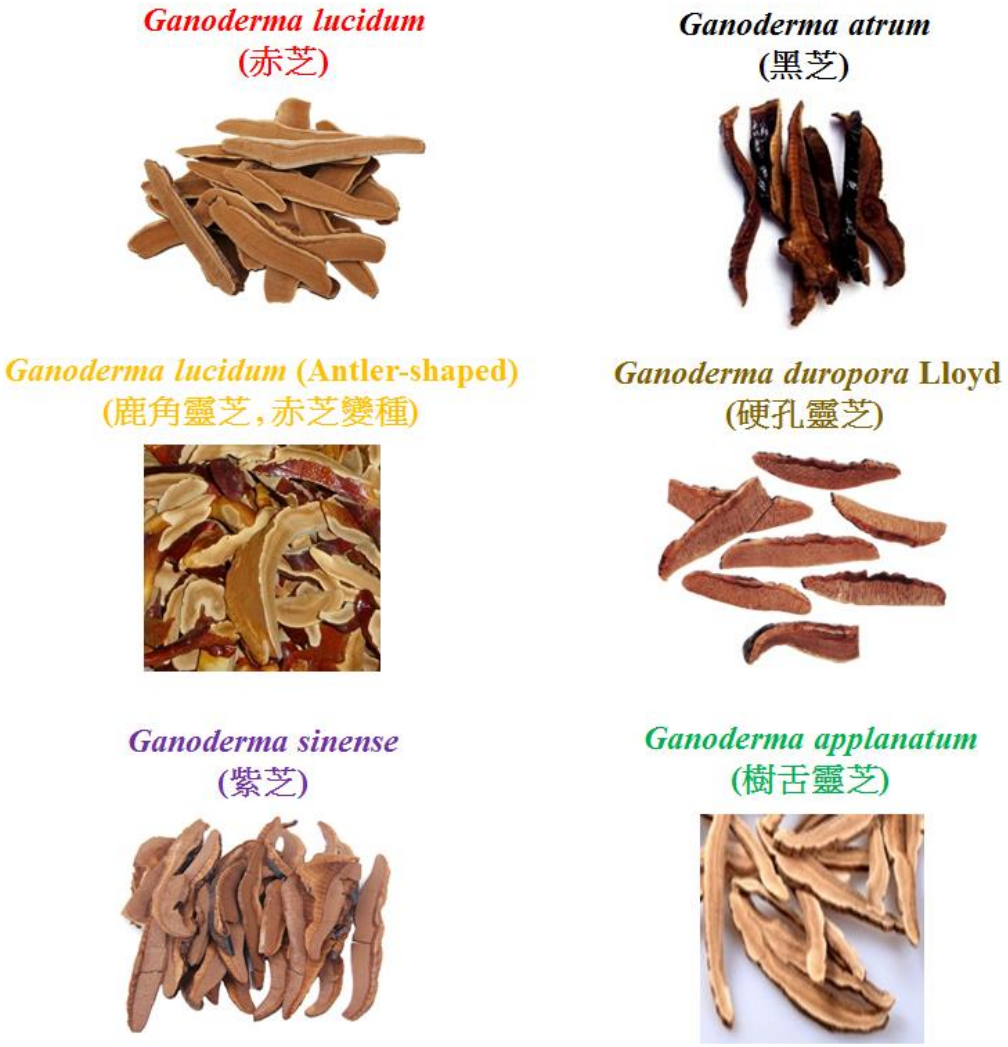


Figure 3.1. Photos of various *Ganoderma* species.

Various methods, including taxonomy [173], morphology [174], microscopy [175], DNA technology [176] and fingerprint chromatography [177, 178], have been developed for species differentiation of *Ganoderma*, Among these methods, fingerprint chromatography can provide precise information about the chemical ingredients present in *Ganoderma*, and thus is commonly adopted for species differentiation of *Ganoderma*. However, this method requires sample

homogenization and extraction, separation of components using techniques such as liquid chromatography (LC) [179], gas chromatography (GC) [180] and capillary electrophoresis (CE) [181], and detection using techniques such as diode array detection (DAD) [182] and mass spectrometry (MS) [183-185], and is relatively time-consuming and laborious. In the present study, direct ionization mass spectrometry (DI-MS), a simple and rapid technique that requires no or only minimal sample preparation for analysis of raw samples [16, 100-103], was developed for rapid differentiation of *Ganoderma* species. Compared to other methods, DI-MS allows direct analysis of raw *Ganoderma* samples, and in a short period of time can generate mass spectra that contain chemical compositions of *Ganoderma* species and can serve as the fingerprints for characterization of the species. As a powerful technique for analysis of herbal medicines, DI-MS has not been used for analysis of *Ganoderma* yet. We demonstrated that DI-MS could unequivocally differentiate official and confused *Ganoderma* species based on their mass spectra. Meanwhile, wild and cultivated *Ganoderma* and potentially *Ganoderma* from different geographical origins could also be distinguished based on principal component analysis (PCA) or hierarchical clustering analysis (HCA) of the spectra.

3.2. Experimental

3.2.1. Chemicals and materials

Samples of *Ganoderma* species from different geographical origins of China were provided by the local farmers and manufactures or purchased from authentic Chinese herbal medicine pharmacies (see Table 3.1 for the sample information). The identities of the samples were confirmed by Mr. Xiang Liu, Mr. Songyun Qin, Mr. Zaibo Yu and Mr. Jifeng Zhao, who are experts in species identification of Chinese medicinal herbs. All the *Ganoderma* samples were sealed in bags and stored in an electronic dry cabinet before analysis. Sodium iodide used for external calibration of mass spectrometer was purchased from Panreac Química (Barcelona, Spain). Methanol and all the other solvents used in this study were of HPLC grade and purchased from Tedia (Fairfeild, OH, USA). All chemicals were used directly without further purification.

Table 3.1. List of the *Ganoderma* samples analyzed in this study.

Sample ID	Species name	Type	Geographical origin (China)
1	*Ganoderma lucidum* (Leyss.ex Fr.) Karst. (赤芝)	Wild	Guangxi
2 - 3	*Ganoderma lucidum* (Leyss.ex Fr.) Karst. (赤芝)	Wild	Sichuan
4 - 5	*Ganoderma lucidum* (Leyss.ex Fr.) Karst. (赤芝)	Wild	Zhejiang
6 - 12	*Ganoderma lucidum* (Leyss.ex Fr.) Karst. (赤芝)	Cultivated	Anhui
13	*Ganoderma lucidum* (Leyss.ex Fr.) Karst. (赤芝)	Cultivated	Chongqing
14	*Ganoderma lucidum* (Leyss.ex Fr.) Karst. (赤芝)	Cultivated	Fujian
15 - 16	*Ganoderma lucidum* (Leyss.ex Fr.) Karst. (赤芝)	Cultivated	Hubei
17	*Ganoderma lucidum* (Leyss.ex Fr.) Karst. (赤芝)	Cultivated	Hunan
18	*Ganoderma lucidum* (Leyss.ex Fr.) Karst. (赤芝)	Cultivated	Jilin
19 - 20	*Ganoderma lucidum* (Leyss.ex Fr.) Karst. (赤芝)	Cultivated	Shandong
21 - 23	*Ganoderma lucidum* (Leyss.ex Fr.) Karst. (赤芝)	Cultivated	Sichuan
24	*Ganoderma lucidum* (Leyss.ex Fr.) Karst. (赤芝)	Cultivated	Zhejiang

25 - 26	*Ganoderma lucidum* (Antler-shaped) (鹿角靈芝，赤芝變種)	Cultivated	Guangdong
27	*Ganoderma sinense* Zhao, xu et Zhang (紫芝)	Wild	Hubei
28	*Ganoderma sinense* Zhao, xu et Zhang (紫芝)	Wild	Anhui
29 - 31	*Ganoderma sinense* Zhao, xu et Zhang (紫芝)	Cultivated	Hunan
32	*Ganoderma atrum* J. D. Zhao, L. W. Hsu et X. Q. Zhang (黑芝)	Wild	Guizhou
33 - 34	*Ganoderma atrum* J. D. Zhao, L. W. Hsu et X. Q. Zhang (黑芝)	Cultivated	Guizhou
35	*Ganoderma atrum* J. D. Zhao, L. W. Hsu et X. Q. Zhang (黑芝)	Cultivated	Sichuan
36	*Ganoderma duropora* Lloyd (硬孔靈芝)	Wild	Zhejiang
37	*Ganoderma duropora* Lloyd (硬孔靈芝)	Cultivated	Guangdong
38 - 39	*Ganoderma duropora* Lloyd (硬孔靈芝)	Cultivated	Guangxi
40	*Ganoderma duropora* Lloyd (硬孔靈芝)	Cultivated	Henan
41	*Ganoderma duropora* Lloyd (硬孔靈芝)	Cultivated	Zhejiang
42 - 43	*Ganoderma applanatum* (Pers.) Pat. (樹舌靈芝)	Wild	Guizhou

3.2.2. Setup for DI-MS analysis of Ganoderma samples

The schematic diagram of experimental setup for DI-MS analysis of *Ganoederma* samples is shown in Figure 1.1. A small piece of raw *Ganoederma* sample was cut into a sharp triangular shape (about 1 cm for the height and about 0.5 cm for the base width) and placed orthogonal to the mass spectrometer inlet by using a metal clip, with distances of 1 cm in horizontal and 0.5 cm in vertical from the sample tip to the MS inlet (see Figure 3.2 for photo of the experimental setup). A crocodile clip was used to connect the high voltage supply of the mass spectrometer to the metal clip. With the application of a high voltage (3.5 kV) and some solvents (10 μL) to the center of the *Ganoederma* sample, spray ionization could be induced from the sharp end of the herbal medicine to generate mass spectra.

Figure 3.2. Photo of the experimental setup for DI-MS analysis of *Ganoderma.*

3.2.3. Mass spectrometric measurements

Mass spectra were acquired on a QToF II mass spectrometer (Waters, Milford, MA) using positive ion mode. Instrumental control and data acquisition were conducted by using MassLynx 4.1 software. Voltage of microchannel plate (MCP) detector, sample cone voltage and source temperature were set at 2.1 kV, 30 V and 40 ^{o}C, respectively. High voltage applied to the samples was typically set at 3.5 kV, a voltage obtained after optimization. Mass spectra were acquired in an *m/z* range of 100 - 1000 Da with an interscan time of 0.1 s and a scan time of 1 s. Typically, mass spectra were generated by accumulating data from the first minute. Before DI-MS analysis, sodium iodide was used for *m/z* calibration of the instrument.

3.2.4. Principal component analysis (PCA) and hierarchical clustering analysis (HCA)

Hierarchical clustering analysis (HCA), another commonly used statistics tool that used to sort samples into groups based on measuring the similarity between the samples to be clustered, and the similarity or dissimilarity between samples is usually represented in a dendrogram for ease of interpretation [186]. HCA plots can give another view of the results which showed a clearer visual of the groupings of the geographical origins of the samples.

Principal component analysis (PCA) and hierarchical clustering analysis (HCA) were carried out using Umetrics SIMCA 14 software. For each DI-MS spectrum, the normalized intensities (absolute intensity of the peak observed / total absolute intensity of all peaks observed in the mass spectrum) of the monoisotopic peaks with signal intensities higher than 5% were input to the software for the analysis.

3.3. Results and Discussion

3.3.1. Optimization for DI-MS analysis of Ganoderma samples

Stable spray ionization with strong signal is crucial for DI-MS analysis of *Ganoderma* samples. The experimental conditions for this were thus optimized before the sample analysis. Important factors, including the configuration of the experimental setup, the high voltage and the extraction and ionization solvent, were optimized for DI-MS analysis of *Ganoderma* samples.

For the distance between the sample and MS inlet and the high voltage applied onto the sample, it was found that when the sample was placed > 1 cm for the height and > 0.5 cm for the base width from the MS inlet or the applied voltage was lower than 3.5 kV, no or very weak ion signals were obtained, and when the sample was placed < 1 cm for the height and < 0.5 cm for the base width to the MS inlet or the applied voltage was higher than 3.5 kV, electrical discharge was observed. Optimal MS signals were obtained when the sample tips were placed perpendicular to the MS inlet with the distances of 0.5cm in y-coordinate and 1 cm in x-coordinate (see Figure 3.2), and with the high voltage of 3.5 kV in positive

ion mode.

The added solvent served for extraction of compounds from the sample and ionization of the extracted compounds during DI-MS analysis. In this study, solvents of different polarities and acidities, including water, methanol/water (1/1), acetonitrile/water (1/1), methanol with 0.1% formic acid, methanol, acetonitrile, ethanol, chloroform, dichloromethane and hexane, were used for DI-MS analysis of a cultivated *Ganoderma lucidum* sample and the resulting spectra are shown in Figure 3.3. Ganoderic acids, the major active components of *Ganoderma,* were detected and the signal intensities of the DI-MS spectra for the cultivated *Ganoderma lucidum* are summarized in Table 3.2. The ion profiles of the spectra varied significantly with the solvents used. Ganoderic acids were abundantly detected as the base peaks when methanol with 0.1% formic acid, methanol and acetonitrile were used. However, the signals of ganoderic acids were significantly suppressed and choline [187] cation was remarkebly observed as the base peaks when more polar solvents, i.e. water, methanol/water (1/1) and acetonitrile/water (1/1) were used, indicating that solvents of higher polarity favored the extraction and ionization of ionic compounds. On the other hand, when less polar or non-polar solvents, i.e. ethanol, chloroform, dichloromethane and hexane were used,

triglycerides [188] would be detected as the base peaks and the signal intensities of ganoderic acids were also significantly suppressed, indicating that solvents of lower polarity favored the extraction and ionization of relatively non-polar compounds. Methanol with 0.1% formic acid was eventually chosen as the solvent for subsequent DI-MS analysis of the *Ganoderma* samples because it allowed detection of ganoderic acids with the highest signal intensity. The added low level of formic acid was believed to facilitate the ionization of the analytes [189].

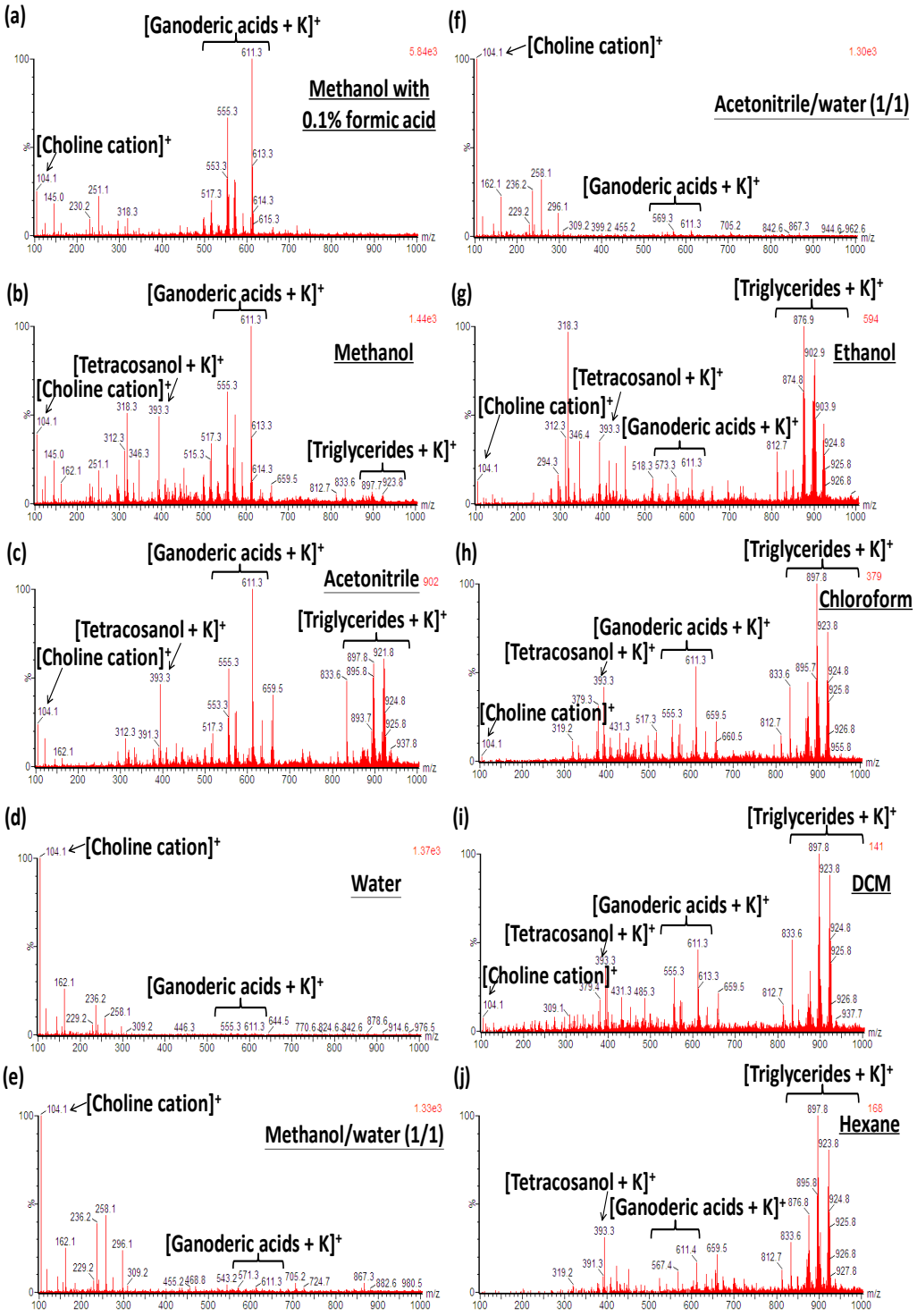


Figure 3.3. DI-MS spectra of cultivated *Ganoderma lucidum* obtained with different solvents: (a) methanol with 0.1% formic acid, (b) methanol, (c) acetonitrile, (d) water, (e) methanol/water (1/1), (f) acetonitrile/water (1/1), (g) ethanol, (h) chloroform, (i) dichloromethane and (j) hexane.

Table 3.2. Summary of the detectability of ganoderic acids and signal intensities of the DI-MS spectra obtained with the cultivated *Ganoderma lucidum* and various solvents.

Solvent	Ganoderic acids	Base peak	Signal intensity
Methanol with 0.1% formic acid	✓	Ganoderic acid	Strongest
Methanol	✓	Ganoderic acid	Strong
Acetonitrile	✓	Ganoderic acid	Medium
Water	✓	Choline	Strong
Methanol/water (1/1)	✓	Choline	Strong
Acetonitrile/water (1/1)	✓	Choline	Strong
Ethanol	✓	Triglyceride	Medium
Chloroform	✓	Triglyceride	Weak
Dichloromethane	✓	Triglyceride	Weak
Hexane	✓	Triglyceride	Weak

3.3.2. DI-MS spectra of the Ganoderma samples

Typical DI-MS spectra of various *Ganoderma* species are shown in Figure 3.4. Ganoderic acids, the major active components used for characterization of *Ganoderma*, could be observed in the DI-MS spectra. For two official *Ganoderma* species stated in the Chinese pharmacopoeia, i.e. *Ganoderma lucidum* and *Ganoderma sinense*, distinct peaks, with masses corresponding to potassium adducts of different ganoderic acids including ganoderic acids E (*m/z* 551.2), AM_1/C_1/J (*m/z* 553.3), A/B (*m/z* 555.3), C/C_2 (*m/z* 557.3), D/M/N (*m/z* 569.3), G/I (*m/z* 571.3), L (*m/z* 573.3), F (*m/z* 609.2), H (*m/z* 611.3) and K (*m/z* 613.3), were observed (see Table 3.3 for the summarized accurate mass data of the observed ions and Table 3.4 for their chemical structures). Choline cation [187] (*m/z* 104.1) and mannitol [190] (*m/z* 221.1) were also detected in the DI-MS spectra. Isomers of each ganoderic acid were detected as a single peak in the DI-MS spectrum, and their differentiation probably could be achieved by using ion mobility mass spectrometry [191]. The predominance of potassium adduct ions in the DI-MS spectra was believed to be related with the presence of potassium salts in the herbal samples and the use of the relatively polar solvent that favored the extraction of these salts.

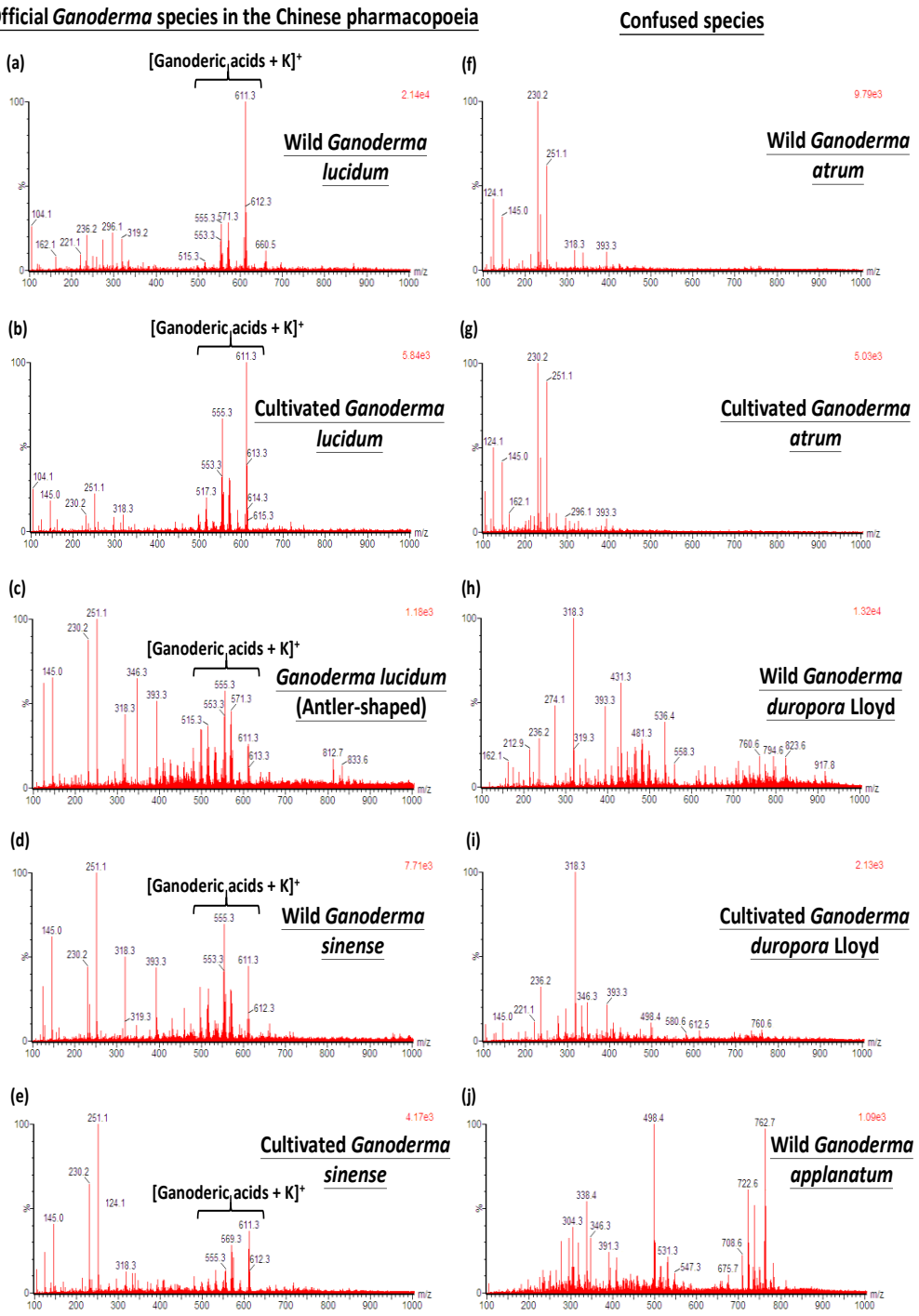


Figure 3.4. DI-MS spectra of the official *Ganoderma* species: (a) wild *Ganoderma lucidum*, (b) cultivated *Ganoderma lucidum*, (c) *Ganoderma lucidum* (antler-shaped), (d) wild *Ganoderma sinense*, (e) cultivated *Ganoderma sinense*; and DI-MS spectra of three easily confused species: (f) wild *Ganoderma atrum*, (g) cultivated *Ganoderma atrum*, (h) wild *Ganoderma duropora* Lloyd, (i) cultivated *Ganoderma duropora* Lloyd, and (j) wild *Ganoderma applanatum*.

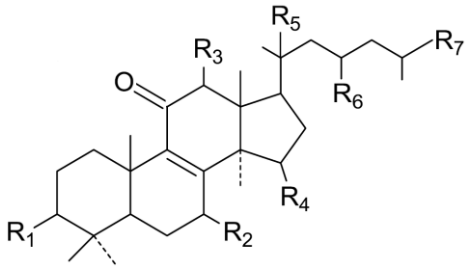
Table 3.3. List of the accurate masses of the ganoderic acids observed in the DI-MS spectra of the two official *Ganoderma* species.

Theoretical mass $[M + K]^+$ (*m/z*)	**Compound [M]**	**Sample**	**Measured mass $[M + K]^+$ (*m/z*)**	**Mass accuracy (ppm)**
551.2406	Ganoderic acid E ($C_{30}H_{40}O_7$)	Wild *G. lucidum*	551.2426	3.63
		Cultivated *G. lucidum*	551.2425	3.45
		G. lucidum (Antler-shaped)	551.2420	2.54
		Wild *G. sinense*	551.2431	4.54
		Cultivated *G. sinense*	551.2424	3.27
553.2562	Ganoderic acid AM_1 / C_1 / J ($C_{30}H_{42}O_7$)	Wild *G. lucidum*	553.2534	- 5.06
		Cultivated *G. lucidum*	553.2540	- 3.98
		G. lucidum (Antler-shaped)	553.2538	- 4.34
		Wild *G. sinense*	553.2533	- 5.24
		Cultivated *G. sinense*	553.2536	- 4.70
555.2719	Ganoderic acid A / B ($C_{30}H_{44}O_7$)	Wild *G. lucidum*	555.2732	2.34
		Cultivated *G. lucidum*	555.2729	1.80
		G. lucidum (Antler-shaped)	555.2726	1.26
		Wild *G. sinense*	555.2733	2.52
		Cultivated *G. sinense*	555.2732	2.34

557.2875	Ganoderic acid C / C_2 ($C_{30}H_{46}O_7$)	Wild *G. lucidum*	557.2895	3.59
		Cultivated *G. lucidum*	557.2892	3.05
		G. lucidum (Antler-shaped)	557.2891	2.87
		Wild *G. sinense*	557.2896	3.77
		Cultivated *G. sinense*	557.2894	3.41
569.2511	Ganoderic acid D / M / N ($C_{30}H_{42}O_8$)	Wild *G. lucidum*	569.2476	- 6.15
		Cultivated *G. lucidum*	569.2480	- 5.45
		G. lucidum (Antler-shaped)	569.2483	- 4.92
		Wild *G. sinense*	569.2477	- 5.97
		Cultivated *G. sinense*	569.2481	- 5.27
571.2668	Ganoderic acid G / I ($C_{30}H_{44}O_8$)	Wild *G. lucidum*	571.2692	4.20
		Cultivated *G. lucidum*	571.2696	4.90
		G. lucidum (Antler-shaped)	571.2689	3.68
		Wild *G. sinense*	571.2697	5.08
		Cultivated *G. sinense*	571.2691	4.03
573.2824	Ganoderic acid L ($C_{30}H_{46}O_8$)	Wild *G. lucidum*	573.2799	- 4.36
		Cultivated *G. lucidum*	573.2802	- 3.84
		G. lucidum (Antler-shaped)	573.2805	- 3.31
		Wild *G. sinense*	573.2801	- 4.01
		Cultivated *G. sinense*	573.2805	- 3.31

609.2460	Ganoderic acid F ($C_{32}H_{42}O_9$)	Wild *G. lucidum*	609.2486	4.27
		Cultivated *G. lucidum*	609.2484	3.94
		G. lucidum (Antler-shaped)	609.2479	3.12
		Wild *G. sinense*	609.2485	4.10
		Cultivated *G. sinense*	609.2481	3.45
611.2617	Ganoderic acid H ($C_{32}H_{44}O_9$)	Wild *G. lucidum*	611.2638	3.44
		Cultivated *G. lucidum*	611.2633	2.62
		G. lucidum (Antler-shaped)	611.2629	1.96
		Wild *G. sinense*	611.2635	2.94
		Cultivated *G. sinense*	611.2630	2.13
613.3345	Ganoderic acid K ($C_{34}H_{52}O_7$)	Wild *G. lucidum*	613.3321	- 3.91
		Cultivated *G. lucidum*	613.3316	- 4.73
		G. lucidum (Antler-shaped)	613.3324	- 3.42
		Wild *G. sinense*	613.3321	- 3.91
		Cultivated *G. sinense*	613.3318	- 4.40

Table 3.4. Chemical structures of the ganoderic acids observed in the DI-MS spectra of the two official Ganoderma species.

Structure	Ganoderic acid	R_1	R_2	R_3	R_4	R_5	R_6	R_7	Reference
	A	=O	β-OH	H	α-OH	H	=O	COOH	[192]
	AM_1	β-OH	=O	H	=O	H	=O	COOH	[193]
	B	β-OH	β-OH	H	=O	H	=O	COOH	[194]
	C	β-OH	β-OH	H	α-OH	H	=O	COOH	[194]
	C_1	=O	β-OH	H	=O	H	=O	COOH	[195]
	C_2	β-OH	β-OH	H	α-OH	H	=O	COOH	[196]
	D	=O	β-OH	H	=O	H	=O	COOH	[197]
	E	=O	=O	H	=O	H	=O	COOH	[197]
	F	=O	=O	β-*O*-Ac	=O	H	=O	COOH	[198]
	G	β-OH	β-OH	β-OH	=O	H	=O	COOH	[199]
	H	β-OH	=O	β-*O*-Ac	=O	H	=O	COOH	[200]
	I	β-OH	β-OH	=O	=O	H	CH_3	COOH	[199]
	J	=O	=O	H	α-OH	H	=O	COOH	[201]
	K	β-OH	β-OH	β-*O*-Ac	=O	H	=O	COOH	[196]
	L	β-OH	β-OH	H	α-OH	β-OH	=O	COOH	[202]
	M	=O	β-OH	β-OH	=O	H	=O	COOH	[203]
	N	=O	β-OH	H	=O	β-OH	=O	COOH	[204]

Three independent measurements were conducted for different slices of the same *Ganoderma* sample to investigate the reproducibility of the technique. Taking sample 5 (wild *Ganoderma lucidum*) and sample 6 (cultivated *Ganoderma lucidum*) as examples, similar peak profiles with comparable ion intensities were obtained in the DI-MS spectra from the three independent analyses (see Figure 3.5), demonstrating a high level of reproducibility of the DI-MS technique.

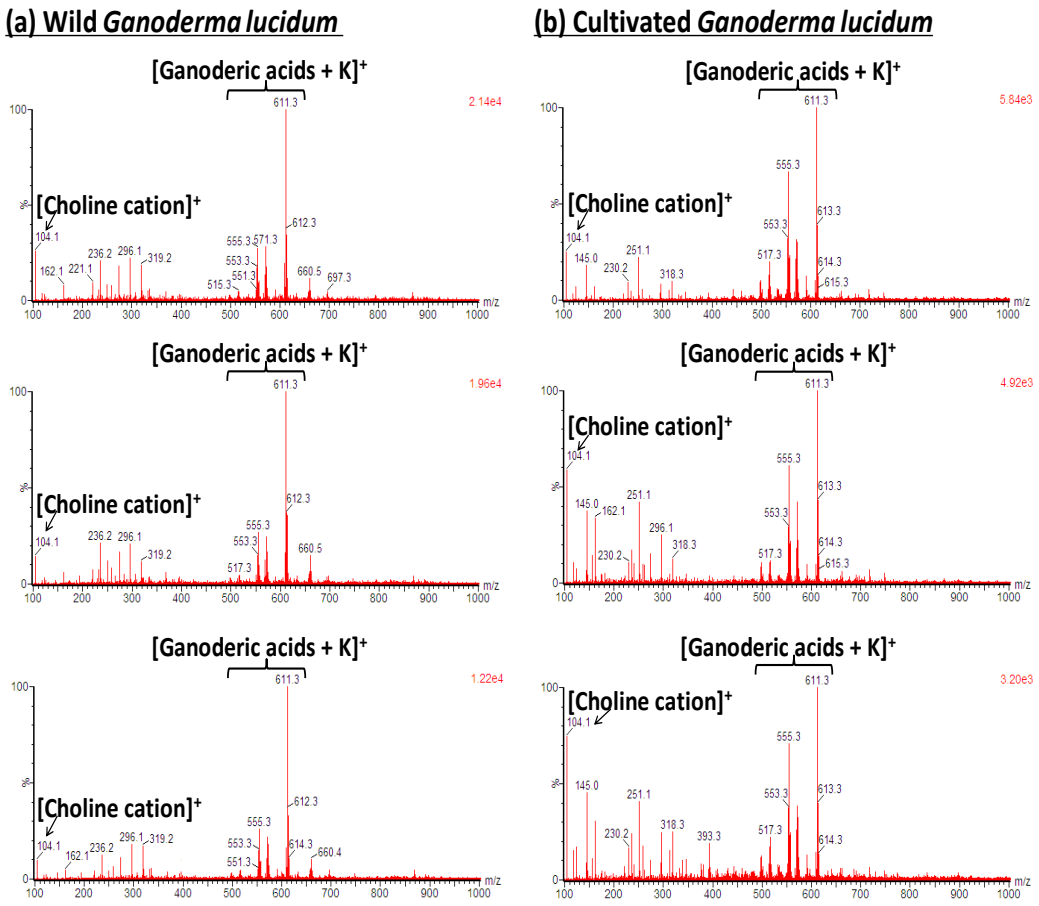


Figure 3.5. DI-MS spectra obtained by DI-MS analysis of different pieces of the same herb samples for (a) wild and (b) cultivated *Ganoderma lucidum* in three independent experiments.

3.3.3. Differentiation of official and confused Ganoderma species

DI-MS was applied to analyze official *Ganoderma* species stated in the Chinese pharmacopoeia, i.e. *Ganoderma lucidum* and *Ganoderma sinense*; and three easily confused species, i.e. *Ganoderma atrum*, *Ganoderma duropora* Lloyd and *Ganoderma applanatum*. As shown in Figure 3.4, the three confused *Ganoderma* species gave spectral profiles that were obviously different from those for the official *Ganoderma* species. Ganoderic acids, the major active components of *Ganoderma*, could not be detected for the three confused species but were abundantly detected for the two official species (Figure 3.4), allowing unambiguous differentiation of the confused species from the official species.

3.3.4. Differentiation of different species, and wild and cultivated species

PCA plot of the two official *Ganoderma* species was generated from the first and second principal components based on their DI-MS data. As shown in Figure 3.6a, the clusters of different *Ganoderma lucidum* and *Ganoderma sinense* species (wild, cultivated and antler-shaped) are well separated in the PCA plot. The clusters of wild *Ganoderma lucidum* and wild *Ganoderma sinense* samples are located in quadrant I and right-hand side of quadrant IV of the PCA plot, respectively, while cultivated *Ganoderma lucidum*, *Ganoderma lucidum* (antler-shaped) and cultivated *Ganoderma sinense* samples are located in quadrant II, III and left-hand side of quadrant IV of the PCA plot, respectively, allowing unequivocal differentiation among them.

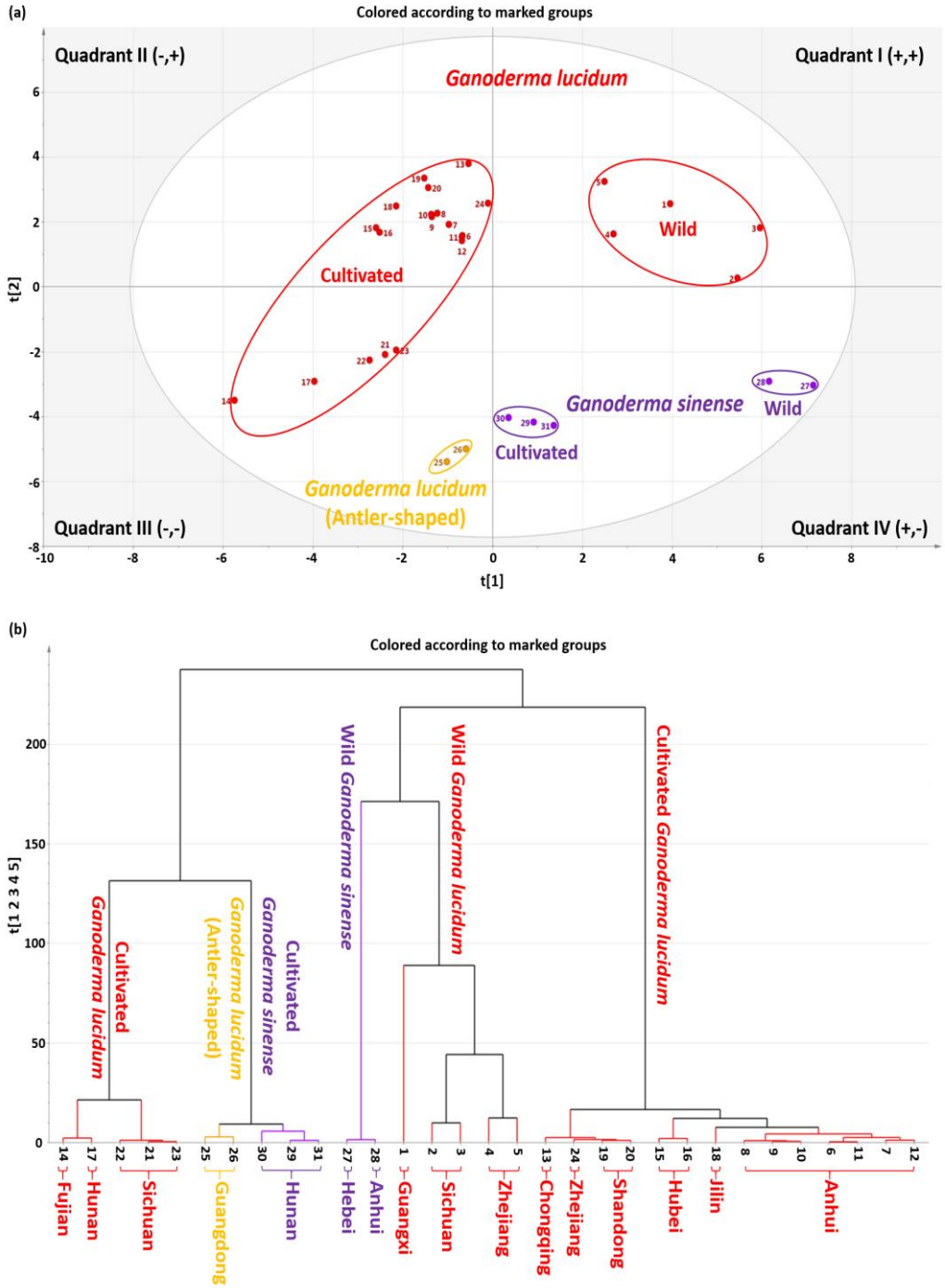


Figure 3.6. (a) PCA plot and (b) HCA plot of *Ganoderma lucidum* (labeled in red) and *Ganoderma sinense* (labeled in purple) samples based on their DI-MS data. Samples 1-5 are wild *Ganoderma lucidum* samples, samples 6-24 are cultivated *Ganoderma lucidum* samples, samples 25-26 are *Ganoderma lucidum* (antler-shaped) samples (labeled in orange), samples 27-28 are wild *Ganoderma sinense* samples, and samples 29-31 are cultivated *Ganoderma sinense* samples.

Similar PCA results were obtained for the confused *Ganoderma* samples, as shown in Figure 3.7a. The clusters of *Ganoderma applanatum*, *Ganoderma duropora* Lloyd and *Ganoderma atrum* samples were located separately in quadrant I, III and IV of the PCA plot, respectively. For *Ganoderma applanatum*, only the wild samples were analyzed in this study, since *Ganoderma applanatum* is seldom cultivated. For *Ganoderma duropora* Lloyd, although the clusters of the wild and cultivated samples were located at the same quadrant in the PCA plot, they were well separated to allow the differentiation. However, the clusters of the wild and cultivated samples of *Ganoderma atrum* were not clearly separated, due to the high degree of similarity of their spectra as shown in Figures 3.4f and 3.4g.

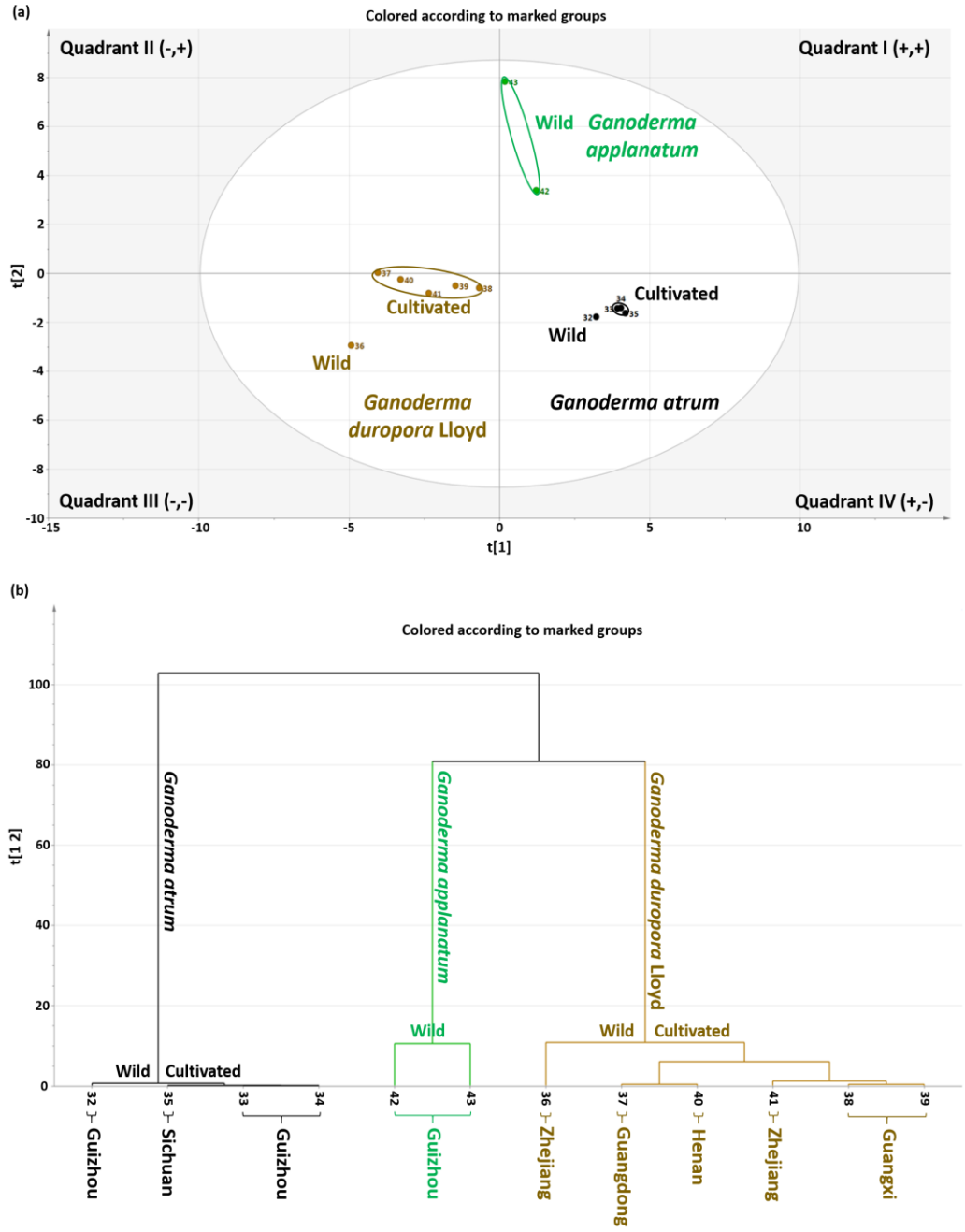


Fig. 3.7. (a) PCA plot and (b) HCA plot of the three easily confused species, i.e. *Ganoderma atrum* (labeled in black), *Ganoderma duropora* Lloyd (labeled in brown) and *Ganoderma applanatum* (labeled in green) samples, based on their DI-MS data. Sample 32 is wild *Ganoderma atrum* sample, samples 33-35 are cultivated *Ganoderma atrum* samples, sample 36 is wild *Ganoderma duropora* Lloyd sample, samples 37-41 are cultivated *Ganoderma duropora* Lloyd samples, and samples 41-42 are wild *Ganoderma applanatum* samples.

HCA, another commonly used statistics tool [205], was also employed to analyze the DI-MS results. As shown in Figures 3.6b and 3.7b, results similar to those obtained from PCA analysis were obtained by using HCA. The dendrograms used in the HCA plots allowed clear groupings of the *Ganoderma* samples.

3.3.5. Differentiation of species from different origins

As shown in Figure 3.6b, the HCA plot of the two official *Ganoderma* species shows that the sample data from the same geographical origins are grouped together within the brackets, indicating that samples from different geographical origins could be differentiated for both wild and cultivated *Ganoderma lucidum* and *Ganoderma sinense* samples. For example, seven cultivated *Ganoderma lucidum* samples from Anhui (samples 6-12) are grouped together, showing clear difference from the others. Similar results could also be observed from the HCA plot of the confused *Ganoderma* species (Figure 3.7b) and the PCA plots of both official and confused *Ganoderma* species (Figures 3.6a and 3.7a). Although only limited numbers of samples were available for each sample source, it can be seen that the sample points of the same sources are clustered together. For example, for wild *Ganoderma lucidum*, two samples from Sichuan (samples 2 & 3) and two

samples from Zhejiang (samples 4 & 5) are each clustered together and separate from each other in the PCA plot (Figure 3.6a), suggesting that the origins of these wild *Ganoderma* samples could be distinguished by DI-MS. However, in general, more samples are needed to further validate differentiation of the sample origins by the method.

3.4. Conclusions

In this study, a DI-MS-based method has been developed for rapid differentiation of *Ganoderma* species. Official *Ganoderma* species stated in the Chinese pharmacopoeia and confused *Ganoderma* species could be unambiguously differentiated based on the detection of the major active components in the DI-MS spectra. Differentiation between wild and cultivated species, and potentially discrimination of species from different geographical origins could also be achieved based on the DI-MS analysis combining with PCA or HCA analysis. The method is simple, rapid and reproducible, and can be easily adopted by researchers in relevant fields. Considering the large market and high analytical demand of *Ganoderma*, this method is expected to have positive impact on the *Ganoderma* industry. The method can also be further expanded for analysis of other herbal medicines as well as other plant and animal samples [206-209], and has great potential applications in the future.

Chapter 4. Mechanistic Studies of Direct Ionization Mass Spectrometry

4.1. Introduction

In recent years, direct ionization mass spectrometry (DI-MS), a new sample preparation and ionization techniques, has been developed to enable direct analysis of raw solid samples [16, 100-103], including Chinese herbal medicines (CHMs), with no or only little sample preparation. In this technique, a small piece of a raw herbal medicine sample is placed in front of the mass spectrometer inlet by using a metal clip. With the application of some solvents and a high voltage, spray ionization could be induced from the herbal medicine to generate the mass spectrum. Active components of CHMs could be detected in their DI-MS spectra, allowing rapid characterization of the CHMs, and the technique can be used for authentication, species differentiation, and discrimination of wild type and cultivated type as well as different geographical origins of CHMs [16, 101]. The analysis of one sample can be completed within minutes. This technique is simple, rapid and reliable, as compared to conventional methods that require comprehensive sample homogenization, extraction, separation and detection and thus are usually time-consuming and labor-intensive. However, its extraction and ionization mechanisms that were still unclear and will be investigated in this study.

Many researchers have tried to investigate the factors that influence the ESI-MS

responses of analytes. Factors that have been investigated include gas phase basicity [210], solvation energy [211] and surface activity (affinity for electrospray droplet surfaces) of the analyte [212, 213], and significant differences in ESI-responses of analytes are usually observed [214]. The investigations indicated that the ease of ionization of analytes is not the only criteria that cause selectivity in ESI-MS as many theories showed that significant difference in surface activity, polarity and acidity of analytes are the important factors that would results in the selectivity of ESI responses [215-219]. In general, analytes that analyzed by ESI-MS should have a polar region so that charge can be resided on the molecules and the rest of molecules may be nonpolar in nature. Analytes with significant nonpolar regions are known to be the "surface-active" ions that have higher ESI responses than the analytes with polar regions only. Several studies have been indicated that surface-active analytes compete more effectively for the excess charge during the ESI-MS process and have higher ESI responses [217, 218]. Apart from that, solvation energy is another important factor that causes the selectivity of ESI responses [211]. The theory suggested that solvation energy, which is the energy that needed to transfer the solvated ions from solution phase to the gas phase, determines the rate of evaporation of analytes from ESI droplets and hence the ESI responses of analytes. However, the mechanism of ESI-MS could not be applied

straightforwardly to the mechanism of DI-MS as their mechanisms are not exactly the same [220, 221]. Therefore, investigation of the extraction and ionization mechanisms of DI-MS is an important task.

In conventional ESI-MS, there are several ionization mechanisms [211, 214, 222] proposed for the desolvation of the observed ions. One is 'ion evaporation' in which ions are released from the droplet surface by the increase in surface charge density leading to the solvent evaporation and generation of a coulombic repulsion that exceeds the liquid's surface tension and cause the ions to eject from the surface [222]. Another proposed mechanism is 'coulomb fission' in which droplets consist of single ions that are released from the large droplets by the increase in surface charge density leading to the offset of the droplet surface tension and solvent evaporation that causes the large droplets divide into smaller droplets and increases the overall surface-to-volume ratio. Most commonly, 'preformed ions' that present as ions in the bulk solution are analyzed by ESI-MS. During the process of ESI-MS, some fractions of the preformed ions are separated from their counter-ions and resided on the surface of the droplets by the applied opposite potential between the capillary and counter electrode. Eventually, free gas-phase ions are formed by ion evaporation [222] or by successive coulomb fissioning leading to single ions

resulted in the ESI droplets [223]. However, in DI-MS, there is simultaneously *in situ* extraction of analytes during desorption and ionization of analytes, which is different from the conventional ESI-MS that only involves desorption and ionization of analytes. Therefore, investigation of the factors governing analytes responsiveness to DI-MS is necessary.

In this study, CHMs with different classes of active ingredients were investigated with solvents of various properties, and the products generated at different extraction and ionization stages were collected, analyzed and compared in order to understand the process and factors governing the signal responses of this direct analysis technique, to understand its extraction and ionization mechanisms, and to enhance the detection of active ingredients of CHMs. Various CHMs including *Ganoderma lucidum*, *Schisandrae chinensis* fructus, *Hylocereus undatus*, *Radix polygoni multiflora*, *Lycoris radiata*, *Psidium guajava* leaves and *Ilex latifolia* leaves (see Figure 4.1. for the photos of the CHMs) were investigated in this study. The analysis of which is of interest because they are commonly found CHMs and consist of different classes of active ingredients including ganoderic acids, schisandrin, kaempferol, emodin, chrysophanol, anthrone, lycorine, galantamine, oleanolic acid, ursolic acid, caffeoylquinic acids, kudinosides and latifolosides,

which belong to different compound types of active ingredients including triterpenoids (tetracyclic), lignan (polyphenolic), flavonoids (ketone-containing compounds), anthraquinones (aromatic organic compound), alkaloids (N-containing compounds, weak bases), terpenes, ester and saponins (amphipathic glycosides). Sample information of the CHMs analyzed in this study was summarized in Table 4.1. Meanwhile, solvent loaded onto the surface of CHMs may be crucial for the observation of desired ion signals as it serves for simultaneous extraction of compounds from the sample and ionization of the extracted compounds in DI-MS. Therefore, solvents of various polarity and acidity were employed to investigate the mechanisms of DI-MS.

In order to further investigate the extraction and ionization mechanisms of analytes during DI-MS analysis of CHMs, the detailed mechanisms of this phenomenon was investigated by comparing the surface activity, polarity and acidity of analytes. Many studies have been conducted and indicated that surface activity, acidity and polarity of analytes may be crucial factors governing ESI-MS responses [224-227]. However, it is impossible to generalize the results of all the previous studies to ESI-MS responses in general because they studied only several similar classes of analytes. Also, it is difficult to extrapolate the ESI-MS responses to DI-MS

responses because of their different mechanisms. Therefore, the results of this mechanistic study of DI-MS would allow us to get more insight into the extraction and ionization mechanisms of DI-MS and would help us to explore the ways to enhance the sensitivity and selectivity of DI-MS.

Figure 4.1. Photos of various Chinses herbal medicines.

Table 4.1. Sample information of the Chinese herbal medicines analyzed in this study.

Chinses herbal medicine	Medicinal parts	Major active ingredients	Compound type
Ganoderma lucidum	Fruiting body	Ganoderic acids	Triterpenoids (tetracyclic)
Schisandrae chinensis fructus	Fruit	Schisandrins	Lignan (polyphenolic)
Hylocereus undatus	Flower	Kaempferols	Flavonoids (ketone-containing compounds)
Radix polygoni multiflora	Root tuber	Emodin, physcion & pterostilbene	Anthraquinones (aromatic organic compound)
Lycoris radiata	Bulb	Lycorine, lycoramine & lycorenine	Alkaloids (N-containing compounds, weak bases)
Psidium guajava leaves	Leaf	Psiguajadials, psidials & guavinosides	Terpenes (multiples of C_5H_8 isoprene units)
Ilex latifolia leaves	Leaf	Caffeoylquinic acids, kudinosides & latifolosides	Ester, saponins (amphipathic glycosides)

4.2. Experimental

4.2.1. Chemicals and materials

Samples of various Chinese herbal medicines, including *Ganoderma lucidum*, *Schisandrae chinensis* fructus, *Hylocereus undatus*, *Radix polygoni multiflora*, *Lycoris radiata*, *Psidium guajava* leaves and *Ilex latifolia* leaves were provided by the local farmers and manufactures or purchased from authentic Chinese herbal medicine pharmacies (see Table 4.1 for the sample information). All the CHMs samples were sealed in bags and stored in an electronic dry cabinet before analysis. Sodium iodide used for external calibration of mass spectrometer was purchased from Panreac Química (Barcelona, Spain). Methanol and all the other solvents used in this study were of HPLC grade and purchased from Tedia (Fairfeild, OH, USA). All chemicals were used directly without further purification.

4.2.2. Four methods for evaluating different extraction and ionization stages of DI-MS analysis of Chinese herbal medicines

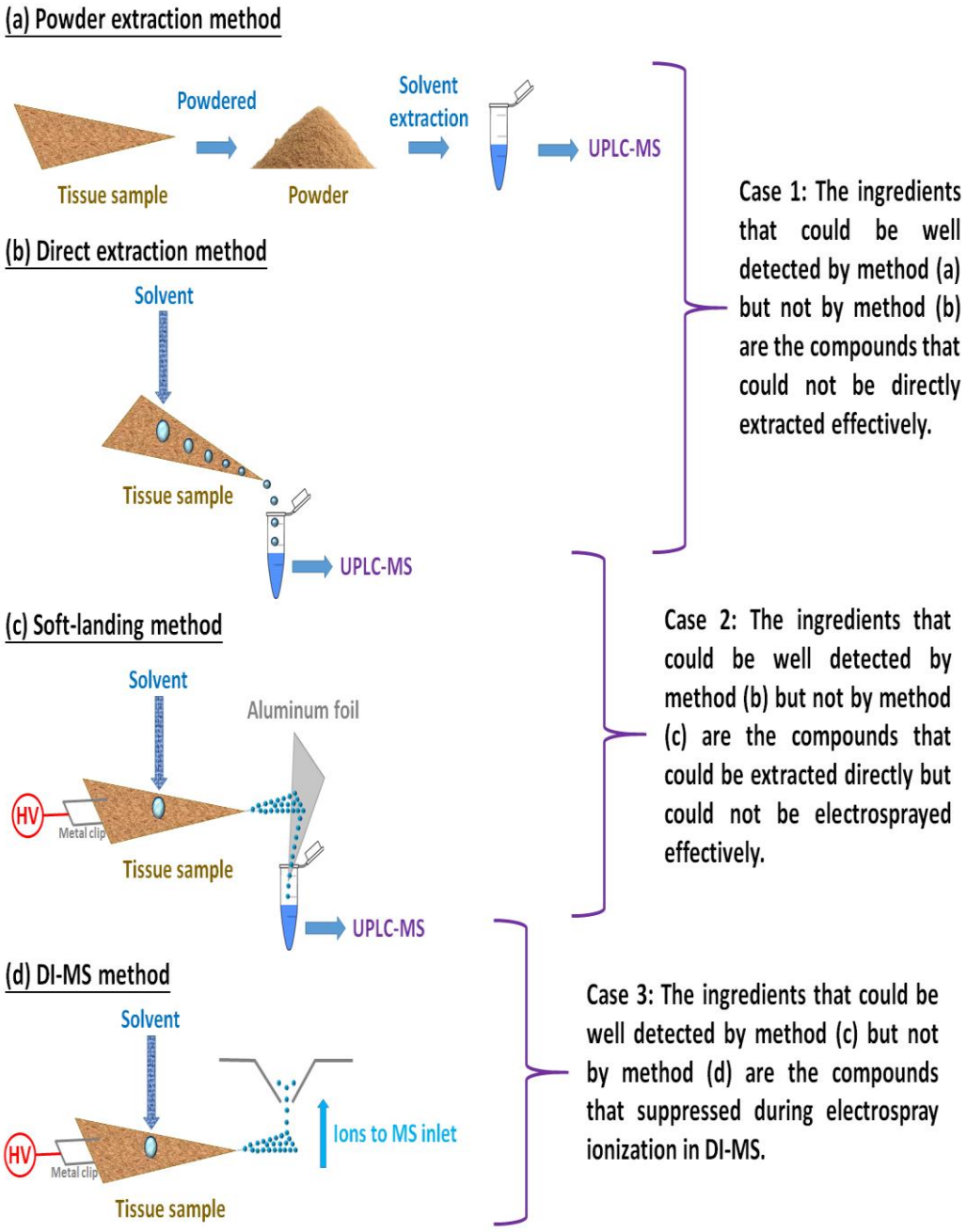


Figure 4.2. Four methods for evaluating different extraction and ionization stages of the DI-MS analysis of CHMs: (a) powder extraction method, (b) direct ionization method, (c) soft-landing method and (d) DI-MS method.

Four different methods were used to evaluate different extraction and ionization stages of the DI-MS analysis of CHMs, i.e. powder extraction method, direct ionization method, soft-landing method and DI-MS method. CHMs with different classes of active ingredients were investigated with solvents of various properties, and the products generated at different extraction and ionization stages were collected, analyzed and compared (see Figure 4.2. for the details). By comparing the results of the powder extraction method and direct ionization method, the ingradients that could be well detected by the powder extraction method but not by the direct ionization method are the compounds that could not be directly extracted effectively. By comparing the results of the direct ionization method and soft-landing method, the ingradients that could be well detected by the direct ionization method but not by the soft-landing method are the compounds that could be extracted directly but could not be electrosprayed effectively. Meanwhile, by comparing the results of the soft-landing method and DI-MS method, the ingradients that could be well detected by the soft-landing method but not by the DI-MS method are the compounds that are suppressed during the electrospray ionization in DI-MS.

In the powder extraction method (Figure 4.2a), raw CHMs were cut into small pieces and powdered, then 0.01 g sample was accurately weighed and 1 mL of solvent was added for extraction. Then, the solution was ultra-sonicated for 30 min and centrifugated for 10 min. Finally, the sample solution was filtrated through a 0.2 μm centrifuge filter before UPLC-MS analysis. In the direct ionization method (Figure 4.2b), 20 μL solvent was directly loaded onto each of the surface of a small piece of raw CHM, this step was repeated 5 times and repeated with 5 pieces of the sample to have 1 mL extract. Then, the solution was centrifugated for 10 min and filtrated through a 0.2 μm centrifuge filter before UPLC-MS analysis. In the soft-landing method (Figure 4.2c), a small piece of raw CHM sample was placed in front of an aluminum foil which was prewashed with 10 μL solvent for 10 times. High voltage was applied to the sample and 2 μL solvent was loaded to each sample surface to induce the spray ionization from the CHM to the Al foil. The solvent was loaded to each sample surface for 10 times and this step was repeated 5 times with different pieces of the sample. The spray was then collected from the surface of the Al foil by loading 10 μL solvent to the Al foil, and repeated 20 times to have 200 μL extract. Afterward, the solution was centrifugated for 10 min and filtrated through a 0.2 μm centrifuge filter before UPLC-MS analysis. In the DI-MS method (Figure 4.2d), a small piece of a raw CHM sample was placed in front

of the MS inlet. High voltage was applied to the sample and 10 μL solvent was loaded to the sample surface to induce spray ionization from the CHM to generate the mass spectrum.

4.2.3. Setup for ultra-performance liquid chromatography-mass spectrometry of Chinese herbal medicines

Chromatographic analysis was carried out using an Agilent 6540 ultra-performance liquid chromatography (UPLC) system equipped with Agilent extend-C18 column (2.1mm × 50mm, 1.8μm), an auto-sampler and a quaternary solvent deliver system. The mobile phase consisted of (A) H_2O with 0.1% formic acid and (B) ACN with 0.1% formic acid and the gradient elution program was as follows: 2 - 30% (B) at 0 - 9 min; 30% to 95% (B) at 9 - 40 min; 95% to 2% (B) at 45 - 46 min and there was 4 min re-equilibrium between injections. The flow rate was 0.3 mL/min and aliquots of 10 μL were injected into the UPLC. A quadrupole-time of flight (Q-TOF) mass spectrometer was connected to the UPLC instrument via an electrospray ionization (ESI) interface. The LC effluent was introduced into the ESI source with high purity nebulizing nitrogen (N_2) gas. Instrumental automatic tuning was applied to optimize all the instrument's parameters. The optimized parameters were as follows: the sheath gas temperature and sheath gas flow were set at 350 °C and 11 L/min respectively.

When relatively low and non-polar solvents were employed as the extraction solvents, i.e. acetone, ethyl acetate, dichloromethane, chloroform, and hexane, reconstitution of solvent was carried out. The solutions prepared by relatively low and non-polar solvents were dried under N_2, then reconstituted into 1 μL MeOH, ultra-sonicated for 10 min and filtrated through a 0.2 μm centrifuge filter before the UPLC-MS analysis.

4.2.4. Setup for DI-MS of Chinese herbal medicines

The schematic diagram of experimental setup for DI-MS analysis of Chinese herbal medicines samples is shown in Figure 4.1d. A small piece of raw CHM sample was cut into a sharp triangular shape (about 0.5 cm for the base width and about 1 cm for the height) and placed orthogonal to the mass spectrometer inlet by using a metal clip, with distances of 0.5 cm in vertical and 1 cm in horizontal from the sample tip to the MS inlet. A crocodile clip was used to connect the high voltage supply of the mass spectrometer to the metal clip. With the application of a high voltage (3 to 3.8 kV) and some solvents (10 μL) to the center of the CHMs sample, spray ionization could be induced from the sharp end of the herbal medicine to generate mass spectra.

4.2.5. Mass spectrometric measurements

Mass spectra were acquired on a QToF II mass spectrometer (Waters, Milford, MA) and a QToF mass spectrometer (Agilent, Santa Clara, CA) using both positive and negative ion mode. Instrumental control and data acquisition were conducted by using MassLynx 4.1 software and MassHunter workstation B.06.00 software respectively. Instrumental parameters, including source temperature, voltage of microchannel plate (MCP) detector and sample cone voltage, were set at 40 ºC, 2.1 kV and 30 V, respectively. High capillary voltage applied to the samples was typically set at 3 to 3.8 kV, the voltages obtained after optimization. Mass spectra were acquired in an *m/z* range of 100 - 1000 Da with an interscan time of 0.1 s and a scan time of 1 s Typically, mass spectra were generated by accumulating data from the first minute. Before DI-MS and UPLC-MS analysis, sodium iodide and Agilent tuning mix were used for *m/z* calibration of the instruments.

4.3. Results and Discussion

4.3.1. Mechanistic Studies of DI-MS analysis of Ganoderma lucidum

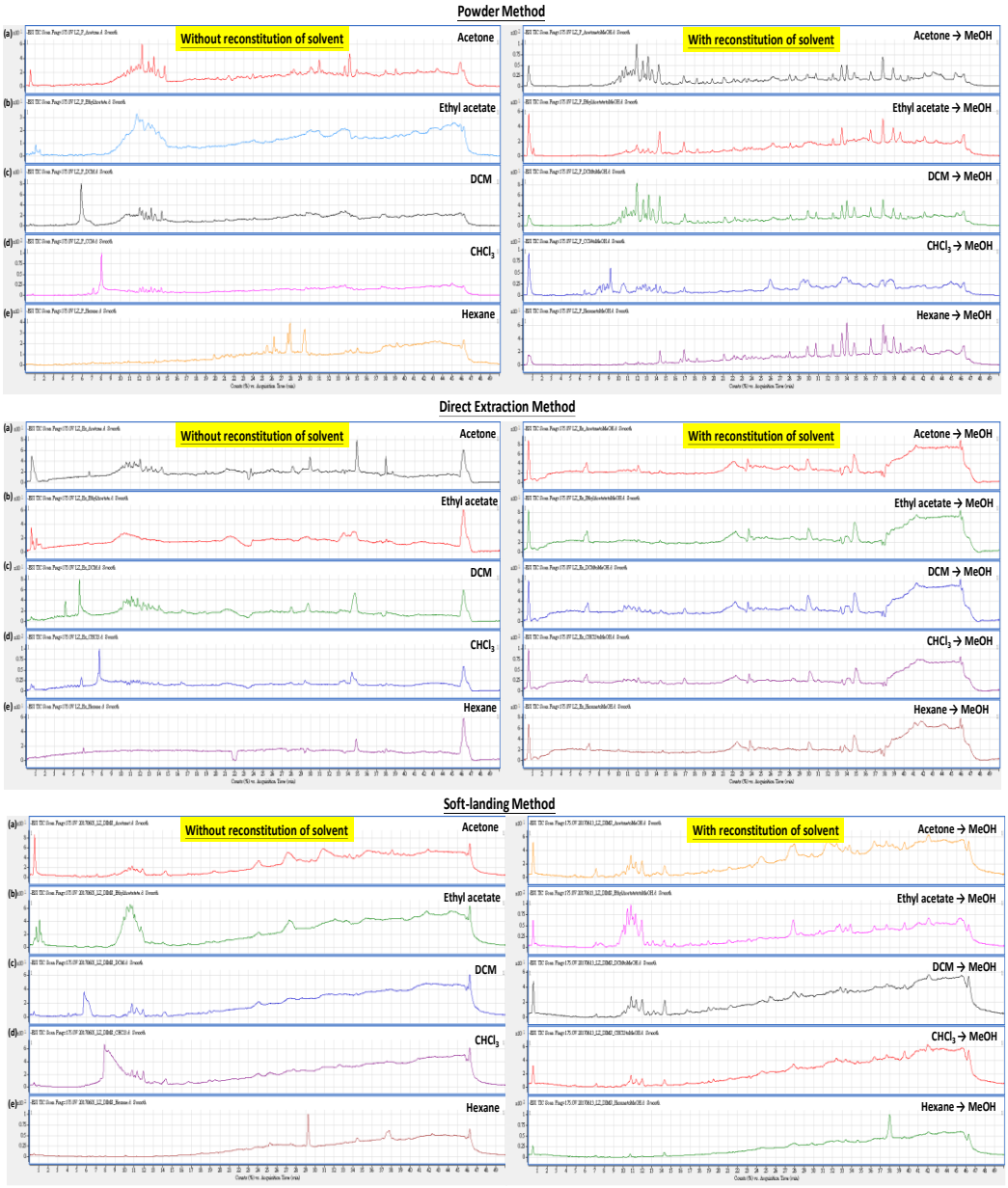


Figure 4.3. Total ion chromatogram (TIC) obtained by UPLC-MS analysis of the *Ganoderma lucidum* samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of different solvents: (a) acetone, (b) ethyl acetate, (c) dichloromethane, (d) chloroform, and (e) hexane without (left), or with (right), reconstitution of solvent into methanol.

By comparing the total ion chromatogram (TIC) obtained by UPLC-MS analysis of the *Ganoderma lucidum* samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of relatively low and non-polar solvents without, or with, reconstitution of solvent into methanol (Figure 4.3), the results showed that with the reconstitution of solvent, the TIC obtained by UPLC-MS analysis of the *Ganoderma lucidum* samples with the application of relatively low and non-polar solvents gave better chromatogram that with shaper peaks and better separation of components. Therefore, reconstitution of solvent was carried out when relatively low and non-polar solvents were employed to the UPLC-MS analysis of the *Ganoderma lucidum*.

Figure 4.4. Total ion chromatogram (TIC) obtained by UPLC-MS analysis of the *Ganoderma lucidum* samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of different solvents: (a) water, (b) methanol/water (1/1), (c) acetonitrile/water (1/1), (d) methanol, (e) acetonitrile, (f) acetone, (g) ethyl acetate, (h) dichloromethane, (i) chloroform, and (j) hexane, where (e) to (j) were reconstituted into methanol.

By comparing the TIC obtained by UPLC-MS analysis of the *Ganoderma lucidum* samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of different solvents (Figure 4.4) and the DI-MS spectra of *Ganoderma lucidum* with the application of different solvents (Figure 4.5), the overall results showed that active ingredients of *Ganoderma* which could be detected by the powder extraction method, the direct extraction method and the soft-landing method, could also be detected by the DI-MS method, indicating that the active components of *Ganoderma* could be directly extracted and electrosprayed, there were no suppression of analytes during DI-MS analysis of *Ganoderma*. The signal intensities of ganoderic acids obtained with the cultivated *Ganoderma lucidum* and various solvents using various methods were further compared quantitatively and summarized in Table 4.2. The overall results showed that the major active ingredients of *Ganoderma lucidum*, i.e. ganoderic acids, were relatively polar that favoured to be extracted with solvents of middle polarity such as methanol and acetonitrile in the powder extraction method, the direct extraction method, the soft-landing method and the DI-MS method. The results indicated that polarity of solvent was a crucial factor for the observation of desired ion signals of the active components of *Ganoderma lucidum*.

Table 4.2. Summary of the signal intensities of ganoderic acids obtained with the cultivated *Ganoderma lucidum* and various solvents using various methods.

Solvent	**Intensity of ganoderic acids**			
	DI-MS	**Powder extraction method**	**Direct extraction method**	**Soft-landing method**
Water	Medium	Strong	Medium	Weak
Methanol/water (1/1)	Medium	Strong	Medium	Weak
Acetonitrile/water (1/1)	Medium	Strong	Strong	Weak
Methanol	Strong	Strong	Strong	Medium
Acetonitrile	Strong	Medium	Medium	Strong
Dichloromethane	Weak	Weak	Weak	Weak
Chloroform	Weak	Weak	Weak	Weak
Hexane	Weak	Weak	Weak	Weak

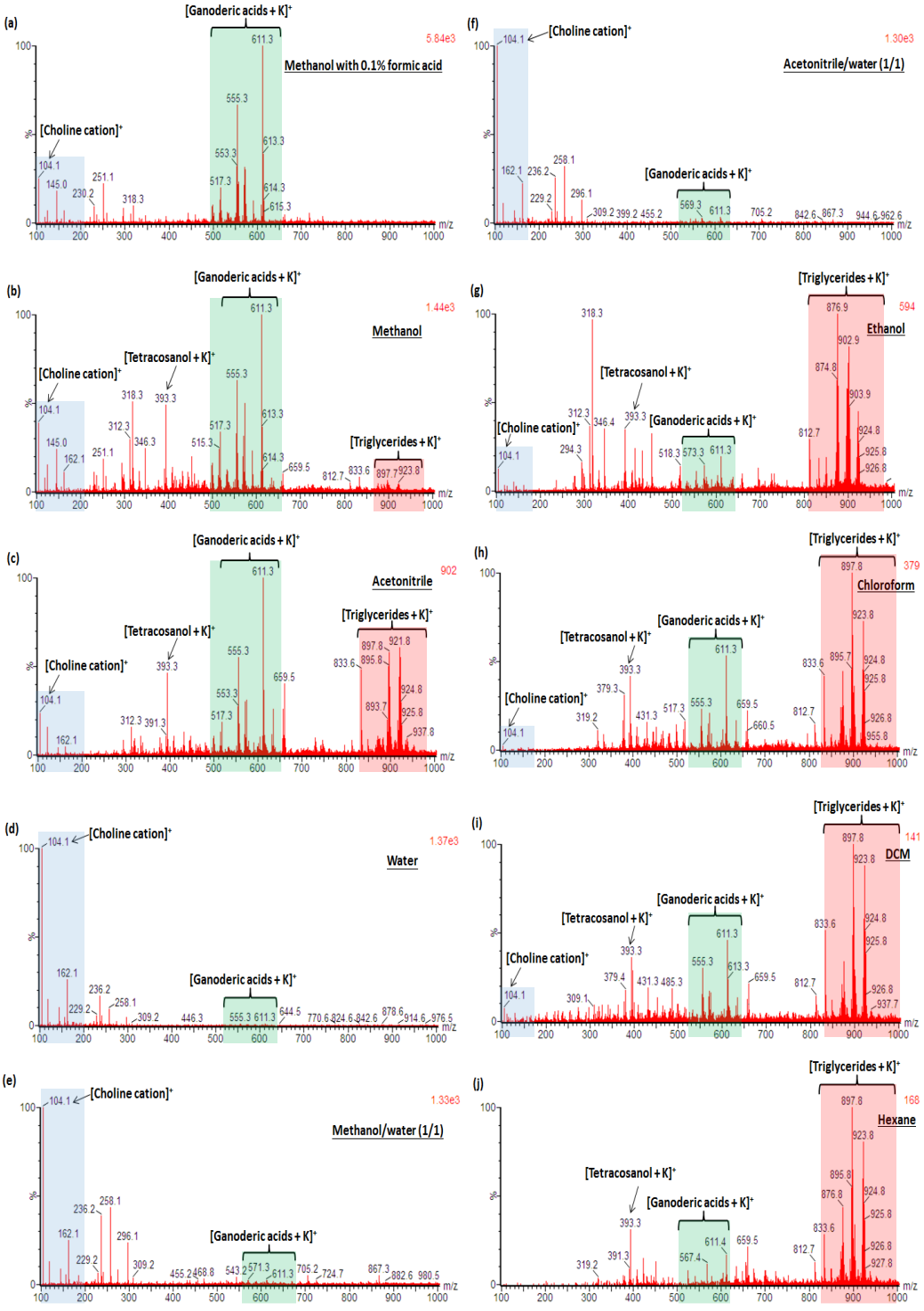


Figure 4.5. DI-MS spectra of *Ganoderma lucidum* with the application of different solvents: (a) methanol with 0.1% formic acid, (b) methanol, (c) acetonitrile, (d) water with capillary voltage of 4 kV, (e) methanol/water (1/1), (f) acetonitrile/water (1/1), (g) ethanol, (h) chloroform, (i) dichloromethane, and (j) hexane.

Moreover, the DI-MS spectra of *Ganoderma lucidum* obtained with the application of different solvents (Figure 4.5) also showed that the spectra varied significantly with the solvents applied. Solvents of different polarities and acidities, including methanol with 0.1% formic acid, methanol, methanol/water (1/1), acetonitrile, acetonitrile/water (1/1), water with capillary voltage of 4 kV, ethanol, chloroform, dichloromethane and hexane, were tested for DI-MS analysis of *Ganoderma lucidum*. The results showed that detection of active ingredients i.e. ganoderic acids could be achieved with all the solvents tested but markedly differences in signal intensities of ganoderic acids were observed. When solvents of higher and lower polarity were used, choline cation [187] at *m/z* 104.1 and triglycerides [188] (PLP at *m/z* 869.8, POP at *m/z* 871.8, PLL at *m/z* 893.7, PLO at *m/z* 895.8, POO at *m/z* 897.8, POS at *m/z* 899.8, OLL at *m/z* 919.8, OLO at *m/z* 921.8, OOO at *m/z* 923.8 and SOO at *m/z* 925.8, where the triglycerides were detected in K^+ adduct form, L: linoleic acid; O: oleic acid; P: palmitic acid and S: stearic acid) would be markedly observed as the base peak of the resulting spectra as they favored the extraction and ionization of relatively polar and non-polar ingredients respectively. The results suggested that solvent loaded onto the surface of *Ganoderma lucidum* was crucial for the observation of desired ion signals of the active components of *Ganoderma lucidum*.

4.3.2. Mechanistic Studies of DI-MS analysis of Schisandrae chinensis fructus

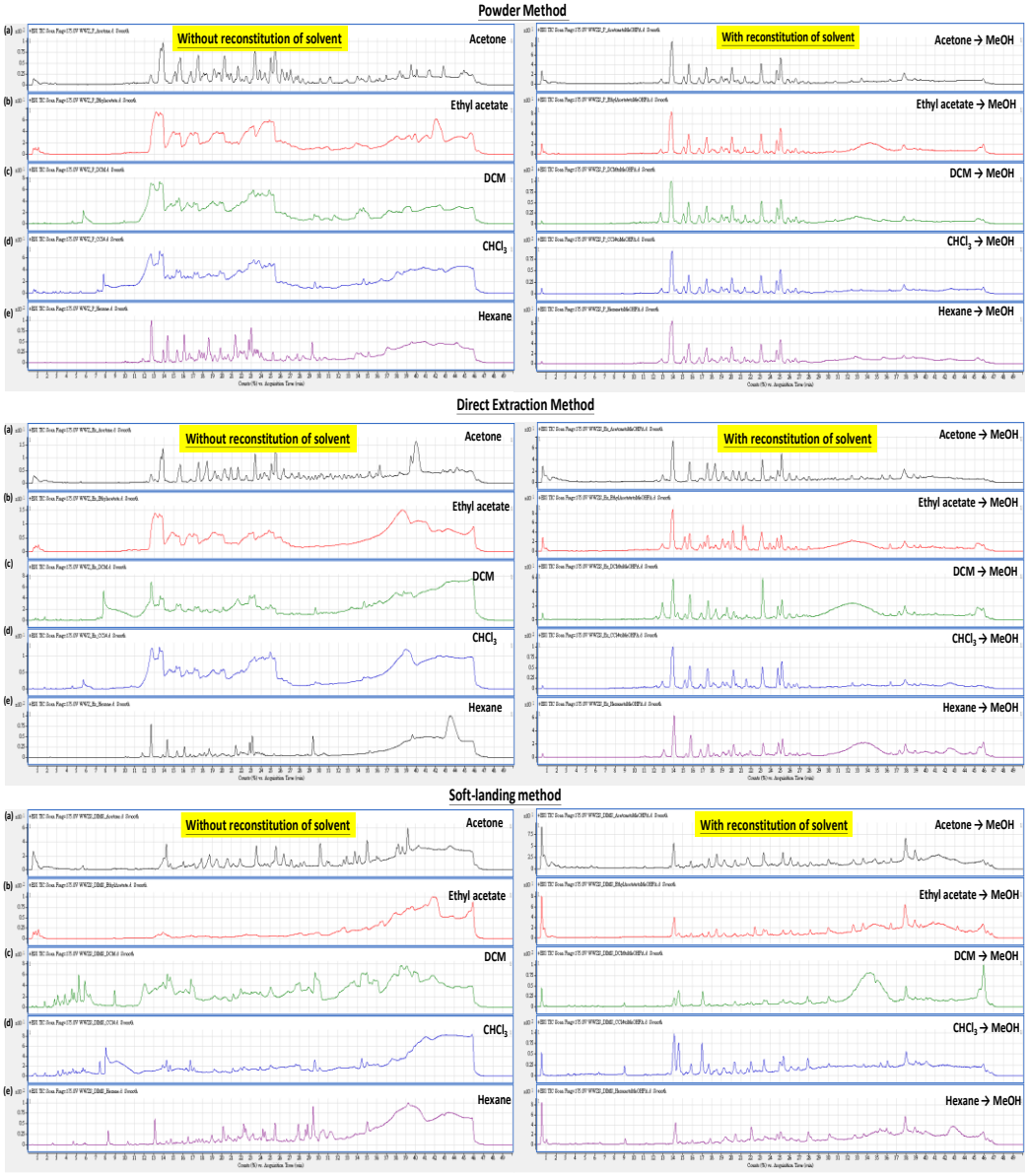


Figure 4.6. Total ion chromatogram (TIC) obtained by UPLC-MS analysis of the *Schisandrae chinensis* fructus samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of different solvents: (a) acetone, (b) ethyl acetate, (c) dichloromethane, (d) chloroform, and (e) hexane without (left), or with (right), reconstitution of solvent into methanol.

By comparing the total ion chromatogram (TIC) obtained by UPLC-MS analysis of the *Schisandrae chinensis fructus* samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of relatively low and non-polar solvents without, or with, reconstitution of solvent into methanol (Figure 4.6), the results showed that with the reconstitution of solvent, the TIC obtained by UPLC-MS analysis of the *Schisandrae chinensis fructus* samples with the application of relatively low and non-polar solvents gave better chromatogram that with shaper peaks and better separation of components. Therefore, reconstitution of solvent was carried out when relatively low and non-polar solvents were employed to the UPLC-MS analysis of the *Schisandrae chinensis fructus*.

Figure 4.7. Total ion chromatogram (TIC) obtained by UPLC-MS analysis of the *Schisandrae chinensis* fructus samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of different solvents: (a) water, (b) methanol/water (1/1), (c) acetonitrile/water (1/1), (d) methanol, (e) acetonitrile, (f) acetone, (g) ethyl acetate, (h) dichloromethane, (i) chloroform, and (j) hexane, where (e) to (j) were reconstituted into methanol.

By comparing the TIC obtained by UPLC-MS analysis of the *Schisandrae chinensis* fructus samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of different solvents (Figure 4.7) and the DI-MS spectra of *Schisandrae chinensis* fructus with the application of different solvents (Figure 4.8**)**, the overall results showed that active ingredients of *Schisandrae chinensis* fructus which could be detected by the powder extraction method, the direct extraction method and the soft-landing method, could also be detected by the DI-MS method, indicating that the active components of *Schisandrae chinensis* fructus could be directly extracted and electrosprayed, there were no suppression of analytes during DI-MS analysis of *Schisandrae chinensis* fructus. The signal intensities of schisandrins obtained with the *Schisandrae chinensis* fructus and various solvents using various methods were further compared quantitatively and summarized in Table 4.3. The overall results showed that the major active ingredients of *Schisandrae chinensis* fructus, i.e. schisandrins, were relatively polar that favoured to be extracted with solvents of middle polarity such as methanol and acetonitrile in the powder extraction method, the direct extraction method, the soft-landing method and the DI-MS method. The results indicated that polarity of solvent was a crucial factor for the observation of desired ion signals of the active components of *Schisandrae*

chinensis fructus.

Table 4.3. Summary of the signal intensities of schisandrins obtained with the *Schisandrae chinensis* fructus and various solvents using various methods.

Solvent	**Intensity of schisandrins**			
	DI-MS	**Powder extraction method**	**Direct extraction method**	**Soft-landing method**
Water	Medium	Strong	Medium	Weak
Methanol/water (1/1)	Medium	Strong	Strong	Weak
Acetonitrile/water (1/1)	Medium	Strong	Strong	Weak
Methanol	Strong	Strong	Medium	Medium
Acetonitrile	Strong	Strong	Strong	Medium
Acetone	Weak	Medium	Medium	Weak
Ethyl acetate	Weak	Medium	Medium	Weak
Dchloromethane	Weak	Medium	Medium	Weak
Chloroform	Weak	Medium	Medium	Weak
Hexane	Weak	Weak	Weak	Weak

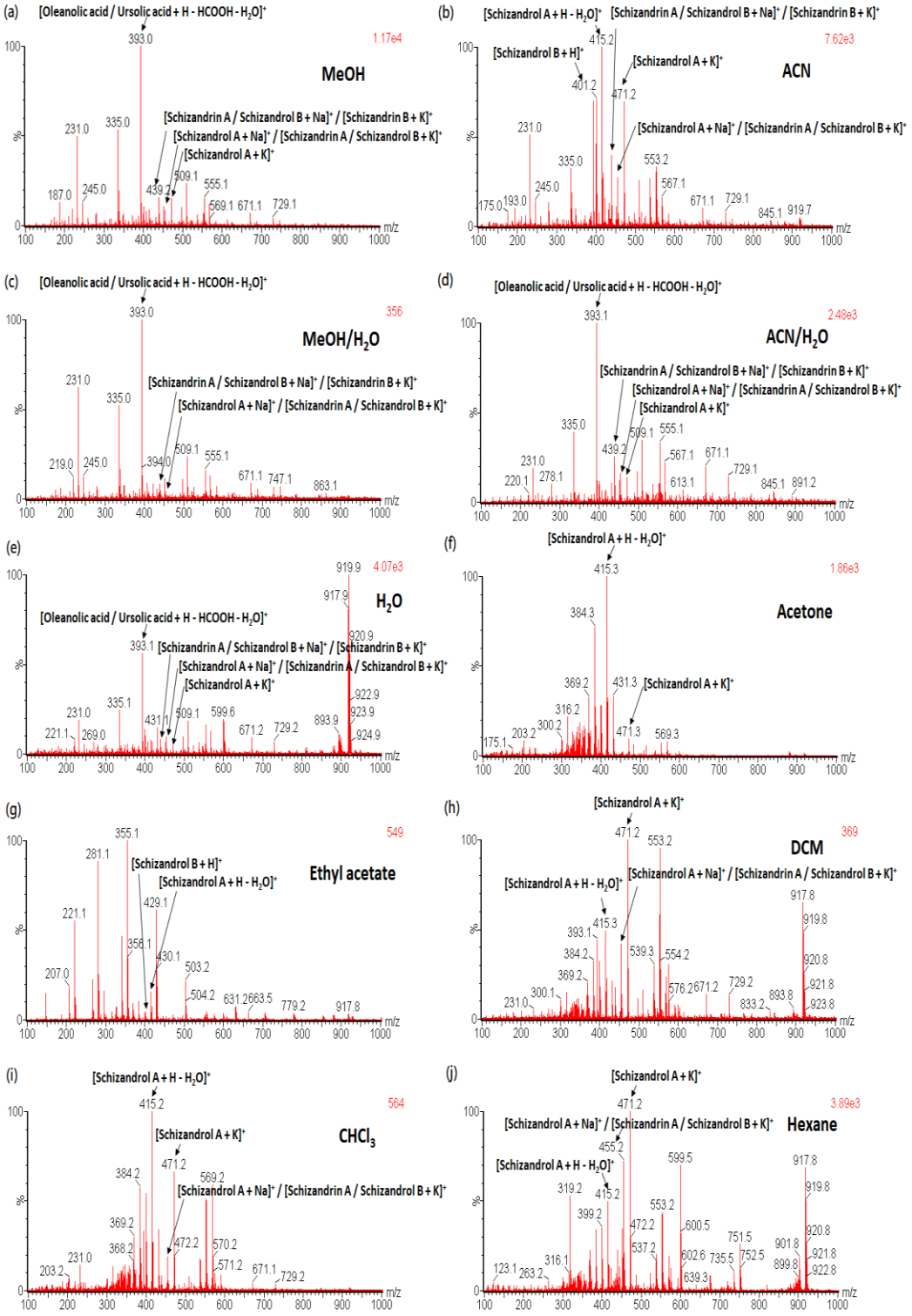


Figure 4.8. DI-MS spectra of *Schisandrae chinensis* fructus with the application of different solvents: (a) methanol, (b) acetonitrile, (c) methanol/water (1/1), (d) acetonitrile/water (1/1), (e) water, (f) acetone, (g) ethyl acetate, (h) dichloromethane, (i) chloroform, and (j) hexane.

Moreover, the DI-MS spectra of *Schisandrae chinensis* fructus obtained with the application of different solvents (Figure 4.8) also showed that the spectra varied significantly with the solvents applied. Solvents of different polarities, including methanol, acetonitrile, methanol/water (1/1), acetonitrile/water (1/1), water, acetone, ethyl acetate, dichloromethane, chloroform, and hexane, were tested for DI-MS analysis of *Schisandrae chinensis* fructus. Detection of [schizandrol B + H]$^+$ at *m/z* 401.2, [schizandrol A + Na]$^+$ / [schizandrin A / schizandrol B + K]$^+$ at *m/z* 455.2 and [schizandrol A + K]$^+$ at *m/z* 471.2 were observed [228-231]. When relatively polar solvents were used, i.e., MeOH/H_2O, ACN/H_2O, MeOH and ACN, significant detection of [oleanolic acid / ursolic acid + H – HCOOH – H_2O]$^+$ at *m/z* 393.0 and [schizandrin A / schizandrol B + Na]$^+$ / [schizandrin B + K]$^+$ at *m/z* 439.2 were observed [228-231]. Meanwhile, when relatively low and non-polar solvents were used, i.e. acetone, ethyl acetate, DCM, $CHCl_3$ and hexane, significant detection of [schizandrol A + H – H2O]$^+$ at *m/z* 415.2 was observed [228-232]. The results showed that detection of active ingredients could be achieved with the solvents tested but markedly differences in signal intensities of active ingredients were observed. The results suggested that solvent loaded onto the surface of *Schisandrae chinensis* fructus is crucial for the observation of desired ion signals of the active components of *Schisandrae chinensis* fructus.

4.3.3. Mechanistic Studies of DI-MS analysis of Hylocereus undatus

Figure 4.9. Total ion chromatogram (TIC) obtained by UPLC-MS analysis of the *Hylocereus undatus* samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of different solvents: (a) acetone, (b) ethyl acetate, (c) dichloromethane, (d) chloroform, and (e) hexane without (left), or with (right), reconstitution of solvent into methanol.

By comparing the total ion chromatogram (TIC) obtained by UPLC-MS analysis of the *Hylocereus undatus* samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of relatively low and non-polar solvents without, or with, reconstitution of solvent into methanol (Figure 4.9), the results showed that with the reconstitution of solvent, the TIC obtained by UPLC-MS analysis of the *Hylocereus undatus* samples with the application of relatively low and non-polar solvents gave better chromatogram that with shaper peaks and better separation of components. Therefore, reconstitution of solvent was carried out when relatively low and non-polar solvents were employed to the UPLC-MS analysis of the *Hylocereus undatus*.

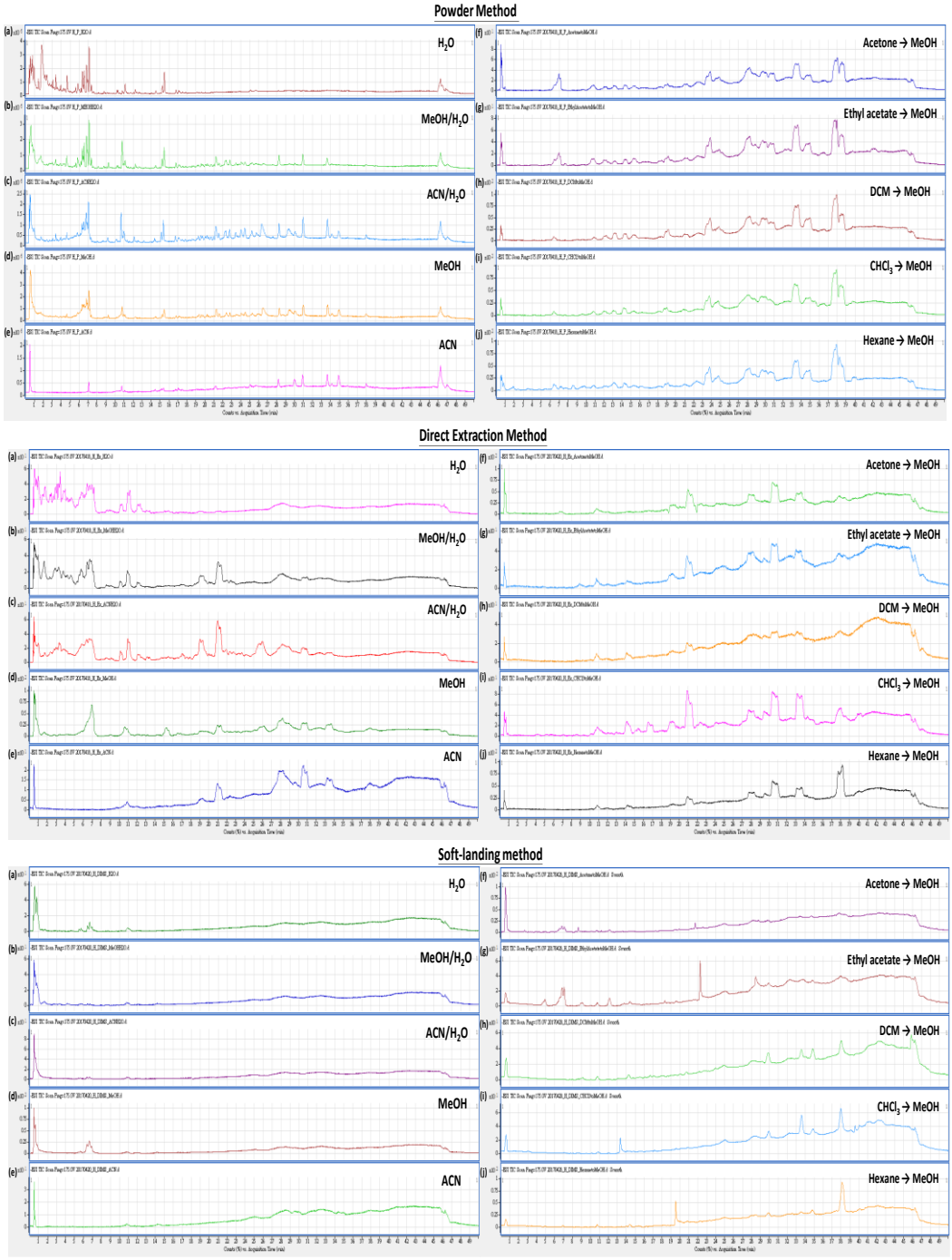


Figure 4.10. Total ion chromatogram (TIC) obtained by UPLC-MS analysis of the *Hylocereus undatus* samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of different solvents: (a) water, (b) methanol/water (1/1), (c) acetonitrile/water (1/1), (d) methanol, (e) acetonitrile, (f) acetone, (g) ethyl acetate, (h) dichloromethane, (i) chloroform, and (j) hexane, where (e) to (j) were reconstituted into methanol.

By comparing the TIC obtained by UPLC-MS analysis of the *Hylocereus undatus* samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of different solvents (Figure 4.10) and the DI-MS spectra of *Hylocereus undatus* with the application of different solvents (Figure 4.11), the overall results showed that active ingredients of *Hylocereus undatus* which could be detected by the powder extraction method, the direct extraction method and the soft-landing method, could also be detected by the DI-MS method, indicating that the active components of *Hylocereus undatus* could be directly extracted and electrosprayed, there were no suppression of analytes during DI-MS analysis of *Hylocereus undatus*. The signal intensities of kaempferols obtained with the *Hylocereus undatus* and various solvents using various methods were further compared quantitatively and summarized in Table 4.4. The overall results showed that the major active ingredients of *Hylocereus undatus*, i.e. kaempferols, were relatively polar that favoured to be extracted with solvents of middle and high polarity such as methanol, methanol/water (1/1) and acetonitrile in the powder extraction method, the direct extraction method, the soft-landing method and the DI-MS method. The results indicated that polarity of solvent was a crucial factor for the observation of desired ion signals of the active components of *Hylocereus undatus*.

Table 4.4. Summary of the signal intensities of kaempferols obtained with the *Hylocereus undatus* and various solvents using various methods.

Solvent	**Intensity of kaempferols**			
	DI-MS	**Powder extraction method**	**Direct extraction method**	**Soft-landing method**
Water	Weak	Strong	Strong	Weak
Methanol/water (1/1)	Medium	Strong	Strong	Weak
Acetonitrile/water (1/1)	Weak	Strong	Medium	Weak
Methanol	Strong	Strong	Medium	Medium
Acetonitrile	Strong	Medium	Medium	Weak
Acetone	Weak	Weak	Weak	Weak
Ethyl acetate	Weak	Weak	Weak	Weak
Dchloromethane	Weak	Weak	Weak	Weak
Chloroform	Weak	Weak	Weak	Weak
Hexane	Weak	Weak	Weak	Weak

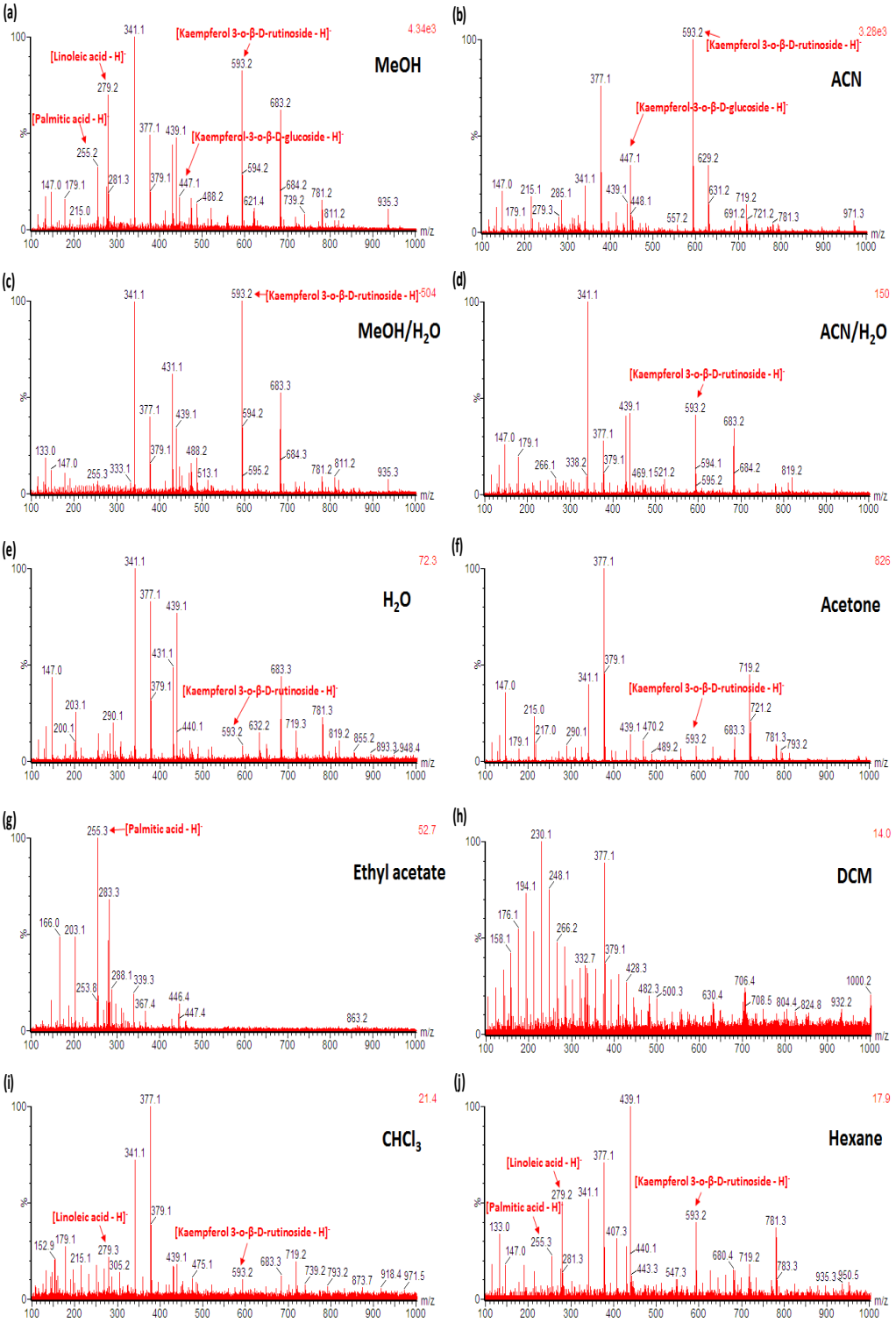


Figure 4.11. DI-MS spectra of *Hylocereus undatus* with the application of different solvents: (a) methanol, (b) acetonitrile, (c) methanol/water (1/1), (d) acetonitrile/water (1/1), (e) water, (f) acetone, (g) ethyl acetate, (h) dichloromethane, (i) chloroform, and (j) hexane.

Moreover, the DI-MS spectra of *Hylocereus undatus* obtained with the application of different solvents (Figure 4.11) also showed that the spectra varied significantly with the solvents applied. Solvents of different polarities, including MeOH, ACN, MeOH/H_2O (1/1), ACN/H_2O (1/1), H_2O, acetone, ethyl acetate, DCM, $CHCl_3$ and hexane, were tested for the DI-MS analysis. Detection of [kaempferol-3-*o*-β-D-glucoside - H]$^-$ at *m/z* 447.1 was observed [233-236]. When relatively polar solvents were used, i.e. H_2O, MeOH, ACN and acetone, significant detection of [kaempferol-3-*o*-β-D-rutinoside - H]$^-$ at *m/z* 593.2 was observed [233-236]. Meanwhile, when relatively low and non-polar solvents were used, i.e. ethyl acetate, $CHCl_3$ and hexane, significant detection of [palmitic acid - H]$^-$ at *m/z* 255.2 and [linoleic acid - H]$^-$ at *m/z* 279.2 were observed with very weak ion signals and undesirable electrical discharge easily [233-236]. Especially, when DCM was applied as the solvent, detection of the active components of *Hylocereus undatus* could not be achieved, only very weak ion signals with undesirable electrical discharge were obtained. The results showed that detection of active ingredients could be achieved with the solvents tested but markedly differences in signal intensities of active ingredients were observed. The results suggested that solvent loaded onto the surface of *Hylocereus undatus* is crucial for the observation of desired ion signals of the active components.

4.3.4. Mechanistic Studies of DI-MS analysis of Radix polygoni multiflora

Figure 4.12. Total ion chromatogram (TIC) obtained by UPLC-MS analysis of the *Radix polygoni multiflora* samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of different solvents: (a) acetone, (b) ethyl acetate, (c) dichloromethane, (d) chloroform, and (e) hexane without (left), or with (right), reconstitution of solvent into methanol.

By comparing the total ion chromatogram (TIC) obtained by UPLC-MS analysis of the *Radix polygoni multiflora* samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of relatively low and non-polar solvents without, or with, reconstitution of solvent into methanol (Figure 4.12), the results showed that with the reconstitution of solvent, the TIC obtained by UPLC-MS analysis of the *Radix polygoni multiflora* samples with the application of relatively low and non-polar solvents gave better chromatogram that with shaper peaks and better separation of components. Therefore, reconstitution of solvent was carried out when relatively low and non-polar solvents were employed to the UPLC-MS analysis of the *Radix polygoni multiflora*.

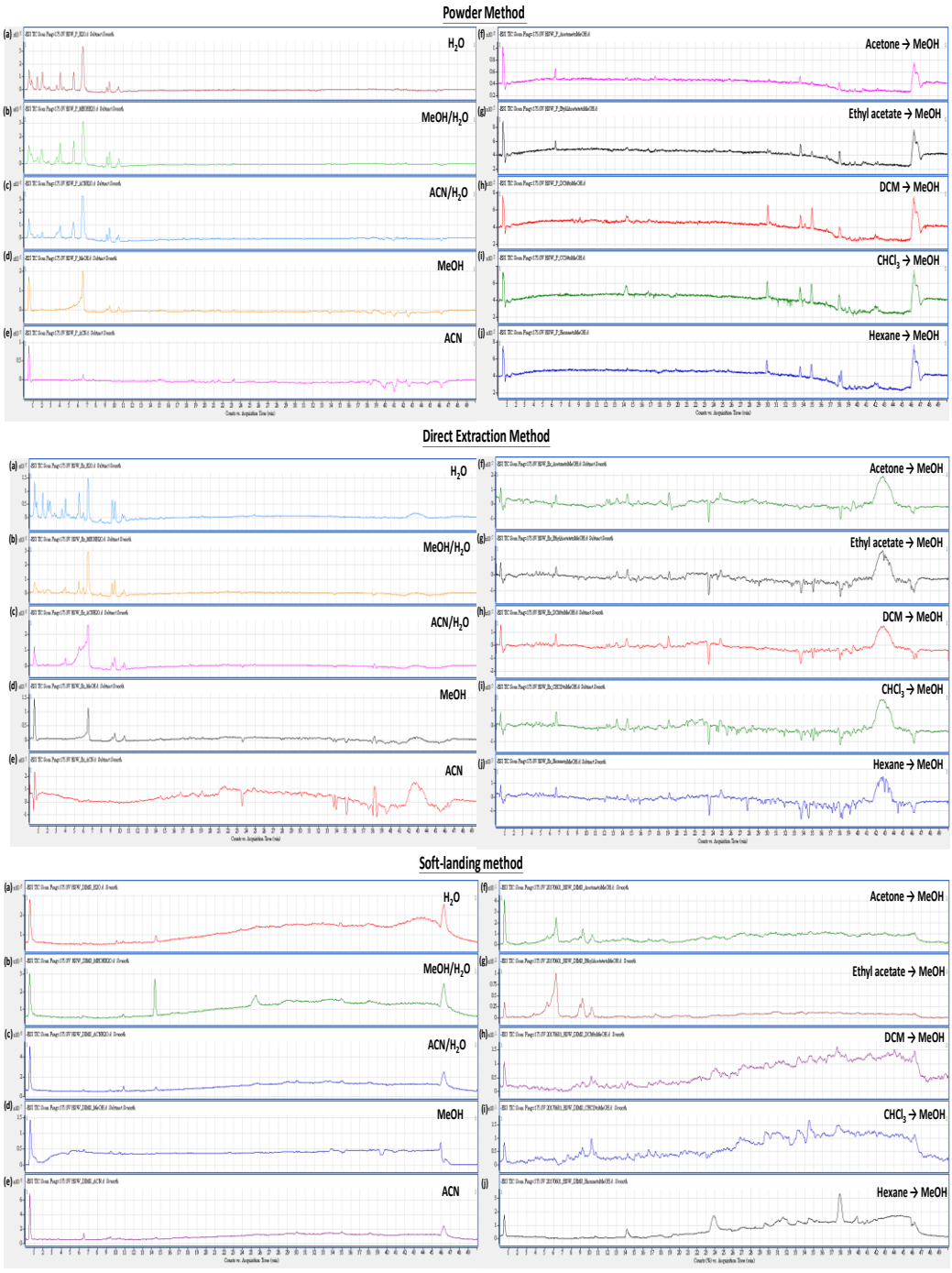


Figure 4.13. Total ion chromatogram (TIC) obtained by UPLC-MS analysis of the *Radix polygoni multiflora* samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of different solvents: (a) water, (b) methanol/water (1/1), (c) acetonitrile/water (1/1), (d) methanol, (e) acetonitrile, (f) acetone, (g) ethyl acetate, (h) dichloromethane, (i) chloroform, and (j) hexane, where (e) to (j) were reconstituted into methanol.

By comparing the TIC obtained by UPLC-MS analysis of the *Radix polygoni multiflora* samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of different solvents (Figure 4.13) and the DI-MS spectra of *Radix polygoni multiflora* with the application of different solvents (Figure 4.14), the overall results showed that active ingredients of *Radix polygoni multiflora* which could be detected by the powder extraction method, the direct extraction method and the soft-landing method, could also be detected by the DI-MS method, indicating that the active components of *Radix polygoni multiflora* could be directly extracted and electrosprayed, there were no suppression of analytes during DI-MS analysis of *Radix polygoni multiflora*. The signal intensities of emodin, physcion and pterostilbene obtained with the *Radix polygoni multiflora* and various solvents using various methods were further compared quantitatively and summarized in Table 4.5. The overall results showed that the major active ingredients of *Radix polygoni multiflora* were relatively polar that favoured to be extracted with solvents of middle and high polarity such as methanol, methanol/water (1/1), acetonitrile/water (1/1) and water in the powder extraction method, the direct extraction method, the soft-landing method and the DI-MS method. The results indicated that polarity of solvent was a crucial factor for the observation of desired ion signals of the active components of *Radix*

polygoni multiflora.

Table 4.5. Summary of the signal intensities of emodin, physcion and pterostilbene obtained with the *Radix polygoni multiflora* and various solvents using various methods.

Solvent	**Intensity of emodin, physcion & pterostilbene**			
	DI-MS	**Powder extraction method**	**Direct extraction method**	**Soft-landing method**
Water	Medium	Strong	Strong	Weak
Methanol/water (1/1)	Medium	Strong	Strong	Weak
Acetonitrile/water (1/1)	Medium	Strong	Medium	Weak
Methanol	Strong	Medium	Medium	Medium
Acetonitrile	Strong	Weak	Weak	Weak
Acetone	Medium	Weak	Weak	Weak
Ethyl acetate	Weak	Weak	Weak	Weak
Dchloromethane	Weak	Weak	Weak	Weak
Chloroform	Weak	Weak	Weak	Weak
Hexane	Weak	Weak	Weak	Weak

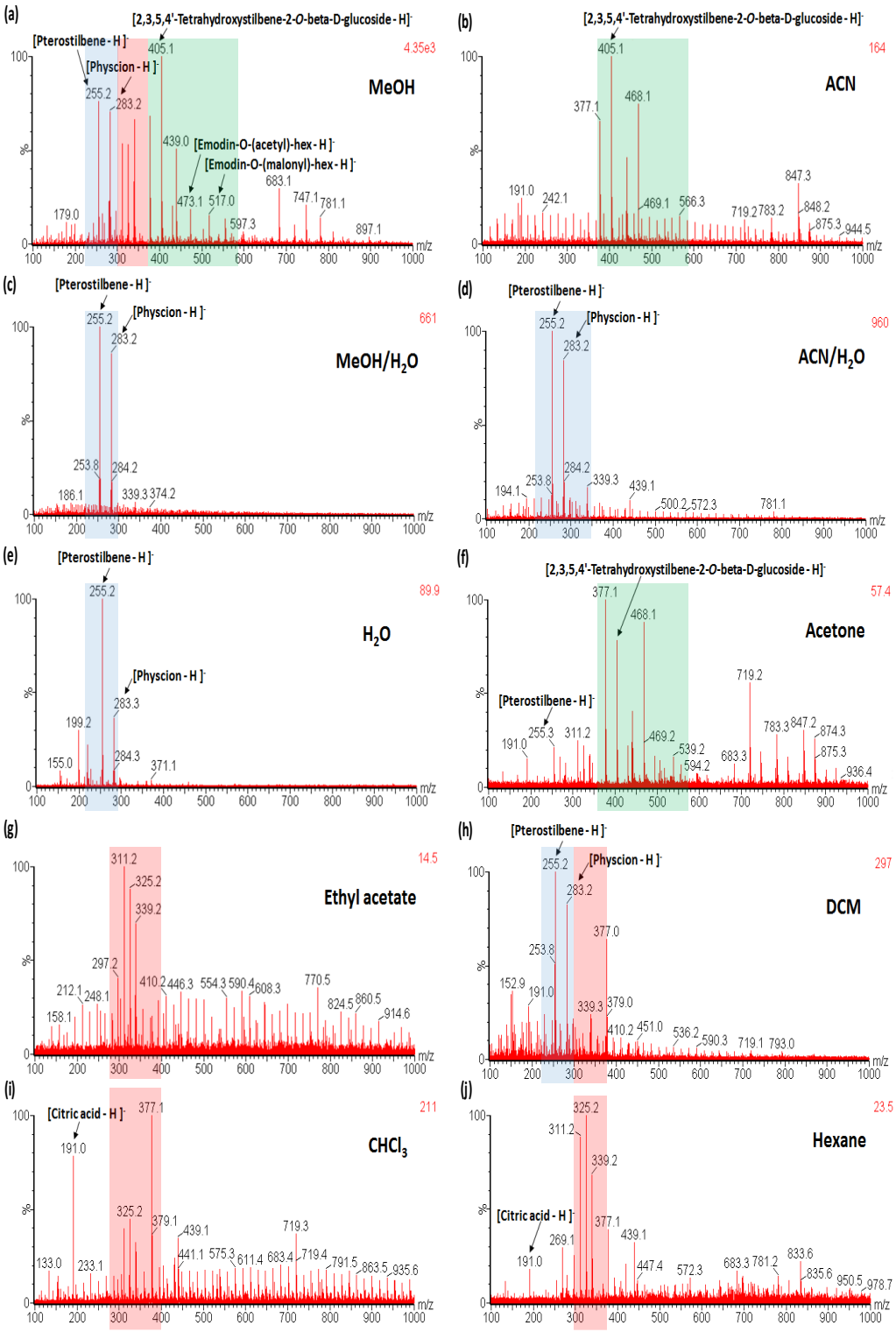


Figure 4.14. DI-MS spectra of *Radix polygoni multiflora* with the application of different solvents: (a) methanol, (b) acetonitrile, (c) methanol/water (1/1), (d) acetonitrile/water (1/1), (e) water, (f) acetone, (g) ethyl acetate, (h) dichloromethane, (i) chloroform, and (j) hexane.

Moreover, the DI-MS spectra of *Radix polygoni multiflora* obtained with the application of different solvents (Figure 4.14) also showed that the spectra varied significantly with the solvents applied. Solvents of different polarities, i.e. MeOH, ACN, MeOH/H_2O, ACN/H_2O, H_2O, acetone, ethyl acetate, DCM, $CHCl_3$ and hexane, were tested for the DI-MS analysis. Detection of [emodin-*o*-(acetyl)-hex - H]$^-$ at *m/z* 473.1 and [emodin-*o*-(malonyl)-hex - H]$^-$ at *m/z* 517.0 were observed [237-240]. When relatively polar solvents were used, i.e. H_2O, MeOH/H_2O and ACN/H_2O, significant detection of [pterostilbene - H]$^-$ at *m/z* 255.2 and [physcion - H]$^-$ at *m/z* 283.2 were observed. When middle polarity solvents were used, i.e. MeOH, ACN and acetone, significant detection of [2,3,5,4'-tetrahydroxystilbene-2-*o*-beta-D-glucoside - H]$^-$ at *m/z* 405.1 was observed [237-240]. Meanwhile, when relatively low and non-polar solvents were used, i.e. ethyl acetate, DCM, $CHCl_3$ and hexane, significant detection of ion peaks at *m/z* 311.2, 325.2, 339.2 and 377.1 with undesirable electrical discharge were observed. The results showed that detection of active ingredients could be achieved with the solvents tested but markedly differences in signal intensities of active ingredients were observed. The results suggested that solvent loaded onto the surface of *Radix polygoni multiflora* is crucial for the observation of desired ion signals of the active components.

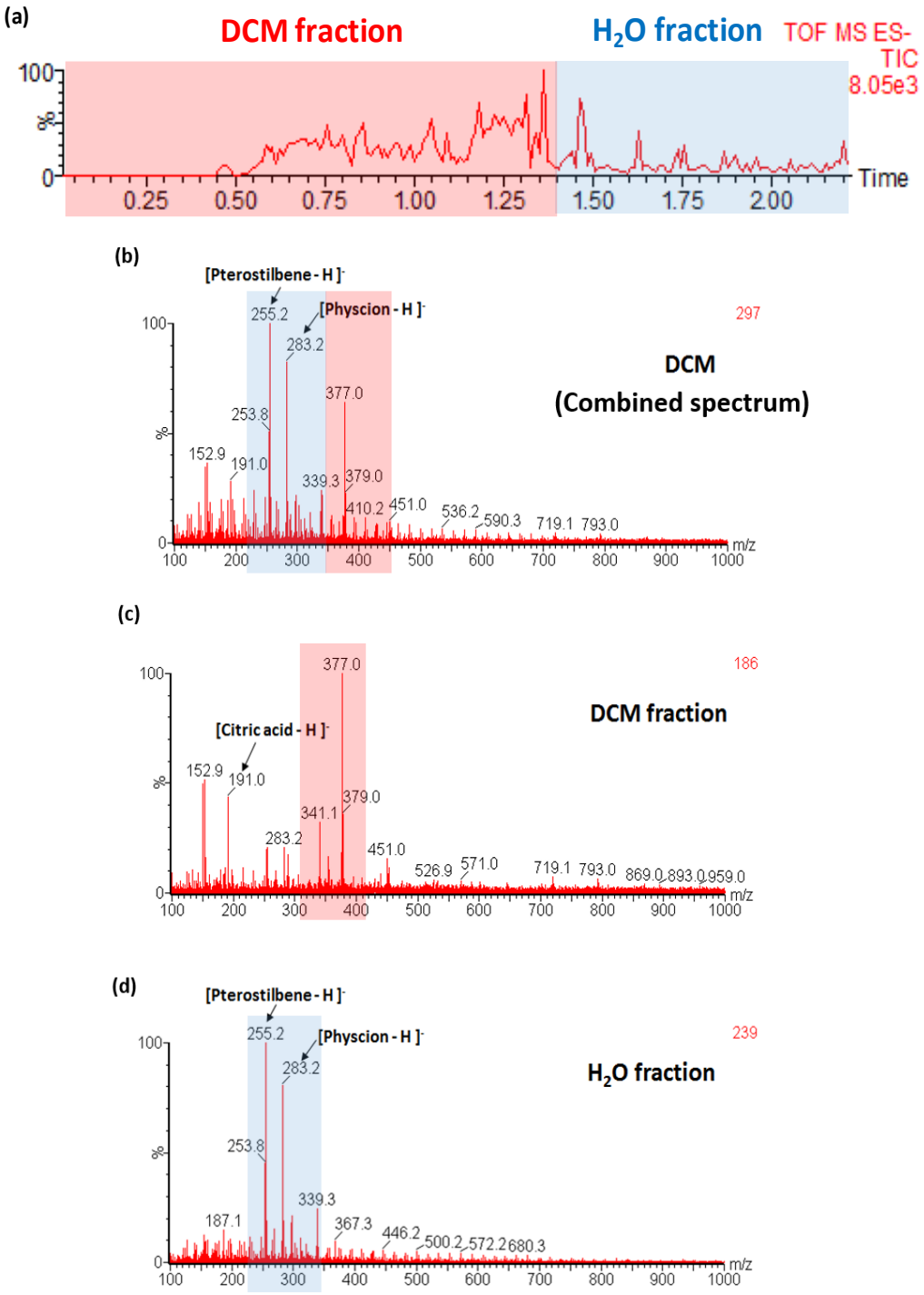


Figure 4.15. (a) Total ion chromatogram (TIC) and DI-MS spectra obtained by DI-MS analysis of *Radix polygoni multiflora* with the application of dichloromethane as the solvent: (b) combined DI-MS spectrum of the whole TIC, (c) combined DI-MS spectrum of the DCM fraction of the TIC, and (d) combined DI-MS spectrum of the water fraction of the TIC.

In addition, when DCM was applied as the solvent for the DI-MS analysis of *Radix polygoni multiflora*, ice dendrite formation on the surface of the tissue sample was observed after the DCM was loaded and evaporated. This process took about 1 min, thus the first minute of the total ion chromatogram (Figure 4.15a) was the DCM fraction of the DI-MS analysis of *Radix polygoni multiflora*, and the corresponding DI-MS spectrum of this DCM fraction was shown in Figure 4.15c. Afterward, the ice dendrite on the surface of the tissue sample melt, water was appeared on the surface of the tissue sample and served as the extraction solvent for further electrospray ionization, this contributed to the H_2O fraction of the total ion chromatogram (Figure 4.15a), and the corresponding DI-MS spectrum of this DCM fraction was shown in Figure 4.15d, which $[pterostilbene - H]^-$ at *m/z* 255.2 and $[physcion - H]^-$ at *m/z* 283.2 were significantly observed when relatively polar solvents were employed. Meanwhile, Figure 4.15b was the combined mass spectrum of the DCM fraction and the H_2O fraction of the total ion chromatogram of DI-MS analysis of *Radix polygoni multiflora* when DCM was applied as the solvent, it showed that both the relatively polar and non-polar components were observed in the combined mass spectrum.

4.3.5. Mechanistic Studies of DI-MS analysis of Lycoris radiata

Figure 4.16. Total ion chromatogram (TIC) obtained by UPLC-MS analysis of the *Lycoris radiata* samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of different solvents: (a) acetone, (b) ethyl acetate, (c) dichloromethane, (d) chloroform, and (e) hexane without (left), or with (right), reconstitution of solvent into methanol.

By comparing the total ion chromatogram (TIC) obtained by UPLC-MS analysis of the *Lycoris radiata* samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of relatively low and non-polar solvents, i.e. acetone, ethyl acetate, dichloromethane, chloroform, and hexane without, or with, reconstitution of solvent into methanol (Figure 4.16), the results showed that with the reconstitution of solvent, the TIC obtained by UPLC-MS analysis of the *Lycoris radiata* samples with the application of relatively low and non-polar solvents gave better chromatogram that with shaper peaks and better separation of components. Therefore, reconstitution of solvent was carried out when relatively low and non-polar solvents were employed to the UPLC-MS analysis of the *Lycoris radiata*.

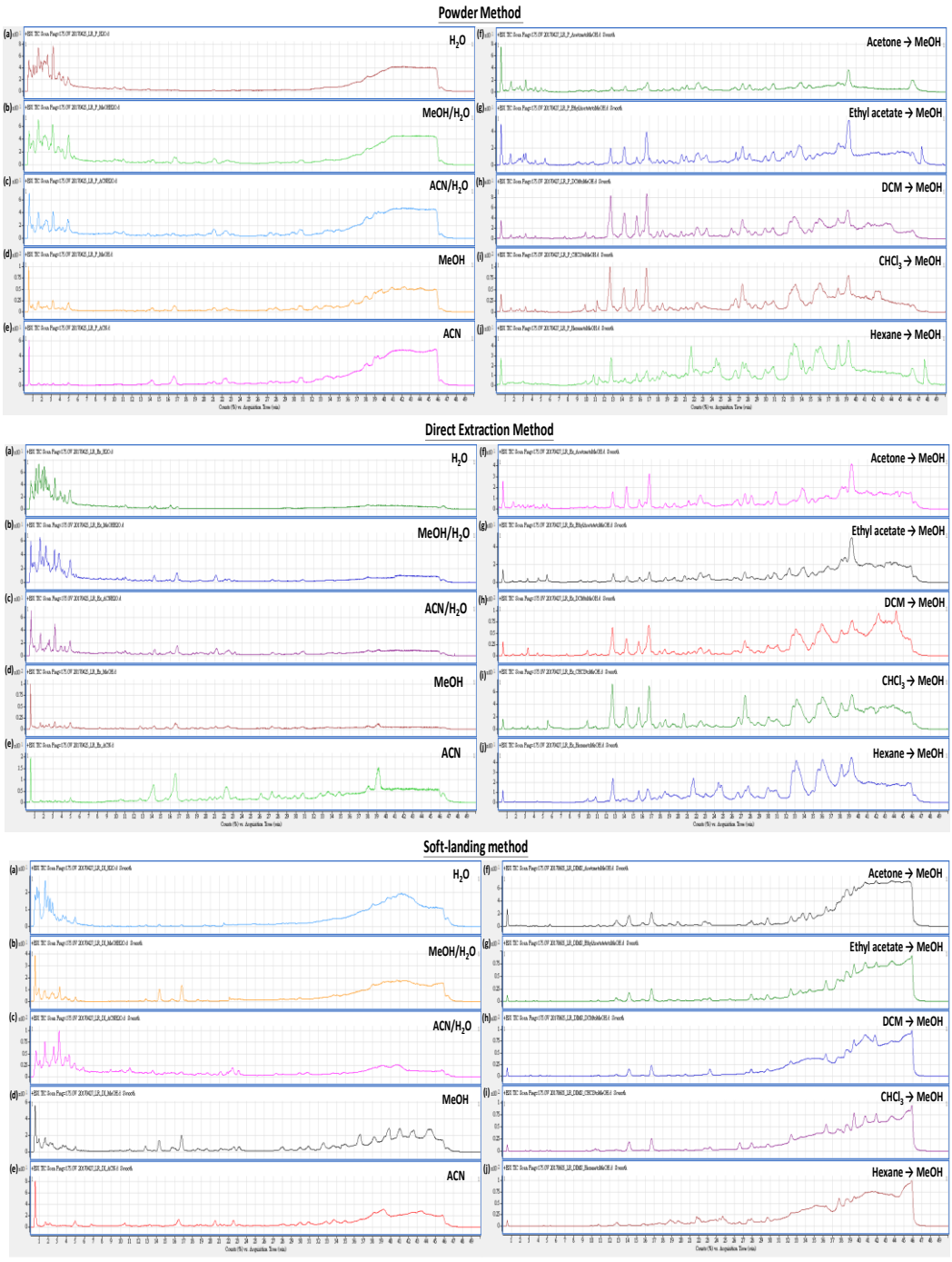


Figure 4.17. Total ion chromatogram (TIC) obtained by UPLC-MS analysis of the *Lycoris radiata* samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of different solvents: (a) water, (b) methanol/water (1/1), (c) acetonitrile/water (1/1), (d) methanol, (e) acetonitrile, (f) acetone, (g) ethyl acetate, (h) dichloromethane, (i) chloroform, and (j) hexane, where (e) to (j) were reconstituted into methanol.

By comparing the TIC obtained by UPLC-MS analysis of the *Lycoris radiata* samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of different solvents (Figure 4.17) and the DI-MS spectra of *Lycoris radiata* with the application of different solvents (Figure 4.18), the overall results showed that active ingredients of *Lycoris radiata* which could be detected by the powder extraction method, the direct extraction method and the soft-landing method, could also be detected by the DI-MS method, indicating that the active components of *Lycoris radiata* could be directly extracted and electrosprayed, there were no suppression of analytes during DI-MS analysis of *Lycoris radiata*. The signal intensities of lycorine, lycoramine and lycorenine obtained with the *Lycoris radiata* and various solvents using various methods were further compared quantitatively and summarized in Table 4.6. The overall results showed that the major active ingredients of *Lycoris radiata* were relatively polar that favoured to be extracted with solvents of middle and high polarity such as methanol, methanol/water (1/1), acetonitrile/water (1/1) and water in the powder extraction method, the direct extraction method, the soft-landing method and the DI-MS method. The results indicated that polarity of solvent was a crucial factor for the observation of desired ion signals of the active components of *Lycoris radiata*.

Table 4.6. Summary of the signal intensities of lycorine, lycoramine and lycorenine obtained with the *Lycoris radiata* and various solvents using various methods.

Solvent	**Intensity of lycorine, lycoramine & lycorenine**			
	DI-MS	**Powder extraction method**	**Direct extraction method**	**Soft-landing method**
Water	Strong	Strong	Strong	Medium
Methanol/water (1/1)	Strong	Strong	Strong	Medium
Acetonitrile/water (1/1)	Strong	Strong	Strong	Medium
Methanol	Strong	Strong	Strong	Medium
Acetonitrile	Strong	Medium	Medium	Weak
Acetone	Medium	Weak	Weak	Weak
Ethyl acetate	Weak	Weak	Weak	Weak
Dchloromethane	Weak	Weak	Weak	Weak
Chloroform	Weak	Weak	Weak	Weak
Hexane	Weak	Weak	Weak	Weak

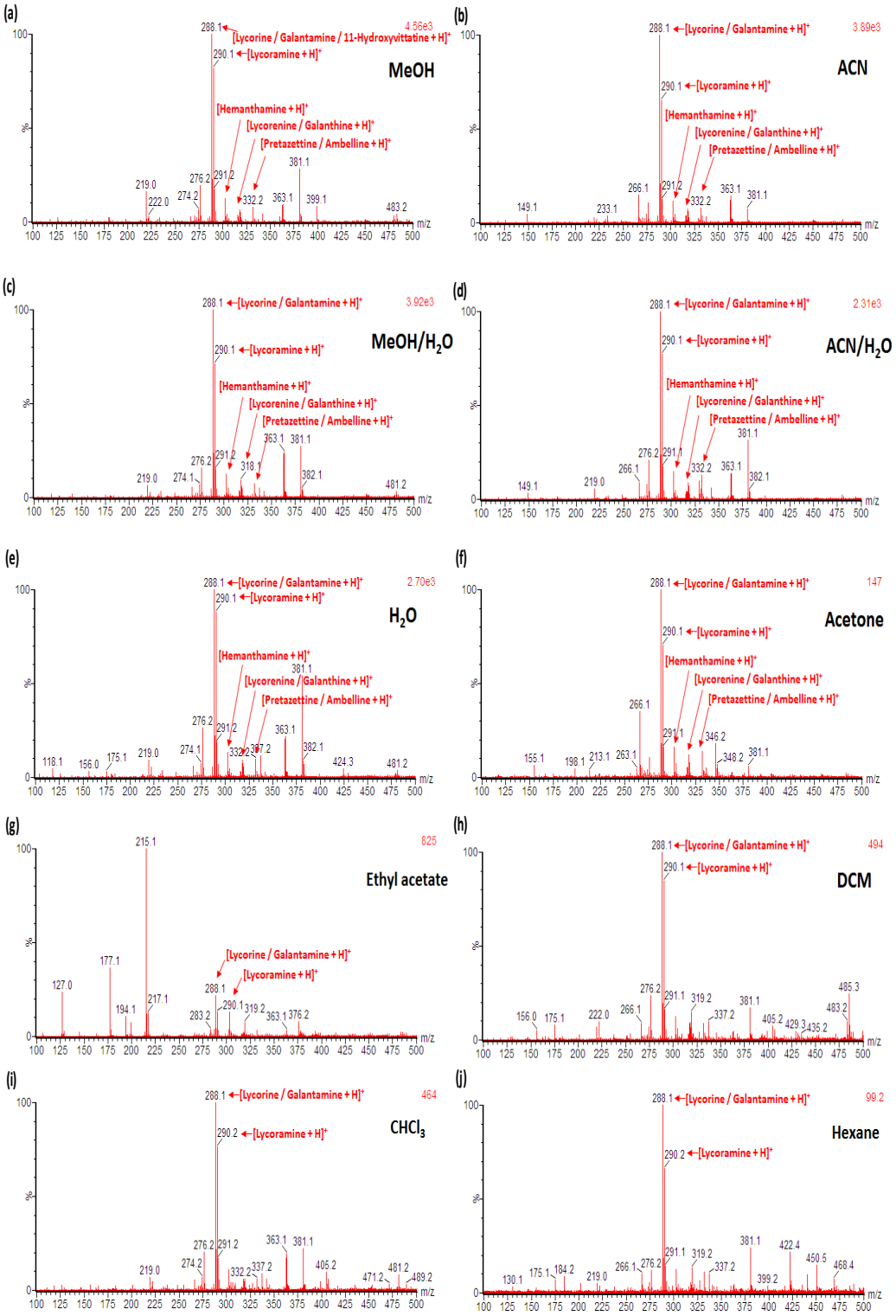


Figure 4.18. DI-MS spectra of *Lycoris radiata* with the application of different solvents: (a) methanol, (b) acetonitrile, (c) methanol/water (1/1), (d) acetonitrile/water (1/1), (e) water, (f) acetone, (g) ethyl acetate, (h) dichloromethane, (i) chloroform, and (j) hexane.

Moreover, the DI-MS spectra of *Lycoris radiata* obtained with the application of different solvents (Figure 4.18) also showed that the spectra varied significantly with the solvents applied. Solvents of different polarities, including methanol, acetonitrile, methanol/water (1/1), acetonitrile/water (1/1), water, acetone, ethyl acetate, dichloromethane, chloroform, and hexane, were tested for DI-MS analysis of *Lycoris radiata*. When relatively polar solvents were used, i.e. H_2O, MeOH/H_2O, ACN/H_2O, MeOH, ACN and acetone, significant detection of [lycorine / galantamine / 11-hydroxyvittatine + H]$^+$ at *m/z* 288.1, [lycoramine + H]$^+$ at *m/z* 290.1, [hemanthamine + H]$^+$ at *m/z* 302.1, [lycorenine / galanthine + H]$^+$ at *m/z* 318.1 and [pretazettine / ambelline + H]$^+$ at *m/z* 332.2 were observed [241-244]. Meanwhile, when relatively low and non-polar solvents were used, i.e. ethyl acetate, DCM, $CHCl_3$ and hexane, significant detection of [lycorine / galantamine / 11-hydroxyvittatine + H]$^+$ at *m/z* 288.1 and [lycoramine + H]$^+$ at *m/z* 290.1 were observed. The results showed that detection of active ingredients could be achieved with the solvents tested but markedly differences in signal intensities of active ingredients were observed. The results suggested that solvent loaded onto the surface of *Lycoris radiata* is crucial for the observation of desired ion signals of the active components of *Lycoris radiata*.

4.3.6. Mechanistic Studies of DI-MS analysis of Psidium guajava leaves

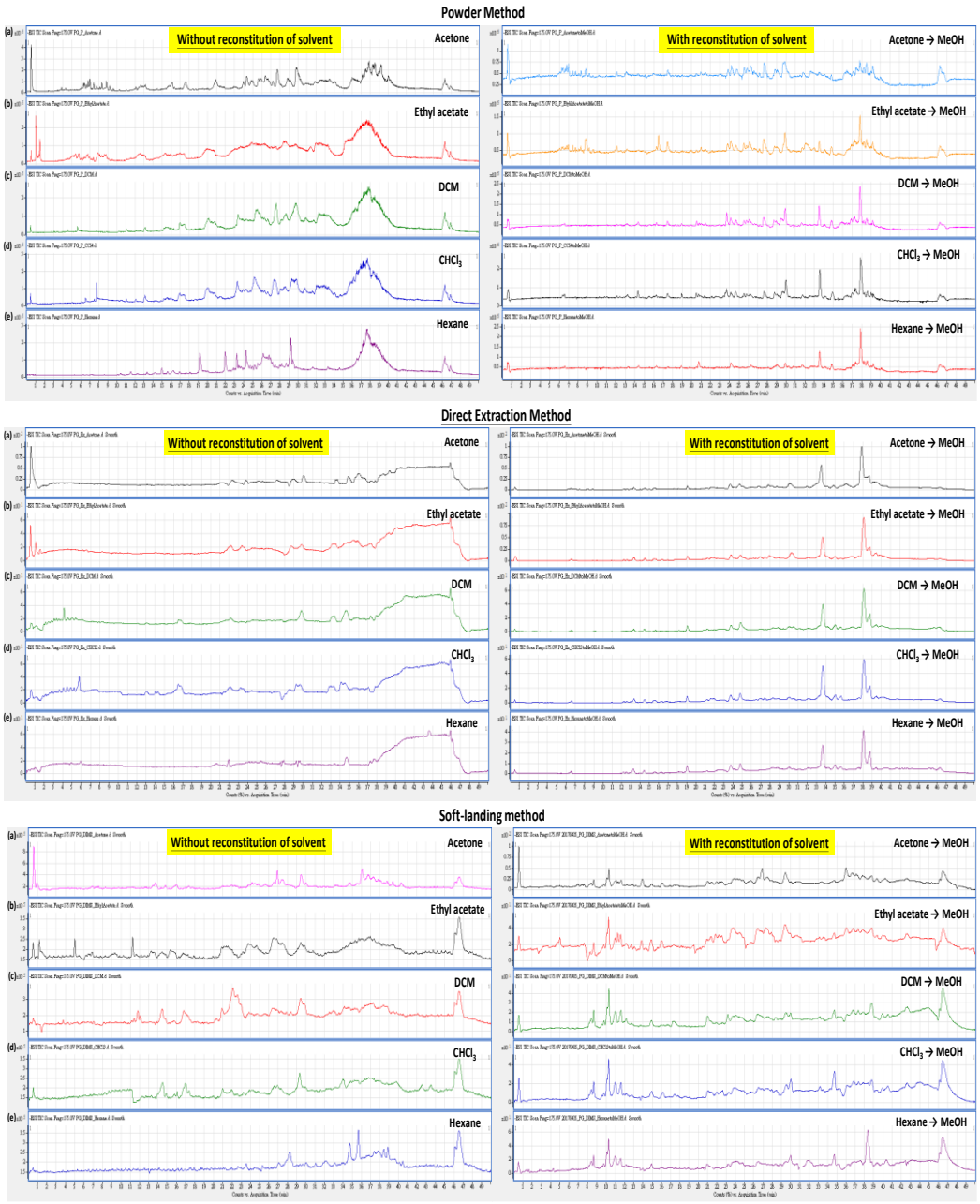


Figure 4.19. Total ion chromatogram (TIC) obtained by PLC-MS analysis of the *Psidium guajava* leaves samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of different solvents: (a) acetone, (b) ethyl acetate, (c) dichloromethane, (d) chloroform, and (e) hexane without (left), or with (right), reconstitution of solvent into methanol.

By comparing the total ion chromatogram (TIC) obtained by UPLC-MS analysis of the *Psidium guajava* leaves samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of relatively low and non-polar solvents without, or with, reconstitution of solvent into methanol (Figure 4.19), the results showed that with the reconstitution of solvent, the TIC obtained by UPLC-MS analysis of the *Psidium guajava* leaves samples with the application of relatively low and non-polar solvents gave better chromatogram that with shaper peaks and better separation of components. Therefore, reconstitution of solvent was carried out when relatively low and non-polar solvents were employed to the UPLC-MS analysis of the *Psidium guajava* leaves.

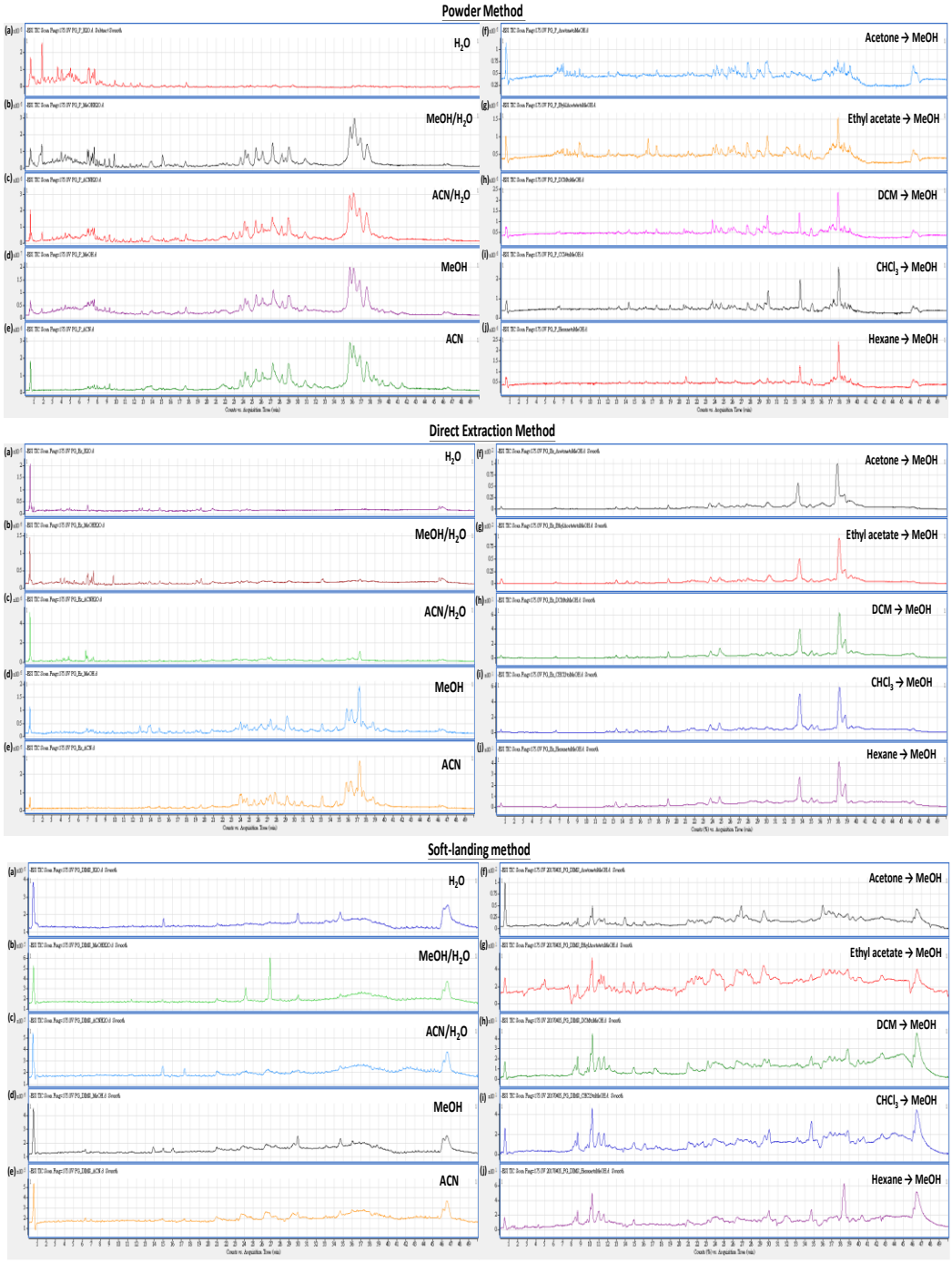


Figure 4.20. Total ion chromatogram (TIC) obtained by UPLC-MS analysis of the *Psidium guajava* leaves samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of different solvents: (a) water, (b) methanol/water (1/1), (c) acetonitrile/water (1/1), (d) methanol, (e) acetonitrile, (f) acetone, (g) ethyl acetate, (h) dichloromethane, (i) chloroform, and (j) hexane, where (e) to (j) were reconstituted into methanol.

By comparing the TIC obtained by UPLC-MS analysis of the *Psidium guajava* leaves samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of different solvents (Figure 4.20) and the DI-MS spectra of *Psidium guajava* leaves with the application of different solvents (Figure 4.21). The overall results showed that active ingredients of *Psidium guajava* leaves which could be detected by the powder extraction method, the direct extraction method and the soft-landing method, could also be detected by the DI-MS method, indicating that the active components of *Psidium guajava* leaves could be directly extracted and electrosprayed, there were no suppression of analytes during DI-MS analysis of *Psidium guajava* leaves. The signal intensities of psiguajadials, psidials and guavinosides obtained with the *Psidium guajava* leaves and various solvents using various methods were further compared quantitatively and summarized in Table 4.7. The overall results showed that the major active ingredients of *Psidium guajava* leaves were relatively polar that favoured to be extracted with solvents of middle polarity such as methanol, and acetonitrile in the powder extraction method, the direct extraction method, the soft-landing method and the DI-MS method. The results indicated that polarity of solvent was a crucial factor for the observation of desired ion signals of the active components.

Table 4.7. Summary of the signal intensities of psiguajadials, psidials and guavinosides obtained with the *Psidium guajava* leaves and various solvents using various methods.

Solvent	**Intensity of psiguajadials, psidials & guavinosides**			
	DI-MS	**Powder extraction method**	**Direct extraction method**	**Soft-landing method**
Methanol	Strong	Strong	Medium	Medium
Acetonitrile	Strong	Medium	Medium	Weak
Acetone	Medium	Weak	Weak	Weak
Ethyl acetate	Weak	Weak	Weak	Weak
Dchloromethane	Weak	Weak	Weak	Weak
Chloroform	Weak	Weak	Weak	Weak
Hexane	Weak	Weak	Weak	Weak

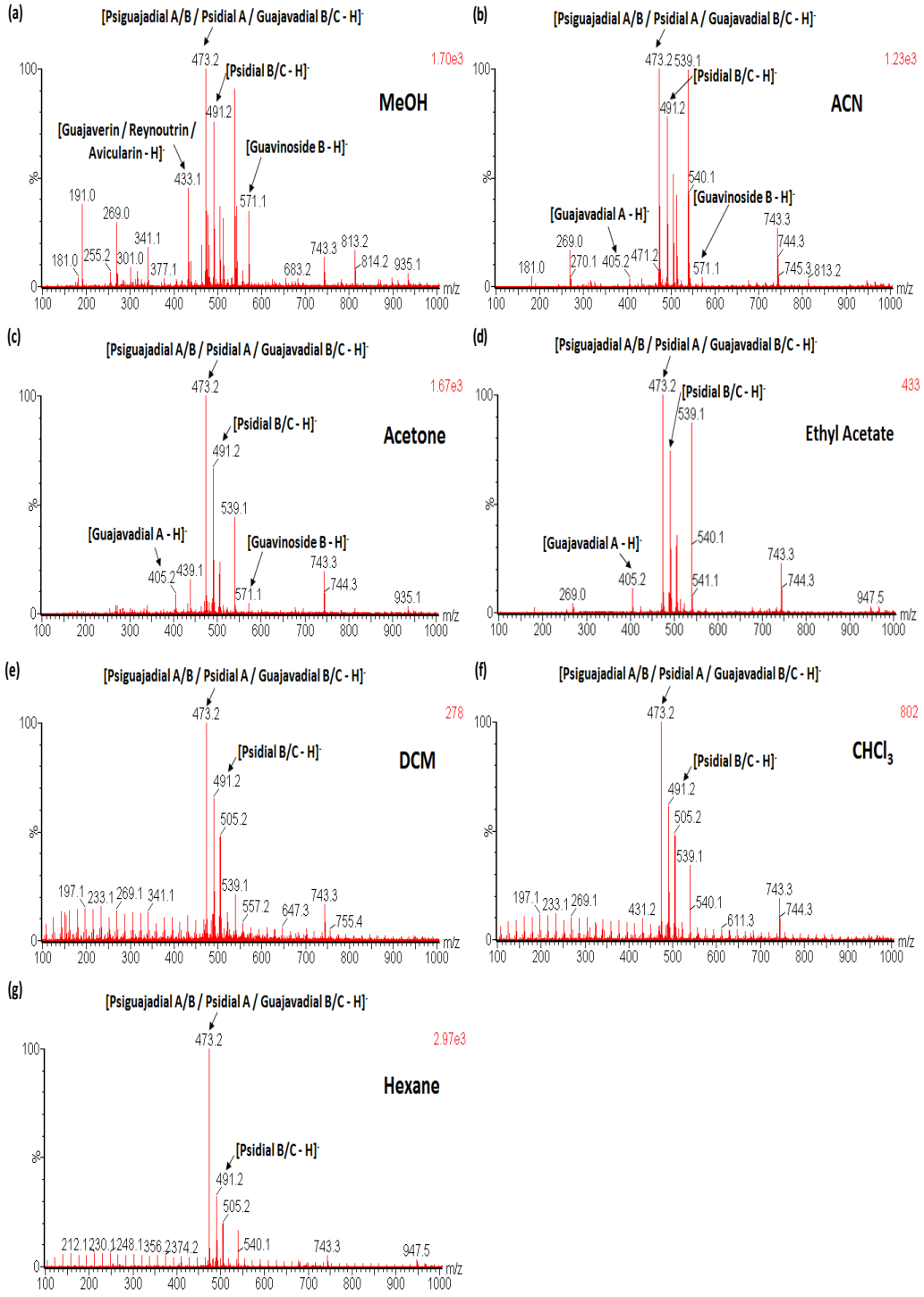


Figure 4.21. DI-MS spectra of *Psidium guajava* leaves with the application of different solvents: (a) methanol, (b) acetonitrile, (c) acetone, (d) ethyl acetate, (e) dichloromethane, (f) chloroform, and (g) hexane.

Moreover, the DI-MS spectra of *Psidium guajava* leaves obtained with the application of different solvents (Figure 4.21) also showed that the spectra varied significantly with the solvents applied. Solvents of different polarities were tested for DI-MS analysis of *Psidium guajava* leaves. When H_2O, MeOH/H_2O (1/1) and ACN/H_2O (1/1) were used as the solvents, the solution loaded would remined as droplets on the tissue surface because of the water-proof leaf surface, and no desirable mass spectra could be obtained or undesirable electrical discharge would be resulted. When relatively polar solvents were used, i.e. MeOH, ACN and acetone, significant detection of [guajavadial A - H]$^-$ at *m/z* 405.2, [guajaverin / reynoutrin / avicularin - H]$^-$ at *m/z* 433.1, [psiguajadial A/B / psidial A / guajavadial B/C - H]$^-$ at *m/z* 473.2, [psidial B/C - H]$^-$ at *m/z* 491.2 and [guavinoside B - H]$^-$ at *m/z* 571.1 were observed [245-248]. Meanwhile, when relatively low and non-polar solvents were used, i.e. ethyl acetate, DCM, $CHCl_3$ and hexane, significant detection of mass peaks at *m/z* 473.2 and 491.2 with undesirable electrical discharge were observed. The results showed that detection of active ingredients could be achieved with the solvents tested but markedly differences in signal intensities of active ingredients were observed. The results suggested that solvent loaded onto the surface of *Psidium guajava* leaves is crucial for the observation of desired ion signals of the active components.

4.3.7. Mechanistic Studies of DI-MS analysis of Ilex latifolia leaves.

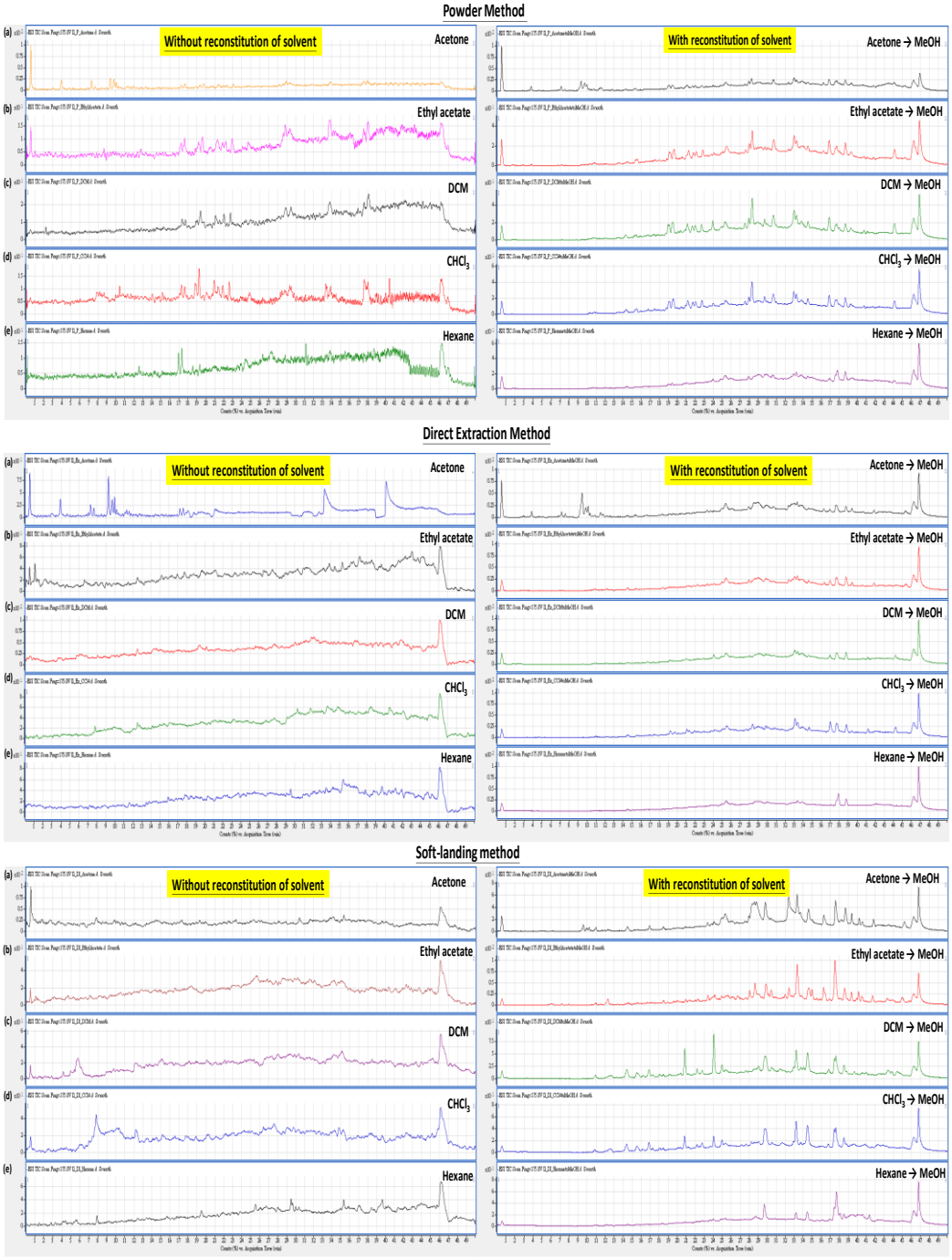


Figure 4.22. Total ion chromatogram (TIC) obtained by PLC-MS analysis of the *Ilex latifolia* leaves samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of different solvents: (a) acetone, (b) ethyl acetate, (c) dichloromethane, (d) chloroform, and (e) hexane without (left), or with (right), reconstitution of solvent into methanol.

By comparing the total ion chromatogram (TIC) obtained by UPLC-MS analysis of the *Ilex latifolia* leaves samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of relatively low and non-polar solvents without, or with, reconstitution of solvent into methanol (Figure 4.22), the results showed that with the reconstitution of solvent, the TIC obtained by UPLC-MS analysis of the *Ilex latifolia* leaves samples with the application of relatively low and non-polar solvents gave better chromatogram that with shaper peaks and better separation of components. Therefore, reconstitution of solvent was carried out when relatively low and non-polar solvents were employed to the UPLC-MS analysis of the *Ilex latifolia* leaves.

Figure 4.23. Total ion chromatogram (TIC) obtained by UPLC-MS analysis of the *Ilex latifolia* leaves samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of different solvents: (a) water, (b) methanol/water (1/1), (c) acetonitrile/water (1/1), (d) methanol, (e) acetonitrile, (f) acetone, (g) ethyl acetate, (h) dichloromethane, (i) chloroform, and (j) hexane, where (e) to (j) were reconstituted into methanol.

By comparing the TIC obtained by UPLC-MS analysis of the *Ilex latifolia* leaves samples collected by the powder extraction method, direct extraction method and soft-landing method with the application of different solvents (Figure 4.23) and the DI-MS spectra of *Ilex latifolia* leaves with the application of different solvents (Figure 4.24), the overall results showed that active ingredients of *Ilex latifolia* leaves, including caffeoylquinic acids, kudinosides and latifolosides, which could be detected by the powder extraction method, could also be detected by the direct extraction method and the soft-landing method, indicating that the active components of *Ilex latifolia* leaves could be directly extracted and electrosprayed. However, some of the active ingredients of *Ilex latifolia* leaves, i.e. kudinosides and latifolosides, which could be effectively detected by the soft-landing method but they were significantly suppressed in the detection of the DI-MS method (see Table 4.8). The results showed that there was significant suppression of analytes during DI-MS analysis of *Ilex latifolia* leaves. The signal intensities of caffeoylquinic acids, kudinosides and latifolosides obtained with the *Ilex latifolia* leaves and various solvents using various methods were further compared quantitatively and summarized in Table 4.8. The overall results showed that the major active ingredients of *Ilex latifolia* leaves were relatively polar that favoured to be extracted with solvents of middle polarity such as methanol, and acetonitrile

in the powder extraction method, the direct extraction method, the soft-landing method and the DI-MS method. The results indicated that polarity of solvent was a crucial factor for the observation of desired ion signals of the active components of *Ilex latifolia* leaves.

Table 4.8. Summary of the signal intensities of caffeoylquinic acids, kudinosides and latifolosides obtained with the *Ilex latifolia* leaves and various solvents using various methods.

Solvent	**Intensity of caffeoylquinic acids**				**Intensity of kudinosides and latifolosides**			
	DI-MS	**Powder extraction method**	**Direct extraction method**	**Soft-landing method**	**DI-MS**	**Powder extraction method**	**Direct extraction method**	**Soft-landing method**
Methanol	Strong	Strong	Medium	Medium	Weak	Strong	Medium	Medium
Acetonitrile	Strong	Medium	Medium	Weak	Weak	Medium	Medium	Weak
Acetone	Medium	Weak	Weak	Weak	Weak	Weak	Weak	Weak
Ethyl acetate	Weak	Weak	Weak	Weak	Weak	Weak	Weak	Weak
Dchloromethane	Weak	Weak	Weak	Weak	Weak	Weak	Weak	Weak
Chloroform	Weak	Weak	Weak	Weak	Weak	Weak	Weak	Weak
Hexane	Weak	Weak	Weak	Weak	Weak	Weak	Weak	Weak

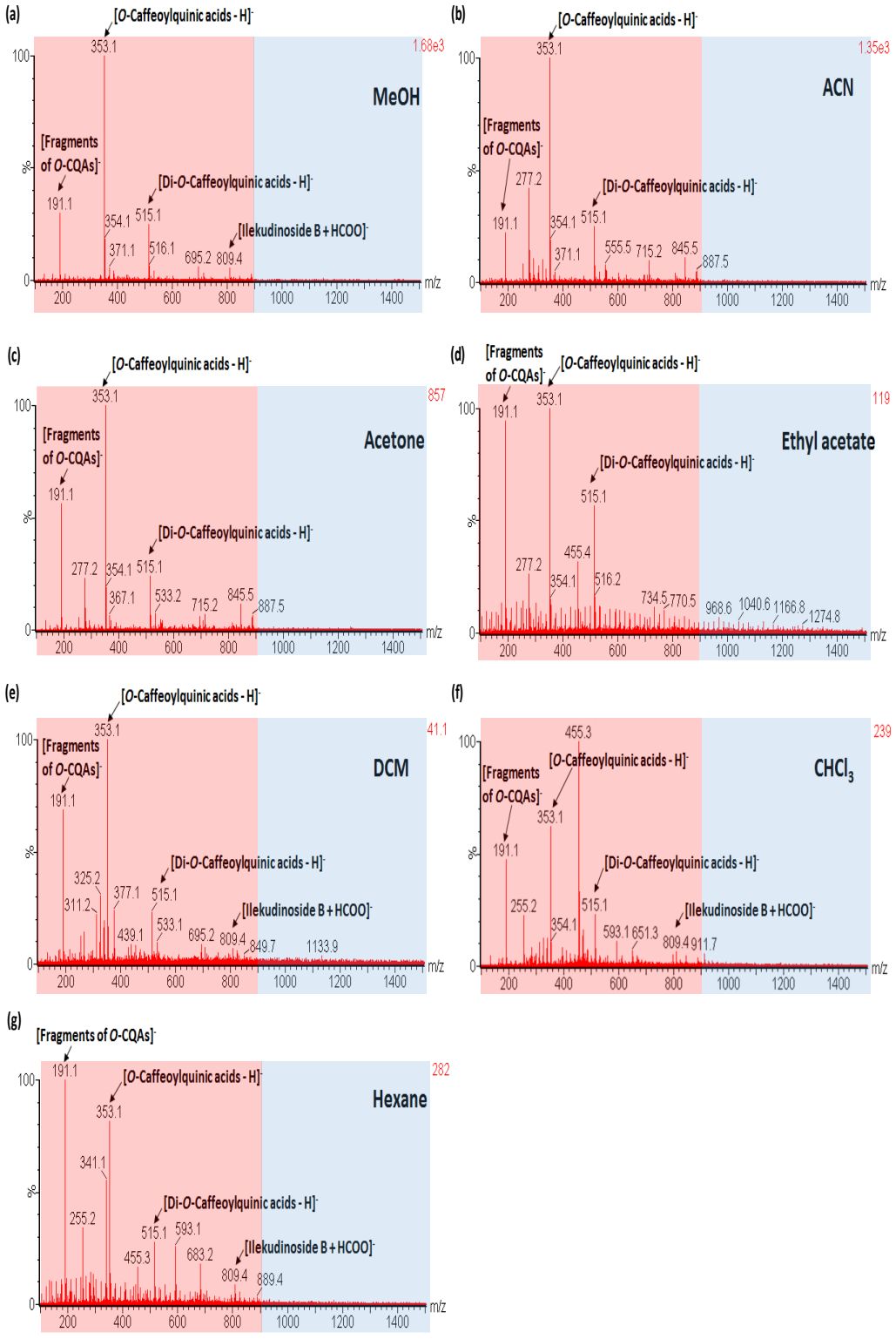


Figure 4.24. DI-MS spectra of *Ilex latifolia* leaves with the application of different solvents: (a) methanol, (b) acetonitrile, (c) acetone, (d) ethyl acetate, (e) dichloromethane, (f) chloroform, and (g) hexane.

Moreover, the DI-MS spectra of *Ilex latifolia* leaves obtained with the application of different solvents (Figure 4.24) showed that there was no significantly variation of the spectra with the solvents applied. Solvents of different polarities were tested for DI-MS analysis of *Ilex latifolia* leaves. When H_2O, MeOH/H_2O (1/1) and ACN/H_2O (1/1) were used as the solvents, the solution loaded would remined as droplets on the tissue surface because of the water-proof leaf surface, and no desirable mass spectra could be obtained or undesirable electrical discharge would be resulted. Significant detection of [fragments of *o*-CQAs - H]$^-$ at *m/z* 191.1, [*o*-caffeoylquinic acids - H]$^-$ at *m/z* 353.1, [di-*o*-caffeoylquinic acids - H]$^-$ at *m/z* 515.1 and [ilekudinoside B - HCOO]$^-$ at *m/z* 809.4 were observed [249-254]. The results showed that detection of active ingredients could be achieved with the solvents tested but markedly differences in signal intensities of active ingredients were observed. When relatively polar solvents were used, i.e. MeOH, ACN and acetone, the detection of the active ingredients showed higher signal intensities. Meanwhile, when relatively low and non-polar solvents were used, i.e. ethyl acetate, DCM, $CHCl_3$ and hexane, the detection of the active ingredients showed very weak signal intensities with undesirable electrical discharge. The results suggested that solvent loaded onto the surface of *Ilex latifolia* leaves is crucial for the observation of desired signal intensities of the active components.

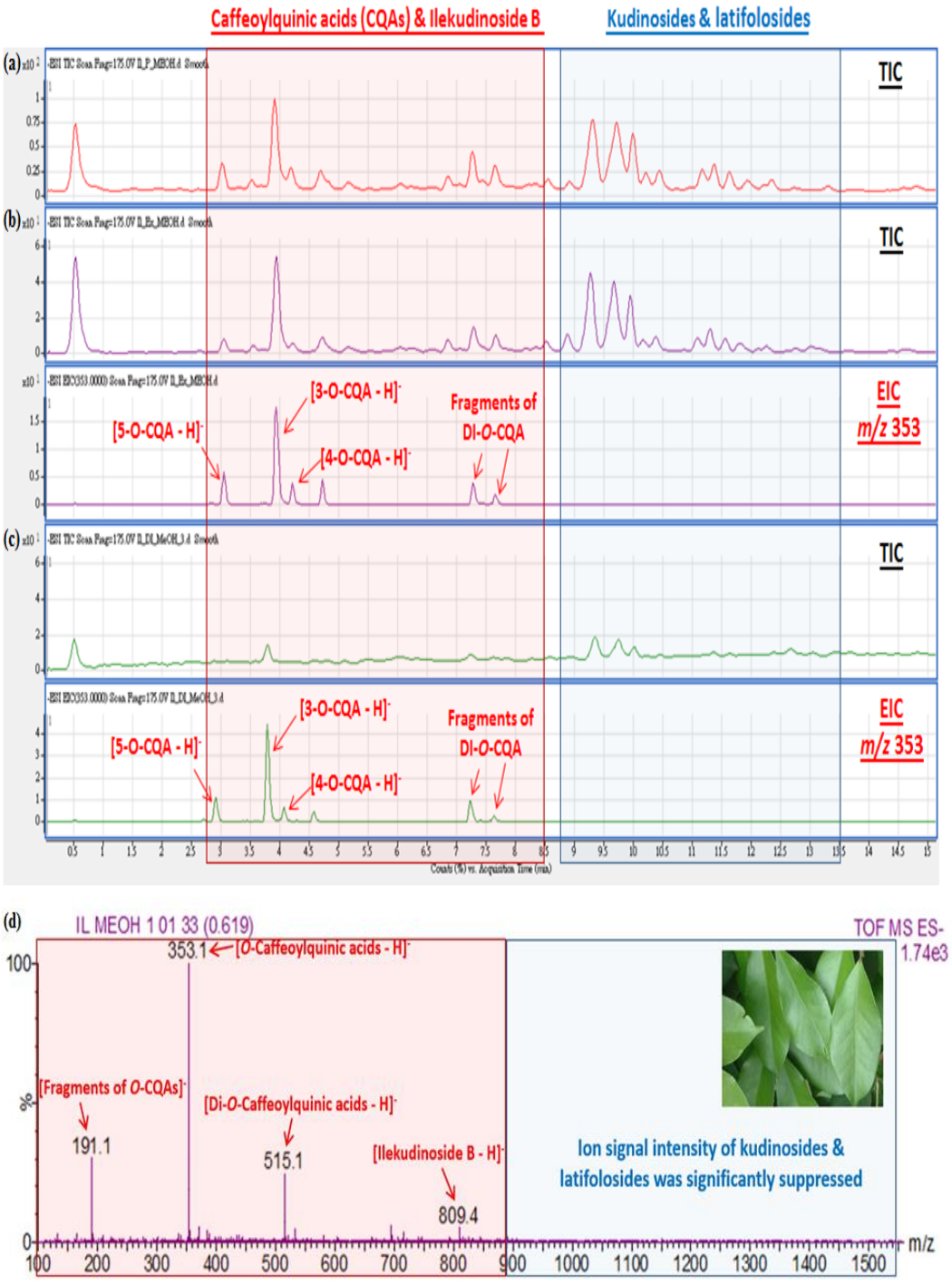


Figure 4.25. Total ion chromatogram (TIC) and extracted-ion chromatogram (EIC) obtained by UPLC-MS analysis of the samples collected by the (a) powder extraction method, (b) direct extraction method and (c) soft-landing method; and (d) DI-MS spectrum of *Ilex latifolia*.

To have a deeper look into the results of the UPLC-MS and DI-MS analysis of *Ilex latifolia* leaves, Figure 4.25 showed the total ion chromatogram (TIC) and the extracted-ion chromatogram (EIC) obtained by UPLC-MS analysis of the *Ilex latifolia* leaves samples collected by the powder extraction method, the direct extraction method and the soft-landing method; and DI-MS spectrum of *Ilex latifolia*, with the application of MeOH as the solvent. UPLC-MS separation and detection of caffeoylquinic acids (*o*-CQAs), di-caffeoylquinic acids (di-*o*-CQAs) and ilekudinoside B, including their isomers: 3-*o*-CQA, 4-*o*-CQA, 5-*o*-CQA, 3,4-di-*o*-CQA, 3,5-di-*o*-CQA and 4,5-di-*o*-CQA, could be achieved at the retention time about 2 to 9 mins. Meanwhile, UPLC-MS separation and detection of kudinosides and latifolosides could be achieved at the retention time about 9 to 14 mins. The TIC obtained by the powder extraction method (Figure 4.25a) and the direct extraction method (Figure 4.25b) gave very similar chromatogram that similar number of peaks with similar peaks intensities were detected. However, the TIC obtained by the soft-landing method (Figure 4.25c) showed less number of peaks and much lower peak intensities were detected. Although some of the peaks seemed could not be observed in the TIC obtained by the soft-landing method, the peaks could be unambiguously observed in the EIC. Taking *o*-CQAs as an example, the peaks of different isomers of *o*-CQAs seemed could not be

observed in the TIC, but they could be unambiguously observed in the EIC of *m/z* 353, i.e. 3-*o*-CQA at about 3.8 min, 4-*o*-CQA at about 4.2 min and 5-*o*-CQA at about 3 min. The overall results of the EIC that obtained by the soft-landing method showed that active ingredients of *Ilex latifolia* leaves, including *o*-CQAs, kudinosides and latifolosides, which could be detected by the powder extraction method and the direct extraction method, could also be detected by the soft-landing method, indicating that the active components of *Ilex latifolia* leaves could be directly extracted and electrosprayed. However, the results of the DI-MS analysis of *Ilex latifolia* leaves (Figure 4.25d) showed that the peaks of fragments of *o*-CQAs at *m/z* 191.1, *o*-CQAs at *m/z* 353.1, di-*o*-CQAs at *m/z* 515.1 and ilekudinoside B at *m/z* 809.4 dominated the mass spectrum with high signal intensities, but the peaks of the other active ingredients of *Ilex latifolia* leaves, i.e. kudinosides and latifolosides were significantly suppressed in the DI-MS spectrum. Since the UPLC-MS results revealed that kudinosides and latifolosides could be directly extracted and electrosprayed, and had similar abundance to the *o*-CQAs detected, they should also be effectively detected in the DI-MS spectrum with similar signal intensities, but the DI-MS results showed a significant suppression of them. Therefore, it is worth to investigate this phenomenon in order to understand the process and factors governing the signal responses of this direct

analysis technique.

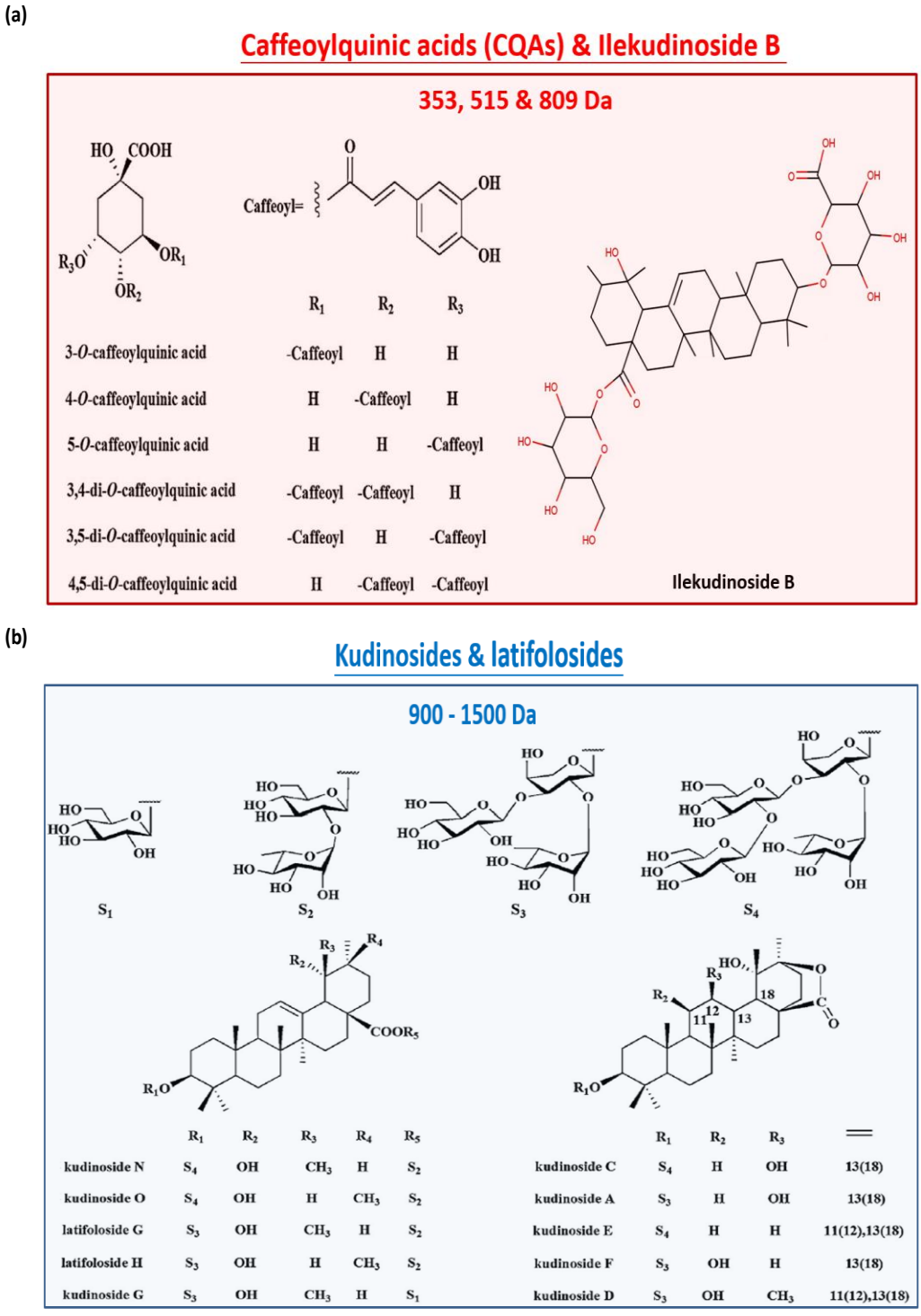


Figure 4.26. Chemical structures of (a) caffeoylquinic acids and ilekudinoside B, and (b) kudinosides and latifolosides.

In order to further investigate the significant suppression of analytes during DI-MS analysis of *Ilex latifolia* leaves, the detailed mechanisms of this phenomenon was investigated by comparing the surface activity, polarity and acidity of the analytes. Figure 4.26 showed the chemical structures of different caffeoylquinic acids, kudinosides and latifolosides that present and detected in the *Ilex latifolia* leaves, including 3-*o*-CQA, 4-*o*-CQA, 5-*o*-CQA, 3,4-di-*o*-CQA, 3,5-di-*o*-CQA and 4,5-di-*o*-CQA, ilekudinoside B, kudinoside A, kudinoside C, kudinoside D, kudinoside E, kudinoside F, kudinoside G, kudinoside N, kudinoside O, latifoloside F and latifoloside G [249-254]. By inputting the chemical structures of the analytes into a molecular modeling program called Marvin software, various physical parameters of the analytes of *Ilex latifolia* such as the polar surface area, van der Waals surface area, solvent-accessible surface area, log *P* and p$K_{\mathbf{a}}$ values could be calculated (Figure 4.27), and are available to MS users to predict the ESI-MS responses.

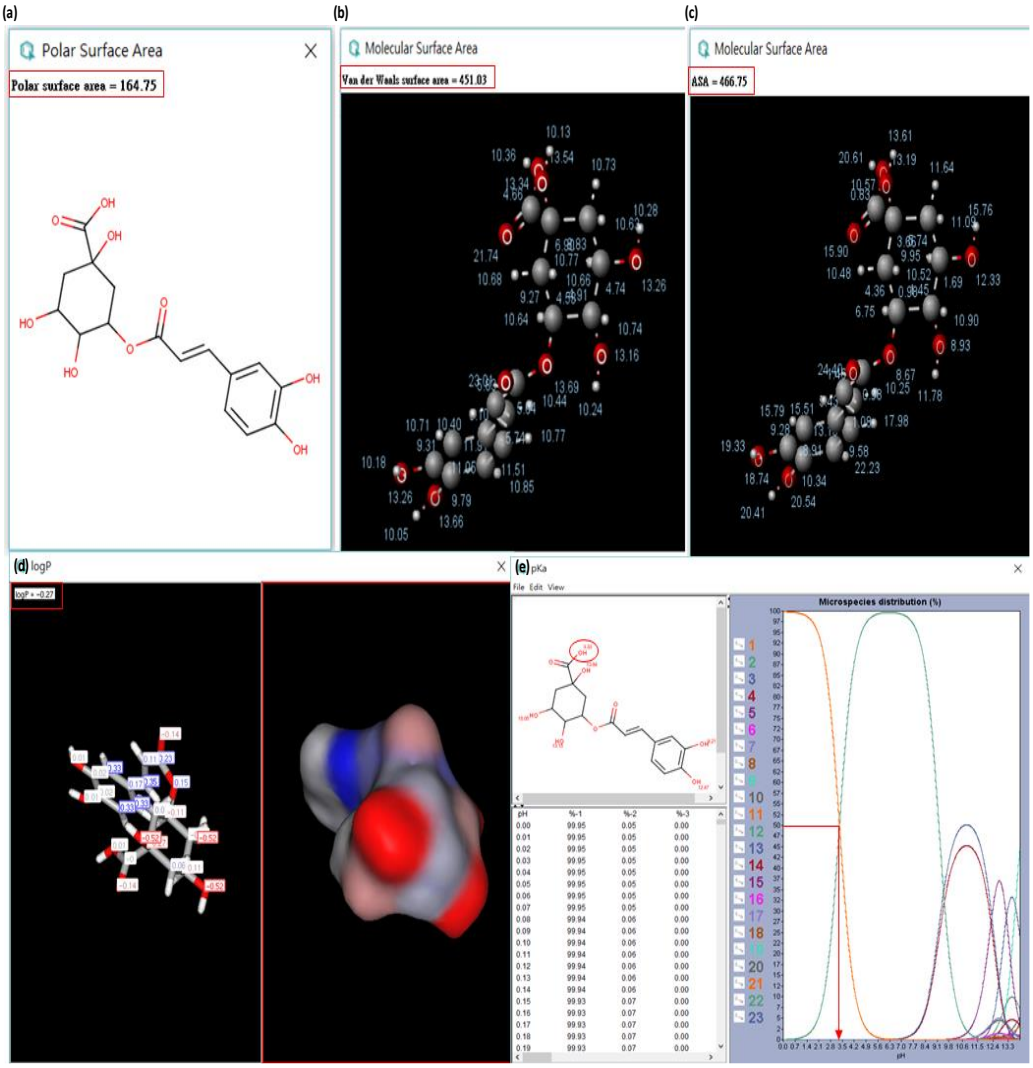


Figure 4.27. A diagram about calculation of the physical properties of the analytes of *Ilex latifolia* with Marvin software: (a) polar surface area, (b) van der Waals surface area, (c) solvent-accessible surface area, (d) log *P*, and (e) pK_a.

Table 4.9. Summary of the physical properties of the analytes of *Ilex latifolia* leaves.

Analyte	Polar surface area (PSA) (Å^2)	Molecular surface area		(PSA / vdWSA) x 100 %	(PSA / SASA) x 100 %	Log *P*	pK_a
		van der Waals surface area (vdWSA) (Å^2)	Solvent-accessible surface area (SASA) (Å^2)				
3-*O*-CQA	164.75	451.03	466.75	36.5 %	35.3 %	- 0.27	3.33 (Acidic)
3,5-Di-*O*-CQA	211.28	643.35	680.05	32.8 %	31.1 %	2.16	3.27 (Acidic)
Ilekudinoside B	253.13	1221.60	864.06	20.7 %	29.3 %	1.63	3.59 (Acidic)
Kudinoside A	283.98	1375.56	962.81	20.6 %	29.5 %	0.02	11.76 (Basic)
Kudinoside C	363.13	1583.29	1078.03	22.9 %	33.7 %	- 1.75	11.76 (Basic)
Kudinoside G	353.90	1590.27	1151.67	22.3 %	30.7 %	- 0.56	11.61 (Basic)
Kudinoside N	491.97	1992.98	1329.41	24.7 %	37.0 %	- 3.05	11.61 (Basic)

After calculation of the physical properties of the analytes of *Ilex latifolia* with Marvin software, including the polar surface area, van der Waals surface area, solvent-accessible surface area, log *P* and pK_a values, Table 4.2 summarized the physical properties of the analytes. Polar surface area (PSA) is formed by polarized atoms of a molecule and its estimation is based on the method described in Ertl et al. [255]. For molecular surface area of a molecule, there are two types of available calculations, i.e. the van der Waals surface area (vdWSA) and the solvent-accessible surface area (SASA), and the calculation methods are based on Ferrara et al. [256]. Log *P*, is the logarithm of the partition-coefficient (*P*) for a molecule between water and octanol, which is used as a measurement of analyte polarity or hydrophobicity, and the calculation method is based on the publication of Viswanadhan at al. [257]. ESI responses of analytes can be correlated to the log *P* values. Analytes with relatively high log *P* values are more favorable for the octanol (non-polar solvent) than the water (polar solvent). An increase in log *P* value would cause an increase in the ions evaporation of the deprotonated molecular ions because the molecules have a higher surface activity in aqueous droplets and migrate faster to the droplet / air interface. Therefore, higher ESI-MS responses are expected for analytes with relatively high log *P* values than analytes with relatively low log *P* values due to their non-polar characters which increase

in the log *P* value would causes an increase in the surface activity of the molecules and hence an increase in the ionization efficiency. Moreover, p$K_{\mathbf{a}}$ value is a quantitative measure of the strength of an acid in solution, it is also known as -log $K_{\mathbf{a}}$ where $K_{\mathbf{a}}$ is the acid dissociation constant [258]:

$$HA + H_2O \leftrightarrow A^- + H_3O^+ \qquad K_a = [H_3O]^+[A]^- / [HA]$$

Based on the equation, analytes with high K_a values, which is acidic in nature, will favor the deprotonation (the smaller the value of p$K_{\mathbf{a}}$, the larger the extent of dissociation) to become anions in solution or in the electrospray droplets. Therefore, it is expected that DI-MS responses in negative ion mode are inversely related to pK_a of analytes.

Studies have also revealed that surface activity is one of the important factors that affect the responses of analytes during ESI-MS analysis. Analytes with significant nonpolar portions have higher ESI responses than highly polar analytes as the polar portions are necessary to enable ion formation, while the nonpolar portions are responsible for increasing the fraction of the analyte that reside on the surface of ESI droplets [213, 217, 259-261]. However, there are no proportionally correlated relation between log *P* value and surface activity since analytes are completely solvated into octanol partition for the calculation of log *P* value, while the analytes

are only partially desolvated into the surface partition of ESI droplets for the calculation of the surface activity. Since log *P* value is calculated for the neutral analytes instead of their ionic forms, the log *P* values of neutral analytes helps to evaluate the molecular tendency to surface partition of ESI droplets. Both log *P* value and surface activity can help to predict the ESI-MS responses.

In order to investigate the factors governing the DI-MS responses, surface activity of the analytes was first exanimated to see whether it contributed to the significant suppression of analytes during DI-MS analysis of *Ilex latifolia* leaves. The percentage of the polar surface area of the analytes was first calculated by dividing the polar surface area (PSA) by the molecular surface area, i.e. van der Waals surface area (vdWSA) and solvent-accessible surface area (SASA) respectively. The smaller the percentage of the polar surface area of the analytes, i.e. the smaller the values of (PSA / vdWSA) x 100 % and (PSA / SASA) x 100 %, the larger the percentage of the non-polar surface area of the analytes, which would give higher signal responses. From Table 4.2, it found that caffeoylquinic acids and kudinosides have similar percentage of the non-polar surface area, there was no significant relationship between the surface activity and the responses of analytes during DI-MS analysis.

Meanwhile, studies have demonstrated that log *P* and pK_a values are also important factors that influence the responses of analytes during ESI-MS analysis [224, 262-264]. From Table 4.2, it also found that caffeoylquinic acids and kudinosides have similar log *P* value, there was no significant relationship between the log *P* value (polarity of analytes) and the responses of analytes during DI-MS analysis. However, it found that caffeoylquinic acids and kudinosides have significant difference of pK_a value. The pK_a values of caffeoylquinic acids and ilekudinoside B were about 3, while the pK_a values of kudinosides and latifoloside were about 11. The analytes with low pK_a values, which is acidic in nature, would favor the deprotonation of the analytes and would have higher signal responses compared to the analytes with high pK_a values which is basic in nature. Thus, the signal responses of kudinosides and latifoloside were significantly suppressed compared to the signal responses of caffeoylquinic acids and ilekudinoside B. The detailed mechanisms of this phenomenon could be elaborated by the acidity of the analytes. A positive correlation between analyte ions responses and acidity (pK_a) was observed when *Ilex latifolia* leaves were analyzed with DI-MS because analytes with lower pK_a values are more responsive to DI-MS analysis in negative ion mode as they have higher efficiency for deprotonation. More acidic analytes are more responsive to the analysis with DI-MS in negative ion mode since they

have a greater tendency to form negative ions.

The effect of acidity (pK_a) on ESI signals is an important factor governing responsiveness to ionization in ESI-MS [227]. In this study, an obvious result was observed of significantly decreasing DI-MS responses at high pK_a value of analytes during DI-MS analysis of *Ilex latifolia* leaves. This observation agreed with the theory that the pK_a value and the ESI responses in negative ion mode have an inversely proportional relationship. The results demonstrated that there was a significant relationship between the pK_a value (acidity of analytes) and the signal responses of analytes during DI-MS analysis of *Ilex latifolia* leaves. But for the analytes tested here, surface activity and polarity were found not to have an influence on DI-MS responses. The results show that surface activity and polarity are not the conclusive parameters for prediction of the relative responses of DI-MS among a series of different classes of active ingredients of CHMs. However, acidity of analytes appears to be useful for this purpose. In general, acidity (pK_a) of analytes would be a significant first parameter to predict the DI-MS responses, particularly when choosing the ionization mode of DI-MS.

4.4. Conclusions

The aims of our study was to investigate the influence of analytes and solvent properties on responsiveness to the analysis of CHMs with DI-MS. The results of this study are important to users of DI-MS in different ways. First, our results revealed that solvent loaded onto the surface of CHMs was crucial for the observation of desired ion signals in DI-MS as it served for simultaneous extraction of compounds from the sample and ionization of the extracted compounds during the DI-MS process. The DI-MS responses of a series of different classes of active ingredients of CHMs were analyzed with different solvents and were generally observed to be higher in relatively polar solvents, i.e. methanol, acetonitrile, acetonitrile/water (1/1), methanol/water (1/1) and water, than relatively non-polar solvents, i.e. acetone, ethyl acetate, dichloromethane, chloroform and hexane, due to the relatively polar solvents have the advantages of hgher conductivity and compatible surface tension with DI-MS analysis. As the solution becomes more aqueous, its surface tension would increase leading to an increase in difficulty to have a stable electrospray during the DI-MS analysis. Also, it is difficult to achieve stable electrospray with the relatively non-polar solvents, due to their very low surface tension, low dielectric constant, and high volatility.

Therefore, relatively polar solvents are generally preferred. Meanwhile, the quantitative responses of the active ingredients of the Chinese herbal medicines investigated were summarized in Table 4.10. In general, the power extraction method gave the strongest signal intensity; the direct extraction method and the DI-MS method gave the medium signal intensity; and the soft-landing method gave the weakest signal intensity. Moreover, among the seven different CHMs analyzed in this study, an obvious solvent effect of analytes was observed during the analysis of *Ganoderma lucidum*, and a significant ion suppression of analytes was observed during the analysis of *Ilex latifolia* leaves.

The work studied here also comprehensively compared the influence of surface activity, acidity and polarity of analytes on the DI-MS responses of CHMs with different classes of active ingredients. Overall, the results demonstrated that the extraction and ionization mechanisms for DI-MS of CHMs were related to the physical properties of the analytes, including the surface activity, polarity and acidity of the analytes, which are useful parameters for the prediction of DI-MS signal responses of CHMs with different classes of active ingredients. The results from this study showed that DI-MS responses were correlated to the acidity of analytes for a number of structurally diverse analytes. Therefore, users of DI-MS

can expect higher ions response intensities in negative ion mode for analytes with relatively lower pK_a valuess. Acidity (pK_a) of analytes is a significant first parameter to predict the DI-MS responses, particularly when choosing the ionization mode of MS. In positive ion mode, protonation of basic analytes to form cations is favored, while deprotonation of acidic analytes to form anions is favored in negative ion mode. It is a useful parameter to predict whether or not the analytes will form negative ions and have intense signals in negative ion mode of DI-MS based on the pK_a values of the analytes. However, it is not always the case that the DI-MS responses can be decided by the analytes' acidity because there are a lot of cases are much more complex that would not be able to be predicted just based on the pK_a values. For examples, some analytes with relatively high pK_a values but have significant non-polar regions may also be able to form negative ions and analyzed responsively in negative ion mode of MS. Conversely, some analytes with relatively low pK_a values but have very polar regions may respond very poorly in negative ion mode of MS. In general, when choosing between positive or negative ion modes of DI-MS, pK_a values of analytes are significant parameters for a first consideration. However, it is difficult to correlate signal responses of analytes to DI-MS with any single parameter due to the complexity of the extraction and ionization mechanisms of DI-MS and the complexity of the analytes

of CHMs. Further experiments on other CHMs may help to have a comprehensive understanding of the extraction and ionization mechanisms of DI-MS. The research conducted here presented some fundamental studies of DI-MS process and compared several significant factors to the responsiveness of different classes of active ingredients of CHMs by DI-MS. The results of this study help the understanding of DI-MS responses depend on the analytes' properties, and provides a basis to predict DI-MS responses of analytes. It would help the users who apply DI-MS for the analysis of CHMs.

Table 4.10 Summary of the Chinese herbal medicines investigated and listed all the active ingredients and their quantitative responses at different stages.

Chinses herbal medicine	Major active ingredients	DI-MS	Powder extraction method	Direct extraction method	Soft-landing method	Solvent effect	Ion suppression
Ganoderma lucidum	Ganoderic acids	Medium	Strong	Medium	Weak	√	X
Schisandrae chinensis fructus	Schisandrins	Medium	Strong	Medium	Weak	X	X
Hylocereus undatus	Kaempferols	Medium	Strong	Medium	Weak	X	X
Radix polygoni multiflora	Emodin, physcion & pterostilbene	Medium	Strong	Medium	Weak	X	X
Lycoris radiata	Lycorine, lycoramine & lycorenine	Medium	Strong	Medium	Weak	X	X
Psidium guajava leaves	Psiguajadials, psidials & guavinosides	Medium	Strong	Medium	Weak	X	X
Ilex latifolia leaves	Caffeoylquinic acids, kudinosides & latifolosides	Medium	Strong	Medium	Weak	X	√

Chapter 5. Overall Conclusions and Prospects

The research in this thesis involves the applications of DI-MS for direct analysis of raw Chinese herbal medicines samples and investigation of the extraction and ionization mechanisms of DI-MS analysis of CHMs.

Analysis of CHMs plays an important role in quality control of medicinal materials. The greatly increased global demand and profitability of CHMs lead to the problems of counterfeiting and species confusion which compromises the safety and efficacy in the use of CHMs. Conventional approaches for the analysis of CHMs are usually labor-intensive and time-consuming. DI-MS is a technique that allows direct and rapid analysis of CHMs with minimal to no sample pretreatment. In this study, we have developed a DI-MS-based method for rapid authentication of *Gastrodiae rhizoma* and rapid differentiation of *Ganoderma* species. Based on the detection of the major active components that are present in *Gastrodiae rhizoma* and *Ganoderma*, genuine and counterfeit *Gastrodiae rhizoma* species could be unambiguously differentiated, and official *Ganoderma* species stated in the Chinese pharmacopoeia and confused *Ganoderma* species could also be unambiguously differentiated. Differentiation between wild and cultivated species and potentially discrimination of species from different geographical origins could also be achieved based on the DI-MS analysis combining with PCA or HCA

analysis. The results also suggested that by using a suitable endogenous compound as the internal reference compound, DI-MS could be efficiently used for quantitative comparison of active ingredients in herbal samples. In brief, our results demonstrated that DI-MS could be a rapid and efficient method for authentication and differentiation of CHMs. The method is simple, rapid and reproducible and can be further applied to analysis of other sample systems. The techniques are expected to play an important role for rapid analysis of various CHMs in the future.

In the last part of this thesis, with the use of CHMs with different classes of active ingredients and solvents of various properties, the products generated at different extraction and ionization stages were collected, analyzed and compared in order to understand the process and factors governing the signal responses of this direct analysis technique, to enhance detection of ingredients, particularly active ingredients, of CHMs. We revealed that the solvent loaded onto the surface of CHMs was crucial for the observation of desired ion signals as it served for simultaneous extraction of compounds from the sample and ionization of the extracted compounds in DI-MS. The effects of various physical properties, including the surface activity, polarity and acidity of the analytes on the extraction

and ionization mechanisms for DI-MS of CHMs were systematically investigated and the physical properties of the analytes were found to be important for the prediction of DI-MS signal responses of analytes. These findings allowed us to get more insight into the extraction and ionization mechanisms of DI-MS, and to explore the ways to enhance the sensitivity and selectivity of DI-MS.

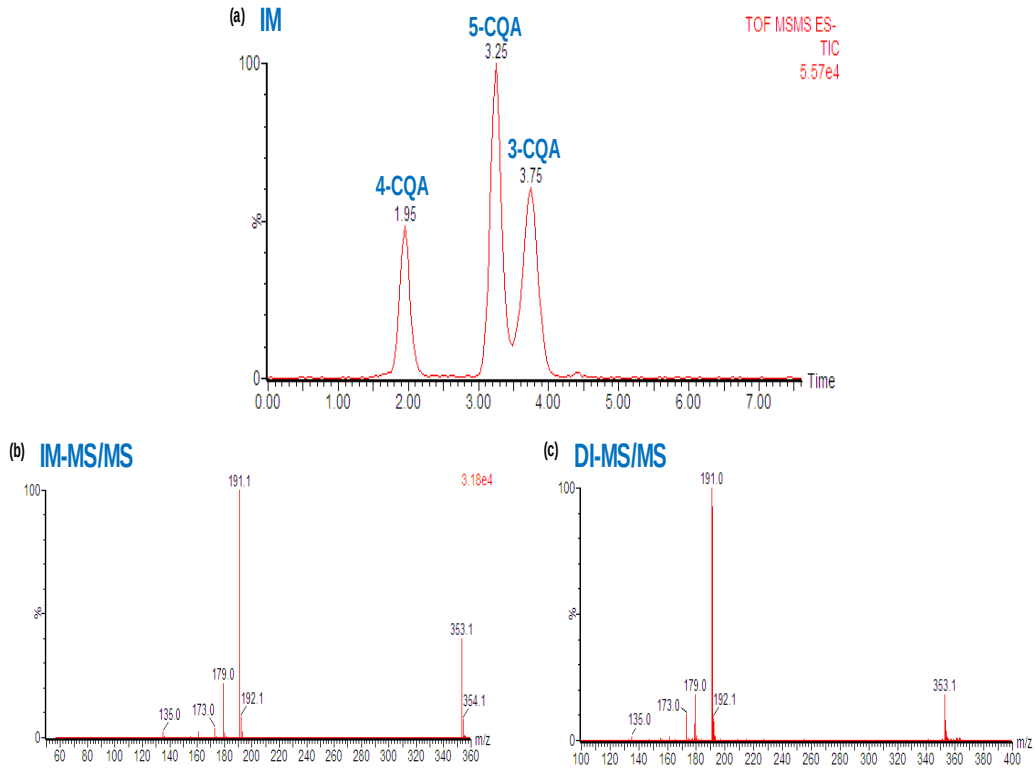


Figure 5.1. (a) Total ion chromatogram of ion mobility-tandem mass spectrometric (IM-MS/MS) separation of three caffeoylquinic acids isomers of *ilex latifolia* leaves extract with collision energy of 20 eV, (b) IM-MS/MS and (c) DI-MS/MS spectra of *ilex latifolia* leaves extract of precursor ion with *m/z* 353.1.

Developing and coupling miniature mass spectrometers with DI-MS would give great and potential applications of DI-MS since DI-MS can be used for rapid, direct and high-throughput analysis with high sensitivity, high reproducibility and requires no or minimal sample pretreatment. These advantages allow DI-MS for on-site analysis when coupled with miniature mass spectrometers. One of the drawbacks of DI-MS is that isomer differentiation could not be achieved since no chromatographic separation of the components is employed in DI-MS. Isomers that co-existed in CHMs would be detected as a single ion peak and overlapped in the DI-MS spectra. Thus, a significant improvement is needed for DI-MS to differentiate isomers.

Ion mobility mass spectrometry is a promising tool for this as it allows separation of molecules based on size and shape which could significantly enhance the detection specificity, and a recent study indicated that a series of chlorogenic acid isomers could be differentiated and detected by using differential ion mobility and tandem mass spectrometry [191]. Chlorogenic acid isomers are also present in the CHM of *ilex latifolia* leaves, and our DI-MS analysis results of the *ilex latifolia* leaves samples showed that the three chlorogenic acid isomers, i.e. 3-caffeoylquinic acid (3-CQA), 4-caffeoylquinic acid (4-CQA) and 5-caffeoylquinic

acid (5-CQA), could be detected but overlapped as a single ion peak in the DI-MS spectrum. Therefore, we tried to apply IM-MS to differentiate the detected chlorogenic acid isomers of the *ilex latifolia* leaf samples without the use of chromatographic separation. Extract of *ilex latifolia* leaves were analyzed with IM-MS and DI-MS respectively, and the results showed that IM-MS allowed unequivocally differentiation of the three CQAs isomer (see Figure 5.1a) and the isomer detected were further confirmed with MS/MS fragmentation. The MS/MS fragmentation pattern of the three CQAs isomer obtained by IM-MS/MS (Figure 5.1b) were the same as that obtained by DI-MS/MS (Figure 5.1c). These experimental findings indicated that the three chlorogenic acid isomers of the *ilex latifolia* leaves extract could be efficiently differentiated and identified by IM-MS/MS. Coupling DI-MS with ion mobility would be a great and potential direction for future study, and it would allow DI-MS to become a more comprehensive and robust analytical tool.

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