THE HONG KONG POLYTECHNIC UNIVERSITY

Department of Applied Biology and Chemical Technology

Development of Electrospray Ionization Mass Spectrometry for Direct Analysis of Complex Samples and an Investigation of Ionization Mechanism of Matrix-assisted Laser Desorption/Ionization Mass Spectrometry

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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

August 2013

CERTIFICATE OF ORIGINALITY

I declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

Wang Haixing

August 2013

Abstract

Mass spectrometry (MS) is a powerful tool for analysis of various samples. Conventional mass spectrometric analysis, however, typically requires extensive pretreatment to raw samples. In recent years, ambient ionization techniques have been developed to enable direct analysis of complex samples with little or no sample preparation. Following our previous research on direct ionization analysis of tissue samples, in this study, pipette-tip electrospray ionization mass spectrometry (ESI-MS) was developed for direct analysis of another common sample form, herbal powder. This new technique combines commonly available and disposable pipette tip with a syringe pump for on-line sample extraction and ionization. We demonstrated that various powders of herbal medicines and foods could be directly analyzed with this technique and closely related herbal species could be clearly differentiated based on the mass spectra thus obtained. This technique offered continuous and stable signals, and its capability in direct quantitation of chemical ingredients in powder samples was also demonstrated in this study. The pipette tip of this technique could be replaced with a commercially available C18 pipette tip (Zip-Tip), which contained C18 absorbent in the tip end. In this way, the technique could be used for direct analysis of raw solution samples, including detection of protein solutions containing high contents of sodium chloride or sodium dodecyl sulfate (SDS) and quantitation of abused drugs in urine.

Abstract

The capability of MS for direct analysis of raw protein samples, particularly membrane proteins, was further explored in this study. Membrane proteins play important roles in biological processes and are targets of many drugs. MS analysis of membrane proteins has been a challenging task due to their poor solubility and that detergents commonly used to improve the solubility are not compatible with MS analysis. In this study, for the first time, extractive electrospray ionization mass spectrometry (EESI-MS) was attempted for direct analysis of membrane proteins containing high contents of detergents, and our results indicated that EESI-MS was more tolerant to nonionic detergents than ESI-MS. In this study, wooden-tip ESI-MS, a technique developed in our group, was also developed for direct quantitation of di-(2-ethylhexyl)-phthalate (DEHP), a hazard plasticizer, in beverages such as fruit juice and sports drink. We demonstrated that wooden-tip ESI-MS was a simple and economical method for rapid screening of DEHP in beverages.

Matrix-assisted laser desorption/ionization (MALDI) is another commonly used ionization technique in mass spectrometry. The ionization mechanism of MALDI is, however, not completely clear yet. In this study, oil-assisted sample preparation (OASP), a MALDI sample preparation method recently developed in our group, and deuterium-labeled matrix and analyte were employed to investigate the interactions between matrix and analyte molecules. The results revealed that all active hydrogens of analyte molecules could be replaced by hydrogens from matrix. The exchange tendency of active hydrogens from different functional groups of analyte and different conformational peptides was investigated. These results allowed us to get new insights into the MALDI ionization process.

Research Publications

Conference Papers

1. Wang H.X. and Yao Z.P. Development of Pipette-tip Electrospray Mass Spectrometry for Direct Analysis of Herbal Powder Samples, *HKSMS Symposium 2013 15th Annual General Meeting*, Tsim Sha Tsui, Hong Kong, 22 June 2013.

2. Wang H.X. and Yao Z.P. Direct Analysis of Herbal Powders by Pipette-tip Electrospray Ionization Mass Spectrometry, *Proceedings of the 61st ASMS Conference on Mass Spectrometry and Allied Topics*, Minneapolis, U.S.A. 9-13 June 2013.

3. Wang H.X. and Yao Z.P Development of Mass Spectrometry for Direct Analysis of Herbal Powders, *The 20th Symposium on Chemistry Postgraduate Research in Hong Kong*, The Chinese University of Hong Kong, Hong Kong, 27 April 2013.

4. Wang H.X. and Yao Z.P Investigation of Membrane Proteins by Extractive Electrospray Ionization Mass Spectrometry, *The 19th Symposium on Chemistry Postgraduate Research in Hong Kong*, The Hong Kong University of Science and Technology, Hong Kong, 14 April 2012.

Journal Publications

5. So, P.K.; Ng, T.T.; Wang, H.X.; Hu, B.; Yao, Z.P., Rapid detection and quantitation of ketamine and norketamine in urine and oral fluid by wooden-tip electrospray ionization mass spectrometry, *Analyst* **2013**, *138*, 2239-2243.

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List of Abbreviations

Full Form	**Abbreviation**
atmospheric pressure chemical ionization	APCI
6-aza-2-thiothymine	6-ATT
chemical ionization	CI
3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate	CHAPS
collision-activated dissociation	CAD
collision-induced dissociation	CID
α-cyano-4-hydroxycinnamic acid	CHCA
daltons	Da
desorption atmospheric pressure chemical ionization	DAPCI
desorption electrospray ionization	DESI
deuteration ratio	DR
di-(2-ethylhexyl)-phthalate	DEHP
2,5-dihydroxy benzoic acid	DHB
3,5-dimethoxy-4-hydroxycinnamic acid	SA

dissononyl phthalate	DINP
direct analysis in real time	DART
n-dodecyl β-D-maltoside	DDM
electron capture dissociation	ECD
electron ionization	EI
electron transfer dissociation	ETD
electrospray-assisted laser desorption/ionization	ELDI
electrospray ionization	ESI
extractive electrospray ionization	EESI
fast atom bombardment	FAB
formic acid	FA
Fourier transform ion cyclotron resonance	FTICR
Fructus Schisandrae Chinensis	FSC
Fructus Schisandrae Sphenantherae	FSS
gas chromatograpy	GC
high performance liquid chromatography	HPLC
hydrogen/deuterium exchange	HDX

3-hydroxy-picolinic acid	3-HPA
internal standard	IS
laser ablation electrospray ionization	LAESI
laser desorption/ionization	LDI
limit of detection	LOD
limit of quantitation	LOQ
mass spectrometry	MS
matrix-assisted laser desorption/ionization	MALDI
methanol	MeOH
multichannel plate	MCP
multiple reaction monitoring	MRM
number of deuterons incorporated	D
n-octyl-β-D-glucoside	OG
oil-assisted sample preparation	OASP
pipette tip-based micro-extraction	TBME
plasma desorption	PD
principal component analysis	PCA

quadrupole	Q
relative standard deviation	RSD
secondary ion mass spectrometry	SIMS
selected reaction monitoring	SRM
signal-to-noise ratio	S/N
sodium dodecyl sulfate	SDS
tandem mass spectrometry	MS/MS
thin layer chromatograph	TLC
time-of-flight	ToF
total ion current	TIC
traditional Chinese medicine	TCM
trifluoroacetic acid	TFA
triple quadrupoles	QqQ
ultra performance liquid chromatography	UPLC

Chapter 1 Introduction

1.1 General Introduction of Mass Spectrometry

Mass spectrometry (MS) is a technique based on generation of positively or negatively charged ions in gas-phase and measurement of the mass-to-charge ratios (*m/z*) of the generated ions in electrical or magnetic field. A mass spectrometer mainly consists of three elements: ion source, mass analyzer and detector (Figure 1-1). Ion source is the component where analytes are ionized and converted into positive or negative ions in the gas-phase. A wide variety of ionization techniques have been invented and applied in mass spectrometry (Table 1-1).[1, 3] These ionization techniques include electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI), electron ionization (EI) and atmospheric pressure chemical ionization (APCI), chemical ionization (CI) and fast atom bombardment (FAB), among which ESI, MALDI, EI and APCI are currently the most popular ionization techniques used in mass spectrometry. Ambient ionization techniques have been developed in recent years,[4-6] and corresponding ion sources such as direct analysis in real time (DART),[7] desorption electrospray ionization (DESI)[8] and laser ablation electrospray ionization (LAESI)[9] are commercially available and widely used. Mass analyzer is the component where the charged ions are sorted and measured by their *m/z* values. Commonly used mass analyzers include time-of-flight (ToF), quadrupole and ion trap. Fourier transform ion cyclotron resonance (FTICR) and orbitrap, two ultra high resolution mass analyzers, are becoming more and more popular due to their high mass accuracy for qualitative analysis. Detector, such as multichannel plate (MCP), is to detect the arrival of the charged ions and measure their intensities. The detected signals are finally converted into a mass spectrum, in which the x-axis represents *m/z* values of the detected ions and the y-axis represents the abundance or intensity of the detected ions.

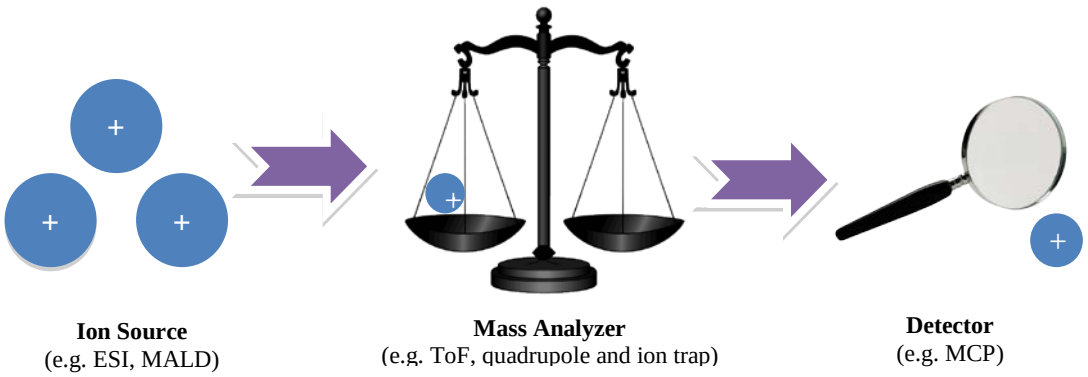


Figure 1-1 Basic components of a mass spectrometer.

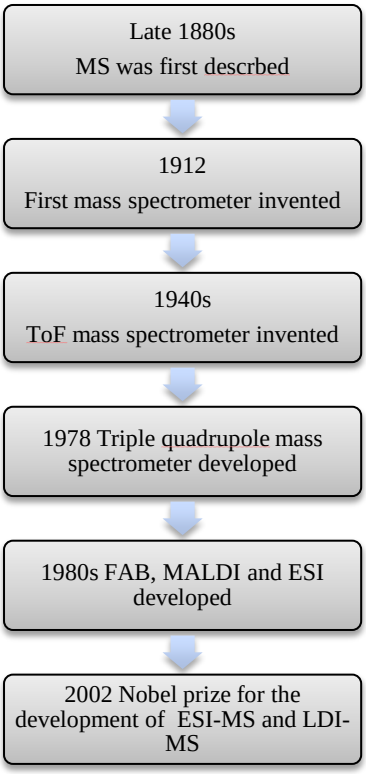


Figure 1-2 Some important events in the history of MS.

MS has a long history of more than a century. The history of MS rooted in the end of nineteenth century (Figure 1-2). In1886, German physicist Eugen Goldstein discovered a new type of radiation, "*Kanalstrahle*", and assumed this ray to be positively charged particles.[2, 10]

In 1898, physicist Wilhelm Wien showed that positive ions could be deflected by strong electric and magnetic fields, and it was also considered that there should be a fixed relationship between charge-to-mass ratios (*m/z*) and parabolic curves.[2, 10]

Inspired by Goldstein's and Wien's research, another physicist Joseph John Thomson of the University of Cambridge constructed the first mass spectrometer in the world and produced the first mass spectrum in 1913. At that time, he predicted that this technique could be a powerful analytical tool in the future.[11]

In 1942, MS was employed as a critical tool for stable isotope analysis.[12] The first commercial mass spectrometer for analysis of organic compounds was manufactured to satisfy the development of the oil industry in the 1940s.[2, 10] After the invention of gas chromatography (GC) in 1952,[13] Holmes and Morrell combined it with MS, i.e., GC-MS, in 1957.[14]

Mass spectrometers in the early stage were only equipped with EI ion sources, which used 70 eV electrons for ionization of compounds. For many compounds, their spectra were predominated with fragment ions with the absence of molecular ions under such a harsh condition. To overcome this problem, CI was invented in 1966.[15] It used softer ionization conditions to allow the observation of molecular ions. However, both EI and CI require thermal vaporization of analytes and thus are only suitable for analysis of volatile, thermally stable small molecules, and not applicable for thermally unstable, nonvolatile compounds.

Plasma desorption (PD), invented in 1974,[16] is another kind of "soft" ionization technique that allows observation of molecular ions of analytes. The applicable mass range of this technique has been enormously improved and biomolecules up to 100,000 Da could be ionized. Soft ionization techniques FAB,[17, 18] ESI[19, 20] and MALDI[21, 22] were introduced in the 1980s. ESI and MALDI were considered as revolutionary techniques in MS and made analysis of large biological molecules (proteins, nucleic acids and large complexes) by MS not just possible but also easy. This was a significant step in the development of mass spectrometry. John B. Fenn and Koichi Tanaka were thus awarded the Nobel Prize in Chemistry in 2002 for their outstanding contributions to the development of ESI-MS and laser desorption/ionization-MS (LDI-MS) for "identification and structure analyses of biological macromolecules" (quoted from the official web site of the Noble prize), respectively.

As a powerful tool in analytical chemistry, MS is applied for qualitative and quantitative analysis of a wide variety of chemical and biological samples, such as proteins, nucleic acids and even viruses and bacteria. MS can be used for determination of elemental compositions of compounds using exact mass measurements, structural elucidation of compounds using tandem MS, identification of unknown compounds, and quantitative measurements of components in mixtures.[2, 6]

1.2 Ionization Techniques

The development of ionization techniques expands the applications of MS from atomic mass measurement to analysis of complex biological mixtures. By now, various ionization technique have been developed in the history of MS.[3] The common ionization techniques for MS are listed in Table 1-1.

Table 1-1 Common ionization techniques for MS.[1]

Ionization technique	Applicable analytes	Mass range	Technology description
Electron inization (EI)	Volatile and thermally stable compounds	Less than 1000 Da	Fragment ions mainly, hard ionization method
Chemical ionization (CI)	Volatile and thermally stable compounds	Less than 1000 Da	Molecular ion available, soft ionization method
Atmospheric pressure chemical ionization (APCI)	Nonpolar to polar compounds, thermally stable	Less than 1000 Da	Molecular ion and fragment ions, less soft ioniztion method
Fast atom bombardment (FAB)	Polar compounds	200-2000 Da (optimal range)	Matrix required, soft ionization method
Electrospray ionization (ESI)	Polar compounds	From very low mass to extrordinarily high mass	Singly charged or multiply charged ions, soft ioniztion method
Matrix-asissted laer desoprtion/ionization (MALDI)	Polar compounds	From very low mass to extrordinarily high mass (up to 500,000 Da)	Singly chareged ions, matrix required,soft ioniztion method

1.2.1 Electrospray Ionization

Generally, the origin of ESI was considered to start from the discovery of electrospray ionization of a polymer in gas-phase by Dole and his coworkers in 1968.[23] However, this ionization technique was not used in mass spectrometer at that time. The first combination of ESI to a mass spectrometer happened in 1984 by John B. Fen and his coworkers.[19, 20] They successfully coupled an ESI ion source to a quadrupole mass spectrometer and detected ions in both positive and negative modes. ESI-MS could be applied for the analysis of nonvolatile organic or inorganic compounds, as well as biological complexes. The predominant ions of this ionization technique are singly or multiply charged molecular ions.

The ion formation of ESI is a physical process.[24] The pathway for the formation of ions in ESI is shown in Figure 1-3. First, a sample is dissolved in a solvent. Methanol, acetonitrile and their mixtures with water are commonly used solvents in ESI. Then, the sample solution is infused into a capillary.

Second, when a high voltage (typically 3-5 kV) is applied to the capillary, a Taylor cone and cone-jet are formed from the tip of the capillary. The term “Taylor cone” was first described by Sir Geoffrey Taylor in 1964.[25] When a voltage is applied to an electrically conductive solvent, the shape of the solvent would change from the common shape induced by surface tension. Along with the increment of the voltage, the solvent droplet

would become elongated and a Taylor cone is formed. When a threshold voltage is reached, a liquid cone-jet is generated at the tip of the Taylor cone.

Third, a plume composed of highly charged droplets is formed following the formation of the cone-jet. With the assistance of a desolvation gas (e.g. nitrogen) usually, the solvent is vaporized and the size of the droplets is further reduced. Along with the evaporation of the solvent, the charge densities on the surface of the droplets become higher and higher. When repulsive forces between the charges on the droplet surfaces reach the limit, coulomb explosions occur, which split one charged droplet into several small charged droplets.

Finally, successive evaporation of droplet solvents and fission of droplets lead to formation of analyte ions in the gas phase. There are two models to explain this process: the ion evaporation model and the charge residual model. In the ion evaporation model, further solvent evaporation results in high repulsive forces of charges on the droplet surfaces again. When these repulsive forces outstrip the surface tension of the droplets, analyte ions would be desorbed into the gas phase one by one.[26, 27] This model is applicable to explain the formation of analytes ions with relatively low masses. The charge residue model is considered to favor ions with relatively high masses.[28] According to this model, successive solvent evaporation eventually leads to remaining of one or no analyte ion.

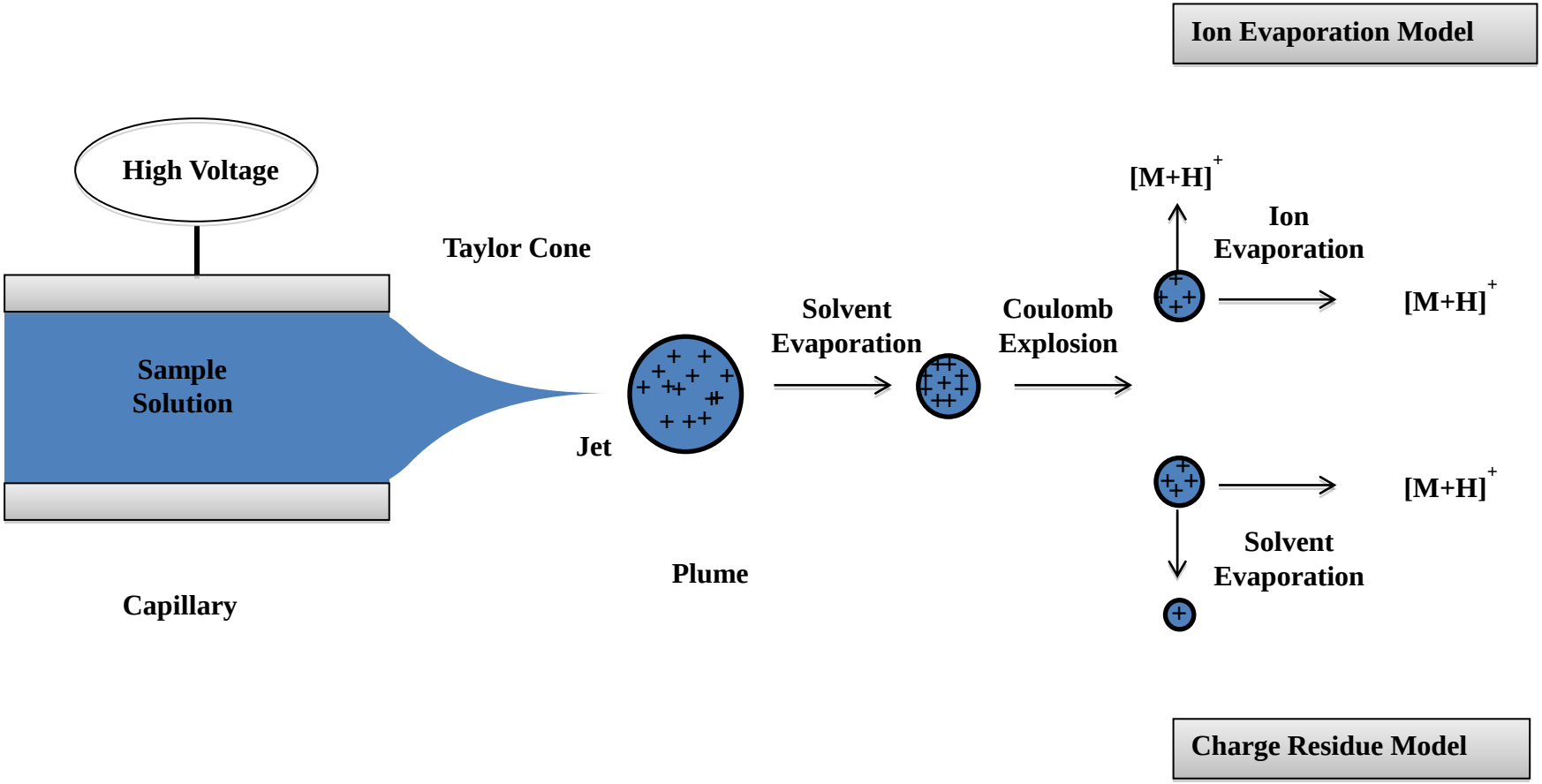


Figure 1-3 Ion formation process in ESI.

1.2.2 Matrix-assisted Laser/Desorption Ionization

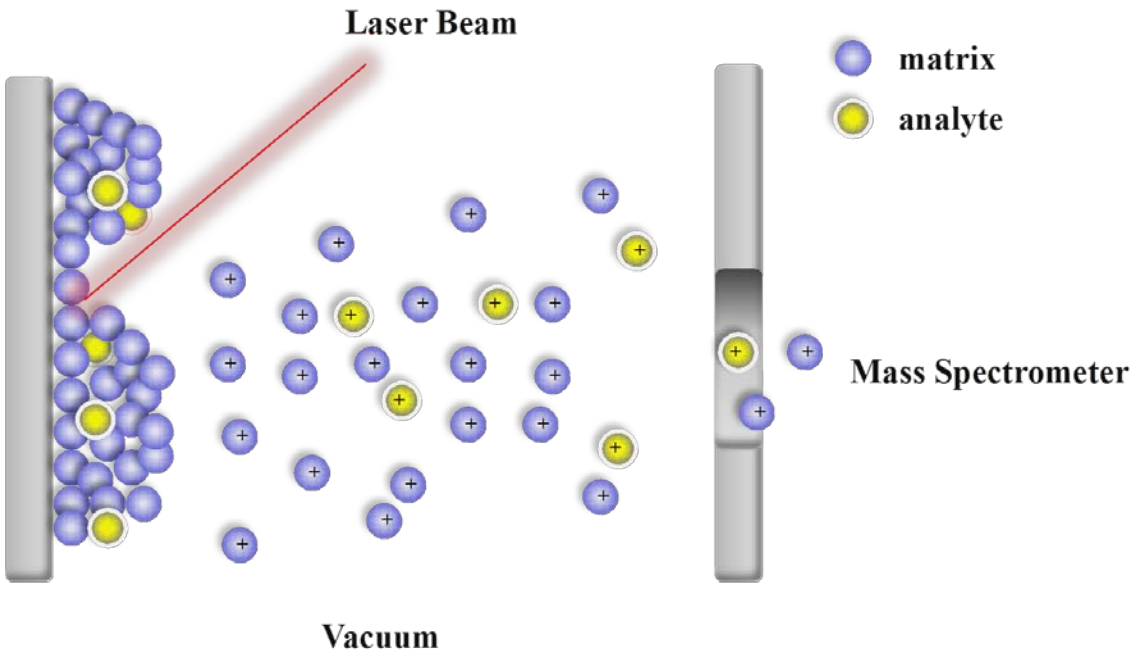


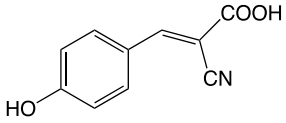
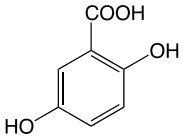
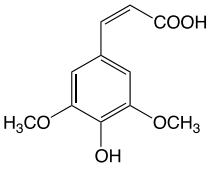
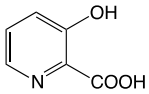
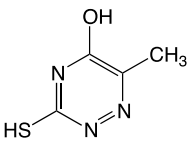
Figure 1-4 Schematic diagram of MALDI ionization.

MALDI is another powerful MS ionization technique, and is widely utilized together with ESI in proteomic research.[29, 30] It can also be applied for the analysis of many other compounds, such as nucleic acids[31] and lipids.[32] MALDI can be utilized for tissue imaging (MALDI imaging), which is an active field in recent years.[33]

MALDI-MS typically requires mixing sample with a matrix. Matrix is important for MALDI ionization; it can absorb laser energy and assist desorption and ionization of analytes as shown in Figure 1-4. There is no single MALDI matrix or sample

preparation method suitable for analysis of all analytes. Some **c**ommonly used MALDI matrices are listed in Table 1-2.

Table 1-2 Commonly used MALDI matrices.[34]

Matrix	**Abbreviation**	**Structure**	**Major applications**
α-cyano-4-hydroxycinnamic acid	CHCA		peptides, proteins
2,5-dihydroxy benzoic acid	DHB		proteins, peptides, carbohydrates, syntheic polymers
3,5-dimethoxy-4-hydroxycinnamic acid	SA		proteins, peptides
3-hydroxy-picolinic acid	3-HPA		Best for nucleic acids
6-aza-2-thiothymine	6-ATT		proteins, peptides, non-covalent complexes

1.2.2.1 Sample Preparation Methods

Sample preparation in MALDI is to mix matrix and analyte together on MALDI plate for subsequent analysis. It is important for getting ideal MALDI-MS spectra. There are many kinds of sample preparation methods and some of them are introduced below.

Dried-droplet (DD) method

This is the most commonly used MALDI sample preparation method. Generally, the sample solution is mixed with a matrix solution, then ~1 μl of the mixture solution is deposited onto a plate well with a pipette and air dried. Co-crystals will be formed between matrix and analyte molecules and they are important for good signals of the analytes. This method is suitable for analysis of a wide variety of samples and its operation is easy. The major disadvantages of this method are the inhomogeneity of the crystals and the appearance of "sweet spots". A "sweet spot" is a spot that gives better signals of the analyte.

Double-layer method

In the double-layer method, matrix solution is first deposited onto the plate well to form a thin layer of matrix crystals. Then a droplet of analyte solution is deposited onto the matrix thin layer. The preformed matrix crystals serve as crystallization nuclei for the analyte molecules and allow the formation of more homogenous crystals on the surface of the plate.

Solvent-free method

The solvent-free MALDI sample preparation method was developed in 2001 and has been applied in analysis of various samples including polymers and biological samples.[35, 36] In solvent-free sample preparation, matrix powder and sample powder are directly mixed together without the application of solvents, and deposited on the plate for MALDI-MS analysis. Compared with the conventional solvent-based MALDI-MS, this technique could offer better sensitivity and resolution for analysis of polymers.

Oil-assisted sample preparation (OASP) method

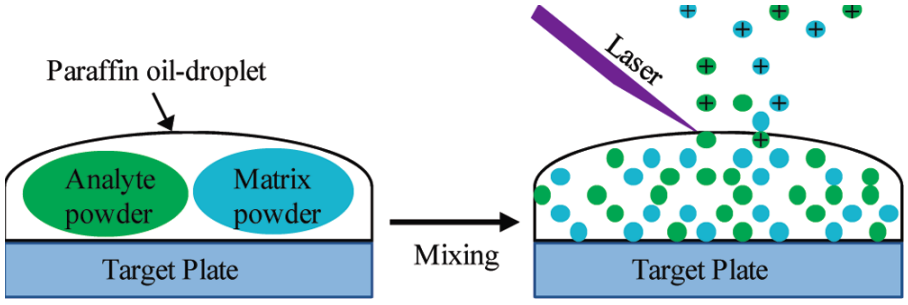


Figure 1-5 Oil-assisted sample preparation method for MALDI-MS.[37]

The oil-assisted sample preparation (OASP) method was developed in our group in 2011.[37] In this method, paraffin oil is used as a medium for the mixing of solid analyte and matrix on a MALDI plate as shown in Figure 1-5. This method simplifies the procedure for solvent-free sample preparation. Many kinds of samples, including poorly

soluble organic compounds and membrane peptides, polymers and solid biological samples, have been successfully analyzed with this method. In this thesis, this method was employed for the investigation of MALDI ionization mechanism, which will be described in Chapter 6.

1.2.2.2 Ionization Mechanism of MALDI

Although MALDI-MS has been developed for more than two decades, the ionization mechanism of MALDI is still poorly understood. In the process of MALDI ionization, there are a great variety of experimental conditions, such as laser wavelength, laser energy, sample preparation method, matrix and analyte property, that could affect the ionization process, and various models have been developed to explain the MALDI ionization mechanism.[38-43] Two popular models for MALDI ionization, the gas-phase protonation model and the cluster ionization model are briefly introduced below.

Gas-phase protonation model

The gas-phase protonation model is so far the most widely accepted model for the MALDI mechanism.[44] In this model, photochemical ionization of matrix molecule is the primary step for the subsequent gas phase proton transfer ionization.[45] Matrix molecule absorbs energy from laser in the form of photon and is ionized as shown below:

$$M \xrightarrow{m(h\nu)} M^{+\cdot} + e^{-} \qquad \textbf{(1-1)}$$

This step is considered to happen on the surface of the sample and matrix crystals. The process for energy absorption of matrix from laser involves multiphoton absorption or energy pooling.[44]

The second step is considered to happen in the gas phase. The resulting $M^{+\cdot}$ and e^- react with other matrix molecules and yield MH^+ and $(M-H)^-$ as shown in Eqs. 1-2 and 1-3 respectively.[44]

$$M^{+\cdot} + M \rightarrow MH^+ + (M-H)^{\cdot} \quad \textbf{(1-2)}$$

$$e^- + M \rightarrow (M-H)^- + H^{\cdot} \quad \textbf{(1-3)}$$

Then photon transfer reactions occur between protonated or deprotonated matrix ion and analyte molecule due to the difference between their gas phase proton affinities (PA) (Eqs. 1-4 and 1-5), leading to formation of protonated analyte molecule AH^+ or deprotonated analyte molecule $(A-H)^+$.

$$MH^+ + A \rightarrow M + AH^+ \quad \textbf{(1-4)}$$

$$[M-H]^- + A \rightarrow M + (A-H)^- \quad \textbf{(1-5)}$$

Cluster ionization model

The cluster ionization model is another popular model for MALDI ion formation; it was first postulated by Michael Karas *et al.* in 2000[46] and further refined in 2003.[39] In this model, analyte molecules are assumed to be pre-charged in the solution and existed as

neutral clusters [AH_n^{n+}+nX$^-$] with their counterions X$^-$. These clusters are preserved and incorporated into the matrix molecules after co-crystallization with the matrix. During the MALDI process, these clusters, together with the matrix molecules, are ablated from the crystal surface by laser irradiation and released into the gas phase in a plume. In this plume, the singly charged positive matrix ion MH^+ goes through a counterion neutralization process and the singly charged negative matrix ion $(M-H)^-$ goes through a analyte deprotonated process, leading to formation of the corresponding analyte ions. The neutralization probability of counterion could significantly increase with the increasing of charge state. So the singly charged ions, which have a sufficiently low neutralization probability, have a chance to survive in a possibly highly efficient ionization process. Due to this reason, this model was thus named as a lucky survivor model in the beginning.[46] The formation of positively charged analyte ion could be described as follows:

$$AH_n^{n+} + nX^- + MH^+ \rightarrow AH^+ + nHX \qquad \textbf{(1-6)}$$

Comparison between gas-phase protonation model and cluster ionization model

The gas-phase protonation model assumes there are neutral analyte molecules in the crystal mixture with matrix, photoionization of the matrix molecules is the first step and charge transfer (protonation/deprotonation) to analyte molecule in gas-phase is the second step. The cluster ionization model assumes analyte molecules are precharged in crystals and the subsequent neutralization with matrix ions leads to the obtained analyte ions. So the major difference between the two models is the origin of the proton added to

analyte molecule. In the gas phase protonation model, neutral analyte molecules are charged by proton transferred from matrix ion in the gas phase; while in the cluster ionization model, it is pre-multiple charged in the solution phase and the singly charged analyte ions survive the process of neutralization.

1.3 New Ionization Techniques

In the past decades, a great variety of new ionization techniques have been developed for direct analysis of complex samples under ambient condition with no or minimal sample preparation.[4, 5, 47-49] The first well-recognized ambient ionization technique, desorption electrospray ionization (DESI) MS, was introduced by Cooks' group in 2004.[8] A lot of the new ionization techniques, including direct analysis in real time (DART),[7] thin layer chromatograph (TLC) ionization,[50] extractive electrospray ionization (EESI),[51] paper spray ionization,[52, 53], probe electrospray ionization,[54] direct electrospray ionization on a tip column,[55] direct analysis of tissue samples,[56-59] and electrospray ionization using wooden tips,[60] have been developed recently. Herein, several ionization techniques related to the study in this thesis are briefly introduced.

1.3.1 Desorption Electrospray Ionization (DESI)

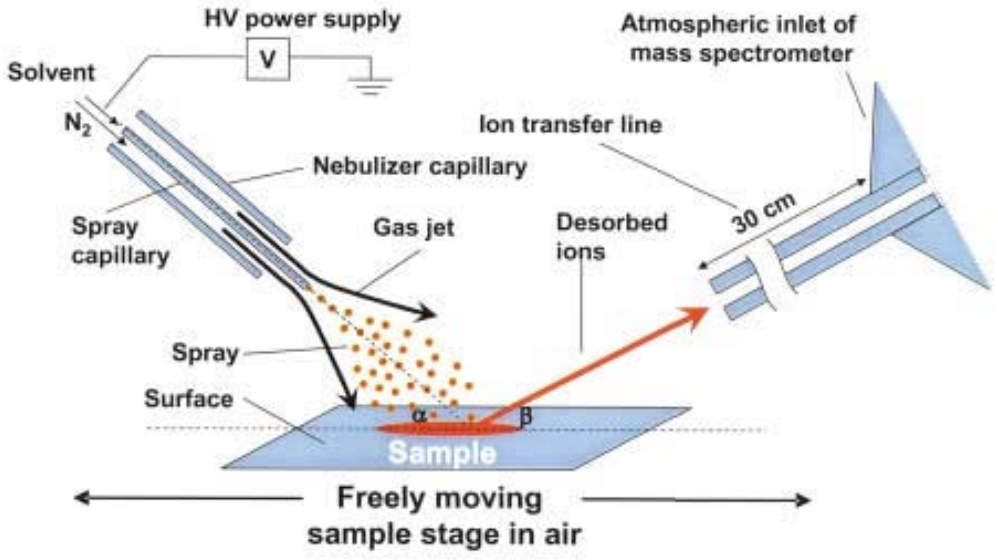


Figure 1-6 Schematic diagram of a typical DESI ion source.[8]

The invention of DESI in 2004 is a revolutionary initiation of ambient MS.[8] As a significant technique in the development of MS, the applications of this novel ionization technique could be summarized as small and large molecules analyses, *in situ* analysis, high-throughput analysis and tissue imaging.[61-63] As shown in Figure 1-6, charged droplets of solvents from an ESI sprayer are directed towards a surface. Analyte molecules on the surface are desorbed and ionized by the charged droplets, and then introduced into the mass spectrometer for analysis. Various compounds can be detected by DESI-MS, and the obtained mass spectra are very similar with those obtained by conventional ESI-MS.

1.3.2 Extractive Electrospray Ionization (EESI)

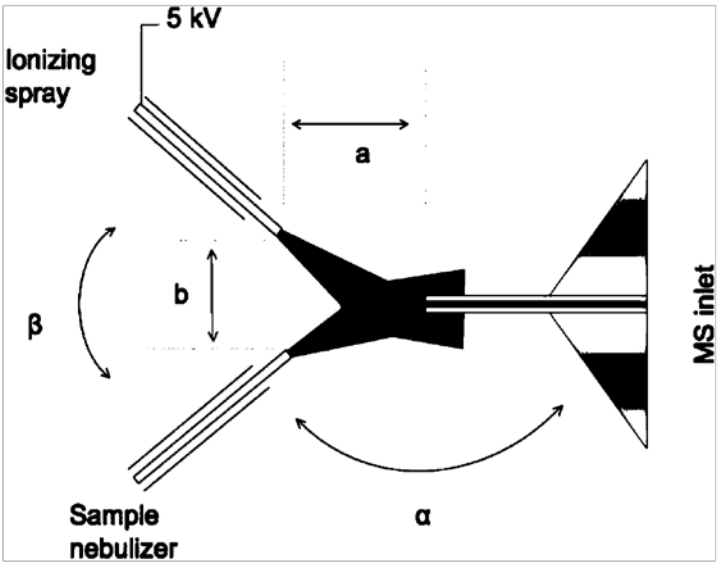


Figure 1-7 Schematic diagram of the EESI ion source.[64]

EESI is a technique based on liquid-liquid extraction between two colliding plumes (Figure 1-7).[51, 64, 65] The colliding plumes come from two separate sprayers, one for nebulizing sample solution and the other for generating charged droplets of solvents. With the interaction of the two plumes, the target analyte molecules are expected to be extracted from the sample solution and ionized by the charged droplets of solvents. This technique has been applied for direct analysis of undiluted urine, milk, native proteins samples and some other complex mixtures.[51, 64, 65] In this thesis, EESI was employed to directly analyze membrane proteins in the presence of detergents. More details about EESI could be found in Chapter 5.

1.3.3 Electrospray Ionization Using Wooden Tips

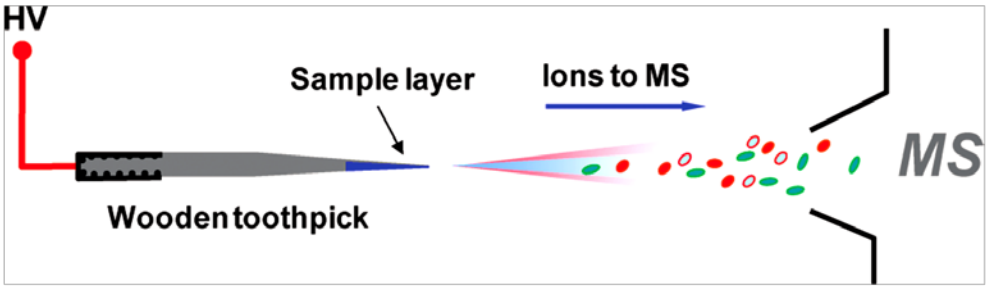


Figure 1-8 Schematic diagram of electrospray ionization using wooden tips.[60]

Electrospray ionization on wooden tips is a novel ionization technique developed by our group.[60] As shown in Figure 1-8, a wooden toothpick, which was very readily available, cheap, and directly compatible with nano-electrospray ion source, was utilized as an electrospray emitter. A high voltage was applied to the wooden toothpick to induce an electrospray for ionization. Sample solution could be directly loaded at the sharp end of the wooden toothpick, which was hydrophilic and porous and could hold the sample solution for electrospray ionization analysis. A variety of samples, including small molecule samples, digested peptides, raw biological liquid samples, powder samples and proteins, were successfully analyzed using this technique. In this thesis, the application of this technique in direct quantitation of raw samples was demonstrated in Chapter 4.

1.3.4 Direct Analysis of Tissue Samples

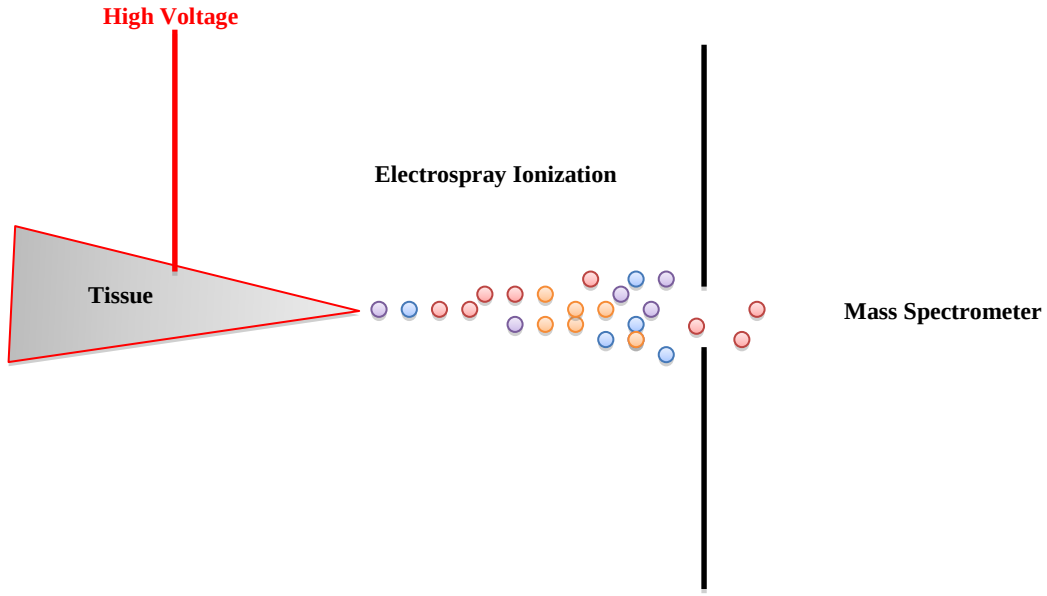


Figure 1-9 Schematic diagram of direct analysis of tissue samples.

Techniques for direct mass spectrometric analysis of biological tissue, including plant tissue[56, 59] and animal tissue,[57, 58] have been developed by our group and other groups in recent years. These techniques have similar setups. As shown in Figure 1-9, a high voltage was directly applied to the tissue sample to induce electrospray ionization at an artificial tip, which was detected by a mass spectrometer. A sharp end, i.e., an artificial or natural tip, is essential for inducing spray ionization in this technique, and only a small piece of tissue is required for analysis.

1.4 Mass Analyzer

Mass analyzer is the main body of a mass spectrometer in which ions are sorted according to their mass-to-charge values. The separation of ions could be achieved in electrical or magnetic field.[1] Commonly used mass analyzers include quadrupole, ToF, ion trap and FTICR. Quadrupole and ToF mass analyzers were employed in the studies of this thesis, and their principles would be briefly introduced.

1.4.1 Quadrupole Mass Analyzer

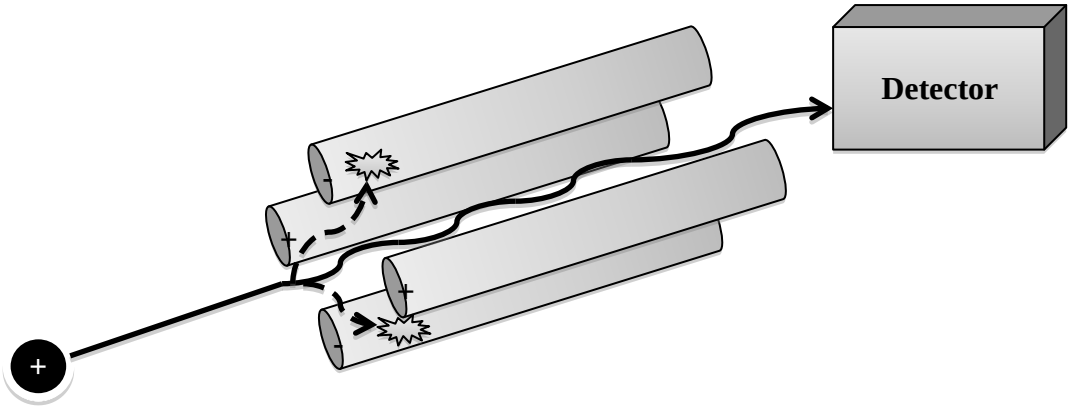


Figure 1-10 Schematic diagram of a quadrupole mass analyzer.

The quadrupole mass analyzer is also called quadrupole mass filter. It consists of four parallel electrical rods. All four rods are connected to direct current (DC) potential and radio frequency (RF) potential (as shown in Figure 1-10).[1] Taking a positively charged ion as an example, after generation in the ion source, it is transferred to the quadrupole

mass analyzer and moved toward the negative rod. Before this positive ion strikes the negative rod, the polarity of the rod is changed to positive due to changes of DC and RF. In this situation, the ion moves forward in the form of oscillation and only “right” ions with a specific *m/z* range can pass through the quadrupole mass analyzer and reach the detector. The “wrong” ions out of the specific *m/z* range collide with the rods in the process of advancing. Eventually, all the ions would be scanned and transmitted to the detector successively with the ramping of DC and RF.

The advantages of the quadrupole mass analyzer include its low cost, easy operation, lower demand for vacuum, and stable signals. However, the major disadvantage of this mass analyzer is its upper limitation of mass range (< 4000 Da). The quadrupole mass analyzer is usually combined with other mass analyzers (including other quadruple mass analyzers) for tandem MS analysis. Triple quadrupole mass spectrometer is widely used for quantitative analysis of target compounds.

1.4.2 Time-of-flight Mass Analyzer

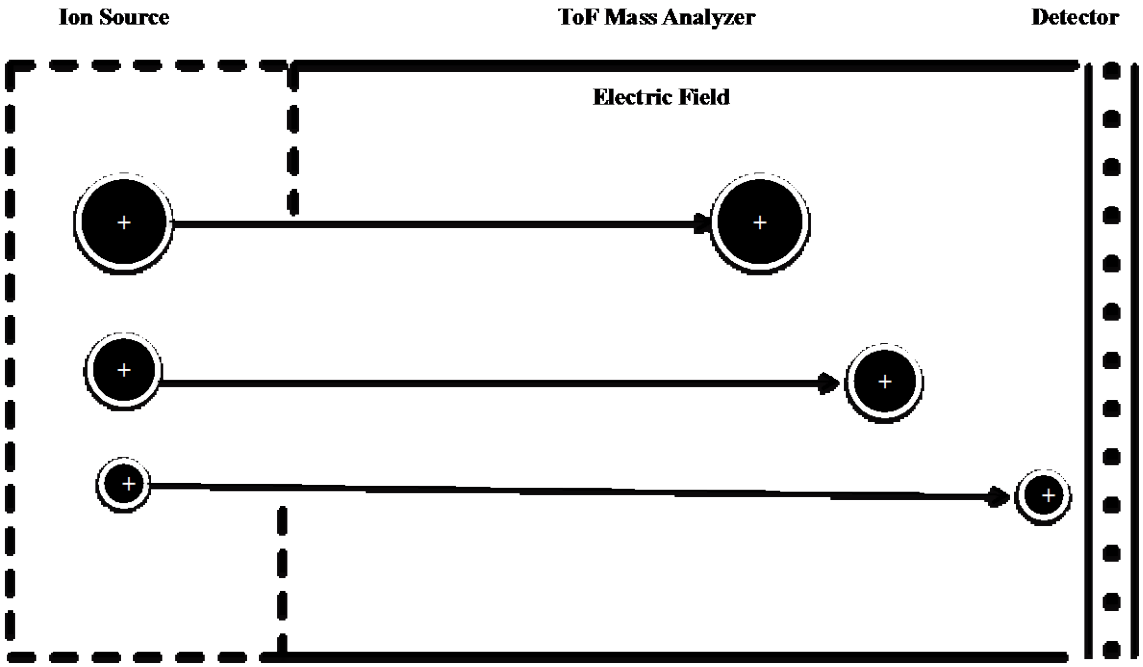


Figure 1-11 Schematic diagram of a ToF mass analyzer.

As shown in Figure 1-11, the principle of a ToF mass analyzer is relatively simple. Ions from the ion source are accelerated with the assistance of an electric filed and directed into a flight tube. Ions with different *m/z* values take different durations to fly through the flight tube to reach the detector. This process can be expressed by the following equation:[66]

$$m/z = t_f^2 2Es/(2s + x) \quad \textbf{(1-7)}$$

where t_f^2 represents the flight time, E represents the applied voltage, s is the length of ion acceleration region, and x is the length of flight tube. This equation can be further simplified to

$$m/z = Kt_f^2 \quad \textbf{(1-8)}$$

where K represents a calibration factor. This equation could clearly illustrate the relationship between *m/z* and the flight time.

The ToF mass analyzer has better resolution than the quadrupole mass analyzer and its *m/z* range is in principle unlimited. However, to satisfy the requirements for free flight of ions in a flight tube, a higher vacuum condition is required. The size of a ToF mass spectrometer is also relatively large. ToF mass spectrometers are more commonly applied for qualitative analysis of samples.[1, 2]

1.5 Tandem Mass Spectrometry (MS/MS)

Tandem mass spectrometry, sometimes called MS/MS, was first described by Fred W. McLafferty in 1981 and is a very useful technique in MS.[67] It is a multiple-stage MS involving fragmentation for analysis of some specific analytes in mixtures. In MS/MS, specific analyte ion is selected by the first mass analyzer, and fragmentation information is obtained in the final mass analyzer after dissociation. This technique has higher sensitivity for analysis of specific compounds. The MS/MS spectra contain structural information of the analytes, and can be used for both qualitative and quantitative

analysis. Currently, MS/MS is widely applied in proteomics, e.g., peptide sequencing for protein identification.[29, 68, 69]

1.5.1 Tandem Mass Spectrometry Classification

Multiple-stage MS/MS can be performed in space or in time. In space, several mass analyzers are connected in series to achieve precursor ion selection, dissociation and subsequent mass measuring of fragment ions (Figure 1-12).[70-72] The quadrupole and ToF analyzers are the most widely used mass analyzers in MS/MS in space. The triple quadrupoles (QqQ) and quadrupole ToF (QToF) analyzers are commonly used tandem mass spectrometers.

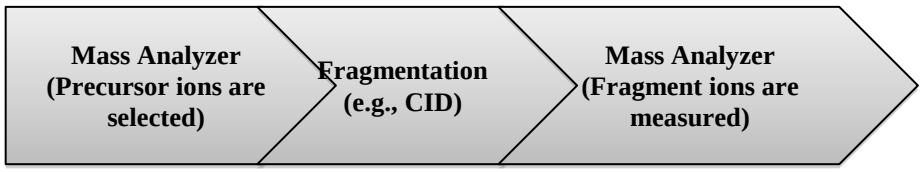


Figure 1-12 Schematic diagram of MS/MS in space.

MS/MS in time means only one space or mass analyzer is employed and the trapped ions are selected and then fragmented successively in the same space (Figure 1-13).[73] Ion trap and FTICR are the common mass analyzers for MS/MS in time. The major advantage of MS/MS in time is that it can perform MS^n analysis. This technique is very useful for structural elucidation.

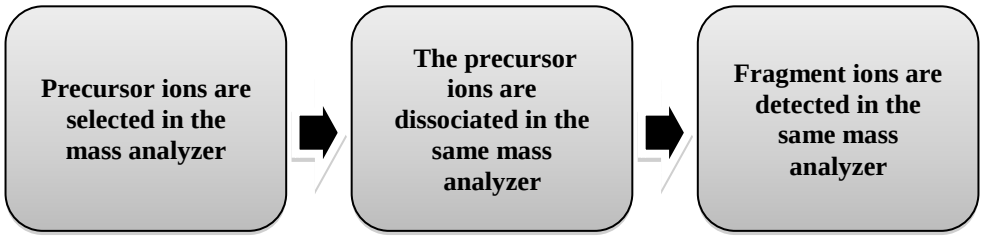


Figure 1-13 Schematic diagram of MS/MS in time.

1.5.2 Fragmentation Methods

Collision-induced dissociation (CID), also called collision-activated dissociation (CAD), is the most popular fragmentation technique.[74, 75] In the process of CID, precursor ions are directed to collide with a neutral gas (e.g. helium or argon) in the collision cell. This collision results in subsequent dissociation of precursor ions due to energy transfer in this process. This process can be described as follows:

$$\boldsymbol{ABH^+ \xrightarrow{Collision\ gas} AH^+ + B\ or\ A + BH^+} \qquad \textbf{(1-9)}$$

where A and B represent two components of the analyte, and H represents proton. Analyte ion ABH^+ collides with collision gas and leads to the fragmentation. According to the energy used for the fragmentation, CID can be divided into low energy (< 100 eV) and high energy CID (> 100 eV).[1] Low energy CID is the commonly used one in QqQ and Q-ToF tandem mass spectrometers. High energy CID can be achieved by some mass spectrometers with high accelerating potentials. It was reported that charge-driven dissociation was predominant in low energy CID. High energy CID can produce

additional ions induced by multiple cleavage, complex charge-driven rearrangement and charge-remote fragmentation.[76]

Apart from CID, there are some other fragmentation methods, e.g., electron capture dissociation (ECD)[77] and electron transfer dissociation (ETD).[78-80] Both ECD and ETD are primarily applied in FTICR mass spectrometers for proteomic analysis.[81, 82] The process of ECD can be represented by the following equation:[83, 84]

$$[\boldsymbol{A + nH}]^{\boldsymbol{n+}} + \boldsymbol{e^-} \rightarrow [[\boldsymbol{A + nH}]^{\boldsymbol{(n-1)+}}]^{\cdot} \rightarrow \boldsymbol{Fragments} \qquad \textbf{(1-10)}$$

A multiply charged cation $[A + nH]^{n+}$ interacts with a free electron e^- to become an odd-electron ion, which is dissociated to form fragment ions. Similar to ECD, ETD induces fragmentation of a cation by transferring an electron to the cation. But in ETD, the electron comes from an anion X^-. ETD can be represented by the following equation:[85]

$$[\boldsymbol{A + nH}]^{\boldsymbol{n+}} + \boldsymbol{X^-} \rightarrow [[\boldsymbol{A + nH}]^{\boldsymbol{(n-1)+}}]^{\cdot} + \boldsymbol{X} \rightarrow \boldsymbol{Fragments} \qquad \textbf{(1-11)}$$

Both ECD and ETD are very useful fragmentation techniques for peptide sequencing and top-down protein identification in proteomic research.

1.5.3 Modes of Tandem Mass Spectrometry

Currently, there are four common MS/MS modes: product ion scan, precursor ion scan, neutral loss scan and selected reaction monitoring (SRM, also known as multiple

reaction monitoring, MRM). These MS/MS modes, except for product ion scan, normally can only be performed in triple quadrupoles mass spectrometers.[86] The schematic diagrams and the symbols[87] for these common MS/MS modes are shown in Figure 1-14.

Product ion scan

Specific precursor ions are selected in the first mass analyzer, and after fragmentation in the second stage, fragment ions are scanned and detected in the final mass analyzer. This technique is for detecting the product ions produced from selected precursor ions and is very useful for structural elucidation and identification of compounds.

Precursor ion scan

Precursor ions are scanned in the first mass analyzer, and after fragmentation in the collision cell, only selected product ions are detected in the final mass analyzer. This scan mode is for detecting precursor ions that can produce the selected product ions.

Neutral loss scan

All the precursor ions are scanned in the first mass analyzer, and after fragmentation in the second stage, only ions corresponding to a neutral loss of the precursor ions are detected in the final mass analyzer. This scan mode is employed to detect a class of compounds that can have the same neutral loss.

Selected reaction monitoring

Specific precursor ions are selected in the first mass analyzer, and after fragmentation in the second stage, only specific fragment ions are detected in the final mass analyzer. This method is very specific and sensitive for quantitation of target analytes in a mixture.

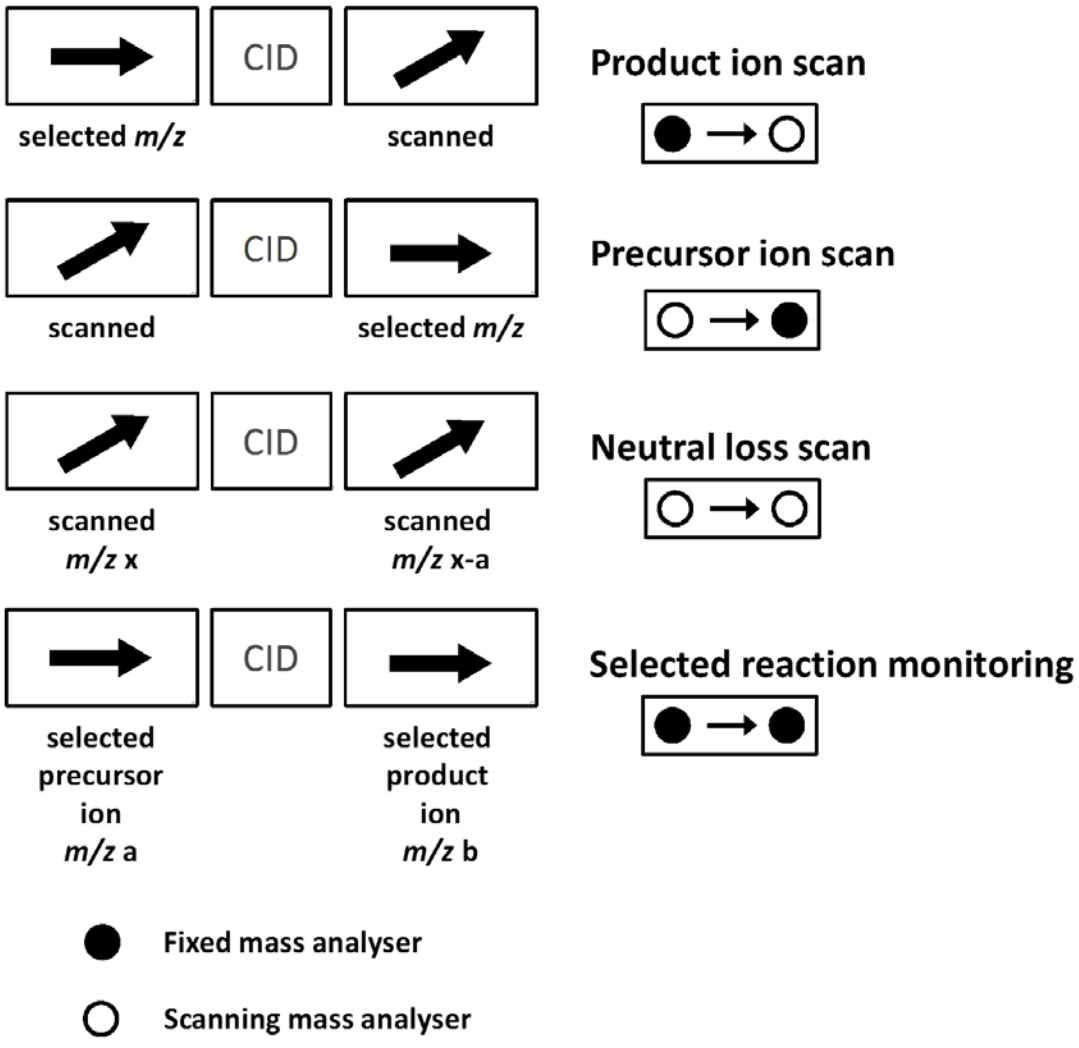


Figure 1-14 MS/MS scan modes.[86]

1.5.4 Tandem Mass Spectrometer

1.5.4.1 Triple Quadrupole Mass Spectrometer

Triple quadrupole mass spectrometer is a commonly used tandem mass spectrometer. The first triple quadrupole mass spectrometer (abbreviated as QqQ) was introduced in 1978.[88] As mentioned previously, triple quadrupole mass spectrometer is normally used for qualitative analysis with product ion scan mode and for quantitative analysis with SRM mode.[86] In this thesis, a triple quadrupole mass spectrometer (Quattro Ultima, Waters) was employed for quantitation analysis. The schematic diagram of such a triple quadrupole mass spectrometer is shown in Figure 1-15**.**

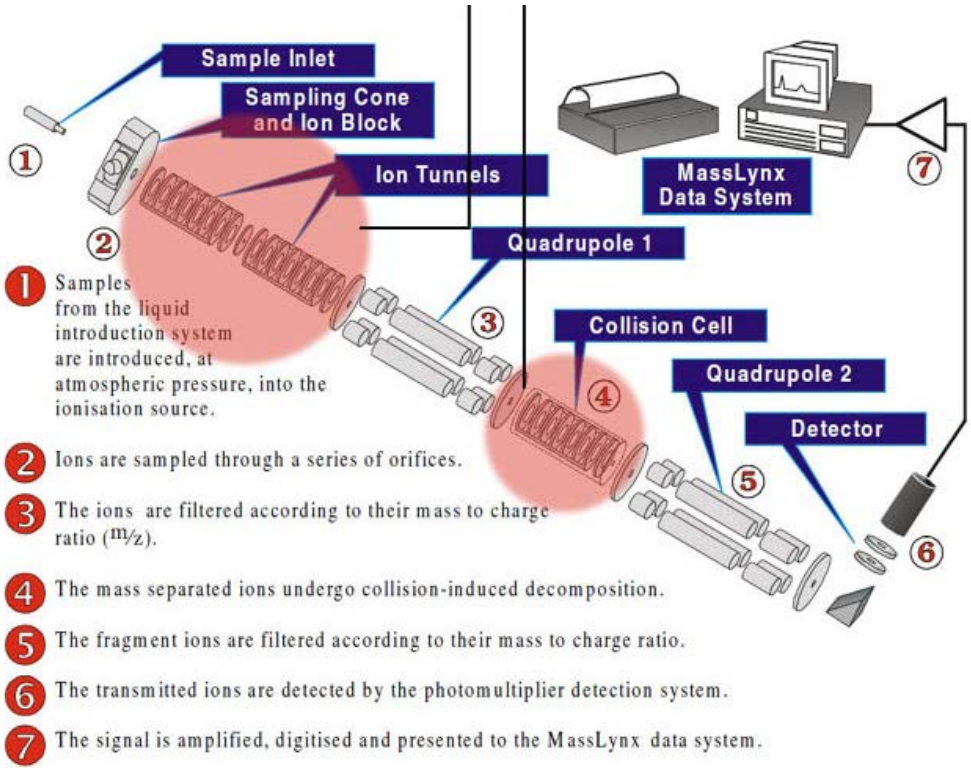


Figure 1-15 Schematic diagram of a triple quadrupole mass spectrometer.[89]

In the SRM mode for quantitation with this triple quadrupole mass spectrometer, sample is ionized at the ion source part (conventional ESI ion source or our self-assembled ion source). Then the sample ions are transported to the first quadrupole for selection of specific precursor ions, then the selected precursor ions are transmitted into the collision cell for fragmentation. Finally, only specific fragment ions are selected to pass through the final quadrupole and detected, and corresponding SRM chromatogram could be obtained for quantitative analysis.

1.5.4.2 Quadrupole Time-of-flight (Q-ToF) Mass Spectrometer

Q-ToF mass spectrometer was introduced in 1996,[90] and is mainly used for qualitative analysis. This mass spectrometer combines a quadrupole mass filter with a ToF analyzer. With this hybrid tandem mass spectrometer, informative mass spectra with high resolution could be obtained. A quadrupole time-of-flight (Q-ToF) tandem mass spectrometer (API QStar Pulsar, PE Sciex, Canada) was employed for qualitative analysis in this thesis.

Figure 1-16 is the schematic diagram of the API QStar Pulsar mass spectrometer. Sample is ionized in the ion source part and the ions are transmitted to quadrupole analyzer RQ1, then collision-induced dissociation is carried out in quadrupole RQ2. The fragment ions are directed to fly through the flight tube in the ToF mass analyzer and are detected. Use of ToF mass analyzer enables Q-ToF to have higher resolution than the triple quadrupole mass spectrometer.

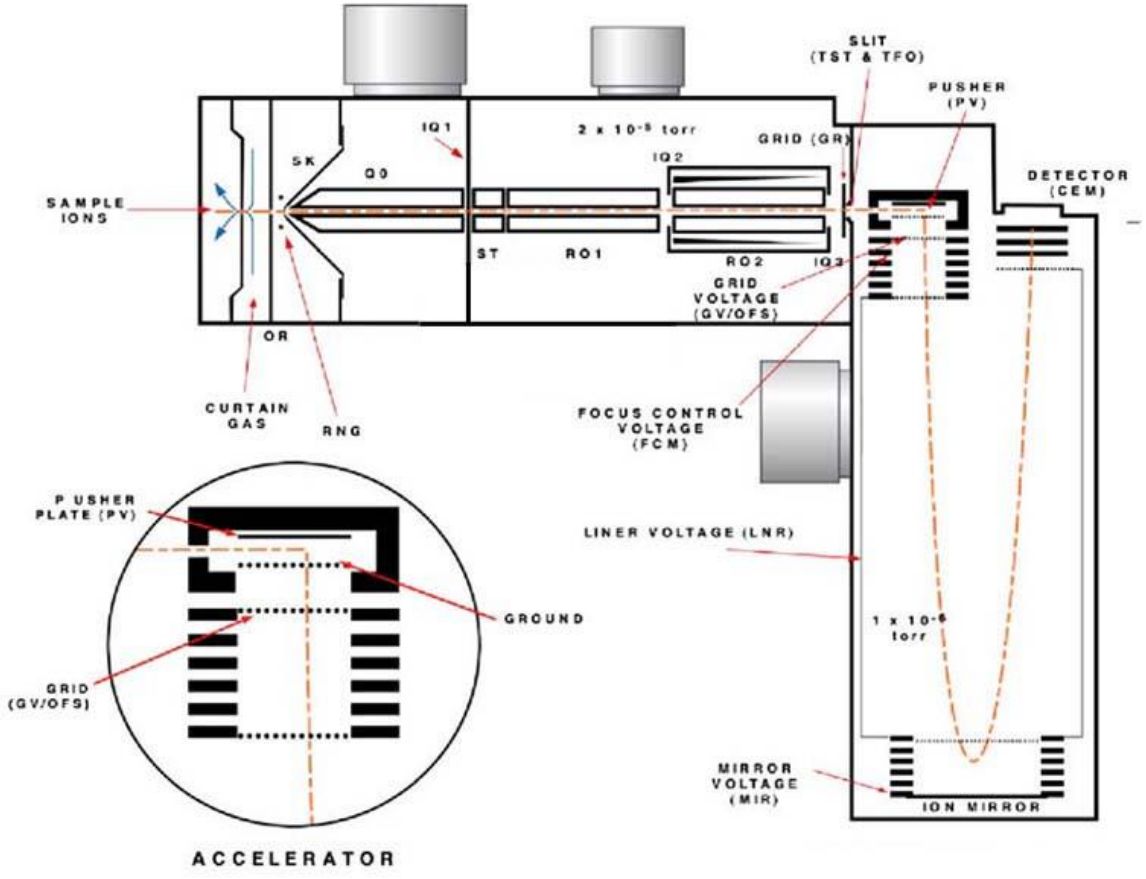


Figure 1-16 Schematic diagram of an API QStar Pulsar Q-ToF tandem mass spectrometer.[91]

1.6 Outline of This Thesis

The research in this study includes two major parts. The first part is about development of new ionization techniques for direct analysis of complex samples by ESI-MS. The second part is about investigation of MALDI ionization process using the OASP method.

1.6.1 Development of New Ionization Techniques for Direct Analysis of Complex Samples by ESI-MS

Following the development of electrospray ionization using wooden tip,[60] direct ionization of biological tissue[57] and EESI,[64] these techniques were further developed and applied for qualitative and quantitative analysis in this study. The related work in this thesis includes:

Chapter 2: a pipette-tip electrospray ionization mass spectrometry was designed and utilized for direct analysis of herbal powders under ambient conditions. The quantitative analysis of chemical components in powders and rapid differentiation of closely related herbal species were investigated as well.

Chapter 3: a C18 pipette-tip electrospray ionization mass spectrometry was described and applied for direct analysis of raw protein samples and quantitative analysis of abused drugs in human urine.

Chapter 4: wooden-tip ESI-MS was applied for rapid quantitation of di-(2-ethylhexyl)-phthalate (DEHP) in beverages.

Chapter 5: EESI was developed for direct analysis of membrane proteins containing detergents

1.6.2 An Investigation of MALDI Ionization Mechanism

Chapter 6: Use of OASP sample preparation method for investigation of MALDI ionization mechanism revealed exchange of active hydrogens on analytes with the hydrogens from matrix.

Chapter 2 Direct Analysis of Herbal Powders by Pipette-tip Electrospray Ionization Mass Spectrometry

2.1 Introduction

Plants are important natural resources for foods and herbal medicines. Analysis of chemical compositions and bioactive components of plants is an essential part of analytical chemistry and natural product research. Such analysis typically involves various experimental steps including extraction, separation and characterization, which can be time-consuming and labor-intensive. Mass spectrometry (MS) is a powerful tool for analysis of various samples. In recent years, great efforts have been made to allow direct analysis of samples, including plant samples, by mass spectrometry. Direct analysis of plant tissue by MS can now be achieved by using techniques such as secondary ion mass spectrometry (SIMS), matrix-assisted laser desorption/ionization (MALDI), desorption electrospray ionization (DESI) and electrospray-assisted laser desorption/ionization (ELDI, or laser ablation electrospray ionization LAESI).[9, 61, 92-94] These techniques allow *in situ* profiling of phytochemicals on surfaces of plant tissue, and the latter two are ambient ionization techniques that can be performed under ambient pressure and require little or no sample preparation.[4-6, 48, 49] Recently, direct ionization techniques were developed for analysis of plant and animal tissues.[56, 57, 59, 95] These techniques involved connection of a high voltage to the tissue sample, and with application of solvents if necessary, spray ionization could be induced from the tip of the tissue sample and a mass spectrum regarding phytochemicals of the sample could be obtained.

Herbal powders are commonly present as food, supplementary products, medicines, etc. For example, many household products such as tea, coffee and pepper are usually sold

and used in powder forms, and many herbal medicines are prepared in powder forms for convenient uses. Development of a simple and rapid method for analysis of these herbal powders is thus highly desirable for their quality control and food safety. However, there have been few studies about direct analysis of herbal powders by mass spectrometry.[60, 96, 97] In our group, attempts have been made to use solid-substrate electrospray ionization mass spectrometry (solid-substrate ESI-MS),[60, 95] e.g., wooden-tip ESI-MS, for direct analysis of herbal powders. Although ion signals with desired intensity could be readily obtained, the reproducibility and duration of signals needed to be further improved, particularly for comparison of different species and for quantitation purpose. In this study, by combining common and disposable pipette tips with syringe pump and ESI-MS (termed pipette-tip ESI-MS herein), we demonstrated that herbal powders could be readily analyzed and stable and continuous signals could be obtained. The method is simple, rapid, only requires a small amount of powder samples, and allows quantitative measurements of chemical components in the samples.

2.2 Experimental Section

2.2.1 Materials and Chemicals

Fructus Schisandrae Chinensis (FSC) and *Fructus Schisandrae Sphenantherae* (FSS) samples were purchased from licensed pharmacy stories in Hong Kong and mainland China and further confirmed by an experienced researcher. Other herbal medicine samples were purchased from licensed pharmacy stores in Hong Kong. Tea samples (green tea, black tea, Pu Erh tea, Iron Buddha tea and jasmine tea) and other herbal food samples were purchased from local supermarkets. Water was distilled water prepared

using a Milli-Q system (Millipore, USA). All the other solvents were of HPLC grade and purchased from Tedia (Fairfeild, USA). Caffeine was purchased from Sigma-Aldrich.

2.2.2 Preparation of Samples and Standard Solutions

The herbal samples used for the analysis in this study were either directly obtained in powder forms or homogenized to powders. The particle sizes of the analyzed samples were typically smaller than 40 mesh. For qualitative analysis, approximate 1 mg of sample powder was filled into a pipette tip for direct analysis.

For direct quantitation of caffeine in the five tea samples using pipette-tip ESI-MS/MS, standard addition method was applied. Caffeine standard solutions used in the standard addition method were prepared at a concentration of 4.00% (w/v) first and then diluted to concentrations of 1.00% and 2.00% with the same solvent (methanol/water, 80/20, v/v). The accurately measured 1 mg of tea powder sample was filled into a pipette-tip and spiked with 1 μl of standard caffeine solutions (0, 1.00%, 2.00% and 4.00%). These moist powder samples with spiked caffeine were dried at 60°C for 5 min and cooled to room temperature for MS analysis.

For quantitation of caffeine in tea samples with ultra performance liquid chromatography ESI-MS/MS (UPLC-ESI-MS/MS), caffeine standard solutions (2, 4, 6, 12, 24, 48 ng/ml) for construction of calibration curve (peak area *versus* caffeine

concentration) were prepared by diluting a 4800 ng/ml caffeine stock solution with methanol/water (50/50, v/v). Each tea sample solution was prepared as follows: 100 mg of accurately weighed tea powder was extracted with 5.00 ml 50/50 (v/v) methanol/water and sonicated for 30 min at room temperature, and then filtered through filter paper. This procedure was repeated twice, and then the filtrate was combined and centrifuged at 4000 rpm for 10 min using a C-28A centrifuge (BOECO, Germany). The supernatant was diluted to exactly 20.00 ml and 150 µl of this solution was further diluted to 1.00 ml, both with methanol/water (50/50, v/v). The final solution was filtered through a 0.20 µm hydrophilic PTFE syringe filter (Millex-LG, USA). All solutions were stored at -20 °C prior to MS analysis.

2.2.3 Instrumentation and Setup

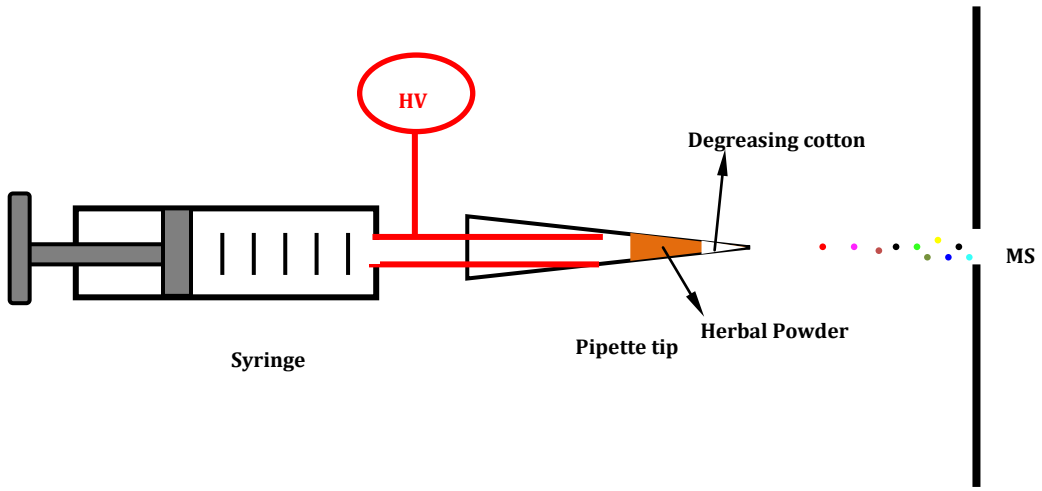


Figure 2-1 A schematic diagram of pipette-tip ESI-MS setup.

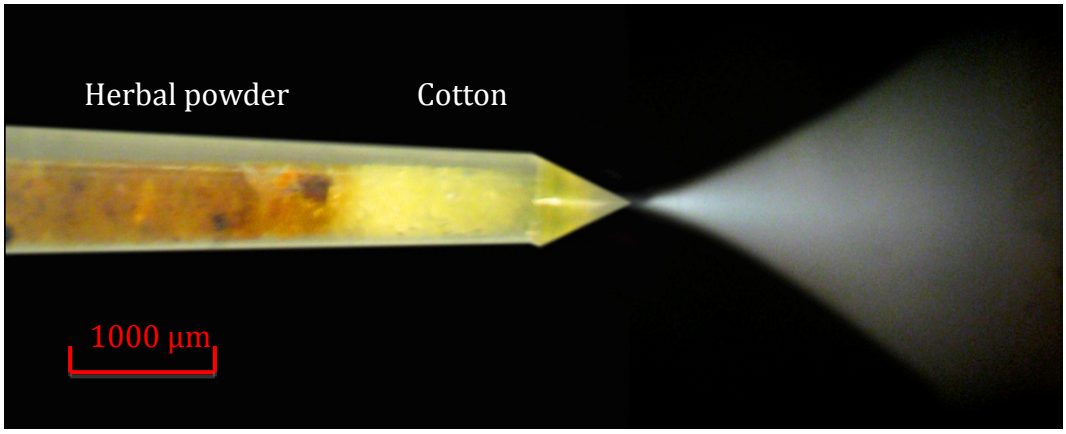


Figure 2-2 A photo of electrospray generated by pepitte-tip ESI-MS in the analysis of the *Rhizome coptidis* powder.

The designed pipette-tip ESI ion source was assembled and installed as shown in Figure 2-1. The blunt point needle (i.d. 410 µm and o.d. 720 µm) of a glass syringe (250 µl, Hamilton) was inserted into the pipette tip (0.1-10 µl, Sorenson™) to form a ~2 µl sealed space which could be filled with a variety of sample powders. A very small degreasing cotton swab was normally placed in front of samples to retain the powders. Methanol/water/formic acid (50/50/0.1, v/v/v) was used as the extraction and spraying solvent if not specified elsewhere. A syringe pump (Harvard Pump 11 Plus) was employed to supply solvents with adjustable flow rates. A high voltage (typically 5.5 kV) was applied to the stainless steel syringe needle and conducted to the pipette tip end through the solvent to induce electrospray ionization (Figure 2-2). Mass spectrometric measurements were performed in positive ion mode unless specified.

For qualitative analysis, the pipette-tip ESI ion source was coupled with a quadrupole time-of-flight (Q-TOF) mass spectrometer (QStar Pulsar, Applied Biosystems, USA). The mass spectrometer was operated with a curtain gas flow of 30 A. U., and the spray voltage (IS), first declustering potential (DP1), focusing potential (FP) and second declustering potential (DP2) were set to optimum values in both positive and negative ion modes. For differentiation of herbal samples, spectra from the first 60 scans (1 min) were accumulated for principal component analysis (PCA) using SPSS software (version 18.0, SPSS Inc., USA).

For direct quantitation of caffeine contents in the tea samples, the pipette-tip ESI ion source was coupled with a triple quadrupole mass spectrometer (Quattro Ultima, Waters) in the selected reaction monitoring (SRM) mode. The capillary voltage, cone voltage and source temperature were set at 4.0 kV, 30 V and 150 °C respectively. The selected reaction *m/z* 195 → *m/z* 138 was monitored with a collision energy of 19 eV and a dwelling time of 0.5 s for quantitation, while another selected reaction *m/z* 195 → *m/z* 110 was monitored with a collision energy of 21 eV and the same dwelling time for further confirmation. The inter-channel delay time and inter-scan delay time were set at 0.02 s and 0.1 s respectively. MRM spectra of the first 120 scans (2 min) was accumulated and processed with MassLynx^{TM} V4.0 software (Waters, USA).

Conventional quantitation of caffeine contents in the tea samples was performed on a triple quadrupole mass spectrometer, which was equipped with a routine ESI ion source and an UPLC system (ACQUITY UPLC, Waters, USA) and operated in SRM mode. A

Waters ACQUITY UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm,) was used for chromatographic separation. The autosampler temperature was set at 10 °C and the injection volume was 2 μl. Milli-Q water containing 0.1% formic acid was used as mobile phase A and acetonitrile containing 0.1% formic acid was used as mobile phase B. The mobile phase flow rate was 0.40 ml/min. The caffeine analyte was eluted with a solvent gradient as follows: 0-12 min 5-26% mobile phase B; 12-14 min 26-65% mobile phase B; 14-19 min 65-5% mobile phase B; 19-20 min 5% mobile phase B.

2.3 Results and discussion

2.3.1 General Properties of Pipette-tip ESI-MS

Figure 2-3a is the total ion current (TIC) chromatogram obtained by analysis of the powder of *Rhizome coptidis*, a common herbal medicine, using pipette-tip ESI-MS. With a flow rate of 3 μl/min for the extraction and spray solvent (methanol/water 50/50, v/v), the signals from 0.5 mg *Rhizome coptidis* powder could last more than 60 minutes. The total ion intensity was relatively stable in the first 10 minutes and gradually declined as the time went on. The obtained mass spectrum was predominated by three species: coptisine at *m/z* 320.1, berberine or epiberberine at *m/z* 336.1, and plamatine at *m/z* 352.2 (Figure 2-3b). The mass spectrum obtained by pipette-tip ESI-MS here was very similar to that obtained by direct ionization analysis of the same herbal species,[57] but the signals obtained by pipette-tip ESI-MS were much more stable and durable.

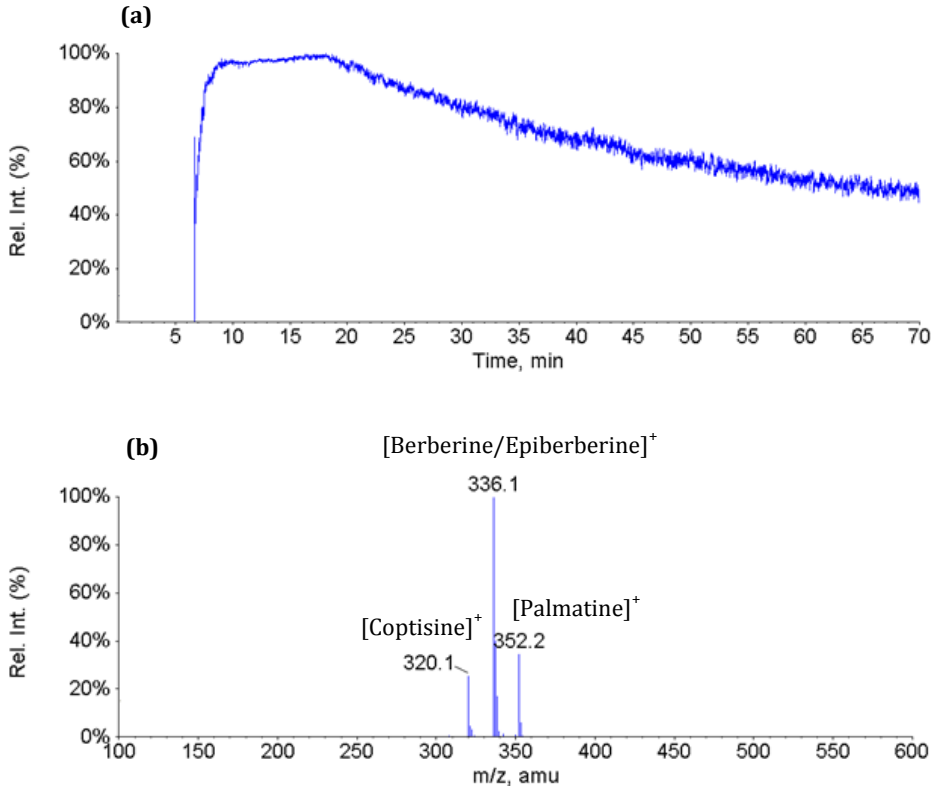


Figure 2-3 Pipette-tip ESI-MS analysis of *Rhizome coptidis*: **(a)** Total ion current; **(b)** Mass spectrum obtained by accumulation of spectra between 55-60 min.

Various solvents, including hydrophilic and hydrophobic organic solvents such as methanol, ethanol, isopropanol, acetonitrile, acetone, hexane, methylene dichloride, chloroform and their mixtures with different proportions of water, were tested as the extraction and spray solvents for pipette-tip ESI-MS. It was found that the acquired spectra were predominated by the same alkaloids for the *Rhizome coptidis* powder with all the solvents tested. The expectation that different extraction and ionization efficiency achieved by solvents of different polarities was not observed. This result might be due to the high ionization efficiency of the alkaloid components in the sample. The limited effects of extraction and spray solvents observed in this study are consistent with

previous results obtained with direct ionization analysis of tissue samples.[57, 59] For pipette-tip ESI-MS analysis of herbal powders, methanol/water/formic acid (50/50/0.1, v/v/v), a common ESI solvent system, was chosen as the extraction and ionization solvent in this study unless specified elsewhere.

The effects of other parameters of the pipette-tip ESI ion source were investigated and the settings were optimized. The distance between the pipette-tip end and the MS inlet was determined to be around 2 cm for optimum signal intensities and to avoid contamination to the MS inlet. Regarding the diameter of the pipette-tip end, it was found that at a fixed high voltage, smaller pipette-tip ends resulted in stronger mass spectral signals. For convenient applications, common 0.1-10 µl pipette tips having an i.d. of ~550 µm and an o.d. of ~ 700 µm at the tip end, which are compatible with the needle of common 250 µl syringe, were directly used without further modification.

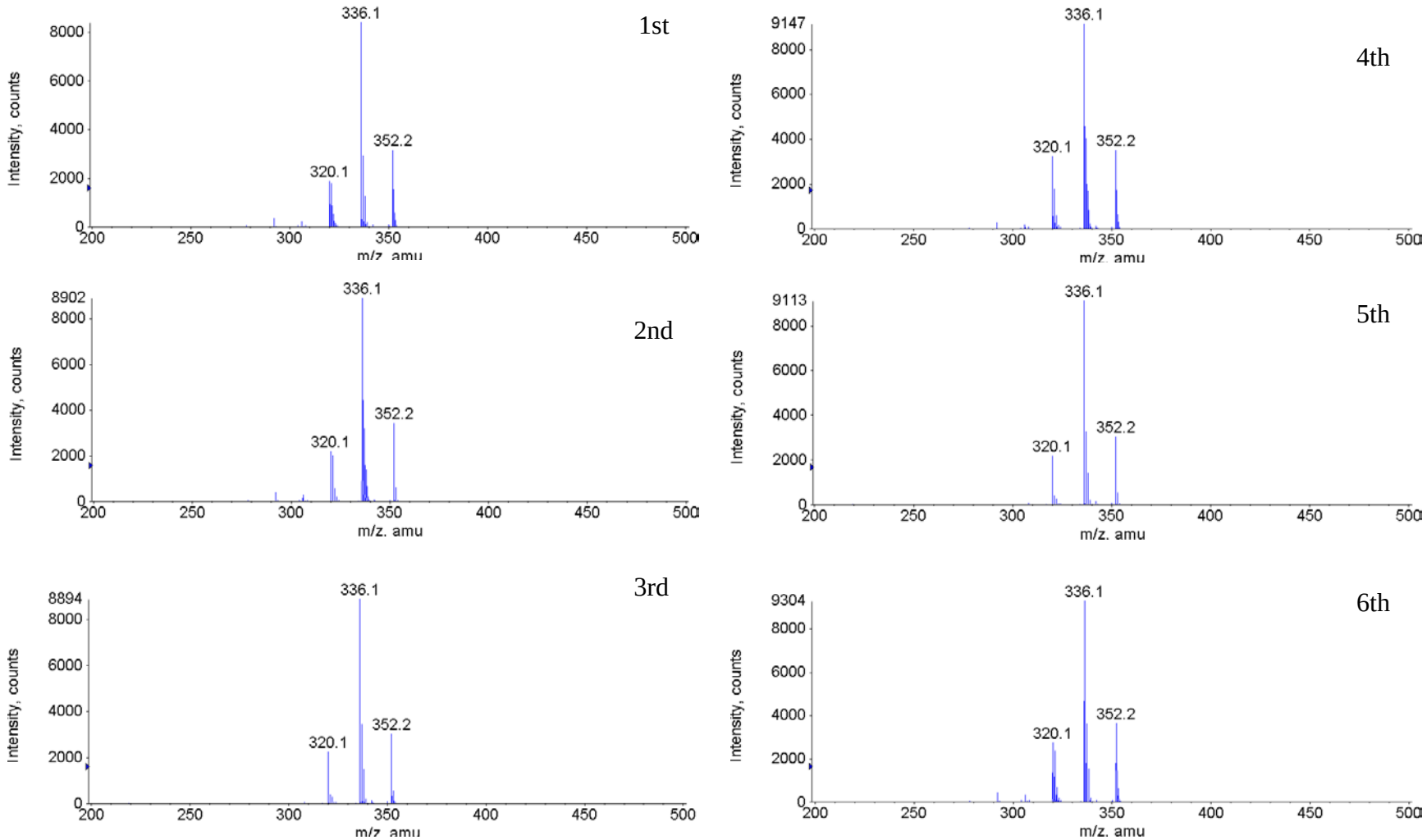


Figure 2-4 Mass spectra obtained by pipette-tip ESI-MS analysis of the *Rhizome coptidis* powder using six individual pipette tips.

Electrospray ionization from disposable pipette tips has been reported previously.[55, 98] However, those designs typically involved insertion of a platinum wire to connect the high voltage to the tip end. Our present technique makes use of the stainless steel syringe needle for delivery of the high voltage, which further reaches the pipette-tip end through the solvents. Solvents have been demonstrated to be good media for delivery of high voltage to induce spray ionization.[99, 100] Pipette tips used in this study have a conical shape and tip end with inner diameter (i.d. 550 μm) smaller than the outer diameter (o.d. 720 μm) of the syringe needle, thus allowing a space between the two tip ends for accommodation of powder samples for analysis. Solvents from the syringe pass through the powder samples, extract the chemicals from the powders, spray out of the pipette tip with application of a high voltage (see Figure 2-2 for a photo of such electrospray), and mass spectra are obtained for the samples. The current setup is simple and easily achievable in common laboratories. The syringe pump allows controllable and reproducible flow of solvents for extraction and ionization and thus acquisition of reproducible spectra. As shown in Figure 2-4, good reproducibility was observed for spectra obtained by analysis of 1.0 mg *Rhizome coptidis* powder with six individual pipette tips at a solvent flow rate of 3 μl/min.

2.3.2 Qualitative Analysis of Various Herbal Powders

Coffee

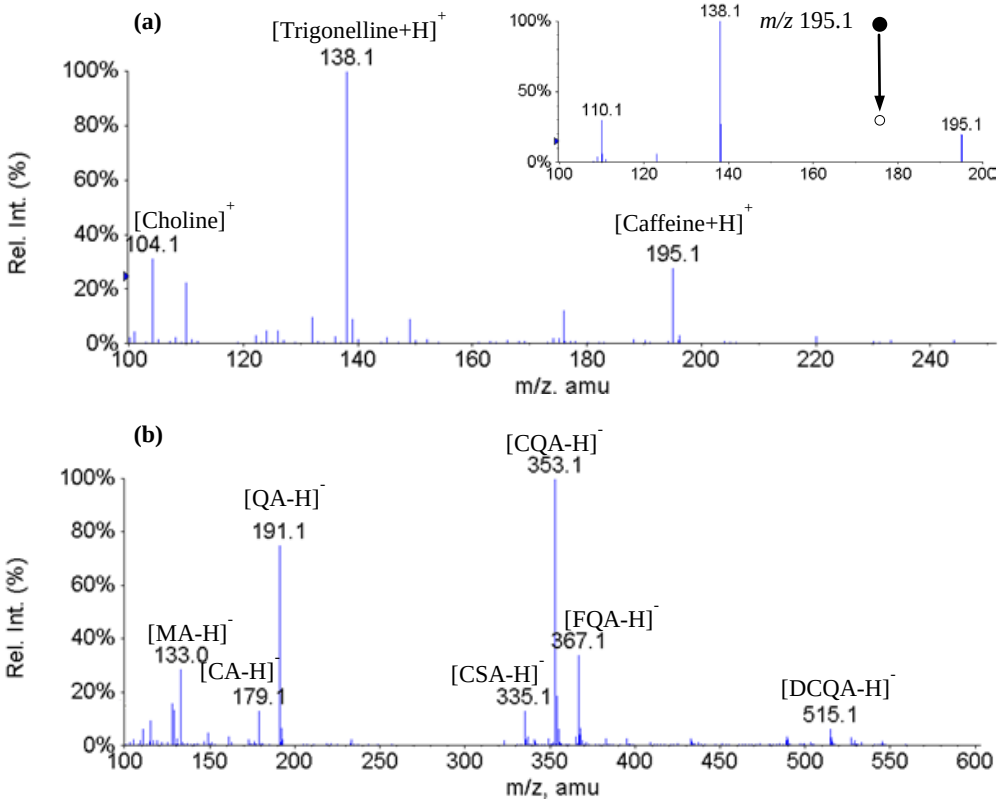


Figure 2-5 Mass spectra obtained by pipette-tip ESI-MS analysis of roasted coffee beans: **(a)** Positive ion mode; **(b)** Negative ion mode. (MA: Malic acid; QA: Quinic acid; CA: Caffeic acid; CSA: Caffeoylshikimic acid; CQA: Caffeoylquinic acid; FQA: Feruloyquinic acid; DCQA: Dicaffeoylquinic acid.)

Coffee is generally the powder of roasted coffee beans from plant *Coffea*, and is one of the most popular brewed beverages in the world due to its wake-up efficacy and special taste. The bitter taste and wake-up consequence of coffee comes from its stimulant compound, caffeine, while the sour taste comes from its acidic compounds.[101-103] A coffee sample was analyzed by pipette-tip ESI-MS with both positive and negative ion modes in this study, and the spectra are shown in Figure 2-5. In positive ion mode, two alkaloids, trigonelline (*m/z* 138.1) and caffeine (*m/z* 195.1),[104] were predominantly detected in the spectrum (Figure 2-5a). In negative ion mode, five phenolic acids: caffeic acid (*m/z* 179.1), caffeoylshikimic acid (*m/z* 335.1), caffeoylquinic acid (*m/z* 353.1), feruloylquinic acid (*m/z* 367.1) and dicaffeoylquinic acid (*m/z* 515.1), and malic acid (*m/z* 133.0) and quinic acid (*m/z* 191.1), two organic acids widely existed in fruits [105], were detected in their deprotonated forms (Figure 2-5b). All of these phytochemicals were further identified by their MS/MS mass spectra. Comparing with the mass spectra obtained by analysis of coffee extracts with conventional ESI-MS,[106] the positive ion spectrum obtained by pipette-tip ESI-MS was simpler and contained fewer peaks, most probably due to the difference in extraction conditions.

Lotus plumule

Lotus plumule is the core of the seed of *Nelumbo nucifera* Gaertn. It serves as a traditional herbal medicine to calm the nervous system.[107] Lotus plumule is commonly characterized and evaluated by alkaloids, its main bioactive components.[108] The doubly and singly charged proton adducts of isoliensinine at *m/z* 306.2 and *m/z* 611.3 respectively, together with the doubly and singly charged proton adducts of neferine at

m/z 313.2 and *m/z* 625.3 respectively, dominated the spectrum obtained by pipette-tip ESI-MS analysis of the lotus plumule powder (Figure 2-6a). These alkaloids were identified by their MS/MS spectra (Figure 2-6b and 2-6c) and also observed by LC-ESI-MS analysis of the lotus plumule extract in a previous study.[109]

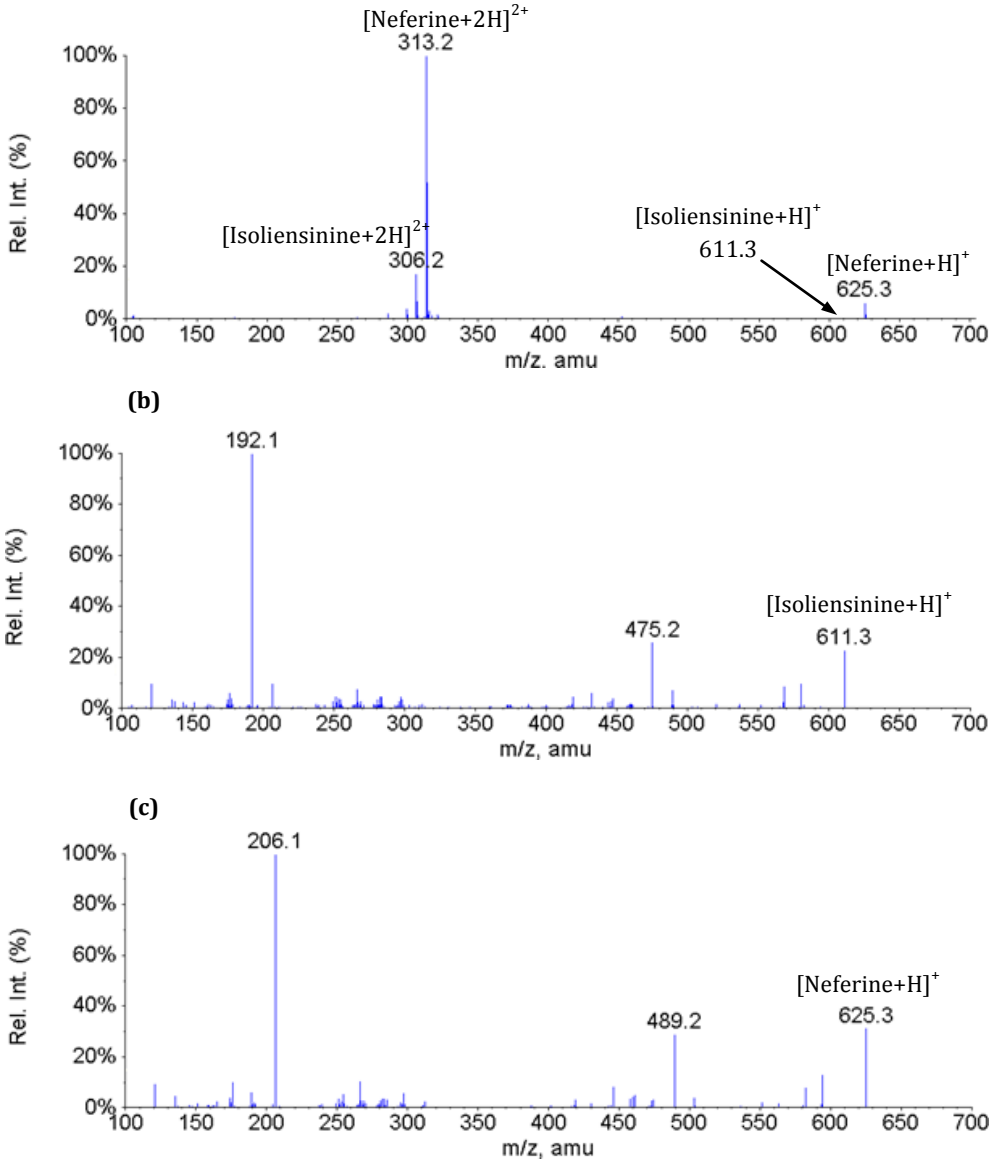


Figure 2-6 (a) Mass spectra obtained by pipette-tip ESI-MS analysis of lotus plumule; **(b)** MS/MS spectrum of *m/z* 611.3; **(c)** MS/MS spectrum of *m/z* 625.3.

Great burdock achene

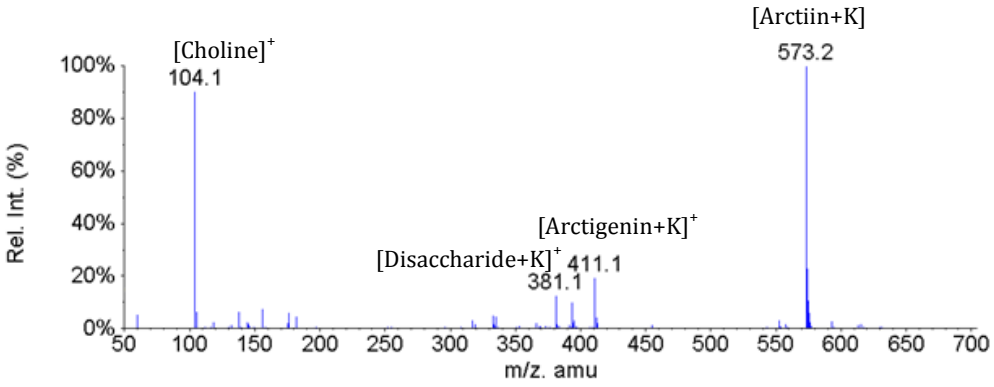


Figure 2-7 Mass spectra obtained by pipette-tip ESI-MS analysis of great burdock achene.

Great burdock achene is the ripe seed of *Arctium lappa* Lim and is also a common herbal medicine. The spectrum obtained for the powder of this sample was predominated with potassium adducts of glycoside arctiin (*m/z* 573.2) and aglycone arctigenin (*m/z* 411.1) (Figure 2-7), which were previously detected by LC-ESI-MS/MS [110] and GC-MS.[111] Both arctiin and arctigenin were considered to be very valuable phytochemicals because of their anti-cancer and other bioactivities.[112, 113] Choline (*m/z* 104.1) and disaccharide (*m/z* 381.1), two common constituents in plants, were also observed in the acquired spectrum (Figure 2-7).

Black pepper

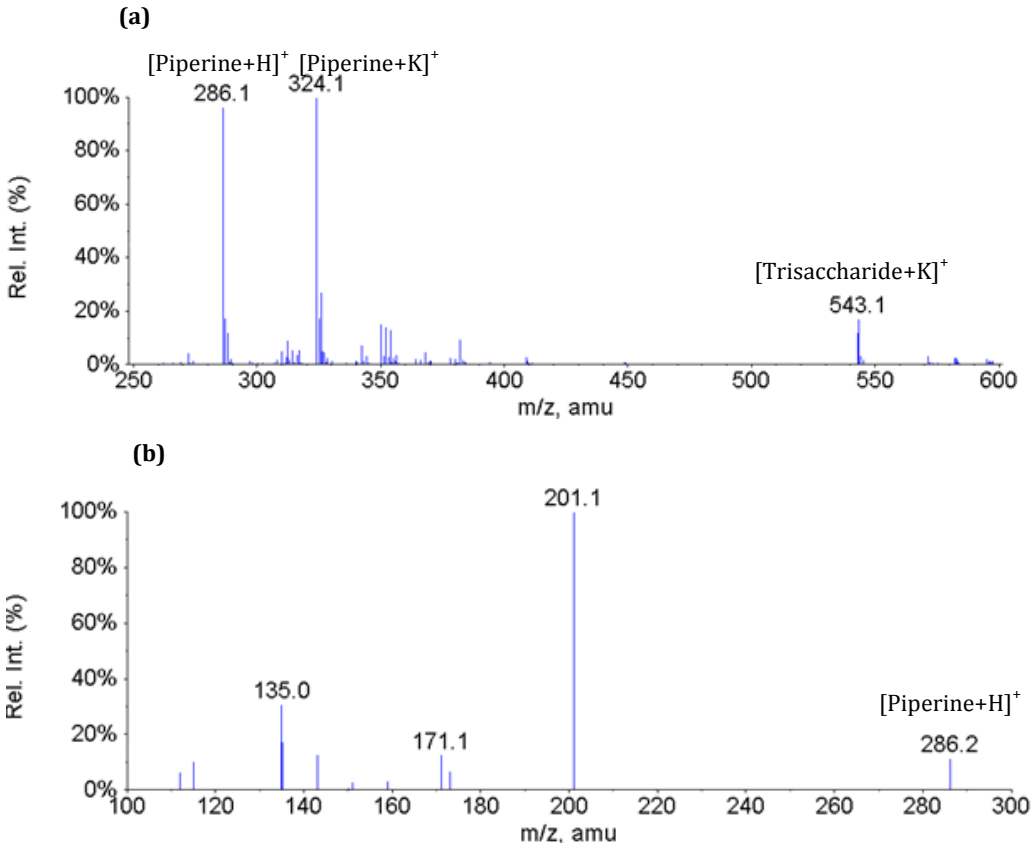


Figure 2-8 (a) Mass spectra obtainied by pipette-tip ESI-MS analysis of black pepper; **(b)** MS/MS spectrum of *m/z* 286.2.

Black pepper (*Piper nigrum*) is a widely used flavoring. The piquancy of black pepper is mainly originated from an alkaloid, piperine,[114] the major component of black pepeper that was reported to inhibit human p-glycoprotein and CYP3A4.[115] Detection of this compound from black pepper normally requires time-consuming solvent extraction.[116, 117] In this study, piperine, observed as the proton and potassium adducts at *m/z* 286.1 and

324.1 respectively, could be readily detectd by direct analysis of the black pepper powder and identified by the MS/MS spectrum with pipette-tip ESI-MS (Figure 2-8). In addition to piperine, trisaccharide was also detected as potassium adduct ion (*m/z* 543.1) in the mass spectrum.

Green tea

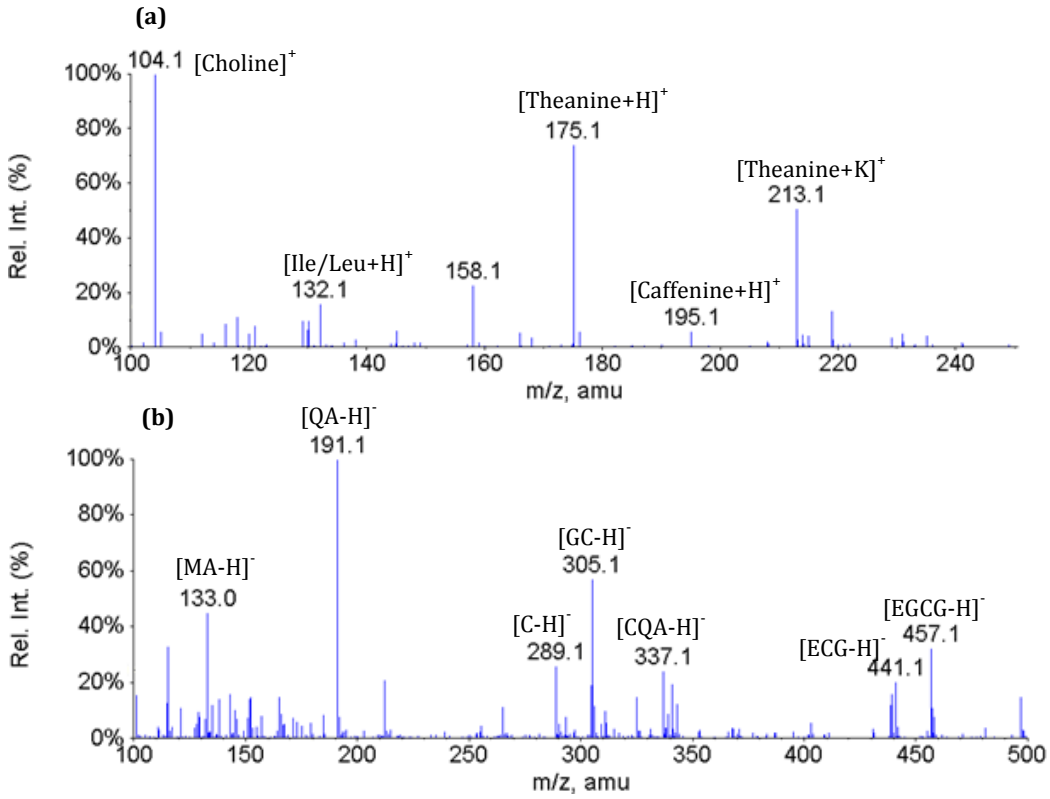


Figure 2-9 Mass spectra obtained by pipette-tip ESI-MS analysis of green tea: **(a)** Positive ion mode; **(b)** Negative ion mode. (MA: Malic acid; QA: Quinic acid; C: Catechin; GC: Gallocatechin; CQA: 4-*p*-Coumaroylquinic acid; ECG: Epicatechin-3-gallate; EGCG: Epigallocatechin-3-gallate)

Tea is another type of popular brewed beverages consumed worldwide, especially in Asia. For convenience, some commercial tea is made into powder and packed in small bags for fast brewing, so powder is a very significant form for tea. In the analysis of green tea powder with pipette-tip ESI-MS, both positive and negative ion modes were employed. In positive ion mode, bitter-tasting caffeine was detected as proton adduct (*m/z* 195.1) and sweet-tasting theanine was detected as both proton adduct (*m/z* 175.1) and potassium adduct (*m/z* 213.1) (Figure 2-9a).[118] Choline and isoleucine (or leucine) (*m/z* 132.1) was observed too. All of these chemical components were determined by their MS/MS spectra and exact mass measurements. In negative ion mode, three organic acids: malic acid (*m/z* 133.0), quinic acid (*m/z* 191.1) and 4-*p*-coumaroylquinic acid (*m/z* 337.1) were identified. The well-known bitter-tasting antioxidative tea polyphenols: catechin (*m/z* 289.1), gallocatechin (*m/z* 305.1) and epicatechin-3-gallate (*m/z* 441.1) and epigallocatechin-3-gallate (*m/z* 457.1), were detected in their deprotonated form (Figure 2-9b).[119]

Panax ginseng, Panax quinquefolius and Panax notoginseng

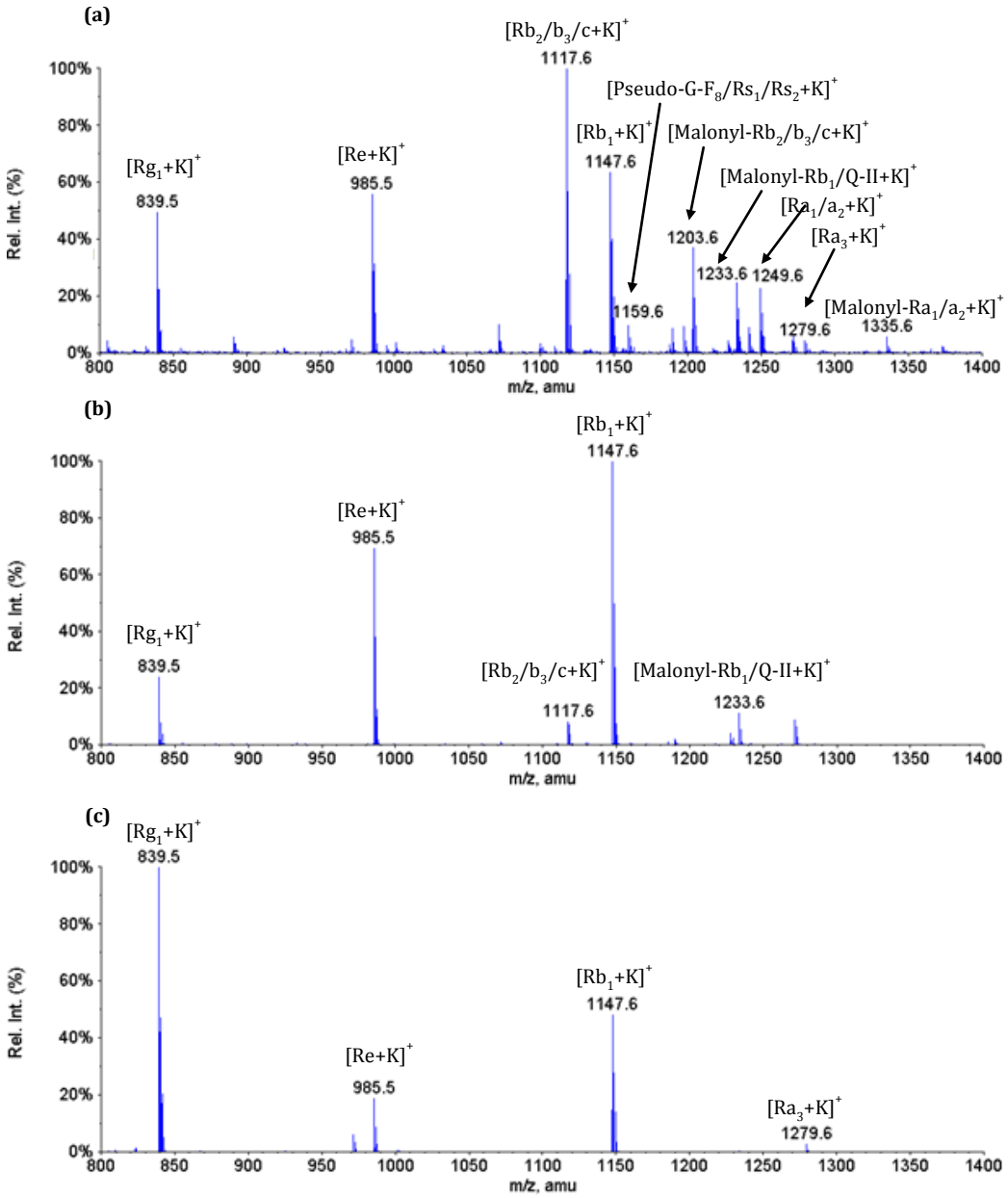


Figure 2-10 Mass spectra obtained by pipette-tip ESI-MS analysis of *Panax ginseng* **(a)**, *Panax quinquefolius* **(b)** and *Panax notoginseng* **(c)**.

Panax ginseng, *Panax quinquefolius* and *Panax notoginseng* are three well known and valuable traditional herbal medicines. They are closely related but have very different pharmacological effects. Such differences were believed to be attributed to their differences in compositions of ginsenosides that were believed to be their bioactive components.[56, 96, 120] For *Panax ginseng*, which is also known as Asian ginseng, as shown in Figure 2-10a, ginsenosides, including Rg_1 (*m/z* 839.5), Re (*m/z* 985.5), Rb_2/Rb_3/Rc (*m/z* 1117.6), Rb_1 (*m/z* 1147.6), pseudo-G-F_8/Rs_1/Rs_2 (*m/z* 1159.6), malonyl Rb_2/Rb_3/Rc (*m/z* 1203.6), malonyl Rb_1/Q-II (*m/z* 1233.6), Ra_1/Ra_2 (*m/z* 1249.6), Ra_3 (*m/z* 1279.6) and malonyl Ra_1/Ra_2 (*m/z* 1335.6), could be detected. For *Panax quinquefolius*, which is also known as American ginseng, as shown in Figure 2-10b, mainly five components, i.e., Rg_1, Re, Rb_2/Rb_3/Rc, Rb_1, and malonyl Rb_1/Q-II, could be detected. For *Panax notoginseng*, which is known as San Qi in Chinese, as shown in Figure 2-10c, only four components, i.e., Rg_1, Re, Rb_1 and Ra_3, were detected. The mass spectra obtained here showed that the three ginseng herbs exhibited different composition of ginsenosides, allowing differentiation of these three closely related herbal medicines. Comparing with a previous MALDI-MS study for differentiation of *Panax ginseng* and *Panax quinquefolius*,[96] the spectra obtained with the current method showed similar patterns of ginsenosides, but much higher signal-to-noise ratio (S/N) for these compounds.

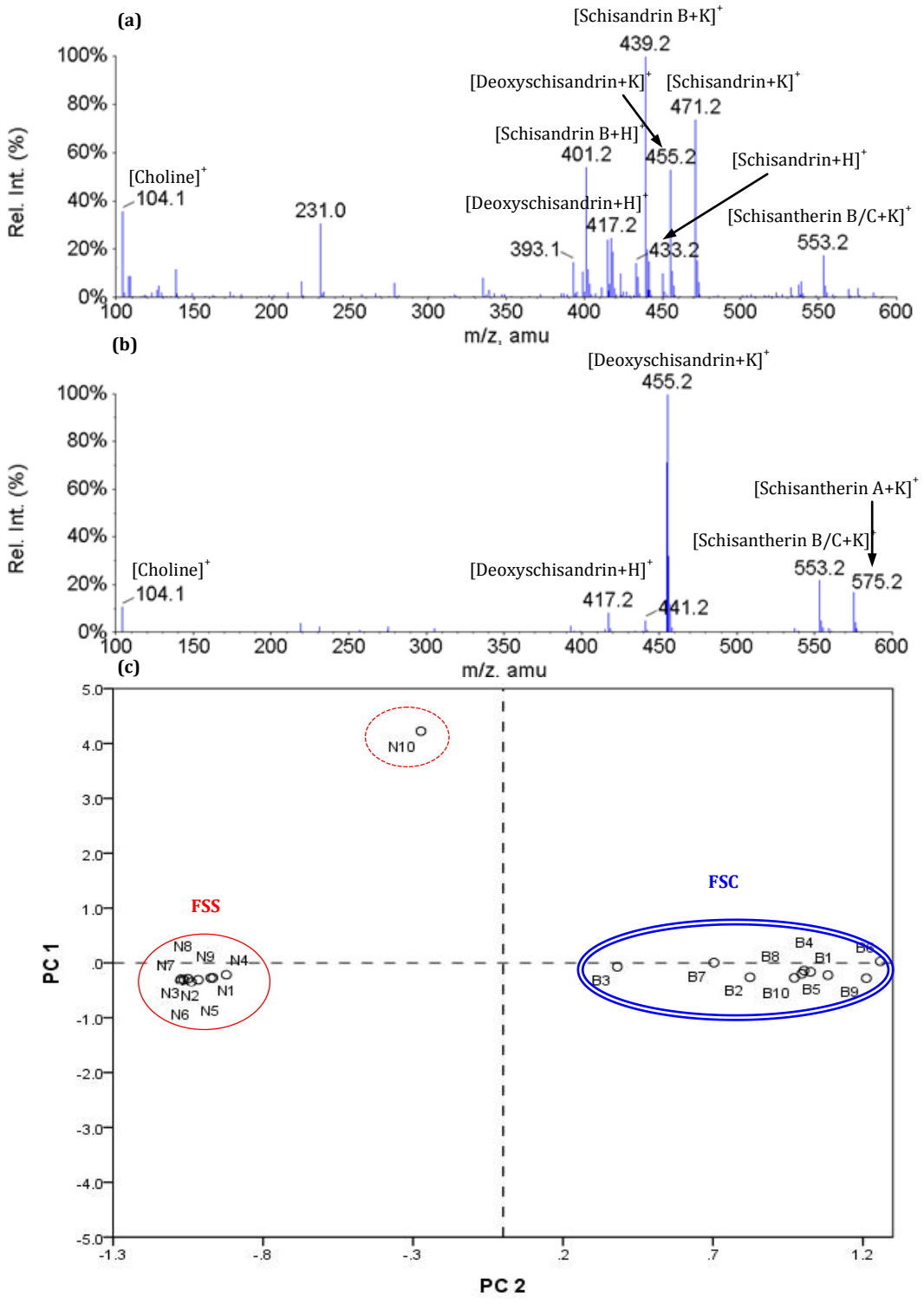


Figure 2-11 Mass spectrum obtained by pipette-tip ESI-MS analysis of FSC **(a)** and FSS **(b)**; **(c)** PCA score plot obtained based on the mass spectra of FSC and FSS.

Fructus Schisandrae Chinensis (FSC) and Fructus Schisandrae Sphenantherae (FSS)

FSC, known as Beiwuweizi in Chinese, is the ripe fruit of *Schisandra chinensis* (Turca.) Baill, which mainly grows in the northern part of China. FSS, known as Nanwuweizi in Chinese, is the ripe fruit of *Schisandra sphenanthera* Rehd. et Wils., which mainly grows in the southern part of China. Both of them could be used as tonic agent in TCM and food in daily life. FSC is considered to be better in quality and efficacy than FSS. FSC and FSS have very similar appearances and thus require analytical techniques for differentiation. The spectra obtained for these two herbal species by pipette-tip ESI-MS analysis with ethanol/water (50/50, v/v) as the extraction and ionization solvent were shown in Figure 2-11a and 2-11b. Lignans, the bioactive components of these species,[121, 122] were predominantly detected and the lignans patterns observed were similar to those obtained by direct ionization analysis of the same species.[57].Deoxyschisandrin ($[M+H]^+$ at *m/z* 417.2 and $[M+K]^+$ at *m/z* 455.2) and schisantherin B/schisantherin C ($[M+K]^+$ at *m/z* 553.2) could be detected in the spectra of both FSC and FSS; while schisandrin B ($[M+H]^+$ at *m/z* 401.2 and $[M+K]^+$ at *m/z* 439.2) and schisandrin ($[M+H]^+$ at *m/z* 433.2 and $[M+K]^+$ at *m/z* 471.2) could only be detected in the spectrum of FSC, and schisantherin A ($[M+H]^+$ at *m/z* 575.2) could only be detected in the spectrum of FSS. In this study, ten batches of each species of samples were analyzed, and each sample was measured three times and mass spectra in the range of *m/z* 100-600 were centered and scaled for PCA analysis. As shown in Figure 2-11c, the PCA score plots (PC1 *versus* PC2) allowed clear differentiation of the two species. One FSS sample subjected to a specifically different manufacturing process was found to have apparently different lignan compositions and thus different PCA results compared to other samples of the same species. The total contribution of PC1 and PC2 represented 83.38% of all variables

after the dimensions reduction treatment in PCA. Compared with differentiation of FSS and FSC using surface desorption atmospheric pressure chemical ionization mass spectrometry (DAPCI-MS),[123] pipette-tip ESI-MS allowed detection of lignans, the active components of these herbal species that are relatively nonvolative and could not be observed by DAPCI-MS.

2.3.3 Quantitative Analysis of Caffeine Contents in Tea Powders

Tea is another type of popular brewed beverages consumed worldwide. Many tea products are made in powders and packed in small bags for fast brewing. Pipette-tip ESI MS spectra of such a green tea sample were shown in Figure 2-9.

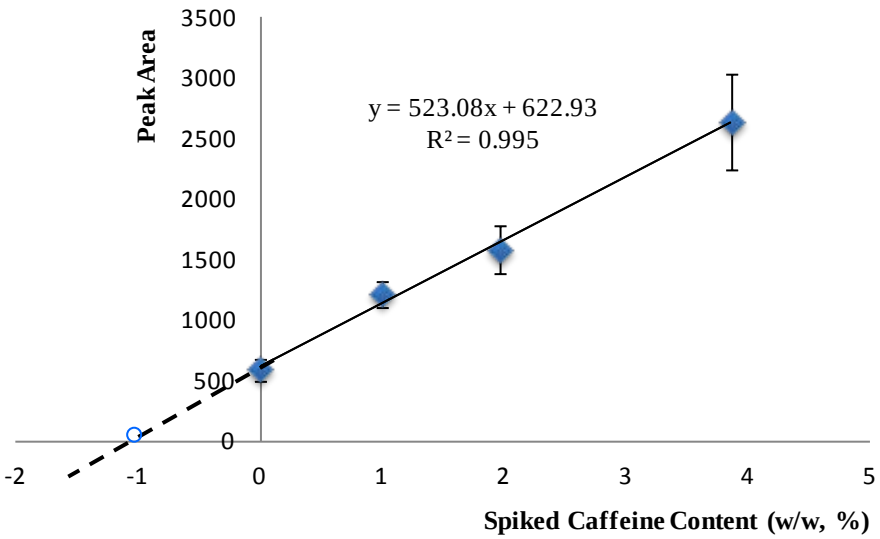


Figure 2-12 Calibration curve for quantitation of caffeine in the green tea (peak area *versus* spiked caffeine content).

Table 2-1 Caffeine contents (w/w, %) in five tea samples determined by pipette-tip ESI-MS/MS and UPLC-ESI-MS/MS.

Sample	UPLC-ESI-MS/MS (Mean ± SD, n=3)	Pipette-tip ESI-MS/MS (Mean ± SD, n=3)
Green tea	1.322 ± 0.068	1.191 ± 0.173
Black tea	2.403 ± 0.115	2.123 ± 0.133
Pu Erh tea	2.960 ± 0.168	2.621 ± 0.336
Iron buddha tea	2.953 ± 0.120	2.376 ± 0.240
Jasmine tea	4.059 ± 0.246	3.149 ± 0.405

The stable and reproducible signals offered by pipette-tip ESI-MS enabled quantitative analysis of components in the powder samples. This was demonstrated by quantitative measurement of caffeine content in tea powders in this study. Standard addition method was used for the measurements. As shown in Figure 2-12, a calibration plot (peak area *versus* spiked caffeine content) was constructed for each tea sample, and the absolute value of the x-intercept was the original caffeine content in the sample. Five tea samples, including green tea, black tea, Pu Erh tea, Iron Buddha tea and jasmine tea, were analyzed and the results were summarized in Table 2-1. As a comparison, these samples

were also analyzed by UPLC-ESI-MS/MS. The caffeine contents obtained from the pipette-tip ESI-MS/MS method were comparable to those from the UPLC-ESI-MS/MS method. This part demonstrated the capability of pipette-tip ESI-MS in rapid quantitation of chemical components in herbal powders.

2.4 Conclusions

A novel technique, pipette-tip ESI-MS, was developed and demonstrated for direct and rapid analysis of herbal powders without prior extraction and chromatographic separation. Powder is a common form for herbal medicine and food in daily life. Phytochemicals in the analyzed samples could be rapidly detected with this new technique. Compared to the recently developed direct ionization of bulky solid tissue, the signals of this technique were more stable, durable and reproducible, due to the continuous and stable supply of spraying solvents and the more homogenous sample form. In this study, pipette-tip ESI-MS was successfully applied for qualitative analysis of herbal medicine and food, allowing differentiation of herbal medicines with similar appearances. Application of pipette-tip ESI-MS/MS for rapid quantitation of herbal powders was also demonstrated by measurements of caffeine contents in five tea samples. The quantitative results were comparable to those obtained by using a conventional method (UPLC-ESI-MS/MS). This novel technique will be further optimized and applied to analysis of wider range of samples, e.g., biological tissues, in the future.

Chapter 3 Direct Analysis of Complex Solution Samples by C-18 Pipette-tip Electrospray Ionization Mass Spectrometry

3.1 Introduction

In this part, the pipette-tip electrospray MS was further extended for direct analysis of complex solution samples. The herbal powder filled into pipette tip end was replaced with sorbent C18 for sample desalting, purification and concentration. Pipette tip-based micro-extraction (TBME) has been reported,[124-126] and such a C18 pipette tip, i.e., ZipTip, is commercially available. In this study, the C18 pipette tip column is combined with syringe and syringe pump for ESI-MS analysis (Figure 3-1). After sample loading and clean-up, analyte molecules bound to C18 bed are directly sprayed out for ESI-MS analysis with the application of a high voltage. Electrospray ionization from C18 pipette tip or similar pipette-tip columns has been reported previously.[55, 98] However, those previous setup required insertion of a metal wire for connection of the high voltage and in some cases additional power supplies. Moreover, typically only about 2 μL of elution solvent was supplied and the signals were usually unstable and could not last a long time. As shown in Figure 3-1, our technique made uses of a syringe needle and solvents[99, 100] for delivery of the high voltage and a syringe pump for supply of solvents. It is simple, easy to assemble, and ensures continuous and stable signals. The elution solvent serves as the mobile phase for elution and subsequent spray ionization of the absorbed analytes. In this study, using rapid analysis of proteins in presence of salts or detergents and direct quantitation of ketamine and norketamine in urine samples as examples, we demonstrated the qualitative and quantitative capabilities of this technique.

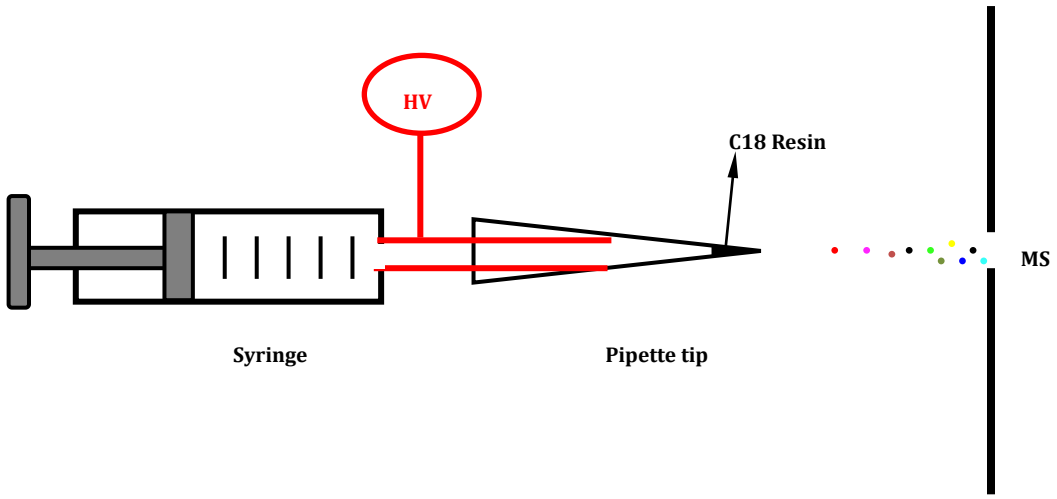


Figure 3-1 The schematic diagram of C18 pipette-tip ESI-MS.

3.2 Experimental Section

3.2.1 Materials and Chemicals

C18 pipette tips, i.e., 10 µL ZipTip containing 0.6 µL C18 resin, were purchased from Millipore (USA). The C18 resin was made of silica of 15 µm diameter and 200 Å pore size. Cytochrome *c* from equine heart, myoglobin from equine heart, α-lactalbumin from bovine milk and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich. Urine was collected from a healthy volunteer. Water was distilled water prepared using a Milli-Q system (Millipore, USA). All the other solvents were of HPLC grade and purchased from Tedia (Fairfeild).

3.2.2 Sample Preparation

For the detection of proteins, 5 μM cytochrome c, myoglobin and α-lactalbumin were prepared in the solution of NaCl (1%) or SDS (0.1%). Then the protein solution was mixed with methanol (0.2% FA) in a volume ratio of 1: 1 for conventional ESI-MS analysis as comparison, and spiked with 0.1% TFA for sample preparation with C18 pipette tip and subsequent C18 pipette-tip ESI-MS analysis.

For quantitation of ketamine and norketamine, standard solutions of ketamine or norketamine were prepared with concentrations of 400, 100, 50, 10, 5, 2 μg mL^{-1} in methanol, and internal standard solution of d_4-norketamine was prepared with a concentration of 50 μg mL^{-1} in methanol. Urine samples containing ketamine and norketamine for quantitative analysis were prepared with the followed procedures: 1 mL urine containing 0.1% trifluoroacetic acid (TFA) was spiked with 1 μL ketamine and 1 μL norketamine standard solutions of various concentrations (2, 5, 10, 50, 100, 400 μg mL^{-1}), then 1 μL of the internal standard solution was added to each solution. Finally, a set of urine samples containing ketamine and norketamine with different concentrations (2, 5, 10, 50, 100, 400 ng mL^{-1}) and d_4-norketamine with a fixed concentration (50 ng mL^{-1}) were obtained. Blank urine sample for limit of detection (LOD) and limit of quantitation (LOQ) measurements was prepared by mixing 1 mL urine containing 0.1% FA with 1 μL of the internal standard solution.

In order to reduce the matrix influences on MS analysis, the C18 pipette tip was used to remove salts, detergents or other non-absorbed compounds in urine, using the following procedures that are similar to the protocol described in the Millipore C18 ZipTip manual:

1. Attach the pipette tip to a 20 µL micropipette with a volume setting at10 µL. Aspirate 10 µL acetonitrile to the pipette tip for wetting and then pipette it out. Repeat this step three times.

2. Aspirate 10 µL water containing 0.1% TFA for equilibration and then pipette it out. Repeat this step three times.

3. Aspirate 10 µL urine sample for sample loading. Repeat this step six times and each time the sample solution was discarded.

4. Aspirate 10 µL water containing 0.1% TFA for washing. This step could remove the salts and some polar compounds to reduce their influences. Repeat this step three times.

After these procedures, the C18 pipette tip was combined with the syringe and syringe pump for direct electrospray ionization MS analysis.

3.2.3 Instrumentation and Setup

As shown in Figure 3-1**,** the setup for C18 pipette-tip ESI-MS is similar with that for pipette-tip ESI-MS as described in the previous chapter. For the analysis of the protein solutions with C18 pipette-tip ESI-MS, the C18 pipette-tip ESI ion source was coupled with a quadrupole time-of-flight (Q-ToF) mass spectrometer (QStar Pulsar, Applied Biosystems). For the analysis of proteins solutions with conventional ESI-MS, the

equipped ESI ion source was coupled with a same mass spectrometer. The mass spectrometer was operated at the same conditions as described in chapter 2.

For quantitation of ketamine and norketamine in urine, the C18 pipette-tip ESI ion source was coupled with a triple quadruple mass spectrometer (Quattro Ultima, Waters), with the pipette tip end located at a position with a perpendicular distance of 3.0 cm and parallel distance of 1.0 cm to the MS inlet. The capillary voltage, cone voltage and source temperature were set at 3.8 kV, 30 V and 80 °C respectively. The elution and spray solvent used was 80% methanol containing 1.0% FA, and the flow rate is 5 μL min^{-1}. The equipment was performed in the positive selected reaction monitoring (SRM) mode. The product ion scan mass spectra of ketamine and norketamine are shown in Figure 3-2, with the potential chemical structures of major fragments labeled according to previous reports.[127, 128] Ion *m/z* 125, the major fragment ion for both ketamine and norketamine, was chosen as the product ion in the SRM scan for the two compounds. The selected reaction *m/z* 238 → *m/z* 125 for ketamine was monitored with a collision energy of 25 eV and a dwelling time of 0.2 s; the selected reaction *m/z* 224 → *m/z* 125 for norketamine was monitored with a collision energy of 20 eV and the same dwelling time of 0.2 s; the selected reaction *m/z* 228 → *m/z* 129 for internal standard d_4-norketamine was monitored with the same conditions as for norketamine. The inter-channel delay time and inter-scan delay time were set at 0.02 s and 0.1 s respectively. The spectra were acquired and processed with the MassLynx^{TM} V4.0 software (Waters, UK).

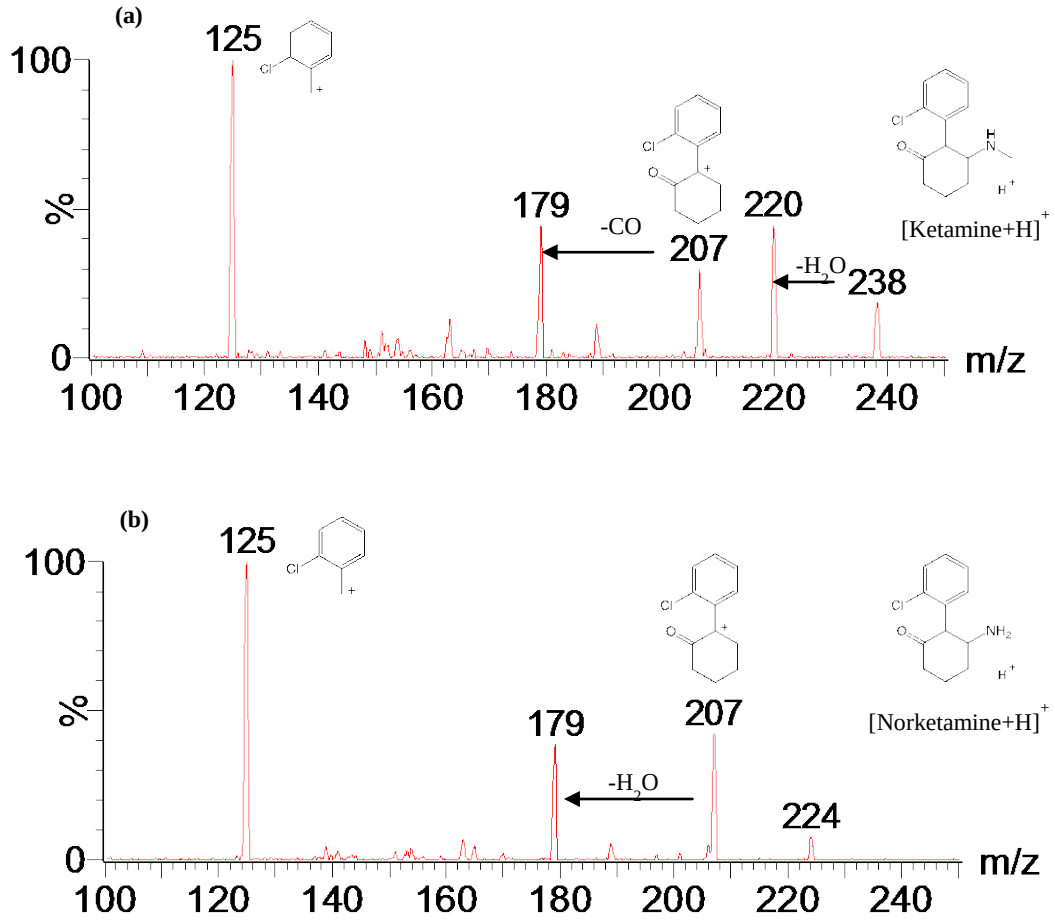


Figure 3-2 Product ion scan mass spectra obtained for 5 µg mL^{-1}ketamine **(a)** and norketamine **(b)** in methanol containing 0.1% FA (potential chemical structures of the major product ions are labeled).

3.3 Results and Discussion

3.3.1 Analysis of Protein Solutions Containing Salt or Detergent

Salts and detergents are commonly present in protein samples.[129, 130] Salts such as sodium chloride are widely used to mimic the physiological environment of organisms for *in vitro* protein research, while detergents such as sodium dodecyl sulfate (SDS) are commonly employed for protein isolation and solubilization, especially for membrane proteins that are usually of poor solubility.[131, 132] Mass spectrometry is the method of choice for analysis of proteins, however, it is not compatible with salts and detergents. Removal of salts and detergents from protein samples is thus essential prior to mass spectrometric analysis.[130, 131, 133] Various methods have been employed for removal of salts and detergents, and these methods are usually time-consuming and laborious.[131, 134] In this study, C18 pipette-tip ESI-MS was attempted for analysis of protein solutions containing salts and detergents, in an effort to develop approaches for rapid detection of proteins in the presence of salts and detergents.

A solution of cytochrome c (5 μM) containing 1% NaCl was analyzed by C18 pipette-tip ESI-MS, in comparison with conventional ESI-MS. As shown in Figure 3-3, only the NaCl clusters and no signals of the protein could be detected in the mass spectrum when the sample was analyzed by conventional ESI-MS. While with C18 pipette-tip ESI-MS, multiply charged ions of cytochrome *c* (*m/z* 1112.7, 1223.7, 1359.6 1529.5 and 1747.7) were clearly observed in the spectrum with almost no signal of the salt. Similar results were obtained when another protein solution, 5 μM myoglobin with 1% NaCl, was analyzed. As shown in Figure 3-4, no peaks corresponding to myoglobin were observed

in the spectrum obtained with conventional ESI-MS; while with C18 pipette-tip ESI-MS, quality spectrum showing a series of multiply charged ions of myoglobin (*m/z* 848.6, 893.2, 942.7, 998.1, 1060.4, 1131.1, 1211.8, 1304.9, 1413.6, 1542.0 and 1696.1) and the heme (m/z 616.2) was obtained. These results demonstrated the capability of C18 pipette-tip ESI-MS for desalting and rapid analysis of protein samples containing salts.

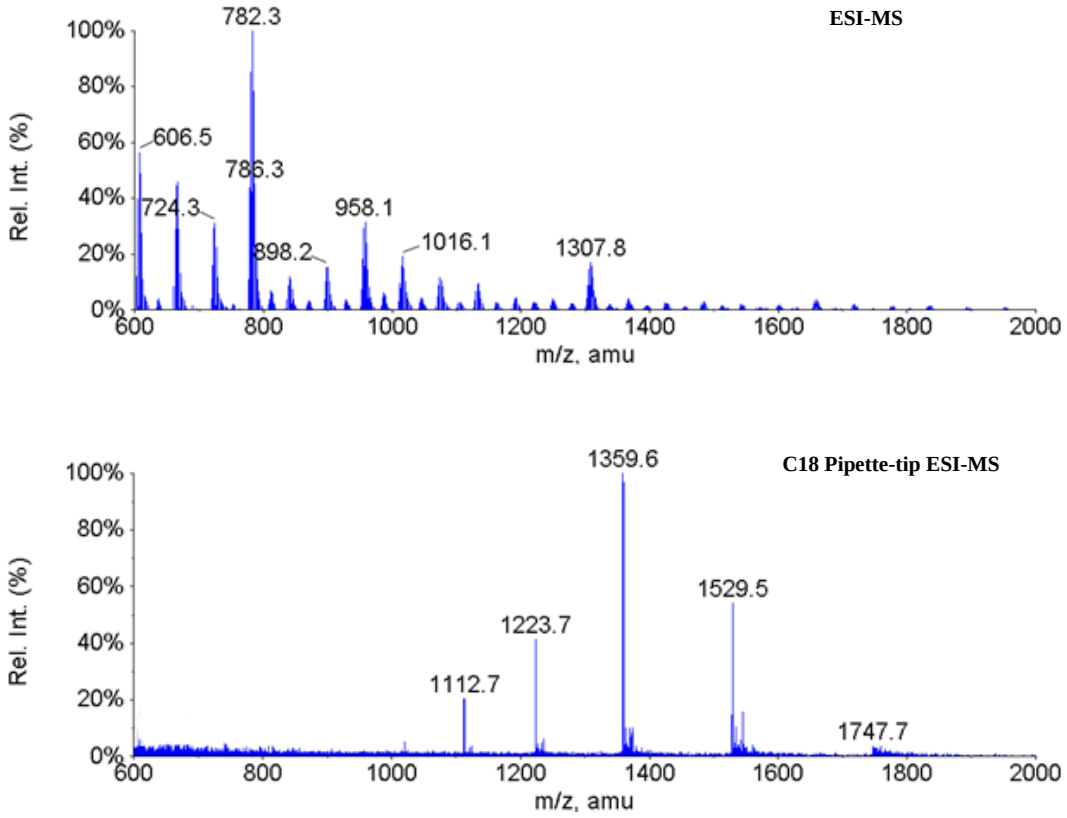


Figure 3-3 Spectra obtained by analysis of cytochrome *c* (5 µM, 1% NaCl) with conventional ESI-MS and C18 pipette-tip ESI-MS.

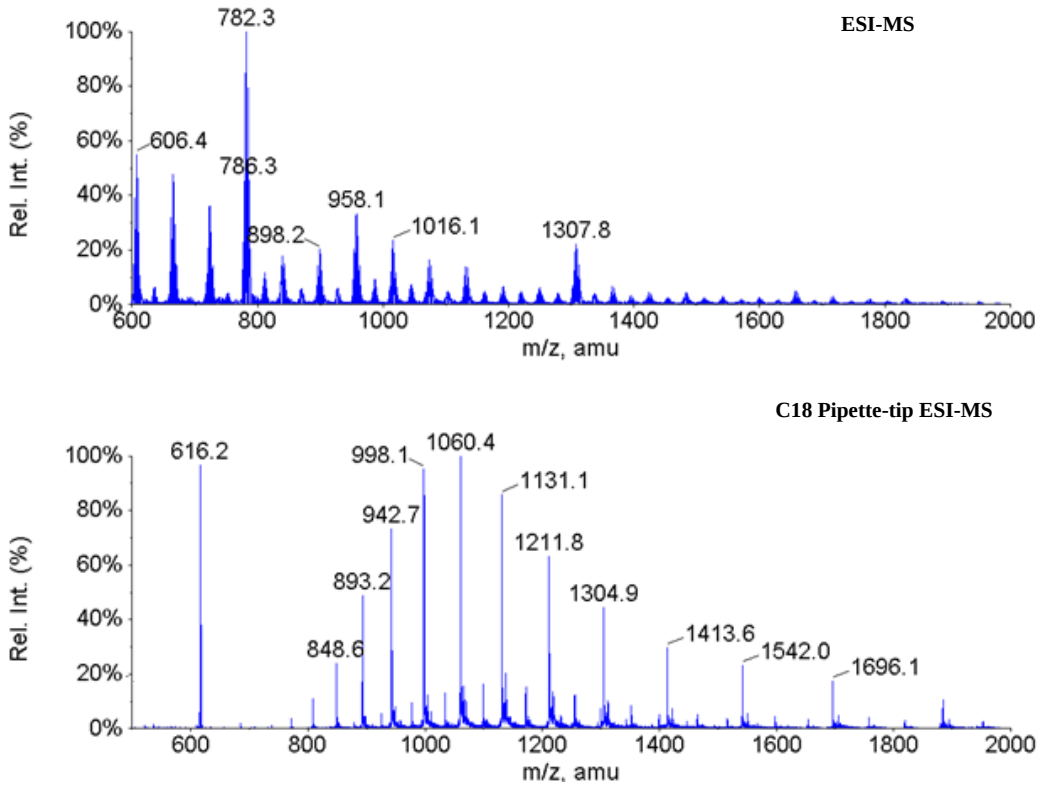


Figure 3-4 Spectra obtained by analysis of myoglobin (5 μM, 1% NaCl) with conventional ESI-MS and C18 pipette-tip ESI-MS.

Protein solutions containing detergents were examined as well in this study, and the spectral results are shown in Figure 3-5. α-Lactalbumin, a common membrane protein, in a solution containing 0.1% SDS was analyzed using both ESI-MS and C18 pipette-tip ESI-MS. Conventional ESI-MS could not detect any protein signals and only the SDS clusters could be obtained. With C18 pipette-tip ESI-MS, the multiply charged ions of α-lactalbumin (*m/z* 1289.9, 1418.8, 1576.3, 1773.2, 2026.4 and 2363.9) could be detected obviously.

During the sample processing with C18 pipette tip, relatively hydrophobic proteins could be readily absorbed by no-polar C18 resin, while hydrophilic NaCl and SDS could not be absorbed and are washed out. This allows rapid and convenient detection of protein from solutions containing salts and detergents by using C18 pipette-tip ESI-MS.

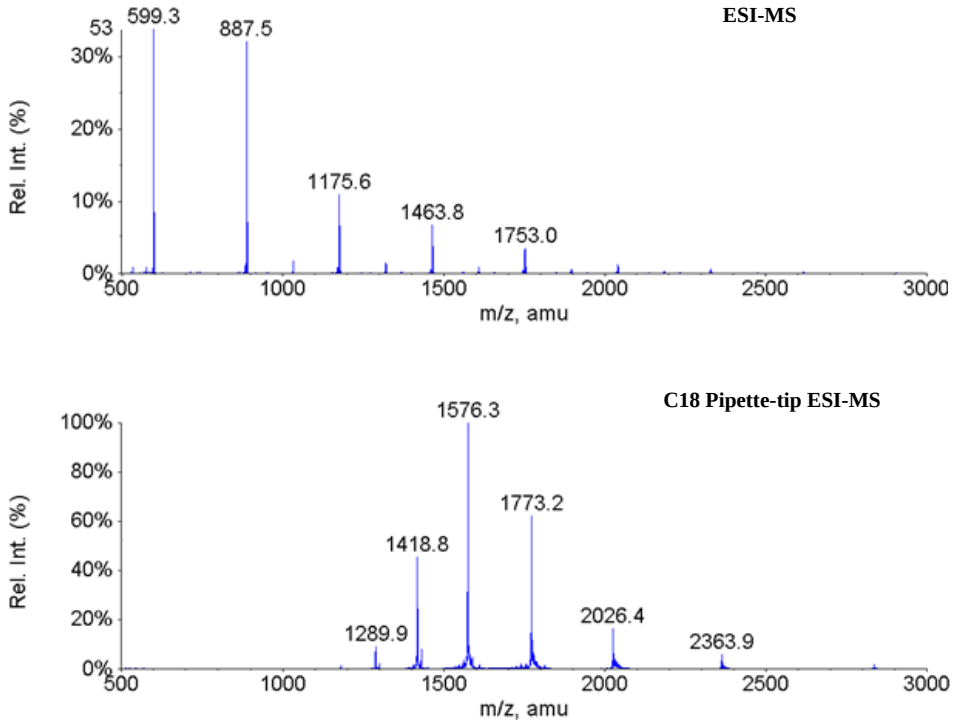
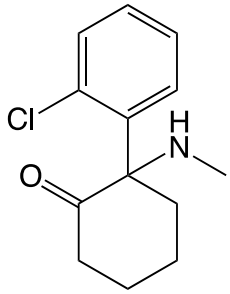
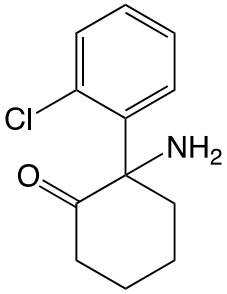


Figure 3-5 Spectra obtained by analysis of α-lactalbumin (5 μM, 0.1% SDS) with conventional ESI-MS and C18 pipette-tip ESI-MS.

3.3.2 Quantitation of Ketamine and Norketamine in Urine



Ketamine
Chemical Formula: $C_{13}H_{16}ClNO$
Exact Mass: 237.09



Norketamine
Chemical Formula: $C_{12}H_{14}ClNO$
Exact Mass: 223.08

Figure 3-6 Chemical structures of ketamine and norketamine.

Drug abuse, especially the abuse of psychotropic drugs, is a serious problem worldwide. Abuse of psychotropic drugs could not only harm the health of the abusers, but also bring a lot of social problems. Many kinds of psychotropic drugs are thus prohibited or controlled in most countries in the world. Ketamine, called as “K powder” in Hong Kong, is a kind of abused psychotropic drug to induce psychedelic effects to the abusers,[135] and norketamine is the major metabolite of ketamine in human body (Figure 3-6).[136, 137] The abuse of ketamine popularly existed in east and southeast Asia, especially in Singapore and Hong Kong,[138] and the involved population has been growing in recent years.[139] Identification of ketamine abusers is critical for drug control, which usually relies on measurements of ketamine residue and norketamine in the abusers’ urine, blood, nail and hair.[140-143] Urine is commonly chosen for the measurement due to the relatively high concentrations of ketamine and norketamine and

larger volume available. For identification of ketamine abusers and judicial practice in drug control, a valid and rapid method for quantitative analysis of ketamine and norketamine in urine is essential.

Quantitation of ketamine and norketamine in urine is usually performed using MS combined with GC or HPLC.[127, 140, 142, 144, 145] In order to reduce interferences of matrices in the urine samples, extensive sample pretreatments are required before chromatographic separation and MS detection.[127, 140, 142, 144, 145] Development of simple, rapid and high-throughput methods are thus highly desirable for the growing analytical demands in beat drugs campaigns.

Ambient MS developed in recent years[4-6] allows direct analysis of complex samples without sample pretreatments and chromatographic separation. These new ionization techniques have been attempted for direct analysis of abused drugs. For example, DESI was applied to directly detect ketamine on skin.[63] Very recently, direct detection and quantitation of ketamine and norketamine in urine and oral fluid was successfully achieved in our group using wooden-tip ESI-MS.[128] The linear range, limit of detection (LOD) and limit of quantitation (LQD) of this technique for quantitation of both ketamine and norketamine in urine were 50-5000 ng mL^{-1}, 20 ng mL^{-1} and 50 ng mL^{-1} respectively.[128] For direct analysis of various real samples, a linear range with lower concentrations and lower LOD and LOQ may be required,[142] and a new method is thus expected. In this study, C18 pipette-tip ESI-MS was examined for direct quantitation of ketamine and norketamine in urine.

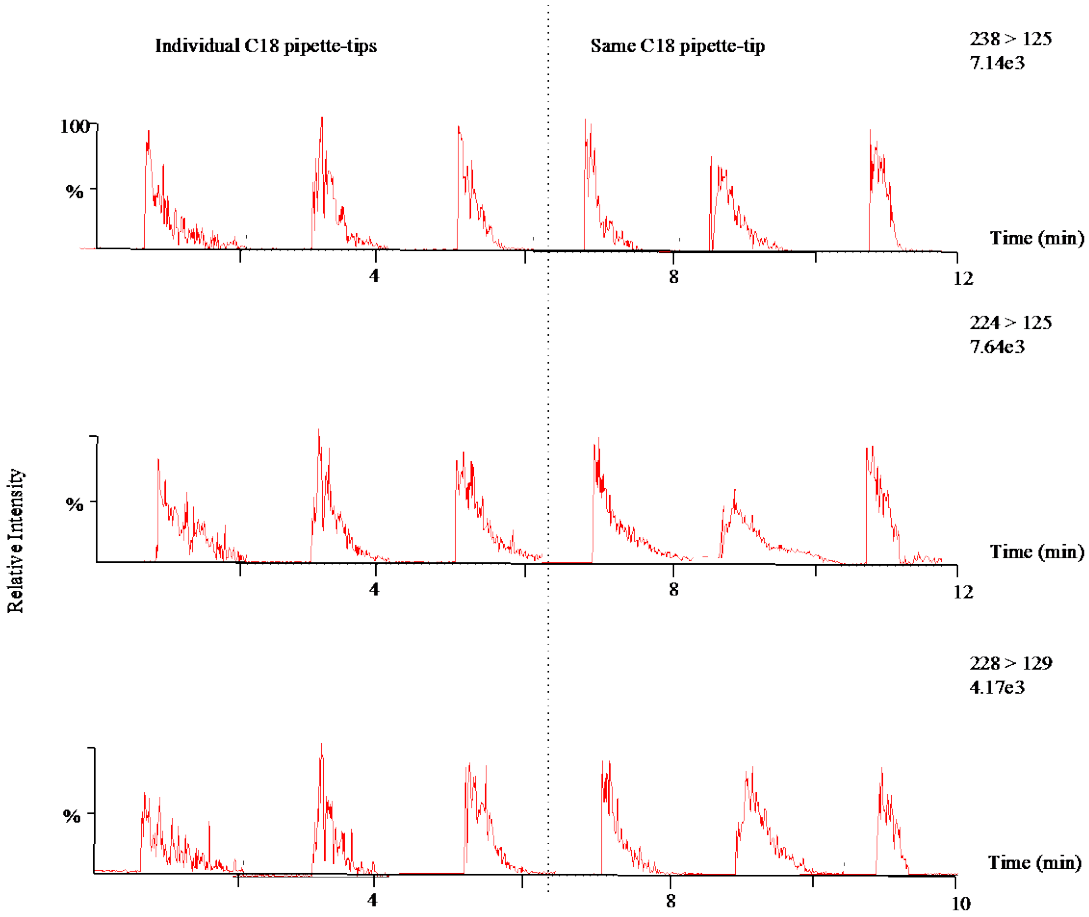


Figure 3-7 The SRM chromatograms of three measurements using three individual C18 pipette tips and the same C18 pipette tip.

We first tested the possibility to repeatedly use one C18 pipette tip for the measurements. The reproducibility using three individual C18 pipette tip or the same C18 pipette tip for three repeat measurements was investigated with a urine sample spiked with 50 ng mL^{-1} ketamine, norketamine and the internal standard, and the obtained TIC spectra are shown in Figure 3-7. For three individual C18 pipette tips the RSD (relative standard deviation) for the three peak area ratios of ketamine and norketamine to the internal standard was 10.2% and 9.8% respectively, which are acceptable for sample analysis.

For the repeat measurements using the same C18 pipette tip, the peak area ratios for ketamine and norketamine were 11.6% and 13.1% respectively, which were comparable with acceptable RSD and indicating no memory effects from the previous measurement. To reduce the experiment costs and make the measurement simpler, one C18 pipette tip was used for the whole measurements. The re-used C18 pipette tip was carefully washed with 200 µL methanol/ water/1% FA (50/50/1, v/v/v) each time before the measurement.

Quantitation of ketamine and norketamine in urine was simultaneously performed with C18 pipette-tip ESI-MS, using d_4-norketamine as the internal standard and selected reactions *m/z* 238 → *m/z* 125, *m/z* 224 → *m/z* 125, and *m/z* 228 → *m/z* 125 for the three compounds respectively. The C18 pipette tip allowed rapid sample enrichment and clean-up. A lower concentration range of 2-400 ng mL^{-1} was thus investigated with this novel technique. The SRM chromatograms for ketamine (*m/z* 238 > 125) and norketamine (*m/z* 224 > 125) in the concentration sequence of 2, 5, 10, 50, 200, 400 ng mL^{-1} and the internal standard d_4-norketamine (*m/z* 228 > 128) with a fixed concentration of 50 ng mL^{-1} are shown in Figure 3-8. A positive correlation between the peak area and analyte concentration was found both in the analysis of ketamine and norketamine. The peak area of the internal standard for different samples did not vary significantly. As shown in Figure 3-8, distinct chromatographic peaks with a time window of 40-80 s could be obtained within two minutes. Since the sample preparation procedure with the C18 pipette tip only needed about 2-3 minutes for each sample, the total time for analysis of each sample could be less than 5 minutes.

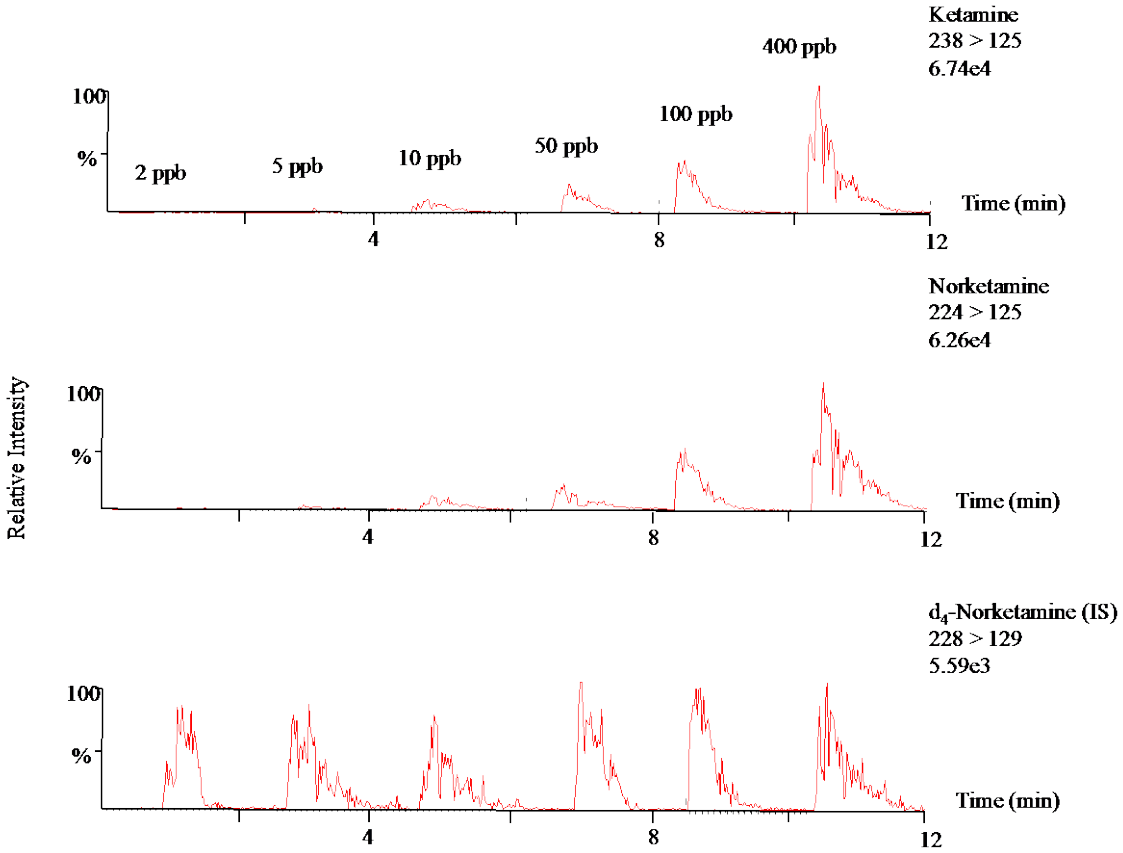


Figure 3-8 The SRM chromatograms for simultaneous detection of ketamine (*m/z* 238 > 125, 2-400 ng mL^{-1}), norketamine (*m/z* 224 > 125, 2-400 ng mL^{-1}) and the internal standard d_4-norketamine (*m/z* 228 > 129, 50 ng mL^{-1}).

Table 3-1 RSD for each sample in quantitation of ketamine and norketamine.

Concentration (ng. mL^{-1})	Ketamine		Norketamine	
	Reak area ratio (analyte/IS)	**RSD (n =3, %)**	**Reak area ratio (analyte/IS)**	**RSD (n =3, %)**
2	0.1066	3.05	0.0978	11.13
5	0.1755	3.02	0.1804	7.60
10	0.3070	3.02	0.4966	4.47
50	1.6935	5.04	2.1889	8.50
100	3.1496	5.82	4.0405	6.15
400	12.8589	5.43	16.7950	8.03

For construction of calibration curves, each urine sample was measured for three times, and the mean values of the peak area ratios between analyte and the internal standard and their relative standard deviation (RSD) were shown in Table 3-1. The calibration curves for ketamine and norketamine, as shown in Figure 3-9, were obtained by plotting peak area ratio against the analyte concentration. For both ketamine and norketamine, the calibration curves displayed excellent linear relationship over the examined concentration range of 2-400 ng mL^{-1}. The coefficient R^2 was very closed to 1 for both ketamine (R^2 =0.9999) and norketamine (R^2 = 0.9998). This linear range covered the lowest detected concentrations for both ketamine and norketamine in drug abusers' urine.[142] This achieved linear range was comparable to the conventional HPLC-MS and

GC-MS in the previous reports.[127, 128, 140, 141, 144, 145] The RSDs for analysis of each sample were listed in Table 3-1, which were acceptable for the measurements.

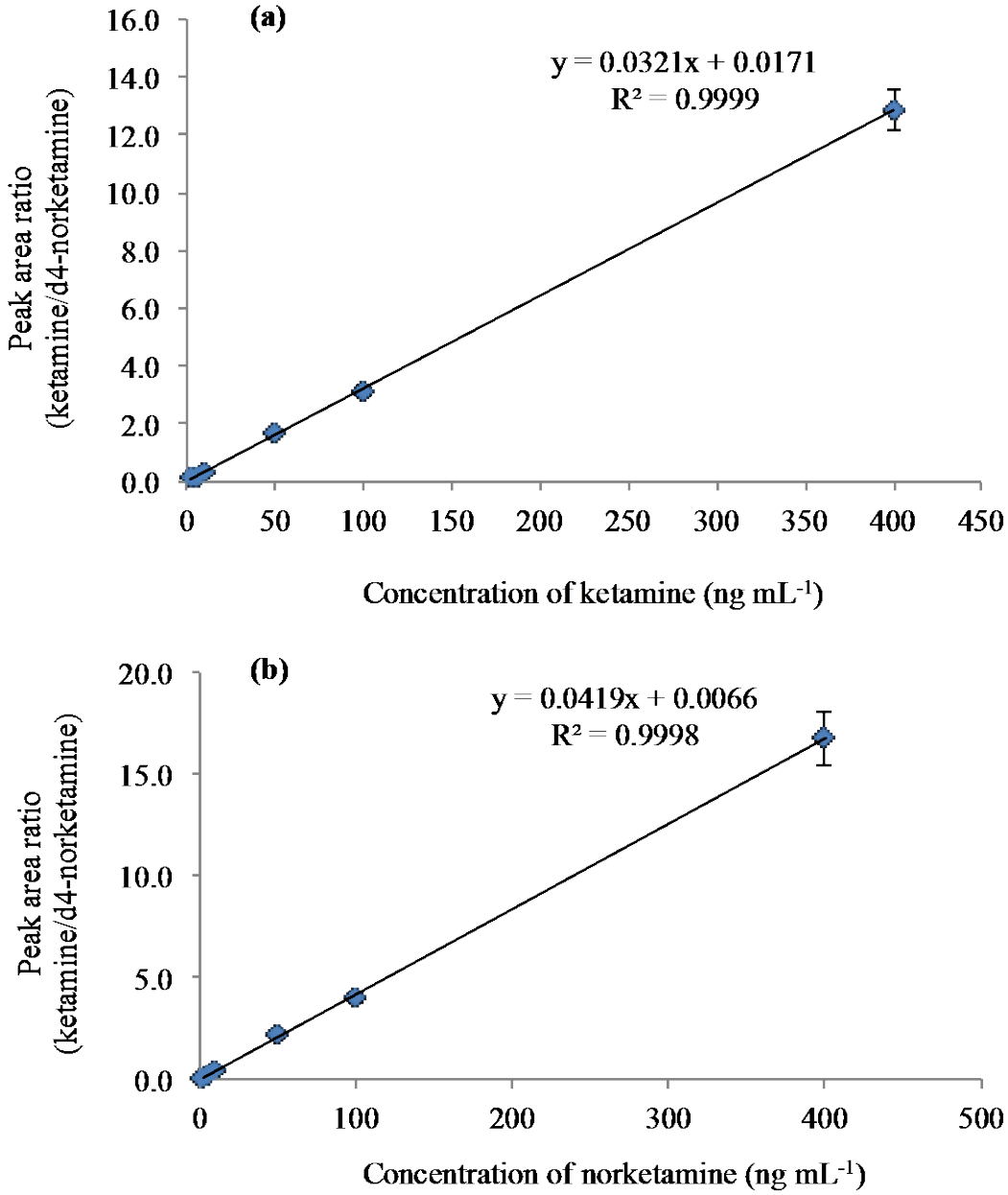


Figure 3-9 Calibration curves for ketamine **(a)** and norketamine **(b)**.

Table 3-2 Precision and accuracy for quantitation of ketamine and norketamine in urine with C18 pipette-tip ESI-MS.

	Ketamine			**Norketamine**		
Spiked Concentration (ng mL^{-1})	**Measured Concentration (ng mL^{-1})**	**Precision (n=6)**	**Accuracy (n=6)**	**Measured Concentration (ng mL^{-1})**	**Precision (n=6)**	**Accuracy (n=6)**
20	18.9	13%	94%	19.6	5%	98%
200	179.2	5%	90%	177.1	7%	89%

The precision and accuracy for quantitation of ketamine and norketamine in urine with C18 pipette-tip ESI-MS were investigated as well (Table 3-2). A low concentration at 20 ng mL^{-1} and a high concentration at 200 ng mL^{-1} were measured. For ketamine, the precisions were determined to be 13% and 5%, and the accuracies were determined to be 94% and 90%, at the two concentrations respectively. For norketamine, the precisions were 5% and 7%, and accuracies were 98% and 89%, at two concentrations respectively. These results were comparable to those obtained with conventional methods.[127, 128, 140-142, 144, 145]

The limit-of-detection (LOD) and limit-of-quantitation (LOQ) of this method were determined by comparing the peak height of the analyte with the average height of the noises after the analyte peak. The LOD and LOQ were defined as the concentration of

the analyte when the signal of the analyte is three and ten times of the signal of the noises respectively. The LOD and LOQ for the present technique were determined to be 0.3 ng mL^{-1} and 0.5 ng mL^{-1}, and 0.8 ng mL^{-1} and 1.0 ng mL^{-1} for ketamine and norketamine respectively (Figures 3-10 and 3-11). These LOD and LOQ were good enough for analysis of real samples.[142]

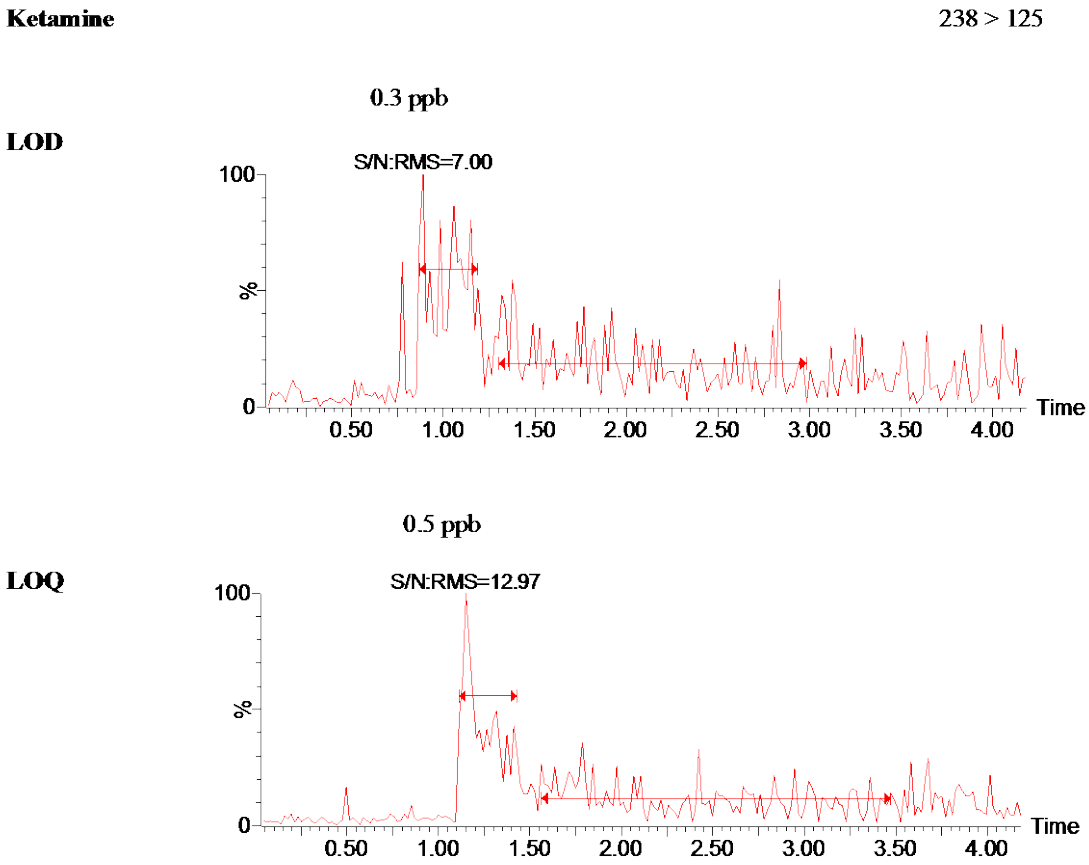


Figure 3-10 The SRM chromatograms of ketamine with concentrations of 0.3 ng mL^{-1} and 0.5 ng mL^{-1} in urine for LOD and LOQ determination respectively.

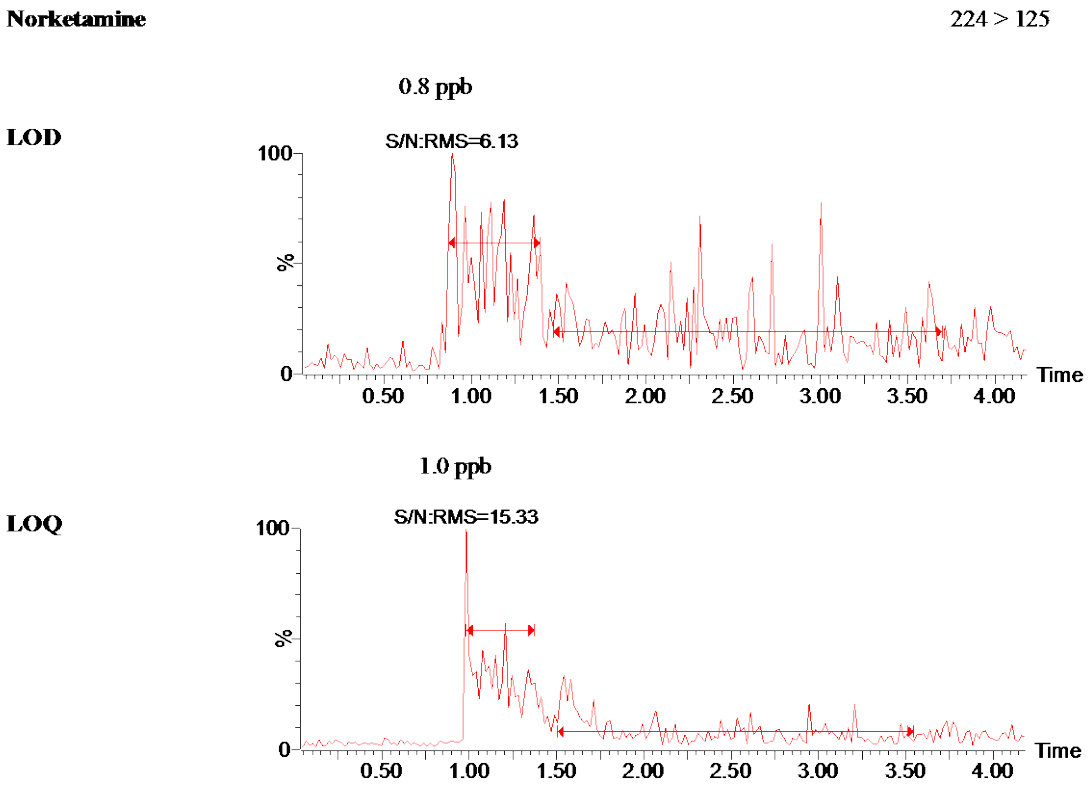


Figure 3-11 The SRM chromatograms of norketamine with concentration of 0.3 ng mL^{-1} and 0.5 ng mL^{-1} in urine for LOD and LOQ determination respectively.

3.4 Conclusions

By combining C18 pipette tip, a device for rapid purification of samples, with syringe and syringe pump, a C18 pipette-tip ESI-MS technique was developed. In this technique, analyte in complex samples was concentrated and purified by C18 pipette tip rapidly and directly eluted to form electrospray ionization for mass spectrometric analysis. Using this novel technique, rapid detection of proteins from solutions containing salts such as sodium chloride or detergents such as SDS, and direct quantitation of ketamine and norketamine in human urine with a lower linear range 2-400 ng mL^{-1}, were successfully

achieved. The linear range, precision, accuracy, LOD and LOQ for both ketamine and norketamine quantitation with this method were well acceptable for analysis of real samples. This method is very simple, easy to operate, cost-effective (the C18 pipette tip could be reused), and thus very useful for rapid analysis of complex samples. The C18 resin can also be replaced with other chromatographic materials and be used for other analytical purposes.

Chapter 4 Rapid Quantitation of Di-(2-ethylhexyl)-phthalate (DEHP) in Beverages by Electrospray Ionization Mass Spectrometry Using Wooden Tips

4.1 Introduction

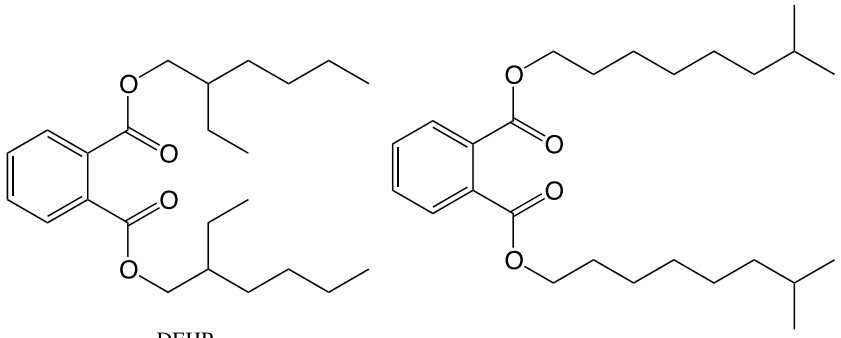


Figure 4-1 Chemical structures of DEHP and DINP.

In 2010, the clouding agent scandal happened in Taiwan alarmed the whole world. This incident originated from a plasticizer di-(2-ethylhexyl)-phthalate (DEHP, see Figure 4-1 for its chemical structure), which was illegally used as a clouding agent. Clouding agents are a kind of food additives used to make beverages naturally opaque and more like high juice products.[146, 147] Palm oil is an important composition of clouding agents. Because DEHP could have clouding effect similar to palm oil, some beverage manufacturers in Taiwan illegally used DEHP to substitute the relatively expensive palm oil in clouding agents to reduce the production cost. The detection of high concentration of DEHP in many food and beverages made this clouding agent scandal shock the whole world.[147]

DEHP, called DOP sometimes, is the most important plasticizer in the family of phthalic acid esters (PAEs).[148] PAEs are dialkyl or alkyl aryl esters of phthalic acid that are mainly used as plasticizers. In addition to DEHP, diisodecyl phthalate (DIDP), di-n-butyl phthalate (DBP) and dissononyl phthalate (DINP) (Figure 4-1) are also widely used. DEHP is a colorless and viscous organic liquid, which is almost insoluble in water, but miscible in mineral oil and some organic solvents. Because of its function of softening plastics and increasing plasticity, DEHP is commonly used to manufacture polyvinyl chloride (PVC) in plastic industry and its content in plastics can be up to 40% (w/w).[149, 150] DEHP can be found in packaging, children's toys, medical devices, preservative film, flooring, agricultural adjuvants, building materials and some other plastic materials.[151]

DEHP is generally considered as a toxic compound. As far back as 1987, International Agency for Research on Cancer (IARC) concluded that DEHP was a potential carcinogen to humans.[152] The DEHP-induced toxicities to kidney, liver, ovary, fetus, thyroid, testes and uterus of animals were demonstrated by various experimental data.[153, 154] It was also reported that DEHP was a sex hormone endocrine disruptor[155] and could change the sexual differentiation of rat by affecting hormone synthesis.[156] As a widely existed contaminant in environment, DEHP has been strictly monitored for its concentrations in food for a very long time.[151, 157, 158] Distinctly, DEHP was strictly forbidden to be used as a food additive in beverages and food, even for food-contact uses in some countries.[159, 160]

Because there is no substantial covalent binding between DEHP (or other phthalates) and plastics, DEHP can slowly transfer to the contact materials and surrounding environment.[151, 161] Since 1999, the European Union has banned the use of DEHP and other phthalates in children's toys and required that the total amount of phthalates should not be greater than 0.1% (w/w) of the plasticized part of the toy.[162] Regarding the potential risk of DEHP for cancer, the U.S. Environmental Protection Agency (USEPA) limited the MCL (maximum contaminant level allowed) of DEHP in drinking water at 6 ng mL^{-1}.[163] With the background of economic globalization, the DEHP scandal happened in Taiwan triggered the food safety crisis of the world. In June 2011, Hong Kong set the maximum threshold value of 1.5 μg mL^{-1} for DEHP in food or medication.[164]

Gas chromatography-electron capture detection (GC-ECD), high performance liquid chromatography coupled with ultraviolet detector (HPLC-UV), gas chromatography-mass spectrometry (GC-MS), and high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) are the techniques of choice for analysis of DEHP. USEPA proposed extraction of DEHP from water samples with methylene chloride followed by measurements of the extracts using GC-ECD.[165] HPLC-UV has been used for analysis of DEHP in blood samples,[166, 167] while GC-MS[168] and HPLC-MS/MS are the major methods for detection of DEHP in various samples, e.g., urine, water, air, toys, soil and food.[63, 169, 170] After the DEHP scandal, the government of Taiwan published the method for detection of DEHP in food using HPLC-MS/MS.[171] Sample extraction and clean-up (e.g., using gel permeation chromatography[172]) as well as chromatographic

separation are the typical procedures for detection of DEHP in samples by these methods. To meet the analytical demands of large volume of samples, simpler, faster and more economic methods are required.

Wooden-tip electrospray ionization mass spectrometry (ESI-MS) (Figure 4-2) is a novel technique developed by our group recently.[60] The disposable wooden tips used in this technique are commonly used wooden toothpicks, which are cheap, readily available, and directly compatible with the nano-electrospray ion source. A small volume (1-5 μL) of sample solution could be loaded onto the sharp tip-end by pipetting directly. A period of successive ion signals could be acquired due to the spray ionization of sample solution at the tip-end induced by a high voltage. The instrumental setup of this technique is very simple and can be readily achieved in various mass spectrometers.

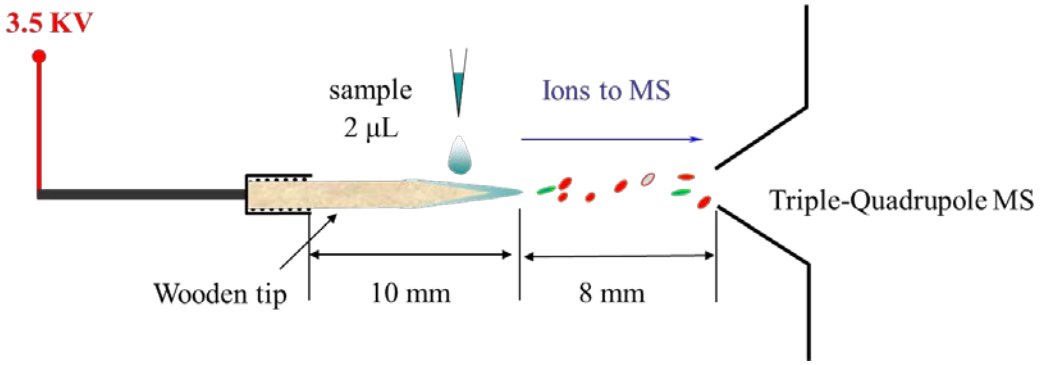


Figure 4-2 Schematic diagram of electrospray ionization with a wooden tip.

This new ionization technique was proved to be effective for analysis of various compounds, including organic compounds, peptides and proteins. Since the sample loading and ionization occurs on the tip surface and interactions of the tip surface with the sample allow reduction of matrix interferences, wooden-tip ESI-MS can be used to directly analyze raw samples, including slurry samples, urine, beverages, etc. In view of these merits and the analytical demand for detection of DEHP in beverages, quantitative analysis of DEHP in beverages using wooden-tip ESI-MS was conducted in this study, which also demonstrated the quantitative capability of this new method.

4.2 Experimental Section

4.2.1 Materials and Chemicals

Di-(2-ethylhexyl)-phthalate (DEHP) and the internal standard (IS) dissononyl phthalate (DINP) were purchased from Sigma-Aldrich. The BEST-buy brand wooden toothpicks used in this study were purchased from a PARKnSHOP supermarket in Hong Kong. Beverages, including Aquarius sports beverage and Mr. Juicy orange juice, were purchased from a PARKnSHOP supermarket in Hong Kong as well. Water was prepared using a Milli-Q system (Millipore, USA). All the other solvents were of HPLC grade and purchased from Tedia (Fairfeild).

4.2.2 Sample Preparation

Stock solutions of DEHP (analyte) and DINP (IS) were prepared as 1mg/mL in ethanol containing 0.1% formic acid and stored at -20 ℃ before use. Working solutions of DEHP in various concentrations, i.e., 0.25, 0.5, 2.5, 5, 10, 25, 50, 100 μg mL^{-1}, were prepared by diluting the DEHP stock solution with ethanol containing 0.1% formic acid. The beverage solutions used for analysis were obtained by mixing beverage and the standard solution in a volume ratio of 1 : 2 firstly, and then 1 mL of each mixture solution was spiked with 20 μL of DINP stock solution.

4.2.3 Instrumentation and Setup

The length of the wooden tips used for the study was ~10 mm. The distance between the wooden tip and the MS inlet was ~8 mm (Figure 4-2). Two μL of each sample solution was loaded onto a fixed position of the wooden tip that was close to the sharp tip-end. Each sample was analyzed three times and the wooden tip was rinsed with enough ethanol before each analysis.

All spectra were acquired in positive ion mode on a Quattro Ultima mass spectrometer (Waters). Selected reaction monitoring (SRM) was operated with the following conditions: capillary voltage 3.5 kV, cone voltage 30 V, collision energy 18 eV, collision cell pirani 5.27×10^{-4} mbar. The selected reaction *m/z* 391 → 149 for DEHP quantitation was monitored with a collision energy of 18 eV and a

dwelling time of 0.2 s. The selected reaction *m/z* 419 → 149 for internal standard DINP was monitored with a collision energy of 18 eV and a dwelling time of 0.2 s. Data acquisition and processing were performed using Masslynx 4.1 software (Waters, UK).

4.3 Results and Discussion

4.3.1 DEHP Quantitation

In order to minimize the experimental errors, DINP, which has a similar chemical structure with DEHP, was chosen as the internal standard and spiked into the samples. SRM scan was used to eliminate the influences from the matrix in the beverages and enhance the selectivity and sensitivity of the detection. Both DEHP and DINP are soluble in ethanol, but not soluble in water. The MS/MS spectra of protonated molecules of DEHP and DINP, obtained using wooden-tip ESI-MS/MS with 1 μg/mL DEHP spiked with internal standard DINP are shown in Figure 4-3. The potential structures of major product ions in the spectra were labeled according to previous reports.[173, 174] Product ions m/z 149, 167, 279 were observed for DEHP, and product ions m/z 149, 275, 293 were observed for DINP. Ion m/z 149, the product ion with the highest signal intensity, was selected for SRM detection of both DEHP and DINP, along with precursor ions m/z 391 and m/z 419 for the two compounds respectively.

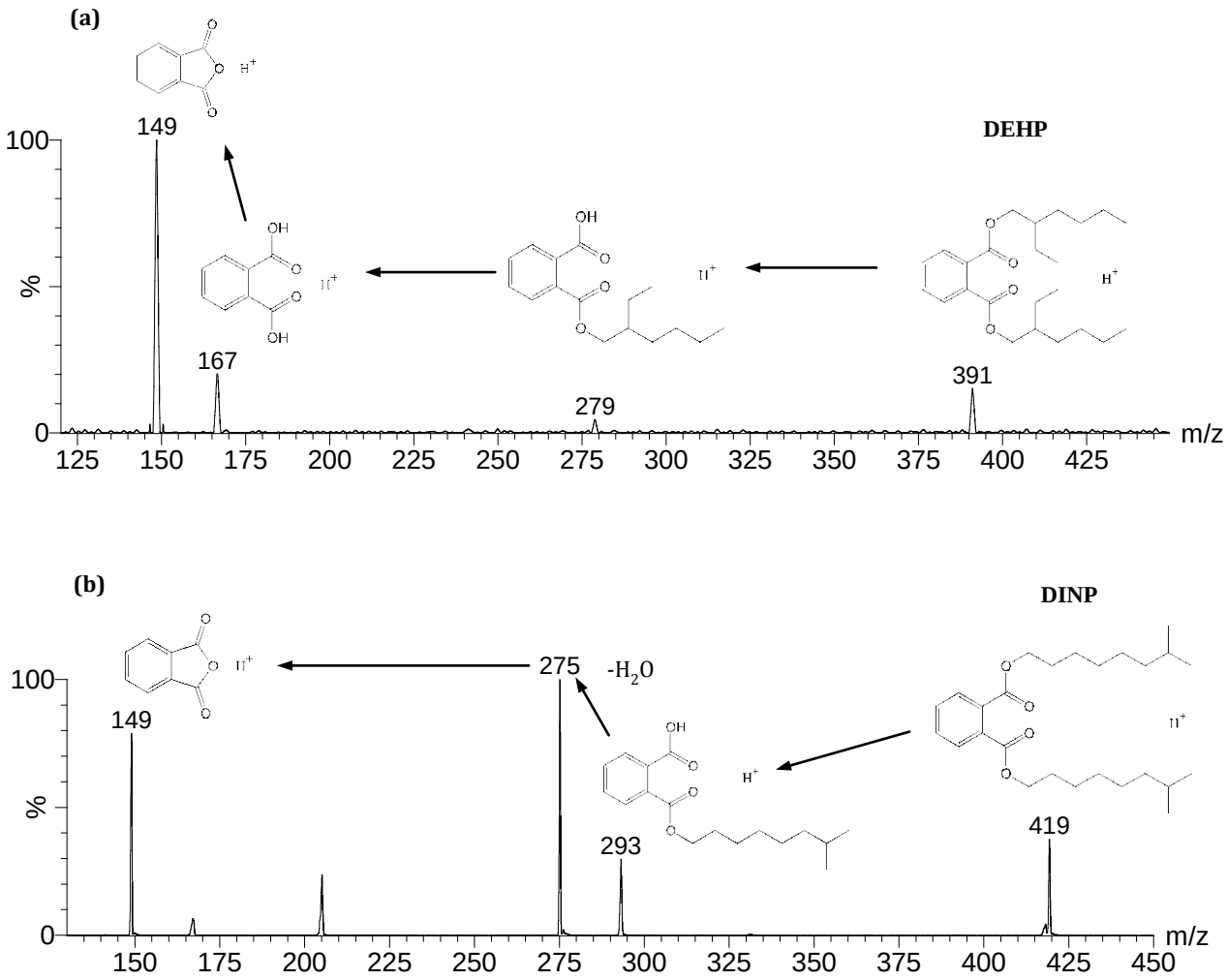


Figure 4-3 The MS/MS spectra of $[M+H]^+$ of DEHP (*m/z* 391) **(a)** and DINP (*m/z* 419) **(b)**.

The SRM chromatograms of DEHP (m/z 391 > m/z 149) and DINP (m/z 419 > m/z 149) are shown in Figure 4-4. After smoothing and peak integration using the Masslynx software, the peak area ratio of DEHP to internal standard DINP was calculated and found to have a good linear relationship against the concentration of DEHP, and the results were very reproducible.

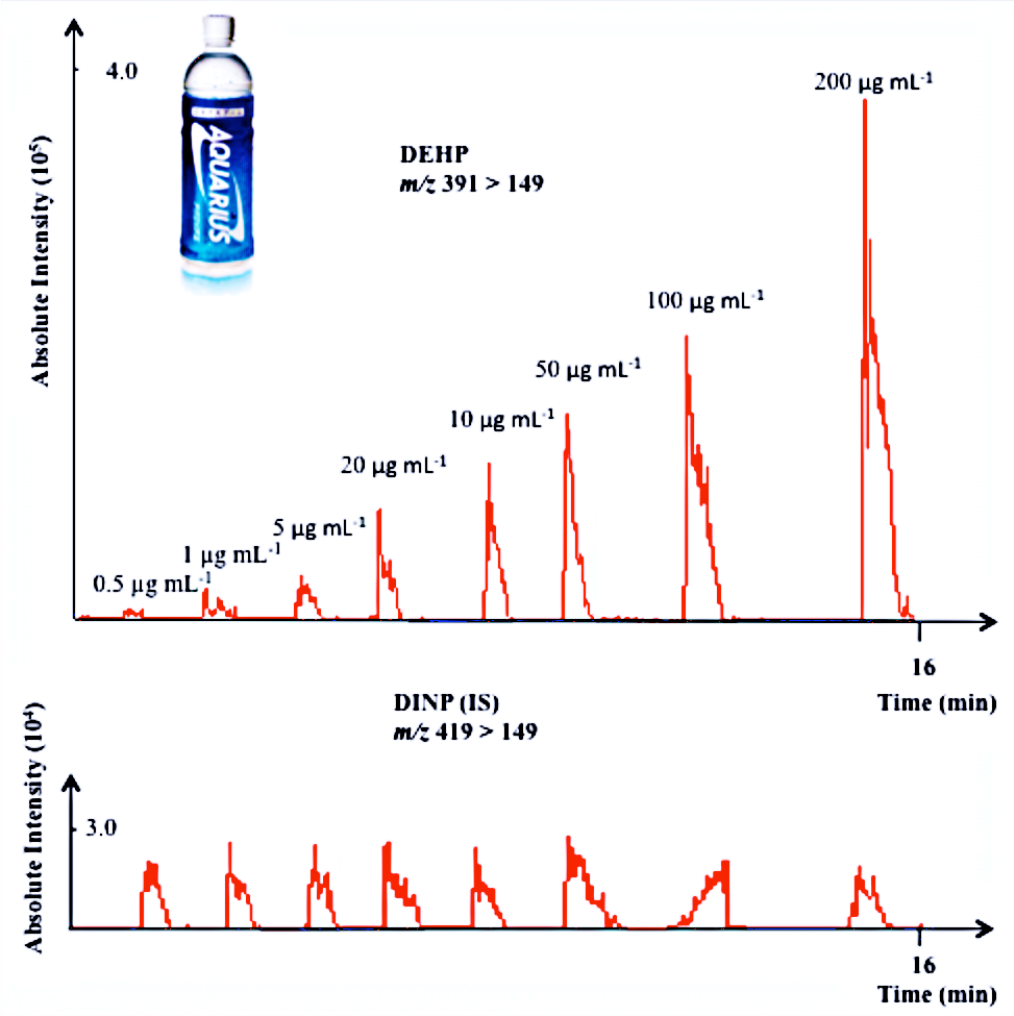
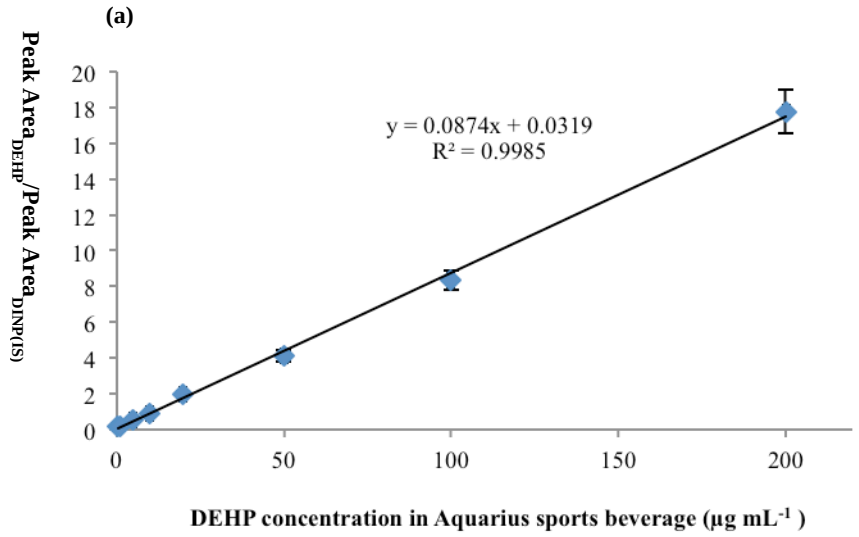
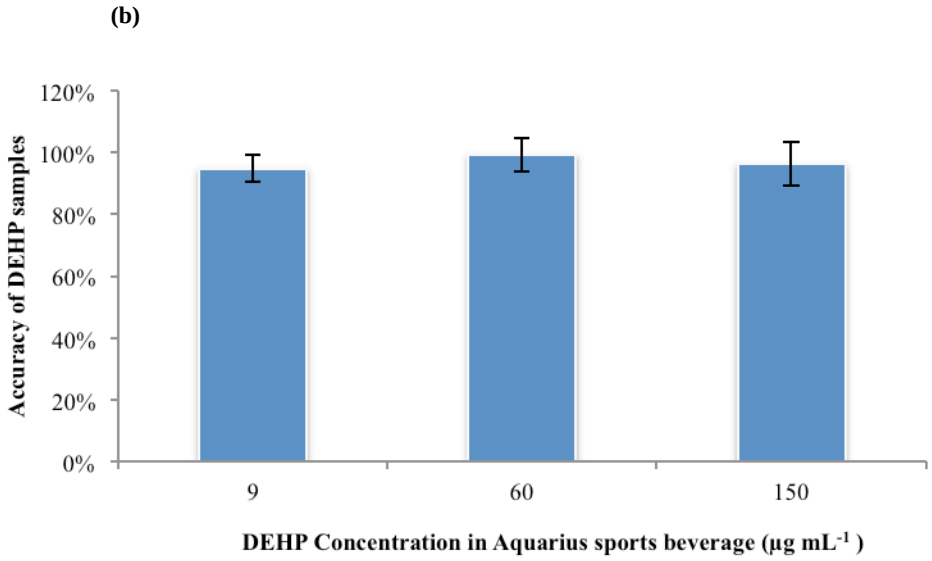


Figure 4-4 SRM chromatograms of DEHP (*m/z* 391 > 149) and DINP (*m/z* 419 > 149) obtained by wooden-tip ESI-MS analysis of the Aquarius sports beverage samples.





To be continued…

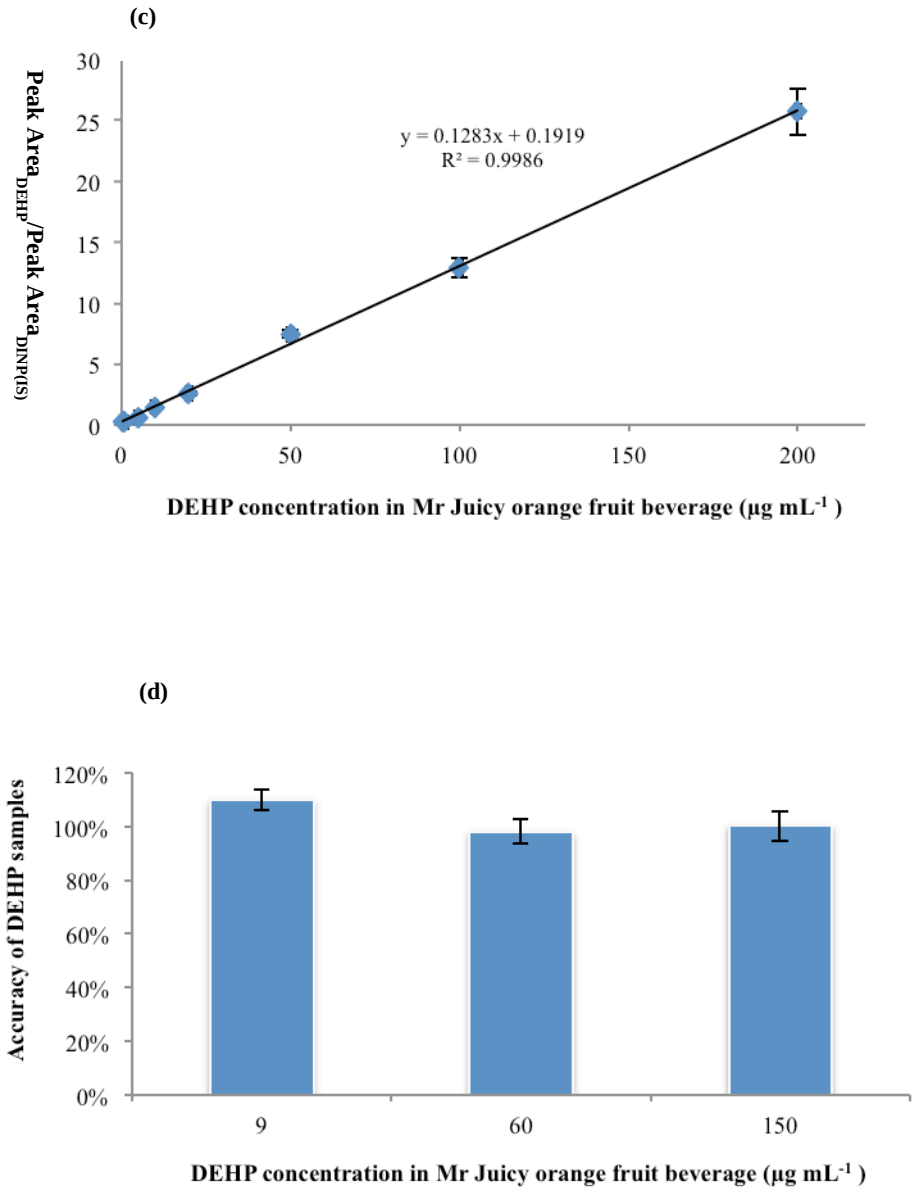


Figure 4-5 Quantitative analysis of DEHP in Aquarius sports beverage and Mr. Juicy orange juice using wooden-tip ESI-MS (with internal standard). (**a**) Standard calibration curve for DEHP in Aquarius sports beverage. (**b**) Accuracy and precision (n=6 replicates at 9 µg mL^{-1}, 60 µg mL^{-1} and 150 µg mL^{-1}) for DEHP in Aquarius sports beverage. (**c**) Standard calibration curve for DEHP in Mr. Juicy orange juice. (**d**) Accuracy and precision (n=6 replicates at 9 µg mL^{-1}, 60 µg mL^{-1} and 150 µg mL^{-1}) for DEHP in Mr. Juicy orange juice.

Table 4-1 Experimental data for determination of accuracy and precision of the wooden-tip ESI-MS in quantitation of DEHP in Aquarius sports beverage and Mr. Juicy orange juice (using DINP as the internal standard).

Spiked concentration (μg mL^{-1})	Aquarius sports beverage			Mr. Juicy orange juice		
	Measured concentration (μg mL^{-1})	Accuracy (n=6)	Precision (n=6)	Measured concentration (μg mL^{-1})	Accuracy (n=6)	Precision (n=6)
9	8.5	94.9%	4.6%	9.9	109.8%	3.5%
60	59.6	99.3%	5.4%	58.7	97.9%	4.7%
150	144.6	96.4%	7.4%	150.3	100.2%	5.5%

The peak area ratio of DEHP to DINP was plotted against the DEHP concentration and linear calibration curves over the concentration range of 0.5-200 μg/mL for both beverage samples were obtained (Figure 4-5a and 4-5c). The correlation coefficients (R^2) for both curves were better than 0.99. According to the standards of the US Food and Drug Administration, the accuracy and precision should be less than 15% for bioanalytical method validation.[175] To investigate the accuracy and precision of the method, the sports and orange beverage samples were spiked with DEHP in 9, 60, 150 μg mL^{-1}, representative of low, medium, and high concentration ranges respectively, and were measured with the wooden-tip ESI-MS method. The measured concentrations were all within 100 ± 9.8% of the spiked concentrations and the relative standard deviations (RSD) of the peak area ratio of DEHP to DINP at all examined

concentrations were less than 7.4% (Figure 4-5b, 4-5d and Table 4-1), indicating that the accuracy and precision of this method were well acceptable.

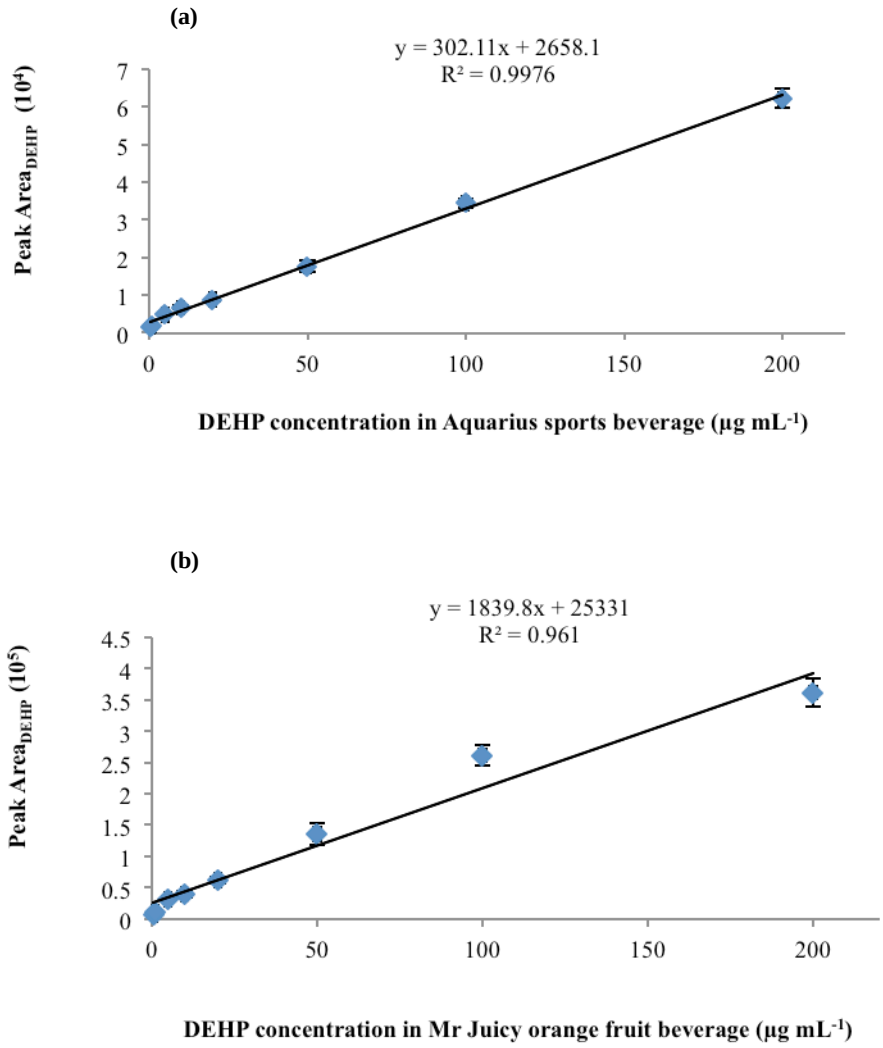


Figure 4-6 Quantitative analysis of DEHP in Aquarius sports beverage and Mr. Juicy orange juice using wooden-tip ESI-MS without internal standard). (**a**) Standard calibration curve for DEHP in Aquarius sports beverage. (**b**) Standard calibration curve for DEHP in Mr. Juicy orange juice.

4.3.2 Effect of The Internal Standard

DINP was chosen as the internal standard for analysis of DEHP because it has a chemical structure and properties similar with DEHP. The internal standard was used to reduce the errors coming from variations in sample processing, sample loading and MS responses during the experiments. With the use of DINP as the internal standard, the accuracy and precision obtained for DEHP in this assay were within well acceptable levels. If the internal standard was not incorporated for the measurement and the peak area of DEHP was directly used for plotting against the concentration of DEHP, the obtained calibration curves are shown in Figure 4-6. Linear relationship could also be achieved for the two beverages over the examined concentrations (0.5-200 µg/mL), however, with a correlation coefficient of 0.9976 and 0.961 respectively. The accuracy measured using the three concentrations (9 µg mL^{-1}, 60 µg mL^{-1} and 150 µg mL^{-1}) was in the range of 86.7-112.8% and the precision was in the range of 8.9-14% (Table 4-2), which were still acceptable for the analysis. These results demonstrated that the use of internal standard could improve the quantitation, and sometimes may not be necessary, particularly for rapid detection and when no suitable internal standard is available.

Table 4-2 Experimental data for determination of accuracy and precision of the wooden-tip ESI-MS in quantitation of DEHP in Aquarius sports beverage and Mr. Juicy orange juice (without internal standard).

Spiked concentration (μg mL^{-1})	Aquarius sports beverage			Mr. Juicy orange juice		
	Measured concentration (μg mL^{-1})	Accuracy (n=6)	Precision (n=6)	Measured concentration (μg mL^{-1})	Accuracy (n=6)	Precision (n=6)
9	8.2	91.1%	10.0%	7.8	86.7%	9.8%
60	65.3	108.8 %	8.9%	67.7	112.8%	14.0%
150	141.3	94.2%	11.4%	139.5	93.0%	10.1%

4.3.3 Limit of Detection and Limit of Quantitation

A TIC peak could be observed even a blank sample, e.g., solvent only, was loaded onto the wooden tip. Such ion signals might arise from the background and electronic noises. In this study, LOD and LOQ were defined as the DEHP concentration that could achieved three and ten times peak area ratio (DEHP/DINP) of the blank sample respectively. As shown in Figure 4-7, the LOD and LOQ were determined to be 0.25 μg mL^{-1} and 0.5 μg mL^{-1} respectively for the fruit beverage, and 0.25 μg mL^{-1} and 0.5 μg mL^{-1} respectively for the sports beverage.

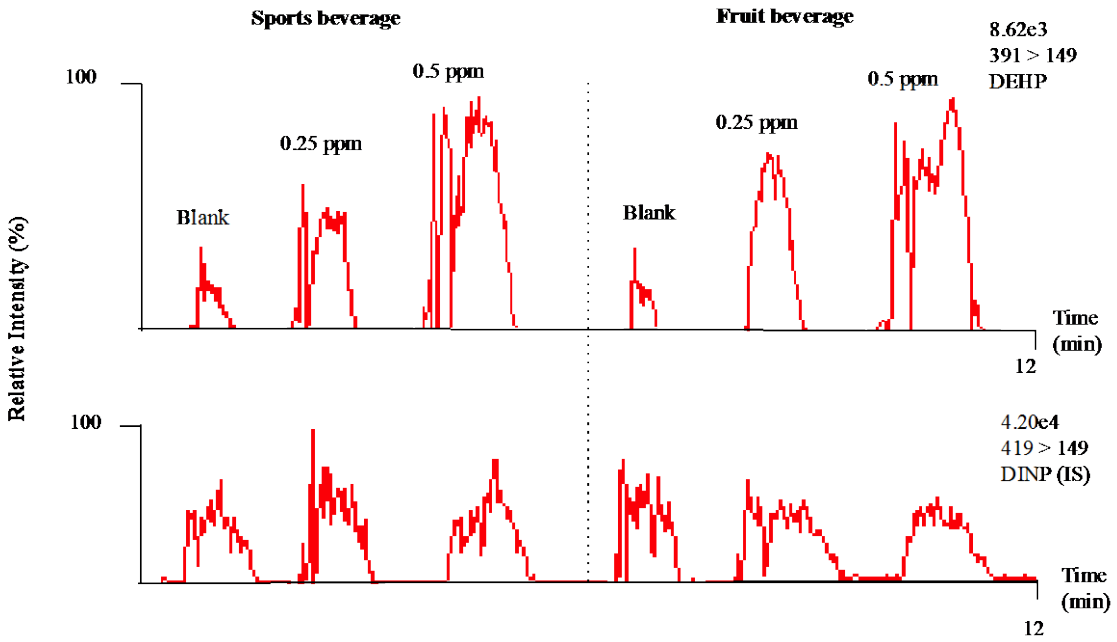


Figure 4-7 SRM chromatograms for the LOD and LOQ determination of the sports beverage and fruit beverage.

4.4 Conclusions

Quality control of beverage is of importance to public health, especially after the DEHP scandal. The growing public concern over tainted food requires methods for fast screening of DEHP or other related contamination compounds in various samples. Herein, a rapid and simple method for quantitative assay of DEHP in beverages was developed. With the use of DINP as the internal standard, the calibration curve showed well acceptable accuracy (94.9-109.8%) and precision (3.5-7.4%). The linear range was 0.5-200 µg/ml with the square regression coefficient (R^2) better than 0.99. The LOD and LOQ were 0.25 µg mL^{-1} and 0.5 µg mL^{-1} respectively for the two beverages

investigated. This technique can also be used for analysis of other similar compounds for food and beverage safety in the future.

Chapter 5 Investigation of Membrane Proteins by Extractive Electrospray Ionization Mass Spectrometry

5.1 Introduction

Membrane proteins are proteins that are attached to or associated with the membrane of a biological cell or organelle. Integral and peripheral membrane proteins are two different kinds of membrane proteins. Integral membrane proteins are permanently attached to the membrane and could be separated from the membranes using detergents or nonpolar solvents; Peripheral membrane proteins are temporarily attached either to the membrane or to intergral proteins and could be dissociated in polar reagents. It was estimated that about 25% of the human proteins were membrane proteins.[176] Membrane proteins play a key role in mediating the interactions of the cell with the surroundings, e.g., ensuring the stability of the cell, allowing cells to identify and interact with each other, mediating cellular responses upon binding of ligands, performing movements of substrates across membranes by utilizing electrochemical gradients or energy from chemical reactions, and catalyzing chemical reactions.[177-180] Many diseases, e.g., epilepsy, cataract, lungedema, hyperinsulinemia, congestive heart failure, livercirrhosis, nephrogenic diabetes insipidus and cystic fibrosis, are related to membrane proteins.[181-183] Membrane proteins are the most popular targets of drugs.[184] It was estimated that about 80% of the detected drug targets belonged to membrane protein family.[185]

For studies of membrane proteins, the major difficulties come from the poor solubility of membrane proteins in aqueous solutions due to the hydrophobicity of membrane proteins. Detergents, which are partly hydrophilic (polar) and partly hydrophobic (non-polar), are commonly used to solubilize, extract and purify membrane proteins.[132] According to their chemical properties, detergents can be divided into two types: ionic

detergents and nonionic detergents. Sodium dodecyl sulfate (SDS) is the most commonly used ionic detergent in proteomics,[132] and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) is another commonly used ionic detergent. *n*-dodecyl β-D-maltoside (DDM), n-octyl-β-D-glucoside (OG) and Triton X-100 are commonly used nonionic detergents.[132] MS is a powerful technique for investigation of proteins. However, the presence of detergents can cause shape distortion of protein signals,[186] adduct formation and signal suppression for MS analysis. Removal of detergents using techniques typically involving extraction or chromatography separation is thus required prior to MS analysis and such removal is usually complicated and time-consuming.[130, 131, 187] One of our research efforts was to develop mass spectrometric techniques for direct analysis of membrane proteins. In our previous study, we attempted to use C18 pipette-tip ESI-MS for analysis of membrane protein samples containing detergents, as described in chapter 3. In this chapter, our new efforts in this respect will be presented.

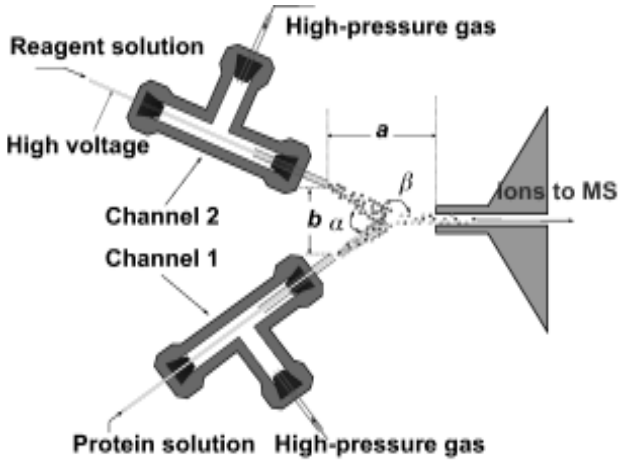


Figure 5-1 Schematic diagram of EESI-MS.[51]

Extractive electrospray ionization mass spectrometry (EESI-MS) is a new technique that allows direct analysis of analytes, including proteins, in complex samples, e.g., milk, urine, saliva and polluted water.[51, 64] In EESI-MS, there are two separate sprayers: one sprayer to nebulize the sample solution without application of a high voltage and the other one to produce charged microdroplets of solvent in a way similar to conventional ESI sprayer (Figure 5-1). The compounds of interest in the sample solution can be extracted from the plume of the sample solution by the charged solvent droplets. EESI could thus be considered as an ESI technique combined with extraction.

Herein, EESI-MS was attempted for direct analysis of membrane proteins in the presence of detergents for the first time. The lab-made EESI ion source, which was stable and adjustable, was successfully coupled with a Q-ToF mass spectrometer (Figure 5-2). Our results showed that compared to ESI-MS, EESI-MS was much more tolerant to some detergents and could be used to directly analyze membrane proteins in the presence of high concentrations of those detergents.

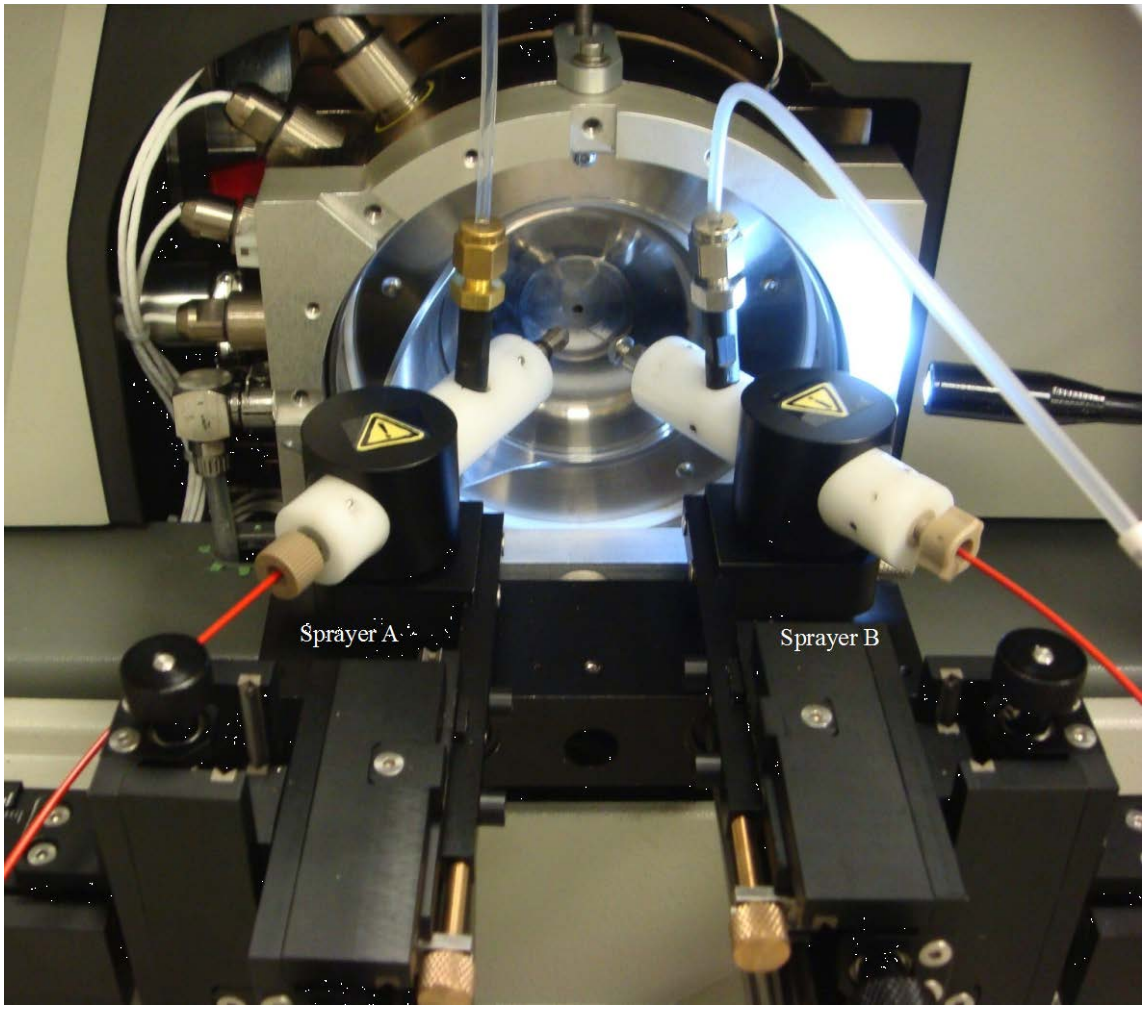


Figure 5-2 Photo of the lab-made EESI ion source installed on a Q-TOF mass spectrometer (sprayer A is the solvent spray connected a high voltage and sprayer B is the neutral spray of sample solution).

5.2 Experimental Section

5.2.1 Materials and Chemicals

Membrane protein α-Lactalbumin and detergents DDM, OG, Triton X-100, SDS and CHAPS were purchased from Sigma-Aldrich and used without further purification. Water was distilled water prepared using a Milli-Q system (Millipore, USA). All the other solvents were of HPLC grade and purchased from Tedia (Fairfeild).

5.2.2 Sample Preparation

α-Lactalbumin was dissolved in 50% methanol (containing 0.1% formic acid) at a concentration of 5.0 μM with different concentrations of DDM (0.2, 2, 20, 50 mM) or other detergents, and infused into sprayer B as sample solution for EESI-MS analysis or directly used for ESI-MS analysis using a syringe pump (Harvard Pump 11 Plus). Solvents were delivered into sprayer A of the EESI ion source by another syringe pump to generate charged droplets of solvents with application of the applied high voltage.

5.2.3 Instrumentation and Setup

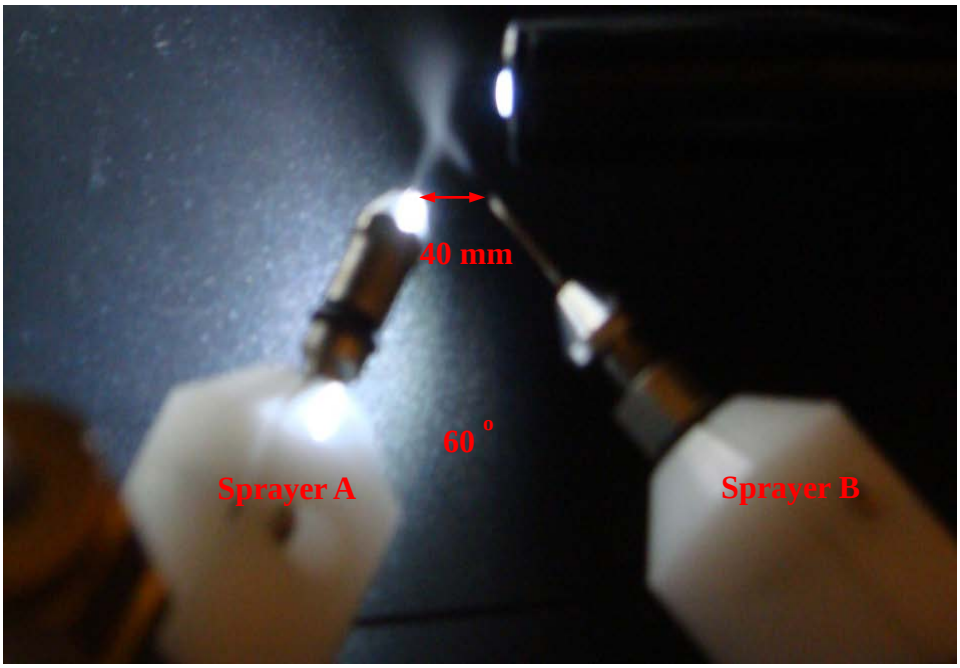


Figure 5-3 Photo of the optimized alignment of the EESI ion source for direct analysis of α-lactalbumin with detergents (the angle between sprayer A and sprayer B was 60°, the distance between the tips of two sprayers was 40 mm and the distance between the tips of sprayer A to MS inlet was 2 cm).

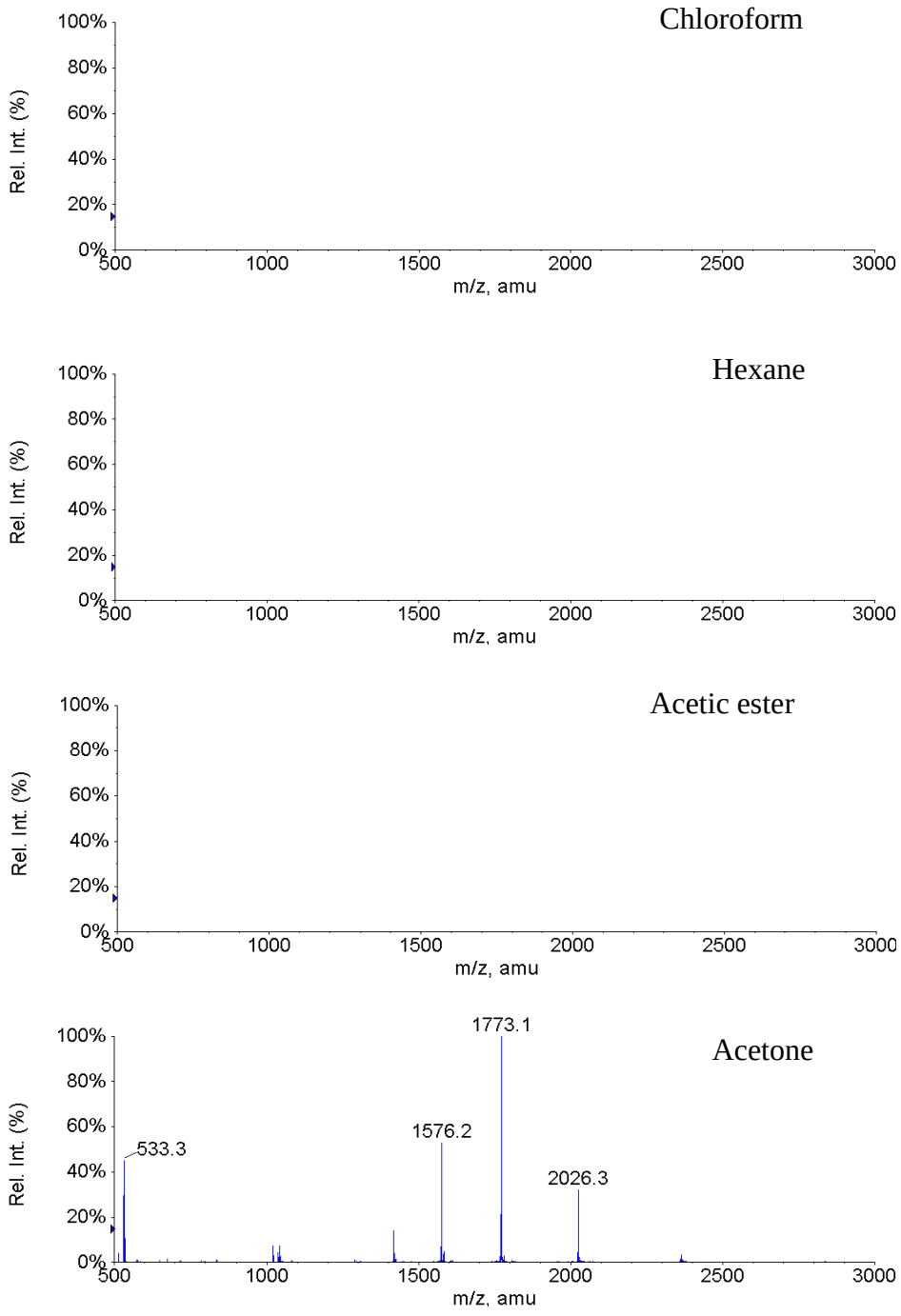
All mass spectra were acquired in positive ion mode using a Q-TOF mass spectrometer (QStar Pulsar, Applied Biosystems) fitted with a lab-made EESI ion source. As shown in Figure 5-2, the EESI ion source has two sprayer, spray A and spray B, both of which are adjustable for accurate movement of going up, down, left, right, ahead and back. After optimization of the ion source setup, the detailed position of each sprayer could be identified by the scale and reproducibly used for subsequent analysis. The high voltage

of sprayer A was provided by the power supply of the QSTAR mass spectrometer. The angles and distances between the two sprayers of the EESI ion source were adjusted as shown in Figure 5-3 to get optimum signals. Parameter settings of the mass spectrometer are as follows: curtain gas (CUR) 25, ion source gas 1 (GS1, connected with sprayer A) 25 A. U., ion source gas 2 (GS2, connected with sprayer B) 30 A. U., ion spray voltage (IS) 5500 kV.

5.3 Results and discussion

5.3.1 Optimization of Extraction Solvent

The major advantage of EESI is that during the desorption/ionization process, specific analyte in a complex sample selectively ionized by using extraction solvents.[64] The choice of suitable extraction solvents is thus crucial for the analysis. In this study, the extraction solvents were optimized firstly using α-lactalbumin, a typical membrane protein, along with DDM, a nonionic detergent, as the model sample.



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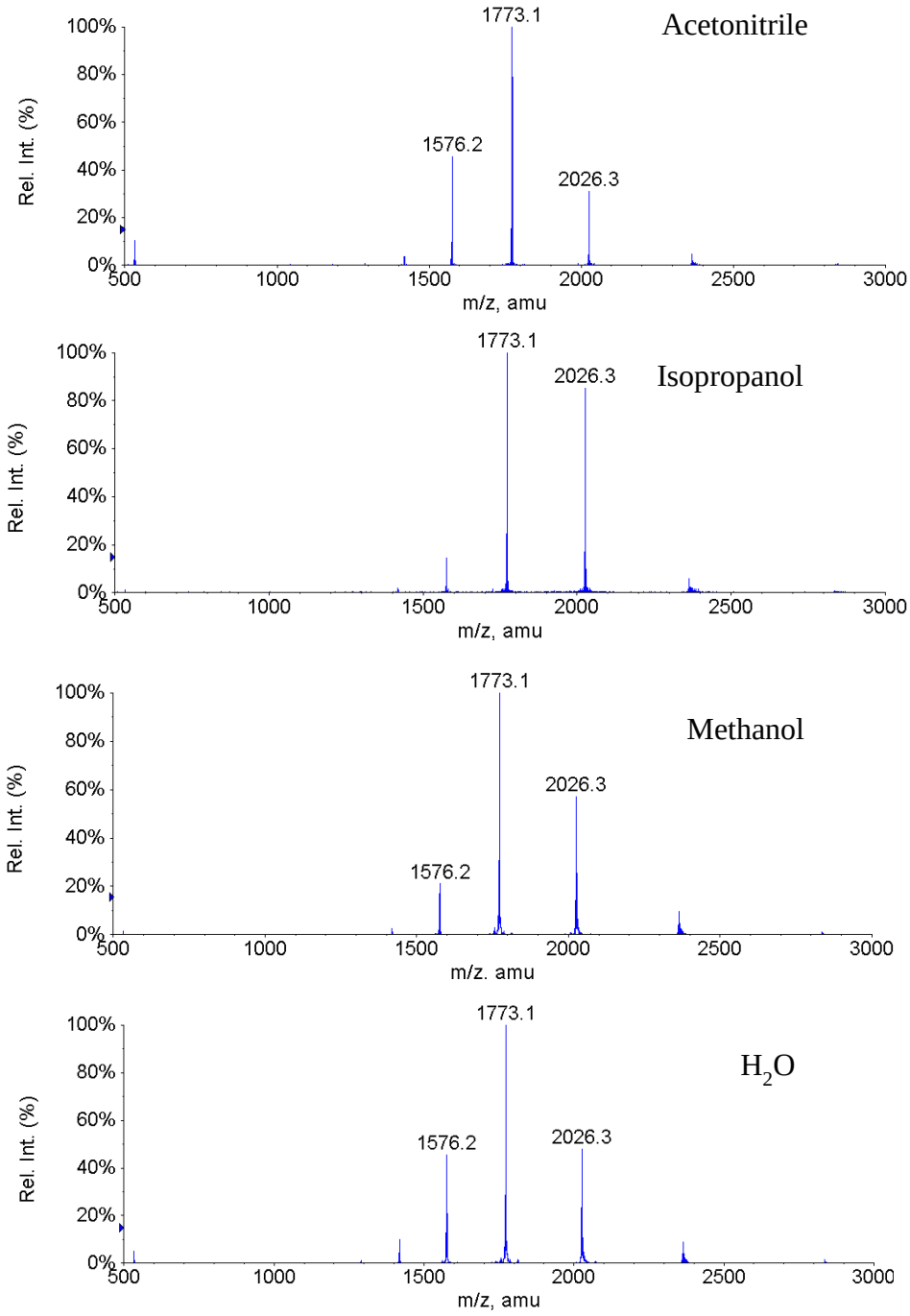


Figure 5-4 EESI-MS spectra of α-lactalbumin (5.0 μM in MeOH/H_2O/FA, 50/50/0.1, v/v/v) in the presence of 0.2 mM DDM obtained with various solvents (each containing 1% FA) as the extraction solvents.

Figure 5-4 showed the EESI-MS spectra of 5.0 μM α-lactalbumin in MeOH/H_2O/FA (50/50/0.1, v/v/v) in the presence of 0.2 mM DDM. The peaks detected at *m/z* 1576.2 (+ 9), 1772.1 (+ 8) and 2026.3 (+ 7) corresponded to the multiply charged ions of α-lactalbumin. DDM were detected at *m/z* 533.3 ($[M+Na]^+$). In order to increase the relative intensity of the membrane protein and reduce the relative intensity of DDM, different kinds of solvents (each containing 1% FA) were employed as the extraction solvents to improve the extraction efficiency of the membrane protein in EESI-MS. As shown in Figure 5-4, quality spectra were obtained when isopropanol, methanol and water were used as the extraction solvents, indicating better extraction and ionization efficiencies of these solvents for the membrane protein. No significant signals were observed when chloroform, hexane and acetic ester were used as the extraction solvents, probably due to their poor ionization efficiencies.

Figure 5-5 shows the spectra obtained using methanol mixed with different portions of water as the extraction solvents. No significant spectral difference was observed when the water ratio in the solvent was increased from 0% to 100%. So 50% methanol (1% FA), a solvent commonly used in ESI-MS analysis, was employed as the extraction solvent in this study.

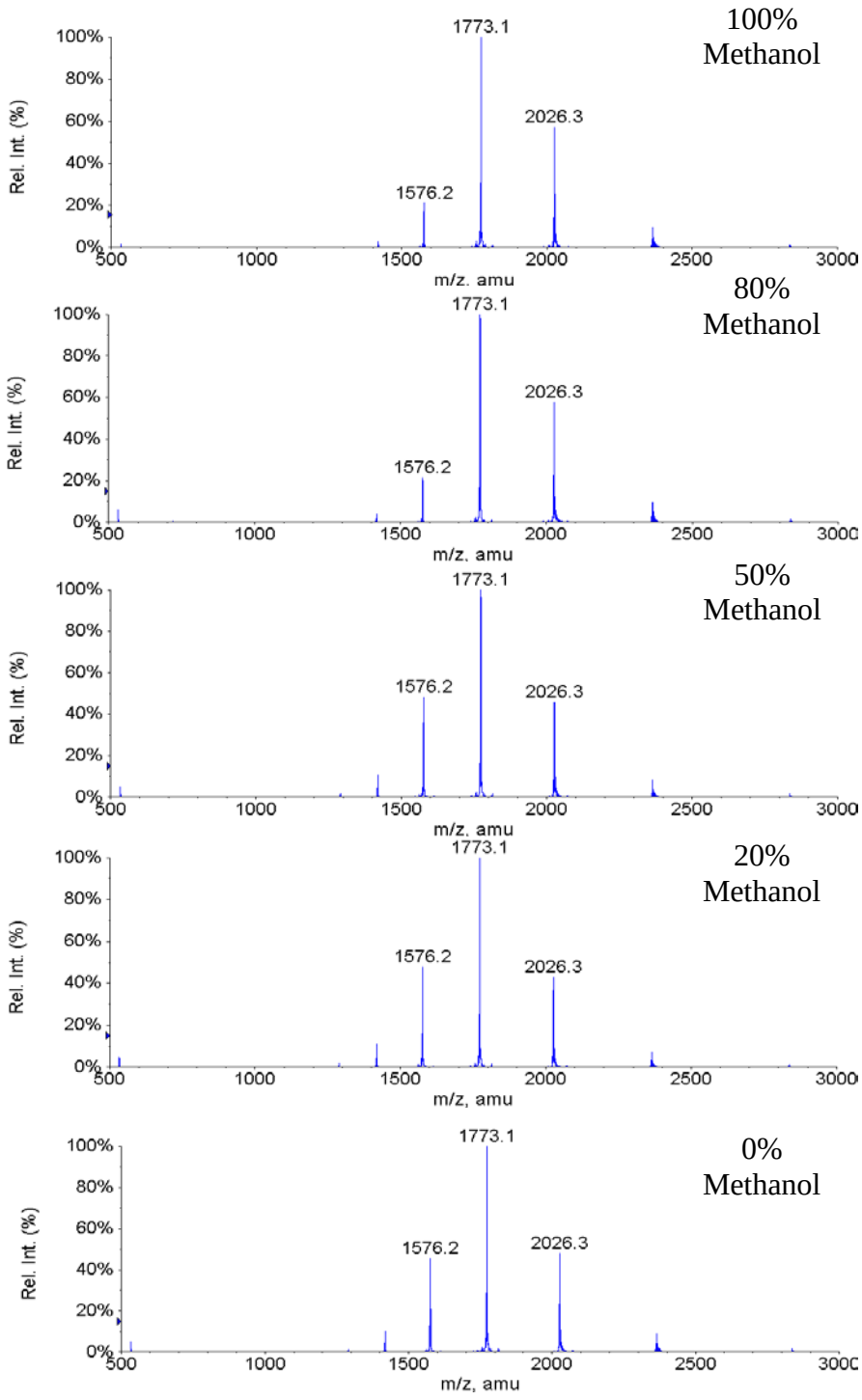


Figure 5-5 Spectra obtained by EESI-MS analysis of α-lactalbumin solution (5.0 μM in MeOH/H_2O/FA, 50/50/0.1, v/v/v) containing 0.2 mM DDM using methanol with different portions of water as the extraction solvents (each containing 1% FA).

5.3.2 Tolerance of EESI-MS to Different Kinds of Detergents

The tolerance of EESI-MS to detergents for analysis of membrane proteins was examined by comparing that of conventional ESI-MS. α-Lactalbumin with nonionic detergent DDM was tseted firstly. As shown in Figure 5-6, α-lactalbumin with different concentrations of DDM was analyzed by both ESI-MS and EESI-MS for comparison. For EESI-MS, 5.0 μM α-lactalbumin in MeOH/H_2O/FA (50/50/0.1, v/v/v) with different concentration of DDM (0.2, 2, 20, 50 mM) was delivered into a sprayer, and 50% methanol containing 1% FA, the optimized spray solvent system, was infused into another sprayer under the application of a high voltage. Comparing the acquired mass spectra, the multiply charged ions of α-lactalbumin could be detected with both conventional ESI-MS and EESI-MS in the presence of 0.2 mM DDM. When the DDM concentration was higher than 0.2 mM, the multiply charge ions of α-lactalbumin could only be detected with EESI-MS but not ESI-MS. These results demonstrated that EESI-MS was much more tolerant to DDM than ESI-MS and allowed direct analysis of the membrane protein samples containing high concentrations of detergents. Using EESI-MS, the time-consuming and laborious procedures for removal of detergents and the sample loss thus caused could be avoided.

Tolerance of EESI-MS to another commonly used nonionic detergent, octyl glucoside (OG), was investigated by comparison with ESI-MS, and the spectra obtained are shown in Figure 5-7. In addition to the multiply charged ions of α-lactalbumin, cluster ions of OG were also observed at *m/z* 310 ($[M+NH_4]^+$), 315 ($[M+Na]^+$), 602 ($[2M+NH_4]^+$), 607 ($[2M+Na]^+$), 1187 ($[4M+NH_4]^+$), 1479 ($[5M+NH_4]^+$) and 1772 ($[6M+NH_4]^+$). Similar to

the investigation of DDM, the spraying solvent for OG analysis with EESI-MS was optimized, and finally 80% methanol (1% FA) was employed as the spray solvent. As shown in Figure 5-7, in the presence of 2.5 mM OG, the protein signals were more obvious to be detected with EESI-MS than ESI-MS. When the concentration of OG was higher than 2.5 mM, i.e., 12.5, 25 and 50 mM, only the OG cluster ions but no protein signals could be observed in the ESI-MS spectra; while in the EESI-MS spectra, the protein signals could still be significantly detected. These results revealed that EESI-MS was much more tolerant to OG than ESI-MS.

Other nonionic detergents, including Triton X-100 and tween-20, were also examined with 50% methanol (1% FA) as the extraction solvent for EESI-MS. As shown in Figures 5-8 and 5-9, EESI-MS showed better tolerance to both of these two nonionic detergents than ESI-MS for analysis of α-lactalbumin.

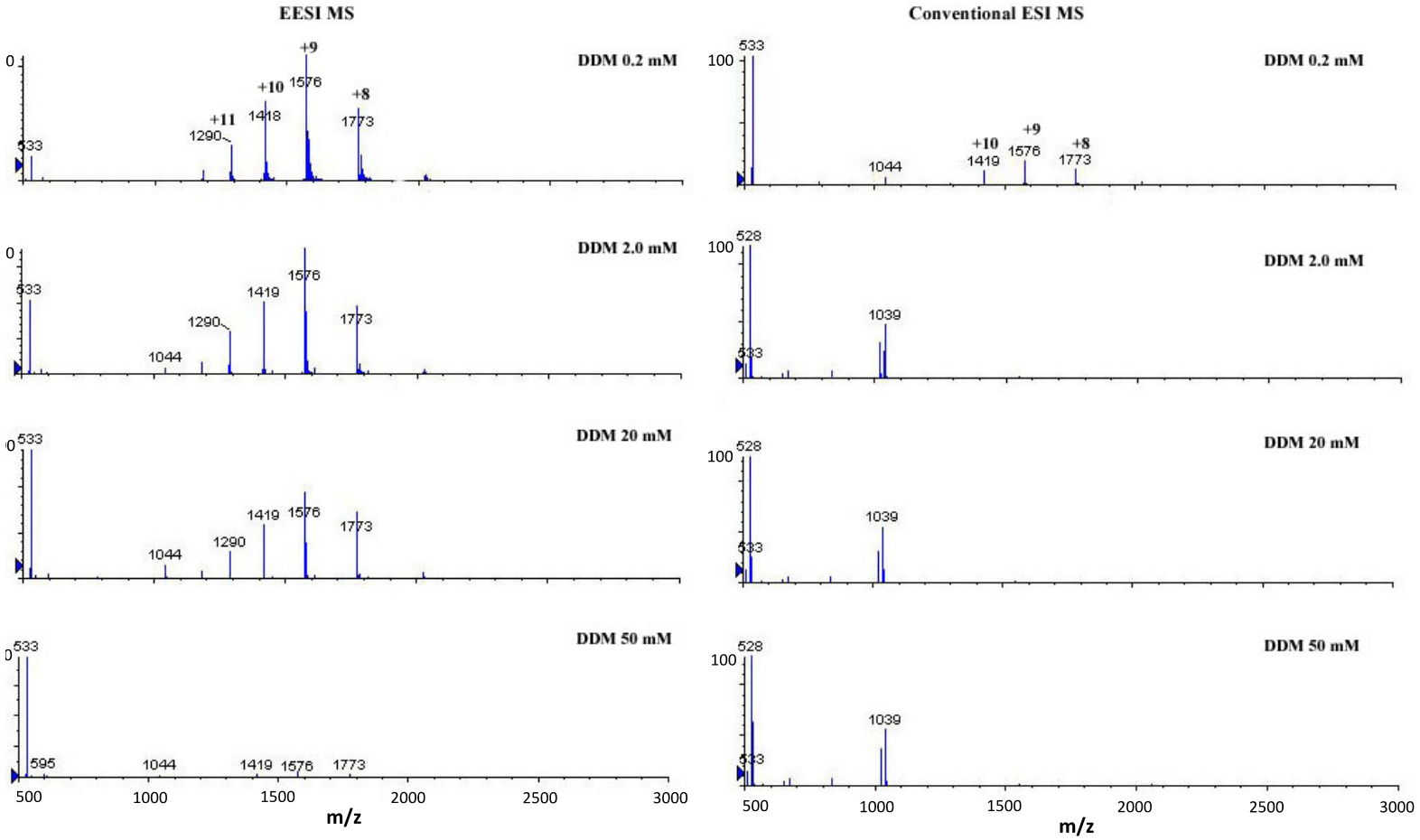


Figure 5-6 Mass spectra of 5.0 μM α-lactalbumin solution in the presence of different concentrations of DDM (0.2, 2, 20 and 50 mM) obtained by EESI-MS and ESI-MS.

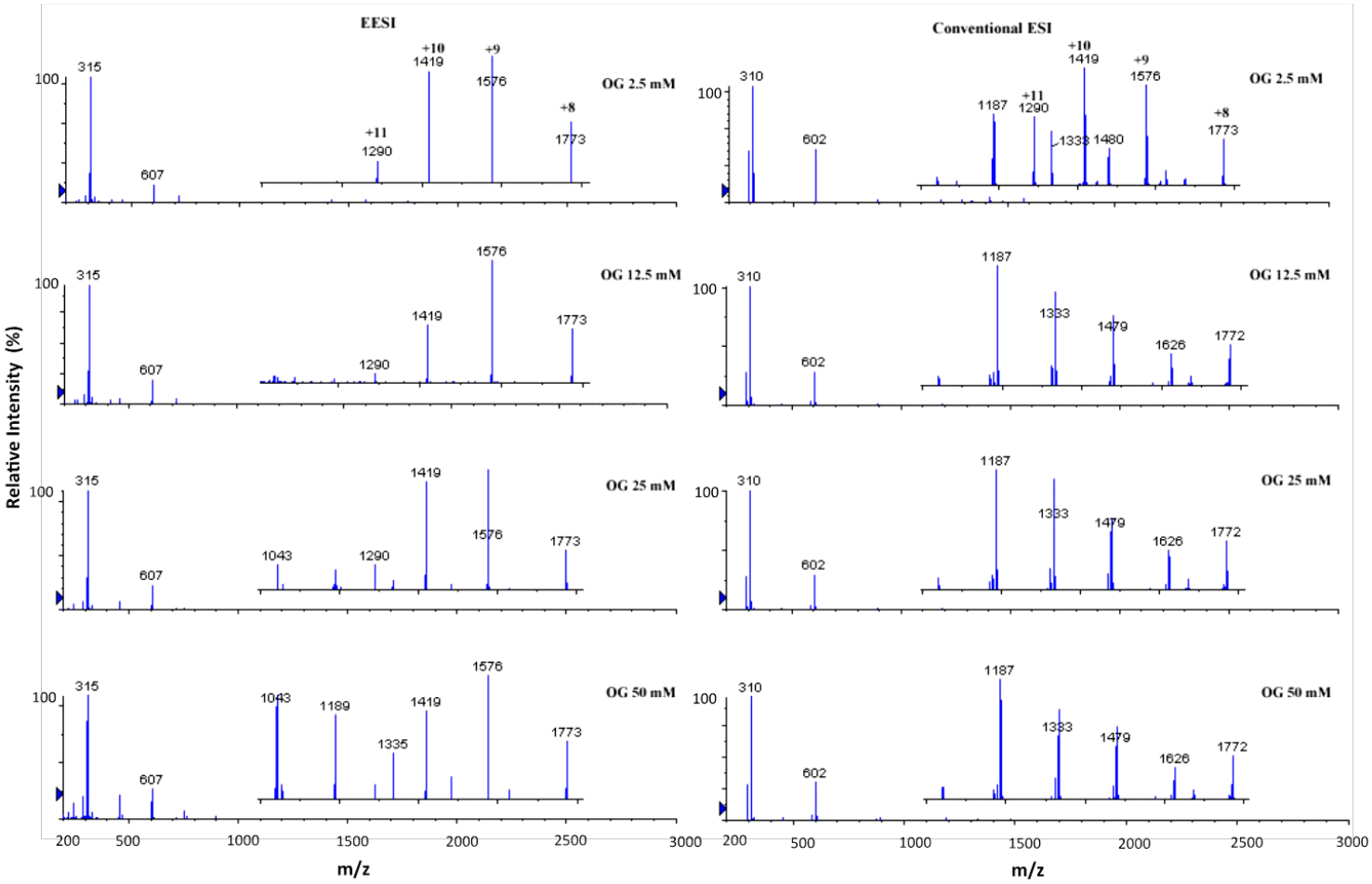


Figure 5-7 Mass spectra of 5.0 μM α-lactalbumin in the presence of different concentrations of OG (2.5, 12.5, 25 and 50 mM) obtained by EESI-MS and ESI-MS.

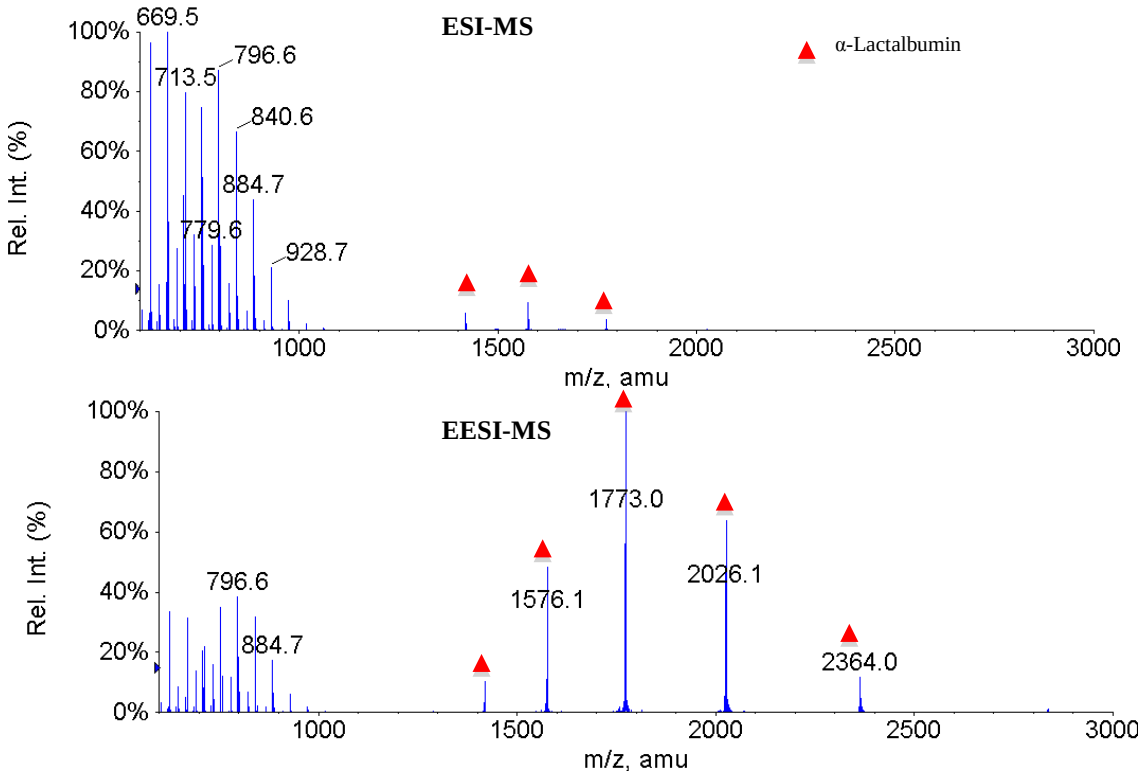


Figure 5-8 Mass spectra of 5.0 μM α-lactalbumin in the presence of 10 mM Triton X-100 obtained by EESI-MS and ESI-MS.

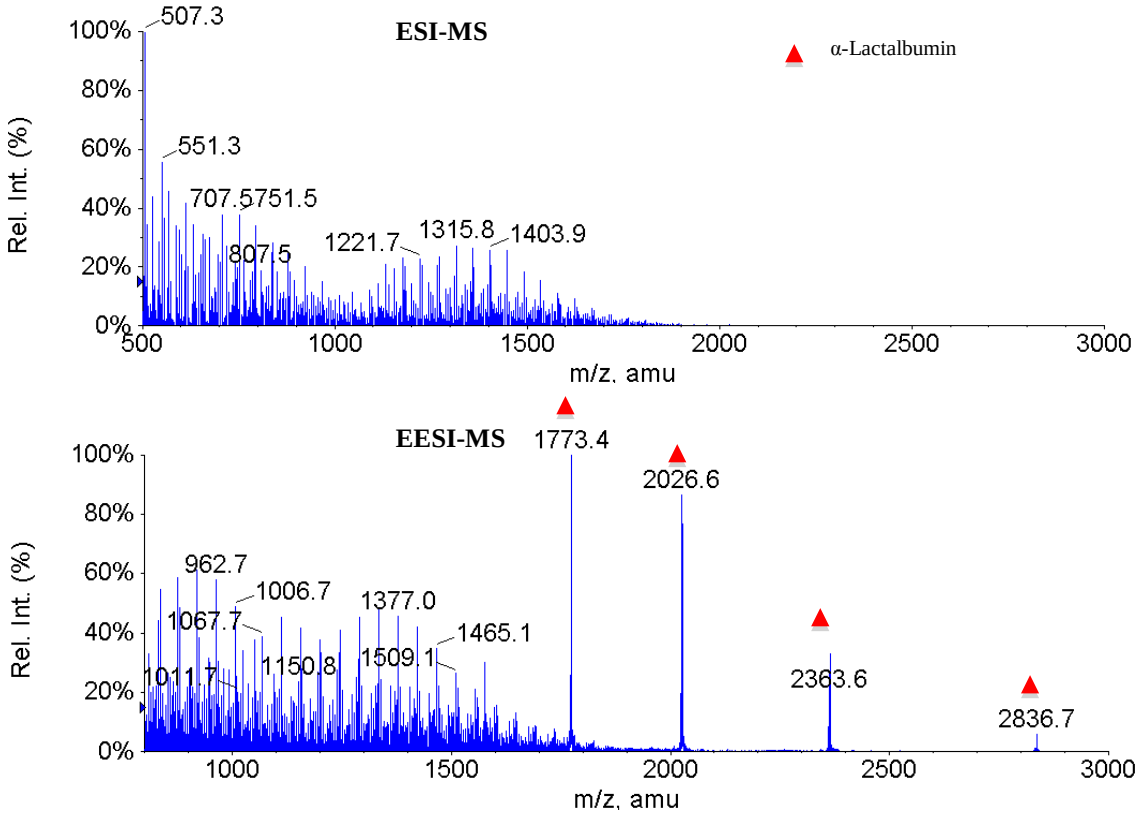


Figure 5-9 Mass spectra of 5.0 μM α-lactalbumin in the presence of 10 mM tween-20 obtained by EESI-MS and ESI-MS.

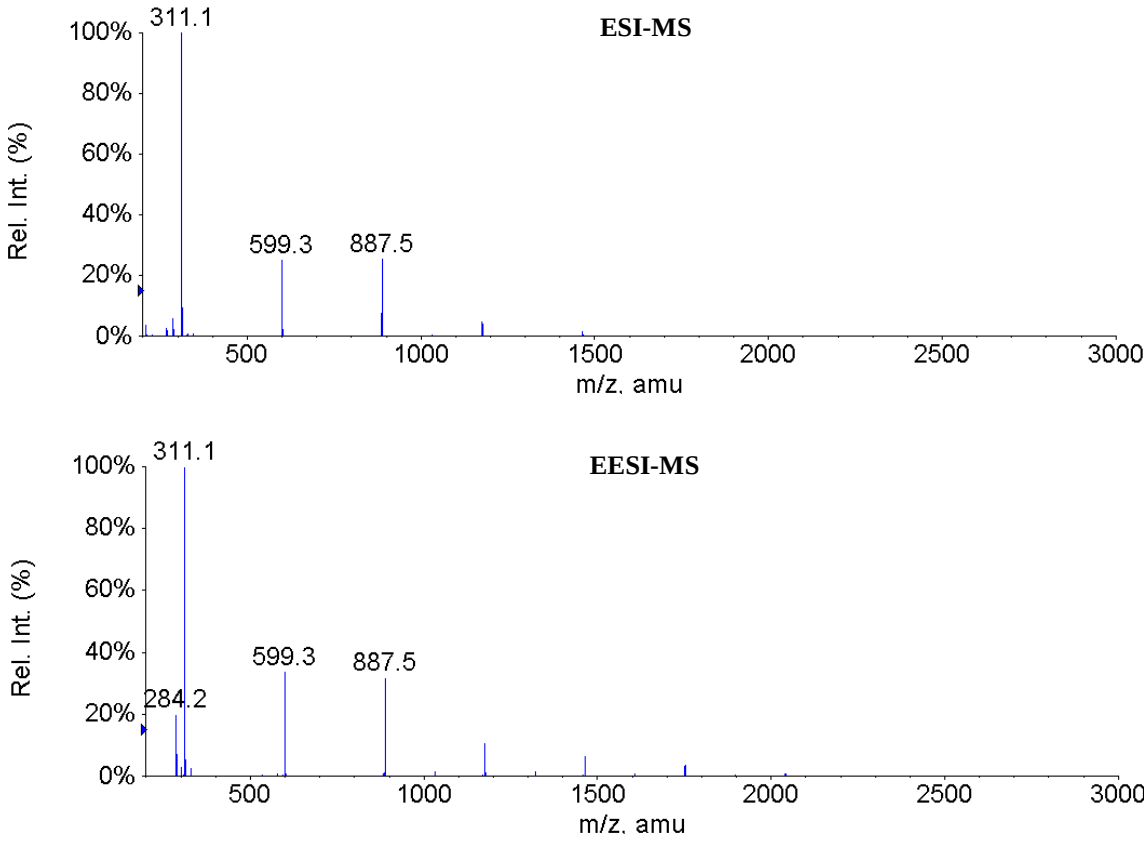


Figure 5-10 Mass spectra of 5 μM α-lactalbumin in the presence of 1.0 mM SDS obtained by EESI-MS and ESI-MS.

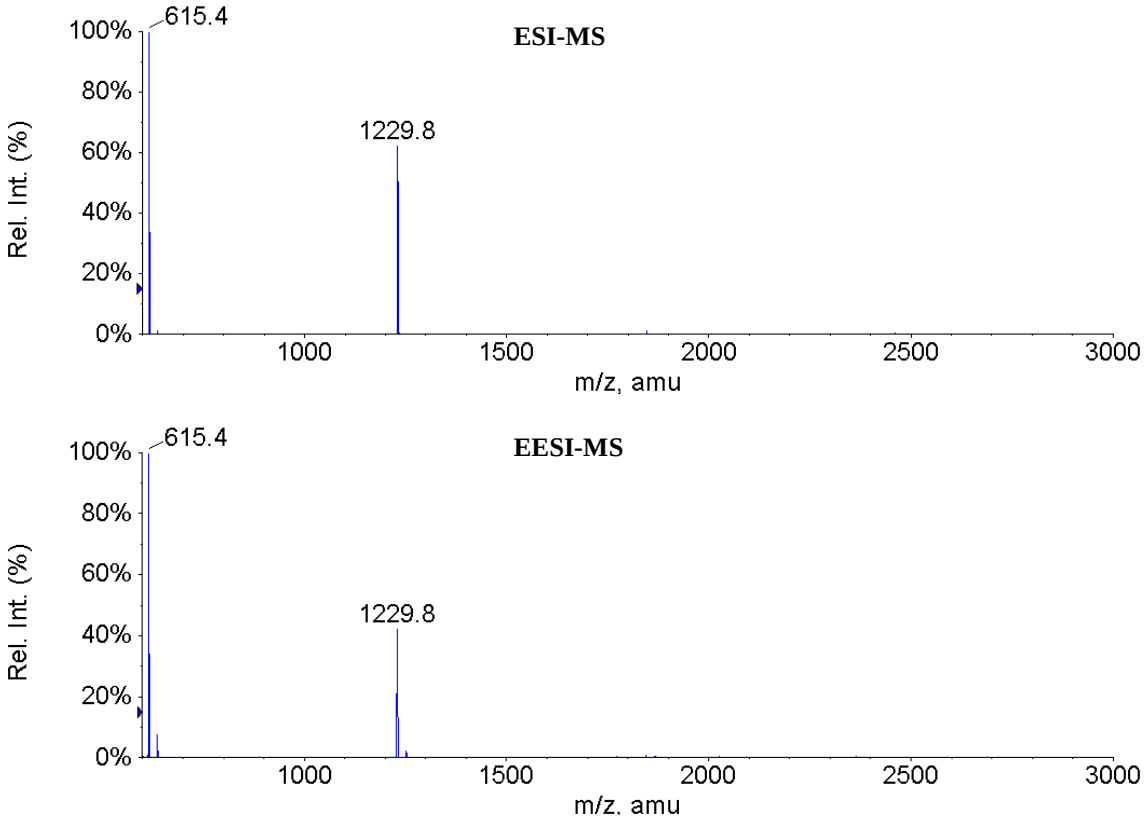


Figure 5-11 Mass spectra of 5 μM α-lactalbumin in the presence of 1.0 mM CHAPS obtained by EESI-MS and ESI-MS.

Tolerance of EESI-MS to two ionic detergents, i.e., sodium dodecyl sulfate (SDS) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), were examined as well, in comparison with that of ESI-MS. 50% methanol (1% F.A.) was used as the extraction solvent of EESI-MS for both detergents. As shown in Figure 5-10 and 5-11, no protein signals were observed in both EESI-MS and ESI-MS for the two ionic detergents even when the detergent concentration was only 1.0 mM. Similar results were obtained when methanol mixed with different portions of water (20% and 80% methanol), other hydrophilic or hydrophobic solvents (each containing 1% FA) were used as the extraction solvents. These results indicated that EESI was not tolerant to

ionic detergents as to nonionic detergents. This might be due to the differences in chemical and physical properties, including the ionization efficiency, solubility and polarity, between the nonionic detergents and ionic detergents. The tolerance of EESI-MS to nonionic detergents might originate from the extractive spraying solvent that could extract and ionize the membrane protein from the sample solution containing the nonionic detergents. In the case of ionic detergents, the ionic detergents had high ionization efficiencies and were difficult to be ruled out from the extraction, and were thus predominately observed in the spectra.

5.4 Conclusions

An adjustable and stable EESI ion source was constructed and coupled with a QSTAR mass spectrometer for analysis of membrane proteins in the presence of detergents in this study. α-Lactalbumin, along with nonionic and ionic detergents, two major kinds of detergents, were investigated, and the mass spectra obtained were compared with those obtained by conventional ESI-MS. The results revealed that EESI-MS was much tolerant to nonionic detergents than conventional ESI-MS for analysis of α-lactalbumin. However, no improvement in tolerance to ionic detergents was observed with EESI-MS as compared to ESI-MS.

Chapter 6 An Investigation of Interactions Between Analytes and Matrices during the Matrix-assisted Laser Desorption/Ionization Process

6.1 Introduction

Although MALDI has been widely used for more than twenty years, its ionization mechanism is not yet fully understood.[188, 189] Many models have been proposed,[38, 39, 41, 42, 44, 190, 191] and there are two major ones for the explanation of the MALDI ionization process: the gas phase protonation model and the cluster model, which have been introduced in the introduction part of this thesis. These two ionization models have a similar photoionization process for matrix molecules as the initial step, but have a different second step for ionization of analyte molecules, which could be described by the following equations (Eqs. 6-1 and 6-2) respectively:

Gas phase model: $$A \xrightarrow{MH^+} M + AH^+ \quad \textbf{(6-1)}$$

Cluster model: $$AH_n^{n+} + nX^- \xrightarrow{MH^+} AH^+ + nHX \quad \textbf{(6-2)}$$

where A represents analyte molecule, M represents matrix molecule and X^- represents a counterion of the analyte ion. As shown in the two equations, the major difference is the origin of the proton for protonation of analyte. In the gas phase protonation model, this proton is transferred to analyte from matrix molecule, while in the cluster ionization model, this proton is from the pre-charged analyte crystals. Very recently, an investigation on the protonation mechanism of analyte was conducted by Karas *et al.* using CHCA-*tert*-butylester-d_9 (see Figure 6-1 for the structure**),** a deuterated CHCA ester, as the matrix.[43, 192] This deuterated matix yielded deuteronated ester [CHCA-*tert*-butylester-d_9 + D]$^+$ and fragment [CHCA-COOD + D]$^+$ in its MALDI-MS spectrum (Figure 6-1). The deuteron in these ions could be transferred to the analyte molecule to

form AD^+ in the gas phase protonation model or to neutralize the counterion to form DX (Eqs. 6-3 and 6-4):

Gas phase model: $$A \xrightarrow{MD^+} M + AD^+ \quad \textbf{(6-3)}$$

Cluster model: $$AH_n^{n+} + nX^- \xrightarrow{MD^+} AH^+ + DX + (n-1)HX \quad \textbf{(6-4)}$$

Their results revealed that both the deuteronated analyte ion in the gas phase protonation model and the protonated analyte ion in the cluster ionization model could be detected and thus demonstrated that both models could be applied to the MALDI ionization process. The ratio of the products from the two models was found to be related to experiment conditions such as laser fluence and natures of analyte and matrix.

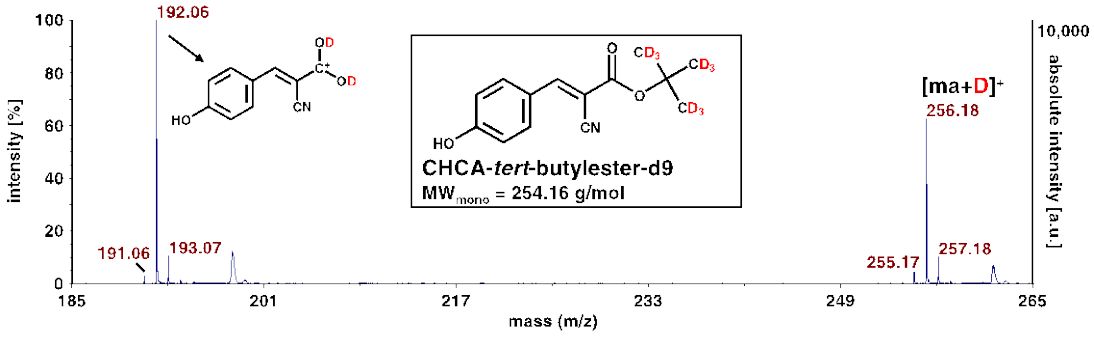


Figure 6-1 The chemical structure of CHCA-*tert*-butylester-d_9 and its MALDI-MS spectrum.[43]

In addition to protonation and neutralization, does matrix have any further interactions with or effects to analyte? There are very few literatures in this respect. In this study,

deuterium-labeled matrix and/or analyte were prepared and used to investigate the molecular interactions during the MALDI process. OASP[37] was employed as the sample preparation for the investigation since this technique allowed convenient analysis of solid samples without any use of solvents and thus avoided the back exchange of deuterium caused by the solvents. Our results revealed that in addition to protonation and neutralization, matrix molecules offered hydrogens to exchange with active hydrogens on analytes. The ionization process related to this was discussed in this study as well.

6.2 Experimental Section

6.2.1 Materials and Chemicals

CHCA was purchased from Fluka. Paraffin oil, Methanol-d and all other chemicals were purchased from Sigma-Aldrich. Water was distilled water prepared using a Milli-Q system (Millipore, USA). All the other solvents were of HPLC grade and purchased from Tedia (Fairfeild).

6.2.2 Preparation of Deuterated Samples

Fifty mg of CHCA was dissolved in 25 mL methanol-d for hydrogen/deuterium exchange (HDX) at room temperature and subsequently blow-dried with nitrogen to generate deuterated CHCA powder. This deuterated CHCA powder was analyzed with OASP-MALDI-MS to measure the number of deuterons incorporated (D). As shown in

Figure 6-2, D was calculated by subtracting the centroid of the undeuterated peaks ($C_{undeuterated}$) from the centroid of the deuterated peaks ($C_{deuterated}$). Deuteration ratio (DR) was obtained by dividing D by the total number of active protons in the sample molecule.

$$D = C_{deuterated} - C_{undeuterated}$$

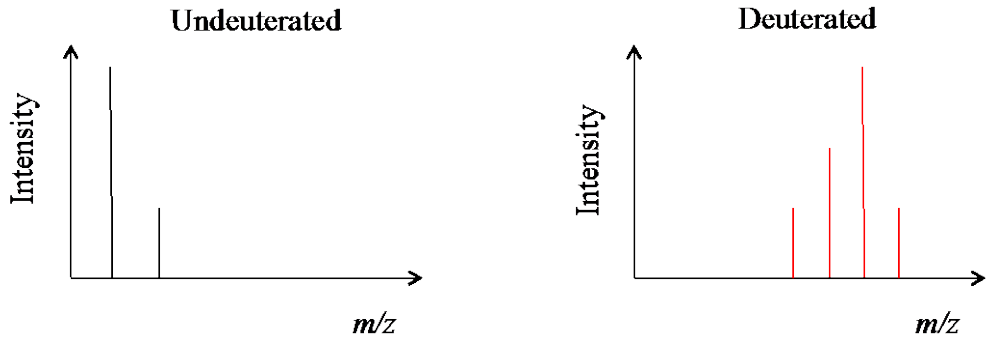


Figure 6-2 Schematic diagram for determination of the D value

To achieve a higher DR value for CHCA, the HDX procedures were repeated for CHCA. The deuterated CHCA was measured each time and the spectral results are shown in Figure 6-3. After repeating the HDX experiment three times, the DR value of CHCA was kept at about 80%. Full deuteration of active hydrogens of CHCA could not be achieved probably due to the back exchange of the incorporated deuteron with the environment moisture during the processing.[193] Deuterated melamine was prepared with procedures similar to CHCA. Confirmation of deuterated melamine was made by direct analysis of deuterated melamine in methanol-d using ESI-MS on a Q-ToF mass

spectrometer. As shown in Figure 6-4, the number of deuterons incorporated (D) was determined to be 5.5, equaling to a DR value of 78.7%.

Each D value and DR value in this part were obtained by averaging the data from three repeat experiments. The RSD was less than 15% in each case.

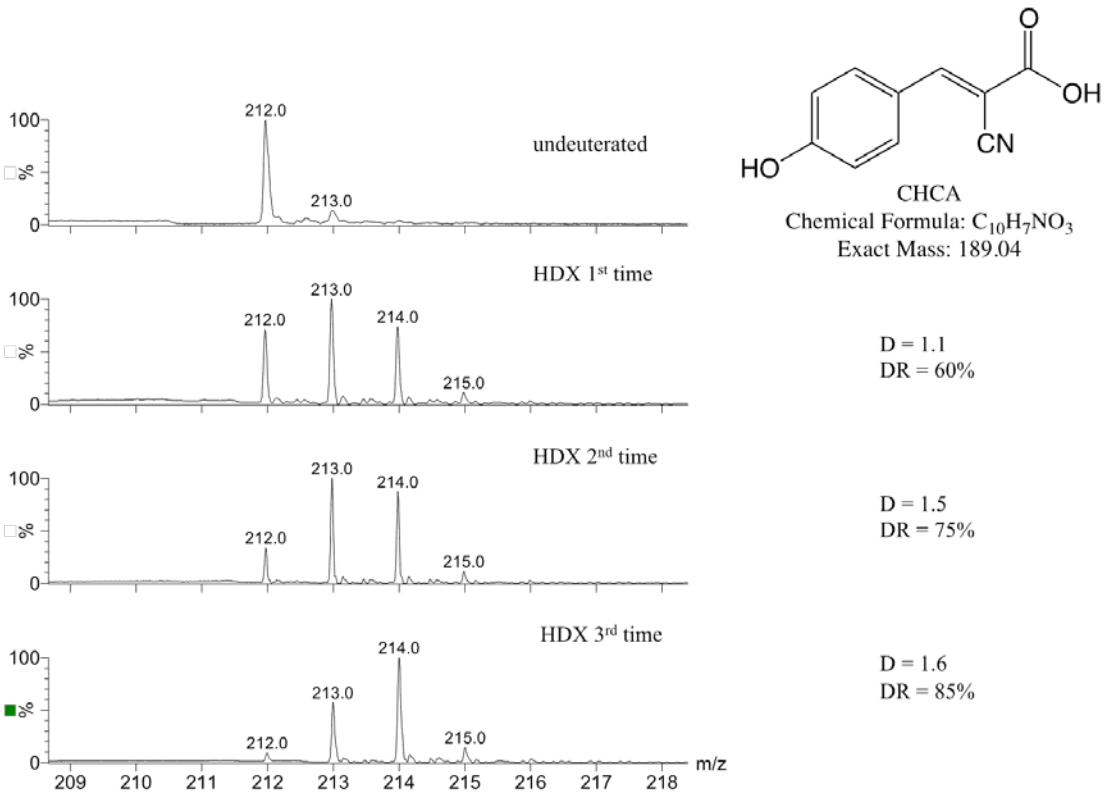


Figure 6-3 OASP-MALDI mass spectra of CHCA with different exchange times.

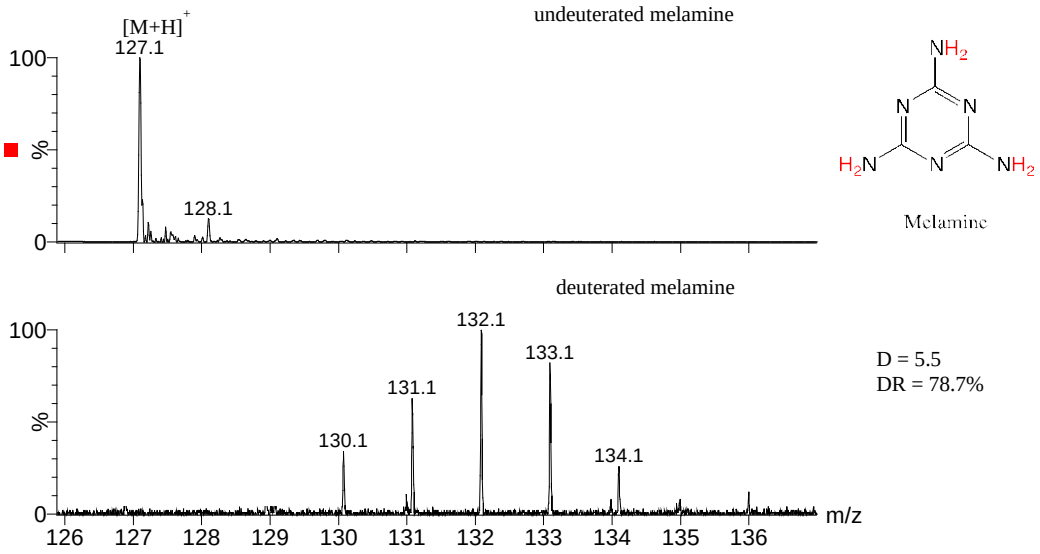


Figure 6-4 MALDI-MS mass spectrum of undeuterated melamine and ESI-MS spectrum of deuterated melamine.

6.2.3 Sample Preparation for MALDI-MS Analysis

CHCA solution was prepared using 10 mg of CHCA dissolved in 1 mL methanol containing 0.1% TFA. For the DD method, sample solution was mixed with the matrix solution at a volume ratio of 1 : 1, and 1 µL of the mixture was loaded on the target and dried prior to MALDI-MS analysis.

For the OASP method, the sample was prepared as described previously.[37] Briefly, a droplet of paraffin oil was deposited on a sample well of the MALDI plate to form a thin oil layer. Then a minimal amount of matrix powder and an approximately equal amount of sample powder were transferred onto the thin oil layer, and mixed together using a microspatula to form a visual homogeneity for subsequent MALDI-MS analysis.

For experiments involving sodium adducts, appropriate amounts of sodium chloride were added to the solutions (DD method) or powders (OASP method) to obtain abundant sodium adduct peaks of the analyte molecules under examination.

6.2.4 Instrumentation

MALDI mass spectra were acquired using a MALDI-ToF mass spectrometer (MALDI Micro MX, Waters) equipped with a nitrogen UV laser (wavelength 337 nm). The MALDI-ToF mass spectrometer was operated in positive reflectron mode with the following settings: flight tube voltage 12 kV, reflectron voltage 5.2 kV and TLF delay 500 ns. The mass spectrometer was calibrated with CHCA and polyethylene glycol 600-2000 for lower mass range (100-1000) and higher mass range (600-2000) respectively.

6.3 Results and Discussion

6.3.1 Hydrogen Exchange Between Matrix and Analyte in MALDI-MS

Melamine (see structure in Figure 6-5) has three amino groups and six active hydrogens. By comparison with the spectra obtained using deuterated and undeuterated CHCA respectively, it was found that 3.6 deutrons were incorporated into the deuterated melamine, i.e., $[M+D]^+$, when the deuterated CHCA was used as the matrix (Figure 6-5). This result indicated that in addition to the 0.8 deutrons (deuterated CHCA was 80% labeled) added to melamine due to the deuteronation, 2.8 more deutrons were

incorporated into melamine. A reasonable explanation for this was that the active hydrogens of melamine exchanged with deuterons produced from the deuterated CHCA.

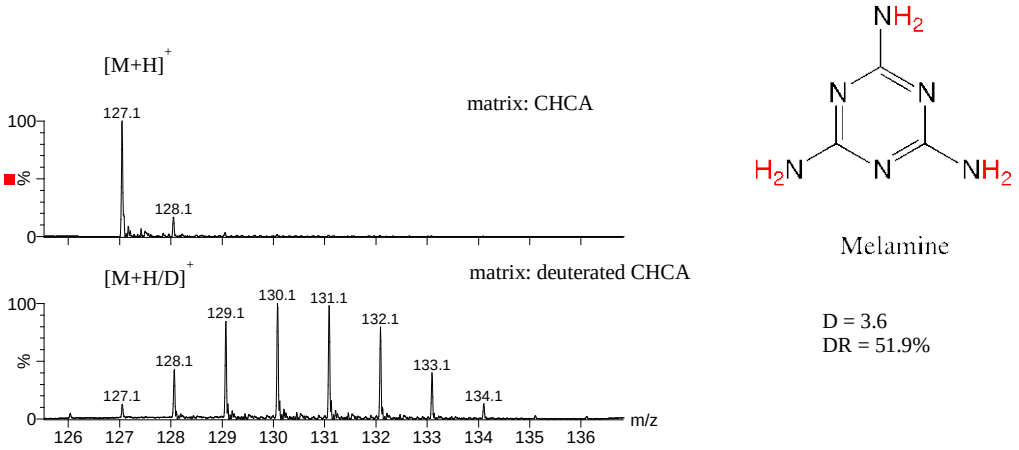


Figure 6-5 OASP–MALDI-MS spectra of melamine with undeuterated CHCA and deuterated CHCA as the matrix respectively.

To further confirm the exchange of active hydrogens between analyte and matrix, deuterated melamine was prepared and analyzed using OASP-MALDI-MS with undeuterated CHCA as the matrix. As shown in Figure 6-6, almost no deuteron was retained on the detected molecular ion of melamine, indicating that the active deuterons previously on the deuterated melamine were almost totally replaced by hydrogens from CHCA.

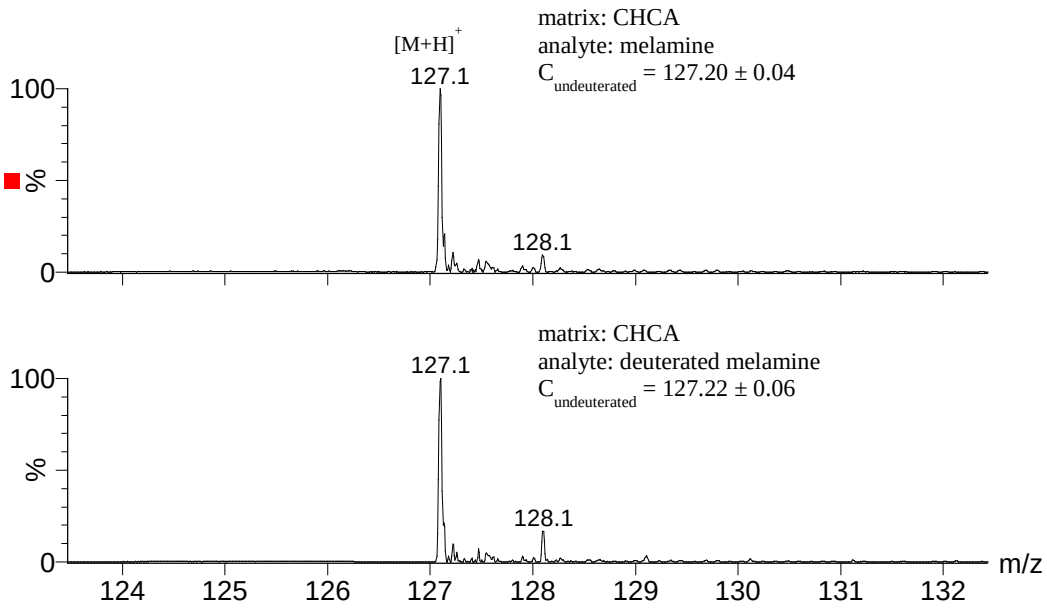


Figure 6-6 OASP–MALDI-MS spectra of melamine and deuterated melamine with undeuterated CHCA as the matrix.

To further confirm that such exchange only occurs with active hydrogens of analytes, betaine, a compound containing no active hydrogen, was analyzed using OASP-MALDI-MS with deuterated CHCA as the matrix. As shown in Figure 6-7, no deuterium was found in the sodium adduct of betaine when deuterated CHCA was used as the matrix (Table 6-1), indicating that stable hydrogens was not involved in the hydrogen exchange.

Similar results were obtained when DHB was used as the matrix. These results revealed that active hydrogens on analytes were replaced by hydrogens generated by the matrix during the MALDI process. This phenomenon has not yet been reported. The OASP

method allowed convenient MALDI-MS analysis of sample powder and matrix powder without any use of solvents. The deuterons could thus be retained during the analysis and the hydrogen exchange between analyte and matrix could be observed. Since the sample and matrix were both in solid phase and the parafilm oil was inert hydrocarbon oil, the hydrogen exchange between analyte and matrix should occur in the gas phase.

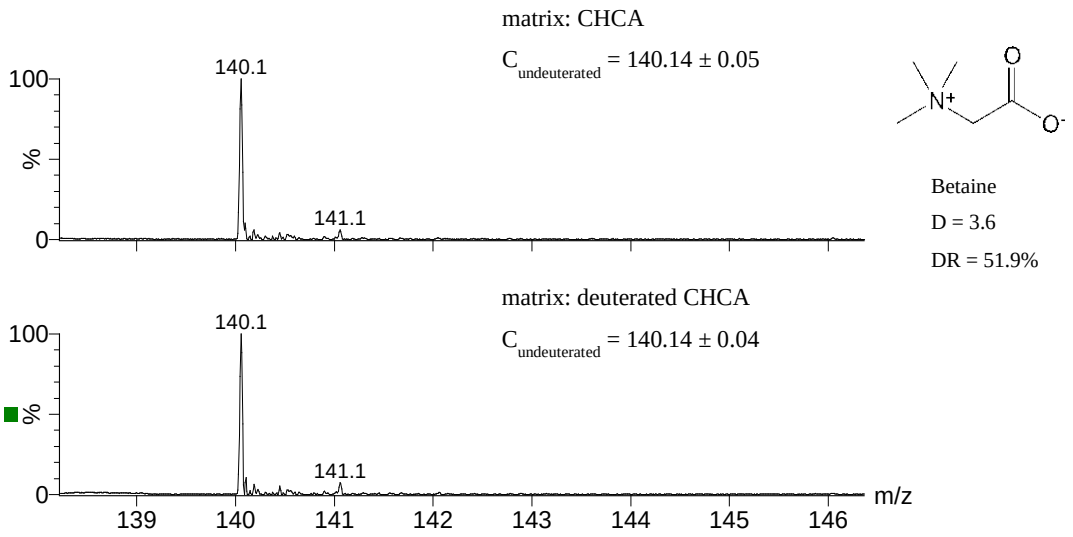
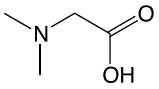
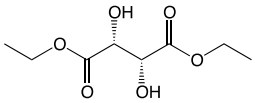
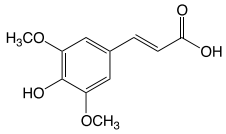
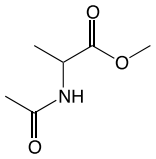
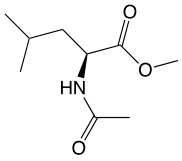
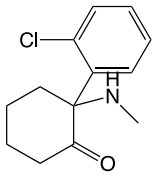
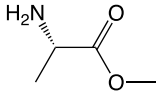
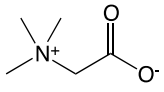


Figure 6-7 OASP–MALDI-MS spectra of betaine obtained with undeuterated and deuterated CHCA as the matrix respectively.

6.3.2 Hydrogen Exchange of Various Compounds

Table 6-1 D and DR values of various compounds obtained using OASP-MALDI-MS and deuterated CHCA as the matrix.

Analyte	Functional group for active hydrogen	Chemical structure	D	DR
N,N-Dimethylglycine	carboxyl		0.49	49%
(+)-Diethyl L-tartrate	hydroxyl		0.82	41%
Sinapic acid	carboxyl and hydroxyl		0.85	43%
Ac-Ala-OMe	amide		0.11	11%
Ac-Leu-OMe	amide		0.10	10%
Ketamine	amino		0.38	38%
H-Ala-OMe	amino		0.80	40%
Betaine	no active hydrogen		0	0

To further investigate the hydrogen exchange between analyte and matrix, compounds with various kinds of active hydrogens were analyzed with OASP-MALDI-MS using deuterated CHCA as the matrix. To eliminate the interference of protonation (or deuteration), the sodium adducts of these compounds were used for calculation of the D value and DR value, and the results are shown in Table 6-1 (mass spectra in Figures. 6-8 - 6-14).

The extent of hydrogen exchange, which could be evaluated by the DR value, was significantly related to the functional group. As shown in Table 6-1, amide hydrogen had the lowest exchange ratio. Hydroxyl hydrogen and amino hydrogen had similar exchange ratios that were higher than amide hydrogen. The exchange ratio for carboxyl hydrogen was the highest among all the active hydrogens investigated. This exchange tendency could also be observed when all these compounds were mixed equally and analyzed with deuterated CHCA in a single experiment. More active hydrogens in the compounds did not affect the exchange tendency. Such exchange tendency was consistent with the basicity of these functional groups and the previous results for gas-phase hydrogen/deuterium exchange.[194 195, 196 197 198]

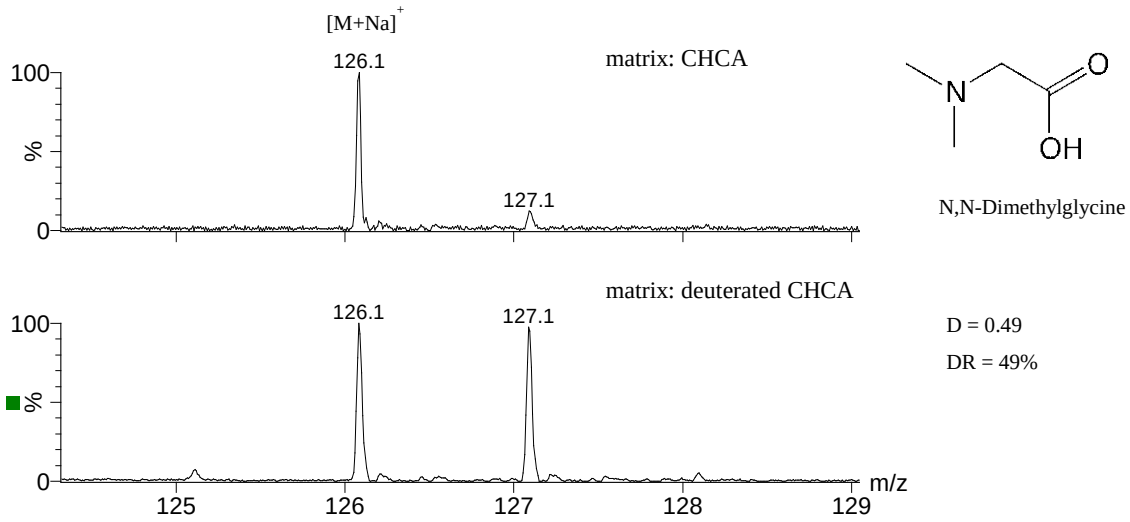


Figure 6-8 OASP–MALDI-MS spectra of N,N-dimethylglycine obtained with undeuterated and deuterated CHCA as the matrix respectively.

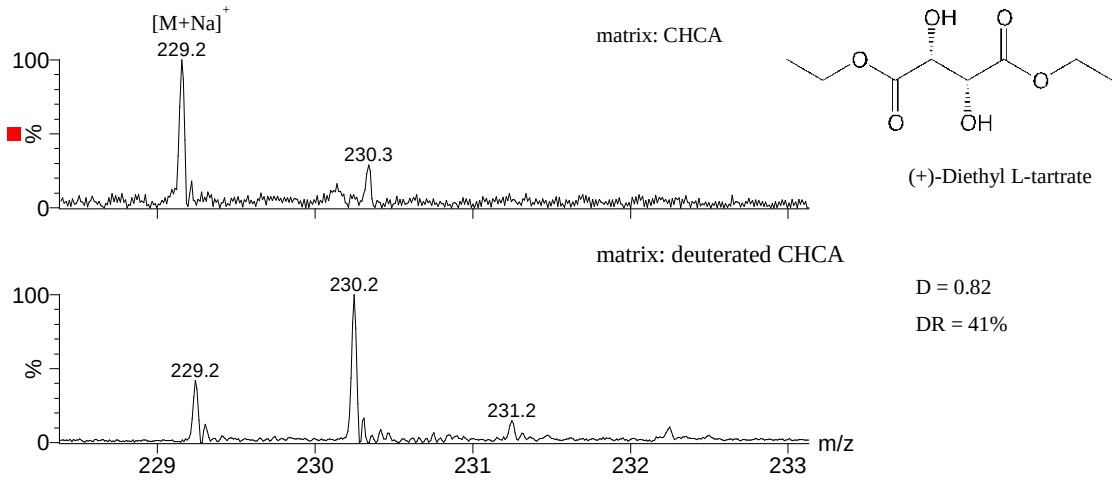


Figure 6-9 OASP–MALDI-MS spectra of (+)-diethyl L-tartrate with undeuterated and deuterated CHCA as the matrix respectively.

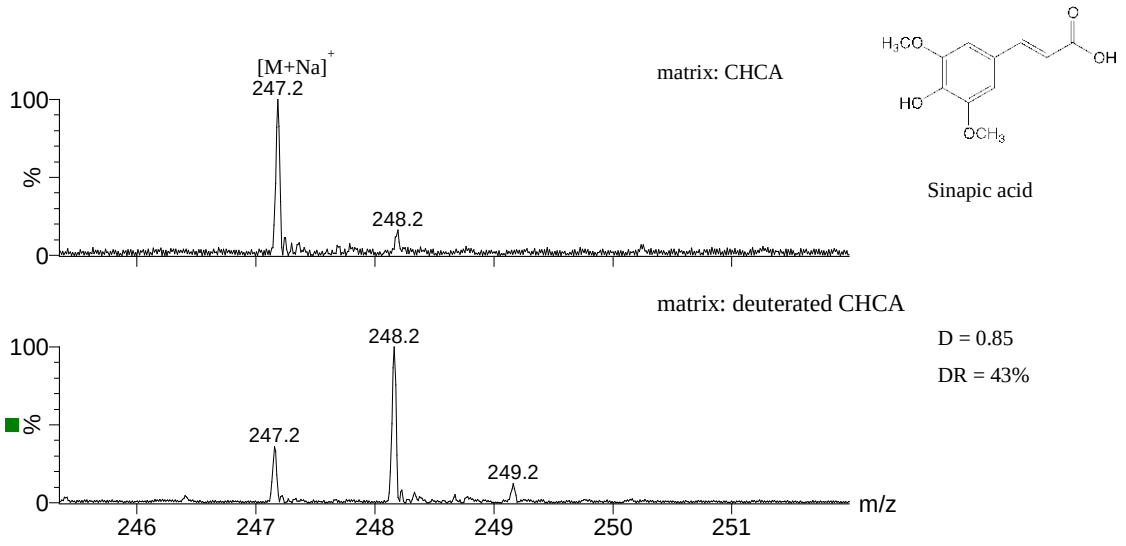


Figure 6-10 OASP–MALDI-MS spectra of sinapic acid with undeuterated and deuterated CHCA as the matrix respectively.

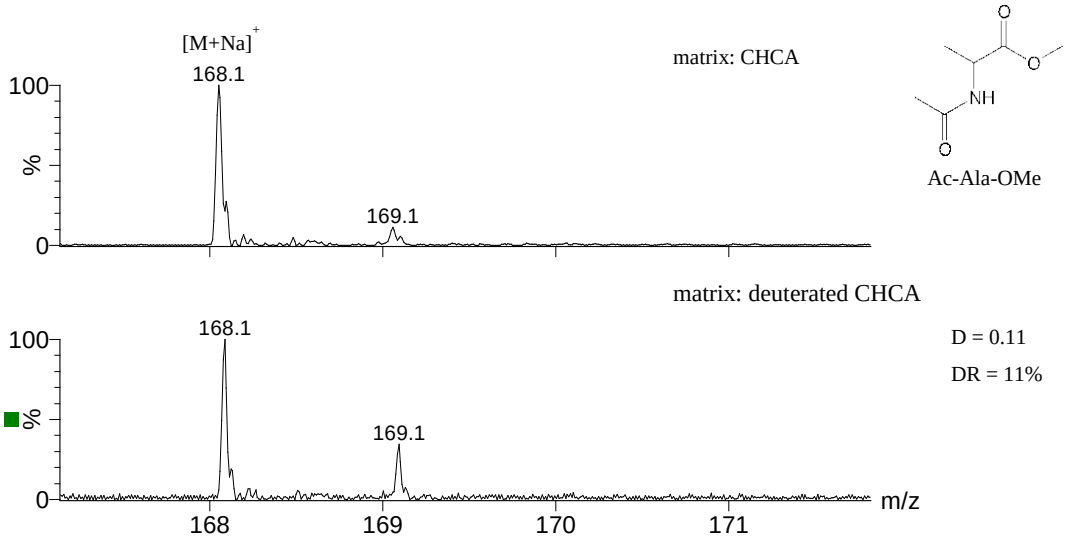


Figure 6-11 OASP–MALDI-MS spectra of Ac-Ala-OMe with undeuterated and deuterated CHCA as the matrix respectively.

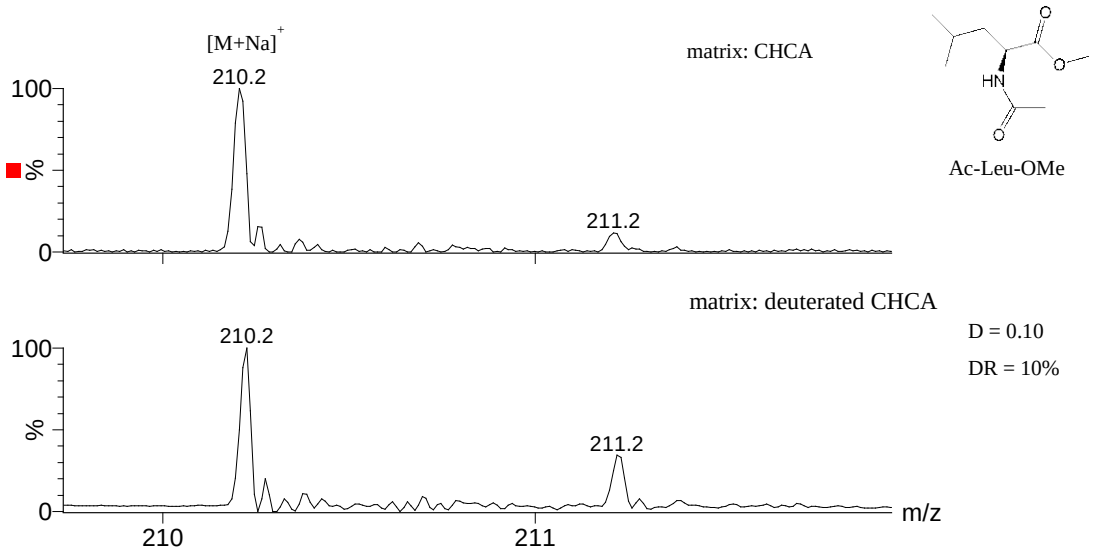


Figure 6-12 OASP–MALDI-MS spectra of Ac-Leu-OMe with undeuterated and deuterated CHCA as the matrix respectively.

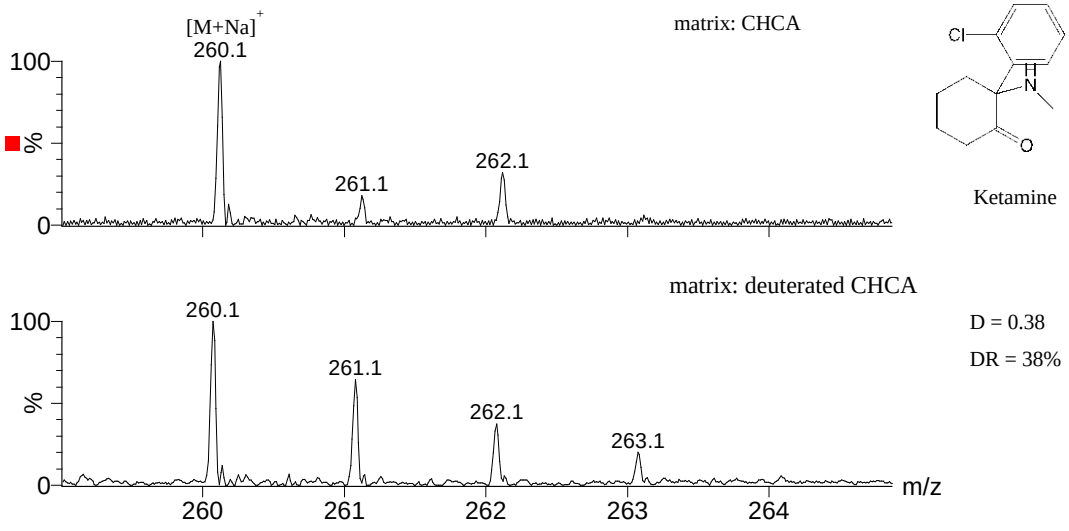


Figure 6-13 OASP–MALDI-MS spectra of ketamine with undeuterated and deuterated CHCA as the matrix respectively.

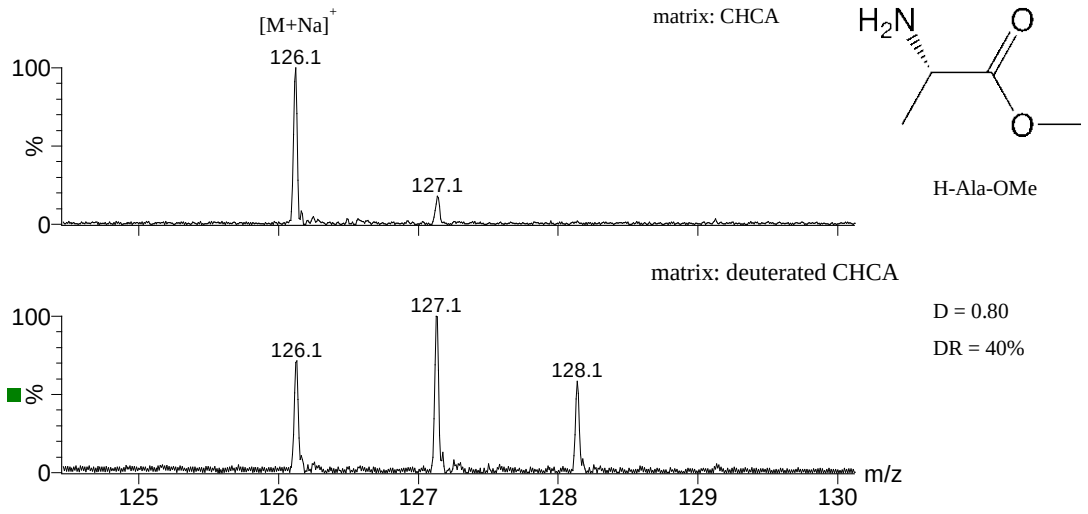


Figure 6-14 OASP–MALDI-MS spectra of H-Ala-OMe with undeuterated and deuterated CHCA as the matrix respectively.

6.3.3 Effect of Peptide Structure on The Hydrogen Exchange

Hydrogen exchange mass spectrometry is a powerful technique for study of conformations of proteins and peptides.[198, 199] In such investigation, the hydrogen exchange could be carried out in solution phase,[199-202] or in gas-phase,[196, 203-205] and the samples used for study are normally in solution form. The observation of hydrogen exchange between matrix and analyte in OASP-MALDI-MS inspired us to further investigate the effect of peptide structure on the hydrogen exchange and the possibility of using this technique for studying peptide structures with solid samples. The primary results are presented here.

Gly-Gly-Gly-Gly-Gly is a linear peptide with four amide hydrogens, two amino hydrogens and one carboxyl hydrogen (see Figure 6-15 for its structure). Comparing with the spectrum obtained with undeuterated CHCA as the matrix, it could be found

that 2.2 deutrons were incorporated into the sodiated molecular ion of this peptide (Figure 6-15) when deuterated CHCA was used as the matrix, corresponding to a DR value of 31%. A similar DR value was obtained for another peptide H-His-Leu-Leu-Val-Phe-OMe, as shown in Figure 6-16.

Figure 6-17 is the OASP-MALDI-MS spectra obtained with gramicidin D as the sample. Gramicidin D is a mixture of gramicidin A, B and C, with gramicidin A is the major component. Gramicidin A is a hydrophobic and compact pentadecapeptide with active hydrogens that are difficult to be accessed by solvents or deuterium reagents.[206-211] As shown in Fig. 6-17, no deuterium was incorporated into gramicidin A when deuterated CHCA was used as the matrix.

The above results showed that the active hydrogens of the loosened peptides Gly-Gly-Gly-Gly-Gly and H-His-Leu-Leu-Val-Phe-OMe could have hydrogen exchange with deuterated CHCA during the MALDI process, while such hydrogen exchange was not observed with gramicidin A due to its compact structure. Because no solvent was used in this process and the application of paraffin oil could isolate sample from air and moisture, so back-exchange could be neglected. These results suggested that this technique could be used to explore the conformations of peptides and proteins. Comparing with conventional HDX, this technique is rather simple. Further development and application of this technique is being undertaken.

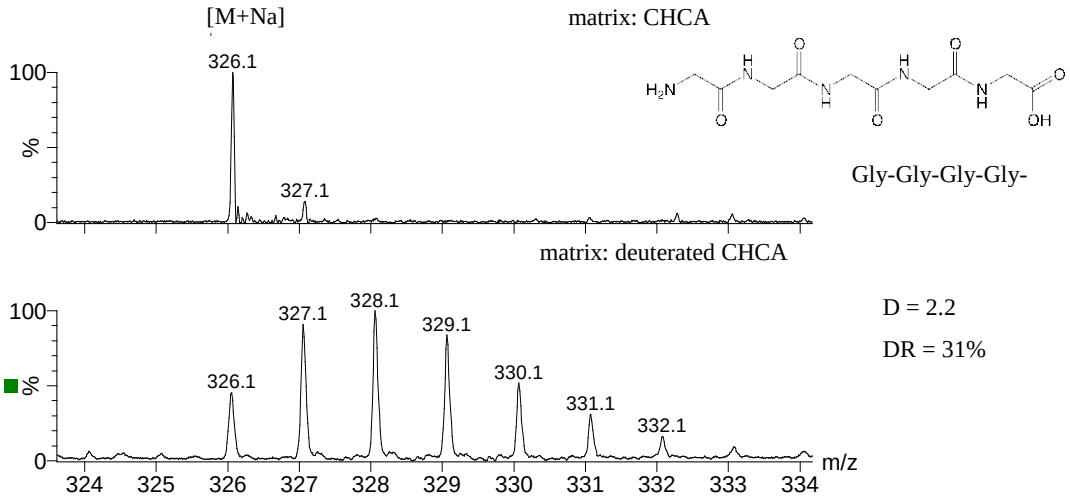


Figure 6-15 OASP-MALDI-MS spectra of Gly-Gly-Gly-Gly-Gly with CHCA and deuterated CHCA as the matrix respectively.

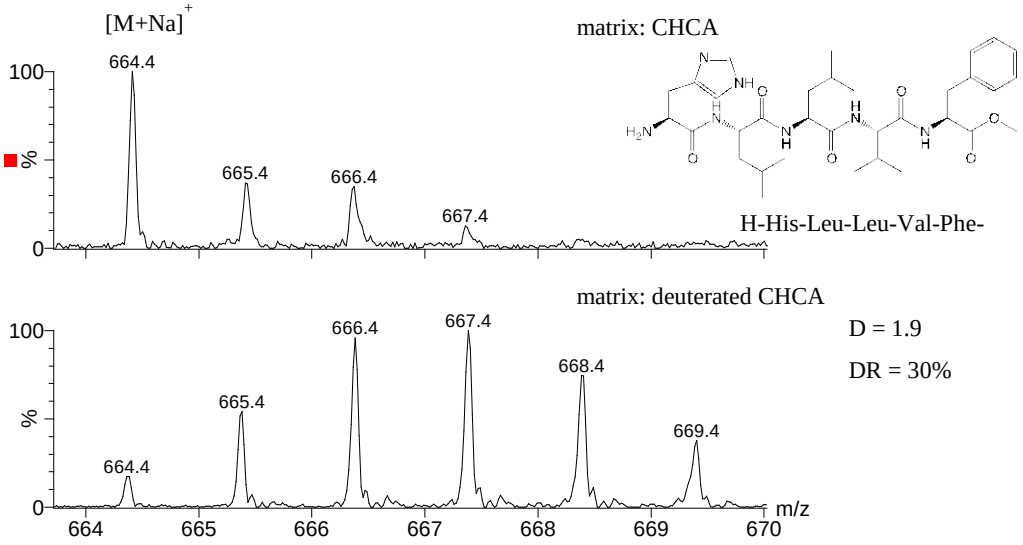


Figure 6-16 OASP-MALDI-MS spectra of H-His-Leu-Leu-Val-Phe-OMe with CHCA and deuterated CHCA as the matrix respectively.

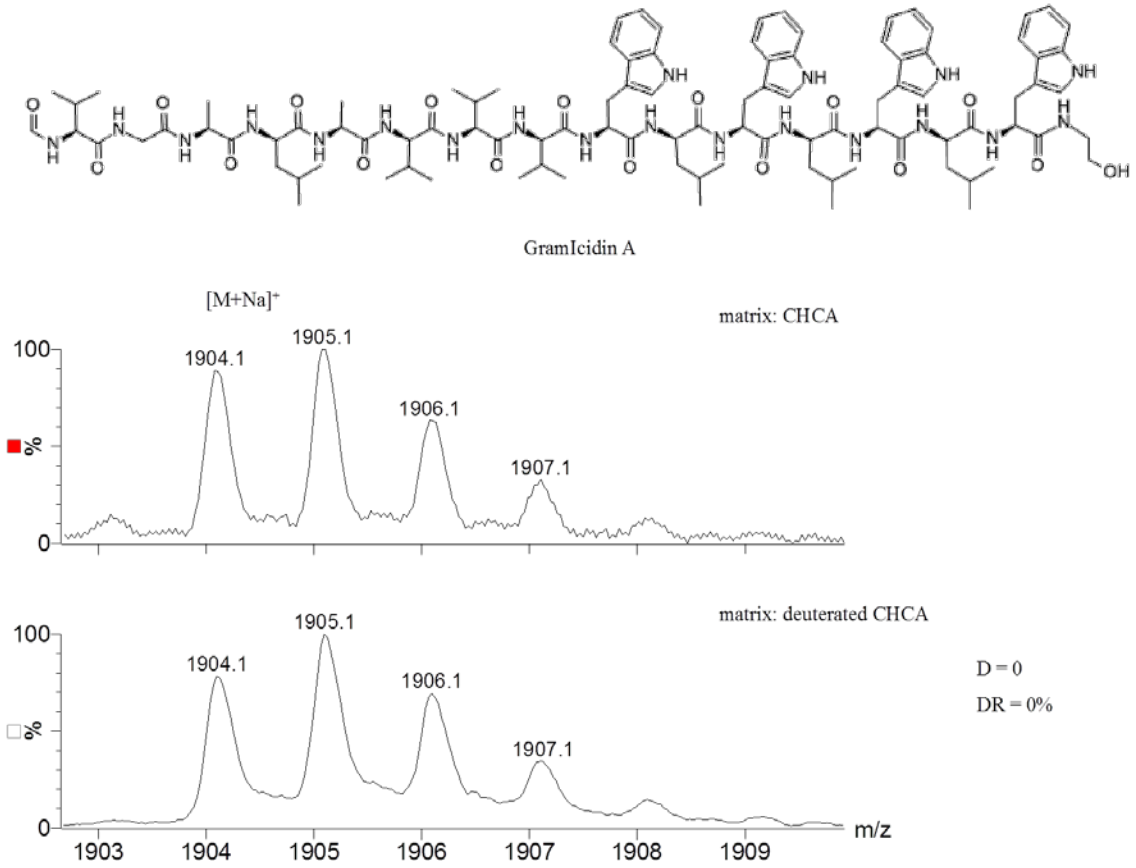


Figure 6-17 OASP-MALDI-MS spectra of gramicidin A with CHCA and deuterated CHCA as the matrix respectively.

6.3.4 Indication to the MALDI ionization mechanism

Our results revealed that active hydrogens of analyte molecules could be replaced by hydrogens from matrix during the MALDI process. This replacement has not been involved in the models proposed for MALDI process. Our results suggest that during the MALDI process, when matrix absorbs energy from laser and becomes activated, active hydrogens of the matrix can be released to form free protons or hydrogen radicals, which can then exchange with active hydrogens on the analyte molecules during the desorption and ionization process.

6.4 Conclusions

Our results revealed that active hydrogens of analyte molecules were replaced by hydrogens from matrix. Among the active hydrogens from carboxyl, hydroxyl, amino, and amide, amide hydrogen has the lowest exchange ratio. Investigation with different peptides indicated that such hydrogen exchange was structure-dependent and might be used to explore conformations of peptides and proteins. The observation of hydrogen exchange between analye and matrix allowed us to get more insights into the molecular interactions during the MALDI process.

Summary

The research in thesis involves development of ESI-MS-based techniques for direct analysis of complex samples and investigation of ionization mechanism of MALDI-MS.

We have developed pipette-tip ESI-MS for direct analysis of herbal powders, a significant form of samples. This novel technique is simple, easy to assemble and operate, and allows direct qualitative and quantitative analysis of various herbal powders. The pipette-tip ESI-MS was then further developed to C18 pipette-tip ESI-MS that allowed purification and enrichment of target compounds from complex sample solutions and subsequently direct ESI-MS analysis. We demonstrated that this technique could be used for analysis of protein solutions containing high contents of NaCl or SDS and for direct quantitation of ketamine and norketamine in human urine with a linear range to very low concentrations. The pipette-tip ESI-MS could be further extended and applied for direct analysis of other raw solid samples, e.g., tissue samples, and liquid samples, e.g., peptide mixture samples containing with salts and detergents. Furthermore, in this study, we developed wooden-tip ESI-MS for rapid detection and quantitation of plasticizer DEHP in beverages, and demonstrated that EESI-MS was more tolerant to nonionic detergents than ESI-MS for analysis of membrane proteins. These studies enabled direct analysis of the raw samples, and the related techniques are expected to play more important roles in the future.

In the final part of this thesis, with the use of deuterated matrix and analytes and by using OASP, a MALDI sample preparation method that allows convenient MALDI-MS analysis of analytes and matrix in their powder forms, we revealed that active hydrogens

of analyte molecules could be replaced by hydrogens from matrix during the MALDI process. This finding allowed us to get more insight into the molecular interactions during the MALDI process and may be used to explore conformations and structures of proteins and peptides in solid phase.

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