RAPID DETERMINATION OF DRUGS-OF-ABUSE IN URINE AND ORAL FLUID AND RAPID AUTHENTICATION OF EDIBLE OILS BY MASS SPECTROMETRY

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

November 2017

CERTIFICATE OF ORIGINALITY

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Abstract

The scope of my study is to develop analytical techniques to facilitate the analysis of two real-life issues, i.e., determination of drugs-of-abuse in urine and oral fluid, and authentication of edible oils. Mass spectrometry (MS), a commonly used analytical tool, has been developed to address these two issues.

Drug abuse is a severe problem worldwide. Detection of drugs-of-abuse in the body fluids of drug abusers is the critical step for the law enforcement as well as the treatment and rehabilitation. To handle the huge analytical demand of drug analysis, drug screening using the testing kits is usually employed as the preliminary analysis to screen the submitted samples, and only the positive samples are further subjected to confirmatory analysis using techniques such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS). However, such preliminary analysis frequently produces false positive or false negative results, and the confirmatory analysis are usually labour-intensive and time-consuming. Development of new analytical techniques for rapid and reliable drug analysis is thus highly desirable. In this study, wooden-tip electrospray ionization mass spectrometry (WT-ESI-MS), a simple and cost-effective technique recently developed by our group, was attempted for

rapid detection and quantitation of six commonly drugs-of-abuse, i.e., ketamine, methamphetamine, cocaine, ecstasy, cannabis and heroin, in urine and oral fluid. The results showed that WT-ESI-MS could be used for detection and quantitation of ketamine, methamphetamine and ecstasy in raw urine and oral fluid, with analysis of one sample within minutes and analytical performances acceptable for analysis of real-life samples. However, the detection and quantitation of cocaine, cannabis and heroin still need to further improve. Rapid extraction and enrichment using solid-phase microextraction (SPME) followed by direct coupling of SPME with electrospray ionization mass spectrometry (SPME-ESI-MS) was then developed for analysis of the six drugs in urine and oral fluid. The results indicated that the limits-of-detection for analysis of the six drugs were much improved with SPME-ESI-MS and could fulfill the requirements of the international standards except for cannabis in oral fluid. Quantitation of all the targeted drugs could be achieved with satisfactory precisions and accuracies by using the SPME-ESI-MS approach.

Authentication of edible oils is crucial for ensuring the quality and safety of edible oils and becomes more important with the emergence of gutter oils. Conventional gas chromatography-flame ionization detection (GC-FID) method for edible oil analysis

requires chemical derivatization and chromatographic separation and is time-consuming. Moreover, there is no universal method for screening of gutter oils yet. In this study, a simplified matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) approach was developed for rapid and high throughput analysis of edible oils. Mass spectra of more than one thousand oil samples were acquired and a spectral database of edible oils was established. The spectra for various edible oils were analyzed and compared, and the characteristic peaks and spectral features of each edible oil were obtained. Edible oils were divided into eight groups based on their characteristic spectral patterns and principal component analysis results. An overall correct rate of 97.2% was obtained for classification of 435 edible oil products using partial least square-discriminant analysis. Differentiation of counterfeit edible oils and gutter oils from normal edible oils could also be achieved based on the MALDI-MS spectra, and analysis of one oil sample could be completed within minutes.

Research publications

Journal papers

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6. Ng, T. T.; So, P. K.; Hu, B.; Yao, Z. P. Rapid detection and quantitation of drugs-of-abuse by wooden-tip electrospray ionization mass spectrometry (submitted to *J. Food Drug Anal.*).
7. Ng, T. T.; Hu, B.; So, P. K.; Yao, Z. P. Detection and quantitation of drugs-of-abuse using direct coupling of solid-phase microextraction with mass spectrometry (under preparation).

Conference paper

1. Ng, T. T.; So, P. K; Yao, Z. P. Rapid differentiation of mixed, recycled or gutter oils from pure vegetable oils by MALDI-MS. HKSMS Symposium 2014 & the 16th Annual General Meeting of The Hong Kong Society of Mass Spectrometry. Hong Kong, 7 June **2014.** (Poster)
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Symposium 2015 & the 17th Annual General Meeting of The Hong Kong Society of Mass Spectrometry. Hong Kong, 13 June **2015.** (Poster)

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List of abbreviations

Full Form	Abbreviation
2,5-dihydroxy benzoic acid	DHB
3,4-methylenedioxymethamphetamine	MDMA
6-acetylmorphine or 6-monoacetylmorphine	6-MAM
6-acetylmorphine-D_3	D-6-MAM
11-nor-9-carboxy-delta-9-THC	THC-COOH
1-nor-9-carboxy-delta-9-THC-D_9	D-THC-COOH
α-cyano-4-hydroxycinnamic acid	CHCA
acetonitrile	ACN
atmospheric pressure chemical ionization	APCI
benzoylecgonine	BEN
charge residue model	CRM
chemical ionization	CI
cocaine	COC
cocaine-D_3	D-COC
collision induced dissociation	CID
Dalton	Da
delta-9-THC-D_3	D-THC
desorption electrospray ionization	DESI
deoxynucleotide acid	DNA
dichloromethane	DCM
direct analysis in real time	DART

direct current	DC
Driving under the Influence of Drugs, Alcohol and Medicines	DRUID
divinylbenzene	DVB
docosahexaenoic acid	DHA, C22:6
eicosapentaenoic acid	EPA, C20:5
eicosenoic acid	Ei, C20:1
electron ionization	EI
electrospray ionization	ESI
electrospray ionization Fourier transform ion cyclotron resonance	ESI-FTICR
erucic acid	Er, C22:1
Ethanol	EtOH
European Union	EU
European Workplace Drug Testing Society	EWDTS
fatty acid methyl ester	FAME
Formic acid	FA
gas chromatography	GC
gas chromatography-flame ionization detection	GC-FID
gas chromatography-mass spectrometry	GC-MS
heroin	HER
heroin-D_9	D-HER
hydrochloric acid	HCl
infrared	IR
internal diameter	I.D.

internal standard	IS
International Union of Pure and Applied Chemistry	IUPAC
ion evaporation model	IEM
ketamine	KET
ketamine-D_4	D-KET
Lauric acid	C 12:0
liquid chromatography	LC
liquid chromatography-mass spectrometry	LC-MS
linoleic acid	L, C18:2
linolenic acid	Ln, C18:3
limit-of-detection	LOD
mass spectrometry	MS
mass-to-charge ratio	*m/z*
matrix-assisted laser desorption/ionization	MALDI
MDMA-D_5	D-MDMA
methamphetamine	MA
methamphetamine-D_5	D-MA
Methanol	MeOH
morphine	MOR
morphine-D_3	D-MOR
myristic acid	C14:0
nor-ketamine	Nor-K
nor-ketamine-D_4	D-Nor-K
oleic acid	O, C18:1

oil-assisted sample preparation	OASP
oral fluid	O.F.
palmitic acid	P, C16:0
partial least square discriminant analysis	PLS-DA
polyacylate	PA
polydimethylsiloxane	PDMS
polyethylene terephthalate	PET
polyvinyl alcohol	PVA
predicted scores	PS
principal component analysis	PCA
radio frequency	RF
ricinoleic acid	12-OH-C18:1
selected ion monitoring	SRM
sinapinic acid	SA
sodium chloride	NaCl
sodium iodide	NaI
solid-phase microextraction	SPME
Substance Abuse and Mental Health Services	SAMHSA
stearic acid	S, C18:0
tandem mass spectrometry	MS/MS
tert-butyl methyl ether	TBME
tetrahydrocannabinol (delta-9-THC)	THC
time-of-flight	TOF
triacylglycerols	TAGs

trimethylsulfonium hydroxide	TMSH
triple-quadrupole mass spectrometer	QQQ-MS
ultra-performance liquid chromatography	UPLC
ultraviolet	UV

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Chapter 1: Introduction

1.1 General introduction of mass spectrometry

Mass spectrometry (MS) is a powerful tool to analyze samples by measuring the mass-to-charge ratios (*m/z*) of gaseous ions. Mass spectrometry provides both qualitative and quantitative information, such as the molecular masses of compounds and concentrations of chemicals in the measured samples. The development of mass spectrometry was initiated by the research of J. J. Thomson, who discovered electron. Thomson constructed the first mass spectrometer in 1912 to measure the *m/z* values of different gases such as oxygen and nitrogen.[1] Various ionization techniques and mass analyzers have been invented later on for analyzing various compounds, and improving the sensitivity and resolution of mass spectrometers. Nowadays, organic and inorganic compounds[2] and even macromolecules such as proteins[3] and deoxynucleotide acids (DNA)[4] can be detected by mass spectrometers. For example, Chen and co-workers demonstrated the detection of coliphage T4 DNA ions which have the molecular weight of 1.1 x10^8 Dalton (Da) by an electrospray ionization Fourier transform ion cyclotron resonance (ESI-FTICR) mass spectrometer.[5]

Mass spectrometers consist of three major components, including ion source, mass

analyzer and detector. Molecules in samples are ionized to form either positive or negative ions in ion sources in mass spectrometers.[6] Various ionization techniques are applied to produce ions of various compounds. Electron ionization (EI) works well in ionization of small and volatile molecules, but in many cases fragment ions rather than molecular ions are observed, as highly energetic electrons (70 eV) are involved in the ionization process. Such fragmentation behavior, however, allows structural determination of targeted analytes without tandem mass spectrometry (MS/MS) analysis.[7] On the other hand, soft ionization techniques including chemical ionization (CI), atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) result in less fragmentation of analyte ions and generate abundant molecular ions, thus allow the determination of molecular masses of targeted compounds and creation of simple mass spectra.[1] Conventional ionization techniques are well compatible with gas chromatography (GC) or liquid chromatography (LC) for analysis of complex samples.[8,9] Gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) are commonly used analytical techniques for compound detection, identification and quantitation, but laborious sample preparation and time-consuming column separation are required to reduce the interferences and separate the

compounds in the samples. Ambient ionization techniques developed in recent years allow direct analysis of samples with little or no sample pretreatment under ambient conditions.[10,11] Rapid detection and quantitation of targeted analytes in complex samples can thus be achieved.[12]

After the generation of analyte ions, the gaseous ions will be directed to the mass analyzers in mass spectrometers for ion separation. Commonly used mass analyzers include time-of-flight (TOF), quadrupole, ion trap and orbitrap.[1,13] In TOF, the *m/z* values of analyte ions are determined by measuring the time taken by the ions to pass through a field-free flight tube located between the ion source and the detector. The relationship between the drift time (*t*) of an ion travelled and its *m/z* is shown in equation 1-1, where *L* is the length of the flight tube and *V* is the accelerating voltage applied to the ion:[1]

$$t^2 = \frac{m}{z}\left(\frac{L^2}{2eV^2}\right) \quad (1\text{-}1)$$

Currently, reflectron TOF rather than liner TOF is more commonly equipped with mass spectrometers as higher mass resolution can be achieved using the reflectron configuration. TOF mass analyzer offers wide mass range, high sensitivity and high mass resolution for detection of analytes, and thus is commonly used in qualitative

analysis.

Quadrupole is another popular mass analyzer. It separates ions by changing the oscillating electric field. Quadrupole consists of four rods (electrodes) which are parallel with each other. The rods are connected with both direct current (DC) voltage and radio frequency (RF) voltage. Only ions with specific *m/z* can travel along the electric field created by the electrodes through a stable trajectory and reach the detector.[1] Coupling of more than one quadrupoles is a common way to allow the mass spectrometers operating with different tandem mass spectrometry modes.[14] Four scan modes are commonly used in MS/MS analysis, which are product ion scan, precursor ion scan, neutral loss scan and selected reaction monitoring (SRM). In product ion scan, the first quadrupole (Q1) is set to select a specific *m/z*, allowing only the ions with the selected *m/z* to pass through Q1 and enter the second quadrupole (Q2). The selected ions are then fragmented in Q2 through collision induced dissociation (CID) or other fragmentation techniques and the resulted fragment ions are finally entered and detected by the third quadrupole (Q3). Structural information provided by product ion scan is very useful for identification of compounds presented in samples and determination of sequences of peptides.[15] Precursor ion scan is just like the reverse of product ion scan,

using Q1 to scan the precursor ions and Q3 to select a specific fragment ion. It is a popular technique for detection of a group compounds such as phospholipids which have the same fragment ions.[16] In neutral loss scan, Q1 scans the precursor ions and Q3 scans the fragment ions with the loss of neutral species such as water molecule from the precursor ions. Only the precursor ions producing such a neutral molecule will be recorded in this scan mode. In SRM, both Q1 and Q3 are operated in selection mode, only the ions producing the precursor ion with selected *m/z* value and the fragment ion with selected *m/z* value can be detected. It is a highly sensitive and specific method for detection of targeted compounds. More than one SRM can be operated in triple quadrupole mass spectrometer (QQQ-MS). Such multiple reaction monitoring (MRM) is a common practice in quantitative analysis using mass spectrometry.[17]

Orbitrap is a new and advanced mass analyzer which offers higher mass resolution and sensitivity than TOF and quadrupole. Orbitrap consists of an outer barrel-like electrode and a central spindle-like electrode. Electric field created by the electrodes forces the ions with different *m/z* values cycle around the central electrode with different trajectories. The image currents included by the ion movement are detected on the outer electrodes and further transformed into *m/z*.[18] Orbitrap mass spectrometer is an

outstanding instrument for accurate mass measurement and proteomics because of its high mass accuracy and high sensitivity.[19]

Mass spectrometry is sensitive, specific and accurate for analysis of organic compounds as well as large biomolecules. Mass spectrometry has been used as a routine analytical method for qualitative and quantitative analysis in many research fields, such as organic chemistry, proteomics, pharmacokinetics, drugs analysis and food safety.[20-22]

1.2 Electrospray ionization

1.2.1 Conventional electrospray ionization

Electrospray ionization (ESI) is one of the most common ionization techniques used in mass spectrometry. Electrospray ionization can ionize a wide range of compounds that are dissolved in solvents, such as organic compounds, synthetic polymers and proteins. The idea of coupling ESI with mass spectrometry is initiated by Malcolm Dole who designed an experiment to determine the molecular masses of synthesis polymers. He and the co-workers nebulized the solution of polymers using a hypodermic needle connected to a high voltage with the assistance of nitrogen gas, and the fine droplets

were rapidly evaporated to create the polymer ions.[23] However, the signals produced by the initial design were too low to be detected by mass spectrometers. John Fenn and co-workers further investigated and improved the ESI system reported by Dole. They applied a countercurrent drying gas after the ESI process which allowed better evaporation of the find droplets, and they further optimized the parameters such as solvent flow, distance between the ESI source and the mass spectrometer inlet as well as the voltage used for ionization. These modifications greatly improved the ionization efficiency of ESI and accomplished the use of ESI with mass spectrometry.[24] They also reported the production of multiple charged ions in ESI for the detection of large biomolecules such as proteins.[25] Modern electrospray ionization can be easily coupled with LC, allowing sensitive analysis of complex mixture samples after sample pre-treatment.

Ion formation in ESI involves four major steps, which include formation of charged droplets, evaporation of the droplets, fission of the droplets and formation of ions (Figure 1-1). With a high voltage applied to the conductive capillary of the ESI source, a strong electric field is produced between the capillary and the counter electrode. The electric field induces a charge accumulation to the liquid passing through the capillary,

thus produces charged droplets at the ESI capillary tip. If the applied voltage is sufficiently high, the liquid could spray out in a fine cone-jet (Taylor cone). The solvent in the fine droplets is evaporated with the assist of desolvation gas and high temperature, causing shrinkage of charged droplets. As the sizes of the droplets reduce, the charge repulsion at the droplet surfaces increases. When the repulsion overcomes the cohesive forces of the droplet surfaces, which is at the Rayleigh charge limit, fission of charged fine droplets occurs to form smaller charged droplets. Such solvent evaporation and droplet fission process continues, ultimately leading to the formation of gas phase ions.[26,27]

There are two widely acceptable models to describe the ion formation after fission of charged droplets. The ion evaporation model (IEM) is mainly used to explain the formation of small analyte ions, and the charge residue model (CRM) is mainly used to explain the formation of large analyte ions. IEM was proposed by Iribarne and Thomson to describe the ion formation from the charged droplets in 1976.[28] It was predicted in the IEM model that with the solvent evaporation, when the radii of the charged droplets are reduced to a certain level, such as less than 10 nm, direct ion emission from the charge droplets becomes possible. It is well supported that small inorganic and organic

ions are produced by this mechanism.[26] CRM is an early assumption for ion formation proposed by Dole. It is proposed that the fission of charged droplets will continue until the charged droplets are extremely small and contain only one analyte ion. CRM is widely accepted as the mechanism for formation of large protein ions which carry multiple charges.[26,27]

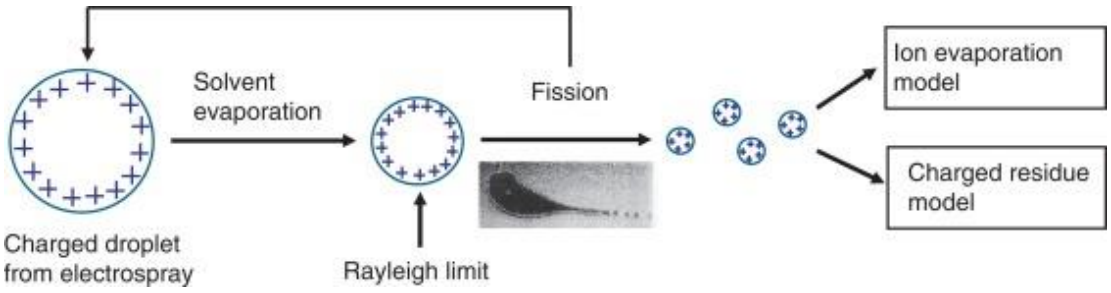


Figure 1-1. The major events for ion formation in ESI (Reprinted from ref[27]).

There are two important features of ESI production of multiple charged ions and concentration dependence of ion formation.[1] Large molecules such as proteins could have several ionization sites for ESI, thus produce multiple charged ions. The *m/z* of multiple charged ions produced from the large molecules falls within the detectable range of the mass analyzers. This allows ESI to be useful for detecting large biomolecules such as proteins. ESI is sensitive to concentrations of analytes in solutions rather than the total quantities of samples injected. This allows modifications of ESI

source such as invention of nano-electrospray ESI, which uses low flow rate and small sample amount but retains similar sensitivity.[1]

1.2.2 Solid-substrate electrospray ionization

Sample pretreatments are typically required to eliminate the impurities and matrices in sample solutions before mass spectrometric analysis. This is a customary practice to protect the LC columns and instruments, and to reduce the background signals. However, the sample preparation steps as well as the column separation can be time-consuming and labor-intensive. Ambient ionization mass spectrometry which requires only little or even no sample preparations before the mass spectrometric analysis is becoming popular for rapid sample analysis.[10,11] Zoltán Takáts and co-workers from R. Cooks' research team as well as Robert B. Cody and co-workers firstly developed two famous ambient ionization mass spectrometry techniques, named desorption electrospray ionization (DESI)[29] and direct analysis in real time (DRAT),[30] respectively. The former technique utilizes an electrospray solvent beam to desorb analytes from samples directly and ionizes the desorbed analytes. Direct analysis of compounds in living tissues and urine samples were demonstrated by DESI.[31] The latter technique, on the other hand, applies high temperature plasma for desorption and ionization of

compounds on the surfaces of substances. Also, analytes from liquids, tablets and gases can be analyzed without solvent extraction.[30,32]

There are over 40 ambient ionization mass spectrometry techniques developed after the innovation of DESI and DART and the number is still increasing.[33] Huang et al. divided ambient ionization into three categories, which are two-step ionization, direct desorption/ionization and direct ionization.[10] In two-step ionization, the analytes are firstly desorbed or released from the samples after laser irradiation,[34] UV-lamp irradaion,[35] thermal heating,[36,37] thermal desorption[32] or nebulization[38,39]. The analytes are then ionized through conventional ionization techniques such as ESI and APCI. On the other hand, direct desorption/ionization and direct ionization allow direct ionization of analytes from the samples. Direct ionization/desorption techniques such as DESI requires desorption of analytes from the samples followed by ionization while direct ionization techniques typically allow direct electrospray ionization of the sample droplets or sample solution on solid substrates.[10] In conventional ESI, liquid samples are typically introduced into MS via a capillary with the assistance of nitrogen gas. Various solid substrates have been used to replace the capillary for sampling and ionization in ESI. The set-up of solid-substrate ESI is usually simpler than other

ambient ionization techniques, since it requires only little modifications to convert the configuration of normal ESI into ambient ionization. Solid-substrate ESI techniques can be compatible with the existing instruments. Extra components such as plasma guns and lasers are usually not required for solid-substrate ESI. The use of solid needle probes,[40] paper,[41] wooden tips[42] and aluminum foil[43] for ESI has been demonstrated. The non-conductive porous materials such as wooden tips can become electrically-conductive after diffusion of solvents through capillary action.[44] Sampling and ionization directly on solid-substrate surfaces avoids the clogging problem of conventional ESI, thus allowing direct analysis of various raw samples. Some of the solid substrates such as wooden tips possess porous surfaces and allow some degrees of chromatographic separation based on properties of the compounds.[44] The sample consumption of ESI using solid substrates can be as low as few microliters. Solid-substrate ESI has already been demonstrated for rapid analysis of targeted analytes in samples containing matrices with little sample preparation and without column separation, e.g., quantitation of therapeutic drugs, sitamaquine and amitriptyline, in blood by paper spray.[45] Integrated solid-substrate ESI with selective and sensitive sample preparation methods, such as solid-phase microextraction (SPME)[46] and surface modified solid-substrate[47] for direct analysis of raw samples could be important issues

in analytical chemistry.

1.3 Matrix-assisted laser desorption/ionization

Matrix-assisted laser desorption/ionization was firstly introduced by Michael Karas and Franz Hillenkamp in 1985. They discovered that the signals of amino acids could be enhanced by using ultraviolet (UV) absorbing tryptophan as matrix in UV laser desorption mass spectrometry.[48] Later on, Koichi Tanaka and co-workers demonstrated the use of nitrogen laser with the assist of nano-cobalt particles in glycerol for ionization of large proteins and he was awarded the Nobel prize in 2002 for this discovery.[49] MALDI offers distinctive advantages for mass spectrometric analysis including short analytical time, high salt tolerance and production of singly charged ions for easy spectral interpretation. MALDI-MS is nowadays a popular technique for peptide and protein analysis.[22] Sample preparation is an important step for successful MALDI-MS analysis.[50] Various sample preparation methods have been established for analysis of different analytes. Apart from conventional solution-based sample preparation methods, many solvent-free sample preparation methods has been developed in recent years for rapid MALDI-MS analysis.[51]

1.3.1 Conventional sample preparation

Dried-droplet method is commonly applied in MALDI sample preparation. The analytes in raw samples are extracted with solvents and purified with pretreatment process such as solid phase extraction or zip-tip treatment. The solution containing analytes is then mixed with large excess molar amount of matrix. Commonly 1μL of mixture is applied onto a spot of the MALDI plate and dried. The analyte molecules are embedded in matrix molecules during drying and formed analyte-matrix crystals. Many samples can be loaded onto different spots of the MALDI plate and introduced into the mass spectrometer for analysis at the same time. There are also some other methods such as the thin-layer method and the sandwich method, in which the matrix layer and sample layer are prepared separately, developed for improving the sensitivity and reproducibility of MALDI-MS analysis.[52]

The choice of matrix is crucial for MALDI analysis. There are still no clear guidelines for the selection of matrix, thus optimization of matrix used for analysis of targeted analytes is normally tested prior to the MALDI experiments. Generally, α-cyano-4-hydroxycinnamic acid (CHCA) is suitable for analysis of peptides, 2,5-dihydroxy benzoic acid (DHB) is used for analysis of carbohydrates, lipids and polymers, and

sinapinic acid (SA) is chosen for protein analysis.[53]

1.3.2 Solvent-free sample preparation

Solvent-free sample preparation method has been developed for the analysis of polymers, organometallic compounds and large organic compounds which are poorly soluble in solvents. The solid analyte and solid matrix is mixed together with a metal ball. The solid mixture is finally affixed onto the MALDI plate by suspension in nonsolvents, double-sided adhesive tape or smearing with micro spatula.[51] Our group has also developed an oil-assisted sample preparation (OASP) for solvent-free MALDI sample preparation. In this protocol, a droplet of inert paraffin oil is spotted onto the MALDI plate. Less than 0.1 mg solid sample and solid matrix are transferred and mixed onto the oil droplet (Figure 1-2).[54] Apart from analysis of insoluble compounds, solvent free sample preparation method reduces the time spent on sample mixing and drying, allowing rapid MALDI analysis.

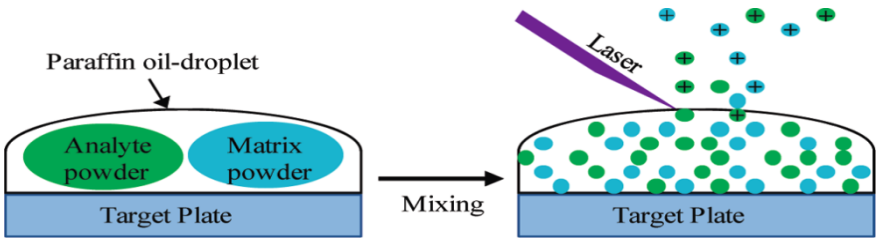


Figure 1-2. Oil-assisted sample preparation for MALDI analysis (Reprinted from ref[54]).

1.3.3 Ionization mechanism

Ultraviolet (UV) lasers such as nitrogen laser (λ = 337 nm) and Nd:YAG laser (λ = 355 nm) are commonly equipped in MALDI. Infrared (IR) lasers, on the other hand, are also used but not very common. When laser is irradiated onto the analyte-matrix crystals, matrix molecules in the crystals absorb the energy and induce rapid heating. The high temperature causes localized sublimation of matrix molecules and entrains analyte molecules in the matrix plume.[55] The ionization mechanism of MALDI is still unclear, but it is known that direct ionization of molecules by photons is energetically unfavorable. Ionization of matrix molecules requires energy from two to three photons, which is not efficient under common MALDI conditions.[56] Therefore, two different models, namely cluster ionization model (or lucky survivor model) and gas phase protonation model, have been proposed to explain the ionization process of MALDI.[57] Cluster ionization model was proposed by Karas and co-workers.[58,59] In this model, analyte molecules and matrix molecules are assumed to be pre-charged and existed with counter ions as neutral clusters in solutions. Once irradiation is applied onto the analyte-matrix crystal surface, the clusters are ablated and vaporized. The mechanical forces of irradiation cause charge separation of the neutral clusters. Charge neutralization could take place in plume of the clusters via proton, cation or electron transfer. The analyte

ions detected are the "lucky survivors" of plume neutralization.[58,59] Gas phase protonation model was proposed by many researchers, but Zenobi and Knochenmuss plotted the major events of this model.[53] In this model, the laser irradiation firstly excites the matrix molecules. Neighboring excited matrix molecules then share the electron distribution and the energy from the irradiation and concentrate onto one molecule. This pooling event causes the photoionization of one matrix molecule and deactivation of other matrix molecules. As MALDI matrices are usually acidic, gas phase proton transfer from matrix ions to analyte molecules can occur, leading to formation of protonated analyte ions. Gas phase cationization and electron transfer can also occur to generate the observed ions.[53,57]

Cluster ionization model and gas phase protonation model could be two complementary ionization mechanisms and explain the ion formation under different conditions. Jaskolla and Karas designed experiments to elucidate the ionization in different scenarios.[60] They used a special deuterated matrix which could supply free deuterium atoms for proton transfer only in the gas phase, while the deuterium atoms were inactive in the solution phase. Therefore, the deuterated analyte ions were expected to be formed in the gas phase and the protonated analyte ions were expected to be formed in the

solution phase. Both deuterated and protonated analyte ions were formed and their signal intensities were varied, depending on the experimental conditions. The authors concluded that gas phase protonation would be dominant when the sample solution was more basic, the laser energy was higher, the proton affinity of matrix was lower and the analyte was smaller.[60]

1.4 Outline of this thesis

The study of this project focuses on the development and applications of new mass spectrometric techniques to solve real-life problems.

In Chapter 1, the basic background of mass spectrometry has been introduced. The fundamentals of two major instruments, ESI-MS and MALDI-MS which are used in the study, have also been introduced. Finally, developments of ambient ionization and sample preparation of MALDI which are the focus of this research have also been discussed.

In Chapter 2, the development of WT-ESI-MS for analysis of six common drugs-of-abuse in Hong Kong, including ketamine (KET), methamphetamine (MA), 3,4-

methylenedioxymethamphetamine (MDMA, commonly known as ecstasy), cocaine (COC), heroin (HER) and tetrahydrocannabinol (THC, the active intergradient of cannabis), and their metabolites, will be discussed. The analytical performances such as limit-of-detections (LODs), precision and accuracy will also be investigated.

In Chapter 3, SPME-ESI-MS, a more sensitive technique for the detection of drugs-of-abuse, will be introduced. The experimental parameters such as the solvent application methods, the SPME extraction time and the elution and ionization will be discussed. Finally, the analytical performances of the developed SPME-ESI-MS for the drug analysis will be investigated.

In Chapter 4, another real-life issue, the quality control of edible oils, will be introduced. The development of a simple MALDI-MS sample preparation method for direct edible oil analysis will be introduced. Over one thousand edible oil samples have been analyzed and the characteristic peaks of each edible oil species will be concluded. Those characteristic peaks are important for the edible oil classification and authentication. Statistical analysis such as principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA) were used to facilitate the spectral analysis. The edible

oils can be divided into eight different group, and 97.2% out of the 435 pure edible oil products could be correctly classified by PLS-DA.

Finally, the research findings will be summarized and the prospects will be discussed in Chapter 5.

Chapter 2: Rapid detection and quantitation of drugs-of-abuse using wooden-tip electrospray ionization mass spectrometry (WT-ESI-MS)

2.1 Introduction

Drug analysis is an essential task in controlling drug abuse, which is a severe problem worldwide. According to the data of Narcotics Division, there are over 5,000 reported drug abusers in Hong Kong.[61] Detection and identification of the abused drugs is the first step for drug treatment and rehabilitation as well as law enforcement. Different drugs can have different metabolic pathways. For example, ketamine will lose one methyl group and convert to nor-ketamine, while an ester group of cocaine will be converted to carboxyl group and become benzoylecgonine in the human body.[62] Most of the drugs metabolize rapidly in the human body, such as the half-life of heroin is only a few minutes after ingestion.[63] Therefore, metabolites of the drugs are always used as the analytical targets of drug analysis, such as morphine and 6-monoacetylmophine are the targets of detection of heroin. However, there are some drugs including methamphetamine (half-life: 6 - 15 h) and MDMA (half-life: 5 – 9h), can remain high concentration in urine and oral fluid after metabolism.[64] They are still detectable 1 – 3 days after ingestion so they can be the analytical targets of the presence

of drugs. Identification of abused drugs is performed by determination of drug residues and its metabolites in biological fluids, such as urine, oral fluid and blood. Analysis of drug residues in hair and finger nail is also important, because of the long detection window and history provided by of these specimens.[65] Drug residues in hair can be detected even after a few years of drug intake while only few hours to few days for biological fluids.[21] However, compared to nail and hair analysis, analysis of biological fluids is of particular advantages because of the relatively high concentrations of drug and metabolite residues in common biological fluids, availability of relatively large sample size and ease of collection.[66,67] Also, analysis of hair requires complex sample preparations including acid digestion and longtime extraction.[68] For the above reasons, biological fluids are still the widely accepted specimens for drug analysis.

Due to the prevalence of the problem of drug abuse, chemical analysis units are required to handle a considerable number of biological fluid samples for law enforcement and healthcare purposes. To deal with the large volume of samples and to ensure the reliability of analytical results, a two-step strategy, preliminary screening followed by confirmatory analysis, is commonly applied.[69-71]

Preliminary screening for the presence of drug residues in biological fluids is commonly performed using commercially available on-site screening devices or immunoassay techniques. Such rapid screening techniques rely on either simple chemical reactions such as Scott test or antigen and antibody interactions, for the detection of drugs residues.[72-74] The current screening technologies possess several drawbacks. First, these screening techniques typically require the use of costly consumables, e.g., cartridges for on-site screening devices and consumable kits for immunoassay. Particularly for immunoassay methods, tedious sample preparations, including loading of sample and drug-enzyme conjugate, incubation, washing, and addition of substrate and stop solution, are usually involved.[75] More importantly, evaluation studies indicated that false positive and false negative results are potentially obtained by commonly used on-site screening devices and immunoassay methods. The performance of those detection devices varies, the accuracy of detection can be as high as 90% for some drugs and lower than 75% for some other drugs.[69,71,75-79] Compounds with the chemical structures similar to illicit drugs would also generate false positive results in the rapid screening techniques. The problem of *cross-reactivity* is commonly encountered in these screening techniques, lowering the specificity of detection.[78] For these reasons, it is generally believed that further confirmatory analysis is required for

the confirmation of positive samples.

Confirmatory analysis is mainly performed by conventional techniques such as GC-MS and LC-MS, which allow highly sensitive and specific detection and quantitation.[75,76,79-81] However, due to the high chemical complexity of biological fluids, extensive sample pretreatments are usually required. Sample pretreatments mainly involve extraction of targeted drugs to reduce the matrix interference and protect the analytical column. The common techniques for sample extraction, mainly including solid-phase extraction and liquid-liquid extraction, are significantly time-consuming and laborious. Particularly for GC-MS, as more of the drugs and its metabolites are non-volatile, tedious derivatization of analytes is often required for effective ionization of analytes.[75,81,82] In addition, typical GC-MS and LC-MS runs usually involve a long period of chromatographic separation, for separating the target analytes from complex matrix, thus are time-consuming as well. Overall, taking the factors of time, cost, and accuracy into account, development of novel analytical methods that are simple, rapid, economical and reliable is highly beneficial to drug analysis.

The objective of this research is to develop a rapid and reliable method for detection

and quantitation of drug-of-abuse in urine and oral fluid by wooden-tip electrospray ionization mass spectrometry (WT-ESI-MS).[42] Six commonly abused drugs in Hong Kong, including ketamine (KET), methylamphetamine (MA), cocaine (COC), MDMA, cannabis (THC) and heroin (HER), have been investigated. The technique of WT-ESI-MS was developed by our research group in 2011. This technique has been demonstrated to be simple, rapid, economical (only include wooden "toothpick" as consumables and low consumption of solvents), easy-to-setup, compatible with different instruments, and applicable to analysis of a wide range of analytes. Particularly, this technique is of high tolerance to impurities, allowing direct analysis of target analyte in complex mixtures, e.g., drug residues in biological fluids, with only little sample preparation and no chromatographic separation. Therefore, the drug analysis could be finished within a few minutes. With these desirable features, this technique could be applied as a one-step method for rapid screening and quantitation of targeted drugs and their metabolites in urine and oral fluid.

2.2 Experimental section

2.2.1. Materials and chemicals

The wooden toothpicks used in this study were BEST-Buy brand purchased from

PARKnSHOP in Hong Kong. The wooden toothpicks are made of birch wood without chemical modification on the surface during manufacturing. Cotton/polyester was cut out from clean laboratory coat from Guangdong Tianyi Co. Ltd. (Zhongshan, China). Melamine foam sponge was purchased from HSK (Korea). Polyvinyl alcohol (PVA) was from Prologic brand purchased from supermarket in Hong Kong. Aluminum foil was purchased from Reynolds (Lake Forest, IL, USA) and stainless-steel needle was purchased from Tongyong Office Co. Ltd. (Shanghai, China). Ketamine hydrochloride, nor-ketamine (Nor-K) hydrochloride, nor-ketamine-D_4 (D-Nor-K) hydrochloride, methamphetamine, methamphetamine-D_5 (D-MA), MDMA, MDMA-D_5 (D-MDMA), cocaine, cocaine-D_3 (D-COC), benzoylecgonine, benzoylecgonine-D_3 (D-BEN), delta-9-THC (THC), delta-9-THC-D_3 (D-THC), 11-nor-9-carboxy-delta-9-THC (THC-COOH), 11-nor-9-carboxy-delta-9-THC-D_9 (D-THC-COOH), heroin, 6-monoacetylmorphine (6-MAM) and 6-acetylmorphine-D_3 (D-6-MAM) standards were purchased from Cerilliant (Round Rock, TX, USA) and morphine sulphate salt solution was purchased from Fluka (St. Louis, TX, USA). HPLC grade methanol (MeOH) was purchased from Tedia (Fairfield, CT, USA) and formic acid (FA) was purchased from Sigma (St. Louis, TX, USA). The blank urine and oral fluid were donated by the research team members.

2.2.2 Sample preparation

Stock solutions of targeted drugs, metabolites and internal standard (IS) were stored according to the instructions of supplier. The standard solutions of targeted drugs and their metabolites used for the preparation of calibration curve were prepared by serial dilution of stock solutions with methanol. Another set of standard solutions with low, medium and high concentrations was prepared for the determination of accuracy and precision of the method at different concentration levels. Finally, the standard solutions of drugs-of abuse, related metabolites and internal standards (250 ng/mL of each deuterated analytes and 500 ng/mL for THC-D_3) were spiked into blank urine and oral fluid and simulated as urine and oral fluid samples for WT-ESI-MS analysis. Only spiked samples were used in this study. All the spiked sample solutions were freshly prepared from stock solution before the analysis.

2.2.3 Instrumental setup

All the experiments were performed on a Waters Micromass Quattro Ultima triple quadrupole mass spectrometer (Milford, MA, USA). For the method optimization, the drug standards were injected into the mass spectrometer through direct infusion with the syringe pump at a flow rate of 5 μL/min. The capillary voltage was set at 3 kV for

positive ionization mode and 2.5 kV for negative ionization mode. The cone voltage was 30 V. The cone gas and desolvation gas flow were 350 L/hr and 100 L/hr respectively. The source temperature and desolvation temperature were 150 ºC and 350 ºC respectively. The collision gas cell pressure was set to 4 $x10^{-4}$ bar for MS/MS analysis.

For the WT-ESI-MS experiments, the ionization source was set into nano-ESI configuration as shown in Figure 2-1. A sharpened wooden-tip (1.5 - 1.7 cm) was mounted onto the capillary holder of the nano-ESI source. The mass spectrometer was operated at 3.5 kV for positive ionization mode and 3.0 kV for negative ionization mode. The cone gas flow and source temperature were 100 L/hr and 150 ºC respectively. The targeted drugs and metabolites were analyzed under MRM mode. The cone voltage and channels used for the analysis are listed in Table 2-1.

For the solid materials, such as polymers and aluminum foil which incompatible with nano-ESI source, were cut into triangular shape and clipped with a pair of forceps. The forceps and the solid materials were placed in front of the inlet of mass spectrometer and connected to ESI source. High voltage was applied to the materials directly. The

mass spectrometer settings were as same as that of WT-ESI-MS.

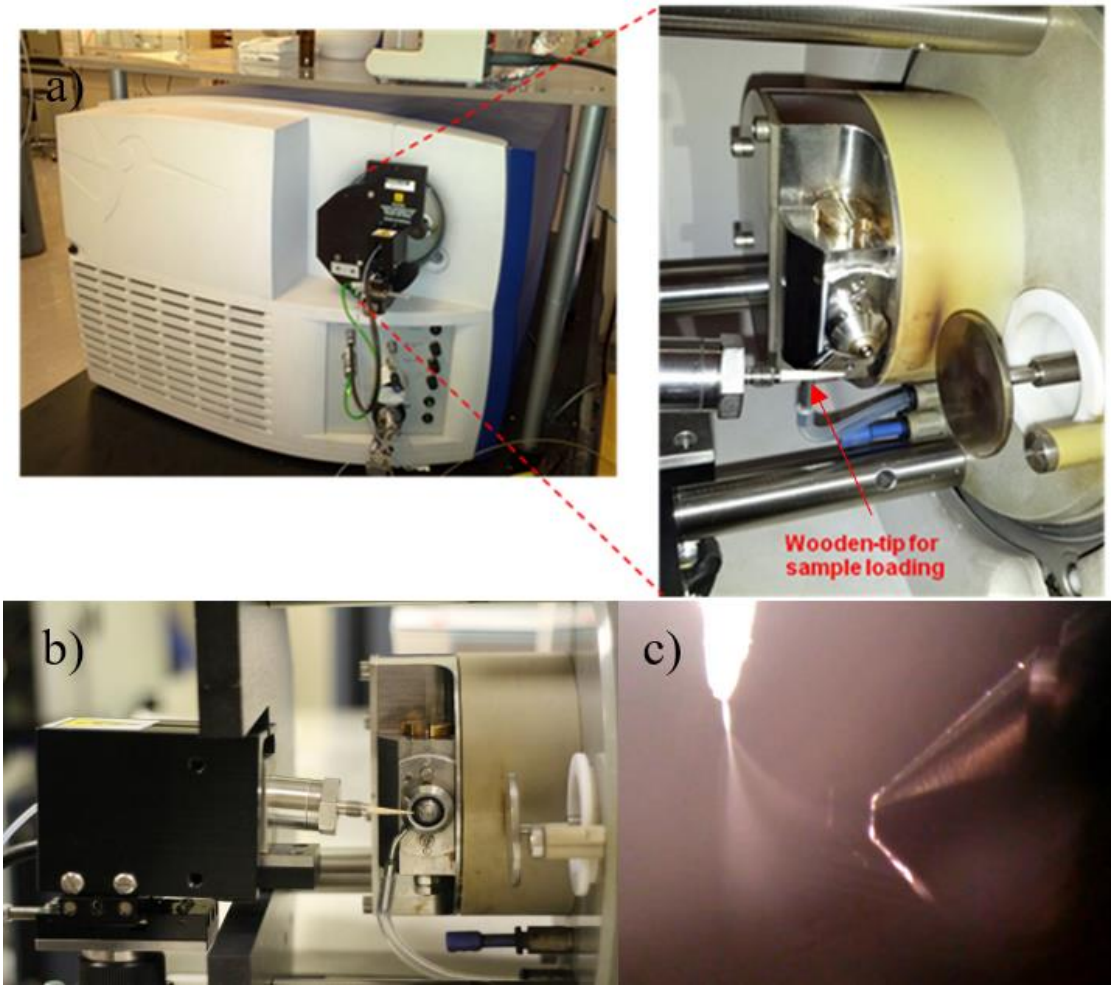


Figure 2-1. (a)The photos of WT-ESI-MS setup on a Micromass Quattro Ultima triple quadrupole mass spectrometer. (b) A close-up of a wooden tip mounted into nano-ESI source for WT-ESI-MS analysis. (c) Electrospray of sample solution applied onto wooden tip.

Table 2-1. MRM conditions and cone voltage of various drugs, metabolites and deuterium labeled internal standards

Analyte	MRM Channel	Collision cell energy (V)	Cone voltage (V)
Ketamine	238 → 125	25	30
Nor-ketamine	224 → 125	20	30
Nor-ketamine-D_4	228 → 129	20	30
Methamphetamine	150 → 91	15	30
Methamphetamine-D_5	155 → 121	10	30
MDMA	194 → 163	8	30
MDMA-D_5	199 → 165	10	30
Cocaine	304 → 182	15	30
Cocaine-D_3	307 → 185	15	30
Benzoylecogonine	290 → 168	18	30
Benzoylecogonine-D_3	293 → 171	18	30
THC	315 → 193	30	30
THC-D_3	318 → 196	25	30
THC-COOH	343 → 299	25	30
THC-COOH-D_9	352 → 308	25	30
Heroin	370 → 268	28	45
6-monoacetylmorphine	328 → 165	35	40
6-acetylmorphine-D_3	331 → 165	32	35
Morphine	286 → 165	38	40

2.2.4 WT-ESI-MS workflow

For each sample, 10 µL of corresponding internal standards, 10 µL of methanol with 0.1% formic acid and 10 µL of spiked urine or oral fluid sample were mixed with vortex for above 10 s. A sample solution with organics-to-aqueous ratio of 2:1 was prepared for WT-ESI-MS analysis. A wooden tip was firstly sharpened and cut into 1.5 to 1.7 cm long and mounted into the capillary holder of the nano-ESI source. The wooden tip was wetted with 4µL of methanol and 2µL of sample solution is then pipetted to the tip end. Upon application of a high voltage (+3.5kV except -3.5kV for THC-COOH) to the wooden tip, spray ionization was induced and ion signals of the analytes were detected under MRM mode. Each sample solution was applied onto the same wooden tip for three times. A new wooden tip was prepared after the analysis of each sample. Figure 2-2 illustrated the WT-ESI-MS work flow for the drug analysis.

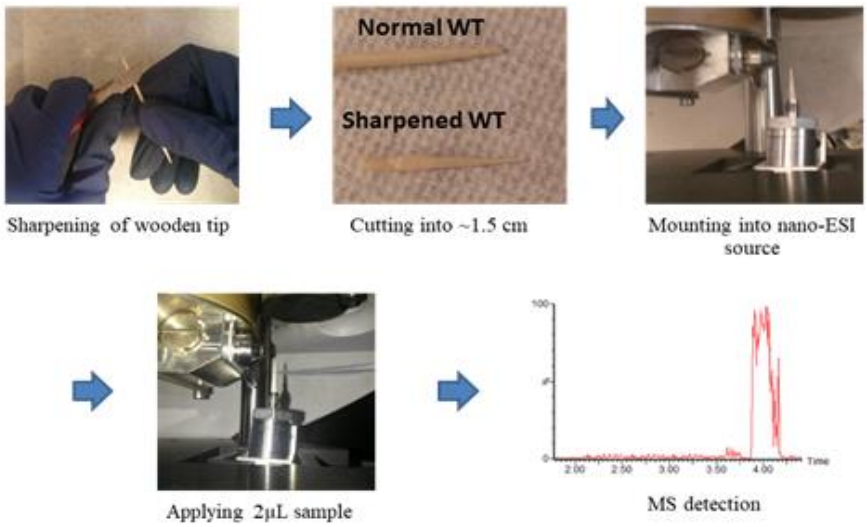


Figure 2-2. Workflow of WT-ESI-MS analysis.

2.2.5 ESI-MS with different solid substrate

To optimize the protocol of WT-ESI-MS method, the ion signals generated from ketmaine, nor-ketamine and nor-ketamine-D_4 in urine using different tip materials including wooden tip, cotton/polyester (35:65), sponge, polyvinyl alcohol (PVA), aluminum foil and stainless-steel needle had been tested.

Wooden-tip surface modification with nitric acid had been investigated to reduce the background of WT-ESI-MS analysis. As described by Su et al., the chemical treated wooden tips were prepared by immersing the sharpened wooden tips in $1.5x10^{-3}$M nitrate acid for 2 hours; then washed the tips with water for 5 min, and repeated six times.[83]

2.2.6 Method validation of WT-ESI-MS

Calibration curves

The calibration curves for quantitation were constructed by averaging three sets of experimental data, while each set of data was obtained by analyzing at least five different concentrations of analytes. The resultant MRM chromatograms were processed using Mass Lynx 4.1 (Milford, MA, USA). The results were smoothed and

peak heights were applied for constructing the calibration curves. The targeted drugs and their metabolites were analyzed at the same experiments, i.e. six experiments were performed for constructing the calibration plots of the six targeted drugs.

Accuracy and precision

The accuracy and precision of WT-ESI-MS method was determined by at least three sets of urine and oral fluid samples spiked with the analytes at low, medium, and high concentrations respectively. Samples at each concentration were analyzed at least five times using individual wooden tips, and the data obtained were averaged for comparison. The accuracy was defined as the closeness of the measured result and the true value according to International Union of Pure and Applied Chemistry (IUPAC)[84] and calculated by:

$$\frac{\text{concentration of analyte determined}}{\text{actual concentration of the analyte in the sample}} \times 100\% \qquad (2\text{-}1)$$

and the precision, i.e., relative standard deviation (R.S.D.), was calculated by:

$$\frac{\text{standard deviation of the concentration determined}}{\text{mean of the concentration determined}} \times 100\% \qquad (2\text{-}2)$$

Limit-of detection (LOD) and limit-of-quantitation (LOQ)

Blank samples were prepared by spiking only the internal standards into blank urine or

oral fluid. The LODs and LOQs were determined by comparing the intensity (peak height) ratio of the analytes and internal standards between the spiked samples and the blank samples [$(I_{analyte}/I_{IS})_{spiked}/(I_{analyte}/I_{IS})_{blank}$] in order to compensate the chemical and electronic noises and variation in instrumental factors. The LODs and LOQs are defined as the concentrations of analytes that could achieve a [$(I_{analyte}/I_{IS})_{spiked}/(I_{analyte}/I_{IS})_{blank}$] value of three and ten, respectively. At least nine measurements were obtained for the determination of LODs and LOQs.

2.3 Results and discussions

2.3.1 Optimization of prototype

Electrospray ionization using non-conductive materials

Our group has demonstrated the use of WT-ESI-MS for detection and quantitation of ketamine in urine and oral fluid in 2013.[85] Herein, different materials were tested for analysis of ketamine in urine as different materials could possess different surface properties for ambient ionization.[44] The material that produced the best signals was selected to use as the solid substrate in this study. Non-conductive materials with porous structures or microchannels could allow solvent diffusion throughout the materials thus resulted in the conduction of electricity.[44] Three of non-conductive materials, sponge,

cotton-polyester mixture and polyvinyl alcohol were used as ESI-emitters in this study and is shown in Figure 2-3. No electrospray and no meaningful ion signals were observed by using those polymers for electrospray ionization. Similar results were obtained by changing the angles to the MS inlet. The porous polymers absorbed the water and solvents in the sample matrix, leading to the high surface tension on the polymer surfaces and failure to generate electrospray from the retained urine samples.

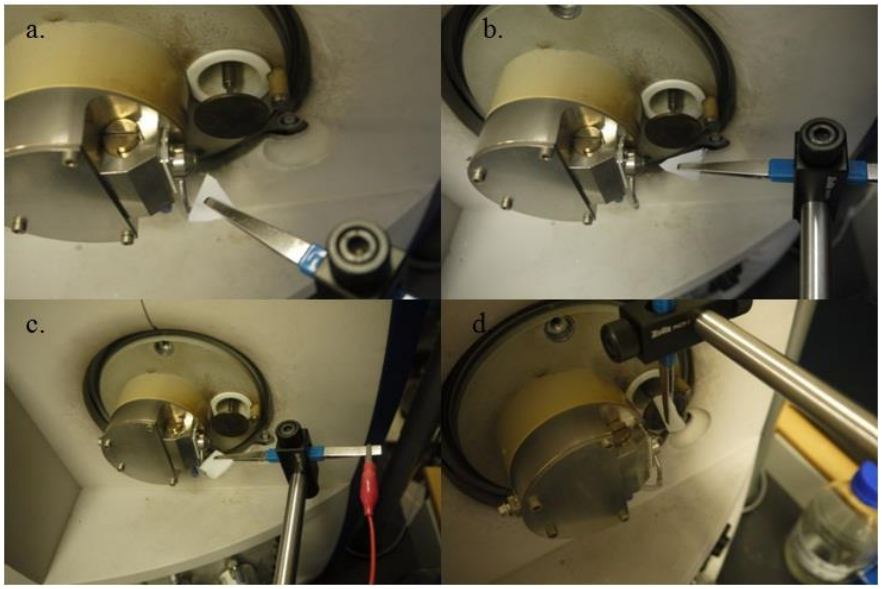


Figure 2-3. (a) and (b) Electrospray ionization using cotton-polyester mixture; (c) and (d) electrospray ionization using sponge.

Electrospray ionization using conductive materials

Hu et al. demonstrated the use of aluminum foil for electrospray ionization[43] whereas Hiraoka et al. demonstrated the use of stainless-steel needle for electrospray ionization.[40] These two conductive materials were also tested in this study. The setup of electrospray ionization using conductive substrates were showed in Figure 2-4. The sharp end and good electrical conductivity of metal substrates allowed charge accumulation on the substrate heads. The non-porous structure allowed the solution to spray out freely. Sharp and strong ion signals were observed (Figure 2-4b and d). However, the water droplet accumulated on the head and obstructed the electrospray ionization. Also, loading of sample solution onto thin needle and soft foil was difficult.

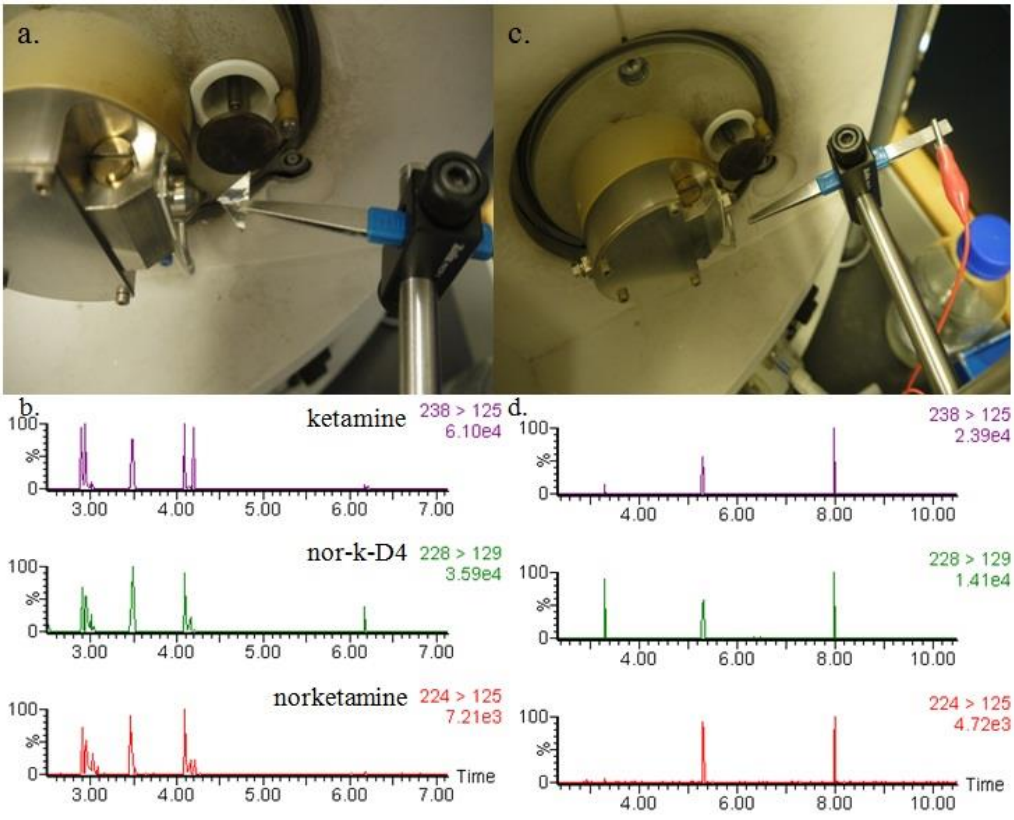


Figure 2-4. (a) and (c) Electrospray ionization using aluminum foil and stainless-steel needle, respectively; (b) and (d) chromatogram showing the ion signal generated by aluminum foils and stainless-steel needles, respectively.

Electrospray ionization using chemical treated wooden tips

Su et al. demonstrated the chemical interference generated by the tissue surface of fibrous materials such as paper could be eliminated by surface treatment such as treating the tissue with dilute nitrate acid.[83] Such chemical treatment may reduce the background and improve the sensitivity of detection. Ion signals generated by normal

wooden tip and chemical treated wooden tips were tested repeatedly (n = 10). Ion signals with signal-to-noise ratio (S/N) ≥ 3 were considered as positive. Typical MRM results for the detection of ketamine and nor-ketamine in urine are shown in Figure 2-5. Both treated and non-treated wooden tips could generate signal easily. However, there were no significant improvements on both the noise elimination and signal enhancement.

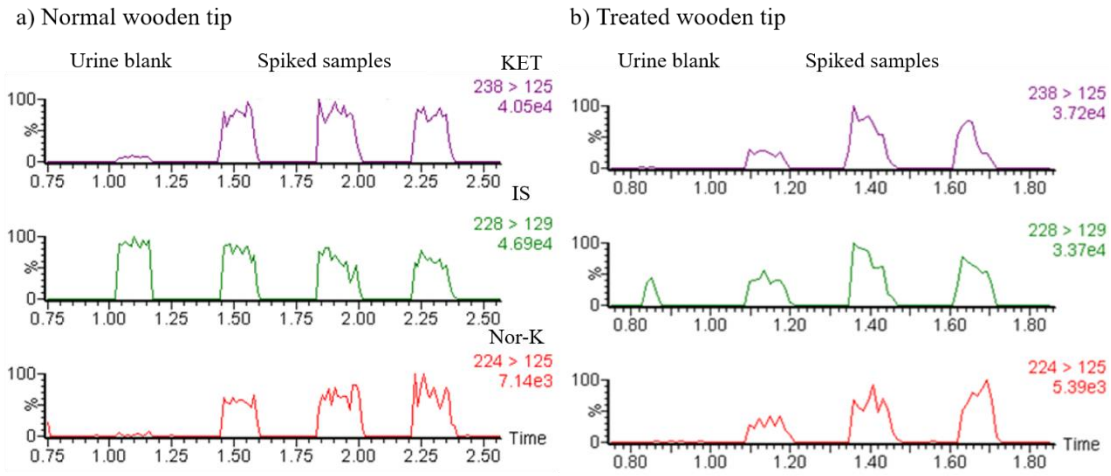


Figure 2-5. Typical ion signals generated for 100 ng/mL spiked ketamine (*m/z* 238 → *m/z* 125), nor-ketamine (*m/z* 224 → *m/z* 125) and internal standard (*m/z* 228 → *m/z* 129) in urine using (a) normal wooden tips and (b) wooden tips treated with diluted nitrate acid.

Summary

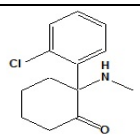
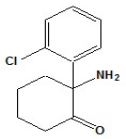
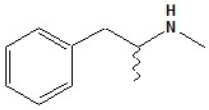
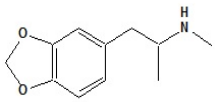
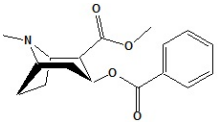
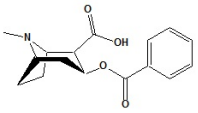
The protocol of electrospray ionization using wooden tips was optimized. Different tip materials, tip orientations and tip modification method had been tested. Polymer materials would retain water onto the surface and into the materials, leading to no meaningful signals were observed. Metal materials gave very sharp signals, but sample loading was difficult and water accumulation at the tip-end obstructed the ionization. Wooden tips gave reasonable toughness and surface areas for sample loading and the fabric structure allowed the sample to retain on the surfaces and diffuse to the tip ends for electrospray ionization. The compatibility of nano-ESI source for the wooden-tip electrospray ionization reduced the difficulty of the experimental set up as well. No significant signal improvements were observed when using the chemically-treated wooden tips.

2.3.2 Optimization of protocol for detection and quantitation of drugs-of-abuse

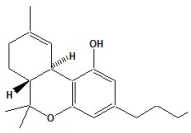
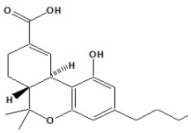
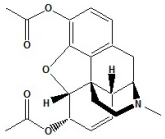
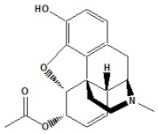
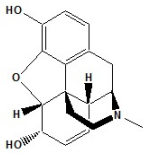
The molecular ions of analytes were confirmed by injecting the standard solutions into the mass spectrometer with normal ESI-MS. The molecular ions were then selected and fragmented after applying the collision energy. The fragment ions were monitored and the collision energy was varied to investigate the effect on the fragmentation. The

fragment ion and collision energy that gave stable and intensive signal were selected for detection and quantitation of the analyte. The structures of the targeted analytes and daughter ions observed in the MS/MS analysis are shown in Table 2-2.

Table 2-2. Structures, molecular formula, molecular ions and daughter ions of the targeted analytes in this study.

Compound	Structure	Molecular formula	Molecular ion	Daughter ions
Ketamine		$C_{13}H_{16}ClNO$	238	220, 207, 179, 125*
Nor-ketamine		$C_{12}H_{14}ClNO$	224	207, 179, 125*
Methamphetamine		$C_{10}H_{15}N$	150	119,91*
MDMA		$C_{11}H_{15}NO_2$	194	163*, 135, 133, 105
Cocaine		$C_{17}H_{21}NO_4$	304	272, 182*, 150, 119, 105
Benzoylecgonine		$C_{16}H_{19}NO_4$	290	168*, 150, 119, 105

(To be continued)

THC		$C_{21}H_{30}O_2$	315	259, 221, 221,193*
THC-COOH		$C_{21}H_{28}O_4$	343	299*, 245, 191, 179
Heroin		$C_{21}H_{23}NO_5$	370	328, 268, 211, 193, 191, 183, 181, 165*
6-monoacetylmorphine		$C_{19}H_{21}NO_4$	328	211, 193, 191, 183, 181, 165*
Morphine		$C_{17}H_{19}NO_3$	286	211, 209, 201 , 185, 181, 173, 165*, 157, 155, 153, 147

* The selected daughter ions for detection and quantitation of targeted analytes under MRM mode.

2.3.3 Detection and quantitation of drugs-of-abuse in urine and oral fluid

Detection of the targeted analytes

Typical MRM results for detection of methamphetamine in urine and oral fluid are shown in Figure 2-6. The wooden tip was firstly wetted with 6 μL of methanol. Sample solution was then applied onto the wooden tip and signals could be generated. Typically, each sample was applied onto the same wooden tip 3 times and each signal could

maintain 10 s to 30 s. The variations of signals are due to the variations of morphology of wooden tips. Although, it was difficult to control the shapes of wooden tips precisely, the signal variations could be compensated by the additional of internal standards. Signals were considered as positive only if the S/N ≥ 3 compared with the blank, and this will be discussed in later part.

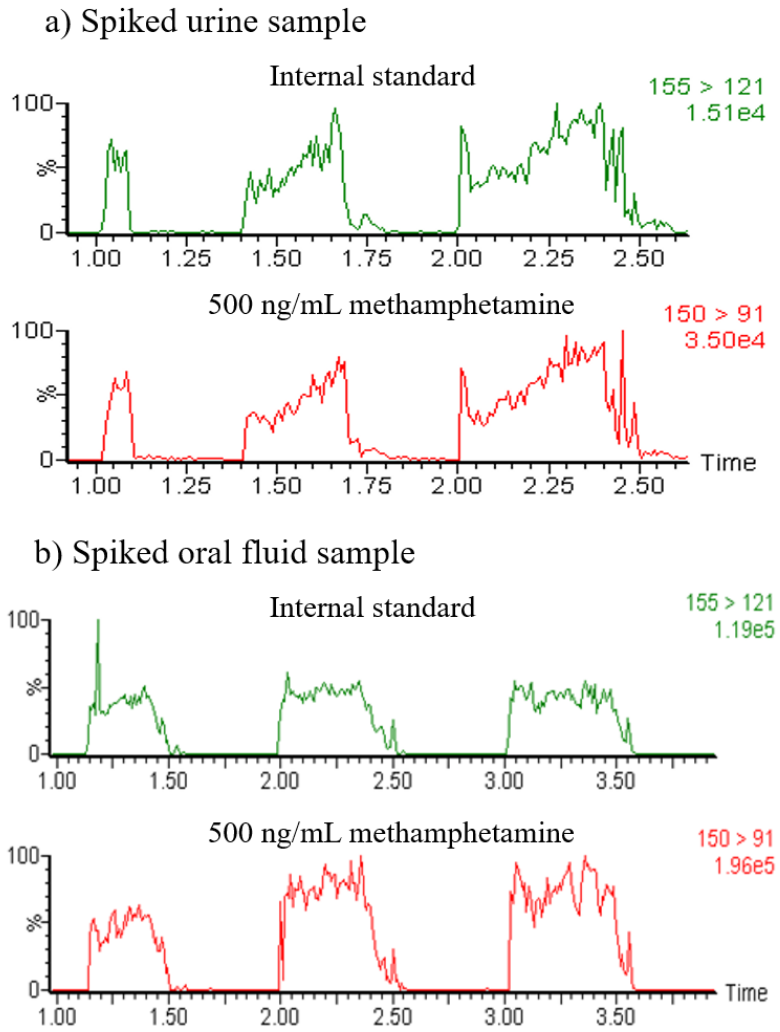


Figure 2-6. Typical MRM results of the detection of 500 ng/mL methamphetamine in (a) urine and (b) oral fluid using WT-ESI-MS.

The same sample solution was repeatedly measured with three induvial wooden tips at the same day and the whole experiment was repeated on another day. The results are shown in Figure 2-7. The chromatograms were smoothed and integrated for measuring the peak heights (peak intensities) of the signals. The absolute intensities of both the 3 measurements using the same wooden tip and all the measurements between different wooden tips varied but still within the acceptable range. The precisions of the 3 measurements using the same wooden tip (no.1 - no.4) were 14.9%, 10.6%, 15.8% and 18.9%, respectively. The precision of all the measurements (n = 12) between different wooden tips (n = 4) was 15.0%. On the other hand, using the relative peak heights (i.e. the peaks heights of the analytes divided by the peaks heights of the internal standard) for the measurements of signals were more reproducible. The precisions of the 3 measurements, in term of relative peak heights, using the same wooden tip (no.1 - no.4) were 7.0%, 7.0%, 9.1% and 9.4% respectively. The overall precision was 6.8%, which was significantly better than that of using absolute intensities for the measurements. Therefore, the relative peak heights were used for the detection and quantitation of targeted analytes in this study.

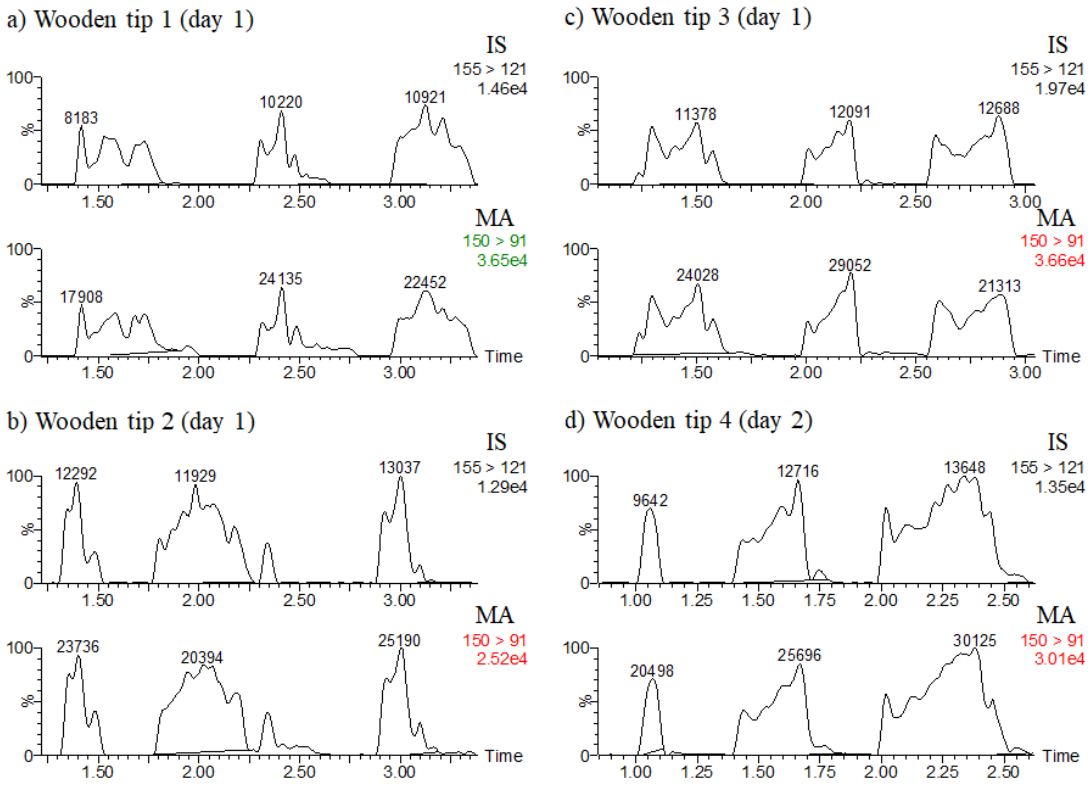


Figure 2-7. Processed MRM results for detection of 500 ng/mL methamphetamine in urine using WT-ESI-MS. (a-c) Repeated experiments using three individual wooden tips within the same day and (d) repeated experiment on another day.

Quantitation of targeted analytes

The calibration curves for the quantitation of targeted analytes in urine and oral fluid were constructed by measuring the signals of at least five spiked samples with different concentrations. Representative data for construction of calibration curves is shown in Figure 2-8. Taking methamphetamine as an example, the intensities of MRM signals for analyte exhibited a positive correlation with the increase in sample concentrations,

while the signals for internal standard, which was at fixed concentration, did not significantly vary between different sample loadings. Construction of a calibration curve with typically five to seven data points could be completed with 10 - 20 minutes. A calibration curve covering a concentration range of 25 – 5000 ng/mL with linearity of R^2 = 0.9996 were obtained. The average standard deviation (average of standard deviation for different sample concentrations), as represented by the error bars, for data obtained in five experiments for construction of the calibration curve was 7.2%, indicating high reproducibility of the present method. Figure 2-8 illustrated the construction of calibration plot using the same wooden tip. However, to prevent memory effect, induvial wooden tips were used to analyze each concentration in this study.

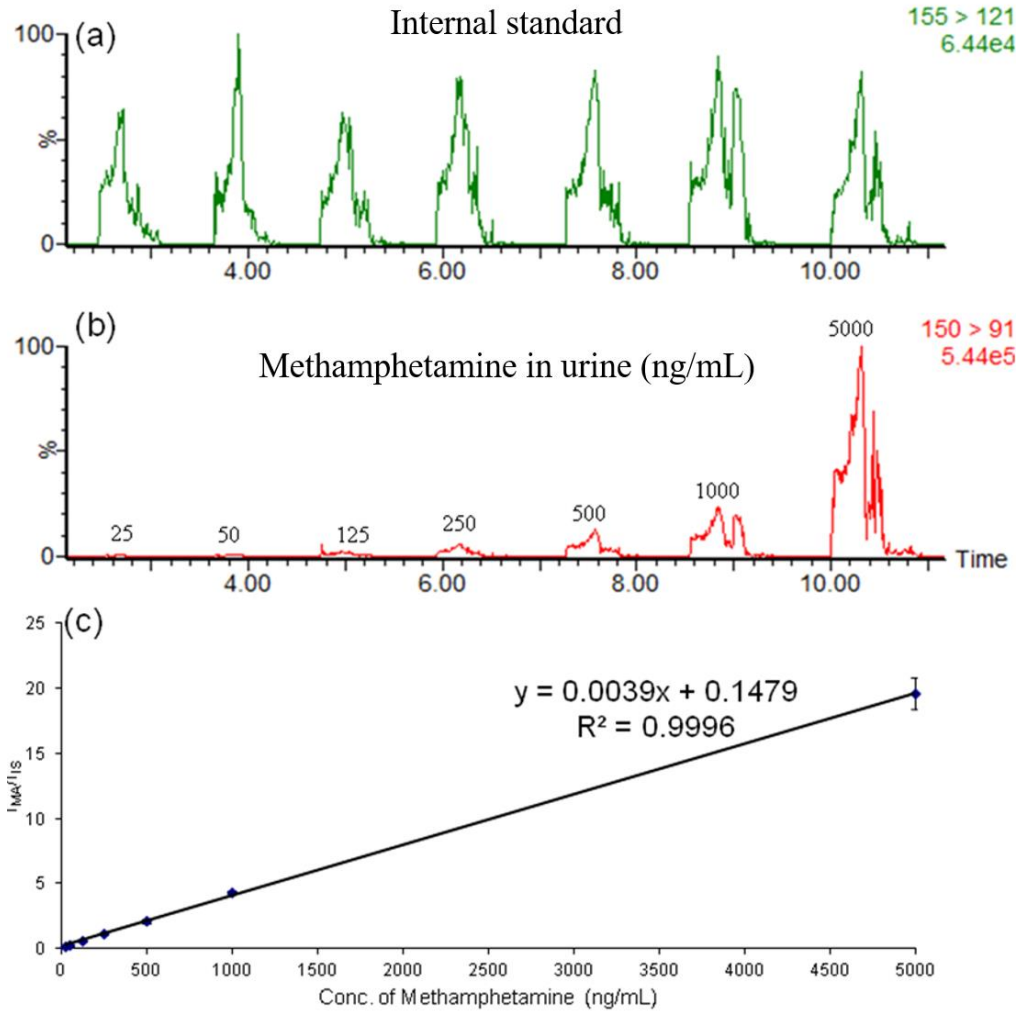


Figure 2-8. MRM signals for (a) 250 ng/mL methamphetamine-D_5 internal standard and (b) different concentrations of methamphetamine in urine. (c) A calibration curve obtained for quantitation of methamphetamine in urine.

The linear range, linearity (in term of R^2) and average R.S.D. of the calibration points of all the targeted analytes in urine and oral fluid are recorded in Table 2-3. The calibration plots for quantitation of the targeted analytes are shown in Figures 2-9 and 2-10. During the study period, deuterated ketamine, heroin and morphine were not available, Nor-K-D_4 and 6-MAM-D_4 were used as their internal standards instead. Generally, the linear range could cover three orders of magnitude (e.g. 50-5000 ng/mL) except that of heroin and its related compounds. The heroin related compounds were hardly detected at low concentrations, thus the calibration points at low concentrations were not available. The calibration range for quantitation of targeted analytes in oral fluid was slightly wider than that of urine. The background signals generated by oral fluid were lower than that of urine, thus leading to better detection of the analytes in oral fluid at low concentrations. The R^2 of the curves were greater than 0.99, indicating good linearity. Overall, the reproducibility of the analytes with its deuterated internal standards were better than those without the deuterated internal standards. The results again indicated that corresponding internal standards were important for WT-ESI-MS analysis. The reproducibility of relative intensities of each analyte was generally better than 20% except morphine which was less reproducible. The signals of morphine were much poorer than most of the analytes and its signals thus were very unstable. The

results showed that the present method was suitable for quantitation of drugs-of-abuse in urine and oral fluid. However, it was found that the ionization efficiency of THC and THC-COOH was poor in the present method, and no calibration curve could be constructed as signals could only be produced when high concentrations of THC and THC-COOH were applied onto the wooden tips.

Table 2-3. Linearity of targeted drugs and metabolites in urine and oral fluid**.**

Compound		Linear range (ng/mL)	R^2	Average R.S.D. of calibration points (%)
Ketamine	Urine	50 - 5000	0.9998	20.6
	Oral fluid	50 - 5000	0.9981	18.4
Nor-ketamine	Urine	50 - 5000	0.9999	10.3
	Oral fluid	50 - 5000	0.9999	18.8
Methamphetamine	Urine	25 - 5000	0.9996	7.2
	Oral fluid	25 - 5000	0.9991	8.6
MDMA	Urine	50 - 5000	0.9998	13.0
	Oral fluid	50 - 5000	0.9996	4.1
Cocaine	Urine	50 - 5000	0.9996	7.0
	Oral fluid	25 - 5000	0.9994	8.1
Benzoylecgonine	Urine	125 - 5000	0.9965	15.1
	Oral fluid	50 - 5000	0.9993	17.0
Heroin	Urine	250 - 10000	0.9985	19.0
	Oral fluid	125 - 5000	0.9990	19.3
6-monoacetylmorphine	Urine	250 - 10000	0.9946	10.1
	Oral fluid	125 - 10000	0.9993	9.1
Morphine	Urine	500 - 10000	0.9967	25.9
	Oral fluid	500 - 10000	0.9903	52.6

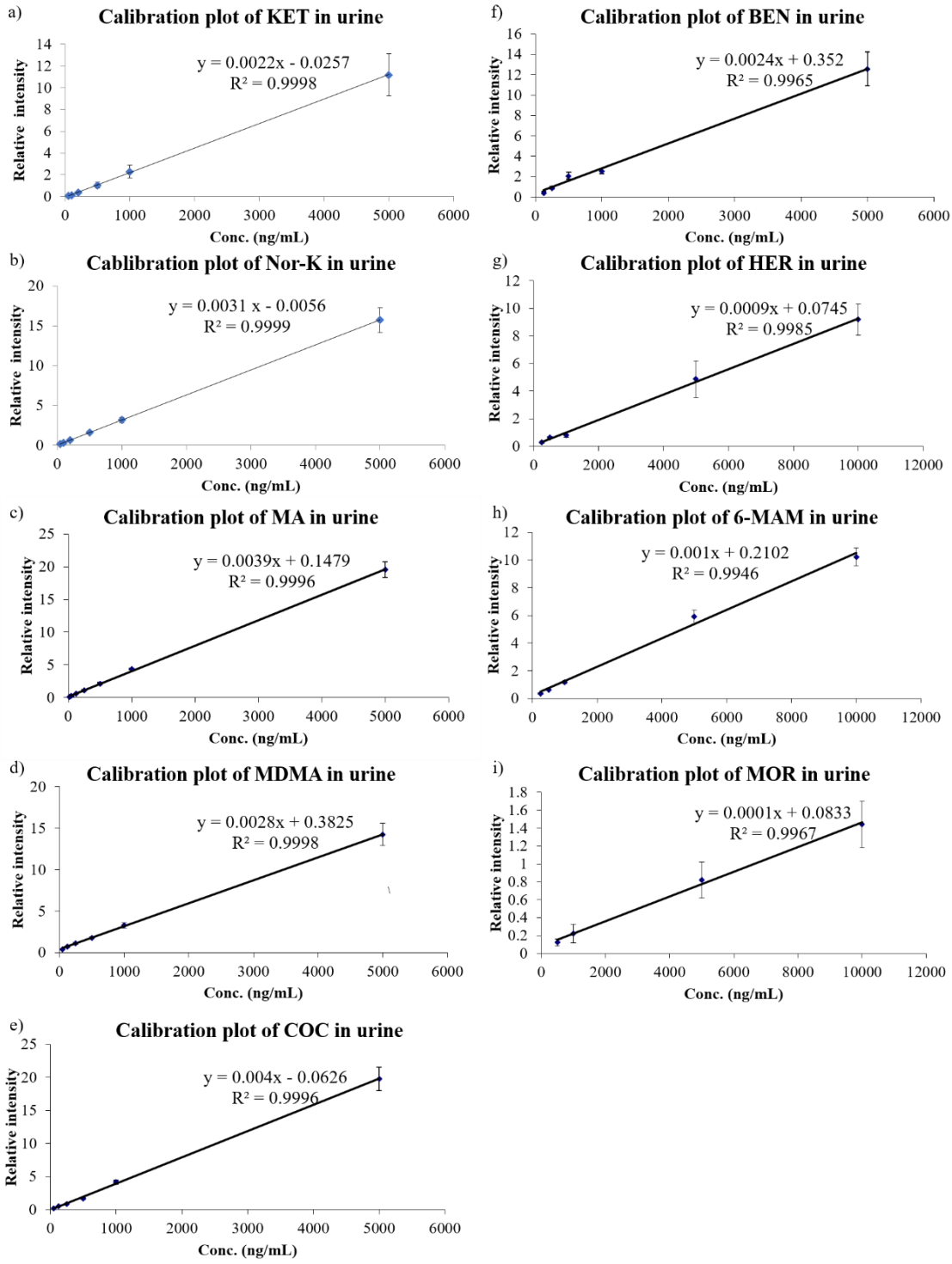


Figure 2-9. Calibration plots for the quantitation of (a) ketamine, (b) nor-ketamine, (c) methamphetamine, (d) MDMA, (e) cocaine, (f) benzoylecgonine, (g) heroin, (f) 6-monoacetylmorphine and (i) morphine in urine.

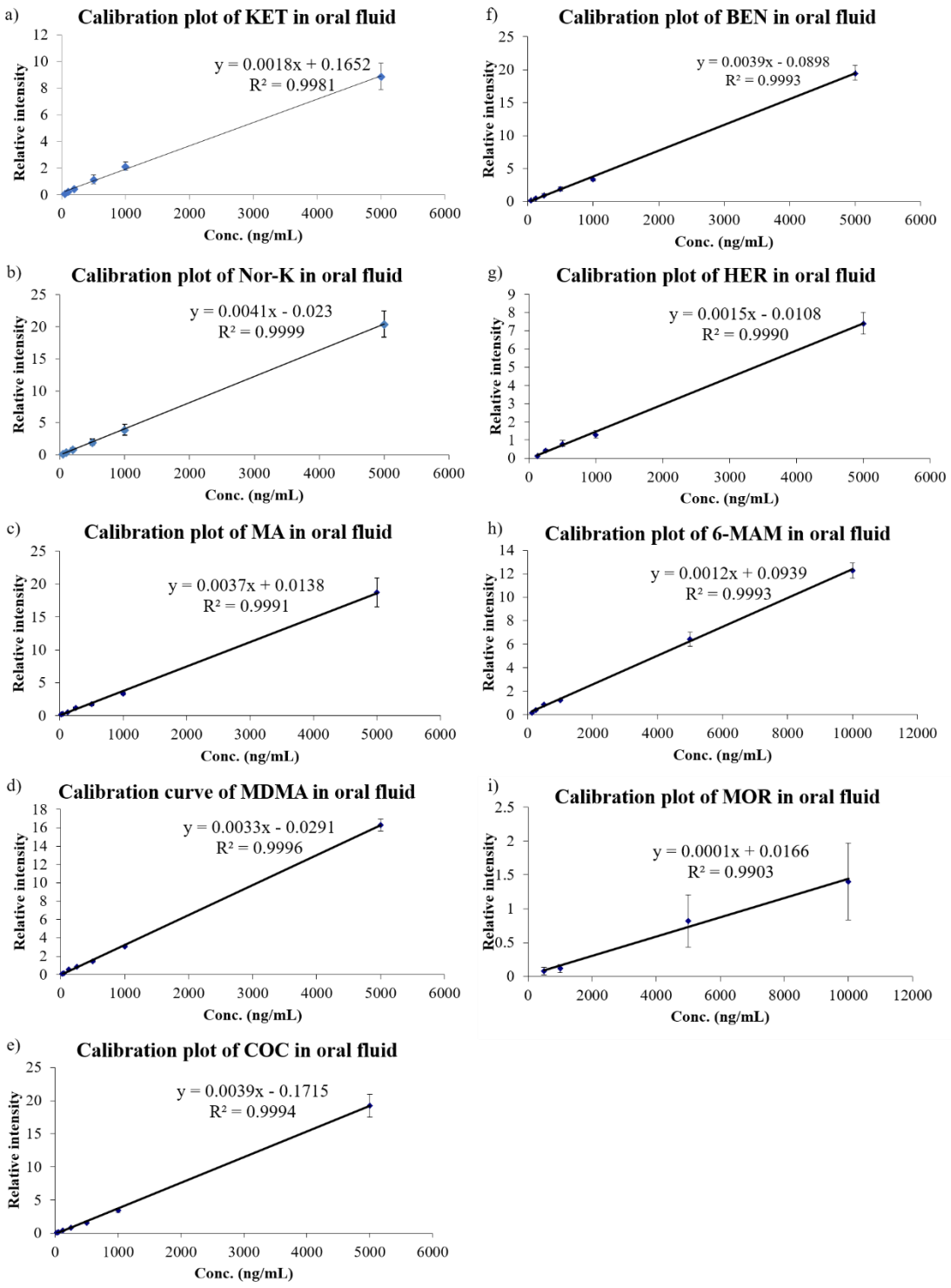


Figure 2-10. Calibration plots for the quantitation of (a) ketamine, (b) nor-ketamine, (c) methamphetamine, (d) MDMA, (e) cocaine, (f) benzoylecgonine, (g) heroin, (f) 6-monoacetylmorphine and (i) morphine in oral fluid.

Accuracy and precision of quantitative analysis

Spiked quality control samples at low, middle and high concentrations of the targeted analytes in urine and oral fluid (O.F.) were tested. The accuracy and precision of the quantitative analysis results are summarized in Table 2-4. For analysis of ketamine, nor-ketamine, methamphetamine, MDMA, cocaine and benzoylecgonine, the precision was in general within 15%, except for methamphetamine in urine with a value (17%) that was slightly higher than other values. For analysis of heroin and its metabolites (i.e. 6-monoacetylmorphine and morphine), the precision determined for analysis of oral fluids was satisfactory (≤ 15%), yet for analysis of urine, the precision was found to be as high as 25%, due to the relatively low absolute intensity for detection of heroin and its metabolites. The accuracy of the present method for analysis of all analytes except heroin and its metabolites was in the range of 82 - 123%, which was very close to the requirement of 80-120% as suggested by the method validation guideline from the US Food and Drug Administration (FDA).[84] The results obtained from morphine were generally the worst, with the accuracy of only 75% even at high concentration. The accuracy and precision determined for analysis of analytes in both urine and oral fluid were generally similar, except better precision was obtained for analysis of heroin and its metabolites in oral fluid. The accuracy and precision data for analysis of THC and

its metabolites were not available because of the poor sensitivity of detection. These data indicated that the present WT-ESI-MS method has acceptable accuracy and precision for quantitation of most of the targeted analytes.

Table 2-4. Accuracy and precision for analysis of different drugs in urine and oral fluid.

Compound	Spiked Quantity (ng/mL)	Determined Quantity± S.D. (ng/mL) (n=5)		Accuracy (%)		RSD (%)	
		Urine	O.F.	Urine	O.F.	Urine	O.F.
KET	100	107±11	84±11	107.4	84.1	9.3	13.1
	300	317±17	349±53	105.8	116.7	5.3	15.2
	600	587±70	690±78	97.8	115.0	11.9	11.3
	3000	3668±248	3162±58	122.3	105.4	6.8	1.8
Nor-K	100	95±10	82±6	95.7	81.8	10.5	7.3
	300	317±34	250±11	105.8	83.4	10.7	4.4
	600	738±62	638±50	123.1	106.3	8.4	7.8
	3000	3314±172	3470±387	110.5	115.7	5.2	11.2
MA	100	105±18	114±7	105.7	114.3	17.0	6.3
	500	498±16	508±64	99.5	101.5	3.2	12.6
	1250	1105±60	1216±61	88.4	97.2	5.5	5.0
	2500	2536±151	2518±169	101.4	100.7	6.0	6.7
MDMA	100	112±8	117±5	112.1	117.1	6.8	4.6
	500	520±56	474±28	104.0	94.8	10.7	6.0
	1250	1186±106	1219±82	94.9	97.5	9.0	6.8
	2500	2492±216	2601±392	99.7	104	8.7	15.1
COC	100	103±11	114±11	102.7	114.4	10.9	9.2
	500	510±46	489±58	102.1	97.8	9.0	11.8
	1250	1366±79	1296±166	109.3	103.7	5.8	12.8
	2500	2517±116	2561±319	100.7	102.4	4.6	12.5
BEN	500	432±37	461±61	86.3	92.1	8.6	13.2
	1250	1047±83	1331±137	83.8	106.4	7.9	10.3
	2500	2314±223	2657±209	92.6	106.3	9.6	7.9
HER	500	569±71	515±47	113.7	103.1	12.4	9.2
	1250	1349±247	977±75	107.9	78.2	18.3	7.7
	2500	2585±412	2346±174	103	93.8	15.9	7.4
6-MAM	500	441±84	467±30	88.1	93.3	19.0	6.4
	1250	1220±62	1024±48	97.6	81.9	5.1	4.7
	2500	2678±267	2822±159	107.1	112.9	10.0	5.6
MOR	500	N.A.	549±80	N.A.	109.9	N.A.	14.6
	1250	1343±342	940±60	107.4	75.2	25.5	6.3
	2500	2456±399	1880±175	98.2	75.2	16.3	9.3

Determination of LOD and LOQ

The LODs and LOQs of different drugs-of-abuse were determined experimentally with the use of spiked urine and oral fluid samples at low concentrations. Three shots of blank samples were firstly applied onto the wooden tip to measure the blank signals. Three shots of spiked samples were then applied onto the same wooden tip and the analyte signals were compared with those of the blank. The LOD and LOQ of an analyte were determined as the concentrations that could generate signals with S/N ≥ 3 [i.e. $(I_{analyte}/I_{IS})_{spiked}/(I_{analyte}/I_{IS})_{blank} \geq 3$] and S/N ≥ 10 [i.e. $(I_{analyte}/I_{IS})_{spiked}/(I_{analyte}/I_{IS})_{blank} \geq 10$]. Determination of LOD and LOQ of methamphetamine in urine and oral fluid are shown in Figure 2-11 and 2-12 as examples. The LOD and LOQ of methamphetamine were determined as 25 ng/mL and 50 ng/mL in urine and 12.5 ng/mL and 50 ng/mL in oral fluid, respectively.

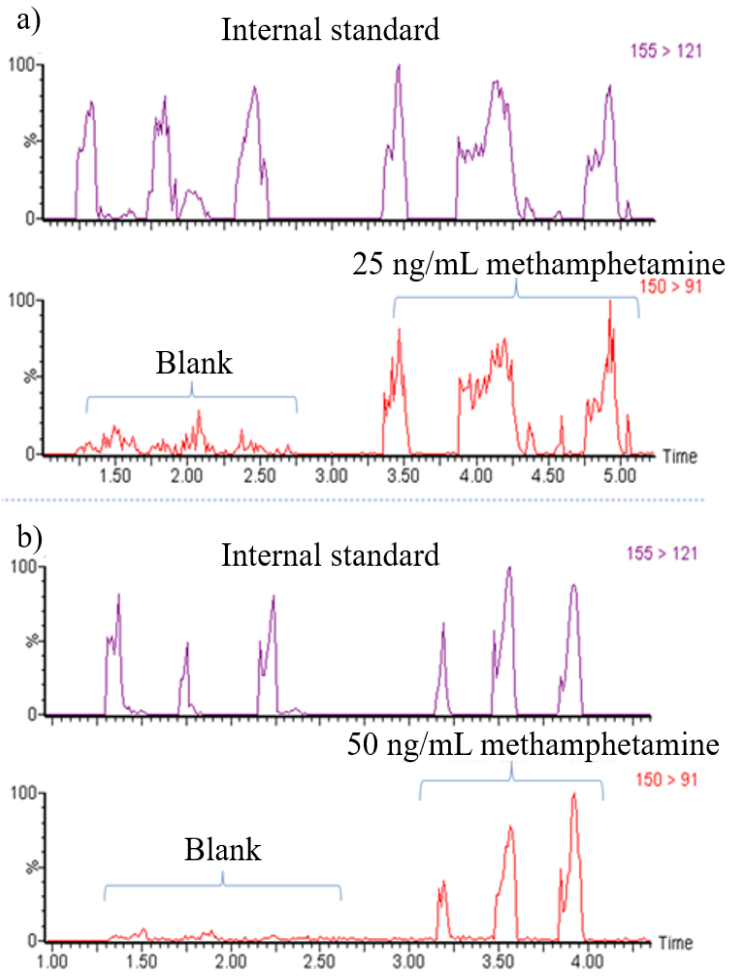


Figure 2-11. Determination of (a) LOD and (b) LOQ of methamphetamine in urine.

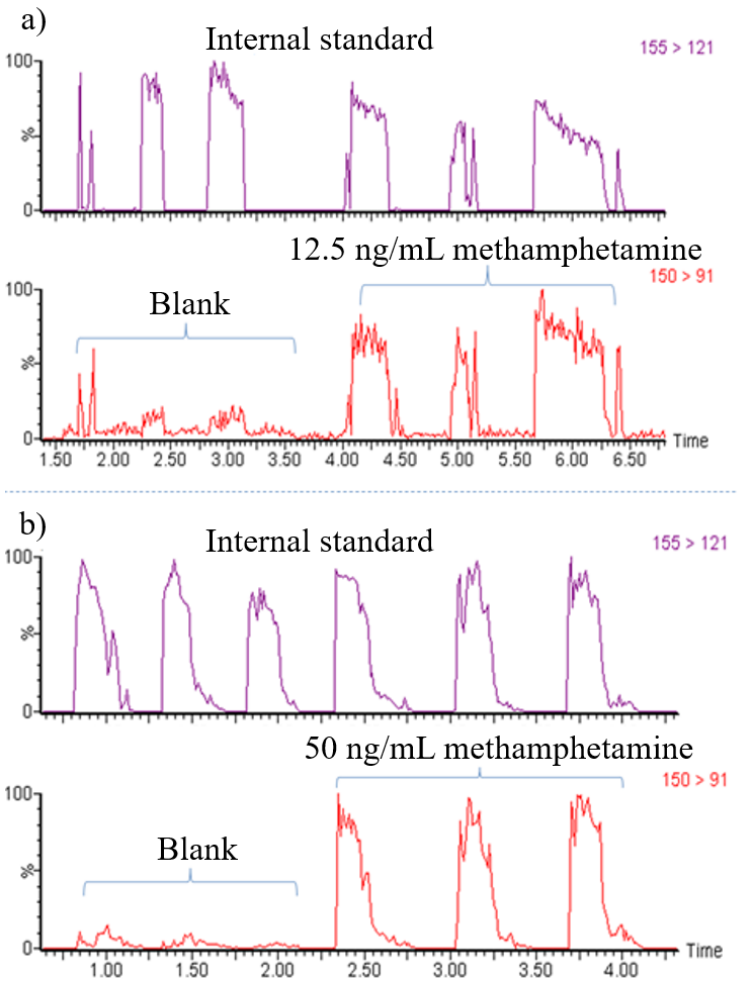


Figure 2-12. Determination of (a) LOD and (b) LOQ of methamphetamine in oral fluid.

The LODs and LOQs of other targeted analytes are summarized in Table 2-5. The LODs and LOQs determined were compared with the cut-off values of international authorities, including Substance Abuse and Mental Health Services (SAMHSA),[86,87] European Workplace Drug Testing Society (EWDTS),[88,89] and Driving under the Influence of Drugs, Alcohol and Medicines (DRUID) project associated with European Union (EU).[62] Generally, the required cut-off values of the analytes in oral fluid are

much lower than those in urine. There are no recommended cut-off levels available for ketamine in the guidelines. However, from the literature reported, ketamine and nor-ketamine found in oral fluid and urine in drug abusers were in the range of 100 – 15,000 ng/mL.[90] The LODs of ketamine in urine and oral fluid were good enough for the detection. For methamphetamine, the LODs were within the recommended cut-off of three guidelines. For MDMA, the LOD of urine generally fulfilled the requirements but the LOD of oral fluid was slightly higher than the recommended level of SAMHSA and EWDTS. The results suggested that the sensitivity of the present method was acceptable for real-life analysis of ketamine, methamphetamine and MDMA in urine and the former two drugs in oral fluid. For analysis of cocaine, the LOD of oral fluid was slightly higher than the cut-off values in SAMHSA and EWDTS while good enough for DRUID. However, its metabolite, benzoylecgonine is selected as the identifier of cocaine in SAMHSA and EWDTS guideline. The sensitivity of detection and quantitation of benzoylecgoine by the present method was not enough for the detection. The detection of heroin related compounds (heroin, 6-monoacetylmorphine and morphine) and THC related compounds (THC and THC-COOH) also needed to improve. Especially, very poor signals were obtained for detection of THC and THC-COOH.

In general, the LODs of the analytes in oral fluid were lower than those in urine, because of the low background signal in oral fluid. Cocaine which is a tertiary amine could give very strong signal and thus its LODs were the lowest. The secondary amines, such as ketamine and methamphetamine, could also be easily ionized and therefore gave low LODs. It is interesting that the LODs determined for cocaine was 10 times lower than its metabolites, benzoylecgonine. The only difference between cocaine and benzoylecgonine is the ester group in cocaine converted to carboxyl group. Similarly, the LODs of heroin and its metabolites became higher when more ester groups were converted to hydroxyl groups. The decrease of the sensitivity of detection (i.e. increase in LODs) might be because the analytes were more favorable to retain onto the surfaces of wooden tips with the increase of carboxyl group and alcohol group, which might be able to interact with the hydroxyl group on the wooden-tip surface. Another reason could be due to the signal suppression as no chromatographic separation in WT-ESI-MS, leading to the signals of those poorly ionized analytes suppressed by the analytes which could be ionized more easily. In fact, the results from the direct injection of the same concentration of cocaine, benzoylecgonine and heroin and its metabolites showed some degree of signal suppression. Both the signals of cocaine and heroin were higher than those of their metabolites, but the signal suppression was not as strong as the

difference of LODs in WT-ESI-MS. THC is a phenolic compound which is hardly protonated thus generated very poor signals. THC-COOH is a carboxylic acid and should generate better signals at negative ionization mode, however, was found to be poorly ionized in WT-ESI-MS at negative ionization mode. The LODs of the targeted drugs in urine could be achieved to lower levels, which could be down to serval ng/mL by using conventional methods.[80] The reduced analytical performances of WT-ESI-MS may due to ion suppression of weakly ionized analytes and the interactions between wooden tip and analytes as discussed previously. The lack of nebulizing gas in WT-ESI-MS for evaporating the solvent and the relatively low conductivity of wooden tip which may reduce charge accumulation at the tip end may also be the possible reasons for poorer performance of WT-ESI-MS.

Table 2-5. LODs, LOQs and recommended cut-off values of various drugs in urine and oral fluid.

Compound	LOD (ng/mL)		LOQ (ng/mL)		SAMHSA cut-off (ng/mL)		EWDTS cut-off (ng/mL)		DRUID cut-off (ng/mL)
	Urine	O.F.	Urine	O.F.	Urine	O.F.	Urine	O.F.	O.F.
KET	20	20	50	50	N.A.	N.A.	N.A.	N.A.	N.A.
Nor-K	20	20	50	50	N.A.	N.A.	N.A.	N.A.	N.A.
MA	25	12.5	50	50	250	15	200	15	410
MDMA	50	50	250	125	250	15	200	15	270
COC	12.5	12.5	50	50	N.A.	8	N.A.	8.	170
BEN	250	100	500	250	100	8	100	8	95
THC	40,000	40,000	N.A.	N.A.	N.A.	2	N.A.	2	27
THC-COOH	N.A.	N.A.	N.A.	N.A.	15	N.A.	15	N.A.	N.A.
HER	250	125	500	250	N.A.	N.A.	N.A.	N.A.	N.A.
6-MAM	500	125	1000	250	10	2	10	2	16
MOR	1000	500	10,000	10,000	2000	15	300	15	95

N.A. = Not available

Further confirmation of presence of drugs by tandem mass spectrometry

MRM is commonly used for detection and quantitation of drugs-of-abuse in sample. It is generally sensitive and specific enough to identify the presence of drugs. The identities of drugs detected could be further confirmed by MS/MS analysis. In MS/MS, the molecular ion of drugs was fragmented to generate product ions. The product ions could be used to confirm the identities of drugs. For example, the identification of methamphetamine in urine is shown in Figure 2-13. The presence of fragment ions of *m/z* 91 and *m/z* 119 was used to confirm the presence of methamphetamine. The fragments observed of the other analytes have been listed in Table 2-2.

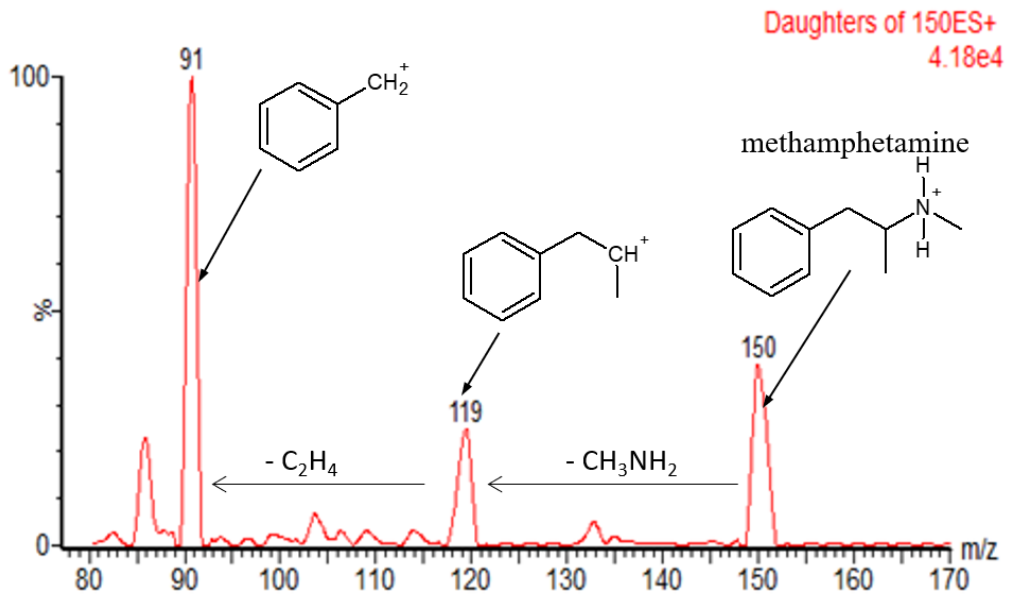


Figure 2-13. MS/MS spectrum obtained of 50 ng/mL methamphetamine in urine.

2.4 Conclusion

The application of WT-ESI-MS for rapid detection and quantitation of drugs-of-abuse in urine and oral fluid has been investigated in this study. Only little sample preparation and no chromatographic separation are required in this method, thus analysis of one sample could be finished within minutes. Moreover, WT-ESI-MS is compatible with existing instrument and no major hardware modification is needed. WT-ESI-MS showed good linearity and wide linear range for quantitation of most of the targeted drugs-of-abuse. High accuracy and precision were also obtained for quantitation of targeted drugs except morphine, THC and THC-COOH. The LODs and LOQs of the targeted analytes were compared with international standards and the present method could fulfill the requirement for detection of ketamine, nor-ketamine and methamphetamine in urine and oral fluid, MDMA in urine and cocaine in oral fluid. Further improvement in sensitivity is required for the analysis of MDMA, benzoylecgonine, heroin related compounds, THC and THC-COOH to fulfill the requirements of international standards. Although the results obtained were not as good as conventional analytical methods, the considerable reduction of analytical time using WT-ESI-MS still showed great potential for rapid screening of some of the drugs, such as ketamine and methamphetamine in body fluid, which could be beneficial to

analytical units.

Chapter 3: Enhanced detection and quantitation of drugs-of-abuse using direct coupling of solid-phase microextraction with mass spectrometry (SPME-ESI-MS)

3.1 Introduction

In the previous chapter, an ambient ionization mass spectrometry technique, WT-ESI-MS, has been demonstrated for rapid analysis of drugs-of-abuse. The results showed the potential of rapid analysis of drugs-of-abuse using ambient ionization techniques. However, the sensitivities for the analysis of some of the targeted analytes, such as benzoylecgonine and morphine, were still not good enough. In this chapter, solid-phase microextraction has been applied for analytes enrichment and the extracted compounds were directly analyzed using ambient ionization mass spectrometry.

SPME is a rapid and efficient extraction and enrichment technique for enhancing the sensitivity of chemical analysis which was invented by Arthur and Pawliszyn in 1990.[91,92] This technique makes use of a micro-tip, usually silica-based tip, coated with various materials on the tip surface for selective extraction and enrichment of analytes in raw samples. The analytes are concentrated onto the SPME tip and thus provide higher sensitivity for the detection. Nowadays, SPME tips with various coating, e.g.

divinylbenzene (DVB), carboxen, polydimethylsiloxane (PDMS), polyacylate (PA), polyethylene glycol, are commercially available, and each tip can be re-used for the analysis of several hundred samples.[93] By selecting an appropriate coating material, analytes can be selectively retained and enriched on the tip and the interfering matrices can be washed out. SPME was initially designed for thermal desorption, which is always coupled with GC or GC-MS for analysis.[94,95] SPME tips for LC analysis are nowadays available, coating such as C18 allows extraction of targeted analytes in biological fluids and the analytes are eluted with solvent or directly desorbed in LC interface.[96,97]

Unlike other extraction techniques, such as solid-phase extraction (SPE) and magnetic solid-phase extraction (MSPE), SPME does not require any vacuum pump, solvent delivery system and magnet for the extraction.[98,99] The analytes in the raw sample are directly absorbed and adsorbed onto the SPEM tip through immersion for non-volatile compounds or headspace extraction for volatile compounds. The impurities in sample matrix can be washed out by rinsing the surface of the SPME tip. The whole procedure is simple which makes SPME ideal for rapid and sensitivity analysis of drugs-of-abuse.

Direct coupling of SPME with mass spectrometry for rapid analysis combines the advantages of both SPME and ambient ionization. The targeted analytes can be concentrated onto the SPME tip and directly analyzed without further sample preparations and column separation.[46] Kennedy and co-works demonstrated the use of SPME for drugs extraction and DESI for desorption, ionization and detection of the extracted compounds.[100] Moreover, the C18 SPME tip fiber is metal alloy which can a conduct electricity and can be used as the ESI emitter directly. Ahmad and co-workers tried to connect the C18 SPME tip with high voltage after sample extraction and directly applied solvent and internal standards onto the SPME tip for analytes elution and ionization.[101] Two analytes were successfully analyzed in their study, but more detailed experiments and optimization are required to adopt SPME-ESI-MS for drugs analysis.

An optimized SPME-ESI-MS protocol is developed for the detection and quantitation of the six drugs-of-abuse and its metabolites as discussed previously, in this study. The extraction of targeted analytes in urine and oral fluid could be finished within ten minutes and multiple samples could be extracted simultaneously. After the extraction, the SPME tip is connected to high voltage and solvents are delivered to the SPME tip by syringe pump and sprayer with nitrogen gas support. The fine droplets from the

sprayer then eluted the extracted compounds and the analytes are finally ionized by ESI. The detection of the targeted drugs is greatly improved using SPME-ESI-MS, when compared with those values obtained by WT-ESI-MS. The LODs of SPME-ESI-MS for most of drugs could fulfill the requirements of the international standards. SPME-ESI-MS is a simple, rapid and sensitive methods for the analysis of drugs-of-abuse in urine and oral fluid.

3.2 Experimental section

3.2.1 Chemicals and materials

The SPME fibers (fused silica fiber) with 60 μm PDMS/DVB, 85 μm PA and 100 μm PDMS coating and SPME LC fiber probe (metal alloy fiber) with 45 μm C18 coating were purchased from Supelco (St. Louis, TX, USA). Ketamine hydrochloride, ketamine-D_4 (D-KET) hydrochloride, nor-ketamine hydrochloride, nor-ketamine-D_4 hydrochloride, methamphetamine, methamphetamine-D_5, MDMA, MDMA-D_5, cocaine, cocaine-D_3, benzoylecgonine, benzoylecgonine-D_3, delta-9-THC, delta-9-THC-D_3, 11-nor-9-carboxy-delta-9-THC, 11-nor-9-carboxy-delta-9-THC-D_9, heroin, heroin-D_9 (D-HER), 6-monoacetylmorphine and 6-acetylmorphine-D_3, morphine and morphine-D_3 (D-MOR) standards were purchased from Cerilliant (Round Rock, TX).

The workplace drug testing urine standards (i.e. urine standards that containing authenticated amount of illicit drugs), Medidrug WDT confirm U -25%, WDT confirm U +25% and Basis-line U were purchased form Medichem (Steinenbronn, Germany). HPLC grade methanol, acetonitrile (ACN) and ethanol (EtOH) were purchased from Anaqua Chemical Supply (Houston, TX, USA) and formic acid, ammonium acetate and ammonium bicarbonate were purchase from Sigma (St. Louis, TX). The hydrochloric acid (HCl), 37% and ammonia solution, 25% used for pH adjustment were purchase from VMR international (Radnor, PA, USA) and International Laboratory (South San Francisco, CA, USA) respectively. Sodium chloride (NaCl) was purchased from Riedel-de Haën (Seelze, Germany). The pH meter calibrants at pH 4, pH 7 and pH 9 were purchase from Inorganic Ventures (Christiansburg, VA, USA). The blank urine and oral fluid were donated by the research team members.

3.2.2 Preparation of spiked urine and oral fluid samples

Stock solutions of targeted drugs, metabolites and internal standard were stored according to the instructions of supplier. The standard solutions of targeted drugs and their metabolites used for the preparation of calibration curve were prepared by serial dilution of stock solutions with methanol. Another set of standard solutions with low,

medium and high concentrations was prepared for the determination of accuracy and precision of the method. The drugs-of-abuse and metabolites were divided into 4 groups for method establishment. Group 1 of ketamine, nor-ketamine, methamphetamine and MDMA. Group 2 of cocaine and benzoylecgonine. Group 3 of heroin, 6-monoacteylmorphine and morphine and group 4 of THC and THC-COOH. The standard solutions of drugs-of abuse and related metabolites in the same group were spiked into a 1000 μL blank urine and 500 μL blank oral fluid and acted as urine and oral fluid samples for SPME-ESI-MS analysis. The pH of the blank urine and oral fluid were measured by a Mettler Toledo pH meter (Greifensee, Switzerland) and adjusted to 6.8 – 7.2 before the addition of standards. Finally, the related internal standards (final concentrations of 200 ng/mL D-KET, D-Nor-K, D-MDMA and D-MA for group 1, 100 ng/mL D-COC and 500 ng/mL D-BEN for group 2, 500 ng/mL D-HER, D-6-MAM and D-MOR for group 3 and 500 ng/mL D-THC and D-THC-COOH for group 4) were spiked into the samples before SPME. All the spiked sample solutions were freshly prepared from stock solution before the analysis.

3.2.3 Instrumental setup

The instrumental setup for SPME-ESI-MS is illustrated in Figure 3-1. An Agilent 6460 triple quadrupole mass spectrometer equipped with ultra-performance liquid chromatography (UPLC) system (Santa Clara, CA, USA) was used in this study. An external high voltage supply (Hengbo Electric Co. Ltd., Zhejiang, China) with 3.5 kV for both the positive and negative ionization mode was used for the ESI. The capillary voltage on the instrument side was set to 100 V, the source temperature was 150 ºC and the source gas flow was 6 L/min. The mass spectrometer was operated under MRM mode. The MRM channels for the detection and quantitation of the targeted analytes are listed in Table 3-11. The Dwell time of each channel was 100 ms. A home-built platform was placed in front of the mass spectrometer inlet for affixing the SPME tip for SPME-ESI-MS. The platform was consisted of a stand and clip for adjusting the height, a glass slide fixed by the clip for supporting the SPME tip and a cushion fixed at the edge of the glass slide for stabilizing the SPME tip in position. A sprayer which is an original ESI nebulizer was removed from the Agilent's mass spectrometer and loaded with spray solvents for eluting and ionizing the analytes absorbed or adsorbed onto the SPME tip. The sprayer was pointed toward the SPME tip. The solvent was supplied by a programmable syringe pump (New Era Pump System Inc., Farmingdale,

NY, USA) with the flow rate of 30 µL/min and the nitrogen gas flow of 3 psi. The sprayer was grounded to protect the operator.

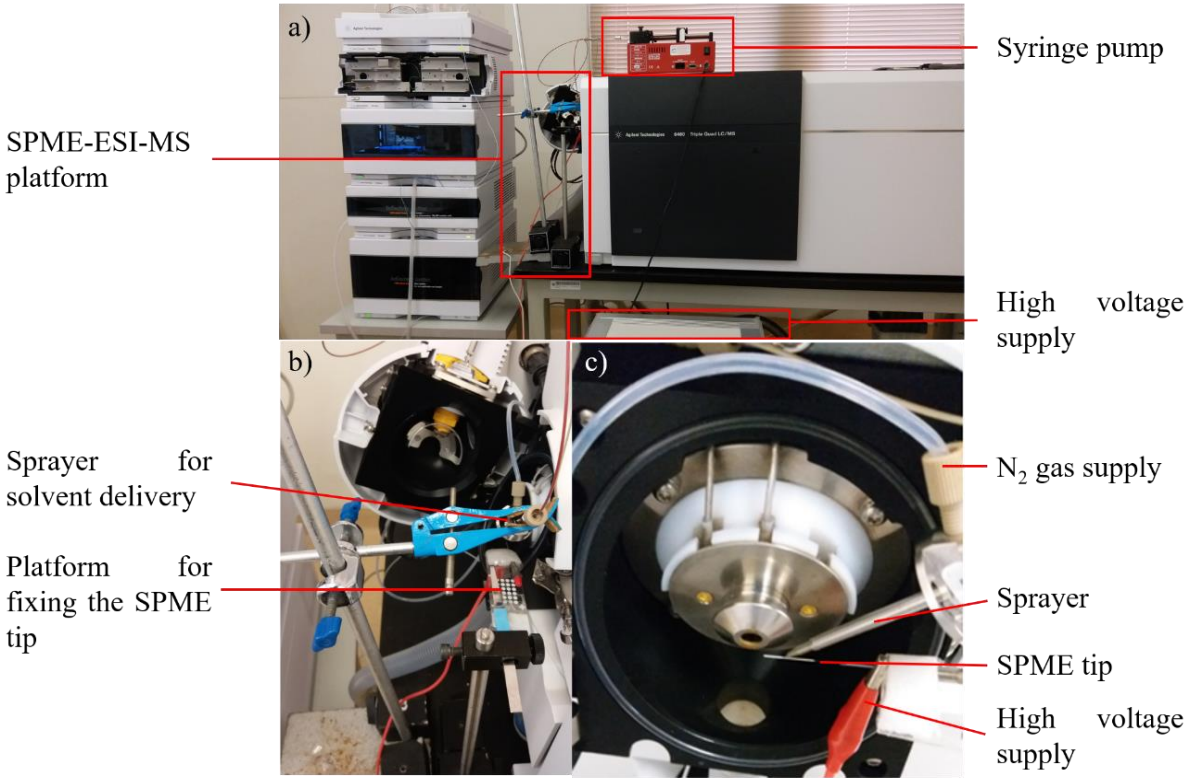


Figure 3-1. (a) The setup of SPME-ESI-MS on an Agilent 6460 triple quadrupole mass spectrometer. (b) Close-up of the platform placed in front of the mass spectrometer for SPME-ESI-MS analysis and (c) Close-up of a SPME tip mounted onto the platform for SPME-ESI-MS analysis.

Table 3-1. MRM condition and fragmentor setting of various drugs, metabolites and deuterium labeled internal standards.

Analyte	MRM Channel	Collision cell energy	Fragmentor
Ketamine	238 → 125*	22	80
	238 → 220	10	80
Nor-ketamine	224 → 125*	20	80
	224 → 207	10	80
Ketamine-D_4	242 → 129	22	80
Nor-ketamine-D_4	228 → 129	20	80
Methamphetamine	150 → 91*	17	80
	150 →119	9	80
Methamphetamine-D_5	155 → 121	9	80
MDMA	194 → 163*	8	80
	194 → 105	22	80
MDMA-D_5	199 → 165	8	80
Cocaine	304 → 182*	15	120
	304 → 82	28	120
Cocaine-D_3	307 → 185	15	120
Benzoylecgonine	290 → 168*	15	120
	290 → 105	28	120
Benzoylecogonine-D_3	293 → 171	15	120
THC	315 → 193*	18	120
	315 → 123	32	120
THC-D_3	318 → 196	20	120
THC-COOH	343 → 299*	15	200
	343 → 245	25	200
THC-COOH-D_9	352 → 308	15	180

(To be continued)

Heroin	370 → 165*	55	200
	370 → 211	30	200
Heroin-D_9	379 → 165	55	180
6-monoacetylmorphine	328 → 165*	40	140
	328 → 211	25	140
6-acetylmorphine-D_3	331 → 165	40	140
Morphine	286 → 165*	48	180
	286 → 153	48	180
Morphine-D_3	289 → 165	45	180

*The channels for quantitation of the analytes.

3.2.4 SPME-ESI-MS workflow

The C18 SPME tips were wetted with 1 mL 1:9 (v/v) H_2O/MeOH for 10 min and conditioned with 9:1 (v/v) H_2O/MeOH for another 10 min before extraction. For silica fibers, the tips were conditioned with 9:1 (v/v) H_2O/MeOH for 10min. Related internal standards were spiked into the urine and oral fluid samples. The SPME tips were immersed into the urine and oral fluid samples for 5 min for the extraction with vortex on a Bench Mixer at ~200 rpm (Benchmark Scientific Inc., Edison, NY, USA). The extraction time for heroin, 6-monoacetylmorphine, morphine, THC and THC-COOH, was 10 min. The SPME tips were rinsed with water for 10 s and ready for SPME-ESI-MS analysis. After the extraction, a SPME tip was affixed at 90^o in front of the mass spectrometer (0.6 - 0.8 cm horizontally and 0.4 – 0.8 cm vertically away from the mass spectrometer) through the SPME-ESI-MS platform. The spray solvent, which acted as

both the elution and ionization solvent, was delivered onto the SPME tip through a sprayer (~0.5 cm away from and ~65° pointed to the middle part of the SPME tip). A ratio of 10:90:0.1 (v/v/v) H_2O/EtOH/FA was used as the spray solvent except 10:90:0.1 (v/v/v) H_2O/MeOH/FA was used for the analysis of heroin, 6-monoacetylmorphine and morphine. The syringe pump was stopped after 10 μL of solvent was applied onto the SPEM tip. The MRM signal generally lasted for 10 – 20 s. For each SPME tip, the solvent was applied onto the SPME tip for three times and three data were recorded. The residues on the SPME tips were removed by washing the tip with 90:10:0.1 (v/v/v) MeOH/H_2O/FA at 40 ºC for 15 min twice. The C18 SPME tips could generally be reused for 10 times and the silica fiber SPME tips could generally be reused for 100 times. The MRM spectra were analyzed using Agilent Qualitative Analysis software.

3.2.5 Optimization of drugs analysis using direct coupling of SPME with mass spectrometry

Analysis using DART-MS

A DART system from Ionsense (Saugus, MA, USA) which is compatible with the Agilent 6460 triple quadrupole mass spectrometer was used in this study. The DART system consists of a DART ion source, a maintain control body, an ion transferring tube

set between the ion source and the ion inlet, a vacuum pump creating the driving force for ion transfer and an auto-sampling platform affixed in front of the MS ion inlet. Both nitrogen gas and helium gas pressure supplied to the DART system were 70 psi. The gas temperature was 250 ºC, the grid voltage was 200 V and the distance between the DART ion source and MS inlet was ~2.5 cm. The capillary voltage of the mass spectrometer was set to 1000 V, the source gas temperature was 250 ºC and the source gas flow was 5 L/min. For the analysis of standard solution, a glass sampling tip provided by Ionsense was immersed into the solution for 10 s. The sampling tip was then placed between the DART ion source and ion inlet for ionization. For SPME analysis, the SPME tip after extraction was placed between the ion source and MS ion inlet for analysis. The MRM channels were the same as those listed in Table 3-1. The data were acquired using Agilent Mass Hunter software and the DART system was controlled by using the software provided by Ionsense. The spectra were analyzed using Agilent Qualitative Analysis software.

Analysis using DESI

The SPME tips after extraction were analyzed using a home-built DESI system on the Agilent 6460 triple quadrupole mass spectrometer. The DESI sprayer for DESI solvent delivery was an ESI nebulizer which is removed from an Agilent's mass spectrometer. The N_2 gas pressure supplied to the sprayer was 50 psi. The DESI solvent, 80:20:0.1 (v/v/v) ACN/H_2O/FA was supplied by a syringe pump with the solvent flow rate at 10 μL/min. The DESI sprayer was located 0.8 cm away from the MS ion inlet and 45° pointed to the SPME tip. The SPME tip was affixed in front of the mass spectrometer and 0.4 cm away from the MS ion inlet. External high voltage supply with 3.5 kV was connected to the DESI sprayer. The capillary voltage of the mass spectrometer was 100 V, the source temperature was 150 °C and the gas flow was 6 L/min. The data were acquired using Agilent Mass Hunter software and the spectra were analyzed using Agilent Qualitative Analysis software.

3.2.6 Optimization of SPME-ESI-MS protocol

Optimization of analyte extraction

Four sets of spiked urine samples including (1) 200 ng/mL of KET, Nor-K, MA and MDMA, (2) 200 ng/mL of COC and BEN, (3) 2000 ng/mL of MOR and 6-MAM and

(4) 2000 ng/mL of THC and THC-COOH, were extracted by immersing the SPME tips into the samples. The extracted analytes were then eluted by immersing the SPME tips in 100 µL 90:10:0.1 (v/v/v) MeOH/H_2O/FA for 30 min in LC vials with glass inert. The extracts were then analyzed by LC-MS in the Agilent 6460 triple quadrupole mass spectrometer. A Waters Acquity UPLC BEH C18 column (1.7 µm in particle size, 2.1 x 50 mm) was used for the LC-MS analysis. The mobile phase and the gradients used are listed in Table 3-2. The capillary voltage and nozzle voltage of the mass spectrometer was 3500 V and 500 V respectively, for both positive and negative ionization. The gas temperature was 300 ºC and the gas flow was 8 L/min. The sheath gas temperature and flow were 270 ºC and 11 L/min respectively. The mass spectrometer was operated in MRM mode with the MRM channels the same as that listed in Table 3-1. Four parameters were tested including the SPME tip selection (PDMS, PDMS/DVB, PA and C18), extraction time (1, 5, 10, 20 and 40 min), extraction pH (5, 7, and 9) and addition of salt (0, 10, 25 and 40% NaCl). Only one parameter was varied for each experiment. The spectra were analyzed using Agilent Qualitative Analysis and peak area was used for the signals comparison.

Table 3-2. LC gradients used at the method optimization step.

Targeted analyte	Mobile phase	LC gradient
1. KET, Nor-K, MA and MDMA	A: 10 mM ammonium acetate B: ACN with 0.1% FA	0 – 1 min 0 – 5% B, 1 – 2 min 5 – 35% B, 2 – 5 min 35 – 40% B, 5 – 7 min 40 – 100% B, 7 – 10 min 100% B, 11 – 13 min 5% B
2. COC and BEN	A: 10 mM ammonium acetate B: ACN with 0.1% FA	0 – 1 min 0 – 5% B, 1 – 7 min 5 – 100% B, 7 – 10 min 100% B, 11 – 13 min 5% B
3. MOR and 6-MAM	A: 10 mM ammonium bicarbonate B: MeOH with 0.1 FA	0 – 1 min 0 – 5% B, 1 – 8 min 5 – 100% B, 8 – 11 min 100% B, 12 – 16 min 5% B
4. THC and THC-COOH	A: H_2O with 0.1% FA B: ACN with 0.1% FA	0 – 1 min 0 – 10% B, 1 – 4 min 10 – 100% B, 4 – 7 min 100% B, 8 – 10 min 10% B

Optimization of SPME-ESI-MS parameters

Different solvent delivery methods were tested, including the addition of solvent using pipette, syringe pump and sprayer. The setup of SPME-ESI-MS using a sprayer was described in Section 3.2.3. The setup of SPME-ESI-MS using pipette was the same as that of using a sprayer, except the sprayer was removed. An aliquot of 1μL of elution and ionization solvent was added onto the SPME tip using a Brand Transferpette S 2.5 μL pipette (Essex, UK). Similarly, the solvent was supplied onto the SPME tip through the syringe pump with PEEK tubing (internal diameter [I.D.] = 0.007") rather than the sprayer in SPME-ESI-MS with the syringe pump. The solvent flow rate supplied by the syringe pump was 15 μL/min. The spray solvent including 10:90:0.1 (v/v/v) MeOH/H_2O/FA, 50:50:0.1 (v/v/v) MeOH/H_2O/FA, 90:10:0.1 (v/v/v) MeOH/H_2O/FA, 90:10:0.1 (v/v/v) EtOH/H_2O/FA and 90:10:0.1 (v/v/v) ACN/H_2O/FA were tested in this study.

3.2.7 Method validation of SPME-ESI-MS

Calibration curves

The calibration curves for quantitation were constructed by averaging three sets of experimental data, while each set of data was obtained by analyzing at least five

different concentrations of analytes. The resultant MRM chromatograms were processed using Agilent Qualitative Analysis software. The signals were manually integrated, and the peak areas were used for constructing the calibration curves. The targeted drugs and their metabolites in the same groups were analyzed simultaneously for constructing the calibration plots of the targeted drugs.

Accuracy and precision

The accuracy and precision of SPME-ESI-MS method was determined by analyzing at least three sets of urine and oral fluid samples spiked with the analytes at low, medium, and high concentrations respectively. Samples at each concentration were analyzed at least six times and the data obtained were averaged for comparison. The accuracy was calculated by equation 2-1 and the precision was represented by R.S.D. which was calculated by equation 2-2 as described in Chapter 2.

Limit-of detection (LOD) and limit-of-quantitation (LOQ)

Blank samples were prepared by spiking only the internal standards into blank urine or oral fluid. The LODs and LOQs were determined by comparing the peak area ratio of the analytes and internal standards between the spiked samples and the blank samples.

The determination of LOD and LOQ was followed the definition of IUPAC,[102] which is given by the equation:

$$x_L = \bar{x}_{bi} + k\, s_{bi} \quad \text{..... (3-1)}$$

where x_L is the smallest measure (signal) that can be detected with reasonable certainty, $\bar{x}_{bi}$ is the mean of the blank measures, s_{bi} is the standard deviation of the blank measures and k is a numerical factor. The LOD and LOQ of an analyte were defined as the concentrations of the spiked samples that can give signal (relative peak area) larger than x_L with the k equal to 3 and 10 respectively. At least nine measurements of the blank and the spiked samples were obtained for the determination of LODs and LOQs.

Extraction efficiency of SPME

The extraction efficiency of SPME was calculated by comparing the signals obtained from the extract of spiked sample with those obtained from standard solution as described by Chou and Lee.[103] One milliliter of spiked urine sample containing 400 ng/mL of each analyte was extracted using C18 SPME tip. The extracted analytes were recovered by immersing the SPME tip into 200 μL of 90:10:0.1 (v/v/v) MeOH/H_2O/FA for 30 min in LC vial. Internal standards of each analyte were spiked into the extract

and the extract was analyzed by ESI-MS. A standard solution of same concentrations of analytes and internal standards was also analyzed for signal comparison.

3.2.8. Analysis of Medichem urine samples

The lyophilized Medidrug WDT confirm U -25%, WDT confirm U +25% and Basis-line U samples were reconstituted with 5 mL of MilliQ water according to the instruction from the supplier. For each sample, 1 mL of the sample were transferred to an eppendorf and the internal standards of all the analytical targets were spiked into the sample. The sample was extracted and analyzed using SPME-ESI-MS method as described in Section 3.2.4 while 15 min extraction and 90:10:0.1 (v/v/v) MeOH/H_2O/FA were used for the analysis. Four time segments, which contained the MRM channels listed in Table 3-1, were used for the detection of the four groups of targeted analytes. Each time segment was lasted for 1 min for the detection of one group of analytes. The spray solvent was delivered at the beginning of each time segment. The experiments were repeated 3 times on the same day and repeated inter-day and inter-week.

For the LC-MS analysis, the samples were extracted by C18 SPME tip for 1 hr. The

analytes were eluted by immersing the SPME tips in 100 µL 90:10:0.1 (v/v/v) MeOH/H_2O/FA for 1 hr in LC vials with glass inert. The extracts were then analyzed by LC-MS in the Agilent 6460 triple quadrupole mass spectrometer. The column used was a Waters Acquity UPLC BEH C18 column (1.7 µm in particle size, 2.1 x 150 mm). The mobile phases were 10 mM ammonium acetate (mobile phase A) and MeOH with 0.1% FA (mobile phase B). The mobile phase flow rate was 0.2 mL/min at room temperature. The elution gradient was 0 – 4 min 5 – 40% B, 4 – 6 min 40 – 50% B, 6 – 8 min 50 – 100% B, 8 – 11 min 100% B, 12 – 18 min 5% B. The capillary voltage and nozzle voltage of the mass spectrometer was 3500 V and 500 V respectively, for both positive and negative ionization. The gas temperature was 300 ºC and the gas flow was 8 L/min. The sheath gas temperature and flow were 270 ºC and 11 L/min respectively. The mass spectrometer was operated in MRM mode and all the channels with the same polarity (i.e. positive or negative ionization) were scanned simultaneously. The Dwell time for each channel was 50 ms. Positive ionization and negative ionization mode were used in separated LC-MS runs.

A spiked urine sample contained all the targeted analytes with the same concentration (900 ng/mL) was prepared by spiking the standard solutions into the blank urine in our

laboratory. The sample was treated and analyzed as same as the Medidrug samples for comparison.

3.3 Results and discussions

3.3.1 Optimization of drugs analysis using direct coupling of SPME with mass spectrometry

Two popular ambient ionization techniques, DART and DESI were tested for the direct coupling of SPME with mass spectrometry before the setup of SPME-ESI-MS. The experimental setups of using DART and DESI for the direct analysis of targeted analytes after SPME are shown in Figure 3-2.

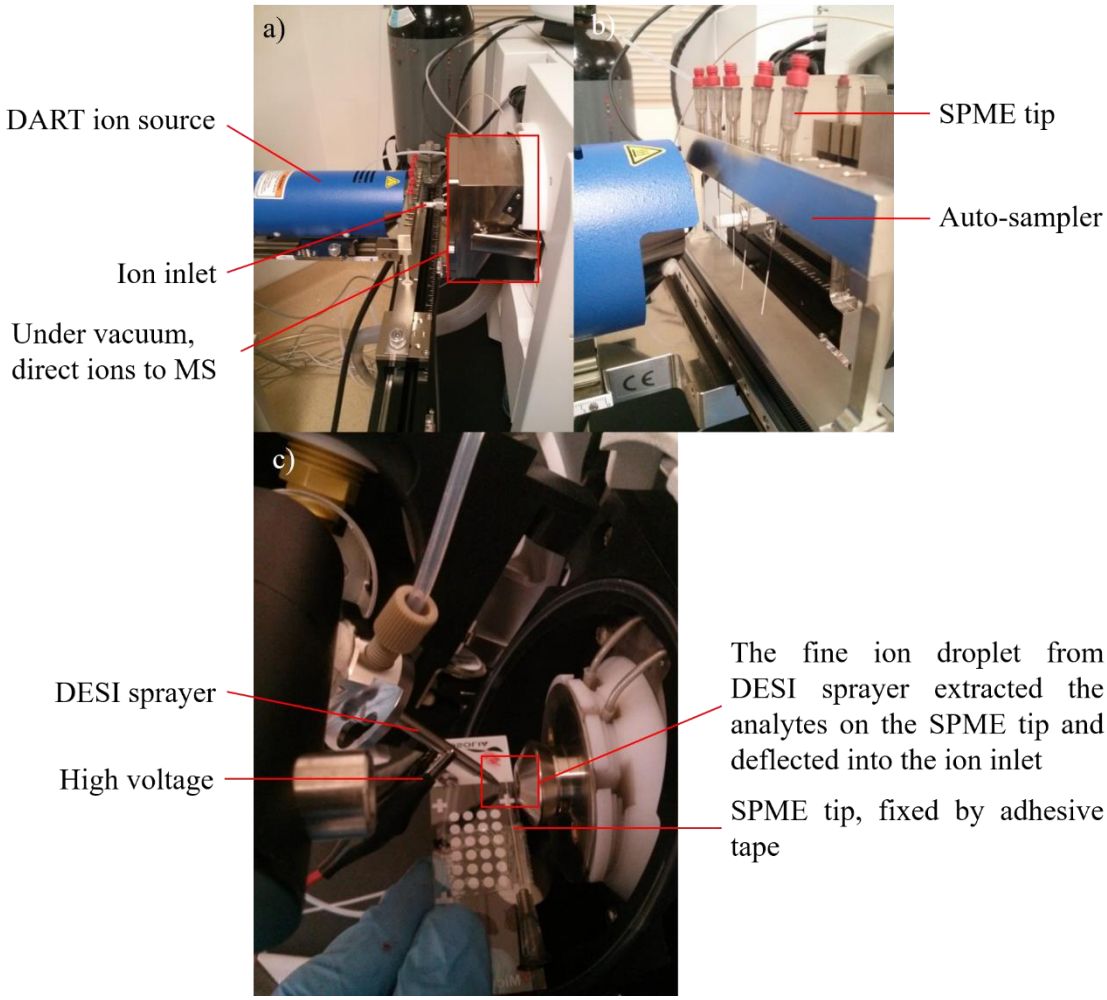


Figure 3-2. Experimental setup of direct coupling of SPME with mass spectrometry using (a, b) DART and (c) DESI.

Direct coupling of SPME with DART

Commercially available DART system is directly compatible with Agilent mass spectrometer interface, the experimental setup provided by the supplier instruction was adopted. The pure drug standards in organic solvent were firstly analyzed by DART directly, without extraction with SPME tip. The molecular ions of most of the drugs could be observed at the concentration of 1000 ng/mL while 5000 ng/mL for the analysis of morphine and 6-MAM. The signal obtained from THC standard was comparable with that of cocaine and was better than the other drugs (see Figure 3-3a and b). However, THC is relatively difficult to be ionized in ESI when compared with other targeted drugs. The reason for the difference could be that THC is more volatile than other drugs, which is easier to be desorbed from the surface during DART process at the given gas temperature. The effect of the volatility of the compounds in DART was also discussed by Maleknia et al. and Hajslva et al., who also concluded that volatile compounds can be desorbed more effectively than semi-volatile compounds in DART.[104,105]

After confirming the ionization of targeted analytes in DART, spiked urine samples were extracted with SPME tip and analyzed using DART. The SPME tips after

extraction were inserted into the auto-sampler of DART, as shown in Figure 3-2b. The auto-sampler was computer-controlled and moved toward the MS ion source and the SPME tips were analyzed one by one on moving by the auto-sampler. The extracted analytes on SPME tips were desorbed by the hot gas and ionized by protonated water cluster.[105] The time for analyzing one sample was approximate 10 s and 20 s was required for the auto-sampler travelling form one sample to another sample. The results of analysis of different drugs in urine before and after SPME are shown in Figure 3-3c and d.

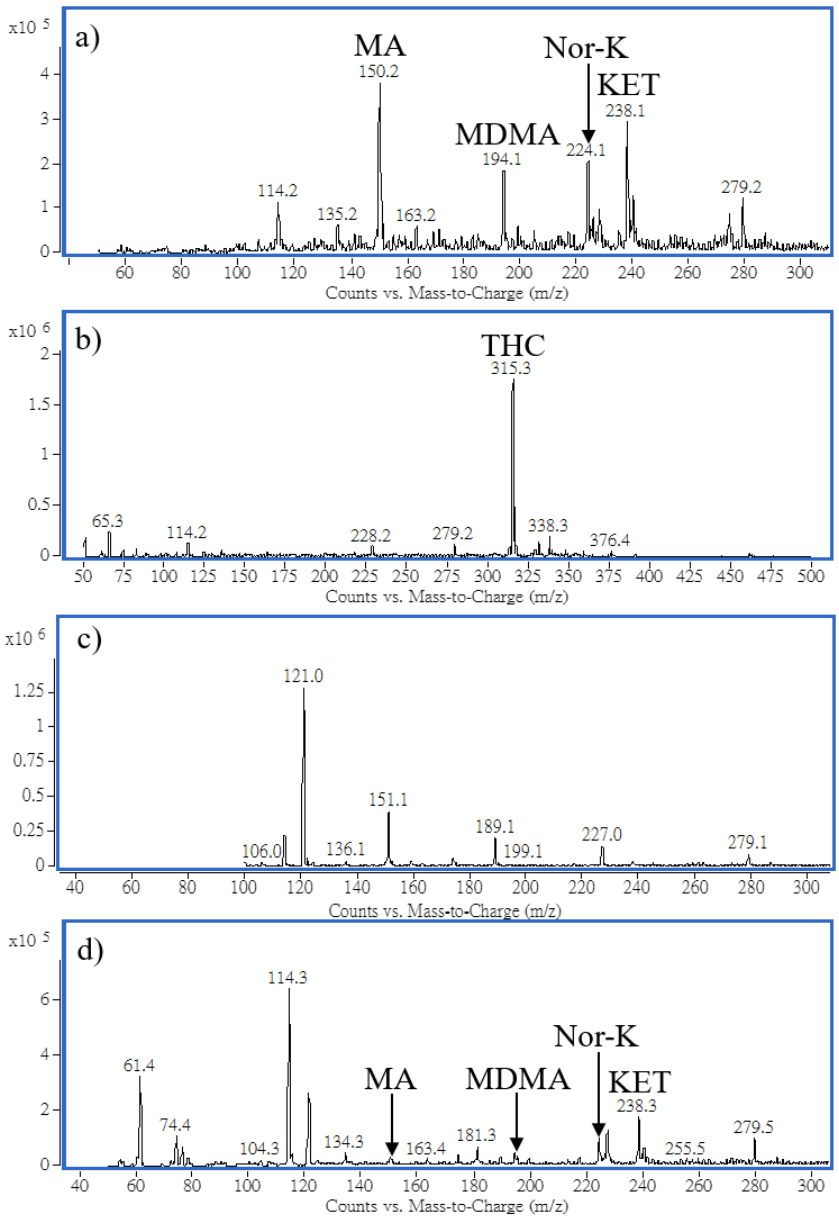


Figure 3-3. The DART mass spectra of (a) 1000 ng/mL of MA, MDMA, Nor-K and KET in methanol and (b) 1000 ng/mL of THC in methanol. The DART mass spectra of 1000 ng/mL of MA, MDMA, Nor-K and KET in urine (c) before and (d) after the extraction with C18 SPME tip.

For the analysis of pure standards using DART, both MA, MDMA, Nor-K and KET produced strong signals. However, for the spiked urine sample without the extraction using C18 SPME tip, no signals were observed. After SPME analysis, the signals of Nor-K and KET were observed clearly and some signals for MA and MDMA were observed with lower S/N ratio. The ionization of MA should be better than other drugs as shown in Figure 3-3a but the C18 SPME tip could extract Nor-K and KET more effectively, leading to better signals for those drugs as shown in Figure 3-3d. The results in Figure 3-3 proved that analytes were enriched onto the SPME tip and interfering compounds could be removed, thus better signals could be obtained. However, the signal intensity after extraction was still weaker than that of the pure standards. The C18 SPME tip also showed different extraction efficiency for different compounds, which would affect the analytical performance of different compounds.

Different concentrations of targeted drugs were prepared and analyzed under MRM mode. The results are shown in Figure 3-4. Only signals of ketamine showed linear correlation even internal standards were used in the analysis. The blank signal of detection of ketamine was also high which would affect the determination of LOD. Lower concentrations of analytes were also analyzed but no significant signals were

produced. For the analysis of other drugs after SPME, such as MOR and 6-MAM in urine, the signals were very poor. Signals of those drugs could only be observed at the concentration as high as 5000 ng/mL under MRM detection. THC, which should be easily detected in DART, however, could not be detected after C18 SPME. They may be due to the poor extraction efficiency of THC in urine.

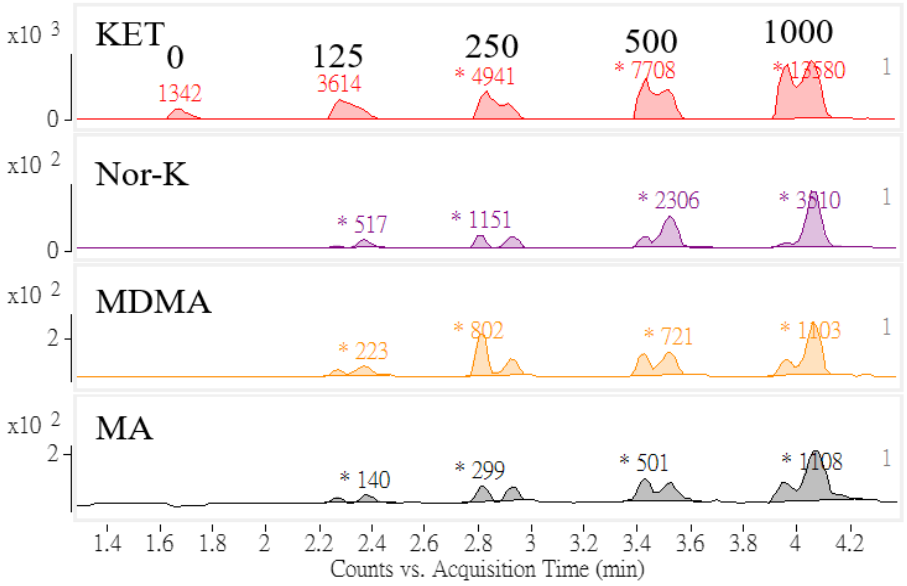


Figure 3-4. The DART MRM results of 0, 125, 250, 500 and 1000 ng/mL of MA, MDMA, Nor-K and KET in urine after the extraction with C18 SPME tip. Only the channels used for quantitation are shown.

The desorption and ionization efficiency of DART should be increased with the increase of gas temperature. However, damage to the C18 SPME tips was observed for the gas temperature higher than 250 ºC. The C18 particles on the SPME tips were burnt at such

high temperature. Therefore, higher gas temperature was not applied for the direct analysis of C18 SPME tips. Another reason for the undesirable performance of direct coupling of SPME to DART is the relatively small surface area of the C18 SPME tips. The C18 SPME tip is too thin and too long compared with the mouth of DART ion source. Not all the C18 coating could be contacted with the desorption gas which reduce the sensitivity of detection.

DART is an attractive technique for direct coupling of SPME with mass spectrometry. The operation is simple and rapid, but the analytical performance still needed to be improved for most of the drugs. Specially designed SPME tip might greatly benefit to the DART analysis.

Direct coupling of SPME with DESI

Since the commercial DESI system coupled with Agilent mass spectrometer interference was not available in our laboratory, a home-built DESI setup was established as described by Kennedy et al.[100] Cocaine which give strongest signal for ESI as described in pervious chapter was used to test the direct coupling of SPME with DESI. Figure 3-5 showed the mass spectrum of using DESI to desorb and ionize cocaine

on the C18 SPME tip which was previously extracted from a spiked urine sample. Signal of cocaine could be observed as shown in Figure 3-5a, however the signal was weak even a high concentration of cocaine was used and the result was not satisfactory. For the detection of COC and its metabolite BEN under MRM mode, the signals were very unstable as shown in Figure 3-5b. The signals of BEN were very low as well.

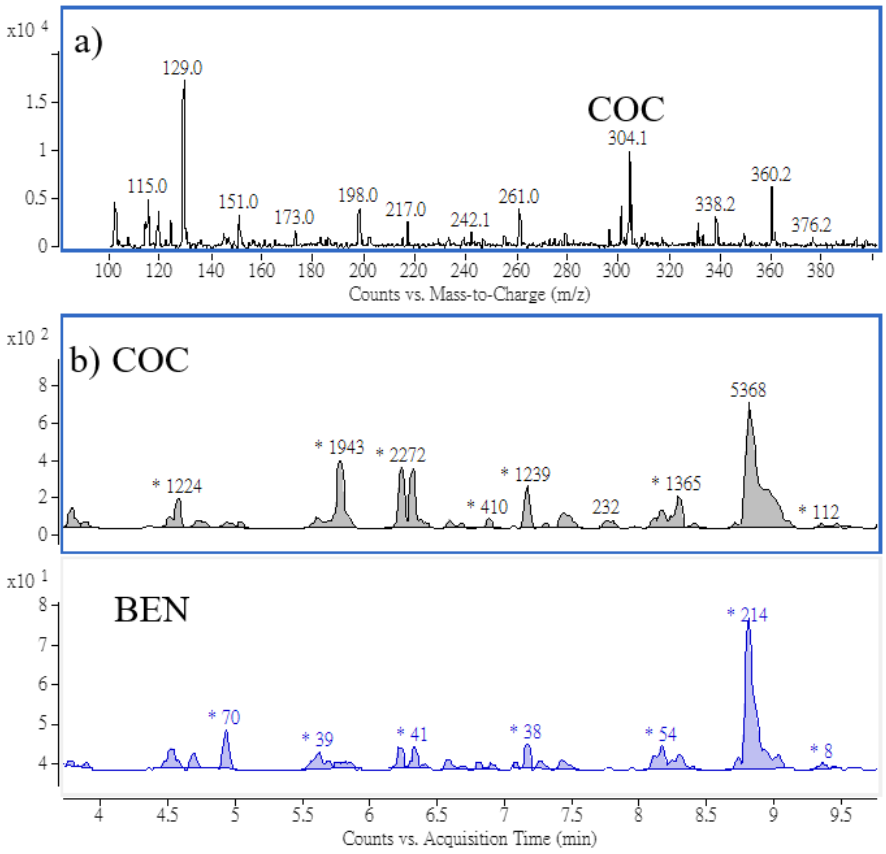


Figure 3-5. (a) The DESI mass spectrum of 2000 ng/mL cocaine in urine after the extraction with C18 SPME tip. (b) The DESI MRM results of 200 ng/mL of COC and BEN in urine after the extraction with C18 SPME tip. Only the channels for quantitation are shown.

There are several reasons for the poor performance of direct coupling of SPME with DESI in this study. In the commercially available DESI system, an ion transfer tube which is extended from the original ion inlet of the mass spectrometer, is located near the sample so as to transfer the desorbed compounds and the ions formed into the mass spectrometer.[100] However, such apparatus is not available in this study. Also, the sprayer for DESI is different from that used for conventional ESI. The inner-diameter of DESI sprayer is smaller for concentrating the desorption spray and higher pressure is used in DESI for creating finer desorption solvent droplet and stronger desorption force. It is proved that special DESI sprayer would also provide better reproducibility of the analysis.[106] Finally, there are many fine adjustments on the geometry parameters including the spray angle, sample collection angle, sprayer to surface distance, ion inlet to surface distance, sprayer to ion inlet distance and the spray tip length which are needed to be optimized for successful DESI experiments. Such fine adjustments were difficult to achieve by using stand and clip only.

3.3.2 Optimization of SPME-ESI-MS protocol

The results obtained from direct coupling of DART and DESI were not satisfactory for the analysis of targeted drugs. SPME-ESI-MS, on the other hand is a technique that is

more easily to setup and can provide better analytical performance for the drug analysis. The results will be discussed in later sections. The optimization of SPME-ESI-MS was divided into two parts: (1) optimization of the extraction of targeted analytes using SPME and (2) optimization of the SPME-ESI-MS setup.

Optimization of analyte extractions

Optimization of extraction conditions of targeted drugs is a crucial step for successful SPME-ESI-MS analysis, as the sensitivity of the detection is highly dependent on the amount of analytes enriched on the SPME tip. Four parameters including selection of the SPME tip coatings, extraction time, extraction pH and addition of salt were optimized for the extraction of each targeted analytes.[107]

Selection of SPME tip coatings is an important step for the optimization of SPME protocol There are four SPME coatings including PDMS, PDMS/DVB, PA and C18 available for LC analysis. PDMS is suitable for the extraction of non-polar volatiles, PA is normally used for the extraction of polar compounds and DVB/PDMS and C18 are more universal, which can be used for the extraction of semi-polar analytes. All of the above SPME tips were tested to extract each targeted analyte in this study. Examples

of the optimizations are shown in Figure 3-6. Both C18 and DVB/PDMS SPME tips could extract KET, Nor-K, MDMA and MA in urine effectively while the performance of C18 was slightly better than DVB/PDMS SPME tip. The extraction efficiency of PDMS was quite low but PDMS SPME tip is always used for drug analysis in GC-MS. It may due to the fact that these drugs became more non-polar and volatile after chemical dativization, leading to higher extraction efficiency of PDMS SPME tip.[94,95] The extraction using PA SPME tip gave very low extraction efficiency. The results obtained from other targeted analytes were similar, both C18 and PDMS/DVB SPME tips could be used for the drug extraction. However, considering the higher extraction efficiency of C18 SPME tip for the extraction of THC-COOH, BEN and 6-MAM than that of PDMS/DVB SPME tip, C18 SPME tip was finally selected in this study.

To optimize the extraction time for targeted analytes, extraction time of 1 – 40 min were tested. The plot of the signal obtained (in term of peak area) versus the extraction time is shown in Figure 3-6b. Peak area was used to do the optimization as it directly reflected the signals observed. The result showed that the extraction rates of KET, Nor-K, MDMA and MA were fastest at 5 – 20 min and reached a plateau after 40 min. Similar results were obtained for the extraction of other analytes, except for COC and

6-MAM, which the highest extraction rate was at 10 – 20 min. Logically, the longer the extraction time, the more the amount of analytes are extracted onto the SPME tip. The deviation between extractions can also be reduced when the extraction is at equilibrium. However, the purpose of this study is to develop a method for rapid analysis of drugs-of-abuse, extraction for more than 40 min is too long for a rapid detection method. Therefore, to balance between the extraction time and the analytical performance, the extraction time for the targeted analytes was set as the shortest time that sufficient amount of analytes were extracted to fulfill the cut-off level of the international standards (i.e. SAMHSA, EWDTS and DRUID). For KET, Nor-K, MDMA, MA, COC and BEN, 5 min extraction was enough for the resultant LODs reaching the cut-off of international standards. For HER, 6-MAM, MOR, THC and THC-COOH, 10 min extraction was required to fulfill the analytical requirements.

The extraction efficiency of SPME should be increased when the targeted analytes are in neutral or undissociated form. Therefore, pH adjustment might help to improve the extraction efficiency. Urine samples at pH 5, 7 and 9 were tested to optimize the pH for the extraction. No further pH values were tested as extreme pH may damage the SPME tips. Most of the targeted analytes such as KET, Nor-K, MDMA and MA as shown in

Figure 3-6c, favored the extraction when pH ≥ 7, except THC-COOH which was better extracted at pH 5. The results were reasonable as most of the targeted drugs are amines and could become ionic at low pH. On the other hand, THC-COOH is a carboxylic acid and could remain neutral at low pH. Sample extraction (including THC-COOH) was normally set at pH 7 in this study as most of the drugs had the optimized extraction at pH 7. Since the pH value of human urine is between 5.5 – 7 which could slightly reduce the extraction efficiency of SPME,[108] measuring the pH of urine samples and adjusting the pH is necessary. In contrast, the pH value of oral fluid is between 6.5 – 7.2, and therefore pH adjustment is generally not essential.[109]

Finally, the salt concentration in the sample solution was optimized. In most of the applications, high salt content can increase the extraction efficiency of SPME through salting-out effect. However, the addition of salt may not improve the performance of SPME in some cases, for example there is no effect for highly polar compounds or compounds with high water solubility. For some cases, addition of NaCl even reduces the performance of SPME as more impurities in the sample solution will also be extracted.[107] The results obtained for the extraction of KET, Nor-K, MA and MDMA with 0, 10, 25 and 40% NaCl content are shown in Figure 4-6d. No significant

improvement was observed for the extraction of all the targeted analytes after the addition of NaCl. The results obtained here are consistent with the study done by Chou and Lee.[103] The possible explanation for this is the addition of salt also increased the extraction of matrix components which did not benefit the extraction of targeted analytes.

In summary, the optimized extraction was obtained by using C18 SPME tip with 5 – 10 min extraction under vortex. The samples solution was adjusted to pH 7 and addition of salt was not necessary. For urine samples, 1mL sample was used as the extraction was done in a 1.5 mL eppendorf. On the other hand, 500 µL sample was used for oral fluid sample as the collection volume of oral fluid was limited, and some of the oral fluid devices could only collect about 500 µL of oral fluid.[110]

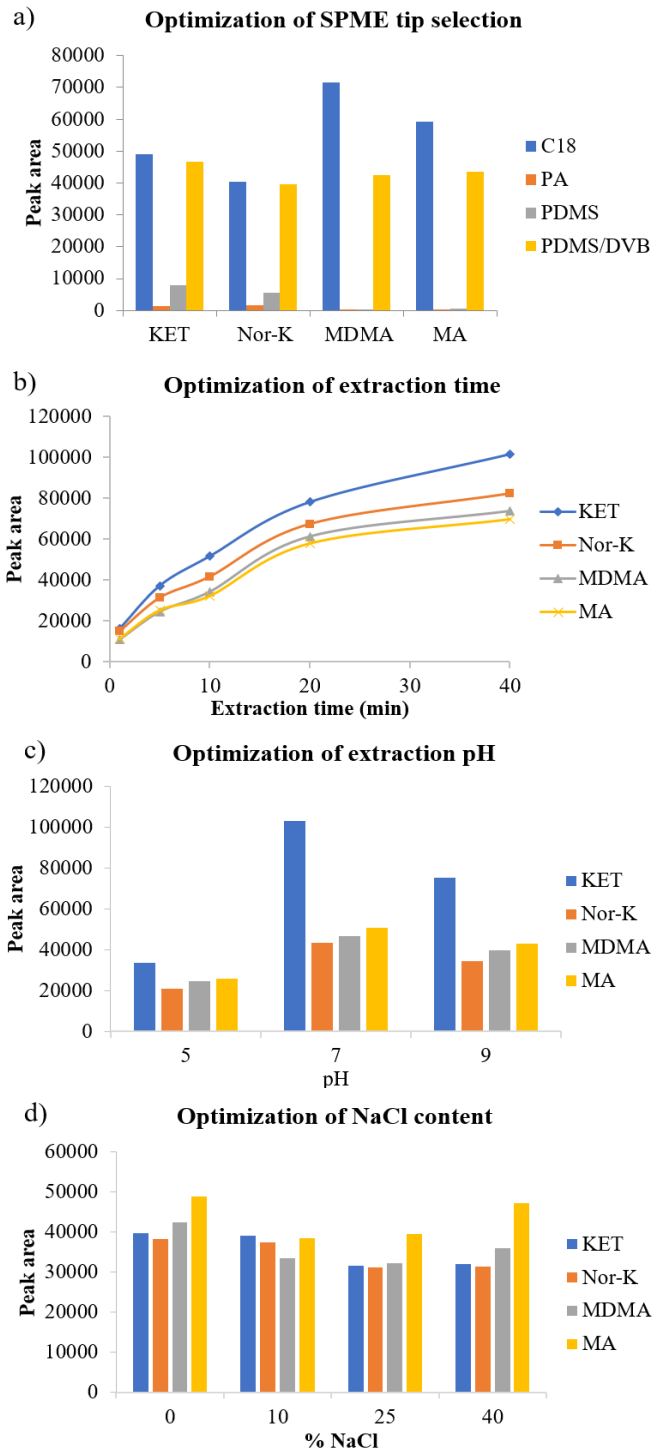


Figure 3-6. The optimization of (a) SPME tip selection, (b) extraction time, (c) extraction pH and (d) addition of salt, for the extraction of KET, Nor-K, MDMA and MA in urine.

Optimization of SPME-ESI-MS setup

There are several parameters that could affect the performance of SPME-ESI-MS including the spray solvent delivery methods, the distance between the SPME tip and the MS ion inlet of the and the spray solvent compositions. The above parameters were tested.

Three different spray solvent delivery methods were tried for SPME-ESI-MS including the addition of solvent using pipette, syringe pump and sprayer. For the pipette method, the solvent was added onto the SPME tip similar to that of WT-ESI-MS. The solvent was pipetted directly onto the SPME tip via a pipette. Signals could be produced but the signal duration was very short, which is less than 6 s. The resultant MRM signals were nearly in triangular shape which was not proper for the analysis. The syringe pump method described by Ahmad and co-workers was also tested.[101] However, the result obtained was quite similar to that of using pipette as shown in Figure 3-7a and b. It was observed that a big solvent droplet was accumulated onto the SPME tip rather than sprayed out. It may due to the surface area of SPME tip was too small and its capacity was limited. Too many solvents may be applied onto the SPME tip using syringe pump and pipette simultaneously. Therefore, a sprayer with solvent supply from the syringe

pump was used for the solvent delivery instead. Finer solvent droplet created by the sprayer was landed onto the SPME-tip and solvent accumulation was prevented, resulting of the production of continuous signal as shown in Figure 3-8c. It was noticed that the actual amount of solvent applied onto the SPME tip was less than the syringe pump as some of the fine solvent droplets were lost to the surrounding. It is not reasonable and necessary to spend longer than 10 min to record one signal. In the final SPME-ESI-MS setup, the solvent supply to the sprayer was stopped after the signal reached its maximum, which was around 20 s (equivalent to 10 μL of solvent at the flow rate of 30 μL/min). Different gas pressures and different solvent flows were also tested. If the gas pressure was too low, no solvent spray could be formed. However, if the gas pressure was too high, the solvent droplets would be too small and evaporated before landing onto the SPME tip, and the solvent loss would also be greater as the spray were more dispersed. The solvent flow rate could also affect the signal duration. A low solvent rate could result in signal similar to that of using pipette while no significant improvement was observed when a higher solvent flow rate was used. When the solvent flow rate was set to very high, such as doubling optimized setting, solvent would accumulate at the tip end and obstruct the ionization process.

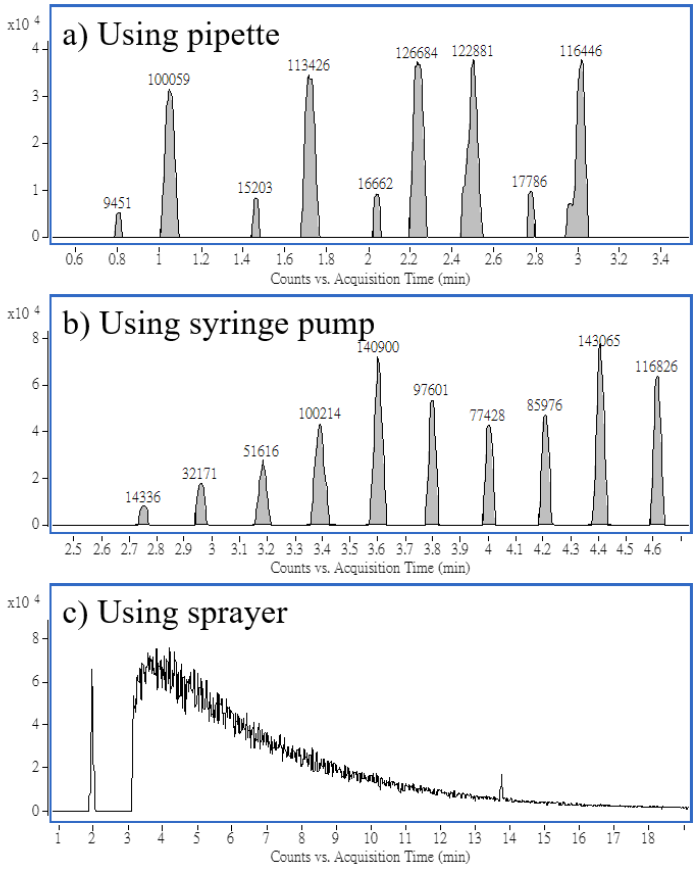


Figure 3-7. The MRM signals (*m/z* 238 → *m/z* 125) obtained for thr detection of 1000 ng/mL ketamine in urine using SPME-ESI-MS with (a) pipette, (b) syringe pump and (c) spray as the solvent delivery method.

The distance between SPME tip and the MS ion inlet were also optimized. The distance was started from 3 x 3 cm (horizontal distance x vertical distance) away from the mass spectrometer. An external high voltage supply was connected to the SPME tip for the ESI. The SPME tip was moved 0.5 x 0.5 cm forward to the ion inlet each time and finely adjusted when strong signal was observed. MRM signals were observed when

the distance was set to 1.5 x 1.5 cm and strongest signals were observed for the distance with 0.6 - 0.8 x 0.4 - 0.8 cm. SPME-ESI-MS using the high voltage supply from the instrument was also tried. However, the high voltage supply of the Agilent mass spectrometer is located on the instrument side, but the SPME tip was located closely to the instrument in order to obtain the signals. Discharge could be easily caused when the SPME tip was set too close to the instrument. Therefore, external high voltage was finally used in this study.

The spray solvent plays an important role for the elution and ionization of the targeted analytes from the SPME tip. Different organic solvent and water solvent systems were tested for SPME-ESI-MS. As shown in Figure 3-8a, the higher the ratio of the organic solvent, the higher the signal intensity was observed. It is reasonable as the targeted analytes could be eluted better in organic solvent and the ionization could also be better due to the fast evaporation of organic solvent than water. 10% of water was still kept in the spray solvent as it is necessary to wet the surface of SPME tip in order to elute and ionize the analytes and to prevent the spray solvent evaporation before it reached the SPME tip. Another set of data which various organic solvents were used is shown in Figure 3-8b. Generally, strongest signals were produced by using EtOH as the spray

solvent, followed by using MeOH. However, the signals obtained by using ACN were significantly lower than that of EtOH and MeOH. 90% EtOH was the most effective spray solvent for SPME-ESI-MS for most of the drugs, except for HER, MOR and 6-MAM, which 90% MeOH could give better signals. Therefore, 90% EtOH was selected as the spray solvent at normal situation except 90% MeOH was used for detection of heroin and its metabolites.

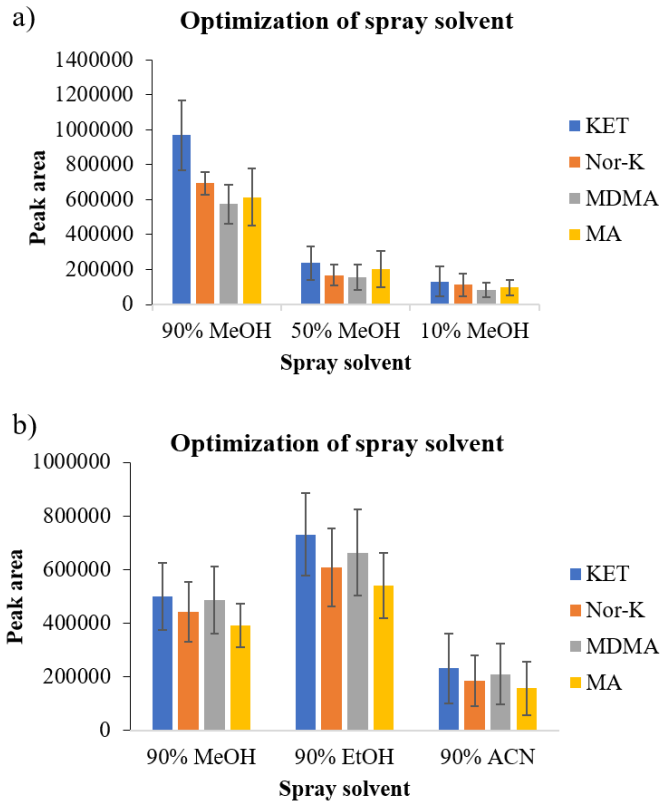


Figure 3-8. The results of optimization of (a) spray solvent with different ratio of organic-aqueous composition and (b) spray solvent with different organic solvent, for the elution and ionization of KET, Nor-K, MDMA and MA in urine.

3.3.3. Detection and quantitation of drugs-of-abuse in urine and oral fluid

Detection of drugs-of-abuse

Typical MRM results for the detection of ketamine in urine are shown in Figure 3-9a. As the core of the C18 SPME tip is a metal alloy, ESI is easily induced after high voltage supply. The spray solvent was applied onto the SPME tip for 20 s each time. Typically, the spray solvent was applied onto the same SPME tip 3 times to ensure the stability of the signals. Signals were considered as positive only if the S/N of the quantifier ion channels is ≥ 3 when compared with that of the blank, as shown in Figure 3-9b. Also, the presence of the analytes was further confirmed by monitoring an additional qualifier ion channel for each targeted analyte. The ion ratio between the quantifier ion and qualifier ion should be within certain value as suggested by EWDTS if a particular drug is present in the samples. The quantifier ion channels and qualifier ion channels used in this study were tested by injecting standard solution in the mass spectrometer and the results were already listed in Table 3-1. The ion ratios (qualifier ion/quantifier ion) for confirming the presence of the targeted analytes are listed in Table 3-3.

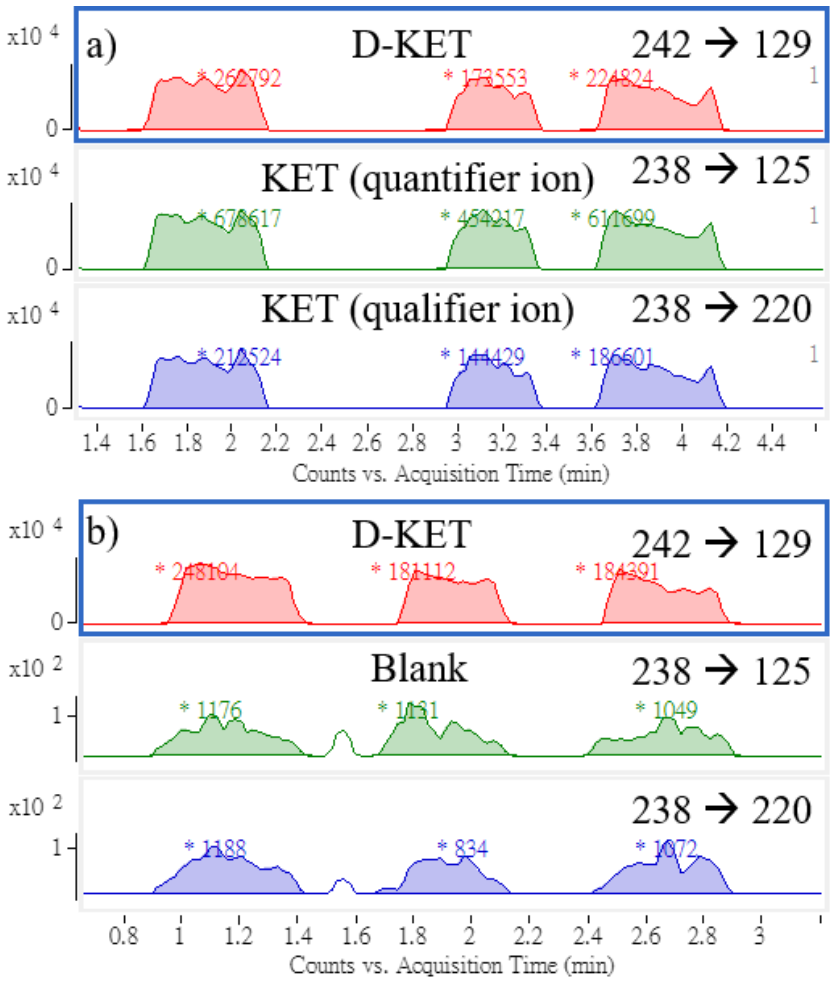


Figure 3-9. Typical MRM results of the detection of (a) 500 ng/mL ketamine in urine and (b) blank urine using SPME-ESI-MS.

Table 3-3. The ion ratios between the quantifier ion and qualifier ion of the targeted analytes.

Compound	Ion ratio (n =12)		
	Mean	**Lower limit***	**Upper limit***
Ketamine	0.31	0.23	0.39
Nor-ketamine	0.56	0.45	0.67
Methamphetamine	0.31	0.24	0.39
MDMA	0.34	0.25	0.42
Cocaine	0.25	0.19	0.31
Benzoylecgonine	0.36	0.27	0.45
Heroin	0.45	0.33	0.56
6- monoacetylmorphine	0.48	0.36	0.60
Morphine	0.88	0.70	1.05
THC	0.85	0.68	1.02
THC-COOH	0.23	0.17	0.29

*The lower limits and upper limits were calculated according to EWDTS guidelines.[88]

Samples with the same concentration were repeatedly measured with three individual SPME-ESI-MS at the same day and the whole experiment was repeated on another day and the results are shown in Figure 3-10. The precisions of the 3 measurements using the same SPEM tip (no.1 - no.4) were 19.8%, 2.9%, 19.1% and 18.5% respectively. The precision of the measurements within the same day (n = 9) was 14.1% and the precision of all the measurements (n = 12) using different SPME tips (n = 4) was 14.4%. On the other hand, using the relative peak areas (i.e. the peaks areas of the analytes divided by

the peaks areas of the internal standard) for the measurements of signals were more reproducible. The precisions of the 3 measurements, in term of relative peak areas, using the same SPME tip (no.1 - no.4) were 2.7%, 1.0%, 6.2% and 2.2% respectively. The overall precision was 5.1% which is significantly better that of using absolute peak areas for the measurements. Therefore, the relative peak areas were used for the detection and quantitation of targeted analytes in this study. The results also demonstrated that the extraction and detection of targeted analytes using the established protocol could be reproducible even the samples were extracted using different C18 SPME tips.

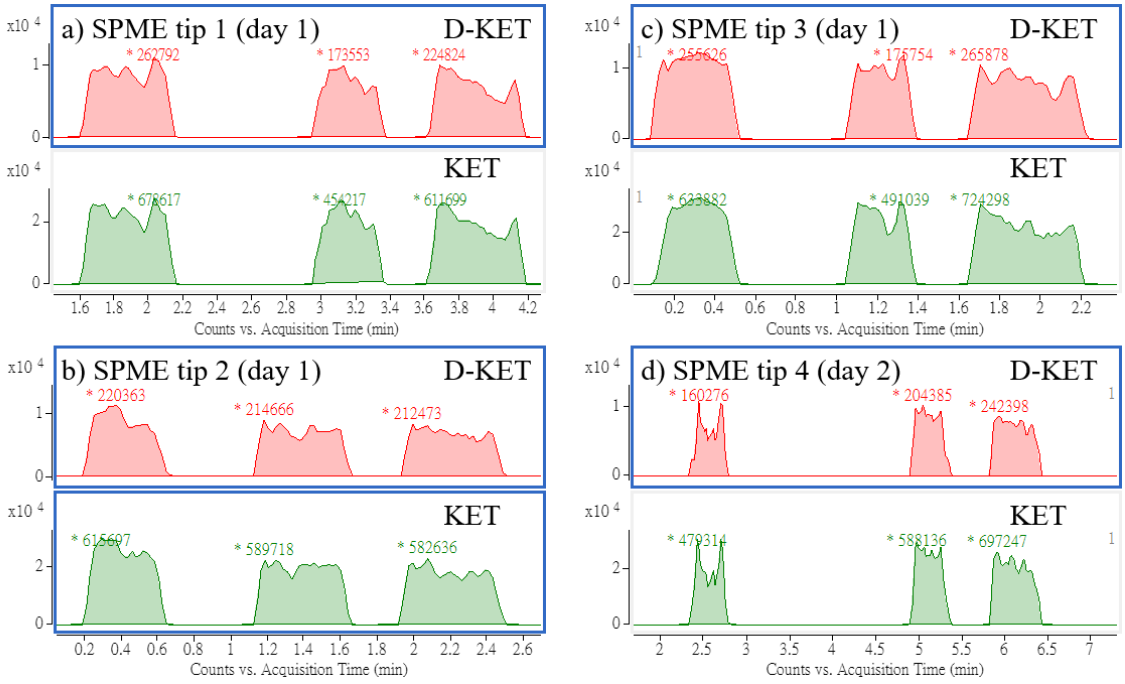


Figure 3-10. MRM results for the detection of 500 ng/mL ketamine in urine using SPME-ESI-MS. (a-c) Repeated experiments using three individual SPME-ESI-MS within the same day and (d) repeated experiment on another day.

Reusability of the C18 SPME tip

The extraction of targeted analytes was achieved using C18 SPME tip. However, the cost of each C18 SPME tip was around HK$ 180 which is relatively expensive compared with other extraction techniques. Therefore, it would be more cost effective if the C18 SPME tip could be re-used after one analysis. The results obtained for analyzing blank urine, 1000 ng/mL ketamine in urine and the washed C18 SPME tip after the analysis is shown in Figure 3-11. By comparing the results of Figure 3-11b and

c, the signals were greatly reduced after washing, it indicated that most of the ketamine residue left on the SPME tip after the SPME-ESI-MS analysis could be removed by washing the tips with organic solvent for 30 min. The residue level after washing was only slightly higher than that of the blank. The ion ratio for the detection of ketamine of the SPME tip after washing was also higher than the acceptable value, which could be considered as no ketamine presence on that C18 SPME tip. Therefore, the results showed that C18 SPME tip can be used after proper washing.

In this study, the total usage time of each C18 SPME tip was recorded and the re-used C18 SPME tips were replaced if the signals obtained were significantly reduced or increased when compared with other tips. Each SPME tip can be generally re-used for 10 analyses. The main reason for the reduced performance of the re-used C18 SPME tips was the detachment of the C18 coating. The repeated solvent washing process or the carelessness of handling the SPME tip may also cause damage to the SPME tips. To avoid misleading results, the SPME tips that was used for the analysis of samples with high concentration were not used for the analysis of samples with low concentration. Also, only the new SPME tips were used for the determination of blank signal level, LODs and LOQs of the analytes.

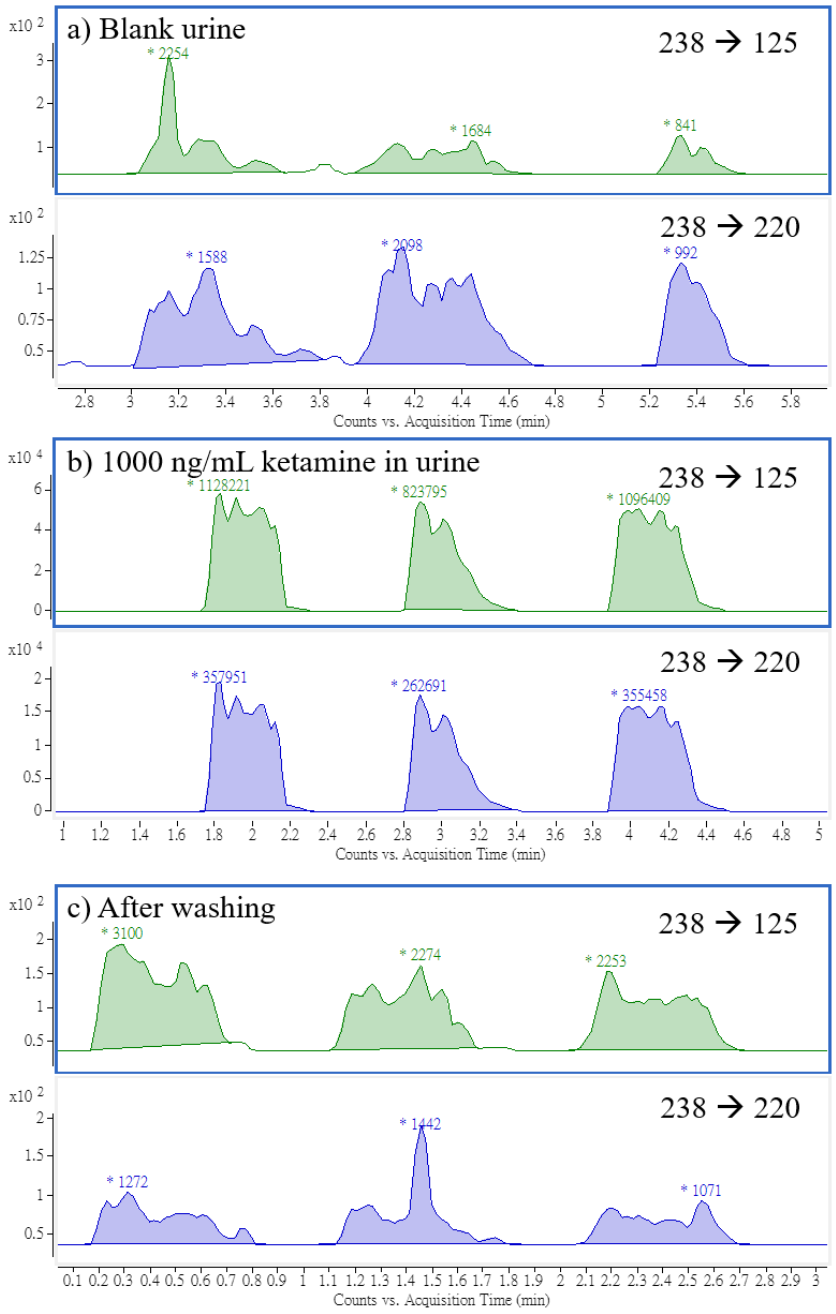


Figure 3-11. MRM results for the detection of ketamine of (a) blank urine, (b) 1000 ng/mL of ketamine in urine and (c) the same C18 SPME tip after washing, using SPME-ESI-MS. *m/z* 238 → *m/z* 125 was the quantifier ion and *m/z* 238 → *m/z* 220 was the qualifier ion for detection of ketamine.

Quantitation of targeted analytes

The calibration curves for the quantitation of targeted analytes in urine and oral fluid were constructed by measuring the signals of spiked samples with at least five different concentrations. The calibration curves constructed for each analyte in urine and oral fluid are shown in Figure 3-12 and Figure 3-13 respectively. All the targeted analytes including THC and THC-COOH which were failed to be analyzed using WT-ESI-MS showed linear correlation between the concentrations and signals obtained in SPME-ESI-MS. Linear calibration plots for quantitation of all targeted analytes were constructed. It is noted that high concentration (500 ng/mL) of internal standards was used for the quantitation of HER, 6-MAM, MOR, THC and THC-COOH in order to produce stable signals.

The linear range, linearity (in term of R^2) and average R.S.D. of the calibration points of all the targeted analytes in urine and oral fluid are recorded in Table 3-4. Generally, the linear range could nearly cover the range of 10 – 1000 ng/mL except that of THC and THC-COOH. It is due to the signals produced by THC and THC-COOH were unstable at low concentrations, thus the calibration points at low concentrations became nonlinear. Calibration points at higher concentrations were not tested as it is difficult to

remove the drug residues on the C18 SPME tips at high concentrations. The R^2 of the curves were greater than 0.99 which indicated good linearity. The reproducibility of relative intensities of each analyte was generally better than 15% except for THC which was less reproducible. The signals of THC were relatively poorer than most of the analytes and its signals thus were not stable at low concentration. In contrast, the performance for quantitation of cocaine was the best, the signals for detection of cocaine at low concentrations were still stable and therefore a wider calibration range was covered. However, benzoylecgonine is always considered as the analytical target for cocaine and the performances of quantitation of benzoylecgonine were not as good as cocaine. The results showed that the present method is suitable for quantitation of drugs-of-abuse in urine and oral fluid and the performance is better than that of WT-ESI-MS.

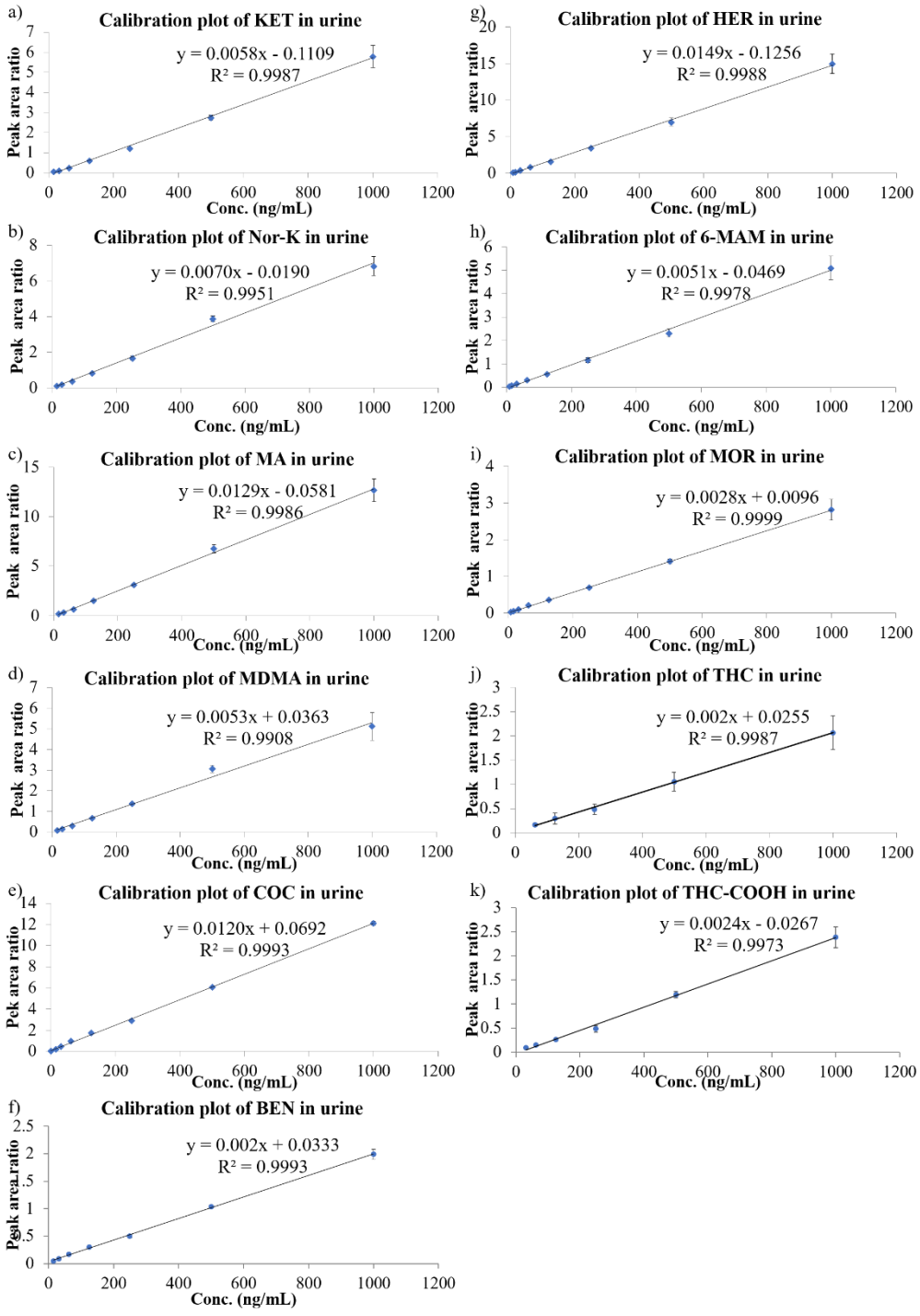


Figure 3-12. Calibration plots for the quantitation of (a) ketamine, (b) nor-ketamine, (c) methamphetamine, (d) MDMA, (e) cocaine, (f) benzoylecgonine, (g) heroin, (f) 6-monoacetylmorphine, (i) morphine, (j) THC and (k) THC-COOH in urine.

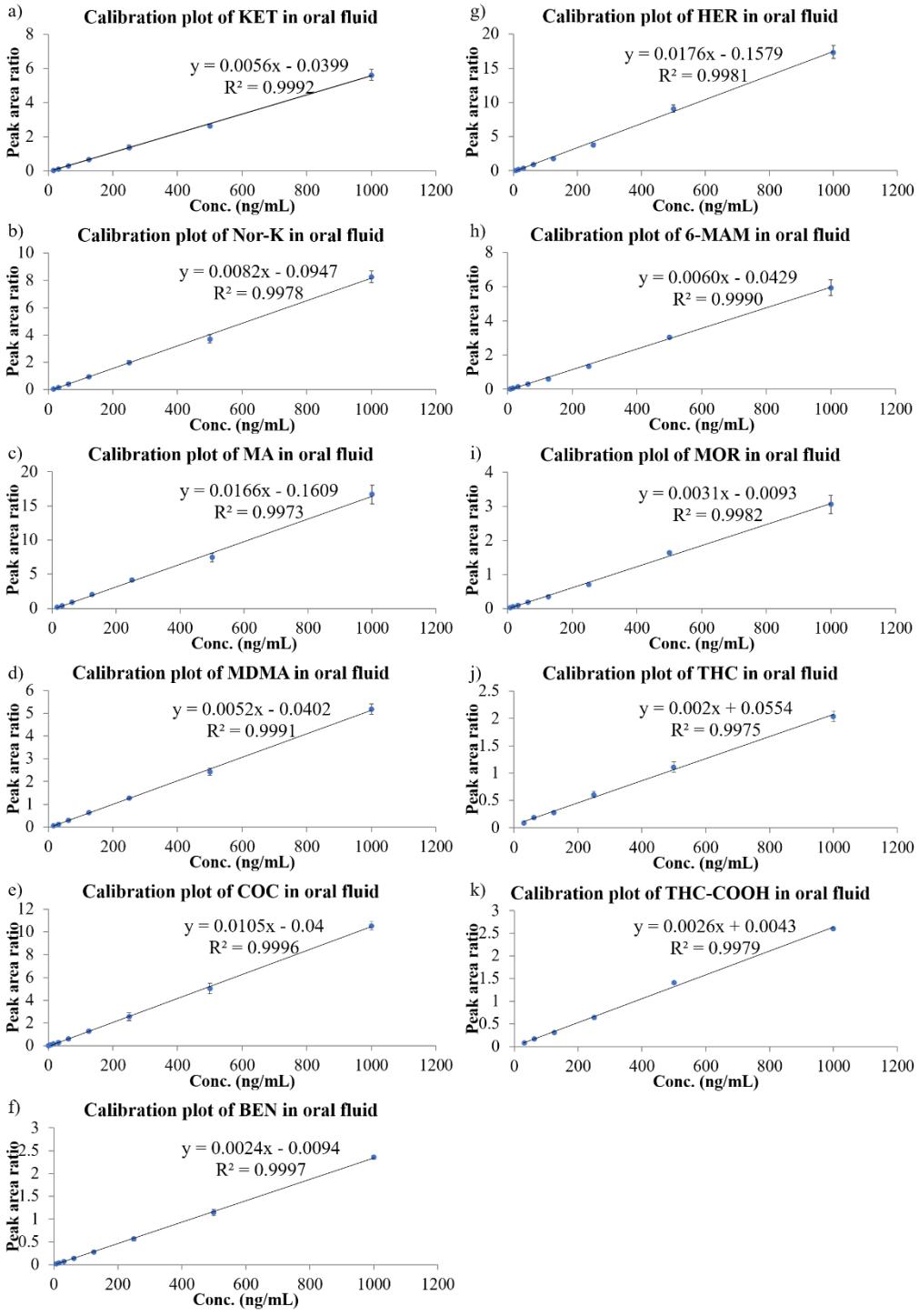


Figure 3-13. Calibration plots for the quantitation of (a) ketamine, (b) nor-ketamine, (c) methamphetamine, (d) MDMA, (e) cocaine, (f) benzoylecgonine, (g) heroin, (f) 6-monoacetylmorphine, (i) morphine, (j) THC and (k) THC-COOH in oral fluid.

Table 3-4. Linearity of targeted drugs and metabolites in urine and oral fluid**.**

Compound		Linear range (ng/mL)	R^2	Average R.S.D. of calibration points (%)
Ketamine	Urine	15.6 - 1000	0.9987	6.0
	Oral fluid	15.6 - 1000	0.9992	14.3
Nor-ketamine	Urine	15.6 - 1000	0.9951	7.1
	Oral fluid	15.6 - 1000	0.9978	15.5
Methamphetamine	Urine	15.6 - 1000	0.9986	7.9
	Oral fluid	15.6 - 1000	0.9973	13.3
MDMA	Urine	15.6 - 1000	0.9908	7.5
	Oral fluid	15.6 - 1000	0.9991	12.7
Cocaine	Urine	0 - 1000	0.9993	4.2
	Oral fluid	0 - 1000	0.9996	11.7
Benzoylecgonine	Urine	15.6 - 1000	0.9993	5.6
	Oral fluid	7.8 - 1000	0.9997	11.9
Heroin	Urine	7.8 - 1000	0.9988	6.8
	Oral fluid	7.8 - 1000	0.9981	8.9
6-monoacetylmorphine	Urine	7.8 - 1000	0.9978	8.6
	Oral fluid	7.8 - 1000	0.9990	8.4
Morphine	Urine	7.8 - 1000	0.9999	9.1
	Oral fluid	7.8 - 1000	0.9982	9.8
THC	Urine	62.5 - 1000	0.9987	22.4
	Oral fluid	31.3 - 1000	0.9975	10.1
THC-COOH	Urine	31.3 - 1000	0.9973	9.4
	Oral fluid	31.3 - 1000	0.9979	12.9

Accuracy and precision of quantitative analysis

Spiked quality control samples at low, middle and high concentrations of the targeted analytes in urine and oral fluid were tested. The accuracy and precision of the quantitative analysis results are summarized in Table 3-5. The accuracy for determining the targeted drugs was satisfactory (within 80 – 120%), except the determination of quality control samples at the lowest concentration, which was 73.4 – 162.9%. For the quantitation of the targeted analytes at high and middle concentrations (i.e. 800 ng/mL and 500 ng/mL), the precision was generally within 15%, except for THC in urine (19.5 – 22.7%) which was slightly higher than other values. The performance of the quantitation of drugs at low concentrations (e.g. 50 ng/mL and 20 ng/mL) varied and was generally within 25%. The precisions for measuring COC, HER and 6-MAM were within 10% and the results were better than that of other drugs. In contrast, the precision for measuring benzoylecgonine at 20 ng/mL was 31.2%, which was not satisfactory. Overall, the results of quantitation of all the targeted analytes at different concentrations were desirable and improved when compared with that of WT-ESI-MS. It is possible to determine the concentration of heroin and related compounds and THC and THC-COOH at low concentrations (e.g. 50 ng/mL and 20 ng/mL) using SPME-ESI-MS. However, the performance for the determination of drugs at low concentration could be

improved. One possible solution is to construct calibration curves which only cover the low concentration range, for the determination of drugs at low concentrations.

Table 3-5. Accuracy and precision for analysis of different drugs in urine and oral fluid.

Compound	Spiked Quantity (ng/mL)		Determined Quantity± S.D. (ng/mL) (n=6)		Accuracy (%)		R.S.D (%)	
	Urine	O.F.	Urine	O.F.	Urine	O.F.	Urine	O.F.
KET	769.2	769.2	605.8±39.1	901.7±62.2	78.8	117.2	6.5	6.9
	487.8	476.2	450.0±33.5	476.2±46.0	92.2	119.9	7.4	8.1
	48.8	47.6	49.2±12.6	58.0±5.4	100.7	121.9	25.5	9.3
	19.8	19.6	15.9±2.1	25.5±3.0	80.4	130.0	13.0	11.7
Nor-K	769.2	769.2	647.2±64.2	670.9±85.6	84.1	87.2	9.9	12.8
	487.8	476.2	475.6±62.5	436.4±45.6	97.5	91.6	13.1	10.4
	48.8	47.6	57.2±10.4	38.5±6.6	117.1	80.9	18.2	17.2
	19.8	19.6	19.0±3.9	14.4±3.5	95.8	73.5	20.3	24.1
MA	769.2	769.2	700.1±62.3	805.6±50.2	87.5	104.7	8.9	6.2
	487.8	476.2	544.7±70.5	549.9±58.9	111.7	115.5	12.9	10.7
	48.8	47.6	46.1±5.4	59.2±5.7	94.5	124.4	11.7	9.6
	19.8	19.6	14.5±3.7	31.9±2.7	73.4	162.9	25.4	8.4
MDMA	769.2	769.2	613.2±34.5	853.0±110.3	79.7	110.9	5.6	12.9
	487.8	476.2	417.2±34.9	460.1±52.3	85.5	96.6	8.4	11.4
	48.8	47.6	49.0±8.2	57.5±9.3	100.4	120.8	16.8	16.2
	19.8	19.6	17.5±2.7	28.5±4.5	88.2	145.2	15.4	15.8
COC	769.2	740.7	661.5±45.3	858.2±45.9	86.0	115.9	6.8	5.4
	487.8	476.2	421.2±14.4	554.8±18.6	86.3	116.5	3.4	3.4
	48.8	47.6	51.8±1.7	54.0±1.1	106.1	113.4	3.3	2.0
	19.8	19.6	23.0±1.0	23.2±0.3	116.3	118.6	4.4	1.3
BEN	769.2	740.7	664.9±48.7	823.0±74.9	86.4	111.1	7.3	9.1
	487.8	476.2	414.7±20.4	533.4±18.0	85.0	112.0	4.9	3.4
	48.8	47.6	43.4±3.6	56.5±2.2	89.0	118.7	8.3	3.8
	19.8	19.6	20.9±6.5	24.5±1.3	105.5	125.0	31.2	5.2
HER	769.2	740.7	817.0±32.1	725.4±68.9	106.2	97.9	3.9	9.5
	487.8	476.2	502.2±63.0	445.5±48.2	103.0	93.6	12.6	10.8
	48.8	48.7	47.1±4.3	48.5±2.7	96.4	99.6	9.0	5.5
	19.8	19.6	26.0±1.9	24.5±1.6	131.2	125.2	7.3	6.6

(To be continued)

6-MAM	769.2	740.7	769.5±63.3	658.2±55.0	100.0	88.9	8.2	8.4
	487.8	476.2	489.4±57.0	441.7±50.4	100.3	92.7	11.6	11.4
	48.8	48.7	48.9±4.1	46.8±3.0	100.3	96.2	8.4	6.5
	19.8	19.6	26.1±2.0	24.3±2.3	132.1	124.2	7.8	9.5
MOR	769.2	740.7	744.8±39.9	634.3±71.4	96.8	85.6	5.4	11.3
	487.8	476.2	476.6±78.1	426.2±50.1	97.7	89.5	16.4	11.8
	48.8	48.7	48.3±3.7	50.1±3.4	98.9	102.9	7.6	6.8
	19.8	19.6	20.5±2.8	24.5±3.9	103.7	125.2	13.7	15.9
THC	787.4	787.4	748.2±145.6	963.8±128.8	95.0	122.4	19.5	13.4
	495.0	495.0	454.9±103.3	461.3±29.3	91.9	93.2	22.7	6.3
	99.0	99.0	83.4±19.3	98.3±9.7	84.3	99.3	23.9	9.9
	49.8	49.8	41.3±7.6	45.4±11.7	82.9	91.1	18.3	25.7
THC-COOH	787.4	787.4	802.7±114.1	775.4±88.5	101.9	98.5	14.2	11.4
	495.0	495.0	510.9±52.2	430.6±50.6	103.2	87.0	10.2	11.8
	99.0	99.0	102.7±11.8	76.4±10.3	103.8	77.2	11.4	13.4
	49.8	49.8	55.9±2.7	38.3±4.3	112.3	76.9	4.8	11.2

Determination of LOD and LOQ

The LODs and LOQs of different drugs-of-abuse were determined experimentally with the use of spiked urine and oral fluid samples at low concentration. However, as it was not possible to apply blank samples and spiked samples onto the same C18 SPME tip for S/N comparison, thus the method for the determination of LOD and LOQ was different from that of WT-ESI-MS. The smallest measurable signals (x_L) for each targeted analyte were firstly determined by measuring the signal obtained from blank samples. The calculation of x_L of each targeted analyte was described in selection 3.2.7, according to the instruction of IUPAC.[102] The LOD and LOQ of an analyte were

then defined as the concentrations of the spiked samples that can give signal larger than x_L with the factor (k) equal to 3 and 10 respectively. The ion ratios between the quantifier ion and qualifier ion for each analyte were also fitted the requirement listed in Table 3-3 for confirming the detection of each analyte. An example for the determination of blank urine signals for cocaine is shown in Figure 3-14a. Generally, the experiments for the blank measurement were repeated 9 times and more than 27 measurements were averaged to calculate the x_L value. Figure 3-14b shows the MRM signals for the detection of 0.5 ng/mL cocaine in urine using SPME-ESI-MS. The averaged signal (relative peak area, 6 experiments and 18 measurements) at that concentration was 0.0045, which was higher than the x_L value for detection of cocaine in urine (0.0039) and the averaged ion ratio was 0.30 which was within the range for detection of cocaine (0.19 – 0.31). Therefore, the LOD of cocaine in urine was determined as 0.5 ng/mL.

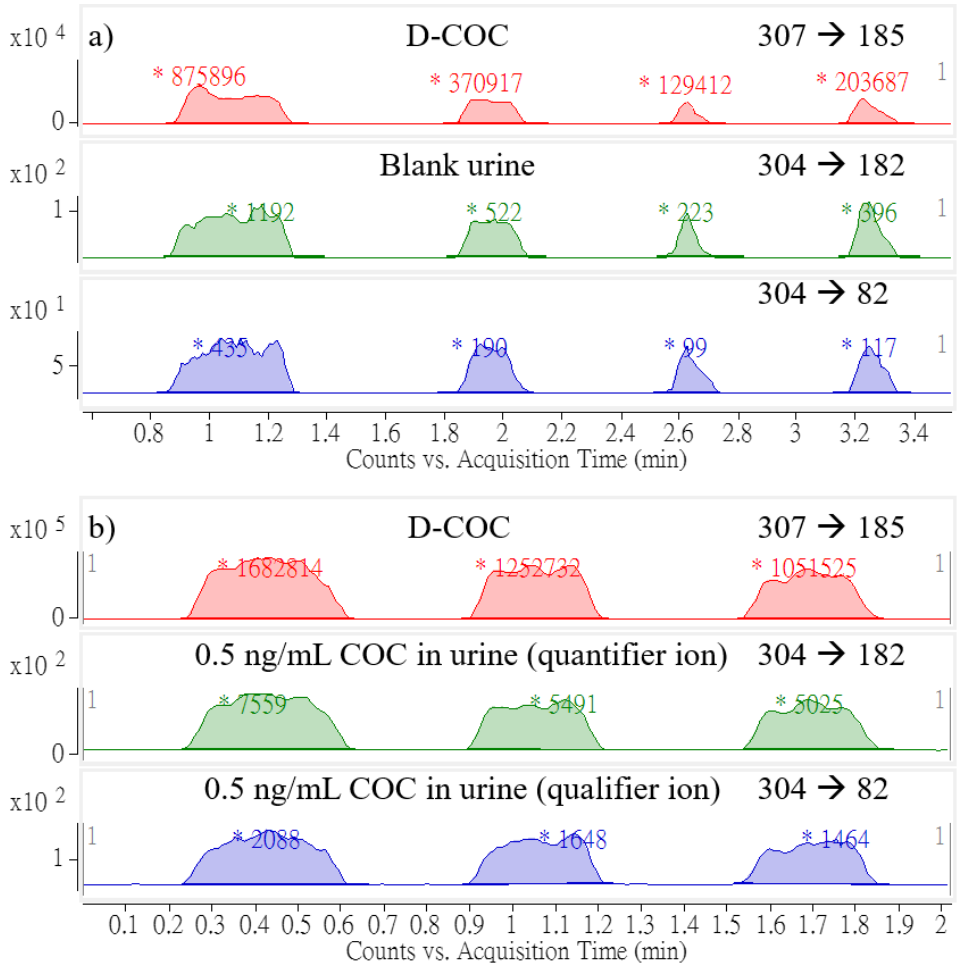


Figure 3-14. MRM results for the detection of cocaine of (a) blank urine and (b) 0.5 ng/mL of cocaine in urine using SPME-ESI-MS.

The x_L values and the corresponding LODs and LOQs of all targeted analytes are listed in Table 3-6. k = 3 was used for the determination of LODs and k = 10 was used for the determination of LOQs. The LOQs of the targeted analytes were normally higher than LODs as higher x_L values were required. The LODs and LOQs of KET and Nor-K in urine were considered as equal, which was an exceptional case. The

signals for the detection of 2 ng/mL of KET and Nor-K were stronger than the x_L values with k = 3. However, the ion ratios for the detection of 2 ng/mL KET and Nor-K in urine were failed to fit within the acceptable range of ion ratios for confirming the presence of KET and Nor-K in urine. Therefore, the LODs and LOQs became equal as the lowest concentration for obtaining acceptable ion ratios of KET and Nor-K in urine was 10 ng/mL. The LOQs of targeted analytes were normally 3.3 times higher than that of LODs when S/N was used for the determination of LODs and LOQs. However, the x_L values obtained in IUPAC method were highly dependent on the standard derivations of the blank signals of the targeted analytes, and no fixed ratios between the LODs and LOQs of the targeted analytes were observed.

Table 3-6. x_L values, LODs and LOQs of the targeted analytes in urine and oral fluid obtained using SPME-ESI-MS.

Compound	x_L (k = 3)		x_L (k = 10)		LOD (ng/mL)		LOQ (ng/mL)	
	Urine	O.F.	Urine	O.F.	Urine	O.F.	Urine	O.F.
KET	0.0025	0.0071	0.0057	0.0198	10	5	10	10
Nor-K	0.0019	0.0082	0.0046	0.0227	10	2	10	5
MA	0.0130	0.0198	0.0340	0.0553	2	2	10	5
MDMA	0.0034	0.0076	0.0091	0.0212	2	2	10	10
COC	0.0039	0.0044	0.0122	0.0112	0.5	0.5	7.8	5
BEN	0.0176	0.0086	0.0455	0.0225	10	8	15.6	15.6
HER	0.0023	0.0119	0.0054	0.0281	1	1	5	5
6-MAM	0.0041	0.0037	0.0096	0.0079	1	1	5	5
MOR	0.0109	0.0103	0.0292	0.0269	5	5	10	10
THC	0.1040	0.0778	0.2874	0.1844	50	50	125	100
THC-COOH	0.0310	0.0395	0.0747	0.1043	15	15	31.3	50

The comparison between the LODs and cut-off levels of international standards of the targeted analytes is listed in Table 3-7. The LODs obtained using SPME-ESI-MS were greatly improved compared with those values obtained using WT-ESI-MS. For the detection of KET, Nor-K, MA, MDMA and COC, which were already good enough for real analysis, there were 2 – 25 times improvement. For the detection of BEN, HER, 6-MAM, MOR, THC and THC-COOH, the improvements were very obvious, from barely or not detectable to clearly detected even at low concentrations. The LODs of most of the drugs obtained using SPME-ESI-MS could fulfill the cut-off levels of international standards except for the detection of THC in oral fluid.

The improvements on the detection were due to the enrichment of targeted analytes onto the C18 SPME tip. The analytes to be eluted were also highly concentrated as only very little amounts of solvent (< 10 μL） was used for elution. Besides, C18 SPME tips are made by metal alloy, which could conduct electricity for better ionization compared with that of wooden tips. C18 SPME tips also possess no porous structures and would not trap the targeted analytes onto the surface that may reduce the sensitivity of detection of the analytes. Moreover, the background signals generated by the SPME tip itself was very low, thus the resultant x_L values were also very low, which benefited

the determination of LODs of the targeted analytes. However, the extraction and ionization efficiency for the detection of THC were still not good enough.

Table 3-7. LODs and LOQs obtained of the targeted analytes using SPME-ESI-MS and recommended cut-off values of various drugs in urine and oral fluid.

Compound	LOD (ng/mL)		LOQ (ng/mL)		SAMHSA cut-off (ng/mL)		EWDTS cut-off (ng/mL)		DRUID cut-off (ng/mL)
	Urine	O.F.	Urine	O.F.	Urine	O.F.	Urine	O.F.	O.F.
KET	10	5	10	10	N.A.	N.A.	N.A.	N.A.	N.A.
Nor-K	10	2	10	5	N.A.	N.A.	N.A.	N.A.	N.A.
MA	2	2	10	5	250	15	200	15	410
MDMA	2	2	10	10	250	15	200	15	270
COC	0.5	0.5	1	5	N.A.	8	N.A.	8.	170
BEN	10	8	15.6	15.6	100	8	100	8	95
HER	1	1	5	5	N.A.	N.A.	N.A.	N.A.	N.A.
6-MAM	1	1	5	5	10	2	10	2	16
MOR	5	5	10	10	2000	15	300	15	95
THC	50	50	125	62.5	N.A.	2	N.A.	2	27
THC-COOH	15	15	31.3	50	15	N.A.	15	N.A.	N.A.

Extraction efficiency of SPME

The extraction efficiency of SPME is shown in Table 3-8. The extraction efficiency of the analytes was not high as the surface area of SPME tip was small, and only little amount of analytes could be attached onto the surface of the tiny SPME tip. The results obtained were consistent with the previous findings of Chou and Lee.[103] Generally, the compounds with hydrophobic structure such as THC, THC-COOH, HER and COC could give better extraction efficiency. The extraction efficiency of compounds with more polar functional groups such BEN and MOR, on the other hand, gave very poor extraction efficiency. The results were reasonable as C18 favor the extraction of compounds with higher degree of hydrophobicity. The compounds with higher extraction efficiency could generally give lower values of LODs, while the LODs of compounds with lower extraction efficiency, such as BEN and MOR were higher. However, extraction efficiency was not the only factor affecting the detection of drugs. THC and THC-COOH could give high extraction efficiency but the detection was poor. It could due to the poor ionization efficiency of THC and THC-COOH. The details will be discussed in later sections.

Table 3-8. Extraction efficiency of analytes using C18 SPME tip.

Compound	Recovery (%)
KET	4.6
Nor-K	5.0
MDMA	2.4
MA	2.0
COC	6.7
BEN	0.4
HER	6.1
6-MAM	2.4
MOR	0.4
THC	19.9
THC-COOH	23.1

3.3.4 Analysis of Medichem urine samples

The results obtained and discussed in the previous sections were relied on the tested samples containing only same types of drugs. However, it is possible that the samples contained more than one types of drugs, if the drug abusers consumed different drugs simultaneously. The present of multiple analytes with different structure at the same sample may affect the analysis results as some of the drugs may be extracted and

ionized more effectively than other drugs, which caused the signal suppression of the weakly ionized analytes. Three commercially available samples from Medichem which contained 29 drugs-of-abuse at different concentrations in human urine, were used to test the ability of SPME-ESI-MS for analyzing complex analyte mixture simultaneously. Medichem urine sample are the reference materials for forensic chemistry and used as same as the patient samples. The protocol used for handling the Medichem samples was the same as the protocol previously developed, but the extraction time was increased to 15 min as 5 or 10 min extractions were not enough to obtain stable signals for the analysis of such complex samples. Four time segments were used for detection of the targeted analytes as the sensitivity was reduced when too many channels are scanned simultaneously. The respective results for analyzing the Medichem Basis-line U sample are shown in Figure 3-15, which contained no drugs-of-abuse in the sample. The SPME-ESI-MS analysis showed negative results (i.e. signals observed $< x_L$ and unfitted ion ratios) for all targeted analytes, which were consistent with the manufacturer manual, as no false positive result was observed.

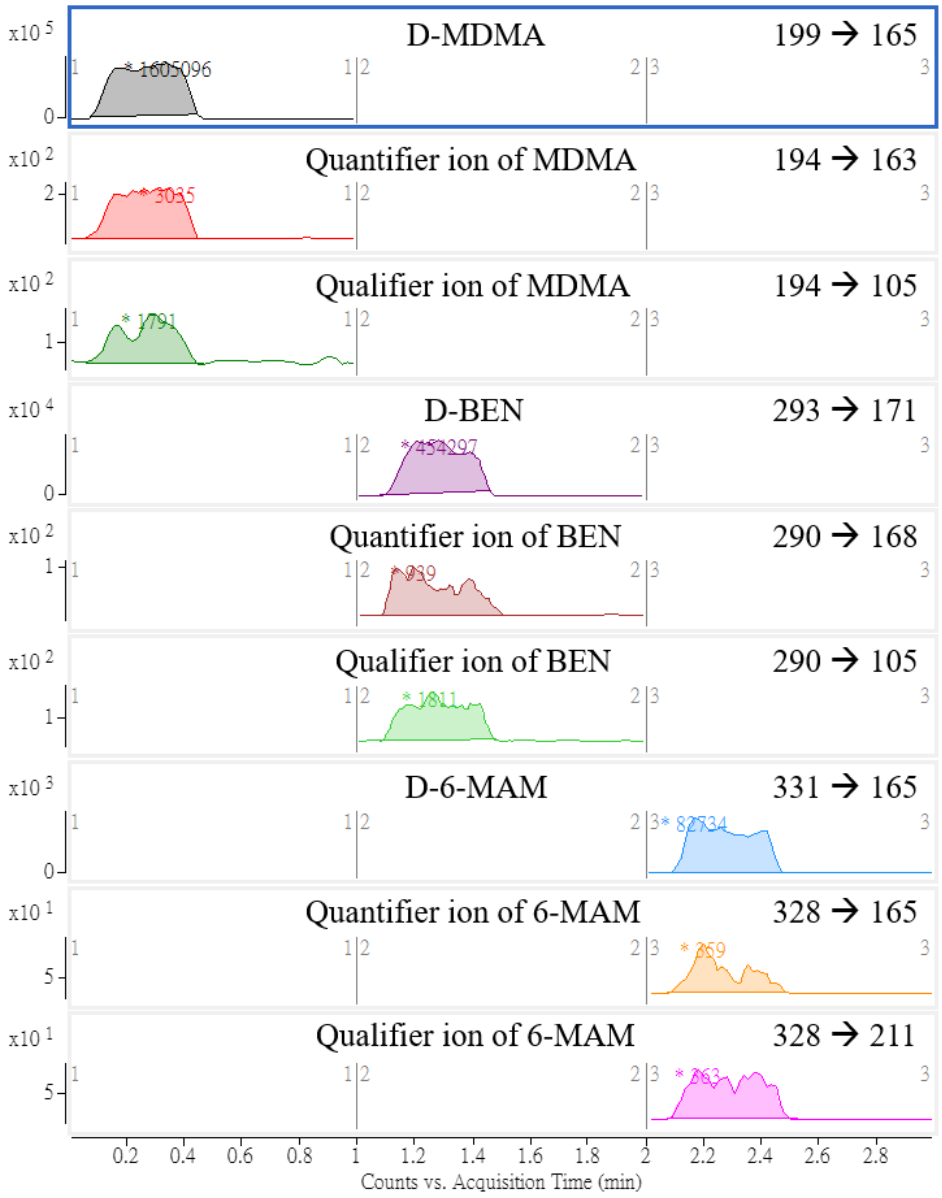


Figure 3-15. MRM signals for the detection of MDMA, BEN and 6-MAM in Medichem Basis-line U sample using SPME-ESI-MS.

Twenty-nine different drugs-of-abuse were contained in the samples of Medichem WDT confirm U -25% and +25%. The concentrations of each drugs-of-abuse varied, which were 25% lower and 25% higher than the EWDTS cut-off levels of that abuse-of-abuse. The Medichem samples were used to investigate whether the established method could detect the drugs-of-abuse according to the EWDTS requirements. Respective results for analyzing the Medichem WDT confirm U +25% sample are shown in Figure 3-16. The targeted analytes that were present in both of the Medichem WDT confirm U -25% and +25% samples with the concentrations higher than the LODs of SPME-ESI-MS which could give positive results in the analysis, except for the detection of THC-COOH. Similarly, for the analytes that should give negative results, the SPME-ESI-MS analysis also gave negative results. The results for the quantitation of targeted analytes in the samples are listed in Table 3-9. The results were within reasonable range except serious under-estimation was observed for the quantitation of morphine, which the accuracy was less than 50% for both samples. Overall, no false positive result was obtained for the analysis of all analytes. False negative results were obtained only for the detection of THC-COOH. In addition, poor accuracy was observed for the quantitation of morphine. Such abnormal results may relate to the presence of other drugs in those two complex samples.

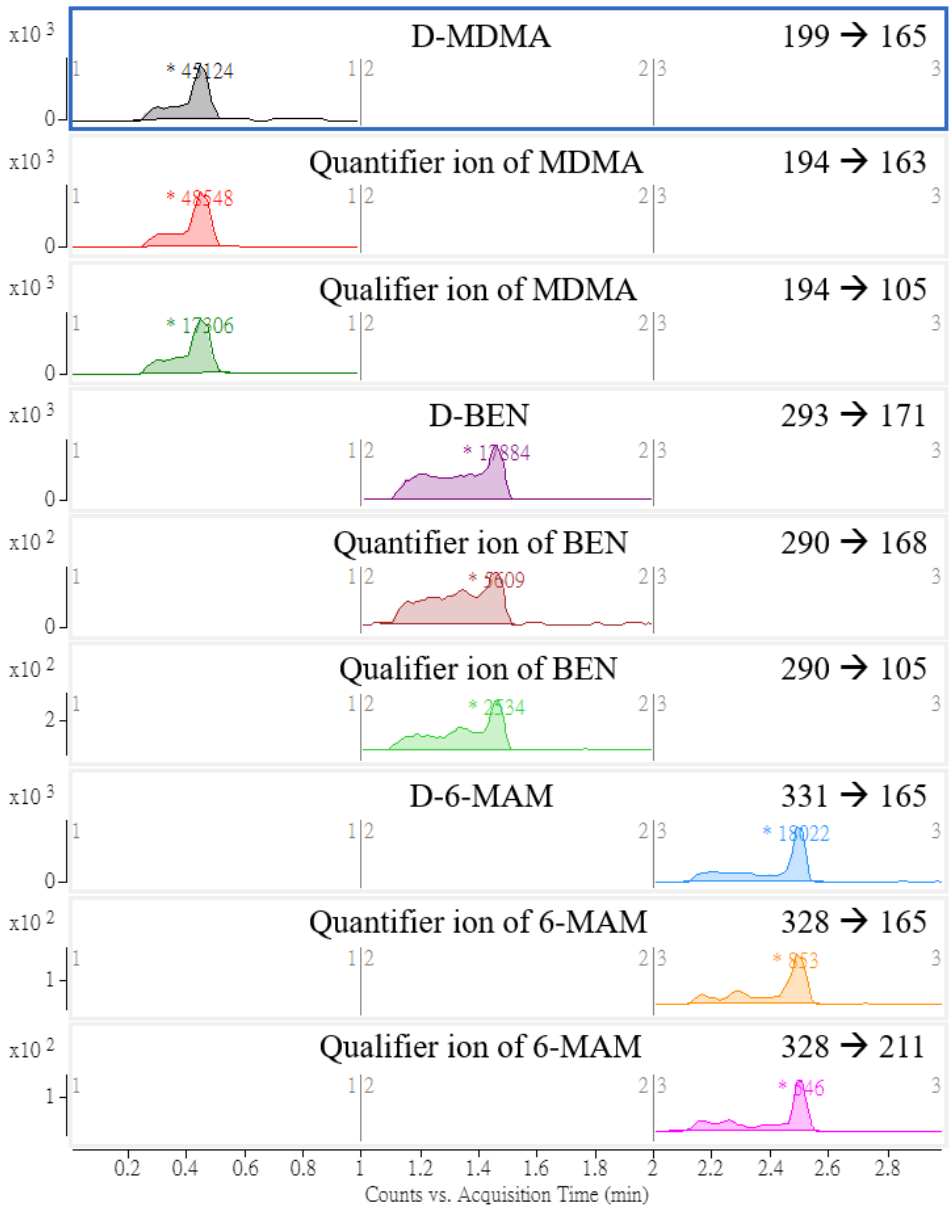


Figure 3-16. MRM signals for the detection of MDMA, BEN and 6-MAM in Medichem WDT confirm U +25% sample using SPME-ESI-MS.

Table 3-9. Results of quantitation for the targeted analytes in the Medichem samples using SPME-ESI-MS.

Compound	Medichem WDT confirm U -25%			Medichem WDT confirm U +25%		
	Actual conc. (ng/mL)	Measured conc. (ng/mL)	Accuracy (%)	Actual conc. (ng/mL)	Measured conc. (ng/mL)	Accuracy (%)
KET	N.D.	N.D.	---	N.D.	N.D.	---
Nor-K	N.D.	N.D.	---	N.D.	N.D.	---
MA	151.3	189.6	125.3	251.3	278.9	115.5
MDMA	143.5	111.2	77.5	241.5	182.0	72.4
COC	N.D.	N.D.	---	N.D.	N.D.	---
BEN	114.7	86.2	75.2	189.2	149.7	79.1
HER	N.D.	N.D.	---	N.D.	N.D.	---
6-MAM	7.9	8.0	101.0	11.9	10.0	84.3
MOR	225.9	107.5	47.6	361.2	153.3	42.4
THC	N.D.	N.D.	---	N.D.	N.D.	---
THC-COOH	10	N.D.	---	17.2	N.D.	---

N.D. = Not detectable (i.e. lower than the LODs)

In the previous studies, both morphine and THC-COOH could be detected at low concentrations when only those drugs and their metabolites were presented in the samples. There are two possible reasons to explain the poor performance for the analysis of morphine and THC-COOH in the Medichem samples. Either the targeted analytes were not able to be extracted by C18 SPME tip or the targeted analytes were poorly ionized, when multiple drugs were presented in the samples. A mass spectrum for analyzing mixture of targeted drugs with the same concentration using conventional ESI-MS is shown in Figure 3-17. It is observed that the order of the ionization efficiency of targeted analytes was: COC > KET ≈ BEN ≈ MA ≈ MDMA > 6-MAM ≈ HER ≈ Nor-K > MOR when all the targeted analytes were ionized together. The signal of THC-COOH was only observed in negative ionization mode. The results indicated that the ion suppression of the weakly ionized analytes by the strongly ionized analytes was possible when they were presented in the same sample. The poor analytical results for the analysis of Medichem samples may due to such ion suppression which reduced the signals of MOR and THC-COOH using SPME-ESI-MS.

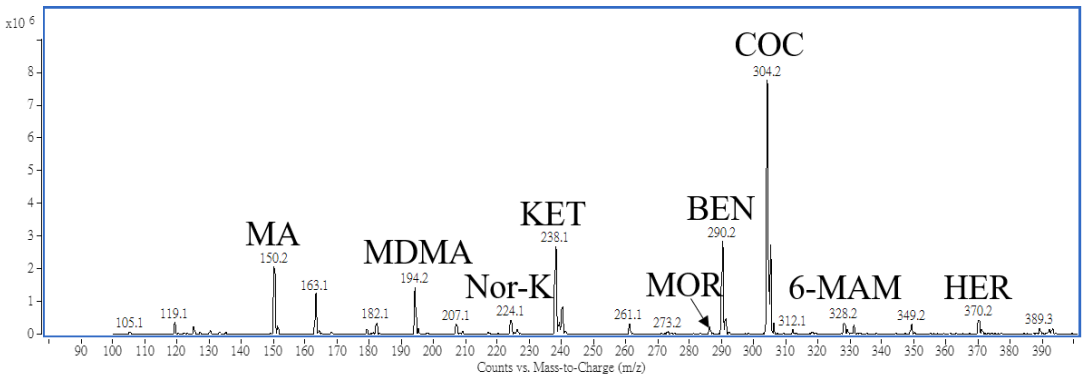


Figure 3-17. The mass spectrum for analyzing a mixture of 2000 ng/mL targeted analytes in MeOH using conventional ESI-MS.

To further investigate the effect on the extraction or ionization of targeted analytes in the samples that contained multiple drugs, the Medichem samples were extracted by C18 SPME tip and eluted in organic solvent for LC-MS analysis. The LC-MRM results for the analysis of MOR and THC-COOH in Medichem WDT confirm U +25% sample using C18 SPME tip for the extraction are shown in Figure 3-18. Signals for the detection of THC-COOH could be observed which indicated C18 SPME tip could be used to extract THC-COOH even when multiple analytes were presented in the sample. The poor ionization should be the reason for the poor analytical performance using SPME-ESI-MS for the analysis of Medichem samples.

The results for the quantitation of targeted analytes in Medichem samples using LC-MS are showed in Table 3-10. The measured amount of all the analytes generally increased when compared with the results obtained by SPME-ESI-MS. The detected amount of morphine significantly increased and THC-COOH became detectable, which again indicated the signal suppression of the weakly ionized analytes analyzed by SPME-ESI-MS may cause under estimation of weakly ionized analytes when multiple targeted analytes were presented in the samples. It is noticed that the ion suppression may not be the only reason for explaining the poor analytical performance of MOR. The 6-MAM in the Medichem samples were at a relative low concentration and its ionization efficiency was also relatively low but it could still give accurate analytical results using SPME-ESI-MS. The poor extraction efficiency of morphine could also contribute to its poor analytical performance.

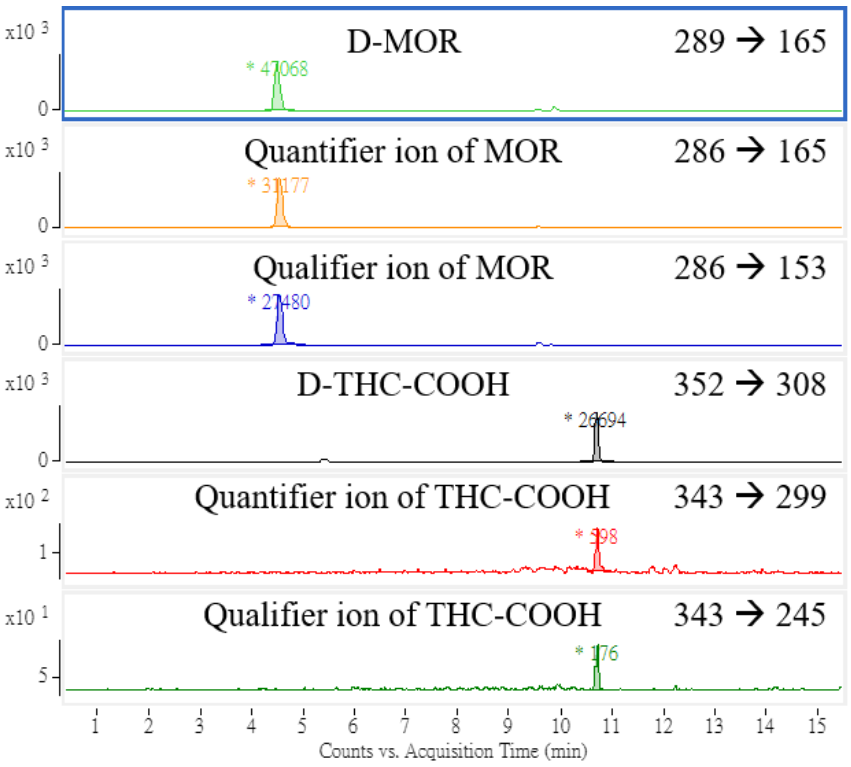


Figure 3-18. The LC-MRM results for the detection and quantitation of MOR and THC-COOH in Medichem WDT confirm U +25% sample using C18 SPME tip for the extraction. Negative ionization mode was used for the analysis of THC-COOH.

Table 3-10. Results of quantitation for the targeted analytes in the Medichem samples using LC-MS.

Compound	Medichem WDT confirm U -25%			Medichem WDT confirm U +25%		
	Actual conc. (ng/mL)	Measured conc. (ng/mL)	Accuracy (%)	Actual conc. (ng/mL)	Measured conc. (ng/mL)	Accuracy (%)
KET	N.D.	N.D.	---	N.D.	N.D.	---
Nor-K	N.D.	N.D.	---	N.D.	N.D.	---
MA	151.3	199.7	132.0	251.3	189.6	75.4
MDMA	143.5	127.4	88.8	241.5	314.8	130.4
COC	N.D.	N.D.	---	N.D.	N.D.	---
BEN	114.7	124.6	108.7	189.2	151.9	80.3
HER	N.D.	N.D.	---	N.D.	N.D.	---
6-MAM	7.9	11.5	146.0	11.9	9.4	78.7
MOR	225.9	144.1	63.8	361.2	233.2	64.6
THC	N.D.	N.D.	---	N.D.	N.D.	---
THC-COOH	10	N.D.	---	17.2	27.6	160.2

N.D. = Not detectable (i.e. lower than the LODs)

The concentrations of targeted analytes in the Medichem samples were very different between different analytes. The poor analytical results of morphine and THC-COOH may also due to the great concentration difference between different targeted analytes. A control sample which contained all targeted analytes at the same concentration was used to investigate whether the poor analytical performance for morphine and THC-COOH in the Medichem samples were affected by the relatively high concentration of other drugs. The quantitation results obtained by SPME-ESI-MS and LC-MS are listed in Table 3-11. The results obtained from SPME-ESI-MS were generally consistent with the results obtained from LC-MS. It indicated that even multiple analytes were presented in the sample, if the weakly ionized analytes were at high and at the same concentration as the strongly ionized analytes, the ion suppression effect may not affect the analytical performance a lot.

It is also observed that the order of the signal intensities of targeted analytes of the same concentration were different from that of conventional ESI. The order of signals obtained by SPME-ESI-MS was COC > KET ≈ MA > Nor-K ≈ MDMA > HER ≈ 6-MAM > BEN > MOR > THC-COOH as shown in Figure 3-19. The order of Nor-K became as high as MDMA and the order of BEN dropped. The signal of MA also

became stronger than MDMA. The changes in the order of signal intensities were because the signals obtained by SPME-ESI-MS were not only controlled by the ionization efficiency of the analytes but also controlled by the extraction efficiency. Nor-K and MA may have higher extraction efficiency and BEN may have lower extraction efficiency when they are extracted by C18 SPME tip, which resulted in the increased and decrease of the signal intensities of SPME-ESI-MS when compared with conventional ESI. MOR and THC or THC-COOH gave both poor extraction efficiency and poor ionization efficiency in SPME-ESI-MS thus it gave the worst analytical performance especially when there were other analytes presented in the samples.

Table 3-11. Results of quantitation for the targeted analytes in the control sample using SPME-ESI-MS and LC-MS.

Compound	Spiked conc. (ng/mL)	SPME-ESI-MS		LC-MS	
		Measured conc. (ng/mL)	Accuracy (%)	Measured conc. (ng/mL)	Accuracy (%)
KET	909.1	867.2	95.4	926.0	101.9
Nor-K		801.5	88.2	747.2	82.2
MA		1241.1	136.5	1262.9	138.9
MDMA		753.7	82.9	775.5	85.3
COC		1291.3	142.0	1200.0	132.0
BEN		1223.9	134.6	1186.9	130.1
HER		653.6	71.9	578.1	63.6
6-MAM		961.4	105.7	731.1	80.4
MOR		596.7	65.6	624.4	68.7
THC-COOH		875.4	96.3	1197.5	131.7

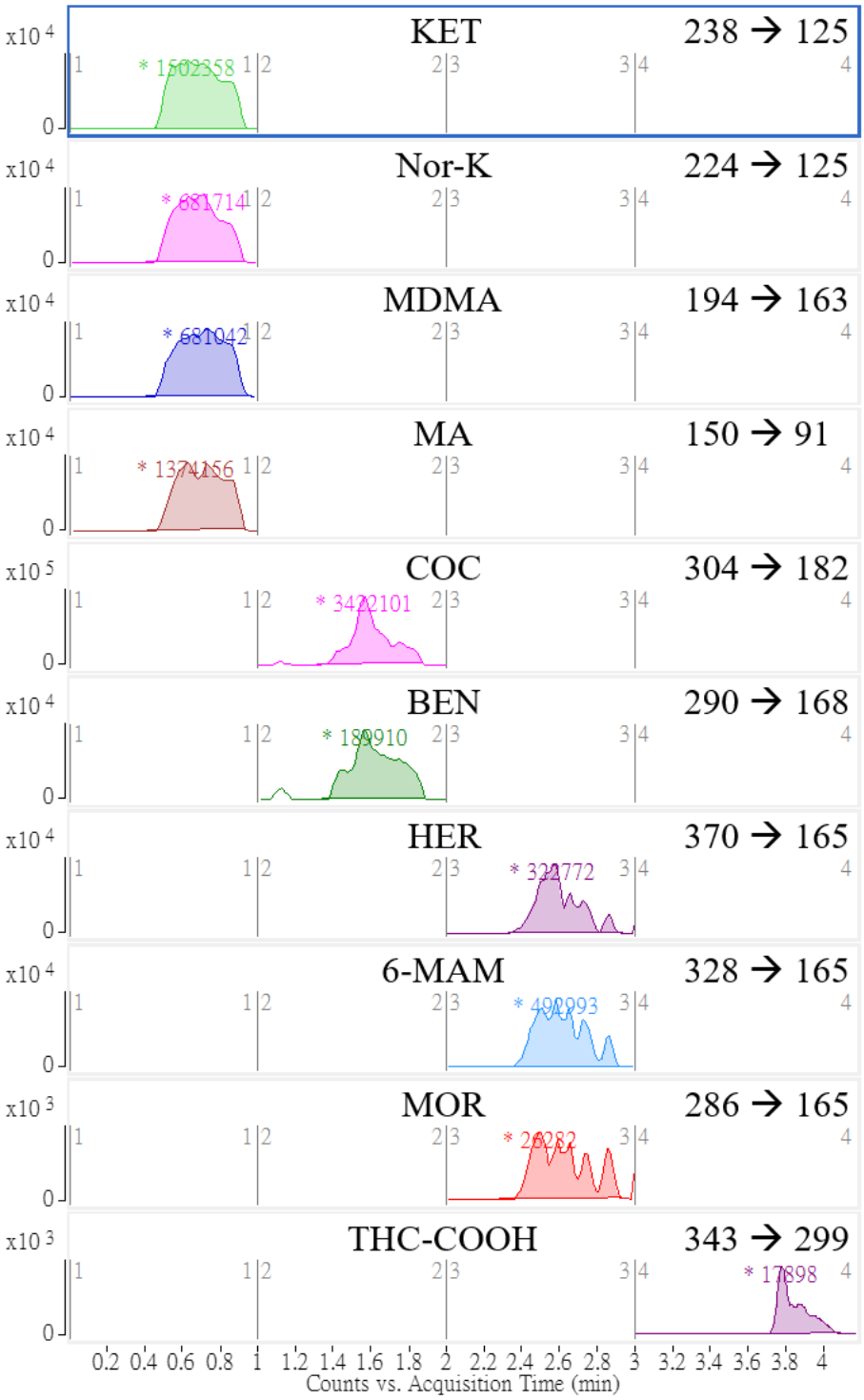


Figure 3-19. The MRM results for the detection of a mixture of 900 ng/mL targeted analytes using SPME-ESI-MS. Only the quantifier ion channels were shown.

3.4 Conclusion

The application of SPEM-ESI-MS for sensitive detection and quantitation of drugs-of-abuse in urine and oral fluid were investigated in this study. The sample preparation of SPME could be finished within reasonable time (5 – 10 min) while longer extraction time is recommended if the samples contained many targeted analytes. No chromatographic separation is required in this method, thus analysis of one sample after SPME could be finished within minutes. In addition, SPME-ESI-MS, compared with other ambient ionization techniques such as DART and DESI, is more compatible with the existing instrument and no major hardware modification is needed. SPME-ESI-MS showed good linearity and wide linear range for the quantitation of most of the targeted drugs-of-abuse. High accuracy and precision were also obtained for the quantitation of all targeted drugs. The LODs and LOQs of the targeted analytes could fulfill the international standards requirement for the detection of most of the targeted drugs in urine and oral fluid, except for THC in oral fluid. However, the analytical performances of some weakly ionized analytes may be reduced when high concentration of strongly ionized analytes are present in the same sample. It was probably due to the ion suppression effect which reduced the signals of the weakly ionized analytes. Introduction of correction coefficients to compensate the signal suppression of poorly

ionized compounds may produce better quantitative results. However, the matrix environment of real life samples could be much more complex than the spiked samples, more studies will be needed to investigate the feasibility of such correction coefficients. The analytes could be enriched onto the SPME tip thus provide better sensitivity for the analysis when compared with WT-ESI-MS. Some of the analytes such as nor-ketamine and methamphetamine could be extracted more effectively by C18 SPME tip thus provided stronger signal while reversed result was observed for benzoylecgonine. In general, SPME-ESI-MS is simple and sensitive enough for the analysis of drugs-of-abuse. However, further improvements for the detection of weakly ionized analytes such as morphine, THC and THC-COOH in complex samples should be done in order to handle all the possible situations in real practice. Furthermore, only spiked samples were used to establish the analytical protocol, the metabolism of drugs in-vivo could be more complex than the simulated situation. For example, morphine can be further glycosylated into morphine-3-glucuronide and morphine-6-glucuronide in human body. It may affect the actual amount of morphine detected in the real urine samples. Therefore, validation of SPME-ESI-MS method using samples collected from real drug abusers could be important before the actual application.

Chapter 4: Rapid authentication of edible oils using MALDI-MS

4.1 Introduction

Edible oils are daily used for cooking and food preparation. Different edible oils have different nutritional values and can have very different market prices.[111] Such as unsaturated fatty acids are considered as healthier than saturated fatty acids. Replacing saturated fatty acids with unsaturated fatty acids in edible oils can reduce the risk of coronary heart disease and reduce the level of low density lipoprotein cholesterol (bad cholesterol) which are proved by many researches.[112,113] For this reason, some of the edible oils, for example olive oil which contains high level of unsaturated fatty acids, is becoming more popular and sold at relatively high price. Counterfeit (i.e. cheaper edible oils are replaced and sold as the edible oils with higher price) and adulteration (i.e. pure edible oils are blended with other types of edible oils and sold as the pure one) of edible oils have been frequently reported.[114-118]

In addition, more public concerns about the quality and safety of edible oils have been raised with the widespread use of gutter oils in recent years. There is still no clear definition for gutter oils (or recycled cooking oils) but they are commonly considered

as the oils that are collected and refined from wasted cooking oils, kitchen wastes, sewers, wasted animal fats or restaurants fryers.[119-123] Such gutter oils are labeled as normal edible oils for sale and use. Gutter oils may contain toxic and carcinogenic compounds and its cooking performance may be reduced when compared with normal edible oils.[120] Gutter oil has become a serious problem in Mainland China and spread to Hong Kong, Taiwan and other regions.[119,121] Nevertheless, there is no widely accepted scientific method for rapid identification of gutter oils. The identification of gutter oils is currently relied on the detection of food residues markers such as capsaicinoids (marker of chilli peppers) and eugenol (marker of seasonings) and detection of toxic substance such as benzo(a)pyrene. [124,125] However, this approach cannot rule out the possibility that the tested sample is a gutter oil even such markers are absent. This is because the cooking oils can be used in different ways and these markers can also be removed from the oils after processing.[111] The marker approach thus can only be used for some special cases. Development of a more general approach for rapid authentication of edible oils and screening out the faked, adulterated and gutter oils has become highly desirable.

Gas chromatography-flame ionization detection (GC-FID) is a standard method for

edible oil authentication.[126] The fatty acid contents in the edible oil sample is determined and matched with the reference values in the standard of Codex Alimentarius. However, chemical derivatization and time-consuming column separation are required for the analysis,[127] which induced the invention of many rapid analytical techniques for edible oil authentication, which require no column separation for the analysis, including Raman spectroscopy,[128,129] laser-induced fluorescence,[130] infrared spectroscopy,[123,131,132] nucleic magnetic resonance (NMR),[118,133] ion mobility spectrometry,[134] electronic nose,[135,136] and different mass spectrometry techniques, such as direct infusion electrospray ionization,[137,138] DART[139] and MALDI-MS,[115,122,127,140-148] have been employed for analysis of edible oils. Among those techniques, MALDI-MS has distinctive features for edible oil analysis, such as high sensitivity, high impurity tolerance, little memory effect and the ability to produce simple and clear mass spectra for direct spectral comparison.

Ayorinde and co-workers firstly investigated the use of MALDI-MS for the analysis of canola, castor and olive oils.[144] Subsequent studies have proved that MALDI-MS spectra of different edible oil species, especially the triacylglyceride (TAG) region, were specific and could be used for the characterization of edible oils.[127,137,140,142,148]

TAGs are the major composition present in edible oils which are the esters of three fatty acid units and a glycerol moiety. The fatty acid contents of different edible oils can be different[149] and the synthesis of TAGs from fatty acids and glycerol-3-phosphate is controlled by specific enzyme activities,[145] thus different oils could have different TAG distributions for edible oil authentication. Recent researches also demonstrated the differentiation of geographical origins of olive oils and detection of olive oil adulteration using MALDI-MS and statistical analysis.[150,151] Ayorinde and co-workers also calculated and compared the fatty acid contents of different edible oils based on their MALDI-MS spectra and GC-FID results, and concluded that the results obtained from the MALDI-MS and conventional methods agreed with each other.[152,153]

Apart from the analysis of pure edible oils, MALDI-MS has also been used to analyze edible oil adulterants and gutter/recycled cooking oils.[115,154-157] Mixing of different edible oils and prolonged heating of edible oils can change the TAG distribution thus edible oil adulterants and recycled cooking oils can be screened out after comparing the MALDI-MS spectra of the samples with the MALDI-MS spectra of the claimed pure edible oils. Prolonged heating of oils could cause degradation of TAGs and formation of compounds such as diacylglycerols, free fatty acids, oxidized TAGs and TAG

polymers,[158] some of the compounds can be detected by MALDI-MS thus provide extra evidence for the differentiation of gutter/recycled cooking oils from the pure edible oils.[154,155,159]

Although MALDI-MS has been extensively used to analyze different edible oils, so far there is no systematic investigation and establishment of a comprehensive MALDI spectral database of various edible oils, which is definitely useful for edible oil authentication through MALDI-MS spectra comparison. Also, the conventional MALDI-MS sample preparation method can be further simplified for rapid analysis of edible oils.

In this study, inspired by the oil-assisted sample preparation method for MALDI-MS we developed previously,[54] a simplified MALDI-MS approach for rapid analysis of edible oils has been developed. In this new approach, oil samples are directly loaded onto the MALDI plate pre-deposited with MALDI matrix, and then introduced into the mass spectrometer for MALDI-MS spectral acquisition. This newly developed direct sample loading method requires no sample extraction and no premixing of matrix and sample solutions, and thus allows high throughput analysis which is an important

consideration for analysis of edible oils due to its high analytical demand. The MALDI-MS spectra obtained by this approach is highly reproducible and the quality is also as good as conventional MALDI-MS approach.

More than a thousand of edible oil samples including more than thirty types of pure edible oils, blended edible oils and gutter/recycled cooking oils were analyzed using the new MALDI-MS approach and a comprehensive MALDI-MS spectral database was then established. The results showed that the classification and authentication of edible oils could be achieved by comparing the MALDI-MS spectra of the samples with the reference spectra in the database and by statistical analysis such as principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA), and such analysis could be finished within several minutes for one sample.

4.2 Experimental section

4.2.1 Chemicals and materials

Thirty types of edible oil products were collected from collaborators and authentic stores in Hong Kong, mainland China and Taiwan. Three bottles were collected for each commercially available oil product. Standards of olive oil, castor oil, corn oil, soybean

oil, sesame oil, coconut oil, peanut oil, linseed oil, palm oil, canola oil, cottonseed oil, sunflower seed oil, safflower oil and lard oil were purchased from Supelco and Sigma-Aldrich (St. Louis, MO, USA). The prolonged heated oil (recycled cooking oil) samples were prepared in the laboratory. The gutter oil samples were provided by Syscan Technology Holdings Limited (Wuhan, China). The complete list of the oil samples is shown in Table 4-1. All samples were sealed and stored in a dry and dark environment before analysis. MALDI matrices 2, 5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnanic acid (CHCA) and sinapinic acid (SA) were purchased from Aldrich (St. Louis, MO, USA). HPLC grade acetone and tert-butyl methyl ether (TBME) were purchased from Acros Organic (Waltham, MA, USA). HPLC grade ACN and MeOH was purchased from Anaqua Chemical Supply (Houston, TX, USA) and HPLC grade dichloromethane (DCM) was purchased from RCI Labscan (Bangkok, Thailand). Polyethylene glycol standards were purchased from Fluka (St. Louis, MO, USA). Sodium iodide (NaI) was purchased from Panreac Química (Barcelona, Spain) and sodium chloride (NaCl) was purchased from Riedel-de Haën (Seelze, Germany). Derivatization reagent, trimethylsulfonium hydroxide (TMSH, 0.25 M solution in methanol) was purchased from Acros Organic, and a mixture of 37 fatty acid methyl ester standards (FAME) was purchased from Supelco.

Table 4-1. List of edible oil samples collected.

Species	No. of product	No. of sample
Almond oil	2	6
Avocado oil	4	12
Butter	7	19
Castor oil	1	1
Camellia oil	9	25
Canola oil (low erucic acid rapeseed oil)	19*	55*
Coconut oil	5	13
Corn oil	36	72
Cottonseed oil	10	10
Fish oil	4	12
Flaxseed oil (linseed oil)	27	47
Grapeseed oil	10	28
Hazelnut oil	5	14
High oleic acid sunflower oil	1	1
Lard	7	13
Olive oil	88	208
Palm oil	2	2
Palm superolein	1	3
Peanut oil	37	71
Perilla oil	4	12
Pine nut oil	3	9
Pumpkin seed oil	5	15
Rapeseed oil	21*	47*
Rice bran oil	28	38
Safflower seed oil	8	10
Sesame oil	55	73
Soybean oil	15	31
Sunflower oil	31	69
Walnut oil	7	19
Wheat germ oil	3	9

(To be continued)

Mixed oil	32	52
Margarine	4	12
Gutter/recycled cooking oil	10	10
Total	**501**	**1018**

*Some of the rapeseed oils were found to be low erucic acid rapeseed oils in the later section.

4.2.2 Preparation of blended olive oils

One brand of pure olive oil, sunflower oil, canola oil and corn oil were selected to prepare the blended olive oils. The sunflower oil, canola oil and corn oil were added individually into the olive oil and different percentages (weight-to-weight ratios) of mixtures were prepared. The oil mixtures were mixed with vortex for 5 min and mixed upside down for another 10 min.

4.2.3 Preparation of prolonged heated oils (recycled cooking oils)

About 1.3 L of soybean oil (Ping Kee Hong, Hong Kong) was poured into a stainless-steel pot and heated to 180 ºC using a hot plate from IKA (Staufen, Germany). The soybean oil was cooked with fresh minced pork for 52 cycles. In each cycle, about 40 g of minced pork was soaked and fried with the heated soybean oil for 3 min. The temperature of the soybean oil was finally equilibrated back to 180 ºC for 20 min. The

steps were repeated until the 52th cycle. About 1 mL of samples were collected after 8 hr (21th cycle), 16 hr (42th cycle) and 20 hr (52th cycle) cooking. Another set of heated soybean oil samples were prepared using the same experimental procedures but without adding any food.

4.2.4 MALDI-MS sample preparation

Direct sample loading method

Aliquots of 0.5 μL DHB solution (100 mg/mL DHB in acetone) were applied onto each sample spot on the MALDI sample plate and allowed to air dry. Thin layers of each oil sample were applied onto the pre-deposited matrix layers by cotton tips. The MALDI sample plate was loaded into the MALDI-MS equipment for MALDI-MS analysis. The protocol is illustrated in Figure 4-1.

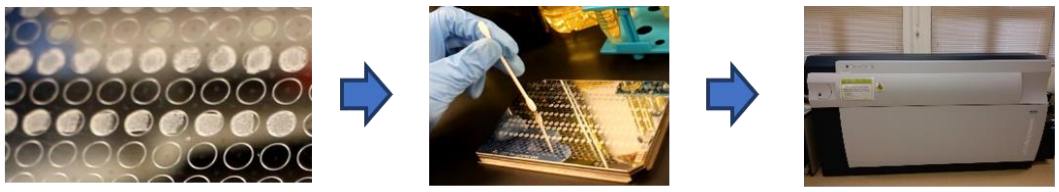


Figure 4-1. Direct loading of edible oil sample on the MALDI sample plate.

Dried droplet method

1 mg of edible oil sample was dissolved in 1 mL of DCM and was mixed with DHB solution (100 mg/mL in 90% MeOH) in the ratio of 1:2 (v/v). 0.5 µL of each sample solution was applied onto the MALDI sample plate and allowed to air dry.

Two-layer method

Aliquots of 0.5 µL DHB solution (100 mg/mL DHB in 90% MeOH) were applied onto the sample spots on the MALDI sample plate and allowed to air dry. Aliquots of 0.5 µL sample solution (prepared as same as the dried droplet method) were applied onto the DHB layer and air dried.

4.2.5 MALDI-MS analysis

An ultrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker, Billerica, MA, USA) equipped with a 355 nm smartbeam-II laser for the MALDI-MS analysis. The mass spectrometer was operated at a frequency of 2000 Hz and in positive and reflectron mode. The matrix suppression cut-off was set to *m/z* 500 and the spectra were acquired with an *m/z* range of 500-2000 Da. The ion pulse excitation was set to 140 ns. The ion source voltage 1, ion source voltage 2, lens voltage, reflector voltage 1 and reflector

voltage 2 were set to 20 kV, 17.75 kV, 7 kV, 21.1 kV and 10.85 kV, respectively. The reflector base detector voltage was set to 2.32 kV and the sample rate was set to 5.0 GS/s. The mass spectrometer was calibrated with the PEG solution mixture (1:2:2:5 (v/v/v/v) PEG600/PEG1000/PEG2000/NaI). For each sample, signals from at least 5 random positions (1000 laser pulse per one position) on the sample spot were acquired manually. The sub-spectra were accepted if the signals in the TAGs region (typically at the region of *m/z* 570-750 or *m/z* 850-920) were observed and the absolute intensity of the top peak of the final spectra obtained by combining at least 5 positive sub-spectra reached $1x10^4$. The mass spectra were processed in flexAnalysis 1.4 program (Bruker, Billerica, MA, USA) under "centroid" peak detection algorithm, "SavitzkyGolay" peak smoothing algorithm (the width equal to 0.2 *m/z* and the cycles equal to 1) and baseline subtraction algorithm "TopHat". Each edible oil sample was analyzed in triplicate. The sodium adducted TAGs were observed in the MALDI-MS mass spectra and their identification were based on the results of the previous studies[144,148,154] and further confirmed with MS/MS analysis in this study. For the TAGs in rapeseed oil, the peak assignments were based on the theoretical calculation of fatty acid contents from the GC-FID results. The actual conformations of TAGs were neglected in the peak assignments.

A Waters Synapt-G2 Si TOF mass spectrometer equipped with MALDI ion source (Milford, MA, USA) was used for the result comparison between different instruments. The mass spectrometer was equipped with a 337 nm N_2 laser. The MALDI laser firing rate was 1000 Hz and the laser energy was set to 380. The flight tube voltage and the reflectron voltage were 10 kV and 3.8 kV respectively. The mass analyzer was operated at "sensitivity" mode.

4.2.6 GC-FID analysis

The procedures for the GC-FID analysis were followed the instructions of ISO 12966-3.[126] About 10 mg of the sample was and dissolved in 500 μL of TBME in a 2 mL glass vial. The derivatizing reagent, 250 μL of TMSH solution was then added to the glass vial and the solution mixture was shaken vigorously for 30 s. One microliter of each sample was injected into an Agilent 7890B gas chromatograph system (Santa Clara, CA, USA) using an Agilent G4513A auto-sampler with 1:100 split ratio. The GC system was equipped with an Agilent DB-WAX column (30 m, 0.25 mm i.d., 0.5 μm film thickness) and a flame ionization detector. The injector temperature and detector temperature were 260 ºC. The initial oven temperature was 80 ºC and held for 2 min. The temperature was then increased from 80 ºC to 160 ºC at 20 ºC/min and held for 2

min and increased to 240 ºC at 4ºC/min and held for 25 min. The total run time was approximately 53 min for each sample. The gas flow of the nitrogen carrier was 1 mL/min and the flow of the FID hydrogen fuel was 30 mL/min. The results were analyzed using Chem-Station program (Agilent, Santa Clara, CA, USA) and compared with the results obtained using the standard FAME mix for identifying the peaks and determination of the fatty acid contents of the samples. No internal standard was added.

4.2.7 Identification of cyclolinopeptides in flaxseed oils

20 µL of flaxseed oil was dissolved in 200 µL DCM and further diluted 10 times with ACN. $1x10^{-4}$ M of NaCl (final concentration) was added into the sample solution before the analysis. The sample solution was then injected into a Thermo Orbitrap Fusion Lumos Tribrid mass spectrometer (Waltham, MA, USA) using a syringe pump with the solution flow rate of 5 µL/min. The mass spectrometer was operated at positive ionization mode with the spray voltage at 3500 V. The sheath gas flow was set to 15 and the sheath gas temperature was 250 ºC. The ion transfer tube temperature and vaporizer temperature was 300 ºC and 35 ºC, respectively. The interested ions were searched using Metlin online database with the mass tolerance set to 5 ppm.

4.2.8 Statistical analysis

The data obtained from the mass spectra of the same edible oil products were averaged. The normalized intensities of monoisotopic peaks of TAGs and cyclolinopeptides in the spectra (absolute intensity of the peak observed / total absolute intensity of all the peaks observed in the mass spectrum) were input into the statistics software (Umetrics Simca 14.0, Andover, MA, USA) for PCA and PLS-DA. The PCA and PLS-DA models were established using "simple mode" and "Autofit" (automatic cross-validation) with the default settings. A training set including 198 pure edible oil products with different brands, collected from different locations and possessed different TAG patterns, were used to establish PLS-DA models. One "grouping" model using the groups as the dummy Y variables and seven sub-models with the species as the dummy Y variables were established. The remaining products were used as testing set to test the performance of the established PLS-DA models.

4.3 Results and discussion

4.3.1 Optimization of MALDI-MS protocol for direct analysis of edible oils

In this study, four parameters including sample preparation methods, matrix selection, solvent selection and addition of additives have been tested to establish a protocol that

can produce good quality and reproducible spectra.

Sample preparation methods and solvent selection

Sample loading methods including dried-droplet, two-layer method and direct sample loading method were tried. Dried droplet method is the routine method for the preparation of MALDI-MS sample.[148] In this method, the matrix solution and sample solution are mixed together and co-crystalized onto the MALDI plate. Two-layer method is modified from dried droplet method, for enhancing the performance of peptide and protein analysis using MALDI-MS.[160] A thin layer of matrix layer was firstly applied onto the MALDI plate, followed by the addition of sample solution onto the matrix layer. The matrix and the sample were finally re-crystallized on the MADLI plate. Direct sample loading method was developed specially for this study.[122] Matrix layers were firstly prepared by applying matrix solution onto the sample spots of the MALDI plate. Acetone was selected as the solvent of the matrix solution as it can rapidly evaporate to form the matrix layers. The edible oil samples were then directly applied onto the matrix layers without solvent dissolution and co-crystallization with matrix.

The results of analyzing the same olive oil using different MALDI sample preparation methods are shown in Figure 4.2. The TAG signals (*m/z* 850 – *m/z* 920) of olive oil could be generated by all the tested methods but less background signals were observed using direct sample loading method. It could be due to no solvents were used for dissolving the samples, which reduced the chance of sample contamination by the solvent and the containers. Also, as no solvent extraction was used, only the major components, which were the TAGs in the samples could be ionized effectively and thus created strong signals.

Acetone is a better solvent for preparing matrix layers in direct sample loading method as it could rapidly vaporize when compared with other solvent system such as different ratios of MeOH or ACN and water mixtures. The time for preparing matrix layers were thus greatly reduced.

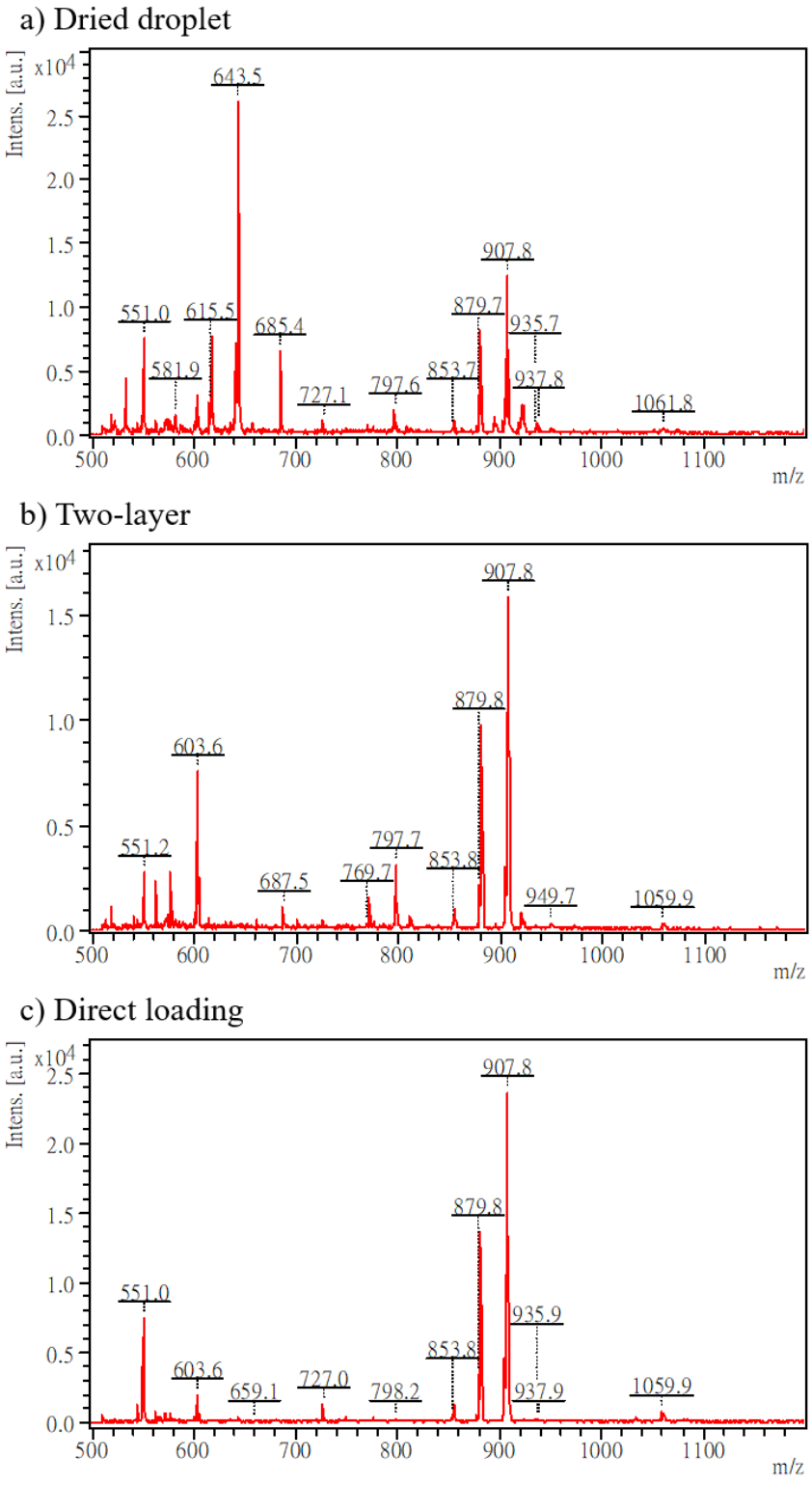


Figure 4-2. MALDI-MS spectra of an olive oil sample obtained by (a) dried-droplet method, (b) two-layer method and (c) direct sample loading method

Matrix selection

Matrix selection plays an important role in MALDI-MS analysis. Different matrices are applied in analyzing different compounds. There are still no rules for the matrix selection and trial and error is commonly applied on signal optimization.[53] Three commonly used matrixes including CHCA, DHB and SA were tested. The MALDI-MS spectra obtained are shown in Figure 4-3. No signals corresponding to the edible oil sample could be obtained by using SA as the matrix. Clear TAG signals were observed for both CHCA and DHB but less noise signals were obtained when DHB was selected as the matrix. Different concentrations of the DHB solution were also tried to create the matrix layer. No significant effects were observed with the increase of concentrations of the matrix solution. Therefore, 100 mg/mL DHB in acetone was selected to use in this study.

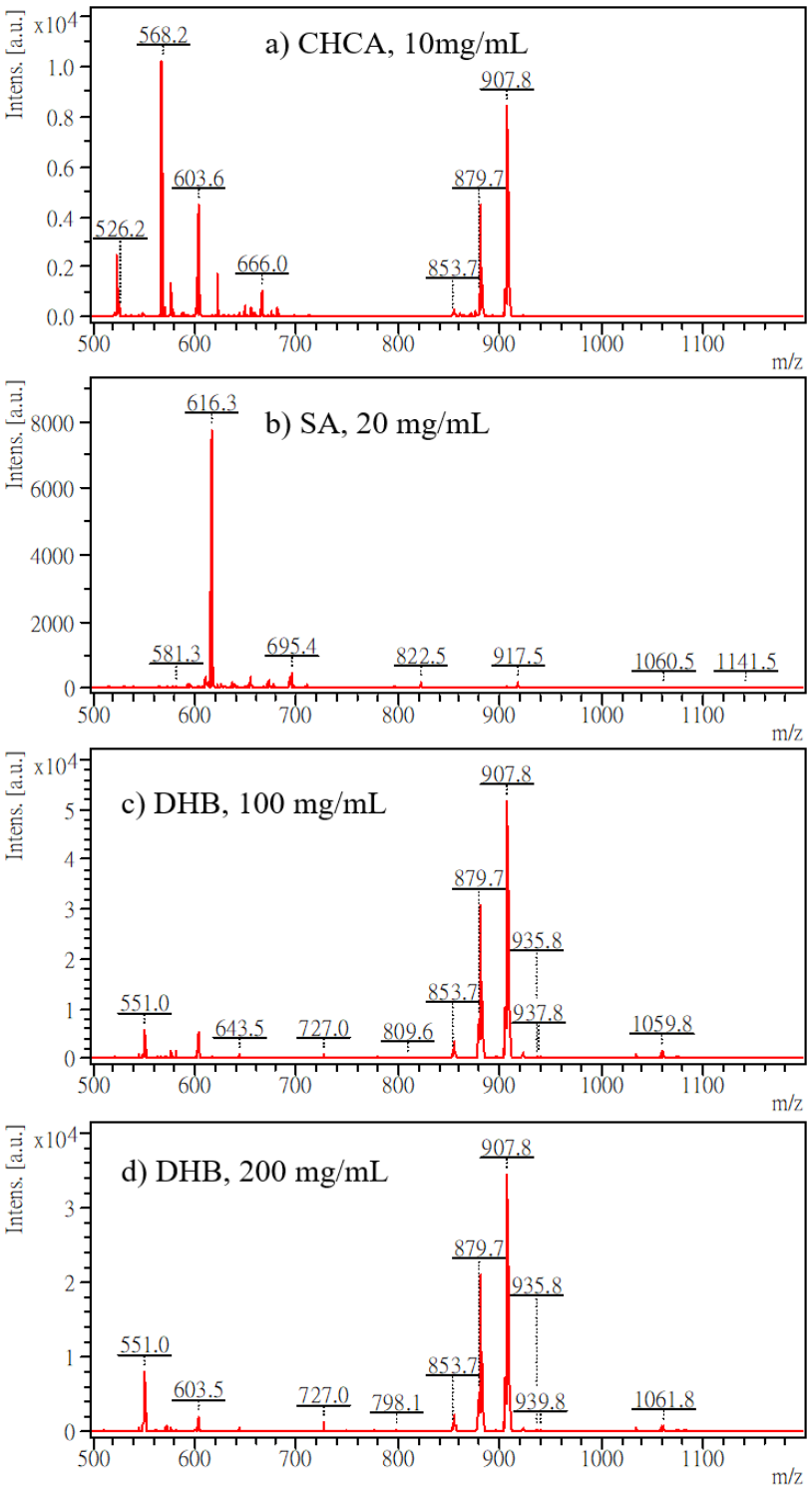


Figure 4-3. MALDI-MS spectra of an olive oil sample obtained using (a) 10 mg/mL CHCA, (b) 20 mg/mL SA, (c) 100 mg/ml DHB and (d) 200 mg/ml DHB as matrix.

Addition of additives

The TAG signals obtained in this study were as the form of sodium adducted ions. Addition of sodium iodide solution into the matrix solution provided extra source of sodium ions and signals might be enhanced. However, as shown in Figure 4-4, the addition of sodium ions in the matrix solution gave no significant improvement in the results obtained. Clear TAG signals could be obtained even no extra sodium ions were provided and the increase in sodium ion concentrations did not improve the S/N of the spectra. Even worst, when the concentration of sodium ions in the solution became very high, such as 0.1 M (final concentration), significant noise signals were generated. Therefore, addition of sodium ions was not considered in this study. It is common that sodium adduct ions could be formed even no extra sodium ions were added on purpose. The free sodium ions presented in the matrices, solvents, containers and samples are generally enough for the formation of sodium adducted ions.[161]

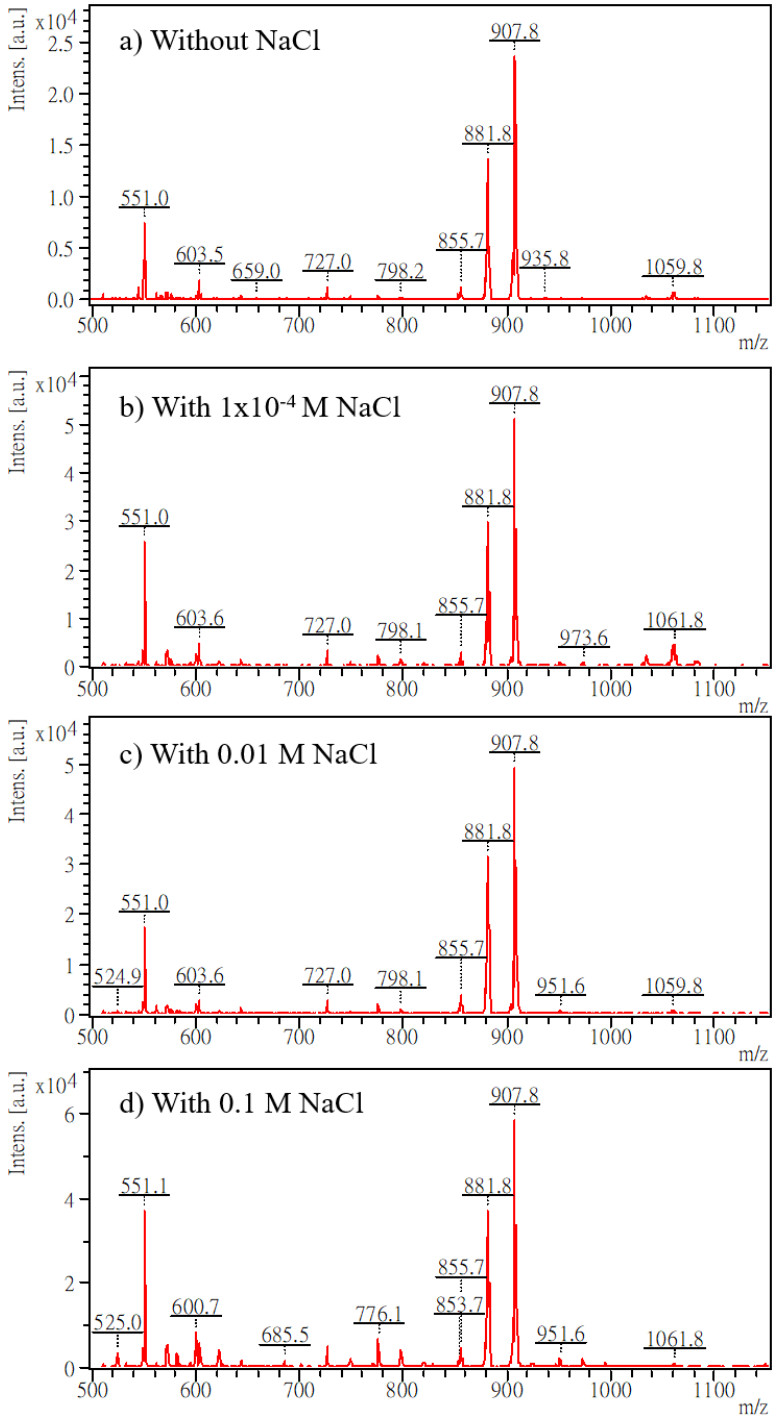


Figure 4-4. MALDI-MS spectra of an olive oil sample obtained with the addition of (a) 0 M, (b) $1x10^{-4}$ M, (c) 0.01 M and (d) 0.1 M sodium chloride solution into the matrix solution (final concentration).

4.3.2 A simple protocol for MALDI-MS analysis of edible oils

After the optimization of MALDI-MS protocol for direct analysis of edible oils using MALDI-MS, a simple protocol for MALDI-MS analysis of edible oils was established. This protocol involved direct loading of oil samples onto the MALDI plate spots pre-deposited with the DHB matrix layers. The dried matrix layers were stable and no significant differences of the resulting spectra were found even after a long time, such as one week. Therefore, the matrix on the plate can be prepared before the analysis, and when oils samples are submitted for MALDI-MS analysis, the oil samples could be directly loaded to the plate. The entire process could be finished within few seconds for each sample. Compared to conventional sample preparation methods that required sample extraction and mixing of sample and matrix, the present protocol is much simpler and easier, and allows high throughput analysis.

The reproducibility of the present method was also very high. The mass spectra of the same olive oil sample obtained within the same day and at another day are shown in Figure 4-5. The characteristic TAG patterns of the olive sample were very similar for both the results. The inter-day and intra-day (3 data from the same day, 1 data from another day and 1 data from another week) precision of the relative intensities of the

TAGs of the olive oil is listed in Table 4-2. The inter-day precision of all the signals of all TAG peaks were good which the precision of all peaks was within 10%. The intra-day precision of the high abundance peaks (relative intensity > 3%) was still as good as the inter-day precision while the precision of the weak peaks significantly reduced to 12.0% - 23.3%. Overall, the characteristic spectral patterns of the same sample were highly reproducible regardless the date of the measurements. The high reproducibility of the spectral patterns obtained by the present method may due to the analytes were dissolved in oil rather than in crystal form. The analytes could be distributed more evenly and the spectra obtained for each laser irritation then could be more reproducible, leading to the highly reproducible resultant MALDI-MS spectra. Such good spectral reproducibility is critical for the establishment of spectral library and for spectral comparison.

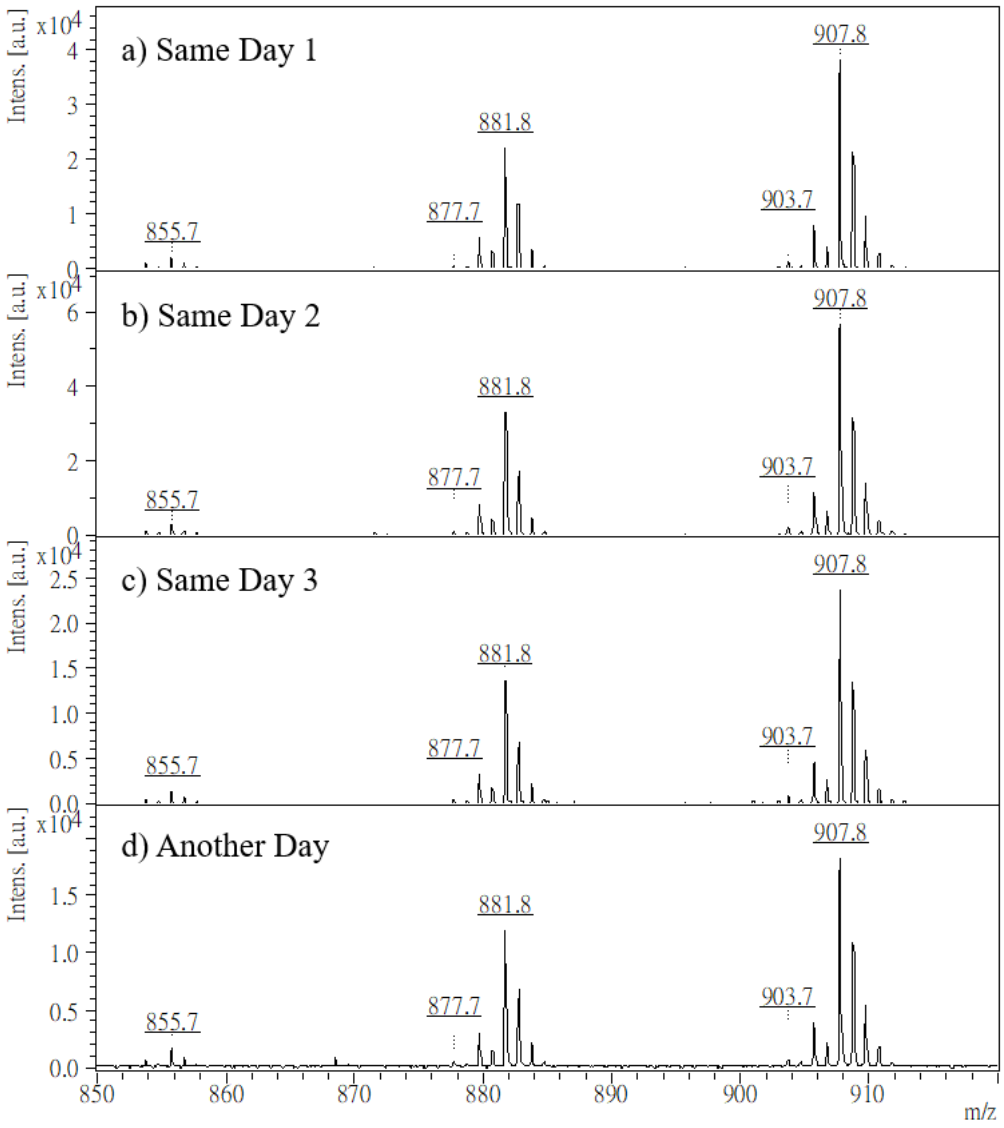


Figure 4-5. MALDI-MS spectra (TAG region) of an olive oil (a-c) analyzed within the same day and (d) analyzed at another day.

Table 4-2. The intra-day and inter-day precision of the relative intensities of TAGs of the olive oil sample.

	Intra-day (n=3)		Inter-day (n=5)	
m/z	**Mean ± S.D.**	**R.S.D. (%)**	**Mean ± S.D.**	**R.S.D. (%)**
853.7	0.99 ± 0.09	9.3	1.12 ± 0.19	17.2
855.7	2.44 ± 0.12	4.8	2.89 ± 0.67	23.3
877.7	0.95 ± 0.03	3.1	1.03 ± 0.13	12.5
879.7	6.67 ± 0.10	1.4	6.71 ± 0.13	2.0
881.8	26.88 ± 0.21	0.8	27.11 ± 0.52	1.9
883.8	4.27 ± 0.23	5.4	4.52 ± 0.39	8.7
903.7	1.63 ± 0.04	2.2	1.70 ± 0.20	12.0
905.8	9.30 ± 0.21	2.3	9.27 ± 0.17	1.8
907.8	46.87 ± 0.39	0.8	45.65 ± 1.78	3.9

The developed MALDI-MS method was universal for all MALDI-MS instruments. The results obtained for the same samples using two different MALDI-MS instruments are shown in Figure 4-6. Same characteristic peaks could be observed in the spectra using both instruments. The spectral patterns were also similar to each other.

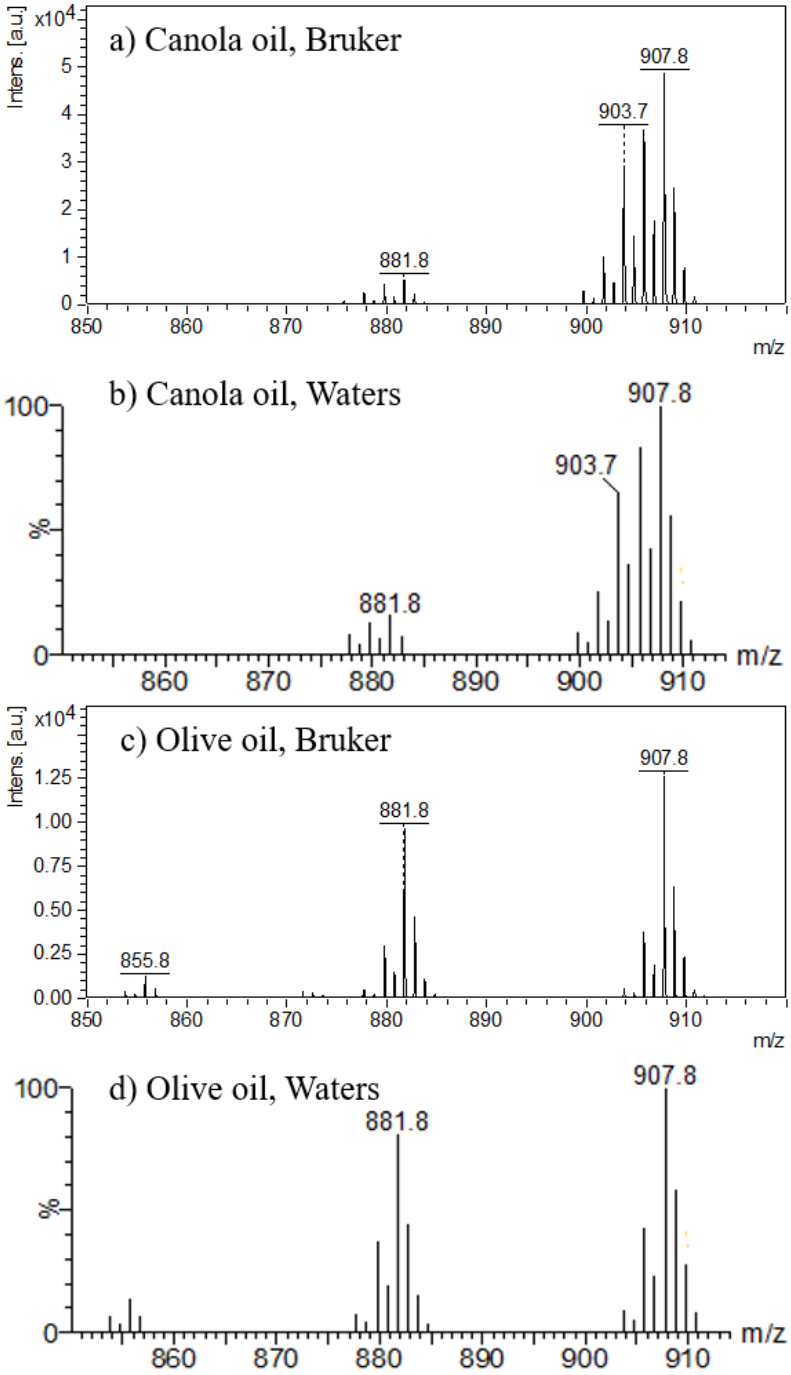


Figure 4-6. The mass spectra obtained of (a) a canola oil and (c) an olive oil using Bruker's MALDI-MS and the mass spectra obtained of the same (b) canola oil and (d) olive oil using Waters' MALDI-MS.

4.3.3 Typical MALDI-MS spectra for edible oil analysis

The peaks observed in the MADLI-MS spectra were assigned and analyzed. Typical mass spectra of a canola oil sample are shown in Figure 4-7. Peaks observed in the lower mass region (*m/z* 500 - 780) were the background peaks produced by the DHB matrix (see Figure 4-7a) and the diacylglycerol-like fragments ($[TAG\text{-}RCOO]^+$) of unstable protonated TAGs produced during laser desorption/ionization process.[162] However, the TAGs of some of the edible oils such as butter and coconut oil were also observed in this region. The TAG signals were detected as the sodium adduct ions in MALDI-MS as shown in Figure 4-7c. The major peaks observed were the TAGs of palmitic acid (P, 16:0), oleic acid (O, C18:1), linoleic acid (L, C18:2) and linolenic acid (Ln, C18:3). The TAG peaks in the spectra were assigned according to the fatty compositions recorded in Codex standard and some literatures.[140,148,154] It could be further confirmed by MS/MS analysis as shown in Figure 4-7d. The MS/MS spectrum of *m/z* 903.7 (LLO) showed the peaks of fragments $[LL+Na]^+$ (*m/z* 621.2) and $[LO+Na]^+$ (*m/z* 623.2) and their protonated forms. The peaks appeared at higher mass region (higher than *m/z* 920) were the oxidized TAGs (i.e. $[TAG + O]^+$ and $[TAG + OO]^+$) and TAG-fragment conjugated products.[154,159] However, characteristic TAGs of rapeseed oil and some marker peaks of flaxseed oil were also found in this region.

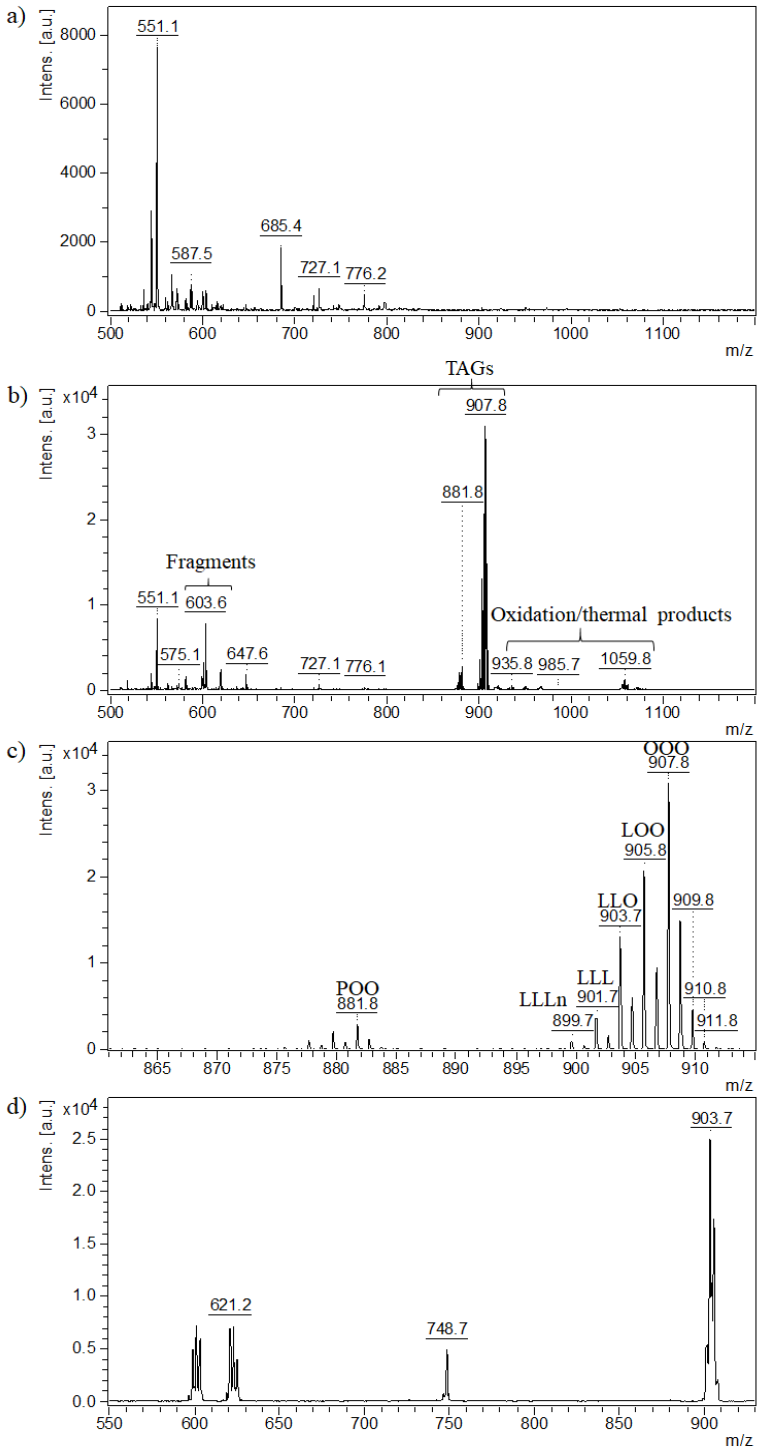


Figure 4-7. (a) Mass spectrum of blank DHB matrix. (b & c) Typical mass spectra of a canola oil sample and zoom in view of TAG region. TAGs were detected as Na^+ adducts in the spectrum. (d) MS/MS spectrum of peak *m/z* 903.7.

4.3.4 MALDI-MS spectra and spectral database of edible oils

For each oil product from the market (i.e. a specific edible oil of a specific brand), typically three bottles of edible oils were collected, which leading to over 900 pure edible oil samples (from 455 different oil products) were finally collected and analyzed in the study. The mass spectra obtained from different bottles of the same oil products were highly similar to each other, with an example of the spectra for peanut oil as shown in Figure 4-8. However, for the same kind of edible oils of different brands, variations were observed for the MALDI-MS spectral patterns. An example for such variation is shown in Figure 4-9. Similar TAG patterns were detected for the peanut oil samples from three different brands, but their intensity ratios varied, e.g., different intensity ratios between *m/z* 903.7 (LLO), *m/z* 905.8 (LOO) and *m/z* 907.8 (OOO) obtained from the different brands of samples. This was consistent with the varied range of C18:1 (35.0 - 69.0%) and C18:2 (12.0 - 43.0%) contents of peanut oils as described in the Codex standards.[149] Variations of TAG patterns were also observed for other edible oils but at different extents, such as olive oil (see Figure 4-10). Some of the edible oils such as peanut oil and olive oil could have larger variations, while some of the edible oils such as canola oil could have smaller variations. The variation could be due to the variation in geographical location, climate, soil type, maturity and genetic of seed and

edible oil processing techniques.[111,163] However, such variations within the same oil species were normally smaller than the differences between different oil species, allowing clear differentiation of different oil species as discussed in the later section.

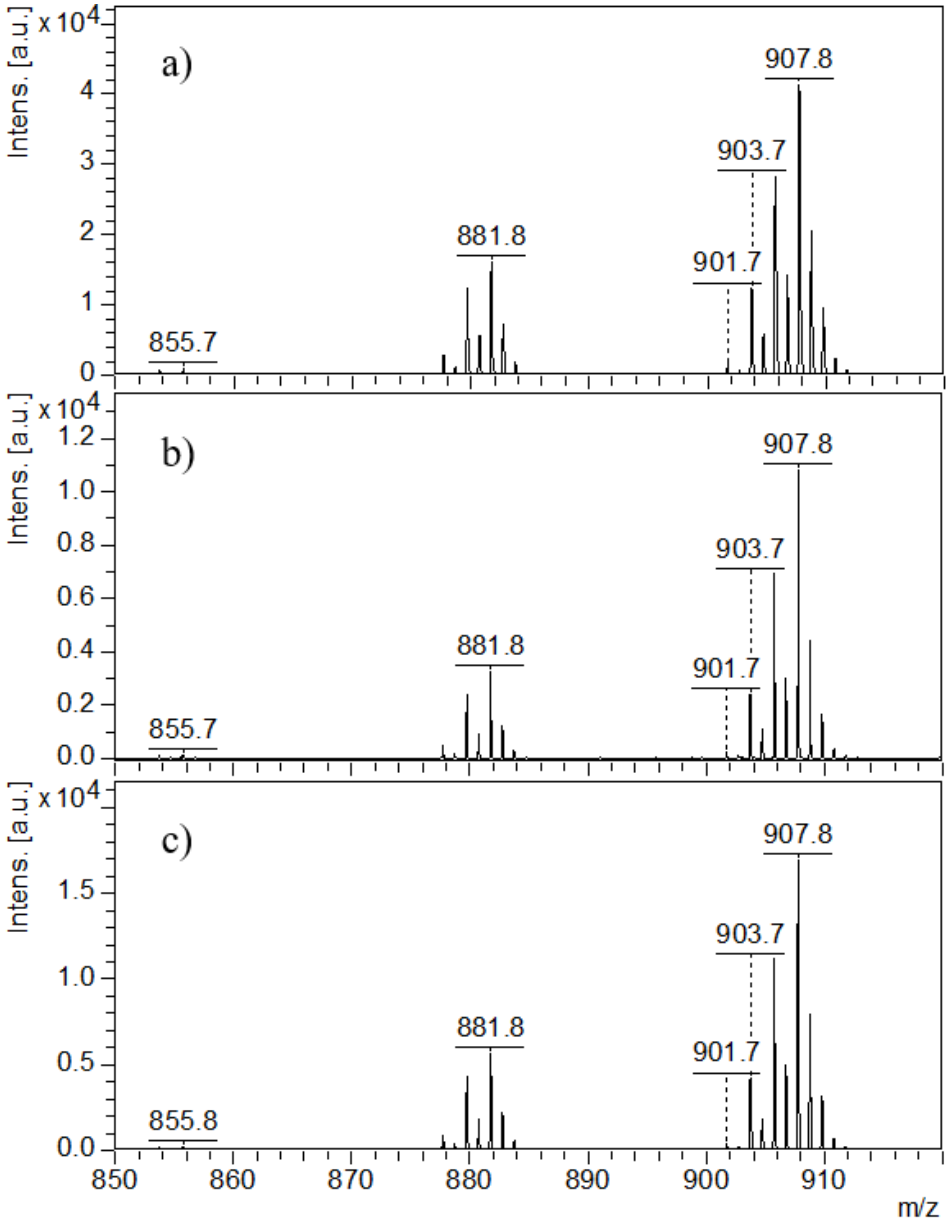


Figure 4-8. (a-c) The mass spectra showed the TAG regions of three different bottles of the same peanut oil product.

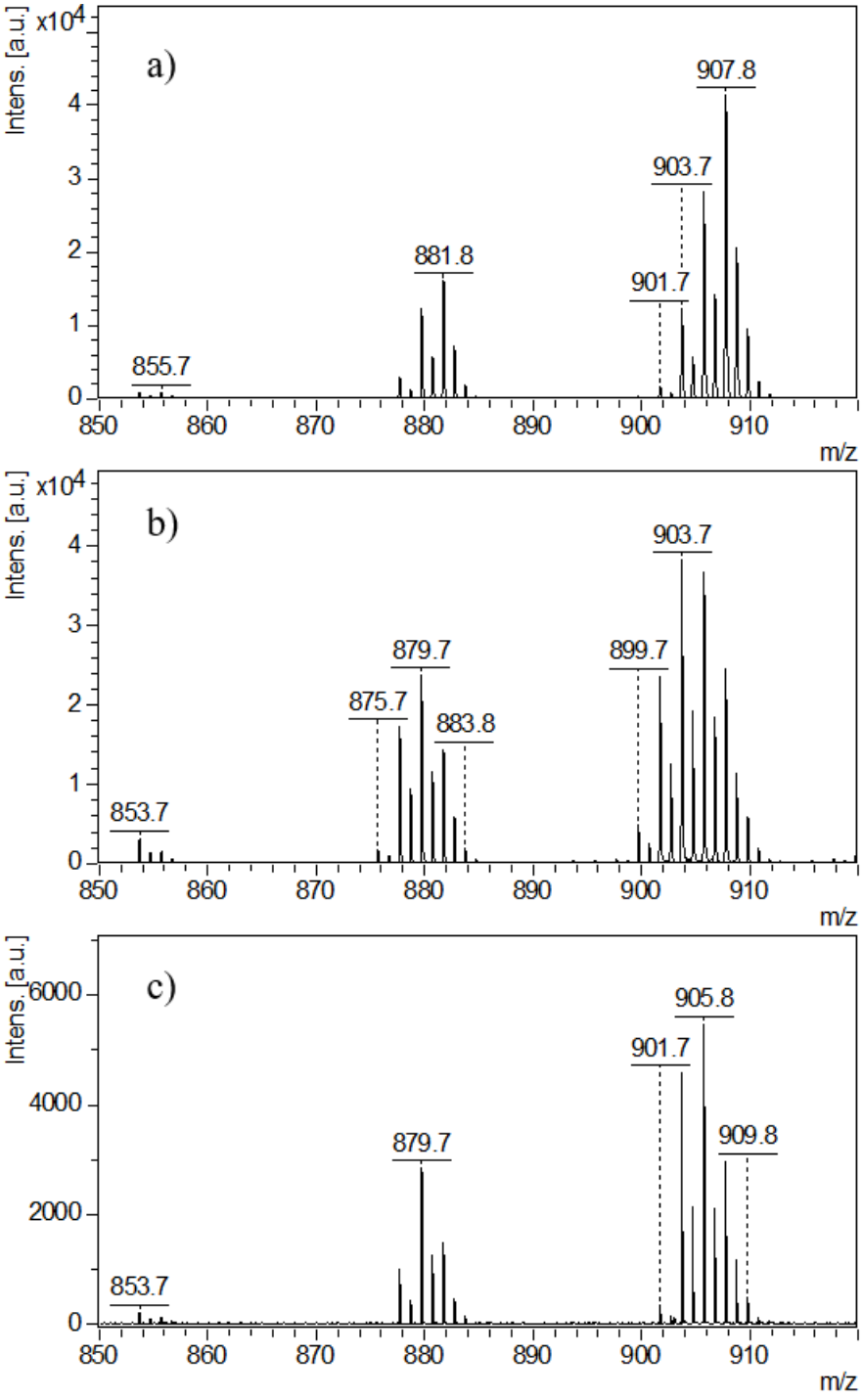


Figure 4-9. (a-c) The TAG region with different spectral patterns of three peanut oil samples collected from different brands.

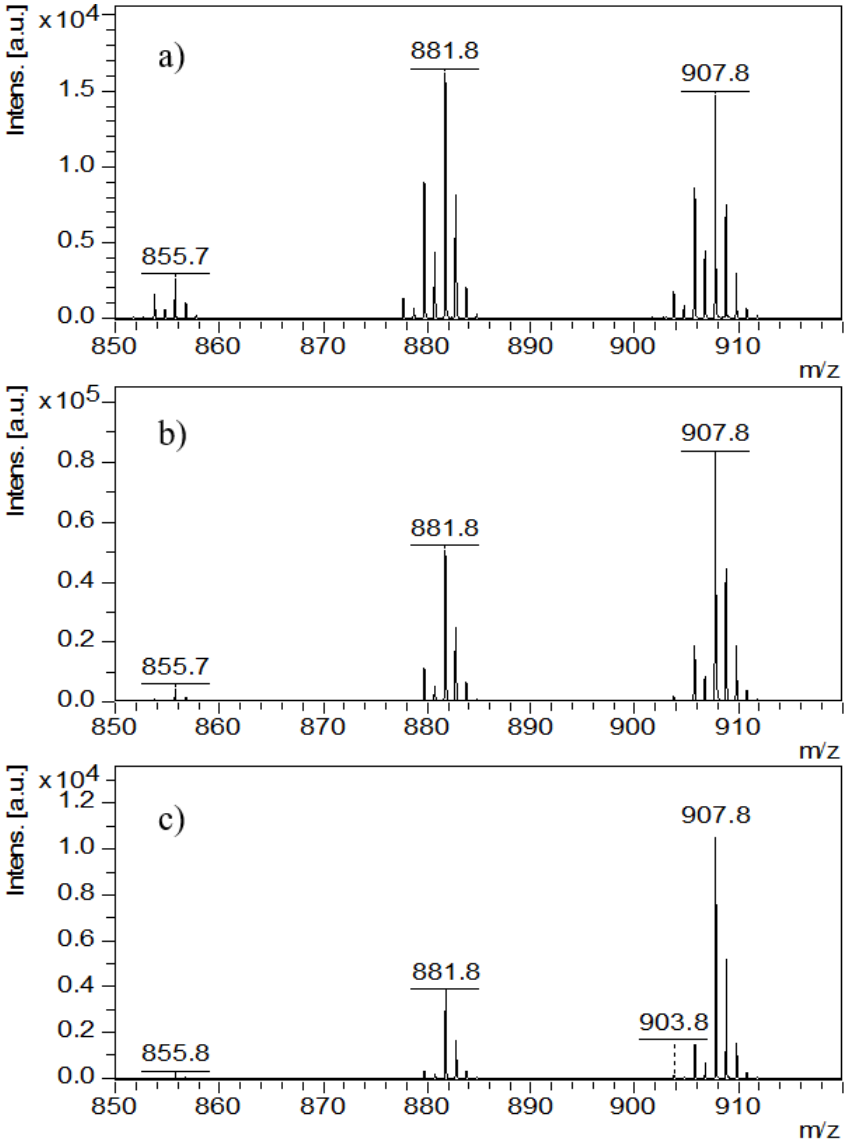


Figure 4-10. The TAG region with different spectral patterns of three olive oil samples collected from different brands.

Special consideration was given to canola oil (low erucic acid rapeseed oil) and rapeseed oil which are two closely related edible oil species. Their fatty acid contents are very similar, except that canola oil contains less than 2% erucic acid (C22:1) and lower eicosenoic acid (C20:1) content. The TAGs of C22:1 were observed in the higher mass region (*m/z* >920) of the spectra as shown in Figure 4-11. The collected rapeseed oil samples were confirmed by conventional GC-FID analysis, and it was found that within the 21 collected "rapeseed oil" products, only 6 contained more than 2% erucic acid and confirmed as rapeseed oil. As the erucic acid content in the edible oils is regulated by law at some regions, such as no more than 5% in Hong Kong,[164] it is better to classify canola oil and rapeseed oil as two different types of oils. The remaining samples thus were counted as canola oil in the later section.

More than 900 pure edible oil samples, including 30 different species of edible oils with different brands, suppliers and origins for each species, were analyzed to construct the MALDI-MS spectral database. For the samples that showed observably different MALDI-MS spectra from those of the same species, confirmatory analysis using the conventional GC-FID method for FAME was performed. The edible oil samples which failed to match the fatty acid contents as required by the Codex standards were rejected

and were not included in the database. Some edible oil samples showed high levels of oxidized/thermal products in their MALDI-MS spectra were also rejected. Finally, 11 edible oil products (13 edible oil samples) were rejected because of the above reasons. The sample information and the MALDI-MS spectra from the authenticated edible oil samples were stored in a home-built database for retrieving individual spectrum according to the oil species.

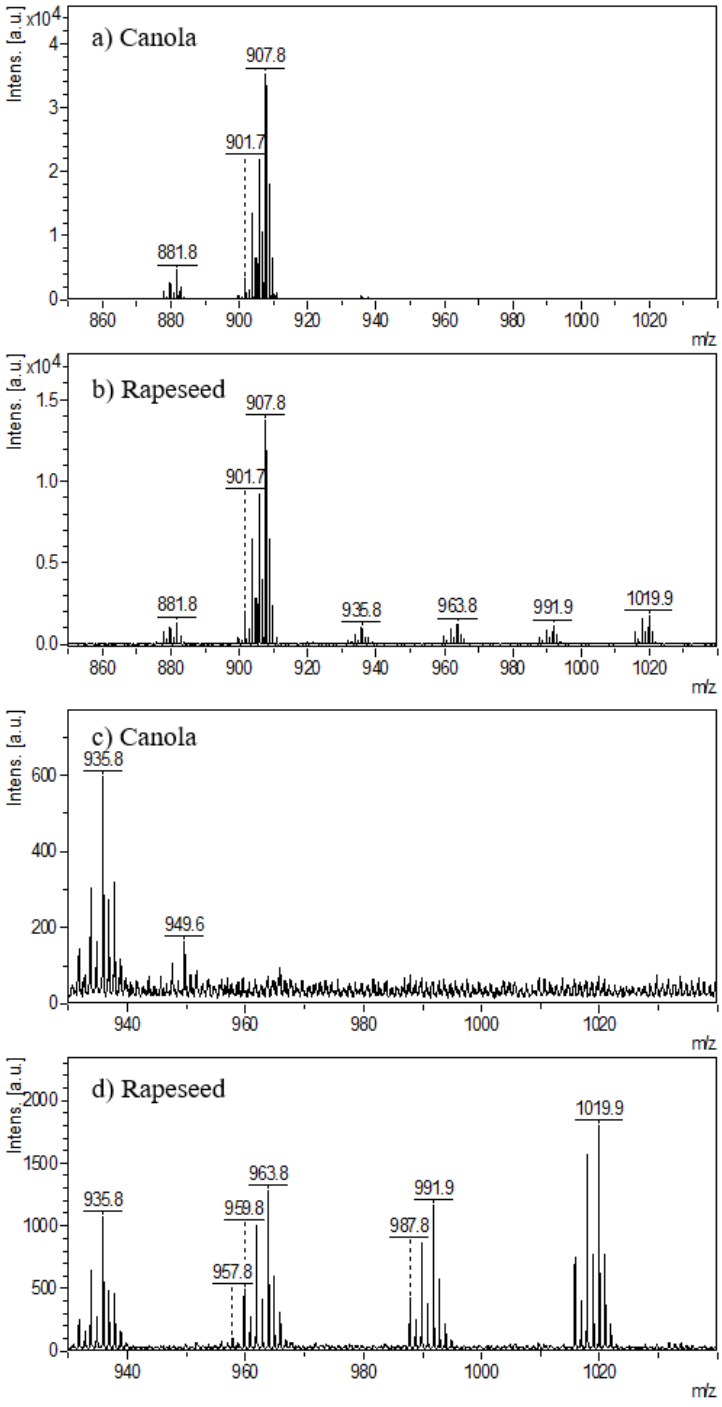


Figure 4-11. The full MALDI-MS spectra of (a) a canola oil and (b) a rapeseed oil and the zoom-in view (m/z >930) of (c) the canola oil and (d) the rapeseed oil.

4.3.5 Characteristic peaks and PCA of the MALDI-MS spectra

Spectra of different edible oils were compared and characteristic peaks have been found for various edible oils, which different edible oil species could show different characteristic MALDI-MS spectral patterns. In general, the MALDI-MS spectra of animal oils were difference significantly with the spectra of vegetable oils. The spectral patterns of animal oils varied while most of the vegetable oils showed TAG peaks at the region of *m/z* 850 – *m/z* 920 with different peak ratios, except for castor oil, coconut oil, palm oil, and rapeseed oil.

Castor oil and fish oil

Special peaks were observed in the mass spectra of castor oil and fish oil as shown in Figure 4-12, which were easily distinguished from those of other edible oils. The mass spectrum of castor oil was very simple and only one major peak at *m/z* 955.7 that corresponds to TAG of ricinoleic acid (12-OH-C18:1) was observed.[144] Fish oil possessed a complex mass spectrum which contained abundant signals for both TAGs commonly observed in vegetable oils and TAGs of docosahexaenoic acid (C22:6) and eicosapentaenoic acid (C20:5) such as *m/z* 981.9 and *m/z* 1010.0.[165] The TAGs of C22:6 and C20:5 were only observed in fish oil among the tested samples. These specific

MALDI-MS patterns allowed castor oil and fish oil to be easily distinguished based on their MALDI-MS spectra.

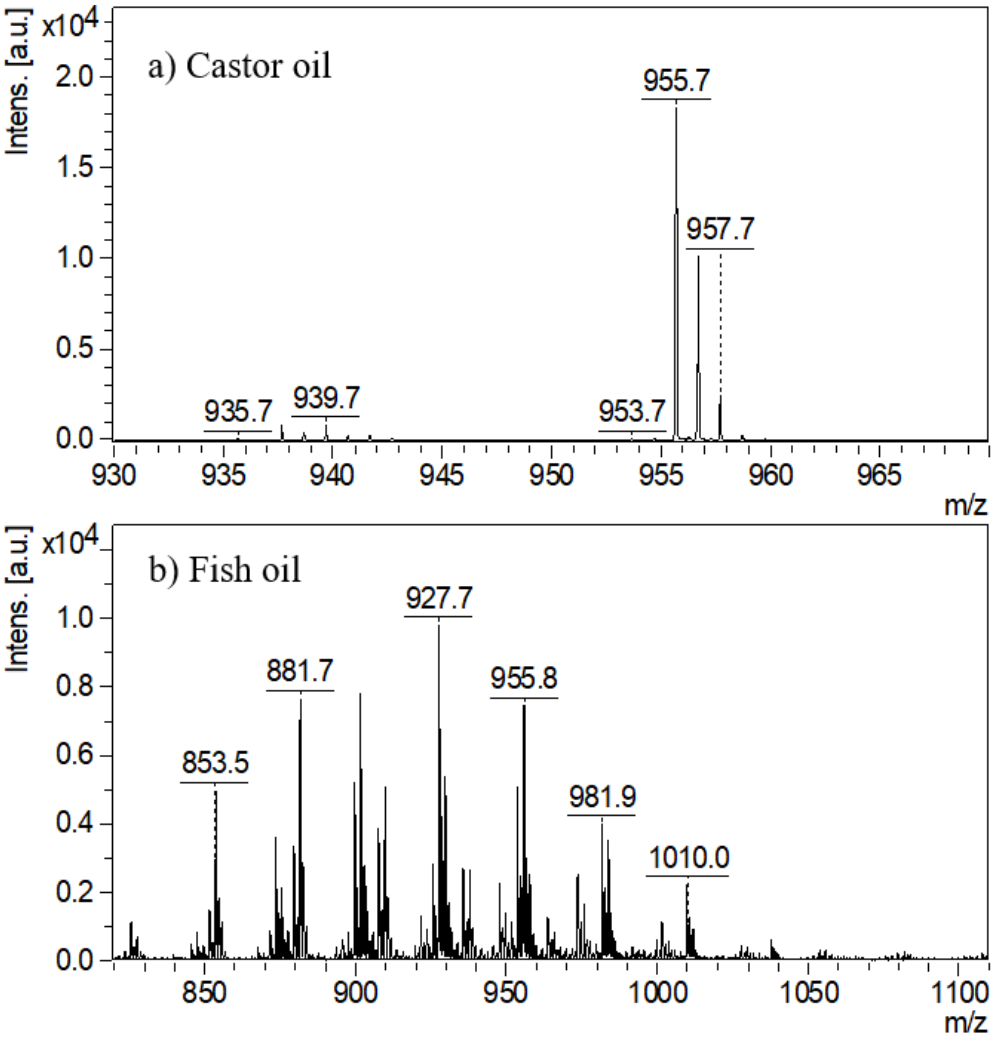


Figure 4-12. The mass spectra for the TAG regions of (a) castor oil and (b) fish oil.

Butter, coconut oil and margarine

The TAGs regions of butter and coconut oil as shown in Figure 4-13 were mainly contained the peaks lower than *m/z* 850. The abundant peaks such as *m/z* 661.5 and *m/z* 689.6 were corresponded to TAGs of lauric acid (C12:0) and myristic acid (C14:0), respectively.[140] These TAGs were only found in butter and coconut oil and could be used for differentiation of butter and coconut oil from other edible oils. The peak ratios of the spectra of butter and coconut oil were different. Coconut oil had higher intensities of peaks at *m/z* 605.5 and *m/z* 633.5 while butter had higher intensities of peaks at *m/z* 771.6 and *m/z* 827.6. It indicated that coconut oil contained more medium chain fatty acids such as C12:0. Margarine is a mixture of vegetable oils but is physically similar to butter, as difficult to be differentiated by naked eyes. As shown in Figure 4-14, the mass spectra of margarine showed stronger signals at higher mass region, such as *m/z* 879.8 and *m/z* 903.7 (i.e. higher level of C18:1 and C18:2), while the mass spectrum of butter contained peaks with lower mass as discussed previously. Such difference at the mass spectra allowed easy differentiation between them.

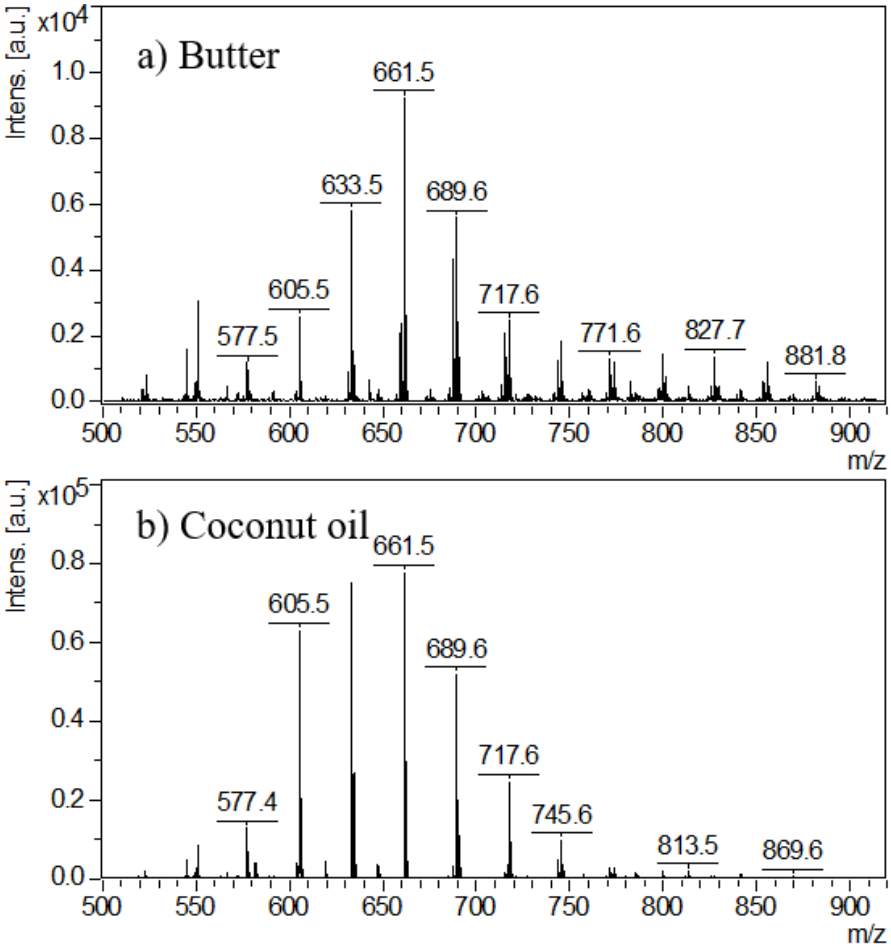


Figure 4-13. The mass spectra for the TAG regions of (a) butter and (b) coconut oil.

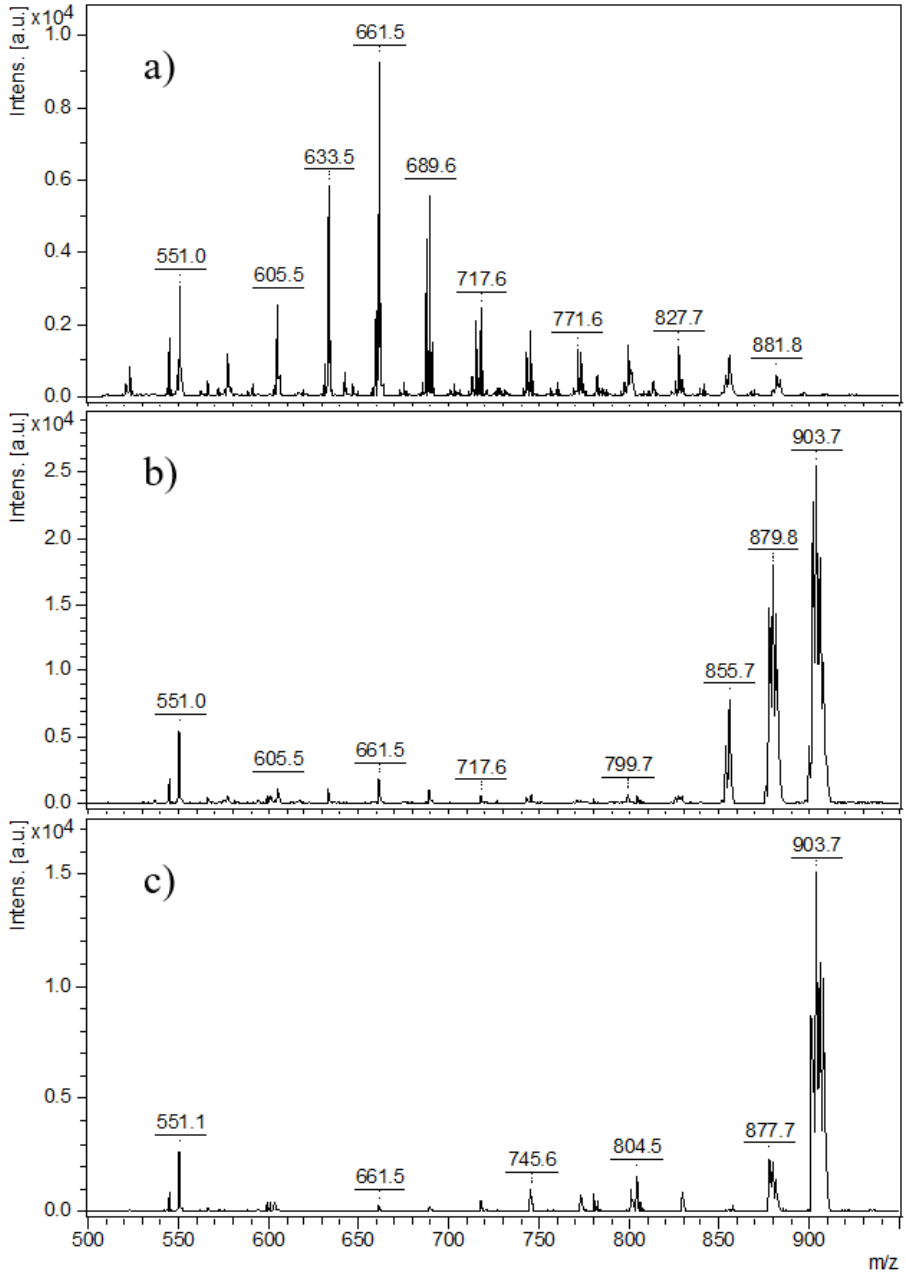


Figure 4-14. The MALDI-MS spectra of (a) a butter sample, (b) a margarine sample (mixture of soybean, palm, coconut and rapeseed oils) and (c) another margarine sample (mixture of sunflower oil and other unspecified vegetable oils).

Perilla oil and flaxseed oil

Perilla oil and flaxseed oil contained high abundance of C18:3 thus showed two specific peaks at *m/z* 895.7 (LnLnLn) and *m/z* 897.7 (LnLnL) in the MALDI-MS spectra as shown in Figure 4-15. High level of such peaks could only be observed in perilla oil and flaxseed and could serve as the markers of these two oils. Peak at *m/z* 899.7 for flaxseed oil showed significantly higher abundance than for perilla oil, allowing simple differentiation between them. In addition, some characteristic peaks such as *m/z* 999.5 and *m/z* 1062.6 were observed only in flaxseed oil (see Figure 4-16). The results from the accurate mass measurement, database searching and MS/MS analysis (see Figure 4-16 c and d for the results) indicated that the most abundant peak (*m/z* 1062.6) in the spectrum was confirmed as the sodium adducted ion of cyclolinopeptide A, a natural product found in flaxseed oil extract.[166] The other high mass peaks at *m/z* 1080.7, *m/z* 1096.7, *m/z* 999.6 and *m/z* 983.5 were corresponded to the sodium adducted ions of cyclolinopeptides B, C, E and J.[167] However, the peaks corresponded to cyclolinopeptide were only detectable in all 25 out of 28 authenticated flaxseed oil products collected. Although the peaks for cyclolinopeptides were not observed in all the flaxseed oil products, the presence of such peaks could provide additional information for characterization of flaxseed oil.

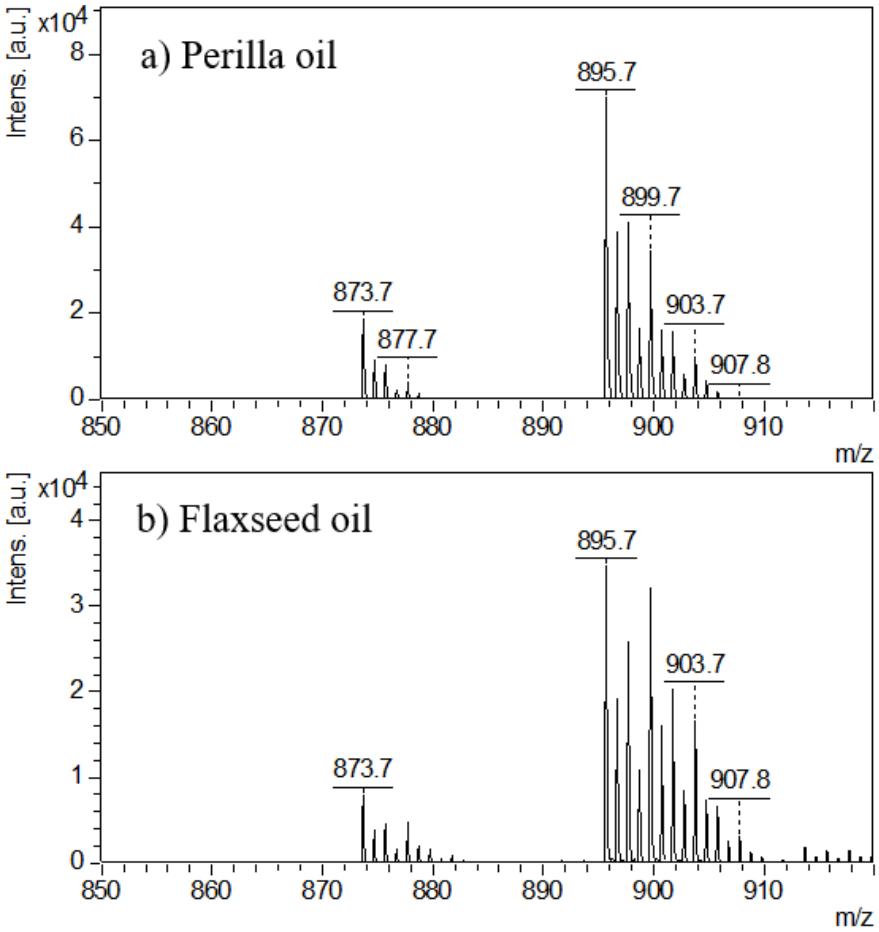
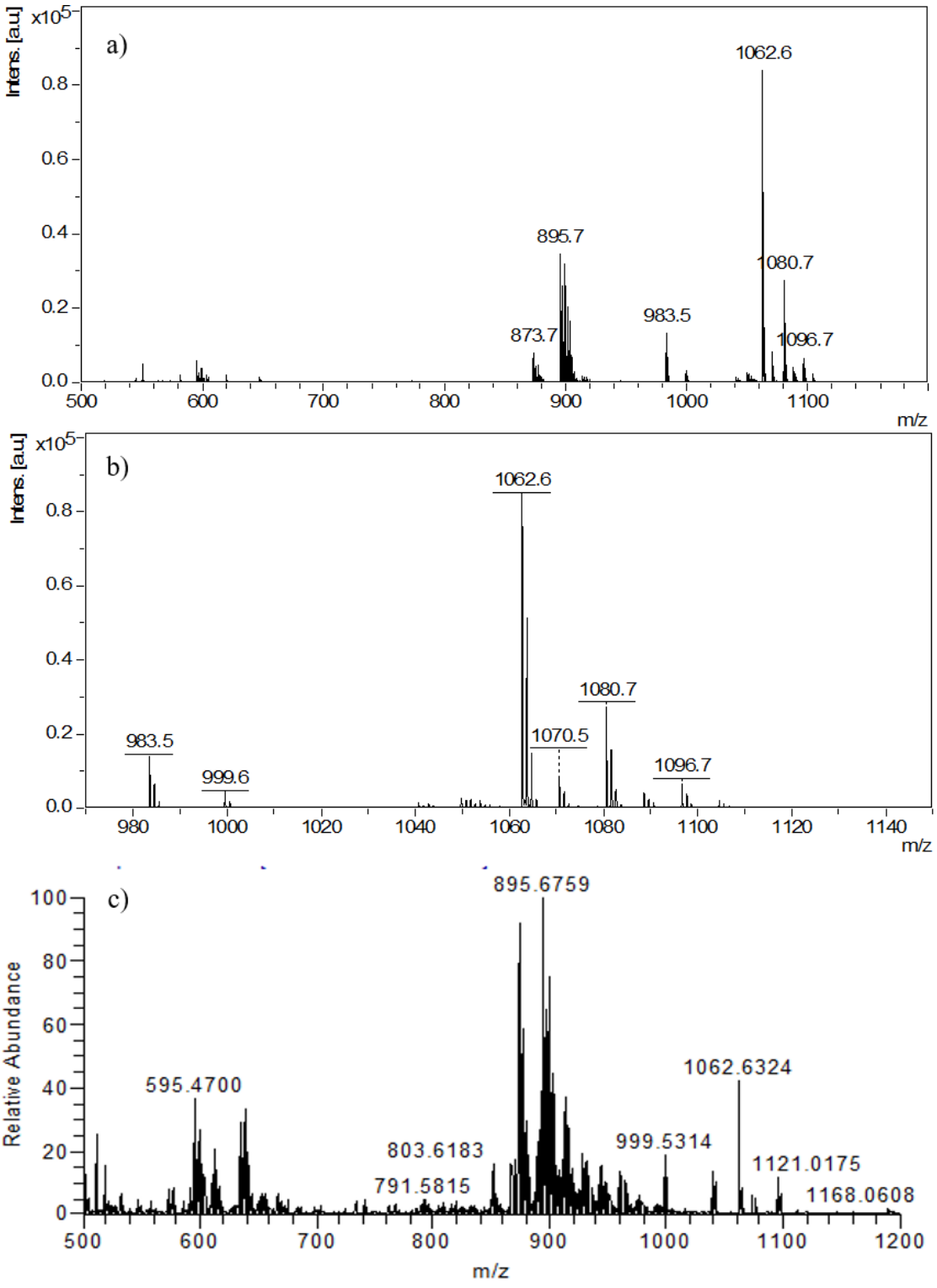


Figure 4-15. The mass spectra for the TAG regions of (a) perilla oil and (b) flaxseed oil.



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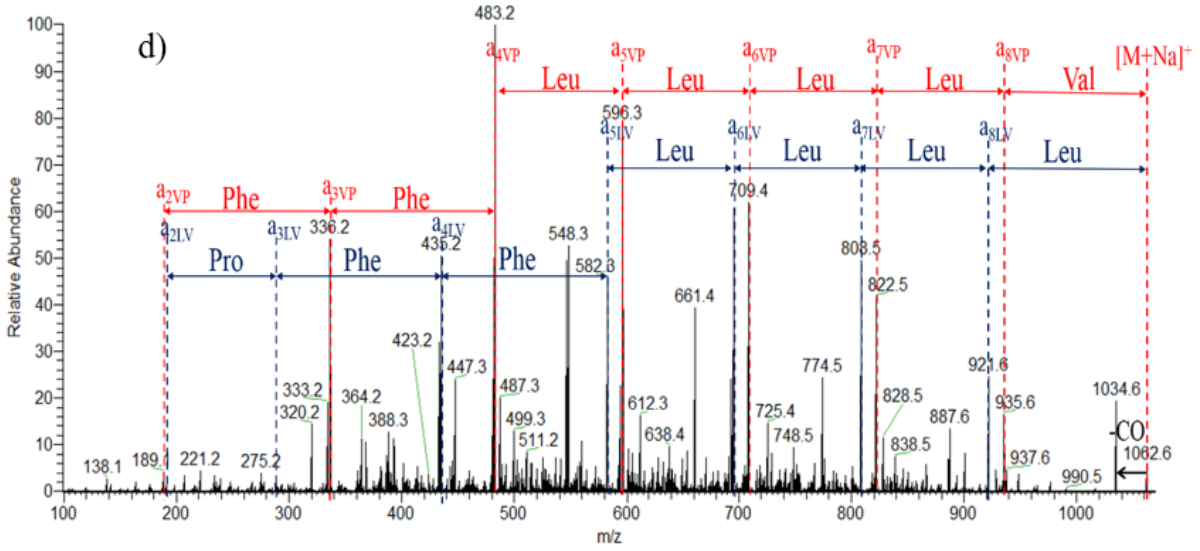


Figure 4-16. (a) The full MALDI-MS spectrum and (b) the cyclolinopeptides region MALDI-MS spectrum of a flaxseed oil sample. (c) The mass spectrum of a flaxseed oil sample analyzed using ESI-orbitrap MS. (d) The orbitrap MS/MS spectrum of ion *m/z* 1062.6 showed two a-ion series of cyclolinopeptide A.

Palm oils and Lard

There are seven types of palm oil derivatives that possess different fatty acid contents.[111] However, only two types of palm oil derivatives, i.e., palm oil (derived from the fleshy mesocarp of the oil palm) and palm superolein (special processed liquid fraction of palm oil), were found in the local markets, although palm oils are very popular in food industry. Palm oil showed higher abundances of peaks at *m/z* 855.7 (PPO) and *m/z* 881.8 (POO) than other common vegetable oils such as canola oil and olive oil as shown in

Figure 4-17. Palm oil and palm superolein were different from the abundance of C16:0 and stearic acid (S, C18:0), thus the later one had weaker peak at *m/z* 855.7 but stronger peak at *m/z* 881.8. Lard shared similar MALDI-MS spectral pattern with that of palm superolein but with slightly weaker peaks at *m/z* 855.7 and *m/z* 907.8.

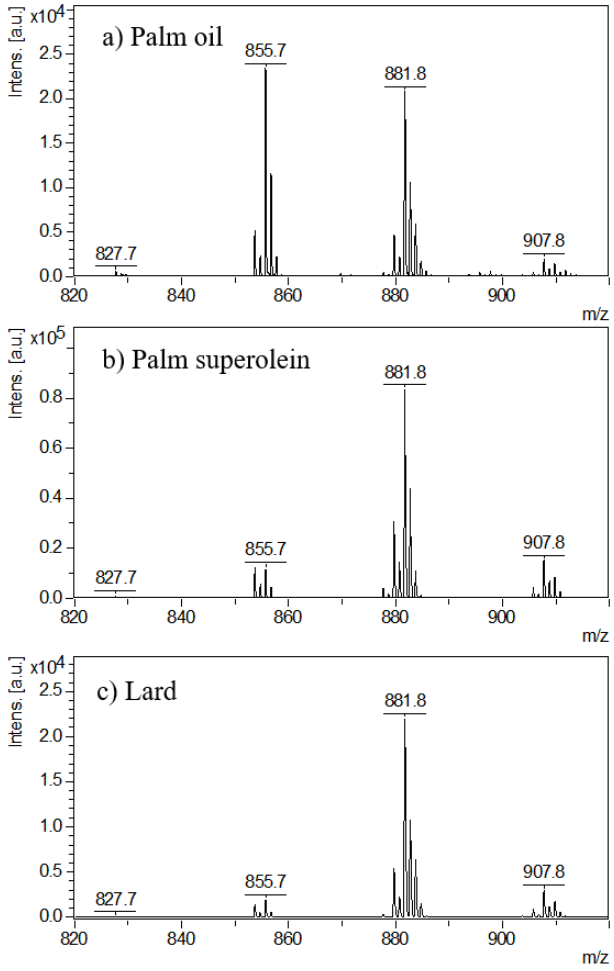


Figure 4-17. The mass spectra for the TAG regions of (a) palm oil, (b) palm superolein and (c) lard.

Rapeseed oil

Rapeseed oil, as discussed in the previous section, shared similar MALDI-MS spectrum with canola oil, except that peaks at higher mass region from *m/z* 931.8 to *m/z* 1019.9 which corresponded to TAGs of C20:1 and C22:1, were observed only in rapeseed oil (see Figure 4-18). However, the intensities of these additional peaks varied at different rapeseed oil products, consistent with the reported C22:1 contents from 2% to 60% in rapeseed oil.[149] The permitted C22:1 content in edible oil products has been regulated in some regions. For example, the threshold level is 5% by weight of fatty acid content in Hong Kong. This MALDI-MS approach is potentially useful for rapid screening of the illegal edible oil products by detecting the TAGs peaks of C22:1, such as *m/z* 1019.9 (C18:1/C22:1/C22:1 or C20:1/C20:1/C22:1) and *m/z* 991.9 (C16:1/C22:1/C22:1)

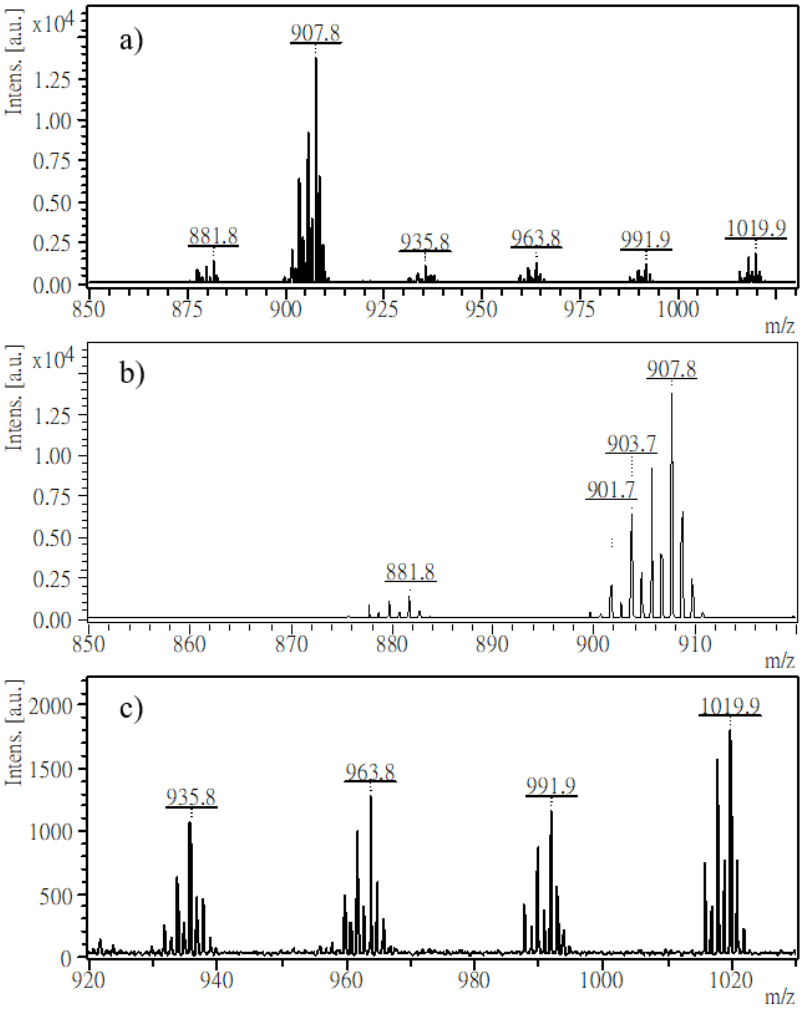


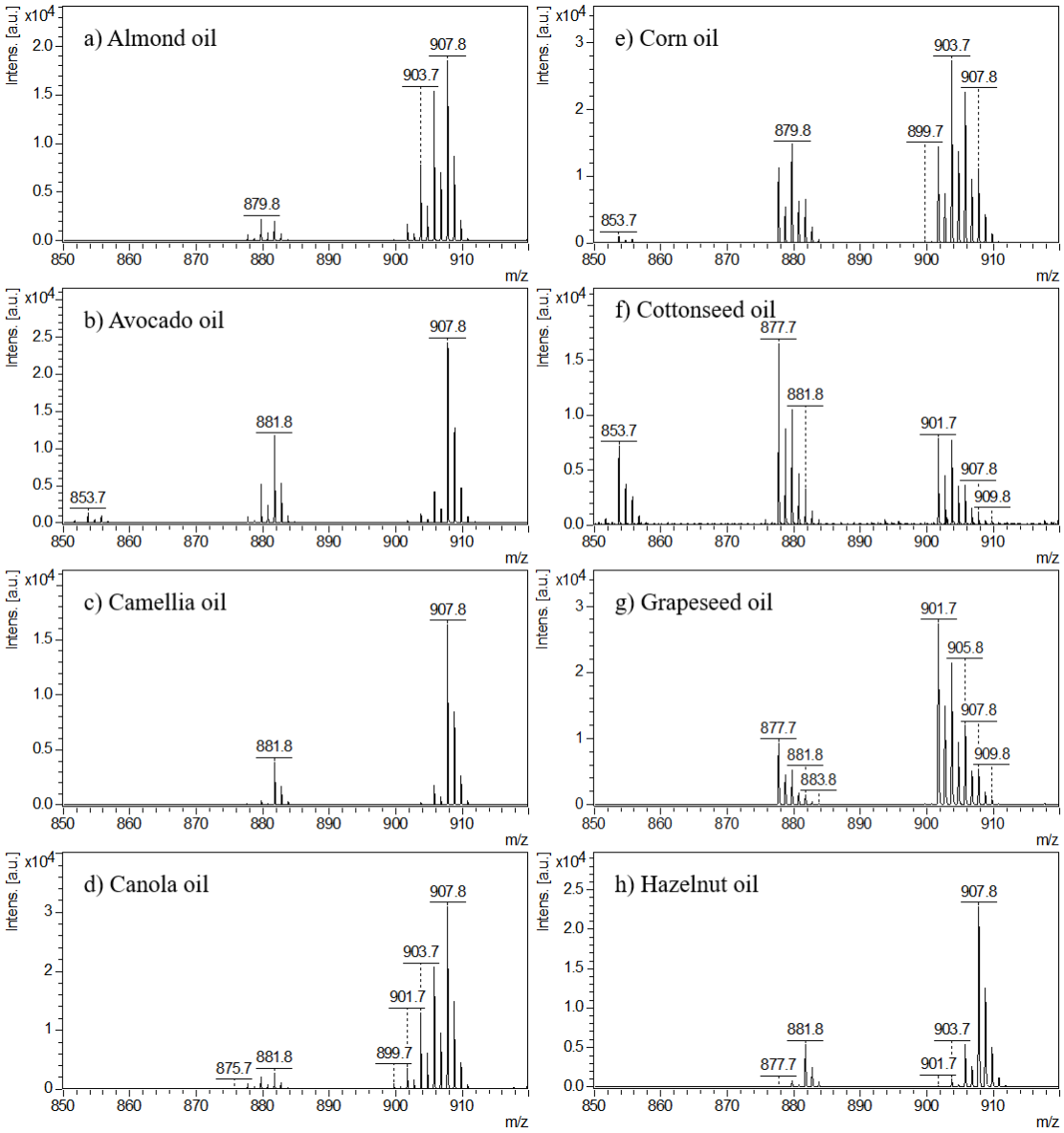
Figure 4-18. The mass spectra for the TAG regions of (a) rapeseed oil and the zone in view of (b) mass region *m/z* 850 – *m/z* 920 and (c) mass region *m/z* 920 – *m/z* 1030.

Other vegetable oils

The mass spectra of the remaining vegetable oils are shown in Figure 19. Except for the edible oil species discussed previously, which showed obvious characteristic peaks, MALDI-MS spectra of the remaining edible oil species were mainly consisted of similar peaks at the region *m/z* 850 – *m/z* 920, such as *m/z* 877.7 (PLL), *m/z* 879.7 (POL), *m/z* 901.7 (LLL), *m/z* 903.7(LLO), *m/z* 905.8 (LOO) and *m/z* 907.8 (OOO), but with different intensity ratios. The first step of the spectral comparison of these oil species was to observe its strongest peaks in the spectra, which were *m/z* 877.7 in cottonseed oil, *m/z* 879.7 in rice bran oil, *m/z* 901.7 in safflower oil, soybean oil, walnut oil and wheat germ oil, *m/z* 903.7 in corn oil, pine nut oil, pumpkin seed oil and sunflower oil, *m/z* 905.8 in sesame oil, *m/z* 907.8 in almond oil, avocado oil, camellia oil, canola oil, hazelnut oil, high oleic acid sunflower oil and olive oil. The most abundant peaks in peanut oil varied as discussed before, which could be either *m/z* 903.7, *m/z* 905.8 or *m/z* 907.8.

These edible oil species could then be differentiated by comparing the peak intensity ratios in their mass spectra. For example, strongest peaks at *m/z* 907.8 were observed in the mass spectra of both olive oil and canola oil, but olive oil showed higher

abundance of peak at *m/z* 881.8, while canola oil showed stronger peak at *m/z* 905.8 and additional peaks at *m/z* 899.7, *m/z* 901.7 and *m/z* 903.7 were observed. Different intensity ratios of *m/z* 881.8 and *m/z* 907.8 were observed for olive oil, avocado oil, hazelnut oil and high oleic acid sunflower oil, allowing differentiation among them.



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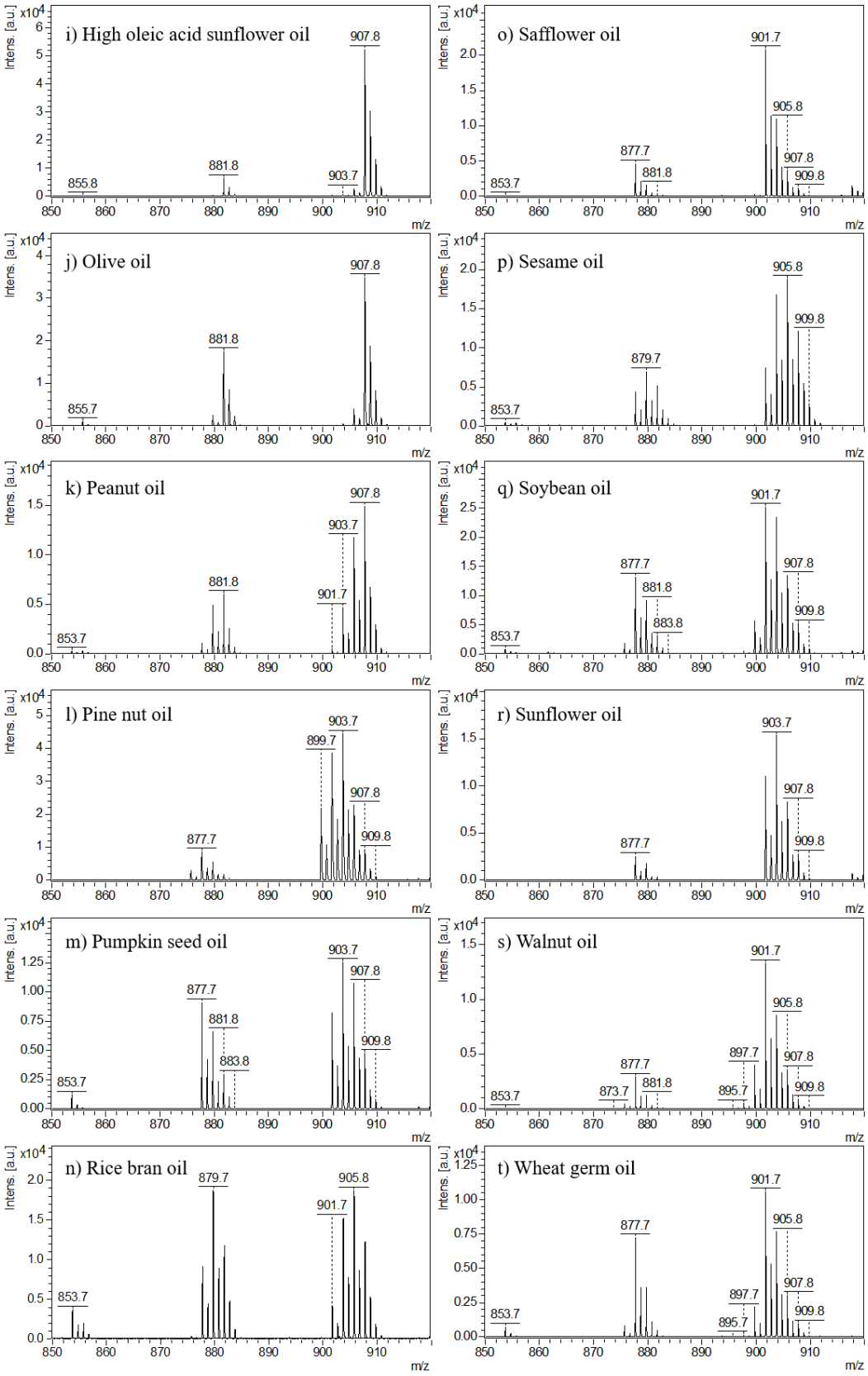
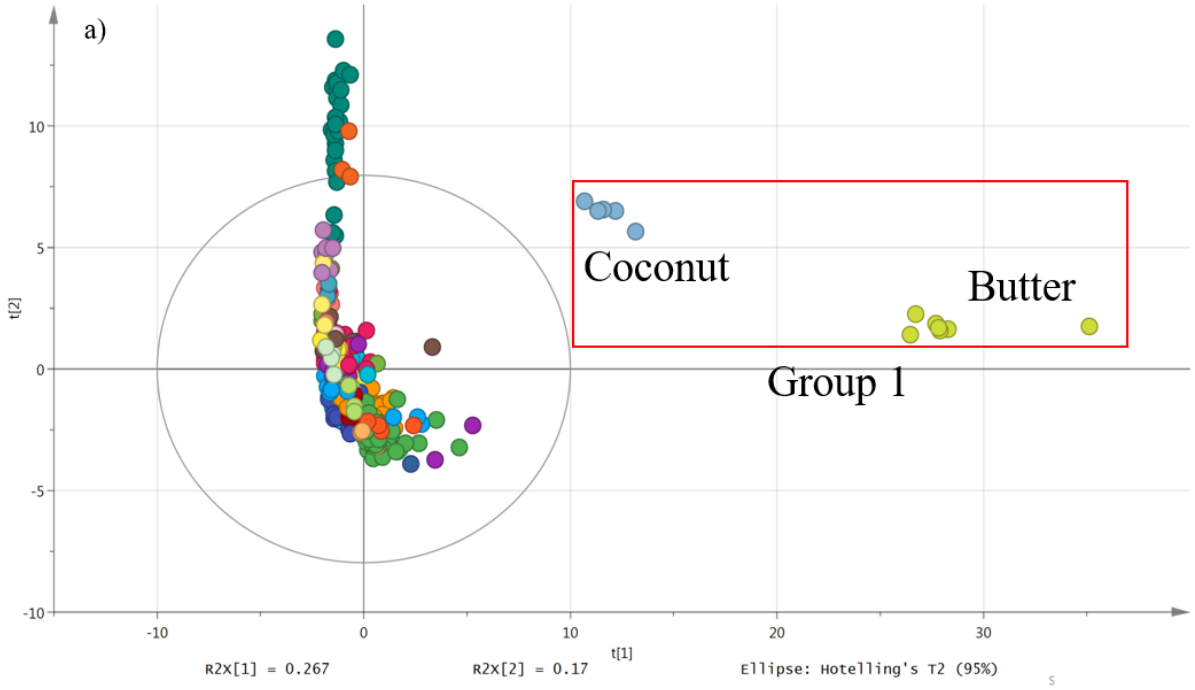


Figure 4-19. (a-t) The mass spectra for the TAG regions of remaining edible oils.

Principal component analysis (PCA)

PCA, a commonly used tool for statistical analysis, is used to facilitate the differentiation and classification of edible oils as demonstrated by Lisa et al. and Lay et al.[148,168] Excluding castor oil and fish oil samples, which showed very special MALDI-MS spectral patterns, spectra of 435 authenticated edible oil products were analyzed using PCA. As shown in Figure 4-20a, butter and coconut oil were clustered very separately from each other and other edible oils, in consistent with their significantly different TAG patterns in lower mass region. PCA after excluding coconut oil and butter revealed two groups of edible oils, with one group containing flaxseed oil and perilla oil and the other group containing palm oil, lard and palm superolein, which were clustered separately from the remaining edible oils (see Figure 4-20b). The reason for the separation also relied on the characteristic peaks presented in the spectra. A new PCA score plot (Figure 4-21a) was obtained for PCA of the remaining edible oils, which could be divided into 4 regions. The separation again was consistent with the differences in spectral patterns as discussed in previous section, such as edible oils showed high abundance of *m/z* 907.8, including olive oil, avocado oil, hazelnut oil and high oleic acid sunflower oil were classified into the same group. Based on these PCA results, the edible oils were divided into seven different groups with one additional

group for castor oil and fish oil. The characteristic features of members in each group are shown in Table 4-3. The edible oils in groups 2, 3, 4, 5, 6 and 7 could be further differentiated with further PCA as shown in Figures 20c-d and 21b-e. It is noticed that although the MALDI-MS spectra of some edible oils such as peanut oil and olive oil showed obvious variations within the same species as discussed previously, such variations were normally still smaller than the differences between different oil species, allowing them to be clearly differentiated from other oil species. However, 3 pairs of edible oils, i.e., olive oil and avocado oil, canola oil and almond oil, and corn oil and pumpkin seed oil, were found difficult to be differentiated based on their TAGs patterns.



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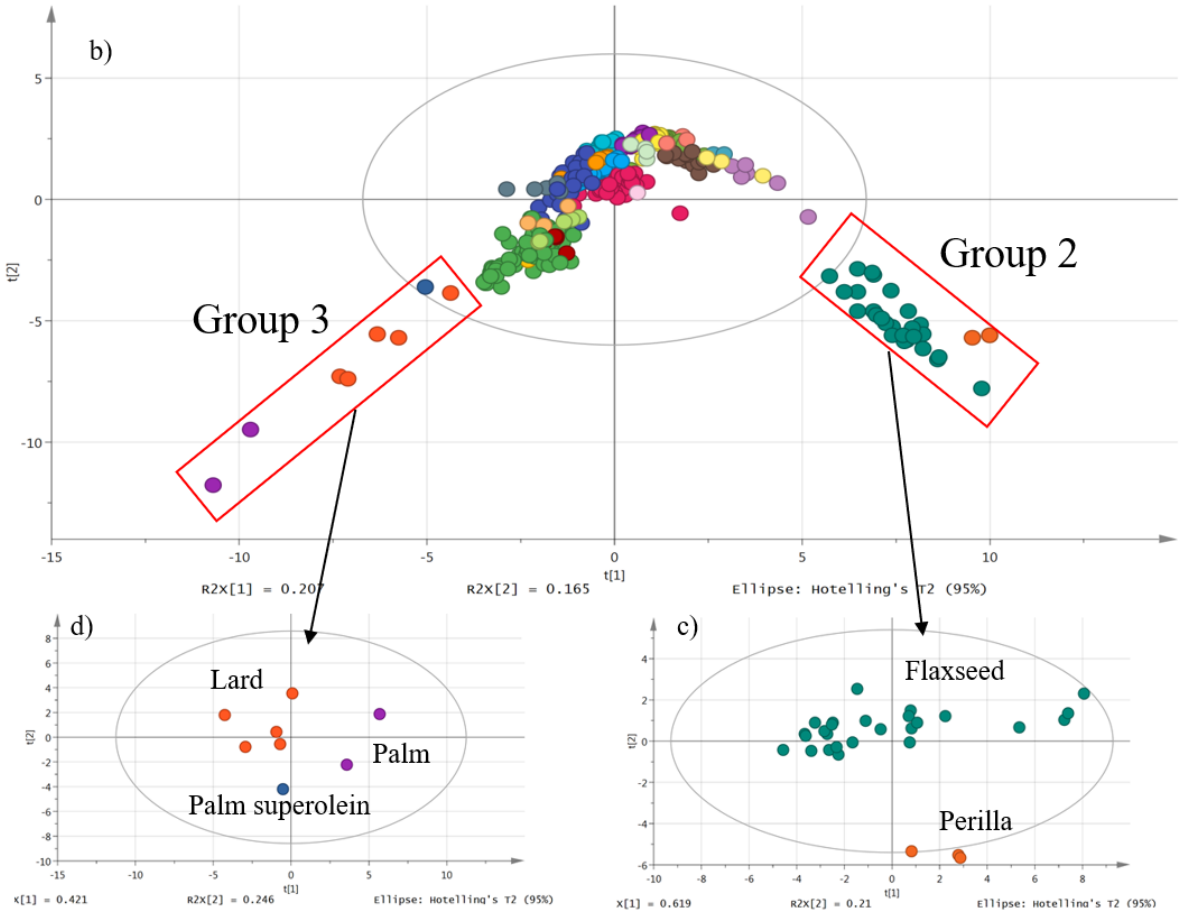

Figure 4-20. (a) PCA score plot for different edible oils (except castor oil and fish oil) based on their MALDI-MS results. The first principal accounted for 27% of variance and second principal accounted for 17% of variance. (b) PCA score plot for different edible oils based on their MALDI-MS results after removing group 1 as well as castor oil and fish oil. The first principal accounted for 21% of variance and second principal accounted for 17% of variance. (c-d) PCA score plots for (c) group 2 and (d) group 3. The first principal accounted for 62% and 42% of variance and second principal accounted for 21% and 25% of variance, respectively.

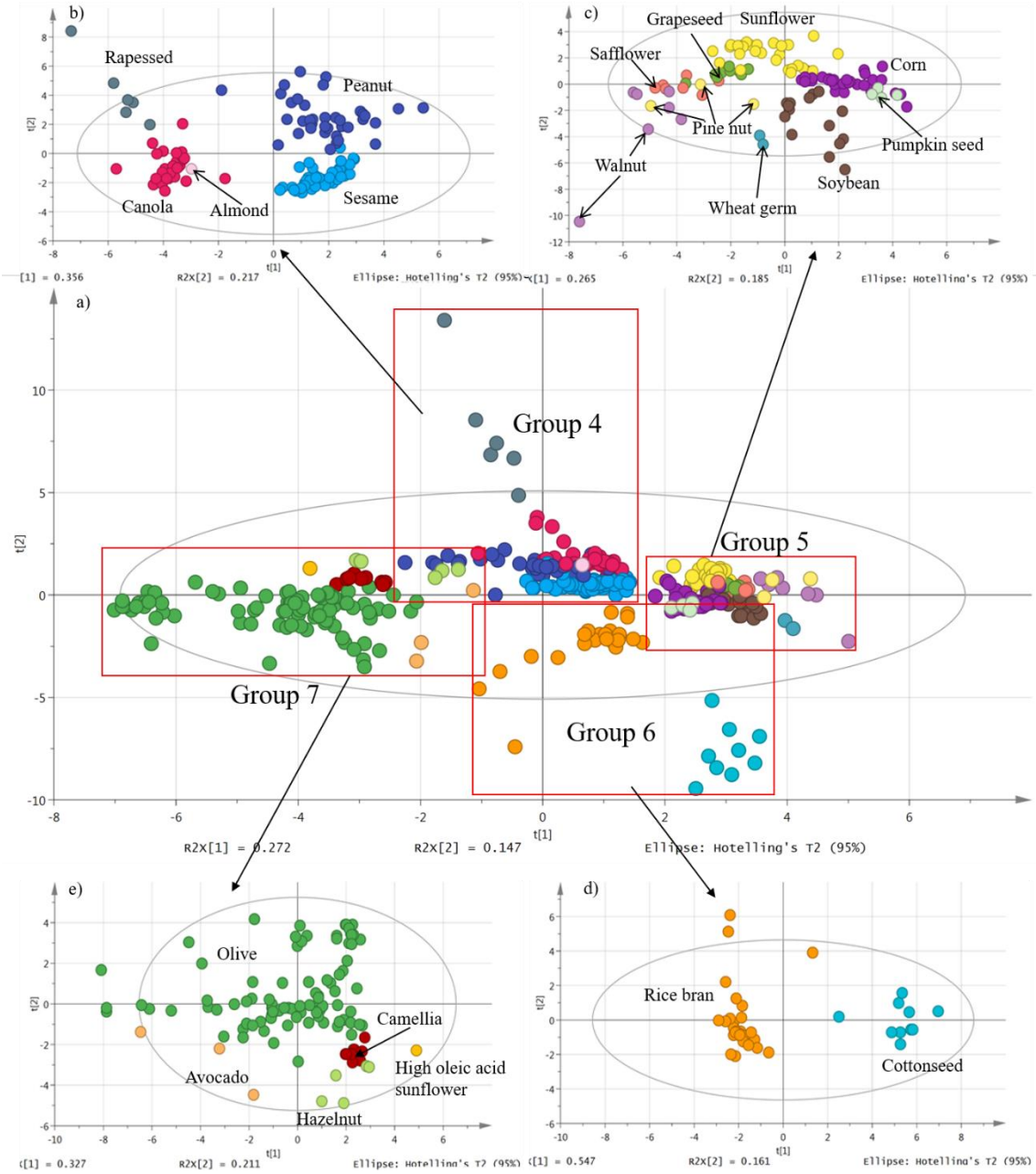

Figure 4-21. (a) PCA score plot for different edible oils based on their MALDI-MS results after removing groups 1, 2 and 3 as well as castor oil and fish oil. The first principal accounted for 27% of variance and second principal accounted for 15% of variance. (b-e) PCA score plots for (b) group 4, (c) group 5, (d) group 6 and (e) group 7. The first principal accounted for 36%, 27%, 55% and 33% of variance and second principal accounted for 22%, 19%, 16% and 21% of variance, respectively.

Table 4-3. Division of edible oils based on their MALDI-MS spectra and PCA results

Group	Oil species	Characteristic peaks in spectra
1	Coconut oil and butter	Abundant peaks at lower mass region (e.g., *m/z* 605.5, *m/z* 633.5 and *m/z* 661.5)
2	Flaxseed oil and perilla oil	Abundant peaks at *m/z* 895.7, 897.7 and 899.7
3	Palm oil, palm superolein and lard	Abundant peak at *m/z* 881.7 (intensity of *m/z* 881.7 higher than that of *m/z* 907.8)
4	1) Sesame oil, peanut oil, almond oil and canola oil	Abundant peaks at *m/z* 905.8 and 907.8
	2) Rapeseed oil	Similar to canola oil, but with additional peaks at *m/z* 963.8, 991.9 and 1019.9
5	1) Grapeseed oil, safflower oil, soybean oil, walnut oil, wheat germ oil	Abundant peak at *m/z* 901.7 (intensity of *m/z* 901.7 higher than that of *m/z* 877.7)
	2) Corn oil, pine nut oil, pumpkin seed oil, sunflower oil	Abundant peak at *m/z* 903.7 (intensity of *m/z* 903.7 higher than that of *m/z* 877.7)
6	1) Cottonseed oil	Abundant peaks at *m/z* 877.7 (intensity of *m/z* 877.7 higher than that of *m/z* 901.7)
	2) Rice bran oil	Abundant peaks at *m/z* 879.7 and 905.8
7	Olive oil, high oleic acid sunflower oil, avocado oil, hazelnut oil and camellia oil	Significantly abundance peak at *m/z* 907.8 (intensity of *m/z* 907.8 much higher than that of *m/z* 905.8)
Other	1) Castor oil	Specific and predominant peak at *m/z* 955.7
	2) Fish oil	Board TAGs distribution from *m/z* 825.7 to 1009.8, abundant peaks at *m/z* 881.8, 901.7, 927.7, 955.8, and specific peaks at *m/z* 981.8 and 1009.8

4.3.6 Classification of edible oils using PLS-DA

PLS-DA is a supervised technique that correlates the *class* (i.e., the edible oil species in this study) and *X-variables* (i.e., the relative intensities of peaks in the MALDI-MS spectra) through the scores calculated by the statistical software.[169] The class of future samples could be predicted after the establishment of PLS-DA models using training samples. This cannot be achieved using the PCA method.[169,170]

Establishment of PLS-DA models

Samples from 198 representative edible oil products were used as the training set for establishment of PLS-DA models in this study. The training set included edible oil products of different species, different collection sources and different MALDI-MS spectral patterns. A two-step approach was employed for the PLS-DA analysis as the results obtained from PCAs. Based on the features of the spectra, the samples were firstly classified into the groups as listed in Table 4-3 using the "grouping" PLS-DA model and then further classified into individual edible oil species using 7 sub-models (model group 1 - 7). The data of samples from training set were input into SIMCA for PLS-DA processing to establish a model with the groups (i.e. group 1 - 7) as the dummy Y variable. Seven sub-models were established using the data of the edible oil samples

in each group and used species as the dummy Y variables. It was found that such two-step approach could provide better sensitivity for the classification of edible oils, especially for sunflower oil, grapeseed oil, avocado oil and wheat germ oil, than conventional approach (i.e. use only one PLS-DA model for analyzing all the samples).

Classification of pure edible oils

Both the data in the training set and in the testing set were input into to the established models for the method validation. The predicted scores (PS) for the matching of each oil species was calculated for each edible oil sample, and the edible oil sample was then classified as the edible oil species which gave the highest score. For example, the PLS-DA analysis of a sample gave PS of 0.01, -0.02, -0.01, 0.06, 0.18, -0.15 and 0.92 for groups 1, 2, 3, 4, 5, 6 and 7, respectively, indicating that this sample was most likely to belong to group 7. The data of this sample was further analyzed using model of group 7 and gave PS of 1.16, 0.03, 0.04, -0.04, and -0.19 for the five edible oils in group 7, i.e., olive oil, camellia oil, high oleic acid sunflower oil, avocado oil, and hazelnut oil, respectively. The oil sample was then determined as olive oil.

As shown in Table 4-4, for the training set, a correct classification rate of 96.0% was

obtained, indicating the effectiveness of the database and approach for classification of edible oils. It was noticed that for the 8 edible oil samples that were misclassified, they were mainly the oil species with sample sizes equal to or less than 3 (e.g. almond oil and high oleic acid sunflower oil), indicating that further sample collection for these oil species might be needed to improve the model establishment and the classification. The misclassification of grapeseed oil was due to the high similarity of the MALDI-MS spectra between grapeseed oil, safflower oil and sunflower oil. As shown in Table 4-5, for the testing set, the correct classification rate was 98.31%, with only 4 tested samples misclassified. All the misclassifications occurred in the grouping step, with 1 sample from group 4 misclassified as group 5 and 2 samples from group 5 and 1 sample from group 8 misclassified as group 4. The spectra of the misclassified samples were re-examined. It was found that although the mass spectra of the two misclassified samples from group 5 (both were sunflower oils) were similar to those of the others in the group, it showed slightly higher peaks at m/z 879.7 and m/z 905.8 than the others (see Figures 4-22 for the spectral comparison). As shown in the PLS-DA loading plot (Figure 4-22c), these two peaks were the important factors for classification of samples in group 4, thus causing the misclassification. Similar explanation could be applied to the other misclassified samples. Such re-inspecting the spectra of the problematic PLS-DA

results might help to reduce the chance of misclassification.

Table 4-4. Results for classification of edible oils in the training set using PLS-DA.

Oil species	No. of products	Number of correct classification (%)	Oil species misclassified as (No. of products)
Almond	1	0	Canola (1)
Avocado	3	2 (67.7)	Hazelnut (1)
Butter	6	6 (100)	---
Camellia	7	7 (100)	---
Canola	12	12 (100)	---
Coconut	4	4 (100)	---
Corn	10	10 (100)	---
Cottonseed	8	8 (100)	---
Flaxseed	11	11 (100)	---
Grapeseed	8	6 (75.0)	Safflower (1), sunflower (1)
Hazelnut	3	3 (100)	---
High oleic acid sunflower	1	0	Camellia (1)
Lard	4	4(100)	---
Olive	28	28 (100)	---
Palm	2	2 (100)	---
Palm superolein	1	0	Lard (1)
Peanut	14	14 (100)	---
Perilla	3	3 (100)	---
Pine nut	3	1 (33.3)	Soybean (1), walnut (1)
Pumpkin seed	4	4 (100)	---
Rapeseed	5	1 (100)	---
Rice bran	17	17 (100)	---
Safflower	6	6 (100)	---
Sesame	11	11 (100)	---
Soybean	10	10 (100)	---
Sunflower	8	8 (100)	--
Walnut	6	6 (100)	---
Wheat germ	2	2 (100)	---
Total	**198**	**190 (96.0)**	

Table 4-5. Results for classification of edible oils in the testing set using PLS-DA.

Oil species	No. of products	Number of correct classification (%)	Oil species misclassified to (No. of products)
Butter	1	1 (100)	---
Camellia	2	2 (100)	---
Canola	22	22 (100)	---
Coconut	1	1 (100)	---
Corn	25	25 (100)	---
Cottonseed	2	2 (100)	---
Flaxseed	15	15 (100)	---
Grapeseed	2	2 (100)	---
Hazelnut	2	1 (50.0)	Canola (1)
Lard	1	1 (100)	---
Olive	60	60 (100)	---
Peanut	23	23 (100)	---
Rapeseed	1	1 (100)	---
Rice bran	11	11 (100)	---
Safflower	1	1 (100)	---
Sesame	41	40 (97.4)	Corn (1)
Soybean	5	5 (100)	---
Sunflower	21	19 (90.5)	Sesame (2)
Walnut	1	1 (100)	---
Total	**237**	**233 (98.3)**	

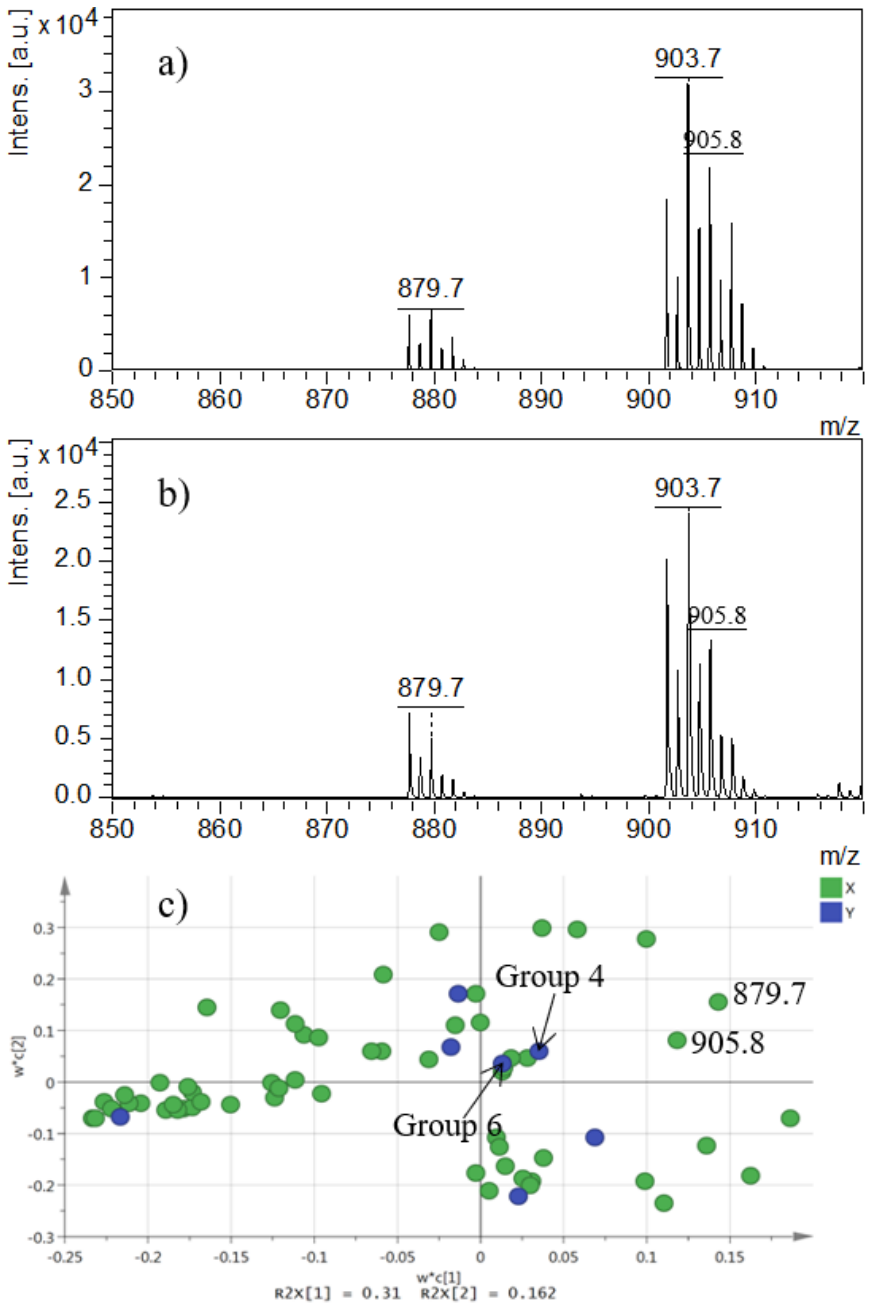

Figure 4-22. Mass spectra for the TAGs regions of (a) a misclassified sunflower oil sample and (b) a typical sunflower oil sample. (c) PLS-DA loading plot for the classification of samples into different groups.

Overall, including both training set and testing set, 97.2% of the tested samples were classified correctly using PLS-DA and only 12 out of the 435 tested samples were misclassified. For commonly used edible oils such as olive oil, peanut oil and canola oil, correct classification rates of 100% were achieved. Such classification could be considered as highly accurate with the high correct classification rate. Most misclassifications were related to the species with small sample sizes, such as pine nut oil and avocado oil in the training set. These could be improved by collecting and analyze more samples for those oil species. The details of the overall classification results are listed in Table 4-6. It is noticed the results obtained from PLS-DA were generally consistent with PCA, as same data sets were used for both analyses. The operation of these two analyses could be simply interchanged using SIMCA software. The results of PLS-DA were better than that of PCA, such as clearer differentiation of corn oils and pumpkin seed oils was achieved in PLS-DA, properly due to the supervised data were used in PLS-DA.

The data set were also analyzed using conventional one-step PLS-DA approach. The overall correct classification rate was reduced to 83.5%, which the classification rates of camellia oil, sunflower oil and corn oil dropped to 67.0%, 45.0% and 68.6%

respectively. The classification rates of grapeseed oil, avocado oil, hazelnut oil, pin nut oil and wheat germ oil were even 0%. The difference between one-step and two-step PLS-DA approach could be due to the small peak ratios different between some edible oil species, e.g. sunflower oil and grapeseed oil, were masked when some characteristic edible species, e.g. coconut oil and flaxseed oil were presented in the pool for spectral comparison. Therefore, the edible oil species without characteristic spectral patterns were difficult to be classified in the one-step PLS-DA approach.

Table 4-6. Overall results for classification of edible oils using PLS-DA.

Oil species	No. of products	Number of correct classification (%)	Oil species misclassified to (No. of products)
Almond	1	0 (0)	Canola (1)
Avocado	3	2 (67.7)	Hazelnut (1)
Butter	7	7 (100)	---
Camellia	9	9 (100)	---
Canola	34	34 (100)	---
Coconut	5	5 (100)	---
Corn	35	35 (100)	---
Cottonseed	10	10 (100)	---
Flaxseed	26	26 (100)	---
Grapeseed	10	8 (80.0)	Safflower (1), sunflower (1)
Hazelnut	5	4 (80.0)	Canola (1)
High oleic acid sunflower	1	0 (0)	Camellia (1)
Lard	5	5 (100)	---
Olive	88	88 (100)	---
Palm	2	2 (100)	---
Palm superolein	1	0 (0)	Lard (1)
Peanut	37	37 (100)	---
Perilla	3	3 (100)	---
Pine nut	3	1 (33.3)	Soybean (1), walnut (1)
Pumpkin seed	4	4 (100)	---
Rapeseed	6	6 (100)	---
Rice bran	28	28 (100)	---
Safflower	7	7 (100)	---
Sesame	52	51 (98.1)	Corn (1)
Soybean	15	15 (100)	---
Sunflower	29	27 (93.1)	Sesame (2)
Walnut	7	7 (100)	---
Wheat germ	2	2 (100)	---
Total	**435**	**423 (97.2)**	

The PS of the tested samples indicated how well the samples were matched with the training samples. PS closer to 1 indicated the better fitting between the models and the tested samples. The ranges, means and the standard deviations of the correctly classified edible oil products are listed in Table 4-7. The PS of butter, coconut oil, flaxseed oil and perilla oil which possessed characteristic peaks in their MALDI-MS spectra were very close to 1, indicating the high agreement between the established models and the tested samples. The PS of most edible oil species were higher than 0.7 except for grapeseed oil, camellia oil and hazelnut oil. The low predicted scores of some edible oil species were normally due to the lack of characteristic spectral patterns of the species, increasing the difficulty in classification of those species. The range of the PS reflected the variations of the mass spectral patterns of the edible oil species. As shown in Table 4-7, peanut oil, walnut oil and soybean oil showed larger variations among all the oil species.

Table 4-7. The predicted scores of the edible oil products that were correctly classified using PLS-DA.

Species	Predicted score	
	Range	**Mean ± SD**
Avocado oil	0.71 - 0.77	0.74 ± 0.04
Butter	0.87 - 1.12	0.98 ± 0.08
Camellia oil	0.34 - 0.40	0.36 ± 0.02
Canola oil	0.54 - 1.11	0.80 ± 0.13
Coconut oil	0.93 - 1.01	0.98 ± 0.03
Corn oil	0.66 - 1.08	0.89 ± 0.12
Cottonseed oil	0.72 - 1.23	0.99 ± 0.13
Flaxseed oil	0.83 - 1.23	1.00 ± 0.09
Grapeseed oil	0.32 - 0.53	0.43 ± 0.07
Hazelnut oil	0.42 - 0.66	0.54 ± 0.13
Lard	0.66 - 1.08	0.84 ± 0.03
Olive oil	0.56 - 1.37	0.99 ± 0.16
Palm oil	0.85 - 0.95	0.90 ± 0.07
Peanut oil	0.54 - 1.39	0.92 ± 0.21
Perilla oil	0.92 - 0.98	0.95 ± 0.03
Pumpkin seed oil	0.54 - 0.69	0.63 ± 0.07
Rapeseed oil	0.60 - 1.20	0.90 ± 0.19
Rice bran oil	0.62 - 1.13	0.99 ± 0.10
Safflower seed oil	0.42 - 0.95	0.75 ± 0.19
Sesame oil	0.63 - 1.13	0.94 ± 0.10
Soybean oil	0.39 - 1.15	0.74 ± 0.24
Sunflower oil	0.57 - 1.03	0.83 ± 0.14
Walnut oil	0.46 - 1.22	0.72 ± 0.25
Wheat germ oil	0.56 - 0.69	0.63 ± 0.09

4.3.7 Analysis of edible counterfeits

Five collected edible oil samples (1 flaxseed oil, 2 sesame oils, 1 sunflower oil and 1 safflower oil), which failed to pass the confirmation using the GC-FID analysis as discussed previously, were analyzed by both the simple spectral comparison and the established PLS-DA models. An example of analyzing edible counterfeit, which was claimed as flaxseed oil, is shown in Figure 4-23. The GC-FID result showed the fatty acid contents of the faked flaxseed oil sample were 7.7%, 5.1%, 27.7%, 58.6% and 0% of C16:0, C18:0, C18:1. C18:2 and C18:3 respectively, which were obviously different from the fatty acid contents of flaxseed oil, i.e. 4.0%-6.0%, 2.0%-3.0%, 10.0%-22.0%, 12.0%-18% and 56.0-71.0% of C16:0, C18:0, C18:1. C18:2 and C18:3 respectively. By comparing the reference MALDI-MS spectrum of the flaxseed oil in the home-built database and the MALDI-MS spectrum of this faked sample, peaks at *m/z* 895.7, *m/z* 897.7 and *m/z* 899.7 were missing and the peaks at *m/z* 903.7, *m/z* 905.8 and *m/z* 907.8 were too high in the faked sample. The edible oil counterfeits thus could be screened out by such simple spectral comparison. As shown in Table 4-8, all 5 edible oil counterfeits were not classified as the claimed edible oil species. These results were consistent with those obtained using the GC-FID analysis, demonstrating the reliability of the PLS-DA models.

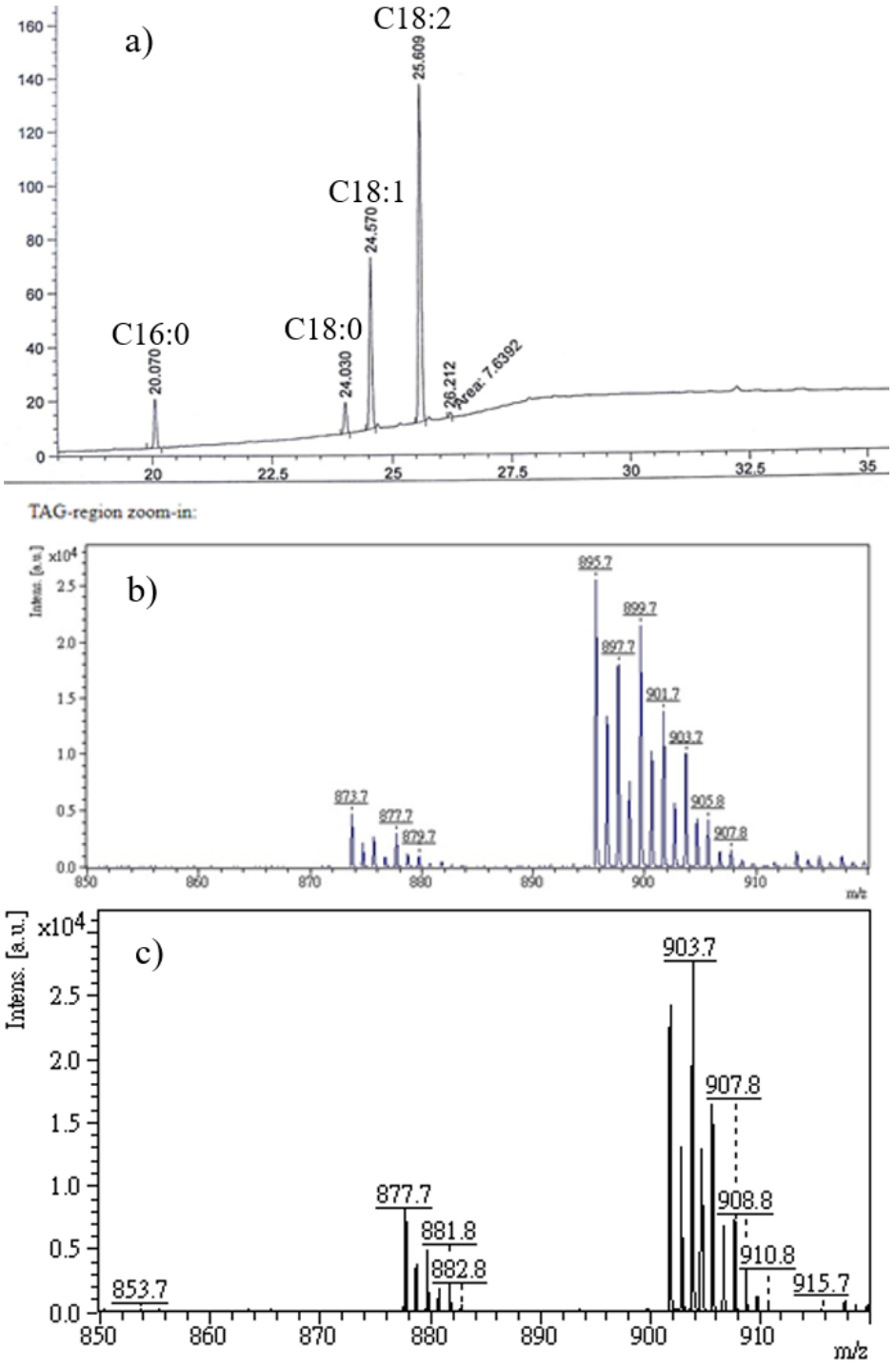

Figure 4-23. (a) The GC-FID result of the faked flaxseed oil sample. (b) A reference MALDI-MS spectrum of flaxseed oil recorded in the home-built database and (c) the MALDI-MS spectrum of the faked flaxseed oil sample.

Table 4-8. PLS-DA results of 5 edible oil counterfeits.

Claimed oil species	PLS-DA result (PS)
Corn oil	Soybean oil (0.99)
Sesame oil	Soybean oil (0.72)
Flaxseed oil	Sunflower oil (0.85)
Safflower oil	Sesame oil (1.43)
Sesame oil	Soybean oil (0.77)

4.3.8 Analysis of edible oil adulterants

Olive oil is considered as one of the most valuable edible oil due to the high unsaturated fatty acid content. Olive oil was mixed manually with sunflower oil, corn and canola oil in different percentage as such blending products are existed in the markets. The mixing of edible oil could change the mass spectra of the edible oils. The blended oils thus could be differentiated from pure oils by spectral comparison as shown in Figure 4-24. Sunflower oil contained higher C18:1 content then olive oil thus stronger peaks of *m/z* 877.7 and *m/z* 903.7 were observed in the spectra. Compared with the pure olive oil, the increases in such peaks in the blended olive oils were clearly observed in the MALDI-MS spectra. The differentiation of pure and blended edible oils thus could be achieved by spectral comparison.

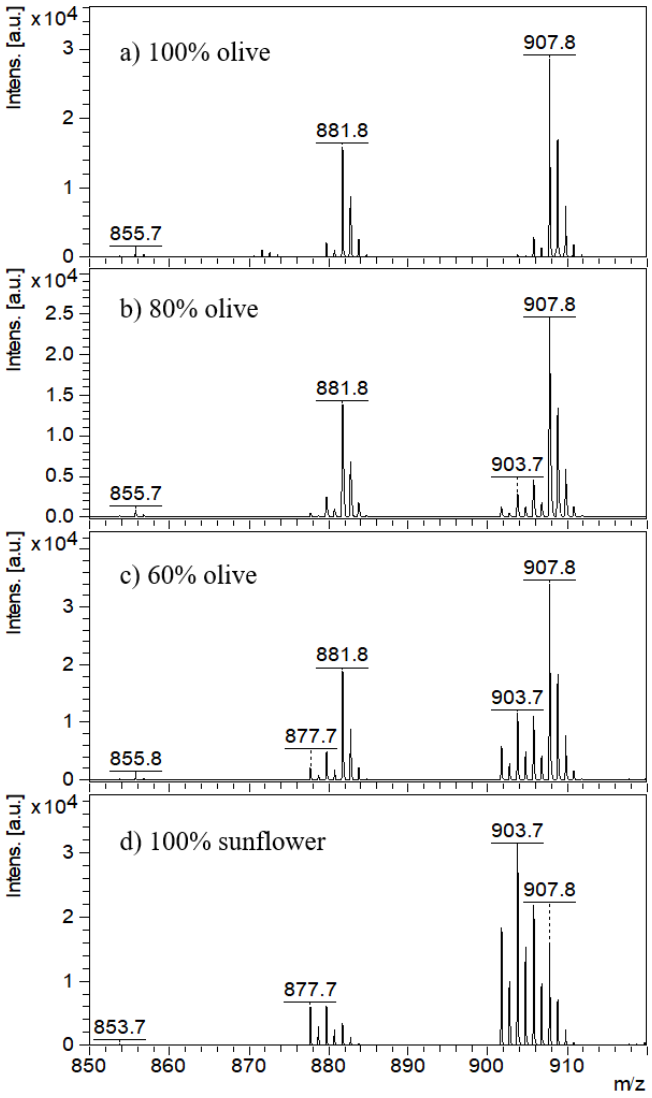

Figure 4-24. Mass spectra showed the TAGs region of (a) 100% olive oil, (b) mixture of 80% olive oil and 20% sunflower oil, (c) mixture of 60% olive oil and 40% sunflower oil and (d) 100% sunflower oil.

The blended oil samples were also analyzed using PSL-DA. It was noticed that a predicted result would be obtained for any data that was input into the PLS-DA models no matter it was a pure edible oil or not. Therefore, both the PS and the mass spectra of the sample should be considered for the result interpretation. It was found that the olive oil samples blended with no less than 20% sunflower oil, 40 % corn oil and 40% canola oil could be differentiated from the pure olive oils as shown in Table 4-9, indicating that PLS-DA should be able to screen out the olive oil adulterants with the above mixing level. Commercially available blended edible oils were also analyzed using the established PLS-DA models. Two blended oils with low olive oil contents could be differentiated from the pure olive oils. The remaining sample, contained unknown olive oil content, was shown to contain significant level of olive oil.

Table 4-9. The PLS-DA results of different olive oil mixtures

Compositions	Matching with olive oil (PS)
Laboratory prepared samples	
5% olive and 95% sunflower oils	Not match
20% olive and 80% sunflower oils	Not match
40% olive and 60% sunflower oils	Not match
60% olive and 40% sunflower oils	Not match
80% olive and 20% sunflower oils	Not match
95% olive and 5% sunflower oils	Matched (0.91)
5% olive and 95% canola oils	Not match
20% olive and 80% canola oils	Not match
40% olive and 60% canola oils	Not match
60% olive and 40% canola oils	Not match
80% olive and 20% canola oils	Matched (0.75)
95% olive and 5% canola oils	Matched (1.00)
5% olive and 95% corn oils	Not match
20% olive and 80% corn oils	Not match
40% olive and 60% corn oils	Not match
60% olive and 40% corn oils	Not match
80% olive and 20% corn oils	Matched (0.59)
95% olive and 5% corn oils	Matched (0.92)
Commercially available products	
Canola oil, palm oil and olive mixture with unknown mixing percentage	Matched (0.86)
20% olive oil and 80% sunflower oil	Not match
10% olive oil and 90% sunflower oil	Not match

* The cut-off PS score was set to 0.56 which obtained from the tested olive oil sample.

4.3.9 Analysis of prolonged heated oils and gutter oils

Spectra of the prolonged heated oils and gutter oil samples are shown in Figure 4-25. In these samples, peaks corresponding to oxidized products, such as peaks at m/z 915-955 were observed, and the TAGs contents in some of the gutter oil samples were relatively low. Gutter oils have diverse compositions and correspondingly there is no universal spectral pattern for their identification, but the differences in the TAGs contents and the presence of extra peaks of the oxidized products could provide clues for distinguishing gutter oils from normal edible oils. As gutter oils are labeled as normal edible oils in order to be sold in the market, identification of mislabeling is a reasonable strategy to screen out gutter oils. In this study, the spectra of the prolonged cooked edible oils and gutter oil samples were exanimated and analyzed using the established PLS-DA models, and the results are shown in Table 4-10. Compared with the predicted scores obtained from the authentic edible oil samples (see Table 4-7), some of the tested gutter oil samples such as s470 and s471 gave either too high or too low predicted scores for the classification, and some of the tested gutter oil samples such as s472 and s473, although classified normally, showed low TAGs content or high level of oxidized products in their spectra (see Figures 4-25d and 4-25e), which were obviously different from those of the authentic edible oil samples. Similar results were

obtained for the prolonged cooked edible oils, which showed additional oxidized products and TAGs patterns that no longer matched with soybean oil.

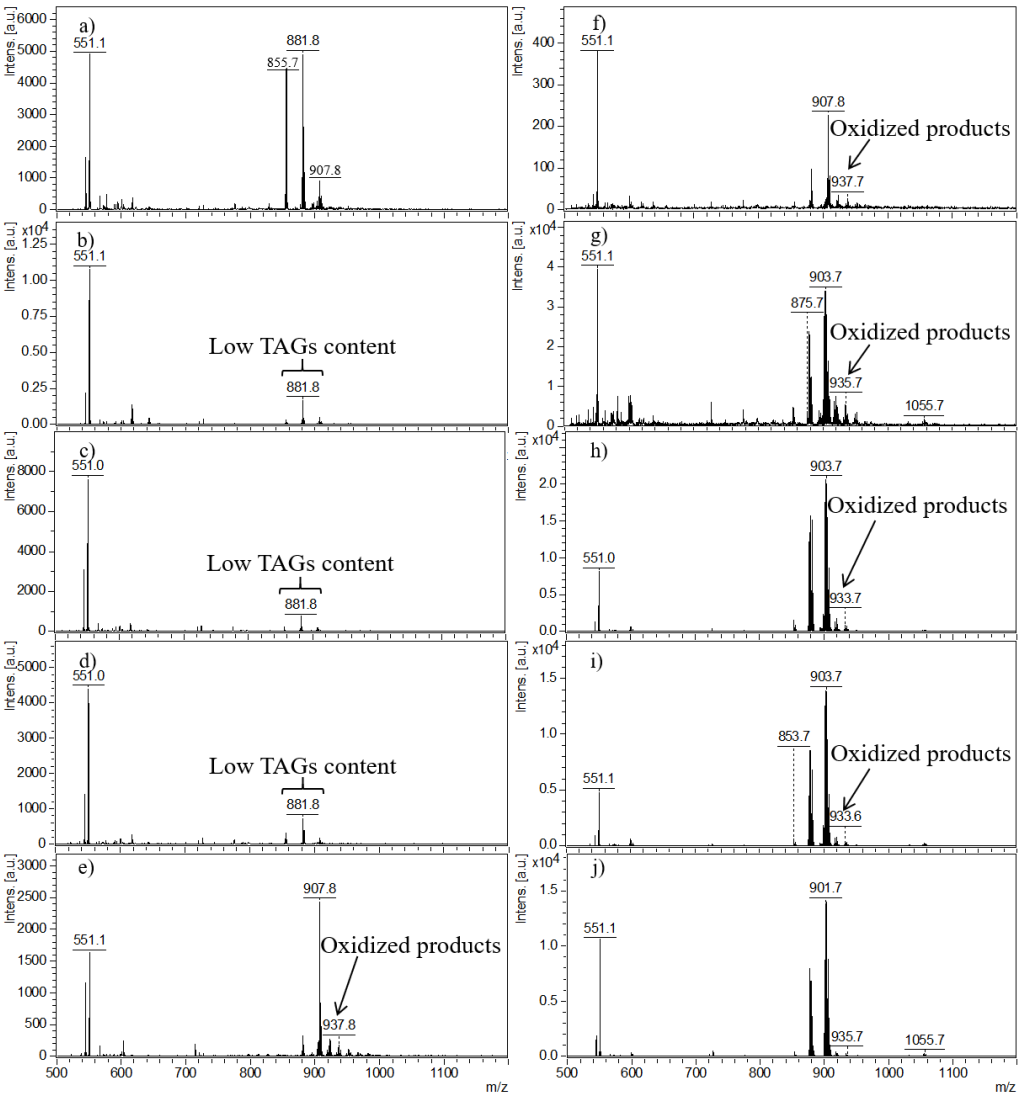

Figure 4-25. The mass spectra of gutter oil samples (a) s469, (b) s470, (c) s471, (d) s472, (e) s473 and (f) s474, and the mass spectra of (g) soybean oil heated for 20 hr, (h) soybean oil cooked with food for 20 hr, (i) soybean oil cooked with food for 16 hr, and (j) soybean oil cooked with food for 8 hr.

Table 4-10. Results for analysis of the prolonged heated edible oils and gutter oils.

Sample No.*	PLS-DA result (PS)	Remarks
s469	Palm oil (0.61)	Score too low
s470	Olive oil (3.31)	Score too high and low TAGs content
s471	Olive oil (2.13)	Score too high and low TAGs content
s472	Lard (0.88)	Low TAGs content
s473	Olive oil (1.13)	High level of oxidized products
s474	Lard (0.84)	High level of oxidized products
s485	Peanut oil (0.72)	High level of oxidized products
s486	Corn (2.27)	Score too high and high level of oxidized products
s487	Corn (1.49)	Score too high and high level of oxidized products
s488	Corn (0.57)	Score too low

* Samples s469-s474 were the gutter oil samples, sample s485 was soybean oil heated for 20 hr, and samples s486, s487 and s488 were soybean oil cooked with food for 8 hr, 16 hr and 20 hr, respectively.

4.4 Conclusion

In this study, a simplified MALDI-MS protocol was established for MALDI-MS analysis of edible oils, and 30 types of edible oils with more than 900 oil samples were analyzed using the present method. Spectral patterns for various edible oils were obtained, and a comprehensive MALDI-MS spectral database was built for analysis of edible oils. Authentication of a labeled vegetable oil could be achieved by comparing its spectrum with those in the database. Based on the spectral patterns and PCA results, edible oils were divided into 8 groups with characteristic peaks for each group. PLS-

DA models were established for classification of edible oils, with overall 97.2% of edible oil products correctly classified from 435 edible oil products. Counterfeit edible oils, adulterated edible oils and gutter oils could also be screened out from normal edible oils using the spectral comparison and PLS-DA models. The whole analytical process only takes several minutes for analysis of one sample. Improved analysis could be achieved with incorporation of more edible oil products, particularly for those species with small sample sizes, into the database. The results of this study showed that MALDI-MS analysis combined with the established spectral database and statistical analysis could be an effective approach for rapid classification of edible oils.

However, there are limitations of the established MALDI-MS method for edible oil analysis. First, it could be difficult to screen out the highly diluted gutter oils and adulterated edible oils with low degree of adulteration, such as lower than 20% as demonstrated in this study. Secondly, some edible oil species, such as almond oil and canola oil shared very similar TAGs patterns, differentiation of those species could be challenging. In those cases, performing complementary analysis using conventional GC-MS method may benefit to the analysis.

Chapter 5: Overall conclusion and prospects

The rapid and improved analysis of two real life issues, i.e., determination of drugs-of-abuse in urine and oral fluid, and authentication of edible oils, using mass spectrometry have been developed in this study.

WT-ESI-MS and SPME-ESI-MS were developed for rapid and sensitive detection and accurate quantitation of six common drugs-of-abuse, including ketamine, methylamphetamine, cocaine, ecstasy, cannabis and heroin, in urine and oral fluid. Only very simple sample preparation and no chromatographic separation are required in the developed methods. WT-ESI-MS is compatible with the existing instruments, thus its setup is simpler than that of SPME-ESI-MS. Analysis of one sample could be finished within minutes by WT-ESI-MS, and took longer time such as 5 – 10 min by SPME-ESI-MS. However, the analysis time of both techniques was still reasonable and much shorter than that by conventional analysis. Both WT-ESI-MS and SPME-ESI-MS showed good linearity and wide linear ranges for quantitation of the targeted drugs-of-abuse with high accuracy and precision, but the results obtained by SPME-ESI-MS for analysis of heroin and cannabis and their metabolites were much better and more suitable for practical analysis. The LODs obtained by WT-ESI-MS could fulfill the

international requirements for detection of targeted analytes except for cocaine, cannabis and heroin. Compared to WT-ESI-MS, SPME-ESI-MS improved LODs 2 – 25 times for analysis of ketamine, methamphetamine, ecstasy, cocaine and their metabolites, and improved more for analysis of heroin, cannabis and their metabolites. The LODs of detection of heroin and its metabolites were improved from a few hundred ng/mL to a few ng/mL after using SPME-ESI-MS and cannabis could only be detected using SPME-ESI-MS with the LODs of a few ng/mL. Overall, detection of all targeted analytes except cannabis in oral fluid using SPME-ESI-MS could fulfill the international requirements. In general, SPME-ESI-MS could be used for sensitive and rapid detection of the six targeted drugs, while WT-ESI-MS could be used for quick detection of samples with high concentrations. However, the analytical performances of SPME-ESI-MS for analysis of morphine and cannabis dropped when other drugs were presented in the samples. One possible solution to improve this is to use SPME tips with different coatings for selective extraction of morphine and cannabis, such as using anion exchange resin for extraction of morphine at high pH. Other ionization techniques can also be used for SPME coupling. For example, DART may be better for ionization and detection of THC. In addition, development of techniques for on-site drugs analysis should be an important direction in the future, especially for the

policemen to inspect suspected drugs drivers. Coupling of SPME with portable instruments such as portable GC-MS may be a feasible way to provide selective and sensitive on-site detection of drugs-of-abuse. However, the performances of such devices for detection of drugs at very low concentrations are still questionable and need further study. Moreover, method validation using real urine and oral fluid samples obtained from drug abusers should be performed before real application.

MALDI-MS has been developed for authentication of edible oils and screening of edible oil counterfeits and adulterants and gutter oils in this study. The edible oil samples could be directly applied on the MALDI-MS plate and the sample preparation time was significantly reduced. A comprehensive spectral database created from over 900 samples and the corresponding PLS-DA models were established for edible oil classification and authentication. The overall correct classification rate of 97.2% out of 435 pure edible oil products has been achieved using the PLS-DA models. However, screening out highly diluted gutter oils and edible oil adulterants with low adulteration levels could be difficult. Such database and models can be improved in future, especially for rare edible oils such as different palm oils and high oleic sunflower oil. The algorithm for the spectral comparison can also be improved, particularly for the

classification of some closely related edible oil species, such as grapeseed oil, sunflower oil and safflower oil. The current data processing procedures still require several manual procedures, such as spectral interpretation, data conversion and PLS-DA analysis. Design of a computer program that can automatically process the data and compare the spectra is highly desirable for the edible oil analysis, and is definitely useful for the non-specialist users from oil industry and other fields.

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