RAPID DETECTION OF PESTICIDES IN HONEY AND JUICE BY ELECTROSPRAY IONIZATION MASS SPECTROMETRY BASED ON FUNCTIONALIZED SOLID SUBSTRATES

YI-CHING CHOI

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The Hong Kong Polytechnic University

Department of Applied Biology and Chemical Technology

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YI-CHING CHOI

A Thesis Submitted in Partial Fulfilment of the Requirements of the Degree of Master of Philosophy

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CERTIFICATE OF ORIGINALITY

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_____ (Signed)

YI-CHING CHOI (Name of student)

Abstract

Detection of pesticide residues in food samples is important to safeguard food quality and safety. Conventional approaches for detection of pesticides in food samples typically involves sample pre-treatment and chromatographic separation before mass spectrometric analysis, and can be time-consuming, labour and material intensive. Electrospray ionization-mass spectrometry (ESI-MS) with solid substrates is a technique that allows direct analysis of raw samples. In this study, we aim to couple ESI-MS with C18 pipette tips (i.e., C18 pipette-tip ESI-MS) and solid-phase microextraction (i.e., SPME-ESI-MS) for rapid detection and quantitation of pesticides in honey and juice.

Honey and juice were chosen as the food samples for the study as they are popularly consumed and pesticide contaminations in honey and juice have been public concerns. Moreover, the large content of sugars in honey and juice, the viscous texture of honey and flesh pulp in juice represent analytical challenges and can serve as interference models for detection of target analytes. Pesticides used for the analysis include atrazine, benalaxyl, carbofuran, dimethoate, imidacloprid, malathion and pirimicarb. C18 pipette tips and SPME tips were used to extract and enrich pesticides in honey and juice samples, and were directly connected to mass spectrometer for qualitative and quantitative analysis.

The analytical performance of C18 pipette-tip ESI-MS and SPME-ESI-MS for analysis of pesticides in honey and juice was evaluated. It was found that the linear ranges varied among pesticides but generally were in the range of 1 – 800 ng mL^{-1}. Linear calibration curves were obtained with R^2 coefficients of 0.943 – 1.000 for C18 pipette-tip ESI-MS

and of 0.951 – 1.000 for SPME-ESI-MS, among which excellent R^2 coefficients (R^2 = 0.993 – 1.000) were obtained for both C18 pipette-tip ESI-MS and SPME-ESI-MS analysis of atrazine and pirimicarb in all food samples. The limits of detection (LODs) and limits of quantitation (LOQs) were 0.3 and 1 ng mL^{-1} respectively for C18 pipette-tip ESI-MS analysis of carbofuran and pirimicarb in honey, and were 0.3 and 0.5 ng mL^{-1} respectively for SPME-ESI-MS analysis of atrazine, benalaxyl, carbofuran, pirimicarb in apple juice. The LODs and LOQs of most pesticides fulfilled the cut-off values (50 ng mL^{-1}) in the international standards. Compared to C18 pipette-tip ESI-MS, SPME-ESI-MS enabled more precise and accurate results, especially for analysis of pirimicarb. The precision and accuracy for analysis of pirimicarb by SPME-ESI-MS were determined to be 3 – 8% and 98 – 100% in honey; 7 – 27% and 91 – 111% in apple juice and 4 – 14% and 100 – 102% in orange juice. These results demonstrated that C18 pipette-tip ESI-MS and SPME-ESI-MS could be used for rapid detection of pesticides in honey and juice, particularly for those pesticides with good responses under ESI conditions.

C18 pipette-tip ESI-MS and SPME-ESI-MS are easy to set up. Analysis of samples by these two techniques requires minimum sample preparation and no chromatographic separation, and results could be obtained within minutes per sample. These techniques could be further extended for analysis of other analytes and sample systems.

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Table 3-5. The precision and accuracy for quantitation of pesticides in orange juice by SPME-ESI-MS.

List of abbreviations

Full form	Abbreviation
aluminium foil	Al foil
apple juice	AJ
carbon 18 pipette-tip electrospray ionization-mass spectrometry	C18 pipette-tip ESI-MS
chemical ionization	CI
Codex Alimentarius Commission	Codex
copper	Cu
dalton	Da
degree Celsius	°C
divinylbenzene	DVB
electron ionization	EI
electronvolt	eV
electrospray ionization-mass spectrometry	ESI-MS
Food and Agriculture Organization	FAO
formic acid	FA
gas chromatography	GC
head-space solid phase microextration	HS-SPME
high-performance liquid chromatography	HPLC
internal standard	IS
kilogram	kg
limit of detection	LOD
limit of quantification	LOQ

liquid chromatography	LC
liquid-liquid extraction	LLE
mass spectrometry	MS
mass-to-charge ratio	*m/z*
matrix-assisted laser desorption/ionization	MALDI
maximum residue limit	MRL
methanol	MeOH
micro	µ
milligram	mg
millilitre	mL
minute(s)	min
multiple reaction monitoring	MRM
orange juice	OJ
pipette tip column electrospray ionization	PTC-ESI
polyacrylate	PA
polydimethylsiloxane	PDMS
quick, easy, cheap, effective, rugged and safe	QuEChERS
relative standard deviation	RSD
signal-to-noise	S/N
solid phase extraction	SPE
solid phase microextraction	SPME
solid phase microextraction-electrospray ionization-mass spectrometry	SPME-ESI-MS
standard deviation	SD

thin layer chromatography	TLC
thin layer chromatography-electrospray ionization-mass spectrometry	TLC-ESI-MS
total ion chromatogram	TIC
wooden-tip	WT
wooden-tip electrospray ionization-mass spectrometry	WT ESI-MS
World Health Organization	WHO

Chapter 1: Introduction

1.1 Pesticides in food

1.1.1 Pesticides and their contaminations in food

Food safety is a global issue which has gained a lot of attention extensively. The human population has raised the awareness of food quality since food contamination is a non-negligible issue nowadays. Pesticides residue is one of the main contaminants found in food products. Pesticides were introduced in mid-twentieth century and since then, they have been widely used in agriculture to reduce the damage caused by pests and also to increase the yield and quality of agricultural products. According to the Food and Agriculture Organization of the United Nations (FAO), World Health Organization (WHO) and The Codex Alimentarius Commission (Codex), pesticides are any substances or chemical compounds that are used to destroy or prevent pests which may damage the crops, unwanted species of plants or animals during the production, storage, transport, distribution, and processing of food.[1] Pesticides can be categorized into different classes according to their functions or the pest that they control. For example, herbicides are used to kill weeds; insecticides are used to kill insects and fungicides are used to kill fungi. Other chemicals such as disinfectants, growth regulator, insect and animal repellent, desiccant, defoliant, fruit thinning agent and sprouting inhibitor are also considered as pesticides. However, fertilizers, plant and animal nutrients, food additives and animal drugs are not considered as pesticides.

The use of pesticides in fields is critical to control diseases in plants. However, the pesticides application must be careful as they are potentially toxic to human and other organisms. Besides, overuse or improper use of pesticides may leave residues in food

products which can be harmful to the consumers. Apart from the direct transfer of pesticides from soil to crops, there are a number of indirect ways for pesticides getting into the food chain.[2] One of the pathways is through the water system. When the pesticides are applied in the field, they could be drifted to soil water or directly to ground water, and can also flow to rivers or sediments. If the contaminated water is used for the crops, field irrigation or animals feeding, pesticides can be transferred to the food products and tertiary transferred to consumers. Some possible ways of pesticides entering the food and water are shown in Figure 1-1.

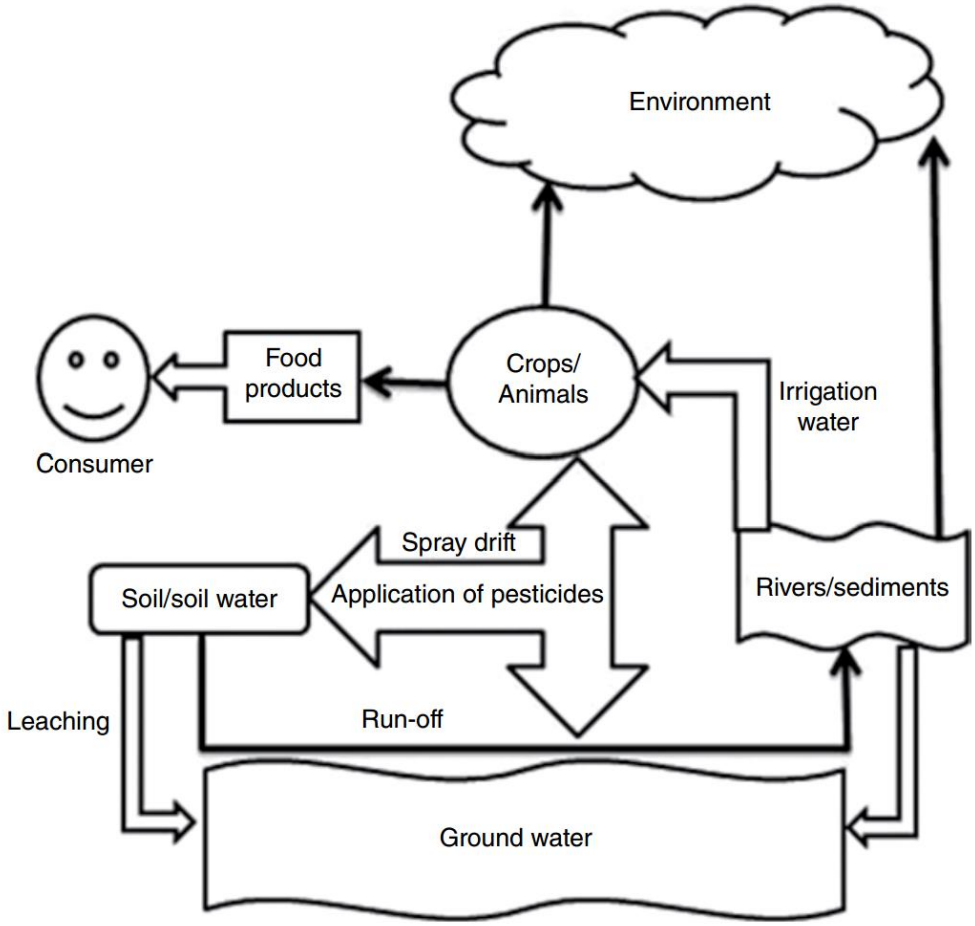


Figure 1-1. Possible routes of pesticides contamination in food and water. (Reprinted from Ref. 2)

1.1.2 Regulations and maximum residue limits of pesticide residues in food

To maintain high level of food quality and confidence of consumer, and to ensure the food provided to the public is safe, there are different international organizations, such as Codex and The European Commission, providing standards, guidelines and code of practice for monitoring pesticide residues in food.[3] The maximum residue limit (MRL) is the highest quantity of a pesticide residue that is allowed in food products (in mg kg^{-1}). In the database provided by Codex, pesticides are categorized into ten classes, including acaricide, aphicide, fumigant, fungicide, generic, herbicide, insect growth regulator, insecticide, nematocide, plant growth regulator, scald control agent, storage scald preventer and synergist. As for the food category, it is mainly divided to food commodities of animal origin and plant origin or processed foods of animal origin and plant origin. For The European Commission, food commodities are basically divided to plants and animal origin. In which plants include fruits, vegetables, cereals, teas, spices and sugar plants whereas milk products and eggs are examples of products originated from animals. The MRLs of some commonly used pesticides in four food categories adopted by The European Commission are listed in Table 1-1.

Apart from international standards, each country may have its own regulation to control and monitor the use of pesticides and tolerance in food production. In Hong Kong, The Pesticide Residues in Food Regulation is implemented in 2012 and is enforced by the Food and Environmental Hygiene Department. A MRL database is designed by the Centre for Food Safety which allows the public to check the MRLs of certain pesticides for specific food commodities. The MRL database of Hong Kong adopts the food classification scheme of the Codex.

Table 1-1. The MRLs (mg kg^{-1}) of seven commonly used pesticides in four food categories adopted by The European Commission.

Pesticide	Fruits/ vegetables	Honey and other agriculture products	Milk	Poultry
Atrazine	0.05-0.1	0.05	-	-
Benalaxyl	0.05-0.3	-	0.05	0.05
Carbofuran	0.002-0.01	0.05	0.001	0.01
Dimethoate	0.02-0.05	-	-	-
Imidacloprid	0.05-5	0.05	0.1	0.05
Malathion	0.01-8	0.05	0.02	0.02
Pirimicarb	0.01-4	0.05	0.05	0.01

1.2 Analysis of pesticides in food: Extraction and detection

There are a number of sample preparation methods for pesticide analysis and the technique should be carefully selected. The selection of an appropriate method depends on the complexity of sample, nature of matrix, property and the concentration of analytes. Sample preparation are sometimes tedious and time-consuming, yet it is crucial for subsequent analysis. In this section, a number of different methods for pesticide residues extraction and detection are introduced. Each method is briefly introduced in this section. Applications of these methods in food analysis will be mentioned in later sections.

1.2.1 Liquid-liquid extraction

Liquid-liquid extraction (LLE) is used to separate analytes from interference by partitioning between two immiscible liquids based on the solubility of the analyte.

Usually one of the phases is organic and the other is aqueous. The hydrophilic analytes tend to dissolve in the aqueous phase whereas hydrophobic analytes tend to dissolve in the organic phase. Analytes which are extracted in the organic phase can be recovered by evaporating the solvent and the analytes extracted in the aqueous phase can be analyzed by techniques such as high-performance liquid chromatography (HPLC).[5]

There are some practical problems of LLE. Firstly, the emulsion of two phases is sometimes encountered especially for fat-containing matrix which a sharp boundary is not seen for separating two phases. Secondary, strong adsorption of analytes to particulate may occur if the sample contains particulate. Thirdly, some of the analytes may bind to very large molecules, such as drugs bind to protein samples. Lastly, the two liquids used may have mutual solubility, making the solutes dissolved in both of them. All of the problems induce lower recovery of analytes from the sample.

1.2.2 Solid phase extraction/solid phase microextraction

Solid phase extraction (SPE) is applied for interference removal and sample purification and enrichment for solid and liquid samples. SPE is often used for the clean-up process, however it can also be used for desalting, solvent exchange and *in-situ* derivatization. In a SPE cartridge, there is a layer of solid packing sandwiched by two layers of frits. The choice of sorbent bed depends on the polarity, acidity and basicity of analyte. Examples of sorbent include silica (for slight to moderately polar analytes), amino and diol (for moderately to strongly polar analytes), quaternary amine (for ionic and acidic analytes) and acids (for basic analytes). Prior to the analysis by SPE, the SPE cartridge is conditioned first. When a sample is analyzed, the sample is passed through the SPE cartridge with solvent added for removing undesired

interferences and stronger solvent used to elute the analytes. The disposable SPE cartridge is designed for single use which reduces the occurrence of sample cross-contamination. Common analytes include organic and inorganic analytes, biological compounds, carbohydrates, metal ions and other classes of analytes. High recovery of analyte is achieved by SPE. In SPE, only small size of sample and organic solvent are required and consumed. However, very volatile analytes may be lost and quantitation is less likely to be achieved. Also, recovery and reproducibility may be an issue.[6]

Solid phase microextraction (SPME), on the other hand, is a similar technique to SPE with the use of coated fibre tip as the stationary phase. The coating material is made of polymer polydimethylsiloxane (PDMS), polyacrylate (PA), Carboxen (CAR) divinylbenzene (DVB), or a mixture of those polymers. The choice of stationary phase depends on the nature of analytes of interest. The SPME fibre tip is inserted into the sample and analytes are partitioned into the coating. When an equilibrium is achieved, the tip is removed from the sample and used for chromatographic analysis. Common analytes include organic and inorganic analytes, biological compounds, drug-of-abuse, pesticides and other classes of analytes. Analytes in complex samples such as animal products, poultry, fish, milk and baby food can also be extrated.[7]

1.2.3 Gas chromatography mass spectrometry

Gas chromatography (GC) is coupled with mass spectrometry (GC-MS) for pesticide analysis. GC is suitable for analyzing volatile pesticide residues in food samples. MS, on the other hand, gives accurate and sensitive structural information of a compound. Combining GC and MS provides specific, sensitive and rapid analysis of a wide range of volatile pesticide residues in food. GC-MS typically consists of sample inlet, GC column, oven, ion source and mass analyzer. A carrier gas is introduced into the column

with a flow rate of 20 - 60 mL min^{-1} and flew through the detector. Inert gases, such as nitrogen, argon and helium, are used as the carrier gas and nitrogen is most commonly used due to its cheap cost.[8]

1.2.4 Liquid chromatography mass spectrometry

Liquid chromatography (LC) is a popular technique for analysing pesticide residues in food samples with high sensitivity. Unlike GC, the analytes do not need to be thermally stable or volatile for LC analysis, therefore relatively thermally unstable pesticides, such as carbamates and organophosphate pesticides, and large and polar pesticide molecules, such as phenoxyl herbicides, rotenoids, dithiocarbamates, can be analyzed by LC.

LC consists of sample injection system, column that is packed with chromatographic material as the stationary phase, solvent as the mobile phase and the detector. The stationary phase is composed of material such as alumina, silica, amino, diol or cyano for normal-phase LC whereas C4, C8 or C18 for reverse-phase LC. In normal-phase LC, the mobile phase uses organic solvents with low to medium polarity such as hexane and isopropanol, whereas reverse-phase LC uses polar solvents such as mixture of water with methanol or acetonitrile. It is not very common to use normal-phase LC for analysing pesticide residues in food due to the instability of column and interference of solvents to the detector. By applying reverse-phase LC analysis, different polarity of pesticide residues can be separated.[9] LC can be coupled with MS for the detection of pesticide residues in food. The details about the detection of MS are discussed in the later chapters.

1.2.5 Quick, easy, cheap, effective, rugged and safe

Quick, easy, cheap, effective, rugged and safe (QuEChERS) is a technique for analysing multi-class pesticide residues in wet food products such as meat, fish, vegetables and fruits. For analysing samples with QuEChERS, a small amount of sample is weighed and mixed with solvent which allows the extraction of pesticide residues in the sample. After the addition of salt and buffer, an aliquot is taken out and gone through dispersive SPE. The added salt separates the organic solvent from the water content originated from the wet sample and also facilitates the extraction of pesticide residues into the organic solvent. After the clean up by the dispersive SPE, the resulting extract is analyzed by HPLC. The analyte recovery is generally high, ranging from 70 - 110%. Apart from food products, environmental samples such as soil, and other classes of compounds such as antibiotics and drugs could also be analyzed by QuEChERS.[10]

1.3 Solid-substrate electrospray ionization mass spectrometry

1.3.1 Electrospray ionization mass spectrometry

MS is a commonly used analytical technique for identification and quantitation of compounds. The mass-to-charge ratios (*m/z*) of ions of an analyte in gas phase and their relative abundances are given in a mass spectrum, which can allow determination of molecular mass, elemental composition and identity of the analyte. A mass spectrometer is comprised of three main components, ion source, mass analyzer and detector. The ion source ionizes the analytes and produces gas phase ions; the mass analyzer separates ions according to their *m/z* ratios and the detector detects and amplifies the signals when the ions reach it and produces a MS spectrum.

MS has been developing and evolving over the last few decades. Different mass analyzers, such as ion trap, orbitrap, time-of-flight, and quadrupole, have been developed. As for the generation of ions, common ionization techniques include electron ionization (EI), chemical ionization (CI), matrix-assisted laser desorption/ionization (MALDI), and electrospray ionization (ESI). Modern MS possesses high accuracy, sensitivity, selectivity and resolution. Recently, portable/miniature mass spectrometers have been introduced.[11]

In this study, electrospray ionization-mass spectrometry (ESI-MS) is focused. ESI is one of the most important ionization techniques used in MS for analysis of various compounds. ESI-MS was first introduced by Fenn et al. in late 1980s for analysis of small molecules and large biomolecules such as oligonucleotides and proteins.[12] In this technique, a liquid sample is introduced by a hollow stainless steel needle (i.e., a capillary) placed in front of the MS inlet. When a high voltage is applied on the needle, electrospray is induced and the sample sprays out to form fine droplets. With the assistance of desolvation gas, the solvents of the droplets evaporate and the droplets shrink, eventually leading to generation of gas-phase ions. The whole process is performed under atmospheric pressure. The ESI apparatus invented by Fenn et al. is shown in Figure 1-2 and the proposed mechanism of ESI is shown in Figure 1-3.

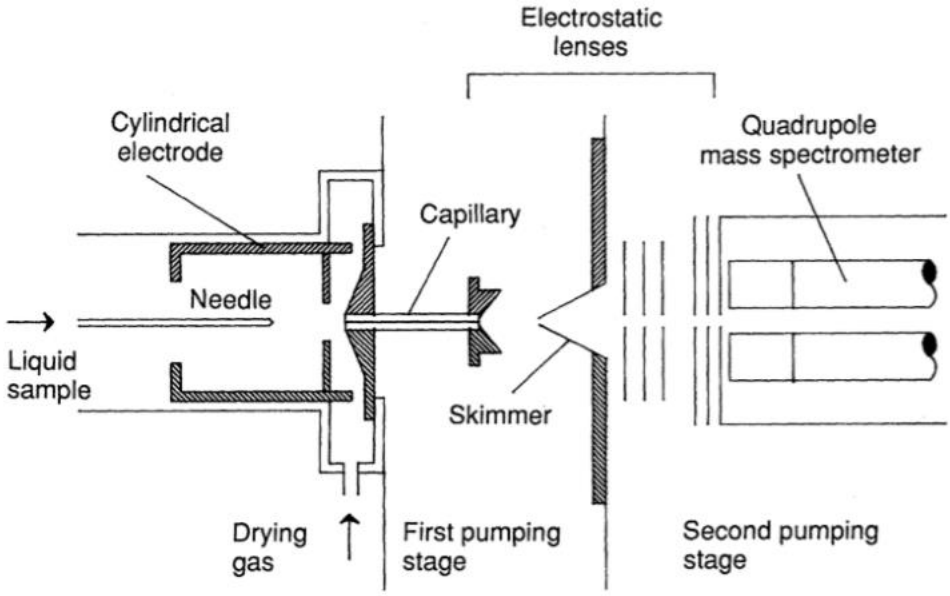


Figure 1-2. A schematic diagram showing the ESI apparatus invented by Fenn et al. (Reprinted from Ref. 12)

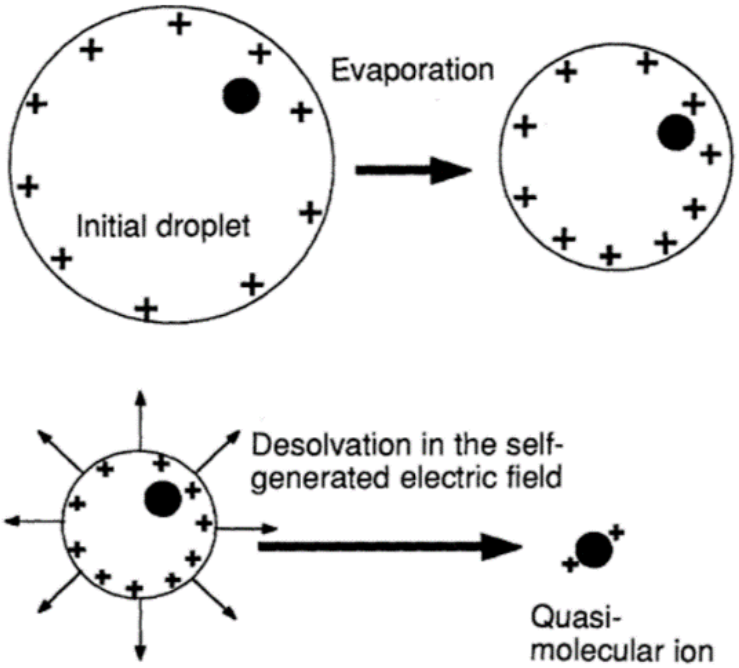


Figure 1-3. The working mechanism of ESI proposed by Fenn et al. The precursor droplets undergo evaporation and desolvation, generating quasi-molecular ions. (Reprinted from Ref. 12)

ESI is considered as a 'soft' ionization technique as only partial residual energy is applied on the analyte and therefore little fragmentation is observed. For molecules which are less than 1000 Da, mainly singly charged ions are generated. As for larger molecules such as proteins, which contain more ionizable sites, multiply charged ions are observed in ESI-MS. For molecules with no ionizable sites, sodium, potassium and acetate adduct ions of analytes are commonly observed.[13]

ESI-MS has been used for analysis of various compounds, including small molecules and large biomolecules, and it can be applied in different fields such as clinical biology,[14-17] biology,[18-19] chemistry,[20] environmental science,[21-22] forensic science,[23] food science,[24-25] plant science[26-28] and other disciplines.[29]

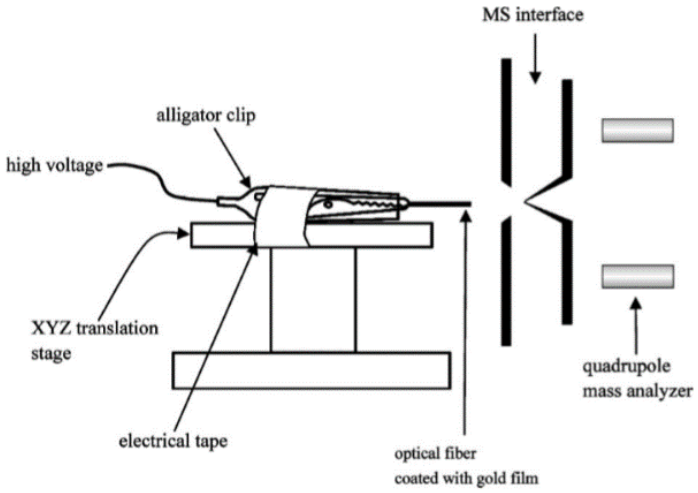
1.3.2 ESI based on solid substrates

Fine capillaries were used for sample introduction and ionization in the early stage of ESI-MS. Since 1990s, scientists have been using other materials, particularly solid substrates, to replace the capillaries. A metallic thin copper (Cu) ring was firstly introduced by Shiea et al. in 1999.[30] A small volume (~1 μL) of sample was directly applied on the Cu ring, and with application of a high voltage onto the Cu ring, ESI was induced. Data showed that the analytical performance was similar to that of ESI-MS using a conventional capillary, but the analytical time required was significantly reduced to ~2 min per sample. The same group also developed surface-modified glass rod as the solid substrate for ESI.[31] In this technique, small quantity of sample was deposited onto a gold-coated fibre tip, and a high voltage was applied onto the fibre to generate ESI. Nanostructured tungsten oxide was also tested as efficient ESI emitter.[32] The diagrams of these solid-substrate ESI-MS techniques are shown in Figure 1-4.

Another group of scientists, Hiraoka et al., proposed the use of solid needles as sampling and ionization probe for ESI-MS analysis of wet samples, including peptides and proteins in 2007.[33-34] Very recently, they developed disposable gel-loading tips for ESI-MS.[35] In this technique, a disposable metal wire was inserted into a gel-loading tip for high voltage connection and inducing electrospray. This device was used for biological tissue diagnosis, with a relatively low flow rate (~100 nL min^{-1}) enough to give a satisfactory result. The schematic diagrams of the solid-substrate ESI-MS techniques invented by Hiraoka's group are shown in Figure 1-5.

(a)

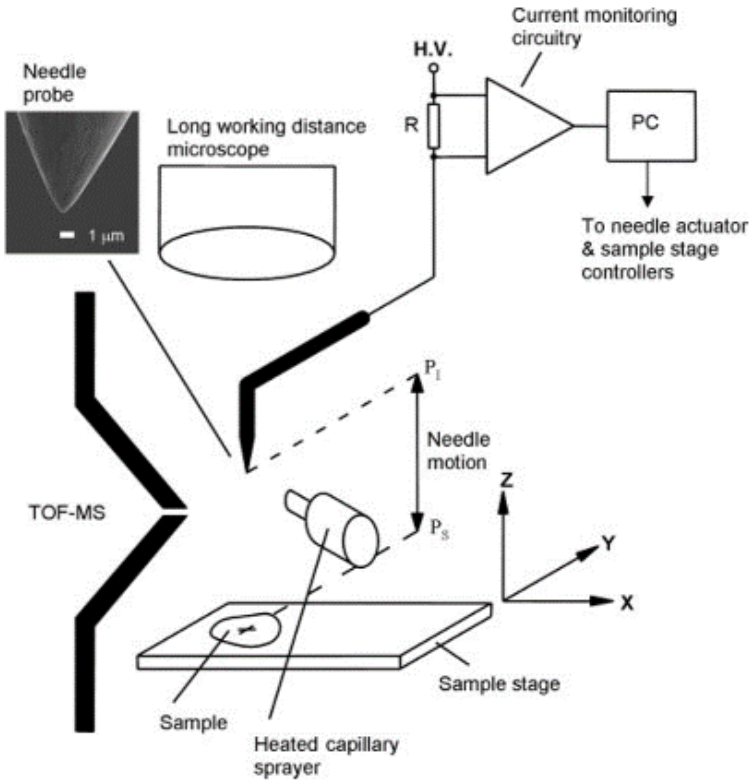
(b)



(c)

Figure 1-4. Schematic diagrams of ESI using solid substrates: (a) copper ring, (b) optical fiber coated with a gold film, and (c) nanostructured tungsten oxide. (Reprinted from Ref. 30, 31 and 32 respectively)

(a)



(b)

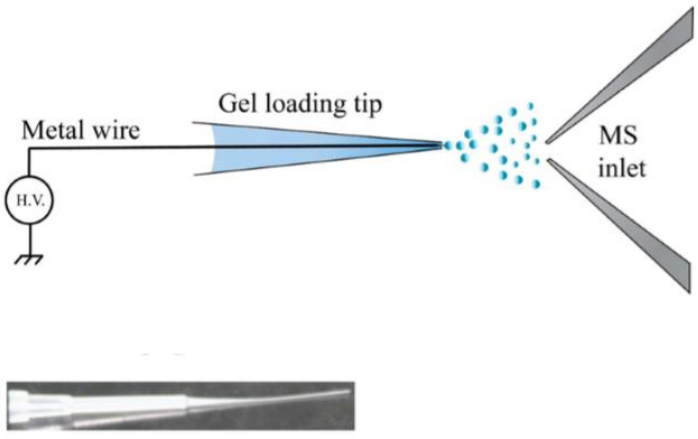


Figure 1-5. Schematic diagrams of ESI using (a) solid needle and (b) gel-loading tip as an emitter. (Reprinted from Ref. 33, 34 and 35 respectively)

Paper spray ionization was introduced in 2010 by Cooks et al. for direct analysis of complex mixtures.[36] In this technique, low-cost paper was used for sample loading and acted as ESI emitter for ionization of analytes. To enhance the ionization efficiency, paper was cut to a triangular shape with an angle of 30°. A metal clipper tip was then connected with the blunt end paper and the sharp triangular end was pointed to the MS inlet. The sample solution was deposited directly onto the paper. With the addition of spraying solvents and a high voltage, ESI was generated. Paper spray ionization was demonstrated for therapeutic drug monitoring,[37] quantitation of pharmaceuticals in blood,[38] direct biomedical analysis[39-41] and clinical diagnosis.[37] The set-up of paper spray ionization is shown in Figure 1-6a.

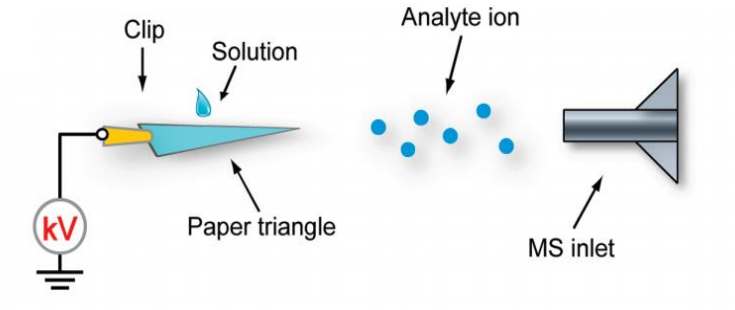
In 2011, wooden-tip electrospray ionization-mass spectrometry (WT ESI-MS) was developed by Hu et al.[42] The WT used was commercially available and disposable wooden toothpicks. To make the WT functions as an ESI emitter, it is firstly cut to produce a sharp tip end and is then mounted onto a nano-ESI ion source. This technique is compatible with most of MS as no hardware modification is required. The sample solution is dipped onto the WT directly. With the application of spraying solvents and a high voltage, ESI is generated. As wood is hydrophilic and porous, sample can adhere on the surface. Proteins, peptide digests, organic compounds and powder samples can be analyzed by this method. The set-up of WT ESI-MS is shown in Figure 1-6b. WT ESI-MS was also applied for detection and quantification of drugs-of-abuse in biological fluids,[43] rapid identification and differentiation of closely-related plant species,[44] quality assessment of Chinese medicine and discrimination of the plant origin by combing with multivariate statistical analysis.[45] When WTs are chemically modified,

it can be used to extract ultra-trace perfluorinated compounds (PFCs) in environmental samples and complex biological samples.[46]

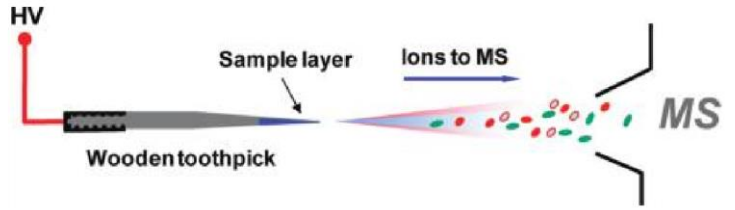
Apart from WT, another technique that uses daily-life material, aluminium foil (Al foil), as substrate for ESI was demonstrated.[47] Al foil is cheap and readily available in the supermarket. It is very soft, easily adjustable and folded for sample holding. The hydrophobic nature of the foil allows sample pre-treatment steps such as extraction and desalting performed on its surface directly. Also, it is conductor of heat and electricity, which allows on-target heating and thermal reactions monitoring. A diagram showing the set-up of ESI using Al foil is shown in Figure 1-6c.

Thin layer chromatography (TLC) plate coupled with MS for direct analysis of raw samples was also developed.[48] The TLC plate could absorb interfering background such as salts and detergents and separate target analytes from complex matrix. TLC-ESI-MS was found to be effective for detection of target analytes in raw biological fluids (illicit drugs in urine) and extracts from plants by both on-line and off-line sampling methods. The set-up of ESI using TLC plate is shown in Figure 1-6d.

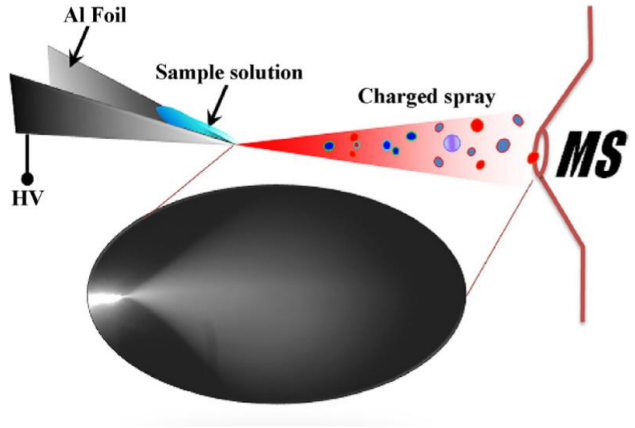
(a)



(b)



(c)



(d)

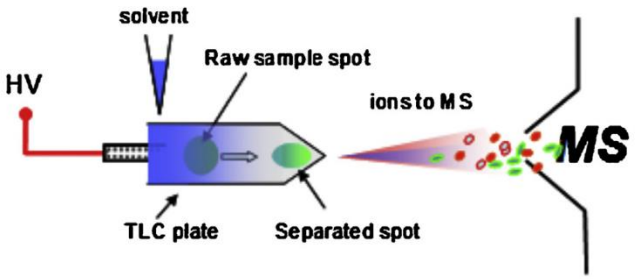


Figure 1-6. Schematic diagrams showing ESI with (a) paper, (b) wooden-tip, (c) aluminium foil and (d) TLC plate. (Reprinted from Ref. 36, 42, 47 and 48 respectively)

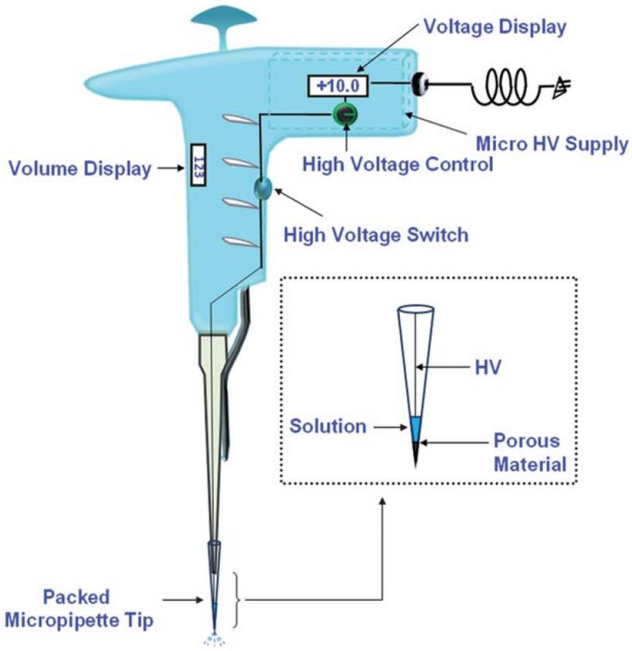
The use of pipette tips as ESI emitters has attracted a lot of attention in recent years. As the pipette tips have a relatively sharp tip end, ionization directly from the pipette tips is possible. Also, the cavity of tip can act as a solvent/sample reservoir, a place for loading the sample or the solvent for ESI. Feng et al. introduced a handheld pipette tip column electrospray ionization source (PTC-ESI source) for ESI-MS analysis of organic compounds and protein in complex mixtures.[49] The whole device involved the use of a modified micro-pipette tip column which looked like a normal pipette tip, but a micro high voltage power supply and its control and switch were also included. The connected pipette tip was packed with porous adsorbent which as used for removing impurities, extracting and concentrating the target analytes. Therapeutic compounds and bioactive chemicals in complex biological samples such as urine and blood were detected and identified using this technique in that study. A schematic diagram showing the set-up of the technique is shown in Figure 1-7a.

Wang et al.[50] developed pipette-tip ESI-MS which combined pipette tips with syringe and syringe pump for analysis of solid samples. In this technique, solid sample was placed inside the pipette tip and retained by a small degreasing cotton swab, which was put in the sharp end of the pipette tip. A syringe containing the extraction solvent was inserted into the pipette tip and connected high voltage supply. Upon the application of a high voltage, ESI occurred. Various power samples such as Chinese medicine, lotus plumule, black pepper, ginseng and coffee beans were tested, and strong and durable signals were detected. The set-up of pipette-tip ESI-MS is shown in Figure 1-7b.

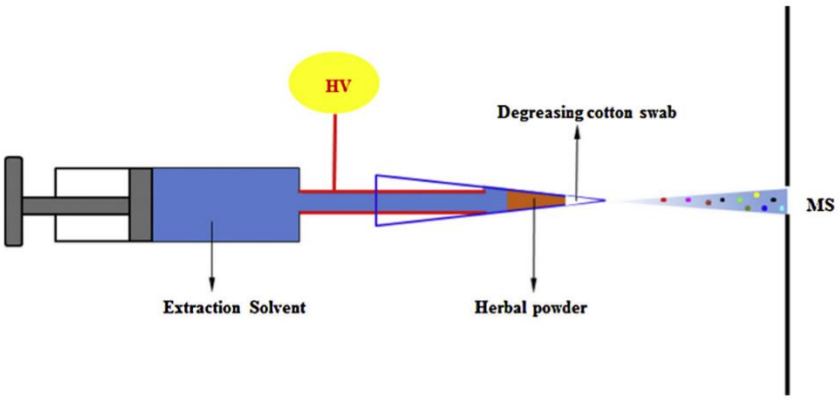
The above technique was later modified for the analysis of raw sample solutions.[51] This time, pipette tips containing C18 sorbent were used. This non-polar C18 resin can be

used for removing common interference such as salts and detergents in protein samples before MS analysis. The hydrophobic protein samples can be retained by the C18 sorbent while the hydrophilic salts and detergents were washed out. C18 pipette-tip ESI-MS was applied for quantitation of ketamine and its major metabolite norketamine in human urine samples. The set-up of pipette-tip ESI-MS is shown in Figure 1-7c.

(a)



(b)



(c)

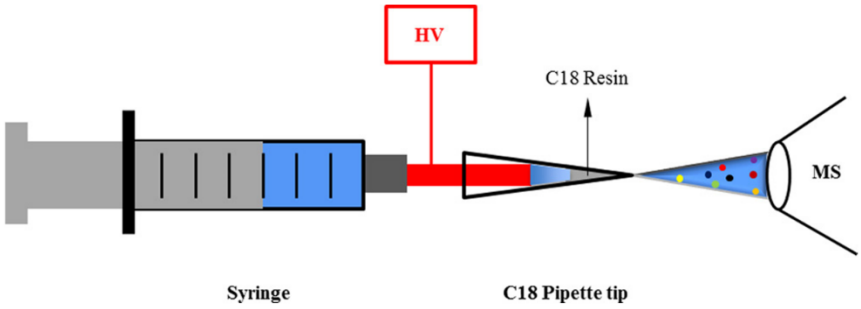


Figure 1-7. The set-up of (a) PTC-ESI source, (b) pipette-tip ESI-MS and (c) C18 pipette-tip ESI-MS. (Reprinted from Ref. 49, 50 and 51 respectively)

1.4 Research objectives

For most of the food samples, sample pre-treatment and chromatographic separation is required before they can be subjected to mass spectrometric analysis. Sample pre-treatment and chromatographic separation could be tedious, time-consuming, labour and chemicals intensive. Fast and efficient methods are highly preferred, particularly for fast screening of pesticide residues in foodstuff, which is important for guarding the food quality and for safe human consumption. In this study, we aim to develop new methods for rapid detection and quantitation of pesticide residues in food samples using solid-substrate ESI-MS.

Honey and juice samples were selected for investigation in this study because of the nature, matrix background and viscosity of the sample. Honey and fruit juice contain many ingredients such as saccharides and flavorloids. For some ready-to-drink fruit juice, they may contain pulps, as the fruit flesh is also nutritious for consumption. Besides, honey has a very viscous gel-like texture. These can serve as interference model for MS detection of pesticides.

This thesis is divided into three parts. In Chapter 2, C18 pipette-tip ESI-MS was developed for rapid detection of pesticides in honey and juice samples. In Chapter 3, SPME was coupled with ESI-MS for rapid detection of pesticides in honey and juice samples. Finally, an overall conclusion is drawn in Chapter 4. The perspectives on the applications of the introduced methods for future study is also included.

Chapter 2: Analysis of Pesticides in Honey and Juice by C18 Pipette-tip ESI-MS

2.1 Introduction

Honey is a popular agricultural product for human consumption due to its sweetness and nutrition. Honey is produced by the honey bees (*Apis mellifera*). The bees work as a group and form a colony. In each bee colony, there is a queen bee and others are worker bees. The queen bee is distinguished in the colony; it is relatively giant and responsible for the continuity of the species. The queen bee fertilizes three kinds of bees, queens (females), workers (females) and drones (males). The fertilized eggs are laid into the honeycomb which later forms worker bees whereas the young queens become larvae. Larvae are fed with royal jelly initially which is produced by worker bees, later on they are fed with honey and pollen. The duties of worker bees include combs construction, hive guardian, food gathering and young bees raising. As for the drones, the main function of them is to fertilize the queen. After that, the worker bees keep them out of the beehive and they tend to die because of starving. The queen bees do not mate with drones generated from their home colony.

Honey bees collect nectar and pollen from flowers to produce honey; they also collect resin from trees to make propolis which is used to build the hive. The collected pollen is stored the comb cells and it supplies rich protein and vitamin for the hive. Pollen contains around 6 – 28% proteins by weight and contains 10 amino acids which are essential for bees. On the other hand, nectar is the carbohydrate supply for the hive as it contains around 5 – 80% sugars but less than 0.2% proteins. Honey bees will convert

the nectar to honey by removing the water inside by the moving their wings swiftly and the addition of enzyme secreted by their salivary glands. The enzyme converts sucrose to glucose and fructose. Only when the water content in the nectar is reduced to 18% or below, honey is formed. “Bee-bread” is a mixture of honey and pollen, and the main source of food for most larvae and bees. Queens larvae are fed with another food called royal jelly, which has content very similar to bee-bread but contains almost triple amount of honey (34% vs. 12%).[52]

Honey is thus a complex substance made when the nectar and sweet deposits from plants and trees are gathered, modified, and stored in the honeycomb by honey bees as a food source for the colony. A number of studies indicated that some species of honey may have the antimicrobial and antiseptic properties.[53-55] The main ingredient of honey are mainly sugars, such as monosaccharides (glucose) and disaccharides (sucrose) and water. It may also contain some minor components such as amino acids, enzyme, proteins, vitamins and flavorloids.[56]

Honey may be contaminated by pesticides by both direct and indirect ways. Direct contamination is due to improper beekeeping practices. Indirect contamination includes environmental and agricultural aspects. For instance, a lot of pesticides are being used to protect the plants from diseases and to increase the agricultural yield and products. The pesticide residues may accumulate in the crops and transported by the honey bee and carried to its hive and consequently contaminate the product, i.e. honey.[57-58] A number of direct and indirect pathways of pesticide contamination in honey are shown in Figure 2-1.

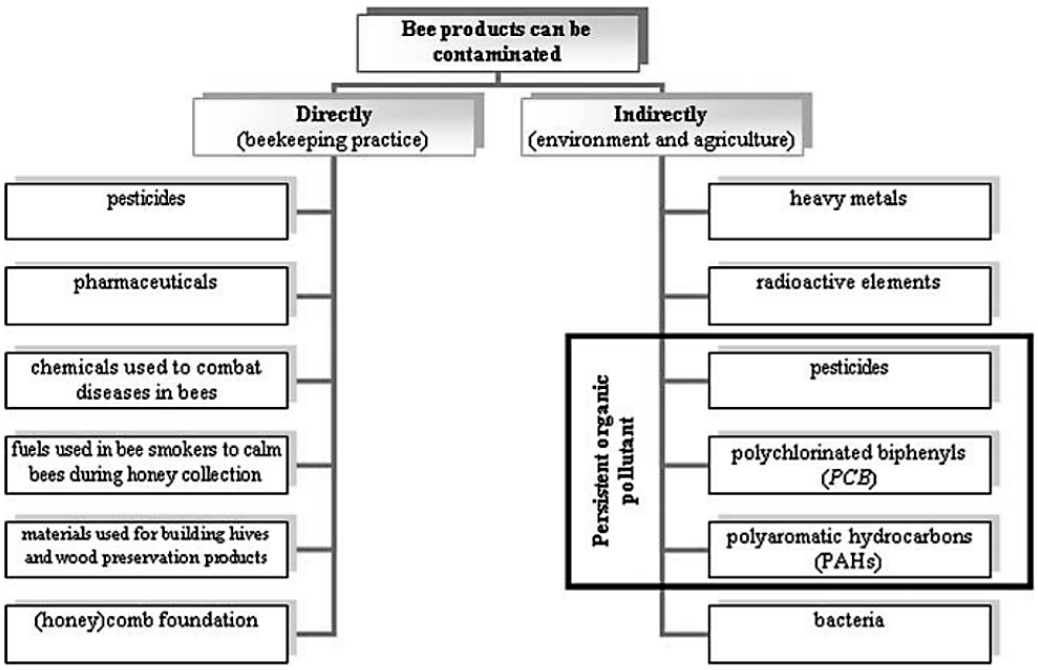


Figure 2-1. Direct and indirect pathways of pesticide contamination in honey. (Reprinted from Ref. 58)

Monitoring of pesticide residues in honey not only ensures the quality of honey for human consumption, but also serves as an indicator of environmental pollution. As the honey bees collect the nectar from plants, they associate with the environment very frequently. Honey bees and bee products can be used to assess the level of environmental contamination with toxic substances such as pesticides, heavy metals and radioactive elements.[58-61]

Fruit juices, on the other hand, are one of the most popular beverages sold worldwide. Examples of fruit juices include apple juice, orange juice, blackcurrant juices, peach juice cranberry juice, mango juice and et cetera. According to Codex, juice is defined as 'unfermented but fermentable juice, intended for direct consumption, obtained by the mechanical process from sound, ripe fruits, preserved exclusively by physical means'.[62] There are many designations for fruit juices and some of them are shown in Table 2-1.

Table 2-1. Some common juice designations (Reprinted from Ref. 62).

Term	Criteria	Remarks
Pure juice 100%	All juice	No adjustment, not from concentrate
Fresh squeezed	Not pasteurized	Held refrigerated, Food safety concerns
Chilled, ready to serve	All juice	Held refrigerated, made from concentrate or pasteurized juice
Not from Concentrate	Single strength	Pasteurized after extraction
From concentrate	Made from concentrate	Reconstituted and pasteurized
Fresh frozen	Unpasteurized	Single strength, frozen after extraction
Juice blend	All juice	A mixture of pure juices
Puree	Pulp-containing	More viscous than juices, totally fruit
Nectar	Pulpy or clear	Sugar, water and acid added, 25 to 50% juice*
Nectar base	Requires reconstitution	Possesses sufficient flavour, acid and sugar to require water dilution for consumption*
Juice drink	Low in juice	Contains 10 to 20% juice*
Juice beverage	Low in juice	Contains 10 to 20% juice*
Juice cocktail	Low in juice	Contains 10 to 20% juice*
Fruit + ade	Lemonade	Contains >10% fruit juice, sugar and water*
Juice extract	Water extract	Fruit extracted by water, then concentrated*
Fruit punch	Token juice	~ 1% juice, + natural flavours
Natural flavoured	Token juice	Usually >1% juice

*Differing country standards for juice solids minimum

Fresh fruit juice is produced by mechanically squeezing of the fruit and filtration of the pulp and seeds. Meanwhile, some of the ready-made fruit juices are prepared by the dilution of concentrated juices with addition of preservatives or other ingredients. Fresh fruit juice is pasteurized before selling out to the market to ensure the bacteria level is safe for human consumption and to extend the shelf life. The apple juice (AJ) used in the study of this chapter was made from concentrated apple juice. The main ingredients of the apple juice are sugar and preservatives. According to FAO, the total sugar content of juice is between 12 –14%, including the initial amount from the fruit itself and the later added in. The chemical and physical properties of fruit juices may be different among species, planting region, environment and conditions.[62]

Pesticide contamination in juice sample has been a public concern. Farmers may apply pesticides to their fruit crops in order to prevent plant disease and the invasion of insects. If the pesticides applied do not degrade properly, pesticide residues may penetrate into the plant tissues and migrate to the fruit pulp and juice. If the pesticides go into the fruit pulp, it is difficult to remove it completely.[63] Also, it is common that the pesticide residue level is higher after juice processing steps, compared with the natural and untreated fruit.

A lot of research has been done on the analysis of multiple pesticide residues in honey and juice. These samples are required to undergo extraction process before MS analysis. Typical extraction methods include liquid-liquid extraction (LLE),[64-65] solid phase extraction (SPE),[64, 66-68] and solid phase micro-extraction (SPME). After the extraction process, pesticide residues can be separated by chromatography such as GC[65-66, 68-74] and LC[63, 74-84] depending on the nature of the pesticides. Development of rapid and

simple methods to avoid complicated sample pre-treatment and chromatographic separation is highly desirable for analysis of pesticide residues in food samples.

In this study, C18 pipette-tip ESI-MS was developed for analysis of pesticides in honey and juice samples. The C18 pipette tips were used for rapid purification and enrichment of pesticides in the raw samples. The sugar contents and other polar components in the tested food samples could be effectively removed, leading to the enhanced detection of pesticides. Since no chromatographic separation and only minimal sample preparation were required in the whole process, the analysis time was significantly reduced and results can be obtained within minutes.

2.2 Experimental section

2.2.1 Materials and chemicals

Pesticide standards including atrazine, benalaxyl, carbofuran, dimethoate, imidacloprid, malathion, pirimicarb and pirimicarb d-6, and reagents such as methanol, acetonitrile (HPLC grade) and formic acid (FA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was prepared by a Milli-Q system (Millipore Laboratory, USA). The C18 pipette tips, i.e., 10 µL ZipTip which contains 0.6 µL of C18 resin, were purchased from Merck Millipore Corporation (Darmstadt, Germany). The C18 resin was made of silica with a diameter of 15 µm and pore size of 200 Å.

2.2.2 Sample preparation

Honey and apple juice sample were purchased from a local supermarket and convenience store (Wellcome and 7-11 convenience store, Hong Kong). The brand of

honey sample was Po Sang Yuen Acacia Flower Honey (100% pure bee honey). Honey sample was diluted 10 times with water before use. The brand of apple juice was Mr. Juicy Fuji apple juice drink. Apple juice and diluted honey and were centrifuged at a speed of 4000 rpm for 15 min to remove the solid particles and to prevent blockage of the C18 pipette-tips. Only the supernatant was collected and used. Sample preparation was made on the same day with the instrumental analysis.

Stock solution (1 mg mL^{-1}) of all pesticides and internal standard (IS) solution of pirimicarb d-6 (1 mg mL^{-1}) were prepared in methanol. Working solutions were prepared by diluting the stock solution in water. For the quantification of pesticides in honey or juice samples, 200 µL of sample was spiked with working solutions of pesticides to concentration of 1, 3, 10, 25, 50, 100, 200, 500, 1000 ng mL^{-1} separately and 2 µL (10 ng mL^{-1}) of internal standard solution was added to each solution.

Pesticides were separated into two groups for quantitative analysis. Atrazine, benalaxyl, carbofuran, and pirimicarb were spiked together whereas dimethoate, imidacloprid and malathion spiked together in samples, because of their similarities in MS response.

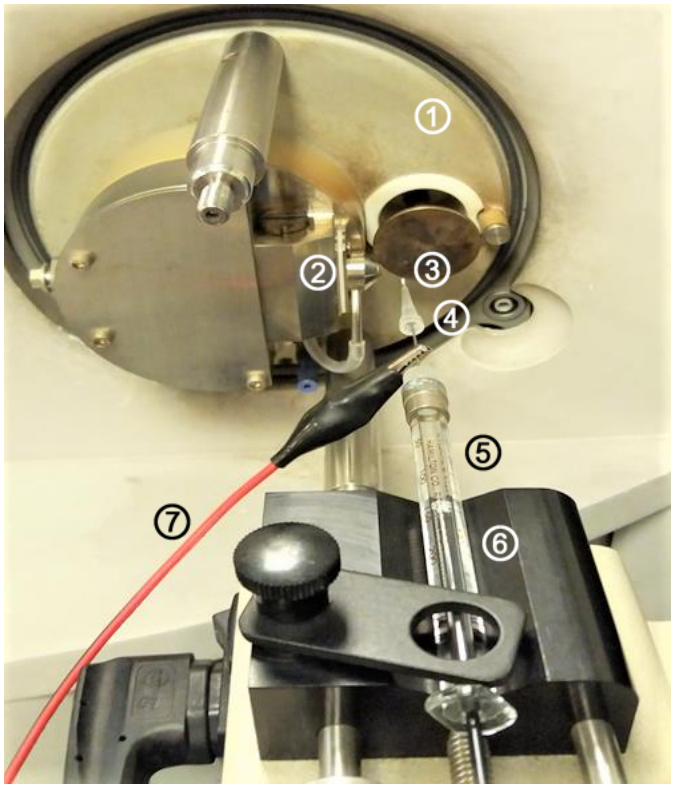
2.2.3 Instrumental set-up and workflow

For conventional ESI-MS analysis of honey spiked with pesticide standards, honey was firstly diluted ten times by water and spiked with pesticide standards (100 ng mL^{-1}) for direct infusion.

For C18 pipette-tip ESI-MS analysis, the C18 pipette tip was firstly pre-washed with methanol and then water each for three times (10 µL each) before use. Then the tip was

inserted into the sample and pipetted in and out for 8 times for sample extraction and enrichment. Finally, the tip loaded with sample was washed with water for 3 times. After the washing step, the C18 pipette-tip was connected to a syringe pump for ESI-MS analysis.

The set-up of C18 pipette-tip ESI-MS is shown in Figure 2-2. In brief, the stainless steel needle (interior diameter: 410 mm; outside diameter: 720 mm) of a glass syringe (250 µL, Hamilton) was inserted into the C18 pipette tip for solvent supply. The C18 pipette tip was placed at 0.5 cm parallel and 1 cm perpendicular to the MS inlet. When a high voltage (+4.0 kV) was applied to the needle by an alligator clip and wire, spraying solvent was also supplied. The spraying solvent used was MeOH: H_2O: FA (60: 40: 0.1 v/v/v) and supplied by a syringe pump (Harvard Pump 11 Plus, USA) at a flow rate of 5 uL min^{-1}. Signals were acquired for around 30 s and after that, the high voltage was turned off. All of the experiments were performed on a triple quadruple mass spectrometer (Quattro Ultima, Waters, USA) in positive ionization mode. The voltage, cone voltage and source temperature were set at 4 kV, 30V and 100 ℃, respectively. The desolvation gas was turned off. Signals were detected under (multiple reaction monitoring) MRM mode. The dwell time for each selected reaction was set at 0.1 s.



Key:

① Mass spectrometer

② MS inlet

③ C18 resin

④ C18 pipette-tip

⑤ Syringe

⑥ Syringe pump

⑦ High voltage supply

Figure 2-2. The set-up of C18 pipette-tip ESI-MS.

2.2.4 Method validation of C18 pipette-tip ESI-MS

Five different concentrations of pesticides were used to construct the calibration curves, and the data of each concentration was obtained by three individual measurements. Peak height was used for plotting the calibration curves. Honey and juice samples were tested following the protocol and samples with the absence of pesticides were used as blank samples in the preparations of standards. A blank sample was tested first in order to confirm that the C18 pipette tips was not contaminated with pesticides and to detect the electronic noises and instrumental variations.

The LODs and LOQs of the pesticides were determined by comparing the peak height of the analytes with the average heights of the background noises. The LODs and LOQs

were determined as the concentrations of the analytes that could give signal-to-noise ratios (S/N) of three and ten, respectively.

The precision were measured by analyzing at least two different concentrations (one low and one high concentration within the linear range) of the pesticides, with each data obtained by five individual measurements. The precision was presented as relative standard deviation (RSD) which was calculated as: (SD of the measured concentrations ÷ mean of the measured concentrations) x 100%.

The accuracy was determined by using the spiked recovery method. In which at least two quantitative levels of the pesticides (one low and one high concentration within the linear range) were spiked into the blank sample separately and performed the analysis, and each was analyzed in five replicates. The detected amount was calculated using corresponding calibration curves. A comparison of the detected amount and the spiked amount demonstrated the recovery of the method, which was an estimate of the accuracy of method. The accuracy (recovery) was calculated as: (the measured concentration ÷ the spiked concentration) x 100%.

2.3 Results and discussion

2.3.1 Effect of C18 pipette-tip extraction on ESI-MS analysis of pesticides in honey

Seven commonly used pesticides were chosen in this study, namely atrazine, benalaxyl, carbofuran, dimethoate, imidacloprid, malathion and pirimicarb. They are classified as herbicides (atrazine) or insecticides (pirimicarb, imidacloprid, carbofuran, benalaxyl, dimethoate and malathion). Honey sample spiked with pesticide standards was firstly analyzed by conventional capillary-based ESI-MS and the result is shown in Figure 2-

3a. As shown in the spectrum, the sugars in the honey sample were predominant and most of the pesticides signals were suppressed. The saccharides in honey were observed in metal adduct forms such as [Glucose + Na]$^+$ at *m/z* 203, [Glucose + K]$^+$ at *m/z* 219, [Sucrose + Na]$^+$ at *m/z* 365, [Sucrose + K]$^+$ at *m/z* 381, [2Glucose + Na]$^+$ at *m/z* 383 and [2Glucose + K]$^+$ at *m/z* 399.

The honey sample spiked with pesticides was then analyzed by C18 pipette-tip ESI-MS. The resulting spectrum (Figure 2-3b) showed that with C18 pipette-tip extraction, all sugar signals were significantly reduced, and the pesticides (in the protonated form) could be predominantly observed. This result demonstrated that C18 pipette tips could be used to effectively remove the sugars in honey and extract the analytes, i.e. pesticides, from the complex matrix and C18 pipette tips could efficiently coupled with ESI-MS for rapid detection of the extracted analytes.

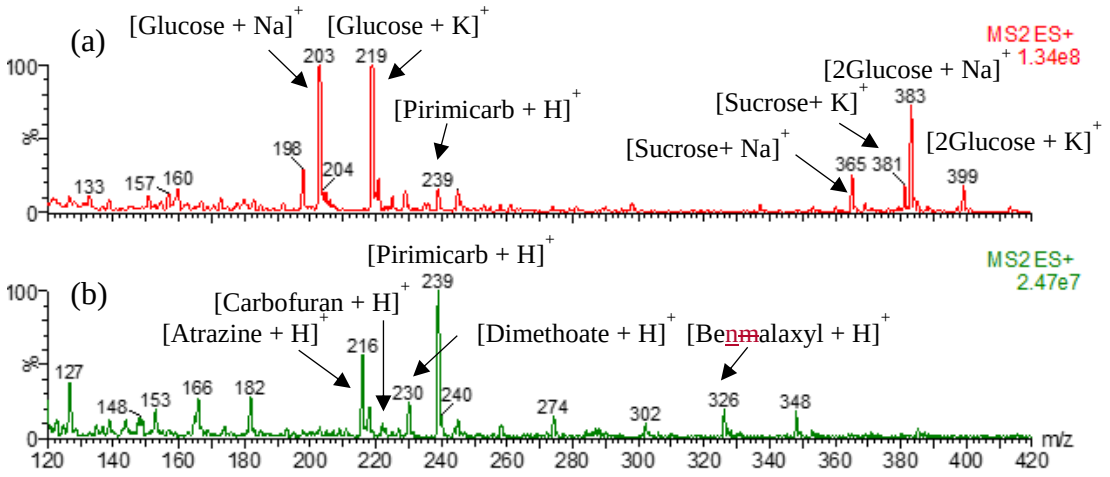


Figure 2-3. Mass spectra of honey spiked with 100 ng mL^{-1} pesticides of atrazine, benalaxyl, carbofuran, dimethoate, imidacloprid, malathion and pirimicarb, obtained by (a) direct infusion ESI-MS and (b) C18 pipette-tip ESI-MS.

2.3.2 Optimization of the MRM conditions

The MRM conditions, i.e. collision energy and the precursor ion/product ion pair, were optimized to achieve optimal sensitivity. The optimization for analysis of imidacloprid is taken as an example herein and the results are shown in Figure 2-4. Initially, the collision energy was set to 2 eV and it was increased gradually until a strong and stable signal of transforming precursor ion to product ion was obtained, and that energy was chosen for the MRM channel of the analyte. For imidacloprid, collision energy of 10, 12, 15 and 20 eV were tested and 12 eV was chosen as the optimized collision energy for imidacloprid; precursor ion *m/z* 256 with product ions *m/z* 209 or 175 were chosen as the ion pair for MRM due to their better responses. The information about the chemical structure, precursor ion, product ion and collision energy of the pesticides are shown in Table 2-2.

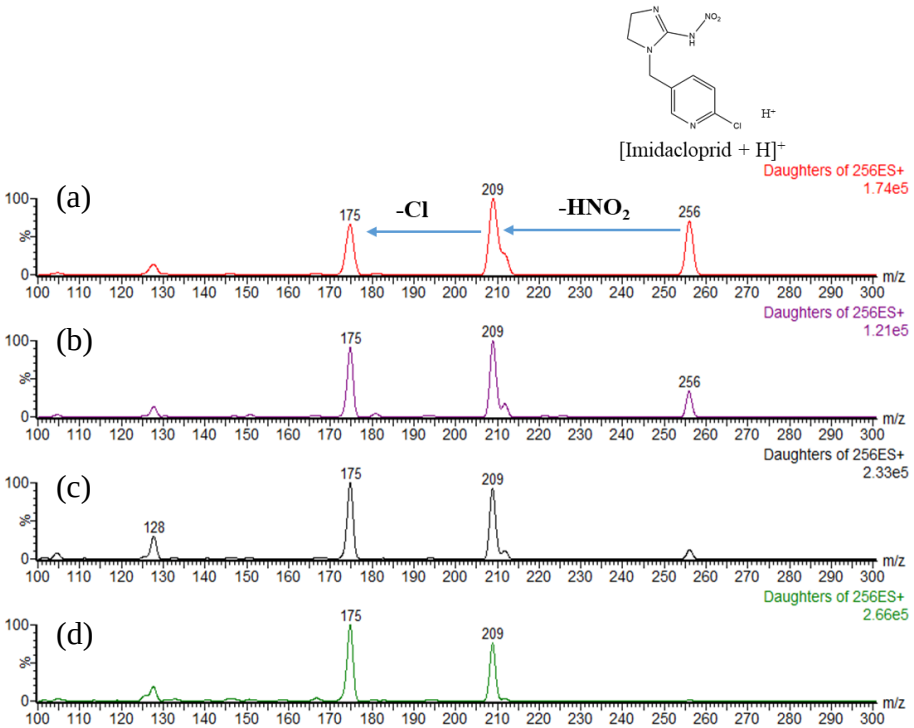
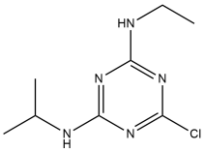
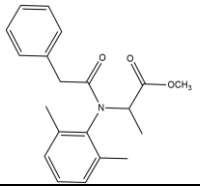
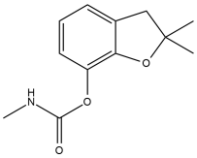
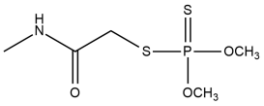
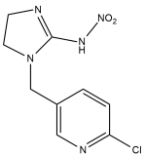
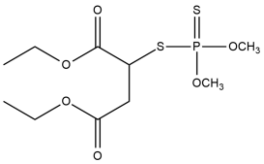
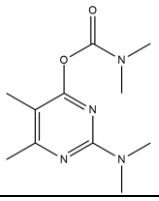
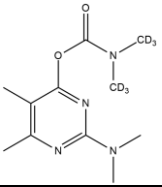


Figure 2-4. The MS/MS spectra of imidacloprid at collision energy (a) 10 eV, (b) 12 eV (c) 15 eV and (d) 20 eV. (Potential chemical structures of the major product ions are labelled)

Table 2-2. The structural information and MRM conditions of the pesticides studied in this project.

Pesticide	Molecular structure	MRM channels	Collision energy (eV)
Atrazine		216 → 146 216 → 174*	20
Benalaxyl		326 → 91 326 → 148*	12
Carbofuran		222 → 123 222 → 165*	10
Dimethoate		230 → 125* 230 → 199	10
Imidacloprid		256 → 209* 256 → 175	12
Malathion		331 → 99 331 → 127*	8
Pirimicarb		239 → 72* 239 → 182	10
Pirimicarb d-6		245 → 78* 245 → 185	15

* The major product ion used for MRM.

2.3.3 Optimization of other experimental parameters

The other parameters tested including the sample volume, composition of spraying solvent, flow rate of solvent, pipette in/out time, capillary voltage, and distance of the tip end to MS inlet and the results are shown in Figure 2-5. Honey sample spiked with 100 ng mL^{-1} pirimicarb was used to optimize the experimental parameters. Optimization of each parameter is discussed below.

Firstly, the composition of spraying solvent was determined. Different compositions of acetonitrile (ACN) and methanol (MeOH) each with 0.1% FA were tested. It was shown that MeOH gave stronger signals than ACN and 60% MeOH gave the optimum signal with smaller deviation (Figure 2-5a). Secondly, the sample volumes of 100 – 500 µL were tested. Volume of 200 µL gave the optimum performance. It was noted that the curve did not show a logarithmic trend (Figure 2-5b). The signal intensity dropped after 200 µL, indicating that increased quantity of the sample did not necessarily increase the signal intensity as the target analytes may not be efficiently adsorbed by the C18 coating. In the 200 µL sample, pipetting in/out for 8 times was adopted for pesticide extraction as it enabled the best signal of the analyte (Figure 2-5c). Similarly, the flowrate of the praying solvent was chosen at 5 uL min^{-1} (Figure 2-5d), capillary voltage at 4 kV (Figure 2-5e), and the distance of the tip end as 0.5 cm parallel and 1 cm perpendicular to the MS inlet (Figure 2-5f).

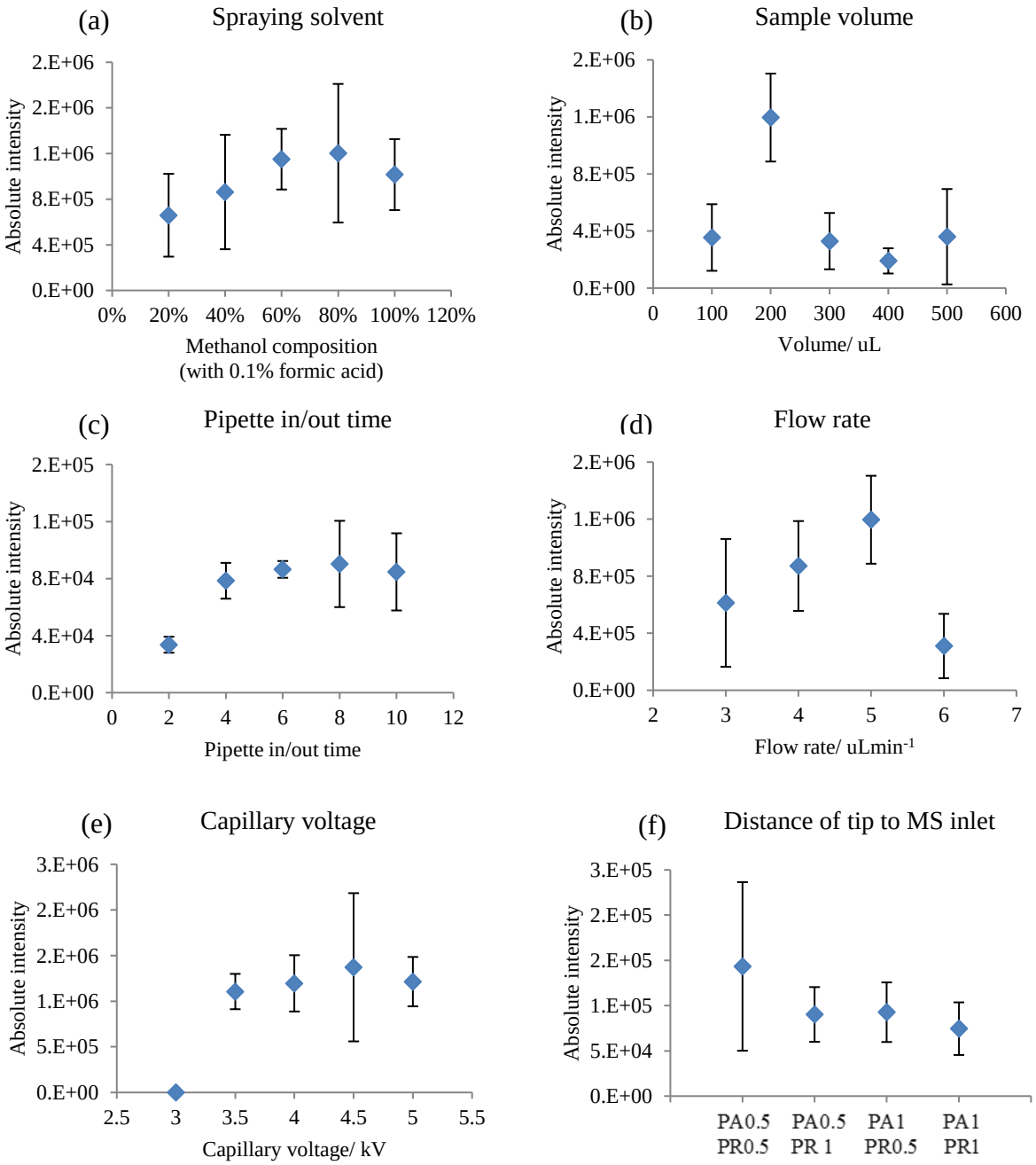


Figure 2-5. Effects of various experimental parameters on C18 pipette-tips ESI-MS analysis of the honey sample spiked with 100 ng mL^{-1} pirimicarb. (a) Solvent composition, (b) sample volume, (c) pipette in/out time, (d) flow rate of spraying solvent, (e) capillary voltage and (f) distance of the pipette-tip end to the MS inlet (PA and PR represent the parallel and perpendicular distance (in cm) of the tip end to the MS inlet, respectively).

2.3.4 Reusability of C18 pipette tips

Normally, C18 pipette tips are designed for single use as the tips can be contaminated after the sample extraction and elution. The reusability of recycled C18 pipette tips for analysis of pesticides was evaluated. Honey sample spiked with 100 ng mL^{-1} of pirimicarb was extracted by the same recycled tip for eight times. From the results shown in Figure 2-6, the absolute intensity of pirimicarb seemed to drop since the third trial, however, after the correction by the internal standard, the differences between the relative intensities of the eight trials were small as the RSD was 11% only (Figure 2-7). Tests showed that no pesticide residues were detected from these washed C18 pipette tips (data not shown), indicating no memory effects from the previous measurement. To reduce the cost of the study, the C18 pipette tips were recycled and reused in this study and each C18 pipette tip was used for a maximum of eight times. After each analysis, the used C18 pipette tip was washed with 100% MeOH for ten times (10 µL each) after each analysis and reused.

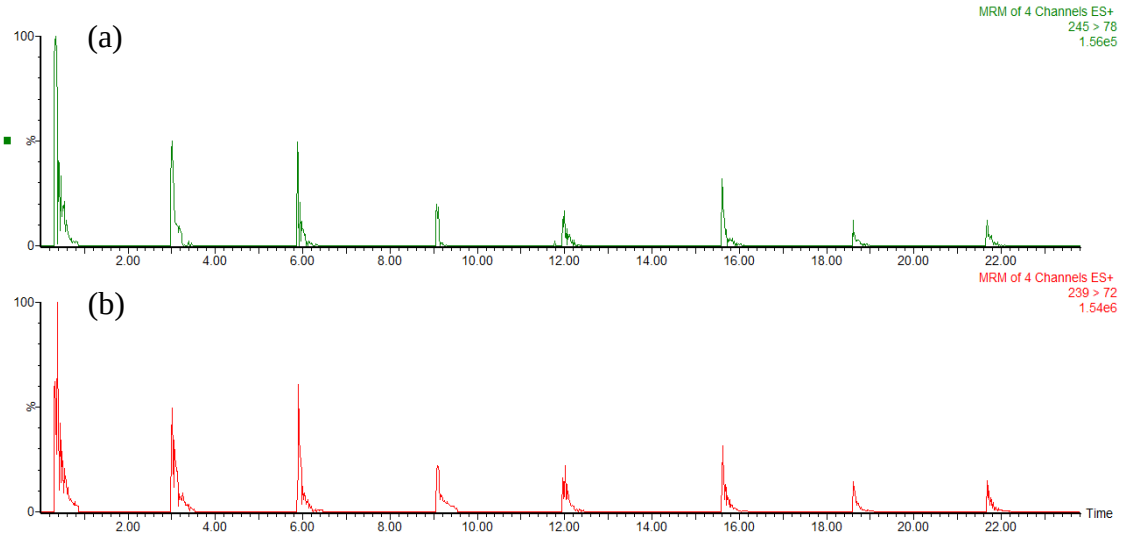


Figure 2-6. TIC chromatograms of (a) pirimicarb d-6 and (b) pirimicarb for analysis of honey spiked with 100 ng mL^{-1} pirimicarb by a recycled C18 pipette tip for eight times.

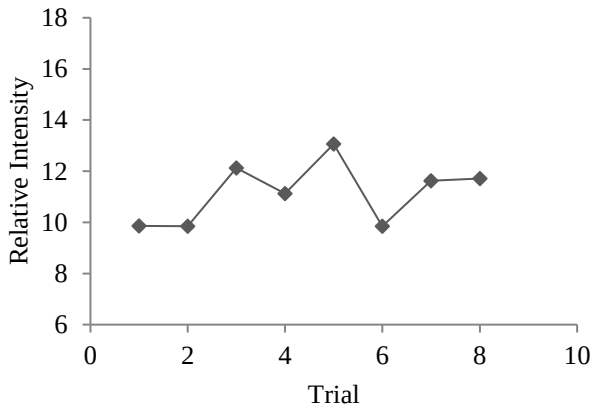


Figure 2-7. Relative intensity of pirimicarb against pirimicarb d-6 for analysis of honey spiked with 100 ng mL^{-1} pirimicarb by a recycled C18 pipette tip for eight times.

2.3.5 Quantitation of pesticides in honey and juice

The LODs and LOQs of the pesticides in the food samples were tested in this study. The LODs and LOQs were determined according to the procedures described in section 2.2.4. The LOD and LOQ values of the pesticides in honey and apple juice are listed in Table 2-3. Generally speaking, atrazine, benalaxyl, carbofuran and pirimicarb gave lower LOD and LOQ values, whereas dimethoate, imidacloprid and malathion gave higher LOD and LOQ values. As the honey sample was diluted 10 times by water before spiking the pesticide standards, in the real sample analysis, the measured LOD and LOQ values should be multiplied by 10. Carbofuran and pirimicarb showed the lowest LODs and LOQs in honey, i.e., 0.3 ng mL^{-1} and 1 ng mL^{-1}, respectively. Atrazine and pirimicarb showed the lowest LODs and LOQs in AJ, i.e., 1 ng mL^{-1} and 3 ng mL^{-1}, respectively.

From the MS spectra shown in Figure 2-8a, pirimicarb gave the strongest MS responses than atrazine, benalaxyl, carbofuran, dimethoate, imidacloprid and malathion when it was analyzed by conventional ESI-MS. With C18 pipette-tip ESI-MS, the MS response order of the pesticides in honey was pirimicarb > atrazine > benalaxyl ~ dimethoate > carbofuran ~ imidacloprid ~ malathion and the order was similar for AJ sample at the same concentration (Figures 2-8b and c).

Different MS responses of the pesticides could be related to their chemical structures, chemical properties and ionization efficiencies. Stronger signals were obtained by atrazine and pirimicarb, probably due to the existence of amine groups in their similar structures. For benalaxyl and carbofuran, their amide functional groups might also benefit the protonation. Organochlorine and organophosphorus pesticides (dimethoate,

imidacloprid and malathion) gave weaker MS responses, but these three pestcides were also studied throughout the project, anticipating that the signals of these pesticides would be enhanced by the new methods.

As the signals of atrazine, benalaxyl, carbofuran and pirimicarb were stronger than dimethoate, imidacloprid and malathion, pesticides were categorized and spiked into samples as two groups based on their signal responses. Such categorization may reduce the signal suppression of the pesticide analytes, which is caused by the very different ionization efficiencies of the analytes.

Quantitation of pesticides in honey and juice was performed by using pirimicarb d-6 as an internal standard (IS) and selected reaction monitoring for each individual pesticide. The calibration curves of seven pesticides, including atrazine, benalaxyl, carbofuran, dimethoate, imidacloprid, malathion and pirimicarb, in honey and AJ were constructed by plotting the peak height ratio against the analyte concentration. Both peak height and peak area were used for the plotting and it was found that there was no discrepancy using either peak height and peak area. Peak height was chosen because peak height was more straightforward and its data were more convenient for processing. The calibration curves for pesticides in honey and AJ are shown in Figure 2-9 and 2-10 respectively. Generally speaking, most of the curves showed good linearity with good R^2 coefficient values. The curves for atrazine, benalaxyl, carbofuran, imidacloprid and pirimicarb in honey and atrazine, benalaxyl, dimethoate, imidacloprid, malathion and pirimicarb in AJ gave better linearity with R^2 coefficients greater than 0.990 (Table 2-3).

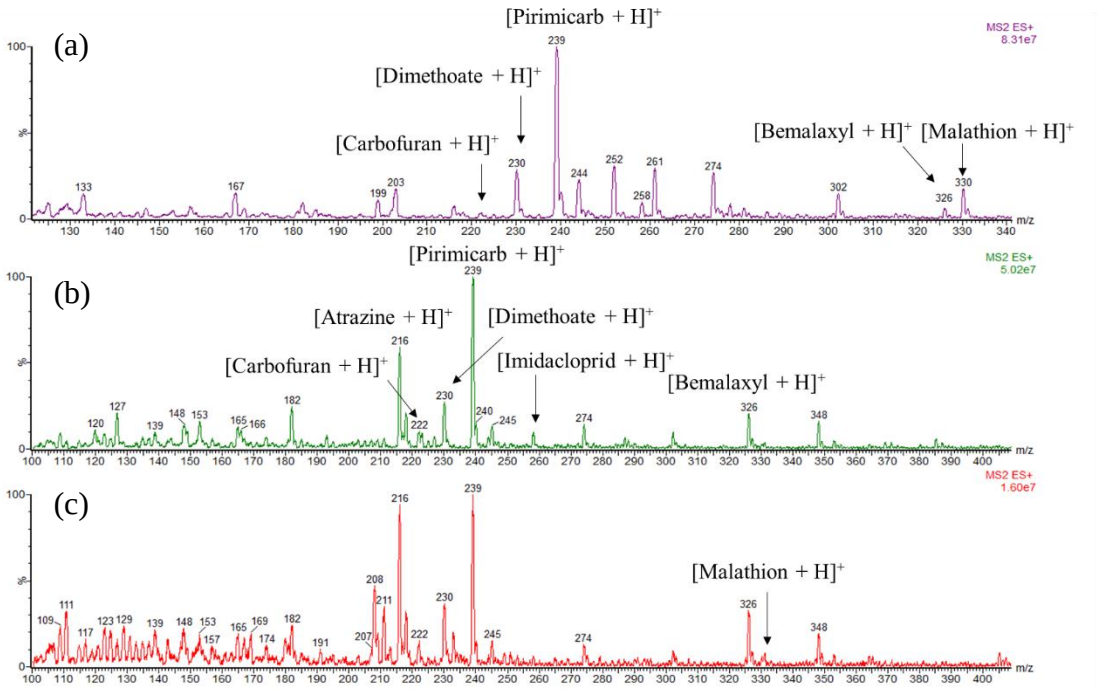


Figure 2-8. (a) Mass spectrum obtained by analysis a mixture of atrazine, benalaxyl, carbofuran, dimethoate, imidacloprid, malathion and pirimicarb (100 ng mL^{-1} each) with direct infusion ESI-MS. Mass spectra obtained by analysis of (b) honey and (c) apple juice containing atrazine, benalaxyl, carbofuran, dimethoate, imidacloprid, malathion and pirimicarb (100 ng mL^{-1} each) with C18 pipette-tip ESI-MS.

Table 2-3. The LOD and LOQ values, linear ranges and R^2 coefficients of the calibration curves for analysis of the pesticides by C18 pipette-tip ESI-MS.

	LOD ($ng\ mL^{-1}$)		LOQ ($ng\ mL^{-1}$)		Linear range ($ng\ mL^{-1}$)		R^2	
Pesticide	Honey	AJ	Honey	AJ	Honey	AJ	Honey	AJ
Atrazine	1	1	5	3	5-500	3-200	0.993	0.993
Benalaxyl	1	3	5	10	5-500	10-200	0.993	0.990
Carbofuran	0.3	3	1	10	1-500	10-200	0.996	0.964
Dimethoate	10	12	25	50	25-500	50-500	0.943	0.991
Imidacloprid	10	20	25	50	25-500	50-500	0.999	1.000
Malathion	10	5	50	10	50-500	10-500	0.989	0.992
Pirimicarb	0.3	1	1	3	1-500	3-200	0.997	0.999

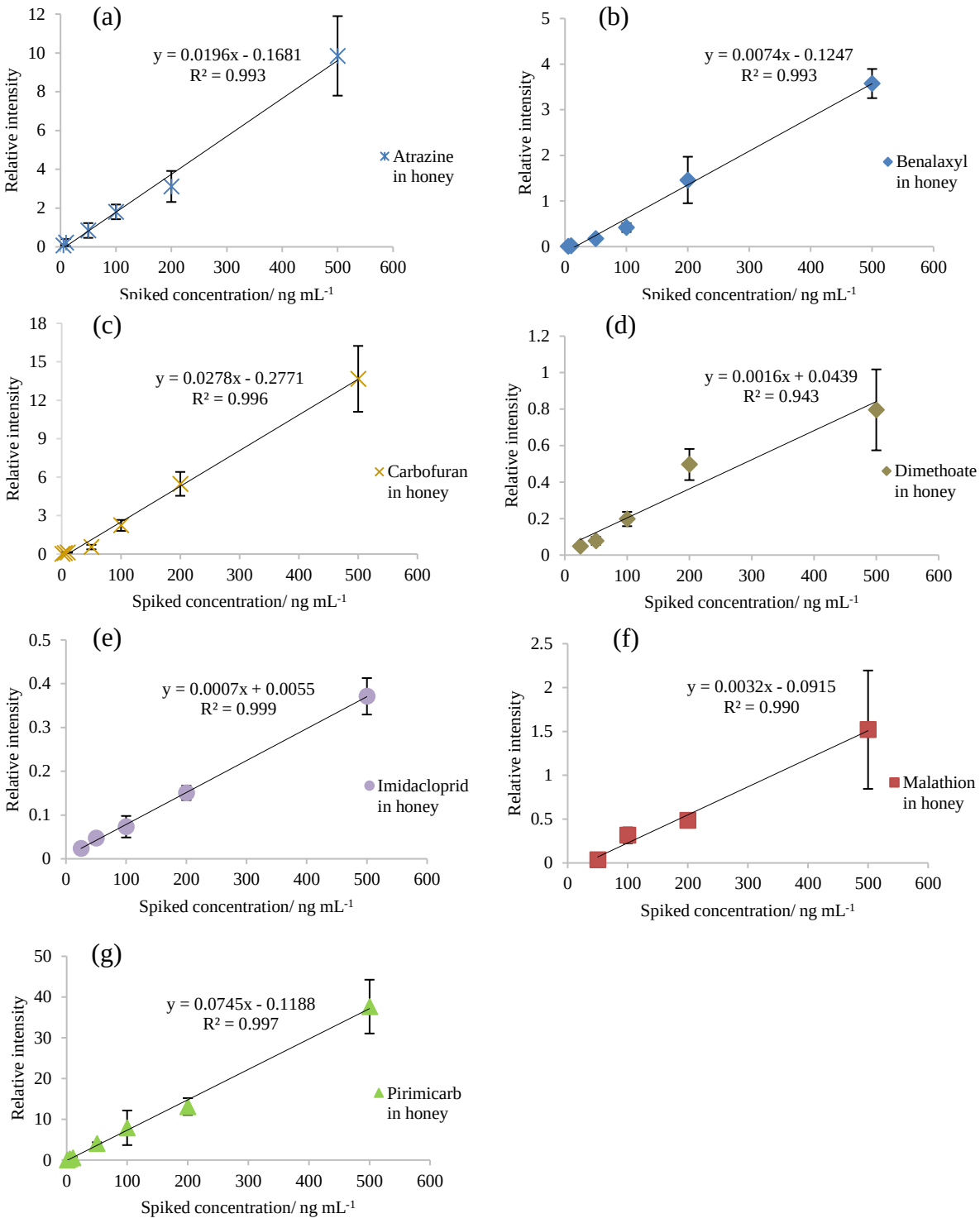


Figure 2-9. The calibration curves for analysis of pesticides in honey by C18 pipette-tip ESI-MS. (a) Atrazine, (b) benalaxyl, (c) carbofuran, (d) dimethoate, (e) imidacloprid, (f) malathion and (g) pirimicarb.

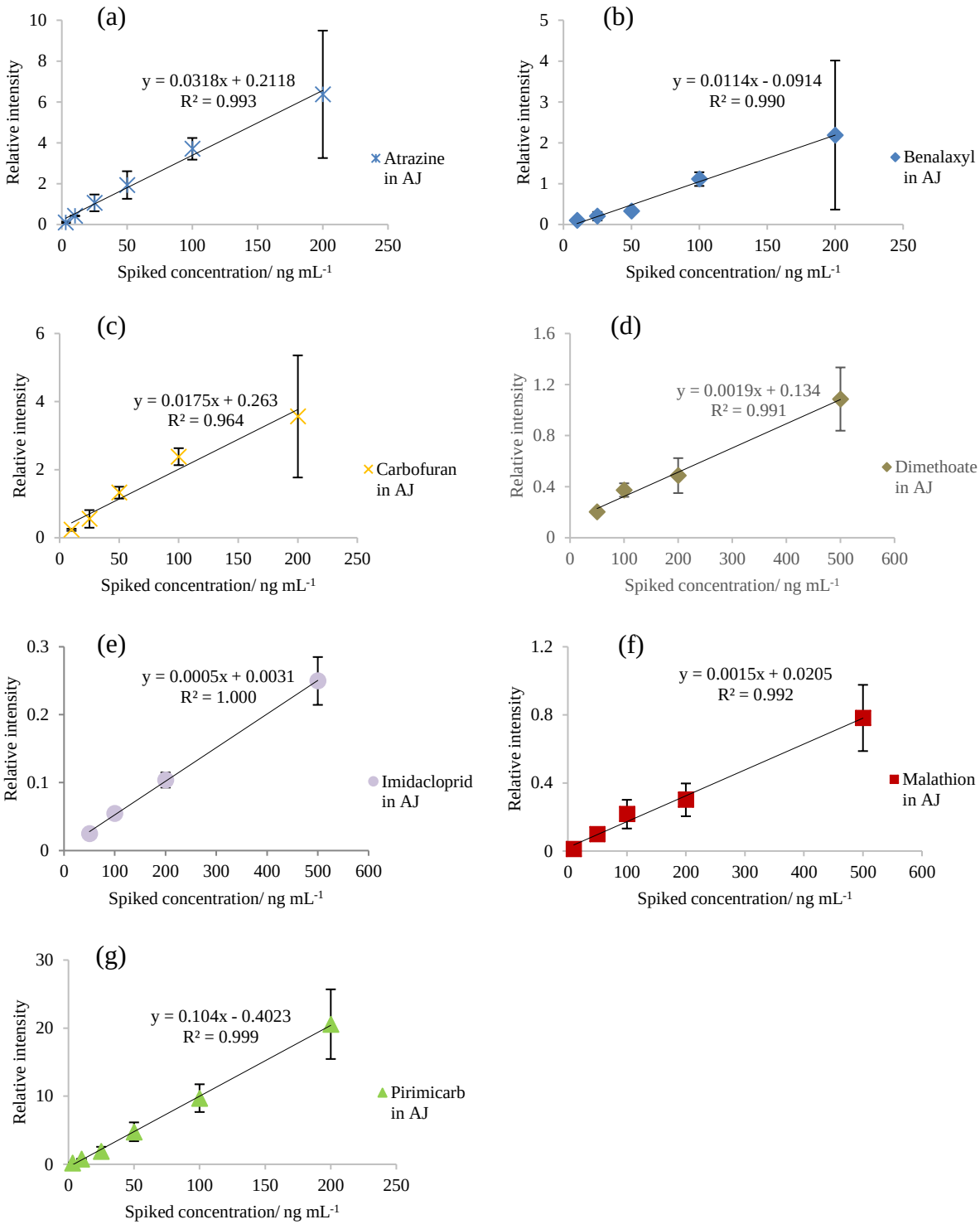


Figure 2-10. The calibration curves for analysis of pesticides in apple juice by C18 pipette-tip ESI-MS. (a) Atrazine, (b) benalaxyl, (c) carbofuran, (d) dimethoate, (e) imidacloprid, (f) malathion, and (g) pirimicarb.

2.3.6 Precision and accuracy

The precision and accuracy for quantitation of pesticides in honey and juice were tested and shown in Table 2-4. The precision and accuracy were measured by spiking samples with at least two different concentrations (one low and one high concentration level) of the pesticides. The accuracy of the method was determined by spiked recovery method. The details of the calculations are described in Section 2.2.4. The results showed that the precision of honey samples varied in the range of 8 – 42%. The most precise results were obtained from pirimicarb, dimethoate and imidacloprid at 250 ng mL^{-1} for honey (8, 11 and 14% respectively). Most of the honey samples (18 out of 21) had a precision below 35% but only 6 samples had a precision below 20% (Table 2-5). The accuracy of two-third honey samples (14 out of 21) was between 100 ± 20%. Accuracy over 150% were observed in some cases, i.e., benalaxyl at high concentration (151%) and imidacloprid at low concentration (150%). However, the performance of AJ was poor. The precision and accuracy of AJ sample varied between 21 – 97% and 40 – 122% respectively. The large variations could be caused by some reasons. Firstly, the competition between target analytes and the components in matrix may affect the precision and accuracy of the quantitative analysis. Some other components in the juice sample could hinder the extraction or compete with the target analytes, resulting in less accurate and precise data. Secondly, as described in the last session, not all the pesticides gave good responses under the experimental conditions. Higher MS responses were observed for atrazine, benalaxyl, carbofuran and pirimicarb, whereas dimethoate, imidacloprid and malathion gave lower responses. The weaker MS response typically made the detection less accurate and less precise. Thirdly, the lack of IS may affect the results. In this study, only one IS (pirimicarb d-6) was used as the IS for all pesticides. The use of isotopically labeled standards for all pesticides were not

realistic due to high costs and unavailability of some standards. The resulted curves of pirimicarb and atrazine were not affected much as they have chemical structures similar to the IS. However, other pesticides with structures and responses very different from the IS might give less accurate and less precise results, particularly that the extraction process of pipetting in/out in the honey or AJ samples for eight times was performed manually, which could induce further variations and errors.

Table 2-4. The precision and accuracy for quantitation of the pesticides in honey by C18 pipette-tip ESI-MS.

Pesticide	Spiked concentration (ng mL^{-1})	Determined concentration (ng mL^{-1}) (*n=5*) (mean±SD)	RSD (%)	Accuracy (%)
Atrazine	75	79±25	32	105
	250	182±43	23	73
	400	378±63	17	95
Benalaxyl	75	72±22	30	97
	250	191±62	32	76
	400	602±191	32	151
Carbofuran	75	84±35	42	112
	250	213±56	26	85
	400	373±79	21	93
Dimethoate	75	95±32	34	127
	250	262±29	11	105
	400	408±78	19	102
Imidacloprid	75	112±32	29	150
	250	326±44	14	130
	400	229±91	20	112
Malathion	75	104±24	23	139
	250	252±108	43	101
	400	442±92	21	111
Pirimicarb	75	77±28	36	102
	250	221±18	8	88
	400	413±99	24	103

Table 2-5. The precision and accuracy for quantitation of the pesticides in apple juice by C18 pipette-tip ESI-MS.

Pesticide	Spiked concentration (ng mL^{-1})	Determined concentration (ng mL^{-1}) (*n=5*) (mean±SD)	RSD (%)	Accuracy (%)
Atrazine	25	16±10	60	63
	75	74±43	58	98
	150	143±73	51	95
Benalaxyl	25	25±10	39	100
	75	73±71	97	98
	150	160±97	61	107
Carbofuran	25	10±7	69	40
	75	73±59	80	97
	150	183±61	33	122
Dimethoate	150	146±54	37	97
	400	261±86	33	65
Imidacloprid	150	132±48	36	88
	400	320±71	22	80
Malathion	150	94±43	45	63
	400	421±173	41	105
Pirimicarb	25	19±5	25	76
	75	62±13	21	82
	150	86±34	39	57

2.4 Conclusions

In this study, C18 pipette-tip ESI-MS was used to analyze honey and apple juice samples containing various pesticides. As no chromatographic analysis and only brief sample pre-treatment were carried out for the analysis, the cost and time could be reduced, particularly with the recycled use of the C18 pipette tips. The results showed that C18 pipette tips could effectively remove sugars and other interference compounds and enrich the pesticides from the honey and juice samples. The coupling of the C18 pipette tips with ESI-MS was simple and allowed rapid detection and quantitation of the extracted and enriched pesticides from the samples.

The results showed that most of the calibration curves had good linearity with R^2 values greater than 0.990 for analysis of pesticides in honey and apple juice samples. The LOD and LOQ values of some pesticides (e.g., carbofuran and pirimicarb in honey) could be down to 0.3 and 1 ng mL^{-1} respectively. The LOD and LOQ values for analysis of most pesticides (except malathion spiked in honey and imidacloprid spiked in AJ) could fulfil the cut-off values (50 ng mL^{-1}) in the international standards. However, the precision and accuracy of this method were not satisfactory. For analysis of the pesticides in the honey samples, the precision was between 8 – 42%, but it was 21 – 97% for the apple juice samples. The accuracy of the method was between 73 – 151% for the honey samples, and between 40 – 122% for the AJ samples. New solid-substrate ESI-MS technique was thus developed, in an effort to improve these.

Chapter 3: Analysis of Pesticides in Honey and Juice by SPME-ESI-MS

3.1 Introduction

Solid phase microextration (SPME) was introduced by Pawliszyn et al. in 1990s.[85] In this technique, a fibre tip coated with a layer of solid sorbent is dipped into a sample for extraction of target analytes. This extraction technique has been widely used to extract various compounds from gaseous, liquid and solid samples. The adsorbed analytes on the SPME tips can be desorbed by thermal mean in GC, or by solvents in LC. There are many advantages for using SPME as an extraction method, including low consumption of solvents, reduced cost and time, simple procedures as well as easy solvent disposal. Besides, the fibre is reusable which further reduces the cost.

In addition to SPME tips with polyimide coating that was originally developed by Pawliszyn et al., SPME tips with various coatings, include divinylbenzene (DVB), polydimethylsiloxane (PDMS), polyacrylate (PA), Carboxen (CAR) or a mixture of those materials, have been commercially available. SPME tips with new coatings, including nano-porous silica,[86] molecularly imprinted polymer, ionic imprinted polymer[87] and immunosorbent and sol-gel coatings,[88] are being developed. As the device is small and compact, it is feasible for field/on-site analysis.[89] After the fibre is exposed to the field sample, it can be analyzed by field portable instruments, or brought back to the laboratory for analysis. Applications of SPME include analysis of volatile organic compounds and polycyclic aromatic hydrocarbons in water samples,[90] pollutants in ground water and soil samples[91-93] and toxins in aqueous samples.[87] SPME

has also been applied in analysis of pesticides in food samples. Modified SPME tips made of PDMS/DVB internal coating with an external layer of PDMS were developed by Pawliszyn et al.[94-95] for extraction of 40 pesticide residues in grape and water samples. It was shown that the modified tips could be used to extract a variety of pesticides in the samples but did not give a satisfactory extraction efficiency for relatively polar and highly hydrophobic pesticides. This was probably due to their low affinities to the tip coating and weak GC responses. The use of SPME for extracting target analytes in other food matrix, such as milk, cheese, oil, fish, meat and plants, can be found in the relevant books and review articles.[96-97]

SPME coupled with GC-MS has been applied for authentication and determination of the botanical and geographical origins of honey samples by comparing the contents of volatile compounds.[98] It was demonstrated that the unifloral honey could be classified and their botanical origins could be discriminated by mass spectrometry-based electronic nose method with SPME headspace sampling.[99] SPME fibres made of DVB/CAR/PDMS coatings were exposed to the headspace for 30 min to extract the volatile compounds and the results allowed 98% correct classification. Advanced GC was also introduced to detect the volatile components in honey. An automated SPME GCxGC TOF-MS was introduced to analyze the volatile components in honey aroma.[100] Head-space solid phase microextration (HS-SPME) using a divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre was combined with two-dimensional gas chromatography–time-of-flight mass spectrometry (GCxGC–TOFMS) to perform the analysis. With the use of a DB-5ms×SUPELCOWAX (5%-polysilphenylenesiloxane) column, a total of 164 volatile compounds were identified in the honey sample and the analysis time was 19 min for

one GC run. Compared with 1D-GC–TOFMS, GCxGC TOFMS allowed higher peak capacity, improved S/N ratio and structured chromatograms.

SPME has also been used for extraction of pesticides in fruit juice.[101-102] In this study, apart from honey and apple juice (AJ), orange juice (OJ) was also selected as one of the food matrices for the investigation.

Although SPME extraction of pesticides in food stuffs is robust, chromatographic separation of the extracted pesticides is normally required prior to the detection by MS. In this study, direct coupling of SPME and ESI-MS is developed for qualitative and quantitative analysis of pesticide residues in food samples. The whole process is very simple and quick, with only minimum sample pre-treatment required.

3.2 Experimental section

3.2.1 Materials and chemicals

Pesticides and reagents used were described in section 2.2.1. Stock solutions of pesticides and serial dilutions were prepared as described in section 2.2.1. SPME-LC fibre probe tips with carbon-18 (C18) embedded in a biocompatible polymer were purchased from Supelco (St. Louis, USA). Each fibre is coated with a layer of C18 with thickness of 45 μm and length of 15 mm. A glass syringe (1000 μL, Hamilton) and syringe pump (New Era Pump System, USA) were used for solvent supply. The information of the honey and AJ samples was as described in section 2.2.2. The orange juice (brand: Minutemaid), which contained orange pulp, was purchased from a local convenience store (7-11 convenience store, Hong Kong).

3.2.2 Sample preparation

Stock solutions of pesticides were made as described in section 2.2.2. Stock solution (1 mg mL^{-1}) of pesticides atrazine, benalaxyl, carbofuran, dimethoate, imidacloprid, malathion, pirimicarb, and internal standard solution of pirimicarb d-6 (1 mg mL^{-1}) were prepared in methanol. Working solutions were prepared by diluting the stock solution in water. For the quantitation of pesticides in honey or juice samples, 1 mL of sample was spiked with standard solutions of pesticides with concentration of 3, 10, 25, 50, 100, 200, 400 and 800 ng mL^{-1} separately and 10 µL of internal standard solution was added to each solution. Pesticides were separated into two groups for quantitative analysis because of their similarities in ESI-MS response. Atrazine, benalaxyl, carbofuran, and pirimicarb were spiked together whereas dimethoate, imidacloprid and malathion were spiked together in the samples. Honey was diluted 10 times with water before analysis. Orange juice was centrifuged at a speed of 4000 rpm for 15 min to remove the pulp, with only the supernatant collected and used. All of the samples were freshly prepared before use.

3.2.3 Instrumental set-up and workflow

The SPME tips were used according to the guideline provided by the manufacturer. In brief, a SPME tip was placed in an eppendorf tube containing 1 mL of MeOH and washed for 5 min, and then washed with 1 mL of water for another 5 min. After the pre-washing steps, the SPME tip was inserted into an eppendorf tube containing 1 mL of sample and vortexed for 10 min for sample extraction. The tip was then quickly washed with water before mass spectrometric analysis. After SPME-ESI-MS analysis, the tip was washed with MeOH for at least 30 min to remove the pesticide residues.

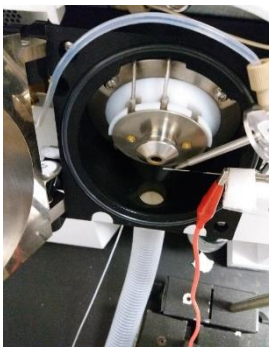
All SPME-ESI-MS experiments were performed on an Agilent 6460 triple quadrupole LC-MS. The workflow and instrumental set-up of SPME-ESI-MS are shown in Figure 3-1 and Figure 3-2, respectively. In brief, the tip was mounted onto a microscopic slide and connected with a wire for high voltage supply (+3.5 kV). A solvent containing MeOH: H_2O: FA (90: 10: 0.1, v/v/v) was supplied by a syringe pump and sprayed onto the SPME tip for eluting the adsorbed analytes. For each sample, signals were lasted for around 10 s and repeatedly analyzed for three times. Those three results were averaged to one data. After data acquisition, the high voltage was turned off. The distance of the SPME tip to the MS inlet was set as ~ 1 cm. Positive ion and MRM modes were used for the analysis. Nitrogen was used as the nebulizer gas, with gas temperature of 150 ℃, gas flow of 6 L min^{-1} and nebulizer pressure of 3 psi. Sheath gas was at 125 ℃ with gas flow of 3 L min^{-1}. Capillary voltage was 3500 V.

1. Samples

2. Pre-washing of the SPME tip with methanol and then water

3. Sample extraction

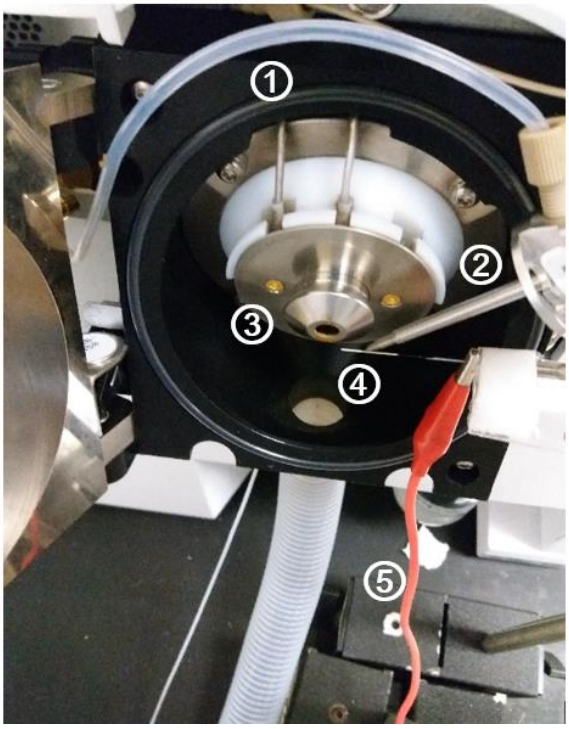
4. Washing with water



5. SPME-ESI-MS analysis

6. Washing with methanol to remove pesticide residues

Figure 3-1. The workflow of SPME-ESI-MS analysis.



Key:
① Mass spectrometer
② Solvent supply
③ MS inlet
④ C18 coating of SPME tip
⑤ High voltage supply

Figure 3-2. The set-up of SPME-ESI-MS.

3.2.3 Method validation of SPME-ESI-MS

Five different concentrations of pesticides were used to construct the calibration curves, and the data of each concentration was obtained by three individual measurements. Peak height was used for plotting the calibration curves. A blank sample was tested first in order to confirm that the SPME tips and food samples were not contaminated with pesticides and to detect the electronic noises and instrumental variations.

The LODs and LOQs of the pesticides were determined by comparing the peak height of the analytes with the average heights of the background noises. The LODs and LOQs were determined as the concentrations of the analytes that could give S/N ratios of three and ten, respectively.

The precision and accuracy of the method were measured by analyzing at least two different concentrations (one low and one high concentration within the linear range), each data was obtained by five individual measurements. The precision was presented as relative standard deviation (RSD) which was calculated as: (SD of the measured concentrations ÷ mean of the measured concentrations) x 100%.

The accuracy was determined by using the spiked recovery method. In which at least two concentration of the pesticides (one low and one high concentration within the linear range) were spiked into the blank sample separately and performed the analysis, and each data was obtained by five individual measurements. The detected amount was calculated using corresponding calibration curves. A comparison of the detected amount and the spiked amount demonstrated the recovery and accuracy of the method.

The accuracy (recovery) was calculated as: (the measured concentration ÷ the spiked concentration) x 100%.

3.3 Results and discussions

3.3.1 Optimization of MRM conditions

As a different instrument was used, the MRM conditions were re-tested. The MRM conditions for analysis of the pesticides studied in this project are shown in Table 3-1.

Table 3-1. The MRM conditions for analysis of the pesticides by SPME-ESI-MS.

Compound	MRM channels	Fragmentor (V)	Collision energy (V)
Atrazine	216 → 146	100	15
	216 → 174*		
Benalaxyl	326 → 91	80	15
	326 → 148*		
Carbofuran	222 → 123	80	5
	222 → 165*		
Dimethoate	230 → 125*	100	15
	230 → 199		
Imidacloprid	256 → 209*	100	10
	256 → 175		
Malathion	331 → 99	100	10
	331 → 127*		
Pirimicarb	239 → 72*	100	20
	239 → 182		
Pirimicarb d-6	245 → 78*	100	20
	245 → 185		

‘*’ The major product ion used for the study.

3.3.2 Optimization of other experimental parameters

The extraction time and volume by SPME were firstly optimized. Honey spiked with pesticides (100 ng mL^{-1} for each pesticide) was used for the optimization. Two volume, 500 µL and 1000 µL, were chosen for the testing, since volumes below 500 µL might not allow full cover of the C18 coating on the SPME tips by the sample during the agitation. Extraction time 5, 10, 15 mins were tested. Extraction time longer than 15 min was considered as too long as we aimed to develop a rapid method for pesticide residue analysis. From the results shown in Figures 3-3a and b, the optimum extraction time was 10 min for both the 500 and 1000 µL samples, and the signal dropped slightly when the sample was extracted for 15 min, indicating the extraction had reached the equilibrium. All of the pesticides were extracted slightly more in the volume of 1000 µL than in 500 µL (Figure 3-3c). However, their signal differences were not so significant, indicating that the sample volume was not a critical factor for the extraction.

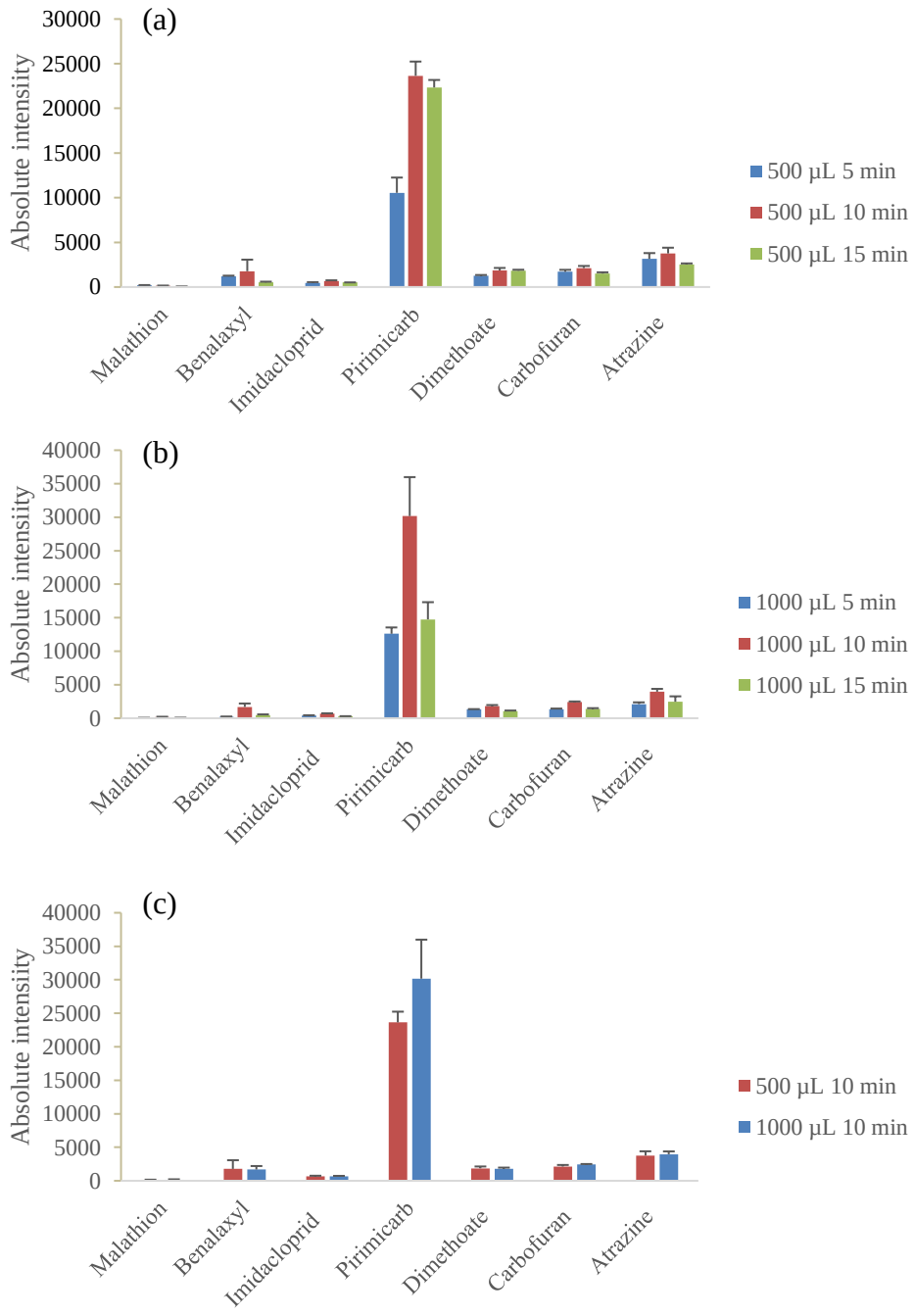


Figure 3-3. Effects of sample volume and extraction time on extraction of seven pesticides by SPME with C18 coating. (a) Sample volume of 500 uL; (b) sample volume of 1000 uL; (c) comparison between the two sample volumes with extraction time of 10 min. Honey spiked with atrazine, benalaxyl, carbofuran, dimethoate, imidacloprid, pirimicarb and malathion (100 ng mL^{-1} for each) were used for the optimization.

The solvent was sprayed onto the SPME tip in order to elute and desorb the adhered analytes. When the solvent was sprayed onto the tip, it was absorbed by the C18 coating and moved toward the tip end, leading to the elution of the adsorbed analytes. The composition of the spraying solvent was optimized. As shown previously in C18 pipette-tip ESI-MS study, MeOH gave better signal responses of the pesticides than acetonitrile. Therefore, MeOH was tested as the spraying solvent. MeOH mixed with water in different percentages (40 – 100%, all with 0.1% FA) were tested. Solvents containing less than 40% MeOH did not give any pesticide signals (data not shown), probably because the high water content of the solvent could not effectively generate spraying due to the high surface tension of water. A solvent composed of 100% MeOH did not give good signals either as it was too volatile and could not reach the tip end for spraying. As shown in Figure 3-4a, 90% MeOH with 0.1% FA gave the strongest signals for all the seven pesticides.

The flow rate of the spraying solvent was controlled by a syringed pump with a glass syringe. Four different flow rates (10, 20, 30, 40 µL min^{-1}) were tested. As shown in Figure 3-4b, the absolute intensities of most of the pesticides increased with the increasing flow rate. It did not reach the maximum even at 40 µL min^{-1}. However, flow rate higher than 40 µL min^{-1} was not favourable since at the very high flow rate and without the assistance of desolvation gas, efficient ionization might not be able to occur and the analytes adsorbed on the SPME tip could also be diluted.

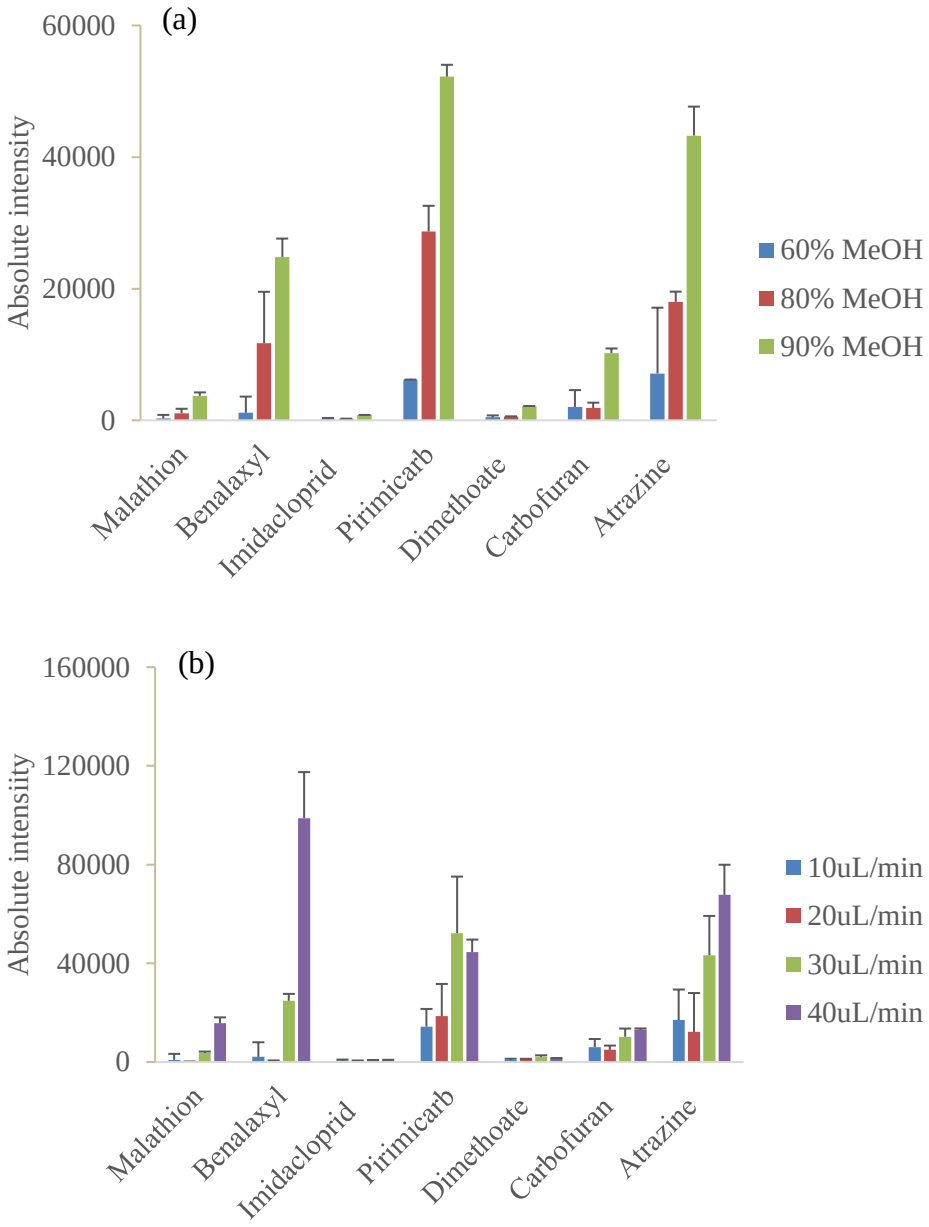


Figure 3-4. Effects of (a) the composition of the spraying solvent (with 0.1% FA) and (b) flow rate on the SPME-ESI-MS analysis.

3.3.3 Quantitation of pesticides in honey and juice by SPME-ESI-MS

The LOD and LOQ values for analysis of pesticides spiked in honey, apple juice and orange juice by SPME-ESI-MS are listed in Table 3-2. Similar to the extraction by C18 pipette tip, atrazine, benalaxyl, carbofuran and pirimicarb gave lower LOD and LOQ values than dimethoate, imidacloprid and malathion. On the other hand, honey and AJ samples generally gave lower LOD and LOQ values than OJ samples for detection of the pesticides. The LODs and LOQs of atrazine, benalaxyl, carbofuran and pirimicarb in AJ were as low as 0.3 ng mL^{-1} and 0.5 ng mL^{-1}, respectively.

The ESI responses of seven pesticides were obtained and compared with conventional ESI-MS (Figure 3-5 a) and SPME-ESI-MS (Figure 3-5b-d) at the same concentration. The order of MS response of pesticides was pirimicarb > benalaxyl > atrazine > carbofuran > dimethoate > imidacloprid ~ malathion in conventional ESI-MS. After the analysis of SPME-ESI-MS, similar orders of MS responses were observed for these three matrices, that pirimicarb gave the strong signal as usual whereas dimethoate, imidacloprid and malathion gave very weak signals. The order of MS response of pesticides in honey and OJ analyzed by SPME-ESI-MS was pirimicarb > atrazine > carbofuran > dimethoate ~ imidacloprid ~ benalaxyl ~ malathion. For AJ, the signal produced by atrazine was greater than pirimicarb. Noteworthily, benalaxyl gave relatively strong signal in conventional ESI-MS but the relative signal was significantly reduced after the SPME extraction, indicating that benalaxyl was not efficiently extracted from these matrices, compared to others. Although the positions of individual pesticides were not consistent, atrazine, benalaxyl, carbofuran and pirimicarb were generally in higher rankings.

Linear calibration curves were constructed for the pesticides detected by SPME-ESI-MS (Figure 3-6 to 3-8). Excellent linearity was shown for pirimicarb in all the three matrices ($R^2 > 0.999$). Atrazine also showed good linearity in all three matrices ($R^2 > 0.993$, Table 3-2). Again, this was likely related to the structural similarity between these two pesticides and the IS. Malathion in OJ gave the least linear curve ($R^2 = 0.951$).

It was noted that atrazine, benalaxyl and pirimicarb normally gave stronger MS response whereas dimethoate, imidacloprid and malathion gave weaker MS response. This was probably due to the difference in ionization efficiency of the pesticide in ESI-MS. Atrazine, benalaxyl and pirimicarb were easier to ionize and therefore produced stronger MS signals, compared to dimethoate, imidacloprid and malathion. These weakly-ionized pesticides produced relatively weak MS signals. Such discrepancy was shown in the mass spectrum (Figure 3-5 a). Large error bars were observed for some higher concentration on the calibration curves, for example, carbofuran, dimethoate, imidacloprid and malathion in honey and AJ as well as benalaxyl, dimethoate and malathion in OJ, which reduced the data reliability and the linearity of the calibration curves, and may resulted in poorer precision and accuracy of the method shown in later sections.

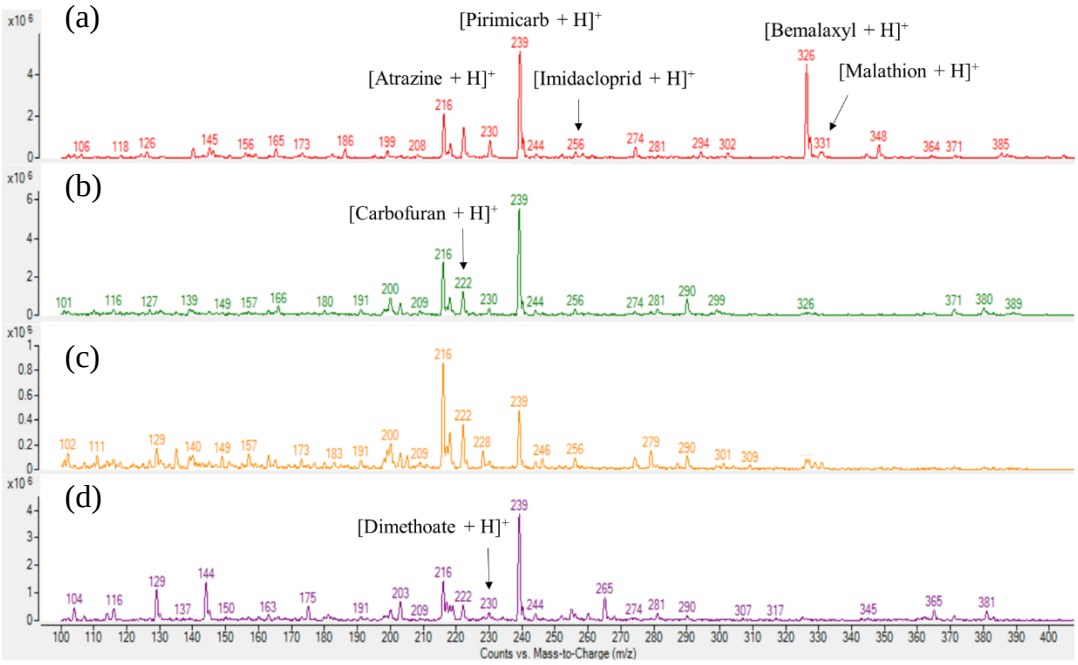


Figure 3-5. (a) Mass spectrum obtained by analysis a mixture of atrazine, benalaxyl, carbofuran, dimethoate, imidacloprid, malathion and pirimicarb (1000 ng mL^{-1} each) with direct infusion ESI-MS. Mass spectra obtained by analysis of (b) honey, (c) apple juice and (d) orange juice containing atrazine, benalaxyl, carbofuran, dimethoate, imidacloprid, malathion and pirimicarb (1000 ng mL^{-1} each) with SPME-ESI-MS.

Table 3-2. The LOD and LOQ values, linear ranges and R^2 coefficients of the calibration curves for SPME-ESI-MS analysis of pesticides in honey, apple juice and orange juice.

	LOD/LOQ (ng mL^{-1})			Linear range (ng mL^{-1})			R^2		
Pesticide	Honey	AJ	OJ	Honey	AJ	OJ	Honey	AJ	OJ
Atrazine	0.6/2	0.3/0.5	1/3	2-200	1-200	3-200	0.993	0.995	0.999
Benalaxyl	1/3	0.3/0.5	3/10	3-200	1-200	10-200	0.994	0.976	0.995
Carbofuran	1/3	0.3/0.5	3/10	3-200	1-200	10-200	0.987	1.000	0.998
Dimethoate	5/20	25/50	20/50	20-800	50-800	50-800	0.986	0.986	0.990
Imidacloprid	20/50	3/10	20/50	50-800	10-200	50-800	0.999	0.987	0.984
Malathion	3/10	10/30	15/50	10-200	30-400	50-800	0.973	0.987	0.951
Pirimicarb	0.5/2	0.3/0.5	3/10	2-200	1-200	10-200	1.000	0.999	1.000

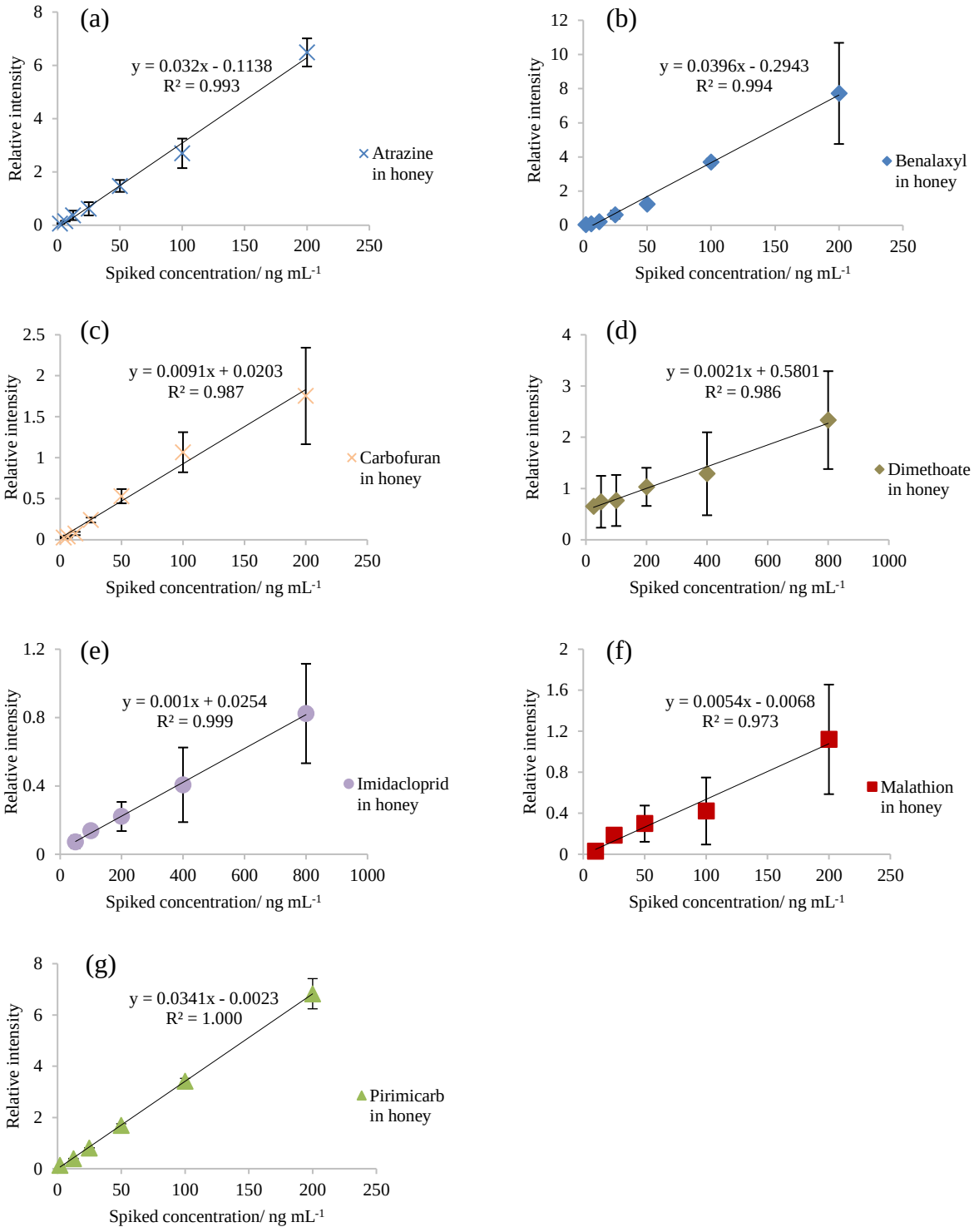


Figure 3-6. The calibration curves for analysis of pesticides in honey by SPME-ESI-MS. (a) Atrazine, (b) benalaxyl, (c) carbofuran, (d) dimethoate, (e) imidacloprid, (f) malathion and (g) pirimicarb.

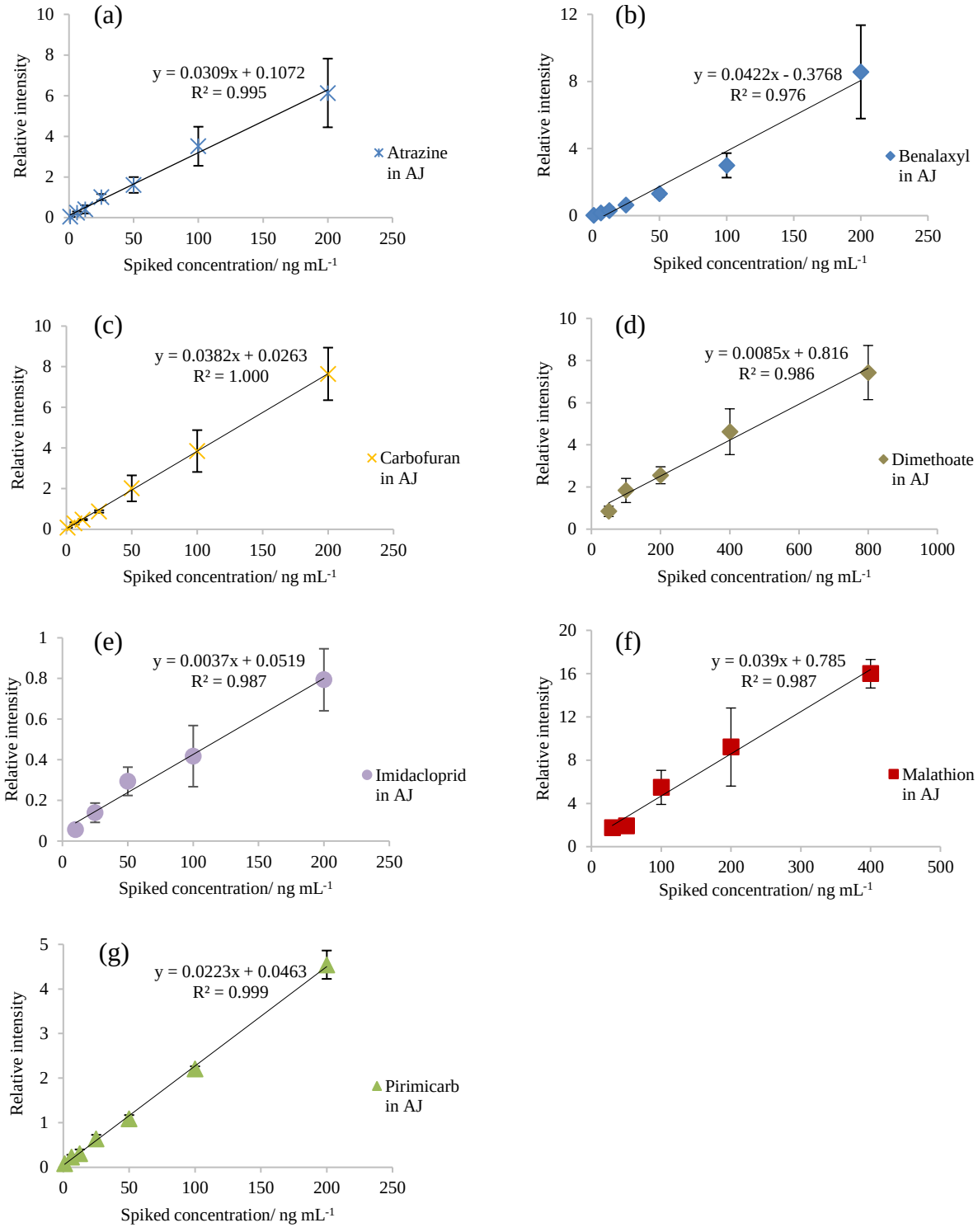


Figure 3-7. The calibration curves for analysis of pesticides in apple juice by SPME-ESI-MS. (a) Atrazine, (b) benalaxyl, (c) carbofuran, (d) dimethoate, (e) imidacloprid, (f) malathion and (g) pirimicarb.

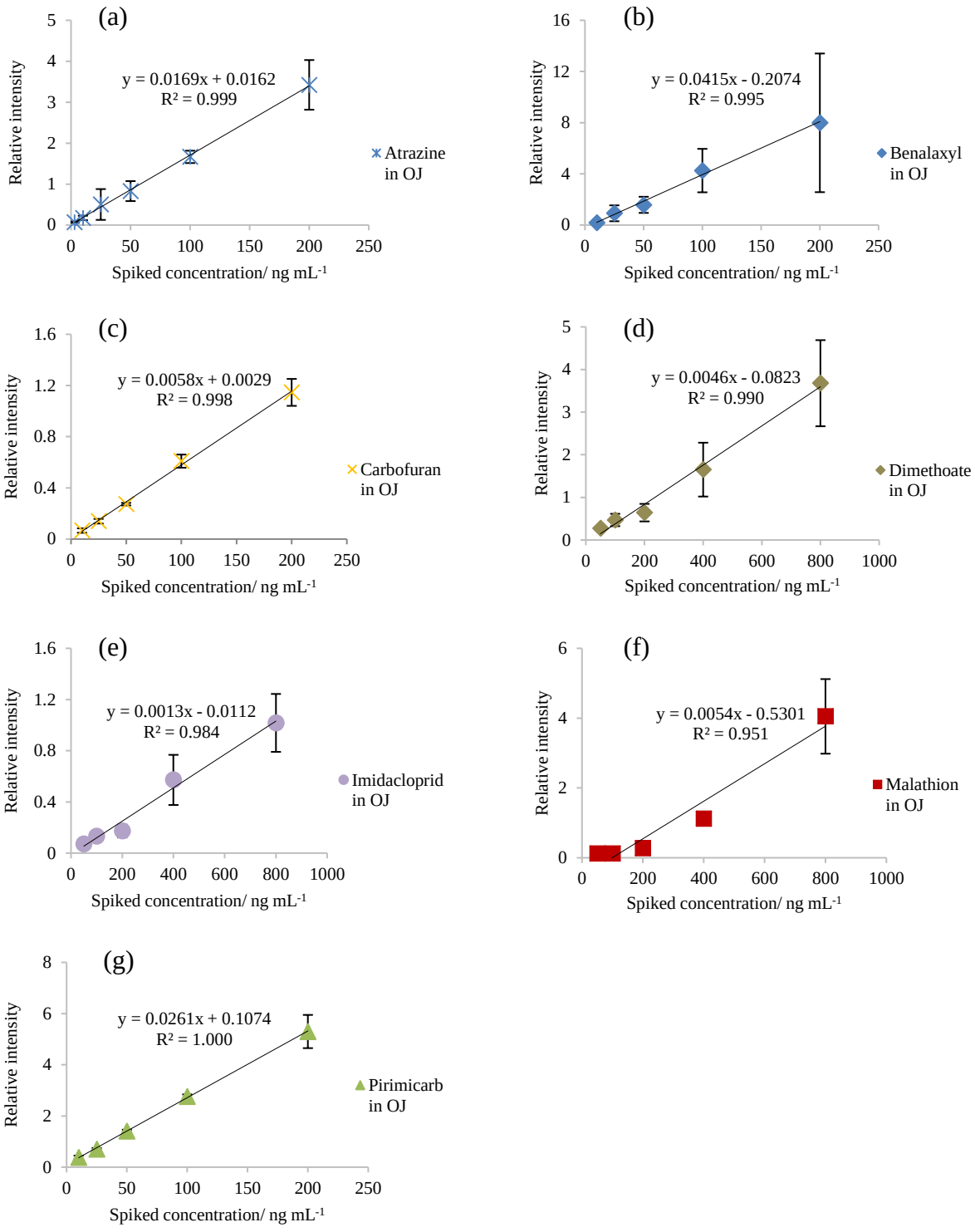


Figure 3-8. The calibration curves for analysis of pesticides in orange juice by SPME-ESI-MS. (a) Atrazine, (b) benalaxyl, (c) carbofuran, (d) dimethoate, (e) imidacloprid, (f) malathion and (g) pirimicarb.

3.3.4 Precision and accuracy

Compared to the study with C18 pipette tips, the precision and accuracy of the present technique were improved with the use of SPME, especially for AJ samples. The improvement was probably also ascribed to the careful control of the experimental parameters such as the extraction time (10 min for all the samples) and the use of a mixer for the agitation. As shown in Table 3-3, Table 3-4 and Table 3-5, the accuracies were between 81 – 126% for honey, 72 – 133% for AJ (except imidacloprid and malathion in low concentration), and 73 – 115% for OJ (except benalaxyl in low concentration).

For the honey samples, the precisions for atrazine, benalaxyl, carbofuran and pirimicarb were in the range of 3 – 32% but dimethoate, imidacloprid and malathion gave less accurate results (up to 69%). More precise and accurate results of dimethoate, imidacloprid and malathion were obtained for the juice samples. This could be due to the easier extraction of the pesticides from the watery texture of juice than from the viscous honey. Pirimicarb gave the best precision among all the matrices (3 – 8% only in honey), with good accuracy as well.

It is noted that those calibration curves with poorer linearities ($R_2 < 0.99$), e.g., dimethoate and malathion in honey (Figure 3-6), generally produced less accurate data (Table 3-3). Also, data points with large standard deviations normally produced less precise results, e.g., dimethoate in honey.

Table 3-3. The precision and accuracy for quantitation of the pesticides in honey by SPME-ESI-MS.

Pesticide	Spiked concentration (ng mL^{-1})	Determined concentration (ng mL^{-1}) (*n=5*) (mean±SD)	RSD (%)	Accuracy (%)
Atrazine	25	25±5	20	99
	100	111±15	14	111
	200	208±40	19	104
Benalaxyl	25	21±5	23	86
	100	96±8	9	96
	200	207±54	26	103
Carbofuran	25	26±6	23	102
	100	97±31	32	98
	200	184±34	18	92
Dimethoate	100	105±72	69	105
	400	402±274	68	121
	800	842±234	28	105
Imidacloprid	100	111±38	34	111
	400	449±152	34	126
	800	650±102	44	81
Malathion	25	24±5	20	96
	100	99±36	36	99
	200	183±80	44	92
Pirimicarb	25	25±1	5	98
	100	100±3	3	100
	200	200±16	8	100

Table 3-4. The precision and accuracy for quantitation of the pesticides in apple juice by SPME-ESI-MS.

Pesticide	Spiked concentration (ng mL^{-1})	Determined concentration (ng mL^{-1}) (*n=5*) (mean±SD)	RSD (%)	Accuracy (%)
Atrazine	25	30±4	12	120
	100	72±21	30	72
	200	259±60	23	130
Benalaxyl	25	24±3	11	95
	100	76±16	21	76
	200	205±90	44	102
Carbofuran	25	23±6	26	91
	100	72±21	30	72
	200	169±70	41	84
Dimethoate	100	133±28	21	133
	400	390±50	13	97
	800	880±330	37	110
Imidacloprid	25	14±3	23	55
	100	111±29	26	111
	200	200±43	22	100
Malathion	50	32±23	71	63
	100	93±42	45	93
Pirimicarb	25	23±6	27	91
	100	108±10	9	108
	200	221±15	7	111

Table 3-5. The precision and accuracy for quantitation of the pesticides in orange juice by SPME-ESI-MS.

Pesticide	Spiked concentration (ng mL^{-1})	Determined concentration (ng mL^{-1}) (*n=5*) (mean±SD)	RSD (%)	Accuracy (%)
Atrazine	25	29±16	55	115
	100	99±7	7	99
	200	197±35	18	98
Benalaxyl	25	16±9	58	65
	100	88±51	58	88
	200	220±75	34	110
Carbofuran	25	21±4	21	84
	100	102±9	9	102
	200	207±20	10	104
Dimethoate	100	103±36	35	103
	400	420±103	25	105
	800	904±212	23	113
Imidacloprid	100	98±30	30	98
	400	388±136	35	97
	800	873±142	16	109
Malathion	100	121±10	8	121
	400	291±32	11	73
	800	850±157	19	106
Pirimicarb	25	25±3	14	101
	100	100±4	4	100
	200	205±15	7	102

3.4 Comparison between C18 pipette-tip ESI-MS and SPME-ESI-MS

3.4.1 The analytical performance

Although the C18 pipette tips and C18 SPME tips used in the study contained the same functionalized material (C18), different analytical performance was observed for their analysis of pesticides due to their differences in designs and operation steps.

In C18 pipette-tip ESI-MS, the C18 pipette tips were packed with 0.6 µL bed of C18 resins with no dead volume, which allows maximum extraction and enrichment of target analyte in a sample. Sample extraction was done by pipetting in/out in the sample manually for eight times and the process was relatively quick. Analytes of interest were extracted and adsorbed on the C18 resins by the interaction between analyte and the non-polar C18 particles. The continuous pipette in/out action allowed maximum extraction of pesticides in a sample. Extracted analytes were eluted when the spraying solvent was applied. As the C18 resins were packed in a pipette tip, they are compact and the extraction can be done for the samples with small quantity.

In SPME-ESI-MS, the C18 materials are coated on the surface of a SPME fibre tip. As the fibre tip is much longer than the C18 pipette tips, a larger surface area of C18 materials on SPME tip is formed, allowing extraction of samples with larger volume. The SPME tip was inserted in sample solution during sample extraction, along with stirring that facilitates the partition of analytes onto the SPME tip and equilibrium. The extraction time of SPME was fixed at 10 mins which was longer than the extraction done by C18 pipette-tip ESI-MS. Upon the application of spraying solvent and high voltage, the adsorbed analytes were sprayed out.

Unlike column separation, a mixture of pesticides was sprayed out simultaneously in both C18 pipette-tip ESI-MS and SPME-ESI-MS. Competition of ionization was likely to occur during the transformation of solution phase to gas phase ions and the production of charged droplets in ionization.

Some different results were observed between C18 pipette-tip ESI-MS and SPME-ESI-MS. Firstly, the orders of the MS response of the pesticides were slightly different for the two techniques. In conventional ESI-MS, pirimicarb gave the strongest MS responses. The order became pirimicarb > atrazine > benalaxyl ~ dimethoate > carbofuran ~ imidacloprid ~ malathion for the honey sample after analysis by C18 pipette-tip ESI-MS. Similar order was observed for the AJ sample at the same concentration (Figure 2-8). For analysis by SPME-ESI-MS, the order was pirimicarb > atrazine > carbofuran > dimethoate ~ imidacloprid ~ benalaxyl ~ malathion for honey and OJ, and similar sequence was observed for AJ except atrazine produced stronger signal than pirimicarb (Figure 3-5). Although the orders were not completely identical, atrazine, benalaxyl and pirimicarb gave stronger signals in both techniques.

Secondly, in general, the precision and accuracy were improved with SPME-ESI-MS. SPME-ESI-MS produced more accurate and precise results for analysis of atrazine, carbofuran, malathion and pirimicarb in honey. The accuracy and precision of atrazine improved from 17 – 32% to 14 – 20% and from 73 – 105% to 99 – 111% respectively. For AJ, the precision and accuracy were improved greatly for every pesticide, e.g., the precision of pirimicarb was improved from 21 – 39% to 7 – 27% and accuracy was improved from 57 – 82% to 91 – 111%. These results indicated that SPME-ESI-MS generally was a better technique than C18 pipette-tip ESI-MS for analysis of the

pesticides in the food samples. However, some results were still not satisfactory even with SPME-ESI-MS. For example, less precise results were obtained for analysis of dimethoate, imidacloprid and malathion in honey samples by SPME-ESI-MS.

Also, the LOD and LOQ values were generally improved when analyzed by SPME-ESI-MS. For example, the LOD and LOQ of atrazine, benalaxyl, carbofuran, and pirimicarb in AJ were reduced from 1 – 3 ng mL^{-1} to 0.3 ng mL^{-1} and 3 – 10 ng mL^{-1} to 0.5 ng mL^{-1} respectively. As for honey, the LOD and LOQ of atrazine, dimethoate, malathion and pirimicarb also reduced.

3.4.2 Limitations of the study

There are limitations in this study. Seven pesticides were chosen for this study and they had different MS responses. Some of them produced strong MS responses and some produced very weak signals. Poorer performance, such as higher LOD/LOQ, poorer linearity, less accurate and precise results, were observed for those pesticides which produced weak signals. It was shown that the extraction of dimethoate, imidacloprid and malathion by both C18 pipette tip and SPME was less efficient than atrazine, benalaxyl, carbofuran and pirimicarb.

Results with large variation were observed in some of the data, including large SD/RSD in the accuracy and precision. The lack of internal standards may affect the accuracy of the results. The IS should be a compound that has a similar structure to the target analyte and can compensate the fluctuations caused by the sample preparation and instrumental measurements. In this study, However, only one IS (pirimicarb d-6) was used in the entire study. Pesticides with similar structure to the IS (pirimicarb and atrazine) would

not be affected, but others, such as dimethoate, imidacloprid and malathion might produce less accurate and precise results. On the other hand, potential competition between these pesticides and the IS may occur, leading to the decrease of formation of ions, ionization efficiency, signal intensity and therefore the data accuracy. Also, results with large variation were observed even an IS was used. The controllability and reproducibility of the techniques should be further improved.

The extraction of pesticides in food samples involved manually-controlled pipetting in/out steps when performing C18 pipette-tip ESI-MS which could not be replaced or controlled by machine. It was anticipated that the loss of analyte during preparation would be compensated with the use of IS in the analysis, however, large variations were still observed even with an IS. Further investigation is thus still needed.

Furthermore, the distance of C18 pipette-tip and SPME tip to the MS inlet may play a role in the signal variation. Unlike the metal capillary in conventional ESI-MS, the position between both C18 pipette-tip or SPME tip end and the MS inlet was manually measured and tuned to the desired position. Even though the position of the tips and MS inlet was optimized and fixed at certain distance, the actual position may shift slightly. Such variation may affect the efficiency of ion formation and cause signal fluctuation and subsequently influence the data accuracy. In order to control the distance more precisely, a configuration can be designed to place the tips at fixed positions and stabilizing the tips. Such system should be easily installed and equipped with the MS. However, this system may also complicate the set-up procedures and more time may be required to spend on the analysis. Also, the cost of the whole study may be increased.

Our results showed that pesticide residues in honey and juice could be effectively extracted by the C18 pipette tips and SPME tips and directly detected by MS. However, no real-life contaminated samples were analyzed during the study due to the difficulty to obtain them. Similar problems are found in the literatures for studies from the other groups. It is anticipated that the analysis of pesticide residues in commercialized honey and juice samples collected from the market using C18 pipette-tip ESI-MS and SPME-ESI-MS will be done in the future.

3.5 Conclusions

SPME is a robust and commonly used method to extract target analytes in a wide range of samples. Conventionally, after the extraction, the tip is washed with solvent to desorb the extracted analytes on the tip. The sample is then analyzed by LC-MS for qualitative and quantitative analysis. In this study, SPME-ESI-MS was introduced for analysis of pesticides in honey and juice samples, with the SPME fibre directly analyzed by MS after the extraction by placing it in front of the MS inlet. The use of SPME as solid-substrate ESI-MS is advantageous. Analytes in complex samples are purified and enriched after SPME extraction. As the head of SPME tip is sharp, it can be served as an ESI emitter and to generate gaseous ions upon high voltage supply. Extracted analytes were desorbed by spraying organic solvent on it at a considerably fast rate and immediate MS results can be obtained. Direct coupling of SPME with ESI-MS allowed rapid identification and quantitation of pesticide residues in food samples with minimum sample pre-treatment.

The results showed that linear calibration curves were obtained with the R^2 coefficients in the range of 0.951 – 1.000 for all pesticides in the honey and juice samples. Excellent

R^2 coefficients (R^2 = 0.993 – 1.000) were obtained for atrazine and pirimicarb in all the three matrices. The LODs and LOQs of some pesticides, i.e., atrazine, benalaxyl, carbofuran and pirimicarb in AJ, were as low as 0.3 and 0.5 ng mL^{-1}, respectively. The LODs and LOQs of all the pesticides in AJ were the lowest, compared to in the other matrices.

The precision and accuracy for analysis of most pesticides have been improved by using SPME-ESI-MS. For analysis of the pesticides in honey, the precisions were between 3 – 32% and the accuracies were between 86 – 111% for atrazine, benalaxyl, carbofuran and pirimicarb. For analysis of pesticides in apple juice, the precisions were between 7 – 30 % and the accuracies between 72 – 130% for atrazine and pirimicarb. For the analysis of the pesticides in orange juice, the precisions were between 4 – 21% and the accuracies between 73 – 121% for carbofuran, malathion and pirimicarb.

Chapter 4: Conclusions and Prospects

ESI-MS is widely used to analyze various compounds, including small molecules and large biomolecules, in different fields such as biology, chemistry, environmental science and food science. In conventional ESI-MS, sample is introduced and ionized via a capillary. Clogging and contamination of the capillary are common problems with capillary-based ESI-MS. The development of ESI-MS using solid substrates avoids these problems and opens new possibilities and new features for analysis of samples.

In this project, we demonstrated the development and applications of C18 pipette-tip ESI-MS and SPME-tip ESI-MS for rapid detection of pesticides in honey and juice samples. Food samples containing pesticides were extracted by C18 pipette tips and SPME tips, and the C18 pipette tips and SPMS tips were directly placed in front of the MS inlet for ESI-MS detection. The C18 pipette tips and SPME tips acted as ESI emitters and gaseous ions of analytes were generated upon application of high voltages. The set-ups of these techniques are simple and easy, and the analysis is quick with only minimum sample preparation involved. As chromatographic separation was not required for the analysis, the results could be obtained within minutes. Both of the C18 pipette tips and SPME tips could be recycled and reused for consecutive analysis of pesticide residues in food samples, which further reduced the costs of the techniques.

Qualitative and quantitative analysis of pesticides in honey and juice by C18 pipette-tip ESI-MS and SPME-ESI-MS were investigated in this project. The targeted pesticides in the food samples could be efficiently enriched by both the C18 pipette tips and the SPME tips, leading to their enhanced detection by MS. LOD of 0.3 ng mL^{-1} could be achieved for analysis of carbofuran and pirimicarb in honey by C18 pipette-

tip ESI-MS, and analysis of atrazine, benalaxyl, carbofuran and pirimicarb in apple juice by SPME-ESI-MS. The LODs for most of the pesticides could fulfil the cut-off values (50 ng mL^{-1}) in the international standards. For most of the cases, SPME-ESI-MS produced more precise and accurate results than C18 pipette-tip ESI-MS.

The methods can be extended to analyze other food contaminants such as food additives and preservatives, residues of antibiotics, drugs and hormones, and different types of food samples such as beverage and drinks, fruits, vegetables and meat products. The use of other solid substrates for selective and sensitive extraction and detection of analytes can also be explored.

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