IN SITU AND REAL-TIME MONITORING AND CHARACTERIZATION OF PESTICIDE RESIDUES ON AND IN FRESH PRODUCE USING SERS

Tianxi Yang
University of Massachusetts Amherst

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IN SITU AND REAL-TIME MONITORING AND CHARACTERIZATION OF PESTICIDE RESIDUES ON AND IN FRESH PRODUCE USING SERS

A Dissertation Presented
by
TIANXI YANG

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2018

Department of Food Science
IN SITU AND REAL-TIME MONITORING AND CHARACTERIZATION OF PESTICIDE RESIDUES ON AND IN FRESH PRODUCE USING SERS

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DEDICATION

To my beloved parents,

Any of my families and friends

who supported me throughout my PhD studies
ACKNOWLEDGMENTS

I would like to first thank my PhD advisor, Professor Lili He for her great support, thoughtful guidance, tremendous patience, and constant encouragement towards me throughout my entire doctoral studies. She set an example of excellence as a researcher, mentor, instructor and she is my role model. I am constantly impressed by her smart and innovative research ideas. Next, I would like to sincerely acknowledge my committee advisors, Prof. John M. Clark, Prof. D. Julian McClements and Prof. Yeonhwa Park for their constructive comments, scientific advice and knowledge and many insightful feedbacks and suggestions for my research.

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ABSTRACT

IN SITU AND REAL-TIME MONITORING AND CHARACTERIZATION OF PESTICIDE RESIDUES ON AND IN FRESH PRODUCE USING SERS

MAY 2018

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Understanding of the behavior and fate of pesticides in fresh produce is of great significance for effectively applying pesticides and minimizing pesticide residues in food. There is lack, however, of an effective method that can monitor and characterize pesticide behaviors including penetration, persistence and translocation. Herein, we developed a novel method for real-time and in situ monitoring of pesticide behaviors in fresh produce based on surface-enhanced Raman scattering (SERS) mapping method. Taking advantage of penetrative gold nanoparticles as probes to enhance the internalized pesticide signals, the internal signals from pesticides were successfully obtained in situ. We found that systemic pesticides (e.g. thiabendazole and acetamiprid) penetrated more rapidly and deeply into fresh produce (e.g. apple, grape and spinach) than non-systemic pesticides (e.g. ferbam and phosmet). Live fresh produce allowed more and faster pesticide penetration than harvested fresh produce due to a higher transpiration level in live tissues. The degradation of thiabendazole on live leaves was detected after 1 week, whereas the
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CHAPTER 1

INTRODUCTION

1.1 Background

In modern agriculture, chemical pesticides are widely used and play an essential role in agriculture production.\textsuperscript{1,2} Most chemical pesticides are designed to be toxic to living things, so by their very nature pose risks to human health and the environment. There is increasing evidence showing an association between pesticides exposures and human health problems, such as cancer and nervous system disorders.\textsuperscript{3,4} Fresh produce is a common source of pesticide exposure and more likely to contain pesticides compared to other food matrices.\textsuperscript{5} Therefore, pesticide residues on and in fresh produce are of great food safety concern.

Pesticides can be classified into non-systemic and systemic pesticides based on their different physical chemistries and abilities to be taken up by plant tissues. Non-systemic pesticides have little or no ability to penetrate plant tissues whereas systemic pesticides can penetrate.\textsuperscript{6} It has been found that the degree of pesticide penetration can affect their persistence in postharvest processes; however, to what degree can they penetrate is not well studied. In addition, limited information is available on their fate on and in plants.

Analysis of pesticide residues in food are traditionally carried out by using chromatographic techniques such as gas chromatography (GC) or liquid chromatography (LC)-based methods.\textsuperscript{7–10} Electrochemical sensors and biosensors have also been used for pesticide detection.\textsuperscript{11–14} However, most of these methods require extraction of the pesticide from sample matrices and thus cannot be used for monitoring pesticide penetration behaviors in plants. Radioactive isotope labeling coupled with mass spectrometry has been developed to monitor pesticide behaviors and determine metabolites.\textsuperscript{15} The drawbacks of
this technique are many and include: expense, the need for pre-labeling, radioactive hazard concern, and limited half-lives of useable isotopes.

Herein, we aimed to develop a novel method that can realize real time and in situ monitoring of pesticide behaviors including penetration, persistence and translocation without pre-labeling using surface-enhanced Raman scattering (SERS) mapping. SERS is an ultrasensitive vibrational spectroscopic technique utilizing Raman spectroscopy and nanotechnology that can detect traces of closely adsorbed molecules on metallic nanostructures (typically Au or Ag). In recent years, SERS has emerged as a powerful analytical tool for biosensing, chemical analysis, biomedical imaging and environmental and food monitoring due to its ultrahigh sensitivity, unique spectroscopic fingerprint and nondestructive data acquisition. Previously, we developed an in situ SERS method coupled with 50 nm citrate coated AuNPs to measure four insecticides on and in fresh tea leaves and apple peels. Interesting, we found the AuNPs were able to penetrate into plant tissues. We took advantage of the fact that gold nanoparticles can rapidly penetrate into plant tissues and apply them as probes to enhance the internalized pesticide signals in situ. Pesticide behaviors can be monitored using the advanced mapping technique with a confocal Raman microscope in real time. Degradation of pesticides can be characterized based on the SERS spectra. Liquid chromatography (LC)-based methods were conducted to validate the number of residues in fresh produce. To the best of our knowledge, this is the first attempt to use SERS beyond pesticide detection but to study the pesticide penetration, persistence, and translocation in situ and in real time. Understanding these processes can help us to develop a better strategy to apply pesticides and reduce
pesticide exposure from fresh produce. Information obtained from this study can also provide a basis for setting residue tolerance levels and other regulatory considerations.

1.2 Objectives

The Overall Objective is to investigate the pesticides behaviors on and in fresh produce in situ and in real time using SERS mapping method. To achieve this, we conducted the following specific objectives:

Objective 1: Investigate the penetration and persistence of pesticides in fresh produce.

For the penetration study, we investigated the penetration of multi-class of non-systemic (e.g. ferbam and phosmet) and systemic pesticides (e.g. thiabendazole and acetamiprid) into different harvested and live fresh produce using SERS mapping method. For the persistence study, we monitored pesticide SERS signals overtime in live plants.

Objective 2: Monitor the pesticide residues on and in fresh produce over time and after different washing methods.

We evaluated the effectiveness of different washing methods including commercial and homemade washing methods in removing both surface and internalized pesticide residues on and in fresh produce.

Objective 3: Investigate the translocation of pesticides in fresh produce.

For the translocation study, we grew live plants in both hydroponic and soil systems with systemic pesticides and monitor the pesticide signals on the different plant tissues in real time.

The completion of these projects will establish innovative methods to monitor and characterize pesticides on and in fresh produce, and gain knowledge on the behaviors and fate of pesticide residues on and in fresh produce. Understanding these processes can help
us to develop a better strategy to apply pesticides and reduce pesticide exposures from fresh produce and food. Information obtained from these studies can also provide a basis for setting residue tolerance levels and other regulatory considerations. The outcome of these studies will greatly benefit to the food chemistry area and long-term stability, safety and sustainability of agriculture and food system.
CHAPTER 2
LITERATURE REVIEW

2.1 Pesticide

2.1.1 Introduction to pesticide

A pesticide is any substance or mixture of substances intended for preventing, destroying, or controlling any pest. There are multiple ways to classify the type of pesticides. For example, on the basis of the target organism, pesticides can be classified as herbicides, insecticides, fungicides, rodenticides, and pediculicides. According to their chemical structure, they can be classified as organic, inorganic, synthetic, or biological pesticides. Pesticides can also be considered as systemic pesticides and non-systemic pesticides based upon their physicochemical characteristics and abilities to be taken up by plant tissues. Non-systemic pesticides have little or no ability to penetrate plant tissues (leaves or roots) and cannot translocate from site of contact to distal parts of the plant. However, systemic pesticides can penetrate plant tissues and be translocated to other parts of plant. Systemicity depends on the physicochemical characteristics of the chemicals mainly including the partition coefficient octanol/water (log Kow) and the coefficient of dissociation (pKa). The parameter of log Kow indicates the lipophilicity of substances which is related to the ability of substances to penetrate through bio-membranes. In order to enter into the plant, chemicals need to cross the plant cuticle. When pesticides are used as root, soil, or seed applications, the sorption of them to plant tissues mainly depends on partition coefficient octanol/water. Researchers have showed that compounds can be considered systemic when their partition coefficient octanol/water goes from 0.1 to 5.4 and maximal cuticle permeability occurs with neutral lipophilic compounds, log Kow being
around between 1 and 2.5. When pesticides are applied as foliar spray, the log Kow and the concentration of the applied formulation also influence uptake via the leaves. Buchholz and Nauen describe two additional parameters that alter cuticle permeability of systemic pesticides: molecular mass and temperature. Molecules with high molecular mass at low temperatures tend to penetrate less. The parameter of pKa indicates if the diluted form of the molecule is a weak or a strong acid. It is important to note that the phloem pH of plants is around 8 and the xylem pH is around 5.5. Almost all systemic compounds are weak electrolytes.

Pesticides are essential in modern agriculture practice due to the benefits of pesticides to control harmful organisms. Farmers can save a lot of money with pesticides to preventing crop losses to insects and other pests. According to the Environmental Protection Agency (EPA) report, the agricultural market shares of total pesticides resulted in $35.8 billion in 2006 and more than $39.4 billion in 2007 in spending expenditure respectively. However, on the cost side of pesticide use there can be costs to the environment and human health. There is increasing evidence showing pesticides may cause acute and chronic health effects in people who are exposed. Pesticide exposure can cause a variety of adverse health effects, ranging from simple irritation of the skin and eyes to more severe effects such as causing cancer and nervous system disorders. Pesticide use results in a number of environmental concerns. Certain pesticides applied to crops eventually end up in non-target species, air, water and soil. In addition, pesticide use reduces biodiversity, cause pollinator decline, and threatens endangered species.
2.1.2 Significance of studying pesticide behaviors

Understanding pesticide behaviors including penetration, persistence and translocation is critically important for effective and safe application pesticides. In terms of pesticide penetration, it has been found that the degree of pesticide penetration can affect their persistence in postharvest processes. For example, one study found it was easier to wash off pesticide residues from olives one day after spraying than after one week, probably due to their increased retention in cuticular waxes over time.\(^40\) Another interesting study reported on the effectiveness of tap water rinsing on reduction of 12 pesticide residues on produce.\(^41\) It was found that residues of vinclozolin, bifenthrin, and chlorpyrifos were not reduced after rinsing, suggesting that pesticide probably penetrated into fresh produce. Thus, the degree of pesticide penetration may determine how well it can be removed by rinsing, even at a depth of several micrometers. However, there is very limited information available regarding the degree (time and depth) of pesticides penetration in different plant tissues, particularly for non-systemic pesticides.

Understanding persistence and degradation of surface and internalized pesticides are meaning for both effectively applying pesticides and minimizing pesticide exposure from food. Information on pesticide degradation can also provide a basis for residue tolerance and regulatory considerations. The metabolic fate of pesticides in plant tissues is dependent on abiotic environmental conditions (temperature, moisture, soil pH, etc.), microbial community or plant species (or both), pesticide characteristics (hydrophilicity, pKa/b, Kow, etc.), and biological and chemical reactions.\(^42,43\) Surface pesticides are mostly influenced by the environmental factors while internalized pesticides are degraded by the plant biochemically, mainly through plant metabolism which involves enzymes, particularly
oxidative enzymes (e.g., cytochrome P450’s, peroxidases, and polyphenol oxidases). Pesticide metabolism in plants generally involves oxidation, reduction, hydrolysis and conjugation which can facilitate detoxification, compartmentalization, sequestration, and/or mineralization. Glucose conjugation to pesticides occurs primarily in plants. The metabolic fate of pesticide molecules vary with many factors such as the species of plants, residence time on and in plant and the degree of pesticide penetration into the plant. Up to now, there is still very limited information on the degradation process and mechanism of pesticides in plants. In addition, there is still challenge to study internalized pesticides without extracting the bound pesticides from plant tissues.

Understanding translocation of pesticides can help us to apply pesticides reasonably and safely. Systematic pesticides are capable of entering plants and being transported into the vascular system. Their distribution in plants can be achieved following foliar application as well as by uptake via roots. Root-to-shoot translocation is more common but less studied due to the technical difficulties associated with root experiments. During the root-to-shoot translocation process, systemic pesticides can be transported to stems or leaves through xylem after root uptake and transpiration was the main force. After pesticides getting into the leaves, leaf veins are the transport system. It is well known about pesticide translocation phenomenon. Nevertheless, it remains unclear the dynamic translocation and the distribution of pesticides in plant tissues such as leaves. Understanding these information is crucial for both effectively applying pesticides and minimizing pesticide residues in plant food.
2.2 Current analytical techniques for studying pesticides and their limitations

Chromatographic techniques are common methods to analyze pesticide residues in food. For examples, gas chromatography (GC)-based methods are routinely used for analyzing organophosphates and pyrethroids residues.\textsuperscript{54} For some new polar neonicotinoid insecticides, such as imidacloprid, are commonly analyzed by liquid chromatography (LC) – based methods, due to their poor thermal stability or volatility.\textsuperscript{55} Recently, analysis of pesticide residues using the Quick Easy Cheap Effective Rugged and Safe (QuEChERS) extraction method in combination with LC or GC and tandem mass spectrometric (MS) detection are very popular for pesticide detections in food.\textsuperscript{56–58} In this dissertation, we will use the QuEChERS extraction and LC or GC-MS/MS as a standard validation method. However, chromatographic techniques have a lot of disadvantages such as time-consuming preparations, laborious manipulations, complex extraction procedures and clean-up. In addition, it is difficult to detect multi-class pesticides of different polarities with one simple extraction method and instrument. Besides chromatographic methods, there are also some other methods developed for detection of pesticides, such as electrochemical sensors and biosensors. For example, Liu. et al. reported an electrochemical sensor for detection of organophosphate pesticides using zirconia nanoparticles as selective sorbents.\textsuperscript{59} Mazzei. et al. presented a bio-electrochemical system for the determination of pesticides by electrochemically monitoring the inhibition of the catalytic activity of the enzyme alkaline phosphatase either in the presence or in the absence of pesticides.\textsuperscript{60} Simonian. et al. also applied nanoparticle-based optical biosensors for the direct detection of organophosphate pesticides.\textsuperscript{61} However, most of these methods can be only applied for specific groups of pesticides or can be influenced a lot from the matrix effects.
There are limited techniques available for monitoring pesticide behaviors and fate in plants. Radioactive isotope labeling coupled with mass spectrometry has been used to monitor pesticide behaviors and determine metabolites.\textsuperscript{15} However, the drawbacks for using radioactive isotopes are very clear: the needs for pre-labeling, expensive, radioactive hazard concern, and limited life time. To the best of our knowledge, there is no such a technique that is capable to analyze pesticides in plant tissues in situ and in real time without pre-labeling. Therefore, it is critical to develop a simple, rapid and sensitive technique that can analyze multi-classes pesticides in complex matrices. Here we propose to establish SERS technique-based methods for analyzing pesticides on and in fresh produce \textit{in situ} and in real time. We will use the established SERS methods for studying pesticide behaviors including penetration, persistence and translocation.

2.3 Raman and SERS spectroscopy

2.3.1 Raman spectroscopy

Raman spectroscopy is becoming one of more promising analytical tools for evaluation of food.\textsuperscript{62–67} Raman signals rely on probing specific molecular vibrations of a sample based on measurements of inelastic light scattering. Specifically, Raman spectroscopy is a scattering technique caused by a substance radiated with a monochromatic light, usually from a laser in the visible, near infrared, or near ultraviolet range.\textsuperscript{68,69} It relies on discrete vibrational transitions that take place in the ground electronic state of molecules. The laser light interacts with molecular vibrations, phonons or other excitations in the system, resulting in the energy of the laser photons being shifted. Most of the scattered light has the same frequency or energy as that of the incident light. Only a slight fraction of the incident light donates or receives energy to contribute to a change in the vibrational and
rotational state of molecules and this constitutes Raman scattering. If the frequency of incident radiation is higher than frequency of scattered radiation, it is Stokes Raman scattering. However, when the frequency of incident radiation is lower than frequency of scattered radiation, it is called anti-Stokes Raman scattering. The difference in frequency of the laser and that of the scattered photon is called the Raman shift (ν in cm$^{-1}$) and is a function of wave numbers. The conventional Raman spectroscopy generally means Stokes Raman spectroscopy because Stokes shifted Raman bands involve the transitions from lower to higher energy vibrational levels and therefore, Stokes bands are more intense than anti-Stokes bands. For a Raman active molecule, it must have distorted electron densities or polarizabilities due to energy exchange.$^{70-72}$ A change in polarizability during molecular vibration is an essential requirement to obtain Raman spectrum of sample. The Raman scattering intensity is proportional to the change of polarizability. Therefore, Raman spectroscopy is considered to be selective in detecting apolar molecules, double-or triple-bonded structures and ring structures.

Raman spectroscopy has a lot of advantages. It provides fingerprint information of compounds and can also be applicable to a wide range of substance including liquid, solid without concern about size, shape or thickness. A Raman spectrum is usually acquired within a few seconds non-invasively. Therefore, no or limited sample preparation is needed. Another major advantage of the Raman method with an advanced instrument and software is that it can be used to generate 2D and 3D maps of the chemical composition of complex materials. A Raman spectrum is acquired at each pixel of an image, and then analyzed to generate artificial color images based on molecular composition and structure. A typical experiment uses sequential sample movement and spectrum acquisition to collect data from
the user defined image area. Raman technique has been used to non-destructively study various materials, including plant tissues and cell biocomponents.\textsuperscript{73–76} However, conventional Raman scatterings are very weak because only one photon in one million scatters light inelastically.

2.3.2 SERS spectroscopy

SERS has attracted a lot of attention for more than three decades because it provides a means to enhance the normally weak Raman signal by several orders of magnitude.\textsuperscript{32,77} SERS is a combination technique of Raman spectroscopy and nanotechnology with lots of advantages such as ultrahigh sensitivity, unique spectroscopic fingerprint identification, and nondestructive data acquisition.\textsuperscript{28,78,79} Placement of sample molecules on nanoscale-roughened surfaces of noble metals (typically silver or gold), which are called SERS substrates, enhances the inherently weak Raman signatures tremendously. The enhancement mechanism lies in the electromagnetic field enhancement attributed localized surface plasmon resonance (LSPR), as well as chemical enhancement due to charge transfer between analyte and substrate.\textsuperscript{80} In electromagnetic field enhancement mechanisms, a great deal of attention has been paid to “hot spots” within SERS substrates, which are nanoscale regions of the SERS substrate that have the strongest local enhancement mechanisms field enhancement.\textsuperscript{81–84} The simplest model of a hot spot is the junction or gap region between two adjacent nanoparticles in a nanoparticle dimer.\textsuperscript{85} This mechanism indicates that when analytes are located at the narrow gap between noble metal nanostructures, the enhancement mechanisms field intensities can be enhanced dramatically (as much as $10^7$) in the gaps relative to the incident intensity. Au NPs are one of the most commonly used SERS substrates. The signal enhancement depends on many
factors, such as composition, size, shape of the noble metal NPs and gap between NPs.\textsuperscript{86} For example, some studies show that the strongest SERS enhancement was produced by Au NPs in the range of 50-80 nm.\textsuperscript{87} In addition, the closer the molecule to the surface, the higher enhancement of the Raman signals. The limit of detection of SERS can go down to the single molecule level in some cases.\textsuperscript{88–90} Among SERS methods, SERS mapping technique is a rising and advanced chemical imaging technique, by which hundreds and thousands of SERS spectra can be rapidly and automatically collected at every pixel of the defined area, and then integrated to generate artificial color images based on the intensity of a designate peak.\textsuperscript{91–93} In recent years, SERS technique has emerged as a powerful analytical tool for molecular detection and characterization in various areas including biosensing, chemical analysis, environmental and food monitoring.\textsuperscript{90,94–96}

### 2.4 SERS applications for pesticide studies

The earliest record of pesticide detection using SERS was performed in 1987 by Alak and Vo-Dinh.\textsuperscript{97} In their article, eight organophosphorus pesticides were characterized using silver-coated microspheres as substrates. Since then, a large number of scientific publications regarding the SERS detection of pesticides have been published.\textsuperscript{93,98–102} Recent development of SERS methods for pesticide detection progressed from detection in simple solvents to complex food matrices.

The detection of pesticides in simple solvents was mainly from the study that intended to demonstrate the potential application of SERS substrates and SERS methods.\textsuperscript{95,101,103,104} For example, Wang. et al. reported the synthesis of silver nanocubes as a SERS substrate for the determination of pesticide paraoxon and thiram (Figure 2.1).\textsuperscript{105} Tang. et al. also fabricated a highly uniform SERS substrate for pesticide (thiram) analysis based on Au
nanoparticles grafted on dendritic $\alpha$-Fe$_2$O$_3$.\textsuperscript{106} Zhang. et al. used silver nanowires as a SERS substrate for the detection of pesticide thiram.

Figure 2.1 Schematic illustration of silver nanocubes as a SERS substrate for the determination of pesticide paraoxon and thiram.

Detection of pesticides in real food matrices involved more studies on the sample extraction and optimization for SERS analysis. Depends on the type of food matrices, i.e. solid or liquid, different methods were explored. For solid matrices like apples, homogenization of the peel or surface swab methods were used to recover pesticides on the surface. For example, homogenization was used to extract three types of pesticides (carbaryl, phosmet, and azinphos- methyl) from apple and tomato peels. The extraction solution was then deposited on a commercial Q-SERS substrate for analysis.\textsuperscript{107} Although the detection limit was not as good as the limits for the pesticides in simple solvents, most of these detection limits meet the regulatory levels. Surface-swab method is a simple method that can effectively recover the pesticides on the surface of fruits like apples and pears. As shown in Figure 2.2, He et al. developed a rapid (within 10 min) and simple method which combines a surface swab capture method and SERS for recovery and
quantitative detection of thiabendazole on apple surfaces. The whole apple surface was swabbed, and the swab was vortexed in methanol releasing the pesticide. Silver dendrites were then added to bind the pesticide and used for enhancing the Raman signals. The accuracy of the swab-SERS method was calculated to be between 89.2% and 115.4%.

Figure 2.2 The illustration of the swab-SERS procedure on apples

Recently in situ detection of pesticides on the surface of vegetables and fruits using SERS method is very popular. A wide range of SERS substrates has been developed such as Au-core silica-shell NPs (Au @ SiO₂ NPs), Ag-coated AuNPs (Au @ Ag NPs) and Au-MNN for SERS applications. Yang et al. fabricated a smart AuNPs dotted magnetic network nanostructure (Au-MNN) as SERS substrate. Au-MNN presenting the large surface and high detection sensitivity enables it to exhibit multifunctional applications involving sufficient adsorption of chemical species for enrichment, separation, as well as a Raman amplifier for the analysis of trace pesticide residues at femtomolar level by a portable Raman spectrometer at various vegetables peels in situ. (Figure 2.3). More recently, we developed an in situ SERS method coupled with 50 nm citrate coated AuNPs to measure four insecticides on and in fresh tea leaves and apple peels.

To advance the capacity of SERS for pesticide studies, herein this project, we used innovative SERS methods which not only can directly measure the surface pesticides, but
also measure the internalized pesticides *in situ*. The SERS methods can be used beyond the detection purpose, but also for studying the behaviors of pesticides, e.g. surface residues, measure the internalized pesticides *in situ*, penetration, persistence translocation, on and in fresh produce.

**Figure 2.3** Scheme for the procedure of the in-situ detection of thiram pesticide residues at vegetable peels monitored by the portable Raman spectrometer using Au-MNN as Raman amplifier.

2.5 Justifications for the fresh produce and pesticides used

2.5.1 Justifications for selecting the target fresh produce

Fresh produce is a common source of pesticide exposure and more likely to contain pesticides compared to other food matrices. Therefore, pesticide residues on and in fresh produce are of great food safety concern.
Fresh and processed fruits and vegetables are top of the total food considered to contain pesticide residues, and fresh products are more likely to contain pesticides. For fresh produce, five representative food matrices, apples, grapes, spinach leaves, basil plant and tomato plant, were chosen to study because they are reported as among the most commonly consumed produce in the US and appear on the list of the fruits and vegetables with the most pesticide residues.\textsuperscript{110} In addition, apple, grape and spinach represents a different type of harvested fresh produce. Apples are examples of fruits that have relatively hard external surfaces with thin peels, which are commonly consumed whole and as peeled or sliced. Grapes are examples of fruits that have relatively soft external surfaces with ultra-thin peels and are usually consumed whole. Spinach leaves are vegetables that have no peel and are consumed whole. Finally, the pesticides residues on the surfaces of these types of fresh produce are more likely to be ingested compared to produce with inedible thick peels (e.g., bananas and oranges). Basil (Ocimum basilicum) and tomato plants (Solanum lycopersicum) was chosen for study as examples of plants due to its association with various pesticide exposures, widespread consumption by humans, and relatively large surface area facilitating carrying out these experiments.

\textbf{2.5.2 Justifications for selecting the target pesticides}

In this study, we focused on multi-class insecticides and fungicides because residues of insecticides and fungicides on and in fresh produce are more likely compared to others such as herbicides due to their higher use pattern on fresh produce in conventional produce farming. 2 insecticides (acetamiprid and phosmet) and 2 fungicides (thiabendazole and ferbam) are selected and their molecular structures and maximum residue levels (MRLs) are shown in Table 2. 1.
Table 2.1 Chemical names, classifications, structures and maximum residue levels (wet weight) of pesticides tested in this study. The data for thiabendazole, ferbam and phosmet are from Electronic Code of Federal Regulations (e-CFR). The data source for acetamiprid are EU Pesticides database.

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<th>Grape</th>
<th>Spinach</th>
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<td>Nonsystemic organophosphate insecticide</td>
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<td>-</td>
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CHAPTER 3

REAL-TIME AND IN SITU MONITORING OF PESTICIDE PENETRATION IN EDIBLE LEAVES BY SURFACE-ENHANCED RAMAN SCATTERING MAPPING

3.1 Abstract

Understanding of the penetration behaviors of pesticides in fresh produce is of great significance for effectively applying pesticides and minimizing pesticide residues in food. There is lack, however, of an effective method that can measure pesticide penetration. Herein, we developed a novel method for real-time and in situ monitoring of pesticide penetration behaviors in spinach leaves based on surface-enhanced Raman scattering (SERS) mapping. Taking advantage of penetrative gold nanoparticles (AuNPs) as probes to enhance the internalized pesticide signals in situ, we have successfully obtained the internal signals from thiabendazole, a systemic pesticide, following its penetration into spinach leaves after removing surface pesticide residues. Comparatively, ferbam, a non-systemic pesticide, did not show internal signals after removing surface pesticide residues, demonstrating its non-systemic behavior. In both cases, if the surface pesticides were not removed, co-penetration of both AuNPs and pesticides was observed. These results demonstrate a successful application of SERS as an effective method for measuring pesticides penetration in fresh produce in situ. The information obtained could provide useful guidance for effective and safe applications of pesticides on plants.
3.2 Introduction

In modern agriculture, chemical pesticides are widely used and play an essential role in agriculture production.\textsuperscript{1,2} Most chemical pesticides are designed to be toxic to living things, so by their very nature pose risks to human health and the environment. There is increasing evidence showing an association between pesticides exposures and human health problems, such as cancer and nervous system disorders.\textsuperscript{3,4} Fresh produce is a common source of pesticide exposure and more likely to contain pesticides compared to other food matrices.\textsuperscript{5} Therefore, pesticide residues on and in fresh produce are of great food safety concern.

Pesticides can be classified into non-systemic and systemic pesticides based on their different physical chemistries and abilities to be taken up by plant tissues. Non-systemic pesticides have little or no ability to penetrate plant tissues whereas systemic pesticides can penetrate.\textsuperscript{6} It has been found that the degree of pesticide penetration can affect their persistence in postharvest processes. For example, one study found it was easier to wash off pesticide residues from olives one day after spraying than after one week, probably due to their increased retention in cuticular waxes over time.\textsuperscript{40} Thus, the degree of pesticide penetration may determine how well it can be removed by rinsing, even at a depth of several micrometers. There is very limited information available, however, for studying the penetration behaviors of pesticides in edible plant tissues. Information on the extent of the penetration of non-systemic and systemic pesticides into plant tissues is a critical need and is crucial for both effectively applying pesticides and minimizing pesticide residues in food.

Analysis of pesticide residues in food are traditionally carried out by using chromatographic techniques such as gas chromatography (GC) or liquid chromatography
Electrochemical sensors and biosensors have also been used for pesticide detection. Most of these methods require extraction of the pesticide from sample matrices and thus cannot be used for monitoring pesticide penetration behaviors in plants. Radioactive isotope labeling coupled with mass spectrometry has been developed to monitor pesticide behaviors and determine metabolites. The drawbacks of this technique are many and include: expense, the need for pre-labeling, radioactive hazard concern, and limited half-lives of useable isotopes. Herein, we aimed to develop a novel method that can realize real time and in situ monitoring of pesticide penetration without pre-labeling using surface-enhanced Raman scattering (SERS) mapping. SERS is an ultrasensitive vibrational spectroscopic technique utilizing Raman spectroscopy and nanotechnology that can detect traces of closely adsorbed molecules on metallic nanostructures (typically Au or Ag). In recent years, SERS has emerged as a powerful analytical tool for biosensing, chemical analysis, biomedical imaging and environmental and food monitoring due to its ultrahigh sensitivity, unique spectroscopic fingerprint and nondestructive data acquisition. In food safety areas, SERS has been widely applied in the detection of chemical toxins and pathogens in foods. 

In situ SERS detection of pesticides on the surface of plants have been demonstrated using Au-core silica-shell NPs (Au @ SiO₂ NPs) and Ag-coated AuNPs (Au @ Ag NPs). More recently, we developed an in situ SERS method coupled with 50 nm citrate coated AuNPs to measure four insecticides on and in fresh tea leaves and apple peels. Interestingly, we found the AuNPs were able to penetrate into plant tissues. This finding inspired us to use the penetrated AuNPs as probes for measuring internalized pesticides. Coupled with a confocal microscope and advanced mapping technique, we measured the penetration
behaviors of ferbam, a non-systemic pesticide, and thiabendazole, a systemic pesticide, into spinach leaves over time. To the best of our knowledge, it is the first study that applied SERS mapping to study pesticide penetration in plant tissues. Understanding pesticide penetration behaviors will help us to develop a better strategy to apply pesticides and reduce pesticide exposure from fresh produce.

3.3 Materials and Method

3.3.1 Materials
Iron(III) dimethyldithiocarbamate (fungicide ferbam) and 2-(4-Thiazolyl) benzimidazole (fungicide thiabendazole) were of analytical reagent grade and were purchased from Sigma-Aldrich (St. Louis, MO). Sodium bicarbonate (NaHCO₃) was purchased from Fisher Scientific (Pittsburgh, PA). Citrate-capped AuNPs colloids were purchased from NANO PARTZ™ Inc. (Loveland CO, USA). Organic baby spinach leaves were obtained from Whole Foods Market (Waltham, MA). All reagents were used without further purification. Ultrapure water (18.2 MΩ.cm) was produced with Thermo Scientific Barnstead Smart2Pure Water Purification System and used for the preparation of all solutions.

3.3.2 Measurement of the SERS characteristic signals of two pesticides
Spinach leaves are chosen as the model edible plant tissue due to its high association with pesticide exposure, high consumption in the US, and relative large and flat surfaces for experiment. These leaves were carefully washed with ultrapure water and dried before experiment. Ferbam and thiabendazole were used as examples of non-systemic and systemic pesticide, respectively, and each was prepared in water with various concentrations. A volume of 50 µL of each solution was then mixed with 50 µL of a 250
ppm 50nm AuNPs solution for 1 min at room temperature to ensure complete pesticide complexation with AuNPs through Au-thiol bond. 5 µL of each of the pre-prepared pesticide solution were then pipetted onto spinach leaves situated on a glass slide and air-dried in a fume hood for 30 min. Solutions of AuNPs without pesticide, ferbam alone and thiabendazole alone were also pipetted onto spinach leaves as control treatments for comparisons. All controls were air-dried in a fume hood as above. For evaluation of feasibility of Raman detection of pesticides on spinach leaves, 5 µL of the pre-prepared pesticide solution and AuNPs solution without pesticide were also dropped onto a gold slide and dried for 30 min in a hood for comparisons. Raman spectra were collected individually for all treatments.

3.3.3 Preparation for in situ study of non-systemic pesticide penetration

To study non-systemic pesticide penetration, 20 ppm of ferbam was chosen as a model. The concentration of 20 ppm pesticide solution was applied to mimic the early stage of pesticide practical applications. Monitoring pesticide residues is meaningful to ensure the good coverage of pesticides on the surfaces as well as to predict the safe date for harvest, from a farmer’s perspective. From a viewpoint of useful guidance for effective and safe applications of pesticides on plants, higher concentration of pesticides is more toxic and meaningful. In detail, a 5 µL aliquot of the 20 ppm ferbam solution was pipetted onto the surface of spinach leaves and put in a fume hood for 30 min. A 5 µL aliquot of a 250-ppm solution of AuNPs was then pipetted onto the same area of the spinach leave where the ferbam solution had been placed. After 1h, bright field light scattering images and SERS mapping images were collected from all samples.
In order to remove any interference caused by pesticide residues on the spinach leaf surface, different washing methods were investigated. After drying the 5 µL aliquot of the 20-ppm ferbam solution that had been pipetted onto fresh spinach leaves, leaves with ~2.0 × 2.0 cm² square area were cut from fresh whole leaves by a sharp knife. Then each leaf was immersed into 20 mL of deionized water or 20 mL of a 10 mg/mL of NaHCO₃ solution. For the water washing method, each leaf was submerged into water for either 10 or 60 min. For the NaHCO₃ washing method, each leaf was submerged into the NaHCO₃ solution for 30, 60, 90, or 120 s and then gently rinsed with 500 mL of deionized water for 30 s, respectively. All washed leaf samples were air-dried at room temperature for 30 min before being treated with AuNPs. After AuNPs solution for drying 30 min, SERS detection was performed on that area. Ten discrete locations were randomly chosen in each leaf sample and scanned. Similar samples without either washing method were also scanned for comparisons.

The penetration behavior of ferbam was monitored during different time periods from 2 hours to 2 days. A 5 µL aliquot of the 20-ppm ferbam solution was pipetted onto leaves and allowed to dry in air at room temperature over different time intervals. Following different exposure periods, the surface residues of ferbam were washed with NaHCO₃ solution and a 5 µL aliquot of the 250 ppm AuNPs solution added as described above. After 1 h for drying, SERS depth mapping images were obtained using the confocal Raman instrument. Similar samples without washing were likewise measured for comparison purposes.
3.3.4 Preparation for in situ study of systemic pesticide penetration

To study accurate systemic pesticide penetration, different washing methods were also used to remove surface thiabendazole residues. Washing treatments were carried out using either water or NaHCO$_3$ solution. For the water washing method, each thiabendazole contaminated leaf was submerged into water for either 10 or 60 min. For the NaHCO$_3$ washing method, each contaminated leaf was submerged into the NaHCO$_3$ solution for 60 s, 90 s, 120 s or 150 s and then gently rinsed with 500 mL of deionized water for 30 s, respectively. Following the NaHCO$_3$ washing method, the leaves were gently rinsed by water for 30 s. Surface thiabendazole residues were then analyzed by pipetting 5 µL of a 250 ppm AuNP solution onto the same leaf area where thiabendazole had been placed prior to washing and determining SERS depth mapping images by Raman instrument as before. The penetration behaviors of thiabendazole were also monitored during different time periods as for ferbam above.

3.3.5 Raman instrumentation and data analysis

A DXR Raman microscope (Thermo Fisher Scientific, Madison, WI, U.S.A.) with a 780 nm laser and a 20× long distance microscope objective was used in this study. Each spectrum was scanned from 400 to 2000 cm$^{-1}$ with 5 mW laser power and 2 s exposure time. For measuring pesticides on both gold slides and spinach leaf surfaces, Raman mapping was applied with a 50 µm slit aperture to maximize the signals. Ten discrete locations were randomly chosen on each sample for analysis. For penetration studies, SERS depth mapping images were obtained with a 50 µm pinhole aperture to control the confocal depths using a scanning depth of 300 µm. Each area was randomly picked up from spinach leaves and vertical to leaves surface with 100 µm × 300 µm area. The step size of
the mapping was 20 µm and one image contained 75 scanning spots. Raman images were integrated based on the characteristic peaks in the pesticide spectra using the atlμs function in the OMINCS software (Thermo Fisher Scientific).

3.4 Results and Discussion

3.4.1 Selection of the AuNPs probe for pesticide penetration study

Previous NPs-plant interaction research determined that AuNPs could rapidly penetrate into plant tissues. As shown in Figure 3.8 (See Supplementary Figures), the penetration of AuNPs of different diameters into leaves were investigated using the in situ SERS mapping technique. The corresponding Raman images were integrated based on the characteristic peak at 1525 cm\(^{-1}\). We observed the penetration of AuNPs started immediately upon pipetting the AuNPs colloids onto the spinach leaf surface. The size of NPs was important for both signal intensity and penetration depth. 15 nm AuNPs had relatively weak signals compared with 50, 70, 90, 150 nm AuNPs, and their signals were lost when they penetrated further than 75 µm. 50 nm AuNPs penetrated to 150 µm within 40 min, further than 70, 90 or 150 nm AuNPs. For 150 nm AuNPs we observed a part of signal at a depth of 250 µm, which is probably due to the various size of 150 nm AuNPs prepared. From this data, we chose 50 nm AuNPs as the probe for studying pesticide penetration.

3.4.2 Measurement of SERS spectra from pesticides on spinach leaf surfaces

The SERS spectra of ferbam and thiabendazole using 50 nm AuNPs were obtained on both the gold coated microscopic slides (Figure 3.9) and spinach leaf surfaces (Figure 3.1A). The characteristic SERS peaks of ferbam were clearly detected at 1371, 1138, 940 and 553 cm\(^{-1}\) (Figure 3.1A-a). The signature peaks for thiabendazole were found at 1275, 1010 and 780 cm\(^{-1}\) (Figure 3.1A-b). Little or no signals were detected from AuNPs, ferbam and
thiabendazole without mixing with AuNPs, or with spinach leaves alone (Figure 3. 1A-c, 1A-d, 1A-e and 1A-f). Moreover, there was not any measurable pesticide from organic spinach leaves obtained. The concentration-dependent SERS spectra of ferbam on spinach leaves were obtained with increasing concentration from 1 ppb to 100 ppm (Figure 3. 1B). The control was spinach leaves without ferbam exposure. Even as low as 1 ppb, Raman signals of ferbam can still be clearly seen, showing the ultra-high sensitivity of the developed SERS method for the detection of ferbam with the detection limit of 1 ppb. Similarly, SERS spectra of thiabendazole on spinach leaves with increasing concentration from 2 ppb to 100 ppm are shown in Figure 3. 1C and the detection limit is determined to be 2 ppb. The ultra-high sensitivity of detection of characteristic Raman signatures of pesticides demonstrates the successful application and the feasibility of using AuNPs for the analysis of ferbam and thiabendazole on spinach leaves. The peaks 1371 cm$^{-1}$ for ferbam and 1010 cm$^{-1}$ for thiabendazole were chosen as the characteristic peaks for monitoring and image integration in the following studies.
Figure 3.1 (A) Raman spectra on spinach leaves. (a) ferbam with AuNPs, (b) thiabendazole with AuNPs, (c) AuNPs, (d) ferbam, (e) thiabendazole and (f) spinach leaves. Concentration-dependent SERS spectra of (B) ferbam and (C) thiabendazole on spinach leaves.
3.4.3 In situ monitoring of the penetration of the non-systemic pesticide ferbam into spinach leaves

Figure 3. 2A shows the schematic illustration of two sample preparation methods used for studying pesticide penetration. In the first method, we pipetted ferbam on the spinach leaf surface followed by the addition of AuNPs colloids. In Method 2, a washing step was employed to remove the surface pesticide residues before applying the AuNP colloids. In detail, an aliquot of 20 ppm ferbam solution was firstly pipetted onto the spinach leaf surface. Subsequently, the leaf was submerged into the washing solution over a period of time and then gently rinsed with deionized water. Before Raman measurements, AuNPs were pipetted onto the same area of the spinach leaf where the ferbam solution had been placed. A bright light scattering image of spinach leaf with AuNPs is shown in Figure 3. 2B-a. For the leaf surface without washing, the SERS mapping image is shown in Figure 3. 2B-b and the corresponding SERS spectra of positions (1-4) from the SERS mapping image are presented in Figure 3. 2B-c which clearly show the fingerprint information of ferbam molecules. These results illustrate a clear penetration of ferbam molecules into spinach leaf at least to 100 µm depth. This finding is unexpected at the first glance as ferbam is a non-systemic pesticide, which means generally it should have little or no penetration abilities. However, it is possible that strong interactions between the ferbam and AuNPs resulted in the co-penetration of the AuNPs-ferbam complex. This finding has three implications. Firstly, the co-penetration of the ferbam and AuNPs may bring up new toxicity issues as the penetrated AuNPs-ferbam complex may have different behaviors and toxicity. Secondly, on the positive side, if it is necessary to alter the non-systemic property of a pesticide, then AuNPs can be used as the delivering matrix for penetration. Thirdly,
for this study, it became apparent that to obtain a reliable measurement of the pesticide penetration, the interference of the pesticide residue on the leaf surface would need to be eliminated. For this purpose, different washing methods were evaluated to ensure complete removal of the surface pesticide residues.

Figure 3.2 (A) Schematic presentation of two methods of monitoring of ferbam penetration by SERS mapping technique: Method 1, direct detection of ferbam in spinach leaves without washing surface residues; Method 2, detection of ferbam in spinach leaves after washing surface residues. (B) (a) Bright light scattering image of spinach leaf with AuNPs,
(b) SERS mapping image of spinach leaf with ferbam and AuNPs (using the intensity of SERS peak of ferbam at 1371 cm\(^{-1}\)). The step size of the mapping is 20 \(\mu\)m and one image contains 75 scanning spots. (c) SERS spectra of selected positions on the mapping image.

**Figure 3.3** (A) SERS spectra of ferbam surface residues after different washing methods. (B) Raman intensity of the band of ferbam at 1371 cm\(^{-1}\) versus the samples corresponding to (a)–(g).
SERS spectra following different washing methods are shown in Figure 3. 3A (b-g). For comparison, a SERS spectrum from a leaf contaminated by ferbam under non-washing condition is also shown in Figure 3. 3A-a. The corresponding Raman intensity of characteristic peaks of ferbam at 1371 cm\(^{-1}\) are presented in Figure 3. 3B. It is clearly seen that after washing with NaHCO\(_3\) solution for 120 s, there is no signal of ferbam detected with our method. This result indicates the surface residues of ferbam are extremely low, less than 1 ppb (detection limit of the developed method) and could be considered negligible.

After washing the leaf surfaces using NaHCO\(_3\) solution for 120 s, no characteristic ferbam signals were detected from 2 hours to 2 days following its application onto spinach leaf surfaces (Figure 3. 4A-a). This result indicates that ferbam is not able to penetrate into the spinach leaves even after 2 days’ exposure. In contrast, the ferbam signals are clearly observed inside the spinach leaves without washing (Figure 3. 4A-b). The penetration depth of ferbam did not correlate to the ferbam exposure time (from 2 hours to 2 days) because the penetration of ferbam is dependent on the AuNPs penetration and this process was the same for all samples. Figure 3. 4B are SERS spectra of selected positions (1-6) on mapping images in Figure 3. 4A, which validates the penetrated signals are from the characteristic SERS peaks of ferbam.
Figure 3.4 Time-dependent penetration of the non-systemic pesticide ferbam and its distribution into spinach leaves using the SERS mapping technique. (A) SERS depth mapping images of ferbam penetration following different exposure time periods (a) with surface washing and (b) without surface washing. Step size is 20 µm and one image contains 75 scanning points. (B) SERS spectra of selected positions on mapping images.
Figure 3.5 (A) SERS spectra of surface residues of thiabendazole using different washing methods. (B) SERS intensity of the band of thiabendazole at 1010 cm$^{-1}$ versus the samples corresponding to (a)–(g).
3.4.4 *In situ* monitoring of the penetration of systemic pesticide into spinach leaves

In order to study the penetration of systemic pesticides into spinach leaves, a washing method for the total removal of surface residues of thiabendazole must be found. As for ferbam, both water and NaHCO₃ solution were examined. As shown in Figure 3.5A, the SERS signals of thiabendazole at 1275, 1010 and 780 cm⁻¹ disappeared and Raman intensity of characteristic peaks of thiabendazole at 1010 cm⁻¹ is very weak after washing with the NaHCO₃ solution for 150 s (see Figure 3.5B). Similarly, this result indicates that the surface residues of thiabendazole is less than 2 ppb and could be considered negligible. Thus, this optimized washing method was used to study thiabendazole penetration into leaves.

The process for monitoring thiabendazole penetration into spinach leaves through removal of surface pesticide residues is given in Figure 3.6A. Figure 3.6B-a shows that thiabendazole can penetrate into spinach leaves even though the surface pesticides had been removed, which reflects its systemic property. The earliest time that we detected thiabendazole is following 6 hours’ exposure. With increasing exposure time, the penetration depths of thiabendazole gradually increased to approximately 150 µm in the depth mapping images. To more accurately estimate the penetration depth following a 2 days’ exposure, the selected areas (100 µm × 100 µm) parallel to leaves surface were scanned at different depths. Figure 3.10-a shows the Raman images at eight different depths (30, 60, 90, 120, 150, 180, 210 and 240 µm) based on the characteristic peak of thiabendazole at 1010 cm⁻¹. We also choose the same position on different depth of mapping images (1-8) and their corresponding SERS spectra are shown in Figure 3.10-b. With increasing depth, the SERS signals of thiabendazole decreased from position of 1 to
8. SERS signals of thiabendazole are clearly observed from 30-210 µm in depth, while no signal was detected at the 240 µm depth, indicating that thiabendazole penetrated into spinach leaves to approximately 210 µm. For comparison, we investigated thiabendazole penetration into spinach leaves without washing surface thiabendazole residues and corresponding mapping results at different exposure times are shown in Figure 3.6B-b. Compared with the Figure 3.6B-a, the penetration depths of pesticides in non-washing conditions are deeper than those after washing. This result may be explained by additional pesticide molecules co-penetrating with AuNPs under the non-washing condition, which allows the pesticide molecules to penetrate further or improves the sensitivity of detection due to larger amount of molecules. To study the concentration effect, various concentrations (0.2 ppm, 2 ppm and 100 ppm) of thiabendazole were tested under the same condition as for the 20 ppm concentration. With 100 ppm thiabendazole, a significantly longer time (i.e. 10 min) was needed to completely remove the surface pesticide residues (Figure 3.11). For lower concentrations of thiabendazole, a shorter time (i.e. 20 s for 2 ppm and 3 s for 0.2 ppm) were needed to completely remove the surface pesticide residues. Figure 3.12 shows that higher concentration of thiabendazole could penetration faster and deeper than the lower concentration. The results obtained here demonstrate the developed SERS method has excellent feasibility for monitoring a wide range of concentrations of pesticide penetration into spinach leaves, which could meet the need to determine realistic low levels of pesticides.
Figure 3.6 (A) Schematic illustration of the monitoring of thiabendazole penetration using SERS mapping technique with washing thiabendazole surface residues. (B) SERS depth mapping images of thiabendazole penetration following different exposure time periods (a)
with washing and (b) without washing. Step size is 20 µm and one image contains 75 scanning points.

3.4.5 Verification of thiabendazole penetration into spinach leaves

To further validate thiabendazole penetration, we developed another method in which we pipetted the pesticides onto one side and the AuNPs onto the other side of the leaf. After 3 days’ exposure, we obtained the SERS depth mapping image based on characteristic peak of thiabendazole at 1010 cm\(^{-1}\) (Figure 3.7). No SERS signals of thiabendazole were observed in the area close to either upper surface or lower surface (Position 1 and 3). Proximity to the center of the leaf, however, we found intense SERS signals of thiabendazole at position 2, indicating the penetrated thiabendazole met the penetrated AuNPs at this position. This data clearly demonstrated that thiabendazole is able to penetrate into the spinach leaves in systemic way.
**Figure 3.7** Verification of systemic pesticide penetration into spinach leaves. The step size parallel to and vertical to leaf surface for depth mapping is 20 µm and 50 µm, respectively. The image contains 75 spots.

### 3.5 Conclusion

We present a novel and simple SERS mapping technique for measuring pesticide penetration into spinach leaves. Using AuNPs, we showed the real-time and *in situ* monitoring of pesticide penetration into plant tissues nondestructively. The tested pesticides, ferbam and thiabendazole, exhibited different penetration behaviors, which were reflective of their non-systemic and systemic properties, respectively. Co-penetration of pesticides and AuNPs were observed when the surface pesticides were not removed before applying the AuNPs. The developed method appears to have the capability of monitoring the fate of pesticides applied onto plant surfaces in a non-destructive and rapid manner. Future studies will focus on the use of this method to study the penetration of a variety of systemic and non-systemic pesticides into different plant tissues.
3.6 Supplementary Figures

![Image of depth profiles of AuNPs penetration into spinach leaves.](image)

**Figure 3.8** Depth profiles of 150, 90, 70, 50, 15 nm AuNPs penetration into spinach leaves.
Figure 3.9 Raman spectra on gold slide. (a) ferbam with AuNPs, (b) thiabendazole with AuNPs, (c) AuNPs and (d) gold slide.
Figure 3.10 (A) SERS mapping images in different depth profile. Step size is 20 μm and one image contains 25 scanning points. (B) Selected SERS spectra of thiabendazole with AuNPs in spinach leaves.
Figure 3.11 (A) SERS spectra of surface residues of thiabendazole after different washing methods. (B) SERS intensity of the band of thiabendazole at 1010 cm$^{-1}$ versus the samples corresponding to (a)–(g).
Figure 3.12 SERS mapping images of penetration of 0.2 ppm, 2 ppm, 20 ppm and 100 ppm thiabendazole into spinach leaves during incubation time for (A) 2h and (B) 10h. Step size is 20 µm and one image contains 75 scanning points.
CHAPTER 4
EVALUATION OF PENETRATION OF MULTIPLE CLASSES OF PESTICIDES IN FRESH PRODUCE USING SURFACE-ENHANCED RAMAN SCATTERING MAPPING

4.1 Abstract
Understanding pesticide penetration is important for effectively applying pesticides and in reducing pesticide exposures from food. This study aims to evaluate multi-class systemic and non-systemic pesticide penetration in three representative fresh produce (apples, grapes and spinach leaves). Surface-enhanced Raman scattering (SERS) mapping was applied for in situ and real time tracking of pesticide penetration over time. The results show that 100 mg/L of systemic pesticides, thiabendazole and acetamiprid, penetrated more rapidly and deeply with maximum depth around 220 µm after 48 h exposure into the tested fresh produce than 100 mg/L of non-systemic pesticides, ferbam and phosmet with maximum depth about 80 µm. The fact that two non-systemic pesticides were also able to penetrate over time into all three fresh produce tested may raise additional food safety concerns. Comparatively, grapes were generally more resistant for pesticide penetration with all pesticides penetration depth below 80 µm compared to apples and spinach leaves. The information obtained here could provide technical support and guidance for accurate, effective and safe application of pesticides and for the reduction of pesticide exposure from fresh produce.

4.2 Introduction
Pesticides play a crucial role in the development of agriculture and are necessary to achieve global food production goals at the present time. Although wide-spread use of
pesticides has played a significant part in increasing agricultural production, concerns of their potential hazards to food safety and human health have increased.\textsuperscript{116–120} United States Environmental Protection Agency (EPA) set tolerance levels for pesticides in food, and the Food and Drug Administration (FDA) tests food produced in the United States and food imported from other countries for compliance with these residue limits. Fresh fruits and vegetables are at the top of the list of food containing pesticide residues.\textsuperscript{121–123} Hence, understanding the behaviors of pesticide residues in fresh produce is critically important in order to minimize pesticide exposures from food.

Based on their physical chemistry, pesticides can be classified as either systemic or non-systemic.\textsuperscript{124,36,125} Systemic pesticides can penetrate into plant tissues and translocate through the plant, while non-systemic pesticides have little or no ability to penetrate into plant tissues (leaves or roots) and cannot generally translocate.\textsuperscript{47,126,127} However, to what degree can systemic and non-systemic pesticides penetrate into plant tissues over time are not well studied. The degree of pesticide penetration into fresh produce may determine the persistence of the pesticide overtime and its residue amounts. The internalized pesticides are less likely to be removed by means of washing during postharvest processes and may present greater risks to public health.\textsuperscript{40,41,128–131} An interesting study reported on the effectiveness of tap water rinsing on reduction of 12 pesticide residues on produce.\textsuperscript{41} It was found that residues of vinclozolin, bifenthrin, and chlorpyrifos were not reduced after rinsing, suggesting that pesticide probably penetrated into fresh produce. In another study, the removal of pesticide residues by washing was found to depend on how long the chemical remained on the plant.\textsuperscript{40} The authors found that it was more efficient to wash the pesticide residue off of olives one day after spraying rather than after one week, probably
due to the increased retention in the cuticular waxes of the plant over time. There is very limited information available, however, regarding the degree (time and depth) of pesticides penetration in different plant tissues, particularly for non-systemic pesticides.

Surface-enhanced Raman scattering (SERS) is a powerful analytical tool with a wide range of applications, particularly for pesticide detection due to its capability of rapid and sensitive recognition of characteristic pesticide signals.\textsuperscript{30,132–136} Previously, we developed a SERS mapping technique to study the penetration behaviors of ferbam, a non-systemic pesticide, and thiabendazole, a systemic pesticide, into spinach leaves over time.\textsuperscript{137} Using gold nanoparticles (AuNPs) as a probe, we were able to detect internal thiabendazole response after removing surface pesticide residues by NaHCO\textsubscript{3} washing solution. Comparatively, ferbam did not show any internal signals after removing surface pesticide residues, demonstrating its non-systemic behavior. In both cases, if the surface pesticides were not removed, co-penetration of both AuNPs and pesticides was observed.

In the present work, we applied this method to study a broader range of pesticides and fresh produce matrices. For pesticides, we selected two insecticides (acetamiprid and phosmet) and two fungicides (thiabendazole and ferbam). Insecticides and fungicides were selected over herbicides due to their higher use pattern on fresh produce in conventional produce farming. In addition, ferbam and phosmet are non-systemic and acetamiprid and thiabendazole are systemic pesticides. The molecular structures and maximum residue levels (MRLs) of these four pesticides are given in Table 1. For fresh produce, three representative food matrices, apples, grapes and spinach leaves, were chosen to study because they are reported as among the most commonly consumed produce in the US and appear on the list of the fruits and vegetables with the most pesticide residues.\textsuperscript{110}
Furthermore, each represents a different type of fresh produce. Apples are examples of fruits that have relatively hard external surfaces with thin peels, which are commonly consumed whole and as peeled or sliced. Grapes are examples of fruits that have relatively soft external surfaces with ultra-thin peels and are usually consumed whole. Spinach leaves are vegetables that have no peel and are consumed whole. Finally, the pesticides residues on the surfaces of these types of fresh produce are more likely to be ingested compared to produce with inedible thick peels (e.g., bananas and oranges). The information obtained from this study should allow us to have a better understanding of the penetration behaviors of pesticides on and in different fresh produce matrices and help us to develop better strategies to minimize pesticide exposure, thereby improving public health.

4.3 Materials and Method

4.3.1 Materials

Thiabendazole (fungicide: 2-(4-thiazolyl)-1H-benzimidazole, ≥99%), acetamiprid (insecticide: (E)-N-[(6-chloro-3-pyridyl)methyl]-N′-cyano-N-methylethanimidamide, analytical grade), ferbam (fungicide: Iron(III) dimethyldithiocarbamate, analytical grade) and phosmet (insecticide: O,O-dimethyl S-Phthalimido Methyl Phosphorodithioate, analytical grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium bicarbonate (NaHCO₃) and glacial acetic acid was purchased from Fisher Scientific (Pittsburgh, PA, USA). Citrate-capped AuNPs colloids (50nm) were purchased from NANO PARTZ™ Inc. (Loveland, CO, USA). Organic baby spinach leaves were obtained from Whole Foods Market (Hadley, MA, USA). Organic apples and grapes were purchased from Stop & Shop Supermarket (Hadley, MA, USA). All reagents were used without further purification. Ultrapure water (18.2 MΩ.cm) was produced using a Thermo
Scientific Barnstead Smart2Pure Water Purification System (Waltham, MA, USA) and used for the preparation of all solutions.

4.3.2 Determination of the characteristic SERS signals from various pesticides applied onto multiple fresh produce

Each test pesticide stock solution of 1000 mg/L (ppm) was prepared with ultrapure water and methanol (v/v, 1:1) and further diluted to desired concentrations with ultrapure water prior to use. A 50 µL aliquot of each pesticide solution was mixed with 50 µL of a 250 mg/L solution of 50 nm AuNPs for 1 h at room temperature to ensure effective pesticide complexation with AuNPs through Au-thiol or Au-amino bond. A 5 µL aliquot of each of the pre-prepared pesticide/AuNPs solutions was pipetted onto fresh produce situated on a glass slide and air-dried in a fume hood for 10 min. Solutions of AuNPs without pesticide and each pesticide alone were also pipetted onto fresh produce as control treatments for comparison. A 5 µL aliquot of either the pre-prepared pesticide/AuNPs solution or AuNPs solution without pesticide were also pipetted onto a gold slide and dried for 10 min in a hood for comparison. Raman spectra were collected individually for all treatments.

In order to analyze the penetration depth of 50 nm AuNPs after placing them onto the external surfaces of different fresh produce, a 1 mL aliquot of a 20 mg/L ferbam solution was mixed with a 1 mL aliquot of a 250 mg/L AuNPs solution for 30 min to form ferbam/AuNPs complex. A 5 µL aliquot of the mixture was pipetted onto apple, grape and spinach, respectively. After air drying for 1h, SERS depth mapping images were obtained using a confocal Raman instrument.
4.3.3 Preparation of fresh produce for the *in situ* determination of pesticide penetration

Organic apples were carefully hand washed with ultrapure water for 3 min and air-dried before experiment to remove surface contaminants. Then a 5 µL aliquot from a 100 mg/L solution of each pesticide was pipetted onto the apple surface. After air-drying the pesticides, apple peels with ~2.0 × 2.0 cm² area were cut from each treated apple using a sharp knife. To determine pesticide penetration with penetrative AuNPs, any interference caused by pesticide residues on the apple surface were removed by washing. Two different washing methods were investigated using either an acetic acid- or NaHCO₃-containing solution. Each peel was immersed into 20 mL of either a 5 % acetic acid or a 1 % NaHCO₃ solution for different times and gently rinsed with 200 mL of deionized water for 10 s, respectively. All washed samples were air-dried at room temperature for 10 min before being treated with AuNPs. After drying AuNPs for 1 h, SERS detection was performed on that area to evaluate washing effects. Ten discrete locations were randomly chosen on each apple peel sample and scanned. Similar samples were prepared without either washing method and were scanned as above for comparisons.

The penetration behavior of each pesticide on apples was monitored over different time periods from 30 min to 2 days. A 5 µL aliquot from each of the 100 mg/L pesticide solutions was pipetted onto apples and allowed to dry in air at room temperature over different time intervals (30 min, 6 h, 24 h and 48 h). Following drying, the surface residues from each applied pesticide were washed off using either the NaHCO₃ or acetic acid solution and a 5 µL aliquot of the 250 mg/L AuNPs solution was added as described above. After 1 h of drying, SERS depth mapping images were obtained using the confocal Raman instrument.
Organic grapes and spinach leaves were carefully hand washed as described above for apples before experiment to remove surface contaminants. In order to study pesticide penetration in grapes, the same two washing methods were used as presented above for apples. Grapes treated with each pesticides solution (100 mg/L) were washed with either a 5 % acetic acid or 1 % NaHCO₃ solution. Washing effects were evaluated using the SERS methods and the penetration behaviors of each pesticide were monitored as for apple above. Pesticides penetration experiments on spinach leaves were carried out as described above for apple and grape.

4.3.4 Raman instrumentation and data analysis

A DXR Raman microscope (Thermo Fisher Scientific, Madison, WI, USA) with a 780 nm laser and a 20× long distance microscope objective was used in this study. Each sample was scanned from 400 to 2000 cm⁻¹ for a 2 s exposure time. For measuring pesticides on both gold slides and fresh produce surfaces, Raman mapping was carried out using a 50 µm slit aperture and 5 mW laser power to maximize the signals. Ten discrete locations were randomly chosen on each sample for analysis. For penetration studies, SERS depth mapping images were obtained using a 50 µm pinhole aperture and 1 mW laser power to control the confocal depths using a scanning depth of 300 µm. Each depth scanning vertical to produce surface was randomly selected in apple peel, grape peel or spinach leaf with an area of 100 µm × 300 µm. The step size of the depth mapping was 20 µm and one image contained 75 scanning spots. Raman images were integrated based on the characteristic peaks in the pesticide spectra using the Atlμs Function in the OMINCS software (Thermo Fisher Scientific). Statistical analysis was conducted using SPSS Statistics 22 software (IBM Corporation, Armonk, NY, USA). The differences among results were calculated.
using an analysis of variance (ANOVA) and a post hoc Duncan test with a confidence level of 95%.

4.4 Results and Discussion

4.4.1 Determination of the characteristic SERS signals from various pesticides applied onto multiple fresh produce

**Figure 4.1** Surface-enhanced Raman spectra (SERS) of each of the test pesticides in the presence of gold nanoparticles (AuNPs) on (A) gold slide, (B) apple, (C) grape and (D) spinach leaf. Raman spectra of AuNPs and all three-fresh produce themselves are shown here as controls.
In order to study the penetration behaviors of pesticides, it is critically important to determine the characteristic peaks of each pesticide used in SERS mapping, and also to determine the detection limits and penetration depth limits of the method. Our previous study determined that AuNPs could rapidly penetrate into plant tissues and the penetration of AuNPs of different diameters into plant tissues were investigated using SERS mapping technique. From these results, 50 nm AuNPs were found to be the best probe for the determination of pesticide penetration because they are effective at produce penetration and SERS signal enhancement. The SERS spectra for each of the test pesticides in the presence of AuNPs were obtained on gold-coated microscopic slides (Figure 4. 1A), apple surface (Figure 4. 1B), grape surface (Figure 4. 1C) and spinach leaf surface (Figure 4. 1D).

The characteristic SERS peaks of thiabendazole were clearly detected at 1575, 1275, 1010 and 780 cm\(^{-1}\) (Figure 4. 1A-a), acetamiprid at 1110 and 633 cm\(^{-1}\) (Figure 4. 1A-b), ferbam at 1373, 1138, 936 and 550 cm\(^{-1}\) (Figure 4. 1A-c), and phosmet at 1192, 1015, 610 and 508 cm\(^{-1}\) (Figure 4. 1A-d). AuNPs alone resulted in only very small signals which could be considered negligible (Figure 4. 1A-e). Each pesticide was also investigated with or without AuNPs on fresh produce surface. As shown in Figure 4. 1B, C and D, when mixed with AuNPs each pesticide produced its characteristic SERS peaks on apple, grape and spinach leaf surface, and little or no Raman signals were obtained from all three fresh produce themselves. By contrast, there were no measurable Raman signals observed from each pesticide on fresh produce in the absence of AuNPs (Figure 4.8, see Supplementary Figures). In addition, no Raman signals were detected from AuNPs alone on each fresh produce. The SERS peaks at 1010 cm\(^{-1}\) for thiabendazole, 633 cm\(^{-1}\) for acetamiprid, 1373 cm\(^{-1}\) for ferbam and 610 cm\(^{-1}\) for phosmet were chosen as the characteristic peaks on each
fresh produce, except 550 cm\(^{-1}\) for ferbam on grape, for monitoring and image integration in the following studies due to their prominent intensities. The use of SERS peak at 550 cm\(^{-1}\) for ferbam on grape is due to the fact that the inherent Raman peak of grape at 1371 cm\(^{-1}\) overlapped with the SERS peak of ferbam at 1373 cm\(^{-1}\) (Figure 4.1C). The concentration-dependent SERS spectra of each test pesticide on the surfaces of three types of fresh produce are presented in Figure 4.9. Even as low as µg/L (ppb) levels, the SERS signals of all test pesticides can still be clearly seen, indicating a high sensitivity of the SERS method for the detection of pesticides on tested fresh produce.

In order to accurately and reliably determine the penetration behaviors of pesticides, the penetration depth of AuNPs into different fresh produce was initially investigated. Our previous study showed that the non-systemic pesticide ferbam at a concentration of 20 mg/L could not inherently penetrate into fresh produce following a 1 h exposure.\(^{137}\) Therefore, ferbam could serve as an ideal indicator to monitor the penetration depth of AuNPs in selected fresh produce by using ferbam/AuNPs complex. More details were given in Figure 4.10 in Supplementary Figures. Using this approach, it was found that AuNPs could penetrate into apple, grape and spinach to depths of approximately 220 µm, 180 µm and 275 µm, respectively after a 1 h incubation. These depths are enough further for the study of pesticides penetration as discussed later.

4.4.2 Real-time tracking of pesticide penetration into apple

In this study, the concentration of pesticides we applied was 100 mg/L, which was lower than the recommended label rate of pesticides (typically 600 mg/L) but have the same order of magnitude, to mimic the early stage of pesticide practical applications. From a viewpoint of useful guidance for effective and safe applications of pesticides on fresh produce, it is
important and meaningful to monitor penetration of pesticide with a higher concentration. Based on our previous study,\textsuperscript{137} prior to the application of penetrative AuNPs, surface pesticide residues must be removed for the accurate determination of pesticide penetration behaviors; otherwise, co-penetration of the ferbam and AuNPs can confound results (Figure 4.11). A schematic illustration was shown in Figure 4.12 for reliable measurements.

Different washing methods were investigated to ensure complete removal of the surface pesticide residues on apple. As shown in Figure 4. 2A, SERS signals of thiabendazole disappeared and the intensity of characteristic peak of thiabendazole at 1010 cm\textsuperscript{-1} was very weak after washing with the NaHCO\textsubscript{3} solution for 12 min, which indicated that the surface residues of thiabendazole was less than 2 µg/L and could be considered negligible. As for the acetamiprid and phosmet, their SERS disappeared and the Raman intensity of characteristic SERS peaks of acetamiprid at 633 cm\textsuperscript{-1} and phosmet at 610 cm\textsuperscript{-1} were very weak after washing with the 1% NaHCO\textsubscript{3} solution for 8 min and 15 min, respectively, again indicating that surface pesticide residues were almost completely removed (Figure 4. 2B and D). In the case of ferbam removal, the 1% NaHCO\textsubscript{3} solution was not effective even after washing for 20 min (Figure 4.13-c). However, after washing for 10 min using the 5% acetic acid solution, the SERS signals of ferbam were absent, indicating almost total elimination of surface residues (Figure 4. 2 and Figure 4.13-d).
Using these washing methods, the penetration of each of the four test pesticides following different exposure times (30 min-48 h) were evaluated on apples using SERS mapping techniques. As shown in Figure 4.3A, all pesticide penetrated into apples after a 48 h exposure. The corresponding SERS spectra of positions 1 to 4 from the SERS mapping images given in Figure 4.3B clearly show the diagnostic fingerprint for each pesticide. The penetration of the two systemic pesticides, thiabendazole and acetamiprid, were observed after a 30 min exposure and the penetration depth gradually increased to approximately 160 µm for thiabendazole and 140 µm for acetamiprid after a 48 h exposure. It is worth mentioning that the terminal depth depends on the color change from light blue to dark blue on each Raman mapping image (See Figure 4.14). In comparison, the non-
systemic pesticides, ferbam and phosmet, were first detected following a 6 h exposure. After 48 h, the penetration depths slowly increased to approximately 80 µm for ferbam and 55 µm for phosmet. These findings are very interesting and unexpected because non-systemic pesticides should have little or no penetration ability. The detection of non-systemic pesticides is significant because internalized pesticides are less likely to be eliminated during postharvest processes and may present greater risks to public health. Until now, there has been no literature reporting that non-systemic pesticide can penetrate deeply into fresh produce. Figure 4.3C summarized the penetration depth of the four test pesticides at different exposure times. The two systemic pesticides penetrated faster than the two non-systemic pesticides and they penetrated deeper than the two non-systemic pesticides after 48 h exposure. Between the two systemic pesticides, thiabendazole penetrated statistically deeper than acetamiprid after 48 h. There is no difference between the two non-systemic pesticides in terms of penetration depth in the initial 6 h. However, Ferbam penetrated statistically deeper than phosmet after 24 h and 48 h exposure. The error bar in Figure 4.3C shows the variability of duplicates by this method is quite slow, indicating the reliability and reproducibility of this method.
Figure 4.3 (A) SERS depth mapping images of the penetration of pesticides following different exposure time periods in apples. Step size is 20 µm and one image contains 75 scanning points. (B) SERS spectra of selected positions on the mapping images. (C) Penetration depths of each pesticide on apples versus different exposure time periods. Samples designated with different capital letters (A, B, C, D) were significantly different (Duncan, P < 0.05) when compared between different exposure time periods (same pesticide). Samples designated with different capital letters (a, b, c, d) were significantly different (Duncan, P < 0.05) when compared between different pesticides (same exposure time).
4.4.3 Real-time tracking of pesticide penetration into grape

Overall the penetration of pesticides on grape was similar to that on apple. Initially different washing methods were evaluated to insure total removal of each pesticide from grape surface (Figure 4.15). The best washing treatments for removing thiabendazole, acetamiprid or phometh were 1% NaHCO$_3$ washing solution for 15 min, 10 min and 18 min, respectively. For elimination of surface ferbam residues, the best treatment was the 5 % acetic acid washing method for 15 min. Subsequently, the penetration behavior for each pesticide was examined by the SERS mapping method. Figure 4. 4A shows the SERS images of each internalized pesticides in grape after different exposure time from 30 min to 48 h. The corresponding SERS spectra from positions 1 to 4 using the SERS mapping images (48 h exposure) clearly show the Raman fingerprint of each pesticide (Figure 4. 4B). Figure 4. 4C summarized the different penetration behaviors of these pesticides on grape. The penetration of thiabendazole or acetamiprid was apparent after 30 min exposure and the overall penetration ability for acetamiprid (about 75 µm in depth) was superior to thiabendazole (about 55 µm in depth) on grapes after 48 h. The penetration of the non-systemic pesticides, ferbam and phosmet, were observed only after 48 h and both of their penetration degrees were shallow (about 20 µm depth). Overall, the two systemic pesticides penetrated faster and deeper than the two non-systemic pesticides. Between the two systemic pesticides, acetamiprid penetrated statistically deeper than thiabendazole all the time. No significant difference was observed between the two non-systemic pesticides in terms of penetration depth.
Figure 4. (A) SERS depth mapping images of the penetration of pesticides following different exposure time periods in grapes. Step size is 20 µm and one image contains 75 scanning points. (B) SERS spectra of selected positions on the mapping images. (C) Penetration depths of each pesticide on grapes versus different exposure time periods. Samples designated with different capital letters (A, B, C, D) were significantly different (Duncan, P < 0.05) when compared between different exposure time periods (same pesticide). Samples designated with different capital letters (a, b, c) were significantly different (Duncan, P < 0.05) when compared between different pesticides (same exposure time).
4.4.4 Real-time tracking of pesticide penetration into spinach leaf

Similar to apple and grape, surface residues of thiabendazole, acetamiprid and phosmet were not detected following washing with the 1% NaHCO$_3$ solution for 10 min, 3 s and 10 min, respectively (Figure 4.16). Surface residues of ferbam were removed by washing with 5% acetic acid solution for 10 min. Following the removal of external pesticide residues using the best washing method for removal each pesticide, SERS depth mapping images were used to investigate pesticide penetration (Figure 4.5A). Figure 4.5B show the corresponding SERS spectra for positions 1 to 4 from the SERS mapping images after a 48 h exposure, which exhibit the signature SERS peaks from each pesticide. The systemic pesticide thiabendazole was detected after a 30 min exposure as seen from the SERS mapping image and penetrated to a depth of ~220 µm by 48 h, indicating that thiabendazole penetrated rapidly and extensively into spinach. In comparison, the other systemic pesticide acetamiprid only penetrated to a depth of 90 µm after a 48 h exposure. The non-systemic pesticide ferbam penetrated to 30 µm after 48 h. This result differs from a previous study$^{137}$ where a 20 mg/L ferbam concentration was used and no internalized signal was observed, which may indicate a concentration-dependent penetration behavior for ferbam. The other non-systemic pesticide phosmet was detected following a 24 h exposure and penetrated to a depth of 70 µm at 48 h. Figure 4.5C summarized the different penetration behaviors of these pesticides on spinach leaves. Again, the two systemic pesticides have significantly stronger ability for penetration then non-systemic pesticides. Thiabendazole penetrated the most and ferbam penetrated the least. It should be noted that in each fresh produce tested (apple, grape and spinach leaf), the penetration depth of each pesticide was lower than that
of AuNPs, indicating that AuNPs appear to be effective and reliable probes for the study of pesticide penetration.

**Figure 4.5** (A) SERS depth mapping images of the penetration of pesticides following different exposure time periods in spinach leaves. Step size is 20 µm and one image contains 75 scanning points. (B) SERS spectra of selected positions on the mapping images. (C) Penetration depths of each pesticide on spinach leaves versus different exposure time periods. Samples designated with different capital letters (A, B, C) were significantly different (Duncan, P < 0.05) when compared between different exposure time periods.
(same pesticide). Samples designated with different capital letters (a, b, c, d) were significantly different (Duncan, P < 0.05) when compared between different pesticides (same exposure time).

**Figure 4. 6** Comparisons of the penetration depths of thiacloprid, acetamiprid, ferbam and phosmet in apple, grape and spinach leaf following different exposure time periods, (A) 0.5 h, (B) 6 h, (C) 24 h and (D) 48 h. Samples designated with different capital letters (A, B, C) were significantly different (Duncan, P < 0.05) when compared between different pesticides (same fresh produce). Samples designated with different capital letters (a, b, c, d) were significantly different (Duncan, P < 0.05) when compared between different fresh produce (same pesticide).
4.4.5 Comparison of the penetration behavior of each pesticide on different fresh produce

Pesticide penetration into fresh produce is a complex process, depending on surface characters of fresh produce, the physicochemical properties of the pesticides and environmental conditions such as temperature or humidity. Figure 4. 6 summarized the penetration of the four test pesticides into apple, grape and spinach. At 30 min, only systemic pesticides were detected in these three fresh produce. Particularly, thiabendazole penetrated to a depth of 100 µm into spinach by such a short time. Two non-systemic pesticides were only detected in apples after 6 h. Phosmet was firstly detected in spinach after 24 h. After 24 h, the penetration depths of thiabendazole for apples and spinach (about 140 and 160 µm) were significantly larger than its depth for grape which was only to about 36 µm. After 48 h, both non-systemic pesticides were first detected in grapes, and ferbam was firstly detected in spinach. This data suggests the penetration behavior for each pesticide is varied based on the nature of pesticide and the matrices of the fresh produce. Generally speaking, systemic pesticides can penetrate into fresh produce faster and deeper (after 48 h exposure) than non-systemic pesticides. Grapes appear to be the most difficult matrix for pesticide penetration.

The different penetration ability on fresh produce is possibly because the differences of cuticle and epicuticular wax on fresh produce. The plant cuticle including of a polymeric cutin matrix and soluble waxes is a nonliving and non-uniform plant structure which acts as a barrier against foreign substance. The cuticle extracellular membrane is composed of cutin and waxes. The cuticular membranes of fruits are generally thicker than those of leaves. Figure 4. 7 shows bright field images of apple, grape and spinach leaf surface.
We can see grapes have a relatively thick wax while the wax on spinach is thin. Moreover, the large stomata on spinach leaves may be beneficial for pesticide penetration. This may probably result in the fact that pesticides penetration on grapes is less likely than apples and spinach leaves. In addition, pesticides penetration may be affected by the pH of the fruit and vegetable matrix and bacterial communities on them.

Figure 4.7 Bright field image of (a) apple surface, (b) grape surface and (c) spinach leaf surface.

4.5 Conclusion

We evaluated the penetration behaviors of four pesticides into three types of fresh produce. We found that systemic pesticides had a quicker and deeper (after 48 h exposure) penetration pattern than the non-systemic pesticide on the tested fresh produce. Although the penetration is slower, we found that the two non-systemic pesticides could still penetrate in all three fresh produce we tested. The furthest penetration depth we observed is 80 µm from ferbam into apple peels. This information may pose us to re-think the potential toxicity issues raised by the unexpected behavior of non-systemic pesticides. For different fresh produce, grape was generally more resistant for penetration when compared with apple or spinach. It is possibly due to the differences of cuticle and epicuticular wax on fresh produce. This information may provide some useful guidance as effective and safe
applications of pesticides based on specific fresh produce, which help us to develop a better strategy to apply pesticides and reduce pesticide exposure from fresh produce. Future work will focus on studying the quantity of penetrated pesticides using liquid/gas Chromatography-Mass spectrometry and also trying to figure out if there is correlation between the SERS data to determine whether SERS imaging is able to quantitative measure the penetrated pesticides. In addition, we will also evaluate the current washing procedure in factory and at home for removing surface pesticides.
**Figure 4.8** Raman spectra of each of the test pesticides alone or AuNPs alone on (A) apple, (B) grape and (C) spinach leaf.
Figure 4.9 Concentration-dependent SERS spectra of each pesticide on (A) apple, (B) grape and (C) spinach leaf.
Figure 4.10 (A) SERS mapping image of ferbam/AuNPs complex penetrated into different fresh produce using the intensity of SERS peak of ferbam at 1373 cm$^{-1}$ on apple and spinach leaf, as well as 550 cm$^{-1}$ on grape. The step size of the mapping is 20 µm and one image contains 75 scanning spots. (B) SERS spectra of selected positions on the mapping images.

Explanation for Figure 4.10: In detail, we first incubated 20 ppm ferbam and 250 ppm AuNPs for 30 min in order to ensure effective adsorption of ferbam onto AuNPs through Au-thiol bonds. Then a 5 µL aliquot of the mixture was pipetted onto different fresh produce and dried for 1h prior to SERS mapping. SERS mapping images were shown in Figure 4.10A and the corresponding SERS spectra of positions 1 to 9 from the SERS images were presented in Figure 4.10B. The position 1 and 2 clearly showed the fingerprint information of ferbam molecules while the positon 3 did not show any SERS peaks, indicating that AuNPs could penetrate into apple about 220 µm after 1h. For grape and spinach leaf, the penetration depth of AuNPs was approximately 180 µm and 275 µm, respectively. The corresponding SERS spectra of positions 4 to 9 from SERS mapping images were given in Figure 4.10B.
Figure 4.11 SERS mapping image of ferbam and AuNPs co-penetration into apple. AuNPs were added onto the surface without removing the surface pesticides. The intensity of SERS peak of ferbam at 1373 cm\(^{-1}\) was used for mapping. The step size of the mapping is 20 µm and one image contains 75 scanning spots.
Figure 4.12 Schematic illustration of monitoring of pesticide penetration using SERS mapping technique with washing surface pesticide residues.
Figure 4.13 SERS spectra of surface residues of ferbam on apple after using different washing methods.
**Figure 4.14** (A) SERS depth mapping images of the penetration of ferbam in spinach leaves. Step size is 20 µm and one image contains 75 scanning points. (B) SERS spectra of selected positions on the mapping images.

Explanation for Figure 4.14: The terminal depth depends on the color change from light blue to dark blue on each Raman mapping image. The intervals are 20 µm. Each signal is actually collected from an area of several square microns. If the signal to noise ratio on the Raman spectrum is more than 3, we think we can detect it.

For example, Figure 4.14-A1 is the ferbam penetration in spinach leaves. The noise of ferbam on the Raman spectrum is about intensity of 10 a.u. which was shown in Figure 4.14-B2 and the corresponding Raman mapping color of dark blue is shown in Figure S7-A2. We cannot see any ferbam fingerprint information in its SERS spectrum thus regard it as non-detectable. The signal of ferbam of 1 in Figure 4.14-A shows the light blue color and the corresponding SERS spectrum with signal intensity of 30 a.u. was shown in Figure 4.14-B1 which clearly show the ferbam SERS peaks. Therefore, the color change from light blue to dark blue really reflect the condition from detection to non-detection.
Figure 4.15 SERS spectra of surface residues of (A) thiabendazole, (B) acetamiprid, (C) ferbam and (D) phosmet on grape after using different washing methods.

Explanation for Figure 4.15: Washing solution was 1% NaHCO₃ for removing thiabendazole, acetamiprid and phosmet and 5% acetic acid for getting rid of ferbam. Figure 4.14 show the SERS spectra of each surface pesticide residues after washing treatment for different times. Finally, we found thibendazole can be almost all removed after NaHCO₃ washing for 15 min because the SERS signals of thiabendazole cannot be seen in this case. For removal of surface residues of ferbam, the washing time is 15 min using acetic acid. Surface acetamiprid and phosmet residues were nearly fully removed after using 1% NaHCO₃ for washing 10 min and 18 min, respectively.
Figure 4.16 SERS spectra of surface residues of (A) thiabendazole, (B) acetamiprid, (C) ferbam and (D) phosmet on spinach leaf after using different washing methods.

Explanation for Figure 4.16: Washing methods were first examined to almost remove each pesticides residues on spinach surface. 1% NaHCO$_3$ solution was the washing agent to get rid of thiabendazole, acetamiprid and phomet. For removal of surface ferbam, 5% acetic acid was used. As shown in Figure 4.15, SERS spectra of each surface pesticide residues were obtained after washing treatment for different times. The SERS signals of thiabendazole cannot be seen after NaHCO$_3$ washing for 10 min, suggesting almost all thiabendazole residues were removed. The treatment time is 10 min using acetic acid as the washing agent for total elimination of surface ferbam residues. When we used NaHCO$_3$ solution to totally wash away surface acetamiprid and phosmet residues, the optimal time were 3 s and 10 min, respectively. It is easy to remove acetamiprid on spinach leaf surface because acetamiprid has a good water solubility on spinach leaves.
CHAPTER 5

INVESTIGATION OF PESTICIDE PENETRATION AND PERSISTENCE ON HARVESTED AND LIVE BASIL LEAVES USING SURFACE-ENHANCED RAMAN SCATTERING MAPPING

5.1 Abstract

Understanding pesticide behavior in plants is important for effectively applying pesticides and in reducing pesticide exposures from the ingestion. This study aimed to investigate the penetration and persistence of pesticides applied on harvested and live basil leaves. Surface-enhanced Raman scattering (SERS) mapping was applied for in situ and real-time tracking of pesticides over time using gold nanoparticles as probes. The results showed that after surface exposure of 30 min to 48 h, pesticides (10 mg/L) penetrated more rapidly and deeply into the live leaves than the harvested leaves. Systemic pesticide thiabendazole and the non-systemic pesticide ferbam can penetrate into the live leaves with depth of 225 µm and 130 µm, respectively than the harvested leaves with depth of 180 µm and 18 µm, respectively after 48-h exposure. The effects of leaf integrity and age on thiabendazole penetration were also evaluated on live basil leaves after 24-h exposure. Thiabendazole (10 mg/L) when applied onto intact leaves penetrated deeper (170 µm) than when applied onto damaged leaves (80 µm) prepared with 20 scrapes on the top surface of leaves. Older leaves with a wet mass of 0.204 ± 0.019 g per leaf (45 days after leaf out) allowed more rapid and deeper penetration of pesticides (depth of 165 µm) than when younger leaves with a wet mass of 0.053 ± 0.007 g per leaf (15 days after leaf out) were used (depth of 95 µm). The degradation of thiabendazole on live leaves was detected after 1 week whereas the apparent degradation of ferbam was detected after 2 weeks. In addition, the removal of pesticides
from basil was more efficient when compared with other fresh produce possibly due to the specific gland structure of basil leaves. The information obtained here provides a better understanding of the behavior and biological fate of pesticides on plants.

5.2 Introduction

The use of pesticides is widespread due to their significant role in increasing agricultural production. However, concerns about their potential hazards to food safety and human health have been raised. To assure a safe food supply, the U.S. Environmental Protection Agency (EPA) regulates pesticide residues in agriculture crops and food products and established appropriate tolerances, which is the legal maximum residue concentration of a pesticide chemical allowed in a crop or food product. Pesticides can be classified as either non-systemic or systemic pesticides and they have different penetration characteristics in plant tissues. The degree of pesticide penetration into plant tissues may be one of the factors determining the persistence of the pesticide over time. Internalized pesticides are not easily removed and may increase the available amount of pesticide when the plant is ingested, which could increase health risk. The biological fate of pesticides on and in plant tissues is determined by their chemical properties and environmental conditions. Understanding pesticide persistence is important to assess their potential exposure and associated risks to human health and the environment.

Pesticide behaviors including penetration and persistence were conventionally conducted by chromatographic techniques such as gas chromatography (GC) or liquid chromatography (LC)-based methods. However, these methods were not efficient, requiring time-consuming extraction processes, qualified personnel and expensive
equipment and more importantly, these methods cannot achieve in situ and real-time detection. Therefore, a new effective method was urgently needed. In our previous work, we developed a novel and simple surface-enhanced Raman scattering (SERS) mapping technique to in situ and real-time monitor both non-systemic and systemic pesticides penetration in different harvested fresh produce such as apple, grape and spinach leaves overtime using gold nanoparticles (AuNPs) as probes with a limit of detection of pesticides at ppb (µg/L) levels.\textsuperscript{137,155} AuNPs penetrate fresh produce and the internalized AuNPs are then available to interact with internalized pesticides, enhancing their Raman signals. One limitation of these previous studies, however, was that the penetration was performed and evaluated on harvested tissues only.

The objective of this current study is to compare pesticides penetration and persistence on both harvested and live plant leaves. In addition, the effects of the leaf integrity and age on pesticide penetration were evaluated for the first time. Basil (\textit{Ocimum basilicum}) was chosen for study as an example of an edible plant, its association with various pesticide exposures, widespread consumption by humans and relative large surface area facilitating carrying out these experiments. Two pesticides, the systemic fungicide thiabendazole, and the non-systemic fungicide ferbam were selected due to their widespread use and their good SERS activities.\textsuperscript{156–161} According to U.S. EPA, annual domestic thiabendazole and ferbam usages are both approximately 150,000 pounds. The molecular structures of these two pesticides are given in Table 5.1 (See Supplementary Table). Each pesticide was applied to the surface of basil leaves over time and monitored using the SERS method. The information obtained from this study should allow us to better understand of the behavior and biological fate of pesticides on plants, which could assist us in the development of
better strategies to effectively and safely apply pesticides on plants and provide a basis for setting residue tolerance levels and other regulatory considerations.

5.3 Materials and Methods

5.3.1 Materials

Thiabendazole (systemic fungicide: 2-(4-thiazolyl)-1H-benzimidazole, ≥99%, analytical grade) and ferbam (non-systemic fungicide: Iron(III) dimethyl dithiocarbamate, analytical grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Citrate-capped AuNPs colloids (50 nm) were purchased from NANO PARTZ™ Inc. (Loveland, CO, USA). Au-coated glass slides were purchased from Fisher Scientific (Pittsburgh, PA). USDA organic basil plants were purchased from Stop & Shop Supermarket (Hadley, MA, USA). All reagents were used without further purification. Ultrapure water (18.2 MΩ.cm) was produced using a Thermo Scientific Barnstead Smart2Pure Water Purification System (Waltham, MA, USA) and used for the preparation of all solutions.

5.3.2 Determination of Characteristic SERS Signals from Pesticides Placed onto Basil Leaf Surfaces

To study the penetration behaviors of pesticides, it is important to determine the characteristic peaks of each pesticide used in SERS mapping and to determine the detection limits and penetration depth limits of the method. In this study, we used 50 nm AuNPs as probes for the investigation of pesticide behavior due to their ability to penetrate plant leaves effectively and to enhance SERS signal based on our previous study.\textsuperscript{137}

Stock solutions of each test pesticide (1000 mg/L, ppm) were prepared using ultrapure water and methanol (v/v, 1:1) and further diluted to desired concentrations with ultrapure water prior to use. A 50 μL aliquot of each pesticide solution was mixed with 50 μL of a
250 mg/L solution of 50 nm AuNPs in a tube by gentle pipetting for 30 s and then placed at room temperature undisturbed for 1 h to ensure effective pesticide complexation with AuNPs through Au-thiol or Au-amino bond. A 5 µL aliquot from one of the pre-prepared pesticide/AuNPs solutions was pipetted onto the top surface of a single basil leaf situated on a glass slide and air-dried in a fume hood for 10 min. Solutions of AuNPs without pesticide and each pesticide alone were also pipetted onto individual basil leaves as control treatments for comparison purposes. Ten discrete locations were randomly chosen on each individual leaf for Raman analysis and each leaf sample had two duplicates. Raman spectra were collected individually for all samples.

To analyze the penetration depth of 50 nm AuNPs after 30 min exposure, a Raman indicator for tracking of AuNPs was first selected. We used 10 mg/L of ferbam as the Raman indicator because it met two requirements. First, ferbam was very SERS active which produced strong SERS signals to facilitate tracking AuNPs penetration. Second, our preliminary results showed that 10 mg/L of ferbam does not penetrate into basil leaves following a 30-min exposure thus it cannot interfere with AuNPs penetration (Results shown in Figure 3B-a). In detail, after placing each pesticide onto the external surfaces of harvested or live basil leaves, a 1 mL aliquot of a 10 mg/L ferbam solution was mixed with a 1 mL aliquot of a 250 mg/L AuNPs solution in a tube for 30 min in the room temperature to form ferbam/AuNPs complex. A 5 µL aliquot of the mixture was pipetted onto harvested leaves or onto the live leaves on the plant. After air drying for 30 min, SERS depth mapping images were obtained using a confocal Raman instrument as detailed below.
5.3.3 Preparation for the Study of Pesticide Penetration and Persistence in Basil Leaves

For the study of pesticide penetration into live basil leaves, 36 intact leaves (approximately 0.2 g per leaf) were selected from six live plants as the experimental target (6 leaves per plant) and carefully rinsed with 450 mL ultrapure water for 30 s and air-dried before the experiment to remove surface contaminants like dust. After rinsing, the clean leaves were used to study of pesticide penetration. The penetration behavior of each pesticide into live leaves was monitored over different time periods at 30 min, 24 h, and 48 h. A schematic illustration is shown in Scheme 5.1 for measurements of pesticide penetration into live leaves. A 5 µL aliquot of a 10 mg/L solution of each pesticide was pipetted onto the top surface of individual leaf and allowed to dry in air at room temperature over different time intervals (30 min, 24 h and 48 h) (Step 1). Some of the pesticides might penetrate into leaves from the surface during the periods. Based on previous study,\textsuperscript{137,155} to accurately determine pesticide penetration with penetrative AuNPs, any interference caused by existing pesticide residues contaminating the leaf surface need to be removed. In this study, ultrapure water was used as a washing agent to wash off surface pesticide residues. After pesticide penetrating into leaves for different time periods, ultrapure water with flow rate of 15 mL/s was utilized to gently rinse the surface of leaves (Step 2). Different rinsing times were then investigated to complete removal of each pesticide residue on the surface. After rinsing of surface pesticide residues, all leaf samples were air-dried at room temperature for 30 min. A 5 µL aliquot of a 250 mg/L solution of AuNPs was then pipetted onto the same area of the basil leaves where the pesticide solution had been placed (Step 3). The video camera in the Raman microscope was used to record the position on the
leaves and help to find the same area for AuNPs deposition. After AuNPs treatment for drying 30 min, SERS detection was performed on that area using the confocal Raman instrument. Each treatment was performed twice.

Scheme 5.1 Schematic illustration of monitoring of thiabendazole penetration on live basil leaves using SERS mapping technique following the removal of thiabendazole surface residues by rinsing. Step 1: addition of thiabendazole on the top surface of live leaf. Step 2: rinse of surface pesticide residues. Step 3: addition of AuNPs and analysis with SERS depth mapping method.

For the study of the pesticides penetration on harvested basil leaves, 36 intact leaves (0.2 g per leaf) were first cut off from petiole on live plants using a sharp knife and carefully rinsed as described above for live leaves. During the experiment, the stems of each harvested leaf were immersed into water to keep the leaf fresh. To study pesticide
penetration into harvested leaves, the same rinsing method and pesticides penetration protocols were carried out as described above for live leaves.

For monitoring the persistence of pesticides in live leaves over time, a 5 µL aliquot from each of the 10 mg/L pesticide solutions was individually pipetted onto a single leaf and allowed to dry in air at room temperature. Different growing time intervals were used to investigate each pesticide behaviors with thiabendazole (30 min, 1 week, 2 weeks and 3 weeks) and ferbam (24 h, 1 week, 2 week and 3 weeks). The first growing intervals of thiabendazole at 30 min and ferbam at 24 h were different. This was because 30-min exposure and 24-h exposure was the first time when thiabendazole and ferbam were able to penetrate into leaves, respectively. After growing over different time intervals, the surface residues from each applied pesticide were rinsed off using water and a 5 µL aliquot of the 250 mg/L AuNPs solution was added on the same position. After 30 min of drying, SERS depth mapping images were obtained using the confocal Raman instrument.

5.3.4 Raman Instrumentation and Data Analysis

A DXR Raman microscope (Thermo Fisher Scientific, Madison, WI, USA) with a 780-nm laser and a 20× long distance microscope objective was used in this study. Each sample was scanned from 400 to 2000 cm\(^{-1}\) for a 2-s exposure time.

For detecting pesticides on basil leaf surfaces, Raman spectra were carried out using a 50 µm slit aperture and 3 mW laser power to maximize the signals. In the area of each dried droplet of AuNPs solution on a leaf, ten discrete locations from the area were chosen and scanned through the Raman microscope. Their SERS spectra were analyzed with TQ Analyst (version 8.0). The final SERS spectrum of each area was averaged by ten independent SERS spectra coming from ten discrete locations. The variances of SERS
spectral data between spots and samples were assessed by principal component analysis (PCA).

For penetration studies, SERS depth mapping images were obtained using a 50 µm pinhole aperture and 3 mW laser power to control the confocal depth using a scanning depth of 300 µm. Each depth area (100 µm × 300 µm) was chosen from the treated spot on the leaf distant from a gland because the gland is the cavity, not a flat surface. The step size of the depth mapping was 20 µm and one image contained 75 scanning spots. Raman images were integrated based on the characteristic peaks in the pesticide spectra using the Atlıus Function in the OMINCS software (Thermo Fisher Scientific). There was one spot on each leaf and one depth area was selected from each spot. For each pesticide depth mapping experiment, there were six duplicates with six leaves coming from three different plants (2 leaves from each plant). Statistical analysis was conducted using SPSS Statistics 22 software (IBM Corporation, Armonk, NY, USA). The differences among results were calculated using an independent sample T-test or analysis of variance (ANOVA) and a post hoc Duncan test with a confidence level of 95%.

5.4 Results and Discussion

5.4.1 Characterization of SERS Spectra of Pesticides on Basil Leaves

The SERS spectra of thiabendazole and ferbam in the presence of AuNPs were obtained on Au-coated microscopic slides (Figure 5.6, see Supplementary Figures) and on non-rinsed basil leaf surface (Figure 5.1A). The fingerprint information of each pesticide in the presence of AuNPs is clearly evident on the leaf’s surface. The peaks at 1010 cm\(^{-1}\) of thiabendazole and 1371 cm\(^{-1}\) of ferbam were chosen as the characteristic peaks for monitoring and SERS mapping in the following studies.
The concentration-dependent SERS spectra of thiabendazole and ferbam on basil leaves are given in Figure 5. 1B and 1C. The controls were leaves without pesticide being applied. Even as low as µg/L levels of pesticide solutions, SERS signals of the test pesticides were clearly seen, showing the ultra-high sensitivity of the currently developed SERS method for the detection of pesticides on plant leaves *in situ.*
5.4.2 Monitoring of Pesticide Penetration on Harvested and Live Basil Leaves Over Time

To accurately and reliably determine the penetration behaviors of pesticides into leaves, we need to determine the penetration depth of AuNPs alone (uncomplexed with pesticide) as well as to determine the most efficient and complete method to remove surface pesticide residues.\textsuperscript{137} The penetration depth of AuNPs alone using live or harvested leaves was determined to be approximately 245 µm and 180 µm, respectively, after a 30-min incubation (Figure 5.7), which was about 30% of the leave thickness. Different methods were then evaluated to ensure the complete removal of surface pesticide residues (Figure 5.8). We found that the SERS signals of surface thiabendazole and ferbam residues were not detected after rinsing with ultrapure water for 8 s and 5 s, respectively, indicating that the concentration of surface residues of thiabendazole and ferbam were lower than 5 µg/L and 10 µg/L, respectively, which could be considered negligibly. Interestingly, these pesticides were easier to remove from basil leaf surfaces when compared with other fresh produce, such as spinach, apple peel or grape peel, when rinsed by water.\textsuperscript{137,155} This finding is probably due, in part, to the specific leaf structure of basil. Basil is an annual aromatic herb belonging to the Lamiaceae family. Like other members of the Lamiaceae, basil leaves have two types of glandular trichomes on their surface, termed peltate and capitate glands, which usually produce volatile oils.\textsuperscript{164–167} When the leaves were held up to light, many tiny glandular structures in the leaves could be seen, which look like holes (Figure 5. 2A). Based on a previous study, the glands can recess into the surface of the leaf and develop as a

**Figure 5.1** (A) Raman spectra on basil leaves. (a) thiabendazole with AuNPs, (b) ferbam with AuNPs, (c) AuNPs, (d) thiabendazole, (e) ferbam and (f) basil leaves. Concentration-dependent SERS spectra of (B) thiabendazole and (C) ferbam on basil leaves.
cavity. Figure 5.2B is the bright light scattering image of basil leaf with the cavity of the gland shown in the center of the image. Figure 5.9 shows more details of the gland on the basil leaf. Figure 5.2C shows that the thiabendazole signals are highest around the cavity, which means pesticide tends to accumulate around the gland. The specific cavity structure of basil leaves likely increases the surface area, which would augment the interaction between water and pesticide residues and thus facilitated the pesticide removal.
Figure 5.2 (A) Photo image of basil leaf surface. (B) Bright light scattering image of the gland on basil leaves added with thiabendazole and AuNPs. (B) SERS mapping image of a basil leaf with thiabendazole and AuNPs (using the intensity of SERS peak of thiabendazole at 1010 cm$^{-1}$). The step size of the mapping is 10 µm and one image contains 100 scanning spots. (c) SERS spectra of selected positions on the mapping image.
After removing surface residues, the penetration of each pesticide following different exposure times (30 min to 48 h) was evaluated on either harvested or live leaves using SERS mapping technique. The mapping images were collected from an area of the leaf that was far away from glands to ensure reliable penetration results because the gland area is not a flat surface. As shown in Figure 5. 3A-a, the systemic pesticide thiabendazole penetrated into both harvested and live leaves following a 30-min exposure. With increasing exposure time, the penetration depths of thiabendazole gradually increased to approximately 180 µm on harvested leaves and 225 µm on live leaves at 48-h after exposure, indicating that thiabendazole penetrated deeper into live leaves versus harvested leaves. The corresponding SERS spectra of positions 1 and 2 from the SERS mapping images given in Figure 5. 3A-b clearly show the diagnostic fingerprint of thiabendazole. Figure 5. 3A-c summarizes the penetration depth of thiabendazole at different exposure times and shows that thiabendazole penetrates statistically deeper into live leaves than into harvested leaves during 30 min to 48 h exposures.

Figure 5. 3B shows the penetration of the non-systemic pesticide ferbam into leaves following different exposure times from 30 min to 48 h. As shown in Figure 5. 3B-a,
ferbam was first detected on harvested leaves after 48 h at a depth of 18 µm, while ferbam on live leaves was first detected at 24 h at a depth of about 50 µm, which increased to 130 µm by 48 h. Figure 5. 3B-b gives SERS spectra from positions 3 and 4 chosen from SERS mapping images (48 h exposure) and clearly show the Raman fingerprint of ferbam. The penetration depths of ferbam at different exposure times are summarized in Figure 5. 3B-c. Similar as with thiabendazole, ferbam penetrated deeper into live leaves versus harvested leaves. This finding may be due, in part, to the higher level of transpiration in live versus harvested leaves since they are still part of a living intact plant. Based on a published study,\textsuperscript{168} penetration of pesticides into leaves proceeds as follows: first pesticide solutes in solution are adsorbed to the surface of the epicuticular wax layer and aggregate there. Pesticide solutes then diffuse through the surface wax and move into the cuticle. An equilibrium of pesticide solutes in solutions, in surface wax and in cuticle is then established. Following the establishment of equilibrium, the amounts of pesticides in these compartments no longer increase. Uptake after this time represents translocation into the leaf cells. This fraction of the pesticide is retained irreversibly, and transpiration is the main force for pesticide translocation. Because live leaves transpire better than the harvested leaves, pesticides translocate more extensively in live leaves compared with harvested leaves. In addition, the penetration depth for each of the test pesticide was less than that of AuNPs, indicating that AuNPs are effective and reliable probes for the study of pesticide penetration.

5.4.3 Effects of Leave Integrity and Age on Pesticide Penetration into Live Basil Leaves

Pesticide penetration was investigated using both intact and damaged leaves on live plants. Damaged leaves were prepared using a sharp knife to scratch the top surface of
intact leaves. Each of the damaged leaves had 20 scrapes on each leaf and the distance between each scrape is 0.2 cm. The detail of damaged leaf is diagramed in Figure 5.10. Figure 5. 4A shows the SERS depth mapping images of intact and damaged leaves after a 24 h pesticide exposure. The penetration depth for thiabendazole applied on intact and damaged leaves was 170 and 80 µm, respectively. The decrease in the penetration depth of thiabendazole in damaged leaves could be the result, in part, of reduced translocation that occurs due to the structural disruption of the damaged leaf. Additionally, the physical damage used to disrupt the intact leaves may have induced certain enzymes, particularly, oxidative enzymes (e.g., cytochrome P450s, peroxidases, and polyphenol oxidases), which are known to metabolize pesticides.\textsuperscript{44,169–174}

![Figure 5. 4](image)

**Figure 5. 4** (A) Representative SERS mapping image of the penetration of thiabendazole on intact and damaged leaves. (B) SERS mapping image of the penetration of thiabendazole on old and young leaves (using the intensity of SERS peak of thiabendazole at 1010 cm\(^{-1}\)). The step size of the mapping is 20 µm and one image contains 75 scanning spots.

To determine the effect of leaf age on penetration, we selected old leaves from plants grown for 45 days with an approximate weight of 0.204 ± 0.019 g for each leaf and young
leaves from plants grown for 15 days with an approximate weight $0.053 \pm 0.007$ g for each leaf. Following a 24 h exposure, thiabendazole penetrated deeper into old leaves (165 µm) versus young leaves (95 µm) (Figure 5.4B). This result was probably due to the fact that older leaves have more and larger stomata cells, resulting in a higher transpiration rate and hence a deeper penetration.
Figure 5.5 A. SERS mapping method for monitoring of thiabendazole persistence overtime. (a) SERS depth mapping images of the thiabendazole following different exposure time periods. Step size is 20 µm and one image contains 75 scanning points. (b) SERS spectra of selected positions on the mapping images. (c) Principal component analysis (PCA) plot of different thiabendazole treatment times. B. SERS mapping method for monitoring of ferbam persistence overtime. (a) SERS depth mapping images of ferbam following different exposure time periods. Step size is 20 µm and one image contains 75 scanning points. (b) SERS spectra of selected positions on the mapping images. (c) Principal component analysis (PCA) plot of different thiabendazole treatment times.

5.4.4 Monitoring of Pesticide Persistence on Live Basil Leaves

Figure 5. 5A-a shows that the penetration depth of thiabendazole decreases overtime following pesticide exposures from 30 min to 3 weeks. The SERS spectra corresponding to each mapping image are displayed in Figure 5. 5A-b, which clearly shows that the intensity of the characteristic peak of thiabendazole at 1010 cm\(^{-1}\) is gradually reduced overtime. The decreased amount of thiabendazole is indicative of increased degradation of the pesticide over the 3-week exposure probably due to enzymes.\(^{45,46,175–177}\) In addition, the SERS patterns of thiabendazole at different time intervals given in Figure 5. 5A-b was variable, and the statistical difference was compared by principal component analysis (PCA) (Figure 5. 5A-c). The evaluation of the four discrete areas indicated that thiabendazole apparently degraded following each exposure time interval.

Similarly, the SERS mapping method was used to monitor internalized ferbam behaviors overtime (Figure 5. 5B-a). As seen with thiabendazole, the penetration depth of ferbam was also reduced following exposure intervals from 30 min to 3 weeks. The SERS spectra obtained from each mapping image showed that the intensity of the characteristic peak of ferbam at 1371 cm\(^{-1}\) was gradually reduced over the exposure interval from 1 day to 2 weeks (Figure 5. 5B-b). After 3 weeks, the SERS characteristic peak at 550 cm\(^{-1}\) was used for ferbam determination due to the fact that the SERS peak of ferbam at 1371 cm\(^{-1}\)
overlapped with the SERS peaks of AuNPs aggregation peaks around 1400 cm\(^{-1}\). The intensity of the characteristic peak of ferbam at 553 cm\(^{-1}\) was very weak, indicating that the concentration of ferbam was low after 3 weeks. The patterns of the SERS spectra for ferbam following different exposure times were different (Figure 5. 5A-b) and PCA was used to assess the difference as before (Figure 5. 5B-c). When the data from 24 h and 1-week exposures were compared, there was some overlap, indicating that the amount of ferbam did not change noticeably during this time interval. This finding may be the result of the reduced penetration ability of ferbam, a non-systemic pesticide, when compared with thiabendazole during the first week following exposure and because plant mechanisms for degradation are mainly effective on internalized pesticides. After 2 and 3 weeks following exposure, there was statistically significant reduction in the SERS pattern of ferbam intensity, indicating that ferbam had obviously degraded.

5.5 Conclusion

In conclusion, we investigated the penetration and persistence behaviors of a systemic fungicide thiabendazole versus a non-systemic fungicide ferbam on harvested and live basil leaves in real-time and \textit{in situ} using the SERS mapping technique. The results showed that these fungicides had a more rapid and deeper penetration pattern on live leaves than when applied onto harvested leaves during surface exposure from 30 min to 48 h. This finding is probably the result of a higher transpiration level in live leaves, which would translocate more pesticide into the plant.

The influence of leaf integrity and age on thiabendazole penetration was also evaluated on live basil leaves. We found that intact leaves allowed for increased penetration when compared with damaged leaves, probably due to impaired transpiration and increased
enzymatic activities leading to degradation. The larger older leaves (45 days) allowed deeper pesticide penetration than the smaller younger leaves (15 days). In the study that evaluated the persistence of pesticides over a 3-week exposure interval, we observed that internalized thiabendazole degraded apparently after 1 week, whereas obvious degradation of internalized ferbam became apparent after 2 weeks. Furthermore, we found an interesting phenomenon that the two tested fungicides were removed to a greater extent following rinsing of basil leaves when compared with these same pesticides applied on other fresh produce, including apples, grapes and spinach leaves. This finding may indicate that the specific gland structures of basil leaves may influence the removal of surface residues. Information obtained from this study will help us to develop a better strategy to apply pesticides and reduce pesticide exposure from fresh produce and it also provides a basis for setting residue tolerance levels and other regulatory considerations. Therefore, the outcome of this study will greatly benefit to the long-term stability, safety and sustainability of agriculture and food system. Future work will focus on studying translocation of pesticides in live plants.
5.6 Supplementary Figures

**Figure 5.6** Raman spectra on the gold slide. (a) thiabendazole with AuNPs, (b) ferbam with AuNPs, (c) AuNPs.
Figure 5.7 (A) SERS mapping image of ferbam/AuNPs complex penetrated into live and harvested basil leaves using the intensity of SERS peak of ferbam at 1371 cm$^{-1}$. The step size of the mapping is 20 µm and one image contains 75 scanning spots. (B) SERS spectra of selected positions on the mapping images.

The explanation for Figure 5.7: In detail, we first incubated 10 ppm of ferbam and 250 ppm AuNPs for 30 min in order to ensure effective adsorption of ferbam onto AuNPs through Au-thiol bonds. Then a 5 µL aliquot of the mixture was pipetted onto basil surface and dried for 30 min prior to SERS mapping. SERS mapping images are shown in Figure 5.7A and the corresponding SERS spectra of positions 1 to 6 from the SERS images are presented in Figure 5.7B. The position 1 and 2 clearly show the fingerprint information of ferbam molecules while the position 3 does not show any SERS peaks, indicating that AuNPs can penetrate into live basil leaves about 245 µm after 30 min. For the penetration into harvested basil leaves, the penetration depth of AuNPs was approximately 180 µm. The corresponding SERS spectra of positions 4 to 6 from SERS mapping images are given in Figure 5.7B.
Figure 5.8 SERS spectra of surface residues of (A) thiabendazole and (B) ferbam on basil leaves after rinsing with water for different times.
Figure 5.9 Bright light scattering image of the gland and stoma on basil leaves.
Figure 5.10 Diagram of the damaged leaf.
## 5.7 Supplementary Table

**Table 5.1** Chemical names, classification and structures of pesticides.

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Chemical structure</th>
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</tr>
<tr>
<td>Ferbam</td>
<td>Nonsystemic dithiocarbamate fungicide</td>
<td><img src="" alt="Ferbam Structure" /></td>
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CHAPTER 6

EFFECTIVENESS OF COMMERCIAL AND HOMEMADE WASHING AGENTS
IN REMOVING PESTICIDE RESIDUES ON AND IN APPLES

6.1 Abstract

Removal of pesticide residues from fresh produce is important to reduce pesticide exposure to humans. This study investigated the effectiveness of commercial and homemade washing agents in the removal of surface and internalized pesticide residues from apples. Surface-enhanced Raman scattering (SERS) mapping and liquid chromatography tandem-mass spectrometry (LC-MS/MS) methods were used to determine the effectiveness of different washing agents in removing pesticide residues. Surface pesticide residues were most effectively removed by sodium bicarbonate (baking soda, NaHCO$_3$) solution when compared with either tap water or Clorox bleach. Using a 10 mg/mL NaHCO$_3$ washing solution, it took 12 and 15 min, respectively, to completely remove thiabendazole or phosmet surface residues following a 24 h exposure to these pesticides, which were applied at a concentration of 125 ng/cm$^2$. LC-MS/MS results showed, however, that 20 % of the applied thiabendazole and 4.4 % of the applied phosmet residues had penetrated into the apples following the 24 h exposure. Thiabendazole, a systemic pesticide, penetrated four-fold deeper into the apple peel than did phosmet, a non-systemic pesticide, which led to more thiabendazole residues inside the apples which could not be washed away using the NaHCO$_3$ washing solution. This study gives us the information that the standard postharvest washing method using Clorox bleach solution for 2 min is not an effective means to completely remove pesticide residues on the surface of apples. The NaHCO$_3$ method is more effective in removing surface pesticides residues on apples. In the presence
of NaHCO$_3$, thiabendazole and phosmet can degrade which assists the physical removal force of washing. However, NaHCO$_3$ method was not completely effective in removing residues that have penetrated into the apple peel. The overall effectiveness of the method to remove all pesticide residues diminished as pesticides penetrated deeper into the fruit. In practical application, washing apples with NaHCO$_3$ solution can reduce pesticides mostly from the surface. Peeling is more effective to remove the penetrated pesticides, however, bioactive compounds in the peels will get lost too.

6.2 Introduction

The use of pesticides in agriculture has led to an increase in farm productivity.$^{115,141,142}$ However, pesticide residues may remain on agricultural produce where they contribute to the total dietary intake of pesticides.$^{99,104,144}$ Concerns about potential hazards of pesticides to food safety and human health have increased, and therefore, it is desirable to reduce these residues.

Washing is the common practice to remove pesticide residues from produce. During the commercial processing of fresh produce, sanitizers are used in the postharvest washing process in order to remove visible soil or organic matter residues as well as to reduce the microbial contamination found on the surface.$^{178,179}$ One of the commercially-available sanitizer washing solutions for produce is Clorox® Germicidal bleach, which is approved by US Environmental Protection Agency (EPA) and is the most commonly used product. The active ingredient is sodium hypochlorite and the solution pH is maintained at 6.5-7.5 with acetic acid. The EPA regulation (EPA Reg. No. 5813-100) for fruits and vegetable washing is that a sanitizing solution of 25 mg/L available chlorine is prepared and fruit or vegetables are submerged for 2 min, followed by a water rinse prior to packaging.
Additionally, many methods have been examined on the removal of pesticide residues from fresh produce during home preparation.\textsuperscript{180–182} Basically, we can use tap water or water containing various chemicals, such as sodium bicarbonate (NaHCO\textsubscript{3}), also known as baking soda, sodium carbonate (Na\textsubscript{2}CO\textsubscript{3}), acetic acid (CH\textsubscript{3}COOH) or sodium chloride (NaCl). The mechanisms for washing pesticides can be described as chemical and physical removal.\textsuperscript{183} In the chemical way, pesticides can be partly decomposed in chemical solutions and the degradation products can be further removed by washing. Physical washing was achieved by removing pesticide directly. Pesticides can be washed away by the combination of chemical and physical forces.\textsuperscript{40,184}

On the basis of our previous studies, pesticides that are applied to the produce surface can penetrate into the produce over time.\textsuperscript{98,137,185,186} Systemic pesticides can penetrate more rapidly and deeper compared to non-systemic pesticides. The objective of this study was to investigate the effectiveness of commercial (Clorox\textsuperscript{®} Germicidal bleach) and homemade (tap water and NaHCO\textsubscript{3}) washing agents to remove surface and internalized pesticide residues on and in apples, respectively. We hypothesize that systemic pesticides will be more difficult to remove from whole apples due to their greater penetrating abilities. After application and washing, we monitored the amount of each pesticide or its degradation products that remained on the surface and penetrated into the apple over time using surface enhanced Raman spectroscopy (SERS) mapping method. If pesticides degraded to other molecules but were still left on the surface, SERS would be able to detect some signals. SERS techniques have been shown to be an effective tool to monitor pesticide residues on fresh produce, both on the surface and internally following penetration, using gold nanoparticles (AuNPs) as probes coupled with a confocal Raman
We have previously shown that AuNPs can penetrate fresh produce and interact with internalized pesticides, improving their Raman signals. A liquid chromatography tandem-mass spectrometry (LC-MS/MS) method was developed to determine the penetration behavior of each pesticide and the effectiveness of the NaHCO₃ washing method in removing each pesticide.

To the best of our knowledge, this study is the first to use the SERS method to investigate the effectiveness of removing surface pesticide residues from apples following washing. Moreover, this work is the first to evaluate the removal of internalized pesticide residues from fresh produce. Understanding the effectiveness of various washing procedures in the removal of pesticides on and in apples will allow us to develop better strategies to minimize pesticide exposure from fresh produce.

6.3 Materials and Method

6.3.1 Materials

Thiabendazole (fungicide: 2-(4-thiazolyl)-1H-benzimidazole, ≥99%, analytical grade), ferbam (fungicide: Iron(III) dimethyldithiocarbamate, ≥99%, analytical grade) and phosmet (insecticide: O,O-dimethyl S-phthalimidomethyl phosphorodithioate, ≥99%, analytical grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Citrate-capped AuNP colloids with different diameters were purchased from NANO PARTZ™ Inc. (Loveland, CO, USA). Sodium bicarbonate (NaHCO₃) was purchased from Fisher Scientific (Pittsburgh, PA). Organic Gala apples and Clorox® Germicidal bleach product with the active ingredient of 8.25 % sodium hypochlorite, yielding 7.85 % available chlorine were purchased from Stop & Shop Supermarket (Hadley, MA, USA). All reagents were used without further purification. Ultrapure water (18.2 MΩ.cm) was obtained using
a Thermo Scientific Barnstead Smart2Pure Water Purification System (Waltham, MA, USA) and used for the preparation of all solutions.

6.3.2 Characterization of SERS Signals from Two Pesticides applied to apples

Gala apple was chosen as the test produce due to the wide range of pesticides used in its cultivation and because of its wide consumptions. Two pesticides, the systemic fungicide thiabendazole, and the non-systemic insecticide phosmet, were chosen to be applied to the apple surface because thiabendazole is commonly used postharvest as a dip or spray and phosmet is used for control of a wide variety of pests.\textsuperscript{158,187–190} The use of AuNPs as SERS substrates was critical in the detection of pesticide residues and thus in the evaluation of the removal effectiveness of different washing agents. The size of AuNPs was important for both signal intensity and penetration depth. Therefore, different sizes of AuNPs (15, 30, 50, 70, 90, 125 nm) as SERS substrates were first evaluated. Ferbam was used as a Raman indicator to monitor AuNPs penetration in this study due to the strong SERS activities of ferbam, thus facilitating the track of AuNP penetration. In addition, our previous study shows that 20 mg/L of ferbam does not penetrate into apples following a 30-min exposure and therefore does not influence AuNPs penetration.\textsuperscript{98} In detail, a 1 mL aliquot of a 20 mg/L ferbam solution was mixed with a 1 mL aliquot of a 250 mg/L AuNPs solution for 30 min to form ferbam/AuNPs complex through Au-amino bond. A 5 µL aliquot of the mixture was pipetted onto apples. After air drying for 30 min, SERS depth mapping images were obtained using a confocal Raman instrument. The AuNPs with the deepest penetration depth was selected as the best probe for the following studies.

Thiabendazole and phosmet stock solutions of 1000 mg/L (ppm) were prepared with ultrapure water and methanol (v/v, 1:1) and then diluted to 100 mg/L with ultrapure water.
before use. A 50 µL aliquot of each pesticide solution was mixed with 50 µL of a 250 mg/L solution of 50 nm AuNPs for 1 h at room temperature to ensure effective pesticide complexation with AuNPs through Au-thiol or Au-amino bond. Apple peels with surface area of ~8 cm² and thickness of about 0.5 cm were prepared using a sharp knife to cut from a whole apple. Then a 5 µL aliquot of each of the pre-prepared pesticide/AuNPs solutions was pipetted onto each apple peel with a concentration of about 125 ng/cm² situated on a glass slide, respectively and air-dried in a fume hood for 10 min. Solutions of AuNPs without pesticide and each pesticide alone were also pipetted onto apples as control treatments for comparison purposes.

6.3.3 Removal of Surface Pesticide Residues by Washing

Different washing methods were investigated to study their effectiveness in removing surface pesticide residues from apples. Organic apples were thoroughly hand rinsed with ultrapure water for 2 min and air-dried before the experiment in order to remove surface contaminants such as dust. A schematic illustration (Scheme 6. 1) shows the washing protocol used for the study of the removal of surface pesticide residues from apples. First, ten 5 µL aliquots of a 100 mg/L solution of each pesticide were pipetted onto the surface of an apple (5 aliquots per half apple), each with an approximate concentration of 125 ng/cm². After the aliquots were air-dried on the apples for 30 min, three different washing agents/methods were investigated: Clorox® Germicidal bleach (25 mg/L available chlorine, pH 8.05), 10 mg/mL NaHCO₃ (pH 9.12) and tap water (pH 6.85). For Clorox and NaHCO₃ solutions, the treated apples were first immersed into 200 mL of a washing solution for 2 min or 8 min and then gently rinsed with 150 mL of deionized water for 10 s. The tap water washing method, which was intended to imitate how people washed their
apples at home, was applied by rinsing treated apples with approximately 1.8 L of tap water for 2 min at a flow rate of 15 mL/s. All washed apples were air-dried at room temperature for 10 min. The intact apple was then cut vertically into two halves with each half apple having 5 aliquots of the treatment pesticide applied before. One-half of apple was used for SERS detection and the other half was utilized for LC-MS/MS detection. For the SERS detection of surface pesticide residues, a 5 µL aliquot of a 250 mg/L AuNP solution was pipetted onto the same position where the pesticide had been previously applied using a camera to record the position. After air-drying the AuNP solution for 30 min, apple peels (approximately 8 cm² area and 0.5 cm thickness) with one 5 µL aliquots of a 100 mg/L solution of each pesticide in the center of the 8 cm² area were cut from each treated apple using a sharp knife and then SERS surface mapping was performed on that area to evaluate washing effectiveness. The detail of aliquots of pesticide on apples is shown in Figure 6.7. Pesticide-treated apples that were not washed and apples with only AuNPs (no pesticides) were also analyzed using SERS surface mapping method for comparison. For each apple sample, the corresponding SERS depth mappings were also collected. Each treatment was repeated three times.
Scheme 6.1 Schematic illustration of SERS mapping method for evaluation of removal effectiveness of pesticide residues on and in apples with commercial and homemade washing agents. The SERS surface mapping image and depth mapping image are integrated with 25 and 75 scanning spots, respectively.

6.3.4 Removal of Internalized Pesticide Residues

SERS depth mapping methods were applied to apples in order to evaluate the effectiveness of different washing agents for removing internalized pesticides that increased over exposure time (Scheme 6.1). The washing treatments were Clorox® Germicidal bleach (25 ppm available chlorine), 10 mg/mL NaHCO₃ solution and tap water. Organic apples were also first washed to remove surface contaminants and then treated by applying each pesticide solution as described for the study of surface pesticide residues above. Apple samples were then air-dried at room temperature over different time intervals (30 min and 24 h) in order to allow for pesticide penetration into apples. Since pesticide residues still remained on the surface, these residues were needed to be removed completely before the effectiveness of different washing methods could be compared in their ability to remove internalized pesticides. 10 mg/mL NaHCO₃ solution was utilized to remove surface thiabendazole and phosmet residues by rinsing apples for 12 and 15 min, respectively. After that, three different washing treatments were examined: 1) Clorox® Germicidal bleach (25 mg/L available chlorine) applied for 8 min; 2) 10 mg/mL NaHCO₃ solution applied for 8 min, and 3) tap water applied for 2 min. The washing process is as follows. For Clorox and NaHCO₃ solutions, the treated apples were first immersed into 200 mL of a washing solution 8 min and then gently rinsed with 150 mL of deionized water for 10 s. The tap water washing method was applied by rinsing treated apples with approximately 1.8 L of tap water for 2 min at a flow rate of 15 mL/s. After apple samples were air-dried
at room temperature, a 5 µL aliquot of the 250 mg/L AuNPs solution was added at the same position where pesticides were applied using the same protocol used in the study of surface residues described above. After 30 min of air-drying, SERS depth mapping images were obtained using the confocal Raman instrument. SERS depth mapping images of apple samples with the only removal of surface pesticide residues were also collected for comparisons. Each treatment was repeated three times.

6.3.5 Raman Instrumentation and Data Analysis

A DXR Raman microscope (Thermo Fisher Scientific, Madison, WI, USA) with a 780-nm laser and a 20× long distance microscope objective was used in this study. Each sample was scanned from 400 to 2000 cm\(^{-1}\) for a 2-s exposure time. For measuring pesticides on apple surfaces, Raman spectra and SERS surface mapping images with an area of 100 µm \(\times\) 100 µm were carried out using a 50 µm slit aperture and 5 mW laser power to maximize the signals. The step size of the surface mapping was 20 µm and one image contained the data from 25 scans from the area where the pesticide aliquot was applied. The SERS spectra obtained from multiple spots of the same sample were averaged and analyzed with TQ Analyst (version 8.0). For penetration studies, SERS depth mapping images were obtained using a 50 µm pinhole aperture and 5 mW laser power to control the confocal depths using a scanning depth of 300 µm. Each depth scanning was collected from the cross-section vertical to apple surface with an area of 100 µm \(\times\) 300 µm and depth of 300 µm. The step size of the depth mapping was 20 µm and one image contained the data from 75 scanning spots. The SERS spectra of 75 spots can integrate to generate artificial color images based on the intensity of a designated Raman peak. Raman images were based on the
characteristic peaks in the pesticide spectra using the Atlas Function in the OMINCS software (Thermo Fisher Scientific).

**6.3.6 Liquid Chromatography/Tandem Mass Spectrometry**

The apple samples were also analyzed with LC-MS/MS method. The limit of detection of LC-MS/MS analysis of phosmet and thiabendazole are both 1 µg/L. The pesticides on and in apple samples were first extracted by QuEChERS standard operating procedure based on a published study\(^{191}\) and Agilent Application Notebook. Extracts were measured on Waters Alliance LC equipped with Waters Acquity TQD MS/MS system at Massachusetts Pesticide Analysis Laboratory. The analytical column was Atlantis T3, 2.1 x 100mm, maintained at 30 °C. Mobile phases consisted of 0.1 % formic acid/water (Phase A) and 0.1 % formic acid acetonitrile (Phase B). We started with 95:5 A:B, held for 0.5min, ramped to 95% B at 7 min, held until 12 min, ramped to 95% A at 13 min, and held until 18 min to equilibrate. The flow rate was kept at 0.2 mL/min and the injection volume was 10 µL. Capillary voltage was set at 3000 V. High purity argon (99.999%) was used as collision gas. Ion source temperature was 250 °C, with nitrogen for desolvation. Chromatograms were obtained in the positive ion and multiple reactions monitoring mode (MRM). MRM conditions: positive ionization ES+, collision gas 0.2mL/min. For thiabendazole, Retention time: 7.85 min, Parent ion 201.96, Quantifying ion 65, Qualifying ion 131. For phosmet, Retention time: 11.70 min, Parent ion 318, Quantifying ion 160, Qualifying ion 133.
6.4 Results and Discussion

6.4.1 Characterization of SERS Spectra from Pesticides applied to Apples

50 nm citrate-capped AuNPs, which can be detected to a depth of approximately 220 µm in apple peel, were chosen as the probe for detection of penetrated pesticides (Figure 6.8). The characteristic SERS peaks for either thiabendazole or phosmet enhanced by AuNPs are shown in Figure 6.1. SERS fingerprint information for each pesticide in the presence of AuNPs is clearly observed and the peaks at 1010 cm\(^{-1}\) for thiabendazole and 606 cm\(^{-1}\) for phosmet were chosen as the characteristic peaks for SERS mapping. The characteristic peaks for either thiabendazole or phosmet were substantially reduced or absent from SERS spectra when AuNPs were absent or from apple samples that received no pesticide or AuNP treatments. The concentration-dependent SERS spectra for either thiabendazole or phosmet on apples were studied in our previous published research where thiabendazole could be detected as low as 2 µg/L and phosmet as low as 10 µg/L, showing the ultra-high sensitivity of the developed SERS method for the detection of pesticides on apples.\(^{98}\)
**Figure 6.1** (A) Raman spectra on apples. (a) thiabendazole with AuNPs, (b) phosmet with AuNPs, (c) AuNPs, (d) thiabendazole, (e) phosmet and (f) apple.

### 6.4.2 Effectiveness of Washing Methods on the Removal of Surface Pesticide Residues

SERS mapping methods were used to evaluate the effectiveness of different washing agents/methods in removing the surface residues of the systemic pesticide thiabendazole (Figure 6. 2A-a–g). Each SERS surface mapping image was integrated 25 locations within each pesticide applied area and gives final artificial color images. Rose color indicates high Raman intensities of pesticides and large amounts of pesticides while white color means pesticides were not detected. For comparison purposes, SERS surface mapping images were obtained from apples that 1) received thiabendazole treatment and AuNPs but were not washed (Figure 6. 2A-a), 2) received AuNPs but no thiabendazole (Figure 6. 2A-h), and 3) received no treatment (Figure 6. 2A-i). The SERS spectra collected from 25 locations in each mapping image were averaged and final SERS spectra are shown in Figure 6. 2B. Figure 6. 2C gives the corresponding Raman intensity of characteristic peaks of thiabendazole at 1010 cm\(^{-1}\). It is clear that after washing the amount of thiabendazole on the surface of the apple decreased. After 2 min washing with different methods (Figure 6. 2A-b, c and d), NaHCO\(_3\) solution resulted in the greatest pesticide loss when compared with either tap water or Clorox solutions. This is because in the presence of NaHCO\(_3\), thiabendazole does not interact well with the epicuticle of apples due to the formation of the higher surface tension of the droplets which has reduced spreading properties on fruit surfaces.\(^{158}\) Therefore, Thiabendazole would be easily rinsed off the fruit. When the washing time was increased to 8 min (Figure 6. 2A-e and 2A-f), there was a substantial decrease in the surface residues of thiabendazole and the NaHCO\(_3\) solution was again the most effective treatment. Upon increasing the NaHCO\(_3\) solution washing time to 12 min
(Figure 6. 2A-g), the SERS signals from surface thiabendazole residues were not detected, which given the detection limit of the SERS method meant that the surface residues were negligible. Apples treated with only AuNPs and apple without AuNPs or thiabendazole did not show any characteristic SERS peaks (Figure 6. 2A-h and 2A-i). These results indicated that increasing washing times resulted in the increased removal of surface thiabendazole residues. The longer washing times caused more loss of surface pesticides. The result also indicated that the standard postharvest washing method with Clorox bleach solution for 2 min did not effectively remove surface thiabendazole.

**Figure 6.2** (A) SERS surface mapping images of apple surface with thiabendazole after different washing treatments. (a) without washing, (b) tap water washing for 2 min, (c) Clorox solution washing for 2 min, (d) NaHCO₃ solution washing for washing 2 min, (e) Clorox solution washing for 8 min, (f) NaHCO₃ solution washing for washing 8 min, (g)
NaHCO₃ solution washing for washing 12 min, (h) AuNPs on apple surface and (i) apple surface alone for comparisons. Step size is 20 µm and one image contains 25 scanning points. (B) Corresponding SERS average spectra of each mapping image. (C) Corresponding Raman intensities in each SERS spectra.

Figure 6.3 (A) SERS surface mapping images of apple surface with phosmet after different washing treatments. (a) without washing, (b) tap water washing for 2 min, (c) Clorox solution washing for 2 min, (d) NaHCO₃ solution washing for 2 min, (e) Clorox solution washing for 8 min, (f) NaHCO₃ solution washing for 8 min, (g) NaHCO₃ solution washing for 15 min, (h) AuNPs on apple surface and (i) apple surface alone for comparisons. Step size is 20 µm and one image contains 25 scanning points. (B) Corresponding SERS average spectra of each mapping image. (C) Corresponding Raman intensities in each SERS spectra.

The apples were also analyzed using the SERS depth mapping method (Figure 6.9). Overall, this analysis showed that thiabendazole was able to penetrate into the apple peel following the 2-min wash and the amount of residue decreased following the 8-min washing (Figure 6.9-b-g). The internalization of thiabendazole is likely to be the result of
two processes; 1) penetration by the pesticide itself and 2) penetration of the pesticide complexed with AuNPs. After washing with NaHCO$_3$ solution for 12 min, all the remaining thiabendazole residues are due to internalized residues as surface thiabendazole residues were reduced below detection limit by this method (Figure 6.9-g). Therefore, even though surface residues can be removed, there was still substantial amounts of thiabendazole in the apple peel, some of which were from the direct penetration of thiabendazole itself. Given this finding, there appears to be a critical need to study the effective removal of internalized pesticides.

Similar results were obtained using the non-systemic pesticide, phosmet (Figure 6.3). Overall, the most efficient washing solution was NaHCO$_3$ solution and tap water were more efficient than Clorox solution. This result also indicated that Clorox-based commercial postharvest washing method was not an effective method to remove phosmet from treated apples in that only a small amount of phosmet was removed. When the washing time was increased to 8 min (Figure 6.3A-e and f), there was an obvious reduction of surface phosmet residues and the NaHCO$_3$ solution was more effective than the Clorox solution. Surface phosmet residues could be essentially removed by further increasing the washing time of the NaHCO$_3$ method to 15 min (Figure 6.3A-g). As with thiabendazole, surface phosmet residues were increasingly removed by increasing the washing times.

Phosmet-treated apples were also analyzed using SERS depth mapping method (Figure 6.10). After different washing treatment (Figure 6.10-b-g), internalized phosmet residues were likewise found in apples. When surface phosmet residues were essentially removed using the NaHCO$_3$ solution and washing for 15 min, SERS depth mapping images still
detected phosmet residues, which meant that phosmet, a non-systemic pesticide, can still penetrate into apples (Figure 6.10-g).

6.4.3 Effectiveness of Washing on the Removal of Internalized Pesticide Residues

SERS depth mapping methods were also applied in order to determine the effectiveness of different washing agents/methods in removing internalized pesticides over time. Surface pesticide residues must be completely removed before addition of AuNPs probes because any contaminating surface pesticide residues would penetrate into apples as AuNPs/pesticides complexes, which will form on the apple surface. Therefore, it is necessary to remove contaminating surface pesticide residues in order to study the removal of internalized pesticides. 10 mg/mL NaHCO₃ solution was applied in order to remove surface thiabendazole and phosmet residues using washing time of 12 and 15 min, respectively, based on the above surface residue removal study. After removal of contaminating surface residues, three washing methods were utilized: 10 mg/mL NaHCO₃ solution with washing for 8 min, Clorox solution with washing for 8 min and tap water with washing for 2 min. The SERS depth mapping images for the penetration of thiabendazole following different exposure times (30 min and 24 h) before removing internalized pesticide (no washing) and after attempting to remove internalized pesticide (after washing) are shown in Figure 6. 4A. The red color displays large amounts of pesticides while blue color means pesticides were not detected. Without the removal of internalized thiabendazole, SERS depth mapping images indicate that thiabendazole penetrated to a depth of 30 µm following the 30-min exposure and this depth increased to approximately 80 µm when the exposure time was increased to 24 h. When tap water was applied as the washing solution for 2 min, there was no obvious decrease in the penetration
depth for thiabendazole compared with the no wash method but the amount of thiabendazole close to the apple surface was reduced at both the 30 min and 24 h exposure times. After washing with either the NaHCO$_3$ or Clorox solutions for 8 min, the penetration depths for thiabendazole decreased slightly following exposures of 30 min and 24 h, respectively compared to no wash method. The thiabendazole residues close to the apple surface also were reduced. The corresponding SERS spectra of positions 1-8 from the SERS mapping images given in Figure 6.4B clearly show the diagnostic fingerprint of thiabendazole.

![SERS depth mapping images](image)

**Figure 6.4** A. (a) SERS depth mapping images of the internalized thiabendazole after different washing conditions following different exposure time periods. Step size is 20 µm and one image contains 75 scanning points. (b) SERS spectra of selected positions on the mapping images.

Based on a published study,$^{168}$ when a pesticide solution was applied on the surface of apples, it was first absorbed onto the surface of epicuticular wax and then diffuse into wax and cuticle. Equilibrium of the pesticide on the epicuticular wax and the pesticide in wax and cuticle is established when the amounts of pesticides in wax and cuticle no longer
increase. Once equilibrium is achieved, pesticides begin to penetrate into the living cells below and this fraction of the pesticide is retained irreversibly. When we applied washing treatments on apples, pesticides that had penetrated into wax and cuticle moved back to the surface of epicuticular wax until equilibrium was re-established and were then removed by the washing treatment. Therefore, the amount of thiabendazole close to the surface was reduced and there was a slight decrease of penetration depth because of the upward movement during the re-establishment of equilibrium. It is noteworthy that the thickness of wax and cuticle of apples varied from 30 to 75 µm. Because thiabendazole was shown to penetrate to a depth of 80 µm after a 24 h exposure, it may have penetrated into the living cells. If true, it would be very difficult to remove pesticides by washing once they enter cells due to the irreversible binding process there.

**Figure 6.5** A. (a) SERS depth mapping images of the internalized phosmet after different washing conditions following different exposure time periods. Step size is 20 µm and one image contains 75 scanning points. (b) SERS spectra of selected positions on the mapping images.

The removal of internalized phosmet residues was studied using the same methods and analysis used for thiabendazole described above (Figure 6.5A). Overall, phosmet, a non-
systemic pesticide, still penetrated into apples. With increased exposure times, the penetration depth of phosmet increased to approximate 20 µm after 24 h in unwashed apples. There was little change in the penetration depth after washing in tap water for 2 min. Using an 8-min wash with either NaHCO₃ or Clorox solutions, no phosmet was detected following a 30 min exposure, which meant all phosmet residues were removed during the wash, indicating that phosmet only penetrated into the wax and cuticle layers of apples during the 30 min exposure. Using an 8-min wash with either NaHCO₃ or Clorox solutions, there were substantial amounts of phosmet present following a 24 h exposure but no apparent change in the penetration depth was seen compared with unwashed apples. Nevertheless, the amount of phosmet close to the surface of the apple was reduced. The corresponding SERS spectra of positions 1-8 from the SERS mapping images given in Figure 6.5B clearly show the characteristic peaks of phosmet.

6.4.4 LC-MS/MS Analysis

LC-MS/MS was used to determine the amount of each pesticide as well as the effectiveness of a 10 mg/mL NaHCO₃ solution in removing thiabendazole residues from apples during a 12-min wash and phosmet residues during a 15 min wash following a 24 h exposure for both pesticides. Under these washing conditions, surface pesticide residues were reduced to below the detection limit for each pesticide. Thus, the amount of pesticide detected was from internalized pesticides only. The relationship between the penetration depth (A) and amount of each pesticide that penetrated the apple (B) as well as the effectiveness of 10 mg/mL NaHCO₃ solution in removing each pesticide (C) are shown in Figure 6.6. These data demonstrated that after a 24 h exposure, thiabendazole penetrated to a depth of 80 µm compared with phosmet that penetrated only to a depth of 20 µm (Figure 6.6A). From the
amounts of each pesticide that penetrated, 20% of applied thiabendazole and 4.4% of applied phosmet penetrated into apples (Figure 6.6B). Thus, the overall effectiveness of the wash method used to remove thiabendazole and phosmet residues from whole apples were determined to be 80% and 95.6%, respectively (Figure 6.6C). These results indicated that the systemic pesticide, thiabendazole, penetrated deeper than the non-systemic pesticide, phosmet. Furthermore, the deeper the penetration depth as seen with thiabendazole, the higher the level of the internalized pesticide, indicating that the wash method used to remove internalized pesticide residues was not complete. In addition, LC-MS/MS was applied to evaluate the degradation of pesticides in the NaHCO₃ washing solution. The results showed that the recovery budget of thiabendazole/metabolites were 96% and almost all the phosmet has degraded.

![Figure 6.6](image_url)  
**Figure 6.6** (A) Penetration depths and (B) penetration amounts of thiabendazole and phosmet as well as (C) the removal effectiveness by 10 mg/mL NaHCO₃ solution washing after 24 h exposure.

**6.5 Conclusion**

In conclusion, we investigated the effectiveness of commercial and homemade washing methods in removing both systemic (thiabendazole) and non-systemic (phosmet) pesticides from the surface of and inside apples using SERS mapping and LC-MS/MS methods. The
results showed that the 10 mg/mL NaHCO₃ solution was most effective in removing thiabendazole and phosmet on and in apples, whereas standard postharvest washing method with Clorox bleach solution and a 2-min wash did not effectively remove these pesticides. We determined that 20 % of the applied thiabendazole and 4.4 % of the applied phosmet penetrated into apples after a 24 h exposure, giving an overall removal efficiency of 80 % for thiabendazole and 95.6 % for phosmet using the 1 % NaHCO₃ solution and washing for 12 and 15 min, respectively. This result showed that the systemic pesticide, thiabendazole, which penetrated deeper, was more difficult to remove compared with the non-systemic pesticide, phosmet, because internalized pesticides that penetrate into the cells below the waxy and cuticle layer of the apple are irreversibly bound there. Although for apples, the peel can easily be remove along with most of the internalized pesticide residues. However, important nutrients (e.g. polyphenolic compounds, fibers, pigments, vitamins and minerals) will also be lost.
6.6 Supplementary Figures

Figure 6.7 Schematic illustration of pesticide on apples.
Figure 6.8 (A) SERS depth mapping images of different size of AuNPs/ferbam complex penetrated into apples using the intensity of SERS peak of ferbam at 1373 cm$^{-1}$. The step size of the mapping is 20 µm and one image contains 75 scanning spots. (B) SERS spectra of selected positions on the mapping images.

Explanation: AuNPs as the SERS substrate was critical to detect pesticide residues and thus evaluate the removal efficacy of different washing solutions. The size of AuNPs was important for both signal intensity and penetration depth. Previous studies of pesticide
penetration on fresh produce showed that AuNPs could rapidly penetrate plant tissues and 20 ppm of ferbam cannot penetrate apples during 30 min. Therefore, in this study, 20 ppm of ferbam was used as the signal molecule to investigate the penetration depth of different diameters of AuNPs into apples using SERS mapping techniques. As shown in Figure 6.8, AuNPs with sizes of 15, 30, 50, 70, 90 and 125 nm was investigated and the corresponding SERS mapping images were integrated based on the characteristic peak of ferbam at 1373 cm$^{-1}$. It showed that 50 nm AuNPs has the deepest penetration of about 220 µm compared with other sizes of AuNPs due to its effectiveness of apple penetration and SERS signal enhancement. From this data, we chose 50 nm AuNPs as the probe for detection of pesticides on apples.
Figure 6.9 (A) SERS depth mapping images of apples with thiabendazole after different washing treatments. Step size is 20 μm and one image contains 75 scanning spots.
Figure 6.10 (A) SERS depth mapping images of apples with phosmet after different washing treatments. Step size is 20 µm and one image contains 75 scanning spots.
CHAPTER 7

REAL-TIME MONITORING OF PESTICIDE TRANSLLOCATION IN TOMATO PLANTS BY SURFACE ENHANCED RAMAN SPECTROSCOPY

7.1 Abstract

Understanding translocation of pesticides is significant for effectively applying pesticides and reducing pesticide exposures from plant food. Herein, we developed a novel method for real-time monitoring of pesticide translocation in tomato plants using surface enhanced Raman spectroscopy (SERS). Systemic pesticide thiabendazole of various concentrations was inoculated in hydroponic system for growing tomato plants. After different time internals, tomato plant organs including leaves, flowers and fruits were picked and measured directly under a Raman microscope after dropping 5 µL of gold nanoparticles (50 nm, citrate coated, 250 ppm) on the surface to enhance the pesticide signals. On the leaves, we found the pesticide signals appeared firstly along the midrib in the lowest leaves and move along to the margin of the leaves. The higher concentration of pesticide applied in the root system caused the less time for pesticide translocation to leaves. Liquid chromatography tandem-mass spectrometry (LC-MS/MS) methods showed that there were 2 µg/g of thiabendazole on leaves after 48 h and 12 h of 10 ppm and 200 ppm translocations, respectively. Translocation of the pesticide into the trichome on the leaves was also observed with the SERS surface mapping method. In addition, we found a unique SERS peak at 737 cm⁻¹ on both tomato leaves and flowers after 4 day and 6 days translocation, respectively when 200 ppm of thiabendazole was applied in the hydroponic system. The peak at 737 cm⁻¹ coming from nicotinamide adenine dinucleotide (NAD) and other adenine-containing materials might be related with plant response to the pesticide toxicity.
Moreover, the effects of hydroponic and soil systems on thiabendazole translocation were also evaluated. We found pesticides applied in hydroponic system allow faster translocation than soil system due to a higher transpiration rate. The SERS method provides a rapid and effective way to study the translocation behavior of pesticides in a plant system. The information obtained here could provide useful guidance for effective and safe applications of pesticides on plants.

7.2 Introduction

Pesticides has been widely used in modern agriculture. As chemicals, they comprise a wide variety of structures, and therefore, differences in their mode of action, uptake, translocation and degradation. Translocation is one of the important behaviors of pesticides and understanding pesticide translocation is significant for effective and safe applications of pesticides on plants.

Systematic pesticides are capable of entering plants and being transported into the vascular system. Their distribution in plants can be achieved following foliar application as well as by uptake via roots. Root-to-shoot translocation is more common but less studied due to the technical difficulties associated with root experiments. During the root-to-shoot translocation process, systemic pesticides can be transported to stems or leaves through xylem after root uptake and transpiration was the main force. After pesticides getting into the leaves, leaf veins are the transport system. It is well known about pesticide translocation phenomenon. Nevertheless, it remains unclear the dynamic translocation and the distribution of pesticides in plant tissues. Understanding these information is crucial for both effectively applying pesticides and minimizing pesticide residues in plant food.
Chromatographic techniques (i.e. gas or liquid chromatography) are traditionally carried out to analyze pesticide translocation in plants. Unfortunately, the complex procedures of sample treatment are time-consuming and it need laborious manipulation. Additionally, these techniques cannot give the information where pesticide translocate in real time and the dynamics of pesticide distribution on plant tissues. Herein, we aimed to develop a novel method that can realize real-time monitoring of pesticide translocation on plants without pre-labelling using surface-enhanced Raman scattering (SERS) method. SERS has become a powerful analytical tool that enables direct identification of analytes in contact or in close proximity with plasmonic nanostructures such as gold (Au) or silver nanoparticles (NPs). SERS has a broad range of applications in food area because of its attractive features such as rapid data collection, easy to use, non-invasive determination and spectroscopic fingerprint identification. We previously reported the use of innovative SERS method for monitoring pesticide penetration behaviour. Multiple classes of pesticide penetration behaviors were investigated real time in different fresh produce (i.e. apple, grape, spinach and basil) with penetrable AuNPs as a probe to enhance pesticide signals. In this study, we will expand the use of SERS as an effective approach to monitor pesticide translocation behavior.

The aim of the present study is to apply innovative SERS techniques for real-time monitoring of pesticide translocation behaviors in plant food and examining the dynamics pesticide distribution on plant tissues. Tomato plant and thiabendazole were chosen for study as an example of the plant and systemic pesticide. This is because tomato plant is widely grown worldwide and thiabendazole is a typical systemic pesticide belong to benzimidazole family which provides good control of tomato diseases. Thiabendazole
of various concentrations was inoculated in hydroponic and soil systems for growing tomato plants. The pesticide was absorbed by root and then translocate to leaves through vascular tissue. After different exposure times, tomato organs including leaves, flower and fruits were measured directly under a Raman microscope after dropping 5 µL of AuNPs which were able to interact with the pesticide molecules. SERS mapping method was performed to display the distribution of the pesticide on trichomes of tomato leaves. Liquid chromatography tandem mass spectrometry (LC−MS/MS) methods was utilized to determine the amounts of pesticides on plants after translocation. To the best of our knowledge, it is the first study that applied SERS method to study pesticide translocation in plants. Understanding these information is important to provide insights into the development of better strategies to effectively applying pesticides and reducing pesticide exposures from plant food.

7.3 Materials and Methods

7.3.1 Materials

Thiabendazole (systemic fungicide: 2-(4-thiazolyl)-1H-benzimidazole, ≥99%, analytical grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Citrate-capped AuNPs colloids (50 nm) were purchased from NANO PARTZ™ Inc. (Loveland, CO, USA). Hoagland modified basal salt mixture was purchased from PhytoTechnology Laboratories (Lenexa, KS, USA). 50 % Hoagland solution was prepared with 0.815 g of Hoagland modified basal salt mixture per liter. Tomato seeds was purchased from W. Atlee Burpee & Co. (Warminster, PA, USA). Vermiculite potting media were provided by Greenhouses Centre at University of Massachusetts (Amherst, MA, USA). Ultrapure water (18.2 MΩ.cm)
was produced using a Thermo Scientific Barnstead Smart2Pure Water Purification System (Waltham, MA, USA) and used for the preparation of all solutions.

### 7.3.2 Plant Culture

Pesticide translocation on tomato plants was performed in both hydroponic and soil systems. In the hydroponic system, tomato seedings were first grew up in a soil system. First, the uniform tomato seeds were placed in small plastic pots filled with vermiculite potting media with temperatures of 25°C (16 hours) and 18 °C (8 hours), respectively, a relative humidity between 50 % and 60 %, and a 16-h photoperiod with light intensity of 200 µmol photons m⁻² s⁻¹ for 30 days. After that, every 12 plants were transferred to a container with 4 L of 50 % Hoagland solution. The container was protected from light with being covered by aluminium foil paper. The roots of the plant were immersed into the solution and the other parts of the plant were outside to receive sunlight. After 7 days, each plant was transferred to a separated and aluminium-foil covered vial with 100 mL of 50 % Hoagland solution containing different concentrations of thiabendazole (200 ppm, 100 ppm, 50 ppm and 10 ppm) for translocation studies. 50 % Hoagland solutions were replenished into each vial after every 8 h to keep the volume of 100 mL. The soil system was established the same way as above for growing plants for 30 days. Then each plant was transferred to an individual pot. Each pot was watered with 50 mL of 50 % Hoagland solutions every 24 h. After 7 days, plants were exposed to 100 mL of 50 % Hoagland solution containing 200 ppm of thiabendazole for translocation studies. 20 mL of 50 % Hoagland solutions were added directly to the surface of the soil after every 8 h. There were four replicate plants per treatment.
7.3.3 Characterization of the SERS Signals of Pesticide on Tomato Leaves

For characterization of SERS signals of thiabendazole on tomato leaves, harvested leaves from organic tomato plants were first carefully washed with ultrapure water and dried before the experiment. Then thiabendazole stock solution of 1000 mg/L (ppm) was prepared with ultrapure water and methanol (v/v, 1:1) and then diluted to desired concentrations with ultrapure water before use. A 50 µL aliquot of 100 ppm of thiabendazole solution was mixed with 50 µL of a 250 mg/L solution of 50 nm AuNPs for 1 h at room temperature to ensure effective pesticide complexation with AuNPs through Au-thiol or Au-amino bond. A 5 µL aliquot of pre-prepared thiabendazole/AuNPs solutions was pipetted onto tomato leaves and air-dried in a fume hood for 10 min. Solutions of AuNPs without thiabendazole and thiabendazole alone were also pipetted onto leaves as control treatments for comparison. Raman spectra were collected respectively.

For evaluation of the feasibility of SERS detection of thiabendazole on tomato leaves, 5 µL of the pre-prepared thiabendazole solution with different concentrations were first dropped onto tomato leaves. Then the leaves were quickly dried with air about 5 min preventing pesticide loss on the surface due to penetration into leaves. After that, 5 µL of AuNPs solution was pipetted onto the same position where pesticides were. After quickly drying for 5 min, SERS spectra were collected on leaves.

7.3.4 Preparation for Study of Pesticide Translocation

To study translocation of thiabendazole in a hydroponic system, 100 mL of 50 % Hoagland solution containing different concentrations of thiabendazole (200 ppm, 100 ppm, 50 ppm and 10 ppm and) were prepared and allowed for root uptake. Thiabendazole signals on leaves were monitored after different time exposure periods from 2 hours to 6 days. After
each time intervals, tomato leaf was cut from the plants using a sharp knife and put onto a glass slide, then 5 µL of 250 ppm AuNPs solution was pipetted onto different positions of leaves surface. After AuNPs dried with air for 10 min, SERS spectra were collected at each position on leaves respectively. Each treatment was performed four times on four different leaves coming from two different plants and two leaves which were nearest to the main stem and on the first branch of the plant were chosen as the target. SERS mapping method was also applied to show the pesticide distribution on trichrome. The pesticide translocation to the flowers and fruits were monitored when 100 mL of 50 % Hoagland solution containing 200 ppm of thiabendazole were prepared for root uptake. SERS spectra were collected overtime after adding AuNPs as on the leaves above.

For the study translocation of thiabendazole in a soil system, 100 mL of 50 % Hoagland solution containing 200 ppm of thiabendazole were prepared for root uptake. Thiabendazole signals on leaves were monitored overtime as in the hydroponic system above.

7.3.5 Raman Instrumentation and Data Analysis

A DXR Raman microscope (Thermo Fisher Scientific, Madison, WI, USA) with a 780-nm laser and a 20× long distance microscope objective, 50 µm slit aperture and 3 mW laser was used in this study. Each sample was scanned from 400 to 2000 cm⁻¹ for a 2-s exposure time. For detecting pesticides of each position on tomato leaf surfaces, within the area of a dried droplet of AuNPs solution from Raman microscope, 10 discrete locations from the area were chosen and scanned. Each discrete location gave one independent SERS spectrum and the final SERS spectrum representing the position was an average spectrum of 10 spectra collected from 10 locations. The average function was analyzed with TQ
Analyst (version 8.0). For detecting pesticides on tomato trichrome, SERS surface mapping method was applied to an area (140 µm × 190 µm). The step size of the mapping was 10 µm and one image contained 300 scanning spots. Raman images were integrated based on the characteristic peaks of thiabendazole at 1010 cm\(^{-1}\) in the SERS spectra using the Atlμs Function in the OMINCS software (Thermo Fisher Scientific).

7.3.6 LC-MS/MS

The amounts of thiabendazole on tomato leaves and fruits were determined with LC-MS/MS method. One fruit from one plant and four leaves coming from four different plants were analyzed. The pesticides on tissues were first extracted by QuEChERS standard operating procedure based on a published study and Agilent Application Notebook.\(^{191}\) Then the extractions were measured on Waters Alliance LC equipped with Waters Acquity TQD MS/MS system at Massachusetts Pesticide Analysis Laboratory. The analytical column was Atlantis T3, 2.1 x 100 mm, maintained at 30 °C. Mobile phases consisted of 0.1 % formic acid/water (Phase A) and 0.1 % formic acid acetonitrile (Phase B). We started with 95:5 A : B, held for 0.5 min, ramped to 95 % B at 7 min, held until 12 min, ramped to 95% A at 13 min, and held until 18 min to equilibrate. The flow rate was 0.2 mL/min and the injection volume were 10 µL. Capillary voltage was kept at 3000 V. High purity argon (99.999 %) was used as collision gas. Ion source temperature was 250 °C, with nitrogen for desolvation. Chromatograms were obtained in the positive ion and multiple reactions monitoring mode (MRM). MRM conditions: positive ionization ES+, collision gas 0.2 mL/min. Retention time: 7.85 min, Parent ion 201.96, Quantifying ion 65, Qualifying ion 131.
7.4 Results and discussion

7.4.1 Measurement of SERS Spectra from Thiabendazole on Tomato Leaf Surfaces

The SERS spectra of 100 ppm of thiabendazole with 50 nm AuNPs were obtained on tomato leaf surfaces (Figure 7.1A). The characteristic SERS peaks of thiabendazole were clearly determined at 1275, 1010 and 780 cm⁻¹ (Figure 7.1A-a). AuNPs alone has some peaks around 1600 cm⁻¹ coming from the AuNPs aggregation in Figure 7.1A-b. Little or no signals were observed from thiabendazole without mixing with AuNPs, or tomato leaf

Figure 7.1 (A) Raman spectra on tomato leaves. (B) Concentration-dependent SERS spectra of thiabendazole on tomato leaves.
alone (Figure 7.1A-c and d). The peak at 1010 cm\(^{-1}\) of thiabendazole was selected as the characteristic peak for monitoring and image integration in the following studies. The corresponding SERS spectra of thiabendazole with various concentrations on tomato leaves were obtained with increasing concentration from 20 ppb to 100 ppm (Figure 7.1B). As show in Figure 7.1B, Raman signals of thiabendazole can still be observed even as low as ppb levels, indicating the ultra-high sensitivity of the developed SERS approach for the determination of thiabendazole on tomato leaves.

### 7.4.2 Monitoring of the Translocation of Thiabendazole in Tomato Plants in a Hydroponic System

Figure 7.2 shows the schematic illustration of preparation methods for studying pesticide translocation in a hydroponic system. First, thiabendazole of various concentrations was inoculated in a hydroponic system for growing tomato plants (step 1). Pesticides were absorbed by root and then translocated to other plant tissues (i.e. leaves) through vascular tissue. After pesticides reaching leaves, leaf veins were the transport system. The amounts of pesticide in leaf veins were higher than other parts of leaves because pesticides first accumulated in veins consisting of xylem and phloem and then translocated to other parts such as spongy mesophyll, palisade mesophyll or epidermis. Target leaf was selected for SERS analysis of pesticide translocation and distribution on leaves. The target leaf was supposed to provide us the earliest time for getting pesticide signals on leaves and based on this, it was selected on the first branch of the plant and the leaf was most close to the stem shown in Figure 7.2. After different exposure times, the target leaf was cut from the plant and AuNPs were directly dropped onto different positions on the leaf surface (step 2). Most of the AuNPs remained on the surface and there might be some AuNPs penetrating...
inside. After AuNPs dried, SERS spectra were collected from each position on leaf surface using a Raman spectrometer (step 3).

**Figure 7.2** Schematic illustration of SERS method for monitoring of pesticide translocation on tomato plants.

SERS results of thiabendazole distribution on the target leaf following different exposure times (20 hours and 4 days) with 200 ppm of thiabendazole are displayed in Figure 7.3. After 20 hours exposure, different positions (1-6) on the leaf were chosen for SERS measurements (Figure 7.3A-a). Position 1, 2 and 3 were selected on the midrib and position 1 was located where is the lowest of the leaf and close to the petiole. Position 4 was chosen on the side vein and close to position 1. Position 5 and 6 were on the lamina. The corresponding SERS spectra of each position in Figure 7.3A-b show the characteristic peaks of thiabendazole are clearly seen on position 1-4 whereas little or no peaks of thiabendazole are presented on both position 5 and 6, indicating that thiabendazole can transport to position of 1-4 through veins and it is difficult to transport to position 5 and 6 at the lamina after 20 hours exposures. After 4 days exposure, eight positions (1-8) are
chosen on the leaf shown in Figure 7. 3B-a. Position 1-4 are the same locations on the leaf as in Figure 7. 3A-a, while position 5, 6, 7 are on the lamina, close to the margin, and
Figure 7. 3 (A) (a) Schematic illustration of leaf structure and different positions (1-6) on leaf surface and (b) the corresponding SERS spectra of selected positions. (B) (a) Schematic illustration of different positions (1-8) on leaf surface and (b) the corresponding SERS spectra of selected positions. (C) (a) Bright light scattering image of the trichome on tomato leaf. (b) SERS surface mapping image of trichome (using the intensity of SERS peak of thiabendazole at 1010 cm\(^{-1}\)). Step size is 10 µm and one image contains 300 scanning points. (c) SERS spectra of selected positions on the mapping image.

position 8 is on the apex. The corresponding SERS spectra of 1-8 are presented in Figure 7. 3B-b, which clearly show the fingerprint information of thiabendazole on position 5-8 and there are no obvious thiabendazole signatures on position 1-4. These results demonstrated that pesticides transport from veins to around margins and tend to accumulate there after 4 days exposure. Interestingly, there were many trichomes on tomato leaf surfaces and SERS mapping method can be utilized to image the pesticide distribution on the trichome (Figure 7. 3C). Figure 7. 3C-a shows the bright light image of trichome with an area of 140 µm × 190 µm following 20 hours exposure of thiabendazole after root uptake. The corresponding SERS surface mapping image is given in Figure 7. 3C-b. Figure 7. 3C-c presents SERS spectra at positions 1-3 chosen from the SERS mapping image. The diagnostic fingerprint of thiabendazole was clearly seen from the SERS spectra on position 1 and 2, while there was no signal seen on position 3 which might because pesticide concentration was very low or the amount of AuNPs was very small there.

Pesticide translocation on flowers and fruits were then monitored in a hydroponic system with SERS method. For monitoring of pesticide translocation to flowers, SERS spectra of 100 ppm of thiabendazole on flowers was the control to show the SERS patterns of thiabendazole on flowers (Figure 7. 4A). At the first 1-2 days, there were no thiabendazole seen from the SERS spectra. After 3 days, the SERS signals of thiabendazole appeared and the SERS signals became more apparent after 4 days, indicating that more
pesticides translocated to flowers. For the determination of pesticides on fruits, we monitored the pesticides signals over time after 10 days, 20 days, 30 days, and 45 days. SERS spectra of 100 ppm of thiabendazole and pure AuNPs on fruits are presented in Figure 7. 4B to show the characteristic SERS peaks of thiabendazole on fruits. We can first get pesticide signals after 45 days. Two positions were picked on the fruit surface and their corresponding SERS spectra are shown in Figure 7. 4B and the small SERS peaks of thiabendazole at 1010 cm$^{-1}$ were observed from the spectra. The results showed that pesticide can translocate into fruits after 45 days.

![Figure 7. 4 SERS spectra on tomato flowers (A) and fruits (B).](image)
7.4.3 Real-Time Monitoring of Different Concentrations of Pesticide Translocation on Tomato Leaves

To real-time monitor different concentration of pesticide translocation on tomato leaves, various concentrations (10, 50, 100, and 200 ppm) of thiabendazole were inoculated in the hydroponic system for growing tomato plants, respectively and SERS method was applied to detect the target leaf signals overtime. The first time when we detect SERS signals of thiabendazole at position 1 on target leaf in Figure 7. 3A-a can reveal the time when pesticide firstly translocate to leaves. We found that the first time for pesticides translocation to leaves was 48, 38, 24 and 12 hours for 10, 50, 100, and 200 ppm of thiabendazole, respectively. The corresponding SERS spectra are displayed in Figure 7. 5A and Raman signatures of thiabendazole are clearly seen. The results showed that higher concentration of pesticides applied on root caused the less time pesticides translocation to tomato leaves. We then monitored the first time when pesticide distribute on the whole target leaf and eight positions (1-8) are chosen for detection as shown in Figure 7. 3B-a. If SERS spectra from all the positions showed the Raman signatures of thiabendazole, it meant that pesticides translocated on the whole leaf. Figure 7. 5B gives the SERS spectra when we first found different concentrations of pesticide translocation on the whole leaf. The results showed that thiabendazole can translocate to the whole leaves after 5, 3, 2 and 1 days with 10, 50, 100, and 200 ppm of thiabendazole exposure, respectively, which also indicated that pesticides with higher amounts in hydroponic systems resulted in the less time for translocation to the leaves.
LC-MS/MS method was then performed to determine the amounts of pesticides transported on the target leaf when we first found pesticide signals using SERS method with 10 or 200 ppm of thiabendazole exposures. The results showed that after 48 hours of 10 ppm or 12 hours of 200 ppm thiabendazole exposures, 2 ppm (µg/g) of thiabendazole
has been transported on the target leaves. The amounts of pesticides determined by LC-MS/MS method was relatively high which seems not correlated with high sensitivity features of SERS method at ppb levels. This was because LC-MS/MS method measured all pesticides on the entire target leaf, whereas SERS method only measured the pesticides presenting at certain positions on the leaf. The amounts of pesticides at the positions are much less than that on the whole leaf. LC-MS/MS method was also used to determine the amounts of pesticides transported on the fruits after 45 days and the results showed there were 0.0079 ug/g of pesticides on fruits. In addition, LC-MS/MS method determined that there were about 15 % of pesticides translocated into the whole plant when 200 ppm of thiabendazole exposure for 6 days.

7.4.4 Response of Tomato plants after Translocation

When we monitored pesticide translocation with 200 ppm of thiabendazole exposure overtime from 2 hours to 6 days, we found leaves gradually turned yellow and the leaf’s condition following 6 days exposures is shown in Figure 7. 6A-b. Compared with a normal leaf in Figure 7. 6A-a, the leaf after pesticide translocation changes the color around margins probably due to the pesticide toxicity to the leaves. SERS spectra collected around the margin of leaves after different exposure time periods are shown in Figure 7. 6B. After 24 hours, the characteristic SERS peaks of thiabendazole were present, indicating that pesticides have translocated to the leaves (Figure 7. 6B-b). After 4 days, the SERS spectrum displayed the SERS peaks of thiabendazole and another peak at 737 cm\(^{-1}\) which might come from other molecules on leaves (Figure 7. 6B-c). The unique peak at 737 cm\(^{-1}\) became more clearly after 6 days exposure and the fingerprint information of thiabendazole was no longer apparently seen (Figure 7. 6B-d) probably because the
amounts of the molecules increased or the interaction between AuNPs with this molecule was stronger than with thiabendazole. On tomato flowers, the same phenomena were seen (Figure 7. 7). After 5 days, the SERS spectra presented the SERS peaks at 1010 cm⁻¹. However, the pesticide peaks disappeared and the specific peak at 737 cm⁻¹ was apparent after 6 and 7 days. Based on the published studies, the peak at 737 cm⁻¹ was attributed to nicotinamide adenine dinucleotide (NAD) and other adenine-containing materials.²¹⁰ These materials belonged to pyridine nucleotide co-enzymes which were essential metabolites for numerous redox reactions in living plants. NAD and its derivative NADP were known as the central metabolites directing plant cellular redox homeostasis. These nucleotides played vital roles in systems controlling adaptation to environmental stresses such as UV irradiation, salinity, heat shock and drought.²¹¹ When pesticides were applied to tomato plants, it might cause stresses. The metabolic balance of the plants was interrupted, and leaves could response to the stress overtime, producing more amounts of adenine-containing materials, and thus the peak at 737 cm⁻¹ turned more apparent overtime.

Figure 7. 6 (A) Photo image of (a) normal leaf and (b) leaf after pesticide translocation. (B) SERS spectra of tomato leaves following different exposure time periods.
Figure 7.7 SERS spectra of tomato flowers following different exposure time periods.

7.4.5 Comparison of Hydroponic and Soil System on Pesticide Translocation into Tomato Plants

Pesticide translocation was also investigated in a soil system on tomato plants with 200 ppm of thiabendazole applications. Figure 7.8 shows the SERS spectra of the lowest midrib on target leaf. SERS spectra of 10 ppm with AuNPs on leaves and SERS spectra of pure AuNPs on leaves were presented as controls. After 4 days, no pesticide signals were seen from the spectra showing pesticide has not translocated into the leaves. The SERS signal at 1010 cm⁻¹ appeared after 7 days, indicating that pesticide can first translocate into leaves after 7 days. Compared with the hydroponic system in the same condition, pesticide can first translocate into leaves after 12 hours in Figure 7.5A. This showed that the hydroponic system allows quicker pesticide translocation than the soil system probably due to plants in the hydroponic system has a better transpiration rate.
7.5 Conclusion

The present study demonstrates the application of SERS for real time monitoring pesticide translocation in tomato leaves, flowers and fruits. Various concentrations of thiabendazole were inoculated in a hydroponic system for growing tomato plants and after root uptake, the pesticide can translocate to leaves through vascular tissue and then distribute on the leaves through leaf veins. The SERS signals of thiabendazole on leaves were determined firstly on the lowest midrib on the target leaf and move along to the margin of the leaves. Pesticides applied with higher amounts in the system caused the less time to translocate into tomato leaves. SERS surface mapping technique was applied to display the pesticide distribution on the trichome after translocation. Interestingly, we found a unique SERS peak at 737 cm$^{-1}$ on leaves and flowers after 4 and 6 days with 200 ppm of thiabendazole application in the hydroponic system, respectively. The peak at 737 cm$^{-1}$ was attributed to NAD and other adenine-containing materials might be associated with plant response to...
the pesticide toxicity. In addition, the effects of hydroponic and soil systems on thiabendazole translocation were also evaluated and we found pesticides applied in hydroponic systems allow faster translocation than soil system due to a higher transpiration rate. The information obtained here could provide insights into effectively applying pesticides and reducing pesticide exposures from plant food.
CHAPTER 8

SUMMARY

We demonstrated SERS as a novel method can realize real time and in situ monitoring of pesticide behaviors including penetration, persistence and translocation with AuNPs as probes. We first developed SERS method for determination of pesticide penetration into spinach leaves nondestructively. The tested pesticides, ferbam and thiabendazole, exhibited different penetration behaviors, which were reflective of their non-systemic and systemic properties, respectively. Co-penetration of pesticides and AuNPs were observed when the surface pesticides were not removed before applying the AuNPs. We then applied the developed SERS mapping method to evaluate the penetration behaviors of four pesticides into three types of fresh produce including apple, grape and spinach. We found that systemic pesticides (thiabendazole and acetamiprid) had a quicker and deeper (after 48 h exposure) penetration pattern than the non-systemic pesticides (ferbam and phosmet) on the tested fresh produce. For different fresh produce, grape was generally more resistant for penetration when compared with apple or spinach possibly due to the differences of cuticle and epicuticular wax on fresh produce. Besides harvested tissues, SERS mapping method can also be applied on investigation of pesticide penetration on live tissues, we then investigated the penetration and persistence behaviors of a systemic fungicide thiabendazole versus a non-systemic fungicide ferbam on both harvested and live basil leaves in real-time and in situ using the SERS mapping technique. The results showed that these fungicides had a more rapid and deeper penetration pattern on live leaves than when applied onto harvested leaves during surface exposure from 30 min to 48 h. The influence of leaf integrity and age on thiabendazole penetration was also evaluated on live basil.
leaves. We found that intact leaves allowed for increased penetration when compared with damaged leaves, probably due to impaired transpiration and increased enzymatic activities leading to degradation. The larger older leaves (45 days) allowed deeper pesticide penetration than the smaller younger leaves (15 days). In the study that evaluated the persistence of pesticides over a 3-week exposure interval, we observed that internalized thiabendazole degraded apparently after 1 week, whereas obvious degradation of internalized ferbam became apparent after 2 weeks.

The penetrated pesticides may bring higher toxicities, so we would like to reduce the risk. Then we further investigated the effectiveness of commercial and homemade washing methods in removing both systemic (thiabendazole) and non-systemic (phosmet) pesticides from the surface of and inside apples using SERS mapping and LC-MS/MS methods. The results showed that the 10 mg/mL NaHCO₃ solution was most effective in removing thiabendazole and phosmet on and in apples, whereas standard postharvest washing method with Clorox bleach solution and a 2-min wash did not effectively remove these pesticides. We determined that 20 % of the applied thiabendazole and 4.4 % of the applied phosmet penetrated into apples after a 24 h exposure, giving an overall removal efficiency of 80 % for thiabendazole and 95.6 % for phosmet using the 10 mg/mL NaHCO₃ solution and washing for 12 and 15 min, respectively. This result showed that the systemic pesticide, thiabendazole, which penetrated deeper, was more difficult to remove compared with the non-systemic pesticide, phosmet. Although for apples, the peel can easily be removed along with most of the internalized pesticide residues. However, important nutrients (e.g. polyphenolic compounds, fibers, pigments, vitamins and minerals) will also be lost.
Finally, we demonstrated the application of SERS for real-time monitoring pesticide translocation in tomato leaves, flowers, and fruits. We found the SERS signals of thiabendazole on leaves were determined firstly on the lowest midrib on the target leaf and move along to the margin of the leaves. Pesticides applied with higher amounts in the system caused the less time to translocate into tomato leaves. SERS surface mapping technique was applied to display the pesticide distribution on the trichome after translocation. Interestingly, we found a unique SERS peak at 737 cm\(^{-1}\) on leaves and flowers after 4 and 6 days with 200 ppm of thiabendazole application in the hydroponic system, respectively. The peak at 737 cm\(^{-1}\) was attributed to NAD and other adenine-containing materials might be associated with plant response to the pesticide toxicity. In addition, the effects of hydroponic and soil systems on thiabendazole translocation were also evaluated and we found pesticides applied in hydroponic systems allow faster translocation than soil system due to a higher transpiration rate.

The information obtained from these studies can help us to develop a better strategy to apply pesticides and reduce pesticide exposure from fresh produce. Information obtained from these studies can also provide a basis for setting residue tolerance levels and other regulatory considerations. Therefore, the outcome of this study will greatly benefit to the long-term stability, safety and sustainability of agriculture and food system.
APPENDIX

PUBLICATIONS

Book chapters


[13] T Yang, B Zhao, L He. Chapter 10: Raman instruments for food quality evaluation. EVALUATION TECHNOLOGIES FOR FOOD QUALITY. (under review)

Journals


[1] B Zhao, X Cao, R De La Torre-Roche, C Tan, T Yang, JC White, H Xiao, B Xing, L He. A green, facile, and rapid method for microextraction and Raman detection of titanium dioxide nanoparticles from milk powder. RSC Adv., 2017, 7, 21380-21388
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