AN ALTERNATIVE ANALYSIS FOR CHLORPYRIFOS RESIDUES IN TEA BY USING SURFACE-ENHANCED RAMAN SPECTROSCOPY

by

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(Under the Direction of YAO-WEN HUANG)

ABSTRACT

Oblique angle deposition (OAD) SERS substrate deposited at a vapor incident angle of 86° demonstrates feasibility of rapid screening chlorpyrifos residues in tea. The standard SERS spectra of chlorpyrifos, an organophosphate pesticide, were established for further analysis. The instrumental detection limit of chlorpyrifos achieves 0.05 ppm. The calibration curves of chlorpyrifos based on Raman areas at bands 419, 691, 1343, and 1575 cm^{-1} have better correlation coefficients (0.9609-0.9918) than that based on Raman intensity (0.9436-0.9731) in the concentration range of 0.2-100 ppm. SERS spectra for chlorpyrifos left on tea samples were demonstrated based on band 1032, 1280, 1342, and1575 cm^{-1}. This study clear differentiate and identify SERS spectra difference between chlorpyrifos and parathion where gas chromatography method has difficulty to separate. Our results suggest that the SERS combined with principle component analysis (PCA) can be used to identify pesticide residues in food systems via identifying the minute different fingerprints.

INDEX WORDS: Pesticide detection, parathion, chlorpyrifos, surface-enhanced Raman spectroscopy, tea.

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DEDICATION

To my parents

Mr. Chau-Jang Huang and Ms. Bi-Shia Lin

Their love and sacrifices make me what I am today.

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CHAPTER 1

INTRODUCTION

Food safety issues regarding pesticide residues in tea have increased concern in the United States due to the growing popularity of tea. The use of chemical fertilizer and agricultural chemicals is rising for modern agriculture in order to protect tea and to produce the high quantity and quality of tea demanded by today's society. However, more and more researches demonstrated that the use of agricultural chemicals result in the pollution and the pesticide residue problems which are threatening the ecological environment and the human health. The problem is becoming serious, especially in developing countries. In June 2000, the EPA banned chlorpyrifos for home lawn and garden use due to its potential risk to children, and restricted its use to certain agricultural products. Chlorpyrifos can cause cholinesterase inhibition in humans, overstimulate the nervous system causing nausea, dizziness, confusion, and at very high exposures can cause respiratory paralysis and death. Chlorpyrifos not only has been widely used in tea but also frequently found residues in tea (*1*).

The standard reference method for the detection of chlorpyrifos residues in food samples is based on gas chromatography (GC). However, there are three limitations that conventional chromatography methods are encountered and SERS has advantages to solve these problems. First, chlorpyrifos residues in tea present many difficulties for conventional chromatography analysis. Tea leaves elute a mixture of aroma components, polyphenols and caffeine. A study has shown poor result for conventional extraction and clean-up of pesticide residues in tea leaves for parathion and chlorpyrifos, which cause peaks overlap in chromatography analysis. It is hard to

differentiate chlorpyrifos from parathion by the conventional chromatography method due to the similarities of polarity for these two pesticides (*2*). Parathion is an extremely hazardous organophosphate pesticide. As a food safety issue, it is important to identify whether parathion or chlorpyrifos residues are present in food. In addition, GC is only applicable to certain types of pesticide not all types of pesticide can be detected by GC method and different detectors need to be applied for further confirmation. For example, a nitrogen phosphorus detector works best for nitrogen or organophosphorus compounds; an electron capture detector works best for chlorinated or organophosphorus compounds. In contrast to chromatography methods, Raman spectroscopy can be used to identify fingerprints of chemical molecules. Raman spectroscopy is a form of vibrational spectroscopy. Because these vibrations involve identifiable functional groups, when the energies of these transitions are plotted as a spectrum, they can be used to identify the molecule (*3*). However, this approach suffers from weak signal making it difficult to examine background. Surface-enhanced Raman scattering (SERS) generates a greatly enhanced Raman signal by exciting vibration transitions in molecules that are adsorbed on certain metal surface such as Cu, Ag, or Au (*4*) with specific geometries. SERS enhancement can be observed in molecule with lone pair electrons or pi clouds. Researchers have reported that aromatic nitrogen, oxygen containing compounds (aromatic amines or phenols), and electron-rich groups (carboxylic acids) are strongly SERS active, and most pesticides possess these chemical structures (*5*). Finally, GC analysis time (5-30 minutes) is longer than SERS analysis time (20 seconds).

The objectives of this work are to evaluate the feasibility of oblique angle deposition (OAD) substrate as an alternative confirmation method to identify and differentiate residues of chlorpyrifos from parathion in tea. We conducted concentration dependence of chlorpyrifos

using OAD SERS substrate to obtain the calibration curve on the selected Raman bands at varied concentration of chlorpyrifos. Further, we identify commercial tea samples, spiked tea samples, and a control sample by their SERS spectra and PCA analysis.

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CHAPTER 2

LITERATURE REVIEW

Popularity of tea

Tea has gained in popularity starting from the eastern countries then imported into the western countries. The demand for tea is increasing. Table 2.1 lists the production data by area for tea from year 2001 to 2006 as reported by the Food and Agriculture Organization (FAO) in 2008. World tea production reached 3.6 million tons in year 2006 (Table 2.1). The figures put world black tea production at 2.5 million tons (Table 2.2) and 1 million tons for green tea (Table 2.3). In 2006, 74.8% of world production was in Far East, the continent of origin of the species. Countrywide, China is the highest production country with 28.7% of world production, closely followed by India with 25.9%. The level of world black tea consumption in 2006 (Table 2.4) was roughly equal to world black tea production. FAO predicted that world green tea production (+4.5 %) is expected to grow at a considerably faster annual rate than black tea (+1.9 %) in 2017 (*1*).

Tea processing

Camellia sinensis, the commercially important Chinese tea plant, has been cultivated by the Chinese for more than 2000 years. Common processing terms are picking, withering (wilting), rolling (bruising), oxidation, kill green (shāqīng), shaping, drying (firing), and curing.

Withering

Newly picked leaves are thinly spread to remove excess water from the leaves and allow a very light amount of oxidation. The leaves can be either put under the sun or left in a cool breezy room to pull moisture out from the leaves. Heated air is forced over the leaves if the climate is not suitable. The leaves sometimes lose more than a quarter of their weight in water during wilting. By the end of this process, the leaves should be pliable enough to be rolled.

Rolling

From the withering racks, the leaves are now twisted and rolled so that the leaf cells are broken up. In order to promote and quicken oxidation, the leaves may be bruised by tumbling in baskets or by being kneaded or rolled-over by heavy wheels. Sometimes shaking is done as well. Oils are released with this rolling process that. This also releases some of the leaf juices, which may aid in oxidation and gives the tea its distinctive aroma. The leaves can be rolled with machinery or by hand. The juices that are released remain on the leaf; a chemical change will occur shortly.

Oxidation

This is the chemical process where oxygen is absorbed. This process began once the leaf membranes were broken during the rolling process. In this process the chlorophyll in the leaves is enzymatically broken down, and its tannins are released or transformed. This process is referred to as *fermentation* in the tea industry, although no true fermentation happens since this oxidative process does not generate energy (this step is also not driven by microorganisms; in other steps of tea processing--aging for example--microorganisms might be used that actually do carry out

fermentation). Oxidation causes the leaves to turn bright copper in color. This process is the main deciding factor whether we have green, oolong or black tea. The tea producer may choose when the oxidation should be stopped. For light oolong tea this may be anywhere from 5-40% oxidation, in darker oolong tea 60-70%, and in black tea 100% oxidation.

Kill-green or shāqīng

This step is done to stop the tea leaf oxidation at a desired level. This process is accomplished by moderately heating tea leaves, thus deactivating their oxidative enzymes, without destroying the flavor of the tea. Traditionally, the tea leaves are panned in a wok or steamed, but with advancements in technology, kill-green is sometimes done by baking or "panning" in a rolling drum.

Shaping

The damp tea leaves are then rolled to be formed into wrinkle strips. This is typically done by placing the damp leaves in large cloth bags, which are then kneaded by hand or machine to form the strips. This rolling action also causes some of the sap and juices inside the leaves to ooze out, which further enhances the taste of the tea. The strips of tea can then be formed into other shapes, such as being rolled into spirals, kneaded and rolled into pellets, or tied into balls and other elaborate shapes.

Drying or firing

In this stage the leaves are dried evenly and thoroughly without burning the leaves. Drying is done to finish the tea for sale. This can be done in a myriad of ways including panning, sunning,

air drying, or baking. However, baking is usually the most common. Great care must be taken to not over-cook the leaves.

Curing

While not always required, some tea required additional aging, secondary-fermentation, or baking to reach their drinking potential. As well, flavored tea are manufactured by spraying with aromas and flavors or by storing them with their flavorants.

Tea is traditionally classified based on production techniques as described above. Basically, three major types of tea differ in the degree to which they have been fermented: green tea and white tea (not fermented), black tea (fully fermented), and oolong tea (semi-fermented). All these tea derived from the Camellia sinensis evergreen plant. What distinguishes each category is the method used when processing the tea leaves (Figure 2.1) (*2*).

Green tea

Green Tea: the processing of green tea is the shortest. After the leaves are plucked, withering is done first for about 8 to 24 hours, but this step might be omitted. The objective of withering is to lets most of the water evaporates. Rolling comes to next, the leaves to break the membranes for oxidation is skipped; hence the oxidation process is also skipped. After rolling, the leaves are pan fried or fired to kill the enzyme to prevent oxidation, polyphenol oxidase, responsible for conversion of the flavanols in the leaf to the dark polyphenolic compounds that color black tea. The last step is to roll the leaves and dry them one last time for its final shape. The green tea leaves usually remain green.

Black tea

The process of black Tea goes through the most stages. Once the leaves are picked, they are left to wither by blowing air on them for several hours. Then black tea are processed in either of two ways, CTC (Crush, Tear, and Curl) or orthodox. The CTC method is used for lower quality leaves that end up in tea bags and are processed by machines. This method is efficient and effective for producing a better quality product from medium and lower quality leaves. Orthodox processing is done either by machines or by hand. Hand processing is used for high quality tea. While the methods employed in orthodox processing differ by tea type, this style of processing results in the high quality loose tea sought by many connoisseurs. After the leaves are rolled, oils from the leaves are brought to the surface. These aromatic oils aid in the oxidation process, which last for several hours. The leaves are *oxidized* under controlled temperature and humidity. This process is also called "fermentation", which is a misnomer since no actual fermentation takes place. Since oxidation begins at the rolling stage itself, the time between these stages is also a crucial factor in the quality of the tea. The last step consists of placing the leaves in an oven with temperatures reaching up to 200 degrees Fahrenheit. Then the leaves are dried to arrest the oxidation process. When the leaves are 80 percent dry, the leaves complete their drying over wood fires. The resulting product is brownish (sometimes black) in color.

Oolong tea

Oolong tea goes through a similar process that black tea goes through. The first two steps are withering and rolling. Instead of rolling, sometimes shaking is done to bruise the outer edges of the leaves. The oxidation period for oolong is half that of black tea. Once the veins become clear and

the edges of the leaves become reddish brown, while the center remains green, the oxidation process is stopped by firing.

Health benefits of tea

Aside from its sensory gualities, tea is also beneficial to human health. Tea, well known for its antioxidant properties, contains many strong antioxidant compounds such as polyphenols including flavonoids, tannins, quercetin, kaempferol, myricitin, and especially catechins. The four major catechins are epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC). Research reports that epigallocatechin gallate (EGCG), primarily in green tea, and theaflavin-3,3′-digallate, a major component of black tea, are the two most effective anti-cancer factors found in tea. In brewed green tea, catechins usually account for 30–42% of the dry weight of the solids. In contrast, black tea contains catechins (10–12%), theaflavins (3–6%), and thearubigins (12– 18%) of solids (*3*). Cao (1996) reported the antioxidant activities of 22 common vegetables, one green tea, and one black tea measured using the automated oxygen radical absorbance capacity assay (ORAC) with three different reactive species: a peroxyl radical generator ($ORAC_{ROO}$), a hydroxyl radical generator ($ORAC_{OH}$), and Cu^{2+} ($ORAC_{Cu}$), a transition metal. The green and black tea had much higher antioxidant activities against peroxyl radicals than all these vegetables (*4*).

Evidence for the bioactivity to affect the pathogenesis of several chronic diseases of tea polyphenols has been provided by numerous in vitro and experimental studies. Studies demonstrated that tea promotes good health, protect against oxidative aging process, and prevent certain disease and cancer. These results indicate that consumption of green tea will help to prolong life by avoiding premature death, particularly death caused by cancer such as skin cancer

(*5-7*), liver cancer (*8*), lung cancer (*9*), gastrointestinal tract cancer (*10*), pancreatic and bladder cancer (*11, 12*), breast cancer (*3*), prostate cancer (*13, 14*), and cardiovascular disease (*15*). In addition, studies show that tea can decrease opportunity for diabetes and obesity, prevent food poisoning, practice good oral hygiene, prevent the increase of cholesterol, control high blood pressure, lower blood sugar, slow the aging process, prevent rheumatoid arthritis, prevent infection, and boost immune function (*3*).

Pesticides use and regulation in tea

Tea has been consumed worldwide for the above documented health benefits. Tea, like fruits and vegetables, is exposed to many pests and diseases. In addition to crop loss, pest damage adversely affects the quality of tea. Polyphenols which contribute greatly to nutrition quality will be much lower due to infestation by thrips, leaf rollers, flushworms, mites, and helopeltis (*16*). The management of a tea plantation must aim to minimize all the possible adverse factors, including pests, diseases, and weeds. Pesticides are used to prevent, control, destroy, repel, and lessen the undesirable effects of target organisms. Therefore, chemical control is necessary to curb localized outbreaks of pests and diseases and to increase quality and quantity of tea production. The application of one unit dollar worth of pesticides saves an estimated 4 unit dollars worth of crops from infestation every year (*17*). However, high use levels of pesticide by some producers who strive to make products more attractive not only damage the environment, but they can also cause serious health problems. With environmental and health consciousness on the rise, pesticide use has been a concern among consumers, farmers, and environmentalists. Extensive monitoring of pesticide residues in fruit and vegetables has been performed in many countries; in contrast, a regulation control of pesticide residues in tea is not well developed.

It is desirable that tea is kept completely free from pesticide but this would be unrealistic in view of the serious pest, disease problems, and the economy of tea production. The only alternative is to keep the residues of pesticides much below the maximum residue limits (MRLs). Global initiative for pesticide management in tea is mainly made by the FAO/WHO Joint Meeting on Pesticides Residues (JMPR) recommending pesticide MRLs for Codex Alimentrius Commission (CAC). Different organizations have various criteria for MRL. For example, MRLs was stimulated by the World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO), the Environmental Protection Agency (EPA), and European Community (EC). Table 2.5 lists some organizations on national and international database of maximum residue levels. Other pesticide regulations such as ADIs are conducted by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR); toxicity classified (hazard ratings) are assigned by the EPA, the WHO, and the EC; and classifications with respect to evidence of carcinogenicity are assigned and published by the International Agency for Research on Cancer (IARC) and by the American Conference of Governmental Industrial Hygienists (ACIGH).

How does tea infusion and processing affect pesticide residues?

Pesticides are applied to tea plants either during cultivation or, occasionally, during storage. Pesticides may dissipate by a number of processing such as volatilization to the atmosphere, washing off by rainfall or overhead irrigation, chemical degradation, growth dilution, and metabolism or excretion (*18*). In spite of these, residue left on dried tea leaves can be transferred to tea infusions and contribute to the dietary exposure of a consumer to hazardous chemicals (*19*). Reports have shown that tea has serious pesticide residue problems. In 2000, India's famous Darjeeling tea was reported with overdose pesticide residues (*20*). In the same year, the tea

exported in China was reported 100% of oolong tea, 44.2% of herbal tea, 21.8% of black tea, and 14.2% of green tea with Fenpropathrin residues (*21*). In 2002, findings show that 60% of tea sold by the leaf in Beijing contained pesticide residues far above legal limits.

Pesticides used on tea plants during cultivation or storage are easy to bind in tea tissue, and hot water may act as a solvent extracting a various group of pesticide residues. Few of organochlorines or pyrethrins end up in the brew because they are not water-soluble, whereas a significant amount of an organophosphates do because they are. Jaggi et al., (2001) found that the transfer of pesticides to the brew can be attributed to their water solubility, partition coefficient, and vapor pressure. Following are some examples of pesticide transfer during infusions. Jaggi et al, (2001) showed 64% of quinalphos used for control of pests in tea gardens is lost during processing and 16% (2.81 ppm) of the pesticide out off the remaining 36% (17.72 ppm) is transferred to cup-infusion. Study observed 2 to 52% transfers of residues of organophosphates into brewed tea, and 3.14% of chlorpyrifos translocation to the tea during infusions (*22*). Propargite residues transferred from manufactured tea to infusion was in range of 23.60–40.00% while 35.71–53.20% residues remained in the spent leaves (*23*). 1.5–14% of bifenthrin was transferred in brew (*24*). Furthermore, manufacturing processes contribute pesticide translocation as well and have different pesticide loss for different types of tea. Comparison manufacturing process between black tea and green tea, green tea involves heating processing which resulted in greater loss of pesticide residues than did black tea (*25, 26*). It was also observed that the increase in brewing time resulted in an increased transfer of pesticides from tea to brew for the cases studied.

Pesticides

Insecticides are categorized into organophosphate**,** carbamate, organchlorine, and pyrethroid (*27*). Organophosphate and carbamate insecticides affect the nervous system by disrupting the enzyme that regulates acetylcholine, a neurotransmitter. Organochlorine pesticides were commonly used in the past, but many have been removed from the market due to their health and environmental effects and their persistence (e.g. DDT and chlordane). Pyrethroid insecticides were developed as a synthetic version of the naturally occurring pesticide pyrethrin, which is found in chrysanthemums (*27*). Among them, organophosphate pesticides have increased in use, because of less damaging to the environment and less persistent than organochlorine pesticides. Commonly used organophosphate insecticides which are registered by the Environmental Protection Agency (EPA) of the United States are given, among them, following have been restricted by the EPA in order to prevent health risk: azinphos-methyl, chlorethoxyphos, chlorpyrifos, coumaphos, diazinon, disulfoton, ethion, ethoprop, ethyl parathion, fenamiphos, fenitrothion, fonofos, isofenphos, methamidophos, methidathion, profenofos, sulfotepp, sulprofos, and terbufos (*28*). Chlorpyrifos is mainly discussed in this study.

The use of organophosphates in private homes, for killing bugs in and around the house and in the garden, forms a large part of the non-occupational exposures in the general population in the United States (*29*). In this circumstance, organophosphate pesticides (OPs) are present in the air in homes, so exposure can occur from inhalation (*30*). OPs can cause acute poisoning symptoms such as abdominal pain, dizziness, headaches, nausea, vomiting, as well as skin and eye problems (*31*). Additionally, long-term health problems such as respiratory problems, memory disorders, dermatologic conditions (*32*), cancer (*33*), depression (*34*), neurological deficits (*35*), miscarriages, and birth defects (*36*).

Chlorpyrifos, an organophosphate insecticide, acaricide, and miticide, used to control foliage and soil-borne insect pests on a variety of food and feed crops, including tea. In 2000, the National Center for Food and Agricultural Policy in Washington, D.C., estimated that up to 3 million pounds (1.4 million kg) of chlorpyrifos was being used in the home-and-garden market each year. Steenland et al., reported that in the United States 82% of adults in the country have detectable levels of chlorpyrifos metabolites in their urine (*37*). Chlorpyrifos can cause cholinesterase inhibition in humans, overstimulate the nervous system causing nausea, dizziness, confusion, and at very high exposures can cause respiratory paralysis and death. Based on animal studies that showed that chlorpyrifos causes higher systemic toxicity in neonates than in adults. A survey of pesticides in foods done in the state of Maryland from September 1995 to September 1996 showed positive results for the residues of organophosphates. Chlorpyrifos was detected in 38.3% of solid food samples out of 75 subjects (*28*). EPA has already taken serious steps to reduce chlorpyrifos exposure in children. Because of its potential danger to humans, chlorpyrifos has been banned for home and garden use by EPA in 2000 (*38*). The EPA stated that the main reason for the ban was to protect children from exposure to chlorpyrifos due to neurological health risks.

The World Health Organization (WHO) estimated a 7.3% mortality for pesticide poisoning incidents (*28*). The major occurrences are in developing countries particularly in Africa, Asia, and Central and South America. In Central America, between 1992 and 2000, the incidence rate of acute pesticide poisoning and mortality had increasing from 6.3 per 100000 to 19.5 per 100000, and 0.3 per 100000 to 2.1 per 100000, respectively (*28*). A survey done in the state of Washington reported that the major means of exposure pesticides for consumers are contaminated produce and fruit juice (*28*). Exposure to organophosphates could happen when

consumers failed to rinse fruits and vegetables thoroughly that had been sprayed while growing or storage. The Food and Agriculture Organization (FAO) confirmed the maximum residue limit in tea commodities of 2mg/kg (2ppm), the acceptable daily intake (ADI) of 0.01 mg/kg body weight and the acute reference dose (acute RfD) of 0.1 mg/kg body weight (*39*).

Conventional methods for pesticide detection

There are two general types of method to determining residues in food and environmental samples: single-residue methods (SRM) and multiresidue method (MRM). SRM is for quantitatively determining a single pesticide in samples of regulatory interest. SRM is generally chosen when the sample is known or potential to contain a residue of an interested pesticide. MRM is capable of detecting and quantifying more than one pesticide in more than one sample. MRMs can rapidly screen whether any pesticide is present, near, or above tolerance level. For examples, OPs and carbamate insecticides can be screened by cholinesterase enzyme inhibition tests; any insecticide residue can be screened by insect bioassaysl; target chemicals or classes of chemicals can be screened by immunoassays (*18*).

No matter which type of method is chosen. Several steps are usually done for sample preparation to remove most part of uninterested interference chemicals before sample determination and detection by selected methods. The sample is extracted to remove as much of the analyte from the matrix as possible, with a minimum extraction of extraneous materials that might interfere in the analysis. After extraction, the sample is followed by cleanup in which analyte is concentrated and purified and the bulk of the interfering coextractives are removed. Some samples may go through a derivitization step in order to enhance extractability (*40*). Physical properties play an important role in choosing the strategy for extraction. Extraction

solvents are chosen based on the polarity of the extractant which matches the polarity of the analyte (like dissolves like) so that the analyte enters the solvent but the matrix remains undissolved. Single or mixture solvents have been employed for extracting pesticides from crop tissue, extracts, derived foodstuffs, and other substances.

Soxhlet extraction method was used in this work. It was originally designed for the extraction of a lipid from a solid material by Franz von Soxhlet in 1879. However, it is not limited to the extraction of lipids. It has been most commonly used in the preparation of tea and perfumes (*41*). Normally, a solid sample is placed inside a thimble made from thick filter paper, which is loaded into the main chamber of the Soxhlet extractor. The extractor is connected to a flask containing the extraction solvent, and a condenser is connected above the extractor. The solvent is boiled, and the standard extractor has a bypass arm that the vapor passes through to reach the condenser, where it condenses and drips onto the sample in the thimble. Once the solvent reaches the top of the siphon arm, the solvent and extract are siphoned back into the lower flask. This cycle may be allowed to repeat several times. After many cycles, the desired compound is concentrated in the distillation flask. The advantage of this system is using less solvent by recycling the same batch of solvent instead of many portions of solvent. After extraction, the solvent is removed by means of a rotary evaporator in warm water bath, yielding the extracted compound. The non-soluble portion of the extracted solid remains in the thimble, and are usually discarded. Theoretically, a Soxhlet extraction is only required that the desired compound has a limited solubility in solvents, and the impurities are insoluble in the solvents. If the desired compound has a higher solubility in the solvents than impurities then a simple filtration can be used to separate the compound from the insoluble substance. No matter what kind of extraction methods used, the main objective is to efficiently remove as much of the

pesticide of interest as possible from the matrix. Usual spiking procedures involve adding a know amount of analyte to a blank or control matrix portion at the point of extraction, and then determining the percent recovery in the prepared extract. In contract to solvent extraction, other extraction techniques do not involve organic solvents, or use minimal organic solvents, such as solid-phase extraction (SPE), solid phase microextraction (SPME), accelerated solvent extraction (ASE), microwave-assisted solvent extraction (MASE), supercritical fluid extraction (SFE), and semi-permeable membrane device(SPMD) (*40*).

The extraction of pesticide residues is sometimes followed by a cleanup step to remove the bulk of the potential interfering coextractives. Potentially interfering coextractives are lipids (waxes, fats, and oils), pigments (chlorophylls, xanthophylls, and anthocyanins), amino acid derivatives (proteins, peptides, alkaloids, and amino acids), carbohydrates (sugars, starches, and alcohols), lignin (phenols and phenolic derivatives), terpenes (monoterpenes, sesquiterpenes, diterpenes, etc.), and miscellaneous environmental contaminants (organic compounds, minerals, sulphur, PCBs, phthalate esters, and hydrocarbons) (*18*). Gel permeation chromatography was performed as a clean-up procedure in this study. It is a separation technique based on the analyte's hydrodynamic radius (Rh) or volume (Vh), not its molecular weight. The separation process takes place in GPC columns that are packed with porous material, such as polystyrene gels, glass beads, silica gel, etc., which will interact with the molecules. The larger molecules cannot enter the pores; thus they pass quickly through the column and elute first. Smaller molecules can enter some pores and take longer to elute. This technique can be used to determine the molecular weight of large biomolecules and polymers, as well as to separate them from salts and small molecules. With or without cleanup, determination is performed in the next step, sometimes after derivatization.

A wide variety of analytical methods have been reported in the literature for pesticide determination the most important of which can be categorized into either physicochemical methods or immunochemical methods. Physicochemical methods include gas chromatography (GC), liquid chromatography (LC), high performance liquid chromatography (HPLC), and thin layer chromatography (TLC). Immunochemical methods include antibody methods and enzyme methods.

Gas chromatography is a common type of chromatography used in organic chemistry for separating and analyzing compounds that can be vaporized without decomposition. Typical uses of GC include testing the purity of a particular substance and separating the different components of a mixture (the relative amounts of such components can also be determined). A gas chromatograph uses a flow-through narrow tube known as the column, through which different chemical components of a sample pass in a gas stream (carrier gas, mobile phase) at different rates, which depend on their various chemical and physical properties and their interaction with a specific column filling (stationary phase). As the chemicals exit the end of the column, they are detected and identified electronically. The function of the stationary phase in the column is to separate different components, causing each one to exit the column at a different time (retention time). Other parameters that can be used to alter the order or time of retention are the carrier gas flow rate and the temperature. A detector is used to monitor the outlet stream from the column; thus, the time at which each component reaches the outlet and the amount of that component can be determined. Generally, substances are identified (qualitatively) by the order in which they elute from the column and by the retention time of the analyte in the column (*42*).

In contrast to GC, liquid chromatography (LC) is a separation technique in which the mobile phase is a liquid. Liquid chromatography can be carried out either in a column or a plane,

which is called HPLC or TLC, respectively. The sample is forced through a column or plane that is packed with irregularly or spherically shaped particles or a porous monolithic layer (stationary phase) by a liquid (mobile phase) LC is historically divided into two different sub-classes based on the polarity of the mobile and stationary phases.

There are, however, some limitations in these conventional methods. GC is most used for pesticides analysis, but GC is only amenable to the separation of volatile and semivolatile compounds or those that can be derivatized to increase their volatility (*43*). Generally, analysis of most pesticide residues is carried out in a sequence of several steps including target extraction from sample matrix, then clean-up and pre-concentration, followed by chromatographic separation and determination. The sample is heated to volatilize; however, some organophosphate compounds decompose at elevated temperatures resulting in misleading results (*44*).

The limitations of liquid chromatography (LC) include the necessary consumption for expensive instrumentation, operation, and high-purity solvents. Hence, the column has difficulties to separate similarities chemical structures and properties, which makes it difficult for conventional analysis to determine many classes or even all pesticides in a class (*43, 45*).

The limitation of immunochemical is that most methods require a clean-up step before analysis because of interfering substances in the food matrix, and different food commodities require different clean-up methods (*46*). The analyte may bind to the sample protein or exist in complexes that do not bind to the specific antibody which may lead to misreading of the sample (*47*). Interfering substances that bind to the antibody can also cause false-positive results (*48*). In pesticide analysis, immunochemical cannot compete with LC and GC because erroneous results

of immunochemical may be caused by a matrix effect. Immunochemical is primarily used only to screen samples (provide yes/no) for pesticide contamination.

The determination of chlorpyrifos residues and its main metabolites in food and environmental samples by different chromatographic methods have been developed (*49-56*). For the determination of organophosphate pesticide in fruits and vegetables both (LC–MS) or (GC–MS) have also been applied, showing LODs between 0.001 and 0.004 mg/kg and 0.005 mg/kg, respectively. Study reports showed LOD of 16 ng/l for the analysis of chlorpyrifos in water by using high pressure liquid chromatography (HPLC) (*57*).

However, chlorpyrifos residues in tea address difficulties for chromatography analysis. Tea leaves are mixture of aroma components, polyphenols and caffeine. Study showed poor result for conventional extraction and clean-up of pesticide residues in tea leaves for parathion and chlorpyrifos, which cause peaks overlap in chromatography analysis. It is hard to identify chlorpyrifos from parathion by the conventional chromatography method due to the physical similarities of these two pesticides (*58*).

Introduction to Raman spectroscopy

In contrast to conventional chromatography based on dispersive, polar, and ionic interaction of chemicals to its stationary and mobile phase, Raman spectra act as identifying molecule fingerprints to diagnose the internal structure of molecules by studying vibrational, rotational, and other low-frequency modes in a system. When a beam of light usually from a laser in the visible, near infrared, or near ultraviolet range is impinged upon a sample, photons are absorbed by the material and scattered. The vast majority of scattered photons has exactly the same wavelength as the incident photons and is known as Rayleigh scatter (elastic scatter). On the

other hand, a tiny portion (approximately 1 in 10^7) of the scattered radiation is shifted to a different wavelength and is known as Raman scatter (inelastic scatter). Most of the Raman scattered photons are shifted to longer wavelengths (Stokes shift), commonly used in spectroscopy analysis, but a small portion are shifted to shorter wavelengths (anti-Stokes shift). Both types of Raman scattering, the energy decays to a different level than that where it started. Stokes Raman scattering occurs when the final energy level is higher than the initial level, while anti-Stokes Raman scattering occurs when the final energy level is lower than the starting level.

For example, a known frequency and polarization of light is impinged and scattered from a sample. The scattered light is then analyzed for frequency and polarization. Raman scattering is frequency-shifted with respect to the excitation frequency, but the magnitude of the shift is independent of the excitation frequency. The energy difference is lost to vibrations of the molecular bonds after the transfer of energy to the molecule. As mentioned above, Raman is a form of vibrational spectroscopy. Because these vibrations involve identifiable functional groups, when the energies of these transitions are plotted as a spectrum, they can be used to identify the molecule (*59*). However, only some excitations of a given sample are Raman active. Hence the frequency spectrum of the Raman scattered light maps out part of the excitation spectrum. Other spectroscopic techniques, such as IR absorption, are used to map out the non-Raman active excitations (*60*).

However, the Raman literature was dominated by physical and structural investigations until 1986. The application of Raman spectroscopy for chemical analysis was hampered for following reasons. The first major impediment to using Raman spectroscopy is the weak intensity. A second problem with Raman spectroscopy is fluorescence interference. The visible light typically used for Raman spectroscopy before 1986 often excites fluorescence of the

analyte of interest or of impurities. Fluorescence is not a scattering process, and fluorescence emission from most liquids and solids does not have the vibrational structure. Even weak fluorescence can be much stronger than Raman scattering, easily overwhelming the weak Raman signal. Before 1986, fluorescence of either the analyte or impurities in the sample in wavelength range 400 to 650 nm light obstacle the observation of Raman scattering in a large fraction of samples. Additional, avoidance of fluorescence was critical to the utility of Raman spectroscopy for practical applications. Third, the inefficient light collection and detection obstructed Raman development as well.

These problems were overcome till 1986 with the introduction of Fourier transform Raman, charge-coupled devices, small computers, and near-infrared lasers (*60*). Significant advantages result showed when FT techniques are combined with laser excitation in the NIR, for example, at 1064 nm. Although the Raman scattering is weaker at 1064 nm compared to visible excitation (e.g., 514.5 nm), the fluorescence background is often orders of magnitude weaker. NIR excitation is sufficiently lower in energy that most of the electronic transitions responsible for fluorescence are not excited. With 514.5 nm excitation the spectrum is completely dominated by fluorescence, while at 1064 nm, the Raman scattering is easily observed with an FT-Raman spectrometer. Regardless of the detection method (FT or dispersive), longer wavelength excitation yields a higher ratio of Raman scattering to fluorescence for the vast majority of samples. Even though the Raman scattering is weaker and detection is more difficult than in the visible region, the reduction in fluorescence permits a much wider range of samples to be examined with Raman spectroscopy.

Raman scattering is a relatively weak process. The number of photons Raman scattered is quite small. Therefore, there are several process which can be used to enhance the sensitivity of a

Raman measurement such as Surface Enhanced Raman Spectroscopy (SERS), Resonance Raman Spectroscopy, Surface Enhanced Resonance Raman Spectroscopy (SERRS), Spontaneous Raman Spectroscopy, etc. Among them, SERS was used as analysis tool in this study.

Introduction to SERS

Surface Enhanced Raman Spectroscopy (SERS) is one of Raman spectroscopic technique that provides greatly enhanced Raman signal from Raman-active analyte molecules effectively adsorbed onto certain special rough metal surfaces such as silver, gold, and copper surface, although aluminum, lithium, and sodium metal surface also gave enhancement with the excitation wavelength near or in the visible region. The morphology of SERS substrates plays an important role in Raman signal enhancement, sensitivity of Raman intensity, and reproducibility of SERS spectra. Popular SERS substrates include colloids (*61*), metal films on dielectric substrates (*62*) , and, recently, arrays of metal particles bound to metal or dielectric colloids through short linkages (*63*).

Surface-enhanced Raman spectroscopy (SERS) has found extensive use in structure and qualitative analysis (*64-67*), but quantitative analysis has falled behind. The quantitative application of SERS is known to be very difficult due to instability of substrate metal degradation, inconsistency of substrate metal enhance ability, and distribution of chemicals absorbed on substrate metal surface. Oblique angle deposition technique (OAD), a simple modification of conventional physical vapor deposition technique, overcomes difficulties of substrate fabrication. First, the metal vapor (silver in this work) was deposited on a substrate at a large incident angel greater than 70° with respect to horizontal glass surface. Silver nanorods

were grown along at this oblique angle. Diffusion of silver condenses onto the substrates and forms individual separate nuclei. As the incident vapor reach on substrate, the taller nanorods capture more atoms than the shorter ones, which results in formation of columns and in the direction of vapor source. This technique presents uniformity, stability, reproducibility of SERS substrates. SERS substrates fabricated by OAD have been shown to be very sensitive (SERS enhancement factor > 10^8) and good SERS spectra reproducibility (*68-70*).

While SERS has noted for provide valuable molecule information based on the vibtational mode enhanced signal to 10^{14}-10^{15} by specific metal (*71*). However, the target molecules have to be attached to or in close proximity to SERS substrates based on electromagnetic theory. Therefore, SERS enhancement cannot be observed for all types of molecule. In other words, SERS enhancement can be observed in molecule with lone pair electrons or pi clouds. Literatures have reported that aromatic nitrogen, oxygen containing compounds (aromatic amines or phenols), and electron-rich group (carboxylic acids) are strongly SERS active.

In spite of sensitive enhancement of SERS, this technique has following limitations (*72*):

1. The analyte is interacting with a metal nanostructure. The analyte need to be efficiently close to roughed surface. The adsorption on solid surfaces according to the strength of bonding between the particle and the substrate.
2. Disappointing reproducibility of active substrate and uniformity of SERS spectra are hindered the development of SERS on quantitative analysis. As the signal is extremely sensitive to a number of factors including any change in adsorbate orientation at the metal surface, the extent of adsorption, and the nature of the surface roughness.
3. Although SERS gives an enhancement up to 10^6, it is possible that small amount of contaminants or impurities within a sample may burst to give sudden signals. In this

case, the spectrum becomes complex to interpret and difficult for quantitative analysis.

SERS effect

The exact enhancement mechanism of SERS is still controversial in literature. Basically, there are two mechanisms explaining SERS enhancement effect: (1) electromagnetic theory, proposed by Jeanmarie and Van Duyne in 1977, which involves enhancements in the field intensity as a result of plasmon resonance excitation and (2) chemical theory, Proposed by Albrecht and Creighton in 1977, which is due to chemical effects such as charge-transfer excited states. The electromagnetic effect is dominant which may be enhanced by factor up to 10^6 (*71*), while the chemical effect enhancement contributes only 10-10^3 (*73*).

Electromagnetic theory, an enhancement occurs because in the electric field, provided by the metal surface, intensity of the Raman signal increase for adsorbents on particular metal surfaces. When the incident light in the experiment strikes the surface, localized surface plasmons are excited. The light incident on the surface can excite a variety of phenomena in the surface, yet the complexity of these phenomena can be minimized and only the dipolar contribution will be recognized by the system. The dipolar term contributes to the plasmon oscillations, which leads to the enhancement. Initially, the field enhancement magnifies the intensity of incident light which will excite the Raman modes of the molecule being studied, therefore increasing the signal of the Raman scattering. The Raman signal is then further magnified by the surface by the same mechanism as the incident light (*59*). Electromagnetic enhancement models can explain several important features related to the SERS effect but still it cannot explain some specific properties of the SERS phenomenon.

On the other hand, chemical mechanism involves charge transfer between the chemisorbed species and the metal surface, forming an adsorbate-metal complex. The incident light strikes the roughened metallic surface resulting in a photon being excited within the metal to higher energy level. From this excited state, a charge transfer process to a vibrational level of the same energy within the target analyte takes place. Variations in vibrational energy states occur resulting in the transfer of a photon of different frequency being passed back to the metallic energy levels, and returned to the ground state of the metal. (*59*).

Application of different types of Raman in pesticide detection

Different types of Raman have been used for pesticides analysis. In the works of pesticide for the quantitative and the identification, the FT-Raman has been widely applied to investigate pesticide formulations (*74-79*). Pesticides determination by SERS (*65-67, 80-82*). Other types of Raman technologies involved to investigate pesticide analysis (*83*).

As mentioned above, the limitations of conventional analysis for pesticide residues in tea are due to similar retention time from interference of caffeine during gas chromatography. Convention methods can cause false positive result. We believe SERS to be an alternative confirmation sensitive analytical tool that can be used to investigate low concentration of pesticide residues and provide unique fingerprint to identify among different pesticides.

Data analysis for classification

Although SERS has mentioned advantages for trace pesticide analysis in tea, objective spectra analysis is hard to achieve by visual analysis. Therefore, chemometrics or multivariate statistical techniques are employed.

To discover the relationships among all samples and variables efficiently, we must process all of the data simultaneously. Chemometrics is the field of extracting information from multivariate chemical data using tools of statistics and mathematics. Chemometrics is typically used for one or more of three primary purposes: to explore patterns of association in data; to track properties of materials on a continuous basis; and to prepare and use multivariate classification models. In spectroscopy, the applications of chemometrics are most often in calibration. Calibration is achieved by using the spectra as multivariate descriptors to predict concentrations of constituents of interest using statistical approaches such as Multiple Linear Regression (MLR), Principal components analysis (PCA), and Partial Least Squares (PLS). To explore the data and identify individual groups based on differences of the SERS spectra, PCA was employed in this study to cluster samples into groups.

Principal component analysis (PCA) is often used as a dimension-reducing technique mathematically reducing similarly correlated variables (dimensionality of data matrix) into groups of principal components (PCs) in which each of the new variables is uncorrelated. The results of PCA are usually discussed in terms of PCs. The PC1 illustrates as much of the variability of the data, and each succeeding PC accounts for as much of the remaining variability as possible. This process can identify the patterns of the data in a way which best explains the variance in the data by highlighting their similarities and differences. Since patterns of data can be hard to find in such high dimension data matrix, PCA is a powerful tool for analysing data. Distingushing similar spectra objectively by visual methods are hard to achieve and unrealistic.

Table 2.1. World tea production (thousand tons)					
	2001-2003	2003	2004	2005	2006
World	**2981.4**	**3035.6**	**3370.1**	**3526.3**	**3645.2**
Africa	**470.0**	**478.3**	**510.9**	**506.1**	**487.4**
Burundi	7.7	7.5	7.5	7.0	6.3
Kenya	293.4	295.9	328.8	332.7	313.0
Malawi	40.2	41.7	50.1	46.9	45.0
Rwanda	16.1	15.6	15.6	16.5	17.6
Tanzania United	27.2	29.5	30.7	30.4	31.4
Uganda	34.5	36.5	37.0	37.7	36.7
Zimbabwe	22.3	22.0	18.7	14.9	15.7
Others	28.5	29.6	22.5	20.1	21.6
Latin America	**85.5**	**85.3**	**82.4**	**88.8**	**92.9**
Argentina	67.1	67.3	69.0	73.0	76.3
Brazil	8.1	8.3	8.3	8.3	8.4
Others	10.3	9.7	5.1	7.5	8.2
Near East	**204.8**	**213.1**	**245.0**	**233.4**	**222.4**
Iran Islamic	55.5	58.1	40.0	27.8	22.3
Turkey	149.3	155.0	205.0	205.6	200.1
Far East	**2109.3**	**2148.1**	**2408.9**	**2573.1**	**2725.3**
Bangladesh	55.8	57.0	55.6	56.0	53.4

China	605.7	631.0	854.0	956.3	1047.4
India	848.2	859.5	895.9	919.4	945.3
Indonesia	169.6	163.0	139.0	165.9	187.9
Sri Lanka	303.9	304.8	309.1	317.2	312.0
Viet Nam	83.7	88.6	93.9	104.0	133.0
Others	42.6	44.2	61.4	54.3	46.3
Oceania	**10.1**	**9.3**	**9.3**	**9.4**	**9.5**
Japan	**87.0**	**87.0**	**100.7**	**100.0**	**91.8**
CIS	**14.6**	**14.5**	**12.9**	**15.4**	**15.8**
Developing	**2866.7**	**2920.5**	**3249.3**	**3407.4**	**3531.8**
Developed	**114.7**	**115.1**	**120.8**	**118.9**	**113.4**

Table 2.2. Black Tea : Actual and Projected Production				
Countries/ regions	Production			
	Actual	Projected	Growth rates	
	2006	2017	1996/2006	2006/2017
	Thousand metric tons		Percent per year	
Total: World	**2565**	**3141**	**2.8**	**1.9**
Total: Developing	**2547**	**3118**	**2.9**	**1.9**
Africa	**480**	**532**	**2.5**	**0.9**
Kenya	311	344	2.0	0.9
Malawi	45	51	1.6	1.1
Uganda	36.7	38	7.8	0.3
Tanzania	31.4	34	4.7	0.7
Others	56.4	65	2.3	1.3
Latin America	**92.5**	**113**	**3.6**	**1.8**
Argentina	76.3	95	4.1	2.0
Other	16.2	18	1.3	1.0
Near East	**222.3**	**236**	**2.1**	**0.5**
Iran	22.3	26	-10.3	1.4
Turkey	200	210	5.7	0.4
Far East	**1744.0**	**2227**	**3.1**	**2.2**
India	945	1175	2.0	2.0

Sri Lanka	307.3	341	1.8	1.0
Indonesia	167.9	197	2.6	1.5
China	156.3	312	12.2	6.5
Vietnam	67	76	13.6	1.2
Bangladesh	53.4	61	1.2	1.2
Others	47.1	65	11.5	3.0
Other developing	**7.9**	**10**	**1.2**	**2.5**
Developed	**18.4**	**22**	**-1.7**	**1.8**
CIS	**12.6**	**17**	**0.8**	**2.5**
Other developed	**5.8**	**5.8**	**-5.5**	**-0.1**

Table 2.3. Green Tea : Actual and Projected Production				
Countries/ regions	Production			
	Actual	Forecast	Growth rates	
	2006	2017	1996/2006	2006/2017
	Thousand metric tons		Percent per year	
World	**968.1**	**1571.1**	**4.7**	**4.5**
China	782.4	1352	5.8	5.1
Japan	91.8	100.5	0.3	0.8
Viet Nam	66.0	106	8.9	4.4
Indonesia	20.0	22.1	-5.7	0.9

Table 2.4. TEA, BLACK: Domestic Utilization				
	Consumption			
	Actual	Forecast	Growth rates	
	2006	2017	1996/2006	2006/2017
Countries/ regions	Thousand metric tons		Percent per year	
WORLD	**2339.6**	**2819.9**	**1.0**	**1.7**
Net Imports	**189.4**	**200.9**	**0.4**	**0.5**
Developing	**483.1**	**491.6**	**0.8**	**0.2**
Far East	**323.3**	**331.5**	**1.2**	**0.2**
Pakistan	115.5	119.1	0.2	0.3
Others	207.8	212.4	1.8	0.2
Other Developing	**159.8**	**160.1**	**0.0**	**0.0**
Developed	**635.7**	**734.1**	**-0.3**	**1.3**
Europe	**233.5**	**241.8**	**-3.3**	**0.3**
EU (25)	**227.0**	**234.7**	**-3.4**	**0.3**
UK	128.0	136.2	-3.4	0.6
Germany	15.0	19.0	-9.3	2.2
Poland	27.1	21.3	-2.0	-2.1
Netherlands	15.0	17.0	-3.4	1.1
France	8.0	8.8	-2.3	0.9
Ireland	9.7	7.3	-2.3	-2.6

Other EU	**24.3**	**25.1**	0.3	0.3
Other Europe	**6.5**	**7.1**	2.2	0.8
Russian Federation	**246.7**	**328.2**	**4.0**	**2.6**
North America	**103.0**	**107.5**	**0.7**	**0.4**
United States	89.1	92.3	0.7	0.3
Canada	13.9	15.2	1.1	0.8
Oceania	**19.6**	**16.1**	**-1.6**	**-1.8**
Australia	15.6	12.1	-1.7	-2.3
New Zealand	4.0	4.0	-1.4	0.0
Other Developed	**32.9**	**40.5**	**-2.2**	**1.9**
South Africa	15.0	19.3	-3.4	2.3
Japan	16.0	20.1	-0.5	2.1
Israel	1.9	1.1	-4.7	-4.9
Domestic Utilisation	**1220.8**	**1594.2**	**1.8**	**2.5**
Africa	**37.2**	**42.5**	**-4.1**	**1.2**
Kenya	14.0	17.1	-0.4	1.8
Malawi	1.1	1.2	1.0	0.8
Tanzania United	8.1	8.6	18.1	0.5
Others	14.0	15.6	-8.2	1.0
Far East	**1166.0**	**1535.0**	**2.2**	**2.5**
India	710.0	906.9	1.4	2.3
Turkey	145.0	189.0	2.6	2.4
China	92.0	115.0	14.6	2.0

Indonesia	68.0	95.0	7.4	3.1
Viet Nam	28.0	37.9	23.5	2.8
Iran Islamic Rep.	64.0	65.0	-3.5	0.1
Bangladesh	44.0	75.0	5.2	5.0
Others	15.0	51.2	-15.2	11.8
Latin America	**15.5**	**13.3**	**-5.2**	**-1.4**
Oceania developing	**2.1**	**3.4**	**8.8**	**4.5**

Table 2.5. National and International Maximum Residue Levels	
Organization	**Link**
The Codex Alimentarius Commission (CAC)	http://www.codexalimentarius.net/mrls/pestdes/jsp/pest_q-e.jsp
Foreign Agricultural Service (FAS)	http://www.mrldatabase.com/
Australian pesticides and veterinary medicines authority (APVMA)	http://www.apvma.gov.au/residues/mrl.shtml
Pest Management Regulatory Agency (PMRA)	http://www.pmra-arla.gc.ca/english/legis/maxres-e.html
European Community (EC)	http://ec.europa.eu/food/plant/protection/pesticides/database_pesticide_en.htm
The Japan Food Chemical Research Foundation	http://www.m5.ws001.squarestart.ne.jp/foundation/search.html
Pesticide Residues Committee	http://www.pesticides.gov.uk/prc.asp?id=1866
USDA/FDA	http://www.epa.gov/pesticides/food/viewtols.htm

Table 2.6 physical characteristic of chlorpyrifos and parathion

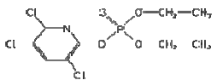
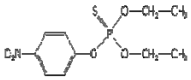
	Chlorpyrifos	Parathion
		
MRL for tea	2 mg/kg (The Codex Alimentarius Commission)	0.1mg/kg (European Community)
Tolerance	0.01-0.05 (USDA)	–
Physical chemistry		
Mol. Wt.	350.6	291.3
M.p.	42-43.5 ℃	6.1℃
V.p.	2.7mPa (25 ℃)	0.89mPa (20℃)
K_{ow}	Log P = 4.7	Log P=3.83
Henry	0.676 Pa m^3 mol^{-1} (calc.)	0.0302 Pa m^3 mol^{-1} (calc.)

Table 2.7 toxicology characteristic of chlorpyrifos and parathion

	Chlorpyrifos	Parathion
Mammalian toxicology		
Oral	LD_{50} for rats: 135-163 mg/kg	LD_{50} for rats: 8 mg/kg
Inhalation	LC_{50} (4-6 h) for rats >0.2 mg/l (14ppb)	LC_{50} (4 h) for rats 0.03 mg/l
ADI (JMPR)	0.01 mg/kg b.w.	0.004 mg/kg b.w.
Toxicity class	WHO (a.i.) II[1] EPA (formulation) II[2]	WHO (a.i.) Ia[1] EPA (formulation) I[2]
[1] WHO Class I a: extremely hazardous; Class II: moderately hazardous; [2] EPA Toxicity Class I: most toxic; Toxicity Class II: moderate toxic		

ADI is acceptable daily intake

[1]The World Health Organization names four toxicity classes as follows:

- Class I – a: extremely hazardous;
- Class I – b: highly hazardous;
- Class II: moderately hazardous;
- Class III: slightly hazardous.

The system is based on LD50 determination in rats, thus an oral solid agent with an LD50 at 5mg or less/kg bodyweight is Class I-a, at 5-50 mg/kg Class I-b, at 50-500 mg/kg Class II, and at more than 500 mg/kg Class III. Values may differ for liquid oral agents and dermal agents.

[2] The Environmental Protection Agency knows four Toxicity Classes. Class I to III is required to carry a Signal Word on the label to warn users of the toxicity. Pesticides are regulated by the Federal Insecticide, Fungicide, and Rodenticide Act(FIFRA).

Toxicity Class I

- most toxic;
- requires Signal Word: "Danger-Poison", with skull and crossbones symbol

  Possibly followed by:

  "Fatal if swallowed", "Poisonous if inhaled", "Extremely hazardous by skin contact--rapidly absorbed through skin", or "Corrosive--causes eye damage and severe skin burns".

Toxicity Class II

- moderate toxic
- Signal Word: "Warning"

  possibly followed by:

  "Harmful or fatal if swallowed", "Harmful or fatal if absorbed through the skin", "Harmful or fatal if inhaled", or "Causes skin and eye irritation".

Toxicity Class III

- slightly toxic
- Signal Word: Caution

  possibly followed by: "Harmful if swallowed", "May be harmful if absorbed through the skin", "May be harmful if inhaled", or "May irritate eyes, nose, throat, and skin".

Toxicity Class IV

- practically nontoxic
- no Signal Word required since 2002.

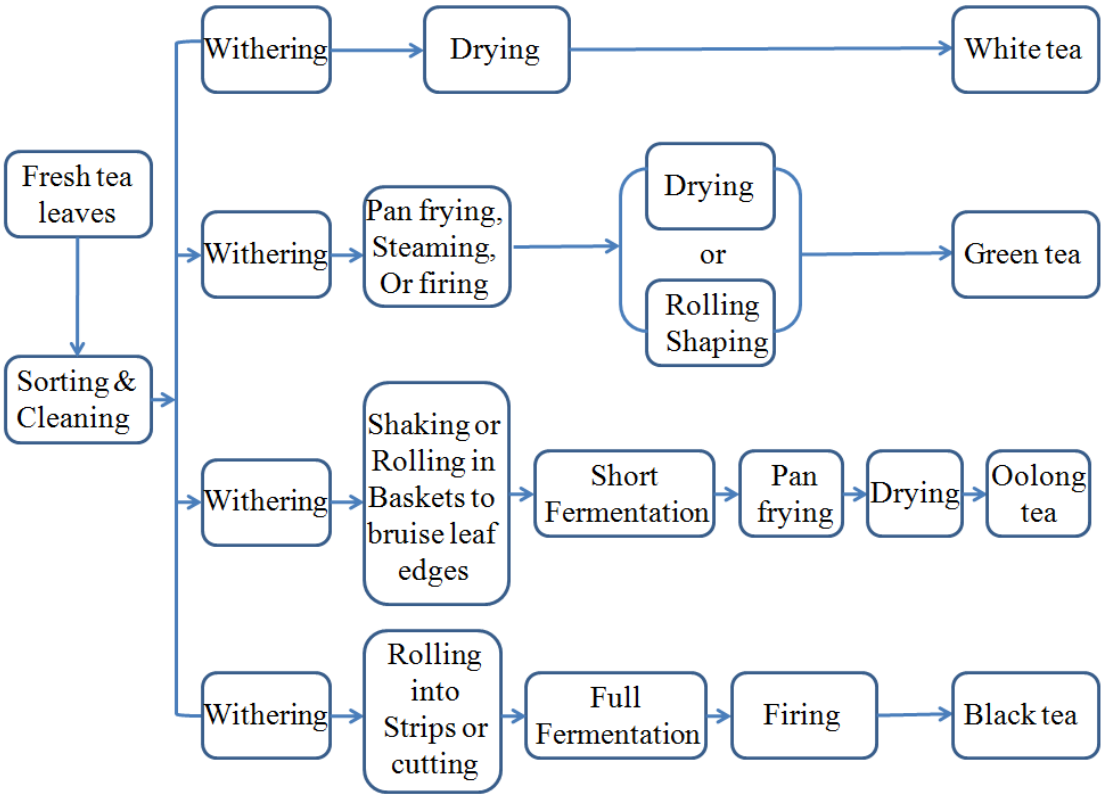


Figure 2.1. Flow chart of tea process

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CHAPTER 3

SERS AS AN ALTERNATIVE CONFIRMATION METHOD TO DISTINGUISH RESIDUES OF CHLORPYRIFOS FROM PARATHION IN TEA[1]

[1] Siao-Ling Huang, Yao-Wen Huang, Jake H. Mulligan, Parshall Bush, and Yiping Zhao. To be submitted to Journal of Agricultural and Food Chemistry.

Abstract

Food safety issues regarding pesticide residues in tea coupled with the growing popularity of tea, have increased concern in the United States. A simple, rapid, and sensitive method for pesticide residue detection employing surface-enhanced Raman spectroscopy (SERS) with Ag nanorod array substrates have been developed. The substrates are prepared by the oblique angle deposition (OAD) method with an 86° vapor incident angle. Chlorpyrifos, an organophosphate pesticide used for many crops and plants including tea, was studied at a laser excitation wavelength of 785 nm. The feasibility of SERS detection technique using Ag nanorod array substrate to rapidly exam pesticide residues in tea is demonstrated. We demonstrated that there is a quantitative relationship between the concentration of chlorpyrifos and the band intensities and integrate area. The calibration curves based on Raman integrate area at bands 419, 691, 1343, and 1575 cm^{-1} have good linearity (0.9609-0.9918) than that based on Raman intensity (0.9436-0.9731) in a chlorpyrifos concentration range of 0.2-100 ppm. The use of SERS spectra for chlorpyrifos residues in tea samples was successfully demonstrated based on bands at 1032, 1280, 1342, and1575 cm^{-1} and clearly distinguished chlorpyrifos from parathion based on SERS spectra, where GC analysis has difficulties to identify because of similar retention time. Principle component analysis (PCA) was objectively used to confirm false or positive pesticide residues. Our results suggest that the SERS combined with PCA can be used to identify chlorpyrifos residues in tea via identifying the minute differences in fingerprints.

Keywords: Ag nanorod arrays, oblique angle vapor deposition, SERS, PCA, organophosphate pesticide, tea

Introduction

Tea, well known for its antioxidant properties, has been shown bioactivity to affect the pathogenesis of several chronic diseases, which were conducted by numerous in vitro and experimental studies. These results indicate that consumption of green tea will help to prolong life by avoiding premature death, particularly death caused by cancers, such as skin cancer (*1-3*), liver cancer (*4*), lung cancer (*5*), gastrointestinal tract cancer (*6*), pancreatic and bladder cancer (*7, 8*), breast cancer (*9*), prostate cancer (*10, 11*), and cardiovascular disease (*12*).

Tea has been consumed worldwide in part due to the above documented health benefits. However, like fruits and vegetables, tea plants are exposed to many pests and diseases attack. Pesticides are applied to tea plants either during cultivation or, occasionally, during storage. In this way, some residue left on dried tea leaves can be transferred to tea infusions and contribute to the dietary exposure to hazardous chemicals (*13*). Chlorpyrifos, an organophosphate insecticide, is one of the major pesticides applied on tea with high frequency found residues in tea (Table 3.1). According to Environmental Protection Agency (EPA) pesticide registration, approximately 11 million pounds of chlorpyrifos are applied in agricultural usage in Unite States per year (*14*). Due to its widespread use, persistency and toxicity, chlorpyrifos has been included in priority lists of pesticides within the European Union (EU) (*15*). Chlorpyrifos can cause cholinesterase inhibition in humans, nausea, dizziness, confusion, and at very high exposures can cause respiratory paralysis and death. Chlorpyrifos was banned for home and garden use in order to protect children from neurological health risks by EPA in 2000. Chlorpyrifos was classified in moderately hazardous by WHO and moderate toxic by EPA. Parathion, also an organophosphate insecticide, is highly toxic pesticide which was banned for all use. However, some farmers still use parathion because of better capacity to kill insects. The Food and Agriculture Organization

(FAO) suggested that the acceptable daily intake (ADI) for chlorpyrifos and parathion of 0.01 mg/kg and 0.004 mg/kg body weight, the acute reference dose (acute RfD) of 0.1 mg/kg body weight and 0.006 mg/kg body weight, and the maximum residue limit in tea commodities of 2 mg/kg and 0.1 mg/kg, respectively (*16*).

Chromatographic methods is the current state-of-art technique to detect chlorpyrifos (*17, 18*). Gas chromatography (GC) and high performance liquid chromatography (HPLC) are the major methods for the analysis of chlorpyriofs residues in fruits and vegetables, with LODs of 0.005 mg/kg and 0.001-0.004 mg/kg, respectively (*19, 20*). However, chromatographic run times are often long because of the need to achieve sufficient chromatographic resolution. Pesticide residues analysis for chromatographic method is generally carried out in a sequence of several steps, such as extraction with organic solvent(s) followed by clean up procedure, then chromatographic separation and determination. Recently, various of different methods are well developed in these areas based on different physics mechanisms and chemical mechanisms from food matrix with reaction of different pesticides (*21*). Soxhlet extraction and gel permission chromatographic (GPC) cleanup are one of commonly used methods for tea samples pretreatment, which were applied in this study.

However, chlorpyrifos residues in tea present many difficulties for conventional chromatography analysis. Tea leaves release a mixture of aroma components, polyphenols, and caffeine. Study showed poor result identifying residues of chlorpyrifos ($C_9H_{11}Cl_3NO_3PS$) from parathion ($C_{10}H_{14}NO_5PS$) in tea, which cause peaks overlap in chromatography analysis. Second, false confirmation arises from caffeine during gas chromatography because caffeine ($C_8H_{10}N_4O_2$) has similar retention time to the two pesticides (*22*). These problems may arise from similar

polarity of these chemicals to column and therefore multipack column is needed for better separation.

In contrast to chromatography methods, Raman spectroscopy can be used to identify fingerprints of chemical molecules in a form of vibrational spectroscopy. When a beam of light is impinged upon a sample, photons are absorbed by the material and scattered. A tiny portion (approximately 1 in 10^7) of the scattered radiation is shifted to a different wavelength and is known as Raman scatter (inelastic scatter) due to the vibration interactions of the probe molecule and the incident light. Because these vibrations involve identifiable functional groups, when the energies of these transitions are plotted as a spectrum, they can be used to identify the molecule (*23*). It is a widely used technique in analytic chemistry to identify unknown chemicals. However, this approach suffers from weak signal making it difficult to examine background. Surface-enhanced Raman scattering (SERS) is a technique that generates a greatly enhanced Raman signal by exciting vibration transitions in molecules that are adsorbed on certain metal surface such as Cu, Ag, and Au (*24*) with specific geometries. Electromagnetic theory and chemical theory are two primary theories to explain enhancement of Raman signal (*25, 26*). Studies showed that silver as substrate has stronger enhancement signal than gold or copper (*27*).

Surface-enhanced Raman spectroscopy (SERS) has found extensive use in structure and qualitative analysis (*28-31*); however, the use of SERS for quantitative analysis has been limited. The quantitative application of SERS is known to be very difficult due to instability of substrate metal degradation, inconsistency of substrate metal enhance ability, and unequal distribution of chemicals absorbed on substrate metal surface. The morphology of SERS substrates plays an important role in enhancement, sensitivity, and reproducibility of SERS spectra. Popular SERS

substrates include colloids (*32*), metal films on dielectric substrates (*33*) , and, recently, arrays of metal particles bound to metal or dielectric colloids through short linkages (*34*). Oblique angle deposition technique (OAD), a simple modification of conventional physical vapor deposition technique, overcomes difficulties of SERS substrate fabrication. This technique can produce uniform, stable, and reproducible Ag nanorod SERS substrates. Those substrates fabricated by OAD have been shown to be very sensitive (SERS enhancement factor > 10^8) and produce good SERS spectra reproducibility (*35-37*).

Different types of Raman have been applied for pesticides analysis. FT-Raman has been widely investigated on pesticide formulations, quantitative, and identification analysis (*38-43*). SERS in pesticides determination and Resonance Raman technology have been used to investigate pesticide analysis (*44*). As mentioned above, conventional methods can cause false positive result for chlorpyrifos residues in tea. We believe SERS to be a sensitive confirmation analytical tool that can be used to investigate low concentration of chlorpyrifos residues and provide unique fingerprint to identify among different pesticides.

The objectives of this work are: (1) to establish, distinguish, and identify the SERS spectra of chlorpyrifos and parathion; (2) to demonstrate the feasibility of OAD substrate; (3) to construct concentration dependence and calibration curves using selected Raman bands for chlorpyrifos; and (4) to differentiate residues of chlorpyrifos from parathion by their SERS spectra and PCA analysis.

Experimental

OAD substrate preparation

Silver nanorod array substrates were made according to the process by Chu et. al (*48*). Glass

microscopic slides (Fisher Scientific), typically 1.2 cm × 1.2 cm, used as the base platform for substrate deposition were washed thoroughly with DI water and then immersed in Piranha solution (20% hydrogen peroxide and 80% sulfuric acid) and heated temperature. The substrates were loaded into a customized e-beam deposition chamber equipped with oblique angle deposition (OAD) set-up for Ag film and Ag nanorod deposition. The source materials for evaporation were Ag pellets (99.999%, Kurt J. Lesker Company, Clairton, PA) and Ti pellets (99.999%, Kurt J. Lesker Company, Clairton, PA). A Maxtek 260 quartz crystal microbalance was used to monitor film thickness. The Ag deposition rate was 0.3 nm/s and the deposition pressure was 1×10^{-6} Torr. 20 nm Titanium film, 500 nm silver film, and approximately 2000 nm of silver nanorod array (at a vapor incident angle of 86°) were deposited in order. The average length of the silver nanorods was ~900 nm, average diameter was ~100 nm, and average density was ~13 rods/μm^2. The average tilting angle of the nanorods was ~71-73° with respect to the substrate normal.

Raman characterization

The Raman instrument used in this experiment was a HRC-10HT Raman analyzer system (Enwave Optronics Inc. Irvine, CA) with a 785 nm narrow linewidth diode laser for Raman excitation. The laser power was set to 40 mW monitored by a power meter (PM 121, Thorlabs Inc., Newton, NJ). The spectra collection time is 20 seconds of averaged scans to obtain a reasonable signal-to-noise ratio (S/N) to the spectrometer equipped with a charge-coupled device (CCD) detector. The spot size of excitation laser beam was 100 μm focused onto the substrate platform.

GC characterization

Two different GCs were used in this work. Gas chromatography/nitrogen phosphorus detector (GC/NPD) and gas chromatography/electron capture detector (GC/ECD). GC/NPD was performed using the Perkin Elmer system with capillary column and program temperature set in 135-275 ℃. GC/ECD was performed using the Tracor 222 isothermal system with packed column and program temperature set in 220 ℃.

Chemicals and reagents

The pure standard pesticides (purity ≧99.6%), chlorpyrifos and parathion, were kindly provided by Agricultural and Environmental Services Lab, University of Georgia. The pesticides were dissolved in methanol for analysis. All other chemicals and reagents were of analytical grade unless otherwise noted. Each pesticide solution (1μl) was transferred onto silver nanorod substrate platform for spectra establishment.

Tea sample preparation and characterization

Two tea samples, Yawaragi Bancha, ITO EN Inc. (coded as T1) and chano ma Hōjicha, ITO EN Inc. (coded as T2), and an organic tea sample, Sencha, Haiku, (coded as O1) were purchased from H-mart in Atlanta, Georgia, USA. The organic tea sample, used as a control sample, is free from chlorpyrifos and parathion residues. For each tea, approximately five grams were weighted for conventional purification (soxhlet extraction (SOP 301) and gel permeation chromatography (GPC) clean up). Ethyl acetate was used in soxhlet extraction and 75 % ethyl acetate / 25 % toluene were used in GPC cleanup.

Three tea samples (T1, T2, O1) were spiked with chlorpyrifos prior to purification procedures at concentration 22.1 ppm, 11.05 ppm, and 11.05 ppm, respectively (coded as T1c, T2c, and O1c). O1 spiked with parathion (11.05 ppm) was used as comparison (coded as O1p).

Data analysis

Enwave Raman analyzer software (Enware Optronics Inc. Irvine, CA) was used for instrumental control and data collection. ASCII data exported from Raman analyzer system into Origin software 8.0 version (OriginLab Corporation, Northampton, MA) for spectra plotting, peak detection, and normalization,.

Principle component analysis (PCA) was carried out by Unscrambler version 9.7 (Camo, AS, Norway) applying the full cross validation. First, SERS spectra data were derivative by Savitsky-Golay method with a 9-point smoothing, first derivative order and second polynominal order. After derivative, area normalization was conducted in next step. Principle component (PC) plots were used for confirmation of positive or negative chlorpyrifos residues.

Results and Discussion

Development of SERS spectra for chlorpyrifos and parathion

Chlorpyrifos was diluted continuously until the weakest significant SERS peaks were observed. The determination of the detection limit is based on the significant peaks of pesticides as the signal required to produce a signal-to noise ratio (S/N) of 3. Five different spectra were collected from various spots of each substrate. Each spectrum represents as average of spectra of eight individual collections to eliminate possible contaminations.

To determine the significant peaks of chlorpyrifos, 1µl of decreased concentrations of

chlorpyrifos were analyzed. Figure 3.1 shows SERS spectra of chlorpyrifos at five varied concentrations (10 ppm, 2 ppm, 0.4ppm, 0.1 ppm, and 0.05 ppm) and background (methanol and blank substrate) in the wavenumber 400-1800 cm^{-1} region. The spectra were collected at excitation wavelength of 785 nm with laser power 40 mW. All the SERS spectra was normalized with respect to its most intense peak to offset spectra variation caused by substrates (*37*), no attempt was made to improve the SERS signal.

Establishing standard SERS spectra of chlorpyrifos is important for identification of chlorpyrifos for further spectra comparison. It is important to ensure the observed SERS peaks are specific to chlorpyrifos and not from background (solvents and substrate). By comparing the SERS spectra of methanol and varied concentrations of chlorpyrifos, the significant peaks of chlorpyrifos are easy to observe at 419, 691, 1343, and 1575 cm^{-1}. The instrumental detection limit (LOD) was established based on these bands with S/N of 3. Based on the selected bands, the LOD for SERS to detect chlorpyrifos can reach 0.05ppm.

The amount of chlorpyrifos per sample spot was calculated based on concentration range 0.05-10 ppm. One drop of 1 μl chlorpyrifos (molar mass of chlorpyrifos is 350.59) with different concentrations, 0.05-10 ppm was applied onto substrate platform individually with perimeter approximately 1.2 cm. The amount of chlorpyrifos coverage on the surface ranged from 1.19×10^{13} /cm^2 to 5.94×10^{10} /cm^2. Thus, the amount of chlorpyrifos distributed on laser spot ranged from 1.19×10^{9} to 5.94×10^{6}. As a result, mole of chlorpyrifos distributed on laser spot ranged from 1.98×10^{-15} M to 9.9×10^{-18} M, which is 6.94×10^{-10} ppm and 3.47×10^{-12} ppm, respectively.

These bands have good proportional relationship between concentration of chlorpyrifos and Raman intensity and are easy to identify. The band at 932 cm^{-1} was notable, which may be an

artifact of the substrate background. This band is also assigned to C-C stretching modes in proteins (*48, 49*), and may be caused from some carbonaceous materials adsorbed onto the SERS substrate during fabrication of OAD method and storage in ambient condition (*37, 50, 51*). Although background signals are frequently shown in SERS detection, they were found to remain unchanged throughout the studies and same position of wavenumber (*37*). According to different concentrations, the significant peaks of chlorpyrifos were located at near 691 cm^{-1} (Cl-ring stretches), near 851 cm^{-1} (assigned to a P-$(O\text{-}R)_2$ stretch), near 1343 cm^{-1} (assigned to a C-N stretch), and near 1575 cm^{-1} (C=C phenyl stretch) (*29*).

Furthermore, to differentiate chlorpyrifos from parathion, standard SERS spectrum of parathion was established at the same Raman conditions for chlorpyrifos. Parathion (1µl) at concentration of 5 ppm was transferred onto silver nanorod substrate platform. Figure 3.2 shows the average normalized SERS spectrum of parathion and chlorpyrifos of eight individual collections. The primary bands for each pesticide are compared, and SERS spectrum of parathion is considerably different from that of chlorpyrifos. The significant peaks of parathion reported by Shende et al. (*29*) are near 647 cm^{-1} (P=S stretch), 1160 cm^{-1} (C-H wag), and 1328 cm^{-1} (NO_2 symmetric stretching mode). The specific chemical vibrational mode for chlorpyrifos and parathion is assigned to Cl-ring (691 cm^{-1}) and NO_2 symmetric stretching (1328 cm^{-1}), respectively. Differences between the two spectra are easily noticeable which provides a unique signature suitable for identification. Therefore, the SERS spectra can clearly differentiate chlorpyrifos and parathion even with high similarities of their chemical polarities.

SERS has following advantages over conventional chromatographic methods: (1) unique SERS spectra of chlorpyrifos and parathion and (2) most pesticides can be detected by SERS. (1) the GC method has difficulties in differentiating chlorpyrifos and parathion. SERS shows unique

spectra for each pesticide. Therefore, SERS can use as an alternative confirmation method to distinguish residues of chlorpyrifos from parathion in tea. (2) GC is most used for pesticides analysis, but GC is only amenable to the separation of volatile and semi-volatile compounds (*52*). Different detectors need to be used to identify different types of pesticide. For example, gas chromatography/nitrogen phosphorus detector (GC/NPD) is sensitive to nitrogen organophophorus compounds; gas chromatography/electron capture detector (GC/ECD) is sensitive to chlorinated organophosphorus compounds. In contrast, SERS enhancement can be observed in molecule with lone pair electrons or pi clouds. Researchers have reported that aromatic nitrogen, oxygen containing compounds (aromatic amines or phenols), and electron-rich group (carboxylic acids) are strongly SERS active, and most pesticides possess these chemical structures (*53*).

Reproducibility of SERS spectra

Our SERS substrates fabricated by OAD deposition have improve several difficulties as follows: (1) uneven distribution of sample onto substrate and (2) poor reproducibility of SERS substrates. First, the irregular distribution of sample results in "hot-spots," where the proximity of adjacent features provides high enhancement, interspersed with large areas of little or no enhancement. The morphology of substrate structure plays a significant role for signal enhancement and reliable spectra data. The adsorption arises on solid surfaces according to the strength of bonding between the particle and the substrate. The analyte need to be efficiently close to roughed surface and interact with a metal nanostructure. Finally, the reproducible of SERS substrates is essential to SERS application. Disappointing reproducibility of active substrate and uniformity of SERS spectra have hindered the development of SERS as a

quantitative analysis technique.

One way to analysis uniformity of SERS substrate and even distribution of sample on substrate is through analyzing the reproducibility of SERS spectra. SERS spectra of chlorpyrifos were collected from separate spots on the same substrate and from different substrates to illustrate reproducible of SERS substrates and even distribution of sample on SERS substrates. The spectra were normalized based on the strongest peak (~930 cm^{-1}), and standard deviations were calculated in the wavenumber range 400-1800 cm^{-1}. If spectra have higher consistency, they have lower standard deviation (SD) or lower percentage of relative standard deviation (RSD). Generally, our results demonstrate a good reproducibility of SERS spectra with n=8 (RSD_{100ppm} < 16%, RSD_{10ppm} < 8%, RSD_{5ppm} < 17%, RSD_{1ppm} < 8%, and $RSD_{0.2ppm}$ < 11%).

From Figure 3.3, the RSD of chlorpyrifos 10ppm is smaller than 8% except at wavenumber 1602 cm^{-1} (RSD = 21%) which may caused by instability of laser excitation from one of spectra. The small Raman intensity variation among individual spectra could contribute from slightly inhomogeneous morphology, subtle degradation of silver substrates, uneven distribution of sample on substrate nanorods, and different contamination from atmosphere (*37*).

SERS technology on quantitative determination is not widespread because of low reproducibility of SERS-active substrates. SERS substrates fabricated by OAD techniques have improved inhomogeneous morphology and have enhanced equal distribution of analyte on substrate. Moreover, the high sensitivity of OAD SERS substrates was reported by Chu et al (*48*).

Concentration dependent of chlorpyrifos

To determine the capability of SERS on quantitative analysis of chlorpyrifos, the bands 419,

691, 1343, and 1575 cm^{-1} were used for conducting the concentration dependent of chlorpyrifos based on their band intensities and band integrate areas. These bands were used because methanol, the solvent, and common substrate background signals do not interfere spectrally. Furthermore, these band are characteristic for chlorpyrifos (*29*). Band intensities and integrate areas were calculated using a two point baseline correction in the range 406-439 cm^{-1} for the 419 cm^{-1} band, 681-708 cm^{-1} for the 691 cm^{-1} band, 1307-1357 cm^{-1} for the 1343 cm^{-1} band, and 1536-1597 cm^{-1} for the 1575 cm^{-1} band. The double peaks were found at 1323 cm^{-1} and 1343 cm^{-1}. The peak at 1343 cm^{-1} was selected using multi-peaks fitting to do integrated area of chlorpyrifos. The calibration data was normalized with most intense band. The calibration curves present excellent linearity with correlation coefficients in the range of 0.9436-0.9731 and 0.9609-0.9918 for band intensity and integrated area measurements, respectively. The linear range was found to be 0.2 ppm-100 ppm for selected bands. From Figure 3.4, the slopes of regression line for concentration of chlorpyrifos and band intensity and integrate area at selected bands are 0.3-0.6 except slope for integrate area at band 691 is 1.6. The regression line for log concentration of chlorpyrifos and log integrate area increases larger at 691cm^{-1} (slope is 1.6) than other bands (slopes are 0.3-0.6).

For quantitative analysis of chlorpyrifos, the use of integrated areas is believed to give more accurate results than the use of band intensities (*41*). As interaction of sample components are more likely to influence the intensities of Raman bands by shifting, whereas the integrate areas are less affected which utilizes a wider spectrum segment for the analysis. For the quantitative analysis of chlorpyrifos, any of the proposed bands may be used. The SERS method (required just 20 seconds) is faster than GC method (5-40 minutes depending on detector type). The applicability of the SERS technique can be further broadened by applying it to other pesticides

analysis through establishing pesticides library for future application.

Chlorpyrifos residues confirmation by GC

Before chlorpyrifos detection by SERS, GC analysis was used to confirm chlorpyrifos residues in tea. Sample pretreatment is generally needed for solid samples and complex matrices such as foodstuffs in chromatography analysis. For each tea, approximately five grams were weighted for conventional purification (soxhlet extraction (SOP 301) and gel permeation chromatography (GPC) clean up). Two types of detectors were used for chlorpyrifos confirmation due to different responses to the pesticide. Since chlorpyrifos possess chlorinated and nitrogen phosphorus compounds, GC/NPD and GC/ECD were performed to exam chlorpyrifos residues. GC/NPD detected no chlorpyrifos for T1 and T2 samples, but GC/ECD, more sensitive than GC/NPD, detected with chlopryrifos residues of 0.29 ppm and 0.20 ppm for T1 and T2 samples. Both ECD and NPD detectors showed no chlorpyrifos residues in organic tea sample (O1).

Analysis chlorpyrifos residues in tea without purification by SERS

Approximately five grams of tea samples (T1 and T2) were extracted in 5 ml methanol using ultrasonicator for 30 minutes. 1μl of tea sample was transferred onto silver nanorod substrates for SERS detection. The spectra were collected at excitation wavelength of 785 nm with laser power 40 mW. However, the fluorescence background is very strong and Figure 3.5 demonstrates no obvious characteristic peaks among various tea samples. The strong fluorescence effect consumes the incident energy, consequently, the scattering from chlorpyrifos is very weak. Several previous papers have reported that Raman spectra of fruits, leaves and

other plants, which also demonstrate the carotene vibration modes at 1521, 1155, 1003 cm^{-1} at Raman 514.5 nm excitation (*43, 54*).

Similarly, there were no significant spectral difference between tea and tea spike with the chlorpyrifos before purification process of tea sample (figure not shown). To purify the interested pesticide, soxhlet extraction and GPC clean up used in conventional GC pretreatment were then incorporated prior to tea detection by SERS. This strong fluorescence background can be reduced by using higher Raman excitation wavelength (1064 nm) or FT-Raman without cleanup required. However, FT-Raman instruments are generally too expensive limiting their applicability. In this study, sample pretreatment is emphasized rather than the use of high cost equipments.

After extraction and cleanup procedures of the tea samples, significant differences in spectral features were readily visible after purification. Moreover, oversize particles or other high concentration of food matrix chemicals may also responsible for no enhancement of SERS signal for tea. Therefore, it is necessary to incorporate purification technique in the sample preparation procedures in order to isolate target chemicals for detection and identification based on SERS. Although SERS can identify pesticide peaks for mentioned pretreatment, a rapid sample pretreatment is needed for future work to improve total analysis time.

Method validation of chlorpyrifos

The limit of detection (LOD), defined as the lowest concentration that the analytical process can reliably differentiate from background levels, was estimated by using principle component analysis (PCA) from the SERS spectra of samples spiked at the lowest analyte concentration tested. In this study, concentrations of chlorpyriofs at 0, 0.155, 0.3125, 0.625, 1.25, and 2.5ppm

were spiked into organic tea sample (O1), respectively. It is hard to visually observe the peaks of chlorpyrifos from the spectra of organic tea because of low spiked concentration of chlorpyrifos. Therefore, PCA was conducted objectively to differentiate the minute spectral difference from spectra of organic tea samples. The detection limit of identifying spiked chlorpyrifos from organic tea is based on the lowest concentration where PCA can identify difference between spiked chlorpyrifos from organic tea. Figure 3.6 shows PCA score plot for different concentrations of chlorpyrifos spiked into organic tea sample. The method reaches detection limit of 0.155 ppm of chlropyrifos residues in tea, which is far below the legal maximum residues limit in tea of 2 mg/kg (2 ppm). The detection limit of our method is sensitive enough and feasible for rapid screening chlorpyrifos residues in tea commodities.

Analysis chlorpyrifos residues in tea with purification by SERS

To test the potential of OAD SERS substrate for trace pesticide analysis, sample pretreatment must be applied in order to reduce fluorescence effect caused by tea pigments. Pesticides were extracted using a soxhlet extractor with ethyl acetate and 75 % ethyl acetate / 25 % toluene mobile phase was used in GPC cleanup. Next, solvents were evaporated from tea sample using nitrogen gas and made up with methanol to original volume (5 ml). Chlorpyrifos was not lost during evaporation procedure. The procedure to evaporate mixed solvents is to remove interference from mixed solvents, which cause complicated bands in SERS spectra. Measurement of SRES spectra for all tea samples (T1, T2, and O1) as previously described.

In order to confirm significant peaks of chlorpyrifos arise from functional groups of pesticide itself, and not from solvent, tea compounds, or substrate, organic tea was conducted as a reference spectrum to exam spectrum of background. The spectrum of organic tea (O1) and two

tea samples (T1 and T2) are alike because they have high similarity of constituents (both are green tea but process in different ways for unique flavor). From qualitative standpoint, comparing four spectra (T1, T2, O1, and chlorpyrifos), the peaks of chlorpyrifos: 1032, 1280, 1342, and 1575 cm^{-1} are shown in two tea samples (T1 and T2) (Figure 3.7), but not in organic tea sample (O1). Distinguishing similar spectra objectively by visual methods are hard to achieve and unrealistic. To explore the data and identify individual groups based on differences of the SERS spectra, PCA was employed to cluster samples into groups.

Analysis chlorpyrifos residues in tea by using principle component analysis (PCA)

Principle component analysis (PCA) mathematically reduces similarly correlated variables (multidimensionality of data matrix) into groups of principle components (PCs), in which each of the new variables is uncorrelated. The results of PCA are usually discussed in terms of PCs. The PC1 illustrates as much of the variability of the data, and each succeeding PC accounts for as much of the remaining variability as possible. An examination was made of the loading plots for the different components in order to explore this database and to identify clusters of similarities. PCs build a link between samples and variables by means of scores and loadings. For each PC, look for variables with high loadings (i.e. close to +1 or –1); this tells you the meaning of that particular PC (useful for further interpretation of the sample scores). This process can identify the patterns of the data in a way which best explains the variance in the data by highlighting their similarities and differences. Since patterns of data can be hard to find in such high dimension data matrix, PCA is a powerful tool for analyzing data.

Organic tea (O1), two tea (T1 and T2), each of their spiked chlorpyrifos (O1c, T1c, and T2c), and chlorpyrifos were investigated by PCA score plot. Figure 3.8 shows the PCA analysis

performed using spectral data in the range of 400-1800 cm^{-1}. The score plot for (PC1, PC2) gives information about patterns in the samples. Samples with close scores along the same PC indicate similarity of their characteristic. On the other hand, sample with opposite scores suggest they have different characteristic from the other one. The hypothesis is to classify tea sample into two categories: with chlorpyrifos residues or without chlorpyrifos residues. The PC contains the greatest variance labled PC1, which can explain 85% of data variance, and the second variance is labled PC2, which can explain 9% of data variance. The score plots for (PC1, PC2) are useful, since these two components summarize 94% variation in the data. From axis of PC1, the clusters are obvious to distinguish from the pesticide and all tea samples (O1, O1c, T1, T1c, T2, and T2c), where pesticide has negative PC1 and all tea samples have positive PC1. Likewise, PC2 can easily identify organic samples (positive PC2) apart from chlorpyrifos and the tea samples with pesticide (negative PC2). The negative value for PC2 of tea samples hints that tea samples prone to have positive-chlorpyrifos residues. moreover, from PC score plot hints that the locations of spiked chlorpyrifos (O1c, T1c, and T2c) are below non-spiked samples. The higher concentration of chlorpyrifos residues in tea, the lower position along PC2 (more negative PC2). This PCA score plot result also confirms GC results that chlorpyrifos residue is higher in T1 (0.29ppm) than T2 (0.20ppm).

Furthermore, by evaluating only tea samples and their spiked samples highlights their differences. Figure 3.9 shows the PCA analysis performed using spectral data in the range of 400-1800 cm^{-1}. The PC contains the greatest variance labled PC1, which can explain 54% of data variance, and the second variance is labled PC2, which can explain 28% of data variance. The score plots for (PC1, PC2) are useful, since these two components summarize 82% variation in the data. PCA score plot clearly distinguish three different types of tea into three clusters (O1, T1,

and T2). The locations of spiked chlorpyrifos have lower position than original tea samples. We can conclude that higher concentration of chlorpyrifos has lower (or negative) PC2. Therefore, to identify the variables (wavenumber) responding to location change of PC2, loading plot of PC2 were investigated. Figure 3.10 shows loading plot of PC2. The variables with high loadings yield to the significant bands of chlorpyrifos (691, 1032, 1125, 1250, 1280, 1343, and 1575 cm^{-1}). Loading plots demonstrate the important variables corresponsed to their difference.

Distinguish residues of chlorpyrifos from parathion in tea by SERS spectra and PCA

Since SERS can detect and differentiate chlorpyrifos residues in tea, it was important to determine whether SERS can distinguish residues of parathion from chlorpyrifos in tea. Parathion was banned for all agricultural use because of extremely hazardous. It is important to rapidly screen and identify if tea has residues of parathion or chlorpyrifos. Organic sample, organic sample spiked chlorpyrifos, and organic sample spiked parathion were analyzed to explore this possibility. Both spiked concentration are 11.05ppm. SERS analysis of these three samples and their respective spectra are displayed in Figure 3.11. While all these samples shared some similar bands because of the same tea components, the significant peaks of different pesticides in the spectra are somewhat different. The SERS spectra for different pesticides residues are unique from one to the other, allowing fingerprint potential for identification purpose. SERS can use as an alternative confirmation method to distinguish parathion residues from chlorpyrifos residues in tea.

To be more objective, PCA was carried out to differentiate pesticide residues. Figure 3.12 shows the PCA score plot using spectra data from organic tea sample, organic tea spiked parathion, and organic tea spiked chlorpyrifos in the 400-1800cm^{-1} range. Based on the PC

scores, the cluster for the organic tea has positive PC1 and PC2; the cluster for the organic tea spiked chlorpyrifos has negative PC1 and PC2, while the cluster for organic tea spiked parathion has negative PC1 and positive PC2. This score plot shows that three different clusters and clearly demonstrates that possibility of SERS to rapid screening and identify pesticides.

Conclusion

This study demonstrates that the combination of SERS pesticide fingerprint with PCA score plot and loading plot can be achieved objectively for the identification of pesticide residues. Developments in surface-enhanced Raman scattering (SERS) methods for chemical applications will aid in the improvement of rapid and precise analysis for a wide range of food items. SERS combined with PCA statistic method has satisfied this need, and it identifies pesticide residues on food objectively by identifying the minute different fingerprints of chemical molecules through their unique spectra. The results from PC score plots and loading plots clearly demonstrate the potential of SRES applied on examination pesticide residues on tea samples for food safety. Future work is focusing on establishing pesticide database and quantitative analysis for pesticide residues in tea according to calibration curve. From these results, definitely, one should be convinced that the method presented can be extended for identification of trace amount of pesticides lest on the food commodities with establishing pesticide spectra database.

Acknowledgements

I would also like to thank Dr. Hsiao-Yun Chu and Dr. Slade Jokela for assistance to fabricate some of the aligned nanorod substrates used in this study and Natalie Bond and Sid

Holems for helping me to prepare tea sample and to run conventional GC analysis, Jeremy Driskell for helping in PCA analysis.

Table 3.1. Survey of pesticide residues in heath tea and herbal tea (*55*)
● Common pesticide residues in herbal tea and health tea: Organochlorine: β-BHC, δ-BHC, op' -DDT, pp'-DDT and pp' -DDE Organophosphorus: DDVP, malathion, MEP, EPN and chlorpyriphos Carbamate: MIPC Organonitrogen: diflubenzuron and pyridaben
● Pesticide residues with high level residues in flower and roast tea: Organochlorine: seldom Organophosphorus: chlorpyriphos, MEP and EPN Organonitrogen: diflubenzuron
● Highly residual pesticides detected in high frequency in all of health tea and herbal tea cultivated in China: op' –DDT, pp'-DDE
● Residues exceed regulation levels based on the Food Sanitation Law: DDT, MEP, EPN and chlorpyriphos

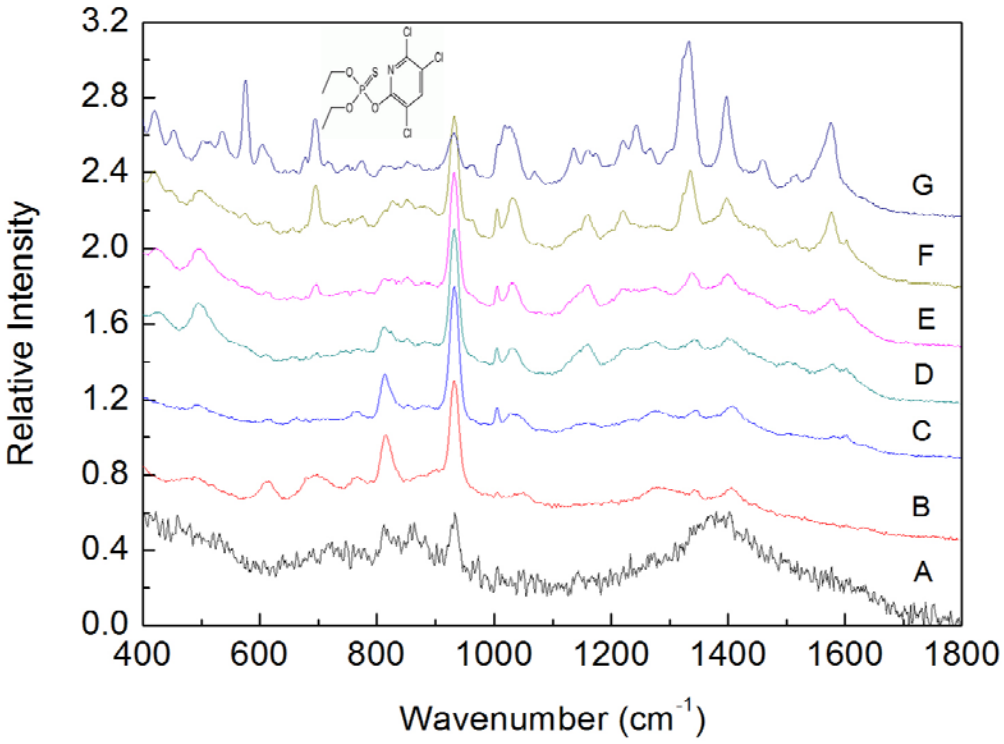

Figure 3.1. normalized SERS spectra of (A) blank substrate (B) methanol (MeOH), chlorpyrifos at concentration (C) 0.05ppm (0.05µg/ml), (D) 0.1ppm (0.1µg/ml), (E) 0.4ppm (0.4µg/ml), (F) 2ppm (2µg/ml), and (G) 10ppm (10µg/ml) in MeOH on silver nanorod array substrates. 785 nm narrow linewidth diode laser for Raman excitation with laser powers of 40mW and collection time of 20 s were used to obtain these spectra. The spectra were recorded over a spectral range from 400 to 1800 cm^{-1}.

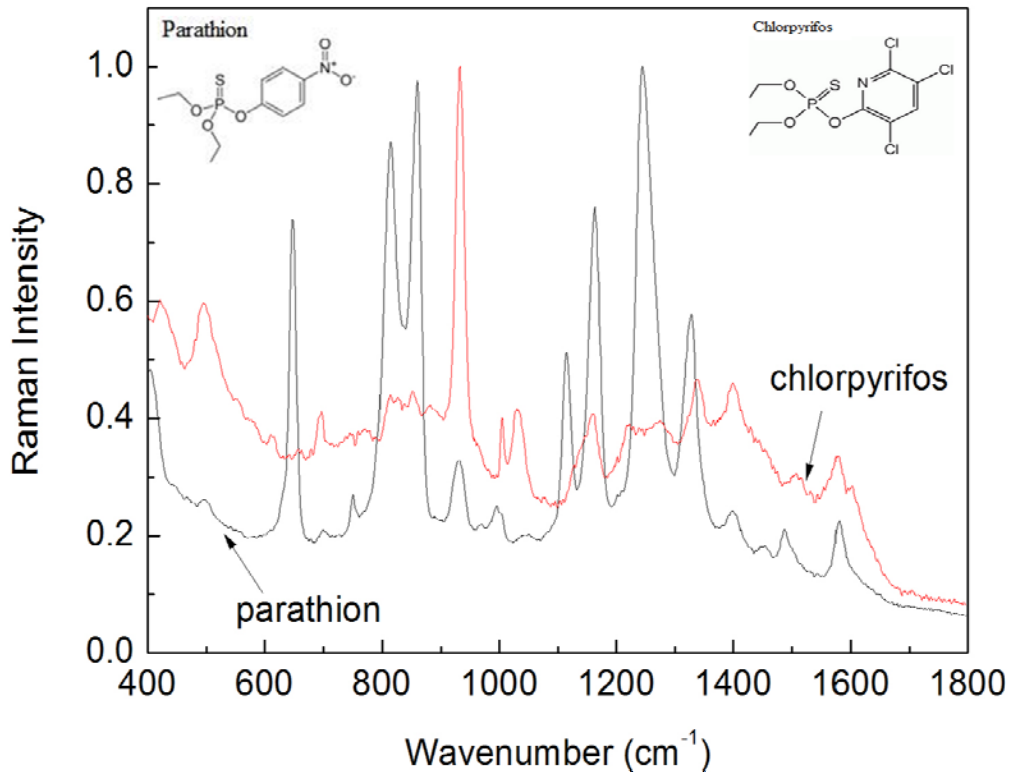

Figure 3.2. Normalized SERS spectra of chlorpyrifos at concentration 5ppm (top) and normalized SERS spectra of parathion at concentration 5ppm (bottom). 785 nm narrow linewidth diode laser for Raman excitation with laser powers of 40mW and collection time of 20 s were used to obtain these spectra. The spectra were recorded over a spectral range from 400 to 1800 cm^{-1}.

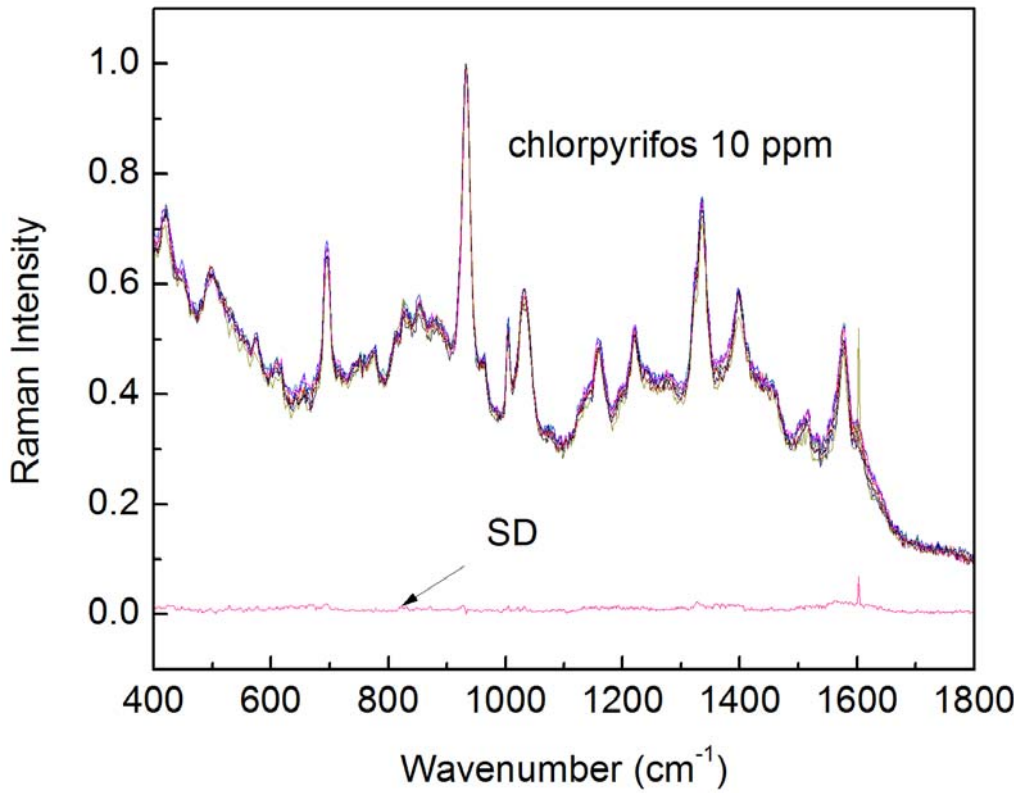

Figure 3.3. The normalized SERS spectra (n=8) of chlorpyrifos 10 ppm. The spectra are overlapped to illustrate the reproducibility of SERS spectra from different spots of the same substrate. The results show good reproducibility of SERS spectra. 785 nm narrow linewidth diode laser for Raman excitation with laser powers of 40mW and collection time of 20 s were used to obtain these spectra. The spectra were recorded over a spectral range from 400 to 1800 cm^{-1}.

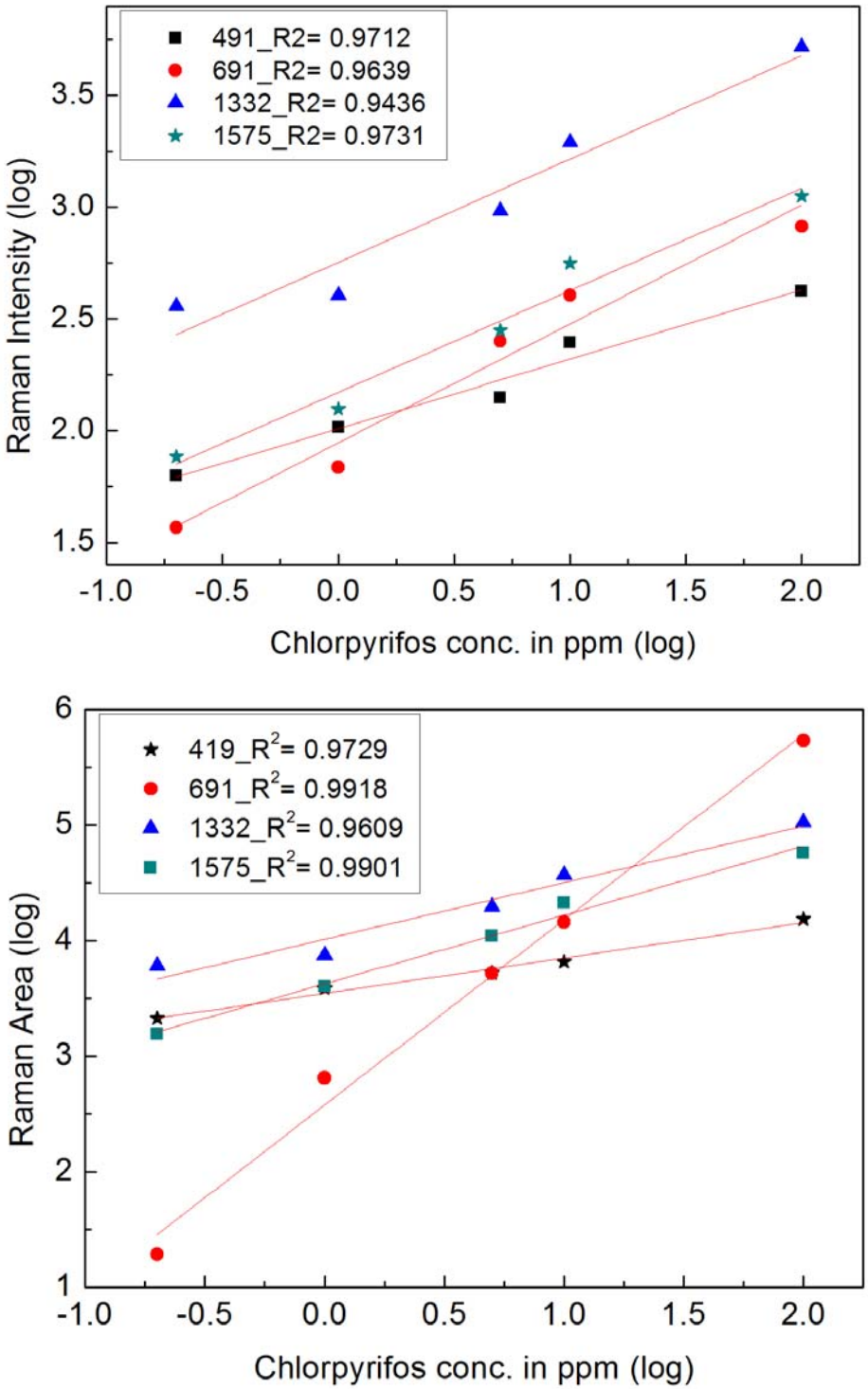

Figure 3.4. Concentration dependant of chlorpyrifos at 419, 619, 1332, and 1575 cm^{-1} between concentration range 0.2 ppm to 100 ppm based on (a) integrate area and (b) band intensity. (a) The unit of X-axis is log base of concentration of chlorpyrifos in ppm, and the unit of Y-axis is

log base of integrate area. (b) The unit of X-axis is log base of concentration of chlorpyrifos in ppm, and the unit of Y-axis is log base of band intensity.

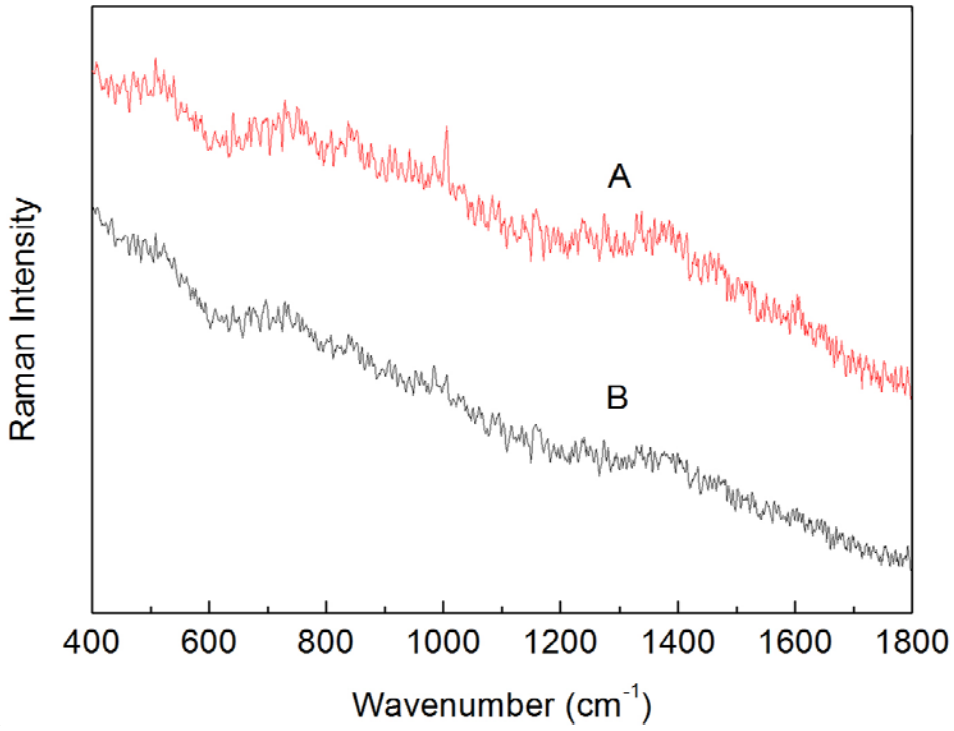

Figure 3.5. SERS spectra of two green tea samples without pretreament. A: T1 and B:T2. 785 nm narrow linewidth diode laser for Raman excitation with laser powers of 40mW and collection time of 20 s were used to obtain these spectra. The spectra were recorded over a spectral range from 400 to 1800 cm^{-1}.

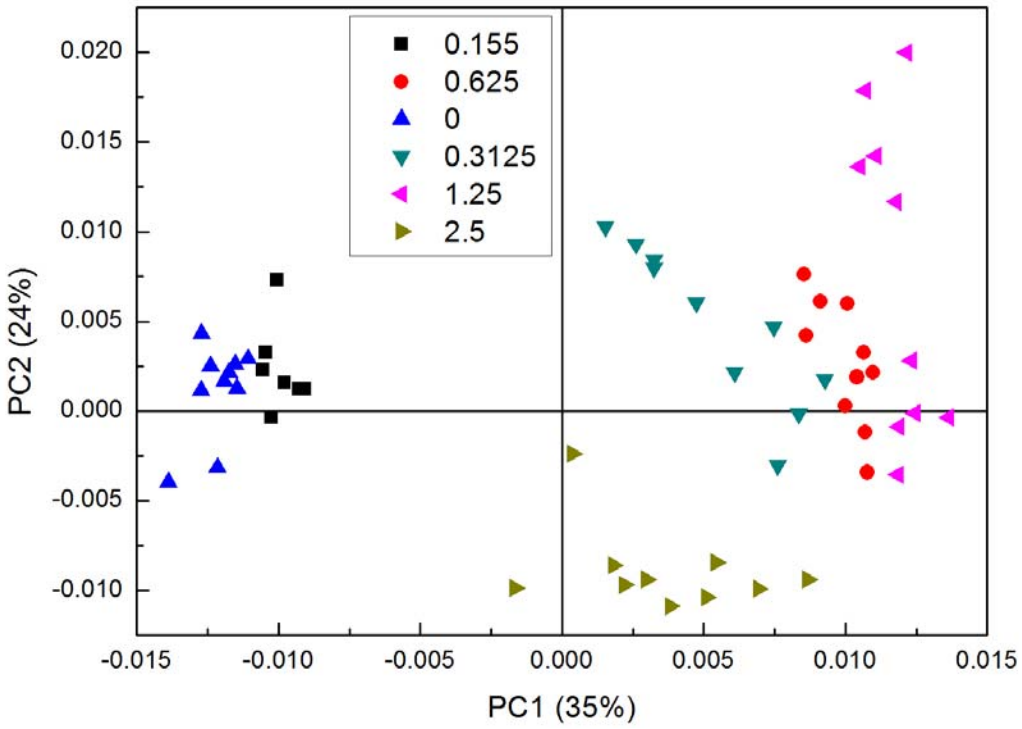

Figure 3.6. Detection limit of chlorpyrifos residues from organic tea by PCA. Six residues concentrations were established: 0, 0.155, 0.3125, 0.625, 1.25, and 2.5ppm. The spectra was within range 400 cm^{-1} to 1800 cm^{-1} for PCA analysis. Incident laser powers of 40mW and collection time of 20s were used to obtain these spectra.

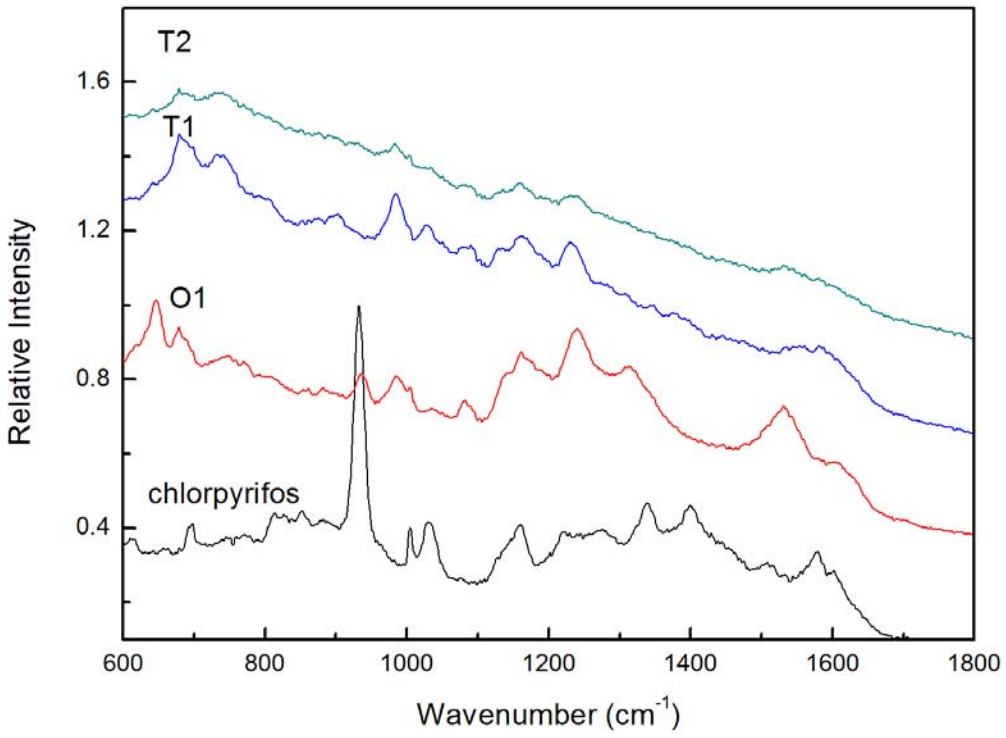

Figure 3.7. Relative SERS spectra of (A) chlorpyrifos 5ppm, (B) O1 (as control sample), (C) T1, and (D) T2. 785 nm narrow linewidth diode laser for Raman excitation with laser powers of 40mW and collection time of 20 s were used to obtain these spectra. The spectra were recorded over a spectral range from 400 to 1800 cm^{-1}.

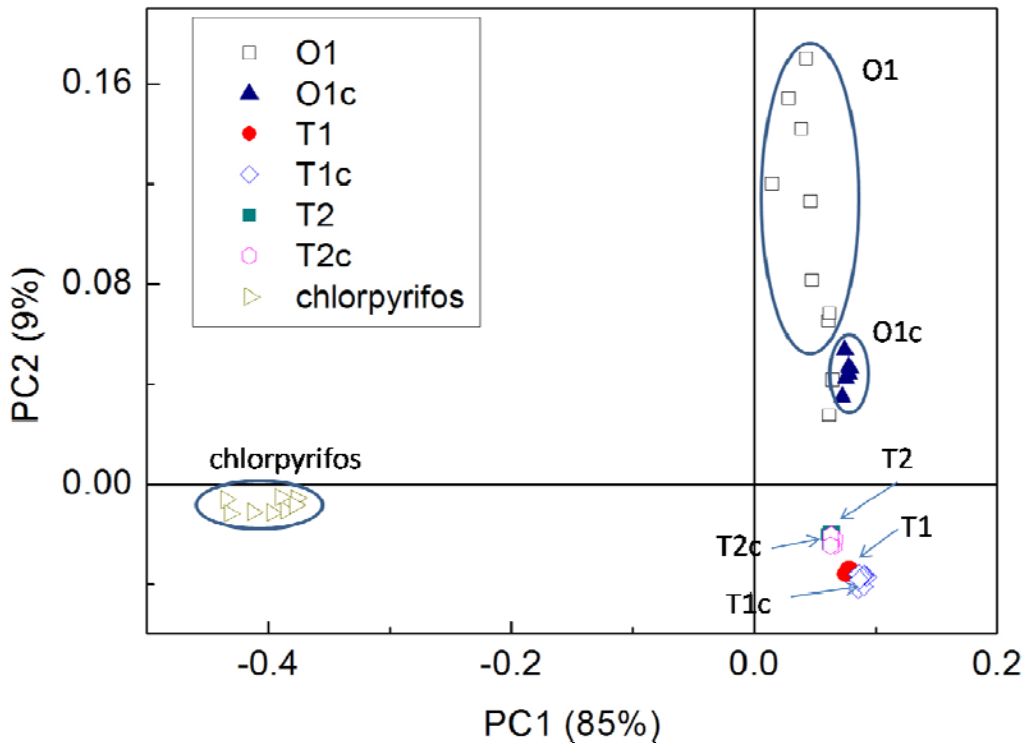

Figure 3.8. PCA classification of two green tea samples (T1 and T2), two tea spike samples (T1c and T2c), an organic tea sample (O1), an organic spiked sample (O1c), and chlorpyrifos. PC1 (85%) and PC2 (9%) account 94% of variability in the data. The spectra was within range 400 cm^{-1} to 1800 cm^{-1} for PCA analysis. Incident laser powers of 40mW and collection time of 20s were used to obtain these spectra.

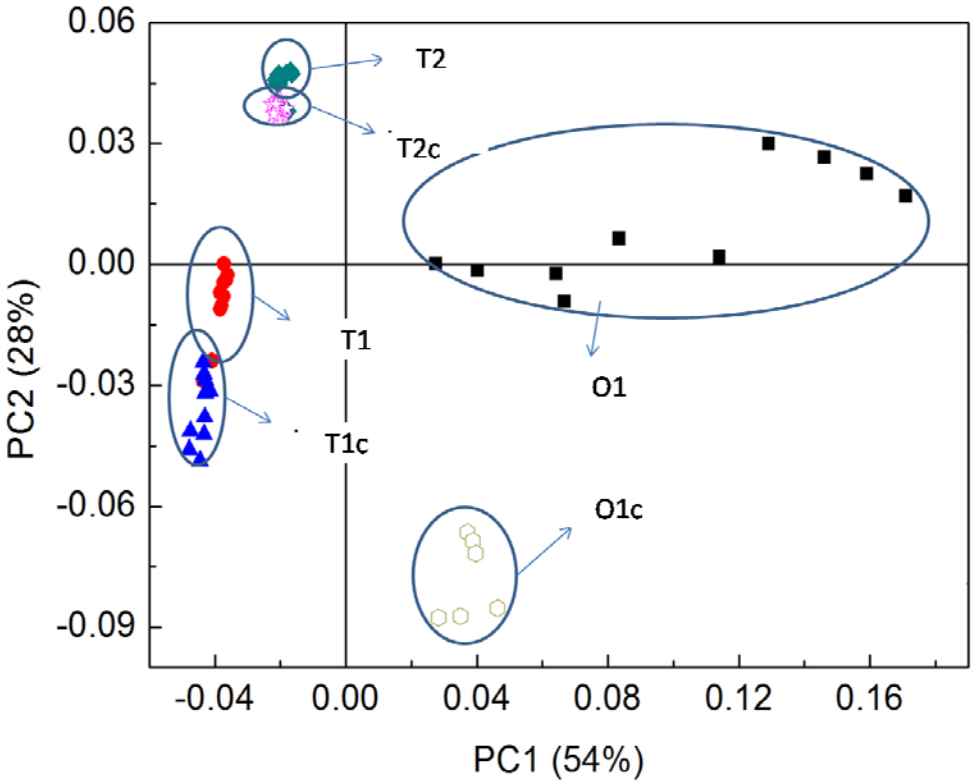

Figure 3.9. PCA classification of two tea samples (T1 and T2), two spiked tea samples (T1c and T2c), an organic tea sample (O1), and an organic spiked sample (O1c). PC1 (54%) and PC2 (28%) account 82% of variability in the data. The spectra was within range 400 cm^{-1} to 1800 cm^{-1} for PCA analysis. Incident laser powers of 40mW and collection time of 20s were used to obtain these spectra.

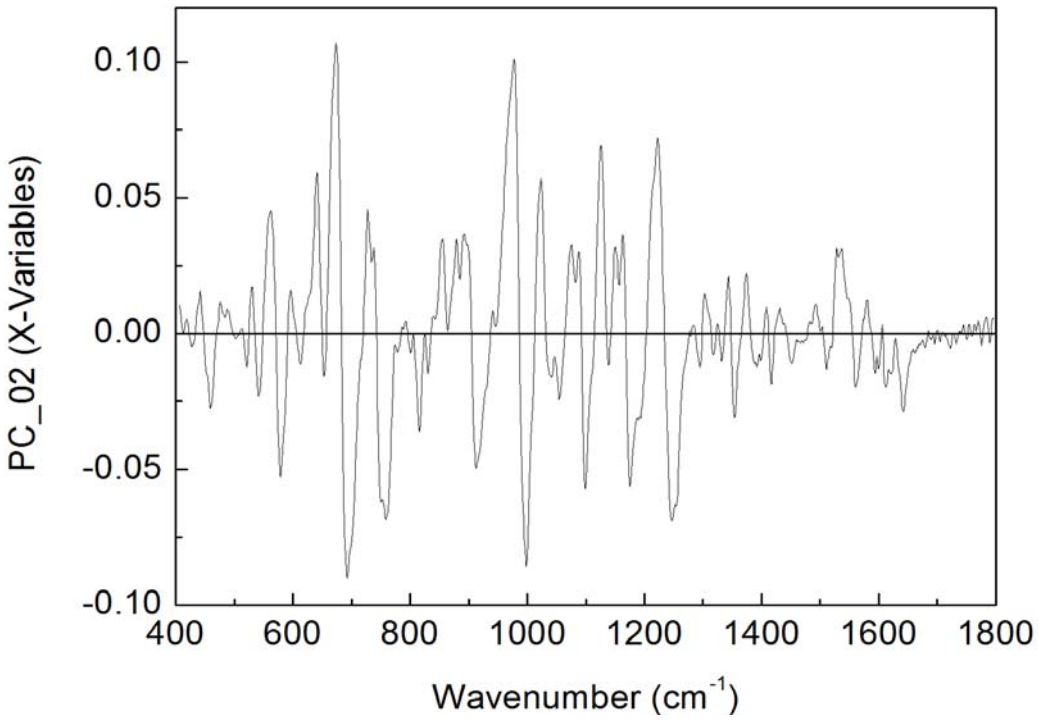

Figure 3.10. PCA loading plot classification of two green tea samples, two tea spiked samples, and one organic tea sample from figure 3.9. The spectra was within range 400 cm^{-1} to 1800 cm^{-1} for PCA analysis. Incident laser powers of 40mW and collection time of 20s were used to obtain these spectra.

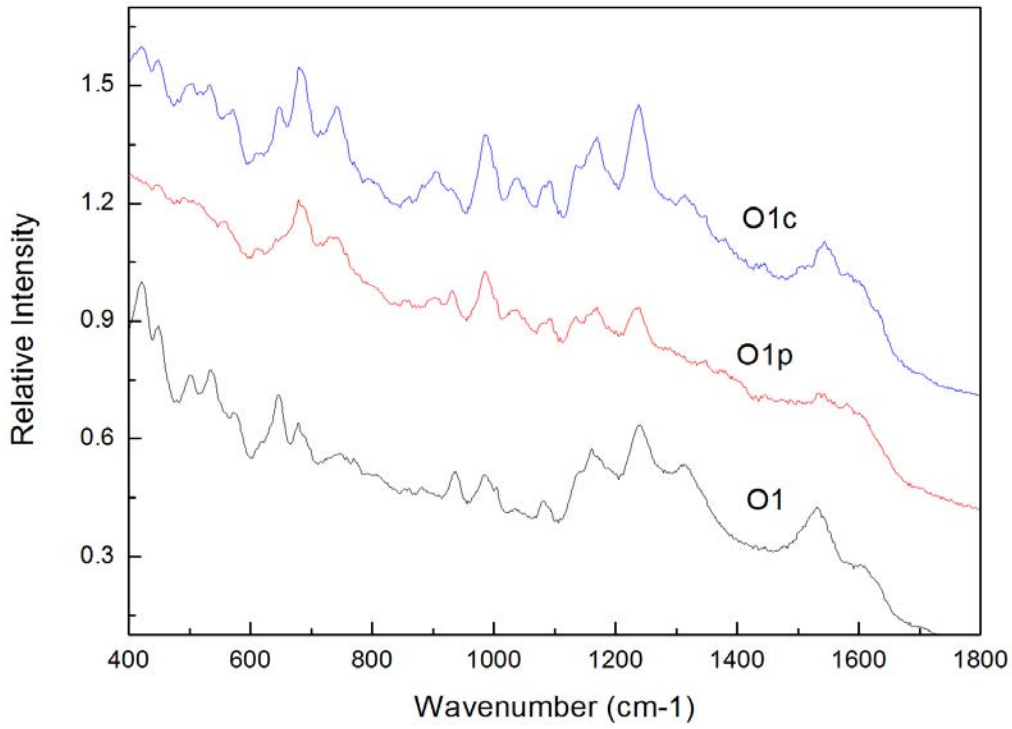

Figure 3.11. Relative SERS spectra from bottom to top are organic tea sample (O1), spiked parathion tea sample (O1p), and spiked chlorpyrifos tea sample (O1c). 785 nm narrow linewidth diode laser for Raman excitation with laser powers of 40mW and collection time of 20 s were used to obtain these spectra. The spectra were recorded over a spectral range from 400 to 1800 cm^{-1}.

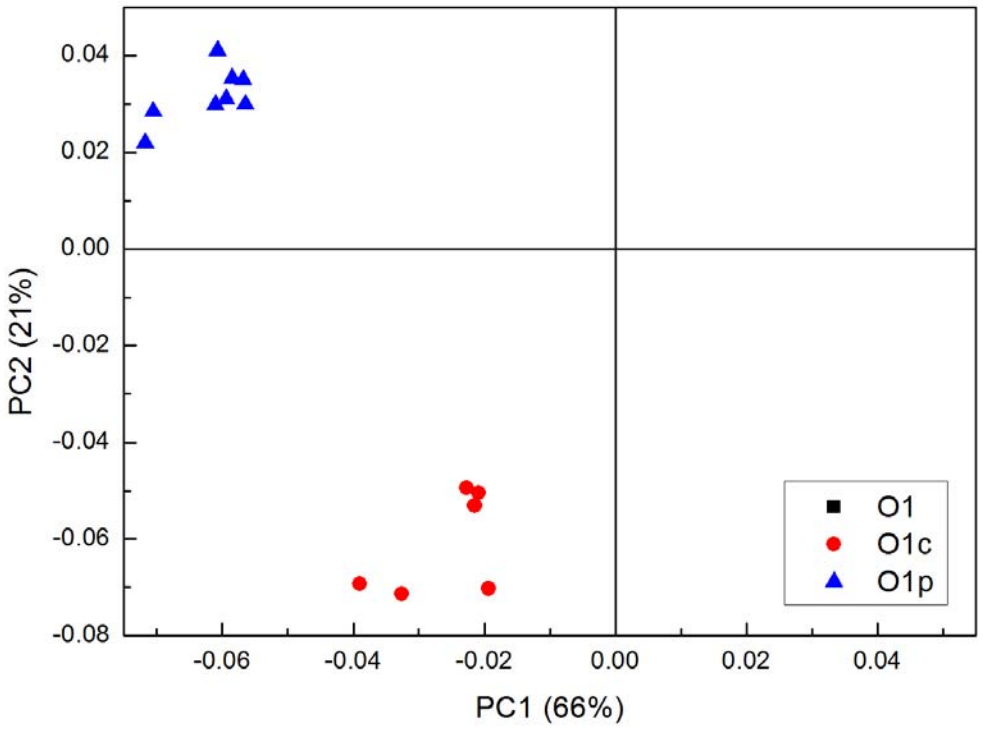

Figure 3.12. PCA classification of organic tea sample (O1), spiked chlorpyrifos tea sample (O1c), spiked parathion tea sample (O1p). PC1 (66%) and PC2 (21%) account 87% of variability in the data. The spectra was within range 400 cm^{-1} to 1800 cm^{-1} for PCA analysis. Incident laser powers of 40mW and collection time of 20s were used to obtain these spectra.

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CHAPTER 4

SUMMARY AND OUTLOOK

Summary

In this work, a new effective method for detection and differentiation residues of chlropyrifos from parathion in tea based on the application of the SERS technique has been developed. Firstly, standard pesticide spectra were established for further spectra comparison. The detection limits of instrument and sample were explored. Additionally, concentration dependent of chlorpyrifos were calculated based on the selected bands. PCA score plot and loading plot were objectively used for spectra data analysis and data characteristics interpretation.

We successfully demonstrated that SERS has advantages for detection of chlorpyrifos residues in tea, where GC has limitation. Firstly, SERS demonstrated clearly unique spectra for chlorpyrifos and parathion. Furthermore, SERS identified chlorpyrifos and parathion residues in tea by PCA score plot and PCA loading plot. Although SERS spectra of these two pesticide residues have minute difference, PCA clearly clustered into two groups: chlorpyrifos residues in tea and parathion residues in tea. Therefore, SERS as an alternative confirmation method can differentiate chlorpyrifos from parathion. Secondly, the detection time for GC (5 minutes-30minutes) is longer than SERS (20 seconds) and different types of GC detectors are required for different types of pesticide detection. Unlike GC, SERS has advantages for pesticide detection in molecule with lone pair electrons or pi clouds, as most pesticides possess these chemical structures. Researchers have reported that aromatic nitrogen, oxygen containing compounds

(aromatic amines or phenols), and electron-rich group (carboxylic acids) are strongly SERS active (*5*).

Outlook

The experimental research carried out in the present work shows that the SERS technique with the OAD fabrication method is quite adequate to detect chlorpyrifos residues in tea samples with application of PCA chemometrics analysis. However, the promising results obtained in the present work would be improved and significantly extended in the future by means of the further works in following directions: (1) to increase the resolution of the SERS spectra of the tea samples, (2) to establish working solution for quantitative analysis, and (3) to establish library of SERS spectra for pesticides.

First, in order to increase the resolution of the SERS spectra, the use of high quality optical and spectroscopic equipments and instruments are worth trying. Furthermore, application of sophisticated software for spectra analysis to increase S/N can improve detection limit of SERS methodology. This is a perspective direction for the increase of the sensitivities of the measurements.

In addition, the quantitative determination of the concentration of chlorpyrifos residues in commercial tea samples can be conducted with the application of PLS (partial least square). To further quantitative concentration of chlorpyrifos residues in tea, working solution need to be established. For example, a series concentration of chlorpyrifos is spiked to control samples in order to establish standard regression line. The bands mentioned in previous work can be used for quantitative analysis combined with partial least square (PLS) to calculate concentration of

pesticide residues in sample based on standard regression line. We can calculate unknown concentration of commercial tea sample by interpolation with the application of PLS.

Finally, detection and identification of pesticides can be done by comparing a standard SERS spectrum of a target against a SERS standard library. Therefore, standard spectra of pesticides are essential for a SERS-based detection method. This SERS spectra library should at least contain frequent found pesticide residues in foods, a background of variety foods, and interfering materials. The measurement conditions such as Raman equipment and SERS substrate need to be standardized. Different Raman equipment and different substrate fabrication have different spectra; therefore, same Raman equipment and substrate condition should be applied for detection.