DESIGN AND FABRICATION OF APTAMER-BASED SERS CHIPS FOR SELECTIVE DETECTION OF PARAQUAT HERBICIDE RESIDUES IN WATER AND SOIL

by

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AUTHOR'S DECLARATION

I, Napatsakorn Kamkrua, declare that the research work carried out for this thesis was in accordance with the regulations of the Asian Institute of Technology. The work presented in it are my own and has been generated by me as the result of my own original research, and if external sources were used, such sources have been cited. It is original and has not been submitted to any other institution to obtain another degree or qualification. This is a true copy of the thesis, including final revisions.

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ABSTRACT

A common herbicide such as paraquat has been countlessly applied in agriculture, and its residues are left in water and soil. Paraquat toxicity highly damages the body, which can be lethal in a later stage. Thus, paraquat detection is important and several techniques have been developed in recent years. Raman spectroscopy facilitates herbicide detection in rapid and non-destructive ways. Furthermore, surface-enhanced Raman spectroscopy (SERS) and bioreceptors are commonly utilized to enhance the sensitivity and selectivity of molecular structure detection, respectively. This study focuses on optimizing SERS-active substrates to improve the selectivity and rapidity of paraquat detection. This work also aimed to develop the combinations of SERS and biosensors for paraquat detection by functionalizing metallic nanostructures with an aptamer that is specific to the paraquat molecule via Raman spectroscopy. Soil and water samples were used for the evaluation of optimized SERS-active substrates. The sensitivity, selectivity, and limit of detection were investigated by Raman measurement. This study optimized the ONSPEC-Prime SERS chip (AgNRs) for paraquat detection in water and soil samples. Without extraction protocol, the chip provided a sensitivity of 638.96 a.u./μM and a detection limit (LOD) of 0.1 μM for the water sample. However, in the soil samples, paraquat extraction protocol with alumina absorbent is required. Furthermore, the aptamer-based SERS chip was successfully fabricated with a sensitivity of 609.15 a.u./μM and LOD of 0.35 μM using 0.1 μM of aptamer immobilized on the ONSPEC-Lite SERS chip (AuNPs).

Keywords: Paraquat, SERS, Aptamer, Biosensor, Raman spectroscopy

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LIST OF ABBREVIATIONS

AgNPs = Silver Nanoparticles
AuNPs = Gold Nanoparticles
AChE = Acetylcholinesterase
ACN = Acetonitrile
C18 = Octadecylsilane
EIS = Electrochemical Impedance Spectroscopy
EM = Electromagnetic
EtOH = Ethanol
GC = Gas Chromatography
GCB = Graphitic Carbon Black
HPLC = High Performance Liquid Chromatography
LC = Liquid Chromatography
LOD = Limit of Detection
LSPR = Localized Surface Plasmon Resonance
MeOH = Methanol
PSA = Primary Secondary Amine
RMSE = Root Mean Squared Error
RSD = Relative Standard Deviation
SERS = Surface-enhanced Raman Spectroscopy/Scattering
SPR = Surface Plasmon Resonance
TCEP = Tris(2-carboxyethyl) phosphine
TE = Tris-EDTA

CHAPTER 1
INTRODUCTION

1.1 Background of the Study

Pesticides are one of the crucial tools in agriculture. The term pesticides explain various chemicals against specific pests such as herbicides, insecticides, fungicides, and rodenticides. They are chemical substances that are used to reduce the density of pests, weeds, and unwanted plants and improve crop yield. Herbicide is defined as a class of pesticides that aim to control the population of weeds that can interfere growth of crops. The ideal herbicides are designed to be less lethal to living organisms (Aktar et al., 2009; Gill & Garg, 2014). They should not harmful to non-targets. In fact, herbicide contamination causes a broad effect on the environment, animals, and humans, including serious health issues, losing habitat, changes in behavior, and extinction risk.

Weeds are undesirable plants that compete against cultivated plants for essential nutrients, sunlight, and water. In favorable environments, weeds can disperse their seeds and grow in many different areas. Uncontrolled weed population eventually causes weed infestation. The effects of weed infestation affect soil fertility, leading to insufficient soil for crop planting as well as the quality and quantity of crops (Kwiatkowski et al., 2020).

The utilization of herbicides to eliminate weeds in cultivated land is a cost-effective method. It is cheaper, easier, and faster when compared with other weed management such as crop rotation, soil tillage, and row spacing (Vencill et al., 2012). Crop rotation is limited to the cost of the machines and seeds. Soil tillage is time-consuming as well as involves more labor. Row spacing needs to be aware of disease pathogens from narrow row spacing (Nielsen, 1997).

The studies reported global pesticide usage has been doubling per decade, especially in developing countries like Thailand and Vietnam (Deguine, 2021; Sharma et al., 2019; Young et al., 1996). Thailand, the main exporter of food products, had a dramatic change in the agriculture industry according to the introduction of heavy machines and chemicals in 1950 (Laohaudomchok et al., 2021; Sharma et al., 2019). Common herbicides like glyphosate and paraquat have been extremely utilized to meet the global food demand. Anyway, paraquat was restricted and banned in several countries because

paraquat is a toxic substance that can cause fatal damage to the human body. Paraquat quickly interacts, readily breaks down, and has a wide range of applications. As a result, paraquat is widespread in developing countries. However, the injudicious use of paraquat can be harmful to farmers and consumers. Paraquat can be exposed to humans by physical contacting, inhaling, and swallowing. The products from chemical reactions between paraquat and organisms can cause severe effects on the nervous, reproductive, cardiovascular, respiratory, and urinary systems. Eventually, multiple organ system failures cause death in the later stage (Ashton & Leahy, 1989).

Therefore, paraquat become a common improper chemical for suicide in Southeast Asia, especially in Korea (Cha et al., 2016; Seok et al., 2009). In Thailand, paraquat was completely banned in 2020 (Kenichi, 2020; Ponnarong Prasertsri, 2020). The reports stated that the maximum residue limits (MRL) of paraquat on all commodities will be zero as well as the chemical residue limits of paraquat in imported foods.

Even though paraquat was already banned, some areas illegally use paraquat to control the weed (Bang et al., 2017). The indiscriminate use of paraquat finally causes the contamination of the environment. To monitor paraquat residues in environments, researchers/scientists have been developing many protocols to detect paraquat residues in food and the environment over several decades (El Harmoudi et al., 2013; Mallat et al., 2001; Van Emon et al., 1987; Zhang et al., 2021). One of the effective methods is surface-enhanced Raman spectroscopy (SERS). SERS takes advantage of nanostructures or rough surfaces of the metallic substrate to identify trace detection of the molecule. Therefore, optimizing SERS-active substrates to improve the selectivity and sensitivity of paraquat detection is necessary to reduce the risk of paraquat exposure and contamination.

1.2 Statement of the Problem

In the last two decades, significant attempts have been made to detect paraquat residues in the environment and food. Chromatography and Raman spectroscopy are two main approaches for paraquat detection. Chromatography was introduced for trace analysis of herbicides because it has excellent sensitivity and selectivity (Stachniuk & Fornal, 2016; Sun & Chen, 2015; Zou et al., 2015). However, chromatography is a laboratory-based approach that is complex, costly, and requires technicians to handle instruments.

On the other hand, Raman spectroscopy is simple, quick, non-contaminant, and non-destructive. They offer little or no sample preparation, high sensitivity, high reliability, and are relatively inexpensive compared to chromatography. Especially, in practical use, rapid and portable detection is crucial for the field sample test. However, due to the limitations of the weak signal from Raman scattering, Raman spectroscopy is not preferred in analyzing molecules at low concentrations (Sur, 2017).

SERS technique can amplify the low signal from Raman scattering and improve the sensitivity of the sensor. The materials and morphologies of SERS substrates are significant factors to enhance the Raman signal. Ag and Au nanostructures are two popular materials for creating the LSPR effect (Chen et al., 2018; Fang et al., 2015; He et al., 2014). NECTEC has been developing SERS chips including Onspec-Prime and Onspec-Lite for rapid and portable applications. Onspec-Prime is SERS chips based on Ag nanorods and Onspec-Lite is based on Au nanoparticles. The SERS chips are multiplexed sensors that can be implemented in various fields of detection which is also a drawback in a specific application. But they lack selectivity in terms of detection.

In this work, an aptamer designed for paraquat detection is conjugated on the substrate to improve the selectivity of the SERS chips. Moreover, the SERS chips are also optimized to enhance the sensitivity and rapidity of the detection. The aptamer-modified SERS chips are tested with paraquat in water and soil samples for real application.

1.3 Objectives of the Study

The overall objective of this research is to improve the detection of paraquat using aptamer-modified SERS substrates which can efficiently detect trace amounts of paraquat residues in both water and soil samples. The specific objectives of the research are given below:

1. To optimize SERS substrates by changing material of nanoparticle, solvent, and the dimension of the nanoparticles.
2. To evaluate paraquat detection efficiency in field samples using the optimized SERS substrates, where the field samples are tap water, drinking water, water from waterlogging, and agricultural water.
3. To evaluate herbicide residues detection efficiency in soil samples using the optimized SERS substrates.

4. To develop a protocol on “the modification of metallic nanostructures with aptamer to increase the selectivity of paraquat detection.”

1.4 Scope of the study

The aim of this research work is to optimize SERS substrates that work using the surface plasmon resonance (SPR) principles of metallic nanostructures for the detection of herbicide residues. The research will explore optimal conditions of a variety of substrates and then evaluation of herbicide detection, where we will target commonly used herbicides in Thailand, viz. paraquat. The research will further explore the application of the plasmonic biosensor in the area of herbicide detection. The proposed study is limited to the development of a plasmonic biosensor prototype that will be evaluated using laboratory experiments, and no on-site field measurements will be conducted during this study.

1.5 Organization of the Study

The study is divided into 5 chapters as the following:

Chapter 1: The introduction describes the background, statement of the problem, objectives, and scopes of the study as well as the organization of the study.

Chapter 2: This chapter is the literature review of the implications of herbicide contamination on living organisms and the environment. In addition, current herbicide detections are also reviewed as well as the SERS technique.

Chapter 3: The chapter explains the method for optimization and modification of the SERS substrate for paraquat detection. Besides, the procedures of creating an aptamer-modified substrate for paraquat sensing are also depicted as well as the investigation of the performance of an aptamer-modified substrate.

Chapter 4: SERS results from the experiments are described in this chapter. The analysis provides an optimum condition for the studies. The sensitivity, selectivity, and detection limit (LOD) of the aptamer-modified substrate are determined and compared with the no-aptamer substrate.

Chapter 5: The summary and recommendation of the thesis are explained here.

CHAPTER 2
LITERATURE REVIEW

For industrial agriculture, high quality and low price of the yield are the heart of the business. In order to maintain them, pests and weeds must be controlled or eliminated. Herbicide application against weeds is a conventional way owing to effective, low cost, and quick action. Anyway, the injudicious use of herbicides caused the contamination of herbicide residues in the environment that are harmful to wildlife and human being.

While herbicides are transported by nature or human activity, herbicides can be degraded by hydrolysis, methylation, and ring cleavage interaction that produce toxic compounds and contaminate the environment (Aktar et al., 2009; Gill & Garg, 2014). Herbicide contamination can occur in several ways. The soil absorbed the remaining herbicides on treated plants; therefore, soil microorganisms can be destroyed, and soil might be insufficient for planting. The leaching of herbicides conducted water pollution. The runoff herbicides are damaging to coastal and other aquatic life. In addition, some herbicides are designed to release into the air to apply to the crop, which can be polluted air.

2.1 Herbicide Impacts on Ecosystems

Herbicides provide significant advantages to humans and other living organisms, which control weeds. The development of herbicides has been increased according to the increasing demand for herbicide applications. Anyway, carelessness of herbicide regulations and overuse of herbicides cause detrimental effects far outweigh benefits, including the evolution of herbicide resistance, clinical health effects, water contamination, and so on. One of the popular herbicides used for the agriculture industry is paraquat, a detecting analyte in this research. Therefore, the following implications are the impacts from paraquat exposure as well.

2.1.1 Target Organisms

2.1.1.1 **Herbicide Resistance.** Herbicide resistance is a severe problem in agriculture, and it depends on the changes in genes, ecology, and herbicide practices. Indiscriminate herbicide usage causes weeds to tend to adapt themselves against the presence of herbicides. Eventually, herbicides are no longer adequate for weeds even herbicide concentration is in the range of recommendation (Sharma et al., 2019).

Moreover, the repeated usage with a high concentration of herbicides conducted the adaptation, causing loss the biodiversity, disturbing local organisms, raising herbicide resistance, and eventually weed resurgence.

2.1.1.2 **Weed Resurgence.** Weed resurgence is also a negative effect of the arbitrary application. Farmers use herbicides for weed control to preserve their profits and reduce the cost. However, applied herbicides might kill non-target, including beneficial plants, causing weed resurgence. Fred and Harold reported increasing row spacing of soybean farming after herbicide applications cause rapid weed resurgence (Fred & Harold, 1991).

2.1.2 Non-Target Organisms

2.1.2.1 **Human.** In addition to herbicides that can harm specific weeds, non-target organisms such as humans are also affected. Farmers and workers are most at risk of herbicide exposure. The excessive herbicide residues cause contamination of treated plants and livestock which can enter the human body through wounds, inhalation, and swallowing. Because of herbicide drift, nearby neighborhoods can be exposed as well. After humans are exposed to the herbicide, the symptoms depend on time exposure and the amount that results in acute and chronic illness.

2.1.2.1.1 Acute Illnesses. The symptoms after being exposed to herbicides in a short time, we called acute illnesses. The patients usually have symptoms such as vomiting, diarrhea, headaches, dizziness, rashes, and nausea that depend on time exposure, mode of application, frequency of use, quantity, and toxicity of the herbicide. However, in the early stage of paraquat exposure, it is usually asymptomatic with less than 10 mL of ingestion (Gil et al., 2014). Higher concentrations result in sore tongue, agitation, shortness of breath, and confusion, eventually associated with acute kidney failure, respiratory failure, or liver failure.

2.1.2.1.2 Chronic Illnesses. For chronic illness, the symptoms are not apparent until a later stage. The frequent exposure of herbicides with significant quantities triggers wild symptoms in the long term that results in adverse effects on the nervous, reproductive, cardiovascular, respiratory, and urinary systems. The study revealed that farmers or workers who work with herbicides, including loaders, mixers, and sprayers, are at the highest risk of exposure, resulting in getting sick later (Bradish, 2012). However, the population is also at risk because of consumption of contaminated diet from herbicides drifting. Finally, chronic toxicity is revealed by chronic exposure to herbicide drifting. Herbicides could damage DNA through DNA methylation and

histone modifications, resulting in chronic diseases like cancer. Herbicides also impact the balancing of reactive oxygen species, causing Parkinson's disease and unbalance of glucose homeostasis (Mostafalou, 2012). For paraquat, it is most severe in primary organs. Especially, a person who is being affected by paraquat intoxication for a long time has likely lung injury and kidney failure.

2.1.2.2 **Wildlife.** In addition to weeds being killed by herbicide application, they are also regulated by their natural enemies including earthworms, and predators. Due to most of the herbicides being designed as non-specific targets and herbicides spread by either humans and nature, thereby eliminating weeds and their natural enemies. Typically, natural enemies take a leading role in controlling the weeds population. Therefore, the decreasing of natural enemies eventually causes weed resurgence and soil structure changes.

These non-target organisms have organized soil structures. For instance, earthworms improve soil fertility by decomposition organic matter, including manure, leaves, etc. They also maintain the soil structure by creating holes for ventilation and draining. The studies revealed herbicides had been detrimental impacts on the growth, reproduction, and physiology of earthworms (Gill & Garg, 2014; Reinecke & Reinecke, 2007). Moreover, predators, such as spiders, wasps, coccinellids, are defined as beneficial organisms for biological control. The study further reported their numbers and species are risky to decline and then extinct in applied or nearby areas while other non-applied sites are conversely.

Besides, pollinators are essential in pollination. Some pollination relies on the carrier such as insects like bees or wind to pollinate the stigma with the stamen from another plant. This mechanism is helpful and valuable in the ecosystem and crops. The adequate population of pollinators affords high yields in agricultural land. Nonetheless, it will be opposed and become ineffective pollination when exposed to herbicides that are toxic to the pollen and nectar. Significantly, herbicides inhibit the foraging and colonization of these pollinators that change their behavior (Henry et al., 2012)(Cullen et al., 2019). For instance, the collapse of honeycomb due to worker bees is influenced by herbicides. It leads to a lack of reproductive ability that turns them into other roles in the colony. The colony has eventually collapsed due to the absence of the pollen collected (Gill et al., 2012).

2.1.3 Pollution

2.1.3.1 Soil Contamination. The injudicious use of herbicides with repeated causes soil accumulation due to the soil organisms being degraded, transported, absorbed, and desorbed by applied herbicides. It results in the alteration of soil diversity, biomass, biochemical reaction, and enzymatic activity. Soil fertility would eventually lose. In addition, beneficial bacteria for soil, fungi, and algae are also affected, causing changes in their growth, colonization, and metabolisms. Furthermore, the adverse effects of changing biochemical reactions such as nitrogen fixation, ammonification, and nitrification cause soil to be insufficient for planting.

2.1.3.2 Water Pollution. After herbicide application, liquid herbicide drifting through runoff into water resources is highly likely to occur, causing contaminated water could threaten aquatic organisms, including fish, dolphins, and so on (Singh & Mandal, 2013). Human is also being threatened by contaminated irrigation. In addition to surface runoff from treated plants, herbicides could reach into water resources in several ways as follows: accidental leaking, washing the related herbicide application equipment, industrial waste, and even aerial spray on crops. The report indicated that aquatic organisms are at risk of to decline in the population, particularly fish (Scholz et al., 2012). Fish is a part of the food web in marine ecosystems. Herbicides have influenced the living being of marine animals, including impacts on olfaction in fishes, resulting in declined productivity, foraging, and homing (Pereira et al., 2013).

2.1.3.3 Air Pollution. Other application forms of herbicides, such as spray drift and volatilization, can induce herbicides to degrade into different forms that make a broad herbicide spreading and aggravate a severe toxic problem. Both droplet sizes and wind speed are two major factors of drifting. After treating plants with herbicides, the volatilization of herbicides depends on time duration, humidity, the surrounding temperature, wind speed, and vapor pressure.

2.2 Current Techniques for Herbicide Detection

2.2.1 Chromatography

Gas Chromatography (GC) has been a popular type of chromatography for vaporized chemicals through separation since the 1990s (Pico et al., 2020). GC introduced herbicides residue analysis because their features provide high sensitivity and selectivity to identify or separate multi herbicides in a single measurement (Jia et al.,

2015). Hogendoorn and van Zoonen reported that GC could detect different herbicide residues up to 300 in food (Hogendoorn & van Zoonen, 2000).

However, due to the dramatic growth of the herbicide industry, novel herbicides have been continuously produced all the time. These modern herbicides are polar, low volatile, thermally labile compounds that are not directly responsive to the GC method. Thereby, highly polar herbicides cannot analyze by GC; they must be converted to become less polar before studying (Amirav et al., 2020). It is a significant limitation of the GC method for quantitative measurement.

Liquid Chromatography (LC) is developed to overcome GC's hurdles (Hogendoorn & van Zoonen, 2000; Stachniuk & Fornal, 2016). High Performance Liquid Chromatography (HPLC) is a standard LC method for classifying herbicides in the matrixes owing to their sensitivity, selectivity, and throughput in one run.

Croes et al. performed a quantitative analysis of paraquat in urine using HLPC. The serum samples were collected and treated by ion-pair extraction. The technique is possible to achieve the Limit of Detection (LOD) of 0.4 ppm as well as serum sample volume requirement is minimal (Croes et al., 1993). Hara et al. explained the utilization of postcolumn reduction conditions for Reversed-phase HPLC to determine paraquat and diquat in human serum as low as 50 and 100 ppb, respectively (Hara et al., 2007). Zou et al. established a method for trace analysis of paraquat in four leafy green vegetables using HLPC and Tandem Mass Spectroscopy, which indicated the capability of sensitive, reliable, and cost-effective detection (Zou et al., 2015).

Nevertheless, these mentioned studies incredibly require sample preparation procedures as well as time-consuming. Some of such sample preparation are deproteinization processes (Hara et al., 2007), ion-pair extraction (Croes et al., 1993), sample homogenization (Zou et al., 2015), solvent extraction, and so on. They also rely on laboratory instruments and specialists, which are limited to the applications of field testing.

2.2.2 Spectroscopy

Even GC and LC are efficient techniques due to high sensitivity; obvious disadvantages are time-consuming, complex operation, and requiring the pretreating sample steps and specialists (Lin et al., 2021). Spectroscopic techniques are based on matter-radiation interaction for analyzing and determining the molecular structures and compositions of

analytes. The results are recorded as a function of wavelength or frequency, which are easy to follow in the discussions and explanations. Spectroscopy has been applied in medical treatment (Di Foggia et al., 2021), heavy metal pollution fields (Eshkeiti et al., 2012; Ouyang et al., 2018) as well as herbicide analysis fields.

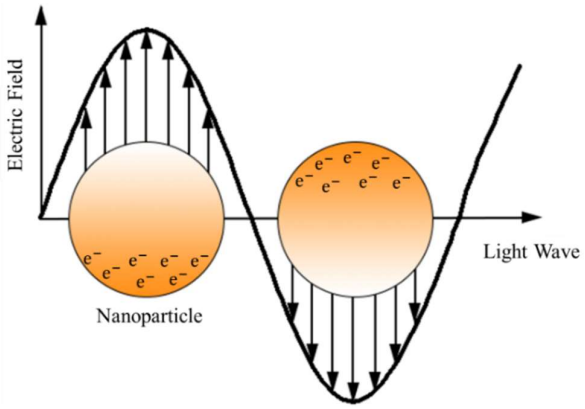
2.2.2.1 Surface-Enhanced Raman Spectroscopy (SERS). A typical spectrometer for the determination of herbicides is Raman spectroscopy, commonly combined by surface enhancing techniques, including roughened surfaces or nanostructures. SERS is based on the combination of surface plasmon resonances (SPR) and the Raman effect in nanostructures.

The photons of incident light interact with electrons in metal surfaces. Electrons are excited with a specific frequency corresponding to a certain angle of the incident light, resulting in light energy absorbed by the collective oscillations of electrons. These excited electrons are called plasmons. Plasmon propagates along the metal surface, generating an electric field for sensing. SPR occurs at the interface of two mediums: a dielectric and metal; whenever there is a change on the sensing surface, such as attaching or binding the molecules, spectrum intensity changes (Mejía-Salazar & Oliveira, 2018; Nguyen et al., 2015).

Similar to SPR, LSPR results from the resonance oscillations of electrons in metallic nanoparticles and incident coherent photons. As a result, collective oscillations are possible to absorb more energy from incident photons than usual, as shown in **Figure 2.1**. Furthermore, unusual electrons generate electromagnetic fields that are sensitive to the changes near metallic nanoparticles. These electromagnetic fields are the main contribution of the SERS enhancement effect.

Figure 2.1

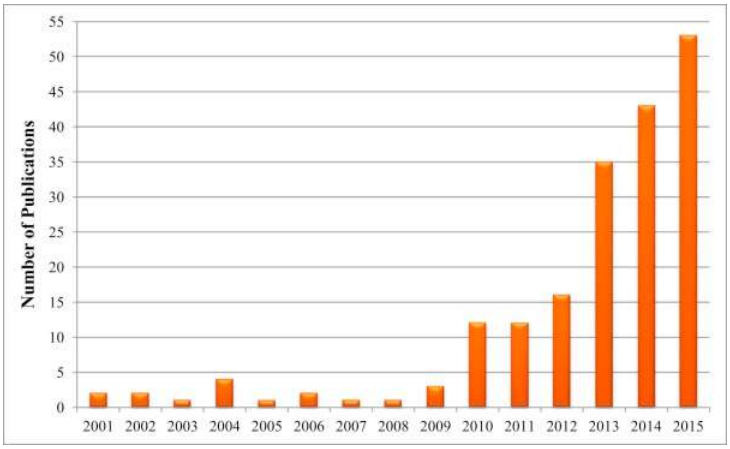
Induced Plasmons by Free Electrons in Metallic Nanomaterial



Performing SERS techniques is likely to be minor damage to the sample and minimal sample preparation. Since SERS enhancement depends on the nanostructures of SERS-active substrate, therefore a single-molecule level is possible to be seen by this technique (Chen et al., 2020), causing the number of publications on pesticide detection based on the SERS technique has been dramatically increased as indicated in **Figure *2.2***. This indicated that how SERS technique has been interesting in this field. The data were recorded and summarized by Pang et al (Pang et al., 2016).

Figure 2.2

Number of Publications on Pesticide Detection by SERS Technique (Pang et al., 2016)



Many studies developed SERS substrate with various structures to show the potential of using SERS technique in herbicide monitoring, including gold nanostars (Lin et al., 2021), silver nanoparticles (Chen et al., 2020), and gold nanoparticles (Chen et al., 2018). Lin et al. fabricated gold nanostar to measure trace amounts of paraquat in tea. The study reported that the spike tips of gold nanostar theoretically offer huge hot spots, which are the intense regions of the enhancements. Gold nanoparticles are developed as a SERS substrate for omethoate and chlorpyrifos detections on apple surfaces by J Chen et al. The study showed the feasibility of study absorption and diffusion of herbicides. However, in addition to precisely controlling the morphology and size of nanoparticles, the parameter such as sensitivity and reproducibility must be considered as well as repeatability. Otherwise, residue wastes from the fabrication processes can harm the environment and cause environmental pollutions later.

2.2.2.2 Electrochemical Impedance Spectroscopy (EIS)

There were some studies related to paraquat determination using electrochemical methods because of their sensitivity and simplicity. One of them is Electrochemical Impedance Spectroscopy (EIS). EIS is based on electrochemical techniques that measure the impedance on the electrode surfaces. EIS allows the separation of the component on the electrode surfaces through current, voltage, and frequency from circuit elements that respond to the electrical impedance of the medium (El Harmoudi et al., 2013). Farahi et al. reported that paraquat binding at the electrode, leading blocking electron transfer resulting in decreased electron transfer resistance which refers to the amount of paraquat (Farahi et al., 2015). Hence, EIS is categorized as a complex technique because it is very surface sensitive to the medium on the surfaces, thereby corresponding solution to the circuit electrode must be stringent (Cartaxo et al., 2015; De Souza & Machado, 2005; Laghrib et al., 2020).

2.2.3 Colorimetry

Color perception by vision is the easiest way for quantification of interest. Colorimetry relies on scientific techniques for determining the concentration of analyte through color changes. Some studies have been highlighted the advantages of colorimetry in herbicide analysis fields. Chuntib and Jakmanee developed a flow injection system to determine paraquat residues in natural water. The system was worked with a LED-LDR colorimeter, which was built as well. The study revealed an alternative system for herbicide detection as low as a concentration of 100 ppb (Chuntib & Jakmunee, 2015).

However, the introduction of nanotechnology into quantitative measurement, inducing distinctive beneficial such as sensitivity, stability, and selectivity, particularly portability that can be possible by controlling the size and density of the structure of nanomaterials. According to Chawla's and Zhang's works, noble metals such as gold and silver provide high sensitivity because they possess unique optical properties, including self-assembly, localized surface plasmon resonance, and light scattering (Chawla et al., 2018; Zhang et al., 2020). These features are quickly sensitive to the changes of the binding events or chemical interactions, even in a small molecule at a poor concentration.

Based on the LSPR method, an incident light interacts with the nanoparticles that are smaller than the wavelength of the frequency of incident light, resulting in the oscillation of electrons in nanoparticles that enhance the electric field near the surface of nanomaterials. This enhancement supplements high spatial and spectral resolution that causes the LSPR method to be an extremely sensitive transducer (Hong et al., 2012).

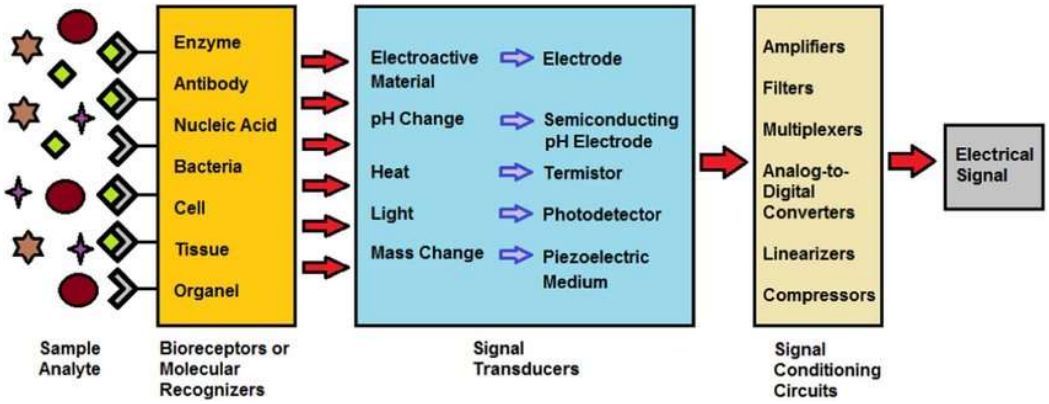
According to the benefits of LSPR, metallic nanoparticles are widely used as sensing probes. Chemical reactions on the sensing probe can divide into two mechanisms. One is enzymatic analytes directly catalyze metal nanoparticles, appearing color changes. Another one is the functional groups of nanomaterials that interact with an enzymatically hydrolyzed analyte. As a result, the changes of color result from the aggregation of nanoparticles. Siangproh et al. fabricated a colorimetric probe using citrate coated on silver nanoparticles to trace paraquat residues (Siangproh et al., 2017). The probe was based on aggregation by the Coulombic attraction between silver nanoparticles and paraquat, resulting color of silica gel being darker, which was easily observed.

2.2.4 Biosensors

Biosensors are incredibly analytical devices for detecting interest, combining a biological recognition element, a transducer, and an electronic system, as shown in **Figure *2.3***. The biorecognition element plays a crucial role as highly specific to an analyte. Since analyte-bioreceptor interactions create a signal, the transducer converts produced signal into a measurable signal. An electronic system processes the signal from the transducer through signal amplifying, signal processing to display as an understandable signal.

Figure 2.3

Main Components of a Biosensor include Bioreceptors, Transducers, and Signal Conditioning Circuits (Ansari et al., 2016)



Hence, the study related to biosensors is famous in many kinds of research because of flexibility, sensitivity, cost-effective measurement, and ongoing monitoring. It is also possible to have the minimum sample preparation. Biosensors can categorize into many ways according to types of bioreceptors and transducers. The reviews from now on are common biosensors regarding herbicide detection.

2.2.4.1 Electrochemical. Electrochemical detection involves using electrodes to detect the response from the sample where the working electrode is sensing, and the counter electrode is to flow the current. Inexpensive, simplicity, and sensitivity, or even miniaturization for portability can be achieved by this method.

Nonetheless, due to using an electrochemical electrode as the sensing, precision and sensitivity depend on influence factors to the electrodes. For example, bare electrodes can cause poor electron transfer rates (Laghrib et al., 2020). Some kinds of electrodes may interact with the sample that formed the incorrect response. In addition, temperature fluctuation is also the main factor that interferes with the sensitivity to the sensor.

The studies utilized silver, gold, and mercury to detect paraquat, providing excellent sensitivity (Cui et al., 2018; De Souza & Machado, 2005; Laghrib et al., 2020). These noble metals are sensitive to electro-oxidation of paraquat. To correct the type of electrodes, the nature and structure of herbicides must be understood. For example, Wei

et al. and Yu et al. developed acetylcholinesterase (AChE) as a bioreceptor that improves the capability of electrodes to quantitative measurement of herbicides in the matrices. The biosensor introduces simplicity and reproducibility, which leads to cost-efficient, rapid, reliable, and high sensitivity as well as excellent detection limit (Wei & Wang, 2015; Yu et al., 2015).

Due to organophosphorus pesticides being toxic to living organisms by damaging the neuro system through enzyme inhibition, the applications of using AChE to detect these herbicides are incisive. Cui et al. immobilized AChE on electrodes that introduce catalytic activity when organophosphorus inhibits AChE, resulting in decreasing thiocholine. It leads to an electrocatalytic reaction between thiocholine and electrode, causing changes that can be detected (Cui et al., 2018). Nonetheless, AChE electrochemical biosensors have been reported for their instability of the biosensors.

2.2.4.2 Immunosensor. Immunosensor is based on the principle of immunoassays that uses antibodies as a bioreceptor combined with transduction systems that convert the recognition signals into detectable signals. Therefore, simplicity, portability, cost-efficient, and high sensitivity can achieve by this assay. Immunosensors can be classified into various classes which depend on the transduction system, including optical, electrochemical, and piezoelectric.

Usually, an antibody is used as a receptor element due to outstanding specificity for an antigen of a target molecule. The binding event between antigen and antibody stimulates an immune response (Fang et al., 2020; Sharma et al., 2016). Hapten molecules are small molecules that formed by antibody binding molecule that is not immunogenic. Most herbicides being haptens and hapten will only induce an immune response when binding with a biomacromolecule like protein. Thus, excellent binding between a couple, antibody, and antigen provides high specificity and sensitivity that are remarkable advantages in the immune-analytical field.

Li et al. generated monoclonal antibodies using an immunochromatographic assay for paraquat determination as low as a LOD of 1 ppb without pretreating sample procedures (Y. Li et al., 2020). Sun et al. concluded that the synthesis of anti-C5a antibodies is possible to be an alternative clinical application for those exposed to paraquat (Sun et al., 2018). Moreover, Garcia-Febrero et al. raised polyclonal antibodies against paraquat in wheat, barley, and potatoes. The study potentially demonstrated a sensitive

ELISA approach for paraquat detection without extraction steps (Garcia-Febrero et al., 2014).

In addition to classified immunosensors, immunosensors could identify as labeled or non-labeled sensors (Aranda et al., 2018). The labeled immunosensors depend on the types of label elements, including enzymes, fluorescent dyes, and metal ions. On the other hand, the non-labeled sensors rely on changing physical properties while antibody-antigen binding events occur. Hence, non-labeled are widely used for quantitative measurement by signal changes through current, voltage, or resistance response.

Moreover, due to various assays for sensing the target, the size of the target molecule is a consideration for the choice of strategies: sandwich or competitive assays. Generally, sandwich assays are suitable for macromolecule, including proteins. The number of recognition events is directly proportional to the presence of analytes. Conversely, competitive assays are more convenient for small molecules detection, including herbicides detection (Fang et al., 2020). The quantity of analyte is conversely proportional to the presence of the analyte in the samples due to the analytes have to compete for binding with the labeled antigen to produce readable signals.

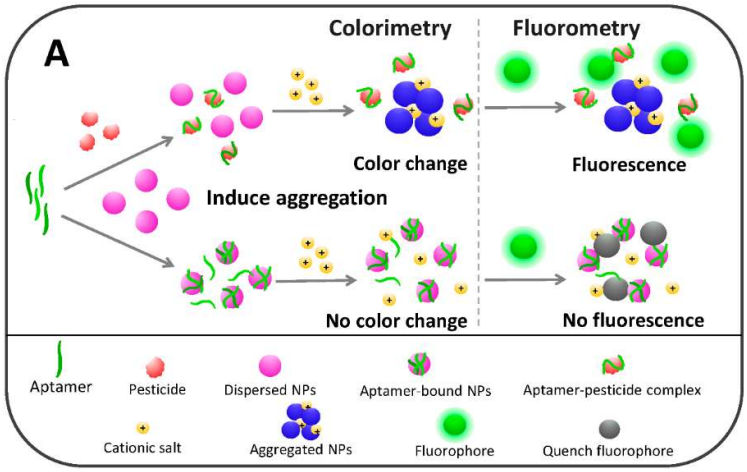
2.2.4.3 Aptasensor. An aptasensor combines aptamer and biosensor to construct an analytical device. Aptamers are single-stranded oligonucleotides that are usually created through the systematic evolution of ligands with exponential enrichment (SELEX). The selected aptamer from the libraries that contain other aptamers has high affinity and selectivity toward interest molecules. Thus, the reproducibility and purity of the selected aptamer are high-level, and aptamer-based biosensors are highly stable. Yang et al. screened aptamer for λ-cyhalothrin residues using the improved Capture-SELEX technique, which applied selected aptamers LCT-1 and LCT-1-39 to the colorimetric sensors. Its LOD of 19.7 ppb and 18.6 ppb are respected.

There were many studies related to aptamer applications (Kuitio et al., 2021; Liu et al., 2019; Nguyen et al., 2015; Wang et al., 2018; Yang et al., 2021). They indicated that aptamer is more cost-efficient than antibodies since antibodies are produced from animal sources and are likely unstable. According to Phopin and Tantimongcolwat reviewed, gold nanoparticles are commonly used as a color indicator in aptsensor applications. Even aptamer is a recognition element; it also inhibits the aggregation of

nanoparticles by electrostatic and steric stabilization, as illustrated in **Figure *2.4*** (Phopin & Tantimongcolwat, 2020).

Figure 2.4

Schematic of Colorimetric Aptasensor Concept (Phopin & Tantimongcolwat, 2020)



Kuito et al. developed an aptasensor for paraquat detection using aptamer-functionalized gold nanoparticles based on the colorimetric assay. They screened aptamers through the SELEX technique. The selected aptamer 77F showed a high affinity toward paraquat as low as a LOD of 0.268 ppm. Aptamer 77F likely soft specific bind to other competitive reagents are low affinities, including dimethyl phosphite, diethyl phosphite, and so on (Kuitio et al., 2021). Moreover, Ouyang et al. developed a simple aptasensor without modification and aggregation of gold nanoparticles (Ouyang et al., 2018). A lead detection via aptamer-gold nanoparticles using the SERS strategy was reported. Gold nanosol was mixed well with aptamer and Pb^{2+}. The combination of Pb^{2+} and aptamer caused the recovery of SERS intensity, which is increased regarding Pb^{2+} concentration. They showed the feasibility of aptamer applications in a variety of research fields in the near future.

Although, aptamer also has significant limitations as well as other bioreceptors. Some cases of studies reported that nanoparticles were not aggregate even there is a presence of interest (Abraham et al., 2018; Chávez et al., 2012). Moreover, the screening processes for a specific aptamer are time-consuming and complicated as well as other

factors easily influence the selection procedures. Guo et al. developed an aptasensor for mycotoxin detection in food (Guo et al., 2020). A variety of types of mycotoxin has been determined. The study revealed the potential of field tests compared to traditional methods like HLPC-MS. Nevertheless, due to different chemical analogs of mycotoxin, it is challenging to select a high specific aptamer properly as well as multi-analysis of analogous structures of analytes is difficult.

2.3 SERS Technique

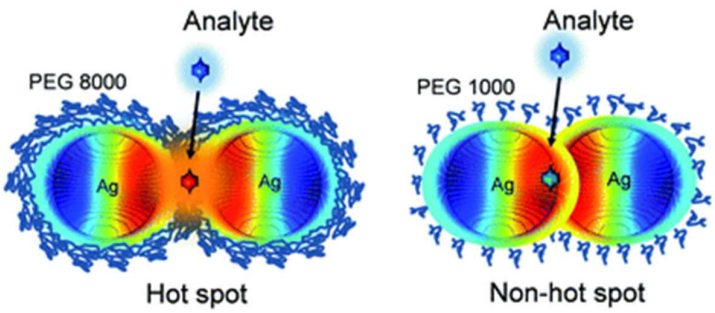
Surface-enhanced Raman spectroscopy is a powerful analytical tool that takes advantage of two different mechanisms. One is the electromagnetic (EM) enhancement mechanism, which is the main contribution to spectra. EM mechanism is referred to the presence of LSPR, which relies on nanostructures and materials of the substrates. The EM mechanism was already explained in **Section 2.2.2.1**. Another one is the chemical mechanism, in which the contribution depends on the experimental conditions related to the charge transfer between metal and absorbed molecule (Cialla et al., 2012; Sharma et al., 2012). Since the chemical enhancement is typically little compared to the EM mechanism, SERS enhancement totally influenced by EM mechanism.

SERS enhancements can get in order of 10^{14} associated with "hot spots", strongly enhanced sites at junctions between the nanoparticles as indicated in **Figure *2.5***. When plasmonic nanoparticles have come closer, the localized regions are occurred, causing the strong electromagenetic field that higher than SPR, called intense local field enhancement (M. Li et al., 2020; Xiao et al., 2021).

As a result, the trace analysis through the contribution of SERS substrate is interesting in the research field related to herbicides. To obtain strong Raman enhancement, several studies focused on different materials and structures for substrate components, including gold nanoparticles decorated on engraved aluminum (Botta et al., 2020), silver nanoparticles on lotus leaf-based SERS chip (Yao et al., 2021), and gold-silver core-shell nanoparticles on gold film (Wang et al., 2016).

Figure 2.5

Hot Spot and Non-Hot Spot Regions of Silver Nanoparticles (Radziuk & Moehwald, 2014)



2.3.1 Different Materials for SERS-Active Substrate

When electrons of nanostructure are collective oscillation with matching incident electromagnetic field at the interface of the metallic nanostructure, this is called LSPR. It results in two consequences. One is the unusual oscillation of plasmons which arise absorption property as shown in **Figure *2.1***. Another one is the enhancement of electromagnetic fields, which is mainly responsible for SERS enhancement. EM enhancement is often dominant in SERS enhancement factors, highly relies on physical properties of nanomaterials such as size and morphology.

Typically, noble metals such as silver and gold have been usually employed as plasmonic substrates because they are stable and provide maximum SERS activity compared to other metal nanostructures. In order to attain significant enhancement, several studies have been studied the effect of sizes and shapes of gold and silver nanoparticles on SERS response. As Butler et al. concluded, large gold nanoparticles are better in observation since their aggregation is more prominent than smaller ones, helping SERS spectral acquisition (Butler et al., 2015). Hong and Li discovered that SERS activity could rise as the size of gold nanoparticles increases and surface area increases (Hong & Li, 2013). The optimal diameter of gold nanoparticles is about 50 nm, providing maximum SERS enhancement factors as well as minimum toxicity effect on the sample.

In addition, some studies indicated the dimension of nanoparticles affects SERS enhancement. Dao et al. fabricated silver nanodendrites coated on silicon wafer. They

succeeded in determining the detection limit as low as 1 ppm of paraquat, which is lower than 100 times of silver nanoparticles coated on silicon wafer. They attributed an excellent sensitivity to nanostructures of SERS-active substrates (Dao et al., 2015). Nanofilbrillar cellulose coated with gold/silver core-shell nanoparticles of Asgari et al. revealed a high distribution of bimetallic nanoparticles to attain the limit of paraquat detection of 46 ppb (Asgari et al., 2020). Botta et al. fabricated sensitive and reproducible substrates through gold nanoparticles decorated on roughened metal to detect paraquat (Botta et al., 2020). Their experiment results corresponded to the computational results, in which the LOD was in the range of 2.7 ppb – 27 ppm.

2.3.2 Solvent Effects on SERS Activity

In addition to the EM enhancement and chemical mechanisms, SERS activity can be influenced by solvent effect, pH, treatment and control conditions, and so on. Due to the different molecular structures of analyte and substrates, a pair of analyte-substrate exhibits a unique charge transfer characteristic of its own. With effort, some studies have been optimized the conditions for obtaining more SERS activity.

Tripathi et al. studied the solvent effect that influences SERS response by changing the analyte mixture with various solvent matrices such as ethanol/water and acetonitrile/water (Tripathi et al., 2018). They concluded that high soluble analytes likely have low SERS intensity due to free analytes not being correctly absorbed on substrates. D. Zhang et al. fabricated flower-shaper silver nanoparticles based SERS sensor (Zhang et al., 2018). Under optimal conditions, the mixture of ethion with acetone provides an excellent Raman measurement as low as 3.8 ppb.

2.3.3 Functionalization of SERS-Active Substrate

SERS technique provides the massive potential for quantitative analysis of small molecules. However, in real samples, it sometimes hardly discriminates the analyte among other molecules whose features are similar to the analyte, resulting in poor selectivity (Yaseen et al., 2018). To tackle this issue, the functionalization of the substrates is essential.

Aptamers are commonly used as probes and combined with several optical techniques since their features are incredibly advantageous. Aptamer binds to the analyte by intermolecular interactions such as hydrogen bonding, van der Waals, and electrostatic

interactions. Moreover, aptamers are possible to change their conformations to bind an analyte(Ogasawara et al., 2009; Phopin & Tantimongcolwat, 2020).

Furthermore, aptamers are capable of reversible denaturation; resulting aptamers are highly thermal stable. The synthesis of an aptamer can perform via polymerase chain reaction or chemical synthesis. The aptamer modification is effortless; therefore, reducing the error from enzymatic degradation and target missing is possible. Above all, aptamer does not need animals or immune systems to generate as high reproducibility and time-saving.

Chen et al. fabricated aptamer-functionalized silver nanorods based on SERS to detect Salmonella Typhimurium, a pathogen in food (Chen et al., 2017). After silver nanorod arrays were fabricated by an oblique angle deposition technique, an aptamer against Salmonella Typhimurium was modified with a C6 thiol modifier and T-linker, then incubated with silver nanorods for conjugation. Then other cells are removed by rinsing with buffer as well as the study reported the detection is limited to 10^8 CFU/mL. Additionally, a colorimetric aptsensor using gold nanoparticles to detect paraquat has been studied (Kuitio et al., 2021). A thiolated aptamer was synthesized and further added gold nanoparticles. After incubation, paraquat and NaCl were added, respectively. Thus, the color of the solution was changed from red to purple, and the LOD of 0.268 ppm was archived.

2.4 Summary

Herbicide contamination has become one of the most severe problems globally since its effects are vastly harmful to humans, animals, and the environment. Paraquat is a poisonous herbicide that causes dangerous symptoms after being exposed. Therefore, different paraquat detections have been established to hurdle.

Chromatography offers ultrasensitivity and reliability, but it is limited to on-site tests, laboratory instruments, and technicians. Spectroscopy such as SERS is highly simple, sensitivity and minimum sample preparation can be achieved. However, SERS configuration such as morphology and size must be concerned to attain tremendous intensity. Biosensors and colorimetry act as integration to other techniques for enhancing a LOD and handling the hurdles. Nevertheless, natural limitations of biomolecules and systems still remain, including enzymatic degradation, stabilization,

and poor sensitivity compared to Chromatography and SERS. **Table *2.1*** shows the advantages and disadvantages of these techniques. Moreover, the summarize table of the current paraquat detection method is shown in **Table *2.2***.

Table 2.1

Advantages and Disadvantages of Current Herbicide Techniques

Method	Advantage	Disadvantage
Chromatography	- Ultrasensitivity - Require small quantity	- Time-consuming - Laboratory-based - Require technicians - Expensive
SERS	- High sensitivity - Minimum sample preparation	- Rely on analyte-metal interactions - Limited selectivity - Poor reusability
Colorimetry	- Real-time applications - Simple observation	- Limited colorless samples - Low sensitivity - Easily interfere
Biosensor	- Cost-effective - Miniaturization - High selectivity	- Poor sensitivity - Degradation - Denaturation
Aptasensor-based on SERS	- High sensitivity - Minimum sample preparation - High selectivity	- Degradation - Short lifetime - Poor reusability

Table 2.2

Summary of Paraquat Detection

Substrate	Method	Matrix	Limit of Detection	Ref
Imida-AgNPs	Colorimetric assay	Water and soil samples	6.27 μM	(Ali et al., 2022)
AgNPs-based lotus leaf	SERS	Water samples	1.2 μg/L	(Yao et al., 2021)
ACN column	HPLC-MS-MS	Vegetables	0.94 ng/g (FW)	(Zou et al., 2015)
Rotating Ag electrode	Voltammetry	Milk	2.8 nmol/L	(Farahi et al., 2014)
AuNPs on Al sheet	SERS	Drinking water	27 μg/L	(Botta et al., 2020)
Au nanostars	SERS	Green tea	0.2 mg/kg	(Lin et al., 2021)
UCNPs and BPNSs	Fluorescence	Green and Black tea	0.18 ng/mL	(Wang et al., 2022)

Note. Upconversion nanoparticles (UCNPs), Black phophorus nanosheets (BPNSs)

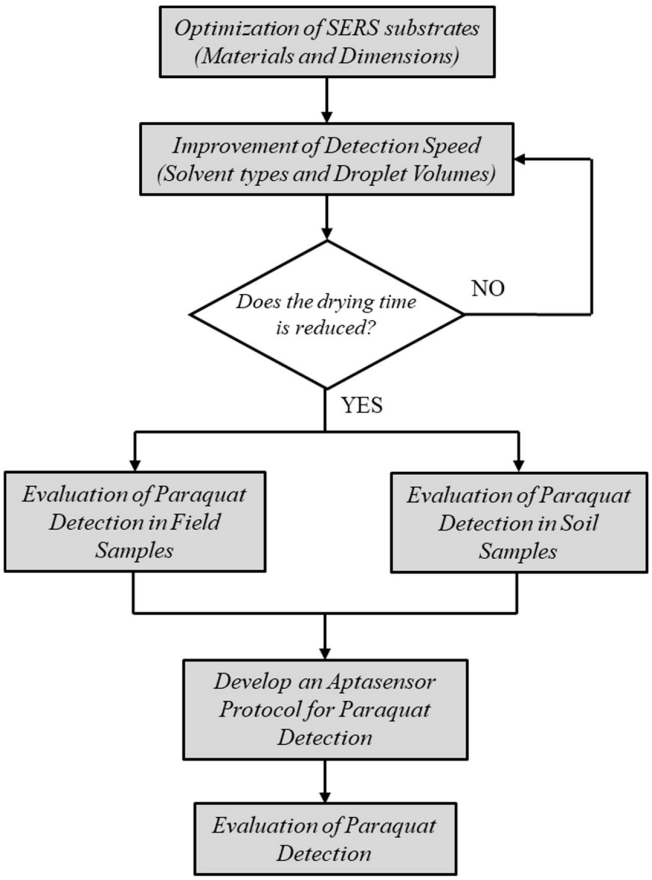
CHAPTER 3
METHODOLOGY

3.1 Research Framework

The methodology of the research is designed to correspond to the objectives as illustrated in **Figure *3.1***. The study started with the optimization of SERS substrates. The parameters such as material, dimension, solvent type, and droplet volume were investigated. The optimum conditions were then used to evaluate the paraquat detection in field water and soil samples. After that, the surfaces of the SERS substrate were functionalized by aptamer that specify to paraquat. In the evaluation of aptasensor for paraquat detection, the sensitivity, the limit of detection (LOD), and selectivity are determined and compared to the no-aptamer substrate.

Figure 3.1

Flow Chart of the Research



3.2 Optimization of SERS Substrates

3.2.1 Selections of Material and Dimension for SERS Substrates

The study experimented with noble metallic nanostructures such as gold and silver. Silver and gold nanostructures (SERS-active substrates) are produced by NECTEC, Thailand, known as ONSPEC SERS chips. They produced a series of ONSPEC substrates such as ONSPEC-Lite, ONSPEC-Prime, and ONSPEC-X^{+}.

In this study, the experiment used ONSPEC-Lite and ONSPEC-Prime. ONSPEC-Lite is a gold-based substrate where gold nanoparticles deposited on the roughened laser-engraved aluminum sheet with magnetron sputtering system, called L-Mark SERS chips. ONSPEC-Prime is based on silver nanorods with a length of 300 nm prepared by physical vapor deposition techniques such as sputtering with glancing-angle deposition. In addition to 300 nm of ONSPEC-Prime, other dimensions of silver nanorods such as lengths of 100 and 150 nm are also investigated.

These substrates such as the ONSPEC-Lite chip and ONSPEC-Prime chip with 100, 150, and 300 nm chips are tested by dropping a 10 μL of 0.01, 1, and 10 μM of paraquat concentration on these chips as shown in **Figure *3.2***. After letting them dry out at room temperature, Raman measurements were performed. Renishaw's inVia Raman spectrometer (UK) with a laser excitation wavelength of 785 nm was used to acquire the signals. For the Raman configuration, each substrate has its configuration as the following: an exposure time of 10 s, a laser power of 10%, and a 50x objective lens for ONSPEC-Prime chip. Meanwhile, 100% of laser power is and 5x objective lens is for the ONSPEC-Lite chip.

Figure 3.2

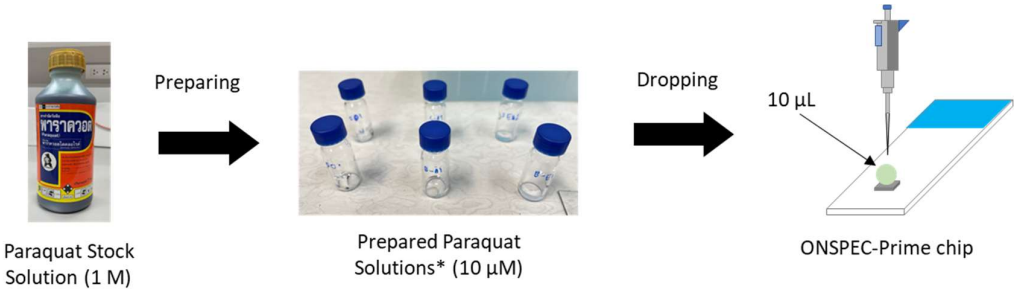
ONSPEC-Lite and ONSPEC-Prime with Paraquat on the Surfaces

3.2.2 Solvent and Droplet Volume Selection for Improvement of Detection Speed

This section aims to minimize the drying time of paraquat detection. Initially, paraquat was prepared at 10 µM with deionized water (DI water). Then paraquat was individually prepared at 10 µM with different organic solvents, including ethanol (EtOH), methanol (MeOH), and acetonitrile (ACN). In addition, 50% of these organic solvents were also prepared without paraquat to be used as control samples. The substrate used in this experiment is the ONSPEC-Prime chip, resulting from the above section. After dropping 10 µL on the substrates, the time for drying for each sample was recorded. Then the spectra of paraquat in prepared solvents were acquired by Raman spectroscopy with the configuration for the ONSPEC-Prime chip. The procedure of solvent selection is shown in **Figure 3.3**.

Figure 3.3

Schematic Diagram of Solvent Selection Procedure



Next, the experiment focused on the effect of droplet volume on drying time. 10 µM Paraquat solution was prepared in deionized water and the volumes of dropping varied to 2.5, 5, and 10 µL. A stopwatch was used to record the drying time. After drying out, acquire the spectra by Raman spectroscopy.

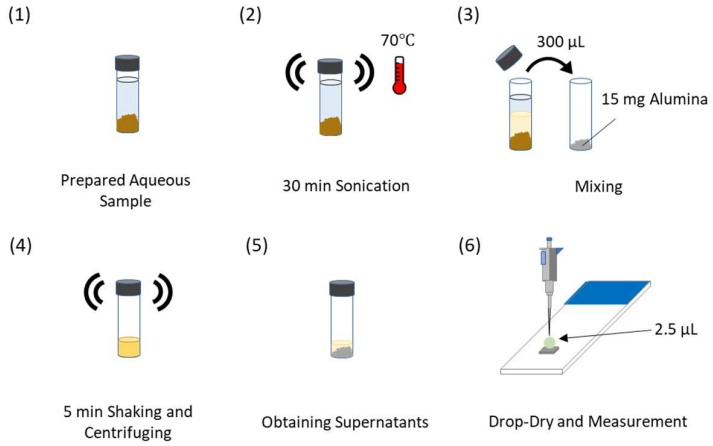
3.3 Evaluation of Paraquat Detection in Field Samples

In order to detect the paraquat in both water and soil samples, the experiment developed a protocol for extracting paraquat from these samples. As shown in **Figure *3.4***, the procedure of extraction is demonstrated. The extraction protocol is starting with both water and soil samples have to prepare in an aqueous solution. To be an aqueous solution, each type of sample has different ways to prepare that will explain in their

sections. After obtaining the prepared solutions, the sample was sonicated at 70°C for 30 min. Then put 300 µL of obtained supernatant to mix with 15 mg of powder alumina (Al_2O_3) which was used as a sorbent. Next, shook and centrifuged for 5 min to obtain the supernatant that was used for the measurement.

Figure 3.4

Diagram of an Extraction Protocol for Field Samples



3.3.1 Paraquat in Water Samples

The extraction is based on QuEChERS extraction using 5 different absorbents. In the early stages, the study focused on the optimum absorbents for the extraction. Therefore, the sample used in the first experiment was not a field water sample. The paraquat solution was prepared at 2.5 µM in deionized water for 50 mL. Then each 10 mL of the prepared sample was sonicated at 70°C for 30 min. Next, 300 µL of the sonicated solutions were collected and mixed with 15 mg of 5 different QuEChERS absorbents. The absorbents used in this experiment were alumina (Al_2O3), octadecylsilane (C18), primary secondary amine (PSA), graphitic carbon black (GCB), and florisil. All 5 samples were shaken to ensure homogenous solutions, followed by a centrifuge for 5 min. Eventually, from a centrifuge, supernatants from 5 samples were obtained. The experiment used these 5 supernatants for the measurements.

After comparing 5 different absorbent samples, the experiment studied the comparison between extraction and non-extracted samples. The water sample was collected from the canal in Solar Park, Thammasart University. For extraction samples, the collected

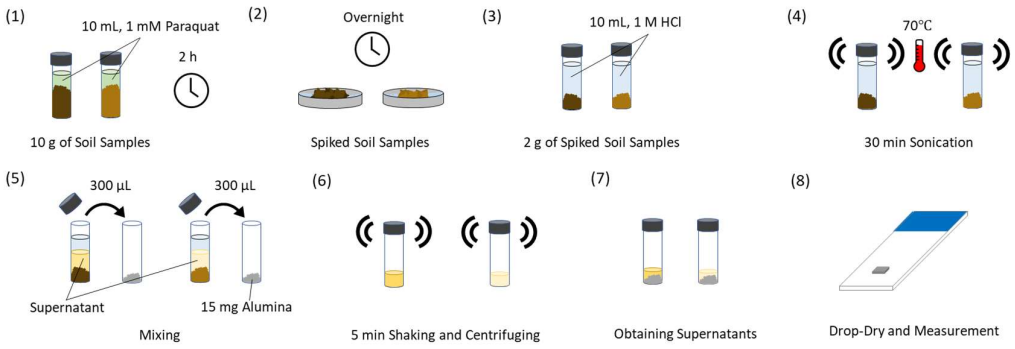
sample was mixed with paraquat to obtain 1 µM paraquat in the water samples. Each 10 mL of the sample underwent the same extraction steps as mentioned earlier but the absorbents used in this experiment were alumina and C18. Two extraction samples were finally obtained. Furthermore, a non-extracted sample was prepared by making 10 mL of 1 µM paraquat in the collected sample. The obtained samples for the experiment were as follows: 1. Extraction with Alumina 2. Extraction with C18 3. Non-extraction. Each sample was taken 2.5 µL to drop on the surface of ONSPEC-Prime chips. After completely drying, all 3 samples conducted the Raman measurement.

3.3.2 Paraquat in Soil Samples

For the evaluation of soil samples, the procedure of extraction was the same as described above but the difference is the preparation of the soil sample. As depicted in **Figure *3.5***, initially, the experiment used 10 g of loam soil that was collected in AIT to immerse in 10 mL of 1 mM paraquat for 2 h. After that, let it dry at room temperature overnight. 2 g of dried soil was mixed with 1 M hydrochloric acid (HCl) to obtain a final volume of 10 mL. The prepared solution was ready for the extraction protocol.

Figure 3.5

Diagram of an Extraction Protocol for Soil Samples



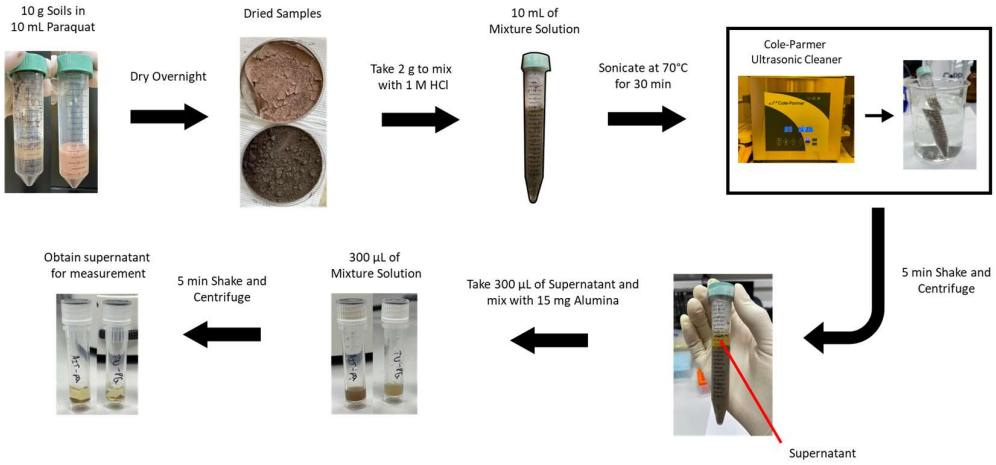
In the first experiment, the prepared solutions were sonicated at 70°C for 30 min to obtain the supernatants. Each 300 µL was mixed with alumina and C18 and then shaken until obtaining homogenous solutions. Centrifuged both solutions for 5 min to obtain supernatants again. 2.5 µL of supernatants were used to drop on the ONSPEC-Prim chips for the measurement. In addition, the sample also underwent filtration for the

comparing with the extraction. For filtration, the prepared solution was filtered by a paper filter. Then dropped a 2.5 µL of filtered solution on the ONSPEC-Prime chip.

Additionally, 5 different soils were also studied, including mountain, red, high sand content, low sand content, and clay soils. These soils were commercial grades and purchased from the grocery and planting shops. The extraction protocol was processed as previously. The schematic of the experiment is shown in **Figure *3.6***.

Figure 3.6

Illustration of Extraction Protocol for Soil Samples



3.3.3 Sensitivity and Limit of Detection (LOD)

The highest characteristic peak, which usually is 1650 cm^{-1}, was used to create the calibration curve to investigate the detection limit (LOD), which can be estimated by using the following **Equation 3.1** (A.J.R. Bauer, 2016):

$$LOD = 3.3 \times \frac{RMSE}{m} \qquad \textbf{3.1}$$

Where root mean squared error (RMSE) is defined as the standard deviation of the differences between predicted and actual values and m is the slope of the calibration curve. For the sensitivity, it can estimate from the slope of the calibration curve.

3.4 Developing an Aptasensor Protocol for Paraquat Detection

3.4.1 Preparation of the Substrate for Surface Functionalization

According to the aptamer sequence adopted from Kuitio's work where the aptamer is based on gold nanoparticles, ONSPEC-Lite SERS chip, gold nanoparticles deposited on engraved aluminum sheets, was selected for the surface functionalization with this aptamer.

In the early stages, the substrate was cleaned to remove the contaminants on the surface by a plasma surface treatment system. The experiment firstly investigated the optimum power for cleaning. The power ranged from 60 to 120 Watt. For other parameters, the system used oxygen gas at 5 psi and 1 min cleaning time. The experiment tested with 10 μM of Rhodamine 6G. After cleaning, the samples were dropped with 2.5 μL of prepared R6G, followed by letting them dry and then performing Raman measurement.

Next, the experiment further studied the effect of cleaning time on the SERS spectrum. The substrate was still the same which was the ONSPEC-Lite chip. The cleaning configuration was the same as above but the power was set at 100 Watt where the cleaning time was varied into 1-3 min. 10 μM R6G was also used for the testing.

3.4.2 Surface Modification of ONSPEC-Lite SERS Chip with Aptamer

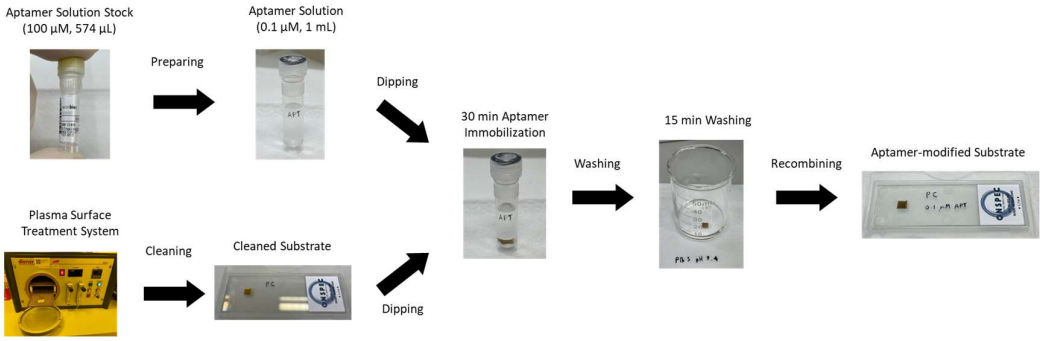
According to Kuitio et al., the aptamer sequence for paraquat detection is 5′-AGG CTT ACA CCT GAA AAG CGG CTT AAT TTA CAC TAC TGT AT-3′ (Kuitio et al., 2021). Aptamer has been modified with a C6 thiol modifier at the 5′ end. As the manual instruction mentioned, the aptamer with a thiol modifier was protected in powder form. Therefore, to deprotect the aptamer, Tris(2-carboxyethyl) phosphine or TCEP reducing agent solution was prepared for the deprotection. Initially, 0.08 g of powder TCEP was mixed with 7 mL of TE buffer or Tris-EDTA buffer pH 8.0 to obtain 7 mL of 40 mM TCEP solution. The solution has adjusted the pH to 7.0 by adding NaOH solution. Finally, obtaining the 14 mL of 20 mM TCEP solution pH 7.0. The obtained TCEP solution was kept in the fridge at -18°C.

The experiment started with the preparation of an aptamer stock solution from powder form. 574 μL of TE buffer pH 8.0 was added into the tube containing the aptamer powder, followed by ensuring that the aptamer solution was mixed well by shaking. The stock solution was kept in the fridge at -18°C. To make the working aptamer solution, the aptamer stock solution and TCEP solution were defrosted at room

temperature. After that, the aptamer stock solution was diluted with deionized water to obtain a double concentration of desired concentration. Then added the TCEP solution with the same volume of aptamer solution, obtaining the desired concentration. Ensuring that the solution was mixed well. The aptamer solution was heated at 90°C for 5 min to create the three-dimensional conformational shape for specific binding (Henri et al., 2019; Zhao et al., 2008), followed by cooling down for 15 min at room temperature. Meanwhile, the substrate was cleaned by a plasma surface treatment system. Next, the cleaned chip was removed and immersed in the prepared aptamer solution for an incubation time. After that, the aptamer-immobilized chip was washed in PBS pH 7.4 for 15 min to remove the excessive aptamer on the surface. Finally, the aptamer-modified substrate was obtained. The brief procedure of development of an aptamer-modified substrate is shown as depicted in **Figure *3.7***.

Figure 3.7

Diagram of Development of Aptamer-Modified Substrate



3.4.3 Determining an Optimal Condition for Aptamer-Modified Substrate

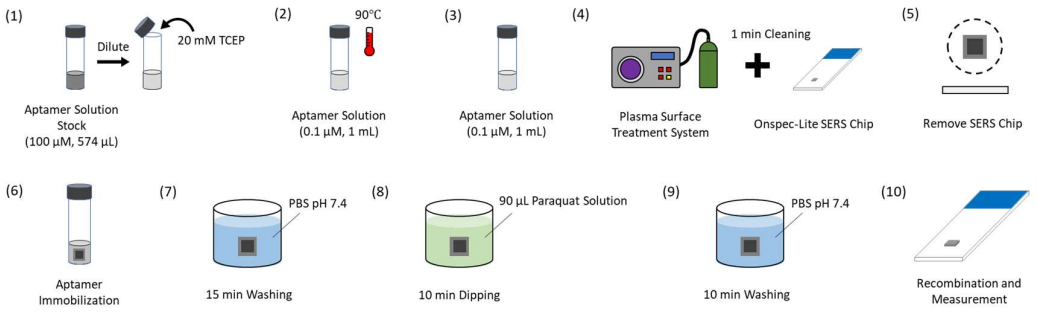
To obtain an optimal condition, the experiment studied both effects of incubation time and aptamer concentration on paraquat sensing. The experiment firstly varied the incubation time for aptamer immobilization. ONSPEC-Lite chips underwent plasma cleaning first. Next, 1 µM of aptamer concentration was prepared from the stock solution. Then the cleaned substrates were incubated in aptamer solution for various incubation times, ranging from 20 to 40 min. After washing and drying the substrates, the substrates were tested with 2.5 µM paraquat, and then acquired spectra.

Next, the experiment focused on the aptamer concentration which was varied into 0.01, 0.1, 1, and 10 µM. Each concentration was used to incubate the prepared substrates for 30 min. The concentration of paraquat used in this experiment was 10 µM. Each substrate was then tested with paraquat, followed by Raman measurements.

Lastly, the study investigated the methods for paraquat detection. Generally, the ONSPEC series is based on the drop-dry method which is dropping an aliquot of the solution on the surface of the chip. Then dry the chip at room temperature, followed by Raman measurement. However, this experiment also aimed to enhance the sensitivity of the aptamer-modified substrate. **Figure *3.8*** shows the overall procedure for the preparation and detection of the aptamer-modified substrate. The aptamer-modified substrate was produced for 2 samples. Paraquat solution was prepared at 2.5 µM for 100 µL. An aliquot of 2.5 µL of the prepared solution was dropped on the first sample and tested with Raman spectroscopy, called the dropping sample. Meanwhile, the second sample was immersed in 90 µL of paraquat solution for 10 min, followed by washing with PBS buffer pH 7.4 for 10 min to remove the loosely bound molecule on the surface. After that, dried the samples at room temperature and conducted the measurement.

Figure 3.8

Schematic of Preparation of Aptamer-Modified Substrate with the Dipping Method



3.5 Evaluation of Aptamer-Modified Substrate for Paraquat Detection

In this section, the aptamer-modified substrates were used to determine the sensitivity, limit of detection (LOD), and selectivity as well as the comparison with the no-aptamer

substrate. The commercial paraquat was prepared from the stock solution to obtain 0.25 to 5 μM paraquat concentration for the studies.

3.5.1 Sensitivity and Limit of Detection (LOD)

For sensitivity, the studied range of the paraquat concentration is 0.5 to 5 μM. The aptamer-modified substrates were prepared according to the developing protocol. For paraquat sensing, the detection method used in the experiment was the drop-dry method with 2.5 μL. After dropping and drying, Raman spectroscopy was used to acquire the SERS spectra. The analysis used the highest intensity of paraquat's characteristics to make the standard curve. This standard curve offered the slope which can be used to estimate the sensitivity. In order to estimate the limit of detection (LOD), **Equation 3.1** was used. The parameters in the equation can be found in the standard curve.

3.5.2 Selectivity

In this experiment, the detection method is the drop-dry method as the same. Two pesticides such as glyphosate and deltamethrin were used. These pesticides were prepared at 10 μM for 200 μL. A 100 μL was tested with the aptamer-modified substrate. Another 100 μL was mixed with 100 μL of 10 μM paraquat to obtain the mixture solution. After obtaining aptamer-modified substrates, 2.5 μL of these solutions were individually dropped on the substrates. For analysis, the intensity of 1650 cm^{-1} from Raman spectra was used to assess the selectivity.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Optimization of SERS Substrates

To interpret the SERS response of paraquat, the fingerprint characteristics of the paraquat molecule were investigated where the bands located at 840 cm^{-1}, 1190 cm^{-1}, 1300 cm^{-1}, and 1650 cm^{-1}. These peaks are assigned to the different vibration and stretching modes as shown in **Table 4.1** where the molecular structure is depicted below in **Figure 4.1**.

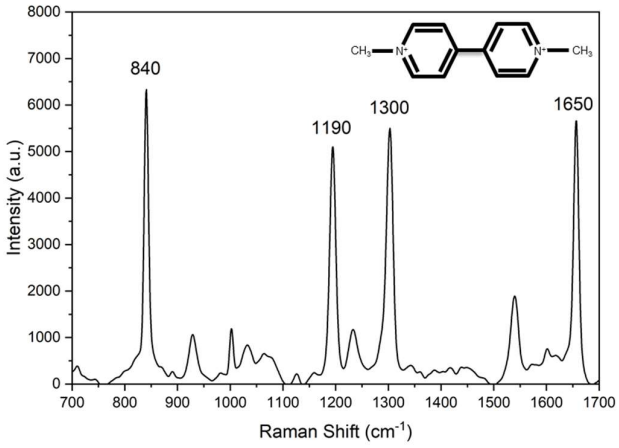
Table 4.1

SERS Characteristics Peaks of Paraquat and its Vibrational Modes.

Characteristic Peak (cm^{-1})	Mode
840	C─N Streching Vibration
1190	C═C Bending Vibration
1300	C─C Structural Distortion
1650	C═N Streching Vibration

Figure 4.1

Molecular Structure of Paraquat and SERS Response of Paraquat on ONSPEC-Prime SERS Chip

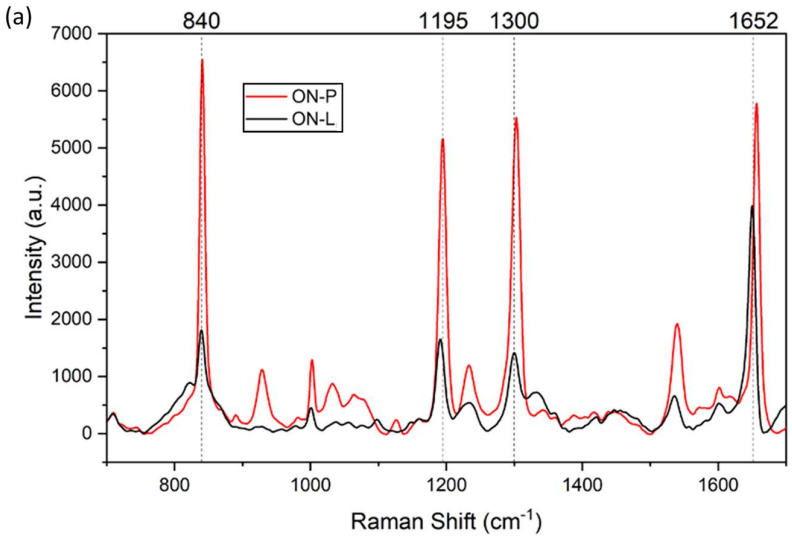


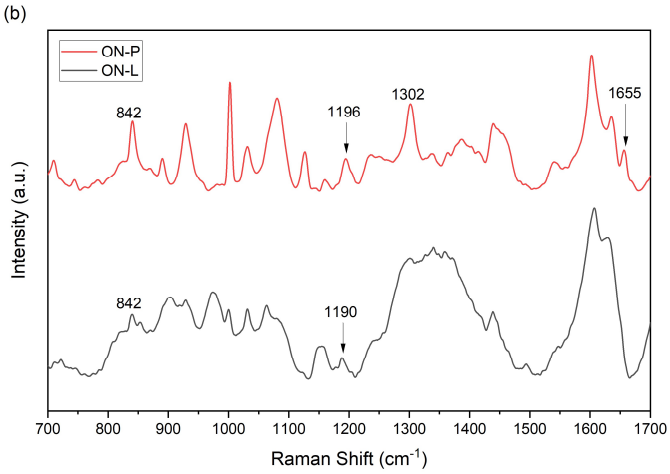
4.1.1 Effects of Material and Dimension of Nanoparticle on Paraquat Detection

For material selections, both of ONSPEC-Prime and ONSPEC-Lite SERS chips have experimented with 10 µM of paraquat. ONSPEC-Prime is made from silver nanorods while ONSPEC-Lite is gold nanoparticles on engraved aluminum. As shown in **Figure *4.2* (a)**, 10 µM of Paraquat on the ONSPEC-Prime chip or the red line in the figure offers apparent paraquat peaks that are more prominent than ONSPEC-Lite chip. When decreases paraquat concentration to 1 µM, paraquat peaks are not completely present on ONSPEC-Lite as in **Figure *4.2* (b)**. The results indicate ONSPEC-Prime chip has a better SERS response than the ONSPEC-Lite chip for paraquat detection. Moreover, ONSPEC-Prime chips with 100 and 150 nm silver nanorods were also studied to find the optimum dimension that offers a good response to paraquat.

Figure 4.2

SERS Signals of 1 and 10 µM Paraquat on ONSPEC-Prime and ONSPEC-Lite SERS Chips



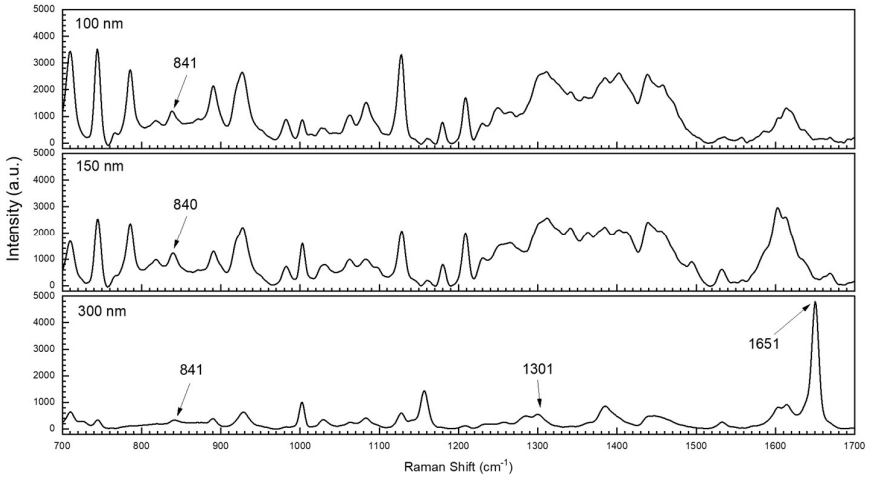


Note. (a) SERS spectra of 10 µM paraquat on both SERS chips with indicated lines of paraquat peaks. (b) Spectra comparison between ONSPEC-Prime and ONSPEC-Lite chips with 1 µM paraquat.

The SERS spectra of 0.01 µM paraquat on 100, 150, and 300 nm of silver nanorods are shown in **Figure *4.3***. The 300 nm can observe paraquat peaks like 841, 1301, and 1651 cm^{-1} as indicated by arrows. For 100 and 150 nm, their responses highly fluctuate. No significant SERS peaks of paraquat are observed except 841 cm^{-1}. This indicates that the response of the ONSPEC-Prime chip with 300 nm to paraquat is preferential.

Figure 4.3

SERS Responses of 0.01 µM Paraquat on 100, 150, and 300 nm ONSPEC-Prime SERS Chips



4.1.2 Improving Speed of Detection by Solvent and Droplet Volume Effects

Typically, the speed of paraquat detection highly depends on the drying time of the analyte on the SERS chip. The effects of paraquat in different solvents on SERS response and drying time are reported in **Table *4.2*** which the SERS spectra of each sample are illustrated in **Figure *4.4***. From the results, paraquat in alcohols, are able to produce paraquat signals as well as fast drying times which is around 15-40 min. However, their signals are not prominent and highly fluctuate in the region of 1550 - 1700 cm^{-1}. As paraquat in deionized water, it shows the clear and sharp peaks of paraquat fingerprints as well as less interference, still, it comes with the longest drying time which is 85 min. Therefore, to decrease the drying time under using the ONSPEC-Prime chip, the effect of different volumes of the droplet of paraquat on the SERS signal was investigated below.

Figure 4.4

SERS Spectra of Paraquat Prepared in Different Solvents and Table of Drying Time

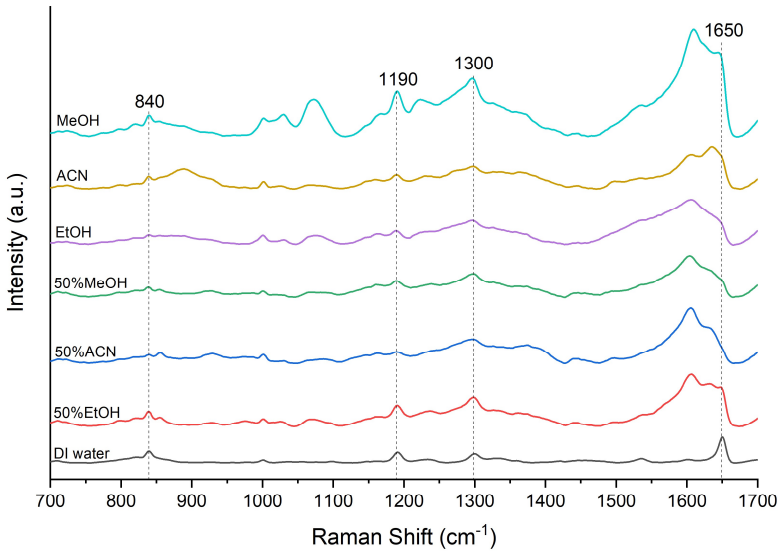


Table 4.2

Table of Drying Time of Paraquat Prepared in Different Solvents

Solvent	Drying Time (min)
MeOH	23
ACN	15
EtOH	21
50%MeOH	35
50%ACN	27
50%EtOH	40
DI water	85

Note. Different solutions were individually dropped on ONSPEC-Prime chips with 10 µL.

In order to reduce the drying time, paraquat was prepared in deionized water at 10 µM and dropped aliquots of 2.5, 5, and 10 µL on the substrates. **Figure *4.5*** depicts the intensity comparison of paraquat peaks with different droplet volumes on ONSPEC-Prime chips and reports the relative standard deviation (RSD) and drying time results in **Table *4.3***. The comparison shows the intensity difference between each volume. At 5 µL(blue column), it gives the strongest intensities with 8-13% RSD, while 2.5 (red column) and 10 (black column) µL are lower intensities. By 2.5 µL possesses the highest RSD which is about 16-34%. Considering the drying time, 2.5 µL consumes 43 min for drying out, while 5 and 10 µL are around 66 and 92 min, respectively.

Figure 4.5

SERS Intensity Comparison between Paraquat Peaks and Droplet Volumes with the Table of RSD and Drying Time

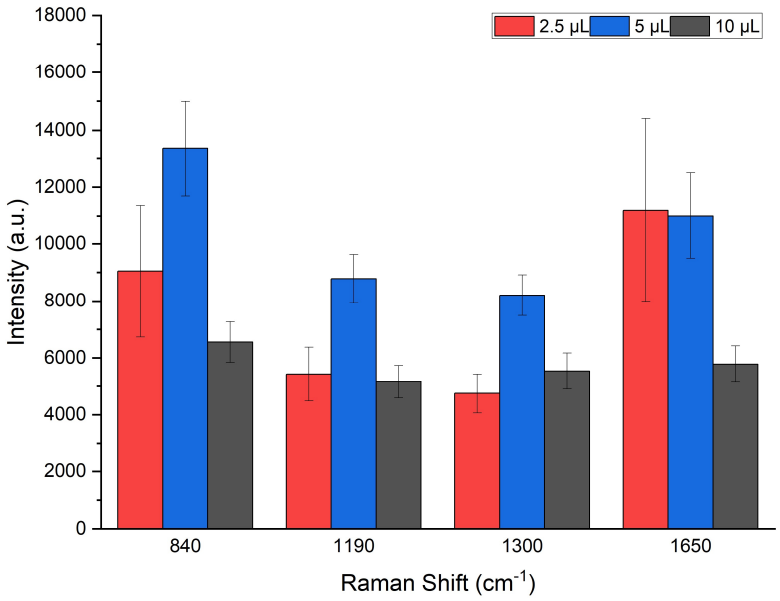


Table 4.3

Table of RSD and Drying Time of Different Droplet Volumes on the Substrate

Droplet Volume (µL)	Percent Relative Standard Deviation (%RSD)				Drying Time (min)
	840 cm^{-1}	1190 cm^{-1}	1300 cm^{-1}	1650 cm^{-1}	
2.5	26.83	19.72	16.73	34.33	43
5	12.36	9.61	8.69	13.66	66
10	10.87	11.19	11.46	11.09	92

Finally, from the experiments, the results indicate that the paraquat sensing of ONSPEC-Prime gave a better signal than the ONSPEC-Lite chip, causing the ONSPEC-Prime chip was suitable for detection. However, due to paraquat being prepared in deionized water, the drying time for detection was up to 85 min. Therefore, the improvement of speed detection by changing the deionized water to organic solvents resulted in 20 – 40 min drying time. Nevertheless, the signals obtained from

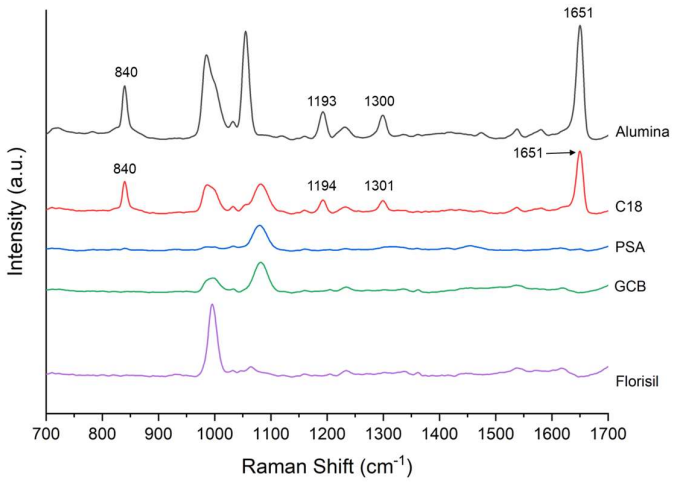
these solvents were quite fluctuations. Hence, the experiment of using deionized water as a solvent and varying the volume of the droplet was conducted. The analysis revealed that the smaller volume gave faster detection.

4.2 Evaluation of Paraquat in Water Sample

To monitor paraquat in real water samples, the extraction protocol was developed by using different sorbents, including GCB, Florisil, PSA, C18, and Alumina. The study focused on selecting the best sorbents for extraction. The extraction is based on QuEChERS extraction. The experiment was conducted with 2.5 µM paraquat prepared in deionized water. After the samples were extracted, the aqueous sample solutions were dropped on the ONSPEC-Prime chips, followed by spectra acquisition. **Figure *4.6*** shows the spectra of extracted paraquat on the ONSPEC-Prime chips. As seen, extracted paraquat with alumina and C18 are present as indicated by significant characteristics of paraquat. Meanwhile, PSA, GCB, and Florisil did not give any peaks.

Figure 4.6

SERS Responses of Extracted paraquat on ONSPEC-Prime chips

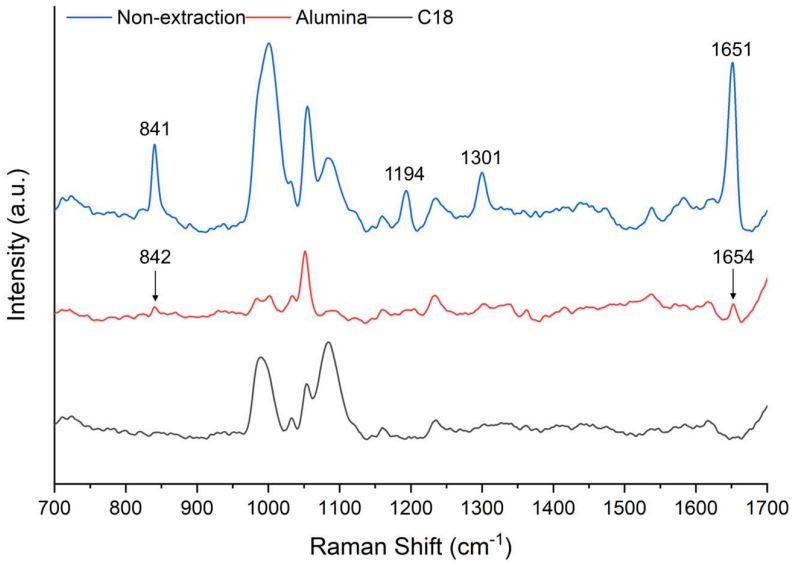


Next, to observe the difference between extraction and non-extraction of paraquat, then the study experimented with the canal water sample from Solar Park, Thammasart University. 1 µM paraquat solutions were prepared in the collected sample with extraction and non-extraction protocols. The spectra of using alumina and C18 for extraction of paraquat in a water sample are compared with non-extraction as revealed

in **Figure *4.7***. All 3 spectra have high noise after signal processing. Using extraction protocol with a practical water sample shows the depleted intensity of paraquat peaks, especially the C18 sample that does not have significant peaks. Therefore, for the detection of paraquat in water samples, the extraction protocol using QuEChERS is unnecessary.

Figure 4.7

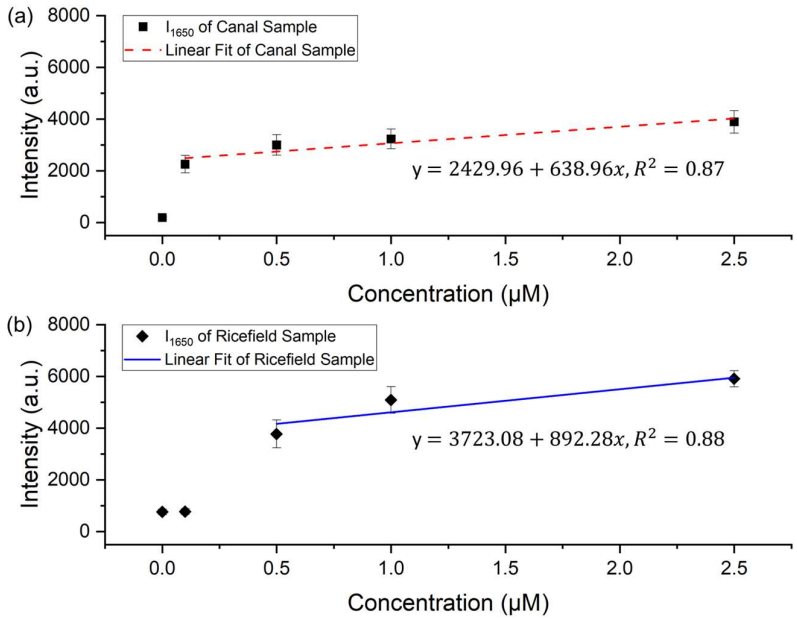
SERS Responses of Extracted paraquat on ONSPEC-Prime chips



Later, the experiment investigated the sensitivity of the ONSPEC-Prime chip for paraquat detection with another type of water sample. In addition to the canal sample, the ricefield sample is collected in Pathumthani. Both samples were prepared with paraquat to obtain 0.1-2.5 µM paraquat in these samples. The intensity of 1650 cm^{-1} is considered the most significant peak of paraquat due to the prominent and intense response. As the calibration curves are plotted in **Figure *4.8***, the slopes in the equations are the sensitivities of ONSPEC-Prime chips that respond to each sample. For the canal sample, the sensitivity is 638.96 a.u./µM while the ricefield sample is 892.28 a.u./µM with the R^2 of 0.87 and 0.88, respectively.

Figure 4.8

Linear Relationships of Paraquat in Different Water Samples



Note. (a and b) The linear relationships between intensity and concentration are plotted to obtain the slopes for determining the sensitivity of the ONSPEC-Prime chip.

4.3 Evaluation of Paraquat in Soil Sample

After the detection of paraquat in the water sample, the study also focused on the detection in the soil sample. At first, loam soil is gathered from AIT. Paraquat was spiked into the soil and then the spiked sample encountered 2 protocols for the experiment: 1. filtration 2. extraction. For the filtration, the soil sample was diluted in deionized water and filtered using filter paper. For the extraction, the soil sample was extracted by using the same protocol as the detection in the water sample. After measuring by Raman spectroscopy, the spectra are compared to each other as demonstrated in **Figure *4.9***. The spectrum of filtration does not have any peaks present, whereas the extractions with alumina and C18 provide the presence of 1190 and 1650 cm^{-1}. As seen in **Table *4.4***, the intensity of alumina is higher than C18, indicating that the extraction with alumina is preferential.

Figure 4.9

SERS Spectra Comparison between Filtration and Extraction with Alumina and C18 for Soil Samples

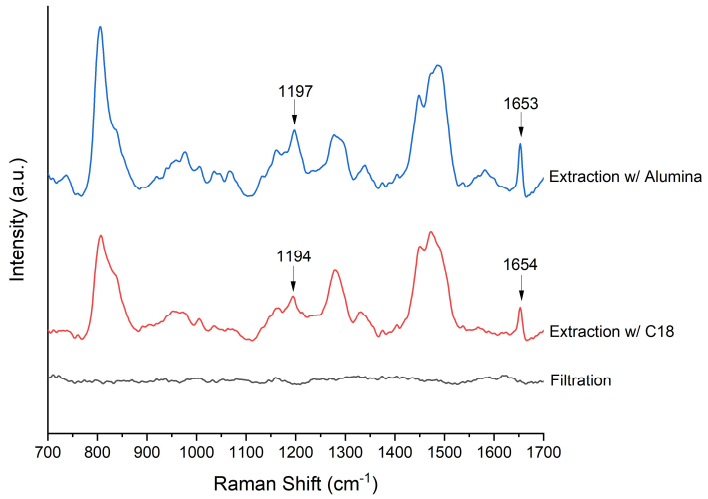


Table 4.4

SERS Spectra Comparison between Filtration and Extraction with Alumina and C18 for Soil Samples

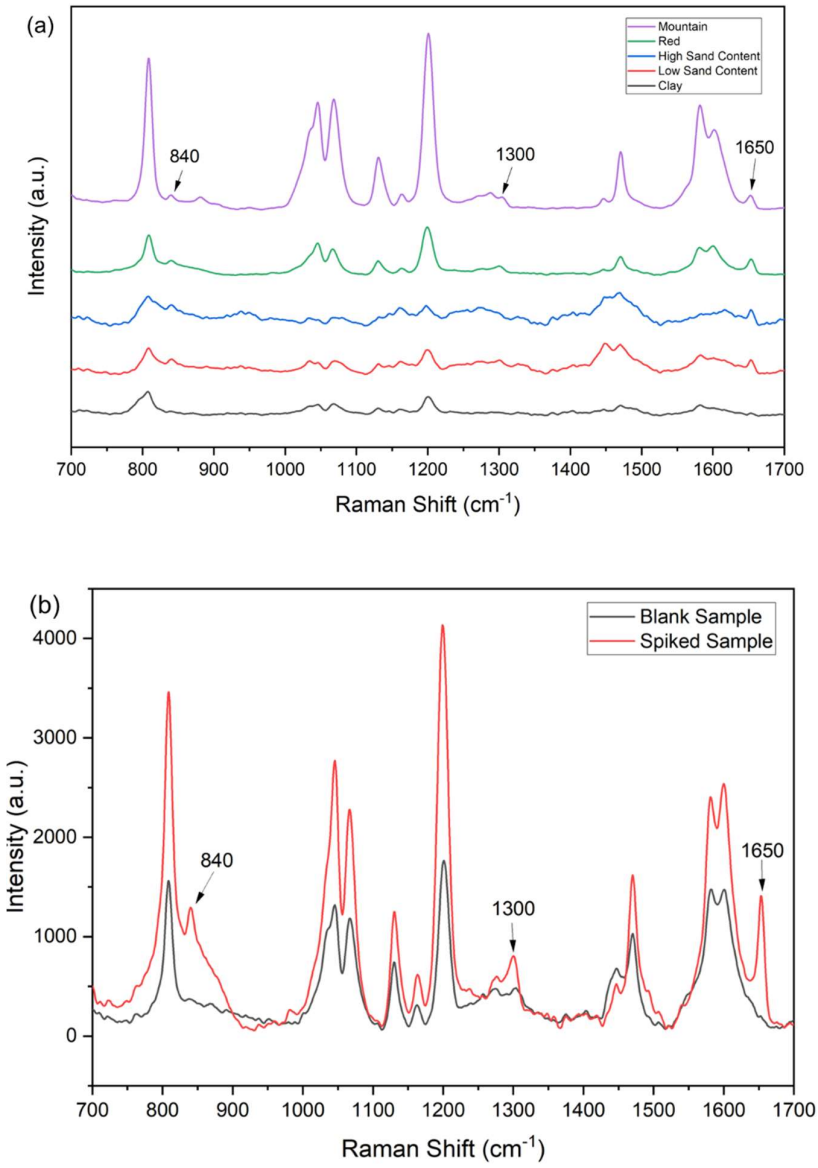
Sample	I_{1190} (a.u.)	$\%RSD_{1190}$	I_{1650} (a.u.)	$\%RSD_{1650}$
Alumina	2230.64	38.39	1798.84	47.72
C18	1498.27	59.91	1153.93	62.83

Besides, the experiment was conducted with different types of soils, including mountain, red, high sand content, low sand content, and clay soils. The spectra of paraquat in these soils are represented in **Figure 4.10 (a)**. As indicated at 840, 1300, and 1650 cm^{-1} of Raman Shift, they reveal the presence of paraquat from the extraction. The paraquat in mountain, red, high sand content, and low sand content soil samples were able to extract by the extraction protocol. For the clay sample, there were no paraquat peaks observed from the sample. In addition, to ensure the company of paraquat, **Figure 4.10 (b)** is illustrated. **Figure 4.10 (b)** is the spectra comparison

between blank and spiked soil samples. It shows that the blank sample is not exposed or related to the paraquat.

Figure 4.10

SERS Spectra of Extracted Paraquat in Different Types of Soil



Note. (a) The spectra of paraquat in the mountain, red, high sand content, low sand content, and clay soils. (b) The spectrum comparison between the blank and spiked samples of the paraquat detection in the soil sample.

For summation, the results of the experiment were summarized as shown in **Table *4.5***. The experiment of paraquat detection in the water sample indicated that the non-extracted sample offered a prominent response over an extracted sample. It was able to detect paraquat at 0.1 µM with a sensitivity of 638.96 a.u./µM. Meanwhile, the detection of paraquat in the soil sample provided a good response from using the extraction protocol. However, the limit of detection of the extraction was high for the real application.

Table 4.5

Table Summarize Results of Water and Soil Samples using ONSPEC-Prime Chip

Samples	Preparation Methods	Brief Result	Sensitivity (a.u./µM)	Limit of Detection
Water Sample	No-Extraction	The spectra were clear and high intensity.	638.96	0.1 µM
	Extraction	The signal was quite poor.	-	1 µM
Soil Sample	Filtration	No response from the signal.	-	-
	Extraction	Alumina provided the best response.	-	1 mM

4.4 Developing a Protocol of Aptamer-modified SERS Chip

In order to enhance the selectivity of the SERS chip, the experiment used a bioreceptor like an aptamer to accomplish this. The aptamer sequence for paraquat detection was adopted from Kuitio's work at Kasetsart University where the aptamer is based on Au nanoparticles. Therefore, the experiment was conducted with the ONSPEC-Lite SERS chip. The investigated parameters for aptamer immobilization were aptamer concentrations and incubation time. The substrates were initially cleaned by using a plasma surface treatment system, followed by aptamer immobilization as below sections.

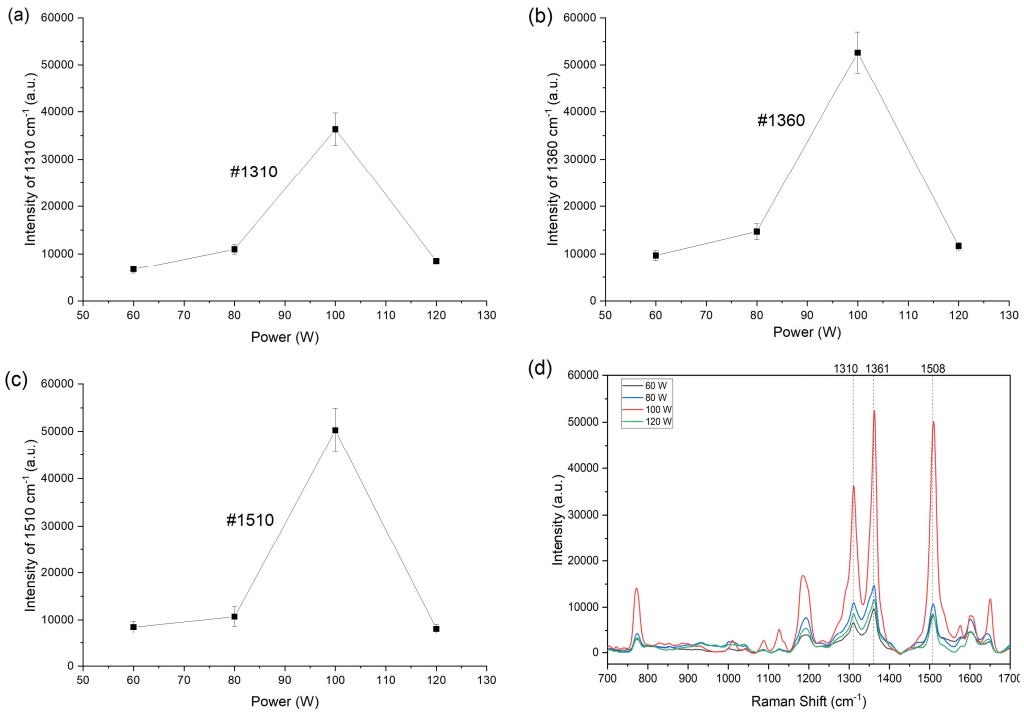
4.4.1 Preparation of the Substrate by Plasma Cleaning

The system uses oxygen gas at 5 psi for cleaning. Cleaning power and time were varied into the ranges of 60-120 Watt and 1-3 min to obtain an optimum condition. The experiments were conducted with Rhodamine 6G as a Raman reporter where the SERS

characteristic peaks of R6G are 1310, 1360, and 1510 cm^{-1}. As shown in **Figure *4.11***, shows the SERS spectra and intensity-power relationships of R6G in different plasma cleaning power. **Figure *4.11* (a-c)** show that intensities of R6G peaks are increased as the cleaning power is increased from 60 Watt to 100 Watt. Then significantly drop when they reach 120 Watt. A further experiment was the variation of cleaning time by fixing the cleaning power at 100 Watt. **Figure *4.12*** illustrates the SERS spectra and intensities comparison of R6G in different cleaning times. **Figure *4.12* (a)** and **(b)** represent the decreasing trend when time is increased. From both results of variations of cleaning power and time, an optimum condition to prepare the substrate for aptamer immobilization is 100 Watt cleaning power for 1 min.

Figure 4.11

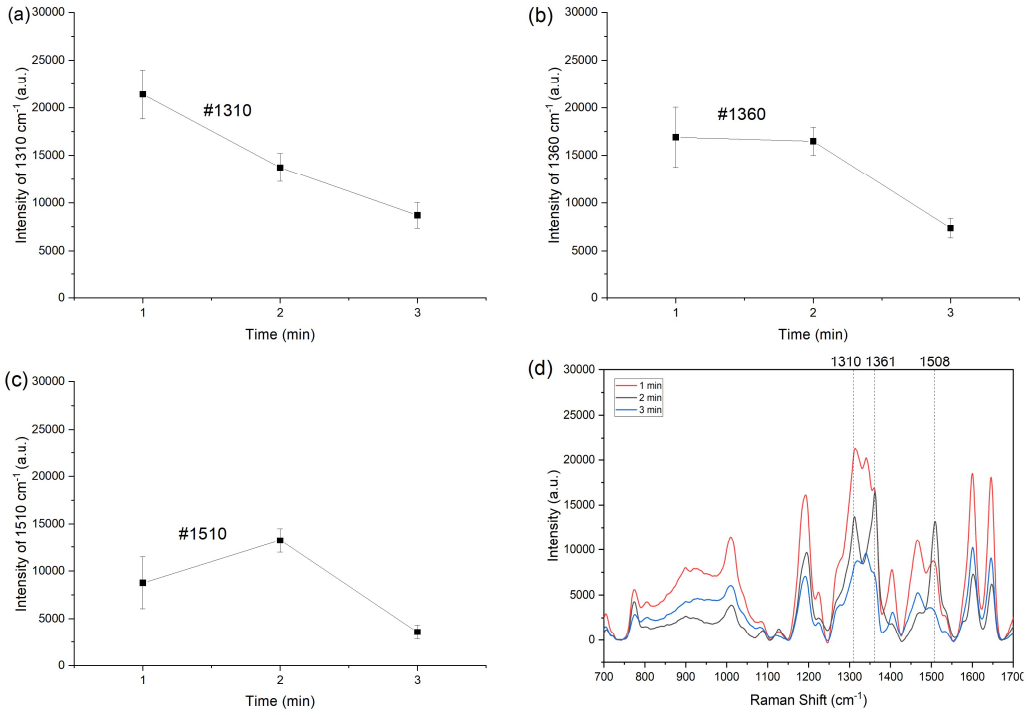
Peak Intensities of R6G with respect to the Plasma Cleaning Power and SERS Spectra of 10 µM R6G on Cleaned Substrate



Note. (a-c) The relationships between the intensity of R6G peaks and cleaning power. (d) SERS spectra of 10 µM R6G on ONSPEC-Lite chip with 60-100 Watt cleaning power.

Figure 4.12

Peak Intensities of R6G with respect to the Plasma Cleaning Time and SERS Spectra of 10 µM R6G on Cleaned Substrate



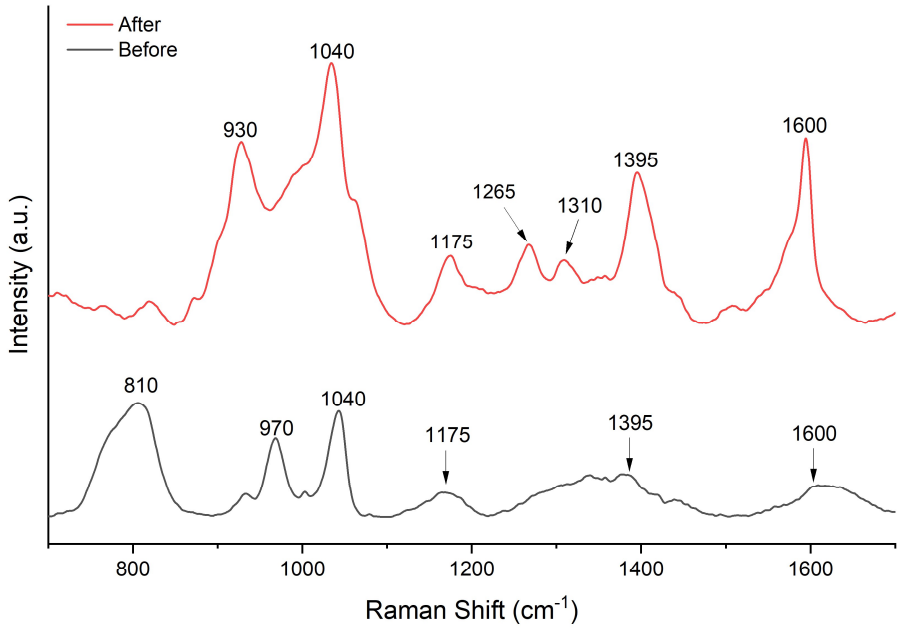
Note. (a-c) The relationships between the intensity of R6G peaks and cleaning time. (d) SERS spectra of 10 µM R6G on ONSPEC-Lite chip with 1-3 min cleaning time.

4.5 Aptamer Immobilization of ONSPEC-Lite SERS chip

After obtaining a cleaned ONSPEC-Lite chip, the experiment further encountered the immobilization and then washing steps to ensure that the aptamer was successfully bonded to the chip. **Figure *4.13*** shows the SERS spectra comparison between before and after immobilization. As a result, observed prominent peaks of aptamer on the chip after the sample which are 930, 1265, and 1310 cm^{-1}. To find out an optimum condition, the experiment started with studying the effects of incubation time, aptamer concentrations, and testing methods, respectively.

Figure 4.13

SERS Signal Comparison between Before and After of Aptamer Immobilization



4.5.1 Effects of Incubation Time and Aptamer Concentration on Paraquat Detection

The incubation time was the first parameter. The experiment used 1 µM aptamer as the initial concentration and fix the incubating temperature at room temperature. Then the study varied the incubation time for immobilization which ranges from 20 to 40 min. **Figure *4.14*** illustrates the spectra of paraquat with 20-40 incubation time for aptamer immobilization. Paraquat peaks are remarked with 4 symbols as shown in the legend of the figure. Considering the amounts of intensity and standard deviation, there is a decreased trend between the intensity of 1650 cm^{-1} and the time as demonstrated in **Figure *4.15***. The figure comes with a linear fitting line and equation in which R^2 (coefficient of determination) is about 0.99. From both figures, the spectrum and intensity of 20 min are the highest. Still, 20 min sample also comes with the largest standard deviation as indicated in **Table *4.6***, while 30 min sample offers high, prominent, and clear peaks as well as the smallest standard deviations as shown in **Figure *4.15*** and **Table *4.6***. As a result, further aptamer experiments were conducted with 30 min incubation time.

Figure 4.14

Signal Comparison of Paraquat on Aptamer-Substrate with Different Incubation Time

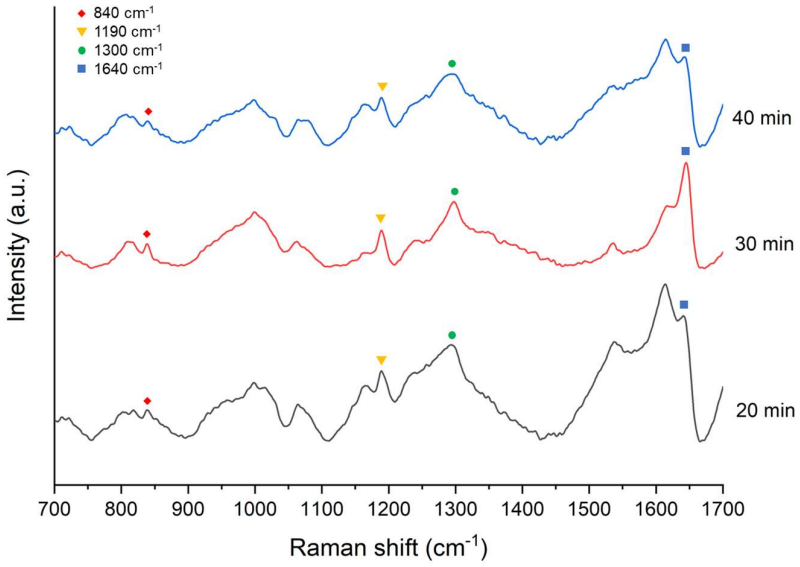


Figure 4.15

Linear Relationship between the Intensity of 1650 cm^{-1} and Incubation Time

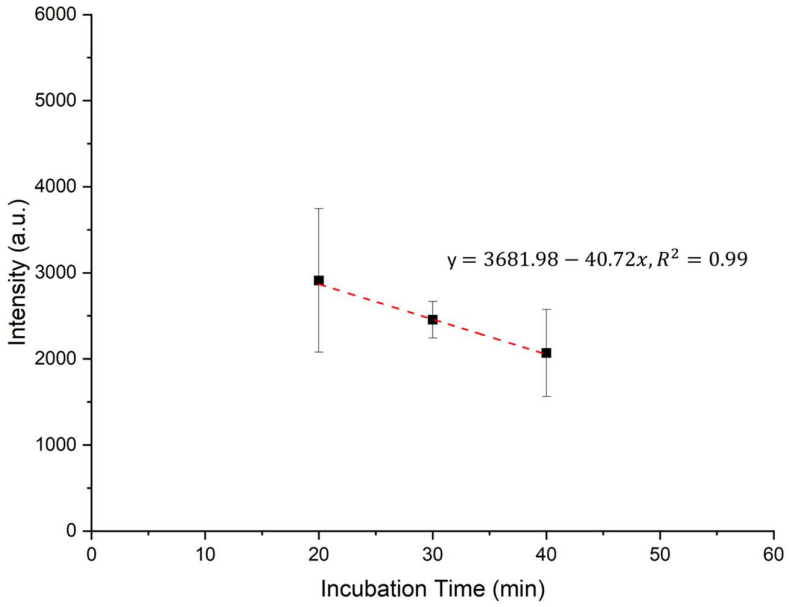


Table 4.6

Table of Intensity and RSD against the Time

Incubation Time (min)	I_{1650} (a.u.)	$\%RSD_{1650}$
20	2911.22	28.66
30	2454.73	8.66
40	2069.28	24.48

After obtaining a time for immobilization, the experiment continuously studied the effect of aptamer concentration. The concentration was ranging from 0.01 to 10 µM. The spectra of different aptamer concentrations are shown as shown in **Figure *4.16***. As shown in **Figure *4.17*** and **Table *4.7***, the intensities of 930, 1265, and 1310 cm^{-1} of different aptamer concentrations are compared together. From the results, even though there are no trends in any peak, RSD was considered to obtain an optimum concentration. It indicates that 0.1 µM is the best consistent with RSD of 7.9, 27.6, and 7.9%, respectively to Raman shift of 930, 1265, and 1310 cm^{-1}. In addition, this experiment was also conducted with paraquat to ensure the results.

Figure 4.16

SERS Spectra of Different Aptamer Concentrations

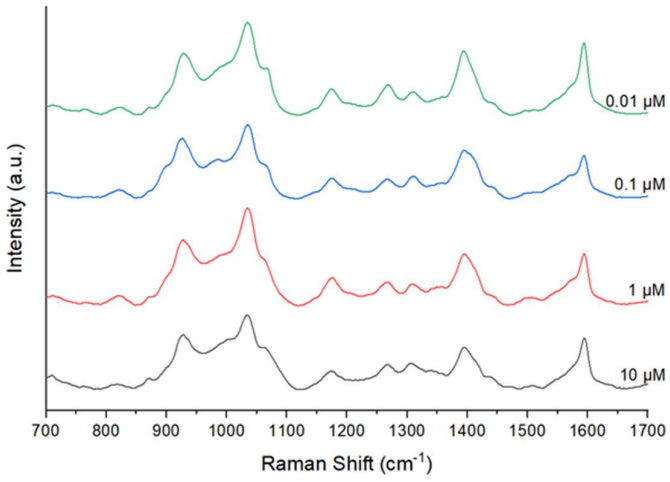


Figure 4.17

Intensity Comparison of Aptamer Peaks and SERS Spectra

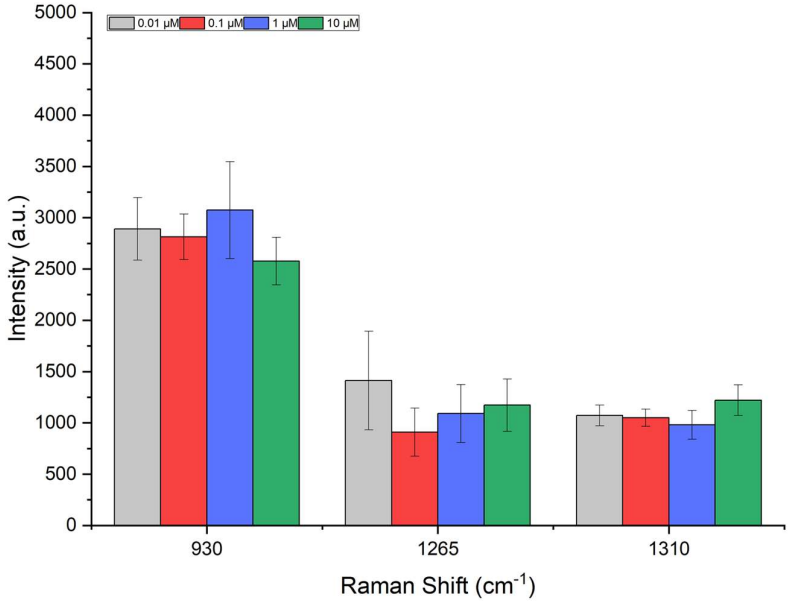


Table 4.7

Table of Intensity and RSD of Aptamer Peaks

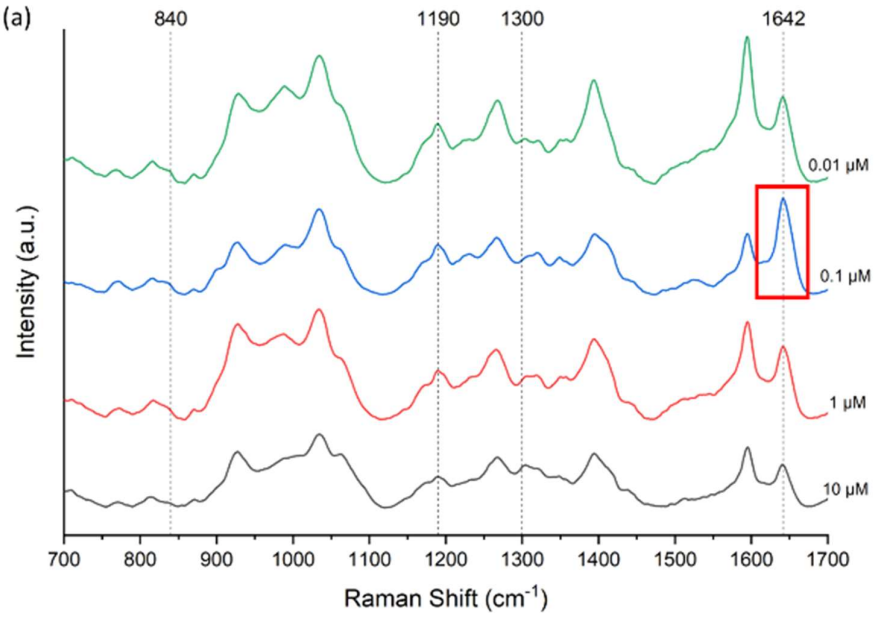
Aptamer Concentration (µM)	#930		#1265		#1310	
	I (a.u.)	RSD (%)	I (a.u.)	RSD (%)	I (a.u.)	RSD (%)
0.01	2890.59	10.5	1412.24	33.9	1071.55	9.4
0.1	2813.04	7.9	885.61	27.6	1052.96	7.9
1	3068.96	15.4	1097.77	26.9	987.74	14
10	2573.9	9	1172.05	21.5	1221.65	12.2

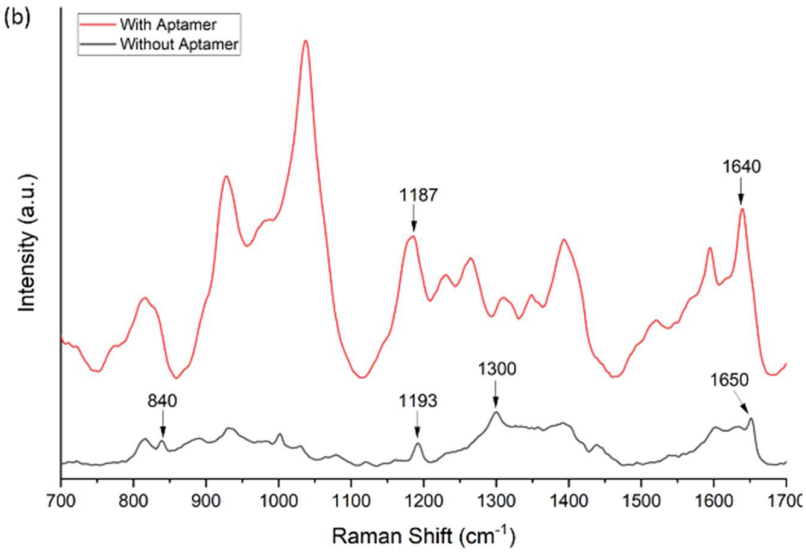
An aliquot of 2.5 µL of 10 µM paraquat was dropped on the substrates, followed by a Raman measurement. **Figure *4.18* (a)** demonstrates the spectra of paraquat on the aptamer-modified substrates. Even though paraquat's fingerprint peaks are present in

each concentration sample, there are no trends observed on any peak of aptamer and paraquat as mentioned above. At 840 and 1300 cm^{-1} in every sample, it does not have clear peaks were observed. On the other hand, at 1190 and 1650 cm^{-1}, they are able to be observed. At 1650 cm^{-1}, it likely shifts from 1650 to around 1640 cm^{-1} as shown in **Figure 4.18 (b)**. From the results, The 0.1 µM sample offers the strongest intensity of 1642 cm^{-1} with low RSD as remarked in **Figure 4.18 (a)** and depicted in **Table 4.8**. The table shows the intensity and RSD of 1190 and 1650 cm^{-1} for considering the optimum conditions. As mentioned in the earlier experiment, with low RSD in aptamer and paraquat, causing 0.1 µM is an optimum aptamer concentration for further experiment.

Figure 4.18

SERS Spectra of Paraquat on Aptamer-Modified Substrates.





Note. (a) Spectra of paraquat on chips with different aptamer concentrations, ranging from 0.01 to 10 μM. (b) SERS responses of paraquat on chips with and without aptamer. It indicates the shift from 1650 to 1640 cm^{-1} of paraquat peak when tested with the aptamer-modified substrate.

Table 4.8

Table of Intensity and RSD of Observed Paraquat Peaks on Aptamer-Modified Substrate

Aptamer Concentration (μM)	I_{1190} (a.u.)	$\%RSD_{1190}$	I_{1650} (a.u.)	$\%RSD_{1650}$
0.01	2086.33	14.8	3052.4	10.65
0.1	1752	10.77	3401.72	10.81
1	1713.92	11.13	2582.84	7.83
10	1086.28	14.57	1518.97	9.32

To indicate the best immobilization protocol for the aptamer, the following procedure is brief and shown below:

- **Step 1**: The plasma surface treatment system was used to clean the ONSPEC-Lite chip by using oxygen gas at 5 psi with 100 Watt plasma power and 1 min cleaning time.
- **Step 2**: An aptamer solution was prepared from the stock solution. Then TCEP reducing agent solution was added to the aptamer solution, followed by heating at 90°C for 5 min. Finally, incubating at room temperature for 15 min.
- **Step 3**: Immersed the cleaned substrate into the prepared aptamer solution for 30 min. The washing with PBS pH 7.4 was performed for 15 min.
- **Step 4**: The chip was dried at room temperature and recombined with the glass slide. The chip was obtained and ready for paraquat detection.

4.5.2 Effect of Testing Method on Paraquat Detection

Next, the experiment focused on the testing method. Typically, ONSPEC SERS chips are SERS substrates based on the drop-dry method. This results in simple testing, fast detection, and diverse applications. The change of testing method from the drop-dry method to the dipping method causes the detection to be longer than 20 min. However, the purpose of the dipping method is to enhance the sensitivity and LOD of the aptamer-modified substrate. The spectrum of paraquat with the drop-dry method is compared with the dipping method as revealed in **Figure *4.19***. The dipping method has a very high and strong signal where paraquat peaks are fully present. For the dropping method, peaks of 1190 and 1640 cm^{-1} are only present. Moreover, the intensities of paraquat peaks of the dipping method are higher and RSD are also lower than the dropping method as indicated in **Table *4.9***. Due to the high intensities and low RSD of the dipping method, therefore the study agrees to experiment with the dipping method.

Figure 4.19

SERS Spectra of Paraquat on Aptamer-Modified Substrates

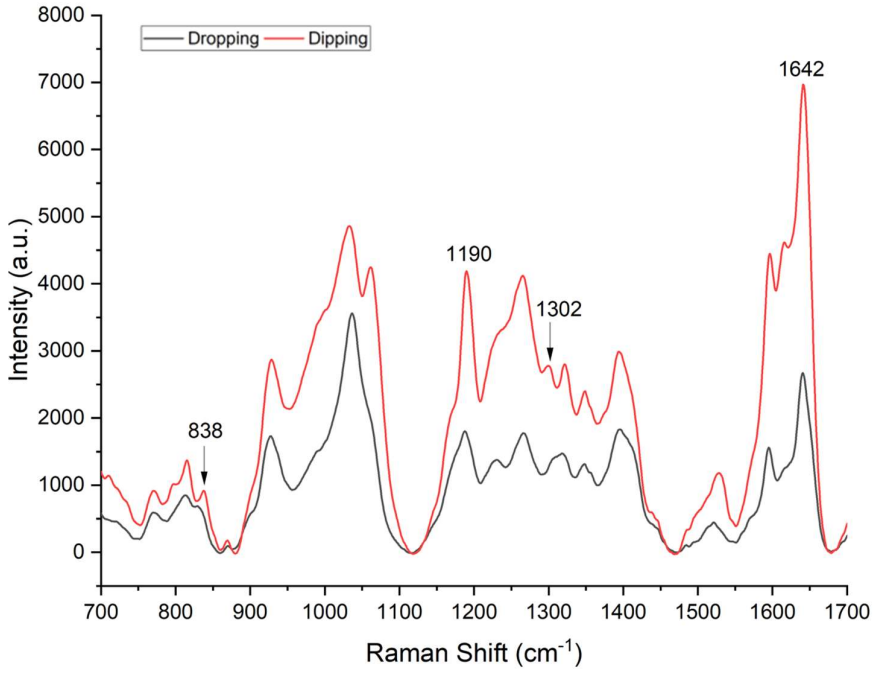


Table 4.9

Table of Intensity and RSD for Method Comparison

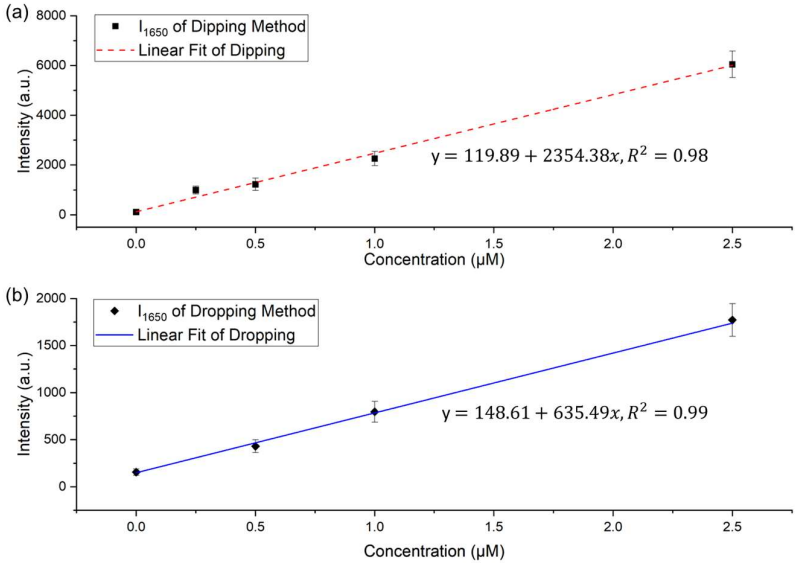
Method	#840		#1190		#1300		#1650	
	I (a.u.)	RSD (%)	I (a.u.)	RSD (%)	I (a.u.)	RSD (%)	I (a.u.)	RSD (%)
Dropping	449.15	22	1806.28	7.6	1368.49	8.99	2672.25	7.66
Dipping	865.67	24.59	4188.66	6	2778.99	7.36	6968.96	3.81

Additionally, the intensities of 1650 cm^{-1} of dropping and dipping methods are drawn against the paraquat concentration where the linear fits are shown in **Figure *4.20***. The dipping and dropping methods show increasing trends of intensity when the concentration is increased as well as offer the R^2 of 0.98 and 0.99, respectively. From the fitting, the dipping method is able to detect at 0.25 µM paraquat while the dropping

method can only reach 0.5 µM. Both sensitivities are obtained from the slopes of linear fits in which the dipping and dropping methods are 2,354.38 and 635.49 a.u./µM, respectively. It is clear that the sensitivity of the dipping method is better than the dropping method.

Figure 4.20

Linear Relationships between the Intensity of 1650 cm^{-1} and Paraquat Concentration



Note. (a and b) The standard curves of the dipping and dropping method are represented as the linear relationships between intensity and concentration.

4.6 Comparison between Aptamer and Non-Aptamer Substrates

In this section, the study focuses on sensitivity, detection limit (LOD), and selectivity of the aptamer-modified substrate. The aptamer-modified substrates were prepared according to the mentioned protocol. For the no-aptamer substrate, the substrate was performed only plasma cleaning.

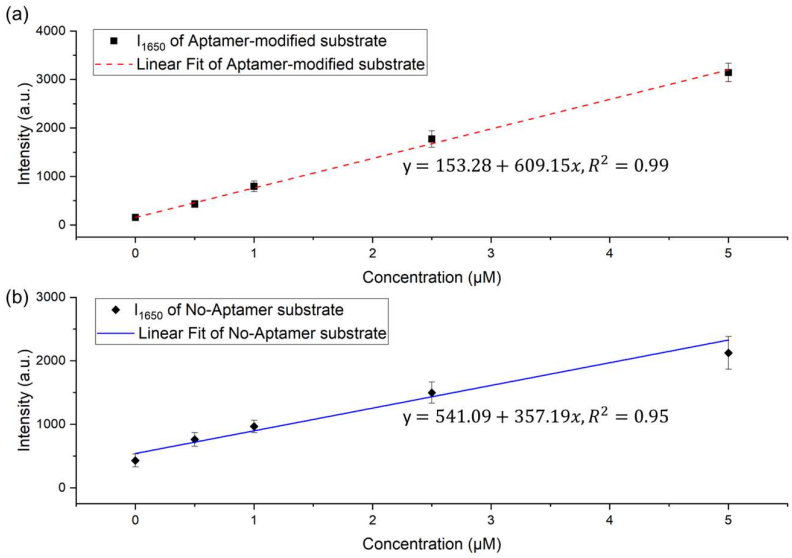
4.6.1 Sensitivity and Limit of Detection (LOD)

For sensitivity, 0.5, 1, 2.5, and 5 µM are paraquat concentrations for the study. The testing method is the drop-dry method. The sensitivities are obtained from the slopes of the calibration curves that are shown in **Figure *4.21***. From the figure, the sensitivity of the aptamer-modified substrate is 609.15 a.u./µM with an R^2 of 0.99 while the no-

aptamer substrate is 357.19 a.u./µM with an R^2 of 0.95. For LOD, both substrates are able to detect at 0.5 µM of paraquat concentration. However, LOD can determine from the calculation by using standard curves in **Figure *4.21*** and **Equation 3.1**. As a result, the LODs of aptamer and no-aptamer substrates are 0.35 and 1.12 µM, respectively.

Figure 4.21

Linear Relationships between the Intensity of 1650 cm^{-1} and Paraquat Concentration



Note. (a and b) The linear relationships between intensity and concentration of aptamer-modified substrate and no-aptamer substrate are plotted to obtain sensitivities from the slopes.

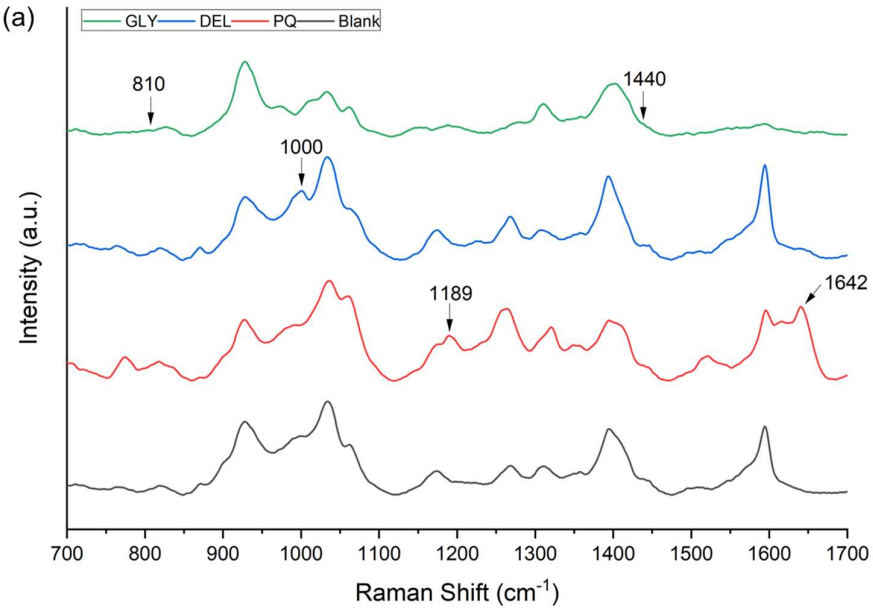
4.6.2 Selectivity

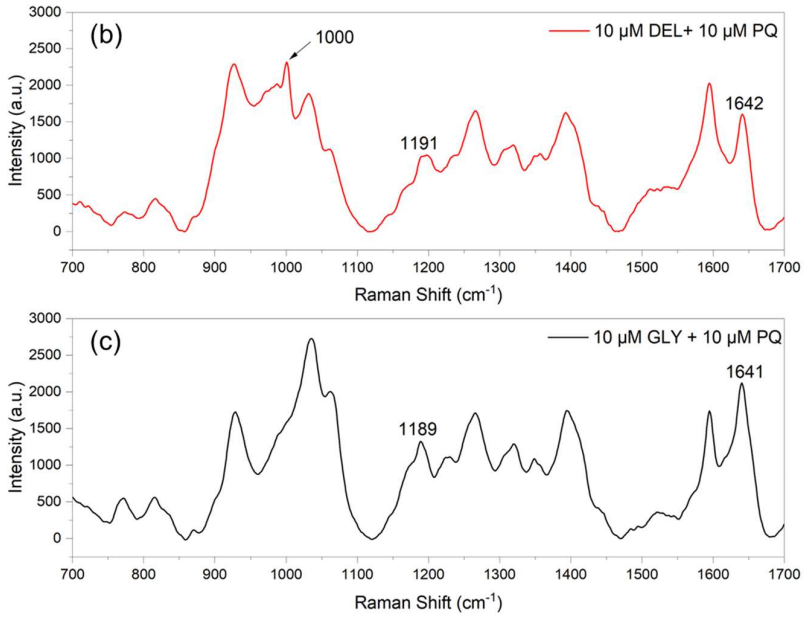
To determine the selectivity, the aptamer-modified substrate was tested with paraquat and two pesticides, including glyphosate (herbicide) and deltamethrin (insecticide). The analytes are prepared at 10 µM and mixed to obtain the mixture solution. The detection method is the same as mentioned above. The spectra of glyphosate and deltamethrin on the aptamer-modified substrates are compared with paraquat and illustrated in **Figure *4.22* (a)**. The indicated arrows are the strong response characteristics of glyphosate, deltamethrin, and paraquat. From the result, the Raman shift of 810 and 1440 cm^{-1} of glyphosate's characteristics are not present in the green line of **Figure *4.22* (a)**. On the other hand, the observed 1000 cm^{-1} in the blue line is the strong wavenumber, indicating the presence of deltamethrin. As shown in **Figure 4.22 (b and c)**, it is the spectra of the

mixture solution between paraquat and deltamethrin/glyphosate. From **Figure 4.22 (b)**, the significant peaks of deltamethrin and paraquat can be observed. Likewise, only paraquat can be monitored in the mixture solution of glyphosate and paraquat as depicted in **Figure 4.22 (c)**.

Figure 4.22

Spectra of Different Analytes on the Aptamer-modified Substrate



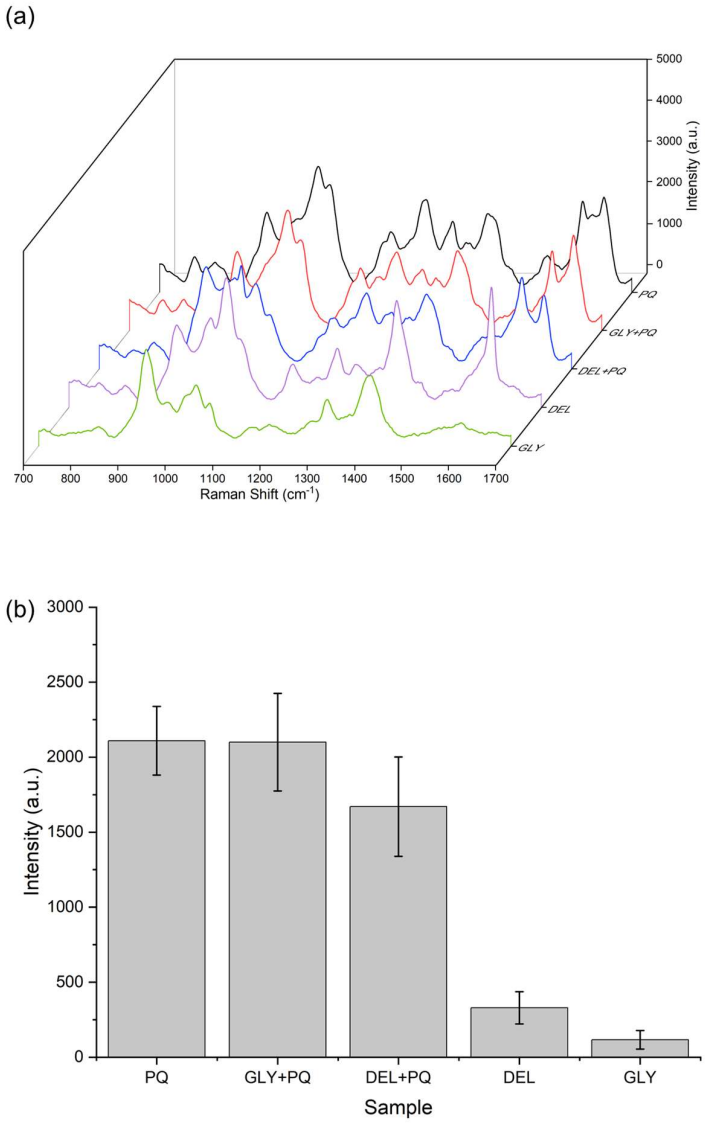


Note. (a) SERS spectra of 10 μM glyphosate, deltamethrin, and paraquat are compared with the spectrum of the aptamer-modified substrate (blank). (b and c) Using the aptamer-modified substrate to detect paraquat in different mixture solutions.

In order to finalize the selectivity, the spectra of glyphosate, deltamethrin, paraquat, and mixture solutions are plotted as in **Figure *4.23* (a)**. The intensity comparison in **Figure *4.23* (b)** selected the intensity of 1650 cm^{-1} for the comparison. As demonstrated in **Table *4.10***, for paraquat and glyphosate/paraquat, the intensity is 2099.73 a.u. with an RSD of 15 %. Meanwhile, the intensity of deltamethrin/paraquat is lower with 1670.22 a.u. and 19.84 % of RSD. In the case of deltamethrin and glyphosate, the amounts of intensity are depleted with high error bars. Hence, these results indicate that aptamer-modified substrate is highly selective to the paraquat in the mixture solution.

Figure 4.23

Spectrum and Intensity Comparison of Different Samples on Aptamer-Modified Substrate



Note. (a) Spectra of paraquat, glyphsate/paraquat, deltamethrin/paraquat, deltamethrin, and glyphosate on aptamer-modified substrate. (b) Intensity comparison for determining the selectivity, with the table of intensity and RSD in the inset of the figure.

Table 4.10

Table of Intensity and RSD of 1650 cm^{-1} of Different Samples

Sample	I_{1650} (a.u.)	$\%RSD_{1650}$
PQ	2108.83	10.85
GLY+PQ	2099.73	15.51
DEL+PQ	1670.22	19.84
DEL	329.26	32.72
GLY	115.79	53.5

CHAPTER 5
CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In the early stages, the experiment successfully optimized the SERS substrate for the detection of real practical samples. Initially, both ONSPEC-Prime and Lite chips were studied the preferential paraquat detection. The results indicate that ONSPEC-Prime offered strong spectra of paraquat. Both chips further experimented with the improvement of detection speed. The effects of solvents and droplet volumes indicated that detection speed depended on these two parameters. Therefore, to achieve rapid detection while maintaining strong SERS responses, the organic solvent should be prevented and the droplet volume for detection ought to be 2.5 µL.

Next, the obtained condition was implemented with the extraction protocol for field sample monitoring. The extraction protocol was based on QuEChERS extraction. Both water and soil samples were spiked with paraquat at a concentration. The extraction protocol was carried out with 5 different absorbents such as alumina, C18, GCB, PSA, and florisil. The extracted solution was then performed by Raman measurement. The results revealed that extraction with alumina offered the best signal in the soil sample when compared with the filtration method. Meanwhile, the extraction did not show a good result for the water sample. Without extraction protocol for paraquat monitoring in the water samples, the canal sample gave a sensitivity of 638.96 a.u./µM lower than the ricefield sample which is 892.28 a.u./µM. However, the detection limit of the canal sample was lower than the ricefield sample which was 0.1 and 0.5 µM accordingly.

In the last experiment, the study successfully developed an aptamer-modified ONSPEC-Lite SERS chip for paraquat detection. The substrate started with cleaning with a plasma surface treatment system. The aptamer selective paraquat with thiol modifier was chosen for the immobilization on the ONSPEC-Lite chip. At first, the optimum incubation time and aptamer concentration were met by the observation of SERS spectra of R6G. Therefore, to immobilize the aptamer on the cleaned chip, the aptamer solution was prepared at 0.1 µM and then immersed the chip in the aptamer solution for 30 min. The obtained aptamer-modified substrate have tested with a different concentration of paraquat to determine the sensitivity and the limit of

detection. The results showed that aptamer-modified substrate with the dipping method for paraquat detection, giving the sensitivity as high as 2,354.38 a.u./μM while the detection limit was 0.25 μM. Moreover, the selectivity test was also conducted with glyphosate and deltamethrin. The intensity of 1650 cm^{-1} was used to indicate the presence of paraquat in the prepared mixture solutions, resulting in an aptamer-modified substrate highly selective to the paraquat.

5.2 Recommendation

In this study, the aim is to create a biosensor that is able to detect paraquat in practical samples. The study focused on utilizing aptamer as a biorecognition and using a SERS technique as a transducer. Due to the combination of aptamer and SERS chip, an aptamer-modified substrate was developed. This substrate has good enough results that show the potential for paraquat detection in real applications.

However, the aptamer-modified substrate is limited to the detection limit which is 0.25 μM. The detection limit is still higher than Australia's maximum residue limit of paraquat in drinking water which is 30 μg/L or 0.11 μM. To achieve a lower limit of detection, the study could improve the aptamer immobilization by varying the incubation temperature and utilizing the blocking agent to ensure that the presence of paraquat on the substrate was the true positive result. Moreover, the aptamer-modified substrate is also limited to the long preparation and laboratory-based. Therefore, the design and development of short preparation, portable, and practical use will make a chance for paraquat monitoring use in real-world applications, to enrich the human being and living organisms as well as the environment.

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