

DETECTION OF FORCHLORFENURON IN GRAPES BY SURFACE-
ENHANCED RAMAN SPECTROSCOPY AND HPLC

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By

HAOBING QIAN

Dr. Mengshi Lin, Thesis supervisor

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The undersigned, appointed by the dean of the Graduate School,

have examined the thesis entitled

**DETECTION OF FORCHLORFENURON IN GRAPES BY
SURFACE-ENHANCED RAMAN SPECTROSCOPY AND HPLC**

Presented by HAOBING QIAN

A candidate for the degree of Master of Science

And hereby certify that, in their opinion, it is worthy of acceptance.

Dr. Mengshi Lin, Food Science Department

Dr. Fu-hung Hsieh, Food Science Department

Dr. Liqun Gu, Biological Engineering Department

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	ii
LIST OF FIGURES	vi
LIST OF TABLES	viii
ABSTRACT	ix
CHAPTER 1	1
INTRODUCTION	1
1.1 Background.....	1
1.2 Objective.....	2
CHAPTER 2	4
REVIEW OF LITERATURE	4
2.1 Food contaminants.....	4
2.1.1 Pesticides.....	5
2.2 Conventional methods for detecting chemical contaminants	8
2.3 Vibrational spectroscopy	10
2.3.2 Surface-Enhance Raman Spectroscopy (SERS).....	14
2.3.3 SERS substrates	18
2.3.4 Applications of SERS in Food Science.....	19
CHAPTER 3	20
MATERIALS AND METHODS.....	20
3.1 Materials	20
3.2 Sample preparation.....	20
3.3 SERS Substrates	21
3.4 SERS Measurement.....	21
3.5 Data Analysis.....	22
CHAPTER 4	25

Results and Discussion	25
4.1 SERS measurement for pure forchlorfenuron	25
4.1.1 SERS spectra vs. normal Raman spectra.....	25
4.1.3 Second derivative transformation.....	28
4.1.4 Partial least squares analysis (PLS).....	29
4.2 SERS measurement for forchlorfenuron extracted from grape skin.....	32
4.2.1 Characteristic SERS spectra pattern for forchlorfenuron extraction at different concentrations	32
4.2.2 Second derivative transformation.....	34
4.2.3 Partial least squares analysis (PLS).....	34
4.3.4 Detection lemit (DL)	36
4.3.5 Recovery based on SERS method.....	36
4.3.6 HPLC verification	37
CHAPTER 5	39
CONCLUSIONS.....	39
REFERENCES	40
VITA	46

LIST OF FIGURES

Figure	Page
Figure 1. The outline of the experimental design.	3
Figure 2. Molecular structure of forchlorfenuron.	8
Figure 3. The major stages in an analytical procedure for measuring pesticide in fruit and vegetables.	9
Figure 4. Rayleigh and Raman scattering processes.	14
Figure 5. Illustration of the localized surface plasmon resonance effect (LSPR) (Tu and Chang 2012).	17
Figure 6. Normal Raman spectra and SERS spectra of 5 ppm forchlorfenuron solution on gold slide and Q-SERS. Black line: forchlorfenuron solution tested on Q-SERS; Red line: forchlorfenuron solution tested on gold slide. Data were processed with smoothing at 4 cm^{-1} cm^{-1}	26
Figure 7. Average SERS spectra (n=5) of different concentrations of pure forchlorfenuron solutions. Spectra were processed by smoothing at 4 cm^{-1} and 2^{nd} polynomial subtraction for baseline adjustment.	27
Figure 8. Second derivative transformation of average SERS spectra (n=5) at 1000 cm^{-1} for different concentrations of pure forchlorfenuron solutions.	29
Figure 9. RMSEP values of the partial least squares (PLS) models with different latent variables.	30
Figure 10. Predicted concentration (ppm) vs. actual concentration (ppm) of pure forchlorfenuron solutions using PLS models. Data processed with smoothing at 4 cm^{-1} , 2^{nd} polynomial subtraction for baseline adjustment, four latent variables, spectral region from $600\text{-}1800\text{ cm}^{-1}$, spectral number = 40.	31
Figure 11. Average SERS spectra (n=7) of different concentrations of forchlorfenuron solutions extracted from grape skins. Spectra were processed by smoothing at 4 cm^{-1} and 2^{nd} order polynomial subtraction for baseline adjustment.	33
Figure 12. Second derivative transformation of average SERS spectra (n=7) for different concentrations of forchlorfenuron solutions extracted from grape skins at 1000 cm^{-1}	34
Figure 13. RMSEP values of the PLS models with different latent variables.	35

Figure 14. Predicted forchlorfenuron concentration (ppm) vs. actual puforchlorfenuron concentration (ppm) extracted from grape skin using PLS models at lowest latent variable. Data processed with smoothing at 4 cm^{-1} , a 2nd order polynomial subtraction for baseline adjustment, seven latent variables, spectral region from $600 - 1800\text{ cm}^{-1}$, spectral number = 35. 35

Figure 15. Calibration curve for forchlorfenuron acquired by HPLC. 38

LIST OF TABLES

Table	Page
Table 1. Band assignments of predominant peaks in SERS spectra of forchlorfenuron .	28
Table 2. Calculation of detection limits (DL) of SERS method for forchlorfenuron	32
Table 3. Recovery of pure forchlorfenuron solutions	37
Table 4. Recovery of forchlorfenuron solutions extracted from grape skins based on HPLC calibration curve	38

DETECTION OF FORCHLORFENURON IN GRAPES BY SURFACE- ENHANCED RAMAN SPECTROSCOPY AND HPLC

Haobing Qian

Dr. Mengshi Lin, Thesis Supervisor

ABSTRACT

The objective of this study was to use surface-enhanced Raman spectroscopy (SERS) for rapid detection and characterization of trace amounts of forchlorfenuron extracted from fruits. Forchlorfenuron is a plant growth regulator widely used in grapes. In this study, gold-coated nanosubstrates (Q-SERSTM) were used for SERS measurements. Partial least squares (PLS) analysis was used as a statistical method for quantitative analysis of the spectral data. Our results demonstrate that enhanced Raman signals acquired from samples exhibited characteristic spectral patterns. The detection limit for forchlorfenuron by SERS is 3.14 ppm at 99.86% confidence interval. The PLS results for quantification of forchlorfenuron were obtained: $R = 0.96$, $RMSEP = 9.336$ ppm. In addition, HPLC was also used to measure forchlorfenuron and verify SERS results. A good linear relationship was observed between 0.1 - 100 ppm with an R value of 0.999 at 260 nm. These results demonstrate that SERS coupled with gold nanosubstrates is a rapid and simple method that requires little sample preparation. It could be a practical and

economical approach to combine SERS with HPLC to screen and analyze large number of food samples for detecting chemical contaminants and residues in fruits.

CHAPTER 1

INTRODUCTION

1.1 Background

Forchlorfenuron or CPPU (1-[2-chloro-4-pyridyl]-3-phenyl urea) is a synthetic cytokinin, approved by the US Environmental Protection Agency (EPA) in 2004 as a plant growth regulator (EPA 2004). Forchlorfenuron is used in grapes, raisins, kiwifruits, and melons together with auxins synergistically to improve fruit size and weight.

Although there are few reports regarding the safety issue of forchlorfenuron except for its kidney toxicity based on a 2-year rat feeding study (EPA, 2004), excessive use of forchlorfenuron can cause exploding of fruits, contamination of the environment and negative long-term effects on human health.

Much attention has been drawn on monitoring the chemical contaminants in fruits and vegetables because most of them are eaten raw, which usually results in higher levels of chemical residues compared to other food groups (Chen and others 2011). Current methods to detect and quantify forchlorfenuron include high-performance liquid chromatography (HPLC) (Chen and others 2011; Hu and Li 2006), mass spectrometry (Valverde and others 2010), immunochromatographic assay (Suárez-Pantaleón and others 2012), and other chromatography-based methods. However, these methods are time-consuming, labor-intensive, and expensive. Therefore, novel analytical techniques

are needed for rapid detection of chemical contaminants in foods with high accuracy and sensitivity.

Raman spectroscopy is one of the vibrational spectroscopic methods and has been considered a useful analytical technique to assess food quality (Lu and others 2011). Inelastic light scattering of the incident light from a sample creates the Raman signals, which is related to characteristic molecular vibrations of analyte molecules. This gives unique Raman shift in the frequency or wavelength resulting in characteristic Raman emission (Kneipp and others 1999).

1.2 Objective

The overall goal of this project was to develop surface enhanced Raman spectroscopy (SERS) methods coupled with novel nanosubstrates and evaluate their performance in detection, characterization, and quantification of forchlorfenuron (CPPU) involved in fruits and vegetables especially from grapes for my project. HPLC was also used to verify and compare with SERS results. Partial least squares analysis (PLS) was used as a statistical method to analyze the SERS spectral data.

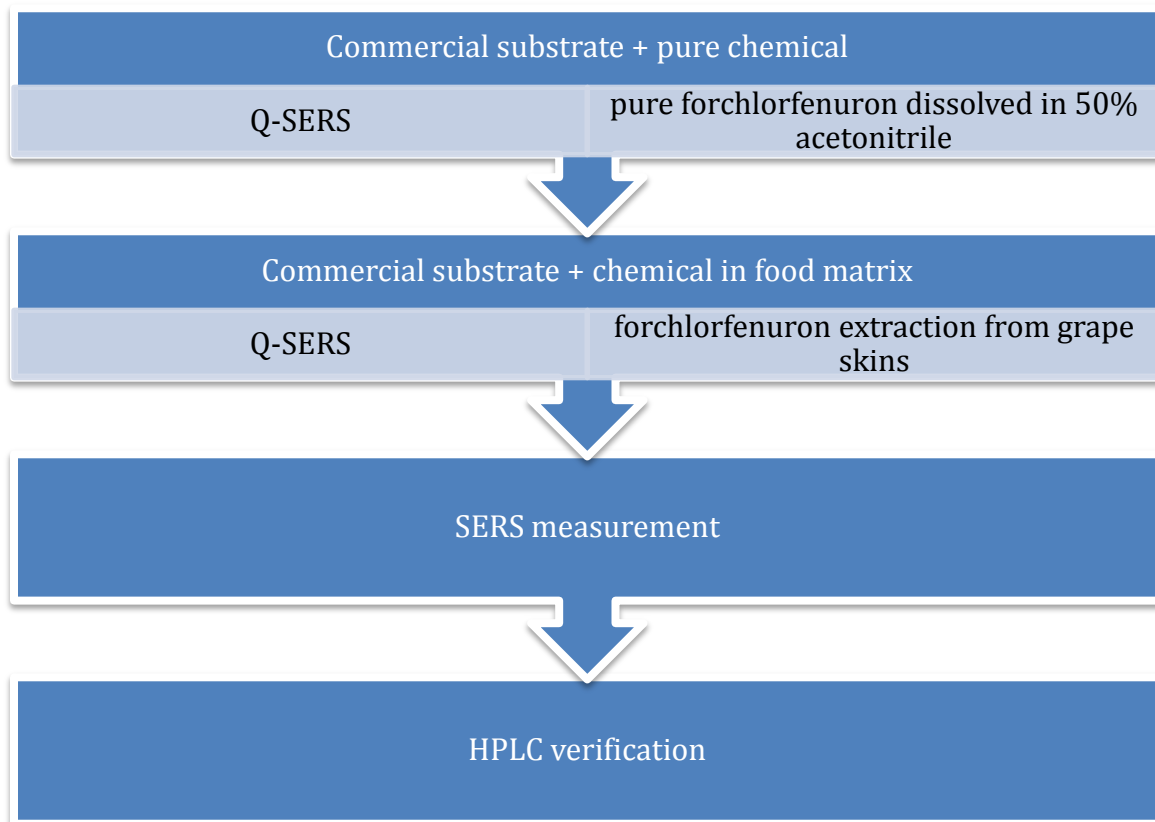


Figure 1. The outline of the experimental design.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Food contaminants

In recent years, there has been increasing concern about extensive use of pesticides, drugs, and other chemicals in foods such as fruits and vegetables, and processed foods. There are numerous types of chemical contaminants in food. They are widely used in various products for different purposes including increasing crop yields, ensuring the quality of crops and foods, and eliminating pests.

For example, contamination of melamine in pet foods and human foods caused a massive recall worldwide in 2007 and 2008 (Brown and others 2007; Cianciolo and others 2008), which led to a global scare for food safety issues (Chan and others 2008; Ingelfinger 2008; Xin and Stone 2008). Melamine is a nitrogen-rich chemical normally used in the production of dyes, fertilizers, fabrics, and other products. However, melamine has been intentionally added into pet foods, infant formula, and dairy products to boost the protein content. Melamine is a toxic compound to humans and animals. They are able to cause serious poisoning effects including chronic kidney inflammation, bladder carcinoma, and nephrolithiasis (Hau and others 2009). There is also a synergistic effect on toxicity when melamine is present with cyanuric acid, which may lead to renal failure in animals (Brown and others 2007; Puschner and others 2007).

Illegal dyes such as crystal violet (CV), fluoroquinolones (FQs), malachite green (MG) and other drug residues have been found in imported seafood from Asia and Mexico

(FDA, 2007a, b). They are inexpensive anti-fungal and anti-parasite drugs used in aquaculture (Alderman 1988). However those chemicals have mutagenic and teratogenic effects on humans. Therefore they are banned for used in aquaculture in most countries (Culp and Beland 1996). Currently, there is a zero tolerance policy for these illegal dyes in fish as set by the FDA.

2.1.1 Pesticides

Pesticides are a major category of food contaminants. Pesticides are a diverse group of chemical compounds used to control pests in fruits and vegetables, and improve the quality and yields of crops and foods. They are widely used for the following purposes:

- (1) Eliminate the pests that destroy the plant;
- (2) Control microorganisms such as bacteria, molds, yeast, algae that cause spoilage in foods;
- (3) Boost production yields;
- (4) Stimulate or delay growing process for special needs.

However, they are one of the most toxic yet environmentally stable substances, thus posing a great risk to consumers (Cairns and Sherma 1992). These pesticide residues not only contaminate the crops on which they are applied, but also the environmental system including soil, water, and air. Pesticides can be divided into organic and inorganic compounds according to their structures. The organic pesticides include organophosphorous, organochlorine, and organonitrogen pesticides; while inorganic pesticides include fluoride insecticides, inorganic herbicides, inorganic fungicides, and

arsenic insecticides. There are clear benefits of using pesticide in fruits and vegetables, but they also pose a great risk to human health because of their toxicity. Nowadays most countries have established regulations for the use of pesticides in agriculture. A common way is to set up a maximum residue level (MRL) for each kind of pesticides in different commodities. For example, in European Union (EU), the limit of detection (LOD) of several particular pesticides (acephate, aldrin, dichlorvos, fenthion) is 0.01–0.05 mg/kg. The LOD of simazine is 0.1 mg/kg rising to 0.25 mg/kg in cherries. An LOD of 5 mg/kg is permissible for malathion in grapes and 7 mg/kg in tomatoes and tangerines (EU, 2013).

2.1.1.1 Forchlorfenuron

Forchlorfenuron or CPPU (1-[2-chloro-4-pyridyl]-3-phenyl urea) is a synthetic cytokinin, approved by the US Environmental Protection Agency (EPA) in 2004 as a plant growth regulator in the chemical class of Phenyl urea. Figure 2 shows the chemical structure of forchlorfenuron. Forchlorfenuron can be applied at early post-bloom by ground sprayer (EPA 2004). Plant growth regulators (PGRs) can grow endogenously by plants themselves or applied externally. They play a crucial role in regulating physiological processes in plant growth, such as cell division, differentiation, enlargement, ripening, germination, reproduction, and the protective responses against stress (Davies 1995; Han and others 2012). PGRs are used at different growth stages by applying foliar spray or bunch dipping to optimize berry growth and ensure the quality with desired size, shape, bunch, and weight. (Chadha and Shikhamany 1999). Forchlorfenuron is widely used in grapes, raisins, kiwifruits, and melons together with auxins (usually gibberellic acid

(GA)). GA is a common phytohormone used in fruits and vegetables synergistically to improve fruit size and weight. It is often applied 48-50 days after fruit pruning according to “*Good Agricultural Practices for Production of Quality Table Grapes*” published by the National Research Center for grape in India. This is done either in dip application or slit dose along with GA or in foliar spray application (Sharma and Awasthi 2003). Although there are few reports regarding the safety issue of forchlorfenuron except for its kidney toxicity based on a 2-year rat feeding study (EPA, 2004), excessive use of forchlorfenuron can cause exploding of fruits, contamination of the environment and negative long-term effects on human health. Different organizations have issued the maximum residue levels for this chemical in different commodities. EPA has a regulation on the tolerance of forchlorfenuron residue in grapes and raisins with 0.06 ppm (GPO, 2010). In EU, the residues of CPPU are regulated at the MRL of 0.01 ppm (EFSA, 2012). Chinese government has set an MRL of 0.05 ppm for forchlorfenuron in grapes (SHFDA, 2012). There has been increasing awareness in recent years about the residues of this synthetic chemical on fruits and vegetables because the FDA found that there is a growing trend of using pesticides and other chemicals on fruits from 2004 to 2007, as indicated by the fact that more domestic fruit samples were contaminated by residues of pesticides and other chemical substances (FDA 2007).

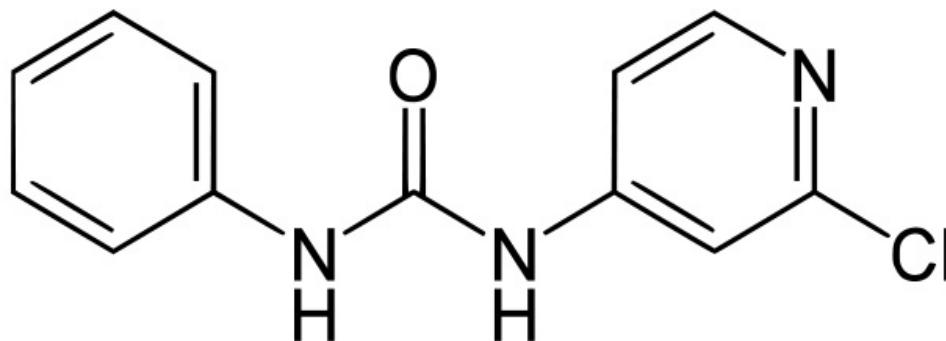


Figure 2. Molecular structure of forchlorfenuron.

2.2 Conventional methods for detecting chemical contaminants

Much attention has been drawn on monitoring the chemical contaminants in fruits and vegetables because most of them are eaten raw, which usually results in higher levels of chemical residues compared to other food groups (Chen and others 2011). There have been well-established traditional methods to detect and quantify forchlorfenuron, including high-performance liquid chromatography (HPLC) (Chen and others 2011; Hu and Li 2006), GC/MS (Valverde and others 2010), immunochromatographic assay (Suárez-Pantaleón and others 2012), and other chromatography-based methods. However, the procedure for detecting chemical contaminants in biological samples is a complex process and involves multiple steps, which are summarized in Figure 3 (Fenik and others 2011).

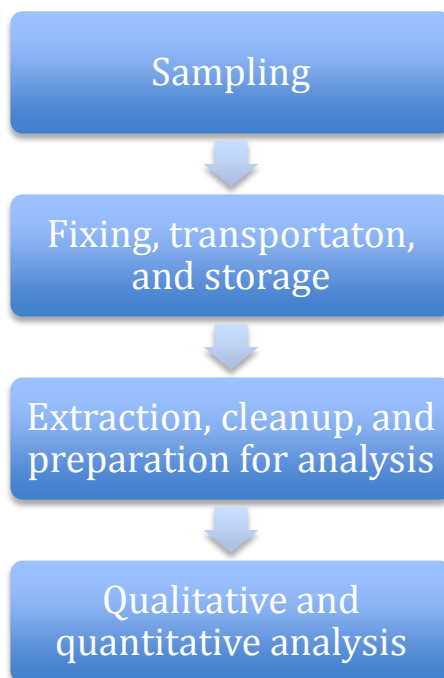


Figure 3. The major stages in an analytical procedure for measuring pesticide in fruit and vegetables.

Gas chromatography (GC) is one of the most commonly used analytical techniques for determining chemical contaminants in foods. GC can detect the residues of all classes of pesticide if equipped with a suitable column and detector. This technique is suitable for testing chemicals that are volatile and thermal stable. The type of chromatographic column is extremely important because it is the key part to separate analytes as well as to qualify and quantify the chemical. An ideal column should be highly efficient and resistant to changes in the separation process that consists of two phases: solid phase and liquid phase. The solid phase should be thermal stable and highly selective to the constituents of the mixture being tested. If more than one pesticide is present in fruits and vegetables, a GC-MS is suitable for this situation due to its extremely efficient chromatographic separation capability, high sensitivity and confirmation power based on electron-impact ionization mass spectra (Fenik and others 2011). Compared to the

conventional GC, GC-MS is more rapid and efficient. HPLC is another powerful technique for detecting and determining various chemical contaminants. It can be used especially when analytes are not suitable for determination by GC. These conventional techniques are well-established and have been widely used as standard methods by the FDA for detection of different types of chemicals in foods. For examples, LC-MS (Turnipseed and others 1998; Andersen and others 2006), HPLC/UV (FDA 2007), LC/MS/MS (Smoker and Krynitsky 2008), and GC/MS (Litzau and others 2008) have been used by the FDA for detection and identification of chemical contaminants and many illegal drugs in different food matrices. Generally, the LOD using chromatography-based methods could reach the ppb level.

However, these methods are time-consuming, labor-intensive, and expensive. Therefore, novel analytical techniques are needed for rapid detection of chemical contaminants in foods with high accuracy and sensitivity.

2.3 Vibrational spectroscopy

Vibrational spectroscopy is a spectroscopic technique based on molecular vibrations. The vibration occurs when the molecule has a constant rotational and translational motion pattern with atoms periodically moving inside. A molecular vibration is excited when the molecule absorbs a quantum of energy. While a fundamental vibration is excited when the energy is absorbed by the molecule when it is in the ground state. There are two categories of vibrational spectroscopy: one is infrared spectroscopy including Fourier transform infrared spectrometer (FTIR) and two-dimensional infrared correlation spectroscopy, the other is Raman spectroscopy.

2.3.1 Traditional Raman spectroscopy

Raman spectroscopy is one of the vibrational spectroscopic methods and has been considered a useful analytical technique to assess food quality (Lu and others 2011). Typically it uses a non-ionizing laser coupled with an incident beam as the excitation source. The incident photons from the laser source can be absorbed, scattered, and pass through the material without interaction. The photons are most likely to be absorbed and the molecule will be promoted from the ground state to the excited state if the energy gap of the molecule between these two states fits the energy of the incident photons (Tu and Chang 2012). The excited molecule then is able to be subsequently relaxed to the ground state by emission. There are two types of emission: stimulated and spontaneous emission. Stimulated emission is almost negligible because it can only occur when there is the incident photon energy and cannot occur after energy relaxation. While the spontaneous emission may occur at any energy level, which accordingly plays a major role in the emission process.

Fluorescence is the most common form that is one of the types of luminescence in order for us to observe the emission of a photon. It is a two-step process and can be described as follows (Le Ru and Etchegoin 2008):

1. An incident (laser) beam at lower level of energy E_L excites an electron from ground state S_0 to excited state S_1 . This is an absorption (dipole allowed) process.
2. The electron then relaxes down the vibrational sub-structure of S_1 through solvent interactions and others within 0.1-10 ps. As a result, it relaxes down to the lowest energy level of S_1 . After that, several transitions may possibly happen. For example, spontaneous

emission may occur with an average lifetime of 1 – 100 ns, which corresponds to the electron relaxation down to a vibrational level in the sub-structure of the ground state S_0 . This emitted photon is associated with the fluorescence process.

Fluorescence occurs at the same time with spontaneous emission. The energies of the fluorescent photons differ from each other and the fluorescence intensity depends on the wavelength which gives the unique spectral pattern of different tested chemicals. The spectrum reflects the fingerprint-like profile of the underlying electronic and vibrational structure of the molecules, which is related to characteristic molecular vibrations of analyte molecules. This gives unique Raman shift in the frequency or wavelength resulting in characteristic Raman emission (Kneipp and others 1999). Therefore, Raman spectroscopy can provide useful vibrational spectral information on various chemicals and biochemical components.

On the other hand, when the electron clouds are distorted by the incident photon, the scattering may take place. There are typically two kinds of scatterings existing in the near-infrared and visible light spectral ranges: Raman and Rayleigh scattering. Rayleigh scattering is a more intense form than Raman scattering and only occur when the electron clouds are distorted. This is an elastic process and no energy exchange happens. Meanwhile if there exists energy transfer triggered by the alteration of the vibrational state of the molecule, Raman scattering takes place, which is an inelastic process. This Raman scattering is usually weak and only one out of 10^6 - 10^8 photons undergoes the inelastic process (Mansour and Hickey 2007; Smith and Dent 2005; Gremlich and Yan 2001), which is indicated by a low Raman signal intensity.

Based on the different direction of energy transfer between the photon and the molecules, Raman scattering can be further divided into two sub-categories: Stokes and anti-Stokes. Stokes scattering occurs when the molecule absorbs energy from the incident photon and promotes itself to a vibrational excited state from a lower energy level. On the contrary, anti-Stokes scattering may take place if the molecule has been already in the vibrational excited state before interacting with the incident photon because of prior external excitation or thermal disturbance. In this situation, due to the fact that scattered photon possesses a higher energy level than the incident photon, the scattered photon will interact with the incident photon by releasing energy and gradually return to a lower energy level. In most cases, as the molecules stay in the ground state at room temperature, Stokes scattering therefore predominates the scattering pattern. The most commonly used unit describing the Raman band shifts, which refers to the energy difference between the scattered and incident photons, is wavenumber expressed in “cm⁻¹” and can be calculated through the following equation:

$$\lambda = E/hc \tag{1}$$

where λ stands for the wavelength; E stands for the energy; h is the Planck constant and c is the speed of light.

Figure 4 is a scheme illustrating the Rayleigh, Stokes and anti-Stokes scattering processes.

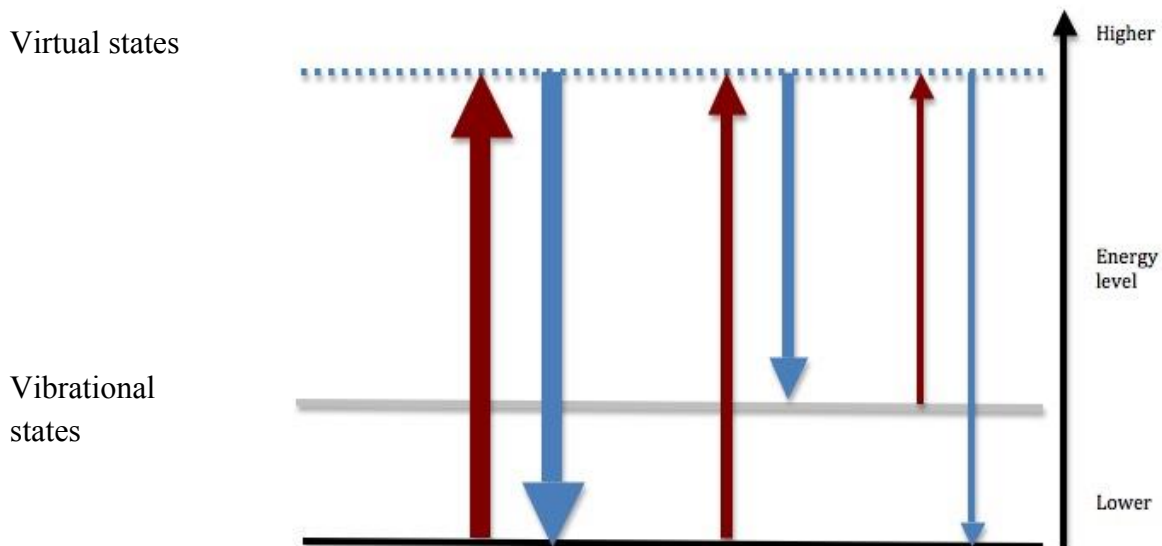


Figure 4. Rayleigh and Raman scattering processes.

One advantage of Raman spectroscopy is that chemicals can be tested in a complex system and is non-invasive. It requires minimum sample preparation with little destruction of samples (Naumann 2000; Li-Chan 1996). However, because only one out of one million photons undergoes Raman scattering, Raman spectroscopy is only suitable for measuring components that are in high concentration (Li-Chan 1996).

2.3.2 Surface-Enhance Raman Spectroscopy (SERS)

Since the 1970s, scientists have discovered and developed surface-enhanced Raman spectroscopy (SERS) that can detect samples based on significantly enhanced Raman signals compared to traditional Raman (Fleischmann and others 1974; Albrecht and Creighton 1977; Jeanmaire and Van Duyne 1977). The signal level is increased by the interaction between the molecule and a nanostructured metal surface. The signal thereby

can be significantly augmented by 10^3 - 10^{14} fold. The enhancement factor (EF) varies depending on the definition of the EF (Le Ru and others 2007; Park and Kim 2010). The most commonly used definition for SERS EF can be calculated by the following equation:

$$EF = \frac{I_{SERS}/N_{Surf}}{I_{RS}/N_{Vol}} \quad (2)$$

Where I_{SERS} and I_{RS} are the SERS and regular Raman intensity, respectively. N_{Vol} is the average number of molecules in the scattering volume for regular Raman, and N_{Surf} is the average number of adsorbed molecules in the scattering volume for SERS (McFarland and others 2005; Van Duyne 1979). For SERS in colloidal solutions, the analytical EF or apparent enhancement factor (AEFs) is calculated by

$$EF = \frac{I_{SERS}/C_{RS}}{I_{RS}/C_{SERS}} \quad (3)$$

where I_{SERS} and I_{RS} are the peak intensities in a SERS spectrum and a normal Raman spectrum, respectively. C_{SERS} and C_{RS} are the analyte concentrations in the SERS measurement and the normal Raman measurement, respectively.

Because of significantly enhanced Raman signals, SERS is suitable for detecting molecules in extremely low concentrations, which makes it attractive for detection of trace amounts of chemical residues in foods.

This significant enhancement is mainly attributed to two contributions: electromagnetic field enhancement and chemical enhancement (Kneipp and others 2002; Haynes and others 2005a).

2.3.2.1 Electromagnetic mechanism (EM)

The electromagnetic field enhancement factor is caused by localized surface plasmon resonance that is excited by electromagnetic radiation and arises the coherent oscillation of the surface conduction electrons (Willems and Van Duyne 2007). When the light is incident on the nanostructured substrate, it drives the electron into collective oscillation and then generates large electromagnetic fields on the surface of the nanosubstrate (Haynes and others 2005b). If the surface is rough, the wave may excite localized surface plasmon resonance (LSPR) on the resurface, resulting in enormous amplification of the electromagnetic fields near the surface.

The LSPR refers to the resonance with the frequency of incident light caused by the collective oscillation of valence electrons in a metal nanoparticle. The excitation of LSPR results in two consequences: scattering of the resonant electromagnetic radiation, and large enhancement of electromagnetic fields at the surface of the roughened transition metal. Figure 5 shows the illustration of LSPR (Haynes and others 2005b).

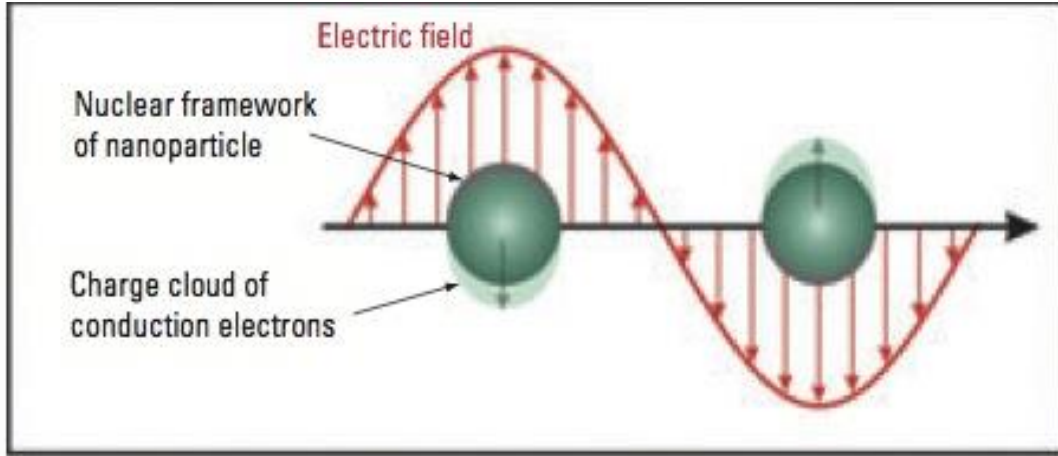


Figure 5. Illustration of the localized surface plasmon resonance effect (LSPR) (Tu and Chang 2012).

Theoretical modeling on the electromagnetic enhancement factor has been extensively established according to different fabrication of metal nanoparticles (Schatz 2001; Jensen and others 1999). The following one is a simplified model with an isolated sphere shaped nanoparticle:

$$E^2 \propto E_0^2 \left| \frac{\epsilon_m - \epsilon_0}{\epsilon_m + 2\epsilon_0} \right|^2 \quad (4)$$

in which E is the electric field magnitude at the surface of the sphere, E_0 is the incident field magnitude, ϵ_m is the wavelength-dependent dielectric constant of the metal composing the sphere, and ϵ_0 is the dielectric constant of the local environment around the sphere (Schatz and Van Duyne 2002).

The electromagnetic enhancement plays a major role in SERS and has an average EF of 10^4 . The size, shape, and roughened surface of the nanomaterial are crucial factors that will alter this enhancement (Haynes and others 2005b).

2.3.2.2 Chemical enhancement mechanism

Chemical enhancement mechanism has an average EF of 100 which is a charge-transfer state created between the metal and adsorbate molecules (Campion and Kambhampati 1998). When the molecule is adsorbed on the surface of metal particle, its magnitude, symmetry and resonant properties from the Raman polarizability of the isolated molecule will be altered. This plays a key role in systems where metal-to-molecule or molecule-to-metal charge transfer occurs where the molecules are directly adsorbed to the roughened surface of the nanosubstrate, which drastically excites the resonances of the system and therefore contributing to so-called chemical enhancement (Moskovits 2005).

2.3.3 SERS substrates

Because SERS signal intensity is greatly influenced by the excitation of LSPR, choosing an appropriate or self-fabricating SERS metal substrates becomes the most important aspect in SERS analysis. There are many factors that can influence the LSPR, which then alter the signal intensity as well as ensure the reproducibility (Haynes and Van Duyne 2001). Factors such as shape, size, dielectric environment, and the inter-particle spacing of the material should be taken into consideration. An ideal SERS substrate can provide the desirable properties such as regularly or periodically arrangement of nanoparticles, reproducible fabrication of the substrate from array to array, homogenous signal enhancement across the surface, long-term stability, biocompatibility with biological chemicals tested like microorganisms, etc (Ciialla and others 2012). Several types of SERS substrates have been developed. For example, metal island films are easy to fabricate and can provide a moderate EF between 10^4 - 10^5 . Colloidal nanoparticles are suitable for solution-phase SERS experiment. Various metallic nanoparticles are one of

the major forms due to their low cost and easiness to fabricate. DNA-based nanoparticle assemblies and nanoparticle clusters are also known for providing hot spots. They are fabricated on the basis of single metallic nanoparticles with nanosized gaps. They have higher EF than single metallic ones (Lee and Irudayaraj 2009) because the intermolecular linkage by DNA or electrostatic interactions (Romo-Herrera and others 2011).

Nowadays, many novel nanosubstrates have been applied in SERS to increase Raman signals because they have a very high surface/volume aspect ratio, which ensures that a great number of probe molecules are captured in the close vicinity of the metal surface (Liu and others 2013a). Gold and silver are two most commonly used nanomaterials for use as SERS substrates (Kneipp and others 2002).

2.3.4 Applications of SERS in Food Science

There has been increasing use of SERS for rapid detection and characterization of different food contaminants, including melamine, cyanuric acid, organophosphosphate, carbamate, and reactopamine (Lin and others 2008; Liu and others 2013b; Zhai and others 2011). The objective of this study was to extract forchlorfenuron from grape skins and to detect and characterize forchlorfenuron by SERS method coupled with gold nanosubstrates. Partial least squares analysis (PLS) was used as a statistical method to analyze the SERS spectral data.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Forchlorfenuron was purchased from Sigma-Aldrich (St Louis, MO, USA). Organic grapes were purchased from a local market and washed to ensure no pesticide residues on grapes.

3.2 Sample preparation

A stock solution of forchlorfenuron (100 $\mu\text{g/mL}$) was prepared by dissolving forchlorfenuron powders in 50% acetonitrile that was used as the solvent system (acetonitrile : deionized water = 1:1, v/v), and stored at 4°C. A serial concentration of forchlorfenuron solutions (0, 0.1, 0.5, 1, 5, 10, 50, and 100 ppm) were prepared by dilution from the stock solution. The 0 $\mu\text{g/mL}$ solution was used as the control group.

The sample preparation procedure and extraction method were based on our previous paper (Liu and others 2013b) with some modifications. Briefly, grapes were weighed and their sizes were measured. The surface area of the fruit was then calculated accordingly. Based on the concentrations of standard forchlorfenuron solutions, the mass of forchlorfenuron that should be spiked on 1 cm^2 of fruit skin was obtained. The samples were peeled and different concentrations of forchlorfenuron solutions were dropped evenly on 4 cm^2 of grape skins. The skins of the samples were then blow-dried using an air dryer, cut into small pieces, and placed in conical tubes containing 4 mL of mixed solvent (acetonitrile/ H_2O = 1:1, v/v). The mixtures were vigorously vortexed for 1 min

and then sonicated using an ultrasonic processor for 5 min. The supernatants were filtered with a 0.45 μm syringe filter. Finally, the filtrates were used for SERS measurement.

3.3 SERS Substrates

The SERS substrates, Q-SERSTM G1, were obtained from Nanova Inc. (Columbia, MO, USA). Q-SERSTM substrates are gold-coated nanostructures fabricated on a silicon wafer. During the measurement, a volume of 0.3 to 0.5 μL of the filtrate was dropped on the surface of a substrate using a micropipette. The substrate was then placed on a hot plate and heated at 35°C until the solvent completely evaporated.

3.4 SERS Measurement

A Renishaw RM1000 Raman spectrometer system (Gloucestershire, UK) equipped with a Leica DMLB microscope (Wetzlar, Germany) was used to test pure forchlorfenuron. This system is equipped with a 785 nm near-infrared diode laser source. During the measurement, light from the high power (maximum at 300 mW) diode laser was directed and focused onto the sample at a microscope stage through a $\times 50$ objective. A 578 \times 385 pixels charge-coupled device array detector detected Raman scattering signals. The size of each pixel was 22 \times 22 μm . Spectral data were collected by WiRE 3.2 software (Gloucestershire, UK). The detection range for forchlorfenuron is from 500 to 1800 cm^{-1} . After measurement optimization, spectra of samples were collected using a $\times 50$ objective with 10 s exposure time, and 300 mW laser power. For forchlorfenuron extracted from the grape skin, the SERS measurement was conducted using a Renishaw InVia Raman

microscope equipped with a Leica Microsystem CMC GmbH using 785 nm near-infrared diode laser source. The extracted samples were tested under the condition of 10 s exposure time, 1 accumulation, and a $\times 50$ objective with 5% laser power. The spectra were collected by Wire 3.3 software (Gloucestershire, UK).

For HPLC test, both pure and extracted samples were pretreated by centrifugation at 10000 rpm at 4°C for 10 min, and then stored at 4°C before testing. The measurement was conducted using Agilent 1100 system with a C₁₈ column. Parameters for measurement included: a mobile phase of methanol/water (63:37 v/v), temperature at 40°C, 260 nm detecting UV light, 1 mL/min flow rate, and 10 μ L injection volume.

3.5 Data Analysis

SERS spectral data were collected and analyzed by Delight software version 3.2.1 (D-Squared Development Inc., LaGrande, OR, USA). Data were first processed by second order polynomial subtraction to offset baseline shift. Gaussian smoothing was used at 4 cm^{-1} to eliminate high frequency instrumental noises. PLS linear regression analysis models were established to predict pesticide concentrations in the samples. The higher an R value is obtained, the better predictability the model indicates (Huang and others 2002). The lowest root mean square error of prediction (RMSEP) value was used to choose the optimum number of PLS latent variables. RMSEP can be calculated in the following equation:

$$RMSEP = \sqrt{\frac{\sum_{i=1}^n (\hat{c}_i - c_i)^2}{n}} \quad (5)$$

C_i' is the predicted concentration (ppm) of forchlorfenuron solutions, C_i is the actual concentration (ppm) of forchlorfenuron. A higher R value or a lower RMSEP value suggests a better predictability of the PLS model.

The detection limit (DL) for forchlorfenuron can be calculated and expressed from the PLS calibration curve according to both the International Union of Pure and Applied Chemistry (IUPAC) and the American Chemical Society (ACS).

$$DL = 3\delta/m \quad (6)$$

Where δ is the standard error in the y-intercept, m is the slope of the PLS model, and 3 represents the model was conducted at 99% confidence interval.

The standard PLS calibration curve of forchlorfenuron was obtained by testing different concentrations of standard forchlorfenuron solutions. Two concentrations (5 and 50 ppm) of forchlorfenuron solutions extracted from the grape skins were selected to determine the recovery percentage, which can be calculated based on the following equation:

$$\text{Recovery\%} = (C_{\text{quantified}} - C_{\text{control}}) / C_{\text{spiked}} \times 100\% \quad (7)$$

where $C_{\text{quantified}}$ is the quantified forchlorfenuron concentration in the spiked grape samples, C_{control} is the forchlorfenuron concentration in the control grape samples, and C_{spiked} is the known concentration of spiked forchlorfenuron calculated according to the spiked amount of the pure forchlorfenuron solution and the weight of grape skins.

CHAPTER 4

Results and Discussion

4.1 SERS measurement for pure forchlorfenuron

4.1.1 SERS spectra vs. normal Raman spectra

Both SERS and normal Raman spectra of 5 ppm forchlorfenuron solution were acquired using Q-SERS substrates and gold slides. As shown in Figure 6, the Raman signals of forchlorfenuron were significantly enhanced on Q-SERS substrates compared with normal Raman spectra. During measurement, a higher power of laser source may provide a better signal-to-noise ratio. However attention was paid to avoid oversaturation that may cause damage to the samples.

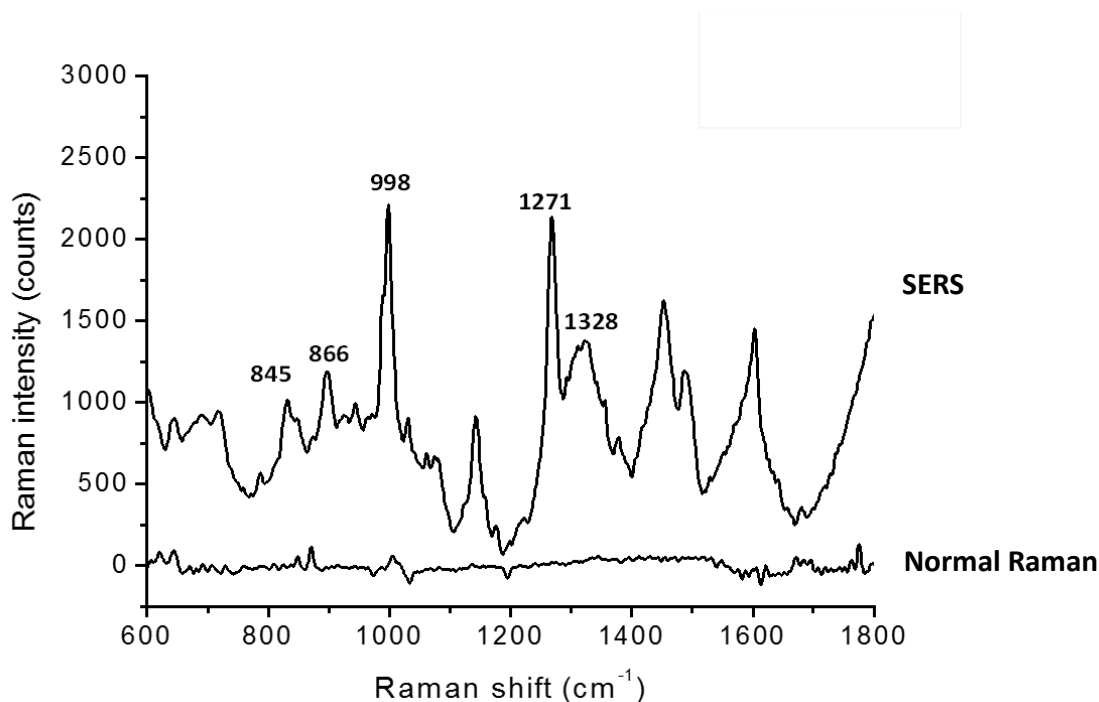


Figure 6. Normal Raman spectra and SERS spectra of 5 ppm forchlorfenuron solution on gold slide and Q-SERS. Black line: forchlorfenuron solution tested on Q-SERS; Red line: forchlorfenuron solution tested on gold slide. Data were processed with smoothing at 4 cm^{-1} .

4.1.2 Characteristic SERS spectra pattern for forchlorfenuron at different concentrations

SERS spectra of different concentrations of pure forchlorfenuron solutions were obtained on Q-SERS. Average SERS spectra ($n=5$) of pure forchlorfenuron are shown in Figure 7. This result is consistent with the spectra of the powder form measured on Q-SERS, indicating that there is little interference from the organic solvent. Band assignments were summarized in Table 1. The most predominant peak of forchlorfenuron is at 998 cm^{-1} due to the symmetric ring stretching of monosubstituted benzene part together with the ring breathing of pyridine part. Other typical peak at 845 cm^{-1} can be attributed to

substituent sensitive vibrations of the pyridine part here referring to the C-Cl linkage; 1271 cm^{-1} due to the C-N asymmetric stretching vibration of monosubstituted benzene; 1328 cm^{-1} due to benzene ring skeletal vibration. As shown in Figure 3, the SERS spectra of forchlorfenuron were distinct compared to the control (acetonitrile solvent system). The Raman intensity of the predominant peaks increased with the increase of sample concentrations.

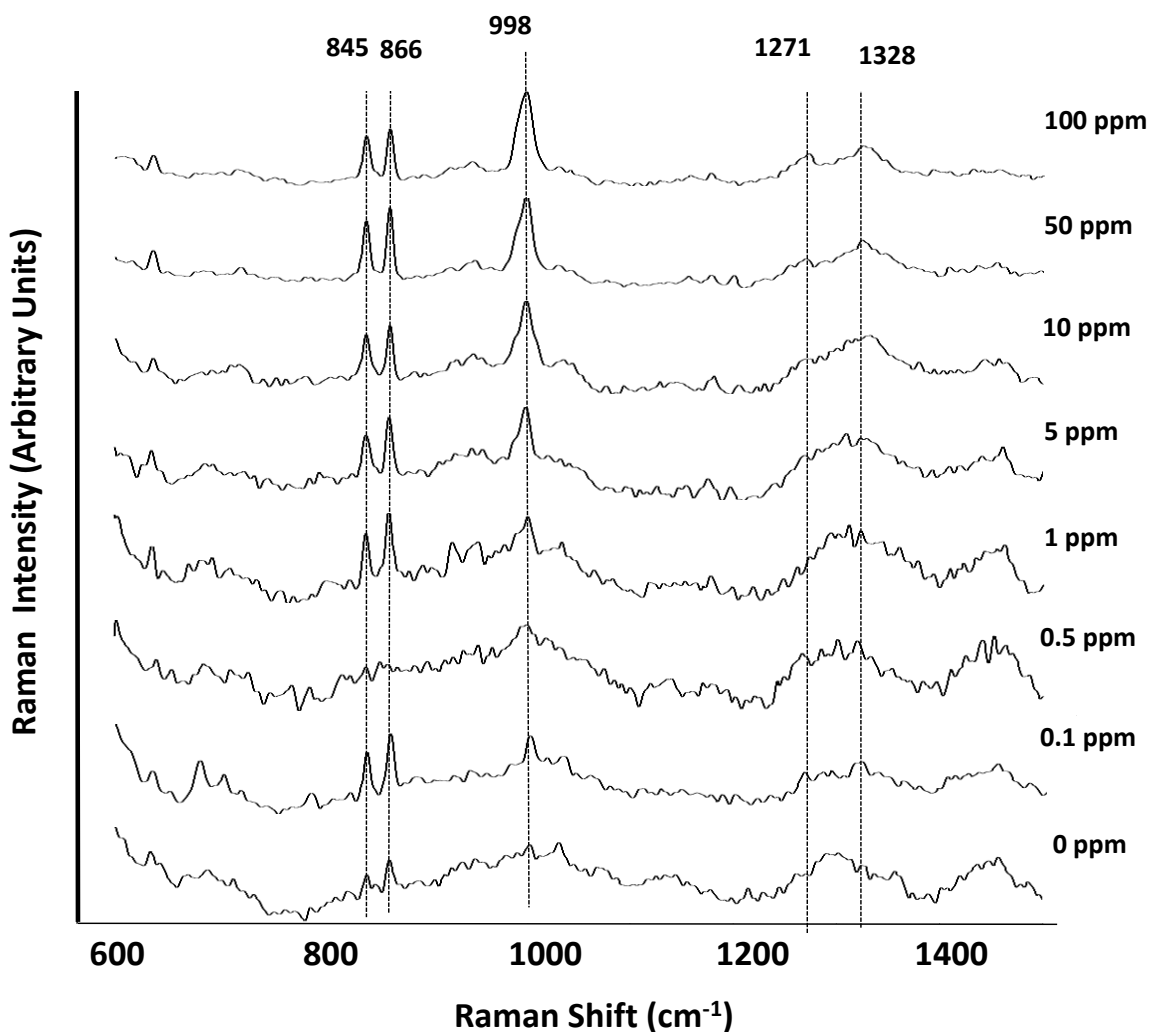


Figure 7. Average SERS spectra ($n=5$) of different concentrations of pure forchlorfenuron solutions. Spectra were processed by smoothing at 4 cm^{-1} and a 2nd polynomial subtraction for baseline adjustment.

Table 1. Band assignments of predominant peaks in SERS spectra of forchlorfenuron

Band (cm ⁻¹)	Assignment
Monosubstituted benzene part	
615 w	Depolarized in-plane ring deformation
998 s	Symmetric ring stretch
1028 m	In-plane CH stretching
1271 m	C-N asymmetric stretching vibration
1328 m	Ring skeletal vibration
1456 w	Ring deformation vibration
1603 m	Ring stretching vibration
Pyridine part (2-substitued)	
845 m	Substituent sensitive vibration
998 s	Ring breathing
700 w	C-Cl stretching
637 w	NCO deformation vibrations

w weak, *m* medium, *s* strong

(Freeman and Lord 1974; Jehlicka and others 2010; Dollish and others 1974; Klots 1995; Klots and Collier 1995)

4.1.3 Second derivative transformation

Figure 8 shows the second derivative transformation of the most predominant peak at 998 cm⁻¹. Second derivative transformation was used to adjust baseline shifts and separate overlapping bands. This figure demonstrates that it can clearly differentiate between different concentrations of forchlorfenuron ranging from 5 to 100 ppm. However, for

those spectral samples with concentrations of less than 5 ppm, it is difficult to differentiate them based on spectral features.

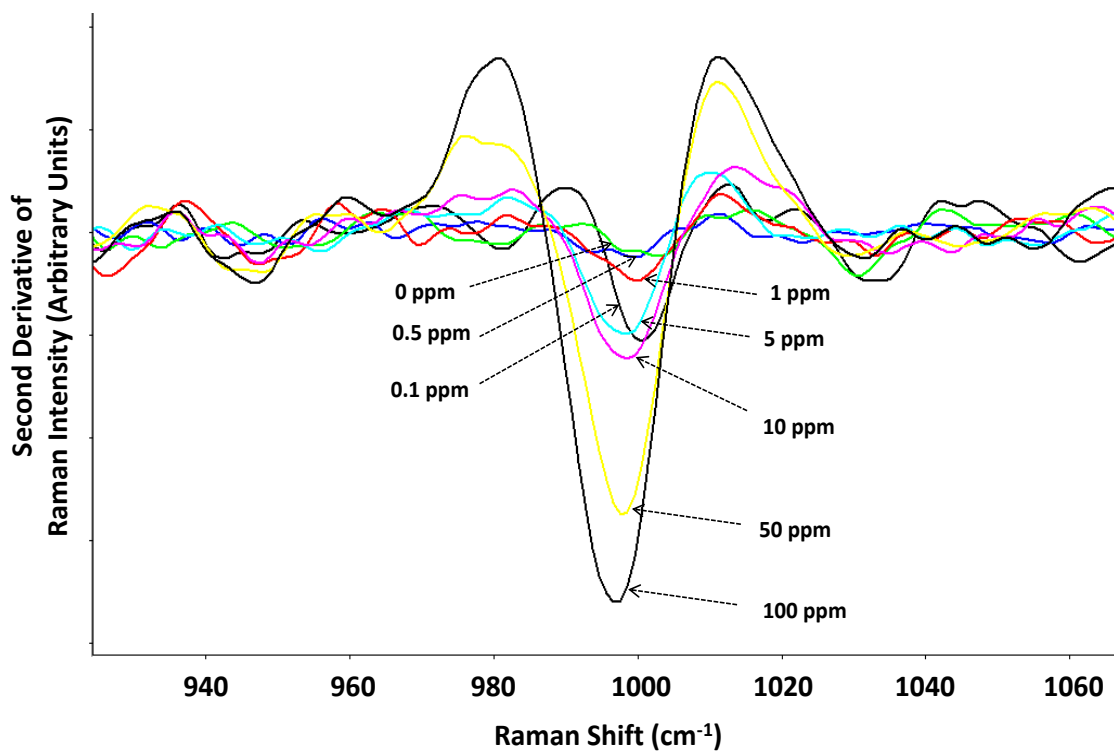


Figure 8. Second derivative transformation of average SERS spectra ($n=5$) at 1000 cm^{-1} for different concentrations of pure forchlorfenuron solutions.

4.1.4 Partial least squares analysis (PLS)

RMSEP values obtained from the PLS model are shown in Figure 9. The lowest RMSEP value was achieved when applying four latent variables, indicating that the optimum RESEP value to construct the PLS model is four. A PLS model was established by plotting predicted concentration verse actual concentration of samples using four latent variables (Figure 10). R value is 0.96 and RMSEP is 0.9314×10^{-5} , suggesting that PLS is a reliable analytical tool for quantification of forchlorfenuron in solution.

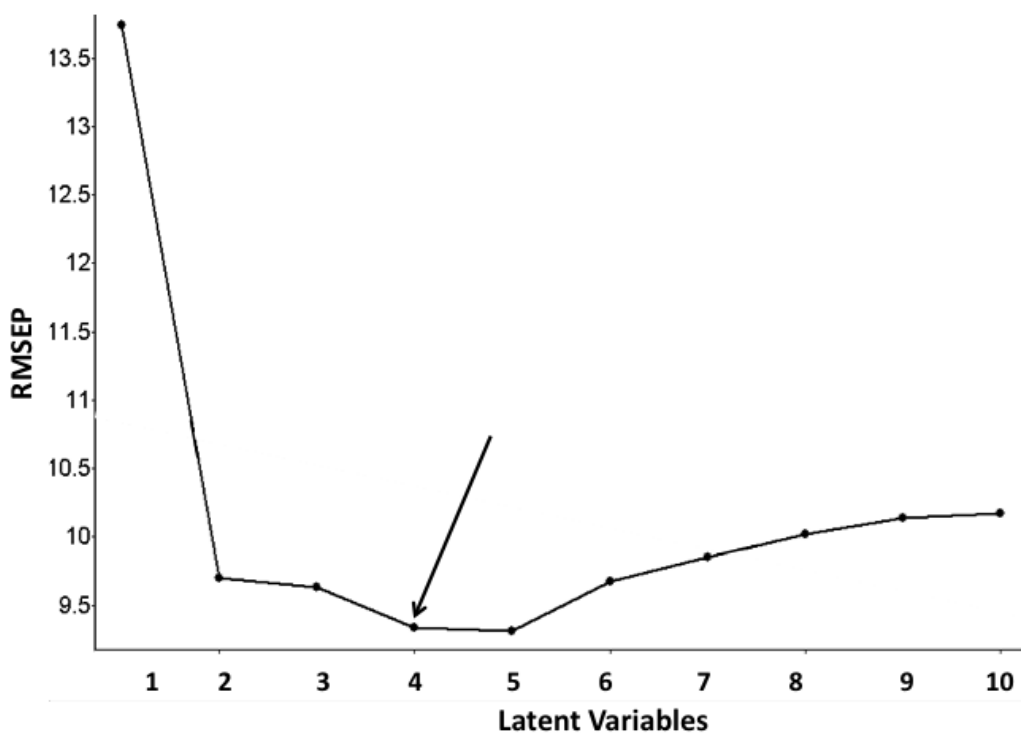


Figure 9. RMSEP values of the partial least squares (PLS) models with different latent variables.

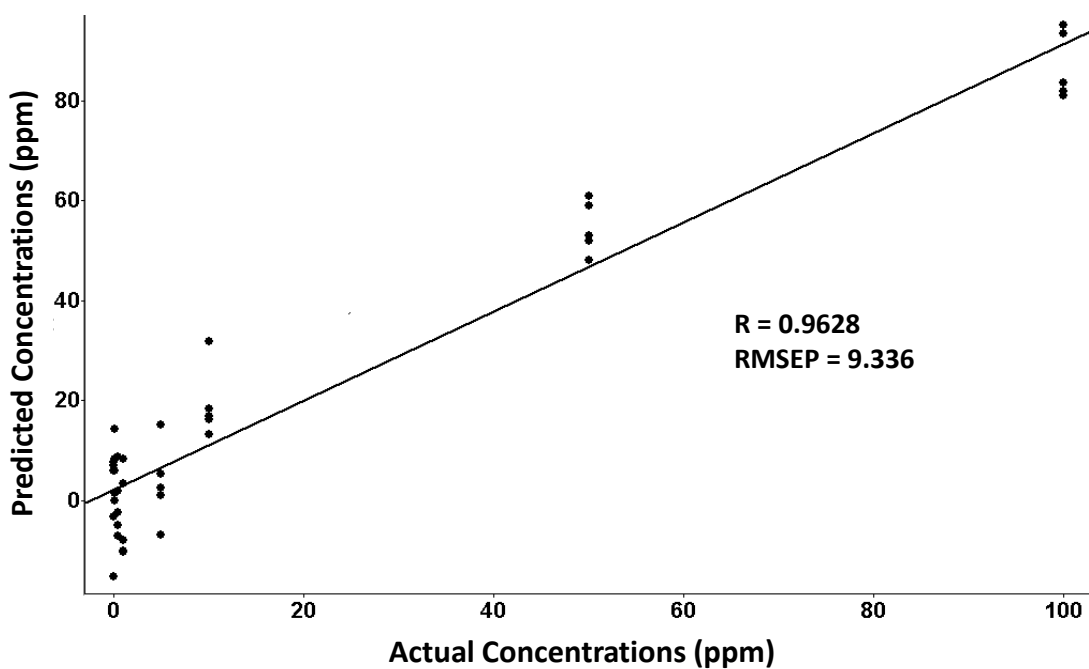


Figure 10. Predicted concentration (ppm) vs. actual concentration (ppm) of pure forchlorfenuron solutions using PLS models. Data processed with smoothing at 4 cm^{-1} , 2^{nd} polynomial subtraction for baseline adjustment, four latent variables, spectral region from $600\text{-}1800\text{ cm}^{-1}$, spectral number = 40.

4.1.5 Detection limit (DL)

The DL of forchlorfenuron by SERS was calculated based on the equation 6 and are shown in Table 2. The DL for detection of pure forchlorfenuron solutions was 3.15 ppm and 4.43 ppm for samples extracted from the grape skin. This is in agreement with Figure 8 as concentrations fall below 5 ppm, the spectra are indistinguishable from each other. Next, the HPLC method was used to in this study further verify these results.

Table 2. Calculation of detection limits (DL) of SERS method for forchlorfenuron

Sample	R	Standard error	Slope	DL (ppm)
Pure Forchlorfenuron	0.96	0.93	0.886	3.15
Forchlorfenuron extracted from grape skin	0.94	1.30	0.880	4.43

To predict the concentration of forchlorfenuron residues and calculate the recovery values, a calibration curve was established (Figure 11). Unlike traditional chromatographic methods of obtaining the calibration curve from pure samples first, and then quantifying the concentrations extracted from food samples accordingly, this method is based on the PLS models and predicts the concentrations of forchlorfenuron directly. As shown in Table 3, the recovery percentage of pure forchlorfenuron with concentrations of 50 and 100 ppm are 111.97% and 79.04%, respectively. While for lower concentrations of samples, the recovery are low (data not shown), suggesting that a better extraction method is needed to establish a more sensitive detection protocol with lower DL.

4.2 SERS measurement for forchlorfenuron extracted from grape skin

4.2.1 Characteristic SERS spectra pattern for forchlorfenuron extraction at different concentrations

We also detected and quantified forchlorfenuron extracted from real fruit sample. Average spectra ($n = 7$) of forchlorfenuron in five different concentrations (1 to 100 ppm) extracted from grape skins were acquired on Q-SERS (Figure 11). Spectral data were preprocessed by the same transformations as the pure samples. The spectral pattern was highly consistent with that from pure forchlorfenuron (Figure 7).

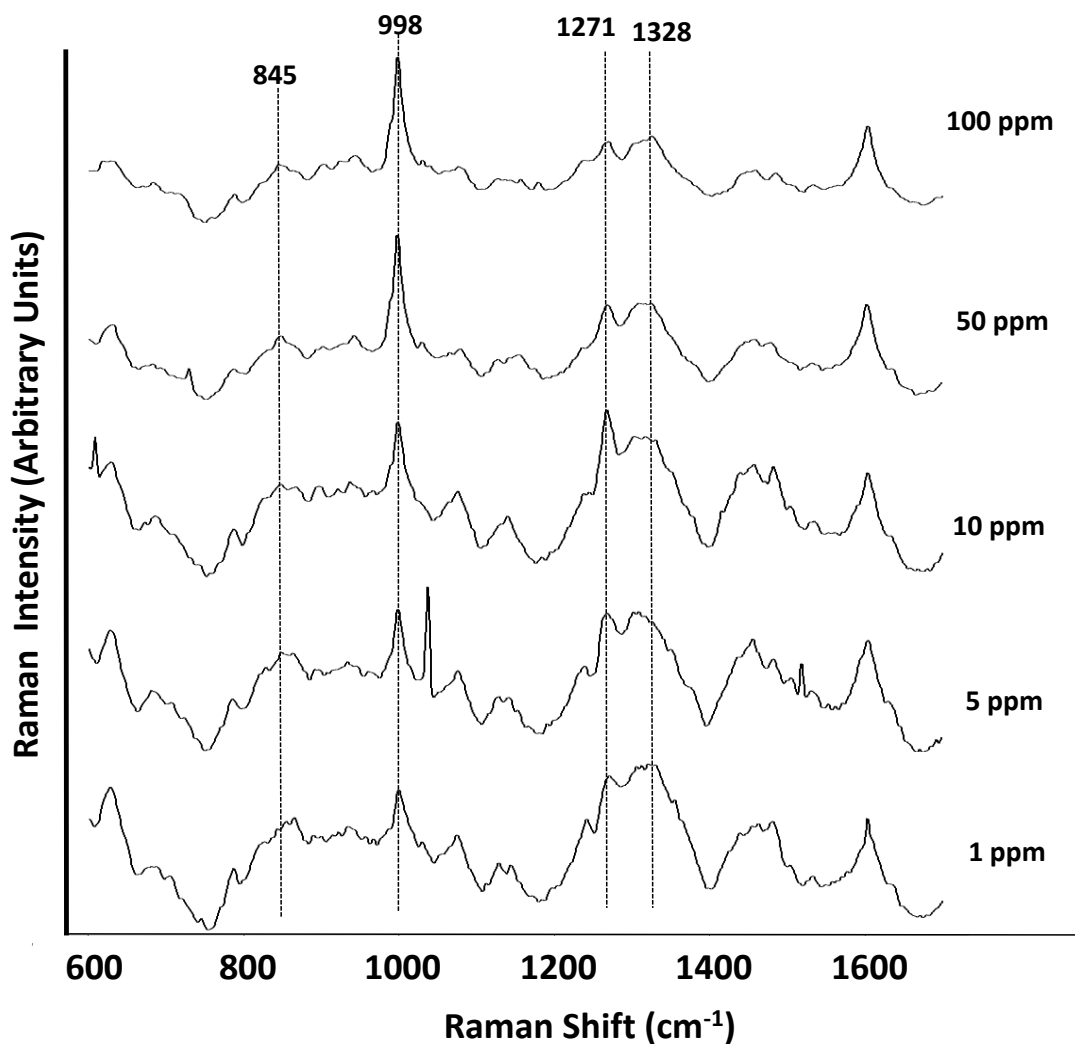


Figure 11. Average SERS spectra ($n=7$) of different concentrations of forchlorfenuron solutions extracted from grape skins. Spectra were processed by smoothing at 4 cm^{-1} and 2^{nd} order polynomial subtraction for baseline adjustment.

4.2.2 Second derivative transformation

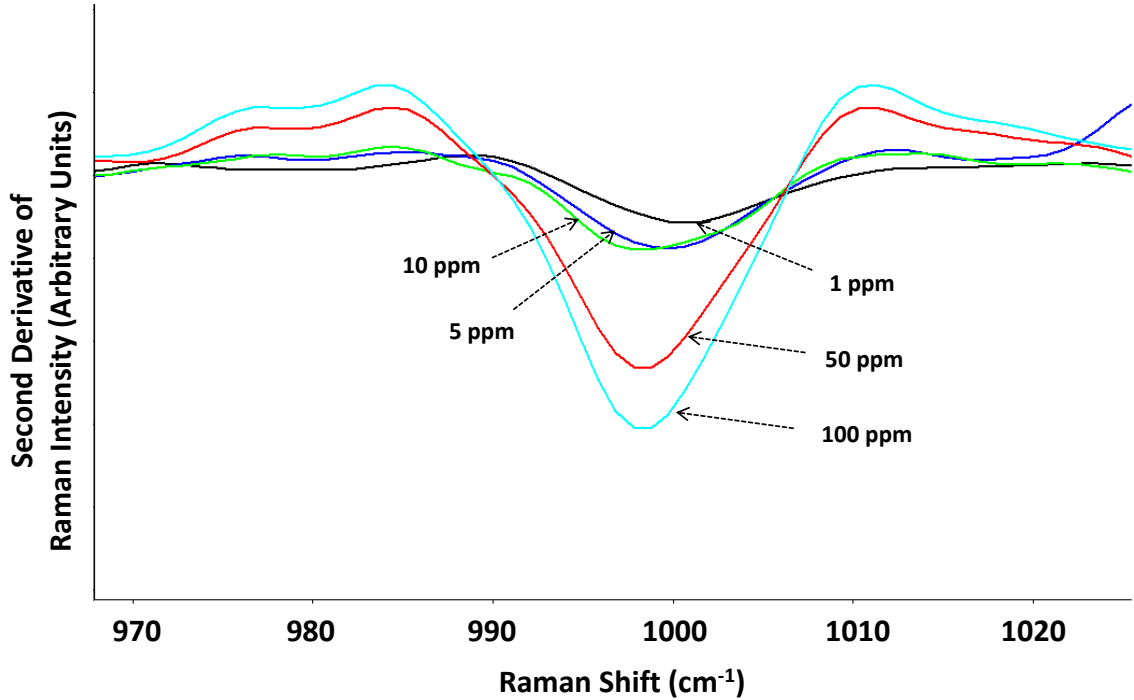


Figure 12. Second derivative transformation of average SERS spectra (n=7) for different concentrations of forchlorfenuron solutions extracted from grape skins at 1000 cm^{-1} .

The second derivative transformation of forchlorfenuron extraction shows the same pattern as the pure chemical with the most predominant peak at 998 cm^{-1} . The signal intensities follow the same trend between different concentrations of forchlorfenuron ranging from 1 to 100 ppm.

4.2.3 Partial least squares analysis (PLS)

The lowest RMSEP value was achieved at six latent variables with R value of 0.94 and RMSEP of 1.305×10^{-5} (Figure 13 & 14).

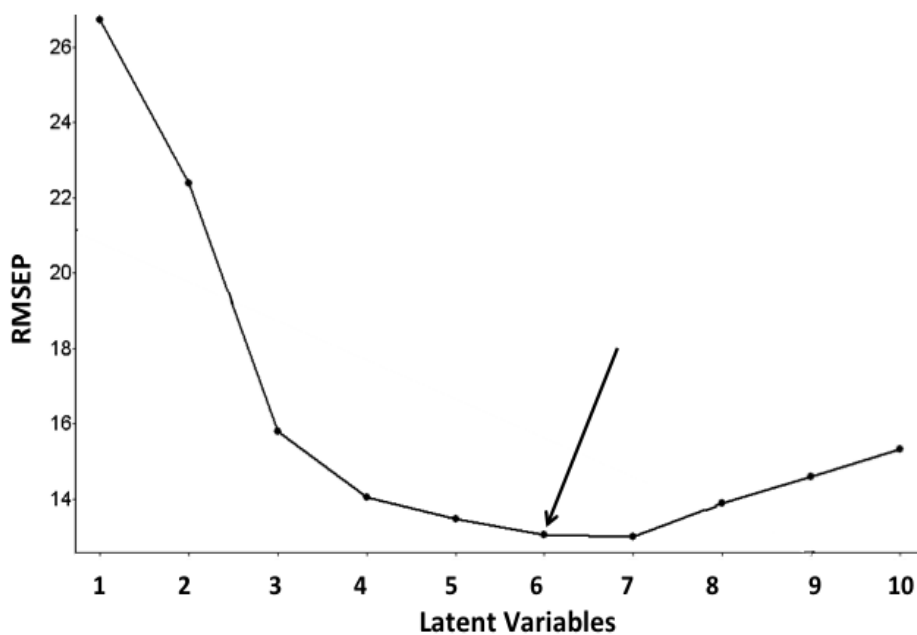


Figure 13. RMSEP values of the PLS models with different latent variables.

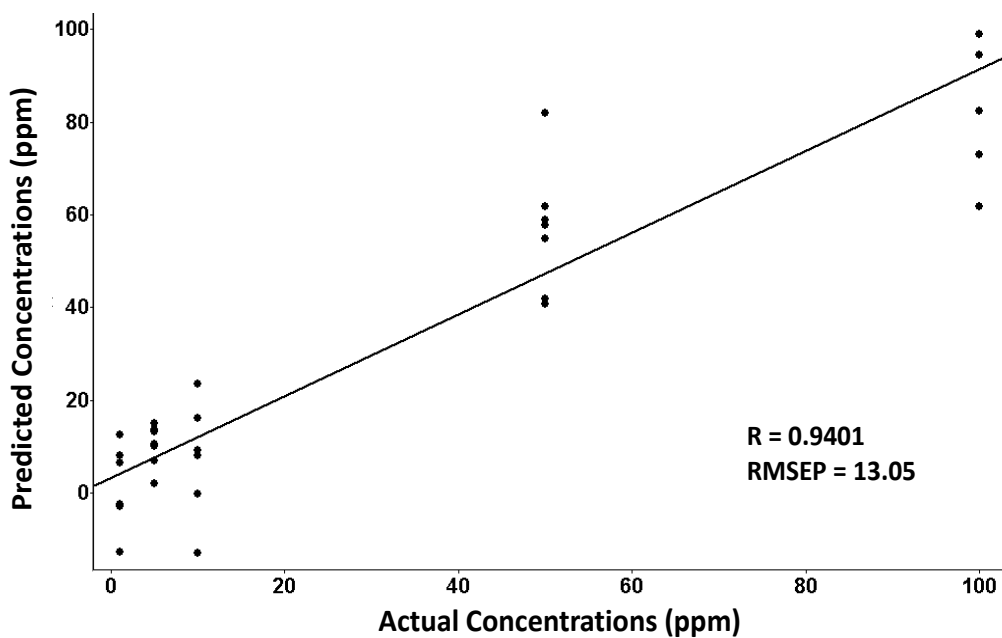


Figure 14. Predicted forchlorfenuron concentration (ppm) vs. actual puforchlorfenuron concentration (ppm) extracted from grape skin using PLS models at lowest latent variable. Data processed with smoothing at 4 cm^{-1} , a 2nd order polynomial subtraction for baseline adjustment, seven latent variables, spectral region from $600 - 1800\text{ cm}^{-1}$, spectral number = 35.

4.3.4 Detection limit (DL)

The DL of forchlorfenuron extracted from grape skin was calculated to be 4.43 ppm (Table 2). This value is a little higher than that obtained from the pure sample, which is reasonable due to the additional extraction procedure.

4.3.5 Recovery based on SERS method

Previous studies suggest that the extracted samples should have the same composition as the standard pure samples (Sentellas and others 2001). Therefore, the recovery of forchlorfenuron extracted from grape skin was established in the same way as the pure chemical. Results show that the recoveries for pure chemical were 111.97% and 79.04% from concentrations at 50 ppm and 100 ppm. For chemical extractions from grape skin the recovery were from 30 to 194.6% for concentrations ranging from 1 to 100 ppm (Table 3).

Table 3. Recovery of pure forchlorfenuron solutions

Sample	Spiked (ppm)	Quantified (ppm)	Recovery (%)
Pure Forchlorfenuron	50	55.99	111.97
	100	79.04	79.04
Forchlorfenuron extracted from grape skin	1	0.30	30.00
	5	9.73	194.60
	10	16.72	167.20
	50	57.06	114.12
	100	86.66	86.66

4.3.6 HPLC verification

HPLC was used for further quantification of forchlorfenuron extracted from grape skin. A calibration curve was established by testing pure chemical solutions ranging from 0 to 100 ppm (Figure 15), exhibiting a linear regression with an R value of 0.99. Five different concentrations of forchlorfenuron from 1 to 100 ppm extracted from grape skin were then tested by HPLC and the recovery percentage was calculated based on the calibration curve, ranging from 72.2 to 158.4% accordingly (Table 4). The DL for forchlorfenuron by HPLC has been reported by previous studies to be 0.02 ppm (Zhang and others 2010), which is lower than that of SERS.

Table 4. Recovery of forchlorfenuron solutions extracted from grape skins based on HPLC calibration curve

Spiked concentration (ppm)	Area (mAU*s)	Actual area	Quantified (ppm)	Recovery (%)
1	26.034	260.34	1.58	158.40
5	49.19	491.90	3.61	72.20
10	94.69	946.90	7.59	75.90
50	599.43	5994.30	51.78	103.60
100	1116.01	11160.19	97.00	97.00

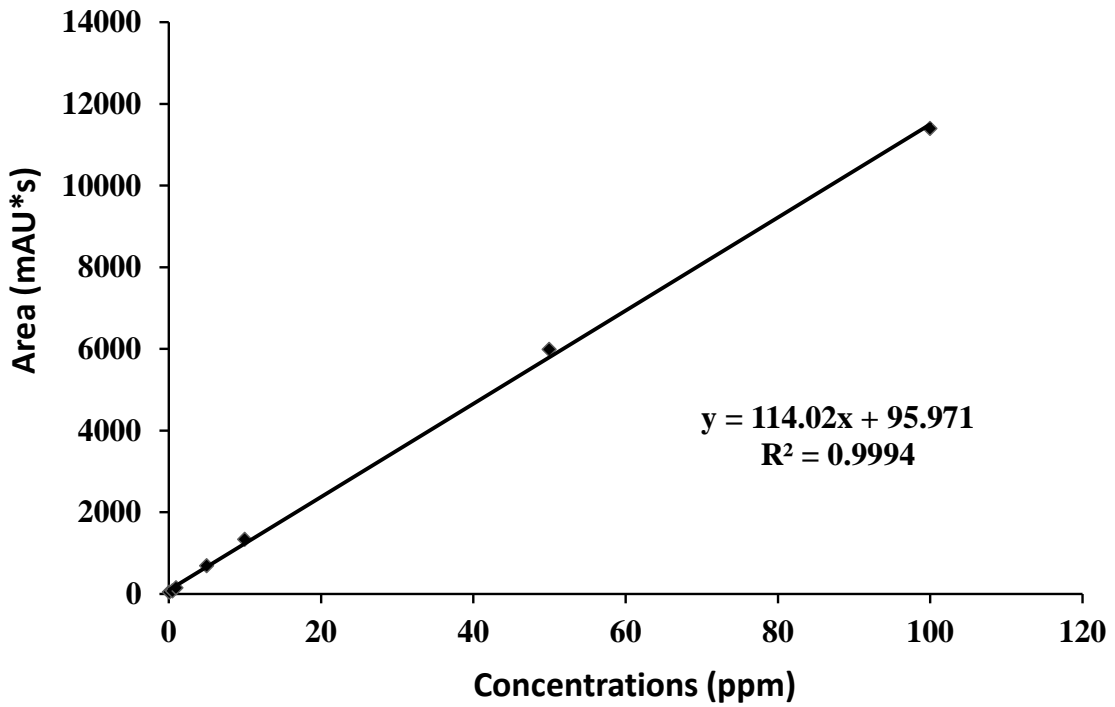


Figure 15. Calibration curve for forchlorfenuron measured by HPLC.

CHAPTER 5

CONCLUSIONS

Forchlorfenuron extracted from grape skins was detected by SERS and HPLC methods. Characteristic SERS spectra of various concentrations of forchlorfenuron (0.1 to 100 ppm) were acquired using gold nanosubstrates. SERS is a rapid and reliable method for detection of chemical contaminants in various food matrices and there is a great potential in using gold-coated nanosubstrates in SERS. Results can be improved if better substrates and extraction protocols are used. SERS in combination with other methods such as HPLC will be a novel approach for rapid detection and discrimination of various chemical and biochemical contaminants in foods. A large number of food samples can be quickly screened by SERS, and the results can then be verified by HPLC.

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VITA

Haobing Qian was born on November 11th, 1989 in Wuxi, Jiangsu, China. She graduated from Shanghai Kongjiang Senior High School in July 2007 and went to Jiangnan University in Wuxi to study Food Science and Technology. In July 2011, she obtained Bachelor of Science degree in Food Science and Technology from Jiangnan University. She joined the Department of Food Science at the University of Missouri in Columbia, Missouri in July 2011 to pursue her Master's degree in Food Science.