

**Molecularly Imprinted Polymers-Based Colorimetric-SERS Dual Biosensor for the
Detection of Atrazine in Apple Juice**

by

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Abstract

Atrazine is a harmful herbicide that can disrupt the hormonal system in humans and animals. Contamination of atrazine in various agri-food products occurs due to illegal uses. Detection of atrazine in foods is time consuming and expensive when the official methods such as gas chromatography-mass spectrometry are applied. In this thesis project, we developed a dual biosensor integrating molecularly imprinted polymers (MIPs) with gold nanoparticles (AuNPs)-based colorimetric assay and surface enhanced Raman spectroscopy (SERS), which can be applied for rapid, high-throughput and sensitive determination of trace levels of atrazine in agri-food products (*e.g.*, apple juice).

This biosensor includes three functions: separation, screening, and quantification. For separation, MIPs were synthesized using molecular imprinting technology by employing atrazine as the template molecule. MIPs-based solid phase extraction (MIPs-SPE) could selectively separate atrazine from apple juice with high recoveries (~93%). AuNPs-based colorimetric assays were able to rapidly detect atrazine due to ligands between atrazine molecules and the surface of AuNPs. High-throughput screening of a large number of samples could be achieved with simple color variation with the limit of detection (LOD) as low as 0.01 mg L⁻¹. For quantification, SERS tests were conducted using AuNPs for Raman spectral collection. Raman spectra of apple juice samples with different concentrations of atrazine were rapidly collected and analyzed by chemometrics. The calculated LOD equals to 0.0012 mg L⁻¹ and limit of quantification (LOQ) was 0.0040 mg L⁻¹, both of which meet the guidelines set up by Health Canada (*i.e.*, 0.005 mg L⁻¹).

Three types of AuNPs with different diameters (*i.e.*, 43 nm, 27 nm, 11 nm) were synthesized and compared for the use of this biosensor. The largest AuNPs worked best for colorimetric assays while the medium-size AuNPs were the most suitable candidate for SERS tests. The extremely low LOD and LOQ strongly validate the potential application of this innovative dual biosensor for accurate and high-throughput determination of atrazine in foods.

Lay Summary

Atrazine is potentially detrimental to human health and the contamination occurs in agri-food products. Because the conventional methods to determine atrazine are expensive, laborious and time-consuming, the development of rapid, accurate, high-throughput and cost-efficient detection techniques is important to food industries and governmental laboratories. In this thesis project, a biosensing system includes separation, screening, and quantification is developed. Using this biosensor, atrazine can be efficiently extracted from food samples and rapidly screened based on simple color variation, followed by accurate quantification. Although many novel techniques are incorporated in the biosensor, it is convenient and affordable to the real-world applications of food safety detection.

Preface

This dissertation is original, unpublished, independent work by the author, Bowen Zhao.

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List of Abbreviations

MIPs	molecularly imprinted polymers
AuNPs	gold nanoparticles
SERS	surface enhanced Raman spectroscopy
MIPs-SPE	molecularly imprinted polymers-based solid phase extraction
LOD	limit of detection
LOQ	limit of quantification
MRL	maximum residue limit
GC	gas chromatography
MS	mass spectrometry
SPR	surface plasmon resonance
UV-Vis	ultraviolet-visible
LSPR	localized surface plasmon resonance
SPE	solid phase extraction
NIPs	non-imprinted polymers
MAA	methacrylic acid
EGDMA	ethylene glycol dimethacrylate
AIBN	2,2'-azobisisobutyronitrile
HPLC	High-performance liquid chromatography
DAD	photodiode array detector
SPME	solid phase microextraction

QuEChERS	Quick-Easy-Cheap-Effective-Rugged-Safe
HAuCl ₄	Chloroauric acid
NaCl	sodium chloride
GPR	gap-plasmon resonance
CCD	charge-couple device
LSD	Least Significant Difference

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Chapter 1: Introduction

1.1 Atrazine contaminations in agri-food products

Atrazine is an artificial herbicide of the triazine class. It was widely used in agri-food industry to control pre-emergence and post-emergence broadleaf weeds and grasses that interfere with the growth of crops (*e.g.*, corn, sorghum, sugar cane). Atrazine used to be the most popular herbicide in the global market due to its advantages of low price, good water-solubility and enduring stability. However, recent studies demonstrated that atrazine could be a factor in disrupting the hormonal system in humans and animals [1-3]. One representative case was the demasculinization of male frogs after exposure to atrazine at extremely low concentrations [4]. Although European countries have banned the use of atrazine since October 2003, persistent pollutions of atrazine in surface-water and groundwater systems occur frequently because of illegal application of atrazine [5], leading to the contamination of various agri-food products (*e.g.*, apple juice, milk, etc.). According to Health Canada, the proposed maximum residue limit (MRL) for atrazine is 0.005 mg L⁻¹. To monitor the trace level of atrazine in food systems, accurate and reliable approaches for detection and quantification are highly required.

1.2 Conventional methods for the determination of atrazine

To determine atrazine in agri-food products, the official protocol proposed by Health Canada includes three steps: sample pretreatment, separation and detection. Liquid-liquid extraction is used to remove macronutrients (*e.g.*, sugar, proteins, etc.) from food samples as the pretreatment. Gas chromatography (GC) is applied to separate the targeted molecule (*i.e.*, atrazine), subsequently

coupled with various detection tools, such as mass spectrometry (MS), flame ionization, electron capture, and nitrogen-phosphorus detectors. By applying this standardized protocol, atrazine can be accurately determined, resulting in a limit of quantification from 0.2 to 1.3 $\mu\text{g L}^{-1}$ (Health Canada, 1993).

However, this official approach does include some significant drawbacks. Because the GC-based techniques lack specificity towards atrazine, interfering compounds (*e.g.*, structural analogues) may not be separated from the targeted molecule and distinguished by the integrated detection tools. In addition, using GC hyphenated methods requires extensive sample preparation and a large amount of organic solvent, making the analytical approach time-consuming, laborious, and not environmental friendly. Another obvious drawback is the high expense for instrument maintenance, which is not affordable by many food industries. Therefore, it is important to develop simple, rapid and cost-efficient techniques for the accurate determination of trace levels of atrazine in agri-food products.

1.3 Emerging technologies for rapid detection of atrazine

1.3.1 Gold nanoparticles (AuNPs)-based colorimetric assays

AuNPs have been widely used for centuries due to their unique optical properties. When AuNPs are exposed to light, a concerted oscillation of free electrons of the gold is induced by the oscillating electromagnetic field of the light. The concerted oscillation exists around the particle surface and generates an electron charge to resonate with a specific frequency of the visible light. The excited resonance is called surface plasmon resonance (SPR). SPR can cause the absorption of the incident

light and thus can be determined using ultraviolet-visible (UV-Vis) spectrometry [6]. The UV-Vis adsorption wavelength and the peak intensity of SPR depend on many factors (*e.g.*, AuNPs size, physical dimensions) because these factors determine the electron charge density on the surface of AuNPs. Specifically, the relationship between SPR and the particle size of AuNPs is theoretically described by the Mie theory [7]. When AuNPs are in small monodispersion (*e.g.*, particle diameter of 30 nm), SPR absorbs light in the blue portion of the spectrum (*e.g.*, wavelength at 450 nm) and the red light (*e.g.*, wavelength at 700 nm) will be reflected, resulting in red color. When the particle size of AuNPs increases or AuNPs are aggregated, the wavelength of SPR-related absorption shifts to a longer wavelength, leading to the absorption of red light and the reflection of blue light, resulting in the AuNPs solution a blue color. Polymers, ligands and biological molecules with donor atoms can induce the aggregation of AuNPs, resulting in color variation from red to blue. Therefore, this unique optical property enables AuNPs to be extensively used in chemical, biological and medical applications [8]. In general, AuNPs that are frequently used have the particle sizes ranging from 10 nm to 500 nm, initially displaying the color of red or pink [9].

Based on AuNPs, colorimetric assays have been developed as simple and straightforward methods for the detection of various analytes, such as nucleic acids [10], proteins [11], viruses [12], cancer cells [13], and herbicides [14]. AuNPs can interact with the analytes by ligands and aggregate rapidly. The degree of the aggregation depends upon the amount of analytes, which is expressed by the changes in color. Higher concentrations of analytes lead to more obvious changes in the color of AuNPs solution from red to blue. The solution will eventually show a clear or translucent color if the concentration of the analyte is high enough to aggregate all the AuNPs. The optical results can be

directly observed by the naked eyes, followed by a further confirmation using UV-Vis spectroscopy. Compared to the conventional dye optical detection methods, AuNPs-based colorimetric assays are more sensitive because of the high absorption coefficient of AuNPs [15]. In addition, AuNPs-based assays have other advantages such as simplicity and a wide detection range. These features have contributed to a rapid development and application of AuNPs-based colorimetric detection in food safety inspection. Determination of various food chemical contaminants, such as melamine [16], clenbuterol [17], and aflatoxin B1 [18], were recently reported.

1.3.2 Surface enhanced Raman spectroscopy (SERS)

Raman spectroscopy is an emerging analytical technique, which is known as a non-destructive and rapid tool to detect and quantify different solid, liquid, and gaseous samples [19]. Upon an interaction between Raman laser light and the analyte, Raman spectroscopic scattering occurs. In the scattering, the absorption and emission of the photons in the light induce transitions among the available energy levels, shifting the frequency of the light. This phenomenon is called Raman shift, which gives information about changes of polarizability of the molecule in different vibrational modes, expressed as different Raman feature bands. Based on these Raman spectral fingerprints, specific molecules can be recognized [20]. However, Raman scattering signal is typically too weak to detect trace levels of compounds because of the small Raman scattering cross-section [21]. Specifically, only a small fraction of incident light produces Raman signals while the vast majority of photons in the light lead to Rayleigh scattering. In Rayleigh scattering, no energy transition occurs, which provides useless information and is considered as the noise against Raman signals. To obtain high-quality Raman signals, devices such as notch filters, tunable filters and laser stop apertures,

have been developed to reduce Rayleigh scattering [22].

SERS is a novel technique based on Raman spectroscopy and noble metallic nanoparticles (*e.g.*, gold, silver). The nanoparticles are used as SERS-active substrates to support localized surface plasmon resonance (LSPR), which excites an electromagnetic field near the surface of the substrates. When the targeted molecules are adsorbed onto the surface of the nanoparticles, significant enhancement of Raman scattering occurs in the electromagnetic field. Consequently, the Raman signals collected by SERS will be enhanced at least 10^6 times than the normal Raman spectroscopy, decreasing the LOD to even single molecule levels [23-24]. Because of the improved sensitivity of Raman spectroscopy, SERS has been widely applied to determine trace levels of chemical hazards in foods. In the recent reports, herbicides such as atrazine [25], paraquat [26], and carbendazim [27] were detected by SERS with various metallic nanoparticles.

1.4 Innovative techniques for the separation of analyte

1.4.1 Molecularly imprinted polymers (MIPs)

MIPs are innovative sorbents with specific affinity to the targeted analytes. This type of polymers are synthesized by mixing a template molecule, a functional monomer, and a cross-linker together to initiate the polymerization, followed by the removal of the template molecule after the polymers have been developed. Specifically, the template molecule interacts with the functional monomer due to the intermolecular interactions (*e.g.*, hydrogen bonds, ionic interactions). The monomer-template complex is polymerized with the cross-linking agent. The chemical bonds between the function

monomer and the cross-linking agent cause the formation of a complex structure around the template molecule. An appropriate solvent is then used to remove the template molecule, leading to the exposure of specific binding sites and cavities in the polymer matrix with chemical and physical specificity to the analyte that shares the same properties as the template molecule [28]. The brief synthesis process of MIPs is illustrated in **Figure 1.1**. The molecular memory is generated and imprinted into these binding sites and cavities, enabling MIPs to selectively recognize and rebind only the template molecules for multiple times. Therefore, MIPs can be used as the sorbents to separate and enrich the targeted analytes from complex biological matrices.

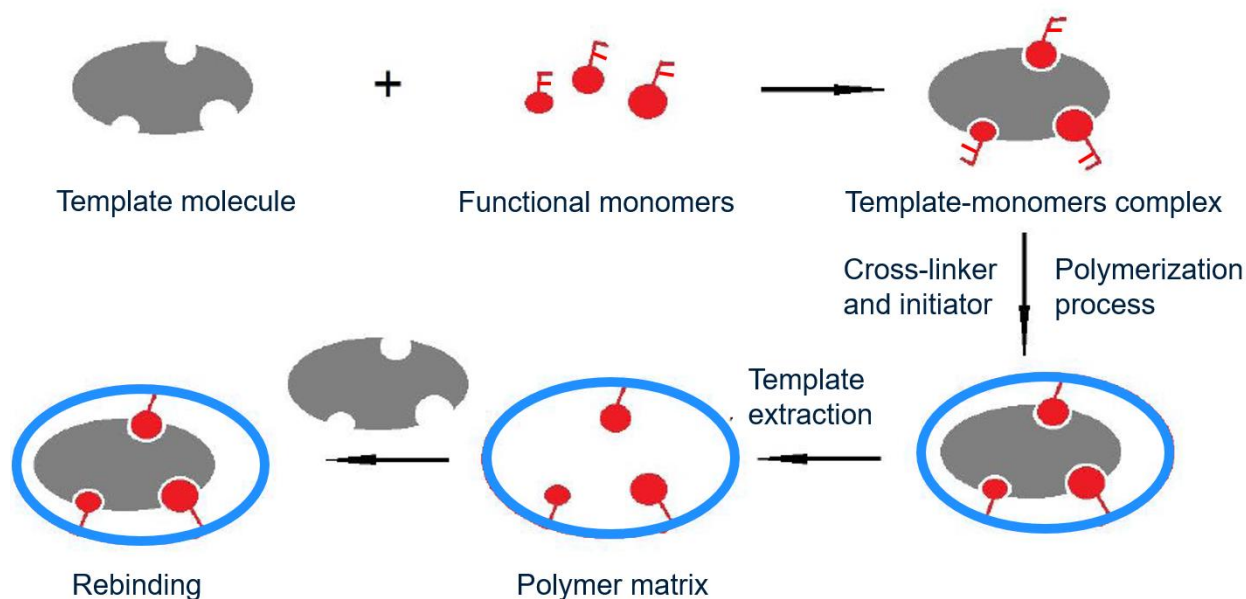


Figure 1.1 Schematic illustration of the synthesis of MIPs

According to the polymerization mechanism, molecular imprinting can be categorized by a variety of bonding types between the template molecule and functional monomer, including non-covalent, covalent, electrostatic/ionic, semi-covalent and metal center coordination [29]. Normally, non-

covalent MIPs and covalent MIPs are the two most common types due to their particular features. In non-covalent MIPs, the dominant interaction between template molecule and functional monomer is hydrogen bonding, which can be easily broken and regenerated within a few simple steps. Non-covalent imprinting is, however, too sensitive to slight disruptions; even the existence of water can influence the interactions. Additionally, heterogeneous binding sites can be generated by non-covalent imprinting, which reduces the specificity of MIPs towards the targeted molecule. On the contrary, covalent MIPs have advantages such as more homogeneous binding sites, more robust binding capacities, and a higher affinity to the template molecule. However, the obvious drawbacks are the complicated synthesis steps of the template-monomer complexes, and less flexibility of bond cleavage, limiting the reusability [30].

Depending on the final applications, MIPs can be fabricated in various physical forms using different approaches, including bulk polymerization, precipitation polymerization and sol-gel polymerization. Bulk MIPs are synthesized as monoliths, followed by grinding and sieving to obtain polymers with specific size ranges. Although bulk polymerization is fast and simple, irregular particle sizes and damaged binding sites can be produced by the time-consuming post-treatment, reducing the loading capacity of MIPs to capture the targeted molecule [30]. Preparation of MIPs by precipitation polymerization can directly obtain fine particles with regular sizes, providing more binding sites. However, a large amount of template molecules and organic solvent are required for the synthesis [31]. The sol-gel polymerization is normally used to produce homogeneous MIPs particles with controllable sizes to fabricate MIPs films [32]. By using novel MIPs films, a rapid equilibration rate of MIPs to adsorb the analyte can be achieved. However, this technique is restricted by the

requirement of complicated instrumentation and materials [33]. Taken together, both advantages and drawbacks of each type of MIPs should be taken into consideration for different application scenarios.

In food safety inspection, MIPs-based techniques are generally used to separate and concentrate trace levels of analytes from food matrices as well as to remove the impurities. One of the most conventional applications is to employ MIPs as the selective sorbents for solid phase extraction (SPE) [34]. To make a MIPs-SPE column, MIPs with specific affinity towards the analytes are packed into a cartridge. After conditioning the column, food samples are loaded and washed inside the column, followed by eluting with appropriate organic solvents. The MIPs-SPE column can retain the analyte when other impurities are washed out, enabling the eluting solution to only contain the analyte. Therefore, the targeted analyte can be efficiently extracted from the complex food matrices. Compared to the SPE column with traditional adsorbing agents, the MIPs-SPE column increases the effectiveness of the extraction due to its specificity towards the analyte, contributing to the subsequent integration with analytical methods (*e.g.*, high performance liquid chromatography coupled with the photodiode array detector, Raman spectroscopy) [34] [35]. In addition, MIPs-based techniques have the advantages such as fast analysis procedures and low-cost preparation, which make MIPs-SPE columns widely adopted to extract different compounds from various agri-food products. In several recent reports, MIPs-SPE methods have been applied to separate fluoroquinolones from milk [36], concentrate patulin from apple juice [37], and extract fenthion from olive oil [38].

MIPs-based techniques have also been widely applied in other areas, such as mimicking natural antibodies for immunoassays [39], substituting natural enzymes for enzymatic catalysis [40], serving as excipients in drug delivery systems [41], and integrating with chemical sensors for clinical diagnostics [42]. The versatility of molecular imprinting technology makes these artificial polymers the most promising materials for molecular recognition.

1.5 MIPs coupled AuNPs-based colorimetric-SERS dual biosensor

Determination of biological and chemical hazards in foods is challenging due to the complexity of the food matrices. To clean up food samples, common methods require a large amount of organic solvents for extraction and have no specificity to the extracted analytes (*e.g.*, liquid-liquid extraction). MIPs-based technique can extract analytes and clean up interferents in food samples due to their specific affinity towards the targeted molecules. To integrate with MIPs-based separation, several emerging detection techniques are being investigated. Novel fluorescent sensors utilized suitable fluorescent monomers to prepare MIPs. Fluorophores inside the recognition sites induced specific transduction from molecular interaction signal into the optical signals, which could be collected by detectors [43] [44]. However, the advantage of MIPs as cost-efficient materials was likely offset by the requirement of unique chemicals (*e.g.*, fluorophores). As another example of the integration, electrochemical sensors employed MIPs-based electrodes to rapidly detect bisphenol A in honey and grape juice samples, but the coating procedures to immobilize MIPs on the surface of probes were time-consuming and sophisticated [45]. In practical terms, food industries and government laboratories demand techniques with the potential of accurate, affordable and fast detection. Therefore, the biosensor to be developed should incorporate MIPs into simple, high-

throughput and rapid detection methods.

AuNPs-based colorimetric assay, as described in **Section 1.3.1**, is a simple and rapid detection technique. AuNPs in a specific size can be synthesized using citrate reduction method [46]. Based on the principle of colorimetric assays, straightforward optical results are obtained within a short time when the color variation of AuNPs is induced by the analyte. These advantages enable AuNPs-based colorimetric assays to screen contaminants from a large number of food samples. As aforementioned in **Section 1.3.2**, SERS is able to determine trace levels of analytes in a sensitive and rapid manner. Collected SERS spectral features can be assigned to different functional groups in the molecules, further recognizing the analytes in food samples. To meet the requirements of food industries and government laboratories, emerging portable Raman spectrometers make this technique more convenient and affordable. Recently, the development of colorimetric-SERS dual sensors improved the efficiency for the detection of mercuric ions in water for environmental analysis [47]. The colorimetric assays were used for rapid and high-throughput screening of analytes in samples, followed by accurate quantifications of the trace level of contamination using SERS. To apply the colorimetric-SERS dual sensors on food hazard analysis, the elimination of interferents in food components (*e.g.*, sugars, proteins, lipids) is critical to the accuracy and reliability of the sensing results. Therefore, MIPs-based separations can be integrated with colorimetric-SERS dual sensors to separate food chemical and microbiological hazards, further exploiting the advantages of the colorimetric-SERS dual sensors.

Various types of MIPs-SERS biosensors have been designed and conducted to determine the

degradation of tocopherol in vegetable oils [48], chloramphenicol in milk and honey [49], melamine in milk [35], Sudan I in paprika powder [50] and histamine in canned tuna [51]. The innovation of integrating AuNPs-based colorimetric assays into MIPs-SERS biosensor is an upgrade of the high-throughput screening. After MIPs-SPE selectively separates the analytes from food samples, AuNPs are employed as the color indicator in colorimetric assays as well as the SERS-active substrate in SERS tests. To the best of our knowledge, this was the first study to couple MIPs and AuNPs-based colorimetric-SERS in one sensing system to rapidly and accurately determine trace levels of chemical hazards in agri-food products.

Chapter 2: Hypothesis and experimental overview

2.1 Rationale

Atrazine is potentially detrimental to human health and its contamination exists in agri-food products. Because the conventional methods to determine atrazine require complex sample pretreatment and instrumentation, the development of rapid, accurate and cost-efficient detection techniques is important to food industries and governmental laboratories.

SERS has been regarded as a rapid and reliable detection method that couples Raman spectroscopy and noble metallic nanoparticles. Atrazine molecule can interact with Raman laser to generate a specific Raman feature band at the wavenumber of 682 cm^{-1} , which is associated with ring-breathing mode 6a [52]. Due to the small Raman scattering cross-section [21], the intensity of the Raman feature band is too weak to determine trace amounts of atrazine residues. By using AuNPs as the substrate, the Raman signals can be significantly enhanced on the surface of the substrate, which is attributed to the electromagnetic field induced by the localized plasmon resonance around the gold nanostructures. Therefore, the enhanced Raman signals can be used for the determination of trace level of atrazine.

To deal with a large number of samples in food safety detection, high-efficient screening methods are necessary for accurate SERS detection. AuNPs-based colorimetric assays are rapid and simple techniques to screen positive samples based on the optical results. With the interaction between AuNPs and atrazine molecules by ligands, the color of AuNPs solution changes from red to blue,

achieving fast screening. However, the accuracy of AuNPs-based colorimetric-SERS dual biosensor can be greatly influenced by impurities and components in food samples. Therefore, it is important to clean up food samples and extract the analyte using effective sample pretreatment before the detection. MIPs, a type of artificial polymers, have specific affinity to the analytes. By using MIPs as the sorbents for sample pretreatment, selective extraction of atrazine from food samples can be achieved. In addition, MIPs are stable enough to be used in extreme *pH* and temperature, and the synthesis of MIPs is simple and cost-efficient, which is affordable to food industry and governmental laboratories.

In this thesis project, a dual biosensor integrating AuNPs-based colorimetric-SERS and MIPs were investigated. Advantages of each part in the biosensor were exploited to achieve the rapid, high-throughput and accurate determination of atrazine in agri-food products. Apple juice was selected as the food matrix because of the accumulation of atrazine in apple crops [53] as well as the wide consumption of apple juice. Since no research has been conducted combining AuNPs-based colorimetric assays, SERS and MIPs to determine atrazine in food products, this study has great importance in improving the convenience, versatility and accuracy of the applied technique for food safety monitoring.

2.2 Objectives

Objectives 1: To synthesize MIPs with specific affinity to atrazine and use MIPs-SPE column to extract trace level of atrazine in apple juice.

Objectives 2: To conduct colorimetric assays to differentiate samples with different levels of atrazine.

Objectives 3: To develop SERS for the quantification of trace level of atrazine.

2.3 Hypothesis

Hypothesis 1: Homemade MIPs can show specific affinity to atrazine and MIPs-SPE separation method can be used to selectively separate atrazine from apple juice.

Hypothesis 2: Homemade AuNPs can be used to rapidly screen apple juice samples containing different concentrations of atrazine.

Hypothesis 3: SERS can apply the same AuNPs as substrates to further validate and quantify atrazine in apple juice.

2.4 Experimental overview

The separation method (*i.e.*, MIPs-SPE) and two detection methods (*i.e.*, AuNPs-based colorimetric assays, SERS) were developed to achieve the objectives and were incorporated to build this biosensor.

MIPs for atrazine were synthesized by bulk polymerization based upon the protocol described in a previous publication with modification [54]. To confirm the specificity of the MIPs towards atrazine,

adsorption capacity of the MIPs was compared with non-imprinted polymers (NIPs) by using different concentrations of atrazine. NIPs were produced without the addition of atrazine during the synthesis. The selectivity of MIPs towards atrazine was verified by comparing the adsorption capacities of MIPs on the same concentration of atrazine, and its structural analog (*i.e.*, thiabendazole and melamine). MIPs were then applied as the sorbents in the SPE columns for the extraction of atrazine from apple juice. The recoveries of atrazine after MIPs-SPE were determined using HPLC-DAD.

AuNPs-based colorimetric assays were conducted on the apple juice eluents from MIPs-SPE. AuNPs of three different sizes were synthesized using the citrate reduction method [17]. The sensitivity of AuNPs was optimized by mixing the appropriate amount of salts. Aliquots of eluents from MIPs-SPE were mixed with AuNPs solution for the colorimetric assays. The optical results were observed by the naked eyes, followed by UV-Vis determination at the wavelength from 400 nm to 800 nm.

SERS tests were performed using AuNPs of three different sizes as SERS-active substrates for comparative analysis. The mixture of apple juice eluent from MIPs-SPE and AuNPs solution was deposited onto the gold-coated slide for SERS spectral collection. A linear regression model was constructed to reveal the correlation between the amount of atrazine in apple juice and the intensity of the SERS feature band of atrazine at 688 cm^{-1} . Finally, LOD and LOQ were experimentally determined.

Chapter 3: Rapid separation of atrazine in apple juice using molecularly imprinted polymers (MIPs)-based solid phase extraction (SPE)

3.1 Introduction

An effective tool that specifically and selectively separates a targeted chemical hazard from complicated food samples is highly required before detection of chemical hazards. We aim to minimize the influence of interfering compounds in food matrices, thus improving the accuracy for further detection.

As the official method for the determination of atrazine in agri-food products, GC is employed as the separation method and MS is subsequently applied as the detection method (Health Canada, 1993). However, as indicated in **Section 1.2**, this technique is laborious, time-consuming, expensive, and requires a large amount of organic solvents. Therefore, it is necessary to develop a rapid, cost-efficient and innovative separation method for the determination of atrazine in foods.

The MIPs-based separation method is an ideal candidate to achieve this goal because it is highly specific, stable, cheap, and can complete the separation in a rapid manner [29]. MIPs are rigid polymers produced by chemical synthesis with specific affinity to the analytes. By packing these polymers into the SPE column, rapid separation of atrazine from complex food matrices can be achieved. The column can retain atrazine when other impurities are washed out. Theoretically, the final eluting solution will only contain atrazine. Compared with other separation methods (*e.g.*, antibody-based methods and aptamer-based methods), it is easier and more convenient to synthesize

MIPs, making MIPs a promising candidate for real-world application.

The objective of this study was to validate 1) whether homemade MIPs have the specificity and selectivity towards atrazine and 2) whether an MIPs-SPE method is able to rapidly separate atrazine from apple juice.

3.2 Materials

Atrazine, melamine, thiabendazole, methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), and 2,2'-azobisisobutyronitrile (AIBN) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, acetonitrile, phosphoric acid and chloroform (all in HPLC grade) were purchased from Thermo Fisher Scientific (Toronto ON, Canada). Sun-Rype brand apple juice samples were obtained from several local grocery stores in Vancouver, BC, Canada. Atrazine standard solutions were prepared using 100% methanol containing different concentrations of atrazine.

3.3 Methods

3.3.1 Synthetic process of MIPs

The procedure for the synthesis of MIPs for atrazine was adopted based upon the protocol described by Matsui and coauthors with modification [54]. Atrazine was used as the template molecule; MAA was used as the functional monomer; EGDMA was used as the cross-linker; AIBN was used as the reaction initiator; and chloroform was used as the porogen. Specifically, 1 mmol of atrazine and 4

mmol of MAA were dissolved in 5 mL of chloroform in a 50-mL round flask under magnetic stir at 22°C for 10 min. Then, 20 mmol of EGDMA and 50 mg of AIBN were added into the mixture before being purged by nitrogen for 10 min. This solution was then sealed and put in a water bath at 60°C for 24 h to initiate thermal polymerization. The monolithic polymer was obtained after polymerization and then crushed, ground, and sieved through a 200-mesh steel sieve, resulting in homogeneous fine particles. Soxhlet extraction was then conducted to remove the template by using 200 mL of methanol/acetic acid (7:3, v/v) for 24 h, followed by using another 200 mL of pure methanol for 24 h until no atrazine was detected from the extraction solution using ultraviolet spectroscopy at the wavelength of 263 nm. Finally, synthesized polymers were dried overnight in a vacuum drying oven at 60°C. As the parallel study, non-imprinted polymers (NIPs) were prepared and synthesized by using the same procedure without the addition of the template molecule.

3.3.2 Tests on adsorption capacity

Tests on the adsorption capacity of MIPs include a static adsorption test, a kinetic adsorption test and a selectivity test. The static and the kinetic adsorption tests were separately conducted on MIPs and NIPs to test the specific affinity of MIPs towards atrazine. Using melamine, thiabendazole, and atrazine, a selectivity test was conducted to determine if MIPs have the selective preference towards atrazine. Different solvents were tested in the preliminary experiment to optimize the adsorption condition towards atrazine because the polarity of the solvent can affect the adsorption performance of MIPs [55].

To determine the static adsorption capacity, 10 mg MIPs or NIPs were mixed with 2 mL methanol

solution (methanol/water, 9:1, v/v) of atrazine at selective concentrations ranging from 10 mg L⁻¹ to 100 mg L⁻¹, followed by shaking at 200 rpm for 3 h at room temperature. The mixture was then centrifuged at 10000 ×g for 10 min and the supernatant was applied through a 0.22-μm nylon syringe filter. The final concentration of atrazine was determined using an Agilent 1260 series high performance liquid chromatography (HPLC) system with the photodiode array detector (DAD) set at a wavelength of 263 nm. In this HPLC system, the volume of sample injection was set at 0.2 μL, the column was a Waters C₁₈ column (particle size: 10 μm, length × inner diameter: 300 mm × 3.9 mm), and the temperature was set at 30°C. HPLC grade solvent of 0.01 M phosphoric acid-acetonitrile (50:50, v/v) was used as the mobile phase with a flow rate of 1.5 mL min⁻¹. Three separate injections were conducted for each sample and the total running time for each injection was 6 min.

The kinetic adsorption test was also conducted. A total of 2 mL of methanol solution (methanol/water, 9:1, v/v) of atrazine at the concentration of 100 mg L⁻¹ was used to dissolve 10 mg of MIPs or NIPs. The mixture was shaken horizontally at 200 rpm during different time intervals (*i.e.* from 10 min to 240 min) at room temperature, followed by centrifugation at 10000 ×g for 10 min. The supernatant was then applied through a 0.22-μm nylon syringe filter before the determination of the final concentration of atrazine using HPLC-DAD at 263 nm. All the parameters set in the HPLC system followed the same protocol as in the static adsorption test.

For the selectivity test, melamine, thiabendazole and atrazine were separately prepared in a 2 mL methanol solution (methanol/water, 9:1, v/v) at the concentration of 100 mg L⁻¹ to dissolve 10 mg MIPs. They were individually incubated under the same condition as indicated in the static

adsorption test before the determination using HPLC-DAD.

3.3.3 Pretreatment of apple juice

Individual apple juice samples (9 mL) were spiked with 1 mL methanol solution of atrazine at different concentrations ranging from 0.005 mg L⁻¹ to 20 mg L⁻¹. Then, 9 mL of hexane was added and each mixture was shaken for 30 s, followed by ultra-sonication for 1 min and centrifugation at 5000 ×g for 3 min. The supernatant (7 mL) was collected and distilled in a rotary evaporator under reduced pressure until the liquid was dried. The remaining components were re-dissolved in 7 mL methanol solution (methanol/water, 1:1, v/v).

3.3.4 Clean-up of samples by MIPs-based SPE (MIPs-SPE)

To set up a MIPs-SPE column, 300 mg of dried MIPs particles were packed into a polypropylene SPE cartridge with two PTFE frits at each end (Agilent, Santa Clara, CA, USA). The procedure for operating the MIPs-SPE was optimized in the preliminary study and then applied to apple juice. Specifically, the MIPs-SPE column was first conditioned with acetonitrile (2 mL), water (2 mL), and 2 mL of methanol solution (methanol/water, 1:1, v/v) in sequence. Then, the pre-treated apple juice sample (3 mL) was loaded onto the column, followed by washing with 2 mL of methanol solution (methanol/water, 3:2, v/v). After that, 3 mL of methanol (100%) was used to elute atrazine. The flow rate was kept consistent at 0.5 mL min⁻¹ for all of the steps. In the end, the eluting solution was analyzed using HPLC-DAD at 263 nm to determine the recovery rate of atrazine. In comparison, all of the procedures were applied using NIPs at the same time.

3.4 Results and discussion

3.4.1 Synthesis and validation of MIPs

The synthesis process of MIPs is illustrated in **Figure 3.1**. Several previous studies were carried out to test the separation of atrazine using MIPs synthesized in different forms, including bulk form [56], precipitate form [54], and sol-gel form [57]. In most cases, MIPs in bulk forms were employed because the procedure of bulk polymerization is simple and fast. However, crushing and grinding monolith MIPs after bulk polymerization can lead to irregular sizes and damage binding sites to particles, further weakening the adsorption capacity of MIPs [29]. Therefore, the fabrication of MIPs in bulk forms for atrazine in this thesis project was adapted and modified based on Matsui's protocol [54]. Processed fine MIPs particles were expected to have less damage on the specificity.

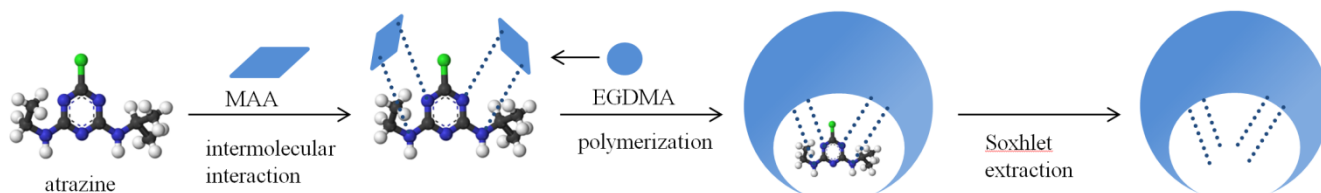


Figure 3.1 Schematic illustration of synthesis process of MIPs for atrazine

Static and kinetic adsorption tests were individually conducted using the synthesized MIPs and NIPs to evaluate the specific affinity of MIPs towards atrazine. Specific binding sites and cavities with the complementary shape and size to the template molecule in the developed MIPs contributed to its

specific affinity. These binding sites and cavities are generated as molecular memory during the bulk polymerization process due to the intermolecular interaction between the atrazine molecule and MAA. Theoretically, this molecular memory enables MIPs to selectively recognize and rebind only atrazine for multiple times, but it can be greatly influenced by the selection of solvent used in the adsorption test. MIPs usually show the best rebinding capacity in the solvent that is also used as the porogenic solvent for the synthesis of MIPs [58]. In this thesis project, chloroform was used as the porogenic solvent because it is one of the best porogens that can facilitate the formation of hydrogen bonds during the synthesis of MIPs [48]. However, chloroform could react with plastic and extract organic plasticizers and stabilizers in the test tubes during the adsorption test. Therefore, other solvents such methanol, ethanol, and acetonitrile were tested in the preliminary study. Methanol was confirmed to serve as the solvent used in the adsorption test, in which the difference in the adsorption capacity between MIPs and NIPs was the most significant.

The static adsorption isotherm curve is shown in **Figure 3.2**. The adsorption capacity (Q) value was used to determine the ability of MIPs to rebind to atrazine. The following equation is used to calculate the Q value [59]:

$$Q = (C_i - C_f) \times V/W \quad (1)$$

C_i represents the initial concentration of atrazine in the solution and C_f represents the final concentration of atrazine in the solution. V is the volume of the solution and W is the mass of the polymers. Adsorption capacity of both MIPs and NIPs increased along with the increase of the initial

concentration of atrazine. Q of MIPs was always higher than Q of NIPs. When the concentration of atrazine reached to 100 mg L⁻¹ in the solvent, Q of MIPs (~0.182 mg g⁻¹) was approximately twice as much as Q of NIPs (~0.096 mg g⁻¹). This difference indicated that the binding sites and cavities with specific affinity to recognize and rebind to atrazine were successfully imprinted in the MIPs particles. NIPs particles could also adsorb a certain amount of atrazine due to the non-specific binding (*e.g.*, electrostatic force) and irregular gaps on the surface of NIPs particles that fit the atrazine molecule.

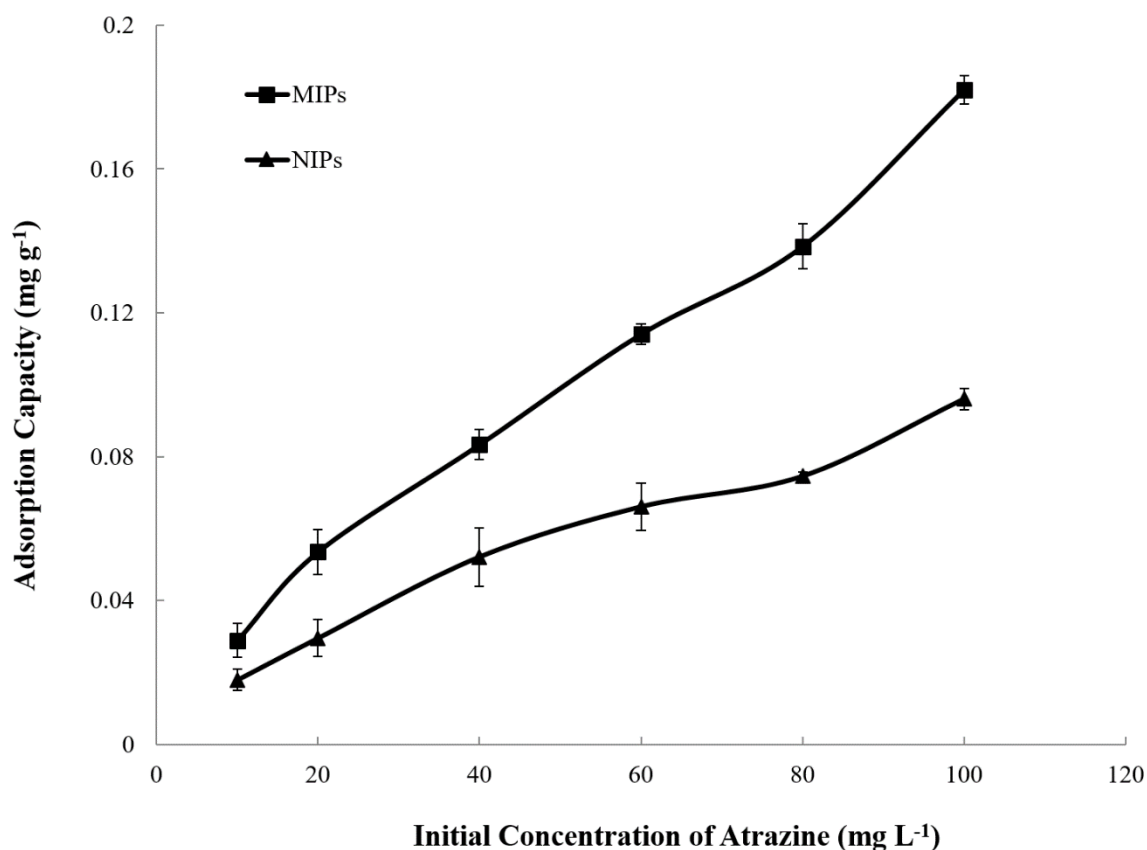


Figure 3.2 Static binding isotherm of MIPs and NIPs for atrazine (reaction time: 3 h). Standard deviation is represented by error bars (n=3).

The kinetic adsorption isotherm curve is shown in **Figure 3.3**. The less time it takes to reach to equilibrium, the faster the MIPs can separate atrazine in the practical application. The equilibration process of 10 mg MIPs and 10 mg NIPs in 2mL of atrazine solution (100mgL^{-1}) was recorded at seven different time points. Both MIPs and NIPs reached to the saturation within 120 min. Due to the large variation in adsorption capacity between MIPs and NIPs, the adsorption rate of MIPs was significantly higher ($P < 0.05$) than that of NIPs. When the incubation time was 10 min, Q of MIPs was approximately 0.164 mg g^{-1} , confirming the high efficiency of MIPs for rapid separation of atrazine in the complex foods matrices.

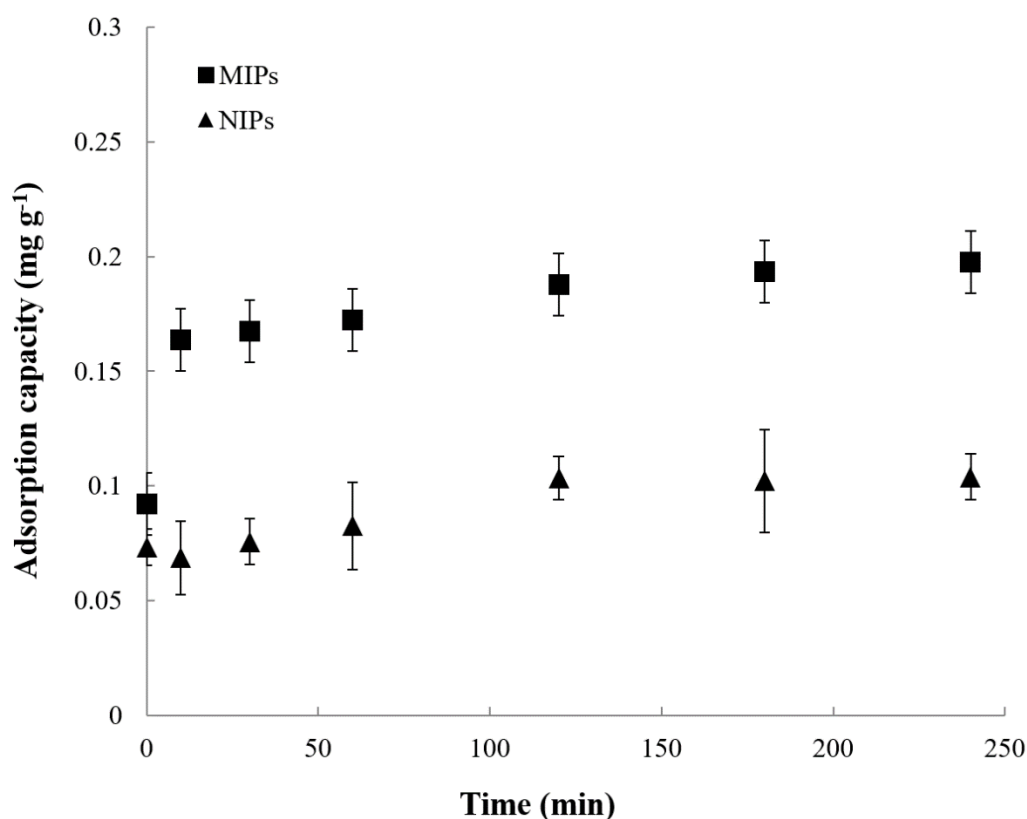
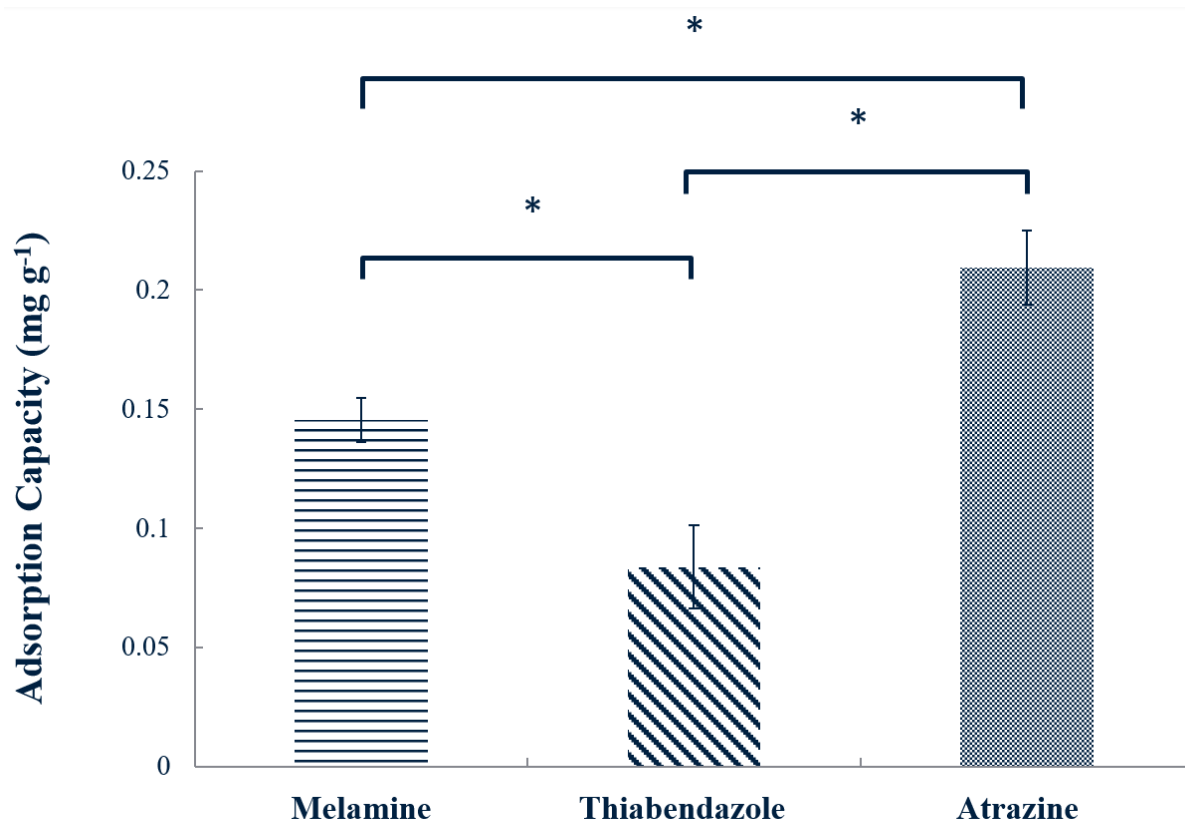


Figure 3.3 Kinetic binding plot of MIPs and NIPs (initial concentration of atrazine: 100 mg L^{-1}). Standard deviation is represented by error bars ($n=3$).

The selectivity test was also performed and the results are shown in **Figure 3.4**. The purpose of this test is to validate the selectivity of MIPs on atrazine instead of other molecules with similar molecular structures or functional groups. Melamine and thiabendazole are two of the most common food chemical hazards [60]. Resembling an atrazine molecule, a melamine molecule contains the triazine skeleton and a thiabendazole molecule has imino groups. In our current study, by using ANOVA with Least Significant Difference (LSD), the significant difference ($P < 0.05$) in the adsorption capacity of MIPs was observed between melamine, thiabendazole, and atrazine, confirming that the synthesized MIPs had selectivity to rebind atrazine. Taken together, all the tests on adsorption capacity validated that the synthesized MIPs could specifically and selectively rebind atrazine.



* denotes significant difference (Single-factor ANOVA and LSD, $\alpha=0.05$, $n=3$)

Figure 3.4 Comparison of the adsorption capacity of the synthesized MIPS towards melamine, thiabendazole and atrazine (initial concentration: 100 mg L⁻¹).

3.4.2 MIPS-SPE for apple juice spiked with atrazine

The procedure for operating MIPS-SPE is demonstrated in **Figure 3.5**. This procedure was designed and optimized by using an atrazine standard solution in the preliminary study, including the adjustment of flow rate and the selection of solvent. To increase the efficiency for practical application of this developed method, the flow rate should be increased to reduce the entire operating time. Acetic acid is commonly used to elute samples from the MIPS-SPE column. The acid

contributes to the breakage of hydrogen bonds between atrazine and MIPs, thus a high flow rate can be achieved with a satisfactory recovery of atrazine. In a previous study, a methanol–acetic acid mixture was applied for MIPs-SPE to extract atrazine from water with a high flow rate of 3 mL min⁻¹ [61], but a time-consuming GC-MS analysis was conducted for the further detection. In our current study, AuNPs-based rapid detection was applied after MIPs-SPE as the proposed sensing element. The existence of acid would destroy the AuNPs used in the tests; therefore, only pure methanol was used for the final elution. The flow rate was relatively decreased and finally set at 0.5 mL min⁻¹ to maintain a high recovery of atrazine (>90%).

After the optimization of MIPs-SPE procedure, both MIPs-SPE columns and NIPs-SPE columns were evaluated by applying apple juice samples spiked with different concentrations of atrazine (*i.e.* 0.5 mg L⁻¹, 1 mg L⁻¹, 5 mg L⁻¹, 10 mg L⁻¹ and 20 mg L⁻¹). These samples were pretreated using liquid-liquid extraction with hexane to remove various macromolecules (*e.g.* sugar, etc.) from apple juice. After that, the pretreated apple juice samples were cleaned up with MIPs-SPE column and atrazine was then collected in the eluting solution. Recoveries of atrazine using both MIPs-SPE columns and NIPs-SPE columns were determined using HPLC-DAD and the results are shown in **Table 3.1**. The recoveries of different concentrations of atrazine in apple juice from MIPs-SPE columns ranged from 91.98% to 93.71%, which were significantly higher than that from NIPs-SPE columns (*i.e.*, 67.26% to 73%).

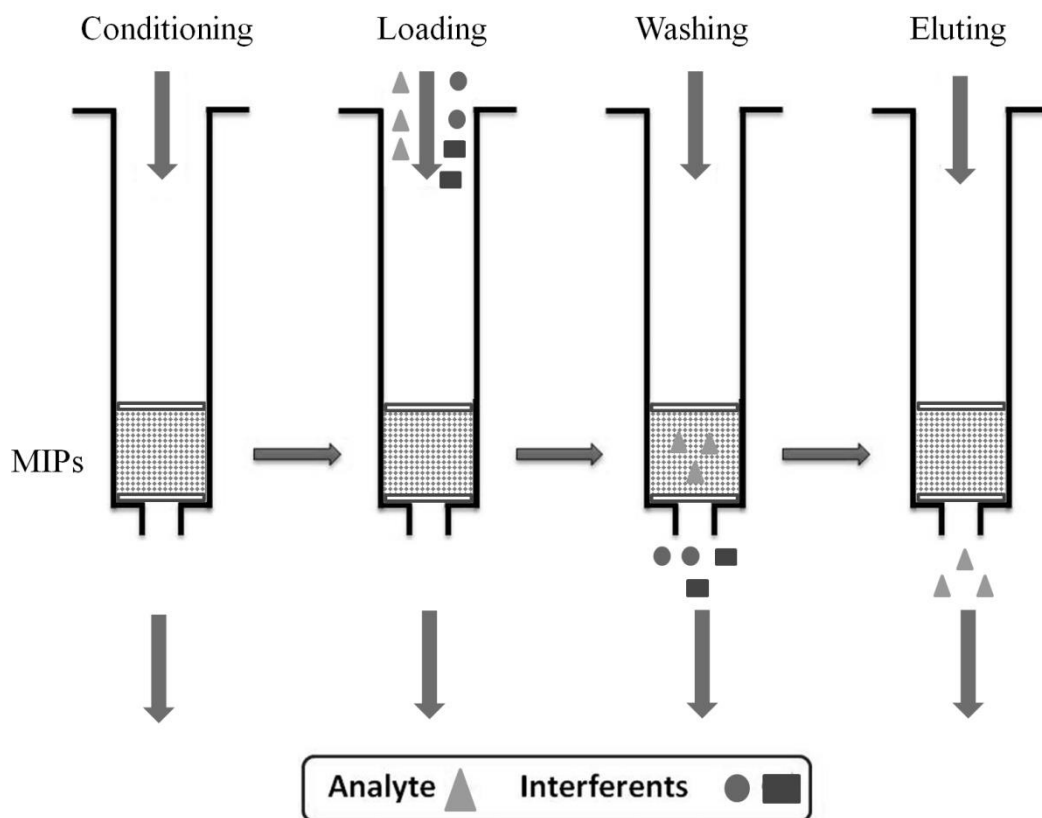


Figure 3.5 Schematic illustration of the procedure for MIPs-SPE to separate the targeted analyte (*i.e.*, atrazine) from other interferents in the food matrices.

Table 3.1 Recoveries of atrazine in apple juice samples by MIPs-SPE and NIPs-SPE (n=3).

Concentration of Spiked Atrazine (mg L ⁻¹)	MIPs-SPE Column (%)	NIPs-SPE Column (%)
20	93.15	72.18
10	92.03	72.32
5	91.98	67.26
1	93.71	73.00
0.5	N/A*	N/A

* HPLC-DAD was unable to detect concentration of atrazine spiked at 0.5 mg L⁻¹.

For the development of analytical methods to determine herbicide residues in the complicated food matrices, simplifying sample pretreatment step is important to improve the separation efficiency. The traditional extraction process for the determination of atrazine requires at least a few hours. It uses dichloromethane to remove macromolecules from the complicated food samples using a liquid-solid extractor, which is time-consuming, expensive, and requires a large amount of organic solvent (Health Canada, 1993). Alternatively, solid phase microextraction (SPME) has been used to simultaneously purify and concentrate herbicide residues from food samples. However, this technique is limited to the aqueous samples and it cannot be applied to highly concentrated analytes [62]. In addition, Quick-Easy-Cheap-Effective-Rugged-Safe (QuEChERS) is another extraction method that has been successfully developed to determine herbicide residues in apple juice. A single extraction can be completed in 10 min with a high recovery rate, but the matrix effect can negatively impact the detection limit [63]. In the current study, by applying MIPs-SPE to clean up the components in apple juice, the MIPs-SPE column was able to separate the atrazine with a high recovery rate. The total time for each sample was about 20 min.

3.5 Conclusion

MIPs with specific affinity towards atrazine were successfully synthesized and confirmed to be effective. These particles were employed as sorbents in the SPE column. The developed MIPs-SPE method could selectively separate atrazine from apple juice with high recovery (~93%) and high efficiency. Moreover, this separation method can be readily integrated with different detection methods to develop novel techniques for the determination of trace levels of atrazine in agri-food products.

Chapter 4: Fast screening of atrazine contaminated apple juice using gold nanoparticles (AuNPs)-based colorimetric tests

4.1 Introduction

In **Chapter 3**, the MIPs-SPE method was able to rapidly and selectively separate atrazine from apple juice. To further determine trace levels of atrazine, novel and rapid detection methods are required to be integrated with the separation step.

AuNPs-based colorimetric assays have been developed as simple and straightforward methods for the detection of various analytes. To detect atrazine, AuNPs are first stabilized in citrate buffer. Upon the addition of atrazine, atrazine molecules replace citrate on the surface of AuNPs and then induce binding and cross-linking of AuNPs with imino ligands. Consequently, AuNPs rapidly change from a monodisperse status to an aggregated status along with a color variation from red to blue that can indicate the amount of atrazine residue. The optical results can be determined by visualization and further confirmed using UV-Vis spectrometry.

Due to the rapid, simple, straightforward and instrument-free features, AuNPs-based colorimetric assays are usually applied as a screening method for qualitative analysis [64]. However, its application in the complicated food matrices is still limited because interferents (*e.g.*, food residues) can also induce the aggregation of AuNPs, leading to false positive and inaccurate results for the targeted analyte. Therefore, we aim to integrate MIPs-SPE with AuNPs-based colorimetric assays as a more reliable sensing platform to determine the trace level of atrazine in foods. Apple juice

samples were cleaned up using MIPs-SPE columns and atrazine was extracted into the eluting solution for subsequent detection. By applying this technology, the analyte (*i.e.*, atrazine) is expected to be the only parameter that can induce the aggregation of AuNPs, thereafter improving the accuracy and the efficiency of the colorimetric assays.

The objective of this study was to develop and integrate the AuNPs-based colorimetric assays with MIPs-SPE for the detection of atrazine in apple juice in a rapid manner.

4.2 Materials

Chloroauric acid (HAuCl₄), atrazine, trisodium citrate dehydrate, and sodium chloride were purchased from Sigma Aldrich (St. Louis, MO, USA). Methanol in HPLC grade was purchased from Thermo Fisher Scientific (Toronto ON, Canada). Apple juice samples spiked with different concentrations of atrazine (*i.e.*, 0 mg L⁻¹, 0.005 mg L⁻¹, 0.01 mg L⁻¹, 0.1 mg L⁻¹, 0.5 mg L⁻¹ and 1 mg L⁻¹) were treated by MIPs-SPE and stored at 4°C. Atrazine standard solutions at various concentrations from 0.005 mg L⁻¹ to 1 mg L⁻¹ were prepared by dissolving atrazine in methanol.

4.3 Methods

4.3.1 Synthesis of AuNPs of different sizes

AuNPs of three different sizes (*i.e.*, defined as small, medium and large) were synthesized using the citrate reduction method. Small size of AuNPs with the expected particle diameters of 10-15 nm were synthesized based upon the protocol described in a previous study with modification [17].

Briefly, aqua regia (HCl/HNO₃, 3:1, v/v) was freshly prepared to completely wash all the glassware and they were then rinsed with deionized water and dried in the oven prior to the following procedures. A total of 0.7 mL of 1% (w/v) sodium citrate solution was rapidly added into a boiling solution of 30 mL of 0.01% (w/v) HAuCl₄ under magnetic stirring. The color of the mixed solution gradually changed from yellow to grey, then to red within 2 min. The mixed solution was continuously boiled under stirring for 20 min before the heating was ceased. The resulting solution was cooled down to room temperature and stored at 4°C. AuNPs with different particle diameters below 50 nm were synthesized by adjusting the ratio of HAuCl₄ to sodium citrate solution [45]. Following the same procedure, AuNPs of medium size (25 nm~30 nm) were synthesized by mixing 0.5 mL of 1% (w/v) sodium citrate solution with 30 mL of 0.01% (w/v) HAuCl₄, while AuNPs of large size (40 nm~45 nm) were obtained by mixing 0.3 mL of 1% (w/v) sodium citrate solution and 30 mL of 0.01% (w/v) HAuCl₄. The AuNPs solutions were all citrate capped and the weight concentrations were 60 mg L⁻¹.

A UV-Vis spectrometer was used to determine the size and number density of AuNPs at wavelengths ranging from 400 nm to 800 nm. Each AuNPs solution was tested 4 times and the wavelength of the highest peak in the UV-Vis spectrum was selected and recorded.

4.3.2 Improvement on the sensitivity of AuNPs

To increase the sensitivity of AuNPs, a preliminary test was conducted to determine the highest concentration of sodium chloride that can be added into the AuNPs solution without causing the aggregation of AuNPs. Different amounts of NaCl (*i.e.*, 10 mmol L⁻¹, 20 mmol L⁻¹, 30 mmol L⁻¹, 40

mmol L⁻¹ and 50 mmol L⁻¹) were added into the synthesized AuNPs solutions, followed by incubation for 20 min at room temperature. The mixed solution was determined using UV-Vis spectrometry at wavelengths of 640 nm and 520 nm.

4.3.3 AuNPs-based colorimetric assays

The procedure of colorimetric assays was optimized and applied to elute apple juice after MIPs-SPE. Specifically, 400 µL of AuNPs solution was mixed with 50 µL of the eluted apple juice samples containing different concentrations of atrazine (*i.e.*, 0 mg L⁻¹, 0.005 mg L⁻¹, 0.01 mg L⁻¹, 0.1 mg L⁻¹, 0.5 mg L⁻¹ and 1 mg L⁻¹). All of the mixed solutions were gently shaken at room temperature for 5 min. Color changes were observed by the naked eyes, followed by UV-Vis spectral collection at wavelengths from 400 nm to 800 nm.

4.4 Results and discussion

4.4.1 Evaluation of size of the synthesized AuNPs

Both size and number density of AuNPs can be determined from UV-Vis spectra due to the relationship between the SPR of AuNPs and UV absorbance [65-71]. In the current study, the average diameter of AuNPs (d) and the estimated number density of AuNPs (N) were calculated using the following equations [66] [67]:

$$d = \begin{cases} 3 + 7.5 \times 10^{-5} X^4, & X < 23 \\ [\sqrt{X - 17} - 1]/0.06, & X \geq 23 \end{cases}, X = \lambda_{max} - 500 \quad (2)$$

$$N = \frac{A_{450} \times 10^{14}}{d^2[-0.295 + 1.36 \exp(-(\frac{d - 96.8}{78.2})^2)]} \quad (3)$$

λ_{max} is the wavelength of the maximum UV-Vis peak in the collected spectra and A_{450} is the absorbance at the wavelength of 450 nm. The calculated results are summarized in **Table 4.1**. The average size of AuNPs met expectations of the predicted sizes for each size of AuNPs. The estimated number density of AuNPs decreased along with an increase of the size of AuNPs.

Table 4.1 Average size and estimated number density of different synthesized AuNPs

AuNPs	Predicted size (nm)	λ_{max} (nm)	A_{450}	Average size (nm)	Estimated number density (dm^{-3})	Standard deviation of average size
Small	10 ~ 15	518	0.70	10.87	5.31×10^{12}	1.00
Medium	25 ~ 30	524	0.67	27.43	2.75×10^{11}	1.50
Large	40 ~ 45	530	0.69	43.43	6.55×10^{10}	2.10

* Values of λ_{max} and A_{450} are averages (number=3) from collected UV-Vis spectra.

Although the synthesis of AuNPs of different sizes was confirmed, it is worth noting that all the calculations were based on two assumptions: 1) the synthesis reaction was complete and the reducing agent was used up; 2) the synthesized AuNPs were monodisperse and perfectly spherical. The first assumption was asserted because no residues of HAuCl_4 could be detected using UV-Vis

spectrometry at wavelengths between 200 nm and 300 nm. For the second assumption, the standard deviation of the particle size (4.8%-9.2%) of AuNPs indicated that the particles were monodisperse and spherical [66]. In addition, the complete cleaning of all glassware with aqua regia was critical to avoid nucleation and aggregation of gold colloid during the synthesis of AuNPs.

4.4.2 Optimization of the addition of NaCl

Addition of the appropriate amount of NaCl in AuNPs solutions can promote ligand exchange between citrate and atrazine at the surface of AuNPs, resulting in an improvement in the sensitivity of the AuNPs [72]. However, excessive amounts of Na^+ and Cl^- ions can modify the surface electric potential of the AuNPs and affect the nanoparticles to lose their zeta potential. Consequently, AuNPs lose their mutual repulsion and concomitantly decrease the inter-particle distance, leading to the conjugation of the particles [73]. The amount of NaCl should be optimized to increase the detection sensitivity of AuNPs without causing the aggregation of AuNPs when there is no atrazine added.

Various concentrations of NaCl from 0 mmol L^{-1} to 50 mmol L^{-1} were individually added into the AuNPs solutions and determined by both naked eyes and UV-Vis spectrometry after 15 min of incubation. The UV-Vis absorbance was measured at wavelengths of 640 nm and 520 nm, and the absorption ratio (640/520) was used to quantify the aggregation level of AuNPs [72]. AuNPs remained stable when the concentration of NaCl was 20 mmol L^{-1} , but an aggregation of AuNPs occurred when the concentration of NaCl was 30 mmol L^{-1} (**Figure 4.1**). The results were consistent for all the AuNPs of different sizes. Therefore, AuNPs solutions used for the colorimetric tests were added with a 20 mmol L^{-1} of NaCl.

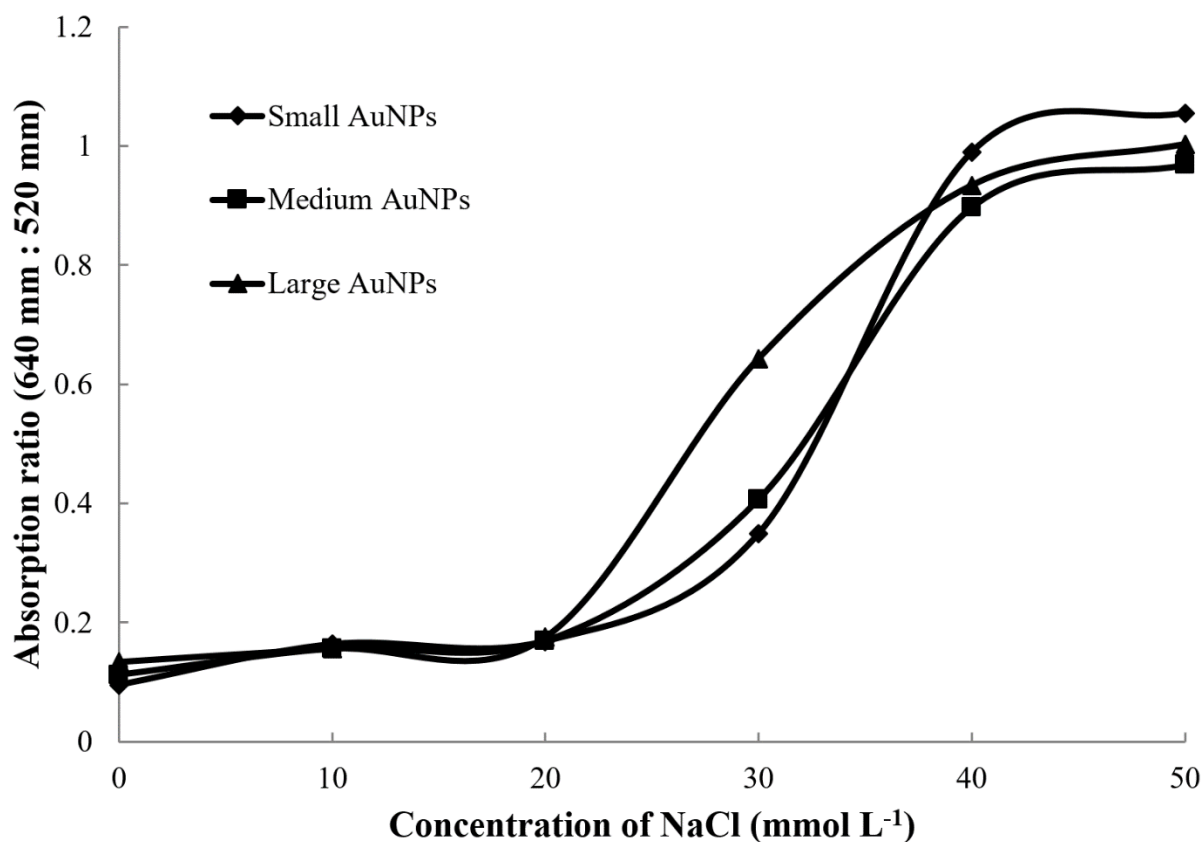


Figure 4.1 Optimization of the addition of NaCl. The concentration of 20 mmol L⁻¹ was selected for all the AuNPs of different sizes.

4.4.3 Colorimetric and UV–Vis spectroscopic analyses of atrazine

The procedure of conducting AuNPs-based colorimetric assays coupled with MIPs-SPE to determine atrazine in apple juice is shown in **Figure 4.2**.

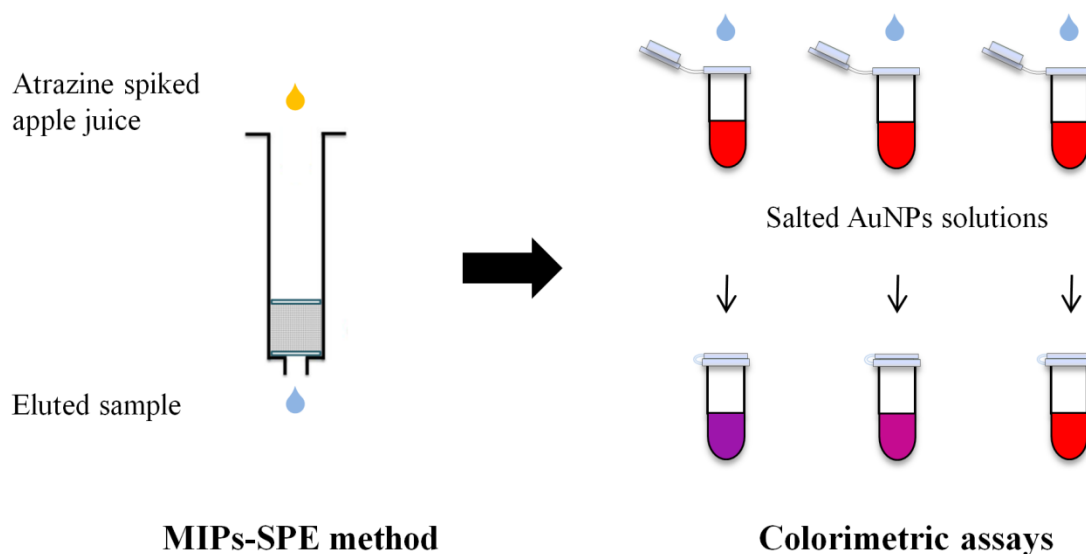


Figure 4.2 Schematic illustration of the determination of atrazine in apple juice using AuNPs-based colorimetric assays coupled with MIPs-SPE separation.

The procedure was designed and optimized (*e.g.*, reaction ratio and time) by using atrazine standard solution and large size of AuNPs. The variation in color observed by the naked eyes was significant when 400 μL of salted AuNPs solution was mixed with 50 μL of atrazine standard solution, followed by gentle shaking for 5 min. The color of these samples gradually changed from red to blue along with the increase in the concentration of atrazine from 0 mg L^{-1} to 1 mg L^{-1} (**Figure 4.3a**). The optimized procedure was then applied to apple juice spiked with different concentrations of atrazine after MIPs-SPE. AuNPs of different sizes were separately used in the colorimetric assays. The visual results of the application of AuNPs with different sizes are shown in **Figure 4.3b, c, d**.

When the AuNPs of large size were used for the colorimetric assays, similar results were obtained between the atrazine standard solution and atrazine-spiked apple juice eluted from MIPs-SPE. These

results validated that atrazine was the only factor to induce the aggregation of AuNPs and MIPs-SPE successfully separated atrazine from apple juice and cleaned up the impurities. By comparing **Figures 4.3b, c, d**, a more distinctive color gradient pattern could be observed by using AuNPs of a larger size in the colorimetric assays. The sensitivity of the colorimetric reaction was improved along with the increase in the size of AuNPs. In the large-size AuNPs-based colorimetric assays, the difference in color could be visually determined between the concentration of atrazine of 0 mg L⁻¹ and 0.01 mg L⁻¹. In the medium-size AuNPs-based colorimetric assays, no significant difference in color was observed among different concentrations of atrazine under 0.05 mg L⁻¹. When the AuNPs of small size were applied, the colorimetric assays were not able to differentiate the samples containing less than 1 mg L⁻¹ of atrazine.

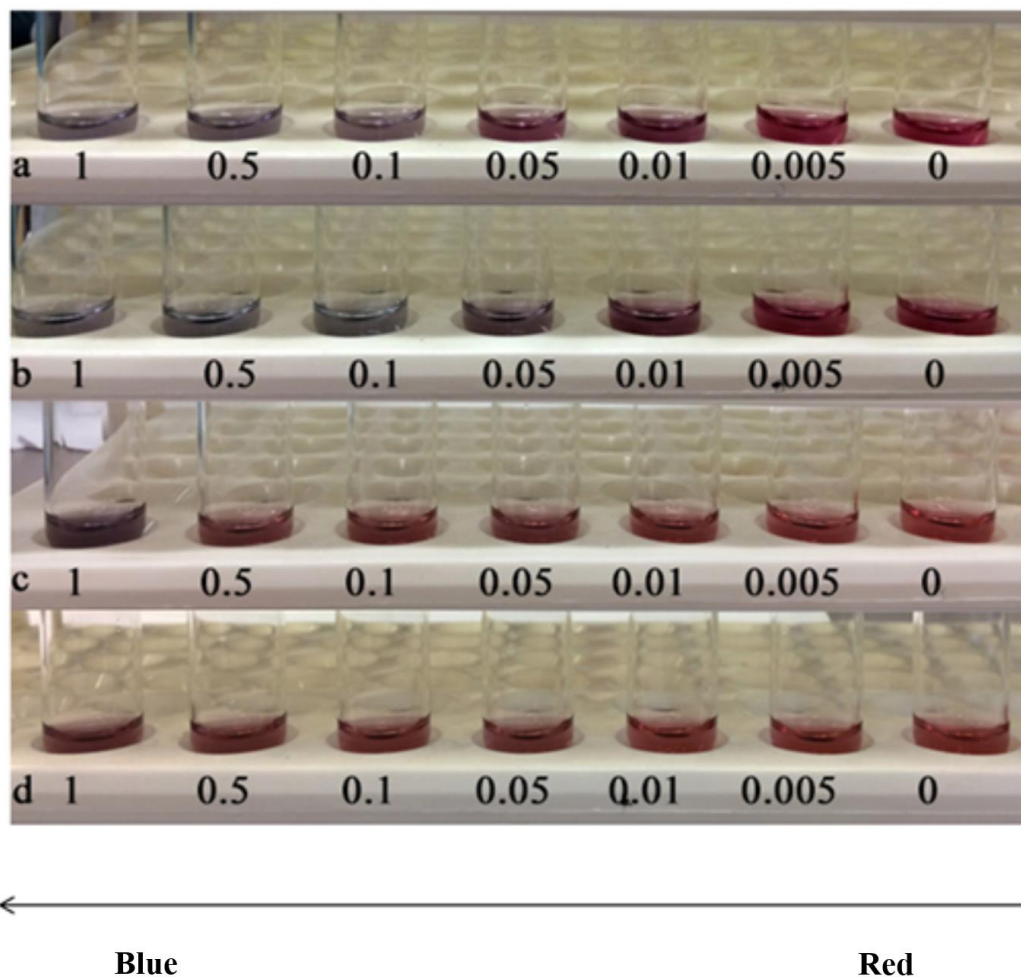
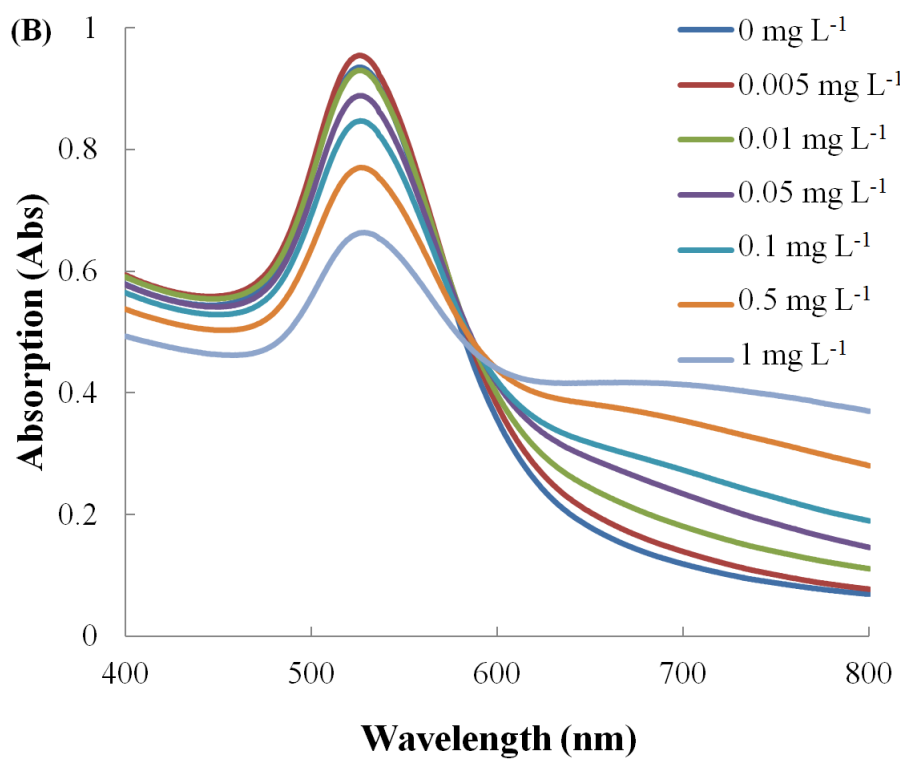
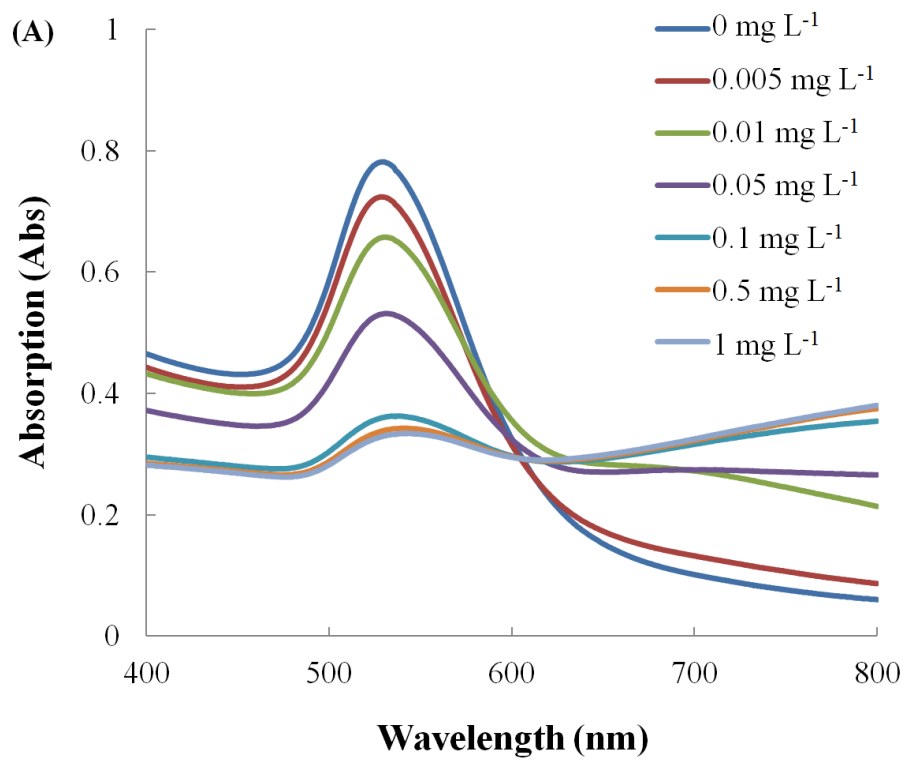


Figure 4.3 Representative visual images of the colorimetric assays on the addition of series of atrazine spiked samples into AuNPs solutions. From right to left are concentrations of atrazine at 0 mg L⁻¹, 0.005 mg L⁻¹, 0.01 mg L⁻¹, 0.05 mg L⁻¹, 0.1 mg L⁻¹, 0.5 mg L⁻¹ and 1 mg L⁻¹. a) AuNPs of large size with atrazine standard solution; b) AuNPs of large size with atrazine spiked apple juice samples; c) AuNPs of medium size with atrazine spiked apple juice samples; d) AuNPs of small size with atrazine spiked apple juice samples.

To further confirm the results from the colorimetric assay, a UV-Vis spectrometer was used to determine the variations among colors. **Figure 4.4** shows the representative UV-Vis absorption spectra of AuNPs of large size (**A**), medium size (**B**) and small size (**C**), under the reaction with different concentrations of atrazine in apple juice eluents after MIPs-SPE.



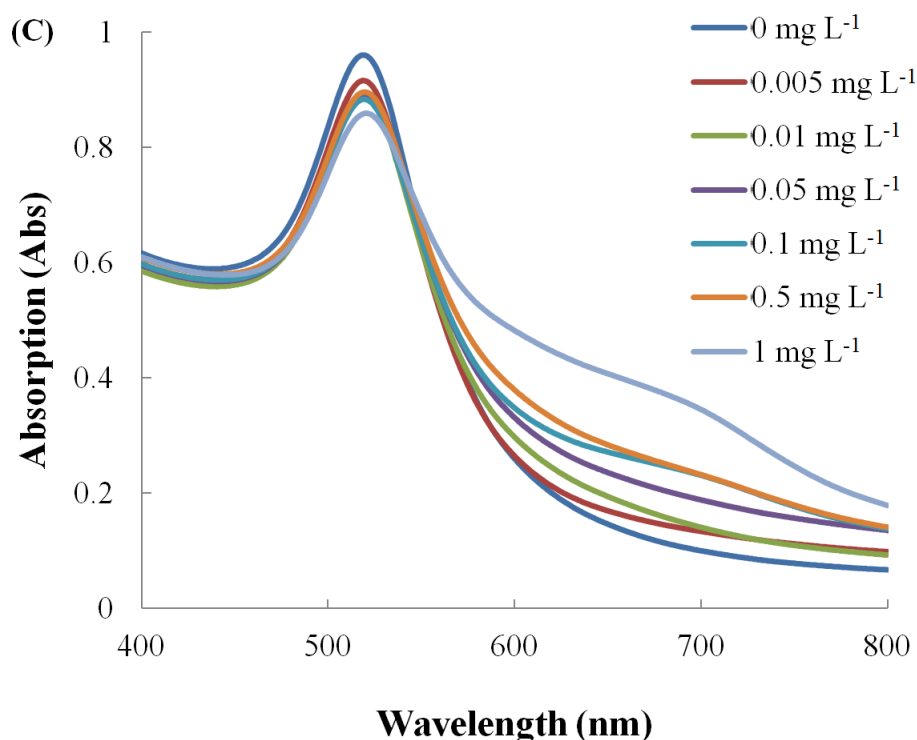


Figure 4.4 Average UV-Vis absorption spectra of AuNPs of large size (A), medium size (B) and small size (C), under the reaction with different concentrations of atrazine in apple juice eluents after MIPs-SPE.

In **Section 4.4.2**, the absorption ratio (640/520) was used to quantify the aggregation of AuNPs, which could be explained by the UV-Vis spectra. Along with the change in color from red to blue, the original UV-Vis absorbance of AuNPs at the wavelength of 520 nm decreased gradually while the absorbance at the wavelength of 640 nm increased noticeably. The absorbance changed due to the variation in SPR caused by the aggregation of AuNPs [35]. Therefore, UV-Vis absorption ratio of 640 nm to 520 nm could be used to represent the color of AuNPs, which further signified the degree of the aggregation of AuNPs [74]. Results of the absorption ratios (640/520) of AuNPs of different sizes used in the colorimetric assays are compared in **Figure 4.5**.

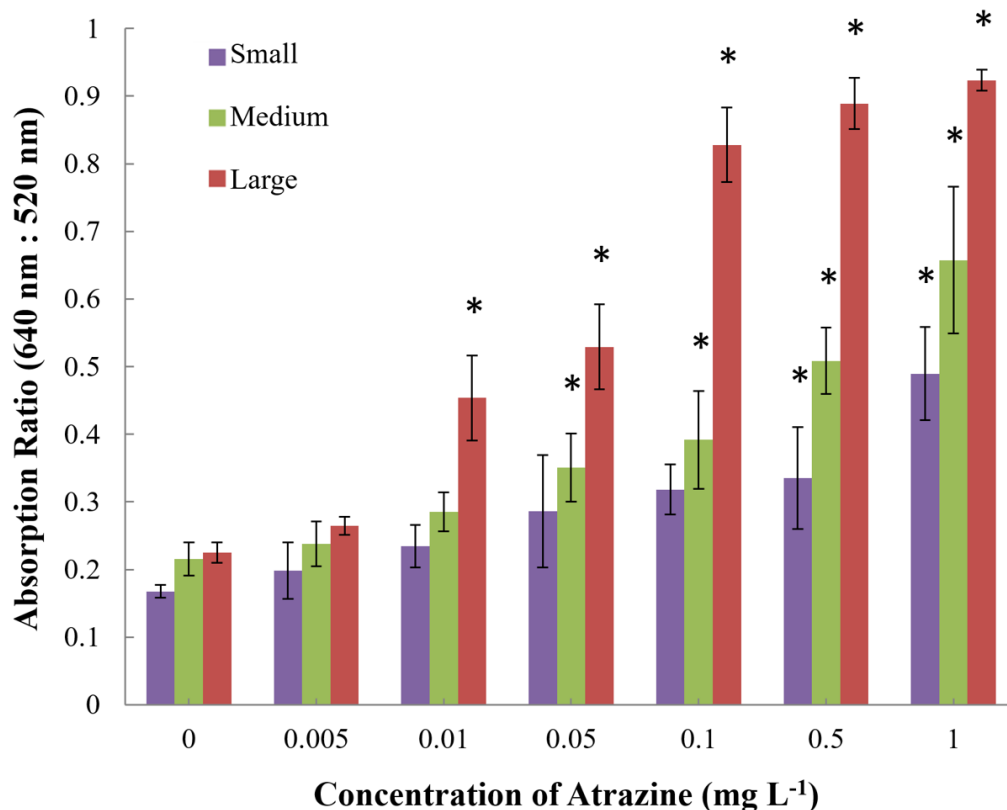


Figure 4.5 The absorption ratio (640 nm : 520 nm) of AuNPs of different sizes versus various concentrations of atrazine spiked in apple juice samples (*i.e.*, 0 mg L⁻¹, 0.005 mg L⁻¹, 0.01 mg L⁻¹, 0.05 mg L⁻¹, 0.1 mg L⁻¹, 0.5 mg L⁻¹ and 1 mg L⁻¹). Stars on the graph indicate the significant difference between experimental groups and the control group (based on the two-tailed T-test).

An increasing trend in the adsorption ratios was observed along with an increase in the concentration of atrazine in the apple juice. The limit of detection (LOD) of atrazine was 0.5 mg L⁻¹ for AuNPs of small size, 0.05 mg L⁻¹ for AuNPs of medium size and 0.01 mg L⁻¹ for AuNPs of large size. Results obtained from UV-Vis spectrometry corresponded to the previous optical results, validating that AuNPs of larger size had a higher sensitivity in the colorimetric assays. Nevertheless, when samples contained high levels of atrazine (*i.e.*, 0.5 mg L⁻¹ and 1 mg L⁻¹), the AuNPs of large size showed no significant differences in color between samples while the AuNPs of medium size could still

differentiate samples. The phenomenon was due to the complete aggregation of AuNPs of large size, eliminating the differences in color caused by SPR [75]. Taken together, the AuNPs of large size were considered as the best candidate for colorimetric assay to determine atrazine.

In the current study, the outstanding qualitative recognition of atrazine from the AuNPs of large size can be attributed to two reasons. First, the large-size AuNPs solution had less number density of particles than the medium- and small-sized ones at the same weight concentration. The reaction of AuNPs could be more sensitive when higher percentage of particles interact with atrazine molecules in a volume. Second, the AuNPs of large size have the better optical performance than the smaller ones. When two nanoparticles are in close proximity, a new type of plasmon resonance called gap-plasmon resonance (GPR) will appear. The peak position of this GPR in the UV-Vis spectrum depends on various factors, particularly due to the particle size. When the particle size of AuNPs is small, there is a spectral overlap between GPR from the conjugating nanoparticles and SPR from a single nanoparticle, leading to insignificant changes in color. When the particle size of AuNPs is large, the spectral overlap is greatly reduced. This enables an increase in absorbance at the wavelength of 640 nm to cause a significant change in color from red to blue, thus directly correlating the color change to the concentration of the analyte (*e.g.*, atrazine) [75].

However, AuNPs of small size (less than 20 nm) were typically reported as the color indicator in the colorimetric assays for complex agri-food products [76] [77] because AuNPs of large size are too sensitive to the influence from impurities, leading to false positive and false negative results. In the current study, this was maximally avoided due to the combination of MIPs-SPE and colorimetric

assays that could clean up the impurities from food samples. Therefore, atrazine was the only factor that can induce the aggregation of AuNPs.

Due to the poor linearity ($R^2 < 0.9$) of the results shown in **Figure 4.5**, the accurate quantification of atrazine could not be obtained by using the AuNPs-based colorimetric assay. In addition, the achieved LOD could not meet the MRL proposed by Health Canada for atrazine in apple juice (0.005 mg L^{-1}). However, the feature of rapid and simple visual determination allowed the assay to simultaneously screen a large number of samples. The total time to obtain an optimized colorimetric result for each sample was ~ 5 min. Therefore, this AuNPs-based colorimetric assay can be used as a high-throughput screening method to integrate with MIPs-SPE.

4.5 Conclusion

The integration of MIPs-SPE and AuNPs-based colorimetric assay was successfully developed.

When atrazine-contaminated apple juice was treated by MIPs-SPE, the AuNPs-based colorimetric assay was able to rapidly detect the analyte by the naked eyes or UV-Vis spectrometry based on color variation. Moreover, the LOD of atrazine was different using AuNPs of different sizes as the color indicator. The LOD was 0.5 mg L^{-1} for the AuNPs of small size ($\sim 10.87 \text{ nm}$), 0.05 mg L^{-1} for the AuNPs of medium size ($\sim 27.43 \text{ nm}$), and 0.01 mg L^{-1} for the AuNPs of large size ($\sim 43.43 \text{ nm}$).

Therefore, the AuNPs of large size were the most efficient color indicators to conduct the colorimetric assay for the determination of atrazine. In sum, MIPs-SPE-AuNPs-based colorimetric assay shows great promise for high-throughput screening of atrazine in agri-food products.

Chapter 5: Fast quantitative analysis of atrazine in apple juice using surface-enhanced Raman spectroscopy (SERS)

5.1 Introduction

As indicated in **Chapter 4**, the integration of MIPs-SPE and AuNPs-based colorimetric assay was developed as an effective tool to rapidly screen a large number of atrazine contaminated apple juice samples. To validate the unknown samples and quantify the positive samples, a further advanced method is required for the accurate quantification of trace levels of atrazine.

Using AuNPs as the active substrate, SERS is an ideal technique for quantitative analysis of various food chemical hazards. With the assistance of AuNPs, weak Raman signals can be tremendously enhanced. The raw Raman spectra can be further analyzed by chemometrics to characterize the analyte, thus resulting in the detection of trace levels of chemicals.

By integrating SERS into the MIPs-SPE-AuNPs-based colorimetric assay, an innovative and efficient dual biosensor was developed. This biosensing system combines separation, screening, and quantification to enable rapid and accurate determination of atrazine in apple juice in a short time period (*i.e.*, 30 min). To the best of our knowledge, this was the first report of a MIPs-AuNPs-based colorimetric-SERS method to determine trace levels of chemical hazards in agri-food products.

The objective of this study was to validate whether AuNPs-based SERS assay can be applied as a method to accurately quantify atrazine in apple juice.

5.2 Materials

Atrazine was purchased from Sigma Aldrich (St. Louis, MO, USA). Methanol in HPLC grade was purchased from Thermo Fisher Scientific (Toronto ON, Canada). Gold-coated slides were purchased from Thermo Electron (Waltham, MA, USA). Homemade AuNPs solutions of different particle sizes were stored at 4°C. Apple juice samples spiked with different concentrations of atrazine (*i.e.*, 0 mg L⁻¹, 0.005 mg L⁻¹, 0.01 mg L⁻¹, 0.1 mg L⁻¹, 0.5 mg L⁻¹ and 1 mg L⁻¹) were treated by MIPs-SPE and stored at 4°C. Atrazine standard solutions at various concentrations from 0.005 mg L⁻¹ to 1 mg L⁻¹ were prepared by dissolving atrazine in methanol.

5.3 Methods

5.3.1 Preparation of samples for SERS tests

AuNPs solution used for SERS tests was not salted. A total of 200 µL of AuNPs solution was individually mixed with 200 µL of the eluted apple juice sample containing different concentration of atrazine (*i.e.*, 0 mg L⁻¹, 0.005 mg L⁻¹, 0.01 mg L⁻¹, 0.1 mg L⁻¹, 0.5 mg L⁻¹ and 1 mg L⁻¹). The mixture was gently blending and reacted for 5 min at room temperature. Then, 2 µL of the mixture was deposited onto a gold-coated slide and dried under laminar flow at room temperature.

5.3.2 Raman spectroscopic instrumentation

Raman spectroscopic analysis was conducted using a confocal micro-Raman spectroscopic system coupled with a 785-nm near-infrared laser. This system includes a Raman spectrometer (Renishaw, Gloucestershire, UK) and a Leica microscope (Leica Biosystems, Wetzlar, Germany). The

spectrometer has an entrance aperture of 50 μm and a focal length of 300 mm, and is equipped with a 1200-line mm^{-1} grating. After complete removal of Raleigh scattering by interference filters, Raman signals were recorded using a 578 $\times$ 385 pixel charge-coupled device (CCD) array detector with a pixel size of 22 μm .

After loading the gold-coated slide with the prepared samples onto the standard stage of the microscope, the SERS spectra were collected using a 50 $\times$ Nikon objective ($\text{NA} = 0.75$, $\text{WD} = 0.37$ mm) over a wavenumber range of 1200-400 cm^{-1} . All samples were prepared at least in triplicate. Each sample was illuminated with 1 mW incident laser power with an exposure time of 10 seconds and at least eight spectra were collected. The procedure of spectral collection was controlled by WiRE 3.4 software (Renishaw).

5.3.3 Chemometric analyses of SERS spectra

OMNIC software version 8.2 (Thermo-Nicolet, Madison, WI, USA) was applied to process the collected SERS spectra. Spectral noise was minimized using automatic baseline correction and smoothing (9-point Savitzky-Golay algorithm). Spectral subtraction of the substrate (*e.g.*, AuNPs) was conducted from all of the collected SERS spectra before further analyses.

A linear regression model was constructed to reveal the correlation between the amount of atrazine in apple juice and the intensity of the SERS feature band of atrazine at 688 cm^{-1} . To establish the linear model, forty-eight spectra were collected from apple juice samples containing different

concentrations of atrazine with eight spectra per each concentration.

5.4 Results and discussion

5.4.1 Analysis of SERS spectra from large-size AuNPs-based experiments

As mentioned in **Chapter 4**, homemade AuNPs were shown to be effective tools to conduct colorimetric assays for the detection of atrazine. In the SERS tests, the synthesized AuNPs were also selected to serve as the SERS-active substrate due to their reliable SERS enhancement factor ($\sim 10^6$) and relatively good reproducibility [78]. Besides gold, silver is another representative SERS substrate. Although SERS enhancement of silver is higher than that of gold, the reproducibility of SERS spectra collected by silver is less stable than that of gold [79]. Another emerging SERS substrate integrates gold and silver into a core-shell particle [80], but complicated steps are required for the synthesis. **Figure 5.1** illustrates the schematic procedure of SERS tests using AuNPs.

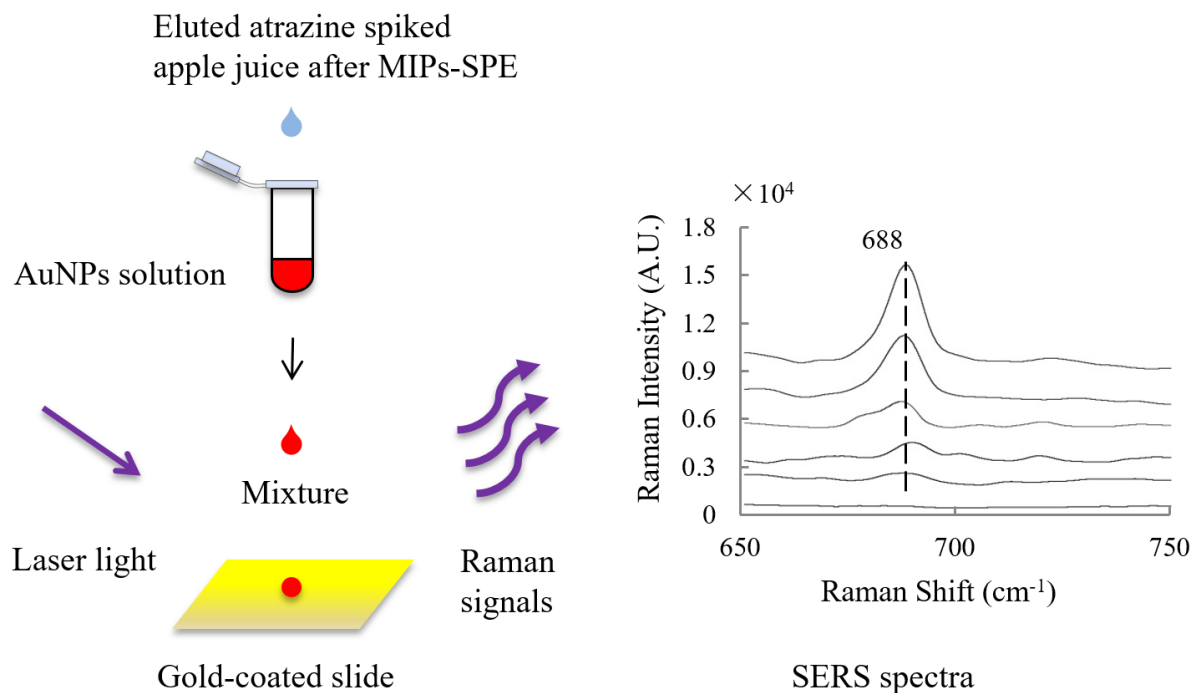


Figure 5.1 Schematic illustration of SERS tests for the detection of atrazine in apple juice after MIPs-SPE.

AuNPs of large size were employed as SERS-active substrates for SERS tests. **Figure 5.2 (a)** shows the Raman spectra collected from the AuNPs solution deposited onto the gold-coated slide. In the wavenumbers of $900\text{--}600\text{ cm}^{-1}$, no obvious Raman signals can be observed, indicating the feasibility of using AuNPs as the SERS-active substrate without interference with the SERS feature band of atrazine. In the same wavenumber, average spectra (number=8) of atrazine crystal determined by normal Raman, atrazine standard solution, and atrazine in apple juice after MIPs-SPE were shown in **Figure 5.2 (b) (c) (d)**, respectively.

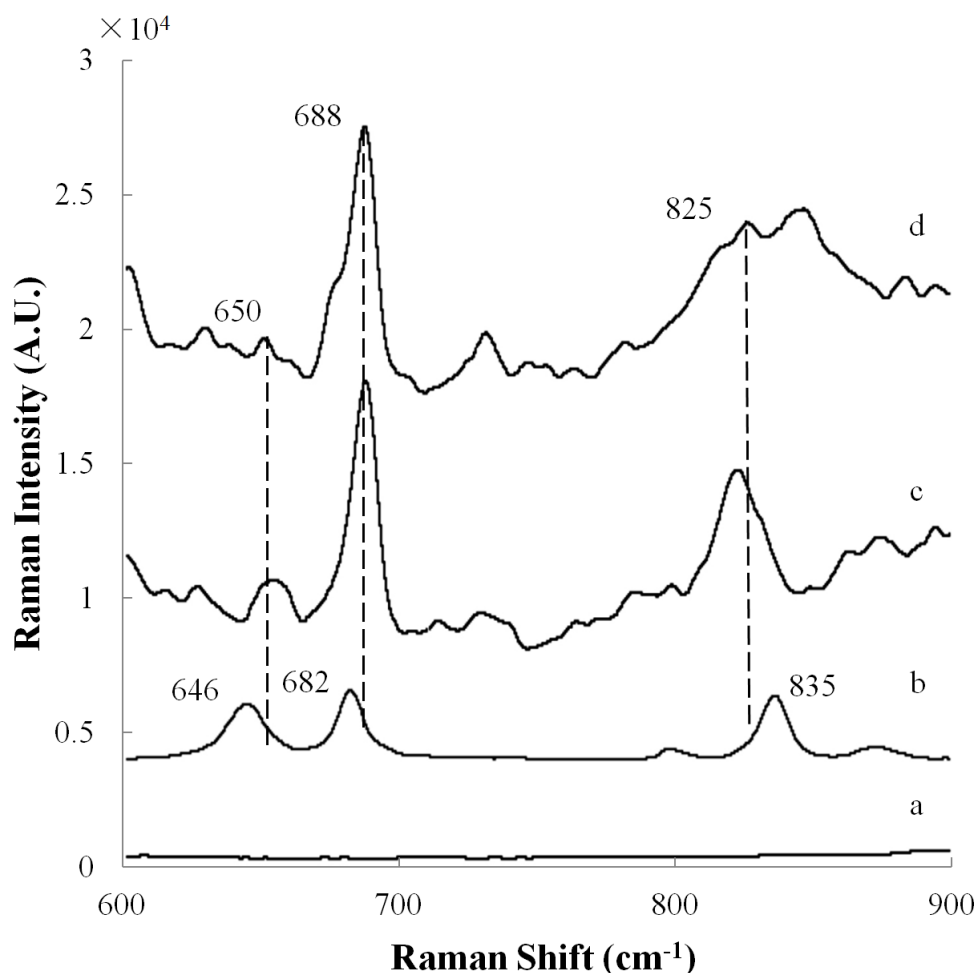


Figure 5.2 Representative spectral features of (a) AuNPs of large size on the gold-coated slide, (b) normal Raman of atrazine crystal, (c) large-size AuNPs-based SERS of atrazine standard solution (10 mg L^{-1}), and (d) large-size AuNPs-based SERS of apple juice spiked with atrazine after MIPs-SPE (10 mg L^{-1}).

In the normal Raman spectra of atrazine crystal, each feature band represents a specific vibrational mode induced by atrazine, thus reflecting the molecular structure of atrazine. Three distinctive Raman bands at wavenumbers of 646 cm^{-1} , 682 cm^{-1} and 835 cm^{-1} can be assigned to CH_2 twisting, ring-breathing mode 6a, and C–C stretching or CH_3 wagging, respectively [81]. These bands are also expected to appear in the spectra collected by SERS. However, in the SERS spectra of atrazine

standard solution and atrazine-spiked apple juice, the band that appears at 835cm^{-1} in the normal Raman spectrum of atrazine crystal shifts to 825cm^{-1} , which is due to a SERS effect [82].

Specifically, when the targeted molecule interacts with SERS-active substrates, the dipole of the targeted molecule can be changed, and consequently shifts the location of SERS feature bands. In the SERS spectra of atrazine-spiked apple juice, the band at 688 cm^{-1} shows a small blue shift compared to the relevant band in the SERS spectra of atrazine standard solution, which may be attributed to the presence of metal ions (*e.g.* Ca^{2+} , Fe^{2+} , Zn^{2+}) in apple juice [83]. Although aforementioned Raman signals are significantly increased due to the SERS-active substrate, the band that occurs at 646 cm^{-1} in the normal Raman spectrum is not enhanced in the SERS spectra. This is because the surface of AuNPs leads the polarization tensor of the atrazine molecule to be orthogonal to polarization of the light field [34]. By comparing the SERS spectra of atrazine standard solution and atrazine-spiked apple juice, the uniformity in the Raman feature bands demonstrated that the MIPs-SPE method effectively extracted atrazine from apple juice.

Next, SERS spectra of apple juice spiked with different concentrations of atrazine (*i.e.* 0 mg L^{-1} , 0.005 mg L^{-1} , 0.01 mg L^{-1} , 0.1 mg L^{-1} , 0.5 mg L^{-1} and 1 mg L^{-1}) after MIPs-SPE were collected. AuNPs of large size were used as the SERS-active substrate. All of the raw SERS spectra were processed, including baseline, smoothing, and spectral subtraction of the substrate. The average spectra in the wavenumber of $750\text{-}650\text{ cm}^{-1}$ are shown in **Figure 5.3**. An increasing trend in the intensity of the feature SERS band at 688 cm^{-1} can be observed along with the increased concentration of atrazine in apple juice.

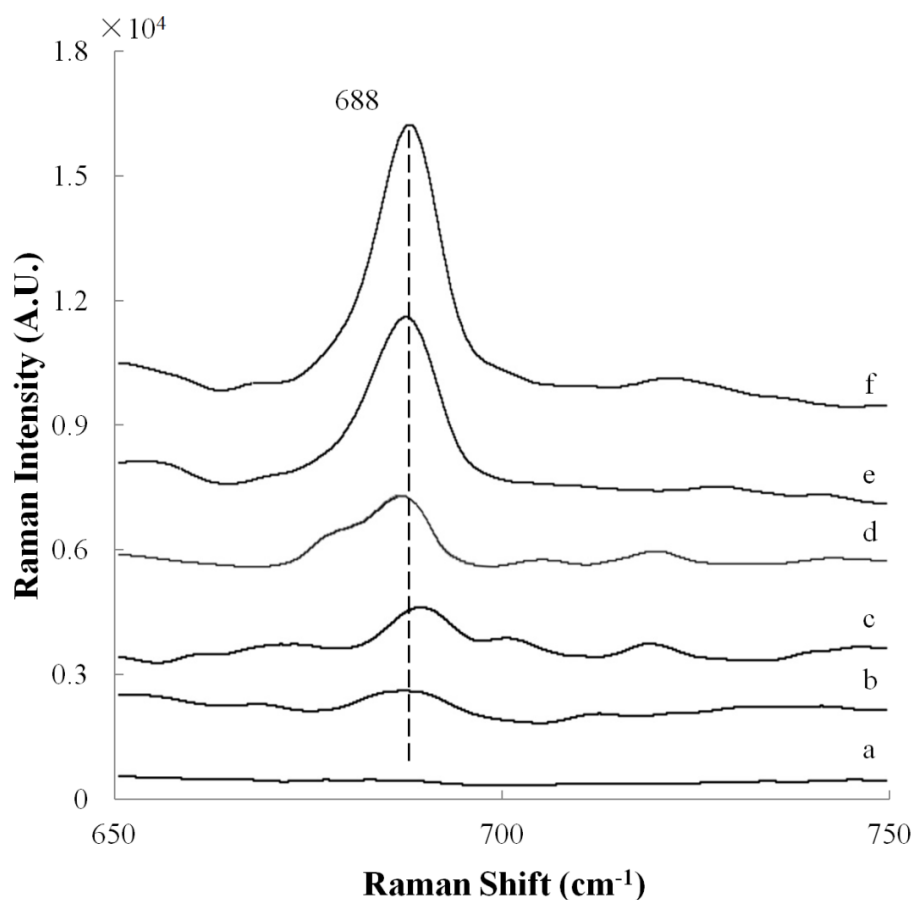


Figure 5.3 Average large-size AuNPs-based SERS spectra of apple juice samples spiked with different concentrations of atrazine after MIPs-SPE: (a) 0 mg L⁻¹, (b) 0.005 mg L⁻¹, (c) 0.01 mg L⁻¹, (d) 0.1 mg L⁻¹, (e) 0.5 mg L⁻¹, (f) 1 mg L⁻¹.

To test the quantitative capability of the SERS, partial least squares regression model is typically constructed to correlate SERS spectral features to the concentrations of the analyte [84]. In the current study, within the selected wavelength region, the band at 688cm⁻¹ was the major parameter to differentiate the spectra of apple juice samples spiked with different concentrations of atrazine. Therefore, it is reasonable to only use the intensity of the SERS feature band at 688 cm⁻¹ to correlate with the concentration of atrazine. A linear regression model was established by using the correlation

between the concentrations of atrazine from 0.005 mg L⁻¹ to 1 mg L⁻¹ and the intensity of SERS band at 688 cm⁻¹. The coefficient of determination (R^2) of this regression model was 0.93, confirming the good linearity. When the regression model was constructed based on the range of atrazine amount from 0 mg L⁻¹ to 1 mg L⁻¹, the R^2 decreased to 0.91 and the prediction became relatively less accurate. Therefore, the regression model was feasible to accurately predict the amount of atrazine in an unknown apple juice sample when the concentration of atrazine in the apple juice samples falls between 0.005 mg L⁻¹ and 1 mg L⁻¹. **Figure 5.4** shows the regression model with a higher R^2 (~0.93).

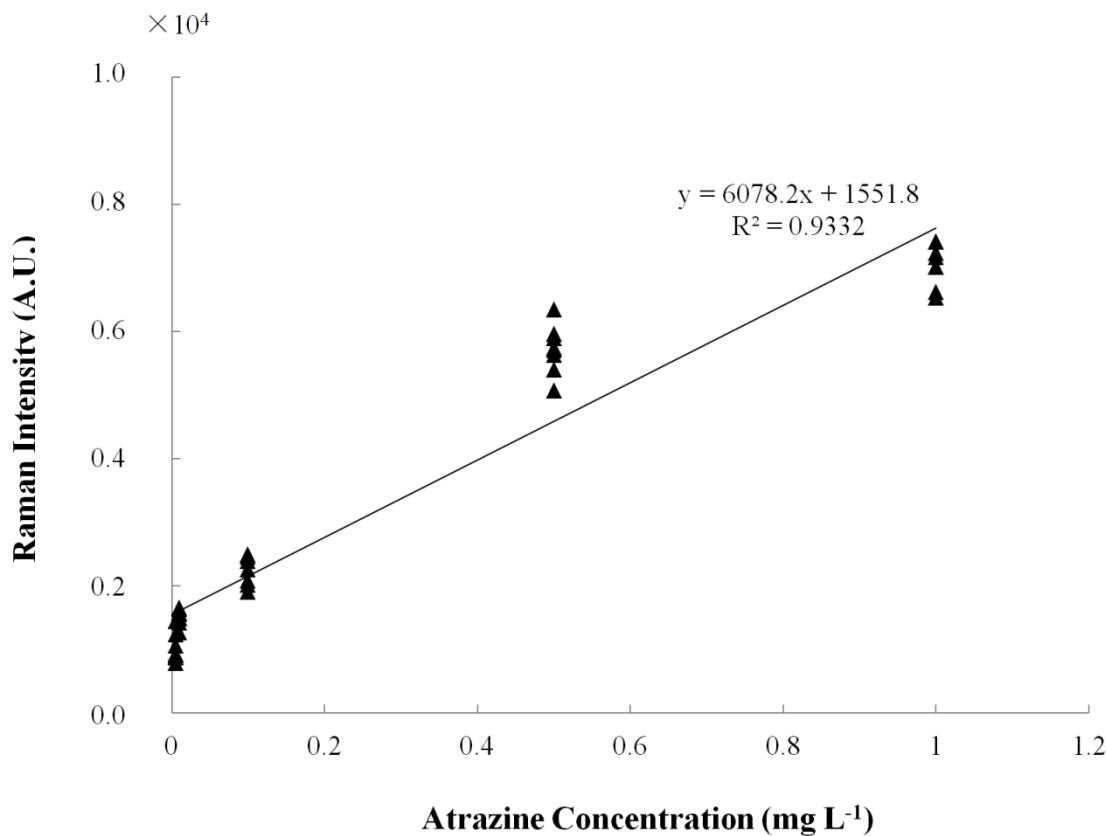


Figure 5.4 Linear relationship between the intensity of SERS band at 688 cm⁻¹ and the spiked concentrations of atrazine in apple juice samples when AuNPs of large size were served as the SERS-active substrate.

Based on the linear regression model, limit of detection (LOD) and limit of quantification (LOQ) were experimentally determined by the non-spiked samples and calculated using the following equations [85]:

$$\text{LOD} = \frac{3S_b}{b} \quad (4)$$

$$\text{LOQ} = \frac{10S_b}{b} \quad (5)$$

S_b is the standard deviation of the intensity of the band at 688 cm^{-1} in SERS spectra of non-spiked samples, and b is the slope of the regression curve. The calculated value of LOD equals to 0.0017 mg L^{-1} and the calculated value of LOQ equals to 0.0058 mg L^{-1} . The achieved LOD of atrazine was lower compared to the linear regression model (0.005 mg L^{-1}) and MRL for atrazine (0.005 mg L^{-1}) proposed by Health Canada, indicating the feasibility and high sensitivity of SERS to detect and quantify trace levels of atrazine. In addition, due to the loss of a certain amount of atrazine during the sample pretreatment and MIPs-SPE, the actual concentration of atrazine determined by SERS detection was lower than the initial level of atrazine spiked in the apple juice. Thus, even a lower LOD and LOQ could be achieved if a better procedure is employed for the sample pretreatment and MIPs-SPE method.

5.4.2 Evaluations on different sizes of AuNPs in SERS tests

AuNPs of different sizes were evaluated for SERS tests to figure out the lowest LOD and LOQ. The AuNPs of medium size and the AuNPs of small size were also applied as SERS-active substrates to conduct the same SERS tests. **Figure 5.5** and **Figure 5.6** show the average SERS spectra of various concentrations of atrazine from 0 mg L^{-1} to 1 mg L^{-1} collected by SERS tests with medium-size

AuNPs and small-size AuNPs, respectively. A similar trend was observed that the intensity of the SERS feature band at 688 cm^{-1} increased with the increasing concentration of atrazine in apple juice samples. However, the intensity of SERS feature band obtained by AuNPs of medium size was much higher than that by the AuNPs of small size.

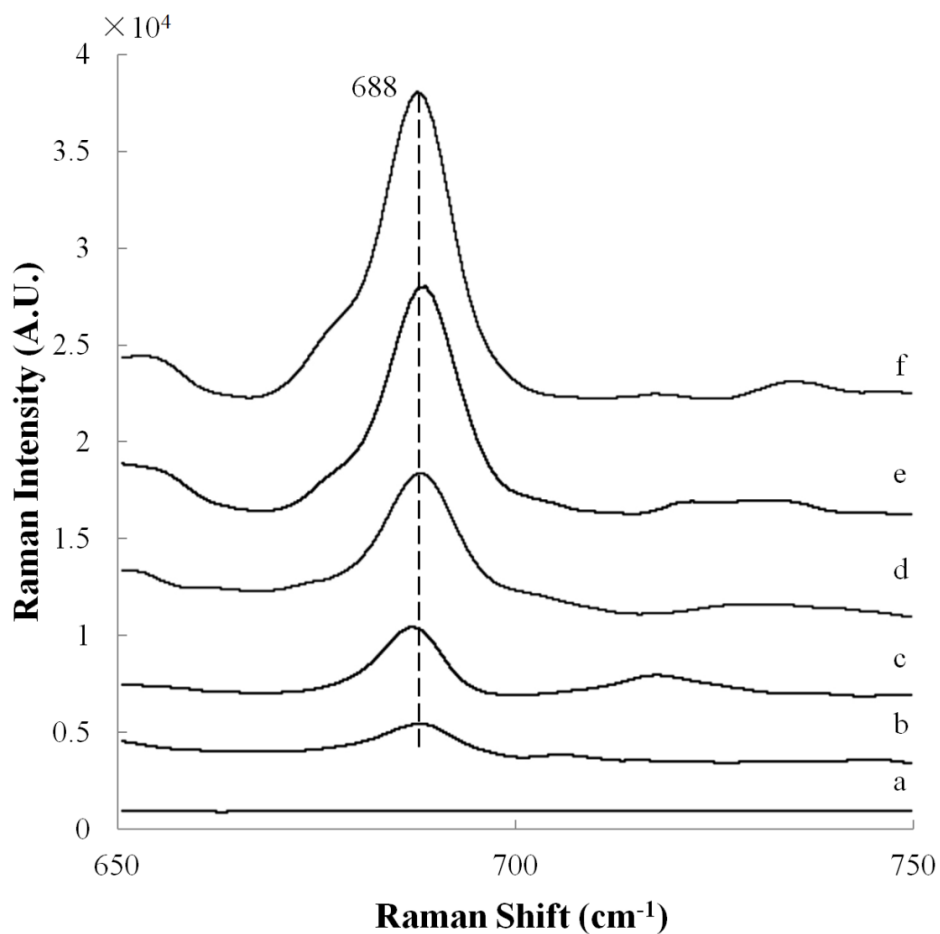


Figure 5.5 Average medium-size AuNPs-based SERS spectra of apple juice samples spiked with different concentrations of atrazine after MIPs-SPE: (a) 0 mg L^{-1} , (b) 0.005 mg L^{-1} , (c) 0.01 mg L^{-1} , (d) 0.1 mg L^{-1} , (e) 0.5 mg L^{-1} , (f) 1 mg L^{-1} .

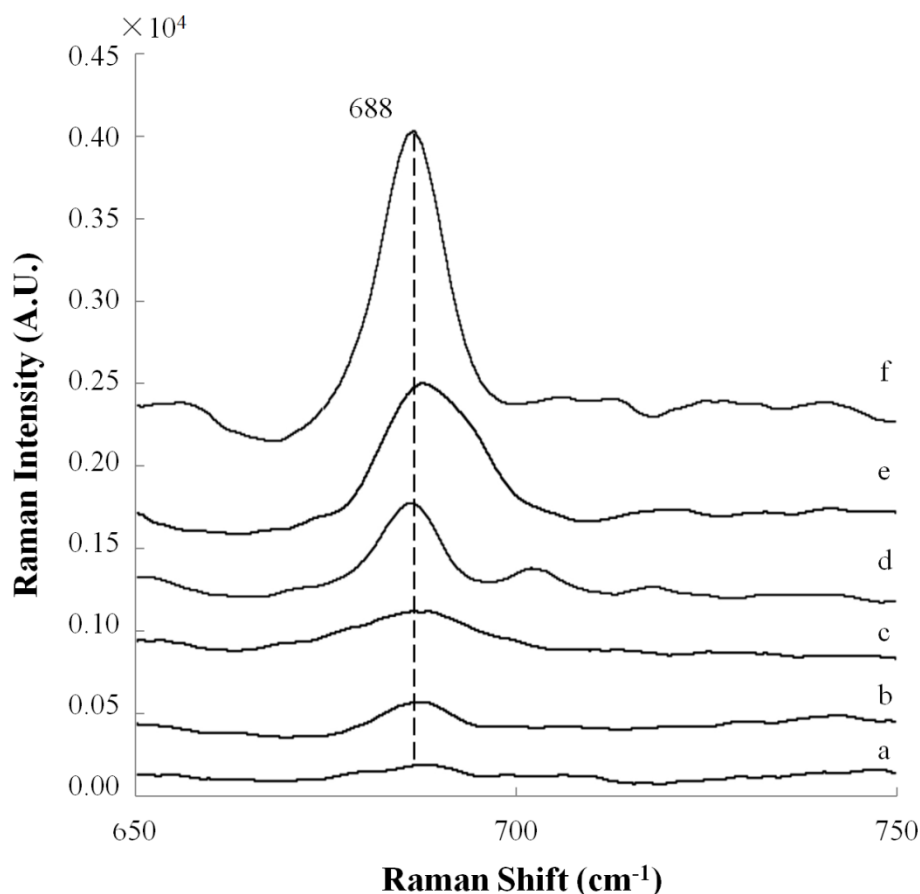


Figure 5.6 Average small-size AuNPs-based SERS spectra of apple juice samples spiked with different concentrations of atrazine after MIPs-SPE: (a) 0 mg L⁻¹, (b) 0.005 mg L⁻¹, (c) 0.01 mg L⁻¹, (d) 0.1 mg L⁻¹, (e) 0.5 mg L⁻¹, (f) 1 mg L⁻¹.

Linear regression models were again constructed by using the intensity of SERS band at 688 cm⁻¹ and the concentration of atrazine from 0.005-1 mg L⁻¹. **Figure 5.7** shows the linear regression model based on the AuNPs of medium size. The R² equals to 0.91 and expresses the good linearity of this regression model. The LOD and LOQ were calculated to be 0.0012 mg L⁻¹ and 0.0040 mg L⁻¹, respectively. The high intensity of the SERS feature band obtained by AuNPs of medium size led to a large value of the slope in the regression curve, and consequently contributed to a lower calculated

LOD and LOQ value based on the equations (4) and (5).

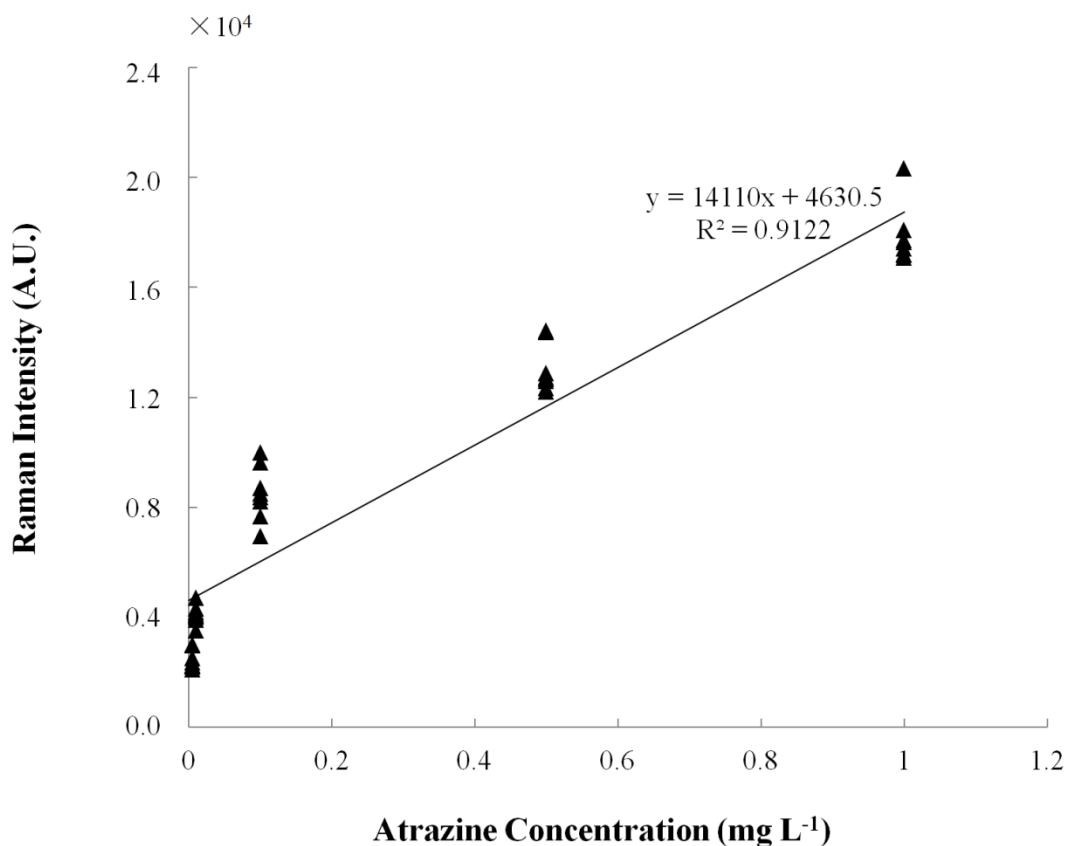


Figure 5.7 Linear relationship between the intensity of SERS band at 688 cm⁻¹ and the spiked concentrations of atrazine in apple juice samples when AuNPs of medium size were served as SERS-active substrate.

A linear regression model with a high R^2 (~ 0.98) is shown in **Figure 5.8** when AuNPs of small size were used in SERS tests. The LOD and LOQ calculated from this regression model equals to 0.017 mgL⁻¹ and 0.056 mgL⁻¹, respectively, which were significantly higher than that obtained from the large-size and medium-size AuNPs. This was attributed to the low intensity of SERS feature band of atrazine obtained by the AuNPs of small size. Taken together, AuNPs of medium size were more suitable to serve as the SERS-active substrate than AuNPs of large size and small size because lower

LOD and LOQ were achieved in SERS tests.

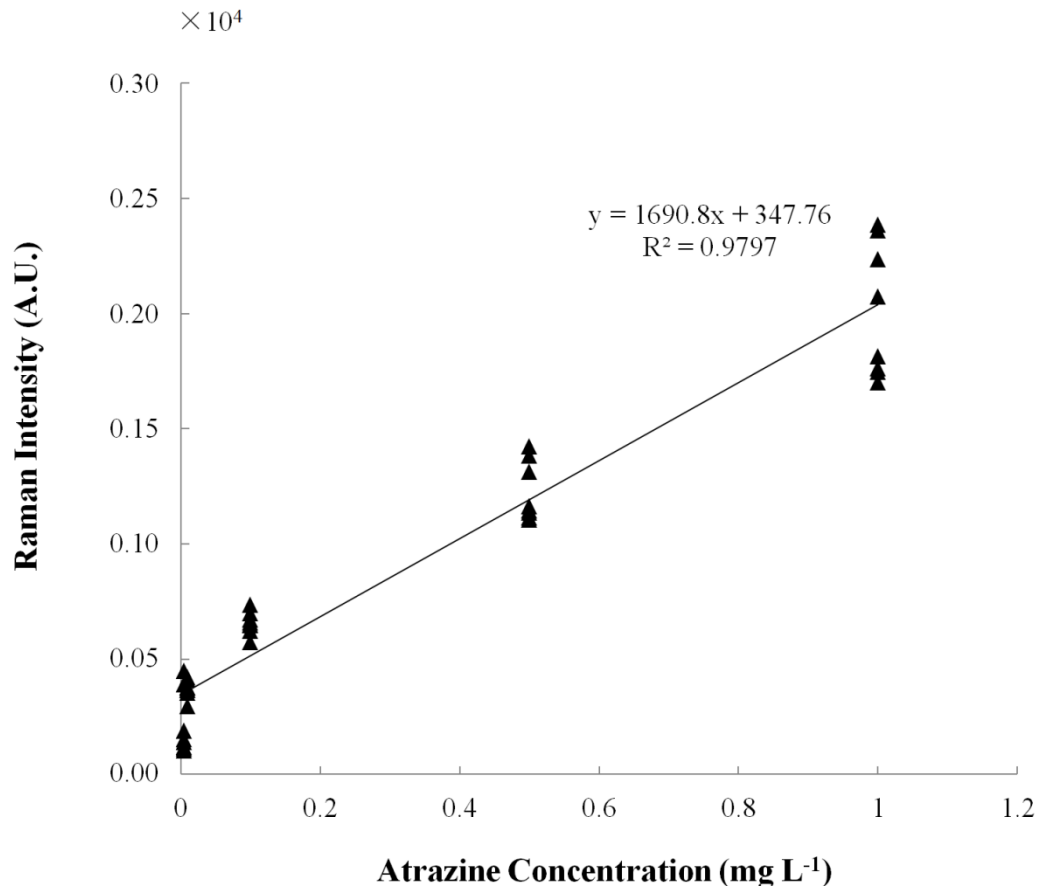


Figure 5.8 Linear relationship between the intensity of SERS band at 688 cm⁻¹ and the spiked amounts of atrazine in apple juice samples when AuNPs of small size were served as the SERS-active substrate.

SERS tests can be a promising tool to detect and quantify atrazine in apple juice at extremely low concentrations by using AuNPs of the appropriate size as the SERS-active substrate. In **Chapter 2**, MIPs-AuNPs-based colorimetric assay confirmed that the lowest LOD of atrazine could be achieved using the AuNPs of large size as the color indicator. However, SERS tests validated that AuNPs of medium size were the best candidate to obtain the highest Raman intensity and lowest LOD and LOQ of atrazine. Thus, the surface area of SERS substrate is not the only factor to contribute to

Raman signal enhancement. The electromagnetic field generated from LSPR is another important factor. Kelly and coauthors discovered that the localized electromagnetic field enhanced along with the increase in the size of the nanoparticles [86]. However, the convex shape of the surface of the nanoparticles turns flatter when the size of the particles becomes larger. Consequently, less light is absorbed and less inelastic scattering occurs on the surface of the nanoparticles, leading to weakening of the electromagnetic enhancement around the surface. The intensity of the SERS feature band will be affected and become weak [87]. Therefore, the largest SERS enhancement will be identified when AuNPs have the maximum LSPR to generate the electromagnetic field without being significantly affected by the shape of the particles. In the current study, the AuNPs of medium size with diameters of ~27 nm were confirmed to contribute to the highest SERS intensity of the atrazine feature band when serving as the SERS-active substrate. This conclusion agrees with a previous report when AuNPs were used for the detection of melamine [72].

The MRL proposed by Health Canada for atrazine in apple juice is 0.005 mg L⁻¹. Owing to the efficient separation of atrazine by MIPs-SPE, both large-size and medium-size AuNPs-based SERS tests can provide an outstanding LOD to meet this requirement for the determination of trace levels of atrazine in apple juice. Moreover, the LOQ obtained by the AuNPs of medium size is also below the official guideline, offering a great promise for the quantitative analysis when the concentration of atrazine is extremely low.

5.4.3 Application of MIPs-AuNPs-based colorimetric-SERS dual biosensor

To fully exploit the advantages of MIPs-SPE, AuNPs-based colorimetric assays and AuNPs-based

SERS tests, we integrated these elements and developed an integrated biosensing system. **Figure 5.9** illustrates the working procedure of this dual biosensor. The MIPs-SPE method is able to clean up apple juice efficiently and selectively extract atrazine with a high recovery. After that, AuNPs-based colorimetric assay can rapidly screen a large number of samples by simple visual determination and the LOD can reach to 0.01 mg L^{-1} when the AuNPs of large size are applied as the color indicator. Positive samples with different concentrations of atrazine will be recognized. SERS tests can be further performed to validate the unknown samples and quantify the positive samples if necessary. The LOD and LOQ can reach to 0.0012 mg L^{-1} and 0.0040 mg L^{-1} when the AuNPs of medium size are applied as the SERS-active substrate. The entire experimental process takes only 20 min for the sample pretreatment and clean up, 5 min for the rapid sample screening and 5 min for the accurate quantification.

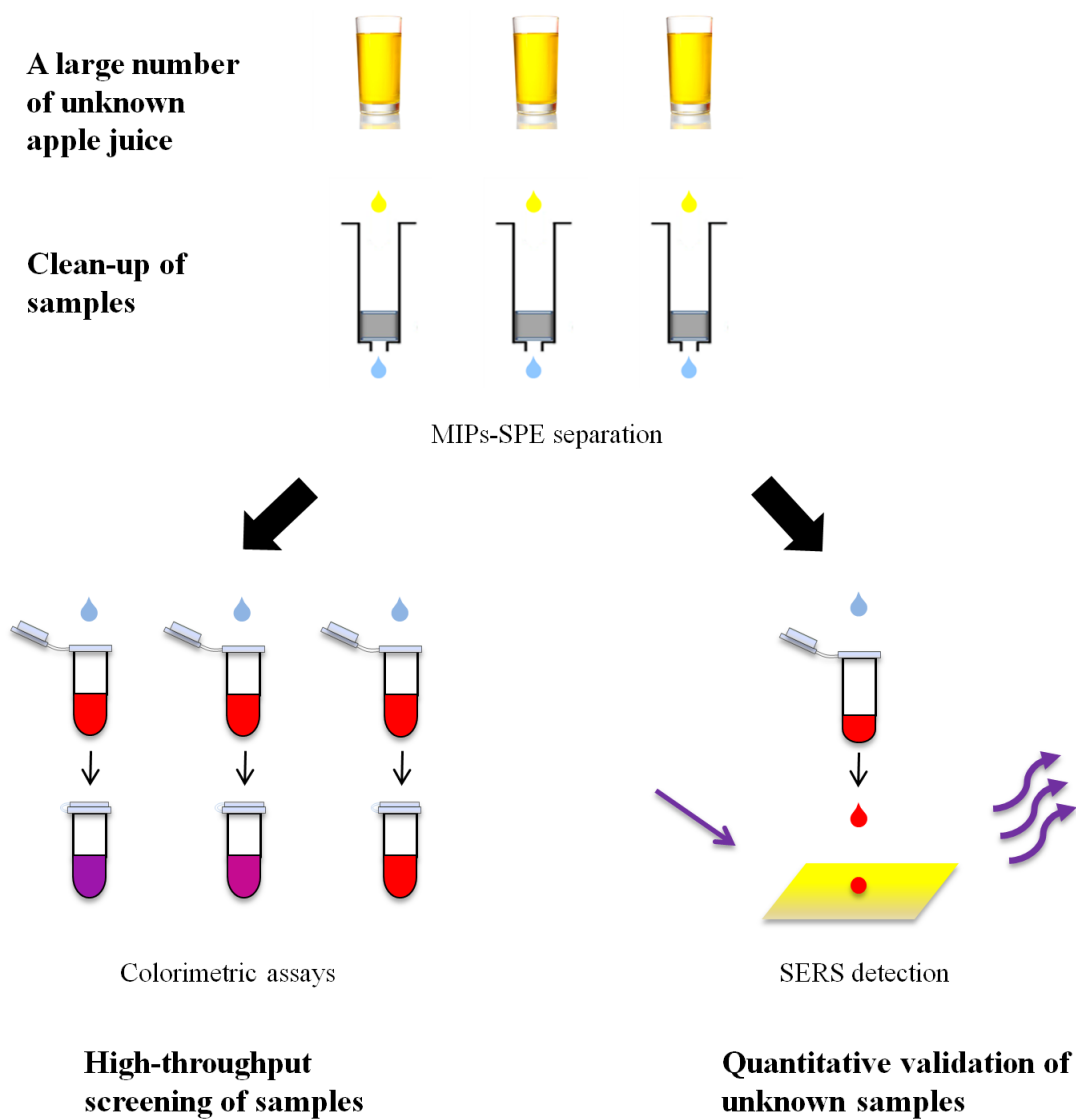


Figure 5.9 Schematic illustration of MIPs-SPE-AuNPs-based colorimetric-SERS dual biosensor for the detection and quantification of atrazine in apple juice.

5.4 Conclusion

In conclusion, MIPs-SERS tests showed an excellent qualitative and quantitative detection capability of atrazine in apple juice. The selection of AuNPs with different sizes to serve as SERS-active substrate could result in different detection limits. For AuNPs of large size, LOD was 0.0017 mg L^{-1}

and LOQ was 0.0058 mg L⁻¹. For AuNPs of medium size, LOD was 0.0012 mg L⁻¹ and LOQ was 0.0040 mg L⁻¹. For AuNPs of small size, LOD was 0.017 mg L⁻¹ and LOQ was 0.056 mgL⁻¹. Thus, AuNPs of medium size were shown to be the most efficient candidate in conducting SERS tests for the determination of trace levels of atrazine in apple juice.

The integration of MIPs-SPE, AuNPs-based colorimetric assay and AuNPs-based SERS test shows a great potential for high-throughput and accurate determination of atrazine in apple juice in a rapid and high-throughput manner (*i.e.*, 30 min). This process can be applied for on-site use or laboratory testing for the government and the food industry when trace-level determination of food chemical hazards is required.

Chapter 6: Conclusion

6.1 Main findings

This research aimed to develop fast, high-throughput and accurate techniques to determine atrazine contamination in agri-food products. The MRL proposed by Health Canada for atrazine in foods is 0.005 mg L⁻¹. GC hyphenated detection methods are applied by official agencies in Canada. However, these techniques require time-consuming and laborious sample pretreatment and instrumental operation with highly trained professional personnel. A biosensing system including separation, screening and quantification was investigated in the current study. MIPs are a type of artificial polymers with specific affinity to the analytes. By using MIPs as the sorbents in SPE to separate atrazine from food matrices (*i.e.*, apple juice), high-recovery extraction could be achieved with reduced sample pretreatment procedure and atrazine extraction time. The atrazine in the eluents from MIPs-SPE could be visualized using the AuNPs-based colorimetric assay. The interaction between AuNPs and atrazine molecule by imino ligands could induce the aggregation of AuNPs, resulting in the change of SPR on the surface of AuNPs and the variation of the color of AuNPs solution from red to blue. Although the accuracy of AuNPs-based colorimetric assay could be influenced by external factors (*e.g.*, pH, interferents), the inherent properties of AuNPs make it possible to apply colorimetric assay to qualitatively recognize the atrazine residue and rapidly screen a large number of samples in a simultaneous manner. For the further accurate quantification of atrazine content in samples after MIPs-SPE, SERS was used to quantify atrazine by collecting Raman scattering signals associated with ring-breathing mode 6a in the molecular structure of atrazine. AuNPs were not only used as the color indicator in the colorimetric assay, but also served as

the SERS-active substrate for SERS tests. Within the electromagnetic field around the surface of AuNPs, Raman signals were significantly enhanced and thus could be used to characterize the atrazine residue at extremely low concentrations. By integrating separation, screening and quantification, the MIPs-AuNPs-based colorimetric-SERS dual biosensor was developed for simple, rapid, high-throughput and accurate determination of the atrazine residue in foods.

Feasibilities of separation, screening and quantification in the dual biosensor were investigated to determine atrazine in apple juice and thus three objectives were developed accordingly. The experimental designs and results of each objective were discussed in **Chapter 3**, **Chapter 4** and **Chapter 5**, respectively. For each objective, a hypothesis was proposed and verified, which was concluded in **Table 6.1**.

Table 6.1 Conclusion of decision for hypotheses proposed for objectives developed in the study.

	Objective 1	Objective 2	Objective 3
	Hypothesis 1	Hypothesis 2	Hypothesis 3
Decision	Accepted	Accepted	Accepted

Objectives 1: To synthesize MIPs with specific affinity to atrazine and use MIPs-SPE column to extract trace level of atrazine in apple juice samples.

Objectives 2: To conduct colorimetric assays to differentiate samples with different levels of atrazine.

Objectives 3: To develop SERS detection for quantification of trace level of atrazine.

Hypothesis 1: Homemade MIPs can show specific affinity to atrazine and MIPs-SPE separation

method can be used to selectively separate atrazine from apple juice.

Hypothesis 2: Homemade AuNPs can be used to rapidly screen apple juice samples containing different concentrations of atrazine.

Hypothesis 3: SERS can apply the same AuNPs as substrates to further validate and quantify atrazine content in apple juice.

For the first objective, **Figure 3.2, 3.3, 3.4** and **Table 3.1** support the acceptance of **Hypothesis 1**. The high adsorption capacity of MIPs to rebind to atrazine in the adsorption tests and the selectivity test confirmed the specific affinity of MIPs to atrazine. High recoveries of atrazine in the SPE procedure validated that MIPs could be used as effective and efficient sorbents to extract atrazine from apple juice samples, successfully achieving **Objectives 1**.

In **Chapter 4**, **Hypothesis 2** was accepted by the demonstrations in **Figure 4.3** and **4.4**. The results obtained by the naked eyes and UV-Vis determination verified the possibility of applying AuNPs as the color indicator to qualitatively screen apple juice samples containing various contents of atrazine. In addition, **Figure 4.5** indicated that AuNPs of large size (~43 nm) was better than AuNPs of medium size (~27 nm) and small size (~11 nm) to achieve a lower LOD (*i.e.*, 0.01 mg L⁻¹) of atrazine. Taken together, **Objective 2** was accomplished.

As the last objective, **Hypothesis 3** was also accepted based on the results shown in **Figure 5.4, 5.7** and **5.8**. The linear regression model was conducted depending on the collected Raman spectra and validated the quantitative capability of SERS to determine trace levels of atrazine in apple juice. In

addition, the comparison among AuNPs of three sizes showed that the AuNPs of medium size were the best candidate to achieve the lowest LOD (*i.e.*, 0.0012 mg L⁻¹) and LOQ (*i.e.*, 0.0040 mg L⁻¹) of atrazine. Because of the acceptance on all of the hypotheses, **Objective 3** was finally achieved.

In summary, MIPs with specific affinity to atrazine were successfully synthesized and the MIPs-based SPE column could selectively separate atrazine from apple juice with the recovery ~93%. By using AuNPs as the color indicator, colorimetric assay could successfully differentiate apple juice eluents containing different concentrations of atrazine by the naked eyes. Different sizes of AuNPs could lead to different LODs of atrazine with UV-Vis determination. The LOD was 0.01 mg L⁻¹, 0.05 mg L⁻¹ and 0.5 mg L⁻¹ for AuNPs of large size (~43 nm), medium size (~27 nm) and small size (~11 nm), respectively. SERS tests were validated to succeed in quantifying different concentrations of atrazine in apple juice eluents after MIPs-SPE. Linear regression models were constructed based on the collected Raman spectra for chemometric analysis. The calculated LOD and LOQ were different when different sizes of AuNPs served as the SERS-active substrate. For AuNPs of large size, LOD was 0.0017 mg L⁻¹ and LOQ was 0.0058 mg L⁻¹. For AuNPs of medium size, LOD was 0.0012 mg L⁻¹ and LOQ was 0.0040 mg L⁻¹. For AuNPs of small size, LOD was 0.017 mg L⁻¹ and LOQ was 0.056 mg L⁻¹. Therefore, the evaluation of three sizes of AuNPs indicated that AuNPs of large size were the best candidate for the colorimetric assay but the AuNPs of medium size were the most suitable candidate for SERS tests. By integrating MIPs-SPE, AuNPs-based colorimetric assay and SERS test into one biosensing system, an innovative technique was developed for rapid, high-throughput and accurate detection of food chemical contamination. The entire experimental process takes only 30 min, including 20 min for sample pretreatment and clean up, 5 min for the rapid sample screening

and 5 min for the accurate quantification.

6.2 Future research directions

In the current study, colorimetric assay based on AuNPs was used as the rapid screening method while it was not applicable for accurate quantification of atrazine in apple juice. However, some reports indicated that colorimetric assays could also be applied for quantitative or semi-quantitative analysis [88] [89]. Therefore, further optimization of the experimental design should be taken into consideration, including reducing the interferents in food matrices for sample pretreatment and optimizing the color indicator in colorimetric assays.

First, although MIPs-SPE was used to remove apple juice matrices, the juice components (*e.g.*, sugars, proteins, etc.) might remain in the treated eluent because of the non-specific affinity of MIPs for atrazine. As introduced in **Section 1.4.1**, different synthesis methods of MIPs (*e.g.*, precipitation, sol-gel, etc.) can increase specific binding sites and cavities in MIPs, leading to improvement in the specific affinity of MIPs to the targeted analytes. By using more effective sample pretreatment, the advanced accuracy of further detection can be achieved. Second, a novel type of bimetallic nanoparticle integrating Ag and Au was recently developed due to its specific optical and physical features. By using Ag as the core and Au as the shell, a strong plasmonic sensitivity of AgNPs and excellent chemical stability of AuNPs can be combined [90]. The accuracy of colorimetric assays will be further improved based on the more sensitive and stable color indicator, subsequently contributing to the quantitative capability. Moreover, as the SERS-active substrate, Ag is excellent for Raman enhancement while Au is known for its good reproducibility [91]. The bimetallic

nanoparticles can improve SERS property and reduce the LOD and LOQ in SERS detection.

Therefore, it is worth of exploring the combination of different types of MIPs, color indicator and SERS substrate to achieve a more advanced biosensing system for separation, screening and quantification of trace levels of atrazine in apple juice.

Although MIPs-AuNPs-based colorimetric-SERS dual biosensor can finish the entire analysis within 30 min, the bench-top Raman system used in this study may limit its application for on-site detection. If portable Raman spectrometer can be integrated into the biosensor, both the convenience and affordability will be significantly improved.

Finally, the methodology proposed in the current study has a great potential to be applied to determine other chemical hazards in food matrices with the availability of specific MIPs. The versatility and the flexibility of this biosensor are of great significance to monitor food contaminants and improve food safety.

6.3 Conclusion

By employing MIPs for separation, AuNPs-based colorimetric assay for screening and SERS for quantification, an innovative dual biosensor was developed for rapid, high-throughput and accurate determination of atrazine in apple juice. In this thesis project, all the objectives were accomplished and all the relevant hypotheses were accepted. Results are summarized in **Table 6.2**. MIPs-SPE was able to selectively extract atrazine from apple juice with high recoveries and clean up food samples with high efficiency. AuNPs-based colorimetric assays could rapidly and qualitatively screen a large

number of samples by simple visual determination with the further confirmation using UV-Vis spectroscopy. SERS tests were feasible to further validate and quantify trace levels of atrazine by analyzing the collected SERS spectra. In addition, three sizes of AuNPs were evaluated in the study both as the color indicator in the colorimetric assays and SERS-active substrate in SERS tests. AuNPs of large size (~43 nm) were most suitable to the colorimetric assay to achieve the lowest LOD of atrazine. SERS tests could obtain the lowest LOD and LOQ of atrazine when the AuNPs of medium size (~27 nm) were applied. The entire experimental process took only 30 min, including sample pretreatment and clean up, rapid sample screening and accurate quantification of atrazine.

In conclusion, MIPs-AuNPs-based colorimetric-SERS dual biosensor is promising to determine atrazine in agri-food products.

Table 6.2 Summary of results of three parts in the MIPs-AuNPs-based colorimetric-SERS dual biosensor

	MIPs-SPE	AuNPs-based colorimetric assays			AuNPs-based SERS		
Key materials	MIPs	AuNPs			AuNPs		
Materials size (nm)	Micro-level	10.87	27.43	43.43	10.87	27.43	43.43
Food matrices tested	Apple juice	Apple juice			Apple juice		
Analysis time (min)	20	5			5		
LOD (mg L ⁻¹)	N/A	0.5	0.05	0.01	0.017	0.0012	0.0017
LOQ (mg L ⁻¹)	N/A	N/A			0.056	0.0040	0.0058

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