

Rapid Detection of Ricin in Liquid Foods Using
Surface-Enhanced Raman Spectroscopy

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Dedication

This paper is a salute to the multitude of people who make it their life's work to ensure the quality and safety of the food we enjoy every day. Yours is the yeoman's work; it is never finished, yet your vigilance endures.

Abstract

The potential for bioterror agents to be distributed using existing supply chains for food with the purpose of causing mass casualty, terror, and/or economic damage is a hazard to homeland security. Therefore, it is necessary that bioterror agent detection procedures be developed which are appropriate and can serve as tools for food companies and other security personnel to monitor the U.S. food supply.

The present research develops an appropriate assay which could be used to detect the toxin ricin in fresh liquid foods, specifically milk and orange juice. Ricin toxin is a category B bioterror agent as defined by the Centers for Disease Control and Prevention due to its potent toxicity and relative ease of access. For this application, an appropriate detection assay was rapid, inexpensive, easy to conduct, and sensitive enough to detect ricin at concentrations below toxic doses if consumed. These criteria were important since food surveillance requires frequent monitoring by personnel who do not necessarily have backgrounds in chemistry nor laboratory techniques. Additionally, tested foods should not proceed through the processing/distribution chain until a negative result was received; therefore the assay must be very rapid.

Research was conducted and procedure developed using an immuno-based separation technique to capture dilute concentrations of ricin, (or a surrogate), from foods spiked with the toxin. Two separation techniques were utilized and evaluated for speed, ease, and final sensitivity. The first separation technique utilized a commercially available polyclonal ricin antibody conjugated to magnetic beads in order to concentrate and separate ricin from a sample. The second separation technique used a single-stranded

DNA aptamer to perform the capture step. Following either separation technique, surface-enhanced Raman spectroscopy (SERS) was used to directly detect the target.

Raman is a mode of vibrational spectroscopy where a sample is exposed to a monochromatic laser and a spectrum of Raman-active vibration modes is obtained. These spectra contain a large amount of information about the sample's molecular structure, and therefore act as 'fingerprints' for a sample. Surface-enhanced Raman exploits cutting edge nanotechnology to significantly increase the sensitivity of Raman analyses by placing a sample on or near noble metal nanostructures.

Immunomagnetic separation (IMS), the first of the separation techniques, produced limits of detection of 4 $\mu\text{g/mL}$ in milk, in less than 20 minutes. According to current toxicological data, these limits of detection are sufficient for providing proper protection in the case of a ricin-related attack. Additionally, the procedure was conducted with a portable, hand-held Raman instrument to validate its compatibility with technology that could be used in a harsh environment.

The second separation technique used an aptamer which had been covalently bound to the nano-enhancing substrate to capture ricin from food. Aptamers are oligomers of single-stranded DNA which can be used like antibodies to selectively concentrate antigens from complex solutions. This separation protocol delivered a final detection limit of 25 ng/mL , 0.1 $\mu\text{g/mL}$, and 0.1 $\mu\text{g/mL}$, in buffer, milk, and orange juice, respectively. This assay could also be completed in less than 20 minutes.

This project has developed novel procedures for the rapid detection of ricin in liquid foods. These procedures were designed to be easily integrated into existing food

quality monitoring programs within food plants or at other sensitive locations, such as national borders or international shipping ports. Additionally, these methods serve as a proof-of-concept since their flexibility allows for easy adaptation to provide detection of other targets as well by replacing the capture molecule.

Table of Contents

Table of Figures	viii
Introduction	1
1.1 Bioterror and bioterror agents	1
1.2 Detection Technology	2
1.3 Statement of the Problem	3
Chapter 2 Review of Literature	6
2.1 Introduction of Ricin	6
2.1.1 Origin	6
2.1.2 Ricin molecule and its <i>in vivo</i> activity	7
2.1.3 Toxicity of ricin	10
2.1.4 Symptoms of intoxication	11
2.1.5 Past examples of bioterrorism	12
2.2 Ricin in Foods	13
2.2.1 Behavior	13
2.2.2 Ricin as a food bioterror agent	16
2.3 Detection of Ricin	20
2.3.1 Overview	20
2.3.2 Immunochemical Methods	21
2.3.3 ELISA	23
2.3.4 Aptamers	25
2.3.5 Official Methods of Detection	28
2.3.6 PCR/IPCR	29
2.3.7 Raman Spectroscopy	31
2.4 Surface-Enhanced Raman Spectroscopy	33
2.4.1 Surface-enhanced Raman scattering phenomena and mechanisms	33
2.4.2 SERS enhancers/nanosubstrates	34
2.4.3 Silver Dendrites	36
2.4.4 Applications of SERS	38
2.4.5 SERS-based detection methods in food	40
2.4.6 Pros and cons	42
Chapter 3 Immunomagnetic Separation coupled with SERS	45
3.1 Materials and Methods	45
3.1.1 Ricin and milk:	45
3.1.2 Preparation of Beads:	45
3.1.3 Preparation of Silver Dendrites:	46
3.1.4 IMS Procedure:	48

3.1.5 SERS Sample Preparation:	48
3.1.6 Raman Instrumentation.....	49
3.1.7 Spectral Data Collection and Analysis:.....	51
3.2 Results	52
3.2.1 Qualitative Analysis	52
3.2.2 Quantitative Analysis.....	55
3.3 Discussion	58
3.3.1 Qualitative Analysis	58
3.3.2 Quantitative Analysis.....	60
Chapter 4 Aptamer-Conjugated Silver Dendrites coupled with SERS	62
4.1 Materials and Methods	62
4.1.1 Ricin B chain, Ricin, and milk/orange juice:	62
4.1.2 Preparation of Aptamers	63
4.1.3 Preparation of Aptamer-Conjugated Silver Dendrites	65
4.1.4 SERS Sample Preparation:	65
4.1.5 Raman Instrumentation.....	66
4.1.6 Spectral Data Collection and Analysis:.....	67
4.2 Results	67
4.2.1 Ricin B Chain (RTB).....	67
4.2.2 Ricin toxin (A & B Chain)	72
4.3 Discussion	82
4.3.1 Ricin B Chain (RTB).....	82
4.3.2 Ricin toxin (A + B chains):	85
Chapter 5 Conclusion	90
5.1 Summary of Methods	90
5.1.1 IMS-SERS.....	90
5.1.2 Aptamer-conjugated Dendrite-SERS.....	91
5.2 Comparison of Methods	91
5.3 Future Study	94
Chapter 6 References Cited.....	96

Table of Figures

Figure 2-1 Left: Flowering castor (<i>Ricinus communis</i>) plant cultivated for landscaping and chemical feedstock; center: castor bean (seed) containing ricin toxin; right: ricin toxin holoprotein indicating RTA (blue) and RTB (orange).	7
Figure 2-2 Diagram of cellular mode of action for ricin toxicity. Reprinted from Pommerville, 2012 (Pommerville 2012).....	9
Figure 2-3 Diagram of Immunomagnetic Separation. A mixture of compounds containing a target analyte is mixed with magnetic beads which are conjugated with antibodies of the target. After immunocapture, a strong magnet is used to concentrate the bead-antibody-antigen complex. Lastly, the supernatant containing unwanted molecules can be removed. Adapted from Qiu et al., 2009 (Qiu et al. 2009).	23
Figure 2-4 Single-stranded DNA aptamer developed by Lamont et al. for the capture and concentration of ricin toxin (E. a Lamont et al. 2011). The 40 residue aptamer shows a stem-and-loop configuration that is capable of binding the ricin molecule.....	27
Figure 2-5 Schematic of Raman scattering and sample spectrum showing shift from the baseline, which equals the frequency of the incident beam.	32
Figure 2-6 Scanning electron microscopic images of silver dendrites generated by the reaction of zinc metal in silver nitrate solution. Reprinted from (Lili He et al. 2010).	37
Figure 2-7 Schematic diagram of the immunomagnetic separation procedure combined with solution-based SERS developed in He et al., 2011 (Lili He, C. L. Haynes, et al. 2011).	42
Figure 3-1 Schematic diagram detailing preparation of silver dendrites (AgD) which will be used as a nanoenhancer in this study.....	47
Figure 3-2 Schematic of IMS-SERS substrate preparation for spectral collection.	49
Figure 3-3 Top: Thermo Fisher Scientific's DXR Raman instrument; bottom: DeltaNU's portable Inspector Raman.	51
Figure 3-4 Stacked, raw SER spectra of positive and negative controls. Also background spectrum of elution buffer (EB). Figure adapted from He et al., 2011 (Lili He, Deen, et al. 2011)	53
Figure 3-5 PCA plot of spectra collected from negative controls (PBS and milk), positive control (ricin), and elution buffer background (EB). Figure adapted from He et al., 2011 (Lili He, Deen, et al. 2011)	54
Figure 3-6 PCA plot of milk spiked with 4 ug/mL and milk without ricin. This data was collected using the portable Inspector Raman instrument. Figure adapted from He et al., 2011 (Lili He, Deen, et al. 2011)	55
Figure 3-7 Stacked, second derivative SER spectra of increasing concentrations of ricin in milk. Figure adapted from He et al., 2011 (Lili He, Deen, et al. 2011).....	56
Figure 3-8 Principle component analysis plot of the varying concentrations of ricin in milk. 4 µg/mL was the limit of detection for this method. Figure adapted from He et al., 2011 (Lili He, Deen, et al. 2011).	57
Figure 3-9 PLS plot used for determining the predictive ability of the procedure for quantifying the amount of ricin present in a sample. Figure adapted from He et al., 2011 (Lili He, Deen, et al. 2011)	58

Figure 4-1 Sample preparations based on concentration of antigen per mL of matrix and the capture agent used in the study	63
Figure 4-2 Modified SELEX process performed by the S. Sreevatsan Lab at the University of Minnesota to produce the SSRA1 aptamer specific for capture and concentration of RTB. Figure is reprinted from electronic supplementary information connected to Lamont et al., 2001 (E. a Lamont et al. 2011).	64
Figure 4-3 Schematic Diagram of the silver-aptamer complex capture method and subsequent SERS detection.....	66
Figure 4-4 Raw SER spectra of aptamer-modified silver dendrites (Ag-Ap), as well as after capturing RTB (Ag-Ap-RTB). Additionally, RTB on silver dendrites has been included to show a 'pure' spectrum. Figure adapted from He et al., 2011 (Lili He, E. Lamont, et al. 2011).	68
Figure 4-5 Left: (A) SER spectra detailing differences in the 985 cm^{-1} shift region; and Right: (B) the 621 cm^{-1} shift region. Figure adapted from He et al., 2011 (Lili He, E. Lamont, et al. 2011).	69
Figure 4-6 PCA plot comparing spectra obtained from RTB-spiked samples (Ag-Ap-RTB), and aptamer without RTB (Ag-Ap). Varying symbols represent different starting concentrations of RTB in PBS. Figure adapted from He et al., 2011 (Lili He, E. Lamont, et al. 2011).	70
Figure 4-7 Top: (A) PCA plot comparing RTB captured from orange juice spiked at 50 ng/mL (crosses) to the negative control (circles). Bottom: (B) PCA plot of RTB in milk at 100 ng/mL (crosses) compared to the negative (circles). Figure adapted from He et al., 2011 (Lili He, E. Lamont, et al. 2011).	71
Figure 4-8 Raw SER spectra of pure RTB (top), and ricin toxin (bottom) directly conjugated onto silver dendrites.	72
Figure 4-9 Stacked, SER spectra of controls and sample spectra collected using aptamer-conjugated silver dendrites (Ag-Ap). Bare silver (Ag) served as a background spectrum. Important spectral differences are boxed. Figure adapted from He et al., 2011 (Lili He, E. Lamont, et al. 2011).	73
Figure 4-10 PCA plot showing clear separation of data sets. Separation denotes a significant difference. Figure adapted from He et al., 2011 (Lili He, E. Lamont, et al. 2011).	74
Figure 4-11 Stacked, second derivative SER spectra of aptamer-conjugated dendrites after capture from varying concentrations of ricin in PBS. Figure adapted from He et al., 2011 (Lili He, E. Lamont, et al. 2011).....	74
Figure 4-12 PLS plot of quantification study using varying concentrations of ricin in PBS. Range of quantification was 0 to 50 ng/mL; no significant spectral difference was found between 50 and 100 ng/mL. Figure adapted from He et al., 2011 (Lili He, E. Lamont, et al. 2011).	75
Figure 4-13 Stacked, second derivative SER spectra of native and denatured (inactivated) ricin directly adsorbed onto silver dendrites (Ag-R, Ag-inR) as well as captured using aptamer (Ag-Ap-R, Ag-Ap-inR). Figure adapted from He et al., 2011 (Lili He, E. Lamont, et al. 2011).	76

Figure 4-14 Stacked, 2 nd derivative SER spectra of whole ricin toxin spiked into orange juice samples. Significant differences associated with the presence of ricin were found in the peak located at 1100 cm ⁻¹ (indicated).	78
Figure 4-15 Focused view of overlaid 2 nd derivative spectra surrounding the peak at 100 cm ⁻¹	78
Figure 4-16 PCA plot of spectra collected from orange juice spiked with varying concentrations of whole ricin toxin.....	79
Figure 4-17 PLS plot showing the platform's ability to predict the concentration of ricin in a sample of diluted/filtered orange juice.....	80
Figure 4-18 SER spectra of whole ricin toxin spiked into 10x diluted milk. Significant difference from the presence of ricin was found at 1100 cm ⁻¹ (indicated).	80
Figure 4-19 PCA plot of 10x diluted milk spiked with varying concentrations of whole ricin toxin.	81
Figure 4-20 PLS plot showing the platform's ability to predict the concentration of ricin in a 10x diluted sample of milk.....	82

Introduction

1.1 Bioterror and bioterror agents

Since the passage of the Biosecurity Act of 2002, the US government has tasked the Department of Homeland Security with implementing strategies which secure the US food supply from acts of bioterrorism. Bioterrorism in the food supply would likely take the form of intentional contamination with a toxic agent in order to cause casualty, terror, or economic damage. Such an attack could be performed by as few as a single person by deploying a bioterror agent into a food at any point in the supply chain and exploiting the food's intact supply and distribution chains to spread the agent.

Bioterror agents are classified by the Centers for Disease Control and Prevention into categories based on their toxicity and ease of dissemination. Though many agents exist, it is those which are easily accessible, extremely toxic, and hard to detect which are the most likely to be used in an attack. An example of such a toxin is ricin—a highly toxic protein which is derived from a widely available plant. Though ricin has never been implicated directly in a food bioterror event, it has reportedly been intentionally added to food to cause harm. Additionally, large amounts of ricin have previously been found in the possession of terrorists whose intentions were unknown.

The food supply is characterized as a vast network of interconnected supply chains which transport and distribute foods to virtually every person. Often times, these supply chains are not well controlled and steps to manage or detect accidental or purposeful adulteration are absent. For this reason, it is unlikely that a contamination

event would be discovered before casualties began to appear and the public health system could ascertain the source. This reactionary approach would be too late for many casualties, and would cause economic damage as consumers lost faith in the safety of the affected food. In fact, during his resignation address in 2004, then Department of Health and Human Services Secretary Tommy Thompson stated his disbelief that terrorists hadn't already attacked the US food supply citing its extreme vulnerability.

With the more recent passage of the Food Safety Modernization Act, the US Government and Food and Drug Administration have demonstrated their adoption of a preventative model to ensure food quality and safety. Unlike the reactionary approach, preventing bioterror agents from entering the food supply is important since not only does it prevent human casualty, but it may bolster consumers' confidence in the safety of their food and the ability of the government to protect it. However, such prevention requires strategic surveillance of the food supply prior to consumption.

1.2 Detection Technology

Detection of bioterror agents in foods requires technologies which are able to distinguish substances present at dilute concentrations in very complex matrices. Many technologies exist which are capable of extremely sensitive detection such as immunochemical assays, chromatography, and mass spectrometry. However, implementation of these platforms for routine detection assays would be difficult for a variety of reasons including cost, expertise required, and duration of the test. Previous study by He et al. (2011) reported that food contaminants could be detected quickly and easily at parts per billion concentrations using surface-enhanced Raman spectroscopy.

This method holds promise for adaptation and optimization to detect bioterror agents in food.

1.3 Statement of the Problem

The present study develops two methods for the detection of ricin in fresh liquid foods. These methods were developed according to certain criteria that would make their implementation feasible while maintaining sensitivity necessary to protect people in case of an attack. Important feasibility criteria included short duration, low cost, ease of procedure, and flexibility in many food matrices. Also important for developing an assay is understanding the space and personnel who would be required to perform such as assay, as well as the location in the supply chain where the assay would occur.

Based on vulnerability studies, it is most likely that a ricin contamination event would occur during distribution of a fresh liquid food product prior to centralization. Such foods, like milk and orange juice, have structurally similar supply chains: raw product is produced at many disparate locations, trucked to a central facility, processed, and then redistributed. Early in the supply chain, prior to centralization, is probably the most vulnerable period as it would be difficult for surveillance mechanisms to ensure that every truck from every farm is unadulterated. Additionally, the fact that a food product is liquid means that if undetected, a single contaminated truck could be centralized where it would mix with uncontaminated product and become impossible to trace back to the originating location. Furthermore, pasteurization of fresh liquid foods such as milk is largely insufficient for the deactivation of ricin's toxicity. Companies'

existing security measures make it unlikely that a terrorist could gain unauthorized access to disperse ricin inside a facility. Also, it would be very labor intensive to contaminate a large number of end-user containers which are often packaged in tamper-evident packaging. Therefore, testing each truckload of product as it arrives at the central processing facility represents the highest probability of uncovering a bioterror event. A detection assay which was designed to operate in such a time and place would be most efficacious.

It was desirable to have the test be completed in the shortest amount of time possible, however <20 minutes was used as the window of time which was defined as 'rapid'. This was used based on an estimate of the amount of time a tanker truck of milk is currently held while quality tests are performed. Because product could not enter the facility until a bioterror assay was completed, the same way that milk cannot enter the distribution chain until an antibiotics test is completed, it was necessary to develop a rapid assay.

It was also necessary that the test be applicable to many different food products. An assay which could only be adapted for a narrow range of products would be less useful than one that could be applied to many. Additionally, a simple procedure and portable, durable technology was favored since the assays would likely not be completed by scientists with expertise in spectroscopy, but rather by quality technicians in a variety of food manufacturing facilities.

Lastly, the assay must have a limit of detection which ensured that a product which was tested could not contain an amount of ricin which was capable of harming an

individual. The scenario where a 20 kg child with the greatest ricin sensitivity (using lowest published LD₅₀) consumed contaminated milk or orange juice was used to establish adequate limits of detection.

Chapter 2 Review of Literature

2.1 Introduction of Ricin

2.1.1 Origin

Ricin is a protein produced by the castor bean plant, *Ricinus communis*, and is a component of the plant's oilseed where it acts as a phytotoxin (Balint and August 1974). The plant is cultivated for the seeds which are pressed in the production of castor oil, a valuable chemical feedstock due to the high concentration of ricinoleic acid used in the production of many industrial and cosmetic products (Mutlu and Meier 2010). The plant is cultivated for industry primarily in India (70%), China, and Brazil, but readily grows as a perennial, medium-to-large flowering bush in tropical climates or as an annual in more temperate climates. Due to the plant's frost heartiness, brilliant red color of some cultivars, and long flowering season, it is often cultivated decoratively in ornamental landscaping applications (Brickell 2008).

After pressing the seeds for their oil, the remaining cake is comprised of approximately 1.5% ricin protein phytotoxin; the rest of the plant also contains this protein however at far smaller concentrations (Mutlu and Meier 2010). Prior to commercial use, the oil is purified to remove traces of ricin and ricinine, a toxic alkaloid component of the seeds, as well as an allergen. The cake may undergo a number of physical and/or chemical treatments to detoxify it depending on the end use (Anandan et al. 2005). Where more pure samples are required, complex solvent extraction and

subsequent affinity chromatography methods exist for the isolation of castor seed components for use as laboratory standards (Olsnes and Pihl 1973).

2.1.2 Ricin molecule and its *in vivo* activity

Ricin toxin is a holoprotein consisting of two polypeptide chains which are nearly equal in molecular weight and are joined by a disulphide linkage. The ricin toxin A (RTA) is a highly ordered polypeptide chain consisting of 267 amino acids and functions as an N-glycoside hydrolase. The ricin toxin B (RTB) polypeptide chain is a largely unordered lectin consisting of 262 amino acids (Michael, M. Roberts, and Robertust n.d.). Both subunits are required to impart toxicity *in vivo* or in cell cultures, however in cell free experiments, RTA alone can impart ribosome-inactivating activity (Wright and Robertus 1987). By itself, RTB shows no toxicity or inhibition of protein synthesis *in vitro* or *in vivo*.



Figure 2-1 Left: Flowering castor (*Ricinus communis*) plant cultivated for landscaping and chemical feedstock; center: castor bean (seed) containing ricin toxin; right: ricin toxin holoprotein indicating RTA (blue) and RTB (orange). Figures adapted from [Wikipedia.org/wiki/Ricin](https://en.wikipedia.org/wiki/Ricin)

Ricin is classified as a type 2 ribosome inactivating protein (RIP) due to its inhibitory effect on protein synthesis and its having more than one subunit. In cell studies, RTB acts as a carrier lectin which preferentially binds galactosyl moieties on the surface of a cell (G. L. Nicolson et al. 1975). Once bound, RTB induces endocytosis within 30-60 minutes and radio-tagged ricin toxin can be located in both endocytotic vesicles and free in the cytoplasm after 60 minutes. Once free in the cytoplasm, the single disulphide linkage between the A and B chain must be reduced (cleaved) in order to impart toxicity (Wright and Robertus 1987). Once separated, RTA is able to enzymatically inactivate the 28S ribosomal RNA subunit by cleavage of a specific adenine residue (Michael, M. Roberts, and Robertust n.d.) (depurination) thereby stopping RNA translation and protein synthesis and causing eventual cell death (G. L. Nicolson et al. 1975) (Olsnes and a Pihl 1973). Nicolson showed the galactose-binding property of RTB which could be reversed 90-95% within 10 min by the addition of 0.1M lactose or galactose leading to the hypothesis that B chain is a carrier protein that binds to cell surfaces involving galactose but that the toxic action of ricin is due to the A chain. Also supporting this hypothesis, it was shown in vitro that toxicity is greatly diminished if the two chains are cleaved from one another using B-mercaptoethanol prior to endocytosis or if reductive cleavage of the ricin subunits is prevented in the cytoplasm (Olsnes and a Pihl 1973).

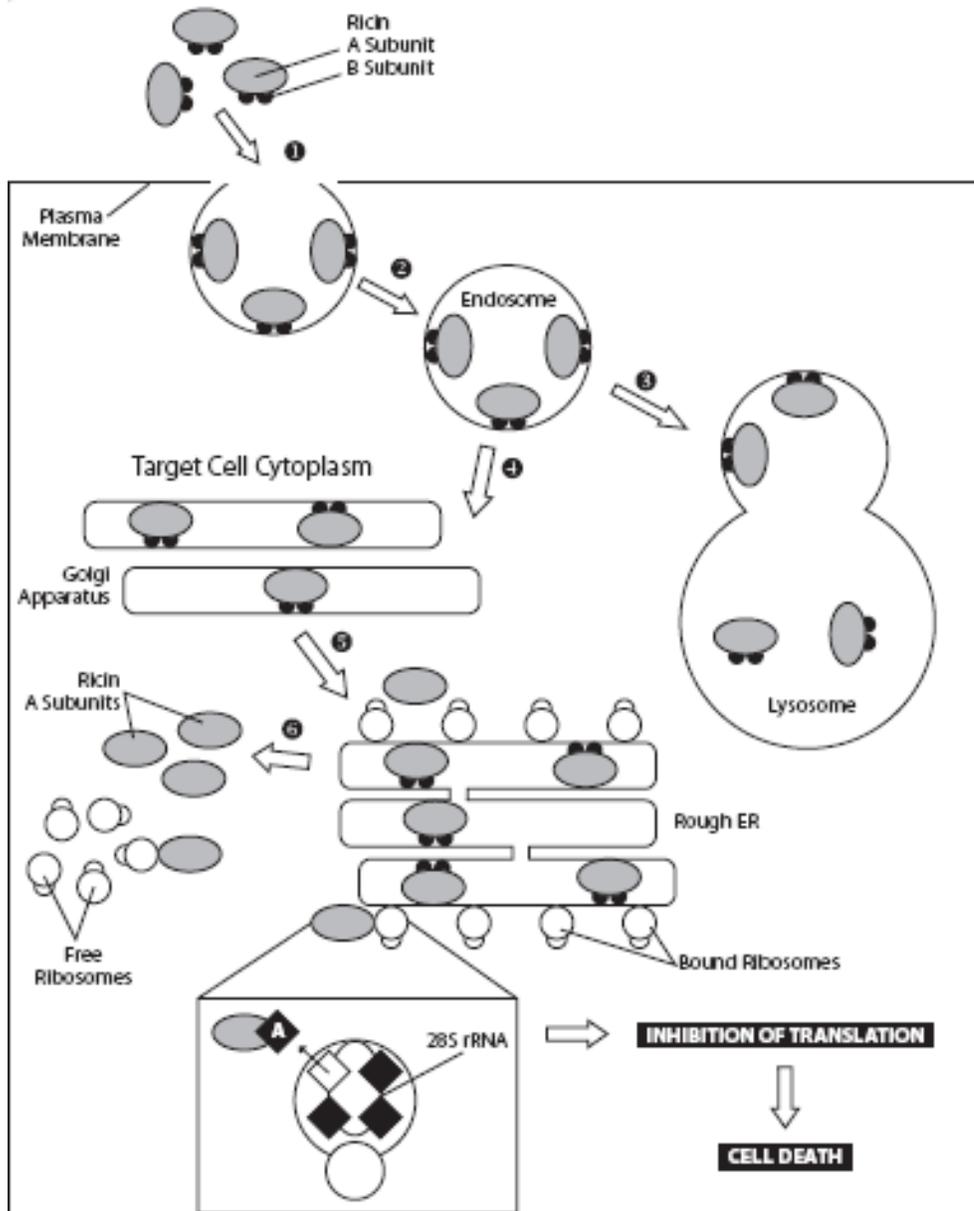


Figure 2-2 Diagram of cellular mode of action for ricin toxicity. Reprinted from Pommerville, 2012 (Pommerville 2012).

2.1.3 Toxicity of ricin

Ricin's toxicity and symptoms of intoxication depend on the route of entry into the body as well as the dose. Toxicity has been demonstrated through parenteral/intravenous, inhalation, and oral absorption of ricin; it is believed that ricin may also impart toxicity by absorption through damaged skin (Balint and August 1974). Animal studies involving mice have demonstrated that the median lethal dose (LD₅₀) of paraneural administered ricin is 5-10 µg/kg body weight (Godal et al. 1984). The LD₅₀ for inhaled ricin is 4-5 µg/kg body weight but it was reported that lethality is highly dependent on the particle size of aerosolized ricin with smaller particles (<5µm) being most lethal as they can deposit deeper into the lungs. LD₅₀ of ingested ricin in mice is 5-20 mg/kg body weight (Garber 2008). Because toxicity testing of ricin is not possible on humans, they are calculated based on toxicological data of animal studies as well as case studies of accidental human intoxications. Based on these reports, the human LD₅₀ for intravenous or inhalation exposure is estimated to be 5-10 µg/kg body weight (CDC 2003b), and 1-20 mg/kg bodyweight administered orally (Audi et al. 2012). Accidental poisoning cases have been reported where ingestion of 0.5-30 beans has resulted in mild clinical symptoms to death.

The reduced toxicity of ingested ricin is thought to be due to the large relative size of the molecule making it poorly absorbed in the gut or degraded by gastrointestinal enzymes (Franz and Jaax 1997). It has been reported that ricin is resistant to the action of the proteases trypsin, pepsin, and papain (Balint and August 1974; Frenoy 1986), possibly leading to the diminished but not complete elimination of toxicity from ingested

ricin. Based on animal models, 25-40% of an oral dose of pure ricin is excreted unchanged in the feces which indicates both its bioavailability as well as its resistance to the action of proteolytic enzymes (Audi et al. 2012).

The toxicity of ingested intact castor beans (misnomer, as the 'bean' is botanically a seed) is much lower than the purified toxin and is reported to be 0.2-14 g/kg body weight due to poor absorption as a result of the undigestible seed pericarp (Balint and August 1974). However, once the seed coat has been broken, the ricin toxin is released and the LD₅₀ decreases with increasing levels of mastication, age, and other comorbidities.

2.1.4 Symptoms of intoxication

Because ricin can technically poison any cell due to its fairly general mode of toxicity, symptoms of ricin intoxication are diverse and depend on the dose and route of exposure, and typically intensify over time (Franz and Jaax 1997). It should be noted that in all of these cases, the symptoms of toxicity begin mild and intensify over time. The non-specificity of symptoms may be confused with more innocuous conditions leading to inaction.

Parenteral administration of ricin may present with flu-like symptoms, pain, and necrosis of muscle tissue at the site of injection (Health 2008). Symptoms of inhalational exposure usually develop within 4-8 hours and may include sudden congestion of the nose and mouth, respiratory distress/failure, difficulty breathing, flu-like symptoms, nausea, and muscle pain. Sub-acute doses of ingested ricin typically present with nausea, vomiting, and cramping within 2-6 hours of ingestion; larger doses may lead to severe

gastrointestinal symptoms, dehydration, and possible kidney and/or liver failure within 10 hours. Injection or inhalation exposures are more likely to become symptomatic sooner and will progress faster than oral exposures to ricin given the same dose (CDC 2005).

Ingested toxin acts mostly on the liver, pancreas, kidneys and intestines because these are the locations that ricin is absorbed and excreted (Audi et al. 2012). Acute toxicity will often include diarrhea, tachycardia, asthmatic symptoms, and eventual multi-organ failure. (Balint and August 1974) describes post-mortem histological evidence of acute damage to the smooth endoplasmic reticulum of liver cells, bleeding in serous membranes, hemorrhage in stomach and intestines, and damage to heart, kidneys, and lymphoid cells of the spleen. Death may occur within 36-72 hours depending on the dose, route of administration, presence of comorbidities, and is typically a result of paralysis of the respiratory and vasomotor centers in the medulla oblongata (Balint and August 1974). There is no specific treatment for intoxication other than supportive care, although there are some vaccines presently in development (Holtermann 2006).

In vivo exposure to ricin leads to the formation of anti-ricin antibodies which have been observed both in mice, as well as in farmers who work with the castor bean plant (Balint and August 1974). It is not known if the presence of ricin antibodies in farmers imparts any resistance to ricin toxicity.

2.1.5 Past examples of bioterrorism

While purified ricin has to-date never been used as a foodborne bioterror agent, castor bean fragments were once placed in a few jars of baby food in Orange County, CA (Gottlieb and Tran 2004). Additionally, ricin has been implicated in a number of

incidents where individuals or small groups of extremist/terror organizations have been found in possession of ricin (Musshoff and Madea 2009). For example, in 2003, a letter containing ricin was mailed to the White House which demanded repeal of changes to US trucking laws and threatened to poison water supplies with ricin (CDC 2003a). In 2004, ricin was found in a package which was sent to the Dirksen Senate Office Building in Washington, DC. Perhaps the most famous case of ricin poisoning was when it was weaponized into a spring-loaded umbrella which was used to assassinate Bulgarian dissident Georgi Markov, thought to have been killed by Soviet KGB (Knight 979).

Should an incident occur where ricin is implicated as a bioterror agent, it is unlikely that the event would be the result of accidental contamination since it is highly unlikely that castor and/or ricin could otherwise enter the food supply. Therefore, all events concerning ricin are considered acts of terrorism until proven otherwise, and trigger a cascade of State and Federal actions including notification of the Federal Bureau of Investigation (FBI), CDC, Federal Department of Health and Human Services, and Department of Homeland Security (DHS) and subsequent investigation (Holtermann 2006). For this reason, it is important that assays for bioterror agents, such as ricin, have a low chance of producing false positive results.

2.2 Ricin in Foods

2.2.1 Behavior

There is inadequate research in the literature pertaining to the behavior of ricin in foods regarding its stability, toxicity, and interference in detection. Detection of ricin in a

food system may be complicated and/or hindered by the presence of constituents native to that product. Belitz et al. describes foods as heterogenous mixtures of many different molecular components which can “bind and sequester toxins, rendering them undetectable” (Belitz, Grosch, and Schieberle 2004). This may be due to certain toxin epitopes, which are identified in pure preparations, being “masked” in a food matrix. Furthermore, matrix components may interfere with the detection method due to certain physical and/or chemical properties such as fluorescence, having similar molecular weight to ricin, binding ricin, solubility, etc. For example, while ground beef interfered little with detection of ricin, liquid egg white and low-fat milk both greatly diminished the ability to detect ricin spiked into a sample (X. He et al. 2008). Furthermore, the fact that matrix components will almost certainly be present in the food in concentrations many orders of magnitude greater than ricin may lead to decreased sensitivity (X. He et al. 2010). In some cases, dilution of a ricin-containing food sample with phosphate-buffered saline (PBS) may reduce the diminished sensitivity of the assay (X. He et al. 2008).

Despite diminished sensitivity of detection assays in the presence of food, it is shown that the toxicity of ricin is not heavily impacted by the food matrix, or even increased. One group of scientists showed that ricin toxicity was highest when delivered in PBS but was not significantly greater than in water, apple juice, or half-and-half and there was no difference between ricin toxicities within non-PBS matrices (Garber 2008). Also, it was shown that there was no decrease in toxicity if these products were spiked with ricin and then stored at refrigeration temperatures for 11 days. Even more

problematic is that the toxicity of ricin may be enhanced by the presence of the food matrix which may contain components or possess physical and/or chemical properties which potentiate toxicity (X. He et al. 2008).

There is very limited information regarding the impact of various food processing steps on the toxicity of ricin, with most pertaining to the thermal stability of ricin. Also the preparation of ricin impacts the thermal stability with crude and A+B chain preparations being more thermally stable than the A chain alone which substantially denatures at 55°C (Argent et al. 2000). In general, pasteurization (typically 63°C for 30 min or 72°C for 15 s in milk), which is designed to decrease the microbiological hazard of foods, is insufficient to render protection from ricin intoxication. Jackson et al showed that the half-life of pure ricin in both a milk-based and soy-based liquid infant formulas was 100 min at 60°C, ~7 min at 75°C, and ~2 min at 90°C (Jackson, Tolleson, and Chirtel 2006). Jackson et al investigated the stability of ricin during the pasteurization process undergone by orange juice, as well as ricin's subsequent half-life during storage in acid media such as orange juice. It was reported that the common pasteurization process was not sufficient to ensure non-toxicity and that the half-life in orange juice was 10±3 days and therefore able to reach consumers (Jackson, Z. Zhang, and Tolleson 2010). Finally, Garber showed that toxicity is not significantly impacted by the pH of the food matrix in which it is delivered in vivo (Garber 2008).

For home-cooked food products such as meat and eggs, the USDA recommends cook temperatures (145°F-165°F) and an average length of cooking which together are not able to completely inactivate intact ricin molecules (X. He et al. 2008). Kumar et al

showed that most high temperature cooking processes (boiling, steaming, baking, frying) conducted in consumers' homes would be sufficient to denature the toxic protein rendering it innocuous since toxicity of native ricin is rapidly lost as it is heated to 100°C (O. Kumar et al. 2010).

Frenoy showed that the tight structural domains of the ricin protein protected it from the action of proteolytic enzymes such as trypsin and pepsin, allowing for ingested toxicity of ricin (Frenoy 1986). Furthermore, the three dimensional shape of the intact ricin holoprotein largely inhibits access of reducing agents larger than B-mercaptoethanol to cleave the disulphide bond linking the A and B chains (Lappi et al. 1978) thereby diminishing its activity.

Overall, the toxicity of ricin is largely unaffected by the identity of the food matrix it is within, however many food matrices complicate the extraction and detection methods which depend greatly on the food. This has led to the development of many assays which can detect ricin in foods.

2.2.2 Ricin as a food bioterror agent

Food terrorism is defined by the World Health Organization (WHO) as “an act or threat of deliberate contamination of food for human consumption with chemical, biological or radionuclear agents for the purpose of causing injury or death to civilian populations and/or disrupting social, economic, or political stability” (Organization 2008). According to the Centers for Disease Control and Prevention, Ricin is classified as a Category B bioterror agent because it is moderately easy to disseminate, can result in moderate morbidity rates and low mortality rates, and require specific enhancements of

CDC's diagnostic capacity and enhanced disease surveillance (Workgroup 2000). One of the main reasons that ricin is controlled is because of the relative ease of its purification. Ricin can be purified from the castor plant using a process that is openly published on the internet and in other literature. Someone possessing even the most basic laboratory experience following such a procedure could be successful at making fairly pure preparations of ricin using basic equipment.

The resulting ricin can be prepared as a liquid (protein dissolved in a buffer), or as a white/gray, crystalline, dry powder. Ricin preparations are odorless, tasteless, and water soluble and stable at ambient conditions. Ricin is non-volatile, but can be prepared as an aerosol. In the case of either inhalation or ingestion toxicity, the resulting symptoms are fairly non-specific and could easily be initially misdiagnosed as respiratory infection or gastroenteritis (Holtermann 2006), leading to increased time of recognition by the public health, and subsequently longer exposure time.

Though the toxicity of ricin has been well documented for over a century, it is only within the past couple decades that authorities have imagined it in the context of bioterror. Following the events of September 11th, 2001 the US government has joined with many partners in the food industry and academia to understand the challenges of monitoring such a vast, diverse system which unmonitored, has the potential to become catastrophic (Gaudio and Salerno 2004). Existing surveillance systems are based on detecting typical physical, chemical and microbiological hazards associated with food; information is collected from past outbreaks/contamination events and a specific countermeasure is implemented. However, we cannot suppose that intentional attacks on

the food supply will resemble anything previously observed and may in fact be engineered specifically to evade existing surveillance systems; thus, our current ability to defend the food supply from such events is limited.

In order for ricin to be used as an agent of mass casualty, it would have to be dispersed as a fine aerosol, or deployed into the food or water supply. The ease of isolation and flexibility of applications means that once a ricin extract was prepared, a terrorist could introduce it into food supply at nearly any place in the supply chain. The complexity of the US food supply provides the necessary disorganization that would allow for such an adulteration to go undetected and/or response by the public health sector to be delayed. The US food supply is comprised of both domestic and imported foods which move from “farm to table” through a dizzying number of producers, brokers, processors, distributors, transporters and other intermediate steps which all serve as opportunities for the terrorist to adulterate a product. Additionally, with increasingly centralized production of foods and nation-wide distribution chains, the opportunity for a terrorist to affect the largest number of people is maximized in a single adulteration event. Finally, the ability of the public health sector to identify the cause and source of an attack is challenged by the huge geographic distribution radius.

Fresh, liquid food products such as milk and orange juice represent the perfect storm for such an attack since raw product is typically collected from a large number of small processors who centralize and process the liquid before bottling and distribution (Khan, Swerdlow, and Juranek 2001; Wein and Yifan Liu 2005). A bioterror agent introduced into a centralized liquid processing facility would quickly disperse throughout

a large volume of product, as much as 60,000 gallons, thereby contaminating a massive number of servings. Also for many fresh liquid products, processing often includes a heat process (pasteurization) in order to reduce the number of pathogenic and spoilage microorganisms. However, pasteurization is considered a relatively gentle procedure (in order to maintain product quality) and is insufficient to inactivate the toxicity of ricin. Since pasteurization does not sterilize a product, fresh products generally have a relatively short shelf-life, are refrigerated through “just in time” distribution, and are typically purchased and consumed within a short time period after production. This would maximize the number of victims before public health institutions could identify and address the problem.

Products which are typically cooked prior to ingestion would be an unlikely route of attack since a more thorough cooking process will inactivate the ricin’s toxicity (O. Kumar et al. 2010). Attacks on fresh-eaten solid foods such as fruits and vegetables are feasible, but less likely as it they would require a more prolonged application of ricin to cover products. Although these types of attacks would be less likely to affect a large number of people, high morbidity may not be the goal of a terrorist attack. Even limited number of cases of adulteration by a bioterror agent such as ricin could be sufficient to cause considerable panic/terror in a population (CDC 2001). As has been the case in nearly all cases of foodborne disease, even a small number of cases can cause irreparable economic damage to a whole sector of the food supply as consumers become mistrustful of its protective measures (Wein and Yifan Liu 2005).

Detection of bioterror agents in the US food system is a novel initiative to food safety and is currently not required to be part of routine testing of any food. Currently available detection methods do not allow for online monitoring of bioterror agents due to the long duration and expense of most assays. For this reason, it is unlikely that a food company would adopt such monitoring schemes unless an attack on the food supply were to actually occur, which would already be too late.

2.3 Detection of Ricin

2.3.1 Overview

Detection of ricin in a sample typically occurs in two general steps: capture/concentration, followed by detection. Because ricin may not be present in a sample at high concentrations, it must first be concentrated by some mechanism and separated from the matrix it contaminates. Traditionally, concentration techniques capitalized on the action of ricin's B chain to bind galactose residues bound to the stationary phase of a liquid chromatography column (Balint and August 1974). Today, immunologically-based methods have been employed for concentrating ricin in biological or environmental samples and have been demonstrated to be flexible, sensitive, and inexpensive (Audi et al. 2012). Still, many other methods have also been developed including varying modes of chromatography such as HPLC and GC coupled with myriad detection techniques.

In the second general step, ricin protein can be detected directly through the use of animal toxicology studies, cell death assays, spectroscopy, mass spectrometry, or

indicating chemical reactions like colorimetrics or chemiluminescence (Audi et al. 2012). These detection methods have virtually replaced the use of radioactive isotopes to label ricin in detection methods. While providing adequate limits of detection, radioactive methods were typically long, expensive, required great expertise, and generate hazardous waste which is complicated to dispose of (Poli et al. 1994). Ricin may also be detected indirectly through one of its indicators such as other Castor plant derivatives which would probably be present in a preparation of ricin toxin. Such derivatives include DNA from *Ricinus communis* or other seed components such as ricinine or ricin agglutinin. Detection methods for these compounds include those indicated above and may also include PCR and agglutination assays. While detection of these compounds may give presumptive indication of ricin toxin, they do not confirm it, and are not necessarily correlated quantitatively.

2.3.2 Immunochemical Methods

Immunological techniques utilize antibodies—globular proteins which have a three dimensional structure able to selectively bind specific parts of molecules using a ‘lock-and-key’ configuration. These molecules are especially useful in biological assays as they can be immobilized on surfaces where they can selectively capture and concentrate a target analyte out of a complex solution. After capture and concentration, there are a multitude of different detection schemes which may be utilized to characterize and/or quantify the analyte of interest. Many such schemes have been developed including immunochromatographic assays, enzyme-linked immunosorbent assays (ELISA), time-resolved fluorescence (TRF) assays, Western blot, and

electrochemiluminescence (ECL) assays (Leonard F Peruski and A. H. Peruski 2003). These methods vary in sensitivity, feasibility, duration, and cost, however all utilize an anti-ricin antibody and are therefore reliant on the antibody's affinity and specificity to the antigen. Evaluating the affinity and specificity of antibodies is necessary to immunologically-based assays and often includes challenging the antibody with a series of molecularly similar compounds or compounds from an environment where samples will be collected (Leonard F Peruski and A. H. Peruski 2003).

Immunomagnetic separation (IMS) is a specialized method of immunochemical extraction which hybridizes the selective binding ability of antibodies with magnetism for concentration of an analyte. IMS utilizes antibodies which have been coated onto the surface of magnetic polymer beads. Beads are then mixed with a sample which contains ricin and then concentrated using a simple magnet while the resonant solution/matrix is decanted. Originally, this method was developed for the isolation of blood cells, however it has since been optimized for the selective concentration of many biological molecules—specifically allergens (Gaudernack et al. 1986; Lea et al. 1985).

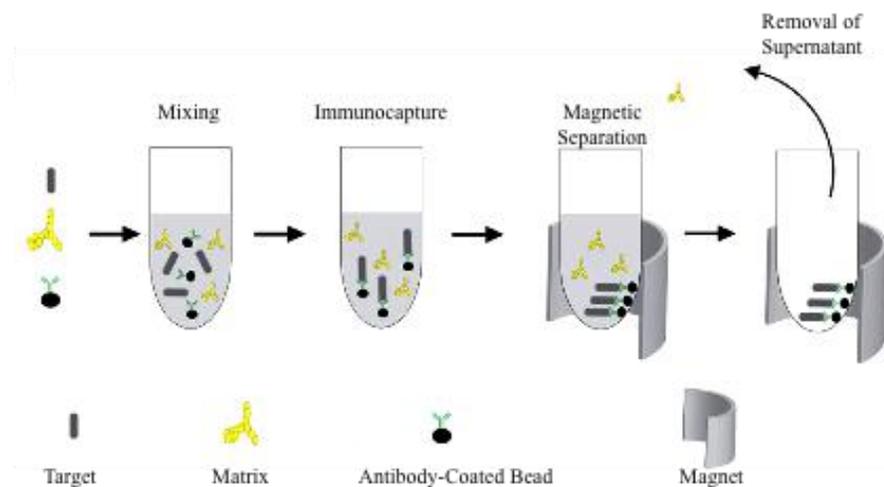


Figure 2-3 Diagram of Immunomagnetic Separation. A mixture of compounds containing a target analyte is mixed with magnetic beads which are conjugated with antibodies of the target. After immunocapture, a strong magnet is used to concentrate the bead-antibody-antigen complex. Lastly, the supernatant containing unwanted molecules can be removed. Adapted from Qiu et al., 2009 (Qiu et al. 2009).

2.3.3 ELISA

ELISA is a specialized antibody-based method for capturing a target antigen from solution. The antibody-antigen binding signal is dependent on the mode of ELISA; typically an enzyme which is bound to the antibody produces a colorimetric or chemiluminescent reaction which can be quantified by absorption at a specific wavelength (Lequin 2005). Many modes of ELISA exist (direct, indirect, sandwich, competitive), however the sandwich format is most commonly used for detecting dilute protein antigens in samples (Kemeny and Chantler 1988).

One of the first studies of using ELISA technology for the capture and detection of ricin reported a limit of detection in rabbit serum of 40 ng/mL (Koja, Shibata, and Mochida 1980). Antibodies purified using affinity chromatography coupled with enhanced detection methods have since lowered the LOD of ELISA techniques used in human and animal urine and serum (Poli et al. 1994). Although ever-more sensitive detection assays are useful, with single-molecule detection being possible using advanced nanotechnology (S. Ding, Gao, and Gu 2010), limits of detection of current industry standard techniques are sufficient for the detection of ricin at concentrations lower than that which would cause toxicity if ingested by a human. Two recent studies have used ELISA kits for the detection of ricin in foods (Garber 2008; Jackson, Tolleson, and Chirtel 2006).

In the food industry, ELISA methods have been used as rapid methods for detecting pathogens or their biomarkers, and other small molecules such as allergens or pesticides. Although not considered an official method for detecting ricin in cases of potential bioterrorism, ELISA technology has become the industry standard for rapid analyses due to adequate sensitivity, not requiring specialized equipment or expertise for reading assays, ease of discarding, and not requiring extensive sample preparation time (Poli et al. 1994). As a result, ELISA kits are available for hundreds of different targets including many toxins, disease biomarkers, microorganisms, allergenic proteins, carbohydrates, and more.

2.3.4 Aptamers

Leveraging the same concept as capture using anti-ricin antibodies, scientists have developed ‘lock-and-key’ capture molecules from single-stranded oligomers of RNA or DNA. Due to hydrogen bonding between complimentary regions, these molecules form unique stems, loops, bulges, hairpins, pseudoknots, and triplexes—3-dimensional structures which form the unique structures allowing for capture of target molecules (Stoltenburg, Reinemann, and Strehlitz 2007). Capture forces are thought to include structure compatibility, van der Waals forces, hydrogen bonding, electrostatic interactions, stacking of aromatic rings, and/or a combination of these effects. Aptamers are able to distinguish between chiral molecules and can identify single epitopes of a molecule; therefore they are useful in distinguishing between closely-related antigens, such as species of bacteria.

Aptamer sequences are chosen from enormous pools of RNA or DNA fragments where it is estimated that only one of every 10^{10} is able to fold into a three-dimensional structure which is able to selectively bind a target (Ellington and Szostak 1990). Aptamers have been shown to possess similar or better affinity and selectivity as compared to protein antibodies and are much more stable to harsh environmental or procedural conditions (Ellington and Szostak 1990; P. Wang et al. 2011). Aptamers may undergo denaturation in these conditions, although it is typically reversible and aptamers will return to their native form following reintroduction to milder conditions where a protein would be permanently denatured. This has led to the application of aptamers in reusable assays, where captured proteins can be eluted from the aptamer by denaturing

the complex, releasing the ligand and removing it, before returning the aptamer to its native formation.

Aptamers are generated first by creating a large pool of RNA (or DNA) oligomers of random sequence that are approximately 100 bases long (Ellington and Szostak 1990). Each of these random sequences is built from a primer/promoter which is used later in the process for amplification to form a pool of approximately 10^{15} possible aptamers. Due to some sequences not being easily amplified, such as those with very high G and C contents, the effective pool of possible aptamers is closer to 10^{13} . This pool can then be challenged using affinity chromatography where the analyte of interest is the ligand bound to the stationary phase of the column. Non-binding aptamers elute off the column first and are discarded; column-bound sequences can be selectively eluted afterward and collected. The collected fraction can then be amplified and reintroduced to the affinity column repeatedly. With increasing repetitions of this process, (usually 6-20 'rounds'), only those aptamers with the highest binding affinity to the target are retained. After the last round of selection, the eluate is amplified and the major sequences present are characterized. This basic process has come to be known as selective evolution of ligands by exponential enrichment, or SELEX.

Having the advantage of being specifically engineered, aptamers can be easily constructed to produce the highest possible binding affinity, either by chemical modification, generation of the aptamers under the same conditions as the final assay, or just by the magnitude of possibilities present in the nucleotide pool. Also, aptamers can be conjugated with a number of reporter molecules such as chromogenic or fluorescent

reporter molecules to aid in detection following capture (Kirby et al. 2004). Additionally, once capture sequences are published, generating aptamers is relatively rapid and inexpensive since they do not need to be generated in a biological system, (as in antibodies), and therefore are conducive to developing specific quantitative assays for industry (Ellington and Szostak 1990; Stoltenburg, Reinemann, and Strehlitz 2007).

Aptamers have been developed for a number of targets including prions (P. Wang et al. 2011), viruses (Tuerk and Gold 1990)(Wongphatcharachai et al. 2013), infectious and non-infectious bacteria (Deisingh and Thompson 2004)(Joshi et al. 2009), dyes (Ellington and Szostak 1990) and many others. Additionally, Lamont et al. has developed a single-stranded DNA aptamer for the capture and concentration of ricin (E. a Lamont et al. 2011). Because of the wide applicability of using aptamers as a biological capture agents, they have been utilized in basic research, detection assays, medical diagnoses and therapies (Stoltenburg, Reinemann, and Strehlitz 2007).

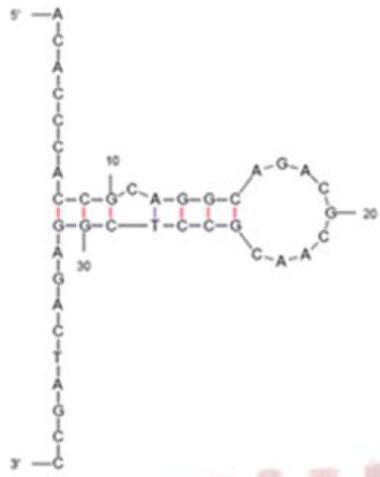


Figure 2-4 Single-stranded DNA aptamer developed by Lamont et al. for the capture and concentration of ricin toxin (E. a Lamont et al. 2011). The 40 residue aptamer shows a stem-and-loop configuration that is capable of binding the ricin molecule.

2.3.5 Official Methods of Detection

Currently there is no routine testing for ricin in the food supply; the only official testing methods for ricin are conducted by the CDC and/or members of the Laboratory Response Network (LRN) (Holtermann 2006). In the case that a sample is suspected for contamination by a bioterror agent, typically a hand held assay (HHA) is first used which is an immunologically-based method of detection. Although rapid, these tests are known for low sensitivity and high rate of false positives compared to other standard methods. For this reason, CDC and LRN do not consider HHAs as official methods. Despite this fact, many first responders still utilize such tests and presumptive positives are sent to the CDC for more rigorous confirmatory testing.

The CDC routinely conducts polymerase chain reaction (PCR) tests as well as time-resolved immunofluorescence (TRF) assays to detect ricin in environmental samples (Holtermann 2006). While these assays are both quite sensitive and do not have high rates of false positives, both TRF and PCR take a minimum of two hours to conduct and have expensive reagents (A. H. Peruski, Johnson, and Leonard Francis Peruski 2002). After the presence of ricin is confirmed, the activity/toxicity of ricin (ability to inactivate ribosomes) may sometimes be confirmed using cell-based kits (Audi et al. 2012).

Additionally, Kalb and Barr have reported the development of a mass spectrometry (MS) method which can quantify the activity of ricin by mixing ricin with an analog of its cellular target and detecting the product (Kalb and Barr 2009). Though very sensitive, MS requires very expensive lab equipment, requires experienced operators, and is not conducive to rapid analyses or high throughput. Ricin is not a typical

environmental contaminant and so the presence of active or inactive ricin would represent an act of bioterror. Therefore, a food processor would not necessarily care about the activity of ricin in a sample. For these reasons, MS, and other toxicity assays would not be feasible for the online detection of ricin in a food processing plant.

2.3.6 PCR/IPCR

Traditional polymerase chain reaction (PCR) technology is a very sensitive method of detection which can be used to amplify sections of DNA or RNA present in a sample in order to identify whether genetic material from a particular source is present. This type of assay has been utilized in the past and would be useful for detecting the addition of crude extracts of ricin which still contain genetic material from the castor plant to a sample. If any genetic material is present, primers will attach to the DNA and create a chain reaction which will make copies of the targeted regions. Repeated many times, the accumulation of these copies can be visualized using gel electrophoresis or sequenced and compared to a library for identification (Belitz, Grosch, and Schieberle 2004). Similarly, real time-PCR incorporates a fluorescent molecule into the DNA copies which allows for more rapid detection and the ability to roughly quantify the amount of starting genetic material in the sample (Holtermann 2006).

Limitations of traditional and real-time PCR methods is that they are based on the presence of a target's genetic material (DNA and/or RNA) and are therefore not true qualitative or quantitative measures of the ricin protein since there is not a constant relationship between the concentration of ricin toxin and ricin DNA in a sample. As

toxicity is not related to ricin DNA and requires only the ricin protein to be present, these methods would not detect purified preparations (Holtermann 2006).

The PCR method has since been hybridized with antibody-based methods giving rise to immuno-PCR (IPCR) which is not limited by the ability to detect only genetic material. Similar to ELISA, an anti-ricin antibody is used to concentrate ricin from a sample, however the reporter molecule is a strand of DNA which is attached to the antibody and is then targeted and amplified using PCR methods. The use of PCR as the reporter system can increase the sensitivity of ELISA by as much as 10,000 fold in buffer and human serum (Lubelli et al. 2006). Additionally, IPCR has been utilized for the detection of ricin in foods with limits of detection of 10 fg/ml in PBS buffer, 10 pg/ml in liquid egg and milk matrices, and 100 pg/ml in ground beef extract (X. He et al. 2010). The limitation of IPCR to other immuno-based methods is the relatively long assay time due to PCR, large consumption of reagents, and high reagent cost.

Overall, antibody-based methods of detection are robust methods for the detection of ricin in food. However, reliance on an antibody may also limit the conditions which may be used during sample preparation since the antibody-antigen complex can be drastically altered by certain extrinsic factors such as temperature, pH, and salinity. Proteins also have been shown to denature on contact with surfaces which can impact antibody binding behavior and limit the applications of antibodies. Also, due to the relative instability of antibodies to degradation over time, commercial test kits have a short shelf life and are prone to denaturation. For this reason, assays utilizing antibodies are rarely reusable. Lastly, it has been shown that other castor proteins, such as castor

agglutinin, may show cross reactivity with anti-ricin antibodies thereby allowing for false positives or cause errors in quantification (Garber 2008).

2.3.7 Raman Spectroscopy

The phenomenon of Raman scattering was first predicted in 1923, and observed in 1928 by Indian physicist C.V. Raman who conducted a simple experiment using sunlight passing through light filters (C. V Raman and Krishnan K 1928). In 1930, Raman was awarded the Nobel Prize in Physics and the scattering effect named after him was later developed into a sensitive detection method. Raman spectroscopy is a mode of vibrational spectroscopy where a sample is exposed to a monochromatic laser and a spectrum of scattered light is collected (C. L. Haynes, McFarland, and Van Duyne 2005). Raman scattering takes place when photons scattered by the sample are a different frequency from the incident beam. The change in frequency is due to energy being transferred from the photons to the sample molecules (Stokes scattering) and yield photons of a lower energy, or energy transferred from the sample to the photons (anti-Stokes scattering) yielding higher energy levels. The resultant Raman spectra diagram the symmetrical Stokes and anti-Stokes scattering, however due to thermodynamic principles, the anti-Stokes spectra have lower intensity and are typically not shown.

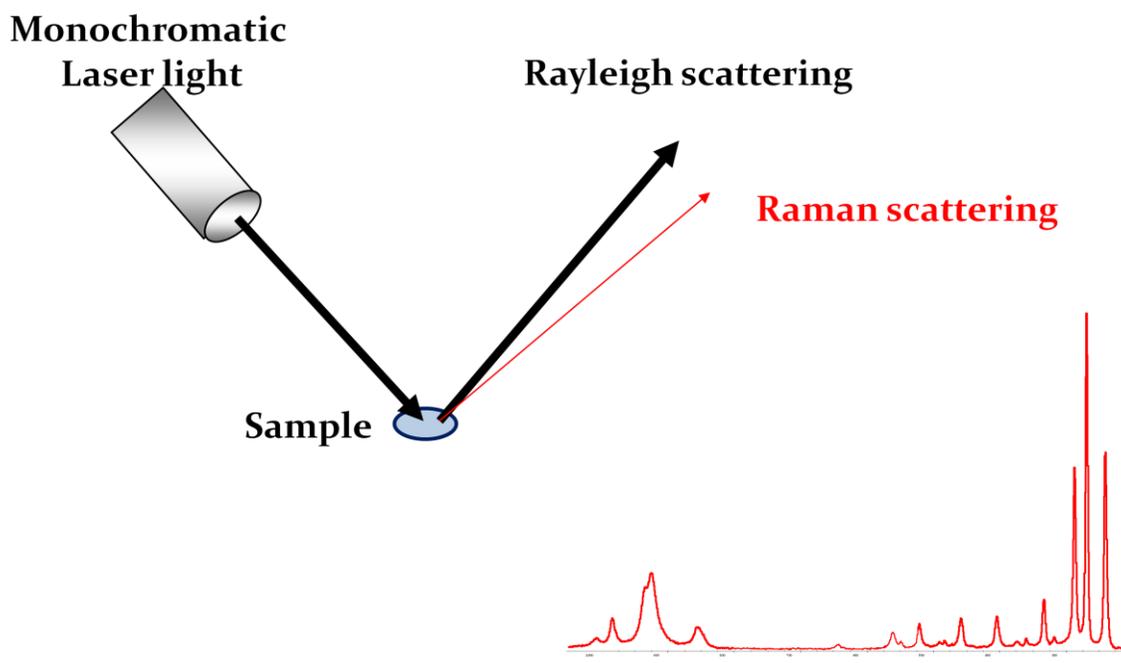


Figure 2-5 Schematic of Raman scattering and sample spectrum showing shift from the baseline, which equals the frequency of the incident beam.

Raman spectra contain a large amount of data related to the structures of molecules present in the sample. Therefore, molecules which have unique structures will produce unique spectra. Scattering is measured based on the magnitude of shifts in the wavelength of the incident beam and are dependent on Raman-active vibrations across covalent molecular bonds as well as the molecular environment of those bonds including secondary/tertiary structure, if applicable. Raman active bonds are those which experience fluctuations in the polarizability of the bond as the atoms become excited by electromagnetic radiation. In this way, Raman spectroscopy is complimentary to infrared spectroscopy (IR), which reports changes in the dipole moment of a bond/functional group as atoms are excited, as Raman-active bonds are typically IR-inactive, and vice versa. For this reason, Raman is a very attractive mode of detection for in situ biological

systems since water is essentially Raman ‘silent’, but will cause large problems in contaminated IR samples. Furthermore, because Raman scattering is not based on transmission of laser light, as in IR, it is not dependant on samples being optically clear. Benefitting from these criteria, Raman detection schemes are very conducive to rapid methods since very little sample preparation is required prior to detection.

The primary limitation of traditional Raman is that only a small fraction (10^{-6} - 10^{-9}) of photons will experience the shift in wavelength (inelastic scattering) with the vast majority scattering at the same wavelength as the incident beam (elastic/Rayleigh scattering) which contain no molecular information. Since 1928 Raman’s limitations have been greatly lessened with improvements in instrumentation, however the inherent weakness of Raman scattering still mean that traditional methods are mostly suitable where analytes are present in high concentrations. This would make Raman spectroscopy incompatible for detecting compounds which may be present in a sample at minute concentrations, and therefore useless for detecting bioterror agents in food which may be highly toxic at still undetectable levels.

2.4 Surface-Enhanced Raman Spectroscopy

2.4.1 Surface-enhanced Raman scattering phenomena and mechanisms

In 1977 it was found that the Raman scattering signal of samples which were placed on or near a roughened noble metal surface was greatly enhanced (Jeanmaire and Van Duyne 1977). The enhancement effect was first described in 1974, however it was not well understood (Fleischmann, Hendra, and McQuillan 1974). Even today, the

mechanism behind the enhancement is still not perfectly understood, however it is believed to be a combination of a chemical effect and an electromagnetic effect (C. L. Haynes, McFarland, and Van Duyne 2005). It is believed that when the nanoscale metal roughness features are excited by the laser, they produce strong, localized electromagnetic fields which dramatically increase the signal generated by the Raman-active bond. This effect has since become known as surface-enhanced Raman scattering (SERS), and has been utilized as a detection mechanism in a wide range of chemical and biological studies including pesticides (Weisenbacher et al. 1997), anthrax (X. Zhang et al. 2005), amino acids (Lin He, Natan, and Keating 2000), and bacteria (Jarvis et al. 2004). Zhao et al recently patented the use of SERS to “determine the presence, qualitatively, and/or quantitatively, and/or differentiating between one or more types of biomolecules, cells, toxins, drugs, viruses, bacteria, explosives, nuclear wastes, contaminants, biohazards, and other chemical and biological compounds of interest...in a sample” (Y. Zhao et al. 2011).

2.4.2 SERS enhancers/nanosubstrates

Although the signal-enhancing mechanism is not completely understood, it is largely agreed upon that the degree of enhancement is dependent on the size, shape, and regularity of the noble metal enhancer’s roughness features. Nanosubstrate enhancers produce characteristic electromagnetic fields surrounding the roughened surface of the metal; these fields effectively function as “enhancement zones.” Proximity of the target analyte to the nanometal substrate is critical to achieve enhancement with exponentially

larger enhancement factors being achievable with increasing proximity to the enhancer (Kennedy et al. 1999).

The magnitude of a substrate's enhancement zone is dependent on the nature of the substrate. It is critical that the material, size, shape, and pattern be uniform and well-characterized by scanning electron microscopy and/or transmission electron microscopy in order to maximize signal strength and guarantee reproducibility (C. L. Haynes and Van Duyne 2001). Substrates are typically fabricated from silver, gold or copper and are synthesized via a number of processes including electron beam lithography, colloid immobilization, chemical synthesis, and template-assisted methods (Culha et al. 2012; C. L. Haynes, McFarland, and Van Duyne 2005).

Synthesis of SERS nanosubstrates typically fall into two categories: "top-down" and "bottom-up" approaches. Generally, "top-down" approaches utilize electron beam lithography or other methods to etch a noble metal surface with a pre-determined pattern of roughness features which are specifically engineered to generate the largest and most intense enhancement zones (Culha et al. 2012). Due to the highly-ordered nature of top-down synthesis methods, they have been exploited to investigate the limits of SERS detection sensitivity, however they are typically very expensive to generate and require very complex instruments. For this reason, such substrates are rarely created by the end user and are very expensive.

Alternatively, "bottom-up" approaches often utilize chemical or electrostatic processes to randomly roughen the surface of a noble metal substrate or immobilize a colloid of metal nanostructures on a surface (Culha et al. 2012). In template-assisted

formats, nanostructures can be synthesized from other compounds and then coated with a noble enhancing metal, or a negative pattern can be placed on a surface, coated with metal, and then removed to leave a pattern or nanostructures. These approaches are much less expensive or tedious since they do not require the same complex instrumentation or expertise and can therefore be performed by the end user. Though randomly generated surfaces can be difficult to characterize and may therefore produce non-uniform or unknown enhancement factors than those created by lithographic means, “bottom-up” substrates are still capable of creating very large enhancement factors. For this reason, SERS substrates used in the detection and characterization of biological molecules have for the most part been synthesized using bottom-up approaches.

Bottom-up approaches were used in the first SERS-based detection methods before mechanisms of enhancement were well understood. However, now that SERS has its own field of science, a subsection of the field deals entirely with the creation and characterization of improved nanosubstrates which typically utilize top-down approaches for improved enhancement characteristics. Because substrates typically have limited stability and are not reusable, it is necessary for scientists to balance an assay’s cost vs. sensitivity before selecting a SERS substrate.

2.4.3 Silver Dendrites

Another method for synthesizing a SERS substrate is by the simple replacement reaction that spontaneously occurs between aqueous silver nitrate and zinc metal. During the reaction, zinc is exchanged in solution for silver which forms dendritic chains of

regularly-shaped crystals. Using field emission scanning electron microscopy, dendrite morphology is determined to be 2-5 μ m long, symmetrical chains of 50 nm diameter hexagonally-shaped nanoparticles (Fang et al. 2008). It was shown that dendrites have acceptable reproducibility, capable of reliably producing $>10^4$ enhancement factors and are compatible for excitation by lasers with frequencies between 600 and 800 nm (Lili He et al. 2010). Also, dendrites have at least a 6 month shelf-life and the reagents required to produce silver dendrites are very inexpensive. For these reasons, silver dendrites represent a very good SERS substrate that provide good enhancement, are inexpensive, easy to fabricate, and will last for a long time.

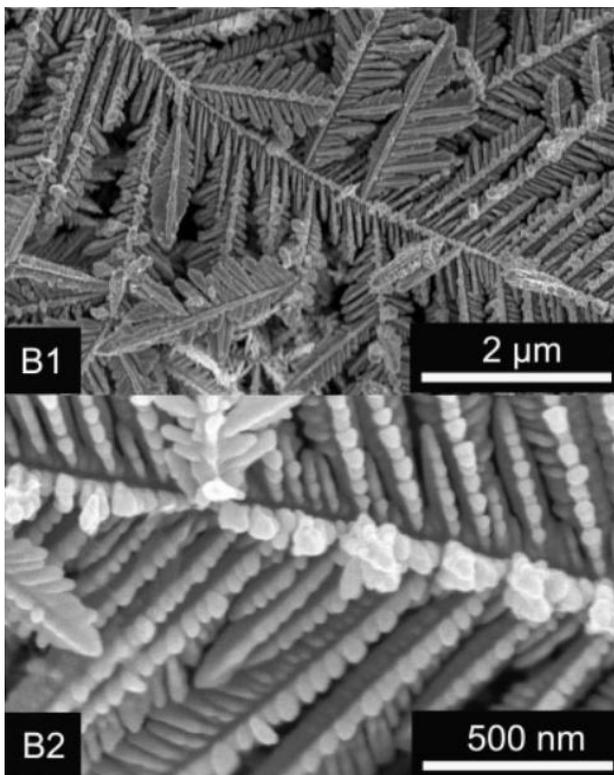


Figure 2-6 Scanning electron microscopic images of silver dendrites generated by the reaction of zinc metal in silver nitrate solution. Reprinted from (Lili He et al. 2010).

2.4.4 Applications of SERS

Applications of Raman-based methods have increased dramatically varying the target molecule, method of concentration, nanosubstrate, and format of SERS detection. SERS can be used to detect a target analyte both directly and indirectly. In the former, spectra generated by the Raman instrument will include peaks which are a result of the target compound's presence in the sample. Sample spectra may be compared to spectra of appropriate positive and negative controls, which may or may not be part of a spectral library/database. In many cases, negative control spectra will be generated which can be "subtracted" from sample spectra so as to not attribute spectral bands associated with the SERS substrate or other reagents to the sample. Subsequently, computer-aided spectral analysis software will focus on these peaks for determining the presence and/or concentration of the analyte. In indirect formats, the Raman spectra generated will include peaks from a Raman-active "reporter" molecule which is indicative of the presence of the analyte of interest. Indirect formats are especially useful when the compound does not generate a strong peak or the peak overlaps with peaks which are part of the sample matrix. Often times, weak peak signals are a result of very dilute analyte concentration or if the molecule does not have strong Raman activity.

SERS may be used as an indirect signal transduction scheme following immunocapture, either directly (where the generated SERS spectra are of the target analyte), or indirectly, where a "reporter" is detected (Cao, Jin, and Mirkin 2002; Doering and Nie 2003; Mulvaney et al. 2003). This type of assay consists of three parts:

capture/concentrating using antibodies, tagging with a SERS reporter (often via another antibody, as in the ELISA sandwich format), and detection using a Raman spectroscope.

Cao et al assembled a multiplex array which was able to detect more than one antigen at a time using an indirect SERS format where gold nanoparticles were detected signaling presence of various targets (Cao, Jin, and Mirkin 2002). In these cases, multiple antibodies to various antigens can be integrated into a single kit to create an all-in-one detection assay or to detect multiple biomarkers which may indicate and confirm a disease diagnosis. SERS is a robust detection method due to its extreme sensitivity with enhancement factors reported of $>10^8$ (Gunnarsson et al. 2001), and has even been shown to allow detection at the single-molecule level (K. Kneipp et al. 1997; Nie and Emory 1997).

Significant spectral differences between SERS may also be used as a signal transduction mechanism to indirectly detect capture events wherein highly Raman-active reporter molecules (“dyes”) may be hybridized onto capture probes such as antibodies or aptamers (Cao, Jin, and Mirkin 2002; Vo-Dinh, Allain, and Stokes 2002). Even with large enhancement factors, it is sometimes necessary to use spectral analysis software to help the user visualize differences between spectra, especially when detecting very dilute analytes. Examples of such analysis are generating 1st and 2nd derivative spectra, spectral smoothing, principal component analysis, hierarchical cluster analysis, and partial least squares analysis.

2.4.5 SERS-based detection methods in food

Although SERS-based techniques are relatively new, their sensitivity and flexibility have garnered a large amount of interest. Not surprisingly, many variations have been developed for a wide range of applications, however those pertaining to applications in food science are focused on here. In the past five years, SERS methods have been adapted for the detection of various target compounds in food. Though many papers study the potential of SERS to detect and differentiate various compounds and/or organisms in food, many are just feasibility studies using PBS and data is extrapolated to in-food detection. Applications of in-food detection include chemical contaminants in fish products (Lili He et al. 2008), melamine in chicken feed, gluten, cake, and noodles (M Lin et al. 2008), and later milk (X.-F. Zhang et al. 2010), and pathogens in spinach (Yuling Wang, Ravindranath, and Irudayaraj 2011), and egg protein in milk (Lili He, Rodda, et al. 2011).

He et al. developed a method of immunomagnetic separation combined with SERS in order to detect hen ovalbumin in milk at 1 $\mu\text{g/mL}$ (1 ppm) (Lili He, C. L. Haynes, et al. 2011). This method was developed for the detection of a protein in a liquid food system which contained other proteins, thus indicating that an allergenic protein from one source could be detected in another food. IMS is useful for such applications because the magnetic beads can be easily mixed into the liquid to capture and concentrate a target. The method utilized silver dendrites prepared on site as the nanosubstrate.

Two formats of SERS substrate were investigated: a substrate-based method, and a solution-based method. In the substrate-based method, silver dendrites are adhered to a

slide and dried, then a few uL of sample solution is placed on the silver spot and allowed to dry. This method was found to have the lowest limit of detection and therefore most appropriate for qualitative detection. In the solution-based method, silver dendrites suspended in buffer are mixed with the sample solution and then co-pipetted onto a slide and allowed to dry. This method, though possessing a lower LOD, could more accurately quantify the amount of protein in solution and would therefore be more useful in quantitative analyses.

The described IMS-SERS method would be very useful for the validation of cleaning and sanitation procedures during changeovers on food manufacturing lines which contain allergenic ingredients. This method for the detection of OVA is a proof-of-concept which could be easily adapted for the detection of other molecules by replacing the capture antibody. Subsequently, the same authors adapted the method to detect ricin toxin from liquid foods, showing that the method could be useful in detecting bioterror agents in food (Lili He, Rodda, et al. 2011). Both procedures directly detect the target molecule and quantify via peaks associated with the targets. Perhaps most importantly, these methods were developed to be rapid which was defined to completed within 20 minutes. This time frame was targeted since milk is currently held prior to centralization for approximately 20 minutes for detection of antibiotics.

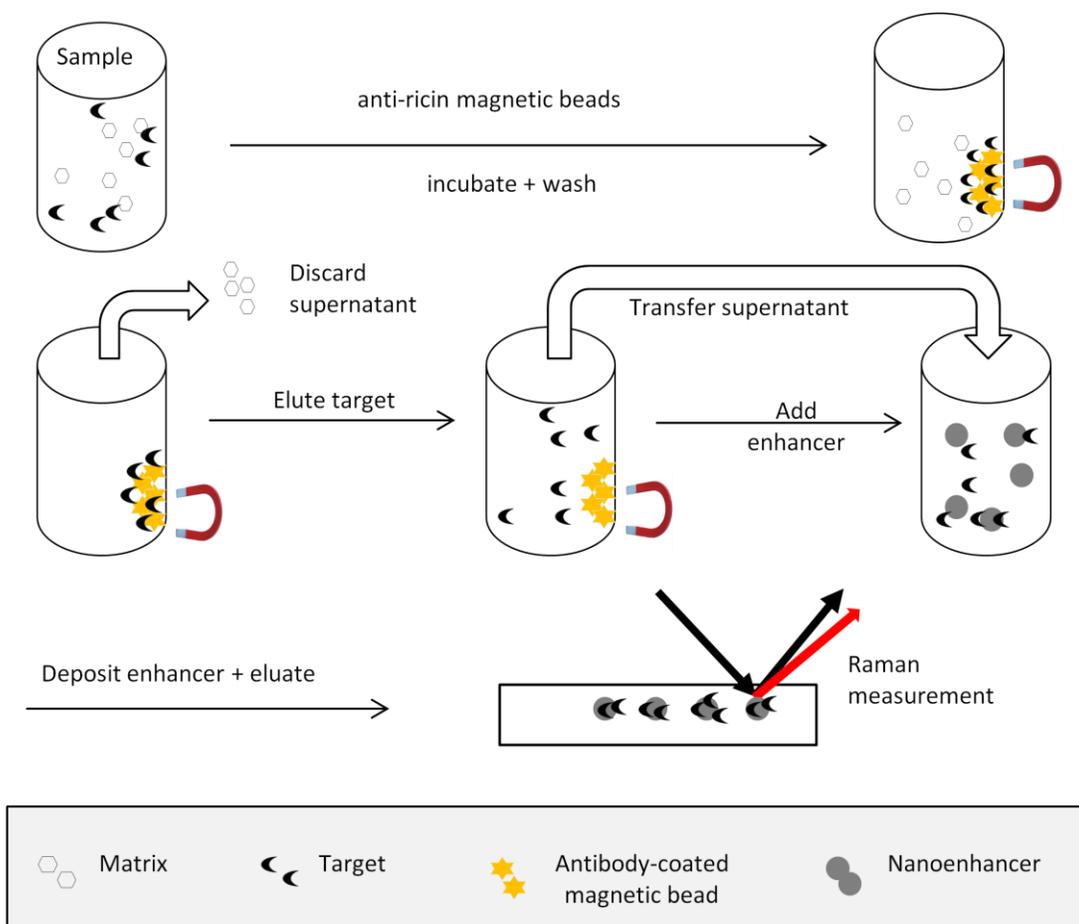


Figure 2-7 Schematic diagram of the immunomagnetic separation procedure combined with solution-based SERS developed in He et al., 2011 (Lili He, C. L. Haynes, et al. 2011).

2.4.6 Pros and cons

As previously mentioned, methodologies utilizing Raman spectroscopy are very useful for *in situ* study of biological molecules due to the compatibility of Raman with rapid methods. The drawback of Raman is that scattering is weak and previously required the analyte to be present in high concentrations. The subsequent discovery of surface-

enhancement of the scattering intensity has opened the door to the development of SERS assays that are both rapid and sensitive.

Another barrier to overcome has been the cost of Raman-based methods. One drawback of SERS is the requirement of having a Raman spectroscope which may be prohibitively expensive and is too large to use outside of a laboratory setting. However, improvements to optical filters, detectors, and fiber optic technology has made smaller, inexpensive, hand-held devices which could be used in the field or in a food plant (Young et al. 2004). Additionally, improvement and maturation of the technologies necessary to fabricate inexpensive, robust nanometal enhancers has also lead to larger interest in SERS-based assays (Burda et al. 2005).

The challenges of SERS methods remain in the signal-to-noise ratio of detecting dilute analytes in complex matrices. Complex biological matrices, such as food, will typically contain many different classes of molecules including nucleic acids, lipids, proteins, carbohydrates, minerals, trace elements and other contaminants. Not surprisingly, the Raman spectra of such a sample will yield many peaks which may mask peaks of interest. This can be especially problematic when the compound of interest is very similar to matrix compounds, such as detecting one protein in a matrix of other proteins.

Similarly, a Raman may have trouble distinguishing the spectra of a protein antibody used in an immunocapture assay from the protein antigen it targets. For this reason development of SERS methods for detecting protein targets, such as ricin, using capture agents other than antibodies are promising. The present study, published in 2011

investigates the use of a DNA aptamer to detect ricin in liquid food (Lili He, E. Lamont, et al. 2011).

Lastly, SERS is a surface based detection, where only the signal of molecular bonds present in the relatively narrow enhancement zone of the nanosubstrate will be enhanced. Therefore, detection of larger targets, such as complex proteins, or whole cells/spores is limited to differences in expression of molecules on the surface of such targets as they are adsorbed onto the nanosubstrate.

Chapter 3 Immunomagnetic Separation coupled with SERS

3.1 Materials and Methods

3.1.1 Ricin and milk:

All preparations of intact ricin molecule were produced in a biosafety level II laboratory at the Department of Food Science and Nutrition at the University of Minnesota (St. Paul, MN). For this study, ricin toxin was purchased from Vector Laboratories (Burlingame, CA). Whole milk fortified with vitamin D was purchased from a local grocery store (Roundy's, Milwaukee, WI). Ricin toxin was spiked into 1 mL aliquots of milk in 1.5 mL eppendorf tubes to give final concentrations of 1, 2, 4, 6, 8, μg ricin/mL milk. Ricin was also spiked into phosphate-buffered saline (PBS) at 10 $\mu\text{g}/\text{mL}$ which served as a positive control. Milk and PBS without ricin were used as negative controls for the study.

3.1.2 Preparation of Beads:

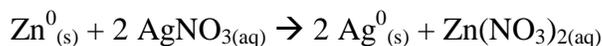
Immunoprecipitation kits were purchased from Invitrogen (Carlsbad, CA). Kits contain 2 mL of 30 mg/mL superparamagnetic polymer beads. Beads ($2.8 \pm 0.2 \mu\text{m}$ in diameter) come in phosphate buffered saline (PBS), pH 7.4, containing 0.1% Tween®-20 and 0.02% sodium azide (NaN_3) and are covalently bound with recombinant streptococcal protein G. Protein G is able to strongly link antibodies to the magnetic beads for use in immunomagnetic separations.

A polyclonal goat antibody to *Ricinus communis* agglutinin I and II was purchased from Vector Laboratories (Burlingame, CA). Antibody was conjugated to

magnetic beads according to the manual: Beads were completely resuspended through agitation for 5 minutes. Then, 50 μL of the provided bead-protein G preparation was separated from the supernatant on a magnetic rack, mixed with 200 μL binding buffer and 5 μg of antibody. This mix was incubated with rotation for 10 minutes at room temperature. Bead complexes were washed with 1 mL aliquots of binding buffer and washing buffer in order to remove excess antibody. These beads can be stored under refrigerated conditions until ready for use.

3.1.3 Preparation of Silver Dendrites:

Silver dendrites were prepared through a simple chemical replacement reaction between zinc metal and aqueous silver nitrate:



200 mM silver nitrate solution was prepared by dissolving AgNO_3 (Fisher Scientific, Rochester, NY) in distilled, deionized (DDI) water. A zinc metal sheet (Fisher Scientific, Rochester, NY) was cut using shears into a strip that was approximately 1 cm by 4 cm. The zinc strip was cleaned in dilute hydrochloric acid ($\sim 0.02\text{M}$) to remove surface contamination/oxidation, rinsed in DDI water, and then dried in a stream of room temperature air. The strip was then held with tweezers and immersed in the silver nitrate solution. In the replacement reaction, silver metal begins to form dendritic chains of crystalline morphologies which are loosely adhered to the surface of the zinc strip. After

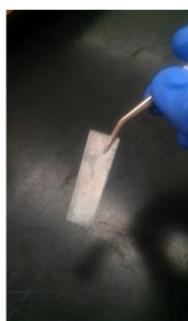
60 seconds, the strip was carefully removed and silver dendrites adhering to the surface were gently scraped off of the zinc strip into a clean glass vial. Dendrites were washed multiple times with DDI water to remove excess Zn^{2+} , Ag^+ and NO_3^- ions. They were washed by adding DDI water and vortexing for 60 seconds, then allowing the dendrites to settle to the bottom of the vial (~5 min), and removing the supernatant. After the final wash, silver dendrites were suspended in DDI water and could be stored at room temperature until ready to use (up to 6 months).



1. Cut zinc



2. Clean in HCL



3. Rinse with water and dry



4. Dip in $AgNO_3$



5. Remove AgD from strip



6. Wash with DDI water



7. Centrifuge AgD to a pellet



8. Store for use

Figure 3-1 Schematic diagram detailing preparation of silver dendrites (AgD) which will be used as a nanoenhancer in this study.

3.1.4 IMS Procedure:

Ricin toxin was captured out of spiked samples by rotating 50 μg of the antibody-coated beads in 1 mL aliquots of sample for 10 minutes at room temperature. After incubation/capture, beads were separated from the resonant solution using a magnetic separation rack (Millipore, Billerica, MA). After capture, the resonant solution was removed, and the bead-antibody-ricin complex was washed 3 times with washing buffer. Next, 20 μL of elution buffer was added to elute antibodies and ricin from the beads and allowed to incubate at room temperature for 2 minutes. Lastly, the tube was placed on the magnetic separation rack and the supernatant (eluate) was removed. This final IMS eluate was used for subsequent SERS sample preparation.

3.1.5 SERS Sample Preparation:

A solution-based method of SERS sample preparation was utilized (Figure 3-2). First, 20 μL of IMS eluate was mixed by gentle shaking with 5 μL ($\sim 20 \mu\text{g}$) of silver dendrites for 30 seconds. After shaking, the tube was left alone while the contents settled to the bottom of the tube; this could also be accelerated by centrifuging (6000 x g) for a few seconds. Next, 5 μL of the pellet was pipetted onto a standard glass microscopic slide (Fisher Scientific, Rochester, NY) and allowed to dry at room temperature (20-30 minutes). Once dry, the silver spots were ready for SERS spectral data collection.

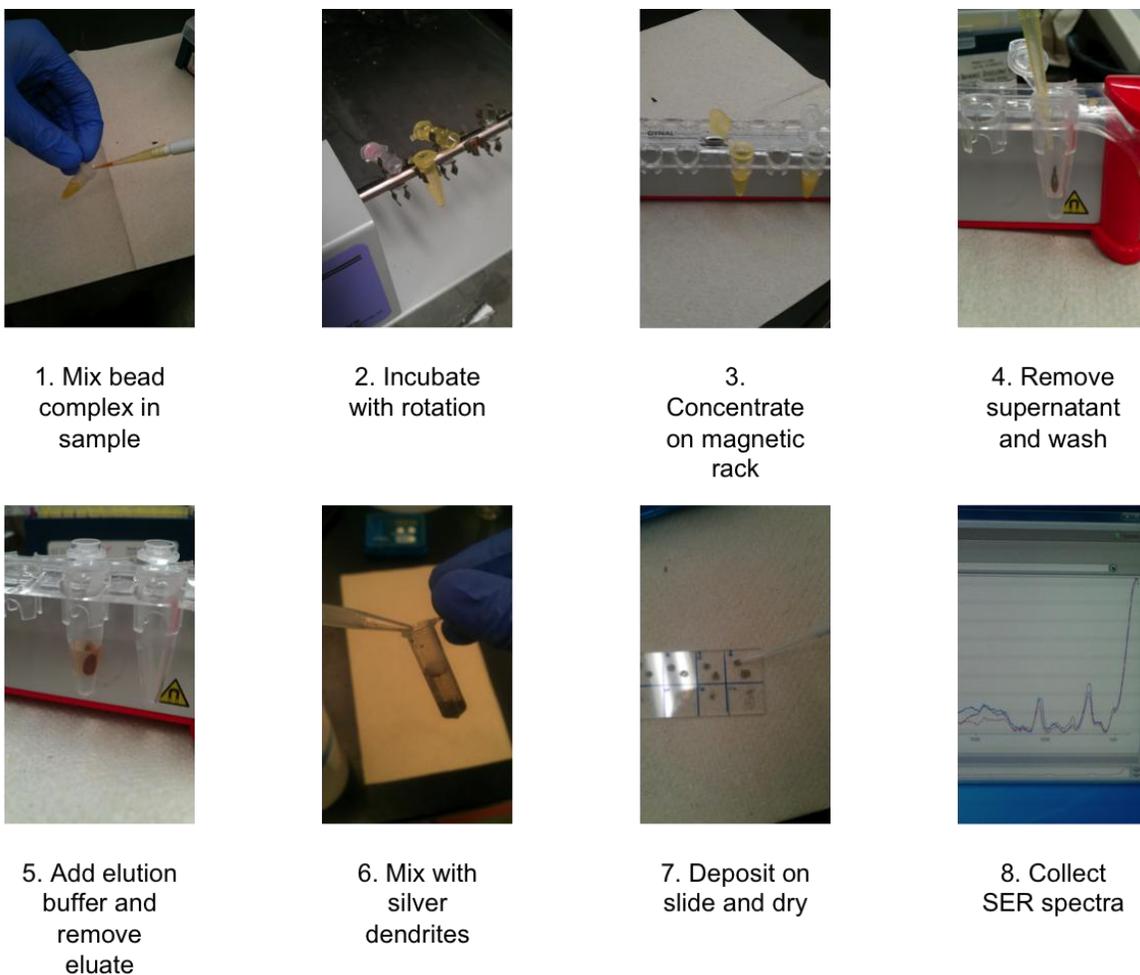


Figure 3-2 Schematic of IMS-SERS substrate preparation for spectral collection.

3.1.6 Raman Instrumentation

A DXR Raman microscope (Thermo Scientific, Waltham, MA) was used to collect spectra in this study. The instrument includes three excitation laser frequencies (532, 633, 780 nm) and a confocal microscope with 5 objectives (4x, 10x, 20x, 50x, 100x). The microscope in this case was used to focus the instrument at the surface of the silver dendrite spots. The 780 nm laser with a 10x objective were used and the resulting laser spot was about 3 μm in diameter with a specific resolution of 5 cm^{-1} . The instrument

also allowed for control of the laser intensity and aperture size; in this study, measurements were taken with a 6mW laser and scattering was collected through a 25 μ m slit aperture. Scattering was collected for 15 seconds per spectrum, in quadruplicate. Data collection could be automated using the instrument's automatic stage and the Thermo Scientific OMNICTM software package. Locations were selected manually within each silver spot and then the software could direct the stage to each location and each silver spot on the glass slide. For this study, 8 locations were selected within each silver spot to minimize the variance due to potential surface irregularities of the silver spots or uneven coverage of the glass slide.

Additionally, a hand-held, portable InspectorRamanTM instrument (DeltaNu, Laramie, WY) was used. It used a 785 nm excitation laser and had a single 100x objective for visualizing the surface of the silver dendrites. The diameter of the laser spots was 35 μ m and the resolution is 10 cm^{-1} . All data (from both Raman instruments) were collected in the Stokes shift range of 200 to 2000 cm^{-1} using a 15s integration time in quadruplicate.



Figure 3-3 Top: Thermo Fisher Scientific's DXR Raman instrument; bottom: DeltaNU's portable Inspector Raman.

3.1.7 Spectral Data Collection and Analysis:

Data analysis of spectra collected from the DXR Raman was performed using the TQ Analyst (v8.0.2.97) software package (Thermo Fisher Scientific) which accompanied the instrument. Delight (v3.2.1) (D-squared Development, LaGrande, OR) was used to analyze data collected using the portable Raman. In both cases, principle component analysis (PCA) and partial least squares (PLS) analysis were performed to analyze/visualize differences between spectra. Prior to spectral analysis, the software was used to perform a small amount of spectral smoothing and the creation of 2nd derivative spectra in order to separate overlapping peaks and to remove baseline shifts.

In PCA, the software program plots spectra according to their variance, or difference score, at two different regions of the spectra. These regions can be selected manually, or the user can allow the computer to select the regions that yield the greatest differences. Difference scores may be changed until the resulting PCA plot yields the best resolution among test preparations. PCA analysis is also useful for determining and discarding outlier spectra.

PLS is a multivariate statistical model which helps to determine whether the spectra contain variance that corresponds quantitatively with varying concentrations of spiked analyte. In PLS analysis, spectra are input and manually assigned by the user to the correct analyte concentration. Then, the computer randomly divides the data points into a group of data points used to construct a calibration curve, and tests the strength of the resulting curve as if it didn't know the analyte concentration of the remaining samples. The root mean square error of calibration (RMSEC) is calculated and reported to the user as a metric to evaluate the robustness of the calibration model where RMSEC values close to 0 indicate a very good model.

3.2 Results

3.2.1 Qualitative Analysis

The eluate resulting from the immunomagnetic separation procedure was mixed with silver dendrites and surface-enhanced spectra were obtained. Figure 3-4 contains raw spectra of negative controls (PBS and milk without ricin), and positive control (ricin in PBS). Elution buffer is added to each sample during the IMS procedure to elute the

antibody and captured antigen from the beads; therefore, the EB spectrum functions as background.

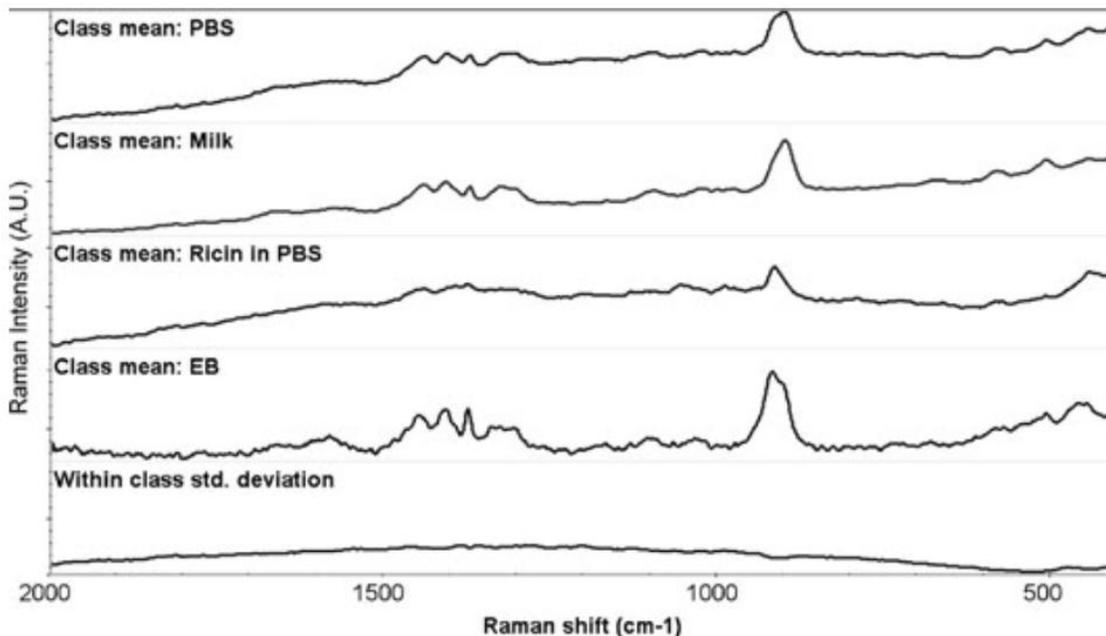


Figure 3-4 Stacked, raw SER spectra of positive and negative controls. Also background spectrum of elution buffer (EB). Figure adapted from He et al., 2011 (Lili He, Deen, et al. 2011)

The positive control spectrum demonstrated significant difference from the others, especially in the region 850 cm⁻¹ to 920 cm⁻¹; this result was confirmed in the PCA plot (Figure 3-5) which is able to clearly separate ricin from the negative controls. Because the data points representing the eluates from PBS and milk without ricin overlap, these two data sets are not significantly different.

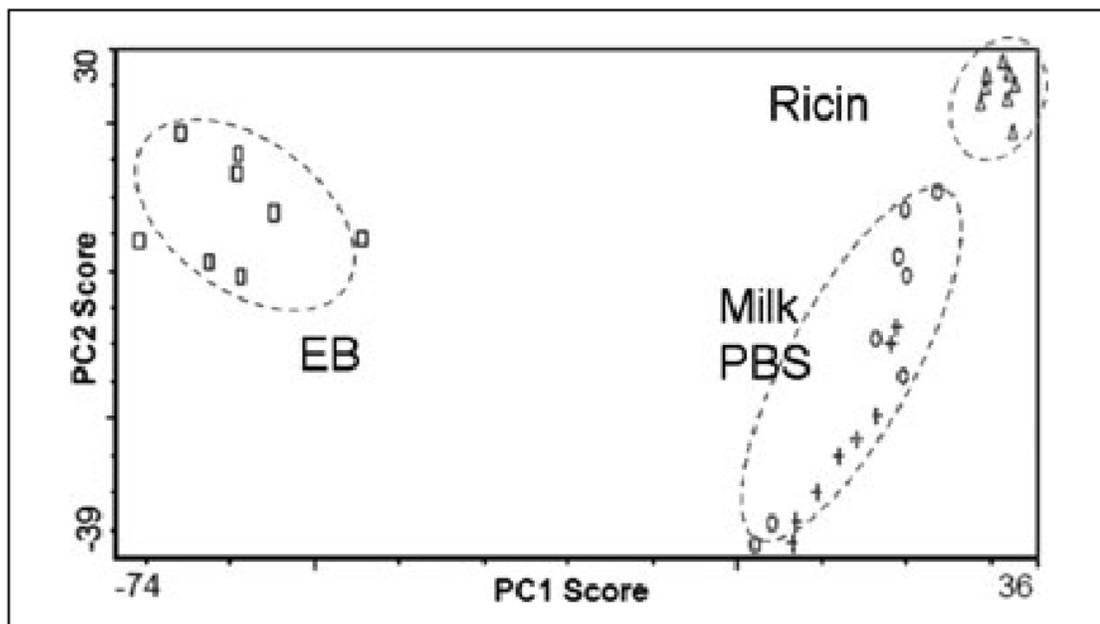


Figure 3-5 PCA plot of spectra collected from negative controls (PBS and milk), positive control (ricin), and elution buffer background (EB). Figure adapted from He et al., 2011 (Lili He, Deen, et al. 2011)

Additionally, the feasibility of the portable Raman instrument to detect ricin in milk was investigated. Figure 3-6 shows a PCA plot of an IMS-SERS procedure conducted on milk spiked with 4 $\mu\text{g}/\text{mL}$ ricin compared with milk without ricin. Though the clusters are less compact, denoting greater variability between spectra, there was clear separation of the data sets and therefore a significant difference. It should be noted that the total time, using previously prepared nanosubstrate coupled with antibody was approximately the same while using either the DXR or portable spectrometer (20-30min).

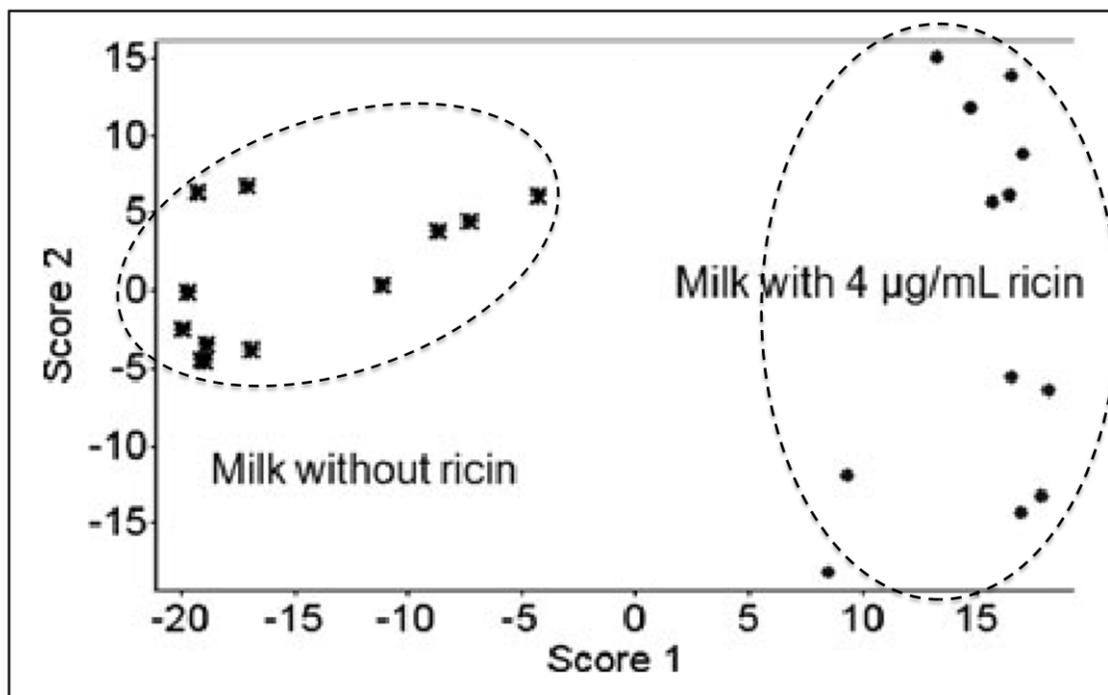


Figure 3-6 PCA plot of milk spiked with 4 $\mu\text{g}/\text{mL}$ and milk without ricin. This data was collected using the portable Inspector Raman instrument. Figure adapted from He et al., 2011 (Lili He, Deen, et al. 2011)

3.2.2 Quantitative Analysis

Varying concentrations of ricin were spiked into milk (1, 2, 4, 6, 8 $\mu\text{g}/\text{mL}$); again, milk without ricin was the negative control and ricin in PBS was the positive control. Stacked, second derivative spectra of these preparations are contained in Figure 3-7. The largest differences were found in the region around 900 cm^{-1} . Most notably, the peak at 900 cm^{-1} decreased in intensity while the peak at 915 cm^{-1} increased with increasing concentrations of ricin in milk.

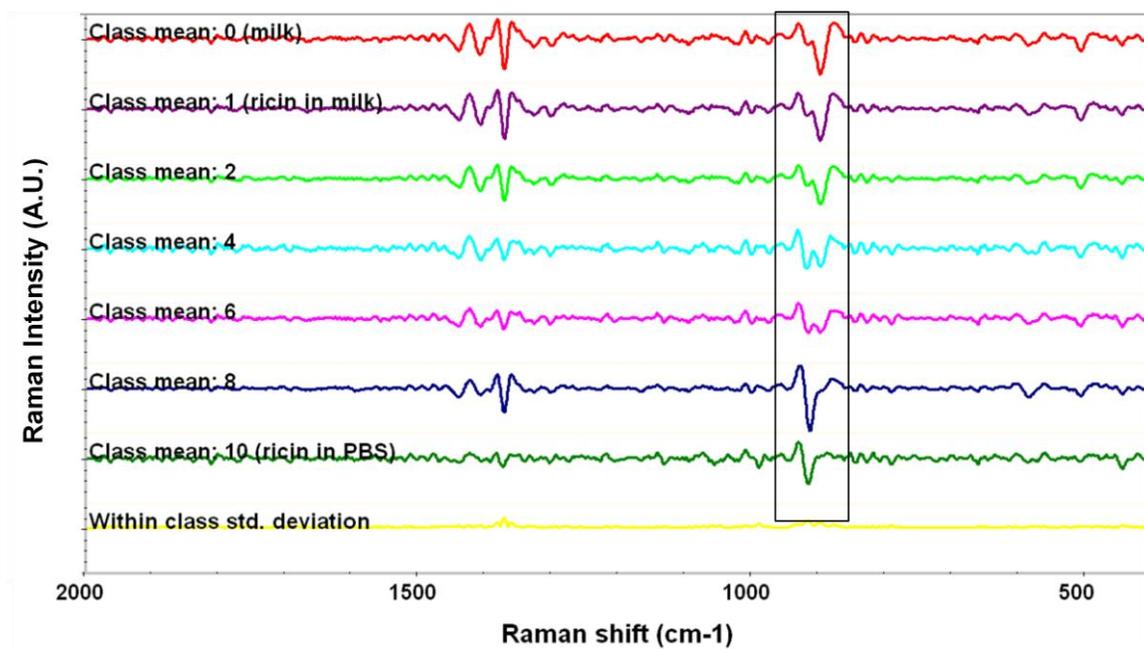


Figure 3-7 Stacked, second derivative SER spectra of increasing concentrations of ricin in milk. Figure adapted from He et al., 2011 (Lili He, Deen, et al. 2011)

Additionally, concentration dependant variability could be attributed to this relationship and principle component analysis and partial least squares analysis were performed to elucidate the differences. In the PCA plot (Figure 3-8), the negative control (0 $\mu\text{g/mL}$), 1, and 2 $\mu\text{g/mL}$ data sets were overlapping and therefore not significantly different. However, the 4, 6, 8 and 10 $\mu\text{g/mL}$ spectra were clustered separately from the negative control and were therefore significantly different. Because 4 $\mu\text{g/mL}$ was the lowest concentration of ricin in milk that was able to be differentiated from the negative control, this was the limit of detection.

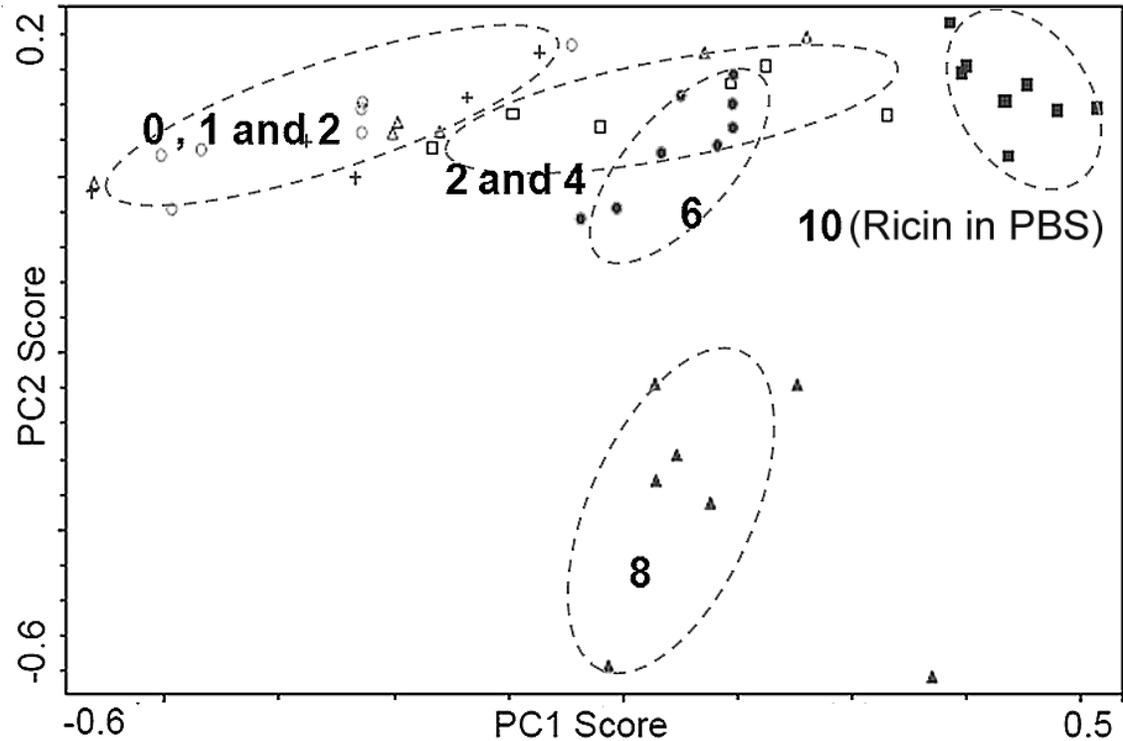


Figure 3-8 Principle component analysis plot of the varying concentrations of ricin in milk. 4 $\mu\text{g/mL}$ was the limit of detection for this method. Figure adapted from He et al., 2011 (Lili He, Deen, et al. 2011).

Partial least squares (PLS) analysis was performed to determine the ability of the method to quantify ricin present in the sample (Figure 3-9). With a high correlation coefficient (0.988) and a good RMSEC (0.538), the constructed model would be considered robust for predicting the amount of ricin present in a milk sample between 4 and 10 $\mu\text{g/mL}$.

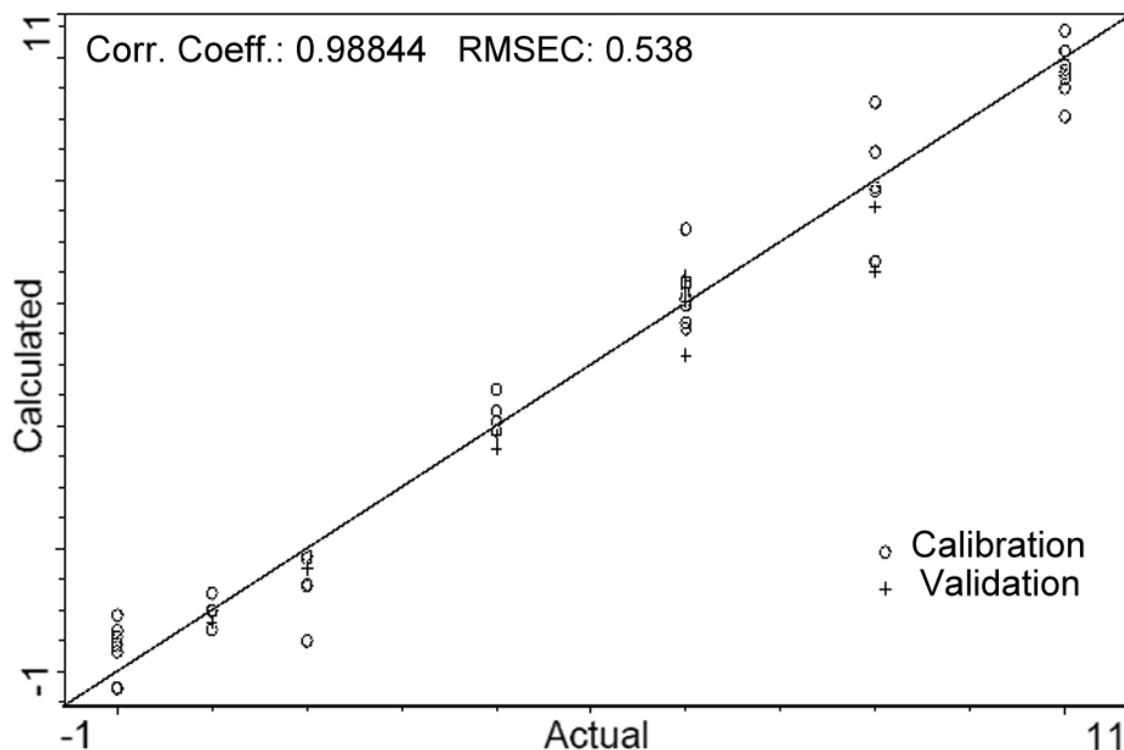


Figure 3-9 PLS plot used for determining the predictive ability of the procedure for quantifying the amount of ricin present in a sample. Figure adapted from He et al., 2011 (Lili He, Deen, et al. 2011)

3.3 Discussion

3.3.1 Qualitative Analysis

Peaks resulting from the presence of elution buffer were present in all IMS spectra and were considered background. The SERS spectra of negative controls (PBS and milk containing no ricin) were not significantly different. This implies that there is no, or low non-specific binding of milk constituents, such as protein and fat, to the magnetic beads or anti-ricin antibodies following magnetic separation. Since there is little non-specific binding, the assumption is that eluate from negative controls would contain

only elution buffer and the antibody and thus the two spectra would be identical and display peaks related to these two constituents. Because the antibody is a protein, peaks commonly associated with proteins, related to their common biochemical elements, are present in the negative control spectra.

The spectrum for the positive control (ricin in PBS) showed a significant difference compared to the negative control spectra, indicating that the antibody-coated magnetic beads are capable of concentrating ricin out of solution and that SERS spectra can distinguish the positive capture event. This means that in addition to the protein peaks present as a result of the antibody, additional peaks, or peak shifts as a result of ricin's presence yield significant difference for detection. A PCA plot helps to elucidate those differences since peak changes are minute in the raw spectra. Additionally, the fact that the data sets for PBS and milk are comingled in the PCA plot corroborates that the antibody is specific to ricin, even in the presence of milk protein and fat.

When detecting ricin at difference concentrations, a PCA plot shows no significant difference between milk without ricin and milk spiked at 1 and 2 $\mu\text{g/mL}$, indicating that these concentrations are below the limit of detection. This is likely due to the minute spectral differences between ricin protein and the protein antibody being too dilute to be significant. With greater concentration of ricin, ($>4 \mu\text{g/mL}$), a significant difference was found between these concentrations and the negative control. The significant difference is indicated by excellent separation of clusters on the PCA plot, and because 4 $\mu\text{g/mL}$ is the lowest concentration whose cluster is discrete from the negative control, this was found to be the limit of detection.

Similarly, SER spectra obtained from the portable Raman instrument at the same limit of detection as the benchtop DXR instrument were analyzed. Though the variability within data sets was much greater, the hand-held, portable Raman was capable of distinguishing between 4 $\mu\text{g}/\text{mL}$ and the negative control. Thus, the limit of detection is at least this level, showing that a SERS platform is useful for detecting ricin in a setting, such as a food plant or National border inspection station, where a large table-top instrument is not appropriate. Additionally, the cost of the portable instrument is a fraction of the large analytical instruments and is more likely to be affordable in such applications.

Overall, the limit of detection for either instrument is at least 4 $\mu\text{g}/\text{mL}$ in milk which is sufficient to render protection in the event of accidental or purposeful contamination. The scenario of a 20 Kg child drinking a pint (about 470 mL) of milk, assuming the most sensitive LD_{50} of 1 mg / Kg body weight, would require that the milk be contaminated at $>40 \mu\text{g}/\text{mL}$ in order to cause death.

3.3.2 Quantitative Analysis

Although it was found that ricin could be detected at sufficiently low concentrations to render protection in a contamination event, the present method was investigated for its ability to predict the level of contamination in a sample. SER spectra from the eluate of ricin spiked into milk at varying concentrations show concentration dependant variability and are therefore useful for building a quantitative model that can predict the concentration of ricin in a sample. A PLS plot of the collected data sets shows

that a good predictive model could be built from the data, the limit of quantification was assumed to be 4 $\mu\text{g/mL}$, and the range of quantification was from 4 to 8 $\mu\text{g/mL}$ in milk.

It is likely that the predictive range of this model is broader, however further investigation was not conducted since ricin is not allowed in foods at any detectable level; therefore being able to quantify ricin in a food sample is not important for online monitoring. This method was built for speed, knowingly trading sensitivity for duration and cost, to increase the likelihood that a food manufacturer would implement such a test into an existing quality monitoring plan. While other previously discussed methods, such as ELISA or mass spectrometry, may detect/quantify ricin much more sensitively, these methods require much greater cost, time, and/or expertise.

The present method represents a novel method for the rapid detection of ricin in food which does not require great expertise to perform, and is likely to be low cost. Additionally, the method could easily be implemented in a variety of settings outside the laboratory due to its flexibility and the availability of portable Raman instruments. Most importantly, the LOD is sufficient to ensure safety of a food product. Though the method is not compatible with quantitative analysis, a simple “yes/no” is all that is required since ricin detected at any level is considered a bioterror event.

Chapter 4 Aptamer-Conjugated Silver Dendrites coupled with SERS

4.1 Materials and Methods

4.1.1 Ricin B chain, Ricin, and milk/orange juice:

All preparations of ricin toxin were produced in a biosafety level II laboratory at the Department of Food Science and Nutrition at the University of Minnesota (St. Paul, MN). For this study, both the purified B subunit of ricin toxin (RTB), as well as the whole ricin toxin molecule (A + B chain connected by a disulfide bond) dissolved in phosphate-buffered saline (PBS) were purchased from Vector Laboratories (Burlingame, CA). Tropicana brand orange juice and whole milk fortified with vitamin D were purchased from a local grocery store (Roundy's, Milwaukee, WI). RTB was spiked into PBS to give final concentrations of 10, 25, 50, 100, and 200, and 500 ng/mL in a 2 mL eppendorf tube. RTB was also spiked into milk at 100 ng/mL and orange juice at 50 ng/mL. Whole ricin toxin was spiked into PBS at 25, 50, and 100 ng/mL. Inactivated ricin was prepared by placing a 2 mL eppendorf tube containing 1 mL of ricin in PBS (100 ng/mL) into a boiling water bath for 30 minutes. Whole molecule ricin toxin was also spiked into milk and orange juice which had been diluted 10x at 10, 25, and 50 ng/mL to functionally give concentrations of 0.1, 0.25 and 0.5 $\mu\text{g/mL}$ in the undiluted product. Orange juice was filtered through a 0.45 μm filter to remove pulp. PBS, milk, and orange juice without ricin were used as negative controls for the study. Varying concentrations of RTB and ricin in PBS and liquid foods were used to determine the limit of qualitative detection as well as the limit/range of quantification for ricin in these

liquids using SERS. Figure 4-1 shows the various RTB and ricin preparations in both PBS and liquid food used in this study.

Capture Agent	Antigen	Matrix	Concentration of Antigen in Matrix
Ag	RTB	PBS	0, 100 ng/mL
	RTB	Milk	0, 100 ng/mL
	RTB	Orange Juice	0, 50 ng/mL
	Ricin	PBS	100 ng/mL
	Inac. Ricin	PBS	100 ng/mL
Ag-Ap	RTB	PBS	0, 10, 25, 50, 100, 200, 500 ng/mL
	RTB	Milk	100 ng/mL
	RTB	Orange Juice	50 ng/mL
	Ricin	PBS	0, 25, 50, 100 ng/mL
	Inac. Ricin	PBS	100 ng/mL
	Ricin	Milk	0.1, 0.25, and 0.5 µg/mL
	Ricin	Orange Juice (filtered)	0.1, 0.25, and 0.5 µg/mL

Figure 4-1 Sample preparations based on concentration of antigen per mL of matrix and the capture agent used in the study

4.1.2 Preparation of Aptamers

The RTB aptamer was selected and characterized in the laboratory of Dr. Srinand Sreevatsan in the Departments of Veterinary Population Medicine and Veterinary and Biomedical Sciences, College of Veterinary Medicine, University of Minnesota. The aptamer was prepared by a modified SELEX process (Figure 4-2) where a vast library of

DNA oligomers are introduced to the RTB molecule and those that bind are separated and amplified. Over multiple generations, diversity of the aptamer pool diminishes as the weakly-binding species are eliminated and the species with the highest binding affinities to the target begin to dominate the pool. When only a few candidates remain, they are sequenced/characterized and the best aptamer is selected. Once the sequence is known, aptamers can be synthesized relatively easily and inexpensively.

The aptamer chosen for this study, deemed *SSRA1*, has been shown to be specific to the RTB molecule in multiple food matrices and was engineered to include a thiol group at its 5' terminus for conjugation to silver dendrites used in the study (E. a Lamont et al. 2011).

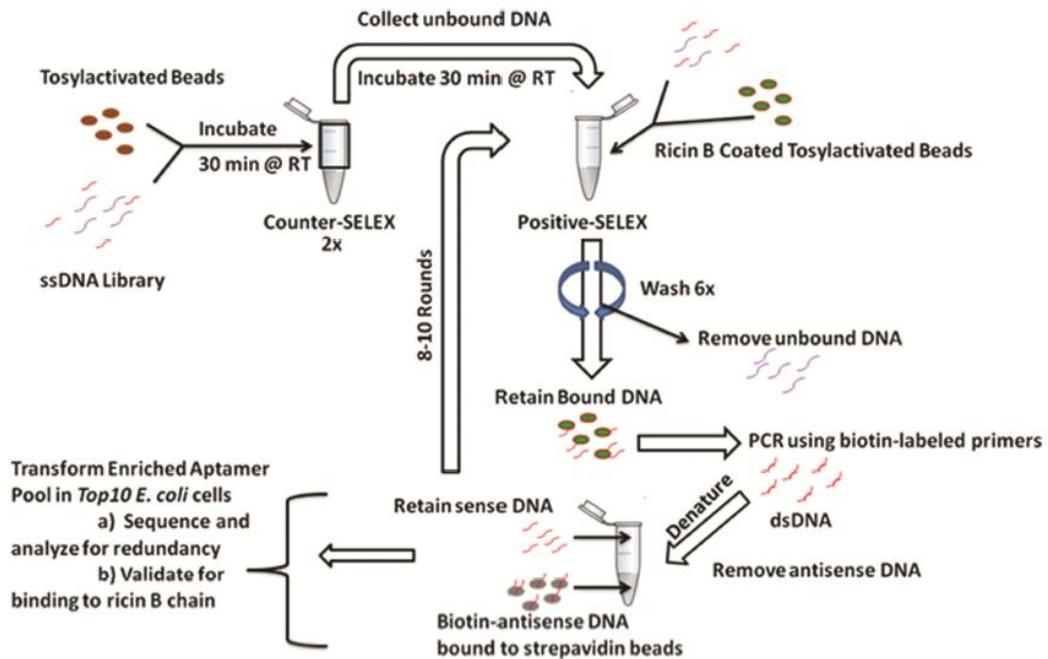


Figure 4-2 Modified SELEX process performed by the S. Sreevatsan Lab at the University of Minnesota to produce the SSRA1 aptamer specific for capture and concentration of RTB. Figure is reprinted from electronic supplementary information connected to Lamont et al., 2001 (E. a Lamont et al. 2011).

4.1.3 Preparation of Aptamer-Conjugated Silver Dendrites

Silver dendrites (AgD) were prepared as described in a previous section. Briefly, a clean, dry zinc metal strip was immersed in a 200 mM AgNO₃ solution for 60 seconds. Dendrites were gently collected from the surface and washed multiple times with distilled, deionized water.

Once prepared, 400 µg AgD were conjugated with constant agitation to 4 µM of 5' thiolated SSRA1 aptamer overnight at room temperature. During the incubation, the thiolated aptamers covalently bond to the surface of the silver dendrites' nanostructure to form aptamer-conjugated silver dendrites (Ag-Ap). After 24 hours, the tubes were centrifuged (6000 x g for 1 min) and the pellet was collected and washed with DDI water. Ag-Ap could be stored in refrigeration until needed.

4.1.4 SERS Sample Preparation:

A solution-based method of SERS sample preparation was utilized: first, 20 µL aliquots of either AgD or the Ag-Ap complex were mixed by gentle shaking for 10 minutes in 1 mL of sample. After capture, the tubes were centrifuged (6000 x g for 1 min) at room temperature and the pellet was collected and washed 3 times using DDI water. Lastly, the pellet was pipetted onto a standard glass microscopic slide (Fisher Scientific, Rochester, NY) and allowed to dry at room temperature (20-30 minutes). Once dry, the silver spots were ready for SERS spectral data collection. Spectral differences between pre- and post-capture spectra served as the basis of detection (Figure 4-3).

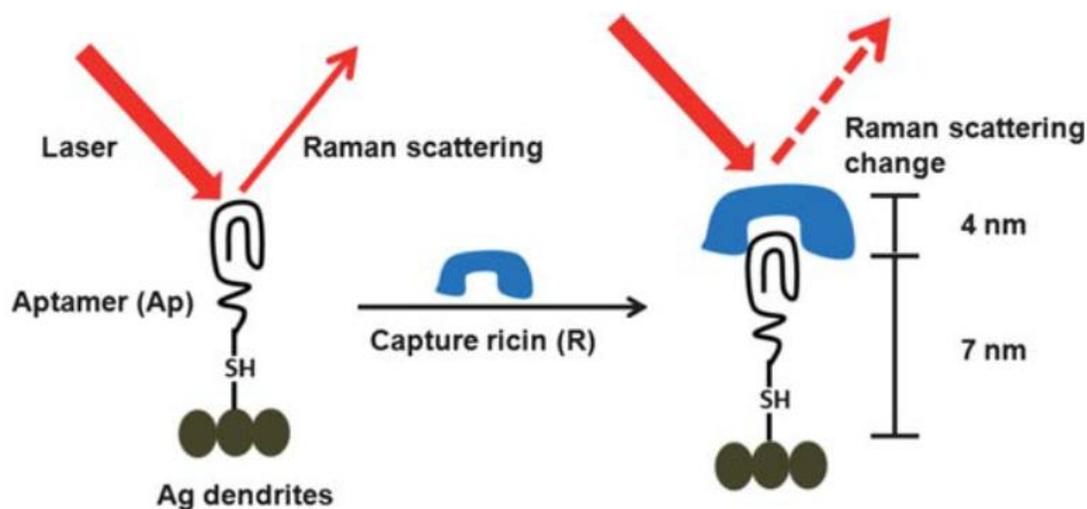


Figure 4-3 Schematic Diagram of the silver-aptamer complex capture method and subsequent SERS detection

4.1.5 Raman Instrumentation

The same DXR Raman microscope (Thermo Scientific, Waltham, MA) was used to collect spectra in this study. A 780nm excitation laser and confocal microscope were used to visualize and collect samples. In this study, measurements were taken with a 4 mW laser and scattering was collected through a 25 μ m slit aperture. Scattering was collected for 15 seconds per spectrum, in quadruplicate. Data collection could be automated using the instrument's automatic stage and the Thermo Scientific OMNICTM software package. Locations were selected manually within each silver spot and then the software could direct the stage to each location and each silver spot on the glass slide. For this study, 25 locations were selected within each silver spot to minimize the variance

due to potential surface irregularities of the silver spots or uneven coverage of the glass slide.

4.1.6 Spectral Data Collection and Analysis:

Data analysis of spectra collected from the DXR Raman was performed using the TQ Analyst (v8.0.2.97) software package (Thermo Fisher Scientific) which accompanied the instrument. Principle component analysis (PCA) and partial least squares (PLS) analysis were performed to analyze/visualize differences between spectra. Prior to spectral analysis, the software was used to perform a small amount of spectral smoothing and the creation 2nd derivative spectra in order to separate overlapping peaks and to remove baseline shifts.

4.2 Results

4.2.1 Ricin B Chain (RTB)

The aptamer-conjugated silver complex was assembled and the spectra in Figure 4-4 were collected and used as a basis for comparing subsequent sample spectra.

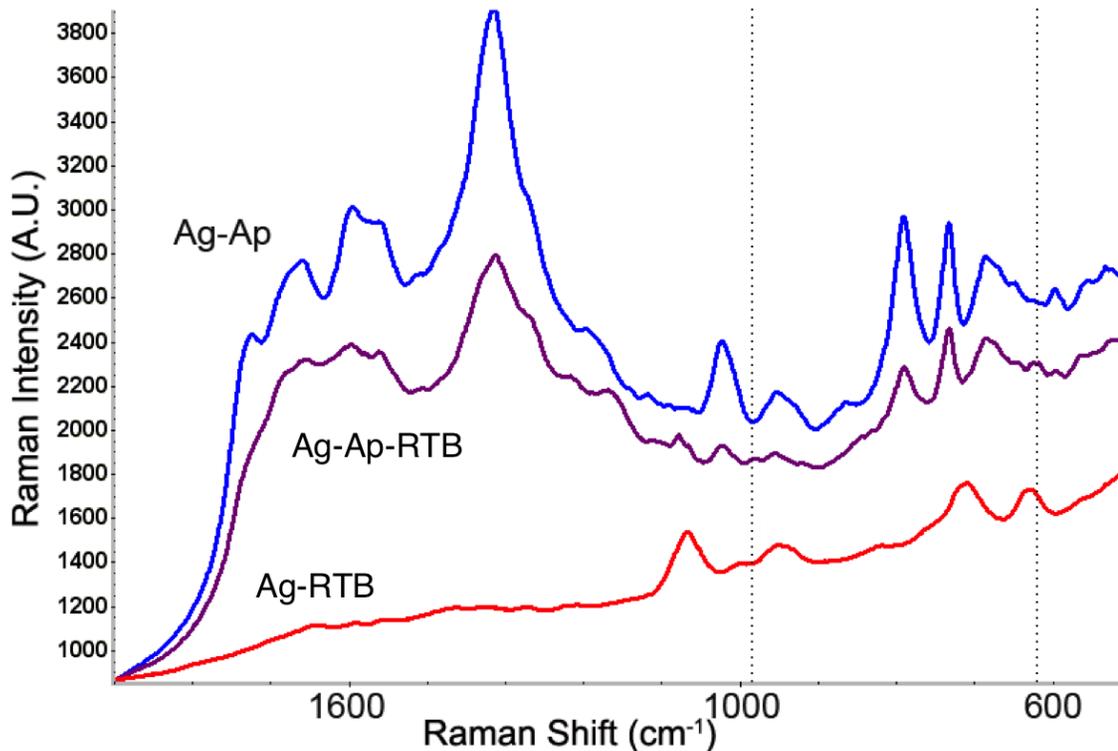


Figure 4-4 Raw SER spectra of aptamer-modified silver dendrites (Ag-Ap), as well as after capturing RTB (Ag-Ap-RTB). Additionally, RTB on silver dendrites has been included to show a ‘pure’ spectrum. Figure adapted from He et al., 2011 (Lili He, E. Lamont, et al. 2011).

The silver-aptamer spectra are similar, however there are two additional small peaks at 985 cm^{-1} and 621 cm^{-1} as a result of capturing RTB. These peaks are elucidated in Figures 4-5A and 4-5B which contain SER spectra focusing on the specified regions showing the additional peaks. To help clarify some of the differences, some spectral processing has been performed as well as creation of second derivative spectra. As a result, peaks of interest appear in the ‘downward’ direction.

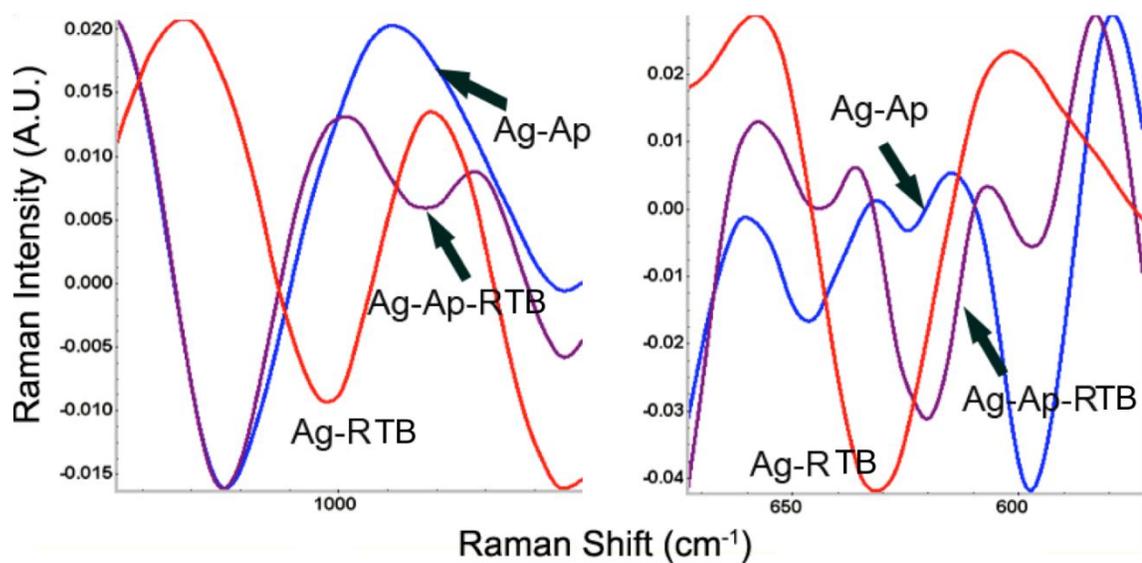


Figure 4-5 Left: (A) SER spectra detailing differences in the 985 cm^{-1} shift region; and Right: (B) the 621 cm^{-1} shift region. Figure adapted from He et al., 2011 (Lili He, E. Lamont, et al. 2011).

Since it was possible to find a significant difference between positive and negative spectra, RTB was spiked into PBS at 10, 25, 50, 100, 200, and 500 ng/mL and SER spectra were collected to determine the limit of detection. Figure 4-6 shows a PCA plot comparing differences between the spiked samples and the negative control. It was confirmed that the aptamer without RTB could be differentiated from the spectra with RTB, however the spectra of positive RTB samples all comingled and were therefore not significantly different between varying concentrations.

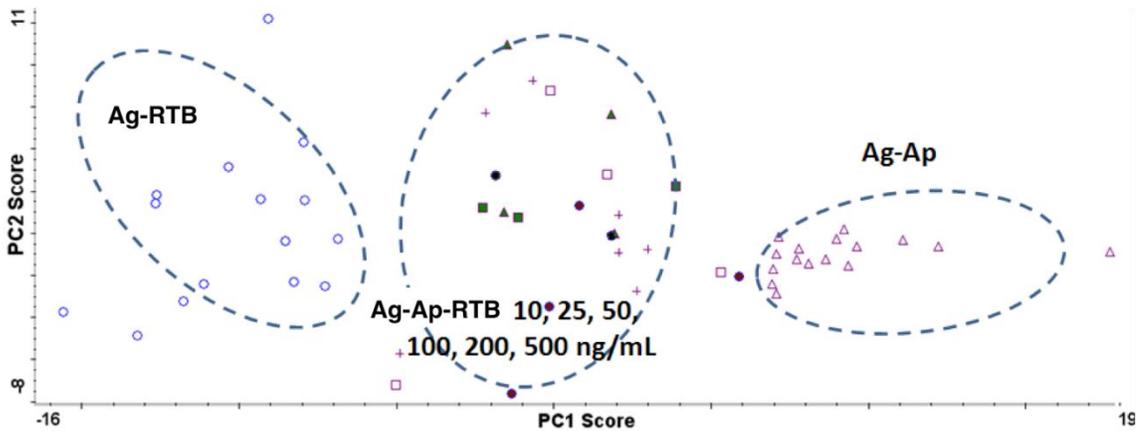


Figure 4-6 PCA plot comparing spectra obtained from RTB-spiked samples (Ag-Ap-RTB), and aptamer without RTB (Ag-Ap). Varying symbols represent different starting concentrations of RTB in PBS. Figure adapted from He et al., 2011 (Lili He, E. Lamont, et al. 2011).

A similar study was conducted with RTB spiked into milk and orange juice.

Figures 4-7A and B display PCA plots summarizing differences between test and negative controls. It was found that the limit of detection in orange juice was 50 ng/mL while the limit of detection in whole milk was 100 ng/mL. These concentrations represent the lowest concentrations of RTB spiked into milk and orange juice whose PCA data sets did not overlap with the negative controls, thus indicating significant difference.

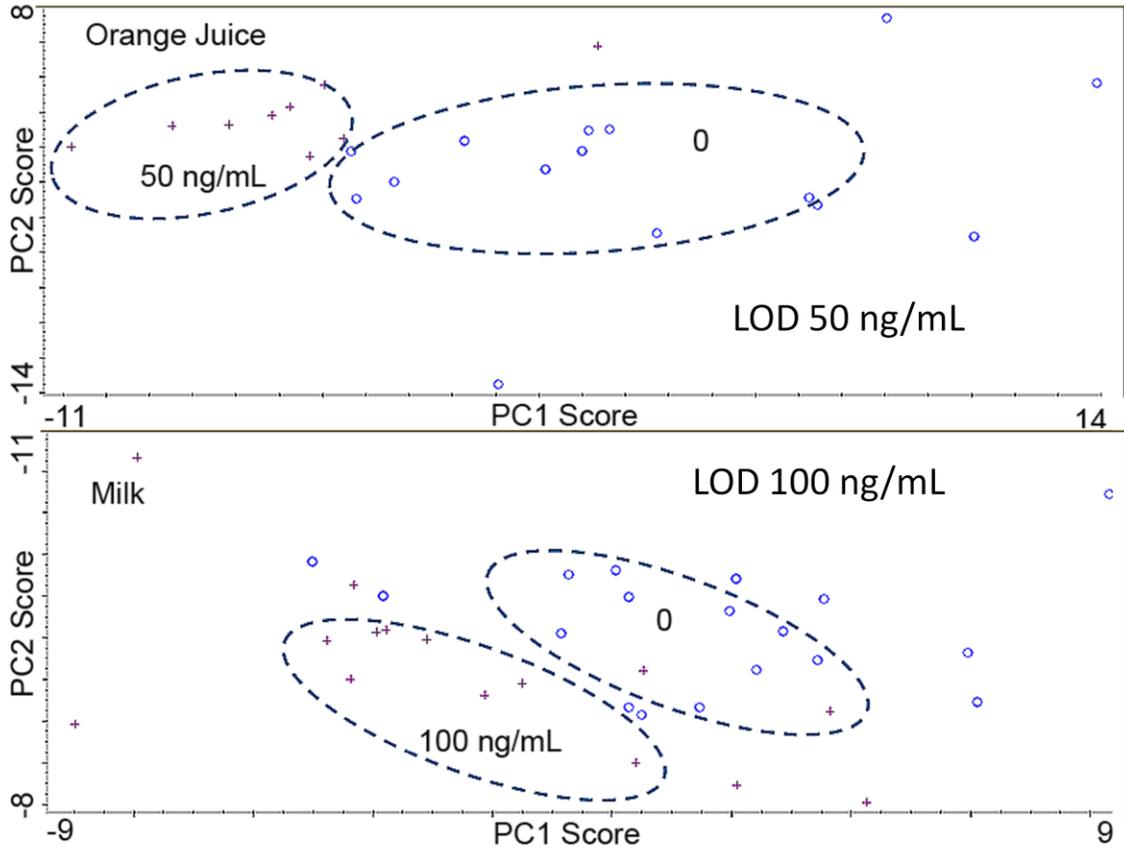


Figure 4-7 Top: (A) PCA plot comparing RTB captured from orange juice spiked at 50 ng/mL (crosses) to the negative control (circles). Bottom: (B) PCA plot of RTB in milk at 100 ng/mL (crosses) compared to the negative (circles). Figure adapted from He et al., 2011 (Lili He, E. Lamont, et al. 2011).

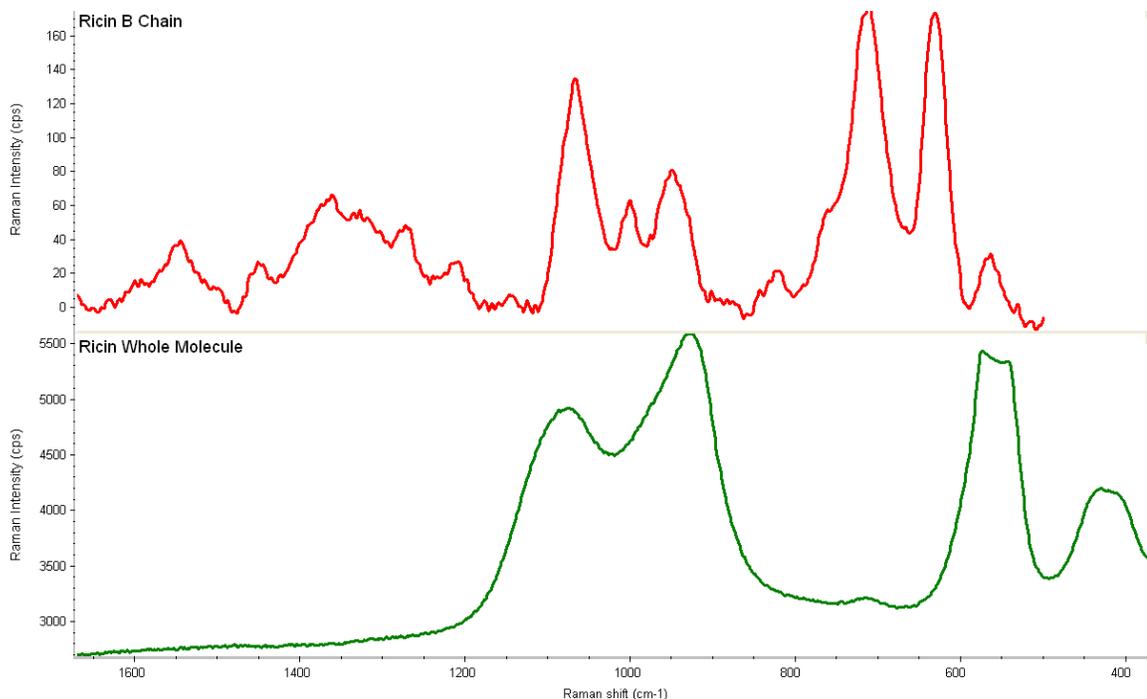


Figure 4-8 Raw SER spectra of pure RTB (top), and ricin toxin (bottom) directly conjugated onto silver dendrites.

4.2.2 Ricin toxin (A & B Chain)

SERS detection studies were subsequently confirmed using the *SSRA1* aptamer against intact ricin molecule (ricin A and B chains bound by a disulfide bond). Figure 4-8 shows the spectral differences between the purified RTB and the whole ricin molecule, most notably in the 500 cm^{-1} to 600 cm^{-1} region. These spectra were used as a basis for locating peaks resulting from ricin in sample spectra.

4.2.2.1 In PBS

After capture using aptamer-conjugated silver dendrites, SER spectra were collected of controls and sample preparations (Figure 4-9). The most important spectral differences between the Ag-Ap spectra and the Ag-Ap-R spectra occurred in the regions

near 1080 cm^{-1} , 920 cm^{-1} , and 550 cm^{-1} (boxed, Figure 4-6). These peaks corresponded with peaks present in the pure ricin preparation. A PCA plot confirms the method's ability to clearly distinguish between sample and controls preparations (Figure 4-10).

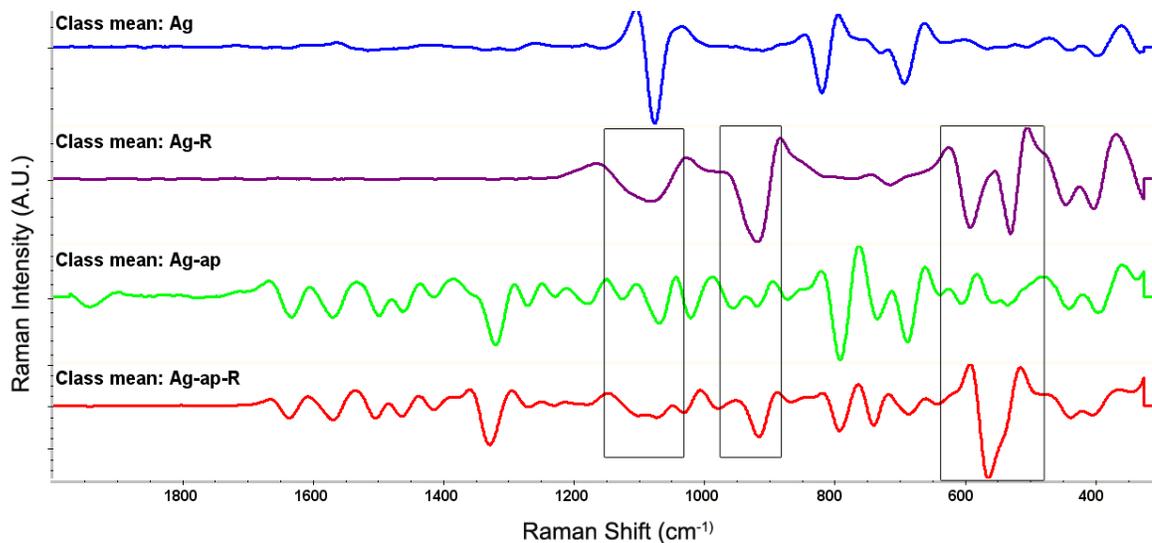


Figure 4-9 Stacked, SER spectra of controls and sample spectra collected using aptamer-conjugated silver dendrites (Ag-Ap). Bare silver (Ag) served as a background spectrum. Important spectral differences are boxed. Figure adapted from He et al., 2011 (Lili He, E. Lamont, et al. 2011).

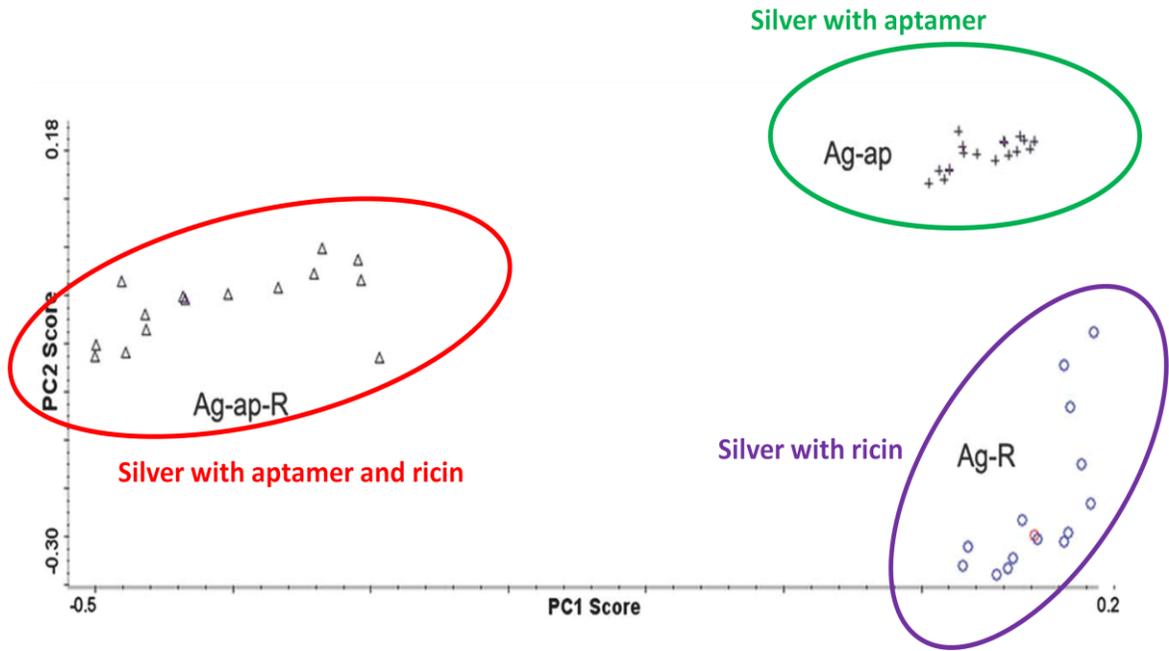


Figure 4-10 PCA plot showing clear separation of data sets. Separation denotes a significant difference. Figure adapted from He et al., 2011 (Lili He, E. Lamont, et al. 2011).

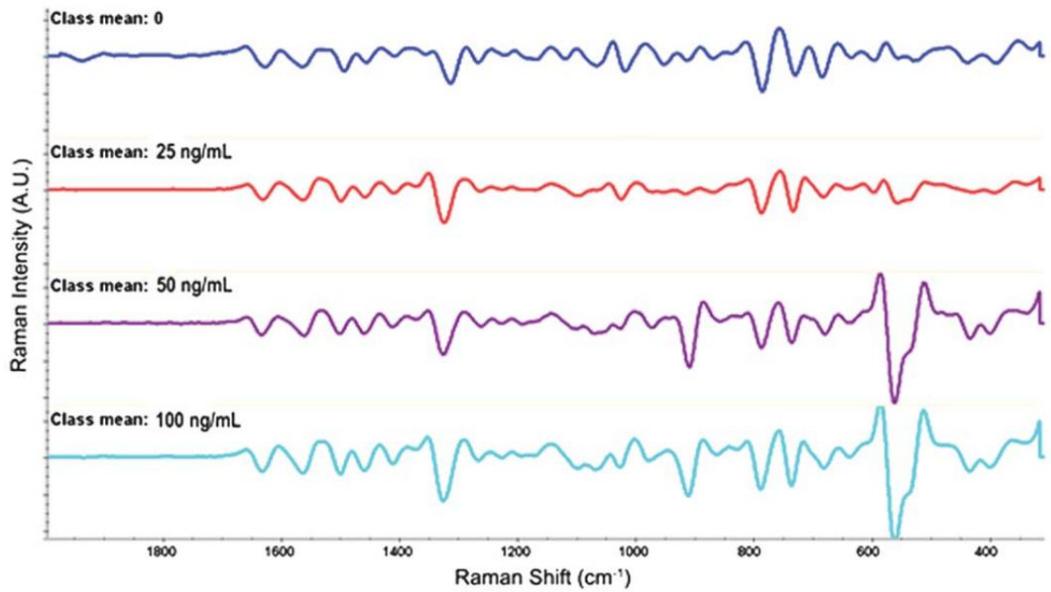


Figure 4-11 Stacked, second derivative SER spectra of aptamer-conjugated dendrites after capture from varying concentrations of ricin in PBS. Figure adapted from He et al., 2011 (Lili He, E. Lamont, et al. 2011)

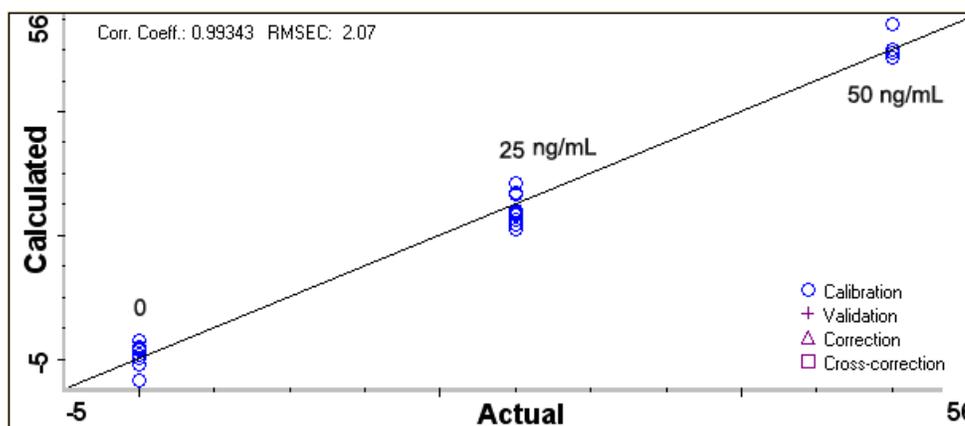


Figure 4-12 PLS plot of quantification study using varying concentrations of ricin in PBS. Range of quantification was 0 to 50 ng/mL; no significant spectral difference was found between 50 and 100 ng/mL. Figure adapted from He et al., 2011 (Lili He, E. Lamont, et al. 2011).

Next, SERS spectra were collected and from Ag-Ap which had been used to capture ricin which had been spiked in to PBS at varying concentrations (0, 25, 50, 100 ng/mL) (Figure 4-11). The limit of detection for this study was ≤ 25 ng/mL as this data set was significantly different from the negative control. Concentration dependant variation was found in the regions around 560 cm^{-1} , 920 cm^{-1} in the range of 0 to 50 ng/mL; no significant difference was found between 50 and 100 ng/mL. A PLS plot showing the strength of predictability was generated in the range of 0 to 50 ng/mL (Figure 4-9).

4.2.2.2 Native and Inactivated

Next, the ability of SERS and the present method to differentiate native (active) and denatured (inactive) ricin protein was investigated. Significant spectral differences were found between native and inactivated ricin which had been directly conjugated onto the surface of silver dendrites without the capture procedure (Figure 4-13). Then native

and denatured ricin were spiked into PBS, capture using Ag-Ap was performed, and SERS spectra were collected. Denatured ricin was captured by Ag-Ap since the spectrum for denatured ricin closely matched the spectrum for native ricin, and not the spectrum for bare Ag-Ap. However, significant spectra differences between the native and inactivated ricin preparations were lost, and therefore the present method cannot differentiate native from inactivated ricin. This fact was elucidated by a PCA plot (Figure 4-14) which had overlap among the native (Ag-Ap-R) and denatured (Ag-Ap-inR) ricin data sets. A significant difference was found between native and inactivated ricin directly conjugated onto silver dendrites, denoted by discrete clusters.

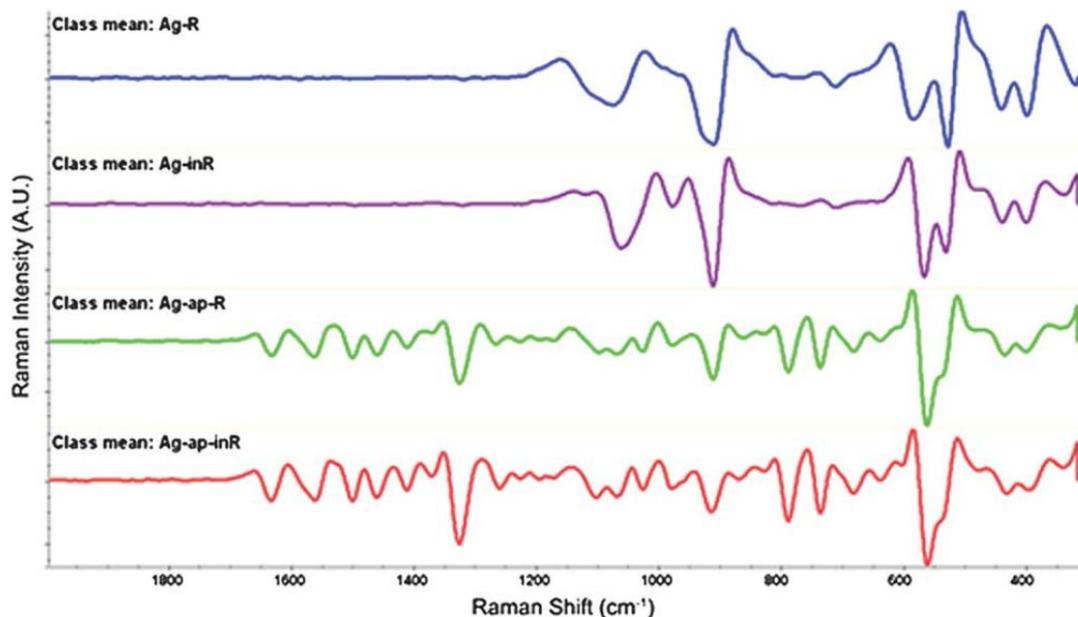


Figure 4-13 Stacked, second derivative SERS spectra of native and denatured (inactivated) ricin directly adsorbed onto silver dendrites (Ag-R, Ag-inR) as well as captured using aptamer (Ag-Ap-R, Ag-Ap-inR). Figure adapted from He et al., 2011 (Lili He, E. Lamont, et al. 2011).

4.2.2.3 In Liquid Food

SERS spectra were collected from orange juice samples which had been spiked with whole molecule ricin toxin. Figure 4-15 shows stacked, 2nd derivative spectra of ricin spiked into orange juice and 0-0.5 $\mu\text{g/mL}$. The most evident difference in the spectra is the appearance of a peak at 1100 cm^{-1} which is not present in the negative control. Figure 4-16 shows this peak in greater detail showing the concentration dependant variability of the peak, where the amplitude increases with increasing concentration of ricin in the sample. A PCA plot of the data sets indicates that each of the 4 preparations is significantly different as they all form discrete clusters from each other (Figure 4-17). The limit of detection of this platform in orange juice is $<10\text{ ng/mL}$ since this is the lowest concentration tested which was distinguishable from the negative control. This corresponds with a LOD of $<0.1\ \mu\text{g/mL}$ concentration in undiluted orange juice.

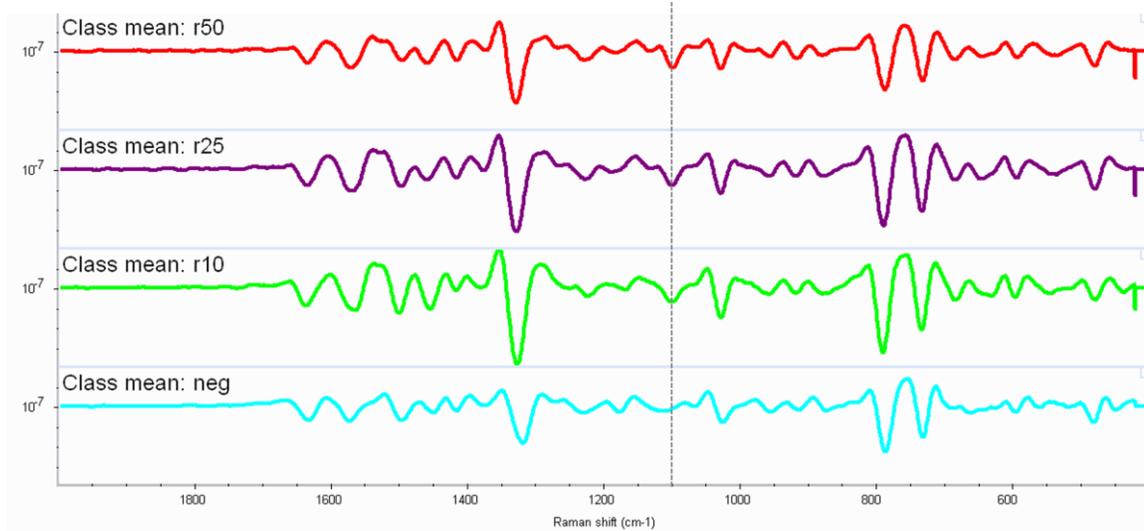


Figure 4-14 Stacked, 2nd derivative SER spectra of whole ricin toxin spiked into orange juice samples. Significant differences associated with the presence of ricin were found in the peak located at 1100 cm⁻¹ (indicated).

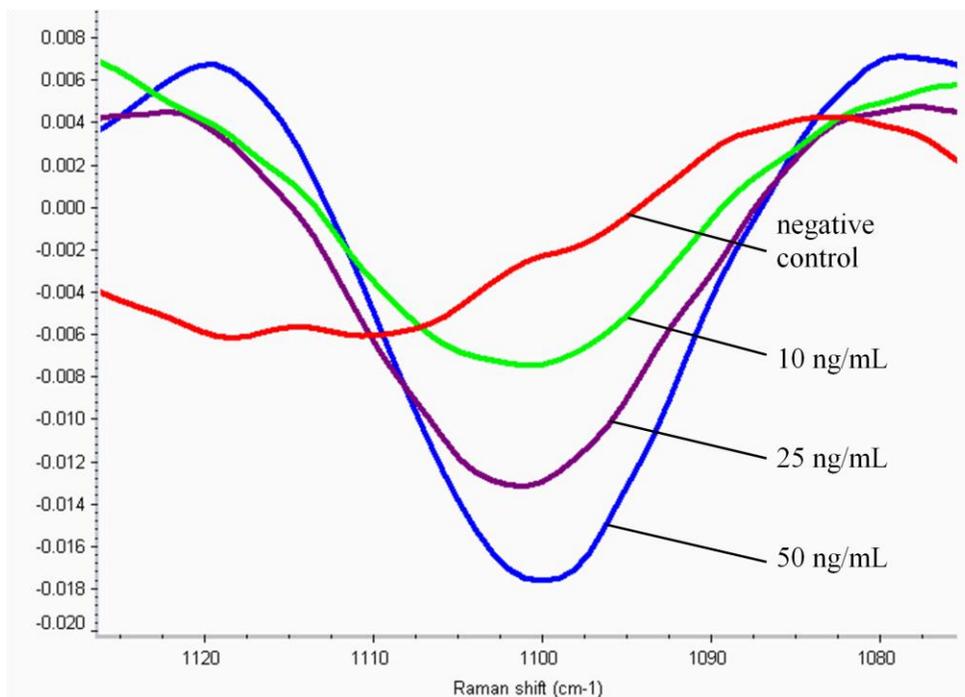


Figure 4-15 Focused view of overlaid 2nd derivative spectra surrounding the peak at 1100 cm⁻¹.

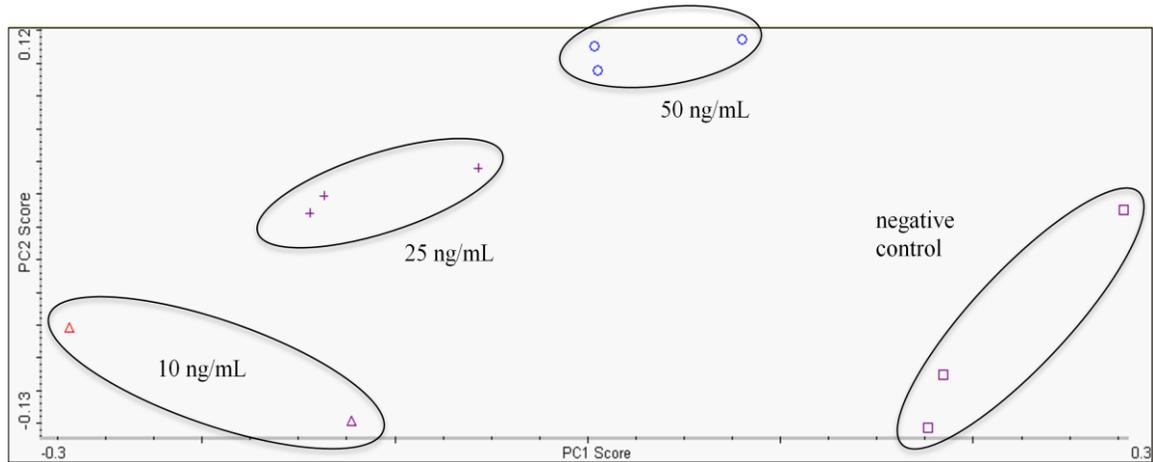


Figure 4-16 PCA plot of spectra collected from orange juice spiked with varying concentrations of whole ricin toxin.

Additionally, a PLS plot indicates the ability of the platform to predict the unknown concentration of a sample (Figure 4-18). The peak at 1100 cm^{-1} was used to create the standard curve as it was resulting from ricin in the sample and showed concentration dependent variability. A strong model was created which had a correlation coefficient = 0.97, and a dynamic range of 10-50 ng/mL. This model could be used to predict the concentration of ricin in an orange juice sample between 0.1-0.5 $\mu\text{g/mL}$.

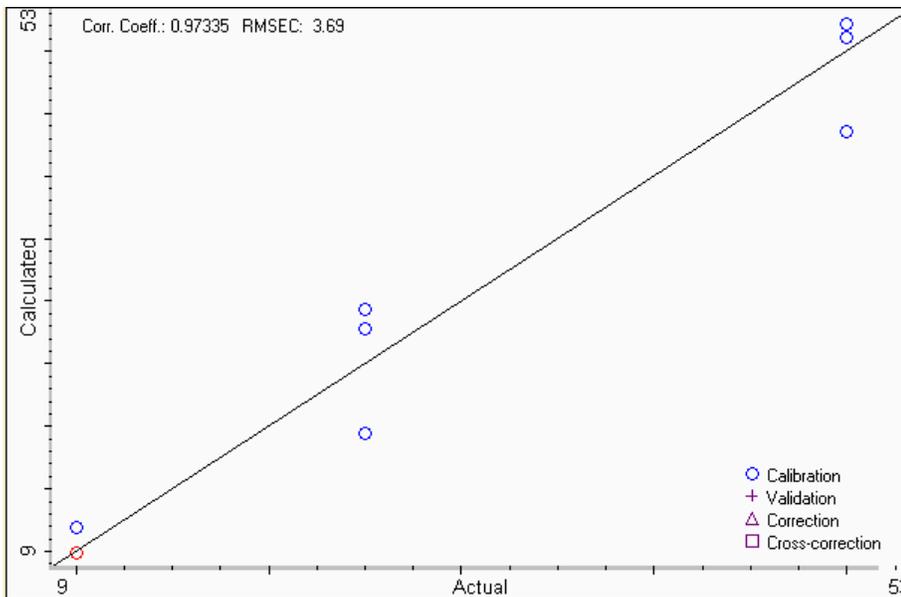


Figure 4-17 PLS plot showing the platform’s ability to predict the concentration of ricin in a sample of diluted/filtered orange juice.

Figure 4-19 shows SER spectra from milk spiked with whole ricin toxin. Again, significant difference was found between all concentrations, as shown by a PCA plot (Figure 4-20).

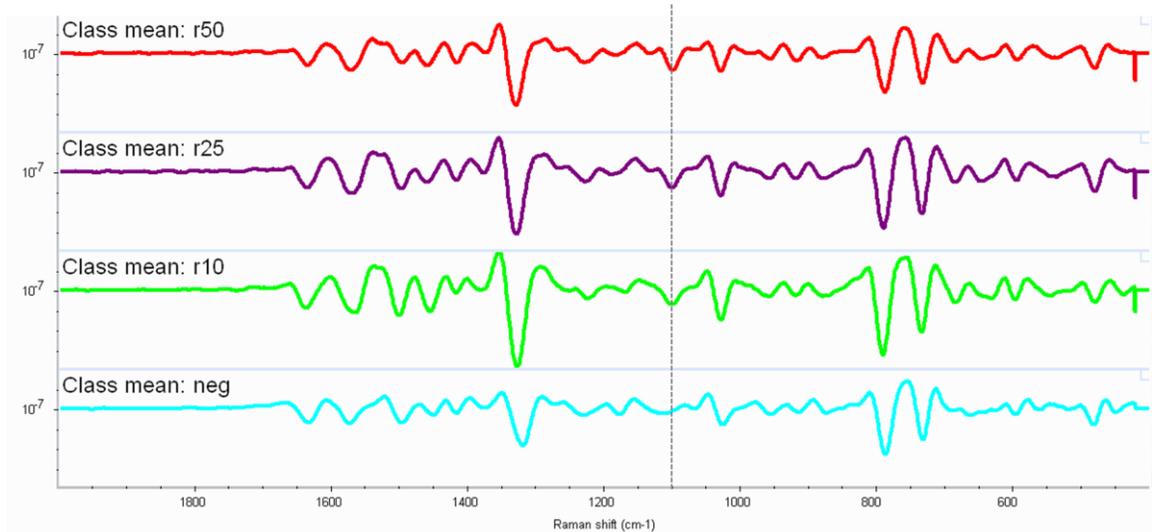


Figure 4-18 SER spectra of whole ricin toxin spiked into 10x diluted milk. Significant difference from the presence of ricin was found at 1100 cm^{-1} (indicated).

The limit of detection for milk was the same as orange juice at <10 ng/mL corresponding to <0.1 $\mu\text{g/mL}$ in undiluted milk. A PLS plot was created which attempted to build a standard curve which could predict the concentration of ricin in an unknown sample of milk. The build model was poor with a correlation coefficient = 0.71 (Figure 4-21).

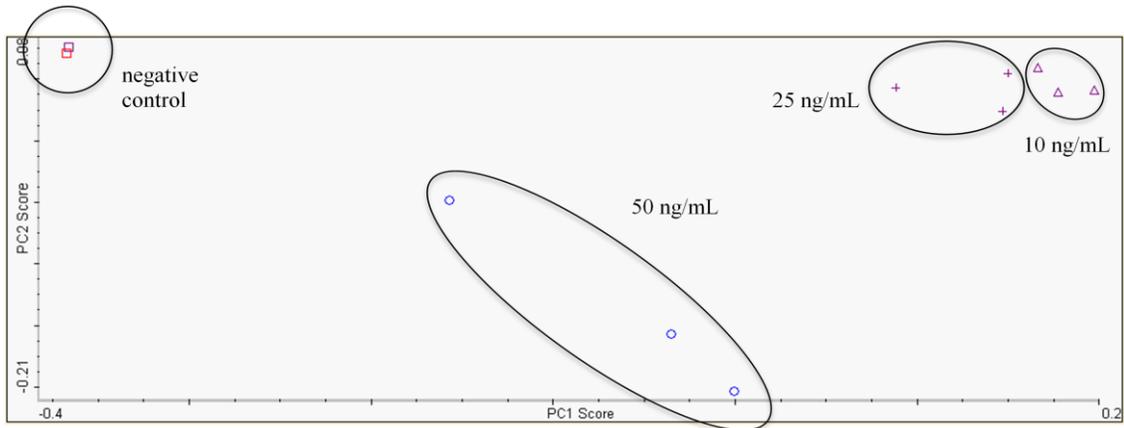


Figure 4-19 PCA plot of 10x diluted milk spiked with varying concentrations of whole ricin toxin.

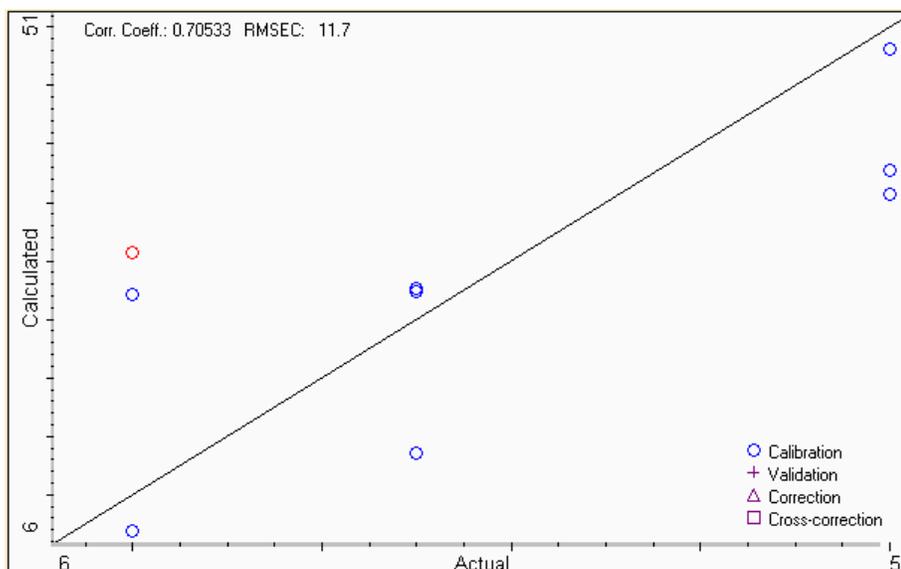


Figure 4-20 PLS plot showing the platform's ability to predict the concentration of ricin in a 10x diluted sample of milk.

4.3 Discussion

RTB was used in this feasibility study as a surrogate for the whole ricin molecule due in part to the fact that the aptamer used was sequenced for use against the RTB molecule, but also due to ricin's potent toxicity. Following promising results in the feasibility study, the study was validated using the whole ricin molecule.

4.3.1 Ricin B Chain (RTB)

SER spectra were collected from aptamer-conjugated silver dendrites (Ag-Ap), and from Ag-Ap which had captured RTB (Ag-Ap-RTB). Differences between these spectra were used as the foundation for discrimination. The addition of two small peaks in the Ag-Ap-RTB spectrum, which were present in the SER spectrum of RTB itself, denote that the method is capable of capturing RTB out of solution. Though these peaks

are small in the raw spectra, they become more prominent following spectral processing and a PCA plot is able to find significant difference between them.

The two additional peaks occurred at 985 cm^{-1} and 621 cm^{-1} , which correlate with peaks in the RTB spectrum at 1001 cm^{-1} and 633 cm^{-1} . Though the spectral shifts are not identical, it is common that small shift variations can occur as a result of the changes in the chemical environment of the detected molecule. For instance, there could be conformational changes in the RTB protein as a result of aptamer capture, or as a result of adsorption to the silver surface and drying.

When capture using Ag-Ap was performed in PBS spiked with varying concentrations of RTB, significant difference was found between all concentrations and the negative control. The lowest concentration of RTB tested was 10 ng/mL , which was discrete from the negative control and so therefore the limit of detection was $\leq 10\text{ ng/mL}$. This aptamer-based method had a much lower limit of detection than the antibody-based IMS method. Since the basis of detection is derived from spectral differences between the negative control and the positive Ag-Ap-RTB complex, the fact that there are very distinct biochemical differences between these two preparations means that detection could be achieved at lower concentrations. The negative control consists of just the DNA aptamer and thus yields a SER spectrum that is very different from the spectrum of an aptamer which has captured the protein RTB. Put another way, while the IMS-SERS method had to detect subtle differences between two proteins (antibody and ricin), the aptamer-SERS method detects differences between DNA and protein.

Qualitative analysis was subsequently confirmed in milk and orange juice, which represented potential food targets for bioterror events, but were biochemically quite different and thus tested the robustness of the method. In both cases, significant differences were found between the test and negative control spectra which yielded limits of detection of 100 ng/mL in orange juice, and 100 ng/mL in milk. The loss of sensitivity compared to detection in PBS is believed to be due to non-specific binding of matrix components onto the surface of the Ag-Ap complex. Additionally, it is possible that the tendency for ricin to bind galactose-containing molecules such as lactose in milk could have an impact the sensitivity of the assay. The presence of such components could confound the ability of SERS to detect at the same sensitivity, though the LOD is still well below the necessary level to render protection in a real-world contamination scenario. Additional washing steps, or an immunoseparation step such as IMS would likely decrease non-specific binding, though these steps would add time and/or cost to the procedure.

While very sensitive for qualitative analysis, all concentrations' data sets (10 through 500 ng/mL) were overlapping in the PCA plot meaning that this platform could not be used to predict the concentration of RTB from a sample. Since surface enhancement is distance dependent, it is believed that the captured RTB molecule is held towards the outer limit of enhancement by the aptamer complex. Therefore subtle concentration-dependent variability was probably lost. Characterization of the *SSRA1* aptamer used in this study shows that it is approximately 7 nm in length, which holds its captured target at a distance which experiences smaller enhancement. It is feasible that

another, shorter aptamer which held RTB closer to the surface of silver dendrites might yield a procedure with the ability to predict a sample's contamination concentration for aptly. Though the ability to quantify RTB in a sample using this method would be convenient, it is not necessary since RTB is not allowable in food at any level. Rather, this method represents a rapid method for getting a “yes/no” answer which is much more sensitive than required to render protection against a ricin-related bioterror event.

4.3.2 Ricin toxin (A + B chains):

A similar study was performed as above but using the whole ricin molecule. Because clear differences exist even between raw SER spectra of RTB and ricin, a validation study was required since it could not be assumed that the intact ricin molecule would behave identically to the RTB chain alone. Also, RTB itself poses no risk to humans since both subunits are required to impart toxicity *in vivo*. Again, spectral differences between spectra of negative control and spiked sample preparations served as the basis of detection.

4.3.2.1 Ricin in PBS

The aptamer selection process was performed with RTB as a target; thus, it was necessary to validate that it could capture and concentrate ricin as well. It is reasonable to assume that it could, since RTB is a component of ricin, but it is possible that conformational changes or steric hindrance from the presence of RTA could affect

aptamer binding. Significant spectral differences were found between spectra for Ag-Ap and Ag-Ap-R, most notably in the regions at 570, 920, and 1080 cm^{-1} . Additionally, a PCA plot confirmed that these preparations were different, thus indicating successful capture of intact ricin toxin from PBS using the RTB aptamer. After validation, Ag-Ap was used to capture ricin from PBS spiked at varying concentrations. Significantly different spectra were found at 0, 25, and 50 ng/mL. Therefore, the limit of detection for the method was found to be 25 ng/mL since this was the lowest concentration which could be discriminated from the negative control. It is possible that the LOD is lower, since no additional concentrations between 0 and 25 ng/mL were investigated.

Partial least squares analysis of the varying concentrations showed that a good model could be constructed which could predict the concentration of ricin in a sample between 0 and 50 ng/mL, making this the dynamic range of quantification. The ability for the present method to quantify ricin in a sample, where it could not for RTB, may be due to the aptamer capturing the molecule and holding regions of it closer to the surface of the silver dendrites thereby increasing the experienced enhancement factor and consequently the spectral resolution. Concentration dependent variance did not extend from 50 to 100 ng/mL. It is possible that the method could not quantify beyond a certain point because aptamer present was saturated and no additional ricin could be captured above 50 ng/mL.

4.3.2.2 Native vs. Denatured Ricin

Most rapid, immunochemically-based procedures cannot distinguish active ricin from inactive; typically this type of analysis requires more complex analyses. Though

unlikely that SERS would be able to detect activity, it was investigated whether SERS would distinguish native ricin from heat denatured ricin.

SER spectra collected from PBS spiked with heat-denatured ricin were significantly different from a negative control indicating that the aptamer was still capable of capturing heat-denatured ricin toxin. However, no significant difference was found between spectra of native ricin and heat-denatured ricin after capture meaning that the present method would not be able to discriminate these two preparations. Because the presence of ricin in a sample, whether denatured or not, indicates a bioterror threat, it is not mandatory that the present surveillance method be capable of making such a distinction.

If knowing whether detected ricin is native or not is necessary, the method can be modified to answer such a question. SER spectra of native ricin and heat-denatured ricin are significantly different from each other when they are adsorbed directly onto the silver surface, not in the presence of aptamer. Therefore, the method could be modified by disrupting the aptamer-ricin complex and separating the two components after capture is completed. Eluate containing the captured material could then be mixed with silver dendrites and SERS analysis would determine whether the protein was denatured or not. Spectral differences found between native and denatured ricin are likely due to hydrophobic amino acid side chains exposed as a result of protein denaturation caused by heat. Such side chains include phenylalanine which, due to the cyclic structure of the side chain, is a very Raman-active molecule. Hydrophobic side chains are typically buried in the center of globular proteins in order to render them water soluble. Since SERS

primarily detects surface moieties, hydrophobic amino acid side chains are typically not detected from water soluble proteins in their native conformation. Once denatured, a protein's hydrophobic side chains are exposed through loss of secondary/tertiary structure and can be detected.

4.3.2.3 Ricin in Food

Lastly, the aptamer-based method was validated for functionality in liquid food systems. Prior to capture, milk was diluted 1:10 in order to limit the interaction of matrix components with the Ag-Ap complex. Similarly, orange juice was diluted and also needed to be passed through a filter (0.45 μm) to remove pulp before capture.

SER spectra of ricin spiked into both milk and juice at any of the tested concentrations were significantly different than the negative controls, indicating that successful capture and detection from food was achieved. Corrected limits of detection were $<0.1 \mu\text{g/mL}$ for both milk and orange juice. These LODs are less sensitive than when capture was conducted from PBS, probably as a result of non-specific binding of matrix components to the Ag-Ap complex. Interfering components limit detection by adsorbing onto the surface of the complex and occluding binding sites for ricin, and by yielding peaks in the spectra which cover up ricin's spectral signature. Because matrix components are present in much higher abundance than ricin in spiked samples, the resulting interference complicates detection/analysis. This may also explain why the PLS model built for ricin in orange juice was much more robust than the model built for milk. Milk contains a greater amount of protein (3.15% in milk vs. 0.68% in OJ), which could

interfere with the detection of ricin protein. Although it was not enough to raise the limit of detection, it could have caused enough interference to make quantification difficult. Because the ability to quantify ricin in a food sample was not the ultimate goal of the developed procedure, further investigation of capture efficiency was not pursued.

Additional washing steps following capture to remove loosely adsorbed matrix material, or a more rigorous separation procedure, such as IMS, could presumably lower the LOD to be closer to that of ricin in PBS. However, because the limits of detection of the present methods are still well below the necessary level to render protection based on ricin's toxicity, the demonstrated sensitivity is sufficient for food surveillance.

Chapter 5 Conclusion

The methods developed in the present study are very useful based on their short duration (<30 minutes), ease of operation, and adequate sensitivity in diverse media. They are based on the direct detection of ricin using SERS following one of two procedures for separation from the food matrix. Following separation, a direct Raman detection scheme was developed because it minimized the chance of false negatives since detection is based on spectral fingerprints rather than an indicator reaction, as in ELISA or PCR. Additionally, these indirect ‘reporter’ reactions often take time to develop, making direct Raman measurement compatible with very rapid procedures.

5.1 Summary of Methods

5.1.1 IMS-SERS

Once a truckload or other large quantity of milk or orange juice arrives at a centralized processing facility, small samples of product could be taken and tested for ricin alongside existing food quality testing protocols. The procedure could be scaled up if required, but the present procedure would require 3-1.5 mL aliquots of product be mixed with magnetic beads which are prepared ahead of time by conjugation with anti-ricin antibodies. After a brief incubation (10 min), a magnetic separation rack would be used to separate the beads from the resonant solution which is discarded. The captured

material could then be eluted into clean water, mixed and incubated with the nanosubstrate (30s), dried (10-20 min), and scanned using a Raman instrument (1 min). The data analysis component present in the present study could be largely automated and programmed into the instrument to create a library of ricin spectra specific to each matrix including standard curves. Therefore, the onsite user would merely have to select which matrix was used and the instrument could provide qualitative and possibly quantitative analysis nearly instantly. This whole procedure could be optimized to take less than 30 minutes.

5.1.2 Aptamer-conjugated Dendrite-SERS

Similar to IMS-SERS, this method would require small aliquots be sampled from larger distribution quantities of homogenous liquid food. Some very simple pre-processing steps would be performed including dilution and filtration. The, prepared aptamer-conjugated silver dendrites would be mixed and incubated in the sample briefly while the aptamer captured ricin. The relatively large silver-aptamer-antigen complexes are then quickly centrifuged to form a pellet which is sampled for Raman measurement. Again, the Raman instrument could be preprogrammed to perform the necessary analyses with a total duration of less than 20 minutes.

5.2 Comparison of Methods

Both methods have two basic steps: in the first step, ricin toxin is separated from the liquid matrix that contains it; in the second step, ricin is detected using surface-

enhanced Raman spectroscopy. Under optimal conditions, SERS-based detection schemes are capable of ultra sensitive limits of detection. However, such schemes typically require rigorous separation parameters, state-of-the-art Raman instruments, and expensive nanosubstrates. In an application that is feasible for food monitoring, such characteristics are impossible, yet SERS remains an attractive method for rapid assays since it requires very little sample preparation.

The differences between the two methods lie in the initial separation step. The first method uses an immunomagnetic precipitation kit to separate ricin from the food. This is a well-developed, commercialized method which utilizes the specific binding capability of antibodies to capture and concentrate the antigen. The benefit of IMS is that it nearly completely separates the antigen from the food matrix components which can interfere with detection sensitivity. This is different from the aptamer-based method which does not have such a rigorous separation procedure and instead uses a combination of dilution, filtration and a brief centrifugation step to separate the dense dendrites from matrix components. Although dilution is not a desirable step, as it also dilutes the amount of ricin present in the sample thereby affecting the limit of detection of the assay, it was a very rapid/easy way of minimizing the interference caused by matrix materials. Even with the dilution step, the aptamer-based method's limit of detection was sufficient to detect ricin at a low enough concentration to render safety.

One of the main advantages of using the aptamer-conjugated silver is that the nanosubstrate is already present at the time of capture, which eliminates the need for an

additional incubation step following capture. This saves time, as the incubation steps are the slowest part of the procedure, and allows for a more rapid assay overall.

Another main advantage is that the capture agent is a piece of single-stranded DNA rather than an antibody. This fact has ramifications on assay cost as aptamers are much more easily synthesized after the sequence is known. Also, the process by which aptamers are generated allow for specific engineering. For instance, they can be generated under conditions (temperature, pH, ionic strength, solvent) that mimic the conditions of the end assay where they will be used. This is largely impossible for antibodies which must be created in biological systems and whose binding affinities can be greatly diminished by non-optimal conditions. Because foods can represent many harsh environments, the use of antibodies may not always be feasible for a detection assay. Additionally, aptamers can be engineered to include specialized functional groups such as groups for covalently binding aptamers to other materials, or with reporter groups such as nanomaterials or enzymes. The aptamer used in this study was conjugated with a thiol group which made it possible to covalently bond the aptamer to the surface of silver dendrites.

However, the use of an aptamer is probably most beneficial since the basis of detection in SERS stems from differences between test spectra and the negative controls. In the present methods, the capture agent will always be present in the sample; therefore, sensitivity of the assay is somewhat based on the computer's ability to discriminate between spectra of just the capture agent, and spectra of the capture agent plus ricin protein. Since Raman spectra contain structural information about molecules present in

the sample, it is logical that similar compounds produce similar spectra. Also, it follows that the more structurally different the capture agent is from ricin, the better the sensitivity of the SERS assay. Since the aptamer is made from DNA, while both the antibody and target are proteins, it is logical that the aptamer is a superior capture agent for Raman-based detection assays. When compared with a study which used silver dendrites conjugated with an anti-ovalbumin antibody, this aptamer-based method showed greater sensitivity (lower limit of detection) by approximately one order of magnitude whether in buffer or milk.

5.3 Future Study

Given the success of the present methods for detecting ricin in food systems, it is important to extend testing the feasibility of detecting different biomolecules in other food systems. Given that ricin is a small, protein molecule, it would be important to test whether a SERS-based detection scheme could rapidly detect other types of biomolecules, such as specific carbohydrates or DNA, or even larger, more complex targets such as a cell. With further investigation and development, it is possible that a multi-analyte bioterror detection panel could be created in order to test a sample for an array of agents.

Additionally, the present method has been developed solely in a laboratory setting. However, the end goal is that it could be deployed in a food manufacturing facility, such as a liquid milk processing facility, and conducted by existing food

quality/safety personnel. Further investigation as to whether the method can retain its processing time and limits of detection in this setting needs to be conducted.

Chapter 6 References Cited

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