Development of Methodology for Rapid Bacterial Detection in Complex Matrices Using SERS

Madeline Tucker

Follow this and additional works at: https://scholarworks.umass.edu/masters_theses_2

Part of the Food Chemistry Commons, and the Food Microbiology Commons

Recommended Citation

This Open Access Thesis is brought to you for free and open access by the Dissertations and Theses at ScholarWorks@UMass Amherst. It has been accepted for inclusion in Masters Theses by an authorized administrator of ScholarWorks@UMass Amherst. For more information, please contact scholarworks@library.umass.edu.
DEVELOPMENT OF METHODOLOGY FOR RAPID BACTERIAL DETECTION IN COMPLEX MATRICES USING SERS

A Thesis Presented

By

MADELINE TUCKER

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

May 2018

Food Science
ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Lynne McLandsborough for her constant encouragement and research advice over the past two years. I am extremely grateful for the opportunities that have arisen during my time as a student in the Food Science department, for which I would not be apart of if Lynne hadn’t considered me for this position. I would like to extend my appreciation to my committee members Dr. Lili He and Dr. Maria Corradini for their time and the role they have played in my education.

I would also like to thank Dr. Lili He for permitting me to work in her lab and for inviting me to be a part of her bi-weekly lab meetings, where I was able to gain valuable insight from my peers in the lab as well as from her advice. Lastly I would like to thank my lab mates in both the McLandsborough and He lab for always being open to answering questions, assisting me with software and providing valuable feedback throughout this experience. This work was funded by the United States Department of Agriculture grant 2015-67021-22993 and hatch MAS00491.
ABSTRACT

DEVELOPMENT OF METHODOLOGY FOR RAPID BACTERIAL DETECTION IN COMPLEX MATRICES USING SERS

MAY 2018

MADELINE TUCKER, B.S., SUNY GENESEO
M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Lynne McLandsborough

Fresh foods, including meats and produce are the fastest growing market in the supermarket and the class of foods most likely to cause a bacterial foodborne illness. As the rate of consumption of perishable products increases, rapid detection of pathogens within the food supply becomes a critical issue. Current methods used for the detection of bacteria that cause food-borne illnesses are time consuming, expensive and often require selective enrichment. In this study we adapted a separation technique originally developed for PCR to extract bacteria from ground beef using β-cyclodextrin (β-CD) and milk protein coated activated carbon (MP-CAC) as filtration agents. The recovered bacteria were bound to a gold slide via a 3-mercaptophenylboronic acid (3-MPBA) sandwich assay and detected with Surface Enhanced Raman Spectroscopy (SERS). The 3-MPBA sandwich assay used with the separation technique allowed detection of Salmonella enterica Enteritidis (BAA-1045), separated from a ground beef matrix, as low as $1 \times 10^2$ CFU/g. Detection at this level was accomplished in less than 8 hours, significantly faster than plate count or enrichment methods that require multiple days. Previously, SERS has been used to detect bacteria within simple matrices; this is the first study to have utilized SERS bacterial detection in a ground beef.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xi</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Justification</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Conventional bacterial detection methods</td>
<td>2</td>
</tr>
<tr>
<td>1.3 Spectroscopic methods</td>
<td>4</td>
</tr>
<tr>
<td>1.4 SERS as a bacterial detection method</td>
<td>5</td>
</tr>
<tr>
<td>1.5 SERS applications in food matrices</td>
<td>6</td>
</tr>
<tr>
<td>1.6 SERS interference from food components within complex matrices</td>
<td>7</td>
</tr>
<tr>
<td>1.7 Methods to Separate Bacteria from Food Matrices prior to detection</td>
<td>7</td>
</tr>
<tr>
<td>2. OBJECTIVES</td>
<td>12</td>
</tr>
<tr>
<td>3. OPTIMIZATION OF BACTERIAL SEPARATION AND SUBSEQUENT DETECTION USING A 3-MPBA SANDWHICH ASSAY WITH SERS</td>
<td>13</td>
</tr>
<tr>
<td>3.1 Introduction</td>
<td>13</td>
</tr>
<tr>
<td>3.1.1 Adaptation of a Separation Technique Designed to Remove PCR Inhibitors from Ground Beef</td>
<td>13</td>
</tr>
<tr>
<td>3.1.2 Detection of Bacterial Cells Recovered with SERS</td>
<td>14</td>
</tr>
<tr>
<td>3.2 Materials and Methods</td>
<td>14</td>
</tr>
<tr>
<td>3.2.1 Bacterial Preparation</td>
<td>14</td>
</tr>
<tr>
<td>3.2.2 Milk Protein Coated Activated Carbon Preparation</td>
<td>15</td>
</tr>
</tbody>
</table>
3.2.3 Ground Beef Preparation

3.2.4 β-cyclodextrin Preparation

3.2.5 Bacterial Extraction Procedure

3.2.6 Bacterial Extraction Recovery Quantification

3.2.7 3-MPBA Coated Gold Chip Preparation

3.2.8 Sample Preparation

3.2.9 Non-Specific Sandwich Assay Procedure

3.2.10 Data Collection

3.3 Results

3.3.1 Efficacy of bacterial separation technique

3.3.2 SE1045 recovery detection with SERS

3.3.3 Influence of β-CD concentration on SERS detection

3.3.4 SERS Detection throughout Extraction Process

3.3.5 Optimization of the 3-MPBA Assay for bacterial detection in complex matrices

3.4 Discussion

3.4.1 Efficiency of bacterial extraction procedure

3.4.2 β-CD’s concentration’s influence on SERS detection

3.4.3 SERS earliest detection in extraction process

3.4.4 Influence of Temperature on 3-MPBA assay

3.5 Conclusion

4. INVESTIGATION INTO THE 3-MPBA SANDWICH ASSAY’S EFFICACY FOR BACTERIAL DETECTION IN GROUND BEEF

4.1 Introduction

4.2 Materials and Methods

4.2.1 General Methods Overview

4.2.2 Sample Preparation for bacterial extraction immediately
followed by SERS detection experiment .......................... 43
4.2.3 Sample Preparation for Detection Limit Experiments .............. 43
4.2.4 Sample Preparation for Capture Efficiency Work .................. 44
4.2.5 Bacterial preparation for strain differentiation ..................... 44
4.3 Results .................................................................. 45
4.3.1 Determination of bacterial detection limits for 3-MPBA assay in complex matrices ................................................. 45
4.3.2 Determination of Capture Efficiency of 3-MPBA assay with complex matrices ......................................................... 49
4.3.3 3-MPBA assay Strain Differentiation Capabilities ............... 54
4.3.4 Bacterial Extraction and Detection Simultaneously ............ 57
4.4 Discussion ................................................................ 59
4.4.1 Limit of Detection Discussion ..................................... 60
4.4.2 Capture Efficiency Discussion ..................................... 62
4.4.3 Strain Differentiation Discussion ................................. 62
4.4.4 Bacterial extraction and subsequent detection discussion .... 64
4.5 Conclusion .............................................................. 64
5. CONCLUSION .......................................................... 66
APPENDIX: SUPPLEMENTARY MATERIALS .......................... 67
REFERENCES ................................................................ 68
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Diagram of bacterial separation methodology adapted from Robert Levin’s bacterial extraction procedure to remove bacteria from ground beef prior to PCR analysis</td>
<td>19</td>
</tr>
<tr>
<td>2. 3-MPBA sandwich assay procedure diagram</td>
<td>23</td>
</tr>
<tr>
<td>3. Bacterial recovery throughout extraction technique illustrating LOG CFU/g loss of SE1045</td>
<td>27</td>
</tr>
<tr>
<td>4. Spectra and chemical mapping deduced from 3 trials performed to detect SE1045 artificially inoculated in ground beef homogenate</td>
<td>29</td>
</tr>
<tr>
<td>5. Spectra and chemical mapping produced from 3 trials comparing β-CD’s influence on SERS detection of bacteria</td>
<td>31</td>
</tr>
<tr>
<td>6. Chemical mapping and spectral images showing optimization of detection throughout extraction process</td>
<td>33</td>
</tr>
<tr>
<td>7. Optical images of inhibitory molecules present on the chips during the bacterial extraction process</td>
<td>34</td>
</tr>
<tr>
<td>8. Optimization of 3-MPBA coating incubation temperature</td>
<td>36</td>
</tr>
<tr>
<td>9. Bacterial detection limit averaged spectra produced from 4 trials (20x20 maps, step size 10 µm)</td>
<td>46</td>
</tr>
<tr>
<td>10. Chemical imaging performed highlighting presence/absence of the ~1023 cm⁻¹ bacterial peak on varied concentration of SE1045 in beef homogenate</td>
<td>47</td>
</tr>
<tr>
<td>11. Relationships between SE1045 concentration and percent of boxes positive for the bacterial peak at 1023 and SE1045 concentration and Raman signal intensity</td>
<td>48</td>
</tr>
<tr>
<td>12. Spectral data and optical images comparing bacterial capture efficiency of SE1045 recovered from beef extract and SE1045 from pure culture</td>
<td>50</td>
</tr>
</tbody>
</table>
13. Capture efficiency of the 3-MPBA assay derived from plate count data………51
14. Capture efficiency of the 3-MPBA assay based on % of pure SE1045 and
% of SE1045 from beef extract cells bound and unbound after 1 hr incubation........53
15. Spectral data comparing 5 strains of nalidixic acid resistant Salmonella
enterica…………………………………………………………………………………………55
16. Chemical mapping comparing 5 nalidixic acid resistant Salmonella Enterica
strains…………………………………………………………………………………………56
17. PCA analysis of spectra illustrating the inability to differentiate between
Salmonella enterica strains based on 1200 spectra obtained for each strain........57
18. Spectral images produced from the average of 6, 400 point maps taken
SE1045 extraction from ground beef…………………………………………………58
19. Chemical imaging performed on samples of SE1045 extracted from
ground beef………………………………………………………………………………59
LIST OF ABBREVIATIONS

AgNR-Silver nano-rods
β-CD- Beta-cyclodextrin
MP-CAC-Milk protein coated activated carbon
SERS- Surface enhanced Raman spectroscopy
SE1045- *Salmonella enterica* subsp enterica BAA1045
3-MPBA-3 Mercaptophenylboronic acid
CHAPTER 1

INTRODUCTION

1.1 Justification

Recently, progressive health and wellness consumers have changed the way Americans are shopping at the supermarket. Progress consumers are no longer concerned with managing chronic conditions or adopting trendy diets, instead they are focused on purchasing and consuming nutritious, minimally processed foods. [1] However, as consumption of minimally processed foods increases the rate of foodborne illnesses also increases. Currently, the CDC estimates that major known pathogens cause 9.4 million foodborne illness cases each year. [2] The vast majority of these illnesses are traced back to minimally processed foods including fruits, vegetables [3], raw meats [4,5] and dairy [6] products. Produce is estimated to cause 46% of food-borne illness cases, while meat (beef and poultry) and dairy products each contribute 22% and 20% respectively [7]. Due to the perishable nature of many of these products, rapid bacterial detection methods are necessary to ensure the safety of the food supply and to optimize the shelf-life of these products.

Current bacterial detection methods are laborious, time-consuming and are incapable of discriminating between viable and non-viable cells. The development of bacterial detection methods which are rapid, cost efficient and can be implemented in industrial food processing settings is necessary to provide quantitative information about the presence of a pathogen in minimally processed food commodities.
1.2 Conventional bacterial detection methods

Bacterial detection methods can be classified into three categories including culture and counting based methods, immunological methods and nucleic acid based methods [8]. Established methods such as the Standard Plate Count for pathogens are inefficient due to the need for the relatively high detection limit. In order to detect the required low levels of pathogens, food samples must undergo selective enrichment followed by a confirmation method either selective plating and biochemical confirmation, or utilization of an immunological or nucleic acid based confirmation. These methods require multiple days for identification and are laborious, making them unfavorable for detection of pathogens within perishable food products [9]. Current techniques including Enzyme-linked Immunoassay (ELISA) and Polymerase Chain Reaction (PCR) both have inherent limitations which make them inadequate for rapid bacterial detection. While PCR is a rapid, sensitive technique, the method relies on nucleic acid amplification and is thus unable to discriminate between viable and non-viable bacterial cells [10]. PCR is also highly inhibited by macromolecules within food matrices such as lipids, proteins, polysaccharides and antibiotics. ELISA is both a highly sensitive and automated detection technique but is limited by the specificity of the monoclonal antibodies employed and often requires an enrichment step [9].

Antibody direct epifluorescence techniques (Ab-DEFT) have been proposed as a rapid bacterial detection method in food matrices. Tortorello et al. compared the sensitivities of Ab-DEFT to a conventional enrichment culture technique. After artificially inoculating *E. coli* O157:H7 into 80% lean ground beef, bacterial cells were
entrapped on a membrane filter and fluorescently labeled polyclonal antibodies to *E. coli* O157:H7 were layered on top of the membrane. Cells were then either quantified by membrane filter microscope factors via epi-fluorescent microscopy or viable plate counts from the enrichment culture method. Both methods were capable of cell quantification down to 16 CFU/g [11]. While the Ab-DEFT quantification method was far more rapid than traditional enrichment methods, Ab-DEFT is limited by the specificity of the polyclonal antibodies which are capable of binding to other microorganisms present in the ground beef [9]. Manual cell counting by this method is also laborious and operator fatigue also limits Ab-DEFT’s efficiency.

Flow cytometry has also been used as a rapid detection method. In flow cytometry, cells are carried by the laminar flow of water, through light which has a corresponding wavelength to that of the dye that the cells were stained in [9]. The main advantages of this method are that it is both rapid and automated. Flow cytometry was used to detect *E. coli* O157:H7 in ground beef as low as $1 \times 10^4$ CFU/mL [12]. However, bacterial cell detection at lower limits was not possible in ground beef or in PBS. This method was limited by the presence of fluorescent particles present in reagents used for the dying procedure.

While there are a plethora of detection methods, none of the current methods meet the criteria of being rapid, non-destructive, highly sensitive and specific, low cost and capable of being adapted into an industrial processing environment.
1.3 Spectroscopic Methods

Spectroscopic methods are becoming increasingly popular due to their rapid detection and non-destructive sample analysis. Raman spectroscopy causes inelastic scattering of molecules resulting in chemical and structural information based on vibrational transitions [13]. Raman scattering uses a laser to irradiate a sample causing the vibrational energy in a molecule to be moved to a high-energy collision state. While most of the molecules relax to the low energy original state, a small number of the excited molecules relax back to excited state releasing photons of a lower frequency than the molecules that returned back to the low energy original state. Emission of a higher frequency photon by molecules that returned back to the original low energy state results in Rayleigh scattering. Emission of the lower frequency photon results in Stokes Raman Scattering. Differences between the frequency of the laser and that of the emitted photon are known as a Raman shift [14].

Vibrational spectroscopic methods provide chemical, structural ‘fingerprints’ of the samples being analyzed. Raman spectroscopy is particularly useful for identification of biological samples due to the weak Raman scattering of water, present in most biological samples [15]. However due to the weak scattering associated with biological samples, signal enhancement is necessary to detect microorganisms.

Surface Enhanced Raman Spectroscopy (SERS) is a Raman technique that utilizes metal particles or surfaces, which are adsorbed to sample molecules, to enhance Raman scattering. This enhanced Raman signaling is due to resonance Mie scattering of light by the metal nanostructures [13]. For years, SERS was used as a chemical detection platform
but the specificity, high sensitivity and relatively low cost of SERS also makes it a promising bacterial detection platform.

### 1.4 SERS as a bacterial detection method

SERS bacterial detection is accomplished by either label-based (indirect) or label free methods (direct) [16]. Label methods detect SERS tags which are cross-linked to bacteria. A variety of SERS tags including antibodies [17, 18, 19], apatamers [20], and glutaraldehyde [21] have been employed in previous bacterial detection studies. Label free methods typically rely on biomarkers produced by the microorganism such as DPA from bacterial spores [22] or from components comprising the cells exterior cell wall including nucleic acids, polysaccharides and proteins [23]. SERS peaks from the cell wall are typically found at 624, 652, 735, 955, 1330, and 1456 cm\(^{-1}\)[24].

SERS has previously been used in microbiology for bacterial detection, discrimination between Gram positive and Gram negative species, differentiation between viable and non-viable cells and comparison of bacterial spores [25, 26]. It is important to note that bacterial detection using SERS produces radically different chemical information when experimental parameters including substrate and chemical reagent vary between experiments [15]. Thus, spectral band information produced by the same microorganism, with the same surface chemistry, but under varied experimental conditions will differ.

Silver nano-rod array substrates were used to detect SERS spectral differences within Escherichia coli, E. coli O157:H7, E. coli DH5α, Staphylococcus aureus, Staphylococcus epidermidis, Salmonella Typhimurium, and bacteria mixtures [25]. In
This study, the most prominent SERS spectral bands were found at 735, 1,330, and 1,450 [25]. Principal component analysis was used to differentiate between Gram positive and Gram negative strains and viable cells were shown to cause higher intensity spectra than nonviable cells.

Gold SERS active substrates were used to differentiate between 5 different Bacillus spores (B. cereus ATCC 13061, B. cereus ATCC 10876, B. cereus, B. subtilis, and B. stearothermophilus) [26]. Principal component analysis was used to discriminate between spectral differences produced from the 5 spores. The limit of detection within these experiments was determined to be as low as a single spore [26].

1.5 SERS applications in food matrices

As SERS bacterial capture and detection methods have improved, SERS has become a possible detection method for pathogens within food matrices. Silica coated magnetic nanoparticles were used in conjunction with antibodies to create a SERS immunoassay [27]. This immunoassay was used to detect S. aureus and S. enterica inoculated with fresh spinach. The bacterial specific immunoassay was able to detect S. aureus as low as $10^2$ CFU/mL using a label method, whereas label free methods were only capable of detecting S. enterica serovar Typhimuriumas low as $10^4$ CFU/mL. Signal intensity for both S. aureus and S. enterica was stronger in PBS than the spinach sample due to interference from the sample matrix [27].

Silver nano-rod (AgNR) array substrates were fabricated by the oblique angle deposition method (OAD) to capture and subsequently detect six different pathogens commonly found on mung bean sprouts. The inoculated mung bean sprout solution
underwent a two-step filtration process then SERS was able to detect *E. coli* O157:H7 recovered from the sprouts at $10^3$ CFU/g [10].

1.6 SERS interference from Food Components within Complex Matrices

While SERS has been used to detect bacteria within simple matrices such as spinach [27], sprouts [10] and fruit juices [28], SERS has rarely been used to detect bacteria within complex matrices such as ground beef, [29,30] ground poultry or soft cheeses. Macromolecules within these complex matrices including polysaccharides, proteins and lipids interfere with the bacteria’s ability to bind to the capture material, thus preventing proper binding and detection of the bacteria. Bacterial detection in food matrices is also inhibited by non-uniform dispersion of bacteria in the matrix, heterogeneity of macromolecule distribution and viscosity differences throughout the food [31]. Separation and concentration methods have been developed via physical, antibody based and chemical based separation techniques to effectively remove detection inhibitors and isolate the pathogens of interest [32, 33]. While the food matrix components cannot be completely removed from a sample, a separation technique that removes the majority of the SERS inhibitors is necessary prior to rapid bacterial detection with SERS.

1.7 Methods to Separate Bacteria from Food Matrices prior to detection

Due to the interference between macromolecules in the food matrix and bacterial capture efficiency, bacterial separation methods are necessary to detect bacteria within food matrices. Wu et. Al used a two-step filtration process to recover *E. coli* O157:H7 from inoculated mung bean sprouts [10]. This two-step filtration process utilized a pre-
filtration step in which large SERS inhibitors were removed and then a second filtration in which a filter membrane captured the target bacteria. The target bacteria were then re-suspended after being vortexed in sterile DI water. This filtration process recovered 74.6% of the inoculated bacteria as determined by plate counts [10].

Electro-spun Au coated polymer mats were utilized to capture *S. aureus* and *E. coli* from liquid matrices including tap water, urine and apple juice and detection was subsequently performed using SERS [34]. Bacterial cells were captured between spaces in the polymer fibers while the fluid passed through the polymer mats and into a vacuum pumped Buchner funnel [34]. While both these methods were rapid and effective, they would perform poorly in foods with more complex matrices.

Immuno-magnetic separation (IMS) has also been proposed as a method to capture bacteria from complex food matrices for pathogen detection. IMS utilizes superparamagnetic beads coated with antibodies against the target pathogen to create immune-magnetic beads (IMB) capable of binding the target bacteria within a matrix. Exposure to a magnetic field then concentrates the food homogenate into a smaller volume. IMS limits interference from macromolecules such as lipids and proteins and is a highly rapid separation technique [35]. IMB’s were capable of capturing 94.4% and 99.8% of *E. coli* O157:H7 from ground beef and whole milk respectively [29]. However, similar to the limitations of ELISA as a detection method, IMB’s are limited by the affinity of the antibodies for the target pathogen and cross reactivity could possibly occur with other bacterial strains. IMS also requires enrichment, limiting its use as a rapid detection method [9].
Other studies have employed centrifugation and filtration techniques to separate and concentrate bacteria from a food matrix. In a study by Cho et al., magnetic nanoparticles were coated to monoclonal antibodies (mAB) specific to *E.coli* O157:H7 which then bound the target pathogen. Separately, gold nanoparticles conjugated with polyclonal antibodies were coated with a Raman reporter (MBA). The mAB pathogen system was then reacted with the pAb reporter. Remaining unbound reactants were then removed with centrifugal filtration. The pathogen coated SERS probe was then localized with a silver enhancement step and subsequently analyzed by SERS. Through this method, detection of *E. coli* O157:H7 was possible at levels as low as 10 CFU/g [30].

While, this method appears to be highly sensitive, the ground beef homogenate spiked with 10 CFU/mL of *E.coli* O157:H7 was incubated at 37 °C for a short 2 hour enrichment prior to analysis. This method is also limited by the specificity of the antibodies employed for their target molecules.

In another study employing centrifugation and filtration techniques *Salmonella enterica* was isolated from ground beef without prior cultural enrichment. β-cyclodextrin (β-CD) is a cyclic polysaccharide with a hydrophilic exterior and hydrophobic interior. The structure of this molecule allows it to capture and bind triglycerides, essentially, allowing bacteria within fat globules to be released and separate into the aqueous phase. β-CD was used in conjunction with milk protein coated activated carbon (MP-CAC) which enhances detection of bacteria by binding PCR inhibitors such as triglycerides [36]. Without fat removal by β-CD and reduction of PCR inhibitors by MP-CAC, conventional PCR could only detect Salmonella at levels of 5.0x10^5 CFU/g. However, after this isolation process, PCR was able to detect Salmonella at 3.0 CFU/g [31]. Thus,
the use of β-CD and MP-CAC improved the extraction and quantification of Salmonella within ground beef. One of the drawbacks of this separation method is the high cost of the reagent β-CD.

In later studies, soluble starch was used in place of β-CD, as a slightly more cost effective method for quantification of Salmonella. Soluble starch used in conjunction with MP-CAC Real time qPCR lowered the detectable limit of Salmonella within ground beef to 1 CFU/g [37]. While this method further increased the sensitivity of detection via PCR, soluble starch was still considered to be a costly component. In an effort to further increase the cost effectiveness of this method, Cossu and Levin performed another study in which they utilized hydrolyzed corn starch, as a more cost effective alternative to β-CD and soluble starch. The corn starch partially hydrolyzed with alpha-amylase used with MP-CAC worked in a similar method to the β-CD, as it disrupted fat globules, allowing the capture of bacteria. Using hydrolyzed corn starch in conjunction with Rti-qPCR allowed the quantification of bacteria down to 1 CFU/g of Salmonella within ground beef and occurred within 4.5 hours [38]. This method was considerably faster when compared to the current enrichment methods which require up to 5 days to confirm a pathogen. Thus, hydrolyzed corn starch with Rti-qPCR was both more cost effective and less time consuming than current methods used in the food industry.

However, the primary disadvantage of utilizing PCR to quantify bacteria is its inability to differentiate between viable and nonviable cells. Thus, while a product may contain a high bacterial load, as inferred from the results of the PCR, many of these cells may have been killed by prior processing methods and pose no threat to the consumer. Another disadvantage of this process is the inability to apply this process to a food matrix
in which different components, such as distinct enzymes or antibiotics, inhibit PCR. Thus each food matrix will require a variety of polymers to isolate the bacteria from substituents within the homogenate. Ultimately, improving this method by replacing PCR quantification with another method that is capable of reporting the difference between viable and nonviable cells, such as SERS, is needed to prevent further foodborne illness outbreaks from contaminated food products.
CHAPTER 2

OBJECTIVES

The objectives of this study are:

(1) The determination of the efficacy of the separation techniques to concentrate and purify bacterial cells inoculated in a ground beef matrix.

(2) Detection of recovered bacterial cells using a 3-MPBA non-specific sandwich assay.

(3) Optimization of both the separation method and detection assay to achieve increased Raman signal intensity.

(4) Determination of the limits of detection for the recovered cells using the 3-MPBA assay.

(5) Quantification of the capture efficiency of the 3-MPBA assay for bacterial cells recovered from a complex matrix.
CHAPTER 3

OPTIMIZATION OF BACTERIAL SEPARATION AND SUBSEQUENT DETECTION USING A 3-MPBA SANDWICH ASSAY WITH SERS

3.1 Introduction

As mentioned in Chapter 1, rapid bacterial detection methods have lower detection thresholds when bacteria is separated, concentrated and purified from complex food matrices. Methods for bacterial separation can be classified as physical, chemical, physiochemical or biological [39]. The separation technique can also be either selective, specifically concentrating a pathogen of interest, or non-selective. Our goal was to develop, or modify an existing bacterial separation technique, to separate and concentrate bacteria from SERS inhibitors within ground beef, specifically lipids.

3.1.1 Adaptation of a Separation Technique Designed to Remove PCR Inhibitors from Ground Beef

To effectively separate and concentrate bacterial cells from ground beef, a technique that was previously developed by Dr. Robert Levin was employed [31]. This technique was used to separate bacterial cells inoculated in ground beef from PCR inhibitors. The method utilizes filtration agents including bentonite, MP-CAC and β-CD used in conjunction with centrifugation to separate, concentrate and purify bacterial cells inoculated in the ground beef. The main PCR inhibitor targeted in this technique was lipids, thus the method would be applicable to remove lipids from ground beef for subsequent bacterial detection with SERS.
3.1.2 Detection of Bacterial Cells Recovered with SERS

After bacterial cell recovery with the previously mentioned separation technique, Raman spectroscopy was used as the bacterial detection platform. This detection platform was chosen due to Raman’s previous success as a bacterial detection platform, its non-destructive sample analysis and time efficiency [13]. To enhance the Raman signaling of biological materials, such as bacteria, metal nanoparticles must be adsorbed to the sample analyte to strengthen inelastic scattering. The non-specific 3-MPBA sandwich assay used for the bacterial detection was previously developed to detect bacteria within simple matrices including spinach and fruit juice. [40, 41]

In this assay, 3-MPBA was used as a capture molecule, to covalently bind 1, 2 and 1, 3 cis diols [42]. The binding is thought to occur between the 3-MPBA capture molecule and glycoproteins present in the cell wall of the bacterial cells.

3.2 Materials and Methods

3.2.1 Bacterial Preparation

*Salmonella enterica* subsp enterica Enteritidis BAA1045<sup>NR</sup>, is a nalidixic acid resistant mutant previously isolated by Dr. McLandsborough’s lab. Using an antibiotic resistant strain allowed for differentiation between SE1045 cells which were artificially inoculated in the beef and microflora naturally present. This strain will hereafter be referred to as SE1045. Frozen cultures of SE1045 were revived in tryptic soy broth (TSB) then cultivated on tryptic soy agar (TSA) slants at 37°C. Fresh slants were made each month from frozen culture. Bacteria cultures for inoculation experiments were prepared by removing a single colony with a loop and transferring to a 9 mL tube of sterile tryptic
soy broth which was grown at 37°C for 18 hours with agitation at approximately 155 rpm.

### 3.2.2 Milk Protein Coated Activated Carbon Preparation

MP-CAC methods were adapted from Dr. Robert Levin’s MP-CAC procedure [36]. The activated carbon was coated with milk proteins at least 4-5 days prior to the experiment to leave ample time for the coated activated carbon to dry. Activated carbon (Calgon Carbon Filtrasorb 200, CAS # 7440-44-000, Pittsburgh, PA) was passed through a Fisher brand 2 mm standard test sieve. Then approximately 44 grams of carbon ranging in size from 0.85-2.00 mm were captured on the top of a Fisher bran 850 µm standard test sieve. The activated carbon was then transferred to a 100 mL flask and washed several times with dH₂O. The carbon was dried at 55°C incubator for 24-36 hours.

Milk proteins isolated from instant nonfat dry milk (Stop & Shop, Landover, MD) were isolated by solublizing 0.45 g in 25 mL of dH₂O in a 250 ml beaker. The milk protein was solubilized in solution by mixing with a magnetic stir bar on medium speed for approximately 2 minutes. The solution was then combined with 50 mL of 95% ethanol and mixed on medium speed with a magnetic stir bar for 2 minutes to precipitate the milk proteins. The precipitated milk protein solutions were pipetted into 250 mL centrifuge bottles. An additional 25 mL of 95% ethanol was used to remove residual milk proteins from the flask, and added to the centrifuge bottles. The samples were then centrifuged at 8000 g (5000 rpm) for 5 minutes at room temperature (20°-25°C). The supernatant was discarded, and the pellet suspended in 150 mL of dH₂O and the pH was adjusted to 9.0 with 0.1 M NaOH.
The dried activated carbon was transferred to a sterile 1 L glass bottle and combined with the milk protein solution. The MP-CAC mixture was placed on a rotary shaker at (150 rpm) at 37°C for 120 minutes. After the incubation period, the excess liquid was poured off and the activated carbon was gently washed 3 times with dH₂O. The MP-CAC was transferred to a 55°C incubator until the mixture had dried (24-48 hours).

### 3.2.3 Ground Beef Preparation

Ground beef (7% fat) was purchased from a local grocery store (Big Y, Amherst, MA) and separated into 25 g portions, which were stored in quart Ziploc freezer bags. Samples were frozen at -20°C until used. Samples were thawed at 4°C approximately 18 hours prior to use.

### 3.2.4 β-cyclodextrin Preparation

β-cyclodextrin (β-CD) (Sigma Aldrich, C4767) 20% stock solutions were prepared by combining 20 g β-CD powder with 100 mL of dH₂O in a 1-L glass bottle. The solutions were heated to approximately 75°C with constant agitation, via a magnetic stir bar, to dissolve the β-CD. Immediately prior to use the solution was cooled to 55°C in a water bath. The β-CD solution is temperature sensitive and would crystallize out of solution when cooled below 55°C.

### 3.2.5 Bacterial Extraction Procedure

The bacterial separation and concentration procedure was adapted from Dr. Robert Levin’s technique to remove PCR inhibitors from ground beef [31]. To study the
recovery efficiency of this technique, 1 mL of the 18h growth SE1045 was added to the
ground beef. Plating occurred throughout the separation technique to quantify the SE1045
cell recovery. For experiments which involved optimization and increasing sensitivity of
the SERS detection, bacteria were added to the beef extract from various steps during or
after the extraction procedure. In Chapter 4, we test the entire processes from inoculated
beef to SERS detection.

Ground beef (25 g) was placed in a Large Whirl-Pak stomacher bag with a filter
and was combined with 75 mL of PBS. The solution was stomached for 120 seconds on
normal speed settings. The cooled β-CD solution (100 mL) was transferred into the
Whirl-pak bag containing the inoculated beef homogenate and stomached for 2 minutes.
The inoculated ground beef homogenate was then pipetted into 50 mL centrifuge bottles,
shaken vigorously and stored on ice for 10 minutes. The cooled solutions were
centrifuged at 1200 rpm for 6 minutes at room temperature (20°C-25°C). At this stage,
the pellet contained beef tissue and β-CD fat complexes.

After centrifugation the supernatant was filtered through a sterile 50 mL syringe,
firmly packed with 0.5 g of glass wool, into a 250 mL centrifuge bottle. The solutions
were again centrifuged at 11,000 rpm for 6 minutes at room temperature. The supernatant
was discarded, and the pellet, containing the bacteria, was suspended in 30 mL of PBS.
The pelleted bacteria were combined with 4.6 g of the prepared MP-CAC and 0.1 g of
bentonite. This mixture was placed on an agitator at room temperature at 150 rpm for 15
minutes.
Following agitation, the solution was filtered through a 50 mL sterile syringe firmly packed with 0.2 g of glass wool into a 50 mL centrifuge bottle. Centrifugation occurred at 12,000 rpm for 6 minutes at room temperatures. The supernatant was removed and the pelleted bacteria were suspended in 30 mL of PBS.

The final 30 mL beef extract sample was either directly used for SERS (if SE1045 were inoculated) or it was divided into 10 mL volumes which were frozen at -20°C. Extract was thawed for approximately 17-18 hours at 4°C prior to use, inoculated with bacteria and used for optimization of SERS assay steps.
Figure 1: Diagram of bacterial separation methodology adapted from Robert Levin’s bacterial extraction procedure to remove bacteria from ground beef prior to PCR analysis.
3.2.6 Bacterial Extraction Recovery Quantification

To determine bacterial recovery through the extraction process, beef was inoculated to a level of approximately $1 \times 10^8$ CFU/g. Plate counts were performed after initial inoculation and at various points in the extraction process by doing a plate count on TSA with naladixic acid 60 µg/ml (TSA$^{\text{Nal60}}$), inhibiting microflora present in the beef samples from growing on this media and inflating cell recovery quantification values. Samples were removed at critical steps within the extraction technique, which will be explained in greater detail in Section 3.1.

3.2.7 3-MPBA Coated Gold Chip Preparation

The sandwich assay and bacterial capture methods noted hereafter were adapted from methods developed by Brooke Pearson [43,40,41]. One day prior to experimentation Au slides (Thermo-Scientific) were cut into approximately 1.5 mm by 2.5 mm rectangles with a glass cutter. Chips were then washed for 30 seconds on each side with dH$_2$O followed by a 100% ethanol washing on both sides for 30 seconds each. The chips were then placed in a solution of 3960 mL of ethanol with 40 µL of 100 mM 3-MPBA (TCI, Portland, OR), in a sterile 5 mL tube. The solution was then placed on a rotary shaker for an 18-24 hour incubation period at room temperature.

After incubation, the 3-MPBA coated gold chips were then washed for 20 seconds on each side with 100% ethanol and placed in a 96 well plate. Chips were covered with 150 µL of 100% ethanol to prevent the 3-MPBA coating from drying out prior to the assay.
3.2.8 Sample Preparation

Ammonia bicarbonate (1 mL of 500 mM) was added to 10 ml of beef extract from section 3.2.5 to increase the sample pH. For the extraction recovery experiments SE1045 was present within the beef homogenate, a 1 mL of the sample was centrifuged at 6500xg for 3 minutes. The supernatant was removed and the sample was suspended in 1 mL of sterile dH₂O. This centrifugation and suspension process was repeated 3 times. After the final suspension, 100 µL of the sample was placed within the experimental wells for SERS analysis.

For optimization and sensitivity experiments, uninoculated frozen beef extract samples were used. Ammonia bicarbonate (1 mL of 500 mM) was added to 10 ml of beef extract from section 3.2.5 to increase the sample pH. In these experiments, 900 µL were removed from the 10 mL beef homogenate/ammonia bicarbonate samples and placed in a 1.5 mL centrifuge tube. To inoculate the beef homogenate, 1 mL of SE1045 was placed in a 1.5 mL centrifuge tube and centrifuged at 6500xg for 3 minutes. The supernatant was removed and the pellet was suspended in 1 mL of sterile dH₂O. This process was repeated 3 times. Then 100 µL from the cleaned SE1045 cells was removed and combined with the 900µL of beef homogenate. After vortexing, this sample was ready for the sample incubation step in the 3-MPBA assay.

3.2.9 Non-Specific Sandwich Assay Procedure

After preparing samples for the assay, the ethanol was removed from each well and the chips were carefully washed 3 times with 200 µL of autoclaved dH₂O. Then 100
µL of each sample created in Section 3.2.8 was pipetted into each well and was incubated in the 96 well plate, covered for 60 minutes at room temperature. During the incubation, the 3-MPBA ammonia bicarbonate layer of the assay was prepared by combining 965 µL of 50 mM ammonia bicarbonate with 10 µL of 10 mM 3-MPBA.

After the incubation, the samples were removed and washed 3 times with 50 mM ammonia bicarbonate. The 3-MPBA ammonia bicarbonate solution was incubated with the chips for 30 minutes at room temperature. The chips were washed with 50 mM ammonia bicarbonate and 100 µL of 60 nm citrate coated Ag nanoparticles (nanoCompsix, San Diego, CA) were applied to the chips. After a 15 minute incubation, the chips were washed 3 times with ammonia bicarbonate and the plate was left open to allow the chips to be air dried for 7-10 minutes. Prior to data collection, the chips were removed from the wells and placed on glass slides to prevent interference between the laser and the walls of the 96 well plates.
Figure 2. 3-MPBA sandwich assay procedure diagram.
3.2.10 Data Collection

After the chips were completely dried and placed on a glass slide, a DXR Raman Spectro-microscope (Thermo Scientific, Madison, WI) was used to analyze the chips. The Raman microscope was set to a 20x microscope objective, 780 nm excitation wavelength and 50 µm slit aperture and a 1 s collection time. The laser power was 5 mW for all maps. The spectral map range was taken from 300 to 2000 nm. For β-CD optimization experiments 100 µm x 100 µm areas were selected with a step size of 5 µm. All other experiments were performed using 200 µm x 200 µm areas with a step size of 10 µm. The resulting SERS spectra was analyzed using Thermo Scientific OMNIC™ software (version 9.1.24) and TQ Analyst (version 9.1.17).
3.3 Results

3.3.1 Efficacy of bacterial separation technique

The main goals of separation techniques are to 1) separate pathogens from macromolecules within the sample 2) remove inhibitory detection molecules and 3) recover the majority of pathogens within the sample without influencing viability of the cells [39]. In order to assess the bacterial separation technique’s utility in our experiments, we first had to determine the recovery rate of the artificially inoculated bacteria from the ground beef.

In this study, nalidixic acid resistant SE1045 was artificially inoculated into 93% lean ground beef. Throughout the procedure, 1 g~1 mL samples were removed 4 times, at critical steps within the separation. The removed samples were then plated TSA<sup>NA</sup>60, to prevent quantification of background microflora present in the ground beef. Plating allowed us to quantitatively determine the bacterial cell recovery throughout the separation process. The first critical step (Step 1) at which plating occurred was after the second stomaching of the solution containing 1 ml SE1045, 75 g PBS, 25 g 7% fat ground beef and ~100 g 10% β-CD. Removal of this sample occurred prior to removing the inoculated beef homogenate from the stomacher bag, this critical step was to determine whether significant bacterial cells remained trapped in large beef tissue which remains in the Whirl-Pak filter bags. At this step an average of 0.17 LOG CFU/g were lost due to the extraction technique.

The second step at which plating occurred was after the 1<sup>st</sup> low speed centrifugation and filtration with glass wool. In these steps, β-CD disrupts the fat
globules within the beef homogenate, releasing trapped SE1045 cells back into the solution [31]. The slow-speed centrifugation then pellets large beef tissue and lipids bound to the β-CD, which is subsequently discarded. Cell quantification was performed in this step to ensure low bacterial losses during the centrifugation. At this step an average of 0.15 LOG CFU/g were lost due to the extraction technique.

The third critical step, (Step 3) at which cells were plated occurred after the solution was incubated with MP-CAC and bentonite and filtered for the second time through glass wool. Quantification was vital at this step because activated carbon has high adsorption capabilities, thus if the carbon was not evenly coated with milk protein, recovered cells would bind the MP-CAC and be removed during the filtration step. At this step an average of 0.19 LOG CFU/g were lost due to the extraction. Lastly, in the fourth critical step (Step 4) cells were plated after the final high speed centrifugation and re-suspension in PBS. This step was performed primarily to determine the overall cell recovery throughout this process. Overall an average of 0.65 LOG CFU/g correlating to 92.8% of inoculated SE1045 cells were recovered (Figure 3). Due to the high percentage of inoculated cells recovered and how rapidly the technique was performed (<2 hours) this separation technique was used for all remaining experiments in which SE1045 was recovered from ground beef for detection with SERS.
Figure 3: Bacterial recovery throughout extraction technique illustrating LOG CFU/g loss of SE1045. In total, 0.649 LOG CFU/g of SE1045 were lost throughout the procedure, resulting in a 93% recovery of SE1045 from artificial inoculation in ground beef. Experimental procedure performed in 3 trials with plate counts performed in duplicate.
3.3.2 SE1045 recovery detection with SERS

After confirming the separation techniques bacterial recovery efficiency, our next objective was to detect the recovered SE1045 cells with SERS. Initially, bacterial detection after the separation technique was not possible. As illustrated in Figure 4, the experimental chip (10 g β-CD) coated with 100 µl of ~1x10^8 CFU/mL SE1045 combined with 900 µl of a previously frozen beef homogenate, which was purified by the separation technique, had no characteristic peaks within the spectra. Specifically the 1023 cm⁻¹ bacteria-MPBA complex peak is absent. In contrast, the positive control spectra, containing 100 µl of ~1x10^8 CFU/mL SE1045 combined with 900 µl of 50 mM ammonia bicarbonate elicited the bacterial peak.
Figure 4: Spectra and chemical mapping deduced from 3 trials performed to detect SE1045 artificially inoculated in ground beef homogenate. In these trials a 10% (10g/100 mL) β-CD solution was used to remove lipids and large tissue from the ground beef. Chips denoted as 10 g β-CD and the positive control both contained ~1x10⁸ CFU/mL SE1045. While the negative control chips contained only beef homogenate without any artificially inoculated bacteria.
The lack of bacterial detection in the experimental chip was further emphasized by the chemical mapping performed on these chips (Figure 4). Chemical mapping creates a simplified grid of the chip of interest illustrating the presence (red) or absence (blue) of a particular peak. In our study, the bacterial-MPBA complex peak at ~1023 cm$^{-1}$ was used to create all chemical maps. Essentially, red boxes on the map emphasize the presence of our characteristic bacterial peak, while blue boxes emphasize the absence of this peak. In Figure 4, the chip with the inoculated beef homogenate has only a 3% presence of the 1023 bacterial peak. In contrast the positive control, containing the same amount of bacteria has a 94% positive presence of the 1023 peak. Thus, it was hypothesized that a component within the beef homogenate was inhibiting bacterial detection.

### 3.3.3 Influence of β-CD concentration on SERS detection

As previously mentioned, separation techniques are capable of removing the majority of detection inhibitors. However, separation techniques are not capable of removing all detection inhibitors. As shown by Figure 4, residual inhibitory compounds remained in the beef homogenate preventing bacterial detection with SERS. In order to detect inoculated bacterial within the beef homogenate, the separation technique was optimized to further reduce residual lipids within the sample. Previously, a 10% β-CD stock solution was used in the separation technique. A 10% stock solution was initially used because it was the concentration that Levin et. al used to remove lipids from ground beef for bacterial detection with PCR [31]. As shown in Figure 5, increasing the concentration of the β-CD stock to 20% and 30% allowed bacterial detection.
Figure 5: Spectra and chemical mapping produced from 3 trials comparing β-CD’s influence on SERS detection of bacteria. Increasing the concentration of the β-CD allowed detection of artificially inoculated bacteria in samples produced from an extraction utilizing 20g and 30g of β-CD. The same amount of bacteria (~1x10^8 CFU/ml SE1045) was used in all concentrations.
The averaged spectra present in Figure 5 represent 3, 20x20 maps, performed at each concentration in 3 separate trials. Beef homogenates procured from the extraction with these varied β-CD concentrations was inoculated with the same amount of bacteria (~1x10^8 CFU/mL SE1045). As shown, Raman signal intensity is strongest when a 20 g β-CD solution was used in the separation technique. While the Raman intensity is similarly strong when 30 g β-CD, using 20 g was more cost effective and similarly effective in removing SERS inhibitors thus it was used for all future experiments. The Raman signal intensity was enhanced from -1.8 to 88.34 when using 10 g and 20 g β-CD respectively in the extraction process.

3.3.4 SERS Detection throughout Extraction Process

Similar to other rapid detection methods, our goal was to detect bacteria as rapidly as possible within our system. Thus, we had to determine whether bacterial detection with SERS was possible earlier in the extraction process. As mentioned previously, inhibitory molecules within complex matrices, specifically lipids prevent bacterial detection with SERS. Thus our goal was to determine the critical step in the extraction process in which the majority of the lipids within the ground beef had been separated from the inoculated bacteria, allowing detection with SERS. In doing so, the 3-MPBA assay was performed on samples removed at 4 steps within the extraction process. The four steps at which detection was attempted were the same steps at which extraction recovery efficiency was measured in section 3.3.1.
Figure 6: Chemical mapping and spectral images showing optimization of detection throughout extraction process. The 3-MPBA SERS assay was performed on samples removed at four steps within the extraction process. Samples were taken at the same steps as those described in Figure 3 & Section 3.3.1. Samples taken at all four steps were artificially inoculated with the same amount of bacteria (~$1 \times 10^8$ CFU/ml SE1045) and 20 g β-CD. Detection of SE1045 in beef homogenate was only possible at Step 4.
As shown in Figure 6, bacterial detection occurred only after the entire extraction process had been performed on the ground beef. Thus, inhibitory compounds including lipids and residues from the filtration agents are present and hinder SERS detection until the final centrifugation step in the extraction process. The presence of inhibitory molecules resulted in chemical mapping which was also unable to detect the bacterial peak until the final step of the extraction process. As noted in Figure 6, 3% or less of the chip exhibited positive signaling for the 1023 bacterial peak in Steps 1-3. Whereas ~83% of the chip exhibited positive signaling for the 1023 peak after the entire extraction process had been performed on the beef homogenate. Ultimately, the extraction process cannot be simplified for more rapid bacterial detection.

**Figure 7:** Optical images of inhibitory molecules present on the chips during the bacterial extraction process. Images produced from samples in Steps 1-3 as compared to the homogenous distribution of bacteria on a chip produced from a sample removed at Step 4.

As shown in Figure 7, chips produced from samples throughout the extraction process were optically different from one another. Chips produced from samples taken at Steps 1 & 2, prior to MP-CAC incubation and filtration had heterogeneously dispersed
clumps of inhibitory molecules which prevented bacterial detection. While the spectra and chemical mapping was not performed on areas covered in these inhibitory molecules, the remainder of the chip lacked the “black dot effect”, which is thought to be colonies or clumps of bacteria [43, 41]. In Step 3, the chip was covered in unknown inhibitory molecules that also prevented bacterial detection. However, chips produced from samples containing the final beef homogenate had a homogenous distribution of the “black dots” or bacterial colonies and clumps. This was the only chip to give Raman signaling at the 1023 cm\(^{-1}\) bacterial peak.

### 3.3.5 Optimization of the 3-MPBA Assay for bacterial detection in complex matrices

After optimizing the extraction process for bacterial detection with SERS, our next objective was to optimize the 3-MPBA assay to achieve enhanced Raman signaling with samples derived from complex matrices. To achieve this, a small alteration was made to the first step of the assay. In the first step of the assay, cut gold chips are incubated in a 3-MPBA ethanol solution overnight. Previous work using this assay incubated the chips on a shaker at room temperature ~20-23°C. However, as noted in Figure 8, after incubating chips at refrigeration temperatures (~4°C) overnight there was enhanced Raman signaling as compared to chips incubated at room temperature. The change in incubation temperature enhanced Raman intensity at the 1023 peak from 30.79 to 110.94.
Figure 8: Optimization of 3-MPBA coating incubation temperature. Spectra produced comparing 3-MPBA coating incubation temperatures influence on Raman signal intensity of SE1045. As shown, signal intensity is approximately 3-4x stronger on chips incubated at 4°C as opposed to chips incubated at room temperature 20-23°C.

3.4 Discussion

One of the biggest drawbacks to rapid method technology is the difficulty in detecting and quantifying low levels of bacteria within a food matrix composed of detection inhibitors including polysaccharides, antibiotics, proteins and lipids. To combat this obstacle, a bacterial extraction procedure is necessary to concentrate and purify cells from a heterogeneous food matrix. Previous studies have employed techniques such as immuno-magnetic separation [44,45] metal hydroxide based concentration [46] and centrifugation and filtration methods [30,36,37] to effectively remove the bacteria from the inhibitory components within a food matrix.
### 3.4.1 Efficiency of bacterial extraction procedure

When a density gradient centrifugation separation technique was used to remove *E. coli* O157:H7 from ground beef, only 20-45% of inoculated cells were recovered [47]. Differential Percoll gradients were used to separate bacteriods from soybean plants. This separation technique was capable of recovering ~90% of cells from the matrix [48]. In our study, the combination of centrifugation and filtration recovered 92.8% of the artificially inoculated SE1045 from ground beef (Figure 3).

In step 1 of our extraction process, there was a possibility for bacterial loss due to adsorption to filtration materials. In a study using crude filtration with cheesecloth, one of the main challenges was the bacterial cells adsorbed to the filter [49]. However, in our technique only 0.17 LOG CFU/g were lost due to adsorption to the Whirl-Pak filter. In step 2, there was a possibility that bacteria would be pelleted with large tissues that bound β-CD and were discarded. To pellet bacteria, a speed of $\geq 3000$ rpm is typically used, thus this centrifugation at 1200 rpm should only pellet minimal amounts of bacterial cells. In this step, 0.145 LOG CFU/g SE1045 cells were lost. In step 3 there was a possibility of SE1045 adsorbing to the activated carbon. Previous studies using MP-CAC, showed a 95.7% cell recovery with a coating of 0.45 milk protein/44 g of activated carbon [36]. In this step 0.185 LOG CFU/g SE1045 cells were lost due to adsorption to the MP-CAC. Overall this extraction process was rapid (<2 hours) and efficient (92.8% SE1045 recovery) enough to be used for all further experiments.
3.4.2 β-CD’s concentration’s influence on SERS detection

After confirming the efficacy of the separation technique, a 3-MPBA assay was used to bind the extracted bacteria to a gold chip prior to SERS analysis. To accomplish this detection, a non-specific 3-MPBA sandwich assay, created by Brooke Pearson, was employed [43,40,41]. Boronic acids are capable of forming cyclic boronate ester bonds by binding 1,2 and 1,3 cis diols present on saccharide structures [50]. In this assay, 3-MPBA which is coated to a gold chip overnight, binds carbohydrate groups on the outer membrane lipopolysaccharides of the bacterial cells [51]. The binding between cell surface structures and the 3-MPBA molecule results in a characteristic peak at ~1023 cm$^{-1}$. This peak is hypothesized to be due to a structural orientation, or change in charge when the 3-MPBA bacterial cell complex is formed [50]. Based on the chemical mapping and the spectral images shown in Figures 4 and 5, produced after increasing the β-CD concentration, it is hypothesized that when higher amounts of lipids are present in the beef homogenate bacteria become trapped within and under these molecules preventing the laser from detecting the bacteria-3-MPBA complex. By increasing the concentration of the β-CD, more lipids are removed during the separation technique and the assay is capable of evenly binding the inoculated SE1045. Thus, all experiments hereafter were performed with a 20 g β-CD solution. While bacterial detection was possible at higher concentrations, choosing to use 20 g was the most cost effective and efficient concentration. Thus, while a 10% β-CD solution was appropriate for bacterial detection with PCR, a 20% β-CD solution is necessary for bacterial detection with SERS.
3.4.3 SERS earliest detection in extraction process

As shown in Figure 6, SERS inhibitory compounds interfere with bacterial detection in Steps 1-3. However in Step 4, the sample has been purified, which removed the majority of detection inhibitory compounds. It is hypothesized that in Steps 1 & 2 the large masses present in images shown in Figure 7 are lipids. Bacteria within the samples becomes trapped within or under these lipids, thus when the laser irradiates the sample the bacteria are undetectable. While no studies have currently investigated lipids inhibitory effect on SERS detection, many studies utilizing rapid techniques have addressed the need to separate inoculated bacteria from inhibitory components within the food matrix due to the food matrix effect in ground beef [52, 53, 54, 55,56]. The food matrix effect is generally bacterial detection interference by one or more compounds present in the food matrix, resulting in low detection sensitivities [52].

In Step 3, the majority of hypothesized lipids are no longer present on the chip; however residues from the filtration agents MP-CAC and bentonite coat the chip impeding bacteria detection. These residues from the filtration agents remain in the solution until they are removed in the final centrifugation step. Similar to the lipids mentioned above, the filtration agent residues coat the gold chip preventing bacteria detection. Ultimately, the entire separation technique must be performed prior to the 3-MPBA assay for bacteria detection via SERS.

3.4.4 Influence of Temperature on 3-MPBA assay

As shown in Figure 8, decreasing the incubation temperature of the gold chips coated in 3-MPBA from room temperature (20-23°C) down to refrigeration temperature
(4°C) increased the Raman intensity of the bacterial peak at 1023 cm\(^{-1}\). Distributors of 3-MPBA, Capot Chemical, suggest the chemical be stored in cool, well ventilated locations [57]. Previous work done utilizing this chemical discussed storing both the powder form and the 3-MPBA solution at refrigeration temperatures however the 3-MPBA coating incubation occurred at room temperatures [43]. It is hypothesized that incubating the 3-MPBA overnight at room temperature results in degradation of the chemical resulting in a heterogeneously distributed monolayer bound to the gold chip. When the chip is incubated at cooler temperatures (4°C) is not degraded and thus forms a more homogenous layer bound to the gold chip. Thus, the cooler incubation temperatures allow a more evenly bound sample layer of bacteria resulting in higher Raman signaling for the chips incubated at 4°C.

### 3.5 Conclusion

Throughout this chapter, a separation technique which extracts bacteria from ground beef was employed and optimized to produce a beef homogenate bacterial sample which was used for SERS bacterial detection. The efficacy of the separation techniques bacterial removal procedure was evaluated based on the bacterial cell recovery. Bacterial cell recovery in this experiment was determined to be 92.8%. Samples produced from this separation technique then were used for a 3-MPBA sandwich assay. Bacterial detection of recovered SE1045 occurred after increasing the concentration of the β-CD stock solution from 10-20%. Both the separation technique and the assay were optimized to achieve the highest Raman signal intensity by investigating the step in the separation technique at which bacteria detection was first possible and by decreasing the incubation
temperature of the 3-MPBA coated gold chips. In the following chapter, objectives 4-5 as noted in Chapter 2 will be explored.
CHAPTER 4

INVESTIGATION INTO THE 3-MPBA SANDWICH ASSAY’S EFFICACY FOR BACTERIAL DETECTION IN GROUND BEEF

4.1 Introduction

There has been continuing development of novel technologies capable of rapid high throughput detection of pathogenic bacteria within food products in the past decade. Ideally, these detection platforms would not only be rapid but also cost effective, highly sensitive and specific to a target pathogen [58]. Previous studies have shown SERS to be a potential rapid bacterial detection platform due to its rapid sample preparation and detection [22,30,25, 23, 59, 60, 61]. In Chapter 3, we discussed optimization of a rapid separation technique that can be used to separate bacteria from inhibitory food matrix components. Thus, preparing a bacterial sample for detection with SERS. Due to the effectiveness of this separation technique combined with SERS detection, a further investigation into the 3-MPBA sandwich assay’s efficacy was performed. Specifically, these experiments determined the bacterial detection limits, the capture efficiency and the strain differentiation capabilities of this assay with bacteria separated from a complex matrix.

4.2 Materials and Methods

4.2.1 General Methods Overview

The bacterial separation technique and 3-MPBA assay were performed as noted in Section 3.2. All experiments were performed with frozen uninoculated beef samples with the exception of the “entire process” experiment in which the raw beef was artificially inoculated with SE1045.
4.2.2 Sample Preparation for bacterial extraction immediately followed by SERS detection experiment

For this experiment, the separation technique was performed with artificially inoculated beef as outlined in Section 3.2.5. For 3-MPBA assay preparation 1 mL of the SE1045 recovered from beef was removed and placed in a 1.5 mL centrifuge tube. The sample was centrifuged at 6500xg for 3.5 minutes and the supernatant was removed and replaced with 1 mL of sterile DiH2O. This wash step was repeated 3 times, then 100 µL was placed in a well with a gold chip in a 96 well plate for 1 h at room temperature. This method varies from the sample preparation outlined in Section 3.2.8 because the SE1045 cells were already inoculated into the beef prior to the separation technique in contrast to experiments in which a frozen uninoculated beef homogenate is combined with cleaned SE1045 cells.

4.2.3 Sample Preparation for Detection Limit Experiments

For detection limit experiments, sample preparation occurred according to the methods outlined in Section 3.2.8. After producing the first sample containing 100 µL of cleaned SE1045 cells and 900 µL frozen beef homogenate the sample was serially diluted in beef homogenate using a series of 1/10 dilutions (100 µL into 900 µL of beef homogenate). A 100 µL sample from each dilution was placed in a well containing a gold chip and the 3-MPBA assay was performed. The initial inoculum was diluted in TSB and plated on TSA N60 for quantification of cells at each dilution level.
4.2.4 Sample Preparation for Capture Efficiency Work

For capture efficiency experiments, sample preparation occurred according to methods outlined in Section 3.2.8. Quantification of the cells placed in each well was performed by removing 100 µL of the SE1045 beef homogenate sample and diluting in a series of 900 µL TSB, then plating on TSA\textsuperscript{NA60}. After the 1 hour 3-MPBA assay sample incubation period elapsed, the 100 µL inoculated sample was removed and placed in 900 µL TSB which was diluted and plated on TSA\textsuperscript{NA60}. Capture efficiency was determined by subtracting the SE1045 cells that were removed after the 1 hour incubation from the total SE1045 cells that were placed in the wells prior to the incubation.

4.2.5 Bacterial preparation for strain differentiation

For the strain differentiation experiment, five different nalidixic acid resistant \textit{Salmonella enterica} strains were employed. The following strains including \textit{Salmonella enterica} subsp enterica BAA1045, BAA711, BAA710, BAA709 and BAA708 were used. Frozen cultures of all 5 strains were revived in tryptic soy broth then cultivated on tryptic soy agar slants at 37°C. Fresh slants were made from frozen culture. Bacteria cultures for inoculation experiments were prepared by removing a single colony with a loop and transferring to a 9 mL tube of sterile tryptic soy broth which was grown at 37°C for 18 hours with agitation at approximately 155 rpm.
4.3 Results

4.3.1 Determination of bacterial detection limits for 3-MPBA assay in complex matrices

One of the primary purposes of performing a separation technique prior to bacterial detection is to improve the sensitivity of the detection platform. Thus, by removing detection inhibitors, the separation technique combined with SERS analysis should be comparable to detection performed on samples with few inhibitors within the matrix. A previous study determined the sensitivity of the 3-MPBA assay to be very low with detection of SE1045 at $1 \times 10^2$ CFU/mL with bacteria inoculated in spinach leaves [43]. Until now, the assay’s sensitivity had not been tested with bacteria recovered from a complex matrix such as ground beef. As shown in Figure 9, detection of SE1045 recovered from ground beef was possible as low as $1 \times 10^2$ CFU/mL.
Figure 9: Bacterial detection limit averaged spectra produced from 4 trials (20x20 maps, step size 10 µm). Bacterial detection was possible as low as $1 \times 10^2$ CFU/mL SE1045 in beef homogenate.

As illustrated by Figure 10, the Raman intensity at the $1023 \text{ cm}^{-1}$ bacterial peak decreases as the concentration of SE1045 suspended in beef homogenate also decreases. Raman intensity decreases due to fewer bacteria present on the chip as the concentration of the SE1045 in the beef decreases. The chemical imaging also illustrates binding variability that is present not only between chips but also between trials.
Figure 10: Chemical imaging performed highlighting presence/absence of the \( \sim 1023 \text{ cm}^{-1} \) bacterial peak on varied concentration of SE1045 in beef homogenate. Each map represents one replicate from four trials (400 point map, 20x20, 10 \( \mu \text{m} \) step size, red indicates presence of 1023 peak).

Based on the data presented in Figures 9 & 10, standard curves were created based on Raman signal intensity and the presence of the 1023 bacterial peak derived from chemical mapping.
Figure 11: Relationships between SE1045 concentration and percent of boxes positive for the bacterial peak at 1023 and SE1045 concentration and Raman signal intensity. Data based on chemical imaging and spectral data shown in Figure 9 & 10.
4.3.2 Determination of Capture Efficiency of 3-MPBA assay with complex matrices

Bacterial detection platforms not only need to be highly sensitive but also must be able to efficiently bind bacteria from a solution prior to detection. To quantify this concentration, capture efficiency studies are performed to show the ability of an assay to bind and concentrate bacteria from a sample. In a previous study, the capture efficiency of SE1045 using the 3-MPBA assay was assessed. In this study, the assay captured 91.7% of the bacterial cells incubated on the chip [43]. However in this study, a pure culture of SE1045, free from detection inhibitors was used. Thus, this does not aptly illustrate the capture efficiency of the 3-MPBA assay used for bacteria recovered from a food matrix.

As shown by Figure 12, SERs signal intensity is stronger for SE1045 removed from a pure culture than that of the SE1045 recovered from a beef extract. As discussed previously, this likely occurs because the SE1045 recovered from a beef extract sample contains SERS detection inhibitors, specifically lipids which bind the bacteria and inhibit detection. While, the separation technique aptly removes the majority of the inhibitory molecules, residual lipids will always be present in the SE1045 recovered from ground beef.

Optical images from 1 of the 4 trials performed indicate visual differences between the SE1045 recovered from beef extract and SE1045 from pure culture. The image from the pure culture containing 5.39x10⁶± 2.10x10⁶ CFU/mL SE1045 appears to have bound a higher concentration of the SE1045 from solution as compared to the SE1045 recovered from beef extract containing 7.44x10⁶ ±2.49x10⁶ CFU/mL SE1045. This is indicated by the presence of more “black dots” on the chip in Figure 12.
Figure 12: Spectral data and optical images comparing bacterial capture efficiency of SE1045 recovered from beef extract and SE1045 from pure culture.
**SE1045 cells bound to the chip & well**

\[ = SE1045 \text{ cells incubated} - SE1045 \text{ cells recovered} \]

**Figure 13:** Capture efficiency of the 3-MPBA assay derived from plate count data. Four trials were performed for both SE1045 from beef extract and Pure SE1045 and plate counts were performed in duplicate.

To further understand the properties of the 3-MPBA assay, a capture efficiency experiment was performed to determine whether the inhibitory molecules present in the SE1045 recovered from beef not only reduced Raman signal intensity, as compared to a pure SE1045 sample, but also if the inhibitory molecules influenced the bacterial chip binding.
After performing the capture efficiency experiment the percent of cells bound to both the chip and well was determined by subtracting the cells recovered (cells unbound) from the initial inoculum which was incubated. Preliminary data showed that 51% and 41% of the SE1045 recovered from beef and SE1045 from pure culture respectively bound to the chip and the well in which the chip was incubated within. Essentially after the 1 hour incubation period 49% and 59% of the cells recovered from the beef and from the pure culture remained unbound and were removed prior to proceeding with the washing step. However, this bacterial binding efficiency quantification neglected to quantify the amount of cells bound to the well compared to the amount of cells preferentially binding the chip.

In determining whether SE1045 from pure culture and SE1045 in beef homogenate preferentially bound the 3-MPBA chip we conducted a secondary control experiment in which we incubated 100 µL of cleaned SE1045 cells in an empty well to determine how many cells bind the well during the incubation period. Raw data derived from plate counts derived from both of these experiments were used to determine the % of cells bound to the well. Based on the data shown in Figure 14, SE1045 preferentially binds the 3-MPBA coated chip as opposed to the well. However, the capture efficiency of this assay is not strong due to the data showing that half or more of the cells incubated did not bind either surface.
Figure 14: Capture efficiency of the 3-MPBA assay based on % of pure SE1045 and % of SE1045 from beef extract cells bound and unbound after 1 hr incubation.
4.3.3 3-MPBA assay Strain Differentiation Capabilities

While the sensitivity of a detection platform is highly important, the specificity of that detection method is equally important. The specificity of a detection platform allows differentiation between different bacterial genus, species and serotypes. The specificity of a detection platform is highly important when targeting a specific pathogen in a food commodity.

In our experiments a non-specific sandwich assay was used to bind bacteria extracted from ground beef. This non-specific assay was capable of binding both the SE1045 which was extracted from the ground beef matrix and the background microflora of the beef. This non-specific sandwich assay had already been shown to bind both Gram negative and Gram positive bacteria [43]. Spectra produced from binding both forms (Gram+/−) were identical. However, no previous work had been performed on strain differentiation with the 3-MPBA assay. For this work, 5 different nalidixic acid resistant strains of Salmonella enterica subsp enterica (BAA1045, BAA711, BAA710, BAA709 and BAA708) at the same concentration, were combined with frozen beef homogenate.

As shown by Figure 15, the spectral patterns produced from the five strains were similar. The Raman intensity produced from the five different strains, which represents an average intensity derived from 3, 400 points maps, was 14.18, 6.61, 17.02, 9.80 and 33.24 for Salmonella enterica subsp enterica BAA1045, BAA711, BAA710, BAA709 and BAA708 respectively. While there was variation in the averaged Raman signal intensity between the strain with the highest signal intensity, BAA708, and that with the lowest signal intensity, BAA711, it is unlikely that this variability would be able to
distinguish between the strains. The inability to differentiate between the strains was further emphasized by the similarities in the chemical mapping performed on the five strains as shown in Figure 16. A PCA analysis was performed to further confirm our inability to differentiate between the five strains. Results are shown in Figure 17.

![Figure 15: Spectral data comparing 5 strains of nalidixic acid resistant Salmonella enterica. Spectral data represents the average signal intensity for 3,400 point maps for each strain.](image)
Figure 16: Chemical mapping comparing 5 nalidixic acid resistant *Salmonella Enterica* strains.
Figure 17: PCA analysis of spectra illustrating the inability to differentiate between 5 \textit{Salmonella enterica} strains based on 1200 spectra obtained for each strain.

4.3.4 Bacterial Extraction and Detection Simultaneously

The majority of the experiments discussed were performed with frozen beef homogenate, which was inoculated prior to the assay procedure, due to the extensive time required for mapping. However, it was critical to ensure that detection was possible after inoculating the beef prior to the extraction technique. While we had already determined that 92.8\% of the cells were recovered from the ground beef, it was vital to show that the bacteria could be separated and detected simultaneously to mimic a rapid detection of a pathogen contaminated ground beef sample.

As shown by the spectra in Figure 18, SE1045 was detected via the 3-MPBA assay after extracting the bacteria from a fresh ground beef sample with an identical spectral pattern as spectral data derived from frozen beef homogenate samples inoculated with SE1045. The Raman signal intensity for the extraction simultaneously followed by
the assay was 15.86 at \( \sim 1 \times 10^6 \) CFU/mL of SE1045. This signal intensity was somewhat lower than the average Raman signal intensity at this bacterial concentration (29.33).

Chemical imaging shown in Figure 19, performed on samples extracted from the ground beef were similar to chemical images derived from frozen beef homogenate samples inoculated with SE1045. For the samples extracted from beef 80.2% ± 15.9% of the area of the chips were positive for the 1023 cm\(^{-1}\) bacterial peak. For samples at the same concentration \( (1 \times 10^6 \text{ CFU/mL SE1045}) \) in which frozen beef was inoculated 86.8%±6.05% of the area chip was positive for the 1023 cm\(^{-1}\) bacterial peak. The bacterial extraction procedure, assay and analytical mapping were performed in under 8 hours for these experiments.

**Figure 18:** Spectral images produced from the average of 6, 400 point maps taken SE1045 extraction from ground beef. The bacterial extraction, assay procedure and detection occurred in less than 8 hours.
Figure 19: Chemical imaging performed on samples of SE1045 extracted from ground beef. Three trials with two replicates in each trial illustrates the reproducibility of the extraction sequentially followed by SERS detection.

4.4 Discussion

While the previous chapter focused on optimizing both the bacterial extraction technique and the 3-MPBA assay when used with complex food matrices, this chapter highlighted the sensitivity, specificity and time efficiency of this rapid detection method. In terms of sensitivity, the extraction procedure combined with the 3-MPBA assay had a lower detection or was equally as sensitive compared to PCR based methods, biosensor based methods and previous SERS bacterial detection work [62, 63, 64, 65, 66, 28, 27]. The 3-MPBA assay was shown to preferentially bind SE1045 cells extracted from the beef matrix and similar spectral patterns were shown for 5 different strains of Salmonella.
*Salmonella enterica* inoculated in beef homogenate. Lastly, the entire process including bacterial extraction, assay incubation and analytical chemical mapping was performed in less than 8 hours, considerably faster than conventional culturing methods.

### 4.4.1 Limit of Detection Discussion

As discussed in Chapter 1, a variety of methods have been employed for bacterial detection in food matrices. One of the most important properties of these detection platforms is the sensitivity of the method. DNA based methods, such as Real time PCR (RT-PCR) have been shown to detect *Listeria monocytogenes* at $1 \times 10^3$ CFU/g in ground beef, *Salmonella enterica* at $1 \times 10^3$ CFU/mL in milk and *E.coli* O157:H7 in ground beef at $<500$ cells/mL [62, 63,67].

Other methods employ biosensors to facilitate the bacterial detection process. Surface Plasmon resonance is an optical biosensor that uses polarized light to generate surface plasmon waves. Due to shifts in resonance, a refractive index of the sample can be generated to determine bacterial density [68]. Previous SPR studies have detected *Salmonella enteritidis* as low as 23 CFU/mL in milk and *Salmonella Typhimurium* at levels of $1 \times 10^2$ CFU/mL in chicken carcasses [69,64]. Electrochemical biosensors such as amperometric biosensors and potentiometric biosensors have been able to detect *Listeria monocytogenes* in milk at $1 \times 10^2$ CFU/mL, *E.coli* O157:H7 in milk at $1 \times 10^3$ CFU/mL [65,66].

For our work, SERS was employed as the bacterial detection platform. SERS has previously been used to detect *E.coli* O157:H7 at levels of $1 \times 10^2$ CFU/mL in apple juice, *Salmonella Typhimurium* at $1 \times 10^3$ CFU/mL in spinach and *E. coli* O157:H7 in mung bean
sprouts at levels of 100 CFU/mL [28, 27, 10]. However, very few studies have used SERS for bacterial detection in complex matrices. In our study, SE1045 was detected at levels as low as 1x10^2 CFU/mL as shown in Figures 9-11. Based on information displayed in Figure 12, it is likely that SERS is actually detecting less than 1x10^2 CFU/mL of SE1045 since the chips only bind approximately 51% of the cells incubated. Thus, the sensitivity of our separation technique used in conjunction with the 3-MPBA assay is quite high. This is further emphasized when considering that our methods, which were performed on a complex matrix with SERS inhibitors, have similar, if not a higher sensitivity than methods performed on simple matrices which contain fewer SERS inhibitors.

As shown in Figure 11, it is hypothesized that the chips become saturated with bacteria at \( \sim 1 \times 10^5 \) CFU/mL. At this concentration and higher the majority of chip is positive for the 1023 cm\(^{-1}\) bacterial peak. Thus, it is difficult to discern concentration differences in chips at concentrations higher than this because a similar amount of the chip will be positive for the bacterial peak. This occurs because on a 20x20 map with a step size of 10 µm there is a total area of 40,000 µm\(^2\). Thus, the area is saturated beyond 4x10^4 CFU/µm\(^2\) and the Raman intensity will continue to increase but the % of area positive for the 1023 bacterial peak will remain steady. However, if a target pathogen was present at concentrations higher than 1x10^5 CFU/mL in a food product, it is likely that a foodborne illness would arise from consumption of this food. Thus, if this system was to be implemented into an industrial food setting it is unlikely that this drawback of our system would be detrimental.
4.4.2 Capture Efficiency Discussion

A previous study performed utilizing the 3-MPBA assay was capable of binding 91.7% of cleaned SE1045 cells exposed to a gold chip [43]. In this previous study, the area of the chips used in this incubation is unknown. In contrast, after performing 4 trials the capture efficiency of the same 3-MPBA assay when exposed to cleaned SE1045 from pure culture and SE1045 in frozen beef homogenate bound 41% and 51% of the incubated cells. For our work, a gold chip 10.5 mm$^2$ in size was placed in a 32 mm$^2$ well. The gold chip only covered approximately 32% of the area of the well. After performing a secondary control experiment we were able to determine that the incubated SE1045 cells preferentially bind the 3-MPBA chip as opposed to the well. Based on the capture efficiency data shown in Figures 13 & 14 it is clear that the capture efficiency of this assay should be improved to bind more than 50% of the cells incubated in the well. Also, another experiment should be performed in which the well control and an uncoated chip in a well is compared to the recovery of a 3-MPBA coated chip.

As more work is performed on SERS bacterial detection, it is likely that the 3-MPBA assay will be adapted with an aptamer or antibody based method in which only target pathogens will bind the gold chips. Thus, future studies should focus on enhancing the capture efficiency of this assay.

4.4.3 Strain Differentiation Discussion

The specificity of a detection platform is one of the most important properties of the detection method. The ability to discriminate between microorganisms at the serotype level is critical for pathogen detection and identification in food commodities. A previous SERS study evaluating silver nanoparticles (AgNP) produced by oblique angle deposition
was unable to discriminate between spectra produced from *E. coli* O157:H7, *E. coli* DH 5α, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Salmonella typhimurium* [25]. The spectral patterns produced by the bacterium were qualitatively similar but the signal intensity varied. A different study which employed silver nano-spheres composed of silver nanocrystals used canonical variate analysis of the Raman spectra to discriminate between *E. coli* O157, *S. typhimurium*, and *S. aureus* [70]. Silver nanosubstrates were also used to differentiate between *E. coli* O157:H7, *S. epidermidis*, *Listeria monocytogenes*, and *Enterococcus faecalis* [23]. In this study cells were treated with sodium borohydride and sodium nitrate which facilitated internalization of the AgNP’s. Another study discriminated between 5 Bacillus strains (*Bacillus cereus* ATCC 13061, *B. cereus* ATCC 10876, *B. cereus* sp., *Bacillus subtilis* sp. and *Bacillus stearothermophilus* sp.) by performing PCA and hierarchal cluster analysis on the Raman spectra [26].

Based on the spectral data obtained in Figure 15, it is evident that the same spectral pattern occurs when performing the 3-MPBA assay on each of the strains incubated in beef homogenate. It is also evident that the binding and approximate area of the chip positive for the 1023 cm\(^{-1}\) peak is similar amongst the five Salmonella strains used, as shown in Figure 16. The PCA analysis shown in Figure 17 further emphasized that we are unable to differentiate between the Salmonella strains used in this experiment. To differentiate between different bacterial strains it is likely that the 3-MPBA assay will need to be modified with either an aptamer or antibody based detection method which only allows specific binding of target organisms.
4.4.4 Bacterial extraction and subsequent detection discussion

One of the most critical properties of a rapid detection platform is how quickly bacteria within a sample can be detected. Many rapid detection platforms such as immunoassays and PCR require an enrichment step at which viable bacterial cells are grown to a detectable level. This enrichment step requires 8-24 hours of growth prior to enumeration [58]. However, in our system an enrichment step is not required and bacterial extraction and detection can occur in less than 8 hours. As shown in Figures 18 & 19, our system is capable of detecting bacteria immediately after extraction from the ground beef matrix. The extraction process requires approximately 2.5 hours followed by 2.5 hours of assay preparation and incubation of samples. The remaining time is used to obtain spectra and perform mapping which can occur more rapidly on new Raman models. Thus in our system, not only can detection occur when SE1045 is inoculated in thawed beef homogenates but also in samples in which the SE1045 is extracted from fresh ground beef.

4.5 Conclusion

In this chapter, properties including sensitivity, specificity, time efficiency and capture efficiency, of our system were elucidated to further understand its usefulness as a rapid detection platform. Detection of SE1045 was capable at levels as low as $1 \times 10^2$ CFU/mL. The extraction procedure and 3-MPBA assay performed sequentially on inoculated ground beef was capable of SE1045 detection in less than 8 hours. The non-specific nature of the 3-MPBA assay prevented discrimination of five different Salmonella strains. Preliminary capture efficiency work showed that ~50% of incubated
SE1045 cells bound the chip and well, thus more work should be performed to optimize the capture efficiency of this assay.
CHAPTER 5

CONCLUSION

The adaptation of a bacterial extraction procedure, originally designed for PCR, used in combination with a 3-MPBA nonspecific sandwich assay followed by SERS detection has been shown to be a powerful detection platform for the detection of SE1045 extracted from ground beef. The bacterial extraction procedure has minimal cell losses throughout the process (0.67 LOG CFU/ml). The extraction procedure has also shown that the majority of SERS detection inhibitors, specifically lipids, are removed by using β-CD concentrations of 20% or higher. Bacterial detection of SE1045 extracted from ground beef was capable at levels as low as 1x10^2 CFU/mL, emphasizing the high sensitivity of our system. Due to the non-specific nature of the 3-MPBA sandwich assay, the assay is incapable of differentiating between five different strains of Salmonella. More work should be performed to better understand and optimize the capture efficiency of the 3-MPBA assay. Further development of this system should focus on 1) utilizing more cost efficient filtration agents in the extraction procedure such as hydrolyzed corn starch 2) improving the specificity of the 3-MPBA assay by introducing an aptamer or antibody based property which targets specific pathogens and 3) adapt the system to other complex matrices including ground poultry and dairy products.
Figure A1: Optical density and standard plate count growth curves of SE1045.
REFERENCES


and isolation of escherichia coli O157: H7 in a food matrix using flow cytometry and
cell sorting. *FEMS Immunology & Medical Microbiology, 19*(4), 267-274.

spectroscopy applied to food safety. *Annual Review of Food Science and
Technology, 4*, 369-380.

(2003). *Vibrational microspectroscopy of food. raman vs. FT-IR*doi:https://doi.org/10.1016/S0924-2244(02)00243-1

using SERS. *Chemical Society Reviews, 37*(5), 931-936.

based surface-enhanced raman scattering for pathogen bacteria detection: A
review. *Biosensors & Bioelectronics, 94*, 131-140.

highly sensitive detection of multi-drug resistant salmonella DT104 using gold
nanoparticles. *Chemical Communications, 47*(33), 9444-9446.

of multi-drug resistant salmonella DT104 through gold nanoparticle–SWCNT
bioconjugated nanohybrids. *Analyst, 139*(15), 3702-3705.

detection of staphylococcus aureus utilizing single domain antibody coated
nanoparticle labels and magnetic trapping. *Analytical Methods, 5*(16), 4152-4158.

typhimurium detection using a surface-enhanced raman scattering-based

SERS tags: Multifunctional platforms toward optical labeling, photothermal ablation

spectroscopic detection of a bacteria biomarker using gold nanoparticle immobilized


