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# Real-time pathogen monitoring during enrichment: a novel nanotechnology-based approach to food safety testing

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ABSTRACT

We describe a new approach for the real-time detection and identification of pathogens in food and environmental samples undergoing culture. Surface Enhanced Raman Scattering (SERS) nanoparticles are combined with a novel homogeneous immunoassay to allow sensitive detection of pathogens in complex samples such as stomached food without the need for wash steps or extensive sample preparation. SERS-labeled immunoassay reagents are present in the cultural enrichment vessel, and the signal is monitored real-time through the wall of the vessel while culture is ongoing. This continuous monitoring of pathogen load throughout the enrichment process enables rapid, hands-free detection of food pathogens. Furthermore, the integration of the food pathogen immunoassay directly into the enrichment vessel enables fully biocontained food safety testing, thereby significantly reducing the risk of contaminating the surrounding environment with enriched pathogens. Here, we present experimental results showing the detection of *E. coli, Salmonella,* or *Listeria* in several matrices (raw ground beef, raw ground poultry, chocolate milk, tuna salad, spinach, brie cheese, hot dogs, deli turkey, orange juice, cola, and swabs and sponges used to sample a stainless steel surface) using the SERS system and demonstrate the accuracy of the approach compared to plating results.



## 1. Introduction

Food-borne illnesses significantly impact society, not only with respect to health, but also health-care costs. The CDC has estimated that each year about 1 in 6 Americans (or 48 million people) gets sick, 128,000 are hospitalized, and 3,000 die of food-borne disease (Centers for Disease Control, 2012). It has also been estimated that food-borne illnesses contribute to $152 billion in health-related expenses each year in the U.S., particularly for bacterial infections caused by *Salmonella, Listeria monocytogenes, E.coli* and *Campylobacter* spp. (Scharff, 2010, 2012). The current level of food safety found in the U.S. is the result of government regulations combined with industry self-monitoring influenced by market incentives, such as legal liability, brand value, reputation, and sales volume. Highly publicized outbreaks of food-borne illness in samples ranging from spinach to cantaloupe to peppers has prompted increased public concern and culminated in the 2011 passage of the Food Safety Modernization Act (Neuman, 2010; Sutton, 2009).

Food pathogen testing may occur on food samples ranging from raw materials to end products or on environmental samples acquired from surfaces, floors, drains, and processing equipment as part of a site's overall Hazard Analysis and Critical Control Point (HACCP) plan (U.S. Food and Drug Administration, 1997). For many samples, a "zero tolerance" requirement is imposed on key human pathogens such as *Listeria monocytogenes* and *E. coli* O157:H7 (Fratamico et al., 2005). The requirement to detect even a single, potentially highly damaged, viable pathogen in a food sample has driven the selection and development of today's state of the art pathogen tests. In particular, all food pathogen testing today requires a culture step to enrich the potentially low levels of pathogens contained in a sample and ensure that the limit of detection of the analytical test is reached. This workflow inherently limits time-to-results, since it is impossible to know *a priori* the starting pathogen load of any given sample. Therefore, the enrichment protocol lengths must be set for the "worst case" scenario (*i.e.* the need to recover one damaged pathogen). As a consequence, samples with higher pathogen loads are cultured longer than may be strictly necessary, leading to a delay in time-to-results. Following the culture step, a portion of the sample is removed and tested by conventional plate-based technologies or, more commonly, by rapid tests, including immunoassays and PCR-based tests (Velusamy et al., 2010).

While culturing food and environmental samples prior to the analytical pathogen test provides the necessary sensitivity, these methods suffer from well-known disadvantages. First and foremost, time-to-results can be long. Methods to reduce these times are currently under development by a number of groups and rely primarily on improving the analytical test by further lowering the test's limit of detection or

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0168-1605/

by detecting pathogen biomarkers such as mRNA that are in higher abundance (Naravaneni and Jamil, 2005; Roka Bioscience, 2012.; Rijpens and Herman, 2002). Attempts to develop improved media that preferentially grow the pathogen of interest over background microflora are also underway (Taskila et al., 2012). There are also attempts at improving target capture efficiency. The Pathatrix® Auto Instrument (Life Technologies, Carlsbad, CA) uses a proprietary technology to concentrate the pathogen by re-circulating the entire sample over a "capture phase" where paramagnetic particles are immobilized (Wu et al., 2004). Target organisms are captured onto the immobilized beads, washed, and eluted in preparation for detection.

Biofunctionalized magnetic nanoparticles (MNP) are increasingly used for concentration and separation of pathogens while biofunctionalized detector particles are used for target detection (Najafi et al., 2014; Huang et al., 2010; Yang et al., 2008; Ravindranath et al., 2009; Zhang et al., 2013; Zhao et al., 2009). R. Najafi et al. describes a method where antibody-conjugated MNPs capture *E. coli* O157 cells spiked into apple juice followed by several washes for capture and separation of the target pathogen (Najafi et al., 2014). SERS nanoparticles are added to the separated MNP nano-aggregate followed by another magnetic separation and three more washes. The final magnetic aggregate is suspended in phosphate buffered saline (PBS) for SERS measurement. Zhao, et al., have described a similar method to simultaneously detect three food-borne bacteria via an approach that combines magnetic microparticles for separation and antibody-conjugated quantum dots as fluorescent markers (Zhao et al., 2009). In both of these approaches, the pathogen-NP-aggregate is removed from the food matrix and detected using an end-point optical measurement.

A key disadvantage of current test protocols is that the sample vessel must be opened after the end of the enrichment period so that an operator can prepare for and conduct the analytical test. If a pathogen is present in the starting sample, the culture step can increase the concentration of the pathogen to as high as $10^8$-$10^9$ CFU/mL, so that opening the sample vessel after culture exposes both the user and the environment to a risk of contamination. This exposure risk may inhibit many food producers from conducting pathogen testing on-site. Many instead choose to send samples to external laboratories for testing, thereby incurring additional cost and time delays.

We report the development of a novel nanotechnology-based immunoassay that uses SERS nanoparticles to incorporate the pathogen test directly within the enrichment container, eliminating the need for wash-steps and allowing the test to be performed directly within a food sample. This particle-based immunoassay uses magnetic beads to repeatedly capture pathogens growing in the culture vessel. SERS nanoparticles are used to generate a signal that can be directly read through the side of the culture vessel without the need for any wash or separation steps. While homogeneous SERS-based immunoassays have been previously reported (Sha et al., 2007, 2008), the inherent compatibility of this assay format within a bacterial culture has never been studied. The results presented here demonstrate sensitive, specific detection of pathogens within a food sample in real time during cultural enrichment.

## 2. Materials and methods

### 2.1. SERS homogeneous assay

The SERS phenomenon has been well-described elsewhere (Campion and Kambhampati, 1998; Le Ru et al., 2007; Moskovits, 2006). In brief, a Raman-active molecule near a noble-metal nanostructured surface (*e.g.* a gold nanoparticle) will undergo enhanced Raman scattering of incident light. Raman scattering is an inelastic scattering process in which the scattered photons are of a different frequency than the incident light, with the energy difference between incident and scattered photons corresponding to the vibrational modes of the molecule. In the work reported here, the Raman signal is provided by a SERS tag, consisting of a gold nanoparticle that is coated with a Raman active reporter molecule ("Raman dye") and then encapsulated in glass (Doering et al., 2007; Mulvaney et al., 2003) (Fig. 1a). The glass encapsulation stabilizes the Raman reporter, protecting it from displacement from the gold nanoparticle surface and ensuring that the Raman signal is stable even in complex biological fluids. The glass also prevents adsorption of proteins, membrane fragments, and other components of a food sample onto the gold surface so that these components do not generate spurious Raman signals of their own. Thus, the measured Raman signal corresponds exactly to the known spectrum of the Raman dye and generates a unique spectral signature for different choices of the Raman dye (Fig. 1b). This spectral barcode can be readily distinguished from other optical signals from the sample such as background fluorescence. Additionally, when the SERS tags are excited at 785 nm, the Raman spectrum is emitted at near infrared wavelengths, where absorption from complex samples such as blood or food is minimal. Furthermore, since each Raman reporter provides a unique spectral signature, the SERS technology is ideally suited for multiplexing.

We have incorporated the SERS tags in a magnetic-capture sandwich immunoassay, as illustrated in Fig. 1c. This immunoassay requires no wash steps and can be performed directly in a food sample. In the assay, affinity reagents such as antibodies are conjugated to SERS tags and also to superparamagnetic microparticles (referred to henceforth as "magnetic particles"). The affinity reagents conjugated to the SERS tags and magnetic particles may be the same or different, so long as they are selected to allow the target of interest (*e.g.* a bacterial pathogen) to be simultaneously bound ("sandwiched") to both a SERS tag and a magnetic particle. When antibody-conjugated SERS tags and antibody-conjugated magnetic particles are mixed with a food sample containing a bacterial pathogen, the pathogen is "sandwiched" between the SERS tag and magnetic particles, with specificity determined by the selection of antibodies (*e.g. Salmonella* specific antibodies). A magnet is then placed against the side of the sample vessel, so that magnetic particles within the field of the magnet are concentrated at the side of the vessel. As the SERS tags are not magnetic, they will only be pulled into the magnetic pellet if they are bound to magnetic particles through a SERS-pathogen-magnetic particle sandwich. Thus, the intensity of the Raman signal from the magnetic pellet is a direct measure of the amount of pathogen present in the sample. The Raman spectrum of the pellet can be read through the side of the sample vessel without the need for any additional wash or incubation steps as is typically required in a sandwich immunoassay.

While SERS-based detection has been previously reported (Sha et al., 2007, 2008), the technology has previously been used on samples removed from a bacterial culture after the completion of enrichment. In the method shown in Fig. 1c, the immunoassay is conducted within the culture vessel repeatedly throughout the enrichment process. We find that repeated formation and dispersal of the magnetic pellet does not inhibit bacterial growth and that the antibodies chosen for this study remain functional for at least 48 hours at temperatures up to 42 °C. Thus, the SERS immunoassay reagents can be used to provide a real-time measure of pathogen concentration. Importantly, since we are using a sandwich immunoassay with specific affinity reagents, we are able to measure specific pathogens, rather than total bioburden, in real-time. A food sample is introduced into a vessel containing SERS and magnetic reagents conjugated to antibodies specific for the pathogen of interest. Liquid growth medium is also added, and the entire vessel is incubated to promote pathogen growth. At intervals throughout the incubation, a magnetic field is applied to concentrate the magnetic particles and any bound SERS tags to the wall of the vessel. A laser is then used to interrogate the concentrated magnetic particle pellet, and any associated SERS signal is detected and quantified on a spectrometer with CCD detector. After the SERS spectrum has been acquired, the vessel is agitated to disperse all particles back into suspension, where they continue to interact with the sample. Early in the culture, pathogen concentration may be too low to be detected, but as the sample continues to incubate, pathogen load will increase, eventually

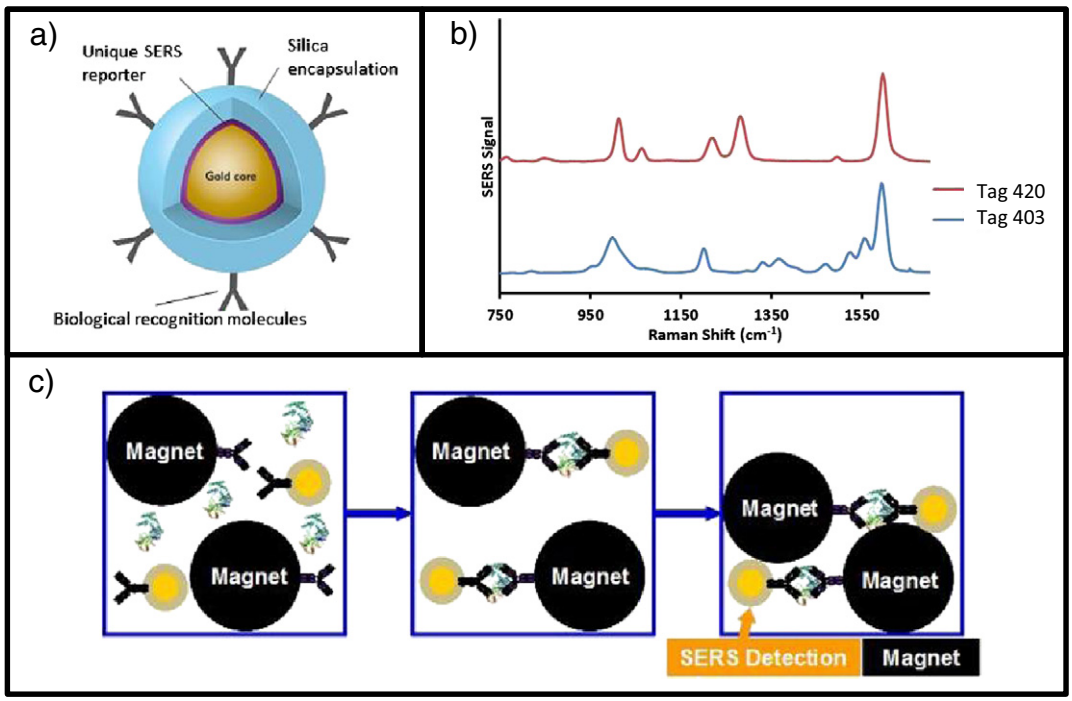


**Fig. 1.** [COLOR]. SERS immunoassay. a) Schematic of SERS nanoparticle tag. b) SERS spectra from SERS Tag 403 and Tag 420 demonstrating the unique spectral fingerprints that enable high levels of multiplexing. c) Representation of the SERS assay format, in which the target pathogen is "sandwiched" between SERS tags and superparamagnetic microparticles conjugated with antibodies specific for the pathogen.

generating a detectable SERS signal. Through repeated pellet formation, measurement, and dispersal at regular intervals of 10-30 minutes, the SERS signal may be monitored over time to provide real-time pathogen detection and quantification.

### 2.2. Synthesis of Raman Active ("SERS") nanoparticles

The SERS tags consist of a 60 nm gold particle core coated with a distinct Raman reporter molecule that is encapsulated by a 30-35 nm protective glass coating (Doering et al., 2007). The colloidal gold was prepared by reducing an aqueous solution of $HAuCl_4$ with sodium borohydride in the presence of a mixture of sodium citrate and hydroxylamine hydrochloride. The gold particles were then coated with 3-aminopropyl-trimethoxysilane (APTMS), and 200 nanomoles of Raman reporter molecule was added per 1000 $cm^2$ of colloidal surface area. Raman reporter molecules used were 5-(4-Pyridyl)-1,3,4-oxadiazole-2-thiol (Tag 403) and 4,4'-Dipyridyl (Tag 420), selected for the strength of their signal and their ability to generate easily distinguishable spectral barcodes. After addition of the Raman reporter, the silica layer was then grown to the desired thickness using the Stöber process (Stöber et al., 1968).

### 2.3. Magnetic particles

Magnetic beads were purchased from Life Technologies (Carlsbad, CA) either pre-conjugated to antibodies (Dynabeads® Anti-*E. coli* O157, catalog #71003) or functionalized (Dynabeads® M-270 carboxylic acid, catalog #14305D) and later conjugated to antibodies as described below.

### 2.4. Antibodies and conjugation methods

Anti-*E. coli* O157:H7 antibodies (MAV119-499) were purchased from Meridian Life Science® (Cincinnati, OH) and were specific for O157:H7. Antibodies to *Salmonella* species were either from Virostat (Portland, ME, catalog #0701) or from SDIX (Newark, DE). The latter are the same as are found in the Romer Labs® Technologies' RapidChek® lateral flow kit, an AOAC validated test kit (AOAC Research Institute Certification No. 080601). Similarly, anti-*Listeria* species antibodies were from the AOAC validated Romer Labs® Technologies' RapidChek® *Listeria* species lateral flow kit (AOAC Research Institute Certification No. 020401). Antibodies were conjugated to thiolated SERS tags using an NHS-maleimide linker (Thermo Scientific catalog #22622). Antibodies were conjugated to carboxylated magnetic particles using standard EDC/NHS chemistry (Nakajima and Ikada, 1995), with the exception of the anti-*E. coli* O157 reagent, which was purchased pre-conjugated from Life Technologies (Carlsbad, CA). Although the Life Technologies reagent detects the O157 serogroup, the measured SERS signal is specific to O157:H7 due to the specificity conferred from the *E. coli* antibody on the SERS nanoparticle. Prior to their use in a bacterial culture, antibody-conjugated SERS and magnetic particles were tested by combining them in buffer, spiking in known amounts of bacteria, and measuring the SERS signal from the magnetic pellet. The analytical limit of detection measured from bacteria titration curves performed this way was approximately $10^5$ CFU/mL.

### 2.5. E. coli O157:H7 spiked food and sponges

*E. coli* O157:H7 (ATCC® 43888) was grown in Nutrient Broth (BD Difco™ 234000) or Buffered Peptone Water (BD 212367) and then diluted into the appropriate medium for each experiment as described below. Sponges were inoculated with 700 CFU *E. coli* O157:H7 in Buffered Peptone Water. Raw ground beef and raw chicken breast were spiked with $10^3$ CFU/g (beef) and $10^6$ CFU/g (chicken) *E. coli* O157:H7 in enriched in Modified Tryptic Soy Broth + Novobiocin ("BAM Media M156: Trypticase Soy Broth Modified (mTSB)," 1998). Orange juice and Coca-Cola were diluted in Universal Pre-enrichment Broth (BD 223510) and spiked with 100 CFU/mL *E. coli* O157:H7. Spinach was rinsed in Butterfield's phosphate buffer (BD 211544), diluted in 2× Buffered Peptone Water and inoculated with 38 CFU/mL *E. coli* O157:H7. All samples were diluted 1:10 (w/v) in growth medium and placed directly in the prototype instrument (described below) for monitoring and detection at 35 °C.

### 2.6. Listeria spiked stainless steel surfaces

Ten stainless steel coupons (1" × 1") were spiked with a mixture of *L. monocytogenes* (ATCC 19115) at 376 CFU/coupon and a 50× concentration of growth competitor, *Staphylococcus epidermidis* (environmental isolate, BD isolate collection). Control coupons were spiked with *S. epidermidis* only. For all coupons, the organisms were spiked in phosphate buffered saline and 10% dried milk. Spiked coupons were allowed to dry overnight to damage the organisms. The next day, environmental swabs were wetted with D/E (Dey/Engley) Broth (BD 281910) and swiped across each coupon surface before being inserted into culture tubes containing RapidChek® *Listeria* 40 Hour Single Step Media System (Romer Labs® Technologies, Inc) and SERS reagents conjugated to SDIX anti-*Listeria* antibodies. The culture tubes were then inserted directly into the instrument for monitoring and detection at 30 °C.

### 2.7. Salmonella spiked raw ground beef

Overnight cultures of *S.* Typhimurium (ATCC 14028) and *S.* Kentucky (BD Isolate Collection) were grown in RapidChek® SELECT™ *Salmonella* Primary medium with supplement (Romer Labs® Technologies, Inc. 7000196). The *Salmonella* cultures were further diluted in the same medium and spiked into raw ground beef at approximately 1 CFU/25 g. The spiked ground beef was then cold stressed for approximately 48 hours at 4 °C. Spiked samples of 25 g were added to 225 mL of RapidChek SELECT™ *Salmonella* Primary medium with supplement, stomached and enriched overnight (approximately 18 hours) at 42 °C. The next morning, portions of the enriched sample were removed for the SERS immunoassay, plating, and lateral flow experiments. For the SERS experiments, 50 μL of the enrichment was added to 5 mL of RapidChek SELECT™ *Salmonella* Secondary medium in culture tubes containing the SERS assay reagents. The samples were then capped and inserted into the instrument for further incubation and detection at 42 °C. Lateral flow experiments were by Romer Labs® Technologies, Inc.'s RapidChek® lateral flow procedure (catalog #7000191), and plating controls were from plating 100 μL of the enriched sample on BD BBL™ CHROMagar™ plates (catalog #214983). In addition, portions of the spiked beef were taken prior to enrichment and sent to an independent testing lab (Chestnut Labs, Springfield, MO) to determine the most probable number (MPN) and the competitor load by aerobic plate counts (APC).

### 2.8. Naturally contaminated raw poultry

The poultry samples were prepared in the same manner as the raw ground beef samples, except that the raw ground chicken and turkey samples were not spiked. Portions of each sample were sent for independent testing (Chestnut Labs, Springfield, MO) to determine MPN, APC and the strain of *Salmonella*, if found.

### 2.9. Listeria spiked foods

Overnight cultures of *L. monocytogenes* (M14, Romer Isolate Collection), *L. monocytogenes* (ATCC 13932), *L. seeligeri* (ATCC 51334), and *L. welshimeri* (ATCC 35897) were grown in RapidChek® *Listeria* 40 Hour Single Step Media System (Romer Labs® Technologies, Inc. 7000176). The isolates were diluted in the same medium and spiked into brie cheese, deli turkey, hot dogs, spinach or tuna salad at multiple spike levels, ranging from 0.3 to 200 CFU/g. The foods were diluted 1:10 (w/v) in RapidChek® *Listeria* 40 Hour Single Step Media and enriched at 30 °C in an incubator. After 22 hours, 5 mL samples of each spiked food were transferred into culture tubes containing the SERS assay reagents. The tubes were then inserted into the SERS instrument for further incubation and detection at 30 °C. SERS results were confirmed by plating 100 μL of the sample on BD BBL™ CHROMagar™ plates (catalog #215085).

### 2.10. Time-to-results vs. pathogen load study

Overnight cultures of *S.* Typhimurium (ATCC 14028) were grown in RapidChek® SELECT™ *Salmonella* Primary medium with supplement (Romer Labs® Technologies, Inc. 7000196). The *Salmonella* cultures were diluted in the same medium and spiked into chocolate milk, peanut butter or onto a 1" × 1" stainless steel surface in 10-fold dilutions to span a wide range of inoculation levels ($10^{-1}$-$10^{6}$ CFU/g or CFU/square inch). The stainless steel samples were allowed to dry overnight to damage the organisms. The next day, environmental swabs were wetted with D/E Broth (BD 281910) and swiped across each coupon surface before being inserted into culture tubes containing 5 mL RapidChek® SELECT™ *Salmonella* Primary medium with supplement (Romer Labs® Technologies, Inc. 7000196) and SERS and magnetic particles each conjugated with polyclonal antibodies to Salmonella (Virostat 0701). The spiked chocolate milk and peanut butter samples were diluted 1:10 (w/v) in RapidChek® SELECT™ *Salmonella* Primary medium with supplement. 5 mL aliquots were transferred to culture tubes containing SERS reagents. Tubes were then inserted directly into the instrument for monitoring and detection at 42 °C.

### 2.11. SERS instrumentation and culture tubes

SERS spectra were generated using a wavelength-stabilized laser emitting 50 mW at 785 nm, a wavelength at which optical absorption by biological materials is minimized. The laser was integrated with appropriate filters and focusing and collecting lenses into a custom read head (Innovative Photonic Solutions, Monmouth Junction, NJ) to provide delivery of laser light and collection of Raman scattered light. The read head coupled light into a 200 μm core multi-mode optical fiber (Leoni Fiber Optics Inc., Williamsburg, VA), which routed the Raman scattered light to a fiber-coupled miniature spectrometer optimized for Raman spectroscopy (Hamamatsu Photonics K.K., Solid State Division, Hamamatsu City, Japan). These components form the core of the SERS detection system. As shown in Fig. 2, the SERS detection system was integrated into a custom incubator that enables continuous agitation of the sample during incubation. At predetermined time points (every 10-30 minutes during the incubation), magnets were applied to the side walls of the culture tubes to form the magnetic pellets. The SERS spectrum was read from each pellet, and the acquired spectrum was compared to a library of reference spectra to calculate a SERS tag weighting factor. This "tag weight" is a scaling factor between the SERS tag component of the acquired spectrum and a known reference spectrum. It reflects the intensity of the measured signal coming from the SERS reporter molecule. The SERS detection system monitored the tag weight over time to generate a growth curve. A SERS tag weight above the baseline level was used to indicate positive detection of the pathogen. After each SERS reading, the magnets were removed from the side walls of the culture tubes. Incubation of the sample was resumed, with the agitation serving to disperse the magnetic pellet. Culture tubes were standard 16 mm diameter by 100 mm long design, with a screw cap. Tubes made of cyclic olefin polymer (COP) were used due to COP's low background signal.

## 3. Results

### 3.1. Proof-of-concept: E. coli O157:H7 spiked food and sponges

Fig. 3 illustrates the performance of the SERS assay in a variety of food matrices and for a variety of inoculation levels. *E. coli* O157:H7 was spiked at high loads into growth media containing 10% of stomached beef, stomached chicken, orange juice, Coca-Cola, spinach or sponge. The SERS signal was initially at baseline level because the load of *E. coli* was below the analytical limit-of-detection (approx. $10^{5}$ CFU/mL). As time passed, *E. coli* levels increased due to bacterial growth, and sandwich formation was enhanced. The SERS signal increased over time,

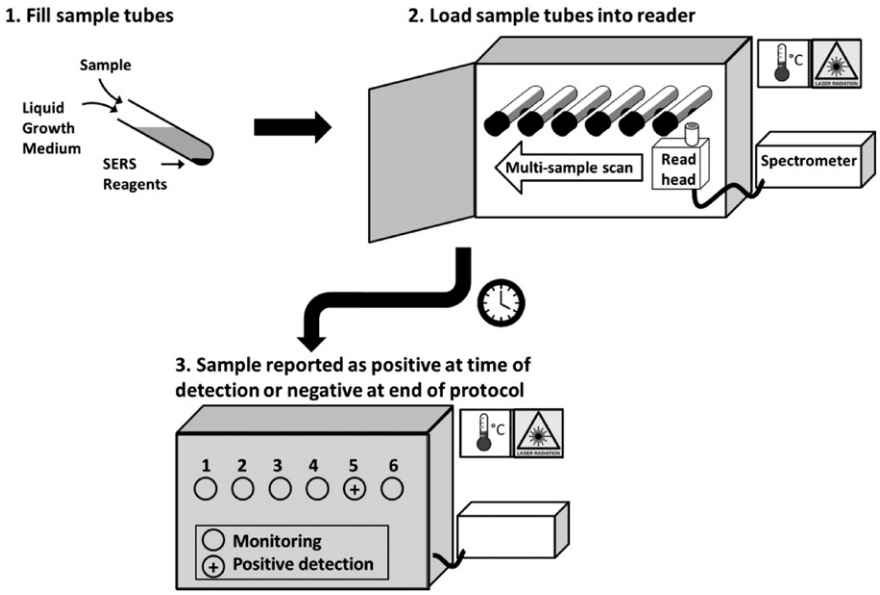


**Fig. 2.** Schematic of SERS reader and workflow. Standard culture tubes with screw caps are loaded with the sample, growth medium, and SERS reagents (step 1). The culture tubes are then loaded into the SERS reader, which incubates the samples at controlled temperature, forms pellets in the samples on a predetermined schedule, and scans the Raman read head across the pellets (step 2). A sample is reported to the user as positive at the moment growth is detected. A sample is reported to the user as negative if growth has not been detected at the end of the protocol (step 3).

reflecting the increasing concentration of pathogen in the sample. In this experiment, SERS readings were taken every 30 minutes. The time at which the curve deviates from baseline is dependent on the initial concentration and damage level of the pathogens in the sample. For example, *E. coli* O157:H7 in chicken was detected within two hours of enrichment because it was inoculated at a high level ($10^6$ CFU/g), while *E. coli* O157:H7 in spinach rinsate was detected around five hours because it had a lower inoculation level (38 CFU/mL in the rinsate).

### 3.2. Listeria spiked stainless steel surfaces

Table 1 shows the performance of the SERS assay for environmental samples. Ten replicate stainless steel coupons were spiked with 376 CFU *L. monocytogenes* (ATCC 19115) in the presence of 50× growth competitor and dried overnight. The damaged *L. monocytogenes* cells were recovered in all 10 replicates, while the two competitor-only coupons gave no indication of *Listeria* growth. The experiment was carried out using a medium that typically requires a 40 hour pre-enrichment

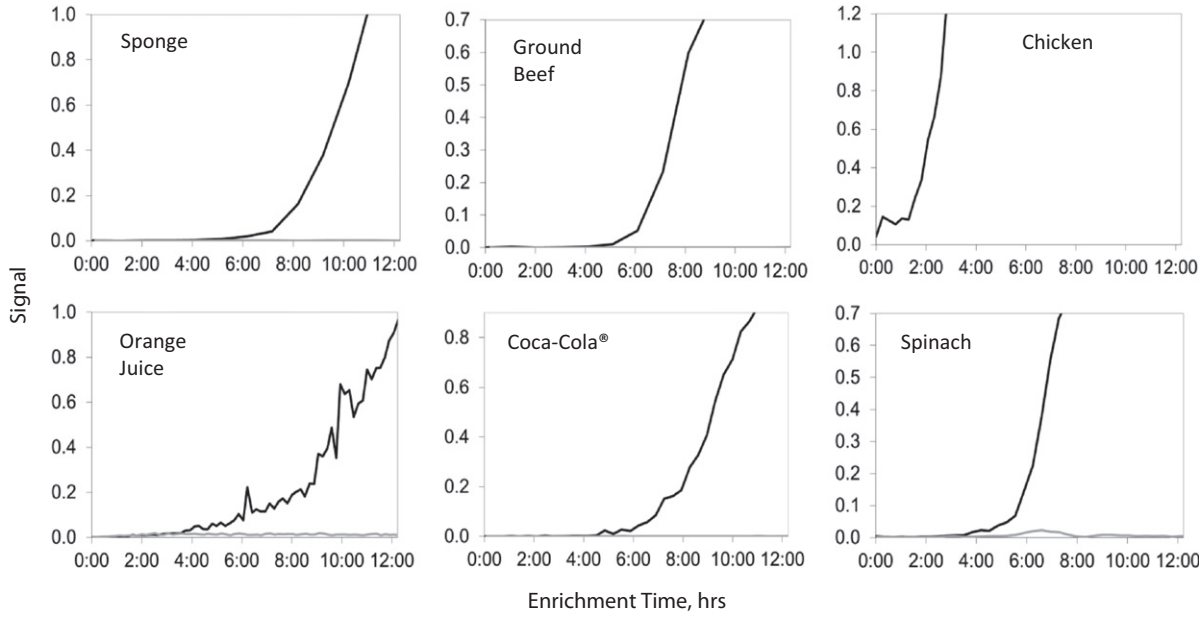


**Fig. 3.** *E. coli* O157:H7 spiked into various matrices. Samples were diluted 1:10 (w/v) with standard *E. coli* O157 media containing the detection reagents and then inserted directly into the instrument for pathogen growth monitoring. Black curves are spiked samples, and gray curves are negative controls. The spike levels were 700 CFU/sponge, $10^3$ CFU/g ground beef, $10^6$ CFU/g chicken, 100 CFU/mL orange juice, 100 CFU/mL Coca-Cola®, 38 CFU/mL spinach rinsate. SERS Tag 420 was used for the experiment. Media were buffered peptone water (sponge), modified tryptic soy broth + Novobiocin (beef and chicken), universal pre-enrichment broth (orange juice and Coca-Cola), and 2× buffered peptone water (spinach rinsate in Butterfield's phosphate buffer).

**Table 1**
Recovery of dried *Listeria* on stainless steel.

| Sample | Time-to-positivity (hours) | cfu Listeria | cfu Competitor |
|---|---|---|---|
| 1 | 22.3 | 376 | 2.00E + 04 |
| 2 | 23.4 | | |
| 3 | 22.4 | | |
| 4 | 25.4 | | |
| 5 | 21.4 | | |
| 6 | 25.5 | | |
| 7 | 24.5 | | |
| 8 | 20.4 | | |
| 9 | 23.5 | | |
| 10 | 24.5 | | |
| 11 | Negative | 0 | 2.00E + 04 |
| 12 | Negative | | |

prior to the pathogen test. However, with real-time monitoring during culture the *Listeria*-positive samples were identified as positive between 20.4 and 25.5 hours, averaging 23.3 hours to positivity.

### 3.3. Salmonella in raw ground beef and poultry

The SERS assay was also used to test for *Salmonella* in raw ground beef and poultry. The beef samples were spiked with either *S.* Typhimurium or *S.* Kentucky, but the poultry samples were unspiked (natural contamination only). In this experiment, we wished to test the technology's ability to detect contamination levels of 1 viable cell per sample. The *S.* Typhimurium samples were spiked at ~1 CFU/25 g (*i.e.* ~1 CFU/sample), while the *S.* Kentucky samples were spiked at <1 CFU/25 g, as determined by the most probable number (MPN) method. The MPN levels of the naturally contaminated ground chicken and turkey ranged from 0 to 115 CFU/25 g. At spike levels near 1 CFU/25 g, it is statistically expected that some samples will be positive and some will be negative, depending on whether an organism is actually present in the tested portion of the sample. Table 2 summarizes the results of the experiment. The third column gives the expected *Salmonella* level in the food, as determined by third-party MPN analysis (Chestnut Labs, Springfield, MO) on a different portion of the same lot. Thus, some discrepancy between the MPN analysis and the other test methods may be expected due to statistical variability about the 1 CFU/25 g spike level (*e.g.* ground beef #8 or ground chicken #6). The last three columns of the table show whether *Salmonella* was detected in the sample by SERS, lateral flow, or plate methods. Since plating and lateral flow were conducted on the same portion of the beef or chicken sample, all three results are expected to be in agreement, as in fact they are. In all cases, the SERS results agreed with the lateral flow and agar plate, demonstrating that pathogens can be detected directly during culture at the 1 CFU/sample limit of detection.

Representative data (growth curves) for spiked raw ground beef and naturally contaminated raw ground poultry are shown in Fig. 4. Note that in these *Salmonella* experiments we mimicked the AOAC approved protocol for the media and antibody reagents (AOAC Research Institute Certification No. 080601) which utilizes a 16-22 hour primary enrichment in one medium, followed by a 6-8 hour secondary enrichment in a different medium. We placed the SERS assay reagents in the SERS culture tube for secondary enrichment and were able to detect *Salmonella* almost immediately after the start of the secondary enrichment and before the positive signal at the end of the 22-30 hour lateral flow protocol. For a single medium system such as was used in Fig. 3, the SERS assay reagents are placed directly in the primary enrichment culture, and real-time monitoring starts from the beginning of the culture process.

### 3.4. Time-to-positivity vs. pathogen load study

To demonstrate the ability of the technology to detect contaminated samples as soon as the enrichment reaches the limit of detection, titrations of *S.* Typhimurium (ATCC 14028) were spiked in three different sample matrices: peanut butter, chocolate milk, or on a stainless steel surface. A single enrichment medium was used, with the SERS assay reagents placed directly in the culture tube. Fig. 5 illustrates the dependence of time-to-positivity on bacterial load. As expected, pathogens are detected more quickly as the inoculation level increases. Compared to the chocolate milk and peanut butter, the samples on stainless steel coupons took longer to detect, presumably due to the highly damaged state of the dried pathogens. Horizontal dashed lines on Fig. 5 indicate the published time-to-detection of industry-leading commercial instruments.

### 3.5. Sensitivity and specificity study for Listeria species

The sensitivity and specificity of the SERS method will depend greatly on the performance of the antibodies and media, which must work together to specifically detect pathogens while suppressing non-pathogenic background organisms. To understand the sensitivity and specificity of the SERS method with high quality reagents, we used AOAC validated antibodies and media from Romer Labs® Technologies' RapidChek® *Listeria* species lateral flow kit (AOAC Research Institute Certification No. 020401). Multiple strains of *Listeria* at different concentrations were spiked into food samples and compared to results obtained by plating the enriched sample onto CHROMagar™. The enriched sample from the SERS experiment was also tested after the full 40 hour enrichment period on Romer Labs® Technologies' RapidChek® *Listeria* species lateral flow kit. Results are summarized in Table 3. In total, 4 *Listeria* strains in 5 food types at multiple inoculation levels

**Table 2**
Fractional recovery of *Salmonella* in food samples.

| Food matrix | *Salmonella* strain | *Salmonella* per 25 g (MPN) | Background organisms per 25 g (APC) | SERS result | LF result | Plate result |
|---|---|---|---|---|---|---|
| Ground beef #1 | Typhimurium 14028 | 1.075 | 1.95E + 06 | + | + | + |
| Ground beef #2 | Typhimurium 14028 | 1.1 | 4.50E + 05 | + | + | + |
| Ground beef #3 | Typhimurium 14028 | 1.075 | 3.00E + 08 | + | + | + |
| Ground beef #4 | Typhimurium 14028 | 1.075 | 9.75E + 05 | + | + | + |
| Ground beef #5 | Typhimurium 14028 | 1.875 | 4.75E + 06 | + | + | + |
| Ground beef #6 | Typhimurium 14028 | 1.1 | 2.18E + 05 | + | + | + |
| Ground beef #7 | Kentucky BD LL | 0.3 | 7.75E + 07 | - | - | - |
| Ground beef #8 | Kentucky BD LL | <0.3 | 1.05E + 08 | + | + | + |
| Ground beef #9 | Kentucky BD LL | 0.9 | 1.30E + 08 | + | + | + |
| Ground chicken #1 | N/A | <0.1 | 3.50E + 05 | - | - | - |
| Ground chicken #2 | Enteritidis (natural) | <0.1 | 1.80E + 05 | - | - | - |
| Ground chicken #3 | N/A | <0.1 | 2.75E + 06 | - | - | - |
| Ground chicken #4 | Heidelberg (natural) | 0.3 | 1.70E + 06 | + | + | + |
| Ground chicken #5 | N/A | <0.1 | 3.50E + 06 | - | - | - |
| Ground chicken #6 | Enteritidis (natural) | 3.725 | 2.40E + 06 | - | - | - |
| Ground turkey #1 | Heidelberg (natural) | 115 | 6.00E + 05 | + | + | + |
| Ground turkey #2 | N/A | <0.1 | 3.00E + 07 | - | - | - |
| Ground turkey #3 | Group C2 (natural) | 1.1 | 1.20E + 05 | + | + | + |

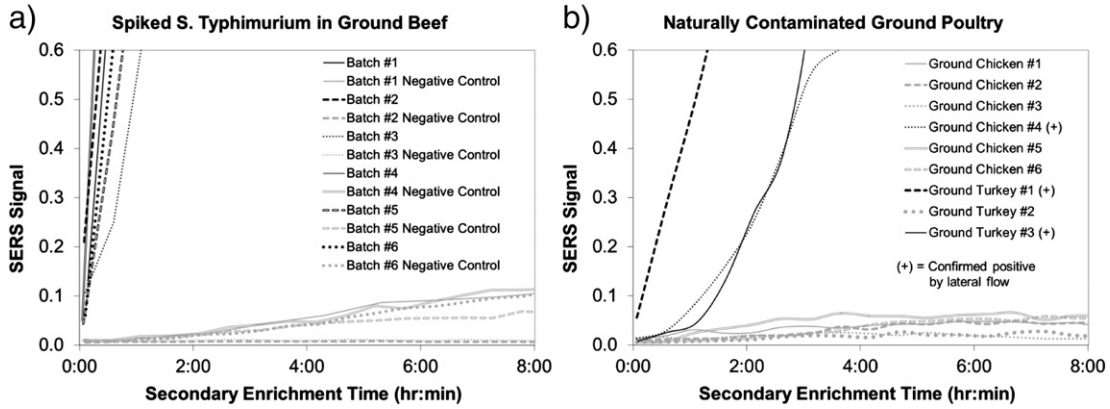


**Fig. 4.** a) *S.* Typhimurium spiked into raw ground beef at ~1 CFU/25 g. The graph shows the secondary enrichment growth curves for the spiked ground beef (dark lines) and negative controls (grey lines). b) Naturally contaminated store-bought ground chicken (n = 6) and ground turkey (n = 3) with MPNs ranging from 0.3 to 115 CFU/25 g. Three of the nine samples at these fractional loads were detected as positive by SERS, plating, and lateral flow (dark lines). The remaining samples were negative by all methods, indicating that no viable organisms were in the tested portion of the poultry (fractional regime). SERS Tag 403 was used in this experiment.

were tested, for a total set of 144 curves (48 negatives and 96 positives). Overall, the SERS assay yielded a 95.3% sensitivity and 97.9% specificity compared to the plate results.

## 4. Discussion

### *4.1. Real-time monitoring of bacterial cultures*

Continuously monitoring a bacterial culture during the enrichment process is a standard practice in clinical applications such as the detection of blood stream infections. In this case, a nominally sterile fluid (blood) is monitored continuously for byproducts of bacterial respiration. A positive result indicates the presence of non-specific bacteria, which is clinically significant in normally sterile fluids. Due to real-time monitoring of the blood culture, a positive result can be reported as soon as it is detected, enabling rapid clinical intervention. For food safety applications, the starting sample is typically highly non-sterile. Background organism counts in a 25 gram sample could exceed $10^6$ CFU/g, as shown in Table 2 for example. Thus, a non-specific bacterial sensor provides little value in determining if a sample is contaminated with a human pathogen such as *Salmonella* or *E. coli*. Immunoassays in contrast offer highly specific, antibody-mediated pathogen detection, but typically require long incubation times and multiple wash steps to remove interferences. Even the methods described by Najafi, et al. and Zhao, et al., while using magnetic separation and optical detection, require multiple wash steps and are therefore not compatible with ongoing bacterial culture. The SERS-based immunoassay reported here allows us to incorporate specific pathogen detection and identification directly into the cultural enrichment, thereby integrating the pathogen test and the culture. This new integrated test allows shorter time-to-results, provides biocontainment of the enriched sample, and simplifies the overall pathogen testing workflow.

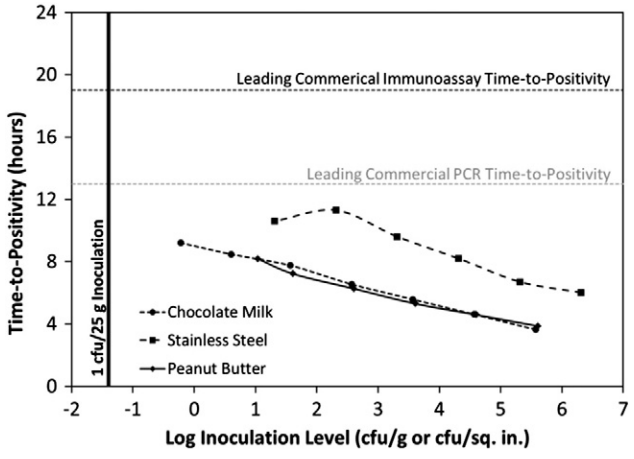


**Fig. 5.** Time-to-positivity versus inoculation level curves for *S.* Typhimurium in chocolate milk, stainless steel (swab), and peanut butter. The detection time decreases with increasing inoculation level. Detection times at the required limit of detection, 1 CFU/25 g (vertical line), are expected to be longer than the detection times plotted here, but still on par with state-of-the-art immunoassays (bioMérieux Vidas®, black dashed line) and PCR assays (DuPont™ BAX®, gray dashed line).

### *4.2. Faster time-to-positivity*

Time-to-results is the time required to determine if pathogens are absent (time-to-negativity) or present (time-to-positivity) in a food sample. Time-to-negativity for pathogen testing is typically determined by four key factors: the inherent sensitivity of the diagnostic method, the prevalence of the detected target, the inherent doubling rate of the target, and the ability of the media to preferentially resuscitate and grow pathogens over high levels of background microflora. For end-point food pathogen tests that are conducted after the completion of cultural enrichment, there is no difference between time-to-positivity and time-to-negativity, as positive results are reported at the end of the testing protocol at the same time as negative results. This concept is indicated by the horizontal dashed lines in Fig. 5, where PCR detection is often faster than immunoassay detection. For real-time monitoring with the SERS immunoassay, negative results are reported at the end of the test protocol, but positive results are reported whenever the pathogen concentration within the detection vial reaches the test's analytical limit of detection. As can be seen in Fig. 5, higher starting loads lead to faster detection times. In addition, samples with highly viable pathogens that grow quickly should be detected before samples with damaged pathogens that require extensive resuscitation. Time-to-negativity for the SERS immunoassay, as for other technologies, is determined by the time required to resuscitate one badly damaged pathogen in the starting sample. This is shown in Fig. 5 by the vertical line at 1 CFU/25 grams. Although Fig. 5 does not show the performance of the SERS immunoassay at this low inoculation level, Tables 2 and 3 show the sensitivity of the technology in detecting positives at the 1 CFU target inoculation level (*i.e.* the "fractional" regime), and provide a comparison with results from AOAC-approved methodologies (*e.g.* plating). It is worth noting that such a comparison, *i.e.* benchmarking the SERS immunoassay against AOAC-approved methods in the fractional regime for test matrices of interest, is the AOAC-established method for accrediting a new pathogen test. As these data show, the SERS immunoassay demonstrated equivalent sensitivity to the AOAC-approved tests.

**Table 3**
SERS assay performance for Listeria spiked food.

| Food matrix | *Listeria* strain | Inoculation cfu/g | Plate SERS LF (# positive/# tested) | | |
|---|---|---|---|---|---|
| Soft cheese 1 (Brie) | *L. monocytogenes* M14 | 0.3-30 | 6/6 | 6/6 | 6/6 |
| Soft cheese 1 (Brie) | *L. seeligeri* ATCC 51334 | 1.8-180 | 6/6 | 6/6 | 6/6 |
| Soft cheese 2 (Brie) | *L. monocytogenes* M14 | 0.3-30 | 0/6 | 0/6 | 0/6 |
| Soft cheese 2 (Brie) | *L. seeligeri* ATCC 51334 | 1.8-180 | 6/6 | 6/6 | 6/6 |
| Deli turkey 1 | *L. welshimeri* ATCC 35897 | 14-56 | 6/6 | 6/6 | 6/6 |
| Deli turkey 2 | *L. welshimeri* ATCC 35897 | 14-56 | 6/6 | 6/6 | 6/6 |
| Hot dog 1 | *L. welshimeri* ATCC 35897 | 14-56 | 6/6 | 6/6 | 6/6 |
| Hot dog 2 | *L. welshimeri* ATCC 35897 | 14-56 | 6/6 | 6/6 | 6/6 |
| Spinach 1 | *L. monocytogenes* ATCC 13932 | 50-200 | 6/6 | 6/6 | 6/6 |
| Spinach 2 | *L. monocytogenes* ATCC 13932 | 50 | 6/6 | 6/6 | 6/6 |
| Spinach 3 | *L. monocytogenes* ATCC 1393 | 50-200 | 6/6 | 6/6 | 6/6 |
| Spinach 4 | *L. monocytogenes* ATCC 1393 | 50 | 6/6 | 6/6 | 6/6 |
| Tuna salad 1 | *L. monocytogenes* M14 | 0.3-30 | 4/6 | 2/6 | 4/6 |
| Tuna salad 1 | *L. seeligeri* ATCC 51334 | 1.8-180 | 6/6 | 6/6 | 6/6 |
| Tuna salad 2 | *L. monocytogenes* M14 | 0.3-30 | 6/6 | 4/6 | 4/6 |
| Tuna salad 2 | *L. seeligeri* ATCC 51334 | 1.8-180 | 6/6 | 6/6 | 6/6 |
| | | Total | 88/96 | 84/96 | 86/96 |

A dramatic example of the benefit of faster time-to-positivity can be seen in the results from testing dried, damaged *Listeria* in the presence of 50× growth competitor on stainless steel. Using the same media and antibodies as a commercial 40-hour *Listeria* method, the SERS method was able to detect dried *Listeria* on a stainless steel surface at ~23 hours for this contamination level. The extra 17 hours' notice is a significant amount of time for food producers to shut down a contaminated production line.

### 4.3. Biocontainment

Standard pathogen tests conducted today require first enriching a food sample and then removing a portion of the enriched material for pathogen testing by *e.g.* plating, PCR, or immunoassay. Opening a highly concentrated sample of enriched pathogen poses a risk of cross-contamination of the testing facility or, in a worst case, the production line. The fear of contaminating production facilities is one factor that contributes to a food producer's decision to outsource pathogen testing. By integrating the pathogen test into a sealed culture vessel, the need to open the sample is eliminated, and the closed vessel can go directly to disposal while remaining fully biocontained. This biocontainment feature may enable food or environmental pathogen tests to be conducted closer to the point of production, thereby eliminating delays and costs due to shipping samples to external facilities with BSL-2 capabilities. Even in laboratories equipped to handle pathogen culture, the reduced risk of cross-contamination between samples may be a significant asset.

### 4.4. Workflow

Integrating the pathogen test into the culture vessel offers an inherently simpler workflow, since there is no need for additional human intervention to conduct the pathogen test following the completion of culture. For environmental and small food samples, the SERS assay reagents may be present in a culture bottle or bag, and the user need only add the growth medium and food sample prior to starting the test. One of the challenges to this approach, however, is the large sample volumes that are required in some segments of the food industry. For example, *E. coli* O157 testing is often performed on 375 gram samples, requiring over 1 L of enrichment media. We have conducted the SERS assay on sample volumes up to 250 mL with good results, so the assay is likely to perform well at these higher volumes. However, issues such as reagent cost would need to be considered. In general, SERS reagent requirements do not scale linearly with volume, and reagent concentrations can be reduced at larger sample volumes, provided enough particles are present to form a magnetic pellet that is large compared to the interrogating laser beam. For example, we observed that a 250 mL sample volume required only 20% of the reagent concentration of a 5 mL sample in order to form a magnetic pellet that could be robustly interrogated. Nonetheless, reagent cost as well as instrument footprint would be important considerations in configuring the assay to handle some of the larger volume samples seen in some segments of the food industry.

### 4.5. Limitations

As with any assay method, the SERS immunoassay has limitations. As an immunoassay, its performance is determined to a large extent by the availability of antibodies with high affinity and specificity for the target of interest. Furthermore, because the antibodies are present in the culture throughout the enrichment, there is an additional requirement that the antibodies be stable in food plus media at temperatures up to 42 or 45 °C. In our testing, the antibodies described here remained functional over these times, and temperatures and were also functional in lower pH samples such as 10% Coca-Cola® or orange juice (Fig. 3). However, it is to be expected that some antibodies will degrade under these conditions. In addition, robustness of the SERS signal is dependent on the ability to concentrate the magnetic particles in the area of the laser. For certain food matrices with large particulates, care must be taken in the design of the instrument and the magnetic pelleting system to ensure that these particulates do not interfere with reproducible concentration of the magnetic particles. Even with robust antibodies and reproducible pelleting, there are practical considerations for large sample volumes. For very large food samples, the cost of reagents may preclude integrating the assay with the full volume of cultured sample. Finally, there is much interest in the food safety industry in reducing time-to-result for food pathogen testing. However, time-to-result is dependent only in part on the sensitivity of the analytical method. The ability of the growth media to resuscitate a single damaged organism plays a critical role in determining how long it will take for bacteria levels to grow to levels detectable by this or any other detection method.

### 4.6. Summary

The potential impact of improved food testing methods is significant. The SERS immunoassay described here represents the first integration of an immunoassay into the bacterial culture, providing specific detection of pathogens without interfering with bacterial growth. Incorporating the pathogen detection reagents into the culture enrichment enables real-time monitoring of pathogen levels, flagging positive samples as soon as they reach the limit of detection and enabling complete biocontainment of the sample. The data presented here demonstrate the potential of this approach for high sensitivity and specificity in a variety of food and environmental samples.

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