Hort Innovation

Final Report

A Revolutionary New Sensor for In-Field Measurements of Food Safety in Leafy Vegetables

Project leader:

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Delivery partner:

University of Sydney

Project code:

VG13073

Project:

A Revolutionary New Sensor for In-Field Measurements of Food Safety in Leafy Vegetables- VG13073

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Summary

The project aim was to construct a working prototype of a new-generation Raman sensor that could detect dangerous microorganisms in leafy vegetables, on farm and in the packaging chain.

This project was successful in producing a compact and portable Raman spectroscopy sensor. Although the Raman technology failed to successfully detect bacteria, the methodology was sound as improvements to the sensitivity were obtained throughout the project, and hence these initial results proved encouraging. The sequence of development and testing made significant progress to achieving the successful outcome, however, the undertaking was ambitious and the necessary development and testing proved to be beyond the scope of this project.

The Raman sensor probe was fitted and integrated to the Ladybird robot and this successfully demonstrated the ability to autonomously inspect vegetables before harvest.

While no tests were undertaken in the packaging chain, the sensor probe developed was sufficiently compact to demonstrate the feasibility of a hand held. Being a real time sensor, there would be no reason for any produce to be delivered to the customer without testing.

Keywords

Raman spectroscopy; PIMMS, leafy vegetables; Salmonella; E. coli; vegetable harvesting; food poisoning.

Introduction

Worldwide there is a growing market for fresh ready-to-eat leafy vegetables such as lettuce and baby leaf crops (Olaimat & Holley 2013). Since most vegetable products are minimally processed or eaten raw, there is increased risk for foodborne disease outbreaks. On-farm pathogen sources may be from soil, animal faeces, irrigation water, dust, inadequately composted manure, wild or domestic animals and humans (Olaimat & Holley 2013). Additional risk of contamination is increasing with the proliferation of grower-processors who are undertaking various levels of processing and packing on the farm. These sanitation practices may not always meet the highest standards required to ensure that there is no biological hazard in the product.

Water Wash treatments such as chlorine, or occasionally peroxyacetic acid, are typically used in the processing to remove pathogens from the surface of produce. This procedure fails if the sanitization process is not precisely chemically monitored or if there is a build-up of biofilms on equipment, as pathogens are difficult to remove from cut and uneven surfaces. Beyond the processing stage, a report from the Centre for Disease Control & Prevention, identified that a major source of foodborne illness is likely to occur at the final stage of delivery, either through handling or storage.

Traditional methods of food monitoring involving laboratory analytical techniques such as ELISA, RT PCR and microbial plating are beyond the scope of on-farm monitoring. Other spectroscopy tools using near infrared cannot capture internal constituent gradients within food products which may lead to discrepancies between predicted and measured composition.

Raman vibrational spectroscopy is a powerful analytical method that can generate a "spectroscopic fingerprint" from the microbial sample. This provides quantitative and qualitative information that can be used to characterise, discriminate and identify microorganisms in both bacteria slurry and at the single-cell level. Commercial devices are available but are bulky, expensive and limited in sensitivity and application.

The PIMMS technology at the University of Sydney (developed by Bland-Hawthorn) can achieve diffraction-limited performance in a compact sized micro-spectrograph. This has been applied to achieve a smaller Raman spectrograph measurement tool for this project.

This project has laid the foundation for a compact photonic Raman spectroscopy to detect the presence of bacteria capable of causing food poisoning in leafy vegetables. It has been made small and portable enough to be used in the field at any stage while the vegetable is growing, in the processing floors where the vegetable is sized and packaged, and on the shelves directly prior to purchase or placed on the table.

Methodology

The project aim was to construct a working prototype of a new-generation Raman sensor that could detect dangerous microorganisms in leafy vegetables. The sensor was integrated into a farm robot (The Ladybird) to further demonstrate the potential of this technology in the field. The methodology here outlines the steps achieved in this project to achieve this aim.

Initial Design

The initial design compromised of the following components assembled as shown in Figure 1:

1. Spectral coverage of 300-2500 cm-1 (800-960nm) with a resolution of 2cm-1
2. 1x19 multicore fibre photonic lantern as the collection fibre
3. 785 nm laser as the Raman excitation source
4. 3D printed custom hosing
5. Off-the-shelf focuser lens,
6. 8M pixel cooled CCD

The experiment was arranged as shown in Figure 2. To obtain the first Raman spectra the excitation source was focused onto a thin layer of powdered paracetamol. The collection fibre was placed directly behind the focus and sample. While not optimal this allowed sufficient coupling to detect the paracetamol Raman signature. Both the raw extracted spectrum and a post-processed spectrum are shown in Figure 1. The post processing is required to remove the Raman signature of the optical fibre and any other weak fluorescence.

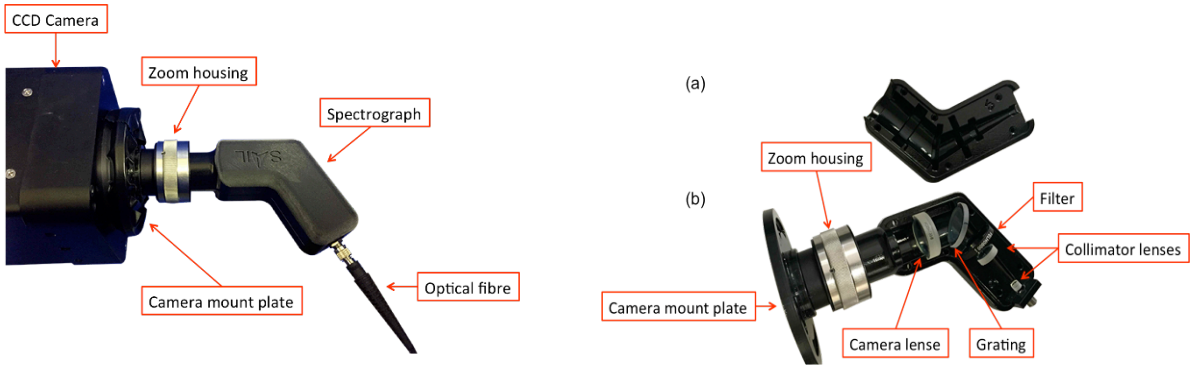

Figure 1 - Raman spectrograph mounted on CCD camera. (Left) Assembled. (Right) Disassembled.

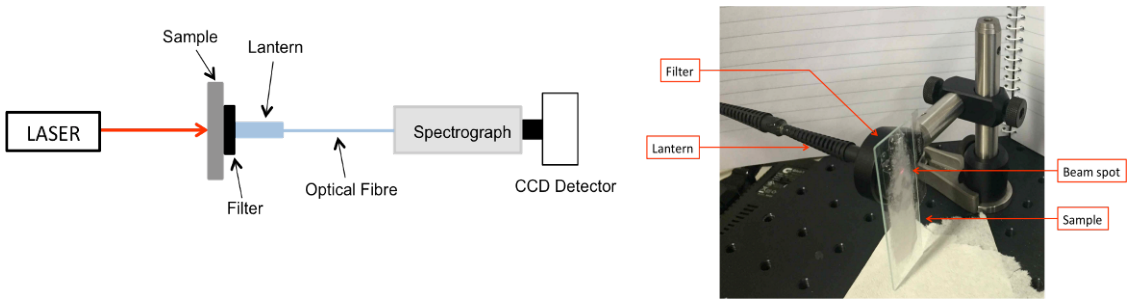

Figure 2 - Raman set-up arrangement (left), illuminated sample and light collection with the lantern (right)

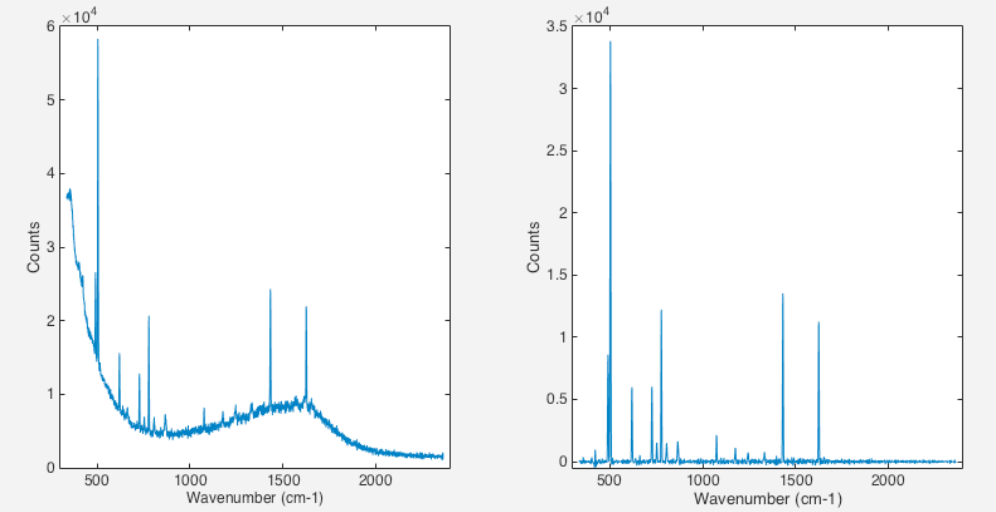

Figure 1 - (Left) Raw Raman signal from Paracetamol. (Right) Signal after post-processing.

Further Refinement

Further testing of the spectrograph revealed that mechanical instability was encountered in the mount between the CCD housing and the spectrograph optics housing. This caused problems in the 1D spectrum extraction procedure, which was not apparent until further testing was done. A modified spectrograph housing that features a more rigid interface to the CCD housing eliminated this issue.

Another significant issue encountered was result repeatability and reduced signal-to-noise in the Raman spectra when delivering the Raman laser through the optical fibre. This was in part due to inadequate delivery and collection optics (difficult when using two separate fibres) and additional background signal generated in the delivery fibre (florescence and Raman signal of silica). Further development of the fibre bundle was required to reduce this issue.

Manufacturing the fibre bundle proved very challenging as fibres were breaking within the Capillary skeleton required for the photonic lantern multi-mode to single-mode converter (Figure 2). After further development fibre bundles were successfully produced (Figure 3). The bundles simplify the positioning/focusing of the excitation laser and collection fibre (as they are permanently collocated). This allowed for simpler collection of the paracetamol signal in the lab.

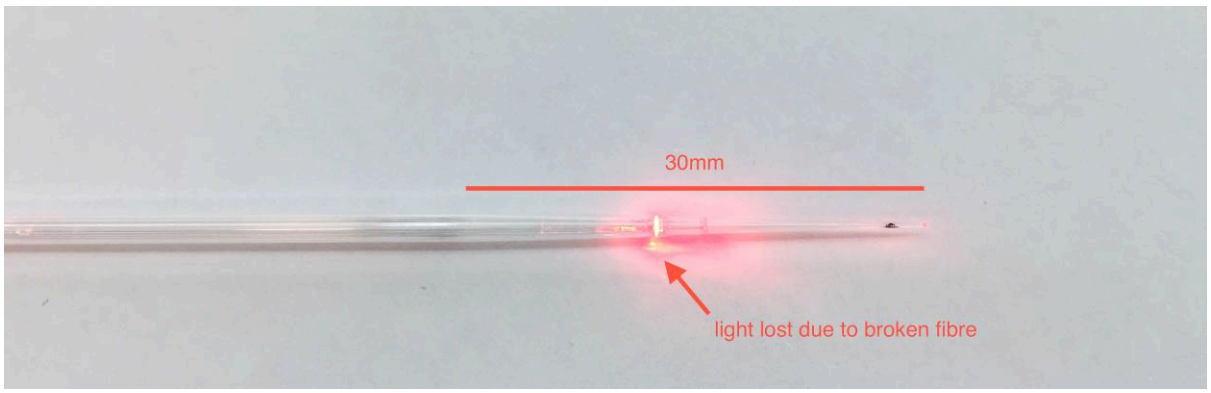

Figure 2 - Image of an unsuccessful fibre bundle. The multimode delivery fibre broke mid transition. As result all the light from the excitation laser is lost.

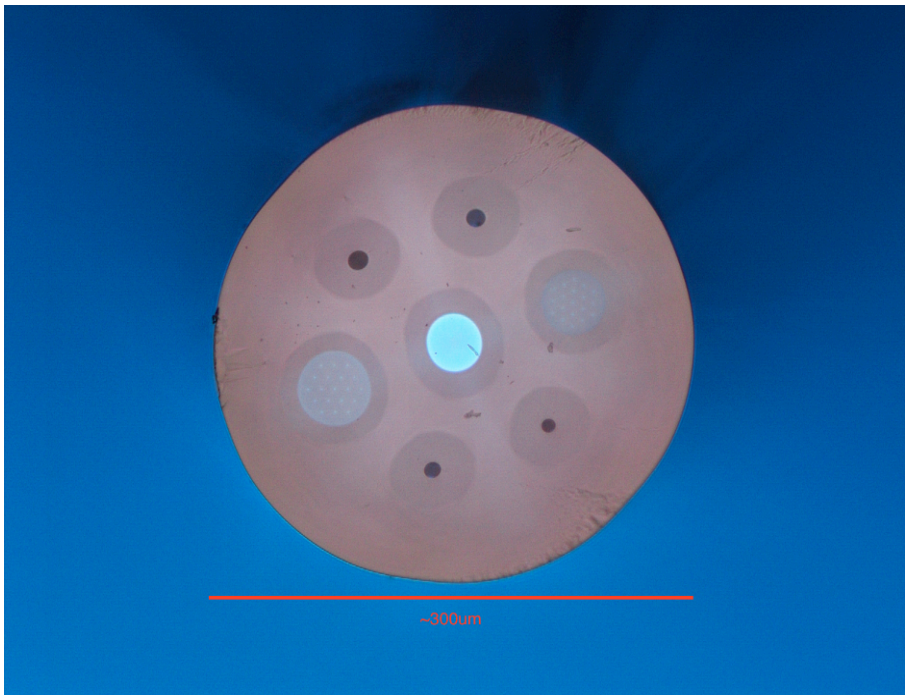

Figure 3 - Our combined Raman delivery/collection bundle. The central feature is the multimode delivery fibre that was coupled to the Raman excitation laser. To either side are the photonic lanterns formed from a multicore fibre (the SM cores are just visible).

During further testing, spectral emission lines proved to be sensitive to the fluorescent lamps in the laboratory. This was mitigated with a probe mount that incorporated a baffle (Figure 4), thus shielding the target area from direct illumination. The baffle extends to a point about 1mm before the focus of the probe optics, which may allow it to be used as a guide for any positioning arm.

Figure 4 - Raman Probe holder, includes baffle to indicate focus position and black stray light.

Laboratory tests for Salmonella

Laboratory testing of spinach leaves contaminated with rifampicin resistant *Salmonella* Sofia was performed on the 17th of February 2016. A total of 60 samples were prepared: 3 concentrations with

15 samples each and 15 control leaves (3 independent preparations). Each leaf was positioned under the probe and a 60 second measurement was taken using the SAIL Raman spectrograph (Figure 5). In a few cases the leaves were affected by the focused laser (this was later avoided by jiggling the leaf during exposure).

Figure 5 - Testing Spinach leaves contaminated with Salmonella Sofia at USYD Faculty of Agriculture and Environment in a PC2 laboratory.

The spectra obtained at first did not show obvious signs of the expected Raman bacteria signature, however further differential analyses (comparing the target spectra with the control spectra) shows two peaks at 650cm^{-1} and 735 cm^{-1} (shown in Figure 6), which are the key markers for Salmonella seen in literature. This signature was seen in 2 of 15 samples of the highest concentration of bacteria. Note there was some error in the exact height the probe was above each sample, which has likely contributed to the lack of detection in the other measurements (i.e. not at optimal focus).

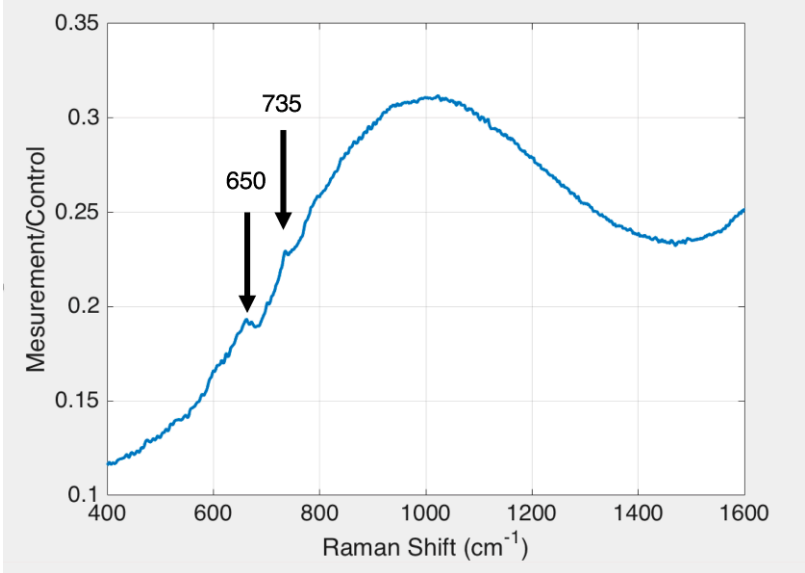

Figure 6 - Differential measurement of Raman spectra obtained of Salmonella contaminated spinach. The two labelled peaks are considered indicative of bacteria presence.

Subtraction of the background signal (primarily Raman signature and fluorescence of delivery fiber) has proven to be a limiting factor in the current design. Further improvements were made to include additional spectra filtering in the Raman probe to complement existing filtering in the spectrograph. This should lower the detection limit by dramatically reducing background signal. Treating this comprehensively is beyond the scope of this project as it will require additional lab testing to build a reliable library of Raman spectra for field-testing.

Mounting on the Robotic Arm

The Raman sensor probe was mounted to the end of a Universal Robots UR10 robotic arm in the ACFR field lab using a custom 3D bracket. The laser, CCD and electronics kit for the Raman sensor was placed on the adjacent bench and fibre-optic cable temporarily attached to the robotic arm. The robotic arm was manipulated to position the sensor probe above a lettuce leaf as shown in Figure 7. A simple program was written to command the arm to visit specific leaves in sequence, with time delay to allow for sampling.

Figure 7 - Raman sensor mounted to the robotic arm and demonstration movement between two sample points.

Further Upgrades to the Raman Probe

The project then focused on undertaking several improvements to the collection optics. This required the design and construction of a new custom Raman probe that incorporates additional optical filters to suppress background light associated with the excitation laser delivery fibre. This was achieved using three filters:

1. A narrow bandwidth notch filter, also known as a laser-line cleanup filter. This filter only allowed a narrow bandwidth centered on the excitation laser wavelength to be transmitted, thereby removing any Raman and/or fluorescence signal generated by the delivery fibre itself.
2. A dichroic mirror/filter. This reflected the excitation laser and transmitted light with wavelengths red-ward of 800nm. This was used to combine the light paths of the collection fibre and laser delivery fibre.
3. A longpass filter. This only transmits light red-ward of 800nm and acts to further suppress any collection of the excitation laser light. This filter was originally included in the spectrograph optical path.

The final layout of the optics in their 3D printed housing (and the light paths) can be seen in Figure 8.

The probe is only slightly larger than the original, but provides a dramatic improvement in the collected Raman spectra. The primary goal of the new probe was to suppress unwanted background signal. The success is evident in the raw spectrum of paracetamol, shown in top panel of Figure 9. The same spectrum obtained with the original probe is shown in the lower panels. Comparing the spectra we see an order of magnitude increase in sensitivity.

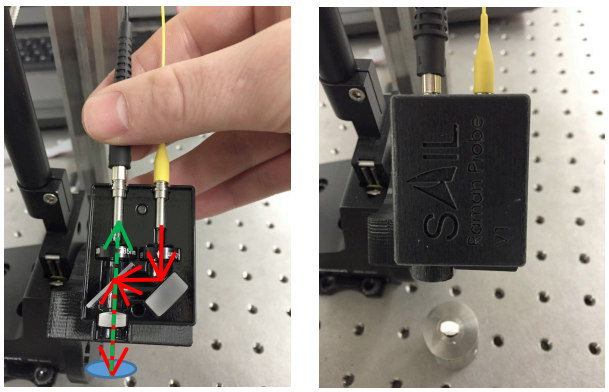

Figure 8 – The upgraded custom Raman probe with and without cover. This probe incorporates optimised collection optics and additional optical filters to reduce background light generated by the excitation laser delivery fibre (which has its own florescence and Raman signature). The optical path is illustrated in the left image. The red path shows the excitation laser, which is brought to a focus on the target sample (shown in blue). The green path shows light that is collected and fed to the Raman spectrograph (Note – Some of the excitation laser light follows this path but is rejected by the optical filters).

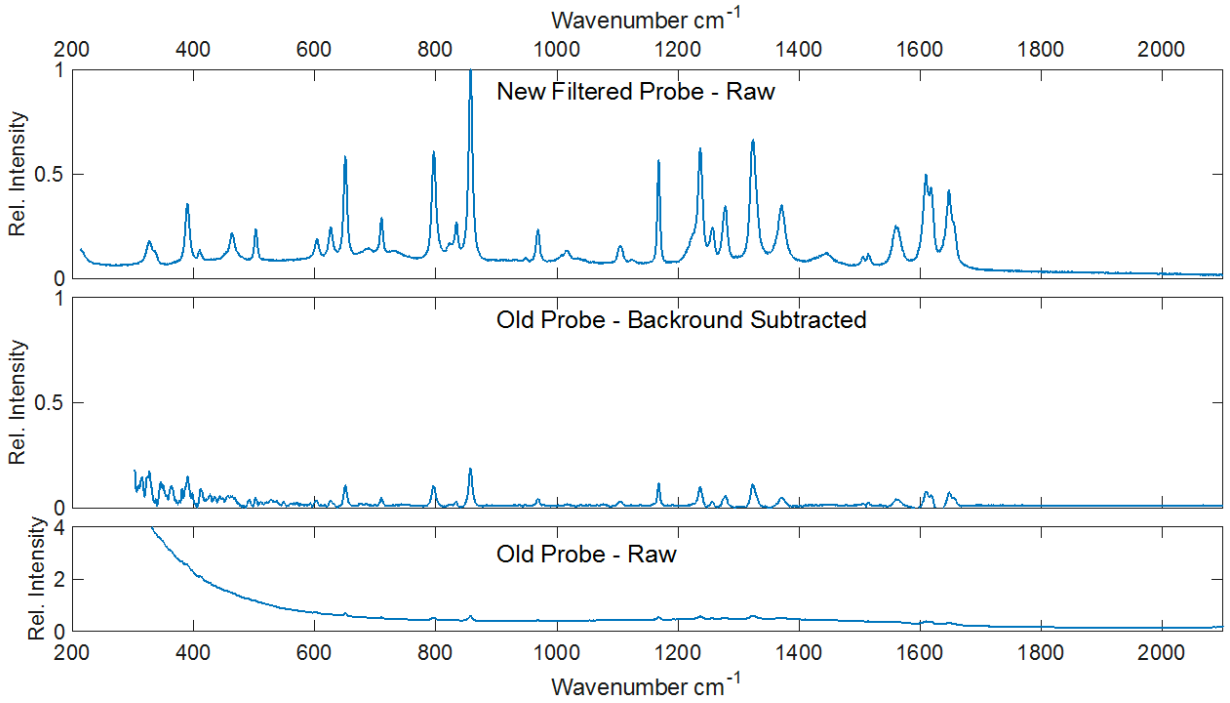

Figure 9 - Raman spectrum of Paracetamol using the new probe and the original probe. All spectra have been normalised by the maximum number of counts in the new probe spectrum. The top panel shows the raw extracted spectrum using the new probe where no background subtraction has been performed. The equivalent spectrum for the original probe is shown in the bottom panel. The background subtracted version of the old probe spectrum is shown in the middle panel. There is an order of magnitude increase in the sensitivity of the new probe with respect to the original probe. We also gained additional spectral information below 600cm^{-1}.

Further lab tests on Salmonella

On Tuesday 17 May 2016, we repeated the testing at the Faculty of Agriculture and Environment. A control and three 100-fold serially diluted suspensions of *Salmonella* Sofia containing 1.64 x 10^7, 1.64 x 10^5 and 1.64 x 10^3 cfu/mL were used and replicated across multiple sets of spinach leaves. These leaves were soaked in bacteria solution and allowed to dry. Each leaf was then positioned at the optimal focus of the probe, and a 60 second exposure was taken.

The spectra showed a clear signature that appears to be associated with the Spinach leaf (similar to the literature, Ref 1). Average spectra of each concentration and the control are shown in Figure 10. There are not any obvious differences between the spectra. A further differential analysis also does not show any significant difference between contaminated leaves and the control in areas associated with bacteria. The difference that are seen seem to scale with concentration, but we are not able to attribute them to lines known to be exhibited by bacteria (or spinach). One challenge is the lack of clear reference spectra in the literature. The appendix shows two samples, which are not fully in agreement.

Further, several spectral lines associated with bacteria are also very close to lines associated with the spinach and indeed any organic matter. It is worth noting that to our knowledge there has never been detection/measurements in the literature that did not make use of SERS (surface enhanced Raman spectroscopy) and/or a Microscopy setup (normally both are used). Further, the inoculated leaves were stored for 20hrs before testing and the viable numbers of cells dropped to 1000 per leaf or about 1.2 per square mm if evenly distributed, this was about 700 times less dense that the cells on leaves in the first experiment which could account for the differences in detection.

For this generation of detector and for current concentrations tested, the bacteria on a spinach leaf appears below our detection threshold and the initial detection seen is likely a false positive.

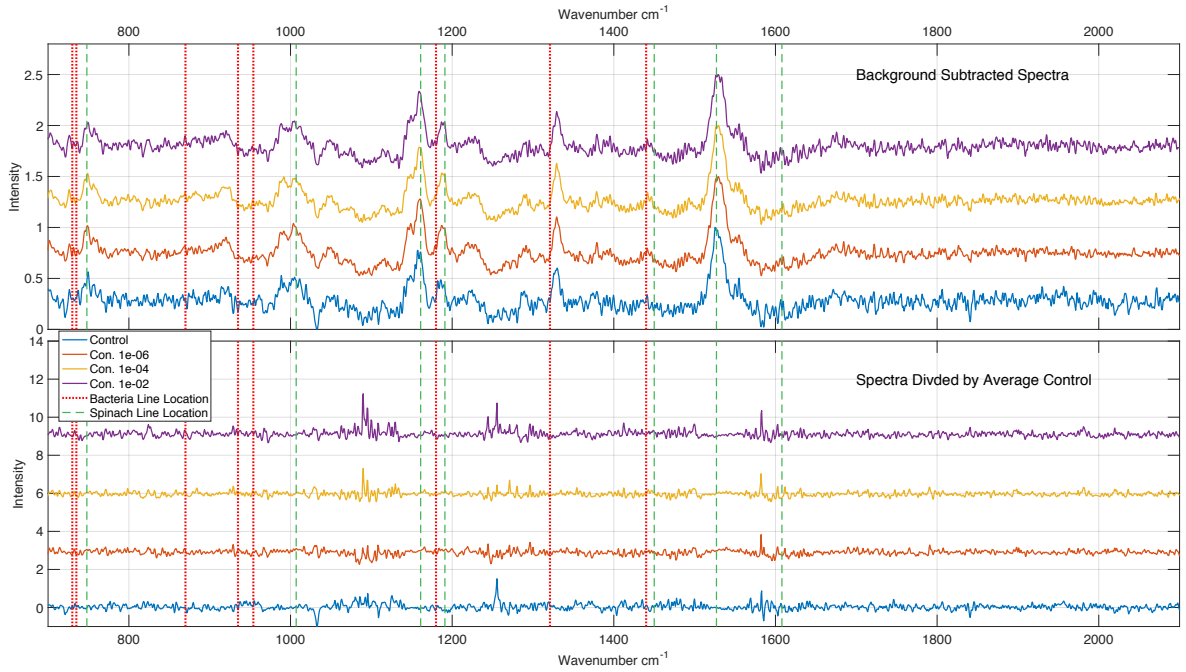

Figure 10 - Top Panel shows the sample background subtracted spectra of the inoculated spinach leaves and the control. Bottom Panel shows each spectrum divided by the average of all control spectra. A difference from the bacteria line would be considered evidence of detection. Shown in both are vertical lines that indicate the position of spectral lines associated with Bacteria (red) and spinach (green).

Field test on Robot

Repairs and modifications were made on the Ladybird robot and on 20 May 2016, a field test was conducted at the University of Sydney Lansdowne farm in Cobbitty NSW (Figure 11). The site had planted lettuce, and three lettuce were marked and inoculated with *Salmonella Sofia* as per the lab tests conducted on the 17 May. Another three lettuce plants were marked as control samples. The Ladybird robot was driven up to each of the plants and the robotic arm was manipulated to position the Raman sensor probe approximately 15 mm above the contaminated leaf. The three control plants were first tested and then the three inoculated plants were tested. Between each inoculated sample, the Raman probe was wiped and sterilised with ethanol. Although a manual task, it was useful in identifying an automated solution that could reproduce this action. The alternative concept of heat element sterilisation remains a practical solution but would require further development.

Operation of the Ladybird and robot arm in this field test were successful and the probe could be placed consistently approximately 15 mm above the leaf. The probe and robotic arm maintained a steady position for the 60 second sample, however, wind would cause the leaf to move a few mm. This slight variation in focal length did not seem to affect the samples gathered.

Again, the results gained in the field were similar to the results gained in the lab and no difference could be observed in the spectra lines between the control and inoculated lettuce leaves. This reaffirmed that further work with the detector and concentration samples is required.

Figure 11 – ACFR's Ladybird Robot fitted with the Raman sensor probe. The laser, CCD and electronics have been integrated with the Ladybird's frame and electrical power distribution.

Figure 12 – Raman sensor probe being positioned above the lettuce leaf.

Outputs

The project achieved the following outputs:

- A compact and portable photonic Raman spectroscopy sensor. Although the sensitivity was not able to confirm the presence of bacteria, the improvements in the sensitivity throughout the project were encouraging to recommend that further work could lead to repeatable successful testing of bacteria.
- The Raman sensor probe was fitted and integrated to the Ladybird robot and this successfully demonstrated the ability to autonomously inspect vegetables before harvest.
- The Ladybird robot mapped the lettuce vegetables detected and further work could integrate the Raman sensor spectrograph data with leaf locations.
- The Raman sensor probe developed was sufficiently compact to prove the feasibility of delivering a hand held version.
- The project progress was discussed with the vegetable industry during the 25/2/16 Advisory Meeting at the ACFR.

Outcomes

The following outcomes were achieved during the development of this project:

Sep 2015

- First design and build of compact Raman Sensor assembly.
- Collection and delivery fibre bundle (6 lanterns with central excitation source delivery fibre).
- Optical relay for collection and delivery fibre.
- Raman spectra of bacteria samples.

Oct 2015

- Second design and build of compact Raman Sensor assembly. This incorporated refinement of the Raman spectrograph design learned from the first design.
- First combined delivery and collection fibre bundle manufactured.

Mar 2016

- A better understanding of the limitation of the Raman sensor and the next steps to improve on design.
- In lab testing for bacteria, initial results determined that there is the potential for detecting *Salmonella* Sofia although further testing required.
- Initial results of the sensor on the arm show that the capability to sense on crops is achievable.

May 2016

- Third design and build of improved Raman sensor probe assembly with significant sensitivity improvements.
- Further lab tests with Salmonella Sofia. These confirmed the limitations with the final design in this project.
- Integration with field robot and infield testings of lettuce inoculated with Salmonella Sofia. This demonstrated how this technology could be adopted in the field.
- Consideration of design implications of the need to sterilise the sensor tip in between samples.
- Identification of further work required to achieve successful testing:
    - Enhance collection optics with new spectral filters,
    - Add multiple collection fibres per original proposal (i.e. larger bundle),
    - Additional lab testing with Salmonella samples,
    - Test a more sensitive CCD sensor with reduced read noise to increase signal to noise of spectra.

Evaluation and Discussion

This project was successful in producing a compact and portable Raman spectroscopy sensor. Although the Raman technology failed to successfully detect bacteria, the methodology was sound as improvements to the sensitivity were obtained throughout the project, and hence these initial results proved encouraging. The sequence of development and testing made significant progress to achieving the successful outcome, however, the undertaking was ambitious and the necessary development and testing proved to be beyond the scope of this project.

As described in the methodology, from the results of initial tests, and the opportunity to learn and make corrections led to several iterations of design. The result in the scope of this project was a significant improvement of the sensitivity of Raman spectra.

Completing the integration with the field robot and conducting inspections in the field was very worthwhile to show the capability of this technology and how it could significantly improve fresh produce quality and potentially eliminate the harvesting of contaminated vegetables.

While no tests were undertaken in the packaging chain, the sensor probe developed was sufficiently compact to demonstrate the feasibility of a hand held. Being a real time sensor, there would be no reason for any produce to be delivered to the customer without testing.

As Defined in original project agreement:

- Economical *and* affordable mechanism to detect microbial problems.
    - The system did not detect microbes
    - The system that was developed used low cost componentry and 3D printing techniques.
- In the field pre-harvest and providing a check point to eliminate the source and prevent cross contamination further along the supply chain.
    - The system did not detect microbes
    - The system was mounted onto the Ladybird robot and demonstrated the capability of infield testing.
- An adaptable hand held Instrument to assist in verifying the chemical efficacy of wash water systems through Identification of microbial load in the wash water.
    - The system developed is small enough to be hand-held, however no testing of the device in this manner was undertaken.
- An adaptable hand held instrument to scan and assess cleanliness of equipment Including harvest machinery, packing crates, boxes, cool rooms, and packing shed floors.
    - The system developed is small enough to be hand-held, however no testing of the device in this manner was undertaken.
- A system to detect microbial contamination in the finished product package, providing greater certainty of a safe product and reducing risk of food safety breach.
    - The system developed is small enough to be hand-held, however no testing of the device in this manner was undertaken.
- Scope for use along the supply chain, e.g. in supermarkets as packages are placed on shelves.
    - The system may be capable of achieving this with further investigation required.
- Extrapolated to other products in the vegetable industry e.g. tomatoes, cucumbers, sprouts.
    - This was not achieved.

Recommendations

Improvement of Raman Technique

- Several spinach Raman lines are very close to expected bacteria lines:
    - A possible solution is to target a smaller spectral bandwidth with increased resolution, thus allowing these to be distinguished.
- Change wavelength of excitation laser:
    - A longer wavelength 1064nm will reduce fluorescence of the target, and can produce more information from biological samples. It however requires more specialised (and expensive) detectors/CCDs.
    - A shorter wavelength (532nm/630nm) can also be explored. While this could increase florescence it will also increase the amount of light that is Raman scattered. This would also push the detection wavelength range to a higher efficient region of silicon CCDs.
- Implementation of a fluorescence mitigation technique:
    - Because the fluorescence signal is not instantaneous, it is possible to gate the capture/measurement in order to bound where the Raman light has scattered but where fluorescence has not begun.
    - This requires high-speed timing between the laser and detector (i.e. Ref 4).
- Include additional spectra filtering in the Raman probe:
    - This would complement existing filtering in the spectrograph.
    - This should lower the detection limit by dramatically reducing background signal.
    - This will also likely require additional lab testing to build a reliable library of Raman spectra for field-testing.
    -

Alternate technologies/options to explore

- Monitor plant health:
    - The results obtain provided a spectra of the spinach and further research could generate a spectra profile for different conditions of the plant.
- Possibility of NIR absorption spectroscopy and/or correlation with Raman spectra.

Practicalities in-Field Testing or During Packaging

As the Raman probe requires close proximity to the vegetable, the potential for contamination of the probe becomes a concern. Sterilisation of the probe will be necessary especially after confirmed bacteria detection results. Sterilisation by heating or spraying and wiping with alcohol are both feasible and would be a matter of further development. Conceivably, this process would be automated if used by the field robot: if the spectrograph confirms the bacteria was present, as a matter of caution the probe would be sterilised automatically with included equipment.

For hand held applications the human operator could spray and wipe the probe.

For both applications it will be very important to design the probe to have only one contact area that could be contaminated. This would likely be a long stiff probe that would ensure that the sensing end would only make contact with the produce.

Scientific Refereed Publications

None to report

Intellectual Property/Commercialisation

No commercial IP generated

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Appendices

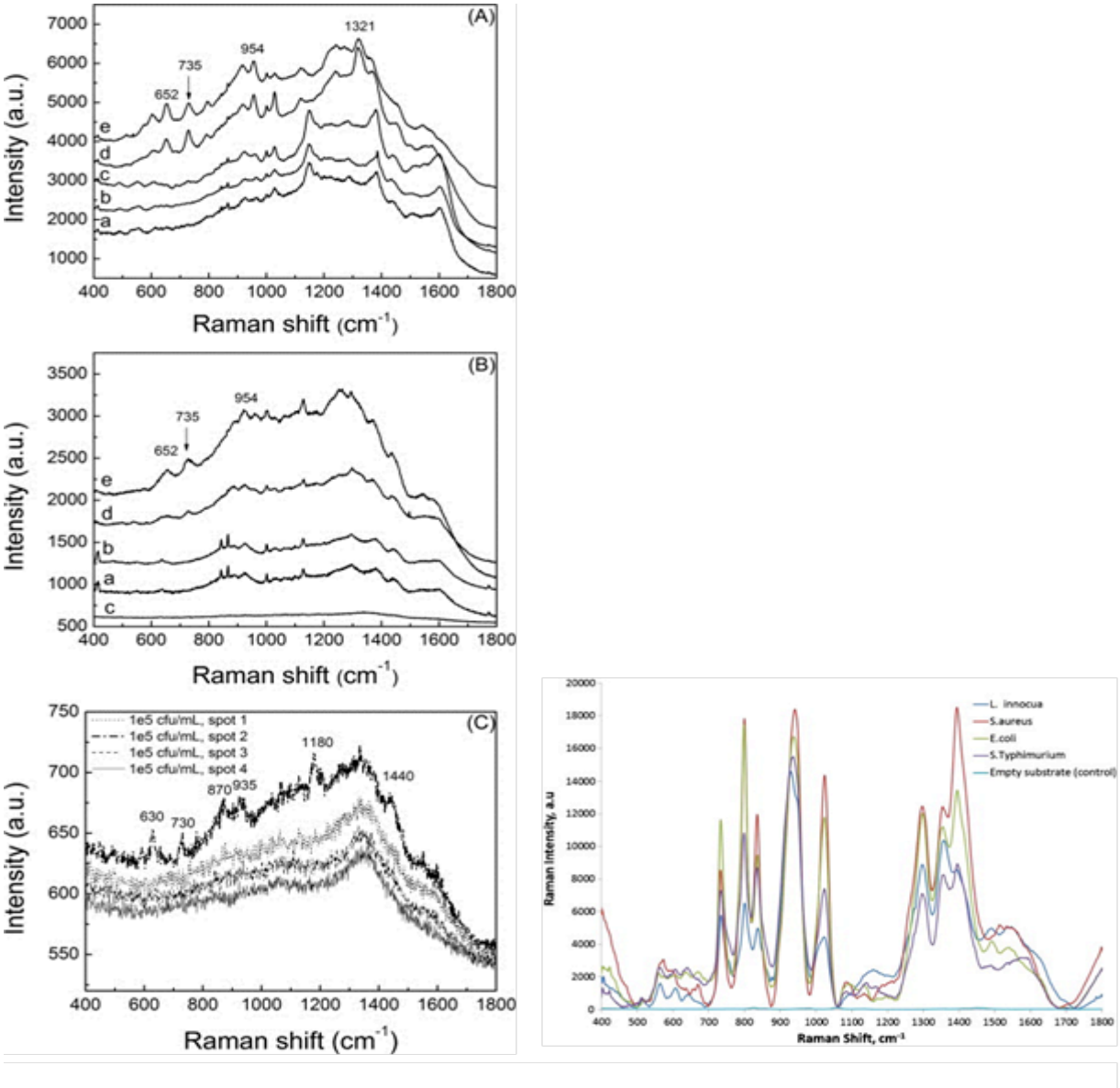

Figure A1 – Reference Raman spectra of bacteria from literature. The Figure on the left is from Ref 2 where a SERS active filter is used to concentrate bacteria and enhance the Raman signal. The figure on the right is from Ref 3 where confocal microscopy is used to target bacteria on SERS substrate.

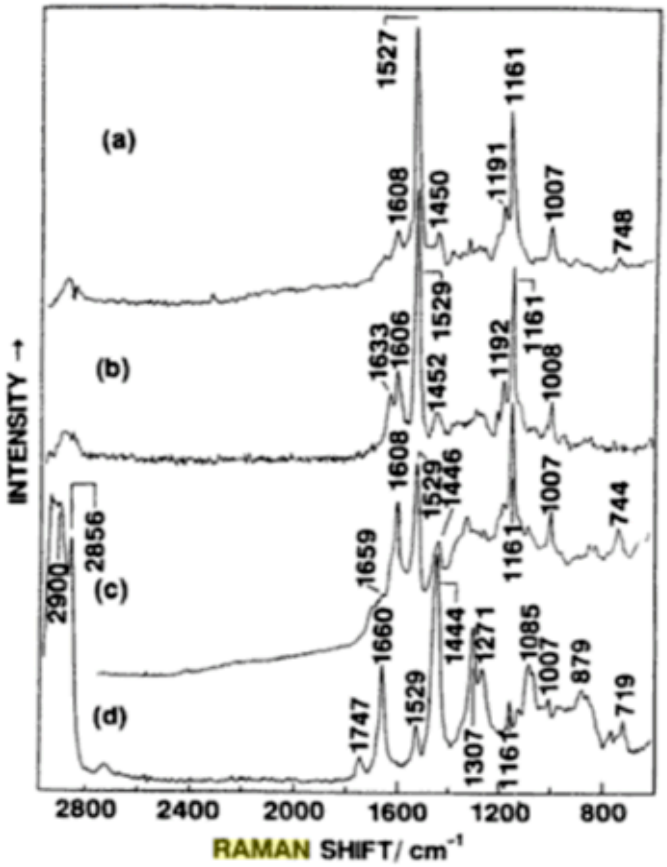

FIGURE 20 FT-Raman spectra of (a) fresh spinach leaf, (b) peel of mandarin orange, (c) new leaf Japanese tea, and (d) hen's yolk. (Reproduced from Ref. 25 with permission. Copyright © 1992, Society for Applied Spectroscopy.)

Figure A2 – Figure copied from Ref 1. Shows spectrum of spinach leaf (a).