Raman Spectroscopy Capabilities of the CBex Handheld Raman Instrument

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Raman Spectroscopy Capabilities of the CBex Handheld Raman Instrument

An honors thesis presented to the
Department of Chemistry,
University at Albany, State University Of New York
in partial fulfillment of the requirements
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and
graduation from The Honors College.

Samantha Ingenito

Research Mentor: Igor Lednev, Ph.D.
Academic Advisor: Paul Toscano, Ph.D.
Abstract

Raman spectroscopy is a reliable, nondestructive method of identifying and distinguishing between different types of biological fluids. However, forensic studies have been restricted to indoor desktop Raman instruments when it would be optimal if Raman spectroscopic analysis could be performed at a crime scene. The CBex Handheld Raman Instrument was developed as a portable tool that could perform Raman spectroscopy outside the lab. Unfortunately, the CBex cannot obtain viable spectra of biological fluids. The handheld was compared to the Renishaw invia Raman Microscope by quantifying the signal-to-noise ratio of each by acquiring Scotch tape spectra. The handheld has a significantly lower detector sensitivity than the desktop and cannot take viable spectra over an integration time of 2.5s without reducing the laser power.
Acknowledgements

I would like to thank Dr. Igor Lednev and the entire lab for their help and support in the completion of my studies. Dr. Lednev was especially active in guiding me in the right path and encouraging me to do my best work. Under his mentorship, I truly felt like my efforts held significance in forensic chemistry research.

Dr. Paul Toscano deserves high recognition for his incredible dedication towards being my research advisor. He has worked day and night to make sure I got the most out of my education. I’m quite grateful that for the last couple of years I have had an advisor who I could undoubtedly rely on and trust that he had my best interests at heart.

I would also like to thank my family and friends for their love and support. They pushed me to overcome any hardships that I have come across and motivated me to keep pursuing a career in chemistry. Lastly, I would like to thank Chris Pollatos for providing a firm foundation and appreciation for scientific research in high school. The knowledge and experience from the science research program gave me the skills and confidence I needed to excel here at UAlbany.
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Introduction to Raman Spectroscopy

Spectroscopy refers to the study of how light interacts with matter. In the field of chemistry, different types of spectroscopy are useful in identifying compounds and their unique properties. Raman spectroscopy is a fairly recent technique credited to Indian physicist, Dr. C.V Raman, in 1928. However, practical use of Raman spectroscopy was not available until the 1960s when laser systems were developed.\(^1\) In the 1970s, Raman spectroscopy was further improved by combining the laser system with the microscope, which increased the focusing capabilities and boosted the detected signal.\(^2\) Today Raman spectroscopy is regarded as one of the most versatile methods of substance identification, making it highly desirable for forensic chemistry applications.

Raman spectroscopy works through the concept of the Raman effect, which is based on molecular deformations in the electric field as determined by molecular polarizability. In other words, when a strong light source, such as a laser, hits a substance there is light scattering that is unique to the substance’s molecular composition. The laser causes the molecules to be excited and vibrate at a certain frequency. After excitation, there are three possibilities when the absorbed electromagnetic radiation is reemitted: Rayleigh scattering, Stokes scattering, and Anti-Stokes scattering.\(^3\)

Rayleigh scattering occurs when a molecule absorbs a photon of a certain frequency and then emits this energy at the same frequency. This is considered elastic light scattering since no energy is lost and makes up for 99.999% of light scattering involved.\(^1\) Raman spectroscopy does not consider Rayleigh scattering as significant data since there is no observable change in frequency. This is why Raman spectroscopy was not practical until the development of the laser...
system as a strong light source. By increasing the strength of the light source, the probability of detecting inelastic light scattering, Stokes and Anti-Stokes scattering, increases enough for applicable use. Stokes scattering is when the emitted photon has a lower frequency than the absorbed photon. This is due to the loss of energy to the Raman-active mode in the molecule. Anti-Stokes scattering is when the emitted photon has a higher frequency than the absorbed photon, due to the absorption of excess energy while already in an excited state. Stokes scattering is much more common than Anti-Stokes scattering, as it it much easier to lose energy than to store excess energy. ³

Rayleigh scattering is a major problem when collecting Raman spectra. It is crucial for Raman instrumentation to have the appropriate wavelength filters and detectors. Otherwise the Raman instrument will be unable to discern stray light from the desired inelastic scattering, and thus be rendered incapable of acquiring viable spectra.¹ Later this will come into play when discussing the capabilities of the new CBex Handheld Raman Instrument, but first the capabilities of the well-established Raman technology should be established.
Applications of Raman Spectroscopy

The ability for a Raman microscope to distinguish between bodily fluids in a quick, non-destructive manner without the need for sample pretreatment makes Raman spectroscopy an invaluable method for crime scene investigations. Our lab has completed many studies to determine the numerous applications of Raman spectroscopy for real-world forensic purposes. There have been studies about differentiating between a wide range of bodily fluids such as blood, sweat, saliva, semen, and vaginal fluid. Mixtures of fluids can be identified through unique characteristics of each component and samples containing contaminants, like sand, dust, and soil, can still be identified. This is important because bodily fluids found at a crime scene are not realistically collected as perfectly isolated samples.

Raman spectroscopy is so specific in distinguishing molecules that it is possible to characterize different types of the same bodily fluid, such as blood. For instance, Raman spectroscopy can distinguish between peripheral blood and menstrual blood based on the slight variation of present proteins. The relative age of the bloodstain can also be determined because blood changes in composition over time. Raman spectroscopy can even differentiate between blood samples of people of different races. Based off of a small sample of blood, Raman spectroscopy makes it possible to contribute invaluable information about the extent of the crime and the people involved.
The Development of a Portable Raman Device

Huge strides have been made in Raman forensic research. However, all of these studies were performed using a desktop Raman microscope, thus begging the question of how Raman spectroscopy could be brought out into the field for a faster analysis of evidence. This began the development of portable Raman instruments that could be used directly at a crime scene. One such instrument is the CBex Handheld Raman Instrument, complete with a 785 nm laser with a maximum power of 50 mW. While not technically a Raman microscope with its absence of an objective lens, the CBex handheld can perform Raman spectroscopy by pointing the laser at the sample in order to produce the necessary inelastic photon scattering.\textsuperscript{11}

Unfortunately, the CBex handheld could not identify bodily fluids, such as semen, unlike a Raman microscope. A previous study of the CBex demonstrated the poor detector sensitivity by comparing the acquired spectra of dried semen between the handheld and the Renishaw invia Raman microscope. The thickness of the accumulation of 18 µL of semen was not enough for the handheld to analyze properly.\textsuperscript{12}
Figure 1: Comparison of semen spectra taken with the Renishaw invia Raman Microscope and the CBex Handheld Raman Instrument

Samples of Scotch tape were used to determine the minimum thickness required for the entirety of the sample to be saturated with photons in order to obtain proper spectra. Scotch tape was used due to its strong Raman effect and that each layer had a known thickness of 78.74 microns. The minimum thickness for the CBex to obtain an effective spectrum was was 1181.1 microns, the equivalent of fifteen layers of tape.
Figure 2: The maximum intensity detected at the highest peak of the Scotch tape spectra (811 cm$^{-1}$). The intensity started to plateau at 15 layers of tape.$^{12}$

This information built the foundation for quantifying the difference in detector sensitivity between the CBex handheld and the desktop Raman microscope. This was done by finding the signal-to-noise ratio (SNR) for each instrument. As the name suggests, the SNR is the measure of how much of a signal can be detected apart from any background noise. As discussed previously, a detector in a Raman instrument must be able to differentiate between an actual signal from background radiation and Rayleigh scattering. Only then can the instrument acquire viable spectra.
Quantifying the SNR of the CBex Handheld and the Renishaw Microscope

Ten glass slides were prepared with a layer of aluminum foil and then fifteen layers of Scotch tape. The aluminum helps to reduce background radiation, which is why most samples in Raman studies are prepared on top of aluminum. The fifteen layers of tape were carefully laid on top of each other to create a sample 1.2 mm thick. This ensured that the laser would saturate the sample with photons and the detector would receive as high of a signal as possible.

The CBex handheld parameters were set to 785 nm laser with 50 mW of power. The Renishaw invia Raman microscope was also set to 785 nm laser with 65 mW of power using the 20X objective. These settings minimized the difference between laser power to ensure that any difference in the spectra was due to the detector sensitivity. All the tape samples were measured at 1s, 2s, and 2.5s integration times to compare the SNR of each device. While increasing the integration time should boost the signal, the SNR should remain the same. Therefore, it was expected that there would be consistency in SNR across any integration time. The integration times would have been more evenly spread out, but the handheld was unable to acquire viable spectra at 3s integration time without chopping off the tops of the most intense peaks. This meant that the sample became oversaturated with photons, rendering the spectra inconclusive. The third integration time had to be reduced to 2.5s to eliminate this problem at this stage. This was an interesting limitation to the handheld that will be discussed more at length later.

In order to obtain spectra, the handheld was clamped to a ring stand while plugged into the computer and the sample was placed on an adjustable stage. The stage had to be perpendicular to the laser for accurate results. To acquire the highest intensity, the stage was
moved up and down to bring the sample into focus. After the laser was in focus, the handheld took three accumulations and the resulting spectra were averaged together. This was repeated for each of the ten tape slides within each integration block. The SNR was calculated for each tape sample and then the SNR values for all ten were averaged together to determine the overall SNR for the integration time. This same procedure was repeated for the desktop microscope.

In order to calculate the SNR from a tape spectrum, the signal and the area of noise had to be chosen. The main focus on the tape spectra was the peak of highest intensity at around 811 cm\(^{-1}\), as determined by previous handheld data. The maximum intensity of this peak was compared to the average noise between 540-752 cm\(^{-1}\), where there are not any peaks as shown in Figure 3, in order to calculate the SNR.

![Sample Tape Spectrum](image)

*Figure 3: Raman spectrum of Scotch tape*

For each spectrum the amplitude of the 811 cm\(^{-1}\) peak was divided by the average amplitude of each peak between 540-752 cm\(^{-1}\). This value was then squared to find the SNR. After the SNR was calculated for each sample and the average SNR was found for each
integration time, the detector sensitivities of the handheld and desktop Raman instruments could be compared to each other.

Results of the Raman Instrument Comparison

Table 1 shows the average values from the ten samples for the peak with the highest intensity and SNR from the CBex handheld trials over 1s, 2s, and 2.5s integration times.

<table>
<thead>
<tr>
<th>Integration Time</th>
<th>Average</th>
<th>St Dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>1s</td>
<td>Max Peak (cm$^{-1}$)</td>
<td>810</td>
</tr>
<tr>
<td></td>
<td>SNR</td>
<td>3.32</td>
</tr>
<tr>
<td>2s</td>
<td>Max Peak (cm$^{-1}$)</td>
<td>811</td>
</tr>
<tr>
<td></td>
<td>SNR</td>
<td>3.18</td>
</tr>
<tr>
<td>2.5s</td>
<td>Max Peak (cm$^{-1}$)</td>
<td>811</td>
</tr>
<tr>
<td></td>
<td>SNR</td>
<td>3.91</td>
</tr>
</tbody>
</table>

Table 1: Average values of SNR of the CBex Handheld Raman Instrument

The average position of the maximum peak occurred at 810.7±0.6 cm$^{-1}$, which was consistent from previous handheld data. The average SNR for all three integration times was 3.47±0.39, showing a fairly consistent ratio across the three integration times as expected.

Table 2 shows the average values from the ten samples for the peak with the highest intensity and SNR from the desktop Raman microscope trials over 1s, 2s, and 2.5s integration times.
<table>
<thead>
<tr>
<th>Integration Time</th>
<th>Max Peak (cm$^{-1}$)</th>
<th>Average</th>
<th>St Dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>1s</td>
<td>808</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SNR</td>
<td>30.27</td>
<td>6.06</td>
</tr>
<tr>
<td>2s</td>
<td>808</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SNR</td>
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<td>3.08</td>
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<tr>
<td>2.5s</td>
<td>808</td>
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</tr>
<tr>
<td></td>
<td>SNR</td>
<td>30.68</td>
<td>4.74</td>
</tr>
</tbody>
</table>

**Table 2: Average values of SNR of the Renishaw invia Raman Microscope**

The average position of the maximum peak occurred at 808.0±0.06 cm$^{-1}$, which suggests a slight difference in calibration between the handheld and desktop. While this should not affect the comparison of SNR between the two instruments, it was important to note as it already suggests a difference in the ability of the detectors.

The average SNR for all three integration times was 30.07±0.73. Again, the ratio was fairly consistent across integration times as expected.
As shown in Figure 2, the desktop Renishaw microscope produces spectra with a higher intensity than the CBex handheld Raman instrument. Since the average SNR values of the handheld and desktop were 3.47 and 30.07 respectively, the handheld is 8.67 times less sensitive than the desktop. This would explain why the handheld has been incapable of acquiring viable spectra of bodily fluids which are not as Raman-active as Scotch tape.

**Figure 4:** Comparison of the average spectra of the 2s integration time block between the handheld and the desktop Raman devices
Attempt at Acquiring Spectrum for Thick Sample

While the signal detected by the CBex handheld was much lower than the desktop Raman microscope, the handheld could still obtain spectra for tape at a thickness of 1.2 mm. While it was understandable that the handheld could not take spectra of bodily fluids from samples of a single application, there was still the question whether a 1.2 mm thick sample could be detected. Theoretically, this was the minimum thickness required to saturate the sample with photons to efficiently obtain spectra. It was then decided to attempt to make a 1.2 mm thick sample of a bodily fluid to test this thought.

First the type of bodily fluid had to be chosen. Since the sample had to be quite thick, the bodily fluid could not be colored. Otherwise the sample would absorb too much light and not provide an accurate spectrum in return. This knocked out the possibility of blood, especially since it also changes color as it ages. The next best choice was semen, as it is a colorless fluid commonly found at crime scenes. Semen was also a sensible choice as it was the fluid used to first demonstrate the difference in signal between the CBex handheld and the Renishaw invia Raman Microscope.

A metal slide was designed to have a circular divot pressed into the surface. This created a flat-bottomed well in the slide that was 3.0 mm in diameter and 1.2 mm in thickness, for a total volume of 8.5 mm$^3$. The same semen sample (LOT # BRH844081) was used to fill the divot and then allowed to dry. Taking a spectrum of a wet sample would not have worked, as the water would not have contributed to the actual thickness of the semen sample. The semen had to be allowed to dry for the most accurate results. Using a pipettor, the semen was inserted into the divot and was refilled as many times as possible before allowing the sample to dry overnight.
Originally the plan was to do only one application per day, but the divot needed to be filled as much as possible to amass the 1.2 mm thickness. The sample was refilled whenever enough water had evaporated.

Unfortunately, the quickened application process still was not enough to completely fill the divot with semen. After three weeks of constant applications, only a small layer of deposit was left behind after all water had evaporated. As this was still the thickest sample produced by the lab, the sample was tested anyway with the CBex handheld. It came to no surprise that the handheld could not obtain a viable spectrum, so the results of this attempt were inconclusive.

As frustrating as this failure was, it further demonstrated the impracticality of the minimum thickness required to get a proper Raman spectrum with the handheld. It became clear that making a 1.2 mm thick sample was unrealistic in a lab setting, let alone the real-world application of crime scene analysis. The detector would have to be improved so that a sample can be less than 1.2 mm thick and still be detectable. In order to improve the detector, more tests were performed on the handheld to establish its full capabilities.
Testing the Integration Time Limitations of the CBex

As mentioned previously, the CBex handheld could not be used past an integration time of 2.5s without the signal becoming oversaturated. This was an interesting discovery because desktop Raman instruments can take higher integration times without a problem. In an effort to fix this problem, the laser power of the handheld was decreased from 50 mW to 30 mW of power. By lowering the power of the laser, the tape samples should not be oversaturated with photons anymore. In order to test this hypothesis, the procedure for acquiring spectra on the CBex handheld was repeated on the same ten tape samples except with the power lowered to 30 mW. First it had to be determined whether lowering the laser power affected the SNR, so the samples were run again at the integration times of 1s, 2s, and 2.5s.

<table>
<thead>
<tr>
<th>Integration Time</th>
<th>Average</th>
<th>St Dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>1s</td>
<td>Max Peak (cm⁻¹)</td>
<td>811</td>
</tr>
<tr>
<td></td>
<td>SNR</td>
<td>3.29</td>
</tr>
<tr>
<td>2s</td>
<td>Max Peak (cm⁻¹)</td>
<td>811</td>
</tr>
<tr>
<td></td>
<td>SNR</td>
<td>3.49</td>
</tr>
<tr>
<td>2.5s</td>
<td>Max Peak (cm⁻¹)</td>
<td>811</td>
</tr>
<tr>
<td></td>
<td>SNR</td>
<td>3.40</td>
</tr>
</tbody>
</table>

Table 3: Average values of SNR of the CBex Handheld Raman Instrument at 30 mW power
The maximum peak held steady at 811 cm$^{-1}$ just as it did for the handheld at maximum power. The average SNR was 3.39±0.10, which was not significantly different from the average SNR of the 50 mW trials (3.47±0.39).

**Figure 5:** The comparison of the SNR from the 50 mW and 30 mW trials at each integration time and the average of the three integration times

The error bars representing the standard deviation from each integration time confirmed that there was no significant difference in SNR within each laser power block. This meant that the SNR remained consistent across integration times as expected. The overlap of error bars when comparing the 50 mW SNR values with the 30 mW SNR values also confirmed that lowering the laser power did not significantly change the SNR between the integration time range of 1-2.5s.
Now that it was confirmed that lowering the laser power did not negatively affect the SNR of the instrument, it was possible to test how much the integration time range increased. Once again, the same tape samples were analyzed with the handheld at 30 mW of power using the same methods to calculate the average SNR for each integration time block.

![SNR at 30mW](image)

**Figure 6: The average SNR for each integration time from 2.5s to 4.5s at 30 mW power**

The CBex handheld was able to acquire spectra at 4.5s integration time without the samples being consistently oversaturated. After plotting the average SNR for each time block, it became clear that the SNR steadily decreases beyond an integration time of 2.5s. Since the $R^2$ value of the linear fit was 0.99, it is possible to approximate the SNR between 2.5s and 4.5s at 30 mW of power. This would be helpful to keep in mind should future experiments using the handheld require a higher integration time of 2.5s.
Conclusions

By quantifying the SNR of the CBex handheld Raman instrument and comparing Scotch tape spectra with the Renishaw invia Raman microscope, it became clear that the detector sensitivity in the handheld was much lower than the desktop. This would explain why the handheld cannot detect biological samples, such as semen, as the desktop can. Without the ability to detect and identify bodily fluids, the CBex Handheld Raman Instrument is not ready for practical use in the field.

An interesting discovery was that the handheld could not acquire viable spectra after an integration time of 2.5s at maximum laser power. It was possible to decrease the laser power to 30 mW and acquire spectra up to 4.5s integration time. While the SNR was not significantly affected from integration times of 1-2.5s, the quality of signal steadily decreases after that range. From this it is important to keep in mind the trade-off between higher integration times and SNR.

While the ability to boost the integration time by lowering the laser power does not help to obtain viable spectra of biological samples, it was crucial to ascertain the full capabilities of the CBex handheld. The next step would be to improve the detector sensitivity and build upon these established capabilities. Ideally, with the eventual modifications, the CBex handheld Raman instrument will be able to detect biological samples out in the field.
Future Studies

Now that it has been determined that the problem with the CBex handheld is its low detector sensitivity and have quantified the level of its sensitivity, it is possible to start the improvement of the device. By monitoring the SNR of the device while making modifications to the detector, we can determine the progress of the device.

When the handheld eventually becomes able to detect biological fluids, it would be interesting to see how it overcomes the ultimate obstacle: the outdoors. The purpose of the handheld is to be able to conveniently analyze biological samples at the crime scene. All the testing performed with the handheld was performed in a darkened room to reduce interference from stray light. It would be interesting to see how the device could be brought outdoors and be used without the unnecessary radiation ruining the results.

It may become necessary to explore other options for portable Raman analysis, such as a portable Raman microscope. While much bulkier than the CBex, the portable microscope has an enclosed space that could block out stray light. More testing needs to be done with our lab’s current portable microscope to determine its detector sensitivity and ability to identify biological fluids. This may end up the more realistic option of portable analysis while the CBex handheld undergoes modifications.
References