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Resolution of Portable Raman Spectrometers and its Effect on the Analysis of Biological Specimens

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Abstract

Body fluid traces are a common and a major form of evidence that can be found at a crime scene, as it is a good source of DNA. As such, it is vital that body fluid evidence is properly detected and identified. Issues with the current methods of identifying body fluids and the large backlog of evidence in most forensic labs call for more accurate on-scene (*in situ*) body fluid identification. Past research has demonstrated that Raman spectroscopy is an advantageous tool for accurately and quickly identifying body fluids, namely in the laboratory. Portable Raman instruments have been created to meet the need for performing this task at the crime scene. Specifically, a number of handheld Raman instruments are commercially available. Our laboratory has previously tested such instruments and found them not suitable for the analysis of trace evidence because of poor spatial resolution. BioTools instrumentation company has developed a first portable Raman microscope, which has a much higher spatial resolutions than handheld instruments. This project was focused on evaluating the capability of this instrument for the identification of biological stains. Specifically, we targeted determining the effects of this instrument's spectral resolution on the analysis of body fluids. For comparison, the spectra of powder and cast film proteins were also collected. The performance of the portable instrument was compared with that of a desktop instrument. We found that the portable Raman microscope was capable of producing spectra of proteins and body fluids of a comparable quality to its desktop counterpart. Overall spectral features were largely similar that allowed for quick identification of body fluid spectra via visual comparison. These results indicated that the BioTools portable Raman microscope can potentially be used for analyzing biological evidence immediately at the scene of the crime and could improve the quality of forensic investigations.

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Introduction

One of the most important steps in a criminal investigation is the collection and analysis of all types of evidence that is found at the crime scene. These evidences can range from explosive debris to controlled substances to footprints, and there a myriad of methods for analyzing each kind. Many of these tests, such as DNA profiling, are performed in forensics labs, and they often require use of costly materials and require a lot of time to run. The Organization of Scientific Area Committees has noted the resultant backlog of evidence at several crime labs that seriously slow the progress of criminal investigations. They have reported that there is a great need in techniques and technologies to remedy this issue [1]. These new methods would need to be accurate and quick, and potentially for use of inspection of various kinds of evidence *in-situ*, or at the crime scene itself.

One such area of evidence is biological (i.e. blood, semen, saliva, etc.). Presently, there are numerous preliminary and confirmatory tests for identifying biological fluids, and most utilize biochemical reactions to detect the presence of the body fluid. These tests, namely the preliminary ones, have been considered relatively inexpensive and user-friendly, and thus compatible with use in the field [2]. However, these tests come with their limitations. They are highly specific to a certain body fluid, so one piece of evidence might have to undergo repeat testing, which would cause the method to be still time-consuming. Additionally, this would result in loss of evidence, since the tests are often destructive to the sample. The tests used at the crime scene are presumptive, because they contain significant amounts of inaccuracy, so the evidence must undergo further testing at the lab, resulting in more time and evidence being used up [3]. Because of these shortcomings, biochemical identification tests are proving to be largely ineffective in dealing with the needs of forensic investigators.

To aid in this quest, research has focused on potential ways to remedy the current obstacles of identifying body fluid evidence. One such technique has gained a lot of notoriety: Raman spectroscopy. Spectroscopy is a group of methodologies that measure various interactions between light and materials. On such subcategory is Raman, which measures the light that is scattered by a material. The energy of the light that is scattered differs from the energy of the light used to irradiate the material, and this shift in energy (measured in wavenumbers or cm^{-1}) is based on the structures of the compounds present in the material. As such, a material will have a distinct profile of Raman signals, or spectrum. these unique spectra can be used to distinguish different substances from each other, which would make this technique highly advantageous in a forensic setting.

Several researchers have already demonstrated the efficiency of Raman spectroscopy in identifying numerous forms of evidence, such as biological specimens, drugs, trace evidence, firearm residue, and even fingerprints [4-7]. Our lab has used Raman to chemically characterize numerous body fluids, which has been used to identify them with great accuracy [3, 8-11]. Raman spectroscopy has also been used for tasks beyond simple body fluid identification, including determining the race, age, and gender of body fluid donors and the time since deposition of a biological stain [12-16]. This bloom in research indicates that Raman could potentially surpass the limitations of the current biochemical identification tests, and even provide more insight on the evidence than these tests.

While these studies are promising, their methodologies are not suitable for *in-situ* analysis. The majority of these studies used lab grade samples, which are free of environmental contaminants, and were performed with large, stationary spectrometers that must be used in the laboratory. As such, they cannot identify evidence out in the field, where the greatest demand for

this technology is. In response, companies have been developing portable and handheld Raman instruments to be used for this purpose. Researchers have found that these compact spectrometers are capable of testing drugs, chemical warfare agents, explosive residues, and some body fluids [17-23].

These studies have demonstrated how advantageous portable and handheld Raman spectrometers can be to crime scene investigation *individually*. They did not, however, determine their effectiveness in comparison to laboratory spectrometers. Portable instruments are known to have lower resolution in their measurements than desktop instruments, which can impact the quality and some features of their spectra. This can further complicate the task of identifying evidence, which is often done using statistical models based on standard spectra. If a spectrum obtained from a portable instrument does not match well to the standard spectra in an identification model, it will be misidentified. This kind of problem can seriously hinder the capability of portable instruments in analyzing crime scene evidence. Weather et. al. have created a methodology to remedy this, by transforming spectra of a chemical from a laboratory Raman spectrometer to resemble a spectrum of the same chemical as collected by a portable spectrometer. This method produced highly accurate identifications by adding these transformed spectra into the identification model, but it was noted that this technique would only work for materials that produce consistent spectra [24]. Because of this, the method described in that study would not be applicable to body fluids. The composition of body fluids varies greatly between donors and even withing different samples from the same donor, which results in highly varied spectra.

Another area of concern when analyzing body fluid samples with portable Raman spectrometers is the low Raman signal of body fluids. Some components in body fluids that are

Raman-active tend to be found in low concentrations, and as such will provide very small Raman signals. This issue can be compounded by the fact that portable instruments have been found to have more difficulty in detecting weak signals and chemicals in low concentrations, as compared to desktop spectrometers [25].

The results of these studies show how important it will be that forensics and other analytical scientists understand how analysis via portable and desktop instruments can vary. In forensics, a piece of evidence may have to be analyzed in the lab after preliminary testing at the crime scene. In terms of spectroscopy, the spectra of this evidence would need to be congruent with those collected at the scene in order to be considered valid. If these spectra differ, due to the differences in resolution and other characteristics between laboratory and portable instruments, the scientists might consider the previous testing invalid and may have to perform further tests to confirm the results of previous analysis. This would again strain the time and resources on the labs, which will not resolve the current problem they have with evidence backlogs and may even contribute to it.

As such, this project seeks to determine the effects of varying resolution of portable and laboratory spectrometers on analyzing biological specimens. The results of this study will help analysts who use Raman to recognize and account for how spectra changes between these types of instruments, and possibly to find methods or tools that can minimize these differences.

Experimental Procedure

Sampling

To quantitatively determine the resolution of the portable and desktop spectrometers used in this study, a known standard for Raman, an argon lamp, was tested with both instruments. Two other standards were also tested (polystyrene petri dish and calcium carbonate) but were used for qualitative comparisons. Additionally, a simple salt (perchlorate) was also used to preliminarily assess the differences in resolution between the two spectrometers. The standards were selected from the Standard Guide for Testing the Resolution of a Raman Spectrometer [26]. For the portable instrument, the solid powder standards (perchlorate and calcium carbonate) were tested on an aluminum slide designed with two 3 mm wells. One well contained a "cheat" sample of dried 1 Molar sugar solution that was used to focus the portable instrument's laser. In the other well was deposited the powder sample. A piece of the petri dish was broken off and placed on top of the aluminum slide for testing.

The next task was determining the effect of resolution on protein analysis. Body fluids are complex mixtures of proteins and several other biological compounds, so to start, a simpler protein sample was tested. The protein used was hen egg white lysosome (denoted here as HEWL), which was stored in powder form. HEWL was tested in a variety of conditions to determine the best method of sample preparation for the analysis of proteins (and further, body fluids). One condition, noted as "unpacked," simply consisted of depositing the HEWL into the well on the aluminum slide until it was full. In the next condition, which was the "packed" condition, the HEWL was deposited into the well and packed down tightly. The well was then refilled, and the powder packed down, until the well could not fit any more powder. The last was the "cast film" condition, where a 1 mg/mL HEWL solution in water was prepared and deposited

onto a glass microscope slide. This was dried overnight, producing a cast film that was then removed from the slide and transferred to the well in the aluminum slide for testing. This was meant to simulate body fluid samples, which dry on one surface and then are transferred to another (like the aluminum slide) for analysis.

The last task was to then test body fluid samples to determine how the effects of resolution on simple protein analysis would translate into complex protein analysis. Three body fluids were tested: blood, semen, and sweat. 20 μL of each substance were deposited onto a glass slide and allowed to dry overnight. The dry samples were then transferred to a well in the aluminum slide. This process was, again, meant to simulate the analysis of real body fluid evidence that would be found in a crime scene.

Instrumentation and Spectral Collection

The portable spectrometers used in this project was the BioTools, Inc. μ-BioRAMAN, with a piezoelectric stage and a 50x objective. It came with a software to control the instrument and collect spectra, and a camera view of the stage. The desktop spectrometer used was the Horiba Xplora Raman microscope, equipped with an externally controlled stage and a software to be used for spectral collection. The instrument's 100x objective was used. For both instruments, the 758 nm laser wavelength was employed.

The standards and the HEWL samples were tested at 100% laser power, which was 100 mW for the μ-BioRAMAN and 110 mW for the Xplora. Each spectrum was collected with one 10-second accumulations. For the standards, only one spectrum was collected after focusing the laser directly on the sample. For the HEWL samples, two spectra were collected for each sample preparation condition. One spectrum was collected after focusing the laser on the "cheat" sugar solution, and another was collected with a laser focus on the HEWL itself.

The body fluid samples were only tested on the portable instrument, since there were pre-existing spectra of these substances collected on the Xplora for another project. Each type of body fluid was analyzed under specific parameters provided by the instrumentation company. Blood samples were tested using 20 mW laser power with 10-10 s accumulations. semen samples were testing using 80 mW for 5-30 s accumulations. Sweat was tested using 60 mW laser power and 10-10 s accumulations.

Resolution Calculations

To calculate resolution, the GRAMS Thermo software (Thermo Fisher, Inc.) was utilized. The spectra of the argon lamp from each instrument was used for these calculations. The peaks were manually fit to a Gaussian curve function, which provided the peak center, height, and width at half-maximum height. The widths of all of the peaks in each spectrum were averaged, and these averages were recorded as the resolution for each spectrometer.

Data Processing

The peaks in the spectra of the Raman standards and the HEWL were naturally well defined, and as such did not require any further processing. The body fluid samples produced a lot of fluorescence, which overshadowed most of the peaks, so these spectra had to be preprocessed. All body fluid spectra underwent baseline correction (Baseline Automatic Least Squares, either 4^{th} or 5^{th} polynomial), normalization, and smoothing (Savitzy-Golay). Preprocessing was performed in the MATLAB software (v. 2010b, Mathworks, Inc.) with PLS Toolbox (v. 7.03, Eigenvector Research).

Results & Discussion

Resolution Determination

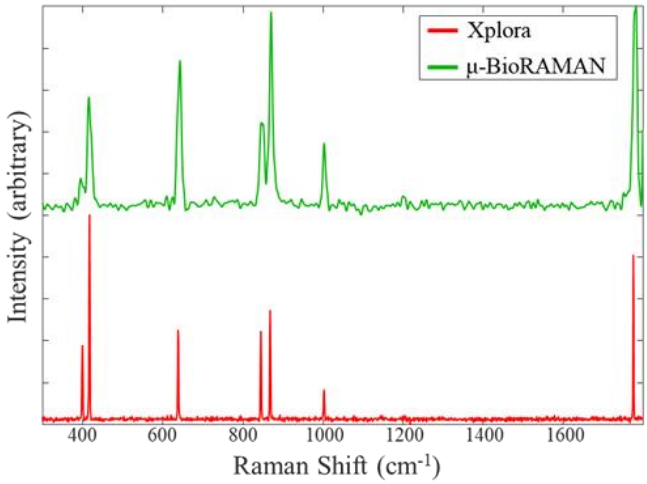
The perchlorate and the Raman standards polystyrene and calcium carbonate provided a good visual confirmation of the differences in resolution between the two spectrometers. Instruments with lesser resolution tend to yield wider peaks in their spectra, and this was observed in the spectra of these standards when collected from the instrument. The same trend occurred with the spectra of perchlorate, which lost peaks at 850 cm^{-1} and 420 cm^{-1} when tested with the μ-BioRAMAN as opposed to the Xplora. This is another trend that is expected for less resolute instruments, so it was treated as further confirmation of varying resolutions between the two spectrometers.

The spectra of these standards were not, however, used to calculate resolution. For peaks that were quite sharp in the Xplora spectra, there was a marked difference in width and intensity when compared to the μ-BioRAMAN spectra. However, for peaks that were already broad, as was the case for most peaks in these spectra, there was no noticeable difference in these characteristics between μ-BioRAMAN and Xplora spectra. Broader peaks do not often change predictably between different instruments, so a difference in resolution would be hard to quantify if using peaks like these, as resolution is determined using peak widths. As such, these standards were no longer considered for resolution determination.

An argon lamp was subsequently used to test the resolution on both instruments. Lamps are known to give very sharp, intense peaks, referred to as “lines.” With the previous standards, it was found that the sharp peaks would differ significantly between the two instruments, so all the peaks in the argon lamp spectra would ideally change consistently, making this material more suitable for calculating resolution. As expected, the spectrum of the argon lamp as collected by

the Xplora differed visibly in peak width from the spectrum collected by the μ-BioRAMAN (Figure 1). The peaks in the μ-BioRAMAN spectra were consistently wider, which caused the peaks at 370 cm^{-1} & 418 cm^{-1} and at 849 cm^{-1} and 871 cm^{-1} to overlap. This was again visual confirmation of the lower resolution of the μ-BioRAMAN spectrometer.

Figure 1. *Stacked comparison of argon lamp obtained from the Xplora (red) and the μ-BioRAMAN (green) spectrometers.*



As discusses above, the GRAMS software was used to calculate the peak widths of each peak in the argon lamp spectra. This program determined the width at half-maximum for each peak, which is demonstrated for the peaks at about 400 cm^{-1} and 418 cm^{-1} in Figure 2. These widths, as listed in Table 1, were averaged for each instrument their respective resolutions. For the Xplora spectrum, the peak widths ranged from 2.0-3.0 cm^{-1}, with an average of 2.7 cm^{-1}. The RSD for these widths was 123 ppt. For the μ-BioRAMAN spectrum, the peak widths ranged from 9-14 cm^{-1}, with an average of 11.4 cm^{-1}. The RSD for these widths was 142 ppt. these values indicated that the Xplora desktop spectrometer had a nearly 5 times greater resolution than the portable μ-BioRAMAN spectrometer. Additionally, it was observed that the precision of the peak widths for the Xplora was also greater, as indicated by the RSD values. They were

relatively high for both instruments, but the RSD value for the portable instrument was much higher. This suggested that instruments with lower resolution can produce more variable peaks widths and size, which could in turn produce more variable spectra.

Figure 2. *Gaussian curve fittings for the ~400 cm^{-1} and ~418 cm^{-1} peaks on the argon lamp spectrum from the Xplora (top) and the μ-BioRAMAN (bottom). Red trace indicates the original spectrum, and green and blue traces indicate the fitted curve.*

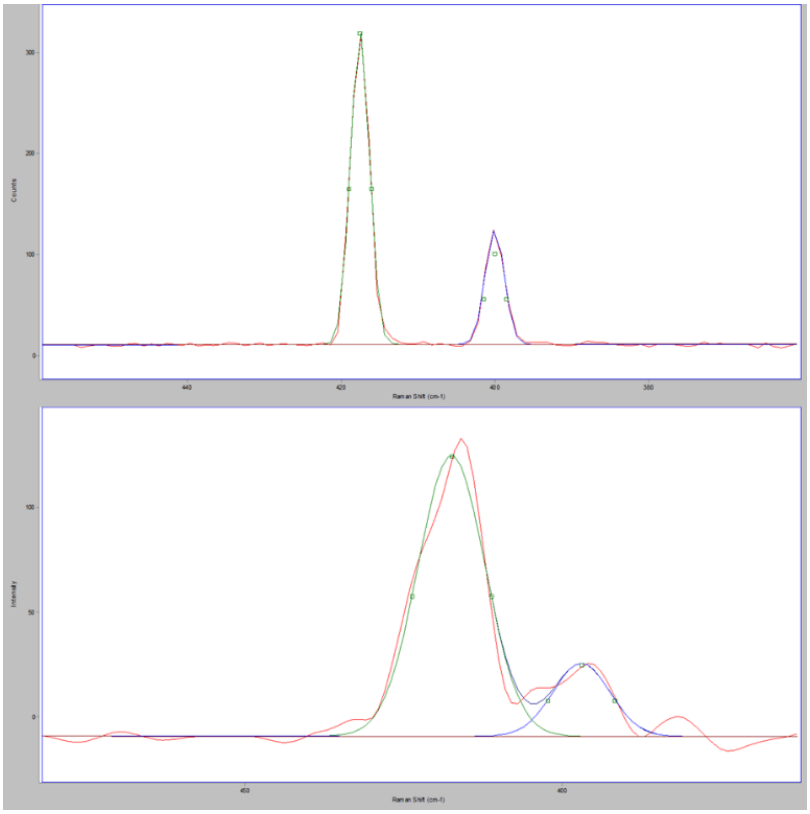


Table 1. *Data from Gaussian curve fitting of all peaks.*

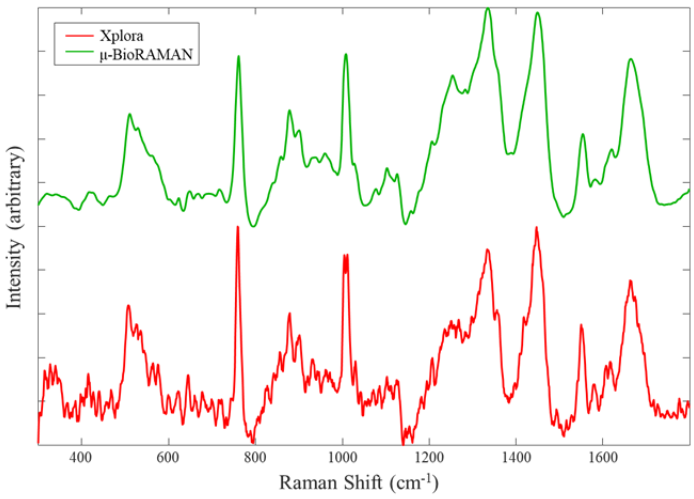
Instrument		**Peak Data**						
μ-BioRAMAN	**Center (cm^{-1})**	370	417	642	849	871	1004	1781
	Width (cm^{-1})	11	12	12	14	10	9	12
Xplora	**Center (cm^{-1})**	400	418	639	846	869	1004	1777
	Width (cm^{-1})	2.9	2.9	2.7	2.8	2.7	3.0	2.0

Single Protein Analysis

A powder protein (hen egg white lysosome, HEWL) was analyzed with both the desktop and portable spectrometers to determine the impact of resolution on protein spectra. HEWL was meant to be a simpler example of biological specimens that could be important in forensic investigations, which can contain numerous, complicated proteins. As discussed before, two focusing conditions were used. In the first condition, the laser was focused on a "cheat" dried sugar solution before analyzing the protein, whereas in the second condition, the laser was focused directly on the sample. These two methods yielded very similar spectra, so only the results from the sample-focused condition will be discussed here.

At a visual level, the spectra of powder HEWL collected on the μ-BioRAMAN and the Xplora spectrometers were very similar (Figure 3). The peak widths and intensities did not change significantly between the two spectra. The Xplora spectrum did contain more noise, most likely due to its greater resolution, but this did not majorly reduce the quality of the spectrum. This resulted demonstrated that the portable μ-BioRAMAN instrument has the capability of producing similar spectra to that of the desktop Xplora, in the case of simple proteins.

Figure 3. *Stacked plot of HEWL spectra from the Xplora (red) and the μ-BioRAMAN (green).*

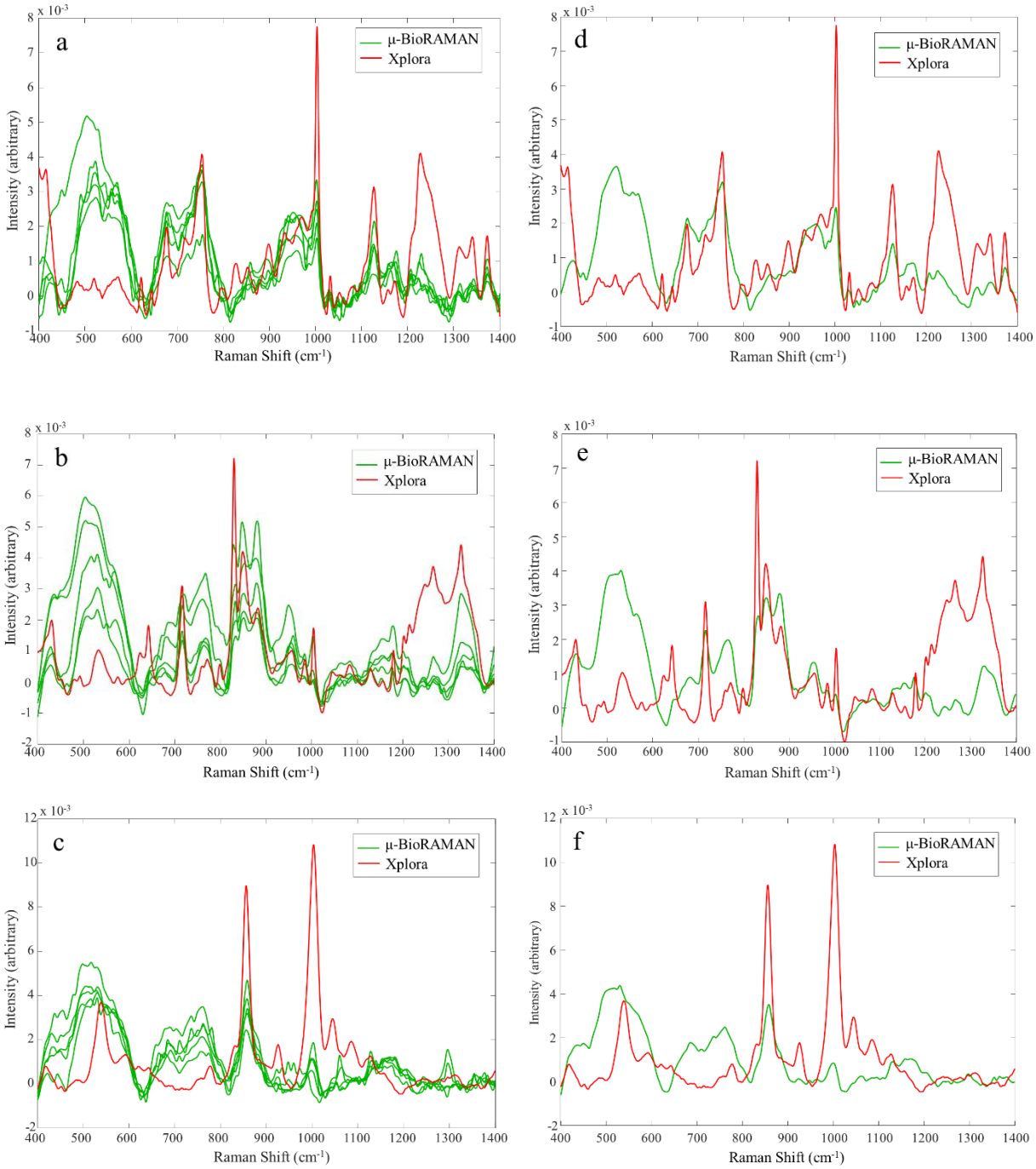


Body Fluid Analysis

When analyzing body fluids with the portable instrument, the same trends observed with the testing of standards were observed again in the spectra of blood, semen, and sweat. Figures 4a-f offer a visual comparison of the μ-BioRAMAN spectra of these body fluids and the respective averaged spectra from the Xplora, previously collected for another project. In both spectra, the peaks were broad and there were a few peaks that overlapped heavily. This is not uncommon for body fluid spectra, seeing as they contain several complex components that can give off signals at nearby wavenumbers. There were also cases of lost peaks, as occurred with the perchlorate standard, such as the peaks found in the Xplora blood spectra at about 650 cm^{-1} and 825 cm^{-1}. There were sizeable differences in the intensities of peaks between the two instruments, where some larger peaks in the Xplora spectrum tended to decrease in size in the μ-BioRAMAN spectra. Conversely, some of the smaller peaks in the lower wavenumber region would increase in intensity and width in the μ-BioRAMAN. The overall features in the spectra for both instruments were still quite similar. This again demonstrates that a portable spectrometer is capable of producing quality spectra of body fluids that can be comparable to those collected from a desktop instrument.

It is important to note that the μ-BioRAMAN spectrometer yielded very variable spectra, where the shape and intensity of the peaks changed frequently between individual spectra. In the Xplora spectra of body fluids, this variability was only minor. This greatly resembles the trend observed when testing the argon lamp, where the peak widths varied more with the portable spectrometer than they had with the desktop. This greater variability results in the averaged spectra from the μ-BioRAMAN looking more dissimilar to the averaged spectra from the Xplora, seeing as it accounts for more irregularities, as demonstrated in the figures below.

Figures 4a-f. Overlapped plot of individual spectra of (a) blood, (b) semen, and (c) sweat collected from the μ-BioRAMAN and the averaged spectra of (d) blood, (e) semen, and (f) sweat collected from the μ-BioRAMAN, compared to averaged spectra collected from the Xplora.



Conclusion

Research has demonstrated that portable spectroscopy technology will be greatly advantageous to the field of forensics. As portable Raman enters the field of criminal investigation, it will be important that technicians understand the way that these instruments will be different than their laboratory counterparts, and how these differences will impact evidence analysis. In this project, a standard method was used to quantify the resolution of portable and benchtop instruments and assess the effects of this important characteristic on analyzing biological compounds. Lower resolution instruments will produce broader peaks relative to the sharp ones observed from a more resolute instrument. Simple biological compounds do not produce sharp peaks, so their spectral features may not change greatly between portable and desktop instruments. There was a noticeable difference in the spectra of body fluids collected by these two spectrometers, likely due to their complex composition. However, the body fluid spectra from both spectrometers were still comparable and could possibly be matched to one another easily through visual comparison. This indicates that the spectra from the μ-BioRAMAN can be effective at analyzing body fluid evidence at a crime scene. These results seem to resolve the issues seen with current methods for analyzing and identifying body fluids *in situ*, which is vital for the field of forensics.

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