The Interpretation of Body Fluid Mixtures Using Raman Spectroscopy in Forensic Investigations

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Abstract

This study aimed to demonstrate the ability of Raman spectroscopy to identify body fluids, both individually and in mixtures. After obtaining informed consent and IRB approval, venous blood, saliva, semen, and urine were collected from volunteers, and analyzed using a Raman microscope kept at a constant wavelength of 780 nanometers. The analysis was performed on individual fluids, mixtures, and in the presence of substrates. DNA profiling was then performed on a selection of samples after Raman analysis. The individual fluids were shown to provide their own unique spectra. Four of the mixtures (blood and semen, saliva and semen, saliva and urine, semen and urine) gave positive results for the detection of both fluids present. Blood was the only fluid detected on substrates, and only on one of the two tested.

1 Introduction

In the course of an investigation, biological stains, which may contain multiple body fluids, can be recovered as evidence. While there has been widespread research into the resolution of DNA mixtures, little has been done to determine an effective method for differentiating between body fluids in a mixed sample. However, Raman Spectroscopy is emerging as an effective, non-destructive method that can be utilized to identify any body fluids present in or on a piece of evidence.

1.1 Current Methods for Body Fluid Detection

The methods currently being used for the identification of body fluids generally use a presumptive test that can be performed in the field, followed by a confirmatory test that is performed in a laboratory environment. There are a wide array of presumptive tests for the presence of blood, and many of these are based on the peroxidase-like activity of hemoglobin [1]. These presumptive tests include the use reagents such as leucomalachite green, and Luminol testing [2]. Confirmatory tests are beginning to move towards immune-chromatographic tests, such as the ABAcard® HemaTrace strip test [3].

For semen, most presumptive tests rely on the presence of an enzyme commonly found in semen known as seminal acid phosphatase (SAP). Once a stain has given a presumptive positive for the presence of semen, it may be checked for the presence of spermatozoa. If there is no sperm present, testing may be performed to check for the presence of prostate-specific antigen (PSA or p30) [1]. However, it has been shown that PSA can also be found in other body fluids, limiting the usefulness of the test [3].

Saliva testing can be performed using enzyme-linked immunosorbent assay (ELISA) tests or the Rapid Stain IDentification (RSID-Saliva) test can target alpha amylase or statherin, enzymes commonly found in saliva [3]. Urine testing is infrequently performed, and tests for the presence of urea or creatinine. These tests are not specific, and therefore are unreliable for urine detection [1].

1.2 Raman Spectroscopy

Unfortunately, these presumptive tests can be prone to false positives, and the confirmatory tests require more time, and consume the sample in question, to be performed. Furthermore, none of these tests are capable of differentiating between multiple body fluids present in a single sample.

Raman spectroscopy is being suggested as a viable alternative to the current methods. The effect known as Raman scattering is utilized in this form of biospectroscopy. A low-intensity laser is fired at a sample. The photons change the vibrational energy of molecules present in a sample, which enter a virtual energy state. Upon leaving this energy state, the photon will be re-emitted from the molecule at a different wavelength than the incident photon [4]. This technique requires virtually no sample preparation, and requires only minimal amounts of a sample for detection to occur [5]. This would allow for analysis of evidence *in situ* and would enable the analysis of trace evidence. However, one of the most important advantages to this technique is that it is non-destructive, leaving the sample and allowing for further analysis such as DNA profiling to be performed post-Raman analysis[5].

2.1 Materials and Methods: Sample Collection

After obtaining approval from an Institutional Review Board (IRB), body fluid samples were collected from volunteers following informed consent. Seven venous blood samples were collected by a registered phlebotomist. A single test tube of blood was collected from each volunteer, and none of the samples were treated with additives of any form. The other three body fluids were collected by the volunteers using sample collection kits and then returned after collection. Saliva was collected by asking participants to spit into 15 mL tubes until they were half full. For semen, participants were asked to ejaculate into a 50 mL tube once and then seal and return the tube. Urine was collected by asking volunteers to urinate into the tube and then seal and

return the tube. After samples were returned, they were stored at 4°C for the duration of the study.

2.2 Materials and Methods: Sample Preparation

All samples were prepared on aluminum-wrapped glass microscope slides as shown in **Figure 1**. The slides were wrapped in aluminum foil to prevent fluorescence from the glass causing interference during analysis. For the individual fluids, 10 µL were taken from the body fluid sample and pipetted directly onto the slide or onto the fabric substrate being tested. The two substrates used in this study were black and white cotton from two different Hanes brand t-shirts. For the mixtures, 20 µL were prepared in varying ratios and then placed onto the slide or substrate. Six different mixtures were prepared: blood and semen, blood and saliva, blood and urine, semen and saliva, semen and urine, and saliva and urine. Two mixed samples of each mixture type were prepared in equal ratios, one sample with the fluids in a 1:4 ratio, and one with the same ratio, but with the major contributor to the mixture switched. All of the samples were prepared under sterile conditions, and were allowed to dry overnight before Raman analysis.
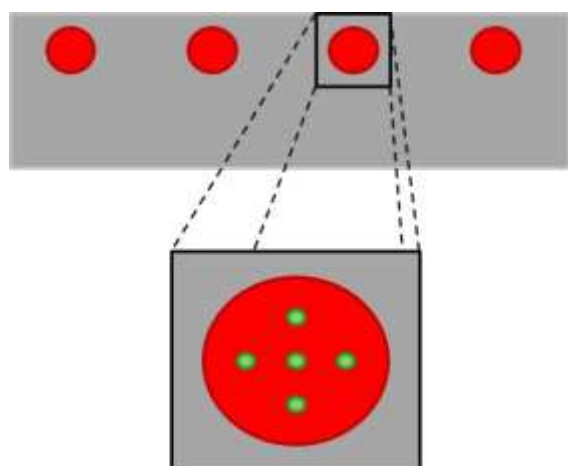

Figure 1: An example of a slide used in this project. The red circles represent the fluid samples. The magnified area shows an example of the typical pattern followed for the sampling areas during Raman analysis.

2.3 Materials and Methods: Raman Spectroscopy

All of the samples prepared were analyzed using a Thermo Scientific DXR Raman Microscope. The microscope was equipped with a 10X objective and the Thermo Scientific OMNIC™ Software. The laser was kept at a constant power of 10 mW and a constant wavelength of 780 nm throughout the duration of the project. The aperture used was a 50 µm slit, and the grating was 400 lines per mm. A polystyrene standard was run every day prior to any samples being run to check the alignment and calibration of the machine. The sample exposures were kept constant at five 20-second exposures per run. Five runs were performed on each sample, taking each set of accumulations from a different area of the sample, as shown in **Figure 1**. After analysis, all the runs from a single sample were averaged using OMNIC to create a mean for that sample. A mean was then calculated using all the samples of an individual fluid type to create an average for that fluid type.

2.4 Materials and Methods: DNA Profiling

After the completion of the Raman analysis, a selection of samples was chosen to be DNA profiled to demonstrate the non-destructive nature of Raman Spectroscopy. The DNA was extracted using a general extraction protocol, and standard STR typing was performed using Ampflister Identifiler Plus.

3.1 Results: Individual Fluids

Shown in **Figure 2** are the body fluid averages for all of the individual fluids on the aluminum slide. It should be noted that blood and semen show consistency with spectra from past research. Saliva consistently gave weak spectra, and there are almost no definite peaks in its spectra. Urine was observed to consistently have a large peak around 1000 cm^{-1}.
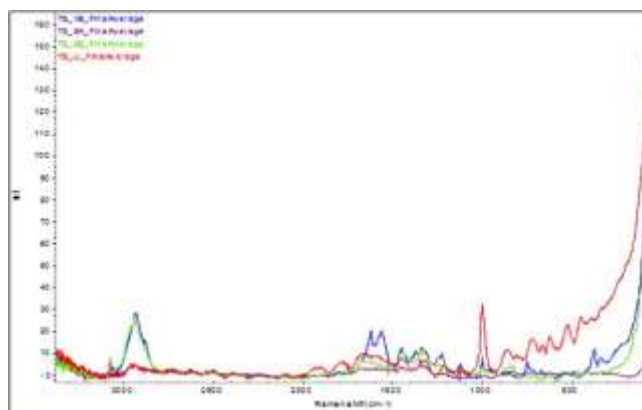

Figure 2: The body fluid averages for the four individual body fluids (Blood: Blue, Saliva: Purple, Semen: Green, Urine: Red).

3.2 Results: Mixed Samples

For the mixed fluids on the aluminum slides, four of the six mixtures were able to be successfully identified and differentiated. Those four mixtures were blood and semen, semen and saliva, semen and urine, and saliva and urine. Seen in **Figure 3** is an example of how the mixtures were interpreted. The mixture's average spectrum was first compared to the body fluid of the two fluids present in the mixed sample. A mean was then calculated using the two individual fluids, which was then compared to the average of the mixed sample. If the mixture spectrum matched the calculated mean more consistently than it did either of the individual fluid spectra it was determined to be a positive differentiation.
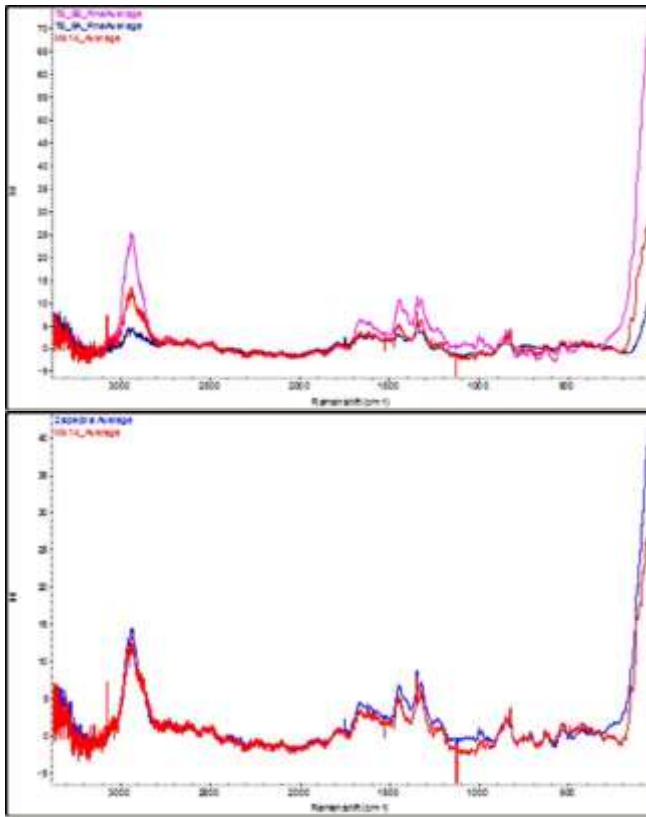
Figure 3: (Top) The average for a semen-saliva mixture (red) compared to the fluid averages for semen (pink) and saliva (blue). (Bottom) The same average (red) compared to a mean calculated using the two individual fluids from the first graph (blue).
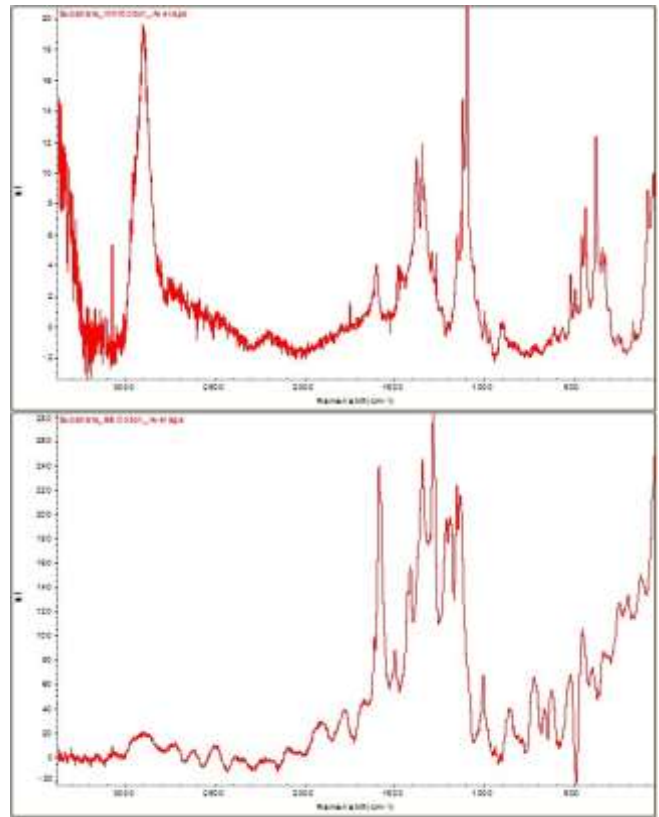

Figure 4: (Top) Average spectrum for neat white cotton substrate. (Bottom) Average spectrum for neat black cotton substrate.

3.3 Results: Substrate Analysis

Shown in **Figure 4** are the average spectra for the two substrates tested in this study. For the individual fluids tested on substrate, only blood was detected, and only it was only detected on the white cotton substrate. The other fluids showed too much similarity on the substrate to be identified. The substrates created too much interference for any of the other fluids to be detected, especially the black cotton, as seen in **Figure 5**. The black cotton also had a tendency to exhibit fluorescence. Furthermore, none of the mixtures were detected or differentiated on either substrate, as seen in **Figure 6**.

3.4 Results: DNA Profiling

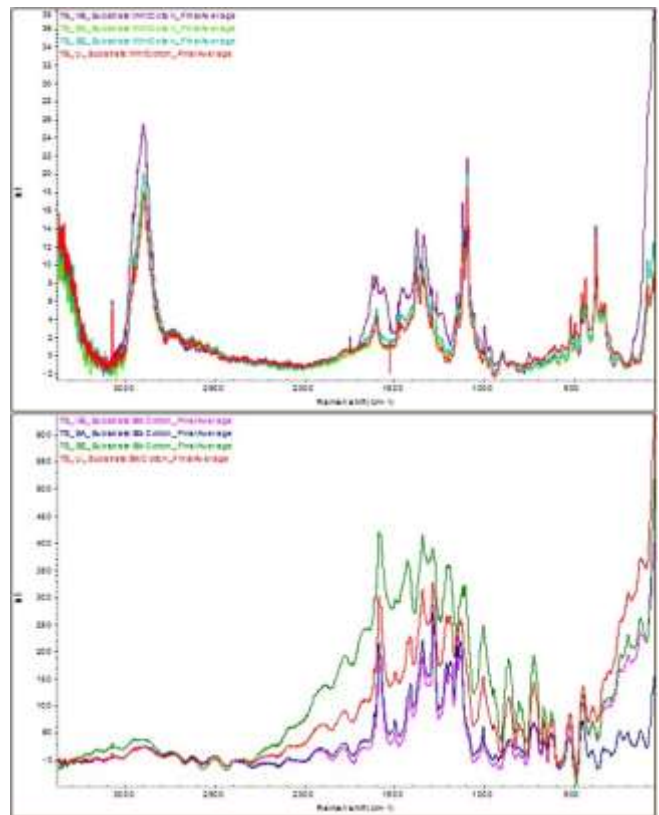Full DNA profiles were obtained from the selection of samples taken after the completion of Raman analysis.

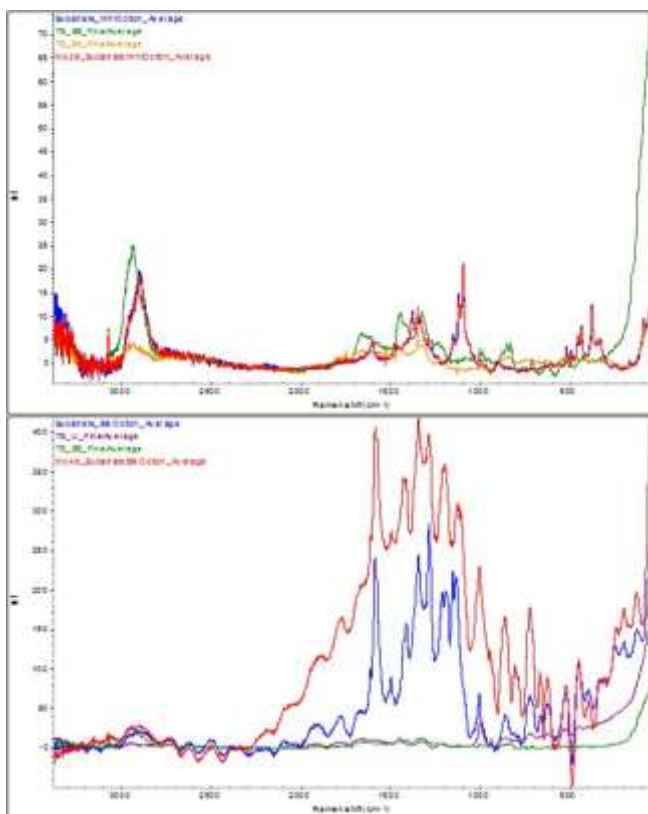**Figure 5** Individual Fluids on white cotton (Top) and black cotton (Bottom).

Figure 6: (Top) A mixture of semen and saliva on white cotton (red) compared to the average for the neat substrate (blue), and the fluid averages for semen (green) and saliva (yellow). (Bottom) A mixture of semen and urine on black cotton (red) compared to the neat substrate average (blue), and the fluid averages for urine (purple) and semen (green).

4 Discussion

While all of the individual fluids were able to be identified by their unique spectra, not all of the mixtures were able to be differentiated. The two that were not (blood and saliva, blood and urine) were most likely not differentiated due to the blood giving too strong of a signal. Saliva and urine gave weaker spectra compared to blood and semen, and as such they may have been overpowered in mixtures containing blood.

One of the largest problems encountered in this project was the large amount of interference the substrates introduced into the spectra. The interference was so strong that spectral subtraction was unable to eliminate the signal from the substrate while preserving the signal from the body fluids. Both substrates almost completely eliminated any signal from the body fluids. There are several possible reasons for this. One reason may have been that simply not enough of the fluid was present to overcome the signal of the substrates, which would account for why blood was the only one to be detected on white cotton, as it usually gave the strongest signal of the four fluids, and the white cotton gave less intense spectra than the black cotton. Another possible reason for the substrate making detection difficult is that the substrate used may have had certain dyes that could have influenced the signal. The fabric was unwashed before being analyzed, and if optical brighteners were present due to the substrates being from commercial products, they could have influenced the signal. Yet another

possible reason for the interference may be nature of the substrate itself. The fabric was cotton, which is heterogeneous in nature. This would make obtaining an average spectrum difficult, as different areas of the substrate would result in different spectra.

Whatever the reason may be for the interference, further research will be required in order to determine the best method for eliminating interference from substrates. One of the current methods used is correcting for fluorescence and then performing spectral subtraction. Another method currently being developed involves changing the source of the laser to a near-infrared source. However, neither of these methods are able to overcome the obstacles that heterogeneous substrates present to Raman Spectroscopy, requiring future research in this area.

5 Conclusion

This proof of concept study has demonstrated that Raman Spectroscopy can be used to identify individual body fluids based on their unique spectra, and established the potential for the technique to differentiate between multiple body fluids present in a single mixed sample.

6 References

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7 Acknowledgments

8. Biography

Tyler Schlagetter of Sidney, Ohio is a Junior Forensic Science and Biology double major with a concentration in Biochemistry. Currently enrolled at the University of New Haven, he is a teaching assistant for the Cell Biology laboratory. Upon graduating, he plans to either go into work at a crime lab, or continue research to further improve the field of forensic science.