Surface-enhanced Raman spectroscopy for forensic analysis of human semen

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Boston University
SURFACE-ENHANCED RAMAN SPECTROSCOPY FOR FORENSIC ANALYSIS OF HUMAN SEMEN

by

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ABSTRACT

Identification of an unknown stain encountered at a crime scene, especially where the context of the case does not provide an indication to the identity of the stain, currently requires a number of time consuming and costly presumptive and confirmatory tests to be performed. Surface-enhanced Raman spectroscopy (SERS) is a vibrational spectroscopic method that could allow crime scene analysts to identify unknown stains rapidly both in the laboratory and in the field. The SERS technique utilizes a laser, which interacts with molecules applied to a gold nanoparticle chip (SERS substrate) that enhances the normal Raman signal, producing a shift in energy characteristic of the vibrational modes present. Therefore, the light scattering spectrum obtained provides the analyst with a unique spectral fingerprint of the molecular components of the sample. The advantages of this SERS based method include its high sensitivity, speed, non-destructive nature, ease-of-use, minimal sample preparation requirement, portability, and multiplexing capabilities.

In contrast to conventional Raman spectroscopy, SERS offers higher sensitivity resulting in small sample volumes (approximately 1 µL or less) being required for sample identification and the ability to process dilute solutions. This allows for the remaining sample to be used for other forensic tests, making the technique an ideal analytical method for use at a crime scene.
It is hypothesized that SERS can be coupled with multivariate statistical methods, such as principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) to be established as a confirmatory technique in the forensic analysis of human body fluids. It was concluded that semen produces a spectral pattern that is consistent and readily distinct from blood, saliva, urine and vaginal fluid. In addition, this investigation identified and characterized semen from four donors, utilizing liquid semen as well as semen stains on cotton swatches and glass cover slips.

Reproducibility was established by analyzing three separate SERS chips for every sample and/or solution. Ten spectra of each chip were obtained, averaged, and then compared to one another. A protocol was designed for the extraction of dried semen stains on cotton swatches and application to a SERS chip. Different extraction conditions were performed, varying both the volume of water used and the time the cutting remained submerged in the water, resulting in optimal signal from 5 µL of water for 5 minutes. Additional parameters including analysis of the perimeter of the stain and the use of saline as an extractant were examined. A second protocol for the extraction of dried semen stains from a glass cover slip was designed and tested, utilizing 1 µL of water. All experimental spectra were subjected to PCA for comparison with neat semen, and determined to be consistent. Additionally, a mixture of semen and vaginal fluid was evaluated. Visual inspection and PCA of the resulting spectra demonstrated that the mixture was a combination of both body fluids. Such samples are of particular importance in sexual assault cases.
In summary, this preliminary study of the identification of semen using SERS demonstrates the potential for the method to be used as an investigative tool for the detection of trace amounts of human body fluids at crime scenes and within forensic laboratories. Not only is semen differentiable from other body fluids, but it is also capable of being extracted from stains and successfully identified by SERS.
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<td>mg</td>
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<tr>
<td>mm</td>
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<td>mW</td>
<td>Milliwatt</td>
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<td>Nd-YAG</td>
<td>Neodymium Doped – Yttrium Aluminum Garnet</td>
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<td>nm</td>
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<td>PCA</td>
<td>Principal Component Analysis</td>
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<td>PCI</td>
<td>Post-coital interval</td>
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<td>PLS</td>
<td>Partial Least Squares</td>
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<td>PSA</td>
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<td>RPM</td>
<td>Revolutions Per Minute</td>
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<td>RSID</td>
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<td>SAP</td>
<td>Seminal Acid Phosphatase</td>
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<td>SEM</td>
<td>Scanning Electron Microscope</td>
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<td>SERS</td>
<td>Surface-Enhanced Raman Spectroscopy</td>
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<tr>
<td>SiO₂</td>
<td>Silicon Dioxide</td>
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<td>Sg</td>
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<td>SVSA</td>
<td>Seminal Vesicle-Specific Antigen</td>
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<tr>
<td>TNT</td>
<td>Trinitrotoluene</td>
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<tr>
<td>µL</td>
<td>Micro liter</td>
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µm  Micro meter  
µM  Micro molar  
UV  Ultraviolet  
VAP  Vaginal Acid Phosphatase
1. INTRODUCTION

Identification of an unknown stain encountered at a crime scene, particularly where the context of the case does not provide an indication to the identity of the sample, currently requires a number of time-consuming and costly presumptive and confirmatory tests to be performed. The establishment of a universal technique to identify stains that is simple, rapid and sensitive would be beneficial to the forensic community. Preliminary studies and methodology development for semen identification via surface-enhanced Raman spectroscopy (SERS) demonstrate a potential new tool for the analysis of stains.

1.1 Biochemistry of Semen

Semen, a product of the male reproductive organs, is a white, viscous liquid composed of two distinct constituents: seminal fluid and spermatozoa (sperm). The identification of semen plays an important role in the processing of forensic evidence and determining the events of a crime, particularly for sexual assault cases; therefore, it is necessary to gain an understanding of its chemical characteristics.

1.1.1 Production of Semen

Spermatogenesis is the process by which sperm are produced within the male reproductive organs. Over the course of approximately 10 weeks, sperm develop within the testes until they become fully mobile and fertile. Once mature, the sperm relocate to the epididymis via the vas deferens where they are stored until ejaculation.\(^1,2\) At the point of ejaculation, fluids secreted by the seminal vesicle, prostate gland and bulbourethral
gland combine with sperm and are expelled (approximately 2-5 mL) from the male reproductive system.\textsuperscript{2,3}

1.1.2 Seminal Components
1.1.2.1 Seminal Fluid

Seminal fluid, the non-cellular constituent of semen, consists of a combination of proteins, sugars, acids, and various elements.\textsuperscript{2,4-8} Three of these components are currently utilized in the forensic identification of semen: acid phosphatase (AP), prostate specific antigen (PSA), also known as antigen p30, and semenogelin (Sg).

AP is an enzyme responsible for the hydrolysis of ester linkages in monophosphate molecules. There are two forms of AP: seminal acid phosphatase (SAP) and vaginal acid phosphatase (VAP). SAP is produced in epithelial cells within the prostate and excreted into the seminal fluid during ejaculation, whereas VAP is present in female vaginal fluid.\textsuperscript{2,9,10} PSA, a glycoprotein produced by epithelial cells within the prostate gland, is responsible for the hydrolysis of Sg (approximately 0.5-2.0 mg/mL) within the seminal fluid.\textsuperscript{2,11,12} Seminal Vesicle-Specific Antigens (SVSAs) are major proteins secreted by the seminal vesicle and epididymis, two of which include SgI and SgII, which are responsible for the coagulation of semen post ejaculation. In addition, PSA and Sg exhibit antigenic properties that contribute to their ability to be detected using chromatographic immunoassays.\textsuperscript{2,9,10}

Additional components not currently exploited for identification purposes include fructose, glucose, lactic acid, citrate, albumin and lysozyme. Fructose and glucose are simple sugars functioning as the primary energy source for sperm where the breakdown
of fructose results in lactic acid; citrate contributes to the overall negative charge of semen.\textsuperscript{2,4–8} The most abundant protein found in seminal fluid is albumin, which is produced in the prostate.\textsuperscript{13} Lysozyme is a single polypeptide chain enzyme that lyses the cell wall of Gram positive bacterial cells and thus provides protection against infection.\textsuperscript{14} Zinc, copper, calcium, sulfur, chlorine, potassium, titanium, iron and nickel are elements found within seminal fluid that are suspected of aiding in the process of spermatogenesis.\textsuperscript{6–8} Urea and ascorbic acid are compounds also found in seminal fluid.\textsuperscript{4,5}

1.1.2.2 Spermatozoa

In addition to the seminal fluid constituents of semen, sperm are the dominant cellular component of semen and are responsible for providing half of the genetic material during reproduction. Intact sperm cells contain a head, mid-piece, and tail (Figure 1). The head is elliptical in shape and measures approximately 4-5 micrometers (\(\mu m\)) in length by 2-3 \(\mu m\) in width. Within the head there is an organelle surrounding the nucleus of the sperm cell that contains enzymes to aid in the fertilization of an egg, referred to as the acrosome. The mid-piece is similar in length to the head of the sperm and contains mitochondria. The tail is approximately 40 \(\mu m\) in length, however, it is often not present during microscopic examination due to its fragility.\textsuperscript{2,3,10}
Morphological abnormalities of sperm are quite common, presenting in approximately 18-60% of total sperm and ranging from irregular head shape to the absence of flagella.\textsuperscript{15} In addition to variations in morphology, sperm concentration differences exist.\textsuperscript{2} A normal healthy male typically has between 10 and 100 million sperm per ejaculate; however, the ejaculate of an individual with oligospermia, a condition referring to low sperm count resulting from genetic disorders\textsuperscript{16}, trauma\textsuperscript{17} or radiation exposure\textsuperscript{18}, contains less than 20 million sperm per milliliter. Azoospermia, also referred to as aspermia, is a condition in which sperm are absent in an individual’s semen.\textsuperscript{2,16,19,20} Such conditions become problematic when attempting to detect semen microscopically through the identification of sperm.

In addition to medical conditions that may cause sperm to be absent from semen evidence, the time elapsed between the deposition and collection can affect the presence of sperm, especially in sexual assault cases. The detection of sperm is highly dependent upon the location of collection, whether it is from vaginal, cervical, rectal, or oral
cavities. Based on post-coital interval (PCI) determinations, sperm are rarely detected after 7 days within the vaginal cavity, after 3 days within the rectal cavity and after 24 hours within the oral cavity.  \(^9,10,21–24\)

1.2 Current Methods for Forensic Detection of Semen

Common protocol for the detection of semen includes a workflow process outlining the order in which testing is performed. Upon identification of a suspected semen stain, presumptive testing that identifies a seminal fluid component is conducted. If positive results are obtained, testing continues with microscopic examination. The identification of sperm confirms the presence of semen; however, the absence of sperm requires further testing for additional components of semen to reach a conclusion.  \(^2,9,10,25,26\)

1.2.1 Identification of Stains

In order to test for the presence of semen, a suspected stain must first be identified. This is most commonly accomplished either at a crime scene or during evidence examination within the crime laboratory through visualization with the naked eye or an alternate light source (ALS).

1.2.1.1 Visualization of Stains

The first method utilized to identify semen is visual inspection with the naked eye. Liquid semen, while rarely encountered at a crime scene, is typically white, grey or yellowish in color and viscous in nature. Once dry, semen stains may become off-white to colorless, and when concentrated are often stiff and crusty. Investigators and
laboratory personnel are trained to inspect evidence for stains that share these same characteristics. Identification of suspected semen via the naked eye allows investigators to readily collect the stain and perform further testing to confirm its presence.\textsuperscript{2,9,10,26}

1.2.1.2 Alternate Light Source

Due to the fact that semen can appear colorless, it may be difficult to visualize and differentiate from the substrate on which it is deposited; thus, an ALS is used to aid in locating any stains. An ALS is a handheld instrument that can illuminate various surfaces in a fashion similar to a flashlight in order to reveal any staining present through a process known as light conversion. This phenomenon occurs when a molecule absorbs the incident radiation resulting electronic excitation. The excited electron then decays back to the ground state, by emitting radiation at longer wavelengths, resulting in fluorescence.\textsuperscript{2,26}

With the use of a complementary colored barrier filter to eliminate the incident wavelength, the fluorescence can be viewed. Semen, as well as saliva, urine and breast milk, all fluoresce in the blue/violet region of the electromagnetic spectrum, 415-530 nanometers (nm), and can be viewed using an orange barrier filter.\textsuperscript{2,26,27} Alternatively, an ALS can be used with longwave ultra-violet (UV) light (365-415 nm) instead of non-UV light sources. While this method does not require a barrier filter, protective goggles must be worn to protect the eyes from harmful UV rays. However, shorter wavelength UV light can cause damage to deoxyribonucleic acid (DNA), thus, care must be taken when illuminating biological samples.\textsuperscript{2,28}
The use of an ALS is common among investigators and laboratory personnel at crime scenes during evidence analysis, as well as during sexual assault examinations of victims due to its portability and ease of use. Using an ALS as a screening method is also beneficial because it is non-invasive and can be used to screen large areas. Even so, the use of an ALS to locate latent stains has limitations. For example, the substrate being examined may have fluorescent properties that mask any stains present, or the substrate may be absorbent and pull body fluids beneath the surface, inhibiting light from reaching it. In addition, other material such as hair, bone, household cleaning supplies and gunshot residue can interfere with the results. Therefore, the use of an ALS is presumptive and must be used in conjunction with other testing methods.

1.2.2 Presumptive Semen Testing

Presumptive identification of semen plays an important role during an investigation of a case, informing investigators of which stains are probative and should be collected and submitted for confirmatory testing and/or DNA analysis. Colorimetric tests for detecting the presence of AP are among the most utilized presumptive screening techniques.

AP, as previously described, is a highly stable protein found in high concentrations in the seminal fluid of semen. While there are two forms of AP, they can be differentiated by electrophoresis due to their different mobility rates. This method is no longer utilized in forensic testing, thus, forensic testing is not specific for SAP. Additionally, because AP is found in low levels in other body fluids such as blood, urine and vaginal fluid, its detection is only presumptive for semen. Colorimetric tests for AP
provide an easy, quick and inexpensive method to screen for semen. In general, alpha-naphthyl phosphate is added to a cutting or swab of the suspected stain, which is broken down by AP to produce free naphthol and sodium phosphate. A colorless azo dye (nitrogen containing compound) is then added and combines with the free naphthol creating a color change. Two commonly utilized colorimetric tests are Diazo Red and Brentamine Fast Blue B. Diazo Red uses Fast Red RC Salt as the azo dye that reacts with free naphthol to create an orange-red color. Conversely, Fast Blue B uses 3,3'-dimethoxybenzidine to react with free naphthol, producing a violet color. Unlike both Diazo Red and Fast Blue B which can be performed as two-step reactions, the commercially available AP Spot test (SERI, Richmond, CA) combines the alpha-naphthyl phosphate and 3,3'-dimethoxybenzidine into one reagent, making it a one-step test (yielding a violet color). This is advantageous from a time perspective.

In addition to conventional AP testing, chemical AP mapping is a technique used when the facts of the case lead investigators to believe a semen stain is present on a particular item that is not conducive to visualization with an ALS (e.g., highly absorbent, white or dark substrates, and materials stained with other substances). A piece of filter paper moistened with water is placed in contact with the item of evidence for 2-5 minutes, then the AP spot reagent is applied. The appearance of a violet color on the filter paper corresponds to the location of the semen stain.

1.2.3 Microscopic Identification of Sperm Cells

After preliminary testing for AP, the confirmation of semen is necessary in order to provide investigators with the most accurate conclusions. Microscopic identification
of sperm based on their morphology is the single best confirmation for the presence of semen, as sperm cells are unique to semen. In order to visualize the sperm, phase contrast microscopy or staining methods are generally used to provide contrast between the background and other cellular material.\textsuperscript{2,3,10}

Phase contrast microscopy is a technique that allows for the visualization of sperm on unstained smear slides. A swab of a suspected semen stain is smeared across a glass microscope slide. A phase contrast filter is applied to increase contrast between the sperm and epithelial cells present and the background. This method is commonly employed on slides obtained from sexual assault evidence collection kits.\textsuperscript{2,3}

Unlike phase contrast microscopy, staining techniques apply dyes in order to improve the contrast between sperm and epithelial cells for visualization. Of the many staining techniques, Kernechtrot picoindigocarmine (KPIC), also known as the Christmas Tree Stain, and hematoxylin and eosin (H&E) are most commonly used. KPIC is a dual stain technique that dyes sperm and other nuclei red and cellular cytoplasm green/blue. A metallic dye, nuclear fast red, is first applied to the sample, staining any sperm heads and epithelial cell nuclei present. This is followed by picroindigocarmine, which stains epithelial cells as well as sperm tails.\textsuperscript{2,10,36} In instances when the sample is old or contaminated, the dyes may abnormally stain the sperm resulting in difficulty visualizing cells. Bacteria, yeast and white blood cells will also be stained red and may cause confusion, necessitating that the analyst be well trained in cell morphology. Similar to KPIC, H&E is a two-stain method. The first stain, hematoxylin, dyes nucleic acids present in the sperm head purple, whereas the eosin stains proteins and cytoplasm of
epithelial cells pink. In this method, there is less contrast between sperm and epithelial cells; thus the KPIC staining procedure is typically preferred.2,37

More recently, fluorescence staining has been employed for the identification of sperm. SPERM HY-LITER™ (Independent Forensics, Lombard, IL), a commercially available fluorescent staining kit, utilizes two fluorescent dyes—Alexa 488, a derivatized mouse monoclonal antibody is used to dye human sperm heads and 4’,6-diamidino-2-phenylindole (DAPI) identifies all cell nuclei. With a 488 nm excitation source and 520 nm barrier filter, sperm can be identified microscopically based on the fluorescence emitted from the stained sample.38,39 While this method is capable of identifying a single human sperm amongst other nucleated cells, it is both time consuming and costly.2,39,40

1.2.4 Chromatographic Immunoassay Testing

Instances where samples are positive for presumptive AP screening but are negative for the identification of sperm require further analysis. In these cases, lateral flow chromatographic immunoassay cards are used to test for the presence of either PSA or Sg. While found in large concentrations in semen, lower levels of PSA have been discovered in male serum, urine,41,42 breast milk43 and tumors42, which may cause false positive results. Similarly, Sg is encountered in various organ tissues and skeletal muscle. Because of this, PSA or Sg testing is not considered a confirmatory method, but may be coupled with other analysis techniques in order to reach a conclusion.2,10

Chromatographic immunoassay cards use antibody-antigen interactions to indicate the presence of either PSA or Sg. To test for PSA, monoclonal antihuman anti-PSA dye-labeled antibodies are present within the test strip. When the sample is
introduced into the sample well and PSA is present, an antibody-antigen complex forms and travels through capillary action to the test area. There the complex binds to immobilized polyclonal antihuman PSA antibodies, becoming visible as a colored band. In addition, there is a control region consisting of immobilized antibodies that must yield a colored band in order to ensure the test is functioning properly. A positive result is obtained when colored bands are observed in both the test and control regions.\textsuperscript{2,44,45} Sg chromatographic immunoassay cards operate in a similar fashion.\textsuperscript{2,46–48} These types of assays are sensitive up to a dilution of 1 in 100,000 \textsuperscript{48,49} and are easy to use; however, extremely high concentrations of PSA or Sg can cause a high dose effect, where excess antigen can lead to a false negative result.\textsuperscript{2,46,47}

1.2.5 Disadvantages of Current Detection Methods

While current detection methods allow investigators to identify semen, there are several drawbacks to the methods. In cases with limited contextual indications, the identification of an unknown stain may require the employment of numerous time consuming presumptive and confirmatory testing methods. In addition to time concerns, current semen identification methods detecting seminal components can exhibit false positive results because some of the components being tested for are also found in other body fluids or tissues.\textsuperscript{2,10,48} Lastly, while the identification of sperm definitively indicates the presence of semen, the lack of sperm does not signify an absence of semen.\textsuperscript{2,10,16,19–24} Currently, a universal body fluid identification method does not exist and the confirmation of semen requires multiple tests. Therefore, the development of a rapid universal identification method with limited false positives and where the presence
of sperm cells is not a determining factor would be a considerable improvement over current methodologies.

1.3 Raman Spectroscopy

1.3.1 Raman Spectroscopy

In the late 1920s while studying light diffraction through various media, the Indian physicist C. V. Raman observed slight changes in wavelength between the incident and scattered light. This phenomenon, namely Raman scattering, sparked considerable interest within the scientific community, eventually leading to the development of modern Raman spectroscopic methodologies.

1.3.1.1 Theory

Raman spectroscopy is a type of vibrational spectroscopy, that is based on the interaction of matter with monochromatic light and results in molecular specific information. Incident optical radiation interacts with molecules causing excitation to virtual excited levels. Unlike fluorescence where emission comes from the electronic excited state, Raman scattering does not need to coincide with an electronic transition and thus any incident light frequency can be used to excite a Raman scattering spectrum. Radiation of the same incident frequency is emitted, returning the molecules to the original, generally the ground, vibrational state and is referred to as Rayleigh (elastic) scattering. Raman scattering, or inelastic scattering, results in the emission of scattered photons at a different frequency than the incident radiation. Two types of Raman scattering transitions are possible: Stokes and anti-Stokes scattering. Stokes scattering,
which is more intense and will be the only type observed in the results described in this study, occurs when the molecule is excited to a higher lying vibrational level due to interaction with the incident photon, and causes the emission to yield scattered photons of a longer wavelength (loss of energy). Anti-Stokes scattering arises due to transitions from thermally populated vibrational levels and thus emits radiation of a shorter wavelength (gain of energy) compared to the incident photon. A spectrum of the intensity of the Raman scattering bands as a function of scattered wavelength is indicative of the molecular vibrations present in an illuminated sample. \(^{51,52}\)

The ability of a molecule to undergo Raman scattering is derived from its nuclear coordinate dependent electronic polarizability. Electronic polarizability is the ability of electronic density to respond to an electric field, which in this case is the incident laser source. The coupling of the induced polarizability to the nuclear motions during normal mode vibrational displacements results in the Raman effect. \(^{52}\) The polarization of the Raman scattered photons relative to the incident light is contingent upon the type of molecular vibration. Therefore, the intensity of the Raman scattering is dependent upon the electronic polarizability the molecule. \(^{51,52}\)

In addition to the nuclear coordinate dependence of the polarizability of the molecule, the intensity of the observed Raman bands is linearly proportional to the incident laser power, the number of illuminated molecules, and the population of initial vibrational levels. When a shorter incident wavelength is used, or the frequency of the incident light is increased, the intensity of the Raman bands generally increases. \(^{51,52}\). The intensity of Raman bands can be concealed in the presence of a generally strong,
broad fluorescence. Fluorescence occurs when light is resonant with an electronic excited state and the excitation energy is emitted back to the ground state. When the much more intense fluorescence emission overlaps the Raman spectral region, it appears on the spectrum as a broad feature that can often limit the ability to observe the discrete Raman bands.52

1.3.1.2 Instrumentation

A modern Raman instrument contains five main components: a laser source, a sample holder, a wavelength selector, a detector and a computer data system. The laser is the monochromatic excitation source and is carefully chosen as to limit the amount of fluorescence. Argon ion, krypton ion, helium-neon, diode, and neodymium doped – yttrium aluminum garnet (Nd-YAG) are among the most commonly utilized laser types. Regardless of the laser type, the mechanism remains the same by which monochromatic light of the near infrared to the UV regions can excite Raman emission from the sample. The sample holder is dependent upon the phase of the analyte, i.e. solid, liquid or gas, the size of the sample, as well as the specific instrument being used. Some examples of simple sample cells include glass microscope slides, glass tubes, capillaries, potassium bromide windows, and calcium fluoride windows.51

As previously described, the sample is excited and emits Rayleigh and Raman scattered photons. Because the Rayleigh scattered photons are much more intense than the Raman scattered photons, they must be filtered out using a notch filter (wavelength selector). The Raman scattered radiation is dispersed onto a charge-coupled device (CCD) detector by a spectrometer.51,52 Barron et al. determined that the use of a CCD
was able to increase the spectral acquisition rate dramatically compared to traditional instrumentation (e.g., photomultiplier tubes or photodiode arrays). A CCD detector is composed of an array of pixels, or photosensitive capacitors, that store the charge resulting from array absorbed photons. The total stored charge, which is directly related to the number of photons that came in contact with the pixels, is measured and digitized by an amplifier. The digital output is then read and processed by a computer data system, producing a spectrum containing the Raman bands.

A modern adaptation to Raman instrumentation includes the addition of a microscope for visualization of the analyte prior to exciting it with the laser and for excitation of near diffraction limited sampling volumes. The microscope can focus the laser to approximately 1 μm, allowing Raman to create chemical maps of heterogeneous materials.

1.3.2 SERS

In an attempt to study electrochemical processes in situ using a spectroscopic probe, Fleischmann et al. discovered that Raman scattering was intensified when pyridine, an organic compound, was adsorbed onto a roughened silver surface. Shortly thereafter, a pair of research groups simultaneously determined that substances in an adsorbed state with a metal surface resulted in enhancement of the Raman signal, prompting the exploration of what is now considered SERS.

1.3.2.1 Theoretical Description of SERS

SERS is a Raman technique in which an analyte is adsorbed onto or in close proximity to (approximately 10 Å) a nanostructured metal surface in order to enhance the
Raman scattering intensity. Due to the mechanism of SERS activity, enhancement of the Raman signal is dependent upon the distance between the metal surface and the analyte, and the orientation of the analyte molecules. Only molecules within approximately 5 nm of the nanostructured metal surface are enhanced.\textsuperscript{58–61} The SERS intensity is also dependent on the orientation of the molecules relative to the metal surface, thus the vibrational bands observed in a SERS spectrum vary from those observed with normal Raman.\textsuperscript{60,61}

There are two SERS enhancement mechanisms that are invoked: electromagnetic enhancement and chemical enhancement. The electromagnetic enhancement is due to the excitation of surface plasmons, or oscillating electron density at the surface of the nanostructured metal surface, (usually gold or silver).\textsuperscript{62,63} The nanoscale roughness causes the resonant electronic oscillations to occur perpendicular to the surface, effectively amplifying both the incident and scattered light in this spatial region, resulting in increased Raman scattering when the incident and scattered radiation is resonant with the surface plasmon resonance frequency. Additional enhancement will occur when surface plasmons are excited at wavelengths similar to those of the analyte’s absorption bands, known as electronically enhanced SERS, and results in the largest enhancement factors.\textsuperscript{51} However, large Raman enhancements (up to $\sim10^8$) can be obtained without this added resonance effect depending on the surface plasmon resonance frequency, the Raman excitation wavelength, and size/shape of the nanometer-sized surface.\textsuperscript{52}

The chemical enhancement effect, or charge-transfer theory, is typically explained in terms of electron transfer between the metal and analyte. Typically, lone pairs of
electrons are shifted from the highest occupied molecular orbital (HOMO) of the analyte, to the metal surface of the SERS substrate, and then to the lowest unoccupied molecular orbital (LUMO).\textsuperscript{62,64} This excitation using the metal as an intermediate for electrons requires less energy, thus allowing the transition to occur in the visible light region of the spectrum (400 to 800 nm).\textsuperscript{65} Unlike electromagnetic enhancement, chemical enhancement is dependent upon the electronic structure of the analyte. For instance, enhancement is reduced for saturated hydrocarbons, whereas the replacement of hydrogen atoms with halogens increases this enhancement mechanism.\textsuperscript{62} The chemical enhancement mechanism is generally considered to be less important than the electromagnetic effect.

1.3.2.2 Advantages

When comparing Raman techniques to other detection and characterization methods, such as gas chromatography-mass spectrometry (GC-MS) and Fourier Transform infrared spectroscopy (FTIR), Raman has several advantages. Unlike other techniques, portable instrumentation can be utilized and minimal sample preparation is required prior to analysis.\textsuperscript{66-68} Consequently, the method is rapid and simple to use. In addition, other spectroscopic methods are masked in the presence of water; however, water has minimal interference with the analyte signal when using Raman.\textsuperscript{51} A significant advantage of the Raman technique is its multiplexing capability. Multiplexing refers to the ability to detect multiple components within one analyte. Such capability allows Raman to identify and characterize more complex samples.\textsuperscript{69}
One of the greatest advantages of SERS compared to normal Raman is the large per molecule enhancement factor, which is typically in the range of $10^6$ to $10^8$. As a result, the detection limit of SERS has been reported to be between $10^{-9}$ and $10^{-12}$ molar (M). Furthermore, single molecule detection has been reported when the laser excitation is both electronically and plasmonically resonant. A second advantage of SERS is the ability to quench fluorescence. Fluorescence is a means by which an excited molecule can return to its ground state through the emission of light; however, when in close proximity to a metal surface, this transition is possible without the production of radiation, thus quenching fluorescence that may cause interference during SERS analysis.

1.3.2.3 Applications

Applications for SERS extend over a wide variety of scientific disciplines, from medicine to explosives detection. Some examples where SERS is utilized include bacterial characterization and diagnostics, identification of cancer proteins, early diagnosis of renal diseases, and detection of environmental contaminants such as pesticides, perchlorate and arsenic concentrations. Within the last decade, the use of SERS has expanded to include forensic applications. Of the most studied is the use of SERS for illicit drug detection and identification. Sagmuller et al. demonstrates SERS coupled with liquid chromatography to identify cocaine, heroin, and amphetamines in forensic samples. Similarly, Trachta et al. uses a combination of SERS and liquid chromatography in order to uncover illicit drugs in spiked biological samples.
slightly different methodology is applied by Bell and Sirimuthu to detect low concentrations of nicotine.\textsuperscript{80}

Other forensic applications include the detection of explosive material, specifically trinitrotoluene (TNT)\textsuperscript{75}, and characterization of a variety of dyes and inks.\textsuperscript{75,81--83} Most recently, SERS has been applied to the identification and characterization of blood. Using a SERS substrate of nickel nanotips coated with silver (Ag) nanoparticles, Boyd et al. was able to detect the presence of human blood up to a 1 in 100,000 dilution and from blood-stained fabrics.\textsuperscript{84} Premasiri et al. utilized gold (Au) nanoparticle covered silicon dioxide (SiO\textsubscript{2}) substrates in order to characterize whole blood, plasma and red blood cells.\textsuperscript{69}

1.4 Research Objectives

Utilization of SERS as a biological sample identification method is based on the premise that each body fluid has a unique chemical composition.\textsuperscript{4,5,7,8} The purpose of this research is to demonstrate the potential use of SERS as a new tool in human body fluid identification for forensic applications. With a focus on the analysis of human semen, objectives of this particular study include the initial comparison of body fluids in order to establish the practicality of the method, identification and characterization of neat semen, methodology development for the extraction of dried semen stains, establishment of reproducibility within and between donors, determination of the sensitivity of the technique, and a preliminary investigation of body fluid mixtures.
2. MATERIALS AND METHODS

The identification of semen within this research was separated into several separate studies. First, SERS analysis of the five major body fluids was performed in order to confirm differentiability. Secondly, neat semen, seminal fluid and sperm were identified and characterized through the examination of samples from four donors, along with a series of dilutions. Next, the development of an extraction method for semen from a porous and a non-porous substrate was achieved through varying time, volume and extraction solution parameters. Finally, a preliminary study of mixtures amalgamating semen with other body fluids was conducted. All of the collected data was subjected to partial least squares discriminant analysis (PLS-DA) or principal component analysis (PCA) in order to create a classification system.

2.1 SERS Substrates

In order for SERS analysis to be performed on experimental samples, specially designed SERS substrates unique to Dr. Lawrence Zeigler’s research group of Boston University’s Photonics Center were produced and quality tested on a weekly basis. The SERS substrates were gold nano-particle coated SiO$_2$ chips measuring approximately 1 square millimeter (mm$^2$) (Figure 2). Synthesis of chips was achieved through a multistep procedure conducted weekly by the research scientist responsible for their development. Each batch produced was stored within a plastic isolation glove box (Plas Lab, Lansing MI) in glass beakers containing chip reducing solution to prevent dehydration.
When selecting SERS substrates, the physical characteristics of each individual chip were taken into consideration. Circular chips that had at least one flat side and exhibited a lustrous surface were chosen. Any substrates that were egg-shaped, fragmented, or transparent (signifying that they lacked the necessary gold nano-particle coating) were discarded. The selection of chips based on appearance was not quantitatively determined, and therefore was a subjective process.

Once a SERS substrate was selected, it was deposited (flat side up) on a cover slip temporarily mounted to a glass microscope slide. Any excess chip reducing solution was removed and 1 µL of the desired sample was applied using a micropipette (Eppendorf, Germany). The chips were then covered with a Petri dish cover and allowed to dry for approximately thirty minutes before being analyzed. Typical analysis consisted of obtaining ten spectra per chip.

Figure 2. SERS substrate. (A) Image of SERS substrate on glass microscope slide with scale; (B) Scanning electron micrograph image of SERS substrate illustrating the Au clusters on its surface.60
2.2 Sample Preparation

Semen used in this research was donated in compliance with established Institutional Review Board protocols or was purchased (Lee Biosolutions, St. Louis MO). Liquid blood, saliva, urine and vaginal swabs were donated from a single source on the day of analysis. Semen stains were prepared by depositing approximately 25 micro liters (µL) onto white cotton swatches measuring 1 square inch in size (TexWipe®, Kemersville NC), mimicking stains on a porous substrate. Each stain was outlined using black ink to indicate the location of the stain once dried. Likewise, 25 µL of semen was deposited on glass microscope cover slips (Thermo Scientific, Tewksbury MA) and placed within miniature culture dishes in order to mimic a stain on a non-porous surface. Cotton swatches and cover slips were allowed to dry for 24 hours within the glove box at constant humidity (approximately 40%) and temperature (approximately 20 ºC).

Upon receipt of each semen sample, efforts were made to limit the number of freeze-thaw cycles that the samples would undergo by preparing substrates and aliquots prior to freezing. Semen was deposited (100 µL) in the center of three white cotton swatches and outlined. Similarly, 25 µL of semen was deposited on three cover slips placed within miniature culture dishes. Cotton swatches and cover slips were allowed to dry for 24 hours within the glove box at the same conditions previously described. Three aliquots of 10 µL were transferred to microcentrifuge tubes and the remaining volume of semen was deposited into another microcentrifuge tube. All of the prepared samples were stored at -20 ºC until use, at which point they were allowed to thaw for
approximately 15 minutes and if liquid, vortexed (Barnstead/Thermolyne Type 16700 Mixer, Dubuque IA) for 15 seconds.

2.3 Comparison of Human Body Fluids

Semen, blood, saliva, urine, and vaginal fluid were analyzed in order to ensure differentiability of the SERS spectra. Due to the fact that the vaginal fluid sample was donated on swabs, it had to be extracted prior to application on a SERS chip. Approximately one eighth of the swab was cut using a disposable steel scalpel (Miltex, Inc., York PA) and placed in a microcentrifuge tube with 5 µL of purified Millipore water (EMD Millipore, Billerica MA). The solution was mixed and allowed to sit for 5 minutes, at which time the extract solution was transferred into a clean microcentrifuge tube. An unused swab was extracted in the same manner, and analyzed as a negative control. Semen, saliva, urine and vaginal fluid extract (1 µL each) were applied to chips (previously described) in triplicate. In the case of blood, 1 µL of the sample was deposited onto the chip and immediately drawn back up without lifting the pipette tip in an attempt to prevent red blood cells from covering the surface, henceforth referred to as “removed samples”.

2.4 Identification and Characterization of Semen

2.4.1 Neat Semen

Immediately upon receiving the semen samples, 1 µL was deposited on three separate chips. All semen samples were examined for sperm using the Raman
microscope. In addition, three neat-removed samples were prepared by applying 1 µL of the sample on three chips and immediately removing it without lifting the pipette in order to determine the affects of viscosity.

2.4.2 Sperm-Free Seminal Fluid and Sperm

For a single donor, a 10 µL aliquot of semen was centrifuged at 10,000 revolutions per minute (RPM) for 2 minutes. The supernatant was carefully removed, transferred into a clean microcentrifuge tube, and 1 µL was applied to three chips for analysis. Saline was then used to wash the pellet three times. This was accomplished by adding 50 µL of saline to the pellet, vortexing the microcentrifuge tube for 15 seconds, centrifuging the sample using the aforementioned parameters, and carefully removing the supernatant. Following the final removal of the supernatant, the pellet was suspended in 10 µL of saline and vortexed for 15 seconds. Three chips were then prepared with 1 µL of each pellet sample.

2.4.3 Dilutions

A series of dilutions was analyzed with the purpose of determining the most efficient analysis method, as well as investigating the sensitivity of the technique. The first set of dilutions was prepared using semen donor #1. Dilutions of 1 in 10, 1 in 100 and 1 in 1,000 were made with Millipore water. A second series of dilutions was performed using semen donor #2. Dilutions of 1 in 10, 1 in 100, 1 in 200, 1 in 500 and 1 in 1,000 were made with high-performance liquid chromatography (HPLC) grade water (Fisher Scientific, Fair Lawn NJ) and again with saline. For the final two donors (#3 and
#4), a single saline dilution of 1 in 10 was prepared. All dilutions were applied to chips in 1 μL triplicates.

2.5 Development of an Extraction Method

A method for the extraction of semen from cotton swatches and cover slips was developed by varying time, volume and extraction solution parameters.

2.5.1 Extraction From Cotton Swatches

In an effort to exploit the speed and ease-of-use of the SERS method, the semen stain extraction was designed to require minimal time, materials, and simplicity in order to facilitate implementation at a crime scene. Cuttings (approximately 2 mm by 2 mm) of the center of the prepared semen stains on cotton swatches from donor #1 were placed in microcentrifuge tubes with varying volumes (5-10 μL) of extractant (Millipore water). The solution was then mixed using a pipette, allowed to incubate at room temperature for a pre-determined amount of time (1-10 minutes), and transferred into a clean microcentrifuge tube. In addition, a negative control (unused cotton swatch) was extracted in the same manner for 5 minutes using 5 μL of Millipore water. Swatches were prepared in triplicate (Table 1).
Once an optimal volume and time were determined, additional considerations were explored. First, a cutting of the perimeter of the semen stain was extracted in order to assess homogeneity. Secondly, the procedure was repeated using saline as the extractant. Finally, the extraction was performed with semen from the additional donors using both Millipore water and saline.

2.5.2 Extraction From Cover Slips

Similarly to the extraction of stains on cotton swatches, the extraction of semen stains on cover slips was developed to retain ease-of-use of the method. Millipore water (1 µL) was deposited onto the stain, pipetted up and down three times without lifting the pipette tip, and subsequently deposited on a chip. This process was repeated using 10 µL of Millipore water and 1 µL of saline. In addition, 1 µL extractions using both Millipore

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Table 1. Volume and time parameters utilized for the extraction of a single donor’s semen stain on cotton swatches. Cuttings of semen stains were combined with varying volumes of extractant in microcentrifuge tubes for varying time periods.

<table>
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<tr>
<th>Extraction</th>
<th>Volume (µL)</th>
<th>Time (minutes)</th>
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water and saline were conducted for the remaining three donors. All extractions were performed in triplicates.

2.6 Preliminary Study of Mixtures

A preliminary investigation into a 1:1 mixture of semen with vaginal fluid was conducted. Approximately one eighth of a vaginal swab was extracted as previously described and 3 µL of the extraction solution was combined with 3 µL of semen in a microcentrifuge tube. The mixture was vortexed for 15 seconds, and substrates were prepared (in triplicate) with 1 µL of sample.

2.7 Spectral Acquisition and Analysis

2.7.1 Instrumentation

Sample analysis was conducted on a Renishaw Raman microscope (model RM-2000, Wotton-under-Edge, Gloucestershire) containing a CCD (400 x 578 pixels) detection system with a 0.25 m monochromator and a 1200 groove/millimeter grating. A 785 nm diode laser (Renishaw model HPNIR785, Wotton-under-Edge, Gloucestershire) was utilized at an incident laser power of 0.34 milliwatts (mW). Scattered light was collected with a 50x microscope objective (Leica Microsystems, Wetzlar, Germany) and the spectral data acquisition time was set at 10 seconds. The instrument was wavelength calibrated using a silicon wafer, which characteristically produces a phonon band at 520 wavenumbers (cm⁻¹). Cosmic ray removal was performed within the WiRE 2.0 software (Renishaw, Wotton-under-Edge, Gloucestershire). Spectral cutting, baseline correction,
peak location determination, and other spectral analyses were performed using an in-house written MATLAB® (v. 2013b) routine. PCA was performed using MATLAB in conjunction with the Partial Least Squares (PLS) Toolbox (Eigenvector Research, Inc, Wenatchee WA).

2.7.2 Spectral Acquisition

Upon introduction of the glass slide containing the SERS substrate onto the microscope stage, an image of the chip was displayed through the WiRe 2.0 software and brought into focus. Acquisition parameters were adjusted to utilize 1% laser power (.34 mW). Alignment of the sample with the focal point of the objective was achieved using the fine adjustment under one second real time acquisitions, referred to as quick scans, at a predetermined spectral window in order to maximize the collection of emitted light. The quick scan spectral window was centered at 730 cm\(^{-1}\) due to the presence of several peaks in the spectrum of semen. Once the sample was focused, 10-second acquisitions were obtained covering a spectral window between 200 and 1800 cm\(^{-1}\).

2.7.3 Initial SERS Substrate Analysis

Prior to analysis of experimental samples, the quality of the SERS substrates was analyzed. This was accomplished through a two-step process. First, each batch of chips was subjected to contamination testing by analyzing one to two substrates from each to determine if a signal was produced. Substrates that produced a signal were deemed contaminated and discarded. Next, the uncontaminated batches of substrates underwent signal testing. One microliter of 100 micro molar (µM) adenine (Sigma Life Science, St. Louis MO) was applied to one to two chips from each batch, allowed to dry, and then
analyzed. The average signal strength recorded from five one-second quick scans was calculated for each sample. It should be noted that the determined signal potential was representative of the batch and not guaranteed with each individual chip. Research quality chips were considered to be an uncontaminated batch that produced an adenine signal greater than 1,000 counts.

2.7.4 Peak Location Determination

The spectra obtained from each chip were imported into MATLAB for analysis. First, spectra were categorized visually for each donor within every experiment. The average spectrum for each category was calculated and the location of peaks for all averages was determined. A peak was defined as using an approximate threshold of 20% above the baseline.

2.7.5 Statistical Analysis

PCA is a multivariate statistical model capable of detecting spectral variations within a dataset. In order to accomplish this, the initial dataset, which may contain related variables, is reduced and transformed into uncorrelated (or orthogonol) variables known as principal components (PCs). Abundances of PCs can be displayed on score plots to graphically view any spectral variation.71,85,86

In this study PCA was chosen to compare experimental datasets due to the ability of the technique to statistically identify any spectral variation, its lower degree of supervision compared to other statistical methods, and because it is commonly applied to vibrational spectroscopic data.71,85,86 Tight grouping of datasets, indicating no significant spectral differences between two or more sets of spectra, is reported as being consistent
with one another. Instances where little to no spectral overlap occurs are considered to contain samples inconsistent with one another.

All body fluid data was converted into barcodes for statistical analysis methods. Barcodes were determined by the sign of the second derivative of the noise filtered spectrum as a function of frequency.\textsuperscript{71} Optimization of the barcode procedure could be accomplished by adjusting the Fourier transform smoothing function and floor cutoff. The barcoded spectra were the input vectors for the multivariate data analysis including PLS-DA. This procedure enhanced the variation and separation between datasets of different sample types by rotating the PCs to create the maximum variance.\textsuperscript{71,87}
3. RESULTS AND DISCUSSION

3.1 Comparison of Human Body Fluids

Upon review of neat semen, blood, saliva, urine, and vaginal fluid, it was determined that each body fluid produced differentiable spectra. Initial conclusions were reached based on visual examination of average spectra for each body fluid. Each of the five spectra contained a unique combination of peaks; thus, each body fluid was capable of being independently identified (Figure 3A). PCA showed overlap between multiple body fluids, potentially due to the same components present in multiple samples. It was determined that PCA was not robust enough to differentiate the body fluids; therefore, further analysis was conducted using PLS-DA. Analysis successfully separated each individual body fluid from the remaining body fluids; the separation of semen is illustrated as a cluster of data points removed from the discriminant line (Figure 3B). In addition, a single semen dataset was input into PLS-DA as an unknown sample and successfully categorized as semen. These results demonstrate that SERS is capable of differentiating human body fluids and has potential to be used within a forensic context.
Figure 3. Comparison of body fluids. (A) Spectral averages of neat semen, blood, saliva, urine and vaginal fluid illustrate visual differences between spectra suggesting differentiability. (B) PLS-DA of semen (red), blood (black), saliva (green), urine (cyan) and vaginal fluid (blue) demonstrating successful separation of semen from the other body fluids; thus, confirming body fluids can be differentiated. In addition, the semen donor input as an unknown (red triangle) was appropriately categorized as semen as it lies above the discriminant line when semen is the predicted class.
3.2 Identification and Characterization of Semen

3.2.1 Neat Semen

As previously mentioned, all semen samples were examined for the presence of sperm. The existence of sperm in all samples limits the variables present in the study and allows for appropriate comparison between all semen samples. An example of positive microscopic sperm identification on a SERS substrate is shown in Figure 4.

![Figure 4. Microscopic view of semen on SERS substrate.](image)

Initial analysis of neat semen was achieved through examination of the ten spectra obtained for each of the chips for all four donors in order to determine homogeneity within a SERS substrate. Average spectra for each chip within a single donor were then compared to ensure reproducibility. In addition, spectra from all three chips for each donor were averaged together and compared to one another to ensure reproducibility.
between donors (Figure 5). Final analysis was completed by averaging all spectra from every donor to produce a single average neat semen spectrum. This process was repeated for each of the experimental categories (data not shown).

Figure 5. Progression of spectral analysis for neat semen. (A) Ten spectra of neat semen from donor 3 obtained from a single SERS substrate with the average spectrum in red. Consistency of the spectra demonstrates homogeneity within a chip. (B) The average spectra of neat semen for all three SERS substrates of donor 3, with the average spectrum in red. Consistency of the spectra demonstrates reproducibility within a single donor (with a few variations). (C) The average spectra of neat semen for each donor (1 in blue, 2 in green, 3 in purple, and 4 in cyan), with the average neat semen spectrum in red. Consistency of the spectra demonstrates reproducibility between donors with minor discrepancies.

Analysis of neat semen for all four donors revealed the presence of two visually distinct spectra, illustrated in Figure 6A. Collection of both spectra occurred throughout
the entirety of this study under numerous circumstances; therefore, the reason behind the existence of two spectra remains unknown. Subsequent analysis of spectra determined that the majority of the samples contained only one of the two spectra during all measurements of a single substrate; however, there were instances where a single chip included both spectra (Figure 6B). While the two spectra are visually distinct, after analyzing peak locations and completing PCA it was determined that the spectra contained majority of the same peaks and no significant spectral differences existed (Figure 6C); therefore, both spectra can be identified as semen.

**Figure 6.** Semen spectra 1 and 2. (A) Comparison of average semen spectrum 1 on top in green and average semen spectrum 2 on the bottom in blue. (B) Ten spectra obtained from chip two of the exterior extraction analysis containing both semen spectrum 1 (green) and 2 (blue). (C) PCA score plot of semen spectrum 1 and semen spectrum 2. The spectral overlap present indicates the spectra are consistent with one another.
Once it was determined that the PCs of spectra 1 and 2 were consistent with one another, the spectra from all four donors were submitted to PCA and showed no significant spectral variations, thus demonstrating reproducibility between donors (Figure 7). Neat semen spectra were used as a comparison for experimental spectra visually and within PCA.

![PCA score plot of four semen donors](image)

**Figure 7. PCA score plot of four semen donors.** PCA score plot of semen donors 1, 2, 3 and 4 displays overlap indicating that there is a lack of spectral variance among donors.

Although the contributing components of each peak are currently unknown, it is suspected that the peaks located at approximately 658 cm\(^{-1}\) and 724 cm\(^{-1}\) are due to the presence of xanthine and hypoxanthine\(^{69}\), respectively, based on the spectra for the two compounds individually (Figure 8). Due to the fact that the other components are unidentified, such conclusions cannot be reached at this time.
In addition to the application of 1 µL of semen to a chip, other chips were prepared in which 1 µL of the sample was deposited and immediately removed without lifting the pipette. When the sample is removed in this manner only a thin layer of semen remains on the SERS substrate, and through comparison of the removed sample spectra to spectra of neat semen, the effect viscosity has on the results can be evaluated. Examination of spectra and PCA score plot for the removed method compared to neat semen revealed consistency between the two (Figure 9). In other words, viscosity did not affect the results. This also demonstrated that in cases where evidence was present in an extremely small volume, the sample can be applied to the chip, removed, and retained for future testing.
3.2.2 Comparison of Neat Semen and Sperm-Free Seminal Fluid

The analysis of seminal fluid and sperm separately was conducted in an attempt to uncover the cause of the two semen spectra. It was hypothesized that one of the spectra was produced by seminal fluid and the other by sperm; however, the results did not support this hypothesis. As observed in Figure 10A, sperm-free seminal fluid resulted in
spectra containing sharp peaks at approximately 445, 724, 962 and 1577 cm\(^{-1}\). Conversely, analysis of sperm resulted in no signal, leading to the hypothesis being rejected. It is possible that sperm produced a signal that was unable to be obtained due to the large concentration of cells preventing the laser from reaching the chip surface or the emitted signal from being detected. In order to examine this in the future, the sperm fraction can be diluted and reanalyzed.

When comparing the average seminal fluid spectrum to that of neat semen, there were consistencies with the aforementioned peak locations as well as other, weaker signals. In addition, PCA indicated that seminal fluid was consistent with neat semen, (Figure 10B). These results aid in the overall characterization of semen.
3.2.3 Dilutions

Upon visual examination of spectra obtained from dilutions utilizing both water and saline (Figures 11A and B) and subsequent analysis of peak locations, evidence of peak loss is observed. Peak loss occurs when an expected peak has become indistinguishable from the baseline. This phenomenon was exhibited in all dilution samples. In addition, the majority of the dilutions follow the general loss of peaks at approximately 483 and 860 cm$^{-1}$. The 658 and 724 cm$^{-1}$ peaks were lost for dilutions of 1
in 500 or lower. While this was not ideal, the dilutions were consistent with neat semen when submitted to PCA. Figure 11C displays PCA comparison of the dilutions of 1 to 1,000 utilizing water and saline to neat semen with minimal outliers, exemplifying the consistency observed. These results do not establish the detection limit for the identification of semen using SERS, however, they do reveal that the method is capable of identifying semen diluted to 1 in 1,000. Overall it was determined that SERS possesses comparable sensitivity (at a minimum) to current semen identification methods.

**Figure 11. Comparison of water and saline dilution sets.** (A) Spectra obtained from the first dilution set utilizing Millipore water. Peak loss is exhibited throughout the series. (B) Spectra obtained from the first dilution set utilizing saline. Peak loss is exhibited throughout the series. (C) PCA score plot of 1 in 1,000 water and saline dilutions compared to neat semen. The spectral overlap present indicates the spectra are consistent with one another.
3.3 Development of an Extraction Method

3.3.1 Extraction from Cotton Swatches

During the development of an extraction method for semen stains from cotton swatches, the quickest and simplest parameters resulting with consistent identification of semen are suggested for future testing. Initial analysis investigating the volume of Millipore water (5 and 10 µL) resulted in consistent spectra (Figure 12A). When subjected to PCA, the spectra from each of the two extractions were consistent with neat semen (Figure 12B). In an attempt to prevent unnecessary dilution, the volume of 5 µL was chosen for further development.

Investigation of extraction time variations demonstrated that when solutions were allowed to extract for 1 minute, difficulty obtaining spectra and an overall loss of features occurred, observable in Figure 12A. During spectral acquisition of samples extracted for 1 minute, a considerable amount of time was exhausted attempting to locate areas on the SERS substrate that contained a signal. This was not the case for longer extraction times using the same batch of chips; therefore, it was concluded the SERS substrates were not the culprit but rather the lack of semen present. Because of this, methods containing such time parameters are undesirable for the extraction of semen stains. Conversely, solutions allowed to extract for 5 minutes or longer yielded spectra consistent with that of neat semen. PCA incorporating feasible time variations resulted in the successful classification of 5-minute and 10-minute extractions as semen; thus, the shorter extraction time was chosen for further development.
Experimental results concluded that extraction parameters of 5 µL of extractant and 5 minutes allowed for extraction provide the simplest and quickest means of obtaining semen from cotton swatches for successful identification using SERS. Such parameters are significantly less time and sample consuming compared to current detection techniques, which require extraction for a minimum of one hour using a cutting
of the stain approximately 20 mm$^2$ \cite{36,44,45}. For this reason, the utilization of SERS would drastically reduce the effort needed to analyze evidentiary samples.

Once extraction parameters were chosen, a cutting from the perimeter of the stain was analyzed to determine if the stain was homogeneous. This was based on the concept that different components travel across the substrate at different rates during the drying process, potentially causing heterogeneity within the stain that could result in a different spectrum. Visual comparison of the perimeter versus the center cuttings extracted under the same conditions showed consistent spectra, labeled as perimeter and 5 µL in Figure 12A. PCA demonstrated a lack of spectral variation between the perimeter and center cuttings; thus, the location from which a cutting of the stain is obtained does not cause spectral variation (Figure 12B).

Saline was explored as a potential extractant under the chosen extraction parameters and compared to the results obtained using Millipore water. It was determined that there was no significant visual difference or variation in peak locations between the average spectra obtained (Figure 12A). Saline spectra were also consistent with the other extraction techniques tested, shown through PCA (Figure 12B). These results demonstrated that there is no advantage to utilizing one extractant over the other in order to identify semen; however, due to the fact that water is already incorporated into typical crime scene investigation kits, it is preferred over saline.

3.3.2 Extraction from Cover Slips

As with the extraction of semen from cotton swatches, parameters were varied in order to establish the simplest extraction method for a non-porous surface. It was
determined that there were no significant spectral variations between two of the three parameters investigated (1 µL Millipore water and 1 µL saline); however, analysis using 10 µL of Millipore water resulted in spectrum 2 (Figure 13A). This was not of concern due to the fact that PCA previously demonstrated classification consistency between the two spectra. In addition, PCA of all three experimental variations were consistent with one another with only slight spectral variation using 10 µL of Millipore water (Figure 13B). While the use of 1 µL of water resulted in slight variation, it is hypothesized that a larger sample size would reduce the deviation; however, further investigation is needed to support this. Despite the variation, the 1 µL of water parameter is still consistent with semen and is therefore preferred due to the smaller volume preventing unnecessary dilution of the stain and the fact that water is already included in typical crime scene investigation kits.

Current collection and identification of dried semen stains on non-porous surfaces relies on the transfer of sample to cotton swabs. Cotton swabs are subsequently analyzed per laboratory protocols. The use of the described extraction method for identification of dried semen on glass cover slips can eliminate the intermediate step, not relying on the transfer of sufficient sample to the cotton swab.
3.4 Preliminary Study of Mixtures

The preliminary investigation into mixtures of semen and vaginal fluid indicated that SERS is capable of categorizing the mixture based on its components. Visual comparison of the average spectrum obtained from the 1:1 mixture to neat semen and vaginal fluid revealed similarities (Figure 14A). For example, the mixture contains peaks at approximately 483 and 1020 cm\(^{-1}\), which are present in semen and vaginal fluid,
respectively. In addition, the mixture contains a peak at approximately 734 cm$^{-1}$ which is present in vaginal fluid and may be responsible for masking the suspected hypoxanthine peak found in semen. The opposite is true of the peak at approximately 1580 cm$^{-1}$, in which semen potentially masks the vaginal fluid peak at approximately 1545 cm$^{-1}$. These results indicate that the mixture spectrum is additive of the two components spectra; this was further supported by PCA, which showed slight overlap between the mixture and semen and vaginal fluid (Figure 14B). However, there is significant clustering of each class of data, which indicates that PCA can identify a mixture of vaginal fluid and semen from its components. This was not surprising as both are components that contributed to the overall composition of the mixture. The next step in evaluating the ability for SERS to differentiate a semen-vaginal fluid mixture is to premix a sample and apply it to a cotton swatch, to be extracted as per the determined parameters. It is also necessary to increase the number of samples, donors, and replicates in order to test the reproducibility of identifying mixtures. Successful categorization of the aforementioned premixed stain as containing semen and vaginal fluid will prompt continued research in this area.
Figure 14. Analysis of semen-vaginal fluid mixture. (A) Spectra obtained from neat semen (red), the mixture of semen and vaginal fluid (black) and vaginal fluid (blue). The mixture contains features from both semen and vaginal fluid. (B) PCA score plot comparing semen and vaginal fluid to the mixture displays significant clustering indicating that PCA can identify the mixture separately from its components.
4. CONCLUSIONS

Through this study SERS has been established as a potential universal investigative tool for the identification of human body fluids. Initial analysis differentiating the five major body fluids provided the support necessary to further explore this technique. Intensive examination into the detection of semen revealed reproducibility within and between donors, which is essential in order to classify unknown samples. In addition, the viscosity of semen was determined to have no affect on SERS analysis, as both the neat and removed samples were consistent with one another. The successful detection of semen at a dilution of 1 in 1,000 gives presumptive indications to the sensitivity of the SERS technique; however, in order to determine the true sensitivity of the method, lower dilutions must be analyzed. Consistency independent of viscosity and dilution demonstrates ability of SERS to identify semen in numerous states.

Methods used for the identification of body fluids in forensic settings require the capability to test dried stains; therefore, investigation into the analysis of dried stains using SERS was conducted, confirming such capabilities. Potential parameters for the extraction of semen from cotton swatches and glass cover slips were determined to be 5 µL of Millipore water extracted for 5 minutes and 1 µL of Millipore water, respectively. While these factors were chosen based on simplicity and speed, and both methods resulted in successful identification of semen, additional parameters must be explored prior to implication of SERS in the field.
Analysis of the ability to identify a mixture of semen and vaginal fluid, conducted in an attempt to further develop SERS as an identification tool, exposed similarities between the spectra. Peaks found in both semen and vaginal fluid were present in the mixture suggesting additive properties, and allowing for the identification of the mixture’s components. Continued exploration into the analysis of mixtures must be conducted in order to explicitly describe SERS capabilities.

In summary, this preliminary study into the identification of semen using SERS demonstrates the potential for the method to be used as an investigative tool for the detection of human body fluids at crime scenes and within forensic laboratories.
5. FUTURE STUDIES

5.1 Semen Identification

5.1.1 Component Analysis

The next step in furthering this identification method is to identify the components contributing to peaks within a semen spectrum. An extensive literature review must be conducted in order to determine previously identified components of semen. Once potential contributors have been identified, they must be analyzed individually and compared to the semen spectrum. If peaks overlap, further analysis of component mixtures must be conducted until the semen spectrum is reproduced. This can also be completed for additional body fluids.

5.1.2 Additional Analysis

One of the most essential continuations of this research is to increase sample size. Only four semen donors and a single donor for blood, saliva, urine and vaginal fluid were examined, limiting the scope of the study. In order to ensure reproducibility between individuals, the number of donors for all body fluids must be increased dramatically. This can also be expanded to include a comparison of spermic and aspermic semen. Additionally, in depth research analyzing donor variables, such as age, health and diet, is another aspect to take into consideration.

5.2. Additional Biological Stains

Due to the fact that the application of SERS to forensic identification of biological fluids is a new concept, there are numerous continuations of this research to consider.
Incorporating other body fluids using similar methodology is necessary in order for this technology to be employed. In addition to further identification and characterization of blood, saliva, urine and vaginal fluid, investigations can include menstrual blood, sweat, tears, breast milk, vomit, earwax, nasal mucus, and feces.

5.3. Sensitivity

Even though two dilution series were analyzed within the current study, the sensitivity of the SERS technique remains undetermined. While sensitivity is dependent upon the specific SERS substrates being utilized, it is important to have an estimation of the detection limits. This can easily be accomplished by preparing and analyzing further semen dilutions as well as other body fluid dilutions using a quality batch of chips.

5.4. Specificity

Specificity of testing is a significant aspect in choosing an identification technique. Methods that lack specificity are unable to act as confirmatory identification methods; therefore, future testing should include comparing SERS analysis of human semen to semen of commonly encountered animals. Such a specificity study also should be completed for all body fluids. In addition, fluids that are commonly mistaken for biological materials at crime scenes, such as wine, ketchup, lotions or apple juice, should be compared to human body fluids to ensure previously determined spectra are unique to the individual body fluid.
5.5. Mixtures

5.5.1. Biological Mixtures

The current study conducted a preliminary investigation of mixtures of semen with vaginal fluid. In order to accurately describe the effect mixtures has on the spectra, further analysis must be conducted. First, the continued analysis of 1:1 mixtures including additional body fluids should be performed. Next, the ratio of the mixture should be varied to include samples where one fluid in is much greater quantity than the other. Finally, mixtures containing three or more body fluids at varying ratios should be prepared and analyzed.

5.5.2. Additional Mixtures

In addition to encountering mixtures of body fluids, biological fluids are also found mixed with other common household items. For example, a crime committed within a garage or unfinished basement may contain combinations of rust and blood, or a crime scene where a cleanup occurred may contain a mix of bleach and blood. Other mixtures may include semen and condom lubricants, as well as vaginal fluid and lubricants. At crime scenes where blood is not visible to the naked eye but is suspected to be present, luminol may be used to detect it. In such cases, the combination of luminol and blood may require analysis. Investigation into such mixtures is necessary in order to employ the SERS technique with forensic samples.
5.6. Forensic Substrates

One of the most forensically relevant investigations required for the employment of the SERS technique in the field is the effect various substrates may have on the ability to identify semen. Within the current study, only white cotton swatches and glass cover slips served as substrates. In the future it is necessary to examine commonly encountered substrates, such as colored fabrics including cotton, polyester, fleece, spandex, untreated and treated wood, a variety of carpet types, tile, linoleum, and paper. In addition, the analysis of stains on washed fabrics can provide useful information as to the capacity of SERS limitations.

5.7. Environmental Conditions

Evidence received by forensic scientists is not always recovered from pristine conditions. For example, a piece of evidence recovered from a fire scene where it was subjected to extreme heat or an item of clothing recovered from decomposed human remains may both contain probative biological stains. Investigations of semen and other body fluids subjected to extreme conditions, such as high heat and strong acidic or alkaline environments, would evaluate SERS limitations and potentially lead to the development of analysis methods for such evidence.

5.8. DNA

A final consideration prior to the implication of SERS in the forensic field is the affect it may have on future DNA testing. Part of the appeal of SERS is the minimal
sample required for analysis; therefore any DNA testing performed would most likely be on a portion of the sample not used for SERS analysis. However, situations may arise where DNA testing may need to be performed on the analyzed sample. Because of this, it is necessary to understand if SERS has a negative impact on obtaining a DNA profile. In order to accomplish this, a method must be developed to remove the analyzed sample from the SERS chips for subsequent DNA analysis.
**LIST OF JOURNAL ABBREVIATIONS**

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**BIBLIOGRAPHY**


CURRICULUM VITAE

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- Presented at AAFS Annual Meeting, Orlando, FL  2015

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