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Project Title: Development of SERS-active forensic evidence swabs for rapid, non-destructive confirmatory serological screening and STR typing of human bodily fluids

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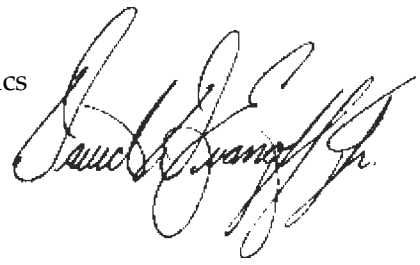


Project Goals and Objectives

In a crime laboratory, evidentiary swabs that may potentially contain human bodily fluids are screened using both presumptive and confirmatory tests. These tests, such as the acid phosphatase (AP) test and the so-called Christmas tree stain for semen and the Kastle-Meyer and Takayama tests for blood, are quite time consuming, expensive, only test for one bodily fluid and are prone to both false positives and false negatives. Recent literature reports however, have indicated that Raman spectroscopy may have a far lower limit of detection than traditional methods and may allow investigators to perform one type of measurement for all body fluids, potentially leading to higher efficiency, more definitive results, and higher accuracy. Another recent report utilized surfaced enhanced Raman spectroscopy (SERS) as a means to decrease the limit of detection of certain forensic samples. SERS, in which an analyte is placed on or near a nanostructured metal surface, has the potential to increase the Raman cross-section of the analyte by many orders of magnitude.

We have worked to develop SERS-active forensic evidence swabs by attaching silver nanoparticles grown via the hydrogen reduction method to the fibers of commercially available swabs. We optimized the synthetic protocol of the swabs that produced Raman signal of human biological fluids such as semen while also ensuring that there were minimal negative effects of the silver swabs on typical DNA extraction and quantification protocols. STR typing of semen samples collected on SERS-active swabs as well as those collected on pristine swabs were performed to determine the effect silver may have on the quality of data that is produced using protocols typical to forensic crime laboratories. We also explored the Raman limit of detection of semen collected on swabs. Likewise, we found little difference in the ability of SERS-active swabs to collect evidentiary material as compared to pristine swabs.

Materials and Methods

The nylon swabs used in this research were Copan FLOQswabs generally acquired from Life Technologies. The silver (I) oxide (99%) was acquired from Strem Chemicals and the silver nitrate (99%) from Fisher Scientific. Hydrogen gas (research grade, 99.9995%) was acquired from Airgas. Ultrapure water was always obtained from a Barnstead NANOpure Diamond system with a 0.2 μm hollow fiber filter, at a measured resistivity of 18.2 MΩ-cm. All chemicals were purchased and used without further purification. Pooled human semen (30 – 40 million cells /mL) was acquired from Lee Biosolutions. For the swabbing of dried seminal fluid

from a microscope slide, the slides were sterilized via a SpectrolinkerTM XL 1500 UV Crosslinker before use. Falcon conical tubes used for storage were acquired from Fisher Scientific. The 5000 mL heavy wall Pyrex vessel was obtained from Ace Glass Inc. Tris(2,2'-bipyridyl)ruthenium(II) chloride (98%) was acquired from Acros Organics. 0.45 μm nylon syringe filters from Fisher Scientific were used for swab fiber filtering in silver quantification analysis. An Applied Biosystems™ PrepFiler™ Forensic DNA Extraction Kit was used for silver analysis and DNA quantification. Likewise, standard protocols using a Quantifiler Trio kit and GlobalFiler kit were used for quantification and STR analysis.

The silver nanoparticles used throughout this research were synthesized using the hydrogen reduction method (Evanoff & Chumanov 2004). Prior to setting up the reaction vessel, the nylon swabs had been prepared for the reaction in a two-step process. The swabs' applicator shaft was clipped to a shorter size and the remaining swab head and applicator were then soaked in ultrapure water for 24 hours to remove any impurities. Secondly, the swabs were soaked in a subsequent 48-hour soak in saturated silver (I) oxide solution to ensure the swabs' surface remains saturated during the reaction and also ensures particles form and continue to grow on the nylon fibers. The swabs were stored in a clean container in ultrapure water before being dried for use in a glovebox. Once a swab had been used, the swab was allowed to dry on a swab drying rack at room temperature until visually dried (typically an hour). A swab that was allowed to dry during Raman measurements was allowed to visually dry on the microscope stage (typically 20 - 30 minutes).

For Raman and SERS measurements, different methods were employed to obtain Raman and SERS signal. For studies with CV solution, swab tips were soaked in the solution prior to the measurement. For studies with [Ru(bpy)3]2+, samples were swabbed in the following manners: solutions of [Ru(bpy)3]2+ were dropped into a petri dish and allowed to dry prior to swabbing. A moistened swab, wet using ultrapure water, was used to collect the dried [Ru(bpy)3]2+ solution. Additionally, dry swabs were also used with [Ru(bpy)3]2+ solutions that had not been allowed to dry for the means of examining the effects of using a wet vs. dry swab. For semen studies, the petri dish swabbing method with either a dry or wet swab was used. Further studies included swabbing semen that had been dried on a hard surface, a glass microscope slide, with a wet swab and also on a soft surface in a similar manner.

To assess the effects of synthesis temperature on amount of silver leached in the lysis step, three swabs from each temperature, 60 °C, 70 °C, 80 °C, 90 °C, 100 °C and, 110 °C were used. Each swab was placed in its own 1.5 mL eppendorf tube and labeled. 300 µL of PrepFiler™ Lysis Buffer was added to each sample along with 5 µL of 1.0 M dithiothreitol (DTT). The samples were briefly vortexed (1-2 seconds) and centrifuged (15 seconds) then placed in a thermal shaker, heated to 70 °C for 90 minutes at 900 rpm. The PrepFiler™ extraction protocol suggests that solid substrate be removed by incorporating an extra centrifuge step following incubation. Each swab was removed using tweezers and placed in a PrepFiler™ filter column which was then placed back into the original centrifuge tube. The samples were centrifuged for 2 minutes at 13,400 rpm. The swabs were then transferred to clean Eppendorf tubes and the filter columns were discarded. The remaining contents in the original centrifuge tubes contained the lysisbuffer mixture and now a pellet of solid silver that had collected at the bottom. The lysis buffer supernatant was aspirated by pipetting and transferred into new Eppendorf tubes. Other trials were performed to quantify the amount of silver in purified DNA extracts.

Project Findings and Data Analysis

The hydrogen reduction method for silver nanoparticle synthesis was first outlined by Evanoff and Chumanov and allows for the synthesis of surfactant-free silver nanoparticles with control of nanoparticle size. Particular to this research, the silver nanoparticles synthesized are attached to the fibers of nylon swabs. Copan FLOQswabs™ are manufactured by spraying nylon onto the tip of the applicator in the presence of an electrostatic field. The nanoparticles attach to the fibers of the nylon swabs as a result of electrostatic interaction by the donation of lone pair electrons from the nylon, a polyamide. In Figure 1 is shown swabs before and after silver nanoparticles attachment as well as electron micrograms of the Ag-modified fibers.

Figure 1. unmodified vs. SERS active swab and representative electron micrographs of swabs used in the study described below

$Ru[bpy_3]^{+2}$ was used as a model compound for initial SERS experiments. An example of the SERS enhancement of 10^{-6}M $Ru[bpy_3]^{+2}$ on SERS swabs can be seen in Figure 2. Reaction temperature was found to play a large role in the amount of silver on the swabs and thus the ability for SERS enhancement. To assess the SERS enhancement provided by swabs fabricated at different temperatures, the spatial point analysis technique described above was used. The swabs were used to collect 180 µL of 10^{-4} M $Ru[bpy_3]^{+2}$, dried, and mounted on microscope slides. Each swab was analyzed at 9 different points, all in the same spectral focal plane, with 457.9 nm excitation provided by an argon ion laser. The resulting spectra from an individual swab were averaged along with the spectra collected from four other swabs of the same condition. Figure 3 shows the variation in the integrated intensities of the 1488 cm^{-1} $Ru[bpy_3]^{+2}$ band for each synthesis temperature swab set. The 1488 cm-1 band integrated intensities for all 45 spectra in a swab set were averaged together and plotted, showing that the 80 ◦C, 90 ◦C, and 100 ◦C give the highest average intensities.

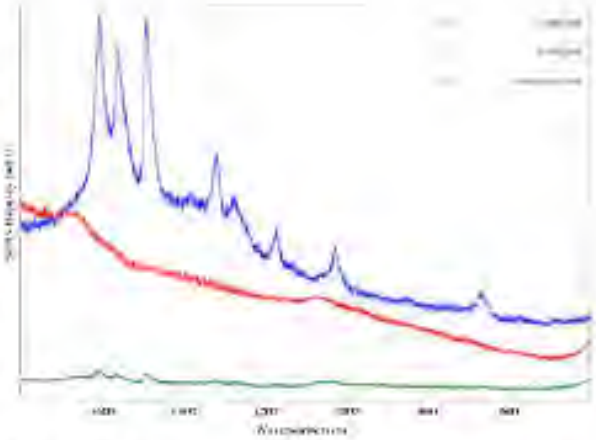


Figure 2. Comparison of [Ru(bpy)3]2+ signal obtained using 1.641 mW and 0.135 mW to that of a silver-less swab using 1.641 mW.

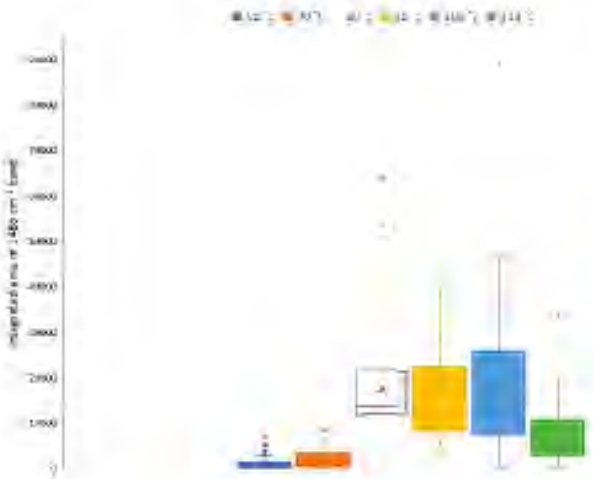
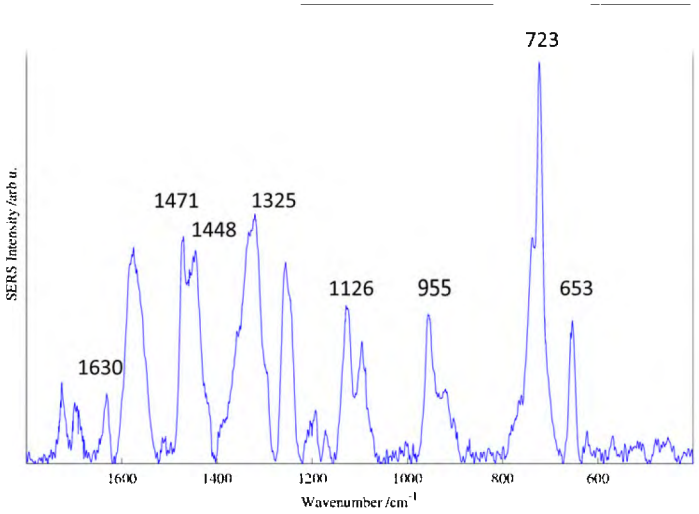


Figure 3. Box and whisker plot showing the variation is integrated area of the 1488 cm^{-1} $Ru[bpy_3]^{+2}$ band between reaction temperatures.

Seminal fluid studies began by first attempting to obtain a Raman spectrum of semen to serve as both a comparison to a literature spectrum and a reference for future studies. However, a conventional Raman spectrum could not be obtained with the available instrumentation using the HeNe laser. The HeNe laser was chosen as the excitation source for SERS of seminal fluid as previous studies in the lab had obtained SERS of seminal fluid using that excitation wavelength. So that they could be compared to the reference SERS spectrum obtained of seminal fluid, all seminal fluid spectra were collected using a 1 second acquisition time and 15 accumulations,



Raman Shift /cm^{-1}	Vibrational Mode
641	Ring deformation (Tyrosine)
715	CN sym. stretching (Choline)
829	Ring breathing (Tyrosine)
958	PO_4^{3-} sym. Stretching (SPH)
1125	CN asym. stretching (SPH)
1179	CH_2/NH_3 rocking (Tyrosine)
1265	Sym. ring deformation (Tyrosine)
1327	Ring stretching (Tyrosine)
1448	CH_2, CH_3 bend (Tryptophan)
1461	CH_2 bending (SPH)
1616	CC stretching (Tyrosine)
1668	Amid I (Albumin)

Figure 4. SERS spectrum of a 100 µL sample and Literature Raman vibrational mode assignments of semen.

and every Raman measurement involved finding the optimum focal plane for measurement by adjusting the stage to maximize signal to the detector. To acquire the initial SERS spectrum of seminal fluid using the swabs, a silver-modified swab was dipped into 100 µL of seminal fluid and analyzed immediately. The bands in the spectrum have been labeled to illustrate the correspondence to that of a literature Raman spectrum, in which bands and vibrational modes have been assigned based on data from other literature sources. Figure 5 shows the overlaid spectra of the seminal fluid SERS signal and nylon SERS signal that has been normalized. The SERS spectra of semen and nylon are similar as semen is a complicated mixture rich with proteins, and nylon is a synthetic protein. However, spectral comparison reveals differences, and the bands around 653 and 723 cm^{-1} can be used as indication for the presence of seminal fluid as they are absent in the nylon substrate. The band indicative of for choline (723 cm^{-1}), a constituent of a neurotransmitter present in semen, is especially significant as it's a unique chemical component to seminal fluid and has been used as a

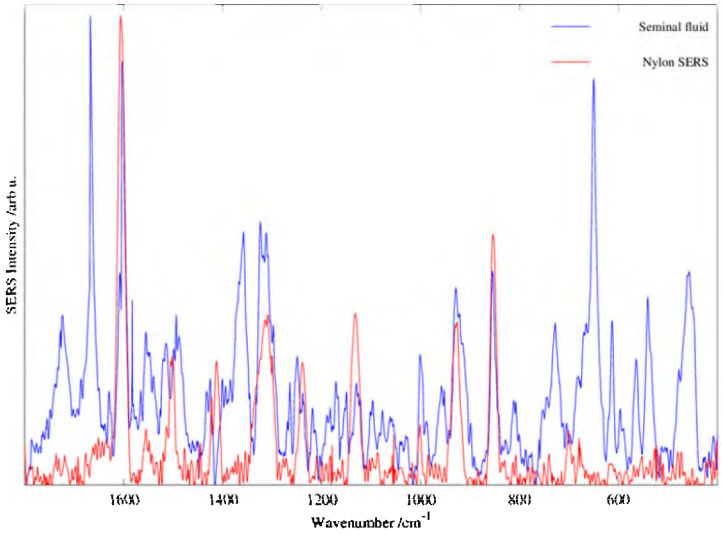


Figure 5. Overlaid spectra of 12 µL of semen with the SERS nylon

chemical identifier for the presence of semen in past forensic investigation techniques. Investigations into the smallest volume of seminal fluid which would reliably be swabbed and give SERS signal was determined to be 12 μL and the resulting spectrum is shown in Figure 5, which has also been normalized.

SERS-active forensic swabs have been shown previously to exhibit SERS enhancement of molecules such as Ru[bpy3]+2 and forensically relevant samples such as semen. However, studies are needed to determine whether swabs have an effect on the DNA workflow downstream. To probe the effect of the silver on the DNA extraction efficiency, a study was developed that compared the extraction of known concentration of DNA doped onto plain and silver-modified swabs. During the lysis step of extraction, it was observed that a significant amount of silver was leached from the modified swabs into the lysis buffer, essentially contaminating the lysate with silver species. As reported previously, low quantities of DNA were obtained from the SERS-active swabs, as shown in Figure 6A. Following extraction, purified DNA concentrations were assessed using the QuantFiler Trio DNA quantification kit form Life Technologies. This kit enables quantification of total human and total male DNA using a large autosomal (214 BP), small autosomal (80 BP), and Y chromosome target. To correct this, a centrifuge step and supernatant transfer to a clean tube were performed early in the extraction protocol to remove silver from the lysate. The results of quantification with this additional step is shown in Figure 6B. As can be seen, the DNA retrieved is similar between modified and unmodified swabs.

To determine whether swabs and/or the Raman measurement had an effect on the remainder of the DNA workflow downstream, the following study was carried out twice. In each instance, the measurements were carried out in triplicate. For each experiment, 20 SERS-active swabs were prepared using our previously described method. We also prepared 3-fold dilution series of human seminal fluid, creating 10 samples with calculated DNA concentrations ranging from 15 ng/μL – 0.7 pg/μL, based on previous extractions of the same semen sample. These ten concentrations were each doped (10 μL each) on 3 different swabs – a plain Copan 4N6 FLOQswabs and two of the SERS active swabs. After all semen samples had dried, 1 set of SERS-active swabs were exposed to a 632.8 nm laser during a standard measurement sequence (15 minutes for a full 9-point analysis),

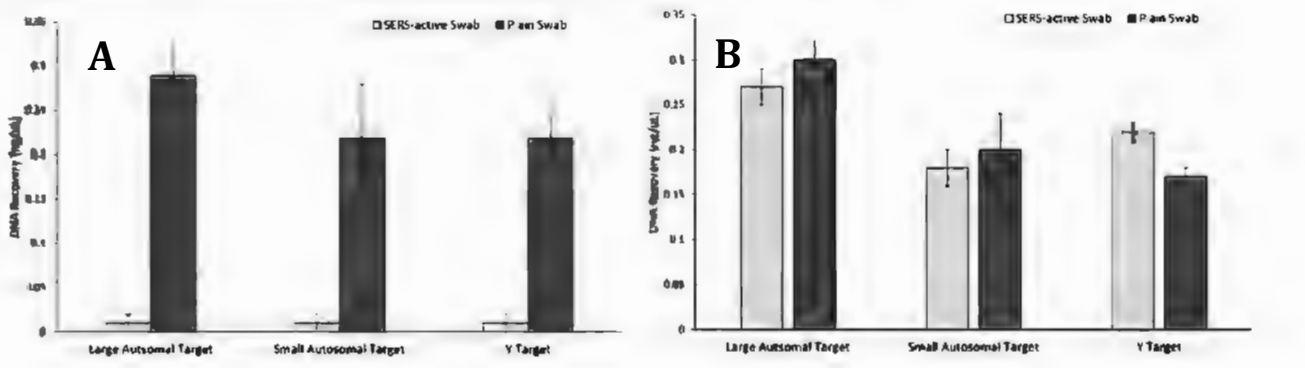


Figure 6. Quantitation of DNA with silver modified swabs and unmodified swabs without (A) and with (B) a supernatant transfer step added to the extraction protocol.

while the other set of SERS active swabs were not exposed to the laser. Next all 30 swabs were put through the workflow, in which DNA was extracted using the PrepFiler™ Forensic DNA Extraction kit, Quantified DNA using the Quantifiler™ Trio kit, and genotyped using the GlobalFiler™ kit. The data shown below are the results of the first of two triplicate experiments, thus a total of 90 swabs were used – 30 plain and 60 SERS-active swabs. The quantifiler Trio kit allows for simultaneous assessment of total human and human male DNA. As we are testing only semen, we would expect to see similar results from each of the three targets available, the male target, as well as the small (80 bp) and large (214 bp) autosomal targets. The results of quantification of the small autosomal target is shown in the figure below. Figure 7 shows the results of each of the ten concentrations used, labeled A – J. At each concentration, there are three reported values. The light grey bars show the results of quantification of DNA extracted from SERS-active swabs (labeled SERS+) and exposed to the Raman laser during a typical measurement (labeled Raman+). The hatched bars show the results of quantification of DNA extracted from SERS-active swabs that had not been exposed to the Raman laser (labeled Raman-), as well as the control experiment of extracting DNA from pristine swabs (black bars labeled as not having been SERS active swabs, nor exposed to the Raman laser. We were able to quantify DNA from all 10 of our concentrations with each swab. The error bars represent the standard deviation of the triplicate measurements. In each case, the three bars appear similar. Additionally, it does not appear as though there is a consistent decrease in DNA recovered when comparing the pristine to SERS-active swabs or when comparing laser exposure to none. In fact, a nonparametric Kruskal-Wallis Test was conducted and rendered a Chi-square value of 0.009. ($df = 2$, $N = 90$), $p > 0.05$ (0.995). The distribution of concentrations for the human small autosomal target are not significantly different. Though not

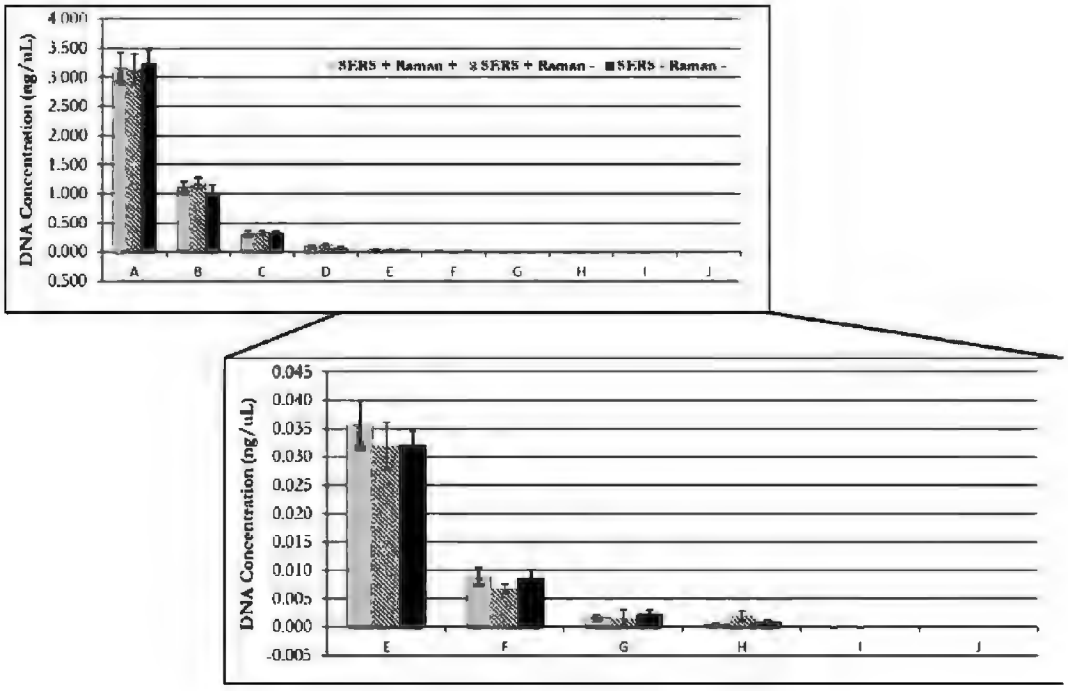


Figure 7. DNA quantification of the small autosomal target. DNA was extracted from pristine nylon swabs (SERS-Raman-), SERS-active swabs that had been exposed to the Raman laser (SERS+Raman+) and SERS-active swabs that had not been exposed to the Raman laser (SERS+Raman-).

plotted here, the large autosomal target and the male target showed similar results. For the large autosomal target the nonparametric Kruskal-Wallis Test was conducted and rendered a Chi-square value of 0.104 (df = 2, N = 90), $p > 0.05$ (0.949), such that the distributions of concentrations for the human large autosomal target are not significantly different. Likewise, for the male target, the nonparametric Kruskal-Wallis Test was conducted and rendered a Chi-square value of 0.005 (df = 2, N = 90), $p > 0.05$ (0.998), again indicating that the distributions of concentrations for the human male target are not significantly different.

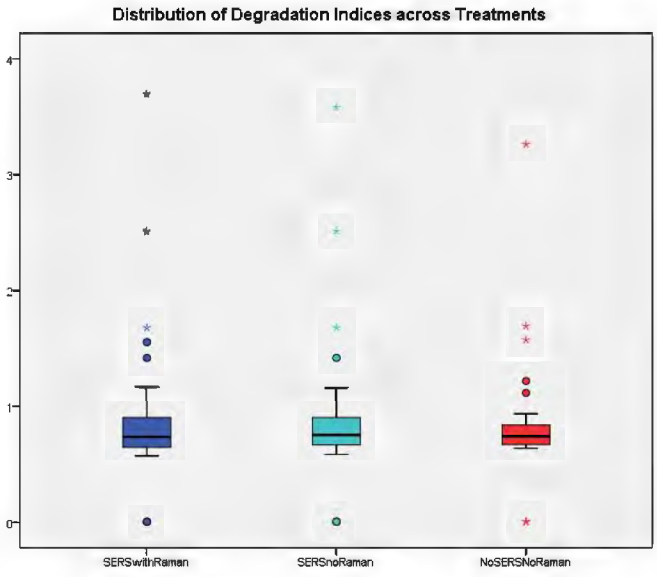


Figure 8. Comparison of Degradation Indices across swab treatments

As mentioned previously, an advantage to using the GlobalFiler Trio kit is the ability to assess DNA degradation. This was quite critical in that there were concerns that the exposure to either silver, laser, or both may have a degrading impact on the DNA present in the bodily fluid. To assess this question, a degradation index (DI) is calculated by dividing the concentration of the small autosomal target by the concentration of the large autosomal target. As seen in Figure 8, it appears that none of the treatments had a noticeable impact on

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the median degradation index, with each being less than 1, meaning no degradation. Generally, it appears that the inclusion of the SERS active swabs has perhaps a slight increase in the spread of the data, as the quartile boxes for the SERS swabs appears larger than that of the pristine swabs. Nevertheless, a nonparametric Kruskal-Wallis was conducted and rendered a Chi-square value of 0.267 ($df = 2$, $N = 90$), $p > 0.05$ (0.875). This would indicate that the D=distributions of degradation indices are not significantly different.

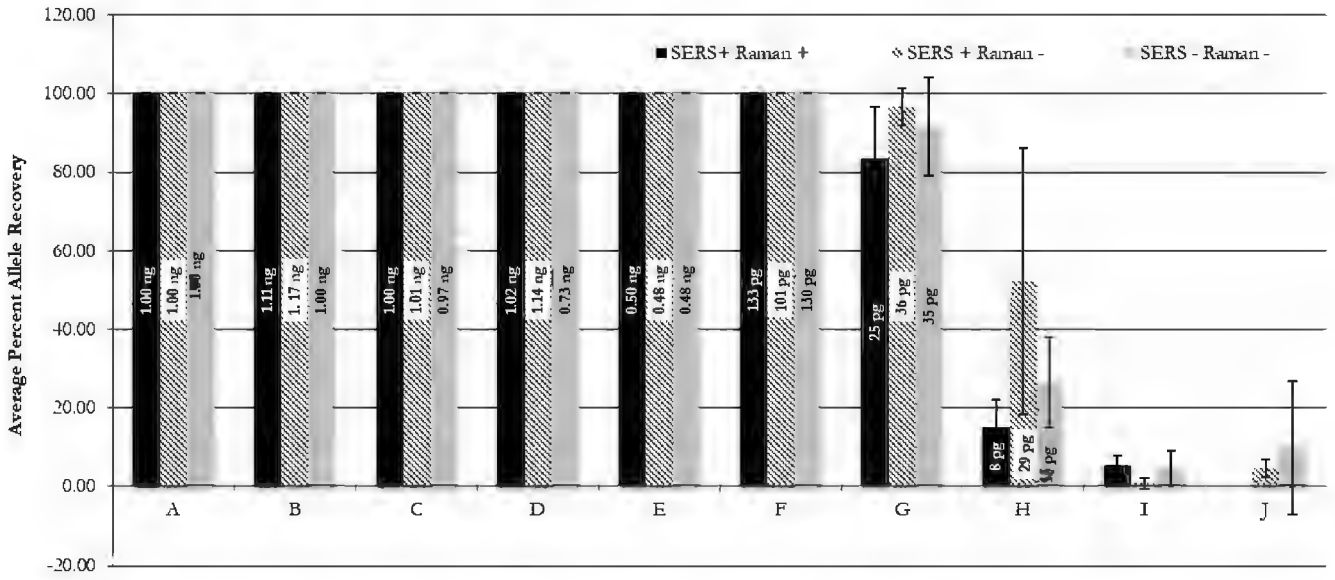

Figure 9. Comparison of Allele recovery during genotyping at each concentration (A – J) for the various swab treatments (Error bars are standard deviation of the triplicate averages).

Given the results of DNA quantification and the knowledge that the SERS swabs, nor the Raman measurement procedure is degrading DNA or inhibiting PCR, the results presented in Figure 9 are not particularly surprising. Allele recovery percentages for the different concentrations and swab treatments are presented. We found no evidence of the 'ski slope effect' in the electropherograms, providing further evidence that degradation of the DNA is not occurring. Likewise, in concentrations A – F, we received practically 100% allele recovery regardless of the swab type used. At the lowest concentrations, allele recovery dropped significantly for all three swab types. Given the extremely small concentrations this is not surprising and we mostly attribute this difference to variations in DNA input due to swab loading rather than due to issues with silver – DNA interactions.

Impact of the project on the criminal justice system

We believe that this project has confirmed our initial hypothesis that SERS-active swabs can be used to detect a human bodily fluid such as semen in a non-destructive way and allow for DNA genotyping without degradation or interference. As such, we believe the results of this project give further credence to the notion that Raman spectroscopy is a valuable tool in forensic science.

At this time, we are certainly not able to say that SERS-active swabs have the potential to be operationalized. Candidly, they are not without their problems. We were not able to solve the issue of silver transfer from the swab to the substrate of the sample to be swabbed. It seems unlikely that the forensic science community would embrace a technique that contaminates an evidentiary field of interest with a metal. Likewise, we have found the swabs to be difficult to reliably fabricate, which leads to issues of spectral data collection. Some researchers in our group had more success than others at fabrication, which leads us to suspect that minuscule levels of contamination can impact the resulting silver surface and thus the ability for SERS enhancement.

However, we believe that our biggest contribution to the forensic science community is likely our proof that the addition of metallic particles to an evidentiary sample is not detrimental to the genotyping of that same sample. Likewise, we proved that laser irradiation does not degrade HBF samples. While we initially set out to collect an evidentiary sample on a SERS active substrate, it may be that silver can be added to the swab post collection. This would eliminate the issue of contaminating the field. A concentrated mist of silver particles, for example, may be sprayed onto a swab, still allowing sample and silver to come into close contact. The addition of the silver immediately prior to Raman measurement would also alleviate contamination issues, as well as some of the issues with heterogeneity of SERS substrates.