3Dconfocal Raman microscopy potentialities for the nondestructive confirmatory forensic identification of semen and postcoital fluid: sexual assault simulation

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ABSTRACT

Background: Violence against women is a global crisis, with 30% of women aged 15+ experiencing sexual or physical violence. Forensic identification of body fluids like semen and vaginal fluid is critical for sexual assault investigations, but current methods are often destructive, costly, or time-consuming. Raman spectroscopy offers a promising alternative due to its non-destructive nature and high specificity for trace analysis. **Aim of the Work**: This study pioneers the use of Raman spectroscopy to characterize post-coital fluid (a natural mixture of semen and vaginal fluid) to simulate rape cases, while also evaluating spectral heterogeneity in semen from different donors. **Methodology:** Post-coital vaginal swabs (15 samples) and semen samples (15 samples) were collected from volunteers, dried on glass slides, and analyzed using a WiTec alpha300 R confocal Raman spectrometer (532 nm laser, 50 mW power). Data were processed via OriginLab for peak identification and spectral comparison. **Results:** Semen spectra exhibited distinctive peaks for choline (715 cm^{-1}), spermine phosphate (1065 cm^{-1}), and tyrosine (641, 1616 cm^{-1}). Post-coital fluid showed dominant urea/lactic acid peaks (577, 1445 cm^{-1}) with residual semen markers (715, 829 cm^{-1}), confirming mixed fluid detection. Donor variability in semen spectra was observed but did not obscure key biomarkers. **Conclusion:** Raman spectroscopy reliably identifies post-coital fluid through unique spectral signatures, combining semen and vaginal fluid markers. This non-destructive method holds potential for on-site forensic applications in sexual assault cases. Further studies should explore environmental and substrate effects.

Keywords: Raman spectroscopy; sexual assault investigations; post coital fluid identification ; Vaginal fluid; semen; Forensic body fluid analsis ; non-destructive forensic methods.

INTRODUCTION

Violence against women has profound implications for the state, victims/survivors, and society at large. Globally, an estimated 736 million women—approximately 30% of those aged 15 and older —have endured physical and/or sexual intimate partner violence, non-partner sexual violence, or both at least once in their lifetimes **(World Health Organization, 2021).** The associated costs can be categorized as tangible or intangible, as well as direct or indirect **(World Health Organization, 2021).**

In forensic science, the identification of bodily fluids and tissues plays a pivotal role, as it provides critical evidence for criminal investigations and aids judicial decision-making **(Sijen and Harbison, 2021).** Among these biological materials, semen is one of the most frequently encountered in forensic casework, particularly in sexual assault investigations **(Shaler, 2002).**

The identification of vaginal fluid, along with other biological fluids, is often essential in forensic investigations of rape and sexual assault **(Sikirzhytskaya et al., 2012).** However, current on-site detection methods for vaginal fluid remain limited to presumptive tests, which provide only preliminary findings **(Gaensslen, 1983).**

The evidentiary value of such samples is significantly strengthened when DNA profiling corroborates the presence of a specific bodily fluid or tissue **(Sijen and Harbison, 2021).** Consequently, it is imperative that all potential biological samples—including semen and vaginal fluid—are collected meticulously and preserved in their original state during initial forensic examination. These fluids can serve as critical evidence in linking a suspect to a crime or identifying a victim **(Best, 2002).**

Most conventional techniques for bodily fluid identification involve lengthy and destructive sample processing, which can compromise limited or trace evidence. When only a minimal quantity of a sample is available, non-destructive methods are essential to preserve material for subsequent analyses, such as forensic DNA typing **(Dissell, 2010).**

A notable limitation of DNA-based evidence is its high cost, as processing and storing samples in forensic laboratories demand substantial financial resources **(Dissell, 2010).** Consequently, there is a pressing need for rapid, non-destructive, and cost-effective analytical techniques that can accurately identify bodily fluids directly at crime scenes, thereby aiding forensic investigators **(Virkler and Lednev, 2009b).**

In this context, Raman spectroscopy has emerged as a promising tool over the past decade, gaining increasing traction in forensic applications due to its non-destructive nature, speed, and precision **(Macleod and Matousek, 2008).**

Raman spectroscopy is based on the principle of inelastic scattering of monochromatic, low-intensity laser radiation by molecular components in solid, liquid, or gaseous samples. A typical Raman spectrum provides a vibrational fingerprint of the sample, enabling the identification of unknown substances. This technique requires only minute quantities of material (as little as picograms or femtoliters) and is non-destructive, preserving samples for subsequent analyses **(Virkler and Lednev, 2009a).**

Recent studies have demonstrated the efficacy of Raman microspectroscopy combined with advanced statistical methods for the forensic identification of trace bodily fluids, including blood, saliva, semen, sweat, and vaginal secretions **(Virkler and Lednev, 2008).**

This study presents, for the first time, a Raman spectroscopic characterization of post-coital fluid to simulate forensic evidence in sexual assault cases. Samples were collected from multiple female donors following consensual intercourse to replicate biological evidence encountered in rape investigations.

The proposed method offers several advantages, including:

Non-destructive analysis (preservation of evidence)

High chemical specificity (accurate identification)

Trace-level detection (picogram/femtoliter sensitivity)

Minimal sample preparation (workflow efficiency)

Additionally, we evaluated the Raman signatures of seminal fluid from different donors to assess how biological variability influences spectral profiles. This investigation addresses a critical gap in forensic spectroscopy by characterizing both post-coital fluid heterogeneity and seminal fluid variability under controlled conditions.

MATERIAL AND METHODS

1 Sample Preparation

Sample Collection

Samples were collected from volunteers after obtaining informed consent. The sample size was determined using G*Power software, which established that 15 body fluids per group (totaling 30 samples) were suitable for the study's aims. Confidentiality was maintained by assigning anonymous codes to all participants.

Body fluids were divided into two groups: semen samples and post-coital vaginal samples. Each group consisted of 15 samples.

Inclusion and Exclusion Criteria

· Inclusion Criteria for Semen Donors: Male volunteers providing semen samples were included.

· Exclusion Criteria for Semen Donors: Any donor with identified semen abnormalities was excluded from the study.

· Inclusion Criteria for Post-coital Samples: Post-coital vaginal swabs were collected from female volunteers after intercourse (within one to six hours) and before douching. These samples were intended to represent the natural mixture of seminal and vaginal fluids and to simulate conditions relevant to sexual assault cases.

Sample Processing

For semen samples, 30 microliters of the sample were placed directly on a glass microscopic slide and left to dry overnight.

Post-coital vaginal swabs were collected using cotton swabs. The samples were then extracted and centrifuged. The resulting material was placed on glass microscopic slides and left to dry before further processing.

2 Raman Microscopy

A 3D confocal Raman imaging microscope with a 50x long-range objective (numerical apertures = 0.75) and a WiTec alpha 300 R confocal Raman spectrometer we conducted to examine the samples. The spectrometer was calibrated with a reference silicon sample, and the resulting readings had a resolution of 1 cm^{-1}. An excitation laser beam of 532 nm was employed. On the dried samples, the laser power was around 50 milliWatts (mW), and the excitation beam spot size was around 50 µm wide when operating in standard confocal mode. As a rule, a 30 milliseconds integration period and 20 accumulations were used to capture the Raman spectrum.

Before applying Raman spectroscopy, we searched the literature and previous results to find the fitting power used in body fluids. However, when this power was used with the WiTec alpha300 R confocal Raman spectrometer, we did not see any results indicating that the sample was burnt or the device was not detected. After trials we found the optimum WiTec Raman system in order to occupy them to identify the different body fluids.

For dried semen samples, and post-coital vaginal swabs, the laser power was approximately 50 mW.

3 Data Analysis

In order to process the data retrieved from the Raman Spectrometry, we used the OriginLab software. The peak analysis of the spectrum was done without any manipulation of the graph. All Raman spectra were processed in Origin Lab Software to eliminate cosmic ray noise. Significant peaks have been detected using the Peak Analyzer feature of the OriginLab software.

RESULTS

We collected the spectra of dry traces of the semen, and post-coital samples from the Raman spectrometer and then imported them to the OriginLab software for drawing a graph of body fluids and peak analysis. Each body fluid is composed of multiple distinctive chemical compositions, making it even more complex and heterogeneous. In order to determine the composition of each body fluid, the percentages of contributions from different mixtures were carefully chosen to identify the corresponding spectra and distinctive peaks. Analysis of the spectra from semen and post coital fluids was done through the shape of the spectrum and the series of unique peaks of each of them. we took the images captured from the charge-coupled device (CCD) camera of Raman Microscopy as viewed in Fig.1.

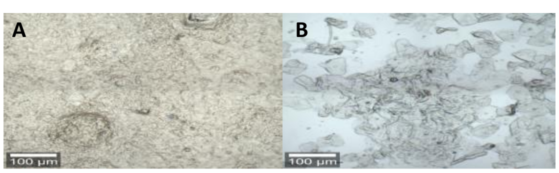

Figure 1 The dried traces of different body fluid traces captured by the CCD camera of Raman Microscopy A) semen B) post-coital vaginal swab.

Peak Analysis of the Raman spectra

Dry traces of semen

Fig. 2 shows the graph of the dry semen spectra and the y-axis represents the intensity of the scattered light, while the x-axis represents the Raman shift. The range of the wavenumber of the Raman shift was from 600-1700 cm^{-1} and that of intensity was from 700-1200 arbitrary units (a.u). The significant peaks are 641, 715, 759, 829, 983, 1003, 1065, 1125, 1264, 1448, and 1616 cm^{-1}.

The chemical assignment of each peak in the spectral analysis is shown in Table 1.

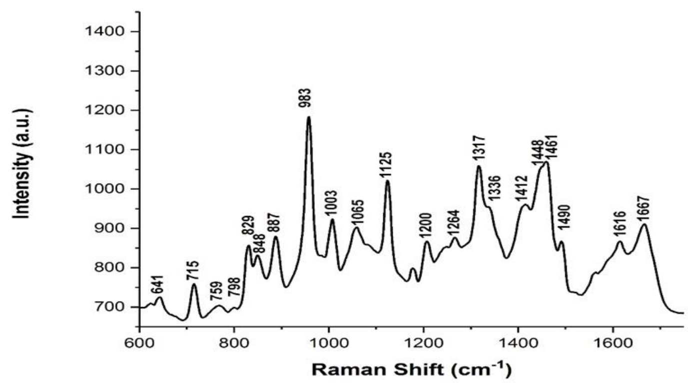

Figure 2 The peak analysis of the Raman spectra of the dry traces of semen.

Table 1: Characteristic Raman spectral peaks and their chemical assignments in pure semen and post-coital vaginal swab sample

Semen		Post-coital Vaginal Swab	
Raman Shift (cm^{-1})	**Chemical assignment**	**Raman Shift (cm-1)**	**Chemical assignment**
641	Tyrosine	481	Urea
715	Choline	577	Urea
759	Albumin	715	Choline
798	Tyrosine	830	Tyrosine , Fructose
848	Tyrosine , Monosaccharaides	853	Amino acids, Lactate
887	Spermine phosphate hexahydrate	930	Acetic Acid
983	Tyrosine	1082	Lactic Acid
1003	Albumin ,Phenylalanine	1250	Proteins
1065	Spermine phosphate hexahydrate	1310	Amide III
1125	Tyrosine , Amide III, Proteins,	1330	Amide III
	Spermine phosphate hexahydrate	1445	Lactic Acid
1200	Amide III	1610	Lactic Acid
1264	Fatty Acids		
1317	Spermine phosphate hexahydrate		
1336	Albumin		
1412	Lipids , Tryptophan		
1448	Albumin , Choline , Tryptophan		
1461	Spermine phosphate hexahydrate		
1490	Tyrosine		
1616	Tyrosine		
1667	Amide I		

(Virkler and Lednev, 2009b),(Casey and Mistek, 2020),(Muro et al., 2016),(Sikirzhytskaya et al., 2023),(Muro and Lednev, 2017),(Movasaghi et al., 2007),(Sikirzhytski et al., 2010),(Sikirzhytskaya et al., 2012),(Zou et al., 2016).

Dry traces of Post-coital vaginal samples

Fig 3. Illustrating the dry traces of post-coital samples with the intensity of the spectrum between 400-600 a.u. The three major peaks were 577, 1002, and 1445 cm^{-1}. The matching peaks with the semen spectrum were 715, and 829 cm^{-1}. We noticed the spectrum is particularly extinguished from the premixed previous samples.

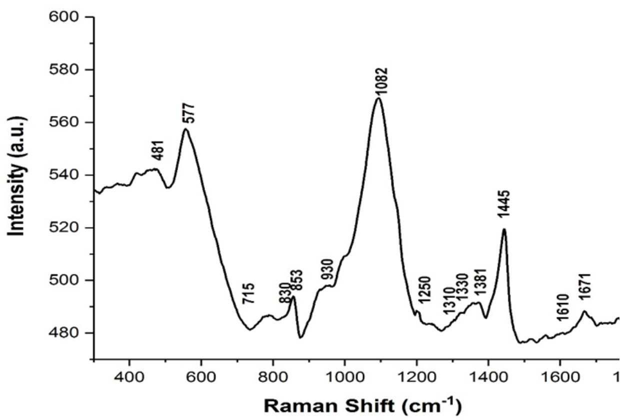

Figure 3 The peak analysis of the Raman spectra of the dry traces of post-coital vaginal swabs.

Series of unique peaks in semina samples and post coital samples

Fig 4. Showing the spectra of all volunteers combined with one graph. There are variations in the intensities of each spectrum.

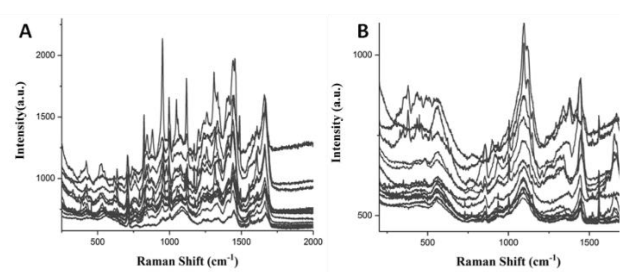

Figure 4. The Raman spectra of the different donors showed variations in intensities. A) Semen spectra, B) Post-coital vaginal swabs.

DISCUSSION

The main objective of this study was to determine the reliability of Raman spectroscopy in identification of human semen mixed with vaginal fluid in the post coital fluid and to find out how much variation present in its spectra from pure semen and pure vaginal fluid, in addition to the introduction of a new technique utilizing Raman microscopy for the detection of body fluids at a single point, rather than through comprehensive mapping.

The second objective of this study was to find out if there is variation in the spectra among different donors.

Based on previous study that was held by **(Virkler and Lednev, Forensic Sci. Int. 2009)** Raman spectroscopy is still a valid technique to determine whether a sample contains semen if there is minimal variation in the spectra between donors.

The utilization of portable Raman spectroscopy has the potential to assist in the detection of body fluids at crime scenes. The spectrum of the semen sample was heterogenic and signified diverse structures and peaks. Semen is a mix of cellular and non-cellular components with contributions of multiple glands in its secretion. The structure's complexity contributes to its heterogeneity **(Das et al., 2021)**, **(Virkler and Lednev, 2009b).**

We collected natural post-coital vaginal fluid by swabs, as both semen and vaginal fluid have a thick consistency. We believed that this mixture would enable us to identify the genuine peaks caused by the actual presence of seminal and vaginal fluid.

As Table 1. Clearly stated, the chemical assignment of the Raman band is revealed in the semen spectrum. There was the existence of choline, spermine, tyrosine, albumin, Amide I, Amide III, tryptophan, protein, and fatty acids. Along with the shape of the spectrum and the chemical assignment of the semen, these results were consistent with the preceding studies done on semen by Virkler and Lednev as they reported that the mixture of three main components: a component consistent with spermine phosphate hexahydrate, a component consistent with tyrosine, and a component including a protein and potentially choline. can be employed as a special spectroscopic signature to determine whether semen is present **(Virkler and Lednev, 2009b).**

Muro et al. stated in their research that the first strong peak, found at 715 cm^{-1}, is due to C–N symmetric stretching in choline **(Muro et al., 2016)** . The choline assignment found at that peak was also consistent with many published studies, including those by **Casey & Mistek. (2020), Sikirzhytskaya et al. (2012), Sikirzhytskaya et al., (2023)** , **Muro & Lednev. (2017)** and **Movasaghi et al. (2007).**

The spermine phosphate hexahydrate (SPH) gave rise to peaks at 887, 1065, 1317, and 1461 cm-1. Those peaks assigned to spermine were in agreement with **Sikirzhytskaya et al. (2012), Sikirzhytskaya et al., (2023).**

In semen samples, tyrosine was significantly found at 641, 798, 848, 983, 1125, 1490, and 1616 cm^{-1}. Previous researchers agreed with these findings**(Casey and Mistek, 2020), (Sikirzhytskaya et al., 2023), (Sikirzhytski et al., 2010), (Muro and Lednev, 2017).**

In post-coital vaginal swabs, tyrosine was pronounced in less peaks at 1179, and 830 cm^{-1} respectively. **Sikirzhytskaya et al.** studied the Raman signature of vaginal fluid and did not mention tyrosine in the chemical assignment of the Raman bands **(Sikirzhytskaya et al., 2012)**. **Sikirzhytskaya et al.** stated that Proteins, urea, and lactic acid were allocated spectral components by the ALS approach, and these contributions to the major components were the largest. High goodness-of-fit findings from the statistical analysis were obtained for this spectroscopic signature, which could be fitted to all the dry vaginal fluid samples and aid in the separation of vaginal fluid from other bodily fluids discovered at crime scenes.

Semen samples exhibited high levels of albumin, while albumin could not be detected in post-coital vaginal swabs.

Tryptophan and phenylalanine were observed in the Raman bands of semen at 1412, 1448, and 1003 cm^{-1}. In post-coital vaginal swabs, we didn't observe peaks for tryptophan or phenylalanine, and this was in disagreement with the research done by **Sikirzhytskaya et al.** that concluded there was the peak at 1002 cm^{-1} in the vaginal fluid related to phenylalanine and urea **(Sikirzhytskaya et al., 2023) .** The lack of these peaks can be ascribed to a modification in the post-coital vaginal swabs that deviated from pure vaginal fluid.

The post-coital vaginal swabs showed domination of urea, lactic acid, and acetic acid with the contribution of tyrosine, choline, fructose, and Amide III proteins. The post-coital vaginal samples had a unique spectrum and showed the same pattern in different subjects. Urea, lactic acid, acetic acid, and proteins were assigned by **Muro et al.** as the main components of vaginal fluid **(Muro et al., 2016)**.

The tyrosine, fructose, and choline peaks were identified in the post-coital vaginal fluid, and the altered spectrum of the post-coital vaginal swab could aid in distinguishing between vaginal fluid and post-coital vaginal fluid **(Muro et al., 2016) , (Sikirzhytskaya et al., 2012)**

CONCLUSION

This study found that the active peaks in semen stains detected by spectral analysis were associated with choline, spermine phosphate hexahydrate and tyrosine, and that the shapes of the spectra in each body mixture were altered by examining individual points in the samples. In particular, distinct peaks associated with post-coital vaginal swabs were observed in the spectrum. By combining the study of their series peaks with a thorough review of the chemical assignment of the Raman shift, it was possible to identify the post coital fluid.

RECOMMENDATIONS AND LIMITATIONS

We recommend the use of different environmental conditions, different substrates, and different concentrations of body fluids. In addition, we recommend using these techniques for the natural combination of body fluids in different circumstances and studying the significance and causes of variations in the intensities of each body fluid from different donors.

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الملخص العربي

الإمكانات التحليلية لمجهر رامان متحد البؤر ثلاثي الأبعاد للتعرّف المؤكد وغير المتلف على السائل المنوي و سائل ما بعد الجماع: محاكاة لحالات الاعتداء الجنسي

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تُعد تقنية مطيافية رامان من الأساليب الواعدة في مجال تحديد العينات الجنائية، ولا سيما سوائل الجسم التي يتم العثور عليها في مسارح الجريمة. وتُعدّ معرفة وجود هذه السوائل أمراً بالغ الأهمية لإجراء اختبارات الحمض النووي اللاحقة بهدف التعرف على الجناة المحتملين. ونظراً للطبيعة الخاصة لحالات الاعتداء الجنسي، فإن احتمال اختلاط السوائل الجسدية وارد بشكل كبير، مما يجعل من الضروري التمكن من تحديد هذه السوائل بدقة. وقد هدفت دراستنا إلى تقييم فعالية المجهرية الرامانية في الكشف عن أدلة وجود السائل المنوي في عينات المسحات المهبلية المأخوذة بعد الجماع. ومن خلال مطابقة القمم الطيفية مع التكوينات الكيميائية لسوائل الجسم، وجدنا أن مكونات السائل المنوي كانت موجودة في جميع العينات المختلطة. علاوة على ذلك، قد تُعد شدة القمم الطيفية مؤشراً على نوع السائل الجسدي الموجود. وتُثبت هذه التقنية قيمتها كأداة فعّالة في التحقيقات الجنائية لما تتمتع به من موثوقية في الكشف عن سوائل الجسم والحفاظ عليها لمزيد من التحليل.