Raman spectroscopic signature of semen and its potential application to forensic body fluid identification

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ABSTRACT

A great potential of Raman spectroscopy for non-destructive, confirmatory identification of body fluids at the crime scene has been reported recently (Virkler and Lednev, *Forensic Sci. Int.* 2008 [5]). However, that analysis was carried out on only one sample of each body fluid and did not take into account any variations that might occur between different donors of the same fluid. This paper reports on the role of heterogeneity within a sample as well as among multiple donors for human semen. Near-infrared (NIR) Raman spectroscopy was used to measure spectra of pure dried human semen samples from multiple donors in a controlled laboratory environment. The major chemical components that contributed to the Raman spectrum of semen were determined and used to tentatively identify the principal spectral components. The issue of potential spectral variations that could arise between different donors of semen was also addressed. Advanced statistical analysis of spectra obtained from multiple spots on dry samples showed that dry semen is heterogeneous and its Raman spectra could be presented as a linear combination of a fluorescent background and three spectral components. The relative contribution of each of the three components varies with donor, so no single spectrum could effectively represent an experimental Raman spectrum of dry semen in a quantitative way. The combination of the three spectral components could be considered to be a spectroscopic signature for semen. This proof-of-concept approach shows the potential for Raman spectroscopy to identify an unknown substance to be semen during forensic analysis.

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1. Introduction

The ability to identify traces of body fluids discovered at crime scenes is a very important aspect of forensic investigations [1–3]. With DNA analysis being one of the most popular and informative forensic techniques, it is imperative that any potential body fluid sample is properly collected and not destroyed during the initial identification process. Fluids such as blood, semen, saliva, and vaginal fluid can be very useful in identifying a victim or suspect [4], and they can also help answer questions regarding the events of a crime. An analytical technique that could identify a particular body fluid rapidly, simply, and non-destructively at the scene of a crime would be a valuable tool for forensic investigators.

We have recently reported that Raman spectroscopy can be potentially used to distinguish different body fluids [5] as well as provide non-destructive, confirmatory identification of body fluids at the scene of a crime [6]. However, this analysis was carried out on only one sample of each body fluid and did not take into account

any variations that might occur between different donors of the same fluid. Since each donor's sample is heterogeneous within itself due to many different chemical components, we would also like to investigate the effect these chemical components have on the Raman spectral components of a body fluid. This paper investigates the role of heterogeneity within a sample as well as among multiple donors for human semen.

In addition to blood, semen is one of the most prevalent body fluids found during criminal investigations, especially in cases involving sexual assault [2]. There are currently several tests, both presumptive and confirmatory, that can be used to identify an unknown fluid found at a crime scene to be semen [6]. An alternate light source (ALS) can be used as a finding aid to detect certain biological materials, including semen [1,7]. Some commercial ALS instruments have been developed such as the Wood's Lamp [8], Bluemaxx™ BM500 [9], Polilight® [10], and the Lumatec Super-light 400 [11], but these are not exclusive to semen identification and can only be used for initial detection. Popular presumptive tests for seminal acid phosphatase (SAP) [1,12] are much more reliable, but they are destructive to the sample and there is still some potential for false positive results. The most widely used methods for confirmatory identification of semen include the

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microscopic visualization of sperm cells using specific stains and immunological tests for prostate-specific antigen (PSA) [1,7]. The staining method will of course not be helpful if the donor is azoospermic, so this technique has limited applications including having to perform the test in a laboratory. Several commercial PSA test kits which can be used at a crime scene have been developed including Biosign® PSA [13], OneStep ABAcard® [14], Chembio, Medpro, Onco-screen [15], PSA-check-1, Seratec® PSA Semiquant [14], and SMITEST [16,17]. Like with the presumptive tests, these PSA test kits do show false positive results and are destructive to the sample. The test for PSA can even be considered as non-confirmatory since there is a potential for a rare false positive result in the presence of blood coming from women that have certain cancers [18].

In most cases there is likely a large amount of unknown sample to be analyzed, so the need for an identification test to be non-destructive might not seem imperative. However, sometimes a very small amount of semen evidence can solve a case if examined properly, so it is crucial that the minute portion of available evidence is processed efficiently and non-destructively so that further analysis, including DNA typing, can be performed [19]. Another issue is the potential of false positive results with current identification techniques [20]. The current easy-to-use test kits do not absolutely confirm the presence of semen either in pure form or as part of a stain. The forensic community is in great need of a reproducible, non-destructive, and portable method that can exclusively identify the presence of semen at a crime scene and distinguish it from other body fluids.

Raman spectroscopy is a forensic technique that has increased in popularity over the last several years [21,22], and it can be used to gain information about the structure and properties of materials based on their vibrational transitions [23]. Some applications being used today involve the identification of fibers [24], drugs [25], and lipsticks [26], as well as ink [27], paint [28], and condom lubricant [29] analysis. The theory behind Raman spectroscopy involves the inelastic scattering of a low-intensity, monochromatic, and non-destructive laser light by a solid, liquid or gas sample. There is little to no sample preparation, and no reagents are needed for analysis. Most importantly, the required amount of sample needed for Raman analysis can be as low as several picograms or femtoliters, and the sample will not be destroyed so that further analysis can still be performed. A typical Raman spectrum reveals a specific vibrational signature of the sample being measured based on the energy of the scattered light, and this feature is very useful in identifying an unknown substance. Raman spectroscopy is also very appropriate for the analysis of disordered and heterogeneous samples [30] which are common properties of body fluids. Finally, Raman spectroscopy shows very little interference from water [31] which makes it a great technique for analyzing body fluids and their traces. Portable Raman spectrometers are available now [32,33], and these designs along with advanced software could be applied to the identification of semen at a crime scene.

To our knowledge, there have been no publications of any experiments involving the identification of semen using Raman spectroscopy. The objective of this study is to determine the heterogeneity of a dried semen sample from one donor as well as analyze the qualitative variation among samples from different donors using NIR Raman spectroscopy. It is important to emphasize that the samples being measured are in pure form and do not represent an actual crime scene situation involving mixtures, dilutions, or substrate contamination. This is a proof-of-concept study, and more investigation is needed to determine the level of real-life applicability of this method. Our hypothesis was that human semen is both chemically and spectrally hetero-geneous causing spectra measured from different spots in the same sample to be significantly different. This variation means that no

single library spectrum of human semen can exist. Instead, a spectroscopic signature must be developed which is a linear combination of the multiple spectral components in semen, and these components can be identified based on the known chemical composition of semen. The characterized principal components could then be used in a "multi-dimensional analysis" of dried semen as opposed to a "single-dimensional analysis" which only involves the comparison of a single average library spectrum. The principal components found from the basis semen sample will be fitted to the average dried semen spectrum obtained from multiple donors to illustrate the capability of a unique spectroscopic signature to be applied to all semen samples. We report here the NIR Raman dried semen component spectra found by analyzing a single semen sample as well as the spectra obtained from analyzing dried semen samples from many donors. Preliminary assignments of major Raman peaks and possible identities of the semen components were made based on literature data.

2. Materials and methods

2.1. Samples

A set of 50 semen samples was obtained from anonymous donors at an in vitro fertilization clinic. One sample was randomly chosen to be a basis sample that would yield the spectroscopic signature, and this signature would be fitted to the remaining samples for comparison. A small 10 μL drop of the basis sample was placed on a circular glass slide designed for use with an automatic mapping stage and allowed to dry completely. The sample was analyzed using automatic mapping that scanned a sample area of 75 μm × 75 μm and measured Raman spectra from 36 random points within the area with six 10-s accumulations at each point. The spectra obtained from this sample were used to determine the number and possible identities of the principal components of semen and to develop the spectroscopic signature. Any of the 50 semen samples could have been chosen as the basis with similar results expected. The remaining samples were also measured using automatic mapping in a similar manner.

2.2. Raman microscope

A Renishaw inVia confocal Raman spectrometer equipped with a research-grade Leica microscope, 20× long-range objective (numerical aperture of 0.35), and WiRE 2.0 software were used. For the automatic mapping, the lower plate of a Nanonics AFM MultiView 1000 system was set up under the microscope, and measurements were taken using Quartz II and QuartzSpec software. A 785-nm laser light was utilized for excitation. The laser power on the dried samples was about 115 mW, and the spot size of the excitation beam was about 5 μm wide using standard confocality mode. The spectral resolution is about 3.5 cm^{-1}, and peak accuracy was assured by calibration with a silicon standard.

2.3. Data treatment

All of the spectra obtained from the automatic mapping of the dried semen samples were first treated using GRAMS/AI 7.01 software to remove any cosmic ray interference. The spectra were then imported into MATLAB 7.4.0 for statistical analysis and normalized to adjust for the varying amount of background interference in each spectrum. The number of principal components in the basis sample was determined using significant factor analysis (SFA), and the individual component spectra were extracted using the alternate least squares (ALS) function. The components found in the original basis sample were used to create a spectroscopic signature, and this signature was fitted to each average spectrum found from the remaining semen samples. The Curve Fitting Toolbox in MATLAB was used to perform residual analysis on the difference between the fitted and experimental spectra, and "goodness-of-fit" statistics were calculated based on how well the signature matched the experimental spectrum.

3. Results and discussions

3.1. Main approach

The main goals of this study were to determine the level of spectral heterogeneity of human semen based on principal components and to find out how much variation there is in the spectra from different donors. If there is very little change in the spectrum from one donor to another, then Raman spectroscopy can be considered to be a reliable method to identify a sample as semen

based on the application of a calculated spectroscopic signature. This signature, which could be fitted to a semen sample collected from any donor, could be produced based on several spectral components found in semen that are present due to the heterogeneous distribution of the many chemical species in semen. A unique signature can ultimately be developed for other body fluids as well so that an unknown body fluid discovered at a crime scene could potentially be identified in a confirmatory manner.

3.2. Single sample heterogeneity

A single semen sample, called *basis* hereafter, was used to develop the spectroscopic signature that would be applied to all samples. Raman spectra acquired at 36 random points on the basis sample were imported into MATLAB, and SFA analysis [34,35] was performed to determine the number of principal components that were present. The results of this analysis (data not shown) indicated six principal components. The ALS function [34,35] was applied to extract the spectra of each of these components, and further examination of the results revealed that there were actually only three unique components that were spectral representations of the chemical species in the semen sample. The remaining components consisted of background fluorescence, a virtual duplicate of one of the three real components, and a component that appeared to just be noise. Fig. 1 shows the average spectrum of the basis sample along with the three principal components. The wavenumber range of 670–1750 cm^{-1} is shown in the figure and will be used to create the spectroscopic signature since this is the region that contains most of the important characteristic peaks. The major Raman peaks that define each component are labeled and are listed in Table 1. Vibrational modes for each peak are also listed in Table 1 based on literature data.

The spectral components of semen are complex, and some have contribution from multiple chemical species. According to several literature sources [36–38], some of the chemical components of semen that are present in the highest concentrations are fructose, choline, spermine, citric acid, acid phosphatase, and albumin. Other chemical components of lower abundance are glucose, inositol, lactic acid, and urea. There are of course many other substances present in semen, but for the purposes of the principal component assignments, they are not as important due to their low Raman scattering ability. It is also important to mention the fact that there are chemical changes that happen after the ejaculation process occurs [38] so the assignments of these components are tentative.
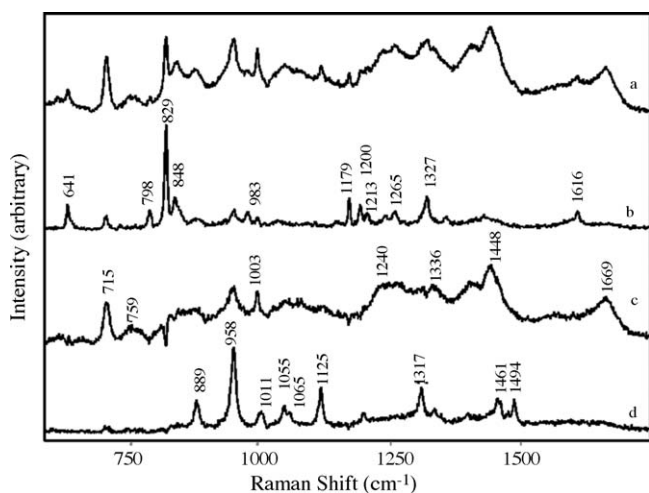

Fig. 1. The average Raman spectrum of the basis semen sample (a), and the Raman spectra of semen spectral components 1 (b), 2 (c), and 3 (d) with major peaks labeled.

Table 1
Raman assignments of dried semen.

Raman shift (cm^{-1})	Spectral component	Vibrational mode
641	1	Ring deformation [62]
715	2	CN stretching [50]
759	2	Ring vibrations (Trp) [63]
798	1	CH$_2$ deformations in ring [62]
829	1	Ring breathing [39,62]
848	1	Ring bending [62,64]
888	3	Phosphate mode [52]
958	3	PO$_4^{3-}$ sym. stretching [53,65]
983	1	CH$_2$ wagging [62]
1003	2	Aromatic ring breathing (Phe) [47]
1011	3	CC stretching [52,62]
1055	3	CN sym. stretching [52]
1065	3	PO$_4^{3-}$ asym. stretching [53,65]
1125	3	CN asym. stretching [52]
1179	1	CH$_2$/NH$_3$ rocking [62]
1200	1	CC stretching [64]
1213	1	CH$_2$ twist and rock [62]
1240	2	Amid III [48]
1265	1	Sym. ring deformation [66]
1317	3	CH vibration [62]
1327	1	Ring stretching [62]
1336	2	CH bending (Trp) [40,48]
1448	2	CH$_2$, CH$_3$ bend (Trp) [31]
1461	3	CH$_2$ bending [31]
1494	3	NH$_3$ sym. bending [53]
1616	1	CC stretching [64]
1668	2	Amid I [48]

Despite the previously mentioned list of dominant chemical species, component 1 shown in Fig. 1 is consistent with the amino acid tyrosine. The peaks at 641, 798, 829, 848, 983, 1179, 1200, 1213, 1265, 1327, and 1616 cm^{-1} are almost exactly the same as peaks depicted in literature sources on the Raman spectrum of tyrosine [39,40]. There are also residual peaks from other chemical species, but these are more dominant in the other two components. It is surprising that a single amino acid would be found to be one of the major principal spectral components of semen instead of a more complex chemical compound, but of the free amino acids known to be in semen, tyrosine is one of the most abundant [41], and its concentration has been shown to increase over time as semen dries [42]. The aromatic ring of the tyrosine side chain is also known to be highly Raman active [43]. In addition, phosphorylated tyrosine residues have been reported to have a protecting effect on the membranes of sperm cells and help stabilize lipids [44], so an abundance of tyrosine as a free amino acid in semen is practical. Finally, it has been found that tyrosine phosphorylation occurs when sperm undergo capacitation which is necessary before fertilization [45]. This assignment is preliminary and not conclusive, and more investigation is needed.

It is obvious at first glance that component 2 is dominated by the polypeptide backbone of a protein due to the presence of amid I and amid III [46] peaks at 1668 cm^{-1} and 1240 cm^{-1}, respectively. It has been reported that the protein albumin contributes about one-third of the total protein content of semen [37], therefore this is a possible assignment as a contributor to component 2. Comparison of literature data on the Raman spectrum of albumin supports this conclusion [47,48], with matching peaks occurring around 759, 1003, 1336, and 1448 cm^{-1} in addition to the amid I and III peaks already mentioned. It is also possible that the enzyme acid phosphatase is contributing to this component due to its protein qualities and large abundance in semen, but there is not much literature data available to compare with, so this assignment is uncertain. At this stage in the investigation, the major contributor of this component will just be referred to as a protein. Finally, choline also appears to be present in component 2. The large peak at 715 cm^{-1} is consistent with the C–N symmetric
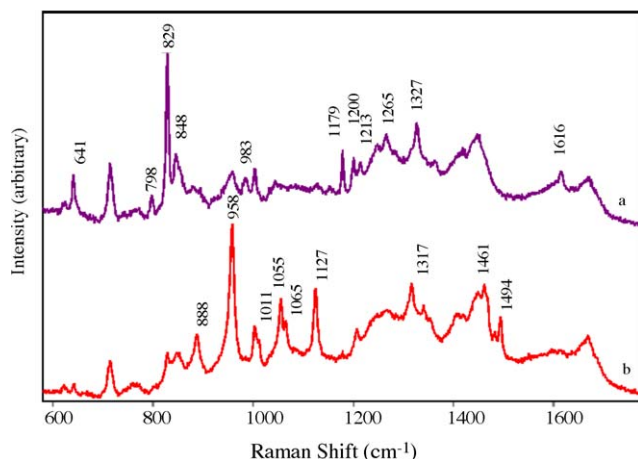
Fig. 2. A dry trace of semen is strongly heterogeneous. Raman spectra acquired from different spots in the same dried semen sample dominated by component 1 (a) and component 3 (b).
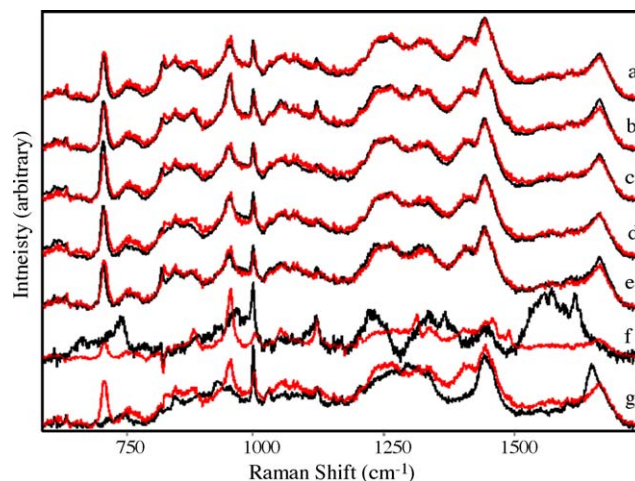

Fig. 3. The average Raman spectra of five semen samples (black) with the fitted spectroscopic signature (a–e), and the Raman spectra of blood (f) and saliva (g) with the fitted spectroscopic signature.

stretch found in choline which has been previously reported [49–51]. It is also likely that the CH_2 scissoring in choline is contributing to the peak at 1448 cm^{-1} [50]. This peak is very large in component 2 so it is probable that more than one chemical species is contributing to it. As with component 1, there are other peaks present that are much stronger in the other two components, so they are not considered to be dominating in component 2.

Component 3 appears to oppose component 1 when comparing spectra from one donor collected from different spots. When the peaks present in component 1 are strong, the peaks for component 3 are weak and vice versa (Fig. 2). The chemical contributor to component 3 appears to be fairly simple like with the case of component 1 (Fig. 1). The spectrum for component 3 is consistent with spermine phosphate hexahydrate (SPH) that has previously been reported [52,53]. The peaks found in component 3 at 888, 958, 1011, 1055, 1065, 1125, 1317, 1461, and 1494 cm^{-1} all appear in the known spectrum of SPH and are listed in Table 1 with vibrational assignments. As previously mentioned, spermine is present in large concentrations in human semen, and it has even been the basis of forensic semen identification in the past [54–57]. The basic nature of spermine causes it to interact with the phosphoric acid groups of nucleic acids and form strong bonds [52,53]. This binding leads to a precipitation of SPH in semen [58]. These crystals were first observed by van Leeuwenhoek [59], and they have since been observed in semen as the fluid begins to dry [60]. These properties regarding the presence of SPH in semen make it an understandable assignment of component 3, but this is not certain and more investigation is needed. As with the other two principal components of semen, component 3 contains residual peaks from chemical species which are more dominant in components 1 and 2.

3.3. Multiple donors

The second objective of this study was to determine the amount of spectral variation from one semen donor to another. The first step was qualitative in nature and involved the visual comparison of the average spectrum from each of the different donors. All of the spectra appeared to be very similar and contained all of the same major peaks. There were changes in the intensity of some peaks for different donors, but this is expected since the relative contribution of the chemical species in semen will likely change with each donor and can even change within the same donor [38]. Fig. 3 shows the average spectra of five semen samples (black lines)

from different donors as an example of their similarities. To demonstrate the spectral differences between body fluids, the spectra of blood and saliva that we have previously reported [5] are also included in this figure. Unlike the slight intensity changes within the semen samples, blood and saliva have major peak differences which make them distinguishable. The other features of Fig. 3 will be discussed in more detail in the following paragraphs.

After achieving visual confirmation that there is consistency among the spectra of semen from different donors, a more quantitative approach was developed. A spectroscopic signature was created that consisted of the three principal components found in the basis semen sample along with a horizontal line and a line with a slope equal to that of the fluorescence background. These five basis spectra were linearly fitted to the average basis semen spectrum, and the two spectra overlapped very well. The spectroscopic signature was also applied to the spectra from each of the remaining 49 semen samples to determine if it could universally be fitted to a sample from any donor. Fig. 3 shows the fitting to only five representative semen samples, but all of the samples had very similar fits. The bottom of Fig. 3 contains the results of fitting the spectroscopic signature to the spectra of blood and saliva, and it is visually obvious that they are very poor matches. These results qualitatively show the specificity of this signature to semen and its potential ability to be used as an identification technique for forensic purposes.

A quantitative statistical analysis was performed to determine how well the spectroscopic signature fit the experimental spectra. Using the Curve Fitting Toolbox in MATLAB, the intensity values for the basis experimental spectrum and fitted spectrum were plotted on an axis as the x- and y-coordinates, respectively. All of the spectra were normalized to a maximum value of 1, so that is the highest value for both the x- and y-axis. Two identical spectra would yield a scatter plot matching a line with the equation of $y = x$, so this line was used for comparison and was fit to the plotted data points of the basis sample evaluation to determine how close of a match the experimental spectrum and signature were. The statistical result was three quantitative goodness-of-fit values which statistically confirmed the qualitative match of the experimental and fitted spectra (Table 2). These values are the sum of squares due to error (SSE), R-square, and root mean squared error (RMSE). The SSE value measures the total deviation of the data points from the $y = x$ line, and a value closer to 0 means there are fewer random errors [61]. The R-square value indicates how

Table 2
Goodness-of-fit statistical results for semen signature fitting.

Sample	SSE	R-square	RMSE
Semen basis	0.0556	0.998	0.00630
1	0.299	0.990	0.0146
2	0.379	0.982	0.0164
3	0.450	0.979	0.0179
4	0.311	0.981	0.0149
5	0.319	0.979	0.0151
6	0.200	0.992	0.0119
7	0.324	0.987	0.0152
8	0.164	0.996	0.0108
9	0.161	0.992	0.0107
10	0.189	0.989	0.0116
11	0.107	0.996	0.00875
12	0.111	0.995	0.00888
13	0.122	0.995	0.00932
14	0.107	0.996	0.00875
15	0.281	0.987	0.0142
16	0.191	0.991	0.0117
17	0.460	0.984	0.0181
18	0.0645	0.999	0.00678
19	0.251	0.990	0.0134
20	0.0736	0.998	0.00725
21	0.118	0.996	0.00919
22	0.220	0.991	0.0125
23	0.0853	0.998	0.00780
24	0.174	0.993	0.0112
25	0.177	0.993	0.0112
Blood	1.38	0.967	0.0313
Saliva	2.25	0.822	0.0401

well the $y = x$ best fit line explains variation in the data, and a value closer to 1 indicates that a higher proportion of the variance is accounted for by the line [61]. A value closer to 1 also means that the fitted signature and experimental spectrum are a better match. Finally, the RMSE value estimates the standard deviation of the random data components. Again, a value closer to 0 indicates that the $y = x$ line is a better fit [61], and that the signature better fits the experimental spectrum.

This same fitting procedure was performed for the remaining 49 samples. The graphical result of a typical fit is shown for sample 22 in Fig. 4A as an example. The top half is a graph showing the fit of the line $y = x$ to the comparison of the signature and sample, and the bottom half is a plot of the residuals which are found by subtracting the best fit line from the scatter plot. A residual plot with random points around 0 that do not form a pattern indicates a good fit [61], as is the case here. The results for SSE, R-square, and RMSE for 25 of the 50 samples are shown in Table 2. As expected, the basis sample results indicated the best match since that sample was the template for the spectroscopic signature determination, but the statistical values for all of the samples are very close and fall within a certain range that suggests a good fit. To put in perspective how well the signature fits the semen samples, it was also applied to the spectra of human blood and saliva which we have already reported [5]. The goodness-of-fit statistics for those fits are listed at the bottom of Table 2, and it is easy to see how poorly the semen signature matches the spectra of the other two body fluids when all three statistics are taken into account. The R-square value for the semen signature fit to blood is not too much different than the average fit to semen, but the SSE and RMSE values definitely indicate a much worse fit. The visual results of the signature fit to the saliva sample are also shown in Fig. 4B. There is a large amount of disagreement between the best fit line and scatter plot in the top graph, and there is an obvious pattern of digression away from 0 in the residual plot.

As revealed in Figs. 3 and 4, and Table 2, the semen signature closely matches the experimental semen spectra and clearly does not fit the blood or saliva spectra. This result shows that a spectroscopic signature created from one basis semen sample can be fitted to multiple other semen samples from different donors,
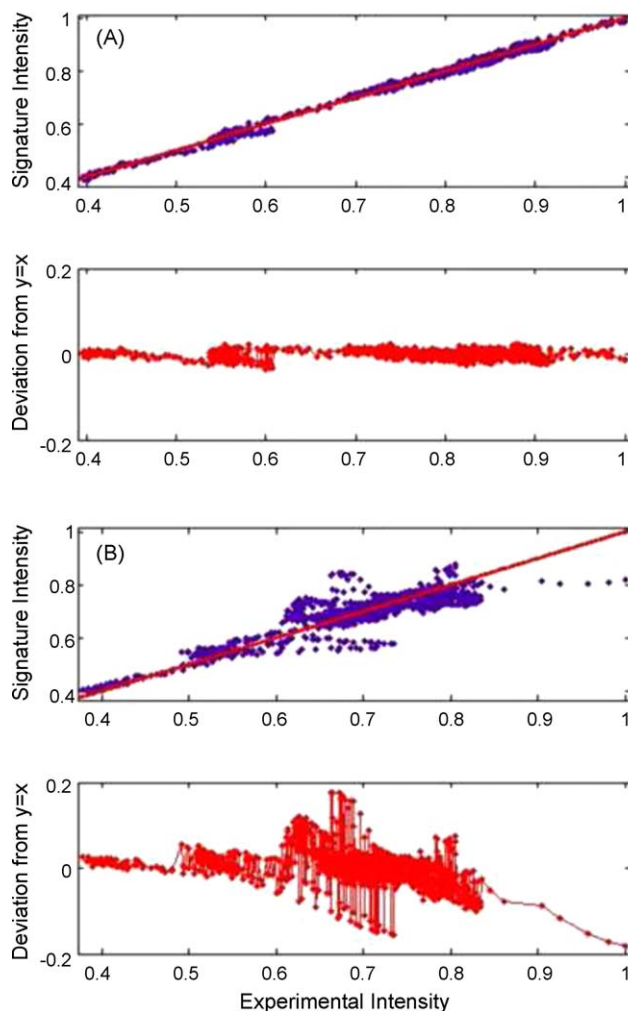

Fig. 4. Quantitative evaluation of the fitting quality. Comparison of the line $y = x$ with the semen signature fit for a semen sample (A, top) along with the residual plot (A, bottom). The semen signature fit for a saliva sample (B, top) along with the residual plot (B, bottom).

and this technique can potentially be used to identify an unknown sample to be semen.

4. Conclusions

A spectroscopic signature for human semen was developed based on the heterogeneous chemical composition of semen using NIR Raman spectroscopy. Statistical analysis found that the spectrum of a dried semen sample contained three major spectral components: a component consistent with tyrosine, a component containing a protein and possibly choline, and a component consistent with spermine phosphate hexahydrate. These assignments are possible suggestions based on known literature data. We also demonstrated qualitatively that there are no significant visual changes in the Raman spectra of dried semen acquired from multiple donors, and we showed that the spectrum of dried semen varies considerably when compared to the spectra of dried blood and saliva. The combination of the three principal components can be used as a unique spectroscopic signature to identify the presence of semen and possibly distinguish it from other body fluids and substances of artificial nature found at a crime scene. The signature's specificity to semen is additionally reinforced by the preliminary determination that two of the three spectral components are consistent with choline and spermine, respectively, and that these chemical

components are unique to semen and have been used as forensic identification techniques for semen in the past. This spectroscopic signature can be fitted to all of the dried semen samples with high goodness-of-fit statistical results, and this outcome shows how the signature can be applied to any human semen sample to potentially identify it.

We envision the potential use of this method for non-destructive detection and confirmatory identification of semen at a crime scene, both in its pure form and even as part of a stain. A forensic investigator would be able to determine the true identity of a suspected semen sample and whether it was pure and not contaminated. Portable Raman spectrometers that are currently being used to identify drugs and explosives [32] are very user-friendly, and successful analysis does not require a knowledge of Raman spectroscopy since the instrument automatically calculates a list of possible matching substances with correlating probabilities. This type of instrument can potentially be applied to body fluid identification. The ability to make these identifications and conclusions, especially at the scene of a crime, would be major progress in the area of forensic semen analysis. This proof-of-concept study involving pure samples in a controlled laboratory setting showed promising results, but many more samples with known demographic information should be investigated. In addition, more real-world studies need to be performed to determine the robustness of this technique, such as semen dilutions, mixtures, and stains on various substrates. The technique introduced in this paper shows the potential for the Raman spectroscopic signature of semen to be useful in identifying semen at crime scenes.

Continuing investigation of semen samples and other body fluids is currently taking place in our laboratory. We hope to develop unique spectroscopic signatures for other body fluids to support the assumption that the different fluids can be distinguished from one another using Raman spectroscopy since they are composed of different chemical components. We are also experimenting with a more advanced statistical method which uses principal component analysis (PCA) to mathematically compare multiple spectra of different body fluids as well as spectra from different animal species of the same fluid.

Acknowledgements

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