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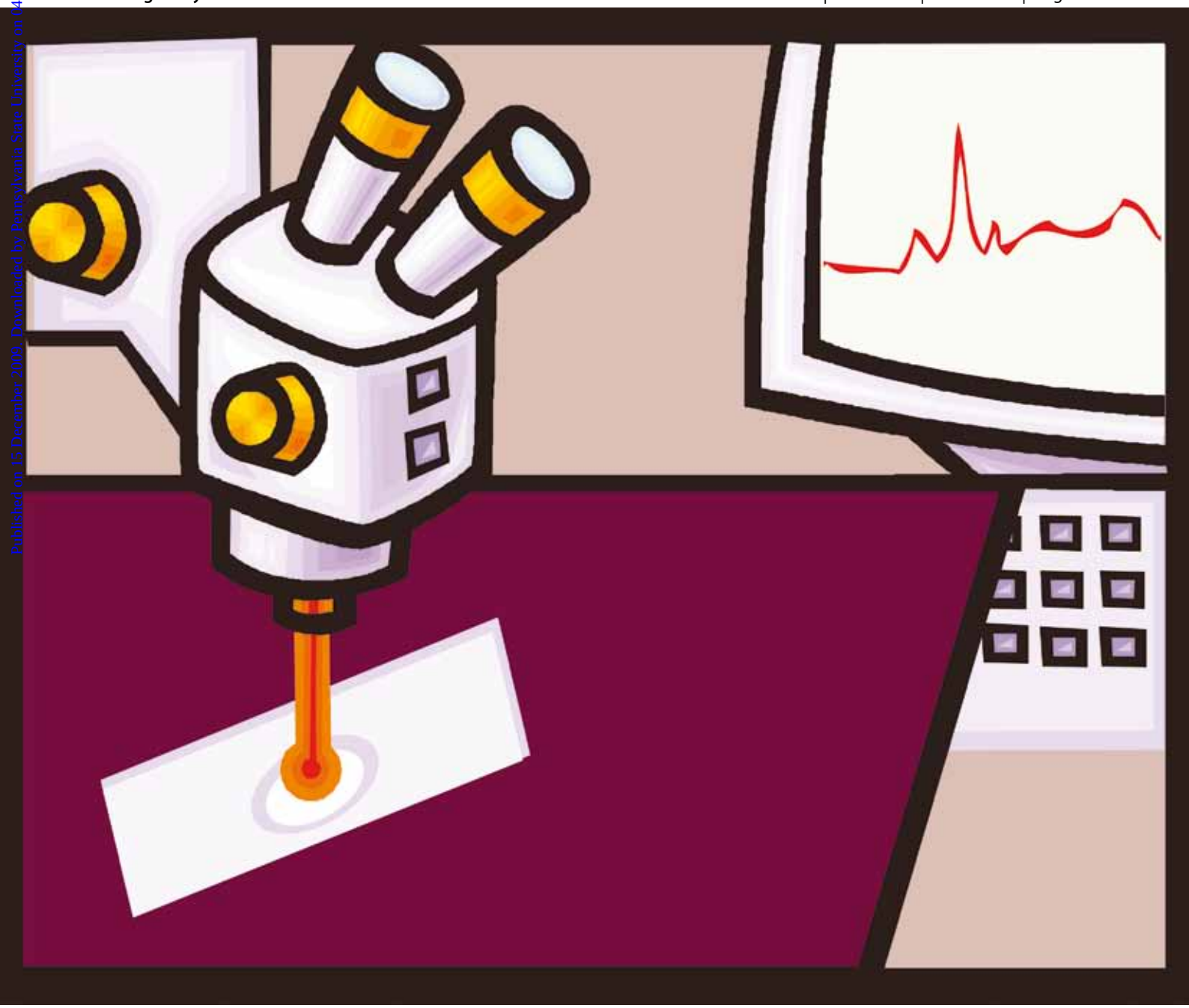

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Forensic body fluid identification: The Raman spectroscopic signature of saliva

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The potential use of Raman spectroscopy for nondestructive, confirmatory identification of body fluids at the crime scene has been reported recently (Virkler and Lednev, *Forensic Sci., Int.,* 2008, **181**, e1–e5). However, those experiments were performed using only one sample of each body fluid and did not investigate the potential for spectral variations among different donors of the same fluid. This paper reports on the role of heterogeneity within a single human saliva sample as well as among samples from multiple donors. Near-infrared (NIR) Raman spectroscopy was used to measure spectra of pure dried human saliva samples from multiple donors in a controlled laboratory environment. Principal component analysis of spectra obtained from multiple spots on dry samples showed that dry saliva is heterogeneous and its Raman spectra could be presented as a linear combination of a fluorescent background and three spectral components. The major chemical components known to be present in saliva were used to tentatively identify the principal spectral components. The issue of potential spectral variations that could arise between different donors of saliva was also addressed. 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 saliva in a quantitative way. The combination of these three spectral components could be considered to be a spectroscopic signature for saliva. This proof of concept approach shows the potential for Raman spectroscopy to identify an unknown substance to be saliva during forensic analysis.

Introduction

Recently, forensic analysis has become one of the largest growing areas of analytical chemistry.[1,2] The ability to identify traces of body fluids recovered at crime scenes is a very crucial part of many forensic investigations.[3–5] With the advancement and popularity of DNA analysis, body fluid evidence must be properly collected and not destroyed during the initial stages of testing, especially if there is only a small amount of sample available. Fluids such as blood, semen, saliva, and vaginal fluid can be very useful in identifying a victim or suspect,[6] and they can also help piece together the events of a crime. A new technique that could identify a particular body fluid rapidly, simply, and nondestructively at the crime scene would be a valuable tool for forensic investigators.[7–9]

Saliva evidence is not encountered as often as blood or semen, but it can still play an important role in cases involving sexual assault,[10] bite marks on food,[11] and even envelopes.[12] DNA can also be extracted from saliva,[3] so it is imperative to recover and identify any saliva that might be present. An alternate light source is a useful finding aid that will cause saliva stains to appear bluish-white under ultraviolet illumination, though this technique will not distinguish saliva from another body fluid.[13] Saliva is also more difficult to locate than other fluids using an alternate light source since there are fewer solid particles in saliva.[5] The most successful and widely-used technique to presumptively identify a saliva stain is a destructive chemical test for amylase.[14] This cannot be considered to be a confirmatory test since amylase is also present in small amounts in other body fluids. Popular tests such as the starch-iodine test and Phadebas® test are based on color changes that occur when amylase breaks down starch.[14] A commercial test kit known as Rapignost®-Amylase that was developed for urine analysis has also been used for saliva detection.[15] These tests all have the potential for false positive results, so confirmatory identification is not a possibility. No confirmatory test for saliva is currently accepted in forensic laboratories, so the development of a new analytical technique for confirmatory saliva identification would be very beneficial to forensic investigators, especially if it was nondestructive and could preserve valuable DNA evidence.[16]

Raman spectroscopy is an analytical technique that has become increasingly popular in forensic applications over the last several years.[17,18] It can be used to gain information about the structure and properties of materials based on their vibrational transitions upon illumination with a monochromatic laser.[19] Some forensic applications of Raman spectroscopy being used today involve the identification of fibers,[20] drugs,[21] and lipsticks,[22] as well as ink,[23] paint,[24] and condom lubricant[25] analysis. The theory behind Raman spectroscopy involves the inelastic scattering of a low-intensity, monochromatic, and nondestructive laser light by a solid, liquid or gas sample. Sample preparation is very minimal, 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 femtolitres, and the sample will not be destroyed so further analysis can still be performed. A Raman spectrum will reveal

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a specific vibrational signature of the sample being measured based on the energy of the scattered laser light, and this spectrum is very useful in identifying an unknown substance. Raman spectroscopy is also very appropriate for the analysis of disordered and heterogeneous samples,[26] which are common properties of body fluids, especially saliva. Finally, Raman spectroscopy shows very little interference from water[27] which makes it a great technique for analyzing body fluids and their traces. Portable Raman spectrometers are available now,[28,29] and these designs along with advanced software could be applied to the identification of saliva at a crime scene.

To our knowledge, there have not been any experiments published that involve the identification of saliva using Raman spectroscopy. The goal of our study is to determine how heterogeneous a single sample of dried saliva is, and we also want to analyze the qualitative variation among saliva samples from different donors using NIR Raman spectroscopy. It is important to emphasize that the samples being measured are pure 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. We have already had success in developing a spectroscopic signature for human semen using this same technique.[9]

Experimental

Samples

A set of 15 saliva samples were obtained from anonymous donors and volunteers. Five samples were randomly chosen as a basis set that would be used to determine the spectroscopic signature, and this signature would be fitted to the remaining samples for comparison. A small 10 μL drop of the each sample was placed on a circular glass slide designed for use with an automatic mapping stage and allowed to dry completely. Each sample was analyzed using automatic mapping that scanned a sample area of 75 × 75 μm and measured Raman spectra from 36 random points within the area with 6 ten-second accumulations at each point. The spectra obtained from the basis set were used to determine the number and possible identities of the principal components of saliva and to develop the spectroscopic signature. Any of the 15 saliva samples could have been chosen as the basis with similar results expected. Five were chosen as a means of capturing more potential variety than what would be found in a single sample so that the spectroscopic signature would contain the most accurate and abundant spectral components.

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 was about 3.5 cm^{-1}, and peak accuracy was assured by calibration with a silicon standard.

Data treatment

All of the spectra obtained from the automatic mapping of the dried saliva 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 set 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 set were used to create a spectroscopic signature, and this signature was fitted to each average spectrum found from the remaining saliva 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. The standard deviations and confidence intervals of these statistics were used as comparison to the goodness-of-fit statistics of other body fluids to show the specificity of the spectroscopic signature to human saliva.

Results and discussion

Main approach

The two main objectives of this study were to determine the principal spectral components of saliva and determine the level of heterogeneity among saliva samples from different donors. If there are only minor changes in the spectra from one donor to another, then Raman spectroscopy can be considered as a potential method to identify a sample as saliva based on the goodness-of-fit of the spectroscopic signature. This signature, which could be fitted to a saliva sample collected from any donor, could be developed based on the principal spectral components found in saliva. With the creation of unique spectroscopic signatures for other body fluids, an unknown sample could potentially be identified based on which signature is the best match.

Single sample heterogeneity

A set of five saliva samples, called *basis set* hereafter, was used to develop the spectroscopic signature that would be eventually fitted to all the samples. Raman spectra measured at 36 random points from each sample of the basis set were imported into MATLAB, and SFA analysis[30,31] was performed on a matrix of all 180 spectra to find the number of principal spectral components. The results of this analysis (data not shown) initially indicated 11 principal components. The ALS function[30,31] was applied to find the individual spectra of each of these components, and further examination of the 11 component spectra revealed that there were actually only 3 unique components that were spectral representations of the chemical species in the saliva sample. The other 8 components consisted of background

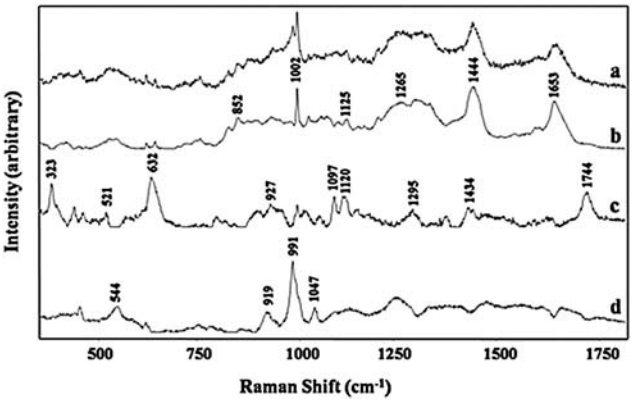

Fig. 1 The average Raman spectrum of the basis set saliva samples (a), and the Raman spectra of saliva spectral components 1 (b), 2 (c), and 3 (d) with major peaks labeled.

Table 1 Raman assignments of dried saliva

Raman shift/cm^{-1}	Spectral component	Vibrational mode
323	2	Endocyclic vibrations[38]
521	2	Exocyclic vibrations[38]
544	3	C–C_3 bending[38]
632	2	Skeletal bending[54]
852	1	C–N stretching[38]
919	3	Uncertain
927	2	C–H bending[54]
991	3	Uncertain
1002	1	Aromatic ring breathing (Phe)[38]
1047	3	C–CH_3 vibration[55]
1097	2	C–O stretching [38]
1120	2	C–C stretching[38]
1125	1	CH_3 rocking, C–O vibration[55]
1265	1	Sym. ring deformation (Tyr)[56]
1295	2	C–O stretching[54]
1434	2	CH_2 bending[54]
1444	1	CH_2, CH_3 bend (Trp)[27]
1653	1	Amide I[37]
1744	2	C═O stretching[54]

fluorescence, duplicates of the 3 principal components, and noise. Fig. 1 shows an average spectrum taken from the basis set along with the 3 principal components. The wavenumber range of 300–1800 cm^{-1} is used in the figure and will be the range used to create the spectroscopic signature since it 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 saliva are complex, and apparently have contributions from multiple chemical species. The assignments given to these components are suggestions based on the known composition of saliva and literature data. More investigation is needed to absolutely determine the identities of these spectral components. According to several literature sources,[32–35] some of the chemical components of saliva that are present in the highest concentrations are electrolytes, mucus, antibacterial compounds, and various enzymes, though water is of course the dominant component. One of the compounds present in the largest amount is mucus, and that consists of glycoproteins, such as mucin, and mucopolysaccharides.[36] There is of course a potential for a large variety of contaminants in any saliva sample due to the eating habits of a particular donor, but these interferences do not appear to affect the spectroscopic signature. This experiment does not study the effects of time on a saliva sample. Whether or not the spectrum of saliva will change relative to the age of the stain is unknown, and this uncertainty will be investigated in the future.

Principal component 1 is present in the largest abundance in the saliva samples and most closely resembles the saliva spectra as a whole. It is apparent at first glance that this component is dominated by the polypeptide backbone of a protein due to the amide I[37] and aromatic ring breathing[38] peaks at 1653 cm^{-1} and 1002 cm^{-1}, respectively. The CH stretching peak at 1444 cm^{-1} is also prevalent in the spectra of various proteins. It is likely that this component represents the strong presence of the previously mentioned glycoproteins that are known to be in saliva, and it could perhaps be from mucin, but further investigation is needed. Some spectra of mucin in the literature[39,40] do appear to be consistent with component 1, so this is a logical possible assignment. Table 1 shows some important peaks from component 1 as well as their vibrational mode assignments.

Component 2 appears to be a mixture of more than one dominant species. There are some peaks, including those in the low wavenumber range of 323–521 cm^{-1}, that indicate the presence of some kind of saccharide. As mentioned before, the mucus in saliva contains mucopolysaccharides also known as glycosaminoglycans.[41] Some literature spectra of saccharides[38,42] contain some peaks consistent with component 2, so this is a reasonable possible assignment. Another species that appears to be present in this component is acetate. The peaks at 632, 1295, 1434, and 1744 cm^{-1} are consistent with the literature spectra of some acetates.[43–45] Acetate has been reported to be found in saliva,[46,47] with one possible reason being carbohydrate fermentation in the mouth.[46] The main peaks of component 2 are also listed in Table 1 with vibrational mode assignments.

Component 3 was the smallest contributor to the overall spectra of the saliva samples and was the most difficult to assign. The best guess at this point is that it is dominated by the amino acid arginine based on the peaks at 544, 919, and 991 cm^{-1}. A database spectrum of arginine[48] is consistent with these peaks. However, the peaks in the higher wavenumber range above 1000 cm^{-1} of arginine are not present in the spectrum of component 3, so this assignment is still debatable. There are free amino acids present in saliva,[49,50] however, so this suggestion is still reasonable. The identity of this component is the most uncertain at this point, but it is still a critical part of the saliva signature and is necessary to make a proper fitting with a known saliva spectrum. Table 1 also lists the peaks of component 3 with possible vibrational modes.

Multiple donors

The second objective of this study was to determine how much the spectrum of a saliva sample would change from one donor to another. The first step was qualitative and involved simply visually comparing the average spectrum from each of the different donors. All of the spectra contained the same major

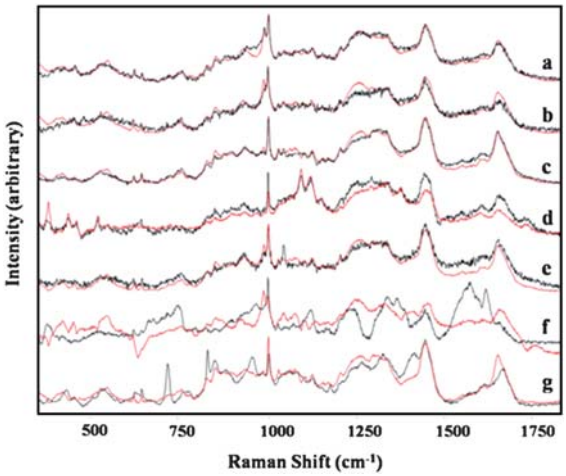

Fig. 2 The average Raman spectra of five saliva samples (black) with the fitted spectroscopic signature (a–e), and the Raman spectra of blood (f) and semen (g) with the fitted spectroscopic signature.

Table 2 Goodness-of-fit statistical results for saliva signature fitting

Sample	SSE	R^2	RMSE
1	0.36	0.986	0.014
2	0.25	0.995	0.012
3	0.26	0.994	0.012
4	0.45	0.985	0.016
5	0.35	0.986	0.014
6	0.37	0.992	0.014
7	0.27	0.991	0.012
8	0.42	0.991	0.015
9	0.30	0.986	0.013
10	0.22	0.992	0.011
11	0.22	0.990	0.011
12	0.22	0.996	0.011
13	0.16	0.997	0.0095
14	0.13	0.998	0.0088
15	0.29	0.994	0.013
99% Confidence Range	≤0.55	≥0.979	≤0.018
Blood	1.7	0.977	0.031
Semen	5.1	0.905	0.053

peaks and in general appeared very similar to each other. Only the intensity of some peaks changed for different donors, and this is understandable since the relative contribution of the chemical species in saliva will likely change with each donor and can even change within the same donor throughout the day.[51] Fig. 2 shows the average individual spectra of five random saliva samples (black lines) as an example of their similarities. To demonstrate the spectral differences between body fluids, the spectra of blood and semen that we have previously reported[7] are also included in this figure. Unlike the slight intensity changes within the saliva samples, the spectra of blood and semen contain major peak differences which make them easily distinguishable. The other features of Fig. 2 will be further explained in the following paragraphs.

After visually confirming that there is consistency among the saliva spectra from different donors, a more quantitative approach was developed. A spectroscopic signature was created that consisted of the 3 principal components found from analyzing the basis set along with a horizontal line and a line with a slope equal to that of the fluorescence background. This signature was linearly fitted to the average spectrum of each basis set sample, and there was a very nice overlap for all five. The spectroscopic signature was also applied to the remaining 10 saliva samples individually to determine if it could universally be fitted to a sample from any donor. Fig. 2 shows the fitting to only five random saliva samples, but all of the samples had very similar fits. The bottom of Fig. 2 shows the saliva spectroscopic signature fit to the spectra of blood and semen, and it is visually obvious that they are very poor matches. These results qualitatively show the specificity of this signature to saliva and its potential ability to be used as an identification technique for forensic purposes.

In addition this qualitative comparison, a quantitative statistical analysis was performed to determine how well the spectroscopic signature fit the experimental saliva spectra. Using the Curve Fitting Toolbox in MATLAB, the intensity values for the each basis set experimental spectrum and fitted spectrum were individually 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 largest value for both the x- and y- axis. Two identical spectra would have equal x and y values yielding a scatter plot matching a line with the equation of $y = x$, so this line was the basis of comparison since it represents the best fit scenario. The $y = x$ line was fit to the plotted data points of the basis set sample evaluations to determine how close of a match each experimental spectrum and the spectroscopic signature were. The result was three quantitative goodness-of-fit values which statistically confirmed the previous qualitative match of the experimental and fitted spectra (Table 2). These values are the sum of squares due to error (SSE), R^2, 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.[52] The R^2 value indicates how 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.[52] 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,[52] and that the signature better fits the experimental spectrum.

This same fitting procedure was performed for the remaining 10 samples. The graphical result of a typical fit is shown for sample 13 in Fig. 3A 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 this best fit line from the scatter plot. A residual plot with random points around zero that do not form a pattern indicates a good fit,[52] as is the case here. The results for SSE, R^2, and RMSE for all 15 samples are shown in Table 2. The statistical values for all of the samples are relatively very close and fall within a certain range that suggests a good fit. This range was found by calculating the standard deviation for each of the three statistics, and the standard deviation was used to find the 99% confidence interval that contained the values expected for a saliva sample. The confidence limits are also listed in Table 2. To put in perspective how well the

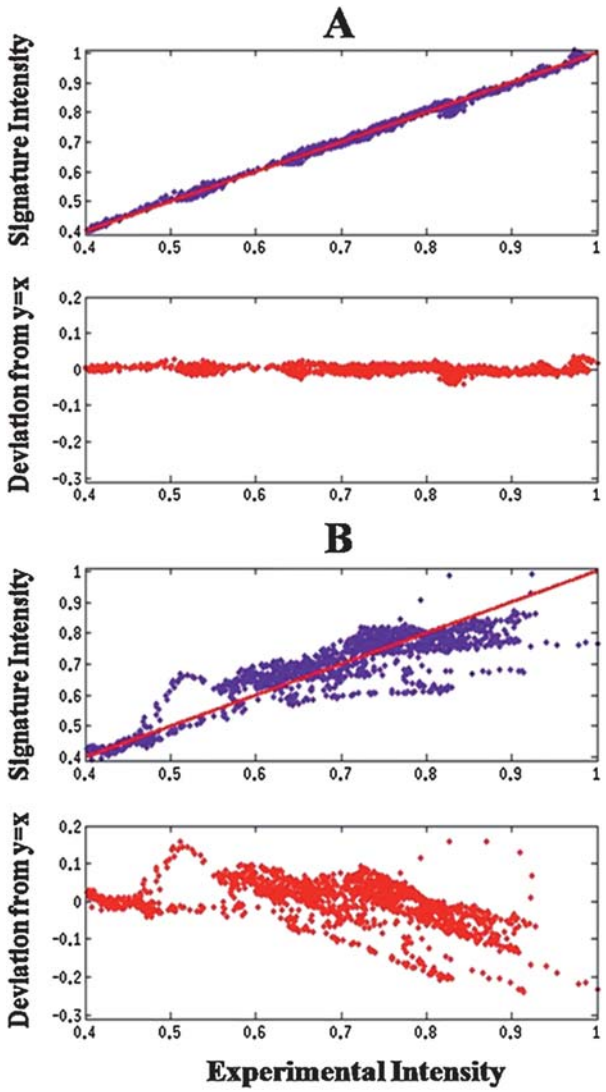

Fig. 3 Quantitative evaluation of the fitting quality. Comparison of the line $y = x$ with the saliva signature fit for a saliva sample (3A, top) along with the residual plot (3A, bottom). The saliva signature fit for a semen sample (3B, top) along with the residual plot (3B, bottom).

signature fits the saliva samples, it was also applied to the spectra of human blood and semen which we have already reported.[7] 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 saliva signature matches the spectra of the other two body fluids when the three values are taken into account. All three statistics for both fluids are outside the range of the saliva samples, and this quantitatively illustrates that the saliva signature does not fit blood or semen. The graphical results of the signature fit to the semen sample are shown in Fig. 3B. 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 zero in the residual plot.

Conclusions

A spectroscopic signature for human saliva was developed based on its heterogeneous chemical composition using NIR Raman spectroscopy. Statistical analysis found that the spectrum of a dried saliva sample contained three major spectral components; a component consistent with a protein, a component containing acetate and a saccharide, and a component somewhat consistent with the amino acid arginine. These assignments are preliminary suggestions based on known literature data. We also showed the qualitative similarities in the Raman spectra of dried saliva from multiple donors, and we demonstrated that the spectrum of dried saliva varies considerably when compared to the spectra of dried blood and semen. The combination of the three principal components can be used as a unique spectroscopic signature to identify the presence of saliva and potentially distinguish it from other body fluids and substances of artificial nature found at a crime scene. This spectroscopic signature can be fitted to all of the dried saliva samples with a specific range of goodness-of-fit statistics, and this outcome shows how the signature can be applied to any human saliva sample to potentially identify it.

Further experiments involving saliva samples and other body fluids are currently being performed in our laboratory. Our goal is to create unique spectroscopic signatures for other body fluids to support the hypothesis that different fluids can be distinguished from one another using Raman spectroscopy since they are composed of different chemical species. The development of a unique spectroscopic signature for semen has already been shown to be possible.[9] We have also recently reported a more advanced statistical method which uses principal component analysis (PCA) to mathematically compare multiple spectra of dried blood from different animal species, and our results indicate that species distinction using Raman spectroscopy is possible.[53]

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