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Whole blood and semen identification using mid-infrared and Raman spectrum analysis for forensic applications

Yun Zou,[a] Pan Xia,[b] Feiyu Yang,[a] Fangqi Cao,[a] Ke Ma,[a] Zhongliang Mi,[b] Xiaochun Huang,[b] Nengbin Cai,[b] Bei Jiang,[c] Xuejun Zhao,[a] Wenbin Liu*[a] and Xianfeng Chen*[c]

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The identification of body fluids is important in forensic science. This paper describes the application of mid-infrared and Raman spectroscopies in the non-destructive identification of human blood and semen, where other detailed information can also be obtained in one single measurement. Samples of human blood and semen were probed and characterized utilizing Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) and confocal Raman spectroscopies. The result shows their ability to identify an unknown substance to be human blood or semen without the use of chemical reagents. Age determination of dried blood and semen spots through their mid-infrared spectra was investigated, which could probably be used during forensic casework. Furthermore, the origin of the Raman scattering peaks of human semen at 2907 and 2968 cm^{-1} is detailedly analyzed, which has not been studied in previous literature. Overall, this optical detection and identification method exhibits advantages over conventional chemical methods in terms of non-destruction, high sensitivity, rapid detection and direct confirmation.

1. Introduction

The identification of traces of body fluids is a very important aspect of forensic science, where good preservation of the sample is crucial for subsequent forensic analysis. With the development and popularity of DNA analysis, body fluids need to be properly collected and retained. Common body fluids, such as blood and semen, are very useful for identifying a victim or a suspect. However, conventional chemical analysis methods are limited, destructive and time-consuming. Thus, identification methods featuring non-destruction and high speed are of great significance and importance in practical applications.

Mid-infrared and Raman spectroscopies are versatile optical analytical techniques. They provide detailed information about the components and molecular structures of a material in terms of its mid-infrared absorption and vibrational transitions. Minimal sample and almost no reagent are needed for such analysis based on these optical detection methods. Bloodstain examination represents a major forensic application during crime scene investigation, which is frequently subjected to infrared and Raman spectroscopies.[1–5] Numerous attempts have been made to estimate the age of bloodstains by means of spectrophotometry, microspectrophotometry and electron paramagnetic resonance (EPR) spectroscopy. However, research on the mid-infrared absorbance of human blood and semen has rarely been reported. Previous studies were mainly concentrated on the investigation of the components of whole blood.[1] Moreover, the relationship between the characteristic spectrum in the mid-infrared region and the sample age has not been investigated. On the other hand, Raman spectroscopy has been widely used to discriminate gunshot residue,[6] fingerprints,[7] ink,[8] drugs,[9] paint,[8] fibers,[10] lipsticks,[11] condoms lubricants[12] and body fluids.[13–15] We re-examined the Raman spectrum of human blood and semen with high spectral resolution. Other than a similar result to the research by Bertino *et al.*,[16] we also found several vibrational modes of the Raman peaks that were not previously presented. The aim of our study is to identify human blood and semen *via* mid-infrared and Raman spectroscopies, and propose a protocol to estimate or determine the sample age.

[a]*Shanghai Key Laboratory of Crime Scene Evidence, Shanghai Research Institute of Criminal Science and Technology, Shanghai 200083, China. E-mail: wbliu1981@163.com*

[b]*Shanghai Key Laboratory of Crime Scene Evidence, Institute of Forensic Science, Shanghai Public Security Bureau, Shanghai 200083, China*

[c]*Department of Physics and Astronomy, Shanghai Jiao Tong University, Shanghai 200240, China. E-mail: xfchen@sjtu.edu.cn*

2. Experimental

2.1 Sample preparation

All experiments with human subjects were carried out in accordance with the guidelines of the National Institute of

Health, Shanghai, China and approved by the Ethical Committee of Shanghai Jiao Tong University. All participants provided their written informed consent for the collection of biological samples. We studied two types of body fluids, *i.e.*, human blood and semen. The samples were obtained in liquid form from anonymous volunteers. A set of 16 blood samples, including four blood types (A/B/AB/O) from both genders, were collected. And a set of three semen samples were obtained from adults. All samples were not treated by any chemical or biological method throughout the entire measurement. For analysis, one drop (approximately 100 μJ) of each sample was deposited on microscope slides. The measurement of fresh samples was carried out immediately after collection. And blood and semen stain samples were tested after they dried naturally at room temperature in different time intervals. Replicate measurements were made, and each group of samples showed similar characteristics and results. Hence, one typical result of each sample group is presented here. All the experiments were carried out at room temperature. Preliminary assignments of major Raman peaks and possible components were made based on literature data.

2.2 Instruments

Mid-infrared spectroscopy. A single reflection ATR-FTIR spectrometer (Nicolet iS50, Thermo Scientific) was employed. Absorbance spectra were scanned in the mid-infrared range between 2.5 and 25 μm with a spectral resolution of 0.4 cm^{-1}. The microscope slides were then pressed with a clamp on a horizontal ZnSe crystal plate of an ATR sampling accessory. A homogeneous contact between the sample surface and the crystal was ensured. Each sample was measured 3 times and the average spectrum was used for analysis. The spectra were recorded without smoothing.

Raman microscopy. A Renishaw in Via confocal Raman spectrometer equipped with a microscope, 50× long-range objective (numerical aperture of 0.75) was used. A 532 nm laser was utilized for excitation. The laser power at the sample plane was measured to be approximately 2 mW, far weaker than any optical damage threshold. The Raman spectrum was typically recorded using an integration time of 10 ms and one accumulation. The spectral resolution was 1 cm^{-1}, and the instrument was pre-calibrated with a standard silicon sample before measurement. Spectra with high signal-to-noise ratio were obtained. And no manipulation was conducted except for the subtraction or addition of a constant background to shift the Raman spectra to combine them in one graph, since the absolute value of the intensity is irrelevant during the analysis.

3. Results and discussion

The experiment demonstrated the possible application of optical mid-infrared and Raman spectroscopies in the discrimination between dried human blood and semen spots, and their spectral evolution in the mid-infrared region with respect to the aging of the samples was studied. The absorbance ratio of two characteristic peaks (1531 and 1635 cm^{-1} for blood, 1240 and 1633 cm^{-1} for semen) is found to change with respect to the age of the stains. This research may be useful for searching and identification of trace evidence. Generally, the measurement of the absorbance spectrum in the mid-infrared region is considered as a confirmation test for a compound, because each substance's absorbance spectrum in the infrared region (the fingerprint region) is unique. The mixture of several compounds is very common in body fluids and the actual case of spectrum analysis may be very complicated. However, it is still possible that the compounds in biological fluids could be distinguished by the combination of mid-infrared and Raman spectroscopies.

Fig. 1 illustrates the mid-infrared spectra of human blood at different time phases, *i.e.*, fresh, after 1/4, 1 and 2 day(s), respectively. Fresh blood was analysed as a liquid form while aged bloodstains were analysed after they had naturally dried. Dry blood exhibited several peaks which were not present in fresh blood, because of water absorption. The result shows that the mid-infrared absorption spectrum of blood is similar to that of serum.[5] It is well known that serum is the main component of blood, which determines the absorption characteristics. The spectrum analysis is as follows: the pronounced peaks at 1635 and 1531 cm^{-1} are caused by the amide I vibration of the peptide chain and amide II, respectively. Peaks at 1238 and 1304 cm^{-1} can be assigned to the amide III vibrations. The hump around 3278 cm^{-1} originates from the O–H stretching. The vibration modes of peaks at 2958, 2931 and 2872 cm^{-1} correspond to the asymmetric vibration of C–H in CH3, asymmetric vibration of C–H in CH2 and symmetric vibration of C–H in CH3, respectively. Peaks at 1082 and 1165 cm^{-1} are induced by vibration of C–O and bending vibration of C–O–H. The vibration of C═O is responsible for the peak at 1392 cm^{-1}. The relative absorbance ratio of the two peaks at 1531 and 1635 cm^{-1} to each other is increasing with respect to the time elapsed from 1/4 day to 1 day, and the ratio tends to be stable after 1 day. This could be used as an indicator in its age estimation. The spectrum of fresh blood seems to be substantially dominated by IR peaks of water, whose bending vibration overlaps the band at 1635 cm^{-1}, thus leading to higher intensity.

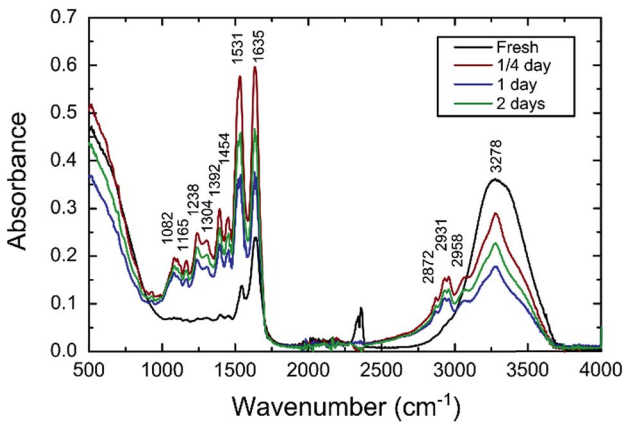

Fig. 1 Mid-infrared spectra of whole blood at different time phases: fresh, 1/4, 1 and 2 day(s), respectively.

Fig. 2 shows the mid-infrared spectra of semen at different time phases, *i.e.*, fresh, after 5/4, 5 and 30 days, respectively. Compared with the mid-infrared spectra of blood, many absorbance peaks are similar, such as peaks around 3275, 2960, 2935, 2877, 1633, 1539, 1390 and 1240 cm^{-1}. The peak at 1454 cm^{-1} disappears in the spectrum of semen, and a new peak at 1057 cm^{-1} appears. From the literature of ref. 15, albumin is the component that exists in both human semen and blood. So the similar absorbance peaks may originate from albumin. The appearance of the peak at 1057 cm^{-1} is possibly attributed to the asymmetric vibration of P–O–C. The relative absorbance ratio of the peaks at 1240 and 1633 cm^{-1} to each other is decreasing with the sample age, and the albumin degradation in semen is possibly responsible for the variation. This could be used as an indicator in its age determination.

The mid-infrared spectra of human blood and semen contain several similar characteristic bands, but the difference is also obvious. Fresh blood samples exhibit a peak at 1531 cm^{-1} which is not observed in the fresh semen spectrum. Other significant differences can also be found between human blood and semen. Peaks at 1082 and 1165 cm^{-1} are unique to the blood spectra, whereas the peak at 1057 cm^{-1} is observed only in human semen. In the experiment of mid-infrared spectroscopy, we focused on the vibration modes of absorbance peaks and the difference in the spectra of different body fluids. The effect of time on samples was also investigated. In terms of the experimental results, fresh and dry samples such as human blood and semen can be differentiated *via* their mid-infrared spectra. In addition, aging induced noticeable differences can be determined by the ratio of absorbance of peaks.

In Fig. 3, the Raman spectrum of dry blood is characterized by several peaks, with the most pronounced being peaks at 1584 and 1637 cm^{-1}, which possibly originate from hemoglobin. The experimental result is in agreement with recent research.[3,17] All Raman scattering peaks of the tested samples from multiple donors of different ages, genders and blood types are the same. The spectral components of human blood are complex. According to the literature, hemoglobin, heme and tryptophan contribute significantly to the overall spectrum of blood. Peaks at 1083, 1130 and 1434 cm^{-1} are tentatively assigned to lactic acid, lactate and acetates, respectively. Major Raman peaks and corresponding vibrational modes are summarized in Table 1.

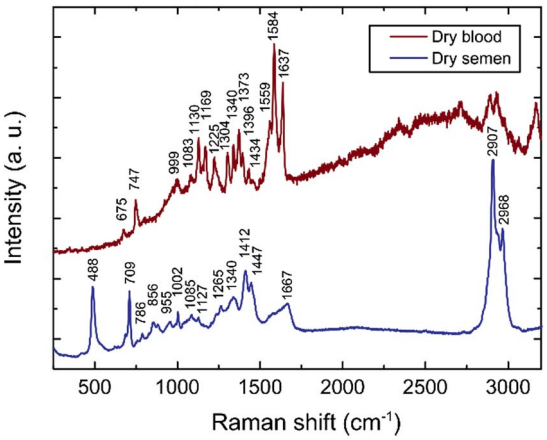

Fig. 3 Raman spectra of dry blood and semen.

Semen is one of the most prevalent body fluid evidence in sexual assault cases. The Raman spectrum of dry human semen is also presented in Fig. 3. Major Raman peaks of human semen and its corresponding vibrational modes are listed in Table 2. Major chemical components in human semen are urea, albumin, spermine phosphate hexahydrate and tryptophane.

Other than similar characteristic bands, human blood and semen stains can be easily distinguished by their Raman spectra too. Semen exhibits peaks at 488, 709, 768, 856, 955, 1265, 1412, 1447, 1667, 2907 and 2968 cm^{-1}, whereas peaks at 675, 747, 1169, 1225, 1304, 1373, 1396, 1434, 1559, 1584 and 1637 cm^{-1} are only observed for blood. This demonstrates that Raman spectroscopy has the ability to differentiate whole blood and semen.

It is worth mentioning that the peaks at 2907 and 2968 cm^{-1} have not been characterized and discussed in previous studies,

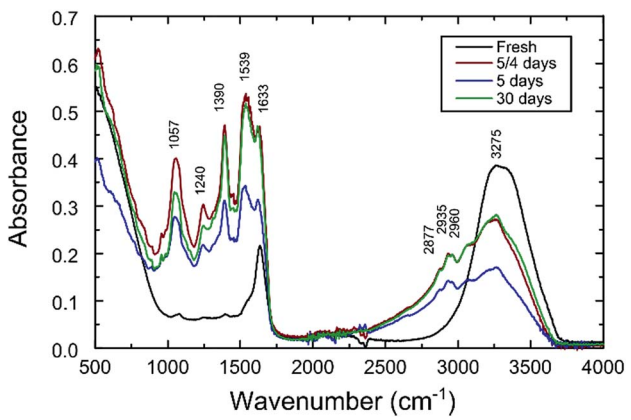

Fig. 2 Mid-infrared spectra of human semen. Mid-infrared spectra of whole blood at different time phases: fresh, 5/4, 5 and 30 days, respectively.

Table 1 Raman assignment of dry blood

Raman shift (cm^{-1})	Vibrational mode	Component
675	C–C–N bending	Hemoglobin[17,18]
747	Ring vibrations	Tryptophan[4]
999	Aromatic ring breathing	Hemoglobin[4]
1083	C–O vibration	Lactic acid[19]
1130	CH3 rocking, C–O vibration	Lactate[20]
1169	C–C stretching	Hemoglobin[21]
1225	Ferrous low spin	Hemoglobin[16]
1304	C–C stretching	Hemoglobin[22]
1340	C–H bending	Tryptophan[23]
1373	CH3 symmetric stretch	Heme[24]
1396	C=N antisymmetric stretching	Heme[22]
1434	CH2 bending	Acetates[25]
1559	Pyrrole ring stretching vibrations	Hemoglobin[21]
1584	C–H bending	Hemoglobin[22]
1637	Ferrous low spin	Hemoglobin[16–18]

Table 2 Raman assignment of dry human semen

Raman shift (cm^{-1})	Vibrational mode	Component
488	N–C–O bending	Urea[26]
709	C–N stretching	Albumin[27,28]
768	Ring vibrations	Albumin[29]
856	C–C aliphatic stretching	Tyrosine[20]
955	PO43-symmetric stretching	Spermine phosphate hexahydrate[30]
1002	Aromatic ring breathing, C–N stretching	Phenylalanine, urea[31,32]
1085	C–O vibration	Lactate[20]
1127	C–N asymmetric stretching	Spermine phosphate hexahydrate[33]
1265	Symmetric ring deformation	Amino acid tyrosine[34]
1340	C–H bending	Tryptophane[23]
1412	CH2, CH3 bending	Acid phosphatase[31,35]
1447	CH2, CH3 bending	Tryptophane[31]
1667	Amide I	Protein[27]
2907	CH asymmetric stretching	Dimethylthio-acetamide[36]
2968	CH2 asymmetric vibration	L-Asparagine[37]

because the intensities of the two peaks were weak compared with those of other ones.[16] However, the peak intensity at 2907 cm^{-1} is the most pronounced in our experiment. And these two peaks can be tentatively assigned to dimethylthio-acetamide and asparagines. The major components of semen have a composite character and could be assigned to urea, albumin, tyrosine, spermine phosphate hexahydrate, phenylalanine, lactate, amino acid tyrosine, tryptophane, acid phosphatase, protein, dimethylthio-acetamide and asparagines.

Although blood and semen (or other body fluids) are complex in ingredients and share similar components, mid-infrared and Raman spectroscopies together can distinguish one from the other; with the two spectroscopies combined together, this could provide their confirmative identification on a substrate or from an unknown substance. And without consequent procedures, the age of dried blood and semen spots through their mid-infrared spectra can also be estimated. Thus, this method could probably be useful during forensic casework.

4. Conclusions

A combination of mid-infrared and Raman spectroscopies has been used to study the composition of whole blood and semen. Aged whole blood and semen stains can be differentiated by mid-infrared and Raman spectroscopies, and a confirmative identification could be validated with a combination of the two. We also found that the relative absorbance ratio of mid-infrared peaks depends on the age of the samples, which could be used as an indicator in the age determination. Different from the previous study, the peak at 2907 cm^{-1} of Raman spectra of semen is the most pronounced. The origin of Raman peaks of semen at 2907 and 2968 cm^{-1} is analyzed. Overall, mid-infrared and Raman spectroscopies show great potential for non-destructive, confirmatory identification of whole blood and semen.

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