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Discrimination of human and animal blood traces via Raman spectroscopy

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ABSTRACT

The characterization of suspected blood stains is an important aspect of forensic science. In particular, determining the origin of a blood stain is a critical, yet overlooked, step in establishing its relevance to the crime. Currently, assays for determining human origin for blood are time consuming and destructive to the sample. The research presented here demonstrates that Raman spectroscopy can be effectively applied as a non-destructive technique for differentiating human blood from a wide survey of animal blood. A Partial Least Squares-Discriminant Analysis (PLS-DA) model was built from a training set of the near infrared Raman spectra from 11 species. Various performance measures, including a blind test and external validation, confirm the discriminatory performance of the chemometric model. The model demonstrated 100% accuracy in its differentiation between human and nonhuman blood. These findings further demonstrate a great potential of Raman spectroscopy to the field of serology, especially for species identification of a suspected blood stain.

1. Introduction

The identification of a body fluid stain is an important and unavoidable aspect of many forensic investigations. There are a variety of presumptive tests that are currently used for identifying a stain as blood. These tests are most commonly oxidation–reduction assays, which are destructive in nature, using reactants such as leucomalachite green, luminol, phenolphthalein and tetramethylbenzidine [1,2]. A common confirmatory blood test is a microcrystal assay (e.g. Teichmann or Takayama assays). The Ouchterlony or similar immunochromatographic assays can then be employed to determine if the blood is nonhuman [1]. It is ultimately preferable to confirm the presence of blood and the species of origin before forensic DNA profiling, but this can be practically problematic.

Since the amount of suspected blood evidence may be miniscule, it needs to be preserved and analyzed efficiently. Identification testing schemes need to be minimally destructive to preserve the sample for DNA analysis [3]. Since both presumptive and confirmatory tests use chemicals that are destructive, their use consumes a portion of the sample. To minimize this effect, a modified testing scheme is employed. Typically, after a stain has been presumptively identified as blood, further characterization is not carried out [4]. If a DNA profile is not extracted from the sample, then the suspected blood is presumed to be of animal origin [4]. This is a problematic testing scheme primarily because there is a lack of confidence that the sample is of human origin and in fact blood. This uncertainty is based on the fact that presumptive tests have known occurrences of false positives. This streamlined testing approach could also be detrimental for crime labs since time and money would be wasted on nonhuman or non-blood samples. Therefore, a non-destructive screening technique to efficiently identify human blood would be highly valuable. Raman spectroscopy is a technique that has the potential both as a non-destructive confirmatory identification for blood [5,6] and as a species of origin assay [7].

Raman spectroscopy has been proven as an effective and versatile analytical technique for a variety of forensic applications [8,9], including identification of lipsticks [10], drugs [11], condom lubricant [12] and fibers [13]. There is often no sample preparation needed for Raman analysis and the sample size can be on the order of picograms. More importantly, Raman spectroscopy is typically non-destructive to the sample which is crucial for forensic applications. This technique is based on the detection of light that is inelastically scattered by a sample upon irradiation from a monochromatic light source. A Raman spectrum contains numerous distinctive bands which correspond to specific molecular vibrational modes [14]. The Raman spectrum of blood in particular

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provides rich detail and has been targeted previously for forensic studies [15,16]. The popularity of Raman spectroscopy has been growing in forensic laboratories especially due to reduced cost of instrumentation and its numerous possible applications.

De Wael et al. was the first to apply vibrational spectroscopy to the problem of species identification of blood samples [17]. They reported the inability to differentiate between blood particles originating from human, cat and dog samples through their infrared and Raman spectra. The infrared spectrum of human and sheep blood has also been reported to be visually indistinguishable [18]. However, a recent study proves that discrimination can be enhanced with chemometric modeling of Raman spectroscopic data [7]. Virkler et al. reported the ability to distinguish blood samples from human, cat and dog using a Principal Component Analysis (PCA) model. Separation between these classes within the PCA model exceeded a 99% confidence interval. Even though there is agreement that the spectra obtained from the three classes appeared very similar, chemometric models are designed to exaggerate minor variations in the data. This method of data analysis enhances the selectivity of Raman spectroscopy and has been used extensively to build similar classification models [19,20].

To expand upon the proof of concept work of Virkler et al., we analyzed blood from a wider survey of species (12 in total). Bearing in mind forensic purposes, the animal (nonhuman) samples were selected to represent three groups: animals that are bred for domestication (cat, dog, horse and cow), those that are consumed as food (chicken, cow, pig, and rabbit), and those that are integrated with human existence (mouse, rat, opossum and raccoon). Analyzing a wider variety of animals enhances the forensic practicality of the study and adds more certainty to model predictions. To account for the increased complexity of the dataset, a Partial Least Squares-Discriminant Analysis (PLS-DA) classification model was used. These models have more class discrimination power than those of PCA and provide automatic soft prediction of classes [21]. A PLS-DA model was used to differentiate a large training dataset of human and animal blood spectra and to classify a set of unknown samples. As an external validation step, predictions were performed on cow spectra which were excluded from the training dataset. The constructed model demonstrates a great ability to discriminate human from animal blood. We demonstrate a more comprehensive and robust method for screening a suspected blood stain to identify human origin. Our analytical approach aims to be used for the rapid and non-destructive identification and characterization of a blood stain at a crime scene.

2. Methods and materials

2.1. Raman microscope and blood samples

A Renishaw inVia confocal Raman spectrometer and a Renishaw PRIOR automatic stage were used for data collection for all blood species experiments. The instrument was calibrated with a silicon standard before all measurements. Spectra were accumulated with a 20× long range objective with 785 nm excitation in the spectral range of 250–1800 cm^{-1}. Laser power at the sample was approximately 4.0 mW. A Raman map consisting of 35 spectra was collected from each of the samples. WiRE software version 3.2 was used to operate the instrument.

Animal (nonhuman) blood from cow, cat, dog, horse, pig, mouse, opossum, raccoon, rabbit, rat and chicken were obtained from Bioreclamation, LLC. For each animal species class, 2 mL of blood were procured from 10 individual donors. Animal blood was collected randomly from both genders to ensure donor diversity. Human blood was also obtained from Bioreclamation, LLC with consideration to gender, race and age diversity. Blood samples were kept frozen until sample preparation. For each blood sample, approximately 30 μL was placed on an aluminum covered microscope slide and allowed to dry for at least 60 min. Raman measurements were obtained within 48 h of initial sample preparation.

2.2. Data preparation and statistical treatment

All data preparation and construction of statistical models were performed with the PLS Toolbox 7.0.3 (Eigenvector Research Inc.) operating in MATLAB version R2010b. The spectra were truncated to the data range 252–1709 cm^{-1}. For each sample, the 35 spectra were baseline corrected with a sixth-order polynomial and normalized by area. These 35 spectra were averaged to form a single spectrum representing one sample. These averaged spectra were imported into a data matrix. The dataset was finally mean centered before models were calculated. The model was cross-validated using the Venetian Blinds method [22].

2.3. Blind test

To evaluate the performance of the classification model, a set of ten unknown samples were prepared from the available blood samples. Five human and five animal samples were arbitrarily chosen in a single-blind fashion (where the analysts were not aware of the identity) and assigned as unknown #1–10.

3. Results and discussion

The main objective of this study is to develop a method to differentiate human blood samples from a wide variety of animal blood samples through their Raman spectra. The experimental design, including laser power, excitation wavelength, data acquisition, data processing, and model selection were made based upon previous studies of body fluid traces in our laboratory. A PLS-DA classification model was built using a training dataset containing Raman spectra from human and selected animal samples (excluding cow). The number of components was selected by choosing a local minimum of total data variance captured using a scree plot (not shown). The PLS-DA model was constructed in a binary manner by classifying each spectrum as either human or animal. The model was tested by attempting to classify ten unknown samples and ten known samples (cow) which were outside of the training dataset.

3.1. Spectral analysis of training dataset

For each blood sample, a Raman spectral map of 35 points was collected. The spectra within each map were preprocessed by baseline treatment, normalized by area and averaged. The training dataset consisted of ten mean spectra from every species considered (excluding the ten cow samples) for a total of 110 mean spectra. The preprocessed training dataset spectra are shown in Fig. 1A. The prominent features in a Raman spectrum of blood correspond to specific vibrational modes related to hemoglobin [23]. From visual inspection, all spectra look to be identical in terms of the number of spectral features and their location. There are variations visible in the relative intensity of many Raman bands which are confirmed by the standard deviation of this dataset (Fig. 1B). A comparison of the animal and human class mean spectra is shown in Fig. 1C. These spectra are the total averaged data for their respective classes after preprocessing. The largest difference between the animal and human spectra is primarily in the shape and intensity variations within the 1220–1300 cm^{-1} spectral range, corresponding to stretching vibrational modes of hemoglobin [23].