Differentiating Donor Age Groups Based on Raman Spectroscopy of Bloodstains for Forensic Purposes

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Supporting Information

ABSTRACT: Developments in analytical chemistry technologies and portable instrumentation over the past decade have contributed significantly to a variety of applications ranging from point of care testing to industrial process control. In particular, Raman spectroscopy has advanced for analyzing various types of evidence for forensic purposes. Extracting phenotypic information (e.g., sex, race, age, etc.) from body fluid traces is highly desirable for criminal investigations. Identifying the chronological age (CA) of a blood donor can provide significant assistance to detectives. In this proof-of-concept study, Raman spectroscopy and chemometrics have been used to analyze blood from human donors, and differentiate between them based on their CA [i.e., newborns (CA of <1 year), adolescents (CA of 11–13 years), and adults (CA of 43–68 years)]. A support vector machines discriminant analysis (SVMDA) model was constructed, which demonstrated high accuracy in correctly predicting blood donors’ age groups where the lowest cross-validated sensitivity and specificity values were 0.96 and 0.97, respectively. Overall, this preliminary study demonstrates the high selectivity of Raman spectroscopy for differentiating between blood donors based on their CA. The demonstrated capability completes our suite of phenotype profiling methodologies including the determination of sex and race. CA determination has particular importance since this characteristic cannot be determined through DNA profiling unlike sex and race. When completed, the developed methodology should allow for phenotype profiling based on dry traces of body fluids immediately at the scene of a crime. The availability of this information within the first few hours since the crime discovery could be invaluable for the investigation.

Analytical chemistry, being a highly valuable measurement science, is widely used in countless areas of research and practical applications. Similar to other fields of science, analytical chemistry is constantly evolving. Over the past decade or so, strong effort has been shown in the development of small, portable, user-friendly devices for a variety of applications ranging from point of care testing (PoCT) to geological in-field investigations and to industrial process control.1 Raman spectroscopy has demonstrated compelling potential for implementation into multtarget, universal, portable instruments for in situ sample analysis. This approach has already provided significant benefits to a variety of applications including forensics, medicine, military, and pharmaceuticals. Regarding forensic science in particular, evidence could be analyzed directly at a crime scene and in its packaging in a nondestructive and confirmatory manner.

The field of analytical forensics is booming, as evidenced by the hundreds of ground-breaking studies published annually and a significant amount of novel research being carried out, in part fueled by criticisms from the National Academy of Sciences 2009 report.2 Within the past decade, major improvements have been made in forensic science. These advancements have offered increased capabilities to obtain more probative information from all types of evidence.

Most recently we have demonstrated the superb potential for Raman spectroscopy to procure information about an individual through phenotype profiling studies. These included determining the sex and race of donors based on unique spectroscopic signatures of body fluids.3−6 In this work we demonstrate a proof-of-concept study to determine the age group of an individual as a means to complete our growing suite of phenotype profiling methodologies. This study has particular importance since a person’s chronological age cannot be determined through DNA profiling unlike a person’s sex and race. When completed, the developed methodology should allow for phenotype profiling based on dry traces of body fluids immediately at the scene of a crime. The availability of this information within the first few hours since the crime discovery could be invaluable for the investigation.

The identification of individuals and differentiation between humans based on certain characteristics has always been an important and intriguing aspect of forensic science. In particular, identifying the age of a victim or a suspect is a crucial part of any forensic investigation. Arguably, human classification began in 1879 with the institution of the Bertillon system of criminal identification.7 Better known as anthropometry, the French criminologist Alphonse Bertillon introduced the ability to “individualize” human beings based on specific physical attributes including dimensions of one’s face, the height of a person, and measurements of other body parts. However, this

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“science” was soon replaced by fingerprint matching and, much later on, DNA profiling became a mainstay. Although each of these techniques are very useful and provide important information, none of them can identify a person’s chronological age.

Typically, estimating the age of an individual can be done using techniques afforded by forensic anthropology, which, in some ways, is similar to anthropometry. For this, a body (or skeleton) is needed to make proper measurements of the bones’ dimensions and structural morphology. However, in the absence of having a body or bones to examine physically, other methods need to be utilized. Eye witnesses may be able to provide information about a suspect or a victim, but including such details in testimony may prove difficult due to lack of validation. Fingerprint evidence analysis can be carried out, but this can require lengthy and sometimes destructive techniques. Also, when performing a fingerprint comparison with a database, such as the automated fingerprint identification system (AFIS), a match needs to be already in the database, which is not always the case.

Other types of evidence collected, such as bodily fluids, can be analyzed as well. For instance, blood found at a crime scene or elsewhere could be used to extract pertinent information. Most often a DNA profile is extracted from bodily fluid evidence and compared to a database, such as the combined DNA index system (CODIS). However, similar to fingerprint analysis, a DNA profile needs to already be present in the database for a match to be confirmed, which severely limits the results of the technique. Not to mention, procuring a DNA profile from any sample is a destructive process. Technology has advanced so that now certain human phenotypes such as eye color, hair color, and skin color can be identified. However, accurate identification (or estimation) of a person’s chronological age is currently not possible from analysis of blood samples by any of these methods.

It is known that human blood differs based on biological sex, chronological age, and health status. Differentiation of old and young healthy human donors has been shown by comparing levels of alkaline phosphatase (ALP) using a biocatalytic assay. Regarding donor age specifically, whole blood from newborns contains fetal hemoglobin (HbF), which differs from adult hemoglobin (HbA) in the volume of red blood cells per 100 mL of blood (hematocrit) and, more importantly, in its structure. Just like HbA, HbF is a tetramer containing four subunits, where both HbA and HbF have two identical α-subunits, but the two β-subunits in HbA are replaced by two γ-subunits in HbF. Therefore, due to its high selectivity, Raman spectroscopy should be capable of differentiating HbF from HbA rather easily. DNA-based approaches for estimating human age, specifically utilizing telomere length as a means for age differentiation, have already been demonstrated.

For the purposes of the method developed here, it is important to note that differentiation of blood donors based on their chronological age (actual numerical age) was targeted, and not differentiation based on donors’ biological age. The reason for this is because a person’s biological age can be affected by things such as health status (presence or absence of diseases), diet, physical fitness, and more. Therefore, two people who have the same chronological age, may not appear to be the same age biologically, due to the aforementioned conditions.

In this study, Raman spectroscopy and chemometrics were used to analyze blood from 45 human donors and differentiate between them based on their chronological age. The donors were split up into three classes consisting of newborns (with a chronological age of less than 1 year), adolescents (with a chronological age of 11–13 years), and adults (with a chronological age of 43–68 years). Spectral variations were first identified to determine specific differences between the age groups. Then, a support vector machines discriminant analysis (SVMDA) classification model was used to confirm the identity of the collected spectra as blood and not one of four other bodily fluids. For chronological age determination, a separate SVMDA model was constructed and then tested for its accuracy in predicting each donor’s age class.

RESULTS AND DISCUSSION

This proof-of-concept study targeted the distinction between human donors based on their chronological age using Raman spectroscopy of dried bloodstains. The specific objectives of the work included (i) confirmative identification of bloodstains as blood, (ii) visual spectral examination with the purpose of distinction between age groups, and (iii) building a classification model for donor age differentiation. For this study, donors were split into three age classes: a “newborn” class consisting of blood donors with a chronological age of less than 1 year, an “adolescent” class consisting of blood donors with a chronological age of 11–13, and an “adult” class consisting of blood donors with a chronological age of 43–68. Bloodstains from healthy Caucasian individuals consisting of 10 (5 male and 5 female) newborns, 15 adolescent males, and 20 (10 male and 10 female) adults were analyzed via Raman spectroscopy. Subsequent to Raman spectral collection from bloodstains of the 45 donors, the spectra were preprocessed and then loaded into a pre-existing SVMDA model for bodily fluid identification. Spectra were then visually scrutinized for similarities and differences, and finally used for calibrating and externally validating a novel chronological age differentiation SVMDA model.

During the initial stages of our human blood differentiation projects we used only male donors, but then expanded the adult data set to also include female donors for increased diversity. It is known that differences exist in the composition of blood between males and females. Overall, certain components (e.g., hematocrit, lipid content, etc.) of whole blood are lower for adult females as compared to adult males. Furthermore, it is known that women begin menopause in their late 40s to early 50s which could have an effect on their blood content and therefore result in spectral variations. We have recently shown that these biological differences are significant enough to allow for distinguishing between adult blood donors based on sex using Raman spectroscopy.

In the current foundational proof-of-concept study, the donor pool of the adolescent class was limited to include only male donors. This was done intentionally because blood from donors in the adolescent age range studied here had not previously been analyzed, and these donors were at, or around, the age when puberty begins. Additionally, research studies based on blood from adolescents of the age range surveyed here dwarf those from adolescents of the age range studied here. Furthermore, the spectral differences that exist between human donors based on biological sex are much smaller than those based on donors’ ages, as is explained in detail below.

Even with this prospective “limitation” in the selection of donors, we were able to obtain superb separation between newborns, adolescents, and adults, allowing for high accuracy in chronological age predictions. In future studies we plan to include female donors into the adolescent age group, as well as
introduce race variability into all three age classes since we have previously shown the ability to distinguish adult donors based on race using Raman spectroscopy. These objectives are beyond the scope of this work, but are important for subsequent studies to determine if in fact any additional changes exist and/or if the results for differentiating blood donors based on chronological age are truly affected by a donor’s biological sex or race.

**Identification of Bloodstains.** Prior to building any statistical models to differentiate blood donors based on chronological age, the stains were confirmed to be blood, and not one of four other bodily fluids (i.e., saliva, semen, sweat, or vaginal fluid). To do this, a recently developed SVMDA classification model was used. The blood spectra collected for this study were tested by loading them into the prebuilt SVMDA model as external predictions. The model demonstrated 100% accuracy in correctly predicting all 1350 blood spectra from all donors in each of the three age groups as peripheral blood and not any of the other four bodily fluids (data not shown). This result is expected since all of the blood spectra looked most similar to that of a standard blood spectrum, especially in comparison to spectra of the other four bodily fluids. More importantly, this demonstrates the selectivity of Raman spectroscopy and that the chronological age of a blood donor should not have an effect on the ability of the pre-existing SVMDA model to identify an unknown stain as blood.

**Donor Age Comparisons of Raman Blood Spectra.** The averaged raw and preprocessed (baseline corrected and normalized) spectra (colored by age class) for all 45 (10 newborn, 15 adolescent, and 20 adult) donors used for the study are shown in Figure S1 and Figure 1, respectively. These spectra are very similar, specifically when comparing the number of peaks and their Raman shift (cm$^{-1}$). This is not surprising, because it is well-known that the peaks in a Raman spectrum of dried blood, analyzed with 785 nm excitation, can be exclusively attributed to vibrational modes of hemoglobin (Hb).27−29 However, variability in the relative intensity and shape of certain peaks exists when comparing the three age groups (Figure 1). This provides good indication that differentiation of bloodstains based on donors’ chronological age should be possible, particularly when incorporating multivariate statistical analysis.

From Figure 1 it is evident that, for newborn blood donors, as compared to the adolescent and adult blood donors, the relative intensity is (i) higher for the peaks at 320, 375 (metHb marker), 1030, 1570, 1620, and 1667 cm$^{-1}$ (amide III) as well as the peaks around 900, 970, 1370 (ν$_s$), 1450, and 1600 cm$^{-1}$ (ν(C$_n$−C$_n$)). These differences are mostly attributed to vibrational modes of the polypeptide portion of Hb, and provide indication that blood composition (and Hb structure) is different for these three age classes. More specifically, these differences can, in part, be attributed to variations in HbA (adults) and HbF (newborns).

The peak at 375 cm$^{-1}$ is a known marker for metHb, which has been identified as being important for determining the time since deposition (TSD) of bloodstains.30,31 The newborn and adolescent donors had a higher relative intensity for this peak as compared to adult donors. From the inset in Figure 1, it is clear that the standard deviation of both the adolescent and adult donors does not overlap at all with that of the newborn donors, demonstrating significant differences between those age groups. The fact that all bloodstains were analyzed in the same manner (i.e., same amount of time in between sample preparation and analysis), yet there was a difference in the average intensity for the peak at 375 cm$^{-1}$ between the three age groups, provides evidence that this peak can also help to differentiate the chronological age of the donor in addition to a bloodstain’s TSD. In fact, the variations with respect to donor age seen for the 375 cm$^{-1}$ peak here are much more pronounced than those we have previously witnessed for bloodstain TSD studies.30,31

The 1210−1270 cm$^{-1}$ spectral region is part of the amide III region, where differences in protein secondary structure can be elucidated: specifically α-helix, β-sheet, and/or unordered polypeptide content.32 Intensity variations for this region are not surprising, because of the known differences in the relative amounts of whole blood components (e.g., red blood cells, lipids, proteins, etc.) based on age of a blood donor.33 More specifically, the known structural differences between HbA and HbF are most likely playing a role here. This strengthens the notion that even though the biological differences in whole blood of these donors are relatively minor, Raman spectroscopy is selective enough to probe for them.

**Blood Donor Age Differentiation.** Since it is evident that spectral variations exist between the newborn, adolescent, and adult blood donors, chemometrics was incorporated to enhance the differentiation and provide statistical confidence levels. SVMDA was used to classify the spectra belonging to the three different age groups. The model was constructed using a calibration data set of 1080 preprocessed spectra from 8 of the 10 total newborn blood donors, 12 of the 15 total adolescent blood donors, and 16 of the 20 total adult blood donors. These donors, used for model calibration, were arbitrarily selected using a random number generator.

The internal cross-validated (CV) prediction results represent the most stringent test for the model, before performing external validation. The CV results demonstrated that there is adequate, but not complete, separation between the blood donors’ age classes. Under CV predictions, 16 (14 being from one donor and two from a different donor) of the 360 adolescent spectra were misclassified as adult, and 4 of the 480 adult spectra were misclassified as adolescent; all of the newborn donors’ spectra were predicted correctly. The number of misclassifications can be identified as a way to indicate the model’s prediction perform-
Table 1. Cross-Validated (CV) and External Validation (ext. val.) SVMDA Prediction Results for Distinguishing between Donors from Three Age Groups: Newborn (Aged Less than 1 Year), Adolescent (Aged 11–13 Years), and Adult (Aged 43–68 Years)

| model parameters | newborn     | adolescent | adult      |
|------------------|-------------|------------|------------|
| sensitivity CV   | 1.00        | 0.96       | 0.99       |
|                  | ext. val.   | 0.95       | 1.00       | 0.98       |
| specificity CV   | 1.00        | 0.99       | 0.97       |
|                  | ext. val.   | 1.00       | 0.98       | 0.99       |
| predicted as newborn CV | 240 | 0         | 0          |
|                  | ext. val.   | 57         | 0          | 0          |
| predicted as adolescent CV | 0 | 344       | 4          |
|                  | ext. val.   | 1          | 90         | 2          |
| predicted as adult CV | 0          | 16        | 476        |
|                  | ext. val.   | 2          | 0          | 118        |

Although the results for model calibration are promising, the true test for any model is to perform external validation. By taking this approach, new blood spectra from completely different donors that the model had never encountered before were analyzed. This can be considered as a type of mock forensic evidence and used to gauge how well the model can predict spectra from completely unknown blood donors as would occur in actual casework. To perform this critical step of testing the prediction accuracy of the model for validation, the 270 spectra from the nine donors (two newborn, three adolescent, and four adult) randomly selected and set aside in the beginning as the validation data set (not included as part of the calibration data set used to build the SVMDA model) were used.

Analyzing CV predictions is the most rigorous step for calibration spectra, but this option is not available for external validation spectra. Instead, the most robust prediction scores come from strict class predictions. Figure 2 shows a strict class prediction scores plot for all spectra (calibration and external validation) from all three age classes. For a strict class prediction scores plot, the results show one overall prediction score (i.e., class) per spectrum. Strict class predictions are determined based on a default threshold of 50%. If a spectrum has a prediction score higher than this threshold for one specific class, it will be predicted as belonging to that class. However, if a spectrum has a prediction score above this threshold for more than one class, or lower than this threshold for all classes, it will be predicted as unclassified (class 0). For SVMDA model building purposes, each class (i.e., newborn, adolescent, and adult) is assigned a “dummy” value, which is a placeholder (in the form of a number) assigned to represent the user-defined class name (on the y-axis of a scores plot). Therefore, spectra receive a qualitative value of 1, 2, or 3, respectively, for class predictions as a newborn, adolescent, or adult donor.

As shown in the strict class prediction scores plot (Figure 2), only seven (four adolescent and three adult) spectra out of the 1080 total spectra in the calibration data set were misclassified, while all 240 spectra from the newborn donors were predicted correctly. Upon external validation, three spectra from the newborn donors were unclassified, and one was misclassified to the adolescent age class, while all 90 spectra from the three adolescent donors were correctly classified, and two spectra from one adult donor were misclassified to the adolescent age class. This resulted in sensitivity values of 0.95, 1.00, and 0.98, and specificity values of 1.00, 0.98, and 0.99, for external predictions of the newborn, adolescent, and adult age classes, respectively (Table 1).

It is important to note that there were no false negative assignments to the newborn blood class. That is, none of the calibration or external validation blood spectra from either the adolescent donors or the adult donors were misclassified to the newborn class. This means that the model is well-trained to identify and differentiate newborn blood from that of older age groups. This is not surprising since it is known that HbF is structurally different than HbA by having two γ-subunits (gamma-subunits) instead of two β-subunits (beta-subunits) as part of their Hb tetramer; the other two subunits for both types of Hb are identical α-subunits (alpha-subunits). The amino acid sequence for the γ-chain in HbF is 28% different as compared to the β-chain in HbA. Also, relative to HbA, HbF has reduced 2,3-bisphosphoglycerate (2,3-BPG) binding affinity, which, in turn, increases the ability of HbF to bind oxygen.16,17 These biochemical differences were evident by the appreciable visual differences in the spectra between newborn blood donors and both adolescent and adult blood donors.

Lastly, the SVMDA model constructed here represents one of hundreds of models that could be constructed for blood donor age differentiation purposes. Therefore, we have performed an additional study, outlined in the Supporting Information, where 45 new SVMDA models were constructed, each using n − 1 donors, from those surveyed here, to build the model and the remaining donor to test the model.

| MATERIALS AND METHODS |
|------------------------|

Blood Samples and Spectral Collection. For this study, 45 human peripheral blood samples, all from Caucasian donors,
were used. All blood samples were purchased from BioreclamationIVT, LLC, where blood was collected via venipuncture from donors who provided informed consent. The blood cohort surveyed consisted of 10 (5 male and 5 female) donors in the “newborn” age range (≤1 year old), 15 male donors in the “adolescent” age range (11–13 years old), and 20 (10 male and 10 female) donors in the “adult” range (43–68 years old). Blood samples were kept frozen until preparation, where 30 μL of blood was deposited onto an aluminum foil covered microscope slide, and allowed to dry overnight, prior to spectral collection. Throughout this study, no unexpected or unusually high safety hazards were encountered.

A Renishaw inVia Raman spectrometer, controlled by WiRE software (version 3.2), was used for sample analysis. The instrument was equipped with a Leica optical microscope, and a 20× long working distance objective was used along with a PRIOR automatic stage. A silicon standard was used to calibrate the instrument daily, before any measurements were taken. A 785 nm excitation source at 5% laser power (~4.0 mW) was used to irradiate every sample 20 times, for 10 s each. Spectra were recorded in the range 250–1800 cm⁻¹ using Raman mapping from 30 different spots for each sample.

**Statistical Analysis.** All blood spectra were imported into MATLAB (version R2013a; MathWorks, Inc.) for statistical analysis. All data preparation and construction of statistical models were performed with the PLS Toolbox (version 8.0: Eigenvector Research, Inc.) operating in MATLAB. Each spectrum was truncated to 300–1800 cm⁻¹, and then preprocessed by baseline correction using an automatic weighted least squares (AWLS) algorithm (6th order polynomial), normalized by total area, and mean centered before building any statistical models.

The confirmation that bloodstains were blood was carried out first. A recently developed methodology²⁶ for differentiation of bodily fluid traces (i.e., peripheral blood, saliva, semen, sweat, or vaginal fluid) using SVMDA was implemented for predicting the identity of bloodstain spectra for each donor. To perform identity predictions for the bloodstains, the preprocessed spectra were loaded into the prebuilt SVMDA model, and results were generated.

The entire data set of 1350 spectra was split into calibration and external validation data sets, subsequent to preprocessing. The calibration data set contained 1080 spectra from 8 (4 male and 4 female) of the 10 total newborn donors, 12 of the 15 total adolescent male blood donors, and 16 (8 males and 8 females) of the 20 total adult donors, collectively. The validation data set contained the other 270 spectra from the 9 remaining donors [2 newborn donors (1 male and 1 female), 3 adolescent male donors, and 4 adult donors (2 males and 2 females)]. A SVMDA model was constructed using the 1080 preprocessed spectra contained in the calibration data set using partial least squares (PLS) compression with five components and 286 significant factors. The model was internally cross-validated (CV) using the venetian blinds method with 10 splits, and then externally validated with the 270 spectra set aside at the beginning.

**CONCLUSIONS**

Here, a novel proof-of-concept method using Raman spectroscopy and chemometrics was developed to distinguish between blood donors corresponding to three different age groups including newborns (aged less than 1 year), adolescents (aged 11–13), and adults (aged 43–68). All spectra, regardless of the donor’s age, were correctly predicted as peripheral blood (and not one of four other common bodily fluids) using a recently developed SVMDA bodily fluid identification technique.²⁶ The spectral differences between the three age groups (classes) surveyed indicated that blood composition varies for donors in these age groups. With the implementation of multivariate statistical classification analysis, specifically SVMDA, the three human age groups could be differentiated with high accuracy. The sensitivity and specificity values varied from as low as 0.95 (external validation sensitivity for newborns) to as high as 1.00 (multiple occurrences).

The results procured indicate the superb selectivity of Raman spectroscopy, enhanced through chemometrics, for differentiating between blood donors based on their chronological age. Although it is known that a person’s diet, health, and other factors can affect their biological age, these variables did not seem to affect our results for correctly predicting a person’s chronological age. However, additional studies will need to be carried out to confirm this, specifically by analyzing blood samples from individuals with disparities in their diet, health status, exercise regimen, and more.

This approach could provide significant benefits to advancing the field of forensic science, specifically serology, to help narrow down a suspect pool or help determine the age of a victim. To expand upon this study in the future, a cohort of donors varying in sex (particularly for the adolescent age group), race, age, and health status should be incorporated to account for a more diverse population. Subsequently, new classification models should be constructed for differentiation purposes. If a significant enough number of donors for individual ages, or specific age groups, are included, regression analysis can be performed for more quantitative age predictions.

It is to be expected that the more diverse the donor pool is, the more variation there will be in the blood spectra. The additional spectral differences should aid in the enhanced ability to distinguish between donors of closer age ranges so long as the variations within a specific age (or age range) are less than those between ages (or selected age ranges). Through the inclusion of these types of donors, a significant advancement of this proof-of-concept methodology would be attainable. Furthermore, by incorporating this work into the continually growing suite of chemometric models our group has developed thus far (i.e., time since deposition of bloodstains, donor sex prediction, and donor race prediction) we can move one step closer to having a more complete “picture” of a person based on spectra collected from a bloodstain at the beginning of an investigation. Ultimately, the methodology will utilize a cascade of chemometric models, each with a high level of statistical confidence, to efficiently provide personally identifiable information in addition to a proposed timeline. This information would substantially contribute to the investigation and help to ensure correct resolution of a crime.

**ASSOCIATED CONTENT**

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.8b00198.

Additional discussion, SVMDA modeling results, and averaged raw spectra (PDF)

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Notes

The authors declare no competing financial interest.

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