Quantification of hemoglobin in human blood to differentiate individuals from one another

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QUANTIFICATION OF HEMOGLOBIN IN HUMAN BLOOD TO DIFFERENTIATE INDIVIDUALS FROM ONE ANOTHER

by

Audrey Jeanne Auleley

A Thesis
Submitted to the University at Albany, State University of New York
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Master of Science

College of Arts & Sciences
Department of Chemistry
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ABSTRACT

Blood is one of the principal sources of evidence in crime scene investigations. The level of hemoglobin in human blood is known to hardly fluctuate over time in a healthy adult patient. Using a method like the sodium lauryl sulfate (SLS) method for hemoglobin level determination is beneficial because SLS converts any other derivates of hemoglobin into methemoglobin. The amount of hemoglobin would be determined using the linear correlation between the absorbance of methemoglobin and its amount in blood samples. Those curves differ significantly from sample to sample. Therefore, a differentiation can be observed between the blood samples of different originators. Ultraviolet-Visible spectrophotometry and an image processing software were utilized to quantify the amount of hemoglobin in different human whole blood samples. The data obtained was used to plot different graphs, each one representing an individual. A distinction between a fresh blood sample and an aged blood sample is determined using enzyme-linked immunosorbent assay (ELISA) by tracking the change in absorbance of horseradish peroxidase (HRP) present in blood serum. Based on the results obtained, a clear difference in change of absorbance between a fresh and one-week old blood serum is observed. Future development and further analysis may lead to the conception of a small device using the method of quantification of hemoglobin proposed in this project, with which forensic investigators would be able to use directly on-site and get an idea of how many people were on a crime scene.
DEDICATION

I would like to dedicate this thesis to my parents, Solange and Guy-Robert, who have given me the opportunity of an excellent education, thank you for your encouragement and guidance throughout my life. This thesis is also dedicated to my brother, Michel, who has always been a person I can look up to and a great friend. Finally, to my family and friends who have shown loved and support throughout my studies, I could not have done it without you.
ACKNOWLEDGMENTS

I would like to heartily express my appreciation to my research advisor, Dr. Jan Halámek, whose guidance from the beginning to the end of this project helped me grow as a graduate student and more generally, as a scientist, and develop my understanding in this subject.

I would also like to offer my regards and blessings to my labmates who supported me in any respect during the completion of my project, and a special thank you to Erica Brunelle for helping me get the materials I needed and Mindy Hair whose contribution was beneficial to part of my project.
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<th>Description</th>
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<tr>
<td>ABTS</td>
<td>2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>KCN</td>
<td>Potassium cyanide</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>m</td>
<td>Meter</td>
</tr>
<tr>
<td>M</td>
<td>Mole per liter</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>μ</td>
<td>Micro</td>
</tr>
<tr>
<td>n</td>
<td>Nano</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>Phosphate Buffered Saline - Tween</td>
</tr>
<tr>
<td>SLS</td>
<td>Sodium Lauryl Sulfate</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet–visible</td>
</tr>
<tr>
<td>1°Ab</td>
<td>Primary antibody</td>
</tr>
<tr>
<td>2°Ab</td>
<td>Secondary antibody</td>
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CHAPTER ONE

INTRODUCTION

1.1 Overview

Blood is one of the principal sources of evidence in crime scene investigations. Deoxyribonucleic acid (DNA) contained in blood is mostly the only component forensic investigators are looking for in blood samples because it is the most widely accepted means of identification (Nelson). However, the analysis of DNA is still mainly a lab-based technique and is very time-consuming. Blood is composed of a lot of other molecules, such as hemoglobin (Hb) and horseradish peroxidase (HRP). The development of on-site methods of analysis using the proprieties of those two compounds present in blood samples found on crime scenes would be beneficial for crime scene investigators and be an alternative to the analysis of DNA.

1.2 Hemoglobin in human blood

The level of hemoglobin in human blood is known to barely fluctuate over time in a healthy adult patient. However, it differs from individual to individual (Gedye 382). Moreover, several methods to determine the level of hemoglobin in blood are known and widely used. Nowadays, those methods are mostly used for medical and clinical research, to detect the presence of anemia for example (Lamhaut 548). One of the most-used methods for measuring hemoglobin levels is based on photometric detection of a specific form of hemoglobin, cyanmethemoglobin. HemoCue can be found as an alternative to this technique and developed a photometric method based on the determination of another form of hemoglobin, azide metahemoglobin (Rosenblit 108).

However, using a method like the sodium lauryl sulfate (SLS) method for hemoglobin
determination is beneficial because it will convert hemoglobin into methemoglobin in the order of
oxyhemoglobin, hemochrome, and methemoglobin with its oxidative activity which does not
create toxic wastes such as KCN unlike the cyanide hemoglobin method, which is part of the well-
known methods for hemoglobin determination (Oshiro 83). The conversion will useful when the
amount of hemoglobin is determinate by UV-Vis spectrophotometry.

Different applications of digital camera in analytical chemistry have been developed in
more than a decade, especially smartphone cameras. Indeed, for example, smartphones are used
for spectrometer for colorimetric biosensing application (Wang 3233), proton concentration
measurements conducted on pH paper (Chang 549) and colorimetric paper sensor array for the
detection and discrimination of explosives using a smartphone (Salles 2047). The spectral
sensitivity of cameras as a function of wavelength can be divided in three peaks: one peak in the
blue region, one peak in green and one peak in the blue region of the visible absorption spectrum
(Jiang 2013).

1.3 Enzyme-linked immunosorbent assay

For more than a century, enzyme-linked immunosorbent assay (ELISA) has been used to
identify blood samples. According to Cattaneo, this assay has been used to detect human albumin,
IgG, beta thromboglobulin in bloodstains and also the stability of protein in dried blood (140).
Nowadays, horseradish peroxidase is commonly used in ELISA due to its production of colored
products, whose absorbances can be analyzed using UV-Vis spectroscopy (Beyzavi 145).

1.4 Purpose/Goal

In this project, it was hypothesized that the amount of hemoglobin from different blood
samples would be determined using the SLS method and differ so significantly from sample to sample that a differentiation can be made between the blood samples of different originators. It was believed that UV-Vis spectrophotometry, as well as image processing analyses, would demonstrate the potential applicability of SLS method combined with a small device to get confirmation as quickly as possible that bloodstains left on crime scenes belong to one or more individuals. The motivation for using a smartphone camera is to determine if it could be used as a field-instrument to replace a UV-Vis spectrophotometer, which is a bulky instrument. Indeed, a smartphone is a very portable device, which can take pictures with great resolution and can run image processing software to possibly get results instantly.

It was also hypothesized that a distinction between a fresh blood sample and an aged one would be determined using enzyme-linked immunosorbent assay by tracking the change in absorbance of HRP present in blood serums (Cattaneo 139).
CHAPTER TWO

QUANTIFICATION OF HEMOGLOBIN IN HUMAN BLOOD

2.1 Materials and methods

Before using the SLS method, human hemoglobin calibration curve was plotted. Human hemoglobin lyophilized powder was purchased from Sigma-Aldrich. Four standard solutions from 0.1 mg/mL to 0.5 mg/mL of hemoglobin in phosphate buffer (pH = 7.41) were prepared.

Normal K2-EDTA whole blood from ten different donors were purchased from ProMedDx. The samples were stored in a freezer.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Sex</th>
<th>Age (years old)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>53</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>52</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>55</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>31</td>
</tr>
<tr>
<td>5</td>
<td>Male</td>
<td>55</td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>64</td>
</tr>
<tr>
<td>7</td>
<td>Male</td>
<td>28</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>28</td>
</tr>
<tr>
<td>9</td>
<td>Male</td>
<td>59</td>
</tr>
<tr>
<td>10</td>
<td>Male</td>
<td>22</td>
</tr>
</tbody>
</table>

Table 1. Blood donors’ sex and age

To determine the amount of hemoglobin in human whole blood sample, the following steps were taken: a 2.08 mM Sodium Lauryl Sulfate-Phosphate buffer (pH = 7.41) working solution was prepared, which was then added to Eppendorf tubes in which different volumes of human blood, 1.00 µL, 1.09 µL, 1.21 µL, 1.35 µL, 1.52 µL, 1.75 µL, 2.06 µL, 2.51 µL, and 3.20 µL, were added to a final solution of 1.6 mL.
Table 2. Dilution factor of each standard solution

<table>
<thead>
<tr>
<th>Volume of blood in tube (µL)</th>
<th>Dilution Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>1600</td>
</tr>
<tr>
<td>1.09</td>
<td>1463</td>
</tr>
<tr>
<td>1.21</td>
<td>1325</td>
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<tr>
<td>1.35</td>
<td>1188</td>
</tr>
<tr>
<td>1.52</td>
<td>1050</td>
</tr>
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<td>1.75</td>
<td>913</td>
</tr>
<tr>
<td>2.06</td>
<td>775</td>
</tr>
<tr>
<td>2.51</td>
<td>638</td>
</tr>
<tr>
<td>3.20</td>
<td>500</td>
</tr>
</tbody>
</table>

The solutions were incubated at room temperature for 15 minutes before adding each solution to the wells of the plates. As a control for image processing, a 3.50 mg/mL of Supernatural Kitchen red food dye solution in deionized water was prepared.

Using clear 96-well plates, absorption curves of hemoglobin and absorbance values for the different volumes of blood were recorded by spectrophotometer SpectraMax Plus 384 Absorbance Plate Reader and pictures of each plate were taken from an iPhone 8 Plus camera and analyzed using the image processing software Fiji ImageJ.

2.2 Results and discussion

Plotting known concentrations of human hemoglobin standard solutions against their absorbances provide to this project its shape.

Figure 1 shows that the strongest absorption peak of absorption of the human hemoglobin solutions peaks at a wavelength of 405 nm. Because there is the most signal there, the following graph only track absorbance at 405 nm (figure 2). Therefore, the absorbance values of the four standard solutions of human hemoglobin were read at 405 nm. Figure 2 demonstrates that the plot
of a calibration curve depending on the amount of hemoglobin is linear, revealing that the relationship between the amount of hemoglobin and the absorbance is directly proportional. The coefficient of determination \( R^2 \) is equal to 0.997. The value is quite close to 1, which demonstrates how well the regression model fits the data obtained. Therefore, the use of UV-Vis spectrophotometry is justified.

UV-Vis spectrophotometry was applied to real human blood samples whose hemoglobin amounts are unknown, however the trendline expected for their curves is linear. Figure 13 shows that the strongest absorption peak of hemoglobin in human blood samples is at a wavelength of 415 nm. Because there is the most signal there, the following graphs only track absorbance at 415 nm (figures 14 to 22). Figures 14 to 24 illustrate the absorbance depending on the volume of blood samples added in the final solution. Each graph follows a linear trendline. Indeed, in figure 14, the trendline of the absorbance versus the amount of hemoglobin in human blood sample 1 has a coefficient of determination \( R^2 \) equal to 0.9905. This value is close to 1, which shows that the linear regression fits the data obtained. In figure 15, the trendline of the absorbance versus the amount of hemoglobin in human blood sample 2 has a coefficient of determination \( R^2 \) equal to 0.9755. This value is close to 1, which shows that the linear regression fits the data obtained, however it is not equal or greater than 0.99, which can be explained by the data point for 1.21 \( \mu \)L of blood in final solution being a bit further away from the trendline. In figure 16, the trendline of the absorbance versus the amount of hemoglobin in human blood sample 3 has a coefficient of determination \( R^2 \) equal to 0.9861. This value is close to 1, which shows that the linear regression fits the data obtained. In figure 17, the trendline of the absorbance versus the amount of hemoglobin in human blood sample 4 has a coefficient of determination \( R^2 \) equal to 0.948. This value is close to 1, which shows that the linear regression fits the data obtained, however it is not
equal or greater than 0.99, which can be explained by the data points for 2.06 and 2.51 µL of blood in final solution being a bit further away from the trendline. In figure 18, the trendline of the absorbance versus the amount of hemoglobin in human blood sample 5 has a coefficient of determination $R^2$ equal to 0.9861. This value is close to 1, which shows that the linear regression fits the data obtained. In figure 19, the trendline of the absorbance versus the amount of hemoglobin in human blood sample 6 has a coefficient of determination $R^2$ equal to 0.9859. This value is close to 1, which shows that the linear regression fits the data obtained. In figure 20, the trendline of the absorbance versus the amount of hemoglobin in human blood sample 7 has a coefficient of determination $R^2$ equal to 0.9907. This value is close to 1, which shows that the linear regression fits the data obtained. In figure 21, the trendline of the absorbance versus the amount of hemoglobin in human blood sample 8 has a coefficient of determination $R^2$ equal to 0.9843. This value is close to 1, which shows that the linear regression fits the data obtained. In figure 22, the trendline of the absorbance versus the amount of hemoglobin in human blood sample 9 has a coefficient of determination $R^2$ equal to 0.977. This value is close to 1, which shows that the linear regression fits the data obtained, however it is not equal or greater than 0.99, which can be explained by the data points for 1.75 and 3.20 µL of blood in final solution being a bit further away from the trendline. In figure 23, the trendline of the absorbance versus the amount of hemoglobin in human blood sample 10 has a coefficient of determination $R^2$ equal to 0.9129. This value is close to 1, which shows that the linear regression fits the data obtained, however it is not equal or greater than 0.99, which can be explained by the fact that three data points, for 1.51, 1.75 and 3.20 µL of blood in final solution, are further away from the trendline. Although small amounts of blood were used, signals were still able to be read, which indicate how sensitive the spectrophotometer is. Each sample solution was measured in quintuplets, and mostly each data
point demonstrates narrow error bars, except the graph in figure 16 and figure 22, where for the last data point at 3.20 µL, the error bars are much broader. To minimize these errors, a more significant number of replicates should be analyzed in the future.

Figure 24 compares the trendline of each sample. Even though a few data points are overlapping, a clear distinction between each line can be observed. This observation leads to believe that quantifying the amount of hemoglobin in human blood can be used to distinguish the blood samples’ originators from one another.

Pictures taken from a smartphone camera (figures 3 to 12) were analyzed using an image processing software, whose ability to measure mean gray area was used to calculate the ratio between control, the red dye, and the blood samples in SLS-Phosphate buffer. Figures 25 to 34 present a trend shared by each sample’s curve. Indeed, the ratio of mean gray values slightly increases as the amount of hemoglobin in human blood increases. Figure 35 demonstrates that each curve can be distinguished from one another. However, due to the difference in lighting and slightly difference between each picture, the quantification of hemoglobin level is so far challenging when using the mean gray values. Indeed, the curve corresponding to sample 1 has a range of ratio of mean gray values lower than the other curves. It can be explained by the different in the lighting in the image taken by the smartphone’s camera (figure 3). Those challenges should be addressed in the future. Having a consistency in the parameters and settings of this process, such as the parameters of the camera and the lighting of the surroundings when taking pictures of the wells, is key to improve this method. The use of a smartphone camera was to determine if it could be used as a field-instrument to replace UV-Vis instrument, that is why smartphone pictures results are compared to the standard curves measured by the UV-vis.
CHAPTER THREE
DISTINCTION BETWEEN FRESH AND AGED BLOOD SAMPLES

3.1 Materials and methods

To design the best model to distinguish a fresh blood sample and an old blood sample, changes in different parameters were made and compared to see which combination of parameters give the most accurate results. Three blood serums were purchased from ProMedDx renamed serum A, B and C. The samples were stored in a freezer. Affinite Pure Donkey anti-human Ig and Peroxidase-conjugated AffiniPure Donkey anit-human IgG were purchased from Jackson ImmunoResearch. Bovine Serum Albumin (BSA) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were purchased from Sigma-Aldrich.

The first step was to compare the change in absorbance in horseradish peroxidase in each fresh blood serum depending on the amount of blood serums used in the enzyme-linked immunosorbent assay. The serums were diluted 10,000 times (10 K), 20,000 times (20 K) and 50,000 (50 K) in Phosphate Buffered Saline. 100 µL of 5 µg/mL Affinite Pure Donkey anti-human Ig, used as the primary antibody (1°Ab) in 0.1 M diluted in carbonate buffer pH = 8.5 were added in wells of a 96-wells plate (triplicates for each blood serum and blank) and left in a fridge overnight. 1°Ab was then removed from the plate and wells washed with 200 µL of 0.2% Phosphate Buffer Saline-Tween three times. 250 µL of 1% Bovine Serum Albumin diluted in PBS were added in each well and left to incubate at room temperature for 1 hour. 1% BSA was removed from the plate and the wells washed with 200 µL 0.2% PBS-T three times. 50 µL of diluted blood serums (10K, 20K or 50K) were introduced in their respective wells. No blood serum was placed in wells for the blanks. The plate was left to incubate at room temperature for 1 hour. Diluted blood
serums were removed from the plate and the wells were washed with 200 µL 0.2% PBS-T three times. 100 µL of 1,000 (1K) diluted Peroxidase-conjugated AffiniPure Donkey anit-human IgG, used as secondary antibody (2°Ab), diluted in PBS were added in each well and left to incubate at room temperature for 30 minutes. 2°Ab solutions were removed from the plate and wells washed with 200 µL 0.2% PBS-T three times. 240 µL of citrate buffer, 30 µL of 1 mM ABTS and 30 µL of 1 mM H₂O₂ were placed in each well. The plate was read by a spectrophotometer SpectraMax Plus 384 Absorbance Plate Reader at 405 nm for 10 minutes with 3 seconds shake before and between reads.

The second step was to compare the change in absorbance in HRP in each fresh blood serum depending on the concentration of 2°Ab used in ELISA. The peroxidase-conjugated AffiniPure Donkey anit-human IgG was diluted 1,000 times (1 K), 5,000 times (5 K) and 10,000 (10 K) in PBS. 100 µL of 5 µg/mL Affinite Pure Donkey anti-human Ig (1°Ab) in 0.1 M carbonate buffer pH = 8.5 were added in wells of a 96-wells plate (triplicates for each blood serum and blank) and left in a fridge overnight. 1°Ab was then removed from the plate and the wells were washed with 200 µL of 0.2% PBS-T three times. 250 µL of 1% BSA diluted in PBS were added in each well and left to incubate at room temperature for 1 hour. 1% BSA was removed from the plate and the wells washed with 200 µL 0.2% PBS-T three times. 50 µL of 10 K of diluted blood serums were added in their respective wells and left to incubate at room temperature for 1 hour. The diluted blood serums were removed from the plate and the wells were washed with 200 µL 0.2% PBS-T three times. 100 µL of diluted 2°Ab (1 K, 5 K or 10 K) in PBS were added in each well and left to incubate at room temperature for 30 minutes. 2°Ab solutions were removed from the plate and wells washed with 200 µL 0.2% PBS-T three times. 240 µL of citrate buffer, 30 µL of 1 mM ABTS and 30 µL of 1 mM H₂O₂ were placed in each well. The plate was read by a
spectrophotometer SpectraMax Plus 384 Absorbance Plate Reader at 405 nm for 10 minutes with 3 seconds shake before and between reads.

Once the right concentrations of blood serum and secondary antibody were chosen, the change of absorbance in HRP in fresh blood serums and in 1-week old blood serums were analyzed. Fresh blood serums were analyzed the day they were removed from the freezer and aliquots of 10 µL of blood serums in Eppendorf tubes were left out of the freezer, at room temperature, for a week for “aged” blood serum. 900 µL of PBS were added in each Eppendorf tube the day of the analysis, prior to adding the solutions into the plate. To achieve complete dilution, they were briefly vortexed. 100 µL of 5 µg/mL of 1°Ab in 0.1 M carbonate buffer pH = 8.5 were added in wells of a 96-wells plate (triplicates for each blood serum and blank) and left in a fridge overnight. 1°Ab was then removed from the plate and the wells were washed with 200 µL of 0.2% PBS-T three times. 250 µL of 1% BSA diluted in PBS were added in each well and left to incubate at room temperature for 1 hour. 1% BSA was removed from the plate and the wells washed with 200 µL 0.2% PBS-T three times. 50 µL of 10 K of diluted blood serums (fresh or one-week old) were added in their respective wells and left to incubate at room temperature for 1 hour. The diluted blood serums were removed from the plate and the wells were washed with 200 µL 0.2% PBS-T three times. 100 µL of 1 K times diluted 2°Ab in PBS were added in each well and left to incubate at room temperature for 30 minutes. 2°Ab solutions were removed from the plate and wells washed with 200 µL 0.2% PBS-T three times. 240 µL of citrate buffer, 30 µL of 1 mM ABTS and 30 µL of 1 mM H₂O₂ were placed in each well. The plates were read by a spectrophotometer SpectraMax Plus 384 Absorbance Plate Reader at 405 nm for 10 minutes with 3 seconds shake before and between reads.
3.2 Results and discussion

Figures 36 to 38 show the change in absorbance of HRP over time at 405 nm in three different fresh blood serums depending on the dilution factor of the antigen. In figure 36, the curve for the 10 K times diluted blood serum A presents a greater change in absorbance in HRP. The curve for the 50 K times diluted serum A has a slightly lower change in HRP. The values of the change in absorbance of HRP in the 20 K times diluted blood serum A is even lower. In figure 37, the curve for a dilution of the 10 K times diluted blood serum B shows a greater change in absorbance in HRP. The curve for the 50 K times diluted serum B has a slightly lower change in absorbance of HRP. The values of the change in absorbance of HRP in the 20 K times diluted blood serum B is quite lower. In figure 38, the curve for a dilution of the 10 K times diluted blood serum C shows a greater change in absorbance in HRP. The curve for the 50 K times diluted serum C has a slightly lower change in absorbance of HRP. The values of the change in absorbance of HRP in the 20 K times diluted blood serum C is even lower. For each blood serum, the three different concentrations of blood serum do not drastically modify the readability of the absorbance signals of HRP. Indeed, none of the graphs overlap the graph corresponding to the blank solution, which means a significant signal can still be observed. However, because the 10 K times diluted blood serum demonstrates greater signals of absorbance for each blood serum, 10 K dilution of serum was chosen in the design of the best model of ELISA to distinguish a fresh blood sample and an old blood sample.

Figures 39 to 41 show the change in absorbance of HRP over time at 405 nm in three different fresh blood serums depending on the dilution factor of the secondary antibody. In figure 39, the curve for a dilution of 1 K of secondary antibody presents a greater change in absorbance in HRP. The curve for a dilution of 5 K and 10 K dilution of antibody overlap the curve of
corresponding to the signals of the blank solution. Figure 40, the curve for a dilution of 1 K of secondary antibody presents a greater change in absorbance in HRP. The curve for a dilution of 5 K and 10 K dilution of antibody overlap the curve of corresponding to the signals of the blank solution. In figure 41, the curve for a dilution of 1 K of secondary antibody presents a greater change in absorbance in HRP. The curve for a dilution of 5 K and 10 K dilution of antibody overlap the curve of corresponding to the signals of the blank solution. For the blood serum A, B and C, the change in concentrations of secondary antibody does affect the readability of the absorbance signals of HRP. Indeed, the graphs corresponding to 5 K and 10 K dilution overlap the graph of blank solution, whereas the graphs of 1 K times diluted secondary antibody solution do not overlap with the blank ones, which means signals of absorbance can be read. Therefore, 1 K dilution of 2°Ab was chosen in the design of the best model of ELISA to distinguish a fresh blood sample and an aged blood sample.

Figure 42 to 44 show the change in absorbance of HRP over time at 405 nm in three different fresh and one-week old blood serums. In figure 42, the graph for fresh blood serum A has a greater change of absorbance in HRP over time than the graph corresponding to one-week old blood serum A. Indeed, the value of absorbance after 10 minutes is around 0.6 for the fresh serum whereas its value is less than 0.2 for the one-week old serum A. In figure 43, the graph for fresh blood serum B has a greater change of absorbance in HRP over time than the graph corresponding to one-week old blood serum B. The value of absorbance after 10 minutes is around 0.4 for the fresh serum whereas its value is less than 0.2 for the one-week old serum B. In figure 44, the graph for fresh blood serum C has a greater change of absorbance in HRP over time than the graph corresponding to 1-week old blood serum C. The value of absorbance after 10 minutes is around 0.6 for the fresh serum whereas its value is less than 0.3 for the one-week old serum C.
Based on those results, a clear distinction between a fresh and an one-week old blood serum can be made. In future studies, a larger span of time should be applied to blood serum to establish if a more precise time since deposition of blood samples would be determined using ELISA to read the absorbance of HRP. Also, each sample solution was measured in triplicates, that is why the error bars appear to be broad. To minimize those errors, a more significant number of replicates should be analyzed in the future.
CHAPTER 4

CONCLUSION

This method shows promising results. Further studies should be conducted to develop the method and support the idea of using hemoglobin level determination on crime scenes to help differentiate blood samples from different individuals.

In order to do so, blood samples from more donors should be analyzed. Moreover, more replicates for each standard should be prepared to lower errors. In this project, it was shown that fresh and aged blood samples can be differentiate. To take a step further, to be able to obtain more realistic results, an analysis and comparison between the amount of hemoglobin in fresh blood and aged and/or dried blood samples should be done. Being able to know how old the blood sample analyzed for hemoglobin quantification is will help with the comparison between bloodstains left on crime scene at different times.

A small device that forensic investigators would be able to use directly on-site and would give them an idea of how many people where on a crime scene would shorten the time spent during investigations, getting samples sent and analyzed to a laboratory. Future research could lead to the adoption of those devices by every forensic investigator in the country and serve as an indispensable tool to solve a crime.
REFERENCES


APPENDICES
APPENDIX A

Figure 1. Absorption spectra of human hemoglobin standard solutions
Figure 2. Concentration of human hemoglobin v. Absorbance (at 405 nm)

\[ y = 4.8951x + 0.0345 \]

\[ R^2 = 0.997 \]
Figure 3. Plate of blood sample 1

Figure 4. Plate of blood sample 2
Figure 5. Plate of blood sample 3

Figure 6. Plate of blood sample 4
Figure 5. Plate of blood sample 5

Figure 6. Plate of blood sample 6
**Figure 7.** Plate of blood sample 7

**Figure 8.** Plate of blood sample 8
Figure 9. Plate of blood sample 9

Figure 10. Plate of blood sample 10
Figure 13. Absorption spectra of blood samples
Figure 114. Absorbance v. amount of hemoglobin in human blood sample 1 at 415 nm

\[ y = 0.2667x + 0.00136 \]

\[ R^2 = 0.9905 \]
**Figure 125.** Absorbance v. amount of hemoglobin in human blood sample 2 at 415 nm

\[ y = 0.2951x + 0.0528 \]

\[ R^2 = 0.9755 \]
Figure 16. Absorbance v. amount of hemoglobin in human blood sample 3 at 415 nm

\[ y = 0.2036x - 0.0433 \]

\[ R^2 = 0.9861 \]
Figure 17. Absorbance v. amount of hemoglobin in human blood sample 4 at 415 nm

\[ y = 0.2411x + 0.0271 \]

\[ R^2 = 0.948 \]
Figure 18. Absorbance v. amount of hemoglobin in human blood sample 5 at 415 nm

\[ y = 0.2036x - 0.0433 \]

\[ R^2 = 0.9861 \]
Figure 19. Absorbance v. amount of hemoglobin in human blood sample 6 at 415 nm

\[ y = 0.2995x -0.018 \]

\[ R^2 = 0.9859 \]
Figure 13. Absorbance v. amount of hemoglobin in human blood sample 7 at 415 nm

$y = 0.376x + 0.0166$

$R^2 = 0.9907$
Figure 21. Absorbance v. amount of hemoglobin in human blood sample 8 at 415 nm

Absorbance (415 nm)

Volume of blood in final solution (µL)

$y = 0.2267x - 0.0092$

$R^2 = 0.9843$
Figure 14. Absorbance v. amount of hemoglobin in human blood sample 9 at 415 nm

\[ y = 0.3314x + 0.0765 \]

\[ R^2 = 0.9777 \]
Figure 15. Absorbance v. amount of hemoglobin in human blood sample 10 at 415 nm

\[ y = 0.3152x + 0.0419 \]

\[ R^2 = 0.9129 \]
Figure 24. Absorbance v. amount of hemoglobin in human blood samples at 415 nm
Figure 25. Intensity ratio v. amount of hemoglobin in human blood sample 1
Figure 26. Intensity ratio v. amount of hemoglobin in human blood sample 2
Figure 16. Intensity ratio v. amount of hemoglobin in human blood sample 3
Figure 17. Intensity ratio v. amount of hemoglobin in human blood sample 4
Figure 18. Intensity ratio v. amount of hemoglobin in human blood sample 5
Figure 19. Intensity ratio v. amount of hemoglobin in human blood sample 6
Figure 20. Intensity ratio v. amount of hemoglobin in human blood sample 7
Figure 21. Intensity ratio v. amount of hemoglobin in human blood sample 8
Figure 22. Intensity ratio v. amount of hemoglobin in human blood sample 9
Figure 23. Intensity ratio v. amount of hemoglobin in human blood sample 10
Figure 24. Intensity ratio v. amount of hemoglobin in human blood samples
Figure 36. Absorbance HRP at 405 nm v. Time Fresh serum A (10K, 20K and 50 K dilution)
Figure 257. Absorbance HRP at 405 nm v. Time Fresh serum B (10K, 20K and 50 K dilution)
Figure 268. Absorbance HRP at 405 nm v. Time Fresh serum C (10K, 20K and 50 K dilution)
Figure 39. Absorbance HRP v. Time Fresh blood serum A (1K, 5K and 10K 2°Ab dilutions)
Figure 40. Absorbance HRP v. Time Fresh blood serum B (1K, 5K and 10K 2°Ab dilutions)
Figure 41. Absorbance HRP v. Time Fresh blood serum A (1K, 5K and 10K 2°Ab dilutions)
Figure 42. Absorbance HRP v. Time Fresh and 1-week old blood serum A
Figure 27. Absorbance HRP v. Time Fresh and 1-week old blood serum B
Figure 44. Absorbance HRP v. Time Fresh and 1-week old blood serum C