Effects of Different Haematocrit Values on Estimation of Time since Deposition of Human Blood Stains Using Diffuse Reflectance Spectroscopy

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Emily Wellington

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Principle Supervisor: Dr Mark Reynolds
Academic Supervisor: Associate Professor James Spears

Murdoch University
Perth, Western Australia
DECLARATION

I declare that this manuscript does not contain any material submitted previously for the award of any other degree or diploma at any university or other tertiary institution. Furthermore, to the best of my knowledge, it does not contain any material previously published or written by another individual, except where due references has been made in the text. Finally, I declare that all reported experimentations performed in this research were carried out by myself, except that any contribution by others, with whom I have worked is explicitly acknowledged.

Signed:

Dated: 23/07/2017
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Part One

Literature Review

Effects of Different Haematocrit Values on Estimation of Time since Deposition of Human Blood Stains Using Diffuse Reflectance Spectroscopy: A Review
Abstract

Blood stains are a relatively common occurrence at scenes of violent crime and are a source of much information including biological, circumstantial, positional and potentially, information on the timeline of the crime. Successfully aging blood on scene could aid in time of death estimation, time since incident or even verify alibies of potential suspects. Methods in use thus far for time since death currently hold high error rates due to fluctuations in environmental factors. Several different techniques for aging of blood stains have been analysed and compared for suitability as well as similar spectroscopic techniques. Some of these aging methods were found to be complimentary, as reflectance spectroscopy has a relatively low error rate for short term aging, followed by a gradual increase, making it unsuitable for long term aging. Whereas, RNA marker analysis which follows the general degradation pattern of select RNA, was able to show a more steady error rate making it a more viable method for long term aging but not well suited to short term. The major variable factors in determining the time since deposition of blood stains include; daily or person to person fluctuations in blood protein percentages, haematocrit values, drugs present and environmental considerations such as temperature, humidity and UV light exposure. In order for any method to be acceptable in court, the full extent to these factors needs determination and full error analysis implemented. Additionally, improvement in portability, efficiency and development of non-destructive methods should be prioritised.
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<tr>
<td>BPA</td>
<td>Blood pattern analysis</td>
</tr>
<tr>
<td>DNA</td>
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<td>HbO₂</td>
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<tr>
<td>Hct</td>
<td>Haematocrit</td>
</tr>
<tr>
<td>L*</td>
<td>Lightness</td>
</tr>
<tr>
<td>a*</td>
<td>Colour continuum of red to green</td>
</tr>
<tr>
<td>b*</td>
<td>Colour continuum from yellow to blue</td>
</tr>
<tr>
<td>Cab*</td>
<td>Chroma</td>
</tr>
<tr>
<td>Hue*</td>
<td>Hue</td>
</tr>
<tr>
<td>n</td>
<td>Sample size</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
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<tr>
<td>HSD</td>
<td>Honestly significant difference</td>
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1. Introduction

Blood stains are a common form of evidence found at violent crime scenes and are highly valued due to potential links to offenders through pattern analysis and DNA profiling (Bremmer, Nadort, van Leeuwen, van Gemert, & Aalders, 2011). DNA identification has been a relatively recent and major breakthrough, enabling the identification of persons of interest who would have otherwise gone unknown. It however cannot provide information on when the biological material was deposited, only its presence (Anderson, Howard, Hobbs, & Bishop, 2005). Blood can not only be useful for suspect and victim identification, but also for determining time since death or injury. Generally, time line establishment is most often limited to eyewitness or victim interpretations (E. K. Hanson & Ballantyne, 2010). However, these are not always available to investigators. Knowing how old a stain is could be essential to on scene Blood Pattern Analysis (BPA) examination where multiple bloodletting events may have occurred, which could aid with prioritisation by distinguishing old from newer stains and whether or not they belong to the crime being investigated (Bremmer, de Bruin, van Gemert, van Leeuwen, & Aalders, 2012). Currently, all techniques for determining the time of a crime or death such as rectal temperature, wound examination and entomology, to name a few, have relatively large error margins (Bremmer et al., 2012).

Estimating the age of a blood stain is essentially estimating the date of trauma which can confirm or deny alibis or narrow parameters for missing persons investigations (Li, Beveridge, O’Hare, & Islam, 2011). As the error parameters commonly increase over prolonged exposure times for many time since death measurements, new methods of determination that can limit this effect would be highly beneficial. Non-destructive methods are sought for analysis in all forensic disciplines so that second opinion research can be conducted (Bremmer et al., 2012). General practice calls for non-invasive, non-destructive techniques to be used primarily, with little to no contact or damage to the substrate. This can then be followed up by more destructive methods if the initial methods were inconclusive; however, destroying the evidence during processing creates complications in the court room if there is not enough for a second opinion test. For this reason, non-destructive, non-contact methods are requiring further development and research to become more effective.

Older methods also tend to be affected by the subjective interpretation of the investigator (Matsuoka, Taguchi, & Okuda, 1995) therefore more recent methodology has centred around exact
determination. Some of these methods are: oxygen electrodes, RNA degradation, electron paramagnetic resonance spectroscopy, high performance liquid chromatography, near infrared spectroscopy and atomic force spectroscopy, however, most of these are not readily available in forensic laboratories or in the field and are expensive and complex, requiring additional sample processing (Li et al., 2011). Visible spectroscopy, which is non-destructive and relatively cost effective, does not require a blood sample to be taken back to the lab. It is based on a simpler method of analysing colour changes as a function of time which relates to the degradation of Oxyhaemoglobin (HbO₂) into its respective derivatives of methaemoglobin (met-Hb) and hemichrome (Hc) (Li et al., 2011).

The main reasons for inaccuracies for many methods are due to environmental factors such as humidity, temperature and light exposure. These all play a part in the aging process of biological compounds and are potential factors in blood stain aging (E. K. Hanson & Ballantyne, 2010). In terms of aging blood stain patterns, all parameters and contributing factors have not yet been investigated and as a result, no technique has shown the precision and reproducibility required to enable use in the field (Bremmer et al., 2011). This review will outline different methods which have been developed to determine the age of blood stains and attempt to identify regions which require further development.

2. Properties of Blood

2.1 Physical

Physical properties of blood include: surface tension, viscosity and relative density (Raymond, Smith, & Liesegang, 1996). These are all relevant to blood pattern analysis and scene reconstruction as fluctuations in these properties may affect results. Surface tension is the inward pull of molecules in the absence of competing forces which minimises the surface area and forms a spherical shape (Raymond et al., 1996). As surface area decreases, molecules are drawn to the centre of the drop in order to decrease surface energy (Raymond et al., 1996). This is disturbed upon impact where the droplet will be forced to redistribute energy upon settling into a concave shape on flat surfaces, and tear drop on angled surfaces.
A Newtonian fluid is considered to have constant liquid viscosity and is the basis for many models in fluid dynamics (Zhao & Yang, 2013). Unfortunately, bodily fluids aren’t uniform due to the electrostatic charge created when the electrolytes in a solution come into contact with a solid surface which causes a redistribution in ions in a charged diffuse layer (Zhao & Yang, 2013). Viscosity is measured as the ratio of shear stress to shear rate which is constant for a Newtonian fluid but dependent upon the shear rate for a non-Newtonian fluid, such as blood (Raymond et al., 1996). This can vary in the human body from day-to-day due to factors such as de-hydration and cellular percentages which can be measured as haematocrit values. As stains age, the morphology changes, initially blood will coagulate, fibrin strands form, creating a clot (Laan, Smith, Nicloux, & Brutin, 2016). De-hydration of the stain occurs as water evaporates, causing the cellular material to flow to the centre of the stain initially then are drawn to the periphery which forms a circular rim (Laan et al., 2016). Cracks can be seen forming from the edges once dried which continue throughout as the drying process is completing, this can be observed in Figure 1 below. The colour change can also be observed in Figure 1 where the dark red lightens during coagulation then darkens again upon drying.
2.2 Biological

The composition of human blood is generally in the ratio of 45% cellular material and 55% plasma. Plasma consists of 91.5% water, 7% proteins with the remaining 1.5% consisting of other solutes (Farley, Hendry, & McLafferty, 2012; Tortora & Grabowski, 1993). Fibrinogen is a glycoprotein found in plasma and is a coagulation factor which is triggered upon inflammation or rupture of a blood vessel where it then acts as an adhesive for platelet aggregation and forms an insoluble fibrin clot at the end of the coagulation cascade (Herrick, Blanc-Brude, Gray, & Laurent, 1999). As a result, when blood is exposed to the environment outside a vessel, the cellular components that clot separate from the plasma and the liquid component left will contain no fibrinogen, this is known as serum. The three main types of cells found in the blood are erythrocytes (red blood cells), leukocytes (white blood cells)
and thrombocytes (platelets). Leukocytes are primarily involved in immune response by recognising and responding to foreign antibodies and can move into different tissues depending on where the chemical signals originated from (Rodak B.F, 2012).

Haemoglobin is a major component of red blood cells and its primary function is to carry oxygen from the lungs to the cells of the whole body as well as carry carbon dioxide from the tissues back to the lungs for exhalation (Rodak B.F, 2012). There are four heme groups inside each red blood cell that form the haemoglobin (Hb) molecule which can reversibly bind with oxygen and it is this double bond that creates the red colour we associate with blood (Rodak B.F, 2012). Approximately 1% of oxyHb is auto-oxidised into metHb within the body which is then manipulated back to Hb by cytochrome b5, a reductase protein (Bremmer et al., 2011; Rodak B.F, 2012) this is demonstrated in Figure 2 below.

![Haemoglobin reaction kinetics inside the body (left) and outside the body (right) modified from (Bremmer et al., 2011).](image)

While Haemoglobin can be found in both oxygenated and deoxygenated forms, when blood leaves the body, oxidation immediately begins, converting any deoxy-Hb into HbO₂ upon exposure to the air (Matsuoka et al., 1995). As this is an extremely reactive process, virtually no deoxygenated version will be present in a sample as what might be expected at a crime scene. HbO₂ contains ferrous iron (Fe²⁺) which will further degrade into Met-Hb having ferric iron (Fe³⁺) which is unable to bind oxygen (Bremmer et al., 2012; Doty, McLaughlin, & Lednev, 2016). Met-Hb is brown to blue in colour and does not turn red when exposed to oxygen like Hb (Rodak B.F, 2012). Met-Hb will advance degradation into Hc which is formed by a central conformation change to the heme group (Bremmer et al., 2011).
et al., 2012). Blood is a unique substance as once it leaves the body it begins to clot and separate cellular mass from the liquid component, plasma. This phenomenon occurs immediately upon exposure to air, therefore, to study many aspects of the whole blood, anticoagulants may be required.

2.3 Haematocrit Variations

The Hct range in adults is generally from 35% to 54% with females dominating the lower range at 35% to 46% and males the high range of 40% to 54% (Rodak, 2002). These averages vary depending on the source but usually only differ by one or two per-cent, for example 37% to 48% in females and 42% to 52% in males are reported average values from 10 years earlier, showing the variation in populations (Cabot et al., 1992). Hct values are determined through the estimation of packed cell volumes as the ratio of the volume of red blood cells to the volume of whole blood and is therefore represented as a percentage (Raymond et al., 1996; Rodak B.F, 2012). The Australian standard of measurement requires centrifugation in a graduated or capillary tube at 12000 rpm for 3-5 minutes and the packed cell volume is measured in the column and divided by the total length of the cells and plasma combined to find the overall percentage (Raymond et al., 1996; Rodak B.F, 2012).

Fluctuations in haematocrit values can be seen from day to day due to diet, hydration, exercise, etc. but are more likely to show larger differences over longer periods of time and different conditions. Some haematological parameters can change over long term training and competitions, generally, during intense periods Hb can decrease up to 8% (Banfi, Lundby, Robach, & Lippi, 2011). Cyclists RBC, Hb and Hct has been tracked over a 15 month period and found that all three percentages decreased during the racing season (Guglielmini et al., 1989). This was confirmed in another study where during non-competition season the mean Hct value of 28 athletes was 45% which decreased to 40.7 by the end of season which increased again as training declined (Mørkeberg, Belhage, & Damsgaard, 2009). This could be due to the increase in extracellular fluids caused by renin-aldosterone axis activation therefore initiating haemodilution (Banfi et al., 2011). Schumacher et al. (2002) also observed seasonal variations where the same three values (Hb, Hct and RBC) were lower in winter than in the summer season. Additionally, the RBC count has been proved to increase in prolonged exposure to high altitudes and can modify plasma volume by 7-10% upon acute exposure (Sharpe et al., 2002).
Evans (2015) investigated the effect that variations of Hct values could have on the dynamics of blood stains and therefore the accuracy of blood pattern analysis calculation methods related to area of origin. The Hct value directly relates to viscosity as well as density which is not constant as the Hct value is also not constant (Raymond et al., 1996). It was concluded that Hct values did not affect the forensic reconstruction methods however, there was a change noted in appearance that may factor into aging stains more than BPA (Evans, 2015).

3. Forensic Value of Blood

Blood evidence is an increasingly useful resource in forensic examinations due to its potential to provide investigators information about DNA, discern gender, identify physical feature possibilities and upon cross reference, provide positive identification of persons of interest. Substances that are able to be carried within the blood stream such as drugs, poisons and their derivatives can also be analysed and identified. This is especially useful when a death or event is suspicious and can confirm witness accounts. One thing that blood has yet to confirm with any finality, is the time since death or bloodletting event. Considering the frequency in which violent crimes have some form of blood pool or spatter present, it is not surprising that many methods have been tested to gain an estimate of age of the stains.

4. Aging of Blood Stains

As blood ages it undergoes various chemical reactions. Inside the body, the natural aging process sees that RBC’s have a life span of approximately 120 days and are broken down and replaced with new cells (Munro, 2009). Outside the body, the coagulation cascade occurs, causing separation of cellular and liquid components before evaporation dries and sets the stain. On a molecular level, several degradation processes are occurring simultaneously. The denaturation of Hb in dried blood stains is primarily directed by chemical and physico-chemical reactions, not by enzymatic, due to the absence of an aqueous environment (Fujita et al., 2005).

Spectroscopic methods express a typical absorption spectrum for human blood such as that shown in Figure 3 where the HbO₂ spectra can be observed as well as its derivatives. A strong peak (off scale
in this image) appears at approximately 415nm and is named the Soret band while the smaller peaks are β at approximately 540nm and α at approximately 576nm (Li et al., 2011). Due to the differing intensities that can be observed over time, the amounts of each of the three compounds can be recorded and compared to standards in order to estimate the time since deposition of a blood stain.

Figure 3: absorption spectra of HbO₂, MetHb and HC as derivatives of degradation of blood stains and key identifying factors in aging processes (Asakura, Minakata, Adachi, Russell, & Schwartz, 1977; Bremmer et al., 2011; Zijlstra, Buursma, & Meeuwen-van der Roest, 1991).

Considering that the absorbance spectra in Figure 3 are quite different, it is not surprising that as these concentration levels change, a colour shift from red to brown occurs (Bremmer et al., 2011). The colour change has been examined using several methods as well as measuring the amount of each derivative present at different stages or oxygen percentages.

5. Age Estimation Techniques

Currently, no methods of blood stain aging are instigated in forensic examinations at this stage and most require samples to be processed within the laboratory (Edelman, Manti, van Ruth, van Leeuwen,
As these are time sensitive cases, it would be beneficial to develop a method which is fast, accurate and portable as well as non-destructive. The problem here is there is currently no established, widely accepted method in existence (Doty et al., 2016). The importance in ageing stains and increasing accuracy lies not in gaining 100% accuracy but developing the method to distinguish between hours, days and months as would be relevant in forensic cases where a timeline requires confirmation (E. K. Hanson & Ballantyne, 2010). Many techniques focus on the deterioration of Hb over time and the resulting changes in spectra, below these will be recapped as well other approaches including colour observation analysis (E. K. Hanson & Ballantyne, 2010).

5.1 Oxygen Electrode

The oxygen electrode method can be used to measure oxygen consumption rates in biological media (Robinson & Cooper, 1970). Time is required for the system to reach equilibrium and ensure probe stabilisation as well as rapid stirring and strict temperature control once the test solution is added (Robinson & Cooper, 1970). The rate of oxygen activity is then picked up by the electrode in the medium and, if calibrated correctly, will correlate with the oxygen saturation percentage of the tested solution.

Matsuoka et al. (1995) stated that methods measuring the degradation of HbO₂ are inadequate to make a precise determination due to the difference between the absorbance spectrum of the sample and the control being very small, with the colour of the sample also interfering with the reflectance spectrum. To counter this statement, a method was developed using oxygen electrode to analyse the amount of fractional HbO₂ in blood stains. Samples were taken and mixed with the anticoagulant EDTA to prevent premature coagulation then aliquots of 200µL were placed into ceramic wells before being dried at temperatures ranging from 5-37°C (Matsuoka et al., 1995). Analysis was undertaken using a polarographic oxygen analyser on the samples which were rehydrated with saline and added to a deoxygenated sample of water (achieved by bubbling nitrogen through the water) to determine dissolved oxygen and assayed by oxygen electrode (Matsuoka et al., 1995). The processed results are represented in Figure 4 where if ambient temperature and fractional HbO₂ is known, the correlating age can be determined.
Exploration into other environmental parameters is required as well as larger sample sizes for accuracy. The sample size was not clear in the text, therefore this could be considered a pilot study for consideration in the future. It is a difficult concept to manipulate into a portable version which can be used on-scene, as solutions need to be made up and a nitrogen source readily available for deoxygenation. Additionally, the blood stain is effectively destroyed in this method, meaning that it should only be used if all other non-destructive techniques fail to produce results. Oxygen electrodes also require calibration to each medium used as it doesn’t measure concentration, only activity and the presence of electrolytes affects the solubility and activity coefficient of oxygen in solution (Robinson & Cooper, 1970). Therefore, regular calibrations will be required onsite if a portable version was able to be produced and a form of auto-calibration would be necessary to develop for ease of use.

One potential benefit that is not able to be tested (thus far) with other procedures, is that this method has the ability to be manipulated to determine carbon monoxide poisoning using oxygen saturated erythrocytes to detect CarboxyHb (Matsuoka et al., 1995). This may be useful in suspicious circumstances where blood aging is the only available alibi check source. An example could be where

Figure 4: standard curves for the estimation of time since deposition using fractional HbO₂ determination (Matsuoka et al., 1995).
blood spatter is present from an accidental death, such as from an individual running a generator in an enclosed space, and therefore foul play cannot yet be ruled out.

5.2 RNA Markers

RNA is used for analysis because many RNA species are plentiful, easily altered and can be species specific via polymorphism analysis (Anderson et al., 2005). Since RNA decays at a slower rate to DNA, it can be more relevant to forensic cases in which a length of time has passed due to increased stability.

Anderson et al. (2005) hypothesised that the types of RNA (mRNA, tRNA and rRNA) may decay at differing rates and therefore age can be determined by observing the differing ratios of each. Samples were deposited onto white cotton and stored at 25°C and 50% humidity during the analysis. Each sample underwent an RNA extraction consisting of several incubations, centrifugations and solvent additions which amounted to approximately 60 minutes. This then was subjected to reverse transcription, real time PCR methods, as well as relative RNA quantification and RiboGreen Quantification. This is a very lengthy process, and considerable time passes before any statistical analysis can even be started, rendering it ineffective for forensic use in time sensitive cases.

The results showed a correlation with aging which may be useful in cases where it is feasible to take a sample to the laboratory for extensive analysis; however, there was a difference between genders as seen in Figure 5 (Anderson et al., 2005). This will require further investigation as male RNA levels appeared to be lower in general to that of the female participants indicating biological variability that is not usually apparent in Hb derivative studies.
Figure 5: Mean change in RNA levels as time since deposition of blood stain estimation with segregation in male and female sample data (Anderson et al., 2005).

The variability seen may be due to hormonal levels of RNA expression which are relevant to the monthly menstrual cycle, possibly affecting the ratio of 18 S to β-actin RNAs which were the only two analysed and compared (Anderson et al., 2005). Therefore, a separate correction model for each sex may need to be formulated if further study is undertaken with larger sample sizes. This model, while a slower process initially, may prove more useful in cases where stains are very old or over a month where other methods appear to decrease in accuracy. Ratios between the two RNA’s remained the same when 1, 5 and 10µL samples of blood were taken, indicating that this can be highly sensitive for biological materials.

Probes generated for use with 18 S and β-actin were species specific, targeting polymorphic regions of the coding region (Anderson et al., 2005). This enabled species differentiation as the β-actin probe only recognised RNA from human samples, however, the 18 S probe still showed a significant signal for pig RNA creating the need to ensure samples are not contaminated with pig DNA. Other animals tested did not have a significant signal and therefore did not interfere with analysis of human RNA.
levels. Further analysis will be required to observe environmental effects on RNA degradation as well as to advance species specific probes and eliminate the potential pig RNA contamination issue.

Another more recent study conducted by Lech et al (2016) looked into biological markers using mitochondrial RNA (mRNA) biomarkers as predictors of time of deposition of blood stains. Using 21 genes and observing levels of expression over 24 hours, Lech et al (2016) were able to establish three time categories based on peak expression; night/early morning, morning/noon and afternoon/evening. Molecular biomarkers were then incorporated and the final prediction model, which included two biomarkers and three mRNA markers in the expectation that a hormone/mRNA combination would be more accurate than either one alone. All of these markers relate to the circadian rhythm and therefore have a limited timeline in which time since deposition can be estimated. This timeline was 24 hours and non-specific to a day/week/month time period (Lech et al., 2016).

The intended forensic application may be for alibi checking where it could become important to know if it was day or night when the bloodletting event occurred to clear or condemn. However, this could also be determined more accurately by methods using haemoglobin derivatives, as it is possible to track the degradation over hours into days and months. While circadian rhythms are generally consistent in a healthy subject, there has been no mention of the need to investigate circumstances involving drugs, diseases or night shift work which can affect the levels of cortisol and melatonin therefore potentially rendering this model irrelevant. This process may be useful for research purposes but for forensic application there are other methods that hold priority for further exploration.

5.3 Chromatographic Techniques

Thin layer chromatography (TLC) is affected by impurities in the samples and bloodstain properties containing benzidine reaction positive substances such as heavy metal ions (Matsuoka et al., 1995). Due to this sensitivity, the method has been tried but deemed ineffective for the aging of stains containing blood as there is no guarantee of the purity of the sample or the substrate it is found on.

High performance liquid chromatography (HPLC) is able to separate and quantify individual components within a mixture and therefore allows for the quantification on Hb derivatives (Bremmer et
The retention time of approximately 7.45 minutes showing a peak described as X protein by Andrasko (1997) can be seen in Figure 6. This is representative of a protein that is not related to heme and increases in intensity over the aging process. The identification of the peak was not investigated at the time; however, this could prove to be a useful indication of time since deposition. HPLC is not currently a portable option and this may be a better method for use in cases which are not time sensitive given that the ratios are found to be non-temperature dependent for Hb derivatives (Andrasko, 1997) despite the rate changes that can occur.

![Figure 6: HPLC retention time of protein X showing changes in peak height over time since deposition on cotton cloth, temperature of 37°C and measured at wavelength 220 nm (Andrasko, 1997).](image)

**5.4 Spectroscopic Methods**

Spectroscopy involves the utilisation of direct and diffuse light in the measurement of absorbance or reflectance that occurs from each substrate. This can be used on solutions in small cuvettes or directly onto the surface of solid substrates. Considering the change in colour from red to brown over time, colour quantification by optical spectroscopy is a method worth pursuing (Bremmer et al., 2012).
These can vary depending on the wavelength being used, for example, in the infrared, UV or visible regions of light.

### 5.4.1 Electron Paramagnetic Resonance Spectroscopy

Electron paramagnetic resonance (EPR) spectroscopy is a non-destructive technique based on measuring the denaturation of hemoproteins where when the intensity is plotted against time, a linear correlation can be observed (Fujita et al., 2005).

Blood samples were taken by Fujita et al. (2005) without added anticoagulant and both gauze and filter paper were saturated with the sample then divided into various groupings of temperature and lighting combinations. Comparison of dried samples at 1 day and 775 days recorded differences in levels of ferric heme, ferric non-heme, low spin ferric heme and free radicals which could be attributed to lipid peroxy radicals formed during degradation of blood components. Of all these components, the low spin heme appeared to have the highest correlation coefficient at 0.97 and was therefore utilised in a model to predict the age of stains up to 698 days. Using this, there was an error range of 25% from the actual age where a 133 day old stain was estimated at 129 ± 26 days which is acceptable if only a broad timeline was required as when the time increased, so did the margin eg. 698 days was determined at 835 ± 236 days. It was discovered that the substrate made a difference in the aging of the stains as the samples deposited onto gauze showed lower EPR signal intensities than that of the filter paper samples. Furthermore, it was discovered that elevation of temperature and increased light exposure does have a major effect on the apparent age of a bloodstain using the EPR method (Fujita et al., 2005).

Given the linear correlation observed over a time period of greater than one year, this is a valid method of estimation of time since deposition of blood stains. It is non-destructive, however, requires a sample size of 10mg of dried blood which can be difficult in cases where small spatter marks are all that are present. Portability may also be an issue in terms of forensic scene of crime analysis. Preliminary work has been performed on the effect of different substrates and found to be a factor that affects this particular method as well as light and temperature which will need to be a consideration in future studies for all methods.
5.4.2 Raman Spectroscopy

The majority of spectroscopic methods used follow the degradation path of HbO₂ as well as the increasing and decreasing levels of met-Hb and Hc. Following several degradation paths increases accuracy as many substrates will contribute to fluorescence profiles and only using one value to predict the age of a stain is not feasible (Doty et al., 2016). This is why all three derivatives are considered in these analyses. Raman Spectroscopy measures the vibrational scattering of the incident light caused by density fluctuations in the media which produces spectra for analysis (Zhang, 2012).

Doty et al. (2016) reported that even after one hour, the met-Hb marker (377 cm⁻¹ band in Figure 7a) was present, indicating that the conversion from oxyHb had already progressed. Over time, this marker increased in peak height while the oxyHb marker (420 cm⁻¹) gradually decreased and smoothed out as demonstrated in Figure 7a.

![Figure 7](image)

**Figure 7:** a) Spectra of stains over time showing changes in fluorescence, b) age estimation of blood stains up to one week, red line is actual fit and green line is an ideal 1:1 fit (Doty et al., 2016).

One key finding was the distinction between times of deposition that can be made from 1 hour to 8 hours to a week, which can aid in the establishment of a timeline and relative affiliation between
stains even if not 100% accurate (Doty et al., 2016). The $R^2$ of the dataset represented in Figure 7b is 0.97 which shows a relatively high correlation, however, the accuracy of predictions decreased after 24 hours therefore once older stains are analysed using this method, the most relevant information (like most methods) will be that of relative time. Unfortunately, Raman Spectroscopy is unable to take accurate readings in strongly fluorescing backgrounds (Edelman et al., 2012). Considering the diverse nature of materials and colours that can be produced and therefore can be covered in blood, it is required that fluorescing backgrounds are compensated for somehow, whether it be mathematically or some form of pre-treatment. Raman has the benefit of being non-destructive but at this point, portability is also an issue.

5.4.3 Near Infrared Spectroscopy

Near Infrared (NIR) Spectroscopy utilises the near-infrared region of the electromagnetic spectrum and has the ability to be used with little experience without the need to sample materials or use reagents, therefore allows analysis of complex matrices in a short time (Hall & Pollard, 1993). It has been used to analyse blood serum and other substances that are light scattering and strongly absorbing (Hall & Pollard, 1993).

Edelman et al. (2012) noted that background colouring with high absorption readings cannot be subtracted from the overall absorption as it dominates the blood stain spectra and the spectral features are indistinguishable. Through NIR spectroscopy using Pearson’s correlation coefficient they were able to determine a wavelength region of 1150-2500nm where background colour did not significantly influence the blood stain spectra. NIR spectroscopy is able to identify blood stains from at least 30 other red/brown substances with 100% sensitivity and specificity with the only overlapping peak being that of water content (Edelman et al., 2012). It was identified that other processes such as Raman Spectroscopy may be able to identify more spectral features of the blood stain as NIR spectroscopy was only able to identify haemoglobin, albumin and globulin due to weak, overlapping and broad absorption peaks making identification of components difficult (Edelman et al., 2012; Hall & Pollard, 1993).

Figure 8 depicts the aging results of this study in which the errors greatly increase along the duration of the investigation. However, when looking at a younger stain the error rates are much lower and
reasonably accurate up to 10 days which indicates that NIR spectroscopy is a useful tool for determining the early stages of aging. The correlation of 0.95 is reasonably high, however, other methods have established a higher correlation.

![Graph showing correlation between estimated age and real age](image)

Figure 8: Age estimation of blood stains on black cotton using wavelengths 1150-2500nm. Dots are averages of three blood stains and whiskers are standard deviation (Edelman et al., 2012).

Environmental effects need to be taken into consideration and compared to other methods to see if the standard deviation at older ages is an issue. Background interference has been identified as an issue which most previous methods have suffered from. Due to the ability of NIR to limit the impact of this interference, it should be investigated further for determining the time since deposition of blood stains.

### 5.4.4 UV-Visible Spectroscopy

UV-Visible Spectroscopy is a method based on the recordings of reflectance and absorbance in the visible and the ultraviolet regions of the electromagnetic spectrum. Substrates can be analysed in both solid state and an extracted liquid state (Dana W. Mayo, 2014).
Hanson and Ballantyne (2010) examined the UV-Visible absorption spectra of Hb in aging blood stains and observed a blue shift in the Soret band that correlated with the relative age of the stains in a controlled environment. The process used involved extraction of the blood stain and analysis of the proteins in solution as opposed to analysis of the dried stain. This becomes a longer and more complicated procedure requiring laboratory processing, therefore eliminating the usefulness of analysis at the scene of a crime. Some temperature ranges have been analysed ranging from -20°C to 37°C as well as humidity from 50% to 90%. As the humidity increases, the shift to the blue Soret band decreases, at 50% humidity and 22°C the rate was 0.028nm/hour whereas at 85% the rate decreased to 0.014 nm/hour. When the temperature was raised to 30°C the effect of humidity was not observed as strongly which allows for the conclusion that higher temperatures lessen the effect of humidity on the aging process of dried blood stains (E. K. Hanson & Ballantyne, 2010). This is a good start, however, in countries like Australia where the temperature can be up at 50°C with low humidity and the road surfaces even hotter, further studies are required to cover higher parameters to meet these conditions.

Through the temperature section of experimentation, it was confirmed that increased temperature increases the rate at which the blue spectral shift occurs and that at -20°C no apparent change is observed, this trend can be viewed in Figure 9b. This holds potential for storage of stains after collection and an assay validation was implemented to confirm that storage for several weeks was able to keep the aging process dormant, however, 8 months storage at -47°C recorded an increase in the shift compared to samples tested at two weeks.
The error rate is reasonably high for this study as demonstrated in Figure 9a and 9b where only a small number of samples fell along the prediction lines. This enables a rough estimation of time like most methods but not specific enough to be ready for field use. The relationship between humidity and temperature has begun to be explored, and the findings that one affects the other in a counterintuitive manner of ‘cancelling out’ will be vital to future research and developing ways to measure this. Additionally, Hanson and Ballantyne (2010) were able to assess a portable spectrophotometer that is durable, lightweight and uses only 0.7µL of buffer extracted sample. Again, the only issue with this is that a small amount of sample needs processing and is therefore destroyed, however 1µL was deemed as effective to produce a spectrum in this case so it is at least possible to minimise the damage.

Hanson et al. (2011) were able to perform a validation on the above method using different materials and temperature rages of 4°C to 37°C as well as confirm human blood presence using the aqueous extract used in the previous study. Denim, polyester and paper were used instead of the white cotton to deposit stains onto and aged for up to 3 months. The Hypsochromic shift was not significantly affected by the different substrates, while temperature did prove to increase both the rate and size of the shift (E. Hanson, Albornoz, & Ballantyne, 2011), indicating an increase in aging rate. Visible spectroscopy generally struggles with background interference (Edelman et al., 2012) which is a major hindrance and something that will need to be developed in order to find a way to neutralise background absorbance readings. This validation confirmed that the effect of substrates is not a

**Figure 9:** a) Effects of different humidity values at 22°C on blue spectral shift of blood stains and b) effects of different temperatures at 50% humidity (E. K. Hanson & Ballantyne, 2010)
significant factor at this wavelength and may be useful for stains on dark materials where direct analysis is likely to be affected by background substrate interference.

5.4.5 Diffuse Reflectance Spectroscopy

Similar to UV/Vis spectroscopy, the diffuse reflectance spectroscopic method uses incident light to determine a material's optical properties by using non-specular reflection of light (Bremmer et al., 2011) instead of the change in transmittance of the light. This can be used to pick up colour changes effectively rather than absorption values which could be forensically useful due to the colour changes described earlier in this review. These colour changes in ageing blood suggest that it may be possible to use colour quantification by optical methods to determine the time since deposition (Bremmer et al., 2011).

Bremmer et al. (2011) used reflectance spectroscopy for determination of time since deposition and found that after one hour, the spectra resembled the shape of oxyHb as seen in Figure 3 (from Section 4) with dips at 540nm and 576nm. These dips appeared to smooth out as the stain aged and started to resemble the spectra of Hc as well as a steep slope at 600-650nm smoothing out during aging. At all-time points measured, there was a unique combination of haemoglobin derivatives and only three were detected as the sum of the three fractions remained constant and close to 1. This has been an important observation as this allows for further investigation into haemoglobin derivatives as an indication of age.

These derivative values can be seen in Figure 10a as relative values have been plotted with line of best fit to demonstrate the day by day differences in concentrations. This is the training set developed by Bremmer et al. (2011) which has relatively high variation and the test set (not shown) also displayed the same pattern. Even though there is high variation, it shows that the majority of areas don’t overlap, especially with the met-Hb which consistently displays lower levels. This means that if it becomes possible to gain consistent readings and use mathematical corrections effectively, it will be possibly to differentiate between different sample ages.
This method of estimation when tested against a validation set of 20 stains gains a rough estimate of
time since deposition but still shows high error margins as can be observed in Figure 10b. Discrimination
of ages can be distinguished using this model to a degree, however, it is likely that
there will be at least 3 days error to either side as an estimation of a 3 day old stain varies between
1.5 and 6 days and the margin increases with age. Refining of this method should be explored as the
combination of haemoglobin derivative analysis with reflectance spectroscopy has potential to be
used effectively in the field. It was stated that 40 samples were measured simultaneously using a
probe fixed at 10 ± 1mm height and perpendicular to the surface. Consecutive measurements are
something that not all techniques are able to promise and portability of the instrument is a feasible
option to look into given the methodology.

Li et al. (2011) used a developed reflectance method with a micro spectrophotometer (MSP) in order
to apply pre-processing and feature selection analysis to the dataset hoping to minimise the
differences between spectra observed at the same age and increase discrimination between different
ages. Stains were made using equine blood onto 6x4cm glazed white tiles and stored at a constant
temperature of 22°C and humidity at 40% (Li et al., 2011). After analysis between 380 and 780nm,
data pre-processing was implemented to reduce the differences in the reflectance values between the
sample and reference points via a de-trending method. Feature selection method and linear discriminant analysis were also performed and overall, the region of the α and β bands (seen in Figure 11) were able to have differences from baseline shifts and scattering reduced, wavelength measurement points for best determination were set at 442nm to 585nm (Li et al., 2011).

Figure 11: Pre-processed reflectance spectra of blood stains at increasing times since deposition (Li et al., 2011).

The α and β peaks in Figure 11, as well as the trough between them, are key points at which maximum differentiation can be applied between ages. Differentiation does become more difficult as the stains age due to the decreased rate of oxidation after the initial exponential phase. Using the leave one out cross validation method, Li et al. (2011) were able to gain a correct classification rate of 91.5% over all samples and 96.9% if the error acceptance was raised to one day with most of the misclassifications occurring after 23 days. When test samples were processed within the model, the total correct classification rate was 31.5% and when divided, 1 to 19 days was 45.3% with 23 to 37 days only having 12.7% of ages estimated correctly (Li et al., 2011).

The importance of spectra pre-processing has been demonstrated with the potential to determine the time since deposition within one day in stains less than 20 days old and within three days for stains aged over 20 days. Differences in the results between the standard and test samples are possibly due to substrate imperfections creating spectral distortions (Li et al., 2011). If this is the case, then method development will need to be reassessed using different background substrates in order to attempt to
render the background effect negligible. Interference may have been a large factor here due to the stain being a smear as opposed to a drip pattern as the analysis could have been performed on the thinner sections where the background may be showing through. Thicker sections of blood stain could not be analysed using this method and substrate combination due to the inability to correct spectral pre-processing. To correct this, a different substrate needs to be analysed or a different method implemented which will still allow pre-processing as well as the usual need for testing with various changes to environmental conditions.

A recent method was developed by Caren (2015) using International Commission of Illumination (CIE) chromaticity values allowed a mathematical detection of colour change and therefore differentiation in aging. Chromaticity values were originally suggested in a study conducted by Patterson (1960) which showed that CIE chromaticity values could be used to accurately measure red, green and blue reflectance values from a blood stain using high precision colorimetry. The greatest colour change was observed in the first three days followed by a more gradual transformation as aging progresses which correlates with general observations about the colour changes of drying blood moving from a dark red to a brown colour (Caren, 2015; Patterson, 1960). Colorimetric determination of MetHb, has also been investigated by Matsuoka et al (1995), however, requires a large sample of 200µL and the absorbance is too small to give the total Hb concentration. Therefore reflectance spectroscopy was used instead to determine age of stains at constant temperature and a high and low temperature measured for comparison (Caren, 2015; Matsuoka et al., 1995).

The Hct value was between 36% and 40% for the duration of the experiment, no anticoagulants were used for age determination studies and white card was used for the background substrate (Caren, 2015). Pre-processing was applied in the form of recursive feature elimination and linear transformation to find that out of seven variables tested, only five were suitable and the correlation between the colour continuum of red to green (a*) and Chroma (Cab*) was 0.9989. These variables were chosen due to showing the least variability between time points in a Tukey HSD test. After linear transformation a correlation of 0.9758 for a* and 0.9774 for Cab* which is quite significant as this was then able to predict the test samples with a correlation of 0.9869. This however did not hold up with the blind trial, as the storage temperatures were different to the standard and test samples but were able to be grouped into rough time frames. Temperature had a considerable effect on the spectra of
the stains, as spectra of 6 and 12 hours at 4°C was of a similar shape to that of the spectra of 1.5 hours at approximately 25°C. The relative difference between the extremes in temperature can be seen in Figure 12 where the mean values of a* have been compared for all three temperatures tested by Caren (2015).

Figure 12: Mean values of a* of aging blood stains at low (4°C), room (25°C) and high (37°C) temperatures over time (Caren, 2015).

This method was greatly affected by temperature despite being relatively accurate at determining the colour changes occurring since deposition. Cunningham (2016) increased the number of samples for the further validation of effect of different temperatures on aging of blood stains using the same instrument parameters and processing methods. The variance seen in time since deposition prediction over temperatures from 10°C to 40°C can be observed in Table 1.
**Table 1:** Predicted and actual values of time since deposition determined at various temperatures (Cunningham, 2016).

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<th>Actual Age (hours)</th>
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There is potential for portability using this method and accuracy within controlled environments appears to be higher than previous methods, however, as with all the methods available, there is still the issue of environmental factors. Countering this fact will require extensive research and recording of all possible combinations and models made for correction for fluctuating environments.

### 6. Factors Affecting the Rate of Aging

There is an ever increasing source of literature on the topic of estimating time since deposition of blood stains and some have estimated ages with acceptable degrees of accuracy. However, all of these studies mention that their model will need confirmation and possibly mathematical adjustment when external factors are considered, making it impractical to implement these methods in the field, for example, on a hot day and outdoors. Furthermore, environmental conditions will not be constant for the majority of scenes during the standing period (Matsuoka et al., 1995). Most criminal offences
will not occur in a controlled environment, nor will the blood always be from a healthy, drug free specimen. Fluctuating temperatures, humidity, sun exposure, moisture, bacteria and other pollutants may affect the stain, therefore it is necessary that all of these possible environmental effects have been considered (E. K. Hanson & Ballantyne, 2010).

6.1 Light Exposure

Ultra violet (UV) light has a detrimental effect on most substrates exposed; this can be observed by merely walking outside and seeing peeling paint or powdered plastic chairs, or even dead grass. Considering UV light is a form of radiation, it is therefore able to create free radicals on contact with biological material (Gomes, Ribeiro, Shaw, Mason, & Raposo, 2009). Treatment for contamination in DNA laboratories can consist of UV exposure as it is a known DNA degradation method and too much exposure on skin cells can cause cancer. The effects of different light exposure intensities is something that needs to be explored in the aging of blood stains to aid in accurate measurements for future reference.

6.2 Temperature

Different temperatures will be present at a scene depending on the geographical location and seasonal shifts therefore producing a finite and potentially restricted range of possible values (E. K. Hanson & Ballantyne, 2010). Conversions of HbO$_2$ to Met-Hb is more rapid at higher temperatures (Matsuoka et al., 1995) making this an important factor to consider. As each region’s weather is recorded and reported by several sources, it will be possible to discern the average conditions of exposure to estimate time since deposition once all combinations have been tested and validated. It may be more difficult in rural areas where recordings are less frequent but it may be possible to place a temperature recorder at the scene if the time since deposition is suspected to be within a few weeks of discovery.
6.3 Humidity

As previously explored by Hanson and Ballanyne (2010), humidity has been shown to have a direct relationship with temperature. Increased humidity can affect the rate of aging which is important when using a model which may have originated, for example, in the north of Australia which has much higher humidity percentages than the southern regions.

6.4 Drug Intake/ Poisons

It is not currently known what effects certain drugs or poisons may have on the aging of blood stains. Carbon monoxide may decrease the initial oxygen saturation of the blood inside the body, decreasing the rate of oxidation and therefore apparent aging. Additionally, many people use prescribed medications. Elderly people may be prescribed blood thinners such as aspirin and warfarin of which the effect is also unknown. Some of the methods developed used blood samples with anticoagulants such as EDTA and Sodium Citrate and no control sample was stated to ensure correct representation of results. The drying dynamics could change a lot with the use of anticoagulants, for instance, Laan et al. (2016) noted the change in colour from dark red to bright red upon coagulation which was not observed in blood with added anticoagulants. This could affect the results dramatically in colorimetric analysis methods.

6.5 Surface Background Interference

Many of these studies have suggested surface background to be an interference factor, especially in spectroscopic methods. The need to eliminate this as a factor is high as many different substrates are witnessed every day, some rough, dark and reflective. It will be luck of the draw at a crime scene and will most likely be something ununiformed.

6.6 Cellular Concentration of Blood

Variability in the texture of the blood stain such as thickness and roughness or cracks may have an influence of the values obtained from both diffuse and specular reflectance (Li et al., 2011). Different
haematocrit values may affect the time taken in drying stains as well as oxygen penetration therefore slowing the aging process for the overall stain. Different viscosities may play a major role in colour determination as the stain will be thicker and smaller in size at higher Hct values. As this is a highly variable factor between people, more so than temperature as many rooms have temperature control, it is important to know that variation in packed cell concentrations and therefore viscosity is not yet another factor that needs adjusting for.

7. Future Direction

For the majority of the techniques reviewed here, the inaccuracies increase over time. Reflectance spectroscopy with the aid of data pre-processing, appears to be leading the way in early age estimation and will require further investigation into ages beyond 20 days to increase the accuracies. RNA analysis errors appear relatively constant making it a viable option for long term age determination and may be able to narrow the error parameters upon investigation with different RNA markers.

A good assessment of the environmental conditions is imperative to the accuracy of estimating the time since deposition (E. K. Hanson & Ballantyne, 2010). As the outside environment is in an ever constant state of flux, all the factors discussed need investigation to discover how high the error margin can reach and to gain knowledge of how to lower these margins. It seems that temperature especially will affect all samples in a similar way, no matter the method of analysis chosen for use, therefore, thorough recording of all temperature variations are required to create a successful mathematical model for error elimination in future predictions. Other changes that may occur over time could be due to the loss of water in the Hb molecule or partial denaturation of proteins which may affect the UV-VIS spectrum and account for subtle differences in readings (E. K. Hanson & Ballantyne, 2010). This links into viscosity and drying patterns which can be analysed through varying the haematocrit values of samples and testing for changes to the readings over time. Additionally, extreme environmental factors should be investigated as well as contact with different substrates and surface temperatures to account for the potential of protein denaturation.
High performance liquid chromatography, electron paramagnetic resonance, oxygen electrode and RNA degradation measurements are more invasive than most spectroscopic methods and can only be performed in the lab (Edelman et al., 2012). The least evasive or destructive method at this stage is reflectance spectroscopy which allows the stains to be examined within the original context and has the potential of portability. Samples which have to be taken to the laboratory for analysis are often required to be dissolved in water or saline solutions in preparations which essentially destroys the sample and disallows for second opinion analysis (Bremmer et al., 2012). As a result, to be accepted in court as a forensic method, the non-invasive techniques require development ahead of invasive ones which can then be developed as a secondary method much like the system which is used in print analysis using visual, physical and then chemical procedures to minimise damage.

Handheld devices are becoming more available for spectroscopic analysis, with portability as well as cost now being a major factor for forensic investigators. The demand for reducing costs, time and processing requirements means that more samples can be analysed in the field and higher chances of accuracy as the age determination can occur instantaneously. There is great potential in the development of time since deposition estimation techniques, but in order for these to be used in court in confidence, the potential factors that may affect rate of aging will need to be addressed (Schumacher, Jankovits, Bültermann, Schmid, & Berg, 2002).

8. Experimental Aims and Hypothesis

Considering the minimal amount of literature available for review regarding the effects of varying Hct values on the aging of blood stains, it would be essential to determine the extent to which haematocrit values alter the rate of aging.

Many methods have been investigated as to their ability to determine time since deposition. The main aim is to determine whether age can be reliably estimated using UV/VIS reflectance spectroscopy over varying haematocrit values. This will provide researchers with information on whether or not this can be a viable method for future use or if a different method will require further development instead.
Experimental Hypothesis

H₀: Changes in the haematocrit value of blood does not affect the ability to accurately determine age of blood stains.

H₁: Changes in the haematocrit value of blood does affect the ability to accurately determine the age of blood stains.
9. References


Cunningham, N. (2016). *The Effect of temperature on estimating the time since deposition of a bloodstain using diffuse reflectance spectroscopy*. (honours in forensic biology and toxicology honours), Murdoch University.


Evans, S. (2015). *EFFECT OF HEMATOCRIT VALUE ON AREA OF ORIGIN DETERMINATION FOR BLOODSTAIN PATTERN ANALYSIS*. (Honours Degree of Bachelor of Forensics in Forensic Biology and Toxicology honours), Murdoch University.


Hanson, E. K., & Ballantyne, J. (2010). A Blue Spectral Shift of the Hemoglobin Soret Band Correlates with the Age (Time Since Deposition) of Dried Bloodstains. *PLOS ONE, 5*(9), e12830. doi: 10.1371/journal.pone.0012830


Part Two

Manuscript

Effects of Different Haematocrit Values on Estimation of Time since Deposition of Human Blood Stains Using Diffuse Reflectance Spectroscopy
Effects of Different Haematocrit Values on Estimation of Time since Deposition of Human Blood Stains Using Diffuse Reflectance Spectroscopy

Emily Wellington¹, Mark Reynolds², James Speers¹

¹Murdoch University, School of Veterinary and Life Sciences, Perth, WA.
²West Australian Police, Forensic Division

Abstract

The estimation of time since deposition of human blood stains is currently unavailable for use in crime scene analysis. This is due to the high degree of variability associated with environmental factors and the requirement for associated mathematical adjustments. Furthermore, physiological factors such as haematocrit (Hct) values are worth considering. This information may not be readily available and any variation due to these factors could further impact on the development of methods to determine time since deposition of blood. This study used UV/Vis diffuse spectroscopy to determine the effect of various Hct concentrations on age determination using colorimetric analyses. A range of 24 % - 61 % was examined under identical conditions at 14 time points, from 1.5 h – 672 h inclusive, and compared using plots of means, Two-way ANOVA and Tukey's HSD post hoc tests. Both plots and ANOVA analyses suggest significant differences between Hct values on all chromaticity values analysed while the Tukey's HSD suggests no significant difference in variance. This conflict could be due to cumulative errors disallowing a distinct separation in data for post hoc analysis. As a result, Hct concentration could not be ruled out as an interfering factor in the determination of time since deposition.

Keywords: Haematocrit, UV/Vis, Reflectance spectroscopy, Age estimation, Blood stain aging, Colorimetry
Introduction

Common forms of evidence found at the scene of a violent crime are blood stains. These are highly valued because they can link potential offenders through blood pattern analysis (BPA) and deoxyribonucleic acid (DNA) analysis (1). Both DNA and BPA are excellent tools for possible identification for who was present and what occurred, unfortunately, these tools are not useful in determining a timeline. The timing of a blood event is an important factor to confirm or deny alibis of persons of interest, which can be difficult when timeline establishment is most often limited to eyewitness or victim interpretations (2). Also, knowing the age of a blood stain could be essential at a scene of crime, especially where multiple bloodletting events have occurred, by distinguishing old from newer stains (3). Current techniques for determining the time of a crime or death such as rectal temperature (4), wound examination (5) and entomology (6), have relatively large error margins (3) and are not relevant to cases of assault where no death has occurred. Accurate blood stain aging can help verify witness statements in circumstances of timeline ambiguity.

Estimating the age of a blood stain is essentially estimating the date of trauma which can confirm or deny alibis or narrow parameters for missing persons investigations (7). As the error parameters commonly increase over prolonged exposure times for many time since death measurements, new methods of determination that can limit this effect would be highly beneficial. Non-destructive methods are sought for analyses in all forensic disciplines so that second opinion research can be conducted (3). As these non-destructive methods are a preference to prevent destruction of the evidence, it is these types that should be developed, minimising contact and disruption to the scene, therefore, preventing excessive evidence loss.

Blood stain aging by the subjective interpretation of the forensic investigator is an unreliable method therefore, recent methodology has centred around exact determination using instrumental techniques (8). These methods have ranged from: oxygen electrodes (8, 9), RNA degradation (10, 11), electron paramagnetic resonance spectroscopy (12), hyperspectral imaging (13), near infrared spectroscopy (14) and raman spectroscopy (15). However, most of these are not readily available in forensic laboratories or in the field and are expensive and complex, requiring additional sample processing (7).
A more promising technique is diffuse reflectance spectroscopy as it is non-destructive and relatively cost effective (16). The method analyses blood stain colour changes as a function of time which relate to the degradation of HbO$_2$ into its respective derivatives of methaemoglobin (met-Hb) and hemichrome (Hc) (7).

A number of these aging methods, if not all, are affected by external, environmental factors and require some form of mathematical adjustment: UV light exposure is known to degrade DNA and is able to create free radicals on contact with biological materials (17). Conversions of HbO$_2$ to Met-Hb is more rapid at higher temperatures (8). Humidity has a direct relationship with temperature, counteracting aging effects of high temperatures when high humidity is also present (2). All of these are subject to fluctuations in the weather in any outdoor environment and in order to account for each one, detailed information for that region must be collected. Additionally, the individual’s drug intake or potential poisoning may affect the rate of aging as could the cellular concentration. Recent studies on UV/Vis reflectance methods have shown that it is possible to estimate the age of a blood stain but that temperature is still a major contributor to error rates (18, 19). As measurements can be acquired for temperature this may still be mathematically adjusted.

Another factor which can affect the physical properties is haematocrit (Hct). This is the cellular percentage of the blood and generally comprises of 35-46% blood volume in females and 40-54% in males (20). This can vary in the human body due to factors such as hydration levels, diet, exercise, etc. on a daily basis, however, larger differences occur over long periods of time. This can be because of anaemia, hemochromatosis, haemodilution or rigorous athletic training regimes and varying altitudes (21, 22).

Currently, there is limited information on the affect that different Hct concentrations could inflict on the aging process of blood stains. Many studies choose to investigate environmental changes first when something internal could potentially present as many difficulties (3). Hct is currently unable to be determined post-mortem due to the evaporation of the water component and requires further investigation as this may not be able to be accounted for and therefore limit the use to crimes where the victim is readily accessible for fresh blood samples. An attempted murder or assault could result in a large volume of blood loss, diluting their bodies Hct percentage temporarily, additionally, in a missing person’s case a sample cannot be acquired at all. Knowing that Hct concentration does not
affect the aging process could be crucial to the development of a viable and portable crime scene analysis method.

UV/Visible diffuse reflectance measurement of chromaticity values established from a system developed by the International Commission on Illumination (CIE) (23) will be utilised in this study to measure colour changes of blood stains of various Hct concentrations at 14 time periods between 1.5 h and 672 h. This method has been shown to be suitable for analysis of biological materials (24) and have been used by Caran (2015) to develop a forensically relevant method. To ensure that the addition of an anticoagulant does not also affect the readings, a neat blood sample will also be tested and treated in the same conditions for comparison as a control.

Materials and Methods

Collection and Preparation of Blood Samples

Venous blood was collected from a 26 year old volunteer. The Hct value of 37 % was pre-determined using standard methods as described in Rodak (2002). From this initial concentration, the packed cell volume required for each desired concentration was calculated and volumes were used as seen in Table 1 where 10 mL was extracted for each sample. The % Hct achieved (Table 1) was measured by the same method as the initial determination and shows that the required concentrations were achieved.

Blood was collected via venous puncture into a 10 mL syringe then transferred immediately into citrated tubes to prevent premature coagulation, with the exception of the control sample which was denoted as neat and did not contain any anti-coagulant. Each 10 mL extraction (with the exception of the neat and 35 % samples) was centrifuged at 4,900 rpm for 10 minutes to ensure complete separation of the blood components. Plasma was then removed as required according to the figures in Table 1, remixed and a 2 mL sample set aside for all except the neat for Hct determination.
Table 1: Volumes of blood components used to achieve desired range of concentration and measured result.

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<th>% Hct required</th>
<th>Cellular material (mL)</th>
<th>Plasma (mL)</th>
<th>% Hct achieved</th>
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<td>24</td>
</tr>
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All samples taken were fresh and aging of stains was timed from the moment the blood left the body. Each dilution was created immediately after centrifugation and blood was transferred using a pasture pipette, spotting onto 20 white BPA cards cut from GSM screen board for each Hct concentration, using a total of 3 drops from a height of approximately 15 cm for each card. This is to ensure a similar diameter and thickness is achieved throughout all 120 samples and represents a passive drip stain that may be found at a crime scene.

2.2 Storage of Blood Stains

A temperature and humidity controlled cabinet (Ningbo Southeast Equipment Co.Ltd. climatic chamber-HWS) was used to store the blood samples prior to analysis. This was set to 23 °C ± 0.5 °C and 80 % ± 7 % humidity which fluctuated briefly when the door was opened to retrieve a sample set for analysis. All samples were stored and transported in the same manner each time.

2.3 Analysis of Blood Stains

Each sample was analysed via UV-Visible reflectance spectrophotometry using a research grade spectrophotometer, Perkin Elmer Lambda 650S UV-Vis in conjunction with Perkin Elmer WinLab software to generate the spectra. The instrument parameters used were developed by Caren (18). D65 tungsten lamp with a 10 degree observer angle, slit width 4 nm, scanning speed 923.59 nm/min within the wavelength range of 780-380 nm in 5 nm data intervals.
Before each analysis, an auto-zero was performed using a calibrated, white Spectralon® reflectance standard and the white BPA card was analysed before each sample set to account for background correction.

The WinLab software was set up to record the spectra and chromaticity values which included seven variables; X-value, Y-value, $L^*$ (lightness), $a^*$ (the colour continuum of red to green), $b^*$ (the colour continuum of yellow to blue), $Cab^*$ (chroma) and $Hue^*$ (25).

Each sample set was measured at 14 time points ranging from 1.5 h to 672 h with the exception of the 24 % Hct value set due to increased drying time from a higher water content. These times were initially staggered at 1.5, 3, 6, 12 and 24 h followed by once a day for a week then weekly readings to 672 h (4 weeks). Each blood stain was scanned twice, rotating clockwise on the second scan to account for the natural variation in blood and to gain an accurate colour reading representation. As a result, each sample set and separate time has a dataset of 40 scans.

2.4 Data Pre-processing

Data sets acquired for each Hct value were processed in Microsoft Excel by identifying and eliminating outliers, if any. This was achieved by testing for homoscedasticity using boxplots, as well as histograms for normality and removing any values that appeared outside the normal range. For some sets this eliminated up to six values of the 40 and others removed none, for consistency, the same parameters were used for all data regardless of variations in standard deviations.

2.5 Statistical Analysis

Data charts were developed in Microsoft excel using the means of the processed data in a scatter graph against time to determine the relationship between each factor and aging. The time scale was spaced logarithmically to space the data for ease of visual understanding.

As there were two independent variables, inferential statistics was then performed in Excel using two-way analysis of variance (ANOVA) with repeated measures analysis on the whole dataset to find if there is any interaction between the two independent variables on the dependent variable. An
individual two-way ANOVA without repeated measures was carried out after determining if the data satisfied the assumptions of equal replicates, normality and homogeneity of variance on each chromaticity value set. Whenever necessary (significant differences found in ANOVA), a post-hoc test was done to identify the differences. Tukey’s honestly significant difference (HSD) post-hoc test was chosen and performed on all chromaticity value datasets using astatsa.com (26) and representative graphs drawn up for ease of observation.

3. Results

3.1 Visual Observations

The edges of each blood stain dried first in all samples and none were fully dry at 1.5 h, however, all excepting the 24 % set were fixed enough to restrict movement and were able to be turned sideways for analysis. As the stains dehydrated, the colour observed darkened and over time moved from red to red-brown. Cracking occurred from approximately 3 h onwards and was more prominent in the higher concentration range. The change in colour is represented in Figure 1 using the neat sample (35 % Hct) where both colour and cracking is visible. A lighter red outer ring is apparent in the sample up to 96 h after which it darkens to a similar shade to the interior of the stain.

![Figure 1](image)

Figure 1: Colour change of neat blood sample over time period of 0 h (top left) to 672 h (bottom right). Photos were taken at time of deposition and at every measured time.
Figure 2: A) Comparison of appearance of blood stains at 0 h (top) and 672 h (bottom) between neat and addition of sodium citrate anticoagulant. B) Comparison of blood stain appearance of high and low Hct concentrations at 3 h (top) and 672 h (bottom).

The physical appearance is very similar between the neat and citrated 35 % samples and Figure 2A shows similar colours and patterns initially, however, the cracking pattern is less uniform in the neat sample once drying has occurred. Initially, there was a large difference in colour when 24 % and 61 % was compared as the lower concentration showed a bright red immediately. Even at the 3 h mark shown in Figure 2B, there is a difference in brightness. This continues throughout the experiment as seen with the 672 h samples at the bottom in Figure 2B.

3.2 Spectral Trends

The reflectance spectra generated followed a trend showing distinct differences between grouped time frames with each measured time showing a slightly different shape and % reflectance (Figure 3). Some cross over was noted between similar times and intersections are more prevalent as the age progresses.
Separation was also clearly perceived between the different Hct values as shown in Figure 4A and 4B which presents one mid-range value from each Hct concentration sampled for 3 h and 168 h respectively. At early aging times the separation appears less clear between 43 %, 56 % and 61 % as well as the neat blood sample. 35 % and 24 % are clearly separated. At the later stages, in this case one week, the separation is clearly represented, indicating that reflectance values are dependent upon cellular concentrations.

**Figure 3:** Spectral form of one blood stain of the neat 35 % dataset over time showing all measured time points.
Figure 4: A) Spectra of Hct concentrations at 3 h B) spectra of Hct concentrations at 168 h. Both ranging from 24 % to 61 %.
3.3 Chromaticity Values as a Function of Time and Hct Concentration

Means of each chromaticity value dataset were determined in Microsoft Excel and graphed against time. The data represents ± standard errors of mean (SEM) with \( n=40 \) per group. To further investigate group variance, a two-way ANOVA with repeated measures was performed to test the hypothesis of equal effects of 6 independent variables at each experimental time point. The reported \( p \)-value was \( 1.24 \times 10^{-61} \) indicating that there was a significant difference leading to a rejection of the \( H_0 \) that Hct concentration does not affect the rate of aging as this is below the alpha level of 0.05. Additionally, the \( F\text{-}stat \) of 55.05 is significantly higher than the \( F\text{-}crit \) of 2.02 further confirming that there is a significant difference between Hct concentration groups. For each individual chromaticity value dataset, a highly significant \( p \)-value was determined as confirmation for \( H_0 \) rejection using two-way ANOVA without repeated measure. These are reported in Table 2 and confirm that Hct concentrations are significantly different over each time point.

![Table 2: Two-way ANOVA without repeated measures \( p \)-values and \( F \)-values of each chromaticity measurement where if \( F\text{-}stat > F\text{-}crit \) and \( p \)-value < alpha of 0.05 the \( H_0 \) is rejected therefore confirming a significant difference between groups.]

<table>
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<th>Chromaticity measurement</th>
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<th>( F\text{-}stat )</th>
<th>( F\text{-}crit )</th>
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<td>( y )-value</td>
<td>( 1.2 \times 10^{-19} )</td>
<td>23.48</td>
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<td>( L^* )</td>
<td>( 7.13 \times 10^{-28} )</td>
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</tr>
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<td>( a^* )</td>
<td>( 1.8 \times 10^{-27} )</td>
<td>98.08</td>
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</tr>
<tr>
<td>( b^* )</td>
<td>( 9.11 \times 10^{-31} )</td>
<td>58.77</td>
<td>1.87</td>
</tr>
<tr>
<td>( C_{ab}^* )</td>
<td>( 1.52 \times 10^{-33} )</td>
<td>84.81</td>
<td>1.87</td>
</tr>
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<td>( Hue^* )</td>
<td>( 2.46 \times 10^{-36} )</td>
<td>7.10</td>
<td>1.87</td>
</tr>
</tbody>
</table>

3.3.1 \( x \)-value

As can be observed in Figure 5, the \( x \)-value decreased over time with an initial exponential decline followed by a stabilising effect as the stain ages. The reflectance recorded is different for each
concentration, indicating a direct relationship with the rate of aging and the percentage of Hct. There is also a difference noted between the 35 % Hct value and the neat sample, which is of the same concentration as neither was diluted after extraction. Some overlapping of data points and SEMs are present, especially at higher Hct concentrations and older time points. The Tukey’s HSD post hoc test for x-value is presented in Figure 6 where each concentrations dataset was compared against each other. This result shows that there is no significant difference between the different Hct percentage data as represented by the colour red.

![Figure 5: Plot of chromaticity X-value as a function of time over varying Hct concentrations ranging from 24% to 61%.](image-url)
Figure 6: Tukey's HSD post hoc test for similarity of mean X-values between different Hct percentages. Red represents no significant difference while green (if present) represents a significant difference. P-values available in the appendix.

3.3.2 Y-value

The y-value data displays inconsistencies in shape between different concentrations of Hct which can be observed in Figure 7. The SEMs overlap at random data points and the shape of each concentration does not correlate with each other. The p-value of $1.2 \times 10^{-19}$ from Table 2 also indicates a significant difference between Hct values. Figure 8 shows the results of the Tukey's HSD post hoc test where no significant differences were found between the different concentrations. This correlated with the data in Figure 7 regarding the cross-over of errors.
Figure 7: Plot of chromaticity Y-value as a function of time over varying Hct concentrations ranging from 24 % to 61 %.

Figure 8: Tukey’s HSD post hoc test for similarity of mean X-values between different Hct percentages. Red represents no significant difference while green (if present) represents a significant difference. P-values available in the appendix.
3.3.3 $L^*$

Data from $L^*$ shows a general downward trend, however, much like the $y$-value data, there are inconsistencies. Figure 9 represents this lack of correlation where the data lines are frequently crossing paths. This is also represented by the $p$-value in Table 2 which is significantly lower than the alpha of 0.05. Tukey’s HSD post hoc test in Figure 10 shows that the majority of data points between concentrations have no significant differences except for those between 56 % and 24 % as well as 61 % and 24 %. This value was highly significantly different due to being lower than an alpha value of 0.01 indicating that these values are independent of each other.

![Figure 9: Plot of chromaticity $L^*$ as a function of time over varying Hct concentrations ranging from 24 % to 61 %](image-url)
Figure 10: Tukey’s HSD post hoc test for similarity of mean $L^*$ between different Hct percentages. Red represents no significant difference while green represents a significant difference. $P$-values available in the appendix.

3.3.4 $a^*$, $b^*$ and $Cab^*$

The $a^*$, $b^*$ and $Cab^*$ values follow the same trend as $x$-value, starting as a negatively exponential curve and smoothing out over time. SEMs also overlap at the intervals at older ages and with higher Hct values, especially with that of the 61 % Hct which crosses over several other samples in the initial phase. $P$-values in Table 2 indicate a significant difference which correlates with observation of the spacing seen in Figures 11, 13 and 15. Figures 12, 14 and 16 show the Tukey’s HSD post hoc test results where no significant difference was seen between data sets on either $a^*$, $b^*$ or $Cab^*$. 
Figure 11: Plot of chromaticity $a^*$ as a function of time over varying Hct concentrations ranging from 24 % to 61%.

Figure 12: Tukey's HSD post hoc test for similarity of mean $a^*$ between different Hct percentages. Red represents no significant difference while green (if present) represents a significant difference. $P$-values available in the appendix.
Figure 13: Plot of chromaticity $b^*$ as a function of time over varying Hct concentrations ranging from 24 % to 61 %.

Figure 14: Tukey’s HSD post hoc test for similarity of mean $b^*$ between different Hct percentages. Red represents no significant difference while green (if present) represents a significant difference. $P$-values available in the appendix.
Figure 15: Plot of chromaticity $Cab^*$ as a function of time over varying Hct concentrations ranging from 24 % to 61 %.

Figure 16: Tukey’s HSD post hoc test for similarity of mean $Cab^*$ between different Hct percentages. Red represents no significant difference while green (if present) represents a significant difference. $P$-values available in the appendix.

3.3.5 Hue*

Representation of $Hue^*$ displays minimal correlation with the aging of blood stains as each Hct percentage in Figure 17 presents a different shape over time. The absence of positive interactions
between time and $Hue^*$ is further solidified by the SEMs which are relatively large in comparison to all previous datasets and still retain minimal cross-over. Tukey's HSD post hoc test in Figure 18 shows non-significant difference for half the Hct interactions. Moderately significant differences are represented by a $p$-value <0.01 and significant differences with a $p$-value <0.05 which appears between the lowest and highest values, therefore, there is still not a complete and clear separation between groups, even in the least correlated dataset.

![Figure 17: Plot of chromaticity $Cab^*$ as a function of time over varying Hct concentrations ranging from 24 % to 61 %](image)
Figure 18: Tukey’s HSD post hoc test for similarity of mean Hue* between different Hct percentages. Red represents no significant difference while green represents a significant difference. P-values available in the appendix.

4. Discussion

Finding a reliable method to estimate the age of blood stains is a difficult task and is greatly affected by changes in environmental factors. Temperature is a well-recognised interfering factor as is humidity in many aging techniques (2, 3) though in the method used here, only temperature has been investigated (19). Another consideration is that of internal factors such as Hct percentages and drug intake. This investigation was undertaken using UV/Visible diffuse reflectance spectroscopy and colorimetric analysis under controlled conditions to determine whether Hct concentrations affect the rate of aging. This method has previously been found effective in estimating time of deposition of human blood stains (18), however, further investigation into factors which affect this method is required.

Previous analyses have determined, with the aid of recursive feature elimination, a* and Cab* to be the most useful colorimetric values for estimation of time since deposition of blood stains (18, 19). During the inferential statistical analysis of this investigation, the graphs generated show the correlation between age and reflectance for x-value, a*, b* and Cab* while the remaining chromaticity values of y-value, L* and Hue* do not show a clear relationship, these are the same trends observed by Caren (2015) (18). The general shape and spacing of the graphs in Figures 5, 11, 13 and 15 were also found to be very similar to those developed from variations in temperature by Cunningham (2016) (19) showing that Hct variation is almost as large a factor as temperature change. There is limited information on the effect of Hct values on estimation of time since deposition as focus has
previously been on external factors making this a relatively novel approach. One study used varying Hct values for BPA analysis in regards to stringing methods and accuracies and found there to be negligible error margins (27). This, however, only refers to the fluids physical behaviour and not chemical changes which were measured here.

A visual difference in colour as well as a mathematical difference in varying Hct values was clearly reported in the current study (Figures 5 to 18). *P*-values reported in Table 2 from Two-way ANOVA suggest that there is a highly significant difference between Hct values as each value is smaller than an alpha value of 0.001. This is slightly contradicted by the Tukey’s HSD post hoc tests performed on the same data which states that there are no significant differences with the exception of *Hue* and *L*; however, this takes into account the overlap in error margins of which are relatively high in this method.

In order to further investigate these results, a secondary study should be conducted with smaller sample sets in at least duplicates. Smaller sample sets will aid accuracy as each scan runs for approximately one minute so by the time all 20 samples are scanned twice, a minimum of 40 minutes has passed between the first and last sample, increasing the error rate, especially at early deposition times. Less time between the first and last scan could decrease the error by up to 25 % at a 3 h time point or 3 % at 24 h. After this point the errors created by the time lapse become negligible. As great care was taken to control the environmental exposure of each sample set and all instruments were regularly calibrated, this is the most likely source of error. This is enforced by observing the SEM bars on each scatter graph above as the margin is largest at the earlier time frames and become much smaller towards the 672 h mark, with the exception of Figure 18 where *Hue* maintains a similar error throughout.

Another factor which was not visually perceived in Figure 1A but can be seen in the data of the chromaticity graphs in section 3.3 as well as Figure 4, is the effect of anticoagulant factors. Many people are prescribed a variation of blood thinners with anticoagulant properties and although sodium citrate was used during this procedure and is not a commonly prescribed medication, there is enough of an effect to warrant investigation. The difference could be due to the specific nature of citrate which creates a bond with calcium preventing coagulation, or due to the increased homogeneity as a result of the prevention of a coagulation cascade (28). This can be observed in Figure 1A where both 35 %
samples are compared at time 0 h and 672 h where the initial colouring is very similar, however, the cracking observed appears smaller and more uniform in the coagulated sample.

One other factor to consider could be the increased drying time in samples infused with the anticoagulants. Drying time was also increased for samples of lower Hct values which contained a higher proportion of water; therefore, this is likely something that would need further research if studies were to continue into the area of colorimetry with UV/Visible spectroscopy. A possible explanation could be that the additional plasma is able to act as a protective insulated layer, delaying initial oxidation of the Hb. In order to determine this, a study on oxygen percentages could be implemented using the oxygen electrode method (9). Tan (29) used Hyperspectral imaging to investigate the drying effects of blood in comparison to imitation blood of the same colour. It was concluded that the effect that was observed was unique to the blood samples and therefore most likely due to a chemical reaction rather than drying. Considering the large variance in Hct values found in the general population and that many aged and also some young people are medicated with some form of anticoagulant to prevent clotting and heart disease, this will require further investigation to determine the extent of the delay in aging of blood stains.

Overall, this study suggests that cellular concentration is likely a factor in the blood aging process when using colour change as the measured value. This means that in order to use this method in the field, background information on the victims regular Hct levels will be required to form accurate age estimation. Caren (18) and Cunningham (19) have investigated the effects of temperature on the chromaticity readings and found significant variations while humidity, a known contributor to variation in aging rates for other methods, has yet to be examined. Overall, the variation in temperature, humidity, light exposure and now Hct values are all requiring testing and development of corrective algorithms for accurate estimations, or even weekly approximations. This could present a problem if the source of the blood pattern is deceased or has recently lost a significant amount of blood as the cellular percentage will have changed due to evaporation or loss of matter.
Acknowledgement

The author would like to acknowledge Associate Professor Robert Mead for his phlebotomy skills and willingness to share advice and knowledge as well as Dr Vineeta Bilgi for her aid in statistical processes. This research was funded by Murdoch University, Perth, WA.

References


19. Cunningham N. The Effect of temperature on estimating the time since deposition of a bloodstain using diffuse reflectance spectroscopy [honours]: Murdoch University; 2016.


27. Evans S. EFFECT OF HAEMATOCRIT VALUE ON AREA OF ORIGIN DETERMINATION FOR BLOODSTAIN PATTERN ANALYSIS [honours]: Murdoch University; 2015.


Appendix Supplementary Figures

### ANOVA

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**Figure A1:** Two-way ANOVA with repeated measures extended result.

**Figure A2:** Tukey’s HSD post hoc test for similarity of mean X-values between different Hct percentages including p-values. Red represents no significant difference while green represents a significant difference.

**Figure A3:** Tukey’s HSD post hoc test for similarity of mean Y-values between different Hct percentages including p-values. Red represents no significant difference while green represents a significant difference.
Figure A4: Tukey's HSD post hoc test for similarity of mean $L^*$ between different Hct percentages including p-values. Red represents no significant difference while green represents a significant difference.

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Figure A5: Tukey's HSD post hoc test for similarity of mean $a^*$ between different Hct percentages including p-values. Red represents no significant difference while green represents a significant difference.

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Figure A6: Tukey's HSD post hoc test for similarity of mean $b^*$ between different Hct percentages including p-values. Red represents no significant difference while green represents a significant difference.

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### Figure A7: Tukey’s HSD post hoc test for similarity of mean $\text{Cab}^*$ between different Hct percentages including $p$-values. Red represents no significant difference while green represents a significant difference.

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### Figure A8: Tukey’s HSD post hoc test for similarity of mean $\text{Hue}^*$ between different Hct percentages including $p$-values. Red represents no significant difference while green represents a significant difference.

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