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Spectral analysis of blood stains at the crime scene

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5 - PRACTICAL IMPLEMENTATION OF BLOOD STAIN AGE ESTIMATION

In preparation

To enable the use of spectroscopy for the age estimation of blood stains in forensic practice, a method applicable for blood stains found on many different backgrounds is needed. We describe a one dimensional light transport model, which corrects for light absorptions of the background. Using this method, we calculate the relative amounts of HbO₂, MetHb and HC in blood stains on coloured backgrounds, based on their reflectance spectra. Additionally, we describe a statistical method to calculate an estimated age within a 95% confidence interval. The increased applicability in casework and its possible value for crime investigations is demonstrated in a case example of a shooting incident where 3 bodies were found dead in a living room. For crime reconstruction purposes, the absolute and relative age of different groups of blood stains were measured using visible reflectance spectroscopy. We analyzed blood stains at two distinct locations in the house: downstairs where the victims were found, and upstairs. The results indicated that the group of blood stains found upstairs was older than the blood stains found in the vicinity of the bodies downstairs. Thus, the blood stains upstairs were probably not related to the current crime. The age estimated for the blood stains downstairs was 1.5-3.2 days old. This time interval includes the moment gun shots were heard by a witness. Combined with other evidence, the age of blood stains may lead to a better reconstruction of the timeline of events.

5.1. INTRODUCTION

Blood stain age estimation techniques can provide valuable information for criminal investigators. Age information gives insight in the sequence of blood shedding events, or even the absolute time of deposition. This intelligence can be useful for the verification of suspect or witness statements, and for the selection of relevant samples for e.g. DNA analysis, reducing the workload in forensic laboratories. In **Chapter 4** it was shown that the age of blood stains on white backgrounds can be estimated using visible reflectance spectroscopy or hyperspectral imaging^{1, 49, 67, 68}. On coloured backgrounds, light absorption by the background influences the measured reflectance spectra. In this chapter, we describe a physical spectral processing model to correct for these background absorptions, which is demonstrated by a case example.

In the last decade several spectroscopic techniques, including visible reflectance spectroscopy (**Chapter 3, 4**)^{1, 18, 49, 65, 67, 68}, near infrared spectroscopy (**Chapter 7**)^{19, 66}, and Raman spectroscopy^{103, 104} have been explored for the analysis of blood stains in laboratory settings. The transition from laboratory measurements, with its ideal experimental conditions, to crime scene analysis however, is complicated by the wide variety of substrates on which the blood may be deposited¹⁰⁵. This problem is often encountered in case work, and may explain why no blood stain age estimation technique has yet routinely been applied in forensic investigations.

We demonstrated in **Chapter 4** how visible reflectance spectroscopy or hyperspectral imaging can be used to measure the concentration change of oxyhaemoglobin (HbO₂), methaemoglobin (MetHb) and hemichrome (HC) – all reaction products of haemoglobin – in a laboratory setup, without destroying or even touching the sample⁴⁹. A reference database of the relative amounts of HbO₂, MetHb and HC for blood stains of different ages stored under controlled ambient conditions (with known temperature and humidity) can in turn be used to estimate the age of a questioned blood stain.

In previous studies on age estimation of blood stains using spectroscopy, possible sources of spectral variation were often controlled or taken constant for all experiments^{1, 67, 68}, e.g. the background colour, the blood

stain thickness, and the sample-detector distance, which is impossible in forensic practice. We now describe a one dimensional light transport model for the calculation of the relative amount of HbO₂, MetHb and HC in the blood stains, which can be applied to correct for these variables, thereby increasing the applicability of blood stain age estimations in forensic practice. Additionally, we describe a statistical method to calculate an estimated age within a 95% confidence interval.

Using reflectance spectroscopy we repeatedly measured equally aged blood stains on a glass slide which were placed on top of several coloured backgrounds. The calculated haemoglobin derivative fractions for blood stains on different background colours were compared. Finally, we describe a criminal case where we estimated the age of blood stains using the light transport model. At the scene of a presumed double homicide followed by suicide, blood stains were found on several backgrounds at two distinct locations. The research questions were twofold. 1) What is the age of the blood stains surrounding the victims? This information gives insight in the moment the crime was committed. 2) Is there an age difference between the blood stains found at two different locations? The answer to the latter question can indicate whether a blood stain is related to the crime. Application of this technique in practice provided investigators with information currently not available, which could be used to create possible scenarios.

5.2. BLOOD STAIN AGE ESTIMATION

5.2.a LIGHT TRANSPORT MODEL

To find the relative fractions of HbO₂, MetHb and HC present in a blood stain a least squares fitting algorithm was employed in combination with a one-dimensional light transport model for multi-layered samples⁸⁶:

$$R = \frac{1 - R_b \cdot (a - b \cdot \coth(bST))}{a - R_b + b \cdot \coth(bST)}, \text{ with } a = \frac{S + K}{S} \text{ and } b = \sqrt{a^2 - 1}, \quad (5.1)$$

where R is the blood stain reflectance (-), R_b the reflectance of the background (-), S is derived from the scattering coefficient (mm^{-1}), and K from the absorption coefficient (mm^{-1}), which are all wavelength dependent^{1, 87}. T is the blood stain thickness (mm). This model describes the reflectance of a homogeneously coloured sample of fixed thickness on top of an absorbing substrate as a function of wavelength. Light is assumed to be diffuse in all directions after penetrating the sample, although the model considers only two net fluxes straight up and straight down. The model used in **Chapter 3 and 4** is the limit of formula 5.1, valid only for blood stains of infinite thickness.

Formula 5.1 is compared to the measured reflectance spectrum using a least squares fitting algorithm. Input data are the reflectance spectrum of the blood stain, the reflectance spectrum of the background, and the optical properties of the haemoglobin derivatives⁸². The output of the algorithm is the:

- relative amount of HbO₂ in the blood stain,
- relative amount of MetHb in the blood stain,
- relative amount of HC in the blood stain,
- blood stain thickness,

based on the best fit of the non-linear function to the measured reflectance spectrum. The relative amounts of haemoglobin derivatives change when blood stains are aging, as shown in **Chapter 4**^{6, 18}. The blood stain thickness determines how much the reflectance spectrum is influenced by light absorption of the background.

5.2.b STATISTICAL ANALYSIS

To estimate the age of a questioned blood stain, the calculated HbO₂ fractions are compared with a reference database of blood stains measured up to an age of 200 days old. The MetHb and HC fractions are disregarded, because of their sensitivity for humidity changes⁵⁶. Because first order reaction kinetics are expected, the HbO₂ fractions are plotted against the natural logarithm of the age in Figure 5.1. In this figure three different phases are observed, with age intervals of less than 1 day, from 1 to 20 days, and more than 20 days. In the first phase, the measured HbO₂ fraction decreases exponentially,

corresponding to the fast oxidation rate of HbO₂ observed by Bremmer et al in the first hours after deposition of the blood stain. After this phase, a slower exponential decay is observed for blood stains with ages between 1 and 20 days, again in agreement with Bremmer et al. The age interval of the third phase was not analysed in Bremmer's report, which explains his reference to a biphasic decay. The change after 20 days may be explained by the decreasing fraction of MetHb around this age, as shown in **Chapter 4**^{1,49}.

For each of the three age intervals, we calculated a linear regression model, which was used to relate the natural logarithm *age* (days) of blood stains to the HbO₂ fractions in order to estimate the age of questioned blood stains (denoted by the subscript q) based on measured HbO₂ fractions [HbO₂]_q:

$$\text{Phase 1) } \ln(\text{age}) = -11.7 \cdot [\text{HbO}_2]_q + 6.9 \quad (\text{adjusted } R^2 = 0.95) \quad (5.1)$$

$$\text{Phase 2) } \ln(\text{age}) = -6.1 \cdot [\text{HbO}_2]_q + 4.0 \quad (\text{adjusted } R^2 = 0.91) \quad (5.2)$$

$$\text{Phase 3) } \ln(\text{age}) = -22.7 \cdot [\text{HbO}_2]_q + 7.4 \quad (\text{adjusted } R^2 = 0.91) \quad (5.3)$$

Independent of the phase, a 95% confidence interval for the predicted age can be determined, as described by Maddala¹⁰⁶, by:

$$\ln(\text{age}) \pm t_{\alpha/2}(n-2) \sqrt{1 + \frac{1}{n} + \frac{([\text{HbO}_2]_q - \overline{[\text{HbO}_2]})^2}{S_{[\text{HbO}_2]}}}, \quad (5.4)$$

in which $\alpha = 0.05$, t is Student's t-value, n is the number of measurements in the reference database, $\overline{[\text{HbO}_2]}$ is the average HbO₂ fraction, and $S_{[\text{HbO}_2]}$ its standard deviation:

$$\overline{[\text{HbO}_2]} = \sum_{i=1}^n [\text{HbO}_2]_i / n,$$

$$S_{[\text{HbO}_2]} = \sqrt{\sum_{i=1}^n ([\text{HbO}_2]_i - \overline{[\text{HbO}_2]})^2 / (n-1)}$$

This confidence interval is influenced by the variability between different blood stains in the reference database.

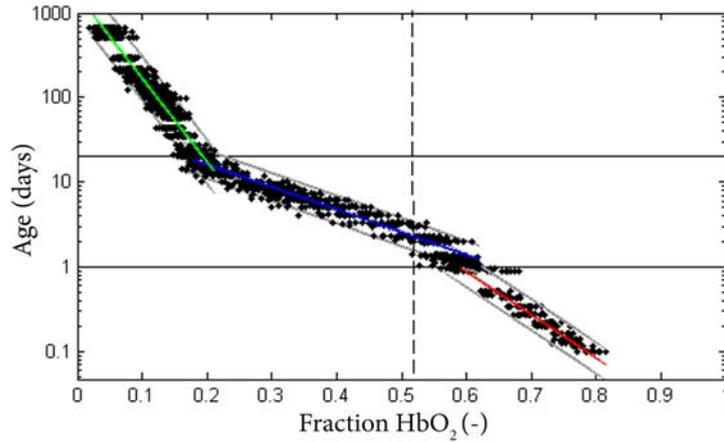


Figure 5.1. The age of blood stains from the reference database plotted against the measured fraction HbO₂ (black dots) on a semi-logarithmic scale. Green, blue and red lines depict the regression lines for three different phases, separated by the horizontal black lines. Their corresponding confidence intervals are plotted in grey. By drawing a vertical line at a measured HbO₂ fraction of a questioned blood stain, its age can be determined (e.g. the striped line).

5.3. LABORATORY TEST

5.3.a MATERIALS AND METHOD

To test background correction of the light transport model, six blood stains were applied onto glass slides, and placed on top of 5 pieces of cotton (white, yellow, pink, blue, and green) with the blood stain facing the cotton (see Figure 5.2). Reflectance spectra of all combinations of 6 blood stains and 5 background colours were measured using a CCD spectrometer (USB 4000; Ocean Optics; Duiven, the Netherlands), a tungsten-halogen light source (H-2000; Ocean Optics; Duiven, the Netherlands) and a non-contact probe (QR400-7-UV/BX; Ocean Optics; Duiven, the Netherlands). These measurements were repeated 10 times, from 2 hours up to 19 days old.

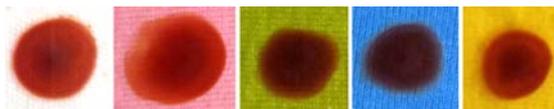


Figure 5.2. Photographs of the samples: blood stains on glass slides, placed on top of white, pink, green, blue and yellow cotton.

Next to the blood stains, reference measurements were collected of the background material. Spectral analysis was limited to the wavelength range of 450 – 800 nm, because of the low detector sensitivity and low power of our light source beyond this range. Each measured spectrum was corrected for dark noise and for intensity variations of the light source using the reflectance spectrum of a white reflectance standard (Spectralon). Reflectance spectra were normalized by applying the standard normal variate algorithm¹⁴. Data analysis was performed using custom-made scripts written in MATLAB (The Mathworks Inc., Natick, Massachusetts, USA).

All corrected reflectance spectra were analyzed using the multi-layer light transport model described above, in which the spectral reflectance of the background was incorporated. The coefficient of determination (R^2) between the measured spectrum and a haemoglobin derivative fit was used as a quality check. This R^2 value approaches 1 if the theoretical fit matches the measurement. Spectra with an $R^2 > 0.999$ were used for further analysis. For all measurements with an R^2 value exceeding this threshold, the relative amounts of haemoglobin derivatives were calculated and plotted against the age of the blood stains.

5.3.b RESULTS

For all combinations of blood stains and background colours, the fitting algorithm was used to deduce the relative amount of haemoglobin derivatives. Figure 5 shows two examples of measured spectra and their theoretical fits for a blood stain on a pink and blue background respectively. This figure demonstrates the difference in fit quality; on the pink background the fit shows a high correlation with the measured spectrum ($R^2 > 0.999$), while the fit clearly deviates from the reflectance spectrum on the blue background ($R^2 = 0.986$).

Based on the threshold of $R^2=0.999$ blood stains on the blue background were disregarded in the further analysis (range of R^2 values: 0.983-0.997). The same applied for blood stains on the green background, which likewise did not pass the quality check (range of R^2 values: 0.986-0.994), contrary to the blood stains on pink, yellow and white backgrounds.

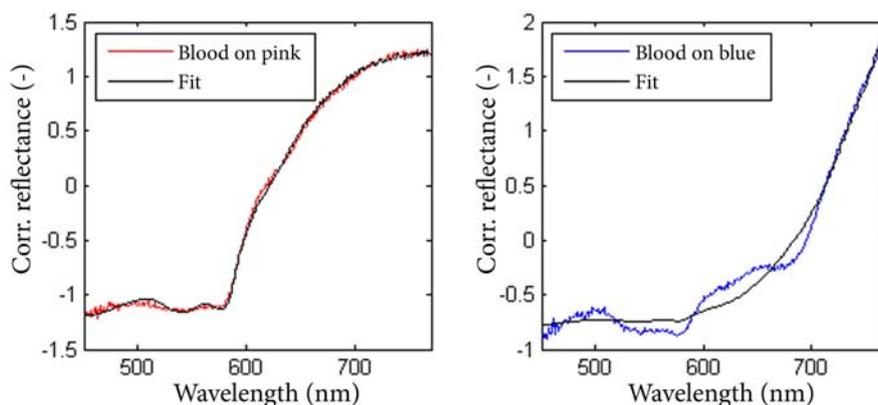


Figure 5.3. Examples of corrected reflectance spectra of a blood stain on a pink background (left, $R^2 > 0.999$) and a blue background (right, $R^2=0.986$) and their corresponding haemoglobin fits.

For these blood stains with an $R^2 > 0.999$ the calculated fractions of HbO_2 , MetHb and HC are plotted against the age in Figure 6. This figure shows overlapping error bars (one standard deviation) of the calculated fractions for the different background, supporting the use of the reference database of blood stains on white cotton for the other colours.

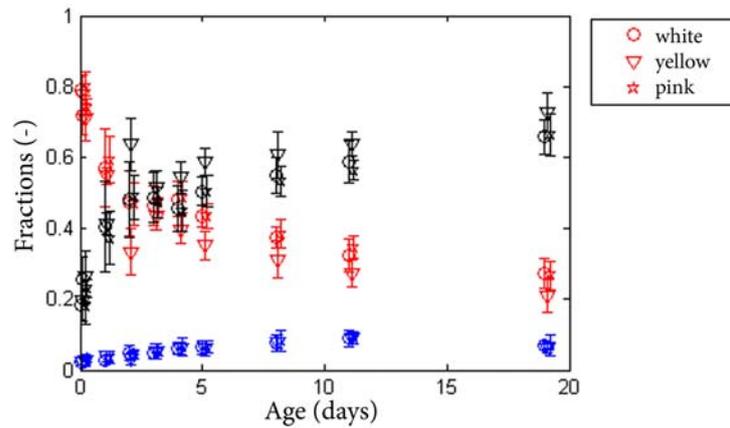


Figure 5.4. Calculated haemoglobin fractions for blood stains of different ages on white, yellow and pink backgrounds. Red data points depict HbO₂, blue corresponds to MetHb, and black data points show the fractions of HC. Error bars illustrate the standard deviations.

5.4. CASE EXAMPLE

5.4.a CASE DESCRIPTION

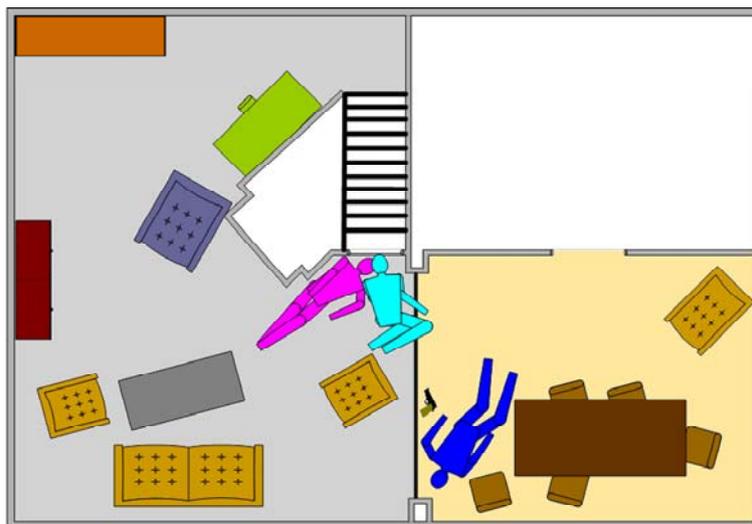


Figure 5.5. Digital sketch of the crime scene, showing three victims in the living room.

On a Sunday morning, a witness reported the sound of gun shots in the neighbouring residence. After entering the house through the attic window, police patrol found two dead men and a woman in the living room (Figure 5.5). A gun was found close to one of the men, who lived in the residence. Forensic investigators measured a rectal temperature difference between this man and the other two victims, indicating that he had died at a later moment. The question arose whether this was a triple suicide, a double murder followed by a suicide, or whether a fourth individual had been involved. In order to reconstruct the events taken place, the blood stain patterns were analyzed. Apart from the blood found around the victims downstairs, several stains were found upstairs (see Figure 5.6).



Figure 5.6. Photographs of blood stains found at the crime scene. Top: blood stains found downstairs (left: next to the man close to the gun, right: around the other two victims), bottom: blood stains found upstairs.

5.4.b MATERIALS AND METHOD

To determine whether these stains were related to the crime, the age of 19 blood stains was estimated using visible reflectance spectroscopy and the light transport model described above. Spectroscopic measurements on the blood

stains were performed 2,5 days after the reported gun shots. DNA evidence showed that the blood stains found upstairs all belonged to the man who lived there. Downstairs, not surprisingly, blood stains with DNA from all three victims were found. Both relative and absolute ages of the blood stains upstairs and downstairs were estimated.

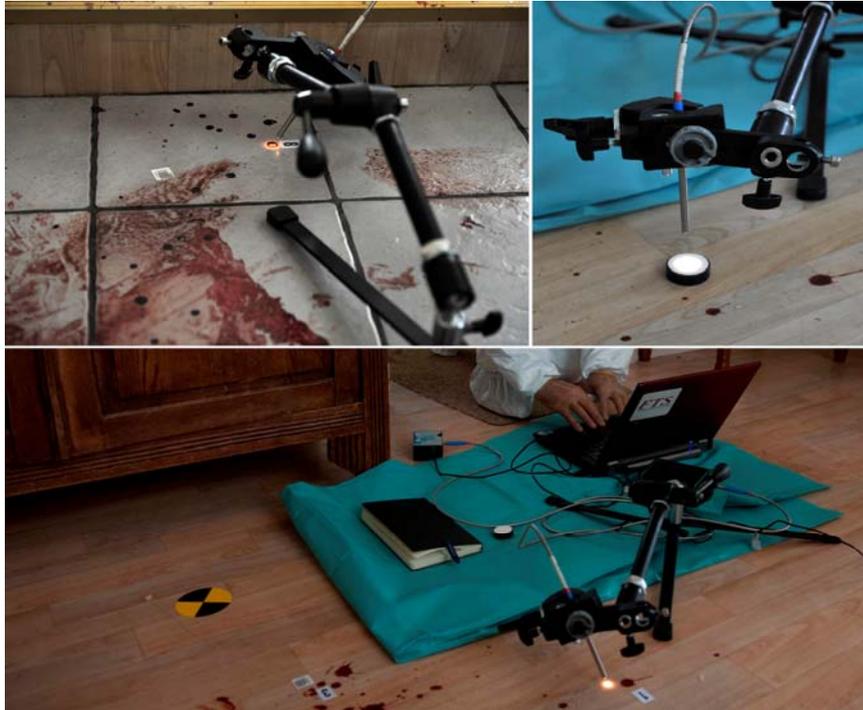


Figure 5.7. Photographs of the measurement setup during reflectance measurements of a blood stain (top left), a white reference (top right) and a clean background (bottom) at the crime scene.

5.4.c RESULTS

Of the 19 selected blood stains in the criminal case, 11 measurements (6 from downstairs, 5 from upstairs) produced an $R^2 > 0.999$ and thus passed the quality check for further analysis. Figure 5.8 (left) shows an example of a measured reflectance spectrum of a blood stain found downstairs and the

corresponding haemoglobin derivative fit. In this spectrum, the absorption characteristics of oxyhaemoglobin (dips at 540 and 576 nm) and methaemoglobin (a dip at 630 nm) are clearly visible. This indicates the relative freshness of the stain. The dip around 720 nm is due to light absorption of the background, which is corrected for. The right spectrum in Figure 5.8 has less characteristic features, indicating that the stain is older.

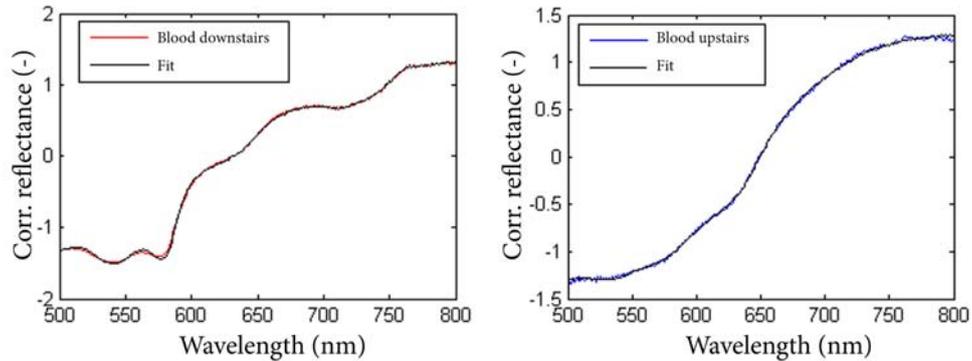


Figure 5.8. Examples of corrected reflectance spectra and their corresponding haemoglobin derivative fits for a blood stain found downstairs (left) and upstairs (right).

The calculated haemoglobin fractions are displayed in Figure 5.9. The error bars of the two blood stain groups do not overlap. This suggests that the blood stains were not created at the same time. More specifically, the lower amount of HbO_2 found for the blood stains upstairs indicates that these blood stains are older, as the amount of HbO_2 decreases in time.

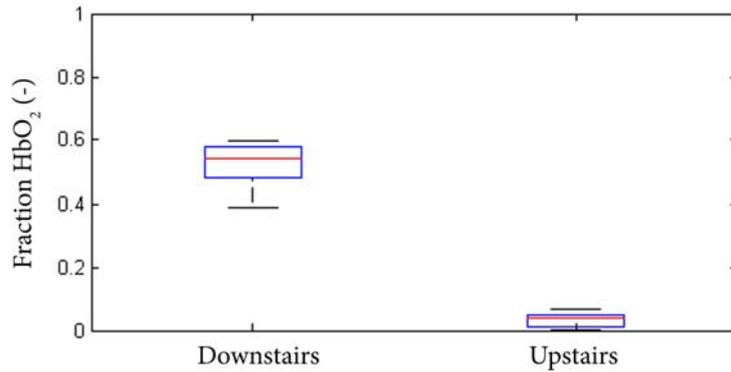


Figure 5.9. Box plots showing the measured HbO₂ fractions for blood stains downstairs and upstairs.

The average amount of HbO₂ for the blood stains downstairs is 0.52 (with a standard deviation of 0.08). The striped line in Figure 5.1 shows that this value corresponds with blood stains in the second phase (2-20 days). Using the second regression model described in formula 5.2, and formula 5.4 we calculated an age of 1.5 – 3.2 days, within a 95% confidence interval. This interval includes the moment a witness claimed to have heard the sound of gun shots. The fraction of HbO₂ found upstairs (average: 0.03, standard deviation: 0.03) does not correspond with these in the reference database at any moment in time, which suggests that the blood stains are very old. With a humidity and temperature comparable to the laboratory in which the reference blood stains were analysed (45 ± 5 % RH and 22.3 ± 0.5 °C respectively), this stage is not yet reached after 200 days. In a warmer atmosphere, reaction rates are faster and the amount of HbO₂ will decay faster⁵⁶. However, even in an environment of 40 degrees Celsius, the measured HbO₂ fraction was not reached after 20 days (unpublished results).

5.5. DISCUSSION

We demonstrated a light transport model which is able to correct for background absorptions (within limits), and therefore widely extends the

applicability of visible reflectance spectroscopy for blood stain age estimation in forensic practice. In a laboratory setup, we showed that the calculated fractions of haemoglobin derivatives were similar for blood stains on white, yellow and pink backgrounds. Finally, we successfully applied the blood stain age estimation technique in a criminal case and calculated 95% confidence intervals of the questioned age based on a statistical method described in this chapter.

The light transport model estimates the measured reflectance spectrum under the assumption that the blood stain is homogeneous and has a constant thickness. This is a simplified model, which will cause some deviations from the complex reflectance spectrum measured in reality. The described model incorporates the reflectance spectrum of the background in a non-linear way and varies the influence of background absorptions by varying the blood stain thickness. In theory, this model is a good estimation for all background colours. However, for green and blue backgrounds, the best fit deviated significantly from the measured reflectance spectrum. Optimization of the least squares fitting algorithm is needed to solve this problem. If the thickness of the blood stains can be measured, this reduces the amount of fit parameters, which may improve the fit quality. Until that time, the R^2 threshold serves as a quality check, to test which measurements can be successfully analysed.

The proposed statistical model uses three linear regression models to describe the data for the different phases in the aging process. These are simple models, which leave room for future improvement, but they describe the data sufficiently, as indicated by the high adjusted R^2 values. The boundaries of the three phases were chosen manually by observing the data, and correspond with the phases described by Bremmer et al⁵⁶. The first two phases have been attributed to fast oxidation of the α chains of haemoglobin and slow oxidation of the β chains. The change to the third phase may be related to the transition from an increase to a decrease of the amount of MetHb^{49, 107}. The optimal change points can be determined statistically in the future¹⁰⁸. How to deal with blood stains with an age around the boundaries of the models is a topic for further discussion.

In our model, we disregard the amounts of MetHb and HC for the age estimation of questioned blood stains, because of their dependence on the ambient relative humidity⁵⁶. If we would take these haemoglobin derivatives into account, a more accurate age estimation is expected, however the uncertainty and thus the confidence interval would be larger. The confidence intervals used are based on individual measurements of the reference database, instead of their averages. This procedure is conservative, but gives a good indication of the uncertainty. Confidence intervals may be decreased using an approach based on average measurements.

The resulting confidence intervals in casework are generally expected to be larger than intervals resulting from measurements performed in a laboratory setup. Variations in illumination intensity, blood stain thickness, background optical properties, environmental temperature and humidity may all cause differences in spectral response. These factors may also have caused the low fit quality of 8 blood stains analyzed in this case and the blood stains on blue and green backgrounds. Detailed knowledge about these influences is useful to create a list of requirements needed for practical applications to be successful, e.g. maximum and minimal stain thickness and blood stain sizes, background colours, etc. More research is scheduled to discover the limitations of the technique.

In the described case, the relative amount of HbO₂ in two groups of blood stains differed significantly. Under the assumption that the temperature upstairs was similar to downstairs, it was concluded that the blood stains upstairs were not created at the same time. The age interval estimated for the blood stains downstairs, 1.5 – 3.2 days old, was in agreement with tactical information (the reported sound of gun shots), and the estimated post mortem interval based on rectal temperature measurements. The blood stains found upstairs were much older. Combined with other tactical and technical evidence, these results indicated that the blood stains found upstairs were not created at the moment of the crime. DNA evidence showed that these stains all belonged to one of the victims, who lived in the residence.

The reported ages of blood stains should be interpreted with caution. Stains found on different locations may have aged under different

environmental circumstances. Humidity and temperature influence the speed of the chemical reactions within the blood stains⁵⁶. Hence, spectral variations are not necessarily caused by age differences, but can also be due to environmental differences. Prior knowledge about these circumstances is needed to correct for differences in chemical reaction rates. In the described case, a weed cultivation site was located in the attic. Although the blood stains were found on other floors, a higher temperature may have accelerated the aging process. However, even at a temperature of 40 degrees Celsius the amount of HbO₂ found upstairs was not yet reached after two weeks in our reference database⁵⁶, supporting the statement that the blood stains found upstairs were created at an earlier moment than the blood stains downstairs.

In the described case, it would have been interesting to know which person was shot first and what the time difference was between the shots. In a laboratory setup, the age of blood stains can be determined with an accuracy of 1 hour within the first day after bleeding⁶⁸. If our measurements would have been performed immediately after discovery of the crime scene, it may have been possible to indicate the sequence of different blood stain patterns created near the victims (different blood stains found downstairs). The chemical reactions taking place in the blood stains are rapid in the beginning, but slower in a later stage⁵⁶. As a result, the accuracy of age estimations decreases with the age of the blood stain. Thus, to culminate small age intervals, it is recommended to perform spectroscopic measurements as soon as possible.

If early measurements are not possible, stains may be collected from the scene and stored deep-frozen to slow down or even stop any further chemical reactions taking place before the analysis is performed. Ideally, stains are collected and frozen on their original background. Traditional collection of blood stains with moistened cotton swabs will alter their chemical composition, as the addition of water to a blood stain is known to induce the transition from HC back to MetHb¹⁰⁹. Further research is needed to find a collection procedure which does not influence the results of the age estimation.

As visible in Figure 5.7, the equipment used in this case was not dedicated for forensic practice. When used at the crime scene, equipment ideally is portable, wireless, easily decontaminated and user-friendly. Instead of

a fibre-optic probe spectroscopy setup, a hyperspectral imaging system may be used to perform measurements remotely, as in **Chapter 4**⁴⁹. An additional benefit of hyperspectral imaging is that the reflectance spectra of multiple blood stains can be recorded simultaneously, which makes the measurements more time-effective, while the spatial distribution of the stains in the scene is registered at the same time. Recent technological advances enable the development of wireless hyperspectral imaging systems which can easily be deployed at the crime scene.

In conclusion, we demonstrated a light transport model to for the age estimation of blood stains on coloured backgrounds, and a statistical model to calculate a 95% confidence interval. The practical applicability of the technique was demonstrated in a recent forensic case. When adopted in daily forensic practice, this innovative technique can add valuable information about the moment of a crime and the sequence of events.