Spectral analysis of blood stains at the crime scene

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4 - HYPERSPECTRAL IMAGING FOR THE AGE ESTIMATION OF BLOOD STAINS AT THE CRIME SCENE

The age estimation of blood stains can provide important information on the temporal aspects of a crime. As previously shown, visible spectroscopy of blood stains can successfully be used for their age estimation. In the present study we evaluated the feasibility to use hyperspectral imaging for this purpose. Visible reflectance spectra of blood stains were recorded using a push broom hyperspectral imaging system. From these spectra, the relative amounts of oxyhaemoglobin, methaemoglobin and hemichrome within the blood stains were derived. By comparison of the haemoglobin derivative fractions with a reference dataset, the age of blood stains up to 200 days old was estimated. The absolute error of the age estimation task increased with age, with a median relative error of 13.4% of the actual age. To test the practical applicability of this method, a simulated crime scene was analyzed, in which blood stains of several ages were deposited. Hyperspectral imaging combined with the proposed analysis provided insight in the absolute age of the blood stains. Additionally, the blood stains were clustered based on their haemoglobin derivative fractions, without the use of a reference dataset. Results demonstrated that the order of formation of blood stains can be determined, even under unknown environmental circumstances, when no proficient reference dataset is available. These findings are an important step toward the practical implementation of blood stain age estimation in forensic casework.

4.1. INTRODUCTION

Knowledge of the time of bleeding is highly significant in many forensic investigations, because this information can be used to determine the moment a crime was committed, or whether a blood stain is crime-related. In a recent murder case in The Netherlands where blood stain age estimation was requested, several blood stains were found in a suspect building. The main question was whether these blood stains all originated from a single event or from multiple events. Information about the blood stain ages would enable investigators to verify testimonies, and to adjust the direction of their investigation accordingly. Several techniques, including high performance liquid chromatography[88], electron paramagnetic resonance[89, 90], atomic force microcopy[91] and RNA degradation measurements[92, 93] have been investigated for this purpose, as reviewed by Bremmer et al[94]. However, none of these methods is yet implemented in forensic practice; most require sample preparation and need to be performed in a laboratory.

Non-destructive blood stain age estimation can be performed using visible reflectance spectroscopy[1, 67]. When blood exits the human body, oxyhaemoglobin (HbO_2) auto-oxidizes into methaemoglobin (MetHb), which in turn denatures into hemichrome (HC)[1]. Previously we have shown that visible reflectance spectroscopy can be used to determine the relative fractions of the haemoglobin derivatives and estimate the age of blood stains by comparing these fractions to reference data[1]. A different approach for processing of the spectral data was proposed by Li et al[67], who applied linear discriminant analysis to estimate the age of blood stains.

A drawback of using spectroscopy is that point measurements of all suspected stains at the crime scene can be time consuming and labour intensive. Hyperspectral imaging integrates conventional spectroscopy and imaging, thereby obtaining both spatial and spectral information from all objects in the field of view. As a result, spectral properties of all objects can be recorded together with information about their location and distribution within the scene, without the need for further documentation. Because current hyperspectral imaging systems are fast and portable, they can be transported to

the crime scene where traces can be analysed and interpreted in the original context, thereby reducing the workload in forensic laboratories and almost instantly providing investigators with valuable information. However, the transition from spectroscopy to hyperspectral imaging means a change in illumination-detection geometry[57], thus different processing of the spectra is required. In **Chapter 3**, we demonstrated the feasibility of hyperspectral imaging of the crime scene to detect and identify blood stains remotely[18]. In that study, blood stains were distinguished from other samples with a sensitivity of 100% and a specificity of 85% using hyperspectral imaging. Even small blood stains with a diameter of approximately 1 mm could be detected from 1.5 meter distance.

The aim of this study is to show the feasibility to use hyperspectral imaging for age estimation of blood stains. Thereto, visible reflectance spectra of blood stains were recorded and spectrally unmixed to obtain the relative fractions of HbO_2, MetHb and HC within the stains. The temporal behaviour of these fractions was used to estimate the age of blood stains up to 200 days old. We investigated the practical applicability of this method by the analysis of a simulated crime scene in which blood stains of several ages were deposited. By hyperspectral imaging, spectral unmixing, and comparison of the haemoglobin fractions to a reference dataset, the absolute ages of blood stains were estimated. Additionally, we proposed a method to determine the relative ages of blood stains, which is useful in forensic investigations where the environmental conditions are unknown.

4.2. DATA ACQUISITION AND ANALYSIS

4.2.a HYPERSPECTRAL IMAGING

Measurements were performed using a push broom line-scanning spectral imaging system (Spectral Imaging Ltd., Oulu, Finland). This system consisted of a rotary stage, a hyperspectral camera operating in the visible and near infrared wavelength range (400-1000 nm) and a halogen broadband white light source. Using this system, we recorded so-called hypercubes (see Figure 4.),

containing two spatial dimensions and a spectral dimension, as introduced in **Chapter 2**. Using the light intensities at all measured wavelengths, reflectance spectra were obtained from all pixels in the hypercube, as described below.
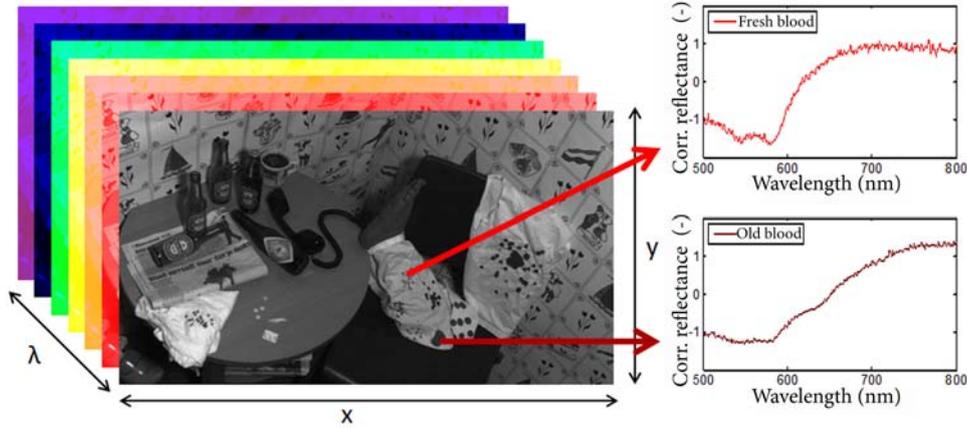

Figure 4.1. Hypercube of a simulated crime scene, with two spatial (x,y) and one wavelength (λ) dimension (left). From the hypercube a reflectance spectrum was obtained from each pixel (right).

4.2.b PRE-PROCESSING

Spectral analysis was limited to the wavelength range of 500-800 nm, because of the low camera sensitivity and low power of the light source beyond this range. The following pre-processing steps were applied to all spectra in the hypercube. First, the dark response ($I_{dark,ij}(\lambda)$) of the camera was subtracted. Depending on the system, this can be pixel and wavelength dependent. Next, to account for wavelength dependent intensity differences in the light source, we divided by the background response of a white reference plane at all wavelengths ($I_{white}(\lambda)$), previously corrected for the dark response:

$$R_{ij}(\lambda) = \frac{I_{ij}(\lambda) - I_{dark,ij}(\lambda)}{I_{white}(\lambda) - I_{dark,ij}(\lambda)},$$

where R is the reflectance, I is the intensity and i and j are horizontal and vertical pixel indices. Finally, all reflectance spectra were corrected using the standard normal variate algorithm[95]. All data analysis was performed using custom-made scripts written in MATLAB (The Mathworks Inc., Natick, Massachusetts, USA).

4.2.c SPECTRAL UNMIXING

After the pre-processing procedure a non-linear spectral unmixing model was employed to find the relative fractions of HbO_2, MetHb and HC. Similar to the way described in **Chapter 3**[65], a one-dimensional solution of the radiative transport theory was employed to translate the measured corrected reflectance spectra into the absorption spectra of HbO_2, MetHb and HC[82, 96]. A non-linear least squares fit was used to estimate the relative fraction of each haemoglobin derivative. The coefficients of determination R^2 between the measured spectra and the fit were used as a quality test. Measurements with an R^2 value below 0.99 were excluded from the further analysis.

4.3. MATERIAL AND METHODS

4.3.a REFERENCE DATASET

To build a reference dataset, blood was drawn from a healthy non-smoking volunteer, deposited on white cotton cloth and stored in a laboratory at room temperature. Hypercubes of the blood were recorded repeatedly for 200 days (almost daily in the first month, thereafter approximately once a month). After pre-processing of the data, a training set was selected consisting of 8 regions of 25 pixels for each age, which were averaged to obtain 8 reflectance blood spectra per age. The spectral unmixing procedure was applied to these spectra to find the fractions of HbO_2, MetHb and HC present in the blood, which were plotted against the age. In between data points, values were interpolated linearly.

4.3.b AGE ESTIMATION

The obtained reference dataset of haemoglobin fractions was used to estimate the age of a test set of 8 reflectance spectra of neighbouring blood stains, again all averaged over 25 pixels, and recorded up to 200 days. As above, the fractions of the haemoglobin derivatives were derived and compared to the reference dataset. A *k*-nearest neighbour (k-NN) algorithm was applied to find the age at which the Euclidean distance to the reference fractions was minimal (k=1)[97]. All estimated ages were plotted against the actual age. The absolute and relative errors were calculated.

4.3.c CRIME SCENE ANALYSIS

A crime scene was simulated and recorded using the hyperspectral imaging system (Figure 4.2). The blood stains present in the scene were donated by 5 different healthy non-smoking volunteers (not including the volunteer whose blood was used to create the reference data set) and deposited on white cotton cloths 0.1, 2, 15, 40, and 200 days prior to the measurements. Before these blood stains were put in the scene, they were stored in a laboratory at room temperature, i.e. under similar conditions as the reference blood stains.

Pre-processing and spectral unmixing were applied to all spectra of the hypercube, as described above. Before analyzing the haemoglobin fractions, it was determined whether a spectrum resembled that of blood, using the automatic detection and identification method described in more detail in **Chapter 3**[18]. By thresholding the R^2 values between the corrected reflectance spectra and the least-squares fit of the absorption spectra of HbO_2, MetHb and HC, a mask was created to isolate all pixels depicting blood stains. For the subsequent age estimation task, two approaches were used, as described below.
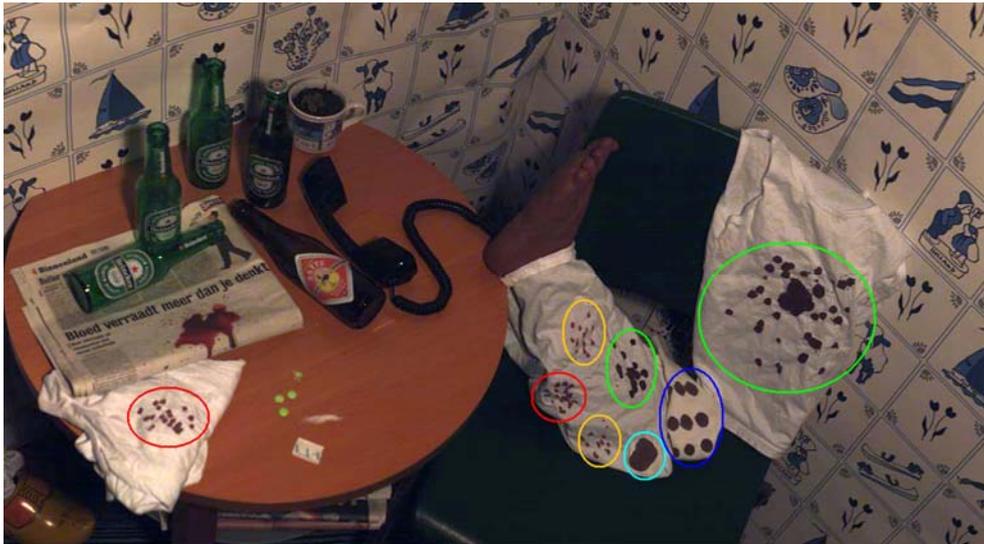
Figure 4.2. Colour image of the simulated crime scene, in which blood stains of various ages were deposited. The locations of blood stains are indicated with ellipses, of which the colour symbols the age; orange = 0.1 day, red = 2 days, green = 15 days, blue = 40 days, cyan = 200 days.

Absolute age

First, the absolute ages were estimated by comparing the fractions of HbO_2, MetHb and HC for blood stains at the crime scene with those of the reference dataset. Within the mask depicting all blood stains, all connected pixels were grouped and labelled to analyse all individual blood stains separately. The reflectance spectra of all pixels within one stain were averaged and spectrally unmixed to find the haemoglobin fractions within the blood stain. For each stain, a k-NN algorithm was applied to find the age at which the Euclidean distance to the reference dataset was minimal (k=1)[97]. Average estimated ages were calculated for each class and plotted against the actual age.

Relative age

In forensic practice, if blood stains are found in different or unknown environmental circumstances, the method described above may lead to wrong

age estimations, as both humidity and temperature are known to influence the chemical reactions within a blood stain[56]. For those cases, we propose a method to estimate the relative age of blood stains. The averaged spectra of all blood stains in the simulated crime scene described above were analysed. K-means cluster analysis[98] was applied to find groups of blood stains with similar haemoglobin derivative fractions. Results were presented by depicting all clusters in different colours overlayed on a greyscale image of the scene. By analysing the haemoglobin fractions of the different clusters, the order of formation was determined.

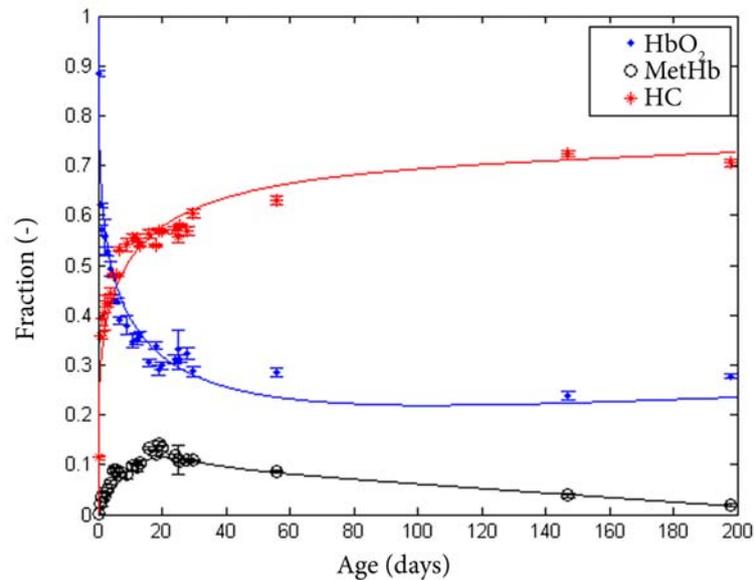## 4.4. RESULTS

4.4.a REFERENCE DATASET

Figure 4.3. Calculated fractions of oxyhaemoglobin (HbO$_2$), methaemoglobin (MetHb) and hemichrome (HC) against the age of the blood stains. The lines through the data points are a guide to the eye.

Figure 4.3 shows the temporal behaviour of HbO_2, MetHb and HC fractions in blood stains up to 200 days old. Within the measured blood stains, the fraction of HbO_2 decreased with age, whereas the fraction of MetHb increased in the first three weeks, followed by a decrease, and the fraction of HC seemed to increase in time.

4.4.b AGE ESTIMATION

By comparison of the haemoglobin fractions with the reference dataset (Figure 4.3), the age of blood up to 200 days was estimated (Figure 4.4). The absolute error of the age estimation task increased with age (Figure 4.5). The median relative error was 13.4% of the actual age.
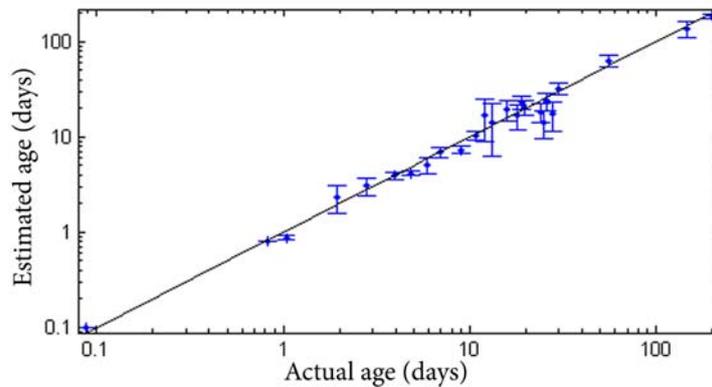

Figure 4.4. Mean estimated age of blood stains versus the actual age for blood stains up to 200 days old. Error bars depict the standard deviation. The solid line is the line of unity.
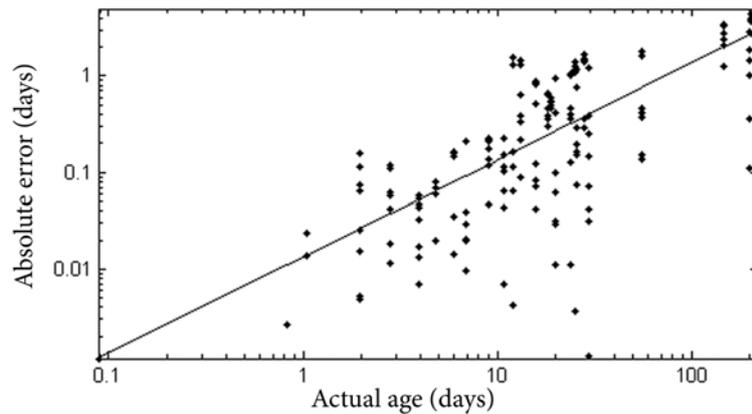
Figure 4.5. Absolute error of the age estimation task versus the actual age for blood stains up to 200 days old. The solid line depicts the median relative error of 13.4%.

4.4.c CRIME SCENE ANALYSIS

Absolute age

The mean estimated age for all blood stains at the simulated crime scene was plotted against the actual age in Figure 4.6. Error bars depict the standard deviation between different blood stains of the same age. An exception was made for the oldest blood stain; because only one stain of 200 days old had been deposited in the scene, 6 different regions within this stain were selected and used to calculate the mean estimated age and the standard deviation. The absolute errors of the age estimation task are given in Table 4.1.

Table 4.1. Actual ages, estimated ages and absolute errors of the age estimation task of blood stains in the simulated crime scene.

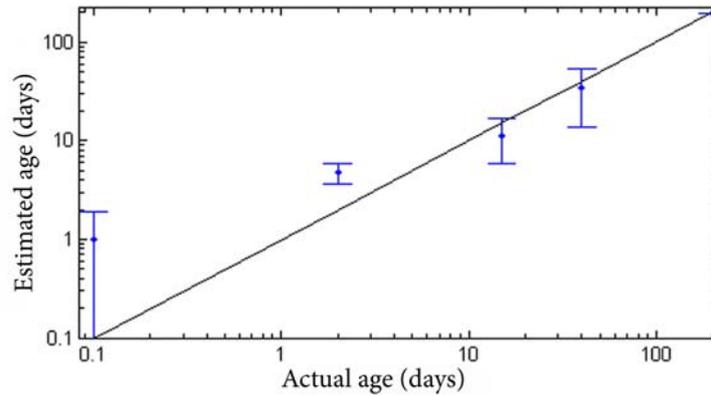| Actual age (days)	Estimated age (days)	Absolute error (days)
0.1	1.0	0.9
2	4.7	2.7
15	10.7	4.3
40	34.0	6.0
200	197.9	2.1

Figure 4.6. Mean estimated age of blood stains at the simulated crime scene versus the actual age. Error bars depict the standard deviation between different blood stains of the same age (0.1, 2, 15, and 40 days old), or the standard deviation between different regions within the stain (200 days old).
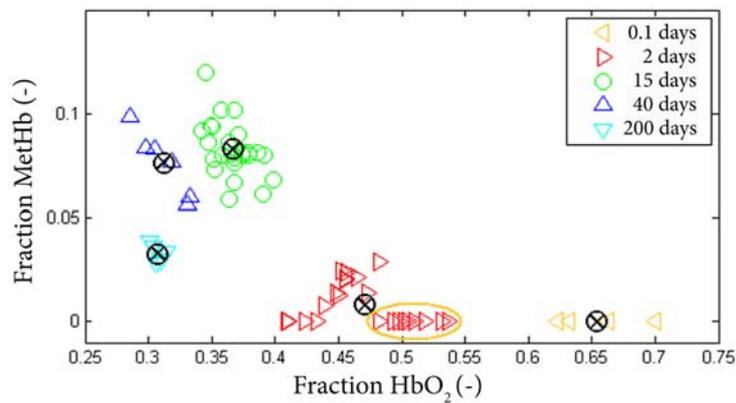
Relative age

Figure 4.7. Scatter plot depicting the HbO2 and MetHb fractions calculated for blood stains in the simulated crime scene. Each blood stain is assigned to a different colour, class number and marker type using k-means clustering. The majority of blood stains was assigned to the correct age group mentioned in the legend. Blood stains in the orange ellipses were incorrectly clustered in the group of 2 days old blood stains, while the actual age was 0.1 days.

The HbO$_2$ and MetHb fractions calculated for all blood stains in the simulated crime scene are plotted in Figure 4.7. Different marker types and colours in this figure indicate the results of a k-means cluster analysis, with k=5. The majority of blood stains was assigned to the correct age group mentioned in the legend. Data points within the orange ellipse belong to blood stains of 0.1 days old, but were incorrectly assigned to the cluster of 2 days old blood stains. The results are presented differently in Figure 4.8, where each cluster is indicated by a different colour. Again, the orange ellipses show the incorrectly clustered blood stains.
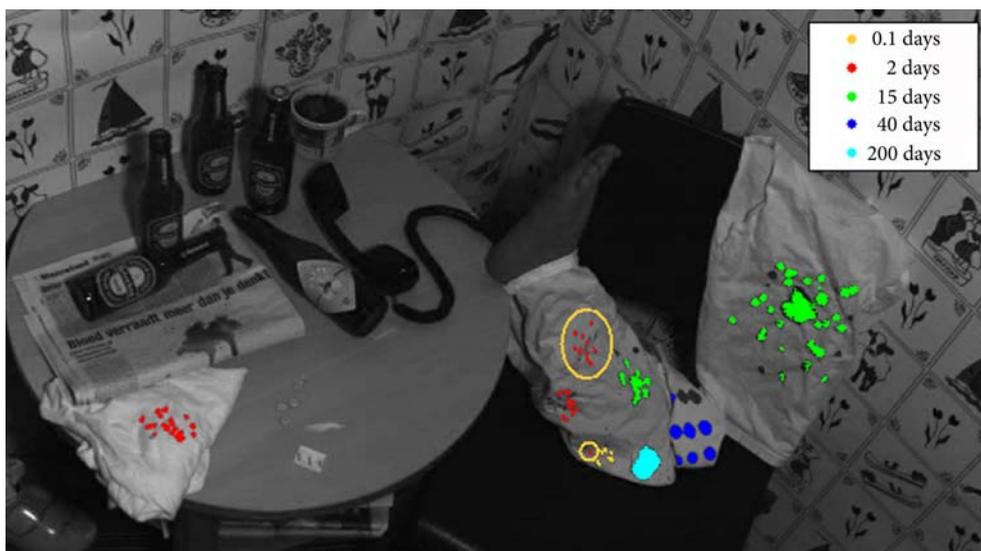

Figure 4.8. Results of the relative age estimation task. The haemoglobin fractions of all blood stains were clustered using k-means clustering. The majority of blood stains was assigned to the correct age group mentioned in the legend. Blood stains in the orange ellipses were incorrectly clustered in the group of 2 days old blood stains, while the actual age was 0.1 days.

Analysis of the HbO$_2$ fraction in the different blood stain clusters (Figure 4.7) indicates the order of formation of the blood stains. The average HbO$_2$ fractions for clusters 1 to 5, indicated by the crosses in Figure 4.7, were 0.65, 0.47, 0.37, 0.31, and 0.30 respectively. Assuming that the HbO2 fraction

decreases in time, as shown in Figure 4.3, the correct order of formation could be predicted using the average cluster fractions.

4.5.   DISCUSSION

We introduced the use of hyperspectral imaging for the age estimation of blood stains at the crime scene. The temporal behaviour of HbO_2, MetHb and HC fractions within blood was measured for blood up to 200 days old (Figure 4.3), and used to estimate the age of other blood stains with a median relative error of 13.4% (Figure 4.4). For stains previously identified as blood, the age estimation task was completely automatic and did not need any human interference. The practical applicability of this technique was demonstrated by the analysis of a simulated crime scene. Within this scene, the age of blood stains could be estimated successfully by comparison with a reference dataset of haemoglobin fractions (Figure 4.6). Additionally, without using any reference dataset, blood stains were clustered in groups with similar ages and the order of formation was determined successfully (Figure 4.8).

The described approach of splitting the spectra into the different chemical components has the advantage that the influence of temperature, humidity or other environmental circumstances can be studied. Bremmer et al demonstrated that the oxidation of HbO_2 is relatively independent of humidity, whereas the transition of MetHb into HC strongly depends on humidity[56]. The influence of environmental factors at the crime scene will make precise estimation of the absolute age of blood stains challenging. If the environmental factors can be reconstructed we are able to study the kinetics of the haemoglobin derivatives, which can be used to estimate the absolute ages of blood stains. Additionally, even in unknown circumstances, we demonstrated the possibility to determine the relative age of different blood stains, under the assumption that they were exposed to similar environmental conditions. Because the fraction of HbO_2 decreases in time, this fraction can be used to determine the order of formation of different blood stains, as demonstrated by the analysis of the simulated crime scene.

To our knowledge, no other application of hyperspectral imaging for the age estimation of blood stains has been reported previously. Two prior studies were reported using a more controlled spectroscopy setup. An advantage of using hyperspectral imaging is the speed of acquisition; within a minute millions of spectra can be recorded. The time needed for the analysis depends on the size of the scene and the calculation power. In this particular case we needed several hours to analyze the scene. The accuracy of our age estimation improved compared to the accuracy reported by Bremmer et al, who used a fibre based spectroscopy device to estimate the age of blood stains[1]. For example, at an age of 3 days, the ages estimated by Bremmer et al varied between 1.5 and 6 days. In comparison, at an age of 2.8 days, we estimated ages between 2.2 and 4 days. Although we cannot test this improvement on significance, it can be explained by the pre-processing procedure we applied prior to analysis, as suggested by Li et al[67]. Application of the standard normal variate algorithm reduced the variance between spectra of blood stains of the same age.

Using spectroscopy combined with pre-processing and linear discriminant analysis, Li et al reported an error of less than one day for blood stains up to 19 days old, after which the accuracy decreased[67]. Although it is expected that the accuracy decreases with age, as chemical changes are faster in the beginning of the aging process, the sudden decrease in accuracy after 19 days can be explained by analysis of the relative fractions of haemoglobin derivatives derived in our study. After an increase of approximately 3 weeks, the relative fraction of MetHb within a blood stain starts to decrease (see Figure 4.3b). This explains why a linear model can successfully be used to approximate the spectral behaviour in the first 19 days, but after that results become inaccurate. In the first three weeks, we achieved similar results as Li et al. However, using the fractions of haemoglobin derivatives, ages of older blood stains up to 200 days could also successfully be estimated. In practice, the accuracy will depend on the actual age of the blood stain and knowledge of the environmental conditions. How this influences the evidential value depends on the hypotheses relevant to the case.

A challenge under field operation is the ability to analyze complex crime scenes, where blood stains are typically recorded with varying distances to the camera, and under varying angles. In this study, the blood stains were randomly distributed throughout the crime scene, so the possible variation caused by different distances and angles was part of the error bars shown in this chapter (Figure 4.6). The exact influence of geometrical properties is subject of extensive further study. The mean absolute difference between actual and estimated ages of blood stains ranged between 0.9 and 6.0 days (Table 4.1). To estimate the relative ages of blood stains in the simulated crime scene, we used cluster analysis with prior knowledge of the number of clusters. Although this number will be unknown in forensic practice, advanced cluster analysis[98] combined with bloodstain pattern analysis may give more insight in the number of events that created the blood stains. Additionally, if prior information about blood stain groups is available (e.g. based on DNA analysis), the amount of errors may be reduced by replacing unsupervised cluster analysis by supervised classification, which makes use of a predefined training set.

Other challenges typically encountered in forensic casework, e.g. contaminated traces found on non-ideal backgrounds, emphasize the necessity to refine and validate the technique in forensic practice. When blood stains are deposited on coloured backgrounds, age estimation using visible hyperspectral imaging is expected to be hampered by the absorption of visible light by the background. The model we used in this study is a general model for light propagation in turbid media, in the limit of a non-absorbing background, in which the only chromophores are the haemoglobin components. Extension to other backgrounds requires adaptation of the boundary conditions. This requires an extensive further study presented in **Chapter 5**, in which both the scattering and absorption properties of the substrate will be accounted for. For some backgrounds, a switch to the near infrared wavelength range may be necessary (**Chapter 7**)[19]. Another topic for further research is the influence of carboxyhaemoglobin on our analysis. Because we analysed blood of healthy non-smoking volunteers who usually have only a small percentage (<4%) of carboxyhaemoglobin[99], the possible presence of this haemoglobin derivative was not taken into account. However, the increased level of

carboxyhaemoglobin expected for smokers, people suffering from sickle cell disease, fire victims or in cases of fatal carbon monoxide poisoning, may influence the results in forensic practice[100-102].

In conclusion, we demonstrated the feasibility to use hyperspectral imaging for the absolute or relative age estimation of blood stains at the crime scene, based on the relative amount of haemoglobin derivatives present in the blood stains. When applied in forensic practice, the described technique provides investigators with valuable information, which can be used to reconstruct the timeline of events.

4.6. ACKNOWLEDGEMENTS