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Non-contact spectroscopic age determination of bloodstains

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Bloodstains can be crucial in reconstructing crime events. However, no reliable methods are currently available to establish the age of a bloodstain on the crime scene. We show that determining the fractions of three hemoglobin derivatives in a bloodstain at various ages enables relating these time varying fractions to the age of the bloodstain. Application of light transport theory allows addressing the spectroscopic changes in ageing bloodstains to changes in chemical composition, i.e. the transition of oxy-hemoglobin into met-hemoglobin and hemichrome. We have found in twenty bloodstains that the chemical composition of the bloodstain with age, called hemoglobin reaction kinetics, under controlled circumstances, shows a distinct time-dependent behavior, with a unique combination of the three hemoglobin derivatives at all moments in time. Finally, we employed the hemoglobin reaction kinetics inversely to assess the age of twenty other bloodstains studied, again over a time period of 0-60 days. We estimated an age of e.g. 55 days correct within an uncertainty margin of 14 days. In conclusion, we propose that the results obtained under controlled conditions demand further evaluation of their possible value for age determination of bloodstains on crime scenes.

CHAPTER 2
**AGE ESTIMATION OF BLOODSTAINS BY HEMOGLOBIN DERIVATIVE
DETERMINATION USING REFLECTANCE SPECTROSCOPY**

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1. INTRODUCTION

1.1 Age determination of bloodstains

Bloodstains at crime scenes have enormous forensic value, ranging from DNA-profiling for verifying the suspect's identity to pattern analysis for reconstructing the crime. However, until now, the potential of using bloodstains to determine the time when a crime was committed has not yet been materialized, despite several attempts. Already in the 1930s Schwarzbacher attempted to correlate the age of a bloodstain with the bloodstain's rate of inhibition of solubility in water, which begins rapid and then decreases slowly as the bloodstain ages [45]. Over the last two decades, many more techniques have been explored for the forensic quest, including oxygen electrodes [57], RNA degradation [35], atomic force microscopy [19], and electron spin resonance spectroscopy [58]. Although all of these approaches confirm that the physical and chemical properties of bloodstains change over time, no technique has yet shown the precision and reproducibility that is needed for age determination in forensic practice.

The color of a bloodstain changes with time from red to brown which suggests that color quantification of bloodstains by optical methods can be a worthwhile approach for pursuing age determination. Patterson was the first to recognize this approach in 1960. He recorded bloodstain's reflectance spectra [1], and observed that the changing color of the bloodstain depends on environmental conditions. Twelve years later, Patterson *et al* managed to quantify absorption bands and suggested to use these spectra for age determination [2]. A similar approach, but only using a small spectral window has been suggested by Blazek and Lins [51].

We propose Diffuse Reflectance Spectroscopy (DRS) for determining the age of a bloodstain. DRS is based on non-specular reflection of light to determine a material's optical properties. This technique has proven its value in various disciplines ranging from materials science [68] to tissue diagnostics and is routinely used in medicine to determine the oxygen saturation of blood [69]. Our approach of DRS elaborates on recent progress in biomedical optics which allows quantifying the fractions of relevant bloodstain chromophores by multi-component spectral fits. Subsequently, these fractions which change with time are then related to the age of the bloodstain.

1.2 Hemoglobin reaction kinetics

Hemoglobin is the iron containing protein responsible for oxygen transport between lungs and tissue. It is the main chromophore in blood, since hemoglobin makes up 97% of the blood's dry content. Hemoglobin can appear in various forms, called hemoglobin derivatives. Conversion between various hemoglobin derivatives is different outside the human body, than inside. Inside a healthy human body, hemoglobin molecules are mainly present in two forms: one without oxygen: de-oxyhemoglobin (Hb) and one saturated with oxygen: oxy-hemoglobin (HbO₂). The average saturation level of arterial blood is >90% and of venous blood >70%. Only a small part (~1%) of HbO₂ is auto-oxidized into a third form, met-hemoglobin (met-Hb) [25], which is reduced back

to Hb by reductase protein cytochrome *b5* [27]. Outside the body, blood will totally saturate to HbO₂ as soon as it comes in contact with the oxygen in the atmosphere. However, due to a decreasing availability of cytochrome *b5*, necessary for reduction of met-Hb, the transition of HbO₂ into met-Hb will no longer be reversed. Once hemoglobin is auto-oxidized to met-Hb it will denature to hemichrome (HC). HC is formed through changes of protein conformation so that atoms endogenous to the protein become bound to the iron at the sixth ligand [23].

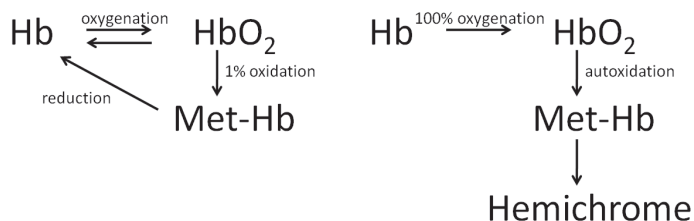


Figure 1. Simplified hemoglobin reaction kinetics inside the body (left hand) and reaction in bloodstains (right hand).

Figure 1 shows a schematic overview of the hemoglobin reaction kinetics, of both *in-vivo* and of extracorporeal blood. Since the absorption spectra of HbO₂, met-Hb, and HC differ from each other (see figure 2) this transition is accompanied by a color change from deep red to dark brown.

We will use DRS to monitor this chemical process in bloodstains and our research question is whether the fractions of the various hemoglobin derivatives allow estimation of the age of bloodstains under controlled environmental conditions.

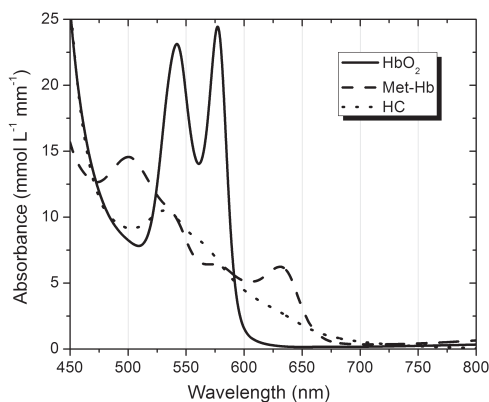


Figure 2. Absorption Spectra of the three hemoglobin derivatives used for the analysis of the bloodstain reflectance spectra. Data of the absorption spectrum of HbO₂ and met-Hb is from [70] and HC data from [32]. Isosbestic points of HbO₂ and HC at 525 nm and 585 nm are used to match the HC spectrum with the HbO₂ and met-Hb spectrum.

2. METHODS

2.1 Sample preparation

We created 40 bloodstains, five stains from each of eight healthy male volunteers. Two tubes (5 ml) of blood were drawn from the donors. One tube, with anti-coagulant, was sent to the hematology department for hematocrit determination and found to be 0.44 ± 0.02 . The second tube, without anti-coagulant, was used to create the bloodstains on white cotton, five stains per donor with a stain diameter of 21 ± 4 mm (see figure 3). The time between the donation of the blood and the creation of the bloodstain was less than 15 seconds. The temperature in the laboratory, monitored with an I-button (Maxim-DS1920) was stable at 22.3 ± 0.5 °C.

2.2 Non-contact Diffuse Reflectance Spectroscopy

The non-contact diffuse reflectance measurements were performed with a combination of a tungsten-halogen light source (Ocean Optics, DH-2000) and a reflection probe in fixed position containing six 400 μ m core diameter delivery fibers, circularly placed around a similar central collecting fiber. The probe was positioned perpendicular to and centered 1 cm above the surface. The illumination spot diameter was 5 mm. The collected light was back scattered into the central collection fiber and sent to a compact CCD spectrometer (Ocean Optics, USB 4000). First, a spectral measurement of a clean spot of the white cotton was taken for reference, R_0 . Thereafter, we measured the reflectance of the bloodstain, R . The ratio R/R_0 is the reflectance signal used for data analysis. In one measurement series we recorded the reflectance signal of all forty bloodstains. In the first ten days, we performed at least one measurement series per day. Thereafter, we measured every two days and after week four, every week. A total of 1412 reflectance spectra were measured in 60 days.

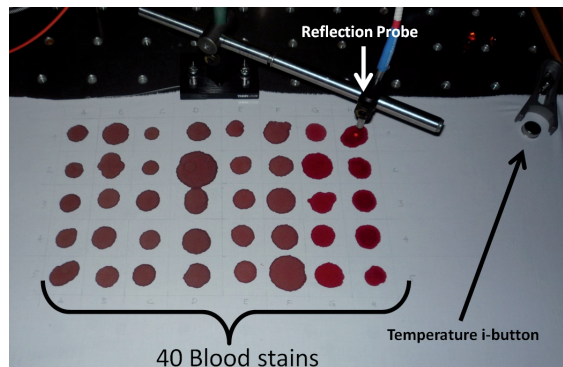


Figure 3. Experimental setup for measuring the bloodstains' reflectance spectra. The forty bloodstains are measured with the reflectance probe. Simultaneously the temperature was monitored with an i-button.

The reflectance spectra were recorded by 4096 pixels in the wavelength range 350–1050 nm; however the optical resolution of the spectrograph was 5 nm. Oversampling of the data was used by averaging data into bins of 10 pixels, which allows calculation of a standard deviation that represents noise within the signal.

2.3 Data Analysis

The two-flux solution of the general radiation transport theory was used to describe the diffuse reflectance signal. This theory is used to relate Kubelka Munk absorption and scattering coefficients with their transport theory equivalents [71-73]. It assumes that the sample possesses (i) inhomogeneities which are small compared to the sample thickness; (ii) that the light field is diffuse; and (iii) that reflections occurring at boundaries can be neglected. For bloodstain reflectance, all three conditions are met, since (i) inhomogeneities are caused by hemoglobin packaged in red blood cells which are much smaller than the bloodstain thickness; (ii) and (iii) are fulfilled when the bloodstain is created on a highly diffuse reflecting background, like cotton. The formulas that were used for the data analysis are given in appendix A.

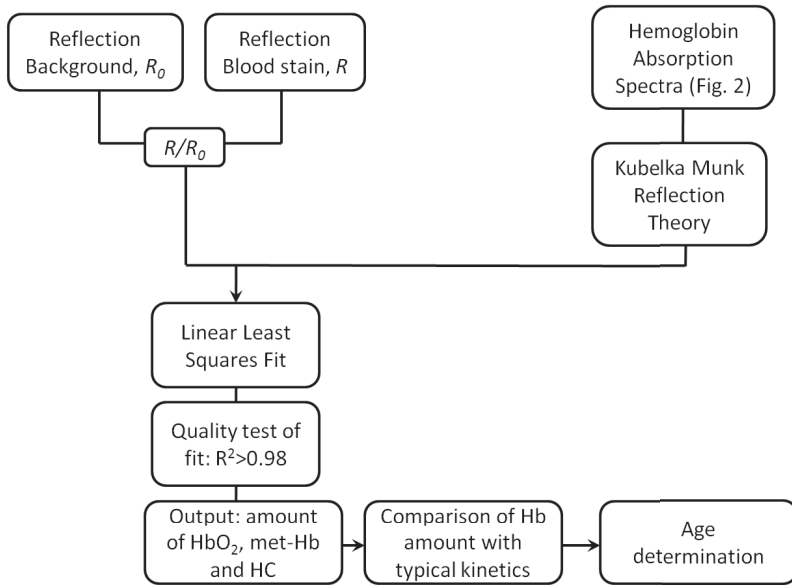


Figure 4. Flow chart of the various steps in data analysis: from bloodstain reflectance measurement to age determination.

The fractions of HbO_2 , met-Hb and HC within the bloodstain were determined by performing a linear least squares (LLS) fitting algorithm between the Kubelka Munk theory, with the known absorption spectra [70], [32] of the three hemoglobin

derivatives (shown in figure 2) as input, and the bloodstain reflectance signal between 450-800 nm. Consequently, we had to account for scattering and spectral flattening. The latter was achieved by assuming chromophore packaging in the erythrocytes, with an erythrocyte radius of 3 μm [74]. To account for scattering by the erythrocytes, a Lorentz-Mie behavior was employed, see Appendix A.

The LLS fitting algorithm varies the amplitudes of the three absorption spectra in order to find the combination of the three with a minimum of difference between the spectrum of the three amplitude fractions and the diffuse reflectance spectrum. The LLS-fitting procedure yields the estimated fractions of HbO_2 , met-Hb and HC. Correct data analysis requires a high correlation between the reflectance signal and the LLS-fit. If the correlation is poor, overcompensation by one of the derivatives may occur, and the outcome of the fitting procedure becomes unreliable [75]. To prevent this, a quality test between data and fit is utilized and we only accepted LLS-fits with correlation coefficient $r^2 > 0.98$; which holds true for 91% of the measurements in this study. The 127 measurements with correlation lower than 0.98 were homogeneously distributed over the range of ages. Finally, all steps described above were scripted into Labview code (vers. 8.6 National Instruments). The data analysis is schematically drawn in a flow chart in figure 4.

2.4 Age determination

The bloodstain samples were divided into two equal subsets, according to the standard approach called one round cross-validation[76]. Accordingly we divided the total set of bloodstains into two groups: twenty bloodstains from four donors in the *training set* and twenty bloodstains from four other donors in the *age estimation set*. First, we determined the fractions of Hb, met-Hb and for all time points in the training set. These fractions averaged over the four donors constituted the lines of laboratory bloodstain calibration kinetics. Secondly, from the age estimation set of twenty bloodstains we also determined the fractions of hemoglobin, met-Hb and HC for all time points. These fractions of Hb, met-Hb and HC, determined at a certain time point, were matched to the laboratory calibration kinetics. The age at which the relative difference between the sum of the three measured fractions and the laboratory calibration kinetics was minimal is the estimated age of the bloodstain. Every measured bloodstain from the age estimation set provides a single estimated age. The range of estimated ages of all bloodstains measured at a single time point indicates the error margin of the age estimation.

3. RESULTS

3.1 Reflectance spectra of bloodstains

Figure 5 plots four typical reflectance spectra with corresponding blood component fit. Figure 5a shows a bloodstain one hour after deposition. The spectrum has two distinct dips, one at 540 nm and one at 576 nm, corresponding to the HbO_2 absorption spectrum (figure 2). This spectrum also has a very steep slope between 600 and 650 nm corresponding to the absence of met-Hb and HC. Figure 2b, at an age of one day, also shows dips in the reflectance spectrum at 540 and 576 nm, according to a large amount of HbO_2 , but the slope between 600 and 650 nm is not as steep as in the spectrum at $t=1\text{h}$. After one day, already a reasonable amount of HC has been formed. As the time increases, the dips in the reflectance spectra at 540 and 576 nm become smaller, and the slope between 600 and 650 nm becomes less steep, as is shown in figure 5c and 5d at ages of $t=7\text{d}$ and $t=63\text{d}$ respectively.

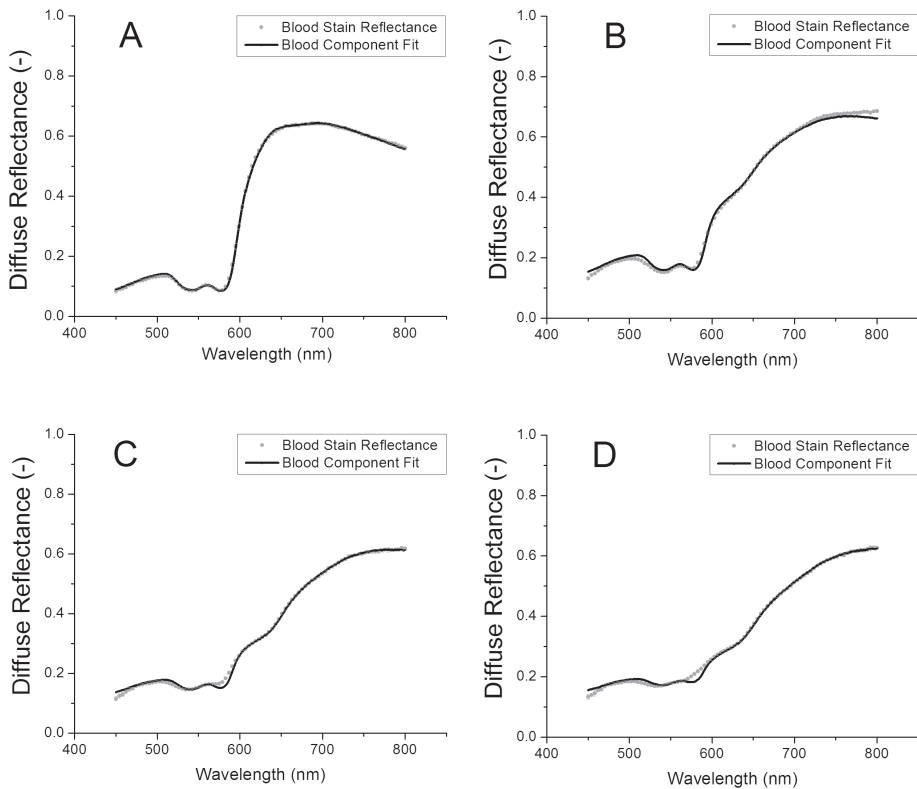


Figure 5. Four typical bloodstain reflectance measurements. A) age = 1h; B) age = 1d; C) age = 7d; D) age = 63d. The gray dots are the recorded reflectance spectrum, the solid black line is the blood component fit.

3.2 Hemoglobin reaction kinetics

Figure 6 plots the hemoglobin fractions of HbO_2 , met-Hb and HC as a function of time. The data points +SD are the average of twenty bloodstains from the training set, measured at that time point. The fractions of hemoglobin derivatives in bloodstains show a distinct temporal behavior. At $t=0$, all hemoglobin appears to be HbO_2 . After deposition of the bloodstains, the amount of HbO_2 decreases, and both met-Hb and HC are formed. The transition rate of HbO_2 into met-Hb and HC is at first rapid, but decreases as the bloodstain ages. HbO_2 shows a biphasic autoxidation curve. For $t < 10\text{d}$ the decay constant is larger than for $t > 10\text{d}$. This biphasic effect is also observed for autoxidation of HbO_2 in aqueous form [77].

The amount of met-Hb is zero at $t=0$, and increases slowly. However, the amount of met-Hb appears to be always smaller than the amount of HC. Met-Hb reaches a maximum fraction of 0.2 at an age of approximately 15 days and thereafter the amount decreases. We found that the sum of the three hemoglobin fractions remained approximately constant over the total time period and close to 1 (not shown). Hence, suggesting all HbO_2 is transformed into met-Hb and HC and no other derivatives are formed. At all moments, we found that a unique combination of these three hemoglobin derivatives exists, which can be utilized for age determination.

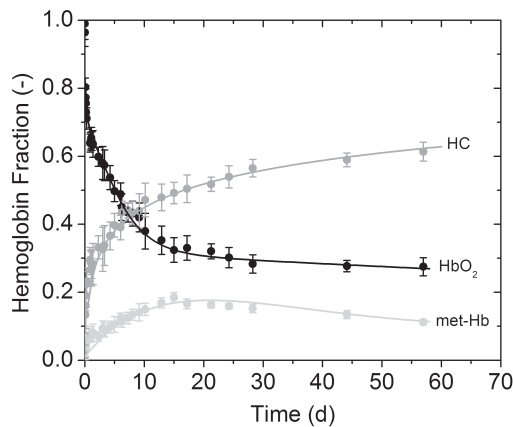


Figure 6. Average fractions of HbO_2 , met-Hb and HC for twenty bloodstains from training set. Error bars represent the standard deviation. Solid lines are smoothed average hemoglobin fractions.

3.3 Age determination

The average hemoglobin fractions, as a function of time are determined on the bloodstains from the training set and are shown as solid lines in figure 6. Subsequently figure 7 shows the comparison between the average hemoglobin fractions from the training set and all measured hemoglobin fractions for twenty bloodstains from the

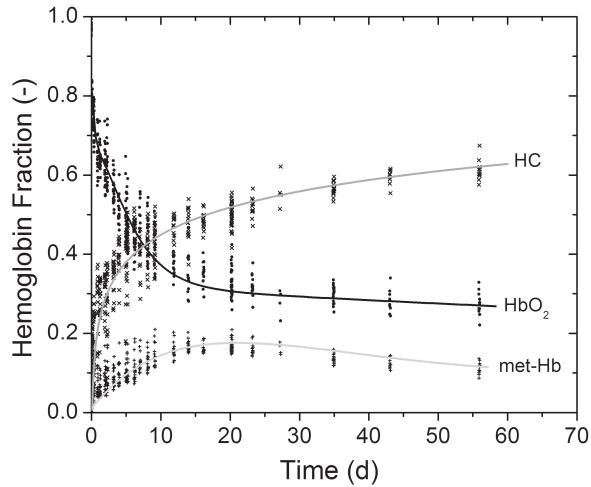


Figure 7. Combination of smoothed average hemoglobin fractions from training set (solid lines) and fractions of HbO₂ (dots), met-Hb (plusses) and HC (crosses) for twenty bloodstains from the validation set. The determined hemoglobin fractions from every bloodstain from validation set is plotted as a single data point.

validation set. The variation among bloodstains from the validation set is observed as the data scatters around the line representing the average hemoglobin fraction.

The estimated age of bloodstains from the validation set follow by comparing the measured hemoglobin derivative fractions with the lines of average fractions from the training set. Figure 8 plots the actual age of the bloodstain against the estimated age, based on the above described procedure. The age estimation satisfies over a wide range of ages (range: 0-60 days). However, the combination of biological variation and small

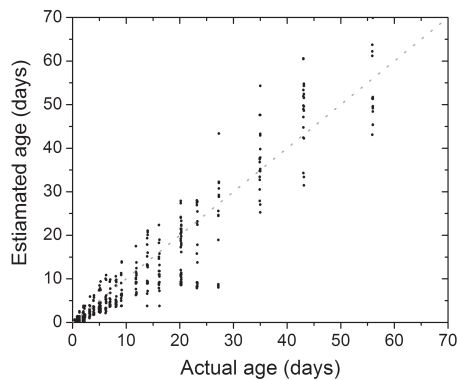


Figure 8. Age estimation of bloodstains of the validation set plotted versus actual age. Data points measured at the same time are scattered to various estimated ages, indicating the error margin of age estimation.

uncertainties in the measurement procedure cause uncertainty in age estimation, this effect is observable in the plot as the data scatters around the line of unity. Discrimination in age between bloodstains of different ages is possible, when the scattering range of the estimated ages do not overlap. To illustrate this, at an age of 3 days, the estimated ages vary between 1.5 and 6 days; at an age of 12 days, the estimated ages vary between 6 and 16 days; and at an age of 35 days, the estimated ages vary between 25 and 55 days. Hence discrimination of age among these three examples is possible

4. DISCUSSION AND CONCLUSION

We showed that age determination of bloodstains is feasible under controlled environmental conditions. The excellent short- and long-term fits (figure 5) of the DRS measurements in oxy-Hb, met-Hb and HC strongly suggests that the oxidation cascade of extracorporeal blood as well as the absorption spectra utilized for these derivatives at a large spectral range are essentially correct. Thus, the combination of oxidation cascade and optical DRS requires further consideration in pursuing age determination of bloodstains found under non-laboratory conditions and perhaps even on crime scenes.

The division of the bloodstains samples into two groups allowed for independent determination of average hemoglobin reaction kinetics and age estimation, according to one round cross-validation[76]. Determination of the average kinetics based on the total sample size of 40 bloodstains, instead of only 20 does not change the average hemoglobin fractions very much, e.g. the average HbO₂ fraction after 1 day for 20 bloodstains is 0.65±0.02 and for 40 bloodstains 0.66±0.02; and the HbO₂ fraction after 17 days for 20 bloodstains is 0.33±0.04 and for 40 bloodstains 0.33±0.03. Alternative method for age determination would be the leave-one-out (n-fold cross-validation) approach, but this approach requires much more calculation and will not improve the age estimation, considering the small differences between average hemoglobin fractions based on 20 or 40 bloodstains.

Our simultaneous measurements of 40 stains required displacement of the probe. For all measurements we kept the probe in same configuration at 10±1 mm height and perpendicular to the surface. In additional serial experiments on one stain with the probe in fixed position over the total measured time period (not shown), we found similar hemoglobin kinetics, but with much lower spreading of data. Thus, in a forensic situation, multiple consecutive measurements can be performed, which allow determination of the hemoglobin fractions and the *in situ* hemoglobin kinetics. Hence evolving age estimation, based on multiple measurements can be more precise than shown in this study.

Owing to the capillary flow [78], the edge of a bloodstain appears slightly darker and thus contains more chromophores. Although this effect is very limited in bloodstains on cotton; we measured the reflectance at the center of the bloodstain. Inhomogeneities within a bloodstain or small bloodstains can still be accounted for when a spectral imaging setup is used. This technique has also been suggested for age determination

of bruises [79] and would be a suited technique for age determination of bloodstains as well.

The diffuse reflectance spectroscopy setup is portable, cheap and non-invasive and non-destructive, all favorable properties when measuring in a forensic setting [62]. However, prior to forensic implementation, we believe that knowledge of the reaction rates of HbO₂ to met-Hb and HC as a function of temperature, humidity and e.g. exposure to sun light needs to be attained and incorporated in the optics analysis. In addition, also the influence of the surface background of the bloodstain on DRS requires further study.

In conclusion, our results suggest that determination of hemoglobin derivatives by means of diffuse reflectance spectroscopy demands further consideration in pursuing for age determination of bloodstains under non-laboratory, forensic conditions. Successful age determination at crime scenes requires more knowledge of the kinetics of the Hb cascade under environmental conditions and their incorporation in the optics analysis method.

APPENDIX A

The Kubelka Munk theory was employed to analyze the recorded diffuse reflectance spectra described in the data analysis section. We used the following formulae:

$$\frac{R(\lambda)}{R_0(\lambda)} = 1 - \frac{K}{S} \left(\sqrt{1 + \frac{2S}{K}} - 1 \right) \quad (1)$$

Here $R(\lambda)$ denotes the bloodstain's reflectance, and $R_0(\lambda)$ is the reflectance of the white substrate surface, both depend on wavelength λ . K and S represent the absorption and scattering of the bloodstain. Here $K = \mu_a / \eta$, with μ_a the absorption coefficient per unit length which depends on wavelength and η being a dimensionless function depending on albedo. Because the chromophores are packed in red blood cells, the absorption spectra as shown in figure 2 are flattened. We corrected for this flattening effect, as described by Finlay and Foster[74], with an erythrocyte radius of 3 μm .

This relation is described in detail elsewhere [71], [73]. For scattering S we assumed Lorentz-Mie scattering:

$$S = S_0 \cdot \left(\frac{\lambda}{\lambda_0} \right)^{-0.4} \quad (2)$$

The scattering S depends on the wavelength λ in nm, λ_0 is 450 nm and the scattering coefficient at 450 nm, S_0 is 13.5 mm^{-1} . We set the scattering to be constant over the total measured time period.