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### Spectral analysis of blood stains at the crime scene

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## **7 - IDENTIFICATION AND AGE ESTIMATION OF BLOOD STAINS ON COLOURED BACKGROUNDS BY NEAR INFRARED SPECTROSCOPY**

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Non-destructive identification and subsequent age estimation of blood stains are significant steps in forensic casework. The latter can provide important information on the temporal aspects of a crime. As previously shown, visible spectroscopy of blood stains on white backgrounds can successfully be used for their identification and age estimation. The use of this technique however, is hampered by dark backgrounds. In the present study the feasibility to use near infrared (NIR) spectroscopy was evaluated for blood stain identification and age estimation on dark backgrounds. Using NIR reflectance spectroscopy, blood stains were distinguished from other substances with 100% sensitivity and 100% specificity. In addition, Partial Least Squares Regression analysis was applied to estimate the age of blood stains on coloured backgrounds. The age of blood stains up to 1 month old was estimated successfully with a root mean squared error of prediction of 8.9%. These findings are an important step toward the practical implementation of blood stain identification and age estimation in forensic casework, where a large variety of backgrounds can be encountered.

## 7.1. INTRODUCTION

Blood stains are important traces in many forensic investigations. After detection of a stain at the crime scene, a technique for blood stain identification is required to prove the presence of blood in the stain. Identified blood stains can subsequently be used for other purposes, like pattern reconstruction and DNA analysis. Most presumptive blood identification tests use chemicals which, in contact with blood, change colour<sup>79</sup>, fluoresce<sup>120</sup> or luminesce<sup>121</sup>. However, these chemicals can lead to a loss of the blood stain patterns<sup>121</sup>, may interfere with subsequent tests, e.g. confirmative identification tests, species tests or DNA analysis<sup>122</sup> and may be harmful for the investigators. There is a need for confirmatory, non-destructive methods which can be used to identify blood stains at the crime scene without the need of sampling and subsequent lengthy laboratory testing<sup>123</sup>. In **Chapter 3** we<sup>65</sup> successfully applied reflectance spectroscopy for the non-destructive identification of blood stains. In the described study, blood stains were distinguished from other blood resembling substances (e.g. ketchup, red wine, lip gloss) deposited on white cotton. However, when blood stains are deposited on dark backgrounds, the identification of blood stains using visible reflectance spectroscopy is severely hampered by the absorption of visible light by the background. Because near infrared (NIR) light is absorbed less efficiently by many dark coloured substrates, NIR reflectance spectroscopy may solve this problem. Absorptions that occur in the NIR region of the electromagnetic spectrum (720-2500 nm) are due to the overtone and combination bands of molecular stretching and bending vibrations of the fundamental absorptions of –CH, –NH, and –OH groups<sup>124, 125</sup>. As a result, NIR spectroscopy provides more information about the chemical structure of samples compared to visible spectroscopy, and is expected to be useful for identification purposes.

After a positive identification of blood stains, it is of interest to estimate the time of bleeding, which may help crime scene investigators to determine the temporal aspects of a crime. Several techniques, including high performance liquid chromatography<sup>126</sup>, electron paramagnetic resonance<sup>89, 90</sup>, atomic force microscopy<sup>127</sup> and RNA degradation measurements<sup>128, 129</sup> have

been investigated for this purpose, as reviewed by Bremmer et al<sup>94</sup>. However, none of these methods is yet implemented in forensic practice, and most require sample preparation and need to be performed in a laboratory. Just like identification, blood stain age estimation can be performed non-destructively using visible reflectance spectroscopy<sup>1, 68</sup>. When blood exits the human body, oxyhaemoglobin auto-oxidizes into methaemoglobin, which in turn denatures into hemichrome<sup>1</sup>. These reactions cause a colour change from red to brown. Visible (Vis) reflectance spectroscopy can be used to measure this colour change quantitatively, determine the chemical components and thereby estimate the age of blood stains (**Chapter 4,5**)<sup>1</sup>. Botonjic-Sehic et al<sup>66</sup> explored the potential of using the NIR region for the age estimation of blood stains. They demonstrated that spectral changes in a broad wavelength band from 1460-1860 nm were useful to estimate the age of a blood stain on glass and on gauze.

In this study we investigated the use of NIR spectroscopy for the identification and age estimation of blood stains on coloured backgrounds. We therefore first inspected typical features in NIR reflectance spectra of aging blood stains and compared these qualitatively to literature spectra of different components of human blood. Next, we studied the sensitivity and specificity to distinguish blood from non-blood stains using NIR reflectance spectroscopy and subsequent analysis. Finally, we explored the use of NIR reflectance spectra to estimate the age of blood stains on coloured cotton backgrounds. For this task Partial Least Squares (PLS) Regression models were created. The accuracy of the age estimation models was evaluated.

## **7.2. MATERIALS AND METHOD**

### **7.2.a SAMPLES**

#### **Pure blood**

To characterize the spectral features of blood, initial measurements were performed on a single blood drop from a healthy male volunteer (volunteer #1) deposited directly onto a sample holder, without a substrate.

## Samples on cotton

In forensic practice blood stains are always deposited on a substrate. Therefore, both blood and non blood samples were deposited on cotton backgrounds. To analyze the influence of coloured backgrounds, cotton with 5 different colours was used. This led to the following sample sets:

- Blood on white cotton) 3 blood stains, drawn from a healthy female volunteer (volunteer #2). Next to this, a plain cotton reference sample was created (see Figure 7.1). Reflectance spectra of these samples were measured repeatedly for 77 days (with varying frequencies of every 2 hours in the beginning up to monthly in the end, 54 measurements in total).
- Blood on coloured cotton) Blood from a healthy female volunteer (volunteer #3) was deposited on black, red, green and blue cotton (3 blood stains each). Next to this, plain cotton reference samples were created (see Figure 7.1). Reflectance spectra of these samples were measured repeatedly up to the age of 28 days (66 measurements in total).



Figure 7.1. Photographs of blood stains on white, black, red, green and blue cotton and corresponding plain reference samples (bottom row).

- Non blood on white cotton) 30 different samples of non-blood substances (specified in Figure 7.2). These samples were selected because they are likely to be false positives when using luminol (bleach) or visible spectroscopy (all other substances) for the detection of blood stains. Measurements on all non-blood substances were repeated after one week.



Figure 7.2. Photographs of non-blood samples containing: 1.tea, 2.coffee, 3.red wine, 4.red grape juice, 5.black currant soda ('cassis'), 6.coke, 7.cherry coke, 8.red berry juice, 9.tomato juice, 10.ketchup, 11.curry, 12.Tabasco, 13.ketjap manis, 14.Worstershire sauce, 15.soy sauce, 16.balsamic, 17.Maggi aroma, 18.red wine, 19.cherry marmalade, 20.forest fruit marmalade, 21.apple syrup, 22.chocolate sauce, 23.red cabbage, 24.red beet, 25.bleach, 26.lipstick, 27.lipstick, 28.lipstick, 29.red food colourant, 30.red food colourant.

## 7.2.b NIR SPECTROSCOPY

### Pure blood

Using a Fourier transform near infrared spectrometer (Bruker, MPA-NIR-FT type), the NIR reflectance spectra (800-2778 nm) of the pure blood stain was measured repeatedly (every 5 min for the first 5 hours, thereafter hourly until the age of 4 days, 240 measurements in total).

## **Samples on cotton**

Vis-NIR reflectance spectra of all samples on cotton and the reference substrates were measured using a FOSS NIR systems 6500 spectrophotometer (400-2500 nm). Samples had a diameter of approximately 3 cm, and were contained in glass covered sample holders. 32 scans from different positions on the sample were averaged to obtain an average spectrum. In between measurements, samples were stored in a laboratory at room temperature ( $\approx 22$  °C).

### **7.2.c PRE-PROCESSING**

All measured reflectance spectra were transformed to apparent absorbance spectra ( $\log(1/R)$ , where R is the reflectance). Next, all absorbance spectra of samples deposited on cotton were corrected for background absorption by subtracting the absorbance spectrum of the reference sample of the same colour. All data analysis was performed using custom-made scripts written in MATLAB (The Mathworks Inc., Natick, Massachusetts, USA).

## **7.3. DATA ANALYSIS**

### **7.3.a BLOOD STAIN CHARACTERIZATION**

To characterize the absorbance spectra of pure blood, the absorbance maxima were compared to reference absorption maxima (see Table 7.1) of the following blood components: oxyhaemoglobin, methaemoglobin, albumin, globulin, triglycerides, glucose, cholesterol, and urea. Reference spectra of fibrinogen were not found, as research is often done on serum, which does not contain fibrinogen. Other components, normally present in lower concentrations in blood, were not taken into account<sup>130</sup>.

### **7.3.b WAVELENGTH SELECTION**

When analyzing blood stains on cotton, the absorbance spectra are influenced by the background. Therefore we corrected these spectra for background absorptions. However, when the absorption of the background is high, the background dominates the spectra and spectral features of the blood stains become negligible. In these cases, the background correction is not optimal and blood stain identification and age estimation will be hampered. To analyze the influence of background absorptions on the corrected spectra, the absorbance spectra of the differently coloured reference backgrounds were compared and Pearson's correlation coefficient between the different backgrounds was calculated. Based on this, a wavelength region was selected in which the colour of the cotton apparently did not influence the spectra. This wavelength region was used for the subsequent identification and age estimation tasks.

### **7.3.c IDENTIFICATION**

For the identification of blood stains on coloured cotton, we used a comparison library and a test set. The comparison library consisted of 3 absorbance spectra of blood stains on white cotton with an age of 1 day, 1 week and 1 month. All other absorbance spectra of blood stains on white, black, red, green and blue cotton (315 in total) formed the test set. Of all spectra, the wavelength region determined above was selected. Spectra of the blood stains from the test set were compared to the library using the coefficient of determination ( $R^2$ ).  $R^2$  values were calculated between all spectra from the library and the test set. For each sample from the test set, the maximum  $R^2$  value was plotted in a box plot. Spectra of all blood stains were expected to be similar to the spectra from the library, which means the  $R^2$  values were expected to approach 1.

Similarly,  $R^2$  values were calculated between all spectra from the library and the non blood samples on white cotton. Again, the maximum  $R^2$  value was plotted in a box plot. For these non blood samples, lower values were

expected. If all  $R^2$  values for non blood samples were lower than the  $R^2$  values for blood samples, blood stains could be identified based on the coefficient of determination.

### **7.3.d AGE ESTIMATION**

To estimate the age of blood stains on coloured backgrounds PLS regression analysis was applied, using the wavelength region selected above. PLS is a useful statistical tool for the analysis of spectroscopic data, as it can handle datasets with more variables than observations, and the data may contain highly correlated predictor variables<sup>131</sup>. PLS makes linear combinations of the original predictor variables to construct new predictor variables, which are the most relevant for estimating the age.

A PLS model was created for each coloured background. Each time, the data was split into a training set and an independent test set. The training set consisted of the absorbance spectra of blood stains on four colours, not including the questioned colour. The test set consisted of absorbance spectra of blood stains on the background colour in question, and was used to predict the ages of blood stains. To evaluate the performance of these predictions, the coefficient of determination  $R^2$  and the root mean squared error of prediction (RMSEP) were calculated for each background.

## **7.4. RESULTS**

### **7.4.a BLOOD STAIN CHARACTERIZATION**

Fourier transform NIR spectroscopy on a pure blood stain showed the temporal spectral changes in the first days of aging. In the first minutes, the spectrum was dominated by water absorption peaks at 1454 and 1940 nm, indicated by the blue lines in Figure 7.3.

After drying (in air at room temperature, for approximately 10 min), several peaks appeared which have been reported in literature as absorption peaks of haemoglobin, albumin, and globulin (see Figure 7.3 and Table 7.1). The slope of the spectrum near the peaks at 1690 and 1740 nm changed in

time. These peaks are also reported in spectra of glucose, but other glucose absorption peaks (around 2120 and 2260 nm)<sup>132-134</sup> were not visible in the blood spectra. Absorption peaks of cholesterol (around 1472, 1718, 1750, 2076, and 2310 nm)<sup>134, 135</sup>, urea (around 2200 nm)<sup>134, 136</sup> and triglyceride (around 1725 and 2130 nm)<sup>137</sup> were not visible.

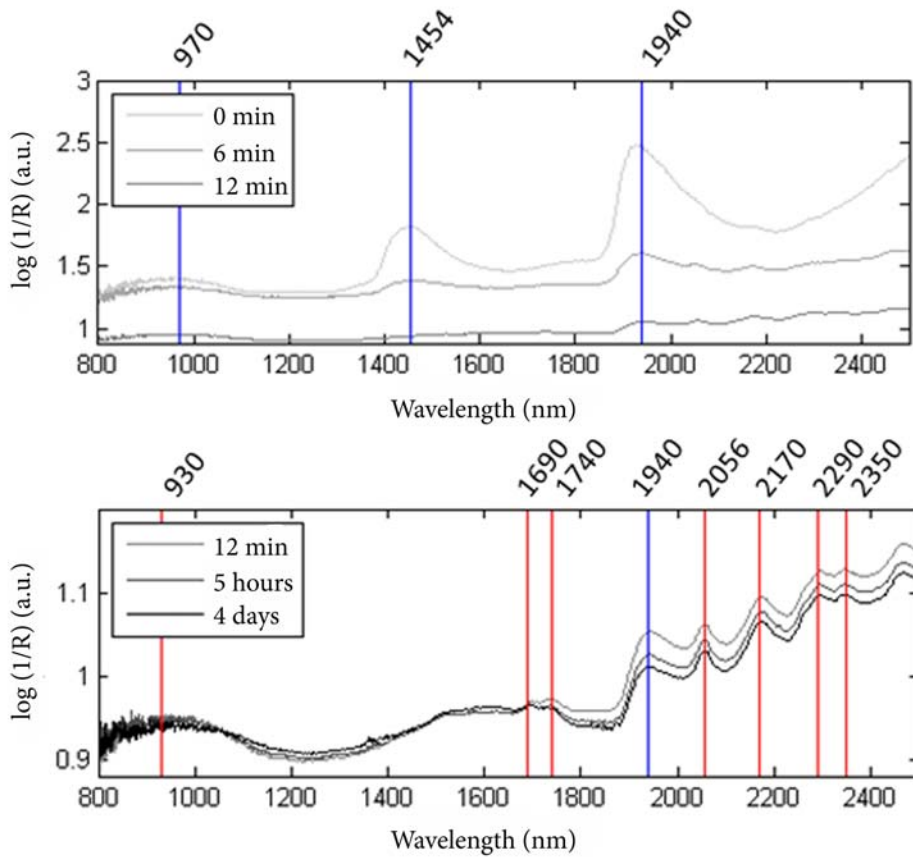


Figure 7.3. Log (1/R) spectra of a wet blood stain of 0, 6 and 12 min old (top) and spectra of the same blood stain after drying with an age of 12 min, 5 hours and 4 days (bottom). Units are arbitrary (a.u.). Lines in the figure show the position of absorption peaks listed in Table 7.1, blue lines referring to water peaks, red lines to peaks from other components present in blood.

Table 7.1. List of absorption peaks of several blood components as reported in literature, which were observed in the spectra in Figure 7.3. Positions of these peaks are depicted in Figure 7.3. Possible chemical origins of the peaks are mentioned, next to the bonds found in the references listed.

Wavelength (nm)	Component	Bond	References
930	Oxyhaemoglobin	Third overtone of –CH and –CH <sub>2</sub> stretching vibrations	138, 139
970	Water	Combination of H-O-H symmetric and asymmetric stretching vibrations	124, 140, 141
1454	Water	Combination of H-O-H symmetric and asymmetric stretching vibrations	124, 138, 140, 141
1690	Haemoglobin, Albumin, Globulin	First overtone of –CH stretching vibration	132, 133, 136, 138, 142, 143, 143, 143-145
1740	Haemoglobin, Albumin, Globulin	First overtone of band at 3477 nm	132, 133, 136, 138, 142, 143, 143, 143-145
1940	Water	Combination of H-O-H bending and asymmetric stretching vibrations	124, 138, 140-142
2056	Haemoglobin, Albumin, Globulin	Combination of amide A and amide II or another combination	125, 133, 138, 142-144
2170	Haemoglobin, Albumin, Globulin	Combination of amide B and amide II or overtone of amide II	125, 133, 138, 142-144
2290	Haemoglobin, Albumin, Globulin	–CH stretching and deformation combinations	125, 133, 138, 142-144
2350	Haemoglobin, Albumin, Globulin	–CH stretching and deformation combinations	125, 133, 138, 142-144

### 7.4.b WAVELENGTH SELECTION

Absorbance spectra of the differently coloured cotton reference samples are shown in Figure 7.4. This figure shows that coloured cotton backgrounds absorbed highly in the visible region, whereas white cotton absorbed almost no light in this region. For wavelengths over 1150 nm, however, the absorbance spectra of different colours almost overlapped, showing typical features of cotton<sup>146</sup>, which was confirmed by a correlation analysis. Pearson's correlation coefficient between the different background spectra from 1150 -2500 nm exceeded 0.999. Therefore, this region was selected for subsequent analyses.

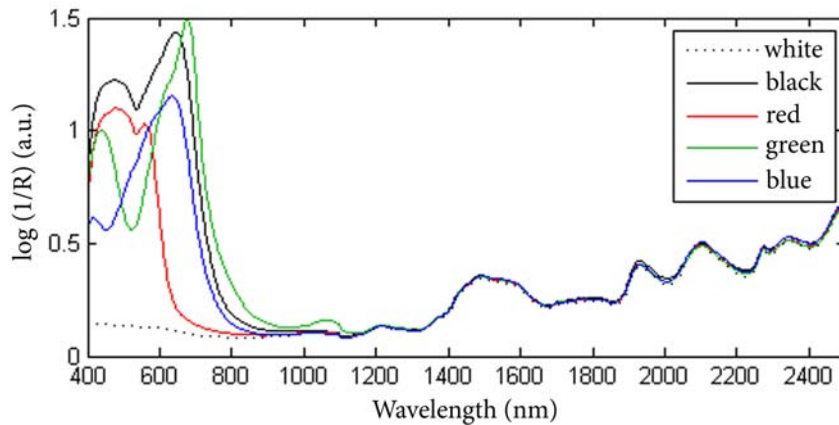


Figure 7.4. Log (1/R) spectra of white, black, red, green and blue cotton backgrounds. This shows that white cotton absorbed almost no light in the visible region, while coloured backgrounds absorbed highly in this region. For wavelengths larger than 1150 nm however, the spectra of different colours almost overlapped.

### 7.4.c IDENTIFICATION

Example absorbance spectra of blood and non blood spectra on white cotton are shown in Figure 7.5.

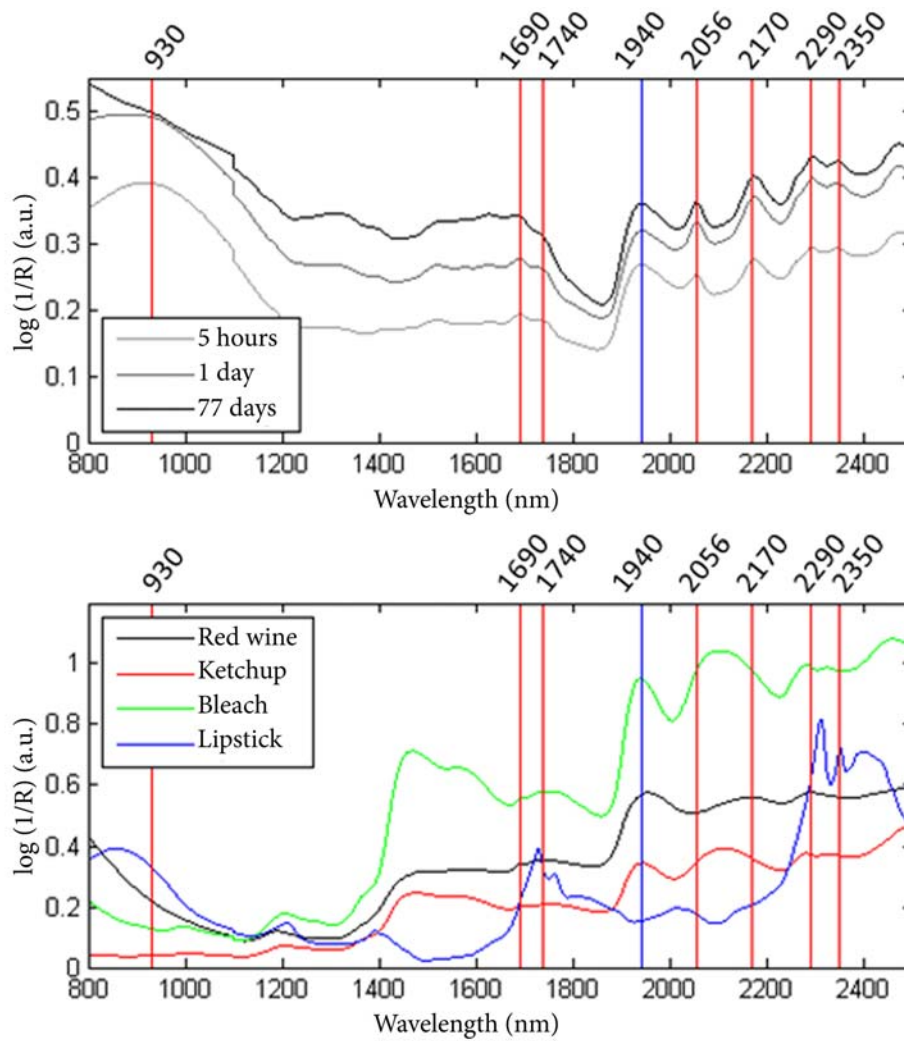


Figure 7.5. Apart from the water peak at 1940 nm, the spectra of non blood stains (lower panel of Figure 7.5) clearly differed from the blood spectra.

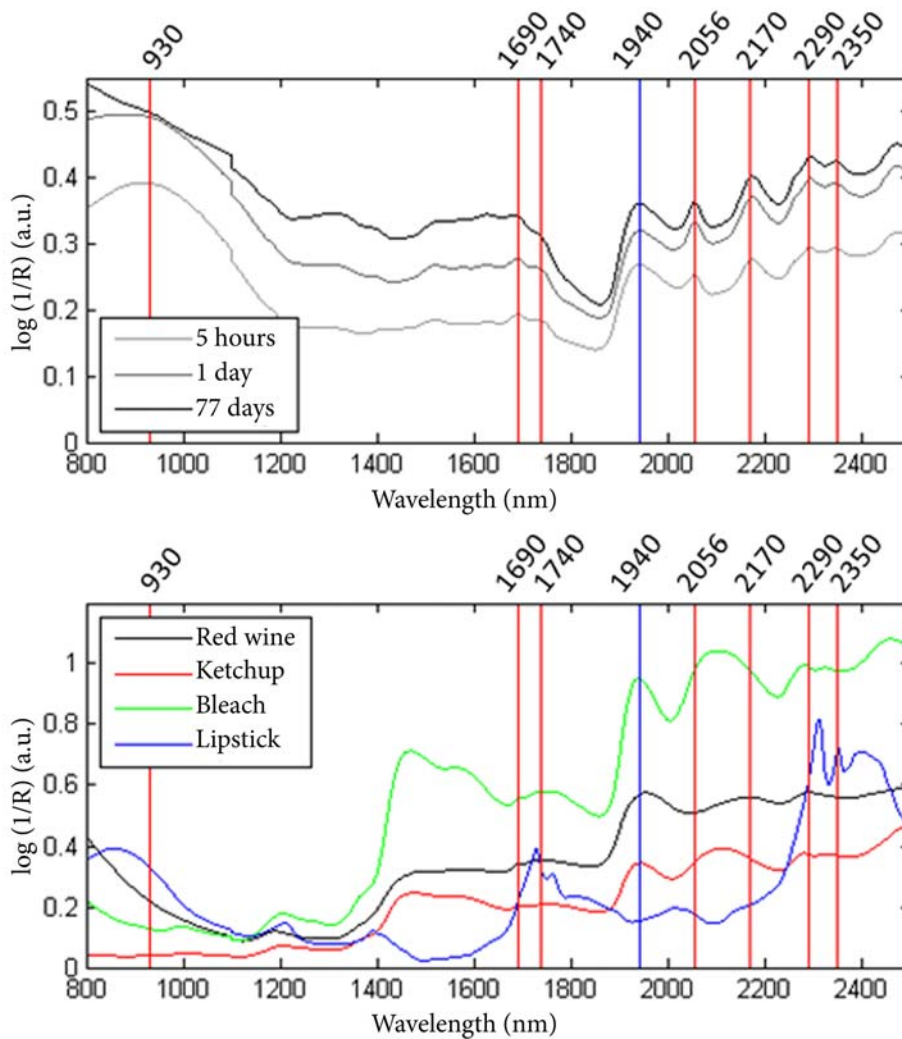


Figure 7.5. Background corrected  $\log(1/R)$  spectra of dry blood stains on cotton of 5 hours, 1 day and 77 days old (top) and of 2 days old samples of red wine, ketchup, bleach and lipstick (bottom). Vertical lines in the figure show the position of absorption peaks listed in Table 7.1.

$R^2$  values between the absorbance spectra of blood stains in the library and spectra from other blood stains on the one hand and spectra from non blood samples on the other hand are depicted by the box plots of Figure 7.6.

This figure shows that a threshold could be chosen which separates all blood stains from all non blood samples, e.g. if a threshold of  $R^2=0.8$  was used to identify blood stains, this resulted in 0 false positives and 0 false negatives. This means blood stains could be identified with a sensitivity of 100% and a specificity of 100%.

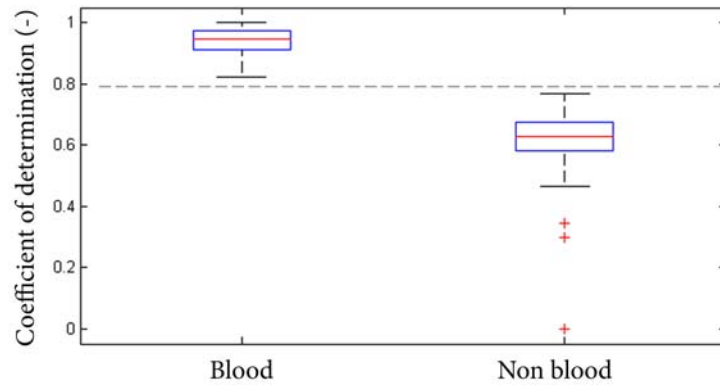


Figure 7.6. Box plots showing the coefficients of determination ( $R^2$ ) between blood stain absorbance spectra from the library and all other blood stain absorbance spectra and non blood absorbance spectra. At a threshold of  $R^2=0.8$  (dashed line) all  $R^2$  values from blood stains can be distinguished from these from non blood stains.

#### 7.4.d AGE ESTIMATION

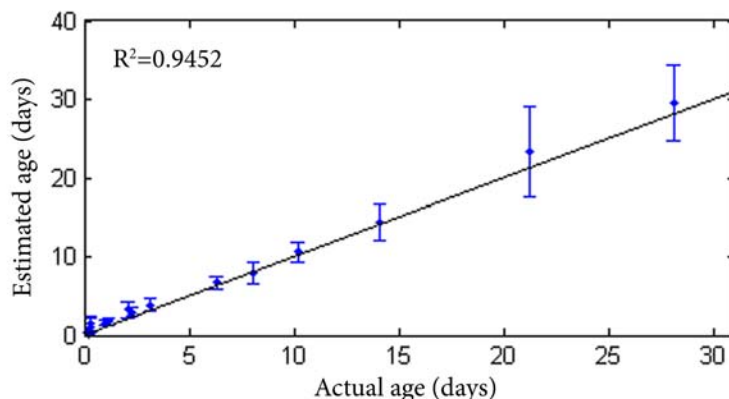


Figure 7.7. Results of the age estimation of blood stains on black cotton using a PLS model in which a part of the NIR region was used (1150-2500 nm). Dots represent average measurements of three blood stains, whiskers depict the standard deviation. The line of unity is plotted for comparison ( $R^2 = 0.9452$ ).

To estimate the age of blood stains on coloured backgrounds, a PLS model was created for each colour, which was trained with absorbance spectra of blood stains on all other backgrounds. Estimated ages of blood stains on black cotton were plotted in Figure 7.7. This figure shows that the accuracy of the estimation decreases with age, which is shown by the larger error bars for older blood stains. Results for the other colours were similar. For each colour, the coefficient of determination  $R^2$  between actual ages and estimated ages and the root mean squared error of prediction (RMSEP) were calculated (see Table 7.2). The RMSEP values are relative errors, the absolute errors increase with age.

Table 7.2. Results of the age estimation task for blood stains on different cotton backgrounds. For each test and training set, the resulting  $R^2$  and RMSEP are given.

Training set	Test set	$R^2$	RMSEP
white, red, green, blue	black	0.9452	8.9%
white, green, blue, black	red	0.9679	6.8%
white, red, blue, black	green	0.9607	8.5%
white, red, green, black	blue	0.9749	9.1%

## **7.5. DISCUSSION**

In this study, we identified NIR spectral features of blood stains which can be used for blood stain identification and age estimation, even when blood is deposited on coloured backgrounds. Using NIR reflectance spectroscopy blood stains can be distinguished from other substances with a sensitivity of 100% and a specificity of 100%, and their ages can be estimated non destructively.

### **7.5.a BLOOD STAIN CHARACTERIZATION**

NIR reflectance measurements on blood stains show that the spectra are dominated by decreasing water absorption peaks in the beginning of the aging process. After drying, absorption peaks from several other components of human blood, e.g. haemoglobin, albumin, globulin are observed. Because NIR absorption bands are characteristically weak, highly overlapping, and very broad<sup>125</sup>, identification of the contributions of different components to the spectrum of whole blood is difficult. The components and bonds mentioned in Table 7.1 are therefore just indicative. Other techniques, e.g. Raman spectroscopy, may be useful for the exact identification of all components. Using Raman spectroscopy, Boyd et al identified scattering peaks characteristic of haemoglobin and fibrin<sup>103</sup>. Identification of more components present in blood could provide interesting information about the chemical processes involved in the aging of blood stains.

### **7.5.b IDENTIFICATION**

In the first 77 days of aging, we found that the NIR absorbance spectra of blood stains correlate highly, and are thus useful for the identification of blood stains. By analyzing the similarity with a library of 3 blood stains of different ages, NIR absorbance spectra of all blood stains were distinguished from a set of other substances. Apparently, the similarity of the spectra of blood stains was high compared to the similarity between blood and our selection of non blood samples. This limited non blood sample set was chosen such that it

contained substances which are likely to be false positives when luminol or visible spectroscopy is used for the identification of blood stains. Other substances with absorption properties resembling blood stains in the NIR wavelength range may exist (e.g. substances containing proteins). Adding other samples to the set may induce false positives and thus reduce the specificity. Regarding the sensitivity, it is unknown if we will be able to identify blood stains older than 77 days, diluted blood stains, or blood from smokers or people suffering from certain diseases.

Compared to chemical blood identification tests, e.g. luminol<sup>121</sup>, the main advantage of spectroscopy based techniques is their non-destructiveness. Therefore, Raman spectroscopy<sup>74, 81, 84, 103, 147</sup> and visible spectroscopy (**Chapter 3**)<sup>65</sup> have been examined recently for the purpose of blood identification. We now added NIR spectroscopy to this range of techniques. All spectroscopic techniques are hampered by certain backgrounds, which can be encountered in forensic practice. While Raman spectroscopy is difficult on strongly fluorescing backgrounds, like fabrics, visible spectroscopy is hindered by coloured backgrounds. We demonstrated that NIR spectroscopy can be complementary to Raman and visible spectroscopy, as we successfully identified blood stains on coloured fabrics. It is expected that this method will be successful on many other backgrounds. Only materials which highly absorb light of many NIR wavelengths are expected to complicate the identification task. Similarly, water absorption peaks complicate the identification of wet blood stains using NIR spectroscopy.

### **7.5.c AGE ESTIMATION**

After a successful identification, the ages of blood stains on coloured backgrounds were estimated using NIR spectroscopy. The RMSE of prediction of the age of blood stains on black cotton up to one month old was 8.9%. We observed a better accuracy for younger than older blood stains, which can be explained because chemical and thus spectral changes are faster in the beginning of the aging process. These results demonstrate that NIR spectroscopy is suitable for short term age estimation, as previously shown by

Botonjic-Sehic et al<sup>66</sup>. While Botonjic-Sehic et al calculated the areas under a small part of the spectra, we used a large wavelength region to estimate the age of blood stains with PLS regression. In addition, we demonstrated the applicability of this technique on coloured backgrounds, where age estimation using visible spectroscopy is hampered<sup>1</sup>. As the sample set used for training the PLS model did not include the background colour of the test set, this method is expected to be successful for other colours not included in our sample sets.

Other methods for age estimation, e.g. high performance liquid chromatography, electron paramagnetic resonance, atomic force microscopy and RNA degradation measurements are more invasive and can only be performed in the laboratory. Although in this study a laboratory setup was used, with two large spectrometers, recent developments in hyperspectral imaging technology, i.e. the combination of spectroscopy and conventional imaging, offer potential for crime scene investigations. The development of portable equipment enables the analysis of an unknown stain at the crime scene without waiting for results from the laboratory.

Before NIR spectroscopy for blood stain age estimation can be applied in practice, key steps in the research process are refining and validating the data to meet the needs of the legal and scientific communities. For example, more research is needed on the effect of environmental conditions. As the aging of blood stains is influenced by factors like temperature, humidity and lighting conditions<sup>56</sup>, the effect on the NIR spectra should be studied.

#### **7.5.d CONCLUSION**

NIR spectroscopy can be employed for the non-destructive identification and age estimation of blood stains on coloured backgrounds. When introduced in forensic casework, the described method can provide investigators with important information; it can give an indication of when a crime was committed, or it can help investigators to determine if a certain stain is relevant to the case.

## 7.6. ACKNOWLEDGEMENTS

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