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Non-contact spectroscopic age determination of bloodstains

Bremmer, R.H.

Publication date
2011

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Citation for published version (APA):

Bremmer, R. H. (2011). *Non-contact spectroscopic age determination of bloodstains*. [Thesis, fully internal, Universiteit van Amsterdam].

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Blood detection and identification at crime scenes are crucial for harvesting forensic evidence. Unfortunately, most tests for identification of blood are destructive and time consuming. We present a fast and non-destructive identification test for blood, using non-contact reflectance spectroscopy. We fitted reflectance spectra of 40 bloodstains and 35 non-bloodstains deposited on white cotton with spectroscopic features of the main compounds of blood. Each bloodstain was measured 30 times to account for ageing effects. The outcome of the blood measurements was compared with the reflectance of blood mimicking stains and various body fluids. We found that discrimination between blood and non-blood deposited on white cotton is possible with a specificity of 100% and a sensitivity of 98%. In conclusion, a goodness of fit between the sample's reflectance and the blood component fit may allow identification of blood at crime scenes by remote spectroscopy.

CHAPTER 3

REMOTE SPECTROSCOPIC IDENTIFICATION OF BLOODSTAINS

Journal of Forensic Science 2011, in print



INTRODUCTION

Identification of bloodstains at a crime scene is of critical importance in criminal investigations. Ideally, traces are judged and interpreted in the original context, at the crime scene [62]. Thus, there is a need for techniques that allow for remote, non-contact identification of evidence. Many screening tests, routinely used in forensic practice, use chemical methods for identification of blood to discriminate it from other body fluids or red substances. Most chemical tests, including tetra-base [80] and Kastle-Meyer [81] employ peroxidase activity of hemoglobin molecules. The peroxidase either causes a color change or induces chemiluminescence. A common example of the latter is the luminol test. By spraying luminol onto the suspected area, the reactant will glow in the presence of blood [82]. This test is especially appropriate for indicating bloodstains after cleaning attempts. Most of these tests are presumptive in nature, not confirmative, since several other substances are reported to catalyze this peroxidase reaction [83]. More reliable, confirmatory tests are based on hemoglobin derivative crystals [84, 85] or RNA markers in blood [86]. However, these tests require advanced sample preparation and microscopic observation, and are therefore not applicable for interpreting traces in its original context, at the crime scene.

Recently, however, optical techniques have been suggested for bloodstain identification. Hemoglobin has specific absorption bands at 420, 540 and 576 nm [31]; it fluoresces at 465 nm, when excited at 321 nm [87]; and heme provides Raman-bands at 1.222 and 1.542 cm^{-1} [88]. All these corresponding techniques have the potential to allow *on field* identification of blood based on the specific spectroscopic features of hemoglobin [88, 89]. Despite promising results, these techniques have not been reported to be implemented in forensic practice. Accordingly, all these techniques have their own drawback. Measuring Raman signals is highly complicated by interference with the fluorescence signal of the trace and its background. A fluorescence signal on its turn is difficult to measure, because of the high absorption properties of hemoglobin in the UV/VIS spectral range. Blood identification based on the absorption properties has been investigated by Kotowski *et al* in a microspectrophotometry setup [90]; De Weal *et al* recently confirmed these findings [89]. Their approach however, requires advanced sample preparation and a laboratory environment for accurate measurements.

We propose a non-destructive and non-contact reflectance spectroscopy technique for the identification of bloodstains, which can be used at the crime scene. Reflectance spectroscopy is a widely used technique to determine the optical properties of turbid materials. Based on spectroscopic analysis of the reflected light, tissue chromophore concentrations can be estimated. In bloodstains, mainly three compounds are present, oxy-hemoglobin, methemoglobin and hemichrome [23]. In chapter 2 we showed that fitting the absorption spectra of the hemoglobin compounds to the measured reflectance spectrum allows determination of the compound fraction estimation of the age of a bloodstain. The goodness of fit between the absorption spectra reported in literature and the measured bloodstain can be expressed in a Pearson correlation coefficient.

In this experiment we have investigated whether blood can be discriminated from other body fluids and substances visually mimicking blood, based on the correlation coefficient between the reflectance spectrum and a blood-component fit. To test the sensitivity and specificity of this method two sets of samples are analyzed: a set of blood samples and a set of red/brown colored substances and various body fluids. Finally, an example is given of a recent forensic case, in which our technique was applied.

MATERIALS AND METHOD

Sample preparation

Forty blood samples were obtained from eight healthy male donors. These samples were prepared by depositing a small drop on a piece of white cotton, creating a stain with a diameter of 21 ± 4 mm. We performed 1200 measurements on the forty blood samples; thirty measurements per sample in the time span from a few seconds after deposition until a year after deposition. When not being measured, the bloodstains were stored in a dark laboratory at 22.3 ± 0.5 °C, up to a year.

The second set contained 35 non-blood samples. Among the samples are 31 samples visually mimicking blood, including ketchup, red wine and fake blood; and four body fluids commonly found at crime scenes: saliva, semen, urine and perspiration. These samples were created similarly to the bloodstains on a piece of white cotton.

Non-contact reflectance spectroscopy

Reflectance measurements were performed with a combination of a spectrograph (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). This probe contains six 400 μ m core diameter delivery fibers, circularly placed around an identical central collecting fiber. Figure 1 shows the schematic of the setup. The probe is positioned 1 cm above the specimen. During measurements, photons emitted by the delivery fibers scatter through

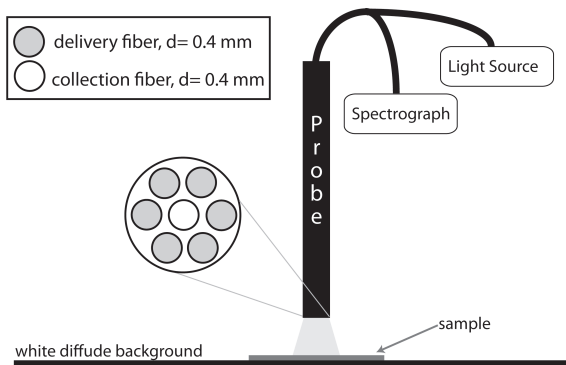


Figure 1. Schematic of the measurement setup. The reflection probe tip shows six delivery fibers, circled around a central collection fiber. The delivery fiber is connected to the light source and the collection fibers are connected to the spectrograph.

the sample, and are collected with the central fiber. The light intensity measured with the collection fiber is the reflectance $R(\lambda)$, which is wavelength (λ) dependent. For each measurement a reflectance spectrum of the sample, $R(\lambda)$ and a reflectance spectrum of the background material (cotton), $R_0(\lambda)$ was obtained.

Analysis of reflectance spectrum

The reflectance ratio of the sample and its background $R(\lambda)/R_0(\lambda)$ were analyzed with a multi-component linear least squares fit. The absorption spectra of the three compounds present in blood: oxy-hemoglobin, met-hemoglobin and hemichrome[23] were used as input. A light transport model was employed to translate the absorption spectra into a reflectance spectrum, which is described in the appendix. The 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 theory and the diffuse reflectance spectrum.

Coefficient of determination

The Pearson correlation coefficient gives information about the goodness of fit between the measured reflectance spectrum and the multi-component blood-fit [91]. The coefficient of determination, r^2 , is the square of Pearson's correlation coefficient. When blood is present and the light transport model is correct, the coefficient of determination between the blood-fit and the specimen will be high and approach one. The r^2 value of all reflectance measurements has been calculated and tested for discrimination between blood and non-blood samples.

ANOVA-test

One way ANOVA tests are performed on the blood samples to test on differences within the total bloodstain population. Three individual, one way ANOVA tests are taken on samples ($n=40$), donors ($n=8$) and ageing. For ageing testing, we grouped the bloodstains in four categories: age < 1 day; 1 day < age < 1 week, 1 week < age < 1 month and age > 1 month. Significance is defined if $p < 0.05$.

RESULTS

Blood samples

The upper part of figure 2 shows two typical diffuse reflectance spectra of blood samples: of one day old (figure 2a) and a year old (figure 2b). The spectrum in figure 2a shows two distinct dips, one at 540 nm and one at 576 nm, corresponding to the oxy-hemoglobin absorption spectrum [31]. The calculated coefficient of determination between the blood reflectance spectrum and the blood component-fit is very high: $r^2=0.996$. The spectrum of figure 2b has fewer features than figure 2a, because of the nature of the absorption spectrum of hemichrome, the main component of old blood.

The reflectance spectrum of all blood samples was obtained and all corresponding coefficients of determination were calculated. For the total of 1200 measurements we found $r^2=0.986\pm0.012$.

Three one-way ANOVA tests were performed to test on differences in sample, donor and ageing. The outcome of the tests show that at a 0.05 level, no significant difference is found among sample variation (F-value = 0.8014, prob >F = 0.8040) and donor variation (F-value = 0.8215, prob >F = 0.569). However, for ageing, a significant difference is found (F-value = 64.4, prob >F = 0). For both ages smaller than one day and ages between a day and a week $r^2=0.99\pm0.01$; for ages between a week and a month and older than one month $r^2=0.98\pm0.01$. This difference in r^2 -value between stains measured within a week and after a week of deposition is found significant at a 0.05 level.

Non-blood samples

The lower parts of figure 2 show two typical reflectance spectra of blood mimicking samples: ketchup (figure 2c) and lip-gloss (figure 2d). The agreement between the reflectance spectrum of ketchup (Albron; The Netherlands) and the blood-fit is poor, especially for $\lambda < 600$ nm, resulting in a relatively low coefficient of determination:

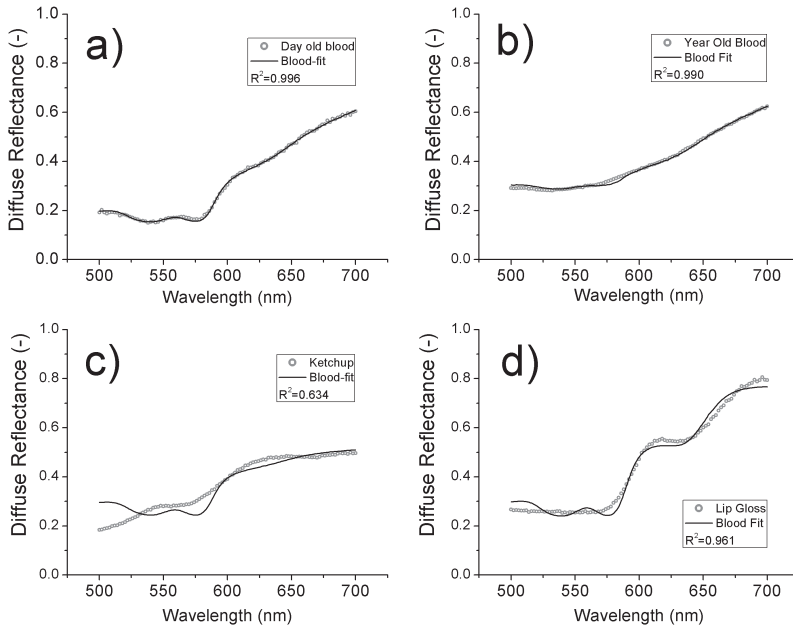


Figure 2. Diffuse reflectance signal (gray dots) with corresponding blood-fit $R(\lambda)/R_0(\lambda)$ (black line). Four typical measurements are shown: a) a blood sample of one day old with $r^2=0.996$; b) a blood sample of one year old with $r^2=0.990$; c) a non-blood sample with relative low correlation: ketchup, $r^2=0.634$; d) a non-blood sample with relative high correlation: lip gloss, $r^2=0.961$.

$r^2=0.634$. The agreement of the reflectance spectrum of lip-gloss (Brown Lipgloss, Etos; The Netherlands) and the blood-fit is relatively high: $r^2=0.961$. This high r^2 -value is because of a shouldered feature in the reflectance spectrum of Lip-gloss around $\lambda=630$ nm. This feature is also found in the absorption spectrum of met-hemoglobin, one of the main compounds present in bloodstains. However, for $\lambda<600$ nm the reflectance spectrum of lip-gloss differs distinctively from the blood-fit since the characteristic dips of oxy-hemoglobin around 540 nm and 576 nm are absent in the Lip-gloss reflectance. Nevertheless, this particular Lip-gloss scored the highest r^2 -value of all non-blood samples. For the total population of 35 non-blood samples $r^2=0.67\pm 0.21$ was found.

Coefficients of determination

Figure 3 shows the coefficients of determination of four typical bloodstains samples and all measured non-blood samples. The blood samples, colored in black in figure 3, have the following coefficients of determination: immediately after deposition, $r^2=0.997$; after one day: $r^2=0.996$; after one month: $r^2=0.982$ and after one year: $r^2=0.984$. The non-blood samples are colored in gray. The non-blood samples with highest r^2 are colored lip gloss: $r^2=0.961$ and red wine: $r^2=0.954$. Non-red body fluids, shown on the right side of figure 3, score low correlations, saliva: $r^2= 0.199$, semen: $r^2=0.476$ perspiration: $r^2=0.669$ and urine: $r^2= 0.381$. The patterned column on the far right resembles the measured r^2 of the case study, which will be discussed below in paragraph 4.

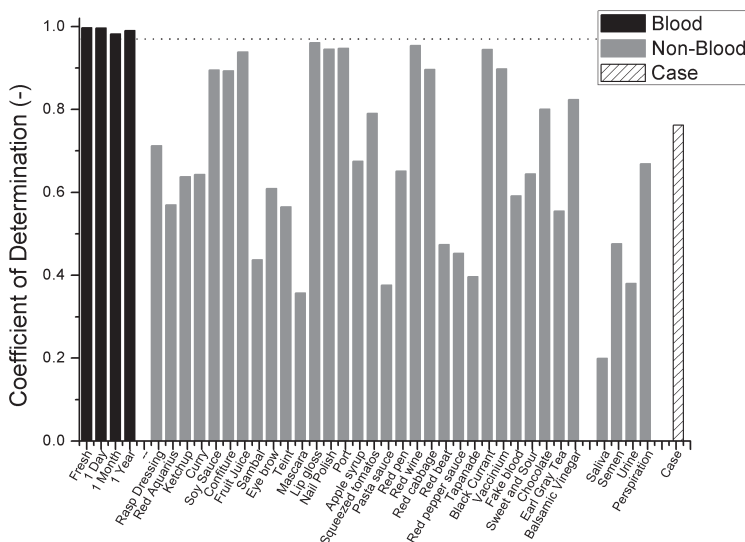


Figure 3. Column plot of coefficients of determination of four typical blood samples (black), all non-blood samples (gray) and case study (patterned).

Figure 4 plots the distribution of the obtained r^2 -values for all samples. The box plot shows the distribution of 25%, 50% and 75% of the samples, the whiskers show 1% and 99% of samples. The vertical axis of figures 4 plots 1- coefficient of determination on a logarithmic scale to enable visualization of the distribution in both blood and non-blood samples.

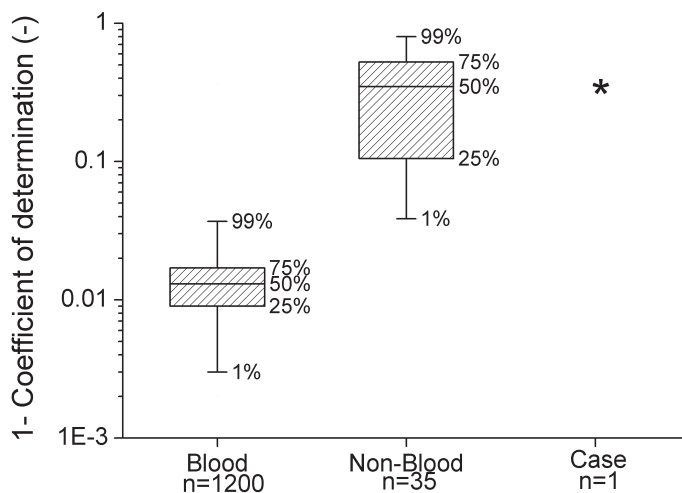


Figure 4. Box plot of the distribution of observed coefficients of determination of all blood measurements ($n=1200$) and all non-blood samples ($n=35$). On the right the outcome of the case example.

Case Example

The method presented in this paper was applied for investigational purposes in a case where someone was suspected of multiple burglary cases. The paint can found at one of the crime scenes contained latent traces of a fingerprint, possibly printed in blood. Confirmation or exoneration was crucial for the processing of this particular crime. Figure 5 shows a photograph of the object found at the crime scene. We obtained a diffuse reflectance spectrum of the object and found an r^2 -value of 0.672, indicative of a non-blood material.

To verify the optical identification for this case example, an additional tetrabase test [80] was performed on the same spot. Tetrabase tests are routinely used in forensic practice. According to the instructions, filter paper was swept onto the paint can. Thereafter the filter paper was treated with the tetrabase chemicals. The filter paper did not color up after deposition of the tetrabase, indicating no presence of blood on the paint can. The tetrabase test was performed twice, once with dry filter paper, and once with wet filter paper. The results of these tests are in agreement with the outcome of the spectroscopic identification.

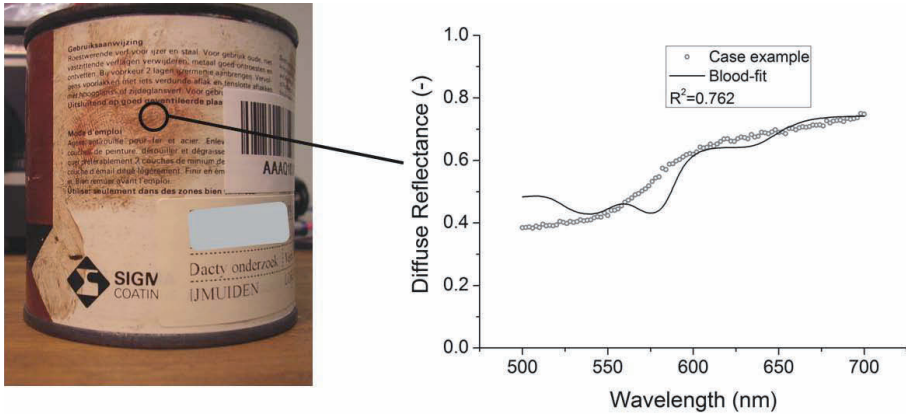


Figure 5, Left: Photograph of paint can of interest for blood test. The black line with open circle shows the spot where the reflectance spectrum is recorded. Right: Diffuse reflectance signal with corresponding linear least squares fit.

DISCUSSION AND CONCLUSION

We report on a remote blood identification test which is fast, non-destructive and applicable on crime scenes. We showed that discrimination between blood and non-blood samples is possible, based on the coefficient of determination between the sample's reflectance spectrum and a multi-component blood-fit. For blood samples $r^2=0.986\pm 0.012$ and for non-blood samples $r^2=0.67\pm 0.21$. Discrimination between blood and non-blood is possible by setting the threshold at $r^2=0.97$. No non-blood samples were reported with $r^2>0.97$ and only 1.9% of the blood samples were found to have $r^2<0.97$. Equally important, an ANOVA-test showed no significant differences between bloodstains from various samples or from various donors. A small significant difference was found among bloodstains, when tested on age of the bloodstains. Yet, these differences did not hamper discrimination between blood and non-blood samples.

For every sample the reflectance ratio of the sample and its background $R(\lambda)/R_0(\lambda)$ was analyzed, with typical examples shown in figure 2. To this, a reflectance spectrum of the background material, $R_0(\lambda)$ was obtained. All measurements in this study were performed on samples deposited on white cotton. Samples deposited on a non-white substrate require a more sophisticated optical sampling and analysis method. For application in forensic practice the influence of background color has to be evaluated. A possible suggestion to overcome the drawback of background color is the use of a hyper-spectral imaging system [63]. Spectral imaging allows imaging of the crime scene and chemical analysis of the imaged object. Although this approach is beyond the scope of this study, it is an interesting topic for future research.

Figure 3 shows the wide variation of non-blood samples investigated. The outcome of coefficients of determination of some samples is remarkable: for instance, visually well

mimicking samples like fake-blood (Vampire in a box, Running Press) scores a relatively low $r^2=0.59$, while lip gloss (Brown Lipgloss, Etos; The Netherlands) scores exceptionally high: $r^2=0.96$. The high correlation of Lip-gloss suggests room for improvement in the fitting procedure for future work. Figure 4 plots the distribution of all samples and enables differentiation between blood and non-blood samples. By setting the threshold at $r^2=0.97$, a specificity of 100% of the investigated samples is realized, combined with a sensitivity of 98%. A higher sensitivity can be obtained by lowering the threshold; however lowering the threshold will decrease the specificity as well.

In conclusion, we showed that discrimination between blood and non-blood sample on white cotton is possible with non-contact spectroscopy. The high sensitivity and specificity indicate that this optical test is close to confirmative. Our blood identification test was successfully applied in a forensic case example. However, before further forensic implementation, future research on colored substrates with various structures can be recommended.

APPENDIX

For analysis of the sample's reflectance, we used Kubelka Munk's theory of reflectance:

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

Here $R(\lambda)$ denotes the sample's reflectance, and $R_0(\lambda)$ is the reflectance of the white substrate. K and S represent the absorption and scattering coefficients. For a blood component fit we take K being the sum of the absorption spectra of oxy-hemoglobin, met-hemoglobin and hemichrome. Because these chromophores are packed in red blood cells, the absorption spectra are flattened. We corrected for this flattening effect, as described by Finlay and Foster[74], with an erythrocyte radius of 3 μm . For scattering S we assumed Lorentz-Mie behavior as function of wavelength λ : with scattering power of -0.4: $S=(\lambda/\lambda_0)^{-0.4}$, with $\lambda_0=450$ nm.