JOURNAL OF FORENSIC SCIENCES

J Forensic Sci, November 2011, Vol. 56, No. 6
doi: 10.1111/j.1556-4029.2011.01868.x
Available online at: onlinelibrary.wiley.com

PAPER

CRIMINALISTICS

Rolf H. Bremmer,[1] *Ph.D.; Gerda Edelman,*[1] *M.Sc.; Tessa Dijn Vegter*[1]*; Ted Bijvoets*[2]*; and Maurice C.G. Aalders,*[1] *Ph.D.*

Remote Spectroscopic Identification of Bloodstains*

ABSTRACT: Blood detection and identification at crime scenes are crucial for harvesting forensic evidence. Unfortunately, most tests for the identification of blood are destructive and time consuming. We present a fast and nondestructive identification test for blood, using noncontact reflectance spectroscopy. We fitted reflectance spectra of 40 bloodstains and 35 nonbloodstains deposited on white cotton with spectroscopic features of the main compounds of blood. Each bloodstain was measured 30 times to account for aging 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 nonblood 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.

KEYWORDS: forensic science, bloodstains, reflectance spectroscopy, presumptive, confirmatory test

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 (1,2). Thus, there is a need for techniques that allow for remote, noncontact identification of evidence. Many screening tests, routinely used in forensic practice, use chemical methods for the identification of blood to discriminate it from other body fluids or red substances. Most chemical tests, including tetrabase (3) and Kastle–Meyer (4), 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 (5). This test is especially appropriate for indicating bloodstains after cleaning attempts. Most of these tests are presumptive in nature, not confirmative, because several other substances are reported to catalyze this peroxidase reaction (6). More reliable, confirmatory tests are based on hemoglobin derivative crystals (7,8) or RNA markers in blood (9). 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 (10); it fluoresces at 465 nm, when excited at 321 nm (11), and heme provides Raman bands at 1.222 and 1.542 cm^{-1} (12). All these corresponding techniques have the potential to allow *on field* identification of blood based on the specific spectroscopic features of hemoglobin (12,13). 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 and Grieve (14) in a microspectrophotometry setup; De Wael et al. (13) recently confirmed these findings. Their approach, however, requires advanced sample preparation and a laboratory environment for accurate measurements.

We propose a nondestructive and noncontact 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 (15). Recently, we showed that fitting the absorption spectra of the hemoglobin compounds to the measured reflectance spectrum allows the determination of the compound fraction estimation of the age of a bloodstain (16). 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

[1]Department of Biomedical Engineering and Physics, Academic Medical Center/University of Amsterdam, Meibergdreef 9, 1109 AZ, Amsterdam, The Netherlands.

[2]Forensic Investigation Department Regional Police Kennemerland, Tiberiusplein 1, IJmuiden, Velsen, The Netherlands.

*Funded in part by a personal grant in the *Vernieuwingsimpuls* program by the Netherlands Organization of Scientific Research (NWO) and the Technology Foundation STW.

Received 21 April 2010; and in revised form 3 Aug. 2010; accepted 10 Oct. 2010.

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 Methods

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 have taken 1200 measurements on the 40 blood samples; 30 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 nonblood samples. Among the samples are 31 samples visually mimicking blood, including ketchup, red wine, and fake blood; 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.

Noncontact Reflectance Spectroscopy

Reflectance measurements were taken with a combination of a spectrograph (USB 4000; Ocean Optics, Duiven, the Netherlands), a tungsten–halogen light source (H-2000; Ocean Optics), and a noncontact probe (QR400-7-UV/BX; Ocean Optics). This probe contains six 400-µm-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 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), $R_0(\lambda)$, were obtained.

Analysis of Reflectance Spectrum

The reflectance ratio of the sample and its background $R(\lambda)/R_0(\lambda)$ was analyzed with a multicomponent linear least squares fit. The absorption spectra of the three compounds present in blood, oxy-hemoglobin, met hemoglobin, and hemichrome (15), 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.

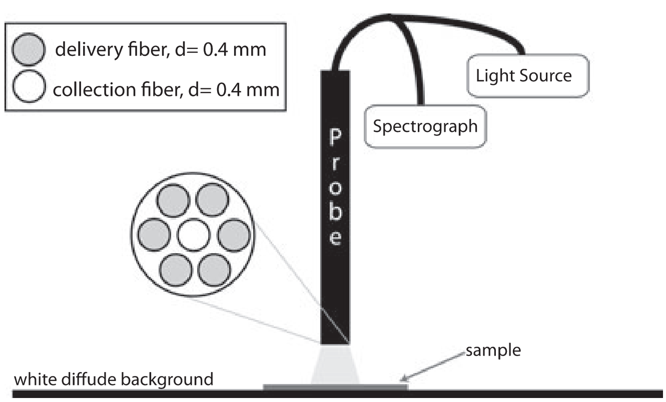

FIG. 1—*Schematic of the measurement setup. The reflection probe tip shows six delivery fibers, circulated around a central collection fiber. The delivery fiber is connected to the light source, and the collection fibers are connected to the spectrograph.*

Coefficient of Determination

The Pearson correlation coefficient gives information about the goodness of fit between the measured reflectance spectrum and the multicomponent blood-fit (17). The coefficient of determination, R^2, is the square of Pearson 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 nonblood samples.

Analysis of Variance Test

One-way analysis of variance (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 aging. For aging 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 Fig. 2 shows two typical diffuse reflectance spectra of blood samples: of 1 day old (Fig. 2*a*) and a year old (Fig. 2*b*). The spectrum in Fig. 2*a* shows two distinct dips, one at 540 nm and one at 576 nm, corresponding to the oxy-hemoglobin absorption spectrum (10). 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 Fig. 2*b* has fewer features than Fig. 2*a*, 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 $R^2 = 0.986 \pm 0.012$.

Three one-way ANOVA tests were performed to test on differences in sample, donor, and aging. The outcome of the tests shows that at a 0.05 level, no significant difference is found between sample variation (F-value = 0.8014, prob > F = 0.8040) and donor variation (F-value = 0.8215, prob > F = 0.569). However, for aging, a significant difference is found (F-value = 64.4, prob > F = 0). For both ages smaller than 1 day and ages between a day and a week, $R^2 = 0.99 \pm 0.01$; for ages between a week and a month and older than 1 month, $R^2 = 0.98 \pm 0.01$. 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.

Nonblood Samples

The lower parts of Fig. 2 show two typical reflectance spectra of blood-mimicking samples: ketchup (Fig. 2*c*) and lip gloss (Fig. 2*d*). The agreement between the reflectance spectrum of ketchup (Albron, De Meern, the Netherlands) and the blood-fit is poor,

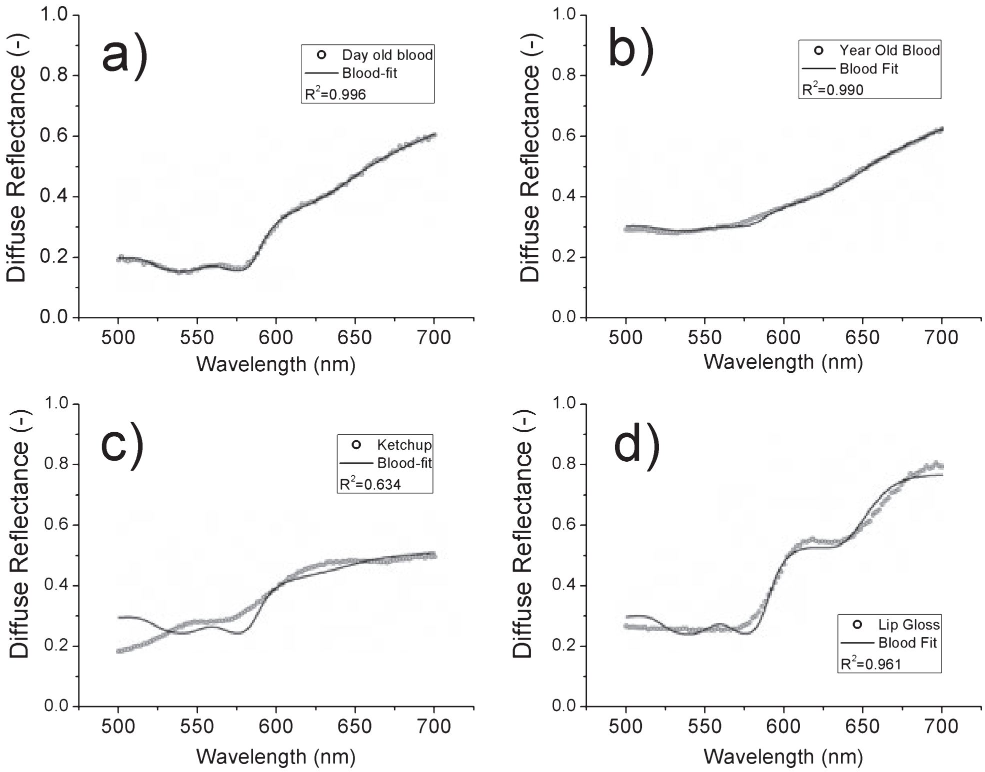

FIG. 2—*Diffuse reflectance signal (gray dots) with corresponding blood-fit* $R(\lambda)/R_0(\lambda)$ *(black line). Four typical measurements are shown: (a) a blood sample of 1 day old with* $R^2 = 0.996$*; (b) a blood sample of 1 year old with* $R^2 = 0.990$*; (c) a nonblood sample with relative low correlation: ketchup,* $R^2 = 0.634$*; (d) a nonblood sample with relative high correlation: lip gloss,* $R^2 = 0.961$*.*

especially for $\lambda < 600$ nm, resulting in a relatively low coefficient of determination: $R^2 = 0.634$. The agreement of the reflectance spectrum of lip gloss (Brown Lipgloss; Etos, Zaandam 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 because 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 nonblood samples. For the total population of 35 nonblood samples, $R^2 = 0.67 \pm 0.21$ was found.

Coefficients of Determination

Figure 3 shows the coefficients of determination of four typical bloodstain samples and all measured nonblood samples. The blood samples, colored in black in Fig. 3, have the following coefficients of determination: immediately after deposition: $R^2 = 0.997$; after 1 day: $R^2 = 0.996$; after 1 month: $R^2 = 0.982$, and after 1 year: $R^2 = 0.984$. The nonblood samples are colored in gray. The nonblood samples with highest R^2 are colored lip gloss: $R^2 = 0.961$ and red wine: $R^2 = 0.954$. Nonred body fluids, shown on the right side of Fig. 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.

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, and the whiskers show 1% and 99% of samples. The vertical axis of Fig. 4 plots 1-coefficient of determination on a logarithmic scale to enable visualization of the distribution in both blood and nonblood samples.

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 nonblood material.

To verify the optical identification for this case example, an additional tetrabase test (3) 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.

Discussion and Conclusion

We report on a remote blood identification test that is fast, nondestructive, and applicable on crime scenes. We showed that

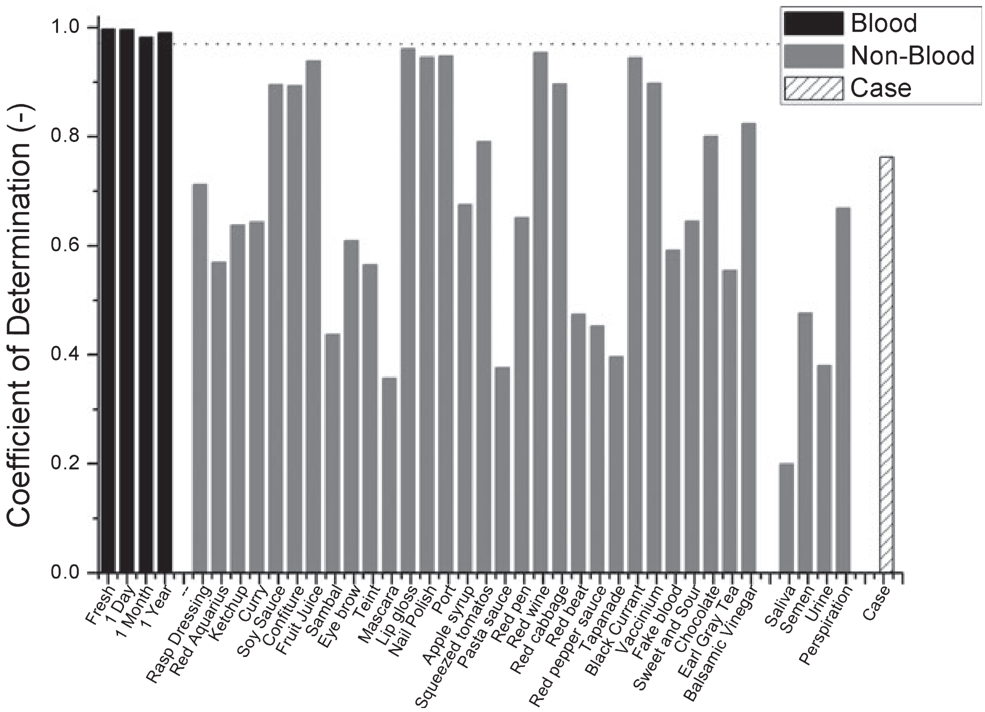

FIG. 3—*Column plot of coefficients of determination of four typical blood samples (black), all nonblood samples (gray), and case study (patterned).*

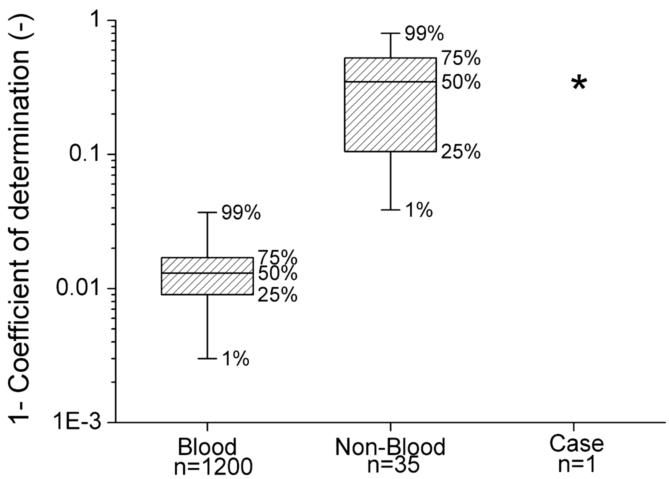

FIG. 4—*Box plot of the distribution of observed coefficients of determination of all blood measurements* (n = *1200) and all nonblood samples* (n = *35). On the right, the outcome of the case example.*

discrimination between blood and nonblood samples is possible, based on the coefficient of determination between the sample's reflectance spectrum and a multicomponent blood-fit. For blood samples, $R^2 = 0.986 \pm 0.012$, and for nonblood samples, $R^2 = 0.67 \pm 0.21$. Discrimination between blood and nonblood is possible by setting the threshold at $R^2 = 0.97$. No nonblood 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 between bloodstains, when tested on age of the bloodstains. Yet, these differences did not hamper discrimination between blood and nonblood 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 Fig. 2. To this, a reflectance spectrum of the background material, $R_0(\lambda)$, was obtained. All measurements in this study were taken on samples deposited on white cotton. Samples deposited on a nonwhite 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 hyperspectral imaging system (18). 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 nonblood 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, New York, NY) score a relatively low: $R^2 = 0.59$, while lip gloss (Brown lipgloss; Etos) 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 nonblood 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 nonblood sample on white cotton is possible with noncontact 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.

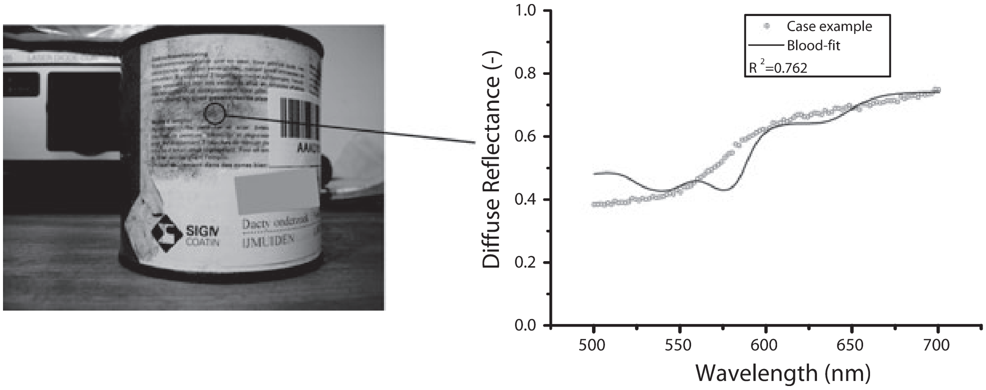

FIG. 5—*Left: Photograph of paint can of interest for blood test. The black line with open circle shows the spot where the reflectance spectrum is recorded. Right: Diffuse reflectance signal with corresponding linear least squares fit.*

Acknowledgments

We would like to thank M.J.C. van Gemert for constructive review of the manuscript. In addition, we would like to thank I.B. Alberink from the Netherlands Forensic Institute for valuable discussions on the statistical interpretation of the bloodstain analysis.

References

1. U.S. Department of Justice. Crime scene investigation: a guide for law enforcement. Washington, DC: U.S. Deparment of Justice, 2000.
2. Virkler K, Lednev IK. Analysis of body fluids for forensic purposes: from laboratory testing to non-destructive rapid confirmatory identification at a crime scene. Forensic Sci Int 2009;188(1–3):1–17.
3. Lomholt B, Keiding N. Tetrabase, an alternative to benzidine and orthotolidine for detection of hemoglobin in urine. Lancet 1977;1(8011): 608–9.
4. Johnston E, Ames CE, Dagnall KE, Foster J, Daniel BE. Comparison of presumptive blood test kits including Hexagon OBTI. J Forensic Sci 2008;53(3):687–9.
5. Blum LJ, Esperança P, Rocquefelte S. A new high-performance reagent and procedure for latent blood stain detection based on luminol chemiluminescence. Can Soc Forensic Sci J 2006;39(3):20.
6. Kent EJM, Elliot DA, Miskelly GM. Inhibition of bleach-induced luminol chemiluminescence. J Forensic Sci 2003;48(1):64–7.
7. James SH, Nordby JJ, editors. Forensic science. An introduction to scientific and investigative techniques, 2nd edn. Boca Raton, FL: Taylor & Francis, 2005.
8. Saferstein R. Criminalistics—an introduction to forensic science, 2nd edn. Englewood Cliffs, NJ: Prentice Hall, Inc., 1981.
9. Zubakov D, Hanekamp E, Kokshoorn M, van Ijcken W, Kayser M. Stable RNA markers for identification of blood and saliva stains revealed from whole genome expression analysis of time-wise degraded samples. Int J Legal Med 2008;122(2):135–42.
10. Zijlstra WG, Buursma A, Meeuwsen-Van der Roest WP. Absorption spectra of human fetal and adult oxyhemoglobin, de-oxyhemoglobin, carboxyhemoglobin, and methemoglobin. Clin Chem 1991;37(9): 1633–8.
11. Nagababu E, Rifkind JM. Formation of fluorescent heme degradation products during the oxidation of hemoglobin by hydrogen peroxide. Biochem Biophys Res Commun 1998;247(3):592–6.
12. Virkler K, Lednev IK. Raman spectroscopic signature of blood and its potential application to forensic body fluid identification. Anal Bioanal Chem 2010;396(1):525–34.
13. De Wael K, Lepot L, Gason F, Gilbert B. In search of blood—detection of minute particles using spectroscopic methods. Forensic Sci Int 2008; 180(1):37–42.
14. Kotowski TM, Grieve MC. The use of microspectrophotometry to characterize microscopic amounts of blood. J Forensic Sci 1986;31(3): 1079–85.
15. Marrone A, Ballantyne J. Changes in dry state hemoglobin over time do not increase the potential for oxidative DNA damage in dried blood. PLoS One 2009;4(4):e5110.
16. Bremmer RH, Nadort A, Van Gemert MJC, Van Leeuwen TG, Aalders MCG. Age estimation of blood stains by hemoglobin derivative determination using reflection spectroscopy. Forensic Sci Int 2011;206(1–3): 166–71.
17. Nagelkerke NJD. A note on a general definition on the coefficient of determination. Biometrika 1991;78(3):691–2.
18. Exline DL, Wallace C, Roux C, Lennard CI, Nelson MP, Treado PJ. Forensic applications of chemical imaging: latent fingerprint detection using visible absorption and luminescence. J Forensic Sci 2003;48(5): 1047–53.
19. Finlay JC, Foster TH. Effect of pigment packaging on diffuse reflectance spectroscopy of samples containing red blood cells. Opt Lett 2004;29(9):965–7.

Additional information and reprint requests:
Rolf Bremmer, Ph.D.
Department of Biomedical Engineering and Physics
Academic Medical Center/University of Amsterdam
Meibergdreef 9, 1109 AZ Amsterdam
The Netherlands
E-mail: r.h.bremmer@amc.uva.nl

Appendix

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

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

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 (19), 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.