Chapter 7
Investigating the Age of Blood Traces: How Close Are We to Finding the Holy Grail of Forensic Science?

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Abstract Blood traces found at crime scenes often comprise pivotal information regarding the events and individuals associated with the crime. Nowadays, even minute amounts of blood allow retrieval of a whole host of such 'profiling' information: e.g. diet, life style, age, gender. However, establishing any forensic value of such traces necessitates a veritable connection to a crime. The age of a blood trace, i.e. the time of its deposition, is crucial in this effort. This far-reaching forensic implication as well as the lack of currently validated and accepted trace dating methods, render blood stain age estimation the *holy grail* of forensic science. In its pursuit, several methods which determine the time since deposition of blood traces by probing different aspects of the trace degradation process have been proposed and explored. The present chapter collates and discusses current research investigating some of these blood trace ageing methods and their practical application in three categories. The first category comprises techniques which require trace sampling and consume these samples in their entirety during the analysis process. Similarly, the techniques in the second category require sampling of the blood trace but leave the sample intact for further analysis. Lastly, the third group of methods requires neither sampling nor contact. This, in turn, allows in situ analysis of the trace in question. The following operational aspects pertaining to these three categories are discussed in more detail: (i) required sample preparation, (ii) practical implementation and (iii) necessary operational skills. These aspects largely determine the suitability for forensic practice. Technology maturity (i.e. practical applicability) is quantified using the Technology Readiness Levels (TRL) as defined by the NASA/Airspace systems.

7.1 Introduction

Blood traces found at crime scenes can contain a wealth of information regarding the events and individuals associated with the crime. Recent studies have significantly

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diversified the accessible types of this 'profiling' information, retrievable from even minute amounts of blood: e.g. diet, life style, age, gender. Prior to such analysis, the blood traces must be localised, identified and chemically analysed, preferably directly at the crime scene, to aid the selection of relevant traces for further laboratory based processing. Accordingly, detection and identification of blood traces represent the first challenges to blood trace-based donor profiling. Moreover, depending on their underlying principle, the methods employed to this end can have implications for ensuing trace analyses.

Currently, the detection and identification of biological evidence both rely on visual inspection by the crime scene investigators (CSI), sometimes with the aid of forensic lights (coloured light sources) to enhance or create contrast between a presumed trace and its substrate. Once a trace is found, it is subjected to one or more presumptive tests for specific biological fluids (e.g. blood, semen, saliva). The most common screening test for blood utilizes a catalytic reaction inducing chemiluminescence (faint glow) or a colour change in the presence of blood as described in the previous chapter. Recently, immunological tests capable of identifying human blood-specific proteins have also become available. These immunological tests, however, require sampling and consume material. Following a positive presumptive test at the crime scene, the trace is sampled for a confirmatory test at the forensic laboratory. This investigation protocol is labour-intensive and carries the double risk of sampling redundant or irrelevant traces while missing relevant traces altogether. Furthermore, it isolates the sampled traces from their respective contextual information.

Blood traces sampled, processed and analysed in this way may contain crucial information regarding the crime; however, in order for a trace to have any forensic value, the determination of a veritable connection to a crime is imperative. The time of deposition, calculated from the 'age of the bloodstain', is an essential parameter in this effort. Furthermore, if bloodstains are clearly crime-related, their age may reveal the time of the crime or even the sequence of events. Owing to this high forensic value and the lack of currently validated and accepted trace dating methods, blood stain age estimation is still considered the *holy grail* of forensic science.

In its pursuit, many techniques have been proposed which probe the blood degradation process in order to determine the age of blood traces. Ideally, such methods should measure a (preferably) donor-independent and predictive (i.e. time-dependent) quantity in situ with known responses to environmental factors such as temperature and humidity. Moreover, the embedding of such a technique within the routine crime scene processing work-flow necessitates compliance with the following field-specific requirements: equipment portability, sample preservation and the operational skills of the CSI. When in situ trace analysis is not possible, techniques which require trace sampling, e.g. for measurements under controlled conditions, but preserve the sample for further processing can be employed. Lastly, destructive methods can be used which not only require trace sampling, but also consume the sample. While this is the least preferable approach, the high forensic value of blood trace ages would usually outweigh the disadvantages of such destructive techniques.

A survey of available literature reveals that many methods lack practical implementation, despite their reported success in the laboratory. The successful translation

from lab technology to field application is not only determined by the aforementioned operational constraints, the robustness of the technique and admissibility in court but also by the possibilities for commercialization.

This chapter aims to provide an overview of current research investigating blood trace ageing methods and their practical application. The research discussed here will be categorized into three main groups: the first category requires sampling and consumes the sample in its entirety during the chemical processing; the second group of techniques requires sampling of the blood trace but preserves the sample for further analysis; the third group requires neither sampling nor contact and therefore completely preserves the trace in its original context allowing in situ analysis of the trace in question. Within these three categories the following aspects will be discussed for each group of techniques: (i) necessary sample preparation, (ii) practical implementation and (iii) required operational skills. These aspects largely determine the acceptance/applicability in forensic practice. Technology maturity (i.e. practical applicability) will be quantified using the Technology Readiness Levels (TRL) as defined by the NASA/Airspace systems. The scale is depicted in detail in Fig. 7.1 and comprises 9 levels, where TRL 9 denotes a fully validated and launched method.

7.2 Lab-Based Techniques

7.2.1 Invasive and Sample Consuming

7.2.1.1 DNA/RNA Degradation

DNA extracted from biological stains can be used to identify the trace donor; this information is commonly used to establish links between the perpetrator, the victim and the scene of a crime. Additionally, the ex vivo degradation of DNA/RNA can be used as a measure of the trace age. The feasibility of this approach has been explored by several research groups. In 2005, Anderson et al. used real-time reverse transcriptase PCR to show that, over the course of 150 days, the ratio of different types of RNA (mRNA versus rRNA) exhibits a linear relationship with trace age [1, 2]. To this end, 10 µl bloodstains were dissolved and consumed in the process, and the authors showed that the possibility to simultaneously extract DNA and RNA is beneficial for further processing. The influence of environmental factors as well as donor gender, age and ethnicity had yet to be studied. In a follow-up study 6 years later, the same group utilized this technique in conjunction with multivariate analysis of the trace age-related ratio of different RNA segments [3]. This approach allowed robust distinction between three blood trace age categories: (i) fresh; (ii) 6 days; (iii) 30 days or more. Some of the 30-day-old samples could be distinguished from 90-day-old samples, while others could not. More recently, in 2012, Simard et al. demonstrated the feasibility of RNA marker degradation-based blood stain age estimation [4]. Fur-

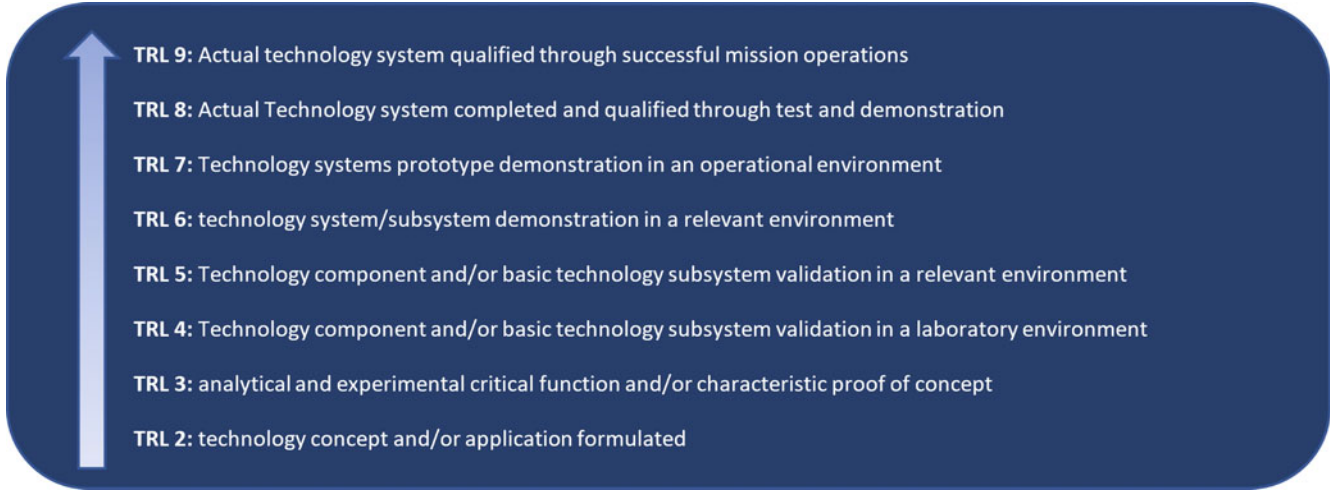

Fig. 7.1 Technology readiness levels (TRL) as defined by the NASA/Airspace systems. This scale is used throughout the chapter to classify the practical applicability of the discussed methods

thermore, the authors found ratios to be ineffective age measures due to the similar decay kinetics of the various markers, rendering such ratios time-independent. This study further showed that sample storage at −80 °C prevented sample degradation. The effects of other storage and environmental conditions were not studied. In 2013, donor gender was found to have a significant influence on the method proposed by Anderson et al., necessitating further research [5]. More recently, in 2017, Alshehhi et al. studied the degradation of three mRNA markers (HBA, PBGD, HBB) and two microRNA markers (miR16, miR451), along with three reference genes (18S rRNA, ACTB mRNA, U6 snRNA) as a blood trace age measure using blood samples from 10 donors [6]. While potential feasibility was suggested, no follow up has been published to date. Finally, Mohammed et al. investigated mRNA levels as a measure for bloodstain ages up to 55 days [7]. More specifically, Caspase-1 and Caspase-3 showed a tendency to decrease over time. All studies discussed here remained within the lab validation phase and revealed ex vivo RNA degradation to be less well understood than its in vivo counterpart. At the time of editing this chapter, no further lab or field validation studies were available, resulting in a TRL score of 3 or 4 for DNA/RNA-based blood stain age estimation. However, potential future introduction of these methods in forensic practice would be facilitated by the existing DNA/RNA facilities and experts in forensic laboratories.

7.2.1.2 Haemoglobin Degradation

Electron paramagnetic resonance (EPR), also called electron spin resonance, studies chemical species (especially metal complexes and organic radicals) with unpaired electrons. Contrary to the more common Nuclear Magnetic Resonance (NMR) spectroscopy, which measures nuclear spin, EPR probes electron spin. EPR is typically performed by placing the sample in a magnetic field, the strength of which is varied, while microwaves of a fixed frequency in the GHz range serve as probe. The studied effects within EPR samples are very sensitive to local environments and, because of the short relaxation times, often necessitate very low measurement temperatures [8]. Miki et al. and Sakurai et al., both assessed the feasibility of EPR-based blood trace dating, following the hypothesis that the spin configuration of the iron atom within the haemoglobin molecule will change over time [9, 10]. They found that the logarithm of the ratio of two specific spin states and the logarithm of sample age exhibited a linear correlation for up to 432 days with an error range within 25%. While this method has to be performed under controlled laboratory conditions, advantageously, the blood trace can be dry sampled, i.e. ‘scraped off the surface’, and transported to the laboratory. The researchers stated that environmental factors such as differences in light exposure and fluctuations of storage temperature did have an influence on the changes of the EPR-active compounds, but these factors were not further investigated. EPR devices are not standard inventory at forensic laboratories and the measurements require highly specialised personnel. Together, these conclusions and the lack of continued method development classifies the technique at TRL 4.

7.2.1.3 Biomolecule Degradation

In 1997, Andrasko investigated the feasibility of using High-Performance Liquid Chromatography (HPLC) for age estimation of bloodstains deposited on clothing [11]. HPLC is a method of separating, identifying and quantifying each chemical component in complex mixtures. To this end, the sample is injected into a flowing solvent and both will be "pushed through" the stationary phase of a separation column. Separation is achieved by specific chemical or physical interactions of the sample with the stationary phase: different chemical components in the sample travel through the column at different velocities and hence exit the column at different times, at which point they will be detected using various means e.g. using their light scattering, absorption or fluorescent properties. Already in 1992, the technique was explored by Inoue et al., who found an 'unidentified decomposition peak', potentially useful for ageing stains [12]. Andrasko studied several other ageing processes and found some suitable for the estimation of the age of blood stains [11]. Notably, and of considerable practical importance, the ratio of the different peaks formed by the ageing process is practically independent of environmental temperatures between 0 and 37 °C. Despite these promising results, the associated costs and required operational skill levels may hamper integration of HPLC into routine forensic practice. With a current TRL of 4, additional research is necessary to advance this technique towards practical implementation.

7.2.1.4 Amino Acid Conversion

Another interesting approach is based on the aspartic acid racemization (AAR) rate in ageing bloodstains. AAR is a common process during the natural ageing of proteins and therefore used in medical applications to assess protein ageing. Although this destructive method is established for the dating of other (slow turnover) tissues, such as tooth tissue and fossils, only one author [13] applied the technique to aging bloodstains. The procedure involves dissolving the present amino acids, followed by a centrifugation step and subsequent gas chromatography (GC) analysis. Initial experiments at various temperatures were successful and revealed a linear relationship between D-aspartic acid (D-Asx) content and age. However, the relationship was also found to be highly sensitive to the environmental temperature. As only a few stains were measured and no follow-up research has been published to date, this approach currently remains at TRL 3.

7.2.1.5 Metabolomics

All methods described in this chapter aim to determine the absolute time of deposition. The temporal resolution can be increased further by determining both the moment of deposition as well as the time of deposition within the circadian (24 h) night/day cycle. The idea to use circadian biomarkers present in the blood was

explored by Kayser et al. in 2010 [14]. Two circadian hormones were found suitable for trace deposition time estimation and in 2016 the same group demonstrated that messenger RNA (mRNA) biomarkers significantly improve the prediction accuracy [15]. This idea was extended in 2018 when the suitability of metabolites, measured using a targeted metabolomics approach, were explored as potential measure for trace age. An integrated approach, using time prediction modelling in conjunction with a combination of these metabolites and the hormone and mRNA biomarkers identified in the previous studies, achieved an improved prediction accuracy reaching AUCs of 0.85, 0.89 and 0.96 respectively [16]. The authors believe that this approach currently is at TRL 3.

Advantages and Limitations Most of the proposed blood trace dating approaches discussed in this section employ analytical chemistry methods. Accordingly, the necessary equipment is generally located in a chemical laboratory, usually expensive and must be operated by specialized personnel. The required sampling and processing steps entail the following risks: trace contamination, material loss, and change of the chemical composition of the sample. Operational risks and practical constraints notwithstanding, such lab-based devices usually outperform most portable techniques in terms of stability, reliability and data quality. Moreover, the required equipment is usually part of the standard inventory in a forensic lab. Lastly, some of these laboratory techniques are slowly progressing towards mobile applications, however, usually at the cost of robustness and sensitivity.

7.2.2 Invasive but Sample Preserving Techniques

7.2.2.1 Exploiting Biomolecule Degradation

Raman spectroscopy is an optical technique based on inelastic (so-called Raman) scattering of light, a process in which photons lose or gain energy upon interactions with vibrating molecules. This, in turn, provides chemical 'fingerprints' of cells, tissues or biological fluids and yields detailed information about the chemical structure of the present (bio)molecules. Consequently, Raman spectroscopy has been explored extensively in the medical field and, more recently, also for forensic applications. To measure a Raman spectrum, the sample has to be illuminated with a light source providing a stable output, usually a diode laser. The optimal wavelength depends on the material under investigation and is a trade-off between strong Raman scattering signals at short (UV-Vis) wavelengths at the cost of more unspecific background (fluorescence) signal, or less disturbance from the background signal at longer wavelengths at the cost of lower Raman signals. The four most widely used wavelengths are 405, 532, 785 and 1064 nm. Recording such Raman signals requires a sensitive spectrograph, which makes up most of the relatively high costs of Raman systems. Many application papers show measurements of pure components on a non-fluorescent, and non-Raman scattering substrate such as aluminium foil. Translational investigations

therefore often rely on sampling and purification. For blood trace age estimation, blood was mostly deposited on aluminium foil-covered microscope slides.

In 2016 and 2017, the Lednev group demonstrated the feasibility of using Raman spectroscopy for blood stain age estimation over a time range of 200 days [17, 18]. Time since deposition predictions were performed employing two statistical regression algorithms: partial least squares regression (PLSR) and principal component regression (PCR). Error analysis revealed an overall accuracy of ~70% for the models to correctly predict the time since deposition at each time point. Besides trace dating, the technique also enables discrimination of human and non-human blood traces [19]. Taken together, these findings indicate that translation of this approach from the lab to forensic practice still requires major technological developments, rendering Raman-based blood stain age estimation TRL 5.

7.2.2.2 Exploiting Structural Conversion (Sample Elasticity)

Atomic force microscopy (AFM) is a technique capable of providing elasticity measurements of red blood cells. This, in turn, can be used to monitor time-related changes in the elasticity of red blood cells in ageing blood traces. Unlike Raman spectroscopy, AFM measurements are not sensitive to substrate interference; however, since AFM measures minute displacements, it is extremely sensitive to vibrations. As a result, AFM measurements have to be performed in a vibration-free environment rendering the development of portable systems challenging. While recent developments show a trend towards increased ease of use and portability, a high level of operational expertise is still required for data acquisition and interpretation. AFM-based bloodstain age estimation was explored by Strasser et al. [20] and Smijs et al. [21] with results indicating potential feasibility. Both groups reported an age-related decrease in red blood cell elasticity, caused by drying and coagulation processes. Dating of bloodstains was possible for up to 8 days with a reasonable accuracy at days 6 and 7. Despite these promising results, many possibly influential factors remain to be investigated before moving to the field validation phase, rendering this approach TRL 4.

Advantages and Limitations Similar to the previous section, the techniques discussed in this section require sampling. However, advantageously, they do not consume or alter the sample allowing subsequent complimentary analyses. Reasons for sampling and transportation of the sample include substrate interference or the necessity to dissolve the blood for the measurement. While the sample preservation allows ensuing additional analyses, the sampling requirement introduces trace contamination risks. Furthermore, sampling may cause a change in chemical properties such as the relative amounts of haemoglobin derivatives. This problem is frequently overlooked, as liquid blood or even dissolved blood samples represent the analytical starting point in some publications, entirely negating any adverse sampling effects. These limitations notwithstanding, some of the techniques presented in this section are potentially portable and could be used in field work, e.g. Raman spectroscopy.

7.3 Portable and Non-invasive Techniques

This section describes the most recent technological developments aimed at providing blood trace age estimates in an entirely non-contact fashion, i.e. in situ and without the need to sample. Such methods are extremely sought after as they preserve the original trace context; prevent trace contamination and destruction, while potentially reducing the required operational skill level and workload by the same token.

Experimentally, all approaches discussed here utilize visible and near-infrared light to probe and monitor the chemical composition of ageing blood traces. The employed data acquisition tools vary broadly in sophistication: from ubiquitous smartphone cameras to portable hyperspectral imaging systems and lab-grade spectrometers. Similarly, data processing strategies range from statistical classification techniques to advanced physical models describing the light-sample interaction. Consequently, some of the associated advantages and limitations are strategy-specific and need to be discussed and weighed separately. In general, non-contact methods employing visible light all probe the degradation state of one of the main constituents of blood, the oxygen-transporting protein haemoglobin. The degradation of this protein is associated with a visible change in blood stain colour (Fig. 7.2), from bright red to dark brown.

Upon contact with air, haemoglobin immediately binds oxygen molecules; this fully oxygenated form of haemoglobin is referred to as oxyhaemoglobin (HbO_2) and is responsible for the bright red colour of fresh blood. Due to the continued exposure to air, HbO_2 subsequently converts (auto-oxidizes) to a molecule known as methaemoglobin (metHb). As a result, the colour of the blood stain changes to a darker, brownish red. Finally, the visible ageing process of haemoglobin is completed by the degradation (denaturation) of metHb to hemichrome (HC), resulting in a dark brown appearance of the blood stain. Techniques which are capable of objectively quantifying such colour changes, i.e. spectroscopic techniques, are therefore, in theory, ideally suited to probing this ageing process.

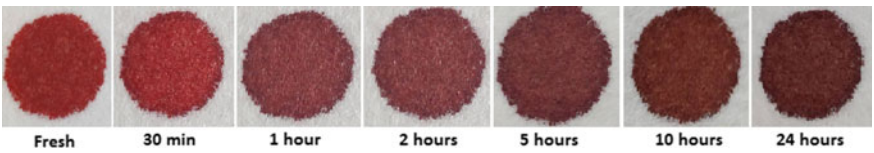

Fig. 7.2 Colour change observed in ageing blood stains

7.3.1 Low-Cost Approaches

7.3.1.1 Smartphones

Some of the most recent investigations into optical blood stain age estimation are geared towards the use of smartphones for both data collection and analysis. If successful, such techniques would be conceivably cost-efficient by lowering the required operational expertise, workload and equipment cost. Consequently, these approaches are particularly attractive in low resource settings.

Recently, two studies explored the feasibility of smartphone-based age estimation of blood stains. The first one [22] utilized digital colour images recorded with a smartphone camera in conjunction with a statistical classification (machine learning) algorithm. All pixels of the recorded digital images comprise information about *three* primary colours: red (R), green (G) and blue (B). These values can be used to monitor the expected change in blood stain colour over time. In this study, however, the recorded RGB values were first transformed to intensities in a complimentary colour model, the four colour (CMYK) model as these exhibited a stronger correlation with blood stain age. The CMYK model is primarily used in the printing industry and represents all visible colours as a combination of *four* primary colours (cyan, magenta, yellow and black) instead of the previously mentioned three. Following the transformation, a so-called Random Forest machine learning algorithm was applied to estimate the age of the blood stains. This approach allowed 100% accurate age estimation for blood stains deposited on filter paper for several time periods between 1 h and 42 days under controlled environmental conditions. For blood stains deposited on six light-coloured common household substrates, the correct classification rate dropped to 83%. In this case, the age estimation task was limited to three categories: younger than one day; between one day and one week old; older than one week. Interestingly, in both cases, the classification algorithm performed best for the youngest and the oldest stains.

The approach pursued in the second, and more recent, of the two studies also employed a combination of digital images recorded using a smartphone and a colour value transformation [23]. Here, RGB values were mapped to brightness values by assigning every RGB triplet the minimum of its three values. This colour value transformation seems to reduce the sensitivity to the visible ageing process, as changes in the brightness value were limited to ages between 0 and 42h.

Advantages and Limitations Clear advantages of smartphone-based methods are that, in principle, they are fast, simple and cheap and require minimal training of the end user. Despite some promising results, however, considerable challenges remain. The perhaps most obvious limitation is that of substrate colour. Dark and otherwise coloured substrates mask age-related variation in the measured colour values, eroding any age estimation potential. Moreover, the experimental set-up of the first study included a purpose-built white foam box containing the sample to be imaged. Imaging of the blood stains was carried out through a small hole at top of the box, presumably to achieve homogenous illumination conditions. This requirement may hamper the

application of this method in forensic field work. Furthermore, the used statistical classification model is device specific and therefore has to be rebuilt specifically for every smartphone. Lastly, the conversion to CMYK values seems to introduce a sensitivity to sample light exposure, as the magenta values measured on blood stains exposed to natural sunlight differed significantly from those of blood stains of the same age not exposed to sunlight. Together, these aspects classify smartphone-based blood stain age estimation as TRL 4.

7.3.2 Portable Lab-Grade Equipment

7.3.2.1 Reflectance Spectroscopy

As mentioned earlier, the colour change observed in ageing blood stains is associated with the successive formation of three haemoglobin derivatives: HbO_2, metHb and HC. Figure 7.3 shows the light absorbing properties of these three compounds as a function of wavelength, i.e. light colour, in the range of 500 nm (green light) to 800 nm (near-infrared light). Light absorption and reflection are natural inverses of each other as increased absorption at a certain wavelength decreases the amount of light available for reflection. There are pronounced differences in these three wavelength-dependent absorption profiles and hence also in the colour of the three haemoglobin derivatives. Furthermore, this graph clearly shows a variety of compound-specific spectral features such as highly localised absorption peaks. Consequently, employing measurement equipment of sufficient wavelength samples and resolution allows more specific monitoring of the blood stain ageing process.

A technique ideally suited to this purpose is reflection spectroscopy as it records the light reflection profile of a sample as a function of wavelength. Accordingly, there

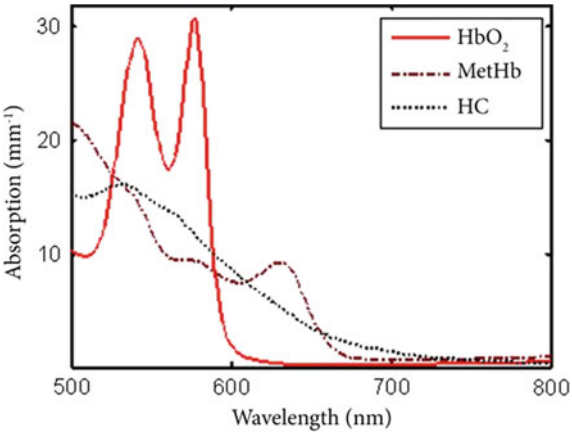

Fig. 7.3 Light absorbing properties of three haemoglobin derivatives formed in ageing blood stains. Reproduced from: G. J. Edelman, "Title: Spectral analysis of blood stains at the crime scene" Ph.D. thesis Faculty of Medicine (AMC-UvA), http://hdl.handle.net/11245/1.416827

have been a number of studies investigating the potential of reflection spectroscopy for blood stain age estimation.

The most recent study investigated the use of visible reflectance spectroscopy in conjunction with chemometric data analysis strategies [24]. Here, three different statistical classification algorithms were benchmarked against each other to determine the most effective age estimation method. Blood stains were deposited on glass slides and reflection spectra were recorded between 500 and 780 nm at ages ranging from 2 h to 45 days. The study found that the most effective age estimation strategy consisted of first reducing the dimensionality (number of data points) of the reflection spectra using partial least squares regression and using this transformed data to build age estimation regression models by means of least-squares support vector machines. This approach achieved a root mean square error of prediction (RMSPE) of 42.792h. Another study investigated the effectiveness of a slightly different strategy for dimensionality reduction and classification, employing Fourier transformation as feature selection tool followed by Linear Discriminant Analysis for the classification task [25]. Using this approach correct classification (±1 day) of blood stains deposited on a white glazed tile and younger than 19 days was possible for 83.3% of all tested blood stains.

As mentioned above, reflectance spectrometers, of sufficient wavelength samples and resolution, should allow the monitoring of compound-specific light absorption features. However, due to relatively complex light-sample interactions (scattering), the measured blood stain reflectance will usually depend on these absorption profiles in a non-linear way (Fig. 7.4). Consequently, reflectance-based quantification of haemoglobin derivatives necessitates a physical model accounting for these light-sample interactions. The use of such models for the purpose of blood stain age estimation has been the subject of several studies in the past. Most notably, one of these methods recently matured to being applied and validated at crime scenes [26]. In this approach, the complex light-sample interactions (including substrate reflectance) are accounted for by employing a so-called light transport model to

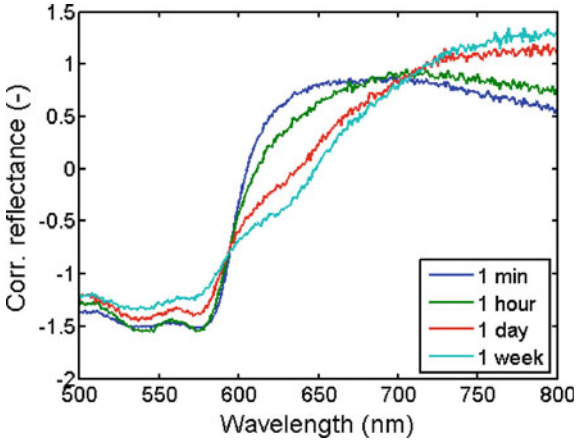

Fig. 7.4 Reflectance spectra of blood stains on cotton of different ages exhibiting absorption features of the different haemoglobin derivatives. Reproduced from: G. J. Edelman, "Title: Spectral analysis of blood stains at the crime scene" Ph.D. thesis Faculty of Medicine (AMC-UvA), http://hdl.handle.net/11245/1.416827

describe the blood stain reflectance. This model is derived by describing blood stains as two-layer systems (blood atop a substrate) and solving one of the fundamental physical equations describing light propagation. By applying known physical constraints (layer thickness etc.), a theoretical prediction of the wavelength-dependent blood stain reflectance can be derived. This, in turn, allows estimation of the contribution of each of the three haemoglobin derivatives by comparing measured and theoretical spectra and determining the best fit. Finally, these estimated contributions yield fractions of the present haemoglobin derivatives. Figure 7.5 shows the change of these fractions as a function of blood stain age, in particular the decrease of the HbO_2 over time, rendering it a suitable measure of blood stain age. In order to use the HbO_2 fraction of an *unknown* blood stain to predict its age, a database was created consisting of HbO_2 fractions of multiple blood stains on cotton measured under laboratory conditions for a period of 200 days. Utilising this data to build a statistical regression model then allows the calculation of a predicted age along with a 95% confidence interval. This method was then tested at a real crime scene where it enabled differentiation of two groups of blood stains likely deposited at different times. Another possible approach compares the three haemoglobin fractions of an unknown stain to a similar database containing all three fractions for different blood stain ages [27]. Finding the smallest difference between the three measured fractions and any database entry then yields a predicted blood stain age with a median relative error of 13.4%. Both methods allow non-contact age estimation for up to 200 day old blood stains.

The reaction kinetics of haemoglobin degradation strongly depend on environmental factors such as temperature and humidity. Specifically, the reaction rate of the entire degradation process increases with temperature, while increased humidity levels decrease the rate of the metHb to HC conversion. As a result, employing either databases or training sets containing age-specific data acquired under a particular set of environmental conditions would lead to incorrect age predictions, if

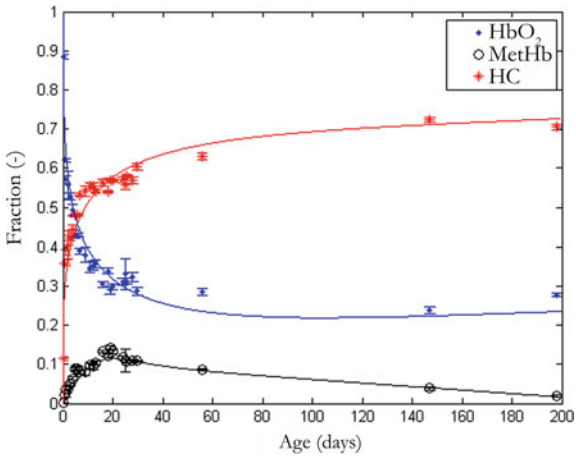

Fig. 7.5 Estimated fractions of the three haemoglobin derivatives as a function of time (reprinted from reference [27] with permission from Elsevier)

used to estimate the age of a blood stain aged under different or unknown conditions. In forensic practice, however, it is often the *relative* age of blood stains which is of interest. Therefore, if environmental conditions are unknown but conceivably identical for two sets of blood stains, comparison of their estimated haemoglobin fractions will still reveal valuable information: significantly different fractions likely indicate different times of deposition [27]. This comparison necessitates estimation of the haemoglobin fractions and hence the use of the light transport model discussed above.

Advantages and Limitations The use of reflectance spectroscopy for the non-contact age estimation of blood stains is associated with several advantages over the low-cost approaches discussed earlier. Firstly, it enables more specific monitoring of the ageing process (haemoglobin degradation) due to the larger number of recorded wavelength (colour) samples and generally increased wavelength resolution; this, in turn, increases the statistical strength of the results obtained in this way. Secondly, using suitable fibre-based probes inherently ensures homogenous sample illumination. Thirdly, to date no sunlight exposure sensitivity has been observed in data recorded using reflectance spectroscopy. Aside from these advantages, and just as in smartphone-based approaches, the use of classification algorithms requires no a priori knowledge about reaction kinetics or advanced physical modelling skills. However, when using reflection spectroscopy, the increased number of recorded data points significantly diversifies data (pre-)processing options, such as data dimensionality reduction. While smartphone-based measurements are perhaps the most intuitive to carry out, spectroscopic measurements are also very simple operations requiring minimal user training. Bedsides its many advantages there are also some limitations to the use of reflection spectroscopic methods for the non-contact age estimation of blood stains. Dark substrates strongly reduce the measured reflectance thereby diminishing the information content of the reflectance spectra. Consequently, no meaningful results can be obtained from visible reflectance spectra measured on dark backgrounds. Coloured substrates pose a problem only in combination with statistical classification methods. Here, libraries would have to be built containing example spectra of blood of different ages on every encounterable substrate. Lastly, hardware requirements render reflectance spectroscopy a more expensive approach than smartphone-based methods. It is important to note that, the use of the light-transport model mentioned above is not hampered by coloured substrates as it explicitly accounts for the substrate influence on the measured reflectance spectra. Moreover, this approach is the only non-contact age estimation method tested in forensic field work to date. Consequently, this approach is the most advanced of all blood trace age estimation techniques at TRL 9.

7.3.2.2 Hyperspectral Imaging

In digital photography, rendering of all perceivable colours is achieved by recording information about three primary colours for every pixel in three separate channels:

red (R), green (G) and blue (B). These types of images are therefore most commonly referred to as RGB images. As discussed earlier in the low-cost approaches section, these three channels do not sufficiently capture the colour changes observed in ageing blood stains. This shortcoming can be addressed by increasing the number of wavelength samples, i.e. colour channels, for instance using a hyperspectral camera. Such cameras successively record reflectance images at numerous wavelengths (colours) creating so-called hypercubes; every pixel of a hypercube therefore comprises an entire reflection spectrum (Fig. 7.6). Selecting regions of interest (ROIs) within the hypercube, e.g. a group of pixels constituting a blood stain, and averaging its spectra yields a reflection spectrum for the selected region, e.g. of the blood stain in question. Consequently, this enables non-contact age estimation of blood stains utilizing either the statistical classification methods or the light-transport model discussed in the reflectance spectroscopy section. The feasibility of such hyperspectral imaging approaches has been demonstrated in two studies in the past.

In the first of the two studies [27], blood was deposited on white cotton and left to age under laboratory conditions. Hypercubes, with a spectral range of 500–800 nm, were recorded of the sample at numerous time points between 0 h and 200 days. ROIs were then selected within these hypercubes to obtain blood stain reflectance spectra at every time point for further analysis. To this end, 8 ROIs comprising 25 pixels each were selected per hypercube. These 25 reflectance spectra were then averaged to yield 8 ROI-specific reflectance spectra per hypercube, i.e. per time point. Subsequently, three haemoglobin derivative fractions were calculated for every ROI and time point employing the light-transport model approach outlined above. Using these age-specific haemoglobin derivative fractions, a database was generated serving as a reference set in the age estimation for a comparable test set of blood stains. This test set was created by repeating the above ROI selection and haemoglobin fraction calculation steps for a set of neighbouring blood stains within the same

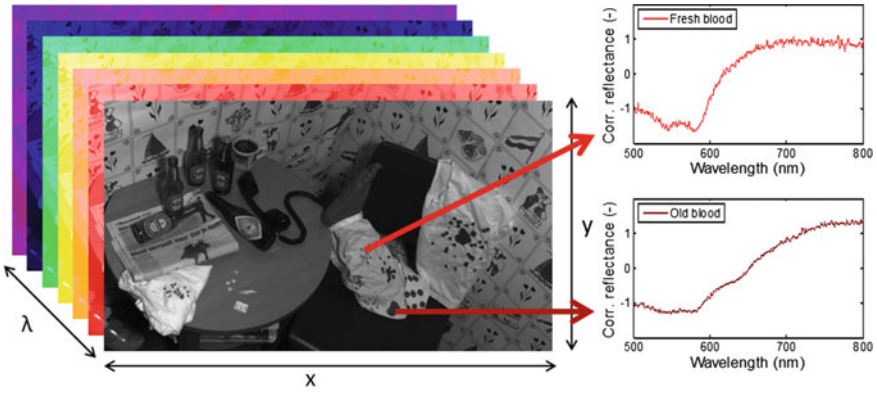

Fig. 7.6 Hypercube (left) of a mock crime scene and the reflectance spectra (right) of two pixels within two blood stains of different ages (reprinted from reference [27] with permission from Elsevier)

hypercubes. Non-contact age estimation was achieved by finding the reference set entry containing the most similar combination of haemoglobin fractions to the test set entry in question. This approach enables non-contact age estimation of blood stains for up 200 days with a median relative error of 13.4% of the true age. Unknown environmental conditions at the crime scene potentially render absolute age estimations inaccurate. In this case, relative ages, which are frequently of forensic interest, can still be determined. This information can also be derived from the haemoglobin derivative fractions using so-called clustering algorithms. These algorithms attempt to divide large data sets into smaller subgroups with shared properties. Here, similar combinations of the three haemoglobin derivatives indicate similar times of deposition. Consequently, blood stains grouped together on the basis of their haemoglobin derivative fractions are likely of similar ages. Figure 7.7 shows the results of this relative age estimation task overlaid on an image of the mock crime scene. All blood stains in the scene were grouped correctly, except for the youngest stains (orange ellipse) which were grouped with 2 day old blood stains while their true age was 0.1 days.

The second study investigated the feasibility non-contact age estimation of blood stains using hyperspectral imaging in conjunction with statistical classification methods [25]. To this end, hypercubes of two blood stains deposited on white printing paper were recorded daily for ages between 10 min and 30 days with a spectral range of 505–600 nm. Here, 10 ROIs of 25 pixels each were selected per blood stain yielding two databases containing 310 reflectance spectra each (10 for each age). One of the databases then served as training set for the classification algorithm while the

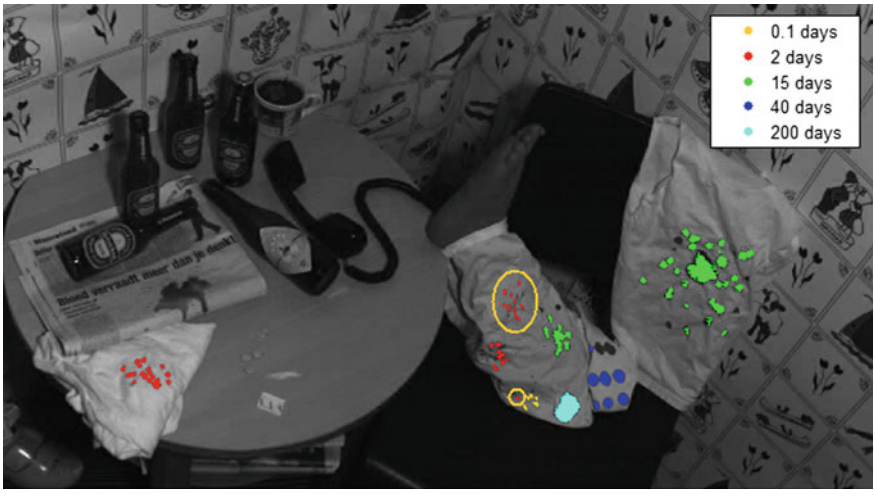

Fig. 7.7 Results of the largely automated relative age estimation task overlaid on an image of the mock crime scene. Almost all stains were grouped correctly, except for the stains in the orange ellipse which were included in the group of 2 day old stains while their true age was 0.1 days (reprinted from reference [27] with permission from Elsevier)

other one was used as a test set to monitor the accuracy of the age estimation. Allowing misclassification errors of one day, this approach achieves correct classification for 89.3% of the test data set. However, this rate significantly decreases for older ages, particularly for blood stains older than 14 days: only 53.5% of blood stains aged between 15 and 30 days are classified correctly.

Besides non-contact age estimation of blood stains another highly relevant forensic application of hyperspectral imaging is the visualization of latent blood traces on dark substrates. This additional capability is a by-product of the spectral range of these cameras, as they usually include the near-infrared part of the electromagnetic spectrum. While many dyes and colourants which strongly absorb in the visible do not absorb light in the near-infrared, some of the haemoglobin derivatives formed in the ageing process do. Consequently, contrast is created between blood traces and dark substrates in reflectance images recorded at near-infrared wavelengths (Fig. 7.8).

Advantages and Limitations Hyperspectral imaging not only records the trace context for later review, but also provides sufficient spectral (colour) information to accurately estimate the age of blood stains. Furthermore, hyperspectral imaging can be combined with standard image processing and computer vision tools, enabling highly objective and fast screening of entire crime scenes. This, in turn, significantly reduces the operational workload, while increasing the rate at which potentially investigation-guiding intelligence becomes available. Consequently, hyperspectral imaging, in conjunction with advanced signal processing, is certainly the most powerful emerging technology in the field of non-contact age estimation of blood stains. Until very recently, one aspect of the technique possibly hampering its adoption in forensic practice was hardware complexity, both physically and operationally. However, recent hardware developments have largely addressed and removed these

Fig. 7.8 Visualisation of latent blood traces on black cotton using hyperspectral imaging. Reflectance images recorded in the visible (left) and the near-infrared (right) part of the spectrum

shortcomings, providing forensic investigators with a highly portable and easy-to-use device requiring minimal to no additional training. Moreover, commercial software packages are being developed enabling forensic investigators to independently carry out non-invasive age estimation of blood stains at the crime scene. One remaining aspect potentially preventing the introduction of this technique in certain settings is the associated cost which exceeds that of smartphones and fibre-based reflection spectrometers. As a result, hyperspectral imaging for non-contact age estimation of blood stains can be classed at TRL 8/9.

7.4 Dark Substrates

7.4.1 Near-Infrared Spectroscopy

All techniques discussed in the previous sections probe and monitor colour changes observed in ageing blood stains. These colour changes, however, may be masked by dark substrates due to their strongly light absorbing properties. Consequently, approaches utilising visible reflectance are not applicable in cases involving such substrates. This limitation can be overcome by quantifying near-infrared reflectance instead, as many dyes and colourants which strongly absorb visible light do not absorb near-infrared light [28–30].

One study investigated the feasibility of non-destructive age estimation of blood stains on dark substrates using near-infrared spectroscopy. To this end, blood was deposited on black cotton and reflectance spectra were recorded between 1150 and 2500 nm. A statistical classification model was built and used to estimate the age of blood stains. This method achieves a relative RMSPE of 8.9% for blood stains on black cotton for ages between 0 and 30 days [28].

Advantages and Limitations If blood stains are found on dark substrates, near-infrared spectroscopy offers possibilities for age estimation where all methods utilising visible reflectance would fail. However, this approach is associated with some practical limitations. Firstly, the required equipment is not portable. As a result, samples have to be transported to a lab rendering it difficult or impossible to analyse blood traces deposited on immobile substrates. By the same token, necessary operational skill levels and associated costs exceed those of visible reflectance techniques. Taken together, these aspects render near-infrared spectroscopy for the age estimation of blood traces on dark substrates TRL 4.

7.5 Conclusions

This chapter showcases the most recent efforts in blood trace age estimation, a discipline frequently referred to as being in pursuit of the '*holy grail*' of forensic science. This designation reflects the unifying character of trace age information as well as its high forensic value by the same token: the age of a trace can establish a veritable connection between the trace and crime. Consequently, trace age effectively serves as a classification measure, reliably distinguishing relevant from irrelevant traces. So far, considerable strides have been made in pursuit of this '*holy grail*'. Numerous age-related processes along with their appropriate probes and potential quantification measures have been identified and investigated. In general, there are three classes of techniques: (i) those that require sampling and consume the material during the analytical process; (ii) those that require sampling but preserve the sample material for subsequent analyses; (iii) those that do not require any sampling and conserve the trace in situ. The associated practical advantages and limitations can be class- or even technique-specific. Accordingly, the most appropriate approach would have to be identified on a case-by-case basis. Overall, while many efforts seem to stagnate in a pre-translational phase, the methods currently within reach of the '*holy grail*', i.e. closest to being embedded in forensic routine- and field-work, utilise portable lab-grade equipment to objectively quantify subtle age-induced blood stain colour changes in situ. Most of these approaches, however, are limited to light-coloured substrates. In conclusion, while there are appreciable advances towards the '*holy grail*' of forensic science, its pursuit must be continued by addressing and overcoming the remaining challenges and hurdles.

References

1. Anderson S, Howard B et al (2005) A method for determining the age of a bloodstain. Forensic Sci Int 148(1):37–45
2. Alrowaithi MA, McCallum NA et al (2014) A method for determining the age of a bloodstain. Forensic Sci Int 234:e30–e31
3. Anderson SE, Hobbs GR et al (2011) Multivariate analysis for estimating the age of a bloodstain. J Forensic Sci 56(1):186–193
4. Simard AM, DesGroseillers L et al (2012) Assessment of RNA stability for age determination of body fluid stains. J Can Soc Forensic Sci 45:179–194
5. Qi B, Kong L et al (2013) Gender-related difference in bloodstain RNA ratio stored under uncontrolled room conditions for 28 days. J Forensic Leg Med 20(4):321–325
6. Alshehhi S, McCallum NA et al (2017) Quantification of RNA degradation of blood-specific markers to indicate the age of bloodstains. Forensic Sci Int Genet Suppl Ser 6:e453–e455
7. Mohammed AT, Khalil SR et al (2018) Validation of mRNA and microRNA profiling as tools in qPCR for estimation of the age of bloodstains. Life Sci J 15(6):1–7
8. Eaton GR, Eaton SS et al (2010) Quantitative EPR: a practitioners guide. Springer-Verlag
9. Miki T, Kai A et al (1987) Electron spin resonance of bloodstains and its application to the estimation of time after bleeding. Forensic Sci Int 35(2–3):149–158
10. Sakurai H, Tsuchiya K et al (1989) Dating of human blood by electron spin resonance spectroscopy. Naturwissenschaften 76(1):24–25

11. Andrasko J (1997) The estimation of age of bloodstains by HPLC analysis. J Forensic Sci 42(4):601–607
12. Inoue H, Takabe F et al (1992) A new marker for estimation of bloodstain age by high performance liquid chromatography. Forensic Sci Int 57(1):17–27
13. Arany S, Ohtani S (2011) Age estimation of bloodstains: a preliminary report based on aspartic acid racemization rate. Forensic Sci Int 212(1–3):e36–e39
14. Ackermann K, Ballantyne KN et al (2010) Estimating trace deposition time with circadian biomarkers: a prospective and versatile tool for crime scene reconstruction. Int J Legal Med 124(5):387–395
15. Lech K, Liu F et al (2016) Evaluation of mRNA markers for estimating blood deposition time: towards alibi testing from human forensic stains with rhythmic biomarkers. Forensic Sci Int Genet 21:119–125
16. Lech K, Liu F et al (2017) Investigation of metabolites for estimating blood deposition time. Int J Legal Med 132(1):25–32
17. Doty KC, McLaughlin G et al (2016) A Raman, "spectroscopic clock" for bloodstain age determination: the first week after deposition. Anal Bioanal Chem 408(15):3993–4001
18. Doty KC, Muro CK et al (2017) Predicting the time of the crime: bloodstain aging estimation for up to two years. Forensic Chem 5:1–7
19. Bai P, Wang J et al (2017) Discrimination of human and nonhuman blood by Raman spectroscopy and partial least squares discriminant analysis. Anal Lett 50(2):379–388
20. Strasser S, Zink A et al (2007) Age determination of blood spots in forensic medicine by force spectroscopy. Forensic Sci Int 170(1):8–14
21. Smijs T, Galli F et al (2016) Forensic potential of atomic force microscopy. Forensic Chem 2:93–104
22. Thanakiatkrai P, Yaodam A et al (2013) Age estimation of bloodstains using smartphones and digital image analysis. Forensic Sci Int 233(1–3):288–297
23. Shin J, Choi S et al (2017) Smart forensic phone: colorimetric analysis of a bloodstain for age estimation using a smartphone. Sens Actuators B Chem 243:221–225
24. Sun H, Dong Y et al (2017) Accurate age estimation of bloodstains based on visible reflectance spectroscopy and chemometrics methods. IEEE Photonics J 9(1):6500614
25. Li B, Beveridge P et al (2013) The age estimation of blood stains up to 30 days old using visible wavelength hyperspectral image analysis and linear discriminant analysis. Sci Justice 53(3):270–277
26. Edelman GJ, Roos M et al (2016) Practical implementation of blood stain age estimation using spectroscopy. IEEE J Sel Topics Quantum Electron 22(3):7200107
27. Edelman G, van Leeuwen TG et al (2012) Hyperspectral imaging for the age estimation of blood stains at the crime scene. Forensic Sci Int 223(1–3):72–77
28. Edelman G, Manti V et al (2012) Identification and age estimation of blood stains on colored backgrounds by near infrared spectroscopy. Forensic Sci Int 220(1–3):239–244
29. Pereira JFQ, Silva CS et al (2017) Evaluation and identification of blood stains with handheld NIR spectrometer. Microchem J 133:561–566
30. Morillas AV, Gooch J et al (2018) Feasibility of a handheld near infrared device for the qualitative analysis of bloodstains. Talanta 184:1–6