Non-contact spectroscopic age determination of bloodstains

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This chapter gives an overview of an extensive search in English literature of techniques that address the quest for age determination of bloodstains. In brief, most techniques are complementary to each other, in short as well as long term age determination. Techniques are compared concerning their sensitivity for short and long term ageing of bloodstains and concerning their possible applicability to be used on a crime scene. In addition, experimental challenges like variation in substrate, interdonor variation and environmental influences are addressed. Comparison of these techniques contributes to our knowledge of the physics and biochemistry in an ageing bloodstain. Further improvement and incorporation of environmental factors are necessary to enable age determination of bloodstains to be acceptable in court.
CHAPTER 1
FORENSIC QUEST FOR AGE DETERMINATION
OF BLOODSTAINS: A REVIEW

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INTRODUCTION

Various traces found at the crime scene are candidates for determining the time a crime was committed. The technique mostly used in forensic practice is, in case of the presence of a body, measuring the rectal temperature [6-8] to relate this to the post mortem interval. Other traces potentially suited and explored for age determination are open wounds [9, 10], bruises [11, 12], latent fingerprints [13, 14], ink on questioned documents [15, 16] and bloodstains, the subject of this review. Some of these techniques are forensic routine, but all dating techniques come with large error margins. Bloodstains are among the traces encountered most frequently at crime scenes [17]. Estimation of the age of a bloodstain can be a first indication to forensic investigators when the crime was committed, which is particularly useful when bloodstains are the only piece of evidence available. If, from other pieces of evidence, the time of committance is known, then bloodstain age determination may confirm or exclude a bloodstain as being relevant to the crime.

The aim of this chapter is to summarize potential techniques, recent developments and future challenges relevant for age determination of bloodstains. It discusses both the fundamental background as well as improvements in bio-analytical methods and developments of novel approaches. This review also evaluates the inaccuracy in age determination for various techniques, and the applicability for on site age determination of each method presented.

COMPOSITION OF BLOODSTAINS

Various compounds of a bloodstain can be used for age determination. Bloodstains originate from droplets of whole blood that are dried out. Whole blood contains blood cells, proteins and amino-acids; suspended in a liquid called blood plasma. There are three major types of blood cells and cell fragments: red blood cells, white blood cells and platelets. This paragraph delineates several of the compounds in bloodstains and discusses how each of these compounds can contribute to determine the age of a bloodstain.

Red blood cells

Red blood cells (RBC) are the most numerous from the three major blood cells. RBCs are donut shaped, approximately 7 μm in diameter, do not have a nucleus, and thus contain no DNA. Scanning electron microscope (SEM) and atomic force microscope (AFM) images of RBCs in bloodstains show an intact shape of the red blood cell after deposition [18, 19]. The main component of RBCs is the oxygen carrying protein hemoglobin, which makes up 97% of the blood’s dry content. Hemoglobin contains iron and is responsible for oxygen transport between lungs and tissue. Hemoglobin is built up of four heme subunits; each containing one iron atom, which can bind one oxygen molecule. Hemoglobin can appear in various forms, called hemoglobin
derivatives. Conversion kinetics between various hemoglobin derivatives differ outside the human body compared to in vivo.

Inside a healthy human body, in vivo, hemoglobin molecules are mainly present in two forms: one without oxygen, de-oxyhemoglobin (Hb) and one saturated with oxygen, oxy-hemoglobin (HbO₂). When unbound to oxygen, the iron in the heme subunit is in the ferrous state. This state is bivalent (Fe²⁺) and paramagnetic. Bound to oxygen, HbO₂ is observed to be diamagnetic [20, 21] and the oxygen molecule becomes a superoxide anion [21]. The precise nature of the oxidation state of iron in HbO₂, Fe²⁺, Fe³⁺ or partial electron transfer, is topic of debate [20-23]. The iron atoms in the hemoglobin molecule, whether oxygenated or deoxygenated, exhibit no electron paramagnetic resonance (EPR) signal [24]. HbO₂ can auto-oxidize into met-Hb [25], which contains iron in the Fe³⁺ state [26] and is paramagnetic (high spin). Because met-Hb is incapable of binding oxygen, only a small part (~1%) of in vivo hemoglobin will be met-Hb in a healthy situation. When met-Hb is formed in vivo it will be reduced back to Hb by reductase protein cytochrome b5 [27]. Met-Hb is paramagnetic and has an EPR absorption peak around g=6 [28].

Outside the body, hemoglobin saturates completely with oxygen in the ambient environment to HbO₂. Due to a decreasing availability of cytochrome b5, necessary for reduction of met-Hb, the transition of HbO₂ into met-Hb will, in contrast to inside the body, no longer be reversed [27]. Once hemoglobin is auto-oxidized to met-Hb it will denature to hemichrome (HC), which is formed by an internal conformation change to the heme group [23]. HC contains a low spin [29] and has an EPR absorption peak around g=2.5 [29]. This process of oxidation and denaturation, HbO₂ → met-Hb → HC, is accompanied by both a change in optical absorption and a change in spin configuration of the iron molecule. A summary of the properties of hemoglobin derivatives as described above is shown in table 1.

Besides a chemical change, also physical changes in the RBC can be used for age determination. The stiffness and adhesive force of a RBC are reported to increase after deposition, which can be measured by atomic force microscopy (AFM) [19, 33, 34].

Techniques that elaborate on changes in red blood cells are: EPR, HPLC, Reflectance Spectroscopy, Oxygen Electrodes and AFM.

White blood cells and platelets

White blood cells are much less numerous than red blood cells (1:700) and contribute to the immune system. Anderson et al and Bauer et al have shown the ability to use the nucleus of the white blood cell for age determination, since it contains DNA and RNA [35-37]. Whereas DNA seems to be stable in dried bloodstains [38] –as long as no bacterial overgrowth occurs; RNA is reported to be rapidly degrading. No results are reported on degradation of the cells themselves for age estimation of bloodstains.

The third group of blood cells are platelets, which are cell fragments rather than cells and responsible for clotting and coagulation of blood. When blood vessels are
damaged, a plug of platelets is formed, enmeshed in a network of insoluble fibrin molecules. The coagulation process starts within a few seconds after bleeding. Because of the fast and complex mechanism of coagulation, platelets do not seem to be very applicable for bloodstain age estimation. Hence, no results are reported measuring platelets for age determination of bloodstains.

A technique that elaborates on changes in white blood cells is: RNA analysis.

Blood plasma

Blood plasma consists of serum proteins and clotting factors. The serum proteins include serum albumin, serum globulins and hormones. The serum proteins degrade after deposition and can be separated by electrophoresis. The ratio of various proteins can subsequently be used as an age indicator of the bloodstain [39, 40]. Additionally, the hormones or biomarkers, such as melatonin, can be used to determine the deposition time of a bloodstain relative to the 24h day cycle [41].

Techniques that elaborate on changes in blood plasma are: protein degradation and biomarker analysis.
TECHNIQUES FOR AGE DETERMINATION

The following paragraphs enumerate various techniques that have been explored for age determination of bloodstains. The order of discussed techniques is similar to the order of the above-mentioned bloodstain compounds. At the end, all techniques are compared on short and long term age determination and forensic applicability.

Early Pioneering

Already in the beginning of the previous century, forensic scientists have dealt with the challenge of finding methods for determining the age of a bloodstain. To our knowledge, the earliest reference in literature is by Louis Tomellini from the university of Genoa, Italy in 1907 [42]. He developed a chart with twelve figures to illustrate color changes of a bloodstain from the moment of deposition up to one year. Three years later, probably independent from Tomellini, Leers described the ageing of bloodstains from a physiological point of view as follows: “When a bloodstain slowly dries, it shows, already after a few days, not anymore the pure HbO₂ spectrum, but the spectrum of met-Hb, or a combination of met-Hb and HbO₂” [43]. Leers concluded this, after comparing bloodstain spectra with the hemoglobin spectra measured in 1901 by Ziemke and Franz Müller [44], see figure 1.

In the 1930s, Schwarzacher [45] attempted to correlate the age of a bloodstain with the bloodstain's rate of solubility in water, which begins rapid and then decreases slowly as the bloodstain ages. A few years later Schwarz [46] used a guaiacum-based assay to determine catalase and peroxidase activity of hemoglobin in bloodstains. This assay showed that the intensity of the reaction's color was inversely related to the age of

![Figure 1. Qualitative absorption spectra of hemoglobin derivatives from 1901; HbO₂ (upper panel), Hb (middle panel), and met-Hb (bottom panel). The black bands depict the absorption bands, the corresponding wavelength range is presented on the left side. Image from [44].]
a bloodstain. In 1960, Patterson applied photo-spectrometry. He recorded bloodstain’s reflectance spectra [1] and observed that the changing color of the bloodstain depends on environmental conditions. Twelve years later, Patterson et al managed to quantify absorption bands in bloodstains to improve age determination [2]. Despite all these impressive results in the 20th century, most work ended after one or two publications and unfortunately did not receive follow up.

Techniques based on changes of red blood cells

*High Performance Liquid Chromatography (HPLC)*

High performance liquid chromatography (HPLC) is one of several separation techniques, able to identify and quantify the individual components of a mixture, including hemoglobin derivatives. Mobilized proteins are transported through a column to the detector, where they arrive at characteristic retention times typical for the various proteins. The proteins can be extracted from the bloodstain after adding deionized water to the bloodstain. Quantifying degradation products of heme by HPLC in bloodstains can be used as a marker for age estimation [47-50]. The detector at the end of the column measures the optical density at various wavelengths; a convenient wavelength for detection of proteins in bloodstains is 220 nm (ultraviolet). Various proteins have different retention times and the integrated peak area of a protein is a measure for the amount of proteins present. Inoue et al have related the ratio of the peak areas of the hemoglobin α-chain and the heme protein to the age of the bloodstain. They found a weak correlation between the age and the peak area ratio [47]. An improved age indicator was found following the discovery of the presence

![Figure 2. Appearance of the ‘X’ peak detected by the HPLC for bloodstains stored on cotton cloth at 37 °C during different time periods. The detection wavelength was 220 nm. Data from [49].](image-url)
of an unknown protein 'X', with a retention time more than 8 times shorter than the retention time of the heme protein [48].

The peak area of protein X increases with age of the bloodstain [48, 49] and is shown in figure 2. This figure also shows that this designated 'X' protein is not present in fresh blood. The retention time in the reverse phase HPLC of 'X' is 8 times shorter than the retention time of the heme protein [48]. Inoue et al related the ratio of the areas underneath the peak of 'X' and the heme peak to the age of the bloodstain. This ratio is 0 for fresh blood and increases to 0.3 for bloodstains stored for 52 weeks in the dark at 37 °C. Andrasko recorded the absorption spectrum of the X-component and found only an absorption peak around 220 nm. The lack of absorption between 250-600 nm indicates that compound 'X' is not related to heme. Andrasko found the presence of additional peaks in the HPLC analysis, which were designated 'Y' and 'Z' peaks. While the origin of these peaks was not revealed, they may assist in age estimation of bloodstains by HPLC.

Reflectance Spectroscopy

The color of a bloodstain changes with time from red to brown which suggests that color quantification of bloodstains by optical spectroscopy methods can be a worthwhile approach for pursuing age determination. Various attempts have been made relating the color, based on the reflectance spectrum of the bloodstain, to its age [1-4, 51]. All these approaches operate in the visible part of the spectral range (450-700 nm), since color changes varying from red to brown refer mainly to this spectral range. This technique requires a white light source and a spectrometer. An established approach for age determination by reflectance spectroscopy is by relating the reflectance spectrum directly to the age of the bloodstain is measuring the reflectance at two wavelengths and determining the ratio.

Kind et al related the age of the bloodstain to the reflectance ratio at 540 and 576 nm, designated the $\alpha$-ratio [2]. Another approach, suggested by Hanson and Ballantyne, is measuring the wavelength of maximal absorption around 414 nm, the so-called Soret absorption band and relating the spectral blue shift of the Soret band relative to 414nm to the age of the bloodstain [3], see figure 3.

Figure 3. Spectral Shift in ageing bloodstains. Spectral profiles from bloodstains stored at room temperature for 15 minutes (solid line) and 1 year (dashed line). Analysis is in terms of difference in absorbance (parameter 1) and spectral shift of absorption maximum (parameter 2). Data from [3].
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Apart from the visible part of the optical spectrum, functional information on the ageing stage of the bloodstain can also be obtained in the near infrared (NIR) region [52, 53]. This approach has recently been explored to discriminate between bloodstain and non-bloodstains by infrared hyper-spectral imaging [54-56]. Analysis of NIR spectra is more complicated than analysis of reflectance spectra in the visible part of the optical spectrum, because the characteristic spectral features do not only originate from hemoglobin derivatives, but also from water, lipids, and various proteins. The loss of water plays a significant role in establishing the initial change in the spectra. The H$_2$O absorption band between 1350-1500 nm is not overlapped by any other chromophore absorption and the area underneath this absorption band vanishes almost completely during the first hour of ageing, see figure 4. After evaporation of water, a consistent series of absorption bands due to proteins appears between 1900 and 2500 nm, but does not reveal much alteration during ageing [52]. The appearance of a spectral band between 1450-1900 nm is attributed to the formation of met-Hb with subsequent binding of water during the ageing of bloodstains. While results appear eliciting, Botonjic

![Figure 4. Representative NIR spectra of a bloodstain on gauze aged over a period of 22 days. Note that the area underneath the H$_2$O curve vanishes almost completely after one hour. Data from [52].](image)

**Figure 4.** Representative NIR spectra of a bloodstain on gauze aged over a period of 22 days. Note that the area underneath the H$_2$O curve vanishes almost completely after one hour. Data from [52].
et al ignore HC in their analysis of the ageing bloodstain spectra [52], which may have caused an overestimation of the amount of met-hemoglobin.

Oxygen Electrodes

The amount of HbO₂ in bloodstains can be established by using oxygen electrodes [57], dissolving the bloodstains in a saline solution and using a polarographic oxygen analyzer. The oxygen is measured from an electron current by a negative electrode in a hydroxide bath. Matsuoka et al have monitored ageing bloodstains for ten days [57]. Figure 5 shows the decay of fractional HbO₂ measured at several temperatures. At room temperature, the decay of HbO₂ is at first rapid, but decreases after a few hours. At T = 5 °C no oxidation was measured at all. Bloodstains stored at higher temperatures reveal a faster decay of HbO₂ [57], while after 24 hours, no further decay is measured.

Electron Paramagnetic Resonance (EPR)

The denaturation of hemoglobin in dried bloodstains is governed by a spin state change of the iron ion in the hemoglobin molecule, which can be measured by EPR (Electron Paramagnetic Resonance) [29, 58, 59]. Bloodstains give four striking EPR signals in the g = 6.2 (g6); 4.3 (g4); 2.27 (H); and 2.005 (R) regions of the paramagnetic center's electronic structure. These four signals represent ferric high-spin (g6), ferric non-heme (g4), ferric low-spin (H) and free radical species (R), respectively. As far as hemoglobin derivatives concern, g6 has been related to met-Hb and H to HC [29]. Yet it remains unclear to which compounds g4 and R should be related to [29]. HbO₂ is diamagnetic and consequently has no EPR spectrum [21, 24]. The EPR signal intensity as a function of age of the bloodstain is shown in figure 6. Fujita et al found that the age of a bloodstain could be related to the ratio of the H/g4 signal. For all four signals,
during the first 30 days an increase in EPR signal was found. After 30 days, the g6, (met-Hb) stays at a constant level, while the H signal (HC) decreases after 30 days. The increment of all four EPR signals during the first 30 days suggests that the sum of the chemical components responsible for the EPR signals is not conserved. This hampers relating the EPR signals to the oxidation and denaturation chain of hemoglobin in a bloodstain.

**Atomic Force Microscopy (AFM)**

Atomic Force Microscopy (AFM) is a high resolution type of scanning probe microscopy that scans a surface with the tip of a cantilever with nanometer resolution. By externally oscillating the cantilever, AFM can determine the elasticity of a surface by assessing the cantilever’s resonance frequency. Subsequently, AFM can determine the elasticity of extracorporeal RBCs. The tensile elasticity, or Young’s modulus of a normal red blood cell is 40 kPa [33]. This relatively low value of elasticity corresponds with a high flexibility, necessary for RBCs to penetrate in the smallest capillaries in the microcirculation. Strasser et al have investigated the elasticity of red blood cells in bloodstains by AFM as a function of bloodstain age [19]. The elasticity of the red blood cells increases when becoming extracorporeal. They found that the elasticity of a red blood cell increases almost 8-fold to 300 kPa when measured 1.5 hours after bleeding. Within 30 hours, the elasticity becomes 600 kPa; and after 30 days it reaches 2.5 GPa. Figure 7 shows the elasticity as a function of the age of the bloodstain. While the elasticity increased, the shape of the red blood cell remained intact. Neither the erythrocytes nor the cracks in the bloodstain showed any morphological alterations during the observation period of 31 days [19].
Measuring Young’s modulus of red blood cells in bloodstains can be worthwhile in age estimation. This method is minimally invasive and one sample can be measured multiple times. However, the large standard deviation of these measurements, in the order of 60% of the measured elasticity, precludes inaccurate age estimation. Wu et al have used AFM measurements to determine RBC cell volume and adhesive forces. They found that the cell volume remains constant after deposition, while the adhesive force shows a sudden increase after seven days [34].

Techniques based on white blood cells

RNA, which can be extracted from the nucleus of a white blood cell, is not as stable as DNA; hence RNA degradation measurements seem a suitable candidate for age determination of bloodstains. RNA degradation in bloodstains has been used for age determination by Bauer et al [35] and Anderson et al [36, 37]. RNA expression can be measured in Ct (cycle threshold) values, which is the value of the nth cycle at which the fluorescence generated within a reaction exceeds a certain fluorescence threshold. Various RNA species are highly abundant in blood. The instability of RNA implies that small changes induce polymorph RNA species. Polymorphism which occurs between RNA species can be exploited to provide species specific tests. Two types of RNA examinations have been explored: 1) semi-quantitative duplex Polymerase Chain Reaction (PCR) [35] for reversely transcribing RNA into cDNA, which is then amplified by PCR and 2) quantitative PCR, used to measure the quantity of a PCR product, commonly in real-time [37]. A relation between the age of the bloodstain and RNA degradation was found when the ratio of 18 S rRNA to β-actin mRNA was investigated. Both 18 S and β-actin are considered “housekeeping genes” which are

![Figure 7. Elasticity of red blood cells versus the age of the bloodstain. The whisker displays the standard deviation of the force curves. Data from [19].](image)
expressed in all cell types at relatively high levels, thus the RNA products from cells are likely to be recovered at crime scenes. The $Ct$ value of $18S$ did not change over the course of 150 days, but the $Ct$ values for $\beta$-actin became significantly reduced as a function of time, thus the relative ratio of $18S$ rRNA to $\beta$–actin mRNA increased over time [36] as is shown in figure 8. The age estimation of Anderson et al was limited to half a year of ageing, while Bauer et al showed that quantification of degraded RNA is possible for bloodstains stored up to 15 years [35]. Improvement in age estimation was found when a multivariate analysis of the RNA species was implemented [37]. Finally, it was shown that the forensic function of mRNA markers in bloodstains are not limited to age estimation, but can also be used for discrimination between blood and menstrual blood [60].

Techniques based on blood plasma

Proteins present in blood, especially those in the serum, will deteriorate gradually when bloodstains are formed. Rajamannar studied the decomposition of various $\alpha$, $\beta$ and $\gamma$–globulins, as well as albumin in bloodstains ranging in age from fresh up to a year old [39]. In this study, the presence of the proteins was determined by immunoelectrophoresis, which is an invasive technique and requires cotton fibers soaked with blood for analysis. It was observed that all globulins and albumin were present in fresh bloodstains. After 15 days, the $\alpha$–globulins and the albumin were decomposed. Thereafter, the $\beta$ and $\gamma$–globulins gradually decomposed, until after a year no globulins were found in the bloodstain. Hence, the presence and concentration of globulins could be an indicator for the age of the bloodstain.

**Figure 8.** RNA ratio in bloodstains as a function of age. Data represent the ratio of $18S$ rRNA to $\beta$-actin mRNA as determined by real-time reverse transcriptase PCR. Each data point represents average ± SD for $N=72$. Data from [36].
UV absorbance photometry is another method to detect the presence of enzyme activity. Tsutsumi et al used this method to quantify the activity of lactate dehydrogenase (LDH), glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) [40]. They found a higher activity of these enzymes in bloodstains than in serum and a comparable activity in completely hemolyzed blood. Age estimation up to 12 weeks after formation of the bloodstain was established by a combination of LDH/GOT and GOT/GPT ratios. This method requires removal of the bloodstain, extraction with saline, labeling of the enzymes and detection at a wavelength of 340 nm. Tsutsumi et al found that the GOT/GPT ratio remains stable over the first weeks, while the LDH/GOT ratio is at first high (>10), and decreases to a ratio of 5 after eight weeks.

A more recent approach, by Ackermann et al, is to estimate trace deposition with circadian biomarkers [41]. By using commercial enzyme-linked immunosorbent assays they show that the characteristic 24-h profile of two circadian hormones, melatonin (concentration peak at late night) and cortisol (peak in the morning) can be reproduced from small samples of bloodstains. This approach differs from all other age determination methods described in this review, because it cannot determine the age of the bloodstain, but only the trace deposition time within the 24-h cycle. It was observed that melatonin is quite stable up to 28 days after storage, whereas decay with storage time was observed for cortisol. The technique requires a 30-80 μl sample of dried bloodstain for in vitro analysis at -80 °C. Figure 9 shows that the melatonin concentration remains <10 pg/ml during office hours. But it reaches a maximum of 60 ± 20 pg/ml around 3 am at night. This approach opens a new field of circadian biomarkers for forensic applications.

Figure 9. Twenty-four-hour profiles of melatonin concentrations in blood samples from six subjects and the mean (±SD) values. Data from [41].
Comparison of techniques

The various techniques discussed can be compared on many levels. First and one of the most important aspects of a technique is the inaccuracy. We quantified the inaccuracy in age estimation of three techniques, based on the reported results for AFM from Strasser et al as shown in figure 7 [19], RNA from Anderson et al as shown in figure 8 (male data points only) [36], and EPR from Fujita et al as shown in figure 6 [58]. We calculated the trend line in ageing for these four techniques and converted the reported standard deviation into an inaccuracy. The conversion was based on the calculated trend. Figure 10 shows the inaccuracy for these three techniques. This figure is based on single measurements, for bloodstains stored under laboratory circumstances. For instance, the tabulated data of RNA ratio of 18S rRNA to β-actin mRNA is reported to be 1.56 ± 0.05 after 30 days (see figure 8) For this specific technique, a standard deviation of 0.05 corresponds to an inaccuracy of 25 days in age estimation, based on the trend line. This means that a bloodstain that is measured to be 30 days may in fact be 5-55 days old.

For all techniques, the inaccuracy ranges from 1 day for AFM to 15 days for RNA for bloodstains of 0.1 days of age. The inaccuracy for RNA remains approximately constant around 20 days, which makes RNA especially suitable for long term age determination. The inaccuracy of AFM only contains three data points, i.e. one day at t=0.1 days, an inaccuracy of 4 days at t=1.25 days and an inaccuracy of 23 days at 31 days. The inaccuracy for EPR increases with age, and has been measured up to 750 days. Final note on the inaccuracy, figure 10 is based on single measurements in laboratory circumstances. Performing age determination based on multiple measurements may improve the accuracy, while going to unknown environmental storage conditions will make age estimation less accurate.

The second important aspect in age determination is whether a technique is sensitive for short term or long term changes. Alterations in hemoglobin derivatives occur already within a few hours, and changes are minimal after two months, suggesting suitability for short term age determination. In contrast, the sensitivity of RNA does not depend much on the age of the bloodstains, and is therefore more suitable for long term age determination.

A third feature for which the techniques differ from each other is the level of invasiveness or destructiveness. Ideally, traces are examined and interpreted in the original context, at the crime scene [61]. For preservation of the crime scene and for securing DNA evidence, non-destructive tests are very much appreciated [62]. Figure 11 compares the degree of invasiveness for several techniques discussed in this review for short term or long term age determination. Non-invasive indicates no sample preparation and a technique applicable on the crime scene. Minimally invasive indicates no sample preparation, but it does require transportation to the laboratory. Intensively invasive techniques require transportation to the laboratory and sample preparation. Often, preparation incorporates dissolving the bloodstain in water, a procedure that can only be performed once and hampers a possible second opinion measurement.

The least invasive technique is reflectance spectroscopy. This technique uses a light source, a fiber probe and a spectrometer, all portable and relatively cheap. This
technique requires no sample preparation, and is non-destructive, because there is no physical contact and the light doses are low. Successful age determination by reflectance spectroscopy however, is yet limited to bloodstains found on a white background. Samples deposited on a non-white substrate require a more sophisticated optical sampling and analysis method. 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 optics-based chemical analysis of the imaged object. So far, most reflectance spectroscopy techniques focus on ages not longer than 60 days [3, 52].

Bloodstain age estimation by AFM does not require sample preparation and can be measured under ambient conditions in the laboratory. However, the background surface has to be non water absorbing. Therefore, bloodstain age determination by AFM is yet limited to bloodstains on tiles, glass and plastic. Strasser et al observed that the AFM measurements showed a high standard deviation (figure 2), which they explained by the non-homogeneous composition of the blood clot [19]. The AFM measurements are performed up to 31 days; the long term ageing effects on the stiffness of red blood cells remain currently unclear.

EPR measurements have to be performed in the laboratory as well. The technique requires no sample preparation, but the bloodstain and substrate have to be cooled down to -196 °C. This does not hamper performing multiple measurements on the same bloodstain, but not all substrates are suitable to resist cryogenic conditions. Fujita et al performed their EPR measurements on bloodstains ranging in age from 2 days up to 2.5 year and found changing EPR ratios over the entire time span [58]. Therefore, EPR seems suitable for both short and long term bloodstain age determination.

Figure 10. Inaccuracy of age determination as a function of the actual age, determined for four techniques: AFM, black squares, data from [19]; RNA, red circles, data from [36]; and EPR, green triangles, data from [58].
HPLC and oxygen electrode are sensitive to short term variations in the concentration of hemoglobin derivatives. Both techniques require dissolving the bloodstain, HPLC in distilled water and oxygen electrodes in a saline solution. Sample preparation for age estimation by RNA [64] or biomarkers [41] requires swabbing of the bloodstain by TRI Reagent B; which is a standard procedure for use in the simultaneous isolation of RNA and DNA from whole blood. RNA measurements are tested over a time span up to 150 days and come with large standard deviations. Biomarkers have the potential to reveal the hormonal level of the donor of the bloodstain, which can be related to the time of the day the bloodstain was deposited. Therefore, biomarker measurements can be worthwhile in combination with other techniques.

Experimental Challenges

Performing a measurement on ageing bloodstains may seem straightforward. But, regardless of the technique, the experimental materials and methods have to be chosen thoughtfully. This section addresses the challenges for experiments on ageing bloodstain that apply regardless of selected technique.

Use of Anticoagulants

To simulate blood at crime scenes as realistic as possible, it is recommended to use untreated whole blood for preparation of bloodstains. Most experiments discussed in this review have used anti-coagulants to prevent early clotting. Without adding anti-coagulant blood will clot within 30 seconds [65]. Many anti-coagulants exist for preventing in vitro blood coagulation, including heparin, citrate, and EDTA. Among these three, EDTA is found to be favorite for preservation of DNA extraction from blood [66]. However, no evidence is reported in the literature of anti-coagulants influencing the
processes associated with ageing of bloodstains. Bauer et al found that the use of EDTA in conventional concentrations does not significantly influence RNA degradation [35].

**Interdonor Variation**

Few reports address the interdonor variation amongst bloodstains. The interdonor variation has been tested for RNA and reflectance spectroscopy. A larger spread in RNA ratios was expected for bloodstains from females due to altered hormonal levels encountered during the monthly estrus cycle. We have performed a one-way ANOVA test on the tabulated RNA ratio of 18 S rRNA to β-actin RNA from [36] between males and females and found no significant difference at the p<0.05 level. The insignificant differences between sexes are confirmed by a larger population study [64]. Chapter 3 will show that for reflectance spectroscopy, no significant interdonor variations were found (p<0.05) among forty bloodstains from eight donors. The amount of interdonor variations will most probably depend on the measurement technique.

**Bloodstain Substrate**

The substrate of the bloodstain is an important parameter in bloodstain pattern analysis [17, 67], yet few reports have addressed the influence of substrate on bloodstain ageing, despite its importance. Most studies described in this review have created the bloodstains on white cotton fabric or filter paper. These substrates adsorb the blood and are diffuse reflectors as well. The first property is useful for extraction to in vitro analysis, while the latter property is advantageous for reflectance spectroscopy. For the AFM study (section 3.1), bloodstains on glass were used, to fulfill the requirement of having a nearly flat surface. Only one study, with HLPC, has compared the effect of ageing bloodstains on two substrates: cloth and paper and in this case no significant difference was found in the age estimation [49]. A candidate for future research is the possible difference in ageing for bloodstains on adsorbing versus non-adsorbing substrates or the difference between hydrophobic and hydrophilic surfaces.

**Environment**

Already in the 1960s Patterson reported that the changing color of the bloodstain depends on environmental conditions, including temperature, humidity and exposure to (sun)-light [1]. Identifying and accounting for the influence of the ambient environment is among the greatest challenges in the quest for age determination of bloodstains. Not only is the influence of the environment complex, it also affects the oxidation of hemoglobin, although probably in a different manner than the decomposition of enzymes or the degeneration of RNA. These differences hamper unification of accounting for the environmental factors among the various age determination techniques. Nevertheless, an overview of the efforts of all studies regarding the influence of environment is summarized in table 2. It shows the measured parameter specific for that technique for a fresh bloodstain and after eight weeks of storage at two temperatures, i.e. at 4 °C and 22 °C. These temperatures are conveniently selected in many studies for storage in the refrigerator and at room temperature. However, not all studies have measured up to eight weeks or at these two temperatures, and if measured otherwise this is mentioned.
Table 2. Influence of temperature, humidity and exposure to light for various techniques. The fourth column shows the quantity of the measured parameter for fresh bloodstains. This value is compared with the measured quantity in the fifth and sixth column at two temperatures. The measured quantity in the 4th, 5th and 6th column is shown in bold face.

<table>
<thead>
<tr>
<th>Study</th>
<th>Technique</th>
<th>Measured parameter</th>
<th>Data Fresh Bloodstain</th>
<th>Data first Temp</th>
<th>Data second Temp</th>
<th>remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Andrasko [49]</td>
<td>HPLC</td>
<td>X-peak / heme peak ratio</td>
<td>0</td>
<td>5 after 56 days at 0 °C</td>
<td>20 after 56 days at 22 °C</td>
<td></td>
</tr>
<tr>
<td>Inoue [48]</td>
<td>HPLC</td>
<td>α-globin</td>
<td>6500</td>
<td><strong>4300</strong> after 56 days at 4 °C</td>
<td><strong>3800</strong> after 56 days at 22 °C</td>
<td>dark</td>
</tr>
<tr>
<td>Inoue [48]</td>
<td>HPLC</td>
<td>α-globin</td>
<td>6500</td>
<td><strong>5900</strong> after 56 days at 4 °C</td>
<td><strong>5000</strong> after 56 days at 22 °C</td>
<td>fluorescent light</td>
</tr>
<tr>
<td>Hanson [3]</td>
<td>Spectroscopy</td>
<td>Abs max (nm)</td>
<td>414</td>
<td>411 after 7 days at 4 °C</td>
<td><strong>409</strong> after 7 days at 22 °C</td>
<td>Humidity =50%</td>
</tr>
<tr>
<td>Hanson [3]</td>
<td>Spectroscopy</td>
<td>Abs max (nm)</td>
<td>414</td>
<td>413 after 7 days at 22 °C</td>
<td><strong>413</strong> after 7 days at 22 °C</td>
<td>Humidity =90%</td>
</tr>
<tr>
<td>Fujita [58]</td>
<td>EPR</td>
<td>Log (H/g4)</td>
<td>1</td>
<td><strong>0.45</strong> after 56 days at 20 °C</td>
<td>-0.8 after 56 days at 40 °C</td>
<td>dark</td>
</tr>
<tr>
<td>Fujita [58]</td>
<td>EPR</td>
<td>Log (H/g4)</td>
<td>1</td>
<td>-0.7 after 56 days at 20 °C</td>
<td>-0.7 after 56 days at 20 °C</td>
<td>sunlight</td>
</tr>
<tr>
<td>Matsuoka [57]</td>
<td>Oxygen electrodes</td>
<td>Fractional HbO₂ (%)</td>
<td>100</td>
<td>50 after 10 days at 5 °C</td>
<td><strong>30</strong> after 10 days at 24 °C</td>
<td></td>
</tr>
<tr>
<td>Tsutsumi [40]</td>
<td>Enzyme</td>
<td>GOT (IU/l)</td>
<td>500</td>
<td>500 after 56 days at 4 °C</td>
<td><strong>500</strong> after 56 days at 37 °C</td>
<td></td>
</tr>
<tr>
<td>Tsutsumi [40]</td>
<td>Enzyme</td>
<td>LDH/ GOT</td>
<td>7000</td>
<td><strong>7000</strong> after 56 days at 4 °C</td>
<td><strong>500</strong> after 56 days at 37 °C</td>
<td></td>
</tr>
</tbody>
</table>
in table 2. Also, comparison between bloodstains stored in the dark and bloodstains exposed to light; as well as comparison between bloodstains stored at medium and high humidity has been inserted into table 2.

The influence of temperature in the results is unanimous: decomposition, oxidation and denaturation of bloodstains are faster at higher temperatures [3, 40, 48, 49, 57, 58]. However, defining a general relation between ageing and temperature is hampered by the small number of only five temperatures included in the studies. Future work is required to indicate whether the relation is linear, contains a threshold temperature or can be derived from first order kinetics.

The influence of exposure to light is even more complicated, with three different and contradicting influences reported from negative to neutral and positive effects on ageing. No study specifies the intensity and the wavelength of the light to which the bloodstain was exposed, yet these are essential parameters to identify the influence of light exposure on ageing bloodstains. Inoue et al found a slower rate of ageing for bloodstains exposed to a fluorescent lamp, compared to those stored in the dark. However, wavelength and intensity were not mentioned [48]. Bauer et al found no difference in RNA degradation between bloodstains exposed to sunlight and those stored under sunlight protection [35]. On the other hand, Fujita et al observed with EPR that bloodstains exposed to sunlight showed signs consistent with an increased rate of ageing. Bloodstains exposed to sunlight at $T = 20\, ^\circ\text{C}$ behaved similar to bloodstains stored at $40\, ^\circ\text{C}$ in the dark [58]. Thus the effect of sunlight on the ageing of bloodstains seems to be method-specific. Finally, the effect of humidity has only been studied by Hanson and Ballantyne with reflectance spectroscopy [3]. By exploring the range of 50-90%, they observed that the spectral blue shift in ageing bloodstains is highest at 50% and lowest at 90% humidity.

Experimental protocol

Without pretending to provide the rules for the ideal experiment, the authors would recommend standardized experimental guidelines for future experiments. Our suggestions are shown in table 3.
Numerous techniques have been explored during the last century to address age determination of bloodstains. All techniques are still in the experimental phase and come along with large standard deviations and inaccurate age estimation. Reducing the standard deviation is very important for implementation into forensic practice and eventually in court.

An additional challenge in the age determination is the relative age of one bloodstain compared to another bloodstain, possibly formed at different points in time. However, little research has been reported addressing the challenge of relative age determination. The order of formation of bloodstains can confirm or exclude specific scenarios, especially in cases of questioned self-defense. The resolution of a technique to discriminate the deposition time of two bloodstains depends very much on the ages of the bloodstains. Yet the environmental factors are less complicating than for absolute age determination and the forensic demand is omnipresent. The side conditions for successful relative age determination are very much case-dependent, since figure 10 shows that the inaccuracy for most techniques increases with age of the bloodstain. We look forward to seeing the first case reports of this new future development, whenever a technique is used for relative age determination in a criminal case.

### Table 3. Suggested guidelines for future bloodstain ageing experiments.

<table>
<thead>
<tr>
<th>Method</th>
<th>Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-coagulant</td>
<td>EDTA</td>
</tr>
<tr>
<td>Amount of stains per measurement</td>
<td>3</td>
</tr>
<tr>
<td>Interdonor variation</td>
<td>No</td>
</tr>
<tr>
<td>Substrate</td>
<td>Cotton</td>
</tr>
<tr>
<td>Measurement time</td>
<td>technique dependent*</td>
</tr>
<tr>
<td>Frequency of measuring</td>
<td>technique dependent†</td>
</tr>
<tr>
<td>Temperature</td>
<td>22 °C</td>
</tr>
<tr>
<td>Humidity</td>
<td>~ 40%</td>
</tr>
<tr>
<td>Light</td>
<td>No, i.e. in the dark</td>
</tr>
</tbody>
</table>

*The recommended measurement time for hemoglobin-based techniques is 10 days, and one year for other techniques.
†The recommended frequency for hemoglobin-based techniques is twice a day, and for other techniques once every month.

**DISCUSSION AND CONCLUSION**

Numerous techniques have been explored during the last century to address age determination of bloodstains. All techniques are still in the experimental phase and come along with large standard deviations and inaccurate age estimation. Reducing the standard deviation is very important for implementation into forensic practice and eventually in court.

An additional challenge in the age determination is the relative age of one bloodstain compared to another bloodstain, possibly formed at different points in time. However, little research has been reported addressing the challenge of relative age determination. The order of formation of bloodstains can confirm or exclude specific scenarios, especially in cases of questioned self-defense. The resolution of a technique to discriminate the deposition time of two bloodstains depends very much on the ages of the bloodstains. Yet the environmental factors are less complicating than for absolute age determination and the forensic demand is omnipresent. The side conditions for successful relative age determination are very much case-dependent, since figure 10 shows that the inaccuracy for most techniques increases with age of the bloodstain. We look forward to seeing the first case reports of this new future development, whenever a technique is used for relative age determination in a criminal case.