Chapter

Forensic Analysis of Bloodstain Color

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Abstract

This book chapter delves into the field of colorimetric analysis of bloodstains in forensic science, focusing on its application in crime scene investigation. Therefore it provides a comprehensive overview of the biological background of age-induced color changes. The chapter begins with an introduction to the significance of blood evidence in solving crimes and the emergence of colorimetry as a valuable tool in blood analysis. The principles of forensic spectroscopy are explored, specifically its ability to provide information crucial to crime reconstruction, such as the age of bloodstains. The chapter discusses the transformation of hemoglobin derivatives over time and the corresponding measurable color changes that accompany aging blood traces.

Keywords: forensic science, blood pattern analysis, absorbance spectroscopy, bloodstain age estimation, influencing factors

1. Introduction

In the fascinating field of forensic science, the analysis of blood evidence is crucial in solving crimes. For centuries, the presence of blood at crime scenes has provided investigators with valuable clues to identify perpetrators and with information for the reconstruction of the crime scene with their sequence of events. With advances in analytical technology, colorimetry in particular has emerged as an invaluable tool in the forensically motivated examination of blood.

This chapter is devoted to colorimetric analysis of blood traces, a technique based on the absorption of light by blood components. We will dive deep into the principles of forensic spectroscopy, which makes it possible to obtain information relevant to crime reconstruction, such as the age of individual blood traces. It will be worked out to what extent the biological components of a blood trace are changed with aging and what measurable color changes accompany this.

We will examine the various components of blood responsible for color formation and address the challenges and limitations of this technique. A special focus will be placed on the manifold influencing factors of this colorimetric analysis, the understanding and mitigation of which is the content of current research.

2. Spectroscopy in forensics

Whether in the structural elucidation of sample material found at the scene of a crime, in the determination of the origin of fibers and paints, in the detection of counterfeit drugs or banknotes, or in the age estimation of biological traces: spectroscopic methods are now established in almost all areas of forensic science. Spectroscopy is a biophysical measurement technique. It provides information about the size, shape, structure, charge, molecular weight, function, and dynamics of the macromolecules under investigation. Specific properties of light are exploited, allowing conclusions to be drawn about the state of the sample under investigation.

2.1 Term classification

Spectrometry or spectroscopy is a collection of physical methods in which the interactions of electromagnetic radiation with surrounding matter are observed and measured. The light can be reflected, transmitted, or absorbed. The interpretation is usually based on recorded spectra. These spectra are recorded with so-called spectrometers and are intensity distributions of radiation as a function of wavelength. They are used to identify substances and provide qualitative information about the quantitative composition of the sample. **Figure 1** shows a schematic picture of absorbance spectroscopy, which is one of these methods [1].

There is a wide range of spectroscopic examination methods in forensics that define or compare the color of trace materials to obtain information about their origin and to determine whether they were altered at the time of the crime. Examples include forensically motivated examinations of soil [69], vehicle paint [70, 71], textile fibers [72], postmortem interval [73], drugs [74], and others.

The spectroscopic method relevant to this chapter is spectroscopy in the ultraviolet and visible range (UV/VIS spectroscopy). It is an analysis method with which electrons are excited in the UV and visible range. It belongs to the invasive analysis methods. On the one hand, advantages are that only a small amount of sample is required, and on the other hand, a substrate-independent analysis is possible. In the case of organic substances, only unsaturated substances can be excited; thus, UV/VIS spectroscopy is a detection method for unsaturated, organic substances. An analysis of the recorded spectra enables making statements about different molecular bonds.

When recording a spectrum, the light coming from the light source is passed through a monochromator, which scans the set wavelength range. Since the visible and ultraviolet regions are being examined, two light sources are often used. These

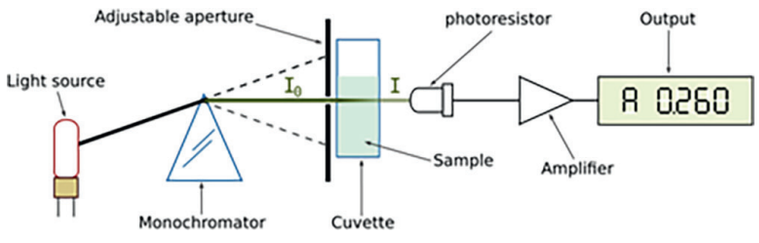

Figure 1.
Schematic setup of an absorbance spectroscope. Monochromatic light is brought to a fixed intensity I0 with an aperture and passed through the sample. The remaining intensity is recorded, amplified, and can finally be read as absorbance value [1, 2].

are usually a tungsten filament lamp for the VIS range and a UV lamp for the other range, which alternate based on a preset wavelength.

So that a sample can be examined, so-called cuvettes are used. These contain the sample dissolved in a suitable solvent. The recording of the spectrum depends on the particular spectrophotometer. There are so-called single-beam spectrophotometers and double-beam spectrophotometers. The former first measures the pure solvent, followed by the measurement of the dissolved sample, which is corrected for the effect of the reference measurement. In double-beam spectrophotometers, the monochromatic light is split, allowing simultaneous measurement of sample and solvent. It is important that the solvent is also measured before each measurement so that the light from the sample and reference beams can be matched.

The absorbed radiation is converted to substance concentration using Lambert-Beer's law. This law gives the radiation intensity Eλ when passing through a medium with an absorbing substance as a function of the layer thickness d and the concentration of the absorbing substance c. The following formula applies:

$$E_\lambda = \varepsilon_\lambda \, c \, d \tag{1}$$

c: Mass concentration of the absorbing substance in liquid (in $\text{mol} \cdot \text{l}^{-1}$).
d: Layer thickness d of the irradiated body (in meters).
ελ: decadic extinction coefficient of the wavelength λ.

As mentioned before, in a UV/VIS spectrophotometer, electrons are excited by light. These are in the ground state and enter the excited state when they absorb light energy. The absorption of certain wavelengths depends on the molecular structure of the substance. The result is an absorption spectrum. Such a spectrum is called a measurement curve, which reflects the absorption of monochromatic light of different wavelengths of a substance [4].

2.2 Electromagnetic spectrum

In the wavelength range from about 380 nm (f = 789 THz) to 780 nm (f = 384 THz), electromagnetic radiation is visible to the human eye. It and sometimes also the adjacent ranges (infrared radiation and ultraviolet radiation), which can also be partially glimpsed due to the diffuse perception limits of the eye, are referred to as light. The actual nature of the underlying waves or particles was almost completely unknown until the beginning of the 19th century and is still debated today. In 1802 Thomas Young confirmed with his double-slit experiment Huygens' principle, which had already been designed in the 17th century. The opinion prevailing until then, that light can be regarded as a bundle of straight rays (ray optics or geometrical optics), was thus replaced by wave optics or physical optics, in which light is treated as a wave and can form wave fronts. However, the laws of geometrical order are still used today to represent relationships in an abstract way.

With the help of the wave-optical approach, properties such as diffraction, polarization, or color could be explained. Color, for example, depends on the wavelength or frequency of the light, which in turn depends on the speed of propagation of the wave in the respective medium. Two special forms are white light, a superposition of many waves, and monochromatic light, which has only one fixed wavelength/frequency. Known interactions of light with matter are, for example, reflection,

scattering, refraction, or absorption. The wavelength λ is calculated from the phase velocity c divided by the frequency f. [3]

3. Blood in forensic analysis and its parameters

Forensic examinations of bloodstains analyze their morphological appearance and variations in their composition. This requires a basic understanding of the physics, chemistry, and biology of blood, which is laid out in this section. Particular attention is paid to the blood color characteristics.

3.1 What is blood?

It is called the divine life element in many places, a symbol of the continuing vitality of the human body. Blood was considered the seat of the soul even in Egyptian times. Ritual ceremonies and healing practices with human or animal blood can be traced back to antiquity with the Mayas or in the Roman Empire and still take place today. In medicine, too, the so-called "red juice of life" plays a central role in anamnesis, since its components ensure the survivability of all body cells and also protect them from pathogens. It is easy to obtain, and any deviations in the composition of its components are important indicators of a present disease of the patient. [5]

3.1.1 Some information about the anatomy and function

Not only is it proverbially thicker than water, and its average temperature of 38°C is about one degree warmer than body temperature (**Figure 2**). Its main function is to support and maintain various bodily functions by transporting substances. To do this, it is pumped through the blood vessels by the mechanical action of the heart, first away from the heart through the arteries and finally back to the heart through the veins. There is an average of about 70–80 ml of whole blood per kg of body weight in the human vascular system, which translates to about 5 to 6 liters for a 70 kg man. This whole blood consists of about 55 percent liquid blood plasma and 45 percent blood cells, which include platelets and red and white blood cells. The latter form the specific and nonspecific immune defense by detecting foreign substances (antibodies) and then binding or lysing them. These lymphocytes are of particular forensic

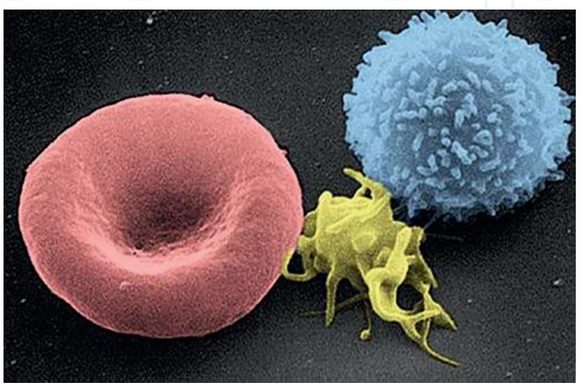

Figure 2.
Scanning electron micrograph of human blood cells. An erythrocyte (left), a platelet (center), and a leukocyte (right) can be seen [6].

relevance because they are the only type of blood cell that has a nucleus, making it possible to extract DNA for genetic fingerprinting. The much smaller blood platelets (thrombocytes), which make up only about one percent of all blood cells, perform the important task of blood clotting by attaching themselves to an injured vessel wall and thus forming a sealing blood clot. At around 96 percent, red blood cells (erythrocytes) make up the largest proportion of human blood in terms of quantity and mass. They transport the oxygen absorbed via the lungs to the organs, and the carbon dioxide accumulates there back to the lungs. Due to the lack of a nucleus, they look like small discs dented in the middle under the microscope [5].

3.2 The colors of the blood

About 90 percent of the dry mass of erythrocytes consists of the oxygen-binding protein hemoglobin. This iron-containing complex gives blood its red color (**Figure 3**). It consists of four subunits (2 "alpha"- and 2 "betachains"), which have a fold characteristic of globulins, in which an iron-II complex, the so-called heme, is bound. **Figure 4** contains a schematic representation of the ID 2H35 of the *protein data bank* (PDB). Under this ID, the theoretically calculated and experimentally proven structure of human hemoglobin can be found on the PDB. This freely accessible database contains information on the arrangement of amino acid chains (primary structure), their basic 3D arrangement (secondary structure), and the 3D structure of the whole chain (tertiary structure) of millions of proteins.

A central position in the heme complex is occupied by an iron atom, which can accept an oxygen molecule without changing its charge.

Within an organism (*in vivo*), hemoglobin is either in the oxygenated form (oxyhemoglobin) on its way from the lungs to the organs, or it is deoxygenated again on its way to the lungs (deoxyhemoglobin). If one oxygen molecule is bound to each of the four iron ions, the overall color changes from dark to light red; deoxyhemoglobin (unloaded) converts to oxyhemoglobin (oxygen-loaded). In rare cases, the already oxidized state undergoes further auto-oxidation, producing methemoglobin, which no longer has an affinity for oxygen. In humans, however, the enzyme methemoglobin reductase ensures that normally never more than 2 percent of the total hemoglobin is present in this stage since; otherwise, the organism's adequate oxygen supply would no longer be guaranteed.

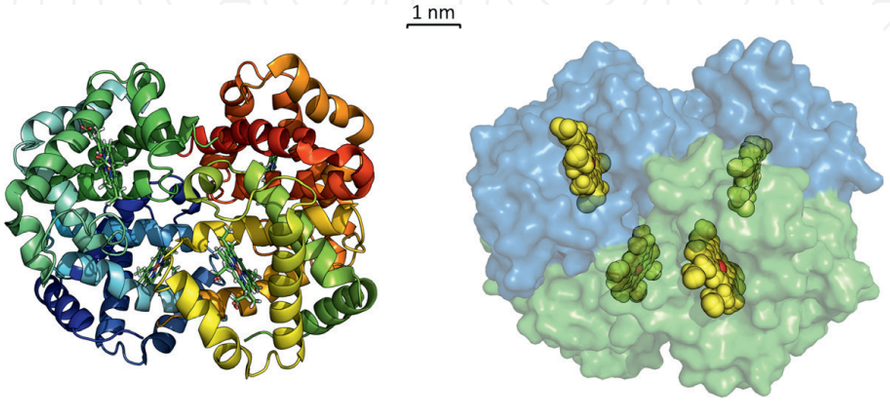

Figure 3.
Hemoglobin. PDB ID 2H35 with secondary structural elements color-highlighted (left) and heme groups with central iron atom schematically illustrated (right). The two colors (blue and green) on the right side represent the two different chains of HB.

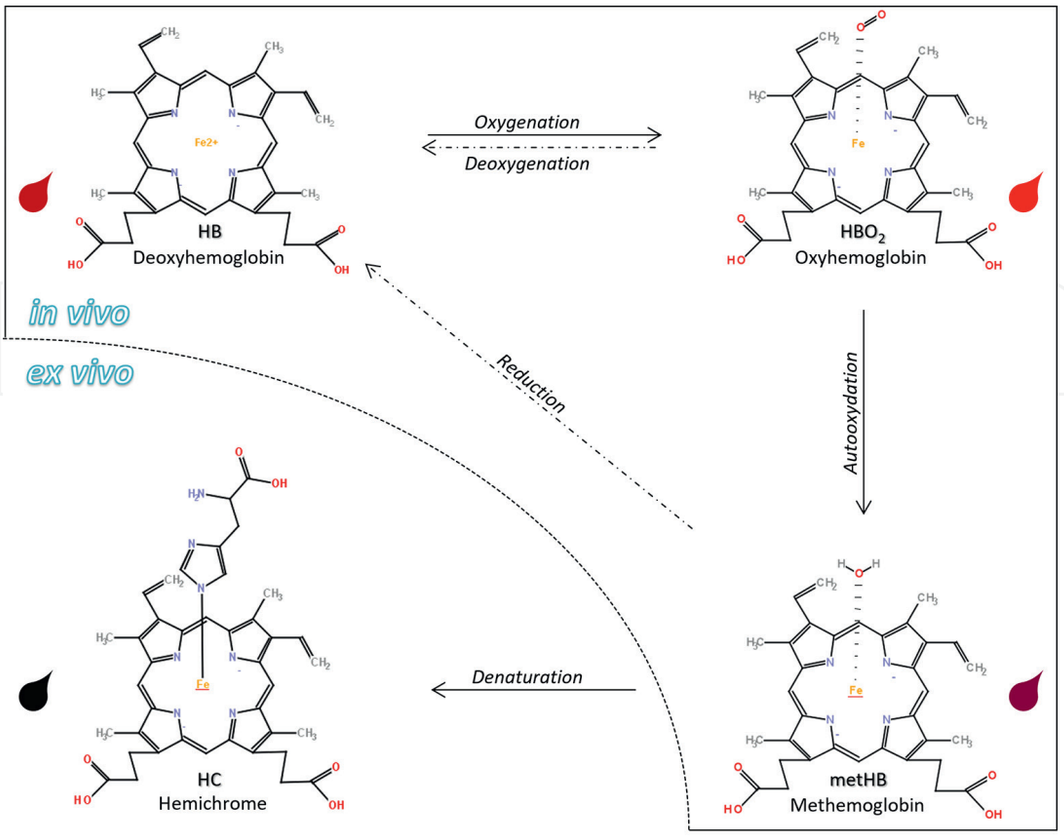

Figure 4.
Hemoglobin derivatives. The bivalent iron of hemoglobin (HBO_2) is oxidized to trivalent iron (metHB), which can no longer function as an oxygen transporter. In the subsequent denaturation, histidine complexes are often formed in which the iron is completely bound (HC). Due to the different peaks of these hemoglobin derivatives in the electromagnetic spectrum (see **Figure 11***), the blood changes color from light red (HBO_2) to brown (metHB) to black (HC).*

The breakdown of old or damaged erythrocytes occurs for the most part in the spleen and liver. Since the deformability of aged or degenerated red blood cells is lower, they are increasingly trapped in the fine meshwork of the spleen, where they can be lysed. Certain scavenger cells of the liver (Browicz–Kupffer cells) can also take up the old cells from the bloodstream and break them down. During this process, the hemoglobin is broken down through several transformation processes to form urobilin (yellow color in urine) and stercobilin (brown color in feces) [7].

When blood is present outside the body (*ex vivo*), atmospheric oxygen oxidizes all hemoglobin deposits to oxyhemoglobin, which then slowly oxidizes further to methemoglobin. However, this process is irreversible due to the absence of methemoglobin reductase ex vivo. Thus, over time, methemoglobin increases at the same rate as oxyhemoglobin decreases.

The methemoglobin concentration increases over several days to a few weeks until the hemoglobin is completely oxidized. As decay progresses, amino acids (often histidine) become tightly attached to the central iron atom starting at two to 3 weeks, rendering the resulting hemichrome completely inactive (**Figure 5**). The temporal denaturation of the blood pigment is also accompanied by a color change. The initially bright red blood (oxyhemoglobin) gradually turns brownish (methemoglobin) until it finally appears almost black (hemichrome). This is, therefore, an oxidation of iron: the blood rusts. The exact process of blood decay ex vivo, unlike blood aging in vivo, has hardly been elucidated because it has no great medical relevance. For criminal

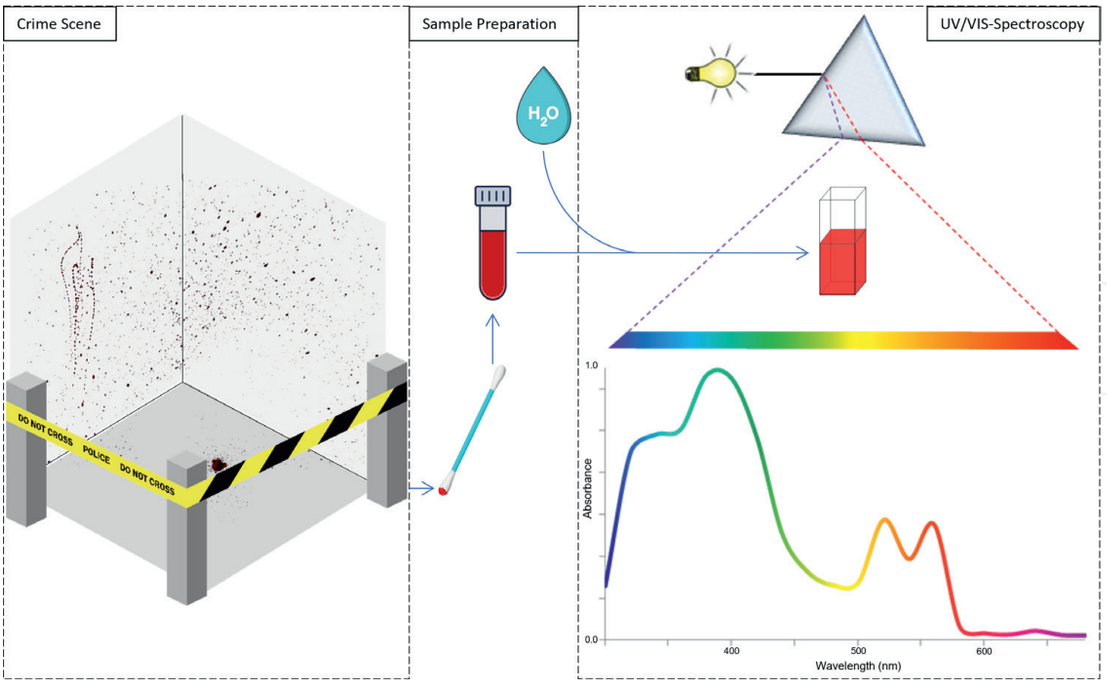

Figure 5.
Schematic overview of a UV/VIS spectroscopic analysis of bloodstains. Wet and dry traces of blood found at the crime scene are secured, for example, using cotton swabs. These samples are diluted with distilled water in the laboratory and transferred to a cuvette. This is then transilluminated with monochromatic light of different wavelengths, producing an image of how much light in which wavelength range is absorbed by the sample.

biology applications, there have been a few publications in recent decades in which the individual phases of blood aging ex vivo were examined in more detail. However, fresh blood was rarely used, or ethylenediaminetetraacetic acid (EDTA) was used as an anticoagulant, which means that the native circumstances can no longer be recreated too accurately. This will be discussed later in this chapter.

A drop of blood freshly discharged from the body thus undergoes a gradual change in composition and, consequently, in color. The speed of this change depends on external influencing factors such as surface properties, temperature, or humidity.

In simplified terms, the degradation of hemoglobin ex vivo takes place in a two-stage reaction, with the respective substeps having the rate constants k1 and k2 [8]:

$$Hb \rightarrow HbO_2 \overset{k_1}{\rightarrow} MetHb \overset{k_2}{\rightarrow} HC \tag{2}$$

3.3 Spectrum of the blood

The method for forensic blood trace age estimation, which is particularly discussed in this chapter, is based on UV/VIS spectroscopy, a form of absorption spectroscopy (see **Figure 1**). In this process, the blood at the crime scene is picked up, for example, with a cotton swab and, diluted with distilled water, measured in the wavelength range between about 200 nm and 700 nm. **Figure 5** shows this process schematically.

As already mentioned, the denaturation of hemoglobin into its derivatives is accompanied by a color change (see Section 2.2). This change can also be followed in an absorbance spectrum of the blood, since the erythrocytes have a large share in it

in terms of quantity. PATTERSON recognized that all hemoglobin derivatives have a strongly pronounced extinction band between about 400 and 425 nm, which is also called the Soret band [9]. Further spectroscopic experiments showed that HBO_2 has two more typical peaks at 542 and 577 nm. According to Zijlstra et al. the specific methHB peak is at 631.8 nm [10]. Thus, in a total spectrum of blood, the peaks of HBO_2 become weaker with time and give way to the peak of methHB (see **Figure 6**).

In addition, the significantly less studied and documented spectrum of HC is of interest. Absorption maxima are seen at wavelengths of 537 and 389 nm. Both maxima have the disadvantage of being overlapped by the absorption maxima of other hemoglobin derivatives in a whole blood spectrum. This maximum overlaps at 537 nm with that of HBO_2 at 542 nm. In addition, the maximum at 389 nm is immediately to the left of the Soret band and is influenced and possibly superimposed by it.

Figure 6 shows the effects of changes in hemoglobin derivatives over time in the whole blood spectrum.

4. Forensic significance

In criminal biology, blood traces have a special significance. They inevitably occur when the human body is violently impacted, for example, by blows or gunshots. The specific evaluation of the internal and external appearance of the stains allows a reconstruction of the crime and thus provides important information to convict

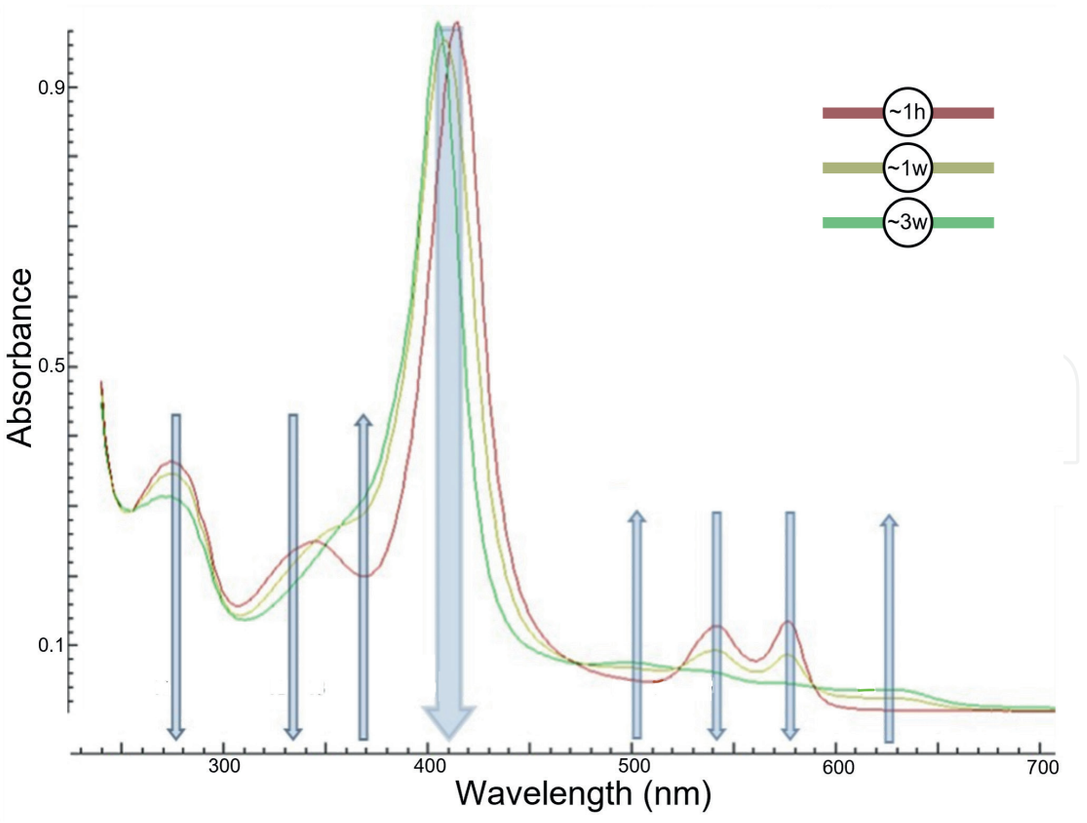

Figure 6.
Resulting blood spectrum. Comparison of spectra from three different age states of bloodstains (ca. 1d, ca. 1w, ca. 3w), color graded by age. Age-correlating changes in the spectrum are shown at different positions. They originate from different concentrations of the hemoglobin derivatives.

possible perpetrators. Forensic analysis of bloodstains can be roughly divided into bloodstain pattern analysis and blood droplet age determination. The methods of blood trace analysis attempt to reconstruct the general sequence of the crime, while blood drop age estimation attempts to narrow down the time of the crime.

4.1 Bloodstain pattern analysis

The morphological examination of a bloodstain often provides information about the direction, type, and intensity of the violent impact, the position of the persons involved in the room, and the distance to the source of the bleeding.

Historically, this way of examining a crime scene has existed since 1956, when the analysis of bloodstains was first mentioned by Lassaigne [12]. This was followed by Piotrowski [13] and Schmidtmann [14], both of whom attempted reconstruction using experimentally generated traces. In 1914, photographic documentation of traces for reconstruction was proposed for the first time by Ziemke [15]. Visualization of blood using 3-aminophthalic acid hydrazide (luminol) has been documented since 1930 [16]. Three years later, a paper on crown formation and the effect of different surfaces on blood stains appeared [17]. In 1939, Balthazard et al. discovered a relationship between the length and width of the stain to the angle of impact [18]. Macdonell postulated another correlation, that of the speed and flight distance of the tracks, in 1971 [19]. In 1975 Brinkmann dealt with the appearance of coughed-up tracks and the blood road crossings [20]. Backspatter traces in gunshot wounds, referred to as backspatter, were also addressed by Macdonell in 1982 [21]. In 1986, the physical background processes involved in the formation of bloodstains were studied for the first time by Pizzola et al. [22, 23]. The first computer-based approach was started by Carter from 1987 [24–26]. Since then, digital analysis systems existed, but routinely they are not used yet. Institutions dealing with bloodstain analysis are, e.g., the IABPA (International Association of Bloodstain Pattern Analysis) or the DGRM (German Society for Forensic Medicine).

Current research in this field is always investigating new physical occurrences that could possibly influence blood. An important requirement of current investigations is the continuous, accurate documentation or archiving of the found samples with high-resolution cameras held vertically over the stain [27]. In this way, one can still refer to this information in the later course of an investigation. The average blood droplet depends on the surface tension and has an average volume of about 50 μl [21], but ranges from 13 to 160 μl [28]. The smaller the volume, the higher the resulting gravitational acceleration. A liquid body always has the lowest surface energy with the largest possible volume/surface area ratio, which is why falling drops always have a slightly oscillating spherical shape or a spherical shape due to gravity [29]. If the trajectory of a formed spot is to be calculated, ballistic laws must be taken as a basis. Thus, Sellier and Kneubeuhl postulated that blood, like any other projectile, is a downward-opened parabola in flight due to the gravitational force of the earth. Typical flight ballistic quantities such as the air resistance or the rotational motion of the projectile (the droplet) must also be taken into account [30].

Since the resulting bloodstains of an act can never be completely identical, each case must, of course, be considered on its own merits. However, there are attempts to categorize or subdivide found bloodstains in order to bring some order to everyday investigation. Bloodstains are officially divided into contact traces and form traces. Contact traces, such as hair or handprints, had prior contact with a specific object that left a specific imprint. Inferences about the clothing worn or the presence of specific

objects can be made when analyzing these prints. In the case of mold marks, the blood has traveled from a source to a specific location at a specific speed due to the application of a force. There, depending on the impact of the force, typical patterns are then created. Examples of mold marks are impact splash marks, drip marks, or coughed-up marks. Another common classification of blood traces is that of the DGRM. This distinguishes between passive traces, transfer traces, projected traces, and miscellaneous traces (see **Figure 7**).

4.2 Bloodstain age estimation

The exact temporal classification of a committed crime is essential in a forensic investigation. The techniques used for this purpose include, for example, rectal measurement of body temperature [32], examination of open wounds [33], or latent fingerprints [34]. Blood also changes in certain ways over time outside the body (see Section 3.2), so age determination is, in principle, possible. Over the last century, there have been many attempts to establish a precise age determination of blood traces. Due to the complexity of the examined source material blood, an enormous variety of starting points for this arose. Then as now, however, the methods were always coupled with a relatively high error rate. However, since blood is often the only trace of a crime that is found or can be used, the current aim is to overcome these inaccuracies.

4.2.1 Historical milestones

The first known mention of this subject in the literature was by the Italian Louis Tomellini in 1907 [35], who designed a color scale on which different age stages (up to 1 year) of a bloodstain could be seen. Three years later, LEERS explained that the

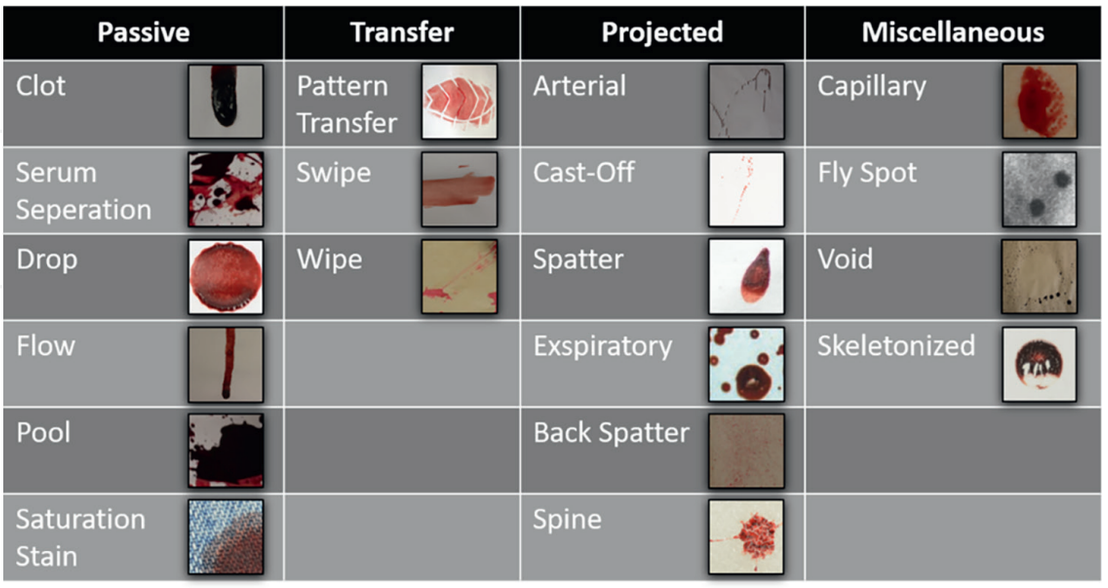

Figure 7.
Blood pattern classification. A schematic depiction of one of the most common classification schemes for bloodstain patterns in Europe. It distinguishes between traces that were generated passively ("passive"), traces that were created actively through transfer ("transfer"), and traces that were created actively through projection ("projected"). Other traces, which are difficult to assign, are assigned to the category "miscellaneous." (mod. From [31]).

spectrum of blood in the visible range changes significantly. The reason for this was the temporal change of hemoglobin [36]. Schwarzacher took a different approach in 1930 and investigated the dissolution rates of old bloodstains, which became progressively more difficult to solubilize [37]. Next, in 1937, the peroxidase activity of hemoglobin was studied using a Guaiacum-based assay. The color response of this experiment was directly correlated with time [38]. The chemical background of the occurring color change during blood aging was investigated and broken down in 1950 [39]. Ten years later, there were the first spectrometric studies showing that the changes were environmentally dependent [9]. The foundation for this was laid by Ziemke as early as 1901 when he studied various hemoglobin derivatives and determined their absorption bands [40]. This methodology was further elaborated and improved by Kleihauer et al. [41] and Patterson et al. [9]. In 1972, Nuorteva proved that blood age determination is also possible with the help of entomology [42]. Bloodstained objects or pieces of clothing are visited by flies like corpses. Their larvae subsequently reveal something about the colonization period.

4.2.2 Current methods of forensic blood age estimation

The list of current techniques for blood age determination is long. There are approaches with several methods for almost all blood components. Due to this diversity, the approaches are subdivided below according to the blood component under investigation.

Since the predominantly clear, liquid portion of blood, blood serum, contains some specific proteins, their observation lends itself to detecting any signs of degradation over time. For example, changes in the various globulins (α-,β-, and γ-globulin) can reveal something about the age of the stain under study. [43] In this immunoeletrophoretic approach, the β- and γ-globulins decayed much more slowly, which served as an approach to calculating age. UV absorption photometry can further be used to quantify the activity of various enzymes (lactate dehydrogenase, glutamate oxaloacetate transaminase, and glutamate pyruvate transaminase), yielding a specific image for each time point as the individual enzymes decompose at different rates [42]. A relatively new possibility for age determination using blood plasma is the use of different degradation rates of contained amino acids. This is accomplished by aspartic acid racemization, which has already been successfully used for rough age estimation of fossils [44]. In 2010, Ackermann et al. created a completely new idea with their ELISA-based (Enzyme-linked-immunosorbent-assay) approach, which, however, answers a different question. With the help of two hormones (melatonin and cortisol), which have a fixed and resolved level in the blood at any time of the day, it is possible to find out the time of the day when the blood spot develops but not the actual age [45].

Since white blood cells, in contrast to red blood cells, have a cell nucleus, so it is possible to extract and analyze RNA here. This is quantified by fluorescence spectroscopy. The ratio of mRNA to rRNA increases slowly and can be converted to ex vivo blood age [46]. This technique can be used for very long postmortem periods, and thus has no upper time limit [47].

4.3 Current colorimetric methods for blood age estimation

The majority of blood age determination methods are based on changes in red blood cells (erythrocytes) and their constituents over time. A frequently used

technology for this is the so-called high-speed liquid chromatography (HPLC), in which the smallest components of liquids can be separated from each other. Hemoglobin and its derivatives or degradation products can be easily detected and distinguished by this equipment. Age correlation using the resulting peaks of the chromoproteins has already been achieved but was not particularly accurate [48]. The peak of a previously unassigned protein (called protein X) allows a better age correlation. The absorbance peaks of this protein, which increase with age, do not lie between 250 and 600 nm, which means that a relationship with hemoglobin can be excluded [49]. The presence of other possibly relevant, as yet unknown proteins (previously named protein Y and Z) was noted but not further investigated. Since the central iron atoms of oxygenated (Oxyhämoglobin→ $Fe2^{+}$) and oxidized hemoglobin (e.g., Methämoglobin→ $Fe3^{+}$) are differently charged and thus have different numbers of free orbitals, they also respond differently to an applied magnetic field. Such fields can be generated with the aid of so-called electron spin resonance spectrometers. Examined blood traces give four specific signals in it, which stand for the different degeneration products of the red blood pigment [50]. The oxyhemoglobin itself has no signal in this process because it is diamagnetic. Due to unexpected values, such as the decrease in the hemichrome peak after about 3 weeks, no further research has been done in this area. Since the concentration of freely accessible (unoxidized oxygen) decreases sharply, especially in the first hours, with the decrease of oxyhemoglobin and increase of methemoglobin, Matsuoka et al. tried to deduce an age relationship from this [51]. In this experiment, a Clark electrode (oxygen electrode) measured the decrease in oxygen atoms in the first 20 hours after blood application. A significant decrease was measured, but it was strongly temperature dependent. Another possibility to examine red blood cells is the so-called atomic force microscopy. In this complex, very high-resolution (down to the nanometer range) technique, the surface of a sample is scanned line by line with an oscillating leaf spring (cantilever). This determines the structure but also the elasticity of the surface. Especially the elasticity of erythrocytes is a helpful parameter here, since it increases enormously in ex vivo stored blood during the first 30 days (from ca.40 kPa to ca. 2.5 GPa) [52]. The fact that the actual structure of the cells hardly changes during this period and that the error rate of age estimation is uncomfortably high also made this research area stand still at first. Since the most noticeable visible change of an aging bloodstain is the transition of the color from red to brown, it seems reasonable to check the absorbance in the visible range (about 390 to 790 nm) for changes. Accordingly, there are some approaches in which this is done with a reflectance spectrometer that records the intensity of reflected rays from a white monochromatic light source. For example, Kind et al. showed that the two typical peaks of oxygen of oxyhemoglobin decrease at 540 and 576 nm and allow estimation of blood age [53].

The rather diffuse Soret band of a blood sample (which is the large peak at about 414 nm) also changes significantly [54]. Imaging of the entire visible region over time provides insight into the decrease or increase in the individual degradation products of hemoglobin and thus also allows for age correlation [55]. In addition to imaging the visible region, peaks arising in the near-infrared region can also help with this problem. NIR scanning can also be used in other contexts with blood, namely to clearly separate it from other blood-like substances [56]. Since 2009, there is now the possibility to investigate the large band between 1350 and 1900 nm. Here, although there is a rapid and highly visible decrease, this is largely due to the decrease of water in the blood, and it subsides after only a few hours. In contrast, the actual protein bands

between 1900 and 2500 nm show little diversity over time [57]. In addition to the qualitative evaluation of spectra, quantitative measurements can also be performed at a specific wavelength range to determine the concentration of an ingredient. A recently published article by Agudelo et al. uses this approach to perform age estimation on both young and older blood samples [58]. The content of this publication is the use of a bipartite bioaffinity assay. Two biomarkers (creatine kinase and alanine transaminase) were thereby added to the buffered blood serum. Both ultimately lead to the build-up of pyruvates, which in turn promote the degradation of nicotinamide adenine dinucleotide hybrid (NADH) to nicotinamide adenine dinucleotide (NAD^+). Measurement of nicotinamide adenine dinucleotide consumption occurs at 340 nm in real time. Because the two biomarkers have different but well-defined denaturation times, the creatine kinase portion of the assay can more accurately date younger blood, and the alanine transaminase portion can more accurately date older blood. An approach, also recently published, allows age determination in the field with the aid of computer-assisted image processing [59]. Here, a cell phone camera is placed on a type of specially constructed darkroom, and the stain to be analyzed is imaged from above. The obtained images are then used to train a classification algorithm (Random Forests). Henceforth, the trained algorithm is to distinguish by age all blood images taken in the same way. Thanakiatkrai et al. not only tested the suitability of different smartphones for this experiment but also simulated different environmental conditions.

In the last 10 years, there have been further developments in almost all areas of spectroscopic bloodstain age estimation. The existing approaches in reflectance spectroscopy have been optimized [62] and adapted so that even the background color no longer produces large measurement errors [63]. Age estimation via NIR has also been optimized so that imaging is now mobile [64]. In addition to method optimization, there has also been further investigation with respect to changes in blood composition *ex vivo* over time. This area has paid particular attention to studies of changes in diverse red blood cell constituents [65–67] to further understand their influence on spectral examination methods. The information on morphological changes, which has also been studied, reveals how much the external appearance depends on environmental conditions [68].

4.4 Influencing factors

Now that some techniques have been presented, the question arises as to which influencing factors alter blood aging and in what way. Such influences are, e.g., solar radiation, humidity, ambient temperature, type, and condition of the soil or also the pretreatment of blood with anticoagulants. All these influences can affect the drying out of a blood trace, which is why it brings with it a certain uncertainty in terms of age estimation. Fresh blood traces are usually still liquid and not completely dried out. In principle, it is already possible to use the determination of blood age for crime detection. Blood dries faster ex vivo than in the body of a corpse.

4.4.1 Temperature, light exposure and humidity

As mentioned in Section 2, HbO_2 in blood exposed to atmospheric oxygen denatures to metHb and later to hemichrome. By analyzing spectroscopically prepared data, Bremmer et al. concluded that the first reaction is strongly temperature dependent: the higher the temperature, the faster HbO_2 is converted to metHb. Humidity

has no influence on the process. The transition from metHb to HC, however, is strongly dependent on both temperature and humidity [8].

The dependencies of air humidity and light exposure are shown as examples in **Figure 8**.

Hanson and Ballantyne investigated another aspect of blood aging in 2010. As part of their research, they recognized that in a spectroscopic examination of aging blood traces, the Soret band of hemoglobin appears to migrate over time. They referred to this phenomenon of leftward shift as blue shift. They found that this shift is strongly

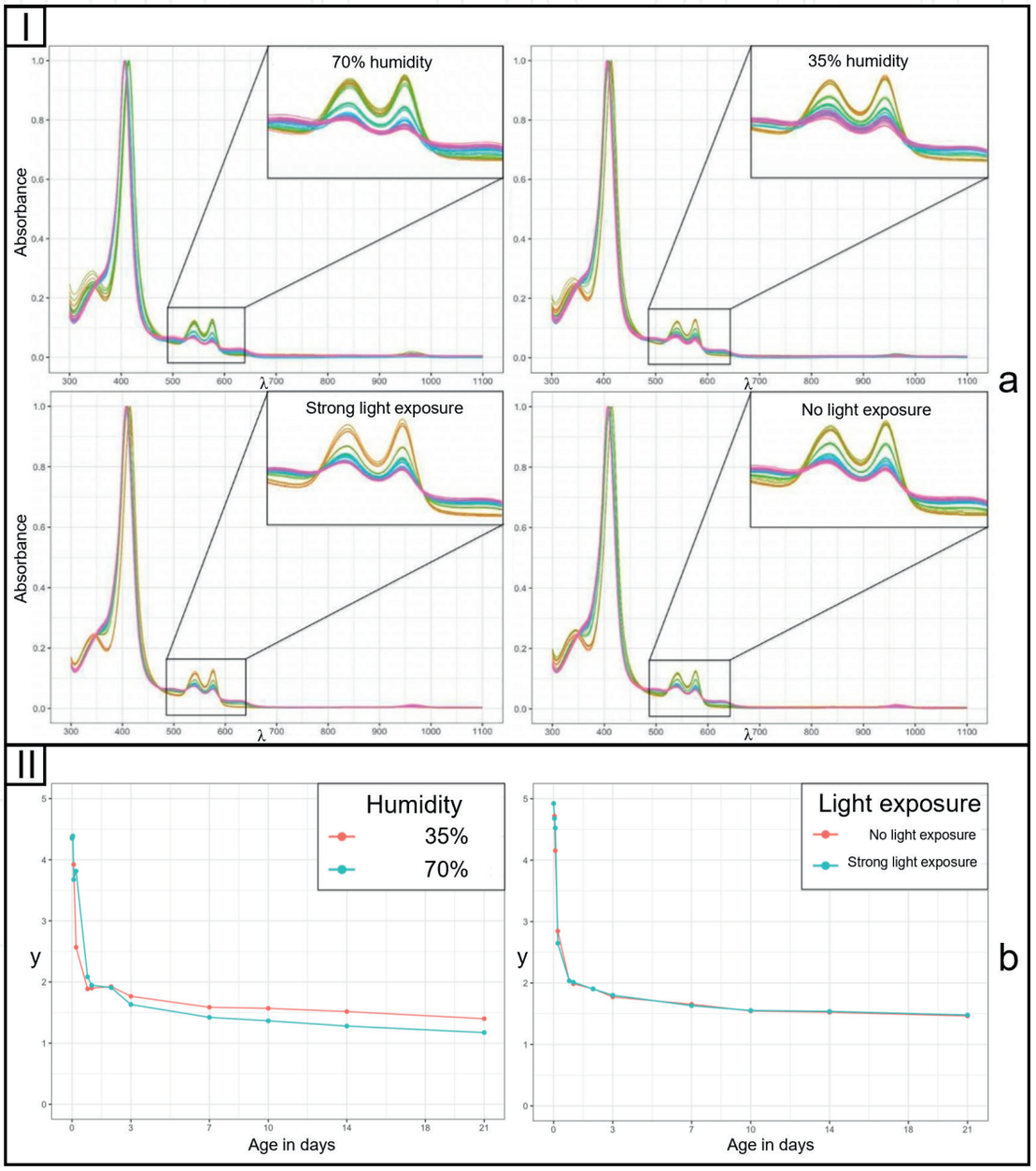

Figure 8.
Influence of light exposure and humidity on bloodstain aging. I] within 3 weeks, blood spectra change due to free atmospheric oxygen ex vivo. This change is influenced by prevailing humidity (a) and solar radiation (b), among other factors. II] a concentration-independent ratio was formed from the major oxy- and methemoglobin peaks (P1 = 542 nm, P2 = 578 nm, P3 = 500 nm, P4 = 532 nm) using the formula y = (P1 + P2)/(P3 + P4). It is noticeable that higher values of humidity favor faster blood aging (a) while there is no significant effect of light intensity on the conversion of HbO_2 to metHB (b).

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dependent on both temperature and humidity. The higher the humidity or the storage temperature, the lower the value by which the Soret peak shifts [54].

Figure 9 shows the shift in the Soret band and its dependence on ambient temperature.

4.4.2 Surface

In 2017, we found that different surfaces (cotton, glass, polyester) show a noticeable influence on spectroscopic results [60]. In the context of investigations carried out in the laboratory, it is therefore advisable to always use the same substrate for

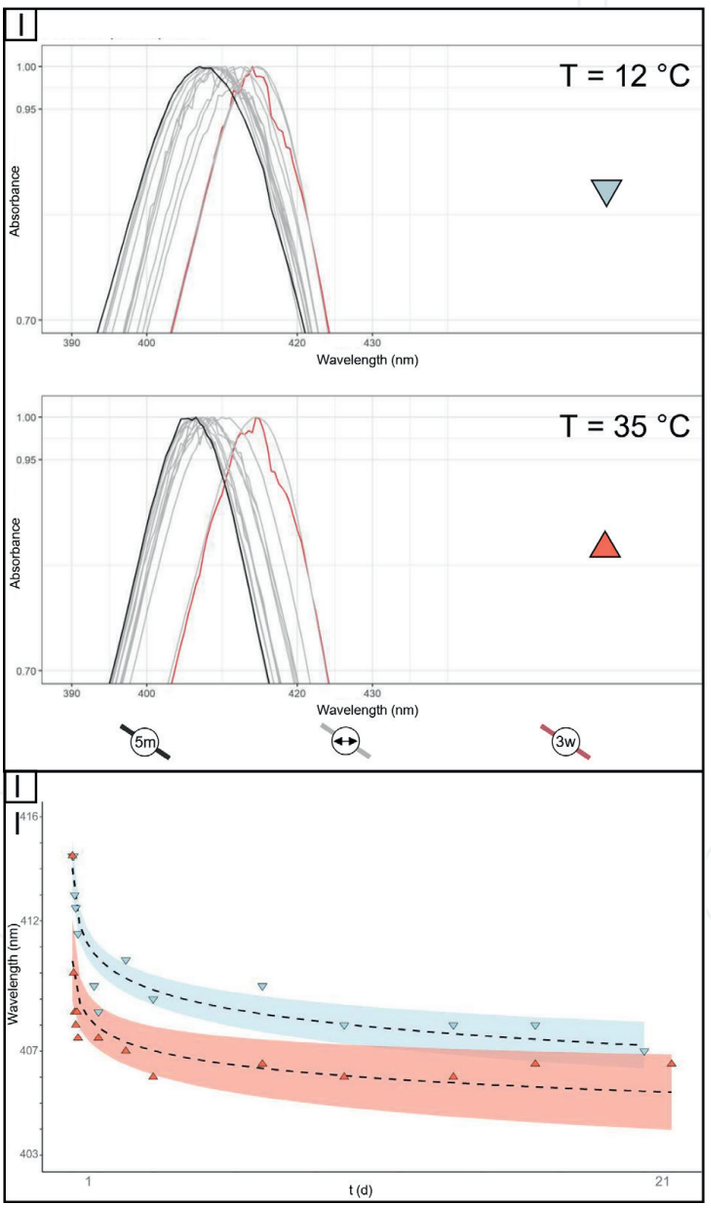

Figure 9.
Temperature dependence of the Soret shift. I] the so-called "blue shift" of the soret band within 3 weeks at 12°C (a) and 35°C (b) and a relative humidity of 35% in each case can be seen. II] from the main oxy- and methemoglobin peaks (P1 = 542 nm, P2 = 578 nm, P3 = 500 nm, P4 = 632 nm) a concentration-independent ratio was formed according to the formula y = (P1 + P2)/(P3 + P4). The differences in the diagram show that higher humidity causes faster "blue drift" or blood aging.

experiments on blood aging. When evaluating case-relevant traces, however, it should always be remembered that blood trace aging proceeds differently on different surfaces.

4.4.3 Anticoagulants

Anticoagulants are frequently used in medicine to prevent potentially dangerous complications during surgery or to reduce the formation of clots in the circulation. Anticoagulants are also popular for obtaining valuable data sets in a forensic context (e.g., generating different blood patterns or recreating crime scenes), as the associated delayed clotting allows blood samples to be used for longer periods of time.

There are different types of anticoagulants such as citrate, hirudin or *ethylenediaminetetraacetic acid* (EDTA), each of which has an influence on different subprocesses of blood coagulation. In 2020, we showed that at least one of them (EDTA) alters the aging of blood in a way that spectroscopic methods for age estimation give false results [61]. This is logical and is also mostly taken into account when evaluating crime-relevant blood traces, but the creation of data sets in the laboratory to recreate the crime or to research the influencing factors discussed here are still frequently carried out with EDTA-amended blood samples for the advantages just described. This should be prevented in the future because otherwise untreated blood drops from the crime scene are compared with EDTA-treated blood drops from previously st6att found experiments.

5. Conclusions

Colorimetric analysis methods have a firm place in forensics. In the specific field of blood trace pattern analysis, they are mainly used for age estimation. The transformation of hemoglobin derivatives observed in this process has been known for over 100 years, but even today, the methods based on it are being optimized to obtain ever more accurate results under all assumed circumstances of the crime. So far, it has been understood that blood color reflects not only information about health status and possible intoxications but also the time the blood has been ex vivo. This is due to the omnipresent atmospheric oxygen ex vivo, which alters the ratio of the hemoglobin derivatives, observable in the wavelength range of about 400 nm - 600 nm. Under real conditions at the scene, the recorded spectral data can be affected by various environmental conditions. In order to correctly interpret the color-coded information, these influences must be investigated and taken into account. The more these external influences are understood and accounted for, the more reliable the blood age estimate will be. For this reason, current scientific publications in this field predominantly deal with optimized ways of calculating the influence of environmental conditions on blood aging rather than completely new methods.

What the future holds is, of course, uncertain, but it is likely that the trend of other forensic disciplines will continue, and parts of blood trace pattern analysis will also be automated using computer-aided methods. Approaches to this have already been made and range from AI-based analysis methods to the so-called "Smart Forensic Phone," a mobile detection system based on a cell phone [59]. Such approaches are not intended to overhaul existing methods but to facilitate their use in everyday forensics. An app under development (not yet published by us) demonstrates this (see **Figure 10**). A clear and easy-to-use interface on a standard cell phone

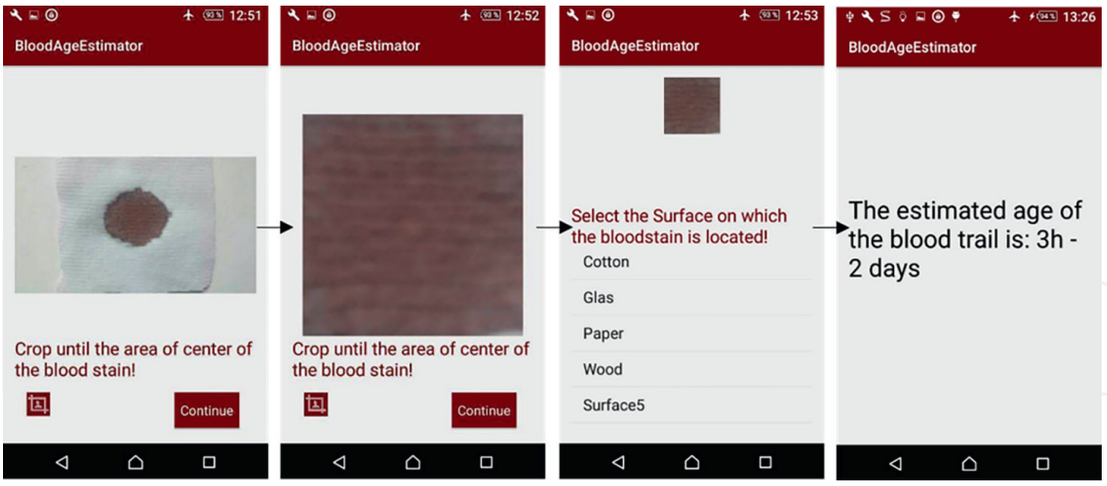

Figure 10.
"Blood age estimator" app. User interface of an application under development for user-friendly recording and age estimation of blood traces.

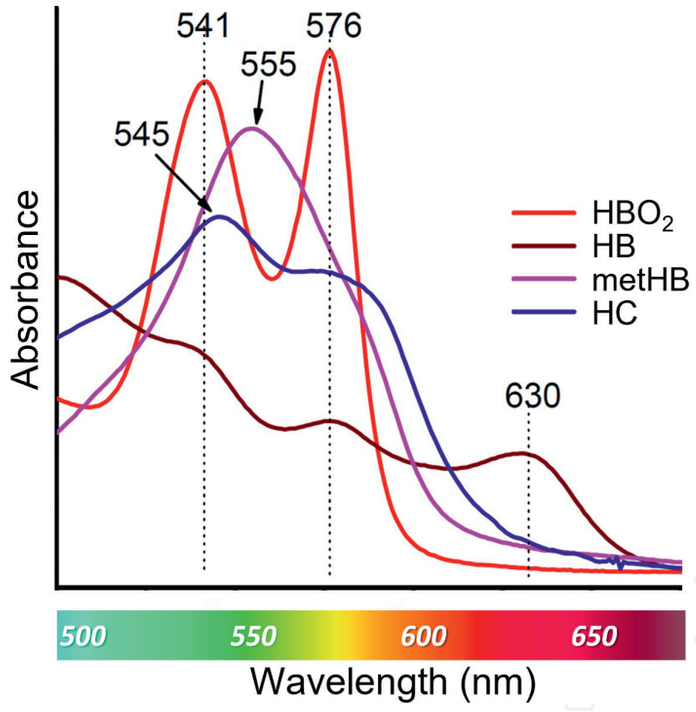

Figure 11.
Hem derivative spectra. Absorbance values of HBO_2 (red), metHB (brown), HB (pink), and HC (blue) in the range from 450 to 700 nm (mod. From [11]).

allows the use of colorimetric methods for forensic blood trace age estimation at almost any location by nontrained personnel.

Conflict of interest

The authors declare no conflict of interest.

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