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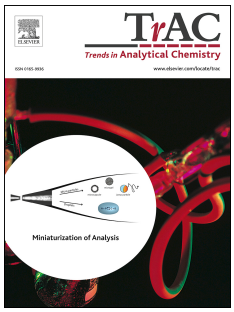

In the pursuit of the holy grail of forensic science - spectroscopic studies on the estimation of time since deposition of bloodstains

Grzegorz Zadora, Alicja Menżyk

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In the pursuit of the holy grail of forensic science - spectroscopic studies on the estimation of time since deposition of bloodstains

Grzegorz Zadora[a,b,*], Alicja Menżyk [b]

[a] Institute of Forensic Research, Kraków, Poland
[b] Institute of Chemistry, University of Silesia, Katowice, Poland
[*]Corresponding author. E-mail address: gzadora@ies.krakow.pl

Abstract

Bloodstains can serve as a source of high-value information, allowing for the reconstruction of bloodshed events or identification of the sample donor. However, from the forensic perspective, the evidential potential of blood traces is not fully exploited. This is because despite significant research efforts, to date, no reliable method for estimating time elapsed since bloodstain deposition has been established. Nonetheless, over the last few years (2011-2017), some noteworthy advances have been made in the field of bloodstain dating, therefore the objective of the following paper is to provide a critical review of recently developed methods, with a particular emphasis on spectroscopy-based approaches. Finally, impediments to applying established procedures in routine forensic practice, along with perspectives to improve the future developments of bloodstain dating techniques, are also discussed.

Keywords:
Bloodstains; Age estimation; Time since deposition; Spectroscopy; Forensic science

Acronym/abbreviation	Description
2D CoS	Two-dimensional correlation spectroscopy
Al	Aluminium
BPA	Bloodstain Pattern Analysis
CCR	Correct classification rate
CMYK	Cyan, magenta, yellow, key (colour model)
deoxyHb	Deoxyhemoglobin
FAD	Flavin adenine dinucleotide
FT	Fourier transform
Hb	Hemoglobin
HC	Hemi- and hemochromes
His	Histidine
HSI	Hyperspectral imaging
HSL	Hue, saturation, lightness (colour model)
HSV	Hue, saturation, value (colour model)
LLS	Linear Least Square (algorithm)
LR	Likelihood ratio
LS-SVM	Least squares support vector machines
metHb	Methemoglobin
MIR	Mid-Infrared
MSC	Multiplicative scatter correction
MSP	Microspectrophotometry
NADPH	Dihydronicotinamide-adenine dinucleotide phosphate
NIR	Near-Infrared
oxyHb	Oxyhemoglobin
PBS	Phosphate buffer saline
PCR	Principal component regression
Phe	Phenylalanine
PLSR	Partial least squares regression
RBC	Red blood cells
RGB	Red, green, blue (colour model)
RH	Relative humidity
RMSE	Root mean square error
RS	Raman spectroscopy
SNV	Standard normal variate
SERS	Surface-enhanced Raman spectroscopy
SIMCA	Soft Independent Modeling of Class Analogy
Trp	Tryptophan
TSD	Time since deposition
UV	Ultraviolet
VIS	Visible
WBC	White blood cells

1. **Introduction**

An abstruse dilemma of time, which can be considered as the fundamental dimension of our existence, has been accompanying mankind since the dawn of human civilization. However, the problem of time is not merely restricted to the philosophical thought. It takes centre stage in many scientific disciplines, yet everywhere - whether in chemistry, physics, archaeology or geology - time plays a distinct role [1]. The situation is no different in forensic sciences. Questions of time remain at the core of forensic investigations. Experts make every effort to reconstruct the events of the past using provided evidential material - in particular its physicochemical properties - which, according to Heraclitus wisdom, undergo continual transformations. Precisely for these ongoing changes of the evidence characteristics, the confrontation with the issue of time may be even considered possible. Encapsulation of these modifications in some measurable parameters, followed by the determination of the relationship between these parameters and time, serves as the foundation for estimating the age of the questioned trace. For a few decades now, the question of time elapsed since bloodstain deposition has been lying at the heart of forensic temporal examinations [2, 3].

Blood, one of the most common body fluids encountered at the crime scene, unquestionably acts as a driving force for the wide array of forensic investigations [4]. Since the early 20th century, scientists were capable of determining the blood type, owing to the discovery of Landsteiner in 1901, which allowed to at least a partial individualization of human blood. However, a real milestone in forensic serology was reached a few decades later. With the discovery of the DNA profiling [5-7], forensic experts have been equipped with another powerful tool to tackle queries that arise during the investigation process - the question of *who?* finally could have been added to the list of *what?*, *where?* and *how?*. Despite the wealth of this obtainable data, blood traces still have much more to offer.

With the methods of bloodstain pattern analysis (BPA) being within reach, information on the activity level can be also provided. Despite recently raised doubts concerning method's objectivity [8], the interpretation of the distribution, size and shape of bloodstains, performed with the aim of recreating actions that gave rise to their origin, may still significantly contribute to the reconstruction of the crime [9]. The consequent combination of BPA results with a DNA profile has proven crucial in many forensic cases, thereby confirming the words of Edward Piotrowski, the pioneer of the BPA discipline, who in 1895 emphasized the significance of the blood evidence: 'It is of the highest importance to the field of forensic evidence to give the fullest attention to bloodstains found at the scene of a crime because they can throw light on a murder and provide an explanation for the essential moments of the incident' [10]. Even though Piotrowski's considerations referred to the reconstruction of bloodshed events based on BPA, they led, in fact, to the notion of an important idea; the idea of bloodstains as a source of extensive information. To this day, however, the potential of blood evidence is not fully exploited.

Verifying the twofold unity of time and place by proving that a suspect was at the crime scene at a certain time, remains the prime objective of forensic investigations [11]. In most cases, demonstration of the link between the alleged offender and the place of a crime, contemporaneously overlooking the temporal aspects, might simply be insufficient to strip the criminal act from its mystery. For instance, this was the issue in Hillstead v R case [12], in which the suspect was accused of murdering his landlord. The key evidence that led to his unjust conviction was bloody palm and finger prints deposited at the crime scene, and the unqualified opinion of the prosecution's expert stating that blood traces were left during the crime event, not seven hours later as the suspect testified. This is just one of many examples demonstrating that the forensic arsenal of analytical methods, especially those facing the questions of time, still remains incomplete. With the development of tools

enabling estimation of time elapsed since blood deposition, one could establish the timeline of events or verify if the preserved trace has relevance to the case in question. However, the significance of the bloodstain evidence usually cannot be determined, as a reliable technique of bloodstains dating is still beyond the reach of forensic experts. As a consequence, in the best case scenario, the evidential value of the blood traces can be called into question when confronted with justified doubts about the age of the preserved evidence. In the worst case though, application of unreliable dating methods accompanied by the insufficient knowledge of the subject may, just as in the example above, give rise to misleading expert evidence, and hence result in wrongful convictions.

It is beyond dispute that forensic experts are well aware of this problem. What at first might have seemed a simple question, within years has turned into a scientific battleground, resulting in dozens of publications facing the issue of bloodstains dating. Early approaches to determine the time of bloodstain deposition were based on the visual comparison of blood colour changes [13, 14], which were shortly afterwards downgraded by more advanced and objective analytical tools, with the spectrophotometric- [15, 16], chromatographic- [17, 18] or spectroscopic-based methodologies [19-22] being just a few examples. Insofar as all of the proposed approaches confirmed the time-dependent behaviour of physicochemical properties of bloodstains, none have demonstrated the reproducibility and the precision, regarded as the absolutely essential condition of the successful implementation of developed methods in the standard forensic practice. The intersection between understanding of the process of blood degradation and coping with this understanding in practical proceedings, remains the main challenge for forensic experts, who should find ways to meaningfully apply the acquired knowledge in routine caseworks. It might therefore appear that forensic science has reached an impasse. According to the recent, and to the best of our knowledge the only review paper that deals with the quest for age determination of blood evidence [2], until 2011 no reliable technique of bloodstain dating has been established. Since that time however, there has been a significant progress toward developing a method aimed at estimation of the time since deposition (TSD) of blood traces, and hence the common opinion on the dating problem, which is viewed as the holy grail of the forensic science, undoubtedly should be revised.

The rationale behind this review is to fill the gap in the scientific literature, which has been systematically deepening since the publication of the Bremmer's paper [2], by providing an update of the current research. During the past few years (2011 - 2017), the field of bloodstains dating has grown intensively with some innovative solutions, among which vibrational spectroscopic methods can be considered as one of the most dynamically developing approaches. The escalating popularity of spectroscopy among forensic practitioners should come as no surprise, since it is capable of producing huge amounts of data over a short period of time. As the obtained spectrum of the bloodstain (or any other sample) is the superimposition of all vibrationally active constituents weighted with respect to their concentration, the pivotal question to be answered is how to extract the desired information from these spectroscopic signatures [23]. Fortunately, the solution is increasingly being provided by application of chemometric and statistical techniques, therefore the importance of vibrational spectroscopy has been already recognized within disciplines dealing with the analysis of complex materials such as biological fluids. The combination of abovementioned attributes with high reproducibility, high sensitivity and, above all, with non-destructiveness, makes it perfectly suited for forensic applications.

This article begins with a brief description of the structure of hemoglobin (Hb) and pathways of its *ex vivo* degradation (Section 2.) aimed at equipping the reader with background information, facilitating the comprehension of the problem of bloodstain dating. Then, the discussion progresses to the substance of this review - spectroscopic approaches of bloodstains age estimation (3. Spectroscopic techniques of bloodstains dating), with special consideration given to papers published between 2011 and 2017. Each of the following subsections will be devoted to characterization of a specific spectroscopic

technique, highlighting certain limitations of proposed methodologies. Finally, considerations will be given to the main impediments to the reliable dating of forensic traces (Section 4.) followed by an outline of future studies directed toward estimation of the time elapsed since bloodstain deposition (Section 5.). The importance of the interpretation of the results in terms of their reliability, and hence the necessity of estimating their evidential value, so frequently discounted during the development of dating methods, will be also discussed.

2. **Hemoglobin structure and pathways of its *ex vivo* degradation**

Whole human blood, a liquid connective tissue, consists of approximately 45% cells and cell fragments - red blood cells (RBC), white blood cells (WBC) and platelets - suspended in a medium known as plasma, which owes its liquid form to the high water content, comprising of nearly 90% plasma composition [4]. Apart from water, plasma contains about 10% of solids including proteins, inorganic salts and a host of organic compounds [24]. Once outside the body, blood undergoes a process of complex transformations, which lead to stabilization of a bloodstain. Initial macroscopic changes, resulting in increased viscosity of the blood, encompass coagulation and water evaporation [25]. The process of bloodstain formation itself has been recently studied by researchers dealing with time line reconstruction of events, with the hope that better understanding of the blood desiccation mechanisms will enhance the assessments based on BPA results [25-28]. After the completion of water evaporation process, the most abundant components in formed bloodstains are RBC, and hence the vast number of developed dating approaches were based on the changing properties, either morphological, mechanical [29-34], or chemical [15-21, 35-36] of erythrocytes. In fact, it is not even RBC that has been given a particular attention, but one of its major constituents, appreciated by forensic scientists for its dynamical nature.

Hemoglobin, referred to above, constitutes almost 90% of the dry weight of RBC [4]. Therefore, most of the bloodstain ageing processes involves structural changes of this protein. The structure of Hb remained unknown until the discovery of Perutz in 1959 [38], and it soon became apparent that unravelling the structure of this complex protein was necessary to understand its spectacular role in biology, chemistry or medicine [39]. For a few decades now, it has been and also continues to be a source of knowledge, which provides an insight into the enigmatic process of bloodstains degradation.

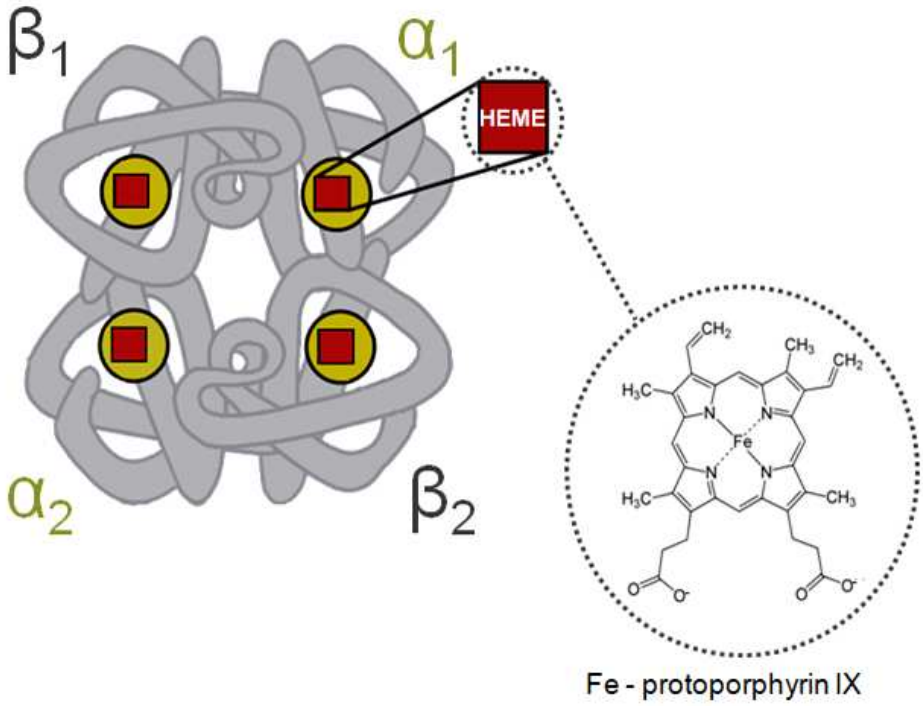

Fig. 1.

Hb, the main blood chromophore, consists of four polypeptide chains, among which two identical α subunits and two identical β subunits can be distinguished, each having 141 and 146 amino acid residues, respectively. As can be seen in Fig.1, within each of these chains one heme molecule, composed of an organic component called protoporphyrin and a centrally placed iron atom, is located. The iron embedded in a protoporphyrin ring of Hb derivatives can appear in different oxidation states, predominantly in ferrous (Fe^{2+}) and ferric (Fe^{3+}) [40]. Depending on this state, oxygen and other small molecules can be bound to iron through its sixth coordination site, giving rise to different Hb-derived molecules [41]. The remaining fifth site is occupied by the proximal histidine (His) from F8 position of its respective polypeptide chain.

In a bloodstream of a healthy individual, two major species of Hb can be recognized - deoxyhemoglobin (deoxyHb), in which the sixth coordination site remains vacant (Fig. 2), and oxyhemoglobin (oxyHb) with a reversibly bonded oxygen molecule (Fig. 2). In the case of deoxyHb, the ferrous ion is placed approximately 0.4 Å outside of the porphyrin plane, whilst upon its oxygenation, electron density is partly transferred from the ferrous ion to the oxygen, leading to the reduction of iron dimensions, and hence enabling its movement into the plane of the organic ring. Even though oxyHb is considered to be a very stable protein, as the iron ion (Fe^{2+}) is protected from oxidizing agents due to its position in a hydrophobic pocket [42], it is subjected to auto-oxidation at a rate of approximately 3% per day, resulting in formation of methemoglobin (metHb; Fig. 2) and a superoxide radical [43]. At this point, however, it should be mentioned that erythrocytes present within bloodstreams are not defenceless against the oxidation-induced deterioration, since they have been equipped with proper enzymes - gluthatione peroxidase and methemoglobin reductase - which recycle the metHb back to deoxyHb [44]. As a consequence, the intracellular amount of metHb should not exceed 1% of total Hb species [16, 45, 61]. Most of the processes of Hb transformations mentioned above are paralleled by alterations in the arrangements of polypeptide chains, leading to conformational changes of Hb molecule. Thus, it should become clear that the dynamical nature of Hb

may be considered as an effect of the rearrangements of the electrons within the iron ion, occurring upon its ligation. Finally, and perhaps most importantly, this Hb dynamic manifests itself also during the *ex vivo* degradation of blood samples.

Unfortunately, contrary to transformations of the Hb that take place *in vivo*, the knowledge about the transitions between different Hb derivatives occurring outside the human body remains superficial. On the other hand though, when the forensic perspective is taken, one could ask if it is absolutely essential to identify all of the components of degrading bloodstains. Examiners developing multivariate dating models based on spectroscopic data usually are not focused on naming individually each component, which gave rise to the observed signal. However, chemometric analysis always should be supported with the chemical interpretation of spectral changes allowing for identifying the relevant variation in spectra, and hence at least a basic understanding of the *ex vivo* Hb transformations should be gained.

Once the bloodstain is created, the cascade of physicochemical processes, leading to blood degradation, begins (Fig. 2). Initially, these ageing changes follow the *in vivo* transformations, involving saturation of deoxyHb to oxyHb with the ambient oxygen, followed by the auto-oxidation of oxyHb to metHb. That, in fact, is where the parallels between the *in vivo* and *ex vivo* transitions cease. Outside of the cardiovascular system, enzymes responsible for converting metHb back to deoxyHb are no longer available, since they become inactivated due to denaturation during the drying process [46]. Thus, the degradation procedure continues with reversible and irreversible [35, 47] formation of hemi- and hemochromes (HC), in which iron, either in the ferric (hemichrome) or the ferrous (hemochrome) low-spin state, coordinates both distal and proximal His residues [40, 45-47]. In order to enable formation of these bis-His complexes, changes of the hydrophobic heme pocket geometry take place, triggering conformational rearrangements in protein chains of Hb [48]. According to Colombo and Sanches [47], some of these conformational changes occurring during the transition from metHb to HC are reversible upon rehydration. The picture that is emerging from this study suggests that dehydratation-induced HC should not be considered as denaturated forms of Hb, but its thermodynamically accessible derivatives. It has been also demonstrated that *in vivo*, under certain conditions, HC may be subjected to further oxidative damage, which results in dissociation of the polypeptide chains and release of the heme moiety [49, 50]. However, little is known about subsequent transformations of denaturated HC during bloodstains ageing. What is certain, is that the sixth coordination site of the iron atom, once again, is where all the action develops. The structural alterations of Hb, evolving during the oxidation and denaturation process, are accompanied by changes of its physicochemical properties, such as magnetic behaviour, spin configuration or optical absorption. Therefore, monitoring of these changing Hb properties with suitable analytical techniques may become an opportunity to decipher the temporal information, hidden within the bloodstain.

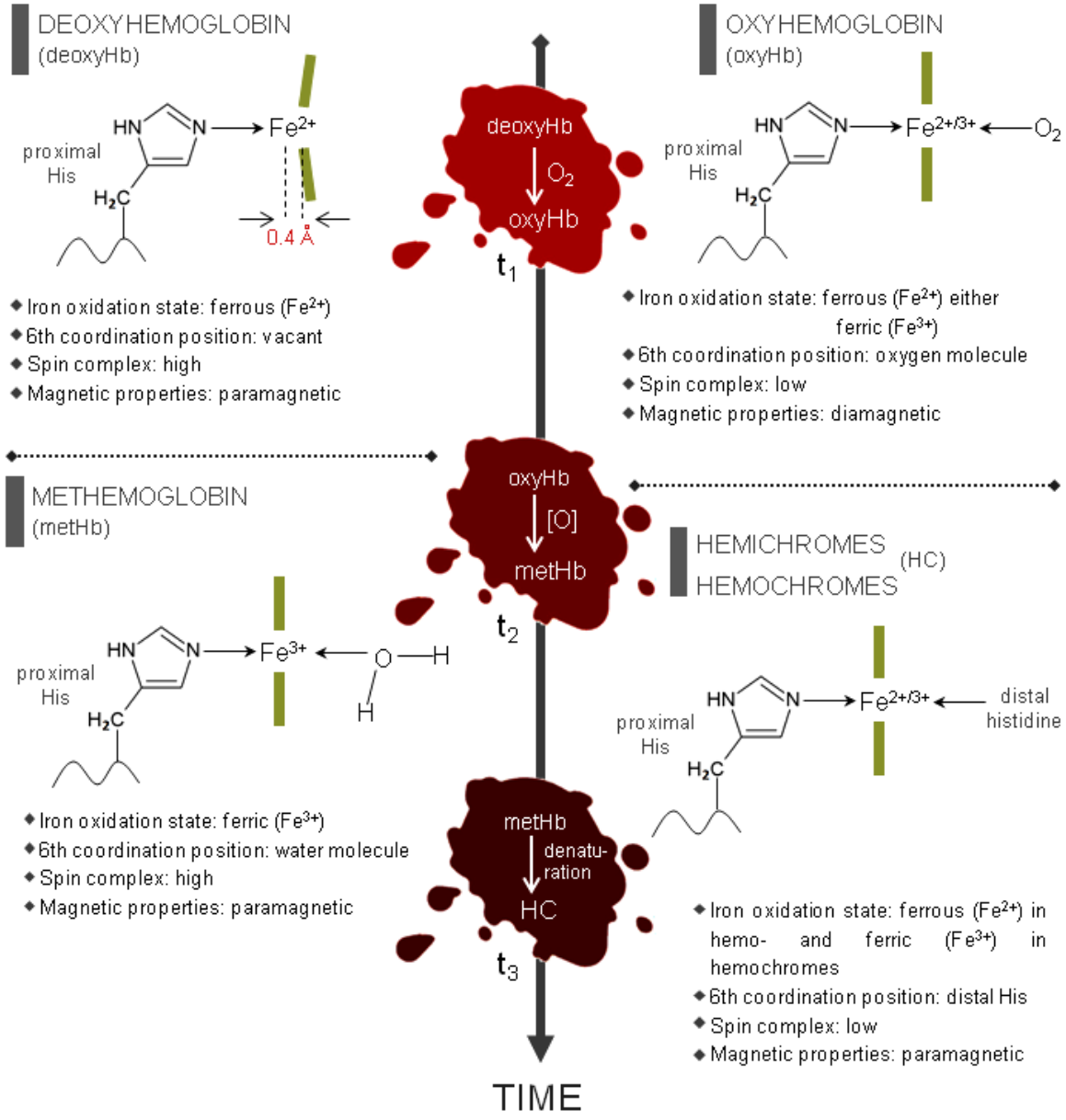

Fig. 2.

2.1 Factors influencing bloodstains degradation

Obviously, time is not the only factor affecting the process of blood decomposition. The real world is not a vacuum, and hence the wide range of external conditions, to which the traces might have been previously exposed, cannot be ignored. Among these contributing factors (Fig. 3), three different groups should be distinguished: differences in blood composition including between- and within-person variance; substrate properties and, perhaps most importantly, storage conditions of the questioned bloodstain. Unfortunately, the already limited numbers of studies investigating this issue have been performed with numerous analytical methods of varying sensitivity, aimed at monitoring different properties of ageing bloodstains, which may partly explain the contradictory conclusions reached by researchers. It should be also clearly stated that this subsection considers only those factors, which may have a direct effect on the process of blood degradation itself. The influence of other method-related parameters that may trigger the process of artificial ageing of bloodstains will be discussed in following sections.

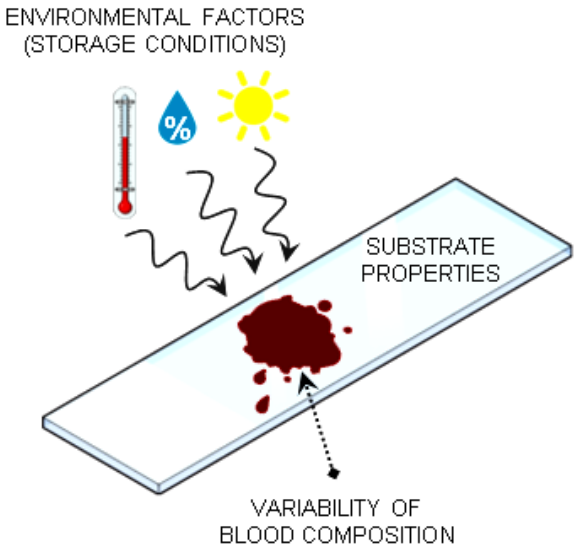

Fig. 3.

2.1.1 Variability of blood composition

In a PhD thesis devoted to bloodstain pattern analysis Larkin noted '... blood can differ greatly from person to person; it is unknown what the significances of such changes in the components of blood will have on the drying bloodstains' [51]. The current state of knowledge on the dependency between the initial blood composition and the rate of its ageing is in a similar position, since a comprehensive evaluation of the influence of intrinsic blood properties on the process of bloodstain degradation is, as of yet, impossible. This is because, in order to avoid introducing additional variability, the overwhelming majority of studies was performed with blood samples collected from a single donor or small groups of people of similar age, biological sex, race, health conditions or habits. Even if the sample population encompassed blood originating from different individuals [19, 21, 52-57], the number of analysed bloodstains was usually insufficient to draw reliable conclusions.

There is no doubt, however, that the aspect of blood variability requires further investigation [3, 57]. Blood biochemistry can be considered as a reflection of health condition of human body. It is not without reason that spectral signatures of blood have been used to monitor progression of numerous diseases such as diabetes, particular types of cancer or malaria [58]. Studies performed by Lekka et al. [59] and Bryszewska [60] evidenced that some of these ailments, for example hypertension or diabetes, can cause changes in the chemical composition of RBC membranes, leading to differences in the erythrocyte stiffness between healthy donors and those suffering from above-mentioned disorders. For example, when elastic properties of RBC investigated with atomic force spectroscopy (AFM) serve as a basis for bloodstains age estimation [29, 30], it would be extremely negligent not to take into account the possible influence of these aberrations. Needless to say that this dependency between blood composition and its ageing may become even more significant, once diseases directly affecting Hb molecules are considered. Methemoglobinemia, a state characterised by the increased content of metHb [43], is a perfect illustration of this. It should be reminded that upon exiting the circulation system of healthy human body, blood rarely contains more than 1% of metHb [16, 44, 45, 61]. In specific cases, this particular form of Hb may be formed *in vivo* in excessive amounts as a result of the genetic defect in the metabolism of RBC, as well as the exposure to various oxidant drugs or toxins [43, 49, 61]. Having looked once again at Fig. 2, our theoretical conclusion

should be that in case of different starting values of metHb, as well as varying physiological conditions regarding the metabolism at the time of the bloodshed, the conversion between Hb derivatives may occur with different rates. However, this kind of hypothesizing must be followed by extensive population studies, which should consider not only blood related disorders, but also other possible sources of human variability including:

- different ages, races, biological sexes of donors [57];
- carboxyhemoglobin levels, which may be elevated in blood of smokers, victims of fire or carboxymonoxide poisoning [3, 62-65];
- intake of medication, in particular blood thinners such as aspirin, which interfere with blood clotting action [21, 66];
- diet [43, 67-75].

When it comes to the impact of dietary factors on blood ageing mechanisms, the evidence supporting this concept is even scantier. Higher levels of metHb in human blood were associated with increased concentrations of nitrates in food [67] and drinking water [64, 67, 74, 75]. Recent results of high resolution Raman imaging of RBC in dried smears have also revealed that, during the drying process, carotenoids of blood plasma tend to surround the RBC, which may give an indication of their contribution to the formation of HC in the periphery of erythrocytes [68]. Even though these preliminary findings are still not solid proof, it cannot be excluded that the degradation of Hb is affected by fluctuating levels of blood components, which have been confirmed to depend on the individual's diet [69]. The point is, if dietary factors, health condition or lifestyle have an established impact on the blood composition, one cannot simply assume that the process of blood degradation will always follow the same pathways, irrespective of the donor.

2.1.2 Environmental factors

The influence of environmental factors on the process of blood degradation is perhaps one of the most substantial impediments to establishing a universal approach for bloodstain dating. Patterson was one of the first to notice that the rate of bloodstain ageing, monitored in this case with reflective measurements of the blood colour changes, is intimately linked to the storage conditions of the trace, such as humidity, temperature and light exposure [15]. However, this kind of study, focusing on the susceptibility of bloodstain degradation process to environmental disturbances, for a long time was rather unpopular among researchers. Only recently, when the implementation of spectroscopic dating methods is within reach of forensic examiners [3], a great effort is put into making the exploitation of these methodologies practical. Such research is sorely needed, since the investigation of the ageing kinetics under various environmental conditions would be critical to the precise estimation of the TSD of a questioned bloodstain [19, 35].

The effect of temperature is, without a doubt, one of the most extensively researched factors affecting the ageing of bloodstains. As with any other chemical processes, the conversion between different forms of Hb, including oxidation of oxyHb to metHb followed by the formation of HC, is temperature dependent [36], and hence the relatively high level of attention paid to the issue of the temperature dependency should come as no surprise. Regardless of the method applied to monitor the blood degradation, results of conducted studies unequivocally demonstrated that elevation of the storage temperature enhances the bloodstain ageing [35, 53, 76-81]. According to Bremmer et al. [80], both transition rates of biphasic oxidation of oxyHb to metHb (Fig. 4), corresponding to fast oxidation of α chains (k_f) and slow oxidation of β chains (k_s), as well as the transition rate of metHb to HC described as k_2, are strongly affected by temperature.

$$oxyHb \xrightarrow{k_1=k_s+k_f} metHb \xrightarrow{k_2} HC$$

Fig. 4.

In case of the fast and slow oxidation, 6-fold and 18-fold increase, respectively, was reported over the temperature range of -20 °C to 37 °C. Moreover, Hanson et al. [35, 79] emphasized that increased temperature contributes not only to a faster rate, but also to a greater extent of these transformations, at least when the hypsochromic shift of Hb Soret band (λ_{max} = 412 nm) is taken into consideration as a parameter depicting the bloodstain degradation. All these observations lead to the conclusion that fluctuations of temperature may result in incorrectly estimating the time since bloodstain deposition, therefore their influence cannot be ignored. It is also worth noticing that both Hanson et al. [35] and Thanakiatkrai et al. [53] demonstrated a certain slow-down in the decomposition of bloodstains stored at low temperature (-20 °C), as, in spite of elapsing time, no significant changes in monitored blood properties were observed. This finding may be a great asset in real-world settings, serving as a method of sample preservation before the analysis. Admittedly, storing bloodstains in deep-freeze conditions cannot be regarded as an entirely non-destructive method of sample preservation [82]. However, halting the post-collection ageing changes, even if quasi-destructive to the evidence, still seems more reasonable than running the considerable risk of reaching a stage of advanced degradation, in which alterations of bloodstain properties are no longer observed.

Apart from the time and temperature, the process of blood decomposition is also a function of ambient relative humidity (RH) [35, 53, 80]. Research of Hanson and Ballantyne [35], as well as Thanakiatkrai et al. [53] exposed the decrease in the rate of ageing with increasing values of RH. Through exploring the ageing behaviour of the samples stored under different humidity ranges at 22 °C and 30 °C, group of Hanson evidenced that the scale of this effect may be also affected by the temperature, suggesting that both of these factors - temperature and RH - may in fact counteract or alleviate each other. Bremmer et al. [80] investigated the influence of humidity even more closely. A reflectance spectroscopy study of ageing bloodstains revealed that the first stage of degradation, the auto-oxidation of oxyHb, was not influenced by the RH, in contrast to the subsequent conversion of metHb to HC, which was highly dependent on the RH. Interestingly, it was observed that for humidity reaching 100%, the k_2 reaction rate constant (Fig. 4) tends to zero, preventing the formation of HC, which is fully in accordance with the previous findings of Tsuruga et al. [83]. The dependency between the RH and the transition between metHb and HC has been also demonstrated by Colombo and Sanches [47], who observed that some of the conformational changes, occurring during this conversion, are reversible upon rehydration. Hence, the concept of extracting bloodstains from the interfering substrate or collecting blood specimens with a moistened cotton swab need to be explored in depth [82], as this method of sample preparation may result in incorrect estimations of TSD.

The effect of electromagnetic radiation on blood decomposition appears to be even more complex, since performed studies brought contradictory results. In order to investigate the influence of illumination, bloodstains were exposed to different sources of irradiation - sunlight either fluorescent light - and compared with those stored in the dark. Experiments of Miki and Ikeya [76], Fuijta et al. [78] and Thanakiatkrai et al. [53] demonstrated that the rate of bloodstain decomposition increased when the stain was subjected to sunlight. Bauer et al. [84], however, obtained completely different results, showing that blood traces exposed to sunlight and those protected from the natural light sources did not exhibit practically any significant differences in the rate of ageing. As already mentioned, apart from exposure to the sunlight, samples were also irradiated with fluorescent light, which is one of the most common forms of artificial lightning. Similar ageing pattern was observed for the groups of samples kept in the dark and under irradiation at room temperature, suggesting that the fluorescent light has little [18] or no effect [53, 76] on the changes in the rate of bloodstain

decomposition. According to Inoue [18], however, differences start to appear with increased temperature, as, after the storage of samples at 37 °C in the fluorescent light, a facilitating effect on the ageing changes was observed in comparison to bloodstains protected from the irradiation. Taking this results into consideration, it seems that it is a combination of temperature and light exposure that may have a decisive impact on the ageing process. Therefore, conflicting conclusions drawn by researchers may have arisen due to the differences in the storage temperature of bloodstains, either, as pointed out by Bremmer et al. [2], the ability to observe the effect of light exposure on the rate of ageing is method-specific.

Finally, it should be borne in mind that bloodstains of questioned age may be also subjected to the outdoor environment, exposed to the natural, hardly ever controlled fluctuations of weather conditions, soil, air pollution or even bacterial growth [35], and hence the influence of these possible sources of contamination must be carefully examined. Even if the wide variety of these contributing factors may seem overwhelming, understanding their impact on the ageing kinetics of bloodstains is indispensable to translate the laboratory results to the forensic practice [80].

2.1.3 Substrate properties

The complexity of the ageing issue may be additionally exacerbated by the blood - substrate interactions. Although the truth is that researchers have attached little importance to the effect of underlying substrate, marginalising it in favour of the investigation of factors discussed above. Indeed, it might appear a reasonable expectation that the surface, on which blood was deposited, should not have a significant influence on the biochemical transformations of ageing bloodstains [57], in contrast to the biophysical properties of RBC [29]. This assumption was partially endorsed by study of Hanson *et al.* [79], which demonstrated no noticeable effect of underlying substrate on the hypsochromic shift of the Hb Soret band, a parameter correlated with TSD. However, these results constitute only partial evidence in support of the investigated hypothesis, as the research involved solely absorbing materials such as cotton, polyester, denim and printer paper. Yet it is known that properties of impacted surface, apart from the composition of the blood and forces acting upon a drop, influence the size, shape and, more importantly, the thickness of the resultant bloodstain [51], which dictates its drying time. What is more, porous surfaces, which allow soaking the blood into the material, generally produces longer drying periods [4, 66]. Since, according to Hanson [35], the oxidation of Hb occurs faster in dried rather than in liquid states of stains, therefore it should be interesting to compare the rate of ageing observed for bloodstains deposited on porous and non-porous substrates, and verify whether the differences in drying time of blood affect the TSD measurements.

Researchers, being aware of the wide range of external factors affecting the sample degradation, in some cases emphasize that the applicability of newly developed methods is limited to bloodstains with known storage history [77, 80, 84]. In practice, however, forensic examiners are usually not provided with information on these factors. Precisely for this reason, development of the universal tool, enabling estimation of the absolute age of the trace may be troublesome, if not impossible to accomplish. One of the possible solutions to this dilemma may be establishing a method of relative dating of a questioned trace, which has been further discussed in Section 4. Moreover, as suggested by Edelman *et al.* [85], in case of the accessibility of a crime scene or, in general, a place where the questioned bloodstain was revealed, it might be reasonable to investigate *in situ* the ageing kinetics of blood. Perhaps, examination of a trace stored under exactly the same conditions, will bring forensic practitioners a step closer to the reliable estimation of TSD.

3. Spectroscopic techniques of bloodstains dating

During last few years, researchers specializing in age examination of bloodstains have focused

mostly on spectroscopic techniques. Among these, approaches based on the visible range of the electromagnetic spectrum have received the greatest attention (approximately 56% of the articles published between 2011 and 2017), whereas the remaining studies have benefited from the fluorescence as well as vibrational spectroscopy (NIR and Raman). Table 1 summarizes papers focused on the estimation of TSD of bloodstains by spectroscopic techniques.

Table 1
Recent spectroscopic techniques (2011 - 2017) applied in the bloodstains dating

In fact, no one should be surprised by this spectroscopy-directed course, taken by the scientific community. It cannot be denied that investigation of interactions between electromagnetic radiation and matter is an effective way of probing not only the chemical composition of samples, but also their physical properties. So what exactly occurs when light encounters the surface of the matter? Depending on the wavelength of the electromagnetic radiation and the physicochemical properties of the investigated material, various kinds of interactions might take place [86]. When the beam of incident radiation strikes the sample, a portion of the radiation undergoes specular reflection (Fig. 5a), which is determined by the refractive index - a measure of bending a ray of incident light, resulting from the difference between optical densities of interfacing media [82]. Since, in this case, the radiation does not penetrate the sample in depth, the interaction with electrons of atoms and molecules of the material is limited, thereby this phenomenon simply cannot offer rich chemical information, and rather serves as an obstacle to measurements of diffusively reflected light. The situation is completely different when penetration of the sample's interior occurs, leading to scattering and absorption of the incident radiation, which can yield valuable insights into the chemical characteristics of the specimen.

Upon irradiation, a photon might be absorbed by the matter and immediately scattered in all directions. In this case, there is no need for the photon to correspond to the energy gap between the ground and the excited state of molecule. When the scattered light has exactly the same wavelength as the incident radiation, the phenomenon is named elastic or Rayleigh scattering (Fig. 5b). Elastic scattering dominates less intense inelastic scattering, named after its principal discoverer as the 'Raman effect' (Fig. 5c), which poses lower or higher wavelengths than the incident light. Differences in energy between scattered and incident radiation corresponds to the vibrational states of the investigated molecule [87], and exactly these varying wavelengths of scattered light constitute a Raman spectrum.

The second of the aforementioned phenomena, an absorption of radiation (Fig. 5d), corresponds to the excitation of the outer electrons of the molecule when occurring within the UV-VIS range; whereas absorption bands observed in the NIR or MIR region originate from vibrations of molecules. Contrary to scattering, the energy, instead of being re-emitted immediately after absorption, may be transformed into the heat (vibrational energy of the matter), transferred to another molecule [82] or released in the form of electromagnetic radiation, giving rise to another type of interaction - photoluminescence (Fig. 5e).

In case of the whole blood, both scattering and absorption (within the wavelength range of 250 - 1100 nm) are predominated by erythrocytes with regard to other blood components by two to three orders of magnitude [88, 89]. When moving beyond 1100 nm, toward the NIR range of the electromagnetic spectrum, blood absorption becomes dominated by the absorption of water molecules. However, as soon as water evaporation completes, small absorption peaks, between 1690 and 2400 nm, assigned to Hb, albumin and globulin [56], can be identified. In essence, if the main contributor to each of abovementioned phenomena are RBC along with Hb molecules, then the interaction of light and investigated matter, which underpins spectroscopic techniques, appears to be an adequate tool to characterize the ageing bloodstains.

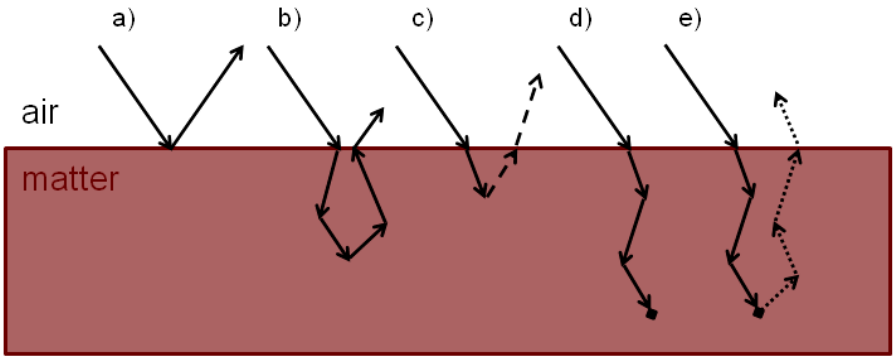

Fig. 5.

It is worth noticing that the real power of spectroscopy lies not only in the possibility of investigating physicochemical properties of the matter, but also in providing highly detailed characterisation of these properties. This is because spectroscopic techniques are capable of producing a huge amount of data. Data, which in some cases can be arduous to handle. Nevertheless, it would be a mistake to refrain from taking advantages of chemometrics, and restrict the interpretation of spectra to monitoring one or two selected variables, corresponding to particular wavelengths or wavenumbers of spectral signature. Considering the example of bloodstain ageing process, it is highly unlikely, as already emphasized by Li et al. [36], that all of the spectral information on the blood degradation will be contained in just a few features of the spectrum. On the other hand though, these crucial variables might be hidden within large spectral regions with low information content, just as in case of the NIR data [91]. Since implementation of poorly informative features into discrimination or calibration models might result in their low performance, application of feature selection tools seems inevitable. Naturally, visual selection of variables for multivariate data analysis should be avoided, since this approach would, at least partially, suffer from lack of objectivity. Not to mention that human sense of sight is not ideally suited for recognising subtle spectral differences, which might be crucial to distinguishing between samples. As a consequence, modern spectroscopic analysis cannot exist without the support of chemometric tools. Obviously, no method of data handling, no matter how advanced and sophisticated, can serve as a substitute for badly performed analysis. However, a competent combination of these two can become a powerful tool in hands of examiners, and hence implementation of chemometric-enhanced spectroscopy within the field of forensic bloodstain dating was only a question of time.

3.1 Colour analysis and ultraviolet-visible spectroscopy

Techniques based on the UV-VIS region of the electromagnetic spectrum have been presumably one of the most widely employed for the investigation of the biological material. In proteins - and thus in the Hb - three types of chromophores can be distinguished: peptide bonds, amino acids within the side chains (such as Trp and Tyr) and, above all, prosthetic groups, with heme being probably one of the most cliché, yet important examples [92]. Porphyrins, constituting numerous prosthetic groups, demonstrate several absorption bands in the visible region of the electromagnetic spectrum, with three distinct maxima - Q band (α and β) between 490 - 650 nm and the most intense Soret band in the region of 400 - 436 nm. These bands are interpreted as $\pi \rightarrow \pi^*$ transitions, deriving from delocalisation of the electrons, which extends throughout the tetrapyrrole ring of porphyrins [92, 93]. The Q band, as well as the Soret band is known to be very sensitive to changes exerted by surroundings, taking place during ligand exchange processes, presumably through the nitrogen atoms of pyrrole rings. Fortunately for dating studies, the VIS spectrum of Hb also demonstrates the

capability to act as an indicator of interactions between the protoporphyrin ring and the surroundings [93]. As a consequence, Hb derivatives exhibit different absorption spectra (Table 2), and hence VIS spectroscopy can be applied in structure - age studies of Hb, aimed at making the question of time elapsed since bloodstain deposition answerable.

Table 2
Absorption maxima of the three main chromophores in bloodstains (oxyHb, metHb and HC)

Since the alterations in the absorption properties of the matter are tightly linked with its colour changes, from the very beginning of blood dating studies, researchers have been making attempts to correlate the colour of the blood trace with its age [15, 16, 35, 80, 94]. In spite of this obvious relationship, a method, which could possibly serve as a reliable dating tool, is not yet available. However, the subfield of bloodstains dating, founded on the broadly defined methods of colour analysis, has come a long way and the recently renewed interest in the exploitation of VIS spectroscopy has brought some remarkable solutions.

In 2011, Li et al. [95] used microspectrophotometry (MSP) in order to register the visible reflectance spectra of degrading bloodstains, which were monitored between 1 and 37 days since their formation. The progressive transformations of the Hb molecule were reflected most distinctively within the 442 nm - 585 nm range of acquired spectra, which included the α and β absorption bands of Hb. Through application of feature selection methods (Fisher's weights and Fourier Transform-based approach) preceded by spectral pre-treatment (de-trending and Standard Normal Variate, SNV), variables, serving for establishing a classification model for TSD predictions, were extracted from among 175 features collected per spectrum. It should be emphasized that this methodology was actually one of the few approaches among published studies, in which particular attention was devoted to the step of spectral processing. Subsequently, a supervised discriminant technique, linear discriminant analysis (LDA), was used to estimate the age of a questioned bloodstain. A point worth mentioning is that the applied statistical model - LDA - is in fact a discriminant technique, categorical in its nature. In this method, a sample is always classified into one of the mutually exclusive subspaces, which are defined within multidimensional feature space by the number of groups distinguished within the data structure. Hence, a bloodstain of unknown age can only be assigned to a previously defined group, corresponding to the particular TSD, which has been used for the model development. However, from the forensic perspective, this kind of categorical reasoning should be avoided, especially when the continuous nature of ageing processes is taken into consideration. Therefore, one should aim at applying soft-modelling techniques - such as soft independent modeling of class analogy (SIMCA), where any new sample can be allocated to one of the defined classes, but also may be an outlier to all the classes - or calibration methods offering regression-based models, even more adequate for the dating studies. Nevertheless, LDA has led to some promising results - up to 99.2% correct classification rate (CCR) were obtained when training as well as test set of spectra derived from the same bloodstain. In the case when separate bloodstains constituted test and training sets, LDA demonstrated poorer accuracy, as the CCR equalled 54.7% up to 19 days elapsed since stain formation, with an average error of 0.71 days. Finally, it should also be highlighted that application of hyphenated instruments that integrate a light microscope with a spectrophotometer might not always be the reasonable choice. It is true, as noticed by Li et al. [95], that MSP allows the observation of small bloodstains and ensures reproducible measurements from well defined location, since spectra can be recorded from areas even as small as 5 μm x 5 μm. However, providing an overall characteristics of an inhomogeneous specimens (such as bloodstains) is a well-established problem that affects not only the MSP-based studies [96], but also any other fields of research, which employ techniques combining microscopic and spectral methods [97-101]. When the area of the specimen being illuminated (e.g. by the laser beam in Raman spectroscopy) constitutes

only a small part of the examined sample, one should always consider the risk of subsampling. To put it differently, the probed area of the specimen might not be representative of the characteristics being investigated. As a consequence, application of hyphenated techniques imposes a necessity to record multiple spectra from different points of the heterogeneous sample. Clearly, the more replicates of the single point measurements are obtained, the more reproducible and accurate information on the state of the sample under examination is delivered. However, collection of large number of spectra requires time, and, through that, it is not always appropriate for monitoring time-dependent properties of bloodstains or any other material. Hence, in this particular case, substituting microscope objectives with fibre-optics systems, which allow to record the spectral signal once from the larger area within a short timeframe, might be preferable.

Indeed, at present, the most widespread method of colour measurements is reflectance spectroscopy utilizing an optical fibre probe, which provides excitation light to the material of interest, collects the reflected signal, and delivers it to the spectrometer. Recently, Bremmer with co-workers [80] exploited the potential of this method to demonstrate that chemical composition of bloodstains, stored under controlled conditions, exhibits a distinct temporal behaviour. Since at each time point, over a time period of 0 - 60 days, a unique combination of three Hb derivatives (oxyHb, metHb and HC) was observed, it was possible to relate these time varying values to time elapsed since bloodstain deposition. In order to extract the values of the oxyHb, metHb and HC fractions from the reflectance spectra, a Linear Least Square algorithm (LLS) was applied, which fitted between experimental spectra of bloodstains registered within 450 nm - 800 nm, and the reflectance spectra of three Hb derivatives, obtained by application of Kubelka–Munk theory with the known absorption spectra of these pure components serving as an input. Eventually, obtained estimates of Hb fractions were plotted versus time, giving rise to ageing curves, which allowed to estimate the age of the bloodstain. However, the estimates were characterized by large errors, especially at the advanced stage of degradation - for example, at an age of 3 days, the estimated TSD ranged from 1.5 to 6 days, whilst for a bloodstain with an actual age of 35 days the estimated TSD varied between 25 and 55 days. The burning question which arises is whether these incorrect estimations resulted from the fact that with prolonged period of ageing only subtle spectral changes were observed, or whether from executing the LLS algorithm on raw data, non-subjected to any pre-treatment methods, aimed at reducing variations due to scattering effects and path length variations. Possibly, combination of both factors contributed to the observed discrepancies, however the predictive accuracy of the proposed methodology might have been, at least partially, improved through implementation of spectral processing tools. Crucially, the authors have also suggested that during the degradation process, no other derivatives of Hb, apart from metHb and HC, are formed, since the sum of the three Hb fractions was close to one, and did not vary over time.

This issue, the aspect of oxyHb auto-oxidation, was properly addressed in the following study of Bremmer et al. [102], where the conversion of oxyHb to metHb and HC was further investigated, with particular attention given to the influence of RH and temperature on these reaction rates. As a consequence, this study deviated from the usual way of proceeding, when empirical methods were employed to interpret the ageing behaviour of bloodstains, and rather focused on understanding the oxidation process and its dependency on external factors, which most of the previous studies appear to have failed to do. According to Bremmer et al. [102], the auto-oxidation of oxyHb follows biphasic decay with a rapid first step, which slows down after approximately 10 hours since bloodstain formation. These oxidation rates can be described as first-order reaction kinetics, and have been proven to be severely affected by temperature, whilst RH appeared to impact only the met-Hb into HC transition. A more detailed description of the dependencies between the rate of blood degradation and its storage conditions can be found in the subsection 2.1.2.

In 2017, Sun et al. [52] followed the path marked out by Li et al. [95] toward application of multivariate chemometric methods in spectral analysis of bloodstains. As stated by the authors, the

ultimate goal of the research was to develop statistical models, which would outperform the solutions proposed in previous studies in terms of their robustness. In order to improve the accuracy of age estimations, instead of discriminating models employed by Li et al. [95], three different regression techniques were implemented to SNV-processed data, namely PCR, PLSR and LS-SVM. Additionally, given the remarks of Bremmer et al. [102] on biphasic decay of Hb, calibration models were developed on three different periods of bloodstain degradation, corresponding to 2 - 24 hours, 1 - 7 days and finally 7 - 45 days elapsed since blood deposition. This modification allowed to significantly improving the predictive capability, yielding the best results for LS-SVM models. Hence, these findings once again have evidenced that proper data handling, and not necessarily development of novel analytical techniques, is an important venue for future research.

Despite these conclusions, researchers obviously continue pursuing new analytical tools. With advances in modern technology, equipment intended to be used at the crime scene is becoming smaller, portable and more user-friendly [90], giving rise to handheld devices, which can be used by people who are neither analysts nor spectroscopists. As a result, a field-deployable instrumentation, such as HSI systems [36, 65] or even smartphones [53, 54], are anticipated to grow rapidly in popularity in the near future [103].

HSI, an emerging field in forensic science [90, 104-110], combines the advantages of optical spectroscopy (from UV to MIR ranges of electromagnetic spectrum) with conventional two-dimensional imaging. In this technique, each pixel of the taken image contains a registered spectral signature. Subsequently, this spectral information is added to the spatial image as a third dimension of values, giving rise to a three-dimensional data cube. Exactly this opportunity provided by the method, the possibility to investigate side by side, both spatial and spectral information, is what makes HSI particularly appealing, especially when the analysis of heterogeneous samples is considered. This is because, within a single examination route, it enables to overcome the major drawback of all spectroscopic single-point measurements, namely an insufficient representativeness of obtained spectrum with regard to the entire surface of the sample.

It cannot be denied that degrading bloodstains fall within the category of non-homogeneous specimens. No wonder, then, that HSI has been applied also in blood dating studies, providing an insight into the chemical changes within the ageing stain. Since the analysis of bloodstains performed in the VIS spectral range had been previously demonstrated to provide valuable temporal information, both Edelman et al. [65] and Li et al. [36] employed visible HSI to characterize the ageing behaviour of blood specimens. The foundation of an approach proposed by Dutch researchers [65] were relative amounts of oxyHb, metHb and HC derived from the hyperspectral images of analysed bloodstains. A non-linear spectral unmixing model allowed to extract the relative fractions of these Hb derivatives, which were subsequently employed to estimate the age of the trace up to 200 days since deposition. Additionally, to verify the applicability of the approach in real case scenarios, the absolute age of bloodstains, revealed at a simulated crime scene, was estimated through its comparison with a reference dataset of Hb fractions; whereas the chronology of bloodstains formation was established by clustering the questioned traces, which exhibited similar values of monitored age-indicators.

With the aim of developing a HSI system simpler than that used by Edelman et al. [65], researchers led by Li [36] established an approach for precise TSD estimation of bloodstains, which did not require high spectral resolution (<5 nm). As in the previous study of Islam's group [95], the development of a statistical model (LDA) was preceded by pre-processing of spectral data (averaging and denoising) followed by the feature selection procedure (Fisher weight's). This tactic has been proven to be profoundly effective, since it enabled estimation of fresh bloodstains with an average error of ±0.27 days for the first 7 days and an overall average error of ±1.17 days up to 30 days. However, the authors themselves admitted that these were only proof-of-concept results, obtained under rigidly controlled conditions. Strictly speaking, their technique may not be suitable for real case scenarios due to possible

lack of precision arising from the influence of environmental factors, which, as yet, has not been properly investigated.

Finally, it is also worth noting that both HSI-based methodologies were proved to work best in different time ranges - Edelman's approach yielded better TSD estimations from one month onwards, while Li's study outperformed the former dating model within the period of first two weeks since bloodstain formation. Consequently, as rightly pointed out by Larkin and Banks [103], these two methods appear to be complementary and, used together, might give better view of the age of a questioned trace. However, it still should be borne in mind that both approaches might not meet the demands of routine forensic casework. The real challenge posed by the field examinations would be the analysis of bloodstains under different geometrical setups - under varying angles and with different distances to the camera. Even though Edelman et al. [65] have managed to reduce the spectral variability resulting from physical conditions during the measurement (e.g. illumination intensity), overcoming the hurdles of substrate interferences in non-destructive manner might be a well-nigh impossible task. The main disadvantage of using the visible HSI is one, which is common to all of the TSD estimations methods operating in VIS range of electromagnetic spectrum, and is a particularly serious one - the absorbing properties of coloured backgrounds are expected to hamper the signal originating from the investigated bloodstain. Accordingly, the influence of strongly coloured substrates, but also materials exhibiting specular reflection, should be promptly investigated, as in both cases, introduced spectral distortions might be difficult to remove, even when using spectral pre-processing techniques [36].

Surprisingly, HSI systems are not the smallest instruments among field-deployable devices. One of the most innovative approaches proposed recently was method developed by Thanakiatkrai et al. [53], which was based on the colour values (RGB, CMYK, HSL, HSV) extracted from the digital images of bloodstains taken with a smartphone camera. When a digital image is formed, light, reflected through RGB colour filters, is converted into the digital signal (e.g. RGB values) by the image sensor. The final colour of each pixel, which is the smallest constituent of the display device, depends on the intensity of these signals. Authors have hypothesized that the colour changes of bloodstains would be quantitatively reflected in their digital images, and that by correlating extracted digital signals (RGB values or their counterparts) with TSD, calibration models for age estimations could have been obtained. Indeed, a machine learning classification algorithm, utilizing all of obtained colour values, gave rise to a classification model capable of estimating TSD up to 42 days with high accuracy (for details see Table 1). The developed model was also proven useful in a crime scene environment, as 83% prediction accuracy for uncontrolled mock casework stains was achieved. Obviously, this analytical approach is not flawless. Methodology developed by the group of Thanakiatkrai is highly dependent on the type of applied camera, illumination system (light source intensity and distance to the bloodstains), and, just as any other method operating in the VIS range, the interferences originating from the coloured backgrounds.

In 2017 Korean researchers led by Shin made another step forward by developing a *Smart Forensic Phone*, a device equipped with a smartphone application [54]. The idea behind this project was to design an inexpensive and manageable tool, which will provide an analytical instrument useful also in the hands of non-professionals. In this newly proposed solution, the V value of HSV colour system, converted from the initially registered RGB values, served as the age-indicator. A rapid decline of the monitored ageing parameter reached a plateau after 42 hours since bloodstain formation, and hence the time frame, for which this methodology is applicable, is significantly shorter than for the approach of Thanakiatkrai. Additionally, contrary to previous findings [53], it was reported that the V colour values were not affected by the varying degree of illuminance or different angles of image acquisition. However, authors have restricted themselves to only vague remarks on this issue, stating that '... the V values were not greatly different', 'the changes in colour intensity ... did not change seriously' or 'no significant difference was detected', without providing any proof of these declarations, failing to explain what these fine statements actually mean. Even though the concept of developing a user-friendly sensor for TSD

estimations is undoubtedly an idea worth spreading, perfectly fitting within current analytical trends [111-115], the importance of proper method validation cannot be diminished. Therefore, the problem of technique robustness should be properly addressed by investigating the dependencies between TSD estimations and various analytical setups. With this background, much more detailed world might be revealed, in which application of portable devices could provide more precise information.

3.2 Fluorescence lifetime measurements

Whole blood contains numerous endogenous fluorophores, which include aromatic amino acids - Tryptophan (Trp), Tyrosine (Tyr), and Phenylalanine (Phe) - NADPH, FAD, and porphyrins [116]. However, from the early studies of fluorescence properties of heme-containing proteins [117, 118], it was evident that the intrinsic fluorescence of Hb is highly quenched for at least two reasons - the inner filter effect and coordinated iron atoms. In spite of the presence of one Trp residue in the α chain and two Trp residues in β chain of Hb, a significant decrease of their fluorescence is observed. This effect has been assigned to properties of heme moieties, exhibiting strong absorption in the range of Trp emission. Since in a Hb tetramer four heme groups can be distinguished, the protein itself can act as an inner filter, reducing the fluorescence intensity of endogenous fluorophores [119]. But if heme can quench the fluorescence by absorbing significant radiation quantities, it should also find a way to release the excess energy. One of the possibilities is to emit the absorbed radiation in the form of visible light. Indeed, metal-free base porphyrins display significant fluorescence in the range from 600 to 730 nm. In case of Hb, however, this intense emission is almost completely diminished upon formation of metallic complexes, as the iron, embedded within the heme moiety, is acknowledged for its quenching abilities [116]. Yet, despite these poor fluorescence properties of Hb, fluorescence spectroscopy, and more precisely fluorescence lifetime measurements, have attracted the attention of researchers investigating the ageing of bloodstains, who gave up the idea of following the changes within Hb molecule in favour of other blood constituents [55, 116, 120]. The backbone of these studies were endogenous fluorophores, Trp and Tyr, which are components not only of Hb subunits, but also albumin and γ-globulin - two major plasma proteins, comprising approximately 95% of the blood's protein mass [3].

Guo et al. [120] hypothesized that the conformational changes of blood Trp-containing proteins, which take place during the bloodstains degradation, might affect the lifetimes of Trp fluorophore. These initial assumptions have been confirmed by the authors, who demonstrated a non-linear decrease of fluorescence lifetimes of investigated molecule over a seven-day period. Apart from distortions of protein conformation, it has been also suggested that the observed changes of the monitored parameter might resulted from the release of iron ions or free amines, which are well known for their quenching properties. However, this fluorescence-based approach is beset with two major problems: firstly, in their study authors employed canine blood without providing any proof that both canine and human blood follow comparable degradation pathways. Secondly, even if canine blood can serve as a reliable surrogate of human tissue, proposed procedure is highly invasive, as sample preparation step consists of dissolving bloodstains in phosphate buffer saline (PBS). Upon recognising these shortcomings, group of Guo upgraded their methodology by introducing a lifetime imaging technique, in which the average fluorescence lifetime of Trp was registered throughout the surface of the bloodstain [116]. Measurements performed in the solid state exposed substantial heterogeneity of the lifetime distribution across the analysed sample, thereby demonstrating that, in case of non-destructive analysis, point measurements certainly cannot allow for a comprehensive exploration of blood ageing processes.

In 2017, the concept of using the fluorescence lifetime measurements for extracting the temporal information from the bloodstains was further explored by Shine et al. [55]. In order to verify

the applicability of the method in forensic scenarios, authors were tracking the behaviour of Trp and Tyr lifetimes not only in pure bloodstains, but also in saliva and blood-saliva mixtures, which usually constitute the stains resulting from the expiration of blood by the victim [121]. This approach allowed to easily differentiating between fresh stains and the old ones due to the significant decrease of fluorescence lifetimes, which plateaued after 91 hours since blood deposition. Most importantly, obtained results of comparison between blood, saliva and blood-saliva mixtures suggested that the observed decrease of fluorescence lifetimes is specific to blood, and therefore it might be also suitable for dating expiratory bloodstains revealed at the crime scene. Unfortunately, the main disadvantage of this technique is its destructiveness, because, likewise in the initial study of Guo et al. [120], PBS solutions were employed to dissolve the blood samples. As a consequence, combination of invasive character of the analysis with the limited availability of fluorescence lifetime spectrofluorometers in forensic laboratories, counts strongly against implementation of this approach in routine forensic practice.

It should be also mentioned that advocates for the use of fluorescence lifetime measurements in studying of bloodstains degradation [55, 116, 120] usually highlight that the time the molecule spends in the excited state before emitting a photon remains unaffected by the excitation wavelength and the concentration of the analyte. According to Shine [55], especially the latter feature of the fluorescence lifetime is particularly desired, as it would ensure that stains of different sizes, which were deposited at the same time, will exhibit similar fluorescence lifetime values. Indeed, any variation in sampling would not influence the measurement; however this variation is not the real problem here. It should be borne in mind that the process of bloodstain degradations itself depends on the amount of deposited blood, e.g. its thickness (see Section 2.1.3), and this dependency between blood volume and the rate of degradation cannot be simply avoided through implementation of an ageing parameter, unaffected by the concentration of the analyte.

Albeit exhibiting some impediments, fluorescence spectroscopy probably should not be treated with disdain. An area worth consideration might be investigation of fluorescence properties of heme degradation products in a function of time, as one of the priorities for the experts who intend to face the problem of bloodstain dating, irrespective of the applied methodology, should be targeting the research at components specific to blood. It should be highlighted that this suggestion of future investigation is quite well-supported by the previous findings of blood traces studies, delivered, what is rather surprising, by Raman spectroscopy. As evidenced by Doty et al. [21] and Lemler et al. [122] upon bloodstain ageing a significant increase of the fluorescence, manifested by the enhanced spectral baseline, is observed. This fluorescence enhancement was linked to the formation and accumulation of Hb degradation products [21, 57, 118, 122-124]. One possible explanation of this effect lies in the dissociation of Hb subunits followed by the release of iron atoms, resulting from the weakening of the iron bond to proximal histidine during the formation of HC [125]. What is more, Nagababu and Rifkind [118], who investigated the formation of fluorescent degradation products during the oxidation of Hb by hydrogen peroxide, found two fluorescent species with excitation wavelengths at 321 nm and 460 nm, and emissions at 465 nm and 525 nm, respectively. Since both free amino acids, as well as the globin cannot be the source of this fluorescence, the observed emissions were associated with heme decomposition products. Admittedly, in the above studies the oxidation of Hb was artificially induced with hydrogen peroxide, however the formation of similar fluorescent degradation species during the natural ageing processes of bloodstain as yet cannot be neither confirmed nor excluded. As a consequence, this direction of studies should not be completely ignored, as it might give rise to a dating tool suited for long-term age estimations.

3.3 Vibrational spectroscopy

The forte of vibrational spectroscopy has been revolutionizing forensic science for a few years now [107, 126, 127]. However, despite being exceptionally informative, spectral data can be also extremely complex, therefore the necessity of data processing with chemometric tools has been well-recognised by the researchers. Fortunately, implementation of mathematical and statistical methods allowed understanding the chemical data and fully appreciating the potential of vibrational techniques. As a result, numerous studies based on two essentially different phenomena, IR absorption (within NIR and MIR range) and Raman scattering, have been applied in examination of forensic traces, also contributing to the field of bloodstains dating [19, 21, 56, 57, 122].

3.3.1. Near-infrared spectroscopy

As previously mentioned, implementation of visible spectroscopy in dating studies of bloodstains might be severely hampered by dark-coloured substrates. However in this case, researchers have also found an alternative solution − a remedy for the background interferences could be reflective measurements performed in NIR range, simply because NIR radiation is absorbed less efficiently by dark coloured materials than VIS light. The basis of NIR spectroscopy is the same as for any other vibrational spectroscopic technique - absorption bands of chemical compounds are the result of molecular vibrations. In the case of NIR, these bands, which are in fact the effect of overtones and combination vibrations of the molecule, occurs in the spectral range from 800 nm to 2500 nm, corresponding to the area of -CH, -OH, -NH, -SH bonds vibrations [128]. As a result, NIR spectroscopy may provide functional information regarding the molecular structure of compounds constituting the sample, and hence this technique can be considered as a potential candidate for monitoring the ageing changes of bloodstains.

The resultant NIR spectral signature of a bloodstain should then be interpreted as the superimposition of all vibrationally active constituents, weighted with respect to their concentration. Therefore, it might be expected that the spectrum of freshly deposited blood would be dominated by two primary water absorption bands - from 1400 nm to 1500 nm and from 1900 nm to 2200 nm. Upon bloodstain degradation, these broad water bands should decrease in intensity, concomitantly revealing other dynamic features, possibly originating from Hb derivatives and other blood components, such as plasma proteins. Indeed, these speculations were proven true by a study from Botonjic-Sehic et al. [19], who noticed that the loss of water plays a crucial role in establishing the initial changes in NIR spectra of bloodstains. It was demonstrated that within the first hour of degradation, the water absorption band (1300 nm - 1600 nm) completely diminished. However, estimation of TSD within an hour since the trace deposition is not particularly useful from a forensic perspective, as the liquid state of a bloodstain indicates quite clearly that the stain has been formed in the recent past, and not, for example, three months prior to its revealing. Consequently, researchers focused on other spectral characteristics, which materialized after completion of water evaporation process. In order to predict the TSD of bloodstains, the time-dependent changes of the band area ranging from 1460 nm to 1860 nm, which had been assigned to the formation of metHb followed by the binding of water, was monitored within 590 hours.

Table 3
List of absorption bands of several blood components along with their possible chemical origins, which were observed in the NIR spectra of bloodstains by Edelman et al. [56]. Data derived from [56].

A few years later, the applicability of the NIR spectroscopy in the sphere of bloodstains dating was reverified by Edelman et al. [56]. In order to define potential age-indicators among spectral features correlated to different components of blood, literature-based qualitative interpretation of NIR spectra was performed (Table 3). The preliminary findings of Dutch researchers reaffirmed the conclusions drawn by their predecessors [19], confirming the dominant contribution of water to NIR

spectra registered within the initial stage of bloodstains ageing. Hence once again, the subsequent research focused on tracking other dynamic spectral features. This time however, in contrast to the subjective methodology applied by Botonjic-Sehic et al. [19], dependencies between TSD and particular wavelengths in NIR spectra, were identified with more advanced methods of data analysis - a PLS regression. The established model enabled estimation of the age of bloodstains up to one month since deposition, with the root mean squared error of prediction (RMSEP) not exceeding a value of 9%. According to the authors, these findings demonstrated the suitability of NIR spectroscopy for short-term age estimations. However, a greatly neglected aspect of data pre-processing should raise our concern.

In the case of the study from Botonjic-Sehic et al., there is no mention of pre-treatment procedures, whereas Edelman did not implement any spectral pre-treatment method, apart from subtracting the absorbance spectrum of the background reference sample. This omission, especially in the case of NIR analysis, might yield some pitiful results. As is well known, pre-processing of the spectra is an essential step undertaken prior to any advanced data analysis. Since the process of spectral acquisition is influenced by distortions in the path length of electromagnetic radiation originating from changes in measurement geometry, sample thickness or its physical properties [129], NIR spectral signatures contain information not only about the chemical composition of the analyte, but also about its physical properties [91]. Therefore, if one aims at obtaining genuinely informative results, different pre-processing tools (e.g. MSC, SNV, spectral derivatives) should be implemented with the purpose of reducing the influence of unwanted physical phenomena. As in the above-mentioned studies this procedure was completely overlooked, then at least a part of the variance observed in the NIR data, interpreted by the authors as the effect of alterations in the chemical composition of bloodstains, might in fact result from scattering effects.

It should also be emphasized that, in the case of NIR spectroscopy, it is not the colour of the substrate, determined by the presence of chromophores, but the chemical composition of the material that might pose a serious obstacle. As it has already been mentioned, NIR spectroscopy is particularly sensitive to specific molecular species containing -CH, -NH, -OH and -SH bonds, which constitute the fundamental chemical structure of the majority of natural and synthetic textiles. As a consequence, blood-absorbing polymeric materials are highly likely to hinder the acquisition of spectra within the NIR range. This might be the reason behind limiting NIR studies entirely to bloodstains deposited on substrates non-interfering with the spectral signature of the specimen, such as glass slides, gauze [19] or cotton [56].

3.3.2. Raman spectroscopy

Water molecules, which severely contribute to NIR absorption bands, are weak Raman scatterers. This gives researchers a perfect opportunity to look directly into the chemical characteristics of body fluids without water interference. What is even more important, of all blood constituents, erythrocytes predominate the scattering of blood with approximately three orders of magnitude due to the difference in refractive index between RBC and the surrounding blood plasma [88]. As a result, inelastic scattering provides direct insight into the state of Hb, and hence RS has been repeatedly applied in blood-related investigations ranging from the understanding of Hb oxygenation [130-136], to monitoring heme aggregation during disorders associated with erythrocytes [137-139], and, most importantly, in the forensic examinations [140, 141].

The 1970s studies on the structural characteristics of Hb initiated the exploitation of RS within the field of blood examination [130-134]. The notion of the fact that Raman spectra of Hb reflect the oxidation and spin state of iron atoms as well as the degree of Hb oxygenation, laid the foundation for future studies [124]. Subsequent research revealed that the vast majority of Raman peaks originate from vibrational modes involving the C–C, C=C, and C–N bonds of the porphyrin ring within the heme structure [132, 142, 143]. Examples of the specific peaks that correspond to three essential types of

vibrational modes of heme proteins are listed in Table 4.

Table 4
Types of vibrational modes observed for Raman spectra of heme proteins [143], illustrated with the oxyHb bands [cm^{-1}] registered with 514.5 nm excitation [147, 157].

Among these, some of the high-frequency vibrational modes are especially sensitive to electron density and structural properties of the protoporphyrin. For example, the bands within the range 1400 - 1300 cm^{-1}, consisting of breathing modes of porphyrin macrocycle, reflect changes of the electron density in the π heme's orbitals. Since during the oxidation-reduction reactions of the central iron, population of these orbitals changes significantly, this spectral region is also known as oxidation state marker line [143]. Features in the high wavenumber region - 1650 to 1500 cm^{-1} - comprising mainly of porphyrin in-plane vibrational modes, are sensitized to porphyrin distortions accompanying alternations in the size of the central porphyrin-core and the spin state of the iron atom, hence this particular line was named the core-size marker line [136, 143]. Given that *ex vivo* transformations of Hb incorporate changes in above-mentioned features, Raman spectroscopy appears to be an appropriate candidate for bloodstains dating studies, as it may provide a molecular insight into the degradation pathways of Hb.

What is more, some of these porphyrin vibrations may be selectively enhanced, enabling precise analysis of heme dynamics. As already mentioned in Section 3.1, the symmetry and electronic structure of heme molecules result in its capability of absorbing VIS light. By tuning the incident laser wavelength to the vicinity of an electronic transition, the resonance Raman effect can be obtained and investigated ranging from UV to NIR frequencies [144]. In fact, the current state of knowledge about Hb geometry and its dynamics is to a large extent derived from these rich resources of resonance Raman data [58]. However, all of the time-related investigations of bloodstains published to this day took advantage of non-resonance RS.

In a 2011 paper on the application of Raman spectroscopy in forensic analysis of blood [145], Boyd with co-workers noticed that the relative intensities of scattering peaks depend on the sample's age. This was presumably the first mention of the time-dependent behaviour of the Raman spectra of bloodstains, and it was tempting to associate these variations with structural changes of Hb, resulting from its *ex vivo* degradation. This finding put forensic scientists on the path leading to the question of what can be accomplished with Raman spectroscopy in terms of bloodstain dating.

Table 5
Band positions, assignments and local coordinates observed during bloodstains analysis using 785 nm excitation [122]

Three years later Lemler et al. [122] followed the direction indicated by the group of Boyd. A two-week investigation of dried stains of whole blood revealed distinct changes in NIR Raman spectra, which were interpreted as the effect of blood aging, triggered by the exposure to ambient conditions. The consequences of this naturally occurring degradation process, reflected in spectral signatures, have been presented in Table 5. Lemler has also managed to remove ambiguities concerning the origin of bands contributing to the spectrum of whole blood, challenging the previous studies reporting the presence of vibrational features corresponding to proteins [149-151] and other blood components [152, 153]. In agreement with previous findings of Sato et al. [154], it was demonstrated that the Raman spectrum of blood, acquired with 785 nm laser, did not provide information about any other constituent of this multicomponent body fluid, but Hb protein; whereas the bands previously assigned to other blood constituents were just spectral symptoms of heme aggregation process.

Since 2016, the leading provider of development in the field of Raman-based bloodstains dating research remains the group of Igor Lednev [155]. In their first study [21], scientists led by Doty combined Raman spectroscopy with statistical modelling in order to estimate the TSD of a bloodstain up to one week old. Several spectral features (summarized in Table 1) were observed to change with time, and their interdependence was investigated in-depth with two-dimensional correlation spectroscopy (2D CoS). The 2D CoS, employed to interpret the dynamics within the region of most prominent spectral alterations (1210 - 1260 cm^{-1}), revealed a negative correlation between two peaks located at 1252 and 1224 cm^{-1}, with the decrease of the 1224 cm^{-1} peak as a first stage, followed by the increase of the intensity at 1252 cm^{-1}. Authors assigned these features to frequencies of amide III band - random coil (1252 cm^{-1}) and β-sheet (1224 cm^{-1}) - concluding that the process of Hb denaturation preceded aggregation. However, these reasoning might be not entirely correct, since the numerous studies of RBC [122, 136, 137, 147, 148, 156, 157] have assigned the band at ~1225 cm^{-1} to the C_m–H methine in-plane bending vibration within protoporphyrin IX moiety (Fig. 6). The region of amide III vibration in turn, which indeed serves as a sensitive probe of peptide conformation, has been defined within 1260 - 1310 cm^{-1}, 1235 - 1242 cm^{-1} and 1240 - 1250 cm^{-1} for α-helix, β-sheet and random coil structure, respectively [158-160], thus it might be an appealing idea to interpret the band at 1252 cm^{-1} as the amide III mode. However, according to Wood et al. [136], the absence of amide I mode (~1650 cm^{-1}), should exclude this assignment from consideration. Instead, several studies of Monash group [136-138, 148, 157] suggest that the whole spectral range between 1200 and 1300 cm^{-1} should be rather interpreted as the signatures of methine vibrations, which also can be severely affected by heme stacking and protein interactions. Hence, ironically enough, doubts regarding spectral alterations occurring between 1210 – 1260 cm^{-1}, which were supposed to be resolved with the support of 2D CoS, still remain, and might require a proper explanation. Nevertheless, ambiguities concerning the root causes of those dynamic spectral changes should not affect the performance of PLSR models developed for time estimations, which eventually allowed to determine the age of bloodstains up to one week since deposition (for details see Table 1). What is even more important, it was demonstrated that the monitored dynamic features did not reach a plateau in the logarithmic time scale, and hence it might be presumed that Raman-based estimation of TSD can be performed not only in scale of hours, but also over longer periods of time.

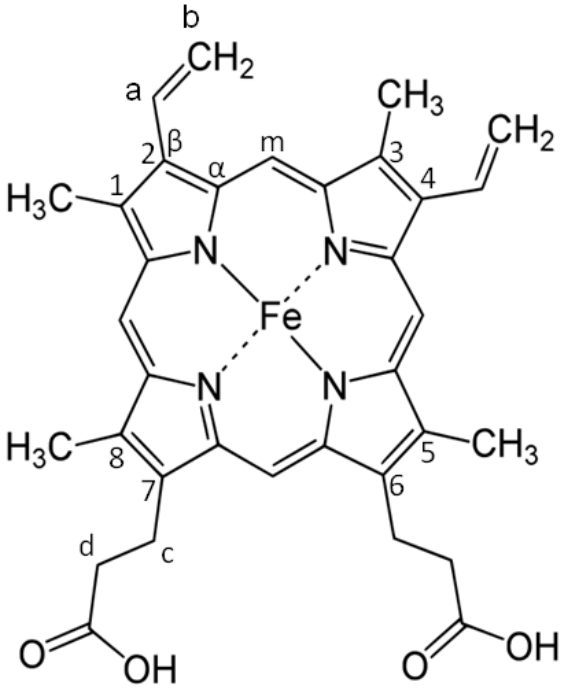

Fig. 6. Labelling scheme of iron protoporphyrin IX [146].

Fortunately, Doty el al. did not keep us too long in a state of suspense. The results of a long-term study published recently [57] evidenced the changes in Raman spectra up to two years elapsed since bloodstain formation (Table 1), however the differences between the spectra corresponding to one- and two-year-old samples were minor. In order to estimate the TSD of a bloodstain, two regression models - PLSR and PCR - were developed. As it could have been expected, the accuracy of predictions decreased as bloodstains aged, simply because spectral signatures of severely degraded stains changed only subtly with progression of time. Long-term predictions of the models were additionally worsened due to poor quality of spectra, resulting primarily from increased fluorescence, which often acts as an impediment to the Raman analysis of body fluids.

Table 5
Vibrational bands of whole blood and red blood cells affected by high laser fluence. Data from [122]

However, this powerful Raman-based temporal information can only be achieved at the cost of intense work devoted to selection of an adequate analytical setup (e.g. laser line, laser power, number of accumulations, time of exposure, etc.), which would allow avoiding an artificial, and therefore undesired, degradation of bloodstains. The exceptional susceptibility of Hb to laser irradiation was established by several researchers [122-124, 161], therefore care should be taken to prevent the photoinduced chemistry of Hb molecules. Unfortunately, ensuring this is not a straightforward task. This is because the spectrum of the ageing bloodstain exhibits the same features as those observed at high laser powers [122], and, by extension, it might be impossible to clearly differentiate between the photoinduced effects caused by the laser irradiation and the non-accelerated Hb transformations, especially when the rate of natural blood degradation remains unknown.

Groups of Ramser [124] and Ahlawat [161], who investigated the effects of laser power in Raman spectroscopy of RBC, have observed three spectral symptoms attributed to the laser-induced processes. It should be reiterated that all of these changes were also noted during natural ageing of bloodstains:
1) A decrease in Raman intensity, which might be an indication of cytoplasm leakage through RBC membrane.
2) A change in Raman spectra indicative of initial deoxygenation of oxyHb, followed by the formation of HC [161] or conversion of oxyHb to metHb [124].
3) An enhancement of fluorescence intensity.

Since both of these studies were performed with 532 nm or 514.5 nm laser lines, which fall within Q-band region of Hb absorption (Table 2), there was a possibility that the observed spectral changes resulted from thermal processes initiated by light absorption. In order to verify this assumption, Ramser et al. [124] carried out calculations of heat transport in RBC. An estimated rise of the temperature at the laser focus equalled ~2.3 °C (for initial laser power 0.7 mW), and hence the thermal degradation was excluded as an explanation of the observed spectral changes, because the actual cell temperature remained well below physiological conditions (~37 °C). As a consequence, photo-induced chemical reactions (e.g. photo-oxidation) were blamed for the observed spectral alterations.

In the second of the mentioned studies, Ahlawat et al. [161] noticed that even a few 10-s accumulations at low laser powers (μW) triggered spectral changes implying transformation of oxyHb toward deoxyHb, which increased with laser power. The effect of power-dependent deoxygenation has been evidenced to be reversible at short (~5 s) exposure to the laser irradiation, however for extended exposure durations, an irreversible derivative of Hb with ferric iron in the low spin state - possibly corresponding to HC - was formed. Since none of these changes were observed in the spectra registered for completely deoxygenated Hb exposed to the same laser powers as oxygenated ones, it was reasonable to conclude that the role of the oxidizing agent, contributing to oxidative damage of erythrocytes, was

played by the free oxygen, which was formed due to laser-induced photo-dissociation. However, these results contradicted the earlier findings of Ramser et al. [124], which suggested the formation of metHb as the final product of the photo-induced changes.

Further evidence in support of the influence of laser irradiation on the Hb chemistry was delivered by Dasgupta et al. [123] and Lemler et al. [122]. Despite employing the laser corresponding to the non-electronically resonant NIR region of Hb, namely the 785 nm line, also in these studies Raman spectra of whole blood were proven to be a function of the incident laser power. Along with the increased intensities of heme aggregation marker bands (972 cm^{-1}, 1248 cm^{-1} and 1366 cm^{-1}), among the symptoms of a photo-damage, Dasgupta et al. [123] also suggested the formation of HC, thereby partly supporting the above-mentioned results of Ahlawat et al. [161]. These bands were also consistent with subsequent observations of the Monash [138, 148] and Ziegler group [122, 156], which attributed the observed Raman signatures to Hb existing in the aggregated form (Table 5).

These differences between results of the aforementioned studies might suggest that mechanisms of laser-induced damage to Hb, occurring at 532/514.5 nm [124, 161] and at 785 nm [122, 123], follow separate pathways. Even though the source of these discrepancies remains undiscovered, one thing is certain - photo-induced denaturation can appear even within the nominally non-resonant region of Hb, therefore it is essential to carefully choose the experimental setup. As suggested by Lemler at al. [122], in order to avoid the photo-damage of RBC, long acquisition times with low excitation power should be preferred over high excitation power coupled with low acquisition times. Another possible solution to decrease the absorption, and hence to reduce the risk of resulting photo-damage is to collect the Raman spectrum while the sample being circulated. This method of analysing exceptionally sensitive specimens has already been proven effective by Woodruff and Spiro [162].

Unfortunately, the impact of the laser power is not the only impediment to the Raman analysis of bloodstains. As demonstrated above, Raman spectroscopy exhibits remarkable capabilities in monitoring the decay of Hb derivatives under controlled conditions. However, the problem of fluorescence and other substrate interferences, encountered in real case scenarios, might constitute an exceedingly complicated issue, posing a significant challenge toward non-invasive estimation of TSD. A research study by McLaughlin et al. [163], devoted to solving the problem of substrate interference in the Raman spectroscopic identification of bloodstains, has already demonstrated that the most troublesome substrates were the absorbing materials of heterogeneous nature (e.g. denim), as well as those contributing both Raman bands and a strong fluorescent background. Therefore, it is imperative that Raman-based research continues with investigation of the substrate effects on the accuracy of age predictions. Due to the incredibly demanding character of dating studies, which often are founded on only subtle time-dependent spectral changes, application of more advanced pre-processing methods than solutions proposed in the research of McLaughlin [163], based on manual subtraction of reference substrate spectrum, would be required.

Arguably, the most important conclusion that emerges from previously mentioned considerations is that for the time being, it is difficult to pinpoint one universal approach, which will be capable of meeting all of the challenges presented by every particular case of bloodstains dating. The assemblage of proposed methodologies focuses on different physical and biochemical symptoms of blood degradation, and hence time scales of their applicability can differ significantly between the dating approaches. These methods might also vary in terms of their susceptibility to the interferences from the substrate. There is thus a possibility that the choice of a suitable tool for establishing TSD would depend upon contextual information, such as the type of the surface on which the questioned bloodstain was formed. In order to obtain an even more complete picture of blood degradation, one might consider implementing various dating approaches, offering complementary information. Fortunately, owing to the rising significance of chemometrics, fusion of data from separate sources no longer poses a problem. Taken together, the reviewed literature also demonstrates how the trail of bloodstain dating research has travelled all the way

from univariate approaches to the present, where multivariate-based methodologies lead the way. It is nothing less than mathematical and statistical power that led to undermining the dominant position of methods focused on monitoring changes of a single variable [19, 55, 65, 80, 102, 116, 120], relegating them to a second priority in favour of the methods tracking multivariate signatures of bloodstains [21, 36, 52, 57, 95]. This trend would most probably continue, since the chemometrics bridge between the lands of severely complex spectroscopic data and their understanding remains under constant development.

4. Estimation of time in forensic sciences - a possible goal or just a chimera?

The starting point for the contemporary studies, addressing the dating issues in criminal investigations, was the realization that the answer to the question of time can be provided through defining a dependency between the time elapsed since trace formation and some measurable parameters, reflecting its degradation. The notion of this possibility was an unquestionable triumph for the forensic community; however it has also given rise to scientific debate about the associated controversies. The rate of growth in our understanding of the ageing processes ironically provides a challenge for establishing a credible dating approach, simply because scientists are becoming increasingly more aware of multiple factors affecting degradation pathways. Indeed, the age of a forensic trace is a peculiar idea. Among all of the suggested concepts of evidence dating, two primary approaches can be distinguished - static and dynamic dating methods [164]. Static approach characterizes the composition of stable components of evidence, which are supposed to be specific to a certain production period. This method is particularly useful in examination of documents, as it allows to reveal anachronisms, just as in the case of Hitler's diaries [11, 165]. Due to the presence of optical brighteners, the authenticity of the diary could have been excluded, as these kinds of components were introduced into the paper manufacturing process after the death of the Nazi dictator. Dynamic dating, in turn, aims at correlating the TSD of a questioned trace with aging processes of its unstable constituents, and can be further divided into relative and absolute approaches. The former methodology, as illustrated with the example of bloodstain dating in Fig. 7, attempts to determine the age of the trace in comparison to the other reference material. However, the serious impediment to establishing the sequence of formation is that only comparison of traces of the same initial composition, stored under the same conditions and deposited on the same substrate, allows one to draw reliable conclusions. These limitations of relative dating pushed scientists toward another approach, which can be performed without providing any reference sample - an absolute dating technique. In terms of the absolute approach, the age of the evidence is thought of as the time that has elapsed since the deposition of the questioned material (e.g. ink entry, stain of body fluid, fingerprint or gunshot residue), which might be defined as its chronological age (Fig. 7). But this is just one of the indicators of the passage of time, which usually cannot be revealed that easily, even with the aid of advanced analytical techniques supported with chemometric tools. This is because the questioned trace may degrade faster or slower, depending on the storage conditions affecting the ageing process, giving rise to the concept of the environmental age of the evidence - revealed during the analytical route, corresponding to the actual state of sample decomposition. What makes absolute dating studies even more complex is that the evidence is not equipped with any metrics, serving as an analogue of our birth certificates, except, perhaps, ink tags - chemicals added purposefully to ink formulations by manufacturers in order to enable identification of the manufacturing date [166]. As a consequence, forensic examiners are forced to base their temporal predictions on previously identified parameters by finding the relation between this age indicator and elapsed time, usually by engaging methods of regression analysis.

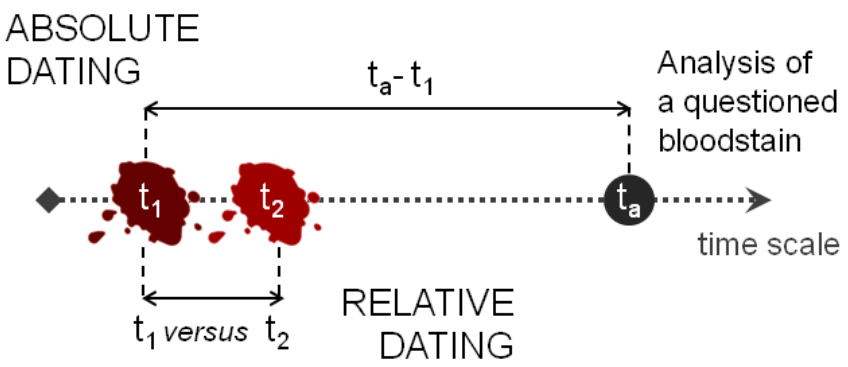

Fig. 7.

Whatever the analysed trace, the parameter serving as the basis for the construction of ageing curves should always be characterised by the following properties [167-169]:

- it has to be persistent and predictable, in other words, it must decrease or increase monotonically with time, preferably over a large time scale;
- it should be applicable to most of the formulations within a particular trace category (bloodstains, fingerprints, inks, etc.). The initial composition of the analysed trace (e.g. ink formulation) is proved to directly affect the decomposition process that might additionally follow different pathways [164]. Therefore, in order to apply the same parameter in dating studies of different objects within the same category, the relation between the ageing indicator and the TSD should remain the same, regardless of the initial constitution of the deposited material. To ensure that the changes of the selected ageing parameter are immune to alterations in the initial composition of the trace, analysis of the representative reference populations should be performed [168];
- it should show reproducible changes over time to enable precise and accurate measurements;
- it should exhibit minimal variability caused by external factors, including the type of substrate or storage conditions, which cannot be controlled in real case scenarios, and which, in most cases, are not known to forensic examiners.

Unfortunately, the last-mentioned condition is hardly ever met. Since an unlimited number of environmental factors can interfere with the degradation process, the information extracted from the evidence by monitoring the ageing parameters, usually corresponds to the environmental age of the trace, not the chronological one, which is the central concern of triers of fact. These two figures, in a majority of instances, do not match up. Therefore, the question arises whether the absolute dating methods, based on age indicators fulfilling these specific demands, are ever attainable.

The problem of reliable dating arises also from lack of available data, since, as it has been already highlighted in the Section 2.1, it is still very uncommon to find comprehensive studies devoted to examination of external factors, influencing a decomposition process of a given trace type [11]. Information regarding the mutual link between the storage conditions of an analysed sample and its ageing kinetics could increase the credibility of the examination. However, it appears virtually impossible, and somehow also unreasonable, to develop in advance dating models for each possible scenario, which can be encountered at the crime scene [167]. Hence, absolute time estimations still prove to be difficult, if not impossible to accomplish, and validity of these results as a form of legal evidence should be questioned [11]. It is not surprising, then, that to this day, no method of absolute dating, irrespective of the trace category, has been found reliable enough to be applied to the actual forensic examination [2, 3, 11, 164, 166, 167, 171, 172].

Given that situation, one could suggest to limit the forensic temporal investigations to a relative dating approach. However, this methodology, as has been already emphasized, is limited in application. Only by avoiding the variability introduced by external factors (section 2.1 Factors

influencing bloodstains degradation), the comparable ageing kinetics can be ensured, and hence the validity of dating models. An antidote to this impediment could be introduction of a dating method, being a sort of variation on the relative approach [167, 170]. Let us imagine a case, for the sake of these considerations, in which the bloodstain serves as the questioned trace subjected to examination. In order to construct an ageing curve, blood of similar volume, freshly drawn from the suspect, placed on an identical surface and stored under similar environmental conditions as those observed at the crime scene during the alleged period of the evidence degradation, should be subjected to the controlled process of ageing. To reconstruct the weather events, in order to establish to what type of storage conditions a questioned bloodstain might have been for the alleged period of ageing, it might be useful to take advantage of the forensic meteorology [173], which uses historic atmospheric data to determine the weather conditions at a specific location and time. If, despite this measure, the decomposition conditions remain unknown, then different alternatives, chosen on the basis of contextual information, should be investigated to maximize the credibility of the model under development. Once the appropriate ageing parameters and models for their monitoring are established, their reliability should be verified by estimating TSD of control bloodstains of different ages. These control samples should also be drawn from the suspect to reduce the inter-donor variability, which might affect the decomposition rate, especially when the evidence of biological origin is considered. Validation procedure should be carried out using samples aged for a time period at least as long as it is relevant to the particular case in question.

Another solution worth attention could be substituting the classical dating approach, based on development of a calibration curve, by so-called comparison problem considered within a likelihood ratio (LR) framework [174-176]. In order to adapt this methodology to dating studies, a bloodstain of a questioned age should act as a recovered material, whereas the role of the reference sample could be fulfilled by blood obtained from the suspect and stored for the period of time corresponding to the suspect's testimony, in exactly the same manner as presented in a previous paragraph. Similar to the example above, apart from the analysis of reference and recovered material, the set of measurements aimed at monitoring the ageing of the bloodstain, should be also carried out. Herein, however, features extracted from the registered spectra, characterising the degrading bloodstains at different time points (e.g. fraction of Hb derivative extracted from reflectance spectrum, colour values derived from digital images or relative intensities of bands observed in Raman spectrum), would serve not as a basis for the construction of a calibration curve, but as a background population dataset, essential to estimation of the parameters such as rarity of observed features, between- and within-object variability, which have to be included into the LR computation. It also has to be clearly pointed out that in this particular case, the object ought to be understood as a group of bloodstains corresponding to the same TSD. Subsequently, the evidence should be assessed in the context of two competing propositions that usually represent the prosecutor's (H_1) and defence's (H_2) standpoints. In the context of this atypical comparison problem, when the TSD of the reference material matches the testimony of the suspect, these hypotheses may be expressed as follows:

H_1: the recovered (bloodstain of questioned age, revealed at the crime scene) and the reference material (bloodstain of known age, subjected to controlled decomposition) are of different ages;

H_2: the recovered (bloodstain of questioned age, revealed at the crime scene) and reference material (bloodstain of known age, subjected to controlled decomposition) are of the same age.

In order to indicate which of these propositions is supported by the obtained spectral signatures of compared bloodstains, conditional probabilities of the registered data, given that any of the hypotheses is true, has to be estimated. The ratio of these conditional probabilities is defined as a likelihood ratio

$$LR = \frac{\Pr(E|H_1,\ I)}{\Pr(E|H_2, I)},$$

where *E* denotes the evidence obtained through the analysis (e.g. spectral data) and *I* corresponds to the background information about the circumstances surrounding the case, e.g. eyewitness testimony, police inquiry or other evidential material accepted before the evaluation of the investigated evidence [Daniel, Aitken]. Imagine, for example, that bloodstains which matched the suspect, charged with murdering his neighbour, were revealed at the crime scene - the apartment of the victim. The results of bloodstain dating studies allowed to estimate the approximate time interval of blood deposition, which was consistent with the time of crime commission. However, in order to reliably assess the value of this finding, other relevant information (I) should be also considered when the probability of observing the evidence (E) under different hypotheses is estimated. For instance, to determine the probability of suspect depositing blood traces during any other event than commission of the crime, the expert would also need the background information, such as testimony of the witnesses regarding the frequency and character of visits paid by the suspect to the victim.

LR can have a value between zero and infinity and is interpreted in the following manner: values of LR above 1 support H_1, while values of LR below 1 provide support for H_2. Moreover, the higher (lower) the value of the LR, the stronger the support for H_1 (H_2), hence, the LR approach provides not only qualitative (which of the propositions, H_1 or H_2, is supported by the evidence), but also quantitative information, being a measure of the strength of the support for one of the considered hypotheses [176]. For example, in the process of comparing the reference and recovered material within a considered case, values of LR above 1 would support the proposition that the bloodstain revealed at the crime scene is not of the same age as the reference material, thereby contradicting the scenario presented by the suspect. To obtain even more balanced evaluation of the evidence, the same experiment should be performed using a bloodstain of the age corresponding to the time (or time ranges) elapsed since the crime event as a reference material. By combining the results of the parallel comparisons based on the LR approach, another step toward a more reliable method of situating the questioned trace in time could be taken (Fig. 8). Another appealing idea might be the application of a multi-class classification approach within the LR framework. Here, the questioned bloodstain would be assigned an LR value, ascribing the sample to one of the categories, defined previously during the process of controlled ageing. In literature, a conceptually similar methodology, based on conducting experiments under controlled conditions in order to assess the evidence in a probabilistic manner, was applied to GSR evidence to discriminate between propositions relating to different distances of firing [178, 179]. A more detailed description of the LR framework is beyond scope of this article and may be found elsewhere [176].

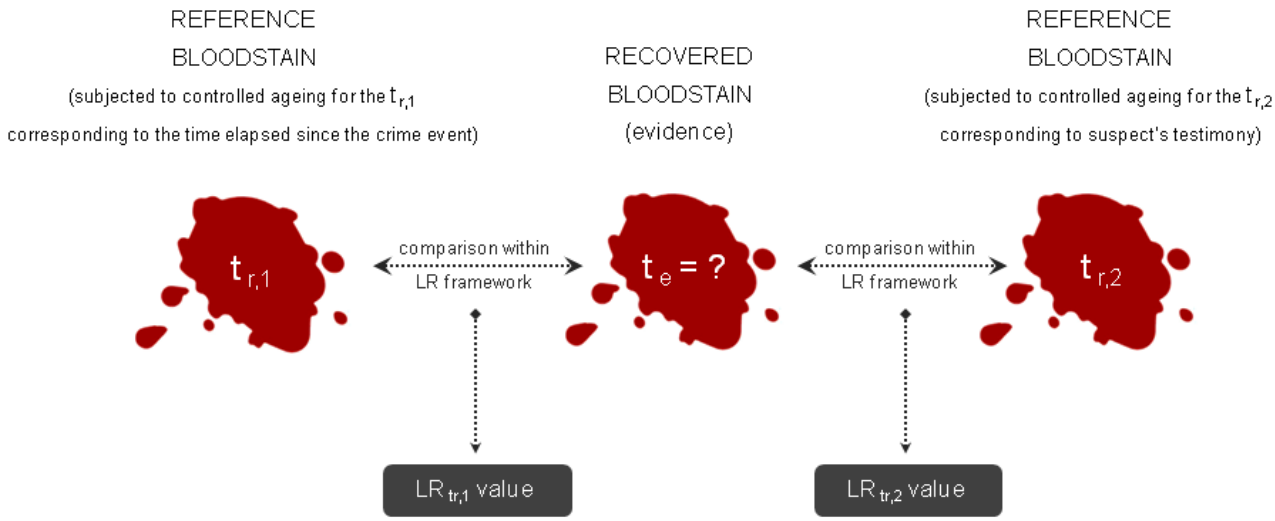

Fig. 8. Implementation of the comparison problem within LR approach aimed at estimation of the TSD of a questioned bloodstain.

Unfortunately, despite their indisputable merits, the dating protocols discussed above exhibit certain drawbacks. First and foremost, they require identification of the donor of the reference material (the suspect or the victim), who should still be alive during the evidence evaluation procedure. If the trace of questioned age is of a biological character, obtaining the reference set of samples might, in some particular cases, pose additional difficulties. As if that were not enough, it cannot be denied that the proposed procedure is incredibly labour-intensive and time-consuming. Yet, this case-suited method of proceeding appears to be the most promising solution, whilst a possibility of reliable estimation of the TSD, provided by this approach, would be the undeniable culmination of undertaken endeavours.

It should also be reiterated that the scientific literature consistently states that methods of forensic dating are still not suitable for implementation into real casework. Nonetheless, numerous forensic experts attempt to situate evidence in time, usually on the basis of their subjective opinions or parameters of debatable reliability [167]. What is even worse, the legitimacy of these testimonies is usually not called into question, as it is simply assumed that forensic experts are, as the name transparently implies, specialized within the particular field and 'they know what they are doing' [180]. Consequently, these one-dimensional opinions are often taken into account by judicature during the decision-making process. This is precisely why the problem of practical implementation of proposed dating approaches should be addressed as promptly as possible, in order to equip forensic practitioners with tools enabling formulation of opinions going beyond *ipse dixit*, simply because 'no one, no matter how well trained or well intentioned, is completely immune to the confirmation bias and to his or her own cognitive blind spots ...' [181].

5. Conclusions and future perspectives

The curtain of obscurity, which for decades has been shrouding the problem of bloodstain dating, now - owing to extensive research efforts - appears to be slightly lifted. As a result, according to Edelman and Aalders [3], some of the spectroscopic methods are one step away from their implementation into routine forensic practice. However, the question is whether the adaptation of developed dating approaches in real-case scenarios is not a hasty decision, since most of these techniques still require verification in terms of their reliability.

Priority should be given to the quest for representative probing of the chemical composition of the heterogeneous surface of bloodstains. Deposited blood hardly ever forms a uniform layer on the substrate, and certainly do not degrade at the same pace within the whole surface of the stain. As a consequence, the dissonance between single-point measurements performed at the micrometer scale, taken at different points of the analysed sample may be disquieting, especially at the initial stage of decomposition. Among published studies using microspectroscopic methods, a dominant approach to dealing with the issue of bloodstains heterogeneity was acquisition of spectral signatures by averaging all spectra collected from a few distinct areas of the sample (100% of reviewed papers, involving only the non-invasive microspectroscopic techniques) - just as in the case of Raman microscopy, where the spectral signature for the each time point was obtained by averaging nine single-point measurements [27, 51]. However, the reliability of conclusions drawn on the basis of averaged spectra should be properly verified before incorporation of this approach into routine forensic use. In order to address this question, it is necessary to prove that the averaged spectral signature truly reflects the degree of blood degradation within the entire surface of the stain. This should be achieved by demonstrating that the averaged spectra can be translated into temporal information with good reproducibility. In other

words, the agreement between the estimates obtained for successive measurements of different blood traces, corresponding to the same TSD, should be exhibited. Since the proposed methodologies still requires proper validation, it remains an open question whether the collection of averaged spectra is the most suitable approach to characterize the molecular content of the degrading bloodstain. Of course the challenge of obtaining meaningful spectra is a long-standing problem, well defined by the spectroscopy community, which gave rise to at least several ways of overcoming the intrasample variation. Two basic strategies for handling inhomogeneous samples are to analyze the entire surface of the specimen or to separate and homogenize it (e.g. by isolating the blood from the substrate and dissolving it in a proper solvent). Unfortunately, most of these solutions are not necessarily suitable for the temporal examinations of forensic traces. The former approach, performed with single point measurements, is incredibly labour intensive and time consuming, and, through that, inappropriate for monitoring the time-dependent properties of the specimen; whereas the latter requires destruction of the evidential material, which is considered highly undesirable by forensic practitioners. Moreover, it has been suggested that addition of water may somewhat reverse the ageing of the bloodstain, inducing the transformation from HC to metHb [47, 82]. Therefore, water-based extraction, if indeed it alters the chemical composition of bloodstains, should not be taken into consideration as a method of sample preparation, since it may result in misestimations of TSD. This is why it is essential to find another alternative to point examinations. The most obvious solution is to record the spectral signal once from the larger area of the bloodstain within a short timeframe. In the case of Raman measurements, it can be achieved through rotation of the sample during the spectral acquisition, application of a low NA objective lens or fiber optic probes. However, this increase of sampling area will be, usually, obtained at a cost of lower signal yield [182], which additionally may be hindered by the intense background signal, generated in the fused-silica fibers [183]. Another remedy for the heterogeneity problem could be HSI, which allows to obtain spatial as well as spectral information from the sample in the field of view [65]. This approach has already been successfully implemented by groups of Li [36] and Edelman [65] to investigate transformations of Hb within the VIS range of the electromagnetic spectrum.

There is also room for improvement in the field of processing recorded data. The spectral acquisition process is affected by distortions in path length of electromagnetic radiation, originating from differences in physical properties of the specimen [129, 184-186]. In consequence, the interaction between bloodstains and light is determined not only by the chemical composition of the analysed stain, but also somewhat reflect its physical properties including the size of the RBC (affecting the scattering of the light), as well as the roughness and thickness of bloodstains (having an influence on specular and diffuse reflectance). Even the optical properties of a substrate and illumination intensity can contribute to distortions between obtained spectral responses, in particular during the measurements based on VIS spectroscopy [82]. Therefore, pretreatment of obtained spectra, implemented in order to remove these undesired non-chemical phenomena and improve the signal to noise ratio, should be an essential step of the analysis, preceding further data modelling. Especially in the advanced stages of ageing, when the rate of changes in spectral characteristics as a function of time becomes less pronounced, the effects of spectral noise may become even more significant. Data pre-processing is often underestimated by researchers. As already pointed out by Li et al. [36], the erroneous estimations of TSD might stem from the inappropriate or completely abandoned procedure of spectral pretreatment. Probably, this was the case with the study of Bremmer et al. [20], in which the incorrect estimations obtained for the older bloodstain, may be linked to the increasing contribution of spectral noise. Hence, it is crucial to apply suitable spectral pre-processing methods, such as SNV or MSC, to reduce non-chemical variations among spectra, and in that way improve the accuracy of the TSD predictions.

However, even if the procedure of spectral pre-treatment prior to data analysis will be improved, the problem of unknown storage conditions remains unsolved. One can only marvel at the

accuracy or the reproducibility of developed methodologies, however upon realizing that most of these techniques have been established under strictly controlled laboratory conditions, questions regarding their reproducibility and transparency in real case scenarios should have arisen. Fortunately, these issues have recently come into sharp focus not only within the field of bloodstains dating, but also in other forensic branches dealing with the question of time [167, 171, 172, 187]. As already emphasized by Weyermann when reflecting on the problem of ink dating [171], the results of an analysis performed on controlled samples under laboratory conditions should under no circumstances be considered equivalent to findings obtained during the examination of real samples. Therefore, the attention of the current research should be focused on determination of such aspects as systematic errors, as well as repeatability and reproducibility of already established approaches, rather than on developing new analytical methods. In order to avoid misleading conclusions, some procedures enabling interpretation of the results in terms of their reliability should be introduced. All these considerations boil down to the one crucial problem, namely the evaluation of the evidential value of physicochemical data.

"Yes or no: was this bloodstain deposited two weeks ago?" Many forensic examiners might find or already have found themselves in this courtroom scenario, faced with the question, which, in fact, cannot ever be precisely answered. This is because, as broadly discussed in section 4, the ageing of bloodstains may proceed at different rates, according to the wide range of external factors. These complex and, as yet, not exhaustively investigated dependencies, in most cases lead to discrepancies between the environmental (the one revealed during the analysis) and the chronological (the one being pursued by the judicature) age of the sample in question. Therefore, in order to obtain reliable results, acceptable as legal evidence, the TSD should be estimated in terms of the probability, after thorough investigation of the ageing kinetics of bloodstains. By studying the blood traces exposed to different environmental conditions, factors that affect the ageing of bloodstains in a predictable manner could be identified [65, 85]. As a result, one could assign probabilities, employing, for example, a Bayesian approach, to the results obtained for different time-points of analysed bloodstains, and by doing so present the uncertainties of the estimate [53]. This methodology of providing court evidence, a probabilistic answer based on Bayesian inference, has recently gained considerable importance in the interpretation of forensic data [188-191], and it has been already implemented in dating studies of gunshot residue [192] and ink entries [171, 172]. Even though most judges still prefer certainty to science, the new rationality, in which the concept of probability is no longer identified with inexperience and ignorance, is progressively taking over. Additionally, in order to facilitate this process, measures should be taken to educate forensic scientists and to integrate a probabilistic reasoning and statistics into the criminal justice system [193]. An essential step is to liberate from the alchemy of binary statements about 'a match' and 'a non-match' with only a single, arbitrary chosen value (e.g. p-value) dividing them. Instead, researchers should embrace uncertainty and take into account variations within the results observed under different circumstances [194].

The challenge of pinpointing the precise time of bloodstain deposition presents all the ingredients of an exceedingly thorny problem. Without doubt, the road leading to this holy grail of forensic science is long and winding, but it is an important one to take, and for a few years now it has been going in the right direction. And even though the problem of time in forensic science continually raises new doubts, isn't it vital to ask and face questions for which no answers are readily available?

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References

[1] I. Prigogine, The end of certainty, The Free Press, New York, 1997.

[2] R.H. Bremmer, K.G. de Bruin, M.J.C. van Gemert, T.G. van Leeuwen, M.C.G. Aalders, Forensic quest for age determination of bloodstains, Forensic Sci. Int. 216 (2012) 1–11.

[3] G.J. Edelman, M.C.G. Aalders, Blood Degradation and Bloodstain Age Estimation, in: E.M.J. Schotsmans, N. Márquez-Grant, S. L. Forbes (Eds.), Taphonomy of Human Remains, Wiley, Chichester, 2017, pp. 53-64.

[4] Introduction to Bloodstain Pattern Analysis, in: S.H. James, P.E. Kish, T.P. Sutton, Principles of Bloodstain Pattern Analysis, CRC Press, Boca Raton, 2005, pp. 1-10.

[5] A.J. Jeffreys, V. Wilson, S.L. Thein, Hypervariable 'minisatellite' regions in human DNA, Nature 314 (1985) 67-73.

[6] A.J. Jeffreys, V. Wilson, S.L. Thein, Individual-specific 'fingerprints' of human DNA, Nature 316 (1985) 76-79.

[7] A.J. Jeffreys, J.F. Brookfield, R. Semeonoff, Positive identification of an immigration test-case using human DNA fingerprints, Nature 317 (1985) 818-819.

[8] S. Gupta, Criminology: Written in blood, Nature 549 (2017) 24–25.

[9] O. Peschel, S. N. Kunz, M.A. Rothschild, E. Mützel, Blood stain pattern analysis, Forensic Sci. Med. Pathol. 7 (2011) 257-270.

[10] E. Piotrowski, Über Entstehung, Form, Richtung und Ausbreitung der Blutspuren nach Hiebwunden des Kopfes, Wien, 1895.

[11] C. Weyermann, O. Ribaux, Situating forensic traces in time, Sci. Justice 52 (2012) 68-75.

[12] Court of Criminal Appeal, 2005, Hillstead v. R, Int. J. Evid. Proof 10 (2006) 145-146.

[13] L. Tomellini, De l'emplol d'une table chromatiqie pour les taches du sang, Arch. d'Antropologie criminelle de Criminol. 14 (1907) 2.

[14] O. Leers, Die forensische Blutuntersuchung, Springer, Berlin, 1910.

[15] D. Patterson, Use of reflectance measurements in assessing the colour changes of ageing bloodstains, Nature 187 (1960) 688–689.

[16] S.S. Kind, D. Patterson, G.W. Owen, Estimation of the age of dried blood stains by spectrophotometric method, Forensic Sci. 1 (1972) 27–54.

[17] H. Inoue, F. Takabe, M. Iwasa, Y. Maeno, Identification of fetal hemoglobin and simultaneous estimation of bloodstain age by high-performance liquid chromatography, Int. J. Legal Med. 104 (1991) 127–131.

[18] H. Inoue, F. Takabe, M. Iwasa, Y. Maeno, Y. Seko, A new marker for estimation of bloodstain age by high performance liquid chromatography, Forensic Sci. Int. 57 (1992) 17–27.

[19] E. Botonjic-Sehic, C.W. Brown, M. Lamontagne, M. Tsaparikos, Forensic Application of Near-Infrared Spectroscopy: Aging of Bloodstains, Spectroscopy 24 (2009) 1-9.

[20] R.H. Bremmer, A. Nadort, M.J.C. van Gemert, T.G. van Leeuwen, M.C. Aalders, Age estimation of blood stains by hemoglobin derivative determination using reflection spectroscopy, Forensic Sci. Int. 206 (1–3) (2010) 166–171.

[21] K.C. Doty, G. McLaughlin, I.K. Lednev, A Raman "spectroscopic clock" for bloodstain age determination: the first week after deposition, Anal. Bioanal. Chem. 408 (2016) 3993–4001.

[22] H. Lin, Y. Zhang, Q. Wang, B. Li, S. Fan, Z. Wang, Species identification of bloodstains by ATR-FTIR spectroscopy: the effects of bloodstain age and the deposition environment, Int. J. Legal Med. (2017), DOI 10.1007/s00414-017-1634-2.

[23] M. Jackson, H. H. Mantsch, Pathology by Infrared and Raman Spectroscopy, in: J.M. Chalmers, P.R. Griffiths (Eds.), Handbook of Vibrational Spectroscopy, Wiley, Chichester, 2001, Volume 4, pp. 5-6.

[24] C. S. Rastogi, Biochemistry, Tata McGraw-Hill Education, Columbus, 2003.

[25] F. Ramsthaler, A. K. Kröll, M. Verhoff, C. G. Birngruber, M. Kettner, Effect of anticoagulation therapy on drying times in bloodstain pattern analysis, Int. J. Legal Med. 131 (2017) 955-961.

[26] D. Brutin, B. Sobac, B. Loquet, J. Sampol, Pattern formation in drying drops of blood, J. Fluid Mech. 667 (2011) 85-95.

[27] F. Ramsthaler, P. Schmidt, R. Bux, S. Potente, C. Kaiser, M. Kettner, Drying properties of bloodstains on common indoor surfaces, Int. J. Legal Med. 126 (2012) 739–746.

[28] N. Laan, F. Smith, C. Nicloux, D. Brutin, Morphology of drying blood pools, Forensic Sci. Int. 267 (2016) 104–109.

[29] Y. Wu, Y. Hu, J. Cai, S. Ma, X. Wang, Y. Chen, Y. Pan, Time-dependent surface adhesive force and morphology of RBC measured by AFM, Micron 40 (2009) 359–364.

[30] S. Strasser, A. Zink, G. Kada, P. Hinterdorfer, O. Peschel, W.M. Heckl, A. G. Nerlich, S. Thalhammer, Age determination of blood spots in forensic medicine by force spectroscopy, Forensic Sci. Int. 170 (2007) 8–14.

[31] Y. Chen, J. Cai, Membrane deformation of unfixed erythrocytes in air with time lapse investigated by tapping mode atomic force microscopy, Micron 37 (2006) 339–346.

[32] T. Smijs, F. Galli, A. van Asten, Forensic potential of atomic force microscopy, Forensic Chemistry 2 (2016) 93–104.

[33] M. Janko, R. W. Stark, A. Zink, Preservation of 5300 year old red blood cells in the Iceman, J. R. Soc. Interface 9 (2012) 2581–2590.

[34] P. Hortola, SEM analysis of red blood cells in aged human bloodstains, Forensic Sci. Int. 55 (1992) 139 - 159.

[35] E.K. Hanson, J. Ballantyne, A blue spectral shift of the hemoglobin Soret band correlates with the age (time since deposition) of dried bloodstains, PLoS ONE 5 (2010) e12830.

[36] B. Li, P. Beveridge, W. T. O'Hare, M. Islam, The age estimation of blood stains up to 30 days old using visible wavelength hyperspectral image analysis and linear discriminant analysis, Sci. Justice 53 (2013) 270–277.

[37] G. J. Edelman, T. G. van Leeuwen, M. C. Aalders, Visualization of Latent Blood Stains Using Visible Reflectance Hyperspectral Imaging and Chemometrics, J. Forensic Sci. 60 (2015) S188-S192.

[38] M.F. Perutz, M.G. Rossmann, A.F. Cullis, H. Muirhead, A. C. T. North, Structure of haemoglobin: a three-dimensional Fourier synthesis at 5.5-A. resolution, obtained by X-ray analysis, Nature 185 (1960) 416-422.

[39] A. J. Marengo-Rowe, Structure-function relations of human hemoglobins, Proc. (Bayl. Univ. Med. Cent.) 19 (2006) 239–245.

[40] Hemoglobin: Portrait of a Protein in Action, in: J. M. Berg, J. L. Tymoczko, L. Stryer, Biochemistry, W. H. Freeman, New York, 2002, pp. 191-210.

[41] A. M. Stadler, I. Digel, G. M. Artmann, J. P. Embs, G. Zaccai, G. Büldt, Hemoglobin Dynamics in Red Blood Cells: Correlation to Body Temperature, Biophys. J. 95 (2008) 5449–5461.

[42] J. M. Rifkind, O. Abugo, A. Levy, J. Heim, Detection, formation, and relevance of hemichromes and hemochromes, Methods Enzymol. 231 (1994) 449-80.

[43] J. Umbreit, Methemoglobin—It's Not Just Blue: A Concise Review, Am. J. Hematol. 82 (2007) 134–144.

[44] O. K. Baskurt, Mechanisms of Blood Rheology Alterations, in: O. K. Baskurt, M. R. Hardeman, M. W. Rampling, H. J. Meiselman (Eds.), Handbook of Hemorheology and Hemodynamics, IOS Press, Amsterdam, 2007, pp. 170-190.

[45] B. Faivre, P. Menu, P. Labrude, C. Vigneron, Hemoglobin autooxidation/oxidation mechanisms and methemoglobin prevention or reduction processes in the bloodstream. Literature review and outline of autooxidation reaction, Art. Cells Blood Subs. Immob. Biotech., 26 (1998) 17-26.

[46] J. Dissing, A. Søndervang, S. Lund, Exploring the limits for the survival of DNA in blood stains, J. Forensic Leg. Med. 17 (2010) 392-396.

[47] M. F. Colombo, R. Sanches, Hydration-dependent conformational states of hemoglobin Equilibrium and kinetic behavior, Biophysical Chemistry 36 (1990) 33-39.

[48] A. Riccio, L. Vitagliano, G. di Prisco, A. Zagari, L. Mazzarella, The crystal structure of a tetrameric hemoglobin in a partial hemichrome state, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 9801-9806.

[49] T. Kanias, J. P. Acker, Biopreservation of red blood cells – the struggle with hemoglobin oxidation, FEBS Journal 277 (2010) 343–356.

[50] E. A. Rachmilewitz, J. Peisach, W. E. Blumberg, Studies on the stability of oxyhemoglobin A and its constituent chains and their derivatives, J. Biol. Chem. 246 (1971) 3356-3366.

[51] B. J. A. Larkin, Bloodstain pattern analysis: scratching the surface (Doctoral dissertation),

2015.

[52] H. Sun, Y. Dong, P. Zhang, Y. Meng, W. Wen, N. Li, Z. Guo, Accurate Age Estimation of Bloodstains Based on Visible Reflectance Spectroscopy and Chemometrics Methods, IEEE Photon. J. 9 (2017).

[53] P. Thanakiatkrai, A. Yaodam, T. Kitpipit, Age estimation of bloodstains using smartphones and digital image analysis. Forensic Sci. Int. 233 (2013) 288–297.

[54] J. Shin, S. Choi, J.-S. Yang, J. Song, J.-S. Choi, H.-I. Jung, Smart Forensic Phone: Colorimetric analysis of a bloodstain for age estimation using a smartphone, Sens. Actuators, B 243 (2017) 221–225.

[55] S. Mc Shine, K. Suhling, A. Beavil, B. Daniel, N. Frascione, The applicability of fluorescence lifetime to determine the time since the deposition of biological stains, Anal. Methods 9 (2017) 2007–2013.

[56] G. Edelman, V. Manti, S. M. van Ruth, T. van Leeuwen, M. Aalders, Identification and age estimation of blood stains on colored backgrounds by near infrared spectroscopy, Forensic Sci. Int. 220 (2012) 239–244.

[57] K. C. Doty, C. K. Muro, I. K. Lednev, Predicting the time of the crime: Bloodstain aging estimation for up to two years, Forensic Chem. 5 (2017) 1–7.

[58] C. G. Atkins, K. Buckley, M. W. Blades, R. F. B. Turner, Raman Spectroscopy of Blood and Blood Components, Appl. Spectrosc. 71 (2017) 767–793.

[59] M. Lekka, P. Laidler, D. Gil, J. Lekki, Z. Stachura, A.Z. Hrynkiewicz, Elasticity of normal and cancerous human bladder cells studied by scanning force microscopy, Eur. Biophys. J. Biophys. Lett. 28 (1999) 312–316.

[60] M. Bryszewska, C.Watala,W. Torzecka, Changes in fluidity and composition of erythrocyte-membranes and in composition of plasma-lipids in type-I diabetes, Brit. J. Haematol. 62 (1986) 111–116.

[61] A. Mansouri, A. A. Lurie, Concise review: methemoglobinemia, Am. J. Hematol. 42 (1993) 7-12.

[62] D. A. Sears, M. M. Udden, J. Thomas, Carboxyhemoglobin levels in patients with sickle-cell anemia: relationship to hemolytic and vasoocclusive severity, Am. J. Med. Sci. 322 (2001) 345–348.

[63] N. J. Wald, M. Idle, J. Boreham, A. Bailey, Carbon monoxide in breath in relation to smoking and carboxyhaemoglobin levels, Thorax 36 (1981) 366–369.

[64] N. R. Woebkenberg, R. A. Mostardi, D. L. Ely, D. Worstell, Carboxyhemoglobin and methemoglobin levels in residents living in industrial and non-industrial communities, Environ. Res. 26 (2011) 347–352.

[65] G. J. Edelman, T. G. van Leeuwen, M. C. G. Aalders, Hyperspectral imaging for the age

estimation of blood stains at the crime scene, Forensic Sci. Int. 223 (2012) 72–77.

[66] T. L. Laber, R. P. Epstein, Substrate effects on the clotting time of human blood, Can. Soc. Forens. Sci. J. 34 (2001) 209-214.

[67] H. Shuval, N. Gruener, Epidemiological and Toxicological Aspects of Nitrates and Nitrites in the Environment, Am. J. Public Health 62 (1972) 1045–1052.

[68] K. M. Marzec, A. Rygula, B. R. Wood, S. Chlopicki, M. Baranska, High-resolution Raman imaging reveals spatial location of heme oxidation sites in single red blood cells of dried smears, J. Raman Spectrosc. 46 (2014) 76–83.

[69] K. Canene-Adams, J. W. Erdman Jr., Absorption, Transport, Distribution in Tissues and Bioavailability, in: G. Britton, S. Liaaen-Jensen, H. Pfander (Eds.), Carotenoids, Volume 5: Nutrition and Health, Birkhäuser Verlag, Basel, 2009, pp. 115-144.

[70] A. Vayá, M. Simó, M. Santaolaria, J. Todolí, J. Aznar, Red blood cell deformability in iron deficiency anaemia, Clin. Hemorheol. Microcirc. 33 (2005) 75-80.

[71] R. T. Card, L. R. Weintraub, Metabolic Abnormalities of Erythrocytes in Severe Iron Deficiency, Blood 37 (1971) 725-732.

[72] E. Nagababu, S. Gulyani, C. J. Earley, R. G. Cutler, M. P. Mattson, J. M. Rifkind, Iron-Deficiency Anemia Enhances Red Blood Cell Oxidative Stress, Free Radic. Res. 42 (2008) 824–829.

[73] L. Fewtrell, Drinking-Water Nitrate, Methemoglobinemia, and Global Burden of Disease: A Discussion, Environ. Health Perspect. 112 (2004) 1371–1374.

[74] J. W. Buenger, V. F. Mauro, Organic nitrate-induced methemoglobinemia, Ann. Pharmacother. 23 (1989) 283-288.

[75] P. Fraser, C. Chilvers, Health aspects of nitrate in drinking water, Sci. Total Environ. 18 (1981) 103-116.

[76] T. Miki, A. Kai, M. Ikeya, Electron spin resonance of bloodstains and its application to the estimation of time after bleeding, Forensic Sci. Int. 35 (1987) 149- 158.

[77] T. Matsuoka, T. Taguchi, J. Okuda, Estimation of bloodstain age by rapid determinations of oxyhemoglobin by use of oxygen electrode and total hemoglobin, Biol. Pharm. Bull. 18 (1995) 1031–1035.

[78] Y. Fujita, K. Tsuchiya, S. Abe, Y. Takiguchi, S. Kubo, H. Sakurai, Estimation of the age of human bloodstains by electron paramagnetic resonance spectroscopy: long-termcontrolled experiment on the effects of environmental factors, Forensic Sci. Int. 152 (2005) 39–43.

[79] E. Hanson, A. Albornoz, J. Ballantyne, Validation of the hemoglobin (Hb) hypsochromic shift assay for determination of the time since deposition (TSD) of dried bloodstains, Forensic Sci. Int. Genet. Suppl. Ser. 3 (2011) e307–e308.

[80] R. H. Bremmer, A. Nadort, T. G. van Leeuwen, M. J. van Gemert, M. C. Aalders, Age estimation of blood stains by hemoglobin derivative determination using reflectance spectroscopy, Forensic Sci. Int. 206 (2011) 166–171.

[81] J. Agudelo, C. Huynh, J. Halámek, Forensic determination of blood sample age using a bioaffinity-based assay, Analyst 140 (2015) 1411–1415.

[82] G. Edelman, Spectral analysis of blood stains at the crime scene (Doctoral dissertation), 2014.

[83] M. Tsuruga, A. Matsuoka, A. Hachimori, Y. Sugawara, K. Shikama, The Molecular Mechanism of Autoxidation for Human Oxyhemoglobin, J. Biol. Chem. 273 (1998) 8607–8615.

[84] M. Bauer, S. Polzin, D. Patzelt, Quantification of RNA degradation by semi-quantitative duplex and competitive RT-PCR: a possible indicator of the age of bloodstains?, Forensic Sci. Int. 138 (2003) 94–103.

[85] G. J. Edelman, M. Roos, A. Bolck, M. C. Aalders, Practical Implementation of Blood Stain Age Estimation Using Spectroscopy, IEEE J. Sel. Topics Quantum Electron. 22 (2016) 415-421.

[86] J. Flammer, M. Mozaffarieh, H. Bebie, The Interaction Between Light and Matter, in: J. Flammer, M. Mozaffarieh, H. Bebie (Eds.), Basic Sciences in Ophthalmology, Springer-Verlag, Berlin Heidelberg, 2013, pp. 21–39.

[87] E. Smith, G. Dent, Introduction, Basic Theory and Principles, in: E. Smith, G. Dent, Modern Raman Spectroscopy - A Practical Approach, John Wiley & Sons, Ltd, Chichester, 2005, pp. 1-21.

[88] M. Meinke, G. Müller, J. Helfmann, M. Friebel, Optical properties of platelets and blood plasma and their influence on the optical behavior of whole blood in the visible to near infrared wavelength range, J. Biomed. Opt. 12 (2007).

[89] N. Bosschaart, G. J. Edelman, M. C. G. Aalders, T. G. van Leeuwen, D. J. Faber, A literature review and novel theoretical approach on the optical properties of whole blood, Lasers Med. Sci. 29 (2014) 453-479.

[90] G. J. Edelman, E. Gaston, C. P. J. van Leeuwen, M. C Aalders, Hyperspectral imaging for non-contact analysis of forensic traces, Forensic Sci. Int. 223 (2012) 28–39.

[91] A. Menzyk, A. Martyna, G. Zadora, Evidential value of polymeric materials - chemometric tactics for spectral data compression combined with likelihood ratio approach, Analyst 142 (2017) 3867-3888.

[92] A. Hofmann, A. Simon, T. Grkovic, M. Jones, UV-Vis spectroscopy, in: A. Hofmann, A. Simon, T. Grkovic, M. Jones, Methods of Molecular Analysis in the Life Sciences, Cambridge University Press, Cambridge, 2014, pp. 15-37.

[93] M. R. Dayer, A. A. Moosavi-Movahedi, M. S. Dayer, Band Assignment in Hemoglobin Porphyrin Ring Spectrum: Using FourOrbital Model of Gouterman, Protein Peptide Lett. 17

(2010) 473-479.

[94] V. Blazek, G. Lins, Spectroscopic age determination of blood stains: new technical aspects, Acta Med. Leg. Soc. (Liege) 32 (1982) 613–616.

[95] B. Li, P. Beveridge, W. T. O'Hare, M. Islam, The estimation of the age of a blood stain using reflectance spectroscopy with a microspectrophotometer, spectral pre-processing and linear discriminant analysis, Forensic Sci. Int. 212 (2011) 198–204.

[96] J. Zięba-Palus, Microspectrophotometry in Forensic Science, in: R. A. Meyers (Ed.), Encyclopedia of Analytical Chemistry: Applications, Theory and Instrumentation 1, Wiley, Chichester, 2006.

[97] S. E. Bell, D. T. Burns, A. C. Dennis, L. J. Matchett, J. S. Speers, Composition profiling of seized ecstasy tablets by Raman spectroscopy, Analyst 125 (2000) 1811-1815.

[98] A. F. Palonpon, J. Ando, H. Yamakoshi, K. Dodo, M. Sodeoka, S. Kawata, K. Fujita, Raman and SERS microscopy for molecular imaging of live cells, Nat. Protoc. 8 (2013) 677–692.

[99] I. W. Schie, L. Alber, A. L. Gryshuk, J. W. Chan, Investigating drug induced changes in single, living lymphocytes based on Raman micro-spectroscopy, Analyst 139 (2014) 2726-2733.

[100] M. J. Romeo, S. Boydston-White, C. Matthäus, M.Miljković, B. Bird, T. Chernenko, M. Diem, Vibrational Microspectroscopy of Cells and Tissues, in: P. Lasch, J. Kneipp (Eds), Biomedical Vibrational Spectroscopy, Wiley, Chichester, 2008, pp. 121-152.

[101] L.-P. Choo-Smith, A. C.-T. Ko, M. Hewko, D. P. Popescu, J. Friesen, M. G. Sowa, Combining Optical Coherence Tomography and Raman Spectroscopy for Investigating Dental and Other Mineralized Tissues, in: P. Lasch, J. Kneipp (Eds), Biomedical Vibrational Spectroscopy, Wiley, Chichester, 2008, pp. 263-290.

[102] R. H. Bremmer, D. M. de Bruin, M. de Joode, W. Jan Buma, T. G. van Leeuwen, M. C. G. Aalders, Biphasic Oxidation of Oxy-Hemoglobin in Bloodstains, PLoS ONE 6 (2011) e21845.

[103] B. A. J. Larkin, C. E. Banks, Recent Advances in Bloodstain Pattern Analysis, in: E. Katz, J. Halámek (Eds.), Forensic Science. A Multidisciplinary Approach, Wiley-VCH, Weinheim, 2016, pp. 263-281.

[104] R. L. Schuler, P. E. Kish, C. A. Plese, Preliminary Observations on the Ability of Hyperspectral Imaging to Provide Detection and Visualization of Bloodstain Patterns on Black Fabrics, J. Forensic Sci. 57 (2012) 1562–1569.

[105] D. L. Exline, C. Wallace, C. Roux, C. Lennard, M. P. Nelson, P. J. Treado, Forensic applications of chemical imaging: latent fingerprint detection using visible absorption and luminescence, J. Forensic Sci. 48 (2003) 1047–1053.

[106] A. Grant, T. J. Wilkinson, D. R. Holman, M. C. Martin, Identification of recently handled materials by analysis of latent human fingerprints using infrared spectromicroscopy, Appl. Spectrosc. 59 (2005) 1182–1187.

[107] E. G. Bartick, Applications of Vibrational Spectroscopy in Criminal Forensic Analysis, in: J. M. Chalmers, P. R. Griffit (Eds), Handbook of Vibrational Spectroscopy, vol. 4, Wiley, New York, 2003, pp. 2993–3004.

[108] M. López-López, M. A. Fernández de la Ossa, C. García-Ruiz, Fast Analysis of Complete Macroscopic Gunshot Residues on Substrates Using Raman Imaging, J. Appl. Spectrosc. 69 (2015) 889–893.

[109] C. S. Silva, M. F. Pimentel, R. S. Honorato, C. Pasquini, J. M. Prats-Montalbán, A. Ferrer, Near infrared hyperspectral imaging for forensic analysis of document forgery, Analyst 139 (2014) 5176–5184.

[110] A. Braz, M. López-López, C. García-Ruiz, Raman imaging for determining the sequence of blue pen ink crossings, Forensic Sci. Int. 249 (2015) 92–100.

[111] S. Choi, S. Kim, J.S. Yang, J.H. Lee, C. Joo, H.I. Jung, Real-time measurement of human salivary cortisol for the assessment of psychological stress using a smartphone, Sens. Bio-Sens. Res. 2 (2014) 8–11.

[112] S. Lee, V. Oncescu, M. Mancuso, S. Mehta, D. Erickson, A smartphone platform for the quantification of vitamin D levels, Lab Chip 14 (2014) 1437–1442.

[113] V. Oncescu, D. O'Dell, D. Erickson, Smartphone based health accessory for colorimetric detection of biomarkers in sweat and saliva, Lab Chip 13 (2013) 3232–3238.

[114] L. Shen, J.A. Hagen, I. Papautsky, Point-of-care colorimetric detection with a smartphone, Lab Chip 12 (2012) 4240–4243.

[115] A.K. Yetisen, J.L. Martinez-Hurtado, A. Garcia-Melendrez, F.D.C. Vasconcellos, C.R. Lowe, A smartphone algorithm with inter-phone repeatability for the analysis of colorimetric tests, Sens. Actuators B: Chem. 196 (2014) 156–160.

[116] K. Guo, N. Zhegalova, S. Achilefu, M. Y. Berezin, Bloodstain age analysis: toward solid state fluorescent lifetime measurements, Proc. of SPIE 8572 (2013) 857214.

[117] J. J. Leonard, T. Yonetani, J. B. Callis, A Fluorescence Study of Hybrid Hemoglobins Containing Free Base and Zinc Protoporphyrin IX, Biochemistry 13 (1974) 1460-1464.

[118] E. Nagababu, J. M. Rifkind, Formation of Fluorescent Heme Degradation Products during the Oxidation of Hemoglobin by Hydrogen Peroxide, Biochem. Biophys. Res. Commun. 247 (1998) 592–596.

[119] Intrinsic Protein Fluorescence, in: D. M. Jameson, Introduction to Fluorescence, CRC Press, Boca Raton, 2014, pp. 251-276.

[120] K. Guo, S. Achilefu, M. Y. Berezin, Dating bloodstains with fluorescence lifetime measurements, Chemistry 18 (2012) 1303–1305.

[121] Evaluating Impact Spatter Bloodstains, in: T. Bevel, R. Gardner, Bloodstain Pattern Analysis, 3rd Edition, CRC Press, Boca Raton, 2008, pp. 199-230.

[122] P. Lemler, W. R. Premasiri, A. DelMonaco, L. D. Ziegler, NIR Raman spectra of whole human blood: effects of laser-induced and in vitro hemoglobin denaturation, Anal. Bioanal. Chem. 406 (2014) 193–200.

[123] R. Dasgupta, S. Ahlawat, R. S. Verma, A. Uppal P. K. Gupta, Hemoglobin degradation in human erythrocytes with long-duration near-infrared laser exposure in Raman optical tweezers, J. Biomed. Opt. 15 (2010) 055009.

[124] K. Ramser, E. J. Bjerneld, C. Fant, M. Käll, Importance of substrate and photo-induced effects in Raman spectroscopy of single functional erythrocytes, J. Biomed. Opt. 8 (2003) 173-178.

[125] S. S. Panter, Release of Iron from Hemoglobin, Methods Enzymol. 231 (1994) 502-514.

[126] C. K. Muro, K. C. Doty, J. Bueno, L. Halámková, I. K. Lednev, Forensic Applications of Vibrational Spectroscopy, in: E. Katz, J. Halámek (Eds), Forensic Science. A Multidisciplinary Approach, Wiley-VCH, Weinheim, 2016, pp. 5-44.

[127] J. M. Chalmers, H. G. M. Edwards, M. D. Hargreaves (Eds), Infrared and Raman Spectroscopy in Forensic Science, Wiley, Chichester, 2012.

[128] Y. Ozaki, Near-infrared spectroscopy - its versatility in analytical chemistry, Anal. Sci. 28 (2012) 545-563.

[129] A. Rinnan, F. van den Berg, S. Balling Engelsen, Review of the most common pre-processing techniques for near-infrared spectra, TrAC 28 (2009) 1201-1222.

[130] T.C. Strekas, T.G. Spiro, Hemoglobin: Resonance Raman Spectra, Biochim. Biophys. Acta – Protein Struct. 263 (1972) 830–833.

[131] H. Brunner, H. Sussner, A. Mayer, Resonance Raman Scattering on the Haem Group of Oxy- and Deoxyhaemoglobin, J. Mol. Biol. 70 (1972) 153–156.

[132] H. Brunner, H. Sussner, Resonance Raman Scattering on Haemoglobin, Biochim. Biophys. Acta. 310 (1973) 20–31.

[133] T. Yamamoto, G. Palmer, D. Gill, I.T. Salmeen, L. Rimai, The Valence and Spin State of Iron in Oxyhemoglobin as Inferred from Resonance Raman Spectroscopy, J. Biol. Chem. 248 (1973) 5211–5213.

[134] T.G. Spiro, T.C. Strekas, Resonance Raman Spectra of Heme Proteins. Effects of Oxidation and Spin State, J. Am. Chem. Soc. 96 (1974) 338–345.

[135] K. Nagai, T. Kitagawa, H. Morimoto, Quaternary Structures and Low Frequency Molecular Vibrations of Haems of Deoxy and Oxyhaemoglobin Studied by Resonance Raman Scattering, J. Mol. Biol. 36 (1980) 271–289.

[136] B. R. Wood, B. Tait, D. McNaughton, Micro-Raman Characterisation of the R to T State Transition of Haemoglobin within a Single Living Erythrocyte, Biochim. Biophys. Acta – Molecular Cell Res. 1539 (2001) 58–70.

[137] B. R. Wood, L. Hammer, D. McNaughton, Resonance Raman Spectroscopy Provides Evidence of Heme Ordering within the Functional Erythrocyte, Vib. Spectrosc. 38 (2005) 71–78.

[138] B. R. Wood, L. Hammer, L. Davis, D. McNaughton, Raman Microspectroscopy and Imaging Provides Insights into Heme Aggregation and Denaturation within Human Erythrocytes, J. Biomed. Opt. 10 (2005) 14005–14013.

[139] B. R. Wood, A. Hermelink, P. Lasch, K. R. Bambery, G. T. Webster, M. A. Khiavi, B. M. Cooke, S. Deed, D. Naumann, D. McNaughton, Resonance Raman Microscopy in Combination with Partial Dark- Field Microscopy Lights up a New Path in Malaria Diagnostics, Analyst 134 (2009) 1119–1125.

[140] K. C. Doty, C. K. Muro, J. Bueno, L. Halámková, I. K. Lednev, What can Raman spectroscopy do for criminalistics?, J. Raman Spectrosc. 47 (2016) 39–50.

[141] K. C. Doty, I. K. Lednev, Raman spectroscopy for forensic purposes: recent applications for serology and gunshot residue analysis, TrAC (2017) https://doi.org/10.1016/j.trac.2017.12.003.

[142] M. Abe, T. Kitagawa, Y. Kyogoku, Resonance Raman Spectra of Octaethylporphyrinatonickel(II) and Meso-Deuterated and Nitrogen- 15 Substituted Derivatives. II. A Normal Coordinate Analysis, J. Chem. Phys. 69 (1978) 4526–4534.

[143] D. L. Rousseau, M. R. Ondrias, Raman scattering, in: D. L. Rousseau (Ed.) Optical Techniques in Biological Research, Academic press, inc., Orlando, 1984, pp. 100-108.

[144] B. R. Wood, D. McNaughton, Resonant Raman scattering of heme molecules in cells and in the solid state, in: P. Lasch, J. Kneipp (Eds), Biomedical Vibrational Spectroscopy, Wiley, Chichester, 2008, pp. 181-203.

[145] S. Boyd, M. F. Bertino, S. J. Seashols, Raman spectroscopy of blood samples for forensic applications, Forensic Sci. Int. 208 (2011) 124–128.

[146] S. Z. Hu, K. M. Smith, T. G. Spiro, Assignment of Protoheme Resonance Raman Spectrum by Heme Labeling in Myoglobin, J. Am. Chem. Soc. 118 (1996) 12638–12646.

[147] M. Asghari-Khiavi, A. Mechler, K. R. Bambery, D. McNaughton, B. R. Wood, A resonance Raman spectroscopic investigation into the effects of fixation and dehydration on heme environment of hemoglobin, J. Raman Spectrosc. 40 (2009) 1668–1674.

[148] B. R. Wood, P. Caspers, G. J. Puppels, S. Pandiancherri, D. McNaughton, Resonance Raman spectroscopy of red blood cells using near-infrared laser excitation, Anal. Bioanal. Chem. 387 (2007) 1691–1703.

[149] M. Casella, A. Lucotti, M. Tommasini, M. Bedoni, E. Forvi, F. Gramatica, G. Zerbi, Raman and SERS recognition of β-carotene and haemoglobin fingerprints in human whole blood Spectrochim. Acta, A. Mol. Biomol. Spectrosc. 79 (2011) 915−919.

[150] K. Virkler, I. K. Lednev, Raman spectroscopic signature of blood and its potential

application to forensic body fluid identification, Anal. Bioanal. Chem. 396 (2010) 525−534.

[151] V. Sikirzhytski, K. Virkler, I. K. Lednev, Discriminant Analysis of Raman Spectra for Body Fluid Identification for Forensic Purposes, Sensors 10 (2010) 2869−2884.

[152] A. J. Berger, T.-W. Koo, I. Itzkan, G. Horowitz, M. S. Feld, Multicomponent blood analysis by near-infrared Raman spectroscopy, Appl. Opt. 38 (1999) 2916−2916.

[153] A. M. K. Enejder, T.-W. Koo, J. Oh, M. Hunter, S. Sasic, M. S. Feld, Blood analysis by Raman spectroscopy, Opt. Lett. 27 (2002) 2004−2006.

[154] H. Sato, H. Chiba, H. Tashiro, Y. J. Ozaki, Excitation wavelength-dependent changes in Raman spectra of whole blood and hemoglobin: comparison of the spectra with 514.5, 720, and 1064 nm excitation, Biomed. Opt. 6(2001) 366−370.

[155] Lednev Research Laboratory, University at Albany. https://sites.google.com/site/lednevlab/ (accessed 15.11.2017).

[156] W. R. Premasiri, J. C. Lee, L. D. Ziegler, Surface-Enhanced Raman Scattering of Whole Human Blood, Blood Plasma, and Red Blood Cells: Cellular Processes and Bioanalytical Sensing, J. Phys. Chem. B 116 (2012) 9376−9386.

[157] B. R. Wood, D. McNaughton, Raman excitation wavelength investigation of single red blood cells in vivo, J. Raman Spectrosc. 33 (2002) 517–523.

[158] J. L. Lippert, D. Tyminski, P. J. Desmeules, Determination of the secondary structure of proteins by laser Raman spectroscopy, J. Am. Chem. Soc. 98 (1976) 7075–7080.

[159] H. Ishizaki, P. Balaram, R. Nagaraj, Y. V. Venkatachalapathi, A. T. Tu, Determination of beta-turn conformation by laser Raman spectroscopy, Biophys J. 36 (1981) 509–517.

[160] A. Rygula, K. Majzner, K. M. Marzec, A. Kaczor, M. Pilarczyk, M. Baranska, Raman spectroscopy of proteins: a review, J. Raman Spectrosc. 44 (2013) 1061–1076.

[161] S. Ahlawat, N. Kumar, A. Uppal, P. K. Gupta, Visible Raman excitation laser induced power and exposure dependent effects in red blood cells, J. Biophotonics 8 (2016) 1–8.

[162] W. H. Woodruff, T. G. Spiro, A Circulating Sample Cell for Temperature Control in Resonance Raman Spectroscopy, Appl. Spectrosc. 28 (1974) 74–75.

[163] G. McLaughlin, V. Sikirzhytski, I. K. Lednev, Circumventing substrate interference in the Raman spectroscopic identification of blood stains, Forensic Sci. Int. 231 (2013) 157–166.

[164] A. Menzyk, G. Zadora, M. Sajewicz, Physicochemical analysis of ink − dating and establishing the sequence of intersecting lines of ink entries, Probl. Forensic Sci. 104 (2015) 279–302.

[165] J. Grant, The diaries of Adolf Hitler, Sci. Justice 25 (1985) 189.

[166] M. Ezcurra, J. M. G. Góngora, I. Maguregui, R. Alonso, Analytical methods for dating modern writing instrument inks on paper, Forensic Sci. Int. 197 (2010) 1–20.

[167] A. Girod, R. Ramotowski, S. Lambrechts, P. Misrielal, M. Aalders, C. Weyermann, Fingermark age determinations: Legal considerations, review of the literature and practical propositions, Forensic Sci. Int. 262 (2016) 212–226.

[168] A. Koenig, C. Weyermann, Ink dating, part I: Statistical distribution of selected ageing parameters in a ballpoint inks reference population, Sci. Justice (2017), http://dx.doi.org/10.1016/j.scijus.2017.08.002.

[169] P. A. Margot, A question of time, Sci. Justice 40 (2000) 64-71.

[170] Altered Bloodstain Patterns, in: S.H. James, P.E. Kish, T.P. Sutton, Principles of Bloodstain Pattern Analysis, CRC Press, Boca Raton, 2005, pp. 179-216.

[171] C. Weyermann, J. Almog, J. Bügler, A. A. Cantu, Minimum requirements for application of ink dating methods based on solvent analysis in casework, Forensic Sci Int. 210 (2011) 52-62.

[172] C. Weyermann, B. Schiffer, P. Margot, A logical framework to ballpoint ink dating interpretation, Sci. Justice 48 (2008) 118-125.

[173] E. Austin, P. Hildebrand, The art and science of forensic meteorology, Phys. Today 67 (2014) 32-37.

[174] C. Aitken, F. Taroni, Statistics and the Evaluation of Evidence for Forensic Scientists, Wiley, Chichester, 2004.

[175] C. Aitken, G. Zadora, D. Lucy, A two-level model for evidence evaluation, J. Forensic Sci. 52 (2007) 412-419.

[176] G. Zadora, A. Martyna, D. Ramos, C. Aitken, Statistical Analysis in Forensic Science: Evidential Value of Multivariate Physicochemical Data, Wiley, Chichester, 2014.

[177] D. Ramos, Forensic Evaluation of the Evidence Using Automatic Speaker Recognition Systems (Doctoral dissertation), Universidad Autonoma de Madrid, 2007.

[178] A. Biedermann, F. Taroni, A probabilistic approach to the joint evaluation of firearm evidence and gunshot residues, Forensic Sci. Int. 163 (2006) 18–33.

[179] Combining gunshot residue and marks evidence, in: F. Taroni, C. Aitken, P. Garbolino, A. Biedermann, Bayesian Networks and Probabilistic Inference in Forensic Science, Wiley, Chichester, 2006, pp. 240-243.

[180] A. Biedermann, F. Taroni, P. Garbolino, Equal prior probabilities: can one do any better?, Forensic Sci. Int. 172 (2007) 85-93.

[181] Law and Disorder, in: C. Tavris, E. Aronson, Mistakes Were Made (But Not by Me): Why We Justify Foolish Beliefs, Bad Decisions, and Hurtful Acts, Harcourt, Orlando, 2007, pp. 126-157.

[182] I. W. Schie, R. Kiselev, C. Krafft, J. Popp, Rapid acquisition of mean Raman spectra of eukaryotic cells for a robust single cell classification, Analyst 141 (2016) 6387-6395.

[183] L. F. Santos, R. Wolthuis, S. Koljenović, R. M. Almeida, G. J. Puppels, Fiber-optic probes for in vivo Raman spectroscopy in the high-wavenumber region, Anal. Chem. 77 (2005) 47-52.

[184] P. Lasch, Spectral pre-processing for biomedical vibrational spectroscopy and microspectroscopic imaging, Chemometr. Intell. Lab. Syst. 117 (2012) 100–114.

[185] R. Gautam, S. Vanga, F. Ariese, S. Umapathy, Review of multidimensional data processing approaches for Raman and infrared spectroscopy, EPJ Tech. Instrum. 2 (2015) 1-38.

[186] N. K. Afseth, V. H. Segtnan, J. P. Wold, Raman Spectra of Biological Samples: A Study of Preprocessing Methods, Appl. Spectrosc. 60 (2006) 1358-1367.

[187] M. Maitre, K. P. Kirkbride, M. Horder, C. Roux, A. Beavis, Current perspectives in the interpretation of gunshot residues in forensic science: A review, Forensic Sci. Int. 270 (2017) 1–11.

[188] A. Michalska, A. Martyna, J. Zięba-Palus, G. Zadora, Application of a likelihood ratio approach in solving a comparison problemof Raman spectra recorded for blue automotive paints, J. Raman Spectrosc. 46 (2015) 772–783.

[189] A. Martyna, G. Zadora, T. Neocleous, A. Michalska, N. Dean, Hybrid approach combining chemometrics and likelihood ratio framework for reporting the evidential value of spectra, Anal. Chim. Acta. 931 (2016) 34-46.

[190] E. Alladio, A. Martyna, A. Salomone, V. Pirro, M. Vincenti, G. Zadora, Evaluation of direct and indirect ethanol biomarkers using a likelihood ratio approach to identify chronic alcohol abusers for forensic purposes, Forensic Sci. Int. 271 (2017) 13-22.

[191] P. Własiuk, A. Martyna, G. Zadora, A likelihood ratio model for the determination of the geographical origin of olive oil, Anal. Chim. Acta 853 (2015) 187-199.

[192] M. Gallidabino, C. Weyermann, F. S. Romolo, F.Taroni, Estimating the time since discharge of spent cartridges: A logical approach for interpreting the evidence, Sci. Justice 53 (2013) 41-48.

[193] C. Aitken, P. Roberts, G. Jackson, Fundamentals of Probability and Statistical Evidence in Criminal Proceedings: Guidance for Judges, Lawyers, Forensic Scientists and Expert Witnesses, Royal Statistical Society, 2015.

[194] J. Leek, B. B. Mcshane, A. Gelman, D. Colquhoun, M. B. Nuijten, S. N. Goodman, Five ways to fix statistics, Nature 551 (2017) 557-559.

[195] W.G. Zijlstra, A. Buursma, W.P. Meeuwsen-Van der Roest, Absorption spectra of human fetal and adult oxyhemoglobin, de-oxyhemoglobin, carboxyhemoglobin, and methemoglobin, Clin. Chem. 37 (1991) 1633–1638.

[196] T. Asakura, K. Minakata, K. Adachi, M.O. Russell, E. Schwartz, Denatured hemoglobin in sickle erythrocytes, J. Clin. Invest. 59 (1977) 633–640.

Figure captions

Fig. 1. Structure of a hemoglobin molecule.

Fig. 2. Pathway of *ex vivo* hemoglobin degradation.

Fig. 3. Factors affecting the rate of bloodstains decomposition process.

Fig. 4. Transition rates of Hb derivatives [102]. The k_1 corresponds to transition rate of oxyHb to metHb, the k_2 represents the transition rate of metHb to HC, whilst k_s and k_f are slow and fast rate constants, respectively, of the k_1.

Fig. 5. The interaction of light with matter may result in different phenomena (a) specular reflection, (b) elastic scattering followed by diffuse reflection, (c) inelastic scattering followed by emission of Raman shifted light (dashed lines), (d) absorption, and (e) absorption followed by photoluminescence emission (dotted lines). Figure modified, acquired from [90].

Fig. 6. Labelling scheme of iron protoporphyrin IX [146].

Fig. 7. Relative versus absolute dating of a questioned bloodstain. T_1 and t_2 corresponds to time of bloodstain deposition, whereas t_a indicates the time of sample analysis.

Fig. 8. Implementation of the comparison problem within LR approach in order to estimate the TSD of a questioned bloodstain.

Table 1
Recent spectroscopic techniques (2011 - 2017) applied in the bloodstains dating

Technique	Monitored characteristics of ageing bloodstains	Sample	Bloodstain formation and storage conditions	Sample preparation for the analysis	Results	Time scale of the method	Ref.
MSP (442 - 585 nm)	Variables extracted from the VIS reflectance spectra with feature selection methods (Fisher weight's and an approach based on Fourier Transform)	Equine blood without addition of anticoagulants or preservatives	- Stains of ~5 cm^2 (volume not specified) created by smearing blood onto 6 cm x 4 cm glazed white tile; - Stored in a laboratory at room temperature (~22 °C) and a RH of ~40%	None; non-destructive analysis	- Most distinctive changes appeared in the region of the α and β absorption bands of Hb, with the bands decreasing in intensity, broadening and merging into one band with increasing TSD; - LDA model, developed to estimate the TSD between 1 and 37 days, allowed to obtain up to 99.2% CCR when training and test set spectra were registered for the same bloodstain; - For separate bloodstains serving as a training and test sets, LDA model was characterized by CCR of 54.7% with an average error of 0.71 days (for estimations up to 19 days since deposition)	-	[95]

VIS Reflectance Spectroscopy (350 - 1050 nm)	Fractions of bloodstain chromophores oxyHb, met-Hb and HC determined by quantitative analysis of reflectance spectra	- Human blood drawn from eight healthy male volunteers; - Without addition of anticoagulants or preservatives	- 40 bloodstains (five stains per donor) of 21 ± 4 mm diameter (volume not specified) deposited on white cotton substrate; - Samples stored for 60 days at 22.3 ± 0.5 °C	None; non-destructive analysis	- Fractions of Hb derivatives (oxyHb, metHb and HC) in bloodstains demonstrated a distinct temporal behaviour; - OxyHb underwent a biphasic auto-oxidation process, rate of oxidation decreased as bloodstains aged; - Sum of the monitored Hb fractions remained approx. constant during the ageing period, indicating that the whole content of oxyHb is transformed to metHb and HC;	- Up to 60 days since deposition	[80]
VIS Reflectance Spectroscopy (450 - 800 nm)	Fractions of oxyHb, met-Hb and HC determined by quantitative analysis of reflectance spectra	- Human blood drawn from healthy, non-smoking volunteer; - Without addition of anticoagulants or preservatives	- First set of samples mesaured three hours after deposition: eight bloodstains created by depositing on with cotton 50 µl of blood diluted with PBS; diluted samples contained following blood volume fractions:	None; non-destructive analysis	- Transformation of oxyHb followed a biphasic oxidation - initially rapid process slowed down after ten hours; - Rates of oxidation processes were strongly temperature-dependent; - Transition from oxyHb to metHb did not depend on RH, in contrary to the subsequent metHb to HC transformation, which was	-	[102]

			0, 0.1, 0.2, 0.4, 0.6. 0.8, 1; - Second set of samples mesaured up to ten days since deposition: five groups of three bloodstains created by depositing 50 µl on white cotton; each group was stored under different temperatures (20 °C, 4 °C, 22 °C, 29 °C, 37 °C) in a dark box at a RH of 45 ± 5%; - Third set of samples measured up to 8 days since deposition: three groups of three bloodstains created by depositing 50 µl on white cotton; each group was stored at varying RH (20%, 50%,		affected by RH (for the RH→100%, no HC is formed)		

			70%), in a dark box at 37 ± 1 °C				
VIS Reflectance Spectroscopy (400 - 1000 nm)	Visible reflectance spectra (multivariate analysis)	- Human blood drawn from eight healthy volunteers; - Without addition of anticoagulants or preservatives	- Eight stains formed by placing 15 µl of blood on glass slides; - Stored in a temperature regulated oven at 37 °C and RH value of ~ 10% for 45 days	None; non-destructive analysis	- PLSR, PCR and LS-SVM models, established on three different periods (from 2 to 24 h, 1 to 7 days, and 7 to 45 days since bloodstain formation), were developed; - The best predictive ability was obtained for the models based on the region of 500 - 780 nm of VIS spectrum, - LS-SVM models outperformed the PLSR and PCR models, achieving RMSEP of 42.7920 hours	- Up to 45 days since deposition	[52]
HSI (in VIS range)	Fractions of oxyHb, metHb and HC - values extracted from visible reflectance images of bloodstains	- Reference database: human blood drawn from non-smoking, healthy volunteer; - Crime scene analysis: human blood	- Reference database: blood (volume not specified) deposited on white cotton fabric and stored under ambient laboratory conditions for 200	None; non-destructive analysis	- Fraction of oxyHb decreased with time, while content of metHb increased within the three weeks since deposition, with subsequent decrease of its value; fraction of HC increased with time; - Owing to the comparison with bloodstains within	- Up to 200 days since deposition (median relative error: 13.4% of the actual age)	[65]

		originating from five healthy, non-smoking volunteers; - Without addition of anticoagulants or preservatives	days; - Crime scene analysis: blood (volume not specified) deposited on white cotton cloth 0.1, 2, 15, 40, and 200 days before the analysis; stored under similar conditions as samples constituting reference database		reference database, TSD of the samples, mimicking evidence found at crime scenes, could have been estimated (absolute dating); - Accuracy of estimation decreased with age from 0.9 days of absolute error, corresponding to the bloodstain of 0.1 day old, to 6.0 days of error for sample analysed 40 days after deposition; - Order of deposition of bloodstains (relative dating), stored under unknown conditions, was established without comparison with database samples;		
HSI (in VIS range, images captured between 505 nm and 600 nm)	Reflectance spectra - four (for 30 days study) and eight (for one day study) variables corresponding to intensities at spectral wavelengths, selected with feature selection method (Fisher test	- Ageing study (within 30 days): equine blood without addition of anticoagulants or preservatives; - Ageing	- Two drops of blood (~20 µl) smeared on white photocopier paper (80 g/m^2) to create two separate stains of ~1 cm^2 each; - Stored in a temperature	None; non-destructive analysis	- Conversion of oxyHb to HC, monitored by the relative intensity of the readings at 550 nm to 520 nm as a function of time, revealed exponential decay, which corresponds to fast oxidation of oxyHb to metHb (complete within three days), followed by	- Up to 30 days since deposition	[36]

	combined with analysis of correlation between variables)	study (within 1 day): human blood drawn from healthy volunteer, without addition of anticoagulants or preservatives;	regulated oven at 30 °C, at a RH of ~40% over a period of 30 days		slow formation of HC (continuing up to 30 days); - LDA model based on selected variables allowed to estimate the TSD of test samples with an average error of ±0.27 days for the first seven days and an overall average error of ±1.17 days up to 30 days		
Digital image analysis	Colour analysis: colour values (CMYK) extracted from the digital images	- Venous human blood originating from four Asian, healthy, non-smoking volunteers of mean age 23.5 ± 2.4 years (one male, three females non-menstruating during the blood donation); - Without addition of anticoagulants	- 50 µl of blood (from four individuals) deposited on filter paper and stored in dark at 25 °C; - Effect of temperature: blood on filter paper stored at 20 °C, 4 °C and 25 °C; - Effect of RH: blood on filter paper stored at room temperature with RH values of 30% and 50% - Effect of light exposure: blood	None; non-destructive analysis	- Magenta colour value (M) was chosen as a parameter for developing a dating model due to its high correlation with TSD (R^2 = 0.966); - Magenta values decreased exponentially with increasing time; - Within- and between-person variations were not observed ($p > 0.05$); - Smartphone camera, temperature, humidity, and substrate colour influence the dating analysis itself, but also the process of bloodstain degradation (T and RH); - Machine learning	- Up to 42 days since deposition	[53]

		or preservatives	on filter paper exposed to direct sunlight, fluorescent lamp and protected from light exposure; - Blind test: four out of forty bloodstains deposited on filter paper were frozen at -80 °C after different periods elapsed since deposition; - Mock case: 24 bloodstains deposited on household objects and stored under uncontrolled conditions		classification algorithm Random Forests™ for age estimation allowed for 100% accurate predictions during the blind test (samples stored under controlled conditions) and 83% correct classifications of mock casework samples		
Digital image analysis	Colour analysis: RGB values per pixel of bloodstain image converted into the V value of HSV (hue, saturation, and brightness)	- Human blood originating from three healthy, non-smoking Asian males (average age	- Drop of blood (~10 µl) deposited onto following materials: wood, slide glass, wallpaper, fabric, and A4 paper at 0° (parallel), 15°,	None; non-destructive analysis	- Changes in the colour of bloodstains were detectable on five different materials up to 42 hours elapsed since bloodstain formation; - No significant difference was observed in colour	- Up to 42 hours since deposition	[54]

		of 30.5 and SD = 1.64); - Without addition of anticoagulants or preservatives	30°, 45°, and 60°; - first group of blood samples stored under following conditions RH=40%, T=23 °C, protected from any light sources apart from the illumination employed in the measurement set up; - second group of samples stored under 10%, 25%, and 55% RH at 23 °C and -10 °C, 5 °C, and 37 °C at 40% RH		intensity values of the bloodstains under the following conditions: 5 °C and 23 °C ; 10, 25, 40, and 55% RH; - Storage temperature influenced the degradation process: differences between V values for bloodstains stored in 10 °C and 37 °C were detected;		
Fluorescence spectroscopy (λ_{ex}: 295 nm, (λ_{em}: 350 nm)	Fluorescence lifetime of tryptophan, endogenous fluorophore of blood proteins	- Canine blood obtained from four different dogs; - Without addition of anticoagulants or	Four blood samples deposited onto plastic Petri dishes and exposed to room environment for the period of three months	Dried scratches of blood dissolved in PBS buffer and centrifuged; separated plasma was subjected to analysis	- Non-linear decrease of fluorescence lifetimes from 4.0 ns for blood analysed immediately after deposition to 2.5 ns, corresponding to two-month-old bloodstains, was observed; -Most rapid changes took	- Up to seven days since deposition	[120]

		preservatives			place within the first 150 hours since bloodstain creation with plateau being reached after approx. 300 hours		
Solid state fluorescence spectroscopy (λ_{ex}: 405 nm, (λ_{em} > 435 nm)	Fluorescence lifetime images of tryptophan	- Expired human blood obtained from blood bank; - Presumably with addition of anticoagulants (non-specified)	- Bloodstains aged for the period of three weeks; - Method of preparation and storage conditions: non-specified	None; non-destructive analysis	- Images of solid state bloodstains demonstrated heterogeneity of the lifetime distributions within the sample surface; - Correlation of the intensity of fluorescence and its lifetime values, corresponding to the different points of bloodstains (fluorescence mapping), with TSD may serve as a more reliable metod of bloodstain dating	-	[116]
Fluorescence Spectroscopy (λ_{ex}: 281 nm, (λ_{em}: 330 nm)	Fluorescence lifetime of tryptophan and tyrosine	-Human blood obtained from six healthy donors; - Without addition of anticoagulants or preservatives	- 3 ml of venous blood from each volunteer deposited in triplicate onto plastic Petri dishes; - Stored uncovered under laboratory conditions (T:	Bloodstains fragments of uncontrolled sizes dissolved in 1 ml of PBS; supernatant, obtained after vortexing, sonication and certifugation of samples, was subjected to analysis	- Non-linear decrease of fluorescence lifetimes over time from approx. 3.0 ns for samples analysed 19 hours after deposition, to approx. 2.0 ns after 50 days of aging was observed; - Decrease was statistically significant ($p < 0.05$) within the first 91 hours of the blood being deposited;	- Up to 91 hours since deposition; - Suitable for distinguishing freshly deposited and older bloodstains	[55]

			23± 2 °C, RH 40±5%, ambient light) for approx. 1200 hours		- Certain degree of inter-donor variation was revealed		
NIR Spectroscopy (1100 - 2500 nm)	Vibrational reflectance spectra of whole dried bloodstains (multivariate signature of blood components)	- Venous and capillary human blood, originating from both genders and various age groups; - Without addition of anticoagulants or preservatives	- Approximately six drops of blood deposited on porous (gauze) and non-porous (glass) substrates; - Stored for a period of one month under ambient conditions (T: 20 °C, RH: not controlled)	None; non-destructive analysis	- Vibrational bands originating from water molecules (~1400 nm and ~1900 nm) were the main contributor of fresh bloodstains; - Substrate affected the rate of bloodstain drying - spectra of blood on gauze exhibited presence of water after one hour since deposition, contrary to blood on glass; - After an hour, bands due to proteins appeared within the range 1800-2500 nm, and a new band, corresponding to formation of metHb, appeared in the 1460-1860 nm; - Standard error of prediction for a model based on area of 1460 - 1860 nm band, fitted with a least squares regression,	- Up to 590 hours since deposition	[19]

					equalled 2.3 hours over a total time of 590 hours		
NIR Spectroscopy (800 - 2778 nm)	Vibrational reflectance spectra of whole dried bloodstains (multivariate signature of blood components, mainly Hb derivatives)	- Human blood obtained from healthy female donors (2); - Without addition of anticoagulants or preservatives	- Three bloodstains (drawn from one of the female volunteers) deposited on white cotton, stored for 77 days; - Three bloodstains (drawn from the second female volunteer) deposited on black, red, green and blue cotton, stored for 28 days; - Samples stored in a laboratory at room temperature (~22 °C, RH: not specified); - Amount of deposited blood was not specified	None; non-destructive analysis	- PLS model created for estimating the TSD, separately for each coloured background, allowed to predict the age of the bloodstain up to one month, with RMSE of prediction varying from 6.8% to 8.9%; - Accuracy of estimation decreased with age	- Up to one month since deposition	[56]
Raman Spectroscopy	Raman spectra of whole dried	- Venous human blood;	- Bloodstain created by	None; non-destructive analysis	- After two weeks elapsed since bloodstains	-	[122]

(laser wavelength: 785 nm)	bloodstains (multivariate signature of blood components)	- With addition of EDTA as an anticoagulant; - Stored in an 8 °C refrigerator prior to the bloodstain formation	depositing a drop of blood on a silicon wafer; - Stored under ambient conditions (details not specified) up to two weeks since deposition		formation, new bands at 667, 747, and 1248 cm^{-1} materialized, whereas markers of Fe-O_2 at bands at 419, 570 and 1638 cm^{-1} disappeared; - Spectrum of a two-week dried bloodstain exhibited the same vibrational features as those observed at high laser powers;		
Raman Spectroscopy (laser wavelength: 785 nm)	Raman spectra of whole dried bloodstains (multivariate signature of blood components, mainly Hb derivatives)	- Peripheral human blood obtained from two healthy donors (male and female); - Without addition of anticoagulants or preservatives	- Bloodstains of approximately 30 µl deposited directly from fingertip onto glass slide covered with aluminium foil; - Stored under laboratory conditions (temperature and RH were not controlled) for a period of 168 hours	None; non-destructive analysis	- Several spectral features changed with time: 1) intensification of fluorescence background toward the low-frequency spectral range during the ageing, possibly due to formation of metHb and HC, 2) 377 cm^{-1} band, identified as a metHb marker, increased its intensity in relation to 420 cm^{-1} (oxyHb marker); 3) intensity of band at 1252 cm^{-1} (part of the amide III spectral region, assigned to random coil) increased , 4) intensity of bands at	- Up to 168 hours (seven days) since deposition; - Suitable for differentiating between “new” (1 hour) and “old” (1 week) bloodstains	[21]

					1637 (O_2 marker band) and 1224 cm^{-1} (part of the amide III spectral region, assigned to β-sheet) decreased; - PLSR analysis with four latent variables was used for developing a predictive model for TSD estimation with cross-validated RMSE and R^2 values of 0.13 and 0.97, respectively		
Raman Spectroscopy (laser wavelength: 785 nm)	Raman spectra of whole dried bloodstains (chemical signature of blood components, mainly Hb derivatives)	- Peripheral human blood obtained from two healthy donors (male and female over 18 years old, both Caucasian); - Without addition of anticoagulants or preservatives	- A small volume (~30 μl) of blood, obtained from fingertip, deposited onto aluminium foil-covered microscope slides using a transfer pipette; - Stored for two years under ambient conditions (T: 22± 3 °C, RH: 50±10%, protected from sunlight	None; non-destructive analysis	- Spectral changes observed with ageing: 1) increase of the fluorescence background toward the low-frequency spectral range, 2) decrease of the 1224 cm^{-1} band and increase of the 1252 cm^{-1} band, corresponding to transformation from β-sheet into random coil (continue up to one month since bloodstain deposition); 3) increase of the 970 cm^{-1} band's intensity, reflecting Hb aggregation; 4) red shift of the band at	Up to 2 years	[57]

			exposure)		520 cm^{-1} to 500 cm^{-1} (after one month elapsed since deposition) with subsequent increase of its intensity, 5) red shift of 676 cm^{-1} and 754 cm^{-1} bands to 660 cm^{-1} and 746 cm^{-1} (after one month elapsed since deposition), 6) diminishing of following bands over time (non-existent in 1-year-old sample's spectrum): 345, 419, 1562, 1600, 1619, 1637 and 1653 cm^{-1}, 7) increase of the intensity of 440 cm^{-1} (novel spectral feature, non-existing in spectra for 1-month-old stains) - Minimal variances between results obtained for different donors; - Minor differences observed between spectra registered for one-year-old and two-year-old bloodstains; - For TSD predictions PLSR and PCR calibration		

					models based on 126 bloodstain spectra of male donor were developed ($RMSE_{PLSR}$=0.29, $RMSE_{PCR}$=0.31, accuracy TSD estimations ~70%)		

Table 2
Absorption maxima of the three main chromophores in bloodstains (oxyHb, metHb and HC)

Hemoglobin derivative	Wavelength of absorption maximum [nm]	Reference
Oxyhemoglobin	414 (Soret or γ band), 542 (α band), 576 (β band)	[195]
	414, 542, 577	[196]
Methemoglobin	406, 500, 632	[195]
Hemichromes	389, 537	[196]

Table 3
List of absorption bands of several blood components along with their possible chemical origins, which were observed in the NIR spectra of bloodstains by Edelman et al. [56]. Data derived from [56]

Wavelength [nm]	Component	Assignment
930	Oxyhemoglobin	Third overtone of –CH and –CH2 stretching vibrations
970	Water	Combination of H–O–H symmetric and asymmetric stretching vibrations
1454	Water	Combination of H–O–H symmetric and asymmetric stretching vibrations
1690	Hemoglobin, albumin, globulin	First overtone of –CH stretching vibration
1740	Hemoglobin, albumin, globulin	First overtone of band at 3477 nm
1940	Water	Combination of H–O–H bending and asymmetric stretching vibrations
2056	Hemoglobin, albumin, globulin	Combination of amide A and amide II or another combination
2170	Hemoglobin, albumin, globulin	Combination of amide B and amide II or overtone of amide II
2290	Hemoglobin, albumin, globulin	–CH stretching and deformation combinations
2350	Hemoglobin, albumin, globulin	–CH stretching and deformation combinations

Table 4
Types of vibrational modes observed for Raman spectra of heme proteins [143], illustrated with the oxyHb bands [cm^{-1}] registered with 514.5 nm excitation [147, 157].

Type of vibrational mode	Local coordinates[a]	Assignment[b]	OxyHb [cm^{-1}]
Skeletal vibrations of heme macrocycle	$\delta(C_\beta C_1)_{sym}$	ν_9	266
	δ(pyr rotation)	ν_{33}	481
	δ(pyr def)$_{sym}$	ν_7	675
	δ(pyr fold)$_{sym}$	γ_5	716
	ν(pyr breathing)	ν_{15}	752
	δ(pyr def)$_{asym}$	ν_{32}	796
	δ(pyr def)$_{asym}$	ν_{46}	931
	ν(pyr half-ring)$_{asym}$	ν_{22}	1121
	$\nu(C_\beta C_1)_{sym}$	ν_{14}	1147
	ν(pyr half-ring)$_{asym}$	ν_{30}	1171
	ν(pyr half-ring)$_{sym}$	ν_{41}	1341
	ν(pyr half-ring)$_{sym}$	ν_4	1375
	ν(pyr quarter-ring)	ν_{20}	1398
	ν(pyr quarter-ring)	ν_{29}	1398
	$\nu(C_\alpha C_m)_{sym}$	ν_{28}	1461
	$\nu(C_\alpha C_m)_{sym}$	ν_3	1504
	$\nu(C_\beta C_\beta)$	ν_{38}	1546
	$\nu(C_\beta C_\beta)$	ν_{11}	1563
	$\nu(C_\beta C_\beta)$	ν_2	1585
	$\nu(C_\alpha C_m)_{asym}$	ν_{19}	1585
	$\nu(C_\alpha C_m)_{asym}$	ν_{37}	1604
	$\nu(C_\alpha C_m)_{asym}$	ν_{10}	1638
Stretching and bending vibrations between	ν(Fe-N)	ν_8	346

the iron and ligands at its fifth and sixth coordination position	ν(Fe-O-O)		422
	ν(Fe-O_2)		568
Vibrations of peripheral substituents at porphyrin ring and their combinations with skeletal modes of heme	$\delta(C_\beta C_c C_d)$		378
	$\delta(C_\beta C_a C_b)$ + $\delta(C_\beta Me)$		422
	$\gamma(C_m H)$	γ_{10}	828
	$\nu(C_c C_d)$		971
	$\gamma(C_a H=)$		998
	$\delta(=C_b H_2)_{asym}$		1058
	$\delta(=C_b H_2)_{asym}$		1090
	prop $\delta(CH_2)$ twisting		1226
	$\delta(C_a H=)$		1305
	$\delta(C_m H)$	ν_{21}	1305
	$\delta(=C_b H_2)_{sym}$		1341
	$\delta(=C_b H_2)_{sym}$		1430
	ν(C=C)vinyl		1619

ν, stretch; δ, in-plane deformation; γ, out-of-plane deformation; sym, symmetric; asym, asymmetric; pyr, pyrrole; p, protein; prop, propionate; deform, deformation

[a] Subscripts α, β, m represent the carbon atoms at the alpha, beta, and *meso* positions of porphyrins; a and b correspond to vinyl group carbons, while c and d to propionate carbons. For detailed scheme of heme labelling, adopted by Hu et al. [146], see Fig. 6.

[b] Assignments based on [142, 146-148].

Table 5
Positions, assignments and local coordinates of bands observed during ageing of bloodstains using 785 nm excitation [122]

Observed band [cm^{-1}]	Vibrational assignment[a]	Local coordinate[b]	Dried 'fresh' bloodstains (~1 hour after deposition) [156]	Aged bloodstains (two weeks after deposition) [122]
347	ν_8	ν(Fe-N)	medium intensity	
377		$\delta(C_\beta C_c C_d)$	weak intensity	increase of intensity
417		δ(Fe-O-O)	medium intensity	absent
570		ν(Fe-O_2)	weak intensity	absent
621	ν_{12}		weak intensity	
667			absent	increase of intensity
677	ν_7	δ(pyr deform)$_{sym}$	medium intensity	decrease of intensity
716	γ_5	δ(pyr fold)$_{sym}$	weak intensity	
747			absent	increase of intensity
754	ν_{15}	ν(pyr breathing)	strong intensity	
788	ν_6	ν(pyr breathing)	weak intensity	
856			weak intensity	

900		p: C-C skeletal	weak intensity	
937	v_{37}		medium intensity	
970	v_{46}	δ(pyr deform)$_{asym}$ or γ(=C$_b$H$_2$)$_{sym}$	absent	increase of intensity
1003		phenylalanine	strong intensity	
1030		δ(=C$_b$H$_2$)$_{asym}$	weak intensity	
1054		δ(=C$_b$H$_2$)$_{asym}$	weak intensity	
1127	v_5		medium intensity	
	v_{22}	v(pyr half-ring)$_{asym}$		
1173	v_{30}	v(pyr half-ring)$_{asym}$	weak intensity	
1224	v_{13} or v_{42}	δ(C$_m$H) prop δ(CH$_2$) twisting	medium intensity	
1248	v_{13}	δ(C$_m$H)	absent	increase of intensity
1311	v_{21}	δ(C$_m$H)$_{asym}$	weak intensity	
1341	v_{41}	v(pyr half-ring)$_{sym}$	medium intensity	
1374	v_4	v(pyr half-ring)$_{sym}$	medium intensity	
1398	v_{20}	v(pyr quarter-ring)	medium intensity	
1450		δ(CH$_2$/CH$_3$)	strong intensity	
1563	v_2 or v_{11}	v(C$_\beta$C$_\beta$)	strong intensity	

1582	ν_{37}	$\nu(C_\alpha C_m)_{asym}$	medium intensity	
1603	ν_{19}	$\nu(C_\alpha C_m)_{asym}$	medium intensity	
1621		$\nu(C_a{=}C_b)$	strong intensity	
1638	ν_{10}	$\nu(C_\alpha C_m)_{asym}$	medium intensity	absent
1654		amide I	weak intensity	

ν, stretch; δ, in-plane deformation; γ, out-of-plane deformation; sym, symmetric; asym, asymmetric; pyr, pyrrole; p, protein; prop, propionate; deform, deformation

[a]Assignments based on [142, 146-148].

[b] Subscripts α, β, m represent the carbon atoms at the alpha, beta, and *meso* positions of porphyrins; a and b correspond to vinyl group carbons, while c and d to propionate carbons. For detailed scheme of heme labelling, adopted by Hu et al. [146], see Fig. 6.

Table 6
Vibrational bands of whole blood and red blood cells affected by high laser fluence. Data from [122]

Low power spectrum (cm^{-1})[a]	High power spectrum (cm^{-1})[a]	Vibrational assignment	Local coordinate
419	-	$\delta(Fe\text{-}O_2)$	$Fe\text{-}O_2$ bend
570	-	$\nu(Fe\text{-}O_2)$	$Fe\text{-}O_2$ stretch
677	667	ν_7	$\nu(\text{pyr deform})_{sym}$
754	747	ν_{15}	ν(pyr breathing)
-	970		$\gamma(C_aH=)$?
1,128	1,123	ν_5	$\nu(C_\beta\text{-methyl})$
-	1,1248	ν_{13} or ν_{42}	$\delta(C_mH)$
1,311	-	ν_{21}	$\delta(C_mH)$
1,374	1,370	ν_4	$\nu(\text{pyr half-ring})_{sym}$
1,398	1,392	ν_{20}	$\nu(\text{pyr quarter-ring})_{sym}$
1,549	1,543	ν_{11}	$\nu(C_\beta\ C_\beta)$
1,582	1,576	ν_{37}	$\nu(C_\beta\ C_m)_{asym}$
1,638	1,629	ν_{10}	$\nu(C_\beta\ C_m)_{asym}$

C_α, C_β, C_m, represent the carbon atoms at the alpha, beta, and *meso* positions of porphyrins, respectively, whilst the pyr denotes the pyrrole ring (see Fig. 6)

[a]The estimated band peak frequency precision is ±1 cm^{-1}

Highlights

- Recent advances in methods of bloodstain dating are comprehensively reviewed.
- Special consideration is given to approaches based on spectroscopic techniques.
- A major problem of situating forensic evidences in time is also discussed.
- A course for the future studies directed towards the bloodstain dating is outlined.