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Research Article

Age Estimation of Concealed and Unconcealed Blood Stains on Different Surfaces – A Chemometric Approach

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Abstract— Blood stains are commonly encountered at crime scenes, either in visible or hidden forms. Establishing the age of these blood stains is critical for effective crime scene investigation. The current study aimed to develop regression models for accurately estimating the age of concealed blood stains on diverse surfaces. Additionally, the study investigated the effects of luminol and concealment on age estimation. The study utilized ATR-FTIR spectroscopy in combination with Chemometric methods, including PCA (Principal Component Analysis) and OPLSR (Orthogonal Signal Correction Partial Least Square Regression Analysis), to analyze blood stains concealed by paint and treated with luminol on three commonly encountered substrates: cement, metal, and wood. The spectral data were analyzed to develop regression models to estimate the age of blood stains for eleven days. The regression models for concealed blood stains exhibited notable differences compared to those for unconcealed blood stains. The predictive Root Mean Square Error (RMSE) values ranged from 0.87 to 1.82, and R-squared values ranged from 0.76 to 0.94. The model for blood stains on cement surfaces was the most accurate in concealed form, while the model for blood stains on metal surfaces was the best fit in unconcealed form. Furthermore, the model for concealed blood stains on wooden surfaces demonstrated the highest prediction error. The results indicated that the presence of luminol and the composition of the substrates on which blood stains were identified significantly influenced the detection of spectral changes associated with age-related alterations observed in blood stains.

Keywords— Blood stains, ATR-FTIR Spectroscopy, Chemometrics, PCA, PLSR, Concealed blood stains, Luminol, Nature of Substrates, Cement, Wood, Metal.

1. Introduction

1.1 Blood as forensic evidence

Biological evidence is pivotal in reconstructing crime scenes, with blood serving as a crucial form of evidence. It furnishes substantial information about various aspects of the crime, particularly in violent cases like murder or sexual assault [1]. The presence of blood can manifest in various forms, appearing on a range of surfaces and within diverse environments, including cement walls, wooden furniture, and automobiles [2][3]. However, the identification of blood stains may pose challenges when the substrate has been deliberately cleansed or exposed to precipitation or moisture. In some instances, trace amounts of blood may result in latent stains that are imperceptible to the naked eye [4]. Perpetrators may seek to conceal the crime by painting over blood stains on different surfaces. Luminol, a highly sensitive blood detection reagent used in forensic investigations, can unveil latent and concealed blood stains [4] [5]. The luminol solutions used for blood stain detection are alkaline and incorporate hydrogen peroxide as an oxidizing agent. The ferric groups of heme moiety in blood would catalyze the oxidation of luminol, a process assisted by the decomposition of hydrogen peroxide in the solution. Blood stains harbor a wealth of information that can aid in crime investigations, including their time since deposition [4].

1.2 Age of blood stains

Blood stains that are encountered at different types of crime scenes hold significant value in forensic investigations, as they can be used to confirm a suspect's identity through DNA profiling and to reconstruct the sequence of events through pattern analysis [6]. However, it is crucial to differentiate between blood stains relevant to the crime and those not. Accidental cuts may cause blood stains that are unrelated to the crime, necessitating a careful distinction in criminal investigations [7]. Determining the time when a blood stain was deposited is important for establishing the timeframe of the crime and can support the account of the events. Various methods have been investigated for determining the timing of blood stain deposition; however, none have exhibited the

level of precision and reproducibility necessary for forensic application [2]. Many techniques such as chromatography, nucleic acid degradation, enzymatic activity, and so on have been used to determine the age of blood stains[8]. Forensic Science emphasizes non-destructive techniques for analysis due to the evidentiary value and limited availability of samples. Currently, spectroscopic techniques are one of the non-destructive techniques that are being explored for age estimation purposes.

1.3 Spectroscopy and Chemometrics

Spectroscopic methods have undergone thorough investigation to discern and identify bodily fluids owing to their expeditious, sensitive, cost-efficient, and non-intrusive attributes. These methods are adept at detecting various bodily fluids and facilitating on-site analysis, thus serving as invaluable assets in forensic scrutiny[9]. There are different types of spectroscopic techniques, finding applications in various domains. Infrared spectroscopy is one of the most powerful analytical techniques used for chemical identification. When in tandem with intensity measurements, this technique may be used for quantitative analysis[10]. Fourier-transform infrared (FTIR) spectroscopy possesses the capability to identify a variety of body fluids present on diverse surfaces, including fabrics, owing to its surface sensitivity and shallow depth of penetration[11]. In addition to detection, FTIR spectroscopy can also estimate the age of body fluids. Attenuated Total Reflection (ATR) FTIR spectroscopy has emerged as a cost-effective method for analyzing body fluids in forensic cases, capable of differentiating all types of body fluids with minimal or no sample preparation and no need for chemical reagents during analysis. However, the large amount of data produced by analytical methods makes the work of experts more demanding and time-consuming and can lead to false positive results from manual examination[12]. Different chemometric techniques have been developed to tackle these challenges and analyze enormous and complex spectral data, providing accurate and timely results. ATR-FTIR spectroscopy, along with chemometric analysis, is thus a powerful tool for estimating the age of blood stains [13]. Research indicates that blood stain age can be accurately estimated with minimal age prediction error up to around 2 years [14]. Nonetheless, there is limited research on blood stains deposited on different substrates under various environmental conditions and no research on age estimation of latent blood stains concealed by paint.

The present study aims to investigate blood stains on three commonly encountered surfaces (cement, wood, and metal) in both concealed and unconcealed settings, estimating their time since the deposition for 11 days. Additionally, the study assesses the influence of luminol, a reagent used to detect hidden blood stains, on the estimation of blood stain age. The analysis utilizes a combination of ATR-FTIR and chemometric techniques.

2. Related Work

2.1 Non-invasive techniques for blood stain age estimation:

Blood is important physical evidence encountered in crime scene investigations, and its biochemical instability in the ex-vivo environment necessitates accurate determination of its age. Estimating the time since the deposition of a blood stain is crucial in forensic analysis [2], [15]. The degradation of blood stains is influenced by a multitude of factors, including temperature, humidity, microorganisms, environmental pollutants, and substrate characteristics. Although initial investigations into the aging of blood stains commenced in the early twentieth century, substantial advancements have been achieved since the early 1990s [16]. Various techniques are employed to assess the age of blood stains, varying from non-invasive to highly invasive methods. Non-contact and non-destructive methods are recommended to minimize contamination and allow independent verification [17]. Historically, blood stain age estimation involved destructive methods but has now transitioned to relatively non-destructive methods such as Infrared spectroscopy, Raman spectroscopy, Electron spin resonance spectroscopy, Reflectance spectroscopy, Atomic force microscopy and UV-Visible spectrophotometry [17], [18]. The major goal was to find age estimation techniques that allowed for further analysis of blood stains, particularly for DNA evidence, leading to research into spectroscopic techniques for Time-Since-Deposition estimation of blood stains.

A study conducted by the Ballantyne group analyzed the oxidative changes in hemoglobin and their impact on DNA extraction from blood stains using UV-Visible absorption spectroscopy [19]. They inferred that these changes did not hinder DNA extraction. They then utilized UV-Visible absorption spectroscopy to estimate the age of blood stains by measuring changes occurring in the Hb Soret band, having a maximum absorbance at 412 nm. A blue spectral shift was observed in this band as the blood stain aged, along with changes in its maximum absorbance with time [20]. The extent of this shift was used as a measure of age to determine the TSD of blood. However, it was noted that this technique is temperature-dependent, with a faster and larger shift in blood stains exposed to heated conditions. Despite being a destructive analysis, the products of UV-Visible absorption spectroscopy can still be used for DNA analysis [19]. This analysis using UV-Visible absorption spectroscopy marks one of the first steps toward non-invasive techniques for age determination of blood stains [21].

Doty and the group conducted a study using Raman Spectroscopy to analyze changes in blood stains for one week. The study involved three steps for spectral analysis. First, they identified all blood stains by comparing their spectra to reconstructed spectra of semen, sweat, blood, saliva, and vaginal secretions. Next, they used 2-D Correlation Spectroscopic analysis (2D CoS) to explore the relationships between spectral changes and natural blood stain aging. Finally, they used Partial Least Squares Regression (PLSR) analysis to predict the age of the blood

stains. The study concluded that freshly deposited blood stains of less than an hour could be easily differentiated from older blood stains. In addition, all blood stains could be identified as blood by matching with spectroscopic signatures. The results showed a strong correlation between several Raman bands and the age of a blood stain. Doty et al. revealed that the peaks at 345, 375, 420, 440, 500, 570, 750, 970, and 1450 cm-1, as well as the spectral regions from 1200–1300 cm-1 and 1520–1670 cm^{-1}, changed most noticeably as blood stains aged. These findings suggest that Raman spectroscopy is a non-destructive tool that can effectively discriminate between blood stains for hours to days [22].

2.2 ATR-FTIR spectroscopy for age estimation:

Hemoglobin serves as a useful indicator for determining the age of blood stains due to its measurable degradation process through various techniques. Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) is a rapid and non-invasive technique that measures the change in the dipole moment of bonds following excitation with electromagnetic radiation. The low IR activity of heme groups allows for non-destructive detection of blood proteins. Analysis of spectra of blood stains using ATR-FTIR reveals the presence of peaks that are characteristic of blood, including 3300 cm-1 (Amide A), 2800-3000 cm-1 (Amide B), ~1650 cm-1 (Amide I), ~1540 cm-1 (Amide II), and 1200-1350 cm-1 (Amide III). ATR-FTIR analysis provides substantial donor information, yet its application in determining the TSD of blood stains is still nascent [23], [24]. Lu examined blood under different humidity levels at 30°C on acrylic and cotton substrates over several months. Notably, changes in the position and intensity of amide peaks were observed due to protein denaturation as blood stains aged. A Partial Least Squares (PLS) regression model was developed to monitor these multivariate changes and estimate the TSD. Increased uncertainty in predicting short-term (1-2 days) versus long-term (4-10 days) blood stain age was noted as spectral result variability intensified [25]. A nonlinear calibration model was recommended by Lu, considering the heterogeneous interaction between blood and substrates. Consequently, substrate type must be considered when devising an aging model. Additionally, Lin et al. utilized ATR-FTIR and chemometric analysis to construct a model for estimating TSD. Fresh, anticoagulant-free blood stains were placed on glass slides and exposed to simulated indoor and outdoor crime scene conditions for up to 85 days. Their PLS regression model accurately predicted blood stain age for up to 7 days, and two PLS-Discriminant Analysis (PLS-DA) models effectively differentiated between blood stains less than a day old and those exceeding one day [26]. Although more research is warranted to unlock the complete potential of ATR-FTIR, its capacity for determining the TSD of blood stains is considerable [21].

Another study conducted by the Raj Kumar group utilized ATR-FTIR spectroscopy to develop different regression models for age estimation of human blood stains and distinguishing between human and animal blood. In this study, various models for age estimation of human blood stains were developed using trained data sets of blood stains aged 1 to 175 days. The models, including Curve Estimation (CE), Multiple Linear Regression (MLR), and Partial Least Squares Regression (PLSR), were developed to determine the most effective prediction model for aged human blood stains. The results indicated that the curve estimation models had the highest dating errors, while the MLR and PLSR models showed excellent age estimation for unknown blood stains. These models had an error of approximately 3 ± 1 days and 4 ± 1 days, respectively, between the actual and estimated dates [27].

Lin et al utilized the Attenuated Total Reflection (ATR)-Fourier Transform Infrared (FTIR) spectroscopic technique along with advanced chemometric methods to assess the age of indoor and outdoor blood stains up to 107 days. They replicated real-time crime scene conditions for blood stain storage. The results revealed that two partial least squares regression models, one for indoor and one for outdoor blood stains spanning 7 to 85 days, demonstrated strong performance during external validation, with low predictive root mean squared error values (5.83 and 4.77) and high R square values (0.94 and 0.96). Additionally, two partial least squares–discriminant analysis classification models effectively distinguished between fresh (≤1 day old) and older (>1 day old) blood stains, providing significant value for forensic investigations. This study underscores the potential of ATR-FTIR spectroscopy along with advanced chemometric methods, as a rapid and non-destructive tool for estimating the age of blood stains in real-world forensic scenarios [26]. The present study has utilized ATR-FTIR spectroscopy along with chemometric tools PCA and OPLSR for age estimation of concealed blood stains in comparison to those unconcealed.

2.3 Role of substrate properties on blood age estimation:

The substrate on which a blood stain is found is a critical factor in blood stain pattern analysis [28]. However, there have been limited reports on how the substrate impacts the aging of blood stains. The interaction between the blood and the substrate could affect the clotting and drying time of blood [29]. These interactions differ based on the porosity of surfaces. While some studies indicate that the substrate does not affect blood stain aging analysis, these methods typically involve extracting the blood stains [20]. However, when examining blood stains in their original location, the substrate nature must be considered. Most studies on the impact of substrates on blood stain aging have utilized white cotton fabric or filter paper, which are beneficial for in vitro analysis and reflectance spectroscopy. Glass surfaces were employed for Atomic Force Microscopy (AFM) studies to simulate a flat surface. One study has compared the effect of aging blood stains on cloth and paper, inferring no evident difference in age estimation [30]. Future research could explore the difference in aging for blood stains on absorbing versus non-absorbing surfaces or the distinction between hydrophobic and hydrophilic surfaces. Certain researchers have conducted experiments with different porous and nonporous substrates and found substantial differences in the aging rates of blood stains [15], [31], [32]. This study

considers the effect of substrate porosity and concealment on age estimation of concealed blood stains.

3. Experimental Method

3.1 Sample preparation

Cement blocks, metal surfaces, and plywood sheets were selected for the study to analyze the impact of substrate nature on the aging of blood stains. All three surfaces were coated with their respective paints to simulate furnished substrates. Cement is naturally porous, while wood, in its unfurnished state, is also porous and becomes non-porous after painting. On the other hand, metal surfaces are non-porous in both states. A single layer of distemper was applied on cement blocks, while the wooden blocks and metal surfaces were painted with a single layer of enamel paint. All surfaces were left to dry at room temperature for one day.

A 5 ml sample of human venous blood was collected from a healthy male volunteer without anemia or a history of blood-related disorders, with the assistance of a phlebotomist and the necessary approval from the Institutional Human Ethics Committee for Human Research (IHEC) Mysore, dated 21.11.2020. Informed consent (written) was obtained from the volunteer before collecting the sample. The collected blood sample was stored in EDTA vacutainers, as previous studies indicated no difference in the aging process of blood stains stored in EDTA containers [14].

3.2 Deposition of blood stains and concealment

The study was designed for eleven days, divided into five time intervals. Each substrate was marked with 5 blood spots, each measuring 100 microliters using a micropipette. These spots represented specific time intervals for blood analysis on days 1, 4, 6, 9, and 11 post-blood deposition. Eight replicates of the blood stains for each time spot were created. Four blocks from each substrate were covered with substrate-specific paint (distemper was applied on cement blocks and enamel paint was applied on wooden and metal blocks), while the four blocks remained uncovered and exposed. All the substrate blocks were stored at room temperature allowing the concealed and unconcealed blood stains to age.

3.3 Detection of concealed blood stains

The detection of concealed blood stains involved the use of Luminol reagent, which produces a bright chemiluminescence upon reacting with the iron component in hemoglobin. The Luminol solution was prepared using the Grodsky formulation by dissolving 5.0 g of sodium carbonate in 100 mL of distilled water, then adding 0.1 g of luminol and swirling to dissolve. The solution was transferred to a spray bottle. 0.7 g of sodium perborate catalyst was dissolved in 100 mL of the prepared luminol solution in the spray bottle just before the application as sodium perborate is highly unstable.

The substrates with concealed blood stains were placed in darkness, and the prepared luminol solution was sprayed uniformly on the surface to observe the pattern of chemiluminescence. This procedure was repeated on each day of sample collection (days 1, 4, 6, 9, and 11), with all the dried concealed blood samples. All the dried blood stains (concealed and unconcealed) were then collected from the substrates using a sterile scalpel and placed in zip-lock bags.

3.4 Instrumental analysis

The IR spectra of the blood samples were obtained using a Bruker Alpha II compact ATR-FTIR Spectrophotometer with a Zinc Selenide (ZnSe) crystal. Each dried blood sample was directly placed on the ATR crystal and scanned in the mid-IR range of 400 cm^{-1} to 4000 cm^{-1} with a spectral resolution of 4 cm-1 and an average of 16 scans [3]. All samples were analyzed with air as the background measurement. The ATR crystal was cleaned with isopropyl alcohol before each subsequent sample analysis, to ensure no contamination and interference. Each sample was analyzed in duplicates. This procedure was performed on days 1, 4, 6, 9, and 11, and the spectral data were collected. The spectral data were collected using Opus software (version 7.5) and imported for further investigation.

3.5 Chemometric analysis:

The spectral data from Opus software was transferred to Excel and subsequently imported into Unscrambler for analysis. The data was chemometrically analyzed using Unscrambler X software, version 10.5 by Camo AS, Norway as follows:

3.51 Data pre-processing:

The spectral data imported was subjected to pre-processing to eliminate undesired scattering effects that could potentially disrupt the construction of the chemometric model. To achieve this, baseline correction was performed using OPUS software and the Unscrambler X software was utilized to perform Standard Normal Variate (SNV), and Orthogonal Signal Correction (OSC) on the data.

3.52 Principal Component Analysis (PCA):

PCA was utilized as an exploratory data analysis method to visualize the interrelationship between samples and variables and to ascertain patterns within the data. Before conducting PCA, the data was mean-centered. The PCA models were developed using the Non-Linear Iterative Partial Least Squares (NIPALS) algorithm to study the potential grouping of samples by their age and their states as concealed and exposed.

3.53 Orthogonal Partial Least Square Regression (OPLSR):

Spectral data points within the range of 1300 cm^{-1} to 3600 cm^{-1} were selected for chemometric analysis based on the findings from the PCA analysis. The data was mean-centered before applying Partial Least Square Regression (PLSR). The OPLSR model was constructed by applying the Kernel PLS algorithm to the data subjected to OSC. Evaluation of the regression model relied on Root Mean Square Error (RMSE) and determination of regression (R^2). Individual models were established for samples on cement, wood, and metal surfaces under both concealed and unconcealed conditions. The developed models were then subjected to full validation (Leave-one-out cross-validation).

4. Results

4.1 Chemiluminescence upon spraying luminol

The time required for the chemiluminescence to develop for blood stains on different concealed surfaces was observed. It was found that the chemiluminescence reaction occurred almost instantly, in less than 2 seconds, when luminol was sprayed over blood stains on wooden and metal surfaces. However, a delay of about 10 seconds was observed for blood stains on cement surfaces. This pattern remained consistent from day 1 to day 11. Moreover, the intensity of the chemiluminescence was observed to increase as the blood stains aged from day 1 to day 11.

4.2 Classification of blood stains based on their time-since-deposition

The PCA scores plot was created to analyze patterns in the spectral data and differentiate blood stains based on their age post-deposition. In Figure 1, the scores plot indicates that the spectra of blood stains from days 1 to 3 were broadly spread across the 1st and 3rd quadrants on the negative side of PC 1 and were segregated. On the other hand, some of the spectra of blood stains from days 3 to 5 were situated on the negative axis of PC 1, while a few were intermingled with the spectra of blood stains older than day 5 on the positive axis of PC 1, and not distinctly separated. The spectra of blood stains from days 5 to 7 could not be differentiated from others, as they were found to be clustered near the origin together with other spectra. However, the majority of spectra of blood stains from days 9 to 11 were distributed across the 4th quadrant on the positive side of PC 1.

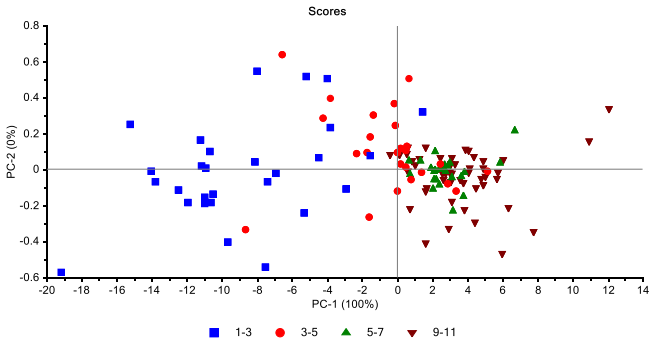

Figure 1: Day-wise PCA scores plot for blood stains

4.3 Discrimination of Concealed and Exposed Blood Stains:

The PCA scores plot in Figure 2 revealed that a complete separation between concealed and exposed blood stains was not attainable. Nevertheless, a discernible pattern was evident among the samples based on their concealed and exposed conditions. The samples were grouped according to their similarities. Two Principal Components explained the entire variation among the samples, with PC1 representing 78% of the initial variation and PC2 representing 5%, resulting in a total variation of 83%. A majority of concealed blood spectra were dispersed on the negative axis of PC1, while most of the exposed blood spectra were situated along the positive axis of PC1. Additionally, the concealed and unconcealed blood spectra were dispersed along both positive and negative axes of PC2.

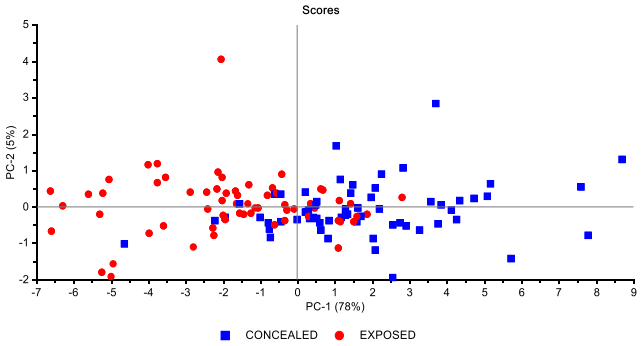

Figure 2: PCA Scores plot for concealed and unconcealed blood stains

4.4 Age prediction using OPLSR models

Distinct OPLSR regression models were developed for blood stains on cement, wood, and metal surfaces using a training dataset of blood samples with two factors from the PLS regression. The first two factors were identified as significant for the model, as they captured a greater amount of variation compared to other pairs of factors. The models were subjected to Standard Normal Variate and Orthogonal Signal Correction using the Kernal PLS algorithm. In total, six models were created, with two models for each surface: one for unconcealed conditions and one for concealed conditions.

The scores plot for blood stains in concealed and unconcealed states on cement, metal, and wooden surfaces (Figures 3-8) revealed that the grouping of blood stains based on their time since deposition was well-defined in concealed conditions compared to those found in unconcealed conditions. In both concealed and unconcealed conditions, the spectra of day 1 to 3 blood stains were distributed along the negative axis of factor 1, while the spectra of blood stains older than day 3 were dispersed along the positive axis of factor 1.

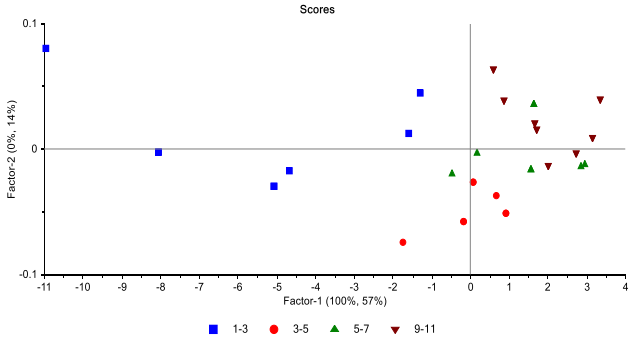

Figure 3: Scores plot for concealed blood stains on cement surfaces

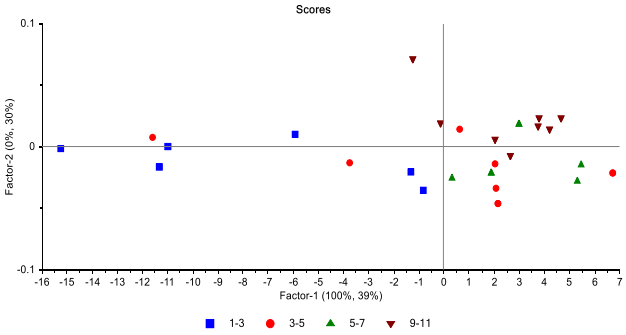

Figure 4: Scores plot for unconcealed blood stains on cement surfaces

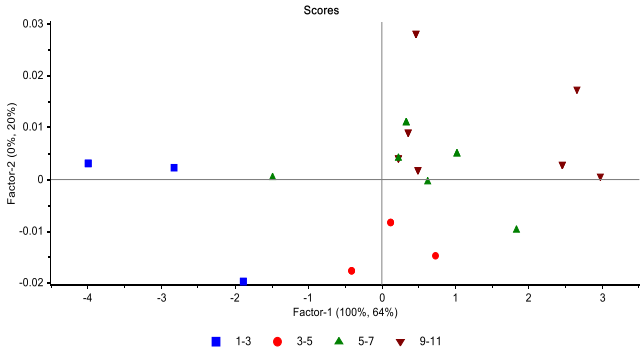

Figure 5: Scores plot for concealed blood stains on metal surfaces

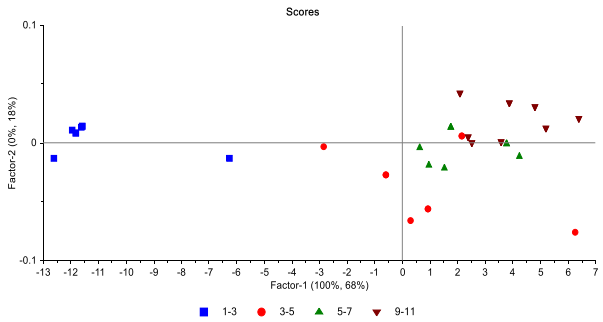

Figure 6: Scores plot for unconcealed blood stains on metal surfaces

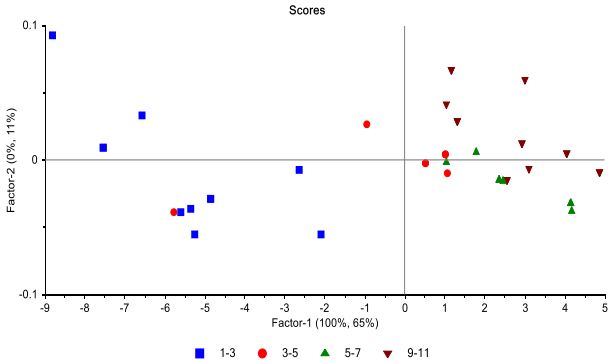

Figure 7: Scores plot for concealed blood stains on wooden surfaces

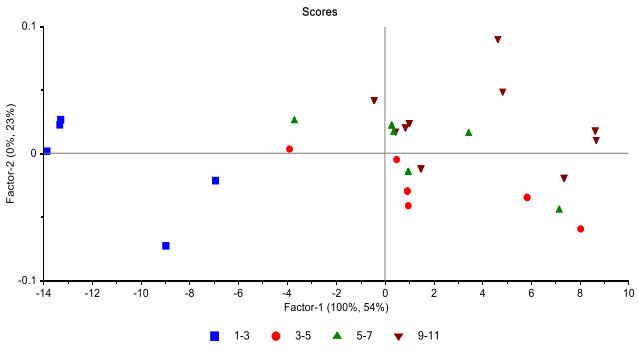

Figure 8: Scores plot for unconcealed blood stains on wooden surfaces

Table 1 summarizes the characteristics of age regression models developed for concealed and unconcealed blood stains on cement, metal, and wooden surfaces.

4.41 Blood stains on cement surfaces:

The X-loading plots exhibit a similar profile to the original data, emphasizing significant areas and variables that offer pertinent information. In the loading plot for concealed blood stains on cement surfaces (Figure 9), Factor 1 demonstrated a positive correlation between 2500 cm^{-1} and 2800 cm^{-1} and a negative correlation between 2800 cm^{-1} and 3000 cm^{-1}. For exposed blood stains (Figure 10), Factor 1 showed a positive correlation from 3100 cm^{-1} to 3150 cm^{-1} and from 3474 cm^{-1} to 3612 cm^{-1}, as well as a negative correlation from 3150 cm^{-1} to 3426 cm^{-1}. The R-square value, representing the goodness of fit for the model, was 0.99 for the calibration set of concealed blood stains and 0.88 for the validation set. For exposed blood stains, the R-square value was 0.97 for the calibration set and 0.81 for the validation set (Figure 11 and Figure 12). The root mean square error (RMSE) for the calibration error of concealed blood stains was 0.31, while for the validation error, it was 1.2. For exposed blood stains, the RMSE values were 0.57 for calibration error and 1.5 for validation error.

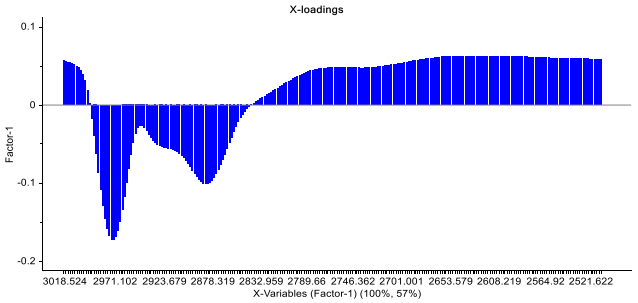

Figure 9: X Loadings plot for concealed blood stains on cement surfaces

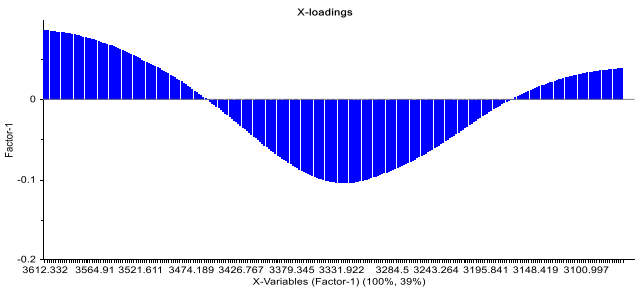

Figure 10: X Loadings plot for unconcealed blood stains on cement surfaces

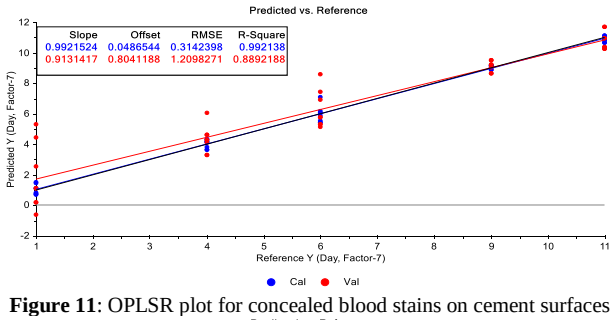

Figure 11: OPLSR plot for concealed blood stains on cement surfaces

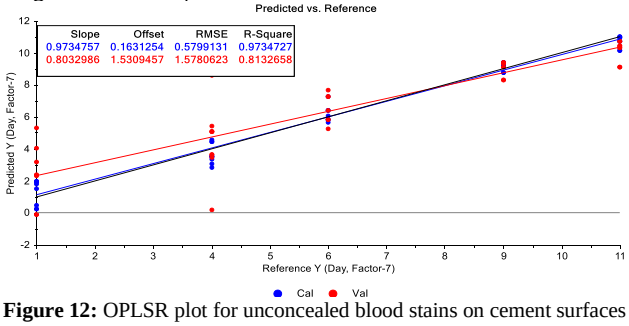

Figure 12: OPLSR plot for unconcealed blood stains on cement surfaces

4.42 Blood stains on metal surfaces:

In Figure 13, the X-loading plot illustrated the positive correlation for Factor 1 between 2500 cm^{-1} and 2800 cm^{-1} for concealed blood stains on metal surfaces. Contrarily, the loading plot in Figure 14 for unconcealed blood stains showed a positive correlation for Factor 1 between 3000 cm^{-1} and 3200 cm^{-1}, as well as between 3450 cm^{-1} and 3600 cm^{-1}. Additionally, it revealed a negative correlation between 3170 cm^{-1} and 3450 cm^{-1}.

Figures 15 and 16 present OPLSR plots, demonstrating an R square value of 0.96 for the calibration set and 0.86 for the validation set of concealed blood stains. For exposed blood stains, the R square value was 0.98 for calibration and 0.94 for validation. The RMSE value for the calibration error of concealed blood stains was 0.63, with a validation error of 1.3. The RMSE values for calibration and validation errors for exposed blood stains were 0.39 and 0.87, respectively.

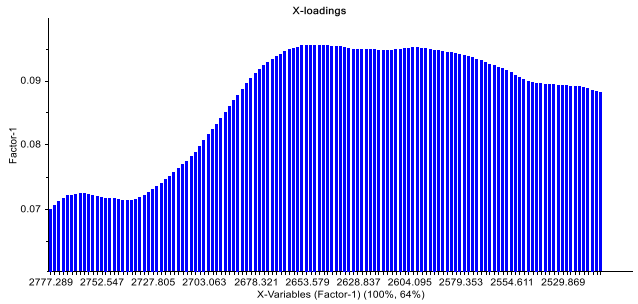

Figure 13: X Loadings plot for concealed blood stains on metal surfaces

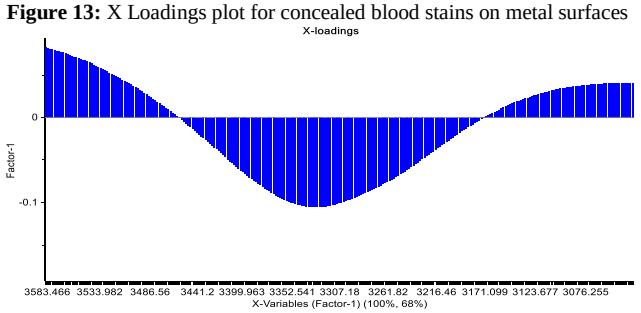

Figure 14: X Loadings plot for unconcealed blood stains on metal surfaces

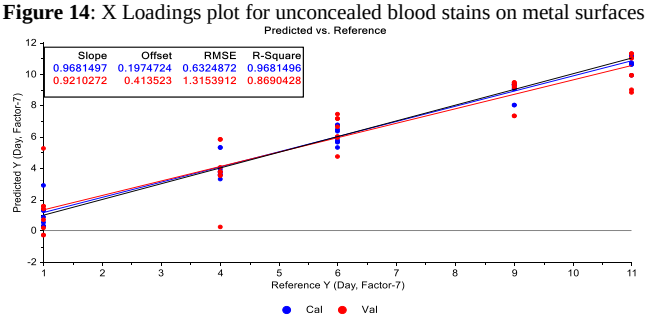

Figure 15: OPLSR plot for concealed blood stains on metal surfaces

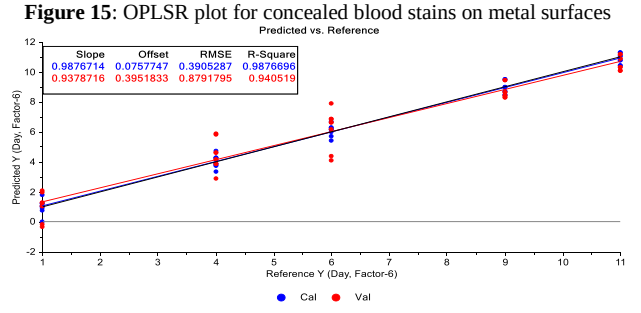

Figure 16: OPLSR plot for unconcealed blood stains on metal surfaces

4.43 Blood stains on wooden surfaces:

Examination of the X-loading plot for blood stains on wooden surfaces indicated that Factor 1 showed a positive correlation from 2500 cm^{-1} to 2840 cm^{-1} and a negative correlation from 2845 cm^{-1} to 3600 cm^{-1} for concealed blood stains (Figure 17). Conversely, the loading plot for exposed blood stains (Figure 18) demonstrated that Factor 1 is positively correlated from 2500 cm^{-1} to 2800 cm^{-1}, 3000 cm^{-1} to 3150 cm^{-1}, and 3500 cm^{-1} to 3600 cm^{-1}, while being negatively correlated from 2850 cm^{-1} to 2950 cm^{-1} and 3150 cm^{-1} to 3500 cm^{-1}. The R-square values obtained for the calibration and validation sets for concealed and exposed blood stains are detailed in Figures 19 and 20, with values of 0.91 and 0.76 for the calibration set of concealed blood stains, 0.99 and 0.80 for the validation set of concealed blood stains, 0.86 and 0.75 for the calibration set of exposed blood stains, and 0.92 and 0.82 for the validation set of exposed blood stains. Furthermore, the root mean square error (RMSE) values for the calibration and validation errors for concealed and exposed blood stains were reported as 1.06 and 1.8 for concealed blood stains, and 0.31 and 1.5 for exposed blood stains.

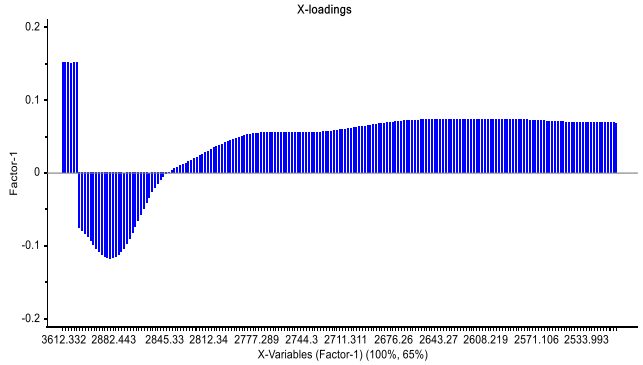

Figure 17: X Loadings plot for concealed blood stains on wooden surfaces

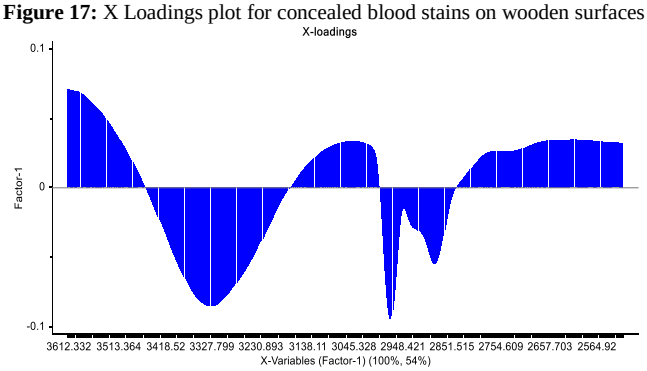

Figure 18: X Loadings plot for unconcealed blood stains on wooden surfaces

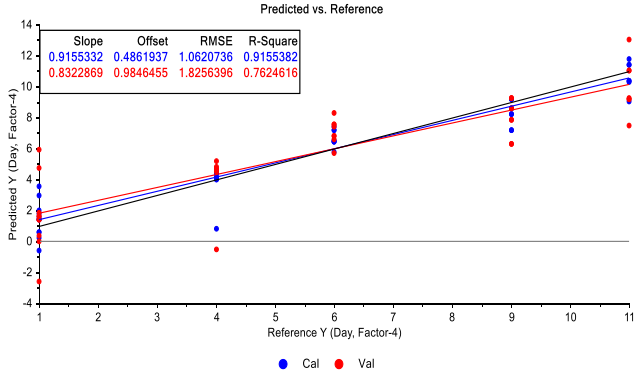

Figure 19: OPLSR plot for concealed blood stains on wooden surfaces

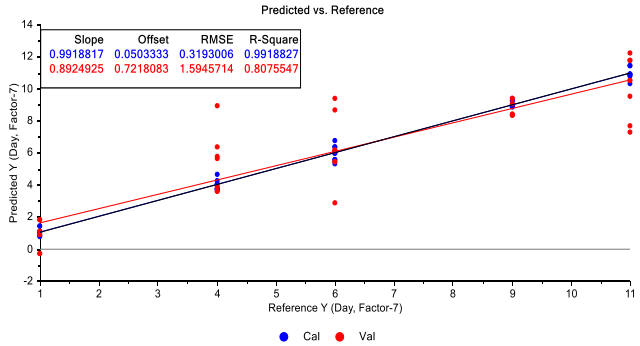

Figure 20: OPLSR plot for unconcealed blood stains on wooden surfaces

Table 1: Regression Model characteristics for blood stains on concealed and unconcealed surfaces

Surface	R Square		RMSE	
	Calibration	Validation	Calibration	Validation
Concealed surfaces				
Cement	0.99	0.88	0.31	1.2
Metal	0.96	0.86	0.63	1.31
Wood	0.91	0.76	1.06	1.82
Unconcealed surfaces				
Cement	0.97	0.81	0.57	1.57
Metal	0.98	0.94	0.39	0.87
Wood	0.99	0.80	0.31	1.59

5. Discussion

An accurate estimation of the time since the blood stain was deposited is essential in forensic investigations. Previous research on blood aging has primarily focused on analyzing changes in various blood components, particularly hemoglobin, over time to determine the post-deposition interval. These studies have utilized spectroscopic techniques and chemometrics to investigate blood aging on different surfaces and under varied conditions. However, an area that merits further exploration is understanding the aging process in concealed blood stains and the potential impact of luminol on age estimation, considering its interaction with the hemoglobin component of blood.

While prior research has emphasized the significance of the bio-fingerprint region (900 cm^{-1} to 1800 cm^{-1}) in blood stains for age estimation, this study discovered that a wider spectral range from 1300 cm^{-1} to 3800 cm^{-1} is influential in capturing variations in age-related changes in blood, whether concealed or unconcealed. Specifically, the spectral range from 2500 cm^{-1} to 3500 cm^{-1} proved to be particularly valuable, as age estimation models developed from data within this range exhibited outstanding predictive performance across all three surfaces under investigation, regardless of whether the blood stains were latent or patent. This finding resonates with established literature, which suggests that this spectral region reflects alterations in the structure of hemoglobin [33]. Notably, no prior studies have delved into the impact of concealment and luminol on age estimation.

In this study, upon comparison of chemiluminescence on wooden and metal surfaces versus cement surfaces, it was observed that chemiluminescence took longer to develop on cement surfaces compared to the instant reaction observed on metal and wooden surfaces upon applying luminol. This observation may be attributed to the nature of the substrate influencing the reaction between luminol and the hemoglobin component of blood stains. Substrates can be classified into absorbent and non-absorbent materials. Absorbent materials have a porous texture and can retain blood for an extended period even after thorough cleaning. Examples include wooden panels, concrete walls, interstitial spaces between tiles or wood objects, carpets, mats, leather, and fabrics. These surfaces are relatively easier to analyze due to their capacity to preserve substantial amounts of blood in relatively undegraded form, thus yielding a strong reaction with the luminol test. This phenomenon is likely due to the quicker drying of blood stains in covered environments, which prevents their degradation by environmental factors. Additionally, absorbent materials can act as a shield, protecting the blood stains from environmental agents such as sunlight, moisture, and attempted cleaning from the perpetrators [34], [35]. Cement being porous, retained blood stains deeper, hence taking a longer time for chemiluminescence to occur. Metal and furnished wood, being non-porous, can be classified as non-absorbent surfaces. These non-absorbent surfaces cannot effectively retain blood and are vulnerable to degradation from various physical and chemical agents. This finding can be supported by Lytle and Hedgecock (1973) [35], who demonstrated that these surfaces can be easily cleaned. Even a mild washing with water and soap can remove blood stains, resulting in minimal reaction with luminol [27], [36].

A comparison of the intensity of luminol chemiluminescence on all three substrates under investigation revealed that it increased with the age of blood stains from day 1 to day 11. Luminol (3-aminophthalhydrazide) is a reagent used to detect concealed blood stains, and it has gained prominence as a presumptive test for blood, notably within the realm of crime scene investigation [37]. A pivotal consideration arises regarding the effect of luminol on the estimation of blood stain ages. The desiccation and aging of blood lead to diverse chemical and biological processes, culminating in the conversion of hemoglobin to methemoglobin due to the oxidation of the iron in the heme groups from Fe^{2+} to Fe^{3+} [38] [34], [39]. The transformation of aged blood stains affects the catalytic properties of blood in reactions that generate luminol's chemiluminescence (CL). Consequently, it has been documented that aged blood stains exhibit more pronounced and enduring chemiluminescence compared to fresh blood stains [34], [39].

The study utilized Principal Component Analysis (PCA) to examine concealed and unconcealed blood samples based on the time since they were deposited on the surfaces. The

results indicated that blood stains deposited from day 1 to 3 could be differentiated from other samples. Samples from days 3 to 5 began to show clustering, while samples from days 5 to 9 did not display a clear pattern. However, samples older than day 9 exhibited a noticeable pattern and were closely associated. This discovery is in line with previous research, underscoring the challenge of distinguishing blood stains younger than 7 days from more recent ones, while older stains can be readily differentiated from fresh ones [40]. It is worth noting that these findings contrast with a previous study related to age estimation of blood stains on cement, metal, and wooden surfaces without concealment, where only blood stains that were 3 days old could be accurately classified, with no distinct pattern observed in PCA beyond that point [3]. This suggests that concealment may act as a protective barrier against age-related changes in blood stains. The clear differentiation between fresh and older blood stains implies that changes in their components begin upon deposition on a surface and become fully apparent after aging for more than a week. To understand the behavior of the samples, the spectral wavelengths shown in the X loadings plot were analyzed. This helped identify the specific spectral regions in blood samples that contributed to the results and represented the maximum variance in the dataset. Figure 21 shows the loading plot for the PCA of blood stains. The variation of the samples was fully explained by PC 1, which exhibited a positive correlation between 1385 cm^{-1} and 2800 cm^{-1} (attributed to C-F, S=O, N-O, C=O, and S-H vibrations), 3000 cm^{-1} to 3120 cm^{-1}(caused by C-H vibrations), and from 3400 cm^{-1} to 3618 cm^{-1}(caused by N-H and O-H vibrations). A negative correlation was observed between 900 cm^{-1} to 1385 cm^{-1}(attributed to C=C, S=O, and C-N vibrations), 2800 cm^{-1} to 3000 cm^{-1} (attributed to C-H vibrations), and 3119 cm^{-1} to 3362 cm^{-1}(attributed to N-H and O-H vibrations).

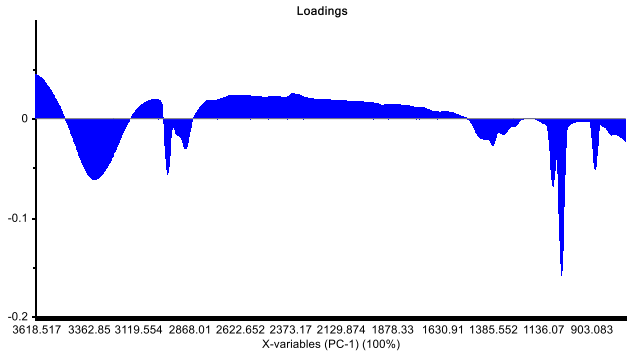

Figure 21: Loadings plot for PCA of blood stains (day-wise)

PCA was also utilized to comprehend the trend of blood samples in both concealed and unconcealed conditions. The results revealed that the samples could be effectively grouped into two categories, irrespective of the type of substrate used. The variability among samples was explicated by two Principal Components. The combined loading plot of PC1 and PC2 (Figure 22) was divided into three regions: - Region I (3500 cm^{-1} to 2900 cm^{-1}) caused by C-H, N-H and O-H stretching - Region II (2800 cm^{-1} to 1800 cm^{-1}) caused by various stretching vibrations including C=O, C-H, C=C=N, S-H and N=C=S - Region III (1300 cm^{-1} to 900 cm^{-1}) attributed to C=C, S=O, C-O, and C-N.

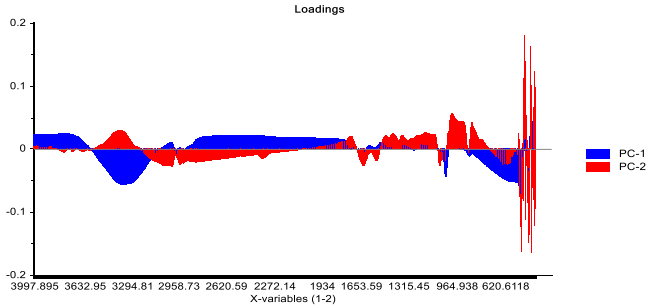

Figure 22: Combined loading plot for concealed and unconcealed bloodstains

The results from the Orthogonal Partial Least Square Regression (OPLSR) models revealed that the predictive ability of the regression models developed for blood stains in both unconcealed and concealed conditions varied based on whether they were subjected to luminol or not, as well as on the nature (whether porous or non-porous) of the substrate. The performance of a model is typically assessed using its R-squared value and RMSE. A higher R-squared value (closer to 1) and a lower RMSE (less than 1) indicate better model performance, with higher predictive accuracy and lower errors. Upon analysis, it was found that the model designed to predict the age of concealed blood stains on cement surfaces had the highest R-squared value of 0.88, implying an 88% accuracy, and an error of 1.2 days between the actual and predicted ages. The model for blood stains on metal surfaces followed with a prediction accuracy of 86% and an error of 1.3 days, while the model for blood stains on wooden surfaces had the least R-squared value of 0.76 and a maximum error of +/- 1.8 days. However, different trends were observed for unconcealed blood stains. The regression model for blood stains on metal surfaces exhibited the greatest predictive accuracy of 94%, followed by cement surfaces with a prediction rate of 81% and wooden surfaces with 80%. The maximum prediction error was observed in the models for wooden and cement surfaces, which was +/- 1.5 days, while the model for metal surfaces had an error of less than one day.

The comparison of regression models for blood stains on concealed and unconcealed surfaces suggests that paint concealment may display a protective effect on the aging process of blood stains. However, this effect appeared to be hindered by the presence of luminol. This was evident in the case of cement, as its porous nature allowed for deeper penetration of blood stains, limiting their contact with luminol, resulting in a better fit model in comparison to unconcealed blood stains. On the other hand, with non-porous surfaces like metal and wood, despite concealment, blood stains came in contact with luminol more readily, potentially interfering with the natural aging process. It was observed that on non-porous surfaces, concealed models had lower predictive accuracy than unconcealed ones. Additionally, luminol was found to negatively impact the age prediction of blood stains on all surfaces. However, concerning cement surfaces, the effect of luminol was not prominent due to the porous nature of the substrate. This observation highlights the need for further discussion on the effect of substrate nature and luminol on the age-related processes of blood stains.

Many researches have been conducted to study how the nature of the substrate affects age estimation [14]. Sharma and group explored the influence of substrate porosity on age estimation. Their findings revealed that subpar spectral peaks of blood were obtained on porous materials like cotton and paper. These substrates absorbed blood, leaving only a small amount on the surface for detection. Another study discovered that the regression model developed for blood stains on metal surfaces provided the most accurate fit compared to those on cement and wooden surfaces [3]. Similarly, in the present study, cement being porous, allowed blood to penetrate deeper into its surface, leaving very little sample to react with luminol in concealed conditions, and acting as a barrier for the reaction between luminol and blood.

Hemoglobin undergoes structural changes over time, which play a significant role in age-related alterations in blood stains. The results of this study indicated that the use of luminol probably interfered with the detection of these changes in hemoglobin's structure. This interference was likely due to the masking effect of the chemicals used in preparing the solution, leading to inaccurate estimations of the age of blood stains based on spectral changes. This conclusion is supported by another study that examined how luminol affects the spectroscopic properties of blood. The study found that luminol affects the ability to detect structural changes in hemoglobin, which are typically determined using spectrophotometry. Barbaro et al. conducted many spectrophotometric tests on blood stains collected from various substrates. They examined the impact of luminol and fluorescein tests and found that when either luminol or fluorescein is present in a sample, it becomes challenging to distinguish the typical absorption peaks of hemoglobin and concluded that this difficulty arises from the reagents utilized in these tests having absorption properties that overlap with the hemoglobin peaks [41].

Several investigations have centered on determining the time since blood has been deposited on various surfaces. However, these studies have utilized a restricted range of substrates when compared to the current study. To date, no investigation has explored the estimation of TSD of concealed blood stains. Consequently, this thorough and definitive research endeavors to provide a clearer comprehension of the age estimation of concealed blood stains on a wide range of substrates using ATR-FTIR spectroscopy and chemometric methods. The study also examines the impact of luminol on the age estimation of concealed blood stains. The findings obtained will serve as a foundation for future research on concealed blood stains.

6. Conclusions and Future Scope

The study concluded that the application of ATR-FTIR spectroscopy along with advanced chemometric techniques is an effective means of determining the time-since-deposition of blood stains on various substrates, whether they are concealed or unconcealed. The research examined the potential impact of substrate nature and luminol on the age estimation of concealed blood stains on different surfaces. The results indicated that the substrate nature and luminol significantly influence the estimation of blood stain age.

The findings revealed that the spectral region ranging from 2500 cm^{-1} to 3500 cm^{-1} was significant in detecting age-related changes in blood stains. Models developed using data from this region demonstrated exceptional predictive accuracy for all three surfaces under investigation, irrespective of whether the blood stains were visible or concealed. The models developed for unconcealed blood stains on metal and wooden surfaces exhibited a higher ability to predict age with minimal error, while the model for concealed blood stains on cement surfaces showed good predictive accuracy compared to those on metal and wooden surfaces. The models demonstrated that concealed blood stains over cement surfaces were a better fit compared to exposed ones. Conversely, the models for exposed blood stains without concealment on metal and wooden surfaces provided better fits compared to concealed blood stains. This underscores the impact of the porous nature of cement surfaces in retaining blood stains more deeply, limiting their contact with luminol.

The findings suggest that luminol may impede the detection of age-related changes occurring in concealed blood stains. Probably this effect was obscured by the presence of paint and the porous nature of the cement surface but was evident on non-porous metal and wooden surfaces. Further research is required to understand the impact of luminol on the spectral changes associated with the aging of blood stains.

The ATR-FTIR technique, along with chemometric methods, presents a promising, non-invasive, and reliable procedure to estimate the age of blood stains. This technique can be easily applied to both latent and patent blood stains found on cement, metal, and wooden surfaces at crime scenes. The study underscores the importance of examining the interaction between luminol and blood stains as well as the influence of luminol on estimating the age of concealed blood stains. Given the diverse factors affecting blood stain aging, conducting comprehensive, long-term research across varied indoor and outdoor conditions on different surfaces using samples from diverse volunteers is crucial.

Data Availability
The spectral data analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of Interest
The authors declare that they have no conflict of interest.

Funding Source
No funding was received for the study.

Author Contributions
Ms. Deepthi Nagesh (Corresponding author) conducted the study, analyzed the data, and prepared the manuscript., Dr.

Nagarajamurthy B supervised the work and Mr. Bhanuprakash R guided with data analysis.

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