

Original Article

Estimation of Age of Bloodstain Spotted on Different Surfaces Using UV-visible Spectroscopy

Suchita Rawat^{1*}, KPS Kushwaha²

¹Ph.D. Scholar, Department of Anthropology, University of Delhi, Delhi, India

²Senior Faculty Biology, Lok Nayak Jayaprakash National Institute of Criminology and Forensic Science, Delhi, India

*Corresponding author email id: suchitarawat1990@gmail.com

ABSTRACT

Estimation of age of bloodstain found at scene of crime is crucial for forensic personnel, which aids in defining time frame in which the individual depositing it was present. Although many methods have been discovered to estimate age of the bloodstain but only few have gained widespread use due to poor time resolution and weak age correlation. The present study used UV-vis spectrophotometric analysis of hemoglobin (Hb) for estimating the age of the bloodstains spotted on different substrates, which are usually encountered at crime scene. The average rate of Hb_{soret} band blue-shift rate varied from 0.0075 to 0.0015 nm/h for bloodstain of environmental exposure while for room temperature exposed it varied from 0.0012 to 0.0002 nm/h. Preliminary blood test (benzidine test), serological test (species of origin) and genetic analysis (DNA extraction) on the same bloodstain were also performed. The benzidine test and osterlony double diffusion test was positive for the samples kept at RT for 73 day while DNA could not be extracted.

Keywords: Bloodstain age estimation, Blue-shift, Hb_{soret} band, UV-vis spectroscopy, Benzidine test

INTRODUCTION

Bloodstains are the most encountered physical evidence found at crime scene. Blood stains are not only used for bloodstain pattern analysis but also reveal the identity of the victim or suspect through DNA fingerprinting. Further another application of bloodstains obtained at crime scene, is to determine when the crime was committed. Extensive work in the field of age determination of bloodstains has been conducted based on changes in the red blood cell^[1]. The pioneer work in age determination of bloodstain was conducted in the 19th century by Louis Tomellini who devised colour chart for estimating the age of bloodstain based on gradual colour changes of the blood after deposition. Further, in 1910, Leers noted that fresh stain characterised by HbO₂ spectrum has greater solubility in water than old stain having metHb spectrum. This was backed by observations of another researcher^[2], while in 1936, Schwarz employed guaiacum assay for

determination of age of blood stain based on enzymes catalase and peroxidase activity^[3]. In 1960, Patterson recorded bloodstain reflectance spectrum using photo-spectrometry observing greatest change in the colour in the fast few hours followed by decreased rate after 72h^[4]. In 1962, Fiori suggested the correlation between age of bloodstain and progressive diffusion of chloride ions around the stain^[5]. Other methods such as, differential extractability of bloodstain in water and transformation of Hb to metHb were proposed by Kleinhauer in 1967 for age estimation of bloodstain^[6]. Besides this, the spectroscopy method was also used for estimation of bloodstain age^[7,8]. In 1974, Nuorteva proposed that based on the length of life cycles stages of insects, age of the bloodstain could be estimated for maggot infested biological evidence^[9]. Further, Rajamannar in 1977 proposed serum protein profile (albumin and globulin) immune electrophoresis technique as function of age of bloodstain^[10]. High-performance liquid chromatography

(HPLC) technique was also applied for age estimation of stain^[11]. The ratio of peak areas α chain of hemoglobin was inversely related heme to age of stain^[12]. Another indicator was, intriguing peak X obtained with retention time 8 times shorter than heme, linearly correlated with stain age^[13]. In 2007, Strasser and his colleagues determined the age of blood spots in forensic medicine by force spectroscopy and also used atomic force microscopy (AFM) for high-resolution imaging of the erythrocytes in the blood sample. They observed that the cell elasticity decreased over time attributed to drying and coagulation process^[14]. Apart from the aforementioned techniques, electron paramagnetic resonance spectroscopy and reflectance spectroscopy was explored for bloodstain age determination^[15,16]. Hanison and Ballantyne in 2010 discovered that the blue spectral shift of the hemoglobin *soret* band correlates with the age and time since deposition of dried bloodstains using UV–vis spectrophotometre^[17]. The concept of quantification of RNA degradation either by semi-quantitative duplex or Competitive RT–PCR as a possible indicator of age of blood stain has also been put forth^[18,19]. The ratio between different types of RNA (mRNA and rRNA) changes over time in linear fashion^[20]. The latest attempt in this field was a preliminary study to correlate the age of bloodstains with aspartic acid racemisation^[21].

The various techniques used for determination of age of bloodstain are complex and require enormous financial input. Hence the current study was conducted to use UV–visible spectroscopy to estimate the age of the blood stain spotted on six different surfaces frequently encountered in the crime scene based on blue-shift of the Hb_{soret} band as proposed by Hanison and Ballantyne 2010.

OBJECTIVES

The study was planned keeping in mind the following objectives: (i) To observe the $\lambda_{max} Hb_{soret}$ blue-shift (shift to shorter wavelength) over time for bloodstain exposed to environmental and room temperature. (ii) To compare the rate of blue-shift (nm/h) of the bloodstain exposed to different temperature. (iii) To perform statistical analysis of the data and obtain logarithmic regression function for bloodstain age estimation. (iv) To check whether preliminary test for blood i.e., benzidine, Ochterlony double diffusion test for species of origin and DNA extraction

could be successfully performed on the oldest (maximum exposed) bloodstain.

MATERIALS AND METHODS

For the preparation of bloodstain, peripheral blood samples were collected in EDTA free vacutainers using the venipuncture method from 2 apparently healthy volunteers after obtaining their consent. Twenty microlitres of venous blood was spotted separately on 6 different substrates (cloth, cardboard, glass, wood, ceramic tile and soil) (Figure 1) and air dried followed by exposure to different temperature i.e. room temperature for two and a half months (collected at interval 2 day, 7 day, 10 day, 21 day, 24 day, 35 day, 62 day, 66 day, 70 day, 73 day) and environmental temperature for a week. After the samples were kept in different exposure period, they were collected for UV–visible spectroscopy. For this analysis, bloodstains were either cut or collected as swab and dipped in 1 ml 0.2M Tris–HCL (PH=8) overnight. The next day, the cut pieces or swabs were discarded and the solution was centrifuged at 10,000 rpm for 3 min and 30 μ l of the supernatant was transferred to another centrifuge tube followed by addition of 970 μ l of 0.2 M Tris–HCL to make up the volume upto 1 ml which was analysed in double beam UV–vis spectrometre at 350–700 nm. The $\lambda_{max} Hb_{soret}$ band was recorded after every exposure and the average blue-shift (nm/h) was calculated. Further correlation between the $\lambda_{max} Hb_{soret}$ and exposure time was also computed. To deduce the relationship between age of bloodstain and Hb_{soret} blue-shift regression equation formulae was generated. All the statistical analysis for the current study was performed on excel stats. Further Ochterlony double diffusion method for species of origin and DNA extraction using the Phenol chloroform method was also performed on the same bloodstain extracts earlier used for spectroscopic analysis.

RESULTS AND DISCUSSION

Table 1 depicts the bloodstain exposed to environmental conditions for one-week period. Observed blue-shift ranged from 1.25 nm (maximum) to 0.25 nm (minimum) while average blue-shift rate ranged from 0.0075 nm/h (maximum) to 0.0015 nm/h (minimum). Table 2 depicts $\lambda_{max} Hb_{soret}$ band for different substrates exposed to room temperature for a period of two and half months. It

Estimation of Age of Bloodstain Spotted on Different Surfaces Using UV-visible Spectroscopy

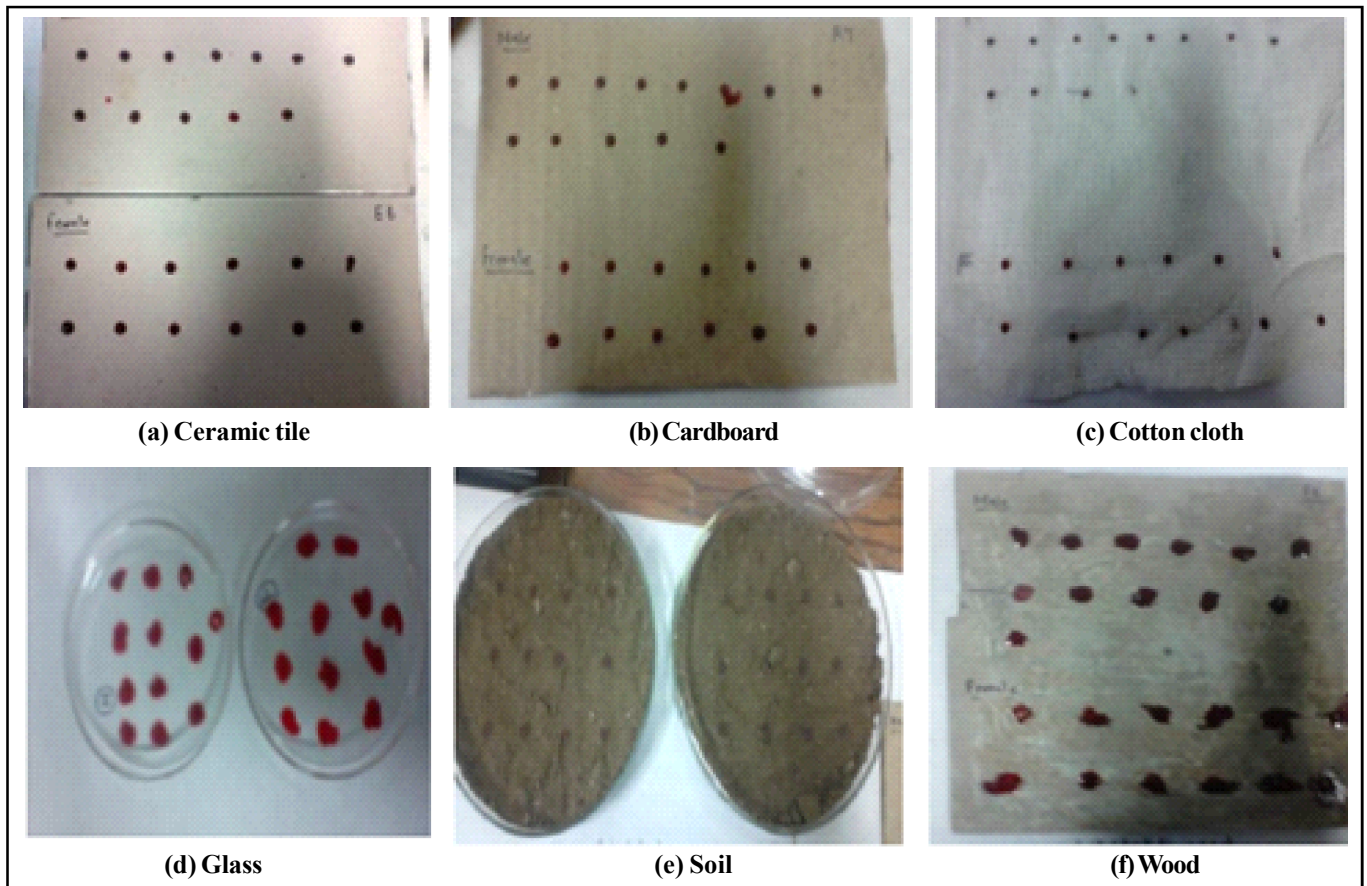


Figure 1: 20 µl of venous blood spotted on different substrate

was observed that blue-shift ranged from 2.10 nm (maximum) to 0.125 nm (minimum) while average blue-shift rate ranged from 0.0012 nm/h (maximum) to 0.0002 nm/h (minimum). The observed Hb_{soret} shift is based on the oxidation chemistry of Hemoglobin with oxyHb in ferrous state gets converted to metHb^[22]. The extent of

the blue-shift was greater in samples exposed to environment conditions than compared to samples exposed to room temperature which could be attributed

Table 1: λmax of Hb_{soret} band for bloodstains exposed to environmental condition for 1 week

Substrate	λmax Hb _{soret} band (nm) after exposure of 2 day	λmax Hb _{soret} band (nm) after exposure of 7 day	Observed blue-shift (nm) (exposure 1 –exposure 2)	Average blue-shift rate (nm/h)
Cotton cloth	405	403.75	1.25	0.0075
Cardboard	404.75	404.5	0.25	0.0015
Wood	405	404.25	0.75	0.0046
Tile	405	404.25	0.75	0.0046
Glass	404.5	403.75	0.50	0.0030
Soil	404.25	357.75*	–	–

Note: All data of λmax Hb_{soret} band are an average of bloodstains from two individual donors (male and female). *Hb_{soret} band was not identified in UV-vis spectroscopy. The moisture in the soil promoted fungal growth causing degradation of the bloodstain.

Table 2: λmax of Hb_{soret} band for bloodstains exposed to room condition

Exposure (in days)	λmax Hb _{soret} band (nm) for bloodstain on different substrate				
	Cloth	Cardboard	Glass	Tile	Wood
Exposure 1(2 day)	407.25	405.25	405.50	406.25	405.50
Exposure 2(7 day)	405.00	404.50	405.00	405.00	404.50
Exposure 3(10 day)	404.75	404.00	405.00	405.00	405.00
Exposure 4(21 day)	405.50	403.50	405.25	404.75	404.50
Exposure 5(24 day)	404.75	405.00	406.00	405.00	405.25
Exposure 6(35 day)	405.25	406.50	404.50	404.00	404.75
Exposure 7(62 day)	404.75	404.00	405.25	405.25	406.00
Exposure 8(66 day)	405.25	404.75	405.75	406.00	404.25
Exposure 9(70 day)	404.25	403.25	405.75	410.00	404.75
Exposure 10(73 day)	404.75	404.00	405.75	405.75	404.25

Note: All data of λmax Hb_{soret} band are an average of bloodstains from two individual donors (male and female).

to sunlight (varying temperature), humidity or air pollutants. For both bloodstain exposed either to environment or to room temperature blood spotted on cotton cloth was maximum while rate was least for cardboard (environment exposure) and wood (room temperature exposure). On computing the correlation between $A_{\text{max}} \text{Hb}_{\text{solet}}$ and Room temperature exposure period, a weak negative correlation was observed in blood spotted on cotton cloth, cardboard and glass while a weak positive correlation in case of bloodstain spotted on wood and tile indicating that the substrate on which the bloodstain is spotted plays vital role due to the surface texture and property. Based on The data generated during the study. Regression equations formulas was generated for each substrate (Table 3). Benzidine test and the species of origin ochterlony double diffusion test was positive for bloodstain kept at oom temperature for 73 day (Table 4). Though DNA could not be extracted owing to the fact that very small quantity (20 μl) of blood was used for preparation of bloodstains. Further the phenol chloroform method of DNA extraction is not sensitive and other sensitive assays could be used. The strength of the study is the use of 6 different substrates frequently encountered in the crime scene which aids in establishing the effect of substrate on bloodstain. The study also had few

limitations; the bloodstains should be exposed to prolonged environmental condition to check the long term effect of temperature.

CONCLUSION

The rate of Hb_{solet} blue-shift is dependent on exposure condition (environmental or Room temperature) which is accelerated with high temperature and also depends substrate on which the bloodstain is present. Preliminary test for blood such as benzidine and determination of species of origin can be performed successfully for maximum exposed bloodstain.

ACKNOWLEDGEMENTS

The authors are greatly indebted to Director LNJN–NICFS and other officers for providing excellent infrastructural facilities including library and laboratory. Further the authors are highly thankful to Dr. J.R. Sethi (academic coordinator chemistry), Shri P. Sharma (SSA Toxicology) and Mrs. Priyanka Mishra (JRF chemistry) for their valuable suggestions.

REFERENCES

- [1] Bremmer RH, de Bruin KG, van Gemert MJ, van Leeuwen TG, Aalders MC. Forensic quest for age determination of bloodstains. *Forensic Sci Int* 2012;216:1–11.
- [2] Schwarzacher. Determination of the age of bloodstains. *Am J Police Sci* 1930;1:374–80.
- [3] Schwarz F. Quantitative untersuchungen der katalase und peroxydase im blutfleck. *Int J Legal Med* 1937;27:1–34.
- [4] Patterso D. Use of reflectance measurements in assessing the colour changes of ageing bloodstains. *Nature* 1960;187:688–89.
- [5] Fiori A. Detection and identification of bloodstains. *Methods Forensic Sci* 1962;1:243–90.
- [6] Kleihauer E, Stein G, Schmidt G. Beitrag zur alterbestimmung von blutflecken. *Arch Kriminol* 1967;140:84–94.
- [7] Kind SS, Patterson D, Owen GW. Estimation of the age of dried blood stains by a spectrophotometric method. *Forensic Sci* 1972; 1:27–54.
- [8] Kind SS, Watson M. The estimation of blood stain age from the spectrophotometric properties of ammoniacal blood stain extracts. *Forensic Sci* 1973;2:325–32.
- [9] Nuorteva P. Age determination of a blood stain in a decaying shirt by entomological means. *Forensic Sci* 1974;3:89–94.
- [10] Rajamannar K. Determination of the age of bloodstains using immunoelectrophoresis. *J Forensic Sci* 1977;22:159–64.

Table 3: Determination of age of bloodstain using blue-shift

Substrate	Average blue-shift (nm) exposure-exposure 10	Average blue-shift rate (nm/h)	R ² (correlation between $A_{\text{max}} \text{Hb}_{\text{solet}}$ and exposure)	Regression equation formulae
Cloth	2.10	0.0012	0.5304	$Y=0.015\text{Ln}(X)+405.72$
Cardboard	0.775	0.0004	0.2970	$Y=0.010\text{Ln}(X)+404.85$
Wood	0.125	0.0001	0.3969	$Y=0.006\text{Ln}(X)+405.14$
Tile	0.55	0.0003	0.4546	$Y=0.026\text{Ln}(X)+404.72$
Glass	0.625	0.0002	0.2031	$Y=0.004\text{Ln}(X)+405.03$

Note: The regression function has the form $y=a\text{Ln}(x)+I$, with $y=\lambda_{\text{max}} \text{Hb}_{\text{solet}}$ band (in nm), a =slope of line, I =intercept, and x =time (in day)

Table 4: Serological and genetic analysis test result performed on the oldest bloodstain (72 day old bloodstain)

Substrate	Benzidine test (preliminary test for Blood)	Species of origin (Ochterlony double diffusion method)	DNA extraction (Phenol–chloroform method)
Cloth	+	+	–
Cardboard	+	+	–
Wood	+	+	–
Tile	+	+	–
Glass	+	+	–

Note: + = Positive result and – = negative result

Estimation of Age of Bloodstain Spotted on Different Surfaces Using UV-visible Spectroscopy

- [11] Andrasko J. The estimation of age of bloodstains by HPLC analysis. *J Forensic Sci* 1997;42:601–07.
- [12] Inoue H, Takabe F, Iwasa M, Maeno Y. Identification of fetal hemoglobin and simultaneous estimation of bloodstain age by high-performance liquid chromatography. *Int J Legal Med* 1991;104:127–31.
- [13] Inoue H, Takabe F, Iwasa M, Maeno Y, Seko Y. A new marker for estimation of bloodstain age by high performance liquid chromatography. *Forensic Sci Int* 1992;57:17–27.
- [14] Strasser S, Zink A, Kada G, Hinterdorfer P, Peschel O, Heckl WM, Thalhammer S. Age determination of blood spots in forensic medicine by force spectroscopy. *Forensic Sci Int* 2007;170:8–14.
- [15] Fujita Y, Tsuchiya K, Abe S, Takiguchi Y, Kubo SI, Sakurai H. Estimation of the age of human bloodstains by electron paramagnetic resonance spectroscopy: long-term controlled experiment on the effects of environmental factors. *Forensic Sci Int* 2005;152:39–43.
- [16] Bremmer RH, Edelman G, Vegter TD, Bijvoets T, Aalders MC. Remote spectroscopic identification of bloodstains. *J Forensic Sci* 2011;56:1471–75.
- [17] Hanson EK, Ballantyne J. A blue spectral shift of the hemoglobin soret band correlates with the age (time since deposition) of dried bloodstains. *PLoS One* 2010;5:12830–40.
- [18] Bauer M, Patzelt D. Evaluation of mRNA markers for the identification of menstrual blood. *J Forensic Sci* 2002;47:1278–82.
- [19] Anderson SE, Hobbs GR, Bishop CP. Multivariate analysis for estimating the age of a bloodstain. *J Forensic Sci* 2011;56:186–93.
- [20] Anderson S, Howard B, Hobbs GR, Bishop CP. A method for determining the age of a bloodstain. *Forensic Sci Int* 2005;148:37–45.
- [21] Arany S, Ohtani S. Age estimation of bloodstains: a preliminary report based on aspartic acid racemization rate. *Forensic Sci Int* 2011;212:e36–e39.
- [22] Horecker BL. The absorption spectra of hemoglobin and its derivatives in the visible and near infra-red regions. *J Biol Chem* 1943;148:173–83.