Damanhour Journal of Veterinary Sciences 12 (1), (2024) 1-9

Damanhour Journal of Veterinary Sciences

Journal homepage: https://djvs.journals.ekb.eg/

E-ISSN 2636-3003 | ISSN 2636-3011

Exploring the Impact of Environmental Variables on Blood stains: Insights into Forensic Analysis of Human and Animal Origin

Dalia H. Samak [1*], Wafa Bader[1], Yasser S. El-Sayed[1], Kadry M. Sadek[2], Hamida Saleh[1]

[1] Department of Veterinary Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Damanhour University, Damanhour, Egypt
[2] Departments of Biochemistry, Faculty of Veterinary Medicine, Alexandria University, Alexandria, Egypt.

Abstract

Forensic investigations utilizing bloodstain analysis have experienced rapid growth. Samples collected from crime scenes may undergo exposure to various conditions before analysis. Several factors, including blood aging, environmental conditions like temperature and humidity, and pH levels, can impact the appearance and preservation of bloodstains. This study sought to assess the impact of different temperature gradients on bloodstain patterns, examining temperature variations (4°C, 37°C, and 50°C) and aging durations (0, 24, 48, and 72 hrs). Using human and cat blood samples, macroscopic changes were observed, followed by phenolphthalein and Takayama tests for blood identification. Results reveal that stains exposed to higher temperatures and longer durations exhibited darker coloration, dryness, and cracking, while those at 4°C remained relatively unchanged, except for increased gelatinous consistency over time. Both phenolphthalein and Takayama tests successfully identified blood in stains across temperature and time variations, with variations in color intensity and crystal morphology observed, particularly at higher temperatures and longer durations. This research underscores the importance of considering environmental factors when analyzing bloodstains for forensic purposes.

Keywords: Bloodstains; Temperature; Human Blood; Cat blood; Takayama Test; Kastel-Mayer Test

*Correspondence: Dalia H Samak
Department of Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Damanhour University, Damanhour, Egypt
Email: dalia_Samak@vetmed.dmu.edu.eg
P ISSN: 2636-3003
EISSN: 2636-3011
DOI: 10.21608/DJVS.2024.286798.1131.
Received: May 02, 2024; Received: May 15, 2024; Accepted: May 27, 2024.
Editor-in-Chief: Prof Dr/Ali H. El-Far (ali.elfar@damanhour.edu.eg).

1. Introduction

Forensic studies play a pivotal role in understanding crime scenes by scientifically analyzing evidence **(Gardner and Krouskup, 2018)**. A significant aspect of this is bloodstain analysis, which provides crucial information about the crime, victim, and potential suspects **(Pokupcic, 2017)**. However, several factors, including blood aging, environmental conditions like temperature and humidity, and pH levels, can impact the appearance and preservation of bloodstains **(Hanslip, 2021)**. Blood trace, along with other biological evidence, is frequently discovered at crime scenes **(Magalhães *et al.*, 2015)**. Typically, the characterization of a bloodstain involves three key steps: visual assessment, presumptive testing, and confirmatory analysis **(Kobilinsky, 2011)**. Blood is a rich source of information,

containing DNA, specific chemical compositions, and patterns that can help reconstruct crime events **(van Oorschot *et al.*, 2019)**. Basic characteristics of blood at crime scenes are determined using serological tests like the Kastle-Meyer test, aiding in preliminary identifications **(Alsheekhly *et al.*, 2019)**. Aging is a significant factor affecting bloodstain properties. As bloodstains age, they tend to become darker and more brittle due to hemoglobin breakdown, making them harder to analyze **(Das *et al.*, 2020)**. This degradation process can also lead to a loss of color, complicating detection efforts **(Bandyopadhyay and Basu, 2015)**.

Environmental conditions, particularly temperature and humidity, also influence bloodstain characteristics. High temperatures cause blood to dry rapidly, resulting in smaller and darker stains. In contrast, low temperatures slow down coagulation, leading to larger and more diffuse stains **(Smith and Brutin, 2018)**. Another crucial distinction lies between human and animal blood. Human blood contains a higher concentration of platelets, causing it to dry faster but coagulate slower. On the other hand, animal blood dries quickly but coagulates at a slower rate **(Doty and Lednev, 2018)**. Differentiating between these blood types is vital as animal blood may not provide relevant crime-related information. Despite technological advancements that have improved bloodstain analysis, challenges persist. Environmental factors remain a significant concern, affecting the integrity and appearance of bloodstains **(Namdee *et al.*, 2015)**. Therefore, the current state of knowledge of forensic bloodstain analysis has advanced significantly in recent years, thanks to the development of new technologies and techniques. However, there are still many challenges in forensic bloodstain analysis, especially when dealing with bloodstains that have been exposed to environmental factors such as humidity and temperature. The development of new technologies and techniques, as well as an understanding of the effects of aging and environmental factors, will help forensic scientists to obtain the most accurate and reliable results from forensic bloodstain analysis. To study the effect of aging, and environmental temperature on forensic bloodstains of human and animal origin, by comparing the results of macro- and microchemical tests among different groups of bloodstains. To understand the preservation and degradation of bloodstains under different environmental conditions. The current experimental study aims to assess the impact of various environmental conditions on bloodstains exposed to different temperature settings and durations.

2. Materials and Methods

2.1. Sample Collection

Bloodstains were collected from human and animal sources (cat blood) in a vacutainer K_2E EDTA tube, in which they were gently shacked 5 times to mix the blood with the anticoagulant reagent. Bloodstains were created by dropping the blood samples on plastic petri dishes and allowed to dry at varying temperatures4°C, 37°C, and 50°C. The blood stains were stored in sealed containers and divided into multiple groups based on the variables of interest: aging for 0, 24, 48 and 72 hrs varying temperatures4°C, 37°C, and 50°C. Blood samples were collected from the jugular vein using a syringe and needle. While cephalic or saphenous venepuncture with a syringe and needle may be better tolerated by cats, the peripheral veins in this species are typically small, which may sometimes prevent the collection of the minimum blood volume required for routine haematology and plasma biochemistry..**(Kamali and Mohri, 2015; Reynolds *et al.*, 2007)**.

2.2. Ethical considerations

The collection of blood samples was carried out under strict ethical guidelines with an issued number (DMU/VetMed-2024/011) and with the informed consent of the donors. Animal samples were collected in compliance with local animal welfare regulations. The study had been conducted in compliance with established ethical guidelines for research involving human and animal subjects.

Chemicals, reagents, and equipment

2 g of Phenolphthalein reagent, hydrogen peroxide (H_2O_2), sodium hydroxide (NaOH)10%, 20 g potassium hydroxide (KOH), 10g zinc Powder mn,100ml distilled water, 3ml saturated glucose solution,3ml pyridine, ethyl alcohol, All other chemicals and reagents were commercially available from local scientific distributors in Egypt.

2.3. Experimental protocol

The blood stains of human and animal origin were divided into four groups: Group 1: Fresh bloodstains (pH 7), kept at room temperature and aged for 0, 24, 48 and 72 hrs. The bloodstains were prepared in four different temperature conditions: Group 2: Bloodstains kept at 4°C in the refrigerator, and aged for 0, 24, 48, and 72 hrs. Group 3: Bloodstains were kept at 37°C in the incubator, and aged for 0, 24, 48, and 72 hrs. Group 4: Bloodstains were kept at 50°C in the oven, and aged for 24, 48, and 72 hrs. The bloodstains in each group were stored under the specified conditions for the duration of the study.

2.4. Macroscopic analysis

Each sample was examined by the naked eye and photographed for the visual identification of bloodstains characters **(Barni *et al.*, 2007)**.

2.5. Chemical analysis

Each blood sample was divided into two halves. Presumptive tests: Kastle Mayer test for blood samples for humans and cats and Takayama test, to determine the presence of hemoglobin, Kastle -Meyer (Phenolphthalein) Test.

Preparation of Kastle-Mayer reagen1-2 g phenolphthalei2-(20 g), potassium hydroxide or Sodium hydroxide (10 - 20 g), zinc powder(100 ml) of distilled water, (ph,ph 2 gm +NaoH 20gm+100cc D.W)→ pink color →boiling → add 10-20 gm powdered Zn→Boiling continued till the fluid became colorless→Cooling and filtration then stored in alight resistant glass bottle. A dry cotton-tipped swab was vigorously rubbed over the bloodstain or blood stain put in Epindorff or petri dish then added the following respectively: one or two drops of ethanol followed by one or two drops of the phenolphthalein stock solution and one or two drops of hydrogen peroxide were added

The Kastle-Meyer test (also known as the phenolphthalein test) is a presumptive forensic test that is used to identify the presence of blood and determine the species of origin. The test is based on the fact that the enzyme glucose-6-phosphate dehydrogenase (G6PDH) is present in red blood cells and when it comes in contact with hydrogen peroxide, it catalyzes the oxidation of NADP to NADPH, and the NADPH produced causes a color change in a phenolphthalein indicator from colorless to pin**(Cox, 1991; Glaister, 1926)**.

2.5.1. Takayama (hemochromogen) Test

The Takayama test is used to confirm the presence of blood and to differentiate it from other possible sources of hemoglobin such as humans and animals. It is a type of microcrystalline test, which means that it is based on the principle that when certain chemicals are added to blood, they form specific crystal patterns. The test is based on the fact that hemoglobin, the protein found in red blood cells, catalyzes the oxidation of a reagent. 1-3 ml NaoH 10%(10 gm NaoH in 100 ml D.W).Add2-3 ml saturated glucose solution followed by 3-3 ml pyridine and 4- 7 ml distal water then put dry stain fragment on slide after that, add cover slide and add 2 – 3 drops takayama reagent followed by gentle heating(or leave for 24 hrs) and finally was examined under microscope (**Stewart *et al.*, 2018**). Several reagents are used such as pyridine (Takayama test), acetic acid (Teichmann's test) or acetone/mineral acid (Wagenhaar's test). Although these are considered as confirmation tests, they cannot distinguish between human and animal blood.(**De Wael *et al.*, 2008**).

3. Results

Forensic science relies on meticulous examination and analysis, particularly in understanding how environmental factors can influence bloodstain patterns. In this comprehensive study, we explore the fascinating transformations that human and cat bloodstains undergo when exposed to varying temperatures: 4°C, 37°C, and 50°C, with different durations of exposure.

3.1. Human blood

3.1.1. Macroscopic observation

- ***Fresh human Bloodstain at Room Temperature***

To provide a baseline for comparison, it is important to note that fresh human bloodstains at room temperature (at a pH of 7) appear as vibrant red and maintain a liquid consistency. This initial observation serves as a reference point for understanding how temperature affects bloodstain properties (Figure 1A).

- ***Effect of temperature blood samples at 4°C***

The fresh bloodstain at room temperature is red-colored and liquid (Fig 1A). At 4°C for 24 hrs, bloodstains become gelatinous, (Fig 1B). After 48 hrs at 4°C and pH 7, bloodstains maintain their red color but exhibit a more pronounced gelatinous consistency, emphasizing time's critical role (Fig 1C). Continuing for 72 hrs at 4°C, bloodstains become even more gelatinous compared to the 48-hour mark, with signs of drying (Fig 1D).

- ***Effect of temperature on bloodstain at 37°C***

The fresh bloodstain at room temperaturae is red-colored and liquid (Fig 1E). When human bloodstains endure 37°C for 24 hrs, they darken significantly to a deep, dark red, lose liquid consistency, and develop a cracked appearance (Fig 1F). Continuing for 48 hrs intensifies changes, maintaining dark red color, increasing dryness, and exhibiting more prominent cracks (Fig 1G). Extending to 72 hrs sustains a dark red color, with some bloodstains transitioning to brownish hue, while drying and cracking effects intensify, surpassing characteristics observed at 24 and 48 hrs (Fig 1H).

- ***Effects of High Temperature (50°C)***

The fresh bloodstain at room temperature is red-colored and liquid (Fig 1I). When human bloodstains are subjected to an elevated temperature of 50°C for 24 hrs while maintaining a pH of 7, noticeable transformations occur. The bloodstains become notably darker, transitioning from red to a deep, dark red hue. Additionally, they lose their liquid consistency, becoming dry and exhibiting visible cracks (Fig 1J). Continuing the experiment for 48 hrs at 50°C and with a pH of 7 intensifies the observed changes. The bloodstains maintain their dark red color but become even drier compared to the 24-hour mark. The most notable transformation is the development of pronounced cracks that resemble crusts on the bloodstain surface (Fig 1K). Extending the exposure to 72 hrs at 50°C further accentuates the effects. The bloodstains remain dark red, and some may exhibit a transition towards a brownish hue. The drying and crusting effects persist, surpassing the characteristics observed at both the 24-hour and 48-hour mark (Fig 1L).

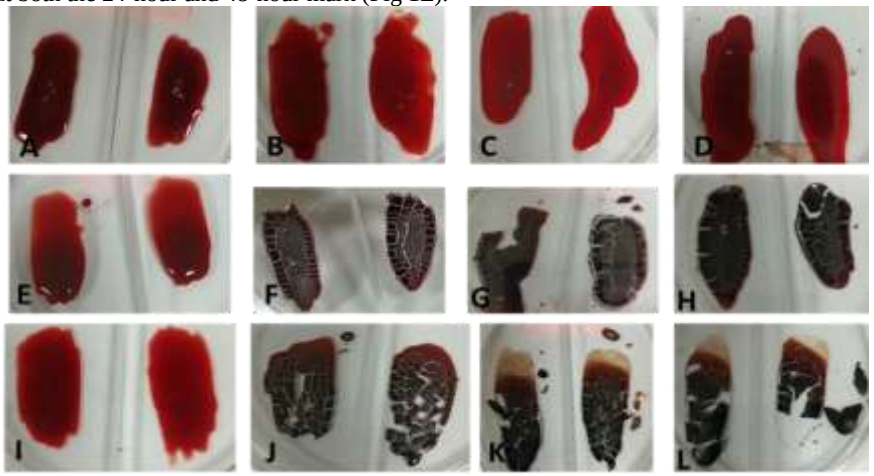

Figure 1. showed the effects of different Temperature degree (4°C, 37°C, 50°C) for zero hrs, 24hrs, 48hrs and 72 hrs on human blood stain. (A)The fresh bloodstain at room temperature is red-colored and liquid; (B) The bloodstain kept at 4°C for 24 hrs is colored-red and become gelati-nous in consistency; (C) The bloodstain kept at 4°C for 48 hrs is colored red and become more gelatinous in consistency than that kept for 24 hrs; (D) The bloodstain kept at 4°C for 72 hrs is still colored red and more gelati-nous in consistency higher than 48hrs and start to dry. (E)The fresh bloodstain at room temperature is red-colored and liquid; (F) The bloodstain kept at 37°C for 24 hrs is dark red-colored, dry and cracked; (G) The bloodstain kept at 37°C for 48 hrs is dark red-colored, dry more than 24 hrs and cracked; (H) The bloodstain kept at 37°C for 72 hrs is still dark red to brownish-colored, dry more than 48 hrs and cracked. (I)The fresh bloodstain at room temperature is red-colored and liquid; (J) The bloodstain kept at 50°C for 24 hrs is dark red-colored, dry and cracked; (K) The

bloodstain kept at 50°C for 48 hrs is dark red-colored, dry more than 24 hrs and cracked like crusts; (L) The bloodstain kept at 50°C for 72 hrs is still dark red to brownish-colored, dry more than 48 hrs and cracked like crusts

3.1.2. Kastle-Mayer test on human bloodstain

The blood stains are subjected to a controlled temperature of 4°C for zero hour, 24 hrs, 48 hrs, and 72 hrs, tested by the Kastle-Meyer test, resulting in a pink color (Fig2A). Over time, the pink becomes somewhat darker with increased time and temperature (Figs.2B, 2C, and 2D). Similarly, at a controlled temperature of 37°C for zero hours, 24 hrs, 48 hrs, and 72 hrs, the bloodstains tested by the Kastle-Meyer test also give a pink color (Fig. 2E). Again, the pink becomes somewhat darker with increased time and temperature (Figs.2F, 2G, and 2H). Finally, when subjected to a controlled temperature of 50°C for zero hours, 24 hrs, 48 hrs, and 72 hrs, the bloodstains tested by the Kastle-Meyer test yield a pink color (Figure 2i). Over time and with increasing temperature, the pink becomes somewhat darker (Figs.2J, 2K, and 2L).

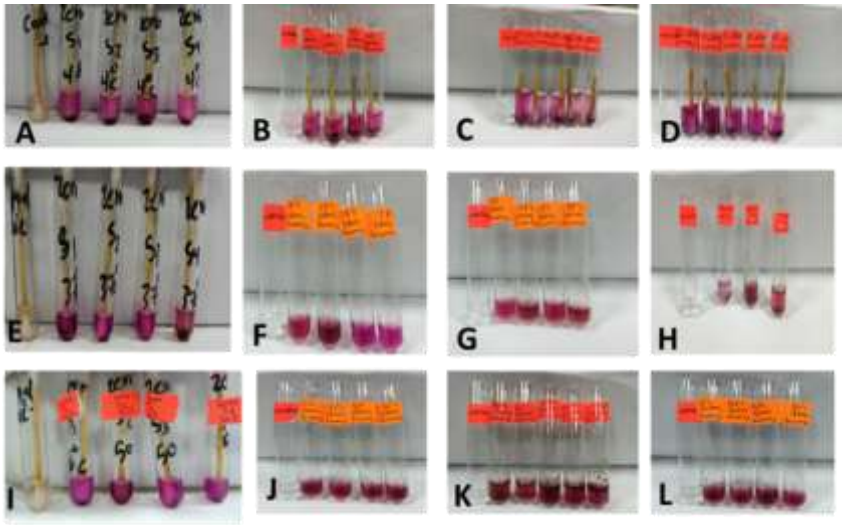

Figure 2. showed the effects of Kastle-Mayer test (phenolphthalein test) with different Temperature degree (4°C, 37°C, 50°C) for zero hrs, 24hrs, 48hrs and 72 hrs on human blood stain. (A) bloodstain at 4°C and pH 7 for zero hr give pink color ; (B) The bloodstain kept at 4°C and pH 7 for 24 hrs give pink color slightly dark than at zero; (C) The bloodstain kept at 4°C and pH 7 for 48 hrs give pink color as at 24 hrs ; (D) The bloodstain kept at 4°C and pH 7 for 72 hrs give dark pink color than at 48 hrs. (E) bloodstain at 37°C and pH 7 for zero hr give pink color ; (F) The blood-stain kept at 37°C and pH 7 for 24 hrs give dark pink color than at zero hr ; (G) The bloodstain kept at 37°C and pH 7 for 48 hrs give dark pink color ; (H) The bloodstain kept at 37°C and pH 7 for 72 hrs give dark pink color. (I)bloodstain at 50°C and pH 7 for zero hr give pink color ; (J) The bloodstain kept at 50°C and pH 7 for 24 hrs give dark pink color than at zero hr ; (K) The bloodstain kept at 50°C and pH 7 for 48 hrs give dark pink color ; (L) The bloodstain kept at 50°C and pH 7 for 72 hrs give dark pink color

3.1.3. Effects of Takayama on human bloodstain

Bloodstains exposed to 4°C for varying durations and tested with the Takayama test show the formation of pink-colored Hemochromogen crystals under the microscope, which become somewhat thicker over time and with increasing temperature (Figs. 3A, 3B, 3C, and 3D). Similarly, bloodstains subjected to 37°C and 50°C temperatures exhibit the formation of Hemochromogen crystals, with their thickness increasing over time and with temperature elevation (Figs. 3E, 3F, 3G, 3H, 3I, 3J, 3K, and 3L).

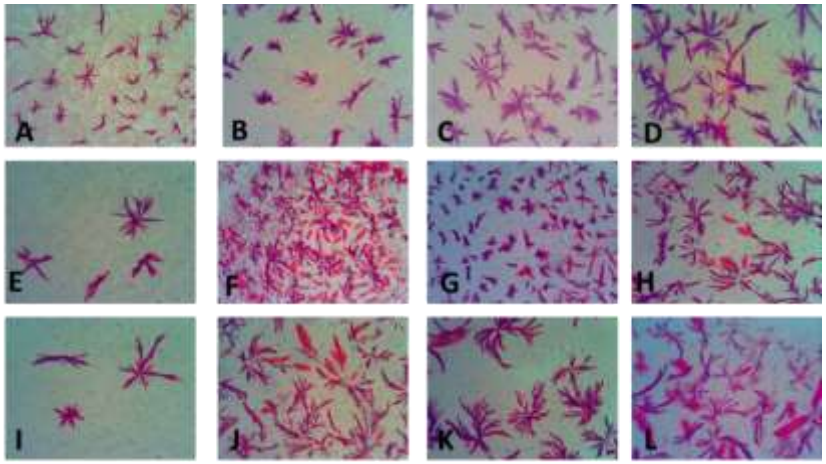

Figure 3. showed the effects of Takayama **(hemochromogen crystal**) test with different Temperature degree (4°C, 37°C, 50°C) for zero hrs, 24hrs, 48hrs and 72 hrs on human blood stain. (A) 4°C and pH 7 for zero hr give Hemochromogen crystals; (B) The bloodstain kept at 4°C and pH 7 for 24 hrs give Hemochromogen crystals dark and thick than at zero hr under microscope; (C) The bloodstain kept at 4°C and pH 7 for 48 hrs give Hemochromogen crystals (dark pink – colored , thick needle) (D) The bloodstain kept at 4°C and pH 7 for 72 hrs give Hemochromogen crystals (dark pink – colored , thick needle) than at 48 hrs under microscope. (E) bloodstain at 37°C and pH 7 for zero hrs give Hemochromogen crystals (F) The bloodstain kept at 37°C and pH 7 for 24 hrs give Hemochromogen crystals (pink – colored , thick needle than at zero hr (G) The bloodstain kept at 37°C and pH 7 for 48 hrs give Hemochromogen crystals (pink – colored , thick nee-dle as at 24 hrs (H) The bloodstain kept at 37°C and pH 7 for 72 hrs give Hemochromogen crystals (pink – colored , thick needle.bloodstain at 50°C and pH 7 for zero hr give Hemochromogen crystals (J) The

blood stain kept at 50°C and pH 7 for 24 hrs give Hemochromogen crystals (dark pink – colored , thick needle than at zero hr (K) The bloodstain kept at 50°C and pH 7 for 48 hrs give Hemochromogen crystals (dark pink – colored , thick needle) (L) The bloodstain kept at 50°C and pH 7 for 72 hrs give Hemochromogen crystals (dark pink – colored , thick needle than at 48 hrs

3.2. Cat bloodstains

3.2.1. Macroscopic examination under different environmental Temperature

- ***Fresh Cat Bloodstain at Room Temperature: Red and Liquid***

To provide a baseline for comparison, it's important to note that fresh cat bloodstains at room temperature (at a pH of 7) appear as vibrant red and maintain a liquid consistency. This initial observation serves as a reference point for understanding how temperature affects bloodstain properties (Fig. 4A).

- ***Effects of 4°C on Cat Bloodstains***

The experiment highlights the effects of temperature and time on the properties of cat bloodstains under controlled conditions. Exposure to 4°C triggers a transition from a liquid to a gel-like state in cat blood (Fig 4A), while maintaining its red coloration at 24 hrs (Fig 4B). As the exposure time increases, the gelatinous consistency intensifies, emphasizing the role of time in altering bloodstain properties. Extended exposure periods, such as 48 and 72 hrs, result in even more pronounced gelatinous consistency and signs of drying. (Figs. 4C and 4D).

- ***Effects of Temperature (37°C)***

The fresh bloodstain at room temperature is red-colored and liquid; (Fig E). Cat bloodstains exposed to 37°C for 24 hrs at pH 7 darken significantly, turning deep red, losing liquid consistency, and developing visible cracks(Fig F). Extending exposure to 48 hrs intensifies changes, maintaining dark red color but increasing dryness and crack prominence, resembling a parched landscape (Fig. G). Continuing for 72 hrs sustains dark red color, potentially transitioning to a brownish hue, with drying and cracking effects (Fig H) persisting and surpassing those at 24 and 48 hrs.

- ***Effects of High Temperature (50°C)***

The fresh bloodstain at room temperature is red-colored and liquid; (Fig I). Cat bloodstains exposed to 50°C for 24 hrs at pH 7 darken significantly, becoming deep red and losing liquid consistency, resulting in a cracked appearance (Fig J). Continuing for 48 hrs intensifies drying and cracking, maintaining a dark red color but becoming even drier compared to 24 hrs (Fig G). Pronounced cracks develop, resembling crusts on the bloodstain surface. Extending to 72 hrs sustains a dark red color, with some bloodstains transitioning to a brownish hue (Fig H). Drying and cracking effects intensify further, surpassing characteristics observed at 24 and 48 hrs.

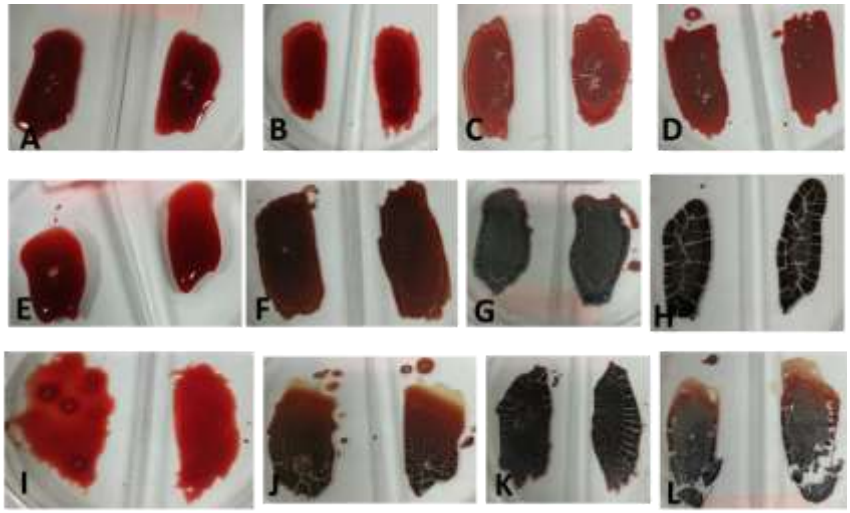

Figure 4. showed the effects of different Temperature degree (4°C, 37°C, 50°C) for zero hrs, 24hrs, 48 hrs and 72 hrs on CAT blood stain. (A)The fresh bloodstain at room temperature is red-colored and liquid; (B) The bloodstain kept at 4°C for 24 hrs is colored-red and become gelatinous in consistency; (C) The bloodstain kept at 4°C for 48 hrs is colored red and become more gelatinous in consistency than that kept for 24 hrs; (D) The blood-stain kept at 4°C for 72 hrs is still colored red and more gelatinous in con-sistency higher than 48hrs and start to dry. (E) The fresh bloodstain at room temperature is red-colored and liquid; (F) The bloodstain kept at 37°C for 24 hrs is dark red-colored, dry and cracked; (G) The bloodstain kept at 37°C for 48 hrs is dark red-colored, dry more than 24 hrs and cracked; (H) The bloodstain kept at 37°C for 72 hrs is still dark red to brownish-colored, dry more than 48 hrs and cracked. (I) The fresh bloodstain at room temperature is red-colored and liquid; (J) The bloodstain kept at 50°C for 24 hrs is dark red-colored, dry and cracked; (K) The bloodstain kept at 50°C for 48 hrs is dark red-colored, dry more than 24 hrs and cracked like crusts; (L) The bloodstain kept at 50°C for 72 hrs is still dark red to brownish-colored, dry more than 48 hrs and cracked like crusts.

3.2.1. Effects of the phenolphthalein test on stained cat blood

The experiment examined the impact of different temperatures (4°C, 37°C, and 50°C) on bloodstains over various time intervals (zero hour, 24 hrs, 48 hrs, and 72 hrs) using the Kastle-Meyer test. At each temperature, the bloodstains initially produced a pink coloration upon testing As time elapsed and temperatures increased, the pink color became progressively darker. as in Figure 5.

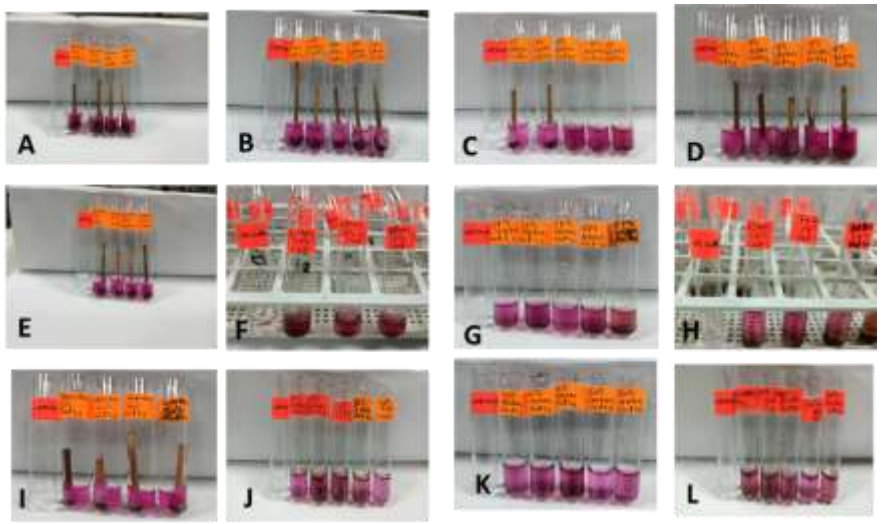

Figure 5. Showed the effects of Kastle-Mayer test (phenolphthalein test) with different Temperature degree (4°C, 37°C, 50°C) for zero hrs, 24hrs, 48hrs and 72 hrs on Cat blood stain. (A)bloodstain at 4°C and pH 7 for zero hr give pink color ; (B) The bloodstain kept at 4°C and pH 7 for 24 hrs give pink color; (C) The bloodstain kept at 4°C and pH 7 for 48 hrs give pink color darker than at 24 hrs ; (D) The bloodstain kept at 4°C and pH 7 for 72 hrs give dark pink color than at 48 hrs. (E)bloodstain at 37°C and pH 7 for zero hr give pink color ; (F) The bloodstain kept at 37°C and pH 7 for 24 hrs give dark pink color than at zero hr ; (G) The blood-stain kept at 37°C and pH 7 for 48 hrs give dark pink color ; (H) The bloodstain kept at 37°C and pH 7 for 72 hrs give dark pink color. (I) bloodstain at 50°C and pH 7 for zero hr give pink color ; (J) The bloodstain kept at 50°C and pH 7 for 24 hrs give pink color to dark pink than at zero hr ; (K) The bloodstain kept at 50°C and pH 7 for 48 hrs give dark pink color ; (L) The blood-stain kept at 50°C and pH 7 for 72 hrs give dark pink color than at 48 hrs.

3.2.2. Effect of Takayama on Cat Bloodstain

The experiment investigated the effects of different temperatures (4°C, 37°C, and 50°C) on the formation and morphology of hemochromogen crystals in bloodstains over various time intervals (zero hrs, 24 hrs, 48 hrs, and 72 hrs). Under all temperature conditions, hemochromogen crystals formed and exhibited similar characteristics, including a pink coloration, needle-shaped morphology, and clustering or rosette arrangement when observed under a microscope. However, as time progressed and temperatures increased, the crystals exhibited a trend of thickening, with higher temperatures generally correlating with more pronounced thickening over time. This observation suggests a relationship between temperature, time, and the structural evolution of hemochromogen crystals in bloodstains, which could have implications for forensic analysis and determining the age of bloodstains at crime scenes (Fig 6). In conclusion, while our study found no discernible difference between human and animal blood in terms of the presence of Hemochromogen crystals, the observation of thinner crystals in humans compared to cats opens up new avenues for inquiry. By delving deeper into these differences, we can broaden our understanding of both comparative physiology and the intricacies of human and animal.

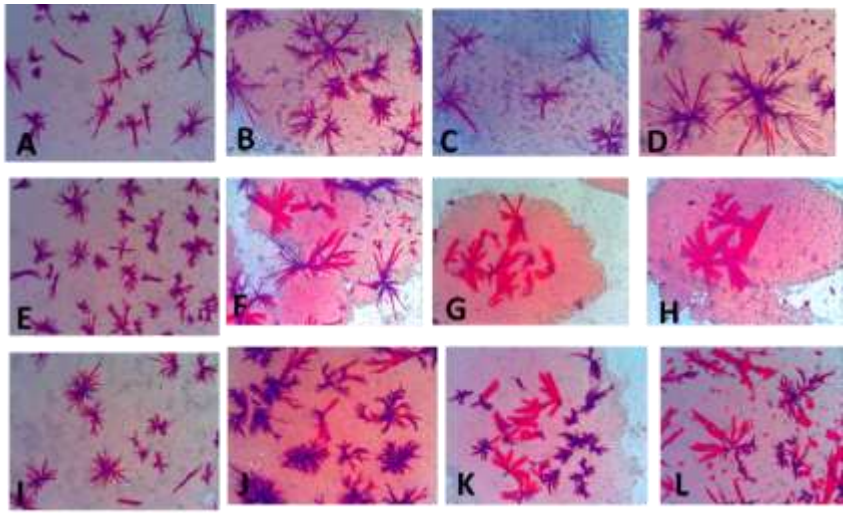

Figure 6. Showed the effects of Takayama **(hemochromogen crystal**) test with different Temperature degree (4°C, 37°C, 50°C) for zero hrs, 24hrs, 48hrs and 72 hrs on Cat blood stain.(A) bloodstain at 4°C and pH 7 for zero hr give Hemochromogen crystals (B) The bloodstain kept at 4°C and pH 7 for 24 hrs give hemochromogen crystals (C) The bloodstain kept at 4°C and pH 7 for 48 hrs give Hemochromogen crystals (dark pink – colored , thick needle) (D) The bloodstain kept at 4°C and pH 7 for 72 hrs give Hemo-chromogen crystals (dark pink – colored , thick needle). (E)bloodstain at 37°C and pH 7 for zero hr give Hemochromogen crystals (F) The bloodstain kept at 37°C and pH 7 for 24 hrs give Hemochromogen crystals (pink – colored , thick needle than at zero (G) The bloodstain kept at 37°C and pH 7 for 48 hrs give Hemochromogen crystals (pink – colored , thick needle) than at 24 hrs (H) The bloodstain kept at 37°C and pH 7 for 72 hrs give Hemochromogen crystals (pink – colored , thick needle) than at 48 hrs.(I) bloodstain at 50°C and pH 7 for zero hr give Hemochromogen crystals (J) The bloodstain kept at 50°C and pH 7 for 24 hrs give Hemochromogen crystals (dark pink – colored , thick needle than at zero hr) (K) The bloodstain kept at 50°C and pH 7 for 48 hrs give Hemochromogen crystals (dark pink – colored , thick nee-dle than at 24 hrs) (L) The bloodstain kept at 50°C and pH 7 for 72 hrs give Hemo-chromogen crystals (dark pink – colored , thick needle) as at 48 hrs

4. Discussion

The objective of the current experimental study is to evaluate the impact of different environmental conditions on bloodstains exposed to various temperature settings (4°C, 37°C, and 50°C) for different durations (zero, 24 hrs, 48 hrs, and 72 hrs). In the current investigation, exposure to different temperature gradients over varying durations significantly influences the macroscopic appearance and the identification process of bloodstains through preliminary and confirmatory tests. Bloodstains exposed to temperature variations (4°C, 37°C, and 50°C) for different durations (zero, 24 hrs, 48 hrs, and 72 hrs) displayed changes in color and consistency. Specifically, bloodstains became darker and drier at 37°C and 50°C, while maintaining a red color and gelatinous consistency at 4°C compared to control stains. These changes are attributed to the slowing down of molecular movement at lower temperatures, leading to increased viscosity. It's crucial to maintain a neutral pH of 7 to isolate temperature effects, as deviations can disrupt the ionization of blood components and yield unpredictable outcomes. Understanding these scientific principles provides insight into the behavior of cat bloodstains under varying environmental conditions

The observed changes in bloodstain appearance can be attributed to the conversion of oxyhemoglobin to methemoglobin, resulting in loss of liquid consistency and dryness at higher temperatures, while molecular movement slows down at lower temperatures. These findings are consistent with **(Abdel Hady *et al.*, 2021)**, who investigated bloodstains subjected to temperatures of 100°C and 50°C over 24 hrs, leading to a darker color compared to control stains possibly due to a shift in methemoglobin concentration. Additionally, the aging of bloodstains results in darker colors due to water loss and increased pigmentation. The exposure to varying temperatures and degrees of burn can indeed have a significant impact on bloodstains, affecting their macroscopic appearance, preliminary test results, and the feasibility of DNA analysis. These outcomes correspond with **(McDonald, 2017)** inquiry, where exposure to air at crime scenes saturates hemoglobin with oxygen, converting it to oxyhemoglobin, which subsequently breaks down into methemoglobin when reaching its oxygen absorption limit which was observed in this study. Another study explored how environmental factors like humidity and temperature affect the blood group system. It found that drying happens faster in warmer temperatures and slower in cooler ones. Since blood evidence in criminal cases often shows up as dried stains, understanding this drying process is critical. Initially, exposed blood clots, followed by more intricate changes during drying, often causing cell structure disruption and protein function loss. Notably, bloodstains at 37°C and 50°C can resemble burnt ones **(Kulkarni, 2015)**.

The phenolphthalein test, also known as the Kastle-Meyer test, is a widely used presumptive catalytic method for detecting blood stains (**Barni *et al.*, 2007**; **Cox, 1991**). When blood is present, phenolphthalein reacts with peroxide in an alkaline solution, causing it to turn pink (**Spalding, 2002**). This test has a sensitivity of 1 part in 10,000 **(Johnston *et al.*, 2008**). However, like other presumptive blood tests, it is not specific to any species and cannot determine if the suspected blood is human or from another animal (**Watson, 2016**). The Kastle-Meyer test primarily relies on the sustained stability of the oxidase enzyme, allowing the phenolphthalein test to detect blood even in aged stains processed months or years later This long-term stability preserves enzymatic activity, facilitating the reaction between blood components and hydrogen peroxide during the test. Catalytic color tests like the phenolphthalein test depend on the peroxidase-like activity of hemoglobin (Hb) molecules found in erythrocytes. In the presence of hemoglobin, the active reagent of the test undergoes oxidation, causing hydrogen peroxide to decompose and resulting in a distinct color change, indicating the presence of blood(Turrina *et al.*, 2008).

In the current study, the phenolphthalein test successfully detected blood in stains exposed to various temperatures (4°C, 37°C, and 50°C) for different durations (zero, 24 hrs, 48 hrs, and 72 hrs), yielding a pink color indicative of a positive result. This outcome underscores the presence of the oxidase enzyme in the blood samples, enabling the reaction of hydrogen peroxide with blood components. These results align closely with the findings of **Abdel Hady *et al.* (2021)**) who similarly explored the impact of environmental factors, including temperature variations and aging times, on blood stain detection using the phenolphthalein test. The persistence of the oxidase enzyme, unaffected by temperature fluctuations, plays a crucial role in determining the test's efficacy. Moreover, the presence of the catalase enzyme in blood enables the reaction with hydrogen peroxide, producing observable oxygen bubbles, serving as a reliable indicator of blood presence. Also, another study investigated the influence of temperature on the ability to detect bloodstains using the phenolphthalein test. Its results showed that blood could still be detected on cotton fabric after exposure to various temperature grades ranging from 100 °C to -20 °C. This suggests that the presence of oxidase enzyme, which is necessary for a positive result in the phenolphthalein test, is not affected by different temperatures.

In the absence of Fe^{+2}, there would be no Fe^{+2} ions available to undergo oxidation to Fe+3. As a result, there would be no color change observed when exposed to high temperature. The color change in this case is typically due to the formation of ferric ions (Fe^{+3}) which have a different color compared to ferrous ions (Fe^{+2}) These findings are consistent with a previous study by **(Khushbu *et al.*, 2017)** who also tested the ability to identify bloodstains under different environmental conditions including temperature and pH variations. Also, (**Prawestiningtyas, 2019**) conducted a study investigating different time frames ranging from sixty days down to three hours, examining the effects of blood deposition on various surfaces. Each surface was covered with blood droplets and subjected to different temperatures, including ambient temperature, -75°C (in a deep freezer), and 150°C (in a hot air oven). This observation suggests that both time and temperature influence the intensity of the pink coloration produced by the Kastle-Meyer test, indicating potential variations in the chemical reactions occurring within the bloodstains under different environmental conditions. Such findings could be significant for forensic investigations, aiding in the determination of the age and conditions of bloodstains found at crime scenes

Another confirmatory test in this study is Takayama test's which is highly sensitive and allows to detection of tiny blood traces, preserving evidence integrity even when stored at room temperature or outdoors, making it suitable for forensic investigations even when exposed to decomposition media (**James *et al.*, 2005**). The test demonstrated a sensitivity capable of detecting as little as 0.001 milliliters of blood or 0.1 milligrams of hemoglobin. Furthermore, it remained effective in positively identifying blood spots up to the age of 20, indicating its reliability in detecting even trace amounts of blood for an extended period. Microcrystalline tests rely on the formation of specific crystals of hemoglobin derivatives like hemoglobin, hematin, and hemochromogen. These tests are suitable for laboratory use and require microscopic examination for observation and analysis. It operates by generating hemochromogen through the application of heat to a dried stain in the presence of glucose

and pyridine within an alkaline environment (**Spalding, 2002**). Despite occasional use under acidic conditions, the formation of needle-shaped crystals is the intended result. This test is preferred due to its advantages, such as decreased sensitivity to heat, as noted.

In the present investigation, the hemochromogen crystal test served as a confirmatory method for detecting blood in blood stains exposed to various temperature grades (4°C, 37°C, and 50°C) for different durations (zero, 24 hrs, 48 hrs, and 72 hrs), resulting in the formation of Hemochromogen crystals. These crystals, characterized by their pink color and needle-shaped, feathery-like appearance arranged in clusters or rosettes, are produced by heating blood with Takayama's reagent. This reagent contains NaOH, which converts the ferrous form of iron in hemoglobin to the ferric form, resulting in alkaline haematin. Haematin combines with pyridine in the reagent to form insoluble colored crystals known as pyridine hemochromogen. The saturated glucose solution in the reagent acts as a reducing agent, reducing the solubility of hemochromogen and promoting crystal formation (**James *et al.*, 2005**; **Katz and Halámek, 2016**). The Takayama crystal assay is utilized in forensic and medico-legal practices to differentiate blood stains from other red-colored marks, even from minute traces of blood. This method is applicable to both fresh and dried blood, aiding in the identification of old blood stains. The interaction between pyridine and the iron atom of the heme group results in the formation of a ferroprotoporphyrin ring, contributing to crystal formation. The Takayama test produced positive results despite variations in color quality. Initially, the crystals appeared brown and gradually transitioned to yellow over a period of up to 720 hours. This color change was consistent across multiple time points, including the examination at 30 minutes (hour 0), 24 hours, and up to 456 hours (19 days). Subsequently, the color shifted to pale yellow at 480 hours (20 days). Additionally, blood spots on cloth submerged in water also yielded positive results, indicating the persistence of blood evidence despite environmental exposure (**Prawestiningtyas, 2019**).

5. Conclusion

Results indicate significant influences on the macroscopic appearance and identification process of bloodstains through preliminary and confirmatory tests. Moreover, confirmatory tests like the Takayama test and microcrystalline tests offer reliable methods for detecting blood traces, even under varying environmental conditions, thus preserving evidence integrity and aiding forensic analyses. Changes in color and consistency were observed in bloodstains exposed to varying temperatures and durations, with darker and drier stains at higher.

Authorship contribution statement: Yasser Elsayed, Dalia Samak, Wafaa Badr Writing – original draft, Formal analysis, Investigation, Validation, Supervision, Writing – review & editing. Yasser Elsayed, Dalia Samak Conceptualization, Methodology, Formal analysis. Dalia Samak, Hamida Saleh: writing – original draft, Validation. temperatures compared to control stains.

Declaration of competing interest: The authors declare that they have no conflicts of interest.

6. References

Abdel Hady R.H., Thabet H.Z., Ebrahem N.E. and Yassa H.A. (2021). Thermal effects on DNA degradation in blood and seminal stains: Forensic view. Academic Forensic Pathology, 11(1): 7-23.

Alsheekhly B., Al-Sadoon T.H. and Al-rawi R.A. (2019). Kastle-Meyer Test Enhancing Diagnosis of Occult Blood in Dentistry. Indian Journal of Forensic Medicine & Toxicology, 13(4).

Bandyopadhyay S.K. and Basu N. (2015). Review of common bloodstain patterns documented at a crime scene in the event of blunt force hit. Am J Comp Sci Inform Technol, 3: 45-63.

Barni F., Lewis S.W., Berti A., Miskelly G.M. and Lago G. (2007). Forensic application of the luminol reaction as a presumptive test for latent blood detection. Talanta, 72(3): 896-913.

Cox M. (1991). A study of the sensitivity and specificity of four presumptive tests for blood. J Forensic Sci, 36(5): 1503-1511.

Das T., Harshey A., Nigam K., Yadav V.K. and Srivastava A. (2020). Analytical approaches for bloodstain aging by vibrational spectroscopy: Current trends and future perspectives. Microchemical Journal, 158: 105278.

De Wael K., Lepot L., Gason F. and Gilbert B. (2008). In search of blood—Detection of minute particles using spectroscopic methods. Forensic Science International, 180(1): 37-42.

Doty K.C. and Lednev I.K. (2018). Differentiation of human blood from animal blood using Raman spectroscopy: A survey of forensically relevant species. Forensic science international, 282: 204-210.

Gardner R.M. and Krouskup D. (2018). Practical crime scene processing and investigation. CRC Press. Pp.

Glaister J. (1926). The Kastle-Meyer Test for the Detection of Blood: Considered from the Medico-Legal Aspect. British Medical Journal, 1(3406): 650.

Hanslip C. (2021). The factors affecting the recovery of bloodstain evidence from buried clothing. *In*. Bournemouth University. pp.

James S.H., Kish P.E. and Sutton T.P. (2005). Principles of bloodstain pattern analysis: theory and practice. CRC press. Pp.

Johnston E., Ames C.E., Dagnall K.E., Foster J. and Daniel B.E. (2008). Comparison of presumptive blood test kits including hexagon OBTI. Journal of forensic sciences, 53(3): 687-689.

Kamali H. and Mohri M. (2015). Effects of heparin, citrate, and EDTA on plasma biochemistry of cat: comparison with serum. Revue Med Vet, 166(9-10): 275-279.

Katz E. and Halámek J. (2016). Forensic Science–Chemistry, Physics, Biology, and Engineering–Introduction. Forensic Science: A Multidisciplinary Approach: 1-4.

Khushbu K., Shalika N. and Rashmi K. (2017). Identification of blood stains under different environmental conditions. Int J Biomed Res, 8(12): 707-710.

Kobilinsky L. (2011). Forensic chemistry handbook. John Wiley & Sons. Pp.

Kulkarni K. (2015). Effect of ageing of environment of north maharastra on ABO Grouping substance of Blood stain. Journal of pharmaceutical,Chemical and Biological sciences, Dec2015-Feb201:3(4): 608-611, 3: 608.

Magalhães T., Dinis-Oliveira R.J., Silva B., Corte-Real F. and Nuno Vieira D. (2015). Biological evidence management for DNA analysis in cases of sexual assault. The Scientific World Journal, 2015.

McDonald T. (2017). Investigating the effect of high temperatures and substrates on the detection of human blood using the ABAcard® Hematrace® kit. *In*. Murdoch University. pp.

Namdee K., Carrasco-Teja M., Fish M., Charoenphol P. and Eniola-Adefeso O. (2015). Effect of variation in hemorheology between human and animal blood on the binding efficacy of vascular-targeted carriers. Scientific reports, 5(1): 11631.

Pokupcic K. (2017). Blood as an important tool in criminal investigation. Journal of forensic science and criminal investigation, juniper publishers.

Prawestiningtyas E. (2019). Takayama Test as A Blood Spot Test Tool in Blood Samples Exposed to Freshwater Decomposition Media. Indonesian Journal of Legal and Forensic Sciences, 9(2): 413017.

Reynolds B.S., Boudet K.G., Faucher M.R., Germain C., Geffre A. and Lefebvre H.P. (2007). Comparison of a new device for blood sampling in cats with a vacuum tube collection system—Plasma biochemistry, haematology and practical usage assessment. Journal of Feline Medicine and Surgery, 9(5): 382-386.

Smith F. and Brutin D. (2018). Wetting and spreading of human blood: Recent advances and applications. Current Opinion in Colloid & Interface Science, 36: 78-83.

Spalding R.P. (2002). Identification and characterization of blood and bloodstains. Forensic Science. CRC Press. Pp. 209-230.

Stewart V., Deacon P., Zahra N., Uchimoto M.L. and Farrugia K.J. (2018). The effect of mark enhancement techniques on the presumptive and confirmatory tests for blood. Sci Justice, 58(6): 386-396.

van Oorschot R.A., Szkuta B., Meakin G.E., Kokshoorn B. and Goray M. (2019). DNA transfer in forensic science: a review. Forensic Science International: Genetics, 38: 140-166.

Watson N. (2016). Analysis of Body Fluids. *In*: White P.C. (Ed.) Crime Scene to Court: The Essentials of Forensic Science. The Royal Society of Chemistry. Pp. 0.