Black Sea Journal of Engineering and Science
doi: 10.34248/bsengineering.1391613

Open Access Journal
e-ISSN: 2619 – 8991

Research Article
Volume 7 - Issue 1: 129-138 / January 2024

# FALSE POSITIVES IN LUMINAL TESTING

**Yakup GULEKCI[1]*, Fatma CAVUS YONAR[2]**

[1]*Kutahya Health Sciences University, Faculty of Engineering and Natural Sciences, Department of Forensic Sciences, 43100 Kutahya, Türkiye*
[2]*Istanbul University-Cerrahpasa, Institute of Forensic Sciences and Legal Medicine, 34500, Istanbul, Türkiye*

**Abstract:** The blood sample is one of the most essential pieces of evidence that helps criminal experts in the elucidation of the crime. However, cleaning the blood found at the crime scene after the crime is committed makes it difficult to detect the crime. Therefore, experts have attached great importance to research on blood samples left at the crime scene. Although many test kits are used, especially in detecting erased blood, the luminol kit is widely used at crime scenes. In the luminol kit, the reaction takes place with the addition of hydrogen peroxide ($H_2O_2$). Since it is known that hydrogen peroxide can react with other substances containing Fe (II) ions that may be present in the environment during the reaction, it was aimed to carry out research to determine which substances luminol interferes with, especially those that may be found in a domestic crime scene in this study. In domestic crime scenes, there are limited substances that can replicate the distinct, enduring luminescence characteristic of a reaction between luminol and genuine bloodstains, while the uncharacteristic glow produced from many other surfaces is likely to be recognised with the naked eye by a good expert, experienced and knowledgeable in the field. In this regard, the prepared samples were treated with luminol in a dark environment, and the reactions occurring in the first seconds were recorded. The results obtained with this reagent are recommended to be supported by other blood test reagents or confirmatory tests, as there are substances that give false positive results with luminol reagents in the present study.

**Keywords:** Luminol, Blood, Chemiluminescence, False positivity, Crime Scene investigation

***Corresponding author:** Kutahya Health Sciences University, Faculty of Engineering and Natural Sciences, Department of Forensic Sciences, 43100 Kutahya, Türkiye
**E mail:** yakup.gulekci@ksbu.edu.tr (Y. GULEKCI)
Yakup GULEKCI https://orcid.org/0000-0001-9643-6850
Fatma CAVUS YONAR https://orcid.org/0000-0001-5941-8434

**Received:** November 15, 2023
**Accepted:** January 01, 2024
**Published:** January 15, 2024

**Cite as:** Gulekci Y, Cavus Yonar F. 2024. False positives in luminal testing. BSJ Eng Sci, 7(1): 129-138.

## 1. Introduction

Forensic biology primarily examines evidential objects found at crime scenes regarding biological fluids (Virkler et al., 2009; An et al., 2012). At a crime scene, an investigator often comes across various types of evidence, among which bodily fluids hold significant evidentiary value. Blood, semen, and saliva are the fluids most frequently encountered, in addition to vaginal secretions and urine. Accurate detection and identification of these fluids at the crime scene can lead to early detection of the offence. For example, while the presence of blood at a crime scene indicates some physical fight or violent assault, the presence of semen makes it possible to focus on the possibility of sexual assault (An et al., 2012). While these definitions assist in guiding the investigation's course, the fundamental objective of identifying bodily fluids lies in pinpointing the exact nature of the fluid and ascertaining the identity of the individual who deposited the biological material at the crime scene, achieved through DNA analysis. In other words, identification can be made by determining the genotypic or phenotypic characteristics of the person to whom the fluid belongs. The main methods used to detect body fluids are screening and confirmation tests. Screening tests are designed to assess the likelihood of the presence of bodily fluids on the evidence. In instances where the result of the screening test is affirmative, a confirmatory test is then employed to ascertain the exact type of fluid with greater certainty (Li, 2015). The diverse screening and confirmatory tests used in identifying body fluids aim to identify one or more components within the body fluid.

Blood is the most commonly found biological fluid at crime scenes and is arguably the most important source for genetic analyses (DNA and RNA analyses) (James et al., 2005). Methods such as visual examination, analyses that determine the presence or absence of blood through the catalytic action of haemoglobin, and confirmatory tests involving antigen-antibody reactions are employed for blood detection. The main problems encountered when applying the methods used to detect blood are the observation of false positive or false negative results. The studies documented in the literature highlight instances of false positives and negatives associated with catalytic colour tests, chemiluminescent substances, and immunoassays. These studies point out various interfering agents that can disrupt accurate blood identification, potentially causing erroneous positive or negative outcomes (Cox, 1991; Tobe et al., 2007; Li, 2015).

Synthesised in 1902 in Germany, the chemical name of luminol, according to IUPAC, is 5-Amino-2,3-dihydro-1,4-phthalazindione, 5 - Amino - 1, 2, 3, 4 - tetrahydrophthalazine – 1, 4 - dione, 3 - aminophthalhydrazide (Barni et al., 2007). Its molecular formula is $C_8H_7O_3N_3$. Luminol is a green-yellow crystalline powder and is odourless. It is dangerous for luminol, which is flammable, to coexist with strong oxidising agents. It emits light in reaction with oxidisers and is sensitive to light (Tajani, 2014). In case of poisoning, it may cause damage to mucous membranes, skin, eyes, and gastrointestinal system (Barni et al., 2007). Since it can maintain its stability for 8-12 hours, it is recommended to prepare it shortly before use (Thorpe, 1987).

Luminol is often used to identify blood that is difficult to see at crime scenes or where attempts have been made to remove it, but trace amounts are still present (Barni et al., 2007; Rogiski et al., 2012). Luminol helps to visualise bloodstain patterns that can be integral to understanding the sequence of events that occur during an attack. In the luminol kit, which is available in commercial formulations for ease of use, the reaction takes place with the addition of hydrogen peroxide ($H_2O_2$). During the reaction, hydrogen peroxide reacts with other substances containing metal ions, such as Fe (II), that may be present in the environment and glow. When an oxidizing molecule like hydrogen peroxide is present alongside a catalyst, luminol emits a bluish-coloured light. This luminescence reaction takes place as follows: metal ions in the medium catalyse the oxidation reaction of luminol with $H_2O_2$ and oxidise it to aminophthalate (Figure 1). Aminophthalate forms a high-energy structure and emits light from the excess energy (Wells et al., 1996). Hydrogen peroxide interacting with blood causes bubbles to form at the edges of the stain (Finnis et al., 2013).

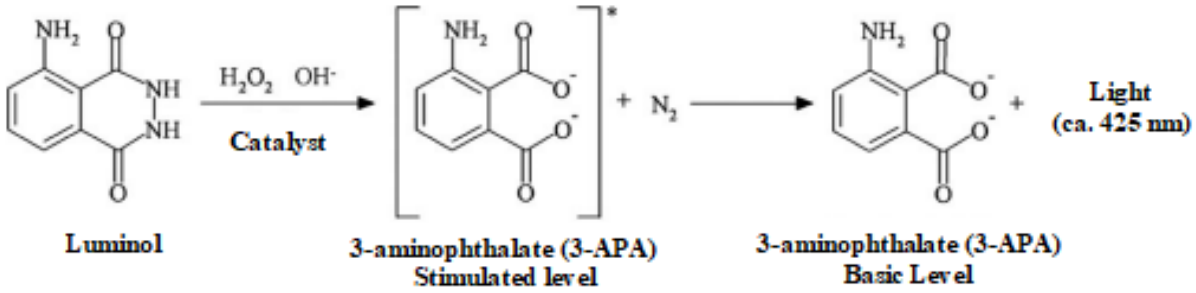


**Figure 1.** Reaction of luminol during blood detection (Stoica et al., 2016).

Iron in haemoglobin (Li, 2015), the protein responsible for carrying oxygen within the cell, which is found in the red cells of the blood called erythrocytes and constitutes by 44% of the blood volume, acts as a metal catalyst and enables this reaction to take place. Haemoglobin, a tetrameric molecule, is composed of four polypeptide chains, comprising two alpha (α) and two beta (β) chains. Embedded in each polypeptide chain is a "haem" molecule, also known as ferroprotoporphyrin, which is a dark red-coloured blood pigment. Central to each haem molecule is an iron ion ($Fe^{2+}$), which has the capacity to bind with oxygen molecules (Marengo-Rowe, 2006; Molnar et al., 2019). The haem molecule of erythrocytes is the most crucial blood component that attracts the attention of forensic sciences in detecting and identifying blood.

The light produced in this reaction, called chemiluminescence, appears bluish in the dark environment (Barni et al., 2007). Therefore, the presence of blood in wiped environments, which is not possible to see with the naked eye, is proved as a result of the reaction. To evaluate the blood stains detected with the help of luminol applied by spraying at crime scenes as evidence, it is essential to photograph the radiation that occurs quickly. A completely dark environment is required during photography (Laux, 2005).

Studies have shown that fresh blood samples show a weaker and shorter chemiluminescence reaction than old, dry and deconstructed blood samples (Klein et al., 2007). The same is true for the comparison of diluted and undiluted blood.

Luminol, an alkaline chemical, includes an oxidizing agent that facilitates the conversion of $Fe^{2+}$ to $Fe^{3+}$. As a result, the transformation of hemin into haematin in bloodstains is amplified when luminol is used. This process involves hematin acting as a catalyst in the breakdown of peroxide and in the oxidation of luminol by peroxide, forming a catalytic cycle (Barni et al., 2007).

Bloodstains that have aged tend to have a higher concentration of haematin compared to recent ones, which explains why luminol exhibits a stronger glow in older bloodstains. The interaction between luminol and haematin in a catalytic cycle is an example of a redox reaction, a type of chemical reaction that involves changes in the oxidation states of certain atoms or molecules. Such reactions encompass both oxidation and reduction processes (Cheyne, 2011).

Initially, in an alkaline environment, haematin's breakdown of hydrogen peroxide results in the formation of hydroxyl radicals (OH-*) and hydroxyl anions (OH-). These by-products then oxidize Haematin ($Fe^{III}P$) in a two-electron oxidation step, leading to the creation of the

hydroxyl-ferryl porphyrin radical ($Fe^{IV}P^{+*}$), a potent oxidizing agent. Subsequently, $Fe^{IV}P^{+}$ catalyzes the oxidation of deprotonated luminol (LH-) to the luminol radical (L-*) through a one-electron oxidation reaction. This radical is then reduced back to hydroxyl-ferryl porphyrin ($Fe^{IV}P$). In turn, $Fe^{IV}P$ undergoes a one-electron reduction back to haematine, a process that occurs concurrently with the oxidation of another luminol molecule that has had a proton removed, forming another luminol radical. This establishes a catalytic cycle that replenishes haematine, enabling it to continue cycling as long as there is a supply of hydrogen peroxide and luminol (as shown in Figure 2). Consequently, repeated applications of luminol can be carried out with minimal reduction in the reaction's intensity. The only significant reduction in intensity arises from the dilution of the bloodstain with the water present in the luminol solution (Barni et al., 2007).

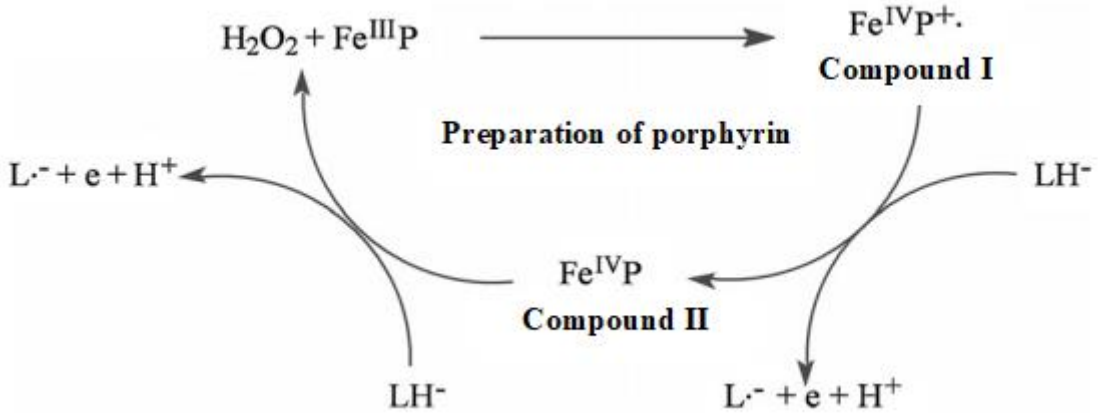


**Figure 2.** The redox cycle involving the iron ion of methemoglobin in the luminol reaction leads to the generation of luminol radicals (Cheyne, 2011).

When blood dries and ages, different chemical and biological changes occur in the blood. These changes cause the formation of methaemoglobin from haemoglobin by oxidation of iron in haem prosthetic groups from Fe (II) to Fe (III) (Patzelt, 2004). This effect affects the catalytic properties of blood in reactions in which luminol is produced. Old blood stains give a more intense and longer glow than fresh ones (Della et al., 2000; James et al., 2005).

Experienced crime scene investigators can often distinguish the luminescence caused by a substance other than blood from the reaction given by blood by paying attention to the luminescence's colour, the luminescence's brightness, and the luminescence's duration.

The main advantages of using luminol are that it can detect dilute blood spots down to $1:10^{10}$, it does not severely damage genetic material, and it can be applied multiple times at different times to visualise a spot (Barni et al., 2007; Rogiski et al., 2012; Chourasiya et al., 2017; Shivangi et al., 2021). One drawback of this technique is that with each subsequent application, the chemiluminescence diminishes due to the decreasing availability of haemoglobin for interaction with the luminol-hydrogen peroxide mixture. This reduction in intensity can pose challenges in photographing and documenting the stain as it is found at the crime scene or in its original location. Furthermore, a significant limitation of luminol is its lack of specificity to human haemoglobin. This is because luminol reacts with various substances including certain metals, animal haemoglobin, plant peroxidases found in fruits and vegetables, and cleaning agents that contain hypochlorite, like chlorine bleach (Barni et al., 2007; Rogiski et al., 2012). Luminol cannot distinguish between human and animal blood as it also catalyses the reaction with haemoglobins belonging to species other than human haemoglobin. If a blood stain found at the crime scene belongs to an animal, no result will be obtained in the DNA analysis stages. In order to prevent this, other tests that can distinguish between human and animal blood are required (Creamer et al., 2003; Quickenden et al., 2004).

Oxidants and plant peroxidases constitute a significant threat in screening tests (Li, 2015). Oxidants can catalyse the chemical reaction even in the absence of haemoglobin, leading to false positive results. Examples include metals such as copper, nickel, cobalt, chromium, manganese, and bleaches and detergents containing hypochlorite ions. Plant peroxidases also catalyse the oxidation reaction, i.e. they can react with reagents similarly to haemoglobin (Seitz et al., 1972; Cox, 1991; Quickenden et al., 2001a; Quickenden et al., 2001b; Ming et al., 2001; Creamer et al., 2003; Creamer et al., 2005; Tobe et al., 2007).

Some studies in the literature have attempted to determine the metals with which luminol reacts. Luminol can make coordination bonds with some metals due to the functional groups in its chemical structure. This allows metals to be detected in samples. Mn (III) containing micro peroxidase 8 (Mn(IIIMP8) plays a catalysing role in the oxidation of luminol with hydrogen peroxide at high pH, making it possible to see the chemiluminescence reaction (Yeh et al., 2003). When these and similar studies are taken into consideration, it

is seen that the studies have focused on the chemiluminescence property of luminol.

Increased knowledge by crime scene investigators about potential false positives/negatives in identifying blood helps ensure that evidential evidence is appropriately collected from the scene and thoroughly analysed. False positives and positive hypothetical results, where stains are identified as blood, can lead experts to waste resources and unnecessary labour by collecting non-evidential evidence from the crime scene or from the person (Petersen et al., 2014). In addition, false negative results that may occur in screening tests may cause evidence that may help the course of the investigation to be left behind. In order to prevent these and similar results, in our study, we tried to identify the substances that give false positive results with luminol, which is one of the frequently used screening tests at the crime scene. No representative experiment has been conducted in Türkiye regarding the substances that give false positive responses with luminol. An experiment was conducted to address which substrates, other than blood, produced or grown in Türkiye can react positively with luminol. There are only a handful of substances capable of replicating the strong, enduring, uniform glow that is characteristic of the reaction between luminol and an authentic bloodstain. Moreover, the atypical glow that luminol produces on various other surfaces is often discernible to the naked eye. Furthermore, the interpretation of patterns at a crime scene contaminated with substances that react positively with luminol requires considerable experience on the part of the forensic scientist.

## 2. Materials and Methods

### 2.1. Preparation of Luminol

Since inhalation or absorption of luminol solution by the skin may cause irritation, personal protective equipment was worn during the preparation of the luminol solution, and the working area was ventilated.

This study used solution B in a bottle labelled LUMINOL16B, powder A in a bottle labelled LUMINOL16A, and SIRCHIE brand luminol (North Carolina, USA) sold as a spray nozzle (Figure 3). Firstly, the cap of solution bottle B was opened and prepared for the procedure. Then, the cap of the bottle containing powder A was opened, and all the powder in the bottle was transferred to bottle B. The spray nozzle supplied with the solutions was attached to bottle B and shaken until all the solids were dissolved. Thus, luminol was ready for use (Sirchie, 2011).

### 2.2. Preparation of Samples

In order to prevent possible contamination, the surface and consumables used were cleaned with Zefirol IM Liquido (Molteni, Switzerland) before the experimental work, and the consumables used were autoclaved and kept under UV for 30 min.

In this study, apple, dried apricot, pineapple, mulberry, grape, celery, parsley, carrot, spinach, curly, bay leaf, purple cabbage, potato, tomato, garlic, onion, horseradish, turnip were used as fruit and vegetable group, detergent powder and bleach cleaning agents, milk, eggs and buttermilk of animal origin, inorganic substances such as naphthalene, iodine tincture, copper powder, tile dust, iron rust, mud and soil samples were preferred. Fruit and vegetable samples were pre-cleaned by washing and drying with distilled water and then crushed in a mortar and pestle. The detergent powder was dissolved in distilled water and naphthalene in methanol (Merck, Germany). Copper powder, tile powder, iron rust and soil were mixed with distilled water. Other selected materials were used directly without any pretreatment.


**Figure 3.** SIRCHIE brand luminol reagents

The 4x5 $cm^2$ calico fabrics, which were sterilised and checked to see whether they reacted with luminol before the application of the samples (blind sample), were used as ground and absorbent surfaces. Stains from the fruit-vegetable and animal foods groups were applied to the calico fabrics in an area of 2x2 $cm^2$. Dissolved detergent powder and mothballs, bleach, iodine tincture, copper powder, tile powder, iron rust, soil and mud samples were applied to the calico with the help of an automatic pipette (Eppendorf, Germany) in 500 µl volume (Figure 4). The prepared samples were allowed to dry for 24 hours at room temperature in a biosafety cabinet (Thermo Heraeus Herasafe KS 15 Class II Type A2 Biological Safety by Thermo Fisher, USA).

### 2.3. Application of Luminol to Prepared Samples

Since the possible irritation of the luminol solution on the skin or respiratory tract cannot be ruled out, a face mask and disposable gloves were worn during the application, and the working area was ventilated. All stages of the study were carried out under a fume cupboard using personal protective equipment.

The luminol prepared as described above was applied in a thin, single coat from a distance of 5-10 cm to cover the target surfaces completely (King et al., 2005). The chemical-resistant liquid with a maximum speed of 1.3 mL/sec was sprayed using a spray pump nozzle

(Divortex, Türkiye) and allowed to dry in a fume hood (Figure 5), (Divortex, 2023).


**Figure 4.** Images of some prepared samples

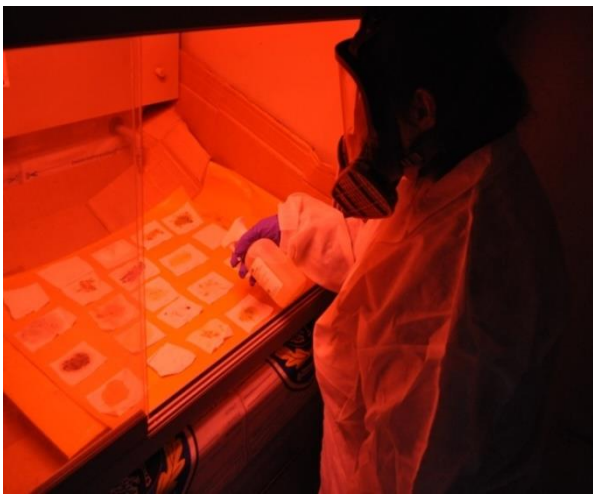

**Figure 5.** Application of luminol to the samples by spraying

**2.2. Chemiluminescence Evaluation**

Chemiluminescence formation after luminol application was visually observed and photographed. The results obtained during the application were evaluated and recorded by an experienced crime scene investigator. A Nikon D7200 camera with a shutter/exposure time of 16 seconds and a light sensitivity of ISO 400 was used for photographic documentation. Visual assessment was performed partly using the sandwich method (superimposing photographs taken in the light and the dark from the same recording position in an image processing program) and partly using the after-flash/backlighting method (a flashlight is thrown onto the ceiling behind the camera, or the ceiling is continuously illuminated with a weak light source while recording brightness in the dark).

It is challenging to visualise differences in the intensity of luminescence reactions, that is, the intensity of the luminescence, with the naked eye or in photographs. During the waiting period of a few minutes during which the reaction takes place, false positive reactions occur at least as spontaneously as true positive reactions but decay much more rapidly (Klein et al., 2007).

Experienced crime scene investigators can distinguish the chemiluminescence produced by real blood from that produced by other substances by evaluating parameters that can be observed with the naked eye, such as emission intensity, duration, and spatial distribution. However, since this approach is subjective, unofficial and without quantitative evaluation, it may lead to misinterpretations. Since the chemiluminescence of some false positive samples may be weaker than that of blood, these samples may be confused with diluted bloodstains (Barni et al., 2007). In such cases, using probabilistic and descriptive reagents for bloodstains helps to select samples that will give reliable results for subsequent DNA analyses (Lytle et al., 1978).

The interpretation of luminol chemiluminescence properties applied at the crime scene should consider the physical structure of the object on which the bloodstains are found, the object's chemical composition containing the stains and other substances present on the substrate. Metals, some paints and varnishes have a distinct and identifiable emission pattern from blood, with the spatial distribution of luminescence and emission intensity (Barni et al., 2007). It is not easy to apply luminol reagent and to obtain high chemiluminescence quality since processes such as wiping and painting to prevent blood from being visible can be applied more easily on non-absorbent surfaces than on absorbent ones (Lytle et al., 1978).

## 3. Results and Discussion

Identification within the scope of forensic biology includes the identification of the available biological fluid and the process of making DNA-based identifications that can reveal the relationships between the event/crime scene/suspect and victim with advanced analysis techniques. While screening and confirmation tests for biological fluid identification are highly beneficial in forensic contexts, it's crucial for scientists and crime scene investigators to consider potential interfering substances that could lead to false positives or negatives. These inaccuracies can stem from the presence of similar identifying elements in other body fluids or tissues, materials from plants or animals, common household products, or due to incorrect storage methods.

While the appearance of bright blue glow (Figure 6) resulting from the reaction was expected to be a blood stain, the brightness of the glow was higher in some substances (strongly positive in apple, dried apricot, pineapple, turnip, detergent powder, bleach, iodine tincture, copper powder, purple cabbage, iron rust and soil) and lower in some substances (positive in onion, horseradish, potato, tomato, tile powder, mud and garlic)

as shown in Table 1. No radiation (negative) was observed in mulberry, grape, celery, milk, parsley, egg, carrot, buttermilk, spinach, mothballs, lettuce, and bay leaf, especially in animal foods. The difference in the degree of positivity in soil and mud samples with similar structures is thought to be due to their heterogeneous nature. Other findings are consistent with the literature (Castello et al., 2002; Adair et al., 2008). The chemical background of the false positive reaction is still unclear. Therefore, not all substances that can trigger the reaction are known. False positive results have been observed in the presence of some metals such as copper or iron ions, dyes (potassium permanganate), cleaning agents/bleaching agents (sodium hypochlorite), plant components (chlorophyll) or vegetables (root vegetables containing peroxidase), photosynthetic microorganisms (Arnhold et al., 1991; Quickenden et al., 2001b). After a detailed examination of the ingredients of the cleaning products, this can be explained by the use of percarbonate or, peroxide or sodium hypochlorite as bleaching agents. A strong positive reaction was observed for the product containing sodium hypochlorite. The same was true for the root vegetable species tested. According to the studies of Quickenden (Creamer et al., 2003) and Creamer (Quickenden et al., 2001b), a positive reaction was observed in parsnip and potato, whereas in the study, a positive reaction was observed in potato and freshly cut horseradish. Possibly very different peroxidase content in fruits and vegetables due to season; fertilisation can explain this (Klein et al., 2007). In addition, these differences may also be due to the different preparation protocols of the luminol kit. In some studies, luminol is prepared as a solution containing only sodium carbonate and sodium perborate (Grodsky's approach), while in some serological or criminal studies, it is usually prepared as a three-component solution of sodium hydroxide and hydrogen peroxide (Weber's approach). This solution should be kept in a cold environment away from direct sunlight (Grodsky et al., 1951; Weber, 1966). Various chemical additives have been used to increase the selectivity of these formulations, but none have found widespread use (Arnhold et al., 1993).

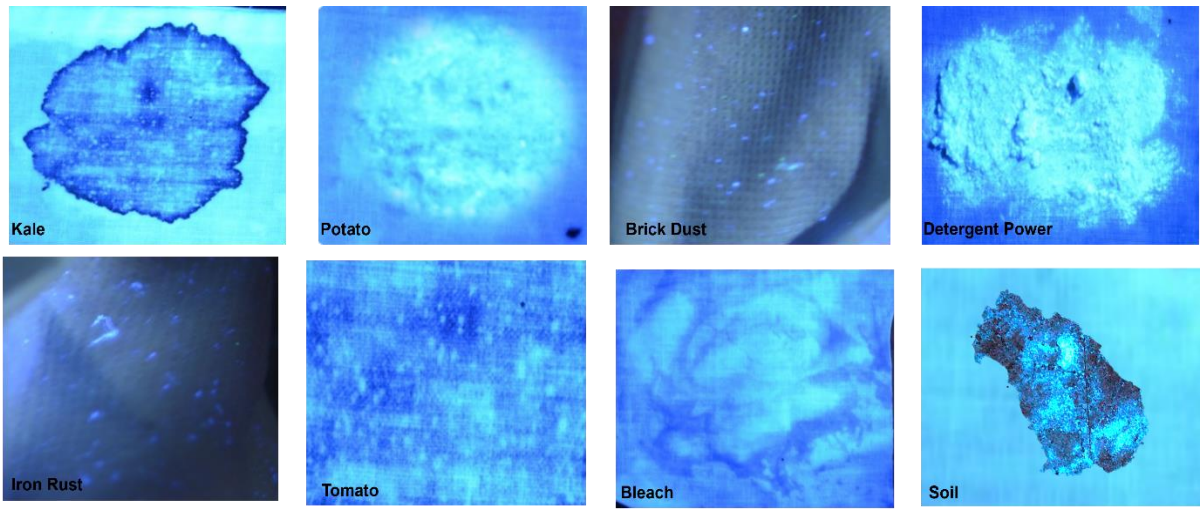


**Figure 6.** Some substances giving positive results with Luminol.

**Table 1.** Chemiluminescence observed after luminol administration

| Samples | Chemiluminescence Occurrence | Samples | Chemiluminescence Occurrence |
|---|---|---|---|
| *Apple* | ++ | *Onion* | + |
| *Dried apricots* | ++ | *Horseradish* | + |
| *Pineapple* | ++ | *Turnip* | ++ |
| *Mulberry* | - | *Detergent powder* | ++ |
| *Grape* | - | *Bleach* | ++ |
| *Celery* | - | *Milk* | - |
| *Parsley* | - | *Egg* | - |
| *Carrot* | - | *Buttermilk drink* | - |
| *Spinach* | - | *Naphthalene* | - |
| *Lettuce* | - | *Iodised tincture* | ++ |
| *Bay leaf* | - | *Copper powder* | ++ |
| *Purple cabbage* | ++ | *Tile dust* | + |
| *Potato* | + | *Iron rust* | ++ |
| *Tomato* | + | *Mud* | + |
| *Garlic* | + | *Soil* | ++ |

"-" indicates negative result; "+" indicates positive result; "++" indicates strong positive result.

In catalytic colour tests, potent oxidizing agents like copper, nickel, and chlorine bleach are capable of catalyzing the oxidation-reduction reaction even without the presence of haemoglobin, falsely indicating the presence of blood. Similarly, plant peroxidases can impact these tests due to their structural resemblance to haemoglobin, which enables them to catalyze the reaction (Novelli, 2020). To effectively deal with potential false positives, it is necessary to be aware of the environment in which the finding is found, to understand what substances may be near or on the suspect stain, and to be aware of appropriate collection and analysis methods. Oxidants can typically be detected by sequentially applying catalytic reagents and observing for any discolouration before introducing hydrogen peroxide. Hydrogen peroxide added to a blood stain will cause bubbles to be produced at the edges of the stain. However, no bubbles were observed for any substance in the experiment, resulting in false positives. Peroxidases found in plants like horseradish, potato, tomato, and onion can be neutralized through the application of high heat before conducting tests. However, this approach is seldom utilized in analyses due to the likelihood of high heat causing damage to genetic material.

BlueStar® Forensic is another screening test based on the principle of chemiluminescence (Dilbeck, 2006; Virkler et al., 2009; Novelli, 2020). This product includes a luminol-based substance in tablet form, which readily dissolves. When mixed with distilled water, this solution is sprayed onto the suspected stain area. The reagent reacts with haemoglobin, producing a blue luminescence that can be both seen and captured in photographs. In a comparative analysis performed by the Scottsdale and Saint Louis police departments, BlueStar® demonstrated superior qualities compared to traditional luminol. These advantages include the ability to detect more dilute bloodstains, better performance on bleach-treated stains, no need for total darkness for visualisation, brighter, longer-lasting chemiluminescence intensity, and no attenuation of the glow with repeated applications. Luminol does not react with other biological fluids except blood (Barni et al., 2007; Adair et al., 2008). Since it contains urea peroxide, a stable oxidant, it can be used days after preparation. It is an easy-to-prepare test that does not damage DNA (Dilbeck, 2006). Nevertheless, researchers recommend confirmation of the luminol reaction with other specific serological tests.

Studies of screening tests that investigate the sensitivity and specificity of blood have reported that the Kastle-Meyer (KM) test, also called phenolphthalein, is the most sensitive method to detect blood diluted to $1:10^{-9}$ (Cox, 1991; Tobe et al., 2007; Chourasiya et al., 2017). The reduced form of phenolphthalein is retained on Kastle-Mayer zinc granules and oxidised back to phenolphthalein by haemoglobin catalysis. It emits pink with positive results (James et al., 2005). However, some studies have contradictory results on leukomalachite green (LMG), one of the screening tests. In one of these studies, positive results were reported up to 1:10.000 blood dilution, while in another one, it was reported that LMG could only be positive up to 1:5000 blood dilution. In addition, interfering agents causing false positives and negatives for KM, LMG, Ortho-tolidine (O-tol) and Tetramethylbenzidine (TMB) tests were also studied. Quebracho extract (a typical skin tannin), sodium percarbonate (the main component of detergents containing active oxygen) and beverages containing vitamin C (ascorbic acid) have been reported to produce varying degrees of false-negative results in catalytic colour tests, chemiluminescent reagents, and immunoassays for the detection of blood (Novelli, 2020).

Immunochromatographic-based tests that are widely used in blood screening are HemaTrace® from Abacus Diagnostics, Seratec® HemDirect, Hexagon OBTI® from Human GmbH and RSID™- Blood from Independent Forensics (Johnston et al., 2003; Misencik et al., 2007). HemaTrace®, Hexagon OBTI® and HemDirect are based on human haemoglobin. The major disadvantages of these tests are their cross-reactivity with ferret or upper primate blood, that is, the possibility of false positive results and the possibility of false negative results if the test input volume is large.

However, due to the possibility that luminol and similar screening tests may disrupt the structure of the genetic materials of blood samples found at the crime scene after misapplication, it is imperative to develop and use new compounds as an alternative possibility in forensic sciences in order to completely eliminate this negativity and disadvantages related to the use of luminol. Studies on the development of luminol analogues with higher specificity and specificity, which can get faster results at crime scenes, meet the common denominator of nanotechnology and forensic sciences (Karabchevsky et al., 2016; Fereja et al., 2019; Fatoki, 2020).

In this study, various substances that have the potential to cause false positive results using luminol, one of the preferred forensic screening tests for the detection of blood, were discussed. The importance of examining any substance that causes false positive or negative results in forensic analyses, especially regarding screening tests, cannot be underestimated. This is the best way to ensure that any findings that may be encountered at a crime scene are accurately analysed most efficiently. The more that is known about a potential problem, the more cautious the expert will be about an uncertain test result in the field or the laboratory.

## 5. Conclusion

If a hypothetical test result for a body fluid is negative, the suspected stain is considered to be of no forensic significance, and no further testing is performed. However, if the stain is blood and there is a condition that prevents the analysis of blood in screening tests, the investigation will be deprived of an important piece of evidence, as confirmatory analyses for the detection of blood cannot be performed. In addition, the presence of

interfering agents at the crime scene, which causes false positive results, will cause the stain to be misidentified, increasing the workload and cost in criminal laboratories in terms of confirmatory analyses. In this case, it causes delays in justice services. In order to prevent the interaction of luminol with the substances that cause false positives identified in this study, it is recommended that the content of the kit components be updated, luminol analogues be identified, or alternative screening tests be considered. Although luminol is frequently used at crime scenes for reasons such as ease of use, it may cause illusions as a result of a false positive result. Accordingly, the stain thought to be blood as a result of luminol application at the crime scene must be confirmed to be blood by a second test such as Kastle-Meyer in the laboratory.

False negative or positive results obtained by screening tests used in the detection of blood, one of the biological fluids frequently encountered at crime scenes, is a subject that has attracted the attention of the forensic sciences community and has recently gained momentum in detailed research. Much more research is still needed to understand better how interfering agents affect screening tests. In particular, the literature has limited information on agents that cause false negativity.

Future studies should be motivated by situations that reflect "real world" conditions commonly found at active crime scenes, as opposed to controlled laboratory settings. In this study, the screening tests were performed on actual items like readily available detergents with active oxygen found in stores, common food products typically present in an average person's refrigerator, and various substrates that one might typically encounter at a crime scene. Future studies could also examine whether genotypic or phenotypic profiling can be developed from stains containing blood contaminated with these interfering agents and on which screening tests have been performed. Such an approach would be beneficial in deciding whether stains yielding positive or negative results for blood warrant further genetic analysis.

**Author Contributions**

The percentage of the author(s) contributions is presented below. The author reviewed and approved the final version of the manuscript.

| % | Y.G | F.Ç.Y |
|---|---|---|
| C | 50 | 50 |
| D | 50 | 50 |
| S | 60 | 40 |
| DCP | 40 | 60 |
| DAI | 60 | 40 |
| L | 40 | 60 |
| W | 40 | 60 |
| CR | 50 | 50 |
| SR | 50 | 50 |
| PM | 50 | 50 |
| FA | 50 | 50 |

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

**Conflict of Interest**

The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

**Ethical Consideration**

Ethics committee approval was not required for this study because of there was no study on animals or humans.

**Acknowledgements**

The authors would like to thank Istanbul University-Cerrahpasa Institute of Forensic Sciences and Legal Medicine, Forensic Sciences Laboratory and Kutahya Health Sciences University, Application and Research Centre Laboratory for providing laboratory facilities and a convenient working environment for this study.

## References

Adair TW, Gabel R, Shimamoto S, Tewes R. 2008. A comparison of the luminol and blue star blood reagents in detecting blood in soil nearly four years after deposition. IABPA News, 24(4): 5-8.

An JH, Shin KJ, Yang WI, Lee HY. 2012. Body fluid identification in forensics. BMB Rep, 45(10): 545–53. https://doi.org/10.5483/BMBRep.2012.45.10.206.

Arnhold J, Mueller S, Arnold K, Grimm E. 1991. Chemiluminescence intensities and spectra of luminol oxidation by sodium hypochlorite in the presence of hydrogen peroxide. J Biolumin Chemilumin, 6(3): 189-192.

Arnhold J, Mueller S, Arnold K, Sonntag K. 1993. Mechanisms of inhibition of chemiluminescence in the oxidation of luminol by sodium hypochlorite. J Biolumin Chemilumin, 8(6): 307-313. https://doi.org/10.1002/bio.1170080604.

Barni F, Lewis SW, Berti A, Miskelly GM, Lago G. 2007. Forensic application of the luminol reaction as a presumptive test for latent blood detection. Talanta, 72(3): 896–913. https://doi.org/10.1016/j.talanta.2006.12.045.

Castello A, Alvarez M, Verdu F. 2002. Accuracy, reliability, and safety of luminol in bloodstain investigation. Canadian Soc Foren Sci J, 35(3): 113-121.

Cheyne M. 2011. Illuminating latent blood. Application methods, fixatives, alternatives and new formulas for luminol. MSc thesis, The University of Auckland, Forensic Science, Auckland, New Zealand, pp: 222.

Chourasiya L, Mahakalkar A. 2017. To compare and study differential detection of human and animal blood using phenolphthalein, tetramethylbenzidine and luminol assays. Int J Recent Trends Eng Res, 3(11): 82-102.

Cox M. 1991. A study of the sensitivity and specificity of four presumptive tests for blood. J Foren Sci, 36(5): 1503-1511. https://doi.org/10.1520/JFS13170J.

Creamer JI, Quickenden TI, Apanah MV, Kerr KA, Robertson P. 2003. A comprehensive experimental study of industrial domestic and environmental interferences with the forensic Luminol test for blood. Lumin, 18: 193-198.

Creamer JI, Quickenden TI, Leah BC. 2005. At tempted cleaning of bloodstains and its effect on the forensic luminol test. Lumin, 20: 411– 413.

Da Silva RR, Agustini BC, da Silva ALL, Frigeri HR. 2012. Luminol in the forensic science. J Biotech Biodiv, 3(4):172-177.

Dilbeck L. 2006. Use of BlueStar forensic in lieu of luminol at crime scenes. J Forensic Identif, 56(5): 706–20.

Divortex, 2023. Kimyasal dayanıklı sıvı püskürtme (Sprey) şişesi 1 Lt. URL: https://www.divortex.com.tr/aksesuarlar/kimyasal-dayanikli-sivi-puskurtme-sprey-sisesi-1-lt. (accessed date: Sep 23, 2023).

Fatoki TH. 2020. In-Silico investigation of luminol, its analogues and improved mechanism of chemiluminescence for blood identification beyond forensics. bioRxiv, 01.

Fereja TH, Kitte SA, Gao W, Yuan F, Snizhko D, Qi L, Xu G. 2019. Artesunate-luminol chemiluminescence system for the detection of hemin. Talanta, 204: 379-385.

Finnis J, Lewis J, Davidson A. 2013. Comparison of methods for visualizing blood on dark surfaces. Sci Justice, 53(2): 178-186.

Grodsky M, Wright K, Kirk PL. 1951. Simplified preliminary blood testing--An improved technique and a comparative study of methods. J Crim L Criminol Police Sci, 42: 95.

James SH, Kish PE, Sutton TP. 2005. Principles of bloodstain pattern analysis: theory and practice, CRC Press, Boca Raton, 1st ed., New York, USA, pp: 533.

James SH, Nordby JJ, Forensic Science: An introduction to scientific and investigative techniques, CRC Press, New York, USA, 2nd ed., pp: 667.

Johnston S, Newman J, Frappier R. 2003. Validation study of the Abacus Diagnostics ABAcard® HemaTrace® membrane test for the forensic identification of human blood. Can Soc Forensic Sci J, 36(3): 173–83. https://doi.org/10.1080/00085030.2003.10757560.

Karabchevsky A, Mosayyebi A, Kavokin AV. 2016. Tuning the chemiluminescence of a luminol flow using plasmonic nanoparticles. Light Sci Applicat, 5(11): 16164-16164.

King R, Miskelly G. 2005. The inhibition by amines and amino acids of bleach-induced luminol chemiluminescence during forensic screening for blood. Talanta, 67(2):345–353. https://doi.org/10.1016/j.talanta.2005.01.034.

Klein A, Feudel E, Türk E, Püschel K, Gehl A. 2007. Luminescence after the use of luminol: Positive or false positive? Rechtsmedizin, 17: 146-152.

Laux L. 2005. The detection of Blood Using Luminol. in: James SH, Kish PE, Sutton TP. (Eds.), Principles of bloodstain pattern analysis: theory and practice. CRC Press, Boca Raton, 1st ed., New York, USA, pp: 369–389.

Li R. 2015. Forensic Biology. CRC Press, New York, USA, 2nd ed., pp: 533.

Lytle LT, Hedgecock DG. 1978. Chemiluminescence in the visualization of forensic bloodstains. J Foren Sci, 23(3): 550-562.

Manna AD, Montpetit S. 2000. A novel approach to obtaining reliable PCR results from luminol treated bloodstains. J Foren Sci, 45(4): 886-890.

Marengo-Rowe AJ. 2006. Structure-function relations of human hemoglobins. Proc Bayl Univ Med Cent, 19(3): 239-245.

Ming Lin J, Shan X, Hanaoka S, Yamada M. 2001. Luminol chemiluminescence in unbuffered solutions with a cobalt(II) – ethanolamine complex immobilized on resin as catalyst and its application to analysis. Anal Chem, 73: 5043–5051. https://doi.org/10.1021/ac010573+.

Misencik A, Laux DL. 2007. Validation study of the Seratec HemDirect hemoglobin assay for the forensic identification of Human Blood. MAFS Newsl. URL: https://pdfs.semanticscholar.org/2c56/c17d14e06a7472f6246596d10f76164844bb.pdf. (accessed date: Sep 25, 2023).

Novelli BC. 2020. A review of substances reported to cause false positives and negatives in forensic blood identification tests. MSc thesis, Boston University, School of Medicine, Boston, USA, pp: 66.

Patzelt D, 2004. History of forensic serology and molecular genetics in the sphere of activity of the German Society for Forensic Medicine. Forensic Sci Int, 144: 185-191.

Petersen D, Kovacs F. 2014. Phenolphthalein false-positive reactions from legume root nodules. J Forensic Sci, 59(2): 481–484. https://doi.org/10.1111/1556-4029.12352.

Quickenden TI, Cooper PD. 2001a. Increasing the specificity of the forensic luminol test for blood. Lumin, 16: 251–253.

Quickenden TI, Creamer JI. 2001b. A study of common interferences with the forensic luminol test for blood. Lumin 16: 295–298. https://doi.org/10.1002/bio.657

Quickenden TI, Ennis CP, Creamer JI. 2004. The forensic use of Luminol chemiluminescence to detect traces of blood inside motor vehicles. Lumin, 19: 271-277.

Rogiski da Silva R, Agustini B, Lopes da Silva AL, Frigeri HR. 2012. Luminol in the forensic science. J Biotech Biodiv, 3(4): 172–177.

Seitz WR, Hercules DM. 1972. Determination of trace amounts of iron(II) using chemiluminescence analysis. Anal Chem, 44: 2143–2149. https://doi.org/10.1021/AC60321A020.

Shivangi G, Apoorva G, Suresh B, Yadav DS, Mahanta P. 2021. Comparative study of presumptive and confirmatory tests for detection of blood on serial dilutions and washed stains. Int J Health Res Medico Leg Prae, 7(1): 59-64.

Sirchie. 2011. Luminol. URL: https://www.sirchie.com/media/resourcecenter/item/l/u/luminol_ti02-48eng-rev10e.pdf. (accessed date: Sep 23, 2023).

Stoica BA, Bunescu S, Neamtu A, Bulgaru-Iliescu D, Foia L, Botnariu EG. 2016. Improving luminol blood detection in forensics. J Foren Sci, 61(5): 1331-1336.

Stott RAW, Kricka LJ. 1987. Biolumin chemilumin symp. John Wiley & Sons Ltd., Chichester, New York, USA, pp: 237–240.

Tajani, A. 2014. Photonics for forensic applications. Photon

Safet Secur, 1987: 368-397.

Thorpe GHG, Kricka L J. 1987. Chemilumin: New Perspectives. Scholmerich JR, Andreesen R, Kapp A, Ernst M, Woods W, editors. Biolumin. Wiley, Chichester, UK, pp: 199–208.

Tobe SS, Watson N, Daéid NN. 2007. Evaluation of six presumptive tests for blood, their specificity, sensitivity, and effect on high molecular-weight DNA. J Forensic Sci, 52(1): 102–109. https://doi.org/10.1111/j.1556-4029.2006.00324.x.

Virkler K, Lednev IK. 2009. Analysis of body fluids for forensic purposes: from laboratory testing to non-destructive rapid confirmatory identification at a crime scene. Foren Sci Inter, 188(1-3): 1-17.

Weber K. 1966. Die Anwendung der Chemiluminescenz des Luminols in der gerichtlichen Medizin und Toxikologie. Dtsch Z ges gerichtl Med, 57: 410–423.

Wells PG, Winn LÇM. 1996. Biochemical toxicology of chemical teratogenesis. Critical Review in Biochem. Mol. Biol. 31 (1): 1-40.

Yeh HC, Lin WY. 2003. Enhancement of chemiluminescence from the oxidation of luminol with hydrogen peroxide catalyzed by Mn (III)-Microperoxidase 8. J Chinese Chem Soc, 50(1): 81-88.