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Effectiveness of various cleaning agents at removing detectable traces of blood

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EFFECTIVENESS OF VARIOUS CLEANING AGENTS AT REMOVING DETECTABLE TRACES OF BLOOD

by

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ABSTRACT

Forensic investigation television shows such as police procedurals, rooted in both fact and fiction, have become an ever-popular staple of modern television in the last 20 years. The popularity of these shows has been blamed for generating higher expectations for forensic evidence by juries across America and may also have had the effect of inspiring criminals attempt to cover up their crimes by destroying potential evidence, particularly bloodstains.

Luminol is a popular blood detection technique because it can be sprayed throughout an area in a dark room and will chemiluminesce when it interacts with hemoglobin. This chemiluminescence is a signal to investigators that latent blood may be located in that spot. Luminol's specificity and sensitivity have long been studied. Luminol is a stable molecule that becomes oxidized when it comes in contact with an oxidant such as hydrogen peroxide and a catalyst. In this excited state, the molecule is unstable and forms 3-aminophthalate. This molecule produces light, and the luminescence slowly dies out as the molecule returns to its ground state.

Chemicals that disrupt the luminol reaction can be considered interferents. These include cleaning agents, biological agents, foods, and drinks, among others. Compounds such as sodium hypochlorite, sodium percarbonate, and hydrogen peroxide are commonly

used as primary cleaning products or as components in popular brands of household cleaners. Such multisurface cleaners, extra strength detergents or other chemicals are readily accessible to someone attempting to clean up a crime scene. Sodium hypochlorite, also known as bleach, has previously been found to cross react with luminol, generating a chemiluminescent reaction whether heme is present or not. Sodium percarbonate is also known as active oxygen and is used in detergents to improve their stain removing capabilities. It can affect the luminol and Bluestar® Forensic tests by causing a negative result, even in the presence of blood. Hydrogen peroxide is a common disinfectant and a necessary component of most presumptive blood tests, however, bulk quantities of it in the luminol reaction stop the reaction from proceeding. Antioxidants, found in many foods and drinks, can inhibit luminescence by preventing heme from being degraded, an important step in order for the luminol reaction to proceed.

The purpose of this investigation is to evaluate the effectiveness of common cleaning agents for removing detectable traces of blood based on published studies. Additionally, an attempt was made to determine if cleaning agents completely remove blood or if they disrupt the luminol reaction when a negative luminol result is obtained. To supplement the literature, a limited experiment was carried out and preliminary data was obtained. This investigation finds that some cleaners interfere with the luminol reaction by altering one or more components in a way that prevents the reaction from fully proceeding, even when blood is still present.

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LIST OF ABBREVIATIONS

KM	Kastle-Meyer/Phenolphthalein
LCV	Leuco Crystal Violet
LMG	Leuco Malachite Green
O-Tol	Orthotolodine
RLU	Relative Luminescence Units
SVU	Special Victims Unit

1. INTRODUCTION

1.1 Forensic science in popular culture

Forensic investigation television shows such as police procedurals, rooted in both fact and fiction, have become an ever-popular staple of modern television in the last 20 years. Shows such as Bones, Forensic Files, True Detective and CSI have stirred in the public an interest in forensic science and presumably inspired a new generation to enter careers in forensic science. The popularity of these shows has been blamed for generating the "CSI effect" in juries across America and may also have had the effect of helping criminals attempt to cover up their crimes.

The CSI effect is the belief that the rise of forensic television shows has raised juror's expectations for relevant scientific evidence, resulting in a notion that almost magical forensic science evidence will be presented at trial that can erase any doubt that the person accused committed the crime (1). Similarly, the CSI effect is thought to cause crime show watching jurors to wrongfully acquit guilty defendants when no solid forensic proof of culpability has been offered (2). The CSI effect could be beneficial either to the defense or to the prosecution. Should jurors have a blind faith on the forensic evidence presented, they may lean towards conviction even if the analysis clearly has faults, helping the prosecution. Alternatively, the jurors might expect to be impressed with the power of the forensic analysis and once that fails to occur, they are more skeptical and move towards acquittal. In the mock case Schweitzer and Saks presented to college students who both were watchers of the television show CSI and its spinoff as well as those who don't watch those shows, 29% of non-viewers voted for guilty verdicts compared to 18% of viewers, which the authors determined not to be statistically significant (1).

The studies on the CSI effect show that there is a strong viewership of this kind of content and indicate that viewers might be learning enough about forensic testing and crime scene investigation to make them believe they could clean up a crime they just committed and essentially get away with murder. In fact, this is often the plot of many suspenseful television shows, where characters may be very close to executing the perfect crime but are eventually caught for one little mistake.

Through these and more accurate shows that depict real cases, such as Forensic Files, individuals learn that they can cover up crimes they either plan ahead for or commit in the heat of the moment. Therefore, it is not unreasonable to believe that upon arriving at a crime scene, there could have been attempts to clean up or destroy blood evidence prior to law enforcement involvement.

When a crime scene is processed, it cannot be assumed that it is exactly as it was at the moment the crime was committed. A cleanup attempt by the perpetrator or a third party may have occurred, and there may be contamination by whoever initially discovered the scene. Observations that are made regarding the presence of cleaning products, empty containers, or chemical odors could affect preliminary blood testing conducted on site. Knowing how effective various cleaning agents are in eliminating bloodstains and how they interact with blood detection techniques could prove to be very helpful to forensic investigators and may influence the decision to conduct further testing.

1.2 Presumptive blood tests

There are several colorimetric tests utilized to detect blood with varying degrees of sensitivity and specificity. Luminol is a popular choice given it can be sprayed throughout an area in a dark room and will chemiluminesce. This chemiluminescence is then a signal that latent blood could be located in that spot. Bluestar® Forensic is a proprietary chemical formulation related to luminol that can be more potent in terms of chemiluminescent reaction and duration (3). Beyond luminol and Bluestar® Forensic, there are Benzidine, Ortho-tolidine, Tetramethylbenzidine (TMB), Kastle-Meyer (KM)/Phenolphthalein, Leucomalachite Green (LMG), Leuco Crystal Violet, and Fluorescein among others.

Cox et al. conducted an investigation into the sensitivity and specificity of commonly used colorimetric tests (4). Kastle Meyer, O-Tol, LMG and TMB were evaluated on blood concentrations between 1:50 and 1:2 million. TMB was determined to be the most sensitive, obtaining a positive result at a 1:2 million blood dilution, followed by KM at 1:1 million; LMG was the least sensitive, not yielding positive reactions beyond concentrations of 1:5000. Specificity of the tests was found to be a tie between KM and LMG, which were highly specific for blood. Conversely, TMB and OT were not very specific, reacting with various different fruits and vegetables.

Benzidine was among the earliest colorimetric tests for blood to be used for forensic cases in the beginning of the 20th century. It is prepared using a combination of acetic acid and ethanol. Adler first published about its use in 1904 (5). Benzidine turns dark blue when it encounters blood, however, it is a known carcinogen (6) so its use and that of its derivatives has declined due to safety considerations. These derivatives include O-Tol and TMB. O-Tol functions under acidic conditions and goes from yellow to blue when in contact with blood. TMB also operates under acidic conditions and turns blue/green in the presence of blood. Hemastix® is a plastic strip test that utilizes TMB as its reagent, going from yellow/orange to dark blue/green when blood is detected (6). Webb et al. (6) proved Hemastix® was very sensitive, up to 1:1,000,000 for blood in solution, easy to use, and a good alternative to luminol when necessary.

The Kastle-Meyer test is also known as Phenolphthalein test. It is performed under alkaline conditions and involves oxidizing Phenolphthalin into Phenolphthalein. Sloots et al. (7) investigated the impact KM reagent has on DNA. KM reagent usage directly on a 10 μl, 1:100 bloodstain rendered it incapable of generating a DNA profile, however, indirect testing by rubbing a swab or filter paper on similar stains wasn't always successful.

Leuco dyes contain molecules that have two forms, one oxidized colored form and reduced colorless form (8). Leucomalachite green is an acidic reagent that forms a green-blue reagent when in contact with blood. Leucocrystal violet is prepared in an acidic medium and turns pink/purple in the presence of blood. It also doubles as an antifungal agent it is oxidized form.

Fluorescein is an alkaline solution that can be used like luminol to detect latent blood. It was used forensically beginning in 1990's. Heme catalyzes the oxidation of the Fluorescin into Fluorescein in the presence of hydrogen peroxide. A yellow-green light is emitted for a positive reaction. Unlike luminol, it requires an alternate light source (ALS) to visualize the stain. Martin et al. (9) investigated whether Fluorescein would have any impact on the extraction of DNA from a bloodstain. Gel electrophoresis was utilized in order detect DNA at one targeted locus, D18S51. DNA was detected for the chosen locus, demonstrating there was no impact.

Having multiple tests available allows for the best one for the situation to be implemented. If a test that is more sensitive but not necessarily specific to detect blood is required, there will be a reagent that fits that mold. Should there be a need for the reverse, another reagent can be chosen. Luminol has been proven to be sensitive and specific with the added benefit of being sprayed over a large area and successfully detecting blood when no blood is clearly visible (6,10,11). Luminol has also been shown to detect bloodstains years after they were deposited, possibly a century after the act (12).

Most of these tests function via redox reactions, where a colorless reagent goes through oxidation and becomes colored or changes color. KM, similar to luminol, involves the oxidation of its main reagent, phenolphthalin, into Phenolphthalein in the presence of hydrogen peroxide and heme, which catalyzes the reaction. TMB derivatives and leuco dyes involve the same process, and all of these tests are conducted in the same general manner. Once a suspected stain has been either swabbed or a piece of the stain cut, the reagent is added first followed by hydrogen peroxide. If the color change occurs prior to the addition of the hydrogen peroxide, it is considered inconclusive. If the expected change occurs immediately or shortly after adding hydrogen peroxide, it is considered positive.

While KM is suitable for testing localized stains, it can't be sprayed over a large area, especially when looking for latent blood. Additionally, it can impact downstream DNA testing if applied directly to evidence. Benzidine derivatives are suspected carcinogens and are more susceptible to false positives. Fluorescein requires additional equipment to visualize the stains, and Leucomalachite green isn't always sensitive enough. Thus, luminol is one of the best general choices to be used in crime scenes suspected of containing latent blood.

2. LUMINOL

2.1 Chemical structure of luminol

Luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione or 3-aminophthalhydrazide) is a cyclic acyl-hydrazide. It possesses a yellow crystalline solid form, is stable at room temperature, sensitive to light, and combustible. It is incompatible with oxidizing agents, strong acids and bases, and reducing agents and has the property of emitting light upon reacting with oxidizers, also known as chemiluminescence (10). While irritating to the eyes and respiratory tract (13), it has been found to pass through mice leaving them unharmed (10). Luminol is sensitive to both light and heat, which can cause it to "expire" faster, rendering it unusable. It has two distinct pKa values of 6.74 and 15.1 that correspond to the loss of the two acyl-hydrazide protons, respectively. In an aqueous solution of luminol, LH2 can be found fully protonated in acidic solutions and in basic solutions can be found as a mono or di-anion (Figure 1). It's the mono cation and mono anion that can undergo keto-enolic tautomerization in a solution and solid state.

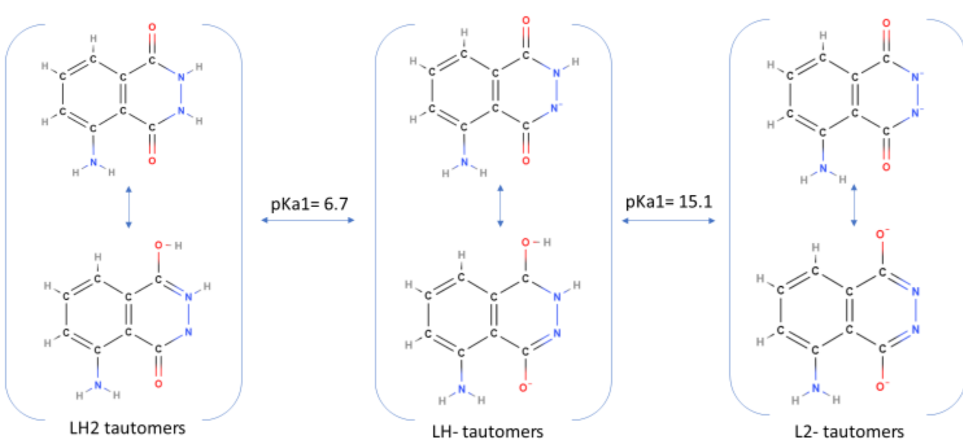

Figure 1: Luminol protonation and tautomerism in acidic, neutral and alkaline conditions, respectively (LH2, LH- and L2). These represent the diprotic, monoanioic and dianionic forms of luminol. Adapted from Barni (10)

Luminol is sensitive enough to detect blood that is no longer visible to the naked human eye. This makes it ideal to spray over a suspected area in a dark room to look for blood. Different formulations have been made throughout the years. Grodsky (14) and Weber (15) are the most commonly employed, and Bluestar® Forensic is a derivative of luminol that is advertised as more potent in comparison to these formulations.

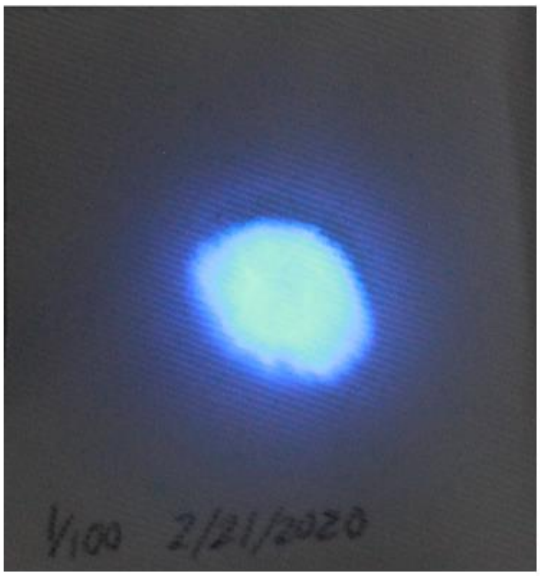

Figure 2: 1:100 blood dilution reacting to luminol.

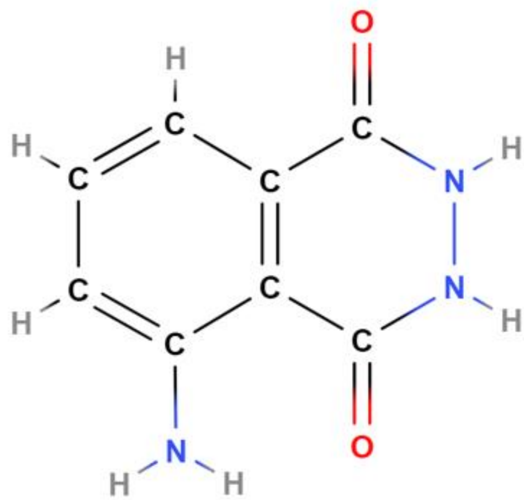

Figure 3: Luminol molecular structure.

The Grodsky formulation is well represented in the literature (6,10,12–14,16–24). It involves mixing sodium perborate, luminol and sodium carbonate in deionized water. Seashols et al. (13) proposed a modified Grodsky formulation involving two solutions, Solution A and Solution B, which can be kept separate and mixed when ready for use (10). Solution A is made with sodium perborate dissolved in distilled water, while Solution B is luminol and sodium carbonate dissolved in distilled water. The Weber (10,15) formulation utilizes separate volumes of Sodium Hydroxide dissolved in water, hydrogen peroxide in water and luminol dissolved with sodium hydroxide in water (25). These 3 stock solutions are then mixed to create the working solution (10). Bluestar® Forensic, while unknown in composition, comes in tablets that can be dissolved in water and placed in the appropriate spray bottle for use (26).

Table 1: Luminol and Bluestar® Forensic formulation preparation.

Grodsky	Weber	Bluestar® Forensic
Weight out 3.5 g of sodium perborate.	Weigh out 8 g sodium hydroxide and completely dissolve them in 0.5 L of deionized water to obtain a 0.4 N solution.	Open Spray bottle; add 125 mL (4 fl. oz) of distilled water.
Add 0.5 L of deionized water.	Measure 10 mL of 30% hydrogen peroxide and add them to 0.49 L of deionized to obtain a 0.176 M solution.	Add a pair of BLUESTAR® FORENSIC tablets. If you need more working solution use 125 mL (4 fl. oz) per pair of tablets.
Stir and Mix until sodium perborate is dissolved.	Weigh out 0.354 g of luminol completely dissolve them in 0.0625 L of 0.4 N sodium hydroxide solution to obtain a final volume of 0.5L (0.004 M).	Mount the plunger onto the spray bottle head and screw on the head of the bottle firmly.
Add in sequence, 0.5 g of luminol and 25 g of sodium carbonate.	Store the 3 stock solutions in glass or plastic containers at 4 C away from direct light.	Allow about 1 to 2 minutes for complete dissolution and mixing of chemicals.
Stir and mix until complete dissolution.	Prepare the test solution by mixing 0.01 L of each of the three solutions to 0.07 L of deionized water to obtain 0.1 L of final working solution.	Stirring gently with a circular motion of your hand.
Decant solution into vaporizer or sprayer and use immediately.	Decant solution into vaporizer or sprayer and use immediately.	DO NOT shake container upside down.

2.2 History of luminol

Luminol's discovery is most commonly attributed to a German scientist named Schmitz around 1908 (10). However, Albrecht is recognized and accepted to be the first to report its chemiluminescence reactions in 1928 (10). Specht, while working in the University Institute for Legal Medicine and Scientific Criminalistics of Jena, Germany studied the role of hemin, a compound containing iron and derivative of heme, and how it reacted with luminol and its potential application in detecting blood (10,27,28).

Proescher et al. (29) predicted the keto-enolic tautomerization of luminol in alkaline solutions as well as how it becomes fully protonated in acidic solutions. They were also among the first to determine that when reacting with dried and decomposed blood, the reaction intensity increased as compared to fresh blood. They also noted that they could re-apply luminol to blood stains for repeated chemiluminescence.

McGrath evaluated the specificity of luminol for biological fluids and found it was very specific for blood and insensitive to other fluids. Nonetheless, he recommended following up with a confirmatory test (10,28).

In 1951, Grodsky proposed his own formulation adding sodium carbonate (Na_2CO_3) and Sodium Perborate ($NaBO_3 * nH_2O$) to luminol mixed with distilled water (10,14). According to the original paper, the adoption of sodium perborate would be advantageous over hydrogen peroxide because the concentration of hydrogen peroxide could be detrimental if it was too little or too much.

The luminol reaction mechanism wasn't immediately apparent when the chemiluminescence reaction was first discovered. Merenyi et al. (30) published a paper in 1981 detailing the luminol reaction mechanism. Additionally, Merenyi et al. released another article detailing the mechanism of action for luminol to become 3-APA in the presence of hydrogen peroxide and heme (31).

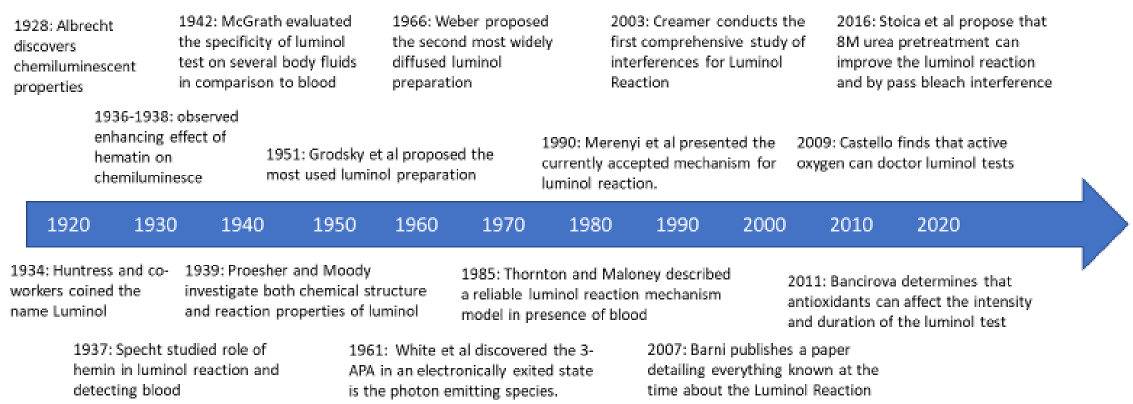

Figure 4: Luminol timeline including recent developments. Adapted from Barni (10) with additional information.

2.3 Environmental impact on luminol testing

Pitarch et al. (32) investigated how environmental conditions and the substrate on which the bloodstain is deposited can impact testing with luminol, KM and LMG. The substrates tested included steel, iron, azulejos (a type of ceramic tile), denim, synthetic cloth, towels, cotton and paper. Conditions were underwater, open air covered and uncovered, buried in soil and in ambient laboratory conditions. Samples were left in the conditions for a period of time between 5 to 125 days and periodically collected for testing. Luminol generally performed well across substrates and environmental conditions. The azulejos, denim, towel, cotton all performed equally well across conditions, having positive test results with Luminol all the way to day 125, although the azulejos were not buried or left underwater due to composition issues. Synthetic cloth only had negative results at day 80 and above when buried. Iron proved to be a poor substrate for retaining detectable bloodstains underwater, only obtaining positive results up to day 5, and exposed in the air uncovered, obtaining positive results up to day 25. Paper proved to be poor underwater and buried in soil, only obtaining positive results up to day 40. Overall, luminol performed well across environments and substrates, outperforming LMG and KM in every scenario.

2.4 Specificity and sensitivity of luminol

Luminol's specificity and sensitivity have long been studied since it began being applied for forensic testing (14). There have been varied limits of detection reported across studies. This can be attributed to factors such as the criteria for a positive result, how the chemiluminescence was quantified if at all, how the samples were prepared, substrates utilized, etc. The following studies compared how specific or sensitive luminol can be.

In 2000, Quickenend and Cooper (33) investigated how they could improve the specificity of the luminol test for blood. They compared the chemiluminescence of blood with the chemiluminesce of bleach. Spectral measurements of the luminescence required the use of a Barr and Stroud CGS2 interference filter, equipped with a microprocessor driven motor in order to be rotated; this allowed for the emited light to be monochromated. The light detector used was an EMI 9635 QAM photomultiplier tube. The signal generated by this was converted to digital and recorded on a computer using a Pico Technology ADC-10 analogue to digital converter. (33). Results demonstrated that luminol reacting with blood generated a peak intensity at 455 ± 2 nm while bleach peaked at 430 ± 3 nm, making them distinguishable.

In 2001, Quickenden et al. (21) investigated how common interferents affect luminol. They used the same set up from their previous study with modifications. The microprocessor was adjusted to scan at 2.5 second intervals, and adjustable gain settings were placed on the operational amplifier, increasing maximum sensitivity 100-fold (21). They found that enamel paint, turnip, parsnip and horseradish produced a strong positive reaction. Fruit and Vegetables including carrot, onion, pumpkin tomato, potato watermelon and banana as well as machine oil, jute, sisal, terracotta, stone and vinyl flooring produced weaker reactions of varying strength. It is recommended by the authors that investigators be aware of what surface the possible blood is on and take a surface sample to test as a blank, while taking note of any possible surface coatings.

In 2006, Webb et al. (34) conducted experiments to compare the performance of BlueStar Forensic® with luminol. The first set consisted of a comparison across substrates and blood dilutions. Substrates included carpet, linoleum, floor board, dry wall, brick, oak, tiles and wood. Blood dilutions were prepared between 1:100 to 1:1,000,000. Two groups were made: a group kept in the laboratory in air conditioned room, while another group was in a non-air conditioned room during summer where temperatures reached 90 degrees Fahrenheit. Stains were made and aged into groups of 4 days, 4 weeks and 7 weeks. The 4 day old stains at dilutions over 1:100,000 were not visible except on dry wall and tile. Luminol performed better on stains from the hot room than the cool room while BlueStar® showed no difference based on temperature. The 4 week study found that stains 1:10,000 and beyond did not produce positive results except for on drywall and tile. The 7 week study found that stains 1:10,000 and above did not produce positive results except on carpet and pre-treated flooring. Age of the stain did not impact results otherwise. BlueStar® outperformed luminol in its ability to detect more dilute stains.

In 2006, Webb et al. (6) investigated how KM, LMG, Hemastix® and Polilight® compared with luminol in terms of sensitivity, safety and toxicity as well as aging of bloodstains. All presumptive tests worked well though luminol proved to be the best in terms of sensitivity reaching 1:5,000,000 in 50 µL bloodstains, blood-soaked cloth and solution, followed by Hemastix® and KM with 1:1,000,000 and 1:100,000 respectively. LMG followed by Polilight® were the least sensitive with 1:1,000 and 1:100 respectively. Polilight® is quite cumbersome to use given the size of the equipment in comparison to a spray bottle or plastic cartridge. Overall, the best choice for a presumptive test was determined to be luminol, followed by Hemastix®.

In 2013, Seashols et al. (13) compared Hemascein®, luminol and BlueStar® Forensic, with regard to sensitivity and specificity on various surfaces. Additionally, blood treated with EDTA was compared to blood that wasn't treated and with synthetic blood in order to establish the viability of using blood preserved with EDTA in future studies. Hemascein® obtained 1:1,000,000 limit of detection on cotton, 1:500,000 for linoleum and over 1:10,000 in nylon. Wood, however, was under 1:10. Luminol reached 1:10,000 in wood and outperformed BlueStar® Forensic by reaching up to 1:100,000 on linoleum. Cotton and nylon both peaked in sensitivity at 1:1,000. Bluestar Forensic outperformed luminol with sensitivity on wood but they performed equally on fabrics. Synthetic blood reacted with the tests, however concentration had to be much higher to get a positive result. EDTA had no negative impact in obtaining a positive result, though sensitivity could be impacted slightly with Bluestar Forensic. Bleach cross reacted with every test and Hemascein® cross reacted with kidney beans, onion, tomato, tomato sauce, floor, carpet and bleach cleaners. All three reagents proved to be highly sensitive but that can be impacted by the substrate, with Hemascein® being particularly sensitive to auto-oxidizing blood samples, possibly due to over spraying.

In 2017, Cassidy et al. (35) designed experiments to determine the limit of detection for presumptive blood tests. A cotton swatch was placed on an embroidery ring and coated with a polyvinyl chloride cement (PVC) in order to concentrate the stain into a defined area. A blood dilution series consisting of 40 samples was prepared using HPLC grade water. The Bluestar® Forensic was made, frozen using liquid nitrogen and subsequently thawed for use in order to maintain chemical activity. The procedure to quantify the chemiluminescent reaction was accomplished through the use of a charged-coupled device camera; long exposure raw images were then evaluated quantitatively for pixel intensity. A Nikon D80 camera with a Nikkor 50 mm f/1.8D AF lens was mounted on an adjustable height optical support rod secured to an optical breadboard. The photographs were converted to DNG. format and imported into MATLAB®. An average background pixel intensity was taken from non-reaction photographs in order to determine the specific value of the chemiluminesct pixels, which were blue due to the luminol emission around 425 nm. The limit of detection was determined to be approximately 1:1,000,000 of blood in HPLC grade water. It was determined that intensity of the chemiluminescent reaction was relative to blood concentration, thus making even a weak signal merit further testing.

In 2018, Stene et al. (12) investigated how long could blood be detected with luminol when exposed to soil. A section of the local law enforcement training facility, which would have limited access to outsiders, was chosen. A 2x2 foot section was poured with 500 mL of horse blood in an X pattern. During the first two years, luminol would be sprayed every two months, and subsequently they tested every 2 years; positive results were obtained every time. After 16 months, surface soil had to be removed to expose the blood, as it would sink to lower strata. The study confirmed that blood can persist on soil exposed to the elements. The authors stressed that the area for testing should be narrowed down to more likely areas before testing and that no DNA testing was conducted.

In 2018, Polacco et al. (36) investigated quantifying the chemiluminescent reaction of ovine blood. They did so through a dilution series of both fresh and dried blood and used a Spectamax M3 multimode microplate reader. Samples were read every 4 seconds for a duration of 3 minutes. The instrument allowed them to assign a numerical value to the luminescence, in relative luminescence units or RLU. When evaluating fresh blood, neat blood samples had a lower RLU value than the others when it came to the maximum chemiluminescence. There was a drop in signal at 1:100,000 though there was still a detectable signal by the instrument, but not by the photographs obtained. The fresh neat blood having a weak signal could be explained because the red blood cells are intact and the iron present in heme has yet to become ferric or ferryl, which catalyzes the reaction of luminol more efficiently. This explains why the dried neat blood had a much higher maximum chemiluminescence in comparison. There was a delay between 12 to 32 seconds depending on concentration to reach maximum luminescence, but this can be attributed to the dried blood needing time to rehydrate in order to have the components available to react.

While limit of detection for blood using luminol varied across studies, an important factor to consider is the lack of standardized quantification of chemiluminescence across studies. For example, a 1:5,000,000 sample rendered a positive result in the study by Webb (6), but it did not specify how this positive result was measured. Cassidy (35) used a CCD camera with a sophisticated program in order to evaluate resulting photographs and quantify the chemiluminescence. This may suggest that the latter study demonstrates a more accurate limit of detection for luminol (1:1,000,000). It's also worth noting that these were conducted under ideal laboratory conditions, which will inevitably stray from what is found in a crime scene.

2.5 Luminol Reaction

In order to better understand how interferents impact luminol, it is important to first understand how luminol works. It is a complicated multistep reaction (Figure 5). Luminol is a stable molecule, however by placing it in contact with an oxidant such as hydrogen peroxide, it becomes oxidized. The new molecule is now unstable enough to react and forms 3-aminophthalate in an excited state. This is the molecule that luminesces, and the luminescence slowly dies out as the molecule returns to its ground state.

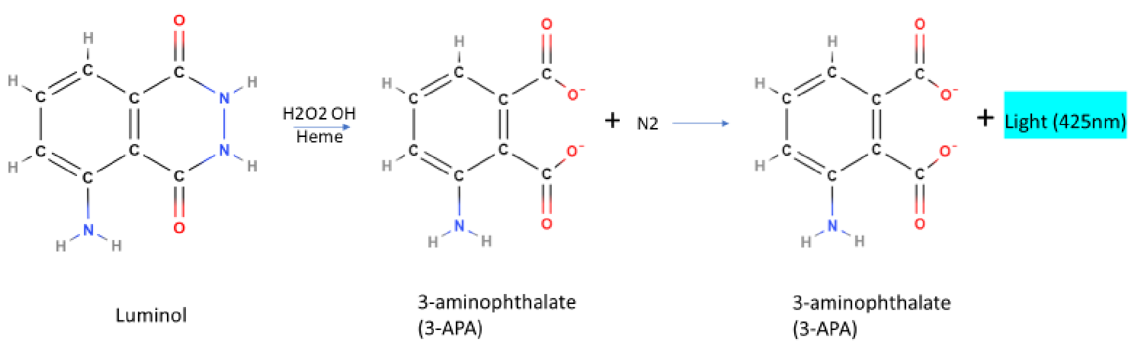

Figure 5: Luminol reaction. Adapted from Barni (10)

Weber's formulation directly uses hydrogen peroxide as part of the solution that is sprayed over the area of interest. However, Grodsky's formulation uses sodium perborate to act as the oxidizer while sodium carbonate is added to dissolve the luminol in solution (10,14). Sodium perborate contains hydrogen peroxide in it's chemical composition, while sodium percarbonate forms hydrogen peroxide, sodium ions and carbonate when placed in water (37). Factors such as pH, temperature and ionic strength of the reaction medium along with the reactive species present have a direct impact in how the reaction proceeds. White et al. (38) were the first to propose that 3-APA in its excited state perfectly matched the chemiluminescent spectrum of luminol. When luminol is in a solvent such as DMSO or H_2O along with a mild to strong oxidant like H_2O_2 and a catalyst that contains a metal ion such as copper or iron, 3-APA is formed in an excited state that returns to its ground state by releasing light. The mechanism of action for luminol turning into 3-APA has been previously reported (24) and is detailed in Figure 6.

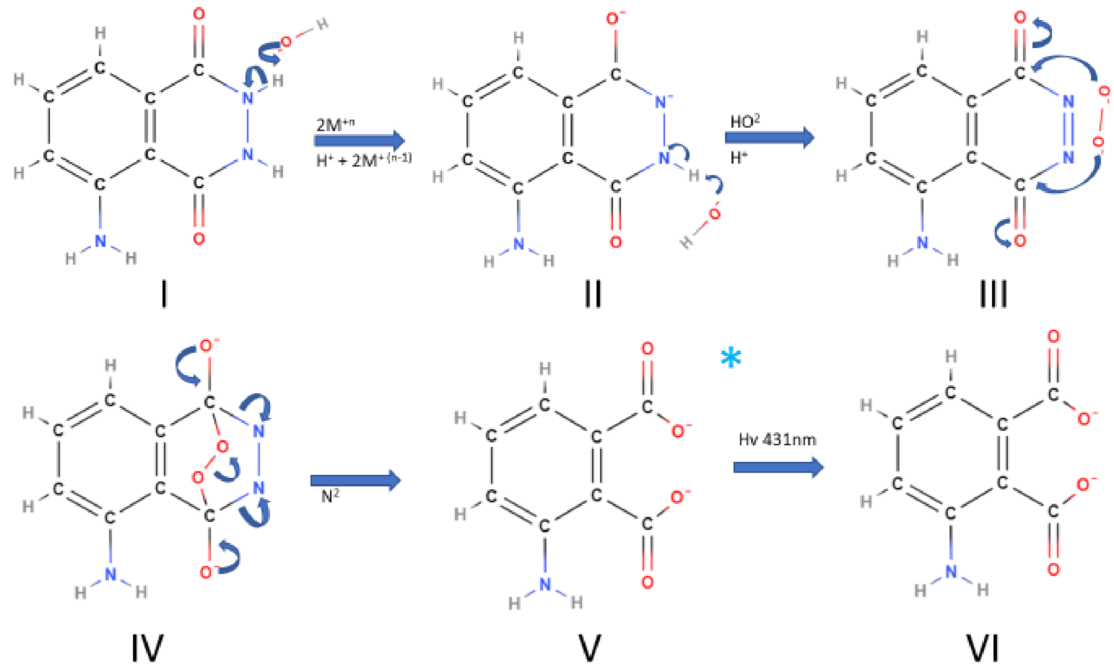

Figure 6. Luminol mechanism of action detailed. Adapted from da Silva (24)

I. Luminol in its initial state as it is applied
II. Luminol becoming oxidized by hydroxide
III. Diazoquinone formed after oxidation
IV. Endoperoxide formation
V. Formation of 3-APA which emits light(*) as it decays into its stable form VI

Hydroxide formed from the intereatction between heme and hydrogen peroxide (Figure 7) interacts with luminol, first removing the hydrogens from nitrogen before hydropexoyl attacks the carbons in order to remove the nitrogen molecules forming N2 and 3-APA. The color of the light varies between blue-violet to blue-green depending on the exact components, but overall tends to be observable at about 425-455nm range. While the exact role of the catalyst is not fully understood, it is known that similar transition metals can carry out the same function and may be dependent on the pH of the reaction in order to proceed.

Hemoglobin possess an Iron (Fe) molecule in its structure. This iron transforms from Fe2+, as it is found ordinarily in blood, to Fe3+ as it is found outside of blood. By becoming 3+, it can bond with OH instead of O_2 as it does inside the body (24,39). This helps turn H_2O_2 into OH (40) which oxidizes luminol and allows for it to interact with reduced H_2O_2 that converts Diazaquinone into 3 aminophthalate, the chemiluminescent product of the luminol reaction.

2.6 Interferents

Any chemicals that disrupt the luminol reaction can be considered interferents. These can be cleaning agents, biological agents, foods, and drinks, among others. In 2003, Creamer et al. (41) conducted a study of industrial, domestic and environmental chemicals that may interfere with the luminol test. In all, 250 different chemicals or substrates were evaluated, and chemiluminesce was measured utilizing a CCD camera with photomultiplier tube (EMI 9635 QAM) and calibrated circular graded interference filter (Barr and Stroud CGS2), and compared to blood. Copper, enamel paint, sodium hypochlorite, dark green spray paint, turnip, parsnip, horseradish, roof lining of certain automobiles and furniture polish generated a signal strong enough that could be compared to that of blood. The mean intensity assigned to blood was 236 ± 12 arbitrary units after 8 replicates, while the values for the chemicals tested ranged from 255 to 44 .

It is apparent from the literature that cleaning products used to remove blood at crime scenes could be preventing subsequent blood detection in one of two ways. Either the bloodstains are in fact being cleaned and completely removed or the cleaner is disrupting the components of the reaction, leading to a false negative despite the presence of residual blood. Castello demonstrated that while the luminol test can be rendered negative in the presence of certain substances (16,19,42), evidence can still be obtained (17,43). As such, it is important to recognize how these interferents work.

Cleaning agents are any common household products that are used to clean floors, walls and other surfaces in the home. They come in a wide array of formulations and potencies for a variety of surfaces. Sodium hypochlorite, also known as bleach, sodium percarbonate, also known as active oxygen, and hydrogen peroxide are commonly used as primary cleaning products or as components in popular cleaning brands. Such multisurface cleaners, extra strength detergents or other chemicals could be found in any home and may be quickly accessible to someone attempting to clean up a crime scene.

2.6.1 Sodium hypochlorite

Bleach, or sodium hypochlorite, is a very common household cleaner and strong basic substance ($pH>11$) (44). It is used in many homes around the world for its potency in removing stains and smells, as well as being an antimicrobial agent (44). Sodium hypochlorite can generate several reactions including saponification, neutralization, and chloramination. Saponification involves the conversion of fatty acids into soap and glycerol. Neutralization occurs when amino acids are neutralized, forming water and salt. Chloramination is the production of chloramine when amino acids and hypochlorous acid interact with sodium hypochlorite. Through these three key reactions, sodium hypochlorite makes for an excellent cleaner that can remove materials on surfaces, act as an antimicrobial and leave a clean smell behind.

In 2005, Creamer et al. (45) investigated the impact bleach has on bloodstains when using luminol. Wet blood and dried bloodstains were compared, with dry stains being more difficult to remove. The cleaning involved using paper towels soaked in either water or bleach and swiping once to clean glazed terracotta tile, each swipe being considered a clean, for a total for 14 "cleanings". The instrumentation utilized by Quickenden and Cooper (33) involving the CCD camera with an interference filter and photomultiplier was used to obtain quantitative measurements of the chemiluminescece. It was found that cleaning with water repeatedly lowered the chemiluminesce intensity with increased washes. Bleach was found to initially generate a drop in chemiluminesce which went right back up and leveled off as more cleaning was conducted. The authors concluded that bleach could generate chemiluminescent reactions that can't be distinguished from blood.

In 2006, Webb et al. (34) conducted three experiments to compare the performance of BlueStar Forensic® with luminol, the latter two involving sodium hypochlorite and how bleach would impact testing. Blood dilutions were deposited on various substrates, and pure Clorox bleach was used to clean the dried stains until they could no longer be seen. Substrates included carpet, linoleum, ceramic, red brick, dry wall, oak, plywood, pine, oak and white wall paneling. Four separate groups were made: control groups of blood being tested with luminol or BlueStar® and groups cleaned with Clorox followed by testing with luminol or BlueStar®. Blood was not detected on any of the substrates in the 1:100,000 and 1:1,00,000 dilutions. In nearly all samples, the luminescence of BlueStar® lasted longer than that of luminol. When bleach was used, a sparkling effect appeared in the wipe pattern. Substrates treated with bleach had weaker results than those that didn't contain bleach. The author determined that bleach had a destructive effect on the blood, especially with lower concentrations of blood, although BlueStar® was better than luminol at detecting blood when bleach was used. The difference in luminescence made it easier to find blood, however , this was only useful when the concentration of blood was higher (1:10 and 1:100 blood dilutions). The final experiment evaluated whether glycine could be added to reduce bleach interference; however, the author did not elaborate why this compound was chosen. Stains were made in a serial blood dilution between 1:10 to 1;1,000,000 and aged between 1 week and 6 weeks. Substrates included drywall, plywood, subflooring, wooden boards, floor board, carpet, paneling and linoleum. Control tests were peformed on blood treated with luminol or BlueStar® that was mixed with glycine. The other tests were conducted on substrates stained with blood and then cleaned with bleach. After cleaning until blood could no longer be seen, the substrates were tested with luminol or BlueStar® mixed with glycine. Glycine did not inhibit the tests' ability to react with blood in the control samples. The addition of glycine caused the reaction with bleach to dull after a few seconds, which allowed for the blood reacting to the the test to be more easily observed. The pH of the solution had to be evaluated prior to usage and converted to 12, which may deter the application of this technique.

In 1991, Anrhold et al. (46) investigated how hydrogen peroxide amplifies chemiluminescence of luminol when reacting to sodium hypochlorite. Similar to Merenyi's (30,31) previous assertion that luminol is oxidized into diazoquinone, which then reacts with hydrogen peroxide to form an endoperoxide that decomposes into 3-APA, a comparable pathway was assumed for luminol's reaction to hypochlorite. Arnhold discovered that while the reaction may occur without adding hydrogen peroxide, adding increasing concentrations of hydrogen peroxide led to increased chemiluminesce. A linear relationship was established between chemiluminescence and the concentration of hydrogen peroxide. Anrhold posited that in order for the chemiluminescece to occur hydrogen peroxide must be included .

In 1993, Arnhold et al. (47) further investigated two mechanisms for inhibition of the luminol reaction by stopping the oxidation of luminol when sodium hypochlorite is present. One mechanism involved the action of catalase, a specific protein found in blood that removes hydrogen peroxide from red blood cells, which was further researched by Nagababu (48) in 2003. The second mechanism involved preventing hypochlorous acid (HOCl/OCl-) from oxidizing luminol into diazoquinone. Without diazoquinone, there is nothing to react with luminol to form the chemiluminescent species. They further determined that hydroxyl radicals, (-OH) aren't involved in the oxidation of luminol when hypochlorous acid is present. Hydrogen peroxide is still a necessary component for the reaction, and it is formed in small amounts in a side reaction during the oxidation of luminol.

Anrhold et al. (47) explained how bleach (Figure 8) causes luminol to chemiluminesce is that it replaced hydrogen peroxide with O-Cl in the oxidation of luminol. Normally, heme and Hydorgen peroxide are required to generate the O-H which will then react with luminol (Figure 7). The hypochlorite ion allows for the reaction to start, then hydrogen peroxide proceeds in turning luminol into 3-APA, which luminesces (Figure 8). This also may explain why the reaction can be brighter and quicker than when blood is present, as the sodium hypochlorite is more available to react. For the reaction to proceed under normal conditions with blood and hydrogen peroxide, heme would have to react with hydrogen peroxide to form hydroxide and hydroperoxyl ions. The reaction would be dependent on the amount of these ions present in the mixture. The hydrogen peroxide depends on the heme to catalyze the reaction, resulting in a slower reaction that lasts longer.

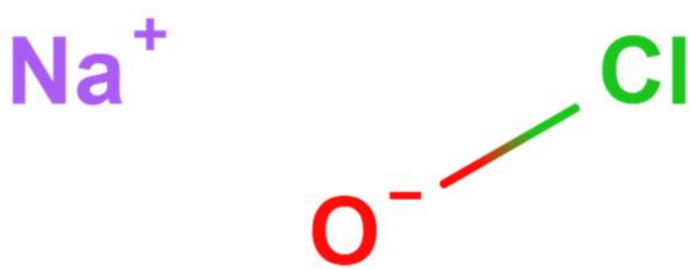

Figure 7: Sodium hypochlorite

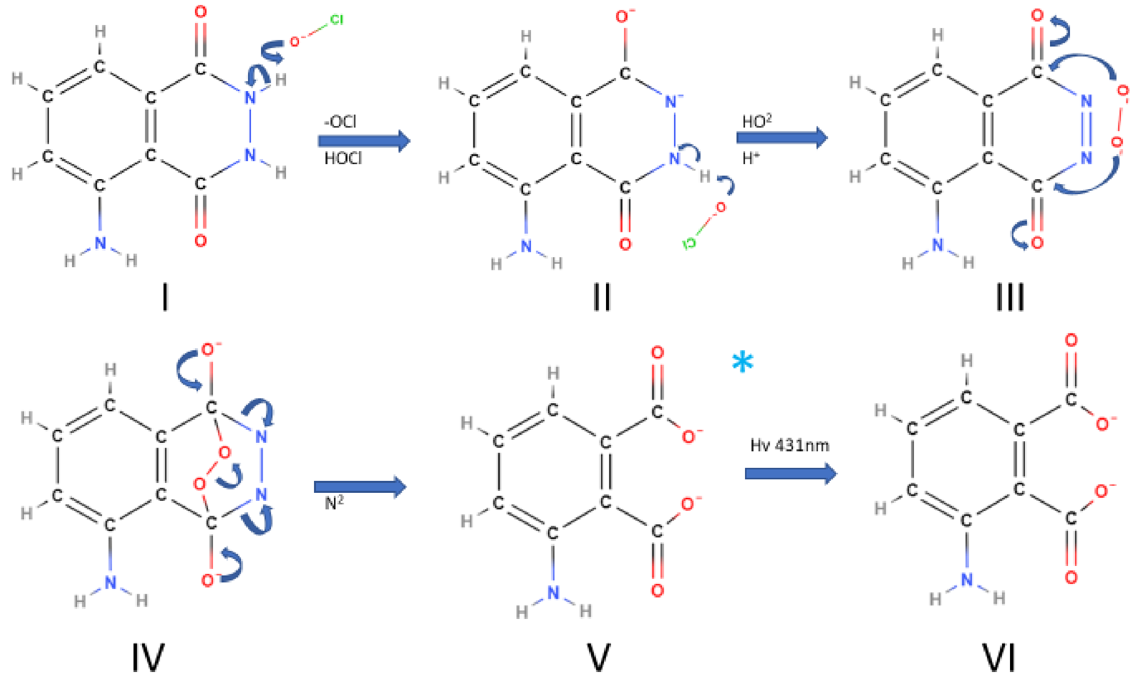

Figure 8: Possible mechanism by which sodium hypochlorite oxidizes luminol.
Adapted from da Silva (24) and Arnhold (46)

I) Luminol in its initial state as it is applied
II) Luminol becoming oxidized by hypochlorite
III) Diazoquinone formed post oxidation
IV) Endoperoxide formation
V) Formation of 3-APA which emits light as it decays into its stable form VI

*Excited state of 3-APA that emits light

Castello et al. investigated how bleach interference in luminol can be impacted by a porous surface (22). Two experiments were done. In experiment 1, only bleach was added to porous brick and cotton fabric then placed outdoors or inside the lab at room temperature. It was checked 1, 3, 5 and 8 days after being in those conditions with luminol. By day 5 it was consistently negative on all, however, bleach may disappear after allowing to dry for a period of time when used on a porous surface. Experiment 2 had bloodstains cleaned with bleach on brick and cotton fabric as in the previous experiment, with the exception of only being held indoors. The positive results on the control were instantaneous while the positive results on the treated samples were either instantaneous or slower. The results of the problem samples in experiment 2 demonstrated that there were two kinds of luminescence: one instantaneous, bright and persistent and one slower to appear, less bright and persistent. The first is attributed to bleach because it was located in a spot that did not contain blood while the other is attributed to blood. Castello concluded that waiting a period of time for the bleach to evaporate deteriorate before testing may be warranted when it's use as cleaner is suspected.

2.6.2 Sodium percarbonate

Sodium percarbonate is also known as active oxygen and is used in detergents to improve their stain removing capabilities (49). It has been found to affect the luminol and Bluestar® Forensic tests by causing a negative result, even in the presence of blood.

Castello et al. (16) discovered that active oxygen/sodium percarbonate containing detergents rendered the luminol, phenolphalein and hemoglobin tests negative for blood. Samples were prepared with 3 substrates (cotton, jeans, and towels) using fresh blood with and without EDTA. At different time intervals between 1 to 40 days, samples would be cut out and washed with a product containing active oxygen (Neutrex brand). The washing step was conducted by adding the product into hot water and leaving the fabric in this mixture for two hours. Subsequently, the fabric was rinsed and left to dry for one day. The stains were visible with the naked eye and UV lights before conducting testing. All control samples gave positive results while all washed samples were negative, regardless of the visibility of the stain. Additional assays were conducted with hot water (40 degrees celcius) without detergent and with cold water and active oxygen detergent. This was done in order to better ascertain the role of active oxygen in the negative test results. Samples without active oxygen were positive and samples with active oxygen despite the cold water were negative. A slight luminescence and formation of a blue line indicative of hemoglobin were noticeable after the time limits set for testing, which wouldn't be considered a positive test result. Detergents that contained sodium percarbonate, along with washing with hot or cold water, prevented bloodstain samples from generating a positive result. Castello concluded that it may be possible that this inhibition was due to the action of hydrogen peroxide, which is generated when sodium percarbonate is dissolved in water, and it's ability to exhaust heme's capacity to breakdown hydrogen peroxide.

A follow up study by Castello et al. examined the impact of sodium percarbonate on DNA analysis (43). Samples were prepared just as the previous study (16) and washed identically. Once results from KM, luminol and Bluestar Forensic were negative, DNA testing was conducted. DNA extraction was conducted via the phenol-chloroform method, and DNA quantification was performed using 2 methods, a horizontal electrophoresis in agarose and then pectrophotometry with a NanoPhotometer. PCR amplification was conducted with the AmpflSTR Profiler PCR amplification kit and run on a GeneAmp PCR System 9700 thermal cycler. Lastly, electrophoresis was run on an ABI PRISM 310 Genetic Analyzer and analyzed using GeneScan and Genotyper. All presumptive tests including human hemoglobin were negative. However, DNA was successfully extracted regardless of some degree of degradation for the samples washed in sodium percarbonate. Time had little impact on the samples, with no specific pattern emerging in DNA concentration. On average, a DNA concentration of 37.8 ng/µl was obtained from the control group while the problem group had an average of 6.4 ng/µl. Castello concluded that while active oxygen can inhibit presumptive testing, DNA testing is largely unaffected, though being unable to identify latent bloodstains can lead to evidence being lost.

Castello et al. followed up their previous research from 2009 by testing sodium percarbonate directly on bloodstains and washing them (19). They compared phenolphthalein, luminol Grodsky formulation, Bluestar® Forensic and Hexagon OBTI using bloodstains washed in hot or cold water, with or without sodium percarbonate. They also evaluated how the concentration of sodium percarbonate impacted the test, starting from the concentration on the detergent label (30%) and halving it into 15%, 7.5%, 3.75%, and 1.875% dilutions. They found that the sodium percarbonate interferes with the reactions at 30% concentration. Water temperature is an important factor, with cold water rendering a positive reaction for every test regardless of the concentration of sodium percarbonate Hexagon OBTI was the exception to this with 30% concentration of sodium percarbonate in cold water rendering a negative result. Hot water was necessary for the sodium percarbonate to be effective, however the concentration needed to be 15% or over to inhibit the presumptive testing. At 7.5% concentration, luminol and BlueStar® Forensic begin to generate positive results. Phenolphthanlein and Hexagon OBTI had positive results in hot water at 1.875% concentration of sodium percarbonate . These results suggest that it's the combination of hot water and sodium percarbonate that can render these tests negative, even if the stain is still noticeable, allowing for evidence to be disposed of because of the negative result.

Oldfield et al. (18) investigated how sodium percarbonate impacts the luminol (Grodsky) test. Four tests were devised to accomplish this. Experiment 1 involved denim stained with blood being washed with detergent that was either biological or non biological in nature at various temperatures (10, 40 and 60 degrees Celsius), with and without sodium percarbonate. Experiment 2 involved the same except it was done to carpet and the washing was done with neutral detergent and scrubbed by hand. Experiments 3 and 4 examined aged bloodstains on denim and carpet, respectively. The stains were aged one, four, seven and fourteen days. The sets of stains were either exposed to lab or environmental conditions. Experiment 1 demonstrated that without sodium percarbonate, detergents did not disrupt the luminol test. Temperatures of 60 degrees were needed along with sodium percarbonate for a majority of samples to generate a negative result. Experiment 2 demonstrated that a neutral detergent with sodium percarbonate had no effect on luminol testing after washing regardless of temperature. Experiment 3 suggested that there is no trend with regard to how long a stain was left to dry on denim before being washed other than it aided in removal of the stain during wash. Samples that had been exposed to the outside environmet had the presecence of detectable blood greatly reduced. Experiment 4 demonstrated that reduced detection of blood on catpet occurred with drying times longer than one day. Additional days, however, had no increased effect. Overall, for sodium percarbonate to disrupt the luminol test when in contact with washed bloodstains, the temperature had to be at least 40 degrees Celsius during the washing. Sodium percarbonate interfered more with the tests on denim because that could be washed at 60 degrees and with various detergents. The carpet could not be washed at 60 degrees Celsius and had to be hand scrubbed with a neutral detergent which also lacked hydrogen peroxide and sodium percarbonate

2.6.3 Hydrogen peroxide

Hydrogen peroxide is a common disinfectant, used to clean cuts and small wounds as part of first aid. It is a necessary component of most presumptive blood tests, however, bulk quantities of it in the luminol reaction stop the reaction from proceeding. This has been noted by Castello (16) and DiCarlo (26). Lysol with hydrogen peroxide rendered tests run by DiCarlo as negative or rather that the chemiluminescent reaction is too weak to the human eye to be considered positive. Preliminary tests conducted as part of this thesis were consistent the results presented by DiCarlo, as shown in Figure 10. Upon further research, Nagababu (48,50) wrote two papers concerning how heme can be degraded by excess hydrogen peroxide. Normally blood is protected from this degradation process by the enzymes catalase and glutathione peroxidase. Outside of blood, red blood cells no longer have this protection; bloodstains that have hydrogen peroxide added to them have degraded heme. This heme can no longer catalyze the luminol reaction, which in turn renders the reaction a negative. DNA could possibly still be recovered as sodium percarbonate still allows for the recovery of DNA despite rendering presumptive blood tests negative. Sodium percarbonate contains hydrogen peroxide as part of its chemical formula, which may explain the similarity of impacting the luminol reaction.

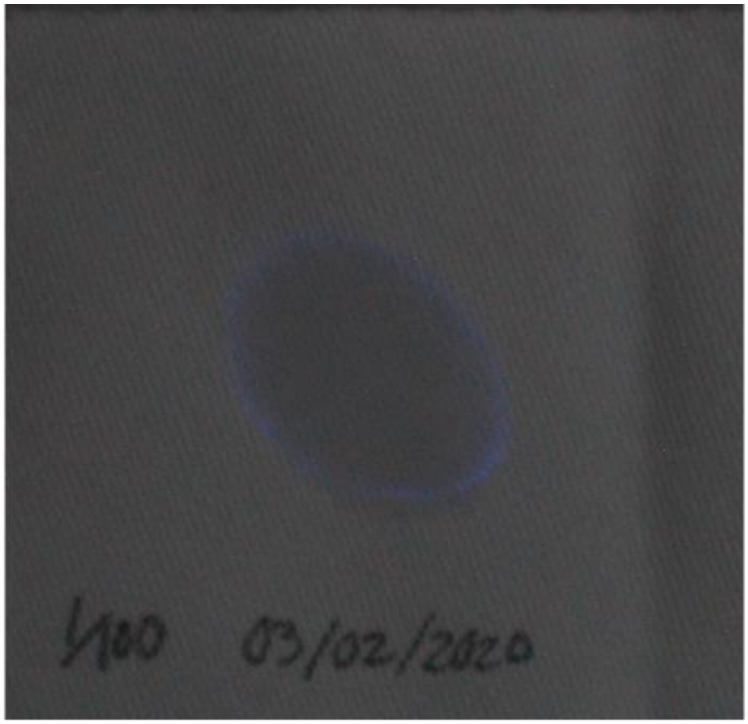

Figure 10: 1:100 blood dilution with hydrogen peroxided added

2.6.4 Antioxidants

Bancirova et al. (51) investigated how antioxidants in green and black tea can impact the detection of blood using luminol. They compared the Grodsky and Weber formulas as well as Bluestar® Forensic. Green tea proved to have more of an impact than black tea. The chemiluminescent intensity was lowered when the tea was present and the reaction was delayed, taking longer to reach a peak. BlueStar® was less impacted, while Weber and Grodsky formulations had a more noticeable impact during the first minute of the reaction. Blood dilutions going from 1:1 to 1:16,777,216 were utilized for comparison. Less than 1:1000 were not visible to the human eye but were detectable by the instrument.

Bancirova et al. (23) continued the previous study by adding white wine and focusing on the Weber formulation and a PBS buffer of pH 7.4. The buffer proved to only weaken luminol intensity by 10 fold. Black and green tea slowed down the reaction and lowered the intensity of the chemiluminescence, while white wine did not negatively impact the reaction, as it is not antioxidant intense enough. Black and green tea can slow down the reaction by 60 seconds, which is critical enough that it may not be considered a positive result.

In the Nagababu study (48), the authors discuss that red blood cells are protected from hydrogen peroxide by catalase and glutathione peroxidase. These are antioxidants, which depending on their concentrations maintain low levels of H_2O_2, preventing heme degradation. It stands to reason that any antioxidant would provide a similar function. Without heme being exposed to the hydrogen peroxide, these two can't convert luminol into 3-APA, or delay the reaction and lower the intensity of reaction. This is consistent with the Bancirova studies (23,51).

2.6.5 Ascorbic acid

Ponce et al. (52) investigated how ascorbic acid impacted O-tol, LMG, TMB and KM. In fresh blood samples, concentrations of 1:2,000 were required for ascorbic acid to render the presumptive tests negative. Dried stain samples had a range of 1:2 to 1:20,000 before the acid interfered with testing. Print samples had a range of 1:2 to 1:2,000, ascorbing acid having an impact starting at 1:20,000. Ascorbic acid may reduce the oxidized reagent, leading to the reaction not happening. Alternatively, the luminol reaction requires an alkaline environment for the reaction to proceed. Hence, this is one of the components that can be disrupted in order to alter the reaction and generate a false negative.

In 2016, Lee et al. (53) investigated the impact of vitamin C and ascorbic acid in delaying the Leucomalachite green reaction to blood. A serial blood dilution was made and tested with LMG, followed by another serial blood dilution with various ascorbic acid concentrations added or beverages with different concenrations of vitamin C added prior to the addition of LMG. The results of these tests showed that LMG can generate a positive result in samples up to 1:10,000 blood dilution. The addition of the ascorbic acid or vitamin C lowered the intensity of the color generated by LMG as the concentration of these interferents increased and the blood dilution was increased. As concentration increased, the reaction would be delayed up to 40 minutes.

2.6.6 Cleaning and tanning products

Multiple studies have investigated whether certain cleaning supplies can interfere with presumptive tests for blood. Ana Castello has contributed heavily to the field, researching how various brands and types of household cleaners cause negative results even if there is blood present.

Table 2: Results of various studies investigating the effects of cleaning products on blood testing.

Cleaner/test	Oxiclean (54)	Lysol Hydrogen Peroxide (26,54)	Arm and Hammer Soda Detergent (54)	Active Oxygen/ Sodium Perborate (16–19)	Quebra-cho (42)	Windex (26)	Bleach (26)	Greenworks (26)
Bluestar Forensic	+	+	+	-	-	+	+	+
Luminol Grodsky				-				
Kastle Meyer				-	-			
Benzidine				-	-			
OBTI Test				-	-			

Adams et al. (54) investigated how Lysol with hydrogen peroxide, Oxiclean and Arm & Hammer impacted Bluestar® Forensic in detecting latent blood. By utilizing ImageJ to detect RGB values in photographs taken of the reaction, a value was determined for comparison. While none of the cleaners were 100% effective in removing blood, Lysol with hydrogen peroxide had the biggest impact by having the lowest difference in RGB values when comparing the chemiluminesce with untreated tiles. The Oxiclean and Arm & Hammer products both contained sodium percarbonate, which Castello (16) had found could render luminol tests negative once a stain has been exposed to it through a washing machine. However, in this study they were found to generate positive results regardless. Because BlueStar® was used, they neglected to use a dark room for testing, which may have impacted results. It is worth pointing out that subsequent studies with sodium percarbonate have also demonstrated that washing the stains along with hot water (60 degrees celcius) has more impact than merely washing them with cold water. Adams concluded that Lysol with hydrogen peroxide is the most effective at removing blood when compared to the others.

DiCarlo et al. (26) investigated how Clorox, Green Works, Lysol and Windex could be used to clean up blood. The experiment involved a quadrant system including one negative control (Q1), one positive control (Q2), one bloodstain wiped once with the cleaner (Q3) and the final bloodstain wiped until it was no longer visible to the human eye (Q4). Presumptive testing for blood was conducted with BlueStar® Forensic. Three surfaces were evaluated: carpet, press-on tile and ceramic tile. Windex and Green Works had no impact on the test, in contrast to Lysol. Quadrant 4 for surfaces cleaned with Lysol with hydrogen peroxide had little or no chemiluminesce. Clorox reacted as expected, having positive results if the Clorox was still present along with the blood. The hydrogen peroxide in the Lysol degraded the heme group in the blood, which is needed to catalyze the Bluestar® Forensic reaction so little or no chemiluminescence was observed.

Castello et al. (42) investigated how quebracho, a plant extract used for the process of tanning leather goods, may interfere with phenolphthalein, benzidine, Bluestar® and Hexagon OBTI. They ran experiments using 2% or 20% quebracho on cotton swatches or 20% on a leather substrate. At 2% quebracho, samples of 1:1 blood could persist for 300 days across every test. Positive results decreased as concentration of blood was lowered in the samples; at 1:1,000 there were no positive results. Samples with 20% quebracho did not yield positive results past 85 days in comparison. Lowering the concentration of blood led to a shorter timeframe from which positive results could be obtained. Overall, higher concentration of quebracho led to a greater interference with the tests. Higher concentrations of blood persisted better than lower concentrations, It's important to take this into consideration when examining leather goods and how long it has been since the blood possibly landed on the leather surface.

2.7 Alternatives to the luminol test

Castello (17) investigated how glycophorin A and human hemoglobin immunoassay testing compared to luminol and Bluestar®. As with previous experiments (16,19,43), four groups were made: hot water with sodium percarbonate (group a), cold water with sodium percarbonate (group b), hot and cold water alone (group c and d, respectively). Group a yielded mostly negatives except for the RSID Blood test, which detects glycophorin A, a protein on the red blood cell membrane. Hexagon OBTI, which targets human hemoglobin, failed to detect anything with group a and b. Test 2 involved blood dilutions (1:50, 1:100, 1:200, 1:400, 1:600, 1:800, 1:1,000) and test 3 tested how concentrations of sodium percarbonate could impact testing. The same groups (a, b, c, d) were made for the blood dilutions and they performed equally in hot water but not as strong at higher blood concentrations for cold water, though 1/200 began showing negative results for the luminol and Bluestar® Forensic. RSID Blood consistently obtained positive results, which could be because it is targeting a different protein than the other tests. Sodium percarbonate may be effective at denaturing the hemoglobin protein, which leads to the

Hexagon OBTI test not detecting it; hence, it was consistently negative across groups and concentrations of blood and cleaner. Glycophorin A was shown to be resistant to the active oxygen in comparison to hemoglobin. Castello posits that because heme has a function with hydrogen peroxide, it will react with it and be degraded by an excess of hydrogen peroxide. Glycophorin A has no function when interacting with hydrogen peroxide and therefore doesn’t react with it..

2.8 Improving the luminol test

Stoica et al. (55) investigated how to improve luminol tests beyond the standard formulations to increase the duration and intensity of chemiluminescence. In order to measure the chemiluminescence, a Genios Tecan plate reader chemiluminometer was used. The chemical additives tested were cyclodextrin derivatives, of which 8 M urea performed the best followed by MCT-beta-cyclodextrin and hydroxy-beta-cyclodextrin. When used as a pretreatment, 8 M Urea led to increased intensity and duration of the chemiluminescent reaction. Additionally, it allows for the removal of the false positive reaction with sodium hypochlorite, and it aided in visualizing stains that could be masked by 30% sodium hypochlorite.

3. CONCLUSIONS

Overall, luminol is a superior tool for detecting latent blood. However, forensic scientists must be aware that bleach and similar compounds can cause false positive results, but these can sometimes be analytically distinguished from a true positive with the right equipment. Likewise, sodium percarbonate, and hydrogen peroxide in higher concentrations can disrupt the luminol reaction and render a false negative. These compounds work by disrupting one or more components of the reaction in order to stop it from occurring. By altering heme to prevent it from catalyzing the reaction, hydrogen peroxide and sodium percarbonate prevent the formation of peroxide ions which are necessary for luminol to be converted into a chemiluminescent species. Other chemical compounds that can either disrupt the hydrogen peroxide or the heme will have a similar effect.

Failure to obtain a positive luminol result after a cleaning product has been used on that surface doesn’t always mean that blood has be successfully removed. The likelihood is that the cleaning agent is actively interfering with the luminol test. As Castello’s extensive research as demonstrated, testing that is not targeting heme can still obtain positive results, such as glycophorin A or DNA testing. While a negative result for latent bloodstains may discourage further testing, if evidence that suggests a cleaning item was used, alternative tests should be considered as well as factors such as time since the cleaning as taken place.

4. FUTURE PROJECTS

4.1 Preliminary results

The original purpose of this investigation was to determine if cleaning agents completely removed blood or if they disrupted the luminol reaction. In order to do so, an experiment was designed and preliminary data was obtained. Cotton swatches were folded into fourths and stained with a serial blood dilution, in quadruplicate (Figure 10). Starting with 1:10 blood up to 1:100,000, the stains were air dried for 48 hours and then one of three cleaning agents was pipetted onto the stain. Another 48 hours were allowed to pass and the stains were tested with luminol, specifically the Grodsky formulation. The results were similar to previous studies reported in the literature (Table 3). Water generated positive results, though 1/10,000 generated a weak positive and 1/100,000 was negative (Figure 11). Bleach caused a positive luminol reaction even in the absence of blood (Figure 12). Lysol with hydrogen peroxide caused negative results for all samples, as chemiluminescence was not consistent with a positive reaction whether blood was present or not (Figure 13).

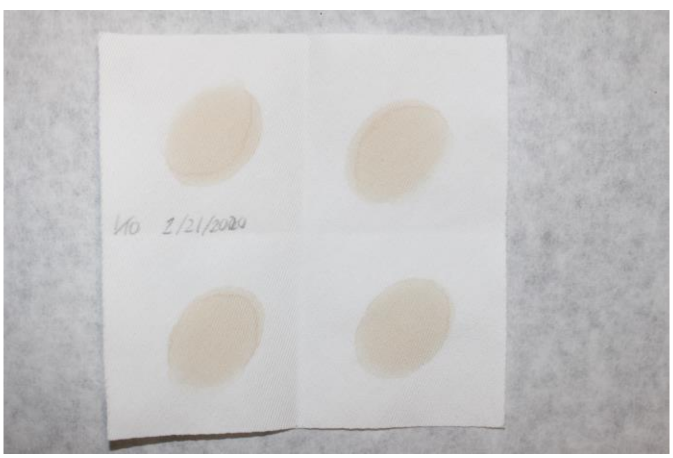

Figure 10: 1:10 blood dilution replicates prior to testing.

Table 3: Preliminary study results.

Serial Dilution and negative control	Distilled Water	Bleach	Lysol with Hydrogen Peroxide
H2O Control	4/4 Negative	4/4 Positive	4/4 Negative
1:10	4/4 Positive	4/4 Positive	4/4 Negative
1:100	4/4 Positive	4/4 Positive	4/4 Negative
1:1,000	4/4 positive	4/4 Positive	4/4 Negative
1:10,000	4/4 weak positive	4/4 Positive	4/4 Negative
1:100,000	4/4 Negative	4/4 Positive	4/4 Negative

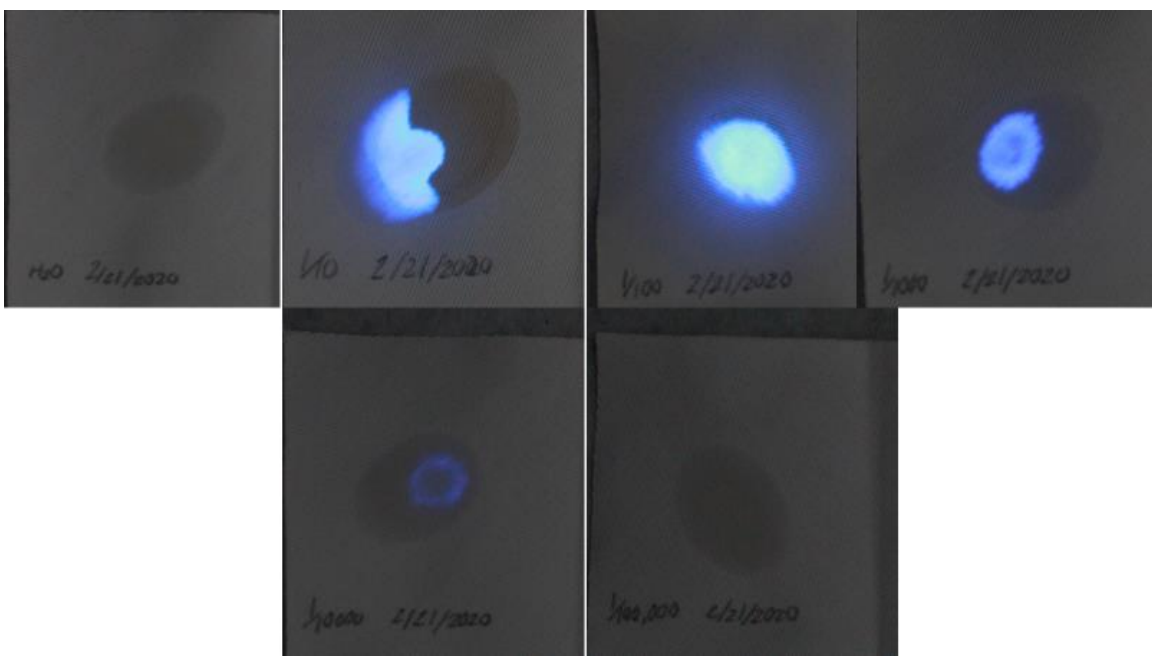

Figure 11: Results of water as a cleaner on bloodstains after the addition of luminol.

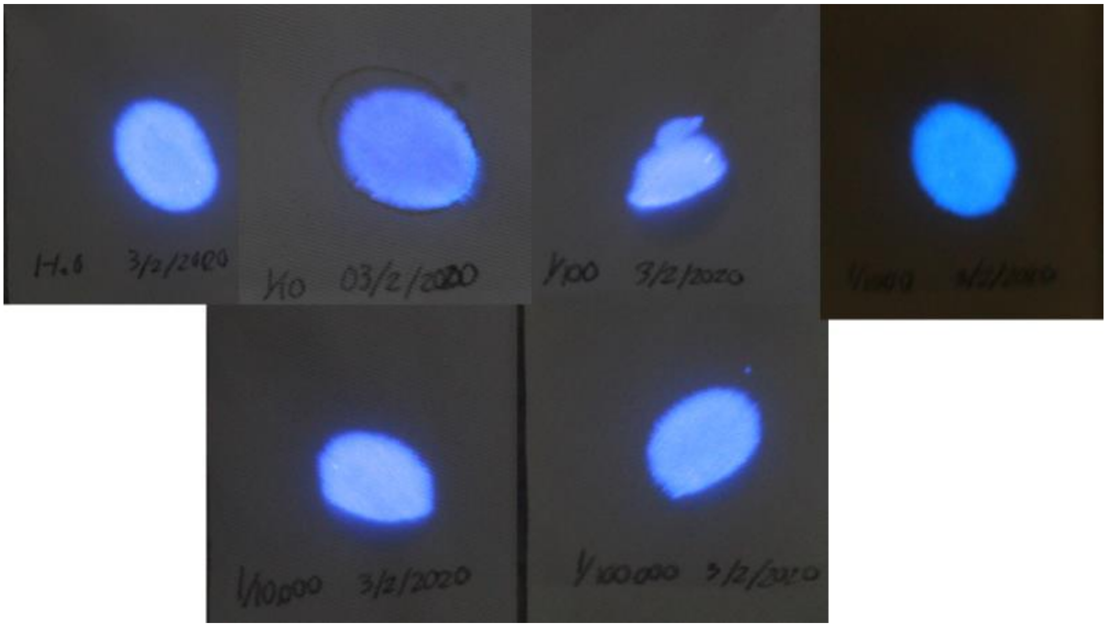

Figure 12: Results of sodium hypochlorite as a cleaner on bloodstains after the addition of luminol.

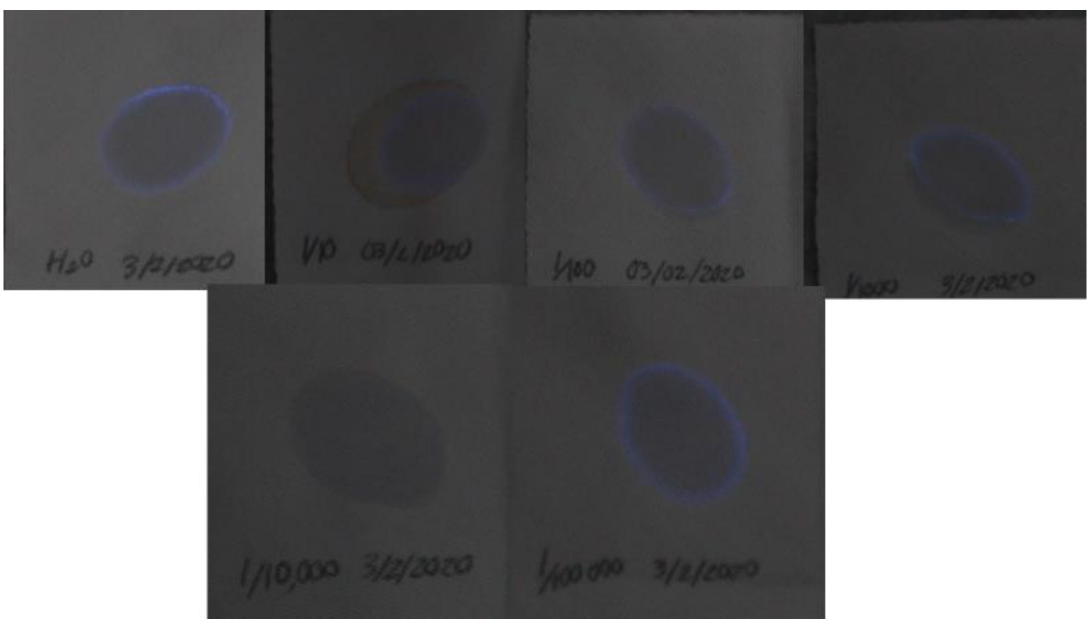

Figure 13: Results of Lysol with hydrogen peroxide as a cleaner on bloodstains after the addition of luminol.

4.2 Groundwork for a new study

Building on completed experiments, cotton swatches would be taken and folded into 4 sides or more depending on the size. Each swatch would have a bloodstain from a dilutions series, possibly starting at neat blood and going through 1:10, 1:100, 1:1,000, 1:10,000 and 1:100,000. It is worth noting that sample chemiluminescence weakened as the dilution increased, with 1:100,000 not rendering a positive for the water group samples. This dilution could be abandoned.

The serial blood dilution was made by adding 10 mL of neat blood to 90 mL of deionized water. Subsequently this mixture was vortexed, and 10 mL were drawn and added to 90 mL of deionized water. This was repeated until all dilutions were made. This stock was then frozen as the blood had no anticoagulants added. Seashols et al. (13) determined that EDTA has no impact on presumptive blood tests, meaning blood already obtained with preservatives can be used and has no repercussions on testing. Impact on being mixed with the cleaning agents could be assessed as part of the study.

The preliminary study used 50 μL of dilute blood, then 50 μL of cleaner pipetted over the stain followed by 50 μL of luminol. The cleaner could be doubled to 100 μL to make sure enough coverage is obtained of the bloodstain. Cassidy et al. (35) treated their cotton swatches with a plastic polymer to concentrate the stain into a specific area. The more dilute the bloodstain is the more it travels, dispersing the stain over a larger area even if it isn't necessarily visible.

The luminol was prepared with the Grodsky formula as prepared by Seashols (13). Solution A is 0.7g of sodium perborate and 50 mL of distilled H20 and Solution B is 5.0g Sodium carbonate and 0.1g of luminol in 50 mL of water. Both are kept separate until the luminol is needed, then equal parts of both are mixed together. Alternatively, it has been shown by Dilbeck (3) that Bluestar® Forensic is easier to mix, doesn't require complete darkness, is more intense after the initial spray than luminol, intensity is maintained in a second spray and lasts a few days after preparation.

The camera settings for this experiment were f 5.6, ISO 1600 and 5" distance, in a totally dark room. This generated excellent pictures. As seen in Figure 911 and 12, the chemiluminescent reaction is very clear. An attempt should be made to capture video instead of just pictures, as this would help determine how long the reaction lasts and how intense it can become. Still images can be taken from the video. Pictures can be evaluated utilizing a computer program such as ImageJ. There can be a light source in the room, if it's not pointed at the sample directly, to aid in obtaining a picture of the whole swatch; a completely dark room provides a clear picture of the chemiluminescence only.

It's important to appropriately label the swatches to understand what the samples are at a glance. Cleaner added should be labeled as well, preferably in a way that can be captured in every sample and not just the whole swatch, in case the pictures are cropped.

Future experiments should involve adding scrubbing as a factor as well as investigating multiple substrates. While initial testing merely assessed whether the cleaner had an impact on the luminol reaction when placed over the blood, a true attempt at cleaning is necessary. Cleaner added to a bloodstain on a substrate such as tile or treated wood could be wiped until it was no longer visible. Seashols (13) experimented with various substrates and these gave different results when it came to the limit of detection. In the Seashols study, the limit of detection was drastically lower on fabrics than surfaces such as linoleum and untreated wood. Following cleaning attempts, a luminol test would be conducted, and any chemiluminescent areas could be swabbed for DNA testing. Further studies should be conducted with other compounds that are suspected of impacting the luminol reaction, as well as other cleaning agents that may disrupt the reaction in unforeseen ways.

LIST OF JOURNAL ABBREVIATIONS

Angew Chem	Angewandte Chemie
Aust J Forensic Sci	Australian Journal of Forensic Sciences
Biochim Biophys Acta - Gen Sub	Biochimica et Biophysica Acta - General Subjects
Br Med J	British Medical Journal
Braz Dent J	Brazilian dental journal
Choice Rev. Online	Choice Reviews Online
Clin Chem	Clinical Chemistry
Cuad Med Forense	Cuadernos de Medicina Forense
Dtsch Z Gesamte Gerichtl Med	Deutsche Zeitschrift für die Gesamte Gerichtliche Medizin
Forensic Sci Commun	Forensic Science Communications
Forensic Science Int	Forensic Science International
J Am Chem Soc	Journal of the American Chemical Society
J Assoc Crime Scene Reconstr	Journal of the Association for Crime Scene Reconstruction
J Biolumin Chemilumin	Journal of bioluminescence and chemiluminescence
J Biotechnol Biodivers	Journal of Biotechnology and Biodiversity
J Crim Law Criminol Police Sci	The Journal of Criminal Law, Criminology, and Police Science
J Emerg Forensic Sci Res	Journal of Emerging Forensic Sciences Research
J Forensic Identif	Journal of Forensic Identification
J Forensic Leg Med	Journal of Forensic and Legal Medicine
J Forensic Science	Journal of Forensic Sciences
J Lab Clin Med	Journal of Laboratory and Clinical Medicine
NIJ Journal	National Institute of Justice Journal

Rev Química	Revista de Química
Sci Justice	Science and Justice

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