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Investigating the Effect of Urea Pretreatment on Bloodstain Detection

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Acknowledgements

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Abstract

Throughout the years, many chemical enhancement methods for bloodstain detection have been developed. One of these chemicals is luminol. Blood detection using luminol and its derivatives, like Bluestar™, have been commonly used at many crime scenes. The pre-treatment of 8M urea on bloodstains was proposed in order to increase the intensity of chemiluminescence of the reaction, and to eliminate false positive reactions that can occur. This study takes a look at bloodstains that are placed on two types of surfaces, at varying dilutions, and analyzed after different amounts of time. These bloodstains were analyzed to see how strong of a reaction is obtained after the addition of both 8M urea and the blood detection reagent Bluestar™ to the bloodstain. The addition of Bluestar™ created a chemiluminescent reaction that can be measured both visually and digitally in terms of its strength. It was determined that bloodstains at higher dilution factors, and bloodstains on non-absorbent surfaces like tile, are more likely to have a stronger and more easily detectable chemiluminescent reaction after the addition of urea. Although some improvements were seen from this study, most of the samples tested did not show any significant trend in increasing the strength of their chemiluminescent reaction. This emphasized the necessity to further explore the feasibility of the method and increase its efficiency through improved methods and testing.

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1. Introduction

1.1 Blood in Forensic Science

Blood is a commonly found bodily fluid at a crime scene and often holds significant probative value in the process of the modern criminal justice system. Above all, it is an inevitable component in the criminal investigation process. This is because it can potentially provide massive amounts of information and provide valuable insights into individualization and crime scene reconstruction. Its nature makes it more frequently encountered at the scenes of violent crimes such as homicides and sexual assaults. The significance of blood is, and will continue to be, integral in the development and expansion of relevant branches of forensic science such as DNA analysis, genetics, and genealogy. As for the present, blood is already in use in many different fields of investigation.

Blood circulates and transports substances throughout the human body and can be deposited at a crime scene when bodily harm occurs. Any unusual substance consumed by the donor of the blood prior to its deposit may be present in the stain as well. These stains could contain valuable personal information of the donor like DNA. Toxicological information can also be obtained from dried blood drops to determine whether the donor was intoxicated by any substance or took any medication at the time they shed the blood (15,17). More recently, however, methods are being developed for dating human blood pools using morphological and physical approaches, which could give blood another function in providing references to establish the time frame in which a crime took place (13).

To utilize blood in these investigation processes, one needs to be able to identify, collect, and examine it. However, the presence of latent bloodstains has complicated this process. This project focuses on the identification of bloodstains using the luminol reaction and explores the feasibility of enhancing the luminol reaction by pre-treating the blood samples with urea.

1.2 Forensic Blood Detection

In forensic science, the process of blood detection is not standardized, however, the use of luminol as the main method of blood detection has emerged across many crime labs in the country. This technique of blood detection in forensic science utilizes the chemiluminescent property of blood. Chemiluminescence is defined as light emission due to a chemical reaction. This process often involves the excitation and relaxation of electrons, where the molecules release an excess amount of energy to its surroundings and return the electrons to the ground state. Some of that energy is released as photons, and therefore chemiluminescence occurs. The general formula is described in figure 1, where [I]* represents an excited state intermediate (1). For the luminol reaction, this excited state intermediate was found to be α-hydroxy hydroperoxide (12). This is a product formed by the oxidation of the luminol molecule, that is then decomposed to emit a photon (12).

$$A + B \rightarrow [I]^* \rightarrow \text{PRODUCTS} + \text{LIGHT}$$

Figure 1: General mechanism of luminol reaction

In forensic practices, when analyzing a potential bloodstain, a mixture of hydrogen peroxide and a luminol solution is used. Blood will catalyze the decomposition

of the hydrogen peroxide thus oxidizing the luminol solution. This reaction produces a chemiluminescence that indicates the presence of blood. The fundamentals of blood detection by luminol were derived from a method to detect iron (Fe) in analytical chemistry (12). It was established by Rose et al., that trace amounts of Fe(II) can be detected by Fe(II) catalyzed chemiluminescence from luminol oxidation by oxygen (12). Additionally, the ability of hematin (which contains Fe(III)) to serve as a catalyst has also been proven (5). Considering both forms of iron co-exist in human blood respectively with oxyhemoglobin and hematin, this luminol formulation was found suitable for detecting trace amounts of blood.

Over the years, many luminol derivatives and formulations have been developed, with some of them dedicated to forensic service. Bluestar™ latent blood reagent, which was used in this project, is a modified luminol-based formulation which claims to be able to provide chemiluminescence with improved intensity even with diluted bloodstains.

1.3 Factors that Influence Forensic Bloodstain Testing

Luminol has had its own criticisms, and it has taken a long time to reach the level of reliability in the forensic science world that it has. In 1951, a study was done to comparatively investigate the methods of blood testing, and it mentioned that at the time, the use of benzidine was the most common method for detecting blood (9). This study mentioned the fact that luminol at the time was considered to have no interference with other substances, and that it was able to solely detect blood (9). However, the benzidine test was criticized in this study for being unable to detect blood in dilute samples and having a low sensitivity level of 1 to 300,000 (9). This paved the way for luminol as a major tool in the forensic science world.

The reliability of luminol is supported by a study done in 2011, which tested the interactions between blood detection reagents (4). This study used reagents such as luminol and benzidine to test their reaction with presumptive and confirmatory tests along with DNA quantitation (4). The results of the study showed that luminol had no detectable interference with the presumptive or confirmatory tests, giving 20 correct results out of 20 trials (4). The DNA quantitation showed no major inhibition on the quantity of DNA extracted from the blood until 120 days after application (4). Another study in 2015 showed how the luminol reagent test did not affect the STR analysis of the DNA in the blood (6). Although, in this study luminol was shown to interfere with the presumptive test in one trial, which was explained by a variety of factors, it still showed consistency in results and reliability in testing (6). These results seem to show extreme success in the luminol reagent's ability to detect blood, which was the catalyst for luminol becoming one of the most commonly used reagents.

On the other hand, studies have recently come out regarding possible interferences with the luminol reagent and creating uncertainty in the reliability around its use. A study done in 2016 tested luminol's interactions with two other presumptive tests, tetramethylbenzidine (TMB) and phenolphthalein (PT) (8). The luminol was concluded to have no negative effect or interference with either substance at dilutions of 1 to 100 or lower (8). At dilutions of 1 to 1,000 or higher, the luminol had a negative interaction with many of the trials of the presumptive tests and caused negative results with known blood samples (8). These results suggest an immediate need to investigate methods to improve or replace the current technique due to interference which can lead to major inaccuracies. Another study done in 2017 investigated the sensitivity of luminol, and was not only able

to get results, but clarify a long-standing debate in the forensic world (3). This study was able to determine that bloodstains of dilution factors less than 1 to 236,000 could be detected by luminol (3). The results of this study show a limitation of the luminol method. This discovery is furthered by Morris et. al.'s claim that 1 to 300,000 is a small sensitivity, even by the standards in 1951 (9). This, along with the author's discussion about the shortcomings of many previous studies show that not only is the method itself in need of improvement, but the studies done on this reagent can be very flawed. The author states that previous studies have had non-reproducible preparation methods, inaccurate blood amounts, uncontrolled application, age effects, etc. (3). This shows that well conducted studies need to be done to both improve the current luminol method and to disprove facts about the reagent that may have come about through inaccurate testing procedures.

1.4 Factors Affecting Chemiluminescence

Another aspect of luminol that has been widely tested is its chemiluminescence when it encounters bloodstains. A 2018 study investigated the strength of this chemiluminescence at different dilutions and how they could be quantified, which has been difficult to do in the past (11). The study used ovine blood and discovered that fresh blood can be more easily detectable at higher dilution factors than dried blood (11). This is one of the first efforts to actually quantify the chemiluminescence of luminol, but it is still, as the study states, very preliminary, and more sensitive testing is needed (11). The study we are conducting using urea may be able to provide this need for better sensitivity in blood detection. Another review done on blood detection techniques, including luminol, was done and stated that luminol is very beneficial in testing for stains in the

dark (16). The review also stated that luminol has been shown to be an important resource for investigating bloodstains (16). It also explains that the weber method for preparing luminol was the best method for ensuring that DNA can still be obtained from the sample (16). It is important to state that the way in which the luminol in this study was prepared did not affect the DNA analysis (16).

Another way in which our study will help improve luminol testing is by eliminating the bleach false positive. Bleach has been the most abundant false positive with luminol, as documented by a 2018 study describing its effects (2). The study described applying luminol to various surfaces that had been cleaned, one of which with bleach (2). The bleached surfaces, even when no blood was applied, showed strong and persistent chemiluminescent reactions (2). Another study was conducted in 2012, assessing how bleach affects DNA profiling and STR analysis (10). The study determined that the bleach had adverse effects on DNA profiling, as DNA was shown to be severely degraded when tested (10). This study made sure to state, though, that luminol itself did not contribute to this, as it was tested and luminol was shown to have no effect on any forensic laboratory testing on its own (10). Bleach is a major false positive that inhibits the ability of luminol to do its job effectively and make studies like the one we will conduct with urea all the more important.

A 2016 study investigated how luminol's chemiluminescence and reliability could possibly be improved with the addition of certain chemical substances, namely 8M urea (14). In this study, researchers added 8M urea to a diluted bloodstain prior to applying the luminol, and the result was an increased chemiluminescence at increasingly dilute bloodstains, even at dilutions that forensic scientists have not been able to see before

(14). Additionally, it nearly eliminated the bleach false positive (14). This provides a promising improvement to the existing luminol reagent, but it cannot be adopted until further testing is done to investigate all possible effects of the reagent. The purpose of our study is to do just that, to explore how luminol interacts with bloodstain detection after pretreatment with urea under a variety of different circumstances including aged bloodstains, diluted bloodstains, and bloodstains on different surfaces.

2. Materials and Methods

2.1 Preparation of Samples

This study aims to investigate how blood of various dilutions and ages found in simulated crime scene scenarios are affected by the pre-treatment of urea before the use of blood detection techniques.

Approval from the Institutional Review Board (IRB) has been granted for the use of human subjects and the extraction of human blood through venipuncture for this study. The blood was drawn by University of New Haven Health Services and was stored in the Forensic Science Department for use at any time. In order to test the effects of urea on diluted blood, the blood obtained from the volunteer was used to create four different dilutions: 1:10, 1:100, 1:1,000, and 1:10,000. Each dilution was made by pipetting the required amount of blood for each dilution into 50mL test tubes. The remaining portions of each test tube were filled with the distilled water solvent to the 50mL line.

Two types of surfaces were used to deposit the blood on: the absorbent and the non-absorbent surface. Dark gray colored “area rug” carpets cut into squares were used as

the absorbent surface, and tan colored pieces of square tile were used as the non-absorbent surface. Approximately 6mL of blood was used for creation of the dilutions.

Each piece of carpet and tile were separated into 5 categories based on how much time was to pass between the deposition and detection. The categories were organized as follows: 48 hours, 1 week, 2 weeks, 3 weeks, and 1 month. For each category, 2 pairs of tiles and carpets were designated, one of which was meant to be treated with urea and was labeled as “urea treated”. The other pair were not meant to be treated prior to detection and was labelled as “not urea treated”. Each piece of tile and carpet was separated into 4 quadrants by pink pieces of tape. Each quadrant denotes one dilution of blood deposited on that type of surface. Each category consisted of one “not urea treated” carpet, one “not urea treated” tile, one “urea treated” carpet, and one “urea treated” tile. Each dilution of blood was deposited in the amount of 2mL on its designated quadrant. All the samples were left to dry in an open and breathable environment for the previously mentioned lengths of time.

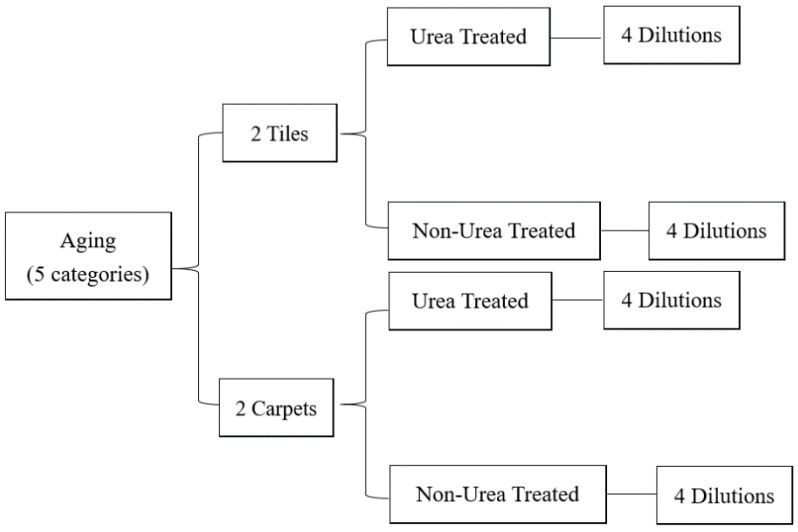

Figure 2: Sample logic

2.2 Detection and Documentation of Samples

After the surfaces in each category were left to dry for their designated periods of time, blood detection with selective urea treatment was conducted. 8M Urea solution was purchased from Sigma Chemical company and prepared according to its instructions. Bluestar™ tablets were also prepared according to instruction from Bluestar Forensic. Aliquots in the amount of 1mL of the 8M Urea solution were applied to each sample of deposited blood on the surfaces designated as "urea treated" surfaces 20 minutes prior to the detection.

A Canon EOS REBEL T3i camera was used to capture the images. With the assistance of a tripod, the camera was fixed above the platform where the detection would occur. Two types of images were taken for each sample, one being the sample under white light/normal lightning condition, which records the sample before the application of Bluestar™; the other being the sample in a dark environment after applying Bluestar™.

In the dark environment, Bluestar™ solutions were applied to the samples using a spray bottle. To achieve maximum chemiluminescence, the spraying was continued until the strength of chemiluminescence stopped increasing; the exposure of images started at the same point.

Camera settings were set to be consistent for each image captured. The detailed camera settings were as follows:

	White Light/Normal Condition	Dark with Bluestar™
Shutter Speed	1/100s	120s
Aperture	f/8	f/8
ISO	800	800
White Balance	Fluorescent	Fluorescent

Table 1: Camera settings

2.3 Digital Brightness Analysis of Chemiluminescence

Besides the subjective rating of all chemiluminescence under the scales of "Very Strong (VS), Strong(S), Moderate(M), Weak(W), or None(N); the photos have undergone a digital analysis method based on HSL color space.

Since the aperture, shutter speed, and ISO were set to constant for all "dark" photos, the amount of light and duration allowed to enter the camera are constant as well. The only factor influencing the brightness in the photos is how much chemiluminescence was generated for that given 120 seconds. This makes all the photos comparable relative to each other in terms of the strength of chemiluminescence.

When looking at digital photos on monitors, the perception of brightness for human eyes is different from directly seeing the chemiluminescence occurring. When looking at an actual reaction, photons generated by the reaction directly interact with receptors in the eyes, causing the contrast of darkness (absence of photon) and brightness (presence of photon). However, digitally, this contrast is caused by a difference in colors presented by monitors. Therefore, an analysis method of quantifying what human eyes perceive as "brightness" on monitors are necessary.

HSL (hue, saturation, lightness) color space is a model for digital display developed in the 1970's, which mirrors the mechanisms of human visual receptors. In these color spaces, colors are created digitally as a mixture of three coordinates of hue,

saturation, and lightness. Lightness can be described using terms such as brightness, brilliance, and strength (7). Maximum light would be considered white, while minimum light would be considered black (7). The specific camera settings used in our experiment fairly represent how human eyes perceive “brightness” of a given color, in this case, the strength of chemiluminescence.

The ultimate purpose of this experiment is to simulate bloodstain samples analyzed both at a crime scene and in a forensic lab. When taking the photos of the results, the camera settings should conform to how the human eyes would naturally perceive chemiluminescence. When determining the L value, which is a digital setting that determines the strength of light in a photo, the brightest area of each photo of the bloodstains was selected and used to determine the average L value of each of the samples. The area selected on each bloodstain was a 3x3 grid of 9 pixels. The sampling area should not be too small, because a small area such as one single pixel would not be an adequate representation of area brightness. The area should not be too large either, since bright points are easy to be spotted by human eyes.

Additionally, it is worth emphasizing that all photos were taken using an exposure period of 120 seconds. Therefore, each chemiluminescent reaction is shown as overlays of light from each 120 second time span. Some isolated small bright points, although obvious in the photos, are unable to be seen in person. This is because the reaction occurs gradually over a short period of time, which is all captured by the long exposure photo.

2.4 Preservation of Samples for Future Research

After each sample was documented with the camera both before and during blood detection, we preserved every sample for future testing. The mixtures of blood, Bluestar™ solution and possible urea solution (depending on sample type) on each quadrant were sampled using sterilized cotton swabs. The resulting swabs were stored in the University of New Haven's Forensic Science Department for potential further study. The purpose of this was to allow future studies to be done on whether DNA would be degraded as a result of adding the urea solution. These swabs are currently in storage and labelled with what dilution of blood it was from, the surface it was swabbed from, whether urea was added to it, and how long after deposition it was tested. The sample log is with the samples in the freezer in which the swabs are kept.

3. Results

3.1 Visual Analysis

After analysis with the Bluestar^{TM} reagent, the strength of the chemiluminescence reactions were reported as being either very strong, strong, moderate, weak, or non-existent. These results are displayed below.

	Carpet				Tile			
	1:10	1:100	1:1,000	1:10,000	1:10	1:100	1:1,000	1:10,000
48-hr non-urea	S	S	N	N	S	S	M	W
48-hr urea	S	S	N	N	S	S	M	M
1-wk non-urea	S	M	N	N	S	S	S	M
1-wk urea	S	S	W	N	VS	VS	VS	VS
2-wk non-urea	S	S	N	N	S	S	M	W
2-wk urea	M	S	N	N	S	S	M	W
3-wk non-urea	S	S	N	N	S	S	M	W
3-wk urea	S	S	N	N	S	S	M	M
1-mo non-urea	S	S	N	N	VS	VS	VS	VS
1-mo urea	S	S	N	N	VS	VS	M	M

Table 2: Chemiluminescence levels of samples. Designations are either Very Strong(VS), Strong(S), Moderate(M), Weak(W), or None(N). Time periods are in Hours(hr), Weeks(wk), and Months(mo).

Below are the photographs from both before and during each sample being tested. All photos will be oriented with 1:10 dilution in the top left quadrant, 1:100 dilution in the top right quadrant, 1:1,000 dilution in the bottom left quadrant, and the 1:10,000 dilution in the bottom right quadrant.

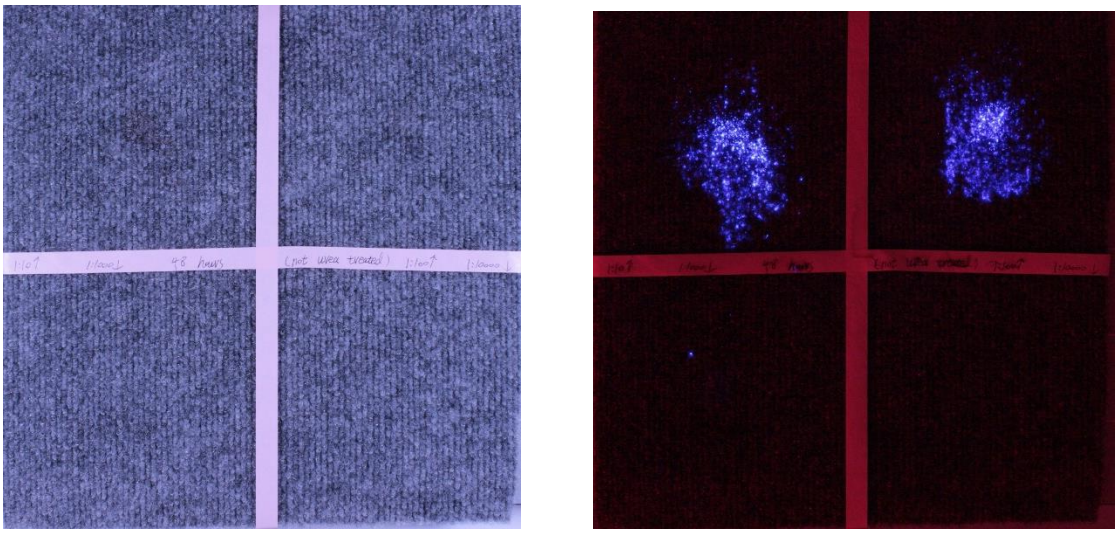

Figure 3: Carpet without urea after 48 hours

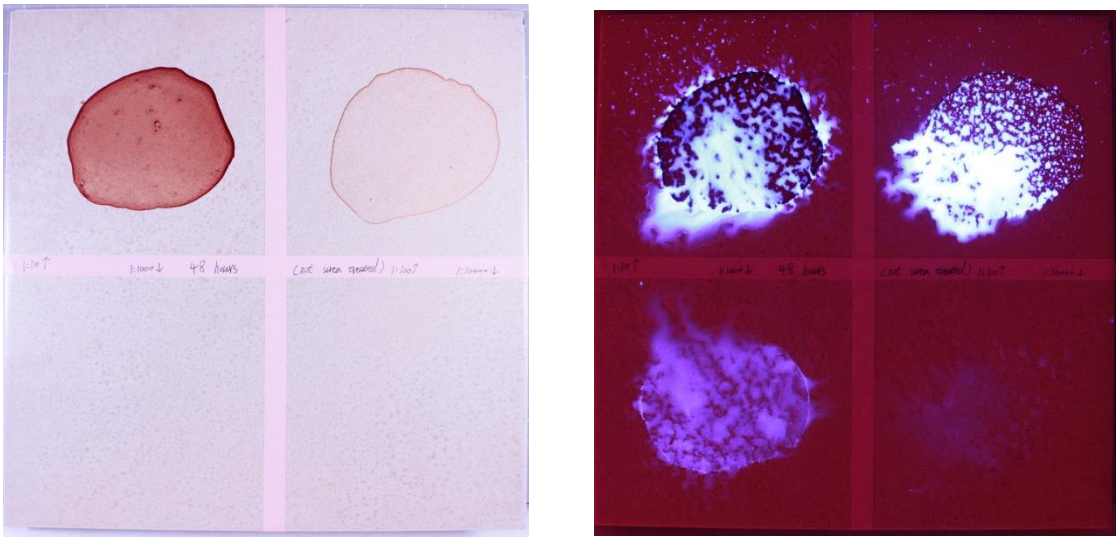

Figure 4: Tile without urea after 48 hours

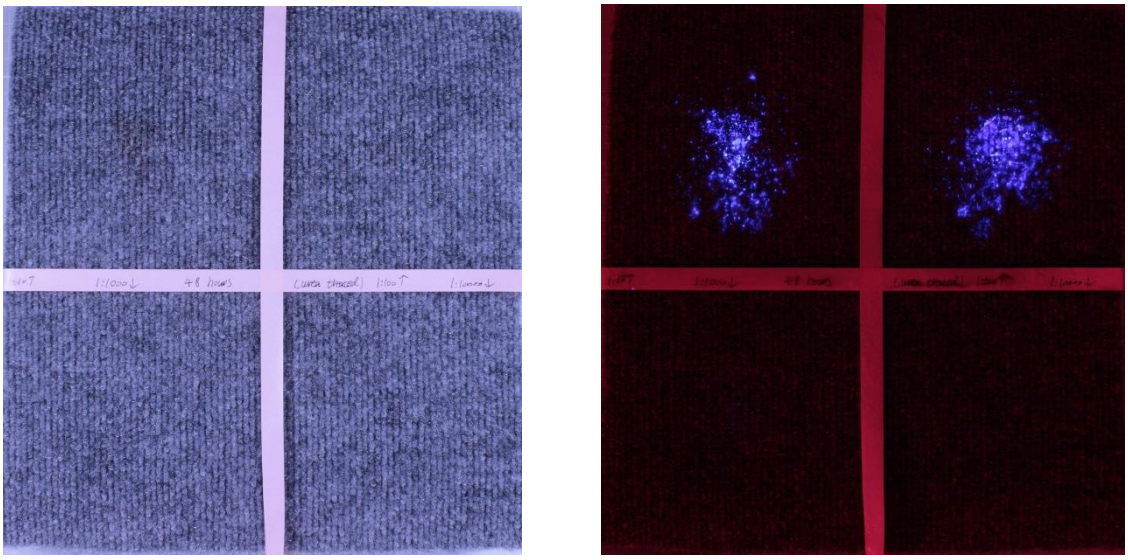

Figure 5: Carpet with urea after 48 hours

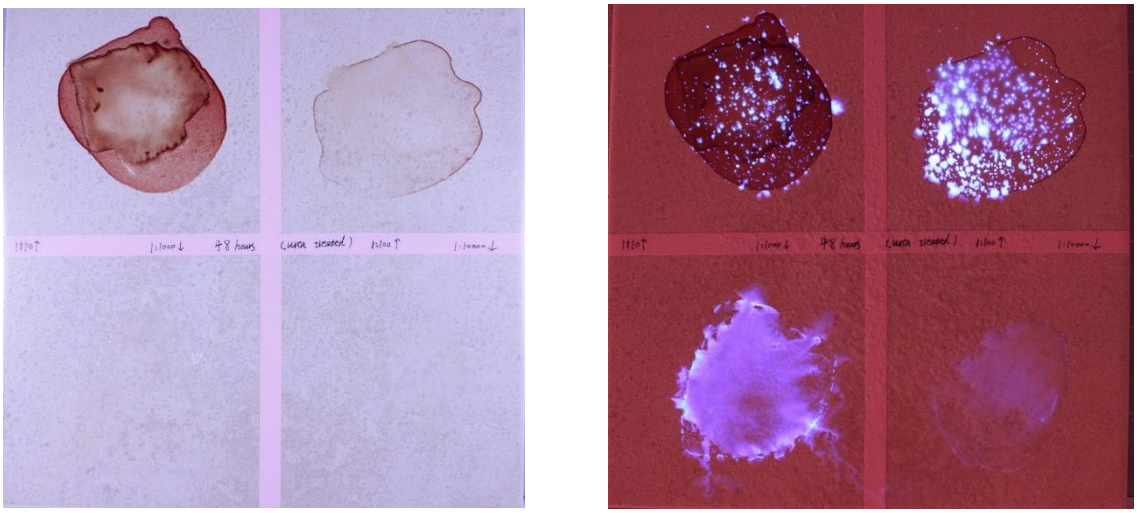

Figure 6: Tile with urea after 48 hours

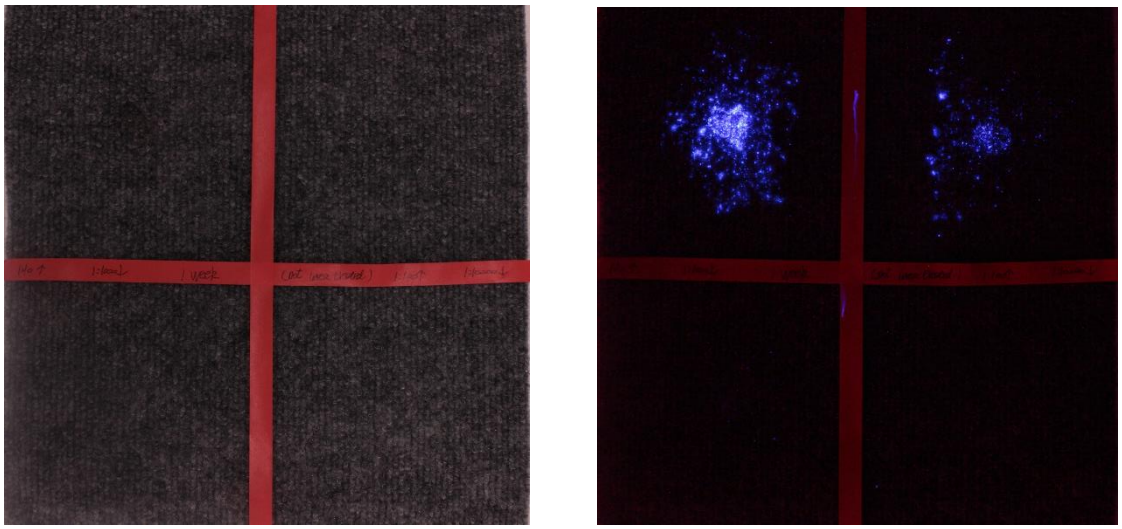

Figure 7: Carpet without urea after 1 week

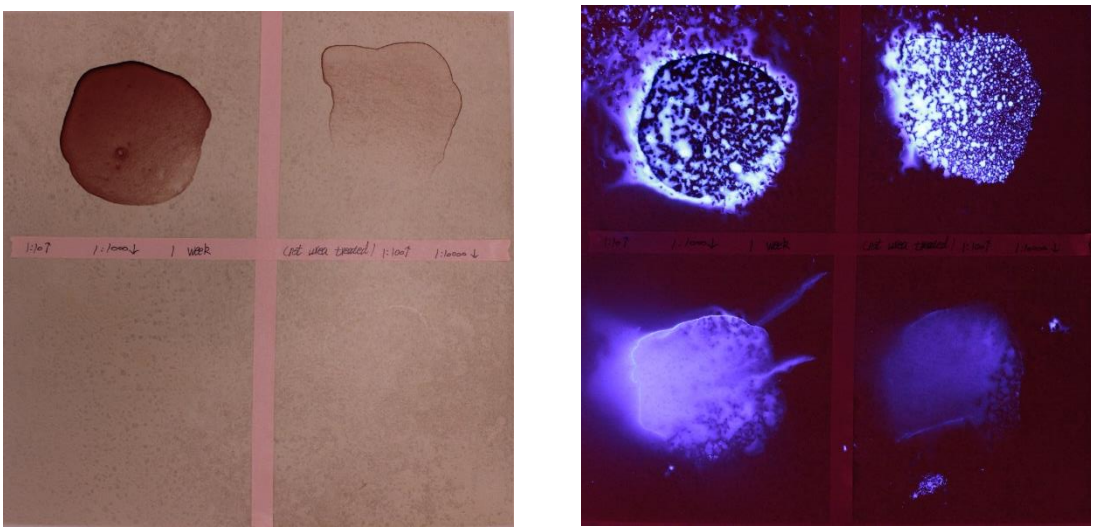

Figure 8: Tile without urea after 1 week

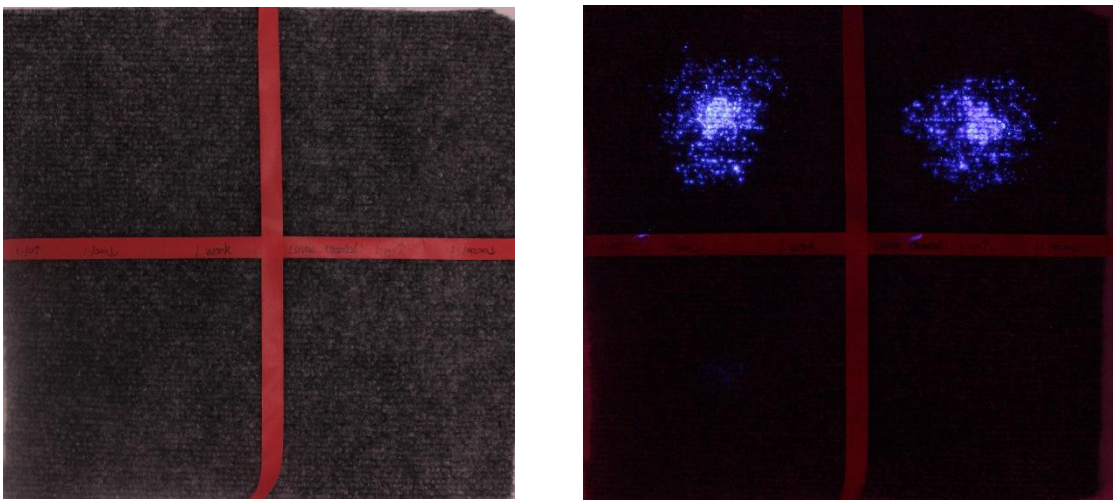

Figure 9: Carpet with urea after 1 week

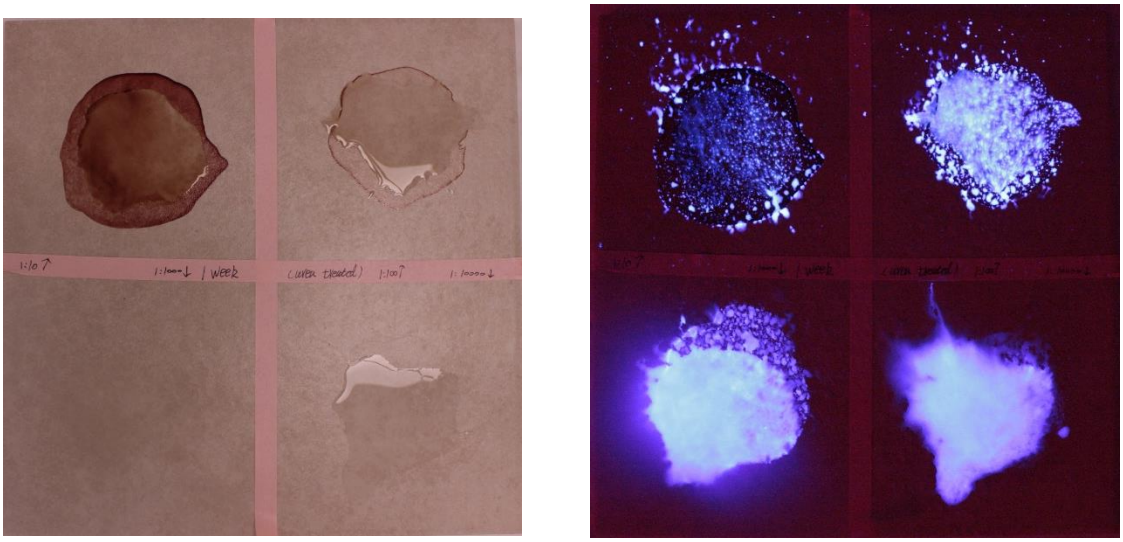

Figure 10: Tile with urea after 1 week

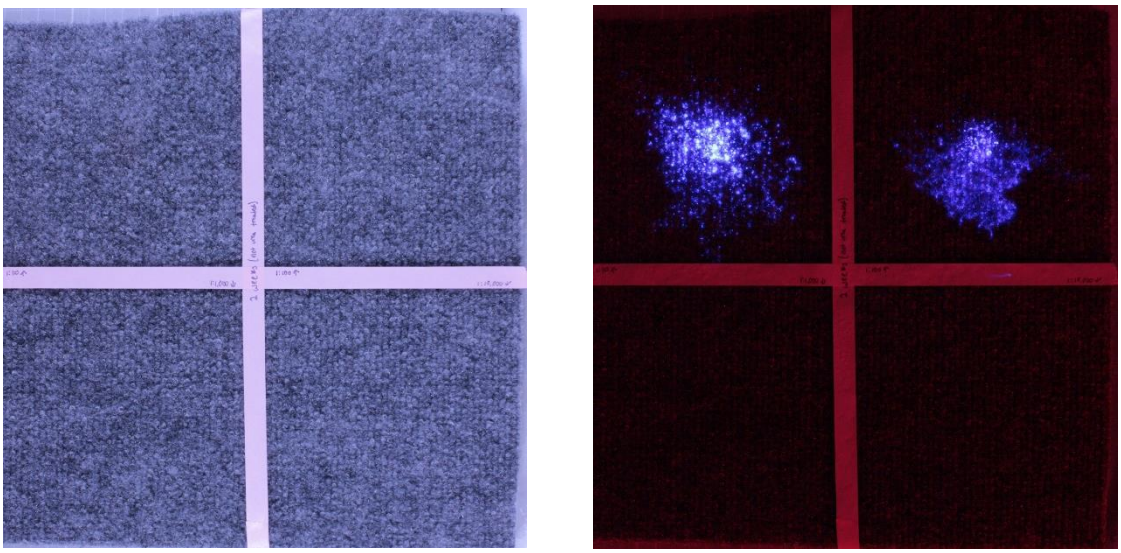

Figure 11: Carpet without urea after 2 weeks

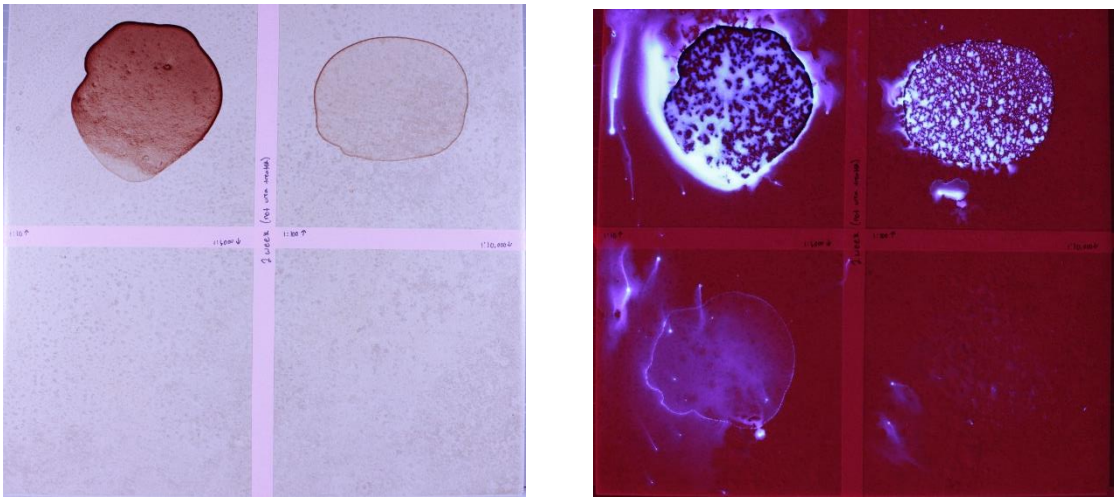

Figure 12: Tile without urea after 2 weeks

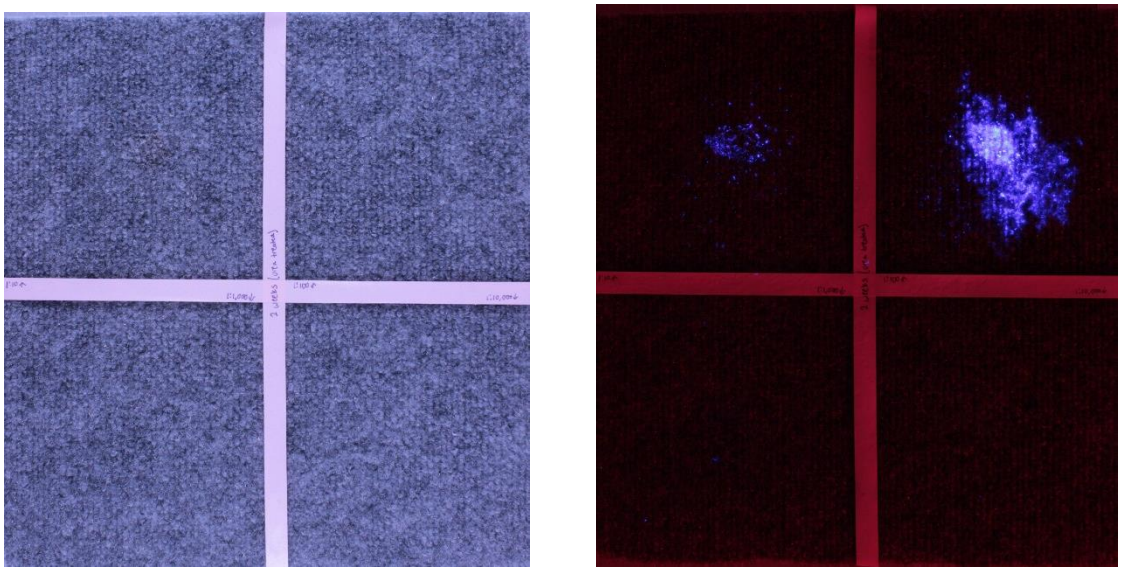

Figure 13: Carpet with urea after 2 weeks

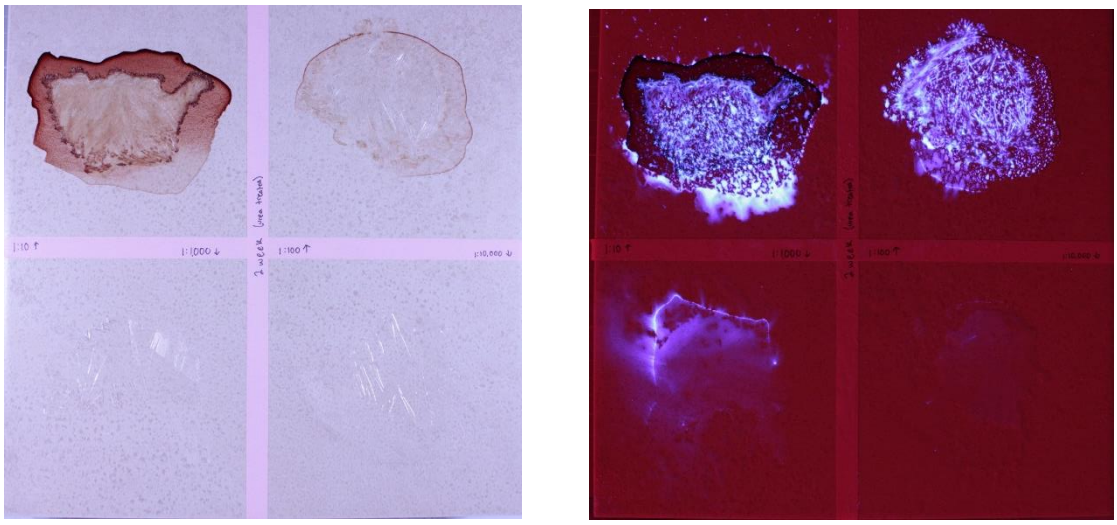

Figure 14: Tile with urea after 2 weeks

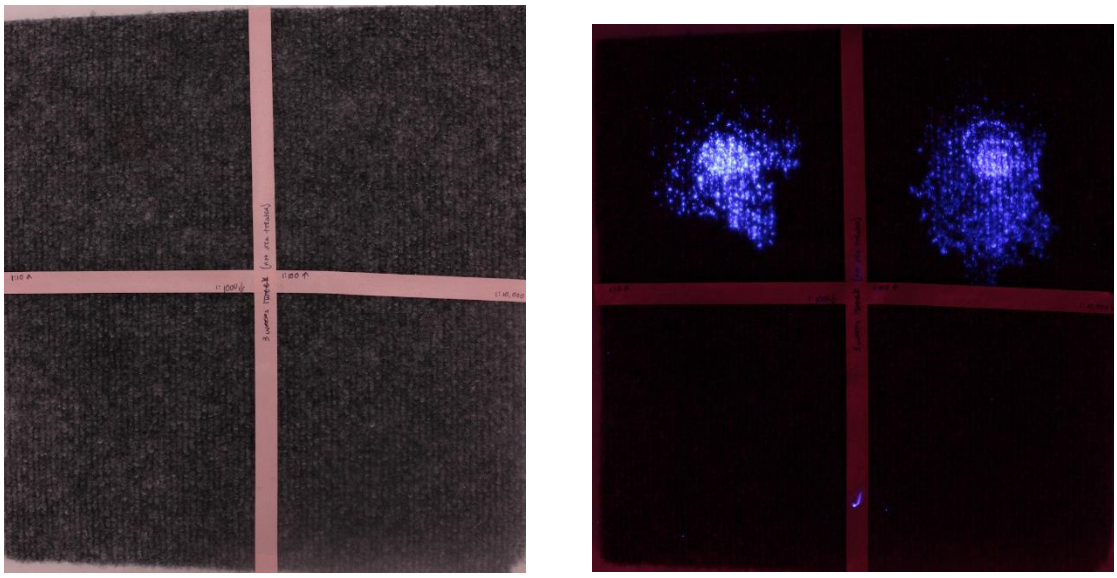

Figure 15: Carpet without urea after 3 weeks

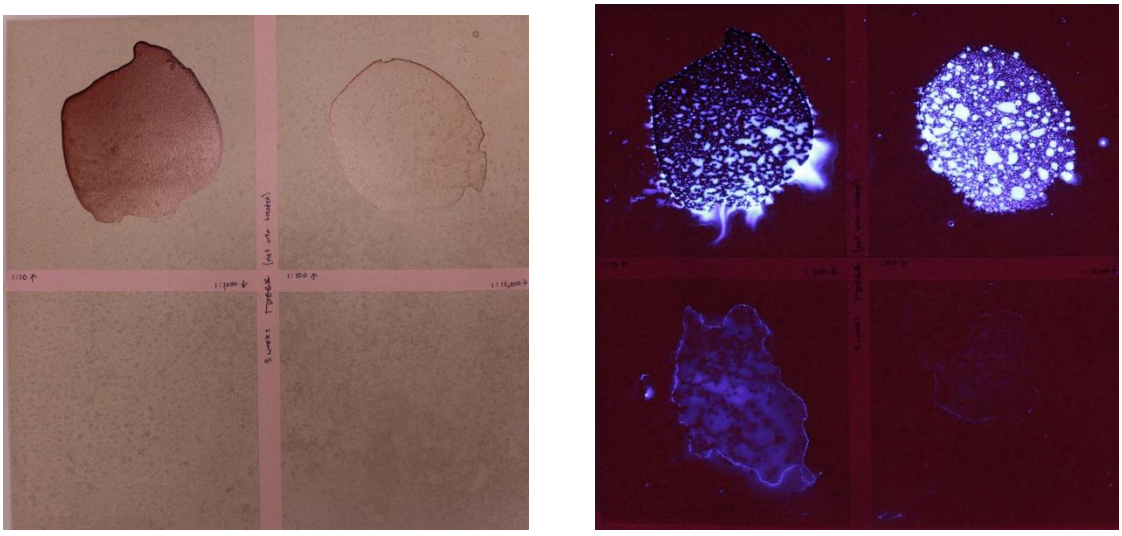

Figure 16: Tile without urea after 3 weeks

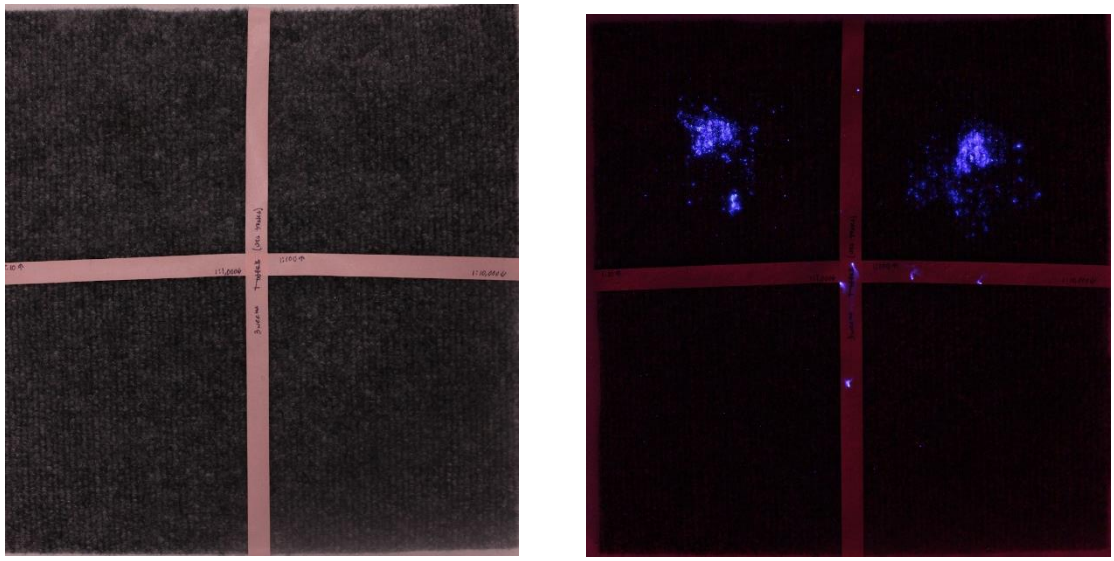

Figure 17: Carpet with urea after 3 weeks

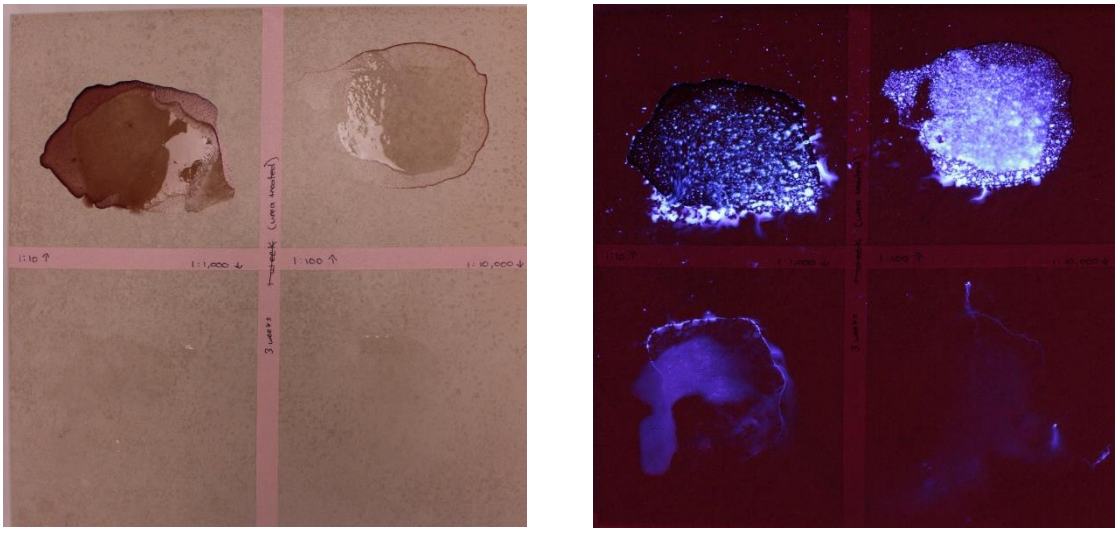

Figure 18: Tile with urea after 3 weeks

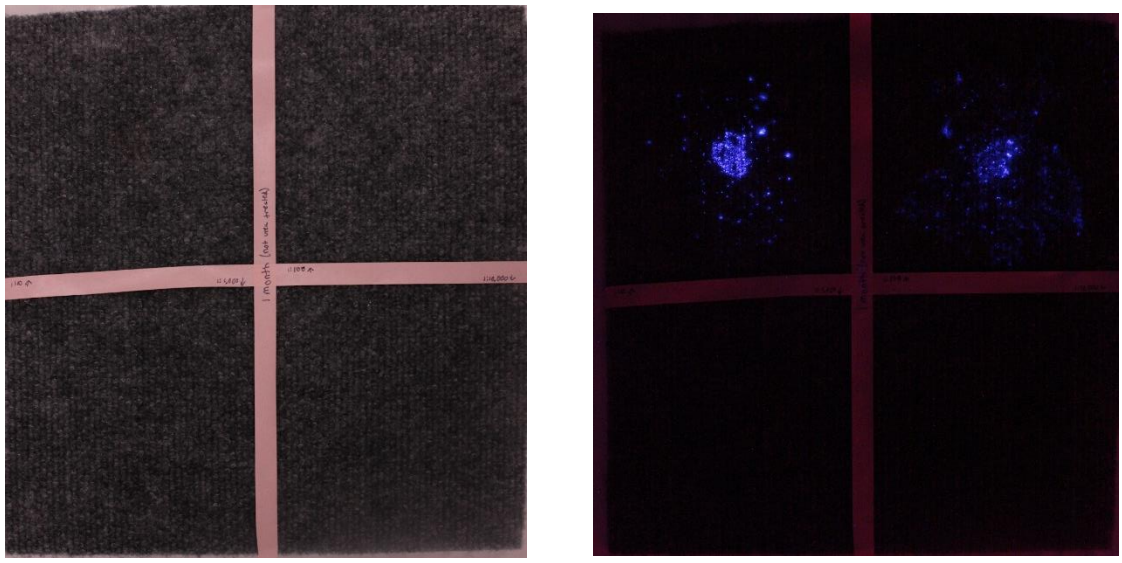

Figure 19: Carpet without urea after 1 month

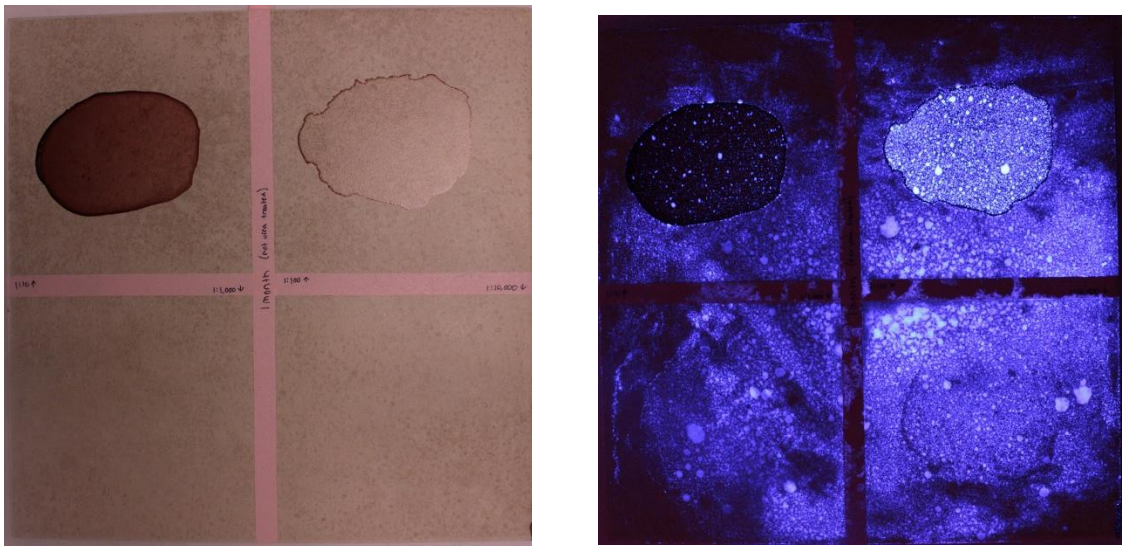

Figure 20: Tile without urea after 1 month

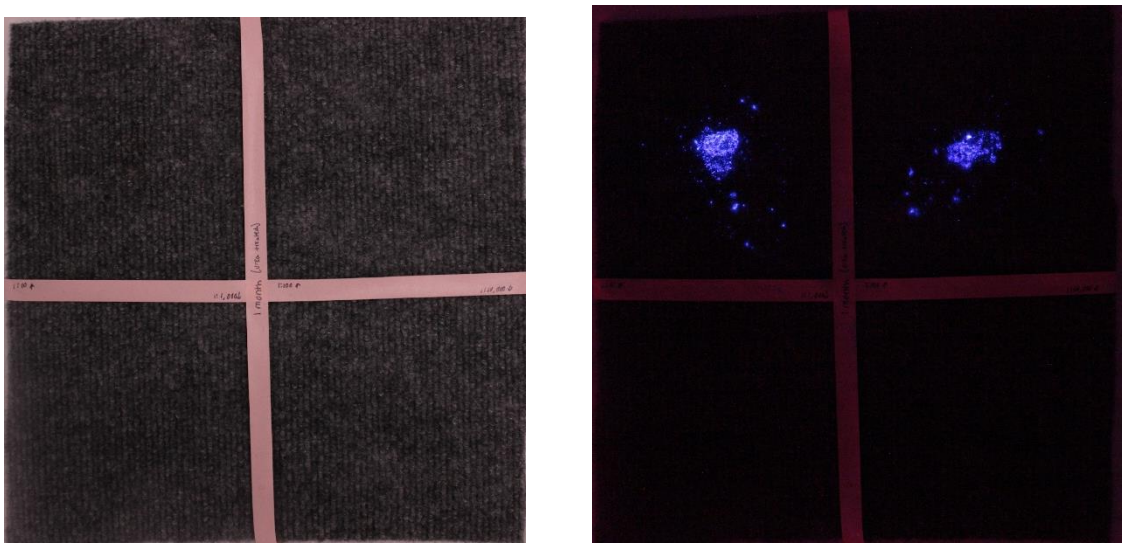

Figure 21: Carpet with urea after 1 month

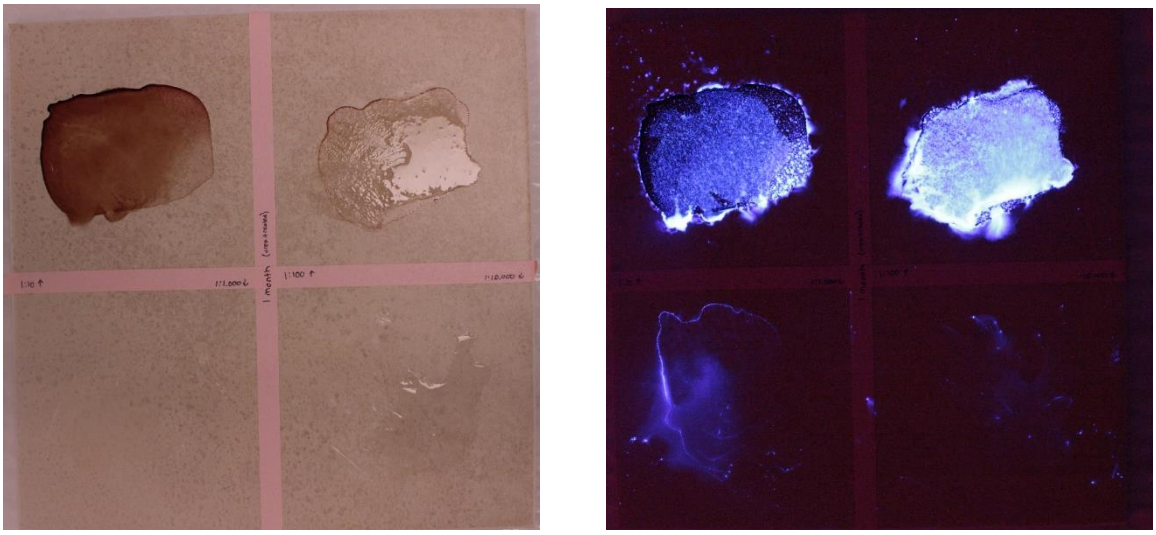

Figure 22: Tile with urea after 1 month

3.2 Digital Analysis

The L values of each sample, arranged by substrate types, sample ages, and dilutions were as follows. The urea-treated samples which yielded stronger chemiluminescence than non-urea treated samples are marked blue.

Tile									
Non-urea Treated					Urea Treated				
Photo#	Camera ID	Age	Dilution	L%	Photo#	Camera ID	Age	Dilution	L%
2	8556	48 Hours	1:10	97	9	8563	48 Hours	1:10	93
			1:100	100				1:100	97
			1:1,000	77				1:1,000	91
			1:10,000	43				1:10,000	58
24	8691	1 Week	1:10	97	28	8695	1 Week	1:10	97
			1:100	99				1:100	94
			1:1,000	83				1:1,000	87
			1:10,000	81				1:10,000	82
18	8572	2 Weeks	1:10	97	14	8568	2 Weeks	1:10	95
			1:100	95				1:100	95
			1:1,000	95				1:1,000	90
			1:10,000	87				1:10,000	29
32	8699	3 Weeks	1:10	100	36	8703	3 Weeks	1:10	100
			1:100	99				1:100	98
			1;1,000	84				1;1,000	79
			1:10,000	35				1:10,000	79
39	8818	1 Month	1:10	80	43	8822	1 Month	1:10	100
			1:100	96				1:100	100
			1:1,000	82				1:1,000	82
			1:10,000	82				1:10,000	72

Table 3: Tile digital analysis results

Carpet									
Non-urea Treated					Urea Treated				
Photo#	Camera ID	Age	Dilution	L%	Photo#	Camera ID	Age	Dilution	L%
5	8559	48 Hours	1:10	89	11	8565	48 Hours	1:10	87
			1:100	91				1:100	84
			1:1,000	0				1:1,000	0
			1:10,000	0				1:10,000	0
22	8689	1 Week	1:10	94	26	8693	1 Week	1:10	94
			1:100	81				1:100	88
			1:1,000	0				1:1,000	0
			1:10,000	0				1:10,000	0
20	8574	2 Weeks	1:10	96	16	8570	2 Weeks	1:10	78
			1:100	85				1:100	90
			1:1,000	0				1:1,000	0
			1:10,000	0				1:10,000	0
30	8697	3 Weeks	1:10	96	34	8701	3 Weeks	1:10	80
			1:100	90				1:100	79
			1;1,000	0				1;1,000	0
			1:10,000	0				1:10,000	0
41	8820	1 Month	1:10	86	45	8824	1 Month	1:10	83
			1:100	82				1:100	89
			1:1,000	0				1:1,000	0
			1:10,000	0				1:10,000	0

Table 4: Carpet digital analysis results

To examine the quality of urea enhancement, the trend of chemiluminescence brightness was plotted on graphs of L value vs. Decreasing blood concentration. The non-urea treated samples are represented by dotted lines, and urea-treated samples are represented by solid lines.

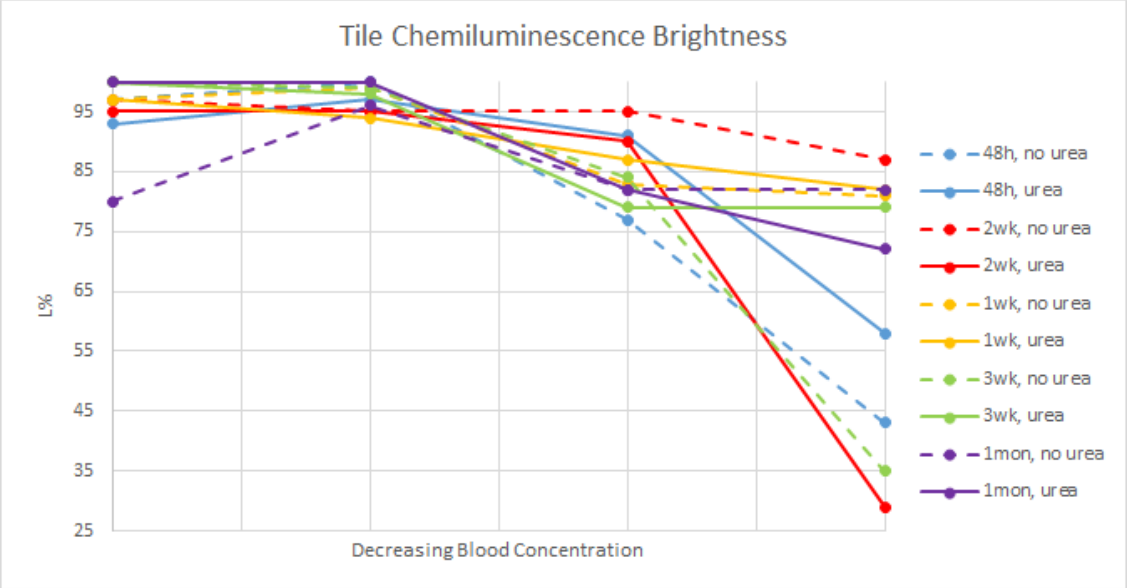

Figure 23: Tile chemiluminescence brightness trend

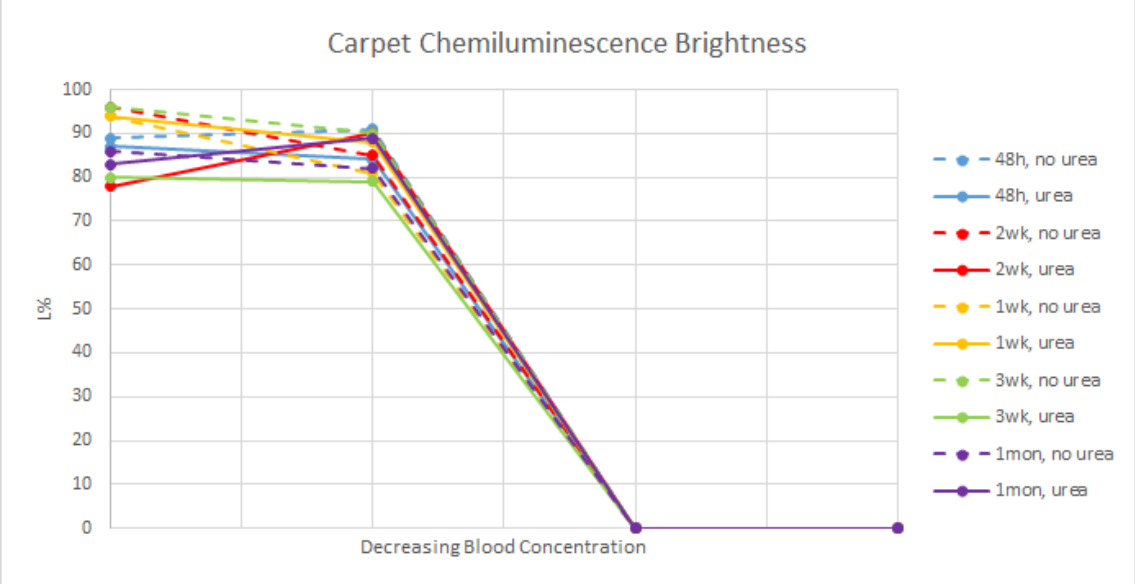

Figure 24: Carpet chemiluminescence brightness trend

Presumably, if urea can significantly enhance the strength of chemiluminescence, we would see a pattern where solid lines lie above dotted lines for one given color. Unfortunately, such a pattern has not been frequently observed for both substrates.

For tile samples, it can be observed that chemiluminescence strengths are relatively clustered at 1:10 and 1:100 dilutions; the strengths do not vary much by dilution nor use of urea. Out of 16 trials, 2 enhancements were achieved at these two dilutions, one of which by 20% and the other by 4%. Strengths are generally spread out when dilution increases to 1:1,000 and 1:10,000, where massive diversity can be observed both in terms of dilution and use of urea. Out of 16 trials, 5 enhancements were achieved at these two dilutions, respectively by 14%, 15%, 4%, 1%, and 44%.

On carpets, samples of all ages, including both urea-treated and untreated, exhibited strong chemiluminescence at 1:10 and 1:100 dilutions. At 1:1,000 and 1:10,000 dilutions, none of the samples exhibit observable chemiluminescence, including those treated with urea. In addition to this, 3 enhancements were observed at 1:100 dilution, by 5%, 7%, and 7%.

4. Discussions

4.1 Dilutions

One of the variables studied was the dilutions of blood placed onto each surface. The dilutions studied and that are shown in the results, include the dilution factors of 1:10, 1:100, 1:1,000, and 1:10,000. The 1:10 dilutions of blood when applied had a very dark reddish-brown color to them and were clearly visible to the naked eye. When Bluestar™ was added to these dilutions, the chemiluminescence across the board had the

strongest reactions. In almost all cases, the chemiluminescence was considered to be either strong or very strong. This is because more blood was present in the samples, meaning more hemoglobin was present for the Bluestar™ to react with. When the 1:10 dilution had the 8M urea added to it, the reactions in all samples seemed to be unaffected by the change. Almost all the reactions, with the exception for two, were found to have the same strength. There was one sample that in fact had a decrease in the strength of its chemiluminescence. In most cases, the 1:10 dilution had the most chemiluminescence than any other dilution. However, in the 2-week carpet sample treated with urea, shown in figure 14, there is a decrease in chemiluminescence. This can possibly be explained by human error. Given that no other samples at no other dilution, time period, or surface experienced any decrease in chemiluminescence, it is thought that the sample could have been contaminated, or an error could have occurred in either sample application or preparation.

On the other hand, the 1:100 dilutions had a consistency more like water with a brownish tinge to it and had a very weak color. As Bluestar™ was added to these samples, the reactions were substantial and in many cases were as strong as the 1:10 dilutions. Additionally, these samples showed about the same level of improvement after using urea as the 1:10 dilutions did. Overall, there were no major changes in the level of chemiluminescence expressed in the 1:100 dilution samples for the most part. However, there were two samples that showed a clear increase in chemiluminescence. Figures 8 and 10 show the increase in chemiluminescence of the 1:100 dilution on the carpet sample after 1 week both before and after the addition of urea. These samples showed how the 1:100 dilution had only a moderate chemiluminescence at first, but then after adding

urea, a much stronger reaction occurred. Additionally, figures 9 and 11 show the increase in chemiluminescence of the 1:100 dilution on the tile sample 1 week after the addition of urea. These samples also showed significant increase in the level of reaction, raising its strength from strong to very strong.

The 1:1,000 dilutions were ones that garnered a much different reaction than the previous two. These dilutions were an almost totally clear liquid, and after drying left no visible stain on the surfaces. When Bluestar™ was added to these surfaces, many of the dilutions did not show any chemiluminescence, and some did but were classified as moderate or weak reactions. For most samples, the level of chemiluminescence stayed generally constant with only two samples showing enough of an improved chemiluminescence to change its strength category. The 1-week carpet sample was shown to have a weak chemiluminescence after treating it with urea when there was not any rection previously. This can be seen in Figure 9. Additionally, an increase in strength from being moderate in Figure 8 to a strong chemiluminescence shown in Figure 10 is shown.

Lastly, the 1:10,000 samples were the most dilute, and again had a consistency like that of water. This dilution was clear with no brown or red tinge and left no visible stain on the surfaces. These dilutions were some of the most variable in terms of the strength of their chemiluminescence. After Bluestar™ was added, the strength of their chemiluminescence, again, was mostly constant with the exception of three samples. All of the dilutions on carpet samples were undetectable, while all samples placed on the tile were detectable at least at a weak level. The difference between Figure 8 and Figure 10 shows how the 1-week tile sample shows the increase in strength of the

chemiluminescence. Additionally, the 3-week tile sample also shows an increase from weak to moderate chemiluminescence.

4.2 Aged Stains

This study also looked at whether or not urea can enhance aged bloodstains. The first bloodstain samples we studied were treated with urea after 48 hours. These bloodstains in this category were shown to have fairly normal levels of chemiluminescence and were consistent with the chemiluminescence levels of the other aged stains. There was an increase in chemiluminescence on the tile sample of this category, increasing the normally weak reaction of the 1:10,000 dilution of blood on the tile, to a moderate level of chemiluminescence.

The next group of bloodstains were analyzed after one week. These stains by far saw the most improvement in their chemiluminescence, especially in the tile sample. The tile sample after being treated with urea had a very strong chemiluminescence for all dilutions. It was even able to make the 1:10,000 dilution on the tile sample increase the most significantly of all samples tested, moving from a moderate strength to a very strong strength in chemiluminescence.

After the 1-week samples, we tested bloodstains that were aged for 2 weeks. These samples saw no improvement whatsoever on any surface or for any dilution. The only change in the strength of chemiluminescence was in the 1:10 dilution of the carpet sample. This, as discussed previously, was likely human error of some sort. Given that no other sample throughout the entire study had its chemiluminescence decreased by urea, it is believed to be human error and an outlier in this experiment.

Next, bloodstains aged for 3 weeks were tested with the Bluestar™ reagent. These results were similar to that of the 48-hour sample. There were mostly not changed except for the 1:10,000 dilution on the tile. This result seemed to indicate that the age of a bloodstain may not be a factor that affects how urea can enhance the chemiluminescence; urea may just be able to increase the chemiluminescence of extremely dilute samples, while not affecting samples that are aged.

Lastly, we tested bloodstains that were aged for 1 month. These results are interesting, as well as an outlier from the rest of the study. The non-urea treated tile sample showed an extremely strong reaction across the entire surface, and even on places where no blood was originally deposited. This, as discussed in a future section, is likely an anomaly, and possibly the result of an unknown contamination event. The tile sample treated with urea did not react in the same way, indicating that the sample is an outlier. This, although an outlier, shows a very important piece of information. This shows that the longer a bloodstain is aged, and the longer a bloodstain goes without being analyzed, the more chances it has to be contaminated. Contamination can, as it did in this experiment, render all analyses unable to be done, and ensure no reliable results can be received.

4.3 Surfaces

Two types of surfaces were used in this study to see how the addition of urea to these surfaces affects the chemiluminescence reaction of Bluestar™. The absorbent surfaces used in the study were the carpet samples. These samples were shown to have a much more condensed area of chemiluminescence. This is likely because the bloodstains were able to absorb immediately into the surface and did not spread out like what we see

on the non-absorbent, or tile, surfaces. In terms of the chemiluminescence reactions, it is shown that almost none of the samples that had increased chemiluminescence occurred on carpet samples. There were only two samples that had visible improvements on carpet, and those improvements are extremely difficult to see with the naked eye. The vast majority of chemiluminescence improvements were seen on the tile samples. In fact, the only sample that had a decrease in chemiluminescence was on a carpet sample. Although this is believed to be human error, it is important to note.

On the other hand, the tile samples that were used in this study saw the majority of improvements. In total there were six instances where the strength of the chemiluminescence reactions were improved. These samples occurred across all ages of stains and dilutions, but were slightly clustered in the 1-week aged stains and the 1:10,000 dilution. This data reveals that the surface on which the bloodstain is deposited significantly affects the ability of it to be detected. In the vast majority of carpet samples, chemiluminescence was not even seen with the 1:1,000 and 1:10,000 dilutions. On the other hand, on the tile samples, all dilutions were able to garner a chemiluminescent reaction, many of them even of moderate strength.

4.4 Enhancement

According to the digital analysis results, for only 10 of 40 sets of comparison, urea-treated samples have reached a higher strength of chemiluminescence than non-urea treated samples, and the overall degrees of enhancement are non-significant. However, it is worth noting that urea's effect can be observed clearly on some samples. For example, on a 2-week tile sample of 1:100 dilution, urea crystal-shaped chemiluminescence can be

observed. Although no enhancement was observed on that particular sample, the formation of such a shape may be further studied.

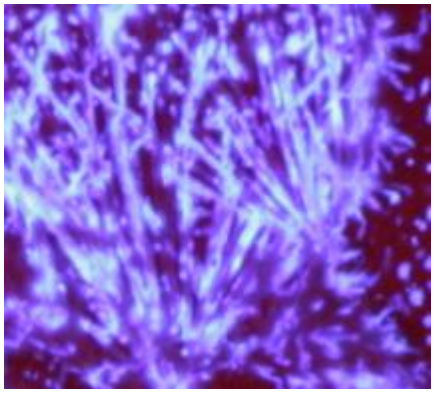

Figure 25: Urea crystal-shaped chemiluminescence

4.5 Errors and Anomalies

One of the most obvious errors seen in the results involved the 1-month tile sample without urea. Normally, and as seen in all other photographs, the chemiluminescence only appears where blood exists in the stain. However, in this sample shown in Figure 20, the chemiluminescence appeared throughout the entire tile and across all dilutions, even in places where no blood was deposited. We believe an unknown and undocumented contamination event occurred that caused the Bluestar™ to react the way it did. This error caused the urea-free sample to have a “very strong” designation and gives no basis from which to compare the results with the 1-month tile sample treated with urea.

Another anomaly seen in the experiment was the decrease in chemiluminescence of the 2-week urea treated carpet sample in the 1:10 dilution section. This is not seen anywhere else in the study and is the only example of where the chemiluminescence

decreased by a significant enough amount to change its designation. This is believed to be an anomaly for that very reason, and that human error could have been a part in it as well.

Overall, the study was conducted without any major issues or abnormal circumstances. It is believed that all other results were obtained free of error and that they are an accurate representation of the treatment of bloodstains with urea.

5. Conclusion

This study investigated how the strength of chemiluminescence of the Bluestar™ reaction with various types of bloodstains can be affected after treating these bloodstains with an 8M urea compound. We analyzed the strength of the chemiluminescence in two ways, visually and digitally. The visual analysis was intended to imitate how a crime scene forensic scientist in the field would perceive the results of the Bluestar™ test. The digital analysis was intended to imitate how a laboratory forensic scientist would perceive the results of the Bluestar™ test. These analytical techniques provided an all-encompassing look at how the pre-treating of bloodstains with 8M urea can affect the strength of the resulting chemiluminescence reactions.

Through visual analysis, we were able to determine that aged bloodstains did not have any significant effect on the ability of urea to improve the chemiluminescence of the samples. There was no data that could specifically attribute the improvement of chemiluminescence to the age of the stain. The only age-related result we obtained was the contamination of the non-urea treated 1-month tile sample. As previously discussed, the contamination of the sample was likely a result of the length of time it was allowed to

sit prior to its analysis. We were also able to determine that the urea treatment can improve very diluted bloodstains. This is shown in the results of the dilutions 1:1,000 and 1:10,000. They are the dilutions with most improvements in chemiluminescence. Lastly, the surface on which a bloodstain is deposited was also found to be an important factor. Six of the improvements in chemiluminescence occurred on the tile samples, while only two were seen on carpet. It was also determined that the carpet samples absorbed much of the bloodstain, which made it much more difficult for the Bluestar™ reagent to detect it.

Digital analysis of the photos of these samples was able to provide a quantifiable result to the study. In the data discussed in previous sections, we see further proof that more improvements are seen in the tile samples as opposed to the carpet samples. Seven improvements are noted on the tile samples, while only three are noted on the carpet samples. Additionally, these results show that almost all improvements across both surfaces and all time periods show a cluster of improvements at the 1:100, 1:1,000, and 1:10,000 dilutions. This furthers the results that higher dilution factors and the non-absorbent surface were the ones to have the most improvement in chemiluminescence after the urea was added.

In this study, we were able to further what Stoica et. al. had done in 2016 by investigating the theory that urea improved chemiluminescence. We did this by using various dilutions of blood, on two different surfaces, after various lengths of time. The results we see are that urea generally does not affect the strength of chemiluminescence, but in certain high dilution factors and non-absorbent surfaces, it can improve the strength of the Bluestar™ reaction. The results give no indication of any consistent decrease in strength or negative effects on the bloodstains that were utilized. Further

study is needed to investigate whether or not urea affects or degrades any DNA present in the sample and whether or not the urea has any use in improving the Bluestar™ method of bloodstain detection.

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