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Fluorescent benzazole dyes for bloodstain detection and bloody fingermark enhancement

Hélio L. Barros [a], Thayse Mileski [a], Crisle Dillenburg [a,b], Valter Stefani [a,*]

[a] Institute of Chemistry, Laboratory of New Organic Materials and Forensic Chemistry (LNMO-QF), Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9500, Caixa Postal 15003, CEP 91501-970 Porto Alegre, Brazil
[b] Instituto Geral de Perícia do Rio Grande do Sul (IGP-RS), Departamento de Papiloscopia, Av. Azenha, 255, CEP 90160-004 Porto Alegre, Brazil

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ABSTRACT

Bloodstains are the most common and useful type of physical evidence in crime investigation. The detection and examination of bloodstains are of great value in the reconstruction of a crime scene and linking a criminal or the victim with the crime scene. There is a wide variety of chemicals available for detecting bloodstains and enhancing bloody fingermarks, however, they have several associated challenges. For instance, they are generally unsuitable for dark and multi-colored substrates. Luminol and other chemiluminescent and fluorescent reagents can be used for dark and often multi-colored substrates, but they have numerous issues. For example, they can cause adverse effects in the detection and subsequent analysis of bloodstains; furthermore, since the oxidation of luminol is a fast and irreversible reaction, the user must act quickly to observe and capture an image or it will be lost. In this study, three fluorescent benzazole dyes were successfully used to detect bloodstains and enhance bloody fingermarks on surfaces of different natures (porous, semi-porous and non-porous) and colors (dark and multi-colored). To assess the efficiency of this approach, comparisons were performed with Amido Black, luminol and cyanoacrylate for the above-mentioned substrates. The proposed dyes have high selectivity and photochemistry stability, enhance and preserve mark details, and use only water as a solvent. In addition, the emitted fluorescence creates a sharp contrast with the bloodstain surfaces, meaning the bloodstains can be clearly visualized and photographed when excited with longwave ultraviolet light (365 nm).

1. Introduction

Luminescence imaging has become an essential tool in forensic science as a method for the detection of latent evidence [1]. Bloodstains are an important type of physical evidence encountered in criminal cases, such as murders, assaults, rapes, abortion and so on. The detection and examination of these marks are important to the reconstruction of the crime scene and linking a criminal or the victim to the crime scene. As criminals now often attempt to clean up the crime scene, the detection of bloodstains is not always an easy task. Diluted blood that is invisible to the naked eye can be detected through presumptive test reagents. Stains that yield positive presumptive test results are subjected to further analyses [2], but for this, it is important that the test reagent does not destroy the genetic material assessed in conventional genetic marker analyses [3]. Even when the identity of a stain may seem obvious to a forensic investigator, absolute confirmation is necessary in order for the evidence to be used in court to either prove or disprove a fact in a criminal case.

The application of luminol as a presumptive test for the detection of bloodstains has been carried out for more than 40 years in forensic science [4,5]. It is considered to be a highly sensitive reagent for detecting latent bloodstains, even when they are quite old, and is useful for investigating large expanses of surfaces and helping to reconstruct the events that may have occurred at a crime scene [6]. It has also been shown that following treatment with luminol, the DNA from bloodstains can be extracted and subsequently analyzed using Polymerase Chain Reaction (PCR) [7–10]. Although, the luminol reagent is widely used for detecting bloodstains, it has certain operational limitations, for example, the luminol reaction must be observed in conditions as dark as possible due to light sensitivity [11,12]. Luminol and its solutions are shown to be thermally unstable, meaning they should be protected from high temperatures [13]. Another important stumbling block when luminol is used for detecting bloodstains is its sensitivity to contaminating oxidizing compounds that produce oxidation in the reagent, giving a positive test both in the presence and absence

* Corresponding author.
E-mail addresses: h.barros@campus.fct.unl.pt (H.L. Barros), mileski.thayse@gmail.com (T. Mileski), crislevignol@gmail.com (C. Dillenburg), vstefani@iq.ufrgs.br (V. Stefani).

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of blood [14]. Furthermore, the oxidation of luminol is a fast and irreversible reaction, so the user must act quickly to observe and capture an image or it will be lost [15].

Non-fluorescent reagents, like Amido Black and ninhydrin, which form dark-colored dye complexes, are useful for detecting bloodstains and enhancing bloody fingermarks as they are compatible with protein fixative agents in acidic media, such as sulfosalicylic acid, to preserve detail, but they are not well suited for dark and multi-colored substrates [16,17].

The fluorescent benzazole dyes reported in this study can overcome some of the limitations inherent to luminol and other commercial dyes, and show high efficiency in the detection of bloodstains and enhancing bloody fingermarks. In our previous work, the dyes were successfully used to reveal latent fingermarks on the sticky side of different types and colors of adhesive tapes [18], and for the visualization of *Oxyuris equi* eggs [19].

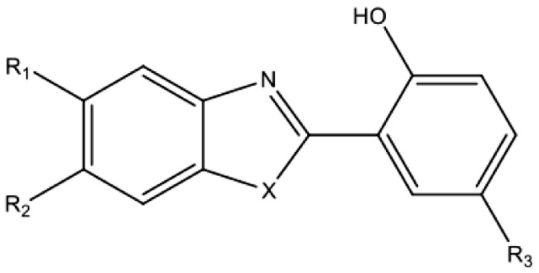
These fluorescent dyes are likely to be selectively anchored in the ridges of the fingermarks via a chemical reaction or electrostatic adsorption of the sulfonic group of dyes with the amine group of proteins or amino acids, constituents of fingermark residues [20]. We decided to use the same dyes for detecting bloodstains based on simple protein labeling and with data reported in the literature, using dyes containing sulfonic groups [20].

2. Materials and methods

All reagents and solvents used in the synthesis and purification of the dyes were purchased from Sigma-Aldrich, Acros or Merck, and were used without further purification. Spectroscopic solvents (Merck) were used to prepare samples for spectroscopic characterization. All dyes were characterized by infrared (IR) spectroscopy, 1H- and 13C-NMR spectroscopy, and mass spectrometry. 1H- and 13C-NMR spectra were obtained with a Varian INOVA-300 or Bruker Avance 400 operating at 300/400 MHz and 50/75 MHz, respectively. IR spectra were obtained using a Mattson Galaxy Series 3000 FT-IR in KBr and the mass spectra in a Shimadzu GCMS-QP20105 with a quadrupole analyzer. An ultraviolet light source with a 365 nm wavelength (black light lamp – 46 W, 50/60 Hz-Golden®) was used for visualizing and photographing developed bloodstains and bloody fingermarks. Throughout this work the term "ultraviolet light" will be used instead of the proper "ultraviolet radiation"; this follows the traditional name of "black light" for such radiation. The blood used was obtained from meat purchased at a supermarket and equine blood samples provided by the Faculty of Veterinary/Federal University of Rio Grande do Sul. These samples were stored at 4 °C and contained EDTA as an anticoagulant. The bloodstains of criminal evidence (a knife) found at the crime scene related to forensic casework (Section 3.9) were human blood samples. The bloodstain samples were treated with three fluorescent benzazole dyes and photographed using a Video Spectral Comparator from Foster Freeman, model VSC-5000, a Nikon photographic camera, model D600 (Fig. 14) or a SONY, model DSC-H10 (Figs. 15 and 16).

2.1. Synthesis of fluorescent benzazole dyes

The fluorescent dyes, named HB-7, HB-9 and HB-11, used in this study (Fig. 1) were previously synthesized, purified and characterized in our laboratory by the present authors and they are patent pending in the National Institute of Intellectual Property (Brazilian Patent Agency - BR102014030942) [21]. All dyes are water-soluble and fluorescent in the green region when exposed to longwave ultraviolet light of 365 nm.



HB-7, X=S, R_1=H, R_2=H, R_3=SO_3H
HB-9, X=O, R_1=H, R_2=CH_3, R_3=SO_3H
HB-11, X=O, R_1=SO_3H, R_2=H, R_3=NH_2

Fig. 1. Structures of the fluorescent benzazole dyes.

2.2. Bloodstains and latent blood fingermark samples

Bloodstains and latent bloody fingermark samples were impressed on surfaces of different natures (porous, semi-porous and non-porous) by three individuals (two males and one female). They were first deposited by placing the finger on the blood sample and then pressing the finger on selected surfaces. To study the sensitivity of these dyes for detecting bloody fingermarks, successive depositions of the same finger were made on the surface to obtain a depletion series of samples, each one presenting subsequently fewer quantities of deposited material. The substrates were stored under ambient conditions. The temperature was varied from 24 to 32 °C and the environment was dry and dusty. All experiments were performed in triplicate.

2.3. Performance on different types of surfaces

Different types of surfaces, common to forensic cases, were used to study the potential of these dyes in revealing bloodstains and latent bloody fingermarks. These included porous surfaces, such as ceramic, wood and polystyrene, paper and semi-porous and non-porous surfaces, such as painted aluminum, stainless steel, glass, polypropylene terephthalate and painted and non-painted polyethylene.

2.4. Evaluation of fixative solutions for blood

There are many blood fixatives that are toxic, carcinogenic and flammable, and some of them are based on methanol formulations. The use of 5-sulfosalicylic acid as a blood-fixing agent is already well known and reported in the literature [22]. However, for methanol-based dyes, 5-sulphosalicylic acid has shown poor results. In this case, an alcoholic solution fixative is more efficient. A aqueous solution of 2% 5-sulfosalicylic acid has been shown to be a safe and effective fixative agent for water-based dyes [22]. Seven different solvents were tested for fixing bloodstains before the use of fluorescent dyes, including some already described in the literature [22]. We tested an aqueous solution with 2% 5-sulfosalycilic acid, a methanolic solution with 2% 5-sulfosalycilic acid, an ethanolic solution with 2% 5-sulfosalycilic acid, an ethanol:water (40:60) solution with 2% 5-sulfosalycilic acid, an ethanol:water (40:60) solution, methanol p.a. and ethanol p.a. ("p.a." stands for the pro analysis grade). For this study, HB-7 was used under the same conditions (the type of surface, time of fixation and dyeing of bloodstains).

2.5. Formulation of staining solutions

Staining solutions (0.1% (w/v)) were prepared by dissolving 0.1 g of each dye in 100 mL of distilled water. The mixture was stirred until the dye was dissolved completely. When necessary, the mixture was heated or sonicated and filtered.

2.6. Protocol for detecting bloodstains and enhancing bloody fingermarks

The bloodstains and latent bloody fingermarks were firstly fixed by a solution of ethanol:water (40:60) with 2% 5-sulfosalicylic acid. The fixative solution was applied by submerging or spraying the substrate, depending on the substrate's nature. The samples remained in contact with the fixative solution for 1 min and then dried at room temperature. They were then washed with water to remove any excess fixative solution on the substrate surface. In the next step, these samples were immersed in or the substrate was sprayed with staining solutions, usually for 5 min. After soaking, the substrates were washed with water to remove any excess dye, and then dried at room temperature or with a dryer. In order to observe the detected bloodstains or bloody fingermarks, the surface was exposed to an ultraviolet light source with a 365 nm wavelength (black light lamp - Golden®). This procedure could be repeated using the same steps in order to improve the quality of the fingermark minutiae. Finally, the detected fingermarks were photographed for comparison, documentation and archiving purposes using a photographic camera.

2.7. Protein labeling test

To evaluate the affinity of the dye with protein, a protein labeling test was performed using a whey protein (NeoNutri®). The protein sample (approximately 40 mg of powder) was fixed on a glass slide with a fixative solution of ethanol:water (40:60) with 2% 5-sulfosalicylic acid, and the dye solution (HB-7) was applied, as described in Section 2.6.

2.8. False positive test

A wide range of substances that might be mistaken in forensic testing for blood was examined. These were preparations of food products, such as Coca-Cola™, tomato ketchup, beetroot juice, pepper sauce, tomato paste and household products considered to interfere with the reaction, which are used as cleaning agents, namely

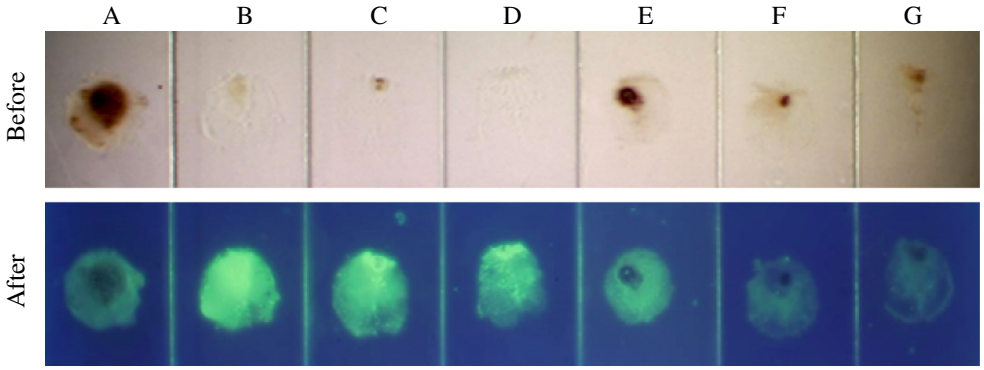


Fig. 2. Images of blood fixed with different solutions, processed with HB-7 and observed under ultraviolet light of 365 nm. (A) Aqueous solution with 2% 5-sulfosalycilic acid, (B) methanolic solution with 2% 5-sulfosalycilic acid, (C) ethanolic solution with 2% 5-sulfosalycilic acid, (D) ethanol: water (40:60) solution with 2% 5-sulfosalycilic acid, (E) ethanol:water (40: 60) solution, (F) methanol p.a. and (G) ethanol p.a.

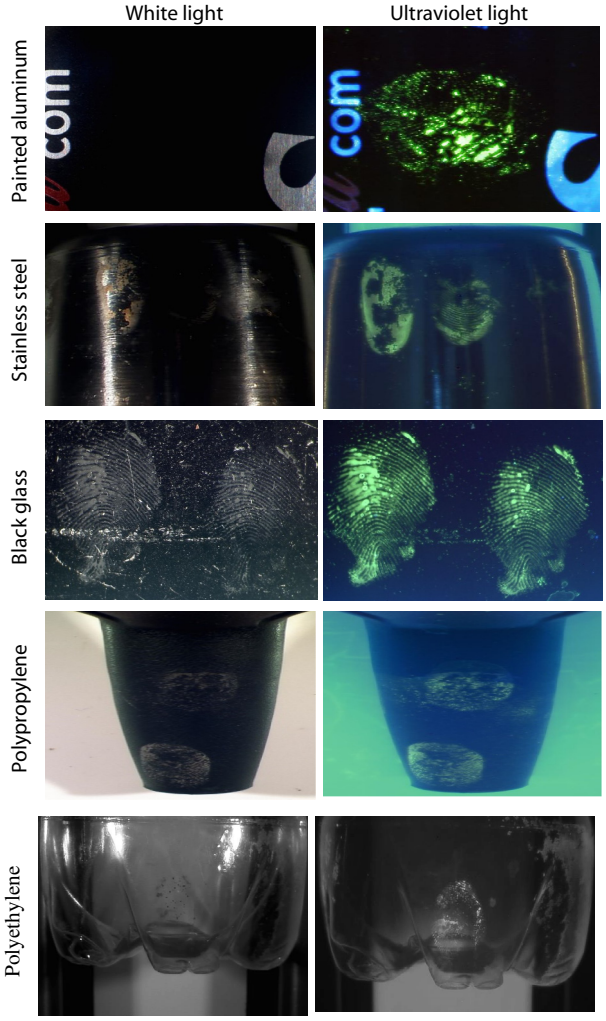


Fig. 3. Images of bloodstains on semi-porous and non-porous surfaces (painted aluminum (soda can), stainless steel, black glass, dark colored polypropylene and light colored polyethylene) processed with HB-7 (concentration of 0.1% w/v) under white and ultraviolet light of 365 nm.

sodium hypochlorite (bleaches) and detergent. All samples were applied on glass slides and exposed to ambient air for one week to complete drying. Samples were fixed using a fixative solution (ethanol:water (40:60) with 2% 5-sulfosalicylic acid) and the solvent was allowed to evaporate at room temperature. The dye solution was applied with a Pasteur pipette and left in contact with the sample for 5 min. The excess dye solution was removed with water and the glass slide was observed and photographed under ultraviolet light of 365 nm.

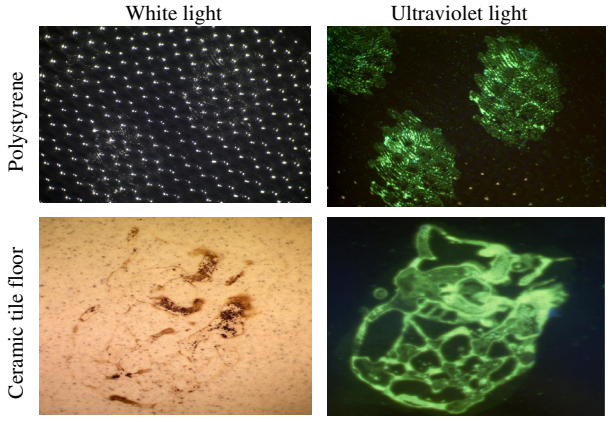


Fig. 4. Images of bloodstains on porous surfaces (ceramic tile floor and expanded polystyrene) processed with HB-7 (concentration of 0.1% w/v) under white and ultraviolet light of 365 nm.

2.9. Technique sequence for detecting bloody fingermarks

This study was performed in order to determine a better sequence to use the developers of bloody fingermarks. Sometimes, it is necessary to use more than one technique for the visualization of latent fingermarks, and the previous technique can not interfere the subsequent analysis. The fluorescent dye HB-7 proposed here for detecting latent bloody fingermarks was tested in combination with the main techniques currently used for this purpose, i.e., Amido Black, cyanoacrylate and luminol. The fixative solution used was ethanol:water (40:60) with 2% 5-sulfosalycilic acid. The dye solution (HB-7) used was prepared as described in Section 2.5. A solution of Amido Black (1% w/v) was prepared in ethanol:water:acetic acid (70:25:5). The detection of fingermarks with cyanoacrylate was effectuated as described in Ref. [1], using the Omega-Print cyanoacrylate fuming compound for latent print development (Sirchie). The blood samples were fixed using a fixative solution of ethanol:water (40:60) with 2% 5-sulfosalicylic acid. Two luminol reagents were used: one was previously synthesized by us and another was purchased from Aldrich. The staining solution was prepared as described by Weber [21]. Initially, the sequential application of HB-7 and Amido Black was studied. The blood samples were fixed on an aluminum surface and processed with HB-7, followed by Amido Black and vice-versa. The samples were observed and photographed under ultraviolet light to visualize the fluorescence emitted by HB-7. The contact time of HB-7 with the aluminum surface ranged from 3 to 30 min and the Amido Black was maintained for 2 min. In the second step, cyanoacrylate, HB-7 and luminol were applied in sequence to detect fingermarks on a soda bottle. To evaluate the adherence of cyanoacrylate on blood, three clean fingermarks and four fingermarks contaminated with blood were placed on glass slides and processed with cyanoacrylate.

2.10. Bloody shoeprints

Shoeprints containing blood were deposited on a ceramic surface and fixed with a solution of ethanol:water (40:60) with 2% 5-sulfosalicylic acid and subsequently processed with the HB-7 dye, as described in Section 2.6. The HB-7 solution was prepared according to Section 2.5.

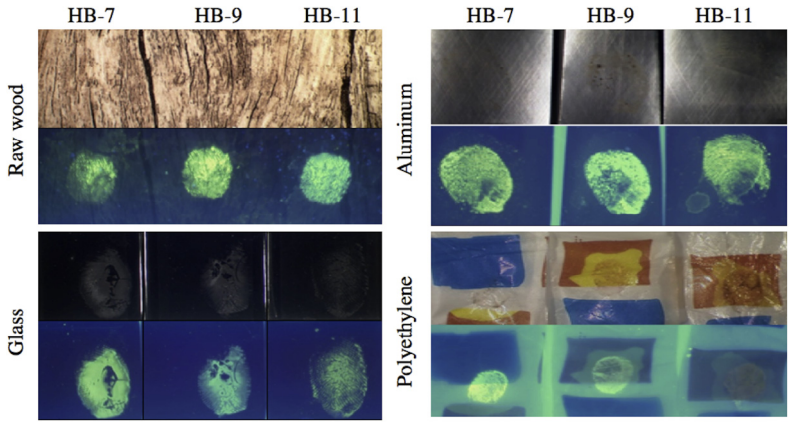


Fig. 5. Images of bloodstains detected with HB-7, HB-9 and HB-11 (concentration of 0.1% w/v) on different types of surfaces and photographed under white light (top) and ultraviolet light of 365 nm (bottom).

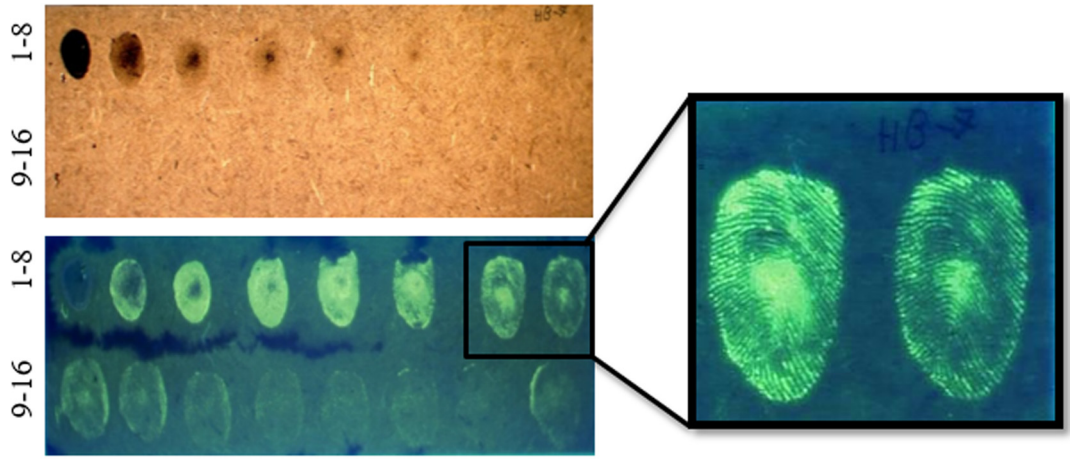


Fig. 6. Images of fingermarks contaminated with blood deposited in sequence (1–16) under white light (top left), detected with HB-7 and observed under white light and ultraviolet light of 365 nm (bottom left). Enlarged image of sequences 7 and 8 (right).

2.11. Forensic casework

A piece of criminal evidence (a paring knife) with human blood was found at a homicide crime scene and taken to the papiloscopy expertise of the Instituto Geral de Pericia do Rio Grande do Sul state (IGP-RS). The evidence was processed with the dye HB-7 following Amido Black. The HB-7 solution and Amido Black were prepared according to Sections 2.5 and 2.9. The evidence was processed as reported in Section 2.6 with the exception of the fixative and dye solutions, which were applied using a Pasteur pipette, not by immersion or spraying.

3. Results and discussion

3.1. Evaluation of fixative solutions for blood

In order to determine the best solution for fixing bloodstains, seven fixative solutions were tested, including some already described in the literature. For this study, HB-7 was used under the same conditions (the type of surface, time of fixation and dyeing of bloodstains). As can be seen in Fig. 2, all the solutions fixed the bloodstains. However, some solutions show better results than others (improved blood fixing capability and/or dilution of blood sample that leads to increasing fluorescence intensity by inhibition of fluorescence reabsorption by the sample).

The methanolic solution with 2% 5-sulfosalycilic acid (B), the ethanolic solution with 2% 5-sulfosalycilic acid (C) and the ethanol:water (40:60) solution with 2% 5-sulfosalycilic acid (D) showed better results, with a greater intensity of fluorescence emitted by HB-7. It can be noted that the solutions containing alcohol generated higher intensity fluorescence, which improved even more in the presence of 5-sulfosalycilic acid. The combination of sulfosalicylic acid with alcohol probably leads to denaturation of proteins in the blood, increasing the contact surface of the sulfonic acid group of the dye with the amine groups of the protein. We recommend the use of an ethanol:water (40: 60) solution with 2% 5-sulfosalicylic acid because this has no methanol in its composition (toxic) and the mixture with water decreases the flammability of the solution.

3.2. Bloodstains detected on different types of surfaces

Different types of surface, such as porous, semi-porous and non-porous, were used to evaluate the efficiency of the dye for detecting bloodstains. Figs. 3 and 4 show bloodstains processed with the HB-7 fluorescent dye on different types of surface and photographed under white and ultraviolet light of 365 nm. Surfaces were subjected to bloodstain detection processes immediately after the bloodstains were deposited (fresh bloodstains). The dye shows high selectivity to the bloodstains and no reaction was observed with the surface background. The porous surfaces in Fig. 4 are not cellulose based surfaces and that these are discussed later.

Fig. 5 shows the comparative results of bloodstains detected with HB-7, HB-9 and HB-11 on raw wood, glass, aluminum and

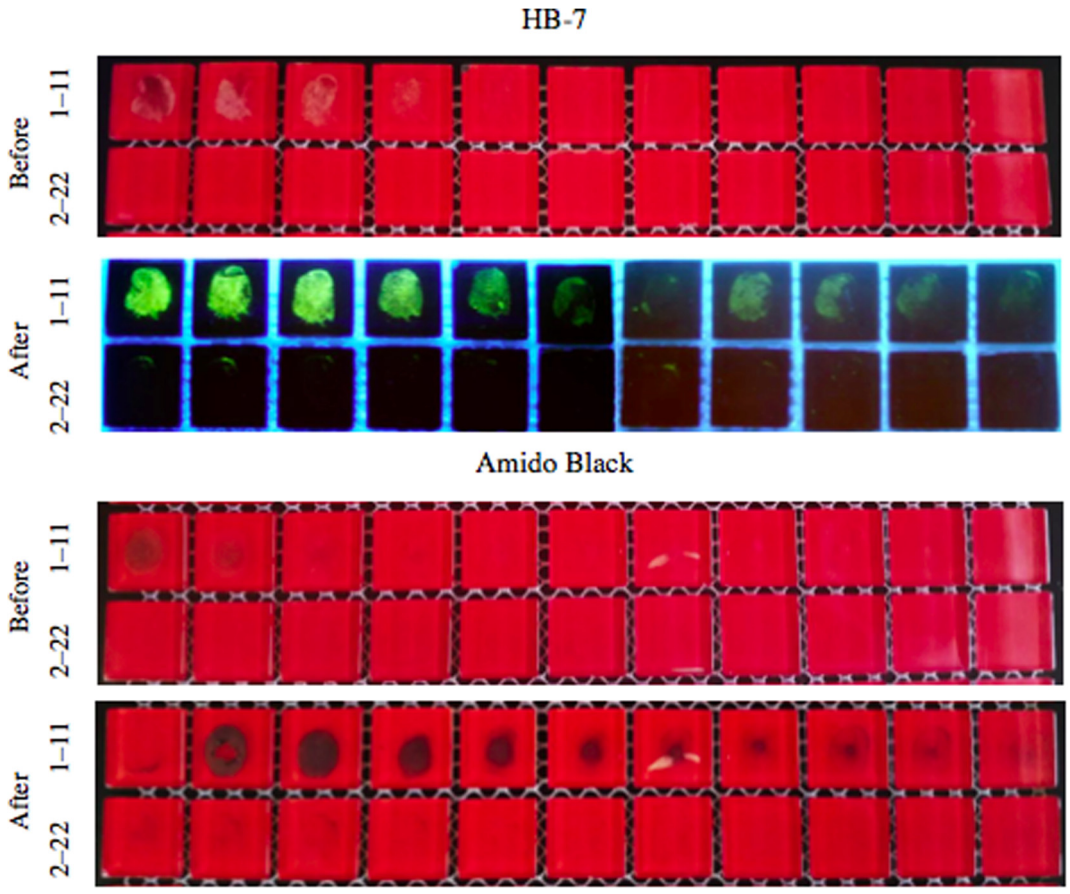


Fig. 7. Images of sequence fresh bloody fingermarks (1–22) detected on a glass surface by HB-7 (top) and Amido Black (bottom).

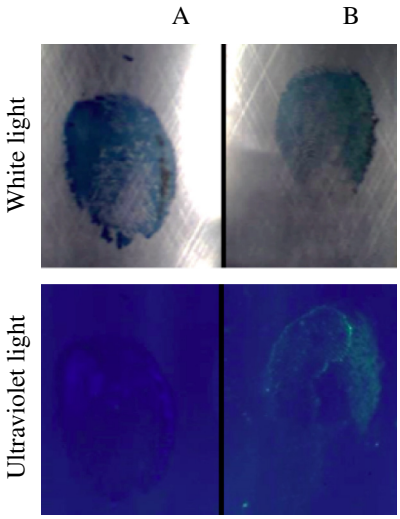


Fig. 8. Images of fingermarks processed with HB-7 and Amigo Black in sequence (aluminum surface) and observed under white light and ultraviolet light of 365 nm. A: HB-7 followed by Amido Black and B: Amido Black followed by HB-7.

painted polyethylene. In general, HB-9 and HB-11 work for the same type of surface tested with HB-7, with the exception of polyethylene. In the case of polyethylene, the poor results can be associated to the ink on the material surface, which was dissolved with the application of dyes interfering with the interaction between dyes and bloodstains or the fluorescence emitted by the dye. The only surfaces that these dyes did not work for detecting bloodstains were the porous surfaces, such as paper and fabric.

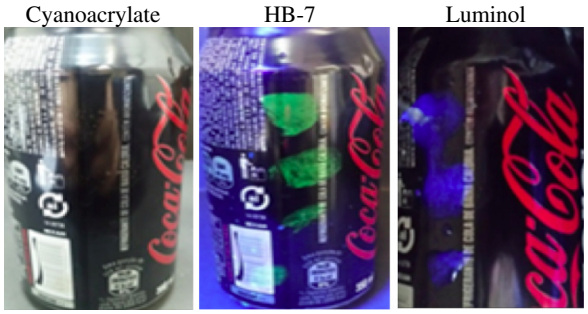


Fig. 9. Sequential analysis using cyanoacrylate, followed by HB-7 and luminol on a soda can (metal surface).

For these materials, the dye solutions were absorbed by the surface causing entire surface staining and not creating the contrast between bloodstains and the background. Furthermore, porous materials that present autofluorescence, such as white papers or fabric, show poor results due to interference of the light emitted by the material surface with the light emitted by dyes. Even using an orange filter, usually applied on these surfaces to eliminate interference of autofluorescence, it was not possible to visualize the bloodstains. This could possibly be solved by using a different excitation source (with a defined wavelength that only allows excitation of the fluorescent dye staining).

3.3. Sensitivity of dyes in the detection of fingermarks in blood

In this study, the sensitivity of the dyes in the detection of fingermarks contaminated with different amounts of blood was eval-

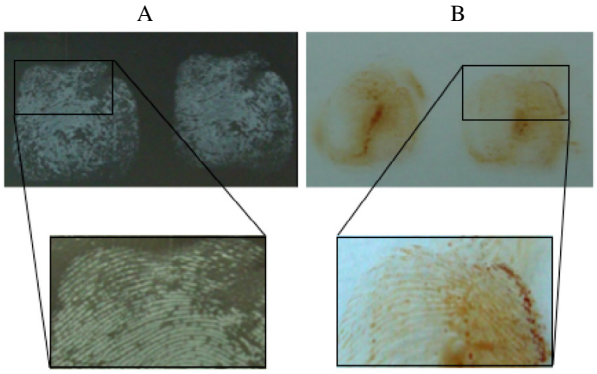


Fig. 10. Images of fingermarks processed with cyanoacrylate on transparent glass surface. A: Clean fingermarks (dark paper was placed under the transparent glass) and B: fingermarks contaminated with blood.

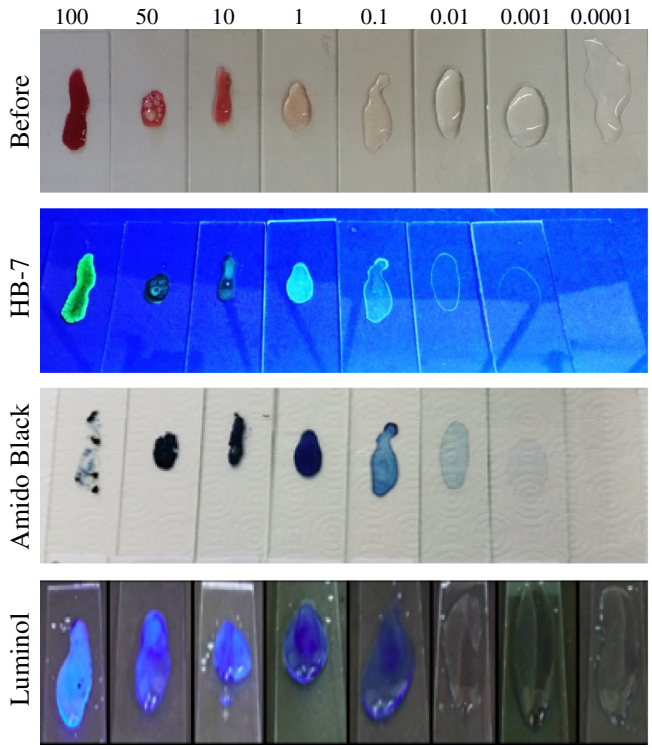


Fig. 11. Images of blood samples diluted in water at different concentrations (100% to 0.0001% (v/v)), applied to glass slides, and processed with HB-7, Amido Black and luminol, obtained under white light and ultraviolet light of 365 nm.

uated. An approach was used where these fingermarks were deposited sequentially on the wood surface, without touching anything between the depositions, to give a series of fingermarks with decreasing amounts of deposited blood residues. These series consisted of sixteen consecutive finger depositions (Fig. 6). After the use of fluorescent dye HB-7, all marks were visible (top left), different from the visual analysis of bloodstains under white light (bottom left) where only marks up to sequence 6 with no details of the fingermarks were shown. A bright fingermark image with good ridge detailing was produced when the fingermarks in blood were treated with HB-7. A slight decrease in detection quality was found for the fingermarks in blood after sequence 11. However, even for the weakest deposits, the image still shows marks of blood. This demonstrates that this fluorescent dye is effective even at low bloodstain concentrations and is sensitive and selective to the

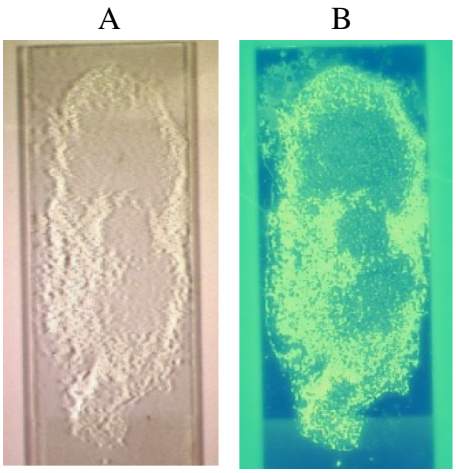


Fig. 12. Images of protein before (A) and after labeling with HB-7 (B) and observed under ultraviolet light of 365 nm.

bloodstain residue. It is important to note that for high concentrations of blood (sequence 1), the details of the fingermarks are compromised probably due to the interference of blood in the emitted fluorescence (by quenching or suppressing the fluorescence). Furthermore, the quality of the detected fingermarks also depends on the way and pressure force exercised when the fingermarks are deposited against the surface. The approaches involving fingerprints were carried out according to the guidelines for the assessment of fingermark detection techniques recommended by the International Fingerprint Research Group (IFRG) [22].

The sequential detection analysis was also performed with Amido Black and compared with HB-7. The fingermarks in blood were deposited sequentially on a glass surface (a series of 22 consecutive finger depositions) and detected with the dyes. Fig. 7 show the results of fingermarks in blood after and before using HB-7 and Amido Black. All images were obtained under white light, except when using HB-7 (the use of ultraviolet light of 365 nm is required to observe the fingermarks). As can be seen, the two dyes exhibit very similar sensitivity. In both cases, it was possible to detect bloody fingermarks up to the 11th sequence (first row). It is important to note that the fingerprints in blood are only weakly perceptible before use of the dye. However, the marks become visible with the use of the dye, allowing their identification. Again, it was possible to observe that the highest concentration of blood can lead to an ineffective result. For example, in the first deposition, after staining with Amido Black, the decrease of bloodstain concentration after washing was observed, making it difficult to observe the marks.

3.4. Technique sequence for detecting bloody fingermarks

This study was performed in order to determine the best sequence of techniques that can be applied to reveal bloody fingermarks without any loss of integrity of the minutiae of the fingermarks. The first combination performed was the use of HB-7 followed by Amido Black and vice versa. As can be seen in Fig. 8, the results showed that Amido Black inhibits the fluorescence of HB-7. The result was poor when the analysis was performed in reverse order, probably because HB-7 interacted weakly with the proteins or almost all active sites of the proteins were already occupied with Amido Black, preventing interaction with HB-7. Even when keeping this reagent in contact with the blood sample for a long time (30 min), the result is not efficient, because only some parts of the prints were marked. If the two reagents compete

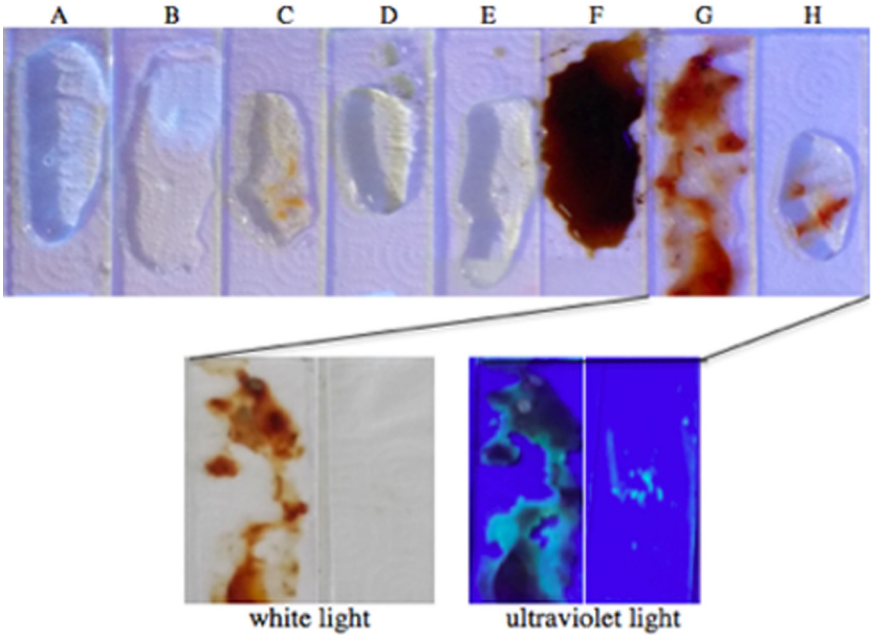


Fig. 13. Samples of food and household products in contact with fluorescent dye solution HB-7 and under ultraviolet light of 365 nm (top). Image of false positive for tomato paste (G) and pepper sauce (H) under white light and ultraviolet light of 365 nm (bottom). (A): Sodium hypochlorite, (B): washing detergent, (C): ketchup, (D): Coca-Cola™, (E): beetroot juice, (F): coffee, (F): beetroot, (G): tomato paste and (H): pepper sauce.

Fig. 14. Images of bloody shoeprints processed with HB-7 under white light and ultraviolet light of 365 nm.

for the same sites of protein, Amido Black probably has the greater strength of interaction than HB-7, because it quickly stains fingermarks, but the opposite does not occur efficiently. Alternatively, the intense dark blue color of Amido Black can block the fluorescence emitted by HB-7. This result shows that HB-7 must be used before Amido Black.

Fig. 9 shows the result obtained with the sequential application of cyanoacrylate, HB-7 and luminol. Amido Black was not used, but it could be applied after HB-7 without affecting the following analysis. After analyzing all combinations of techniques, we determined that the best sequence is cyanoacrylate, followed by HB-7 and luminol. This sequential combination allowed for the detection of latent fingermarks without affecting their integrity. An interesting work was described by Volders, in which he explored the possibility to improve visualization of blood traces, after they were detected with luminol, by exposing them to other chemicals, such as leuco crystal violet (LCV), acid yellow 7 and/or hungarian red [23].

As can be seen, fingermarks were poorly observed on objects analyzed using the cyanoacrylate fuming. Therefore, we performed a test to check his adherence on blood samples. Samples were prepared by placing a dirty thumb with and without blood on a transparent glass surface. After the use of cyanoacrylate, only fingermarks without blood were revealed (appearance of a white cover on the ridges of fingermarks, as expected), proving fingermarks contaminated with blood cannot be revealed with cyanoacrylate. In case of fingermarks sample without blood, dark paper was placed under the glass to ensure that maximum fingermarks details are visualized. This procedure was not necessary for bloody fingermarks due to the dark color of the blood that produces a good background development. It is important to note that cyanoacrylate does not interfere in other analyses, but other reagents can interfere in the analysis of cyanoacrylate to detect latent fingermarks. For this reason, cyanoacrylate must be the first reagent used, followed by HB-7, Amido Black and luminol, as previously determined in this study.

Fig. 10 shows the positive result of the detection of pure fingermarks with cyanoacrylate (A) and the negative result for fingermarks contaminated with blood (B).

3.5. Sensitivity comparison between HB-7, Amido Black and luminol

The sensitivity of HB-7, Amido Black and luminol was evaluated using these dyes in bloodstains (at different concentrations) on a glass surface. These dyes were applied by the optimum sequence already defined in this work. Fig. 11 shows the results obtained with the three dyes. It is possible to observe the fluorescence emitted by the HB-7 up to a concentration of 0.001% (v/v). Again, it was possible to observe that HB-7 and Amido Black have a similar sensitivity. This sensitivity is between about 1:10,000 and 1:100,000. Luminol showed lower sensitivity when compared with HB-7

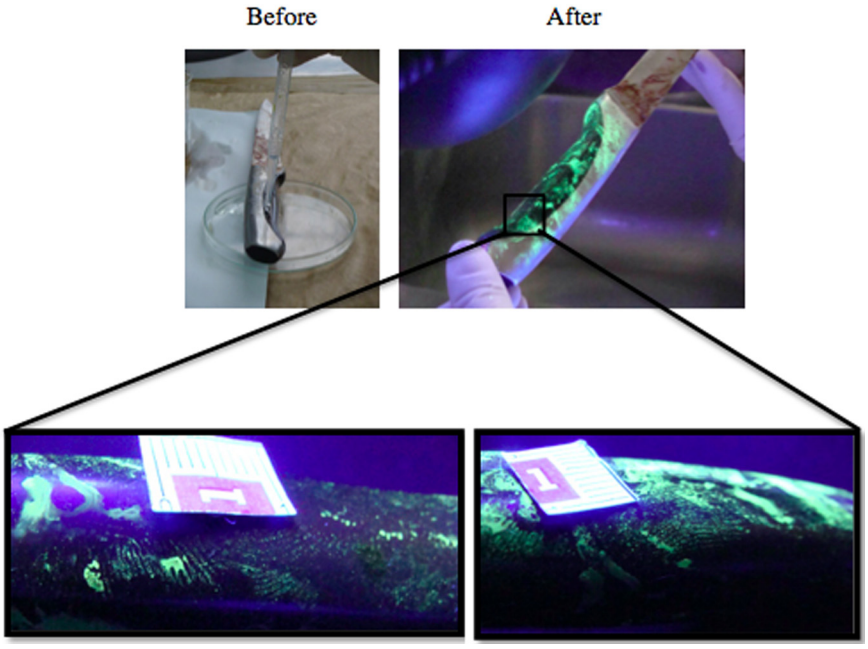


Fig. 15. Images of knife processed with HB-7 and observed under white light and ultraviolet light of 365 nm.

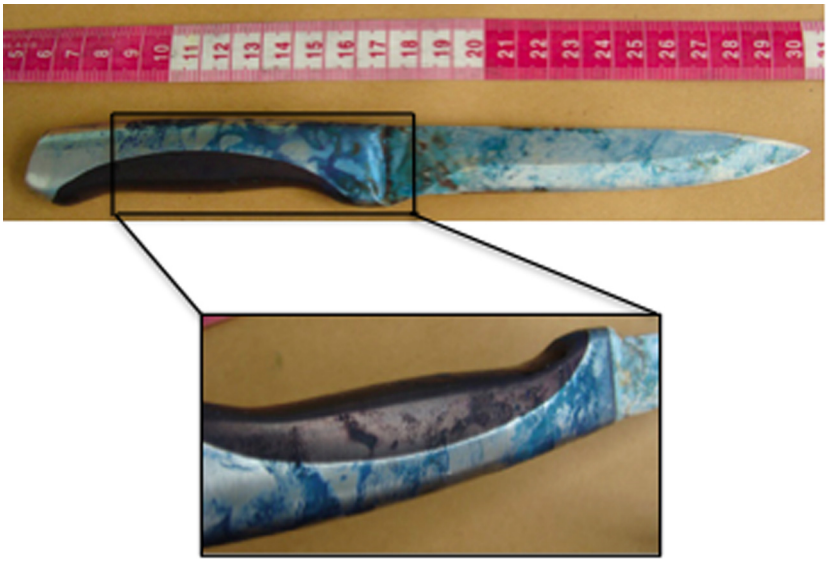


Fig. 16. Image of knife processed with Amido Black and observed under white light.

and Amido Black (about 1:1000). As expected, the fluorescence observed decreased with increasing dilution of the sample. It is important to note that the study was performed by applying the dyes in sequence in order to determine if there is any influence on the sensitivity results of the subsequent analysis.

3.6. Protein labeling test

We suspect that the dye interacts with proteins in blood based on studies reported in the literature [20], where others dyes containing sulfonic group were used. It is proposed that the interaction occurs via a chemical reaction or electrostatic adsorption of the sulfonic group of dyes with the amine group of proteins or amino acids, constituents of blood residues [20]. We performed a simple protein labeling test with HB-7 to evaluate its affinity. Initially, the protein sample (approximately 40 mg of powder) was fixed using a fixative solution and then the fluorescent dye solution was applied and the sample was washed with water, as described in Section 2.6. As can be seen in Fig. 12, the protein shows an intense emission of fluorescence after it was processed with HB-7, confirming that these dyes have a high affinity for proteins.

3.7. False positive test

Currently, a variety of presumptive blood tests have been described [20]. The purpose of this study was to investigate the existence of false positives by products that are potentially present during the detection of blood traces and that might be mistaken in forensic tests for blood. For this, eight different substances were reported and the results are shown in Fig. 13. As shown, only

two of the tested substances produced a level of fluorescence detectable when processed with fluorescent dye HB-7 and exposed to ultraviolet light of 365 nm. This can be explained by the fact that plant substances have proteins in their constitution and therefore can generate false positives due to the interaction of sulfonic acid groups of the dyes with the amino groups of proteins [24]. The other substances were not adhered to the surface of the slide when fixed with the fixing solution (ethanol: water (40:60) in 2% 5-sulfosalicylic acid) and consequently were detached with washing after processing with the fluorescent dye solution. This is probably due to the absence of proteins in these substances, which would be denatured and fixed by protein fixative agents in acidic media, such as sulfosalicylic acid, to preserve detail.

3.8. Use of HB-7 for detecting bloody shoeprints

The fluorescent dye reported here (HB-7) was used to detect shoeprints containing blood that are not visible to the naked eye, generally found at crime scenes. As can be seen in Fig. 14, the bloody shoeprints cannot be observed in white light. However, when they are processed with HB-7 and exposed to ultraviolet light, the shoeprints become clearly visible due to the fluorescence emitted by the dye. The dye shows high selectivity to the bloody shoeprints and no reaction was observed with the material surface where the shoeprints are located.

3.9. Use of HB-7 in forensic casework

Amido Black has been used successfully on light colored surfaces. It is not typically used on dark colored surfaces due to its intense dark blue color (without any luminescence). Therefore, the search for new reagents that can be successfully applied for the detection of bloodstains and bloody fingermarks on dark surfaces is an important area of research.

The fluorescent dyes reported here show excellent results in the detection of bloodstains and bloody fingermarks on surfaces of different natures (porous, non-porous and semi-porous) and colors (light colored and dark surfaces). Fig. 15 shows the result of the processing of a piece of criminal evidence (a paring knife) containing human blood with fluorescent dye HB-7. The evidence was found at a homicide crime scene and taken to the papiloscopy expertise of the Instituto Geral de Pericia do Rio Grande do Sul (IGP-RS). In the case of Amido Black, its previous application would show a poor result because of the dark color of the knife handle. Therefore, we follow the optimum sequence determined in this article for detecting bloodstains and bloody fingermarks, i.e., fluorescent dye HB-7, followed Amido Black. Firstly, the bloodstains were fixed by ethanol: water (40:60) in 2% 5-sulfosalicylic acid (fixative solution) and then processed with the HB-7 dye. As can be seen, the dye showed excellent results, allowing good visualization of bloodstains and some fingermarks on the knife handle. In a second step, the Amido Black was applied to ensure that everything had been revealed (Fig. 16). Unfortunately, it was not possible to identify the criminal because the fingermarks did not have enough minutiae to proceed to the fingermark confrontation. However, it is well known that the identification of fingermarks does not always depend on the reagents used, but the way that the object is held or handled by the individual.

4. Conclusions

It has been demonstrated that the fluorescent dyes reported in this study can be successfully applied for detecting bloodstains and latent bloody fingermarks on different types and colors of surfaces. Furthermore, our proposed method for the detection of latent fingermarks has been proved sensitive, selective and effective, even when a much diluted dye solution is used. It does not require complex instrumentation, pre or post treatment of the processed surface or special storage conditions. Another advantage is the fact that these fluorescent dyes are soluble in water. This avoids the use of toxic organic solvents, which are a risk to the health of those who frequently handle them. The high chemical and photochemical stability of the dyes, as well as the low cost of their synthesis and purification, make these new dyes a simple, versatile and excellent forensic tool. A study in order to confirm the reaction mechanism of these new dyes with bloodstains and to assess whether the use of these dyes can damage samples, and prevent further processing, such as DNA analysis, is still in progress and will be published in due course.

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