The Effect of Mark Enhancement Techniques on the Subsequent Detection of Semen/Spermatozoa

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Highlights

- Strategy to maximise evidence recovery (semen and mark enhancement) and efficiency
- 6 out of 7 techniques did not affect the subsequent detection of semen/spermatozoa
- Iron-oxide powder suspension was detrimental to the detection of semen/spermatozoa
- Protein stains resulted in loss of AP test; however, sperm heads were detected
Abstract

Fingermarks, footwear marks, blood and semen are amongst the most commonly encountered types of evidence at crime scenes. Previous work has extensively investigated fingermark and blood enhancement techniques and a sequence developed to maximise evidence recovery; however, there is limited research as to the effect of these techniques on the subsequent detection of body fluids such as semen.

In this study, seven fingermark and blood enhancement techniques (e.g. powder suspension, cyanoacrylate fuming and acid violet 17) were employed followed by the subsequent detection of semen/spermatozoa. Other variables included in the study were the use of two substrates (white ceramic tiles and grey laminate flooring), a depletion series and ageing periods of 1, 7, 14 and 28 days. The effect these techniques had on the subsequent detection of semen was assessed by visual and fluorescence examination followed by presumptive and confirmatory testing for semen and spermatozoa.

Results found that protein stains (acid violet 17 and acid yellow 7) caused a loss in presumptive test reactivity; however, sperm heads were still observed using microscopic examination after extraction and staining. The use of black magnetic powder, Bluestar® Forensic Magnum luminol, Lumicyano™ 4% and cyanoacrylate fuming followed by basic yellow 40 staining did not hinder subsequent presumptive and confirmatory tests for semen and sperm heads. Powder suspension caused a loss in both presumptive test reactivity and sperm heads from the substrate. In general, the enhancement techniques resulted in the improved visualisation of the semen stains under white and violet/blue light. The results from this study aim to provide a strategy to maximise evidence recovery and improve efficiency in an integrated forensic approach.

Keywords: semen, spermatozoa, blood, fluorescence, finger/footwear mark enhancement, integrated forensic approach
Introduction

Fingermarks, footwear marks and biological fluids are routinely collected from crime scenes to aid criminal investigations in the identification of offenders [1-2]. Current methodology for the recovery of latent (not visible to the naked eye) fingermarks and footwear marks can adversely affect the subsequent detection of biological fluids and vice versa. Despite the importance of these types of evidence, an examination of the literature and discussions with forensic practitioners has indicated that, currently, there are no guidelines or recommendations for the maximum recovery of latent marks and biological fluids. If an item of evidence is suspected of having latent fingermarks on it as well as semen deposits, the application of two different tests will be required; however, which forensic test is applied first and whether the application of one test affects the other has not been fully investigated.

Speculative searching for semen

Dry stains of semen will fluoresce under excitation light sources of wavelengths 300-480nm [3]. Fluorescence is useful as an initial and non-destructive tool that may be used for speculative searching of semen; however, certain light sources and wavelengths may be damaging to DNA. The use of Quaser high intensity light sources has been proven non-destructive to DNA at all wavelengths for exposures of up to 30 minutes [4].

Presumptive test for semen

The acid phosphatase (AP) reagent was developed in 1957 by Stuart Kind [5] and is widely used as a presumptive test for semen by reacting with AP present in seminal fluid to give a purple colour. The AP reagent is only considered as a presumptive test for semen due to the number of potential false positive reactions including those from semen-free vaginal material, faecal material, foods and beverages such as tea [6]. The use of the AP test assists in narrowing down areas for further examination and testing for seminal material which can be used for subsequent DNA analysis. The AP reagent consists of sodium acetate, acetic acid, α-naphthyl phosphate disodium salt and brentamine fast black K salt. In the presence of acid phosphatase, α-naphthyl phosphate is hydrolysed to produce α-naphthol. This then combines with the fast black K to produce a purple azo dye [7]. Since first described by Kind in 1957, the application process of the AP reagent has remained largely unchanged. Until recently, it was believed that two minutes was sufficient time to allow for a purple colouration to
develop and indicate a positive AP reaction. Additionally, a negative reaction was generally recorded if no purple colour was developed or if the time required for the colour to develop was greater than 2 minutes. Recent studies have suggested that this two minute cut off was insufficient to detect some dilutions of semen as high as 1 in 20 [7]. Based on a number of studies [7-9], it is now suggested that reaction times of at least 10 minutes, and up to 15 minutes, be allowed as to not overlook potential dilute seminal evidence.

AP is water soluble and often found in high concentrations in seminal fluid; however, this concentration will vary day to day in individuals and between different individuals (intra and inter person variation). There are two methods of AP testing samples from a substrate; direct and indirect. In the direct method, a water-moistened filter paper is pressed onto the area of interest or a moistened swab is rolled over an area of interest and AP reagent is then added to the filter paper or swab. There is also the possibility of applying the AP reagent directly onto the substrate; however, there is an increased possibility of false positives associated with this [10]. For indirect testing, the area of interest may be swabbed using a moistened swab and an extract made from this, containing both seminal fluid and spermatozoa. A drop of the extract is then applied to filter paper before applying AP reagent. AP reagent can be applied as a drop, spray or by aerosol with recent studies suggesting that application by aerosol is the most effective method of application for detection [10].

**Confirmatory test for semen**

A common method of confirmatory testing for the presence of spermatozoa in semen is microscopic examination. Following a positive AP reaction, the area of interest can either be cut out (if fabric or similar material) or swabbed (if item cannot be easily cut). The fabric or swab head can then be placed into a microcentrifuge tube and distilled water added. Combined action of the water plus agitation will remove many of the sperm heads (if present) from the fabric or swab head. The resulting supernatant can then be centrifuged to form a sperm pellet, which can then be mounted on a slide for examination [10]. A drawback of this method is that sperm tails are often lost due to the mechanical forces involved [8] and as a consequence it is common to find sperm heads without tails.

Sperm heads, and tails if present, need staining before microscopy to provide contrast and allow for identification. In the UK, this procedure is generally performed using haematoxylin and eosin (H&E) staining. These stains will colour the heads of the sperm purple, and the
remaining cellular material a pink colour. Once stained, the slide can then be examined by microscopy at 400x magnification and the presence and concentration of any sperm heads (with or without tails) can be recorded. Alternative staining methods include the ‘Christmas tree stain’, which uses nuclear fast red and picroindigocarmine to stain sperm a distinctive green and red colour [11-12]. There may be instances where there is a positive AP reaction, but no sperm heads are present during microscopic examination. This may indicate a potential false positive AP reaction, or it may be a result of an oligospermic or azoospermic semen sample (i.e. little or no spermatozoa in the semen). In these situations, alternative confirmatory tests are available in the UK including the choline test [13] and prostate specific antigen (PSA) kits, which are more sensitive than the choline test [8].

**Integrated Forensic Approach**

Previous research has set out recommendations and considerations that should be given in terms of sequencing fingerprint enhancement techniques in order to maximise efficiency, the most prevalent of these being the Manual of Fingerprint Development Techniques (MoFDT) [4]. It is clear that an integrated forensic approach to different types of evidence is necessary to maximise evidence recovery, also discussed in the recently released Fingermark Visualisation Manual by the UK Home Office Centre of Applied Science and Technology (CAST) which supersedes the MoFDT [14]. Examples of work discussed in the new manual include items that are suspected to contain fingerprints that are latent and in blood. Such an example is the use of vacuum metal deposition or powders for the detection of latent fingerprints which does not hinder subsequent treatment of marks in blood with protein stains. The use of cyanoacrylate fuming for the detection of latent fingerprints generally hinders subsequent protein staining (water/ethanol/acetic acid formulation) for the enhancement of fingerprints in blood. The use of methanol-based protein stains; however, assists to penetrate the cyanoacrylate polymer to effectively enhance the blood.

There is limited, although on-going, research with regard to the effect of fingerprint enhancement techniques on the subsequent detection of other types of evidence and vice versa. This may result in evidence being missed or destroyed as well as reduced efficiency. A recent study [15] investigated the effect of ninhydrin on the subsequent serological testing of envelopes for the detection of saliva. It was reported that the ninhydrin process did not impact the results of serological testing of the envelopes. Other work assessed the effect of
fingermark detection techniques on the subsequent recovery and analysis of explosive residues [16], the effect of chemical, biological, radiological, and nuclear (CBRN) decontamination on the detection of fingermarks on glass [17], the effect of bacteria on fingermark detection [18], the effect of chemical warfare agents on footwear enhancement techniques [19] and the effect of formaldehyde gas on fingermark evidence [20].

This study aims to contribute to this growing area of research of an integrated approach. Seven fingermark and blood enhancement techniques were employed in this study including: acid yellow 7 (AY7), acid violet 17 (AV17), Bluestar® Forensic Magnum luminol, cyanoacrylate fuming with subsequent basic yellow 40 (BY40) treatment, Lumicyano™ 4%, black magnetic powder and black iron-oxide powder suspension. In order to determine the effect of these enhancement techniques on subsequent detection of semen, three methods of detecting semen/spermatozoa were applied: visual examination, acid phosphatase (AP) reagent and microscopic examination following haematoxylin and eosin (H&E) staining. The sequence of mark enhancement followed by the detection of semen/spermatozoa was applied because fingermarks and footwear marks are fragile and any attempt of an AP presumptive test or swabbing for body fluids may result in a loss of detail recovered. Consequently, this study looked at the sequence of mark enhancement followed by the presumptive AP test and confirmatory test for semen.
Materials and Methodology

Preparation of substrates

White ceramic tiles and grey laminate flooring were used as substrates in this study. Tiles were obtained as new from a local hardware store whereas the laminate was previously used in a family home kitchen. Tiles measured approximately 15cm x 15cm and did not require any cutting; however, the laminate obtained was large in size and was cut using an electric saw to an appropriate size (approximately 15cm x 8cm). Substrates were then thoroughly washed with hot soapy water, rinsed under running tap water, wiped with blue paper towel and left to dry completely before use. Substrates were divided into ten equal parts using a black china marker before use to aid with sample deposition (figure 1).

![Figure 1 - Layout used for creating a depletion series on substrates.](image-url)
Sample Deposition

No fingermark deposition was used in this study but fingermark and blood enhancement techniques were used to assess the effect on the subsequent detection of semen/spermatozoa. This study used one male donor 24 years of age and fresh ejaculate was obtained on the morning it was required for deposition. The semen sample was stored in a glass screw top vial and kept at room temperature before use. A stamp was made using a plastic tube with a circular section of 3.14cm² of used rubber shoe sole material adhered to the end. Prior to each use, the stamp was dipped into a beaker containing a 1% Virkon disinfectant solution, then dipped into a beaker containing distilled water before being dried by dabbing onto clean blue paper towel. The semen sample was poured into a small, clean petri dish and the stamp dipped in the dish three times before a depletion series was made. The depletion series consisted of ten marks being made one after the other, without more semen being added to the stamp (figure 1). The stamp was then washed and dried as previously described before the next depletion series was made. All marks in the depletion series were observed under white light and fluorescence; however, marks 1, 6 and 10 in the depletion series were investigated further through presumptive and confirmatory tests for the detection of semen/spermatozoa.

Controls

A control was performed to ensure the presence of sperm heads in the donor’s ejaculate by taking a 2.5µL aliquot of neat semen and pipetted onto a glass slide. The sample was then heat fixed to the slide using a hot plate at approximately 60°C until completely dried. Following heat fixing, the sample was stained using haematoxylin and eosin (H&E) staining before observed under a Leica and Olympus BX51 microscope at 400x magnification.

Positive and negative controls were prepared for each technique, substrate and ageing period. These were used for comparison to samples which had undergone fingermark or blood reagent treatment. These positive controls consisted of a depletion series of semen from the same ejaculate without the treatment of enhancement techniques. Negative controls were used to ensure that any subsequent detection of semen was not a result of the fingermark or blood technique. This was achieved by treating the substrates with the appropriate fingermark or blood enhancement technique without any semen deposited before being examined for presence of semen/spermatozoa.
Ageing of samples

Once the depletion series of semen had been deposited onto the substrates, ageing periods of 1, 7, 14 or 28 days were investigated. Two depletion series in semen (10 marks) were prepared for each enhancement technique, substrate and ageing period; a test sample (which was treated with fingerprint or blood reagents before testing for semen) and a control sample from the same ejaculate that was not treated with fingerprint or blood reagents. Substrates were left to age after deposition on an open bench in the laboratory away from direct sunlight; however, the temperature and humidity were not controlled.

Examination and photography

Each depletion series was photographed before and after enhancement using a Nikon D5100 digital SLR camera with a 55mm lens and manual settings. A Mason Vactron white Crime-Lite (400-700nm) was used for white light examination and a Quaser 2000/30 was used for fluorescence examination. Semen examination, before and after the application of enhancement techniques, was carried out using a violet/blue excitation source (band pass filter 400-469nm at 1% cut-on and cut-off points respectively) and viewed with a yellow long pass 476nm filter (1% cut-on point).

Chemical formulations and treatment of articles

Acid violet 17 (AV17) and acid yellow 7 (AY7) [14]

Protein staining was performed via a three step process involving fixation, staining and destaining. The fixing solution was prepared by dissolving 5-sulfosalicylic acid dihydrate (23g, Acros) in distilled water (1L) while agitating with a magnetic stirrer for at least 30 minutes. The solution was used to fix the items to be treated by immersion for a minimum of 5 minutes. The staining solution was prepared by dissolving the appropriate protein stain (1g of AV17 or AY7, BVDA) in acetic acid (50mL, Fisher), ethanol (250mL, Fisher) and distilled water (700mL). Following fixation, the items were immersed in the staining solution for a minimum period of 10 minutes. The de-staining procedure consisted of thorough rinsing in a solution of acetic acid (50mL, Fisher), ethanol (250mL, Fisher) and distilled water (700mL). The items under examination were then allowed to dry overnight before photography. AY7 fluorescence was observed with a blue excitation source (band pass filter 385-509nm at 1% cut-on and cut-off points respectively) and viewed with a long pass yellow/orange 510nm filter (1% cut-on point).
**Luminol**
Bluestar® Forensic Magnum was used in this study by thorough mixing of the three tablets and 125mL of liquid supplied with delivery through an Ecospray® fine mist sprayer (nozzle diameter: 0.70mm, flow rate: 0.45ml/s) at a distance of approximately 15-20cms. The prepared solution was used within 24 hours of mixing and substrates were then left to dry for 24 hours before further examination.

**Lumicyano™ 4%**
Lumicyano™ is a one-step fluorescent cyanoacrylate technique. Lumicyano™ solution was removed from refrigeration and warmed to room temperature while shaking before use as per the manufacturer guidelines. 2g of Lumicyano™ solution was weighed followed by 80mg of Lumicyano powder™ to make a solution of 4% weight by weight. The contents of the dish were then gently swirled to mix, ensuring the powder had fully dissolved to produce a pink/orange mixture. Substrates to be tested were placed into a fuming chamber (Air Science CA305) which had been thoroughly cleaned using detergent and water before use. The cabinet was then set to run at 80% humidity and a hot plate temperature of about 100°C for 45 minutes to ensure full evaporation of the Lumicyano™ 4%. 45 minutes running time ensured that 99.99% of the Lumicyano™ 4% was evaporated. After fuming, Lumicyano™ fluorescence was observed by exciting with a blue/green light (band pass filter 468–526 nm at 1% cut-on and cut-off points respectively) and viewed with an orange long pass 529 nm filter (1% cut-on point).

**Cyanoacrylate fuming/BY40 [14]**
2g of superglue (CSI equipment Ltd, UK) was placed into a new foil dish and positioned on a clean support ring on a heat source of about 100°C in the fuming chamber (Air Science CA305). The fuming cabinet conditions were set as described for Lumicyano™. The fuming process was followed by immersion of the articles under examination in a BY40 solution for 1 minute followed by thorough rinsing under running tap water and left to dry at room temperature before fluorescence examination. Basic yellow 40 (Sirchie) dye was prepared by dissolving 2g in ethanol (1L, Fisher). Fluorescence was observed using a Quaser 2000/30 by exciting with a violet/blue excitation source (band pass filter 350-469nm at 1% cut-on and cut-off points respectively) and viewed with a yellow long pass 476nm filter (1% cut-on point).
Black magnetic powder

Black magnetic powder (CSI Equipment, UK) was applied by means of a magnetic brush.

Iron-oxide black powder suspension [14]

Iron (II/III) oxide (20g, Fischer I/1100/53) was weighed and poured into a 100mL glass beaker. Stock detergent solution (20mL) was added slowly whilst stirring with a soft squirrel hair brush until no lumps remained. The stock detergent solution was prepared by measuring Triton X100 (250mL, Acros) and adding ethylene glycol (350mL, Acros) whilst stirring slowly for 10 minutes. Distilled water (400mL) was added and stirred for a further 10 minutes. The iron-oxide powder was applied with a small, animal hair brush and left for a period of 10-15 seconds and then washed under slowly running, cold tap water until all the excess powder was removed from the background. The articles were allowed to dry at room temperature before examination.

AP reagent [7]

Sodium acetate 3-hydrate (20g, Fisher) was dissolved in 1L of deionised water followed by the addition of glacial acetic acid (5mL, Fisher), α-naphthyl disodium phosphate salt (1g, Sigma) and fast black K salt (2g, Sigma). After thorough mixing by means of a magnetic stirrer, the solution was vacuum filtered, transferred to a dark glass bottle and stored at approximately 4°C until required. The reagent was allowed to warm to room temperature before using.

Eosin stock solution

Stock solution: Eosin disodium salt (0.5g, Sigma) was completely dissolved in 10mL of ultra-pure water by means of a magnetic stirrer. The solution was then transferred to a dark bottle and kept refrigerated at 4°C until required.

Working solution: 1ml of eosin stock solution was dissolved in 10mL of ultra-pure distilled water and used immediately after preparation.

Haematoxylin

Mayer’s Haematoxylin solution was obtained ready made from Sigma-Aldrich. This solution was stored at room temperature and filtered before use.
Extraction of semen from surface of substrate [10]

Following visual and fluorescence examination/photography post treatment, semen stains were extracted from the surface for further testing. A cotton swab was dipped into a beaker containing distilled water once to moisten the swab. The area to be extracted was then thoroughly swabbed using the moistened swab head. The swab head was cut off into a labelled 2mL centrifuge tube followed by the addition of 200µL of distilled water using a P1000 automatic pipette. The tube was then manually agitated for 2 minutes before the swab head was removed and placed into a 1.5mL centrifuge tube. The 2mL centrifuge tube was then centrifuged at 10000g for 4 minutes and the supernatant was placed into a separate 1.5mL centrifuge tube without disturbing any pellet which had formed.

The 1.5mL centrifuge tube containing the swab head then had a small hole pierced in the bottom and was placed inside the 2mL centrifuge tube containing the pellet to form a “spinaroo” set up. This set up was then centrifuged at 8000g for 4 minutes, with the lower speed allowing the two tubes to be separated after centrifuging. The 1.5mL tube containing the swab head was then discarded and the 2mL tube centrifuged at 10000g for 4 minutes. Once centrifuged, the supernatant was removed and taking care to not disturb any pellet which had formed, combined with the previously removed supernatant. 20µL of this recombined supernatant was then used to re-suspend the pellet using a P20 automatic pipette, by adding it to the 2mL tube used for the “spinaroo” set up and using the pipette to withdraw and eject the supernatant and pellet several times. The remaining supernatant was then used for AP testing.

AP testing [7]

200µL of the combined supernatant was pipetted onto a 55mm circular filter paper (Fisher) and left to dry. Whilst drying, the previously prepared AP reagent was allowed to come to room temperature prior to use. The AP reagent was then poured into a glass bottle and applied through an Ecospray® fine mist sprayer (nozzle diameter: 0.70mm, flow rate: 0.45ml/s) at a distance of approximately 15-20cms and the time for a reaction to occur recorded. A positive reaction was classed as the first instance of a weak, moderate or strong purple colour being observed. If no colour reaction was observed within 15 minutes, then a no reaction (NR) result was recorded. The positive control consisted of 200µL of neat semen and the negative control consisted of 200µL of distilled water being pipetted onto separate filter papers.
Microscopic examination [10]

2.5µL of the re-suspended pellet was pipetted onto a glass slide using a P20 automatic pipette. The slide was then heat fixed at 60°C until the stain had completely dried. A drop of haematoxylin was applied to the stain and left for at least 2 minutes before gently rinsing the slide under running tap water. Once excess water was removed, a drop of eosin working solution was applied to the stain and left for 30 seconds before rinsing the slide under gently running tap water. Following staining, the slide was then placed back onto the hot plate to dry completely. Once dried, the slide was then viewed under a microscope at 400x magnification as well as other magnifications and the concentration of spermatozoa was recorded using the scale presented in table 1. Each grade in table 1 was assigned a numerical value to calculate the average grade: grades 1-4 were assigned the values 1-4 respectively, the ‘negative’ grade as 0 and the ‘trace’ grade as 0.1. Once observations for all substrates and ageing periods were recorded, the total value was calculated by adding all grades observed and dividing by the total number of marks examined to give the average grade for each technique.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Numerical value</th>
<th>Characteristics of grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>0</td>
<td>No spermatozoa visible</td>
</tr>
<tr>
<td>Trace</td>
<td>0.1</td>
<td>10 or fewer spermatozoa visible</td>
</tr>
<tr>
<td>1+</td>
<td>1</td>
<td>Spermatozoa present, but hard to find</td>
</tr>
<tr>
<td>2+</td>
<td>2</td>
<td>Some spermatozoa in some fields, easy to find</td>
</tr>
<tr>
<td>3+</td>
<td>3</td>
<td>Many or some spermatozoa in most fields</td>
</tr>
<tr>
<td>4+</td>
<td>4</td>
<td>Many spermatozoa in every field</td>
</tr>
</tbody>
</table>
Results & Discussion

Controls

The positive controls for semen, prior to any chemical treatment, demonstrated the expected fluorescence when observed with a violet/blue excitation source (band pass filter 400-469nm at 1% cut-on and cut-off points respectively) and viewed with a yellow long pass 476nm filter (1% cut-on point). A positive AP reaction was classed in the first instance with a weak, moderate or strong purple colour being observed. Positive controls, observed under x400 magnification, ensured the presence of spermatozoa in the ejaculate. A negative control did not reveal any fluorescent stains, confirming that the fluorescence was due to the presence of semen and not due to the substrate. A negative AP reaction was recorded if no colour reaction was observed within 15 minutes. Microscopy of negative controls did not reveal any spermatozoa as expected.

Visual and fluorescence examination

The observation of the marks in semen under white light prior to treatment was difficult, more so for the marks further down the depletion series; however, this was improved with the use of oblique lighting. All possible combinations of excitation and viewing filters were tested for the fluorescence of semen and the violet/blue excitation source (yellow filter) provided the best contrast. In general, fluorescence was significantly weaker on laminate flooring possibly due to the texture, colour and material of the substrate. Nonetheless, the fluorescence before and after enhancement generally remained the same, indicating that the enhancement technique was not hindering the fluorescence of semen. Figures 2 and 3 demonstrate some examples of the depletion series of semen marks after treatment with the various techniques on tiles and laminate after an ageing period of 14 days. Figures 4 and 5 represent the average number of marks detected before and after treatment using the various techniques and lighting conditions across both substrates and all ageing periods.

AV17 and AY7, together with acid black 1 (AB1) are the protein stains recommended for the enhancement fingermarks in blood [14]. Such protein stains are not blood-specific and will stain proteins in other body fluids and other materials such as semen and milk. The use of protein stains in this study resulted in an increase of the number of marks detected in the depletion series. A strong purple colouration was observed when substrates were treated with AV17 and the treatment hindered the fluorescence of semen; however, marks were still
visible due to the contrast between the marks and the background. AY7 stained the marks yellow and the marks fluoresced when examined with a violet/blue light (yellow filter) for the presence of semen on tiles but not on laminate. Nonetheless, since AY7 is a fluorescent protein stain, the marks fluoresced strongly when examined with blue light (yellow/orange filter). Overall the strong colouration from the protein stains increased the contrast with the background using both white and fluorescent light.

Figure 2 - Semen marks 6-10 in the depletion series on grey laminate flooring aged 14 days and viewed under white light (top) and violet/blue light with yellow filter (bottom) post treatment: (a) before enhancement; (b) AV17; (c) AY7; (d) Lumicyano 4%; (e) cyanoacrylate/BY40; (f) black magnetic powder; (g) iron-oxide powder suspension
Figure 3 - Semen marks 6-10 in the depletion series on white ceramic tile aged 14 days and viewed under white light (top) and violet/blue light with yellow filter (bottom) post treatment: (a) before enhancement; (b) AV17; (c) AV7; (d) Lumicyano 4%; (e) cyanoacrylate/BY40; (f) black magnetic powder; (g) iron-oxide powder suspension
Figure 4 – Average number of semen marks observed before and after enhancement under white light across both substrates and all ageing periods.

Figure 5 – Average number of semen marks observed before and after enhancement under violet/blue light (yellow filter) across both substrates and all ageing periods.
Bluestar® Forensic Magnum luminol increased the average number of marks observed on each substrate when using white light; however, when using violet/blue light (yellow filter), the average number of marks observed remained unchanged. Although a fine mist sprayer was used for the delivery of luminol, the diffusion of the marks was apparent. This can be explained by the fact that the substrate were vertical and left in this position to dry after the application of luminol. The increase in the average number of marks visible when using white light was caused by an improved contrast between the semen mark and background substrate post treatment, particularly when manipulating the angle of viewing with white light. This may be due to the hydrogen peroxide in luminol reacting with the semen and/or substrate and increasing the contrast of the semen stains with the background. In comparison to blood, no chemiluminescence was observed after the application of luminol to semen due to the absence of the haem group.

Lumicyano™ 4% increased the average number of marks visible on both substrates. The use of a blue/green light (orange filter) for the observation of Lumicyano™ fluorescence produced additional results on semen marks on tiles (but not on laminate) to that observed under violet/blue light (yellow filter) as demonstrated in figure 6e. Both cyanoacrylate fuming and Lumicyano™ resulted in a build-up of cyanoacrylate polymer on the depletion series of semen. Marks treated with Lumicyano™ developed a light pink cyanoacrylate polymer coloured due to the incorporation of a dye which fluoresced strongly when viewed under blue/green light (orange filter). Additionally, fluorescence could still be observed through the polymer build-up when using violet/blue light (yellow filter) for the presence of semen on tiles; however, the fluorescence for the presence of semen was not observed on laminate with Lumicyano™ (figures 2d and 3d). This is not necessarily due to the technique since the fluorescence on laminate prior to any treatment was very weak or not observed at all (figure 2a). Fluorescence of the semen under violet/blue light post both cyanoacrylate techniques was weaker for all marks in the depletion series on laminate in comparison to tiles; however, this trend was also observed on the control samples prior to fuming. The conventional two-step cyanacoacrylate and staining method using BY40 also improved the average number of marks observed on substrates under white and fluorescent light. Cyanoacrylate fuming developed the semen marks with the build-up of a white polymer which become stained a light yellow after immersion in BY40. The use of ethanol-based BY40 is not recommended for use at crime scenes due to the flammability concerns of ethanol; however, water-based BY40 may not be as effective. If an item needs to be treated at
the scene, the use of water-based basic red 14 offers a suitable, safe and effective alternative [22]. The fluorescence settings for BY40 are the same as that used for the detection of semen and the marks fluoresced strongly when observed post BY40 treatment under violet/blue light (yellow filter). Nonetheless, it was also noted that when substrates were examined after cyanoacrylate fuming, but before BY40 staining, the fluorescence of semen could still be observed through the cyanoacrylate polymer (figure 7).

Figure 6 - Semen marks 6-10 in the depletion series on white ceramic tile (top) and grey laminate flooring (bottom) aged 28 days and viewed: (a) under white light before Lumicyano fuming; (b) under violet/blue light (yellow filter) before Lumicyano fuming; (c) under white light post Lumicyano fuming; (d) under violet/blue light (yellow filter) post Lumicyano fuming; (e) under blue/green light (orange filter) post Lumicyano fuming
Figure 7 - Semen marks 6-10 in the depletion series on white ceramic tile (top) and grey laminate flooring (bottom) aged 28 days and viewed: (a) under white light post cyanoacrylate fuming; (b) under violet/blue light (yellow filter) post cyanoacrylate fuming; (c) under white light post cyanoacrylate fuming/BY40; (d) under violet/blue light (yellow filter) post cyanoacrylate fuming/BY40
Using black magnetic powder on the substrates used in the study resulted in an increase of the number of marks detected in the depletion series when observed under white light which is due to the powder adhering to marks; however, the powder hindered fluorescence examination under violet/blue light (yellow filter), possibly due to the powder masking some of the fluorescent properties of semen. Application of powder suspension to the substrates improved the average number of marks observed under white light. This is due to the powder suspension adhering to semen residues, allowing for marks that are easier to see when using white light. Marks observed under violet/blue light (yellow filter) after iron-oxide powder suspension treatment showed no fluorescence, although the contrast did improve slightly.

Generally the ageing period did not have an effect on the average number of marks observed after treatment with the different enhancement techniques using either white or fluorescent light. This is due to the residues from semen deposition reacting with the enhancement techniques and improving the contrast with the background.

**AP testing**

Figures 8 to 10 represent the average times recorded for a positive AP reaction for the semen marks 1, 6 and 10 across the various enhancement techniques, substrates and ageing periods. AP reactivity was lost in all samples extracted following AV17 and AY7 treatment. This may be explained by the fact that the acid phosphotase in semen is water soluble and fixing with water based 5-sulfosalicylic acid resulted in a negative AP test. A preliminary trial performed on 7 day aged samples on both substrates resulted in a positive AP test when methanol was used as an alternative fixative process to water based 5-sulfosalicylic acid. When using methanol as a fixing agent, mark 1 of 10 showed a longer average time for a positive AP reaction when compared to control samples from the same ejaculate. However, AP testing was still possible and a positive reaction occurred within 15 minutes. Additionally, a recent study [21] reported that methanol-based acid black 1 had no detrimental effect on subsequent DNA analysis; however, consideration would need to be given to health and safety as well as flammability concerns of methanol use. Nonetheless, if items are suspected to contain semen and blood deposits, the use of methanol-based fixing, staining and rinsing may maximise evidence recovery. The negative AP test following the use of water-based fixation and staining may result in subsequent seminal evidence being overlooked since no confirmatory tests would be carried out.
All marks in the depletion series that were treated with Bluestar® Forensic Magnum luminol, Lumicyano™ 4%, cyanoacrylate/BY40 and black magnetic powder demonstrated a positive AP reaction within 15 minutes. On average, the time required for a positive AP reaction took longer for marks that had been subjected to the enhancement techniques when compared to the controls. For the luminol process, mark 1 of 10 showed a shorter time for a positive AP reaction compared to control samples from the same ejaculate. Samples post Lumicyano™ 4% treatment showed a longer average time to achieve a positive AP reaction with approximately 3 minutes for Lumicyano™ 4% treated samples compared to 1.5 minutes for control samples from the same ejaculate. The two-step cyanoacrylate and staining method required thorough rinsing under running tap water to remove the excess dye. It was expected that the water rinsing process might have an effect on the AP reagent; however, a positive AP test was observed for all marks treated with cyanoacrylate followed by BY40 staining. Cyanoacrylate fuming appeared to fix the semen marks with the development of white cyanoacrylate polymer that did not hinder the AP process.

The use of black magnetic powder increased the time required to observe a positive AP reaction compared to control samples from the same ejaculate. However, presumptive AP testing was still possible and reaction times were still within 15 minutes, which has been reported as a more appropriate time to allow for reaction compared to the traditional two minutes [5]. Samples extracted following iron-oxide powder suspension treatment showed no reaction (NR) within 15 minutes when using AP reagent as a presumptive test for semen. This is due to water in the powder suspension formulation as well as the thorough rinsing under running tap water post-treatment.

The different ageing periods in this study had little effect on observations and the small variations observed may be due to the changing AP concentrations day to day in the ejaculate. Additionally, the AP results for marks 1, 6 and 10 appeared to be consistent although the time taken for a positive reaction for marks 6 and 10 was longer than mark 1.
Figure 8 - Average times recorded for a positive AP reaction for semen mark 1 in the depletion series across the various enhancement techniques, substrates and ageing periods.

Figure 9 - Average times recorded for a positive AP reaction for semen mark 6 in the depletion series across the various enhancement techniques, substrates and ageing periods.
Figure 10 - Average times recorded for a positive AP reaction for semen mark 10 in the depletion series across the various enhancement techniques, substrates and ageing periods.

Microscopic examination

Figures 11 to 13 represent the average grades for the detection of spermatotzoa in semen marks 1, 6 and 10 across the various enhancement techniques, substrates and ageing periods. Post AV17 and AY7 treatment, the first mark in the depletion series on both substrates revealed an average grade of 2.65 and 1.7 respectively. This result is of interest when considering a sample that has a negative presumptive AP test. It is also noted that AV17 appears to have stained sperm heads a blue/purple colour, and appears to have blocked or masked any attempted staining of the sperm by H&E staining (figure 14). Following AV17 testing, extracted samples also showed a high number of sperm with tails still attached - a relatively rare occurrence in extracted samples (figure 15). This may suggest that the protein fixing solution or the AV17 staining solution improves the resilience of the tails on the sperm, allowing for easier identification. In contrast, AY7 yielded fewer heads with tails attached than AV17. Compared to control samples from the same ejaculate and aged under the same conditions, the average grade observed after AY7 was less than the control samples. AY7 also showed improved overall visualisation meaning it may still be of use in examinations. Contrary to AV17, samples treated with AY7 appeared to react normally with
H&E staining. A preliminary trial on 7-day aged stains demonstrated that methanol allowed for successful extraction and H&E staining of sperm after treatment. Results obtained showed no notable difference in average grade between methanol treated samples and control samples with both having an average grade of 4. It was also noted that samples extracted following methanol treatment showed a larger number of tails attached to head than control samples which may suggest methanol improved the integrity of whole sperm during the extraction process. There was no direct correlation observed between ageing period and average grade observed during microscopic examination; however, the average grade of spermatozoa decreased from mark 1 to marks 6 and 10.

Figure 11 - Average grading for the detection of spermatozoa for semen mark 1 in the depletion series across the various enhancement techniques, substrates and ageing periods.
Figure 12 - Average grading for the detection of spermatozoa for semen mark 6 in the depletion series across the various enhancement techniques, substrates and ageing periods.

Figure 13 - Average grading for the detection of spermatozoa for semen mark 10 in the depletion series across the various enhancement techniques, substrates and ageing periods.
The use of Bluestar® Forensic Magnum luminol did not have an effect on subsequent extraction and microscopic examination of semen. Samples post treatment and control samples both showed an average grade greater than 3, with samples post treatment having a slightly lower average than the controls. It was also noted that some samples extracted after treatment with luminol still showed tails attached to heads, aiding in identification of spermatozoa within a sample. There was no detrimental effect on the microscopy of spermatozoa with both cyanoacrylate techniques, Lumicyano™ 4% and cyanoacrylate/BY40, with an average grade post treatment slightly lower than the control samples from the same ejaculate. It was also noted that H&E staining performed normally following cyanoacrylate fuming as shown in figure 16. Similar to the observations from AP testing, the white polymer appeared to protect the spermatozoa for further microscopy examination.

Figure 14 - Microscopic examination of spermatozoa aged 14 days on grey laminate flooring, mark 1 in the depletion series following AV17 treatment and H&E staining. Recorded with Olympus BX51, 200x magnification.
Figure 15 - Spermatozoa heads and tails aged 7 days on white ceramic tile, mark 1 in the depletion series observed following AY7 treatment and H&E staining. Recorded with Olympus BX51, 500x magnification.

Figure 16 - Spermatozoa heads aged 7 days on white ceramic tile, mark 6 in the depletion series observed following Lumicyano 4% treatment and H&E staining. Recorded with Olympus BX51, 200x magnification.
Results demonstrated that the average grade of spermatozoa observed between samples extracted post treatment with black magnetic powder and control samples from the same ejaculate was similar. The average grade observed for both was approximately 2 for mark 1, indicating that black magnetic powder does not hinder subsequent detection of spermatozoa. It was however noted that black magnetic powder present on the substrate was also extracted and may therefore be transferred to the slide during preparation which may lower the grade obtained. Iron-oxide powder suspension was the only technique used in this study that resulted in a negative detection for sperm heads present compared to an average grade greater than 3 from control samples of the same ejaculate. When extracted material from substrates treated with powder suspension was stained and subsequently examined, only black powder was observed. This is potentially due to factors such as water/detergent present in the powder suspension formulation as well as post treatment rinsing, resulting in sperm heads being lost.

In summary, table 2 records the observations recorded from the effects of the various enhancement techniques on the subsequent detection of semen/spermatozoa.
Table 2 – A summary of the effects of the enhancement techniques on the subsequent detection of semen and spermatozoa.

<table>
<thead>
<tr>
<th>Method</th>
<th>Improved visual examination</th>
<th>Positive AP test</th>
<th>Confirmatory test</th>
</tr>
</thead>
<tbody>
<tr>
<td>AV17 (water based)</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>AY7 (water based)</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Methanol (preliminary)</td>
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<td>✓</td>
<td>✓</td>
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<tr>
<td>Bluestar® Forensic Luminol</td>
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<td>✓</td>
</tr>
<tr>
<td>Lumicyano™ 4%</td>
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<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Cyanoacrylate/BY40</td>
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<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Black magnetic powder</td>
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<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Iron-oxide powder suspension</td>
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<td></td>
</tr>
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</table>
Conclusion

This study demonstrated that the different enhancement techniques improved the visualisation of semen under white and violet/blue light (yellow filter), more prominently on tiles than on laminate. Water based formulations of protein stains caused a loss in AP reactivity and resulted in a negative AP test; however, sperm heads and tails were still observed after the extraction procedure. A preliminary trial using a methanol-based fix and stain gave a positive AP test with subsequent successful extraction. Bluestar® Forensic Magnum luminol, Lumicyano™ 4%, cyanoacrylate/ BY40 and black magnetic powder yielded a positive AP test with successful detection of spermatozoa. Iron-oxide powder suspension caused a loss in both AP reactivity and subsequent microscopic examination and was thus the most detrimental of the techniques examined in this study.

This study considered the effect that enhancement techniques had on the subsequent detection of semen. The opposite sequence as to the effect that semen detection techniques have on subsequent finger and footwear mark enhancement may be considered; however, since such marks are fragile, a loss of the fine detail may occur. Additional research will assess further enhancement techniques, substrates and other variables for items that are suspected of having latent marks and semen. The results obtained show that the majority of the enhancement techniques have no detrimental effect on the subsequent detection of semen/spermatozoa. A loss of presumptive and or confirmatory testing capabilities caused by some of these enhancement techniques may result in evidence being over looked or lost, reducing efficiency within forensic investigations in terms of time, costs and maximisation of evidence recovery. The research will also expand to the effects of enhancement techniques on other body fluids and other types of evidence such as saliva and gunshot residue.
References


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