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The effect of mark enhancement techniques on the presumptive and confirmatory tests for blood

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Abstract

An investigation into the effects of physical and chemical enhancement on subsequent presumptive and confirmatory tests for human blood is presented. Human blood was deposited onto porous (white 80gsm paper and brown envelope) and non-porous (tile and linoleum) substrates in a depletion series (30 depletions on non-porous and 20 on porous) and subjected to three ageing periods; 1, 7 and 28 days. A number of enhancement techniques were tested [fluorescence, black magnetic powder (BMP), iron-oxide black powder suspension (PS), cyanoacrylate (CA) fuming, acid violet 17 (AV17), acid yellow 7 (AY7), ninhydrin, DFO and Bluestar Forensic Magnum (BFM) luminol] to evaluate their potential effects on subsequent presumptive and confirmatory tests. AV17 and Bluestar provided the best enhancement and fully enhanced all depletions in the series. The sensitivity of the Kastle-Meyer (KM) (presumptive), Takayama and RSID-Blood tests (confirmatory) was initially investigated to determine the range of detectable depletions. The KM test detected all depletions, whereas the Takayama test detected up to depletion 6 and RSID-Blood detected up to depletion 20 (paper), 10 (envelope), 15 (tile) and 9 (lino). The abilities of these tests to detect blood after enhancement were then observed.

A number of techniques resulted in little to no effect on any of the blood tests, whereas adverse effects were observed for others. Ninhydrin and CA fuming caused weak but instantaneous positive KM results whereas methanol-based AV17 and AY7 delayed the reaction by as much as 1 minute. The Takayama test was not very sensitive, therefore, its performance was easily affected by enhancement and negative results were often observed. RSID-Blood tests were largely unaffected by chemical enhancement although a drop in positive results was observed for some of the techniques when compared to positive controls.

Using a standard procedure for DNA extraction, all the tested blood samples (before and after enhancement) gave a detectable quantity of DNA and were successfully profiled. Out of the 45 samples processed for DNA profiling, 44 gave full profiles, while the remaining showed allele drop out in one or two loci.

Keywords

Integrated forensic approach; KM test; Takayama; RSID-blood; DNA

Introduction

It is important to maximise the recovery of evidence during a scene of crime examination and from items received into the forensic laboratory. Issues arise when multiple types of evidence are found in combination on the same substrate, such as latent marks, body fluids and inks. There is a growing body of literature assessing the impact of multiple types of analysis [1–4]. A sequential order for treating multiple types of evidence should be established to maximise the evidence recovery. If a biological stain is not visible, speculative swabbing may be detrimental to any latent marks present. Enhancement techniques may diminish the amount of DNA recovered; however, a recent study [5] reported that cyanoacrylate fuming of latent marks on cables resulted in a larger amount of DNA when compared to latent marks that had not been treated suggesting that the enhancement process provides a target area for swabbing that can result in a higher yield of DNA recovery. This study aims to present the effect of routinely used latent enhancement techniques on the visualisation and subsequent detection of blood by means of presumptive and confirmatory tests.

Presumptive tests for blood

Presumptive tests for blood are notably fallible, capable of producing false positive and negative results [6,7]. Therefore it is important to have an understanding of potential interfering factors, including the effects of enhancement techniques. Such preliminary tests are very sensitive, capable of detecting haemoglobin in blood dilutions ranging from 1 in 10,000 [8,9] to even 1 in 1,000,000 [10]. Despite the sensitivity, such tests are not human specific and haemoglobin's peroxidase activity is not exclusive to blood alone [11]. As such, they are only capable of *indicating* the presence of blood, or excluding a stain as being blood if a negative result is produced [11,12]. One of the most common presumptive tests used is the Kastle-Meyer (KM) test, also known as the phenolphthalein test [6]. A positive result is observed when the colourless phenolphthalein rapidly changes colour to a bright magenta pink after H_2O_2 is added. There is a range of documented reaction times with some practitioners only accepting an immediate reaction [9,13], or within 5-20 seconds [6,10,14], <1 minute [15] and even as long as 4 minutes when the negative control changed beyond that time [8]. The reaction time was observed to increase as blood dilution increased, taking 45 seconds with $\times 10^{-4}$ dilution and 1 min 30 sec to 2 min 30 sec for $\times 10^{-5}$ dilution [8]. Other blood presumptive tests include leuco malachite green (LMG), tetramethylbenzidine (TMB) and Hemastix®. The KM test has been reported to be more sensitive than the LMG test [8,10,16] but LMG is reported to be more

specific to blood due to less false positive reactions with other substances, although it is known to be DNA destructive [8]. Although the KM test is very sensitive, detecting blood dilutions as low as $\times 10^{-6}$ [10], diluted or degraded samples contaminated with even small amounts of an oxidising or reducing agent may produce false positive and negative results respectively [7].

Alternative light sources

Many materials and body fluids other than blood will fluoresce when excited by high intensity light in ultraviolet and violet regions of the spectrum. However, blood does not fluoresce but instead absorbs light at wavelengths across the entire electromagnetic spectrum. The optimal absorption band of haemoglobin, known as the Soret band, is found in the violet (380-450 nm) and UV (100-400 nm) regions of the electromagnetic spectrum [17], with peak absorption at approximately 412 nm [18–20]. A 350-450 nm light source is said to be the most effective to visualise blood [17], which remains dark against the fluorescing background. It is recommended to avoid using shortwave UV light to speculatively search for blood marks if subsequent DNA-STR analysis is desired as 30 second exposure was shown to have a destructive effect [21]. In another study, however, it was observed that treating marks with superglue fuming protected DNA from degradation due to UV exposure [22]. Blood also absorbs light in the infrared (IR) region of the spectrum and can provide good contrast of blood traces on dark surfaces.

Confirmatory tests for blood

Confirmatory tests, such as the Takayama test, are much less sensitive than presumptive tests, detecting dilutions up to only 1 in 10 [11], but are specific for haem found in the haemoglobin in blood. Yet, they are still subject to potential false negative results with trace amounts of blood [23] or false positives from interfering substances such as leghaemoglobin in root nodules, purified catalase or peroxidase [24]. The Takayama test forms characteristic red/brown feathery needle-like pyridine ferroprotoporphyrin (haemochromogen) crystals [15] (figure 1). Lateral flow immunochromatographic test kits, such as ABACard® HemaTrace® and Hexagon OBTI, use anti-human haemoglobin antibodies to specifically detect the haemoglobin of primates (humans) [9]. A Rapid Stain Identification (RSID) kit for blood, developed by Independent Forensics, is reported to be confirmatory and specific for human blood with no false positive results with other substances and body fluids [25]. It is quick and highly sensitive, capable of detecting as little as 100 nL of blood within 10 minutes [26].

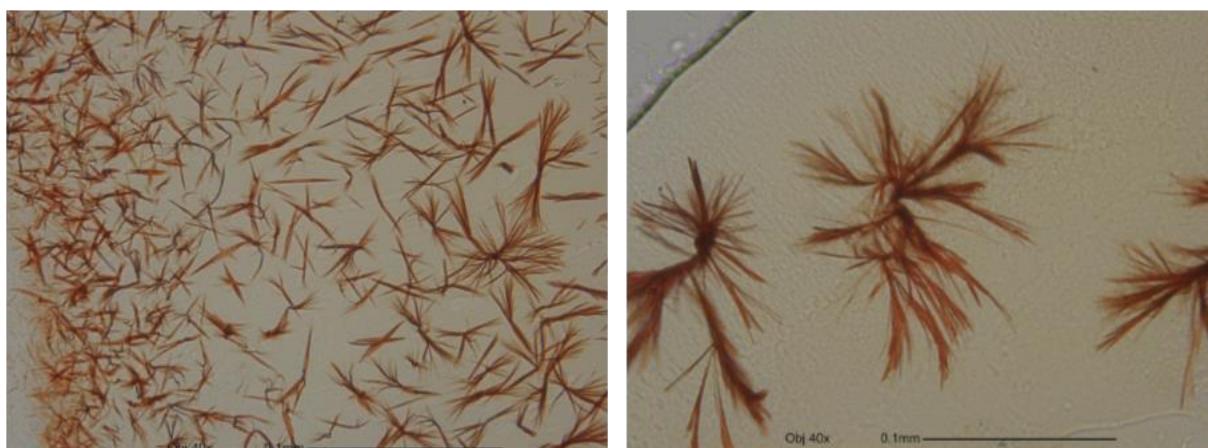


Figure 1 - Haemochromogen crystals formed with Takayama reagent and human blood

Other confirmatory tests currently in use for the identification of peripheral blood include messenger RNA (mRNA) profiling [27,28], microRNA analysis (miRNA, miR) [29,30] and DNA methylation [31,32]. In addition to being highly specific and sensitive, mRNA profiling has the benefit of being incorporated into a DNA run, which is beneficial for associating body fluid and sample type. MicroRNAs play important roles in the down-regulation in cells. Their short sequence (~22 nt) and relatively high abundance make them excellent candidates for samples that are degraded and low quality [29,30]. Similar to mRNA analysis, microRNAs can also be incorporated into a DNA run through extension of primers [33–35]. The DNA methylation technique utilises the addition of a methyl group onto the 5' position of cytosine using DNA methyltransferases. DNA methylation patterns in a body fluid can be determined through bisulfite treatment or through the use of methylation n-restriction sensitive enzymes [31,36–38].

Integrated forensic approach

Identifying a crime stain as blood by visual examination alone is legally insufficient as blood changes colour from red to brown and even black as it dries, ages and with exposure to environmental conditions. If a blood stain is not visible under white or fluorescent light, speculative swabbing may be detrimental to latent marks [39]. Ideally, the blood should be treated to allow visualisation; however, such treatment may affect subsequent blood tests. There is very little research into the effects of enhancement techniques on subsequent blood tests, although the need for such research is recognised [40] and a working knowledge of the effects of enhancement techniques on the blood evidence is crucial when determining which techniques to use and in which order [41].

Previous research indicates that enhancement techniques do have the ability to affect the subsequent detection of blood, in certain circumstances. Both luminol and Bluestar Luminol reportedly have no negative effects on the KM test; however, dilutions of 1 in 1000 on untreated wood resulted in negative results [42,43]. Luminol was found to have no effect on the Takayama test [44] and no adverse effects on the RSID-blood test were observed with blood treated with Bluestar or even blood mixed with Bluestar with the kits detecting blood volumes as low as 250 nL aged 1-3 days [45]. In another study, blood depletions treated with leucocrystal violet (haem reactive) produced negative KM results on all six depletions in a series whereas methanol based acid black 1 provided a positive response on all. Acid yellow 7 (water/ethanol/acetic acid formulation) and acid black 1 (aqueous) only produced negative results with the final sixth depletion [46]. In another study, superglue, black powder, and ninhydrin were found to have no effect on the Takayama test or LMG [44].

Further to the effect of mark enhancement reagents on presumptive and confirmatory tests for blood, DNA analysis also needs to be considered. Various studies [47,48] have investigated the effects of enhancement techniques and presumptive tests on subsequent DNA analysis, with the aim of integrating DNA profiling within the workflow of biological samples analysis. In criminal cases, the evaluation of DNA from fingerprints is generally considered when marks are not suitable for comparison or when fingerprints are stained with other biological material. With fingerprints enhancement, several studies [49–54] have shown that most of the reagents commonly used to develop latent marks, such as aluminium powder, black magnetic powder, fluorescent powders, wet powder suspension and 1,2-indanedione do not affect subsequent recovery of DNA. Other specific chemicals such as cyanoacrylate and ninhydrin may have a negative impact on the process as they can either degrade or sequester the DNA or inhibit the PCR reaction [55,56]. Another study [5] reported that cyanoacrylate fuming of latent marks on cables resulted in a larger amount of DNA when compared to latent marks that had not been fumed with cyanoacrylate. A number of studies [22,57,58] have investigated the effects of enhancement techniques for the visualisation of body fluids to assess their impact on the process of DNA profiling. Frégeau *et al.* [57] investigated the effect of seven blood enhancement reagents (including amido black, DFO, LMG and luminol). It was reported that short-term exposure to the chemicals did not have an adverse effect on the PCR amplification; however, long-term exposure (particularly with Crowle's Double Stain and Hungarian red) resulted in a reduced STR amplification efficiency.

This study aims to expand the current research for an integrated forensic approach by investigating the effects of a number of enhancement techniques on presumptive and confirmatory tests for blood. The enhancement techniques included were; fluorescence, black magnetic powder (BMP), iron-oxide black powder suspension (PS), cyanoacrylate (CA) fuming, acid violet 17 (AV17), acid yellow 7 (AY7), ninhydrin, DFO and Bluestar Forensic Magnum (BFM) luminol. The effect of each enhancement technique on the subsequent detection of blood was assessed by the Kastle-Meyer, Takayama and RSID-Blood tests. In addition, their effects on obtaining a usable DNA profile was investigated using extract derived from the RSID-Blood tests.

Materials and Methodology

Preparation of Substrates

Four substrates were used; two non-porous (white ceramic tile, blue linoleum) and two porous (80 gsm white paper, brown envelopes). To aid deposition each substrate was sectioned into equal portions: 30 for non-porous and 20 for porous. A smaller number of marks were left on porous substrates as more blood is deposited per mark due to absorption. To avoid contamination, both the tile and linoleum were cleaned with hot, soapy water and air dried prior to use. The paper and brown envelopes were kept sealed in the original packaging until required.

Deposition of Blood

Fresh human blood was drawn from a single donor on the day of application. Blood was drawn from the median cubital vein by means of venepuncture using a butterfly needle and a K₂EDTA vacutainer collection tube (10 mL), containing 18 mg ethylenediaminetetraacetic acid (EDTA). The blood was used within an hour and poured into a petri dish to enable a rubber stamp to be dipped in the blood ensuring full coverage. With a single application, the stamp was allowed to drip once then pressed onto the relevant substrate in a continuous manner until all depletion marks were deposited. Three test depletions (blood to be enhanced) were prepared to obtain more robust data for the KM test as well as one positive control depletion (blood without enhancement) for each of the four substrates, ten enhancement techniques and three ageing periods.

Ageing of Samples

The prepared depletions were subjected to three ageing periods: 1 day, 7 days and 28 days. After sample deposition, all depletions were stored and allowed to air dry in the laboratory; however, the temperature and the humidity were not strictly controlled.

Controls

Numerous positive and negative controls were in place to ensure tests were working as expected and without contamination. Positive controls included blood sample depletions running parallel to test depletions, which were subjected to blood testing without prior enhancement. Negative controls included background swabs of substrates prior to sample deposition to ensure the substrate was sufficiently clean, tested using the KM test. For the purpose of KM testing, a positive control of fresh blood acquired by pinprick sampling was applied directly to a piece of filter paper and negative controls of filter paper dipped in distilled water and a background rubbing of the substrate after enhancement were included. For the Takayama test, a negative control of the extraction medium (distilled water) on a cotton bud was tested to ensure no contamination. A positive control of fresh blood, acquired by pinprick sampling, on a cotton bud extracted with distilled water was tested to ensure the reagent was working correctly.

Visual and Initial Fluorescence Examination

All depletions, for each enhancement technique, substrate and ageing period were visually examined. Examination was conducted under white light and a Violet Crime-Lite®82S [10% band width 395–425 nm with a 410 nm peak and viewed with a pale yellow long pass 435 nm filter (1% cut-on point)]. The effectiveness of each technique for blood enhancement was assessed by recording the appearance of the substrate and blood marks and the number of additional marks visible after enhancement.

Physical and Chemical Enhancement

In addition to initial fluorescence examination, ten enhancement techniques were used; black magnetic powder (BMP), iron-oxide black powder suspension (BPS), cyanoacrylate (CA) fuming, two formulations [methanol (MeOH) and water/ethanol/acetic acid (WEAA)] of both

acid violet 17 (AV17) and acid yellow 7 (AY7), ninhydrin, DFO, and Bluestar Forensic Magnum (luminol). BPS, AV17, AY7 and CA were used on non-porous substrates only, and both ninhydrin and DFO on porous substrates only as recommended by CAST [39]. BMP (CSI Equipment, Ltd., UK) and Bluestar were suitable for all substrates. Solutions were prepared in accordance with the Fingerprint Visualisation Manual [39] and manufacturer guidelines.

Black Powder Suspension

After gently pre-rinsing the substrate with cold tap water, the iron oxide black powder suspension working solution was then applied with a soft, animal hair brush and left for about 10 seconds before careful rinsing under running tap water and drying overnight.

Cyanoacrylate Fuming (Superglue)

Test depletions were fumed using 2g of CA (CSI equipment Ltd., UK) in an Air Science CA30S fuming cabinet for 45 minutes at 80% RH. Following basic yellow 40 (BY40) staining, fluorescence was observed using a blue Crime-Lite[®] 82S [10% bandwidth 420–470 nm with a 445 nm peak and viewed with a yellow long pass 476 nm filter (1% cut-on point)].

AV17 and AY7 (MeOH formulation)

Test depletions were fixed in methanol (Fisher) for 1 hour then immersed in the staining solution for at least 3 minutes. The excess dye was rinsed off with a solution of acetic acid (100 mL, Fisher) and methanol (900 mL, Fisher).

AV17 and AY 7 (WEAA formulation)

Test depletions were fixed in 5-sulphosalicylic acid (23 g, Acros) dissolved in distilled water (1 L) by immersion for at least 5 minutes then immersed in the staining solution for at least 3 minutes. For both formulations, AY7 fluorescence was observed with a blue excitation source (band pass filter 385–509 nm at 1% cut-on and cut-off points, respectively) and viewed with a long pass yellow/orange 510 nm filter (1% cut-on point).

Ninhydrin and DFO

The porous substrates were drawn through the ninhydrin and DFO working solutions once using tweezers and the excess liquid allowed to drain back before drying on a cardboard sheet. Substrates treated with ninhydrin were then placed in a Gallenkamp humidity controlled oven

(BR185H) for 4 minutes at 80 °C and 65% RH whereas those treated with DFO were placed in a Thermo Scientific Heraeus oven (UT6200) for 20 minutes at 100 °C. DFO fluorescence was observed by using a green excitation source (band pass filter 473–548 nm at 1% cut-on and cut-off points respectively) and viewed with a band-pass 549 nm filter (1% cut-on point).

Bluestar Forensic Magnum

Bluestar Forensic Magnum solution was prepared as instructed by the manufacturer and dissolving the three tablets into the 125 mL liquid supplied. The solution was then delivered through an Ecospray® fine mist sprayer (nozzle diameter: 0.50 mm, flow rate: 0.27 mL/s) at a distance of approximately 15–20 cm.

Preliminary Limit of Detection Studies

Preliminary studies were conducted to determine the limit of detection (LOD) for the three blood tests to ensure marks selected for testing were within the detectable range. Depletions were prepared as per the main blood deposition protocol on all four substrates and aged for 14 days. Alternating marks were tested, for example marks 1, 3, 5 and so forth, starting from a depletion number already known to produce positive results. The results of the LOD studies are stated in the methodology sections for each blood test.

Kastle-Meyer Test

A phenolphthalein working solution was prepared and stored in the refrigerator for a maximum of 1 week [59]. To test the blood marks with the KM reagent, a 55 mm filter paper was folded into four, the point dampened with distilled water and either rubbed (on non-porous) or pressed (on porous) over the whole mark. 1-2 drops of KM reagent were then applied to the centre. After ~5 seconds, 1-2 drops of 3% hydrogen peroxide (H₂O₂) was added [11]. A result was considered positive if the KM reagent changed from clear to magenta pink *after* the addition of H₂O₂ and only if this reaction occurred *before* the negative control changed colour.

Three marks, from each control depletion and the three test depletions, were selected and tested. The selection protocol consisted of: mark 1 on the edge of visible/latent, mark 3 on the edge of detection (after enhancement), and mark 2 between marks one and three. The LOD study determined that KM detected the full depletion series on all substrates. One KM test consisted of: one of the three selected test marks from all three test depletions, a background rubbing from each test depletion, the corresponding mark from the control depletion (no enhancement),

a positive control, and a negative control. Each KM test was video recorded using a laminated labelled template (figure 2) and a cut-off time of 2 minutes and 30 seconds for a visual record of reaction time. Preliminary testing of two brands of filter paper, Fisherbrand 55 mm and Whatman 55 mm, was carried out to determine the time taken for the reagent to naturally change colour due to oxidation with the air. The Fisherbrand filter paper produced a relatively obvious pink colour after approximately 30 seconds following the addition of H_2O_2 , whereas the Whatman filter paper took at least 2 minutes and 30 seconds to produce a very pale pink colour. Fisherbrand filter paper was selected as the most appropriate due to its extended time and weak negative colour change to allow for a better assessment of a positive reaction and reaction time with blood.

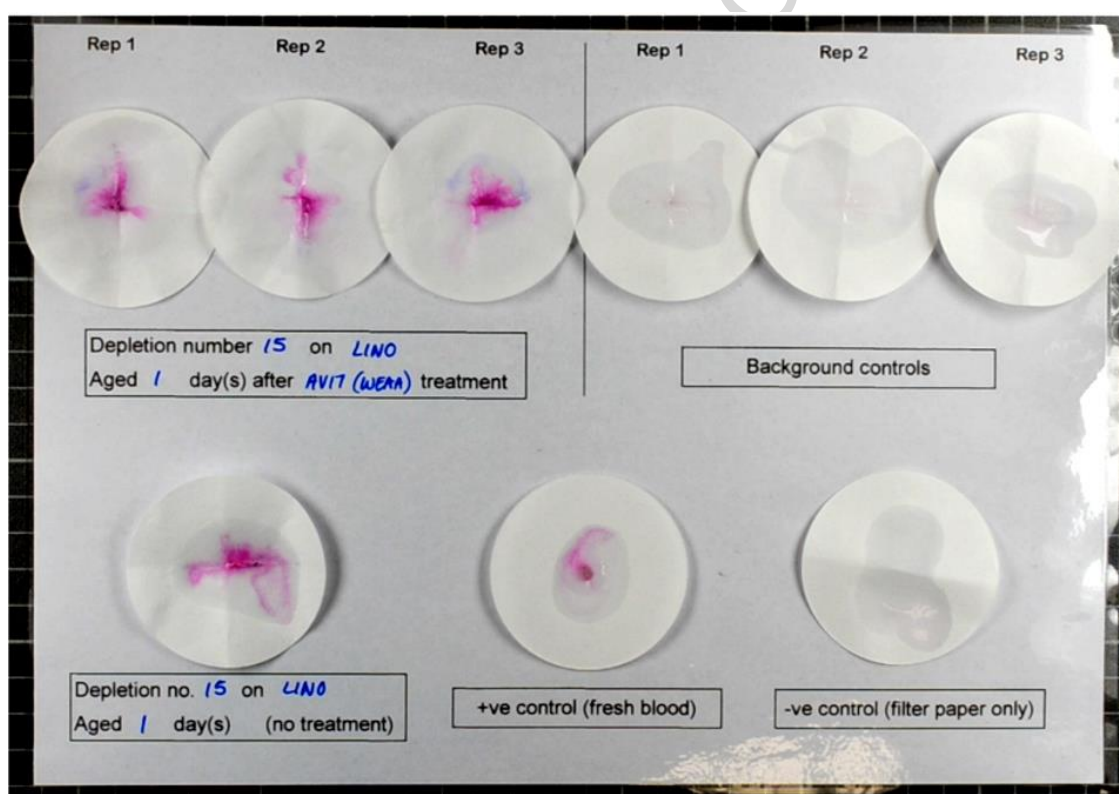


Figure 2 - Exemplar template for one KM test

Takayama Test

A working solution of Takayama reagent was prepared and used fresh [23]. The preliminary LOD study determined the limit of detection for the Takayama tests was depletion 6 for both porous and non-porous substrates. Two marks near the edge of the detectable range were selected for testing, such as depletions 3 and 5 or 4 and 6, from the control depletion and the

first test depletion. An additional preliminary study was conducted to develop an extraction and testing protocol for optimal detection. Test marks on the *non-porous* substrates were swabbed with a cotton bud dampened with distilled water. The head of the swab was cut and placed in a glass vial. Five drops of distilled water were added to the vial over the swab and gently shaken to aid extraction of the blood from the swab. Test marks on the *porous* substrates were cut out and added to a glass vial. Ten drops of distilled water were added directly over the surface and the vial gently shaken. A few drops extract were added to a microscopy slide and allowed to dry on a hotplate for <1 minute to encourage the formation of the haemochromogen crystals. The slide was initially viewed under x100 magnification then x400 magnification with a Leica DM E basic compound microscope. The presence of even a single red needle-like feathery crystal was considered a positive result and graded according to a grading scale developed for the purpose of this study (Table 1).

Table 1: Takayama grading scale

Grade	Characteristic of Grade
Negative	No crystals visible
1	<10 crystals in field
2	10 - 20 crystals in field
3	Many crystals in field, spread out
4	Many crystals in field, compounded
Standard (e.g. 2)	Crystals are small; feathery or simple shape
+ grade (e.g. 2+)	Large, feathery crystals

*when viewed under x400 magnification

RSID™ - Blood Kits

RSID-Blood test strips contain a sample well and a result window with a control line (C) to indicate the test is working correctly, and a test line (T) to illustrate a positive result (Figure 3). Results are not quantitative and are reported as either positive, negative or inconclusive. One mark was selected for testing from both the control depletion and the first test depletion (same depletion number). The LOD study determined the maximum depletion numbers detectable for each of the substrates and were as follows; up to depletion 20 on paper (full series), depletion 10 on brown envelopes, depletion 15 on tile and depletion 9 on linoleum. A mark on the edge of detection, visible after enhancement and within the detectable range was selected. Positive and negative controls were tested prior to sample testing to ensure no issues or contamination occurred during the extraction process. All RSID procedures for non-porous surfaces were

conducted in accordance with the manufacturer's suggested extraction protocol [60]. Although the manufacturer suggests taking a punch or cutting for porous surfaces (20 mm²), preliminary trials showed that swabbing as per non-porous surfaces yielded better sensitivity. An RSID™ Reader was used to objectively analyse the RSID test results. The remaining 100 µL of extracted aliquots were then frozen until subsequent DNA analysis was performed.



Figure 3 - RSID-Blood kit - appearance of a positive and negative result

DNA extraction and quantification

All blood samples analysed with the RSID-Blood test were subjected to DNA analysis. The remaining 100 μL of RSID buffer solution was added to 200 μL of PBS and subjected to the DNA extraction using the PureLink™ Genomic DNA mini kit following the manufacturer's instructions (Invitrogen, UK). For the positive control, 200 μL of blood was used while for the negative control, 200 μL of deionised water was used. All samples were eluted in 30 μL of elution buffer and then stored at -20°C until ready for further analysis.

DNA extracts were quantified using the Investigator® Quantiplex™ kit (Qiagen, UK). Minor modification in the protocol included preparation of samples using half the volumes to give a total reaction volume of 12.6 μL . The samples were run on a LightCycler®96 instrument. Data was acquired during the annealing/extension of the run and analysed using the absolute quantification, while ensuring that the R^2 value was greater than 0.99. Following analysis, the quantification value (C_q) of each sample in the VIC channel was initially checked to evaluate the presence of inhibitors. Samples that showed a VIC positive result had their quantification value considered.

DNA profiling

Out of the 154 samples processed, 45 samples were selected for DNA profiling. Selected samples focused on 7 and 28 days ageing including controls (no enhancement) and those after enhancement to assess the quality of the DNA profiles obtained. DNA profiles were generated using the AmpFℓSTR® NGM™ kit (Applied Biosystems) following the manufacturer's instructions and using a final reaction volume of 12.5 μL .

PCR amplicons were then mixed with 10 μL formamide and 0.25 μL of GeneScan™-500 LIZ® size standard and loaded on a GA 3130. Data generated from electrophoresis was analysed using the GeneMapper v3.7. DNA profiles were assessed for their quality and categorised as full, partial or no profile. Artefacts such as drop-ins, heterozygous peak balance and level of stutter were also noted.

Results and Discussion

Examination of blood marks before and after enhancement

Overall, the use of a Violet Crime-Lite® 82S during the initial fluorescence examination did not provide an increase in the number of marks observed under white light; however, in some case the contrast was improved. The number of marks that were observed before and after enhancement were counted and are presented in table 2 as averaged across the three ageing periods, the number of total depletions on porous and non-porous substrates was 20 and 30 respectively.

Table 2 – Number of additional marks visible after enhancement

Enhancement Technique	Substrate	Before	After	No. of additional marks visible after enhancement
BMP	Paper	12	12	0
	Envelope	12	12	0
	Tile	10	10	0
	Lino	5	5	0
PS	Tile	10	20	10
	Lino	5	10	5
CA	Tile	10	10	0
	Lino	5	5	0
AV17 (weaa)	Tile	10	30	20
	Lino	5	28	23
AV17 (MeOH)	Tile	10	30	20
	Lino	5	25	20
AY7 (weaa)	Tile	10	30	20
	Lino	5	25	20
AY7 (MeOH)	Tile	10	30	20
	Lino	5	25	20
NIN	Paper	12	20	8
	Envelope	12	20	8
DFO	Paper	12	20	8
	Envelope	12	20	8
Bluestar® Forensic Magnum	Paper	12	20	8
	Envelope	12	20	8
	Tile	10	30	20
	Lino	5	30	25

Effects of Ageing and Substrate on Blood Tests

There was little to no difference observed on the presumptive and confirmatory tests for blood before and after enhancement across each ageing period, with the exception of the Takayama test, which showed a significant drop from the ageing period of 7 to 28 days (figure 4). Comparing the control (before enhancement) and the blood marks after each enhancement technique showed that the ageing, substrate (figure 5) and technique had a minimal effect on the KM and RSID tests; however, the effects were more pronounced for the Takayama test. Both figures 4 and 5 demonstrate an average view of the data; however, figures 6-8 present the data collected in this study by the enhancement technique.

Effects of Enhancement Techniques on Subsequent Blood Tests

Figures 6-8 summarise the results observed for the three blood tests (KM, Takayama, RSID) before and after enhancement across substrates and ageing periods. This data is presented in such a way due to the limited effect of ageing and substrate on the blood tests used in this study. For the KM test, all control samples (without enhancement) were positive whereas some of the samples treated with the different enhancement techniques were affected as shown in figure 6. Takayama tests were severely affected by the enhancement technique (figure 7); however, RSID showed minimal effects after treatment, except for ninhydrin (figure 8).

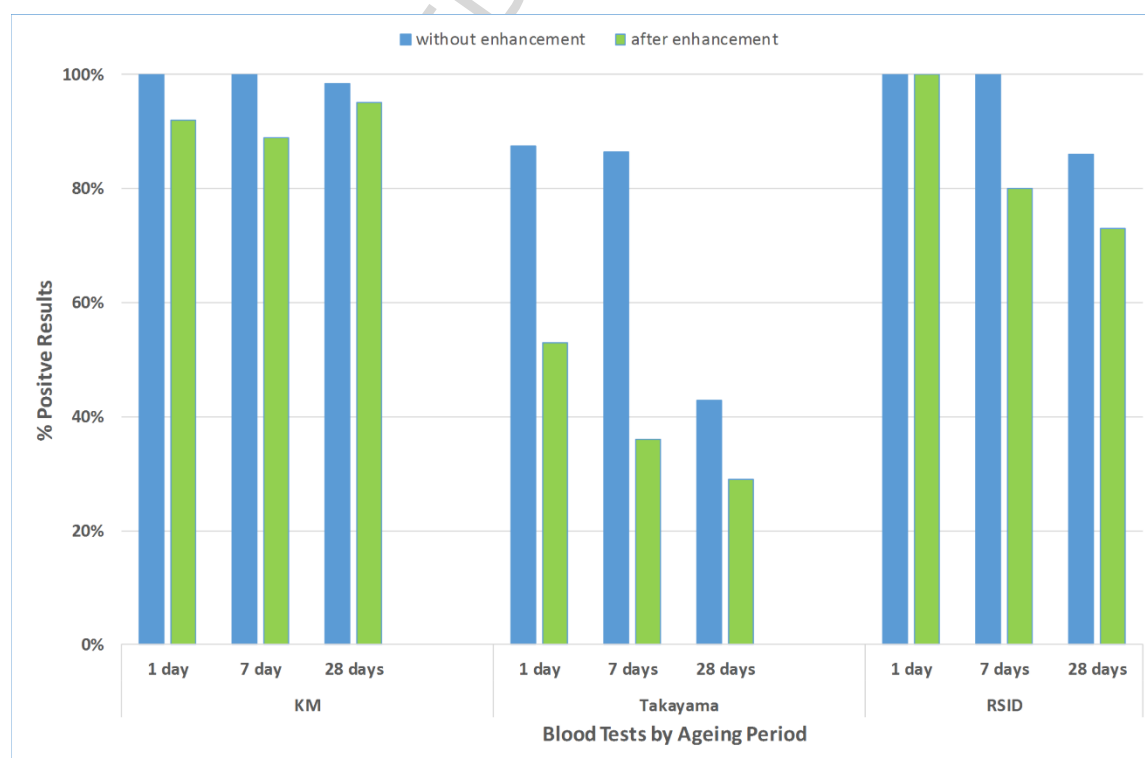


Figure 4 - Effect of ageing on blood tests across all substrates and enhancement techniques, before and after enhancement

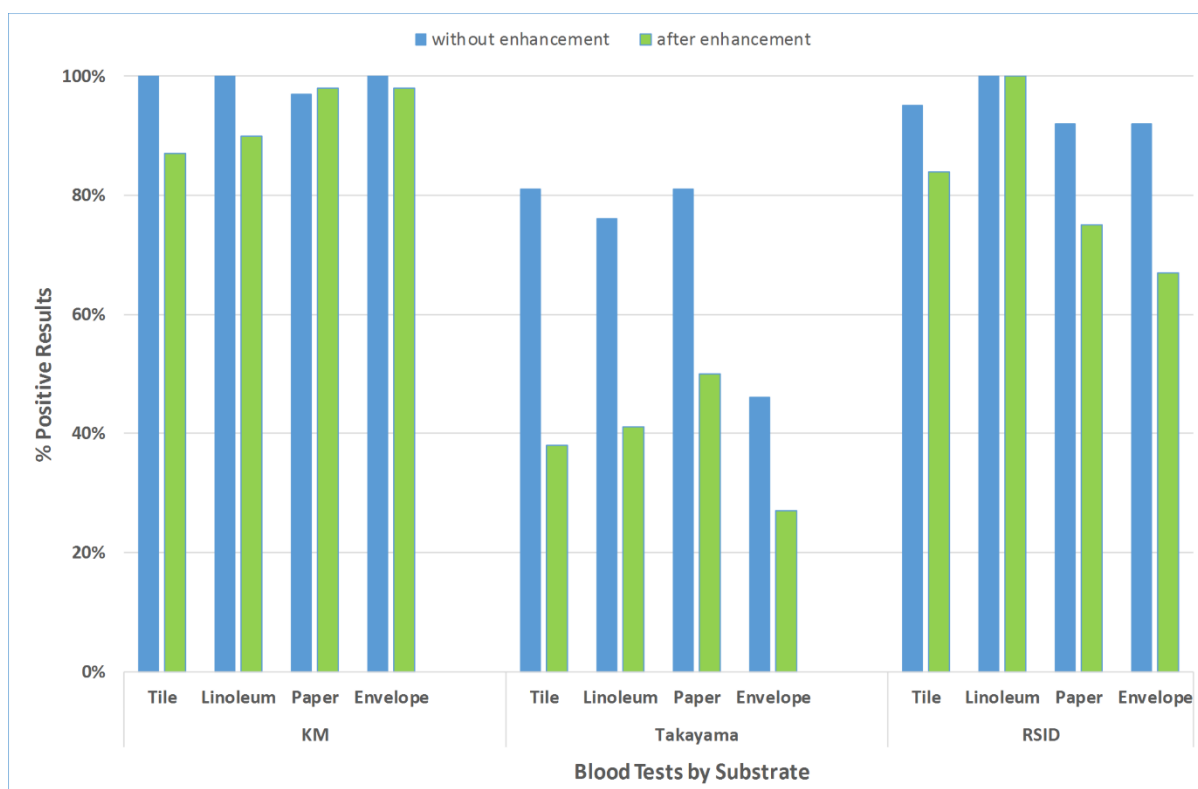


Figure 5 - Effect of substrate on blood tests across all ageing periods and enhancement techniques, before and after enhancement

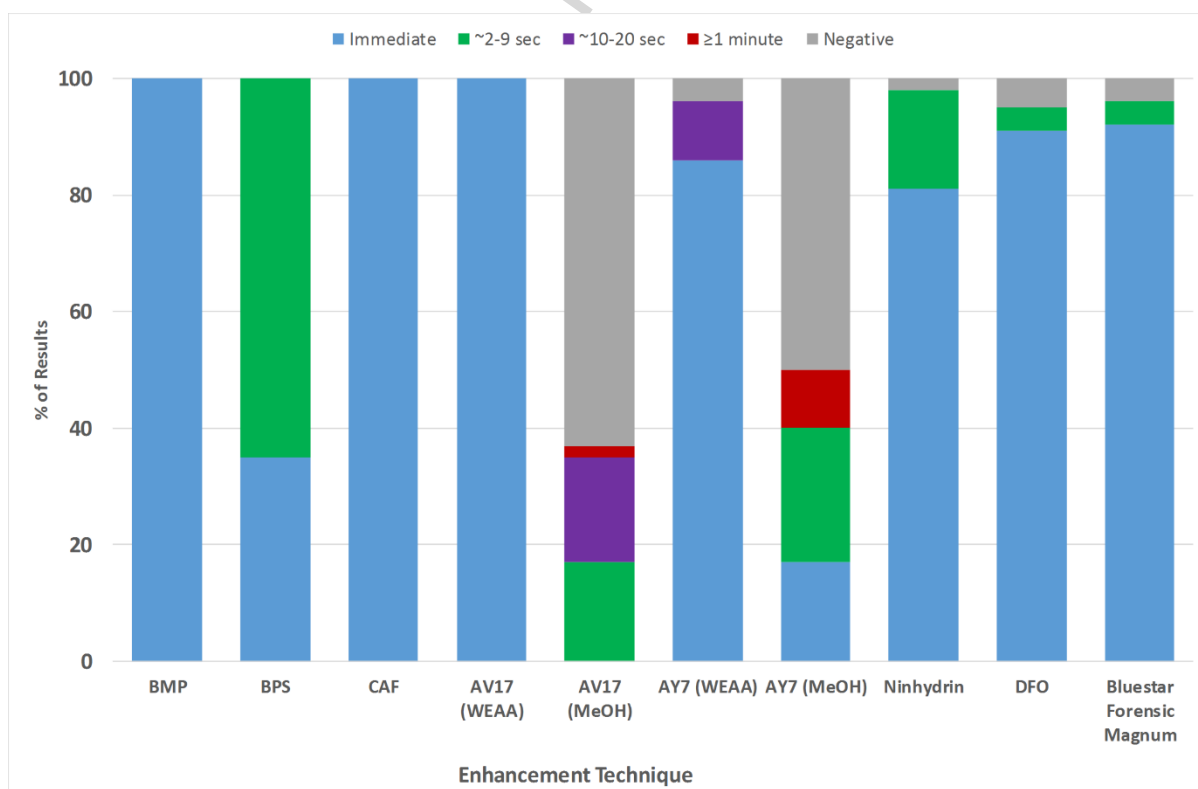


Figure 6 - Effect of enhancement technique on the KM test after enhancement

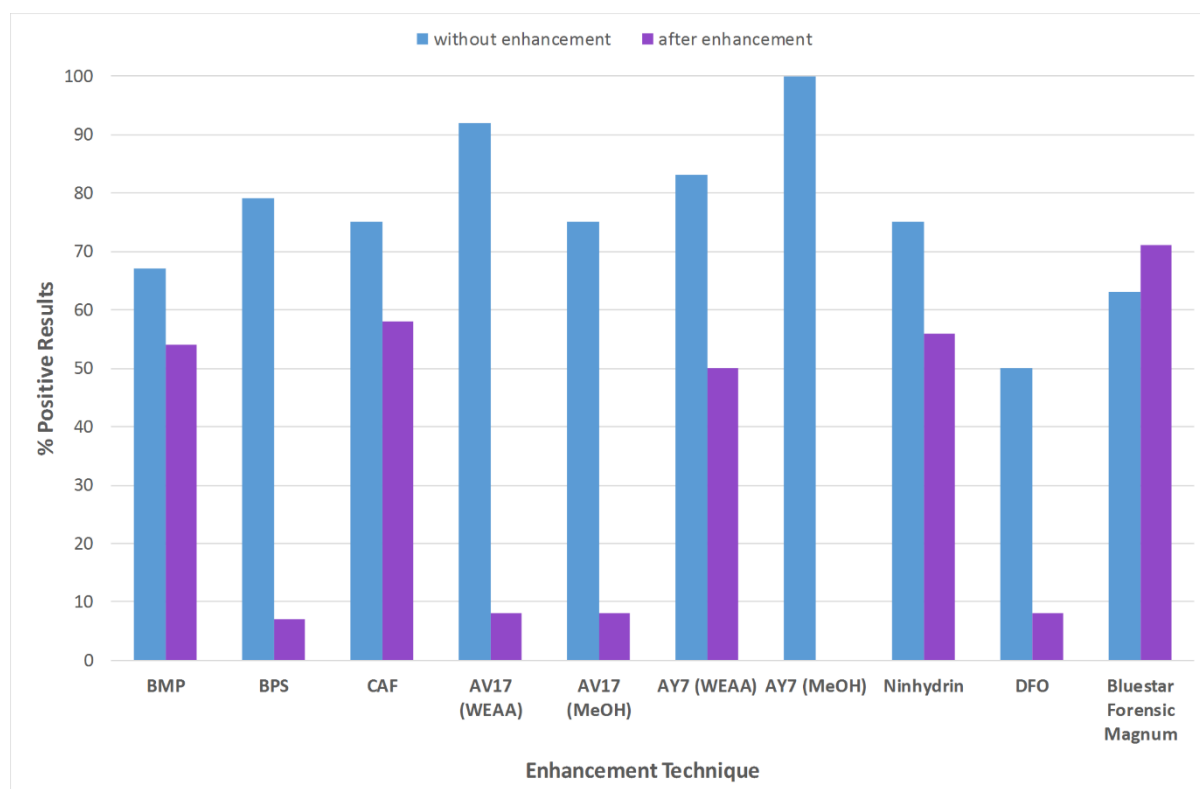


Figure 7 - Effect of enhancement technique on the Takayama test, before and after enhancement

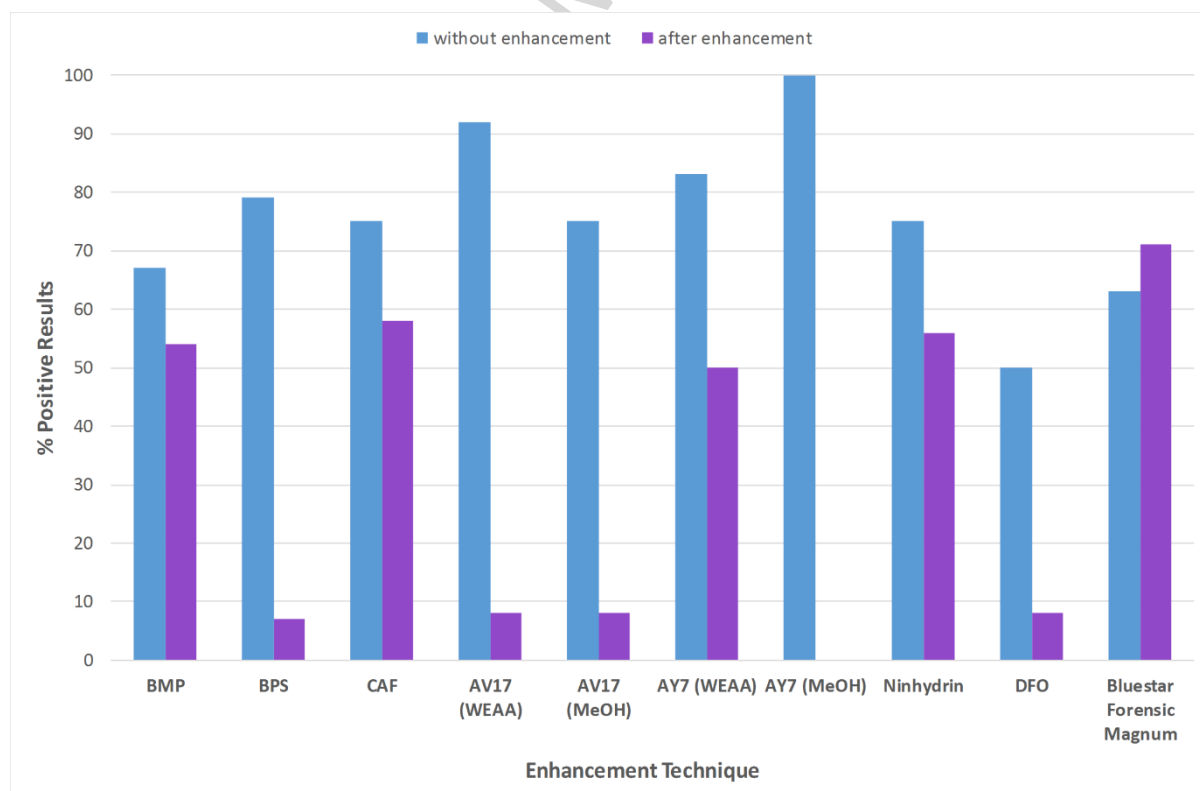


Figure 8 - Effect of enhancement technique on the RSID test, before and after enhancement

Black Magnetic Powder

BMP did not have any major adverse effects on any of the three blood tests. All KM tests were positive with an immediate colour change, although marks visually on the very edge of detection produced a small or weak pink colouration. Only a slight reduction in positive results with the Takayama and RSID tests occurred after enhancement compared to no treatment. Negative Takayama results were most likely due to poor sensitivity of the technique.

Black Powder Suspension

All test marks for both tile and linoleum produced positive KM results. However, not all results were immediate with 78% of marks tested from tiles and 52% from linoleum taking approximately 2-5 seconds to produce a positive colour change. Blood residue may be removed during the rinsing stage to remove excess powder suspension. Previous work [8] observed an increase in KM reaction time as the dilution factor of blood increased which may explain why there was a slight delay in reaction with some of the KM tests in this instance. Almost half of the marks tested from linoleum reacted immediately. The loss of blood after the rinsing stage proved especially problematic for the Takayama test. Positive results were obtained with the RSID test before and after enhancement.

Cyanoacrylate Fuming

All marks treated with cyanoacrylate fuming and BY40 produced an immediate positive KM result. The hard superglue polymer caused some issues with extraction of blood as it was difficult to rub off with the filter paper, which mostly affected weaker depletions. Positive Takayama results were obtained after superglue fuming, albeit from the lower depletion 3 and not from depletion 5. Cyanoacrylate fuming had no adverse effects on subsequent RSID tests, with all marks producing positive results.

Acid Violet 17 and AY7 (WEAA and methanol-based)

The WEAA formulation for both techniques appears to have little, if any, adverse effects on KM results (figure 5); however, the MeOH formulations inhibited the KM reaction with only 37% of marks tested after AV17 (tile and linoleum) producing positive results after enhancement. It also appears that the reaction time increased as the depletion number increased as it took approximately 5-7 seconds on numbers 13 (linoleum), 14 and 21 (tile), whereas depletion 30 (tile) took 10 seconds and depletions 20 and 28 (linoleum) took approximately 10-15 seconds. A number of depletions took over 1 minute to show a pink colouration.

Depletion 30 on the 1-day aged tile took more than 1 minute to change colour; however, since the negative control had not changed colour and a pink colouration formed in the centre of the filter paper this was accepted as a positive result. This contrasts with another study [46] where AY7 WEAA only produced a negative KM result with the final sixth depletion and a positive response for all depletions with methanol based acid black 1. There are clear differences in the number of depletions as this other study [46] only used six depletions compared to the 30 in this study. The results will also depend on the cut-off time for including a positive result.

As the WEAA solution consistently produced positive results, it was hypothesised that the use of methanol in the other formulation accounted for the negative and delayed results obtained. To test this theory, a set of experiments was carried out with blood depletions treated with methanol only. All marks showed that methanol does not inhibit the KM test and an instant positive reaction was observed, albeit slightly fainter than the control without methanol treatment. Phenolphthalein is an acid-base indicator and is colourless in an acidic environment, therefore it may be possible that the acetic acid in the dye solution prevents or delays the colour change of oxidised phenolphthalein. Approximately 10% of the methanol dye solution is acetic acid, compared to a lesser 5% for the WEAA solution, therefore it is plausible that this could be the cause. Further trials were carried out with 30 blood depletions on white ceramic tiles and treatment with a 5% and 10% solution of acetic acid in distilled water. All depletions gave a positive KM result for both concentrations. A further trial of a 10% solution of acetic acid in methanol gave mainly negative KM results except for the heavy blood depletions 1-5. These results suggest that the negative KM results for the methanol formulation of the protein stains is due to a combination of acetic acid and methanol.

Both formulations of each protein stain had an adverse effect on subsequent Takayama testing. The crystals from the mark on tile treated with WEAA were of a considerably lesser grade (graded 1+) compared to its counterpart control mark (grade 4+), which may indicate that staining reduces blood volume (figure 6). Positive results were obtained from samples aged for only 1 day, therefore it may be possible that ageing of blood may also have an effect on the performance of the test in conjunction with the use of protein stains. Both formulations of AV17 and AY7 had no adverse effects on the RSID-Blood tests as all treated marks produced positive results. The extract did stain the test strip purple after AV17; however, this did not reduce visibility of the control and test lines.

Ninhydrin and DFO

During KM testing, blood depletions treated with ninhydrin and DFO made it difficult to rub off with filter paper. Extraction from envelopes appeared to compound issues with ageing as all samples aged 1 and 7 days for both techniques produced immediate results, whereas all envelope samples aged 28 days had a delayed reaction of 1-2 seconds after ninhydrin and a delay of 5 seconds was observed for depletion 20 after DFO. A single mark from both paper and envelope (aged 28 days) was directly tested instead of using filter paper, which produced immediate positive results, although it was difficult to see the colour change on the brown envelope. Furthermore, marks were rubbed more vigorously, without ripping the filter paper, for ninhydrin samples aged 1 day, which successfully produced immediate positive results. Most marks on paper treated with DFO produced immediate positive results. Positive Takayama results were obtained after ninhydrin, however a reduction of ~20-30% was observed compared to control marks. DFO had an adverse effect on the Takayama test since a very small number of positives was observed when compared to the controls without treatment. Contrary to the Takayama test, DFO produced more positive RSID results than ninhydrin. Ninhydrin and DFO showed the highest decrease in RSID sensitivity across all chemical techniques tested.

Bluestar Forensic Magnum Luminol

Bluestar Forensic did not adversely affect KM tests with a small number of negative results originating from depletions 28, 29 and 30 on the 28-day aged tile. This is in line with other studies [42,43], which reported that Bluestar Luminol did not have negative effects on the KM test; however dilutions of 1 in a 1000 on certain substrates did have some negative results. According to the manufacturer, Bluestar Forensic Magnum is “three times more powerful than the regular Bluestar Luminol. The Takayama test, although specific to blood, is not sensitive and in this instance, slightly more positive results were obtained from test marks than control marks from tile and linoleum. Another study [44] reported that luminol had no effect on the Takayama test. A small number of blood depletions treated with luminol resulted in negative RSID results, which originated from samples aged 28 days. This reflects results reported elsewhere [45].

Effect of Enhancement techniques on subsequent DNA analysis

From the 154 samples that were subjected to DNA extraction and quantification with the Investigator® Quantiplex, 152 gave a detectable quantity ranging from 0.002 ng/μL to 5.096

ng/ μ L with the pure blood sample (not treated) giving a quantity of 51 ng/ μ L. The lowest quantities border the sensitivity range given by manufactures instruction of 0.001 ng/ μ L [61] although another study indicated that the assay sensitivity can go down to 0.0003 ng/ μ L [62]. Looking at the internal control (IC) the average IC Cq value for the treated samples was of 27.89 (s.d. 1.54) which was not statistically significantly (P-value >0.05) different from the IC Cq of the standards that were prepared alongside the treated samples (P-value >0.05). This indicates that the treatment of samples did not result in any inhibition for the PCR. These results reflect other studies [49–54] which reported that reagents commonly used to develop latent marks do not adversely affect subsequent recovery of DNA.

Following quantification, the 45 samples selected for amplification with the AmpF ℓ STR[®] NGM[™] kit gave useable DNA profiles (i.e. profiles that provide useful information for comparison and identification). All samples gave full DNA profiles with the exception of 4 samples that gave partial profiles in which 15 or 14 loci were genotyped. The success in the amplification of the DNA from these samples was not unexpected particularly because the sensitivity studies showed that even with 0.062 ng of DNA full profiles can be obtained. In this case full profiles were obtained with 0.13 ng DNA. Artefacts such as heterozygous peak imbalance and allele drop outs were observed mostly when the amount of DNA used was less than 0.1 ng.

The samples that were selected for this study were test samples from day 7 and day 28 of each treatment. No particular relationship was observed between the quality of DNA profiles and the substrate, enhancement treatment or number of days from treatment. Even the four samples that gave partial profiles were from different substrates, ageing periods and treatment i.e. ninhydrin treatment on envelope (7 days), AY7 (MeOH) on lino (28 days), AV17 (MeOH) on tile (7 days) and BFM Luminol on tile (28 days). Negative RSID-Blood results in this study yielded a successful DNA profile, except for one partial profile. Despite this, this study gives an indication about the success of DNA analysis after treatment and the possibility that DNA analysis can be included as part of the work flow after analysis of body fluids and enhancement techniques.

Summary

Table 3 summarises the results observed as to the effects of the different enhancement techniques on the subsequent presumptive, confirmatory and DNA analysis on blood stains. The amount of DNA material in a latent blood stain is higher than what would be found in a

latent fingerprint, and the results from this study do not imply that DNA will be recovered from latent fingerprints when treated with these enhancement techniques.

The work presented in this study aims to provide information to help decision-making when developing a strategy to ensure the maximum recovery of evidence. Any visible finger/footwear mark or blood stain should be photographed and sampled before the use of enhancement techniques. The results demonstrate the high sensitivity of the KM test that can yield positive results up to the 30th depletion although a delayed colour reaction was observed after the use of certain enhancement techniques, most specifically after the methanol-based protein stains. It is also important to note that KM is a presumptive test and users should be aware of possible false positive and negative results. The Takayama test is cheap and specific to blood, however its sensitivity is very poor and in this study was only able to detect blood up to the 6th depletion. This test is also laborious and uses pyridine, a chemical classified as highly flammable, harmful and an irritant. RSID-blood tests are more expensive than both the KM and Takayama tests; however, it is specific to human blood. It possesses a better sensitivity than the Takayama test but less than the KM test. The remainder of the RSID extract is suitable for DNA analysis. The use of enhancement techniques did not affect DNA extraction or profiling when compared to positive controls.

Table 3 - Effect of the enhancement techniques on the subsequent presumptive and confirmatory tests for blood

Enhancement Technique	Improved Visual Examination	KM Testing	Takayama	RSID™ Blood	DNA Analysis
Black Magnetic Powder	X	✓	✓	✓	✓
Iron-oxide PS	✓	✓	Reduced	✓	✓
Cyanoacrylate Fuming/ BY40	X	✓	✓	✓	✓
Acid Violet 17 (WEAA)	✓	✓	Reduced	✓	✓
Acid Violet 17 (MeOH)	✓	Reduced	Reduced	✓	✓
Acid Yellow 7 (WEAA)	✓	✓	✓	✓	✓
Acid Yellow 7 (MeOH)	✓	Reduced	X	✓	✓
Ninhydrin	✓	✓	✓	Reduced	✓
DFO	✓	✓	Reduced	✓	✓
Bluestar® Forensic Magnum	✓	✓	✓	✓	✓

Conclusion

This study considered the effect that enhancement techniques have on the subsequent presumptive and confirmatory tests for blood. The visualisation of blood marks by latent enhancement techniques provides a target area for swabbing for subsequent presumptive, confirmative and DNA tests. If the stains are not visualised in the first instance, then speculative swabbing may be detrimental to the fine detail of latent marks. The results demonstrate that KM is largely unaffected by latent enhancement techniques although the methanol-based protein stains resulted in a delayed reaction of up to 1 minute. A number of protocols recommend the KM test result as negative if the reaction is not instantaneous which may result in a number of false negatives. The Takayama test is not sensitive, therefore, its performance was easily affected by the enhancement technique and ageing periods resulting in negative results. Although RSID-Blood tests are specific to human blood, its sensitivity is inferior to KM. These tests were largely unaffected by chemical enhancement; however, a drop in positive results was observed for some of the techniques when compared to positive controls.

The use of enhancement techniques did not affect DNA extraction or profiling when compared to positive controls. Out of the 45 samples processed for DNA profiling, 44 gave full profiles, while the remaining showed allele drop out in one or two loci. The amount of DNA material in a latent blood stain is higher than what would be found in a latent fingerprints and the results from this study do not imply that DNA will be recovered from latent fingerprints when treated with these enhancement techniques.

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Highlights

- Strategy to maximise evidence recovery (mark enhancement and blood) and efficiency.
- KM test considerably more sensitive than the Takayama and RSID tests.
- KM test largely unaffected by most enhancement techniques.
- Methanol-based protein stains affected the KM and Takayama tests but not the RSID test.
- DNA recovery from bloodstains was still possible after enhancement.

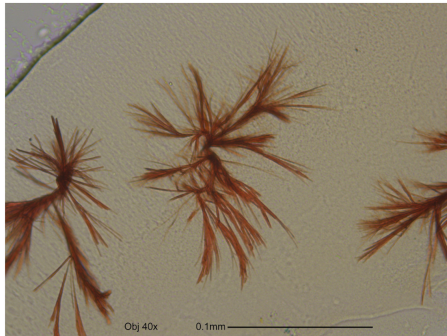


Figure 1

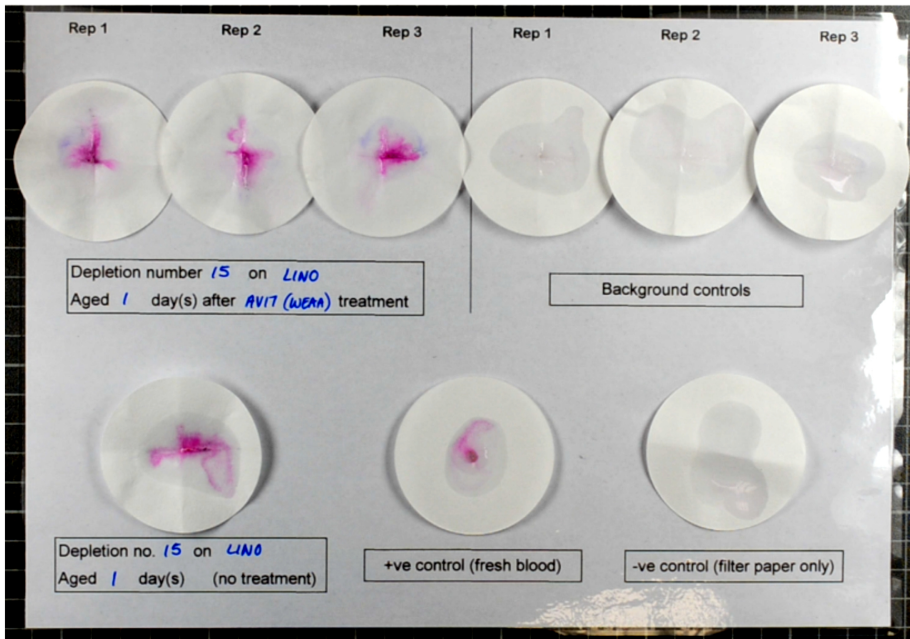


Figure 2



Figure 3

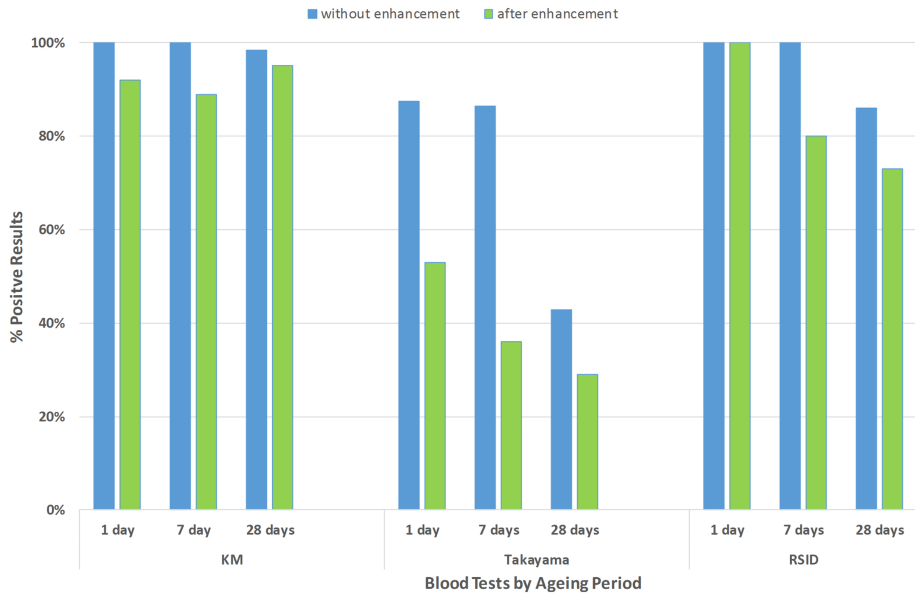


Figure 4

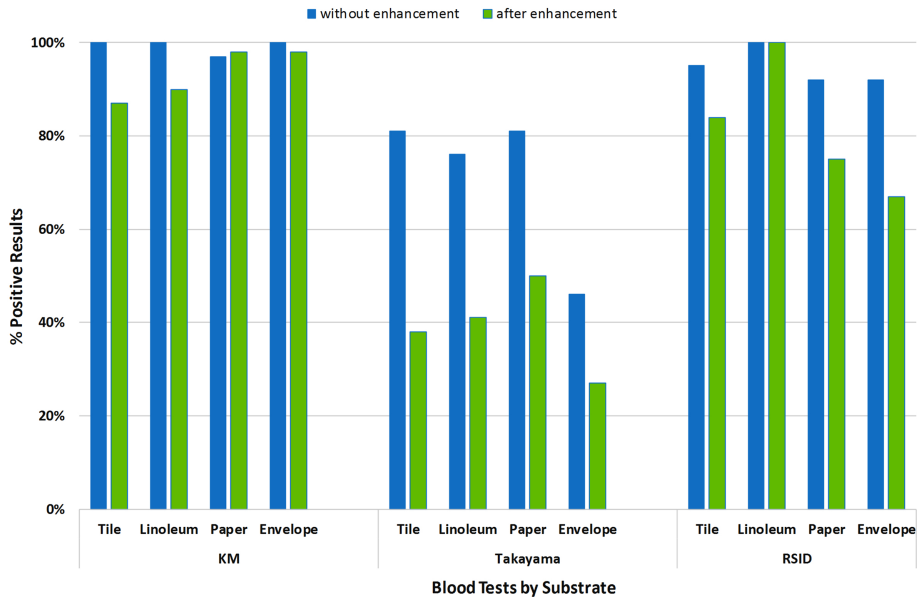


Figure 5

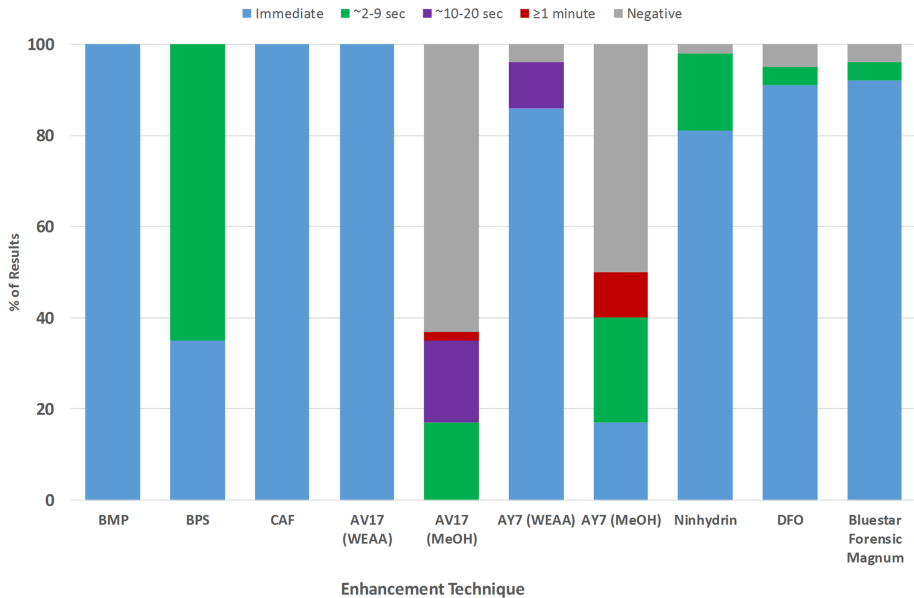


Figure 6

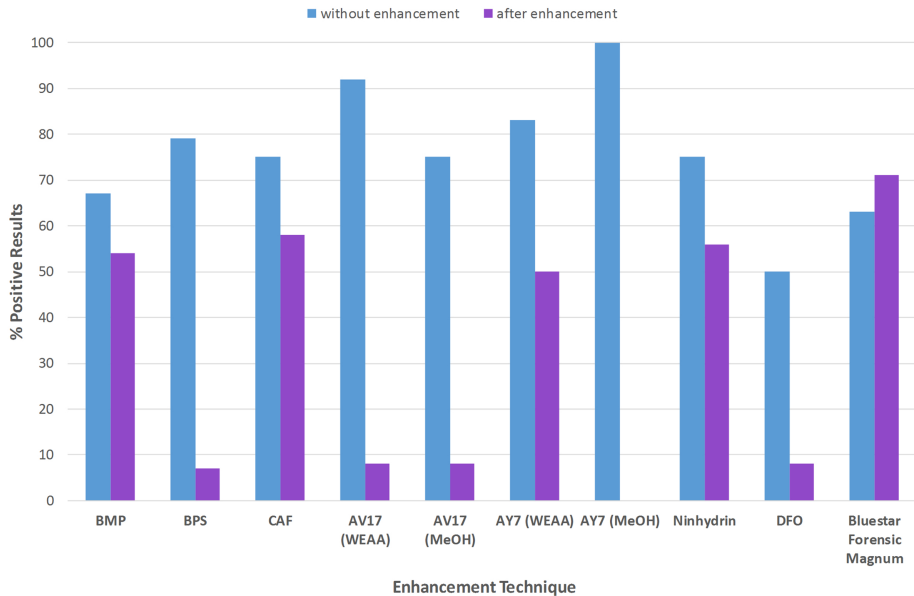


Figure 7

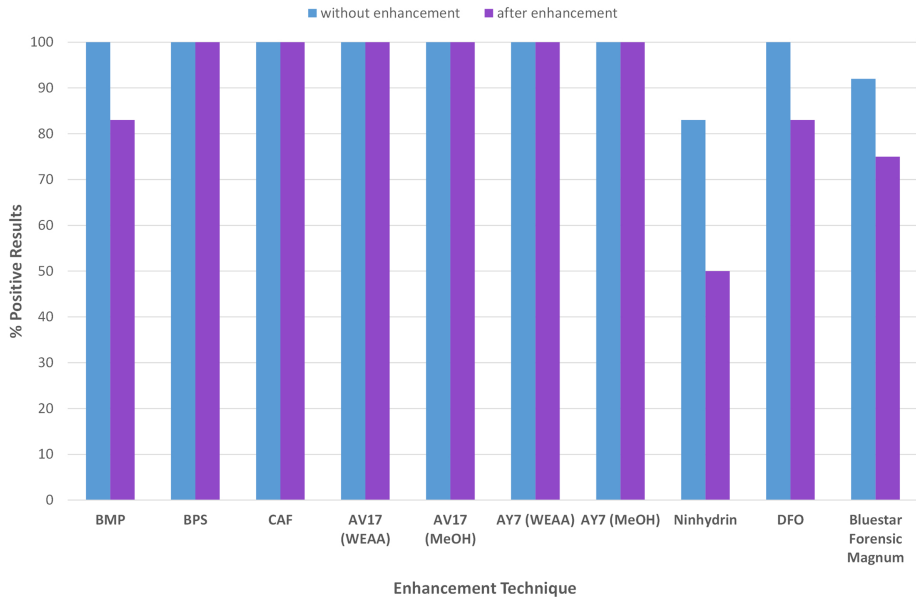


Figure 8