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In depth investigation of the capabilities and limitations of combined proteomic-MALDI MS based approach for the forensic detection of blood

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ABSTRACT

For the past 7 years, Matrix Assisted Laser Desorption Ionisation Mass Spectrometry (MALDI MS) based methods have been developed and published for the forensic detection of blood in stains and fingermarks. However, in the view of adoption in an operational context, further investigation into the capabilities and limitations of this approach must be conducted. The refinement and testing of this approach must also be tailored to the requirements of the end users, enabling them to address the specific circumstances most encountered in a forensic scenario. The present study delves deeper into the assessment of the applicability of MALDI MS based strategy for the reliable and robust detection of human blood through: (i) a semi-qualitative assessment of the sensitivity of the method, (ii) a wider investigation of the compatibility of the method with the prior application of commonly used presumptive tests and (iii) assessment of the specificity of the method (when blood is present in mixture with other biofluids) and of its robustness, by assessing blood detection from a range of porous materials. The findings strengthen the evidence supporting the adoption of MALDI MS based approaches as a confirmatory test for the forensic detection of human blood in an operational context.

1. Introduction

The rapid and confirmatory detection of blood recovered from the scene of a crime can be a vital tool in gaining intelligence around the nature of the crime committed, additional to blood being a source of identifying DNA information. The initial determination of blood presence is carried out using presumptive blood testing. The nature of the surface on which the blood is initially located, the volume of blood and the environmental conditions that this biofluid may have experienced will determine the type of presumptive testing to perform. Leucomalachite green (LMG), Luminol and Kastle-Meyer (KM) (the latter also known as the Phenolphthalein test), together with the commercially available reagent strip Hemastix, are haem-reactive compounds. They are most used for the detection of bloodstains [1] and are followed by the use alternative light sources (ALS) such as the Crime-lite™ [2]. For the detection of blood marks, several acid dyes can be used, with Acid-Black 1 (AB-1), Acid Violet 17 (AV-17) and Acid Yellow 7 (AY-7) belonging to Category A processes (recommended process for routine operational use) within the Fingermark Visualisation Manual (FVM) published by the Home Office UK [3]. AB-1 and AV-17 are protein stains commonly used for the enhancement of blood marks that are effective for several different surfaces from non-porous to porous substrates; conversely, AY-7 is only effective on non-porous surfaces.

As very well documented by Tobe et al [4] and Bossers et al [5] and illustrated in some circumstances by Kennedy et al 2020 [6], all presumptive blood testing suffers from specificity issues with occasional false positives. The most well-known false positive is the reaction of haem-reactive tests to bleach, though reaction with some plant material and horseradish has also been observed [4,5]. Protein dyes by their nature will not distinguish between blood and other protein containing biofluids such as semen and saliva. Microbial growth in sink/bath drains can occasionally yield false positives to the application of haem-based detection methods, potentially misleading investigators in speculating that bloody wastewater may have passed through the drains (*Chris Gannicliffe, personal communication*). This instance was already reported in 1973 where bacterial and soil extracts were shown to yield positive phenolphthalein reactions [7].

For these reasons, confirmatory testing should be performed, though this is not routine standard practice by many forensic providers in casework; generally, a positive presumptive test from a stain *that looks*

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like blood, is considered sufficient to report the stain as 'blood' by most forensic practitioners/providers. However, cases such as *Chamberlain v. The Queen* (1984) [8], where incorrectly claiming the presence of blood contributed to the wrong conviction of Lindsay Chamberlain, call for a more serious consideration of the use of confirmatory testing. This is desirable, at the very least, in those instances where a positive presumptive blood test is obtained from an area where blood cannot be visualised (where only traces are present, for example on a wiped weapon or at a crime scene after attempts by the perpetrator to wash blood away). Alternatively, confirmatory testing could have a greater role in those instances where it is recognised that false positive presumptive test reactions are an issue - such as sinks and drains, or where peroxidase activity from other interfering substances such could be present. Notwithstanding, even some currently available confirmatory tests have limitations. For example, the Takayama [9] and Teichman [10] tests, though specific, due to the use of antibodies for the recognition of human blood, are destructive and prone to false positives when in the presence of peroxidases and catalases at sufficient concentration [8].

When confirmatory testing is considered appropriate, rather than the Takayama and Teichman tests, in general many forensic providers tend to utilise a second blood detection method based on a biochemistry different to that of the initial presumptive test – for example, after a positive KM test (a result based on the peroxidase-like haem activity of the blood present), they may deploy a lateral flow antibody method that detects human or primate haemoglobin (eg Hexagon OBTI or Hematrace) or detecting the red cell membrane antigen glycophorin A (eg the RSID blood test). Nevertheless, specificity issues remain with these tests – for example a generally widely recognised risk related to the use of any antibody test is cross reactivity, which has been, for example, observed with the Hematrace™ test [8,11], where false positive human blood results have been yielded from both primate and ferret blood [8]. False negative results are also still observed, for example with the Hexagon OBTI test [12]; in addition, sensitivity issues connected to age and storage above 4 °C are also observed with this test [13]. Whilst false positives are clearly undesirable, false negatives, reviewed by Castello et al [14] may also be dangerously misleading but may be counteracted by the application of a confirmatory test too.

A confirmatory test that is fast, multi-informative and that has high specificity, sensitivity, low or no cross reactivity, is highly desirable. For the past eight years, MALDI MS Profiling (MSP) and MS imaging (MSI) have been investigated as an alternative forensic test for the confirmatory detection of human blood and animal blood [6,15–20] demonstrating its capability to detect a false positive from presumptive testing [6].

The technique is interesting as it can quickly determine the presence of blood in MSP mode from a stain or can visualise it on a mark in MSI mode, effectively reconstructing molecular images of fingermarks [15–16,18–19], compatibly with the prior application of some of the blood enhancement techniques currently used [15,16,18]. Thus far, MALDI MS has been found to offer a plethora of additional intelligence when used to detect blood, namely *(i)* as a rapid and confirmatory test for human blood [15–18], *(ii)* to determine blood provenance within a range of animal species [6,19–20] *(iii)* to assist in suspect or victim identification through the detection and mapping of Haemoglobin variants [21] and enabling the subsequent application of DNA typing [15]. The most recent work published by Kennedy et al [22] has additionally shown that, in the case of animal blood, a distinction can be made between whether the animal blood originated from the jugular of the animal or a different source such as packaged meat; this intelligence may additionally help in the reconstruction of the events leading to the presence of blood at the scene. Due to the significant body of knowledge available and the compatibility with several CSI and crime labs enhancement techniques, MALDI MSP/MSI has been recently promoted from Category C in the 2014 Home Office edited FVM [3] to Category B processes in the next Edition (*Helen Bandey FVM Editor, personal communication*). A personal communication from the Editor recites: *a Cat B process is an "established process […] likely to offer benefits […] for occasional operational use […] and when all Cat. A options have been exhausted". "Category B processes are generally more established and therefore more likely to be used in casework"*.

Nonetheless, to the best of the authors' knowledge, MALDI MSP/MSI for detection and mapping of blood for forensic purposes is not accredited to ISO/IEC 17025:2017 for use by any academic lab or forensic provider. Whilst accreditation may be desirable, it is very challenging to achieve for an academic laboratory. However, whilst most legal jurisdictions do not require a laboratory to be accredited to ISO/IEC 17025:2017 for that specific method to be admissible in court, it is reasonable that the courts would expect a technique to be fit for purpose and to be assured that the results can be relied upon. Validation of the process is the most effective way of demonstrating this, and the Forensic Science Regulator in England and Wales has stated that *'It is the expectation of the Forensic Science Regulator that all methods routinely employed within the Criminal Justice System, whether for intelligence or evidential use, will be validated prior to their use on live casework material'* [23].

To this end, Kennedy et al have published a preliminary study, in which samples of different nature (human/animal blood, other biofluids, blood/biofluid unrelated matrices), both unenhanced and enhanced with three different blood enhancement techniques, present on a single surface, have been analysed in a blind fashion [6]. Biomarkers of human and animal (bovine, porcine, chicken) blood, (as well as of semen) have been recovered and used to validate the process on another cohort of blind samples obtaining: 1) 0 % false positive rate for human blood detection 2) 0 % false positive rate for blood/biofluid unrelated matrices 3) 100 % correct identification rate for semen. There was one instance of a false negative for human blood and this related to trace amounts resulting from the last blood mark in a depletion series. A second blind study is underway and is further exploring the robustness of the process by including a higher number of surfaces of deposition, blood enhancement and detection techniques and increased sample complexity. The study presented here aims to provide additional validation data by further investigating the sensitivity, the robustness, and the specificity of MALDI MSP/MSI. There are several factors that could negatively impact on the feasibility and operational use of MALDI-based approaches for the detection of human blood, amongst which are: the volume of blood, the nature of the deposition surface, the non-human origin of blood and the potential mixing of human blood with other biofluids. The present study has assessed MALDI MSP performance against the aforementioned factors. The aim is to provide a larger body of evidence which: (i) strengthens the confidence for an operational use and assessment against the general criteria of an accredited process and (ii) supports further promotion as a Category A process, defined in the FVM as a "standard process for routine operational use", although "routine", in this case, refers to the application of the process only in major/violent crimes [3].

2. Methods and materials

2.1. Materials

Trifluoracetic acid (TFA), α-cyano-4-hydroxycinnamic acid (α-CHCA) and Millipore ZipTips containing C18 stationary phase were purchased from Sigma Aldrich (Poole, UK). Acetonitrile (ACN) was purchased from Fisher Scientific (Loughborough, UK). Sequencing grade modified lyophilized Trypsin was purchased from Promega in 20 μg vials (Southampton, UK). Sigma dry tubed swabs were sourced from Medical Wire (MWE) (Wiltshire, UK) and Rapigest was obtained in 1 mg vials from Waters (Wilmslow, UK). Polylysine slides were obtained from Thermo Scientific (Altrincham, UK). Human blood was collected by pricking a clean finger with a 28G safety press and single use lancet (VWR, Leicestershire, UK) under full ethical approval (HWB-

ER13034924). Blood was then collected from the bleeding fingertip into a Vacutest EDTA tube (Kima, Italy); a sufficient volume was collected to perform all experiments included in this study. BlueStar (Luminol) and Kastle-Meyer tests (with hydrogen peroxide, H_2O_2) were purchased from SceneSafe (Essex, UK). Phadebas ® paper was purchased from Phadebas AB (Kristianstad, Sweden).

2.2. Methods

2.2.1. Investigation of the impact of blood volumes and co-presence of other biofluids on blood detection

A blood dilution series was prepared (1:5000, 1:10,000, 1:15,000 and 1:20,000) using human blood (stored in an EDTA tube to prevent anticoagulation prior to dilution preparation) and distilled water. This set of dilutions was used for all experiments in Section 2.2.1.

Untreated blood dilutions - Rapigest (0.1 % v/v in 50 mM ammonium bicarbonate solution) was added to Trypsin to reconstitute it in a 20 μg/mL solution. Ten μL of each blood dilution was added to 40 μL of 40 mM Ammonium Bicarbonate and to 9 μL of trypsin solution at a concentration of 20 μg/mL and Rapigest (0.1 % v/v). Each sample was then incubated for 1 h at 37 °C and the proteolytic digestion was stopped with the addition of 2 μL of 5 % TFA (aq).

Kastle-Meyer treated blood dilutions - Ten μL of each blood dilution was deposited on a clean aluminium slide to generate a blood stain from each dilution and left to dry. Each dilution stain was then swabbed with 70:30 ACN and H_2O. Upon drying, one drop of the Kastle-Meyer reagent was applied to each swab head and left for 5 s, followed by one drop of H_2O_2. The swab head from each respective dilution stain was removed using scissors and transferred into a 1.5 mL Eppendorf where 1 mL of 70:30 ACN: H_2O solution was added prior to sonication for 10 min. The samples were subsequently subjected to proteolysis under the same conditions as for the untreated blood dilutions.

Luminol treated blood dilutions - Ten μL of each blood dilution were spotted on a clean filter paper to generate a blood stain from each dilution. The Luminol reagent was then applied to each dilution stain in a dark room for visualisation of which dilutions gave a positive result. The filter papers for each dilution stain were then transferred into separate 1.5 mL Eppendorf tubes where 1 mL of 70:30 ACN: H_2O solution was added prior to sonication for 10 min. The samples were subsequently subjected to proteolysis under the same conditions as for the untreated and KM treated blood dilutions.

2.2.2. Blood detection from sweat/saliva deposits on clothing

A *t*-shirt was worn over a period of five days by the same donor of the human blood used for the experiments (in evenings, overnight and to mid-morning) in the attempt to accumulate natural deposits of saliva and sweat from moderate and day-day activity.

For the experiments with saliva deposits, one piece of fabric from the right-hand sleeve was sampled and two from the neckline. The Phadebas ® testing paper was applied to these samples of fabric to indicate the positive regions for the presence of amylase, which is an enzyme generally found in high concentration in saliva. The Phadebas paper contains starch microspheres with a blue dye that is cross linked to the starch. When amylase is present, this enzyme breaks down the starch and releases the blue dye into solution, resulting in a colour change [24]. Once location of amylase was identified, 5 μL of blood was spotted on each piece of fabric, specifically on those areas of amylase positive reaction. The fabric samples were then sonicated in 1 mL of 70:30 ACN: H_2O solution for 10 min. The samples were subsequently subjected to proteolysis under the same conditions as detailed in Section 2.2.1.

For the samples of sweat on clothing, fabric from each underarm area and from the back of the *t*-shirt was excised for analysis and 5 μL of blood were spotted on each sample of fabric. The samples were subsequently subjected to proteolysis under the same conditions as detailed in Section 2.2.1.

2.2.3. Blood detection from porous materials (cotton, wool, and polyester)

Neat blood spots of 50, 75 and 100 μL were deposited on either wool, cotton, or polyester (each sample of material was approximately 7.6 × 7.6 cm in dimension). These spots were allowed to distribute/spread on the fabric for 30 min. The radius from the centre to periphery ranged from ~ 0.2 cm to 0.5 cm in the wool stains, ~0.5 cm to 1.0 cm in the cotton stains and ~ 0.5 cm to 2 cm in the polyester stains. Subsequently, a sample from the centre and one from the periphery of each blood spot on each fabric was cut out and sonicated in 1 mL of 70:30 ACN: H_2O solution for 10 min. All cut-outs were 10 mm^2 in size for consistency. The samples were subsequently subjected to proteolysis under the same conditions as detailed in Section 2.2.1.

2.3. Matrix and application

Ten mg/mL α-CHCA in 70:30 ACN: 0.5 % TFA ($_{aq}$) was deposited by spotting 0.5 μL on top of 0.5 μL of the analyte solution for all MALDI MS experiments.

3. Instrumental conditions

3.1. MALDI MS and MS/MS

All MALDI MS analyses were carried out using the Waters MALDI-QTOF Synapt G2 HDMS instrument (Waters Corporation, Manchester, UK). Data acquisition was performed within the *m/z* range 600–2500 Th in positive sensitivity mode. The Nd: YAG laser repetition rate was set to 1 kHz for all analyses.

3.2. Data processing of MALDI MS data

Mass spectra were viewed in Mass Lynx and were then exported to mMass, an open-source multifunctional mass spectrometry software [25,26] upon conversion of the raw spectra into.txt files and only the peaks with S/N of 10 or above were annotated. Mass lists of known matrix (or matrix cluster, adduct) and trypsin autolysis *m/z* peaks were generated and used as an exclusion list for peak annotation. For putative protein identifications, candidate blood proteins were selected for *in silico digest*; namely: haemoglobin α and haemoglobin β (α Hb and β Hb) chains, erythrocyte membrane protein band 4, haptoglobin, ceruloplasmin, apolipoprotein, myoglobin, glycophorin A, complement c4 and albumin; UniProt (https://www.uniprot.org/) was used to search for protein sequences of interest and imported into mMass using "sequence tool" where the *in-silico* proteolysis with trypsin and automatic peak assignment were performed. For peptide assignments a MASCOT PMF (peptide mass fingerprint) search was preliminary launched selecting "monoisotopic MH+" values, peptide tolerance of 10 ppm, 2 missed cleavages and trypsin as the proteolytic enzyme.

4. Results and discussion

4.1. MALDI MS analysis of blood dilutions

A preliminary evaluation of the feasibility of human blood detection through the concomitant detection of the peptides at nominal *m/z* 1275 and 1530 (βHb and αHb peptides respectively-the human biomarkers panel according to Kennedy et al 2020 [6]) was conducted for all the blood dilutions, from 1:5,000 to 1:20,000, with no prior application of any presumptive testing. For the purposes of evaluating the sensitivity of this method, only Hb signals were considered for the assessment reported in Sections 4.1 and 4.2, due to their consistent detection in MALDI MS spectra of trypsinised human blood.

The 1: 5000 and 1:10,000 blood dilutions yielded an Hb peptide, additional to the two human blood Hb markers, whereas the 1:15,000 dilution yielded the two human blood Hb markers (Table 1).

Initially, the 1:20,000 dilution did not yield any Hb peptide

Table 1
Putative blood peptides detected in untreated blood dilutions. In addition to the two positive human blood markers (*m/z* 1275 and *m/z* 1530 belonging to βHb and αHb respectively), other Hb signals were detectable in the dilutions tested within a mass accuracy of −6.7 to 7.0 ppm.

Dilution	Experimental *m/z*	Mass Accuracy (ppm)	Peptide Sequence	Hb subunit
1:5,000	1274.727	−1.4	LLVVYPWTQR	βHb
	1529.742	−5.4	VGGHAAEYGAEALER	αHb
	1833.898	−3.5	TYFPHFDLSHGSAQVK	αHb
1:10,000	1274.734	−6.7	LLVVYPWTQR	βHb
	1529.742	−5.3	VGGHAAEYGAEALER	βHb
	1833.879	7.0	TYFPHFDLSHGSAQVK	αHb
1:15,000	1274.72	4.6	LLVVYPWTQR	βHb
	1529.742	−5.2	VGGHAAEYGAEALER	αHb
1:20,000 (zip tipped)	1171.663	4.4	VLSPADKTNVK	αHb
	1274.721	3.5	LLVVYPWTQR	βHb
	1314.661	2.9	VNDEVGGEALGR	βHb
	1449.79	4.2	VVAGVANALAHKYH	βHb
	1529.731	2.1	VGGHAAEYGAEALER	αHb
	1669.883	4.6	VLGAFSDGLAHLDNLK	βHb
	1797.979	3.7	KVLGAFSDGLAHLDNLK	βHb
	1833.888	2.1	TYFPHFDLSHGSAQVK	αHb
	2058.9385	4.5	FFESFGDLSTPDAVMGNPK	βHb

signatures. For this reason, the remaining solution was purified by means of a C18 ZipTip and re-analysed yielding the expected Hb peptides at nominal *m/z* 1275 and 1530 (βHb and αHb respectively) along with several additional Hb signals (Table 1) indicating that purification of all blood dilutions would have also yielded additional Hb peptides besides the two human Hb markers. Desalting via zip-tipping is very common in proteomics and the authors have found this purification step to be crucial in this and previous research [6]; as such desalting via ZipTip is operationally recommended for the detection of human blood. The reverse phase nature of this "chromatography in a tip" proves effective also to remove other potential blood contaminants such as grease and soap.

In healthy individuals, as an average across the two sexes, haemoglobin ranges between 122 and 172 g/L [27]. Therefore, the ability to confirm the presence of human blood in a 1:20,000 dilution means that haemoglobin is detectable in this study between 6.1 and 8.6 μg/mL. This observation appears to make MALDI MS more sensitive than hyperspectral imaging (HSI) given that Cadd et al reported the ability to detect blood for dilutions only up to 1:15,000 [28]. This semi-qualitative assessment is significant in depleted blood scenarios, for example when a bloody fingertip touching surfaces multiple times (generating a depletion series), or in an attempted clean-up of the scene, as well as for blood recovered outdoors after exposure to rain.

The same series of blood dilutions was also subjected to Kastle-Meyer (KM) testing prior to enzymatic digestion and MALDI MSP analysis.

KM is generally used presumptively in an indirect manner – a piece of folded filter paper is rubbed against the query stain or area, and then KM and hydrogen peroxide solution are sequentially applied to the material transferred to the filter paper. However, in instances where only trace limiting amounts of blood may be present, then one possible strategy can be to swab the query material, apply KM and hydrogen peroxide solution directly to the swab, and if a positive reaction is obtained then the swab can be retained for further tests later at the laboratory. In that instance, any confirmatory blood test therefore must be compatible with the blood traces remaining on the swab post-KM testing and, even more importantly, that it does not interfere with DNA testing. Although the latter aspect should be investigated in depth in the future, original work published by Kennedy et al have already showed that the application of MALDI MS to acid black 1 enhanced blood marks does not prevent successful DNA typing [15].

In this study (and in the manner the test was applied), the KM test yielded a positive result for blood for every dilution tested which was also confirmed by the subsequent application of MALDI MSP as well as indicating the presence of *human* blood through the detection of the two human blood Hb biomarkers (in addition to other Hb peptides) (Table 2).

It was observed that the untreated 1:20,000 blood dilution yielded peptide signatures characterised by a lower intensity (Fig S1A) compared to the KM-treated 1:20,000 blood dilution (Fig S1B) (by ~ 3.6 times for the ion signal at nominal *m/z* 1275 and by ~ 5 times for the ion

Table 2
Putative blood peptides detected in the Kastle-Meyer treated dilutions. In addition to the two positive human blood markers (nominal *m/z* 1275 and 1530, from βHb and αHb respectively), other Hb signals were detectable in each of the dilutions tested within a mass accuracy of −4.6 to 10.3 ppm.

Dilution	Experimental *m/z*	Mass Accuracy (ppm)	Peptide Sequence	Hb subunit
1:5000	1171.668	0.3	VLSPADKTNVK	βHb
	1274.720	4.5	LLVVYPWTQR	βHb
	1449.790	4.3	VVAGVANALAHKYH	αHb
	1529.735	−0.4	VGAHAGEYGAEALER	βHb
	1833.900	−4.6	TYFPHFDLSHGSAQVK	βHb
	2058.937	5.2	FFESFGDLSTPDAVMGNPK	βHb
	2341.159	10.3	TYFPHFDLSHGSAQVKGHGKK	αHb
1:10,000	1274.721	3.7	LLVVYPWTQR	βHb
	1449.790	4.6	VVAGVANALAHKYH	βHb
	1529.735	−0.7	VGAHAGEYGAEALER	αHb
1:15,000	1274.727	−1.5	LLVVYPWTQR	βHb
	1314.664	0.5	VNVDEVGGEALGR	αHb
	1529.736	−1.2	VGAHAGEYGAEALER	βHb
	1833.881	5.7	TYFPHFDLSHGSAQVK	βHb
1:20,000	1274.720	4.0	LLVVYPWTQR	βHb
	1449.791	3.3	VVAGVANALAHKYH	αHb
	1529.723	7.5	VGAHAGEYGAEALER	βHb
	1833.891	0.4	TYFPHFDLSHGSAQVK	βHb

signal at nominal *m/z* 1530).

This may be due to the additional extraction step for the KM-treated dilutions (sonication in 70:30 ACN: H_2O for 10 mins) which may have improved protein extraction prior to digestion.

In this study, the treatment of blood dilutions with Luminol yielded positive detection of blood for all the dilutions up to 1:10,000, but no luminescence was observed from the 1:15,000 and 1:20,000 dilutions. It is challenging to compare these results with "what should be expected". This is because the literature reports a wide range of sensitivity of the Luminol test, probably due to variations in the Luminol solution formulation, the nature of the blood used (stains or liquid solutions), and most critically the extent of darkening of the room and attenuation of the operator's eyes, to ensure even the slightest luminescence is detected. Even for the Bluestar formulation used in this study there is significant variation across three orders of magnitude in reported sensitivity; Finnis et al [29] have reported a sensitivity of 1:1000 for dried bloodstains, Blum et al [30] a sensitivity of up to 1:10,000, and Tobe et al [4] have reported a sensitivity of up to 1:100,000.

The application of MALDI following the use of Luminol (and other presumptive tests) on evidential items has been explored by Seraglia et al [31]. It was shown that, in their hands, samples previously treated with Luminol prevented the detection of blood when α-CHCA matrix was used as in this study. However, in their study, intact αHb and βHb were investigated instead of enzymatic digestion-generated peptides. When MALDI-MSP was performed on blood dilutions preliminarily treated with Luminol, using α-CHCA as a matrix, the spectra were of considerably lower quality in comparison to both untreated and KM-treated dilutions, indicating interference of Luminol with the gas phase ion–molecule reactions between the reactant matrix species and the neutral peptides, as also proposed in the study by Seraglia et al [31]. However, some level of compatibility with this further presumptive testing is still shown as human blood could be detected for the Luminol-treated 1:5000 and 1:10,000 blood dilutions (Table 3).

However, for the 1:15,000 Luminol-treated blood dilution, only one of the two human blood biomarkers, specifically the ion signal at nominal *m/z* 1530, was detected. According to Kennedy et al [6] this result would have been reported as a negative for blood (false negative) just as Luminol has yielded a false negative for this dilution. Purification of this blood dilution (after enhancement and enzymatic digestion and prior to MALDI MSP) did not improve spectral quality and did not enable the detection of both two human blood peptide biomarkers. A similar scenario is observed for the 1:20,000 Luminol-dilution where the absence of the two human blood biomarker peptides (here probably due to the presence of significant polymer contamination) prevented the confirmation of blood presence. In practical terms, and in a real forensic scenario, these results mean that, for blood dilutions of 1:15,000 or higher, Luminol would yield false negatives, which the subsequent application of MALDI MSP would not rectify.

Table 3
Putative blood peptides detected by MALDI MSP in the Luminol-treated blood dilutions. In addition to the two positive human blood markers (*m/z* 1275 and *m/z* 1530 from βHb and αHb respectively), other Hb signals were detectable in the dilutions assessed within a mass accuracy of −4.9 to 4.4 ppm.

Dilution	Experimental *m/z*	Mass Accuracy (ppm)	Peptide Sequence	Hb Subunit
1:5000	1274.720	4.2	LLVVYPWTQR	βHb
1:5000	1529.735	−0.7	VGGHAAEYGAEALER	αHb
1:5000	1833.901	−4.9	TYFPHFDLSHGSAQVK	αHb
1:10,000	1274.720	4.3	LLVVYPWTQR	βHb
1:10,000	1314.664	0.5	VNDEVGGEALGR	βHb
1:10,000	1449.790	4.4	VVAGVANALAHKYH	βHb
1:10,000	1529.735	−0.3	VGGHAAEYGAEALER	αHb
1:15,000	1529.732	1.8	VGGHAAEYGAEALER	αHb

4.2. Detection of human blood in mixture with other biofluids

MALDI MSP of blood present on clothing in mixture with saliva deposits. The sampled regions of fabric from the *t*-shirt that tested positive for amylase deposits using the Phadebas paper (showed as blue stains in Fig S2) were subsequently spotted with 5 μL of blood, excised, extracted, and enzymatically digested prior to MALDI MSP.

MALDI MSP permitted for the recovery of the two human blood biomarkers (in addition to other Hb peptides) (Table 4), showing effectiveness of this confirmatory test even when blood is in mixture with another biofluid, as well as being compatible with amylase testing.

MALDI MSP of blood present on clothing in mixture with sweat deposits - The armpits, neckline, and chest regions of the *t*-shirt were deemed to be the most likely to contain natural deposits of sweat after wearing. These regions were stained with blood and then excised, extracted, and enzymatically digested prior to MALDI MSP. Similarly, to the experiment of blood mixed with saliva, the typical panel of human blood biomarkers was observed in every case (amongst other Hb peptides) (Table 5), showing, in this instance too, the specificity and feasibility of the proposed MALDI-based confirmatory test for human blood detection, even when mixed with another biofluid (sweat in this case).

Table 4
Putative blood Hb peptides detected in the clothing samples after the addition of blood on the areas testing positive for amylase. The two human blood markers (*m/z* 1275 and *m/z* 1530 from βHb and αHb, respectively), were detected with other Hb ion signals within a mass accuracy between −9.4 and 9.6 ppm.

Left Neckline			
Experimental *m/z*	**Mass Accuracy (ppm)**	**Peptide Sequence**	**Hb subunit**
952.5065	3.5	VHLTPEEK	αHb
1071.5604	−5.7	MFLSFPTTK	αHb
1171.6736	−4.7	VLSPADKTNVK	αHb
1274.7285	−2.4	LLVVYPWTQR	βHb
1314.6582	5.0	VNDEVGGEALGR	βHb
1449.7878	5.7	VVAGVANALAHKYH	βHb
1529.7352	−0.7	VGAHAGEYGAEALER	αHb
1833.8907	0.6	TYFPHFDLSHGSAQVK	αHb
2058.9527	−2.3	FFESFGDLSTPDAVMGNPK	βHb
2228.1590	3.6	SAVTALWGKVNVDEVGGEALGR	βHb
Right Neckline			
Experimental *m/z*	**Mass Accuracy (ppm)**	**Peptide Sequence**	**Hb subunit**
952.507	3.5	VHLTPEEK	αHb
1071.561	−5.8	MFLSFPTTK	αHb
1087.626	−0.1	LRVDPVNFK	αHb
1171.674	−4.6	VLSPADKTNVK	αHb
1274.729	−2.7	LLVVYPWTQR	βHb
1314.675	−7.5	VNDEVGGEALGR	βHb
1449.804	−5.7	VVAGVANALAHKYH	βHb
1529.735	−0.7	VGAHAGEYGAEALER	αHb
1833.909	−9.4	TYFPHFDLSHGSAQVK	αHb
2058.954	−2.8	FFESFGDLSTPDAVMGNPK	βHb
2228.180	−5.7	SAVTALWGKVNVDEVGGEALGR	βHb
Right Hand Sleeve			
Experimental *m/z*	**Mass Accuracy (ppm)**	**Peptide Sequence**	**Hb subunit**
952.507	3.0	VHLTPEEK	αHb
1171.659	7.8	VLSPADKTNVK	αHb
1274.713	9.6	LLVVYPWTQR	βHb
1314.659	4.4	VNDEVGGEALGR	βHb
1529.736	−1.0	VGAHAGEYGAEALER	αHb
2058.932	7.6	FFESFGDLSTPDAVMGNPK	αHb

Table 5
Putative blood peptides detected in the clothing samples that accumulated deposits of sweat prior to staining with human blood. The two human blood markers (*m/z* 1275 and *m/z* 1530 from βHb and αHb respectively), were detected with other Hb ion signals within a mass accuracy between −5.9 and 10.0 ppm.

Left Underarm			
Experimental *m/z*	**Mass Accuracy (ppm)**	**Peptide Sequence**	**Hb subunit**
1171.667	1.3	VLSPADKTNVK	βHb
1274.723	1.7	LLVVYPWTQR	βHb
1314.662	1.8	VNDEVGGEALGR	βHb
1529.743	−5.9	VGGHAAEYGAEALER	αHb
1833.892	−0.2	TYFPHFDLSHGSAQVK	αHb
2058.942	2.8	FFESFGDLSTPDAVMGNPK	βHb
Right Underarm			
Experimental *m/z*	**Mass Accuracy (ppm)**	**Peptide Sequence**	**Hb subunit**
1171.666	1.8	VLSPADKTNVK	βHb
1274.717	7.0	LLVVYPWTQR	βHb
1314.662	1.8	VNVDEVGGEALGR	βHb
1449.790	4.5	VVAGVANALAHKYH	βHb
1529.726	5.2	VGGHAAEYGAEALER	αHb
1833.874	10.0	TYFPHFDLSHGSAQVK	αHb
2228.165	0.9	SAVTALWGKVNVDEVGGEALGR	βHb
Back			
Experimental *m/z*	**Mass Accuracy (ppm)**	**Peptide Sequence**	**Hb subunit**
1171.666	1.7	VLSPADKTNVK	βHb
1274.722	2.7	LLVVYPWTQR	βHb
1314.666	−0.8	VNVDEVGGEALGR	βHb
1449.792	3.1	VVAGVANALAHKYH	βHb
1529.734	0.2	VGGHAAEYGAEALER	αHb
1833.890	0.8	TYFPHFDLSHGSAQVK	αHb
2058.942	2.6	FFESFGDLSTPDAVMGNPK	βHb
2228.165	0.9	SAVTALWGKVNVDEVGGEALGR	βHb

4.3. Detection of human blood from porous materials

The presence of blood on various porous fabrics from items such as bedding, curtains, and clothing, is commonly encountered at the scene of violent scenes. The dispersion of blood on these types of materials has been a phenomenon investigated within the forensic community. Li et al [32] reported the effect of different yarn structures in cotton fabrics and the impact they can have on stain shape formation. Whilst still wet, blood stains can be distorted by capillarity; the combination of the differential chemical/physical interaction with the target surface may cause differential migration of blood molecules characterised by different hydrophobicity and affinity for the target surface. If this is the case, then it is feasible/plausible that MALDI false negatives for blood may arise depending on the area sampled. To test robustness of the MALDI-based approach in such scenarios, the bloodstains were generated on polyester, cotton, and wool, at different volumes of 50, 75 and 100 μL, and allowed to disperse across the surface for 30 min. Each stain was sampled from its centre and periphery and subjected to extraction and enzymatic proteolysis prior to MALDI MSP analysis.

All analyses showed that human blood could be detected through the expected two blood Hb peptide signatures (nominal *m/z* 1275 and *m/z* 1530 from βHb and αHb respectively), regardless of the sampling region, from all materials investigated (Table S1).

However, it was observed that generally the intensity of the Hb signals was greater in the centre of the stains compared with the periphery of the stains. This may be an indication that there is a higher abundance of the red blood cells in the centre of the stains which decreases moving towards the edges of the stain. However, this has not been corroborated by the detection of glycophorin A and EBP4.2 proteins, red blood cells-specific proteins, which did not show differential distribution.

MALDI MSP of central and periphery regions of blood stains on polyester - The MALDI MSP analysis corroborated the presence of human blood through the expected panel of two biomarkers irrespective of the sampling region as detailed in Table S1. For the polyester sample (100 μL stain), which is the material on which the most 'spreading' of the blood could be visually observed, several blood serum protein peptide signatures were putatively identified in the centre of the stains and considerably less observed (in number) in the periphery of the sample (Table S2) pointing again to some level of impact of the chemical nature of the surface on the interaction with these proteins.

MALDI MS of central and periphery regions of blood stains on cotton - Similarly to the blood stains on polyester, MALDI MSP confirmed the presence of human blood through the expected panel of two biomarkers regardless of the sampling region (Table S1). Nonetheless, similarly to the polyester surface, the relative intensity of these ion signals was considerably higher in the MALDI MS spectra of the centre region of the bloodstains compared to the periphery region. However, contrary to the observation for the polyester material, when 100 μL blood was deposited, a higher number of serum protein signatures was observed in the periphery of the cotton stain samples (Table S3).

This observation suggests that there may be a somewhat easier migration of these proteins to the peripheral regions on cotton due to possibly a chemistry-dependant lower interaction with this surface.

MALDI MS of central and periphery regions of blood stains on wool - MALDI MSP confirmed the presence of human blood in the stained wool examined, regardless of the volume of blood used and of the sampling region (Table S1). This time, the relative intensity of the two human blood peptide biomarkers (nominal *m/z* 1275 (βHb) and 1530 (αHb)) was very similar for both the centre and the periphery of the stain and for both the 50 and 75 μL blood volumes. A regional difference became noticeable for the 100 μL stain in which the two human blood biomarkers exhibited higher intensity of these signals observed in the centre of the stains. This observation ties in with the lowest blood spreading observed on wool compared to polyester and cotton and the highest blood volumes facilitating the most spreading. Lower dispersion is most likely due to the thickness of the fabric and the more open knit of the wool yarns in the fabric, compared to the woven cotton and polyester fabrics. Fig. 1 summarises the differential detection of the two human blood biomarker peptides in the centre and periphery of the stains for the three blood volumes investigated (50, 75 and 100 μL) and the three porous materials under consideration.

In terms of blood serum protein distribution in the 100 μL wool sample, there was no difference observed between the number of putative peptide identifications made between the periphery and centre of this sample (Table S4).

If the protein distribution is even in both the centre and in periphery, then it could be hypothesised that there is uniform spreading of serum proteins and not a restricted migration. This is corroborated by the lesser spreading of the blood observed in all wool stains. While interesting, these observations would need to be corroborated by a similar investigation carried out with multiple donors.

5. Conclusions

For a new method to be proposed as confirmatory test for the forensic detection of human blood, an extensive investigation of the capabilities, versatility, robustness, specificity and sensitivity needs to be undertaken. Previous work has shown that Matrix Assisted Laser Desorption Ionisation Mass Spectrometry (MALDI MS), especially in combination with bottom-up proteomics, can detect the presence of human and animal blood in a stain using MALDI MS Profiling (MALDI MSP) and provide molecular images of blood fingerprints exploiting human/

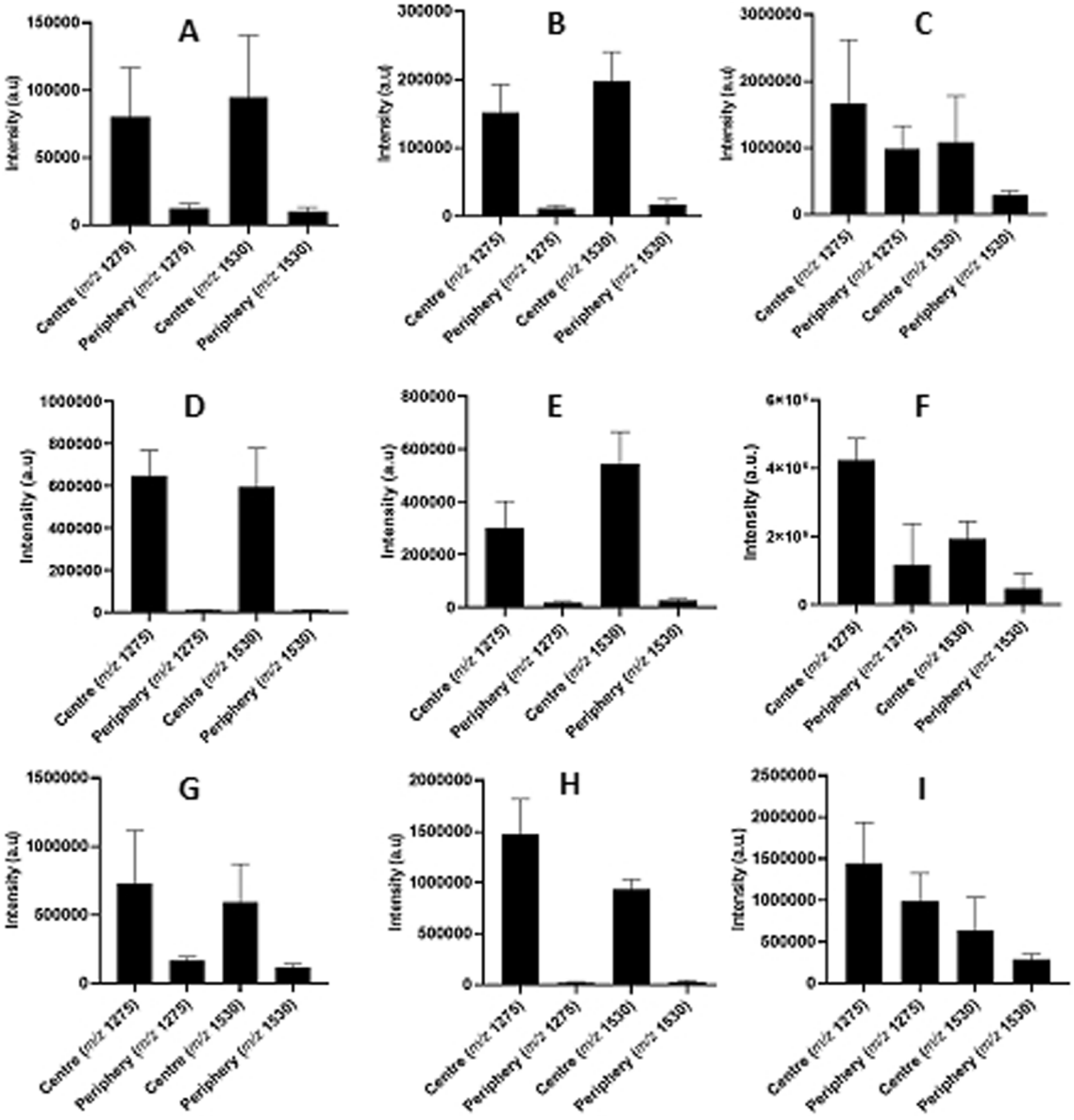

Fig. 1. Bar graphs comparing the intensity of *m/z* 1275 (βHb) and *m/z* 1530 (αHb) for 50, 75 and μL blood stains on polyester, cotton and wool detected from the centre and periphery of each stain in triplicate spectra. (A) 50 μL cotton stain; (B) 50 μL polyester stain; (C) 50 μL wool stain; (D) 75 μL cotton stain; (E) 75 μL polyester stain; (F) 75 μL wool stain; (G) 100 μL cotton stain; (H) 100 μL polyester stain and (I) 100 μL wool stain. (*Note that y axis has not been normalised across bar graphs as doing so would completely suppress observation of some of the column data.*).

animal blood specific peptide biomarkers through MALDI MS Imaging (MALDI MSI). Importantly, compatibility with several blood testing techniques as well as with subsequent DNA typing has been shown. Additional information could be recovered with this process such as the presence of Haemoglobin variants and an indication of how blood originated. MALDI MS effectiveness as a confirmatory test has also been successfully assessed through a pre-validation blind study.

The present follow up study has provided additional understanding as to the capabilities and limitations of this approach. Further compatibility of this potential confirmatory test for human blood has been shown with two more presumptive blood testing, namely the Kastle-Meyer and Luminol tests and for blood dilutions up to 1:20,000 and 1:10,000, respectively.

An investigation of the specificity of this test was also undertaken by mixing blood with other biofluids, such as saliva and sweat on clothing). The study has shown that the presence of other biofluids, in conditions mimicking a real scenario, does not prevent the detection of human blood. Additionally, when in mixture with saliva, this is also possible after the application of Phadebas paper presumptive testing targeting amylase, an enzyme present in high concentrations in saliva. The mixture with other biofluids, including semen and vaginal blood should be investigated in the future and preliminary data already suggest that it is possible to detect human blood in mixture with semen (as well as detecting semen in that mixture).

In this study, it has been observed that, whilst the mixture with saliva and sweat does not prevent human blood detection, the surface of deposition does matter, and these experiments should be repeated with other types of fabric too in addition to the polyester *t*-shirt used in this study (as well as challenging the method with bloody fabric washed with water or with soap/detergent). This consideration has led to the last part of the present study which investigated the recovery of human blood in stains deposited on a range of porous materials namely polyester, cotton and wool. As other studies indicated that the yarn structure and weave of the material plays a key role in the stain morphology, it was investigated

whether the recovery of the human blood biomarkers was dependent on the sampling region and on the volume of the blood generating the stain. The MALDI MS spectra showed that, whilst detectable throughout the stain, the expected human blood peptide signatures in bloodstains on polyester and cotton were more abundant in the centre of the stain than in the periphery across all the blood volume tested (50, 75 and 100 μL). For bloodstains on wool, the two human blood peptide biomarkers were equally present in the centre and periphery of the stain for the 75 and 100 μL blood volumes experimented. This observation ties with the lowest blood spreading visually observed on this type of fabric. Some level of differential distribution can only be observed with a greater blood volume trialled suggesting that the yarn and chemistry of the surface have a higher impact on blood proteins distribution as the volume of blood increases. These observations inform the sampling strategy for MALDI MS analysis. Additionally, given the differential distribution of serum proteins, future experiments could investigate the impact of blood protein size and chemistry of interaction with a given surface on the differential migration.

The findings described here contribute to the body of validation work that is essential for operational deployment of this process as confirmatory test for human blood and its ultimate promotion to Category A in the UK Fingermark Visualisation Manual.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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