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Proteomics goes forensic: detection and mapping of blood signatures in fingermarks

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List of standard abbreviations:

A1AT	Alpha-1-Antitrypsin
AHSP	Alpha-Haemoglobin-stabilizing protein
Albumin	Albumin
APOA1	Apolipoprotein A-1
C3	Complement C3
EPB3	Erythrocyte Protein Band 3
EPB4.2	Erythrocyte Protein Band 4.2
αHb	Haemoglobin alpha
βHb	Haemoglobin beta
α-2-M	Alpha-2-Macroglobulin

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Abstract

A bottom up *in situ* proteomic method has been developed enabling the mapping of multiple blood signatures on the intact ridges of blood fingermarks by Matrix Assisted Laser Desorption Mass Spectrometry Imaging (MALDI-MSI). This method, at a proof of concept stage, builds upon recently published work demonstrating the opportunity to profile and identify multiple blood signatures in bloodstains via a bottom up proteomic approach. The present protocol addresses the limitation of the previously developed profiling method with respect to destructivity; destructivity should be avoided for evidence such as blood fingermarks, where the ridge detail must be preserved in order to provide the associative link between the biometric information and the events of bloodshed. Using a blood mark reference model, trypsin concentration and spraying conditions have been optimised within the technical constraints of the depositor eventually employed; the application of MALDI-MSI and Ion Mobility MS have enabled the detection, confirmation and visualisation of blood signatures directly onto the ridge pattern. These results are to be considered a first insight into a method eventually informing investigations (and judicial debates) of violent crimes in which the reliable and non-destructive detection and mapping of blood in fingermarks is paramount to reconstruct the events of bloodshed.

Statement of significance of the study

The *in situ* shotgun method illustrated in this paper represents a novel advancement of previously published work in the area of reliable and robust blood detection. In particular, the ability to map blood protein signatures directly on the ridge pattern of fingermarks will provide a crucial associative link between the biometric information and the event of bloodshed. This will inevitably have a significant impact on the overall criminal justice system as it will inform investigations and strengthen judicial debates.

1 Introduction

The Fingermark Research Group at Sheffield Hallam University has provided an extensive body of knowledge within the chemical analysis of latent fingermarks by MALDI MS based methods [1–3]. These protocols enable the detection and mapping of a range of endogenous (substances naturally present in sweat), semi-exogenous (metabolite substances excreted in sweat) and exogenous species (contact substances) directly on the fingerprint ridges. This forensic opportunity provides investigators with a link between the biometric information (fingerprint molecular images) and specific intelligence on the lifestyle of the suspect and, potentially, the circumstances of the crime. Amongst recoverable forensic intelligence, the reliable and robust detection of blood is highly desirable as this biofluid is frequently encountered at the scene of violent crimes or when criminals break in, as they may cut themselves, and may aid with the reconstruction of the chain of events taking place during the crime. As it is also the case for latent fingermarks, the presence of blood may not be obvious to the naked eye; the blood might have been concealed (e.g. by attempts of the perpetrator to clean the crime scene) or could be present in invisible amounts; even "red stains" on their own or in association with fingermarks need to be confirmed as blood as opposed to other matrices. For this reason, blood enhancement techniques are primarily applied by investigators in compliance with the protocols described by the Fingermark Visualisation Manual [4] produced by the Home Office UK.

Blood enhancement techniques currently employed **-** While several techniques are available for the enhancement and detection of blood at a crime scene and have been reviewed [4–7], they are only presumptive, meaning that they may lead to false positives [8–14]; for example, acid dyes, used as one of the blood enhancement means, target proteins and, as such, they would stain positive for blood, semen and saliva, because in all of these biofluids proteins are found in high abundance. The most commonly used blood enhancement techniques which rely on the catalytic peroxidase activity of the ferrous ion in the Haem group, are also presumptive and they are prone to both false positives (given that substances other than blood are capable of peroxidase activity) and false negative (due to the presence of substances that inhibit the reaction with Haem). Both false positives and false negatives generate incorrect information potentially misleading the investigations and Court cases. The majority of the blood enhancement techniques are also destructive of the ridge pattern. For example luminol, a fluorescent Haem-reactive chemical frequently used to visualise blood traces at a crime scene, is likely to cause ridge diffusion due to the lack of a fixative step [15]. Immunogenic tests also belong to this category of techniques as they

require swabbing or cutting prior to blood extraction [16]; as such, these methods cannot be employed for blood marks.

Blood visualisation analytical techniques - For the aforementioned reasons, the analytical community has invested significant efforts into developing alternative methods enabling the reliable visualisation and identification of blood using molecular or "analytical" markers. Recent approaches employ spectroscopic techniques such as Raman spectroscopy [17–23], Fourier-transform-infrared spectroscopy (ATR FT-IR) [24], or Hyperspectral Imaging [25,26].

Raman spectroscopy yields scattering peaks characteristic for blood by exciting the sample at a wavelength of 752 nm [27] or 785 nm [28]. These peaks correspond to: (i) (Oxy)Haemoglobin (1000, 1368, 1542 and 1620 cm^{-1}) and probably fibrin (967, 1248, 1342 and 1575 cm^{-1}) [27] for excitation at 752 nm. It has been reported that OxyHaemoglobin and Haemoglobin denaturation products (419, 570, 677, 754, 1128, 1311, 1374, 1398, 1549, 1582, 1638 cm^{-1}) are also detected at 785 nm excitation and at low laser power (1.9 mW). These peaks are however subjected to shifts as a function of higher laser power and age before and after drying [28]. Hyperspectral imaging (HSI) records the reflectance spectrum of a sample in the visible light region, where blood exhibits characteristic absorption band: a strong, narrow absorption band centred at 415 nm (Soret band) and two weaker, broader bands between 500 and 600 nm (β and α bands) [26]. The technique is not only capable of identifying substances based on their reflectance spectra, but also of generating images mapping their distribution. This was investigated by Edelman *et al.* for application to various forensic traces [29] and was trialled on mock crime scenes for the detection and identification of blood [30]. However, limitations are the unsuitability of red and dark substrates and the requirement for a reference spectrum which, in a crime scene scenario, cannot be guaranteed to be free of blood [26]. Furthermore, in theory, a non-blood substance with a sharp absorption band at 415 nm would give rise to a false positive result, though to date such a substance has not been reported. Finally, the Soret band shifts to shorter wavelengths by an appreciable amount as blood ages – it is this hypsochromic shift that researchers are using to date blood. This would give rise to false negatives if one is only looking for this peak in addition to the fact that the α and β bands disappear within some months.

ATR FT-IR peaks correspond to the vibrational stretching of structural bonds and functional groups. In the analysis of blood, peaks are produced by Amides A (3292 cm^{-1}), I (1651 cm^{-1}), II (1540 cm^{-1}) and III (1350-1220 cm^{-1}), methyl stretches of plasma lipids (2956 cm^{-1}), methyl bending of amino acid sidechains, lipids and proteins (1456 cm^{-1}), Fibrinogen/methyl bending of amino acid side chains, lipids and proteins (1359 cm^{-1}) and carbohydrates (1250-925 cm^{-1}). ATR FT-IR can be also used in imaging mode to generate maps of distribution of analytes in various general biological systems [31] including breast cancer tissue [32], as

well as in forensic applications investigating illicit substances in lifted [33] and unlifted fingerprints [34] and questioned documents [35], and has potential to be used to map blood distribution as well. However, the aforementioned species are not exclusive to blood, thus introducing potential for false positives, with the added complication that confident analyte identification can be difficult in complex samples.

Similarly, Raman spectroscopy can be used to produce images as described and reviewed by Steward *et al.* [36] and has been employed in determining the sequence of ink crossings [37] as well as in conjunction with HSI [38]. However, it should be noted that while Raman is a non-destructive technique capable of producing images, the detection of blood is solely dependent on Haemoglobin and its denaturation products [28], making the identification approach less reliable and robust.

Reports on blood detection using Mass Spectrometry and in particular MALDI MS Profiling had been present in the literature since 2004 [39–41]. This work based the confirmatory test on the presence of blood by detecting either a number of distinct blood proteins and peptides [39], Haemoglobin (α- and β-chain) [40] or its bioinorganic prosthetic group Haem [41]. This provides a more specific means to claim the presence of blood, although the analysis of intact Haemoglobin could pose a problem in aged and contaminated samples; it has been shown that the use of luminol prevents the detection of the Haemoglobin α- and β-chains with the usually employed MALDI matrices (such as α-cyano-4-hydroxycinnaminic acid), although identification was possible with the use of a different matrix, 2,6-dihydroxyacetophenone (DHAP) with di-ammonium hydrogen citrate (DHAC) [40].

Application of MALDI MS based techniques for the reliable visualisation and identification of blood **-** Inspired by the work published using MALDI MS, our laboratory undertook method development to adapt and apply these methods for the direct profiling and imaging of blood in fingermarks [42]. In this work the authors demonstrated the opportunity to map both Haem and Haemoglobin directly on the fingermark ridges, keeping their integrity and thus enabling, in a real forensic scenario, the preservation of the link between the biometric information and the event(s) of bloodshed. Such an approach was possible for both fresh and aged (7 days) fingermarks, enabling further mass spectrometric confirmatory tests for the presence of Haem. This approach also enabled the detection of additional blood specific proteins besides Haemoglobin, allowing specificity and confidence in the determination of the blood presence to be further enhanced. Though a clear advancement in terms of both reliable confirmation of blood and the preservation of the integrity of the fingerprint evidence, an even higher level of specificity of the analytical method would be desirable in order to robustly inform both investigations and Court cases. Top down proteomics could be an alternative approach given that the blood profile is very specific. Constant instrument developments may also offer the opportunity of post-source

fragmentation enabling partial protein identification. However, instrumental capability for this type of analysis is not widespread; furthermore the sheer number of protein present in blood, the differential protein concentration spanning several orders of magnitude and the presence of lipids affecting protein ionisation, could render the application of this technique problematic. The use of a bottom up proteomic approach is much more consolidated and would indeed increase reliability of protein identification as it is well known that the mass accuracy that can be achieved on the protein-derived peptides is much higher (in the order of parts per million) than that achievable for intact proteins. The literature already contains many reports attempting to map the proteome of plasma and serum though none of the approaches had involved the direct application of MALDI MS on enzymatically digested blood [43–47]. This is understandable as in all of the previous reports the aim was to map the entirety of the blood proteome for medical and diagnostic purposes. However, in a forensic context, the detection of a handful of blood specific proteins via the more reliable bottom up proteomic approach using MALDI MS would be more than appropriate. This research hypothesis was developed in our laboratories into a study demonstrating the opportunity to recover multiple blood protein signatures in as little as 5 minutes of sample preparation [48]. This work also showed that molecular signatures enable provenance discrimination and that they can also be retrieved in very old samples which were pre-treated with blood enhancement techniques, thus opening a new investigative avenue for cold cases.

Scope and results of the study presented - The work outlined in this paper is complementary to work of both Bradshaw *et al.* [42] and Patel *et al.* [48], bridging the gap between the two; in particular a proof of concept has been achieved through the step-wise development of an *in situ* proteolysis method in order to detect and map blood-specific proteins in fresh blood marks and analysis via MALDI-MSI directly on the ridge pattern. In this work, blood marks were digested *in situ* using trypsin and incubated for 3 hours, prior to MALDI-MSI and Ion Mobility-MS/MS (IMS-MS/MS) analyses. Although any alteration, even minute, to the chemical and physical state of a fingermark is to be considered destructive (even optical methods though they have the lowest degree of destructivity), the application of this *in situ* proteomic protocol enables the ridges to keep their integrity and original pattern including second level details (*minutiae*), thus also allowing the biometric information to be conveyed. Furthermore, compared with spectroscopic techniques, the application of MALDI MS based methods can generate additional and more specific information as it relies on "molecular signatures" rather than "analytical signatures", which can also be verified by MS/MS methods as shown in this work. In particular, the use of Ion Mobility has been particularly crucial for the reliable identification and confirmation of blood signatures, thus adding the required level of confidence in judicial debates.

2 Materials and Methods

***In situ* tryptic digestion of blood fingermarks for MALDI MS Imaging (MALDI-MSI).**

For trypsin spotting experiments, blood fingermarks were prepared by pricking a clean finger with a Unistik® 3 Neonatal & Laboratory single use lancet (Owen Mumford, Oxford, UK) under full ethical approval (HWB-BRERG23-13-14). A droplet of blood was forced out the bleeding fingertip which was rubbed against another clean fingertip. This second finger was then used to deposit blood marks onto ALUGRAMSIL G/ UV254 aluminium sheets (Sigma-Aldrich, Dorset, UK) pre-treated as previously described [49].

For trypsin spraying experiments, a clean finger was pricked using Accu-Chek Multiclix kit (Boots, Sheffield UK) according to the method for distributing material across the fingertips previously described. [42]. Trypsin was employed to enzymatically digest blood directly on a blood mark using different methods. Initially trypsin was spotted at concentrations of 125 µg/mL, 250 µg/mL, 500 µg/mL, 1 mg/mL, 2 mg/mL and 3 mg/mL in 50mM Ammonium Bicarbonate (AmBic), at pH 8, containing 0.1% RapiGest™ SF, by depositing 0.5 µL onto the blood mark.

In another experiment, quarter split blood marks underwent acoustic spotting of trypsin using the automatic spotter Portrait® 630 (Labcyte Inc., Sunnyvale, USA) at trypsin concentrations of either 100 µg/mL, 150 µg/mL, 200 µg/mL or 250 µg/mL in 50 mM AmBic at pH 8 containing 0.1% RapiGest™ SF to determine the most efficient one. In the work reported here, 50 cycles were performed, with one droplet per position deposited onto the split blood marks during each cycle. A total of approximately 8.5 nL of trypsin solution was therefore acoustically printed onto the mark, per spot. Additional blood mark quarters underwent differential trypsin spray coating, as reported for the acoustic ejection, using a SunCollect autosprayer (KR Analytical, Sandbach, UK); here 9 layers of trypsin were delivered at a flow rate 2 µL/min and a nitrogen pressure of 3 bar. All trypsinised samples were placed on polystyrene floats in a Coplin jar half-filled with 50:50 methanol:H_2O, sealed with parafilm and incubated for 3 hours at 37°C, 5% CO_2. The jar's lid was wrapped in paper tissue to prevent condensation forming on the glass and dropping onto the sample.

Matrix Deposition

After incubation, all the digested blood fingermark samples were sprayed using the SunCollect (KR Analytical, Sandbach, UK) with 5 layers of 5 mg/mL CHCA in 70:30 ACN:0.2%TFA_{aq}, containing equimolar amounts of aniline to CHCA (i.e., one mL of 5 mg/mL CHCA solution contained 2.4 µL aniline) at a flow rate 2 µl/min and a nitrogen pressure of 3 bar.

Instrumentation and data acquisition Mass spectrometric images of blood marks using manually spotted trypsin were obtained in the mass range 650-3000 Da using a modified Applied Biosystems API "Q-Star" Pulsar *i* hybrid quadrupole time-of-flight (QTOF) instrument (Concord, Ontario, Canada). The orthogonal MALDI source of the Q-Star instrument has been modified to incorporate a SPOT 10 kHz Nd:YVO_4 solid-state laser [50] (Elforlight Ltd., Daventry, UK) with a wavelength of 355 nm, a pulse duration of 1.5 ns and producing an elliptical spot size of 100 × 150 µM. Images were acquired at a spatial resolution of 150 × 150 µM in raster mode, using 'oMALDI Server 5.1' software supplied by MDS Sciex (Concord, Ontario, Canada) and data processed using BioMap 3.7.5 software (Novartis, Basel, Switzerland). MALDI MS images of blood marks either sprayed with trypsin (SunCollect, KR Analytical, Sandbach, UK) or robotically spotted with this enzyme (Portrait 630®, Labcyte Inc., Sunnyvale, USA) were acquired in positive ion mode in the mass range 600 - 3000 Da using a SYNAPT™ G2 HDMS system (Waters Corporation, Manchester, UK) operating with a 1 KHz Nd:YAG laser, at a mass resolution of 10,000 FWHM (sensitivity mode) and at a spatial resolution of 150 µm. Calibration over a 600-2800 Da mass range was performed prior to analysis using phosphorous red. The laser energy was set to 250 arbitrary units in MS mode and increased to 270 arbitrary units for MALDI Ion Mobility-MS/MS experiments. In particular MS/MS analyses were conducted *in situ* on the peaks exhibiting a S/N of at least 14. Fragmentation was carried out in the transfer region of the instrument, post ion mobility separation, therefore product ions retain the same drift time as the precursor ion. Collision energies ranging between 60-80 eV were used to obtain the best signal to noise ratio for product ions.

Data analysis Mass spectra opened using MassLynx™ (Waters Corporation, Manchester, UK) were either converted into txt files and imported into mMass, an open source multiplatform mass spectrometry software [51], or processed directly performing peak smoothing, baseline correction and peak centroiding. UniprotKB (http://www.uniprot.org/, UniProt release 2015_11) was employed to generate *in silico* peptide lists of known proteins present in blood. Mass lists were generated by selecting "*monoisotopic*", "*MH+*", "*trypsin higher specificity*", "*2 missed cleavages*", "*methionine oxidation*" *and taxonomy "human"*. Peptide lists were imported into mMass to create an "in house" and local reference library. Data analysis of mass images was performed either within BioMap 3.7.5 software (Novartis, Basel, Switzerland) or the HDI software (Waters Corp. Manchester UK).

Prior to peak assignment search, spectra were smoothed and de-isotoped. Peak assignment was not accepted if the S/N was lower than 3:1. Spectral processing consisted of smoothing, baseline correction and lock mass based mass correction. Prior to performing an MS/MS Mascot (Matrix Science, London, UK) search, spectra were processed using MassLynx™

with the MaxEnt 3 algorithm to deisotope and enhance the S/N. Queries were searched against the "Swiss-Prot" database (*release 2015_11*) with parent and fragment ion tolerances set to 50 ppm and 0.1 Da respectively. Two missed cleavages were also selected.

3 Results and Discussion

The ability of MALDI-MSI to spatially map the distribution of proteins and peptides in fingermarks opens up the potential to establish the link between the event of bloodshed and the biometric information, thus linking a suspect to a crime. For this reason, this study aimed to optimise the *in situ* proteolytic digestion of blood marks for analysis via MALDI-MSI.

Optimisation of trypsin concentration: MALDI-MSI of enzymatic digestion spots deposited on blood marks - Due to the high protein concentration in blood [1], it was necessary to adapt the amount and concentration of trypsin used for blood proteolysis, as the 20 µg/mL trypsin frequently reported in the literature for on tissue and fingermark digests [2–4] did not yield any peptides when applied to blood fingermarks (data not shown). In the initial experiments aimed to determine the optimal trypsin concentration, a human blood mark was spotted with concentrations of 125 µg/mL, 250 µg/mL, 500 µg/mL, 1 mg/mL, 2 mg/mL and 3 mg/mL trypsin and digested at 37°C for 3 hours before matrix application and the acquisition of MALDI-MSI data. Analysis of this data revealed multiple blood peptide peaks localised only in the spotted digest regions such as ApoA1 at *m/z* 1529.83 (theoretical monoisotopic *m/z* 1529.78), Complement C3 at *m/z* 1337.73 and 1567.82 (theoretical monoisotopic *m/z* 1334.71 and 1567.88 respectively), α-Haemoglobin (αHb) at *m/z* 1274.77 and 1529.83 (theoretical monoisotopic *m/z* 1274.72 and 1529.73, Serotransferrin at *m/z* 1529.83 (theoretical monoisotopic *m/z* 1529.75) and α2-Macroglobulin at *m/z* 1334.73 and 1394.67 (theoretical monoisotopic *m/z* 1334.72 and 1394.68 respectively); Figure 1 reports peptide images at *m/z* 1274.72, putatively identified as a α-Haemoglobin and at *m/z* 1567.82, putatively identified as a Complement C3 peptide (theoretical *m/z* 1567.9) as an example. The blood fingermark area digested with 250 µg/mL trypsin showed the highest intensity peaks for those peptides, indicating that this would be the most promising concentration to bring forward into subsequent imaging experiments. The use of high trypsin concentrations of 500 µg/mL and above was discontinued, as a significantly lower intensity and/or absence of blood peptide signatures was attributed to an unsuitable trypsin:protein ratio, whereas trypsin concentrations between 100 and 250 µm/mL were chosen for further investigation on quarters of a blood mark split into four. In terms of the performance of the different trypsin concentrations tested, these results were reproducible through different

repeats, though peptide intensities varied according to the different amounts of blood present within each repeat affecting the ratio trypsin:blood proteins. It is in fact important to remember that despite the optimisation of the reproducibility in the deposition of blood marks (see Materials and Methods section), slightly different amounts of blood could have been deposited within each repeat. The amount of blood in the droplet could only have been measured and kept consistent if an exact amount of blood was pipetted off the finger after squeezing and pipetted back onto an uncontaminated finger. This method was trialled by the donor, however it was not possible to deposit marks with clear ridge detail due to the blood beginning to coagulate and dry in the pipette tip. The first blood fingermark generated by contact with the relevant surface was employed for the study each time (primary marks).

Optimisation of trypsin deposition for mapping blood signatures on the ridges of blood marks - In order to spatially map blood peptides on the fingermark ridges, subsequent experiments made use of the acoustic reagent multispotter (Portrait 630®) to deposit discrete trypsin nano-droplets at a spot-to-spot spatial resolution of 200 µm.

However, when a blood mark quarter deposited on an aluminium slide was initially spotted with 100 µg/mL trypsin at a resolution of 200 µm, it was observed that spots were spreading into each other (Fig 2B); trypsin spots surrounding the mark are also visibly merged and distorted in certain areas of the aluminium slide (Fig 2B). Lower volumes of trypsin were trialled (as low as 1.7 nL per spot) in combination with different concentrations, though none of the combinations avoided the merging issue. In order to test the hypothesis that it was the particular surface of deposition to cause this issue, a polylysine-coated glass slide was used as a sample support (Fig 2C). Again, the trypsin nano-droplets were observed to merge across the blood mark resulting in a pool of protease. Conversely, the Portrait® deposited the trypsin solution in a precise manner where blood was not present (Fig 2D); other deposition surfaces, such as lifting tape and a stainless steel MALDI target plate were investigated and all exhibited the same issue suggesting the combination of surface of deposition and, majorly the viscosity of blood to be the problem causing spot merging due to insufficient drying of trypsin during the various spotting cycles. The spot merging issue eventually led to discount the Portrait® as a suitable trypsin depositor for this particular application.

The next best option was therefore the use of an automatic sprayer for the deposition of trypsin (and subsequently matrix) and the Suncollect automated pneumatic sprayer was employed with the intention to deposit trypsin concentrations at 100 µg/mL, 150 µg/mL, 200 µg/mL and 250 µg/mL on 4 different quarters of a blood mark.

It was observed however that with increasing trypsin concentrations, the spray was less and less uniform leading to capillary blockage and syringe breakage, most likely due to increased

viscosity and back pressure build-up respectively. For this reason, only three quarters of the digested blood mark was sprayed with a maximum usable trypsin concentration of 200 µg/mL. In order to increase the trypsin concentration further when using the pneumatic depositor, further method development work including minor instrumental modifications is planned to counteract the potential increase in viscosity with higher protease concentrations.

Examination of the imaging data obtained revealed blood peptide signatures on the blood fingermark ridges for all the three trypsin concentrations investigated and Figure 3 shows example images for 5 peptide species (*m/z* 767.4079, 886.5072, 953.5492, 974.5294, 1068.5814) putatively assigned to Complement C3 (2 peptides), Hemopexin, αHb and Serotransferrin respectively. These species have been reported in Table S1. To prove that these are genuine blood signatures, a control experiment was performed by spraying trypsin at a concentration of 100 µg/mL on a latent fingermark ("control", not blood contaminated) from the same donor, subsequently subjected to MALDI-MSI. Spectra of the control latent mark extracted from the MALDI MS images generated are reported in Fig S1 (supplemental material) as a comparison with the spectra extracted from the MALDI-MS images generated from a blood mark (shown in Fig 3). Figure S1 shows that the aforementioned blood signatures (in red in the spectra of the blood mark) are absent in the latent mark and therefore not endogenous but exclusively present when blood is present. With respect to Figure 3, the spectrum showing the ion signal at *m/z* 1274.7379 assigned to αHb was additionally reported as present in the blood mark and absent in the latent mark. This peptide generated a speckled image which is why was not previously reported in Figure 3. This control experiment demonstrates not only specificity but selectivity of the method. Finally, to prove that the putatively identified peptide sequences could not belong to other proteins (other than the reported blood proteins) with which they might have sequence homology, a BLAST search has been performed for each of the interested species. The search has revealed 100% sequence homology exclusively with the putatively reported blood protein peptide derived species (data not shown). To further prove the exclusive presence of blood peptides on the mark's ridges, an overlay of each peptide image with a matrix peak image at *m/z* 1066.1158 is also reported in Figure 3.

Of the peptides identified, two produced the most abundant signal when digested with 200 µg/mL, whereas two were most abundant when digested with 150 µg/mL trypsin and one appeared to be equally abundant with both trypsin concentrations. While this may complicate the determination of the most suitable concentration, it is a result to be expected given the differential abundance of the interested proteins in blood; while some proteins are present in blood in high concentrations, such as albumin (30-50 mg/ml in healthy individuals), others have been reported with concentrations as little as 0-5 pg/ml for interleukin 6 [43]. This circumstance gives rise to sub-optimal substrate:enzyme ratio for

some proteins. In fact, though some peptide peaks are still present, the overall TIC is much reduced with higher trypsin concentrations (above 250 μg/ml). This result is reproducible within the same conditions which here refer to the blood amount being utilised. Blood amounts were controlled as much as possible by using the same lancet depth each time on the Accu-Check Multiclix device (see Methods Section). Differential abundance may also negatively impact on the uniformity of distribution across the blood mark and eventually on the ridge pattern molecular image reconstruction. This may result in seemingly inferior results in one area, where in fact this area may have not contained "optimal" amount of the target species to begin with, thus resulting in non-uniform distribution both within the same and between the different quarters. As for the spotting experiments, the spraying method was found to be reproducible through different repeats with regards to the proteolytic efficiency of trypsin used in the different concentrations trialled.

Key findings - Data presented here suggest that both 150 μg/mL and 200 μg/mL trypsin are suitable for the *in situ* digestion of blood marks, however taking into consideration the results of the manually spotted image it would be beneficial to also evaluate 250 μg/mL. However this will require some modification of existing instrumentation. In the data obtained so far, additional ion signals could be observed and matched to blood peptides; they did not generate a continuous fingermark ridge pattern when imaged but instead showed a speckled distribution. Despite the known difficulties in successfully performing *in situ* MS/MS experiments, these were optimised and Figure 4 shows an example confirming the presence of the α and β chains of Haemoglobin by selecting the precursor ions at *m/z* 1274.7255 and 1529.7342 respectively.

4 Concluding remarks

Mapping of blood signatures onto to the ridge pattern of a fingermark can provide crucial information in a forensic investigation, linking the suspect to the events of bloodshed. Here a sample preparation method has been developed to undertake trypsin proteolysis *in situ*, thus enabling blood peptides to be mapped in a fingermark and hence facilitating reconstruction of "molecular blood images of the ridge pattern". Results also indicate that the use of the acoustic ejector is unsuitable as the high viscosity of blood causes trypsin spot merging, thus preventing mapping of blood signatures onto fingermark ridges. On the contrary, the automatic sprayer employed here allowed successful imaging of the fingermark ridges. Further instrument developmental work is planned to enable the deposition of higher concentrations of trypsin with the intention to improve the quality of molecular images and

the blood peptide signal intensity. A separate and in depth study is required, trialling the method and adjustments to it (trypsin concentration) needed for different amounts of blood, in order to determine the blood amount range for which a certain trypsin concentration still yields the desired blood peptide signatures. Once this study has been carried out, at this stage, in order to select the best trypsin concentration, it is only possible to combine the associate findings with an estimation of the blood present within the interested evidence on the basis of the lesson learnt from the study itself.

While these authors have already demonstrated in MALDI profiling bottom up proteomic experiments that it is possible to retrieve blood signatures in blood evidence as old as 9 years, future work will include testing the optimised sample preparation/imaging methods on blood fingermarks of different age to prove analytical robustness.

The work illustrated here also opens up new avenues of investigation; similarly to the concept previously demonstrated by this group with regards to establish the order of deposition of fingermarks and condom lubricants using with MALDI-MSI, it may be possible to determine whether blood peptides are present exclusively on the ridges, which would suggest that a mark was left by a bloodied finger, or on the entire sample surface including ridges and valleys, which would suggest a mark having been deposited onto a bloodied surface (or contaminated with blood after deposition). Differentiating between these scenarios would then enable investigators to reach a more confident conclusion and establish a stronger link between the fingermark donor and the event of bloodshed.

Legends

Fig 1. MALDIMS Imaging of *in situ* proteolysis of a blood fingermark. Figure shows molecular images of blood specific peptides within trypsin spotted in 6 different concentrations on the blood mark. Trypsin appeared to be most efficient when used in concentration of 250 µg/mL.

Fig 2. Acoustic spotting of trypsin on blood marks. Figure shows the visual image of the blood mark prior to splitting into quarters (A). Trypsin spots within the mark and surrounding the mark are visibly merged and distorted in certain areas of the aluminium slide (B). A distorted ridge merged blood mark was also visible on a polylysine glass slide after trypsin deposition (C) with a zoomed region showing an unaffected area (no spot merging) where blood was not present (D).

Fig 3. MALDIMS Imaging of *in situ* proteolysis of a blood fingermark. Figure shows molecular images of blood specific peptides generated by spraying trypsin in 4 different concentrations (100, 150, 200 and 250 µg/mL) on the blood mark using the SunCollect. The trypsin concentration of 250 µg/mL could not be delivered to due limitations in the sprayer capabilities. Each peptide image has also been overlaid with the matrix signal at *m/z* 1066.1158. The figure suggests that the best ridge reconstruction performance could be achieved using a trypsin concentration of/between 150 and 200 µg/mL.

Fig 4. MALDI-IMS-MS/MS of tryptic peptides at *m/z* 1274.7255 (4A) and *m/z* 1529.7342 (4B), identified via Mascot as βHb and αHb respectively with both *b* and *y* ions annotated.

Fig S1. Comparison of spectra extracted from MALDI-MS images of a latent mark (control) versus a blood mark. By enzymatically digesting a latent fingermark (not contaminated by blood) using 100 µg/mL of trypsin, the peptide blood signatures previously mapped and shown/reported in Figure 3 are absent.

Table S1. Putatively identified blood peptide signatures through MALDI-MSI of blood marks.

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