Refinement and Validation of Multi-Source Blood Detection for Operational Use

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CANDIDATE DECLARATION

I hereby declare that:

- I have not been enrolled for another award of the University, or other academic or professional organisation, whilst undertaking my research degree.

- None of the material contained in the thesis has been used in any other submission for an academic award.

- I am aware of and understand the University's policy on plagiarism and certify that this thesis is my own work. The use of all published or other sources of material consulted have been properly and fully acknowledged.

- The work undertaken towards the thesis has been conducted in accordance with the SHU Principles of Integrity in Research and the SHU Research Ethics Policy. The MALDI MS data in Chapter 2 was acquired in part by Cameron Heaton (a statement of contributions for this work is attached as an appendix at the end of Chapter 2). The LC-MS/MS data in Chapter 2 was acquired by Tom Clark. The DNA profiling data in Chapter 3 was acquired by Dr Yael Herman and Carla Oz at Israel Police. The MALDI MS/MS data in Chapter 4 was acquired by Dr. Matthias Witt at Bruker Daltonik (Bremen, Germany). I played a major role in the preparation of these samples; in addition, the data analysis and interpretation are entirely my own work.

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ABSTRACT

The operational deployment of any technique in forensic casework must undergo thorough investigation of its capabilities and limitations. At Sheffield Hallam University for the past 14 years, the Francese Group have focused on developing the utilisation of Matrix Assisted Laser Desorption Ionisation Mass Spectrometry (MALDI MS) for forensic applications. Not only has this technique shown potential in several different areas (MALDI MS analysis of latent fingermarks, condom lubricants and drug detection in fingermarks and hair as some examples), also being deployed in casework, but in more recent years, the detection of blood also.

The end users, namely police and forensic providers have shaped the programme of work on blood detection by letting priorities and needs drive the research. The validation studies conducted in this thesis offer evidence of robustness and versatility as well as further insights as to what circumstances MALDI MS would be most suited/needed for the investigation of blood at a scene of a violent crime.

Currently, crime scene investigators (CSI) and crime labs employ commercially available presumptive tests for the detection of blood when recovered from crime scenes. However, these tests are not specific and occasionally yield false positives. Furthermore, confirmatory testing is not routinely performed. The risk of a false positive result could potentially result in a wrongful conviction or dismissal in court and brings into question the validity of these tests when their findings are heavily relied on in judicial proceedings.

The utilisation of MALDI MS Profiling (MSP) and MS Imaging (MSI) for the rapid and informative detection of blood in this thesis has shed light on the multiplex nature of this alternative approach. A great deal of the work presented has shown the capability to not only distinguish between human and animal blood, but to determine blood provenance down to the species level (Chapter 2). In terms of animal blood detection, even more intelligence has been gained within this strand of the project, highlighting that a distinction can be made between intravenous animal blood and traces of animal blood from packaged meat (Chapter 4). If animal blood was detected at a crime scene, being able to offer insight on the

manner in how the blood was shed could offer considerable assistance in reconstructing the dynamics of the crime.

The compatibility and robustness testing performed in this project, which has been demonstrated across several strands of the project (Chapters 2,3 and 5) has additionally shown that MALDI MS can be used in conjunction with several blood enhancement techniques (BET), deposition surfaces, in the co-presence of other biofluids, and in tandem with DNA typing post MALDI MSI analysis. This body of knowledge significantly contributes to further advance implementation of MALDI based approaches for the forensic analysis of blood in stains and fingermarks in an operational environment and to the further promotion of MALDI as a Category A technique in the Home Office Fingermark Visualisation Manual after its recent promotion to Category B.

CONTENTS

ABBREVIATIONS

A1AT	Alpha-1-antitrypsin
A2-MG	Alpha-2-macroglobulin
AB-1	Acid Black-1/ Amido Black
AC	Alternating current
ACN	Acetonitrile
AFIS	Automated Fingerprint Identification System
AFM	Atomic Force Microscopy
ALS	Alternative Light Sources
AmBic	Ammonium Bicarbonate
AP	Atmospheric Pressure
ApoA1	Apolipoprotein A-1
ATR FT-IR	Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy
AV-17	Acid Violet 17
A.u.	Arbitrary Units
AY-7	Acid Yellow 7
BET	Blood Enhancement Technique
CID	Collision Induced Dissociation
CE	Collision Energy
CV	Crystal Violet
Com C3	Complement C3
α-CHCA	α-cyano-4-hydroxycinnaminic acid
DC	Direct current
DESI-MS	Desorption electrospray ionisation-mass spectrometry
DFO	1,8-Diazafluoren-9-one
DNA	Deoxyribonucleic acid
Da	Dalton
EDTA	Ethylenediaminetetraacetic acid
EPB3	Erythrocyte Protein Band 3
EPB4.2	Erythrocyte Protein Band 4.2
ESI	Electrospray ionisation
EtOH	Ethanol
FLS	Forensic light source
FTICR	Fourier-Transform Ion Cyclotron Resonance-Mass Spectrometry
FWHM	Full width half maximum
GC-MS	Gas chromatography-mass spectrometry
G	Gram
GSR	Gunshot residue
Hbα	Haemoglobin-alpha
H_2O_2	Hydrogen peroxide
Hbβ	Haemoglobin-beta
HDMS	High definition-mass spectrometry
HPLC-MS	High performance liquid chromatography- mass spectrometry
HR	Hungarian Red
HIS	Hyperspectral Imaging

IMS	Ion mobility separation
ISO	International Organisation for Standardisation
ITO	Indium Tin Oxide
IR	Infra-Red
KM	Kastle-Meyer Test
kDa	Kilodalton
kV	Kilovolts
LC	Liquid Chromatography
LC-MS	Liquid-Chromatography Mass Spectrometry
LCV	Leucocrystal Violet
LDI	Laser Desorption Ionisation
LMG	Leucomalachite Green
M	Molar
MALDI-MS	Matrix assisted laser desorption ionisation-mass spectrometry
MALDI-MSI	Matrix assisted laser desorption ionisation-mass spectrometry imaging
MALDI-MSP	Matrix assisted laser desorption ionisation-mass spectrometry profiling
mDa	Milli Dalton
MS	Mass spectrometry
MeOH	Methanol
Mg	Milligram
mL	Millilitre
Mm	Millimetre
mM	Millimolar
Mg	Microgram
miRNA	microRNA
µL	Microlitre
µM	Micrometre
mRNA	Messenger RNA
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MSI	Mass Spectrometry Imaging
MSP	Mass Spectrometry Profiling
m/z	Mass to charge ratio
Nd: YAG	Neodymium-doped yttrium aluminium garnet (laser)
NIR	Near-Infrared Raman
NIN	Ninhydrin
Ng	Nanogram
nL	Nanolitre
Nm	Nanometre
NMR	Nuclear Magnetic Resonance
PCA	Principal Component Analysis
PCA-DA	Principal Component Analysis-Discriminant Analysis
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PLS-DA	Partial least squares- discriminant analysis

PMF	Peptide mass fingerprint
PTM	Post translational modifications
q-ToF	Quadrupole Time of Flight
qRT-PCR	Real time quantitative reverse transcriptase polymerase chain reaction
RF	Radio frequency
RT-PCR	Reverse transcription endpoint polymerase chain reaction
RNA	Ribonucleic acid
ROI	Region of Interest
RP	Ruhemann's Purple
SA	Sinapinic Acid
SELDI	Surface Enhanced Laser Desorption Ionisation
S/N	Signal to Noise Ratio
SERS	Surface Enhanced Raman Scattering
SIM	Selected Ion Monitoring
SIMS	Secondary Ion Mass Spectrometry
STR	Short tandem repeat
TFA	Trifluoroacetic Acid
TIC	Total Ion Current
ToF	Time of Flight
UPLC	Ultra-performance liquid chromatography
UV	Ultraviolet
V	Volts
v/v	Volume to Volume
w/v	Weight to Volume
w/w	Weight to Weight
WPS	Wet powder suspension

LIST OF PROTEINS INVESTIGATED

Abbreviation (if used)	Protein
α-2-M	Alpha-2-macroglobulin
Actin	Actin
ApoA1	Apolipoprotein-A1
Beta-Enolase	Beta-Enolase
Carbonic Anyhdrase-3	Carbonic Anhydrase-3
Ceru	Ceruloplasmin
EPB4.2	Erythrocyte Protein Band 4.2
Fructose-biphosphate aldolase	Fructose biphosphate aldolase
Glycophorin A	Glycophorin A
GAPDH	Glyceraldehyde-3-dehydrogenase
αHb	α-Haemoglobin
βHb	β-Haemoglobin
Hpt	Haptoglobin
Hpx	Hemopexin
Mb	Myoglobin
Serum Albumin	Serum Albumin
Semenogelin-1	SEM-1
Semenogelin-2	SEM-2
Trfe	Serotransferrin

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1.0 INTRODUCTION

1.1 Biological Properties of Blood

Blood consists of 55% intercellular material (plasma), 45% formed cellular elements (Anderson et al. 2016; Koen et al. 2017) and makes up 8% of the total human body weight (James et al. 2005). The plasma in blood is comprised of water, inorganic salts, and proteins, and the cellular fraction of blood includes erythrocytes, leukocytes, and platelets. The predominant functions of blood during its circulation are the transportation of carbon dioxide, oxygen, nutrients, hormones, and heat around the body (James et al. 2005 and Reynolds et al. 2008).

Erythrocytes, which are also more commonly known as red blood cells, account for 98.5% of the cellular component of blood (Reynolds et al. 2008). Their biconcave shape and extremely small diameter (~7.5 µM) mean that they are effective at transporting various essential products around the body. The disc-shape of erythrocytes also facilitates in the optimal transportation of carbon dioxide and oxygen around the body (James et al. 2005). In terms of forensic DNA analysis, red blood cells (which contain the protein Haemoglobin (Hb) giving it its red colour) do not contain a nucleus, therefore DNA is sourced from nucleated leukocytes or white blood cells.

'Leukocytes' can be used as an umbrella term for the several other cellular components; granulocytes (basophils, eosinophils, and neutrophils) and agranulocytes (monocytes and lymphocytes). The primary function of leukocytes is the digestion of pathogens in combatting infection and regulating the immune system. Platelets, which are the smallest cellular components of blood (~2µm) (Handtke et al. 2020) aid in maintaining homeostasis through thrombus formation and coagulation (Reynolds, 2008). The blood plasma, which is a clear yellow fluid consists primarily of water (90%), plasma proteins (7% which are albumins, globulins, and fibrinogen), nutrients and salts (3%). The main function of plasma is the transportation of electrolytes, nutrients, and hormones in addition to fluid, pH, and body temperature regulation (Brown and Davenport 2012).

Being the most frequently encountered biofluid at crime scenes, either as stains, spatter or contaminated fingermarks, the detection and examination of blood provides a rounder understanding of the crime taking place (or of foul play in the absence of a body), the timeline of the events and the sequence in which they occurred (Anderson et al. 2016).

In terms of blood spatter, the distribution of this biofluid at the scene, the surfaces it has been deposited on and the volume of blood present can offer a plethora of intelligence as to how the crime occurred (Rohrig et al. 2008). Additionally, the shape, size, and distribution of a blood spatter pattern can assist in reconstructing a crime scene (Lee et al. 2020). Similarly, trajectory analysis of blood drops/spatter can offer a 'retrospective insight' into the spatial positioning of an injured party at the scene (Home et al. 2021). As with fingermark analysis, blood spatter analysis requires a similar expert interpretation to elucidate the way the blood was generated (James et al. 1999; Akin 2005 and Bevel et al. 2008). It is therefore paramount to detect and confirm the presence of blood to undertake blood spatter analysis.

The formation of blood contaminated fingermarks is something that, if understood more, could offer better understanding as to the sequence of crime scene events. The mechanism of deposition of blood within a fingermark is subjectively assessed based on the experience of forensic experts. As such, there is a real danger of misinterpretation of the evidence and miscarriage of justice. The potential scenarios for blood finger mark deposition are as follows: 1) the fingertip was contaminated with blood prior to contact with a surface (blood fingermark), 2) an uncontaminated fingertip contacted a surface that already had blood present (fingermark in blood), or 3) blood has contaminated a fingermark that was already present on an object (coincidental association). Understanding the differences between these scenarios would ensure that the timeline of a crime is accurately interpreted and understood (Creighton et al. 1997 and Huss et al. 2000).

It is of critical importance that each possible scenario for the formation of a blood contaminated fingermark is investigated so that justice is always served (Bone et al. 2012). However, while very few articles have investigated the challenges of such studies (Geller et al. 2018; Langenburg et al. 2008; Praska et al. 2012 and

Becue et al. 2010) only one has attempted (but failed) to develop an objective and quantitative approach to the determination of the mechanism of blood deposition within a fingermark (Deininger et al. 2017).

However, irrespective of the mechanism, the underlying piece of knowledge necessary to undertake any further research on their differentiation sits, in this case too, with the reliable detection and confirmation of the presence of blood in/within a mark.

1.2 Visualisation of Blood (Stains and Fingermarks) using Forensic Light Sources

Before any chemical enhancement of potential blood marks at a crime scene, illumination through optical absorption can be used in the first instance as a non-destructive means to locate this biofluid (Chuena et al. 2010). Blood absorbs light across the visible spectrum and, has a strong absorption peak at ~415 nm due to the presence of Haemoglobin (Stoilovic et al. 1991).

Forensic light sources (FLS) are often used to locate regions where biological fluids are potentially present at a crime scene. As blood can absorb light across an extremely broad region (300 to 900 nm), this intrinsic property can be exploited to visualise the biofluid *in situ* (Stoilovic et al. 1991). However, other substances may have a strong absorption band at 415nm, and as such, this is not a particularly specific test.

An FLS generally refers to some type of illumination system; many of which are available for forensic use (e.g., Foster and Freeman Crime Lite System or the Polilight ®). As blood does not fluoresce under light unless treated with specific BET, the use of an FLS simply improves the contrast of the blood against the substrate it is deposited on (Shenkenberg et al. 2009). This is especially evident when blood is located on darker substrates (Virkler et al. 2009; Stoilovic et al. 1991; Wagner et al. 2003; Lennard et al. 2004 and Vandenberg et al. 2006).

In some cases, perpetrators will attempt to conceal blood evidence under paint or other materials. Howard et al. (2010) investigated bloodstain detection using FLS and infrared (IR) photography with blood under different painted surfaces (of different paint colours). IR photography proved the most effective at detecting

blood under maroon paint as the blood absorbed the light (appearing black) and the paint appeared white creating a clear contrast, whereas the bloodstains under white paint were not as easily located. In the work conducted in collaboration with the Israel Police in Chapter 3, the devised MALDI MS approach for blood detection showed that detecting blood under paint was feasible and indicates the versatility of this approach in forensic practice for blood recovered under different circumstances.

1.3 Blood Enhancement Techniques

With blood being the most encountered biofluid at crime scenes, there are several BET available for the enhancement of partial/latent bloodstains or marks. Only chemical enhancement techniques will be discussed in this section as they were the only type used in the experiments included in the thesis.

Various techniques are available for the enhancement of blood marks to better locate and image them at the scene (Sears et al. 2001). The aim of enhancement is two-fold: reveal the presence of blood and that of level 1 (ridge flow) and level 2 details (*minutiae)* so that a match to a fingerprint record can be found as well as establishing a link with the circumstances of the crime. The selection of the method of enhancement depends considerably on the substrate on which it is deposited which varies across crime scenes.

Patent blood marks (marks which are visible to the naked eye) are straightforward to locate, but in the event of partial blood marks or extremely faint blood marks being present at a scene, the use of a BET can not only indicate the possible presence of blood in all types of blood marks (patent, partial and latent) but also help improve visualisation greatly, especially with latent and partial blood marks. During the drying process of blood in a fingermark, cells are mostly destroyed meaning that the proteins and amino acids present in the blood can then be targeted to stain (reveal) the blood and improve visualisation (James et al. 2005). Generally, protein-staining dyes, amino acid-reacting dyes or haem-reacting compounds are the most used reagents for blood fingermark enhancement. Various FLS can be used post chemical BET application for improved visualisation (the light source used will depend upon the BET applied and surface of deposition) (Figure 1).

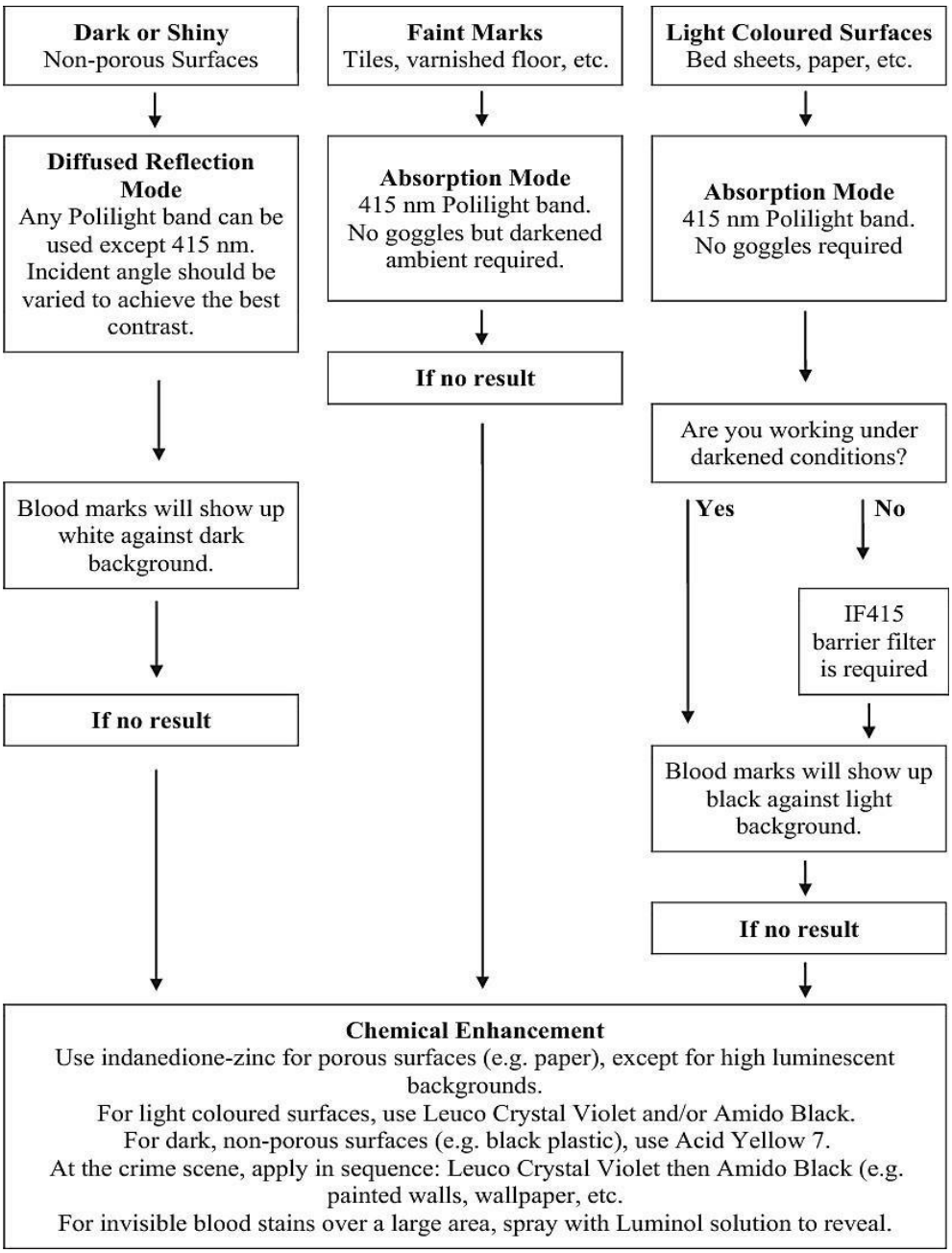

Figure 1: Workflow for blood finger mark enhancement accounting for surface of deposition and working conditions (reprinted from Stoilovic et al. 2010 and in line with guidance provided in the Fingermark Visualisation Manual (2014)).

Despite many instances in which blood fingermarks display clear ridge detail, (better contrast between the ridges and interridges) there are cases in which blood is too abundant which in turn produces smudged marks, instances in which only a trace amount of blood is deposited within the mark, or the blood is diluted prior to mark deposition. In the latter case, there are a range of different BET that

can be used for enhancement as summarised by Bossers et al. (2011). However, it must be considered that a blood fingermark may undergo DNA typing, therefore techniques that do not interfere with DNA profiling are preferable.

1.3.1 Haem-Reactive Techniques

Haem is a prosthetic group of haemoglobin which is abundant in red blood cells. Its presence is targeted/ exploited by several techniques to enhance blood fingermarks. The active site in the haem molecule contains an iron atom which is at the centre of a porphyrin macrocycle (Rogers, 2022).

Haem therefore can catalyse oxidation reactions and in the context of BETs, can react with a colourless solution to stain blood traces or cause reagents to luminesce. It is standard practice to also use hydrogen peroxide (H_2O_2) in these reactions to facilitate the oxidation reaction. BETs of this type have a specific affinity for blood, unlike some others (detailed in sections 1.3.2 and 1.3.3) as it is reliant upon the presence of the haem group so could therefore be classed as both a presumptive test for blood and means of enhancement in one.

Leucocrystal Violet (LCV) is a colourless crystalline solid which dissolves in water to produce a colourless solution. In the presence of H_2O_2, the haem acts as a catalyst in the oxidation of LCV to Crystal Violet (CV) turning the blood residue into a deep purple colour. The CV formed is soluble in lipid material which holds the product in place and enhances the print. This BET is suitable for all surface types (Bandey et al. 2014). Rinses are usually needed after LCV treatment, as, due to photoionisation from oxygen in the air, significant background staining can occur, so photographs of the enhanced marks should be taken immediately after application of this BET (Bandey et al. 2014).

1.3.2 Amino Acid-Reactive Dyes

Amino acid reagents are widely used, due to amino acid abundance in blood. However, these reagents are also used for enhancement of latent, non-blood fingermarks as they are present in natural secretions so do not have a specific affinity for blood. However, as there are typically more amine-containing substances in blood contaminated finger marks versus latent marks, the intensity of the reaction observed with blood is generally greater (Bleay et al. 2021). The reagents react with the amino acids present resulting in a colour change that can

be observed on the mark to aid visualisation (Champod et al. 2004; Bowman et al. 2009 and Stoilovic et al. 2010).

There are several amino acid reacting dyes available for forensic application. As with the protein-staining and haem-reacting reagents, the surface of deposition will partly dictate the enhancement selected. The three common amino acid-reacting compounds are Ninhydrin (NIN), 1,8 Diazafluoren-9-one (DFO) and 1,2-Indanedione. Only NIN will be discussed as it was the only amino-reactive BET used in this body of work.

NIN was the first amino acid reagent used for the enhancement of latent blood marks on porous surfaces e.g., paper and cardboard (Oden and Von Hofsten 1954 and Becue et al. 2010). Ninhydrin reacts with amines (including amino acids and proteins that are excreted by the eccrine glands) to form Ruhemann's Purple (RP) complex. Humidity (50-80%) is essential for this reaction, as too high a temperature can result in the NIN reacting with the surface as opposed to the residues. One disadvantage with NIN is that when the RP is formed, it cannot be viewed under fluorescence as it is not luminescent, so is not suitable on darker surfaces. One promising study also showed that visual differences between latent fingermarks versus blood-contaminated fingermarks can be visually distinguished when enhanced with NIN (Bentolila et al. 2017).

1.3.3 Protein-Staining Dyes

Protein stains are commonplace with blood fingermark enhancement as they interact non-covalently with the protein content in blood and aid in their visualisation. The protein dyes electrostatically interact with the cationic groups of the proteins and the negatively charged sulfonate groups of the dye when under slightly acidic conditions (Platt et al. 2006). It must be highlighted however that protein stains are entirely non-specific to blood so will theoretically enhance anything that contains protein. As a result of this, care must be taken in performing a full blood search of a crime scene prior to wholesale protein staining. If protein staining is performed prior to a thorough blood search, there could be several enhanced marks visualised, some of which could be a latent fingermark in sweat (evidentially less valuable) or in blood (evidentially significant) which becomes hugely important (*Personal Communication*, Chris Gannicliffe, Scottish Police Authority).

Acid Violet 17 (AV-17) (Sears et al. 2001), Acid Yellow 7 (AY-7) (Sears et al. 2005) and Acid Black 1 (Amido Black) (AB-1) (Sears and Prizeman 2000) are the most used dyes in forensic practice (Bandey et al. 2014) with additional dyes such as Hungarian Red and Coomassie Blue also available for use. Only AB-1 and AY-7 will be discussed here as they were the only protein stains used in the work presented in this thesis.

AB-1 is the most used protein stain for blood fingermark enhancement. This dye is a disulfonic acid that reacts with the lysine, histidine, and arginine groups in haemoglobin (Marchant and Tague 2007). The dye stains the proteins in blood to produce a blue-black colour and can be applied to marks on both porous and non-porous surfaces. Expectedly, the greatest contrast with this dye is observed on light-coloured surfaces.

AY-7 is a fluorescent protein dye that enhances blood marks in the same way that AB-1 does and is the most sensitive protein dye due to its fluorescent properties (Forsythe-Erman et al. 2001 and Bandey et al. 2014). It is very effective at enhancing marks on dark non-porous surfaces (Marchant and Tague 2007 and Barker 1999), so its use is generally limited to these surface types. In the study by Sears et al. (2005) it was found that AY-7 was the most performing dye for non-porous substrates, even more so than the most used AB-1. Marks enhanced with this dye can be visualised under fluorescence as AY-7 has an excitation maximum at 460 nm (Zhu et al. 2005) and emission peak at 530 nm (Baxter et al. 2008). Caution must be taken however, as if there is an abundance of blood on the mark, quenching effects will reduce the fluorescence observable.

The specificity of the MALDI MS approach (as shown in the data from Chapter 2) has been highlighted in the blind study. Some of the samples (beet juice, horseradish, and egg yolk) were stained with the protein dyes, which indicated to the analyst that the sample was potentially blood when it was in fact not. The MALDI MS analysis then indicated these samples were in fact not blood as the BET result may have otherwise indicated. These data are presented in Chapter 2 demonstrating the usefulness of this technique as confirmatory test.

1.4 Sequential Processing of Blood Fingermarks

As blood enhancement techniques (BET) are regularly used in forensic practice as first port of call, whether at the crime scene or remotely at the crime lab, it is important to understand their compatibility with the different presumptive blood tests available too. Stewart et al. (2018) investigated this concept by depositing blood depletion marks on a range of different surfaces (30 depletions on non-porous surfaces and 20 on porous), some of which were aged and then enhanced with various BET (fluorescence, cyanoacrylate fuming, AV-17, and NIN as some examples) followed by subsequent presumptive testing (explained further in Section 1.7) by the application of the Kastle-Meyer (KM) test and subsequently of confirmatory testing (illustrated further in Section 1.8) using the Rapid Stain Identification Blood (RSID) and Takayama tests. It was observed that both NIN and CA fuming produced weak but rapid positive results from the KM test whereas the AV-17 and AY-7 treated depletions delayed the result by 1 minute. Similarly, Streeting et al. (2022) compared the efficacy of the Hematrace and RSID confirmatory blood tests after enhancement with LCV or Luminol. The results indicated that the RSID test was greatly inhibited by the application of Luminol, and to a lesser extent with LCV also, so their use in tandem is not recommended. It is vital that any blood testing performed can be used in tandem with the common BET and that any alternative blood detection technique has been tested regarding its compatibility with the various BET used operationally. The devised MALDI MS approach presented in this thesis has shown compatibility with several common BET and some presumptive blood tests as further elaborated in Chapters 2, (AB-1, AY-7, and LCV) 3 (NIN and AB-1) and 5 (KM and Luminol).

1.5 DNA Typing after BET Application

Stains and fingermarks contaminated with blood or other biofluids will normally be retained for DNA extraction and profiling after their enhancement. An important consideration in any forensic workflow is the use of minimally destructive techniques to limit the alteration or destruction of any biological material. It is well documented that most of the BET can be used in conjunction with DNA typing; AB-1, AY-7 and AV-17 were shown to be compatible with subsequent DNA typing (Stein et al. 1996). Further to this, AB-1, LCV and AY-7

were utilised compatibly with DNA and RNA profiling in Fox et al. (2014), but findings indicated that LCV seemed to impact the messenger RNA (mRNA) which should be considered when selecting the appropriate BET.

Compatibility of DNA profiling in Zamir et al. (2000) shown with Hungarian Red, DFO, Ninhydrin and AB-1 (amongst other less routinely used techniques) had no adverse effects on PCR amplification when blood was treated with these BET. However, longer exposure of blood marks to Hungarian Red and Crowle's Double Stain (a less commonly used BET) slightly decreased amplification of the longer STR loci in the DNA profiles (Frégeau et al. 2000). Interestingly, Azoury et al. (2002) also demonstrated that 1,2-Indanedione can be used compatibly with DNA typing, but only when the STR typing is performed within 6 days, as DNA could not be recovered after this point.

Some interesting and very recent findings from Harush-Brosh et al. (2021) indicated in their study that AB-1 showed potential in amplifying touch DNA solely from blood contaminated fingermarks. This blocking of the blood-originating DNA using AB-1 means that a distinction can be made between the DNA profiles generated from the touch DNA (from the latent fingermark) versus from the blood itself. Operationally, blood fingermarks that contain no ridge detail are classed as 'dead-evidence' as the fingermark and touch DNA cannot be used to make human identification, but this study proposes that AB-1 can be used to yield a touch-DNA profile when using AB-1 as a blocker for the blood-deriving DNA.

Most operational workflows require that any blood evidence is retained post enhancement for DNA extraction and profiling, and the study presented in Chapter 3 has indicated that MALDI MS analysis can be used to yield a positive blood result in NIN and AB-1 enhanced blood fingermarks whilst not hindering the retrieval of a DNA profile after undergoing MALDI MSI, an extremely promising finding in the movement towards MALDI being integrated into current operational workflows for forensic blood analysis.

1.6 Blood Mark Enhancement: Alternative Lifting/ Enhancement Methods on Different Substrates

Research into alternative lifting methods and BET for blood stains/ fingermarks is very important for the scientific community to try and combat the known limitations with the current techniques used.

Bentolila et al. (2016) suggested that, when lifting blood marks from dark synthetic silk, alginate gel poses as a promising lifting technique. In a similar application, Munro et al. (2013) assessed the use of alginate lifting of blood fingermarks from a range of surfaces, differently aged marks, and the pre-application of chemical BET with pre and subsequent alginate-lifting. This lifting technique was found to be incompatible with glass and tile surfaces and the lifting of marks post BET produced rather weak marks when compared to non-lifted marks. However, when the alginate was mixed directly with the BET prior to its application, the marks were then both enhanced on the surface and lifted in an alginate cast. In the case of blood marks made on porous surfaces, this technique shows great potential.

A very interesting study delved into the enhancement of blood fingermarks on fruit and vegetables (Rae et al. 2013). A selection of protein-reacting dyes, haem-reacting compounds and amino-acid reacting compounds were assessed against a variety of differently aged marks and depletions. The findings suggested that the protein-staining dyes (e.g., AB-1) were the most effective BET tested. Aronson et al. (2011) investigated the development of blood fingermarks on the adhesive side of duct tape, finding that AB-1 was also the most effective protein dye for mark enhancement on this substrate.

Although there are already BET protocols for most surfaces, there are several other surfaces (more rarely encountered) that require further research into the most suitable BET to use. Hong et al. (2015) tested LCV, AB-1, and Hungarian Red for enhancing blood marks on thermal paper. LCV and Hungarian Red were both found to be inadequate due to blurring of the mark on the substrate and observed bubbling on the substrate. AB-1 was the best performing BET on thermal paper, with blood dilutions up to 1 in 20 being successfully enhanced.

Au et al. (2010) explored the use of wet powder suspensions (WPS) as an additional enhancement agent for blood marks. Used in tandem with the common protein dyes (AB-1, AY-7, and AV-17), blood marks were deposited on a range of surfaces and subsequently enhanced with a WPS. The findings indicated that, despite a significant improvement in overall mark quality, the WPS interfered with subsequent DNA extraction, yielding a 91% decrease in the quality of the DNA profiles collected and as such, the adoption of WPS more widely is unlikely.

Similarly, Bergeron et al. (2003) showed that the use of titanium oxide and methanol can be an effective alternative to enhance blood fingermarks on dark substrates producing white ridge detail. However, it would need to be determined as to whether this technique affects subsequent DNA recovery.

The use of alternative dyes for blood enhancement, as investigated in Barros et al. (2017) demonstrated the potential of using benzazole dyes in enhancing bloodstains and blood fingermarks. The photochemical stability of these fluorescent dyes means that they could be visualised under UV light on a variety of different surfaces. However, for a protein-dye to be used widely by forensic investigators, pseudo-operational trials would be required to test its robustness, as one study cannot support the deployment of this technique.

Ongoing research has also explored alternative natural dyes to use as BET instead of the standard chemical dyes. A study by Thomas et al. (2013) explored the applicability of Genipin and Lawsone to enhance blood contaminated fingermarks on different paper types. In their study, blood fingermarks were deposited in split depletions on paper types of different porosities and colour, then aged for different time periods (1,2,4 and 6 weeks). Contrary to NIN, neither Genipin nor Lawsone enabled visualisation of blood fingermarks on the different paper types. A recent study investigated the use of another natural substance namely *laccifer laca,* also known as lac dye (which derives from resin extracted from the lac insect; native to Southeast Asia), utilised to enhance blood fingermarks on a range of different porous and non-porous surfaces in comparison with reference marks enhanced with AB-1 (Chingthongkham et al. 2019). The results indicated that Lac dye and AB-1 showed similarity in their enhancement capability on non-porous surfaces in terms of the colour grade and ridge detail that could be observed. On porous surfaces, the Lac dye did not perform well, indicating its potential as a natural product for blood fingermark development on non-porous surfaces only.

For enhancing latent blood marks on plastic bags, Meng et al. (2020) conducted research on the viability of silica nanoparticle suspension in efforts to develop latent blood marks and Xu et al. (2019) demonstrated the enhancement of latent blood marks via enzyme-linked chemiluminescence immunoassay and pattern recognition.

1.7 Presumptive Blood Testing

In the US, before 1967, police investigators would assume that any stain that appeared to be blood at a crime scene was in fact blood. The U.S Supreme Court Case (*Miller v. Pate*) prompted the wider deployment of presumptive blood testing when a criminal deposited red paint on clothing to mislead an investigation (Rohrig et al. 2008). This was an overdue requirement also due to perpetrators' attempts in concealing blood evidence by cleaning up the scene as well to instances of false positives yielded by some of the available tests. Particularly the latter circumstance may result in the questioning of their admissibility and possibly leading to a case being overturned (*Personal Communication*, Ravell Bengiat, Israel Police).

Due to several biofluids having a similar appearance, preliminary visualisation or presumptive testing is not sufficient, hence the use of confirmatory testing or serological testing is required to ascertain the nature of the substance but also, in the case of blood, its provenance. However, confirmatory blood testing is not in fact routine forensic practice (*Personal Communication,* Chris Gannicliffe, Scottish Police Authority).

The location of the blood, environmental conditions the evidence was exposed to, presence in a stain, fingermark, palm print or footwear impression, and deposition surface will altogether dictate the test selected for use. Table 1 details all the currently available presumptive tests for blood detection with some examples of the substances that can yield a false positive result.

Presumptive Test	Chemical Reagent	Colour Change (when + for blood)	Mechanism	Substances that can cause false positives	References
Benzidine Test	Benzidine + H_2O_2	Blue	Hb catalyses the oxidation of the reduced benzidine to produce oxidised benzidine (blue coloured derivative).	Oxidants, catalysts, and vegetable peroxidases (e.g., horseradish, beetroot, and cauliflower).	Vittori et al. 2016
BlueStar ®	Commercial alternative to Luminol Test (3-aminophthalhydrazide + sodium carbonate+ sodium perborate + H_2O_2)	Fluorescent Blue/White	The peroxidase activity of iron complexes in the haem molecule present in blood catalyses the oxidation of the BlueStar reagent to produce a chemiluminescent blue.	Iron rich soils, copper, iron oxides, iron rust, carpet, bleaches, varnishes, paints, vegetable peroxidases (radishes, leeks, bananas, and tomatoes).	Fish et al. 2013; Gardener et al. 2005; Bevel et al. 2002; Blum et al. 2006; Saferstein et al. 2002; Seashols et al. 2013; Tobe et al. 2007; Barni et al. 2007; Watkins et al. 2006.
Fluorescein	Fluorescein + UV	Fluorescent Green	When blood is exposed to light (420-490nm) the fluorescein reagent reacts to the iron complexes and Hb in the blood producing a fluorescent green colour.	Cleaning products (e.g., bleach), urine, iron, copper, and strong oxidisers	Cheeseman et al. 1995

Hemastix ®	diisopropylbenzene dihydroperoxide and 3,3',5,5'-tetramethylbenzidine.	Green	Hb in blood cleaves oxygen molecules from the H_2O_2 which catalyses the reaction from the reduced (colourless) form of 3,3',5,5'-tetramethylbenzidineto the oxidized green form.	Iron rust, bleach, sink rot, tomato, potato, and uric acid	Casali et al. 2020
Hemident ® (LMG)	LMG + H_2O_2	Blue/Green	Hb in blood cleaves oxygen molecules from the H_2O_2 which catalyses the oxidation of the reduced LMG to oxidised LMG giving a blue green colour change.	Saliva, semen, tomato, red onion, horseradish, ascorbic acid, cupric sulfate, nickel chloride	Tobe et al. 2007
Phenolphthalein Test (Kastle-Meyer (KM) Test)	Phenolphthalein + H_2O_2	Pink	Hb in blood catalyses the oxidation of the reduced KM reagent giving a bright pink colour change.	Vegetable peroxidases such as broccoli, horseradish, and cauliflower.	Petersen and Kovacs 2014
Leucomalachite Green (LMG) Test	LMG + H_2O_2	Blue/Green	Hb in blood cleaves oxygen molecules from the H_2O_2 which catalyses the oxidation of the reduced LMG to oxidised LMG giving a	Green beans, potato, and horseradish	Cox et al. 1991

			blue-green colour change.		
Luminol	3-aminophthalhydrazide + sodium carbonate+ sodium perborate + H_2O_2	Fluorescent Blue/White	Luminol solution contains both Luminol ($C_8H_7N_3O_2$) and H_2O_2. The H_2O_2 reacts with iron complexes in the blood to produce oxygen. The oxygen produced then reacts with Luminol producing a chemiluminescent response.	Enamel paints, parsnip, turnip, terracotta surfaces, polyurethane varnishes, household cleaning products and jute/sisal matting.	Cassidy et al. 2017; Barni et al. 2007 and DiCarlo et al. 2016

Table 1: Details of commonly used presumptive blood tests in forensic practice.

If presumptive testing must be performed on a suspected bloodstain, the test selected should be sensitive, user friendly (especially in the cases of testing being performed *in situ)* and specific.

Butler et al. (2019) compared a series of presumptive tests (Luminol, Leucocrystal Violet, Benzidine and the Combur Test ® (another benzidine test)) for detecting whole and diluted blood as well as blood stains generated by object transfer (a key with blood traces was moved and blood was transferred) on a range of surfaces (socks, tea towels, dark cotton sheets, carpet, and car mats). The efficiency of these blood tests was better understood through testing these various conditions and it was found that Luminol was the preferred test for diluted blood, having the highest sensitivity (76-96%). The use of powdered bleach was utilised to test specificity, as it is well documented that chemiluminescent blood tests can be prone to false positives from household cleaning products (Farrugia et al. 2011; Nilsson et al. 2006; Creamer et al. 2005; Kent et al. 2003; Quickenden et al. 2001; Céspedes et al. 2021 and Castello et al. 2009). Despite the sensitivity of Luminol being well documented, Cheeseman et al. (1999) found Fluorescein to be considerably more sensitive than Luminol when tested on a variety of both porous and non-porous substrates. As this was only one study, the frequency of using Luminol has not changed.

Webb et al. (2006) found Luminol to be the most sensitive of the tests investigated in their study (Luminol, KM, LMG, Hemastix and alternative forensic light sources) with Hemastix being regarded as an appropriate alternative to Luminol if this test cannot be used (such as an environment that cannot be in total darkness). These findings were aligned with what was observed in Tobe et al. (2007) where Luminol was determined to be the best presumptive test for blood. In some cases, a blood sample may need to be moved from the original item/substrate it was found on (e.g., a blood stain is transferred onto a swab or filter paper for application of the KM test). When this is required, the blood detection limit may decrease or be altered depending on the surface from which the blood is tested (Seashols et al. 2012). The KM reagent also is susceptible to false positive results from some vegetable peroxidases as detailed in Petersen and Kovacs (2014).

It has also been found within several investigations that Luminol can yield false

positives from several different substances such as vegetable peroxidases, household cleaning products and some metals (Thorpe et al. 1985 and Creamer et al. 2003), with similar observations seen with the Benzidine Test (Garner et al. 1976 and Cox et al. 1991). The Hemastix ® test was also found to be rather unspecific in the study conducted by Tobe et al. (2007) reacting to a range of different matrices, namely tomato, meat, onion, salts, and saliva.

Although false positives are certainly damaging to an investigation, false negatives for the presence of blood should be minimised too. Indeed, several inhibiting substances in the presence of blood can prevent the oxidation step that is necessary in some presumptive tests. Substances such as vitamin A, vitamin C (ascorbic acid), vitamin E, beta-carotene, and selenium are examples of these inhibiting agents that have been reported in the literature (Novelli 2020). Ponce and Pascual (1999) investigated different mixtures of ascorbic acid (Vitamin C) and blood and then applied various presumptive tests, with the KM, Benzidine and LMG tests producing false negatives. Similarly, Vennemann et al. (2014) investigated the effects of ascorbic acid on the KM and LMG tests, concluding that both tests were impacted by the presence of ascorbic acid. Pascual et al. (1995) saw similar observations with the Benzidine test when mixing blood with lemon juice in different quantities. Until 50% lemon juice was added to the blood, no false negatives were observed.

Lee et al. (2016) investigated more specifically the effects of vitamin c found in beverages as opposed to the chemical form of ascorbic acid (vitamin c). The study indicated that not only does ascorbic acid (in its chemical form) yield false negative results with the LMG test, but this is also observed with beverages containing the vitamin c form (e.g., orange, grapefruit, tomato juice or vitamin infused drinks). Additionally, when accounting for the interference from antioxidants, Bancirova (2012) showed that false negative results can be yielded from the BlueStar ® and Luminol test when found in some herbal teas. A decrease in chemiluminescent response was observed in the presence of green and black tea, suggesting their potential to act as inhibiting agents in the oxidation process of these tests.

With such variability in the effectiveness of some of these presumptive tests, operationally, there is a requirement for a technique that is not affected by

environmental factors and does not yield false positive results from substances that could be located at crime scenes (foodstuffs, cleaning products etc.).

1.7.1 Presumptive Blood Testing: DNA Typing after Test Application

Successfully yielding a DNA profile from a biological fluid does not confirm the identity of the biofluid from which it was retrieved. As a result of this, some courts in the US and Australia consider whether to admit presumptive test evidence based on whether DNA typing has been possible on the sample or not (Koen et al. 2017). If the biofluid is identified through testing and DNA typing is possible, this makes the evidence far more robust (Peel and Gill, 2004). Despite this being well-acknowledged within the forensic community, several forensic laboratories do not perform confirmatory human blood tests, which can ultimately cause legal challenges further down the line, as observed in *People v. Lovejoy* (2009) and *Palmer v. State* (1994) (Koen et al. 2017).

Almeida et al. (2010) investigated the viability of confirmatory blood testing and DNA profiling post presumptive test application. This study assessed the effect of the reagents in the Luminol, BlueStar and Benzidine tests (120 days after use) on retrieving a positive blood result from the human antiglobulin test, human haemoglobin immunochromatographic test and subsequently on the total DNA concentration. Luminol and BlueStar did not prevent a positive result in the confirmatory blood tests, nor did they promote DNA degradation. A similar study by Jakovich et al. (2007) corroborated this by showing Luminol, BlueStar and Fluorescein did not prevent STR profiling. Additionally, Santos et al. (2009) also showed that the application of Luminol prior to DNA analysis had minimal interference on serological testing followed by DNA typing and extraction. Several other studies document the compatibility of the fluorescent based tests with DNA typing, as outlined in Barbaro et al. (2004); Donachie et al. (2015) and Muralidhar et al. (2019).

In the Almeida et al. (2010) study however, the Benzidine samples could not be further analysed with confirmatory tests and 48h after application, the DNA had begun to degrade. Luminol started to cause DNA degradation 30 days post application, and by 120 days post-test application, all treated samples showed DNA degradation, including the BlueStar samples.

Anderson et al. (2016) performed suitability testing on a range of different presumptive blood tests (LMG, Luminol and BlueStar) and concluded that LMG with a 3% H_2O_2 concentration displayed the clearest colour change in the presence of blood and displayed the highest sensitivity and lowest number of false positive reactions. The Luminol and BlueStar displayed similar results when tested on various matrices but in practice Luminol did not show as many false positives as BlueStar when in the presence of different metals where no false positive results were observed for Luminol. As these two tests do degrade DNA, it was advised that they should only be used if necessary to locate blood at a crime scene.

1.8 Confirmatory Blood Testing

If a stain or fingermark tests positive for blood through presumptive testing, further testing should then be performed to confirm its presence and to determine whether it is of human origin. However, confirmatory testing is not routinely used in operation but could assist in circumstances where risk of a false positive result from the presumptive test is high, such as in sinks and drains, or where peroxidase activity from substances such as some vegetables and household cleaning products could interfere with the results of these tests (Koen, 2017).

The detection of human blood could be conducted with either the Takayama (Kerr and Mason, 1926) or Teichmann test (Miller, 1969), with further serological testing required if the blood is non-human. However, other confirmatory tests for human blood are available as detailed in Table 2.

Confirmatory Test	Mechanism	Substances that can cause false positives	
Takayama Test	Hemochromogen crystals are formed in the presence of blood due to the iron complex at the centre of the haem group (in haemoglobin) reacting with the Takayama reagent forming pink feathery crystals in the presence of blood.	Catalases and peroxidases at sufficient concentration- (metals. some vegetables	Koen et al. 2017
Teichman Test	The haem group reacts with sodium chloride (NaCl) and glacial acetic acid producing hematin chloride (ferriprotoporphyrin chloride) which is observed through the formation of brown rhomboid-like crystals.	Catalases and peroxidases at sufficient concentration- (metals. some vegetables)	Koen et al. 2017
Hexagon OBTI (Immunochromatographic test)	Human haemoglobin reacts with the hexagon reagent (consisting of red coloured particles and anti-human Hb antibodies). If present, the complex moves up the strip test and is immobilized by a second antibody (anti-human Hb) which forms a positive pink line if blood is present.	Cross-reactivity with ferret and primate blood	Koen et al. 2017

Rapid Stain Identification (RSID) Lateral Flow Strip Test (Immunochromatographic test)	The test allows the detection of Glycophorin A, which is a red blood cell membrane antigen, using two anti-human Glycophorin A monoclonal antibodies in the same lateral flow strip test format.	None reported in literature	
ABA Card Hematrace (Immunochromatographic test)	This test detects the presence of human Hb through an antigen-antibody reaction. If human Hb is present, the antigens in the blood will react with the mobile anti-human Hb antibodies, and a pink line will form if positive for blood.	Cross-reactivity with ferret and primate blood.	Koen et al. 2017

Table 2: Most common confirmatory tests for human blood detection.

Although confirmatory testing is far more specific and sensitive than presumptive tests, with several validation studies performed on them (Hochmeister et al. 1999; Johnston et al. 2003; Hermon et al. 2003; Schweers et al. 2008 and Turrina et al. 2008), there are still potential risks with using these tests, with some reporting's of cross reactivity and false positives in the literature (Koen et al. 2017). The subsequent application of confirmatory tests is also time consuming, limiting the throughput time in the processing of the evidence.

With respect to establishing blood provenance, the use of precipitin technology in forensics can be used to assist in discriminating between human and animal blood. The mechanism of the test works by measuring the immune response of animal blood by producing anti-human antibodies to protect from proteins in human blood. Generally, the process of the precipitin test will be the injection of human blood into an animal, the animal will produce anti-human antibodies, a sample of the animal blood is collected and then the animal blood can be used to test other blood to identify whether it is human or non-human blood (Lewis, 1912). The antibodies that are present in the animal blood being tested will detect the foreign pathogens from the human blood which will induce the production of anti-human antibodies from the animal blood that will attack the foreign human antigens.

1.9 Spectroscopic Methods for Blood Detection

Due to their non-destructive nature, there has been a great deal of research carried out on the use of spectroscopic techniques for the detection of blood and other biofluids. Additionally, some of these techniques have shown scope to determine blood provenance too which could be extremely helpful intelligence in a crime scene scenario. Despite not being deployed widely or operationally, some of these techniques offer potential solutions to some of the commonly encountered issues of sensitivity and specificity with the current blood detection, but also hold some caveats themselves.

1.9.1 Atomic Force Microscopy (AFM)

Strasser et al. (2006) detail the use of atomic force microscopy (AFM) for the high-resolution imaging of erythrocytes and measurement of elasticity changes in a sample of blood for confirmatory identification and age determination. The red blood cells can be easily detected with this approach due to their distinctive

shape. In terms of their morphology, no change was observed 4 weeks after collection of the blood, but the elasticity of the erythrocytes displayed a decrease over time, to be expected upon blood drying. In an operational context, the use of AFM for age determination of a blood sample could be challenging, as the blood in this study was collected under controlled conditions. In a real forensic scenario, blood can be subjected to different conditions which could have an impact on the elasticity observed in the erythrocytes.

1.9.2 Raman Spectroscopy

Raman spectroscopy is a non-destructive vibrational spectroscopic technique that provides information on the presence of functional groups from as minute as a microgram to nanogram of sample (Movasaghi et al. 2007). Studies into the applicability of this method in determining blood provenance for forensic purposes have been carried out (McLaughlin et al. 2014) (Fujihara et al. 2016). McLaughlin et al. (2014) investigated the possibility of differentiating human blood from non-human blood sources using Raman, through the development of a training dataset of IR spectrum for 11 different species. Conducting a blind study and external validation, the model showed 100% accuracy in being able to successfully discriminate the human blood samples from the non-human blood samples. This method suggests further scope for investigating non-humanblood and identifying characteristic absorption peaks for each species; thus, improving taxonomic resolution and specificity of this approach overall.

To assess the robustness of Raman for specific human blood detection, a sensitivity and specificity study was conducted by Boyd et al. (2010) investigating Raman scattering when variables such as the dilution factor of the blood, the age of the sample, the deposition surface, and the donor of the sample are changed. The results suggested that when blood samples are recovered from substrates that are not strongly luminescent e.g., metal, dry wall, or plastic, the Hb peaks that were detectable in all samples analysed were considerably more intense when recovered from these surfaces. Issues arise with aged samples as the relative intensity of the oxyhaemoglobin peak decreased significantly with samples age; this circumstance indicated that Raman may not be a useful

detection technique unless blood evidence is relatively fresh and contained in EDTA tubes to prevent coagulation.

However, not only has Raman showed the ability to detect blood, but also other biofluids that may be found at a crime scene. A non-destructive and confirmatory method for the rapid identification and discrimination of biofluids has been detailed in the study carried out by Sikirzhytski et al. (2010) using multi-dimensional spectroscopic IR Raman signatures combined with statistical analysis. The use of these spectroscopic signatures detected for semen, blood and saliva coupled with discriminant analysis allowed for biofluid discrimination with 100% probability. Despite this very promising result, this probability result is only yielded under laboratory conditions. In a real forensic context, biofluids present at a crime scene can be subjected to different environmental conditions which could potentially affect these spectroscopic signatures. The work presented in this thesis has indicated that even blood when extremely diluted, in the co-presence of other biofluids (Chapter 5) and pre-treated with some BET (Chapter 2 and 3) and presumptive tests (Chapter 5), it can still be detected via MALDI MS. The compatibility of some spectroscopic techniques with these variables has not yet been tested extensively which shows the advancements that MALDI has made in recent years in comparison.

1.9.3 Attenuated Total Reflection-Fourier Transform Infra-Red (ATR-FTIR) Spectroscopy

Mistek et al. (2015) researched the use of ATR-FTIR; a non-destructive spectroscopic technique for discrimination between human blood and non-human blood samples using statistical analysis. Using partial least squares linear discriminant analysis (PLS-DA), complete separation between the human and non-human blood samples, with further separation and quick discrimination between human, cat, and dog blood achieved. An added benefit of using this technique is that it is widely commercially available and portable so blood evidence can be analysed *in situ* at crime scenes. Similarly, to Raman microscopy, ATR FT-IR has also been able to successfully detect other biofluids such as semen and blood through the identification of several characteristic scattering peaks for each biofluid without the prior use of chemical reagents (Zou

et al. 2016). In real forensic practice however, it would be necessary for a technique to be compatible with the common BET's and thus a range of different chemical reagents, a factor not considered in this study.

1.9.4 Near Infra-Red Spectroscopy

NIR spectroscopy is an analytical technique that is used for investigating the vibrational properties of a sample and covers the interval between 4000-12,500cm^{-1} (Siesler et al. 2008). Edelman et al. (2012) detailed a study into the use of NIR spectroscopy for blood stain identification and age estimation on dark surfaces. Using NIR reflectance spectroscopy, blood was distinguishable from other substances when deposited on coloured surfaces. However, as blood could plausibly be recovered on a range of different surfaces at crime scenes, investigation into this technique's suitability with different deposition surfaces (both light and dark) would be required more extensively. In addition, the age determination of bloodstains using this technique showed better accuracy in younger stains (<1 month) indicating it may not be as useful in bloodstains that are older. The devised MALDI MS approach used in this PhD programme has been shown to successfully detect blood in both a 9-year-old bloodstain (Patel et al. 2015) and a 37-year-old NIN enhanced blood mark (Deininger et al. 2017) showing its high sensitivity in comparison to other techniques.

1.9.5 Hyperspectral Imaging (HSI)

Visible wavelength hyperspectral imaging (HSI); a non-destructive and non-contact technique proven useful for the detection of blood has been researched by different groups (Edelman et al. 2013; Cadd et al. 2016; Cadd et al. 2016; Crowther et al. 2021 and Zulfiqar et al. 2021). In the studies from Cadd et al. (2016), despite the visualisation of blood (based on the Soret γ band absorption of Hb between 400 and 500nm) in latent blood marks in up to 15,000-fold dilutions, visualisation of ridge flow in 20-fold dilutions and on a range of porous/non-porous surfaces of different colours, these results only suggest the possible presence of blood and does not distinguish between human and non-human blood. There could also be other substances that show absorption within the 400-500nm band so this result could not be reported as confirmatory.

1.9.6 Nuclear Magnetic Resonance (NMR)

In 2013, Scano et al. introduced the application of NMR for differentiating blood, semen, saliva, and urine. From the data collected, metabolites such as lactate, citrate and some other amino acids were assigned to spectral bands as a means of discriminating between the biofluids under investigation (Zapata et al. 2015). With statistical treatment of the data via principal component analysis (PCA), the four biofluids could be differentiated and four visible clusters could be seen from the PCA scores plot. For operational deployment of this technique, further verification and validation would need to be conducted and assessment of whether mixed biofluid samples could be successfully examined by the same means.

2.0 Molecular Biology Blood Detection Methods

The retrieval of DNA from a biofluid can be used to identify an individual whilst mRNA is biofluid-specific so can be used as confirmation of a specific biofluids' presence at the crime scene. A commonly observed defence in a court environment is the plaintiff disputing the provenance of DNA, attributing it to a different biofluid. For example, saliva or sweat could be legitimately found at a crime scene; as such, the use of mRNA would effectively refute this testimony.

The review published by An et al. (2012) reported the use of mRNA, micro-RNA (miRNA) and DNA methylation techniques for the detection of biological fluids, some of which is also reported in the more recent review by Harbison et al. (2016).

Zubakov et al. (2008) reported that when mRNA is isolated, it retains its stability for long periods, and therefore offers scope to be used to forensically differentiate biological fluids; a finding also reported in Juusola et al. (2003 and 2005). Using whole genome gene expression, blood specific mRNA showed stable expression in stains after 180 days of storage, with amplification of blood-specific mRNA possible in even older blood stains of 16 years old**.** Bauer et al. (2003) and Haas et al. (2012) also demonstrated the viability of isolating mRNA from biological stains and yielding a successful donor profile match. Using a multiplex approach, it is also possible to detect multiple biofluids in one biological stain and such intelligence could facilitate the understanding of the dynamics of a

crime (Haas et al. 2009 and Vennemann et al. 2010). The use of reverse transcription endpoint polymerase chain reaction (RT-PCR) and real time quantitative reverse transcriptase PCR assays (qRT-PCR) yielded body fluid specific markers showing the applicability of this multiplex approach to biofluid identification also (Haas et al. 2009; Haas et al. 2012; Juusola et al. 2005; Juusola et al. 2007; Nussbaumer et al. 2006 and Fleming et al. 2009).

Jakubowska et al. (2013) investigated the feasibility of developing a specific test for vaginal fluid by detecting several proteins specific to both vaginal fluid and menstrual blood. The specificity of these markers was tested when attempting to detect vaginal fluid in mixtures with both semen and blood. This Hexaplex PCR approach showed that biofluids in stains (up to 2 years old) could be detected successfully.

Several other studies have explored the identification of biofluids through cell specific mRNA (Bauer et al. 2007) (Gomes et al. 2011), which can be offered as a specific method for biofluid detection and discrimination.

2.1 Mass Spectrometry: General Principles

Mass spectrometry is an analytical technique that is used for the detection/confirmation/identification/quantification of molecules by measuring their mass-to-charge (*m/z*) ratio. To determine the *m/z* of a species, the analyte of interest must ionise into the gas phase and there are several ways in which ionisation can be induced (those employed in this PhD research are explained in greater detail in sections 2.1.1 and 2.1.2). Once analyte ions have been generated, they are separated by a mass analyser or multiple mass analysers to improve detection, resolution or for undertaking tandem mass spectrometry by inducing fragmentation of the parent ion. Ions are finally detected to generate a mass spectrum of their *m/z* versus their relative abundance.

Figure 2 shows a basic schematic of a mass spectrometer and the different components. Vacuum techniques are those in which the source and the mass analysers are kept under high vacuum to avoid fragmentation and only induce it under controlled conditions.

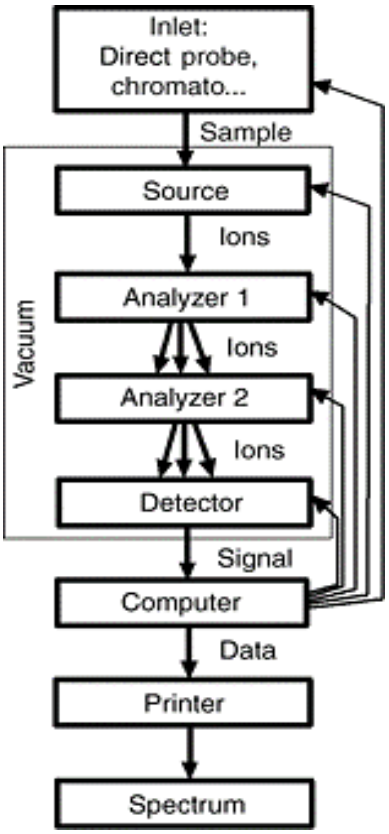

Figure 2: Schematic of common mass spectrometry system set up (reprinted from Hoffmann and Stroobant 2001).

2.1.1 Electrospray Ionisation Mass Spectrometry (ESI-MS)

Electrospray Ionisation Mass Spectrometry (ESI-MS) is a soft ionisation technique, (which in the work presented in this thesis was coupled to a LC) that generates ions through the application of a high voltage to a liquid sample to produce an aerosol under atmospheric pressure (Ho et al. 2003). The ESI process produces multiply charged ions, and as a result, detection of molecules can range from <100 Da (small molecules) to kDa (proteins, protein complexes and polymers) and even mDa, with the detection of the intact MS2 viral capsid at 2.5 mDa shown to be possible using ESI-MS (Tito et al. 2000). The detection of intact proteins and their associated polypeptide fragments has also been reported (Ho et al. 2003; Hoffmann and Stroobant 2007 and Pitt et al. 2009). As this was not the primary analytical technique used in this project, only a general overview will be provided.

ESI occurs in three stages: droplet formation, desolvation and gas phase ion formation. The solution sample is ejected from the capillary under a high voltage (~3-6 kV) electric field producing a charged droplet. The charged droplets then enter the spray chamber and are evaporated by the heated nitrogen gas resulting in the diameter of the droplets becoming smaller. When the charge density increases and the repulsive force between the charged droplets is sufficient to counteract the surface tension in the droplet (reaching the Rayleigh instability limit), the droplets then undergo fission and separate into smaller charged droplets (Coulombic explosion). When the charged droplets reach the nm size, they release gas phase ions (Figure 3) (Ho et al. 2003).

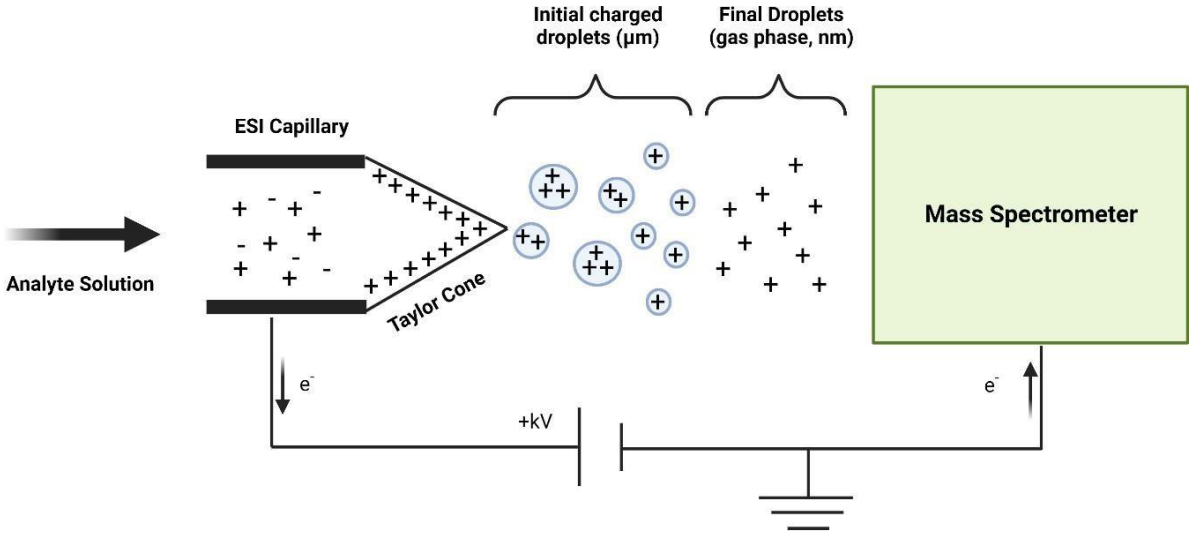

Figure 3: Schematic of ESI mechanism (redrawn from Konermann et al. 2012).

For the LC-ESI-MS/MS data presented in this thesis the Waters Xevo q-ToF G2- XS (coupled with the Waters Acquity UPLC system) instrument was used in positive ion mode.

2.1.2 Matrix Assisted Laser Desorption Ionisation Mass Spectrometry (MALDI MS)

Matrix assisted laser desorption ionisation mass spectrometry (MALDI MS) was first developed by Karas and Hillenkamp in 1988 (Karas et al. 1987; Karas and Hillenkamp 1988) with the pioneering work on soft laser desorption carried out by Koichi Tanaka (for which he received the Nobel Prize in Chemistry in 2002) assisting in its adoption for protein analysis (Tanaka et al. 1987; Karas and Hillenkamp, 1988). MALDI is a soft ionisation technique originally developed for the detection of large, non-volatile, and thermally labile biomolecules such as proteins, oligonucleotides, and other macromolecules such as synthetic polymers (Hoffmann and Stroobant 2007; Li et al. 2009) and is one of the most used ionisation methods for the analysis of high molecular weight species (Wenzel et al. 2005 and Rader et al. 2014). However, the technique has significantly grown into including the analysis of small molecules and peptides too. Within the work presented in this thesis, a bottom-up proteomic approach was adopted for the detection of blood peptide signatures which will be discussed further in Section 2.2. For the detection and identification of proteins, this approach is generally better performing due to higher sensitivity, mass resolution and mass accuracy afforded.

The technique is reliant on the use of a matrix (mixed in some way with the analyte), which is normally a small molecular weight organic compound that has a strong absorption at the laser wavelength (e.g., Nd: YAG laser has a wavelength of 355 nm). In positive mode, the matrix must be a suitable proton donor to allow for proton transfer from the matrix molecules to the analyte molecules during ionisation. The matrix selected for analysis is dependent upon the analyte of interest (Table 4), with α-CHCA being the most appropriate for peptide and protein analysis.

Analyte	Matrix	Abbreviation
Peptides and proteins	α-cyano-4-hydroxycinnamic acid	α -CHCA
	2,5-dihydroxybenzoic acid	DHB
	3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic)	SA

	Nicotinic Acid	NA
	2-mercaptobenzothiazole	MBT
Lipids	Dithranol	DIT
Organic Molecules	2,5-dihydroxybenzoic acid	DHB
Inorganic Molecules	Trans-2-(3-(4-tert-butylphenyl)-2-methyl-2-propenyliedene) malononitrile	DCTB
Carbohydrates	2,5-Dihydroxybenzoic acid α-cyano-4-hydroxycinnamic acid 3-Aminoquinoline (acidic) 2,4,6-Trihydroxyacetophenone	DHB α -CHCA (No abbreviation) THAP
Glycoproteins	2-(4-Hydroxyphenylazo) benzoic acid	HABA
Glycopeptides	5-chloro-2-mercaptobenzothiazole	CMBT
	2,6-Dihydroxyacetophenone	DHAP
Oligonucleotides and DNA	Picolinic Acid 3-Hydroxypicolinic acid 3-Aminopicolinic acid 6-Aza-2-thiothymine	PA HPA, 3-HPA 3-APA ATT
Fullerenes and derivatives	9-Nitroanthracene	9-NA
	Benzo[a]pyrene	(No abbreviation)

Table 3: Common MALDI matrices for different applications (merged from Gross et al. 2006 and Hoffmann and Stroobant 2007).

The MALDI matrix is essential as in large excess, it protects the analyte from the direct impact of the laser (which would fragment the molecule) by absorbing the laser energy. The analyte- matrix mixture is dried prior to analysis, during which time the analyte and matrix will co-crystalise.

For ionisation to occur, the sample is ablated with laser pulses which cause the crystals to rapidly heat whilst the matrix molecules undergo vibrational excitation; the key step involved in primary ionisation and induced upon laser irradiation (Knochenmuss 2006; Hoffmann and Stroobant 2007). The desorption is induced by the firing of the laser (in the UV region) on the sample which is facilitated by the matrix (a UV absorbing molecule). The matrix crystals absorb the laser energy, and as a result, the rapid heating of matrix crystals causes their

sublimation and the conversion of the matrix and analyte molecules (which are mixed with the matrix crystals) into gas phase molecules. Intact analyte ions are generated in this plume as discussed in detail in the next section where the two most developed MALDI ionisation mechanism models are reported.

2.1.2.1 MALDI Ionisation Theory

The formation of ions in MALDI is still not entirely understood (Hoffmann and Stroobant 2007) though few theories have been published to explain the ion formation mechanism in MALDI (Knochenmuss 2021) with the *Gas Phase Protonation Model* and *Lucky Survivor Model* being the most well-known.

Knochenmuss and Zenobi (Zenobi 1998; Knochenmuss and Zenobi 2003 and Knochenmuss 2006) proposed that the generation of analyte ions occurs in two steps, the first being the photoionisation of matrix molecules from laser irradiation of the sample (matrix and analyte mixed) producing matrix ions; this theory is reported in other studies (Chang et al. 2007; Jaskolla et al. 2011 and Bae et al. 2012). The second step is charge transfer from the matrix ions to the neutral analyte species generating charged analyte ions (Figure 4). It is proposed that the charge transfer occurs when the analyte molecule collides with a matrix ion in the gas phase (Chang et al. 2007). This model is known as the *Gas Phase Protonation Model*.

The '*Lucky Survivor'* model; the second model proposed for the first time by Karas et al. (2000), generally refers to proteins as it suggests that, due to their amphoteric nature, the analyte molecules are already charged when mixed with the matrix molecules. In this model, the sample (incorporated with the matrix) is ionised upon laser irradiation producing clusters of ions (including matrix, analyte, and counterions) from the analyte/matrix mix. It is proposed that within the ion plume, most ions are re-neutralised by their respective counterions, and it is only those ions that are not neutralised that are ultimately detected, hence the name '*Lucky Survivor'* (Karas et al. 2000 and Jaskolla et al. 2011). This theory offers some explanation as to why only singly charged species are detected via MALDI as they have the highest chance of 'surviving' (Karas and Kruger 2003; Jaskolla et al. 2011). In the refined '*Lucky Survivor'* model (Jaskolla et al. 2011) the same principles are used from the original model where the analyte molecules are pre-charged within the analyte/matrix mix. However, the difference with this

model is that the matrix molecules are incorporated within desorbed clusters as ionised species, contributing to the neutralisation process, and leading to the formation of singly charged analyte ions from incomplete neutralisation of multiply charged ions (Figure 4) (Jaskolla et al. 2011 and Awad et al. 2015).

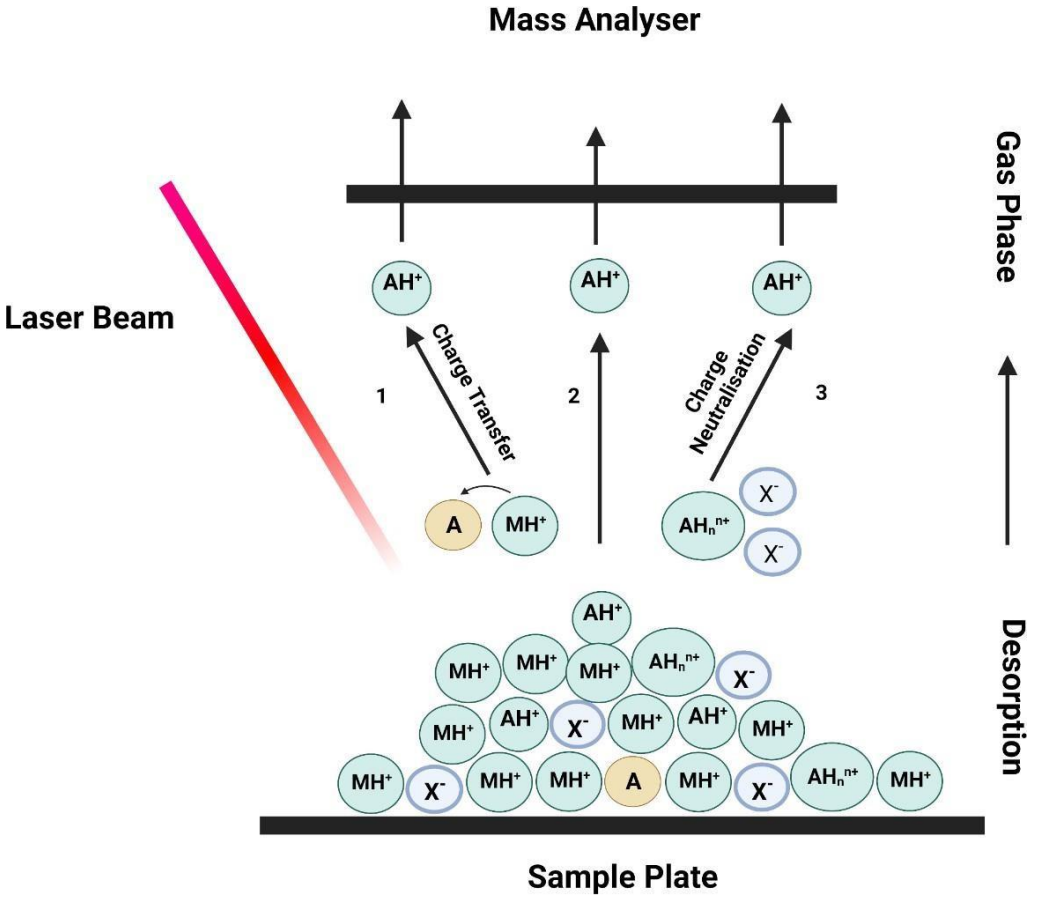

Figure 4: Two proposed ionisation theories (2nd in two parts) for MALDI MS in positive ion mode; 1) gas phase protonation model showing the charge transfer from the matrix ion ($MH^{+)}$ to the analyte (A); 2) lucky survivor model showing the desorption of a monoprotonated analyte ion (AH^+) that 'escaped' neutralisation in the plume; 3) lucky survivor model for multiply charged ions (AHn^{n+}) where incomplete neutralisation by the counterions (X^-) occurs within the gas phase to produce singly charged ions (AH^+) (A= analyte, M=matrix, X^-= counterion). Redrawn from Awad et al. (2015).

2.1.3 Instrumentation: Mass Analysers

Once ionisation of the sample has occurred, the ions are then sorted in the mass analyser based on their *m/z.* As all data presented in this thesis have been acquired on q-ToF mass spectrometers, only the two mass analysers (Time of Flight (ToF) and quadrupole mass filters (q)) will be discussed.

2.1.3.1 Time of Flight (ToF)

In a ToF analyser the *m/z* of an ion will dictate the time it takes for it to travel through a field free drift tube of known length; the higher the *m/z,* the longer it will take to reach the detector (Mamyrin 2001). A ToF can be operated in linear or reflectron mode, as Figure 5 illustrates. In linear mode, the ions travel in a straight line through the ToF and hit the detector at the end of the journey. In theory, a pure, single analyte sample, should produce a very narrow peak as ions of the same *m/z* should hit the detector at the same time. However, upon ionisation, the Boltzmann distribution of energy determines a spread of kinetic energy causing ions of the same *m/z* to reach

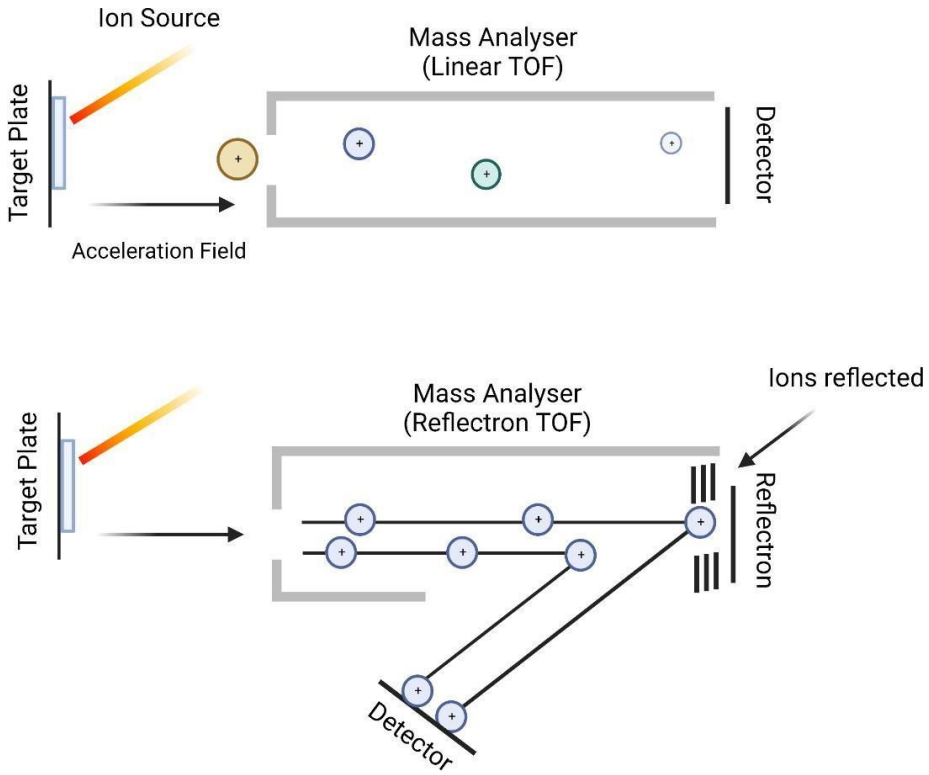

Figure 5: Schematic of linear and reflectron time of flight (TOF) mass analysers in positive ion mode. Reflectron mode is preferable for peptide analysis as the reflection of ions prior to reaching the detector means that differences in kinetic energy between ions is corrected before their detection.

the detector at different times. This circumstance explains why a linear ToF instrument is characterised by low mass resolution. A reflectron ToF can be used to correct the difference in kinetic energies and thus improve resolution and mass accuracy.

A reflectron ToF consists of a series of ring electrodes (mirrors). To each pair of mirrors, a voltage is applied. The voltage increases with the depth of the reflectron. Ions of the same polarity are repelled by this voltage and curve their trajectory hitting a second detector, extending the flight path. Ions with higher kinetic energy will penetrate the reflectron more so than ions with a lower kinetic energy, meaning that the differences in kinetic energy are corrected (amongst ions of the same *m/z*) thus explaining the improved resolution (Gross et al. 2006, Boesl et al. 2017). The reflectron detector is most suitably used when detecting species <3000 Da, as sensitivity decreases with higher molecular weight species analysed in this mode due to reflected ion trajectories deviating from the required flight path, meaning ions do not reach the detector (Fancher et al. 2000). Generally, TOF instruments operate at a mass resolution of 40,000 full width half maximum (FWHM: a means of measuring the mass resolving power required to observe the separation of peaks) (Waters, 2010).

In the state-of-the-art MS instrumentation, mass resolving powers of >1,000,000 at FWHM have been possible, with Bowman et al. (2020) reporting up to 1,600,000 using an FT-ICR system. In more recent instrumentation, Waters Corp. have reported >200,000 FWHM on the newly released Select Series Multi-Reflecting ToF (MRT) system (Towers et al. 2022), far greater than some of the older instrumentation such as the Waters G2 HDMS (used in the work illustrated in this thesis) and G2 Si which have a FWHM of 40,000 and 50,000 respectively.

2.1.3.2 Quadrupole (q)

A quadrupole filter mass analyser (which can be coupled with two more quadrupoles, or in a hybrid manner with a ToF, an Ion Trap, an IMS Cell or an Orbitrap) consists of four parallel conducting rods that have a circular or hyperbolic cross section (Mellon et al. 2003) and was first developed by Paul and Steinwedel (1953). Each pair of rods (of opposite polarity) is electrically connected by fixed direct current (DC) to which an alternating current (AC) is superimposed (Figure 6).

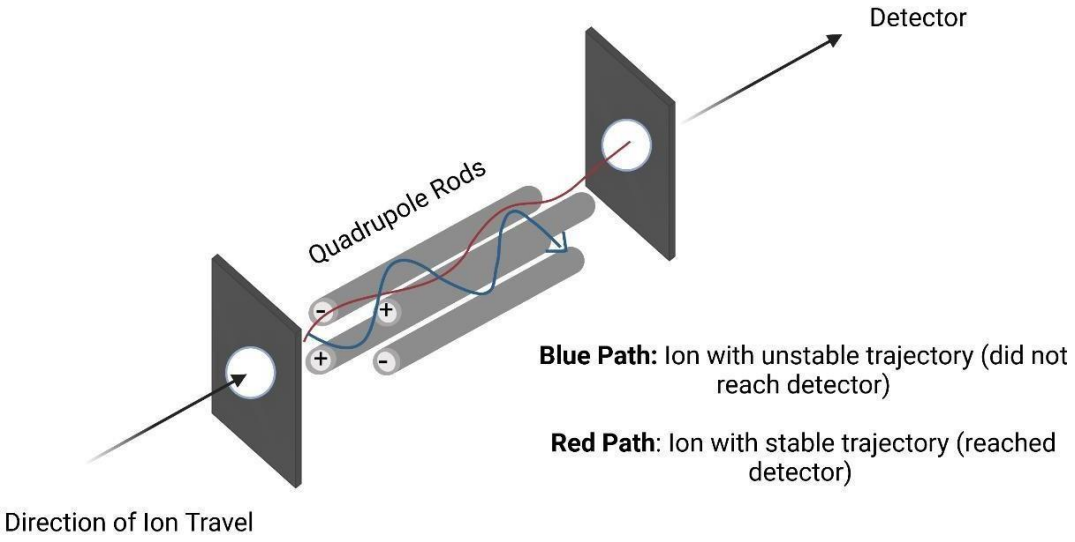

Figure 6: Schematic of quadrupole mass analyser.

The combination of these two voltages produces an oscillating electric field which dictates the oscillatory motion of the ion travelling through the quadrupole. The *m/z* of the ion will determine the stability of its trajectory through the quadrupole; if the ion *m/z* is off resonance with the radiofrequency applied, it will collide against the rods and will be undetected (Dawson et al. 2013). The quadrupole radiofrequency can be tuned to only allow an ion of a particular *m/z* to reach the detector ('selected ion monitoring' mode, generally abbreviated to SIM mode). This analyser can be operated in two different modes: "full scan" or in SIM. With the full scan mode, by changing the DC and RF voltages, the analyser allows ions of different *m/z* to travel through the quadrupole within a specified *m/z* range.

For the SIM mode, the quadrupole will be fixed at a set voltage meaning that only ions of a specific *m/z* reach the detector. This is a mode commonly used for tandem MS (MS/MS) experiments in triple quadrupole instruments where only ions of interest are selected in the first quadrupole prior to their fragmentation. Quadrupoles generally have low mass resolution (2000 at FWHM), an upper mass range limit of 4000 Da and a mass accuracy of 100 ppm hence why they are normally coupled with other mass analysers to improve mass resolution and accuracy (Hoffmann and Stroobant 2007).

2.1.3.3 Hybrid Mass Spectrometers: Quadrupole-Time of Flight (q-ToF)

The Synapt G2 HDMS instrument, which was used for most of the work shown in this thesis, has two main modes of operation: T-Mode (ToF) and M-Mode (Mobility ToF). Within ToF mode, there are three further modes: sensitivity mode ('V' optics), resolution mode ('V' optics) and high-resolution mode ('W' optics) with 'V' optics equating to reflectron mode and 'W' optics indicating double reflectron mode. For all the work carried out, sensitivity mode was used as this was found to be the most performing mode for peptide analysis in terms of mass accuracy and peak resolution, with the instrument having a mass resolving power of 10,000 at FWHM in positive sensitivity mode. In the Synapt configuration, the ToF analyser is set orthogonally to the axial path of the ions. The formed ions are transmitted into a 'pusher' region where packets of ions are removed, then accelerated into the orthogonal ToF by means of a pulsed voltage. The horizontal path of the ions followed by the orthogonal reflectron ToF analyser is what creates the 'V' optics ion trajectory (Hoffmann and Stroobant 2007).

The instrument houses a TriWave region (blue cell in Figure 7) where mobility separation occurs. The TriWave consists of three consecutive, gas filled, stacked ring ion guides to which confining RF and offset DC voltages are applied along with a continuous train of DC pulses (travelling waves) (Waters, 2010). In the Trap Ion Guide, the ions accumulate and are periodically released into the second ion guide (IMS, which has a low-pressure helium cell at the front to reduce fragmentation) where they are separated based on their cross-sectional area and *m/z* when moving through these travelling waves. Once the ions have been separated in the IMS cell, they are accelerated through the third ion guide

(Transfer Ion Guide) which maintains their separation prior entering the ToF region of the instrument.

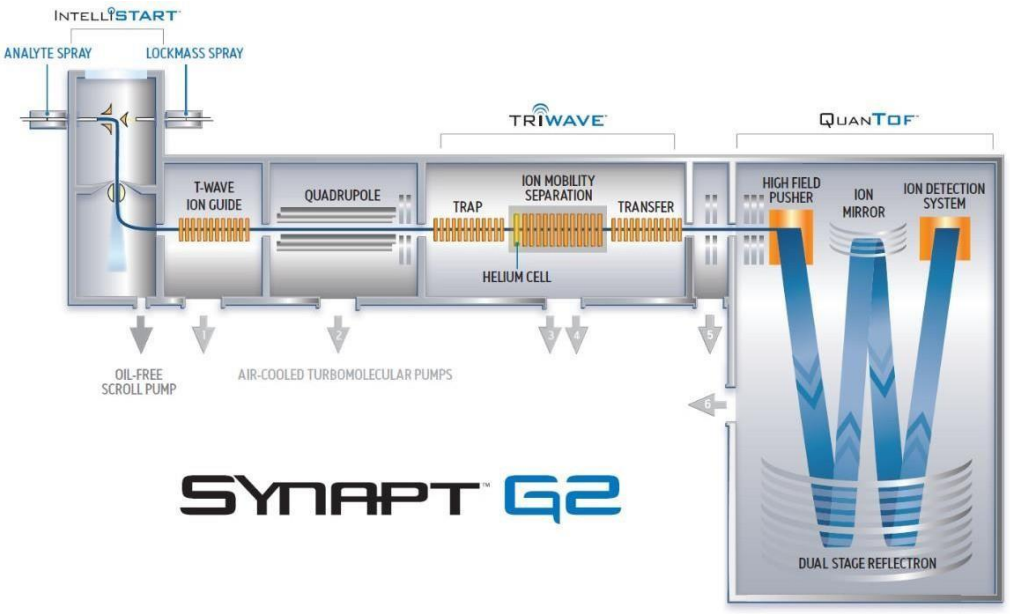

Figure 7: Schematic of the Synapt G2 HDMS instrument (Waters Corp, Manchester, UK)

2.1.4 MALDI MS profiling (MSP) and imaging (MSI) modes

Generally, MALDI MS profiling (MSP) is the modality in which MS spectra are collected at specific MS locations either in a manual or automatic fashion. It is generally performed using a welled steel target plate as illustrated in Figure 8. The analyte and matrix are mixed in solution and spotted directly onto the wells. The solution is then left to dry to allow for matrix-analyte co-crystallisation prior to the laser firing to induce ionisation.

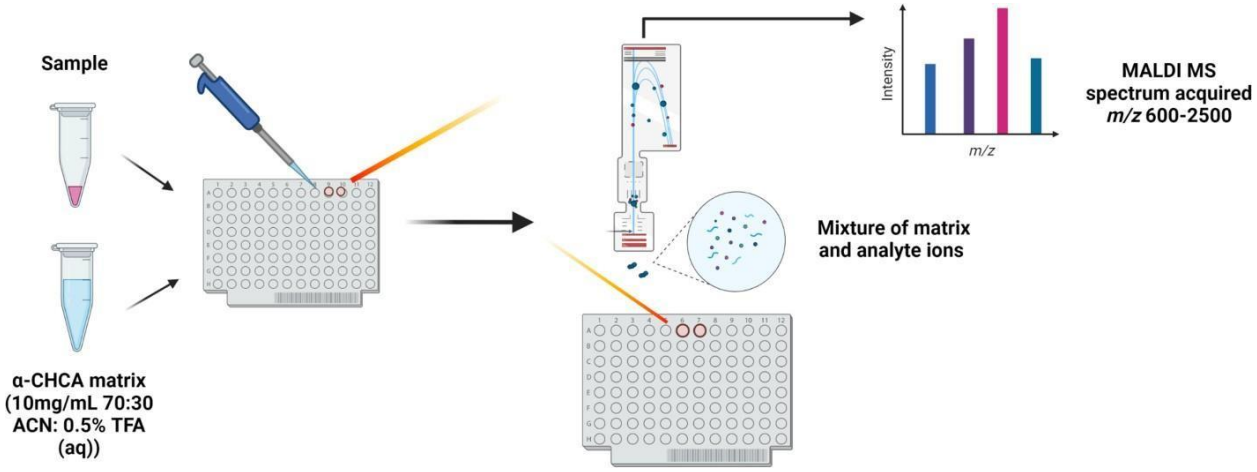

Figure 8: Schematic of classic MALDI MSP workflow on a q-ToF instrument (redrawn from Clark A. E. et al. 2013).

MALDI MS Imaging (MALDI MSI) was first reported by Spengler et al. (1994) but developed by Caprioli's group and first published in 1997 (Caprioli et al. 1997) and further demonstrated in the work published by Chaurand (Chaurand et al. 1999 and Chaurand et al. 2002), Stoeckli and colleagues (Stoeckli et al. 2001) and Rohner et al. (2005). In this modality, MALDI enables spatial information to be acquired *in situ* from intact samples (of both endogenous and exogenous species); this is especially important in biological samples as it provides the link between location and function. In forensics, namely fingermark analysis, it enables the link between the identity of a perpetrator (through the reconstruction of their fingerprint image) and the circumstances of the crime (through the molecules that have generated the image and that could be forensically relevant). In the MSI modality, the sample will require a homogenous coating of matrix which co-crystallises on the surface with the analyte. The MALDI MSI spectra are then acquired from the sample at predefined *x* and *y* coordinates, with each pixel generating a spectrum reflecting the molecular composition of the sample at that specific location upon irradiation from the laser (Figure 9). A mass spectrum is then generated which defines the molecular composition of the sample at known *x, y* coordinates (Francese et al. 2017). This is often referred to as the mass microprobe mode of MALDI MSI (Trim et al. 2012). The data that is acquired can then be analysed to observe the distribution of different molecular

species (through different *m/z* values) across an entire sample using various software tools in both 2D and 3D.

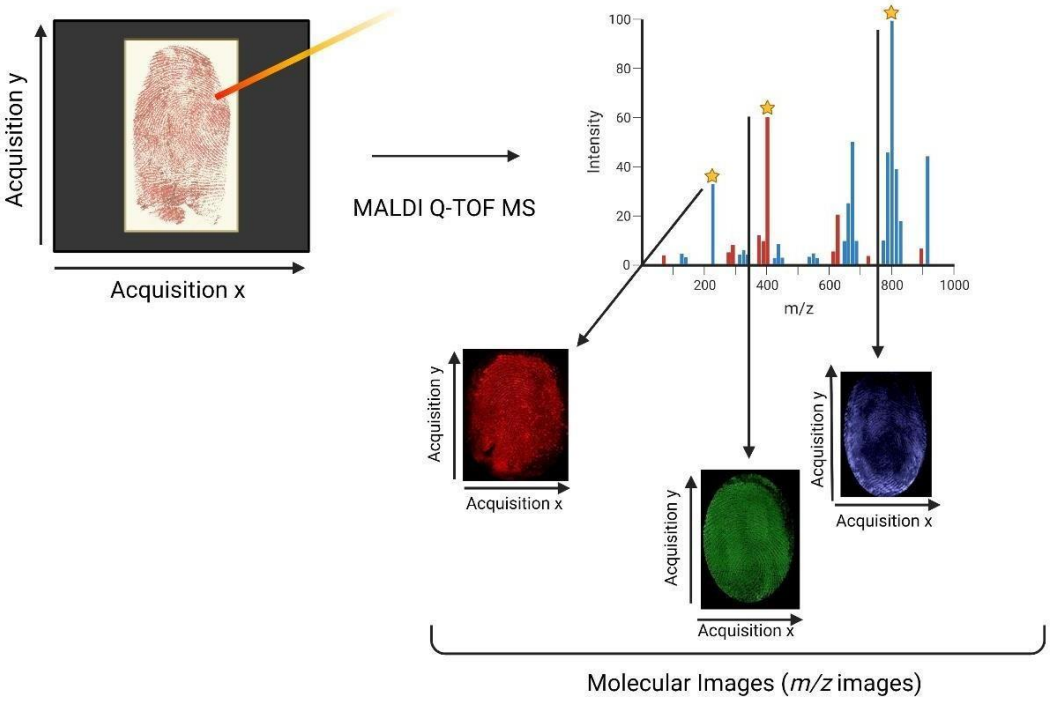

Figure 9: Schematic of MALDI MSI data acquisition process in relation to a blood fingermark.

Due to the high sensitivity that has been reported with MALDI MSI, species can be detected down to the low-femtomole and attomole concentrations (Caprioli et al. 1997; Cheng et al. 2010; Aichler et al. 2015 and Mesbah et al. 2017). Spatial resolution generally ranges from 10 µm to 150 µm for MALDI MSI, but more recent advances and instrumentation have shown capability to image between 1 µm (Zavalin et al. 2015 and Kompauer et al. 2017) and 5-10 µm with some more recently developed instrumentation from Bruker Daltonik (Bremen, Germany) and Waters Corp. (Manchester) (Scheffer, 2022) (Towers et al. 2022).

In the case of the work presented in this thesis, the distribution and mapping of the haem group and of blood specific peptide signatures was obtained using a combined bottom-up proteomic MALDI MSI approach (Appendices I and II).

2.2 Bottom-Up Proteomics

A proteome is defined as the 'complete set of proteins expressed by an organism' (Scitable, 2022). Proteomics is the large-scale study of proteomes and their different functions which can be performed through mass spectrometric analysis. As proteins are one of the main components found in biological evidence at crime scenes, they can be utilised to identify biofluids and convey genetic information from a sample (Parker et al. 2021). The different proteomic approaches, namely top-down and bottom-up (Figure 10) offer different ways in which to yield the proteomic profile of a sample based on its complexity and/or on the expected protein content.

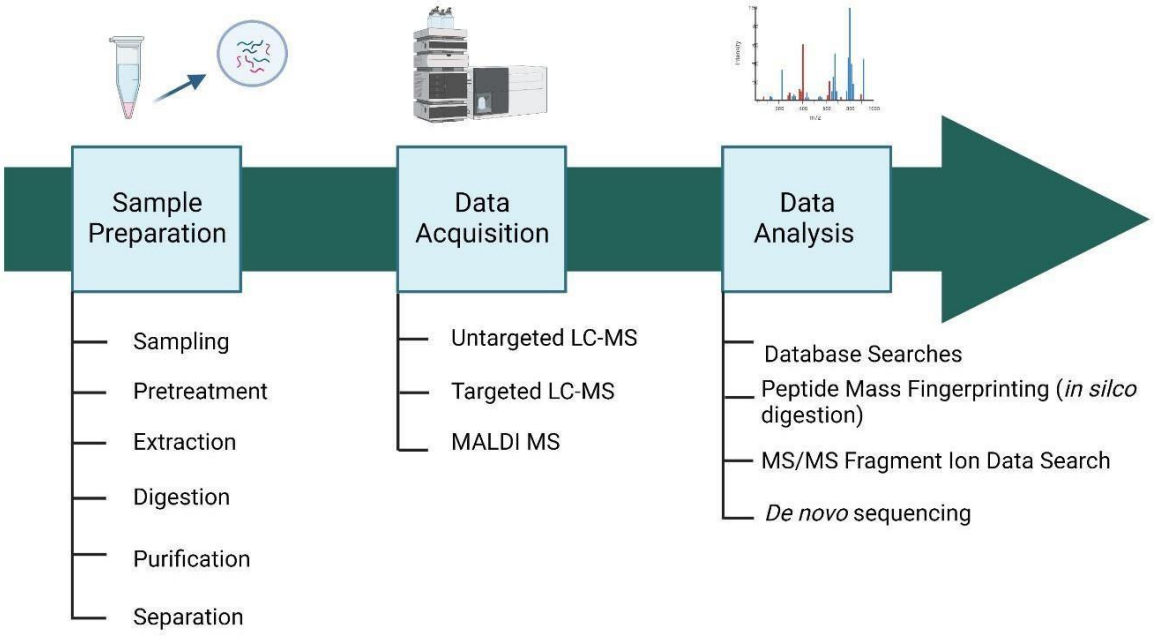

Figure 10: Bottom-up proteomics workflow (re-drawn and adapted from Duong et al. 2021).

There are several different proteolytic digestion methods, but the two that will be discussed in this section are a simplified in solution method and *in situ* (directly on the sample) with proteolysis times generally ranging from ~3-16 h (Merkley et al. 2019). For in solution digestion, reduction alkylation and steps are often reported prior to the addition of the trypsin, but for the work presented in this thesis, these steps were not undertaken in the attempt to obtain the minimum useful information with minimum effort and preparation time. Reduction breaks

the intra and intermolecular disulfide bonds (Ludwig et al. 2018) and alkylation prevents the sulfhydryl groups from reforming disulfide bonds. Whilst this sample preparation (often preceded by protein denaturation) yields many more peptides, it is very time consuming. Particularly in MALDI MSI, these procedures are never carried out due to the need to keep the specimen intact. Proteins are digested with an enzyme, normally trypsin as it is one of the most efficient due to its high peptide yield. Additionally, as peptide analysis is normally carried out in positive mode and as arginine and lysine are basic residues, the generated peptides are positively charged by at least one residue (arginine or lysine), with multiply charged peptides detectable through LC-MS analysis (Tyers and Mann 2003) which can be confirmed more easily through collision-induced dissociation (CID) tandem MS analysis (Dupree et al. 2020). Trypsin cleaves at the C-terminal site of the amino acids' arginine and lysine and peptides are usually detected within the mass range 800-2000 Da which excludes interfering signals from the matrix and many lipids as well as several trypsin autolysis signals. In the work presented in this thesis however, MALDI MSP acquisitions were made up to 2500 Da as some important signals were detected above 2000 Da, therefore an exclusion list of trypsin and matrix signals was made for the mass range of 600- 2500 Da for the work shown in Chapters 3,4 and 5.

After proteolysis, sample purification can also be performed by using C18 zip tips (reversed phase chromatography in a tip). This purification step generally increases the ion population detectable in MALDI as well as the relative abundance. This strategy was shown to be effective when applied to proteolysed blood samples prior to MS analysis (MALDI or LC-ESI MS) by Marshall et al. (2003) for the purposes of ascertaining whether the peptide profiles between healthy serum versus serum from myocardial infarction patients can be distinguished from one another. The effectiveness of utilising the C18 zip tips was also demonstrated in the work shown in Chapters 2 and 5 especially where in the case of the latter, the zip tip purification permitted for the detection of human blood deriving peptide signatures in a 1:20,000 dilution; signals that were not observed in the initial data acquired were the sample had not been initially purified via C18 zip tip.

2.2.1 Bottom-Up Proteomics combined with MALDI MSP and MALDI MSI: Sample Preparation and Data Acquisition Workflow

In the context of work presented in this thesis, in solution proteolysis (in combination with MALDI MSP analysis) was performed by using trypsin to digest proteins (present in blood or other biofluids) into peptides which were detected at high mass accuracy either by LC MS or MALDI MS and then confirmed by tandem mass spectrometry (MS/MS) (Figure 11). When MALDI MS is used in imaging mode, trypsin is routinely sprayed *in situ* onto the sample specimen, carefully with specific protocols devised that aim to avoid analyte migration (Figure 11).

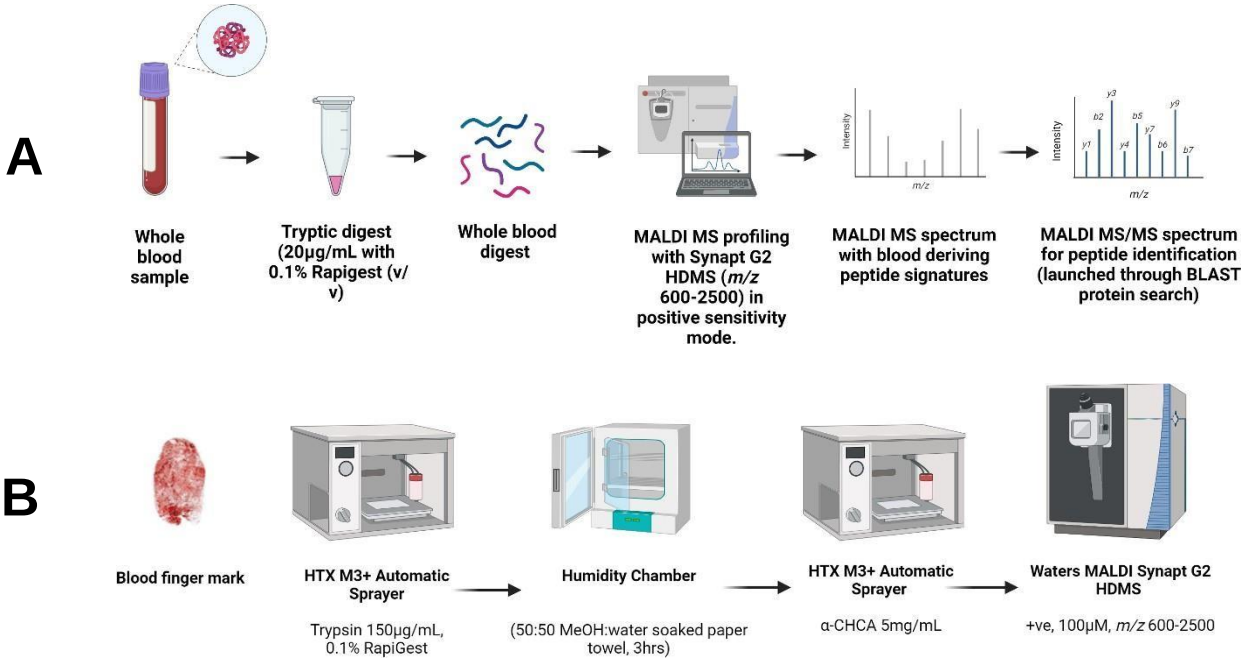

Figure 11: (A) Example of in solution proteolysis and MALDI MSP workflow; (B) Example workflow for MALDI MSI sample preparation using the HTX M3^{+} sprayer and imaging on the Waters MALDI Synapt G2 HDMS (deposition of enzyme, proteolysis, and matrix application).

The enzyme can be sprayed by different means, though in the work described in this thesis, two automatic sprayers were used, namely the SunCollect (SunChrom, Germany) or the HTX TM Sprayer (HTX Technologies, South Carolina). There are different types of HTX TM sprayer, and the data presented in this thesis were generated with either the HTX M3+ or SunCollect sprayers.

After proteolysis, the specimen is homogenously coated with the selected matrix taking care, once again to avoid analyte delocalisation. This practice usually requires optimisation of several parameters which vary according to the sprayer used, such as: number of layers, velocity and height of the nozzle, temperature, and nitrogen pressure.

Once the MALDI MS, MSMS and MSI data of peptides have been acquired, interpretation can be performed manually, in the case of a targeted analysis, or using search engines such as MASCOT (https://www.matrixscience.com) using *in silico* digestion tools. The process of identifying proteins from the peptides generated via bottom-up proteomics is named Peptide Mass Fingerprinting (PMF). Specifically, when using search engines, protein databases exist, databases such as Swiss Prot and NCBI (amongst other) are employed to search the peak lists generated from the spectra against all the protein sequences contained within, selecting parameters specific for the search of interest. Parameter settings includes in a non-exhaustive list: the enzyme that was used to cleave the protein, any known post translational modifications (PTM), taxonomy, mass tolerance, missed cleavages and ion charge state. The engine generates a list of potential protein candidates fitting the parameters set based on a probabilistic scoring system.

For both manual and automatic data interpretation, MALDI MSP spectra presented in this thesis have been processed using the open-source mass spectrometry software mMass (Strohalm et al. 2008 and Strohalm et al. 2010) by exporting raw spectra as .txt files and importing into the software for spectral processing and PMF searches.

MALDI MSI data presented in this thesis were processed using either BioMap (Novartis, Basel, Switzerland) or HDI (Waters Corp, Manchester). Using either software, peak lists could be generated from the MS image, image normalization performed, the overlaying of multiple *m/z* images and specific ROIs could be generated and exported so that spectra for specific regions of a molecular image can be viewed. In the case of MALDI MSI of blood fingermarks, normalization was not performed in every case. The main aim of the MSI experiments in the context of blood fingermark analysis was the visualisation of the ridge flow and of the *minutiae* present. It is the successful mapping of one *m/z* across the entirety

of a mark as opposed to localisation of multiple *m/z*'s on one mark (and comparisons in signal intensity) that is more pertinent.

2.2.2 Forensic Applications of Proteomics

Proteomics has been used in forensics for biofluid detection and discrimination as a more robust and specific alternative to the normally deployed methods of presumptive and confirmatory blood testing (Duong et al. 2021). When compared to the immunoassays available for biofluid identification, the higher specificity, selectivity, reduced cost, and analysis time of a proteomic approach appears to be more suitable in a forensic context and more able to withstand court scrutiny. The adoption of a proteomic approach via subsequent LC-MS and MS/MS analysis for biofluid identification is the gold standard for proteomic analysis as it offers the most informative/rapid/high throughput approach. When applied in forensics, as stated in Wilke et al. (2021), the detection of proteins can reveal specific tissues or body fluids, which in turn can provide further context around a case, especially in the case of sexual assaults.

Van Steendam et al. (2012) investigated in depth the use of LC-ESI q-ToF MS for the identification of different biological fluids and whether this approach is compatible with subsequent DNA profiling. The different biofluid samples (blood, semen, saliva, vaginal fluid, nasal secretions, and faeces) were swabbed and digested with trypsin to yield characteristic peptide signatures for each biofluid. Protein markers were identified for all biofluids, validated through a blind sample set, and differentiation between human and non-human biofluids was also possible through this approach, as was DNA profiling using the extract from the first wash step.

A three staged comparative strategy adopting a proteomic approach was proposed in the work carried out by Legg et al. (2014) using proteome fractionation and subsequent HPLC-MS analysis for the discovery of several unique protein biomarkers as specific indication of a range of biofluids. The use of a proteomic approach serves as a considerably more specific alternative to the currently used tests as specific markers have been identified; a feature that the current presumptive and confirmatory tests do not have. A follow-up study by Legg et al. (2017) performed verification of 23 protein biomarkers for a selection

of biofluids (peripheral blood, vaginal/menstrual blood, semen, urine, and saliva) in 50 human samples using a q-ToF mass spectrometer.
Illiano et al. (2018) reported the use of multiple reaction monitoring (MRM) using LC-MS for the profiling of different biofluids to yield unique peptide fingerprints for each body fluid type. The results indicated that, through the monitoring of four specific biomarkers for blood, identification is possible when blood is detected on its own or when mixed with another biofluid (50:50).

In more recent work, McKiernan et al. (2021) endeavoured to validate amultiplex proteomic assay for the detection of biofluid-specific peptide fragments to distinguish between five biofluids in both pure and mixed stains. The results of the validation studies indicated only a small number of samples could not be identified (e.g., semen when mixed with lubricants). An MS approach supersedes the current methods in place for biofluid detection in many respects, but these results also emphasise the need for high sensitivity instrumentation. A reality in forensic scenarios would be the potential mixing of biofluids with other substances so the need for high sensitivity is imperative to improve the likelihood of detecting biomarkers even in situations where samples are contaminated or of low concentration.

Wild et al. (2001) also demonstrated the ability to detect 99 haemoglobin variants across 250 blood samples (via detection of α and β Hb chains and hybrid Hb's) using a direct infusion-ESI-MS approach, with 15 of the variants discovered being novel. In this study, most Hb variants were detectable within 24h (as some tryptic digests require 12-15h) with a 10 µL sample volume used. In the case of variants of low abundance, an additional purification step was required which extends the analysis time by another 24h. In a forensic context this is rather time consuming and ideally, a technique that is more rapid would be preferable.

More recent work was published on the detection of Hb variants using a bottom-up proteomic approach coupled with MALDI MS (Heaton et al. 2021). This study showed that only one or two proteotypic peptides from the selected variants were sufficient to detect and map them in blood fingermarks via an MSI approach, offering a more rapid alternative to the ESI approach (Wild et al. 2001).

Table 4 reports additional MS studies, mostly LC-MS, conducted with a view of investigating the respective proteome for other biofluids (in varying circumstances) and the different instrumentation used.

Biofluid	Mass Spectrometer	Reference
Vaginal Fluid	LC-ESI-ToF-MS	Igoh et al. 2015
	Nano-LC-MS/MS	Klein et al. 2008
Saliva	LC-Q-Orbitrap MS	Grassl et al. 2016
	Nano-UPLC-MS/MS	Murr et al. 2017
Menstrual Blood	Nano-HPLC-MS, MALDI-ToF-ToF-MS	Yang et al. 2012
Semen	MALDI-ToF-MS or LC-MS/MS	Fung et al. 2004
	LC-MS/MS	Pilch et al. 2006
Sweat	LC-MS/MS (Orbitrap)	Yu et al. 2017
Urine	LC-MS/MS	Zhao et al. 2018

Table 4: Additional studies reporting the use of LC-MS and MALDI-MS for exploration of the proteome for different biological fluids.

2.2.2.1 MALDI MS combined with Proteomics: Forensic Applications

As MALDI has been the primary technique used in this thesis, it is important to assess the current state of the field regarding its development within forensic proteomics.

Several studies have been carried out assessing the potential of MALDI for forensic use, namely the specific detection of blood and other biofluids. Jiang et al. (2019) developed a mass spectrometric method to distinguish between biofluids (blood, semen, sweat, urine, and saliva) using MALDI, and detected several small molecules that could be implemented as discriminatory markers for different biofluids. PCA analysis permitted for 75% clustering of all the biofluid

types investigated, and thus can effectively discriminate between five different biofluid types, with total sample preparation and analysis time coming in at under ten minutes. Similarly, Yang et al. (2013) utilised HPLC and MALDI MS to detect multiple protein markers that are unique to a range of different biofluids (blood, semen, and saliva). Bradshaw et al. (2014) demonstrated the use of MALDI MS and MSI for the specific detection of blood in fingermarks, through the detection and mapping of the haem group and intact Hbα and Hbβ chains. Bradshaw et al. (2014) has also shown MALDI MS to be compatible with AB-1 enhanced blood fingermarks; a dye that is routinely used in forensic casework for enhancing blood fingermarks. This again corroborates the findings from similar studies that have, in part, assessed the compatibility of MALDI MS with blood enhancement techniques (further demonstrated in Chapters 2 and 3).

In addition to the development of a bottom-up proteomic approach for the rapid and confirmatory detection of blood, Patel et al. (2015) reported a shotgun proteomic approach employing the use of hydrophobin, a cysteine-rich fungal protein that can form a hydrophobic coating on the surface of the target plates used for MALDI profiling and thus, adapted to immobilise the trypsin. Despite the conventional bottom-up approach yielding additional blood deriving peptide signatures, the Hydrophobin approach was a far more rapid method, with total sample preparation time of only 5 minutes.

Deininger et al. (2016) showed the potential of MALDI MS for specific blood detection and the mapping of blood deriving peptides directly onto the identifying ridges of blood fingermarks using a bottom-up *in situ* proteomic approach. The thesis from Deininger et al. (2017) also provided indications of the multi-informative nature of adopting a combined bottom-up MALDI MS approach for the detection of blood, with initial validation studies conducted on distinguishing between human and animal blood; a body of work that was significantly taken over and carried out successfully within this thesis (Chapter 2).

In terms of blood provenance, the research conducted to date has shown it is possible to differentiate between human and non-human blood samples across some species via MALDI (Patel et al. 2015; Kamanna et al. 2017; Deininger et

al. 2017 and Witt et al. 2021). However, little work has been carried out investigating the specific differences between species peptide signatures and the subsequent marker identifications that would be required to specifically differentiate animal species via MALDI MS. Kamanna et al. (2017) reported on the differentiation of human and non-human haemoglobins using MALDI ToF-MS with both 'bottom- up' and 'top-down' approach. Intact haemoglobin from human blood and the blood from several Australian native mammals were investigated in this study. Kamanna et al. (2017) detailed the challenges of attempting species determination using a 'top-down' (intact) approach, which is most likely due to the decrease in mass resolving power and sensitivity when attempting to detect proteins rather than peptides. Indeed, adopting an *in-situ* bottom-up proteomic approach coupled with tandem MS permitted for the detection of Hb in blood from humans and a range of Australian native animals through the detection of the respective Hb deriving peptides. The MS signals detected from the intact Hb's however showed significant variance between the measured and theoretical *m/z*'s when using a linear ToF, indicating a bottom-up approach is preferable. In this work, MALDI MSI experiments were conducted on human haemoglobin, and imaging was compatible with the prior enhancement of the bloodied fingermarks with AB-1 and magnetic powder.

Kamanna et al. (2018**)** also reported on the viability of successfully performing MALDI MS analysis on fibres plucked from a micro swab used to collect vaginal fluid and subsequently perform PCR on the remaining fibres for DNA analysis. Performing PCR prior to MALDI analysis was also successful. A dual complementary approach from a single swab was developed where proteomics-based approaches followed by MALDI would assist in understanding the circumstances of the crime and DNA analysis would provide human identification. It is evident that there is clear scope to investigate whether *in situ* analysis from the same sample permits for the application of subsequent DNA analysis through PCR at each sample preparation and analysis step within the MALDI workflow. However, insights were provided by Kamanna et al. (2018) in that haemoglobin could no longer be detected from the purified DNA extract when classic DNA profiling was performed, due to the procedure removing trace proteins. The work

presented in Chapter 3 illustrates the compatibility of MALDI MSI of enhanced blood fingermarks (NIN and AB-1 treated) with subsequent DNA profiling. The data in this study are extremely important as they indicate that, under those conditions, the implementation of MALDI would not hinder the DNA analysis which would routinely be performed after BET treatment or presumptive blood testing.

2.3 Method Validation, Accreditation and Future Perspectives

A vital consideration in the field of forensic proteomics is the rigor and care that must be taken when analysing evidence from a crime scene. The difference between proteomics, versus forensic proteomics is the potential presentation of findings in a court of law. The chain of custody, handling of evidence and tests/analysis performed must be robust and well documented within the scientific community. Any results yielded must be able to stand in a court of law, to avoid inadmissibility of the evidence. As such, any technique used to analyse forensic biofluids must also be supported by a significant body of peer-reviewed reports and have undergone validation and pseudo-operational trials to assess its robustness, specificity, sensitivity, and compatibility with current operational workflows. As much as it is important to understand the capabilities of a method, it is also important to know where the limitations or pitfalls lie; all of which should be shared within the forensic community as Jarman et al. (2019) also report. As reiterated in Koen et al. (2017), issues with presumptive blood tests are well known and as such can have a negative impact if forensic inference on the facts is based on those results only.

As Duong et al. (2021) outlined, an initial proof of concept must be undertaken (protocol development, determining sensitivity, specificity, and robustness testing to inform on where the caveats with the technique may lie). Verification and validation experiments will then be performed, with operational trials tailored to the requirements of the end-user to elucidate operational capabilities and limitations with the method. Only when SOPs and end-user guidelines have been outlined based on these findings, and a technique has scope for being widely deployed for forensic use, the developing laboratory may decide to undergo International Organisation for Standardisation (ISO) accreditation. Conversely, for techniques that are not widely used, a laboratory may find very

challenging to achieve an accredited status. A technique must undergo compatibility testing in multiple laboratories from multiple locations to collate enough outputs in the scientific community to support its submission for accreditation (Nicora et al. 2019).

In the case of validating a bottom-up proteomic MALDI MS method for the detection of blood at crime scenes, the data presented in this thesis amount to a considerable body of new knowledge of the capabilities of this approach, and where some of the limitations lie. Further verification and validation are nevertheless required to a) increase applicability of MALDI analysis of blood evidence and b) assist in taking it over the 'frequently used test' threshold; an assessment used to decide whether a technique is utilised enough to justify the application for a laboratory to undergo accreditation. This process takes years and a considerable amount of testing. Nevertheless, the data shown in this thesis help take some of those much-needed steps on the path to wider deployment of this technique through the generation of new knowledge to better inform the forensic community on the suitability of this technique for different forensic applications.

2.4 Project Aims and Objectives

This PhD project was fully funded by Defence Science and Technology Laboratory (Dstl) and aimed to further refine and test the developed MALDI MS method for the reliable and rapid detection and mapping of blood for operational deployment. The main aims of the project are outlined as follows:

***1) Development of a strategy for the detection and discrimination of human blood and animal blood through a blind validation study*:** The first aim of this project was to devise a strategy for the detection of human, bovine, porcine and chicken blood through the identification of unique markers for each species from a blind sample study using a combined approach of bottom-up proteomics and MALDI MSP analysis (Chapter 2). From this initial blind sample study, there was clear scope to delve further into the strand of blood provenance. Markers were subsequently identified to distinguish between intravenous animal blood and packaged meat animal blood with the aid of multivariate statistical analysis.

***2) Testing the devised bottom-up proteomic approach against commonly encountered BET, deposition surfaces, presumptive tests and in the co-presence of other biofluids*:** Across three chapters in this thesis (2,3 and 5), several variables have been tested against the developed MALDI MS approach that must be considered and tested to better understand the most appropriate circumstances in which MALDI can be implemented for the detection of blood. One of the core aims of the project was to assess whether the prior application of common BET's, presumptive tests and deposition surfaces did not interfere with the retrieval of a positive blood result from the MALDI MS analysis (Chapters 2,3 and 5).

***3) Testing DNA typing post MALDI MSI analysis*:** This work, illustrated in Chapter 3 was designed and conducted in collaboration with the Israel Police. An important consideration when intending to incorporate a new technique into a forensic workflow is to ensure it does not hinder the application of subsequent analysis. Accordingly, the use of MALDI MSI (for the mapping of haem and blood peptide signatures) in tandem with DNA typing required testing to assess whether MALDI prevents successful DNA extraction and profiling.

4) Increase the body of knowledge around capabilities and limitations of MALDI MS for human blood detection to better inform forensic providers and end-users on this approach: For any technique to be widely deployed amongst forensic providers, there must be a considerable body of knowledge available to the forensic community to understand the capabilities and limitations of said technique. The publication of these findings is important to inform the end-users and judicial debates. This work will eventually contribute to the necessary evidence required for validation of this technique and promotion of it to a higher category in the Fingermark Visualisation Manual (Home Office, UK).

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Chapter 2

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Pre-validation of a MALDI MS proteomics-based method for the reliable detection of blood and blood provenance

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The reliable identification of blood, as well as the determination of its origin (human or animal) is of great importance in a forensic investigation. Whilst presumptive tests are rapid and deployed in situ, their very nature requires confirmatory tests to be performed remotely. However, only serological tests can determine blood provenance. The present study improves on a previously devised Matrix Assisted Laser Desorption Ionisation Mass Spectrometry (MALDI MS)—proteomics based method for the reliable detection of blood by enabling the determination of blood provenance. The overall protocol was developed to be more specific than presumptive tests and faster/easier than the gold standard liquid chromatography (LC) MS/MS analysis. This is considered a pre-validation study that has investigated stains and fingermarks made in blood, other biofluids and substances that can elicit a false-positive response to colorimetric or presumptive tests, in a blind fashion. Stains and marks were either untreated or enhanced with a range of presumptive tests. Human and animal blood were correctly discriminated from other biofluids and non-biofluid related matrices; animal species determination was also possible within the system investigated. The procedure is compatible with the prior application of presumptive tests. The refined strategy resulting from iterative improvements through a trial and error study of 56 samples was applied to a final set of 13 blind samples. This final study yielded 12/13 correct identifications with the 13th sample being correctly identified as animal blood but with no species attribution. This body of work will contribute towards the validation of MALDI MS based methods and deployment in violent crimes involving bloodshed.

The reliable detection of bloodstains at the scene of violent crimes is of crucial importance to both reconstruct the dynamics of the crime and to provide, if present, associative evidence. Particularly the presence of blood in fingermarks yields such associative evidence.

Crime scene investigators and forensic laboratories employ a range of blood enhancement techniques (BET) applicable to either stains or fingermarks. As previously reported[1,2], these methods may be prone to false positives (and are therefore considered presumptive). This is due to the relatively unspecific nature of their molecular targets.

The most commonly known false positive example is the reaction of luminol (haem reactive test) to bleach. A less reported example is represented by the false positive reaction of acid dyes to biofluids other than blood, such as semen and saliva. In this respect, the lack of specificity in the identification of blood is worsened by the risk of not detecting other types of biofluids which are important indicators of the crime being committed. The detection of other biofluids would permit a comprehensive reconstruction of the events. For example, in a homicide crime scene, the presence of semen on the victim, or around a body may indicate that some level of sexual activity has also occurred (from consensual intercourse to rape).

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Other important aspects to consider are sequential workflows to gather a body of intelligence from a single evidential source. Blood tests such as the presumptive haem specific Luminol, BlueStar and confirmatory tests such as the Takayama Test, have been shown to result in DNA degradation at 30 days post application; this may be an issue for cases when further DNA analysis is required[3] but the analysis is not conducted immediately after collection. DNA provides a profile of the individual to whom the blood belongs to (if blood is present). It can relay extremely valuable information to both an investigation and judicial debates but does not provide information on the nature of the biofluid that it may be found within. As such, even if the identity of the perpetrator can be retrieved, the detection of blood may be missed and thus, valuable information may still be lacking around the nature and dynamics of the crime. Due to the limitations of the presumptive tests and DNA techniques, it becomes very important to have validated techniques characterised by high specificity in the detection of blood (and better still of other biofluids also) that, ideally, do not interfere with the subsequent process of DNA sampling and analysis.

Deininger et al. reviewed spectroscopic techniques for blood detection including Raman and hyperspectral imaging (HSI)[2]. Despite these techniques showing great promise for blood detection and distribution mapping, they are not be suitable when blood is recovered from red and dark substrates. Furthermore the need to acquire a reference spectrum at a crime scene without any blood would be challenging, as blood contaminated stains or fingermarks can often be latent at crime scenes.

Mass spectrometry (in different forms) has the advantage of being much more specific compared to spectroscopic methods as it detects the compound of interest by measuring the specific mass-to-charge (m/z) ratio; the compound identity can also be confirmed by tandem mass spectrometry experiments. The gold standard for detecting protein biomarkers such as those specific to blood is still currently mass spectrometry-based proteomics through liquid chromatography (LC) hyphenated with Tandem mass spectrometry (LC MS/MS). This is due to both the superior number of protein species detected and the confirmatory value of MS/MS experiments (which can be conducted on single and multiply charged ions). Iliano et al.[4] reported on the use of LC MS/MS to discriminate different biofluids, by identifying several semen, saliva and blood specific markers. In particular, blood specific proteins detected in the samples analysed included haemoglobin (HB), hemopexin and haptoglobin.

However, LC MS/MS remains rather time consuming from both a sample preparation point of view and data acquisition. In biomarker discovery studies, the benefits outweigh the downsides, due to the necessity to map the entire proteome and much more relaxed time constraints.

Nonetheless, one should carefully evaluate the analytical context and the type of information sought for the selection of the analytical technique to be used. In the context of forensic identification of blood and biofluids, a technique that yields only a handful of blood/biofluid biomarkers, but that is faster and more user friendly, may potentially be more suitable than LC MS/MS.

MALDI MS could represent one such alternative technique. This hypothesis is supported by the work of Yang et al.[5] in 2013 who devised a MALDI MS and MS/MS method for the differentiation of blood and other bodily fluids. However the method also included prior chromatographic separation, making the whole procedure possibly even more laborious. Jiang et al. uniquely used MALDI FT ICR MS followed by multivariate statistical analysis to differentiate blood and other biofluids[6]. In the study by Kamanna et al.[7] published in 2017, MALDI MS was employed to detect blood and vaginal fluid biomarkers with or without enhancement using a BET. Blood was confirmed by the presence of haem and HB most abundant peptides (as well as intact HB analysis). Bradshaw et al.[8] demonstrated in 2014 that MALDI MS Imaging (MALDI MSI) was successful in detecting intact haem and HB visualising them in blood marks with and without prior enhancement. Patel et al. and Deininger et al.[2,9] further investigated blood detection in human blood stains and blood fingermarks using a MALDI MS and MALDI MSI based proteomic approach. Within both of these studies, the most abundant and specific blood proteins such as haemoglobin α (αHB) and β (βHB), hemopexin, serotransferrin, complement C3, alpha-1 antitrypsin, apolipoprotein A1, alpha-2-macroglobulin, erythrocyte membrane protein band (EPB) 3 and 4.2 were detected, with many of these proteins being highly specific to blood and with EPB 3 being specifically found in the human red blood cell membranes.

The inclusion of multiple blood-specific proteins is a more specific approach compared to those previously described as, although HB is specific to blood, it may be found as a trace contaminant in other biofluids. Furthermore, Bradshaw et al.[8] Patel et al.[9] and Kamanna et al.[10] demonstrated the possibility to determine blood provenance which can be valuable in an investigation.

Taken together these studies indicate MALDI MS Profiling (MALDI MSP) and MALDI MS Imaging (MALDI MSI) based proteomic approaches as a viable and quicker alternative to LC MS/MS methods and as confirmatory tests for both blood detection and provenance. However, despite the potential that MALDI MS has shown for its operational use in blood detection and provenance, no method validation has been conducted, hence the operational implementation both for live and cold cases is somewhat hindered.

In the present study, the authors report on a "pre-validation" study where the methods developed by Patel et al. and Deininger et al.[2,9] (in which the reduction and alkylation step were removed) are improved and applied to a variety of stains and fingermarks. These samples were deposited on aluminium slides and included blood, other biofluids (human semen, saliva and sweat) and non-biofluids related matrices (ketchup, egg, yolk, body lotion, steak sauce, beetroot juice). All samples were prepared unenhanced or enhanced using three BET namely Acid Yellow-7 (AY-7), Leucocrystal Violet (LCV) and Acid Black 1 (AB-1).

With respect to blood samples, these were either human or animal (porcine (both domesticated and wild boar), bovine and chicken) and in the case of human samples, some contained Ethylenediaminetetraacetic Acid (EDTA) as an example of anti-coagulant. Crucially, all of the samples were prepared blind to the analysts in order to validate the overall method whilst eliminating any possible interpretative bias. The identity of the individual samples was only disclosed once the data were processed and the analyst made the "identity claim".

An iterative process has been used to "learn" the most efficient way to process the data for the subsequent determination of the nature of the sample "at glance" in a final set of 13 pre-validation samples. Whilst MS/MS has been undertaken to facilitate strategy development, the 13 final pre-validation samples were correctly identified for the presence of human and animal blood, as well as for the presence of semen, without performing MS/MS experiments, thus enabling a quick screening method. This pre-validation work has advised and informed on the best approach for further refinement and optimisation of the study design and methods so that full validation of this MALDI-based approach will be feasible in the near future.

Materials and methods

All experimental protocols were approved by Sheffield Hallam University (HWB-BRERG23-13-14 and ER13034924) and performed in accordance with relevant guidelines and regulations. An informed consent was obtained from the donor of the human samples prior to sample donation.

Materials. Trifluoroacetic acid (TFA), α-cyano-4-hydroxycinnamic acid (α-CHCA) and Millipore ZipTips containing C18 stationary phase and TLC sheets were purchased from Sigma Aldrich (Poole, UK). Acetonitrile (ACN) and formic acid were purchased from Fisher Scientific (Loughborough, UK). Sequencing grade modified lyophilized Trypsin was obtained 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). Intravenous blood samples (bovine, chicken and porcine, 1 mL defibrinated and 1 mL with EDTA for each animal) were purchased from TCS Biosciences (Buckingham, UK). All blind stain and fingermark samples were donated by Elite Forensic Services (Minnesota, USA) and used under the Ethics Applications (HWB-BRERG23-13-14 and ER13034924) granted by Sheffield Hallam University. Samples included human and animal blood (bovine, porcine, chicken, and wild boar) human biofluids (semen, saliva and sweat), non-blood/biofluid related matrices (beetroot juice, ketchup, egg white, steak sauce). Human blood, biofluids and fingermarks were donated by 1 male donor at one collection time. Samples were presented both as non-enhanced and enhanced using blood enhancement techniques such as Acid Yellow 7, Acid Black 1 and Leucocrystal Violet and were prepared on TLC aluminium slides. They were subsequently packaged in glass slide containers, kept at room temperature prior to shipping via priority mail to the research group at Sheffield Hallam University.

Instruments and instrumental conditions. All mass spectrometric analyses were carried out using a Waters MALDI-QTOF Synapt G2 *Si* instrument (Waters Corporation, Manchester, UK). Data acquisition was performed within the *m/z* range 600–2000 Th in positive reflectron mode. The MALDI QTOF G2 *Si* instrument is supplied with a repetition rate Nd: YAG laser which was set to 1 kHz for these experiments. A 0.5 µL spot of saturated phosphorus red solution in ACN was used as the internal calibrant in the *m/z* range 600–2500 Th for each sample by acquiring a spectrum in the same acquisition instance as the sample. MALDI MS/MS spectra were obtained using argon as the collision gas; the trap collision energy was set at 100, laser power 300 and low mass resolution at 14.6.

LC MS/MS analyses were performed on a Xevo G2-XS QTOF (Waters Corp, Manchester, UK) equipped with an Acquity UPLC system (Waters Corp, Manchester, UK). For UPLC analysis, the injection volume was 5 µL; mobile phase **A** consisted of 0.1% formic acid in deionised water and mobile phase **B** of 0.1% formic acid in Acetonitrile (HPLC grade). Samples were run on an Acquity Ultra Performance Liquid Chromatography (UPLC) HSS T3 100 Å, 1.8 µm, 2.1 × 100 mm column at a flow rate of 0.2 mL/min starting with 3% B. The mobile phase went from 3% B to 95% B in 49 min and ramped to 98% B in the subsequent minute and was kept constant for 2 min. The mobile phase returned to 3% B in 4 min for an overall run duration of 56 min. Column temperature was set to 45 °C. MS/MS analyses were conducted in data dependent scan mode within a *m/z* range 100–1800 Th. The cone voltage was set to 40 V and Collision voltage was set to 30 V for all samples.

Data processing. UniProt (https://www.uniprot.org/) was used to search for protein sequences of interest. In silico proteolysis with trypsin was performed by using the tool "peptide mass". In silico peptide lists were generated by selecting "monoisotopic", "MH^+", "2 missed cleavages" and "variable methionine oxidation" in the *m/z* range 600–2000 Th. Microsoft Excel tables were generated reporting the theoretical *m/z* and the sequence of peptides generated from (amongst other) haemoglobin (HB, α and β chains), erythrocyte membrane protein band 4, haptoglobin (Hpt), apolipoprotein, myoglobin, glycophorin A and albumin. Theoretical *m/z* values for the above peptides were reported for both human and the animal species investigated. Macros were generated to rapidly highlight proteotypic peptides present in the spectral peak list and those peptides identical in sequence, shared amongst the multiple species under investigation. Blood proteins were investigated from human, chicken, porcine, bovine and wild boar. UniProt does not make clear whether *sus scrofa* as taxonomy refers to the wild boar or domestic pig. Proteins from both these animals were all identified as deriving from *sus scrofa*, therefore a distinction between domestic pig and wild boar could not be made. Mass spectra were viewed both in MassLynx, (Waters Corporation, Manchester, UK) and in mMass, an open source multifunctional mass spectrometry software[11,12], upon conversion of the spectra into .txt files. Firstly, peak centroiding was carried out in MassLynx. Spectra were then exported in mMass and only the peaks with S/N of 10 or above were labelled. Mass lists including known matrix (or matrix cluster, adduct) and trypsin autolysis *m/z* peaks were generated and used in mMass to exclude from the peak labelling irrelevant *m/z* signals. The spectral mass lists were then searched against the Excel tables. Peak assignment was automatically performed using an Excel macro. A peptide match was confirmed in mMass within a mass accuracy of 30 ppm. These signals were then ultimately checked in MassLynx and confirmed if within a relative error of 15 ppm.

MS/MS spectra opened in MassLynx were converted in .txt files and viewed in mMass for smoothing and peak labelling prior to launching an automatic Mascot MS/MS search from within the software. As search parameters, *Chordata* or *mammalia* were chosen as taxonomy when in the suspected presence of chicken (*chordata*), bovine and porcine (*mammalia*) blood respectively. Mass Tolerance was set at 40 ppm for the precursor ion and as 60 ppm for the ion fragments. MH^+ and monoisotopic ions were selected and 2 missed cleavages and variable methionine oxidation were included in the *y*, *b*, *a* and *c* ion fragment search.

Methods. *Extraction, and proteolytic digestion and purification of blind and reference samples.* The trypsin Gold and RapiGest solution was prepared just before proteolysis. RapiGest (0.1% v/v in 50 mM ammonium bicarbonate solution) was added to Trypsin Gold to reconstitute it in a 150 µg/mL solution. Stains and marks were extracted and digested by adapting a previously published method by Patel et al.[9]. Each blind stain/fingermark sample was swabbed with 70% ACN: H_2O. The swab head was removed using scissors and transferred into an eppendorf where 1 mL of 70% ACN: H_2O solution was added prior to sonication for 10 min. Ten µL of the 1 mL extract were 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). The 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. Digests were stored at − 80 °C until analysis. Prior to analysis samples were pre-purified using C18 ZipTips according to the standard protocol and peptides were eluted in 5 µL of 50:50 ACN: 0.1% TFA.

Commercially available intravenous animal blood samples from bovine, porcine and chicken (both with and without EDTA) were also subjected to in solution proteolytic digestion in order to obtain reference spectra. Five microliters of each blood reference sample was diluted 1 in 200 with H_2O and 10 µL of the diluted blood were subjected to enzymatic digestion and purification protocols as described for the blind samples.

Matrix and application. Ten mg/mL α-CHCA in 70:30 ACN: 0.5% TFAaq was deposited by spotting 0.5 µL on top of the sample for profiling experiments.

Results and discussion

Blind samples consisting of stains and fingermarks were prepared on aluminium slides in the following matrices: human semen, saliva and sweat, matrices unrelated to any biofluid (egg yolk, beetroot, lotions, steak sauce and ketchup), human blood, animal blood (porcine (pig and wild boar), bovine and avian (chicken)). All of the samples were supplied either as unenhanced or enhanced using three blood enhancement techniques (BET) such namely Acid Black 1 (AB-1), Acid Yellow 7 (AY-7) and Leucocrystal Violet (LCV). To the authors' knowledge this is the first study of its kind to address validation of a technique for blood detection and provenance determination using blind samples, and of considerable diversity, in combination with the prior application of BET.

Within the selected pool of animal species and biofluids, the data acquired from the blind samples can theoretically enable five levels of identification (ID levels I–V) namely (I) confirmation of presence/absence of blood; (II) determination of blood provenance (human or animal); (III) determination of blood provenance at animal species level; (IV) determination of the presence of biofluid other than blood; ((V) determination of the type of biofluid. This study focussed on the first three levels of identification. However, during the study, semen biomarkers were also identified thus tapping into ID levels IV–V.

Development of the interpretative strategy. The main interpretative hypothesis was that by targeting proteins specific to human or animal blood, it was possible to both detect and source attribute this biofluid. MALDI MS, MALDI MS/MS and LC MS/MS were employed for a selected sample subset to both determine and confirm the identity of relevant ion signals. Once the analysts had made an identity claim on the sample analysed, its true identity was disclosed to assess effectiveness of the interpretative strategy. Figure 1 summarises the type of samples investigated and the general strategic approach. Retrospective analyses of samples that had been incorrectly classified allowed for strategy refinement in an iterative manner. This refinement included the re-definition of the S/N threshold and the mass accuracy for peak picking (the latter based in part on the instrumental calibration and in part on average instrumental performance) which were eventually set at 10 and 15 ppm respectively. For animal blood attribution, sample preparation was modified with respect to the initial experimental protocol by introducing a pre-purification step using C18 ZipTips after proteolysis. Sample purification led to a better S/N ratio and more peptide ion populated spectra when in the presence of animal blood. Initially, for animal blood attribution, in addition to identifying the frequency of the putative presence of proteotypic peptides across the animal species investigated (within the subset of blood proteins taken in consideration), the frequency of putatively detected *shared* peptides per species was used as an additional interpretative criterion. However, this criterion confounded data interpretation leading to the wrong claim and was therefore discounted.

ID level I: detection of blood. Initially, detection of blood was based on the detection of the two most abundant haemoglobin (HB) peptides at *m/z* 1274.725 (βHB LLVVYPWTQR) and at *m/z* 1529.734 (αHB VGGHAAEYGAEALER). Within the system under investigation, these peptides are shared between human, porcine and bovine species only; therefore, if chicken blood was present, this would go undetected. However, an unexpected chicken blood marker was later detected (see "ID level III: influence of blood collection method and animal blood species attribution" section), thus permitting ID Level I when in presence of any of the blood sources investigated.

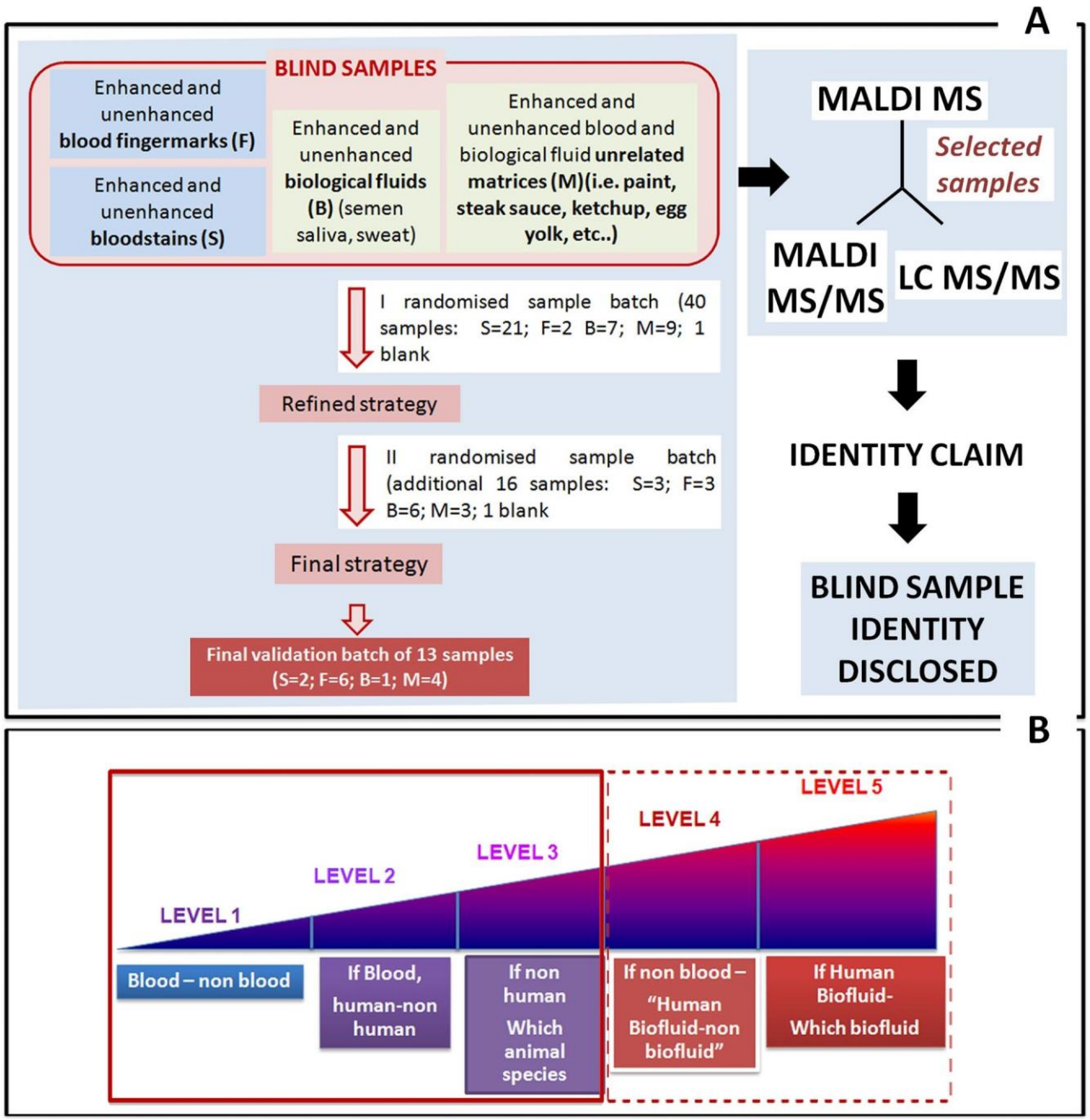

Figure 1. Blind samples investigated and general experimental and analytical approach. (**A**) shows the subdivision of the blind samples and their investigation in a stepwise approach within a refining strategy validated through a final subset of 13 blind samples. Analyses were performed by MALDI MS with some selected samples submitted to MALDI MS/MS and/or LC MS/MS for protein identification/identity confirmation. (**B**) shows the five levels of identification theoretically permitted by the data. Of these levels, this study pursued the first three (continuous line) and generated some knowledge towards levels 4 and 5 (dotted line).

ID level II: discrimination between human and animal blood. The distinction between human and animal blood was not straightforward. The two most abundant peptide markers at *m/z* 1274.725 and *m/z* 1529.734 were used again as positive blood markers as a starting point.

The βHB peptide at *m/z* 1274.725, despite being clearly at its highest intensity in human blood mass spectra, is also shared with porcine and bovine blood. Similarly, the αHB at *m/z* 1529.734 is present in both human and bovine blood. Therefore, the simultaneous presence of both peptides could only mean that the sample did contain blood and could be of either human or bovine origin (in this study, where the blood was known to have single provenance).

To determine whether the blood was of human versus animal (bovine) origin, identification initially relied on the detection of human blood protein derived proteotypic peptides such as that at *m/z* 932.520 (βHB peptide),

Sample no	BET & reaction	I ID level (blood?)	II ID level (if blood, human/animal)	III ID level (animal species?)	True identity	Correct claim?
1 S	None	Yes	Human	N/A	Bovine	No
2 S	None	Yes	Human	N/A	Human	Yes
3 S	None	Yes	Human	N/A	Human	Yes
5 S	AB-1 +	Yes	Animal	Bovine	Bovine	Yes
6 S	None	Yes	Human	N/A	Human	Yes
7 S	None	Yes	Inconclusive	Inconclusive	Porcine	No
12 S	None	No	N/A	N/A	Sweat	Yes
13 S	LCV+	No	N/A	N/A	Porcine	No
14 S	AB-1 +	No	N/A	N/A	Human	No
16 S	None	Yes	Human	N/A	Human	Yes
17 S	None	Yes	Human	N/A	Human	Yes
18 S	None	Yes	Animal	Porcine	Wild boar	Yes
26 S	LCV−	No	N/A	N/A	Saliva	Yes
27 S	None	No	N/A	N/A	Semen	Yes
28 S	None	No	N/A	N/A	Bovine	No
29 S	AY-7 +	No	N/A	N/A	Chicken	No
30 S	AB-1 +	No	N/A	N/A	Egg yolk	Yes
31 S	LCV+	Yes	Human	N/A	Human	Yes
34 S	LCV+	Yes	Human	N/A	Human + EDTA	Yes
35 S	None	No	N/A	N/A	Chicken	No
36 S	None	No	N/A	N/A	Ketchup	Yes
37 S	None	Yes	Human	N/A	Human	Yes
40 S	None	No	N/A	N/A	Sweat	Yes
41 S	AB-1 +	No	N/A	N/A	Ketchup	Yes
49 S	AB-1 +	No	N/A	N/A	Saliva	Yes
53 S	None	Yes	Human	N/A	Human + EDTA	Yes
56 S	None	No	N/A	N/A	Porcine	No
57 S	None	No	N/A	N/A	Paint	Yes
59 S	None	Yes	Human	N/A	Human	Yes
60 S	AY-7 +	No	N/A	N/A	Saliva	Yes
61 S	None	Yes	Human	N/A	Human	Yes
63 S	LCV− faint	No	N/A	N/A	Chicken	No
78 S	None	No	N/A	N/A	Lotion gold bond	Yes
79 S	None	No	N/A	N/A	Blank	Yes
122 F	AY-7 +	Yes	Human	N/A	Human	Yes
141 F	AB+ (spotty)	No	N/A	N/A	Ketchup	Yes
160 F	AY-7 −	No	N/A	N/A	Saliva	Yes
162 F	AB-1 +	Yes	Human	N/A	Human	Yes
165 F	AY-7 +	No	N/A	N/A	Egg white	Yes
175 F	LCV−	No	N/A	N/A	Egg white	Yes

Table 1. Initial MALDI MS proteomic analysis of 40 blind samples randomly selected and in order of analysis. S (stain), F (fingermark). BET indicates "blood enhancement technique" and the corresponding column shows "none" for none applied or reports the name of the technique with the enhancement result; "–" indicates no enhancement whereas "+" indicates enhancement. AB-1 (Acid Black 1); LCV (Leucocrystal Violet); AY-7 (Acid Yellow 7).

m/z 1087.553 (EPB42, UniProtKB–P16452) and 1378.694 (Hpt UniProtKB–P00738). However, these additional ion signals were only occasionally detected and often of a low intensity and resolution or borderline acceptable mass accuracy. Therefore, eventually, an ID Level II claim was made and human blood was identified if both of the two signals at *m/z* 1274.725 and 1529.734 were present, with the caveat that there might have been instances of false (human blood) positives considering that these peptides are found in bovine HB amino acid sequence too. In practice, it has been verified that in all the samples examined, both of the above signals are present together only in human blood. Therefore, this ID Level II criterion was brought forward for interpreting the final set of 13 pre-validation samples (see "Analysis of the final validation set of blind samples" section). The reliance on the simultaneous detection of the signals at *m/z* 1274.725 and *m/z* 1529.734 for human blood resulted in a 1/40 false positive for human blood (Sample 1 S was bovine blood) (Table 1).

Table 1 also shows that out of 15 human samples, only sample 14 S (in which the signals at nominal *m/z* 1275 and 1530 were absent) resulted in a false negative for human blood. Other *m/z* proteotypic peptides for human blood previously identified were also absent. A new protein digest was prepared from sample 14 S extract yielding the same results.

As the original sample was no longer available and given that this is the only false negative for human blood, it is speculated that sample mislabelling occurred (a different analyst prepared the sample at the time). As Table 1 shows, the method also works for human blood mixed with EDTA (chemical agent preventing blood coagulation). Furthermore, correct human blood identifications were made compatibly with the prior application of blood enhancement techniques (LCV, AY-7 and AB-1). These tests correctly indicated the presence of blood but it is important to bear in mind that they cannot discriminate between blood of human and animal provenance.

When in the presence of matrices unrelated to blood or to any biofluid (such as ketchup, egg yolk, paint etc.), the samples were correctly classified, using the MALDI MS based strategy, as "non-blood" (9/9 correct classification). Interestingly, contrary to MALDI MS results, the presumptive test incorrectly indicated blood presence in a few instances (e.g. sample 41—ketchup; sample 30—egg yolk; sample 165—egg white).

ID level III: influence of blood collection method and animal blood species attribution. The adoption of HB and other animal blood specific protein markers (through proteotypic peptides) largely failed to discriminate the provenance of animal blood. Table 1 shows that for 7/9 animal blood samples, the MALDI MS based strategy yielded an incorrect "non-blood" claim with 3/3 false negatives for chicken blood, 3/4 false negative for porcine blood and 2/3 false negatives for bovine blood. In order to understand why animal blood classification was unsuccessful, blood reference samples for bovine, porcine and chicken were investigated to determine the peptide targets to search for within the animal blood blind sample spectra. It was quickly determined that, for all the species, the commercially available blood reference spectra were very different from the corresponding blind animal sample spectra. Figure 2 shows an example comparing the MALDI MS spectra from commercially available chicken blood and from the blind chicken blood sample 10. As it can be seen, HB ion signals at *m/z* 999.487, 1036.561, 1164.648, 1288.736, 1302.645, 2226.129 (βHB, UniProtKB–P02112) and at *m/z* 1645.776, 1847.900, 2121.142 and 2249.224 (αHB, UniProtKB–P01994) could only be putatively observed for the reference blood. At this stage it was disclosed that, while human blood was collected through phlebotomy, the animal blood collected for the blind study was obtained from a butcher in the US by harvesting any volume of blood that had collected in the chest cavity which then was pooled with a large volume syringe (one per animal).

The collection of chicken blood was reported to be the most challenging due to significantly less blood present and the quick dilution with water during the cleaning/processing of the animal. This could explain the lack of detection of HB signals in the blind sample 10. At this stage, only the peptides at *m/z* 1321.746, 1537.754, 1580.817, 1662.858, could be putatively assigned in the blind sample 10 spectrum and were attributed to apolipoprotein 1 (ApoA1, UniProtKB–P08250). Of these 4 assignments, only the ApoA1 signal at *m/z* 1580.817 (1–22, IRDMVDVYLETVK) was found in both sample 10 and the blood reference spectrum.

Notably the most intense ion signals at *m/z* 1391.746, and 1749.798 and 1778.923 could not be assigned to any of the most abundant/blood specific proteins under investigation nor could a Mascot search identify these peaks.

The same type of spectral comparison is reported in Supplementary Fig. S1 for bovine (A) and porcine (B) blood. The blind samples exhibited a much greater complexity in terms of ion population.

Furthermore, porcine and bovine HB signals, clearly present in the reference spectra (from commercially available blood) could not be detected in the corresponding blind sample spectra. The reference blood had been collected for all animals through an incision of the jugular vein with the blood subsequently stored in EDTA. This would explain why HB peptide signals are the most intense in the reference blood spectra.

As blind samples exhibited a different mass spectral profile from the corresponding intravenous blood, it was hypothesised that a different method of collecting blood, closer to how the blood samples were prepared, could be more suitable than intravenous blood to act as a reference blood. Consequently, it was investigated whether the blood collected from packaged meat and blood residues originating from butcher prepared meat had mass spectral profiles superimposable with those from the blind samples or the reference spectra. This investigation was crucial to the ability to discriminate animal species and it became even more interesting because, if the spectra were different, then the difference would be strictly correlated to the way in which blood was harvested.

For blood detection studies, this circumstance would imply that it is incorrect to just target the most abundant proteins that one would normally expect to find in blood. If different blood protein compositions are possible, these different "systems" need to be understood. Wounded animals perhaps resemble the closer scenario to intravenous blood; blood traces from touching the meat prepared directly from a butcher is possibly a more common scenario at crime scenes and, with the widespread purchasing and handling of packaged meat, bloodstains or blood fingerprints could also derive from this form of animal blood contamination.

Determination of a chicken blood biomarker. As mentioned above, for chicken blood, the signal at *m/z* 1749.798 detected in the blind sample 10 was one of the most intense unassigned signals. This ion signal was also present in packaged and butcher freshly prepared meat but not in the intravenous blood. Initially, a MALDI MS/MS analysis was performed on this ion at nominal *m/z* 1750 in the freshly prepared meat, the packaged meat and blind samples. All but the blind sample yielded the identification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (UniProtKB P00356), through the peptide L**V**SWYDNEFGYSNR (theoretical *m/z* 1749.787), with a Mascot score of 117 and 62 respectively which are of statistical significance. The subsequent use of LC MS/MS enabled confirmation of GAPDH within the blind sample 35 S (as sample 10 S was no longer available) (Fig. 3), through the doubly charged ion at *m/z* 835.397 eluting at 15.06 min, with a Mascot score of 92.

Table 2 summarises the MS/MS GAPDH identifications from the three samples analysed.

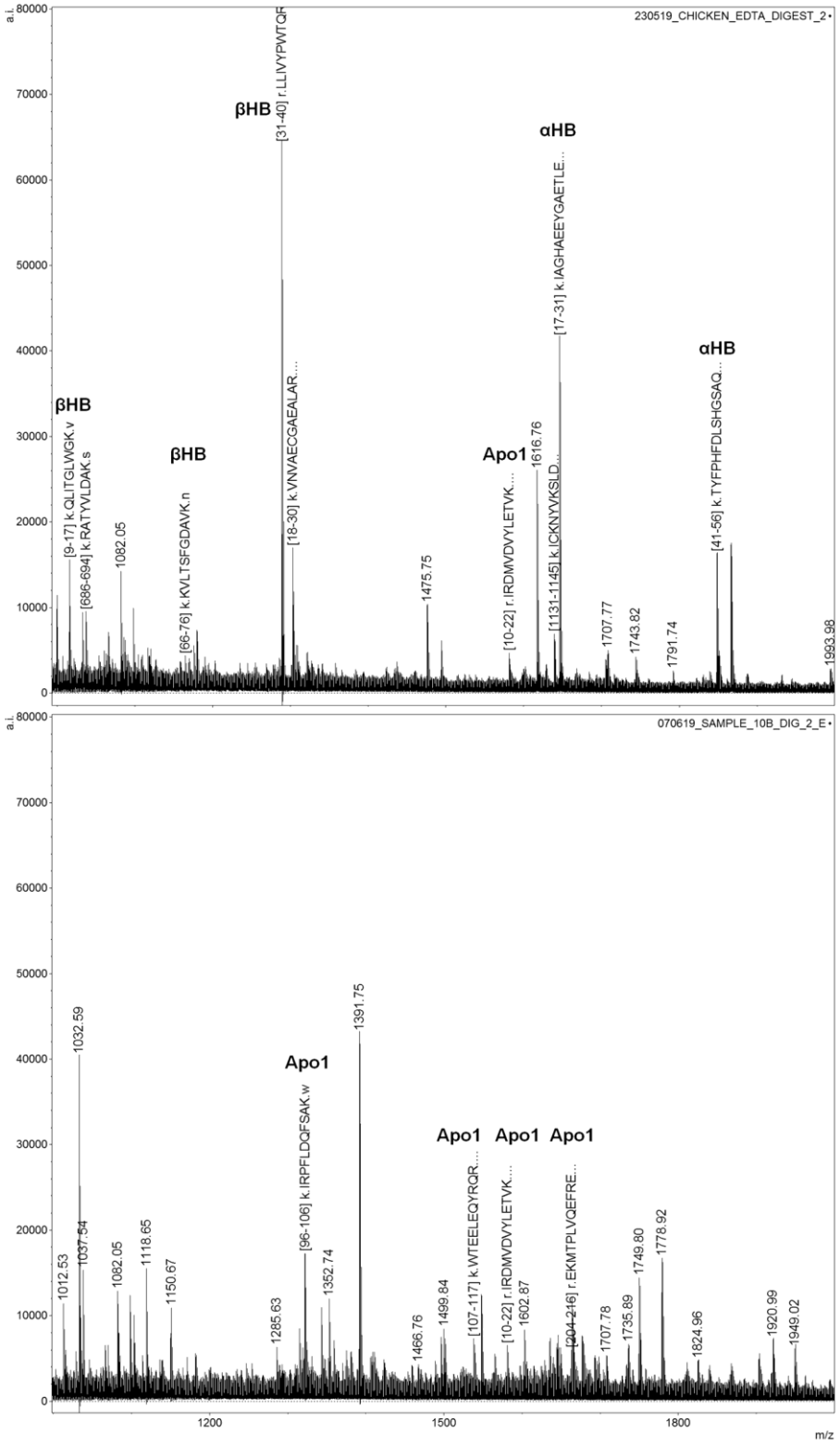

Figure 2. MALDI MS spectra of commercially available chicken blood (top panel) and of blind sample 10 (chicken bloodstain, bottom panel) showing overall different spectral profiles.

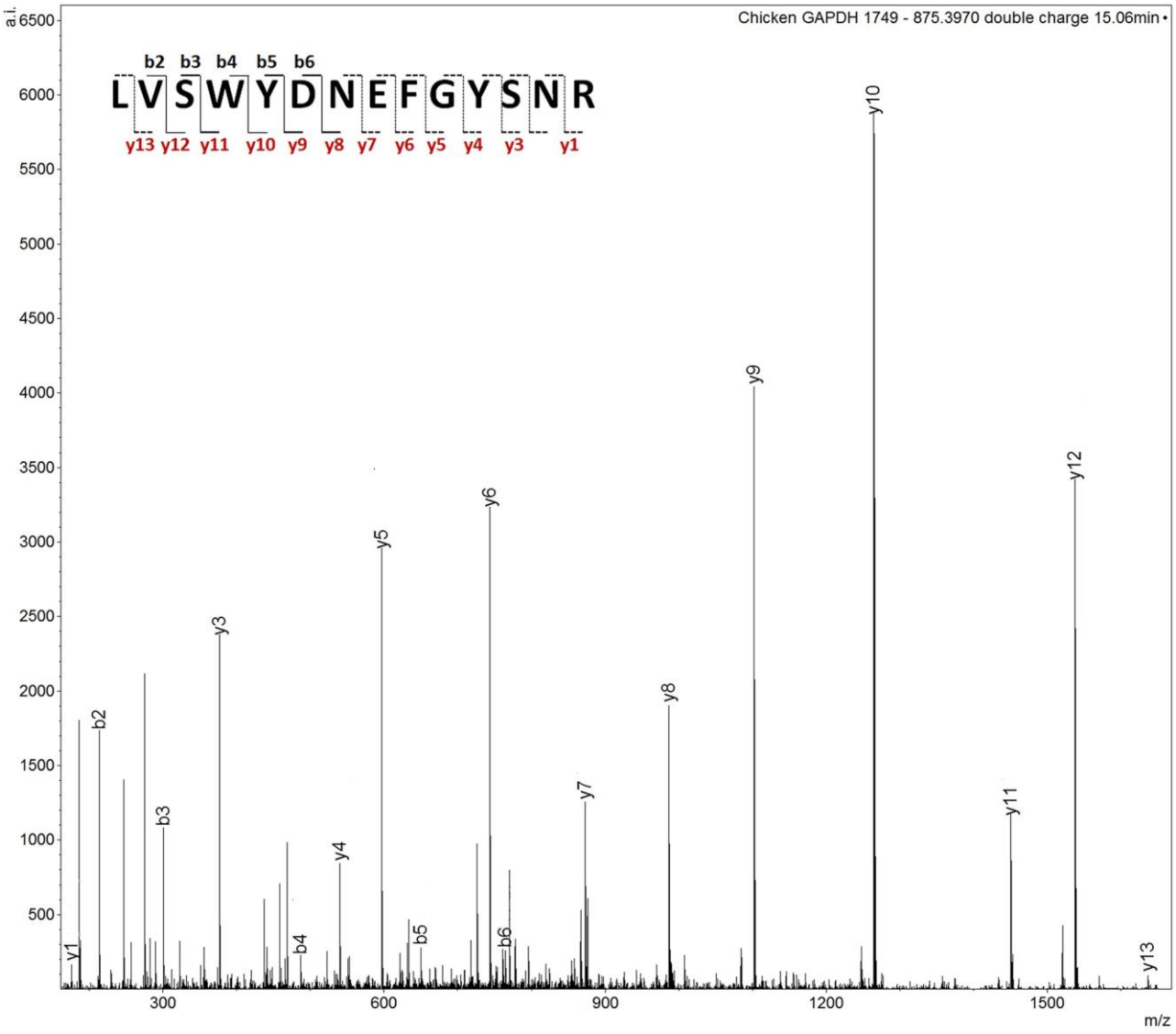

Figure 3. LC MS/MS spectrum of the doubly charged ion at *m/z* 875.397 in sample 35 S. This spectrum yielded the identification of chicken GAPDH with a Mascot score of 92. The fragment ions not annotated on the peptide sequence in the figure indicate that they have not been detected.

Ion signal *m/z* (Th)	Analysis	Mascot Score	ID protein and UniProt accession number	Sequence	Ion fragments detected
Chicken packaged meat					
1749.855	MALDI MS/MS	62	GAPDH–P00356	LVSWYDNEFGYSNR	y1–y12; b8–b9
Chicken butcher freshly prepared meat					
1749.793	MALDI MS/MS	117	GAPDH—P00356	LVSWYDNEFGYSNR	y1–y12; b6–b8
Chicken sample 35 S					
875.397	LC MS/MS	92	GAPDH—P00356	LVSWYDNEFGYSNR	y1, y3–13; b2–b6

Table 2. Summary of selected sample submitted to MS/MS analysis and yielding identification/confirmation identity of a chicken blood biomarker (GAPDH).

Following the peptide identification at nominal *m/z* 1750, other GAPDH signals were searched for in the spectra of both sample 35 S and 10 S (chicken blood) and the additional GAPDH signals at a nominal *m/z* of 795, 805, 1033, 1359 and 1646 were assigned with a mass accuracy ranging between 13.2 and 3.6 ppm.

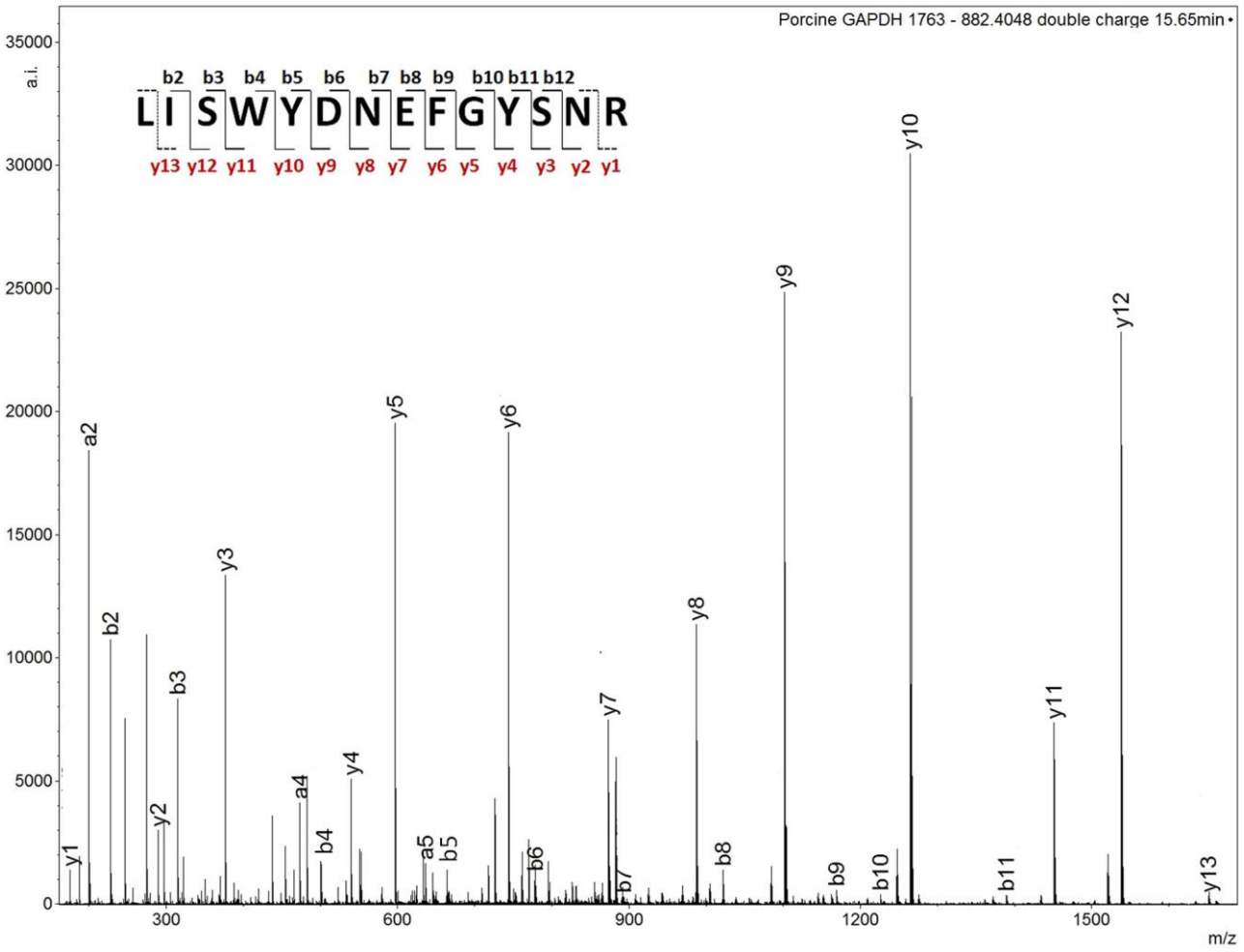

Figure 4. Annotated LC MS/MS spectrum of *m/z* 882.405 from sample 7 S confirming the presence of porcine GAPDH. Missing annotation on the peptide sequence denotes lack of detection of that particular b/y ion fragment.

Determination of bovine and porcine blood biomarkers. *Glyceraldehyde-3 phosphate-deydrogenase (GAPDH)* The signal at *m/z* 1749.787 was absent in bovine and porcine blind blood samples. However, a signal at *m/z* 1763.802 was consistently present in the bovine and porcine blind sample spectra but absent in the corresponding intravenous blood. MALDI MS/MS analysis and Mascot searches were performed on this ion at nominal *m/z* 1764 for porcine sample 7 S and packaged meat, yielding the identification of porcine GAPDH (UniProtKB–P00355) with a statistically significant Mascot score of 69 and 122 respectively, through the peptide of sequence L**I**SWYDNEFGYSNR. This sequence differs from that found in the chicken GAPDH protein by an isoleucine replacing a valine (mass difference of 14 units) in the second position of the peptide. The presence of GAPDH in sample 7 S was additionally confirmed with a Mascot score of 93, through LC MS/MS of the doubly charged ion at *m/z* 882.405 (Fig. 4) eluting at 15.65 min.

Bovine GAPDH (UniProtKB-P10096) was confirmed through LC MS/MS of blind bovine sample 38 S with a MASCOT score of 100 by fragmenting the doubly charged ion at *m/z* 882.405.

As for the chicken blood, the blood spectral profiles for the blind bovine and porcine samples (38 S and 7 S respectively) reported in Fig. S1 were re-investigated. With reference to these spectra, GAPDH was putatively detected via multiple peptides in both the bovine and porcine blind samples but not in the corresponding intravenous blood. However, the only ion signal consistently present across all of the bovine and porcine samples analysed to that point was that at nominal *m/z* 1764.

GAPDH is normally used as a housekeeping gene or 'internal control in experiments' due to its relatively constant expression at high levels in skeletal muscle under changing conditions[13] (in chicken, GAPDH is reported to have the highest expression in skeletal muscle). The expression of this protein explains why these signals were only detected in the blind samples and not in the intravenous blood.

Therefore, ultimately, the hypothesis that blood has a different protein profile, depending on how "blood was generated/collected" has been verified, at least with reference to the way the blood was collected for the blind animal sample preparation and the animal intravenous blood.

Sample	Ion signal *m/z* (Th)	Analysis	Mascot Score	Protein	Sequence	Ion fragments detected
Porcine blood						
7 S	1763.811	MALDI MS/MS	69	GAPDH	LISWYDNEFGYSNR	y1–y9; b3, b8
7 S	882.405	LC MS/MS	93	GAPDH	LISWYDNEFGYSNR	y1–y12; b2–11; a2, a4–a5, a8, a11
Packaged meat	1763.786	MALDI MS/MS	122	GAPDH	LISWYDNEFGYSNR	y1–y12; b2–b6, b8–b11; a2
Bovine blood						
38 S	882.405	LC MS/MS	100	GAPDH	LISWYDNEFGYSNR	y2–y12; b2–b3, b5, b8; a2, a4
38 S	796.921	LC MS/MS	93	Myoglobin	VEADVAGHGQEV-LIR	y1, y7–13; b2, b4–b5, b11–b12, b14; a2
1 S	1592.822	MALDI MS/MS	58	Myoglobin	VEADVAGHGQEV-LIR	y7–y8, y10–y11,y13; b8–b8,b11,b13; a8, a12; c9

Table 3. Summary of selected sample submitted to MS/MS analysis and yielding identification/confirmation identity of porcine and bovine blood biomarkers.

Table 3 reports and summarises the selected samples submitted to MS/MS and yielding GAPDH biomarker identification of porcine and bovine blood.

The identification of the signals at nominal *m/z* 1750 and 1764 permitted a data interpretation strategy refinement; when blood presence is confirmed and human blood is excluded, the presence of the ion at nominal *m/z* 1750 indicates presence of chicken blood via the protein GAPDH. Should the ion at nominal *m/z* 1750 be absent, the GADPH peptide at *m/z* 1763.802 confirms the presence of animal blood but cannot distinguish between porcine and bovine blood.

Myoglobin- Further investigation into the spectra of the blind bovine blood samples that had been incorrectly classified (Table 1) allowed for the putative identification of other bovine blood markers. Bovine blood exhibited the presence of the signals at *m/z* 1669.837 and *m/z* 1592.822 which were putatively attributed to myoglobin (UniProtKB–P02192), initially on the basis of their *m/z* and their mass accuracy (< 15 ppm). Myoglobin peptide at nominal *m/z* 1593 was confirmed (VEADVAGHGQEVLIR) in the blind bovine blood sample 38 S through LC MS/MS by fragmenting the doubly charged ion signal at *m/z* 796.924 eluting at 11.18 min, with a MASCOT score of 93 (Fig. 5).

This identification was also confirmed in sample 1 S (previously incorrectly classified) via MALDI MS/MS of the ion at *m/z* 1592.829, with a statistically significant Mascot score of 58 (Table 3). It was not possible however, to assign definitively the peptide at *m/z* 1669.837 by either MALDI MS/MS or LC MS/MS analysis due to an insufficient number of ion fragments. However, this signal was still used as a putative identification for bovine myoglobin. Although the signal at nominal *m/z* 1593 appears in all of the bovine samples, that at nominal *m/z* 1670 does not. The former ion signal shares the same sequence in porcine myoglobin though it is never detected in the porcine blind samples. Another signal at nominal *m/z* 649 was identified as porcine myoglobin (UniProtKB–P02189) on the basis of the *m/z* and mass accuracy (< 10 ppm). This signal was present in all but one porcine blind samples analysed.

Therefore: (i) the presence of bovine blood was claimed if markers at nominal *m/z* 1764, 1593 and/or 1670 were present (within the set mass accuracy); (ii) the presence of porcine blood was claimed if the signal at a nominal *m/z* 1764 was present and signals at nominal *m/z* 1593 and/or 1670 were absent. Confidence in making this claim was increased if the signal at nominal *m/z* 649 was also present.

Following the identification of myoglobin through the peptide at nominal *m/z* 1593, this protein was also retrospectively identified in bovine sample 38 S (but not in the corresponding intravenous blood) though multiple peptides with a mass accuracy ranging between −11.2 and 2.5 ppm.

Supplementary Table S1 summarises the identifications of haemoglobin, GAPDH and myoglobin biomarkers in blind samples 7 S (porcine blood) and sample 38 S (bovine blood) versus the corresponding intravenous reference blood showing marked differences in the presence of these proteins. The presence and nature of GAPDH, and myoglobin in the blind samples aligns with the location from which blood was harvested.

Within the "system" investigated, altogether these results indicate the opportunity of performing further source attribution (ID Level III) by differentiating chicken, bovine and porcine blood through a refined strategy.

Identification of semen: a glimpse into ID levels IV and V. A further sample had been incorrectly classified as human blood. This was one of the earliest blind samples analysed and the claim was incorrectly based on the presence of only one signal, the αHB peptide at *m/z* 1529.734 (mass accuracy 0 ppm).

Upon disclosure of the sample identity, the spectrum was inspected more carefully and an intense signal at *m/z* 1714.849 was observed. MALDI MS/MS analysis was performed on this precursor ion and a Mascot search was launched. Through this search semenogelin-1 (SEM-1 UniProtKB–P04279) was identified through the peptide sequence GLRPSEFSQFPHGQK, with a statistically significant score of 99. The MS/MS spectral annotation of the ion fragments is shown in Fig. 6.

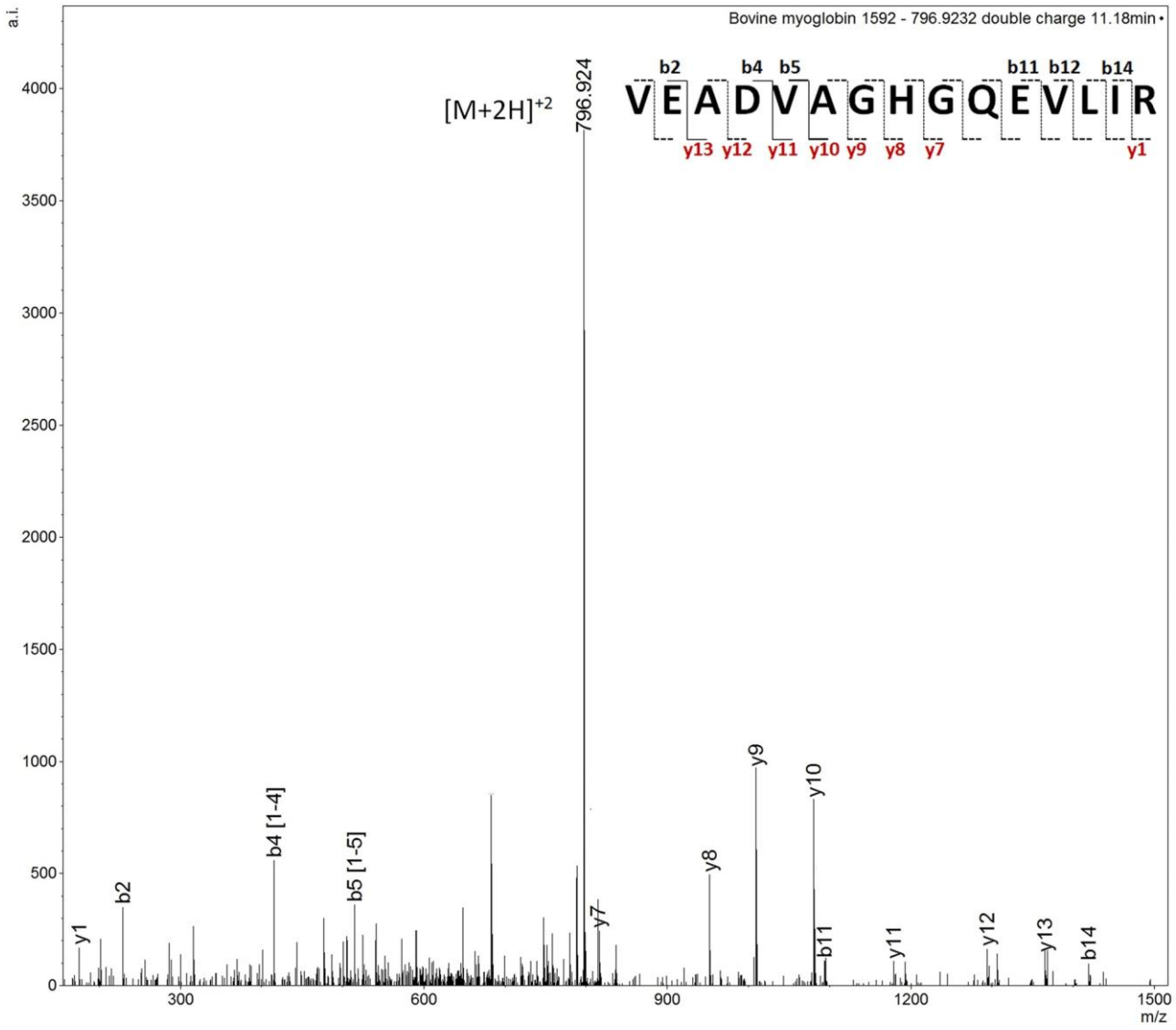

Figure 5. Annotated LC MS/MS spectrum of doubly charged ion at *m/z* 796.924 from sample 38 S confirming the presence of porcine GAPDH. Missing annotation on the peptide sequence denotes lack of detection of that particular b/y ion fragment.

SEM-1 is produced in the seminal vesicles[14] and it is abundant in semen. Semenogelin was also identified by Iliano et al. by LC MS/MS[4] and it is the target for a semen confirmatory test employing a lateral flow immunochromatographic test strip containing colloidal gold-conjugated anti-human semenogelic monoclonal antibodies[15] (Rapid Stain Identification (RSID)).

Additional ion signals were putatively assigned to this protein at *m/z* 1444.764, 1501.744 and 1801.918 with a mass accuracy of 6.6, 0.5 and 13.1 ppm respectively (Supplementary Fig. S2).

Encouraged by these results, a thorough search of other semen specific proteins was performed on the basis of the literature available and Semenogelin-2 (SEM-2, UniProtKB–P04279) was also putatively detected (Supplementary Fig. S2) (*m/z* 1883.936, mass accuracy 0.3 ppm; *m/z* 1444.764, shared with SEM-1, mass accuracy 6.6 ppm; *m/z* 1554.779, mass accuracy 8.6 ppm). SEM-2 has additionally been reported as a protein biomarker for semen detection through mass spectrometric based techniques and also using miRNA[16–18]. Of these ion signals, those at nominal *m/z* 1445 (SEM-1/SEM-2) and 1555 (SEM-2), were consistently found in the semen samples analysed; therefore these markers were also implemented for the rapid identification of remaining semen samples in the study. The putative presence of the signals at *m/z* 1529.734 (αHB) and 1314.682 (βHB) does not allow for the exclusion of the (weak) co-presence of blood within semen. This instance was important for the consideration that, within real crime scenes, it is possible that blood and semen may both be present in a stain; therefore confirmatory MS/MS analyses would be needed given that the finding would be of significant relevance to an investigation.

Upon retrieval of the SEM-1 marker at nominal *m/z* 1715, subsequently semen samples (blind to the analyst) were not only dismissed as blood but also correctly identified as semen (Table 4).

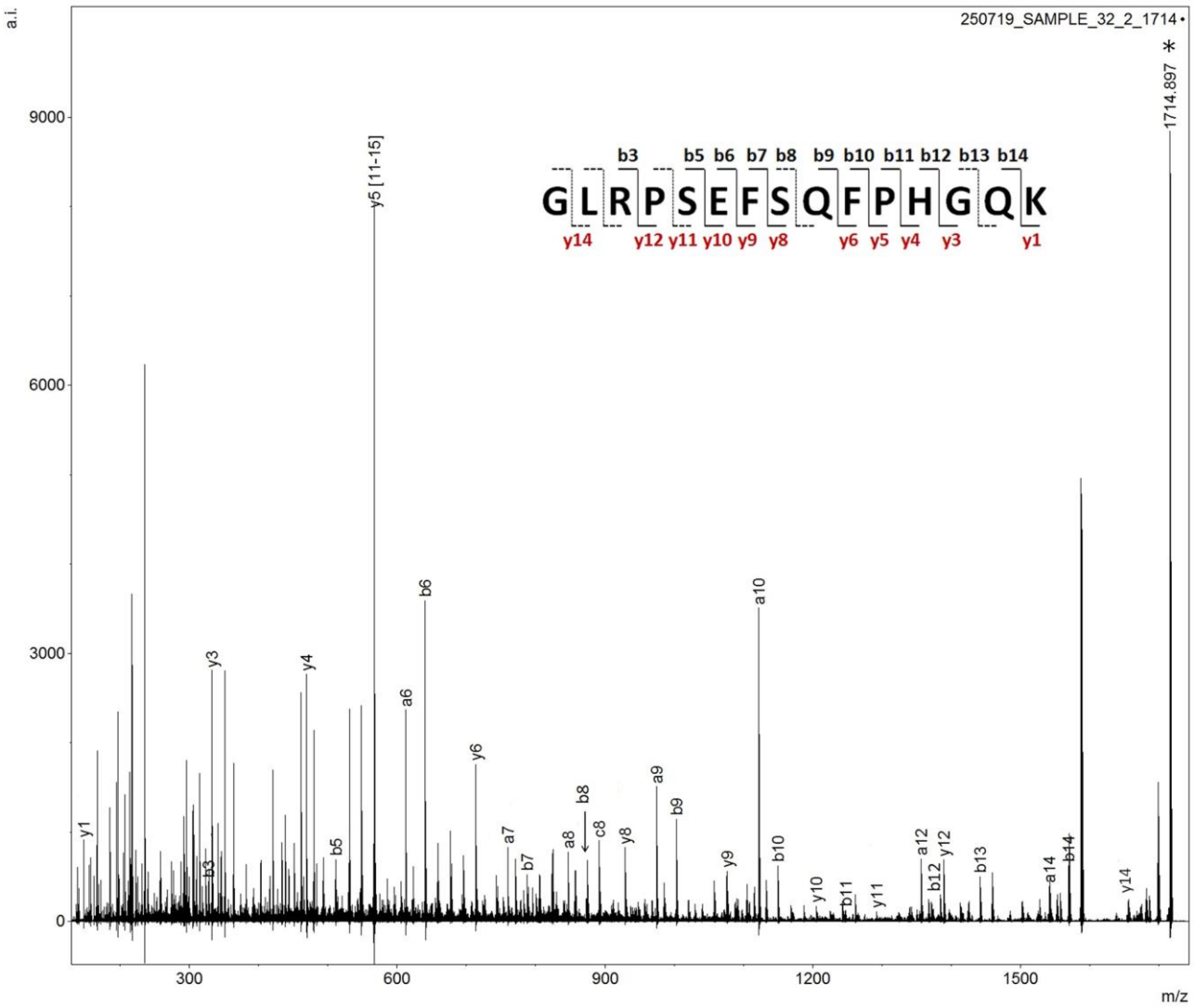

Figure 6. MS/MS spectrum and ion fragment annotation of SEM-1 detected in sample 32. b and y ion are annotated and also shown on the peptide sequence; a and c fragment ions are only annotated in the spectrum. Missing annotation on the peptide sequence denotes lack of detection of that particular b/y ion fragment.

Sample no.	BET	I ID Level (Blood?)	II ID level (if blood, human/animal	III ID level (which animal species?)	True identity	Correct claim?
32 S	LCV−	Yes	Human	N/A	Semen	No
Following retrieval of semenogelin-1 marker at nominal m/z 1715						
27 S	None	No	N/A	N/A	Semen	Yes
58 S	AB-1 +	No	N/A	N/A	Semen	Yes
132 F	LCV (−1 area/ residue)	No	N/A	N/A	Semen	Yes
158 F	AB-1 +	No	N/A	N/A	Semen	Yes

Table 4. Analysis and identification of semen blind samples. S (stain), F (fingermark). BET indicates "blood enhancement technique" and the corresponding column shows "none" for none applied or reports the name of the technique with the enhancement result; "–" indicates no enhancement whereas "+" indicates enhancement. AB-1 (Acid Black 1); LCV (Leucocrystal Violet).

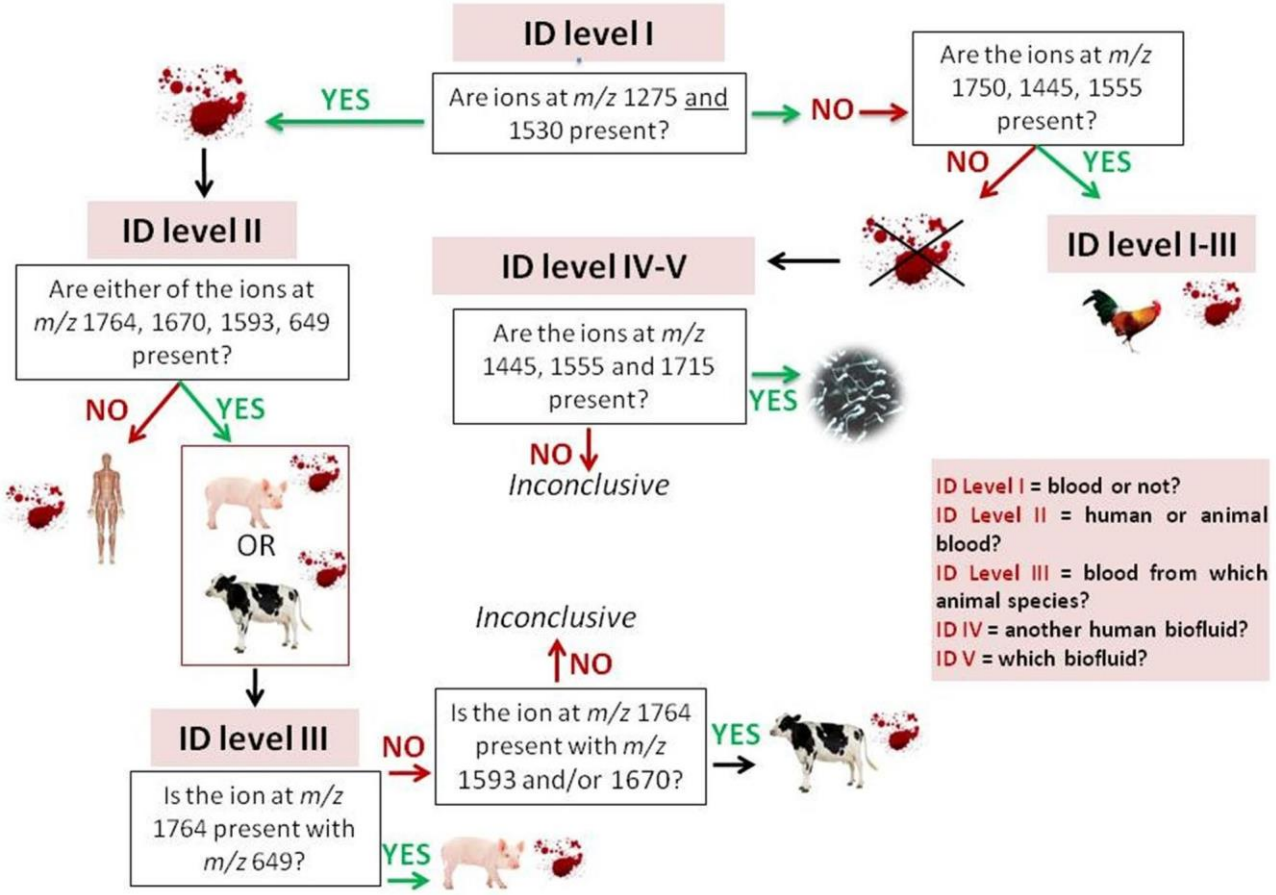

Figure 7. Refined blind sample spectra data interpretation strategy. This strategy enables the determination of the ID Levels I–III of animal provenance down to species as well as the determination of the presence of semen (ID Level IV and V). The *m/z* values are nominal. Their presence is verified with a mass accuracy < 15 ppm.

Semen was correctly classified even when the sample was pre-enhanced with Acid Black 1 (AB-1) or Leuco-crystal violet (LCV). Crucially, where AB-1 gave a positive reaction (samples 58 S and 158 F), MALDI analyses correctly refuted the results of the presumptive test identifying semen instead.

Figure 7 reports the interpretative strategy that was finalised to identify blood samples (from human and animal species) and semen. This strategy was used for revisiting some of the samples incorrectly identified and 11 additional blind samples as described in the following section.

Blind sample cohort summary of results after identification of animal blood markers and partial re-visitation of some samples. Following the identification of bovine/porcine and chicken blood markers, some of the samples incorrectly classified were either re-acquired or re-prepared (extraction, proteolytic digestion and purification). Also, 11 new blind samples were analysed encompassing a mixture of human and animal blood, biofluids and non-biofluids.

Table 6 reports the identification results for a total of 56 samples including the 44 reported in Tables 1 and 4 (with some of the samples being re-visited as explained above) and the additional 11 samples. As it can be seen from Table 3, a 0% false positive rate for human blood was obtained.

One more false negative for human blood occurred for sample 170 F (total 2/13 false negatives), the spectrum of which exhibited none of the ion signals characteristic of blood. A case of mislabelling is still plausible. However, upon disclosure of sample identity, it was revealed that it was a human blood "trace", meaning that it originated from the last mark of a depletion series deposited from a nearly dry and exhausted blood source on the fingertip.

The deposited mark was barely visible (as visible as a latent mark) and it is possible that the blood transfer was so minimal that the instrumental sensitivity was insufficient.

When blind non biofluid-related samples were analysed (beetroot juice, paint, ketchup), again a 0% false positive rate for blood was achieved (0/12 samples). When in the presence of biofluids, semen was correctly identified every time (100% correct identification rate) and a 0% false positive rate for blood was achieved overall (0/13 samples).

When in the presence of animal blood, whilst prior to discovery of the GAPDH and myoglobin marker, 7/9 samples were incorrectly classified as "non blood", after deployment of the refined strategy (Fig. 7) the false negative rate decreased from 77.7% (7/9 samples) to 6.7% (1/15 samples). In particular, before strategy refinement a

false negative rate of 100% (3/3 samples), 75% (3/4 samples) and 66.7% (2/3 samples) was yielded for chicken, porcine and bovine respectively. After strategy refinement, the false negative rate decreased to 0% (0/5 samples), 16.7% (1/6 samples) and 0% (0/4 samples) for chicken, porcine and bovine respectively. The additional new 11 samples analysed after strategy refinement shown in Table 5 (in bold) were all correctly classified, whether enhanced or not.

Analysis of the final validation set of blind samples. Confirmed markers for human blood, animal blood and semen were taken forward in a "final validation" of the method applied to a representative set of additional 13 enhanced and non-enhanced blind samples including a range of stains and marks in blood, semen, other biofluids and non-biofluid related matrices.

Out of these 13 samples, 12 were correctly identified (Table 6). Once again, there was an instance where a presumptive test incorrectly indicated blood when the sample was instead correctly identified by MALDI MSP as semen (sample 176 F).

Sample 138 F was correctly classified as animal blood; the myoglobin peptide marker at nominal *m/z* 1593 was detected in this sample but the marker at nominal *m/z* 1764 was not. As a result, the claim of bovine blood was not made. From this, it can be surmised that, in bovine blood, the GAPDH marker at nominal *m/z* 1764 does not need to be present in addition to the myoglobin signal at *m/z* 1593, in order to correctly identify this animal species as the source of blood.

From a forensic perspective, it is important to highlight that these samples were, at the point of the proteolytic digestion and analysis, 3 years old. Therefore the successful application of the strategy devised to these samples opens up the avenue for investigation of cold cases.

Conclusions

This study aimed at providing validation data to support the previously reported application of MALDI MS and proteomics-based methods for the detection and provenance of blood. The rationale behind the development, improvement and validation of such methods are the reduced analysis time, the easier data acquisition and the user friendly data interpretation. A range of validation samples were prepared blind to the analyst team (including the design of sample collection). Samples included fingermarks and stains prepared with a biofluid (human semen, saliva or sweat), human blood, animal blood (domestic pig, wild boar, chicken and bovine) or biofluid—unrelated matrices. A sample subset was pre-enhanced with either Acid Black 1, Acid Yellow 7 or Leucocrystal Violet. The work presented in this study focuses on an adapted protocol for sample extraction and in solution digestion. However, the approach can easily be transferred to in situ proteomics for both profiling (for pure detection of blood and its provenance) and imaging purposes (when the information of blood presence and provenance is visualised directly on the ridge pattern), as our previous studies have shown.

The present study demonstrates that the overall analytical approach developed enables, regardless of the prior blood enhancement technique used (within the system under investigation): (a) determination or exclusion of blood presence; (b) discrimination between human blood and animal blood; (c) determination of animal blood at a species level; (d) determination of presence (or co-presence) of semen. Notably, in some cases the method was able to correctly refute the positive indication on the presence of blood given by the presumptive test.

Given that semen markers were also found, the MALDI based method is on track to become a multiplexed approach for the screening of biofluids.

A key finding was made with respect to the determination of blood provenance at animal species level. Contrary to our initial hypothesis, that the most abundant and blood specific proteins could be used as molecular targets to prove/disprove the presence of blood and indicate provenance, the relevant markers may instead change according to how the blood was "generated" and "collected". For example, it was rather surprising that haemoglobin signals in chicken (blind) samples are not detected and only one is detected (poorly) for bovine and porcine blood; conversely, GAPDH a non-blood specific protein is detected in the animal blind samples but not within the blood deriving from intravenous collection.

Indeed, the animal mass spectral profiles of the blind samples (collected by a butcher from the blood filling the heart cavity of the animal) greatly differed from (i) those obtained for the corresponding intravenous blood, (ii) those of the blood residues from packaged meat and (iii) those of the blood originating from butcher prepared meat. It was also evident that for the last two types of "blood systems", a greater instrumental sensitivity is desirable due to the extreme blood dilution and the use of preservatives (for packaged meat). For animal species determination, the sample preparation design for the next piece of research should consider the most common scenarios in which animal blood may be found. Perhaps the most commonly encountered scenario is blood contamination from handling packaged and butcher prepared meat and, in a less common scenario, use of tools for hunting and dressing game. However, animals being shot or stabbed at a crime scene would produce a "blood system" resembling more the composition of intravenous blood. All of these "blood systems" require a thorough investigation and multivariate statistical analysis could provide more rapid answers as to the provenance markers which can then be subsequently identified. In a subsequent full validation study will use the knowledge generated by the pre-validation study presented here, in a much larger project. This will include a higher number of surfaces of deposition, donors, blood enhancement techniques and the possibility to lift blood fingermarks for imaging purposes when the surface of deposition is not directly amenable to MALDI MS Imaging. An exploration of blood composition and biomarkers from additional animal species will provide further versatility to the applicability of the method.

Sample no.	BET	I ID level (blood?)	II ID level (if blood, human/not)	III ID level (which animal species?)	True identity	Revisited?	Correct claim?
1 S	None	Yes	Animal	Bovine	Bovine	Yes	Yes
2 S	None	Yes	Yes	N/A	Human blood		Yes
3 S	None	Yes	Yes	N/A	Human blood		Yes
5 S	AB- +	Yes	Animal	Bovine	Bovine		Yes
6 S	None	Yes	Human	NA	Human	Yes	Yes
7 S	None	Yes	Inconclusive	Inconclusive	Porcine	Yes	Yes
12 S	None	No	N/A	N/A	Sweat		Yes
13 S	LCV+	No	N/A	N/A	Porcine	Yes	Yes
14 S*	AB-1 +	No	N/A	N/A	Human		No
16 S	None	Yes	Human	N/A	Human		Yes
17 S	None	Yes	Yes	N/A	Human blood		Yes
18 S	None	Yes	ANIMAL	Porcine	Wild boar		Yes
26 S	LCV−	No	N/A	N/A	Saliva		Yes
27 S	None	No	N/A	N/A	Semen		Yes
28 S	None	No	N/A	N/A	Bovine	Yes	Yes
29 S	AY-7 +	No	N/A	N/A	Chicken	Yes	Yes
30 S	AB-1 +	No	N/A	N/A	Egg yolk		Yes
31 S	LCV+	Yes	Human	N/A	Human		Yes
32 S	LCV−	No	N/A	N/A	Semen	Yes	Yes
34 S	LCV+	Yes	Human	N/A	Human + EDTA		Yes
35 S	None	No	N/A	N/A	Chicken	Yes	Yes
36 S	None	No	N/A	N/A	Ketchup		Yes
37 S	None	Yes	Human	N/A	Human		Yes
40 F	None	No	N/A	N/A	Sweat		Yes
41 S	AB-1 +	No	N/A	N/A	Ketchup		Yes
49 S	AB-1 +	No	N/A	N/A	Saliva		Yes
53 S	None	Yes	Human	N/A	Human blood + EDTA		Yes
56 S	None	No	N/A	N/A	Porcine	Yes	Yes
57 S	None	No	N/A	N/A	Paint		Yes
58 S	AB-1 +	No	N/A	N/A	Semen		Yes
59 S	None	Yes	Human	N/A	Human		Yes
60 S	AY-7 +	No	N/A	N/A	Saliva		Yes
61 S	None	Yes	Human	N/A	Human		Yes
63 S	LCV− faint	No	N/A	N/A	Chicken	Yes	Yes
78 S	None	No	N/A	N/A	Lotion gold bond		Yes
79 S	None	No	N/A	N/A	Blank	Yes	Yes
122 F	AY-7 +	Yes	Human	N/A	Human blood		Yes
132 F	LCV (− 1 area/residue)	No	N/A	N/A	Semen		Yes
141 F	AB-1 +(spotty)	No	N/A	N/A	Ketchup		Yes
158 F	AB-1 +	No	N/A	N/A	Semen		Yes
160 F	AY-7 −	No	N/A	N/A	Saliva		Yes
162 F	AB-1 +	Yes	Human	N/A	Human		Yes
165 F	AY-7 +	No	NO	N/A	Egg white		Yes
175 F	LCV−	No	N/A	N/A	Egg white		Yes
4 F	**None**	**Yes**	**Animal**	**Chicken**	**Chicken**		**Yes**
24 S	**None**	**No**	**No**	**N/A**	**Egg**		**Yes**
25 S	**LCV+**	**Yes**	**ANIMAL**	**BOVINE**	**Bovine**		**Yes**
41 S	**AB-1 +**	**No**	**N/A**	**N/A**	**Ketchup**		**Yes**
44 S	**None**	**Yes**	**ANIMAL**	**PORCINE**	**Wild boar**		**Yes**
53 S	**None**	**Yes**	**HUMAN**	**N/A**	**Human blood + EDTA**		**Yes**
72 S	**None**	**No**	**N/A**	**N/A**	**Steak sauce**		**Yes**
76 S	**AY-7 +**	**No**	**N/A**	**N/A**	**Semen**		**Yes**
107 F	**None**	**Yes**	**Animal**	**Porcine**	**Porcine**		**Yes**
144 F	**None**	**No**	**N/A**	**N/A**	**Blank**		**Yes**
170 F**	**None**	**No**	**N/A**	**N/A**	**Human blood**		**No**

Table 5. MALDI MS proteomic analysis and identification after strategy refinement encompassing re-visitation of some previously incorrectly classified samples. The Table reports a total of 56 samples, 45 of which were reported in Tables 1 and 2. Originals of samples 13 and 14 were no longer available and could not be re-processed. *S* stain, *F* fingermarks. Rows in bold indicate additional new samples analysed after strategy refinement. *Probable sample mislabelling. **Blood in trace amounts for which instrumental sensitivity might have been insufficient. BET indicates "blood enhancement technique" and the corresponding column shows "none" for none applied or reports the name of the technique with the enhancement result; "–" indicates no enhancement whereas "+" indicates enhancement. AB-1 (Acid Black 1); LCV (Leucocrystal Violet); AY-7 (Acid Yellow 7).

Sample no.	Blood presumptive test	Presumptive test result	Claim	True identity
15 S	None	NA	Human blood	Human blood
22 S	AY-7	+	Human blood	Human blood
104 F	None	NA	Chicken blood	Chicken blood
121 F	None	NA	Not blood	Saliva
127 F	None	NA	Semen	Semen
128 F	None	NA	Bovine blood	Bovine blood
129 F	AY-7	+	Chicken Blood	Chicken blood
138 F	**AY-7**	**+**	**Animal blood − species inconclusive**	**Bovine blood**
146 F	None	NA	Not blood	Beet juice
147 F	None	NA	Semen	Semen
155 F	AY-7	+	Porcine blood	Porcine blood
156 F	None	NA	Porcine blood	Porcine blood
176 F	AY-7	+	Not blood	Semen

Table 6. Final validation set of blind samples carried out post strategy change for species determination, showing identity claim and true identity of each sample: 92% correct identification rate Sample in bold indicates a sample correctly indicated as animal blood though animal species was inconclusive. *S* stain, *F* fingermark. The symbol + (in the presumptive test result column) indicates that the sample tested positive for the presence of blood.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

K.K. and C.H. have jointly prepared and analysed via MALDI the blind samples and wrote the methods for the manuscript. T.C. has performed and processed LC MS/MS data. K.K., C.H. and S.F. have processed and interpreted the MALDI MS data. S.F. has interpreted the MALDI MS/MS data. C.H., K.K. and S.F. have written introduction, results and discussion. S.F. has supervised the lab work, composed all the figures, coordinated manuscript writing, wrote Abstract and conclusions. S.F., G.L., M.R.C., V.S. and S.F. have conceptualised the study and devised the design of experiments and analytical strategy amendments. L.M.C. trained the students on data acquisition and quality checked spectra acquired. M.S., R.M. and G.L. have provided input and feedback from a forensic and operational point of view at all stages of the work and manuscript preparation. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Pre-validation of a MALDI MS proteomics-based method for the reliable detection of blood and blood provenance

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Tables

Intravenous BOVINE blood -peptide *m/z*	Mass accuracy (ppm)	Blind sample 38 (BOVINE) - peptide *m/z*	Mass accuracy (ppm)
αHB 1071.554	0.6	---	
αHB 1529.734	0.4	---	
αHB 1833.892	-0.4	---	
βHB 1265.828	-2.2	---	
βHB 1274.723	-1.9	**βHB** 1274.706	15
βHB 1422.716	-7.6	---	
βHB 1477.801	-0.7	---	
βHB 1752.898	-0.6	---	
βHB 1868.951	-1.6	---	
		GAPDH 795.418	-3.0
		GAPDH 805.432	1.1
		GAPDH 1032.595	-6.6
		GAPDH 1358.681	-9.5
		GAPDH 1369.743	-5.5
		GAPDH 1369.743	-5.5
GAPDH 1499.776	-8.2		
		GAPDH 1499.789	-7.2
		GAPDH 1615.880	2.5
		GAPDH 1763.802	2.5
		myoglobin 629.343	3.9
		myoglobin 748.433	-3.2
		myoglobin 1271.668	3.8
		myoglobin 1393.821	3.8
		myoglobin 1592.838	-0.7

		myoglobin 1669.834	-1.3
Intravenous PORCINE blood -peptide *m/z*	**Mass accuracy (ppm)**	**Blind sample 7 (PORCINE) - peptide *m/z***	**Mass accuracy (ppm)**
αHB 1041.540	-3.6	---	
αHB 1115.631	-10.1	---	
αHB 1422.704	-3.2	---	
αHB 1628.904	-4.8	---	
αHB 1876.893	-2.3	---	
αHB 1935.972	-3.2	---	
βHB 1265.830	-2.2	---	
βHB 1274.726	-1.9	---	
βHB 1449.796	-7.6	---	
βHB 1866.012	-7.2	---	
		GAPDH 795.422	4.3
		GAPDH 977.547	5.4
GAPDH 1499.778	7.4		
		GAPDH 1763.813	6.1
		myoglobin 1592.820	-11.9

Table S1. Putative identification of haemoglobin, glyceraldehyde 3-phosphate hydrogenase (GAPDH) and myoglobin in blind porcine and bovine samples versus the corresponding intravenous blood samples.

Figures

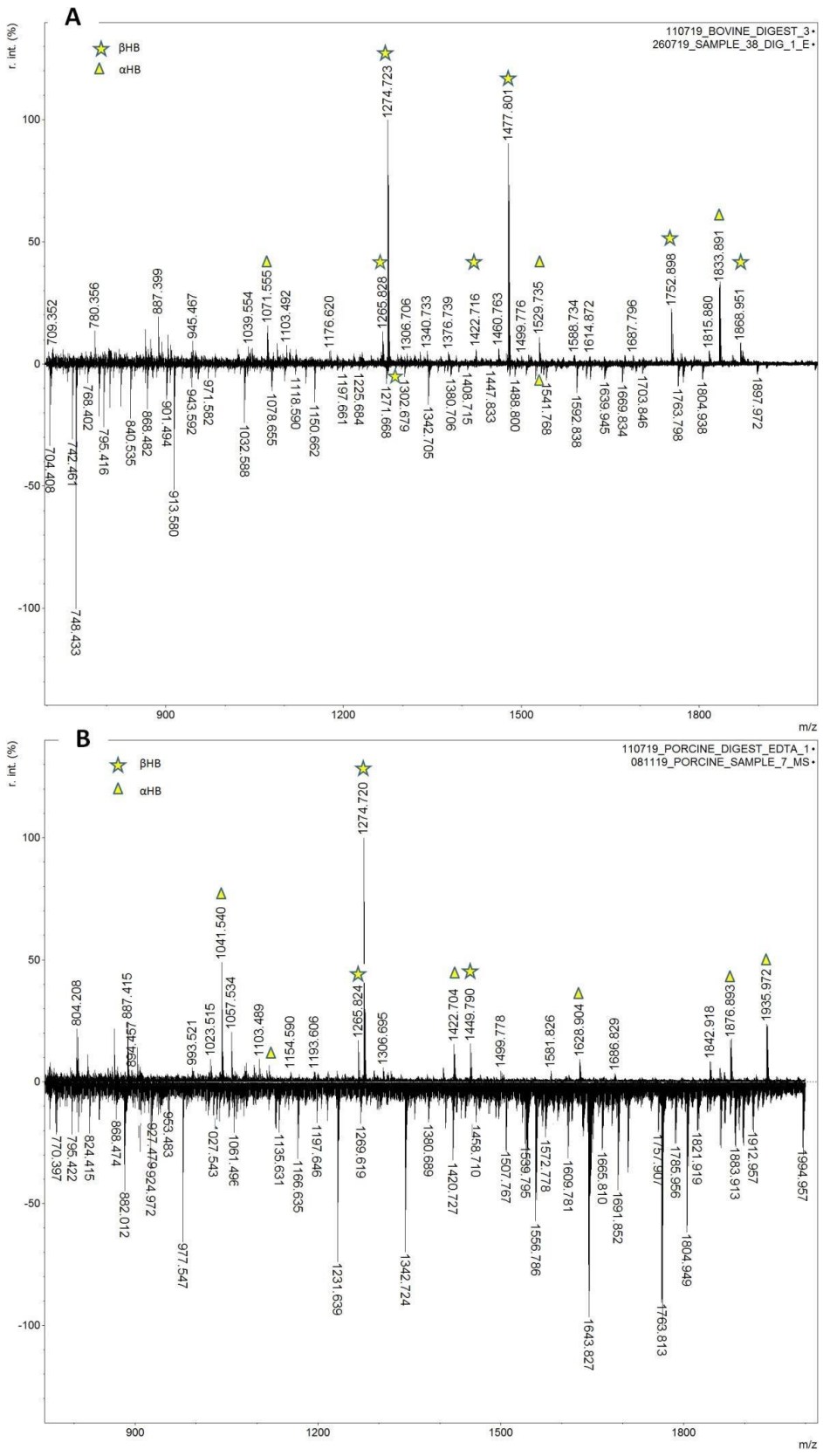
A
βHB
αHB
110719_BOVINE_DIGEST_3
260719_SAMPLE_38_DIG_1_E
r. int. (%)
m/z
B
βHB
αHB
110719_PORCINE_DIGEST_EDTA_1
081119_PORCINE_SAMPLE_7_MS
r. int. (%)
m/z

Fig. S1. MALDI MS spectral comparison of intravenous reference blood and blind blood samples for bovine (A) and porcine (B). The spectral profiles appear different in both cases with haemoglobin (HB) signals only present in the intravenous samples but not in the blind samples.

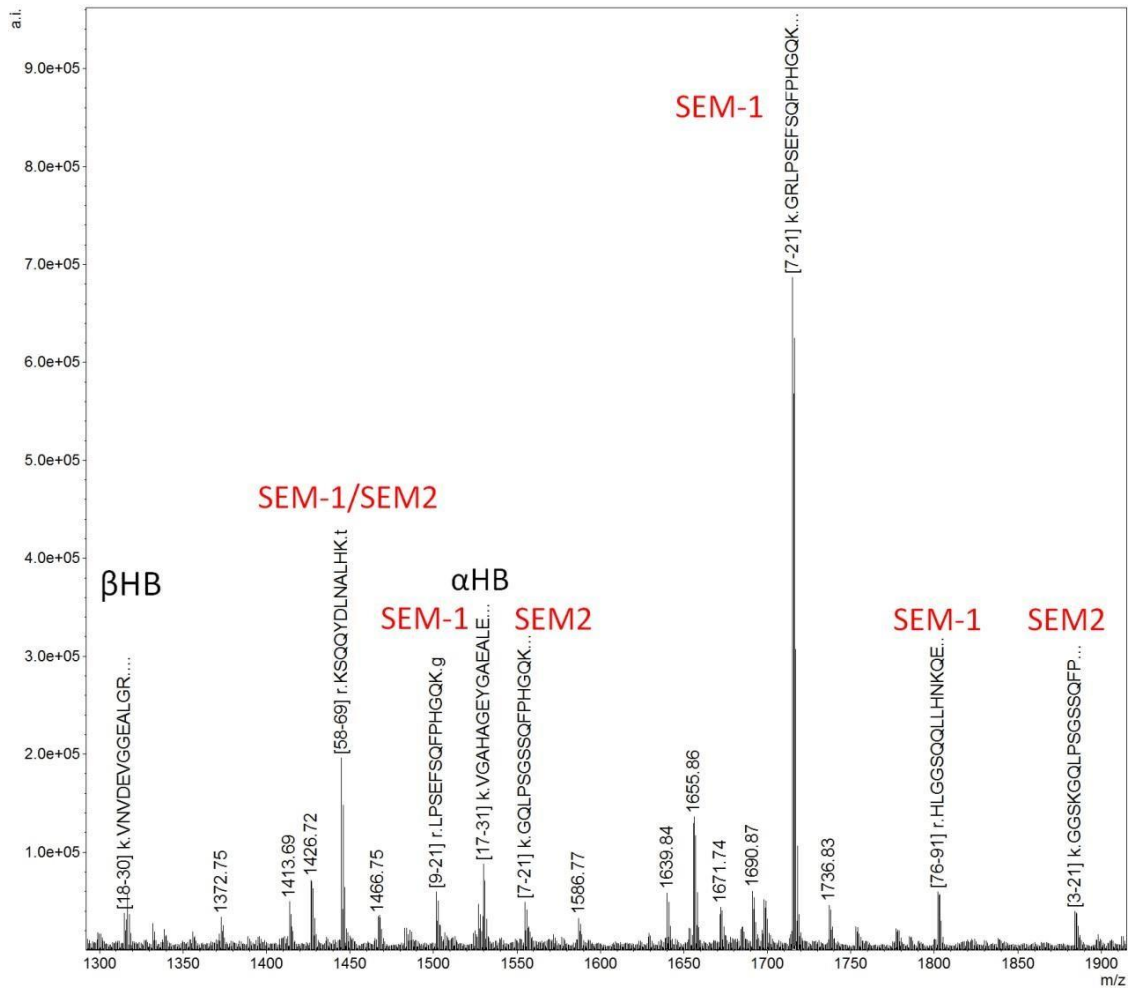

Fig. S2. MALDI MS spectrum of blind sample 32 (Semen). Haemoglobin and semenogelin 1 and 2 ion signals were putatively assigned except for *m/z* 1714.85 which was confirmed by MS/MS.

Accession numbers list of putatively identified and confirmed proteins

Bovine GAPDH (UniProtKB-P10096)

Bovine Haemoglobin (alpha chain) (UniProtKB - P01966) Bovine Haemoglobin (beta chain) (UniProtKB - P02070)

Bovine Myoglobin (UniProtKB - P02192)

Chicken GAPDH (UniProtKB- P00356)

Chicken Haemoglobin (alpha chain) (UniProtKB - P01994) Chicken Haemoglobin (beta chain) (UniProtKB - P02112) Human Erythrocyte protein band 4.2 (UniProtKB - P16452) Human Semenogelin -1(UniProtKB - P04279)

Human Semenogelin-2 (UniProtKB - P04279)

Human Haemoglobin (alpha chain) (UniProtKB - P69905) Human Haemoglobin (beta chain) (UniProtKB - P68871) Porcine GAPDH (UniProtKB - P00355)

Porcine Haemoglobin (alpha chain) (UniProtKB - P01965) Porcine Haemoglobin beta chain) (UniProtKB - P02067) Porcine Myoglobin (UniProtKB - P02189)

CHAPTER 2: STATEMENT OF CONTRIBUTIONS

Date	Work Completed
21.3.19	Started the analysis of blind samples **with Cameron Heaton** (in solution digest protocol training provided by Cameron + supervisors) In solution digests and MALDI MSP of samples 40,57,58,59
22.3.19	In solution digests and MALDI MSP of samples 30, 31, 32, 34
2.5.19	In solution digests and MALDI MSP of samples 5, 10, 12, 13
7.5.19- 10.5.19 (ALL work detailed after this day was completed by **me only**)	In solution digests and MALDI MSP of samples 18, 20, 23, 28, 29, 44, 46, 49, 51, 63, 66.
22.5.19-23.5.19	Chicken, porcine, and bovine blood in solution digests (purchased from TCS Biosciences) and MALDI MSP analysis.
5.6.19-6.6.19	In solution digests and MALDI MSP of Samples 107, 114, 121, 140, 154, 23, 29, 10, 20, 63, 17, 28, 46, 66 (some samples were re-analysed from previous batches).
8.7.19	In solution digests from the original blind samples, followed by MALDI MSP: 1, 4, 7, 14, 24, 30, 32, 35, and 36, 37 (Trypsin + Rapigest), 37 (digest), 41, 44, 56, 72, 76, 79, 104, 107, and 170.
9.7.19	MALDI MS/MS on semen sample and repeat of in solution digest for sample 30.
10.7.19	Zip-tipping packaged meat digests (bovine, porcine and chicken) - Identified *m/z* 1764, *m/z* 1670 and *m/z* 1750
18.7.19	MALDI MS/MS of species markers in packaged meat (bovine, porcine and chicken: m/z 1750 and 1764), semen markers (1445 and 1715) in Sample 32.
23.7.19	In solution digests and MALDI MSP of samples in final validation set: 15, 22, 25, 27, 38, 46, 51, 55, 66, 121, 127, 128, 129, 138, 144, 146, 147, 155, 156, 176.
28.7.19	Re-zip tipping digests and MALDI MSP of Samples: 27, 38, 55, 121,

	128, 129, 144, 146, 147, 155, 156, and 176.
29.7.19	MS/MS of blind study species markers *m/z* 1444, 1714, 1912, 1763 and 1749.
12.9.19	Attempt of *in situ* digest and MALDI MSI on sample 108
25.9.19	Hydrophobin approach with chicken, porcine and bovine reference blood samples and MALDI MSP.
11.10.19	Hydrophobin approach with packaged meat blood and animal reference blood samples and MALDI MSP.
18.10.19	*in situ* digest and MALDI MSI on sample 109.
23.10.19	Hydrophobin approach with directly swabbing packaged meat, and MALDI MSP.
24.10.19	Hydrophobin approach with zip-tipped and non-ziptipped packaged meat digests and MALDI MSP.
29.10.19	*in situ* digest and MALDI MSI on sample 109.
29.10.19	Butcher meat digests and MALDI MSP (bovine, porcine and chicken).
1.11.19	MS/MS (*m/z* 1764 *and m/z* 1750) in butcher meat packets.
23.11.20	MS/MS of blind samples via XEVO (Tom Clarke did this work).
9.12.19	Sample 119 *in situ* profiling and imaging attempts

Chapter 3

Kennedy K, Bengiat R, Heaton C, Herman Y, Oz C, Elad ML, Cole L, Francese S. "MALDI-CSI": A proposed method for the tandem detection of human blood and DNA typing from enhanced fingermarks. Forensic Sci Int. 2021 Jun;323:110774. doi: 10.1016/j.forsciint.2021.110774. Epub 2021 Apr 22. PMID: 33930825.

"MALDI-CSI": A proposed method for the tandem detection of human blood and DNA typing from enhanced fingermarks

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"MALDI-CSI": A proposed method for the tandem detection of human blood and DNA typing from enhanced fingermarks

Abstract

Matrix Assisted Laser Desorption Ionization Mass Spectrometry Profiling and Imaging (MALDI MSP and MALDI MSI), in combination with bottom up proteomics, have proven to successfully detect and map blood-derived peptide signatures in blood fingermarks, with high specificity and compatibility with a number of blood enhancement techniques (BET). In the present study, the application of MALDI MSP and MSI to blood marks has been investigated further. In particular, the MALDI based detection and visualisation of blood has been explored in tandem with DNA typing. This investigation has been undertaken in a scenario simulating blood fingermarks on painted walls. In the present study, two sets of marks were analysed with each set comprising of a depletion series of four marks deposited on a surface treated to simulate painted walls: Set I - developed with Ninhydrin (NIN) and Set II- developed with Acid Black-1 (AB-1). For both sets, the application of MALDI MSP was successful in detecting haem and human specific haemoglobin peptide markers. MALDI MSI also provided molecular images by visualising haem on the ridge pattern enhanced by BET. The feasibility of successful and subsequent DNA profiling from the recovered fingermarks was also assessed for marks that had undergone enzymatic *in situ* digestion and MALDI MSI; it was observed that in 73% of the samples analysed, a DNA profile suitable for comparison was obtained. Based on these results, a possible operational workflow has been proposed incorporating the use of a MALDI MS based approach as a confirmatory test for human blood enabling subsequent DNA typing.

Keywords: MALDI, blood enhancement techniques, fingermarks, STR, DNA typing, crime scene.

Introduction

In violent crimes, such as murder, blood-contaminated fingermarks may be key evidence for crime scene investigators (CSI), as they can provide associative evidence by directly linking the suspect to the crime. These marks, which are commonly found on the walls of crime scenes, may be visible, partially latent or latent, with the latter requiring the application of enhancement techniques to visualise the ridge detail. Ninhydrin (NIN) and Acid Black 1 (AB-1) react with either the amino acids or the proteins in the blood, respectively [1] and are amongst the most commonly used enhancement agents for operational application on surfaces such as painted walls.

A fingermark suspected of being contaminated with blood may provide extra evidential weight if it meets three essential requirements: 1) determination of the presence of human blood in the mark to connect it to the crime; 2) sufficient ridge detail to provide the identity of the suspect by fingermark comparison; 3) full DNA profiling from the blood mark for confirmation of the victim's (most likely) identity.

The forensic determination of blood in marks or stains is generally conducted by using two main presumptive field tests: 1) Kastle-Meyer test to detect haem groups; and 2) Immunological tests, such as the Hexagon OBTI test for the identification of human blood. The former is not specific for human blood and prone to false-positives; the latter is considered more specific but may lead to false-negative results, especially after certain enhancement techniques are applied such as AB-1 and NIN. In addition, it has been reported that the sensitivity of the Hexagon OBTI test decreases with the ageing of the sample and when stored at temperatures above 4ºC [2], which may also affect the result of the test. Additionally, due to the presumptive nature of other tests employed for blood enhancement, a reliable, sensitive, robust and legally admissible method is necessary to confirm the presence of human blood in order for such evidence to be confidently presented in court.

One of the most published techniques for the analysis of latent fingermarks in recent years is Matrix Assisted Laser Desorption Ionisation (MALDI) Mass Spectrometry, in both profiling and imaging modes (MALDI MSP and MALDI MSI) [3]. Since 2014, these techniques have been adapted and extended as confirmatory tests for the detection of blood in stains and fingermarks in laboratory settings. Initial work enabled confirmation of blood presence through the detection of haemoglobin and haem [4] at concentrations of 1000 and 250,000,000 times lower than the physiological value, respectively. In one example reported by Francese [5], it was shown that, where NIN failed to enhance blood in the third mark of a depletion series, MALDI MSI yielded an image of the ridge pattern through the visualisation of haem. Compatibility with the prior application of AB-1 and NIN was demonstrated in fresh as well as aged specimens, such as a 9 year old blood palm print [6] and a 37 year old NIN enhanced mark on fabric [5]; there, a bottom up proteomic approach was employed, yielding the detection of multiple blood specific proteins thus increasing the specificity of the method. These proteins were visualised onto the ridge detail of blood marks in both fresh and 4 year old fingermarks [7,8]. Kamanna *et al* [9] adapted the approach to generate MALDI MS images of haemoglobin tryptic peptides discriminating between human blood and blood from a range of Australian mammals. Recent work by Kennedy *et al* [10] demonstrated extended compatibility with blood enhancement techniques (BET) and, in a blind study, confirmed the ability to discriminate blood from other biofluids and its provenance, down to animal species, within the system investigated. Importantly, it was also demonstrated that these MALDI

based methods correctly reported on the presence/absence of blood when the presumptive tests exhibited false positive/negative results. Given MALDI MSP and MSI capabilities as confirmatory tests for the detection of human blood from fingermarks, it is important to fully elucidate its potential use in a comprehensive forensic workflow where BET application is followed by DNA typing for downstream analysis.

Whilst MALDI MSP has shown, in a rare application of this technique, an ability to detect DNA fragments [11,12], it failed to provide full DNA profiles from blood or any other source [11]. In another report, Kamanna *et al* [12] showed that MALDI MS was unsuccessful when applied to blood detection following classic DNA profiling, due to the latter procedure removing trace proteins. Therefore, from an operational perspective, the application of MALDI MS based analyses downstream has been dismissed here and the hypothesis has been investigated that the application of this technique to blood marks does not prevent successful retrieval of DNA for further short tandem repeat (STR) analysis. It is known that short UV light wavelengths induce DNA degradation and should be avoided when subsequent DNA profiling is required [14]. However, for UV MALDI it is not known whether the irradiation at 337 or 355 nm (typical MALDI UV laser wavelengths used) is detrimental to DNA typing.

In this work, the first of its kind, MALDI MSP and MSI were evaluated for their compatibility and effectiveness in a multi-sequence processing of blood fingermark evidence, including DNA recovery and typing. At real crime scenes, the blood and the mark may come from different individuals. In the case of blood marks, a connection of the suspect to the crime is made by matching the mark to a fingerprint of perpetrator and the blood to that of the victim (which is generally present in much larger amounts). In this study the same donor was used to produce the blood and the mark as the sole purpose was to assess whether DNA profiling is possible at all after the application of MALDI MSP and MSI, employed to determine the presence of blood and its human origin. This work has been conducted in a simulation of a "real crime-scene" scenario in which blood marks are present over a simulated painted wall, and have been subjected to a workflow where they have been: 1) enhanced with either NIN or AB-1, 2) analysed by MALDI MSP and/or MSI and 3) finally subjected to DNA extraction, quantification, amplification and profiling.

Methods and Materials

1.0 Materials

Trifluoroacetic acid (TFA) and α-cyano-4-hydroxycinnamic acid were purchased from Sigma-Aldrich (Poole, UK). Acetonitrile was purchased from Fisher Scientific (Loughborough, UK). Trypsin Gold was obtained from Promega (Southampton, UK). RapiGest^{TM} was obtained in 1 mg vials from Waters (Wilmslow, UK). Aluminium plates were purchased from Sigma Aldrich (Israel) and acrylic matte white-wash water-based paint was purchased from Nirlat (Israel). Ninhydrin (NIN), ethyl acetate, glacial acetic acid and ethanol were purchased from Bio-Lab (Israel) and HFE-7100 was purchased from 3M (Israel). 5-sulfosalycilic acid was purchased from Sigma-Aldrich, (Israel) and Acid Black-1 (AB-1) was purchased from Spectrum (Israel). VACUETTE (R) Tubes containing EDTA (K3EDTA) were purchased from Greiner Bio-One and sterile gauze pads 100% cotton were obtained from NISSAN Medical Industries, (Israel).

2.0 Instrumentation and Instrumental Conditions

2.1 MALDI MSP and MSI Analysis

Two MALDI quadrupole time-of-flight (QTOF) systems were employed in this study; the Synapt G2 HDMS mass spectrometer (Waters Corp. Manchester, UK) and the QStar Elite mass spectrometer (Applied Biosystems, Concord, Ontario, Canada). The former is equipped with a Nd:YAG laser set at repetition rate 1 kHz at a wavelength of 355 nm and the latter equipped with an SPOT 10 kHz Nd:YVO4 solid state laser (Elforlight Ltd, Daventry, UK), operating at 1 kHz at a wavelength of 355 nm, with an elliptical spot size of 100 x 150 µm and a pulse duration of 1.5 ns. Prior to any analysis, 0.5 µL of phosphorus red saturated solution in acetonitrile was used as calibrant for each instrument. MALDI QTOF instruments were calibrated in the range *m/z* 600 - 2500 (Synapt G2) and 100 - 1000 (QStar Elite). *MALDI MSP*- when using the Synapt G2 instrument, MALDI MS spectra were acquired in positive sensitivity mode in the range *m/z* 600 - 2500; positive mode was also selected on the QStar Elite although the aquisitionrange was set between *m/z* 100 - 1000; on the QStar Elite, the declustering potential was set at 15 and the focussing potential at 20 a.u, respectively. *MALDI MSI* - When using the MALDI Synapt G2, MALDI MSI data were acquired in positive sensitivity mode at a mass resolution of 10,000 FWHM with ion mobility separation enabled over the calibrated mass range. MALDI MS images were acquired in the *m/z* range 600 - 2500, at 100 x 100 µm spatial resolution, using variable IMS wave velocity, with the initial wave velocity and an end velocity (m/s) set at 900 and 100, respectively. When the MALDI QStar Elite was employed, all images were acquired in the *m/z* range 100-1000, in ‘slowest’ raster mode at a 100 x 150 µm spatial resolution.

3.0 Data Processing

Mass spectra were viewed both in MassLynx™, (Waters Corp., Manchester, UK) and in mMass, an open source multifunctional mass spectrometry software [17,18], upon conversion of the spectra into .txt files. Peak assignments were performed using an excel macro to aid manual assignments of all the peaks characterised by S/N>3:1. Ion signal annotations in mMass were made using an in-house database consisting of all theoretical *m/z* values for the blood specific peptides for human blood.

4.0 Methods

4.1 Preparation of deposition surface

Aluminium plates were coated with two layers of an acrylic matte white-wash water-based paint with each layer left to dry for at least 24 hrs prior to applying the second coat of paint. The plates thus prepared simulated a "painted wall" and were used to deposit blood fingermark depletion series. For blood marks deposited underneath the paint, a third coat of paint was applied over the blood fingermark (FM) 24 hrs after deposition. The blood fingermarks were generated by the same donor providing the blood and the "fingertip stamp" to produce this evidence.

4.2 Deposition of blood-contaminated fingermarks

Blood was collected from one donor in 4 mL sterile test tubes (BD Vacutainer) containing EDTA to prevent blood-clotting. A sterile medical pad was soaked with blood. Subsequently a fingertip contacted the pad prior to touching the painted surface in a depletion series of 4

fingermarks (FM). The samples were left to dry for over 24 hrs prior to development. Two sets of blood fingermarks were analysed two weeks after preparation: Set I - developed with NIN; Set II- developed with AB-1. In addition, two undeveloped control blood fingermarks were made over the paint.

4.3 Fingermark development

Development by NIN was performed by spraying a solution containing 5 g of NIN in 2 mL of ethyl acetate, 5 mL glacial acetic acid, 45 mL ethanol and 1 L HFE-7100. Development by AB-1 was performed in three stages: 1) spraying the samples with a fixing solution containing 20% aqueous 5-sulfosalycilic acid and waiting 15 min for denaturation to occur; 2) spraying with the development solution comprising of 1 g AB-1, 50 mL glacial acetic acid, 250 mL ethanol and 700 mL water; 3) spraying a washing solution containing 50 mL glacial acetic acid, 250 mL ethanol and 700 mL water to reduce the background staining. The developed blood fingermarks were allowed to dry overnight in a closed dark box at room temperature of 23.5°C and 42% relative humidity and then visualised under white light and photographed using the DCS5 system (Foster & Freeman). The images were saved in a 1:1 scale and 1000 dpi greyscale mode as per the standard requirements for operational work.

4.4 Extraction and enzymatic digestion of enhanced blood fingermark depletions.

Trypsin Gold and RapiGest™ were prepared immediately prior to proteolysis as at 20 µg/mL in 50 mM ammonium bicarbonate, containing 0.1% RapiGest (v/v). RapiGest™ (0.1% v/v in 50 mM ammonium bicarbonate solution) was added to Trypsin Gold (150 µg/mL in 50 mM ammonium bicarbonate solution) for *in situ* proteolysis and subsequent MALDI MSP analysis. For MALDI MSI analyses, 9 layers of the enzyme and surfactant solution were sprayed using the SunCollect automatic sprayer at a flow rate of 2 µL/min for each layer (SunChrom, Germany). The samples were then incubated for 3 hrs at 37ºC and the proteolytic digestion was stopped with the spray-coating of α-CHCA matrix. For *in-solution* digests, Eeach blood fingermark sample was swabbed with 70/30 ACN:H_2O. The swab head was removed using scissors and transferred to a 1.5 mL eppendorf containing 1 mL of 70:30 ACN:H_2O prior to sonication for 10 minutes. Subsequently, 10 µL of the extract was added to 40 µL of 40 mM Ammonium Bicarbonate and to 9 µL of trypsin. The *in-solution* extract was incubated for 1 hr at 37ºC and the proteolytic digestion was stopped with the addition of 2 µL of 5% TFA. Digests were stored at -80ºC until analysis.

4.5 Matrix application

α-CHCA matrix at a concentration of 10 mg/mL in 70:30 ACN:0.5% TFA was deposited by spotting 0.5 µL on top of the sample for MALDI MSP experiments. For MALDI MSI experiments, 5 mg/mL α-CHCA was sprayed post-enzymatic digestion using the SunCollect automatic sprayer, depositing 4 layers at a flow rate of 4 µL/min, at a nitrogen pressure of 3 bar.

4.6 DNA extraction, quantification, amplification and data analysis

DNA extraction from blood fingermarks and the positive control DNA (007) was carried out using the PrepFiler BTA™ Forensic DNA Extraction Kits (Life Technologies, Foster City, CA) [19] according to sample type, as determined by the operational protocols. Quantification was carried out using the Investigator Quantiplex Pro Kit® (QIAGEN®) on a 7500 Real-Time PCR System™ device (Applied Biosystems®, Israel) according to the manufacturer's

protocol. Quantification results were processed in Microsoft Excel. Amplification of extracted and quantitated DNA was performed using the VeriFiler™ Plus PCR Amplification 24 Kit (Applied Biosystems®, Israel) on the GeneAmp® PCR System 9700 cycler (Applied Biosystems®,Israel) [20]. Electrophoresis was performed using Performance Optimized Polymer (POP-4™) on a 3500xL Genetic Analyzer™ (Applied Biosystems®, Israel) Fragment analysis was conducted using GeneMapper@ ID-X software (Applied Biosystems®,Israel).

Results and Discussion

The present study initially assessed MALDI MSP and MSI based approaches for the detection/mapping of blood in marks 1) over a simulated painted wall, enhanced by either NIN or AB-1 and 2) underneath the paint. Subsequently, MALDI MSP and MSI analyses were followed by DNA extraction, quantification and profiling. This further investigation gained insights into whether or not MALDI MSP/MSI interfered with a subsequent DNA STR analysis. Two sample sets were prepared as depletions series of four blood marks (Fig. 1) each as follows: Set I - blood marks over the paint developed with NIN; Set II- blood marks over the paint developed with AB-1 (Fig. 1A). The first blood mark of each depletion series was split into 3 sections to carry out a number of different analyses (Fig 1B); generally, the bottom half was reserved for extraction and MALDI MSP analysis. The extract was either directly analysed in the low mass range to detect haem (MALDI QTOF QStar Elite), or proteolysed and analysed in the peptide mass range (to detect blood specific protein-deriving peptides (MALDI QTOF Synapt G2). The upper left and right quarters were reserved to direct haem imaging (QStar Elite) or digested *in situ* and then imaged in the peptide range (Synapt G2), respectively (Fig 2C). Each 2nd mark of the depletion series was split into two halves and reserved either to haem imaging (QStar Elite) or peptide imaging (Synapt G2); the 3rd and 4th marks of each depletion series were submitted to MALDI MSP only (on both MALDI QTOF instruments).

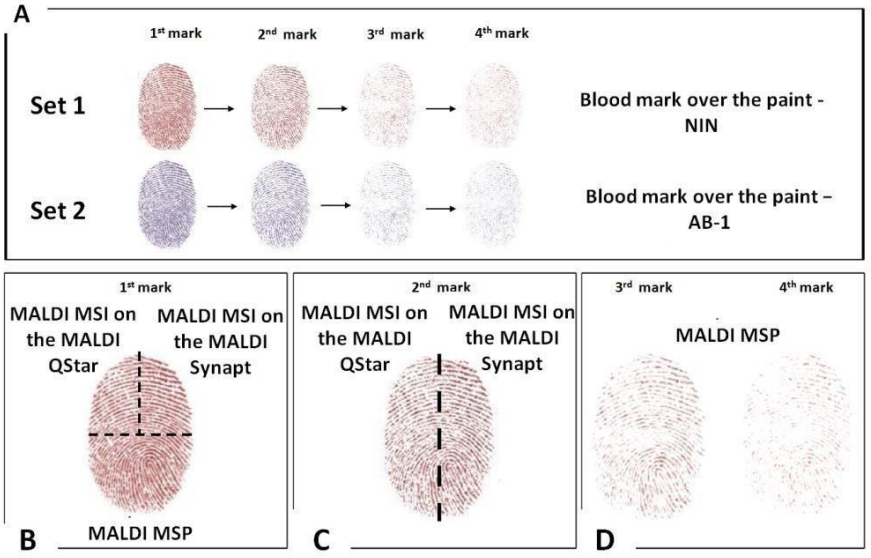

Fig. 1 Preparation of blood marks and analyses performed. (A) Schematic of the blood fingermarks depletion series (sets 1 and 2) prepared for the study. (B) Schematic of the splitting of the 1st blood mark of each depletion series for: MALDI MSP analysis on both

MALDI QStar Elite and Synapt G2 instruments, MALDI MSI of upper left quarter on MALDI QTOF instrument and MALDI MSI of upper right quarter, after enzymatic digestion on MALDI Synapt G2 *Si*. (C) Schematic of the splitting of the 2nd blood mark of each depletion series into two halves for: MALDI MSI on the QStar Elite and Synapt G2 instruments. (D) The 3rd and 4th mark of each depletion series were reserved for MALDI MSP of haem (on the QStar Elite) and blood derived peptides (on the Synapt G2) after enzymatic digestion.

MALDI MSP and MSI of untreated marks - Initial profiling analysis of the paint yielded a densely ion populated spectrum in the *m/z* range 600-2000 exhibiting the expected α-CHCA matrix clusters. However, in addition, a PEG based polymer was also detected (Fig. S1) across the entire mass range acquired, indicating a potential interference or ion suppression of blood molecules of interest, especially peptides, when analysing blood mixed with paint. However, the presence of this polymer was variable across the three replicates of the paint analysed, likely as a result of differential co-crystallisation with the matrix due to varying levels of "polymer segregation".

This is a known phenomenon in MALDI and was reported in previous work on polymeric condom lubricants [21]. It is possible that PEG based polymers could be used as additives and paint preservatives and contribute to the water-based nature of some paints. If that is the case, the detection of PEG was not surprising.

Two untreated (control) blood marks over the paint were analysed by either MALDI MSP or MSI. Prior to the MALDI MSI analysis, the mark was enzymatically digested. The recent study by Kennedy *et al.* [10] has determined that, within the system investigated, if both signals at *m/z* 1274.726 and 1529.734 were present (β and α haemoglobin, respectively) not only can blood presence be claimed but also human provenance can be confirmed. Therefore, particular focus was given to the detection of the two peptides at *m/z* 1274.726 and 1529.734 for detection and confirmation of human blood in enzymatically digested samples.

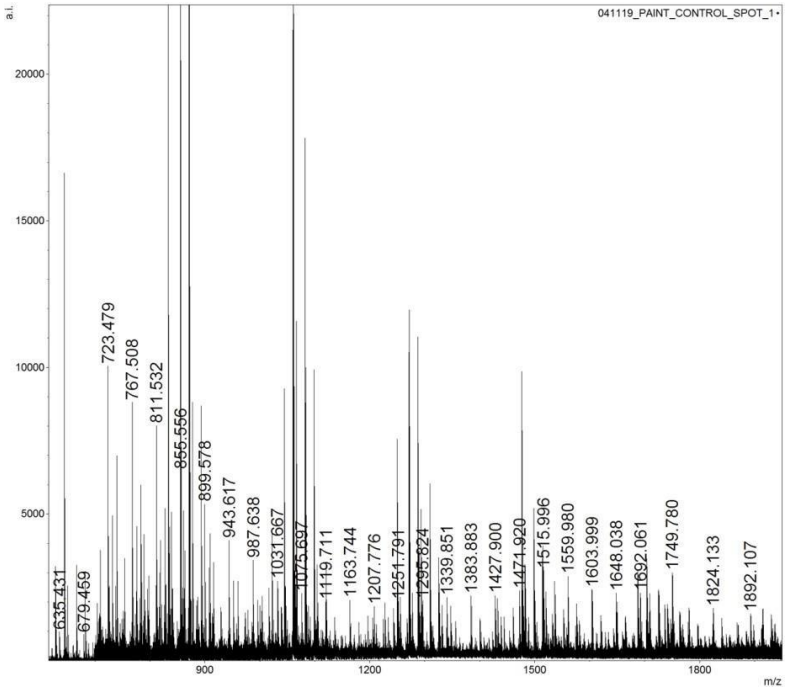

Fig. S1 MALDI MSP spectrum of acrylic paint used to simulate blood marks on walls in this study. The spectrum shows the presence of PEG polymers through ion signals distanced 44 units apart.

In profiling mode and on the Synapt G2, it was possible to detect both haem at *m/z* 616.094 and haemoglobin peptides, including those at *m/z* 1274.736 and *m/z* 1529.727 (Fig. 2A). Another blood mark (half) was subjected to MALDI MSI in the range *m/z* 100 - 1000 and a molecular image of haem at *m/z* 616.2 was obtained and superimposed onto the optical image of the developed blood mark (Fig. 2B). Haem co-localised with the areas where blood presence was revealed by NIN. However, the molecular image of haem, obtained (on the Qstar Elite) by normalising against on ubiquitous signal at *m/z* 855.6 did not permit visualisation of the ridges that were observable in the optical image. An analysis of the matrix deposition showed that the MALDI matrix was not deposited homogeneously across the mark and whilst molecular information is still detectable, images, in this circumstance, are generally compromised.

The non-homogeneity of the matrix could be due to the presence of the PEG based polymer, disrupting crystallisation. Another hypothesis is a potentially undetected discontinuous matrix spray of the automatic sprayer employed.

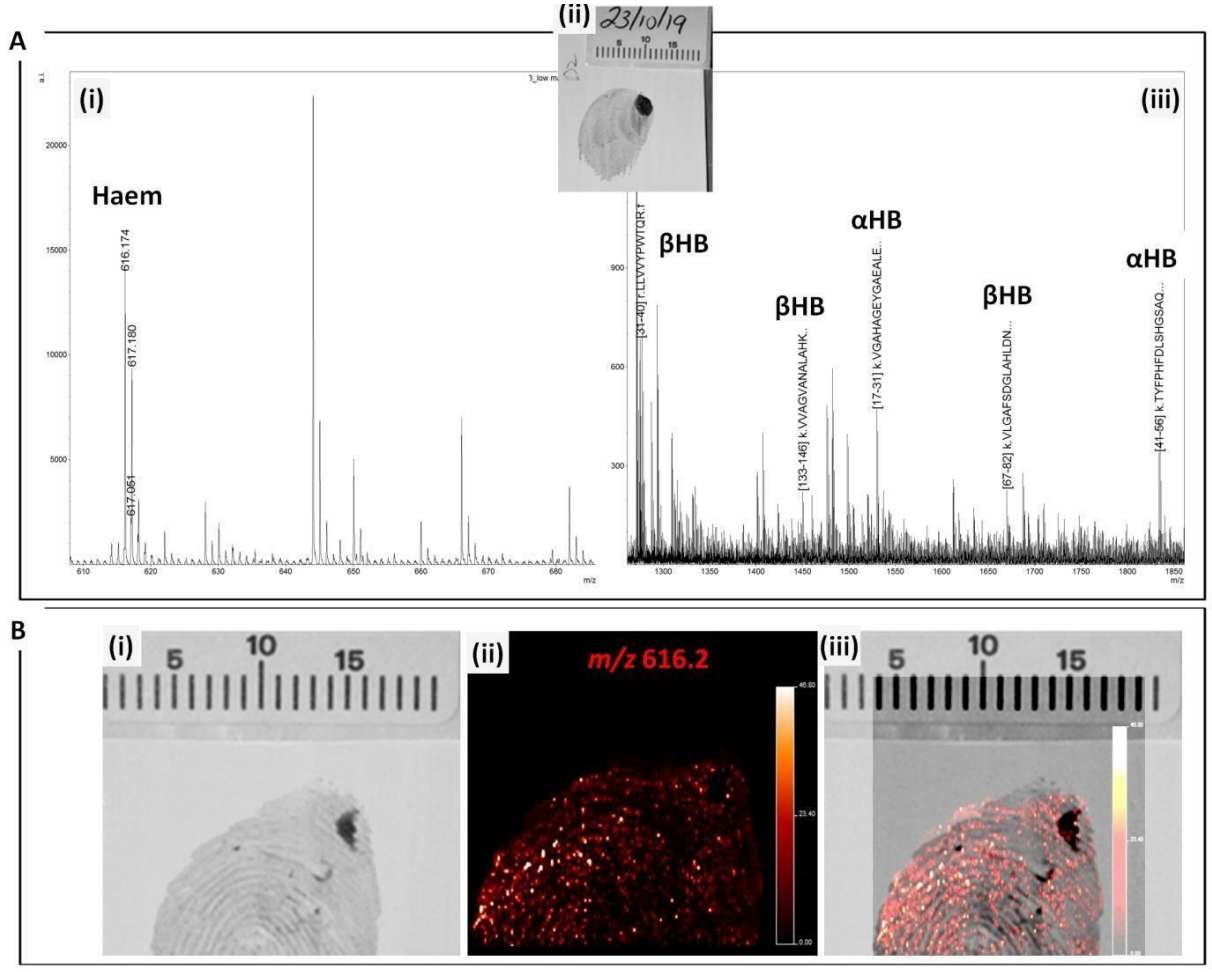

Fig. 2 MALDI MSP and MSI of two control blood marks (undeveloped) over the paint. A: MALDI MS spectra of haem (i) and haemoglobin peptides (iii) from an untreated control

blood mark (iii). B: Images of a second undeveloped blood mark over paint: (i) optical image, (ii) MALDI MS image of haem at *m/z* 616.2 acquired on the MALDI QStar Elite and (iii) the overlay of the MS image of haem with the optical image of the NIN developed blood mark.

MALDI MSP and MSI of enhanced marks over the paint - The application of MALDI MSP aimed to assess whether blood could be detected from enhanced blood marks over paint and, specifically, from which of the marks within the two depletion series investigated. All MALDI MSP analyses were run in triplicates. However, in this paper, only one replicate is shown. MALDI MSI was employed to additionally assess whether blood specific protein deriving-peptides could be visualised on the ridges of the mark, compatibly with the application of either NIN or AB-1, as observed in previous work [5-6,10], as well as "filling in the ridge pattern gaps" left by the enhancement through these presumptive tests.

Set I: *Blood fingermarks over paint enhanced with NIN (FMO-NIN)* - Haem was detected both at nominal *m/z* 616 (expected) and 617 across FMO-NIN-1, FMO-NIN-3 and FMO-NIN-4 marks (Fig. 3). The difference of 1 mass unit is due to the reduction of the haem iron from Fe^{3+} to Fe^{2+} and this has been observed previously [22]. The HB peptides at *m/z* 1071.557, 1529.749, 1833.898 (αHB, 2.8, 9.7 and 3.1 ppm mass accuracy respectively), 1274.729, 1314.669, 1449.794 (βHB, 2.7, -3.3 and -1.5 ppm mass accuracy respectively) and one Complement C3 protein deriving peptide at *m/z* 1930.079 (-1.9 ppm mass accuracy) have been detected in the 1st mark of the NIN depletion series. The 3rd and the 4th marks of the depletion series showed increasing PEG based polymer presence to the extent in which the 3rd mark (FMO-NIN-3) only exhibited 3 HB peptides, just above the S/N 3:1, at *m/z* 1529.730 and 1833.895 (αHB, -3.1 and 1.9 ppm mass accuracy, respectively) and 1274.716 (αHB, -7.3 ppm mass accuracy) and no peptides were detected in the 4th mark (FMO-NIN-4) (Fig. 3).
Overall, MALDI MSP indicated the presence of blood in the three marks of the NIN depletion set examined. For FMO-NIN-4, the presence of blood (through detection of haem) could be established but not its provenance.

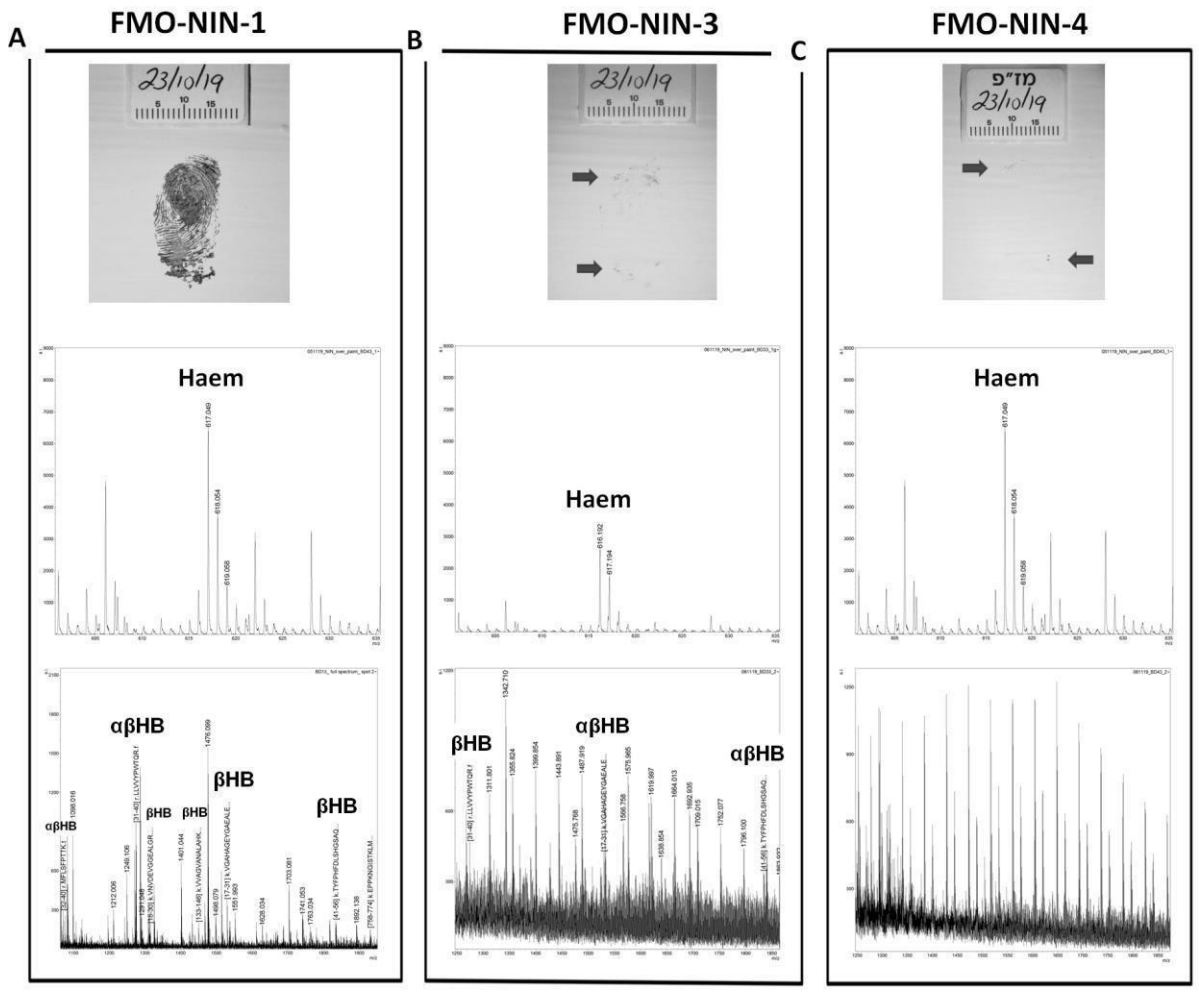

Fig. 3 MALDI MSP of 1st (FMO-NIN-1), 3rd (FMO-NIN-3) and 4th (FMO-NIN-4) mark of the Set I of blood fingermark depletion series deposited over the paint and enhanced with NIN. MALDI MS spectra of haem and HB peptides are shown for each mark.

MALDI MSI was applied to the 1st (FMO-NIN-1) and 2nd mark (FMO-NIN-2) of Set I depletion series. Imaging capabilities were confirmed for NIN enhanced blood marks "over paint" where molecular images of haem at *m/z* 616.2 could be generated albeit with no additional ridge detail (Fig. 4).

The marks were deposited without ensuring clarity of the ridge detail; therefore originally smudged areas could not be resolved by MALDI MSI. The overlay of the haem images with the optical images of the NIN enhanced blood marks shows haem localisation where NIN revealed the presence of blood, thus acting as a confirmatory test. Haem images were generated with and without normalisation against the total ion current (TIC). Normalisation yields a true reflection of the presence/location of the species that is being normalised. However, occasionally, depending on the distribution of the species against which normalisation occurs, this process may compromise clarity of some areas of the ridge pattern. The provision of both normalised and non-normalised images yields complementary ridge flow/details. Haem was confirmed by the presence of its in-source ion fragment at nominal *m/z* 557 which was also previously observed by Bradshaw *et al.* [4].

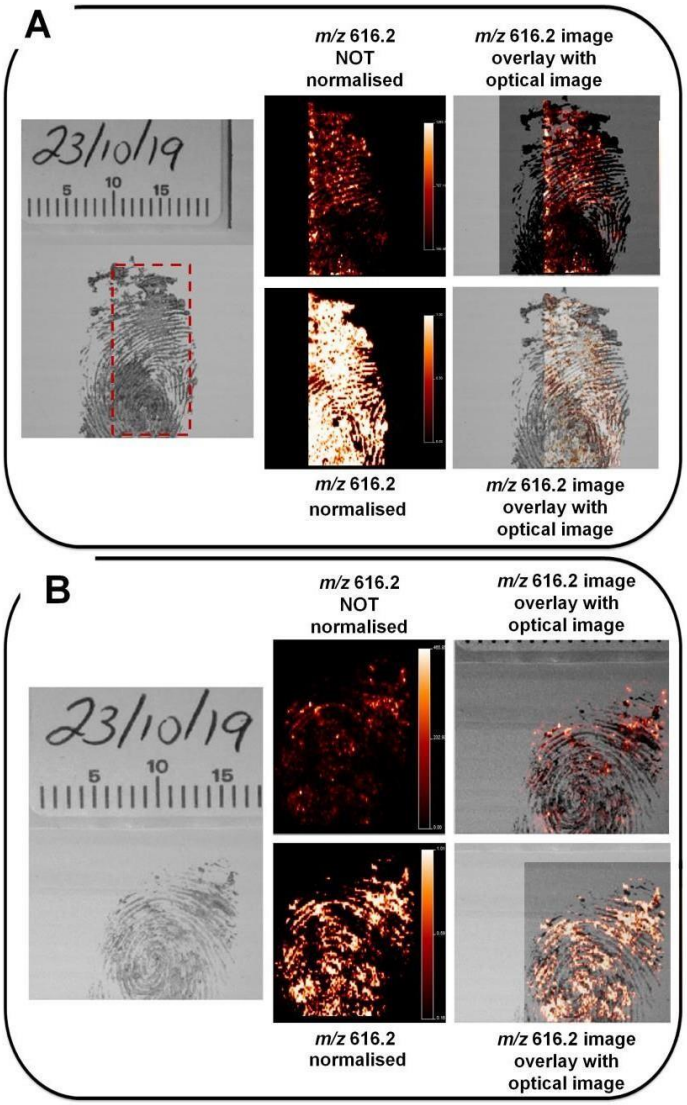

Fig. 4. MALDI MSI of NIN treated depletion series marks (SET I) over the paint. **(A)** 1st mark of the depletion series (FMO-NIN-1), submitted to MALDI MSI. The red dashed frame on the optical images of the NIN developed marks indicate the areas that have been imaged by MALDI MSI. Two molecular images of haem at *m/z* 616.2, both non-normalised and normalised against the Total Ion Current (TIC), are shown followed by the overlay of the MALDI MS image with the optical image. **(B)** 2nd mark of the depletion series (FMO-NIN-2), submitted to MALDI MSI. Two images of haem at *m/z* 616.2, both non-normalised and normalised against the TIC, are shown followed by the overlay of the MALDI MS image and the optical image.

On this occasion, the imaging data, acquired for the other half of the mark that underwent *in situ* enzymatic digestion prior to MALDI MSI on the Synapt G2 instrument, were poor and did not yield relevant blood specific protein-deriving peptides. A sensitivity issue was generally observed, with the instrument only yielding a reasonable molecular image for haem at *m/z* 616.17 (Fig. S2).

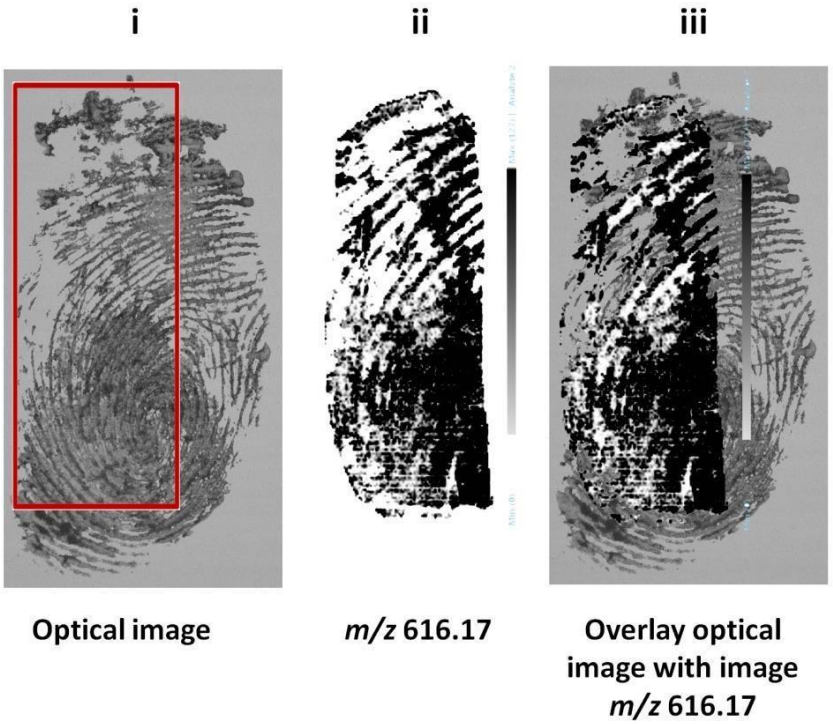

Fig. S2. MALDI MSI of FMO-NIN-1 on the Synapt G2 instrument. (i) Optical image of the mark showing the area imaged by MALDI MSI in the red frame; (ii) Molecular image of haem at *m/z* 616.7 superimposed with the optical image (iii)

Set II - Similarly for Set I, within the AB-1 enhanced blood mark depletion series deposited over the paint (FMO-AB-1), MALDI MSP was able to detect haem in all of the three marks of the depletion series, namely the 1st, the 3rd and the 4th mark of the series (FMO-AB1-1, FMO-AB1-3 and FMO-AB1-4). α and β HB peptides at nominal *m/z* 1275 and 1530 were detected in the 1st and 3rd mark for this depletion series (FMO-AB1-1 and FMO-AB1-3), together with additional HB peptides and other blood specific proteins although to a much lesser extent in FMO-AB1-3 (Table S1). The MALDI MS spectrum of the 4th mark of this depletion series (FMO-AB1-4) is heavily dominated by the PEG based polymer (in 2/3 replicates) and only exhibits the putative presence of Complement C3 protein (Table S1).
As for Set I, these MALDI MSP analyses enabled the confirmation of blood presence across the whole depletion series although it was, again, not possible to establish the presence of human blood in the 4th mark of the depletion series.

Protein	FMO-AB1-1		FMO-AB1-3		FMO-AB1-4	
	m/z	**Mass accuracy (ppm)**	*m/z*	**Mass accuracy (ppm)**	*m/z*	**Mass accuracy (ppm)**
αHB (P69905)	1071.555	0.5				
	1087.621	4.6				
			1129.741	4.6		
	1171.741	-8.0				
	1529.741	4.6	1529.741	-9.1		
	1833.899	3.7				
βHB (P68871)	952.508	-1.8				
	1274.835	7.6	1274.74	1.7		
	1314.667	2.0				
	1378.702	1.1				
	1449.805	6.4				
	1797.973	-7.2				
EMBP 4.2 (P16452)	1161.578	8.1				
Complement C3 (P01024)	960.556	9.3				
			1638.858	-1.3	1638.871	6.7
	1690.892	-1.7			1690.905	-2.7

Table S1. Putative blood protein assignments following MALDI MSP of digested bloodmarks on paint pre-enhanced with Acid Black 1. The 1st, 3rd and 4th marks of the depletion series (FMO-AB1-1, FMO-AB1-3, FMO-AB1-4 respectively) were analysed. Protein names are accompanied by Uniprot accessions numbers (https://www.uniprot.org/).

Figure 5 shows the optical images of the three AB-1 enhanced blood marks and representative mass spectra of haem and haemoglobin peptides indicating the presence of blood.

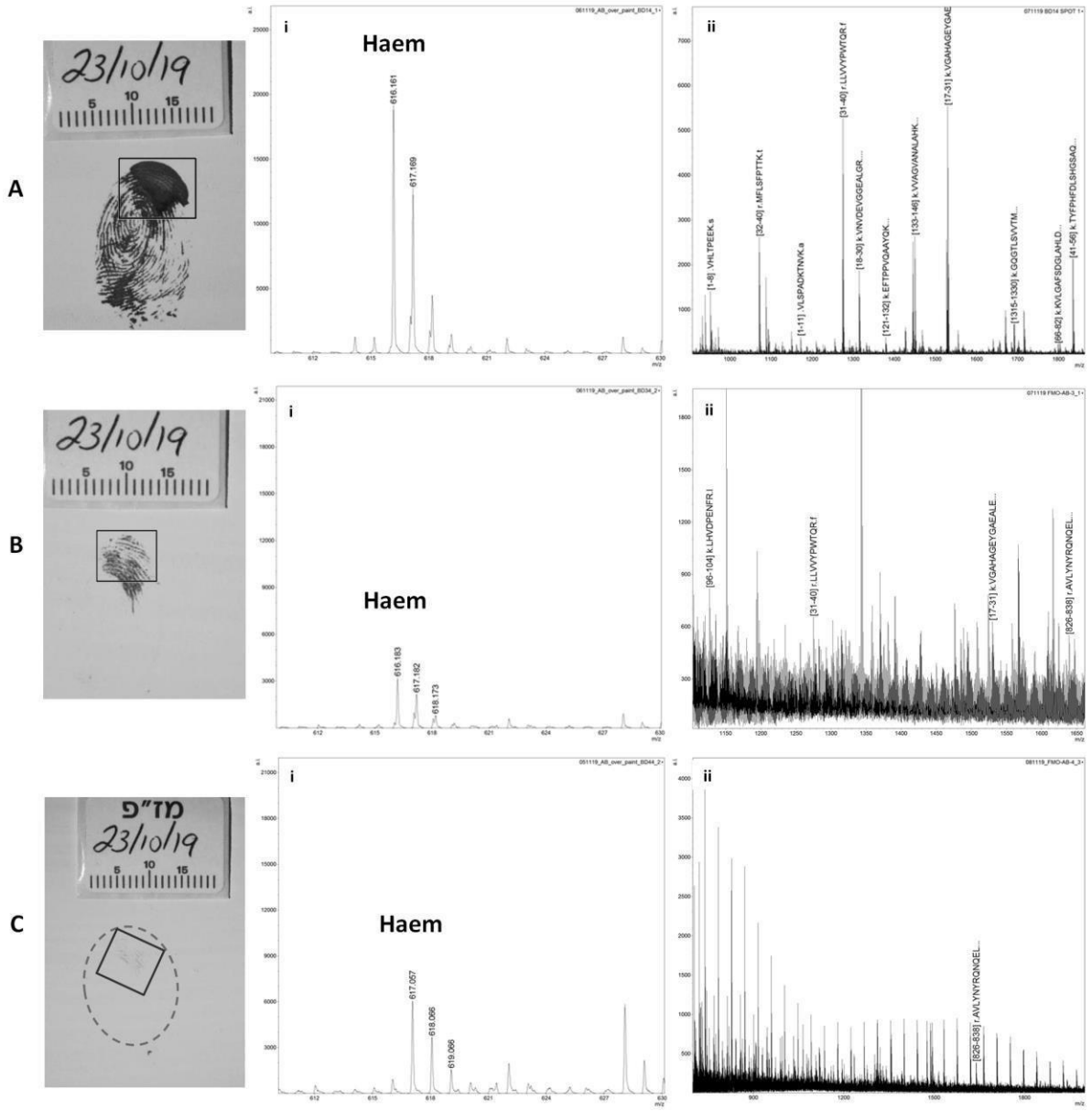

Fig. 5 MALDI MSP of 1st (FMO-AB1-1), 3rd (FMO-AB1-3) and 4th (FMO-AB1-4) marks of Set II (A, B and C respectively) deposited over the paint and enhanced with Acid Black 1 (AB-1). The squares in the optical images indicated the areas submitted to MALDI MSP. MALDI MS spectra of haem (i) and blood protein-deriving peptides (ii) assignments are shown for each mark.

MALDI MSI experiments were subsequently carried out. As for Set I, imaging analyses were performed on the 1st and 2nd mark of the AB-1 depletion series, namely FMO-AB1-1 and FMO-AB1-2 (Fig. 6).

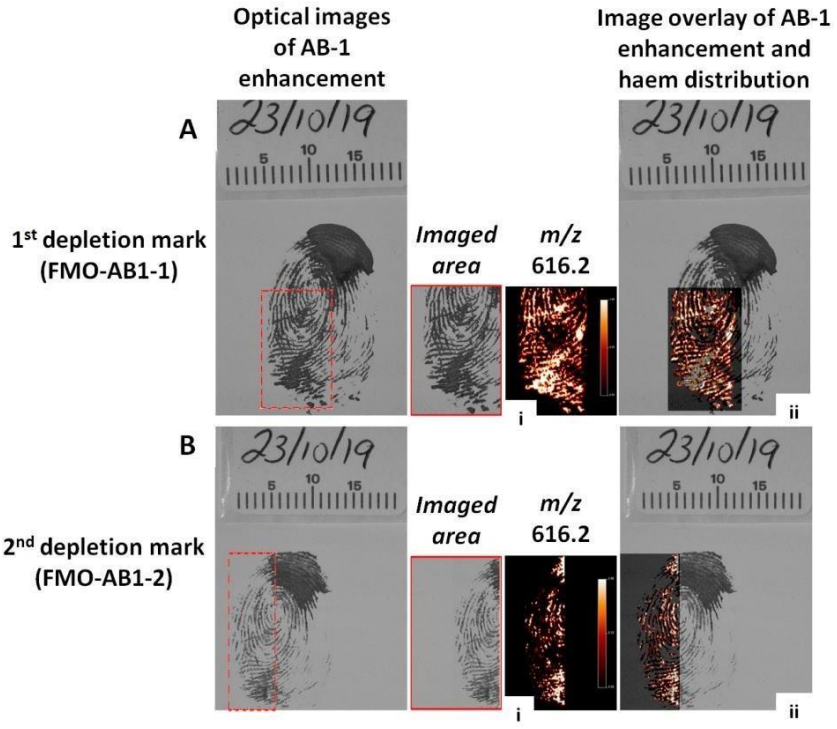

Fig. 6 MALDI MSI (QStar Elite) of AB-1 treated 1st and 2nd blood marks of a depletion series (Set II) over the paint (FMO-AB1-1 and FMO-AB2-2). (A) Optical image of FMO-AB1-1 showing imaged area highlighted with a dashed red frame, accompanied by (i) the MALDI MS image of haem at *m/z* 616.2 and (ii) and subsequent overlay with the optical image and.(B) The optical image of the FMO-AB1-2 mark is accompanied by the MALDI MS image of haem at *m/z* 616.2 (i) and (ii) the overlay between the two images

As it can be seen for FMO-AB1-1, haem was successfully and compatibly mapped on the ridge pattern in the areas where AB-1 presumptively indicated the presence of blood, with the MS analysis confirming the presence of this biofluid. Similarly to Set I, the Synapt G2 instrument underperformed and did not yield suitable images of the blood mark through the relevant blood specific protein-deriving peptides.

Insights into detection of blood under the paint

MALDI MSP analysis was also performed to obtain an initial evaluation of the feasibility of detecting blood after paint had been applied over it. This experiment simulated the scenario in which the perpetrator is attempting to conceal the blood on walls. MALDI MSP spectra show that it was possible to detect blood under three coats of paint through the detection of both haem (*m/z* 616.183, with a mass accuracy of 9.7 ppm) and several haemoglobin peptides (αHB at *m/z* 1071.554, 1529.734 and 1833.892, mass accuracy 1.1 and 0.5 and 1.2 ppm respectively; βHB 1126.564, 1274.726, 1314.665, 1378.700, 1449.789, 1669.876

and 1797.986, mass accuracy, 1.0, -0.5, 1.9, 2.9, 1.8, 1.5 and 7.3 ppm respectively) despite the presence of a PEG based polymer (in 2/3 replicates) (Fig. S3).

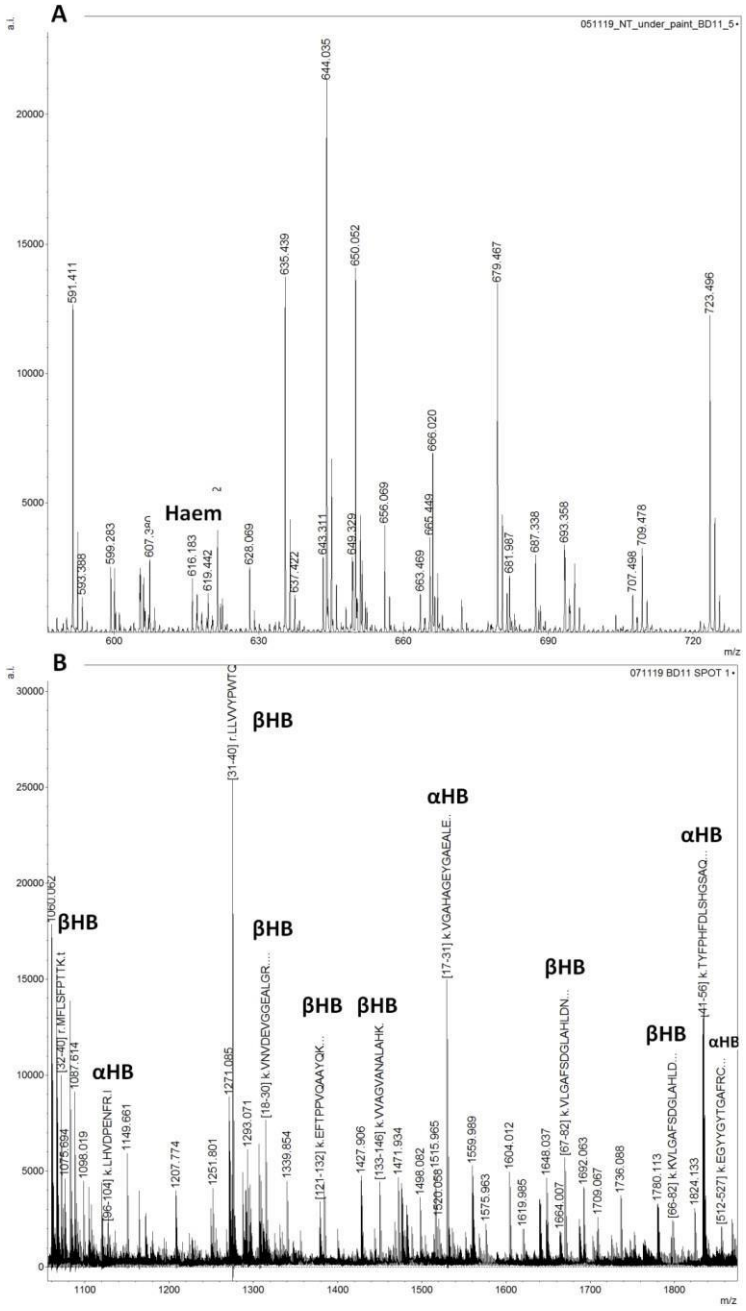

Fig. S3. Synapt G2 MALDI MSP spectra of blood "under the paint". Both haem (A) and haemoglobin peptides (B) could be detected.

PEG based polymers were detected throughout this study except within blanks and other control samples. As PEG-based polymers are known to ionise particularly well in MALDI, the detection of blood despite the PEG polymer was reassuring.
However, in future work, sample purification would be desirable to ensure maximum sensitivity for blood detection in depleted marks.

DNA Profiling from imaged marks

The blood fingermark extracts/in solution digests from Sets I and II that underwent MALDI MSP along with the 1st and 2nd depletion intact marks from the same sets that were imaged, were taken to the Israeli Police Biology & DNA lab for DNA profiling. DNA extraction, quantification and profiling were carried out on these samples. This sequential analysis was important to assess the feasibility of DNA testing *after* MALDI MS based analyses. All original extracted samples (portion of 1st depletion mark, entire 3rd depletion mark and 4th depletion mark in both Sets II and III), showed that the DNA concentration obtained was just below the recommended value required for subsequent PCR amplification (>0.0147 ng/µL) in operational work. This is a recommended DNA concentration for each commercial STR multiplex kit that has been employed in the study. Lower concentrations may still provide DNA profiles, but may result in partial profiles. It is possible that the extensive MALDI analysis performed on the digests has negatively impacted on the amount of DNA available for DNA testing.

DNA amplification was still attempted from the intact marks, even if its concentration was lower than the recommended value, to assess whether a compatible DNA profile for comparison purposes could be achieved. The profiles obtained were compared to the reference blood used to generate blood marks. Indeed, in 73% of these amplifications from the intact fingermark samples that underwent MALDI MSI, comparable profiles originating from the donor were yielded. This result means that a profile of at least 2/3 of the loci tested needs to be achieved, and in the case of the intact samples (FMO-NIN-1, FMO-NIN-2, FMO-AB1-1, FMO-AB1-2) complete profiles were achieved. From the half marks analysed for haem imaging, only FMO-NIN-1 and FMO-AB-1 had a DNA concentration above the recommended value, at 0.35 ng/µL and 0.25 ng/µL respectively. With reference to the half marks analysed for peptide imaging, only FMO-AB-2 had a DNA concentration above the recommended value, at 0.25 ng/µL. The remaining samples did not have DNA concentration above the recommended value but still provided full profiles (Supplementary Table S2).

Instrument	Sample	DNA Concentration (ng/µL)	
		Short	Long
QStar	FMO-D1	0.80	0.58
QStar	FMO-NIN-1	0.35	0.10
QStar	FMO-NIN-2	0.06	0.02
QStar	FMO-AB-1	0.25	0.11
QStar	FMO-AB-2	0.14	0.07
Synapt	FMO-NIN-1	0.12	0.03
Synapt	FMO-NIN-2	0.07	0.01
Synapt	FMO-AB-1	0.09	0.03
Synapt	FMO-AB-2	0.48	0.21

Table S2. DNA extraction quantification following MALDI MS based analyses. Blue rows indicate all the DNA concentrations that are suitable for DNA amplification. These findings are the first of their kind indicating a reasonable compatibility of DNA profiling following the application of MALDI MSI on enhanced blood marks.

The data confirm that exposure for a period between 1-3 hours (QStar Elite) and 10-20 hours (Waters Synapt G2) to the laser wavelength employed (355 nm) enables quantities of DNA that are suitable for further STR testing following MALDI analyses (as opposed to known detrimental exposure of short UV wavelengths commonly employedin some forensic analyses).

However, advanced MALDI instrumentation is capable to image a mark in around ten minutes at the same spatial resolution used here; therefore it is reasonable to speculate that it may be possible to obtain and amplify even higher quantities of DNA.

The DNA profiles were evaluated with respect to peak height and allele balance per locus, and compared to that of the donor, as per accepted analysis methods in the DNA lab within the Israeli Police. The limit of detection (LOD) threshold for minimum peak height of heterozygous alleles was set at 280 RFU, and the stochastic threshold for homozygous allele calls was made at 900 RFU (Internal Validation for VeriFiler™ Plus).

Figure 7 shows the histograms of parts of the recovered DNA profiles from the control (unenhanced) blood marks over paint (Fig. 7a) and blood marks developed with AB-1 imaged using the QStar Elite (Fig. 7b) and Synapt G2 (after proteolysis) (Fig. 7c). There was no difference in the quality of the profiles achieved after AB-1 enhancement.

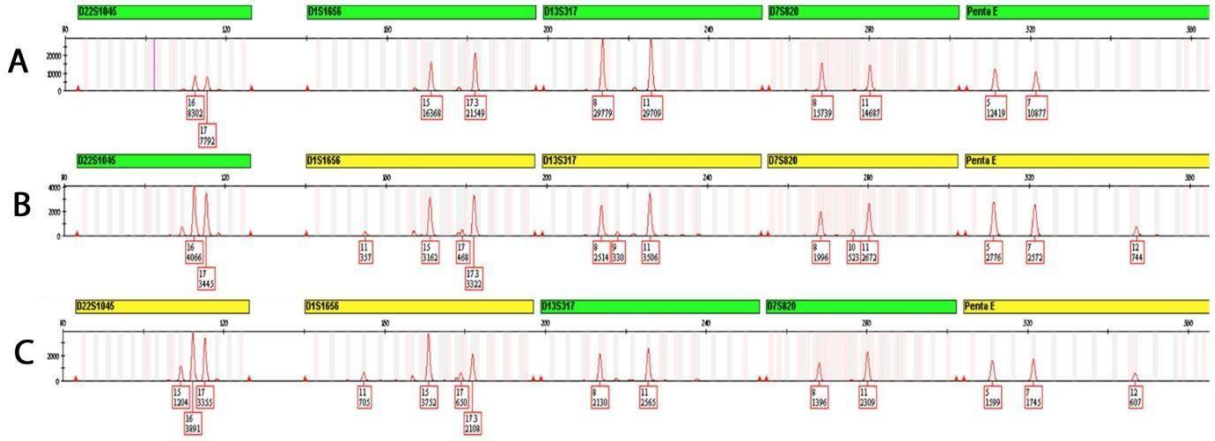

Fig. 7: Part of the DNA profiles recovered from 1st depletions of blood-contaminated fingermarks over paint.
A: no mark development; after ~1 h MSI (QStar Elite) a complete DNA profile of a single origin (23 full STR loci and XX Amel alleles) was retrieved; B: mark development by AB-1; after ~1 h MSI (QStar Elite) a complete mixed-dominant DNA; C: development by AB-1; after MSI acquisitions of up to 30 h (Synapt G2) following *in situ* proteolysis shows a complete mixed-dominant DNA.

The results from DNA recovery and STR amplification of blood fingermarks are summarised in Table 1 and indicate that, whilst a full single source profile was achieved from an undeveloped mark, the developed blood marks provided mixed DNA profiles. However, crucially, comparable profiles were obtained and whether the profile obtained was single source or a mixture is unimportant here as no precautions were taken to operate in a sterile environment. *Set I*, developed using NIN, yielded mixed partial profiles that were not suitable for comparison purposes. Although it is known in the literature that DNA testing can be

Depletion[a]	Development of blood-contaminated fingermarks								
	No development			Ninhydrin			Acid Black-1		
	DNA amount[b]	Number of STR loci[c]	DNA profiles suitable for comparison	DNA amount	Number of STR loci	DNA profiles suitable for comparison	DNA amount	Number of STR loci	DNA profiles suitable for comparison
1-Q	+	24	+	+	24	+	+	24	+
1-S	N/A	N/A	N/A	-	24	+	+	24	+
2-Q	N/A	N/A	N/A	-	21	+	-	24	+
2-S	N/A	N/A	N/A	-	20	+	+	24	+

Table 1: DNA recovery and STR amplification from the fingermarks after MALDI-MSI analysis. [a] 1-Q refers to 1st depletion imaged on QStar Elite, 2-S refers to 2nd depletion imaged on Synapt G2. [b] **+** indicates the DNA quantity found was above the lowest range required for PCR amplification (minimum of 0.25 ng/µL), **-** indicates the quantity below lowest range required for PCR amplification (less than 0.25 ng/µL). [c] The number of STR loci refers to the number of STR loci amplified.

carried out following NIN treatment [23], the Israeli Police reports of previous casework in which NIN has occasionally interfered with subsequent DNA profiling (Dr. Ravell Bengiat *personal communication*) thus reinforcing the hypothesis that it was this enhancement technique and not the MALDI MS based approach that prevented a DNA match with the reference profile. The DNA profiles obtained from the AB-1 samples (Set II) exhibited a dominant profile originating from the donor from both a simple extract and in the proteolytic digest following the extraction. Based on the results obtained, the Israeli Police has suggested a possible simplification to their current sequential processing protocol used when marks are suspected to be contaminated with blood (Fig. S4).

In this suggested alternative workflow: (i) the routinely used non-destructive process is applied first; (i) NIN or AB-1 are used if there is a positive indication for the presence of blood; (iii) if ridge detail is required, the sample is partially scraped/swapped, split and submitted to DNA profiling (victim/perpetrator blood match) and MALDI MSI; (iv) if ridge detail is not needed, sample is entirely scraped/swabbed, split and separately submitted to DNA profiling (victim/perpetrator blood match) and MALDI MSP of haem; (iv) if haem is present, full proteomic analysis by MALDI MSP is undertaken as a confirmatory test of human blood. Further studies including the investigation of sterile conditions and use of faster imaging mass spectrometers might lead to revision of this suggested protocol and implementation of MALDI prior to DNA testing where necessary.

The integration of MALDI MSP/MSI removes extensive cross examination during court proceedings with respect to the validity of presumptive tests as they would be followed by confirmatory mass spectrometric analyses whilst still allowing DNA testing.

Conclusions

MALDI MS Profiling and Imaging have confirmed their capability to detect and image blood in fingermarks after the prior application of Ninhydrin (NIN) or Acid Black 1 (AB-1), this time, on a surface simulating a painted wall. The data demonstrate that the common acrylic paint employed to simulate blood marks on walls(despite containing PEG based polymer) does not hinder the detection of blood. However, as these conclusions are specific to the type of paint used, further work should include investigation of other types of paint.

Blood marks were examined in depletion series and this blood was reliably detected in all the 1st, 3rd and 4th marks of the depletion series "over the paint".

Additionally, for the first time, it was demonstrated that DNA recovery and profiling are possible after the application of MALDI MSI following both AB-1 and NIN development. While DNA profiles were obtained after the application of both AB-1 and NIN development, the data suggest that AB-1 is less destructive to DNA than NIN enhanced marks in which only partial profiles, not suitable for comparison purposes were obtained.

These results are very promising as they show that the short UV wavelengths of the MALDI laser, which until now were assumed to be detrimental to DNA, may still allow a successful recovery and profiling of DNA. Moreover, should MALDI MSP and MSI be recommended for operational work in Biology laboratories, protocols using sterile conditions should be adopted. However, whilst it will be possible to prepare samples for MALDI MS based analyses in sterile conditions, if contamination occurs inside the mass spectrometer, it will not be not possible to remove it.

On the basis of the results obtained, the authors suggest an alternative forensic workflow for suspected blood marks on painted walls, integrating for the first time the use of DNA profiling and MALDI MSP/MSI. Initial results also indicated the possibility to detect blood in marks under the paint (with reference to concealment attempts) and these promising results will be investigated further in future studies.

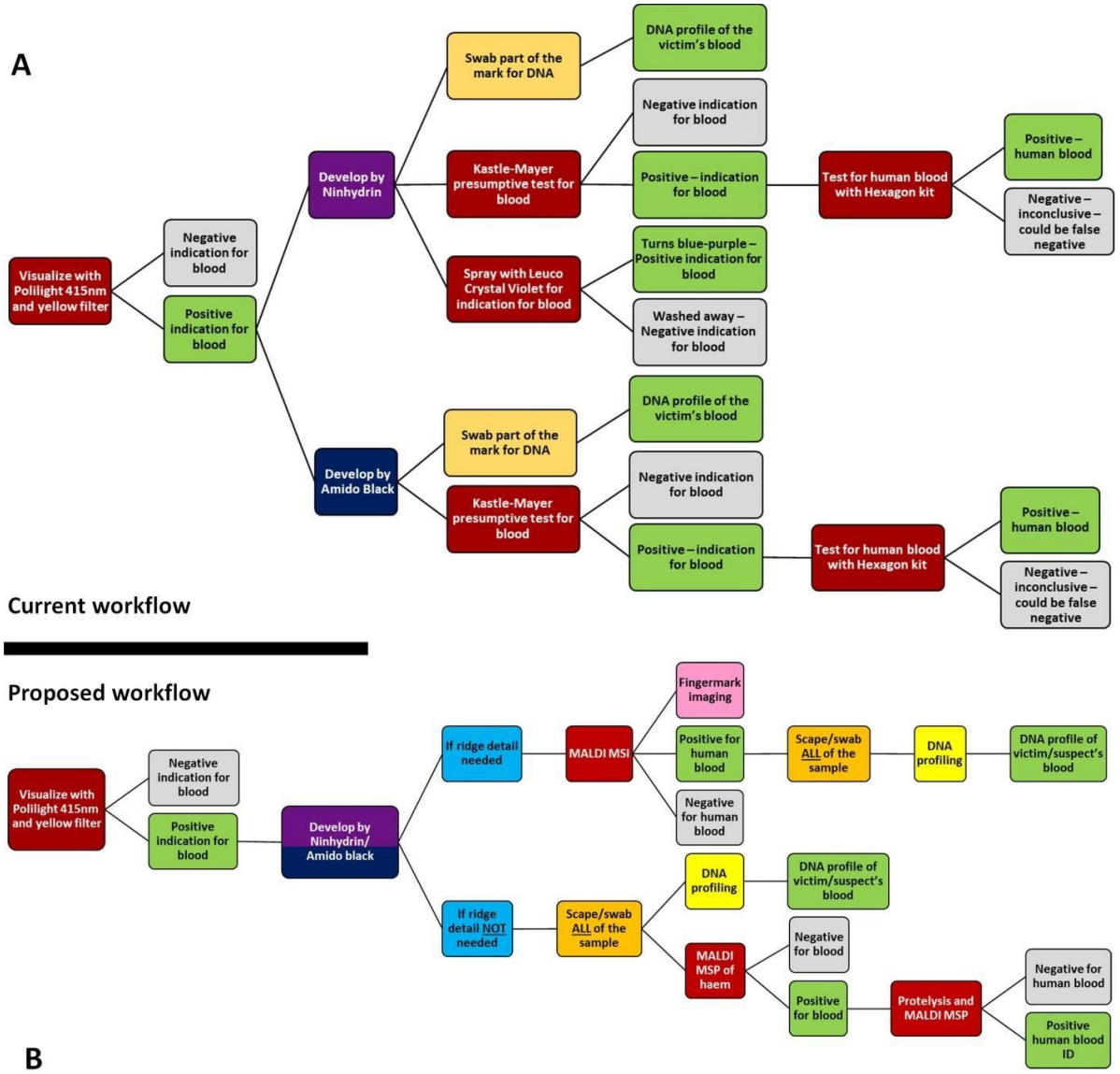
A
Visualize with Polilight 415nm and yellow filter
Negative indication for blood
Positive indication for blood
Develop by Ninhydrin
Swab part of the mark for DNA
DNA profile of the victim's blood
Kastle-Mayer presumptive test for blood
Negative indication for blood
Positive – indication for blood
Test for human blood with Hexagon kit
Positive – human blood
Negative – inconclusive – could be false negative
Spray with Leuco Crystal Violet for indication for blood
Turns blue-purple – Positive indication for blood
Washed away – Negative indication for blood
Develop by Amido Black
Swab part of the mark for DNA
DNA profile of the victim's blood
Kastle-Mayer presumptive test for blood
Negative indication for blood
Positive – indication for blood
Test for human blood with Hexagon kit
Positive – human blood
Negative – inconclusive – could be false negative
Current workflow
Proposed workflow
Visualize with Polilight 415nm and yellow filter
Negative indication for blood
Positive indication for blood
Develop by Ninhydrin/ Amido black
If ridge detail needed
MALDI MSI
Fingermark imaging
Positive for human blood
Negative for human blood
Scape/swab ALL of the sample
DNA profiling
DNA profile of victim/suspect's blood
If ridge detail NOT needed
Scape/swab ALL of the sample
DNA profiling
DNA profile of victim/suspect's blood
MALDI MSP of haem
Negative for blood
Positive for blood
Protelysis and MALDI MSP
Negative for human blood
Positive human blood ID
B

Fig S4. Current and suggested operational sequence of methods for development and testing of suspected blood-contaminated fingermarks in Israel. **A**: workflow includes non-destructive methods for blood visualisation, presumptive tests for blood of increasing specificity and DNA profiling. **B**: application of non-destructive methods first followed by blood enhancement techniques, DNA profiling and MALDI MSP and MSI of human blood.

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Chapter 4

Kennedy K, Cole L, Witt M, Sealey M, Francese S. Forensic Discrimination of Differentially Sourced Animal Blood Using a Bottom-Up Proteomics Based MALDI MS Approach. Molecules. 2022 Mar 22;27(7):2039. doi: 10.3390/molecules27072039. PMID: 35408438; PMCID: PMC9000818.

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Forensic Discrimination of Differentially Sourced Animal Blood Using a Bottom-Up Proteomics Based MALDI MS Approach

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Abstract: Recently published work has reported the development and application of a bottom-up proteomic approach to distinguish between human and animal blood (down to animal species level), by rapid screening using Matrix Assisted Laser Desorption Ionisation Mass Spectrometry (MALDI MS). In that study, it was additionally observed that intravenous animal blood exhibits different spectral profiles from blood collected within the animal chest cavity as well as from the diluted blood collected within packets of meat. In this follow-up study we explored the resulting hypothesis that, depending on how blood is shed or collected, protein biomarker profiles vary to the extent of systematically permitting a distinction between possible sources of blood (for example, flesh wound versus packaged meat). This intelligence may be important in reconstructing the dynamics of the crime. The combination of statistical analysis and tandem mass spectrometry has yielded additional animal blood markers as well as confirming the ability to correctly determine the animal species from which blood derived, regardless of the retailer selling it (amongst the five investigated). These data confirm the initial hypothesis and demonstrate the opportunity for the proteomics-MALDI combined approach to provide additional intelligence to the investigation of violent crimes when examining blood evidence.

Keywords: blood; MALDI; forensic

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1. Introduction

The specific detection of blood and other biofluids at crime scenes is of vital importance to correctly understand the circumstances and the nature of a violent crime. The currently deployed presumptive tests, such as BlueStar Forensic (Luminol), Kastle-Meyer test and some acid dyes for blood stain/mark enhancement can, on occasion, give rise to false positives [1], which may result in a case being dismissed due to the validity of these tests' results being brought into question [2]. Additionally, without the use of a serological test, blood provenance, which is important additional intelligence, cannot be determined. Whilst liquid chromatography tandem mass spectrometry (LC MS/MS) remains the gold standard method to provide comprehensive proteomic information, over recent years, Matrix Assisted Laser Desorption Ionisation Mass Spectrometry (MALDI MS) has been explored as (i) a rapid confirmatory test for the presence of blood [3–6], (ii) as a source of additional intelligence around blood provenance [4,7–9], (iii) to aid suspect or victim identification [10] and (iv) to visualise the presence of blood in fingermarks [2,3,10,11] compatibly with the prior application of blood enhancement techniques and, recently, with the subsequent application of DNA typing [2].

With regards to establishing blood provenance, Bradshaw et al. [3] and Patel et al. [4] first showed the possibility of determining blood provenance in blood marks discriminating

between equine, bovine and human blood using a direct MALDI MS Profiling/Imaging (MSP/MSI) approach or combined with bottom-up proteomics. Kamanna et al. [9] successfully showed that detection and visualisation of both human and animal blood (from a selection of native Australian animals) in fingermarks was possible using intact and bottom-up proteomic approaches with direct MALDI MSI analysis of alpha (α) and beta (β) Haemoglobin (Hb) chains and their derived peptides. A study by Lecrenier et al. [12], in addition to detecting several blood specific proteins including Hb (α and β chains), Serotransferrin, and Apolipoprotein A1, also found specific bovine blood markers.

In a pre-validation study, Kennedy et al. [7] established the feasibility of MALDI MS-based methods to act as a confirmatory test, distinguishing between human and animal blood, down to species level (bovine, chicken and porcine), also confirming some of the markers detected by Lecrenier et al. [12]. In this study [7], animal blood was sourced from the (i) jugular vein, which in the present paper will be referred to as "intravenous", and from (ii) packaged meat. An interesting observation was made in that for each of the selected animal species, the MALDI mass spectrum of the digested blood differed according to where the blood was sourced from, both in signal intensity and in terms of peptide profiles. Ultimately, mass spectral differences appeared to relate to the location from where blood was collected. For example, the mass spectral profile of intravenous blood was not superimposable to that of blood collected from the chest cavity of the animal. This occurrence led to the hypothesis that, in addition to the ability to detect blood and to differentiate between human and animal blood (down to animal species), it may also be possible to provide intelligence with respect to how the animal blood was generated at the crime scene through a specific panel of markers. The body of knowledge derived on animal blood detection and source discrimination from the Kennedy et al. work [7] has been adapted and integrated in a new "identification levels" schematic (Figure 1) hypothesising the potential to gain further insight surrounding the circumstances of animal bloodshed.

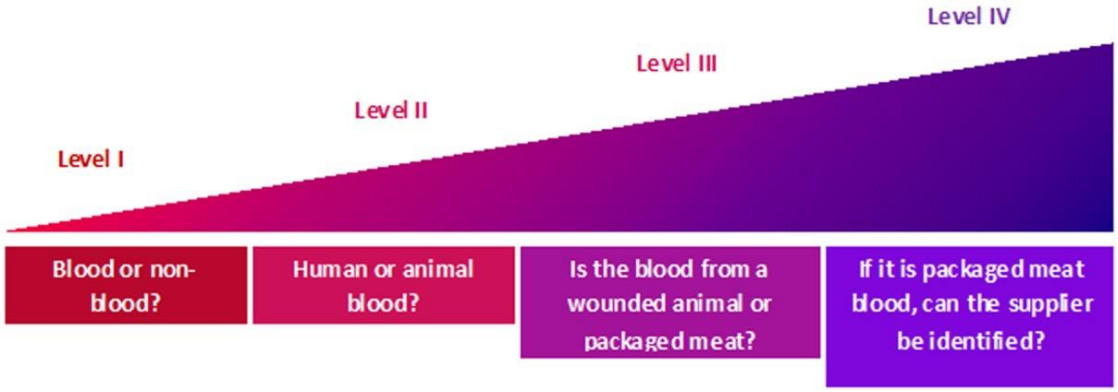

Figure 1. Schematics of the possible levels of identification for the detection of human and animal blood using the bottom-up proteomic MALDI MS based approach. Revised from Kennedy et al. [7].

The ability to ascertain whether animal blood derived from a wounded animal at the scene or from someone handling raw meat may be important to some investigations. In the latter case, as an example, this information would have been helpful in the UK murder case of Susan May (Regina vs. Susan May), to corroborate or disprove her statement, claiming that if blood was indeed present in her fingerprint on the bedroom's wall, it would have been of animal origin, and specifically from raw packaged meat, due to her handling it to prepare lunch for her aunt.

The 1996 US case, State v. Leuluaialii, Nos. 96-C-08256-9, 97-C-01391-3 (Wash., King County Super. Ct. 16 September 1998) is an example where blood from a wounded animal was crucial evidence. Within that double homicide case, the blood of the dog shot dead together with its owners was recovered from the clothing of the offenders [13]. Whilst in that case, canine DNA could be recovered from the bloodstain placing the suspects

at the scene (and aiding conviction), in instances where DNA typing is not possible, the determination of the presence of blood, its animal origin and down to animal species would provide the same intelligence.

In the present adaptation of the Kennedy et al. work [7], identification level III describes the discrimination of blood provenance (within each animal species), between intravenous and packaged meat. Whilst the former scenario refers in our work to the case of wounded animals, the latter scenario refers to an individual handling packaged raw meat and, thereby, contaminating their fingertips with what is most likely a mixture of diluted blood, preservatives and proteins/other biomolecules released directly from the flesh into the blood as a result of severe muscle damage. Level III is the predominant focus of the present follow-up study, but the discovery of additional biomarkers of bovine, chicken and porcine blood was also pursued to increase robustness and reliability of the method.

Identification level IV delves further into species and source discrimination for blood in packaged meat. The robustness of the method was confirmed by establishing that blood originating from packaged meat could still be correctly assigned to the corresponding animal species, regardless of the food retailer from which it was purchased. A subsequent hypothesis that was addressed concerned the assessment of whether blood from packaged meat could be traced back to a specific supermarket (given that the food processing and supply chains could be different for different supermarkets). This type of intelligence may be important to corroborate/disprove the defendant's statement or for gathering additional evidence (such as shopping receipts, reviewing CCTV recordings) by tracing the provenance of the blood in the packaged meat down to a retailer. However, this type of intelligence could only be partially obtained in the present study and indicated that there may only be a few differences in the chain supplies/food processing for the different UK supermarkets investigated.

2. Results and Discussion

2.1. ID Level III: Differentiation between Blood Simulating a Wounded Animal (Intravenous) Versus Blood in Packaged Meat

In the study by Kennedy et al. [7], a method for in solution proteolysis and MALDI MS profiling of chicken, porcine and bovine blood was developed and pre-validated yielding blood biomarkers for each species. However, the study focused on the identification of protein markers in blind samples originating from blood present in butchered meat (which was collected from the chest cavity of the animal), rather than blood sourced directly from the jugular vein (intravenous). The spectral profiles acquired from the chest cavity of the butchered animals were not superimposable with the spectra from the blood found in the packaged meat. These observations suggested that it may be possible to establish the "source" or "type" of blood encountered at the scene. The ability to distinguish between intravenous and packaged meat blood (and other types) would facilitate reconstruction of the crime scene dynamics and the events leading to the presence of animal blood, where involved. For this reason, in the present study, intravenous blood was further investigated using statistical analysis to assess MALDI spectra profiles and candidate markers were searched for in the m/z range 1100–2000 (to minimise matrix/trypsin peaks as well as variability of ion signals above m/z 2000).

2.1.1. Animal Species Determination from Intravenous Blood Simulating a Wounded Animal (Collected from the Jugular Vein)

Principal Component Analysis (PCA) and Principal Component Analysis–Discriminant Analysis (PCA-DA) were performed to objectively demonstrate the difference in blood biomarker composition between intravenous bovine, chicken, and porcine blood. The blood marker panels observed seemed to vary, with the most intense proteotypic signal(s) for each species (within the animal species and proteins being investigated) being present alongside a group of additional peptides, amongst which only some were shared across multiple species. It is this combination of markers, as opposed to the individual theoretical peptides from the in-silico digestion, that is truly discriminatory and acts as a 'species

identifying' panel of ion signals. The ion signals mostly responsible for the different clusters resulting from PCA of the mass spectra of intravenous animal blood were subjected to MALDI MS/MS analyses and identifications are discussed in the following Sections. These analyses permitted the expansion of the panel of identified markers indicative of bovine, porcine and chicken intravenous blood (Supplementary Table S1 and Figure S1). As shown in Figure 2, the signal at nominal m/z 1329 was the most discriminatory signal for bovine blood in the loading plot, although the ion signals at nominal m/z 1102 and 1530 were also included in the biomarker panel for bovine intravenous blood.

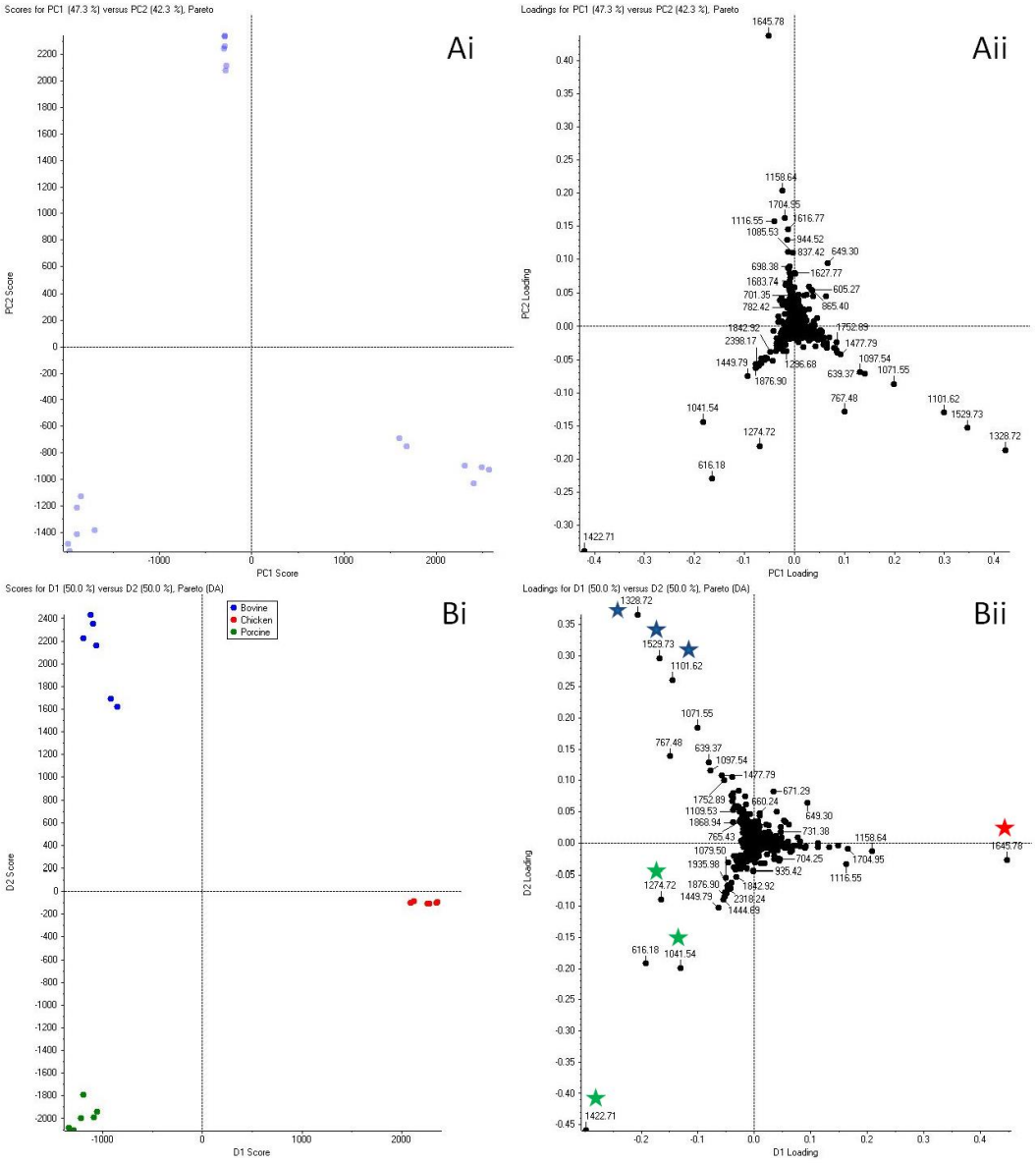

Figure 2. Principal component analysis of bovine, chicken and porcine blood collected from the jugular vein of the animal ("intravenous"). Six replicate spectra from each animal blood in solution proteolytic digests were used for PCA. Clear and distinct grouping can be observed with all three species in both the unsupervised (**Ai**,**Aii**) and supervised PCA-DA analyses (**Bi**,**Bii**). In (**Ai**), the purple dots on the scores plot are to show the variance and spatial relationship of the group replicates from an 'un-supervised', non-bias way hence no color has been pre-assigned to them. The unsupervised score plot in (**Ai**) clearly aligns with that of the supervised PCA-DA validating the rational for labelling. The star symbol in (**Bii**) indicates the ion signals subjected to MALDI MS/MS analysis. The colors in (**Bii**) refer to the animal species as color referenced in (**Bi**).

The ion signal at nominal m/z 1646 was by far the most discriminatory for the intravenous chicken blood. In porcine blood, the signal at nominal m/z 1423 was most responsible for the clustering of porcine blood away from the other animal blood, although the ion signals at nominal m/z 1042 and 1275 were also included in the porcine intravenous blood biomarker panel.

ID Level III: Intravenous Bovine Blood Marker Identification

The only blood peptide signatures detected in the digested intravenous bovine blood were putatively assigned to Hb and characterised by a mass accuracy between −3.7 and **4.7** ppm (Table 1).

Table 1. Bovine intravenous blood peptides putatively identified with a mass accuracy ranging between −3.7 and 4.7 ppm.

Experimental *M/z*	Theoretical *M/z*	Putative Peptide Match	Mass Accuracy (ppm)	Proteotypic	Protein
639.394	639.394	VKAHGK	0	NO	βHb
767.487	767.489	VKAHGKK	2.6	NO	βHb
950.506	950.509	AAVTAFWGK	3.2	YES	βHb
1071.554	1071.554	MFLSFPTTK	0	NO	αHb
1101.627	1101.629	VLSAADKGNVK	−1.8	YES	αHb
1225.626	1225.625	KVLDSFSNGMK	−0.8	YES	βHb
1274.724	1274.726	LLVVYPWTQR	−1.6	NO	βHb
1328.715	1328.717	VKVDEVGGEALGR	1.5	YES	βHb
1477.795	1477.802	VVAGVANALAHRYH	4.7	NO	βHb
1529.733	1529.734	VGGHAAEYGAEALER	0.7	NO	αHb
1752.900	1752.899	MLTAEEKAAVTAFWGK	−0.6	YES	βHb
1833.890	1833.891	TYFPHFDLSHGSAQVK	0.5	NO	αHb
1868.961	1868.954	NFGKEFTPVLQADFQK	−3.7	YES	βHb
2089.953	2089.953	FFESFGDLSTADAVMNNPK	0	YES	βHb
2284.129	2284.126	TYFPHFDLSHGSAQVKGHGAK	−1.3	YES	αHb

Several of these signals were proteotypic to bovine blood. However, despite several signals observed in the intravenous bovine blood being proteotypic (within the species and proteins investigated), very many were not responsible for the clustering of the bovine blood in the PCA; therefore, only the ion signals indicated by the PCA as discriminatory, at nominal m/z 1102, 1329, 1530 MS/MS were subjected to MS/MS analysis (Supplementary Table S1). The signal at m/z 1328.727 was the most intense and putatively assigned to bovine βHb. MALDI MS/MS analysis and an automatic MASCOT MS/MS search confirmed its identity as foetal bovine βHb (VKVDEVGGEALGR) despite the putative identification indicating adult bovine βHb (Supplementary Table S1 and Figure S1A). Indeed, this sequence is found in both bovine foetal βHb and adult bovine βHb and is proteotypic to bovine blood. The signal detected at m/z 1529.745 and corresponding to αHb (VGGHAAEYGAEALER) was also indicated in the PCA loading plot as one of the signals responsible for bovine intravenous blood discrimination. However, this peptide is also found in the human αHb sequence; therefore, only when the signals at nominal m/z 1329, 1102 and 1530 are present together can they be utilised as the (combined) unique protein signature for 'intravenous' bovine blood. Notwithstanding, due to the low intensity of the signal at nominal m/z 1102, MS/MS analysis was not possible, and the identity of this m/z signal remains unconfirmed.

ID Level III: Intravenous Porcine Blood Marker Identification

As for the intravenous bovine blood, several porcine blood proteotypic signals were detected and putatively assigned within a mass accuracy between −3.8 and 8.3 ppm (Table 2).

Table 2. Putatively identified peptides from the MALDI MS spectra of proteolysed porcine intravenous blood.

Experimental *m*/*z*	Theoretical *m*/*z*	Mass Accuracy (ppm)	Peptide Sequence	Proteotypic	Protein
767.487	767.487	0	VKAHGKK	NO	βHb
1041.542	1041.544	1.9	MFLGFPTTK	YES	αHb
1115. 643	1115.642	−0.9	VLSAADKANVK	YES	αHb
1238.680	1238.685	4.0	AHGQKVADALTK	YES	αHb
1243.679	1243.679	0	YELDKAFSDR	YES	αHb
1265.826	1265.830	3.2	LLGNVIVVVLAR	NO	αHb
1274.724	1274.726	1.6	LLVVYPWTQR	NO	βHb
1314.670	1314.665	−3.8	VNVDEVGGEALGR	NO	βHb
1422.700	1422.708	5.6	VGGQAGAHGAEALER	YES	αHb
1449.784	1449.796	8.3	VVAGVANALAHKYH	NO	βHb
1628.908	1628.912	2.5	VLSAADKANVKAAWGK	YES	αHb
1813.977	1813.981	2.2	VLQSFSDGLKHLDNLK	YES	βHb
1876.897	1876.898	0.5	TYFPHFNLSHGSDQVK	YES	βHb
1935.967	1935.978	5.7	AAWGKVGGQAGAHGAEALER	YES	βHb
2045.920	2045.927	3.4	FFESFGDLSNADAVMGNPK	YES	βHb
2237.156	2237.167	4.9	AVGHLDDLPGALSALSDLHAHK	YES	βHb
2318.244	2318.250	2.6	VLQSFSDGLKHLDNLKGTFA K	YES	βHb
2398.167	2398.167	0	TYFPHFNLSHGSDQVKAHGQK	YES	αHb
2445.216	2445.234	7.4	VGGQAGAHGAEALERMFLGFPTTK	YES	αHb

ID Level III: Intravenous Chicken Blood Marker Identification

Every putative peptide match in the MALDI MS spectrum of intravenous chicken blood was assigned to αHb or βHb (Table 3); these peptides were proteotypic to chicken blood (within the animal species and blood specific proteins being investigated) and having a mass accuracy ranging between −1.8 and 14.6 ppm.

The ion at m/z at 1645.791 is a suitable positive marker for intravenous chicken blood due to (i) causing the most clustering of chicken blood in the PCA plot, (ii) its high intensity and (iii) it being proteotypic to chicken blood (within the proteins and animal species being investigated). Its assignment to αHb (IAGHAEEYGAETLER) was confirmed through MALDI MS/MS analysis (Supplementary Table S1 and Figure S1C).

Table 3. Putatively identified peptides from the MALDI MS spectra of proteolysed chicken intravenous blood. All protein matches found in the intravenous chicken blood were proteotypic to this species.

Experimental *M*/*z*	Theoretical *M*/*z*	Putative Peptide Match	Mass Accuracy (ppm)	Proteotypic	Protein
920.489	920.495	LSDLHAHK	6.5	YES	αHb
1036.560	1036.567	VLTSFGDAVK	6.8	YES	βHb
1085.533	1085.534	MFTTYPPTK	0.9	YES	βHb
1288.728	1288.741	LLIVYPWTQR	10.1	YES	βHb
1302.635	1302.647	VNVAECGAEALAR	9.2	YES	βHb
1645.778	1645.782	IAGHAEEYGAETLER	2.4	YES	αHb
1704.959	1704.964	VLSAADKNNVKGIFTK	2.9	YES	αHb
2121.124	2121.155	VVAALIEAANHIDDIAGTLSK	14.6	YES	αHb
2226.142	2226.138	FFASFGNLSSPTAILGNPMVR	−1.8	YES	βHb

2.1.2. ID Level III: Statistical Analysis Discrimination between Intravenous Blood (Mimicking a Wounded Animal) Versus Blood in Packaged Raw Meat

Once discrimination between all animal species could be established through the MALDI MS profile of their intravenous blood it became important to verify the hypothesis made by Kennedy et al. [7] that it may be possible to distinguish between intravenous blood and blood found in packaged meat.

Figure 3 shows both the unsupervised and supervised PCA of intravenous and packaged meat blood for chicken, porcine and bovine animal species, with packaged meat sourced from five different supermarkets (Aldi, Asda, Morrison's, Tesco and Sainsbury's). PCA shows a clear separation between intravenous blood and blood sourced from packaged meat. The clustering does not change significantly between the unsupervised and supervised analyses, thus further verifying the hypothesis made.

2.2. ID Level III: Species Determination from Blood in Packaged Meat

The PCA shown in Figure 3 was "deconstructed" to delve deeper into the intelligence that could possibly be gained in relation to the potential of narrowing down the origin of the packaged meat blood to a retailer. It was preliminarily assessed whether animal species clustering could be observed for blood in packaged meat purchased from only one supermarket. Figure 4 shows the PCA plots for the MALDI MS profiles of blood in packaged chicken, bovine, and porcine meat from one randomly chosen supermarket (Aldi). Clear clustering is visible in both PCA plots indicating that animal species differentiation for packaged meat is also possible (within the system investigated). The ion signals most responsible for animal species clustering and identified in the loading plots (starred in Figure 4) were putatively identified and then subjected to confirmatory MALDI MS/MS analysis (Table 4).

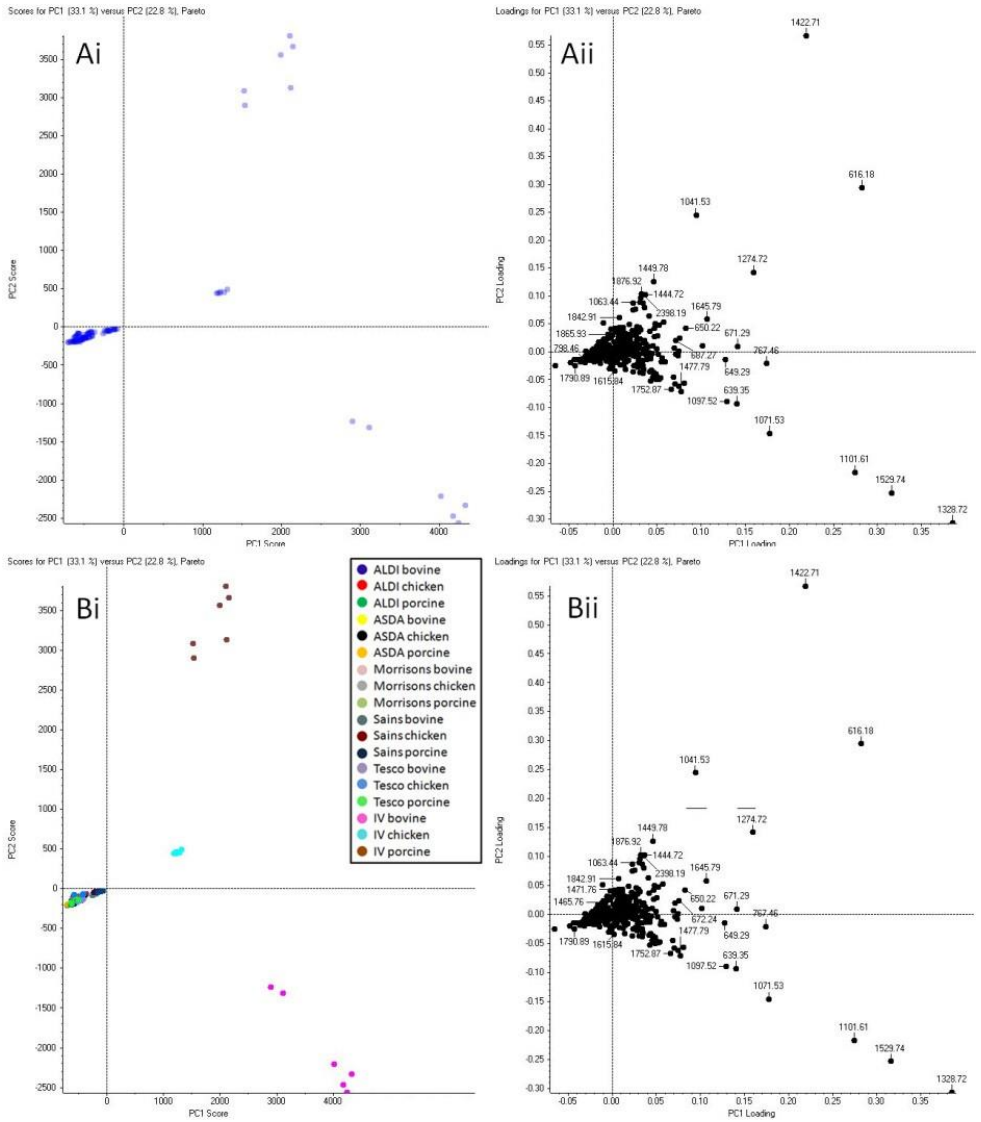

Figure 3. PCA of bovine, chicken and porcine blood collected from the jugular vein of the animal versus bovine, chicken and porcine blood collected from packaged raw meat for each species (from the same five supermarkets). Six replicate spectra from each in solution animal blood proteolysis were used for the statistical analysis. (**Ai**,**Bi**) show the unsupervised and supervised PCA score plots whereas (**Aii**,**Bii**) the unsupervised and supervised loading plots, respectively, where (**Bi**,**Bii**) are the outputs of the PCA-DA.

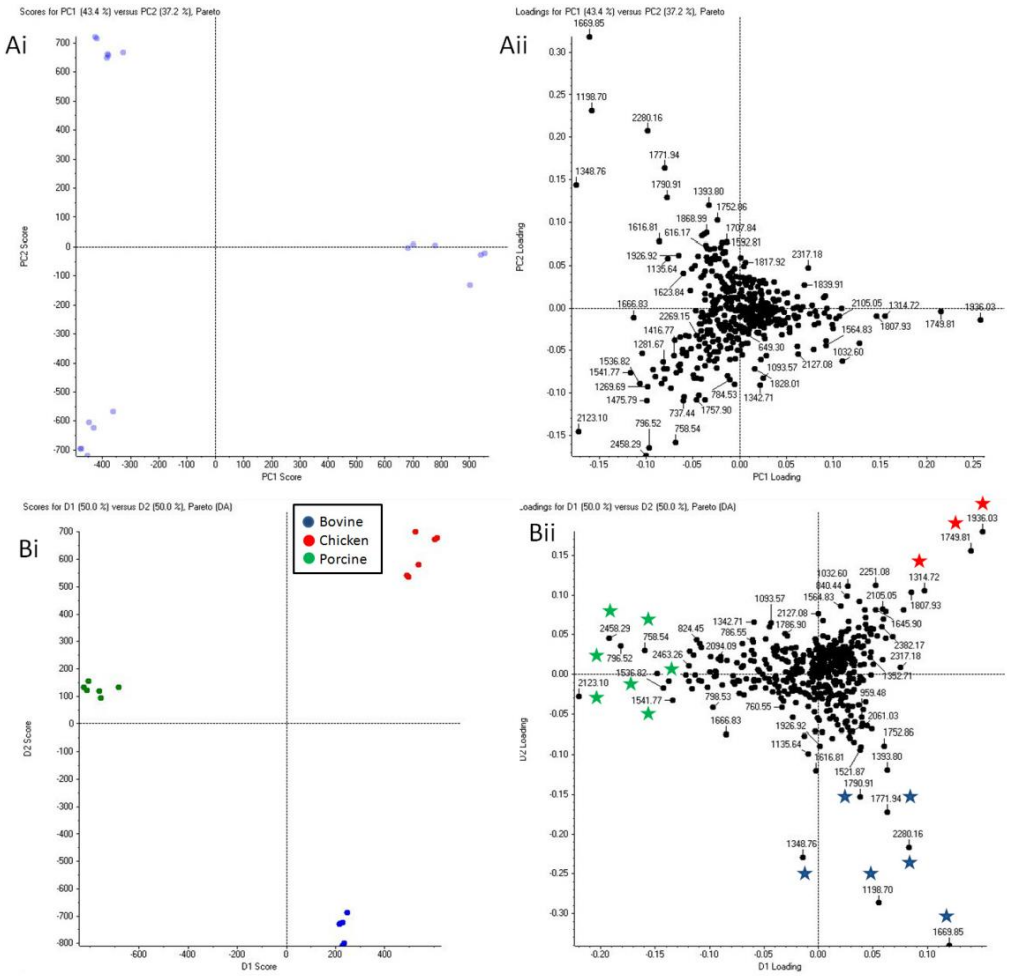

Figure 4. PCA of bovine, chicken and porcine blood collected from packaged raw meat (from just one supermarket namely Aldi). Six replicate spectra from each in solution digest were used for PCA. (**Ai**,**Bi**) show the unsupervised and supervised PCA score plots whereas (**Aii**,**Bii**) the unsupervised and supervised loading plots, respectively. The color stars in (**Bii**) refer to the animal species as color referenced in (**Bi**). The star symbol indicates the m/z ion signals selected for MALDI MS/MS analysis.

2.2.1. ID Level III: Bovine Packaged Blood Marker Identification

For bovine packaged blood, the signals at nominal m/z 1199, 1349 and 1670 were the most discriminatory and were putatively identified as Actin, Carbonic Anhydrase 3 and Myoglobin respectively and were also confirmed by MS/MS analysis (Table S2). The ion signals at nominal m/z 1791 and 2280 were putatively identified as Actin and Myoglobin respectively; though the signal at nominal m/z 1772 could not be putatively identified, it was confirmed to belong to Carbonic Anhydrase 3 through MALDI MS/MS analysis. The detection of both Actin and Myoglobin for blood in packaged meat is expected as they are found in muscle tissue and skeletal muscle tissue respectively. Carbonic Anhydrase 3 is a metalloenzyme present in tissues where a high rate of oxygen consumption occurs [14]. This protein catalyses the reversible hydration of carbon dioxide to bicarbonate and proton and has been reported to play an antioxidant role in the presence of oxidative stress/damage [14]. In Kennedy et al. [7], ion signals at nominal m/z 1593 and 1670 belonging to Myoglobin were indicated as positive markers for bovine blood collected from the chest cavity of the animal.

Table 4. Animal species discriminatory supervised PCA ion signals (from Figure 4) selected putative identification and MS/MS confirmation for blood in packaged meat. (NI: Not Identified; NA: not applicable).

Species	Experimental *m*/*z* (Th)	Putative ID and UniProt Accession No.	Mass Accuracy (ppm)	MS/MS ID (and Accession No.)	Peptide Sequence
Bovine	1198.718	Actin (P62739)	−10.4	Actin (P62739)	AVFPSIVGRPR
Bovine	1348.768	Carbonic anhydrase 3 (Q3SZX4)	−6.2	Carbonic anhydrase 3 (Q3SZX4)	NWRPPQPIKGR
Bovine	1669.851	Myoglobin (P02192)	−8.6	Myoglobin (P02192)	ALELFRNDMAAQYK
Bovine	1771.932	NI	−4.3	Carbonic anhydrase 3 (Q3SZX4)	TLYSSAENEPPVPLVR
Bovine	1790.916	Actin (P62739)	−13.5	Actin (P62739)	SYELPDGQVITIGNER
Bovine	2280.178	Myoglobin (P02192)	- 8.2	Myoglobin (P02192)	ALELFRNDMAAQYKVLGFHG
Porcine	758.576	NI		(Lipid)	NA
Porcine	796.533	NI		(Lipid)	NA
Porcine	1536.823	NI		NI	NA
Porcine	1541.766	Beta-Enolase (Q1KYT0)	−1.2	Beta-Enolase (Q1KYT0)	LAQSNGWGVMVSHR
Porcine	2458.314	NI		NI	NA
Porcine	2463.248	NI	−0.3	Beta-Enolase (Q1KYT0)	AAVPSGASTGIYEALELRDG DKSR
Porcine	2123.125	NI		Fructose-bisphosphate aldolase (Q6UV40)	IGEHTPSSLAIMENANVLAR
Chicken	1314.710	NI		NI	NA
Chicken	1749.799	GAPDH (P00356)	−7.0	GAPDH (P00356)	LVSWYDNEFGYSNR
Chicken	1936.042	NI		NI	NA

2.2.2. ID Level III: Porcine Packaged Blood Marker Identification

For the packaged porcine blood, the ion signals at nominal m/z 758, 796, 1537, 1542, 2123, 2458 and 2463 were observed as the most discriminatory and selected for putative and MALDI MS/MS identification. The ions at m/z 758.576 and 796.533 were clearly identified as phosphatidylcholines through accurate mass and the presence of a phosphocholine headgroup ion fragment at m/z 184.074 (data not shown). Despite being a relatively high intensity and densely ion populated MS/MS spectrum, it was not possible to identify the ion at m/z 1536.823, likely due, from inspection of the spectrum, to more than one parent ion being selected for fragmentation. The ion at m/z 1541.766 was putatively identified as Beta-Enolase (LAQSNGWGVMVSHR) and was confirmed through MALDI MS/MS analysis with a mass accuracy of fragments between −13.6 and 2.1 ppm. Despite no putative identification of the ion at m/z 2463.248, upon MALDI MS/MS analysis, an automatic MASCOT MS/MS search identified this signal as the sequence AAVPSGASTGIYEALELRDGDKSR, also belonging to Beta-Enolase, with a mass accuracy across the ion fragments ranging

between −10.9 and 10.7 ppm. This protein is found in striated muscle and is involved in a sub-pathway of glycolysis. The ion signal at m/z 2123.125 has been identified by MALDI MS/MS as the sequence IGEHTPSSLAIMENANVLAR belonging to Fructose-Bisphosphate Aldolase; another protein also involved in a sub-pathway of glycolysis. The ion signal at m/z 2458.314 could not be identified putatively or through MS/MS analysis.

2.2.3. ID Level III: Chicken Packaged Blood Marker Identification

For the packaged chicken blood, the ion signals selected from the PCA (Figure 4) as the most discriminatory were at nominal m/z 1315, 1750 and 1936. Of these ion signals, only that at m/z 1749.799 could be identified and assigned to the Glyceraldehyde-3-Phospate Dehydrogenase (GAPDH) sequence LVSWYDNEFGYSNR already detected in the blood from the chest cavity of the animal by Kennedy et al. [7]. Where ions could not be identified by MS/MS analysis, this was due to a combination of (i) the known challenge in performing tandem MS on singly charged ions using MALDI (especially for higher m/z ions), as opposed to using LC MS/MS analysis, and (ii) the simultaneous selection of multiple precursor ions yielding mixed MS/MS spectra. Subsequently, we sought to ascertain whether the blood from different animal species could be correctly discriminated regardless of the retailer source. For this investigation, five major UK retailer sources were used. From the PCA plots reported in Figure 5, it can be observed that whilst the unsupervised analysis shows no clustering (Figure 5(Ai,Aii)), the supervised analysis shows clear separation between all the chicken, porcine and bovine blood regardless of the source (retailer) of packaged meat (Figure 5(Bi,Bii)). Figure 5(Ci,Cii) show supervised PCA performed with grouping based on supermarkets to ascertain whether the blood from all packaged meat, across all animal species, could cluster according to the supermarkets of provenance. In this case, there was no clustering except for the Sainsbury's packaged meat. This observation may be beneficial intelligence in a forensic scenario as it could help identify a specific supermarket from which the animal blood could have originated and help corroborate the suspect's testimony or investigative evidence.

2.3. ID Level IV: Assessment of the Potential to Identify the Retailer for the Animal Blood in Packaged Meat

In a final investigation, to provide further assistive intelligence to violent crime investigations it was sought to ascertain whether it was possible to determine the provenance in terms of supermarket retailer from which the packaged meat blood originated. Figure 6 shows both unsupervised and supervised PCA of blood spectral profiles in packaged meat from five supermarkets, but only for bovine blood as an example.

PCA plots of blood collected from packaged meat purchased from five different supermarkets are shown in Figure 6(Bi,Bii) groups. Some level of clustering can be observed for the blood from Morrison's packaged bovine meat and for the blood from Aldi's packaged bovine meat. However, whilst Morrison's and Aldi's can be distinguished from each other, they could be easily mistaken for Asda's and Tesco's, respectively. Bovine blood from Sainsbury's packaged meat remains, in this analysis too, clearly distinguishable from the bovine blood deriving from other retailers in their corresponding packaged bovine meat. From these observations, it can be speculated that Sainsbury's may have a different meat supplier and/or rely on different food processing. The same speculation can be made for Morrison's and Aldi, although, from these analyses, they seem to share supplier/food processing with Asda and Tesco respectively.

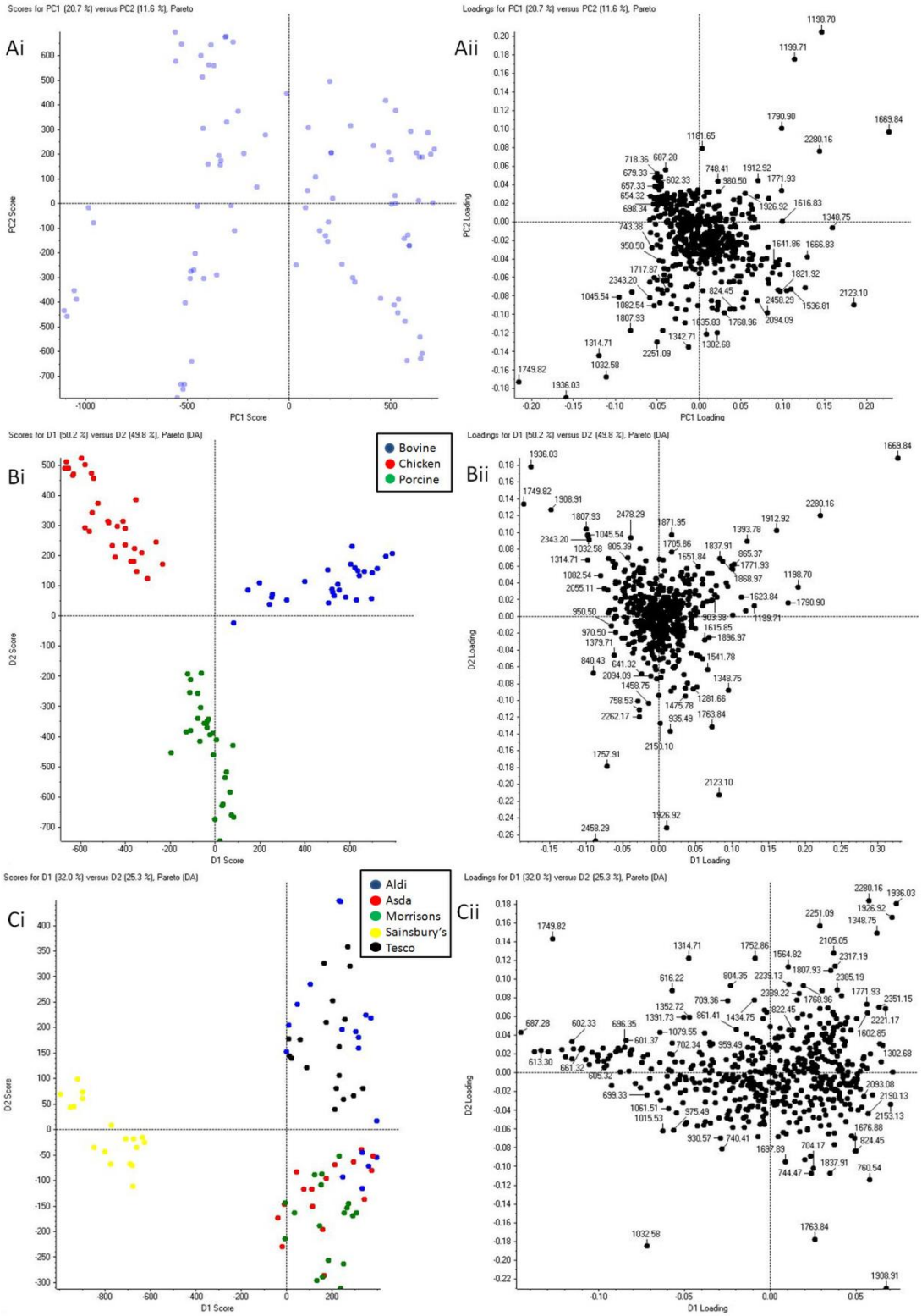

Figure 5. Principal component analysis of bovine, chicken and porcine blood collected from raw packaged meat (from five supermarkets in total: Asda, Aldi, Morrison's, Tesco, and Sainsbury's). Six replicate spectra from each in solution packaged meat animal blood proteolysis were used for the statistical analysis. Panels (**Ai**,**Aii**) are the unsupervised PCA plots; Panels (**Bi**,**Bii**) are the supervised PCA plots with grouping based on meat types; Panels (**Ci**,**Cii**) are the supervised PCA plots with grouping based on supermarkets.

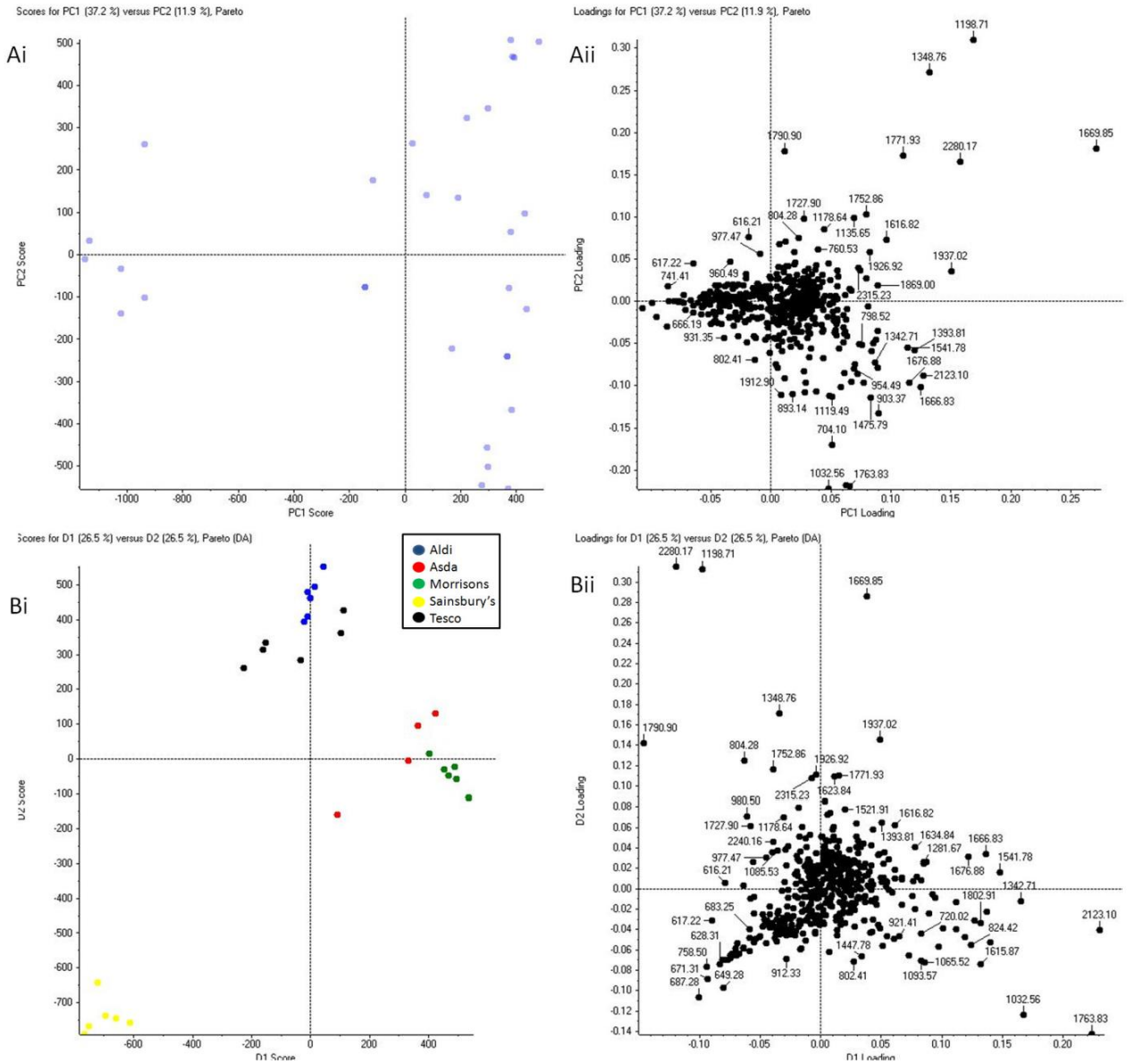

Figure 6. Principal component analysis of bovine blood collected from raw packaged meat (from five supermarkets in total: Asda, Aldi, Morrison's, Tesco, and Sainsbury's). Six replicate spectra from each in solution packaged bovine blood proteolysis were used for the statistical analysis. Panels (**Ai**,**Aii**) are the unsupervised PCA plots; Panels (**Bi**,**Bii**) are the supervised PCA-DA plots with blood provenance grouping based on supermarket of origin.

3. Materials and Methods

3.1. Materials

Trifluoracetic acid (TFA), α-cyano-4-hydroxycinnamic acid (α-CHCA) was purchased from Sigma Aldrich (Poole, UK). Acetonitrile (CAN) 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). Intravenous blood samples (bovine, chicken and porcine, 1 mL defibrinated and 1 mL with EDTA for each animal) were purchased from TCS Biosciences (Buckingham, UK). Packets of chicken, rump steak and pork chops were sourced from Sainsbury's, ASDA, Tesco, Morrison's, and ALDI (Sheffield, UK). The white meat was sourced from the breast

of the chicken, the rump steak was taken from the hindquarter and muscle above the hipbone, and the pork chop was from the loin of the animal, which is located from the hip to the shoulder of the pig. All meat acquired for this study was fresh and in date and kept refrigerated with all analyses performed on the day of purchase.

3.2. Methods

Enzymatic Digestion of Blood

Rapigest (0.1% v/v in 50 mM ammonium bicarbonate solution) was added to trypsin to reconstitute it in a 20 µg/mL solution. For the animal blood from the jugular vein of each animal, 10 µL of blood were spotted onto a polylysine slide and then swabbed with 70:30 ACN: H_2O; for packaged meat, the diluted blood at the bottom of each packet was collected using a swab. The swab head was removed using scissors and transferred into an eppendorf where 1 mL of 70:30 ACN: H_2O solution was added prior to sonication for 10 min. Ten µL of the 1 mL extract were 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). The 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.

3.3. Instrumental Conditions

3.3.1. MALDI MS and MS/MS

All MALDI MS spectrometric 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.

MALDI MS/MS spectra for intravenous animal blood markers were obtained on the MALDI-QTOF Synapt G2 HDMS instrument and on a timsTOF flex system (Bruker Daltonik, Bremen, Germany) for packaged meat blood markers.

For the Synapt MALDI instrument, argon was used as the collision gas. The trap collision energy was set between 30 eV and 90 eV depending upon the precursor m/z (higher collision energy for higher m/z). The laser power and low mass resolution were set at 250 and 14.6, respectively. A 0.5 µL spot of saturated phosphorus red solution in ACN was used as an internal calibrant in the m/z range 600–2500 Th prior to analysis.

For the timsTOF flex MALDI instrument, data acquisition was performed in the m/z range 100–3000 Th in positive ion mode. The instrument was equipped with a smartbeam 3D laser operated at 10 kHz, with the laser focus set for MS/MS experiments. The laser power was set between 35–45% and 500 to 1000 laser shots per scan were acquired (10–20 scans were added for generation of final spectrum). The collision energy was set between 40 eV and 125 eV again depending on the m/z of the precursor.

3.3.2. 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 sample for MALDI MS and MS/MS experiments on the MALDI Synapt instrument. For the timsTOF flex instrument, 1 µL of the sample solution was spotted on an AnchorChip™ target plate and dried at room temperature, then 0.5 µL of 1.4 mg/mL α-CHCA in 85:15 ACN: H_2O, 0.1% TFA (aq) was spotted on top of the sample for MALDI MS/MS experiments and dried. Then the MALDI spots were washed with 1 µL of 1 mM ammonium dihydrogen phosphate ($NH_4H_2PO_4$).

3.3.3. Data Processing of MALDI MS Data

Six replicate spectra were acquired for each animal blood type investigated (obtained intravenously or from packaged meat). For the purposes of protein detection and identification in each animal blood type, only one spectrum was used to search for protein matches whilst all replicates were used for statistical analyses and discrimination of blood across the three animal species. Mass spectra were viewed in Mass Lynx, (Waters Corporation, Manch-

ester, UK) and Data Analysis 5.3 (Bruker Daltonik, Bremen, Germany). Spectra were then exported to mMass, an open-source multifunctional mass spectrometry software [15,16] 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 digestion; namely: αHb and βHb chains, Erythrocyte Membrane Protein Band 4, Haptoglobin, Ceruloplasmin, Apolipoprotein, Myoglobin, Glycophorin A, Complement C4 and Albumin. UniProt (https://www.uniprot.org/, accessed 12 January 2022) 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 blood in packaged meat a MASCOT PMF (peptide mass fingerprint) search was preliminary launched selecting "monoisotopic MH^+" values, peptide tolerance of 15 ppm, two missed cleavages and trypsin as the proteolytic enzyme, chordata or mammalia as taxonomy when in the presence of chicken or bovine and porcine blood, respectively. Subsequently the same strategy used for intravenous blood was applied to blood in packaged meat.

MALDI MS/MS Spectral Identification

MS/MS spectra acquired on the MALDI qTOF Synapt HDMS system instrument were opened in MassLynx and then converted in .txt files to be viewed in mMass for smoothing and peak labelling prior to launching an automatic MASCOT MS/MS search using the same parameters as for the MASCOT PMF search. The mass tolerance for the parent ion and the ion fragments was set at 20 ppm and 30 ppm, respectively. MS/MS spectra acquired on the MALDI timsTOF flex instrument were visualised using the Data Analysis 5.3 software. Profile spectra were exported as text files. The files were then viewed in mMass for MASCOT MS/MS searches and spectral annotation.

Data Processing: Principal Component Analysis

Prior to statistical analysis, SpecAlign (Oxford, UK), an open access software tool, was used to pre-process all spectra acquired [17], which were imported as .txt files for spectral pre-processing consisting in baseline correction, noise removal, normalization to the total ion count (TIC) and removing negative peaks, prior to generating an average spectrum for spectral alignment using the PAFFT correlation method (maximum shift set to 20). Post Specalign .txt files were transposed into table format and then imported into MarkerView software 1.2 (Applied Biosystems/MDS Sciex, Concorde, ON, Canada). A minimum intensity of 0.1 was selected, with a maximum number of peaks of 20,000 and only monoisotopic peaks were selected for both unsupervised and supervised PCA.

4. Conclusions

In silico digestion of blood specific proteins enables the prediction of proteotypic peptides that may be detected (and then experimentally observed) and used, or species discrimination. This was the strategy used by Kennedy et al. [7] in developing a method for the detection and discrimination of blood provenance, distinguishing between human and three animal species. Confirming or refuting the origin of a questioned blood stain as animal, may be crucial finding in an investigation and would assist with reconstructing the dynamics of the bloodshed, as, for example the UK case Regina v. Susan May and the US case, State v. Leuluaialii, Nos. 96-C-08256-9, 97-C-01391-3 suggest. Given the importance of this intelligence, a more objective and quantitative analysis is desirable. Such analysis was involved in this study with the use of statistical analysis to determine discriminatory panels of species-specific blood markers.

The present study integrated principal component analysis (PCA) in the Kennedy et al. approach [7] showing that following proteotypic peptide predictions exclusively reduces the discriminatory accuracy of the method. Interestingly, the findings from this integrated approach also confirmed the hypothesis made by Kennedy et al. [7] that it is possible to

distinguish the origin of animal blood whilst still enabling animal species differentiation. In particular, within the system investigated, intravenous blood can be distinguished from blood found in packaged meat. This intelligence could assist in crime reconstruction by ascertaining the reasons why animal blood, and of a particular origin, was found at the scene. In the present study, PCA plots indicated the panel of biomarkers distinguishing (i) intravenous blood from chicken, porcine and bovine sources; (ii) packaged meat blood from chicken, porcine and bovine, as well as distinguishing packaged meat blood from intravenous blood. Some of these markers were also identified through MALDI MS/MS analyses, thus improving robustness of the blood detection and provenance method developed by Kennedy et al. [7]. An additional study revealed that, for packaged meat blood, these discriminatory capabilities are retained, regardless of the supermarket from which the meat was purchased. Finally, the potential to determine the origin (supermarket) of the blood in the packaged meat was assessed. Bovine packaged meat blood was investigated as an example and data revealed that out of the five supermarket retailers used in the study, it is possible to determine provenance from Sainsbury's. This could be due to this supermarket using a different food processing/supplier than the other four supermarkets. Morrisons' and Aldi's spectral profiles of bovine blood in their packaged meat also appear to cluster away from Sainsbury's and from each other. However, they would be indistinguishable from Asda's and Tesco's, respectively (though the analysis of the packaging may reveal additional intelligence on provenance). Altogether this intelligence could assist further in the investigation of violent crimes where animal blood is potentially involved by proving/disproving the defendant's claim and in reconstruction of the facts under scrutiny.

However, whilst the findings from the present study are original and contribute to potentially new knowledge, some caution is necessary when evaluating their immediate transferability to real life forensic scenarios. Firstly, it would be important to investigate, in a more comprehensive study, reproducibility of the results by performing and analysing multiple protein digests from (i) the same blood source, especially from blood in meat packets, (ii) blood from multiple packets of the same cut as well as from different animal parts and (iii) blood from both fresh meat and meat that had been frozen. Additionally, it is important to bear in mind that it is not yet possible to assimilate definitively the intravenous blood collected from the animal jugular to the blood in a scenario in which the animal is shot at a scene, although jugular blood is the closest approximation in this paper until such studies can be performed. It would also be important to investigate the MALDI spectra profiles of blood originating from animals shot in different body locations, and with a different tissue/muscular tissue depth, to definitely establish differentiation from the spectral profiles of blood from packaged meat.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules27072039/s1, Figure S1: Annotated MALDI qTOF Synapt HDMS MS/MS spectra of intravenous animal blood markers at nominal m/z 1329 (**A**-bovine blood), 1423 (**B**-porcine blood) and 1646 (**C**-chicken blood). Table S1: List of m/z ions submitted to MS/MS analysis selected from the supervised PCA analysis. Table S2: MALDI MS/MS spectral identifications of discriminatory ion signals selected from the PCA analysis in Figure 4, for packaged meat blood from different animal species.

Author Contributions: Conceptualization, S.F.; methodology, K.K., L.C., S.F. and M.W.; formal analysis, K.K., S.F. and M.W.; investigation, K.K., L.C., S.F. and M.W.; writing—original draft preparation, K.K.; writing—review and editing, S.F., K.K., L.C., M.S. and M.W.; supervision, S.F., L.C. and M.S.; project administration, S.F.; funding acquisition, S.F. and M.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Not applicable as animal blood was sourced commercially and from supermarket packaged meat.

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Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data. They did revise the manuscript and provided approval to publish the results.

Sample Availability: Samples of the compounds are not available.

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Supplementary Information

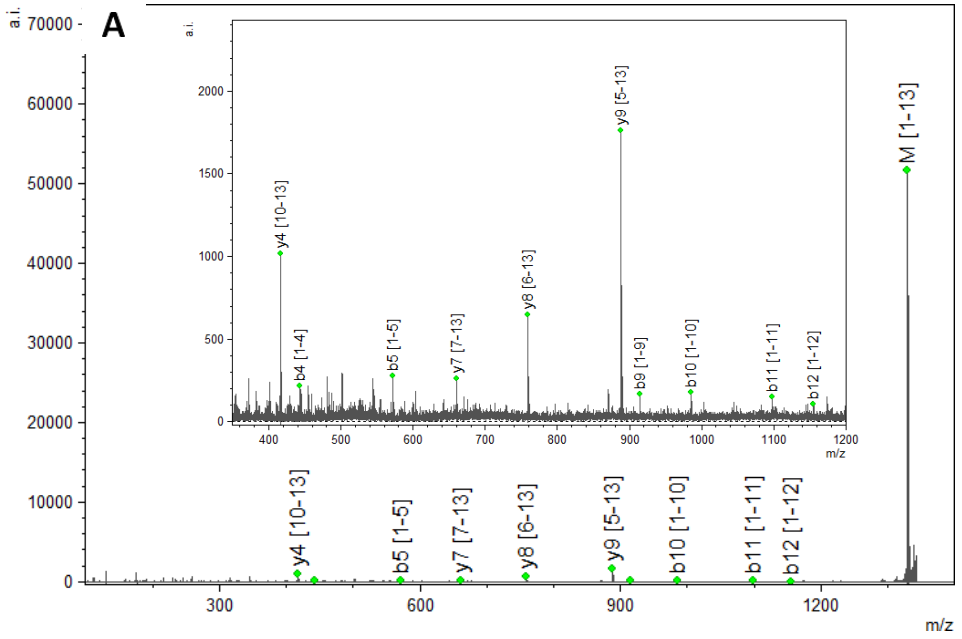
Table S1. List of *m/z* ions submitted to MS/MS analysis selected from the supervised PCA analysis. Identification/confirmation identity of intravenous chicken, bovine, and porcine blood Hb biomarkersis reported.

Species	Ion Signal *m/z* (Th)	Protein and Peptide Sequence	Ion fragments detected
Bovine	1328.727	βHb VKVDEVGGEALG R	y4; y7-y9; b5; b10-b12
Chicken	1645.791	αHb IAGHAEEYGAETL ER	b5-7; b9-10; b12-14;y3-7; y7-9; y11; y13
Porcine	1422.714	αHb VGGQAGAHGAEAL ER	b4-b14; y3-y14
Bovine	1529.745	αHb VGGHAAEYGAEAL ER	y1; y4; y7-y8; y11-y12; b4-b14
Porcine	1274.734	βHb LLVVYPWTQ R	y2-y3, y5-y7, b4-b5,b7-b9

Table S2. MALDI MS/MS spectral identifications of discriminatory ion signals selected from the PCA analysis in Figure 4, for packaged meat blood from different animal species.

Species	Ion signal *m/z* (Th)	Protein and Peptide Sequence	Ion fragments detected
Bovine	1198.718	Actin AVFPSIVGRP R	b2-b10; y1-y3; y5-y9
Bovine	1790.916	Actin AVFPSIVGRP R	b2-b5; b7-b13; y1-y2; y5- y14;
Bovine	1348.768	Carbonic anhydrase 3 NWRPPQPIKGR	b2-b3; b6; b8-b10,y1-y3; y5; y7-y10
Bovine	1771.932	Carbonic anhydrase 3 NWRPPQPIKGR	b2--b12; b14-b15; y1-y4; y7-y16; y15
Bovine	1669.851	Myoglobin ALELFRNDMAAQ YK	b2-b13; y2-y13
Bovine	2280.178	Myoglobin ALELFRNDMAAQYK	b2-b13; b15-b19; y3; y5-y13;

			y16-y17
Porcine	1541.766	Beta-Enolase LAQSNGWGVMVSH R	a9,a13; y1-y4,y8,y10; b8-b13
Porcine	2123.125	Fructose-bisphosphate aldolase IGEHTPSSLAIMENANVL AR	b4-b6; b8-b19; y4-y8; y9- y18
Porcine	2463.248	Beta-Enolase AAVPSGASTGIYEALELR DGDKSR	y1,y5,y7-y8,y11,y15; b18-b19,b21,b23; c18-c19
Chicken	1749.799	GAPDH LVSWYDNEFGYS NR	a11;y1-y12;b3,b5-b11



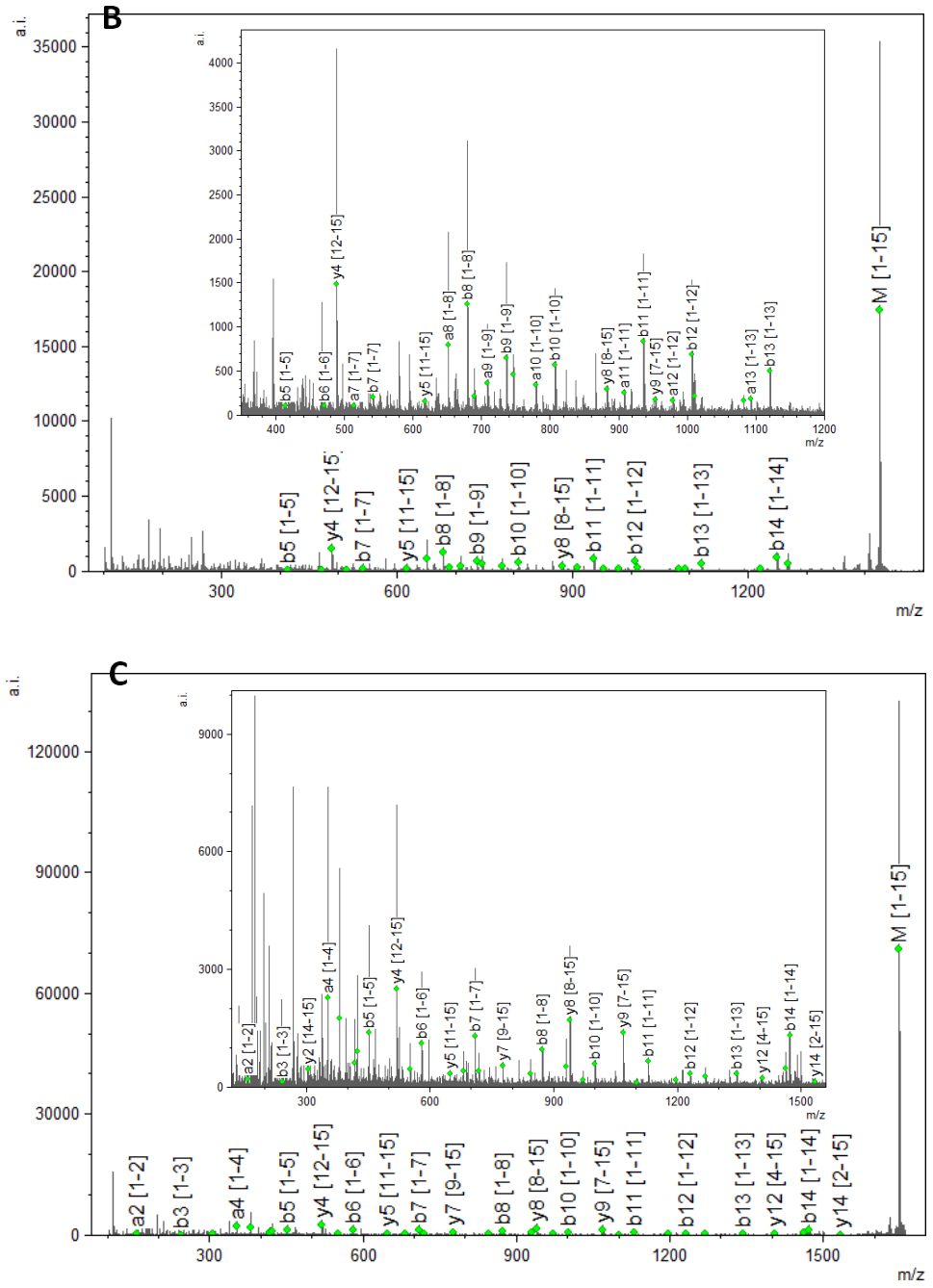


Figure S1. Annotated MALDI qTOF Synapt HDMS MS/MS spectra of intravenous animal blood markers at nominal *m/z* 1329 (**A**-bovine blood), 1423 (**B**-porcine blood) and 1646 (**C**-chicken blood). Annotations for b and y ions are reported and zoom in insets are shown for each MS/MS spectrum.

Chapter 5

Kennedy, K., Gannicliffe, C., Cole, L. M., Sealey, M., & Francese, S. (2022). In depth investigation of the capabilities and limitations of combined proteomic-MALDI MS based approach for the forensic detection of blood. *Science & Justice*.

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

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.

Keywords: blood, forensic, validation, MALDI, compatibility, biofluids.

1.0 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 have 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 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].

2.0 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 seconds, 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 was 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 x 7.6 cm in dimension). These spots were allowed to distribute/spread on the fabric for 30 minutes. 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.

3.0 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.

4.0 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 to 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).

Dilution	Experimental *m/z*	Error (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

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 ßHb and αHb respectively), other Hb signals were detectable in the dilutions tested within a mass accuracy of -6.7 to 7.0 ppm.

Initially, the 1:20,000 dilution did not yield any Hb peptide signatures. For this reason, the remaining solution was purified by means of a C18 zip tip 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 zip-tip is operationally recommended for the detection of human blood. The reverse phase nature of this “chromatography in a tip” prove 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 to 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 mark 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).

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

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 ßHb 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.

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 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.

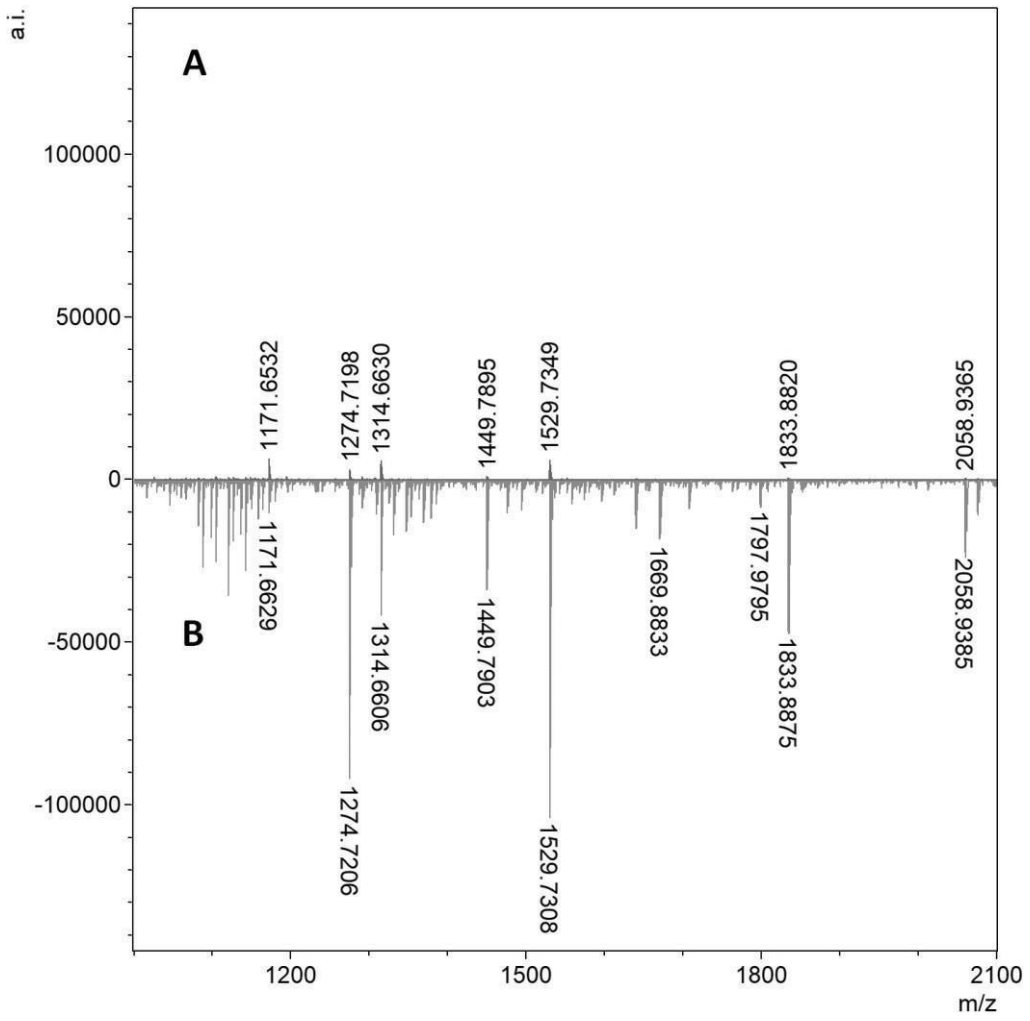

Figure S1: MALDI MS spectra comparison of KM-treated and untreated 1:20,000 blood dilution. The untreated 1:20,000 blood dilution yielded positive MALDI MS spectra for the presence of blood via the peptides at nominal *m/z* 1275 and 1530 purification via zip tipping (A). However, KM-treated blood dilution yielded these two human blood biomarkers at a significantly higher relative intensity with respect to the corresponding untreated dilution (B).

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).

Dilution	Experimental *m/z*	Error (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

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.

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.

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.

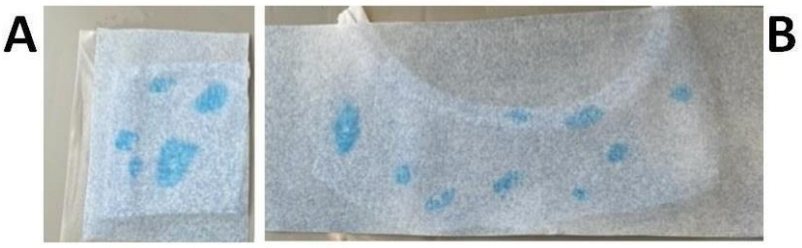

Figure S2: Phadebas paper test for the presence of amylase in saliva. *A:* image of right sleeve showing regions testing positive to amylase; *B:* entire neckline area showing regions testing positive to amylase. Positive areas were subsequently spotted with blood, then excised, extracted, and digested with trypsin prior to MALDI MSP analysis.

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.

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 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.

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).

Left Underarm			
Experimental *m/z*	**Mass Accuracy (ppm)**	**Peptide Sequence**	**Protein**
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**	**Protein**
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	SAVTALWGKVNVDEVGGEAL GR	βHb
Back			
Experimental *m/z*	**Mass Accuracy (ppm)**	**Peptide Sequence**	**Protein**
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	SAVTALWGKVNVDEVGGEAL GR	βHb

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.

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).

Centre Stain Samples									
Theoret. *m/z* (αHb)	50 µL Cotton	75 µL Cotton	100 µL Cotton	50 µL Polyester	75 µL Polyester	100 µL Polyester	50 µL Wool	75 µL Wool	100 µL Wool
1071.554	✓	✓	✓	✓	✓	✓	✓	✓	✓
1087.626	✓	✓	✓	✓	✓	✓	✓	✓	✓
1171.668	✓	✓	x	✓	✓	✓	x	x	x
1529.734	✓	✓	✓	✓	✓	✓	✓	✓	✓
1833.892	✓	✓	✓	✓	✓	✓	✓	✓	✓
2213.089	✓	✓	✓	✓	✓	✓	✓	✓	✓
2341.185	✓	✓	✓	✓	✓	✓	✓	✓	✓
Theoret. *m/z* (βHb)	50 µL Cotton	75 µL Cotton	100 µL Cotton	50 µL Polyester	75 µL Polyester	100 µL Polyester	50 µL Wool	75 µL Wool	100 µL Wool
767.489	x	x	x	x	x	x	x	x	x
932.520	x	x	x	x	x	x	x	x	x
952.510	x	x	x	x	x	x	x	x	x
1149.674	x	x	x	x	x	x	x	x	x
1274.725	✓	✓	✓	✓	✓	✓	✓	✓	✓
1314.665	✓	✓	✓	✓	✓	✓	✓	✓	✓
1449.796	✓	✓	✓	✓	✓	✓	✓	✓	✓
1669.891	✓	✓	✓	✓	✓	✓	✓	x	x
1797.986	✓	✓	✓	✓	✓	✓	x	x	x
2058.948	✓	✓	✓	✓	✓	✓	✓	✓	✓

2228.167	✓	✓	✓	✓	✓	✓	✓	✓	✓
Periphery Stain Samples									
Theoret. *m/z* (αHb)	50 µL Cotton	75 µL Cotton	100 µL Cotton	50 µL Polyester	75 µL Polyester	100 µL Polyester	50 µL Wool	75 µL Wool	100 µL Wool
1071.554	×	×	✓	×	×	×	✓	✓	✓
1087.626	✓	✓	✓	✓	✓	✓	×	✓	×
1171.668	✓	✓	✓	✓	✓	✓	×	×	×
1529.734	✓	✓	✓	✓	✓	✓	✓	✓	✓
1833.892	✓	✓	✓	✓	✓	✓	✓	✓	✓
2213.089	✓	✓	✓	×	✓	✓	✓	✓	✓
2341.184	×	×	✓	×	×	×	✓	✓	✓
Theoret. *m/z* (βHb)	50 µL Cotton	75 µL Cotton	100 µL Cotton	50 µL Polyester	75 µL Polyester	100 µL Polyester	50 µL Wool	75 µL Wool	100 µL Wool
767.489	×	×	×	×	×	×	×	×	×
932.520	×	×	×	×	×	×	×	×	×
952.510	×	×	×	✓	✓	✓	×	×	×
1149.674	×	×	×	×	×	×	×	×	×
1274.725	✓	✓	✓	✓	✓	✓	✓	✓	✓
1314.665	✓	✓	✓	✓	✓	✓	✓	✓	✓
1449.796	✓	✓	✓	✓	✓	✓	✓	✓	✓
1669.891	✓	✓	✓	✓	✓	✓	×	✓	×
1797.986	✓	✓	✓	✓	✓	✓	×	×	×
2058.948	✓	✓	✓	✓	✓	✓	✓	✓	✓
2228.167	×	×	✓	×	×	×	✓	✓	✓

Table S1: Putative MALDI MS identifications of Hb peptides from blood deposited on cotton, polyester, or wool. The greyed boxes indicate the instances where the corresponding peptide was not present in all three replicates but in one/two out of three (all peptide assignments made within +/- 10 ppm). The two human blood biomarkers are highlighted in bold.

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.

Centre			
Experimental *m/z*	**Mass Accuracy (ppm)**	**Peptide Sequence**	**Protein**
1149.6174	-2.1	LVNEVTEFAK	Serum Albumin
1296.7181	-10.5	LAKTYETTLEK	Serum Albumin
1432.7602	-4.5	LKECCEKPLLEK	Serum Albumin
806.4106	-7.8	GPTQEFK	A2-MG
820.4081	9.5	QSSEITR	A2-MG
1215.6528	-1.2	VTAAPQSVCALR	A2-MG
1511.7538	10.7	AAQVTIQSSGTFSSK	A2-MG
1604.8476	-2.8	IAQWQSFQLEGGLK	A2-MG
2096.9512	7.4	DMYSFLEDMGLKAFTNSK	A2-MG
959.5354	-9.0	QKVSVNER	Haptoglobin
2392.1874	-3.6	FTDHLKYVMLPVADQDQCIR	Haptoglobin
Periphery			
Experimental *m/z*	**Mass Accuracy (ppm)**	**Peptide Sequence**	**Protein**
959.5273	-0.5	QKVSVNER	Haptoglobin
1378.6866	5.1	YQCKNYYKLR	Haptoglobin
1545.7291	7.1	KCSTSSLLEACTFR	Serotransferrin

Table S2: Putatively identified blood serum peptides from the centre and periphery of proteolysed blood sample regions on a 100 µL polyester stain within a mass accuracy between -10.5 and 10.7 ppm.

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).

Centre				
Experimental *m/z*	**Theoretical *m/z***	**Mass accuracy (ppm)**	**Peptide Sequence**	**Protein**
1296.700	1296.705	3.5	LAKTYETTLEK	Serum Albumin
1322.689	1322.677	-8.8	CLKDGAGDVAFVK	Serotransferrin
1855.868	1855.868	0.2	EGYYGYTGAFRCLVEK	Serotransferrin
1109.519	1109.508	-9.7	YQCKNYYK	Haptoglobin
1334.671	1334.685	10.4	NANFKFTDHLK	Haptoglobin
Periphery				
Experimental *m/z*	**Theoretical *m/z***	**Mass accuracy (ppm)**	**Peptide Sequence**	**Protein**
1252.660	1252.6572	-2.2	FPKAEFAEVSK	Serum Albumin
806.397	806.4043	8.8	GPTQEFK	α2 Macroglobulin (α 2-MG)
1620.807	1620.8241	10.8	DNSVHWERPQKPK	(α 2-MG)
642.315	642.314	-2.0	INHCR	Serotransferrin
686.324	686.329	7.1	EACVHK	Serotransferrin
1068.540	1068.5506	9.9	EACVHKILR	Serotransferrin
1545.726	1545.74	9.3	KCSTSSLLEACTFR	Serotransferrin
1855.877	1855.8683	-4.7	EGYYGYTGAFRCLVEK	Serotransferrin
959.524	959.5269	2.9	QKVSVNER	Haptoglobin
1378.693	1378.6936	0.4	YQCKNYYKLR	Haptoglobin
620.287	620.2886	2.6	AETGDK	Ceruloplasmin

Table S3: Putatively identified blood serum peptides from the centre and periphery of proteolysed blood sample regions on a 100 µL cotton stain detected within a mass accuracy between -9.7 and 10.8 ppm.

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. Figure 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.

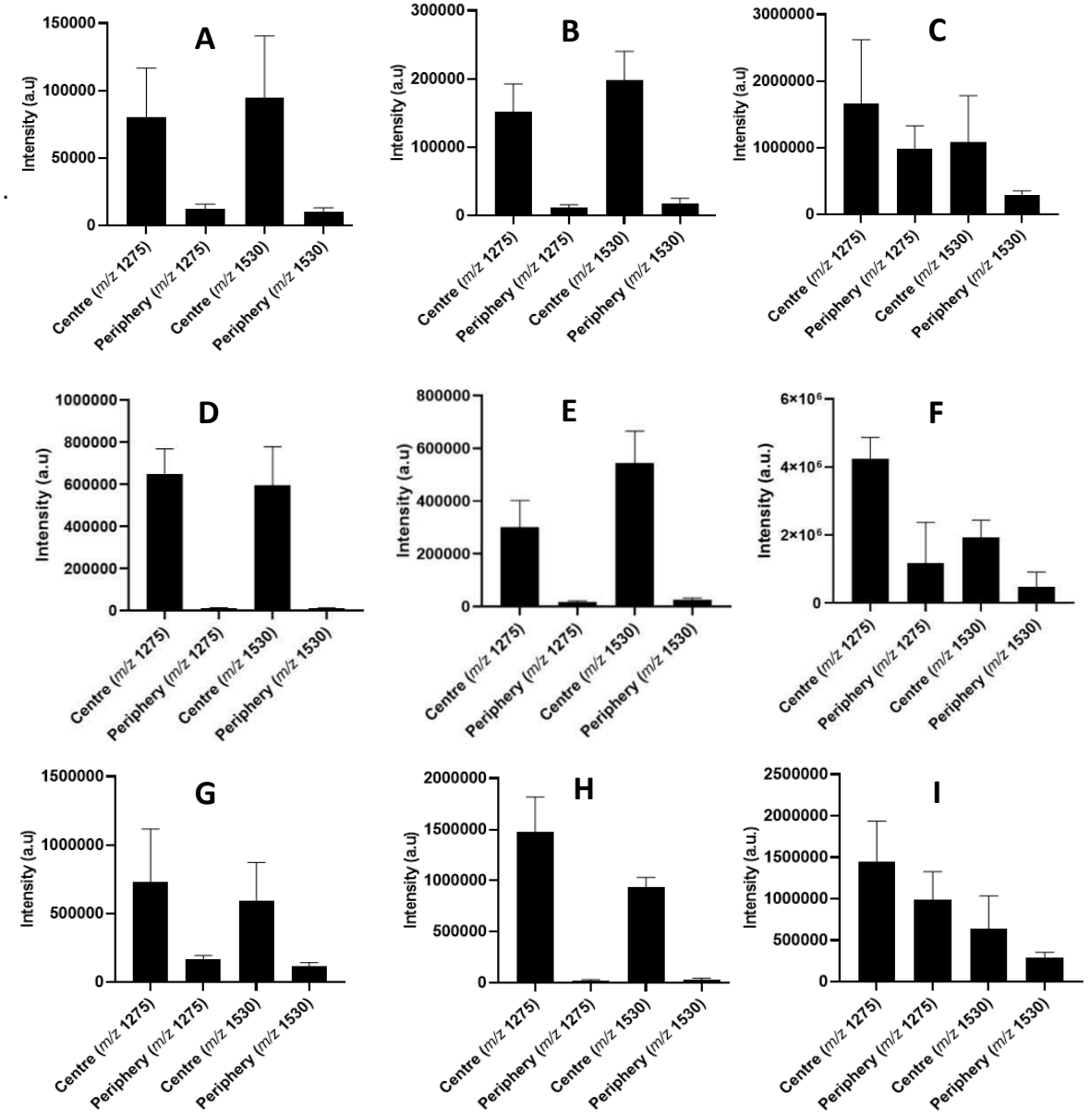

Figure 1: Bar graphs comparing the intensity of *m/z* 1275 (βHb) and *m/z* 1530 (αHb) for 50, 75 and µL blood stains on polyester, cotton and wool detected from the centre and periphery of each stain in triplicate spectra. (A) 50 µL cotton stain; (B) 50 µL polyester stain; (C) 50 µL wool stain; (D) 75 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.*)

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).

Centre 100uL				
Experimental *m/z*	**Theoretical *m/z***	**Mass Accuracy (ppm)**	**Peptide Sequence**	**Protein**
1149.569	1149.576	6.0	DAHKSEVAHR	Serum Albumin
1252.664	1252.657	-5.3	FPKAEFAEVSK	Serum Albumin
1296.701	1296.705	3.1	LAKTYETTLEK	Serum Albumin
1623.805	1623.788	-10.8	DVFLGMFLYEYAR	Serum Albumin
1545.734	1545.740	3.8	KCSTSSLLEACTFR	Serotransferrin
1635.774	1635.790	9.8	NTYEKYLGEEYVK	Serotransferrin
1855.868	1855.868	0.2	EGYYGYTGAFRCLVEK	Serotransferrin
1109.5144	1109.508	-5.4	YQCKNYYK	Haptoglobin
1334.6773	1334.685	5.8	NANFKFTDHLK	Haptoglobin
Periphery 100uL				
Experimental *m/z*	**Theoretical *m/z***	**Mass Accuracy (ppm)**	**Peptide Sequence**	**Protein**
1252.654	1252.657	2.8	FPKAEFAEVSK	Serum Albumin
1296.698	1296.705	5.0	LAKTYETTLEK	Serum Albumin
1623.804	1623.788	-10.2	DVFLGMFLYEYAR	Serum Albumin
940.456	940.456	-0.4	ASYLDCIR	Serotransferrin
1545.733	1545.740	4.5	KCSTSSLLEACTFR	Serotransferrin
1635.789	1635.790	0.6	NTYEKYLGEEYVK	Serotransferrin
1855.866	1855.868	1.2	EGYYGYTGAFRCLVEK	Serotransferrin
1109.515	1109.508	-5.9	YQCKNYYK	Haptoglobin
650.285	650.281	-5.5	QEDMK	Alpha-2 Macroglobulin

Table S4: Putatively identified blood serum peptides from the centre and periphery of proteolysed blood sample regions on a 100 µL wool stain detected within a mass accuracy between -10.8 and 9.8 ppm.

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.

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/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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Chapter 6: Discussion and Final Conclusions

6.0 DISCUSSION AND FINAL CONCLUSIONS

Following from pioneering work by the Fingermark Research Group (FRG) at Sheffield Hallam University on the chemical imaging of fingermarks via MALDI MS based approaches, the body of work presented in this thesis represents further novel research, this time into using MALDI MS Profiling (and MALDI MS Imaging) as a multiplexed and confirmatory analysis for blood detection. Overall, the body of knowledge generated by the FRG to date has been assisted by policing and forensic providers in the UK and around the world. However, operational deployment requires a significant body of knowledge informing as comprehensively as possible on the capabilities and limitations of the technique. In other words, validation is needed to allow the end users to make an informed decision when electing to use this technique in casework.

Forensic providers and end-users who have collaborated in this project have relayed the issues of the current blood tests available, and the ever-increasing risk of cases been thrown because of unreliable tests being depended upon in court (*Personal Communication*, Ravell Bengiat). These factors were the two main driving forces behind the work presented in this thesis.

Bradshaw et al. (2014) first reported on the direct detection of blood in fingermarks by MALDI MSI through the mapping of the haem group and intact Hb chains (α and β) as well as compatibility with the prior application of AB-1. Patel et al. (2015) followed on from this work and demonstrated the improved forensic opportunities of a bottom-up proteomic approach for the rapid detection of blood deriving peptide signatures from both human and equine blood in addition to the detection of blood in a 9-year-old bloodstain. The work completed by Deininger et al. (2016) and Deininger thesis (2018) delved further into the feasibility of MALDI MSP and MALDI MSI (following *in situ* digestion) as a confirmatory test for the detection of blood, specifically by mapping blood specific peptide signatures onto the identifying ridge detail, with some initial exploratory work into the detection of animal blood. Given the very limited amount of data generated within that blind study, one of the main focusses of this PhD programme was to develop an iterative strategy to distinguish between human and animal blood from different species through the identification of species-specific blood peptide markers.

Within the first study published in 2020 in Scientific Reports (Kennedy et al. 2020, Chapter 2) the development of a strategy and a confirmatory test for the detection of both human and animal blood was demonstrated showing the ability to discriminate between chicken, porcine and bovine blood (within the system investigated). This pre-validation study was conducted on a range of samples (all deposited on aluminium slides), namely, human, bovine, porcine and chicken blood, additional biofluids such as sweat, semen, saliva, and a range of non-blood related matrices such as egg, beetroot, paint, lotion; some of the substances that have been reported to yield a false positive with the current presumptive tests. To develop the initial strategy for discriminating between the different sample types, five levels of identification were created (*ID Levels I-V* as named in Chapter 2). *ID Level I*: determine whether the sample was blood or non-blood; *ID Level II*: if the sample is blood, is it human or non-human; *ID Level III*: if the sample is animal blood, what is the provenance; *ID Level IV*: determine the presence of biofluids other than blood and *ID Level V*: identify the specific presence of another biofluid (e.g., semen, sweat or saliva). To develop a strategy in which these ID levels could be applied and tested, an initial blind sample set of 40 samples (21 bloodstains, 2 blood fingermarks, 7 biological fluids, 9 non-blood related matrices and 1 blank) were processed (as detailed in Chapter 2) and MALDI MSP data acquired. From these initial data, and prior to the development of the refined strategy for sample identification, a 2.5% false positive result for blood, 6.7% false negative result for blood and 0% false positive result for blood with the non-blood related matrices were achieved. Regarding animal blood, a 100% false negative result for chicken blood, 33.3% false negative result for bovine blood and 66.7% false negative result for porcine blood were yielded, with an incorrect 'non-blood' claim made for 66.7% of the animal blood samples analysed. The initial strategy of utilising proteotypic peptide signatures to assign the correct provenance to the animal blood samples was ineffective and as such, the strategy was re-examined. After the identification of the species markers, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) at nominal *m/z* 1750 for chicken blood, nominal *m/z* 1764 used for porcine blood and bovine blood; with the additional presence of the Myoglobin (Mb) marker at nominal *m/z* 1593 that had to be present in combination with the GAPDH marker at nominal *m/z* 1764 in order to make a positive bovine blood claim, the identification of the animal blood samples was

far simpler. After the refinement of the strategy for blood provenance establishment and deployment of the new markers, the results improved considerably overall. A randomised batch of 16 samples were analysed. From these, a 0% false positive rate for human blood, 66.7% false negative result for human blood, 0% false positive result for blood with all non- blood related matrices, 100% correct semen sample identifications and a 0% false positive result for blood with all other biofluid samples. In terms of animal blood, after the deployment of the GAPDH and Mb markers, false negative results of 0%, 16.7% and 0% were achieved for chicken, porcine and bovine blood respectively.

The detection of non-blood specific protein markers, coupled with the knowledge that the animal blood was collected from the chest cavity of these animals, has indicated there is further intelligence that can be yielded from animal blood analysis with this MALDI based approach. The way the blood is shed or generated will impact the protein biomarker composition that is observed in the MALDI MS analysis. A distinction can therefore be made between intravenous animal blood versus packaged animal blood/ animal blood collected from the chest cavity of the animal. In a forensic context, the intravenous blood would be the closest simulation to an animal that has been shot for example, and the packaged and chest cavity blood (both had similar MS profiles) would be present at a scene due to the potential handling of raw meat. In the real case of Susan May, a woman who was convicted for the murder of her aunt in 1993 based on three blood marks on a wall at the crime scene, this intelligence could have provided some much-needed clarity. Susan May claimed that the blood marks were present on the wall as she was preparing lunch for her aunt and had been handling raw meat. It later surfaced that the blood testing that was conducted on the stains at the time of the crime were inconclusive and did not determine whether the blood was human or animal. This is one example where the work presented in this thesis would have been of great assistance to eliminate the ambiguity around the origin of the blood marks and either refute or corroborate May’s claim.

In addition to the development of the blood provenance strategy, the study also offered insight into the specificity and multiplex nature of this approach. Not only could blood be detected, but the identification of semen-specific peptide markers permitted for the confirmatory detection of this biofluid also through the detection

of Semenogelin (SEM-1 and SEM-2) deriving peptide markers. This indicates further scope to investigate the feasibility of marker identification for other biofluids such as sweat, saliva, urine, and menstrual blood as examples. Not only this, but a potential occurrence could be biofluids mixed, in which multiple could potentially be resolved in one sample. In cases of sexual assault, the presence of both semen and blood together is plausible. Further work would involve the MALDI MS analysis of mixed biofluid samples (in different ratios) to observe how the spectral profiles differ when another biofluid is also present. It may be the case that expected signals for say human blood and semen are detected at far lower intensity or are entirely absent. As a result, reference spectra for these mixed biofluids could be obtained, and combined with multivariate statistical analysis, training sets for these mixed biofluid samples could be acquired. In a real forensic setting, the environmental conditions from which the sample is retrieved will mean great variability across different crime scenes and as such, a wide range of mixed biofluid samples in varying ratios would generate an extremely helpful body of new knowledge for forensic investigators.

The pre-treatment of some samples in this study with common BET (Acid Black-1, Leucocrystal Violet and Acid Yellow 7) also showed the compatibility of this technique with currently used enhancement agents; an extremely important capability that needed to be assessed to enable consideration of the technique for operational use. In the instances where the BET enhanced the sample and would have in practice, indicated the possible presence of blood, the MALDI MS analysis did not corroborate this and indicated non-blood. Samples 30 (egg yolk), 41 (ketchup) and 165 (egg white) all yielded a 'positive' result with AB-1, AB-1, and AY-7 respectively. As discussed in the introduction however (Chapter 1), as the protein stains do not have a specific affinity for blood, it is certainly unsurprising that some of these samples indicated the presence of blood despite them in fact being non-blood. The use of LCV which could be classified as both a presumptive test and BET due to its affinity to haem did not enhance any non-blood samples, with the MALDI MS analysis corroborating this.

In the final validation sample set (13 samples analysed after strategy refinement) 12/13 of the samples were correctly identified, with only one sample (138- bovine blood) being identified as animal blood, but the species not specified. In the

MALDI MS data for this sample, only the signal at nominal *m/z* 1593, indicating the possible presence of bovine blood, was detected. The implemented strategy required the presence of both nominal *m/z* 1593 (Mb) and 1764 (GAPDH) to claim the presence of bovine blood. The data acquired from this sample is an example of what could potentially cause confusion amongst end-users with this approach. The markers that have been identified for species discrimination thus far have been detected from prepared samples under controlled laboratory conditions. The reference spectra acquired for both intravenous and packaged meat blood samples were also prepared in the laboratory. In forensic practice, this would not be the reality. A sample could have been subjected to harsh environmental conditions, be recovered from outdoors or be aged. The robustness of these markers requires thorough testing as a result. Sample 138 was in fact bovine blood but only one of the two markers required from the strategy was detected. Depending on the amount of blood within the sample or whether it is diluted may impact the intensity at which the signals are observed or whether they are even observed at all. This result brought into question other factors that may cause variability in the signals observed, such as how the animal blood is shed and how this will affect the spectral profiles.

This led onto the work presented in Kennedy et al. (2022), published in *Molecules* (Chapter 4). The study delved further into the hypothesis, that, the way animal blood is shed will have a direct impact on the protein markers detected via MALDI MS analysis, so that these scenarios can be identified and discriminated between. Animal blood from packaged beef, chicken, and pork from five supermarkets and animal blood collected from the jugular vein (also from the same animals) were subjected to proteolysis and MALDI MSP. Markers were identified through PCA and subsequent tandem MS experiments; in this way it was possible to distinguish between animal blood collected from the jugular vein and animal blood from packaged meat. Investigation into whether the supermarket from which the meat was purchased could be determined was also endeavoured, but due to several of the supermarkets in the UK using the same supplier, clustering of supermarkets was observed in the PCA and PCA-DA. Detecting animal blood down to source level (i.e., the supermarket it was purchased from) albeit helpful information if retrievable, would face real scrutiny as it would be easy to discredit. Due to the shared

suppliers across multiple supermarkets in this country, the defence could claim that the meat was possibly purchased from a different shop, and with some markers being shared across species, claiming a source (supermarket) for the animal blood would be challenging and difficult to definitively claim.

Following on from the issue of shared biomarkers; examples of which are nominal *m/z* 1199; an Actin bovine marker shared with human, chicken, porcine and nominal *m/z* 1542; a Beta-Enolase marker shared with human and bovine, it is not individual markers that can identify a species but rather a panel/ particular combination of peptide signals that are indicative of a species. It was shown in Kennedy et al. (2022), that, through the use of multivariate statistical analysis it was possible to distinguish between species in both intravenous blood and blood from packaged meat, but as highlighted in Kennedy et al. 2020 (Chapter 2) the panels of markers must be challenged under different conditions. For example, meat that is not fresh or has not been analysed on the day of purchase, meat that is out of date and defrosted meat could be processed and analysed with the same approach to compare differences. Additionally, the inclusion of other animals such as canine and feline would be beneficial as they would be the animals most likely to be found in a household. Identifying specific biomarker panels for these animals would assist further in reconstructing the dynamics of the crime scene when animal bloodshed has occurred and would further extend the range of species which can be identified with this approach.

As highlighted in section 2.3 of the introduction (Chapter 1), one of the most important requirements working towards the wider deployment of any technique in an operational setting is the need for a continuous collaboration with the end users. Those who would be using the technique operationally are the best equipped to shape and drive the research, for example by suggesting experimentation to answer questions arising from real forensic scenarios. Throughout this PhD programme several such stakeholders have provided input and technical assistance namely Dr. Glenn Langenburg from Elite Forensic Services (Minnesota, US), Dr. Ravell Bengiat and Michal Elvin Elad from the Israel Police, Chris Gannicliffe from the Scottish Police Authority (SPA) and Mark Sealey, Defence Science and Technology Laboratory (UK). The research in collaboration with Elite Forensics has been previously discussed and published

in Scientific Reports in 2020 (here in this Chapter and in Chapter 2). The work published in collaboration with the Israel Police (Kennedy et al. 2021, Chapter 3) highlighted the variety of scenarios in which blood evidence may be found, namely the instances when blood is located on a painted surface, and sometimes has been concealed by the perpetrator, so is located under paint (*Personal Communication*, Ravell Bengiat, Israel Police). In this study, two sets of blood fingermark depletions on paint (four fingermarks per depletion set) were provided by the Israel Police, in which enhanced blood fingermarks were deposited on acrylic-painted aluminium slides. As illustrated in Chapter 2, the first set was enhanced with Ninhydrin (NIN) and the second with Acid Black 1 (AB-1). Additional samples of untreated blood fingermarks under paint were also provided to assess whether blood could still be successfully detected when retrieved from under a painted surface.

The results from the MALDI MSP analysis indicated that the detection of blood deriving peptide signatures from AB-1 and NIN enhanced blood marks on acrylic paint was possible down to the 3rd depletion in both sets (Chapter 3). The unenhanced blood mark retrieved from under paint also successfully yielded human blood deriving peptide signatures with MALDI MSP indicating the presence of the paint did not hinder the detection of the expected human blood markers. This was very important, as the concealment of blood marks with paint has been reported by the Israel Police, thus a method that permits for its confirmatory detection in these circumstances is extremely helpful. One consideration however is the type of paint that is used to conceal the blood mark. With these samples, an acrylic paint was used. A different paint formulation, such as oil, gloss or eggshell are commonplace in households so could feasibly be used to conceal a blood mark at the scene of a crime. To test robustness further, testing the MALDI MS approach for detecting blood under different paints would be beneficial to better understand its compatibility with these different formulations. Similarly, a white paint was used for the preparation of these samples, so testing this approach with coloured paints (which will contain colourants/dyes) to assess whether any of the additives prohibit the detection of the expected human blood peptide signatures (namely nominal *m/z* 1275 (βHb) and *m/z* 1530 (αHb)) would also be beneficial.

It additionally explored the integration of MALDI in a more complex workflow for blood evidence detection and examination. Following the application of either NIN or AB-1, MALDI was used in both profiling and imaging mode as confirmatory tests and subsequently DNA typing was undertaken. It was of extreme importance to ascertain whether MALDI, if adopted by this laboratory, could be integrated into the current workflow without hindering the subsequent retrieval and profiling of DNA. This workflow would permit presumptive testing, confirmatory testing, detection of identifying DNA simultaneously with its attribution to blood origin when human blood is present.

The blood fingermark samples that initially underwent in solution proteolysis and MALDI MSP (a portion of depletion 1 and the entirety of depletions 3 and 4 from both sets) were retained for DNA extraction and profiling. The DNA concentration that was obtained from these samples was below the recommended value that is necessary for PCR amplification and as such, meant that DNA profiles could not be yielded from these samples. As these samples had not actually undergone the MALDI MSP analysis, only the preparation steps prior to MALDI, it suggests the stages involved in the proteolysis have had a negative impact on the DNA quantity available. There is the additional extraction step with the in-solution proteolysis process that is not performed within the MALDI MSI sample preparation which may have contributed to the poor DNA yield. In contrast, despite some of the DNA quantities yielded being lower than the recommended amount, DNA extraction and profiling from the intact blood marks (depletions 1 and 2 of both sets) was performed. From the four samples that were subjected to *in situ* proteolysis and MALDI MSI, 73% of the amplifications yielded comparable profiles that originated from the blood donor. In order to be deemed a comparable profile, 2/3 of the loci tested had to be achieved. For the four samples, complete DNA profiles were achieved, despite all samples not having the recommended DNA quantity (Chapter 3). Subjecting the blood marks to several hours of laser irradiation (with a wavelength of 355nm); which was a concern of the collaborators as short UV wavelengths have been reported to have detrimental effects on DNA profiling (*Personal Communication*, Ravell Bengiat, Israel Police), and still achieving comparable DNA profiles, was one of the most promising revelations of this PhD programme. With the instrumentation used in this study, MSI experiments ranged from 3h to 20h. With advancements in MALDI MS

instrumentation and far improved imaging capabilities, the exposure to laser wavelength for such long periods of time could be avoided with more developed instrumentation, with the imaging time drastically reduced at the same spatial resolution. This could mean that a higher quantity of DNA could be potentially yielded if the sample's exposure time is reduced.

Another potential issue that was revealed upon further interrogation of the DNA profiles was that, despite yielding comparable donor profiles with all the intact marks, the DNA profiles were in fact mixed. There was no attempt to maintain a sterile environment for these experiments as the sample preparation was performed in a shared laboratory and this would have proved challenging. Additionally, the MS instrumentation is used by several research groups running different sample types, so it cannot be ruled out that DNA contamination could occur within the instrument. For this technique to be used operationally, the need for a sterile environment would be essential. Not only this, but regular cleaning of the instrument source would be necessary to reduce the risk of DNA contamination once the sample has been inserted into the instrument. Regarding verification and validation of this technique, and the MALDI MS results to be admissible in court, the maintenance and upkeep of the instrument would be of real importance to minimise the risk of any scrutiny with the results yielded.

As a result of these findings overall, the Israel Police has suggested a novel workflow for the examination of blood evidence integrating the use of MALDI (Chapter 3). The dissemination of these findings to the wider scientific community will likely encourage other police forces and providers to consider the adoption of a MALDI based approach for confirmatory blood detection.

In a similar way, the work carried out in collaboration with the SPA (Kennedy et al. 2022, Chapter 5) is another example of refined and targeted research aiming to address practitioners' scenarios commonly encountered in real forensic settings. MALDI's effectiveness as a confirmatory test was assessed by investigating its application following the deployment of presumptive blood tests such as Luminol and KM to diluted and to blood in the co-presence of other biofluids.

At scenes where a potential blood trace has been located outdoors and has been exposed to rain would mean that the blood will present itself as extremely diluted.

The dilution series reported in Chapter 5 was used to pre-apply the KM test and the Luminol test prior to proteolysis and MALDI MSP. The results indicated that the expected human blood peptide signatures were detectable in all dilutions tested (highest dilution of 1:20,000). For the Luminol treated dilutions, a positive human blood result could only be achieved up to the 1:10,000 dilution. The blood peptide signal intensities observed in all the Luminol treated samples were considerably lower than the KM equivalent samples indicating that this presumptive test causes signal suppression and would prevent successful MALDI MS analysis in higher diluted blood samples (as seen with 1:15,000 and 1:20,000). Despite the interference of the Luminol, both tests have been shown to be compatible with the MALDI MS analysis albeit in varying degrees. The findings from this section also highlight that, not only is it important to relay the capabilities of an approach but to also communicate where the technique would not be of benefit to deploy; both of which are equally important and from this section, it would be recommended to the analyst that MALDI may not be of assistance with extremely dilute Luminol treated blood samples.

Blood found on clothing in the presence of sweat and saliva is commonplace amongst individuals such as habitual drug users who have not been changing their clothing regularly (*Personal Communication*, Chris Gannicliffe, SPA). Therefore, determining whether these natural deposits of sweat and saliva interfere in the detection of the blood is important. The in-solution proteolysis and MALDI MSP of clothing samples containing blood and natural deposits of these biofluids permitted for the detection of the expected panel of human blood peptide signatures in every case. As this portion of the study was aimed at investigating blood in the presence of natural deposits of sweat and saliva, the actual quantities present on the clothing samples were not known and will have differed in each sample. For a more robust assessment of the co-presence of biofluids with the MALDI MS blood detection method, a more quantitative study could be performed whereby sweat and saliva are deposited on the clothing at known volumes in the presence of a known blood volume. Different clothing types should also be tested to determine whether the material impacts the analyses. This would assist in better understanding the volume at which these other biofluids may cause

interference with the blood analysis and whether the material of the clothing impacts the detection of the blood. For the purposes of this study however, it was only of interest to assess whether arbitrary deposits of sweat, and saliva would interfere with the analysis.

In addition to this, the work presented on investigating the migration of blood on different porous materials (cotton, wool, and polyester) and whether the effect of capillarity has any impact on successful blood detection is another scenario that, realistically, would be encountered often. The discovery of blood on porous materials such as curtains, bedding and carpets is regularly encountered by forensic analysts. With some of these material types, the yarn/ weave of the material can distort the morphology of the stain which impacts its interpretation, as a significant amount of intelligence can be gathered from the shape/positioning of a bloodstain. However, it is still not entirely understood what is happening to the blood as it disperses on these material types. This study aimed to better understand what is happening on the molecular level, whether there is migration of specific blood components to different regions of the stain. The bloodstains produced on cotton, wool, and polyester (at 50, 75 and 100 µL) were sampled from the centre and periphery for in solution proteolysis and MALDI MSP. All MALDI MS analysis achieved a positive human blood result, indicating that the region in which the stain is sampled does not affect the confirmatory detection of blood. Additionally, a comparison of Hb signal intensity (nominal *m/z* 1275 and 1530) was made, with the MALDI MS spectra indicating that higher signal intensity for these peptides was observed within the centre of the stain versus the periphery for polyester and cotton. In the wool samples however, the intensity of the two human Hb markers did not differ considerably between the centre and periphery. These findings aligned with what was visually observed with the stains. The wool samples showed the least blood spreading whereas the polyester and cotton samples showed more blood spreading. Further experiments could be performed where larger bloodstains are generated on these materials. It may be that the volumes used in this experiment were not sufficient to observe an intensity difference in the wool samples and that in larger stains, there would be an intensity difference observable between the centre and periphery. Additionally, time may also be a factor, as these stains were only left for 30 minutes to ‘spread’ prior to sample preparation. More time elapsed prior to

sampling may result in different observations regarding blood serum protein distribution and Hb signal intensity. Further to the distribution of blood serum proteins, the putative identifications made with the 100 µL bloodstains on all materials indicated a higher number of putative identifications in the centre for polyester, periphery for cotton and no difference between the two regions in wool. These results did not provide much additional intelligence, and as such, further work would be required to assess this concept, namely additional porous materials, multiple blood donors and a larger range of stain sizes. Despite the inability to comment more on what is occurring on a molecular level with blood deposited on these materials, blood could be detected compatibly when extracted from both the centre and periphery from all stains and materials; knowledge that contributes to the body of validation data required for operational deployment of this technique.

Although the body of work presented in this thesis has offered a significant amount of knowledge and greater understanding as to the effectiveness of MALDI MS based approaches as a confirmatory test for blood detection and for the provision of additional intelligence, its deployment is still subjected to further research and to informed uptake by the end users. The upcoming version of the Fingermark Visualisation Manual edited by Dstl promoting MALDI to a Category B technique, will encourage this process through further verification and validation studies from labs across the UK and the world. Additionally, as communicated by Chris Gannicliffe from the SPA, if a technique is not widely used, it is assessed against the ‘frequently used test’ threshold, and only if it is above this threshold the laboratory will be considered to undergo accreditation. Whilst lack of accreditation would not prevent MALDI from being used in an investigative setting, expert witness testimony in court may prove extremely challenging in the UK and it is inadmissible in the US. Nevertheless, performing these validation studies, compatibility tests and forming collaborations with end users puts MALDI very much on the road to wider operational use. Attending conferences where police forces, forensic companies and providers will be in attendance offers the opportunity to forge new partnerships and effectively ‘spread the word’ on MALDI and what the technique can offer in a forensic scenario. The work carried out in this project has not only shed light on the multi-informative nature of using a combined bottom-up proteomic MALDI MS

approach for blood analysis, but it has also highlighted the areas that need further investigation. The huge body of knowledge acquired throughout this PhD programme has answered many questions but also raised more than initially set out to answer. The number of publications generated since the start of the FRG truly indicates the capabilities that MALDI has in assisting with several different forensic challenges with the author of this thesis co-authoring a total of 4 peer-reviewed articles and 2 application notes (Kennedy et al. 2020; Kennedy et al. 2021; Kennedy et al. 2022; Kennedy et al. 2022; Witt et al. 2021 and Kennedy et al. 2021). Continuing to publish findings into the forensic and scientific communities will only have a positive impact and will help greatly in making the journey to operational implementation of MALDI a much shorter one.

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Chapter 7: Publications, Oral Presentations and Poster Presentations

7.0 PUBLICATIONS, ORAL PRESENTATIONS AND POSTER PRESENTATIONS

Poster

(1) International Mass Spectrometry School (IMSS): 15th-20th September. Title: Optimisation of trypsin concentration for MALDI MS analysis of blood volumes. (Sitges, Barcelona, 2019)

Poster

(2) British Mass Spectrometry Society (BMSS): 3rd to 5th September. Title: Optimisation of trypsin concentration for MALDI MS analysis of blood volumes. (Manchester, UK, 2019)

Poster

(3) American Society of Mass Spectrometry (ASMS): 1st-12th June. Title: Pre-validation of a bottom-up proteomic MALDI MS approach for the detection of blood and blood provenance. (Virtual, 2020)

Talk

(4) British Mass Spectrometry Society (BMSS): 7th-9th September: Title: "MALDI CSI": The reliable detection of blood at crime scenes using MALDI mass spectrometry. (In person, Sheffield, UK) Accepted for talk but could not deliver due to contracting COVID-19.

Poster

(5) American Society of Mass Spectrometry (ASMS): 31st Oct-4th Nov Abstract Title: "MALDI CSI": The reliable detection of blood at crime scenes using MALDI mass spectrometry. (Virtual, 2021)

Poster

(6) OurCon 2021: 12th-13th October Abstract Title: Direct detection and mapping of peptide signatures in human and animal blood finger marks using the HTX M3+ sprayer. (Virtual, 2021)

Talk

(7) World Police Summit: 14th-17th March. Title: "MALDI CSI": The reliable detection of blood at crime scenes using MALDI mass spectrometry. (Dubai, UAE)

Publications

(1) Kennedy, K., Heaton, C., Langenburg, G., Cole, L., Clark, T., Clench, M. R., ... & Francese, S. (2020). Pre-validation of a MALDI MS proteomics-based method for the reliable detection of blood and blood provenance. *Scientific reports*, *10*(1), 1-18.

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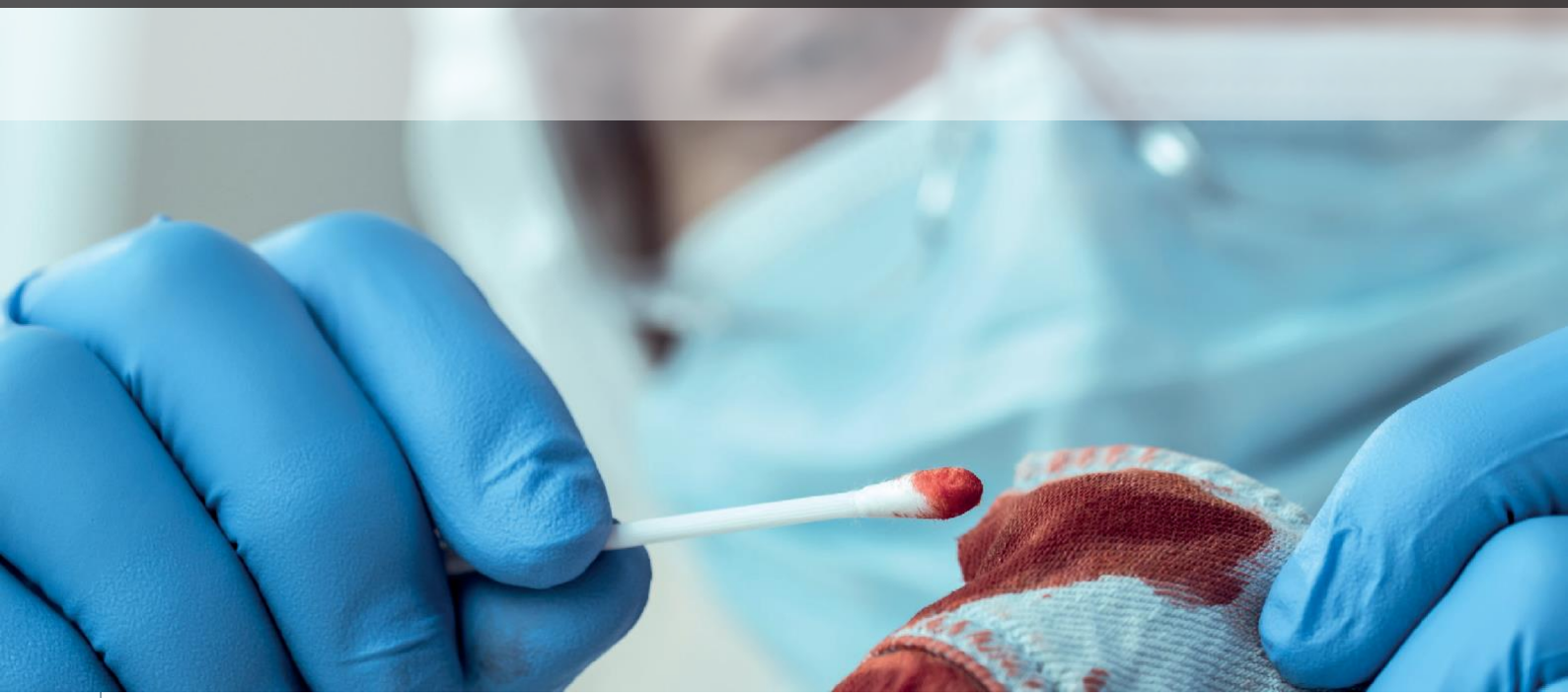
Appendix I



Forensic visualisation of blood and blood provenance in old fingermarks by MALDI MS Imaging

Blood can be often found at the scene of violent crimes. Whether visible or latent, it is critical that its presence is detected, confirmed and distinguished from other biofluids as well as determining its provenance.

Abstract

This intelligence collectively aids in the reconstruction of the dynamics of the crime and indicates the nature of the crime itself. Over the years Matrix Assisted Laser Desorption/Ionisation (MALDI) Mass Spectrometry Profiling and Imaging (MSP and MSI) have demonstrated to be suitable analytical tools as confirmatory tests for blood stains and blood marks. MALDI MSP and MSI can prove or disprove the results of the currently applied presumptive tests deployed at the scene. Additionally, fingermark molecular images can be obtained through the mapping of human blood specific proteins in fresh fingermarks. Here, for the first time, we demonstrate the mapping of biomarkers of human and animal blood detected in four year old fingermarks.

Keywords: Molecular fingerprinting, SCiLS, timsTOF fleX, Imaging

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Introduction

Reliable confirmation of the presence of blood in stains and marks is crucial to reconstruct the dynamics of a violent crime. MALDI MS Profiling (MALDI MSP) and MALDI MS Imaging (MALDI MSI) have been shown to be established confirmatory tests to this effect [1-4]. However, additional and important information is based on the attribution of the source of blood (animal or human), as a few murder cases in the public domain demonstrate. The Fingermark Research Group at Sheffield Hallam University, recently published on the validation of a *MALDI MSP* method to detect and distinguish between human and animal blood (down to the animal species level) in enhanced blood marks and stains analysed in a blind fashion [5] (Figure 1).

Here we use a combination of bottom-up proteomics and MALDI MSI to visualise blood of bovine or human origin in 4-year-old finger-marks. These are unused marks originating from the batch of blind samples on which the Kennedy et al. publication [5] was based.

Methods

The fingermarks investigated were generated by loading the fingertip with approximately 40-50 μL of either human blood or bovine blood and by contacting the fingertip with an aluminium slide after 10-15 seconds. Once dried, in the case of the bovine blood mark, Acid Black-1 (AB-1) was applied using the same protocol employed by crime labs for the enhancement (visualisation) of blood marks.

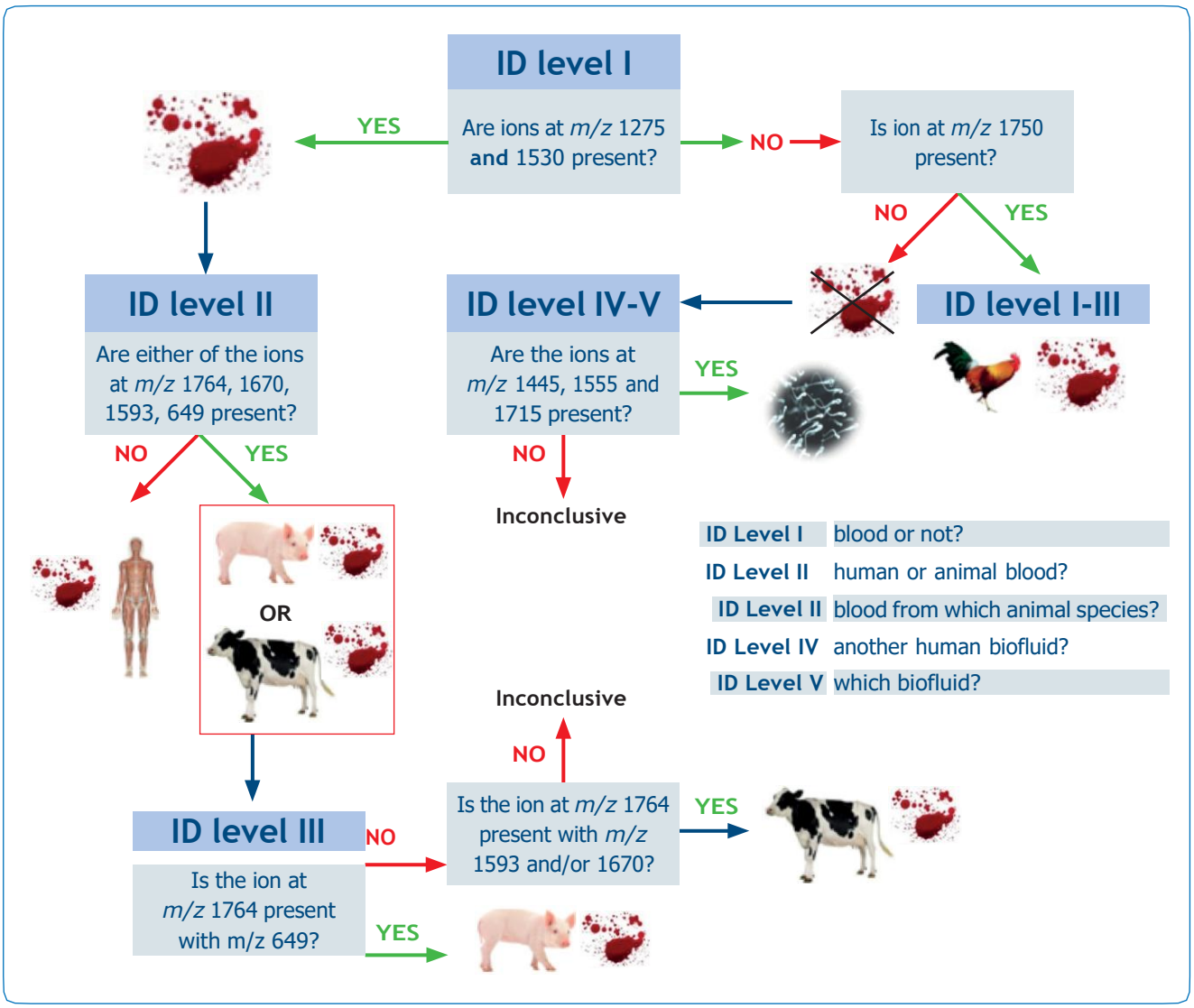

Figure 1. Blind sample spectra data interpretation strategy. This strategy enables the determination of human and animal provenance down to species (as well as the determination of the presence of semen). The m/z values are nominal. Their presence is verified with a mass accuracy <15 ppm. (Reproduced from Kennedy et al. Scientific Reports, 2020 [5] under the Creative Common Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/.)

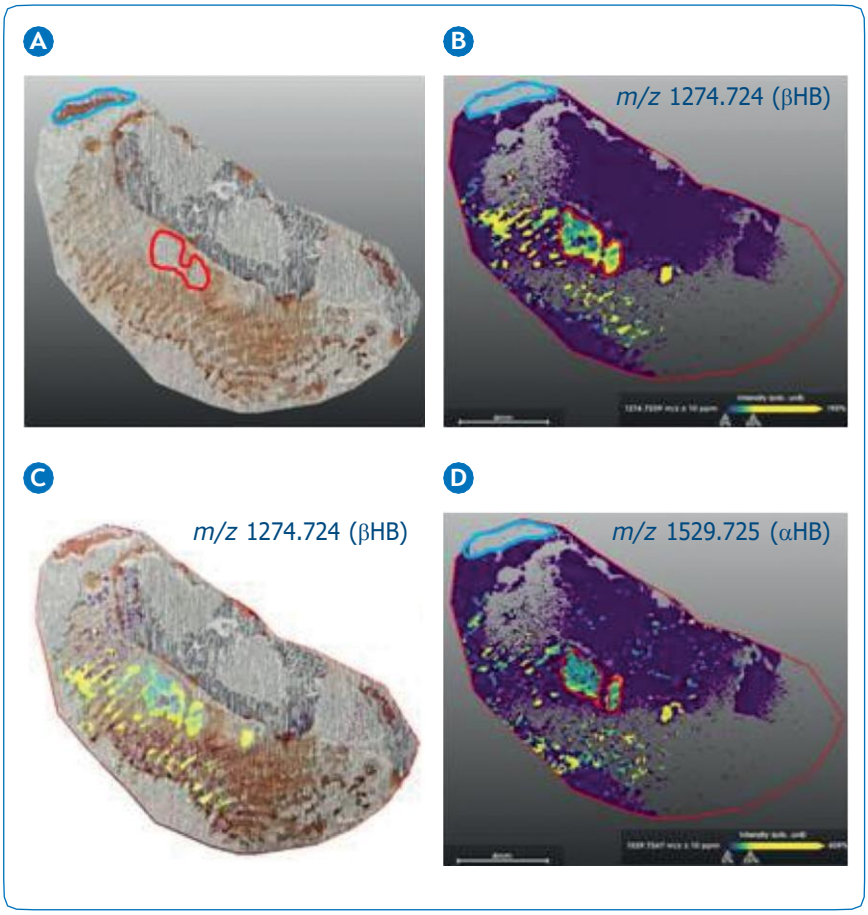

Figure 2. MALDI MSI of a human blood fingermark of 4 years of age stored in uncontrolled ambient conditions. (A) optical image; (B) and (D) MALDI MS image of the HB ions at m/z 1274.724 (βHB) and 1529.725 (αHB) respectively, measured with a 10 ppm mass accuracy. (C) Overlay of the image at m/z 1274.724 with the optical image. Blue and red framed highlight: regions of the mark where blood is visible or not visible respectively, on the optical image.

The unenhanced human blood fingermark and the enhanced bovine mark were digested *in situ* using the SunCollect (Sunchrom, Germany) by employing trypsin at a concentration of 200 µg/mL containing 0.1% RapigestSF (Waters Corp, UK). Nine layers of trypsin were sprayed at a flow rate of 2 µL/min. After incubation at 37°C, 5 mg/mL of α cyano-4 hydroxy cinnamic acid (HCCA) in 70/30 acetonitrile/TFA (0.5%)aq were sprayed at a flow rate of 4 µL/min for a total of 5 layers.

The blood fingermarks were imaged on a timsTOF fleX mass spectrometer (Bruker Daltonik GmbH, Germany) at a lateral resolution of 50 µm in the mass range *m/z* 100-3000. Images were processed using SciLS™ Lab software (Bruker Daltonik GmbH, Germany) and were all RMS normalized.

Results and discussion

The investigated human blood mark exhibits poor ridge detail (Figure 2). This is somewhat common in many violent crime scenes, where blood marks have a scarce amount of *minutiae* permitting an identification. Although no imaging technique can improve the quality of an originally poor/smudged ridge pattern, the MALDI MSI analysis on the timsTOF fleX instrument was able to accurately visualize Haemoglobin (HB) onto the visible ridge pattern in this 4-year-old human blood mark (Figure 2). In addition, some ridge flow was reconstructed and found to be super-imposable with that exhibited by the optical image.

It is important to note that MALDI MSI revealed the presence of blood (through HB) where this was not visible on the optical image (red frame highlight, Figure 2). However, in the region where blood was visible by the naked eye (blue framed), MALDI MSI did not yield the HB signals. These occurrences reflect the delicate nature of the optimal enzyme : substrate ratio. This observation indicates that, as the presence of haemoglobin may vary greatly across the blood mark itself, visualisation of this biofluid will be dependent on its local ratio with trypsin.

The simultaneous detection of the ion signals at *m/z* 1274.724 (βHB) and 1529.725 (αHB) indicates the presence of human blood, according to the findings of Kennedy et al. [5] and within the system that they investigated.

Additional (and aspecific) HB signals and those from other blood specific proteins previously detected [4] were also visualised. These are namely: Erythrocyte Band Protein 4 (EBP42), Haptoglobin (Hpt) and Serotransferrin (Figure 3) with a mass accuracy ranging between -3.1 to 0.9 ppm.

However, in some cases, the distribution maps were weak, reflecting the low abundance of these proteins and the lower ionisation yield of the corresponding peptides (compared to those of Haemoglobin).

In order to improve the quality of the biometric information, lipids can also be imaged within the same analysis. Figure 4 illustrates an example for two lipids at *m/z* 727.554 and 741.526 respectively, yielding molecular images of the mark in which additional ridge coverage can be observed.

A bovine blood fingermark has been subsequently imaged to assess the possibility to map the bovine blood biomarkers discovered by Kennedy et al. [5] using MALDI MSI. The mark has been produced with no specific intention to generate ridge detail but simply mimicking a crime scene scenario in which a fingertip containing much blood is contacted carelessly with a surface.

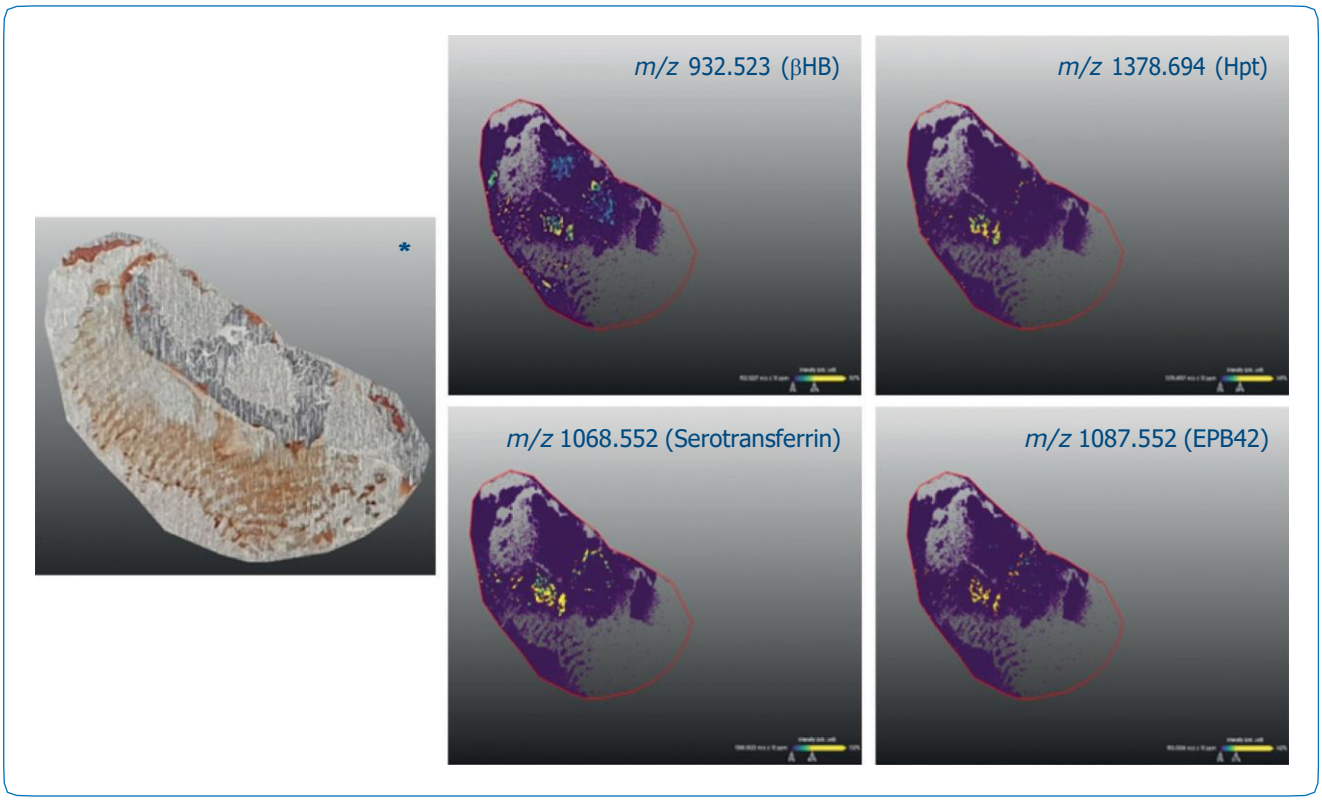

Figure 3. MALDI MS images of blood specific protein deriving peptides in a human blood fingermark. Panel shows again the optical image of the human blood mark that was imaged. Many ion signals indicate the presence of blood through the detection of HB, Hpt, Serotransferrin and EBP42.*

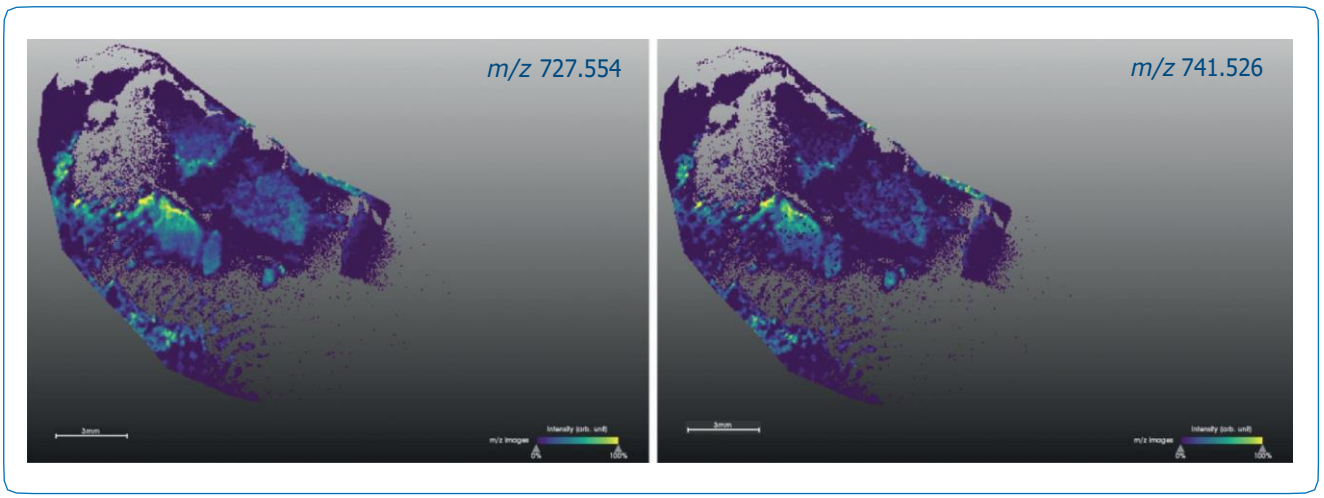

Figure 4. MALDI MS images of lipids at m/z 727.554 and 741.526 in a human blood fingermark. These ion signals show additional ridge coverage with respect to the blood specific protein-deriving peptides.

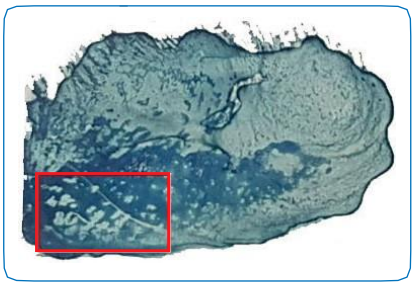

Figure 5. Optical image of an Acid Black 1 (AB-1) enhanced bovine blood fingermark. The red frame shows partial ridge pattern.

Figure 5 shows such a bovine blood mark in which blood has irregularly pooled. The red frame in the figure highlights the presence of partial ridge detail.

As it can be seen in Figure 6, the three peptide markers indicating the presence of *bovine blood* at *m/z* 1592.843 (Myoglobin), 1669.832 (Myoglobin) and 1763.799 (Glyceraldehyde-3 phosphate dehydrogenase) have been, for the first time, successfully imaged showing coherent distributions.

The ions were imaged with a mass accuracy of 20 ppm. Additional (aspecific) Myoglobin and GAPDH ion signals were also mapped. Despite being aspecific, these ion signals support the putatively identified presence of these bovine blood biomarkers.

Particularly the images of the ions at *m/z* 1669.832, 1784.434 and 1477.798 depict the partial ridge detail that was observed in the optical image (red rectangle).

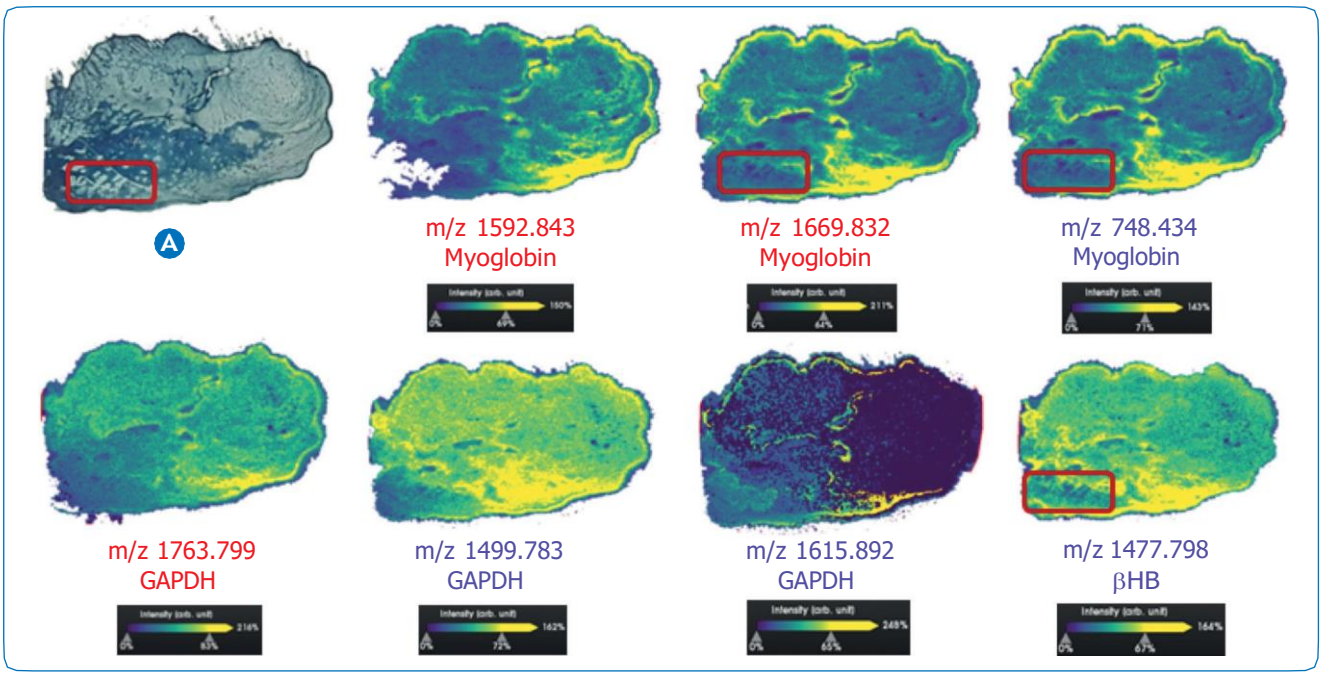

Figure 6. Many ion signals were recovered indicating the presence of blood through Haemoglobin, Myoglobin and GAPDH. A selection of such images is presented here. (A) shows the optical image of the AB-1 enhanced bovine blood mark. The red rectangle indicates the presence of ridge flow in both the optical image and some of the MS images. The m/z values in red indicate the proteotypic bovine blood peptide biomarkers within the system investigated by Kennedy et al. [5].

Conclusion

This short investigation confirmed the ability of MALDI MSI to image blood fingermarks and yield ridge detail in old fingermarks. This capability was contextual to detecting and imaging, for the first time, biomarkers enabling discrimination of blood from different origin. As the marks were 4 years old by the time they were imaged, these results show that, by using a high end mass spectrometer capable of high sensitivity, spatial and mass resolution, it may be possible to perform MALDI based confirmatory tests for blood marks recovered at the scene of violent crimes that can sometimes be accessed at a much later time after the crime has been committed.

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Appendix II

APPLICATION NOTE

#60 Sequential Application of Trypsin and Matrix for Forensic Analysis of Blood Fingermarks

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Application & Background

Detection, confirmation and visualisation of blood at crime scenes informs on the dynamics of the bloodshed and on the nature of the crime. It may be crucial to also indicate foul play in a murder without a body. Current presumptive methods used by crime scene investigators are prone to false positives and hence a reliable confirmatory test is paramount. MALDI MS Profiling and Imaging (MALDI MSP/I) have proven to be suitable confirmatory tests to detect and map the presence of blood and its origin (human or animal)[1] in fingermarks and reconstruct the ridge pattern for suspect identification.[2-3] Specifically, a method has been developed combining the use of bottom-up proteomics and MALDI MSP/I to detect and visualize blood through blood specific proteins and in particular from the peptides generated by the *in situ* enzymatic digestion.

In this application, trypsin is sprayed at a low flow rate across the blood mark using the HTX M3+ Sprayer. A lower flow rate allows to minimise enzyme consumption. Following trypsin application and incubation, the sample is returned to the sprayer for matrix application. Matrix application is performed at a higher flow rate, but with the same pump used for enzyme spraying, without needing any manual modifications to the instrument. The automated PumpScripts™ on the HTX M3+ Sprayer (**Figure 1**) provide thorough and hands-free cleaning of the Sprayer between enzyme and matrix spray cycles, which is essential for optimal application of these two distinct chemicals. An overview of this workflow is shown in **Figure 2**.

Spray conditions specific for blood fingermark samples are needed for both the enzyme and the matrix application in this workflo due to the blood fingermark being less securely attached to slides compared to tissues. As the ridge pattern and details provide biometric information, spray conditions such as gas pressure, velocity, and track spacing must be optimized to preserve the ridge detail integrity.

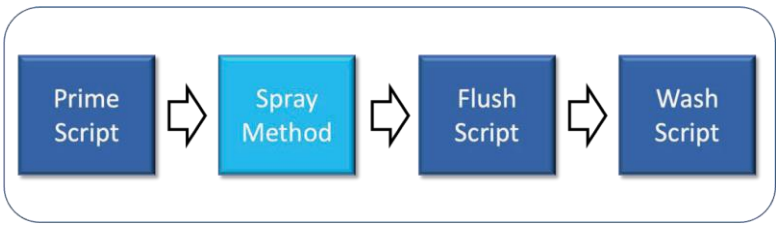

Figure 1. Automated PumpScripts™ attached to each spray method.

Experimental

Experimental Design & Fingermarks

Human blood fingermarks were prepared on poly-L-lysine coated slides. Human blood was obtained by pricking a clean finger with a single-use lancet and contained in EDTA tubes. Ten microliters of blood were spotted on a slide to wet a silicon fingertip which consequently generated a blood mark by contact with another clean glass slide. The blood mark was fixed in 100% methanol for 1 hr before being left to dry.

Enzyme and Matrix Application using the HTX M3+ Sprayer

Using the HTX M3+ Sprayer, trypsin (**Table 1**) was applied to the slide using the parameters displayed in **Table 2**. After trypsin spraying, the slides was placed "face up" in a humidity chamber consisting of a silicon sealed tub and containing tissue paper saturated with a 50:50 methanol:water solution. The sample was left to incubate for 3 hrs in a 37°C oven.

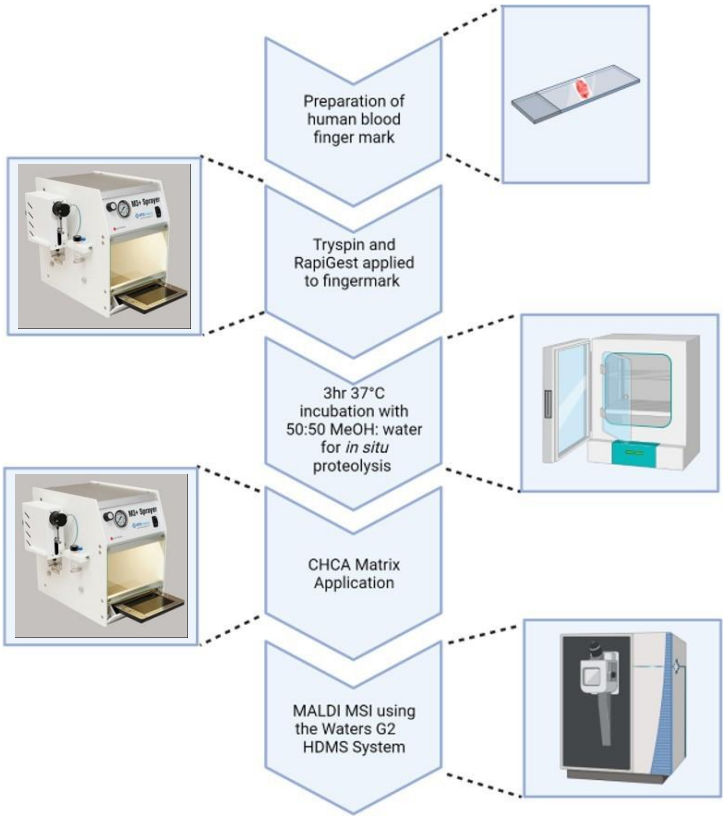

Figure 2. Workflow for peptide MALDI MSI analysis on blood fingermarks.

The slide was then removed from the humidity chamber, and the bottom of the slide was dried using a KimWipe. Using the HTX M3 + Sprayer, α-cyano-4-hydroxycinnamic acid (CHCA) matrix (**Table 1**) was applied to the slide using the parameters displayed in **Table 2**.

MALDI MSI

All imaging experiments were performed on a QTOF G2 HDMS Synapt MALDI mass spectrometer (Waters Corporation, Manchester, UK) in positive ion mode using a 1 KHz Nd:YAG laser. Each pixel was collected at a 100 µm spatial resolution.

Data Analysis

The data were collected and visualized using HD Imaging (HDI) software (Waters Corporation, Manchester, UK). Putative peptide identifications were manually made using an in-house blood peptide database. Blood specific peptide-deriving proteins such as hemopexin, erythrocyte protein band 4.2 (EPB 4.2), serotransferrin, prothrombin, apolipoprotein A1, serotransferrin, ceruloplasmin and complement C3 α were searched;. Images were all normalized to the total ion current (TIC); and to aid visualization of the ridge detail, brightness and contrast were then adjusted by the same level in each image shown. Exclusion mass lists, including matrix/ matrix cluster/ adduct) and trypsin autolysis *m/z* peaks were generated and used to prevent the assignment of irrelevant *m/z* signals.

Table 1. Preparation protocols for all solutions used in MALDI MS blood fingermark peptide imaging workflow.

	Recipe	Final Concentration
Enzyme Buffer	50mM ammonium bicarbonate with 0.1% Rapigest SF (Waters)	50mM AmBic 0.1% Rapigest pH = 8
Trypsin	150µg of trypsin in enzyme buffer for total volume = 1mL	0.15 mg/mL
CHCA	30mg CHCA in 6mL of 70% ACN/0.5% TFA	5 mg/mL

Table 2. Spraying parameters for sequential enzyme and matrix deposition using the HTX M3+ Sprayer.

	Trypsin	CHCA
Solvent	50mM AmBic, 0.1% Rapigest	70% ACN, 0.5% TFA
Concentration (mg/mL)	0.150	5
Flow rate (mL/min, FR)	0.030	0.100
Velocity (mm/min, V)	750	1200
Nozzle Temperature (°C)	30	75
Track Spacing (mm, TS)	2	4
Number of Passes (NP)	8	4
Nitrogen Pressure (psi)	10	10
Spray Pattern	CC	HH
Drying Time (s)	10	0
Nozzle Height (mm)	40	40

Results

Haem and blood specific peptide signatures were imaged in blood fingermarks. Each ion signal *m/z* can be visualized as heatmaps across the slide, and an overlay is also shown, **Figure 3**. Quality of fingermarks clarity, continuous ridge flow, and details (*minutiae*) varied depending on the species imaged with haem providing a grade 4 mark (according to the Home Office grading scheme).[5] While haem is not a result of the trypsin digestion, its visualization enables to appreciate the high quality of matrix application across the fingermark. The presence of hemoglobin alpha and beta peptides at nominal m/z 1275 and 1530 can be used to confirm the human provenance of the blood detected.

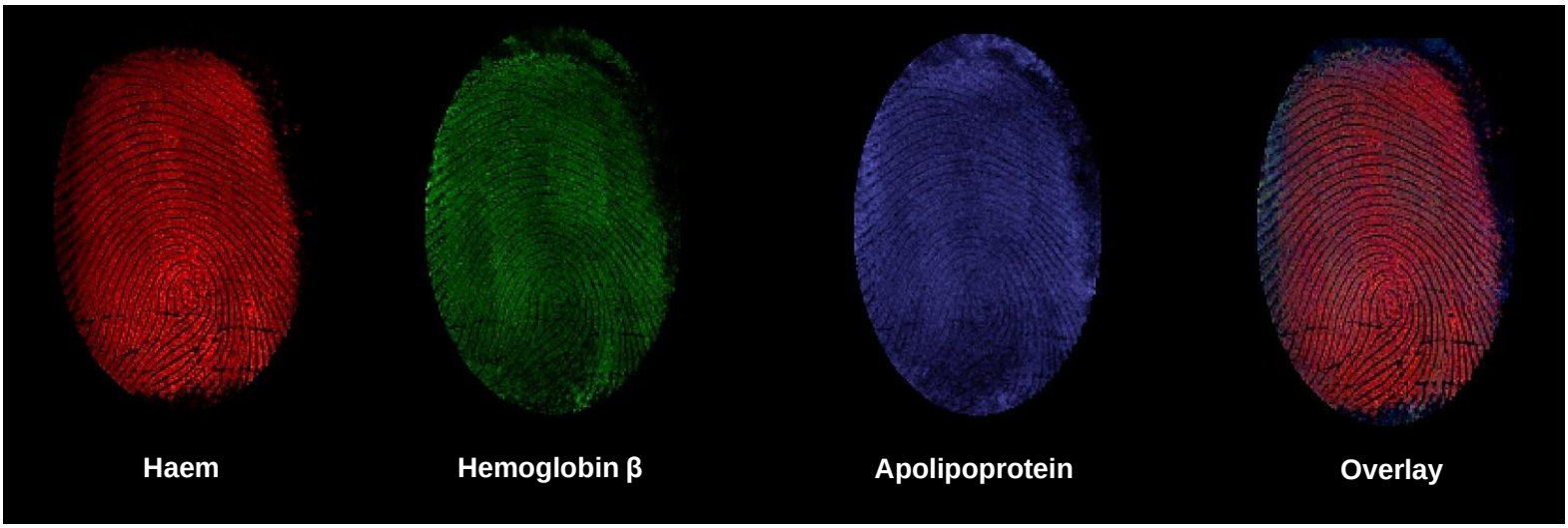

Figure 3. Haem and blood specific peptides from a human blood fingermark following application of trypsin and CHCA matrix. Images shown are haem at *m/z* 616.17 (red), hemoglobin β at *m/z* 1149.56 (green), and apolipoprotein at *m/z* 1215.6 (blue). The 4th and last MS image from the left is a superimposition of these 3 species.

Table3. Key human blood peptides detected from blood fingermarks following tryptic digestion.

Experimental *m/z*	Peptide Sequence	Protein
616.17	Haem	
1529.70	VGGHAAEYGAELER	Human Hemoglobin Alpha
1149.56	VVAGVANALAHK	Human Hemoglobin Beta
952.43	VQKYRMK	Human Hemoglobin Beta
1065.53	MDKVGKYPK	Human Complement 3 Alpha
1171.59	VLSPADKTNVK	Human Hemoglobin Alpha
1215.60	ATEHLSTLSEK	Human Apolipoprotein A1
1194.63	ELLESYIDGR	Human Prothrombin
1065.54	MDKVGKYPK	Human Complement 3 Alpha

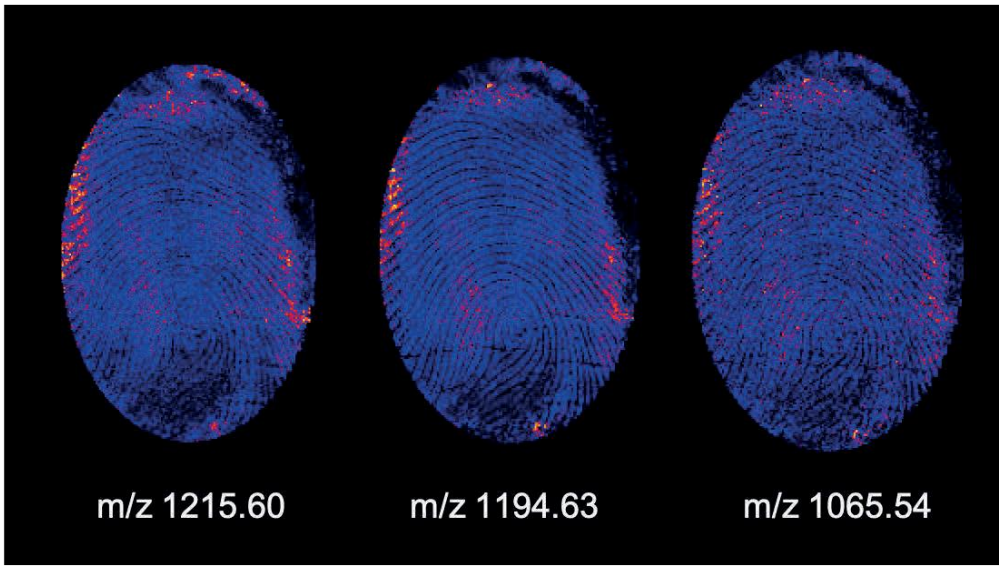

Figure 4. Example blood peptides detected from a human blood fingermark. Peptides shown are apolipoprotein A1 at *m/z* 1215.60 (ATEHLSTLSEK), thrombin at *m/z* 1194.63 (ELLESYIDGR), and complement 3 alpha at *m/z* 1065.54 (MDKVGKYPK).

In all cases, in addition to *minutiae*, level 3 details (pores) were also observed. Additional blood peptides observed were apolipoprotein A1, complement 3 alpha, and prothrombin, **Table 3.** A selection of corresponding images are shown in **Figure 4**, in which, again high quality ridge detail could be generated from each of these tryptic peptides.

While the results shown here indicate successful detection and identification of blood and some peptides specific to human blood, this method could also be used to detect non-human blood peptides for species identification at the scene of a crime. The combination of haem for biometric information and blood peptides detection and localization makes MALDI MSI a suitable confirmatory test generating a wealth of intelligence to utilize in forensic investigations.

Conclusions

We demonstrate the use of the HTX M3+ Sprayer for tryptic blood protein enzymatic digestion and matrix coating on human blood fingermarks for MALDI imaging. This workflow for peptide analysis in blood fingermarks requires two distinct spray methods for reagent application. The HTX M3+ Sprayer provides a simple platform for analyses requiring multi-method workflows. No manual switching of lines or cleaning is required due to the automation of the Cadence Pump 8-way valve and PumpScripts™ on the HTX M3 + Sprayer. Enzyme consumption is also minimized due to the advanced fluidics control of the pump and sprayer.

The data presented here report high resolution images of blood fingermarks prepared on the HTX M3+ Sprayer. High quality ridge detail is retained in the fingermarks following multi-method spray cycles of enzyme and matrix. Key human blood peptides are detected contextual to the biometric data yielded by the ridge flow and *minutiae*. This capability is of great value in crime scene investigations for informing on the nature and the dynamics of the bloodshed at a scene of a violent crime.

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Key Characteristics

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- Unique use of temperature and nitrogen flow to control evaporation rate and matrix crystal formation
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- Continuous matrix coverage as needed for high-resolution imaging
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Appendix III

Additional MS/MS Spectra with *m/z* annotations (corresponding to Figures 3-6) for Paper in Chapter 2 (Kennedy et al 2020, Sci. Reports)

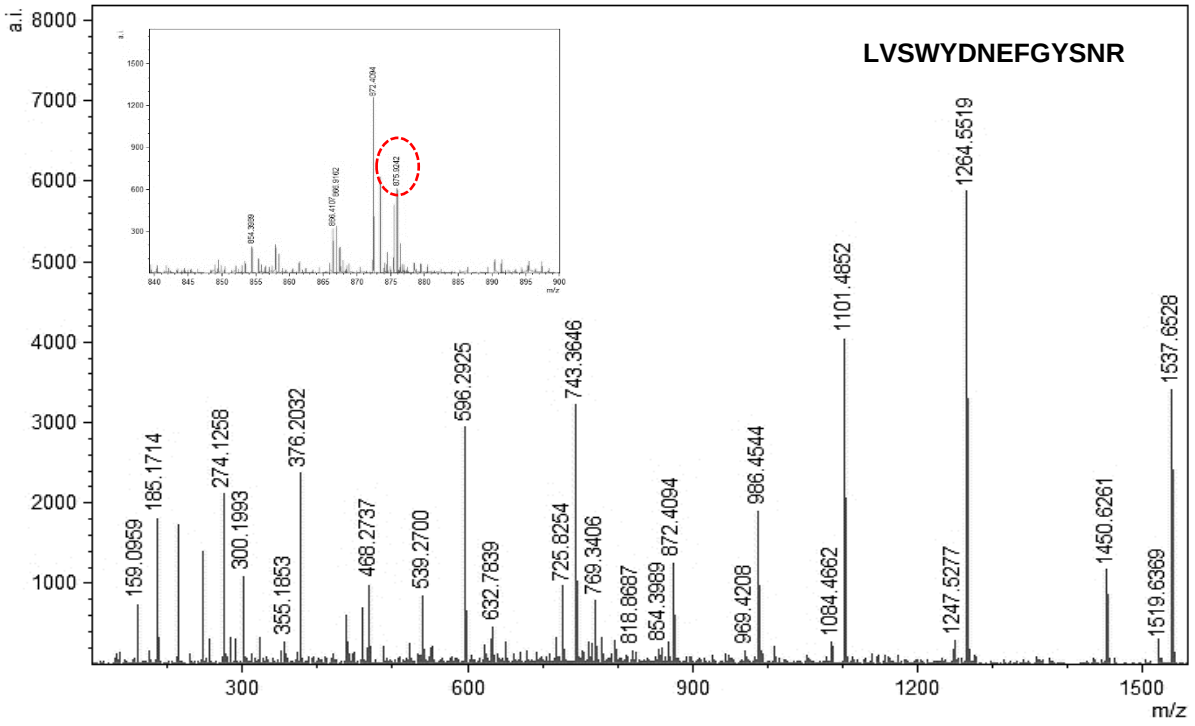

Figure 1: LC-MS/MS spectrum of GAPDH peptide (from chicken sample 35S) from doubly charged peptide at nominal *m/z* 876 (Figure 3 in Paper).

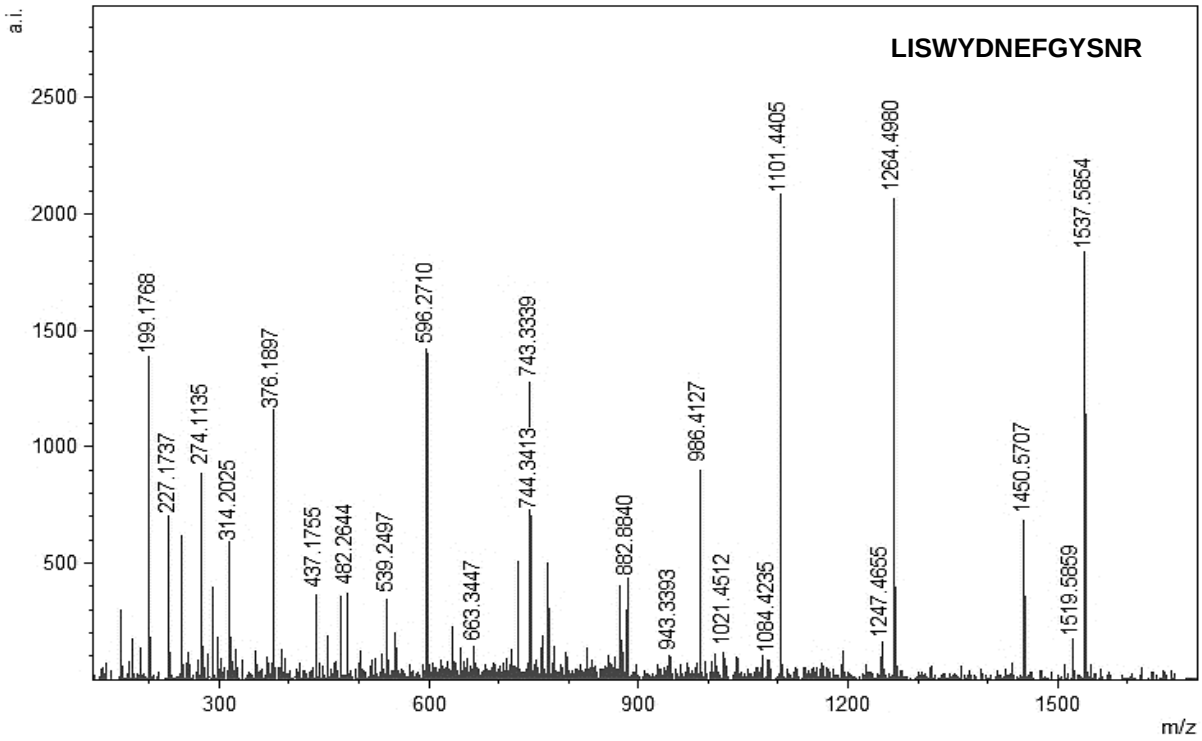

Figure 2: LC-MS/MS spectrum of GAPDH peptide (from porcine sample 7S) from doubly charged peptide at nominal *m/z* 883 (Figure 4 in Paper).

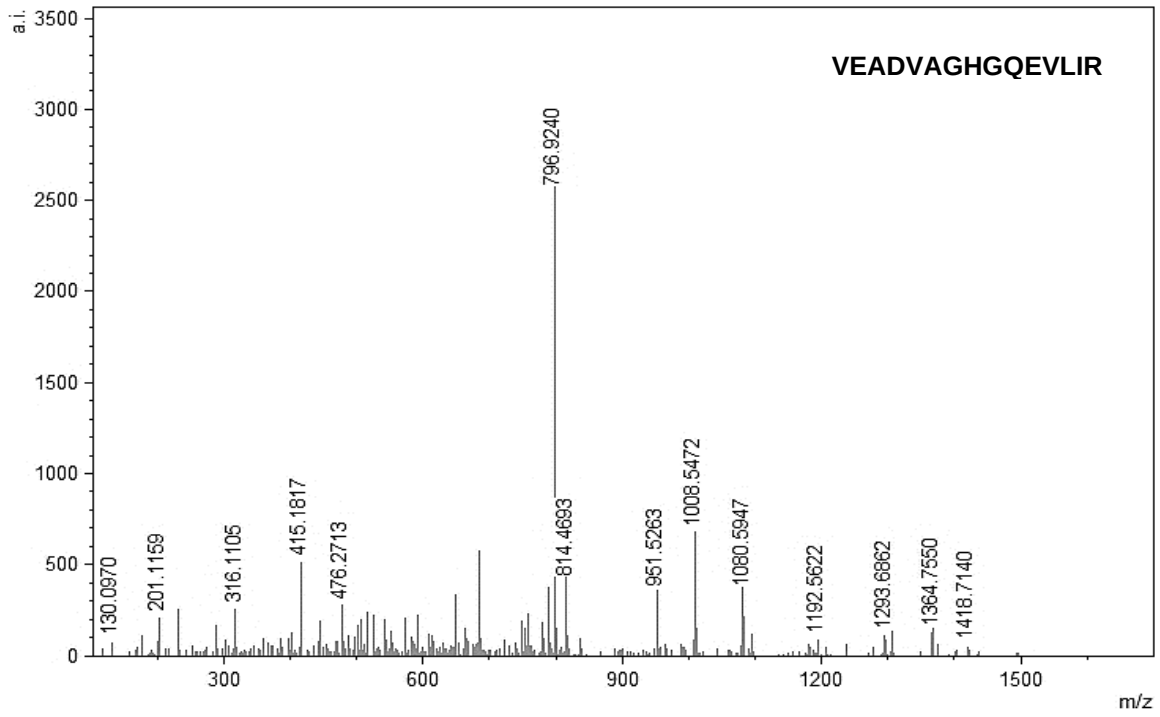

Figure 3: LC-MS/MS spectrum of Myoglobin peptide (from porcine sample 38S) from doubly charged peptide at nominal *m/z* 797 (Figure 5 in Paper).

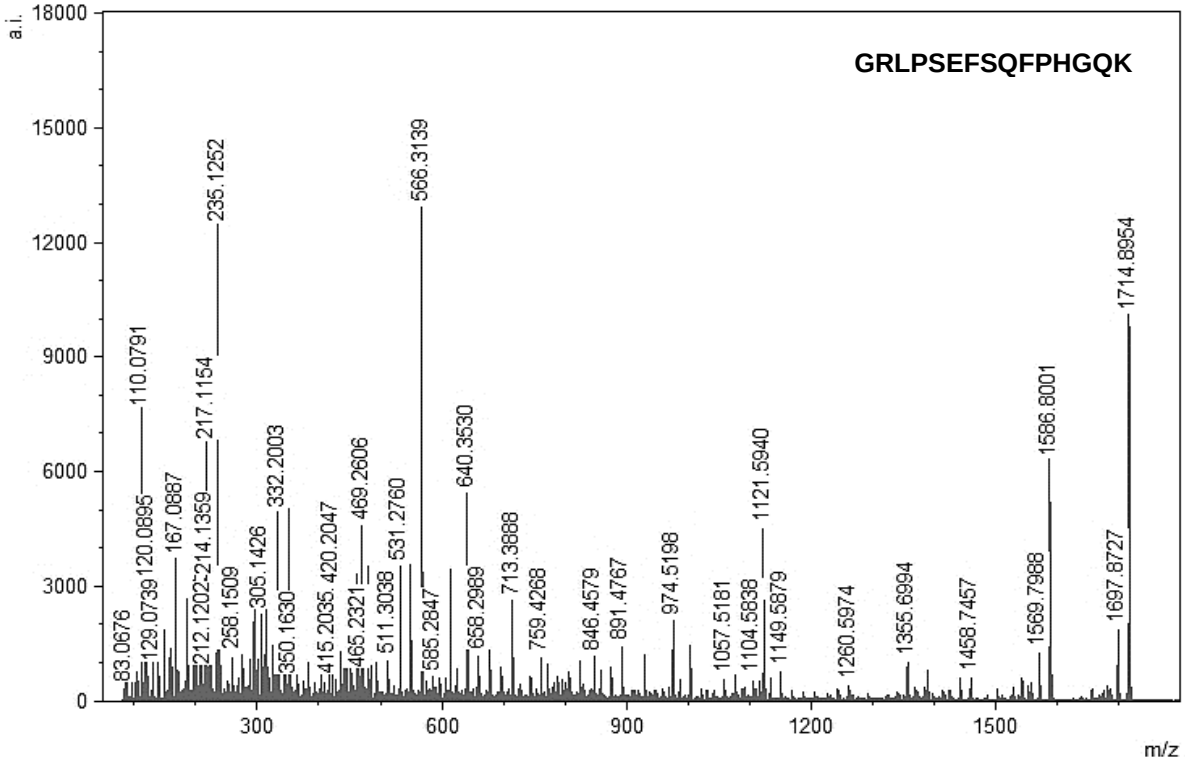

Figure 4: MALDI MS/MS spectrum of SEM-1 peptide marker for semen sample identification at nominal *m/z* 1715 (Figure 6 in Paper).

Intact Haemoglobin Molecular Weights and Monoisotopic Masses

	Molecular Weight (Da)		Monoisotopic Mass $[M+H]^+$	
Species	**Hbα**	**Hbβ**	**Hbα**	**Hbβ**
Human	15258	15998	15259	15999
Bovine	15184	15954	15185	15955
Porcine	15039	16166	15040	16167
Chicken	15429	16466	15430	16467
Amino Acid Sequence of Haemoglobins				
Species	**Hbβ**		**Hbα**	
Human	MVHLTPEEKSAVTALWGKVNVD EVGGEALGRLLVVYPWTQRFFE SFGDLSTPDAVMGNPKVKAHG KKVLGAFSDGLAHLDNLKGTFA TLSELHCDKLHVDPENFRLLGN VLVCVLAHHFGKEFTPPVQAAY QKVVAGVANALAHKYH		MVLSPADKTNVKAAWGKVGAHA GEYGAEALERMFLSFPTTKTYFP HFDLSHGSAQVKGHGKKVADALT NAVAHVDDMPNALSALSDLHAHK LRVDPVNFKLLSHCLLVTLAAHLP AEFTPAVHASLDKFLASVSTVLTS KYR	
Bovine	MLTAEEKAAVTAFWGKVKVDEV GGEALGRLLVVYPWTQRFFESF GDLSTADAVMNNPKVKAHGKK VLDSFSNGMKHLDDLKGTFAAL SELHCDKLHVDPENFKLLGNVL VVVLARNFGKEFTPVLQADFQK VVAGVANALAHRYH		MVLSAADKGNVKAAWGKVGGHA AEYGAEALERMFLSFPTTKTYFP HFDLSHGSAQVKGHGAKVAAALT KAVEHLDDLPGALSELSDLHAHK LRVDPVNFKLLSHSLLVTLASHLP SDFTPAVHASLDKFLANVSTVLTS KYR	
Porcine	MVHLSAEEKEAVLGLWGKVNV DEVGGEALGRLLVVYPWTQRFF ESFGDLSNADAVMGNPKVKAH GKKVLQSFSDGLKHLDNLKGTF AKLSELHCDQLHVDPENFRLLG NVIVVVLARRLGHDFNPNVQAA FQKVVAGVANALAHKYH		VLSAADKANVKAAWGKVGGQAG AHGAEALERMFLGFPTTKTYFPH FNLSHGSDQVKAHGQKVADALTK AVGHLDDLPGALSALSDLHAHKL RVDPVNFKLLSHCLLVTLAAHHP DDFNPSVHASLDKFLANVSTVLT SKYR	
Chicken	MVHWTAEEKQLITGLWGKVNVA ECGAEALARLLIVYPWTQRFFA SFGNLSSPTAILGNPMVRAHGK KVLTSFGDAVKNLDNIKNTFSQL SELHCDKLHVDPENFRLLGDILII VLAAHFSKDFTPECQAAWQKLV RVVAHALARKYH		MVLSAADKNNVKGIFTKIAGHAEE YGAETLERMFTTYPPTKTYFPHF DLSHGSAQIKGHGKKVVAALIEAA NHIDDIAGTLSKLSDLHAHKLRVD PVNFKLLGQCFLVVVAIHHPAALT PEVHASLDKFLCAVGTVLTAKYR	

Table 1: Intact Haemoglobin (α and β) molecular weights, monoisotopic masses and amino acid sequences for all species investigated in this study.