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An update on MALDI Mass Spectrometry based technology for the analysis of fingerprints - stepping into operational deployment

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Abstract

Since in 2009, when Matrix Assisted Laser Desorption Ionisation Mass Spectrometry Imaging (MALDI MSI) was firstly reported for the molecular mapping of latent fingerprints, the range of information and operational capabilities have steadily increased. Pioneering work from our Fingerprint Research Group exploited different modalities, including Profiling (MALDI MSP), tandem mass spectrometry (MS/MS) and Ion Mobility MS/MS; a number of methodologies were also developed to conquer a main challenge, namely profiling the suspect and their actions prior to or whilst committing the crime. Suspect profiling here is no longer based on behavioural science but complements this discipline and the investigations by detecting and visualising the molecular make-up of fingerprints onto the identifying ridges. This forensic opportunity provides the link between the biometric information (ridge detail) and the corpus delicti or intelligence on the circumstances of the crime. In 2013, a review was published covering the research work and developments of four years supported by the Home Office, UK and the local regional Police with some insights (and comparison) into similar research being reported employing other mass spectrometric techniques. The present review is an extensive update on the MALDI MS based methods' achievements, limitations and work in progress; it also offers an outlook on further necessary research into this subject. The main highlights are the increased number of possible information retrievable around a suspect and the more extended compatibility of this technology. The latter has allowed MALDI MS based methods to integrate well with current forensic fingerprinting, leading to the investigation of real police casework.

1.0 Introduction

For more than a century, fingermarks have been the most powerful means of biometric identification. Even with the advent of DNA, The Yorkshire and Humber Regional Police in UK (including Humberside, South Yorkshire, North Yorkshire and West Yorkshire Police) reports that $\frac{3}{4}$ of the suspect identifications are still due to the process of matching a crime scene fingermark to a fingerprint record held in the National Database (Neil Denison, Head of Identification Service, Humber and Yorkshire Police, personal communication). This process is named fingerprinting and it is based on the matching of level 2 features called minutiae which are local characteristics of the ridge pattern.

However, there are a number of scenarios where fingerprinting fails¹ and they are due to factors such as the age of the mark, environmental conditions to which it was exposed, choice of enhancement/development processes, objective state of the mark (which could be smudged overlapped, faint, partial and generally distorted due to non-linear contact) and how "good or bad" is the donor from sweat point of view. All these factors may also interplay thus further affecting the recovery of ridge detail.

Additionally, first time offenders will not have a fingerprint record, thus preventing apprehension on the basis of fingerprinting, unless an arrest is made.

Classically, fingermarks have only been exploited from a "physical" point of view, that is, by visualising the ridge pattern by optical, chemical or physical means.

However, in the scenarios described above, this investigation angle is clearly insufficient. Starting from 2007, the analytical community had started to realise that fingermarks are more than just "beautifully twisting lines" and can offer much more than physical information; in fact, as it was already known that fingermarks are the result of a molecular transfer from the fingertip to a surface upon contact, this molecular content is potentially the carrier of important forensic intelligence.

For latent marks, e.g. those invisible to the naked eye, the major biological matrix being transferred is sweat which is excreted through the sweat pores present in the fingertips. This type of secretion is called eccrine and contains mainly water, electrolytes and in minor part by amino acids and proteins^{2,3}. These are called endogenous secretions. However, fingertips become contaminated due to the regular contact of the fingertips with other body areas, such as the face, presenting a different type of glands (i.e. sebaceous); this is why fingermarks are also composed of fatty acids and lipids⁴.

For patent marks, that is, marks that are visible to the naked eye, a "contaminant", enabling the observation of the mark without applying any process, will be present in larger abundance; examples of contaminants are blood, paint and grease⁴; contaminants of this type are exogenous species as they are not secreted in sweat but have come in contact with the fingertip. Semi-endogenous substances are the third category of molecules and refer to molecular species present in sweat as metabolites; they result from the introduction in the body via inhalation or ingestion of compounds such as drugs and medications as well as ingestion of food and drinks.

In both of those cases where a fingerprint match cannot be obtained, and even if a suspect is identified successfully through fingerprinting, it is desirable to have a technology to help catch the criminal based on what the chemical make-up of their fingermark can tell us. Essentially, through detecting and/or mapping endogenous, exogenous and semi-endogenous substances present in fingermarks, it is possible to reconstruct multiple images of the fingermark itself, which we can either superimpose or inspect, to find the best quality image enabling to obtain the most level 2 features.

Furthermore, exogenous and semi-endogenous molecules will enable the reconstruction of suspect's lifestyle and/or activities prior to committing the crime, while endogenous molecules can potentially tell personal information about the individual themselves such as the sex, the ethnicity and potentially diet and medical conditions.

This forensic opportunity gives rise to a sort of criminal profiling, no longer based on behavioural science but on the chemical content of the mark. In addition, the possibility of associating the detection of a molecule with a precise location (molecular imaging), for example on the fingermark ridges only, as opposed to fingermark valleys, provides associative evidence by linking the crime with the biometric information identifying the criminal.

As anticipated, since 2007 the analytical community has shown a huge interest towards pinpointing the fingermark chemical composition, as it became clear that its exploitation would have a huge potential to enhance the value of forensic evidence, significantly contributing to empower investigations and strengthen judicial debates.

In 2015⁵, a chart was reported representing the efforts of the analytical community towards fingermark chemical analysis, via the number of publications by year covering the use of specific analytical techniques with imaging capabilities. Here this chart has been updated and presents a more comprehensive outlook as it includes also techniques that are not considered to have an operational or imaging character, such as Gas-Chromatography (Fig 1). This is

because these kinds of techniques are still presently used to gain a deeper understanding of the changing fingerprint molecular make-up and can be particularly useful in studies determining the age of a fingerprint.

In addition to GC-MS, seven analytical techniques are featured having both profiling (e.g. detection capabilities) and imaging capabilities. These include spectroscopic techniques such as FTIR and ATR FTIR and Raman, as well as mass spectrometric techniques such as Desorption Electrospray Ionisation (DESI), Direct Analysis in Real Time (DART), Secondary Ion Mass Spectrometry (SIMS), Surface Analysis Laser Desorption Ionisation (SALDI) and Matrix Assisted Laser Desorption Ionisation (MALDI) Mass Spectrometry, with DESI being the first mass spectrometric technique being employed for imaging latent fingerprints⁶.

Finally, the chart also groups under "other techniques": (i) those that have shown ability to detect fingerprints' molecular composition but are not considered operational, such as; Liquid Chromatography -Mass Spectrometry (LC MS), Electrospray Ionisation Mass Spectrometry (ESI-MS) and Liquid Chromatography/Atmospheric Pressure Chemical Ionisation Mass Spectrometry (LC/APCI-MS), Capillary Electrophoresis Mass Spectrometry (CEMS) and (ii) those that have been recently published showing a number of additional benefits for the analysis of latent fingerprints namely: Nanostructured imaging mass spectrometry (NIMS)⁷, Laser Desorption Ionisation (LDI)⁸, Soft-Landing Ion Mobility of Silver Clusters - LDI, and gold nanoparticles enhanced target (AuNPET) - LDI⁹, MeV-SIMS¹⁰, Xray photoelectron spectroscopy (XPS)¹⁰, Liquid Extraction Surface Analysis (LESA)¹¹, Laser Activated Electron Tunnelling (LAET)¹² and Easy Ambient Sonic-Spray Ionization Mass Spectrometry (EASI MS)¹³. This latter (ii) category of techniques, despite some limitations, could overcome some difficulties presented by some of the technologies outside the "other techniques" group; for example, AuNPET - LDI⁹ enables fingerprint visualisation on surfaces treatable via gold sputtering, yielding the biometric information, whilst allowing mass spectrometric imaging analysis to be performed avoiding the use of an organic matrix (used in MALDI). This would reduce the chemical interferences at low molecular weight (100-1000 m/z) that the use of a matrix may, in some instances, present. However, as opposed to MALDI, AuNPET - LDI has not been proven to enable the detection of higher molecular weight species; in fact, in our experiments, peptides and proteins could not be desorbed from fingerprints (data not shown).

Similar advantages are exhibited by Soft-Landing Ion Mobility (SLIM) of Silver Clusters - LDI¹⁴; Silver nanoparticles also enable fingerprint visualisation and mass spectrometric

analysis as silver particles are capable of equivalent photon energy absorption from the incoming laser. Ag sputtering has shown to generate very neat and high resolution images of latent fingerprints¹⁵. However, detection and imaging capabilities were only, so far, shown for lipids and ditallowdimethylammonium ions. Additionally, both this technique (AgLDI) and SLIM of Silver Clusters - LDI may have the same limitations in terms of exploitable mass range as the AuNPET - LDI, as well as inapplicability to marks found on large surfaces that cannot be removed from crime scenes. However, AgLDI has very recently demonstrated potentially wider capabilities by being successfully applied to marks being deposited on a lifting tape¹⁶.

LAET is another interesting technique enabling chemical imaging on a MALDI mass spectrometer. As reported¹², fingerprints were impressed on nanoparticles of bismuth cobalt zinc oxide compressed on a conductive metal substrate (Al or Cu sticky tape) under 10 MPa pressure. Irradiation with an UV laser on the thin film instantly yields photoelectrons that can be captured by the organic molecules present in the fingerprint and, subsequently, cause electron-directed ionization and fragmentation. In Imaging mode, this would provide detection of molecular species and structural information in one analysis useful to confirm their identity.

However, in addition to the limited mass range as for AuNPET - LDI and SLIM of Silver Clusters - LDI, there is presently a strong operational limitation in that fingerprints will have to somehow be transferred from their location to the thin nanoparticles film. In fact, all of the techniques reported under "other techniques" have not, so far, been reported within a significant body of work demonstrating operational capabilities. This is true especially with regards to the compatibility with fingerprint enhancement techniques (FET) that must be used by crime scene investigators and crime lab analysts as first step in fingerprinting.

Techniques that have instead shown, in various degrees, imaging capabilities associated with this type of compatibility are Raman, FT IR, SIMS and MALDI MS and all of them have been included in the recently launched Fingerprint Visualisation Manual edited by the Home Office¹⁷. In particular these techniques have been classed as Category C, Technology Readiness Level 3. This label refers to techniques that have shown potential to be included within the operational fingerprinting processes though their full capabilities have yet to be demonstrated.

As it can be seen in Figure 1, amongst these techniques, classic MALDI MS (in both profiling and imaging mode) is the most published analytical tool applied to the analysis of

fingermarks (peer reviewed papers, patents, application notes) and the technique that by large has the most proven and reported compatibility with FET.

Invented to aid the ionisation of large non volatile and labile molecules, the capabilities of this technique have rapidly expanded to enable the ionisation of a vast range of molecule classes including low molecular weight drugs, metabolites, polysaccharides and lipids. In brief, in its classic implementation (UV-MALDI), this soft mass spectrometric techniques generates intact ions in the gas phase through desorption caused by a laser firing at the same in the UV region assisted by the presence of a low molecular weight non volatile organic molecules (an acid when working in positive mode), exhibiting a high conjugation of double/triple bonds enabling the absorption of the laser energy in the UV region;

two theoretical model exist to explain the ionisation - the gas phase protonation model¹⁸ and the Lucky Survivors model¹⁹. The former proposes desorption of clusters of matrix and analyte in the gas phase triggered by vibrational excitation induced by the absorption of laser energy by the matrix. Contextually, primary ionisation of the matrix occurs; the photo-excited matrix molecules are stabilised through proton transfer (or, in suitable circumstances, cation attachment) to the analyte thus generating mainly protonated molecules, making the interpretation of the mass spectra very simple.

The second model, initially referred to the ionisation of peptides and proteins, postulates that analytes are already incorporated within matrix molecules as charged species as originally present when in solution. In the case of peptides and proteins prepared at typical conditions, they would exist in the matrix crystal as positively charged species together with their counter-ions. Positive gas phase ions would be generated upon 1. disruption of the matrix-analyte co-crystals; 2. subsequent charge separation conferring the cluster a bet charge. Counter-ions deficit would prevent complete neutralisation leading to protonated ions which are called Lucky Survivors if they escape neutralisation through absorption of photoelectrons or electron from the stainless steel target²⁰.

Hillenkamp and Karas were the first to coin the term and report on MALDI MS in 1985²¹. Since, MALDI MS had been operated in profiling mode, that is by detecting molecular species through the application of discrete droplets of matrix and manually firing laser, until Gusev et al. reported, in 1995, the very first example of MALDI MS imaging, which was employed in this modality to image thin layer chromatography lipid spots²².

In this modality, the sample is generally coated in the organic matrix and the laser is automatically fired at a raster of points defined by x,y coordinates. As the laser fires, a mass spectrum is generated and eventually an array of mass spectra is obtained defining the

molecular composition of the sample at known x,y coordinates. The software can be then interrogated yielding 2D (or 3D) molecular maps of the detected ions. Since 1997, when Caprioli published the first article describing the principles and the advantages of MALDI MSI in the biomedical field²³, MALDI MSI had been mainly employed for biomedical and pharmaceutical applications. Only in 2009 our group reported the first fingerprint molecular images using this technique, opening up the development of MALDI MSI in fingerprinting⁴. In order to obtain intelligence and/or molecular images of the fingerprint ridge pattern, three matrix application modes have been applied/developed. The first two modes overlapped with the existing methods, namely the dried droplet application and matrix coating. The former enables a better analyte extraction yielding better signal/to noise ion peaks which could be related to forensically relevant substances. However, molecular imaging is not possible with this method and ridge merging prevents the opportunity to demonstrate that the particular molecule of interest is or not localised on the ridges; this circumstance precludes any associative evidence.

Matrix coating, in particular pneumatic spray coating, enabled the generation of molecular images at a resolution capable to yield fingerprint ridge pattern and minutiae visualisation⁴. While this opportunity was a breakthrough, already at that time, the question of how this technique could be operational was posed. In fact, no matrix coating and no MALDI MSI analysis can take place if the mark is not preliminarily visualised.

To solve this problem, the dry-wet method of matrix application was proposed²⁰. Here the MALDI matrix itself (α -cyano-4 hydroxycinnamic acid in the majority of our applications) is employed to powder fingerprints on different surfaces using a brush, just as the CSI use specific brush and powders to powder marks at crime scenes. Ferguson et al.²⁴ found that the matrix adhered to the ridges enabling the mark to be visualised by the naked eye. Given the spectroscopic properties of the matrix, in addition to optical images, UV and fluorescent images can be captured to be employed for fingerprint database search and suspect identification. The mark is subsequently evenly sprayed with a solvent mist enabling the dissolution of both the matrix and the analytes and their co-crystallisation. MALDI MSI can finally be applied to such treated mark to yield multiple molecular images of the ridge pattern. The dry-wet method provided one of the first examples of double imaging as images of the ridge pattern can be obtained both prior to and after the application of mass spectrometry. Further to this work, by using both SEM and MALDI MSI, it was subsequently demonstrated that the dry-wet method is not only much more reproducible, fast and efficient than the matrix

spray-coating one, but also that the lower the matrix particle size, the higher the quality of the resulting fingerprint molecular images (Fig. 2)²⁵.

Additionally a second organic molecule, namely 1,7-bis-(4-hydroxy-3-methoxyphenyl)-hepta-1,6-diene-3,5-dione (curcumin) was found to behave as an effective MALDI matrix thanks to the presence of an extensive system of conjugated double bonds²⁶. Curcumin was employed within the dry-wet method, both in laboratory settings and in pseudo-operational trials. As previously reported by Garg et al.²⁷, this substance was observed to yield a strong enhancement of the fingerprint, possibly due to hydrogen bond formation between the fatty acids/glycerides of sebum contained in the mark and the carbonyl and hydroxyl group of the curcumin. This hypothesis would fit well with the observation that for very old marks, in which the lipid content is greatly diminished, the adherence is very much decreased (S. Francese, personal communication).

In our report²⁶ we demonstrate that curcumin is suitably acting as a MALDI matrix for lipids and other small molecules, peptides and proteins demonstrating to be versatile as well as, in many occasions, an even better matrix than α -cyano-4 hydroxycinnamic acid. However, despite exhibiting these advantages, in pseudo-operational trials, the application of the dry-wet method employing curcumin, presented a potential liability issue; in fact, the strong adherence of curcumin to fingerprints resulted in deposition surfaces being extremely difficult to clean. In many cases, the stains persisted and this is not neglectable from an operational point of view. For this reason the use of curcumin as dry powder at crime scenes was discontinued.

In the UK, the dry-wet method represented a huge drive for the Home Office, and subsequently the regional West Yorkshire Police (WYP) to initiate a collaboration in order to support the development of the MALDI MS based techniques to an operational stage. This collaboration triggered the development of a number of fingerprint MALDI MS applications with the aim of implementing the relevant methodologies in real casework.

In 2013, after 5 years from the pioneering development of MALDI MS based techniques for the analysis of fingerprints, a review was published by this group reporting on the state of the art of capabilities and range of applications²⁸.

In the present review, an update is given covering the of body of work undertaken since and documented in the literature. In particular, the increased range of applications, the compatibility with FET and operational capabilities of MALDI MSP and MALDI MSI based technology utilising an organic matrix will be discussed.

2.0 Detecting and mapping fingerprints' molecular make up by MALDI MSP and MALDI MSI - an application update

Since the first peer reviewed paper on the application of MALDI MSI for the analysis of latent fingerprints⁴, several classes of molecules have been reported as detected and/or mapped. They include lipids^{10,25,29}, drugs^{30,31,32,33} and other small molecules^{34,35}, pharmaceuticals^{36,37}, explosives^{33,37}, peptides^{38,39}, proteins³⁸ and polymers⁴⁰.

However, not in all cases structural analysis was employed to confirm molecular identity and mass accuracy was not sufficiently high to rely solely on the m/z. For example, the analysis of explosives requires further investigation; following a publication in 2006 demonstrating the detection by MALDI MS of explosives using β -cyclodextrin to form a complex with these species⁴¹, only one other group published on this subject in 2013^{33,36}. Specifically Kaplan et al. reported on the detection of TNT and RDX through the use of a conventional MALDI matrix and in a tandem workflow with two FET Laboratory samples were produced by transferring the explosive dry residue from the initial surface of deposition to an aluminium slide by fingertip contact. MALDI MS images of TNT through its $[M-H]^-$ ion at m/z 226 and of RDX ion fragment $[M-CH_2N(NO_2)_2]^-$ at m/z 102 were reported.

Though these data would be better reported in section 3.0 of this review, in our view, there are not enough information in the papers from Kaplan et al.^{33,36} to corroborate successful detection of these species; the authors state that the target molecules were detected following all the enhancement/recovery processes mentioned above but no data are shown to illustrate this occurrence. Images of the ions at m/z 226 and 102 bear an insufficient mass accuracy to confirm the identity of these ions in MS mode and no MS/MS analyses have been performed for this purpose.

Explosives have proven to be a particularly challenging class of molecules to ionise via MALDI and our group has not even been fully persuaded by the data acquired in our laboratory that detection/imaging via MALDI, using a conventional matrix such as α -cyano-4-hydroxycinnamic acid, is indeed possible.

Pharmaceuticals detection and imaging also require further investigation. Sundar and Rowell³⁷ and Kaplan et al.³⁶ have both reported on the detection and/or imaging of pharmaceuticals such as paracetamol, aspirin and ibuprofen, pseudoephedrine, and procaine using MALDI MSI. While exciting, these data would benefit from additional analysis. As an example, the detection in MSP mode of aspirin (acetylsalicylic acid) was reported in un-lifted fingerprints as contact substance, following development with cyanoacrylate fuming,

exposure to acetone and powdering with the MALDI matrix dihydroxybenzoic acid, DHB³⁷. Two ions are shown in the spectra which could refer to the sodiated and potassiated adducts at m/z 203.154 and 219.132 respectively. However, no MS/MS analyses were conducted to confirm these data; the mass accuracy on such small molecules is insufficient (relative error is 601 and 571 ppm respectively) and it is at least puzzling that only the sodiated and potassiated adducts exist and not the protonated ion.

In this section, the detection and/or imaging, via MALDI MSP and MSI, of a vast range of molecules, macromolecules and biomolecules is reviewed to the current state of the art. Classes of molecules are discussed as belonging to either one of the three categories, namely: endogenous, exogenous and semi-endogenous substances, the nature of which has been described in section 1.0.

2.0.1 Endogenous substances

Lipids. Throughout the development of MALDI MS based methods for the analysis of latent fingerprints, lipids of different classes have been continuously detected and imaged by our group and others^{9, 14, 25, 29}. Their abundant presence in fingerprints has been, in our group, predominantly exploited to reconstruct the fingerprint ridge detail; this contributes to the generation of physical information which could be used for database record comparison and match, from a single fingerprint or via the separation of two fingerprints⁴².

However, already in 2009, our group suggested the possibility to exploit the detection and mapping of lipids to attempt fingerprint dating, that is, establishing the age of a mark since time of deposition. Establishing this time lapse is a significant piece of intelligence, as it would then enable the crime and the suspect to be placed in time and at the scene respectively, thus corroborating or disproving legitimate access.

Other authors had already published studies (and some are very recent⁴³) using different techniques such as GC-MS⁴⁴⁻⁴⁶ and Fourier transform infrared microscopy (μ -FTIR)⁴⁷, demonstrating significant changes in lipid composition over time. Though these fundamental studies are hugely important to understand how a mark may age, not knowing the reference levels of the endogenous lipids at the time of deposition (every donor is different and every donor may present a different composition at different times of the day) does not make these studies readily implementable.

Our group focussed on a "relative ratio" approach instead. Within this approach, it was hypothesised, that for a certain lipid degrading into other molecular forms in time, the relative

ratios between the ion intensity of the original species and its degraded forms could be used as age markers, thus becoming independent from the reference (initial) levels. In 2009, we suggested that the molecular maps of oleic acid, dehydrated oleic acid and a third ion deriving from the further loss of 18 Da, could be focussed on as their intensity ratios were clearly distinctive for marks of same age⁴. Unpublished data acquired three years later, were also promising as they showed distinctive ion intensity ratios (extracted from the three different fingerprint ion maps) for marks of different age, aged under the same environmental conditions.

However, when statistical analysis was performed (on a small scale study) on fingerprint replicates of different age and aged under different conditions, there was no age group clustering, suggesting that this approach was not robust enough. Therefore data were revisited considering the entirety of the marks' lipid dataset⁴⁸. This dataset was preliminarily processed by PCA analysis, enabling to observe clustering of the different ageing times which were separated using three principal components. The data were then processed employing PLS regression where the age information was used as a part of the model. This analysis generated a linear model with $R^2=0.989901$, which indicates a good representation of the data (Fig 3).

These results are certainly encouraging but must be considered as a very initial stage of an investigation that requires a long time and concerted efforts in order to establish feasibility. In fact, the study described was conducted on fingerprints generated through a silicon fingertip, a fingerprint rig (to generate reproducible marks) and a sebaceous reference lipid pad. Therefore the successful results of the study bear the constraint of a very basic experimental design which was devised to gain insights into feasibility of the method overall.

Also, it is important to remember that there are other factors beside the age of a mark which could accelerate/slow lipid degradation. These factors include nature of surface deposition and environmental variability determined by temperature, humidity, presence of debris and contaminants, exposure to soil, fire, water, etc.

These factors can, individually, and through their interplay, influence the ageing process. It is evident therefore that, in order to correctly and robustly establish the age of the mark, any technique would have to benefit from data processing accounting for all of these factors, which should be ideally be parametrised in the given statistical model.

Peptides and small proteins. In 2012, our group reported on the opportunity to detect intact peptides and small proteins in fingerprints via MALDI MSP within the mass range 1000-13000 Da³⁴. Interestingly, it was shown that the detection of these species could be

exploited to discriminate between fingerprints of male and female donors. Others had already attempted to obtain such intelligence exploiting the lipidic content of the marks but the reports concluded that the methods were either not conducive to the desired information or the data were not statistically significant^{49,50}.

In our hands, the combination of MALDI MSP for the detection of small molecules and peptides and partial least squared discriminant analysis (PLSDA) enabled sex discrimination with 85% accuracy.

However, these data were acquired within a cohort of 80 people, with restriction criteria for recruitment of participants and (ungroomed) fingerprints were analysed without the prior application of any enhancement techniques.

A study is underway in collaboration with West Yorkshire Police (WYP), whereby 200 donors have been recruited with no restriction criteria; natural fingerprints were analysed as collected or following development through Vacuum Metal Deposition (VMD).

While the first set of marks is currently being processed, it was already possible to observe that VMD developed marks, at large, did not yield peptide/protein ion signals and therefore data did not undergo statistical modelling. In many spectra, whilst there were no peptide signals or very few, gold nanoclusters were observed in the analysed mass range.

Although gold nanoparticles have been reported to enhance the mass spectrometric signal for a large range of molecules and macromolecules⁵¹, the authors have found that they worked well for small molecules such as drugs and lipids in marks enhanced by using Vacuum Metal Deposition (VMD) (with the metals being gold and zinc)³⁰. In this instance, it was apparent that gold nanoparticles, delivered through VMD, actually suppressed the peptide/protein signals, presumably due to an unsuitable thickness of the gold nanolayers overall. Therefore VMD developed crime scene marks may be an unsuitable sample to extract sex information.

It is well known that peptides and proteins can act as biomarkers of pharmacological/physiological/pathological states of individuals and MALDI MSI has been extensively used to analyse biopsies to pinpoint the biological indicators of many types of diseases, most of all cancer.

Since the report from Ferguson et al.³⁸, demonstrating the opportunity to detect such class of molecules from fingerprints of non-cancer patients via MALDI, it was hypothesised that further developments and refinements of the method could well lead to the detection of cancer biomarkers from fingertip smears.

Towards this research direction, our group preliminarily devised an in situ bottom up proteomic approach in healthy subjects in order to detect, in future studies, a potential set of biomarkers through peptide mass fingerprinting of sweat peptides/proteins³⁹ in cancer patients. This approach involved the screening of a number of non-ionic detergents in order to aid protein unfolding and therefore improve the efficiency of the enzymatic digestion of trypsin leading to a higher number of detected proteins.

Amongst the detergents used, while the ionic surfactant RapiGest^{SF} (Waters Corp, Manchester) showed to be promising with further protocol refinements, the addition of the non-ionic detergent N-Octanoyl-N-methylglucamin (MEGA-8) clearly improved proteolysis (over the proven use of n-Octyl β -D-glucopyranoside (OcGlu)).

The systematic evaluation of the detergents' performance was based on the inclusion of ion signals m/z falling within the fractional range 0.4-0.8 (i.e. only those signals were considered peptides). Figure 4 illustrates the efficiency of MEGA-8 above the other detergents tested, when incorporated in the trypsin solution which was homogeneously sprayed on the mark. From this chart, it was possible to observe that out of the three detergents tested, 2% MEGA-8 yielded reproducible and the highest peptide numbers across the three replicates as demonstrated by the narrow ranges.

Amongst the proteins identified with the two approaches, psoriasin, antibacterial LL-37, dermicidin and Beta-defensin 103 precursor and Human calmodulin like protein were particularly interesting as the literature reports them implicated in tumour onset/progression of human malignancy, particularly expressed in breast cancer⁴⁸⁻⁵²⁻⁵⁶ (Table 1).

These data are encouraging for the development of a fast non-invasive breast cancer screening, detached from the biometric information; such investigations are in progress in our labs and represent a cross-over of forensic into the biomedical world.

2.0.2 Contact (or) exogenous substances

Our interest towards exogenous substances was directed by the range of forensically interesting substances that can be encountered at crime scenes and could potentially be storytellers. In the past years, our group has therefore concentrated on the detection and mapping of condom lubricants, drugs of abuse and blood.

Condom lubricants - The interest towards condom lubricants originated from some 2004 statistics⁵⁷ according to which the number of rapists or sexual assailants using condoms when committing the crime, to avoid leaving incriminating biological traces behind, was on the rise.

PROTEIN	PEPTIDE m/z	SEQUENCE	RELATIVE ERROR (PPM)
Psoriasis	1384.7194	KGTTYLADVFEK	6.2
Keratin type I	1060.5605	TLLDIDNTR	-2.6
	1323.6725	IKFEMEQNLR [Met Ox]	0
Keratin 1B	967.4723	DVDAAYVSK	-0.8
Antibacterial protein LL-37 134-170	1365.6610	WALSRGKR	-8.5
Adrenomedullin	1060.5605	SIGTFSDPCKDPTRITSPNDPCLTGK	-7.7
Beta-defensin 103 precursor	703.3621	EEQIGK	4.8
	933.4896	CAVLSCLPK	-2.3
Dermicidin	676.3829	SSLLEK	-6.9
	725.3932	GAVHDVK	-1.1
	1128.5365	ENAGEDPGLAR	7.6
	1459.7622	LGKDAVEDLESVVK	-1.0
	1466.7872	GAVHDVKDVLDSVL	1.6

Table 1. Peptide Mass Fingerprinting and Putative Protein Identifications from In Situ Fingerprint Digests Performed by Spraying a Trypsin Solution Containing 2% MEGA-8 as a detergent. (Reproduced from Ref 39, Patel et al., J. Am. Spectrom. Soc., 2015, (<http://creativecommons.org/licenses/by/4.0/>))

However, the very act of putting on a condom leads to transfer of the polymeric material to their fingertips and eventually leads to condom-contaminated fingerprints in the event of contact with a surface. In our first publication, we reported the opportunity to detect and map in fresh and aged fingerprints the presence of the polymer PEG and the spermicidal nonoxynol-9 originating from Condomi max Love and Trojan-Enz condoms respectively³⁴.

This forensic opportunity enables a link to be established between the biometric information (reconstruction of the ridge pattern through visualising the distribution of the condom lubricant(s) on the ridges) and the corpus delicti (the detection of the presence of the condom lubricant(s)). Taking stock from our first report³⁴, we demonstrated that it is possible to extend the technique and the methodology to a wider range of condoms and a good level of

integration with other techniques was also illustrated⁴⁰ (Fig. 5). MALDI was applied as main investigative tool primarily in imaging but also in profiling mode when a higher sensitivity was required.

Indeed, this additional modality offered a better sensitivity towards the detection of PDMS in condom contaminated marks. However, in this instance, detection was readily achieved only upon artificially "ageing" of the marks at 37°C for 10 minutes. It was speculated that this occurrence was due to the ageing treatment causing a reduction of more volatile species acting as a suppressant of PDMS (which per se does not have very high ionisation efficiency) in non-aged marks. Therefore in order to detect multiple polymers, the ageing treatment could be a necessary methodological step prior to MALDI MS and MSI analysis.

Using MALDI MSI, it was possible to map all the condom lubricants from the condoms investigated on the fingerprint ridges. Furthermore, the ability to detect/map multiple condom lubricants, which can be routinely part of the formulation, enables the different condom types and/or brands to be, at least in principle, discriminated with mass spectrometry. This was the case for all of the condoms investigated, with the Durex brand being generally the richest in different species.

In order to provide as much information as possible, Raman and ATR FTIR were also employed with the view of providing, together with the MS information, a "spectroscopic fingerprint of the condom contaminated fingerprint". These data could be used to populate a database integrated with a statistical package enabling the operator to obtain a score list of the potential condom/brand type being analysed. Despite being successful overall, Raman spectroscopy could not be integrated in an analytical workflow with MALDI MSI for marks that require tape lifting prior to analysis, due to the spectroscopic interferences originating from the tape. Conversely the use of ATR FTIR in series with MALDI MSI was demonstrated and it is described in section 3.0.

In addition to the above work, insights into the possibility to discriminate between marks in lubricant and lubricant-contaminated marks via MALDI MSI were provided. The former instance relates to the case in which the condom lubricants were already present on a surface and the defendant had simply come in contact with the surface with clean hands, not having handled any condoms. In the second instance, the defendant would have handled the condom, contaminated their fingertips and then left "condom contaminated fingerprints" upon contact with a surface. As it can be appreciated, these circumstances depict a very different forensic scenario and the ability to correctly discriminate between the two could be crucial to the outcome of a court case. Within the first scenario, if the condom lubricant was originally

present on the surface touched with clean fingertips, it is reasonable to hypothesise the condom lubricant(s) to be visualised by MALDI MSI both in the valleys and the ridges of the mark and this circumstance was confirmed upon analysis of a mark generated to simulate this scenario (Fig 6).

Since MALDI MSI can detect thousands of molecules in one analysis, there is also a way to confirm that the above observed polymer distribution is not in fact an artefact; the molecular map of other compounds, for example of an endogenous lipid, as opposed of the polymer molecular map, showed its presence only on the ridges (Fig 6).

In the second instance, it is reasonable to hypothesise the presence of the lubricants only on the ridges and again the MALDI MSI analysis of a mark generated to simulate this second scenario confirmed the hypothesis (Fig 6).

This was one of the first examples demonstrating the possibility to use MALDI MSI to also reconstruct the events that took place at the crime scene and dynamics aspects of the crime being investigated.

Further development of these studies are in our view to be identified in: i) the possibility to distinguish those two scenarios from the coincidental association scenario (where a clean mark was left first on the surface and the condom lubricant ended up on top in some ways) and ii) the characterisation of a vast number of condoms within and outside the UK in order to build a database of identifying spectroscopic information.

Blood - Blood is a forensically relevant biofluid often encountered at the scene of violent crimes. Its reliable detection helps with blood pattern analysis, to reconstruct the events of the bloodshed and therefore the dynamic aspects of the crime. Our interest on the detection and visualisation of blood, both in stains and in fingermarks, stemmed from the presumptive nature of the currently employed blood enhancement techniques (BET).

As well documented by Bossers et al.⁵⁸ and summarised by Bradshaw et al.⁵⁹, BET infer the presence of blood, have different degrees of specificity but all are prone to false positives as several substances and biofluids behave like blood within the working mechanisms of each.

Conversely, a technology and a methodology enabling the detection of blood at a molecular level by revealing and mapping the presence of a set of blood specific proteins would offer a much higher level of specificity in addition to the link between the biometric information and the corpus delicti in the case of blood fingermarks. Our first attempt in this context was the development of MALDI MSI sample preparation protocol enabling the detection and visualisation of Haemoglobin and Haem (the prosthetic group of Haemoglobin) on the ridges of a blood fingermark.

While the detection of these species via MALDI was not new^{60,61}, the novelty lay in the mapping of these species through a MALDI Imaging intact analysis on the ridge of a "fresh" and a 7 days old mark (Fig 7). This "intact approach" also enabled to determine and distinguish blood provenance originating from bovine, equine and human sources on the basis of the slightly different primary sequence of the Haemoglobin in the different cases, with a mass accuracy sufficient to allow confident discrimination.

Furthermore the experimental set-up allowed to derive that it was possible to detect blood in blood contaminated marks through detecting haem and haemoglobin at a concentration 1000 times and 250000 times lower respectively than those observed in physiological samples.

Though the method represented a good step forward towards providing reliable and sound evidence to inform investigations and judicial debates, the use of a bottom up proteomic approach increases reliability of protein identification as the mass accuracy that can be achieved on the protein-deriving peptides is much higher than that achievable for intact proteins. This approach also enables the detection of additional blood specific proteins, besides haemoglobin, allowing specificity and confidence in the determination of the blood presence to be further enhanced. This research hypothesis was supported by the Home Office, UK, that, at the time, indicated the specific and reliable detection of blood as one of its priority challenges.

The literature already contains many reports covering the detection of the proteome of plasma and serum, though none of the approaches had involved the direct application of MALDI MS on enzymatically digested blood. This is understandable as in all of the previous reports, the aim was to map the entirety of the blood proteome for medical and diagnostic purposes.

Conversely, in a forensic context, the detection of a handful of blood specific proteins via bottom up proteomics and MALDI MS could be more than appropriate. Furthermore, in forensic science, provided that reliability of the evidence is not compromised, speed is paramount to investigations and the hyphenated methods reported can be very labour intensive.

A dual strategy was therefore devised⁶² and applied to bloodstains first in order to detect blood protein signatures through: i) a more efficient classic "in-solution digest" (incorporating the use of a relatively new detergent called Rapigest^{SF}) and ii) "a lab on plate (LOP)" approach involving immobilization of trypsin on Vmh2 hydrophobin coated MALDI sample plate⁶³. The methods are complementary as whilst the former generally yields more identifiable proteins (as blood biomolecular signatures), the latter yields a better S/N and is extremely rapid (5 minutes). Both approaches required the extraction of blood from the

surface The classic in-solution method required a maximum incubation time of 1 hour, whereas the LOP method only require 5 minutes to produce the most efficient digest. Both methods generated multiple human blood signatures including Haemoglobin α and β chains, Complement C3, serotransferrin, EBP 4.2, Haemopexin, Apolipoprotein I and Alpha-1 antitrypsin and EPB 3. However there were instances in which signatures were detected by only one of the two methods.

Using MALDI Ion Mobility MS/MS (MALDI-IMS-MS/MS), although generally, a higher ion signal intensity was observed within the classic in-solution digest, both approaches enabled the confident discrimination of blood provenance even when two different blood sources were mixed together thanks to the unique presence of identifying peptides only found in one species' blood and not the other (Table 2)

While these methods were found to be suitable in laboratory settings to reliably detect blood in stains and in enhanced palm impressions as old as 9 years (see section on "Compatibility with FET"), the next logical step forward was to devise a protocol to map these blood signatures using an in situ proteomics approach whereby they could be detected and visualised directly on the ridges of a blood mark. The development of such protocol has been recently reported by Deininger et al.⁶⁴.

This protocol underwent extensive refinement mainly in terms of i) concentration and quantity of trypsin; ii) selection of the appropriate trypsin depositor; iii) spraying parameters on the automatic sprayer employed to keep ridge integrity and iv) selection of the suitable surface of deposition for these initial studies. In terms of points ii) and iv), the acoustic ejector nanospotter Portrait® (Labcyte Inc., Sunnyvale, USA) and the pneumatic sprayer SunCollect (KR Analytical, Sandbach, UK) were tested, whereas, glass slides, and aluminium slides were selected as surfaces of deposition. The combination of the use of the Portrait® with either of the two deposition surfaces proved to be unsuitable due to spot merging likely caused by blood viscosity, surface chemistry and insufficient time for the trypsin to dry prior to a subsequent spot deposition. For these reasons, the use of Portrait® was discarded. The Suncollect proved to be a suitable device to spray trypsin (and then spray-coat matrix) on blood marks deposited on aluminium slides. Different concentrations of trypsin were tested to determine the most efficient one. Each concentration was tested on a quarter of the four in which the blood fingermark had been split, for a direct comparison of the proteolytic efficiency. Unfortunately the viscosity of the trypsin solution increased with the concentration, and this occurrence caused a blockage of the capillary within the SunCollect. For this reason, the highest trypsin concentration under investigation (250 $\mu\text{g}/\text{mL}$) could not

Human proteins	Peptide m/z	Sequence	In-solution Relative error (ppm)	Lab-on-plate Relative error (ppm)
Hemoglobin subunit beta	767.4886	⁶¹ VKAHGKK ⁶⁷	-4.5603	-10.8144
	952.5098	² VHLTPEEK ⁹	-4.5143	-5.5642
	1274.7255	³² LLVVYPWQR ⁴¹	-1.8827	-4.0793
	1314.6648	¹⁹ VNVDEVGGEALGR ³¹	-4.3357	0.1521
	1378.7001	¹²² EFTPPVQAAYQK ¹³³	2.8287	-10.0094
	1449.7961	¹³⁴ VVAGVANALAHKYH ¹⁴⁷	-3.5177	-3.1728
	1669.8907	⁶⁸ VLGAFSDGLAHLNLK ⁸³	-5.0901	-10.7192
	1866.0119	² VHLTPEEKSAVTALWGK ¹⁸	-1.1253	-
	2058.9477	⁴² FFESFGDLSTPDVAVMGNPK ⁶⁰	-2.7198	-2.3312
	2228.1669	¹⁰ SAVTALWGKVNVDVGVGEAL GR ³¹	-2.2439	-2.4683
2529.2190	⁸⁴ GTFATLSELHCCLKLHVDPEN FR ¹⁰⁵	-0.0790	-8.1052	
Hemoglobin subunit alpha	1071.5543	³³ MFLSFPTTK ⁴¹	-1.7731	-1.6798
	1087.6258	⁹² LRVDPVNFK ¹⁰⁰	-1.6549	-0.5516
	1171.6681	² VLSPADKTNVK ¹²	-6.9132	-
	1529.7342	¹⁸ VGAHAGEYGAELER ³²	-4.5105	-3.7915
	1833.8918	⁴² TYFPHFDLSHGSAQVK ⁵⁷	-2.3447	-3.7624
	2043.0042	¹³ AAWGKVGGAHAGEYGAELER ³²	-5.9226	-3.1815
	2341.1836	⁴² TYFPHFDLSHGSAQVKGHGK ⁶²	-2.6055	-2.5200
	2582.2707	¹⁸ VGAHAGEYGAELERMFLSFPTTK ⁴¹	-1.1230	-6.5059
2996.4894	⁶³ VADALTNVAHVDDMPNALSALSDLHAHK ⁹¹	-3.5374	-3.1370	
Myoglobin	1685.8679	¹³⁵ ALELFRKDMASNYK ¹⁴⁸	-	-5.1012
Complement C3	887.4581	⁸⁴² NEQVEIR ⁸⁴⁸	-3.0423	-3.2677
	1334.7096	⁶⁷² SVOLTEKRMDK ⁶⁸²	8.1665	-6.6681
	1087.6357	¹⁵⁹² EALKLEEK ¹⁶⁰⁰	-10.7572	-9.6539
Apolipoprotein A-I	1215.6215	²²⁰ ATEHLSTLSEK ²³⁰	-4.1131	-
	1230.7092	²⁴⁰ QGLLPVLESFK ²⁵⁰	-0.9750	-2.1938
	1723.9449	¹⁴ QKVEPLRAELQEGAR ¹⁵⁵	-3.7704	-4.0024
	1815.8507	⁴⁸ DSGRDYVSQFEKALGK ⁶⁴	7.2693	7.8200
	1833.8918	⁴² TYFPHFDLSHGSAQVK ⁵⁷	-2.3447	-3.7624
	1908.9847	¹⁵⁸ LHELQEKLSPLGEEMR ¹⁷³	-4.0859	-
Alpha-1-antitrypsin	1318.6758	²⁴⁸ LGMFNIQHCKK ²⁵⁸	-0.3033	5.4600
Hemopexin	965.4430	⁴⁰³ VDGALCMEK ⁴¹¹	-5.9040	9.4257
	1060.5785	⁸⁴ ELISERWK ⁹¹	-	-1.8857
	1070.5741	²¹⁴ GEVPPRYPR ²²²	-	2.6154
Serotransferrin	1068.5506	⁶¹ KASYLDCIR ⁶⁹	-	9.7328
	1855.8683	⁵³¹ EGYYGYTGAFRCLVEK ⁵⁴⁶	-0.1616	-0.6465
EPB4.2	949.4771	⁴⁵⁴ EKMEREK ⁴⁶⁰	5.0554	8.3203
	1048.5455	⁴⁵¹ VEKEKMER ⁴⁵⁸	-0.1907	5.2453
	1079.5745	²⁰⁵ WSQPVHVAR ²¹³	-9.4481	-
	1113.4881	⁴²⁸ CEDITQNYK ⁴³⁶	1.7063	-
	1258.7001	⁴⁴⁶ EVLERVEKEK ⁴⁵⁵	-2.3834	1.9861
EPB3	949.4771	²⁸⁴ AAATLMSEK ²⁹²	5.0554	8.3203
	1328.6852	⁷³¹ SVTHANALTVMGK ⁷⁴³	-	-2.7847
Alpha 2-Macroglobulin	1334.7215	³⁵⁰ LSFVKVDSHFR ³⁶⁰	-0.7492	-

Table 2. Peptide mass fingerprinting of whole human blood from in-solution and lab-on-plate digests. Reproduced from Ref 62 with permission from the Royal Society of Chemistry.

not effectively be tested and currently a larger capillary diameter is being used to deliver a higher concentration of trypsin. Within the study⁶⁴, it could be observed that both of the trypsin concentrations at 150 µg/mL and 200 µg/mL yielded blood signature images for Complement C3, Haemopexin, αHb and Serotransferrin (Fig. 8), with complementary efficiencies with respect to the different peptides detected and mapped; this result is to be expected given the differential abundance of the interested proteins in blood which directly

affects the substrate/trypsin optimal ratio. Furthermore these ratios have only been tested for the model blood mark selected by attempting the deposition of reproducible volumes of blood. For blood marks showing a higher volume of blood ("up to pools of blood"), the trypsin concentration may change again. A study is underway to investigate the blood volumes constraints under which the trypsin concentrations employed in our study are still viable.

The study from Deininger et al⁶⁴ was the first in its kind to demonstrate the opportunity to link the presence of blood onto the identifying mark ridges.

A similar research question arisen from condom contaminated marks can apply to blood contaminated marks; it would in fact be desirable to distinguish between marks in blood, blood marks and blood marks in coincidental association. Given the data shown by Bradshaw et al.⁵⁹ for condom contaminated marks, it is possible to hypothesise that MALDI MSI can again distinguish between the first two scenarios exploiting the localisation of blood signatures in the valleys and ridges of the mark or just on the ridges. However, this group is now exploring a non-mass spectrometric technology to evaluate the coincidental association and its discrimination from the first two scenarios.

Drugs of abuse - The detection of drugs of abuse in fingermarks could provide important forensic intelligence in order to reconstruct a lifestyle profile of the suspect and of their activities prior to committing the crime. The presence of these species could indicate drug dealing or drug consumption.

Given the relevance of the associated intelligence, methods have been developed in order to detect and map them in fingermarks by MALDI MS based technologies.

The detection and mapping of cocaine was already accomplished in our laboratories in 2010 but it was unreported until publication of an application note in 2014³⁵. In this work, the authors demonstrated the possibility of detect and map cocaine on the fingerprint ridges of a mock up sample as well as visualising its presence on a mark which was lifted, following powder enhancement, from a stolen and recovered laptop. The authors also illustrated a way to use MALDI Tandem Mass Spectrometry Imaging to confirm the identity of this drug and discriminate it from isobaric molecules, as a possible alternative to High Resolution or Ion Mobility Mass Spectrometry.

Following on this work, in collaboration with the Netherlands Forensic Institute, a more extensive study took place to develop MALDI MSP and MSI protocols in order to detect and map a wider range of drugs as well as metabolites³⁰. A reference table (Table 3) was generated indicating the working mass spectrometric conditions optimised to enable detection and structural through MS/MS analysis.

Drug	MALDI-QTOF-MS		MALDI-QTOF-MS/MS	Ref	MALDI-QTOF-MSI			
	Observed [M+H] ⁺ & IS fragment	Difference (amu.)*	m/z precursor → product ions**		LOD ng/μL	LOD*** ng/cm ²	Handling ng/cm ²	Abuse ng/cm ²
Amphetamine C ₉ H ₁₃ N	136.1371 [M+H] ⁺ 91.0751 [ISF]	-0.0245	136 → 119, 91	[21]	10	2,96	189.5	18.95
4-MeAmph C ₁₀ H ₁₅ N	150.1315 [M+H] ⁺	-0.0032	150 → 133, 105, 91	[21]	-	-	-	-
MethAmph C ₁₀ H ₁₅ N	150.1315 [M+H] ⁺	-0.0032	150 → 119, 91	[21]	-	-	-	-
MDMA C ₁₁ H ₁₅ NO ₂	194.1458 [M+H] ⁺ 163.1017 [ISF]	-0.0277	194 → 163, 135, 105, 58	[21, 22]	1	0,88	189.5	
MDA C ₁₀ H ₁₃ NO ₂	180.1367 [M+H] ⁺ 163.1015 [ISF]	-0.0343	180 → 163, 135, 105	[21, 22]	1	0,88	189.5	18.,95
MDEA C ₁₂ H ₁₇ NO ₂	208.1664 [M+H] ⁺	-0.0327	208 → 163, 135, 105, 72	[21, 22]	-	-	-	-
Cocaine C ₁₇ H ₂₁ NO ₄	304.1907 [M+H] ⁺ 182.1474 [ISF]	-0.0359	304 → 272, 182, 150, 105, 82	[21, 23]	0,01	0,0088	189.5	0.19
BZE C ₁₆ H ₁₉ NO ₄	290.1708 [M+H] ⁺ 168.1270 [ISF]	-0.0316	290 → 272, 168, 150, 105, 82	[21, 23]	0,1	0,088	189.5	1.895
EME C ₁₀ H ₁₇ NO ₃	200.1548 [M+H] ⁺ 182.1495 [ISF]	-0.0262	200 → 182, 168, 150, 82	[23]	0,1	0,088	189.5	1.895
Methadone C ₂₁ H ₂₇ NO	310.2474 [M+H] ⁺ 265.1706 [ISF]	-0.0303	310 → 265, 247, 223, 159, 105	[21]	-	-	-	-
Heroin C ₂₁ H ₂₃ NO ₅	370.1841 [M+H] ⁺ 310.1846 [ISF]	-0.0187	370 → 328, 310, 286, 268, 211, 193	[21, 24]	0,1	0,088	189.5	1.895
6-MAM C ₁₉ H ₂₁ NO ₄	328.1948 [M+H] ⁺ 268.1733 [ISF]	-0.0400	328 → 310, 286, 211, 193, 165	[21, 24]	0,1	0,088	189.5	1.895
Morphine C ₁₇ H ₁₉ NO ₃	286.1737 [M+H] ⁺	-0.0294	286 → 268, 211, 193, 165	[21, 24]	0,1	0,088	189.5	1.895
THC C ₂₁ H ₃₀ O ₂	315.2712 [M+H] ⁺	-0.0389	315 → 315, 300, 259, 193, 135, 123	[21]	10	2,96	189.5	18.95
THCA C ₂₁ H ₂₈ O ₄	345.2463 [M+H] ⁺	-0.0397	345 → 327, 299, 257, 229, 193	[21]	10	2,96	189.5	18.95
2 CB C ₁₀ H ₁₄ ⁷⁹ BrNO ₂	260.0612	0.0326		[25]	-	-	-	-
C ₁₀ H ₁₄ ⁸¹ BrNO ₂	262.0647	0.0381	262 → 245, 230, 164					
mCPP C ₁₀ H ₁₃ ClN ₂	197.0926 [M+H] ⁺	-0.0075	197 → 181, 154, 119, 91	[25]	-	-	-	-

* Calculated difference between measured m/z and calculated monoisotopic [M+H]⁺

** MS/MS product ions obtained for structure elucidation, supported by previous studies

*** ng/cm² as determined by dividing the amount of drug (volume/concentration) over the area of the applied spot (cm²)

ng/cm² as determined by dividing the amount of drug (pure amount) over the area of a fingermark (~2.64 cm²)

Table 3. List of the m/z values detected for drugs, their metabolites and their associated ion fragments via MS and MS/MS as well as the limits of detection observed in the two MALDI imaging experiments (spotted drug and high/low concentration spiking). The table also reports MS/MS conditions and the literature references supporting structural confirmation and molecular identification. *Calculated difference between measured m/z and calculated monoisotopic [M+ H]⁺. **MS/MS product ions obtained for structure elucidation, supported by previous studies. ***ng/cm² as determined by dividing the amount of drug (volume/concentration) over the area of the applied spot (cm²). #ng/cm² as determined by dividing the amount of drug (pure amount) over the area of a fingerprint. (Reproduced and adapted from Ref 30, Groeneveld et al., Scientific Reports, 2015, (<http://creativecommons.org/licenses/by/4.0/>))

Drugs and metabolites were used to homogeneously spike fingertips for the generation of drug-contaminated fingerprints. The amount of drug spiked took into account previously acquired limit of detection data³⁰ and, differently from similar studies⁶⁵⁻⁶⁶, it was related to two different scenarios, namely the "handling" and "abuse" scenarios. Within the former, a reasonable assumption was made that the amount of drugs in fingerprints is higher than for the latter scenario. Therefore fingertips were spiked with either "high" (0.5 µg) or "low" drug amounts (ranging from 0.0005 to 0.05 µg, respectively) prior to analysis by MALDI MSI. The abuse scenario referred to the circumstance by which, upon drug abuse, the intact parent drug is excreted, as well as metabolites in fingerprints.

Results from this study showed that for the handling scenario, molecular maps could be generated by both or either/or the parent ion and the in-source fragments. This was the case for amphetamine, cocaine, heroin, morphine, MDA and THC. The molecular maps of marks in which these drugs were present as ~190 ng/cm², allowed for reconstruction of the ridge detail with a quality between 1-4 according to the CAST, Home Office grading scheme⁶⁷, as shown in Figure 10. Within the abuse scenario, cocaine and heroin still yielded suitable images of the fingerprint ridge pattern (Fig. 9) at amounts as low as 0.19 and 1.9 ng/cm² respectively.

No molecular maps were obtained for MDA and THC with the amphetamine in-source fragmentation ion barely visualised. However, even for just partial or no fingerprint molecular images, the presence of these drugs could still be confirmed by performing post-imaging MS and MS/MS profiling.

These results enabled subsequent investigations into their detection and visualisation in real marks²⁷ and marks which had been enhanced with FET (mock and crime scene samples^{68,32}). These studies will be discussed in sections 2.0.3, 3.0 and 4.0.

2.0.3. Semi-exogenous substances

By semi-exogenous substances, our research group had indicated those species which have entered the body by various means (ingestion, i.v. injection, skin absorption etc..) and are excreted through sweat, and can therefore be observed in fingermarks, either in their un-metabolised or metabolised form.

In the first case, it would be practically impossible, in the case of drugs of abuse or medications, to know if the compound has been handled only or actually entered our body. This is why detecting the presence of metabolites would be a clear indication that it is not a touch chemistry scenario. It can be argued that metabolites could also hypothetically be found in fingermarks as a result of contact transfer, that is, fingermarks of those individuals whose fingertips have just simply come in contact with someone else who had, for example, abused a drug.

This circumstance could pose a limitation to the distinction of the forensic scenarios. As it can be appreciated, this would be a general analytical issue. The investigation of these scenarios and their discrimination has begun in 2014 in our laboratories and the work is ongoing. However, preliminary data would initially suggest that the concentration of these metabolites by contact transfer is too little to enable their detection via MALDI MSI. This means that when a detection is made, it would only be related to the actual drug/medication consumption by the individual whose fingermarks are being investigated.

A preliminary study was reported by Groeneveld et al.³⁰ reporting on the successful optimisation of the MS and MS/MS detection conditions of 4 metabolites including ecgonine methyl ester (EME), benzoylecgonine (BZE), 6-acetylmorphine (6-MAM), morphine and 11-nor-9-carboxy-THC (THCA). Morphine is used in medicine but is also a metabolite of heroin. As data on morphine were presented in section 2.0.2, they will not be covered again in this section.

Similarly to the parent drugs, these metabolites underwent a preliminary LOD study before being subsequently employed to spike fingertips in amounts equating to 0.5 µg ("handling" scenario) or ranging from 0.0005 to 0.05 µg, ("abuse" scenario).

Within the handling scenario, suitable fingerprint ridge pattern molecular maps could be obtained for EME, BZE and 6-MAM. However, within the abuse scenario, while a partial molecular map depicting the ridge pattern could be obtained for BZE, EME and 6-MAM only yielded a speckled image, indicating the presence of a fingerprint, but with no recoverable minutiae. These metabolites were detected at 1.9 and 0.19 ng/cm² in the "handling" and "abuse" scenarios, respectively. THCA only yielded a few pixels for both the "handling" and the "abuse" scenarios. Therefore, although this was not useful for not even claiming that a fingerprint was present, its LOD could be calculated as 1.9 and 0.19 ng/cm² for the two scenarios, respectively. These data are illustrated in Figure 10.

The possibility to detect metabolites in fingerprints, at amounts never reported with any other mass spectrometry imaging technique, triggered another study, in collaboration with Universities and Research Institutions across the UK and the Netherlands Forensic Institute, to investigate the presence of drugs in marks from patients in rehabilitation clinics. In this case though, only Profiling and not Imaging experiments were performed. Oral fluids were analysed in parallel in order to cross-validate the results obtained using MALDI-IMS-MS/MS, SIMS and DESI.

The case of a particular patient was reported³¹ to demonstrate techniques' capabilities. Results showed a closer opportunity to use the aforementioned techniques for crime scene fingerprints and certainly for probation services, prisons, courts and other law enforcement agencies. Being in a drug rehabilitation clinic, the patient was expected to have been administered with methadone; therefore methadone was thought to be the only likely substance to be found in their oral fluids and in fingerprints.

While SIMS was not able to provide any significant information due to scarce selectivity, DESI yielded the detection of cocaine, BZE and EME. Together with no sample preparation and the portability, DESI demonstrated to be a very valuable analytical tool for this kind of investigations. However, it must be said that a number of parameters need optimising every time, with particular reference to source settings including spray conditions, impact and collection angles and distance from the inlet.

Though MALDI does require the use of a matrix and it is not portable, this technique performed well using the Ion Mobility separation and transfer fragmentation experiments. In particular cocaine and BZE were detected, whereas the presence of EME was not actually investigated. In the case of BZE, its detection was proven by demonstrating that the drift time

of product ion at m/z 168.1033 of BZE standard coincided with the drift time of the same BZE product ion excreted in the fingermark as a result of cocaine consumption (Fig. 11).

Although the presence in the mark was not quantified, it was possible to establish that MALDI had a good enough sensitivity to detect the BZE metabolite present at a concentration above 16 ng/mL in the oral fluid.

These results showed potential for MALDI MS based techniques to be used for detecting drug of abuse in crime scene marks in operational conditions. This was later demonstrated and described in section 4.0.2

3.0 Compatibility with Fingerprint Enhancement Techniques (FET)

Classic forensic fingerprinting has remained more or less unchanged in the past hundred years. Apart from a very few novel enhancers/developers that are being currently evaluated and the improvement of existing processes, the armoury of optical, physical and chemical means to visualise a fingermark has stayed the same. This circumstance means that it is very unlikely that one or more of these processes will be replaced any time soon by any analytical technique, even if this should prove beneficial and operational. For this reason, in order for any analytical technique to be employed in the field, it is important that capabilities can be demonstrated or developed, as applied to fingermarks that have been previously treated by the CSI or the Crime Lab analyst through one or more FET.

We have shown many examples in which MALDI MS based methods has been made compatible with FET^{30,32,40,59,62,68} and the work described was often supported both intellectually and financially through the collaboration with the Home Office and/or West Yorkshire Police (WYP) UK.

The first attempt was reported by Bradshaw et al. in 2013 within the context of MALDI MSI analysis of condom lubricant-contaminated fingermarks⁴⁰.

Here a workflow was devised on the hypothesis that either condom lubricant formulations or the surface on which they have been deposited may fluoresce under the excitation of an appropriate wavelength light source. Two condoms were found to contain a fluorescent chromophore and fingermarks were produced by contaminating the fingertips with these two condoms separately. Under an excitation light at 539 nm the marks could be located and photographed for exhibition in a Court of Law. In the tandem workflow described⁴⁰ for the analysis of a Condomi Max Love-contaminated mark, the now located mark could then be

lifted through a BVDA gelatine lifter and submitted to ATR-FTIR revealing the presence of polydimethylsiloxane (PDMS) on the ridges of the mark; a secondary tape lift of the fingerprint residue was submitted to MALDI MSI which enabled the detection and mapping of endogenous substances as well as Polyethyleneglycol (PEG) as well as endogenous compounds. Therefore this workflow enabled both the biometric information and the associative evidence to be gathered by detecting polymers typically found in condom lubricants, directly on the fingerprint ridges (Fig 12).

Not long later after that, a significant body of work was published reporting on the compatibility with a number of most frequently used FET⁶⁸. FET taken in consideration for the study included the most common enhancement/development process such as: powders (aluminium and TiO₂ powders), powder suspension (TiO₂ based), cyanoacrylate fuming followed by Basic Yellow 40 staining (CAF-BY40), Vacuum Metal deposition (VMD), ninhydrin and 1,8-Diazafluoren-9-one (DFO). A number of porous, semi-porous and non-porous deposition surfaces were also investigated including white photocopy and brown envelope paper, an aluminium sheet and a ceramic tile, a clear Low Density Polyethylene (LDPE) bag and a glass slide, a Polyethylene (PET) bottle, a black bin bag and back side of a parcel tape, the adhesive side of a parcel tape. In the case of marks deposited on aluminium sheets, ceramic tiles and a PET bottle, the dry-wet method was employed enabling double imaging.

It was observed that in many cases where MALDI MSI was applied as a standalone technique, the quality of the fingerprint images rivalled that of or were superior to FET images for fresh and aged marks. However, it was clear that MALDI could not be used as a standalone technique as the mark would have needed to be localised prior to matrix coating the relevant area; the dry-wet method confer the technique an operational character as it could be used at the crime scene by powdering the MALDI organic matrix on a range of different non porous and semi-porous surfaces.

However, as discussed above, it would be naive to think that any time soon classic FET could be entirely replaced; in addition there will be deposition surfaces for which FET outperform the dry-wet method as the level of success of this method is not surface independent.

More crucial was therefore the investigation of several workflows where a fingerprint is subjected to a FET and subsequently to MALDI MSI⁶⁸. Even when a suspect match is found through a FET application, MALDI MSI may add additional intelligence, by detecting and mapping compounds of forensic relevance directly onto the identifying fingerprint ridges.

In other cases, from a strictly physical point of view, the multiple images obtained via MALDI MSI may "fill in the gaps" left by the application of a FET or even provide significant ridge detail where a preliminarily applied FET yields none. An example of the latter scenario was provided by the VMD-MALDI MSI workflow applied on a mark deposited on a LDPE black bin bag⁶⁸. However this workflow, though additionally yielding chemical information, does not work for lifted marks, presumably due to inefficient molecular material lifting once gold/zinc has been applied. Powders and powder suspensions also worked successfully in tandem with MALDI, though a degree of ion suppression is observed following the application of these enhancing agents. The ninhydrin - MALDI MSI workflow on fresh marks yielded dot-like molecular images, mirroring the quality of the ninhydrin image (due to the nature of ungroomed marks which may mostly exhibit molecular content in correspondence of the sweat pores). Conversely, no molecular images were obtained following the application of DFO which generates an ion suppression effect, possibly by sequestering the energy of the laser⁶⁸.

CAF-BY40 did allow subsequent MALDI MSI images to be retrieved, though those lipids and external contaminants detected through other workflows did not yield any molecular images in this instance. Ninhydrin, VMD and TiO₂ powdering were subsequently selected to work in tandem with MALDI for the analysis of 10 days old marks exposed to different temperature and relative humidity conditions⁶⁸. Even in this instance, not only was FET compatibility demonstrated again, but MALDI images rivalled FET images possibly showing even higher quality image for the VMD-MALDI MSI workflow (Fig 13).

CAF-BY40 and VMD were further investigated in 2015 by Groeneveld et al.³⁰ in tandem with MALDI MSI as applied to fingermarks that had been spiked with a mixture containing 1.25 μ g each of (i) Amphetamine (AMP), (ii) Cocaine (COC), (iii) Heroin (HER) and (iv) Δ 9-Tetrahydrocannabinol (Δ 9-THC). The marks were subsequently cut in half to produce 'split marks to be imaged within the same analysis and a comparison between the quality image of non-FET developed and FET-MALDI MSI workflows was undertaken. CAF-BY40-MALDI MSI workflow was preliminarily investigated under different conditions to determine which workflow yielded the highest fingermark image quality and provide recommendations (Fig. 14).

It was observed that CAF-MALDI MSI workflow did yield molecular images of the drugs and cholesterol (the latter taken as a reference for the endogenous fingermark content); nonetheless, with the exception of cholesterol, the CAF-MALDI workflow images were of

inferior quality compared to the undeveloped fingermark half. The exposure to acetone fumes of the CAF developed split mark, did not improve the ridge detail upon MALDI MSI, except ever so slightly for the mapping of cocaine. This result contrasts with a previous paper published by Sundar and Rowell³⁷ in which it was reported that acetone vapours enabled the lifting of the mark and the subsequent detection of the drugs investigated by SALDI, MALDI MSP and MSI. It was concluded that if depolymerisation did occur by acetone vapours exposure, as claimed by Sundar and Rowell³³, in our experiments, this was not sufficient to "free" the four drugs and cholesterol, enabling molecular images to be obtained, having the same quality as those obtained with no prior CAF development.

As recommended by the Fingermark Visualisation Manual¹⁷, CAF was followed by BY40 staining and therefore the CAF-BY40-MALDI MSI workflow was also tested.

In this case, while on a first observation, this workflow seemed not to yield any image compared to the undeveloped fingermark half, an increase in brightness revealed the fingermark ridge pattern for cocaine, heroin and cholesterol, with the two drugs yielding a clearer ridge pattern than their undeveloped reference half mark (Fig 14). However, even for cocaine and heroin the drop in signal intensity was very significant. It was hypothesised that being a good UV chromophore, BY40 may leave little laser energy to enable analyte ionisation; furthermore small molecule depletion might have occurred upon immersion of the mark in stain and subsequent washing of the excess stain with deionised water.

Overall, although FET application does affect the quality of the MALDI MS images, fingermark ridge pattern can still be obtained and intelligence can still be provided upon the FET-MALDI workflow; in fact, detection and confirmation of the presence of the drugs could be obtained through post-imaging MALDI MSP and MS/MS analyses.

The Fingermark Visualisation Manual recommends the use of VMD following CAF-BY40 development to further improve the quality of the ridge detail if necessary; therefore in a second set of experiments three different CAF-VMD-MALDI workflows were investigated and compared namely: VMD-MALDI MSI, CAF-VMD-MALDI MSI and CAF-BY40-VMD-MALDI MSI and results are shown in Figure 14. With the exception of THC, which is a challenging molecule to analyse in positive mode MALDI MS, all the species yielded higher intensity/quality images when MALDI MSI followed VMD, in agreement with the notion that gold nanoparticles are capable to store the energy of the laser and ultimately boost ionisation⁵¹. The CAF-BY40-VMD-MALDI MSI workflow shows results similar to those obtained by the CAF-BY40-MALDI MSI workflow confirming that the addition of BY40

does suppress ionisation though in some instances, molecular images and chemical intelligence through post-imaging analyses can still be obtained.

Another group investigated the compatibility workflow between CAF and TOF SIMS⁶⁹. It was shown that TOF SIMS only yielded the detection of very few species⁶⁹. Low efficiency of detection for this technique was somewhat predicted due to the fact that SIMS is a surface technique and the cyanoacrylate polymerisation generates a layer of polymer which render access to the below surface very challenging. The same study⁶⁹ also reports on the application of a range of FET on fingerprints located on a grenade handle including CAF-crystal violet, VMD and small particle reagent (SPR). While only SPR yielded some ridge detail, TOF SIMS was able to visualise very clear ridge pattern within the small area imaged. However, this was not a sequential workflow as TOF SIMS was applied on undeveloped marks.

Two more workflows were investigated pertaining the detection and mapping of blood in fingerprints and palm prints following the application of blood enhancement techniques (BET); unpublished data had already indicated the opportunity to use MALDI MSI to detect and image haem (the prosthetic group of Haemoglobin) at m/z 616 in fingerprint depletion series previously developed with ninhydrin. Additionally, while ninhydrin could not enhance the mark in the second and third mark deposition, not only was MALDI MSI compatible with this developer but could also map the presence of haem on the ridges up to the third deposition.

Additionally, in 2014 Bradshaw et al.⁵⁹ reported on the ability to image a blood mark that had been previously developed using Acid Black 1 one of the protein stains used for blood visualisation in fingerprints. Intact haemoglobin ion signal was employed to obtain the molecular distribution of blood which was found to co-localise with the blood locations as revealed by the application of Acid Black 1 (Fig 15). Though this result clearly demonstrated FET compatibility and "validity" of the MALDI MS images, only the "pools of blood" within the mark yielded a molecular image, indicating a lack of sensitivity for those regions exhibiting a lower amount of blood, that is, the ridges. This circumstance is not particularly surprising as MALDI sensitivity, particularly in imaging mode, decreases with the increase of the analyte's molecular weight. Additionally there might still be a certain level of interference exerted by the Acid Black 1. For this reason, as described in the previous section, our group embarked in the development of a proteomic approach to blood protein detection and mapping.

The first attempt to evaluate compatibility of this approach with a BET was undertaken by investigating a 9 year old blood palm print on a ceramic tile, which at the time was visualised using Acid Black 1⁶². Here two approaches were tested: the improved classic in solution digestion and the "lab on chip" approach, both described in Section 2.0.2.

A number of relevant blood protein-derived peptides were detected including multiple α Hb and β Hb.

Both approaches were successful and allowed the presence of blood to be claimed; however within the in solution digestion method both EBP 4.2 (indicating that the blood may be of human origin) and Complement C3 were identified by one peptide only each. The lab-on-plate approach enabled the detection only one EBP 4.2 peptide.

This result confirmed compatibility of MALDI MSP with this BET agent and opens up the avenue for the review of cold cases. The study of the compatibility of MALDI MSI with BET to retrieve molecular maps of blood protein-derived peptides directly on the ridges of a mark is currently in progress.

Apart from a recently published study using AgLDI Imaging for latent fingerprint analysis¹⁶, the second of its kind, no other studies involving other mass spectrometric techniques are reported describing their compatibility with FET. Therefore, MALDI MS based techniques are arguably the closest to operational deployment, considering the significant body of work reported on the use of these techniques for latent mark analysis, the sheer amount of different classes of substances that is possible to image/detect, the wider compatibility with single and multi-sequence FET and the successful undertaking of operational and pseudo-operational trials.

4.0 Operational capabilities

Before MALDI MS based techniques/methods could be assessed in terms of operational capabilities, many factors regarding the collection, transport and storage of the crime scene marks had to be evaluated and protocols established.

Initially, occasional access to volume crimes scenes was granted to test the dry-wet method on surfaces unrelated to the actual location of the crime. Additionally, primary fingerprint lifts which were deemed of too scarce quality to be submitted to the Automated Fingerprint Identification System, (a biometric identification system employing digital imaging technology to obtain, store, and analyse fingerprint data) were also made available to test

MALDI MS based methods. These lifts were obtained by the CSI after enhancing the marks (primary lifts) with mainly aluminium, white or black powders.

Understandably, in these conditions, testing but not development of the protocols was possible. These analyses did allow some variables to be investigated and their effect elucidated; this was the case for storage conditions, especially for primary lifts. After a few trials, it was clear that storing powder enhanced marks at -80°C prior to use, by spray-coating the matrix, in preparation to MALDI MS or MSI analysis, severely impinged on the quality and quantity of the ions detected and imaged. Therefore enhanced marks on a tape lift were stored at room temperature (as CSI would do).

It was also possible to understand that in principle, MALDI MS based methods could be used outside laboratory settings as separation of fingermarks was for example achieved. In other cases, if imaging the mark failed, yielding no ridge detail on an evidently "empty" mark, molecular information could be retrieved. This instance was exemplified by the detection, confirmation by Tandem MS and visualisation of cocaine on an "empty" mark enhanced by aluminium powder on a stolen and found laptop³²; at least one of its metabolites (benzoylecgonine) was also detected and mapped, though the low ion intensity did not allow successful MS/MS confirmation of the presence of this species.

Despite these encouraging results, this could not be a systematic approach or lead to the development of refined protocols as it was not informative enough.

Furthermore, CSI primary lifts which were archived and retained as evidence for both investigations and court cases were not made available for analysis; therefore the best conditions for efficiently recovering fingermark residues left on the surface (secondary lifts) needed to be established. As it was reasonable to hypothesise that the majority of the fingermark material was present in the first lift, comparative studies were also needed to factually demonstrate the need for the mass spectrometrists to analyse primary lifts instead. Other questions needed an answer concerning, for example, the best way to apply the matrix, the most suitable matrix and which FET in a real scenario are more compatible with MALDI MS based methods.

Collectively these studies were undertaken between 2013 and 2014 and some of the data are described in the 4.0.1 subsection. Prior to these studies, our group was granted access to tag along low profile crime scenes whilst on "attachment" with CSI officers within the constraints of pseudo-operational trials. This collaboration provided an opportunity to understand the factors involved in the fingermark collection. Secondary lifts were mainly

analysed and occasionally primary lifts, when ridge detail was deemed to be too scarce for an identification.

As a systematic study on the collection of primary and secondary lifts at the time could not be carried out, the group's main focus was to develop a way to transport fingerprint lifts and investigate whether a lag between the mark collection and analysis would affect the quality of the resulting data. A more recent means to transport and store crime scene marks was developed by a Fingerprint Expert at West Yorkshire Police⁷⁰. This system comprised a customised box storing in a non-contactless manner several pinned fingerprint lifts⁶⁶. Here an acetate sheet is stuck on the inside wall of the box lid to protect fingerprint lifts from debris.

Finally some consideration was given to the circumstance that in the majority of the cases, fingerprint lifts could be passed by the CSI to the mass spectrometrist by placing the backing sheet back on the powdered and tape lifted mark in order to protect it.. Therefore it was important to assess that it was still possible to obtain ridge detail and/or chemical information from fingerprint lifts removed from the acetate backing sheet. To assess feasibility, a small study was devised in which fingerprints, enhanced with either Aluminium powder or white powder, were lifted from an aluminium slide and then split in halves (A and B). Half A was spray coated in matrix and submitted to MALDI MSI; fingerprint half B was stuck on an acetate backing sheet which was then peeled off again and the tape lifted mark B was spray coated in matrix and submitted to MALDI MSI. These unpublished data are reported in Supplementary Figure S1. The figure shows that both halves of the mark preliminarily enhanced with the aforementioned powders yield the same quality image; they would be both graded as "4" according to the grading system proposed by CAST, Home Office UK⁶⁷. This result suggests that the acetate backing sheet does not affect the recovery of ridge detail/chemical information.

4.0.1 Development of operational protocols for the analysis of primary and secondary fingerprint lifts by MALDI-MS imaging

One of the most important aspects affecting the success of a MALDI MS is the way the mark is treated prior to lifting once localised at the scene of a crime. For secondary lifts and in the context of marks enhanced by powders, there could be in fact four ways to treat the fingerprint residue on the surface following a primary lift: i, tape lift of the mark residue

upon powdering and spray-coat of the MALDI matrix, ii re-enhancement of the mark residue using the same powder enabling a secondary lift followed by MALDI matrix spray-coat, iii re-enhancement and treatment of the mark residue via the dry-wet method of matrix application and iv re-enhancement via matrix brushing (first step only of the dry-wet method) and matrix spray coat following lifting.

Matrix sublimation has been suggested by Lauzon et al.¹⁴ as an additional way to apply matrix to fingerprints; this method was previously reported to yield a very homogeneous coating of the matrix, enabling the detection of small molecules and proteins with high spatial resolution^{71,72}. In their report, Lauzon et al.¹⁵, subjected a range of known MALDI matrices to sublimation in order to visualise and analyse fingerprints, showing the ability to map diacyl and triacylglycerols (DAGs and TAGs) onto the fingerprint's ridges. However, this method may potentially bear a limitation to operational deployment. The method was in fact, proposed for marks deposited on Indium tin oxide (ITO) coated conductive glass slides which could then be readily inserted in the mass spectrometer; this instance suggests that this method could potentially be inapplicable for crime scene marks and using instruments with a TOF-TOF configuration of the kind employed in that study, as marks should be located on this very special conductive support. Therefore this method was not investigated in our laboratories following the publication of this report and the methods i- iv were investigated instead.

It was evident that establishing the conditions yielding the most ridge detail and/or chemical information for secondary lifts, was a necessary step prior to investigate crime scene marks in operational conditions⁷⁰.

Therefore in a first set of experiments, methods i- iv were compared by using one split ungroomed and natural fingerprint divided in quarters; three powders, namely Aluminium, White and Black powders were investigated within this set of experiments and results are shown in Figure 16. One very clear observation was that the dry-wet method was the least efficient of all the sample preparation methods investigated; grade 0 images were produced for all fingerprint samples at large. These data do not conflict with previous instances reported in which the dry-wet method proved to be successful in yielding both ridge detail and chemical information^{24,25}. In fact, previous studies only applied the dry-wet method to undeveloped marks. From observation with the naked eye, the fingerprint residue left upon primary lift, still shows the presence of the powder used for enhancement. This would prevent full adherence of the dry matrix powder leading to scarce ion signal and poor or no

molecular images. Therefore this method was not carried forward in the subsequent set of experiments.

Aluminium powder developed ungroomed and natural marks (Grade 3) showed similar ridge detail quality in each of the three remaining sections suggesting suitability of all the trialled methods (i)-(ii) and (iv). The white powder developed ungroomed marks provided most ridge clarity in the section re-enhanced using the initial powder (Grade 2), whereas for the TiO₂ enhanced natural mark the best results were obtained from the sections re-enhanced using the initial powder and matrix powders, respectively (Grade 2). Therefore for white powder enhancement, a suggestion can be made to re-use it prior to the secondary lifts to cover different types of marks. Similar results were obtained for the black powder.

In a second set of experiments, ungroomed and natural marks were again employed as well as the same three enhancement powders. However this set of experiments aimed to factually demonstrate the importance of analysing primary fingerprint lifts as opposed to secondary lifts. Full data set is described in Bradshaw et al.⁶⁷. In brief, while for the aluminium powder developed natural mark and TiO₂ powder developed ungroomed mark, a similar ridge pattern quality was observed in all the sections (except that re-enhanced using the a-CHCA matrix), in the remaining instances, the primary lifted marks performed significantly better than all the other secondary lifted mark sections, as to be expected.

A suggestion could then be made that, although in some cases secondary lifts can still provide physical/chemical information, primary lifted marks remain a "safer bet".

This observation was at large repeated for fingerprints spiked with a contaminant which is known to be readily ionised in MALDI and very often found in fingerprints (dimethylbenzylammonium ion (DMA)), thus confirming the proposed operational suggestion.

Overall these experiments enabled a better understanding of the sample preparation approach to employ for secondary lifts and persuaded the Police partner to grant mass spectrometric access to primary lifts. This study and the overall outcome enabled a leap forward towards both a structured and informed approach to crime scene marks as well as towards the opportunity to move from pseudo-operational to operational casework as described in the following section.

4.0.2 Recent operational Police Casework

Under the Home Office UK Innovation Fund enabling the collaboration between the Fingerprint Research Group at SHU and West Yorkshire Police, MALDI MS based protocols

were deployed in fingerprint related operational casework. The results of some of these analyses were recently reported in Analyst³².

Amongst the seven casework investigated, four cases were reported³² relating to high profile crimes (drug dealing, murder and harassment). These four cases were found to provide the best preliminary insight into both operational capabilities and limitations of MALDI MS based techniques/methods. Nothing more than a first insight was expected as fingerprints may present themselves for MALDI analysis in a very diverse range of conditions and are subjected to a range of different intrinsic and external variables which may affect composition, enhancement and recovery.

This is why, while compatibility with FET has been greatly investigated and protocols have been outlined, in order to fully evaluate MALDI capabilities in an operational context, accounting for the extreme fingerprint variability, a large number of crime scene marks (primary and secondary lifts) must be analysed.

Within the four examples of casework reported by Bradshaw et al.³², one mark was directly analysed on the surface of deposition (a drug packet); three marks were primary lifts, of which one was given for analysis as stored on the acetate backing sheet (common way to preserve and store fingerprint lifts by CSI) and two were transported to the lab in a customised box.

Here, one case of negative result and one of positive result are summarised and the reader is redirected to the full article for full details.

One of the marks showed an insight into one of the limitations of MALDI MSP and MSI, though it is not clear how much the limitation is technique and/or mark dependant. In fact, the state of the mark, might have severely impacted on the MALDI MS outcome. The mark in question was present on a textured surface and was evidently contaminated with an unknown orange substance. The enhancement with TiO₂ powder by the CSI did not yield any useful ridge detail and the subsequent MALDI MSI analysis failed to deliver both chemical and physical information (ridge pattern). Evidently, the ridge pattern cannot be generated if it is not present originally. Lack of chemical information could have been due to an inefficient lift (scarcity of material) and not necessarily due to lack of sensitivity.

The positive example of casework confirming the MALDI capabilities observed in laboratory settings gave a promising indication that MALDI MS based methods could be upgraded to a higher category and "technology readiness level" in the Fingerprint Visualisation Manual¹⁴, eventually leading to an official deployment in forensic casework.

This example of casework related to a harassment investigation. The mark in question was located on the interior side of a window framed and presented an oily consistency at a visual inspection. Carbon black powder was used to enhance the mark and the subsequent visual inspection did not reveal useful ridge detail; the mark, subsequently stored on an acetate backing sheet, appeared smudged, with faint detail and areas of merged ridges. Therefore, as for the first case discussed, there was no expectation that MALDI MSI could improve the ridge detail and this was in fact confirmed upon analysis. However, it was apparent that the molecular information present could be successfully retrieved and cocaine was one of the substances of interest immediately revealed and confirmed by its in-source fragment at m/z 182.2. The post-imaging application of ion mobility tandem mass spectrometry employing transfer fragmentation for both the sample and the relevant metabolite standards, enabled to confirm the presence of several cocaine metabolites at m/z 290.1240 (benzoylecgonine), m/z 200.1551 (ecgonine methylester), m/z and 290.1392 (norcocaine).

The presence of metabolites confirmed drug consumption and is indicative of the state of mind of the suspect. Even more interestingly, while performing this type of analysis, a fourth metabolite was detected and confirmed, namely cocaethylene at m/z 318.1158 (Fig 18). This metabolite can only form if cocaine is consumed together with alcohol⁷³, with the latter greatly potentiating the effects of the drug, thus telling even more about the state of mind of the suspect.

When interrogated at the Police Station, the suspect was tested positive for cocaine but firmly denied consumption of alcohol which then could not be proved. At our laboratories we provided solid evidence of his false testimony, without knowing the content of his interrogation or any of the crime circumstances and acquired intelligence. Besides the intrinsic value of the evidence that it was possible to provide, it must be also noted the versatility of the MALDI which can be applied in different modalities in both a targeted and an untargeted approach. The untargeted approach enables to maximise the information retrieved from the analysis of a crime scene mark.

5.0 Summary and Outlook

The development of MALDI MS based methods for the analysis of fingermarks has come a long way since it was first reported in 2009. A number of applications have been developed ranging from the detection and/or mapping of contact, endogenous and semi-exogenous substances in fingermarks, thus enabling a profile of the offender to be built. More importantly, the drive for this research not to remain an academic exercise has led to a

significant collaboration with end users such as the Home Office and West Yorkshire Police UK, and the Netherlands Forensic Institute.

These collaborations have resulted in protocols enabling compatibility of MALDI MS based methods with the most often employed fingerprint enhancement techniques. Compatibility has enabled pseudo-operational and operational trials to be undertaken demonstrating feasibility of application of MALDI MS based techniques at a level ranging from high potential to demonstrated retrieval of forensically relevant intelligence. However, many challenges remain and there are many years of research ahead in order to validate the use of this technology.

We would like to think that our research has acted as the springboard triggering additional and complementary research from other groups in the analytical community. For example, the determination of sex from a mark and age of a mark require much more concerted efforts in order to build a robust analytical system capable to withstand court scrutiny.

The operational capabilities in the detection and mapping of forensically relevant substances in crime scene marks require a large number of such specimens to be analysed; the higher the number, the higher chance to investigate marks subjected to several types of cross-talking variables (intrinsic and environmental).

Therefore, in order to evaluate the robustness of the protocols as well as determining their best use in an operational context, it is vital that forensic research is supported by funding bodies other than the end users, something that, despite the clear successes and progress made in this particular field, has not happened in our case in the UK.

The technology has been reported in the Fingerprint Visualisation Manual edited in 2014 by CAST, Home Office as category C, Technology Readiness Level 3-4; this indicates a technology with high potential to be employed in casework but still needs research to assess full capabilities. The goal is, in the next few years, to move up MALDI MS based methods to category A processes, indicating a technology the use of which is recommended by the Home Office for operational casework.

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Legends

Figure 1. Histogram showing the number of publications featuring the use of either GC MS, FT IR, ATR FTIR, Raman (in its various forms including SERS and CARS), DESI, DART, SIMS, SALDI or MALDI MS (in profiling and/or imaging mode) for the analysis of fingerprints with a final category including "other techniques". Figures in the histogram account for peer-reviewed papers in large part with few instances of on-line magazines, technical articles, application notes and patents. Some papers may appear in more than one group. For example, a paper reporting the application of both MALDI and SIMS and LESA for the analysis of latent fingerprints will be counted in the "MALDI", "SIMS" and "other techniques" groups. This information have been obtained by searching both Pubmed and Scopus databases using the keywords fingerprints, fingerprints, GC MS, MALDI, ATR FTIR, Raman, DESI, DART, SIMS, SALDI and MALDI.

Figure 2. Investigation of the impact of matrix particle size on fingerprint mass spectrometric image quality when employing the dry wet-method. Panels A1–E1 report a 700x magnification image of the dusted fingerprints with the spray coat (SC) and the dry–wet (D-W) methods and different matrix particle size (A1 = S-C method, B1 = D-W method and un-ground matrix, C1 = D-W method and manually ground matrix, D1 = D-W method and mechanically ground and sieved matrix, E1 = D-W method and mechanically ground matrix (≤ 10 μ m particle size)). Panels A2–E2 show a 3000x magnification of panels A1–E1. Panels A3–E3 show the corresponding MALDI MS images of the ion at m/z 283.3. Panel E4 reproduces the same mark reported by panel E1, which has not undergone normalisation. Reproduced from Ref 25 Efficiency of the Dry-Wet Method for the MALDI MSI analysis of latent fingerprints, *J. Mass Spectrom.*, John Wiley and Sons.

Figure 3. PLS regression analysis of the MS data for day zero, 1, 4, and 8 day silicon model fingerprints. The model discriminated well the fingerprints on the basis of their age. Reproduced from Ref 48, doi: 10.1016/j.scijus.2015.10.001, (<https://creativecommons.org/licenses/by-nc-nd/4.0/>)

Figure 4. Column graph of the number of peptides with fractional mass between 0.4 and 0.8 for OcGlu, DDM, and MEGA-8 at three concentrations (a). Graphical representation of peptide intensity:matrix intensity ratios for each detergent at three concentrations. Statistical analysis calculated a significant increase between MEGA-8 and OcGlu at 1%, as denoted by the asterisk. (Reproduced and adapted from Ref 39, Patel et al., J. Am. Spectrom. Soc., 2015, (<http://creativecommons.org/licenses/by/4.0/>))

Figure 5. MALDI MS images of condom contaminated fingermarks. Three ion signals of the polymeric Gaussian distribution per condom lubricant were imaged for each condom. Where possible, tentative identification were made. All the ion series detected belong to ethoxylate monomer based polymers. Reproduced from Ref 40 with permission from the Royal Society of Chemistry.

Figure 6. MALDI MSI analysis of Trojan Enz contaminated and non-contaminated fingermarks. The ion signal at m/z 639.5 (9-mer nonoxynol-9) is solely distributed on the ridges of a condom contaminated fingermark deposited on a non condom contaminated surface. The same ion signal is distributed both on the ridges and in the valleys of a non condom contaminated fingermark deposited on a condom contaminated surface. In this case, ridge detail can be obtained by imaging another species such as the endogenous fatty acid at m/z 311.2. Reprinted from Science and Justice, 40, Bradshaw et al., Direct detection of blood in fingermarks by MALDI MS profiling and Imaging, 110-117, Copyright (2013), with permission from Elsevier

Figure 7. MALDI MS Imaging of fresh and aged bloodied marks. Haem (m/z 616.2) was detected and imaged together with its in-source fragment ion (m/z 557.2) (A) in a fresh mark. Successful detection of haem was also achieved for a 7 day old bloodied mark (B). Presence of haem was further confirmed by the product ions at m/z 557.2 and 498.2 in the MS/MS spectrum of the parent ion at m/z 616.2 taken from a small area of the mark (Bi). Furthermore presence of human haemoglobin was ascertained by MALDI MSP analysis on the same mark in the higher mass range (Bii). Reproduced from Ref 59 with permission from the Royal Society of Chemistry.

Figure 8. MALDI IMS MS Imaging of in situ proteolysis of a blood fingerprint. Figure shows molecular images of blood specific peptides generated by spraying trypsin in four different concentrations (100, 150, 200 and 250 $\mu\text{g}/\text{mL}$) on the blood mark using the SunCollect. The trypsin concentration of 250 $\mu\text{g}/\text{mL}$ could not be delivered to due limitations in the sprayer capabilities. Each peptide image has also been overlaid with the matrix signal at m/z 1066.1158. The figure suggests that the best ridge reconstruction performance could be achieved using a trypsin concentration of/between 150 and 200 $\mu\text{g}/\text{mL}$. Reproduced from Ref 64, Proteomics goes forensic: Detection and mapping of blood signatures in fingerprints, Proteomics, John Wiley and Sons.

Figure 9. MALDI MS images of ‘high’ (“handling” scenario) and ‘low’ (“abuse” scenario) spiked fingerprints contaminated with amphetamine, cocaine, heroin, morphine, MDA and THC. All images were normalised against the matrix peak at m/z 190. (Reproduced and adapted from Ref 30, Groeneveld et al., Scientific Reports, 2015, (<http://creativecommons.org/licenses/by/4.0/>))

Figure 10. MALDI MS images of ‘high’ (“handling” scenario) and ‘low’ (“abuse” scenario) spiked fingerprints contaminated with BZE, EME, 6-MAM and THCA. All images were normalised against the matrix peak at m/z 190. (Reproduced and adapted from Ref 30, Groeneveld et al., Scientific Reports, 2015, (<http://creativecommons.org/licenses/by/4.0/>))

Figure 11. Detection of BZE from a fingerprint from a drug user in a rehabilitation centre by MALDI IMS-MS/MS. (Fig. 1A) Drift scope plots of BZE standard and BZE in the fingerprint (generated through transfer fragmentation experiment). (Fig. 1B) MS/MS spectra of BZE standard and BZE in the fingerprint after the selection of the BZE parent ion and product ion within the same drift time plume in the fingerprint and in the standard. Selection of the ion product at m/z 168.1033 in common to the BZE standard and BZE in fingerprint showing superimposable drift time chromatograms. Reproduced from Ref 31 with permission from the Royal Society of Chemistry.

Figure 12. Analytical workflow combining Laser irradiation (1), ATR-FTIR imaging (2) and subsequent MALDI MSI analysis (3) of a Condomi Max Love contaminated fingerprint. (1). The laser was shined at a wavelength of 532 nm using an orange viewing filter (549 nm). The mark was therefore located and lifted with a BVDA gelatin lifter. (2) reference FTIR spectra

of vinyl PDMS (at 1258 cm^{-1}) and PEG 3000 (at 1365 cm^{-1}) superimposed with the spectrum of a BVDA gelatin lifter. Panels B and C show the ATR-FTIR image of PDMS at 1258 cm^{-1} and the ATR-FTIR image of PEG at 1365 cm^{-1} present on the same ridges of a Condomi Max Love contaminated fingerprint selected region. Two ATR-FTIR spectra from the ridge (D1) and the valley (D2) (high and low concentration of the two polymers respectively). (3) MALDI MS images of 32-mer, 33-mer and 34-mer PEG ion signals, the complete ridge pattern provided by the image of the total ion current (TIC), as well as a small sample of the many fatty acids detected. Reproduced and adapted from Ref 40 with permission from the Royal Society of Chemistry.

Figure 13. MALDI molecular images of 10 day aged fingerprints following FET enhancement. Fingerprints were deposited: (A) on white copier paper and developed by Ninhydrin (SET 1); on the back side of parcel tape and preliminarily enhanced by VMD (SET 2); on aluminium sheet and enhanced by TiO_2 powder (SET 3). The enhanced fingerprints were subsequently subjected to MALDI MSI analysis and three ions were compared (m/z 283.2, m/z 311.2 and m/z 638.3). A fingerprint mass image with the best ridge detail was also reported in an inset for each analysed mark. Reprinted from Forensic Science International, 232, Bradshaw R et al., Towards the integration of matrix assisted laser desorption ionisation mass spectrometry imaging into the current fingerprint examination workflow / 111-124, Copyright (2013), with permission from Elsevier.

Figure 14. MALDI MS images of split fingerprints spiked with a mixture of 4 drugs and analysed with and without prior FET development. All images were normalised to total ion current, but each entire fingerprint image was adjusted to optimal brightness and contrast. A: MALDI MS images acquired after CAF, CAF + acetone vapour exposure and CAF + BY40 staining. The last row of the panel shows the brightness increased to reveal enhanced 2D maps of cocaine, heroin and cholesterol. B: MALDI MS images acquired after VMD, VMD + CAF and CAF + BY40 staining + VMD. For both panels A and B, the last row of the panels shows brightness increased to reveal enhanced 2D maps of cocaine, heroin and cholesterol. (Reproduced and adapted from Ref 30, Groeneveld et al., Scientific Reports, 2015, (<http://creativecommons.org/licenses/by/4.0/>))

Figure 15. Acid Black 1-MALDI MSI workflow. A mark developed by Acid Black 1 revealing the presence of blood through the blue stained areas (A) was subsequently analysed by MALDI MSI that provided a superimposable image of distribution of β HHb on the mark (B). Reprinted from Science and Justice, 59, Bradshaw et al., Direct detection of blood in fingerprints by MALDI MS profiling and Imaging, 110-117, Copyright (2013), with permission from Elsevier

Figure 16. Comparison of MALDI MS images of “secondary” lifts obtained from (A) ungroomed and (B) natural latent fingerprints which had been pre-developed using conventional fingerprint powders. Reproduced from Ref 70 with permission from the Royal Society of Chemistry.

Figure 17. Comparison of MALDI MS images of “primary” and “secondary” lifts of (A) ungroomed and (B) natural latent fingerprints which had been pre-developed using conventional fingerprint powders. Reproduced from Ref 70 with permission from the Royal Society of Chemistry.

Figure 18. MALDI-IMS-MS/MS analysis of the ion at m/z 318.1158 suspected to be cocaethylene (COCE) within a primary fingerprint lift recovered on the interior of a window frame following enhancement with carbon black powder. Panel A shows the drift scope plots of COCE standard (i), COCE in 3 locations of the marks (ii-iv) and 1 location outside the mark (matrix, (v)) that were generated through transfer fragmentation experiments. Panel B shows the MS/MS spectra of the ion at m/z in the COCE standard and in the 4 locations (3 in the mark and one outside it) (vi-x) after the selection of the parent ion and products ion within the same drift time plume in the fingerprint and in the standard. Selection of the ion product at m/z 196.1481 in common to the COCE standard and COCE in fingerprint and matrix shows superimposable drift time chromatograms (Panel C xi-xv). Reproduced from Ref 32 with permission from the Royal Society of Chemistry.

Supplementary Figures

Figure S1. Investigation of acetate backing interference within the MALDI MSI analysis of primary lifts. Fingerprint enhanced with either Aluminium or White powder were lifted and then split in halves (A and B). Half A was prepared and submitted to MALDI MSI;

fingermark half B was stuck on an acetate backing sheet which was then peeled off and the tape lifted mark B was then also prepared and submitted to MALDI MSI. Both halves of the marks yield the same quality image, suggesting that the acetate backing sheet does not affect the recovery of ridge detail/chemical information.

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