Sample treatment for tissue proteomics in cancer, toxicology, and forensics

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Sample Treatment for tissue proteomics in Cancer, Toxicology and Forensics

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Abstract

Since the birth of proteomics science in the '90, the number of applications and of sample preparation methods has grown exponentially, making a huge contribution to the knowledge in lifescience disciplines. Continuous improvements in the sample treatment strategies unlocks and reveals the fine details of disease mechanisms, drug potency and toxicity as well as enabling new disciplines to be investigated such as forensic science.

This chapter will cover the most recent developments in sample preparation strategies for tissue proteomics in three areas namely, cancer, toxicology and forensics, thus also demonstrating breath of application within the domain of health and wellbeing, pharmaceuticals and secure societies.

In particular, in the area of cancer (human tumour biomarkers), the most efficient and multi-informative proteomic strategies will be covered in relation to the subsequent application of Matrix Assisted Laser Desorption Ionisation Mass Spectrometry Imaging (MALDI MSI) and Liquid Extraction Surface and Analysis (LESA), due to their ability to provide molecular localisation of tumour biomarkers albeit with different spatial resolution.

With respect to toxicology, methodologies applied in toxicoproteomics will be illustrated with examples from its use in two important areas: the study of drug induced liver injury (DILI) and studies of effects of chemical and environmental insults on skin i.e. the effects of irritants, sensitizers and ionising radiation. Within this section, mainly tissue proteomics sample preparation methods for LC MS/MS analysis will be discussed as: *(i)* the use of LC MS/MS is majorly represented in the research efforts of the bioanalytical community in this area and *(ii)* LC MS/MS still is the gold standard for quantification studies.

Finally, the use of proteomics will also be discussed in forensic science with respect to the information that can be recovered from blood and fingerprint evidence which are commonly encountered at the scene of the crime. The application of proteomic strategies for the analysis of blood and fingerprints is novel and proteomic preparation methods will be reported in relation to the subsequent use of mass spectrometry without any hyphenation. Whilst generally yielding more information, hyphenated methods are often more laborious and time consuming; since forensic investigations need quick turnaround, without compromising validity of the information, the prospect

to develop methods for the application of quick forensic mass spectrometry techniques such as MALDI MS (in imaging or profiling mode) is of great interest.

1.0 Tissue Preparation and treatment in Oncological Proteomics.

The preparation and pre-treatment of tissues for the study of proteomics in cancer is both unique and challenging considering the information desired by the clinician or research scientist. This is true regardless of sample origin, whether this be a patient clinical biopsy, from experimental animal models or novel synthetic tissues used in the field of oncology. Preparation is a key factor in order to extract maximum protein/peptide yield to ultimately generate that 'characteristic molecular snap shot', a true indication of proteomic involvement in the pathway to disease.

This account aims to discuss various perspectives of sample preparation and pre-treatment of human tumour tissue samples essential to study proteomics using advanced analytical techniques. The methodologies and considerations herein are applicable to the techniques of: Matrix Assisted Laser Desorption Ionisation Mass Spectrometry Profiling and Imaging (MALDI-MSP and MALDI-MSI respectively) and Liquid Extraction Surface Analysis (LESA).

Fresh frozen tissue - There are many challenges and factors to consider when selecting fresh frozen (FF) tissue as the storage method of choice prior to sample analysis. Experimental design that involves FF human tissue must not only account for ethical approval but sample availability, sample handling and also the duration between sample harvest and snap freezing, a factor where time really is of the essence.

In order to generate reproducible data from FF tissue that is believed a true, accurate proteomic snapshot, rigorous measures will need to be adhered to minimise potential proteolytic degradation. *Ex - vivo* tissue will still continue to undergo changes in molecular integrity after removal from the site of biopsy. Such post-sampling will result in ischaemic effects which could induce apoptosis and *in situ* coagulation, all conditions which encourage protein degradation [1].

Good technical skills, laboratory procedures and storage facilities will collectively aid in the preservation of molecular integrity of FF samples for future proteomic analysis. Unlike paraffin embedded tissue that has various stages to tissue preparation (wax removal and antigen retrieval), if good laboratory practices are carried out and the FF tissue treated carefully, there should be minimal loss of target species.

The main advantage of analysing molecular targets from human tissue in general, could be the potential of translational, even personalised, medicine that could help advance disease diagnostics.

Tryptic peptide analysis - One of the main potential pitfalls of *in situ* tissue tryptic digestion is protein delocalisation. A factor which, when occurs, prevents the analysis of spatial protein distribution. To ensure good tryptic digest MALDI-MSI, a homogeneous coating is essential during trypsin application. Other issues to consider would be the tissue type, section size and the selection of a suitable flow rate, when using a pneumatic sprayer for trypsin deposition. Too much trypsin sprayed per cycle could lead to 'over wetting' of the tissue and subsequent protein delocalisation. An example of a successful *on tissue* tryptic digest can be seen in Figure 1.

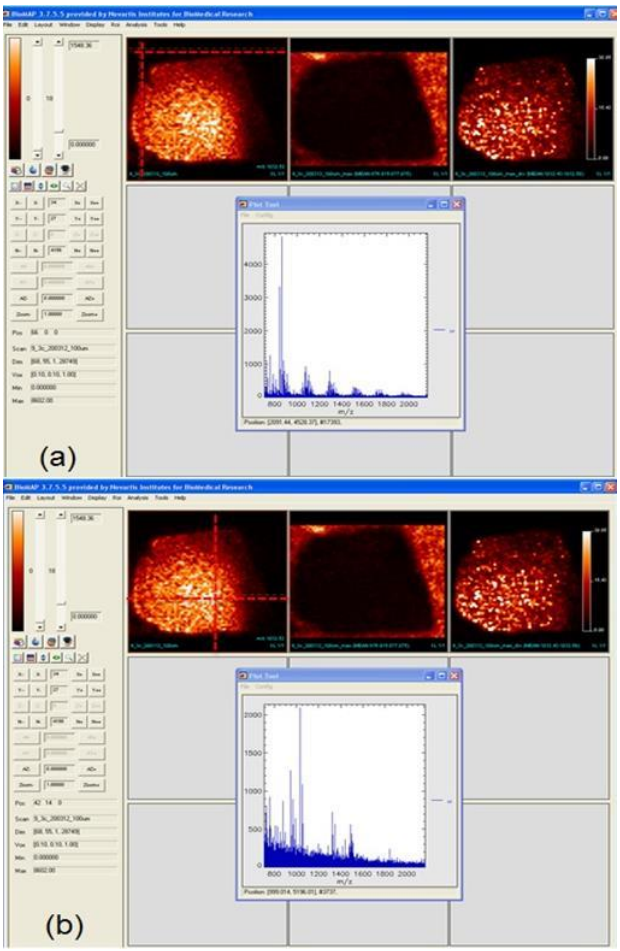

Figure 1: Screen shots of a successful *on tissue* tryptic digest using MALDI-MSI, visualised using BioMap imaging software. Screen shot (a) displays the corresponding spectrum in relation to the position of the red cross hairs which lie in a region that should be predominantly MALDI matrix. Similarly screen shot (b) indicates the peptide mass fingerprint typical of an *on tissue* tryptic digest, with the red cross hairs in the digested tumour tissue region.

A parameter not always considered during enzyme application to a sample like tumour tissue is not only the 'x and y' uniformity but the depth and degree of enzyme seepage within the 'z' plane of the

sample. This other 'z' dimension could also reflect how efficient an *on tissue* is or on the other hand be indicative of "noisy" spectra due to degradation products of an over digested sample.

A summary of issues and influential factors that potentially effect *in situ* tissue tryptic digestion and MALDI-MSI is presented in Figure 2.

Figure 2. Preparatory issues and factors to consider when performing on tissue digestion for MALDI imaging experiments.

The general molecular heterogeneity of tissue samples means that it is vital to maintain the spatial integrity of proteins, especially post-enzymatic digestion. There are numerous studies that have been performed using mass spectrometric techniques on fresh frozen tissues, each study contributing a novel addition to the era of tissue proteomics using mass spectrometry [2-5].

As with all published research in a similar scientific field, each study critically evaluates another body of research against their own data output in order to optimise and progress methodologies. A two

centre study performed in 2014 held in Leiden, Netherlands and in Munich, Germany did one such comparison. Both groups embarked on a comparative set of experiments and produced the output entitled; Multicenter Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Imaging (MALDI MSI) Identifies Proteomic Differences in Breast-Cancer-Associated Stroma [6]. The groups aimed to undertake a *'center-to-center'* comparability study of MALDI-MSI experiments to assess both user variability and the proteomic profile of the tumour stroma in breast cancer. The samples were from two different tissue banks and comprised of FF breast cancer patient tissue, described as being of the ductal subtype.

The groups reported various differences within the proteomic signatures observed from this multicentre study with four proteins identified by the Munich group to be significantly associated with the tumour stromal region and three of these signals were also evident in the Leiden data set. The complex MALDI-MSI data produced was validated using immunohistochemical staining and a range of statistical tests including hierarchical cluster analysis.

It is reported within the article that dissimilarities were expected due to the differing sample treatment methods, some of which are detailed within Table 1.

	Leiden	**Munich**
Tissue washing steps	60% methanol (1 min), 100% methanol (1 min)	70% ethanol (1 min), 100% ethanol (1 min)
Matrix (application system)	sinapinic acid (image prep device)	sinapinic acid (image prep device)
Mass spectrometer (mode)	UltrafleXtreme (positive linear)	Ultraflex III (positive linear)
***m/z* range**	2000–28000	2520–25100
Lateral resolution	150 µm	70 µm
Haematoxylin &Eosin co-registration	same slide	same slide
Maximum peak shift (ppm)	1000	1000
% match to calibrant peaks	20	20

Table 1: Methodologies employed in the two centre study between Leiden and Munich (Adapted from [6]).

Research scientists often use a range of multidisciplinary workflows to aid the validation of proteomic biomarkers. Frozen samples do not exclusively describe a mounted tissue section and increasingly mass spectrometry is being used for needle biopsy analysis. MALDI-MSI has been used within work-flows to assess proteins and match morphological with molecular data in Cytological smears [7]. Following a top-down study by Pagni *et al* (2016) [7], zones of interest from thyroidectomy specimens were preselected by a pathologist (using a May-Grunwald Giemsa stained image) in order for the mass spectrometrist to perform a virtual microdissection of that region of interest post - MALDI-MSI. The spectra within the area chosen were then exported for data analysis. The proteins observed could potentially be correlated to malignant or benign regions of the specimen. The tissue preparations detailed within the publication were minimal due to the top down nature of the experiments. Prior to matrix deposition, the sample slides were thawed, desiccated (30 min), then fixed and washed with ethanol followed by another desiccation step (10 min). Sinapinic acid (10 mg/mL in 60%/0.2% v/v acetonitrile/TFA) was applied using the Image Prep (Bruker Daltonik, GmbH, Bremen, Germany).

Ensuring that experimental methods and techniques deliver maximum data yield is vital and the opportunity to reuse and diversify samples would allow flexibility in a workflow and reap financial benefits. Yet, does this ideal exist in tissue proteomics?

A recent study performed by Randall *et al* (2016) presented a combination of MALDI and LESA for the investigation of lipids, drugs, and proteins all from a single tissue section [8]. This collaborative study used animal brain tissue but, the methodologies could potentially be translational to human tissue samples. The analysis indicated how sampling with LESA altered the corresponding MALDI spectra and integrity of the tissue sample. To help define such changes the group employed multivariate statistical models to assess each sampling point post - LESA and subsequent molecular yield. To perform liquid extraction surface analysis mass spectrometry, pipiette 'z coordinate' was set to a height 0 mm, by doing this the sampling tip came into contact with the tissue surface rather than a non-contact micro-junction method; which is usually set to ~0.2 mm (using the TriVersa Nanomate chip-based electrospray device (Advion, Ithaca, NY)). The solvent for liquid extraction used for LESA MS imaging was methanol, water, and formic acid (69.3:29.7:1). MALDI-MSI post LESA sampling was then performed after matrix deposition.

Although LESA imaging has its own limitations in terms of spatial resolution and the ever increasing need for smaller pixel size, the perception that a single tissue section can be used for multiple analyses such as drug-lipid-protein sampling is a very appealing prospect indeed.

Formalin Fixed Paraffin Embedded **-** Preparing FFPE tissues for mass spectrometry profiling and/or imaging presents many challenges, some of which include; detachment of tissue from the microscope slide, interference from remaining paraffin wax, delocalisation and loss of target species of interest. These factors affect reproducibility and increase the requirement for biological

replicates in addition to technical repeats. However, tissues from patient biopsies are often embedded in paraffin after formalin fixation either for immediate histological analysis or retained for tissue bank archiving. The formalin fixed paraffin embedded (FFPE) tissue protocol has been extensively used for tissue preservation based on its methylene bridge cross linking properties [9-11]. This method allows room temperature storage of patient tissue enhancing sample longevity and storage flexibility.

There are studies that have been performed on sample archival time, the impact of sample 'block age' and potential impact of sample variability. Craven *et al* (2013) performed one such study on patient renal tissue for the application of LC-MS/MS using a label free, bottom up approach [12]. These data generated proteins using historical FPPE archived tissues dating from 2001 - 2011. Interestingly, block age was not found to be the limiting factor in terms of peptide yield and protein hits but established the importance of tumour grading to inform and reflect disease heterogeneity. This study considered not only experimental design but many influential factors that could impact on downstream results. Sample type and histopathology prior to mass spectrometry experiments were defined with factors that included; age, gender, haemorrhagic appearance, necrosis and features indicating inflammation. With around 2000 proteins identified from each sample, the results provided reassurance that archival tissues can be analysed to assess proteomics of diseases like renal cell carcinoma and ultimately yield clinically relevant data.

In addition to pre-experimental histological selection, the tissue preparations employed in the above Craven *et al* (2013) study can be transferrable to other mass spectrometry techniques i.e. MALDI and LESA. The methods describe how 10 µm tissue sections were cut and dewaxed in the standard way (xylene and hydration varying ethanol concentrations). After these stages, a 5 cm^2 area from the sample was macro-dissected using a scalpel, with stained haemotoxylin and eosin (H and E) serial tissue sections providing areas of interest were included. This histological staining as described here could be employed to help focus and reveal regions of interest prior to performing LESA experiments. The next steps in this proteomics experiment would involve some form of enzymatic digestion in a bottom up approach i.e. to observe tryptic peptides. For LESA and MALDI-MSP applications direct from the tissue surface, and then the methods described earlier in the *'Fresh Frozen'* section of this account would be applicable in terms of the enzyme application.

To be applicable to MALDI-MSP, the material from the macro-dissection, once in lysis buffer solution (62.5 mM Tris-HCl, pH 6.8, 4% w/v SDS, 100 mM DTT, 10% v/v glycerol), could be treated using the following described preparations even though originally designed for LC-ESI-MS/MS; the sample protocol that Craven *et al* (2013) employed was extensive, but, in doing this, their efforts resulted in being able to claim that block age did not correlate to a decreased protein yield.

Post - sample macro-dissection, the group reported that lysates were incubated at 105 ^{0}C for 45 min then on ice (5 min) before being pushed through a syringe in order to shear DNA. Subsequently, the solution was microfuged for 10 min at 4 ^{0}C and then, to minimise sample degradation, stored at −80 ^{0}C until required. To perform tryptic digestion, lysates were heated (95 ^{0}C, 2 min) after which the

group reported that a UA buffer solution (50mM ammonium bicarbonate containing 8 M urea) containing ~250 µg of protein was then digested by filter-aided sample preparation as performed by previous studies [13, 14]. Filter-aided sample preparation (FASP) describes a method where anion exchange pipette tip-columns are used. In the sequential sample steps that are detailed in Figure 3, the additions involved in the solution preparations during FASP, the flow through was discarded after any centrifugation steps introduced.

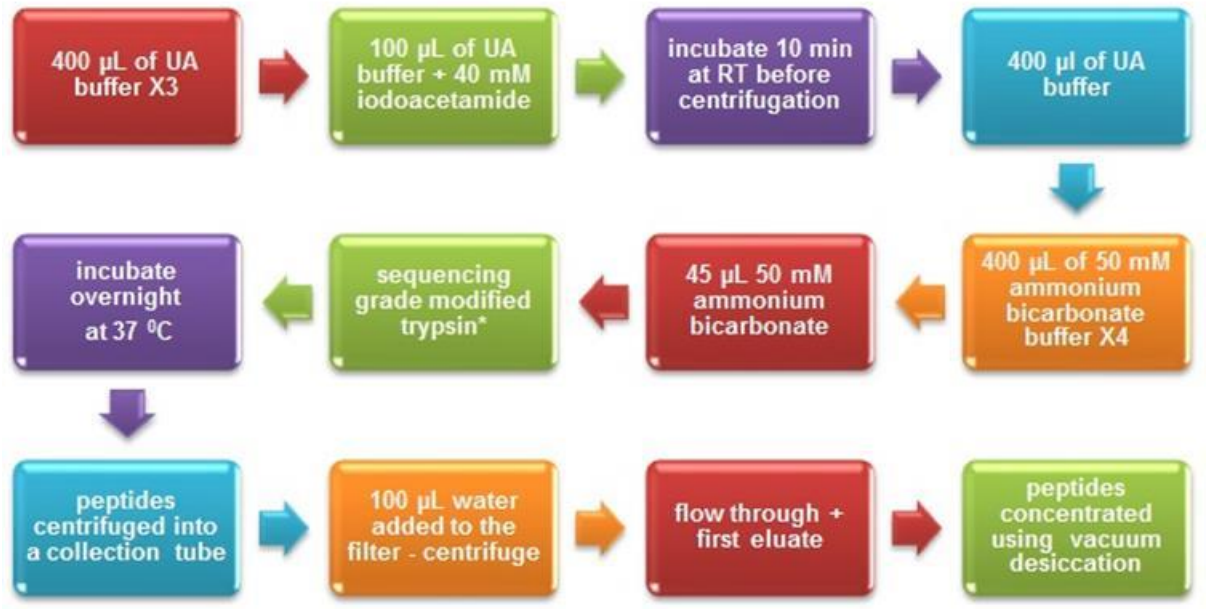

Figure 3. This workflow details the sequential steps used in a FASP experiment to yield tryptic peptides for proteomic analysis. (*20 µg of lyophilised trypsin was re-suspended in 80 µL 50 mM ammonium bicarbonate buffer to protein ratio of 1:50).

Immunohistochemical staining of FFPE patient samples is routinely performed within the clinical setting and within research to observe protein targets in both control and diseased tissue. To unlock and enable access to such biomarker targets within an excised tissue biopsy, the process of antigen retrieval (AR) is deemed a crucial stage in sample treatment. This reversal of the protein cross-linking process induced by FFPE can be considered a key factor in maximising peptide yield, thus improving the volume and quality of signals from subsequent peptide mass fingerprints.

Djidja *et al* (2017) published a study within this developmental field of tissue AR treatment using *ex vivo* human breast tumour tissue preserved by the FFPE method [15]. The resulting peptide yield in the *in situ* digestion spectra was quite profound after performing AR compared with that from *in situ* digestion in the absence of AR (see Figure 4 (a) and (b) respectively). To aid explanation of the AR data further, a graphical representation (Figure 4 (c)) indicates the average peak intensities of peptide ion signals from peptides belonging to actin (m/z 1198.7) and retinoic acid early transcript

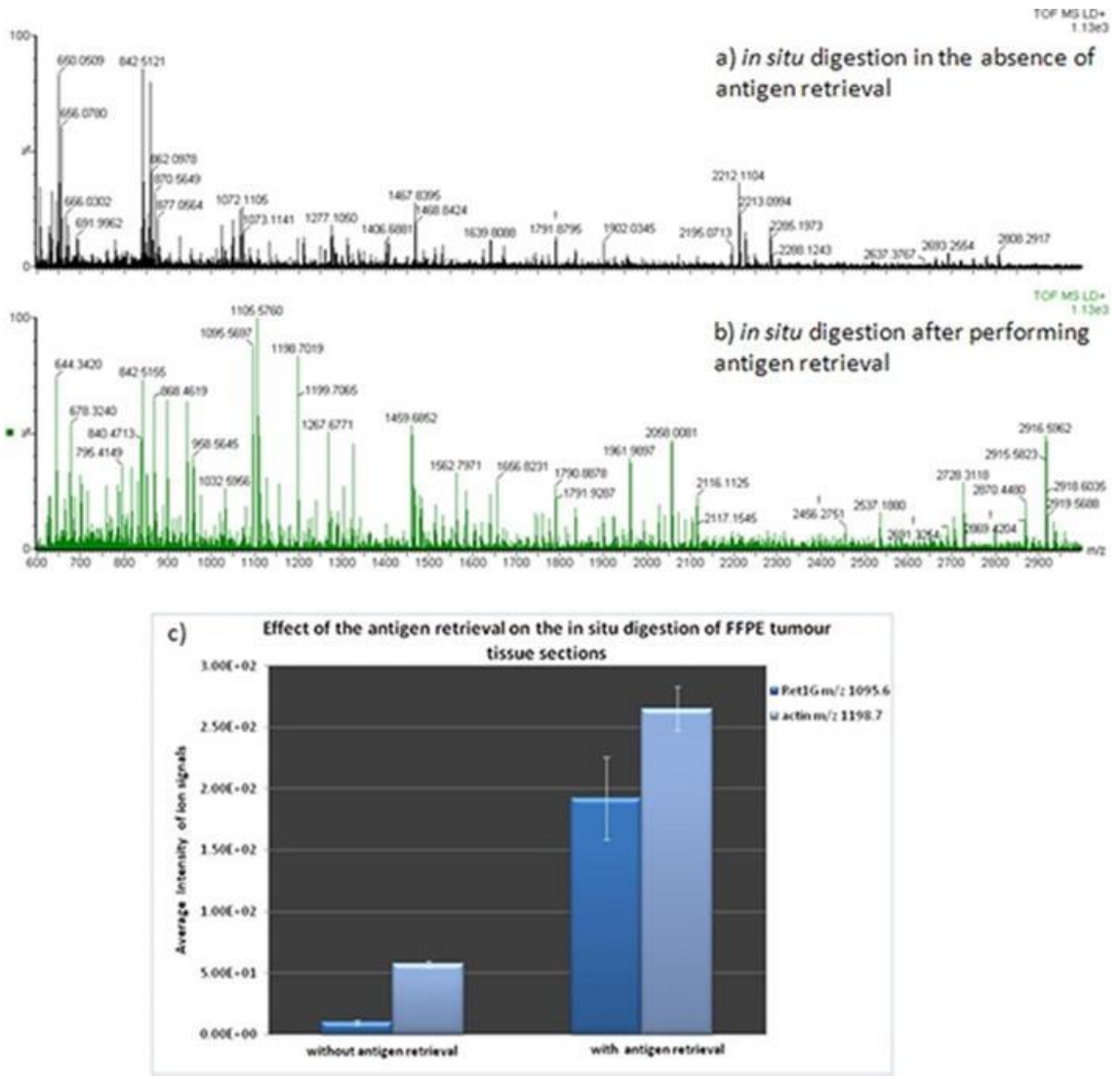

Figure 4. The effects of using antigen retrieval prior to *on tissue* tryptic digestion. Peptide mass fingerprint obtained (a) in the absence of AR and (b) after performing AR respectively. (c) The average peak intensities of peptide ion signals of actin (m/z 1198.7) and retinoic acid early transcript 1G protein (m/z 1095.6) are shown in the absence and use of AR prior to in situ digestion (n = 3). Reprinted from [15] Biochim Biophys Acta 1865, M.C. Djidja, E. Claude, P. Scriven, D.W. Allen, V.A. Carolan, M.R. Clench, "Antigen retrieval prior to on-tissue digestion of formalin-fixed paraffin-embedded tumour tissue sections yields oxidation of proline residues". Pages 901-906, Copyright (2017) with permission from Elsevier.

1G protein (m/z 1095.6), with and without the AR step in the tissue treatment. It is also worth mentioning that the subsequent protein identifications (see Table 2) were remarkable, considering that

Protein name/accession number	Mass (Da)	Observed *m/z*	Mass error (ppm)	Sequence	MOWSE score	Location and function
Actin, cytoplasmic 1/P60709	41,710	976.43	−13.5	AGFAGDDAPR	21	Cytoplasm, cell mobility
		1198.69	−9.60	AVFPSIVGRPR	12	
Collagen alpha-1(I)/P02452	138,827	836.43	−3.75	GPAGPQGPR	55	Extracellular matrix, secreted.
		852.43	−0.76	GPPGPQGAR, oxidation (P)	31	Fibril organisation, blood vessel development
		1105.57	−8.06	GVQGPPGPAGPR, oxidation (P)	24	
		1546.79	−0.49	GETGPAGPAGPVGPVGAR	75	
		1585.76	−4.98	GANGAPGIAGAPGFPGAR, 3 oxidation (P)	34	
		1775.87	2.8	GARGEPGPTGLPGPPGER, acetyl (N-term); 2 oxidation (P)	11	
Collagen alpha-2(I)/P08123	129,209	868.45	−18.3	VGAPGPAGAR, oxidation (P)	14	
		960.45	−11.7	AGVMGPPGSR, oxidation (M); Oxidation (P)	16	
		1562.79	−1.9	GETGPSGPVGPAGAVGPR	26	
		1580.73	−19.9	GPPGESGAAGPTGPIGSR, oxidation (P)	79	
		1751.80	−16.2	GPPGAVGSPGVNGAPGEAGR, 3 oxidation (P)	50	
		1775.87	−3.52	RGPNGEAGSAGPPGPPGLR, 2 oxidation (P)	39	
		1833.89	3.97	RGPNGEAGSAGPPGPPGLR, Acetyl (N-term); 3 oxidation (P)	28	
Collagen alpha-1(III)/P02461	138,479	836.43	−3.75	GAPGPQGPR	43	
		852.43	−0.76	GPPGPQGAR, oxidation (P)	25	
		1094.58	−9.87	GPPGLAGAPGLR, 2 oxidation (P)	37	
		1111.59	−4.6	GRPGLPGAAGAR, 2 oxidation (P)	41	
		1154.56	−1.11	GLAGPPGMPGPR, oxidation (M); 2 oxidation (P)	33	
		1303.61	4.85	GSPGGPGAAGFPGAR, 3 oxidation (P)	48	
		1320.66	−7.12	GPPGPAGANGAPGLR, 2 oxidation (P)	18	
		1508.72	9.67	GESGPAGPAGAPGPAGSR, oxidation (P)	20	
		1702.79	2.81	GEMGPAGIPGAPGLMGAR, 2 oxidation (M); 2 oxidation (P)	13	
		1833.89	−9.76	GPPGPQGLPGLAGTAGEPGR, 3 oxidation (P)	12	
		2104.07	−3.70	GSPGAQGPPGAPGPLGIAGITGAR, 3 oxidation (P)	25	
Collagen alpha-1(X)/Q03692	66,117	1466.67	−9.59	GLNGPTGPPGPPGPR, 6 oxidation (P)	21	
Heat-shock protein beta-1/P04792	22,768	987.61	−1.22	RVPFSLLR	26	Cytoplasm, nucleus, involved in stress resistance
		1163.61	7.54	LFDQAFGLPR	31	
Histone H2A.Z/P0C0S5	13,545	944.53	−5.55	AGLQFPVGR	41	Nucleus, gene regulation
Histone-lysine *N*-methyltransferase/O14686	563,831	1095.55	0.21	GGAHGGRGRGR, acetyl (N-term); oxidation (HW)	14	Nucleus, acts as a coactivator for estrogen receptor, activates transcription
		1286.64	−6.24	NLTMSPLHKR, acetyl (N-term); oxidation (HW); oxidation (M); oxidation (P)	23	
Keratin, type II cytoskeletal 7/P08729	51,386	1406.68	−18.8	SIHFSSPVFTSR, acetyl (N-term)	40	Cytoplasm, structural molecule
Obscurin-like protein 1/O75147	152,786	1111.59	5.44	NGAVVTPGPQR, oxidation (P)	33	Muscle development

Table 2. Tryptic peptides that were identified after *in situ* digestion of FFPE breast tumour tissue sections using direct MALDI- MS/MS. Reprinted from [15] Biochim Biophys Acta 1865, M.C. Djidja, E. Claude, P. Scriven, D.W. Allen, V.A. Carolan, M.R. Clench, "Antigen retrieval prior to on-tissue digestion of formalin-fixed paraffin-embedded tumour tissue sections yields oxidation of proline residues". Pages 901-906, Copyright (2017) with permission from Elsevier.

these data were acquired by means of MALDI-MS/MS directly from FFPE breast tumour tissue sections, hence omitting a pre-separation step. Furthermore, d*e novo* sequencing revealed proline hydroxylation and, as a result, the authors report that this may be due to the occurrence of chemical derivatisation introduced during AR [16].

After the conventional paraffin removal and hydration steps using xylene and graded ethanol, the AR the method included the immersion of tissue in a hydrogen peroxide solution (3% H_2O_2 in methanol, 12 min at room temperature) [15]. The latter treatment was the speculative proline hydroxylation stage mentioned above, knowing of the potentiation of proline to quench hydroxyl radicals therefore initiating diverse compounds such as hydroxyproline to arise [17, 18]. Pre-enzymatic digestion, the samples were placed in tri-sodium citrate buffer at (0.01 M, at pH 6.3) and heated in a microwave oven (13 min until simmering). After which the tissue sections left to equilibrate to room temperature prior to trypsin and matrix deposition.

In the quest to maintain a true insight into the proteomic signature of a diseased tumour section, or likewise the ability to screen the chemical biology from patient excised tissue, it is imperative to encourage the momentum of research into molecular target availability. Although MALDI matrix sublimation is now a widespread technique, one such study by Lin *et al* (2018) aimed to push the boundaries of molecular extraction one step further [19]. As the title of the application note describes, their method of matrix deposition by sublimation claims to enhance MALDI-MSI by introducing a simple sonication step to improve proteomic signals. The authors report that an additional step of hydration post matrix deposition considerably improves ionisation efficiency of proteins. The article is based on the fact that sublimation aims to minimise matrix *'hot spots'* and promote a homogeneous coating, desirable for imaging mass spectrometry experiments [20, 21]. In addition to this, the publication also recognised that sublimation is notoriously poor for efficient protein extraction. After sublimation and hydration stages adhering to protocols similar to those used by previous studies, sonication of the sample sealed in a petri dish chamber occurred at 37 kHz for either 2 or 5 min. Differences in the temperature between sonication parameters were recorded; after 2 min the change in temperature increased from 24 °C to 26 °C and to 30 °C after 5 min of sonication. Interestingly, they conclude an improvement in signal to noise particularly in the higher mass *m/z* range (> *m/z* 5000), with 2 min being the optimal sonication duration. The results showed differences in signal intensity from protein to protein with effects of sonication providing no significant advantage up to 5 min.

The proposed explanation for the improved signal post - sonication was described to be in alignment with previous studies including findings by Je *et al* (2006) [22]. The rationale suggested was that the hydration step alone post - sublimation could extract small and hydrophilic species allowing co-crystallisation. Conversely, sonication permitted mobilisation of larger proteins thus allowing fair competition for matrix crystal free-surface. The publication also describes an analogy that envisages a micro-jet mixing of the liquid-solid interface due to the ultra-sonic wave pressures.

Tissue micro-arrays (TMA) can be regarded as being one step closer to achieving *'higher throughput'* mass spectrometry imaging. Djidja *et al* (2010), previously reported this proof-of-concept nearly a decade ago where the group employed MALDI-MSI to observe peptides in a TMA tumour classification study using human pancreatic tissue cores [23]. The TMA imaged in this study comprised of 60 adenocarcinoma tissue cores (30 patients in duplicate). To ensure proteins were not delocalised during enzymatic application as mentioned previously in the fresh frozen section, a series of trypsin layers were pneumatically sprayed (SunCollect™ automatic sprayer, SunChrom, Friedrichsdorf, Germany); first layer of trypsin was a flow rate 1 µl/min, the second at 2 µl/min and the final three layers were sprayed at 4 µl/min. The group also used a similar order of layering to deposit the MALDI matrix post digestion.

Recently, a TMA study but on a larger scale was performed by Kriegsmann et al (2017), looking at the differentiation and proteomic molecular signatures of adenocarcinomas; colon versus lung cancer

[24]. In this study, 383 FFPE tissue specimens were analysed from individual patients with primary adenocarcinoma. The cohorts included adenocarcinoma of the colon (n = 217) and primary adenocarcinoma of the lung (n = 166). Although on a larger scale, the tissue treatments before mass spectrometry experimental work are comparable only differing by the trypsin and matrix deposition instrumentation being the Image Prep (Bruker Daltonik, GmbH, Bremen, Germany) in this study.

2.0 Sample preparation for tissue proteomics in Toxicology

The application of proteomics to studies in toxicology has been termed "toxicoproteomics". In the following section the application of and methodologies applied in toxicoproteomics will be illustrated with examples from its use in two important areas: the study of drug induced liver injury (DILI) and studies of effects of chemical and environmental insults on skin *i.e.* the effects of irritants, sensitizers and ionising radiation.

2.1 Drug Induced Liver Injury.

Drug-induced liver injury (DILI) is defined as liver injury caused by drugs, leading to abnormalities in liver tests or liver dysfunction with the reasonable exclusion of other causes [25]. DILI is one of the leading causes of acute liver failure, accounting for 13% of cases of acute liver failure in the USA. DILI is thought to occur via a number of mechanisms: direct impairment by the drug itself of the structure and functional integrity of the liver; production of a drug biotransformation product (metabolite) that alters liver structure and function; production of a reactive drug metabolite that binds to hepatic proteins to produce new antigenic drug-protein adducts, (which are targeted by immunological defences); and initiation of a systemic hypersensitivity response (i.e. an allergic response) that damages the liver. DLI poses a major challenge to pharma for drug development and safety. Alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and total bilirubin are currently the only approved DILI biomarkers in clinical practice. While fulfilling an important role in disease diagnostics, these biomarkers are not specific for hepatotoxicity, as their levels increase in practically all liver conditions [26]. Therefore, in order to find more specific biomarkers, proteomics based studies of DILI are ongoing.

It has been postulated that DILI is influenced by underlying environmental and genetic factors as well as the chemical properties of the administered drug. This was the subject of a study conducted in 2015 by Ramm et al [27]. The study provides an excellent illustration of the use of proteomics in the study of DILI. An initial LC-MS/MS proteomics screen followed by a targeted LC–MS/MS (MRM) proteomics approach was used to identify liver and plasma proteins modulated by bacterial endotoxin

(LPS), diclofenac (Dcl) or LPS/Dcl co-treatment. The overall toxicoproteomics strategies employed are shown in Figure 5.

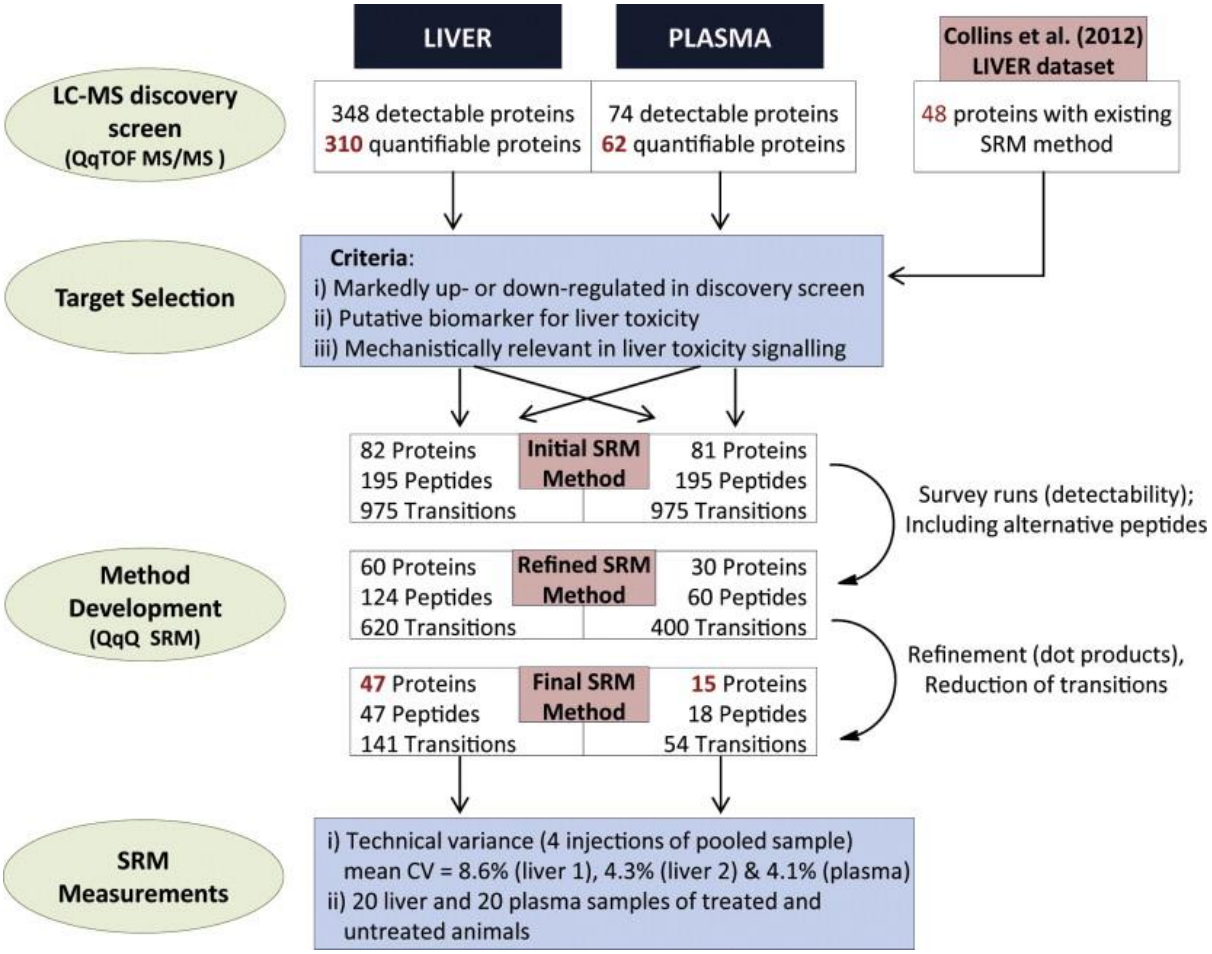

Figure 5. Toxicoproteomics method development workflow for the study of DILI. The proteins selected for monitoring by MRM were derived from an initial label-free LC–MS discovery proteomics screen of 5 liver and 5 plasma samples (pooled), a set of liver proteins previously studied by and examination of the literature on potential candidate hepatotoxicity biomarkers. The initial MRM transition list was evaluated by injection of 1 liver and 1 plasma sample (pooled) to determine the detectability of peptides mapping to proteins of interest. Based on this data a refined MRM method was built, including alternative peptides for the so far undetectable proteins. For the final MRM method peptide signals of good quality (determined by comparison with library MS/MS spectra: dot product calculation) were kept and the number of transitions for each peptide reduced from 5 to 3. The optimized MRM liver (split in 2) and plasma methods were then applied to measurements in entire sample set. Reprinted from [27] Toxicology, 331, S. Ramm, B. Morissey, B. , C. Rooney, S.R. Pennington and A. Mally, "Application of a discovery to targeted LC–MS proteomics approach to identify deregulated proteins associated with idiosyncratic liver toxicity in a rat model of LPS/diclofenac co-administration" Pages 100-111, Copyright (2015) with permission from Elsevier.

Data from the initial screening experiment carried out label free in DDA mode on a QqTOF instrument was analysed using Progenesis QIP software (Non-Linear Dynamics) and the results combined with those from previous studies and the literature to select 47 proteins for MRM based quantification. The quantification experiments were carried out on a QqQ instrument with unique peptides for quantification selected using Skyline (https://skyline.ms) and three transitions per peptide used in the final experiment.

In order to prepare the liver samples for proteomic analysis a protocol was adopted which is widely applicable and is illustrated in Table 3.

- Homogenize 50 mg frozen liver tissue on ice with 120 µl lysis buffer I (10 mM Tris (pH 7.5), 1 mM EDTA, 0.2 M Sucrose, 1.25 U Benzonase per 120 µl buffer (Calbiochem), 1 µl protease inhibitor cocktail (Calbiochem) per 20 mg liver tissue).
- After homogenization, add 880 µl lysis buffer II (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 4 mM DTT, 20 mM spermine) (and re-pipette 30 times to aid suspension).
- Incubated protein extracts at room temperature on a rotary shaker at 500 rpm for 1 h (to ensure complete cell lysis and solubilization of proteins).
- To separate membranous components and other insoluble debris, ultra-centrifuged samples for 30 min at 10 °C and 74,000 × *g*. Aliquoted the supernatant and store at −80 °C.
- Denature liver and plasma protein extracts (200 mg) by re-suspending to a final concentration of 50% trifluoroethanol (TFE), 10 mM dithiothreitol and 50 mM ammonium bicarbonate for 60 min at room temp in a 5 kDa Mw spin filter device (Sartorius Stedim Biotech GmbH, Goettingen, Germany).
- Add Iodacetamide (IAA) to a final concentration of 20 mM and incubate for a further 60 min at room temperature in the dark.
- Diluted denatured and alkylated protein samples 10 fold with wash buffer (5% TFE, 50 mM ammonium bicarbonate buffer) and subsequently concentrate in the 5 kDa Mw spin filters by centrifugation at 3000 × *g* for 60 min at 4 °C.
- The retained protein containing samples should now be transferred to a separate container and digested with the addition of 0.2 µg/µl trypsin to achieve a protein: trypsin ratio of 1:50.
- Incubate in a thermomixer at 500 rpm for 18–24 h at 37 °C.
- Dry the digested samples in a SpeedVac and re-suspend in buffer A (3% (v/v) ACN, 0.1% (v/v) formic acid) to 0.5 µg/µl prior to nLC–MS/MS.

Table 3. Protocol for the preparation of liver tissue for proteomic analysis by nLC-MS/MS: Adapted from Ramm et al [27]

The experimental protocol detailed above can be taken as typical of those used for studies of this nature and gives a good starting point for any proposed work.

Acetaminophen (paracetamol) is the drug that has been most widely studied in DILI. Its effects have been studied in their own right and it has also been used as a model system for DILI studies. An interesting recent paper [28] reports the study of acetaminophen-induced hepatotoxicity carried out using $^{18}O/^{16}O$ labelling for quantitative proteomics. Here comparison of liver tissue isolated from low (100 mg/kg) and high (1250 mg/kg) dose rats was performed. Whilst the methodology employed for the generation of protein digests for LC/MS/MS is comparable to that used by Ramm et al [27] it is the use of $^{18}O/^{16}O$ labelling for quantitative comparison that is of interest. A detailed protocol for preparing samples for $^{18}O/^{16}O$ labelling has been published by Castillo et al [29]. In summary protein extracts for comparison are enzymatically digested in parallel in $H_2{}^{16}O$ (i.e., standard deionized water) and $H_2{}^{18}O$ (^{18}O labelled water). Since the digest is a hydrolysis reaction, in the digest carried out in ^{18}O water, two atoms of ^{18}O are incorporated into the carboxyl terminus of each peptide generated in the digest. Comparative proteomic studies are performed by mixing the sample digest containing unlabelled peptides (generated in normal water) and with digest performed in the isotope-labelled water which contains ^{18}O peptides and analysing the resultant peptide pairs by mass spectrometry.

Relative quantification is derived from ratios of the isotope pairs. Conventional MS/MS experiments can then be used to identify the proteins. Given the relative simplicity of this procedure it is impressive that approximately 1000 proteins were identified and quantified in each sample studied. The overall outcome of this study was the identification of heme oxygenase 1 as a potential plasma biomarker of DILI.

In 21st century toxicology there is a great deal of interest in moving away from conventional animal based studies to in-vitro experiments [30]. For the study of DILI acetaminophen-treated three-dimensional liver micro-tissues were employed in an interesting study reported by Bruderer *et al* [31]. The liver micro-tissues used were a co-culture of consisting of primary human hepatocytes and primary human non-parenchymal cells. These were obtained commercially from InSphero AG (Switzerland) as 3D InSight™ Human Liver Microtissues. For the preparation of the microtissues the protocol employed is summarised in the table 4.

In this study the mass spectrometric methodology employed was a novel data independent acquisition method (DIA) method for hyper monitoring (HRM) was employed (https://biognosys.com).
Due to regulatory, economic and societal issues concerning the use of animals in toxicity studies (summarised under the 3Rs reduce, replace, refine) it is clear that the use of 3D cell culture and organoids will increase. The work carried out, to date, using proteomics to study organoids has been reviewed recently by Gonneaud et al [32].

- Cultivate microtissues in GravityTRAP™ plates with 70 µl 3D InSight™ Human Liver Maintenance Medium (InSphero AG) per well. Treat at day 5 with 0, 1.5, 4.5, 13.7, 41.2, 123, 5, 370.4, 1,111.1, 3,333.3, and 10,000 µM APAP (dissolved in the medium) for 3 days without redosing.
- Measured biological triplicates of each concentration with the CellTiter-Glo® ATP-assay (Promega).
- Pool 12 single microtissues from each condition were in an Eppendorf tube.
- Spin at 200g for 5 min at room temperature and then wash twice with PBS with the same spinning regime between washes.
- Lysis should be carried out in 20 µl of 10 M urea, 0.1 M ammonium bicarbonate, and 0.1% RapiGest, by sonication for 3 min followed by centrifugation at 16,000 g for 2 min at 18 °C.
- Reduced the lysate with 5 mM tris(2-carboxyethyl)phosphine for 1 h at 37 °C followed by alkylation with 25 mM iodoacetamide for 20 min at 21 °C.
- Diluted to 2 M urea and digest with trypsin at a ratio 1:100 (enzyme to protein) at 37 °C for 15 h.
- The samples should now be spun at 20,000 g at 4 °C for 10 min.
- Peptides can now be desalted using C18 MacroSpin columns.
- Re-suspend after drying, in 1% ACN and 0.1% formic acid.

Table 4. Protocol for the preparation of liver microtissues tissue for proteomic analysis by nLC-MS/MS: Adapted from Bruderer et al [31]

2.2 Skin Toxicoproteomics.

The skin is the largest organ of the human body. In addition to its major functions the prevention of desiccation and provision of protection against environmental hazards such as bacteria, chemicals and UV radiation, it is a metabolising organ, a route of excretion and aids the maintenance of body temperature (via the presence of sweat ducts) [33]. Whilst proteomic analysis of skin may be carried out for a number of reasons, the study of skin diseases has accounted for the majority of the work that has been reported to date (Table 5).

Application	Techniques Used	Reference
(i) Disease Studies		
Atopic Dermatitis	MRM	[34]
Melanoma	MSI, nLC/MS/MS, DIA	[35-37]
Contact Dermatitis	Review [16], DIGE, nLC/MS/MS	[16, 38, 39]
Cutaneous T Cell Lymphoma	Review	[40]
Psoriasis	nLC/MS/MS, DIA	[41-43]
Psoriatic Arthritis	SCX, nLC/MS/MS, DIA,MRM	[42, 44]
(ii) Aging	DIGE, MALDI-MS	[45]
(iii) Radiation Exposure		
Ionising radiation	nLC/MS/MS	[46]
UV	nLC/MS/MS LC-MALDI-MS	[47-49]
(iv) Skin Metabolism	GeLC-MS, DIA, MRM	[50]
(v) Wound Healing	MSI, GeLC-MS,DIA	[51-56]
(vi) Skin Irritation	GeLC-MS, ITRAQ	[57-58]
(vii) Skin Sensitization (mostly skin cells)	MSI	[38, 59, 39, 43, 60]

Table 5: Applications of Proteomics in Skin Research

Studies have also been carried out on the presence and effects of metabolising enzymes [50] and wound healing (including the effect of blast, infection and wound healing treatments) [61,51-52].

The paper published by Van Eijl et al [50] on metabolizing enzymes in 2012 provides an excellent general methodology for skin proteomics. Here the object of the experiments conducted was to quantitatively compare the levels of metabolising enzymes in human skin and the 3D reconstructed skin models Epiderm-200 (MatTek Corporation, Ashland, MA, USA), RHE (SkinEthic Laboratories, Lyon, France), and EpiSkin (SkinEthic Laboratories). The strategy adopted was the pre-fractionation of the proteins extracted from cystolic and microsomal fractions of samples using the GeLC-MS approach [62]. In this approach prior to LC/MS/MS analysis the protein extract is separated using 2D SDS-PAGE. The gel is not used to isolate individual proteins but rather as simply a way of sub-

dividing the complex extract into 10-20 fractions by physically cutting the gel lane into equal length strips prior to enzymatic digest. For the preparation of human skin for analysis the overall strategy adopted is summarised in Table 6.

In skin toxicoproteomics a lot of effort has gone into the study of proteomic responses to irritants and sensitizers this will be the focus of the following section. Skin irritants are defined as substances that lead to the production of reversible damage to the skin following application for up to 4 hours. In contrast skin sensitizers are substances that can lead to an allergic response following contact with the skin, such a response is termed allergic contact dermatitis (ACD) in humans. Sensitisation evolves in two phases: the first phase is the induction of specialised immunological memory in an individual following exposure to an allergen. The second phase is elicitation, i.e. the production of a cell-mediated allergic response by exposure of a sensitised individual to the same allergen. [https://eurl-ecvam.jrc.ec.europa.eu/validation-regulatory-acceptance/topical-toxicity/skin-sensitisation]. A major research effort into the study of skin irritation and sensitization was stimulated by European legislation, Directive 76/768 EEC which detailed with the upcoming prohibition of animal testing in the cosmetic industry.

An important outcome of this work and of relevance here was the development of the peptide reactivity assay [63]. This assay makes use of the concept of a molecular initiating event (MIE) [30] to assay a test substance for its potential to be a skin sensitizer based on its binding to model peptides containing an appropriate neutrophile.

The application of proteomics in the study of chemical-mediated allergic contact dermatitis has been reviewed recently by Höper et al [38]. As is highlighted in this review the complexity of whole tissue extracts has led much proteomics studies of skin sensitization to use specific cells lines rather than whole tissues [38].

Proteomics studies of whole skin for the study of skin irritation have however been conducted [57-58]. In 2014 Parkinson *et al* carried out a comprehensive proteomics study of the response of human skin to exposure to the known chemical irritant sodium dodecyl sulphate (SDS) of interest here is that studies were carried out both on *ex vivo* biopsy samples and on biopsy samples which were treated *in vivo* on the skin of healthy volunteers. In this study the samples were analysed qualitatively using a GeLC-MS very similar to that described above but this was followed up with a quantitative comparison between treated and untreated samples using the isobaric tag for relative and absolute quantitation (ITRAQ) technique [64]. ITRAQ is a chemical derivatization strategy that produces MS/MS "reporter" ions in the low *m/z* (*m/z* 114-121) area of the mass spectrum from a peptide. This area of an MS/MS spectrum obtained from a peptide has minimal background, and hence the relative areas of these peaks can be used for relative quantification since they correspond to the proportions of the labelled peptides. In this study the authors, probably correctly, claim to have produced "the most comprehensive qualitative and quantitative data set of skin proteome to date".

- Skin samples (obtained with appropriate ethical consent) should be collected immediately following surgery, chilled on ice during transportation to the laboratory and then stored at -80^{o}C until processed.
- Remove subcutaneous tissue carefully and cut skin samples into small pieces, then homogenize in 1.5-volumes of ice-cold 250 mM potassium phosphate buffer, pH 7.25 containing 150 mM KCl and 1 mM EDTA.
- Prepare microsomal and cytosolic fractions by differential centrifugation and store at -80^{o}C until required.
- Separate proteins in the microsomal and cytosol fractions on 10% NuPAGE Novex bis-tris gels (Invitrogen Ltd., Paisley, UK).
- Stained with Instant- BlueH and each sample-containing lane should then be manually cut into a series of 20 regions based on the position of molecular weight markers (SeaBlue MarkerH, Invitrogen Ltd., Paisley, UK) and the distribution of proteins observed in the gel.
- Digested each gel piece with trypsin, extract peptides and dry.
- In this study: Dried samples were reconstituted, injected onto a reverse phase column and the eluted peptides analysed by liquid chromatography tandem mass spectrometry on-line using an Agilent 1200 LC series (Agilent Technologies UK Ltd., Berkshire, UK) and a Thermo LTQ linear ion trap MS (Thermo Scientific, Hemel Hempstead, UK) as described previously.
- In this study: data analysis was restricted to those proteins with a putative role in xenobiotic metabolism and if this is required these can be selected in an automated fashion using PROTSIFT (https://github.com/jcupitt/protsift)

Table 6. Protocol for the preparation of human skin for proteomic analysis of proteins involved in xenobiotic metabolism by GeLC-MS/MS: Adapted from Van Eijl *et al* [50]

Mass spectrometry imaging (MSI) has also been applied to toxicoproteomics. Hart et al [59] utilised MALD-MSI for the study of *ex vivo* human skin in a search for biomarkers of skin sensitization and protocols for the preparation of skin for imaging have been published [65-67]. Wound healing has been the subject of a number of MSI publications [35, 38, 68, 54, 69]. Taking as illustrative of these the 2016 study of burn wounds by Taverna *et al* [54] the methodology used can be summarised as Table 7 shows and it is a typical methodology for intact protein imaging in skin.

- Sectioned frozen tissue specimens were at 12 µm in a cryostat, transfer to ITO conductive glass slides, and wash with isopropanol (2 × 30 seconds) to remove salts, lipids, and contaminants that can cause signal suppression.
- Robotically spot MALDI matrix, sinapinic acid onto regions of interest (ROI) within the tissue to be profiled for proteins.
- Identify the wound bed (WB) and adjacent dermis (AD) ROIs via histological evaluation of H&E stained sections.
- Acquire and export MALDI MS tissue profiles from selected wound bed (burn in this study) samples and normal skin (controls), baseline correct, and normalize to the total ion current (to generate spectra to be used in statistical analysis).
- For imaging mass spectrometry analyses, matrix sublimation followed by a quick (4 minutes, 85 °C) rehydration and recrystallization step can be used to prepare the samples.

Table 7. Protocol for the preparation of human skin for direct protein analysis by MALDI-MSP and MALDI-MSI adapted from Taverna *et al* [54].

The study of wound healing is another area where the use of 3D cellular models, "living skin equivalents" (LSE) seems set to help to progress the 3Rs agenda [70]. Whilst there are no reports of proteomics studies of wound healing in 3D models to date, the metabolomics work reported by Lewis et al [70] indicates that this would fruitful area for further research.

3.0 Proteomics goes forensic: sample treatment for blood and fingermarks proteomics

It is only in the recent years that proteomics has been employed to answer questions of forensic science nature. Perhaps this is due to the fact that the vast majority of information sought in forensic inquiries derive from the detection and identification of small molecules (drugs of abuse, medications, metabolites, alcohol) or from DNA recovery and analysis. However, as it will be discussed further in this section, proteins can also be an important source of forensic intelligence and this justifies the development and application of sample treatment strategies for proteomic analysis of some forensic specimens.

Although there is a wide range of forensic traces that can be retrieved at crime scenes, fingermarks are the most common and, at least in the UK, 2/3 of suspect identifications are still made through fingerprinting[1] despite the advent of DNA (Mr Neil Denison, Head of Identification Services, Yorkshire and Humber region, UK, personal communication). Whilst fingermarks are not strictly considered "tissue" by the Human Tissue Act, this section will focus on this specimen because they contain residual cells and DNA, as well as because of the pivotal role often played during investigations and judicial debates. In particular, sample treatment strategies will be discussed in order to obtain additional forensic intelligence from this type of evidence.

Whilst fingermarks are common to virtually every crime scene, blood is the most encountered type of evidence at a scene of a violent crime. Its detection can be paramount to reconstruct the dynamics of the crime and a timeline of the events as well as proving/disproving defendant statements and "legitimate access". For this reason, this section of the chapter will also include a discussion of proteomic strategies applied to blood with the aim to provide reliable and robust detection of this biofluid and contribute to a correct course of justice.

In particular, sample treatment strategies will be discussed which do not include the use of liquid chromatography prior to the mass spectrometric analysis.

The hyphenation with liquid chromatography, typically with electrospray mass spectrometry, ensures a much higher yield of protein identification, enabling a much more efficient tandem mass spectrometry to be applied for confirmation of identification. However, this technique bears the disadvantage to be more complex, time consuming and labour intensive. For this reason, *in situ* proteomics and sample treatment for subsequent analysis by Matrix Assisted Laser Desorption Ionisation Mass Spectrometry (MALDI MS) will be covered instead as it offers rapid analysis and simpler sample preparation.

[1] The process of matching a crime scene fingermark to a fingerprint record held in National Databases.

3.1 *In situ* proteomic strategies for fingermark evidence

Fingermarks are the result of the transfer of sweat and contaminants on the fingertip to a surface upon contact. Despite the advent of DNA, they are still a very important means of biometric identification in investigations leading to an arrest and/or a conviction. This type of evidence has been significantly investigated at a molecular level since 2008 thus creating a line of analysis parallel to conventional (physical) police examination based on the ridge detail and local characteristics (*minutiae*) making every fingermark unique.

Ifa et al were the first scientists to report the use of a mass spectrometry technique, namely Desorption Electrospray Ionisation Mass Spectrometry) to detect and map the molecular content of a fingermark directly onto the identifying ridge details [71]. Rowell's and Francese 's groups followed shortly after with the development of Surface Assisted Laser Desorption Ionisation Mass Spectrometry (SALDI MS) and MALDI MSP/MALDI MSI respectively [72-73]. However, out of the three techniques, MALDI MS has been significantly the most extensively published [74] and the only one that has been included in the Fingermark Visualisation Manual edited by the Home Office UK [75] (as Category C, Technology Readiness level 3). Through the application of MALDI, mainly, and other mass spectrometric techniques, a variety of classes of molecules have been investigated within this specimen, from lipids to amino acids and inorganic compounds [76-79], ingested drugs or medications (and metabolites) [80-81] as well as external contaminants such as explosives [82-83], condom lubricants [84-85], blood [86-88], contact drugs and metabolites [89]. The detection and mapping of these substances may provide intelligence around the suspect, their lifestyle and possibly activities connected to the circumstances of the crime as well as crime dynamics.

The first example of peptide and protein studies in fingermarks was provided by the work of Ferguson et al in 2012 [90]. Here the authors exploited the presence of characteristic peptides and proteins normally present in sweat to establish the sex of the donor on the basis of the differential profiles exhibited in men and women. The accuracy of prediction was 85% using a cohort of 80 donors excluding donors over 40 years old, smokers and donors taking of drugs/medications 2 weeks prior to the study. Partial Least Square Discriminant Analysis was employed with the discriminating species having a molecular weight in the range between 2000-9000 Da. The only sample treatment applied to the ungroomed marks, prepared as previously described [73], was the spotting of the MALDI matrix to enable MALDI MSP analysis. The most efficient MALDI matrix composition was 5 mg/mL α-CHCA in 25 : 25 : 50 acetonitrile–ethanol–0.5% TFAaq. Whilst promising and proving that the method was sensitive enough to detect material from a depleted specimen (ungroomed marks), the accuracy of prediction must be improved and "natural fingermarks" would need to be investigated in a study with no exclusion criteria. This work is in progress in our laboratories and will give further insights into feasibility of application. The discriminating peptides and small proteins mostly are antimicrobial species (such as dermicidin and cathelicidin); they are produced in sweat and present on skin as a response to a variety of pathogenic microorganisms-defence. These species had already been

detected in prior studies using either Surface Enhanced Laser Desorption Ionisation Mass Spectrometry (SELDI MS) or MALDI MS, though sweat and not fingermarks was the investigated specimen [91-93].

The detection of such species directly in fingermarks and the prospect of contributing to important forensic intelligence, were the basis for subsequent development work involving *in situ* "tissue" proteomics. In particular, the development of such methods was aimed to: *(i)* confirm the identity of the discriminating species and *(ii)* possibly enhance the prediction of accuracy by using smaller *m/z* peptide/protein deriving peptide sex biomarkers. Additionally, a thorough inspection of the literature indicated that many of the peptide/protein species that it is possible to detect in fingermarks, including psoriasin and dermicidin (DCD) derived peptides, may also act as biomarkers of breast cancer and other tumours[94-96]. This occurrence suggests the possibility to both provide a higher level of forensic intelligence (sex and possible disease detection) and of a cross-over with the biomedical world in the field of diagnostics. For these reasons, the development and optimisation of proteomic strategies to detect peptide and protein species from fingermarks was pursued by Francese's group with great interest.

The only prior example of proteomic strategy for the identification of antimicrobial proteins was published by Baechle et al in 2006 [93]. Here sweat, and not fingermarks, was separated on SDS page and the bands of interest excided and digested *in situ* using trypsin as proteolytic enzyme. It is important to note that no surfactant was employed within the trypsin solution.

It has been common knowledge for many years now that for in gel, in solution and on tissue digestions, the use of detergents in low concentrations greatly reduce steric hindrance enabling trypsin to cleave proteins (especially hydrophobic proteins) at many more specific cleavage sites than when a detergent is not employed [97-99]. For this reason Patel et al [100] focussed on the research and optimisation of the most efficient detergent within trypsin digestions, particularly of fingermarks. The yield in the peptide ion population and ion abundance generated by the different detergents and detergent concentrations were assessed using a systematic approach and a quantitative evaluation. Ungroomed fingermarks were employed to simplify sample preparation as peptide ion suppression is avoided through the prior depletion of lipids within this specimen. However, from our unpublished studies, if natural fingermarks are employed, washes with denatured ethanol and chloroform in separate instances are recommended.

Firstly, a range of detergents was chosen for evaluation [100]. n-Octyl ß-D-glucopyranoside (OcGlu) was primarily selected as it was at the time, at large, the most used detergent for trypsin digestion. Three additional non-ionic surfactants were also selected namely, n-Decyl ß-D-maltoside (DDM), and N-Octanoyl-N-methylglucamin (MEGA-8) (and combinations thereof). Additionally the anionic surfactant sodium 3-[(2-methyl-2-undecyl-1,3-dioxolan-4-yl)methoxyl]-1-propanesulfonate, marketed under the trade name of RapiGest SF was also trialled; this detergent had been already reported for in solution digestions [101-102]. The structures of the investigated surfactants are shown in Figure 6

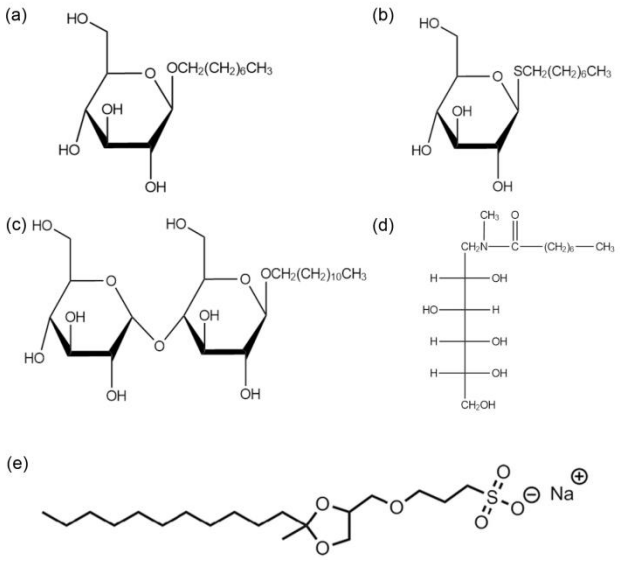

Figure 6. Chemical structures of non-ionic surfactants; (a) n-Octyl ß-D-glucopyranoside (OcGlu), (b) N-Octyl 1-thio-ß-Dglucopyranoside (OcThio), (c) N-Decyl ß-D-maltoside (DDM), (d) N-Octanoyl-N-methylglucamin (MEGA-8) and the anionic surfactant; (e) sodium 3-[(2-methyl-2-undecyl-1,3-dioxolan-4-yl)methoxyl]-1-propanesulfonate (RapiGest SF). Reproduced and adapted (panel e) from ref. [100] Patel, E., Clench, M.R., West, A., Marshall, S., Marshall, N., Francese, S.: Alternative Surfactants for Improved Efficiency of *In Situ* Tryptic Proteolysis of Fingermarks. J. Am. Soc. Mass Spectrom. **26**, 862–872 (2015) under the constraints of a Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0/legalcode)

A number of variables were investigated concurring to the determination of the most efficient detergent namely *(i)* the mode of application of trypsin; *(ii)* the concentration of the detergent (optimised according to *(i)*) and *(iii)* the combination of multiple detergents to encompass the different unfolding capabilities in relation to different proteins in a complex mixture.

With respect to *(i)* the mode of deposition, namely by spotting or homogeneously spraying the enzyme, is dictated by the type of desired outcome; if the purpose is to detect the proteome in discrete areas of the marks, then spotting is the method of choice. If the purpose is to detect and map peptides onto the fingermark ridges via MALDI MSI, then trypsin must be sprayed, although in the work by Patel et al, no fingermark imaging analysis was eventually attempted. In both deposition modes, the trypsin was used at a concentration of 20 μg/mL containing either 10 mM OcGlu, OcThio, DDM, MEGA-8 or RapiGest SF, in concentrations varying from 0.5% to 2% w/v. When these solutions were spotted, 0.5 μL droplets were manually deposited onto the fingermarks. When these solutions were sprayed onto fingermarks using the SunCollect pneumatic sprayer, seven layers of trypsin were deposited at a flow rate of 1.5 μL/min.

In another experiment, 10 mM of each of three detergents, namely OcGlu, MEGA-8, and DDM were mixed in the ratios of 1:1:1, 1:2:1, and 1:1:2 and 0.5% w/v of this detergent mixture was finally added to 20 μg/mL trypsin solution.

The detailed overall conditions of proteolysis and further preparation for MALDI MSP analysis are summarised in Table 8.

Data Evaluation - A manual inspection of all the spectra, produced under the different conditions reported above, enabled preliminary observations on the efficiency of the different detergents used in different concentrations. However, to enable a more objective and quantitative evaluation, following the analysis of the *in silico* digest of all the protein targets, ion signals were considered as "peptides" only if their *m/z* fell within the fractional range 0.4–0.8 (in line with what the proteomic community considers the fractional range of peptides to fall within). The *m/z* and intensity of these ion signals were used to enable a uniform relative comparison between the performances of the different detergents within the two deposition scenarios.

As an example, Figure 7 reports the mass spectra obtained upon trypsin spotting and proteolysis incorporating separately the four non-ionic detergents at a given concentration (0.5%). Figure also show the number of peptides within the fractional range 0.4–0.8 per given detergent (with range). With respect to the evaluation of the ion abundance, a reproducible ion matrix peak was selected and the ratio between the sum of the peptide intensity and this matrix peak was plotted per given detergent (with range). This type of data evaluation enabled to conclude that in spotting experiments repeated in triplicates, all the detergents yielded a comparable number of peptides which is to be expected given the fairly similar detergent structures. However, OcGlu, followed by OcThio, showed the largest range (lower reproducibility) as opposed to DDM and MEGA-8 showing the highest reproducibility in the yield of the ion population. However, when the ion abundance was examined, whilst OcThio produced the most intense peptides, it also showed the lowest reproducibility and was therefore not taken forward in subsequent experiments in which the detergent containing trypsin solution was sprayed rather than spotted.

In the "spraying" experiment, different concentrations of the detergents and their performance were evaluated in the same way. Results are summarised in Figure 8 showing that when used at a 2% concentration, MEGA-8 generated the most reproducible peptide numbers out of the three detergents tested together, with highest number of peptides.

This observation indicated MEGA-8 at 2% as the highest performing detergent in spray deposition conditions, despite, in these conditions, for all the other detergents, 0.5% concentration yields the highest peptide intensity:matrix intensity ratio. It is speculated that for MEGA-8, a better ratio detergent/endogenous proteins and/or trypsin/endogenous proteins occurs in spray conditions over spotting experiments.

	detergent employed	detergent conc. added to trypsin solution (% w/v)	trypsin solution, application and incubation conditions	Matrix deposition
Spotting deposition method	OcGlu	0.5	-20 µg/mL in 50 mM ammonium bicarbonate, pH 8; -upon detergent addition 0.5 µL manually spotted. -incubation in a parafilm-covered jar containing 50:50 H_2O:MeOH for 3 h at 37°C (5% CO_2).	spotting of 0.5 µL of 10 mg/mL α-CHCA in 50:50 ACN:0.5% TFAaq containing equimolar amounts of aniline to α-CHCA
	OcThio	0.5		
	DDM	0.5		
	MEGA-8	0.5		
Spraying deposition method	OcGlu	0.5 1 2	-20 µg/mL in 50 mM ammonium bicarbonate, pH 8; -upon detergent addition seven layers of trypsin were sprayed at a flow rate of 1.5 µL/min -incubation in a parafilm-covered jar containing 50:50 H_2O:MeOH for 3 h at 37°C (5% CO_2).	5 layers of 5 mg/mL α-CHCA in 50:50 ACN:0.5% TFAaq containing equimolar amounts of anilin, sprayed at a flow rate of 1.5 µL/min.
	DDM	0.5 1 2		
	MEGA-8	0.5 1 2		
	OcGlu:MEGA-8:DDM 10 mM each in ratios: 1:1:1, 1:2:1, 1:1:2	0.5		
	Rapigest SF	0.1		

Table 8. Summary of the protocols employed for trypsin proteolysis of fingermarks as reported by Patel et al [100]

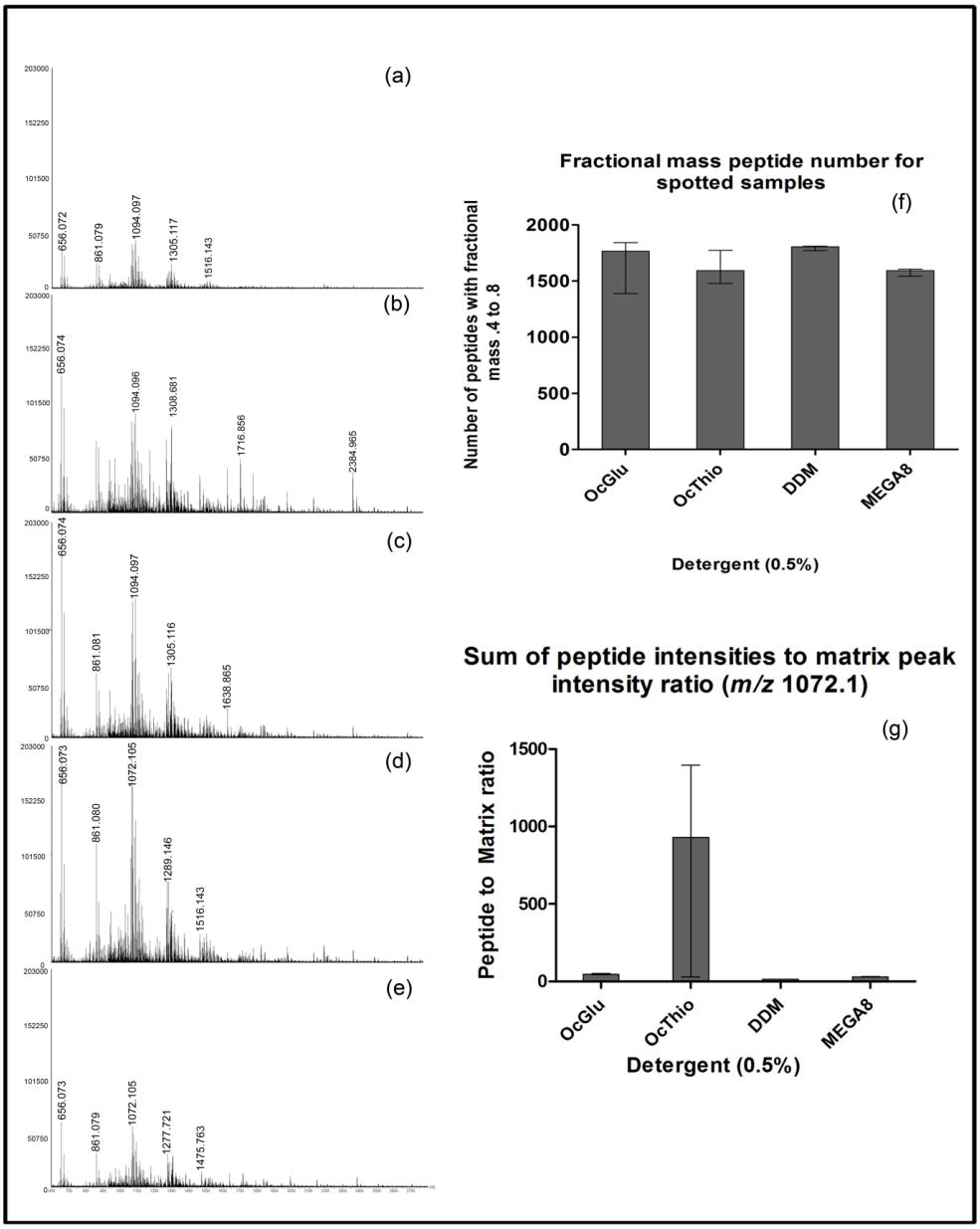

Figure 7. MALDIMS peptide profiles from *in situ* digests of ungroomed fingermarks spotted with 20 μg/mL trypsin alone in 50 mM ammonium bicarbonate pH 8.04 (a), or containing 0.5% concentration of the detergents (b) OcGlu, (c) OcThio, (d) DDM, or (e) MEGA-8. Column graph showing the number of peptides with fractional mass between 0.4 and 0.8 (f) and corresponding peptide:matrix intensity ratio for each detergent (g). The consistently detected matrix peak at m/z 1072.1047 ([CHCA – 4H + 4Na + 1 K]+) was selected for the calculation of this ratio. Reproduced from ref. [100] Patel, E., Clench, M.R., West, A., Marshall, S., Marshall, N., Francese, S.: Alternative Surfactants for Improved Efficiency of *In Situ* Tryptic Proteolysis of Fingermarks. J. Am. Soc. Mass Spectrom. **26**, 862–872 (2015) under the constraints of a Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0/legalcode)

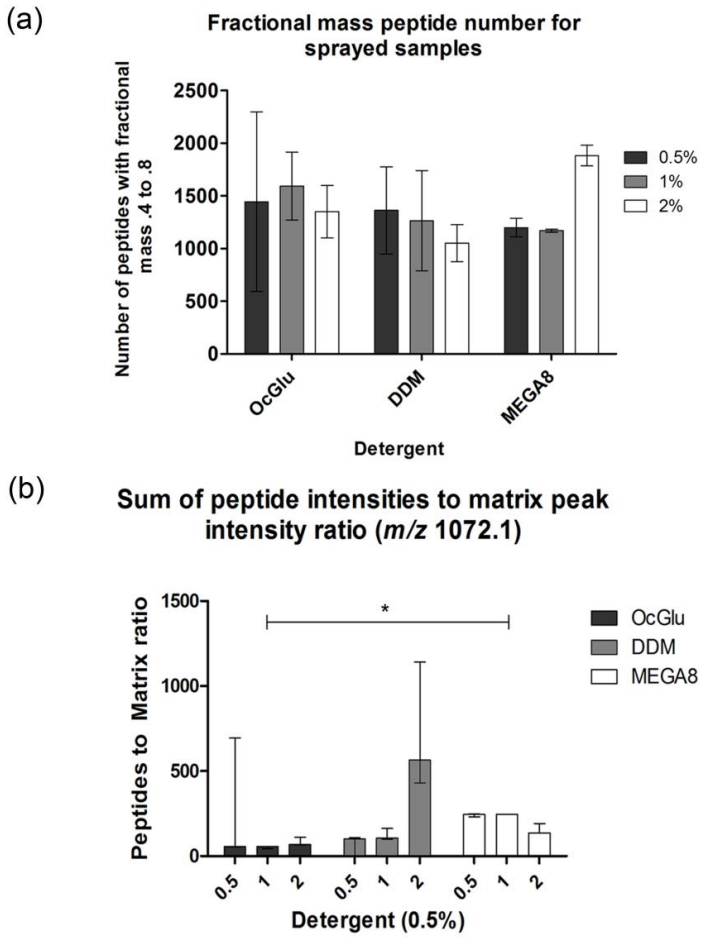

Figure 8. Column graph of the number of peptides with fractional mass between 0.4 and 0.8 for OcGlu, DDM, and MEGA-8 at three concentrations (a). Graphical representation of peptide intensity:matrix intensity ratios for each detergent at three concentrations. Statistical analysis calculated a significant increase between MEGA-8 and OcGlu at 1%, as denoted by the asterisk. Reproduced and adapted from ref. [100] Patel, E., Clench, M.R., West, A., Marshall, S., Marshall, N., Francese, S.: Alternative Surfactants for Improved Efficiency of *In Situ* Tryptic Proteolysis of Fingermarks. J. Am. Soc. Mass Spectrom. **26**, 862–872 (2015) under the constraints of a Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0/legalcode)

When the three detergents were used in mixture in different ratios, the main observation for the optimal 0.5% concentration, was an increase in the ion population beyond the *m/z* 1800 yielded by the individual detergents. This occurrence does verify the initial hypothesis of a synergy of the three surfactants and justifies further investigations into the investigation of higher concentrations.

Rapigest SF, the only anionic detergent tested, also showed an increase in the peptide numbers when compared to the peptide yield using no detergents in the trypsin solution. It was possible to observe that this surfactant produced a similar number of ion signals as for the 2% MEGA-8 digests albeit of lower intensity, except in the 1000–1200 Da mass region. This result is promising and worthy of further investigations of different concentrations other than the one recommended by the manufacturer for in solution digestions (0.1% w/v).

The systematic approach adopted to identify the most efficient surfactant(s) within the sample treatment conditions that a fingermark may be subjected to, led to the proteomic identification of several species that are normally found in sweat. In particular, putative identifications in high mass accuracy have been reported for the most efficient detergent within the studies conducted, namely MEGA-8. A targeted approach was employed in which in *silico* digests of proteins previously identified in sweat using non-*in situ* proteomic approaches, were preliminarily generated and then surveyed [103-104]. Table 9 shows a comparison of the proteins identified when MEGA-8 was spotted or sprayed respectively. It is interesting to note that the putatively identified proteins differ between the two sample treatment approaches and that dermicidin peptides are only detected when MEGA-8 is used in the higher concentration and when the trypsin is sprayed rather than spotted. Clearly, further method development is necessary to identify the set of proteins encompassing both subsets obtained through the two different methods. However the spray-coat method does seem to identify a higher number of low abundance proteins in contrast with the use of the trypsin spotting approach.

	0.2% MEGA-8 spotted	PEPTIDE m/z and sequence	2% MEGA-8 sprayed	PEPTIDE m/z and sequence
PROTEIN				
Adrenomedullin			√	1060.5605 SIGTFSDPCKDPTRITSPNDPCLTGK
Alpha-2-glycoprotein 1 Zinc	√	2035.0434 IDVHWTRAGEVQEPELR		
Antibacterial protein LL-37 134-170			√	1365.6610 WALSRGKR
Aspartate aminotransferase mitochondrial	√	2216.9593 NLFAFFDMAYQGFASGDGDK [Met Ox]		
Beta-defensin 103 precursor			√	703.3621 EEQIGK 933.4896 CAVLSCLPK
Calmodulin-like protein 3	√	2117.0760 ELGTVMRSLGQNPTEAELR [Met Ox]		
Corenodesmosin	√	2750.2882 SIGTFSDPCKDPTRITSPNDPCLTGK		
Dermicidin			√	676.3829 SSLLEK 725.3932 GAVHDVK 1128.5365 ENAGEDPGLAR 1459.7622 LGKDAVEDLESVGK 1466.7872 GAVHDVKDVLDSVL
Filamin B	√	2064.0684 SPFEVSVDKAQGDASKVTAK		
Human Serum Albumin	√	1898.9799		

		RHPYFYAPELLFFAK 2346.9887 TYETTLEKCCAAADPHECYAK		
Kallikrein-11	√	2102.9956 GFECKPHSQPWQAALFEK 2403.0369 CENAYPGNITDTMVCASVQEGGK [Met Ox]		
Keratin 1B			√	967.4723 DVDAAYVSK
Keratin type I	√	2187.0196 SDLEMQYETLQEELMALK [Met Ox]	√	1060.5605 TLLDIDNTR 1323.6725 IKFEMEQNLR [Met Ox]
Psoriasin	√	2750.2882 ENFPNFLSACDKKGTNYLADVFEK	√	1384.7194 KGTNYLADVFEK

Table 9. Peptide mass fingerprinting and putative protein identifications from *in situ* fingermark digests performed by spotting or spraying a trypsin solution containing 0.5% MEGA-8 or 2% MEGA-8 respectively as a detergent.

3.2 Proteomic strategies for blood detection, identification and provenance

Background - Blood is a very common biofluid found at the scene of violent crimes and its reliable detection is extremely important to reconstruct events that have taken place at the crime scene as well as for the detection of forensically interesting substances potentially carried by blood. This biofluid can be found in stains and in association with fingermarks. However, its detection and confirmation is not as obvious as it may seem even when a red colouration is observed; furthermore blood can be present only in invisible traces as a result of an attempted "clean-up" of the scene by the perpetrator or because originally already present in minute amounts. It is then down to the sensitivity of the technique applied whether or not blood evidence can be recovered and confirmed. For stains it is somewhat easier than fingermarks as commonly haem reactive reagents like luminol can be used in generous amounts by spraying the environment and observing fluorescence in the dark (should blood be present). In fact, luminol would also show an attempt to clean up the scene. However, the use of this reagent would destroy the ridge detail of the mark. In addition, all the blood enhancement techniques (BET), whether targeting stains of blood fingermarks, are only presumptive, that is, they provide an indication that blood may be present or not but they are not confirmatory and they may lead to false positives. These methods have been extensively reviewed and all were reported to exhibit a lack of specificity [105]; even haem-reactive compounds, the most specific class of blood reagents, may give false positives as, for instance, horseradish, leather and other extracts from plant material show the same peroxidase activity exhibited by haem in human blood. As a further example, protein dyes are used as BET, working on the attraction of the dyes' negatively charged sulphonate group to the cationic group of proteins. Whilst it is true that proteins are present in great abundance in blood, since they are also present in other biofluids such as semen and saliva, these agents could well lead to false positives.

It is therefore paramount to develop a methodology enabling the specific (and therefore reliable) determination of the presence of blood. Though Haem and Haemoglobin (respectively) had already been analysed and detected by mass spectrometry, Francese's group was the first to use MALDI MSP and MSI for the intact detection of these molecules as markers of blood presence in a forensic context [86]. Furthermore, the presence of these molecules was visualised directly onto the identifying ridge detail of fingermarks (fresh and seven day old) in non-enhanced and BET - enhanced marks (or previously BET - enhanced stains), thus providing the link between the biometric information and the circumstances of the crime. It was possible to calculate that, on the basis of average concentration of Haemoglobin (and Haem) in healthy patients, Haemoglobin and Haem could be detected down to 1000 times and 250 million times, respectively, lower than physiological concentration. A few more experiments were reported in this published work [86] showing

the possibility to determine blood provenance by distinguishing between human, equine and bovine intact Haemoglobin exploiting the subtle differences in the amino acid sequence of the protein.
This methodology involved little sample treatment and exclusively related to the preparation for MALDI MSP and MSI analysis; for fingermarks, they were simply sprayed with a 5 mg/mL solution of α-cyano 4-hydroxycinnamic acid in 70/30 acetonitrile/TFAaq 0.5% whereas stains were preliminarily tape lifted and spotted directly with the same matrix solution, albeit in concentration of 20 mg/mL ready for MALDI MS analysis.

This work represented a step forward in terms of the possibility to provide investigations and judicial debates with more reliable evidence. In fact, the presence of blood is claimed no longer by inferring it from a catalytic property or through simple aspecific changes in colouration but based on the actual determination of the presence of characteristic blood molecules. However, despite the easy and quick sample treatment, the mass accuracy on large biomolecules such as haemoglobin is limited and may not permit adequate detection and blood provenance, especially with mixed blood sources. The mass accuracy that can be achieved on the protein-deriving peptides is much higher (few parts per million), hence a greater specificity (hence reliability of the information) can be achieved by using a bottom up proteomic approach which would also enable the detection of additional blood specific proteins.

When surveying the literature in 2015, there were already many reports attempting to map the proteome of plasma and serum, which were extensively reviewed by Liumbruno et al [106] and 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. All the proposed approaches consisted in the hyphenation of some kind of separation technique with mass spectrometry (with LC-ESI MS/MS being the most proficient); individual approaches yielded typically a few hundred proteins leading to more than 10500 blood/serum proteins being identified in total so far. The most elegant approach yet was proposed by Martin et al [107] by coupling Liquid Extraction Surface Analysis (LESA) with LC-ESI MS/MS; in their study dried blood spots were investigated and the approach yielded the identification of over 100 proteins in an untargeted approach. Here sample extraction from the DBS and digestion was performed robotically and 0.1 μg/μL trypsin solution (not containing any surfactant) was employed to digest the blood proteins for 1 hour prior to transfer to the HPLC autosampler and analysis by LC MS/MS.

However, in a forensic context, the reliable detection of a handful of blood specific proteins via bottom up proteomic approach, using MALDI MS if possible, would be more than appropriate. Furthermore, in forensic science, provided that reliability of the evidence is not compromised, speed is paramount to investigations; the hyphenated methods reported can be very labour intensive and

time consuming, especially since some of them employ preliminary purification to deplete blood of the most abundant proteins. The literature offers information on the most abundant and blood specific proteins [108-111] which are expected to be detected detect and that can be used in a targeted proteomic approach. Haemoglobin, Haptoglobin, Fibrinogen, Transferrin, α-1-Antitrypsin, α-2-Macroglobulin, to name a few, are amongst the most abundant.

In 2017, an Australian research group published a report on the use of an *in situ* proteomic approach targeting Haemoglobin and enabling the discrimination of human and animal blood through the detection of proteotypic peptides [112]. The work described both sample extraction and *in situ* sample treatments for blood stains and blood marks and the possibility to retrieve MALDI molecular images of the Hb peptidic signatures for the different species. However, the image resolution and the localisation of proteotypic peptides on the mark were poor due to the deposition method (spotting of a large volume of enzyme on the mark) and the low spatial resolution with which the data were acquired.

Prior to this work, in 2015, supported by the Home Office Centre for Applied Science and technology (UK), Francese's group embarked in a research programme for the development of a multi-informative and specific methodology for detection of blood in fingermarks and stains via MALDI MS based strategies. Differently from the 2017 report, the methodology targets multiple blood specific proteins, thus increasing the overall method specificity. A method for a homogenous and fine deposition of the proteolytic enzyme has also been devised enabling high resolution mapping of blood signature onto the fingermark ridge details. Sample treatment protocols and the effect on the reliability, robustness and versatility of the methodology will be discussed in the next section.

Sample Treatment for proteomic analysis of blood stains - Initial protocol design was optimised for bloodstains with the view to cover the analysis of this type of trace evidence but also to adapt the methodology for the non-destructive detection of blood in fingermarks. Whilst direct, *in situ* analysis can be performed on blood stains, general analytical community findings are that a preliminary extraction followed by an in solution digest typically yield a higher number of peptides. Furthermore, differently from fingermarks, swabbing is a much more commonly applied forensic practice for blood stains. Because of the latter, and also due to the challenges connected with an efficient tape lift for older and/or BET enhanced blood stains, a sample treatment involving extraction of the blood trace was mainly developed for this type of blood evidence.

Depending on the state in which the evidence is available, two types of blood retrieval techniques and subsequent treatment were applied [87]: *(i)* pipetting 70 µl of 50% ACN_{aq} solution directly onto the dried blood regions; the extract was then transferred to an eppendorf where 50/50 ACN/H_2O was added up to 1 mL in volume and subsequently sonicated. Forty µL of 40 mM Ammonium Bicarbonate (AmBic) (pH 8) were then added to only 10 µL of the blood extracts; *(ii)* swabbing a small blood

containing area (of a nine year old Acid Black 1 enhanced palm print) using a swab previously wetted with 70/30 ACN/H_2O; the tip of the swab was then cut and sonicated in 1 mL 70/30 ACN/H_2O (same solution employed to pipet and extract blood in case *(i)*) to release the proteins. Twenty μL of the supernatant were then dried and re-dissolved in 20 μL of 50 mM AmBic (pH 8) in preparation for proteolysis. Many of the commonly used swabs contain PEG material. This occurrence was uncovered much later down the line of our research and in order to avoid peptide ion suppression, polymer-free swabs only are now used in our labs; *(iii)* rolling silica-free aluminium slides containing dry blood into a glass vial and immersion in 1 mL 50% ACN solution and sonicated. Table 10 summarises sample treatment conditions prior to enzymatic digestion.

Mode of blood recovery	**Extracting solution**	**Extracting conditions**	**Further treatment prior to proteolysis**	**Digestion method**
***(i)* pipetting** extracting solution of blood containing surface	50% ACN_{aq} solution	addition of 50/50 ACN/H_2O up to 1 mL to the transferred 70 μL extracting solution - ultrasonication for 10 min at 45 Hz frequency	addition of 40 mM AmBic (pH 8) in a volume ratio 1/4 extract/Ambic solution (50 μL in total)	In solution
***(ii)* swabbing (with a swab pre-wetted with a 70% ACN_{aq} solution)**	70% ACN_{aq} solution	ultrasonication in 1 mL of 70% ACN_{aq} solution for 10 min at 45 Hz frequency	50 mM AmBic (pH 8) 1/1 with the blood extract (40 μL in total)	In solution and lab-on-plate
***(iii)* immersion (of a rolled specimen containing blood)**	50% ACN_{aq} solution	ultrasonication in 1 mL of 50% ACN_{aq} solution for 10 min at 45 Hz frequency	---	Lab-on-plate
(iv) Undeposited Fresh blood	NA	NA	NA	In solution and lab-on-plate

Table 10. Sample treatment for the recovery and extraction of blood prior to proteolysis

In terms of the actual enzymatic digestion, two approaches were initially compared and contrasted: *(a)* the classic in solution digest and *(b)* the lab on plate approach.

In case *(a)* 9 µL of 20 µg/mL Trypsin Gold including 0.1% Rapigest™ SF were added to a 10 µL blood extract *(i)*or fresh blood. One hour of incubation time was sufficient to achieve most efficient proteolysis whilst minimising trypsin autolysis. Rapigest™ SF was previously trialled in *in situ* digestion of fingermarks [100] and brain tissue sections indicating a viable alternative to the use of the classic detergent octylglucopyranoside.

In case *(b)* typically 1 µL ($1/10^{th}$ of the volume in case (*a*)) of blood extract *(iii)* or fresh blood was spotted on Vmh2-adsorbed enzyme MALDI plate wells contained immobilized trypsin prepared as previously described [113]; the proteolysis was carried out for 5 minutes (1/12 of the time necessary for the optimised in solution digest).

Vmh2 belongs to the class I hydrophobins. These proteins have demonstrated to homogeneously self-assemble on hydrophilic or hydrophobic surfaces [114] and to be capable of strongly binding enzymes such as trypsin (and other proteins) in their active form. In particular, the use of Vmh2 has been proposed as an easy and effective desalting protocol [115]; the method bears the advantages of a much shorter proteolysis time for protein in the nano-femtomolar range and a significant increase of the resulting peptides signal-to-noise (S/N).Table 11 reports trypsin digestion conditions using the classic in solution and the lab on plate approaches.

	In solution digest	**Lab on plate**
Trypsin, Trypsin concentration and additives	20 µg/mL *Trypsin Gold* including 0.1% Rapigest™ SF	6 mg/mL *Bovine Trypsin*
Volume of blood/extracted blood	10 - 20 µL	1 µL
proteolysis conditions	37 °C and 5% CO_2	Room Temperature - (non controlled conditions)
proteolysis time	60 min	5 min

Table 11. Proteolysis conditions for the in classic solution digest *(a)* and the lab on plate approach *(b)*.

In preparation for MALDI MS and MS/MS analysis, an α-CHCA matrix in 50/50 ACN/0.5% TFAaq containing 4.8 µL aniline was either spotted directly on the Vmh2 coated trypsin immobilised MALDI plate well (lab on plate approach) or mixed 1/1 with the digested blood extract/fresh blood on the well of an untreated MALDI plate.

The two approaches enabled the confirmation of the blood presence through numerous blood peptide signatures (between 1 and 11 per protein) belonging to 10 proteins including: Haemoglobin alpha and

beta chains, Complement C3, Myoglobin, Apolipoprotein A1, Haemopexin, α-Anti-trypsin 1, Serotransferrin, EBP 4.2 and EBP 3 and α-2 Macroglobulin. These blood specific proteins were detected using peptide mass fingerprinting and predominantly exploiting the mass accuracy (expressed as relative error in ppm) as a match acceptance criterion, based on the mass accuracy of the mass spectrometer upon calibration prior to sample data acquisition; mass accuracy ranged between 0 to 11 pm for both the classic in solution digest and the lab on plate approach. On a first observation, whilst the peptide intensities were higher using the former digestion method, the lab on plate approach provided generally better mass accuracy. However, it must be noted that higher peptide intensity was only observed in the case of human blood; for equine blood this occurrence was inverted with the lab on plate approach showing higher peptide intensity. This can be explained by the complexity of the sample; horse blood was in fact defibrinated and therefore it is less complex than the whole human blood that was investigated in parallel.

Additionally, although both approaches yielded the detection of the same proteins, the classic in solution digest generally yielded more peptide signatures per identified protein. Whilst, on these bases, it may be obvious to choose the classic in solution digest, the much reduced digestion time within the lab on plate approach (1/12) may outweigh the greater number of peptides detected with the in solution method. This is something that is worthwhile to investigate in a validation study in parallel with LC-MS/MS analysis to verify robustness of the non-hyphenated methods.

Both approaches were also employed to determine the presence of blood in swabbed sample from a 9 year old acid black 1 developed palm print present on a ceramic tile. From this old and already enhanced specimen, only three proteins were detected, namely Hb (αHb and βHb peptides) EBP 4.2 and Complement C3 with a total of 12 peptides. However Complement C3 (which is not highly specific to blood) was only detected through 1 peptide and only via the in solution digest albeit with very high mass accuracy (1.3 ppm). Conversely, EBP 4.2 which is a highly blood specific protein was detected by both approaches and through two different peptides (one per approach). Both peptides are proteotypic and indicate blood of human origin. From these analyses it was therefore clear that these methods may open a new avenue for the re-examination of cold cases. At this stage, the lab-on-plate approach would be recommended first due to its rapidity, though, if positive, a confirmatory test would be advised through the classic in solution digest. In fact, *in situ* MS/MS analysis would be an even better direct confirmatory test. This type of analysis was attempted in this context and proved very useful not only to confirm the presence of blood but also to confirm the presence of blood of mixed provenance. While the *m/z* assignment had already provided the indication that blood was originating from both human and equine source, Ion Mobility MS/MS allowed confirmation of the presence of equine blood through the αHb peptide at *m/z* 1499.7237.

These approaches were considered highly promising and further developed for blood in association with fingermarks.

Sample Treatment for proteomic analysis of blood marks **-** As for bloodstains, BET currently used to visualise blood marks are largely presumptive. However, spectroscopic non-destructive techniques such as Raman [116-122], and Hyperspectral Imaging [123] have been reported for their ability to detect and map blood in a fingermark. Whilst they might be in some cases more specific than BET, they all present other limitations including peak shifts as a function of laser power and blood age (Raman), unsuitability of red and dark substrates and the requirement for a reference spectrum of a non-blood containing area (Hyperspectral Imaging) which, in a crime scene scenario, cannot be guaranteed to be available.

For blood marks, it would be highly beneficial to maintain the integrity of the evidence (ridge pattern) whilst proving the association of a fingermark with blood. If the integrity of the sample is preserved, a crucial associative link between the biometric information and the event of bloodshed can be established. Therefore, a technique capable of producing molecular images of a blood mark is desirable; whilst Hyperspectral Imaging can produce maps of analytical signatures, the above reported limitations make the development of an alternative technique/method desirable. As previously mentioned, MALDI is capable of imaging molecular signatures (much more specific than analytical signatures) and an appropriate methodology was developed on the basis of the work by Bradshaw et al and Patel et al [86-87] bridging the gap between the two studies.

Whilst the adaptation of the lab-on-plate approach is certainly more challenging and is presently under investigation, the initial focus of subsequent work was the adaptation of the sample treatment developed for blood stains in order to carry out an *in situ* (rather than in solution) analysis of blood marks.

It is important to bear in mind that there are three types of association relating to three different dynamics at the scene: *a)* blood mark: the fingertip was covered in blood and made contact with a clean surface; *b)* mark in blood: the blood was present on a surface and a clean fingertip made contact with the biofulid contaning surface; *c)* mark in coincidental association with blood: a clean fingertip made contact with a surface and the blood landed on it as a result of blood spatter. This type of information is extremely important for the building of a case. However, this section will not be covering such work in progress as this is being pursued with non-proteomics based techniques. The first step remains the demonstration that blood and no other biofluids or blood-resembling substances are associated with a mark.

In order to maintain intact the ridge pattern, the proteolytic enzyme must be sprayed over the blood mark in a manner that does not delocalise molecules or cause ridge merging. Not only will the

conditions of a homogeneous spraying need to be devised (number of trypsin layers, speed of spraying, distance between the nozzle and sample to be sprayed) but it is paramount that the concentration of trypsin is also optimised. As it is well known, there is an optimal ratio between trypsin and the sample to digest (50:1 typically), above which, the mass spectrum may be very densely populated by trypsin autolysis ion signals and below which the proteolysis is incomplete/inefficient. Inevitably, this task can only start by using reference samples containing approximately the same amount of blood and spread as homogeneously as possible on the same surface area. Of course, crime scene marks in association with blood may be very varied in both the volume of blood present and in the differential distribution over the mark itself. With respect to the different volumes of blood, a study in progress is systematically investigating the yield of peptide blood signatures in relation to a fixed trypsin concentration and varied volumes of blood. This will establish validity of the use of a certain trypsin concentration for a range of defined blood volumes before concentration of the enzyme has to be raised in order to efficiently digest the next blood volumes range. At this stage, for real crime scenes, blood volumes will have to be correlated with visual appearance of the blood and the estimation of the volume will then correlate to the concentration of trypsin to employ. The choice of sample treatment conditions becomes more complex with differential volumes of blood present in different areas of the same mark, which is the norm in a forensic scenario. In these cases, if the aim is to provide molecular images of blood specific proteins by visualising them on the ridge pattern, it is suggested to privilege the choice of the trypsin concentration which is deemed to be appropriate for the volume of blood estimated to be present in the area where ridge pattern is shown rather than where blood pools are.

The optimisation of the trypsin concentration for complex biological fluids has its limitations due to the fact that blood specific proteins vary greatly in concentration or "accessibility" (e.g. EBP proteins which are erythrocyte membrane proteins). Therefore it is to be expected that the combination of this type of limitation, that deriving from varying blood volumes and use of an *in situ* technique with no hyphenation would yield only a handful of blood specific proteins.

In the work by Deininger et al [88], following preliminary spot concentration tests, the concentration of (gold) trypsin was trialled on reference samples at values of 100, 150, 200 and 250 μg/mL (from 5 to 12.5 times higher than the classic in solution digestion) incubating the sample for 3 hours (3 times as much as the in solution digest to aid trypsin accessibility for a non-in solution sample) and the overall proteolysis conditions are reported in Table 11.

In particular, each blood mark was split in quarters and each quarter treated with a different trypsin concentration. Following on proteolysis, the blood marks were sprayed, using the automatic sprayer SunCollect, with five layers of 5 mg/mL CHCA in 70:30 ACN:0.2%TFAaq, containing equimolar amounts of aniline to CHCA at a flow rate 2μL/min and a nitrogen pressure of 3 bar.

	In solution digest
Volume of blood/extracted blood	~100 - 200 µL
Trypsin, Trypsin concentration and additives	100 or 150, 200 or 250 µg/mL *Trypsin Gold* in AMBIC 50 mM, pH 8, including 0.1% Rapigest™ SF
Spraying conditions on a SunCollect Autosprayer	9 layers of trypsin at a flow rate 2 µL /min and a nitrogen pressure of 3 bar
proteolysis conditions	Samples placed on polystyrene floats in a Coplin jar half-filled with 50:50 methanol:H_2O, sealed with parafilm; The jar's lid was wrapped in paper tissue. 37 °C and 5% CO_2
proteolysis time	180 min

Table 11. Proteolysis conditions for *in situ* digest of a blood mark.

The samples were then analysed in MALDI MSI mode on a Synapt G2 HDMS (Waters Corporation, Manchester, UK).

As a whole, the optimised analytical conditions have led to the detection and clear mapping of four blood proteins namely, Haemoglobin α-chain (αHb), Hemopexin, Serontransferrin and Complement C (2 peptides) with a relative error ranging between 3.2 and 18 ppm (Fig 9).

A control experiment using the same donor's fingermark, which was blood-free, had been performed at a trypsin concentration yielding blood protein signatures in the blood marks. The absence of these signatures in the blood-free mark demonstrated that those ion signals did arise from blood presence in the blood mark. Although MS/MS could not be extensively applied due to the very low intensity of the relevant peptides, the application of Ion Mobility MS/MS did confirm the presence of both Hb α and β chains. Three out of 4 trialled trypsin concentrations, namely 100, 150 and 200 µg/mL, yielded molecular images of blood peptide signatures, although the 150 µg/mL and 200 µg/mL concentrations seemed to perform better. The 250 µg/mL concentration of trypsin caused a delivery capillary blockage due to the high viscosity of the solution and the corresponding quarter was therefore not appropriately trypsinised thus yielding no molecular images.

Recently acquired data show that by using a larger internal diameter capillary (ID 100 µm), a trypsin concentration of 250 µg/mL could be delivered and that in fact was the most suitable concentration for the type of controlled samples used so far (Lisa Deininger, PhD Thesis, submitted January 2018).

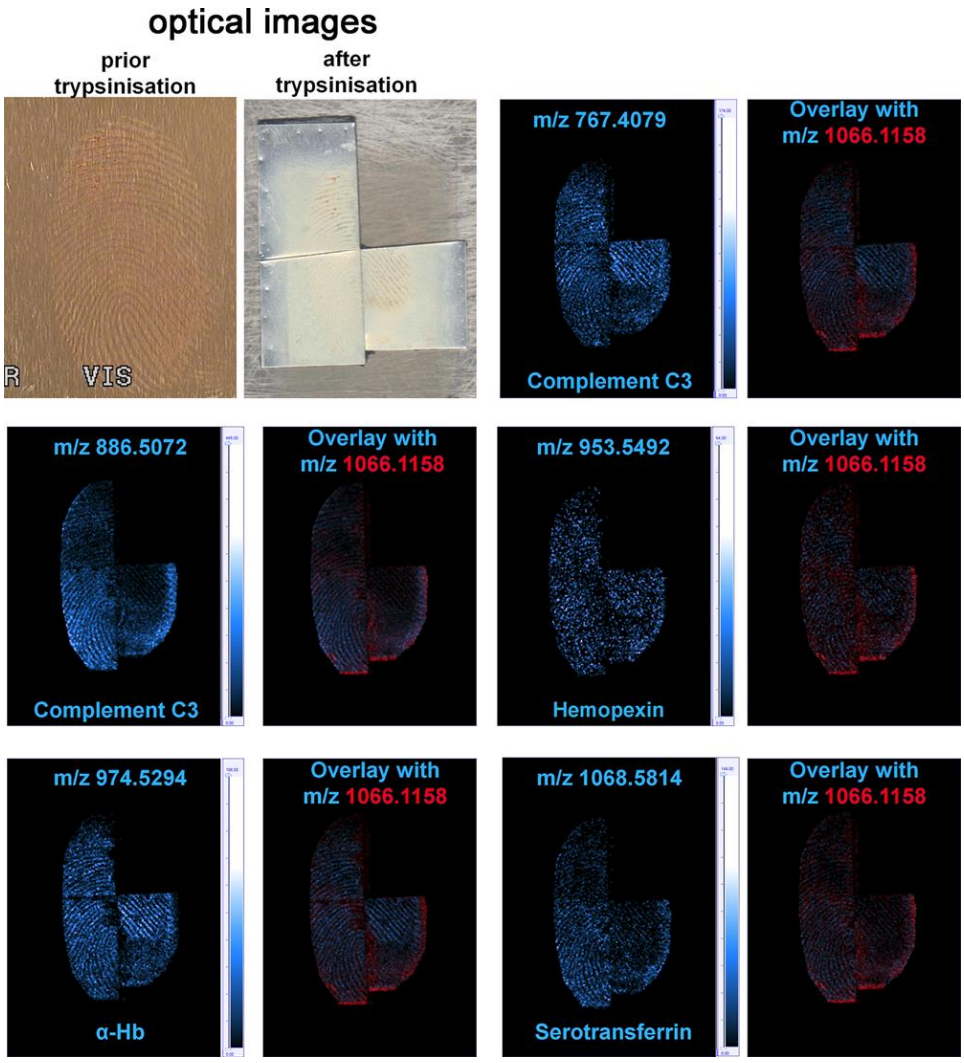

Figure 9. MALDI IMS Imaging of *in situ* proteolysis of a blood fingermark. Figure shows molecular images of blood specific peptides generated by spraying trypsin in four different concentrations (100, 150, 200 and 250 µg/mL) on the blood mark using the SunCollect. The trypsin concentration of 250 µg/mL could not be delivered to due to the internal diameter of the delivery capillary. Each peptide image has also been overlaid with the matrix signal at m/z 1066.1158. The figure suggests that the best ridge reconstruction performance could be achieved using a trypsin concentration of/between 150 and 200 µg/mL. Reprinted from ref [88] Deininger, L., Patel, E., Clench, M.R., Sears, V., Sammon C., Francese, S.: Proteomics goes forensic: Detection and mapping of blood signatures in fingermarks. Proteomics **16**, 1707–1717 (2016) with permission from John Wiley and Sons.

The results from this study further demonstrated the possibility to contribute to forensic investigations involving the determination of the blood presence. However, the next step for blood mark imaging will be the application of the developed methodology to blood marks that have been lifted from surfaces, if the surface is not thin and flat enough to be inserted in the mass spectrometer. To date, the

methodologies developed for bloodstains have been applied to an extract from a swab of 30 year old blood mark on fabric enhanced using ninhydrin. MALDI MS spectra show the presence of Haem and peptides from both Haemoglobin alpha and beta chains (Lisa Deininger, PhD Thesis, submitted January 2018). This occurrence showed the potential to image the whole mark by preliminarily digesting *in situ* and preparing it for a direct insertion in the mass spectrometer (when the surface of deposition is fabric). Though for very old marks, it would not be expected more than Haemoglobin peptides, presence of Hb peptides would pave the way to direct and *in situ* blood fingermark analysis as well as demonstrating again the possibility to contribute to cold cases.

Currently, data from a validation study, applying the methods developed for bloodstains and blood marks to blind samples, are being evaluated. The samples contain blood as well as other biological matrices (human biofluid and other matrices which may test positive for blood) and are both in a BET enhanced and non-BET-enhanced form. The determination of both negative and false positives will enable a first strong evaluation of the applicability of this method in an operational context.

4.0 Conclusions

Although a widely practised technique, on tissue digestion imaging still has many potential courses for optimisation to meet the demands of cutting edge proteomics. The requirement for the best buffer, enzyme, solvent and matrix could indeed reveal numerous concealed biological molecules, essential in the understanding of cancer, response to drugs and also in the recovery of useful intelligence in forensic investigations.

With continuous advancements in tissue preparations and treatments for proteomics involving techniques such as MALDI-MS and LESA together with the gold standard LC MS/MS, these methods have the potential to forge a place in the systematic workflow of clinical diagnostics, drug discovery and forensic science.

In cancer proteomics and in toxycoproteomics, within the assessment of proteins that are pathologically relevant and therapeutic targets respectively, it is important to appreciate how drug treated tissues potentially affect dose response relationship data. The effects of post translational modifications could mask a biomarker during a time course i.e. phosphorylation of peptides due to having a serine/threonine/tyrosine present in the sequence or having under gone methylation/acetylation. The latter being other bounds to conquer during pre-treatment protocol design.

Toxicoproteomics is a field that has received limited attention to date and indeed it has been argued [124] that metabolomics may be a more fruitful area to pursue for this application. However the

studies that have been conducted to date have produced useful data that particularly in case of DILI and skin sensitization has yielded new biomarkers and a test protocol respectively.

In forensic science and with respect to evidence such as fingermarks and blood, the sample preparation challenges to conquer are the minute amount of proteins in the former and the high dynamic protein range in the latter, where extremely abundant proteins, such as albumin and haemoglobin, may mask the presence of lower abundance proteins which are specific to blood. For both these specimens the ultimate challenge remains the design of optimised sample treatment protocols enabling the application of non-hyphenated techniques or *in situ* analysis for fast turnaround of intelligence.

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