APPLICATION NOTE

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

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Application & Background

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

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

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

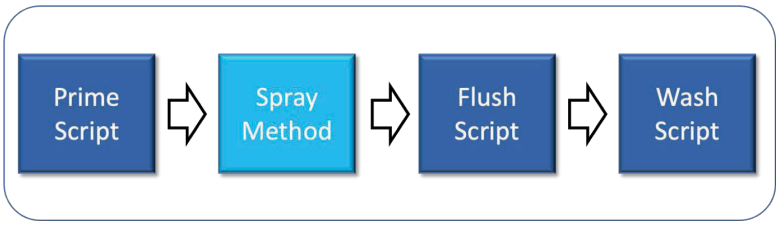

Figure 1. Automated PumpScripts™ attached to each spray method.

Experimental

Experimental Design & Fingermarks

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

Enzyme and Matrix Application using the HTX M3+ Sprayer

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

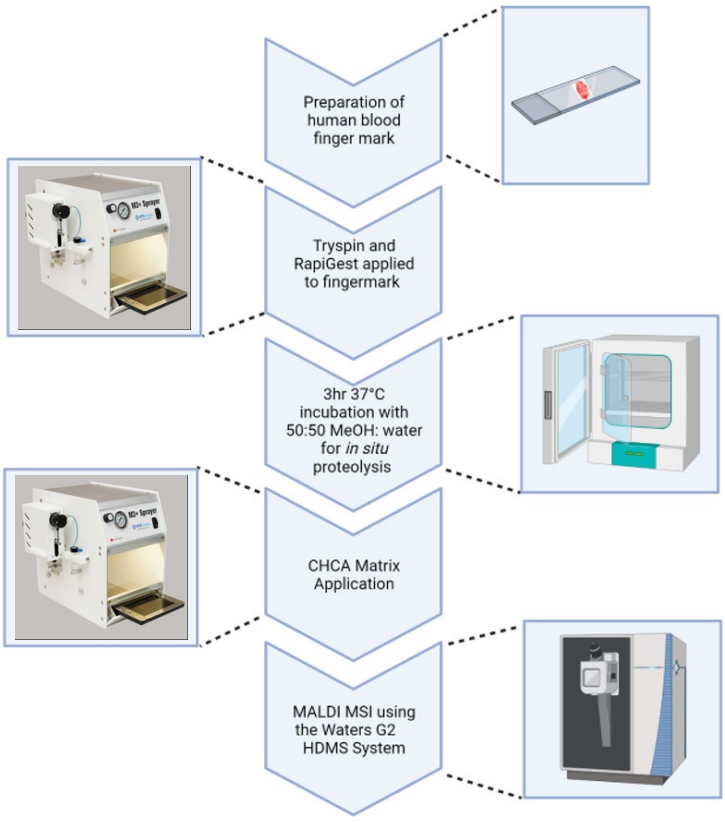

Figure 2. Workflow for peptide MALDI MSI analysis on blood fingermarks.

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

MALDI MSI

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

Data Analysis

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

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

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

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

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

Results

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

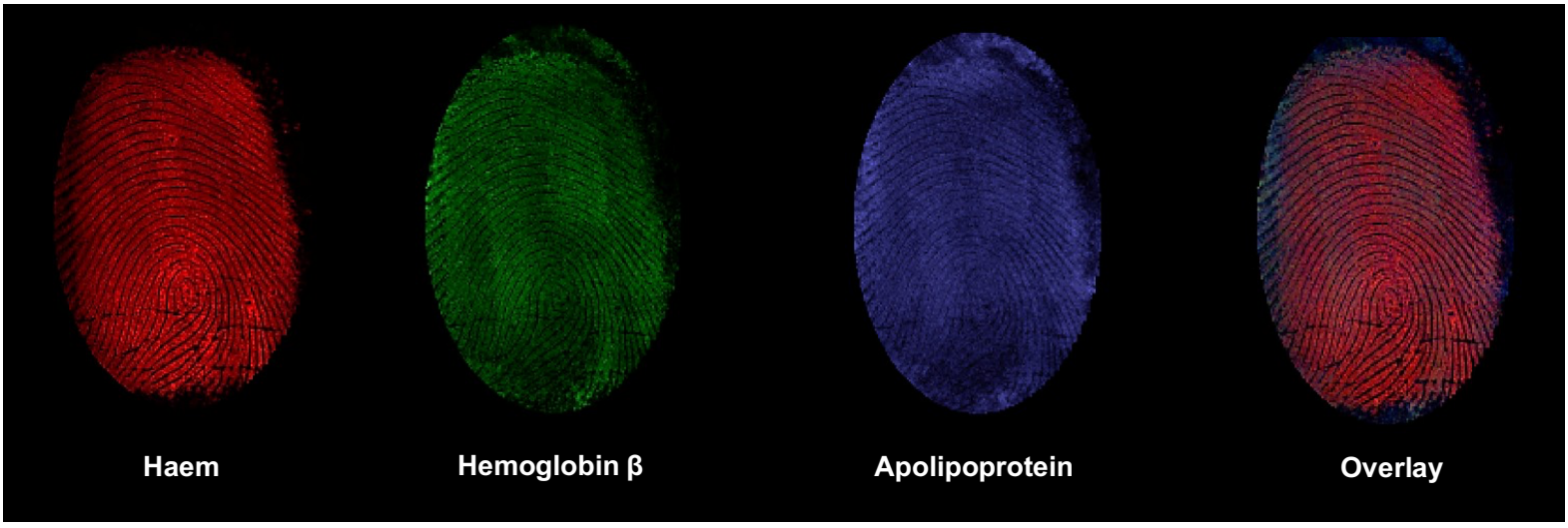

Figure 3. Haem and blood specific peptides from a human blood fingermark following application of trypsin and CHCA matrix. Images shown are haem at *m/z* 616.17 (red), hemoglobin β at *m/z* 1149.56 (green), and apolipoprotein at *m/z* 1215.6 (blue). The 4th and last MS image from the left is a superimposition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

Table 3. Key human blood peptides detected from blood fingermarks following tryptic digestion.

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

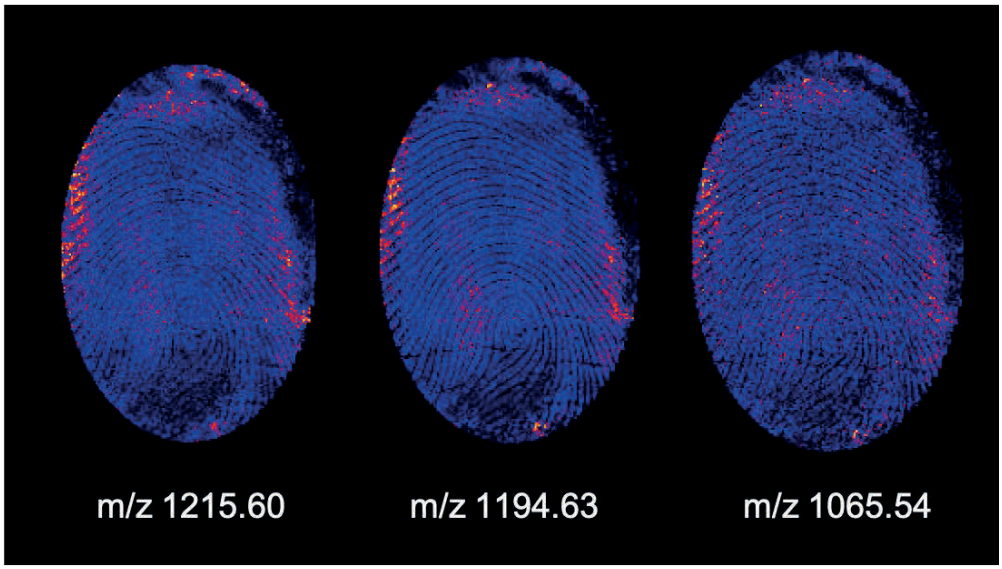

Figure 4. Example blood peptides detected from a human blood fingermark. Peptides shown are apolipoprotein A1 at *m/z* 1215.60 (ATEHLSTLSEK), thrombin at *m/z* 1194.63 (ELLESYIDGR), and complement 3 alpha at *m/z* 1065.54 (MDKVGKYPK).

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

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

Conclusions

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

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

References

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(3) Witt M, Kennedy K, Heaton C, Langenburg G, Francese S. 2021. Forensic visualisation of blood and blood provenance in old fingermarks by MALDI MS Imaging. *Bruker Daltonik Application Note.* MSI-22.

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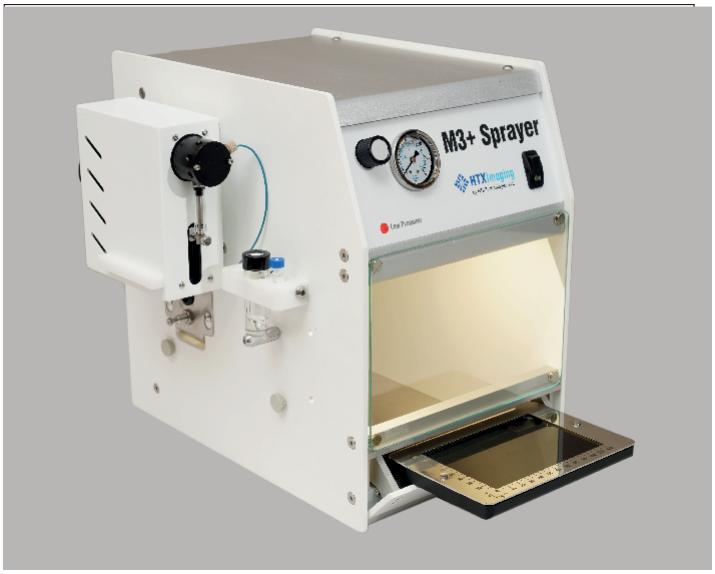
(5) Bandey HL. 2004. Fingerprint Development and Imaging Newsletter: The Powders Process, Study 1, Police Scientific Development Branch, Home Office, Sandridge, Report No. 54/04

HTX M3+ Sprayer™ MALDI Imaging Sample Preparation System



HTX M3+Sprayer™ System is an Automated MALDI Matrix Deposition System Offering High Reproducibility and Superior Data Quality for Imaging Mass Spectrometry

The HTX M3+ Sprayer™ is an easy-to-use, versatile spraying system that provides automated processes for sample preparation in imaging mass spectrometry.

The proprietary spray technology of the HTX M3+ Sprayer™ guarantees a very fine, uniform and consistent matrix coating crucial for high-resolution imaging and relative quantification of analytes.

The unique ability to control liquid and propulsion gas temperature creates a fine solution mist that can be deposited in a precise and adjustable pattern over all or part of any MALDI plate.

Spray characteristics (wet or dry) are easily adjustable via the intuitive operator interface. Users can create and save methods for reproducible operation.

Key Characteristics

- Proprietary technology providing very small matrix droplets (<5 microns)
- High flow rate and fast sample prep (2 to 18 minutes per slide)
- Highly consistent matrix deposition across entire sample area (+/- 3% by weight)
- Unique use of temperature and nitrogen flow to control evaporation rate and matrix crystal formation
- More than 30 validated protocols covering trypsin and most matrices (e.g.: SA, CHCA, DHB, DAN, 9-AA, DHA, CMBT, THAP)
- Validated protocols for enzymatic digestion of FFPE and frozen tissues
- Continuous matrix coverage as needed for high-resolution imaging
- Robust operation and automated clean-up

Addressing the Matrix Deposition Challenge

The main challenge when preparing samples for MALDI Mass Spectrometry Imaging is to balance the positive effects of the matrix solution penetrating the tissue and co-crystallizing with the analyte, and the negative effects of analyte delocalization.

The all-new M3+ chassis, high velocity stage and sample holder drawer contribute to a greater user experience and expanded process capabilities including.

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HTX M3+ Sprayer™ and M5 Sprayer™ are available worldwide exclusively from HTX Technologies, LLC. To request further information, please visit **www.htximaging.com** or contact us at **info@htximaging.com**.