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Optimisation of Matrix Deposition for Ink Analysis and Molecular Fingerprinting in Questioned Document Examinations using MALDI MSI

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Abstract

In this study, we have explored the capabilities of Matrix-assisted laser desorption/ionisation mass spectrometry imaging (MALDI-MSI) as a powerful tool for Questioned Document Examination (QDE) which can offer crucial forensic chemical insights where optical based conventional methods may be inconclusive. However, successful MALDI MSI analysis of complex questioned document samples, such as those containing overlapping inks and/or fingerprints, is strongly dependent on the matrix deposition method, which influences ionisation efficiency, reproducibility, and spatial resolution. This study presents a systematic optimisation of matrix deposition, using sublimation, to enhance the simultaneous analysis of inks and fingerprints deposited on paper. Parameters such as matrix quantity, sublimation duration, and the choice of recrystallisation solvent have been optimised using controlled

samples comprised of printed inks, ballpoint pen inks, and natural latent fingerprints. The optimised sublimation protocol was benchmarked against two established matrix spraying methodologies on a mock forged document. The method enabled the successful differentiation of two optically similar, but chemically distinct, ballpoint pen inks used to alter a date on a document, while concurrently imaging latent fingerprints to provide valuable biometric and chemical intelligence relating to two separate fingerprint donors. This work establishes a validated framework for achieving reliable, high-resolution molecular analysis in complex QDE scenarios, strengthening the analytical power and reliability of MALDI-MSI in forensic investigations.

Keywords: MALDI MSI; Forensics; Questioned Document Examination; Ink; Fingerprints; Sublimation

1. Introduction

Questioned document examination (QDE), is increasingly recognised in published literature for its role in uncovering crime-related facts and supporting forensic evidence during investigations and judicial debates. Documents analysed by forensic document examiners (FDE) often include (in a non-exhaustive list) cheques, identity documents, letters, contracts, wills, and currency.[1] Such analyses often aim to investigate any alteration of the documents whether through page replacement, forged signatures or through modification/addition/deletion of written content, logos, stamps etc. Intersecting lines are also often examined, substrates (e.g., paper) are characterised, and inks analysed from pens and printers.[2]

In QDE, non-destructive techniques that maintain the integrity of samples are preferred, particularly when they may be required as evidence in legal proceedings. Therefore, the "conventional" approaches involve visual examination, which are often used in the initial step of QDE. These can include naked-eye inspection, optical and electron microscopy, enabling FDE to assess physical characteristics such as ink line indentations, writing pressure and paper fibres[3,4]. Advanced examination includes the subsequent use of spectroscopic techniques, such as Raman spectroscopy, enabling chemical characterisation of specific molecular classes contained within the samples (such as dyes) that can be used for their differentiation.[5] Spectroscopic techniques are an important component of forensic workflows; however, their molecular specificity is limited, and dyes and pigments can produce high fluorescence backgrounds and overlapping or poor spectra.[6] These limitations may result in inconclusive findings that necessitate further examination.

The application of mass spectrometry (MS) in QDE allows for improvements in molecular specificity, thus providing higher reliability of chemical information in cases where initial

results may be inconclusive. In last year's bibliometric analysis using visual knowledge mapping, "mass spectrometry" was identified as one of the most frequently occurring keywords in QDE, highlighting its growing significance as an advanced analytical methodology in the field.[7] They found that while broad terms such as forensic science and QDE are commonly used across the field, more specific keywords such as handwriting and writer identification appeared with the highest frequencies. Among these terms, ink (42 occurrences), mass spectrometry (29 occurrences), Raman (25 occurrences), and feature extraction (23 occurrences) were also among the most frequently occurring keywords, highlighting their relevance in QDE research.

Previous research shows numerous MS techniques that have been successfully applied to QDE-type samples. Some examples include secondary ion mass spectrometry (SIMS)[8-10] , direct analysis in real time mass spectrometry (DART-MS)[11,12] and desorption electrospray ionisation (DESI-MS)[13-15]. In addition to these methods, matrix-assisted laser desorption ionisation mass spectrometry (MALDI MS) has also been applied to questioned documents, particularly for characterising various types of inks.[16,17] Previous work by the Francese group highlighted the ability of this technique, in conjunction with machine learning, to discriminate between different pens[18], and more recent studies extended this application to molecular identification of ink constituents, including specific dyes.[15]. Tibljas et al (2025), successfully examined several ink types including stamps, ballpoint pen, and printed ink across different paper substrates to assess whether the materials had been produced and signed simultaneously. The most recent work by the same group demonstrated an innovative application of this knowledge, applying it to inks and substrates of whisky product labels as a powerful tool for counterfeit detection.[19]

The imaging capability of MS techniques can serve as valuable forensic intelligence, showing the distribution of specific chemical components within an intact sample. For example, the

chemical imaging of fingerprints using MALDI-MSI, a process referred to as "molecular fingerprinting", is a powerful technique for providing both biometric (ridge characteristics/patterns) and chemical information on endogenous and exogenous molecules contained within the residue. This methodology can also allow for visualisation of fingerprints in cases where conventional methodologies may have been unsuccessful, due to the ability to detect and visualise molecules that were not being targeted by the applied conventional enhancement methods.[20] Spatial mapping of the molecules not only supports fingerprint identification but also provides insights into the lifestyle and habits of a potential suspect, by visualising the presence of molecules from their fingerprint residue, thereby enhancing forensic investigations. This could be the presence of illicit drugs and/or their metabolites in drug related cases, the presence of explosive residues linking to terrorist activities, and even the presence of certain pharmaceutical drugs stating the underlying medical conditions which can be used for narrowing down the suspect list [21-23]. The UK Home Office and the Defence Science and Technology Laboratory (Dstl) have officially recognized molecular fingerprinting for its operational value. Initially included as a Category C technique in the international Fingermark Visualisation Manual in 2014, its proven effectiveness led to its promotion to Category B in 2023[24]. For many years, the Francese group has collaborated with law enforcement, using their expertise in MALDI-MSI to analyse fingerprint samples and aid criminal investigations. Building on this expertise, the present study aimed to evaluate MALDI-MSI for QDE samples containing fingerprints, to explore its potential for simultaneous chemical and spatial analysis in a forensic context.

Questioned documents normally contain various types of inks as well as potential fingerprints, therefore when applying MALDI MSI, each would require different matrix and solvent conditions when using conventional deposition methods (such as spraying), since solvent choice can strongly influence analyte extraction and selectivity. Alternatively, sublimation is a

solvent-free matrix application in which a solid matrix is heated under reduced pressure, so it vaporises and deposits as a thin layer of small crystals on the sample surface.[25] This technique is advantageous for QDE by MALDI MSI as it avoids solvent spraying, thereby minimising the risk of analyte delocalisation. This study shows a systematic investigation of sublimation as a matrix deposition technique on questioned documents, focusing on the effects of matrix quantity, deposition time and the choice of recrystallisation solvents on controlled samples containing (a) printed ink, (b) ballpoint pen ink and (c) latent fingerprints. The best resulting protocol was then compared with two standard spraying methods previously developed by our group[15,26]. To evaluate the applicability of the method to real samples, a mock forged document containing both inks and fingerprints, was analysed by MALDI MSI using the optimised protocols for matrix deposition. Sublimation as a matrix deposition technique enabled the collection of both chemical and physical information, highlighting the enhanced analytical capabilities of MALDI-MSI for questioned document analysis which could provide additional intelligence in forensic investigations in comparison to conventional methodologies.

2. Experimental

2.1 Chemicals

Trifluoroacetic acid (TFA), α-cyano-4-hydroxycinnamic acid (α-CHCA) and Methyl Violet (MV) were purchased from Sigma-Aldrich (Poole, UK). Formic acid and HPLC grade acetone, methanol (MeOH) and acetonitrile (ACN) were obtained from Fisher Scientific (Loughborough UK). Double-sided conductive carbon tape was from TAAB (Aldermaston, UK). MiliQ water was produced in-house.

2.2 Matrix Coating

Sublimation-based deposition of α-CHCA was performed using a HTX SubliMATE™ system (HTX Technologies LLC, Chapel Hill, USA). The preheat step was performed at 185 °C for

one minute, with ice cooling the SubliMATE top throughout (measured between 6-9 ºC). For spray-coating, a HTX M3+™ Sprayer (HTX Technologies LLC) was used with 5 mg/mL α-CHCA in either: (a) 70:30 (MeOH: 0.1% TFA_{aq}) (flow rate 100 µL/min, and velocity 1300 mm/min) and (b) 70:30 (ACN: 0.1% TFA_{aq}) (flow rate 100 µL/min, and velocity 1200 mm/min) at 8 psi for a total of eight passes.[15,26]

2.3 Mass Spectrometry Data Acquisition and Data Processing

MALDI MSI and profiling were performed using a SYNAPT G2 HDMS Q-ToF instrument (Waters Corporation, Manchester, UK), from *m/z* 50–1,200 using positive ionisation and sensitivity mode. Default RF parameters and automatic quadrupole profile was used. Imaging analyses were conducted using a laser intensity of 150 (arbitrary units), scan time of 0.2 seconds and a pixel size of 100 x 100 µm. MALDI-MS/MS experiments were performed with an optimised collision energy of 50 eV. Prior to each analysis, calibration was performed using Red phosphorus (mean prediction error <0.5 ppm).

Data were acquired using MassLynx™ (v4.2, SCN991) and HDI (v1.6). All the imaging data was processed and visualised using HDI (v1.8) with the following parameters: MS resolution of 20,000 *m/z* window of 0.02 Da, and the number of most intense peaks set to 1,000. No TIC normalisation was applied as it produced imaging artefacts when multiple experimental conditions were represented in the same dataset. The ion image intensity was adjusted to maximise the quality of each image. All the regions of interest (ROIs) were of identical pixel number and were exported using MassLynx™ (v4.2) for spectral analysis and HDI (v1.8); as an MVA file for statistical analysis. Lock mass correction was applied using the known matrix ion at *m/z* 190.0504.

2.4 Statistical analysis

Ion intensity variation was analysed in Prism software. Data normality was assessed, followed by one-way ANOVA (unpaired) with multiple comparisons, in which the mean of each group was compared against the means of all other groups.

2.5 Sample preparation

QD samples containing printed ink, ballpoint pen writing, and a natural fingerprint were prepared under controlled laboratory conditions using *bizhub C659* printer from Konica Minolta (Tokyo, Japan), blue ballpoint pen (BIC), white office paper A4 75 GSM (Woodland Trust, UK). Fingertips of the donors were rubbed together to evenly distribute the fingerprint residue prior to deposition.

A mock QD sample was prepared which contained the signature "Jane Doe" and date "3/9/2003", written with a ballpoint pen. The date was altered to "8/9/2008" with a different ballpoint pen. Two natural fingerprints were deposited over the text, from two donors (male and female).

2.6 Sublimation optimisation

Matrix quantity, sublimation duration, and recrystallisation solvents were optimised. Samples were divided into four sections, each containing a laser-printed ink block, a blue ballpoint pen stroke, and a portion of a fingerprint. Additional fingerprint-only samples were analysed, containing no ink. Each section was subjected to a different set of conditions for the parameter under investigation. Once sublimation had been conducted, the sections were reassembled and imaged as a single composite region. The experiments were also replicated, altering the order of the quadrants, to address any potential variability in fingerprint homogeneity. Optimised sublimation was tested against two different optimised matrix spraying conditions. A schematic for the sublimation optimisation process is presented in Fig. S1.

Matrix concentration- α-CHCA was dissolved in 1.5 mL of acetone at four different quantities: 5 mg, 10 mg, 15 mg, and 20 mg before conducting sublimation at 300 °C for five minutes.

Sublimation time -The optimal matrix quantity (10 mg α-CHCA) was used. Sublimation was carried out at 300 °C for four different durations: 2, 3,4 and 5 minutes.

Recrystallisation – The sublimation conditions were set to those optimised in the previous tests (10 mg α-CHCA, 2 minutes). Following sublimation, recrystallisation was performed by adding 1 mL of different solvents (isopropanol, ethanol, acetone) to a piece of filter paper within a Petri dish. The sample was attached to the underside of the lid, and it was placed into an oven at 60 °C for 1 minute to create vapour.[27] This was compared with no recrystallisation.

2.7 Sublimation vs spraying

Optimised sublimation was compared with two established spraying methods for fingerprint studies[26] and QDE.[15]

2.8 Selection and investigation of Fingerprint types

Four types of fingerprints were analysed (groomed, ungroomed, eccrine and natural). Groomed fingerprints were prepared by rubbing fingertips across the forehead, nose, and chin five times to collect an abundance of endogenous material, producing a sebum-rich mark.[21,28] To generate ungroomed fingerprints, both hands were first cleaned with alcohol wipes, and a 15-minute period of normal work activities was performed before deposition onto the substrate. Eccrine prints were generated by cleaning both hands with a 50% aqueous ethanol solution prior to placing them inside a plastic bag for 15 minutes; subsequently the hand was removed from the bag and a fingerprint was deposited. Natural prints required no prior preparation, other than rubbing the fingertips together before deposition. A quarter from each print was used to form one composite fingerprint which was then prepared using the optimised conditions prior to MALDI MSI.

2.9 Degradation Analysis for Ball Point Pen Inks

To investigate the extent of degradation that occurs due to temperature differences in sublimation and spraying, the ratio of the degradation ion intensity to the corresponding parent ion intensity within ball point pen ink was calculated. The intensity values for both parent and degradation ions were extracted from the MS spectra (6 scans each) for each replicate under both matrix deposition conditions.

3. Results and Discussion

To assess the effects of matrix concentration, sublimation time, and the most effective solvent for recrystallisation, a consistent sample preparation protocol was employed. For each set of experiments, one representative ion is shown for the fingerprint, printed ink, and pen ink to illustrate analyte distribution and intensity. These ions were selected because they exhibit trends consistent with the majority of ions within each dataset.

3.1 Sublimation Optimisation

A natural fingerprint was deposited over two ink types (ballpoint pen and printed ink) and the sample was then split into four quadrants (Fig. 1B). Each quadrant was subjected to sublimation using a different quantity of α-CHCA: 5 mg (top left), 10 mg (top right), 15 mg (bottom left) and 20 mg (bottom right) prior to MALDI MSI (Fig. 1C-E). α-CHCA was selected due to this matrix being used extensively on these sample types previously by our group.[15,19,26]

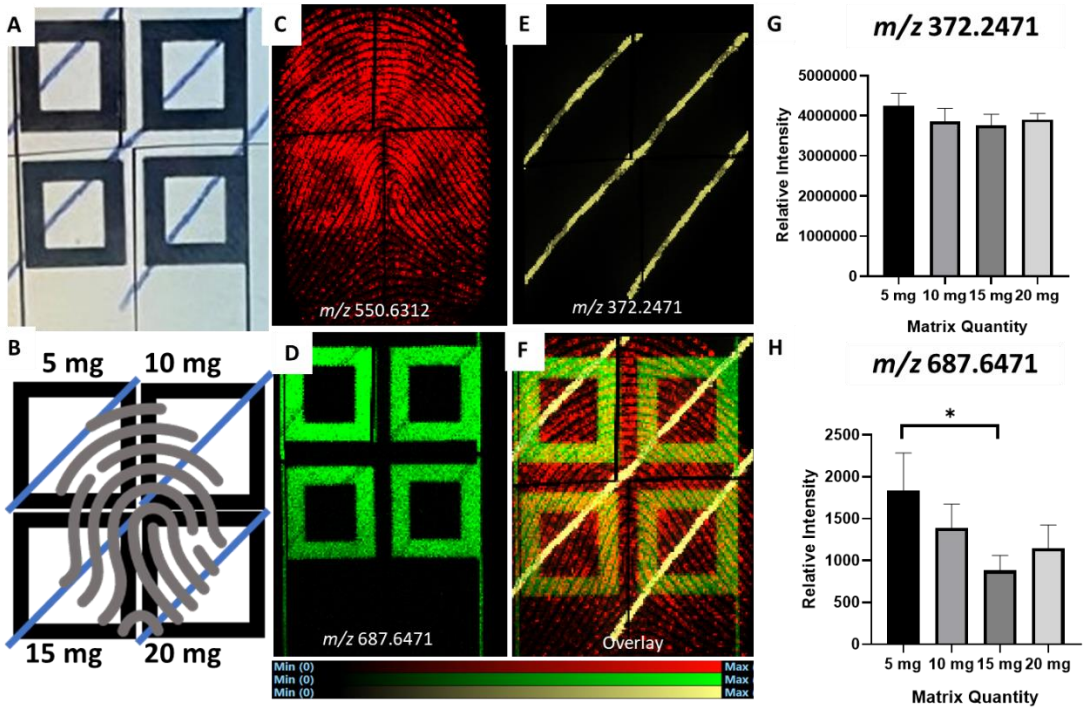

Figure 1. A: Optical image of a sample containing four identical printed ink squares, each containing a single blue ballpoint pen stroke and a natural fingerprint which covered all four

regions B: schematic representation of the experimental set up: Each quadrant was prepared with varying α-CHCA matrix quantity (5- 20 mg). Individual molecular images showing C: a fingerprint derived ion at m/z 550.6312, D: printed ink ion at m/z 687.6471 and E: Ball point pen ink ion at m/z 372.2471. F: Overlay of molecular images for representative ions within printed ink, ballpoint pen ink and fingerprint. Statistical analysis of intensity variations between ions representative of G: ballpoint pen ink (m/z 372.2471) and H: a printed ink constituent (m/z 687.6471) across different matrix quantities.

Matrix Quantity - Individual molecular images (Fig. 1C-E) are shown for dimethyldioctadecylammonium ion ($C_{38}H_{80}N$) at *m/z* 550.6312 (4.90 ppm) for the fingerprint (red), *m/z* 687.6471 (unidentified) for the printed ink (green), and triarylmethane compound $(C_{25}H_{30}N_3)^+$ at *m/z* 372.2471 (-9.94 ppm) for the ballpoint pen ink (yellow). An overlay of the same ion images is shown in Fig. 1F. Statistical analysis showed that varying the amount of α-CHCA (5-20 mg) had no significant effect on detection of ions within ballpoint pens (Fig. 1G). However, statistical analysis of printed ink ion (*m/z* 687.6471) within the fingerprint shows increased signal intensity within the quadrant prepared using 5 mg of matrix (Fig. 1H).

To further validate the matrix-quantity optimisation, an additional experiment was performed (Fig. S2) on a sample containing fingerprint only, thereby eliminating any signal overlap from ballpoint pen or printed ink which could potentially cause ion enhancement/suppression of fingerprint constituents. A fingerprint was deposited and analysed to assess ridge clarity and ion signal distribution at different matrix quantities. The expected matrix peak at m/z 212.2354 is shown alongside common fingerprint ions at m/z 230.2472, 258.2786, 283.2634 oleic acid $(C_{18}H_{35}O_2)^+$ (-1.05 ppm), 311.2949, and 326.3780 didecyl dimethyl ammonium bromide fragment $(C_{22}H_{48}N)^+$ (-2.14 ppm) [28,29]. While some ions exhibit a slight decrease in intensity at 5 mg compared to 10 mg, some other common fingerprint ions (m/z 283.2634, 311.2949, and 326.3780) show no difference between the two matrix quantities. Ridge clarity remains

consistent between all matrix quantities. Considering these findings, and as 10 mg performed well for both ballpoint pens and printed inks, this was determined to be the optimal matrix amount.

In a subsequent experiment, sublimation time was investigated, which included 2 min (top left), 3 min (top right), 4 min (bottom left) and 5 min (bottom right) prior to MALDI MSI (Fig. 2), whilst using the previously optimised condition of matrix quantity (10 mg).

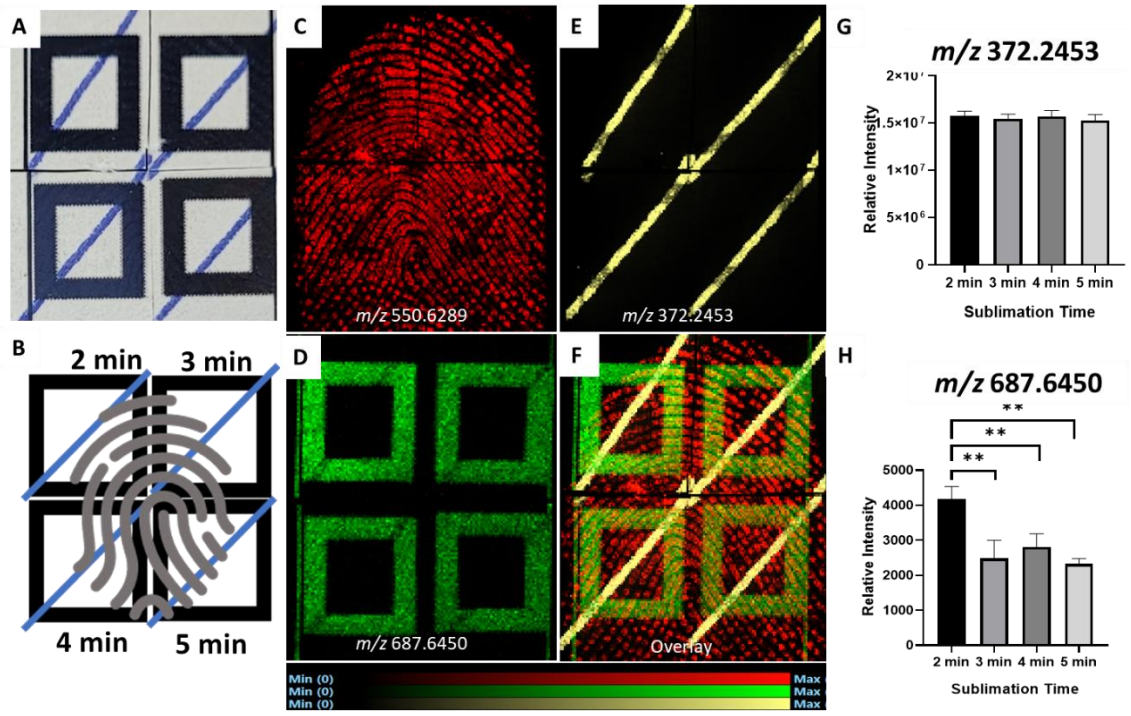

Figure 2. A: Optical image of the sample containing four identical printed ink squares, each with a single blue ballpoint pen stroke and a natural fingerprint covering all four regions. B: schematic representation of the experimental set up: Each quadrant was prepared with varying sublimation duration (2-5 minutes). Individual molecular images generated for C: A fingerprint derived ion at m/z 550.6289, D: printed ink derived ion at m/z 687.6450, and E: Ball point pen ink derived ion at m/z 372.2453. F: Overlay of molecular images for representative ions within printed ink, ballpoint pen ink and fingerprint. Statistical analysis of intensity variations between ions representative of G: ballpoint pen ink (m/z 372.2453) and H: printed ink (m/z 687.6450).

Sublimation time - The distribution of dimethyldioctadecylammonium ion ($C_{38}H_{80}N$) at *m/z* 550.6289 (0.726 ppm) corresponding to the fingerprint (red), *m/z* 687.6450 localised within printed ink (green), and *m/z* 372.2453 corresponding to $(C_{25}H_{30}N_3)^+$ (5.10 ppm) in ballpoint pen ink (yellow) are displayed in Fig. 2 C-E. The overlay of the same ion images is also shown in Fig. 2F. The shorter sublimation time of 2 minutes provided ion images with higher intensity for the printed ink, which is supported by statistical analysis (Fig. 2H), and for the fingerprint. Statistical analysis showed that sublimation time had no significant effect on detection of ions within ballpoint pens (Fig. 2G). This indicates that sublimation of 10 mg α-CHCA at 300°C happens rapidly, therefore requiring a smaller time frame. Indeed, to avoid potential thermal degradation of molecules within the sample, shorter sublimation times are more favourable. Further studies are necessary to establish whether the sublimation time could be reduced further.

The final modification to sublimation optimisation considered the impact of different recrystallisation solvents following sublimation with the parameters optimised in the initial experiments. The different solvents include isopropanol (IPA) (top left), acetone (top right), ethanol (EtOH) (bottom left) and no recrystallisation (N/A) (bottom right) (Fig. 3B).

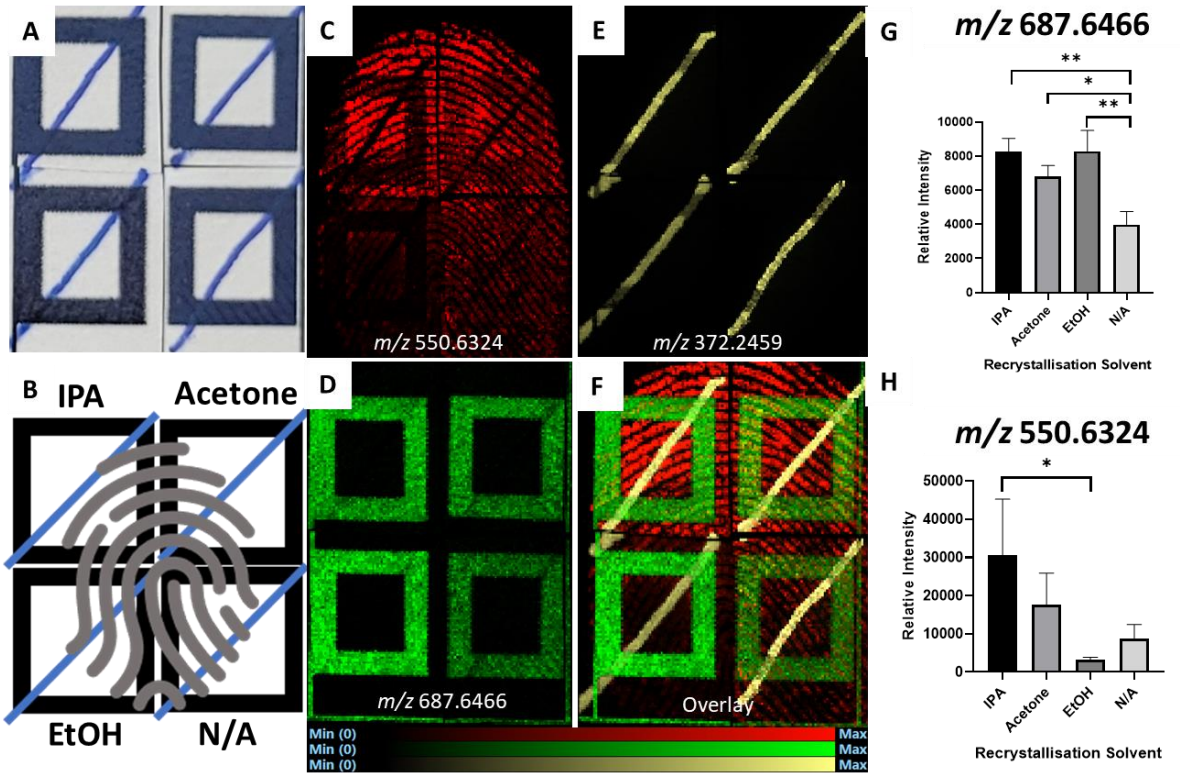

Figure 3. A: Optical image of the sample containing four identical printed ink squares, each with a single blue ballpoint pen stroke and a natural fingerprint covering all four regions. B: schematic representation of the experimental set up: Each quadrant was treated with a different recrystallisation solvent (IPA, acetone, EtOH) or no recrystallisation (N/A). Individual molecular images for each analyte showing C: A fingerprint derived ion (m/z 550.6324), D: Printed ink ion (m/z 687.6466) and E: ballpoint pen ink ion (m/z 372.2459). F: Overlay of molecular images for representative ions within printed ink, ballpoint pen ink and fingerprint. Statistical analysis of signal intensity variations between ions corresponding to G: printed ink (m/z 687.6466) and H: fingerprint (m/z 550.6324).

Recrystallisation - The molecular images of dimethyldioctadecylammonium ion ($C_{38}H_{80}N$) at *m/z* 550.6324 (7.08 ppm) within the fingerprint (red), *m/z* 687.6466 present in printed ink (green) and the triarylmethane compound $(C_{25}H_{30}N_3)^+$ at *m/z* 372.2459 (6.72 ppm) within ballpoint pen ink (yellow) are displayed in Fig. 3 C-E. An ion overlay image is displayed in Fig. 3F. These results showed that IPA provided greater ion intensity for the fingerprint (Fig. 3C) and ballpoint pen ink (Fig. 3E), while EtOH facilitated the detection of printed ink ions (Fig. 3D). These findings are corroborated by the statistical analysis shown in Fig. 3G. The ion corresponding to the printed ink component at *m/z* 687.6466 showed a significant increase in signal intensity for all recrystallisation solvents compared to the non-recrystallised condition. Similar findings were observed for fingerprint ions. For example, Fig. 3H, *m/z* 550.6324 shows significant increase of signal intensity when using IPA in comparison to EtOH. Therefore, IPA was selected as the optimal recrystallisation procedure. The varying results in ion intensity and image clarity as an effect of recrystallisation solvent could be due to several factors such as 1) solubility/extraction of analytes, 2) volatility of the solvent, and 3) possible side reactions.[30]

In summary, the comprehensively optimised sublimation protocol for QDE and molecular fingerprinting using MALDI MSI was established as 10 mg of α-CHCA dissolved in 1.5 mL of acetone, preheated at 185 °C for 1 minute, followed by sublimation at 300 °C for 2 minutes, and recrystallised with IPA for 1 minute at 60 °C (Table 1).

Table 1. Summary of optimised α-CHCA matrix sublimation parameters for QD samples containing fingerprints

HTX SubliMATE Parameter	Optimised Condition
Matrix	α-CHCA
Preheating Temperature	185°C
Sublimation Temperature	300 °C
Matrix Amount	10 mg
Sublimation Time	2 minutes
Recrystallisation Solvent	Isopropanol (IPA)

3.2 Sublimation vs spraying

The optimised sublimation protocol for QDE was tested against two established matrix spraying techniques for subsequent MALDI MSI analysis (Fig. 4). The top left quadrant was prepared by spray coating the sample with 5 mg/mL α-CHCA in 70:30 MeOH:0.1% TFA (aq) at a nozzle temperature of 60 °C, conditions previously developed for ballpoint pen analysis.[15] The bottom left quadrant was prepared by spraying 5 mg/mL α-CHCA in 70:30 ACN:0.1% TFA (aq) at 75 °C, previously shown successful for fingerprint analysis.[26] Both quadrants on the right were prepared under the optimised sublimation conditions.

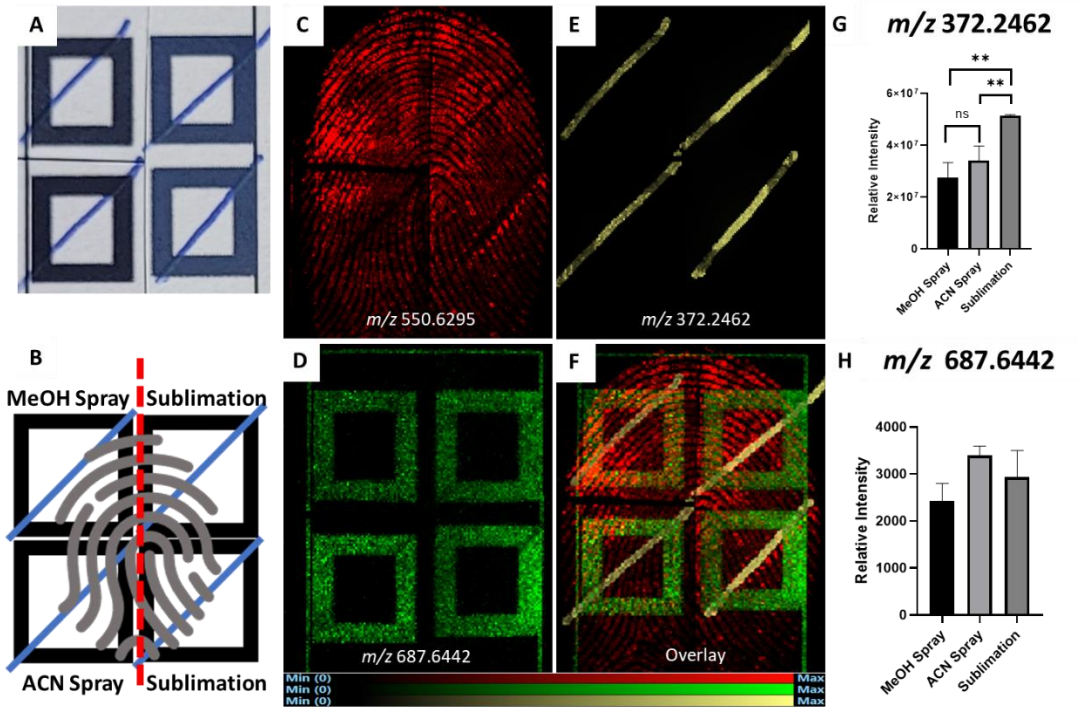

Figure 4. A: Optical image of the sample. B: schematic representation of the experimental set up: The top left quadrant was prepared by spraying 5 mg/mL α-CHCA in 70:30 MeOH:0.1% TFA (aq) at a nozzle temperature of 60 °C, the bottom left was sprayed with 5 mg/mL α-CHCA in 70:30 ACN:0.1% TFA (aq) at 75 °C. Both quadrants on the right were prepared using the optimal sublimation conditions. Individual molecular images generated for C: a fingerprint derived ion at m/z 550.6295, D: a printed ink ion at m/z 687.6442 and E: a ball point pen ink ion at m/z 372.2462. F: Overlay of molecular images for representative ions within printed ink, ballpoint pen ink and fingerprint. Statistical analysis of intensity variations between ions representative of G: ballpoint pen (m/z 372.2462) and H: printed ink (m/z 687.6442).

Molecular images are shown for the dimethyldioctadecylammonium ion ($C_{38}H_{80}N$) at *m/z* 550.6295 (1.82 ppm) within the fingerprint (red), *m/z* 687.6442 in printed ink (green), and triarylmethane compound $(C_{25}H_{30}N_3)^+$ at *m/z* 372.2462 (7.52 ppm) within ballpoint pen ink (yellow) (Fig. 4 C-E). An overlay of those ions is represented in Fig. 4F. The signal intensity for ions derived from ballpoint pen ink, printed ink and the fingerprint is higher in the samples prepared by sublimation than in the cases where matrix was spray coated onto the samples. For

346 the statistical analysis, ROIs were extracted from the two sprayed regions and the sublimated
347 region for the printed and ball point pen inks. Indeed, statistical analysis of the triarylmethane
348 compound, commonly found in ballpoint pen inks, revealed a significant increase in signal
349 intensity when prepared by sublimation in comparison to both spraying techniques, while no
350 significant difference was observed between the two sprayed quadrants (Fig. 4G). For the
351 printed ink ion at *m/z* 687.6442, a similar intensity was observed across all sample preparation
352 conditions (Fig. 4H).

353 However, to rule out the possibility of the printed inks, and/or ball point pen ink affecting
354 ionisation of fingerprint constituents, a separate fingerprint (without printed or ball point pen
355 ink deposition) was performed, comparing the two spraying methods with the optimised
356 sublimation method (Fig. S3). The fingerprint ions at m/z 283.2646 oleic acid $(C_{18}H_{35}O_2)^+$
357 (3.17 ppm), m/z 311.961, m/z 326.3792 didecyl dimethyl ammonium bromide fragment
358 $(C_{22}H_{48}N)^+$ (1.53 ppm), and m/z 550.6317 dimethyldioctadecylammonium ion ($C_{38}H_{80}N$) (5.81
359 ppm) (Fig. S3 C-F) were investigated.[27,28] The results highlight that the fingerprint ion intensity
360 is much higher for sublimation compared to both spraying methods, which was confirmed
361 statistically in Fig. S3 G and H for *m/z* 283.2646 and *m/z* 311.2961, respectively.

362 The efficiency of the developed sublimation protocol was tested on 4 different fingerprint
363 types, namely: groomed, ungroomed, eccrine and natural (Fig. S4). The signal intensity of
364 fingerprint ions, such as those at *m/z* 284.3334 and *m/z* 550.6343, (Fig. S4 C and D) reflected
365 expected sebaceous abundance, with higher signal intensities in groomed and natural
366 fingerprints compared to ungroomed and eccrine (Fig. S4 G), likely due to reduced lipid
367 content and the presence of polar or ionic eccrine components.[31,32] All fingerprint types yielded
368 grade 4 images, important for biometric identification.[33] A ballpoint pen ink ion at *m/z*
369 372.2473 (Fig. S4 E) showed uniform intensity across all four quadrants (Fig. S4 H), whereas
370 a printed ink ion at *m/z* 687.6492 (Fig. S4 F) exhibited enhanced intensity within the eccrine

371 fingerprint region. Ballpoint pen inks typically contain dyes that are readily ionised by the
372 MALDI process[34], and their ionisation seems to be unaffected by the presence of eccrine salts
373 or lipids.

374 Importantly, to address any potential difference in ion abundance due to fingerprint
375 homogeneity, some of the optimisation experiments were replicated, with a different order for
376 each of the quadrants. In all instances, the results obtained from these replicates was consistent,
377 showing that the results from each optimisation parameter is not affected by the location of the
378 quadrant (data not shown).

379

3.3 Degradation Study for Ball Point Pen Inks

To determine whether the elevated temperature of the sublimation chamber (300 °C) caused degradation of components contained within ballpoint pen ink, the ratio of the degradation product ion intensity to the corresponding parent ion was investigated. The same degradation ions have previously been explored by various groups.[35,36,37] In samples prepared using sublimation, higher degradation rations were observed. This was characterised by the degradation of *m/z* 372.2423 representing $(C_{25}H_{30}N_3)^+$ to its daughter ion at *m/z* 356.2142 as well as for *m/z* 358.2277 $(C_{24}H_{28}N_3)^+$ and its daughter ion at *m/z* 342.2013 (Figure 5). The fragments of methyl violet were identified and confirmed separately using MSMS experiments (Fig. S5).

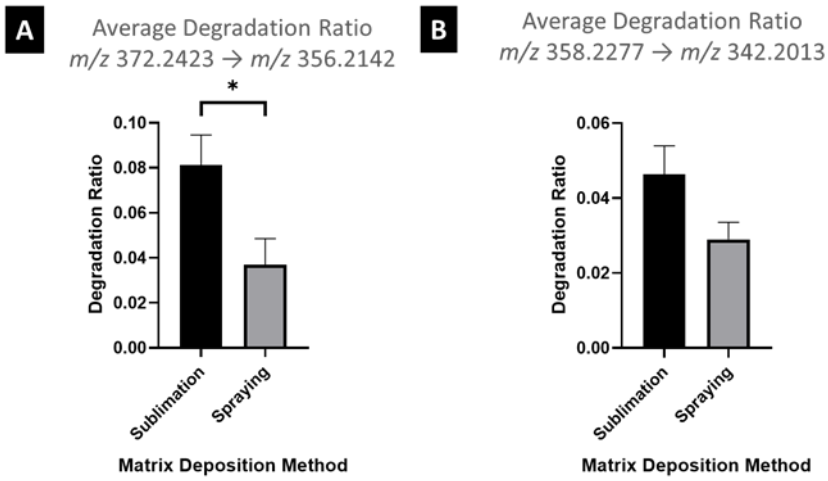

Figure 5. Comparison of analyte degradation ion ratios for sublimation vs. spraying matrix deposition in MALDI-MS. (A) shows the average degradation ratio for the analyte fragment m/z 356.2142 from the precursor ion m/z 372.2423. (B) shows the average degradation ratio for the analyte fragment m/z 342.2013 originating from the precursor m/z 358.2277. Error bars on the bar graphs represent the standard deviation of three replicate measurements.

The observed variation in analyte degradation can be attributed to the fundamental differences in how each matrix deposition method interacts with the analyte. Sublimation is a solvent-free

technique that involves heating the matrix under vacuum, causing it to deposit as a thin, uniform layer of small crystals. [25] However, the high temperatures required for sublimation may expose the analyte to thermal stress, leading to increased thermal decomposition and subsequent degradation product. This was expected as previous studies on ink ageing have demonstrated that exposures to high temperatures promote formation of dye fragments.[37] The temperature-dependent generation of degradation ions could also be due to the very close proximity of the heating wafer and the samples inside the HTX SubliMATE. This configuration causes radiative heat to affect the samples even though the samples are in vacuum and cooled from the top. Further studies could investigate the impact of different sublimation and sample cooling temperatures on analyte degradation. In contrast, the spraying method relies on a solvent to dissolve the analyte. The subsequent rapid evaporation of the solvent during spraying leads to the formation of co-crystals. The results here indicate that spraying is a "softer" process from a degradation standpoint. Even though the nozzle tip is heated to 75°C, the distance between the nozzle and the sample, the constant solvent mist being generated, and the ambient nature of the sprayer provides a cooling effect which reduces any heat induced degradation. The lower degradation ratios imply that for the specific analyte studied, the potential for degradation from the spraying process (e.g., from solvent-analyte interactions) is less pronounced than the thermal degradation experienced during sublimation. This can be crucial for future studies if trying to understand the age of a sample by monitoring analyte degradation. Additionally, degradation of these molecules has been known to be caused by factors including humidity and light, Therefore, minimising additional degradation through the appropriate matrix deposition method is imperative.[38] In such studies, spraying-based matrix deposition techniques would be the preferred approach to ensure greater reliability. Ongoing research in our department is focused on studying the effects of various environmental factors, including heat, humidity, and light, on inks.

3.4 Mock QDE Analysis

The optimised sublimation method was applied to a mock QD sample containing a "Jane Doe" signature with a forged date, written using two different ballpoint pens, as well as two fingerprints from different donors (Fig. 6 A). The sample was subjected to MALDI MSI to show the capability of the systematically optimised sublimation method mentioned in this paper. This analysis demonstrates the molecular-level distinction between the inks used in the forgery, while also providing vital biometric and chemical information from the fingerprints present on the examined site (Fig. 6 B-H).

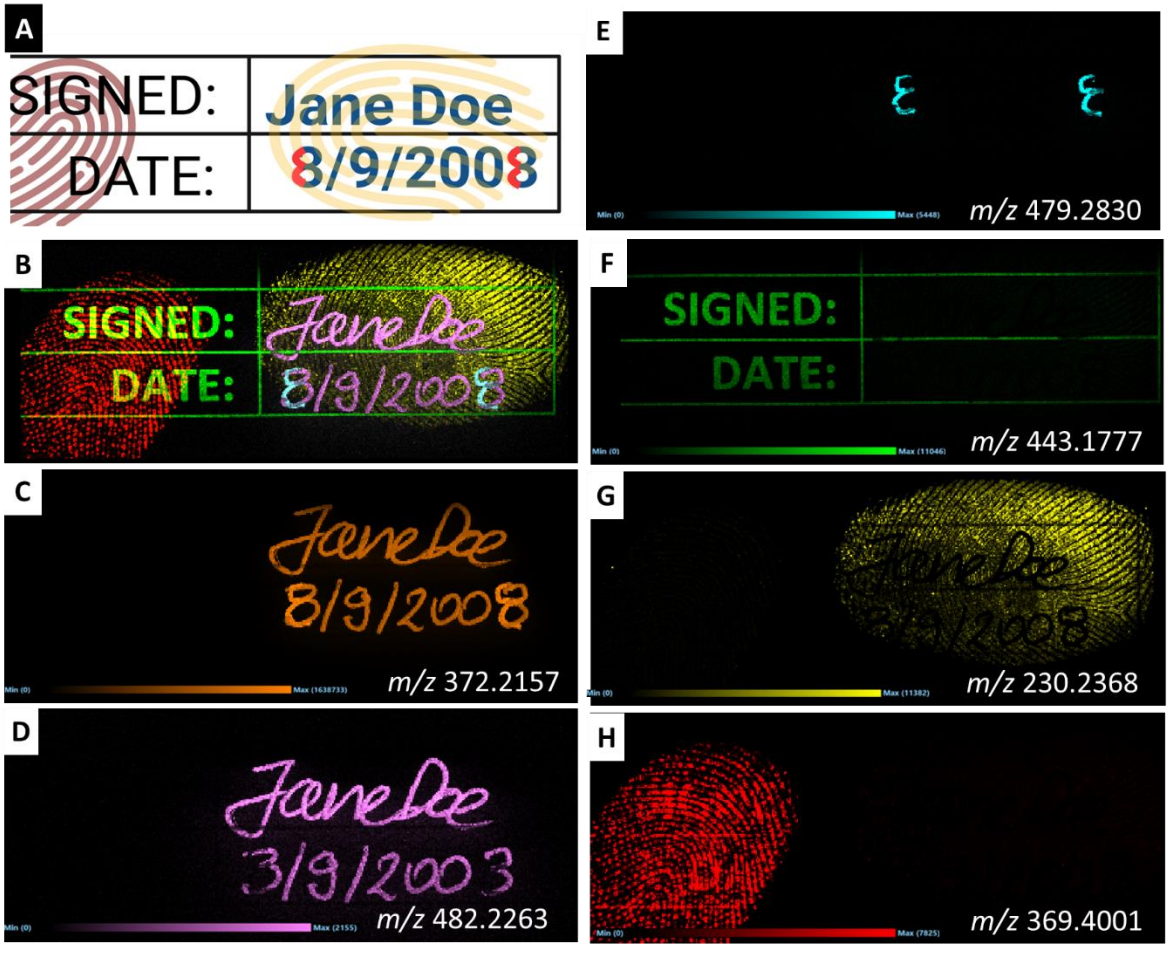

Figure 6. The Mock QD sample analysed using MALDI MSI. (A) Schematic overview of the sample (B) Overlay of the ions images at m/z 482.2263, m/z 479.2830 (ball point pen inks), m/z 230.2368, m/z 369.4001 (fingerprints), and m/z 443.1777 (printed ink). (C) Methyl violet

435 at m/z 372.2157 (D) The original signature and date deposited by the donor using m/z
436 482.2263. (E) the forged part of the date at m/z 479.2830, (F) printed ink at m/z 443.1777,
437 (G&H) two fingerprints at m/z 230.2368 and m/z 369.4001.

438 The overlay MALDI MS images of *m/z* 482.2263 and *m/z* 479.2830 (ballpoint pen inks), *m/z*
439 230.2368 and 369.4001 (fingerprints), and *m/z* 443.1777 (printed ink) effectively highlights
440 distinct ions and their characteristic spatial distributions (Fig. 6 B). Fig. 6 C shows that both
441 pens contain the methyl violet ion at *m/z* 372.2157, however the selection of unique ions to
442 each of the pens reveals modification to the handwritten text within the date (*m/z* 482.2263 and
443 *m/z* 479.2830, respectively) (Fig. 6 D and E). In Fig. 5F, the ion image at *m/z* 443.1777 shows
444 the molecular distribution of the printed ink, highlighting the capability of the optimised
445 methodology to analyse multiple ink types within a single sample. The fingerprints deposited
446 onto the document can be visualised using *m/z* 230.2368 and *m/z* 369.4001 (Fig. 6 G and H).
447 Here, it was possible to not only obtain biometric information from two separate fingerprints,
448 but also observe differences in chemical composition of these fingerprints. Signal enhancement
449 of m/z 369.4001 was observed over the printed ink region and suppression of signal of m/z
450 230.2368 over the ball point pen ink region. This observation was previously discussed in the
451 paper along with its potential underlying causes. Further investigation of the complex
452 interactions that occur between these analytes within QD samples is necessary to understand
453 the instances in which signal suppression or enhancement might be observed.

454 MALDI-MSI has been previously employed for the imaging of forgery samples, and
455 fingerprints separately.[15,26] Fingerprints have been imaged using MALDI from currency notes,
456 and even paper substrate in the past.[39,40] However, this study is the first of its kind where
457 multiple forensic evidence is assessed in specimen (ball point pen inks, printed ink and
458 fingerprints on paper) and where sublimation was used as the matrix deposition method.

Application of matrix is a crucial part of the MALDI imaging workflow, and the results from this study indicate how different matrix deposition parameters can impact the data obtained.

4. Conclusion

The forensic analysis of questioned documents is frequently complicated by the presence of multiple, overlapping evidence types, such as different inks and fingerprints, which can challenge conventional analytical methods. This study aimed to develop and validate a robust MALDI-MSI workflow to overcome these challenges by systematically optimising a novel approach to matrix deposition for these sample types. Standard sample preparation methods for forensic samples such as QDs is performed with a spray-based approach, using organic solvents to deposit the matrix. In this study, the solvent-free matrix deposition approach of sublimation was tested and optimised for analyte signal intensity, spatial information, reproducibility, and creation of any degradation products.

The results shown in this work highlight that the optimised sublimation technique provides a reproducible methodology that can offer a wealth of molecular information from printed ink, ballpoint pen ink, and fingerprint residues on a paper substrate. While sublimation avoids the presence of solvent in the matrix deposition step, short exposure to solvent vapor via recrystallisation was found to increase detection of certain analytes within these samples. In fact, the signal intensity of specific analytes was found to be higher from sublimated samples than sprayed samples, a feature that would become especially important in samples with low abundant analytes. There were some limitations observed, for example an increase in thermal degradation was observed following the high temperature used within the sublimation-based approach and should therefore be carefully considered in any future research aiming to establish the document age based on the ink molecules and its degradation products. The practical use of this method was demonstrated in a simulated forgery scenario, where the

proposed workflow not only enabled the differentiation of two visually similar inks used to alter a document but also provided high-quality molecular images of latent fingerprints. In conclusion, by optimising matrix sublimation, this research has enhanced the operational applicability of MALDI-MSI for questioned document examination. This dual capability to generate both chemical intelligences to detect forgery and provide biometric information about the handler from a single analysis establishes a new framework for analysing complex questioned documents and paves the path for future research on MALDI-MSI's role as a tool for questioned document and fingerprint analysis.

5. Author Contributions

Veronika Tibljas: Investigation, Formal analysis, Writing – original draft, Writing – review & editing. Rohith Krishna: Investigation, Formal analysis, Writing – original draft, Writing – review & editing. Simona Francese: Supervision, Writing – review & editing. Alyson Black: Writing – review & editing. James Langridge:Writing – review & editing. Emmanuelle Claude: Conceptualization, Writing – review & editing. Robert Bradshaw: Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

6. Conflicts of Interest

Rohith Krishna reports financial support was provided by Waters Corporation. James Langridge and Emmanuelle Claude are employees of Waters Corporation. The remaining authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

7. Data Availability

Data will be made available on request.

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