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A multimodal mass spectrometry imaging workflow for ballpoint pen ink analysis and "forgery" detection†

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Inks, including ballpoint, gel, fountain pen, laser toner, inkjet, and security inks, have unique chemical formulations and require specific examination methods based on their properties and the surfaces they are deposited on. Despite the increasing use of digital signatures, ballpoint pens are still used for signing legal documents. Common forensic approaches involving visual examination are crucial in forensic analysis due to their non-destructive nature. However, often their effectiveness can rely on the expertise of the forensic analyst. Other common approaches, such as thin layer chromatography (TLC), require sample extraction which is destructive. This work shows a multimodal imaging workflow, including the application (in order of increasing destructiveness) of Desorption Electrospray Ionisation Mass Spectrometry Imaging (DESI MSI) followed by Matrix Assisted Laser Desorption Ionisation Mass Spectrometry Imaging (MALDI MSI) enabling comprehensive ink and substrate analysis *in situ*. It is important to note that this work was performed in preparation for a real casework study submitted to our group for analysis. The application of DESI MSI in negative ion mode and MALDI MSI in positive ion mode in principle allows for detection and mapping of diverse chemical species (*e.g.* dyes, polymers, *etc.*), providing a comprehensive understanding of the chemical composition of the sample. Indeed, it is well documented that each ionisation technique can enable targeting of different molecular species. Statistical approaches used to interrogate the data, allows in-depth analysis of ink/substrates. This approach offers a robust and reliable methodology, which can complement or offer additional information for forgery determination in forensic cases, where the results obtained from conventional methodologies may have not been successful.

Examining inks on documents to determine their origin and authenticity is essential, when illegal and fraudulent alterations are suspected. Questioned document analysis include contracts, cheques, passports, residence, and identity documents.[1] To maintain the security of users, preserve the integrity of the legal and financial systems and to safeguard intellectual property, it is crucial to be able to identify fraudulent activities.

A variety of inks with different chemical formulations are used in writing and printing, including ballpoint pen ink, gel pen ink, fountain pen ink, laser printer toners, inkjet printing ink, and security ink. Each ink type and surface require a tailored methodology for inspection based on their chemical properties. The two most common types of pens are oil-based, which are more viscous and partially absorbed by paper, and water-based, which soak deeper into the substrate.[2] Ballpoint pens, developed by László Bíró in the 1930s, are mostly oil-based, while fountain pens, rollerball pens, and gel pens are water-based.

Analysing ballpoint pen compositions presents a challenge due to their inherent complexity. The inks consist of many chemical components, including pigments, dyes, solvents and additives.[3] The diverse formulations used by manufacturers further compounds the complexity, as each pen type and/or brand may employ unique combinations and abundance of ingredients.[4] Often, forensic analysis of inks involves optical

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† Electronic supplementary information (ESI) available: Table S1 – DESI and MALDI parameters employed in the multimodal workflow. Fig. S1 – Schematic representations of Sample set 1, Sample set 2 and Sample set 3. Fig. S2 – Example of data extraction for statistical analysis of Sample set 1. Fig. S3 – Ions of interest generated by PLS-DA analysis of the word "forgery" in 7 different pens following DESI MSI analysis with the corresponding MSI images. Fig. S4 – MS/MS of oleic acid, a common ink lubricant. Table S2 – Theoretical and observed ions with calculated mass accuracy. Fig. S5 – MS/MS analysis of the triarylmethane compounds and their expected fragmentation patterns. Fig. S6 – Statistical analysis of intensity variations between ions in fresh *vs.* aged inks observed with DESI (A and B) and MALDI MS (C and D). See DOI: **https://doi.org/10.1039/d5an00217f**

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examination including microscopy frequently coupled with spectroscopic methodologies such as infrared (IR), ultraviolet (UV) and Raman. Visual examinations remain important in forensic analysis due to their non-destructive nature. However, these methodologies rely on the experience of a forensic analyst,[5,6] highlighting the necessity for development of analytical approaches to determine chemical composition of samples to support forensic investigations. To date, a range of advanced analytical approaches have been suggested in the literature, which include Secondary Ion Mass Spectrometry (SIMS),[7–12] Direct Analysis in Real Time (DART),[13–15] Capillary Electrophoresis (CE),[16] Ion Mobility Spectrometry (IMS),[17–19] Liquid Chromatography (LC)[20–22] and Gas Chromatography Mass Spectrometry (GC-MS).[23–25]

Over recent years, Matrix Assisted Laser Desorption Ionisation mass spectrometry (MALDI MS) has increasingly demonstrated its robustness as an analytical tool, within various forensic applications such as analysis of hair,[26] blood[27–29] and fingermarks.[30–33] Interestingly, the work by Scotcher *et al.* (2018)[34] on latent fingermarks deposited on polymer banknotes allowed molecular images to be obtained not only from the fingermarks, but also the surface chemistry of the banknote. Numerous publications report on the use of MALDI MS for ink analysis and various matrices including 3-nitrobenzonitrile (3-NBN),[35] 1,2-dicyanobenzene(1,2-DCB),[35] 1,5-diaminonapthalene (DAN),[36] 2,5-dihydroxybenzoic acid (DHB),[36] silver nanoparticles,[36] α-cyano-4-hydroxycinnamic acid (α-CHCA),[37] and 2-(4-hydroxyphenyl-azo) benzoic acid (HABA),[38] have undergone testing within ink/substrate applications, including a European collaborative exercise[39] in which different printing techniques and types of paper were examined. Here, it was found that the homogeneity of MALDI matrix deposition was inconsistent on different types of paper. Furthermore, the lack of a MS ink database and statistical processing reduced the techniques effectiveness. Chen *et al.* (2005) successfully identified and characterised various ballpoint pen inks extracted from paper.[40] Extraction of inks prior to thin layer chromatography (TLC) is destructive to the sample and impractical in some forensic cases where sample integrity is essential. In 2007, Dunn *et al.* applied MALDI MS to pen ink, (red rollerball) directly on paper, enabling detection of multiply charged dyes, which was not possible using laser desorption ionisation (LDI).[38] Weyermann *et al.* (2006) employed LDI and MALDI MS to study the degradation of dyes and the influence of environmental factors.[41] The degradation of methyl violet and ethyl violet dyes in ballpoint pen strokes was studied under laboratory conditions influenced by light, wavelength, heat, and humidity, and compared to a one-year natural ageing analysis in the dark, and under light exposure. Degradation of the analysed dyes was strongly dependent on exposure to light and humidity, however the effect of heat was not as influential. Huynh *et al.* (2016) explored MALDI-MS coupled to direct analyte–probe nanoextraction (DAPNe) for characterisation of dyes from both pens and inkjet printer cartridges and reported DHB as the most proficient matrix.[36] Most recently, a method has been developed incorporating MALDI-MSI and machine learning (ML) for ink classification using α-CHCA as matrix.[42] Ultimately, it is evident that there is no single, robust methodology in place for the analysis of inks by MALDI MS.

Desorption Electrospray Ionisation mass spectrometry imaging (DESI-MS) is less destructive than MALDI MS, as sampling is performed using a charged solvent and it does not require sample pre-treatment. Therefore, it has significant potential to assist in forensic analysis of questioned documents, whereby forensic integrity may need to be maintained. Khatami *et al.* (2017) used DESI MSI and paper spray ionisation for examining thermochromic erasable ink pens.[43] They found that specific chemical components were only present in either the invisible and reappeared state of the erasable pen ink and could confirm the act of misconduct in documents. Lee *et al.* (2021) used nano-DESI MS for analysis of ink on paper and detected polyanionic dyes with minimal fragmentation. They used these ions to differentiate between inks in a simulated forgery experiment.[44] Most recently, Sun *et al.* (2022) investigated document ageing by examining Crystal Violet and related dyes in ballpoint pen inks and polyethylene glycol (PEG) derivatives in gel ink.[45] Throughout these various studies, different DESI solvent composition have been utilised, including 100% methanol,[43] ethanol and water (1 : 1, v/v)[44] and methanol–water–formic acid (90 : 10 : 0.1%, v/v/v).[45] This highlights that successful application of DESI-MS is dependent on the solvent composition which impacts the type of molecules that are desorbed and ionised. Importantly, incorrect choice of solvent could result in analyte delocalisation during data acquisition which ultimately affects the integrity of the sample, therefore necessitating thorough optimisation.

In the work presented here, a multimodal approach has been explored on inks deposited onto paper. Analysis was performed in order of increasing destructiveness (DESI MSI followed by MALDI MSI) to maximise the intelligence recovered from the sample. DESI MSI was employed in negative ion mode and MALDI MSI in positive ion mode, enabling detection of diverse chemical species and comprehensive understanding of the sample's chemical composition. This workflow was established following development of each ionisation methodology on these sample types. Analysis was conducted on state-of-the-art multi reflecting time-of-flight (MRT) instruments which are capable of mass resolution ≥200 000 FWHM, ppb mass accuracy and lateral resolution of 15 µm without oversampling.[46] To the best of the authors knowledge, this is the first instance of a multimodal imaging analytical approach being applied to ink/substrate analysis on the same sample.

Experimental

Chemicals

Trifluoroacetic acid (TFA) and α-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Sigma-Aldrich (Poole, UK). Formic acid and HPLC grade acetone, methanol (MeOH) and acetonitrile (ACN) were obtained from Fisher Scientific

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(Loughborough UK). Double-sided conductive carbon tape was purchased from TAAB (Aldermaston, UK). MiliQ water was obtained from the inhouse system. Seven different blue ballpoint pens (various manufacturers) and 2 brands of white A4 office paper were obtained from different sources.

Instrumentation

MALDI-MS and DESI-MS analyses in both profiling and imaging mode were conducted on a Waters Corporation SELECT SERIES Multi Reflecting Time-of-Flight (MRT) instrument (Wilmslow, UK). Both instruments were run with 0.2 s scan time, 50 µm pixel size and raster rate of 250 µm s^{-1}. Continuous Lockmass Correction (CLMC) was implemented during the acquisition for both instruments during analysis. For MALDI-MS in positive ion mode, CLMC was applied on the matrix peak at *m/z* 212.0324 and for DESI-MS in negative ion mode, CLMC was applied on Leu–enkephalin peak at *m/z* 554.2615. Complete parameters for both DESI and MALDI methods can be found in the ESI (Table S1†). Matrix application was performed using a HTX M3+ Sprayer™ (HTX Imaging, North Carolina, USA) (Table S1†).

The quad profile was set to Automatic for data acquisition in a full mass range (0–2400 *m/z*). The data acquisition and processing were conducted using MassLynx version 4.2, HDI version 1.7, (Waters Corporation, Wilmslow, UK). Images were normalised by the total ion count (TIC) and contrast and intensity were set to maximise quality of each image. Mean prediction error for the DESI-MS and MALDI-MS analysis following calibration for both instruments were <0.2 ppm.

Tandem MS was performed using either the Waters Corporation SELECT SERIES Cyclic operated using Electrospray ionisation (ESI) in negative ion mode (Wilmslow, UK) or the SELECT SERIES MALDI MRT in positive ion mode. The collision energy was increased (4–50 V) until optimal fragmentation of each analyte was achieved.

Sample preparation

Sample set 1 was prepared by writing numbers with two different blue ballpoint pens on white A4 office paper (Paper 1). The pen that was used to write the number “1000” is referred to as pen 1 and the pen used to modify the number to “1800” is pen 2.

Sample set 2 consisted of seven different blue ballpoint pen inks, each used to write a different letter to spell out the word “forgery”, onto white A4 office paper (Paper 1).

Sample set 3 was prepared with fresh and aged ink. The “fresh” experiment used the same blue ballpoint pen to sign the name “Leo” onto two different brands of white papers. The two signatures were deposited simultaneously, and analysis was performed immediately after ink deposition. In the “aged” experiment, a week-old signature was deposited on the top (Paper 1) and a fresh signature on the bottom (Paper 2) using the same pen. Schematic representation of the samples is shown (Fig. S1†).

Prior to MALDI-MSI analysis, 5 mg ml^{-1} of α-CHCA matrix in 70 : 30 MeOH : 0.5%TFA (aq) was spray-coated the using HTX M3+ Sprayer™ (HTX Imaging, North Carolina, USA) using a solvent flow rate of 100 µL min^{-1} and a N_2 pressure of 8 psi, for 8 layers, at a velocity of 1300 mm min^{-1}.

In all three experiments the same sample underwent initial DESI MSI analysis in negative ion mode, followed by MALDI MSI in positive ion mode. The samples were cut to size and secured to a single glass slide using double sided-carbon tape prior to analysis. No further sample preparation was required for DESI MSI analysis.

Statistical analysis

Mass spectra from regions of interest (ROI) were exported for each ballpoint pen and the paper substrate using the (MVA) tool in HDI 1.7. Subsequently, the data was imported into MetaboAnalyst 6.0 for further analysis. One-factor statistical analysis was conducted, with Pareto scaling applied to all datasets. Principal component analysis (PCA) and partial least squares – discriminant analysis (PLS-DA) was applied to evaluate the underlying patterns in the data. The process for spectral extraction is presented in Fig. S2.† Statistical analysis of ion intensity variations was performed using Prism software. A Gaussian normality test followed by parametric *T*-test (unpaired) were conducted on five regions of interest (ROI) from fresh ink samples and compared to aged ink samples, for both MALDI and DESI MSI data.

Results

Sample set 1 – DESI MSI analysis

To test the applicability of the method, a text alteration experiment was conducted on 2 blue ballpoint pens deposited onto white paper. Pen 1 was used to write the number “1000” and pen 2 was used to alter this number to “1800”. Ifa *et al.* (2007) previously employed DESI for imaging different ink, showing success in observing characteristic peaks for different ink dyes.[47] DESI MSI, in negative ion mode, was first employed as a part of a multimodal workflow. The collected data was subjected to statistical analysis. The PCA scores plot (Fig. 1A) showed clear separation between the extracted mass spectra of paper, pen 1 and pen 2. Furthermore, the PCA loadings plot (Fig. 1B) highlighted ions that contributed to the separation of the datasets. The spatial distribution of a selection of the outlying ions are shown within the DESI-MS images, which shows ions that were present in the paper only (*m/z* 260.8752), both inks (*m/z* 521.4025) and ions unique to pen 2 (*m/z* 457.4420 and *m/z* 468.4330) and pen 1 (*m/z* 251.0053 and *m/z* 248.9896) (Fig. 1C–H).

Sample set 1 – MALDI MSI analysis

Following DESI MSI analysis in negative ion mode, the same sample was sprayed with the MALDI matrix and analysed in positive ion mode using MALDI MSI. Similarly to the data obtained following DESI MSI, the two ballpoint pens and paper could be differentiated within the PCA scores plot (Fig. 2A) and the PCA loadings plot highlighted ions representative of each group (Fig. 2B). The spatial distribution of specific ions was used to visualise the paper substrate (*m/z*

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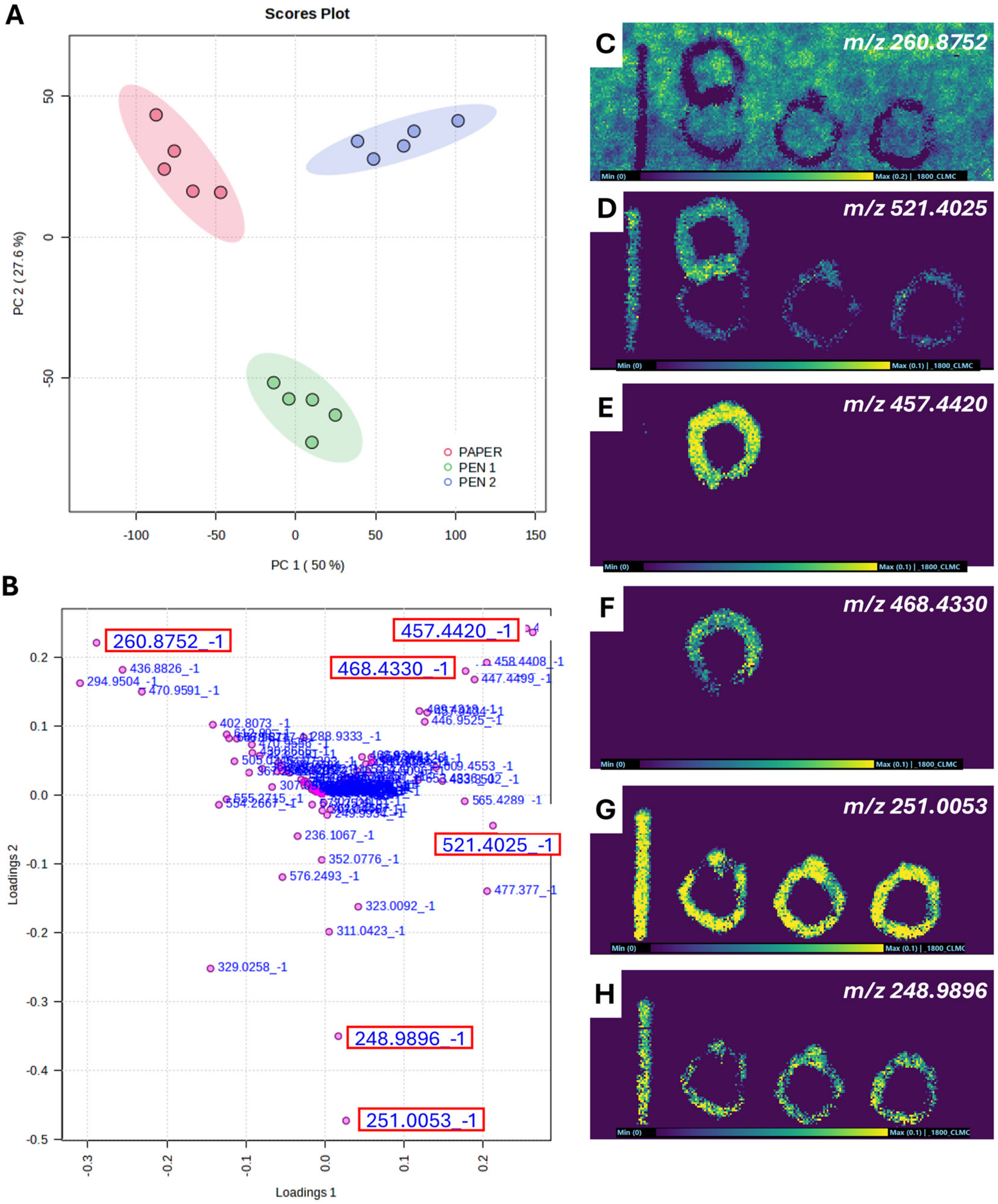

Fig. 1 (A) PCA scores plot displaying separation between the ROIs extracted from the paper, pen 1 and pen 2, (B) loadings plot with highlighted ions that allowed differentiation between the different groups, and DESI MS images of (C) the paper substrate (*m/z* 260.8752), (D) an ion in both pens (*m/z* 521.4025), (E and F) pen 2 (*m/z* 457.4420 and *m/z* 468.4430) and (G and H) pen 1 (*m/z* 251.0053 and *m/z* 248.9896).

650.0475), both pens (*m/z* 559.2719 and *m/z* 470.2603), pen 2 (*m/z* 316.1817) and pen 1 and (*m/z* 232.1702) (Fig. 2C–G). As an example of the suitability of MS/MS analysis for identification purposes, *m/z* 470.2603 was identified as Basic Blue 26 (mass accuracy of 2.616 ppm) through the observed fragmentation pattern (Fig. 2H).

Sample set 2 – DESI MSI analysis

To further test the ability of this method to differentiate between various ballpoint pens, negative ion DESI MSI was applied to seven different ballpoint pen inks deposited onto paper, spelling the word “forgery”. Unique ions were observed for each pen ink in the molecular images (Fig. 3A–H), and the generated PLS-DA 3D scores plot (Fig. 3I) enabled differentiation of each of the 7 different ballpoint pens. The letters “f”, “g”, and “e” revealed most similarity in their chemical compositions.

To interrogate the chemical composition of each of the pen inks further, an important feature list (IFL) was generated through PLS-DA (data not shown). It was revealed that many of the ions were shared between 2 or more different pen inks,

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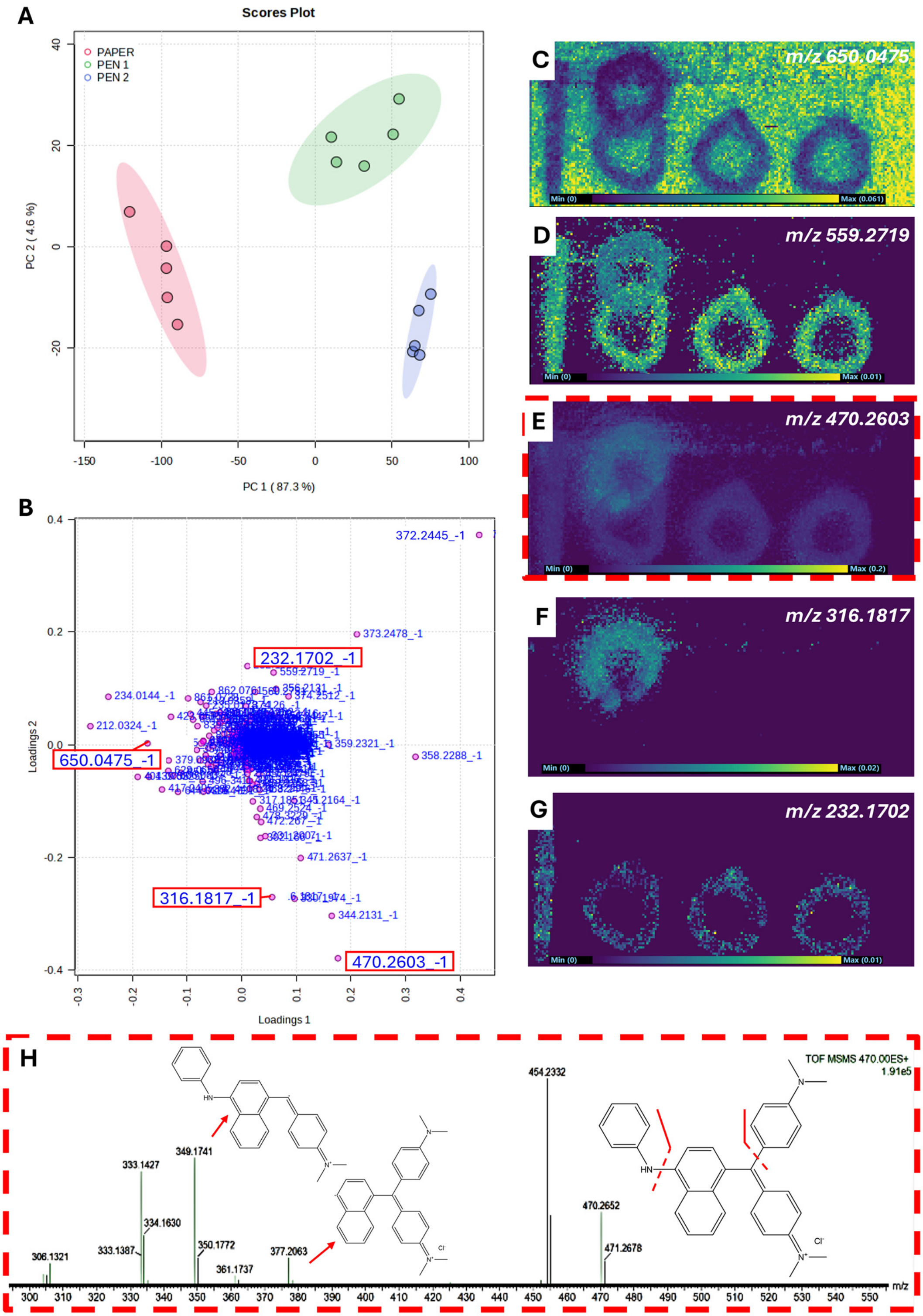

Fig. 2 (A) PCA scores plot displaying separation between the ROIs extracted from paper, pen 1 and pen 2, (B) loadings plot with highlighted ions that allowed differentiation between the different groups, and MALDI MSI images of (C) the paper substrate (*m/z* 650.0475), (D and E) both pens (*m/z* 559.2716 and *m/z* 470.2603), (F) pen 2 (*m/z* 316.1817) and (G) pen 1 (*m/z* 232.1702). (H) A representative example of tandem mass spectrometry analysis of Basic Blue 26 at *m/z* 470.2652.

though the intensity of these ions varies between the different letters. An example of this can be seen for linoleic acid (*m/z* 279.2402) which was identified through the expected product ions at *m/z* 267.0862, *m/z* 261.1416, *m/z* 243.1323, and *m/z* 235.1803 following tandem mass spectrometry analysis (ESI Fig. S3†).

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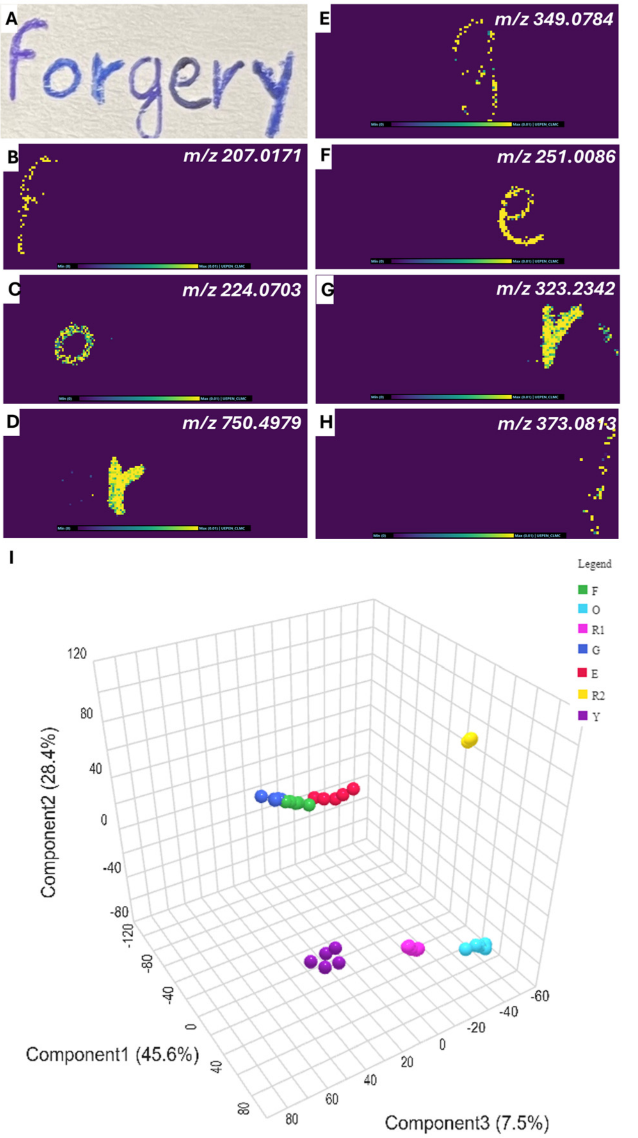

Fig. 3 DESI MSI and statistical analysis of imaging data from seven different blue ballpoint pens used to spell the word "forgery". (A) Optical image of the analysed sample, DESI MS images of (B) *m/z* 207.0171 in letter "f", (C) *m/z* 224.0703 in letter "o", (D) *m/z* 750.4979 in r1, (E) *m/z* 349.0784 in letter "g", (F) *m/z* 251.0086 in letter "e", (G) *m/z* 323.2342 in r2 and (H) *m/z* 373.0813 in letter "y", (I) PLS-DA 3D scores plot of the extracted data.

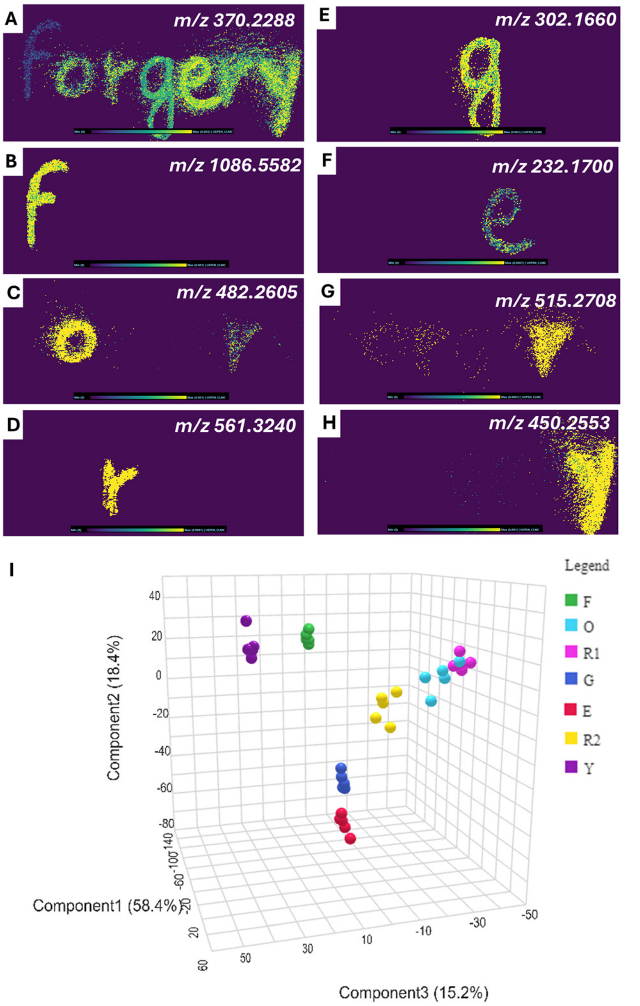

Fig. 4 MALDI MSI and statistical analysis of seven different blue ballpoint pens used to write the word "forgery", including (A) *m/z* 370.2288 present in all 7 pens, visualising the word "forgery", and (B–H) *m/z* 1086.5582, *m/z* 482.2605, *m/z* 561.3240, *m/z* 302.1660, *m/z* 232.1700, *m/z* 515.2708 and *m/z* 450.2553 (I) PLS-DA 3D scores plot of the extracted data.

Sample set 2 – MALDI MSI analysis

Following DESI-MSI analysis, the sample was subjected to MALDI analysis in positive ion mode. Multiple ions were observed that were shared amongst all 7 pens, an example shown at *m/z* 370.2288 (Fig. 4A). Unique ions were also observed in individual letters, for example, *m/z* 1086.5582 for letter "f", *m/z* 561.3240 for letter "r1", *m/z* 302.1660 for letter "g", *m/z* 232.1700 for letter "e" and *m/z* 450.2553 for letter "y". When selecting ions contained within the letters "o" and "r2", they were also observed within other letters, albeit at a lower intensity (Fig. 4B–H). Specifically, for letter "o", *m/z* 482.2605 could also be observed within "r2". Similarly, *m/z* 515.2708 within "r2" displayed a relatively weaker intensity within some of the other letters.

Sample set 3 – DESI MSI paper analysis

A blue ballpoint pen was deposited on two different brands of white paper, indistinguishable through visual examination, prior to the application of the multimodal workflow. Some ions were shared between paper 1 and 2 (*e.g.* *m/z* 329.0311) (Fig. 5B), though differentiating ions could be observed within paper 1 (*m/z* 367.2935) (Fig. 5C) and paper 2 (*m/z* 675.0354) (Fig. 5D). A scores plot generated following PCA analysis confirmed that the same pen was deposited onto the 2 different paper brands of paper (Fig. 5E). Mass spectra extracted from paper 1 and paper 2 are shown in Fig. 5F, whereby *m/z* 367.2935 can only be observed in paper 1 and *m/z* 675.0354 in paper 2.

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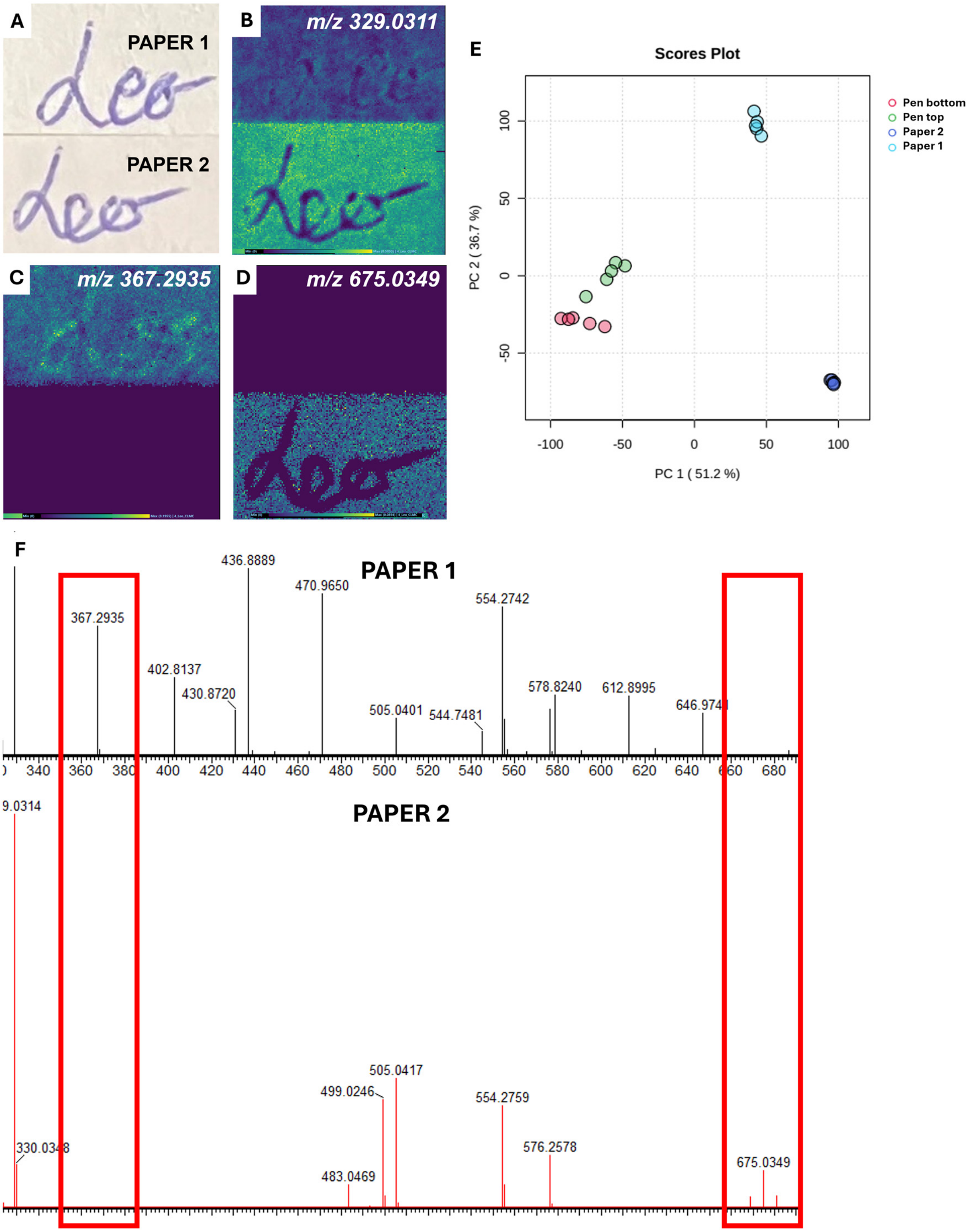

Fig. 5 DESI MSI and statistical analysis of the same blue ballpoint pen deposited onto two different brands of white paper, including (A) an optical image of the analysed samples, (B) an ion present in both paper 1 and paper 2 (*m*/*z* 329.0311), (C) a ion in paper 1 only (*m*/*z* 367.2935), (D) an ion in paper 2 only (*m*/*z* 675.0354), (E) a PCA scores plot of the extracted data, (F) extracted mass spectra showing unique ions for paper 1 (*m*/*z* 367.2935) and paper 2 (*m*/*z* 675.0354).

Sample set 3 – MALDI MSI paper analysis

Following DESI MSI, the same sample was analysed using MALDI MSI. Similar outcomes to the DESI MSI experiment were observed, whereby some ions were shared by the two different papers (*e.g.* *m*/*z* 855.0551) (Fig. 6B) and some ions were unique to paper 1 (*e.g.* *m*/*z* 862.0762) (Fig. 6C). Interestingly, there were no unique ions within paper 2,

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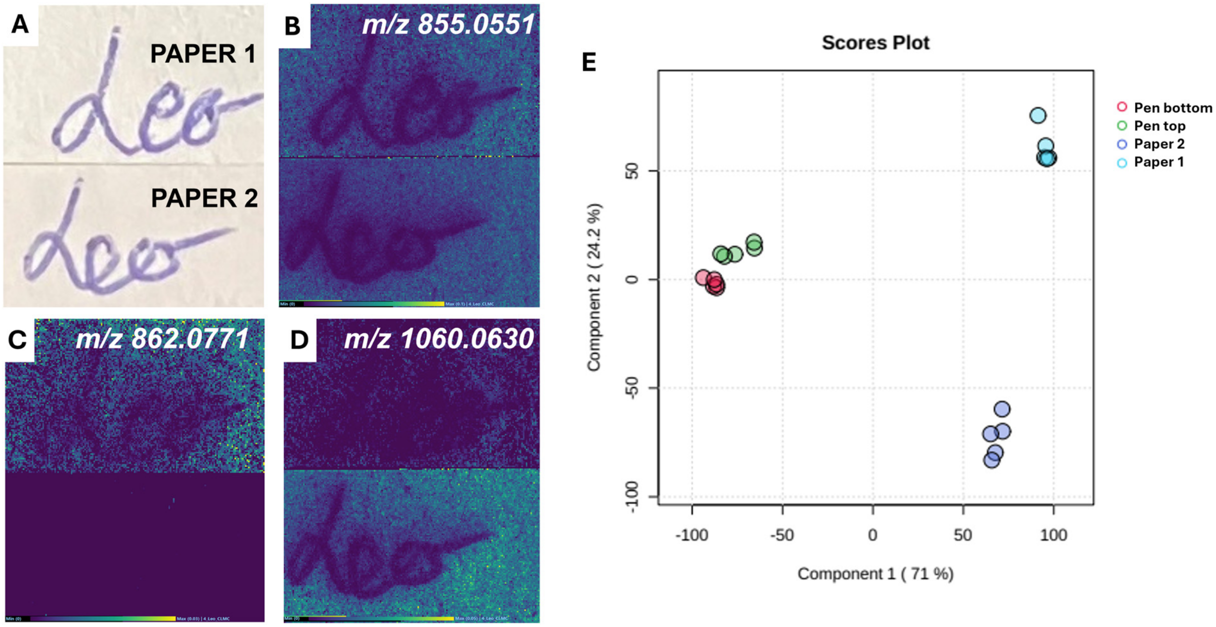

Fig. 6 MALDI MSI and statistical analysis of the same blue ballpoint pen deposited on two different brands of white paper, including (A) an optical image of the analysed sample, (B) an ion present within both paper 1 and paper 2 (*m*/*z* 855.0551), (C) an ion in paper 1 only (*m*/*z* 862.0771), (D) an ion in both papers but showing higher abundance in paper 2 (*m*/*z* 1060.0630), (E) a PCA scores plot of the extracted data.

though some ions differed in abundance, for example *m*/*z* 1060.0640 which had a greater intensity signal for paper 2 but was still present in paper 1 (Fig. 6D). A PCA scores plot for this dataset confirmed the data obtained following DESI MSI, whereby data extracted from the ink deposited onto both paper brands showed a similar chemical composition, but the paper could be differentiated (Fig. 6E).

Sample set 3 – DESI MSI ink ageing analysis

To simulate a scenario of document forgery, whereby ink was deposited with the same pen but at different times, signatures were made onto two different brands of paper simultaneously with the same pen (fresh *vs.* fresh) and compared to a 1-week-old signature and a fresh one (aged *vs.* fresh) before being analysed by DESI-MSI. For the comparison of fresh samples (outlined in red), no unique ions in the chemical profile of the ink were observed (Fig. 7A–E). Interestingly, ions at *m*/*z* 446.4569, *m*/*z* 477.3832 and *m*/*z* 521.4105 are present in both signatures, however all are showing higher intensity signal in the bottom signature, deposited on paper 2. A representative example of a mass spectrum for an ion at *m*/*z* 521.4105 is shown (Fig. 7I) whereby the same ion can be observed within the ink deposited onto both brands of paper. When considering the comparison of fresh and aged ink depositions (outlined in blue), the ion at *m*/*z* 446.4487 remained present in both aged and fresh signatures (Fig. 7F), however, the molecular images of ions previously detected in the fresh signatures at *m*/*z* 477.3753 and *m*/*z* 521.4022 are no longer present in the aged sample (Fig. 7G and H, respectively). This is further highlighted by the representative mass spectrum for the ion at *m*/*z* 521.4022 which shows that this peak is no longer present in the aged ink (Fig. 7J).

Sample set 3 – MALDI MSI ink ageing analysis

The same samples were then analysed by MALDI-MSI and a series of peaks at *m*/*z* 372.2444, *m*/*z* 358.2287, *m*/*z* 344.2130, *m*/*z* 330.1973 and *m*/*z* 316.1817 could be visualised within both the aged and fresh inks (Fig. 8B–F). In all instances, there was a similar signal intensity between the aged and fresh samples, apart from the ion at *m*/*z* 330.1973, which is displaying a higher intensity signal in the 1-week-old signature. The mass accuracy measurements of these ions have also been included in ESI Table S2.†

Further interrogation of this dataset revealed an increase in signal intensity of selected ions in 1-week old ink, in comparison to the fresh ink. Specifically, *m*/*z* 372.2080 and *m*/*z* 358.1924 which displayed a weak signal in the fresh samples (Fig. 9B and C) were much more abundant in the aged ink (Fig. 9G and H). Furthermore, *m*/*z* 356.2131 and *m*/*z* 342.1974 which showed a similar signal intensity in the fresh ink deposited onto the 2 brands of paper (Fig. 9D and E), displayed a greater intensity in the aged samples (Fig. 9I and J).

Discussion

Sample set 1

This experiment was designed to simulate a common type of forgery by altering the number "1000" to "1800" with a different pen. This reflects a potential real-world scenario involving falsification or alteration of a document.

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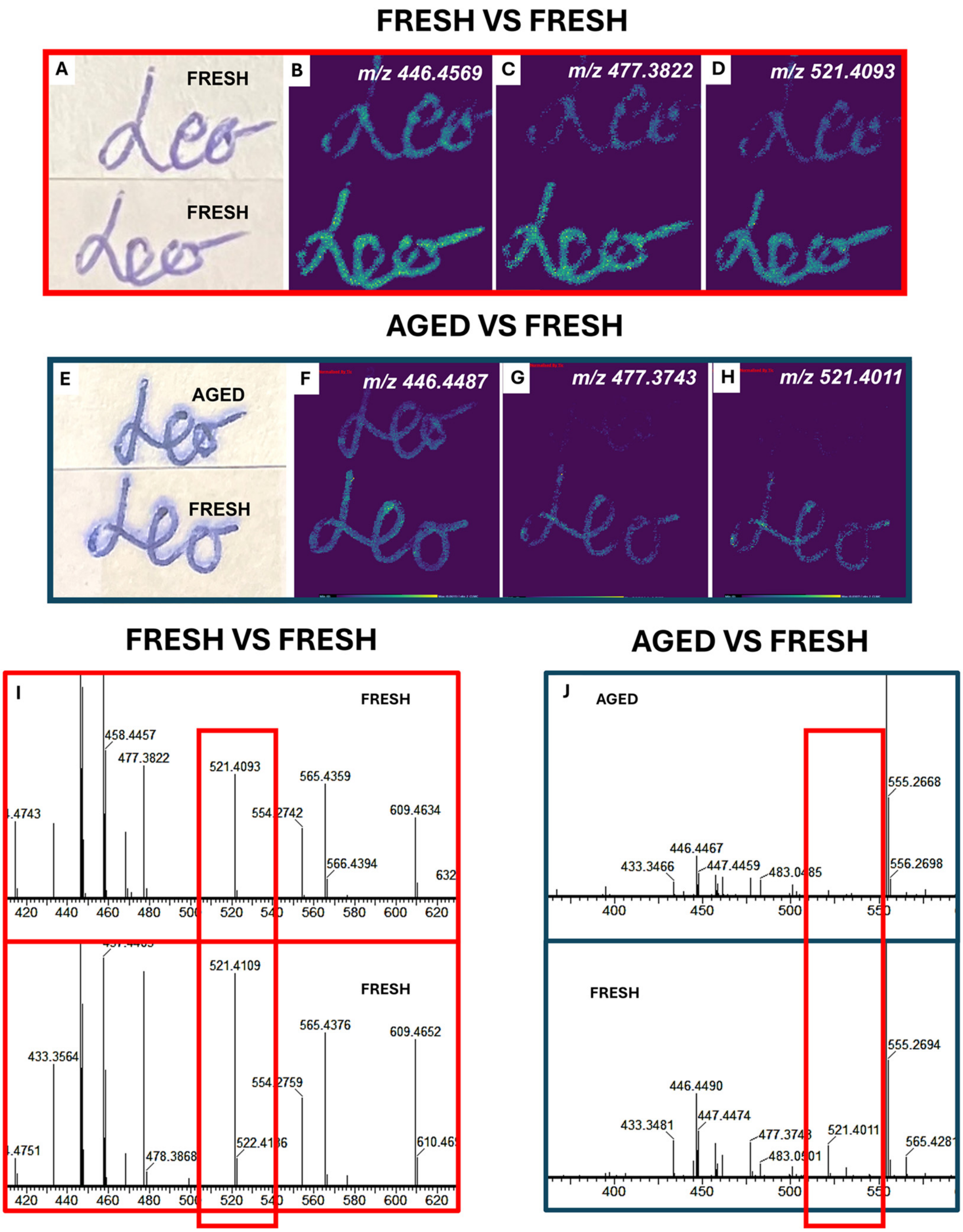

Fig. 7 DESI MSI analysis of a simulated forgery scenario where the same blue ballpoint pen was deposited on two different brands of white papers simultaneously in fresh *vs.* fresh (outlined in red) and a week apart in aged vs fresh (outlined in blue). Fresh samples: (A) an optical image of the analysed sample and DESI MS images of (B–D) *m/z* 446.4569, *m/z* 477.3832, and *m/z* 521.4105. Fresh vs Aged samples: (E) an optical image of the sample and DESI MS images of (F–H) *m/z* 446.4487, *m/z* 477.3753, and *m/z* 521.4022. (I) Mass spectra highlighting the ion at *m/z* 521.4105 present in both fresh ink samples of fresh *vs.* fresh and (J) mass spectra extracted from aged and fresh ink highlighting the same ion at *m/z* 521.4011.

Following DESI MSI analysis, 5 sets of spectra were extracted from the paper, and the ink deposited by pen 1 and pen 2, before conducting PCA analysis. The scores plot (Fig. 1A) showed distinct grouping of extracted spectra, highlighting differences in chemical composition of the paper and the 2 pen inks. The loadings plot (Fig. 1B) revealed ions responsible for the differentiation between the datasets. Within the DESI MS images, some ions were only distributed within the substrate (Fig. 1C), previously observed in the analysis of polymer banknotes by MALDI MSI, whereby the ions contained within substrate could be visualised.[34] Additionally, Adams (2011)[48] showed that printing and writing papers could be characterised and differentiated by Direct Analysis in Real Time Mass Spectrometry (DART-MS), though these results were

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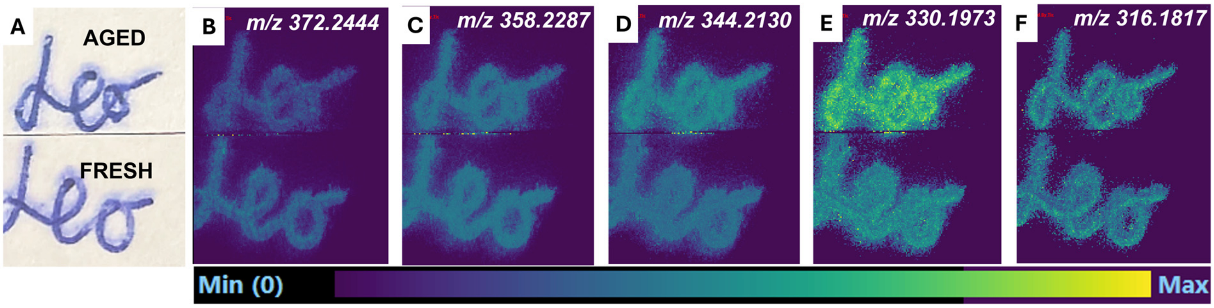

Fig. 8 (A) optical image of an aged and fresh signature made with the same ballpoint pen and (B–F) MALDI MS images of ions at *m*/*z* 372.2444, *m*/*z* 358.2287, *m*/*z* 344.2130, *m*/*z* 330.1973 and *m*/*z* 316.1817.

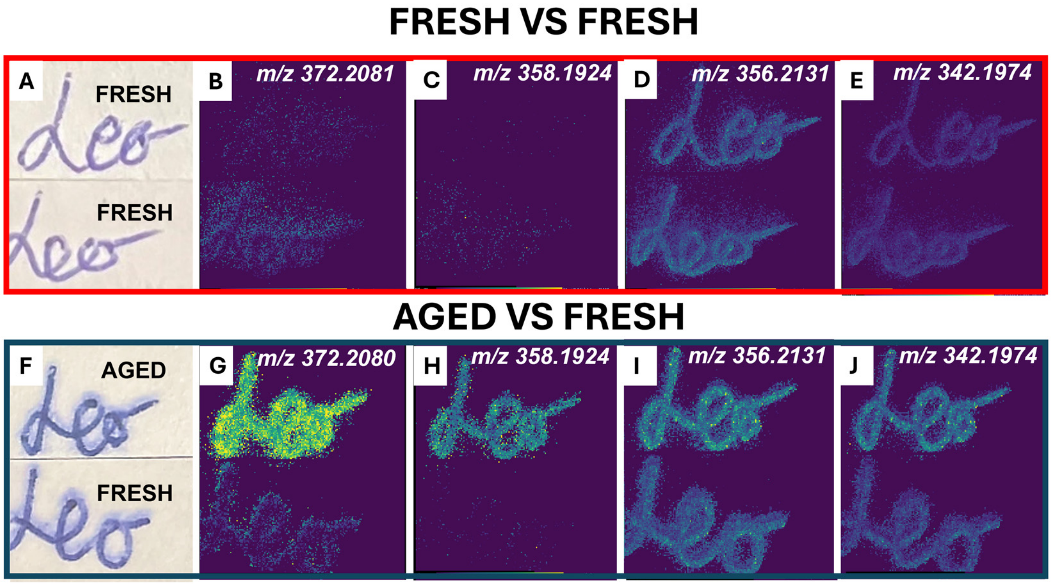

Fig. 9 MALDI MSI analysis within a simulated forgery scenario where the same blue ballpoint pen was deposited on two different white papers simultaneously in fresh *vs.* fresh (outlined in red) and a week apart in aged vs fresh (outlined in blue). Fresh samples: (A) optical image of the analysed sample and (B–E) DESI-MS images of *m*/*z* 372.2081, *m*/*z* 358.1924, *m*/*z* 356.2131 and *m*/*z* 342.1974. Aged *vs.* Fresh samples: (F) optical image of the analysed sample and MALDI MS images of (G–J) *m*/*z* 372.2080, *m*/*z* 358.1924, *m*/*z* 356.2131 and *m*/*z* 342.1974.

obtained from extracted samples. Importantly, the MSI capabilities of the instruments used in this present study enable acquisition directly from the sample, providing comprehensive information of both substrates and inks in their original state.

When considering ballpoint pen inks, some constituents were present in both pens (Fig. 1D), but unique ions were observed for both pen 1 and 2. Pen 2 revealed ions at *m*/*z* 457.4420 and *m*/*z* 468.4330 (Fig. 1E and F), indicating modification to the original number. These ions have been previously reported by Ng *et al.* (2002)[49] in analysis of black ballpoint pen inks by ESI MS, suggesting these ions represent Solvent Orange 25, the structure of which is unknown. Ions observed within only pen 1 at *m*/*z* 251.0053 and *m*/*z* 248.9896 (Fig. 1G and H) allow for visual representation of the original number prior to text alteration.

The same sample was then analysed by MALDI-MSI, followed by statistical analyses. This allowed for differentiation between the paper and 2 pens within the scores plot (Fig. 2A) and revealed ions of interest within the loadings plot (Fig. 2B). Visualisation of ions within the sample showed some ions contained within both pens at *m*/*z* 559.2719 and *m*/*z* 470.2603 (Fig. 2D and E). Basic Blue 26 ion $(C_{33}H_{32}N_3)^+$ (ref. 50) (theoretical *m*/*z* 470.2591) was identified with the mass accuracy of 2.616 ppm, which displayed higher intensity within pen 2 compared to pen 1. Tandem MS analysis of this ion (Fig. 2H) displayed characteristic product ions at *m*/*z* 454.2332, as a result of a loss of a methane (CH_4), *m*/*z* 334.1630 due to a loss of a dimethylaniline and *m*/*z* 377.2063 and *m*/*z* 349.1741 which correspond to eliminating a molecule of aniline or dimethylaniline from the parent ion, respectively.[51] Pen 2 could be visualised by selection of *m*/*z* 316.1817 representing magenta – II $(C_{21}H_{22}N_3)^+$ (ref. 9) (theoretical *m*/*z* 316.1808) with 2.783 ppm mass accuracy. An ion representing pen 1 (*m*/*z* 232.1702) displayed the original number before text alteration.

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Sample set 2

This experiment was designed to evaluate the multimodal approach to differentiate between multiple ballpoint pens on paper. Specifically, 7 different pens were used to write the word “forgery”, and the sample was analysed using DESI-MSI followed by MALDI-MSI.

DESI MSI allowed unique ions to be observed for each pen ink. The PLS-DA 3D scores plot allowed for clear separation of each of the letters based on chemical composition (Fig. 3I). The complexity of the ink formulations evidenced here ultimately assists in the ability to differentiate them. However, due to this complexity, the analytical approach would certainly benefit from the development of an ink composition database and/or the implementation of a ML algorithm to interpret the data.

To further evidence the complexity of the dataset, ESI Fig. S3† highlights ions present in multiple pens, but with variable intensity. For example, *m*/*z* 281.2559 was highlighted within the dataset showing highest intensity within the letter “e”, followed by “g” and finally “f” (Fig. S3B†). This ion, representative of oleic acid $[M - H]^-$ is a common ink lubricant.[52] Tandem MS of this ion is shown in ESI (Fig. S4†), whereby the characteristic fragmentation pattern is observed through the loss of CH_2 molecules. Conversely, the ion at *m*/*z* 279.2402, representative of linoleic acid $[M - H]^-$ is present in the same letters however displaying the highest intensity in “g” (Fig. S3D†). Tandem MS analysis of this ion has resulted in expected product ions (Fig. S3H†).[53] Despite acids being commonly used within different ink formulations,[54] there is a lack of research focusing on identifying these molecules in this type of sample.

MALDI MSI analysis in positive ion mode revealed that all 7 letters showed the presence of methyl violet 10B, methyl violet 2B and methyl violet 6B (data not shown). Unique ions for most of the seven different pens were observed, presenting additional confidence that this method could be used in real-world scenarios. While the same ions were present in the letters “o” and “r2”, the differing signal intensities and abundances between the two ballpoint pens suggest the potential for differentiation using a ML-based approach in the future.

Identification of other molecules (*e.g.* lubricants, additives, resins) would provide additional information in forgery cases which would be useful considering the likely scenario where pen inks share the same pigments/dyes.[55,56] This underscores the importance of a multimodal approach, as DESI MSI analysis in negative ion mode better ionises solvents and additives like oleic and linoleic acid, while MALDI MSI in positive ion mode is more effective for ionising pigments and dyes such as Crystal Violet and Basic Blue 26. It has previously shown that DESI and MALDI in MSI demonstrate complementarity as techniques for other types of molecules such as lipids.[57] It is important to note that choice of solvent and/or matrix is critical for these approaches, as it has been shown to influence the types of molecules being targeted, and therefore extensive optimisation of both techniques employed in the multimodal workflow was conducted in this current study.[58]

Sample set 3

The final set of experiments tested the effectiveness of the multimodal workflow in differentiating between different paper substrates and the ability to observe changes in ink composition due to ageing. Substrate analysis involved examining two different brands of white paper with signatures deposited using the same pen. An ink ageing experiment was also conducted on the same two types of paper, with ink deposits made with the same pen, but with one of the signatures deposited 1 week prior to analysis. This experiment was done in preparation for a real case study submitted to our group for analysis, in which it was questioned whether the signatures on a document had been made at different times and if the paper itself have been changed (data not shown). This analysis is crucial in forgery scenario whereby determining any text alterations or the timing of signature deposition could be critical to establish authenticity.

Despite challenges noted in DART-MS negative-ion analysis of papers, DESI MSI in negative ion mode successfully identified unique ions for each paper,[48] providing valuable forensic information for tracing and authenticating documents. The analysis of fresh ink on both papers showed similar chemical compositions, with slight differences in signal intensities attributed to paper properties.

Analysis of aged ink samples revealed that certain ions were reducing in intensity over time in comparison to the fresh samples. The same findings were reported by Jones *et al.* (2013) using DART MS[59] where it was observed that during the first few months after ink deposition on paper, the more volatile components of the ink were lost. Ng *et al.* used electrospray ionisation mass spectrometry (ESI-MS) to show that natural ageing in the absence of light does not alter the ESI-MS profiles of inks, but light exposure can cause dealkylation of polyalkylated dyes.[49] This emphasises the complexity of ageing studies, due to the differing effects of environmental conditions.

MALDI MSI analysis of ink deposited on two different papers revealed ions derived from triarylmethane compounds (Fig. 8) and the corresponding mass accuracy of each molecule can be found in the ESI (Table S2†). These ions were previously reported by Dunn *et al.* (2007),[38] who noted that these peaks represent distinct components of the ink rather than fragmentation of the *m*/*z* 372 ion. Further MS/MS analysis of the triarylmethane compounds yielded expected fragmentation patterns, detailed in the ESI (Fig. S5†). Similar findings were reported by Goacher *et al.* (2021)[9] in their Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) analyses of ballpoint pens.

In contrast to the reduced ion intensity observed in DESI MSI, MALDI MSI detected increasing intensity of degradation ions due to ageing. Statistical significance of the variability between the signal intensity of fresh *vs.* aged inks are shown on some ions of interest (Fig. S6†). Future research will examine how different paper, colours of ink and environmental conditions (such as temperature, humidity and light) influence ink ageing, offering valuable insights into document authenticity and potential alterations over time.

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Conclusion

This multimodal imaging workflow not only extracts information in order of increasing destructiveness but also generates diverse chemical species, providing a comprehensive overview of the sample's composition and enhancing discriminatory ability, therefore building on the research of other groups in this area. Analysis of Sample Sets 1 ("1800") and 2 ("forgery") distinguished entries made with various ballpoint pens. For Sample Set 3, where the same pen was used a week apart, the aged ink was differentiated from the fresh. This paper has clearly evidenced that the two techniques employed have worked in a complimentary manner to target different analytes of interest to provide a comprehensive overview of the chemical composition of the sample. DESI MSI showed ions disappearing over time, while MALDI MSI revealed the appearance of degradation ions. The methodology also successfully differentiated paper substrates, aiding forgery detection. Statistical tools like PCA and PLS-DA helped interpret data by grouping spectra with similarities and separating those with chemical differences. The MRT instrumentation identified specific ions like Basic Blue 26 with high mass accuracy, and MS/MS allowed for identification of additional ions. Ultimately, the work presented here shows how the comprehensive analysis of inks on paper could provide invaluable evidence in forensic analyses of questioned documents. Future work will focus on developing a comprehensive ink database, exploring machine learning models for detecting text alterations, and applying the proposed multimodal approach for counterfeit analysis within printed inks.

Author contributions

Conceptualization, R.B., S.F.; methodology, R.B., S.F. and V.T., software, V.T., M.A.; validation, R.B., S.F.; formal analysis, V.T.; investigation, V.T.; resources, R.B., S.F., data curation, V.T.; writing – original draft preparation, V.T.; writing – review and editing, V.T., R.B., S.F., M.A., visualization, V.T., R.B.; supervision, R.B., S.F., M.A.; project administration, R.B., M.A., S.F.; funding acquisition, R.B. All authors have read and agreed to the final version of the manuscript.

Data availability

The data supporting this article have been included as a part of the ESI.†

Statistical data analysis for this article was conducted using MetaboAnalyst 6.0, a web-based platform for comprehensive data analysis and integration, available at **https://www.metaboanalyst.ca**.

The data generated from the application of this methodology in a real casework study are not publicly available due to legal confidentiality restrictions.

Conflicts of interest

There are no conflicts to declare.

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