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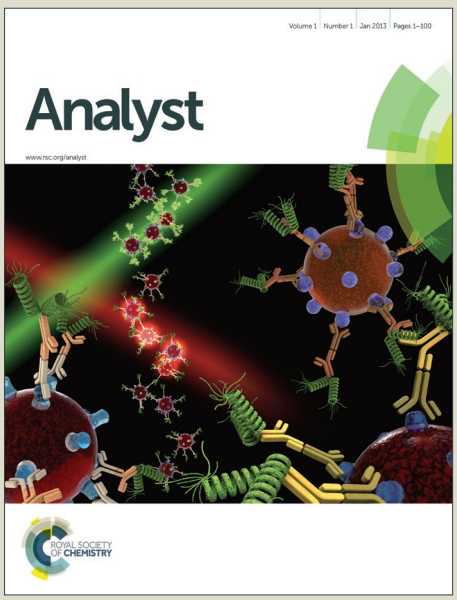

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Research Article

Investigation of falsified documents via direct analyte-probed nanoextraction coupled to nanospray mass spectrometry, fluorescence microscopy, and Raman spectroscopy

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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Microscopy with direct analyte-probed nanoextraction-coupled to nanospray ionization mass spectrometry (DAPNe-NSI-MS) is a direct extraction technique that extracts ultra-trace amounts of analyte. It has been proven to extract ink from documents with little to no physical or chemical footprint. In this study, DAPNe has been coupled to Raman spectroscopy, fluorescence microscopy, and NSI-MS to determine if an ink entry from a document was falsified. A handwritten number was altered using a different ink pen to test if the aforementioned techniques could discriminate the original number from the altered number, qualitatively and/or quantitatively. Chemical species from part of the original number, altered number, and a point at which both inks intersect were successfully differentiated by all techniques when using different pens. DAPNe coupled to fluorescence microscopy and Raman spectroscopy was not able to discriminate the forged ink entry when the exact same pen was used to modify the text (due to the same ink formula). However, DAPNe-NSI-MS successfully discerned that the pen was dispensed on different days by quantitating the oxidation process.

1. Introduction

The field of questioned document analysis is an important area in forensic science, particularly fraud and forgery of handwritten ink entries. The analyses of ink entries on these falsified documents include looking for added ink to the original written ink entry, thus, changing the meaning of the text (e.g., changing the number four into a nine). Discrimination and dating of inks can provide sufficient information, aiding scientific evidences and clues for determining the authenticity of the manuscript [1].

Material evidence found at crime scenes must be kept in its pristine state in order to preserve its evidentiary value. However, a non-destructive method is difficult to achieve when analyzing ink markings. Useful but destructive techniques have been previously applied to forged documents, include mass spectrometry methods such as laser desorption ionization mass spectrometry (LDI-MS), matrix-assisted laser desorption ionization (MALDI), and secondary ion mass spectrometry (SIMS). Utilizing MS techniques instead of chromatographic separation reduces both time and solvent needs [2-6]. Weyermann et al. [7] analyzed 30 black gel pen inks using LDI-MS, where the sample was fixed to a solid steel plate with glue. Even though the samples were directly analyzed without the addition of a matrix, reducing preparation time, the samples were cut in order to fit the plate. Similarly, Wu et al. [8] cut 5 cm ink entries into small pieces and extracted the ink by 1.0 mL of dimethyl formamide (DMF) for 12 h and then filtered through a 0.22 µm Millipore film prior to LDI-MS analysis, both destructive and time-consuming. LDI and MALDI techniques are limited to the size of the sample plate and can have an extended sample preparation time. Denman et al. [9] analyzed 24 blue ballpoint pens using time-of-flight (ToF)-SIMS and prepared the samples by drawing a 3 cm line on paper with each spectral analysis performed over a 100 x 100 µm raster area. Although surface analysis by ToF-SIMS provides ballpoint pen ink discrimination and is non-destructive, like the previous techniques, it is also limited to the size of the sample holder. Other common

procedures such as micropunch [10], removal of ink-bearing fibers from the paper [11], and cutting a small strip from the document [2,12-13] have been used for the extraction of ink from manuscripts. A less destructive approach is needed among ink analysts in order to extract and analyze the ink chemistry of documents while still preserving their original forms.

Direct analyte-probed nanoextraction coupled to nanospray ionization mass spectrometry (DAPNe-NSI-MS) is a technique that extracts ultra-trace amounts of analyte and has already proven to extract ink from documents with minimal to no destruction in addition to providing a quantitative and qualitative advantage with increased sensitivity and resolution [14]. DAPNe uses a device comprised of a nanomanipulator mounted on the stage of a high-powered microscope. Nanomanipulation has been used for many forensic and biological applications including ultra-trace drug identification [15], extraction of a peptide from an individual library of beads [16], extraction of lipid content directly from organelle, preparations of plant tissues [17], and ultra-trace molecular analysis [18].

The advantage of DAPNe-NSI-MS for the analyses of ink and document examination is that it keeps the document in its pristine form. Through coupling a piezoelectric-controlled positioner of the nanomanipulator to a pressure injector, extractions of ultra-trace analytes (as low as 300 attograms) can be achieved [19]. As shown in Figure 1, a solvent droplet is placed on the ink of interest, diffusing components of the ink into the droplet. The ink-infused droplet is then extracted into the nanospray tip for further MS analysis, leaving a water mark at most [14]. The high surface tension of the water droplet holds until the extraction is complete. This is because the ink creates a barrier between the paper and the droplet, increasing the time it takes for the droplet to soak into the paper and allowing enough time for extraction. The nanomanipulator can effectively be coupled to NSI-MS, achieving picomolar sensitivity. The sample can be analyzed using MS directly after extraction, reducing sample preparation and time. This technique is minimally destructive and the working space is capable of examining full documents, including books. In a previous study [14], DAPNe-NSI-MS was able to extract inorganic and organic components from iron gall ink and carbon-based inks, respectively, as well as utilizing the data for an age determination mechanism for the oxidation of polyethylene glycol (PEG), a stabilizer found in inks. By changing the chemistry of the extraction solvent, the DAPNe technique can target specific components in the ink. For example, a chelator was added to the extraction solvent in order to target metal ions (Fe and Mn) within iron gall ink.

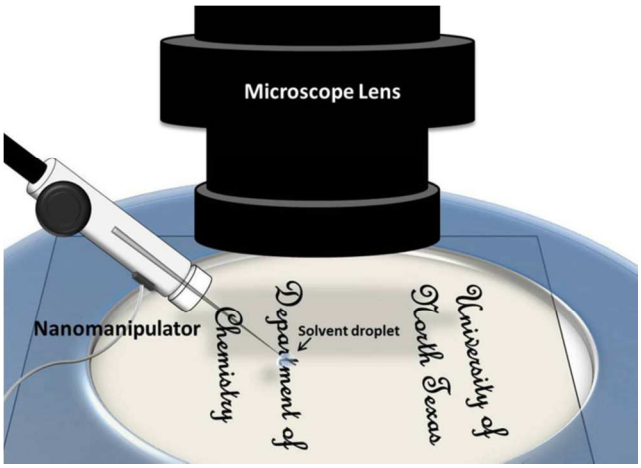

Figure 1. A schematic of the DAPNe technique extracting ink from a document. Ink diffuses into a solvent droplet placed on the paper. A nanospray tip then extracts the solvent and ink.

Although DAPNe-NSI-MS can identify whether different ink pens or the same ink pen was used in a particular handwritten document, initially locating where in the text the modification has occurred may be difficult. Therefore, other techniques need to be implemented to locate the falsified ink entries. Other non-destructive techniques such as Raman spectroscopy [20-22] and fluorescence microscopy [22] can be applied prior to ink extractions in order to image where the alterations have been made. Raman spectroscopy has become increasingly popular in analyzing inks from forensic cases because it is chemically selective, non-destructive, and no sample preparation is required [20]. Braz et al. [23] examined blue crossing ink lines via Raman imaging and determined that the longer the time separating the application of the inks, the easier it was to discriminate the order of the ink lines drawn. Mazzella et al. [24] demonstrated Raman spectroscopy as a general technique for gel pen ink analysis. Different brands and models of 55 blue gel pen inks were examined and identified two main pigments, pigment blue 15 and pigment violet 23. A Video Spectral Comparator (VSC) is a non-destructive way to analyze ink through various energy sources (tungsten, halogen, and fluorescent lamps). Silva et al. [25] analyzed different types and brands of blue pen inks in cursive handwriting using a VSC ranging from 400-1000 nm. The authors were able to distinguish between the different types and brands of blue pens. Reed et al. [26] also utilized a VSC to discriminate 42 different gel inks (blue, red, and black). Raman imaging and fluorescence are useful techniques that provide a chemical footprint of inks which is crucial circumstantial evidence in cases of counterfeit documents. These methods are able to

discriminate between the original ink and the added ink of fraudulent documents.

As technology and direct ionization progress, new direct analysis techniques are coming to light. These include desorption electrospray ionization (DESI), direct analysis in real time (DART), and liquid extraction surface analysis (LESA). DESI provides a rapid and direct approach to ultra-trace analysis by releasing charged droplets onto the surface for ionization of analytes [27]. DART is another high throughput surface method that releases excited state gas molecules for ionization of the analytes on the surface [28]. LESA uses an automated sampling probe for direct analysis [29]. LESA is the most similar technique to DAPNe in that they both interact directly with the surface at the liquid junction. DESI and DART requires a large amount of surface area for analyte ionization and in turn are more destructive. LESA currently has an enclosed workstation with limited workspace which confines document size. The other major weakness with LESA is that it uses pipette tips with a diameter of 800 µm, producing a surface area wetted with extraction solvent of 1-3 mm [30]. DAPNe uses tips with an inner diameter of 1 µm, minimizing the surface area wetted to 5 µm or less. DAPNe's platform strength comes from the coupling to high magnification microscopy and imaging spectroscopy to aid in chemical find, and reduce document destruction.

In this study, DAPNe has been coupled to Raman spectroscopy, fluorescence microscopy, and NSI-MS for the examination of counterfeit or forged manuscripts. These techniques have shown the capability to characterize and differentiate between different pens of the same color. Unfortunately, when the exact same pen is used to alter a document the same chemical species are present, making spectroscopic discrimination more difficult. By using DAPNe-NSI-MS, the oxidation process of the same ink dispensed at different times can be quantified.

2. Experimental Methods

2.1 Reagents and Solvent Preparation

Millipore water was obtained from Milli-Q Plus (Millipore;Billerica,MA) with 18 MΩ resistivity. The glacial acetic acid was purchased from Mallinckrodt Baker Inc. (Phillisburg, NJ). Optima LC/MS methanol, toluene, chloroform, ammonium hydroxide, and analytical grade ammonium acetate were acquired from Fischer Scientific (Fair Lawn, NJ). BIC® cristal, Xtra BOLD (1.6mm), pens were obtained by the pack, with 10 assorted inks (Shelton, CT). Pilot G2, Bold 1.0mm, pens (premium gel rollers, item # G21C4001) are manufactured from ©Pilot Corporation of America (Jacksonville, Fl). Uni-ball vision, fine 0.7mm, assorted pens (item #1823944) and black waterproof (item #1824106) pens are made from ©2013 Newell Rubbermaid office products (Oak Brook, IL). A black Pigma Micron archival pen (0.20mm, Sakura Color Products of America) was also used. All pens are commercially available.

The extraction solvents that were used are methanol:water (1:1, v/v) with 1% acetic acid, toluene:methanol (1:10, v/v) with 0.1% ammonium acetate, methanol:chloroform (1:1, v/v) with 0.1% ammonium hydroxide, and methanol:chloroform (1:1, v/v) with 0.1% ammonium acetate.

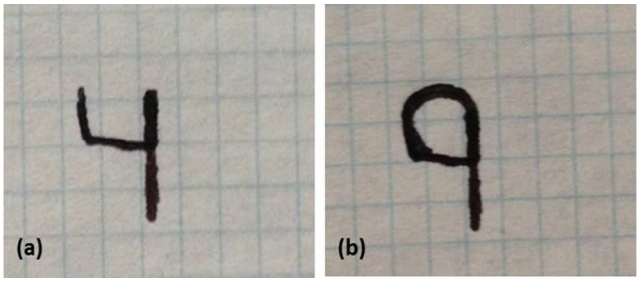

Figure 2. An illustration of a number being altered: (a) a number four was modified into a (b) nine.

2.2 Modified Ink Analysis

Several replicates of the number four was written on A4 type grid paper (ensuring reproducibility), denoted as ink pen 1, and altered to a number nine two days later with a different pen, known as ink pen 2 (Figure 2). A few hours after alteration, DAPNe-NSI-MS, fluorescence microscopy, and Raman spectroscopy were all used to analyze the alteration.

2.3 Instrumentation

A Nikon AZ 100 (Nikon Instruments Inc.; Melville, NY) microscope was equipped with an L200 nanomanipulator (Zyvex; Richardson, TX), mounted on the microscope stage. The nanospray tip is maneuvered by a joystick controller, with a fine spatial resolution up to 5 nm. The injection and extraction of the nanospray tip is controlled by a PE2000b four channel pressure injector (MicroData Instruments Inc.; S.Plainfield, NJ). The nanomanipulator has been previously detailed [14-16,19] and the direct analytical scheme used for the extraction of ink from documents is described thoroughly by Huynh et al. [14] The mass spectrometric analysis was conducted on a LCQ DECA XP Plus equipped with a nanospray ionization source (Proxeon Biosystems; Odense, Denmark). The Nikon AZ 100 microscope is also equipped with an Intensilight fiber illuminator, utilizing a mercury light source, suitable for fluorescence observation (Nikon Intensilight C-HGFI). Filter cubes (or optical blocks) are used to selectively isolate fluorescence emission of certain wavelengths. The Nikon fluorescence filter cubes (Nikon

Instruments, Inc. Melville, NY) include Epi-fluorescence interference and absorption filter combinations. These filter cubes include an excitation filter, dichcromatic beamsplitter, and a barrier filter to satisfy the excitation and emission requirements of the fluorescent compounds. The filter cubes are easily interchanged to match the spectral excitation and emission characteristics of chromophores in the ink. Raman measurements were performed using an Almega XR Raman spectrometer equipped with an Olympus BX51 microscope and mapping capabilities controlled by Omnic for Almega 7 software (Thermo Fisher Scientific Inc., Madison, USA).

2.4 Raman Imaging

Raman mapping was conducted on two different samples: (i) A black Micron pen was used to write the four (ink pen 1) and a black pilot pen (ink pen 2) was used to modify the four into a nine. (ii) A black pilot pen was used to write the four and then used again to alter into a nine. An Almega XR Raman spectrometer equipped with Olympus BX51 microscope with mapping capabilities and spatial resolution down to 1 μm was used. An excitation source of 780 nm (30% of 40 mW), single transverse mode, high brightness diode laser was used. The laser power was not high enough to visibly damage the paper. The Raman signal was collected over the range of 4000-100 cm^{-1} using a 10x microscope objective (0.25 NA).

2.5 DAPNe-NSI-MS

The nanospray tip was filled with the desired extraction solvent prior to inserting into the nanopositioner. A 2 μL droplet of Millipore water is dispensed onto the ink. The nanospray tip is then positioned into the solvent droplet and injected with a tiny amount of extraction solvent using an injection pressure of 15 psi and allowed to sit for 15-20 s, letting the ink diffuse into the droplet. It is important to note that propelling the extraction solvent into the water droplet encourages the components of ink to dissolve into the droplet. The droplet is finally extracted with a fill pressure of 35 psi. The analyte contained in the nanospray tip is directly analysed through NSI-MS without further modification.

Extractions were conducted on three different locations of the fraudulent ink entries (Figure 2(b)): (i) ink pen 1 (part of the four), (ii) ink pen 2 (part of the nine), and (iii) where the ink from the four and nine intersect. The extractions were accomplished using methanol:H_2O (1:1) with 1% acetic acid and mass spectra were scanned from a range of *m/z* 50-1500 in positive mode with a spray voltage of 2.5kV.

2.6 Oxidation of Altered Text

A black Uni-ball (waterproof) pen was used to write the number four and then set to dry for 24 hours. Using the exact same pen, the four was then changed to a number nine and extractions were conducted 1-2 hours later using methanol:H_2O (1:1) with 1% acetic acid. After the first extraction, another extraction was completed subsequently every 24 hours for a total of 5 days. Oxidation was then quantified by calculating the percent relative peak area (RPA) [14, 31-35] with three repetitions, defined as

$$RPA_i = \frac{A_i}{A_{tot}} \, x \, 100\%$$

where A_i is the area of peaks of interest at m/z=i and A_{tot} is the summation of all the significant signals above a certain intensity threshold. The overall concentration cannot be controlled, but relative amounts of extracted analytes are consistent within a given extraction solvent. Mass spectra were scanned from a range of *m/z* 50-1500 in positive mode.

2.7 Nanospray Solvent Chemistry

The solvent chemistry was evaluated by using different extraction solvents in the nanospray tip. Depending on the extraction solvent, glycols or dyes could be extracted separately. Several combinations of extraction solvents were used to change the selectivity of which component is being extracted from the ink as well as optimizing the intensities. This was conducted by drawing several lines of ink on A4 type grid paper and then set to dry for 1-2 hours before extraction. The extraction solvents discussed in section 2.1 were used.

3. Data and Discussion

3.1 Fluorescence Microscopy

The emission of inks is shown in Figure 3. Noticeable alterations can be observed from Figure 3 (a, b). Figure 3(b) shows three different red shades of emission from the paper, ink 1, and ink 2. This is because two different pens of the same color were used to modify the number; dissimilar components from each pen fluoresce differently. Figure 3 (c, d) show no discrimination, indicating time does not have a significant effect on fluorescence intensities of the same pen. Fluorescence microscopy was unable to observe a fluorescent difference when the text is altered using the same pen.

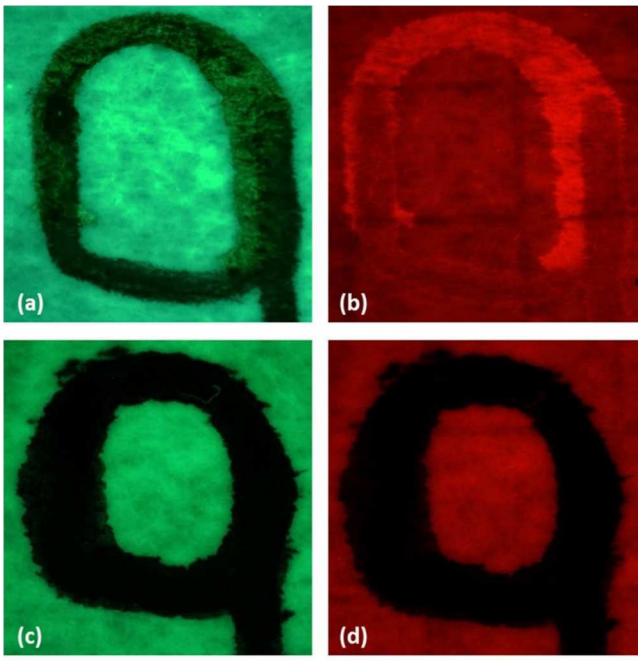

Figure 3. Fluorescent images of the altered text where (a) and (b) was modified with two different black pens, and (c) and (d) was modified with the same black pen. (a) and (c) used a blue excitation filter (420-495 nm) and (b) and (d) used a green excitation filter (510-560 nm).

3.2 Raman Imaging

Raman spectroscopy is a valuable technique utilized in the detection of document falsification because it is highly specific for chemical identification that can discriminate molecular species in inks [20, 36].

Although a valuable technique, the fluorescence interference from both the ink and paper make distinguishing peaks difficult. Paper contains approximately 33% of fluorescent whitening agents (FWAs) or also known as optical brightening agents (OBAs), approximately 80% of which are based on stilbene compounds [37]. Stilbene compounds are chemically similar to anionic direct dyes due to their planar/linear structures containing delocalized π electron systems and one or more sulphonic acid groups (-SO_3H), indicating emission at short visible wavelengths (400-500 nm) [37]. Inks contain dyes, pigments, resins, and binding agents that absorb light strongly in the visible region [38]. They contain structural characteristics that are present in the chromophore, such as electron donating groups (e.g., –OH, –NH_2, –OCH_3) that may increase the quantum yield. The fluorescence interference is an issue because it can cover the anticipated chemical footprints of the sample. Using a 780 nm laser greatly reduced the fluorescence interference compared to a 532 nm laser. Therefore, a 780 nm laser was used throughout all experiments [39].

Figure 4 shows the result of the altered four where the sample in Figure 4(a, b) was altered using different pens and the sample in Figure 4(c, d) was modified using the same pen. The images in Figure 4(b, d) are overlaying the actual written sample. Human error in making these samples cause variations in the scale. The Raman mapping analysis was conducted at 10x magnification. A magnification at 10x was preferred because the spectral variances due to the paper's irregular topography were minimized and the resulting spot size covered a more representative area of ink [23].

The contour map is represented on a color scale from red to blue, where red represents very high intensities of a chemical species at a wavenumber and blue represents very low intensities of a different chemical species at the same wavenumber. At 283 cm^{-1} (Figure 4(a, b)), high intensities from the compounds in the pilot pen (ink pen 2) are similar to the compounds found in paper; hence, ink pen 2 blends in with the paper. This is because both the paper and ink pen 2 contain a type of C-C aliphatic chain. Ink pen 1 contains dissimilar components in its ink formula than ink pen 2, creating the image of only the four. Thus, successfully discriminating the two different pens used in forging written ink entries. Raman mapping was not successful in identifying fraudulent ink entries when using the same pen to modify the text (Figure 4(c, d)). There were also peaks at 3610, 1625, 1428, 769, and 721 cm^{-1} (not shown) with Raman images that looked similar to the Raman image at 497 cm^{-1}.

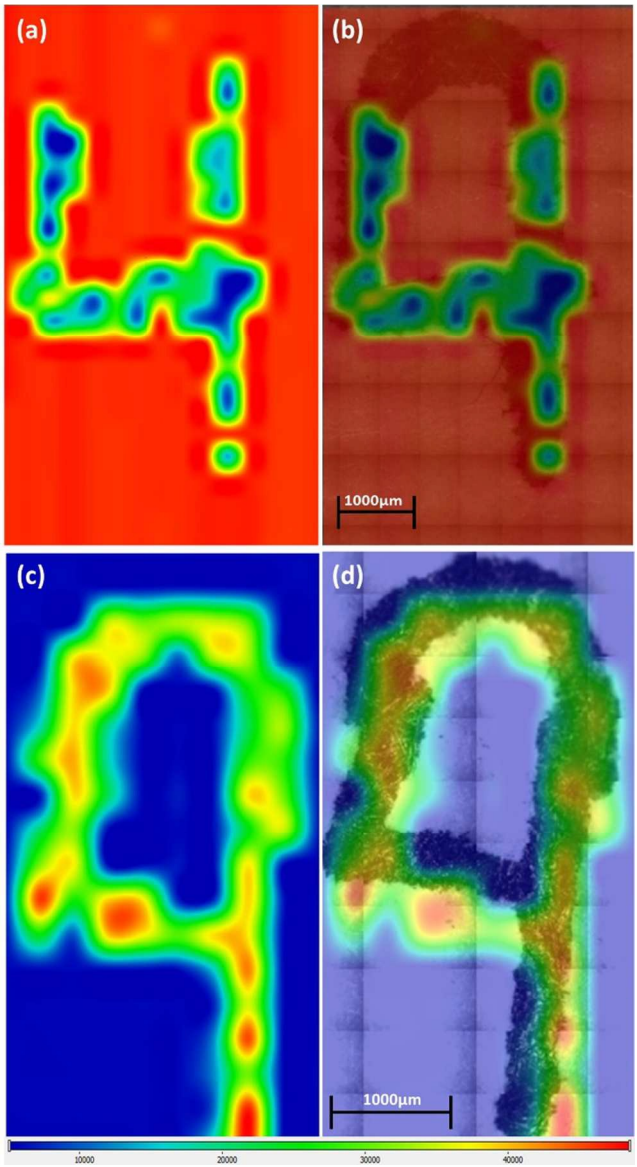

Figure 4. Raman mapping of the number four altered into a nine where (a, b) was modified using different pens (displayed at wavenumber 283 cm^{-1}) and (c, d) was modified using the same pen (displayed at wavenumber 497 cm^{-1}). (b) and (d) are the Raman images overlaying the actual sample. The map intensity scale bar ranges from 5181 to 48426.

3.3 DAPNe-NSI-MS

DAPNe-NSI-MS successfully identified the original ink, ink used to alter the number, and the point at where both inks intersect. In Figure 5, successful extractions indicate that black ink pen 1 (Black BIC pen) contains Crystal Violet and black ink pen 2 (Black Uni-ball pen) contains PEG, a binding agent and stabilizer. Stabilizing polymers prevent dyes or pigment particles from clumping together and give inks a smoother flow [33]. Components from both black ink pens are shown in Figure 5(c), where the extraction was conducted at the intersection of ink pen 1 and 2. Similar results were observed when two different red pens were used. Components from both red pens were successfully extracted at the intersection (Figure 6(c)). Red ink pen 1 (Red Pilot pen) consist of triethanolamine (*m/z* 150.13 ($[M+H^+]$) and 172.20 ($[M+Na]^+$)) and New Fuschin or also known as Basic Violet 2 (*m/z* 329.50, $[M-Cl]^+$), where M is the molecular species. Dunn et al. characterized red dyes found in ballpoint pens using laser desorption mass spectrometry, including New Fuschin found at *m/z* 330 [40]. Ink pens can become too acidic; therefore, triethanolamine is often used to regulate the pH of the ink preventing damage to the pen [41]. Red ink pen 2 (Red BIC pen) contains Rhodamine 6G dye, corresponding to peaks at *m/z* 443.53 ($[M-Cl]^+$) and 415.46 ($[M-C_2H_5]^+$).

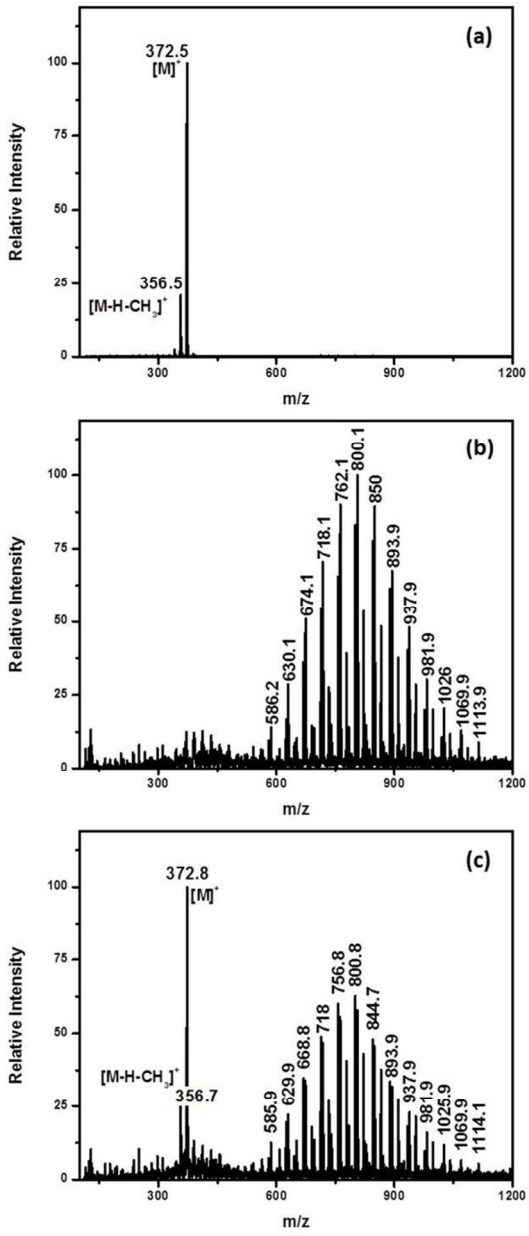

Figure 5. Spectra of (a) black ink pen 1, (b) black ink pen 2, and (c) the intersection of black ink pen 1 and 2 using DAPNe-NSI-MS.

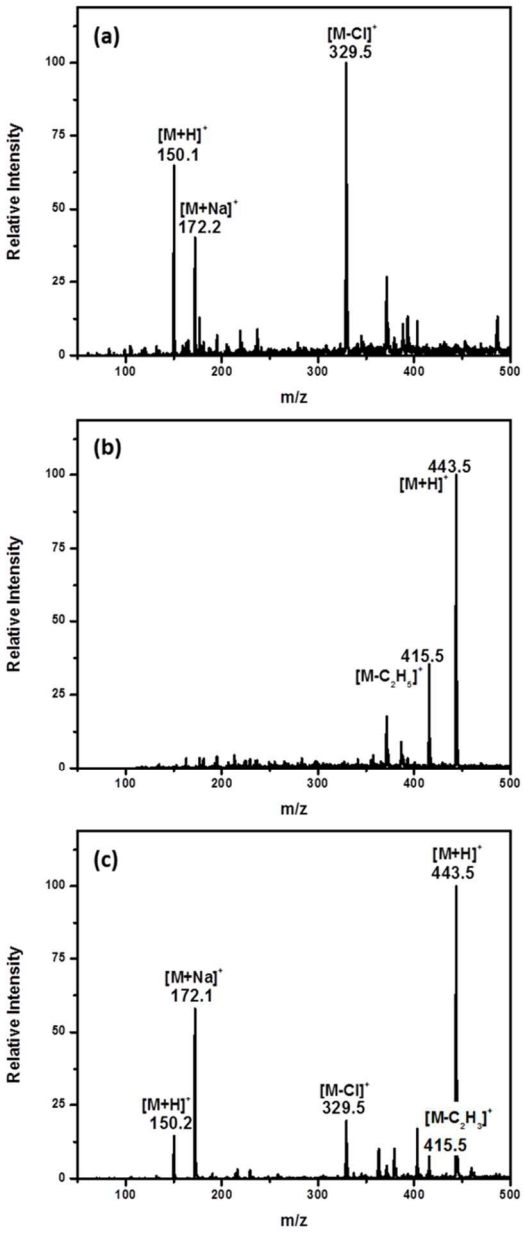

Figure 6. Spectra of (a) red ink pen 1, (b) red ink pen 2, and (c) the intersection of red ink pen 1 and 2 using DAPNe-NSI-MS.

3.4 Oxidation of Altered Text

Figure 7 illustrates the distribution change of PEG from the same black pen dispensed on different days. PEG has a melting point of 13°C and can be severely degraded by air through thermal degradation, inducing a random chain scission oxidation

mechanism [42]. In excess air, PEG and oxygen react to form PEG peroxide (random chain scission process), leading to the formation of several low-molecular-weight oxygenated products (e.g., formic esters). Han et al. compared fresh PEG with PEG aged in a vacuum and almost no degradation occurred [43]. It was found that the oxidation reaction caused by air can effectively be suppressed by adding antioxidants. PEG ions have a distinct distribution with a 44 u separation between each signal and can be detected as protonated or sodiated species, $[M+H]^+$ or $[M+Na]^+$ respectively. The 44 u separation is denoted by a repeating monomeric unit, $(-OCH2CH2-)_n$ where n represents the number of monomeric ions.

The *m/z* peaks are labeled in pairs, having a 5 u difference from each other. The 5 u difference results from the removal of water (18 u) and a sodium adduct (23 u). The ratio of the *m/z* peak pairs asymptotically approaches one after four to five days (Figure 7(a)). The more intense peaks of these pairs oxidize into smaller peaks, increasing the intensity of the less intense peaks. For example, m/z 757 and 801 will degrade in to m/z 762 and 806 respectively. Thus, the intensity of m/z 762 and 806 will increase over time. This observation was quantitated by calculating the percent RPA values of the monitored peaks, m/z 757, 762, 801, and 806 shown in Figure 8.

There is a 3.01±0.14% and 3.33±0.16% difference from day 1 to day 2 for m/z 757 and 762, respectively. For peaks *m/z* 801 and 806, there is a 0.39±0.04% and 3.41±0.20% change from day one to day two separately. The oxidation process generally has the largest difference after the first day. For *m/z* 801, the largest difference occurred from day two to three, 2.57±0.17%. The oxidation processes for inks can occur for several reasons: (i) resin polymerization, (ii) dye degradation, and (iii) solvent loss [43-44]. In this case, PEG is thermally degrading, leading to chain length reduction and lower molecular weight [42-43, 45]. Upon application, the inks used to modify the number similarly follow the oxidation trend seen in Figure 8 [14]. This oxidation process allows the analyst to determine which ink was placed last because it will have a higher RPA value. After examining the modified four, the ink from part of the nine had a higher RPA value. The four has an RPA value of 9.12±0.52% and the nine has an RPA value of 13.40±0.18% for m/z 757 on day 1, indicating the nine was forged. Calculating the RPA value is not limited to the peaks chosen in this paper; any peaks may be used as long as the RPA_i equation is correctly used. Furthermore, modified text using the same pen can be distinguished because the ink was placed on different days.

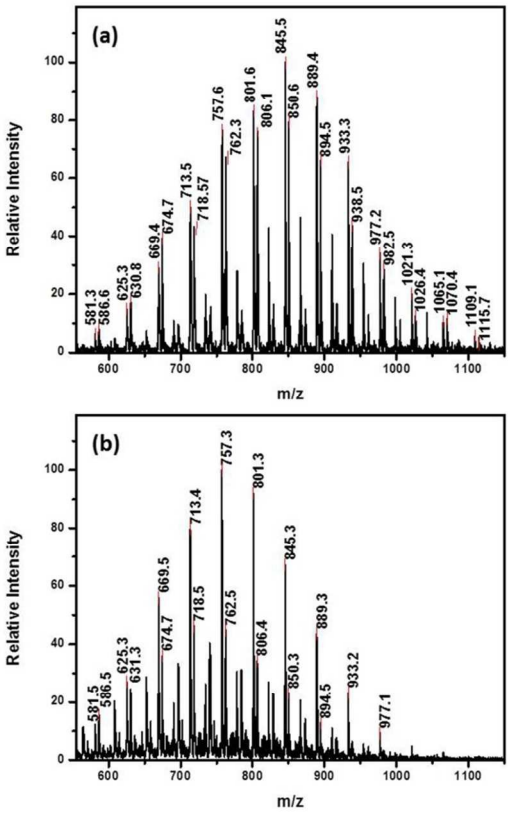

Figure 7. Spectra comparison of the modified number where (a) is the ink from the number four (24hours old ink) (b) is the ink from the nine (fresh ink) using the same black pen. Extractions were conducted using DAPNe-NSI-MS.

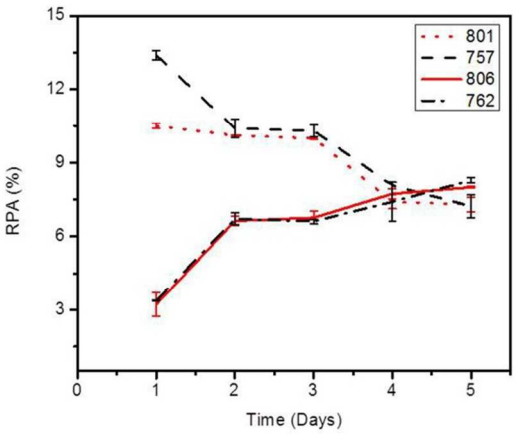

Figure 8. The percent RPA of monitored PEG peaks (*m/z* 757, 762, 801, and 806) from a black pen over a 5 day period (n=3).

3.5 Nanospray Solvent Chemistry

The extraction solvent used to fill the nanospray tip influences which component of the ink is being extracted and the intensities of the molecular ion peaks in a mass spectrum. For example, PEG and Crystal Violet was extracted from Uni-Ball black (waterproof) ink separately. The extraction of Crystal Violet and PEG is achieved when using methanol:chloroform (1:1) with 0.1% ammonium acetate and methanol:H_2O (1:1) with 1% acetic acid as the extraction solvent, respectively (Figure 9). In a previous study, EDTA was incorporated into the extraction solvent in order to chelate Fe and Mn ions from iron gall ink [14]. Being able to extract different components from the same ink entry is very important, especially when the same ink pen has been used to forge documents. Once deposited on paper, dyes are more stable than vehicles, taking longer to oxidize than PEG. Upon application to document forgeries, selectively extracting PEG gives analysts the opportunity to track its oxidation process which aids in differentiating if ink from the same kind of pen was placed at different times.

In many cases, the same component from the ink sample is extracted by using different extraction solvents. Although the spectrum looks similar, the intensities of certain peaks will differ. The variation of peak intensities is compared by converting the relative peak intensities into points via a rating scale, as shown in Table 1. The peak intensities are first normalized and then assigned points based on the rating scale. The rating scale is based on normalization to 100 but this can relatively be altered. For example, in Figure 7(b) the peak at *m/z* 801.3 has a relative intensity of 92.1 which converts to 9 points.

Relative peak intensities	Points
<10	0
11-19	1
20-29	2
30-39	3
40-49	4
50-59	5
60-69	6
70-79	7
80-89	8
90-100	9

Table 1. A rating scale used to convert relative peak intensities into points.

Three different solvents were used on eleven different pens to illustrate how the intensities are affected by the extraction solvents. Table 2 shows the intensities converted into points for each ion obtained from the ink of a blue BIC pen. The Crystal Violet ions were validated by J. Siegel et al. [46] when analysing a blue BIC pen. The three degradation ions that belong to Crystal Violet in Table 2 have resulted from natural aging. The natural aging of Crystal Violet successively losses methyl groups which are then replaced by solvent protons, losing 14 mass units each time [46]. Basic Yellow 2 was also confirmed by J.A. Denman et al. [9] when analyzing a blue BIC pen. After the intensities were converted into points for the ions, they were then totaled together to determine which solvent gave the highest amount of points, indicating which solvent is optimal for that ink. For blue BIC pen, methanol:H_2O (1:1) with 1% acetic acid and toluene:methanol (1:10) with 0.1% ammonium acetate received a total of 18 and 19 points, respectively, which is 6-7 points higher than extracting with chloroform:methanol (1:1), with 0.1% NH_4OH. In this case, two of three solvents would be suitable for blue BIC pen. Table 3 lists only the total points for each pen. All three solvents, in Table 3, extracted PEG from Uni-Ball black (waterproof) pen, but each solvent enhanced different peaks For example, m/z 800 could equal 9 points and m/z 844 could equal 2 points for methanol:H2O (1:1) with 1% acetic acid. However, they'd equal 4 points and 9 points, respectively, when using methanol:chloroform (1:1) with 0.1% ammonium hydroxide. Depending on which PEG peaks are being monitored, the total points can vary for this ink. Black BIC pen received 11 points for all three solvents, indicating all three solvents are suitable. Red pilot pen received 23 points when using toluene:methanol (1:10) with 0.1% ammonium acetate compared to the other two solvents which both totaled to 16 points. Selectivity for the analytes being extracted can easily be controlled by solvent alteration.

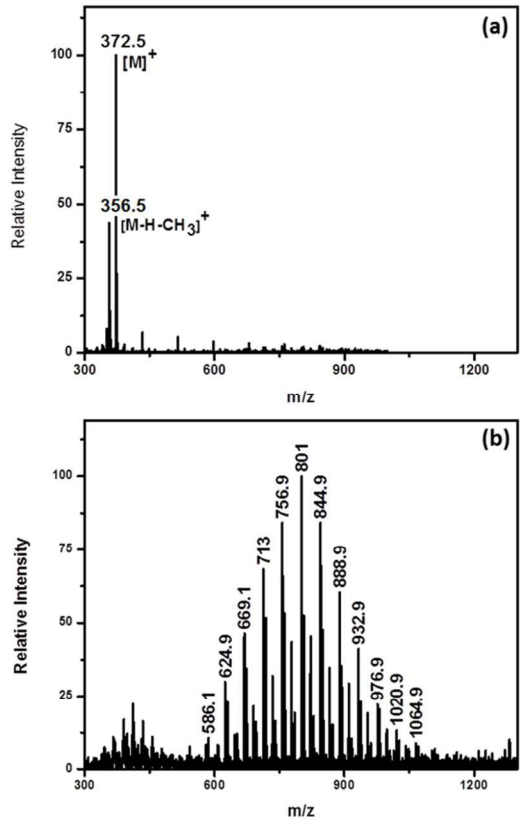

Figure 9. Spectra of Uni-ball black (waterproof) pen using two different extraction solvents, (a) methanol:chloroform (1:1) with 0.1% ammonium acetate and (b) methanol:H_2O (1:1) with 1% acetic acid.

Conclusions

The nanomanipulator is a versatile tool, especially in the field of forensic document examination. Its ability to couple with other document examination techniques, jobs of document analysts may be made simpler. DAPNe with NSI-MS, fluorescence microscopy, and Raman spectroscopy are advantageous couplings because these techniques do not leave destructive chemical or physical foot prints, keeping the document intact. Fluorescence microscopy and Raman spectroscopy demonstrate direct characterization without sample preparation and initially identify areas of different inks or areas of altered text. Upon extraction by nanomanipulation, mass spectrometry can be employed to detect the presence of a dissimilar ink and prove if alteration occurred. Raman spectroscopy detects chemically-active components of ink, fluorescence microscopy offers detection of emission profiles for colorants and additives, and NSI-MS is able to characterize different components of ink with a simple solvent preparation. Oxidation can be effectively quantitated using the RPA equation, especially if the overall concentration cannot be controlled, but relative amounts of extracted analytes are consistent within a given extraction solvent. Due to the non-destructive nature of these techniques and their ability to confirm if a text has been modified, the forensic community will be able to further their studies in chemical composition of fraudulent documents.

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Acknowledgments

This project is funded by the National Institute of Justice grant No. NIJ-2013-3361.

Notes and references

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			Solvent		
m/z	Ions	Component	Methanol:H_2O(1:1), with 1% CH_3O_2H	Toluene:Methanol(1:10), with 0.1% $NH_4C_2H_3O_2$	Chloroform:Methanol (1:1), with 0.1% NH_4OH
268.3	$[M-Cl]^+$	Basic Yellow 2	9	9	9
344.0	$[M+2H-(CH_3)_2]^+$	Crystal Violet	0	1	0
358.4	$[M+H-CH_3]^+$		3	4	1
372.5	$[M]^+$		6	5	2
		Total	18	19	12

Table 2. A comparison of mass spectrometric intensities from a blue BIC pen using three different solvents, based on a rating scale.

		Solvent		
Pen Color	Brand	Methanol:H_2O(1:1), with 1% CH_3O_2H	Toluene:Methanol(1:10), with 0.1% $NH_4C_2H_3O_2$	Chloroform:Methanol(1:1), with 0.1% NH_4OH
Pink	BIC	11	10	11
Blue		18	19	12
Red		12	14	12
Green		12	9	9
Black		11	11	11
Black	Uni-ball	49	61	66
Green		19	21	18
Light Blue		11	16	15
Red	Pilot	16	23	16
Black		25	16	15
Blue		43	9	24

Table 3. A comparison of a variety of mass spectrometric intensities from several different pens, using three different solvents, based on a rating scale.

Graphical Abstract