Document Title:	**Microscopy with Direct Analyte Probe Nanoextraction (DAPNe)-Coupled to Nanospray Mass Spectrometry for Localized Chemical Analysis of Document Inks**
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Microscopy with Direct Analyte Probe Nanoextraction (DAPNe)-Coupled to Nanospray Mass Spectrometry for Localized Chemical Analysis of Document Inks

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Abstract

A method for the extraction and analysis of ink samples was developed using microscopy with direct analyte probe nanoextraction coupled to nanospray mass spectrometry (DAPNe-NSI-MS) for localized chemical analysis of document inks. Nanomanipulation can be effectively coupled to nanospray ionization mass spectrometry providing picomolar sensitivity, the capability to analyze femtograms to attograms of material and reduce the required sample volume to as low as 300 nL. This new and innovative technique does not leave destructive footprints on the surface of a document. As proof of concept, analysis of inks from various eras were tested, iron gall ink and modern inks, as well as the capability to detect the oxidative products of polyethylene glycol (PEG), a common binding agent in India inks. The experimental results showed that DAPNe-NSI-MS was able to chelate iron (II) and manganese (II) ions of iron gall ink and organic components of modern and carbon-based inks. Regardless of whether the ink composition is modern or ancient, organic or inorganic, this new approach is able to identify and characterize the ingredients by modifying the extraction solvent, illustrating the potential diversity of the extraction chemistries.

DAPNe also 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 similar ink formula). However, DAPNe-NSI-MS successfully discerned that the pen was dispensed on different days by quantitating the oxidation process.

The research for this proposal will take the sensitive nanomanipulation technique and combine it with spectroscopic techniques for ultra-trace analysis of chemical inks in document analysis. To establish the proof of concept, reproducibility, sensitivity, and qualitative and quantitative nature, the developed techniques will be used for forensic science trace analysis on paint, ink, and trace document residue from transfer. This will demonstrate the versatility of the techniques, and broad spectrum uses for basic and advanced document forensic analysis.

Table of Contents

Executive Summary

Problem

Investigation of forged documents is crucial when determining authenticity. There have been many cases where checks, notes, contracts, or other important written certificates have been falsified. Forensic document examiners (FDEs) conduct physical and chemical analyses on these documents in order to decipher if a signature was altered or a number was changed and when the alteration occurred. Numerous procedures have been used for the extraction and analysis of ink from documents. Traditional, non-destructive methods involve a visual examination of the document by microscopy and/or Video Spectral Comparison (VSC). VSC relies on a visual comparison of grey levels, but fails to discern between inks in suspected alterations. Common procedures in analyzing these documents, such as separation techniques, are destructive. Although chromatographic methods give significant information about the ink, the extraction of ink from the document is harmful. Ink analysis plays a significant role in questioned and authentic documents. The courts prefer nondestructive methods so most forensic approaches focus on nondestructive means of analysis. 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.

Purpose

The proposed method was to develop a forensic science technique and instrumentation for the trace and ultra-trace analysis of inks, dyes, paints, and transferred chemistries on documents. This method focused on direct sampling and microphase extraction of chemical residues to isolate and analyze key components present in inks and transfer on documents. This was combined on a single package adapted to multiple platforms with broad range capabilities for the field of forensic science. The multistage workstation developed in our group has wide-ranging applications in the biological and chemical sciences. The workstation consists of a platform with 2-nano-positioners that hold end-effectors and capillaries used to manipulate, probe, and characterize objects of interest. The multistage workstation has been coupled to nanospray mass spectrometry allowing for precise and accurate analysis of trace chemical analytes, localized chemical extraction-coupled with sub picomolar sensitivity (<100 attograms).

Figure 1 shows a setup of the nanomanipulator with conventional bright field and Raman microscopy. In general, samples are placed under the microscope for viewing and probing of chemical residues. A metal fixture is used to secure samples in position. Viewing and probing of each sample takes place with multiple objectives. Image capturing is done on NIS Elements software, which is coupled to the microscope with a CCD camera. The nanomanipulator mounted to the microscope consists of 2-nanopositioners controlled in either coarse or fine mode. The range of motion and the resolution achievable is dependent on the mode of manipulation. The coarse mode has a range of motion of 12 mm in the X and Z-axes and 28 mm in the Y-axis with a translational resolution of 100 nm. In the fine mode of operation, the range of motion is 100 µm in the X and Z-axes and 10 µm in the Y-axis. The course mode utilizes nanospray capillary tips coated with a Pd-Au alloy that perform the injection and extraction of the analytes. The PE2000b pressure injector provides the pressure required to do the injection/aspiration process through the nanospray capillary tips.

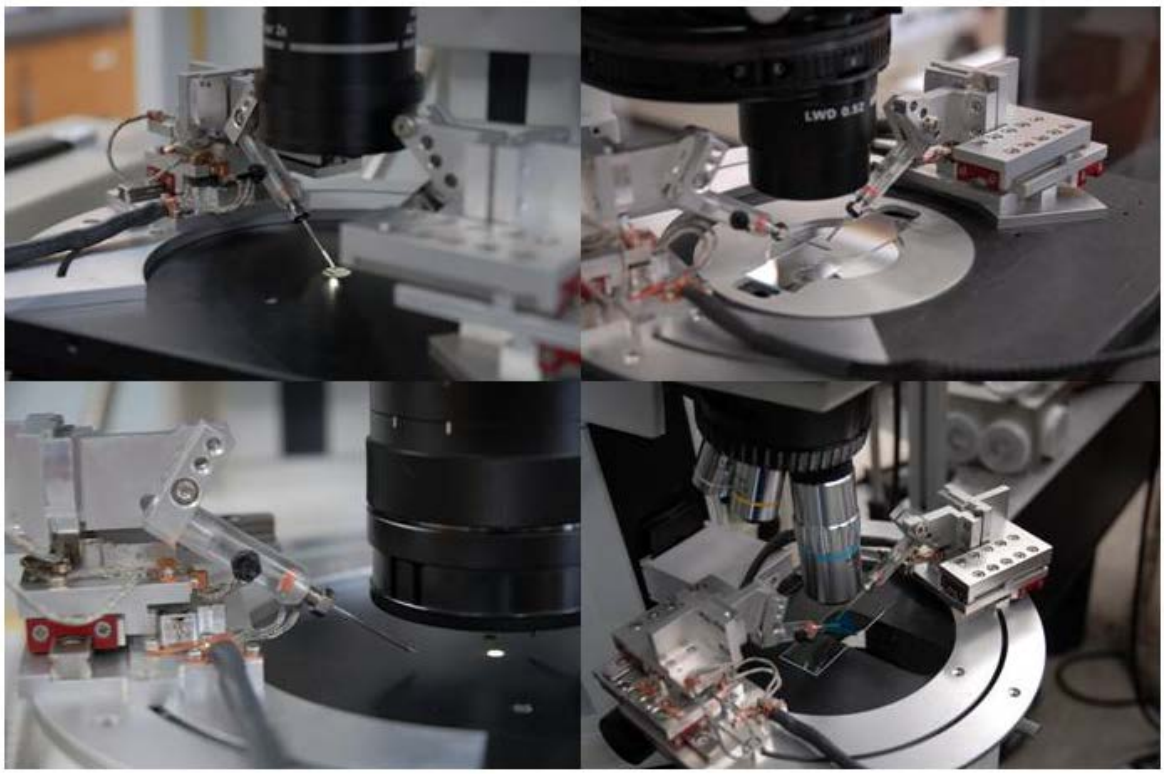

Figure 1. Image of 2-positioner prober on Beta Platform mounted to a Nikon AZ-100 microscope (top left). Image of complete Beta version on prober with injector, Nikon AZ-100 (top right). Enlarged image of nanoprober (bottom left). Image of nanoprober under large working distance 50x for Raman imaging (bottom right).

Development of techniques which couple nanomanipulation (for trace analysis) with spectroscopic imaging (for chemical analysis) has proven that it would be a great benefit to the forensic science field. This method would allow sampling of small (<5um) area with minimal sample destruction. Small areas, where document modifications are made may be difficult to isolate with current technologies. Coupling these techniques will determine the authenticity of the document and most importantly, allow sampling of a small area using extremely small sample volumes. The key advantage of using a nanomanipulation technique for the extraction of ink from the document is that the process does not leave visible destructive foot prints on the surface of the document, which enables the analyst to retain the document in the original form and maintain its integrity.

Research Design and Results

The nanomanipulator is favorable due to its simplicity and short analysis time. For example, the nanospray tip can easily be removed from the nanomanipulator to the mass spectrometer in seconds. This allows for a one step analysis where extracting is the only crucial step. Transferring the tip requires no sample loss or additional steps between removing the tip from the nanomanipulator to the nanospray source of the mass spectrometer.

Other additions to make this application possible are fluorescence microscopy and Raman imaging. Solid surface luminescence and chemical specie variations of minuscule

amounts from matrices can be achieved. With these techniques, the need for sample preparation is eliminated (saving time) while maintaining detection limits in the picogram range. This will demonstrate the versatility of the techniques, and broad spectrum uses for basic and advanced document forensic analysis.

The milestones for this proposal were accomplished in three milestones:

Task 1: *Coupling of nanomanipulator to fit relevant microscopies, specifically Raman and IR for chemical localization, and for other unconventional spectro-microscopy techniques.*

Coupling of the nanomanipulation technique with spectroscopy allowed us to maximize the efficiency of analysis and sample throughput. The primary aim of this line of research was to develop a front-end instrument that incorporates the quick-screening capabilities of Raman and infrared microscopy with the rigorous qualitative analysis of nanoextraction and NSI-MS. This basic approach to sample analysis has multiple benefits. First, spectroscopic mapping of the samples will identify areas of interest, thus narrowing the search for areas on which to perform extractions for NSI-MS. This reduction of sampling will lessen the amount of consumable resources used in the NSI-MS analysis. Finally, pre-screening of samples provided valuable spectroscopic data that, coupled with mass spectrometric analysis, will aid in the identification of unknown compounds without the need for additional instrumentation. While this presented an opportunity for the coupling of the aforementioned techniques, some modification to the current nanomanipulation design was necessary.

One of the key components accomplished was to develop a high-magnification, long-working-distance microscope objectives. Of particular use in Raman analysis, objectives with magnifications of 50x and a working distances of 20 mm or greater allows for the analysis of irregularly shaped surfaces. More importantly, the extended working distance allows a space for the placement of nanoextraction capillaries and end-effectors over the sample without compromising the spatial resolution provided by similar inverted configurations (Figure 2).

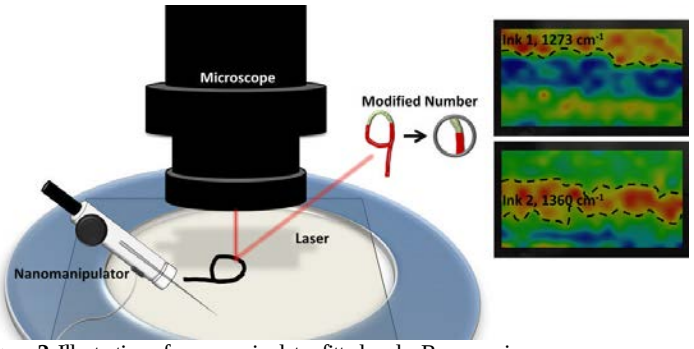

Figure 2. Illustration of nanomanipulator fitted under Raman microscope.

Task 2: *Characterize reproducibility, sensitivity, limits of detection for technique, and develop direct SOP for handling document forensic evidence.*

Though these direct applications are great proof-of-concepts, reproducibility and standardization of the techniques were performed. This task focused on making this broad spectrum front end more robust. Clear tolerances and limitations of the technique was identified. A myriad of statistical analysis for reproducibility, limits-of-detection, and ease of implementation was performed and disseminated during Task 2.

Polyethylene glycol (PEG) is a common base used in inks as well as 2-phenoxyethanol, benzyl alcohol, and other glycol ethers. Ballpoint pens with oil-based inks were first introduced in the 1940s, presenting a new kind of writing apparatus and ink. Before this time, water-based inks were used with fountain and dip pens [1]. PEG-based inks began replacing the oil-based ink formulas in 1952. Advancing through history, many

different pen apparatuses and ink formulas were introduced; but PEG has managed to remain constant in many ink formulations today. Ink formulas of ballpoint pens may contain up to 50% glycol-based solvent or benzyl alcohol [2]. The polar nature of PEG has allowed a wider range of dyes to be used and were found to adhere firmly to cellulose fibers where inks can neither be smudged nor transferred [3]. It also helps pigments to stay dispersed in solution. The molecular structure of PEG contains a repeating oxyethylene unit ($-C_2H_4O-$), making the oligomer distribution spaced 44 u apart [4]. The structural formula of PEG is $H(C_2H_4O)_nOH$, where n represents the number of repeating units. For mass spectral analysis, PEG ions are observed to have proton, sodium, or potassium adducts, $[HO(C_2H_4O)_nH + H]^+$, $[HO(C_2H_4O)_nH + Na]^+$, or $[HO(C_2H_4O)_nH + K]^+$, respectively [5].

Although the amount of PEG extracted from ink cannot be quantified; the amount oxidized over time can be monitored. The monitoring of these peaks were completed by using the relative peak area (RPA) equation. Once deposited on paper, dyes are more stable than vehicles, taking longer to oxidize than polyethylene glycol. Upon application to document forgeries, selectively extracting PEG gives analysts the opportunity to track its oxidation process, in a short amount of time, which aided in differentiating if ink from the same kind of pen (e.g., same brand, color, type) was placed at different times. The process of oxidation was sped up by applying incandescent heat to the ink. After 12 hours of incandescent heat exposure, PEG was shown to completely oxidize from the high mass region (m/z 400-600) and nanomanipulation was able to show the sensitivity differences through absolute intensity comparisons. Reproducibility and sensitivity was accomplished with the analyses of PEG and other dyes found in inks.

The analyte injection/extraction process is carried out with 10 µL of solvent loaded into the nanospray capillary tips. After the analytes are probed, the nanospray capillary tip is landed at ~1 µm away. The appropriate solvent mixture is injected at a pressure of 25 psi for dissolving the analyte. After 10 seconds of dissolution, the solvent/analyte solution is aspirated into the nanospray capillary tip at a pressure of 40 psi. The nanospray capillary tip can then be transferred to the nanospray ionization source for nanospray ionization mass spectrometric analysis (NSI-MS). An ionization voltage of 2.5 kV is applied for analysis on the mass spectrometer.

Task 3: *Develop tip chemistry and methods for the analysis of paints, ink, paper chemistry and document analysis.*

One of the most important issues in successful extraction and nanospray-MS is the choice of solvent used. Not only must the compound of analysis be dissolved in the solvent, but the solvent also requires properties like low surface tension, polar features, reasonably volatile, and must provide a stable spray and acceptable spectra. For the detection of complexes, solvent often plays a critical role. The solvent condition should be maintained in such a way that the interaction is not disturbed by the pH or the high organic content of the solvent. The solvent also needs to promote the ionization of the interested analyte to be detected, especially when compositions of the analytes are unknown and there might be the possibility of having more than one compound. For example 1:1 methanol-water with acetic acid promotes the ionization of proteins and peptides, and the majority of organic drugs, compared to 1:1 chloroform-acetonitrile with sodium acetate which is a better lypophile. However, the usage of non-polar solvents like hexane, tetrahydrofuran, and toluene is not feasible for the better spray of the analytes, limiting the available choices of solvents. One

of the primary metrics for success of this task is solvent selection to address a multitude of chemistries-of-interests.

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 was 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. Ethylenediaminetetraacetic acid (EDTA) was incorporated into the extraction solvent in order to chelate Fe and Mn ions from iron gall ink. 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.

Conclusions

The remarkable ability of nanomanipulation-coupled with nanospray ionization-mass spectrometry (NSI-MS) as an ultra-trace analytical tool has provided an immediate and effective way of doing chemical analysis. The results obtained are both meaningful and sufficient for various types of trace analytical work. This method of analysis has been applied by the PI's laboratory to various forensic science based analysis and has proven to be effective in detecting analytes while eliminating matrix effects.

We have demonstrated this technique by probing chemical residue (drugs and biological) from individual fibers, electrostatic lifts, and direct biological samples and analyzing them using nanospray mass spectrometry. Using a single instrument to manipulate, probe, and characterize an analyte minimizes the need of having multiple devices and instruments; this transitions simple microscopy into a localized chemical analyzer for document analysis. Extraction of chemical residue in combination with the document data (i.e., handwriting) has provided invaluable information that facilitates action for local police authorities in their investigations.

This research has significant impact for trace analysts in the field of forensic science. The analysis of trace and ultra-trace residue within documents provided evidence of other chemicals present that would normally be unseen or too difficult to detect with other instrumental methods or techniques. The analysis method was conducted in a timely manner as to avoid extensive preconcentration methods that are time consuming in nature, and not cost effective. Chemical signature data from extracted residues exhibited high sensitivity and results are very reproducible to traditional methods of analysis. The transfer of trace samples collected from the documents to the laboratory would not be an issue in regards to the amount obtained as well as contamination of the sample from the environment.

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. 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. Using microscopy with direct analyte probe nanoextraction coupled to nanospray mass spectrometry for localized chemical analysis of document inks provide the advantages of analysis with small sample volume, high resolution, low limits of detection, and direct analysis, eliminating further sample preparation and saving analysis time. The procedure does not leave any visible footprint on the surface of the document, at most a water mark. This is advantageous for the analysis of historical, governmental, and/or other documents where maintaining the integrity is crucial.

Policy and Practice

This method of analysis also has the potential to be deployable to any crime laboratory due to its multi-platform nature, which would provide more immediacy toward obtaining results. This deliverable will be ideal for both archival and forensic science labs, and will be tested on trace and ultra-trace chemicals pertaining to forensic science based analysis with local crime laboratories. Document expert Michael Weldon from Weldon & Associates, board certified forensic document examiner, has brought documents from clients to be examined in our lab. We were able to collaborate with Michael Weldon to aid in the authenticity of contracts, and our method has assisted Michael Weldon to test the authenticity of documents.

This research can have an immediate impact for forensic investigations. The analysis of trace residue within document and handwriting data could pinpoint or eliminate potential suspects, and lead to acceptable court resolution. Chemical signature data from the extracted residue can offer the document database another avenue to group and characterize documents. The majority of inks offer chemical signature pointing to manufacturing that could be acquired using this method, thus linking group and distribution chains together with documents.

Main Body

1. Introduction

Developing an analytical technique for the study of antique and ancient documents must satisfy the specific requirement of integrity preservation during ink extraction. Ink analysis is of great importance whenever there is a question regarding verification or age determination of documents under differing conditions such as temperature, humidity, light and pest exposure, and any chemical alterations that may occur over time [6,7]. However, a major problem arises when obtaining accurate results without altering the original contents of the document; hence, a minimal to non-destructive approach is of great necessity.

Numerous procedures have been used for the extraction and analysis of ink from documents. Traditional, non-destructive methods involve a visual examination of the document by microscopy and/or Video Spectral Comparison (VSC) [8,9]. Thin-layer chromatography (TLC) is also a widely used method because it is inexpensive and shows

good discrimination, but its discrimination is reduced for many gel inks and only serves to verify their pigmented nature [8,10,11]. However, the subjective nature of these techniques limit their usefulness. VSC relies on a visual comparison of grey levels and fails to discern between inks in suspected alterations, and TLC results are dependent on either simple side-by-side comparisons of color bands or retention factors calculated by an analyst [10,12–14]. Common procedures such as use of a micro punch [15], removal of fibers from the paper containing ink [14,16], or cutting a small strip from the document [13,17,18] have been used for the extraction of ink from manuscripts. Although TLC [10,11], high performance liquid chromatography (HPLC) [18–21], and capillary electrophoresis (CE) [21,22] are common analytical methods for the separation and analysis of ink components, a less destructive approach using nanospray ionization - mass spectrometry would provide a quantitative and qualitative advantage. By eliminating the need for chromatographic separation, both time and solvent needs can be significantly reduced [11,13,23–25].

The advent of a new approach using microscopy with direct analyte probed nanoextraction coupled to nanospray ionization mass spectrometry (DAPNe-NSI-MS) has allowed the forensic science community to use extremely small sample volumes for trace analyses with increased sensitivity and resolution [26–29]. Nanomanipulation has already been proven to be a valuable technique. The nanomanipulator is able to extract cocaine trace particles directly from a single rayon fiber and extract ultra-trace drug residues retrieved from an electrostatic lifting process [27,28]. The cleavage and extraction of peptides from resin beads and extracting the lipid contents directly from organelle preparations of plant tissues has been proven to be successful using the nanomanipulator [26,29]. Nanomanipulation can be effectively coupled to nanospray ionization mass spectrometry providing picomolar sensitivity and having the capability to analyze femtograms to attograms of material [28]. Nanospray ionization has been able to reduce the required sample volume to as low as 300 nL. Once the extraction is complete, the sample can be directly analyzed using MS, thereby reducing the sample preparation procedure and time. The key advantage of using the nanomanipulation technique for the extraction of ink is that the process does not leave visible destructive foot prints on the surface of the document, which enables the analyst to retain the document in its original form.

This innovative approach is able to facilitate the analysis of modern inks and inks from different eras. Modern ink is made from organic pigments and dyes with sometimes small amounts of metal present, along with solvents and other additives [15]. Compositions using carbon were utilized four to six thousand years ago (India ink), and Japanese Sumi ink came later during the 1300-1400B.C. [30,31]. Carbon-based inks are typically made out of ground soot or charcoal with other additives and binding agents to form a liquid, whereas commercially available India inks use shellac and borax [32]. Another popular ink, iron gall, was developed during the Middle Ages and consists primarily of tannins obtained from plant galls mixed with iron (II) sulphate and natural gum [33,34]. The ink contains a large amount of iron (II) ions along with some significant Zn, Mn, Ca, and Mg ions [35]. Unfortunately, free iron ions present in the iron gall ink can cause corrosion of the paper and results in damaged historical manuscripts. Identifying the cause of corrosion can lead to the proper treatment and preservation of historical documents; in this case, the phytate process has been used to stabilize documents written with iron gall inks [36]. The metal ions present in iron gall ink can be efficiently extracted using chelating agents and subsequently analyzed. Regardless of whether the ink composition is modern or ancient,

organic or inorganic, this new approach is able to identify and characterize the ingredients, illustrating the potential diversity of the extraction chemistries.

DAPNe-NSI-MS can also identify whether pens of different ink (e.g., different brand and type) or pens of similar ink (e.g., same brand, color, and type) was used in a particular handwritten document. Determining that inks are similar could also mean that these similar inks are from different pens (e.g., where pen 1 and pen 2 are two different pens but are characterized as a black BIC Uni-Ball, 0.7 mm, roller fine-point pen). 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 [37–39] and fluorescence microscopy [39] 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 [37]. Braz et al. [40] 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. [41] 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. [42] 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. [8] 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

2. Methods

2.1. Materials

Millipore water was obtained from Milli-Q Plus (Millipore; Billerica, MA) with 18 MΩ resistivity. The glacial acetic acid, ethylenediaminetertraacetic acid (EDTA), and potassium hydroxide (KOH) were purchased from Mallinckrodt Baker Inc. (Phillisburg, NJ). Optima LC/MS methanol from Fischer Scientific (Fair Lawn, NJ) was used and iron gall ink was purchased from John Neal Books (Greensboro, NC). Sharpie ultra-fine point permanent markers (red, blue and black) were purchased from Newell Rubbermaid office products (Oak Brook, IL), the Japanese Sumi ink was purchased through Shopatron, Inc. and Yasutomo and Company (Burlingame, CA) and Higgins Calligraphy India inks were purchased from Chartpak Inc. (Leeds, MA). The extraction solvent for iron gall ink consisted of 0.0021g of EDTA dissolved in 4 mL of water and 350 μL of 0.2 M KOH. The solution was then stirred for complete dissolution of EDTA and denoted as the EDTA extraction solvent. The extraction solvent for carbon-based inks and modern inks was made using 1:1 MeOH:H_2O (v:v) and 1% glacial acetic acid (MeOH/H_2O/1%Ac).

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

2.2. 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 [26,28,43,44] and the direct analytical scheme used for the extraction of ink from documents is described thoroughly by Huynh et al. [43]. 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).

2.3. Direct Extraction Analytical Scheme

A schematic representation of the nanopositioner maneuvered over the document, using a joystick controller, is shown in Figure 3(a). Nanospray capillary tip was loaded with 10 μL of the extraction solvent and inserted into the nanopositioner. A small droplet was made with 2 μL of Millipore water on top of the letter for extraction. The droplet is injected with a tiny amount of extraction solvent using an injection pressure of 15 psi and allowed to sit for 15-20 seconds, letting the ink dissolve into the droplet. The droplet is then extracted with a fill pressure of 35 psi. The extracted solvent is directly analyzed in the mass spectrometer without any further modification. Figure 3(b) illustrates the direct extraction of ink from a document placed on top of the Nikon AZ 100 microscope stage.

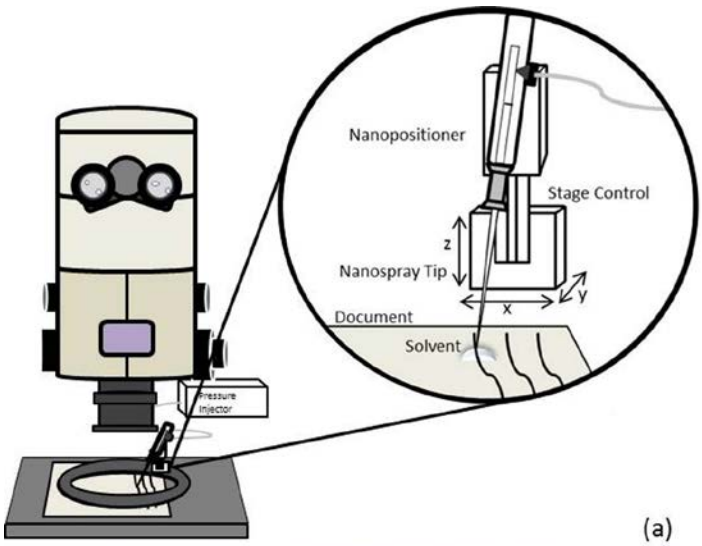

Figure 3. (a) A schematic representation of the nanopositioner maneuvered over the document, using a joystick controller and (b) the direct extraction of ink from a document placed on top of the Nikon AZ 100 microscope stage.

2.4. Analyzing Modern and Ancient inks

Iron gall ink classification

The electrospray ionization method was used for the initial identification of peaks in iron gall ink and then tested on a document using the DAPNe-NSI-MS. A 1 mg/mL solution of iron gall ink was prepared in water. A background spectrum was collected using a 1:1 (v:v) mixture of the EDTA and MeOH/H_2O/1%Ac extraction solvents. After the background was collected, 500 µL of the iron gall ink solution was added to 500 µL of the 1:1 (v:v) EDTA:MeOH/H_2O/1%Ac solvent solution and analyzed in negative ionization mode since both EDTA and metal-EDTA complexes have negative charge. A spray voltage of 3.5 kV and a flow rate of 5µL/min were used for ESI-MS, spray voltages of 2.5kV were used for DAPNe-NSI-MS and mass range of *m/z* 130 to 500 was used for all spectra.

Organic dyes and carbon based inks classification

The nanospray ionization technique identified and characterized the resins and vehicles in Sharpie Ultra-fine permanent markers (red, blue, and black), India ink (MPN: 4415, 4425, and 44314), and Japanese Sumi ink. The extractions were analyzed in positive mode and had a set extraction voltage of 2.5 kV. The scan ranges for the dyes and Japanese Sumi ink were *m/z* 50 to 500 and *m/z* 100 to 1000 for the carbon based inks.

Oxidation of polyethylene glycol

The oxidation of polyethylene glycol (PEG) was simulated by applying incandescent heat every four hours up to twenty-four hours on waterproof and non-waterproof India inks (MPN:4415 and 4425), where a sample was taken between each four hour increment. The maximum temperature resided between 37-40˚C with a 60 watt bulb.

2.5. Analyzing forged documents

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).

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 analyzed through NSI-MS without further modification.
Extractions were conducted on three different locations of the fraudulent ink entries: (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.

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) [43,45–49] 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.

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 2.5 were used.

3. Results

3.1. Analyzing modern and ancient inks

Solvent Selection

An appropriate solvent should be selected to optimize extraction and the integrity of the document. The extraction solvent can be modified to target different chemistries in different inks. An EDTA extraction solution was proven to chelate iron (II) and manganese (II) ions, and MeOH/H_2O/1%Ac successfully extracted organic components of ink. Other extraction solvent components such as dichloromethane (DCM), acetonitrile (ACN), ethanol and dimethylformamide (DMF) can be used for inks [50]. In addition, an appropriate chelator may be added to the extraction solvent for targeting a particular metal. Weyermann *et al.* analyzed Blue Parker ® ballpoint pen entries extracted with DCM [51] and Laporte *et al.* [52] used ACN as the extraction solvent for over 600 black and blue ballpoint inks from 60 different companies. Li *et al.* [53] analyzed 30 black gel pen ink samples collected from different factories in China, spanning different brands for any given factory, in which all were extracted by methanol. DMF showed successful ink extractions from 69 red ink pastes of seals which contained dyes or pigments as colorants, hydrocarbon, plant or mineral oil as vehicles, and other additives [50]. The compatibility of extraction solution with the MS and dissolution of the ink are the limitations of the solvent selection. The nanospray ionization solvents and the solvent extraction technique are valuable assets and more efficient than other minimally-destructive techniques. LDI-MS is a harmful technique due to the ink entry paper being cut into small pieces prior to analysis [13][17]. However, Matthews *et al.* [14] has shown promise with imperceptible damage to the surface of the document by removing a single ink bearing paper fiber. The technique involves an individual extricating a fiber loose and then obtaining it by tweezers, increasing the potential for human error. DAPNe-NSI-MS is very competent due its precise movements using a joystick controller. Although both techniques are almost equally non-destructive compared to each other, removing single ink bearing paper fibers is more time-consuming and less efficient during sample extraction. Moreover, Giurato *et al.* [54] uses a home-modified AP/MALDI-MS system for the investigation of organic dyes and pigments used to print books from the early 1900s. The sample preparation involves depositing a matrix aerosol over 2-3 mm^2 surface area, waiting for the solvent to evaporate and matrix to crystallize before analysis. No visible alterations were observed on the analyzed regions, making this technique eligible for comparison. DAPNe-NSI-MS requires only femtograms to attograms of material and requires a sample volume as low as 300 nL; conversely, the ionization process of AP/MALDI-MS requires excess matrix to be maintained and utilizes a larger sampling area.

Determination of iron gall ink from a document

Iron gall ink has been characterized in Figure 4 and the ions summarized in Table 1. The background spectrum obtained a distinct peak at *m/z* 291.00, as shown in Figure 4(a). The results

obtained indicate that iron gall ink has iron (II) and manganese (II) ions present, confirming that both metal ions can be efficiently chelated using EDTA (Figure 4(c)) [55]. These results are consistent with the study conducted by Bulska *et al.* [56] on written heritage using laser ablation inductively coupled mass spectrometry. Despite the fact that the ink contains more iron than manganese, the spectrum shows relatively higher intensity of manganese complex. This may be that most of the iron has been oxidized leaving less iron for chelation.

Method	Ion	*m/z*	Relative Intensities
ESI-MS	$[EDTA-H]^-$	291.00	16.57
	$[EDTA-4H+Fe]^-$	344.75	13.13
	$[EDTA-3H+Mn]^-$	343.82	99.57
DAPNe-NSI-MS	$[EDTA-H]^-$	291.00	5.54
	$[EDTA-4H+Fe]^-$	344.75	12.32
	$[EDTA-3H+Mn]^-$	343.82	99.37

Table 1. Summarized ions and components in iron gall ink obtained by an EDTA extraction solvent using ESI-MS and DAPNe-NSI-MS.

Determination of Organic Dyes from a Document

Sharpie ultra-fine point permanent markers were used to study the extraction of organic dyes. Currently, red pen ink is known to contain one or both Rhodamine B and Rhodamine 6G dyes. During photodegradation, Rhodamine B and Rhodamine 6G would expect to lose CO_2 (*m/z* 399) and an ethyl group (*m/z* 415), respectively [57][58]. Figure 5(a) indicates that the red Sharpie contains Rhodamine 6G where *m/z* 443.24 is the parent peak and *m/z* 415.10 represents the loss of an ethyl group. Basic Blue 7 is a commonly used dye for blue inks which gives a peak at *m/z* 478.00 as shown in Figure 5(b). A peak at *m/z* 372.53 signifies the extraction of another commonly used blue dye called Basic Violet 3 and gradual N-demethylation for Basic Violet 3 produces peaks at *m/z* 330.60 and 316.00 [15]. Unfortunately, no specific dye peaks could be assigned to the extraction of black ink but diethylene glycol (DEG) was successfully extracted and gave a peak at *m/z* 129.80.

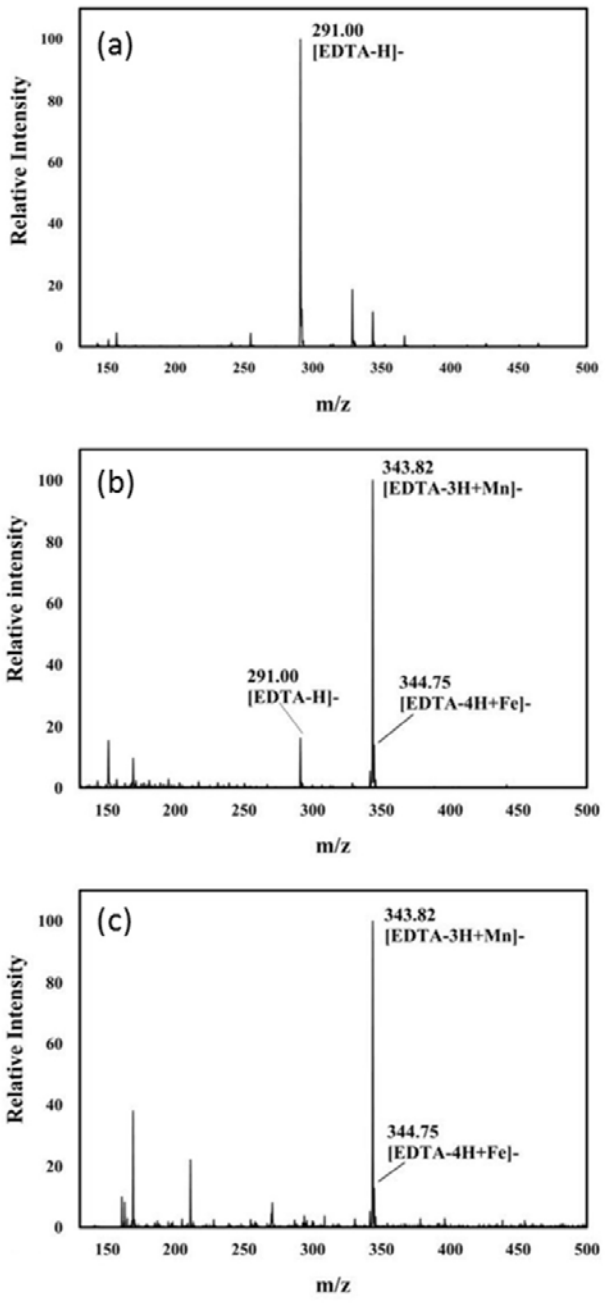

Figure 4. (a) Background spectrum of EDTA extraction solution. (b) ESI-MS spectrum of prepared iron gall ink solution. (c) NSI-MS spectrum of iron gall ink extracted from paper.

Determination of carbon based ink from a document

All the analyzed carbon-based inks contained polyethylene glycol (PEG), except the Japanese Sumi ink. Polyethylene glycols are commonly used as binders and thickening agents in the recipe of ink pens [17]. The PEG ions were detected as sodiated and protonated species ($[M+Na]^+$ and $[M+H]^+$) with a distinctive separation of 44 amu between each PEG signal [16,17]. The separation of 44 amu is derived from the monomeric ions of PEG ($-OCH_2CH_2-$) ions. Each ink is shown to produce a footprint that uniquely characterizes it. In Figure 6(a), the waterproof drawing ink (4415) contains the only protonated PEG peak at *m/z* 327.09, $[HO(C_2H_4O)_7H+H]^+$. The non-waterproof India ink (4425) produces a signal at *m/z* 156.89 which indicates another binding agent, ethoxy diglycol with a sodium adduct, in addition to PEG (Figure 6(b)). The

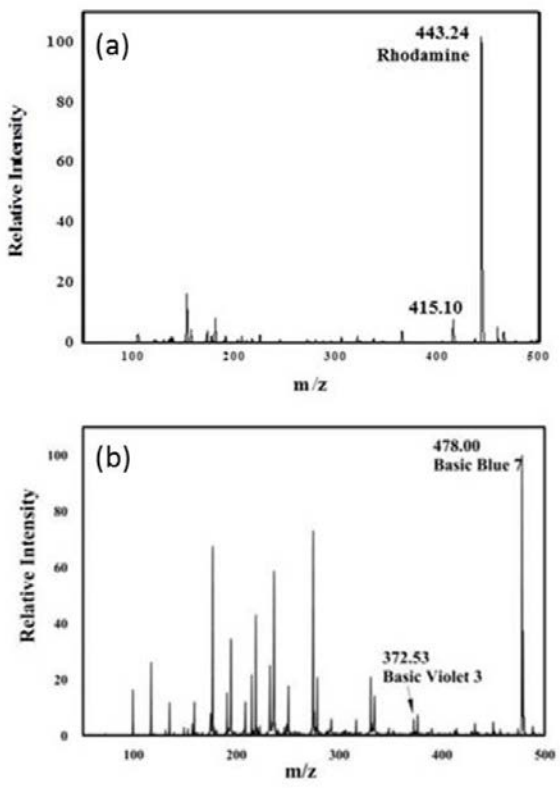

Figure 5. NSI-MS spectra of (a) red Sharpie signifying the presence of Rhodamine 6G and (b) blue Sharpie indicating Basic Blue 7 and Basic Violet 3.

waterproof calligraphy ink (44314) is shown to only contain PEG as its main binding agent, shown in Figure 6(c). Figure 7 confirms that Japanese Sumi ink contains diethylene glycol and ethoxy diglycol as its main binder and thickening agent. The peaks present at *m/z* 129.86 and *m/z* 156.89 verify sodiated adducts of diethylene glycol and ethoxy diglycol, respectively.

Oxidation of PEG in carbon-based inks

The degradation of PEG in 4415 (waterproof ink) and 4425 (non-waterproof ink) was observed as heat was applied over time. Applying incandescent light to ink strokes speed up the process of aging, inferring a simulated aging study [6].The degradation was calculated by the relative peak area (RPA) for each peak.

In Figure 8, the peaks monitored were *m/z* 613, 656, and 700 which concluded a 25.20%, 24.63%, and 55.93% decrease of PEG in the waterproof ink, respectively; however, the non-waterproof ink which shows a 74.39%, 78.64%, and 80.64% reduction. Figure 8 illustrates the different rate of oxidation caused by the waterproof and non-waterproof characteristic of the ink. Han *et al.* [59] investigated the mechanism of thermal degradation of PEG in air at 80˚C and reported that PEG and linear polymers undergo molecular weight reduction and diminution of chain length during the process of degradation caused by a random chain scission oxidation mechanism, resulting from the bond scission in the backbone of the macromolecules. Upon application, inks begin to age and oxidize once deposited on the paper surface due to solvent loss, resin polymerization and dye degradation [60]. As proof of concept, DAPNe-NSI-MS has the capability to detect the oxidation products of PEG.

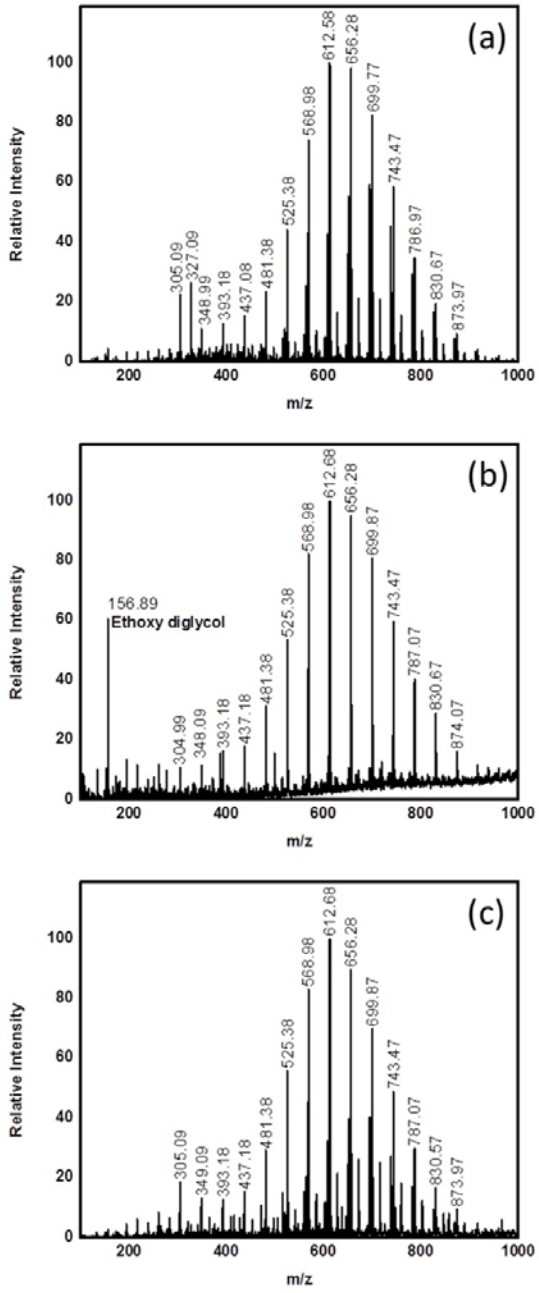

Figure 6. Mass spectra of India inks: (a) waterproof drawing ink (4415), (b) non-waterproof ink (4425), and (c) waterproof calligraphy ink (44314).

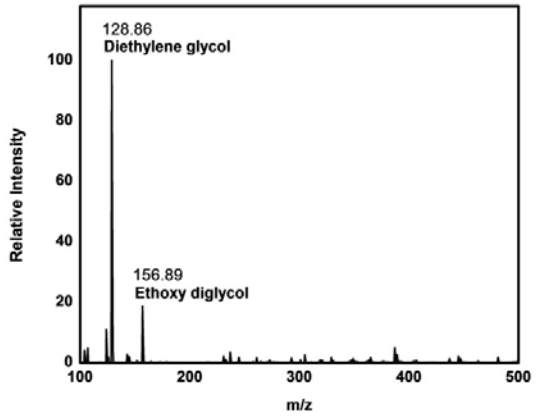

Figure 7. Mass spectrum of Japanese Sumi ink showing two binding agents.

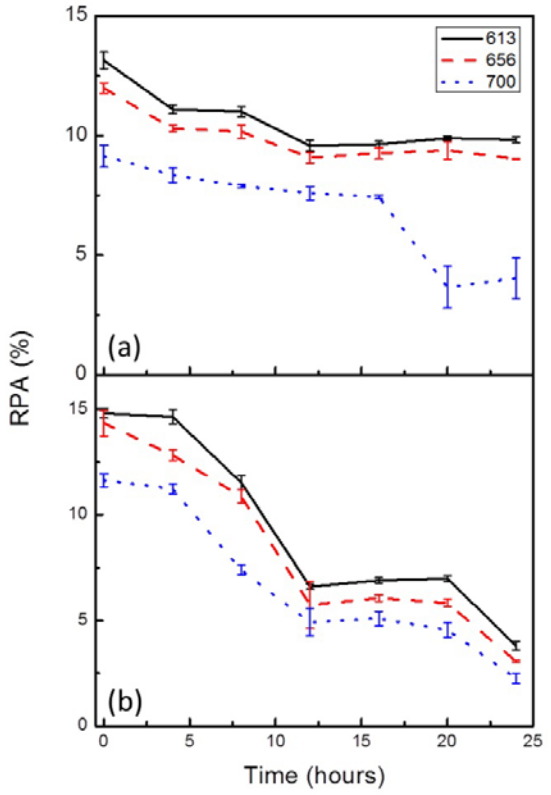

Figure 8. Percent RPA of (a) waterproof (4415) and (b) non-waterproof (4425) drawing ink.

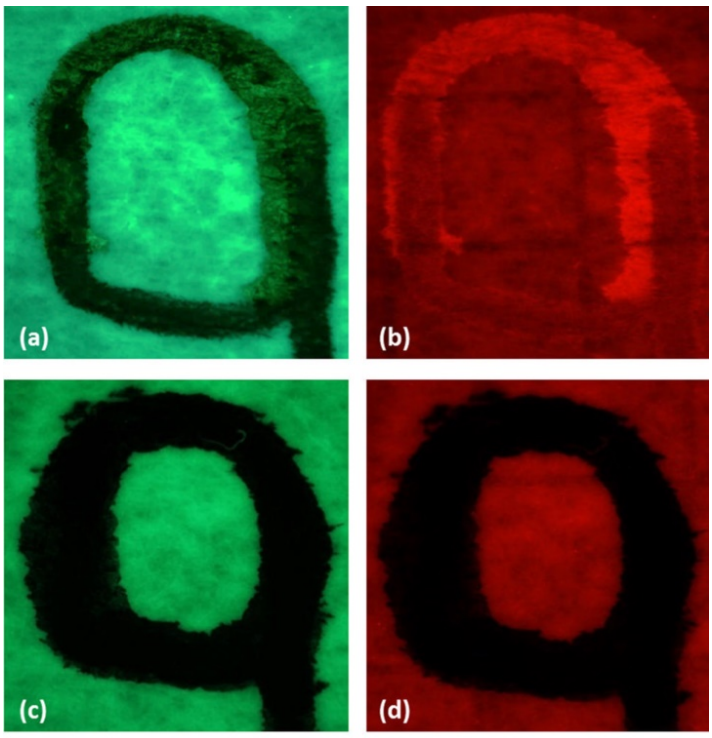

Figure 9. Fluorescent images of the altered text where (a) and (b) was modified with two different black pens, and (c) and (d) was modified with the same black pen. (a) and (c) used a blue excitation filter (420-495 nm) and (b) and (d) used a green excitation filter (510-560 nm).

3.2. Analyzing forged documents

Fluorescence Microscopy

The emission of inks is shown in Figure 9. Noticeable alterations can be observed from Figure 9 (a, b). Figure 9(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 9 (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 (e.g., same brand, type, and color).

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 [37,61]. 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 [62]. 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) [62]. Inks contain dyes, pigments, resins, and binding agents that absorb light strongly in the visible region [63]. 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 [64].

Figure 10 shows the result of the altered four where the sample in Figure 10(a, b) was altered using different pens and the sample in Figure 10(c, d) was modified using the same pen. The images in Figure 10(b, d) are overlaying the actual written sample. Human error in making these samples cause variations in the scale. The Raman mapping analysis

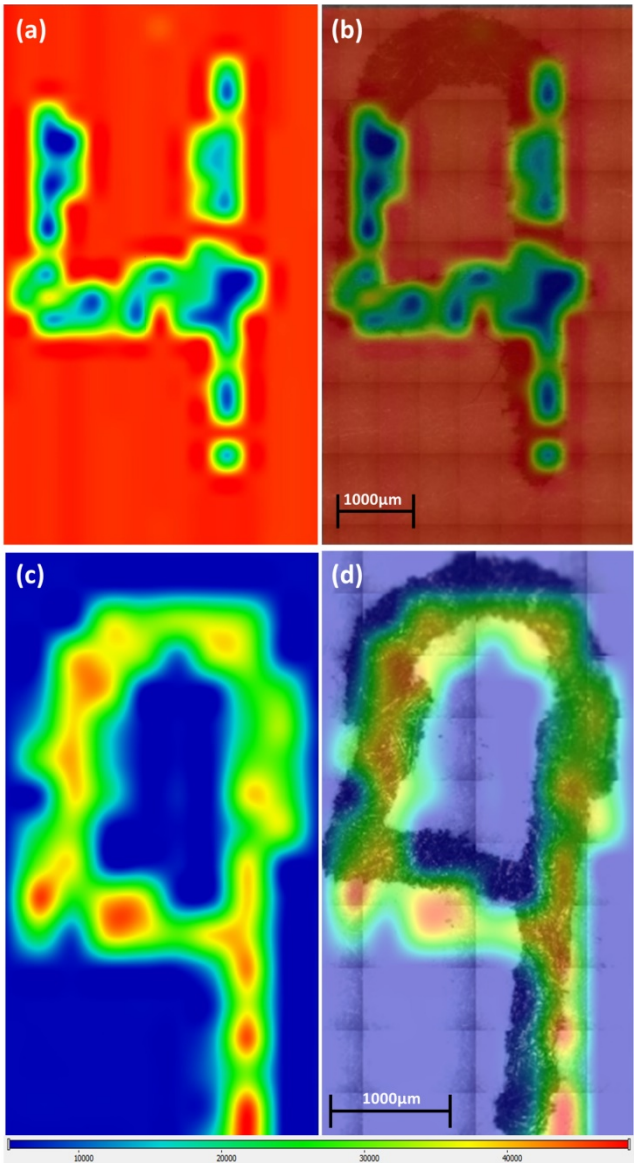

Figure 10. Raman mapping of the number four altered into a nine where (a, b) was modified using different pens (displayed at wavenumber 283 cm^{-1}) and (c, d) was modified using the same pen (displayed at wavenumber 497 cm^{-1}). (b) and (d) are the Raman images overlaying the actual sample. The map intensity bar ranges from 5181 to 48426.

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 [38].

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 10(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 10(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}.

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 11, 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 [6]. Components from both black ink pens are shown in Figure 11(c), where the extraction was conducted at the intersection of ink pen 1 and 2. Similar results were observed when

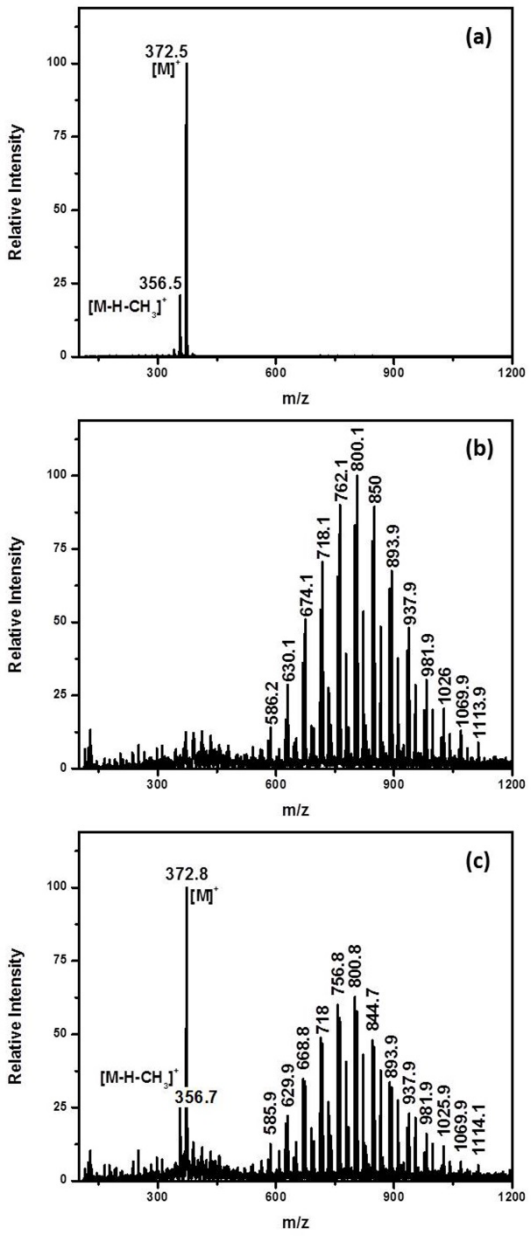

Figure 11. Spectra of (a) black ink pen 1, (b) black ink pen 2, and (c) the intersection of black ink pen 1 and 2 using DAPNe-NSI-MS.

two different red pens were used. Components from both red pens were successfully extracted at the intersection (Figure 12(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. [65] characterized red dyes found in ballpoint pens using laser desorption mass spectrometry, including New Fuschin found at m/z 330. Ink pens can become too acidic; therefore, triethanolamine is often used to regulate the pH of the ink preventing damage to the pen [65]. 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-C2H5]+).

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 [66]. 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. [66] compared fresh PEG with PEG aged in a vacuum and almost no degradation occurred. 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 13(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 14.

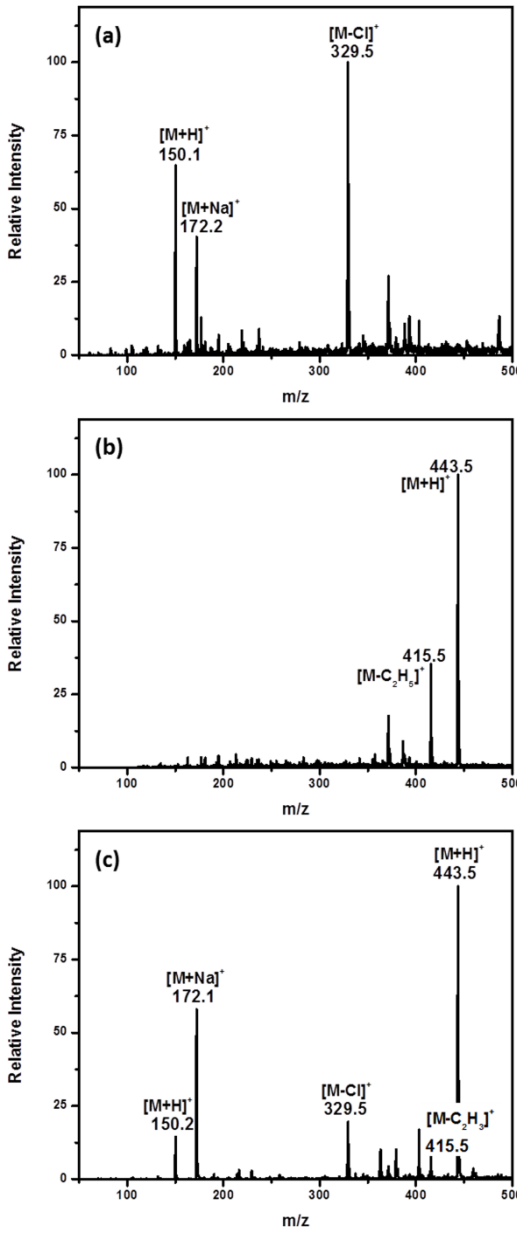

Figure 12. Spectra of (a) red ink pen 1, (b) red ink pen 2, and (c) the intersection of red ink pen 1 and 2 using DAPNe-NSI-MS.

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 [59][60]. In this case, PEG is thermally degrading, leading to chain length reduction and lower molecular weight [60][59][67]. Upon application, the inks used to modify the number similarly follow the oxidation trend seen in Figure 14 [43]. 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 similar pens can be distinguished because the ink was placed on different days.

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:H2O (1:1) with 1% acetic acid as the extraction solvent, respectively (Figure 15). In a previous study,

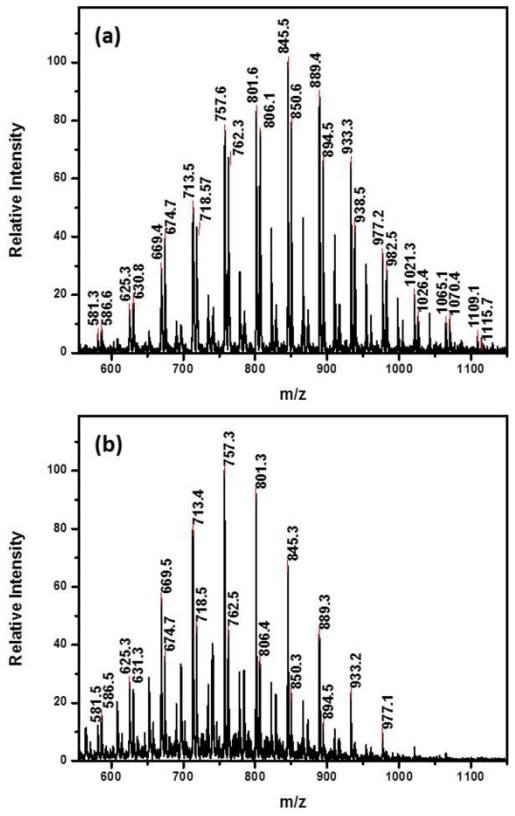

Figure 13. Spectra comparison of the modified number where (a) is the ink from the number four (24hours old ink) (b) is the ink from the nine (fresh ink) using the same black pen. Extractions were conducted using DAPNe-NSI-MS.

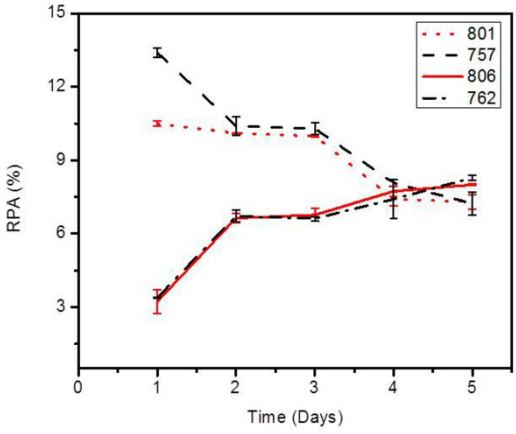

Figure 14. The percent RPA of monitored PEG peaks (*m/z* 757, 762, 801, and 806) from a black pen over a 5 day period (n=3).

EDTA was incorporated into the extraction solvent in order to chelate Fe and Mn ions from iron gall ink [43]. Being able to extract different components from the same ink entry is very important, especially when similar ink pens have 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 2. The peak intensities are first normalized and then assigned points based on the rating scale. The rating scale is based on a normalization to 100 but this can relatively be altered. For example, in Figure 13(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 2. A rating scale used to convert relative peak intensities into points.

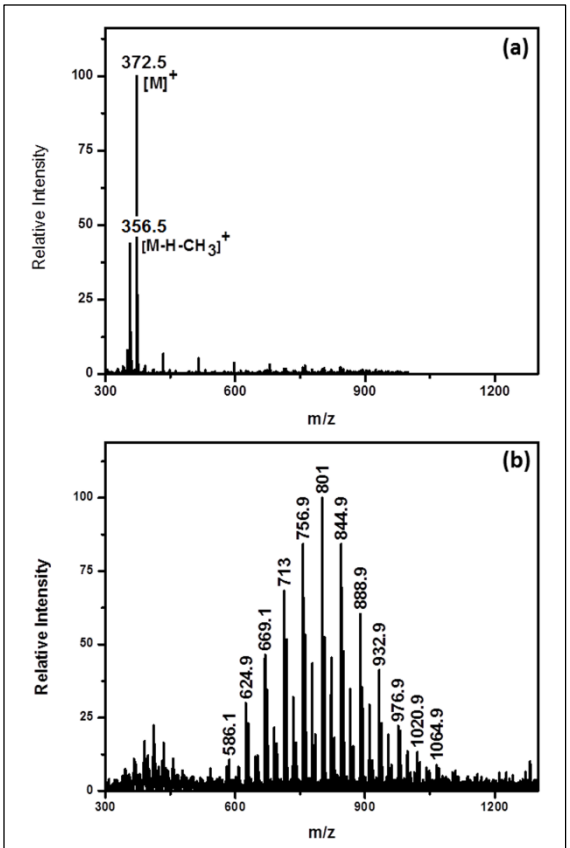

Figure 15. Spectra of Uni-ball black (waterproof) pen using two different extraction solvents, (a) methanol:chloroform (1:1) with 0.1% ammonium acetate and (b) methanol:H_2O (1:1) with 1% acetic acid.

Three different solvents were used on eleven different pens to illustrate how the intensities are affected by the extraction solvents. Table 3 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. [57] when analyzing a blue BIC pen. The three degradation ions that belong to Crystal Violet in Table 3 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 [57]. Basic Yellow 2 was also confirmed by J.A. Denman et al. [68] 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 4 lists only the total points for each pen. All three solvents, in Table 4, 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:H_2O (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.

			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	$[M\text{-}Cl]^+$	Basic Yellow 2	9	9	9
344.0	$[M+2H\text{-}(CH_3)_2]^+$	Crystal Violet	0	1	0
358.4	$[M+H\text{-}CH_3]^+$		3	4	1
372.5	$[M]^+$		6	5	2
		Total	18	19	12

Table 3. 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 4. A comparison of a variety of mass spectrometric intensities from several different pens, using three different solvents, based on a rating scale.

4. Conclusions

A new technique for the extraction and analysis of ink and its components from the questioned documents has been developed. Using NSI-MS provides the advantage of analysis with limited sample volume. It offers high resolution, low limits of detection and direct analysis eliminating further sample preparation and saving analysis time. The procedure does not leave any visible footprint on the surface of the document. This is advantageous for the analysis of historical, governmental or other documents where maintaining the integrity. As can be seen from the spectra, the metal ions present can be efficiently extracted using a chelating agent like EDTA. This approach can also be applied for extraction of trace metal ions from different substrates for analysis. 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. When similar ink (e.g., same brand, type of pen, and color) is involved, the oxidation of certain components in the ink formula may be monitored in order to discriminate that the inks were placed at different times. It is also important to note that similar inks may not be exactly alike when still inside the pen, even if they were manufactured to be exactly alike. 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 as well as identifying the characteristics of the ink alone, the forensic community will be able to further their studies in chemical composition of fraudulent documents.

4.1. Implications for Policy and Practice

Benefit to the Crime Lab

This research can have an immediate impact for forensic investigations. Document expert Michael Weldon from Weldon & Associates, board certified forensic document examiner, has brought documents from clients to be examined in our lab. We were able to collaborate with Michael Weldon to aid in the authenticity of contracts, and our method has assisted Michael Weldon to test the authenticity of documents. The analysis of trace residue within document and handwriting data could pinpoint or eliminate potential suspects, and lead to acceptable court resolution. Chemical signature data from the extracted residue can offer the document database another avenue to group and characterize documents. The majority of inks offer chemical signature pointing to manufacturing that could be acquired using this method, thus linking group and distribution chains together with documents.

Educational Impact

The novel instrumentation developed will greatly add to the research and educational services within the North Texas area and the scientific community at large. The research project encompassed multiple novel research areas in chemical, biochemical, forensic, and materials science disciplines. This instrumentation led to the development of other novel devices and broadened the current field of portable instrumentation. The PI provided opportunities for trainees with diverse educational and cultural backgrounds, including female and minority students, high school students and teachers, undergraduate students, in addition to graduate students and postdoctoral fellows. UNT is a major research university with over 33,000 students; over 100 graduate students (mostly Ph.D.) are currently enrolled in the Department of Chemistry. UNT is home to the renowned Texas Academy of Math and Science (TAMS) early college-entry program for high school students who are outstanding in mathematics and science.

4.2. Implications for Further Research

Now that the examination of inks have been established, the analyses of falsified official government certificates needs to be determined. The authenticity of financial records, contracts, and other important documents is of great importance; especially if they are in question. These forgeries become even more crucial when the written text is masked with a sharpie or disguised with other writing devices. In addition, official government identification cards (IDs) have been duplicated illegally. The need for examining counterfeit articles is essential.

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6. Dissemination of Research Findings

6.1. Presentations

Verbeck, G.F., Trace Analysis of Illicit Chemistries using Direct Analyte Probe Nanoextraction (DAPNe), Sanibel Conference on Security and Forensic Applications of Mass Spectrometry, Clearwater Beach, FL, January 2015, INVITED.

Verbeck, G.F., *"Nanomanipulation: Identification of Fraudulent Documents through Analysis of Ink, Paper, Paint, and other Counterfeit Materials"*, Document Security Alliance Meeting, Washington, D.C., October 2014, INVITED.

Huynh, V.; Sasiene, Z. J.; Mach, P. M.; Golden, T. D.; Verbeck, G. F., SciX, featuring FACSS, in Providence, Rhode Island *Fall 2015*, Speaker Presentation. Direct analyte-probed nanoextraction (DAPNe)-coupled to nanospray ionization mass spectrometry applied to document analysis.

Huynh, V.; Williams, K. C.; Golden, T. D.; Verbeck, G. F., Southwest Regional Meeting, hosted by American Chemical Society (DFW local section) in Fort Worth, TX *Fall 2014*, Speaker Presentation. Forensic application of nanomanipulation applied to document inks.

Huynh, V.; Leveille, J. M.; Joshi, U.; Golden, T.D.; Verbeck, G. F., American Chemical Society meeting in Dallas, TX *Spring 2014*, Poster Presentation. Nanomanipulation-coupled to nanospray mass spectrometry applied to document and ink Analysis.

Huynh, V.; Williams, K. C.; Golden, T. D., Verbeck, G. F., Toulouse Graduate School: The Graduate Exhibition Poster Session, *March 1, 2014*. Nanomanipulation-Coupled to Nanospray Mass Spectrometry Applied to Document and Ink Analysis. Placed 3rd in physical and mathematical sciences division.

Huynh, V.; Leveille, J.M.; Joshi, U.; Golden, T.D.; Verbeck, G. F., Toulouse Graduate School: The Graduate Exhibition Poster Session, *March 3, 2013*. Nanomanipulation-Coupled to Nanospray Mass Spectrometry Applied to Document and Ink Analysis.

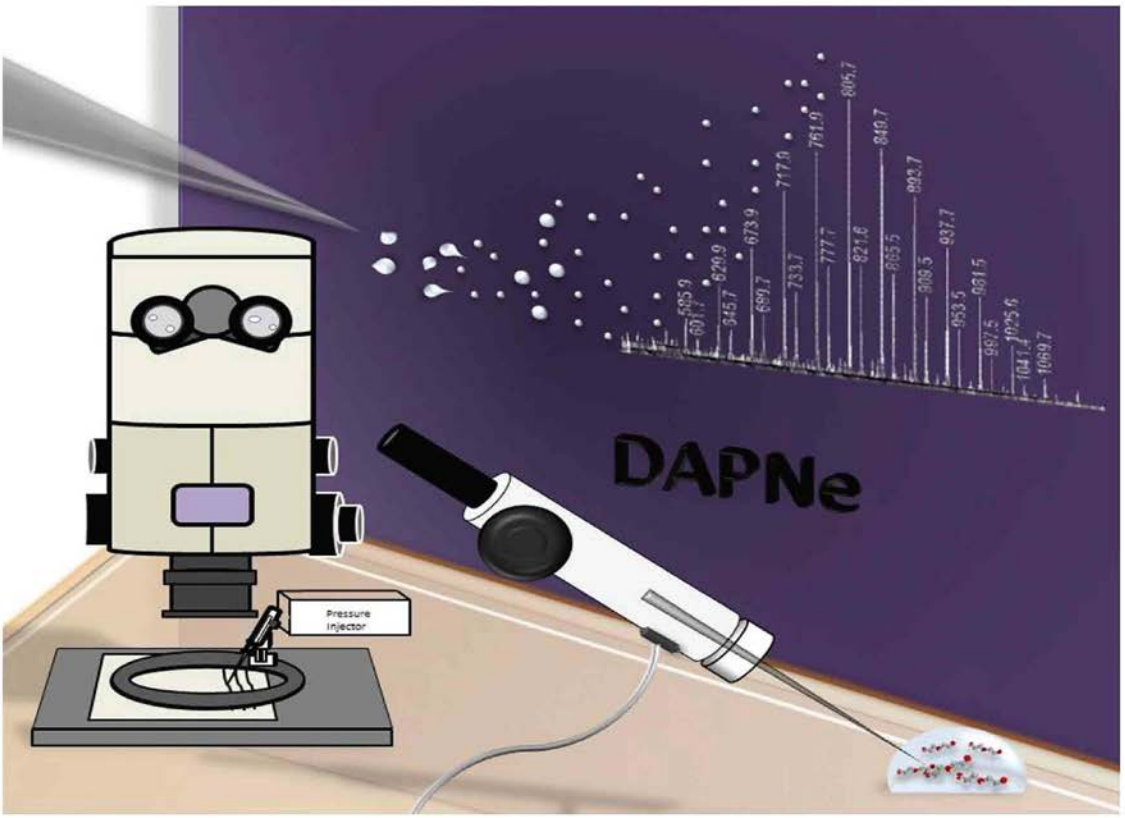
6.2. Publications

Huynh, V.; Sasiene, Z.J.; Mach, P.M.; Golden, T.D.; Verbeck, G.F., "Laser Ablation-Coupled with DAPNe-NSI-MS Applied to Redacted Documents", *Sci. & Just*. Accepted (January 2016)

Huynh, V.; Williams, K. C.; Golden, T. D., Verbeck, G. F., Investigation of falsified documents via direct analyte-probed nanoextraction coupled to nanospray mass spectrometry, fluorescence microscopy, and Raman spectroscopy, *Analyst* 140 (**2015**) 6553-6562.

Huynh, V.; Joshi, U.; Leveille, J. M.; Golden, T. D.; Verbeck, G. F., Nanomanipulation-Coupled to Nanospray Mass Spectrometry Applied to Document and Ink Analysis, *Forensic Science International* 242 (**2014**) 150-156.

Analyst Cover:

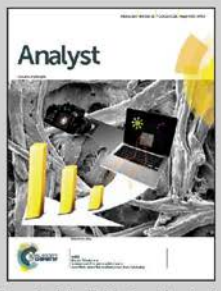


Showcasing research on direct analyte-probed nanoextraction coupled to nanospray ionization mass spectrometry (DAPNe-NSI-MS) applied to document ink analysis from the team of Professor Guido F. Verbeck at the Department of Chemistry, University of North Texas, Denton, Texas, USA.

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

The non-destructive DAPNe technique extracts ultra-trace amounts of ink from full documents, and coupling to NSI-MS achieves higher resolution and picomolar sensitivity, requiring as low as 300 attograms of analyte. The direct extraction method utilizes a piezoelectric-controlled nanopositioner with a removable AuPd-coated nanospray tip, i.d. of 1 µm, where extraction/aspiration is executed *via* pressure injector. The extraction solvent used to pre-fill the nanospray tip can be altered to target specific constituents in the ink such as dyes, organic stabilizers, and inorganic metals.

As featured in:



See Guido F. Verbeck *et al.*, *Analyst*, 2015, **140**, 6553.

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