THE DETECTION OF INK DYES BY LASER DESORPTION MASS SPECTROMETRY COUPLED WITH THIN-LAYER CHROMATOGRAPY AND THE USE OF PHOTOCHEMISTY FOR DYE CHARACTERIZATION

By

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ABSTRACT

THE DETECTION OF INK DYES BY UV LASER DESORPTION MASS SPECTROMETRY COUPLED WITH THIN-LAYER CHROMATOGRAPHY AND THE USE OF PHOTOCHEMISTRY FOR DYE CHARACTERIZATION

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UV laser desorption mass spectrometry (LDMS) has proven to be a viable technique for the detection and characterization of ink dyes directly from a paper substrate. Advantages to using LDMS include high sensitivity and selectivity for detecting dyes, minimal sample preparation, short analysis time, and minimal sample destruction. The most important aspect of the technique in terms of questioned document examination is that written lines of a single pen stroke can be analyzed directly from paper; hence no extraction step is necessary.

Thin-layer chromatography (TLC) is a technique that compliments UV LDMS in identifying ink dyes. TLC is a simple analytical technique that provides information on the color and the number of dyes present in the ink. TLC was indirectly coupled with LDMS. Direct coupling of TLC eliminates the extraction step and preliminary experiments showed the method has potential when coupled with LDMS.

The use of photochemistry with subsequent LDMS analysis has been successfully utilized to characterize ink dyes. Generally, structural information for characterizing the dyes used in this research is limited in LDMS. When dyes are photochemically induced to react they can form photodegradation products and can be detected by LDMS and are structure dependent, hence, they can be used to characterize the dye.

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D.J., I couldn't have survived these insane months without you by my side. You gave me strength and love when I needed it the most. I thank you for not giving up! I give thanks to God for watching over me and bringing us together.

Finally, I thank the National Institute of Justice for funding this research project for two years. We couldn't have successfully completed the project without the financial support.

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Chapter One: Introduction

Dye Classification

The classification of colorants (dyes and pigments) can be found in the Colour Index (CI). The latest edition was revised in 1971 and classifies over 10,500 dyes. Colorants are classified based on their chemical composition and coloring application[1,2]. The color and the application of the dye are represented by the CI name and the chemical composition is the foundation for assigning each dye a unique five digit CI number. The Colour Index recognizes the following 15 application classes: acid dyes, reactive dyes, metal complexes, direct dyes, basic dyes, mordant dyes, disperse dyes, pigment dyes, vat dyes, anionic dyes and ingrain dyes, sulphur dyes, solvent dyes, fluorescent brighteners, food dyes, and natural dyes[3]. The colorants used in this thesis research encompass singly-charged (cationic or anionic) and neutral dyes, specifically, basic and acid dyes. A particular metal complex pigment dye was examined as well and was used as the mass spectrometric calibrant.

Dyes are aromatic, organic compounds that absorb electromagnetic radiation including visible light with wavelengths 350 – 700 nm[4] and UV radiation which allow for easy detection in UV laser desorption mass spectrometry. Chromophores and auxochromes that are contained in the dye structure are responsible for the color of the compound and are the chemical constitutes that distinguish the dyes (20-30 various groups)[3,4]. Typical chromophores include -C=C, -C=N, -C=O, -N=N, and $-NO_2$, and quinoid rings[4,5]. Principal, functional groups such as $-NH_3$, -COOH, $-SO_3H$, and -OH comprise auxochromes[4,5] (the hydrogen atoms are frequently substituted). Some of the

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dye groups include azo (monoazo, diazo, triazo, etc.), anthraquinone, phthalocyanine, tirarylmethane, dirarylmethane, indigoid, azine, oxazine, thiazine, xanthene, nitro, nitroso, methane, thiazole, indamine, indophenol, lactone, aminoketone, and hydroxyketone dyes[3,5].

This research has focused on acid and basic dyes used in ballpoint pen inks and a pigment dye, specifically, a metal complex, used in gel ink. In general, acid dyes are anionic containing one or more sulfonic acid ($-SO_3H$) groups and are the largest class of dyes. The CI lists about 2300 different acid dyes, but roughly 40% of these compounds are used currently in industry[5]. Azo, anthraquinone, and triarylmethane compounds comprise the majority of the acid dyes. Metainil Yellow (Acid Yellow 36) is a monoazo, acid dye containing one sulfonic acid group which was examined in this research. Basic dyes are cationic and frequently contain amine functional groups. About 5% of the dyes listed in the CI correspond to basic dyes[5]. The most common basic dyes include diarylmethane, triarylmethane, anthraquinone, and azo compounds[5]. The basic dyes examined in this research include Crystal Violet (Basic violet 3) and Victoria Blue B (Basic blue 26). Both dyes are triarylmethane compounds. Also, Rhodamine B (Basic violet 10) and Rhodamine 6G (Basic red 1) are two xanthene, basic dyes that were examined extensively and are the main examples used in this research for characterizing dyes using photodegradation and laser desorption mass spectrometry. Copper Phthalocyanine (Pigment Blue 15) is classified as a pigment dye. Specifically, the dye is a phthalocyanine metal complex containing copper. Phthalocyanine metal complexes as well as azo compounds represent the majority of pigment dyes[5]. Pigment dyes are insoluble, non-ionic compounds or insoluble salts making them suitable as colorants in

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gel ink. The molecular qualities of Copper Phthalocyanine were studied to demonstrate the basic characterization information that can be acquired as well as the dye served to be an excellent LDMS calibrant for this research. Both the non-ionic nature of the complex which allows the dye to be detected in both positive and negative-ion modes and the molecular weight of the complex make the species an ideal candidate for calibrating the mass spectrometer.

Dye Application

The application in which the dye will be used depends on the molecular structure and how the molecule interacts with the substrate. For example reactive dyes form covalent bonds (strongest interactions) with -OH, -NH, or -SH groups in the substrate material[5], the acid and basic dyes interact with corresponding ionic species (acid dyes interact with cations and basic dyes interact with anions) that are contained in the substrate through ionic bonding, and pigment dyes have the weakest intermolecular interactions with the substrate. Manufacturers take advantage of the intermolecular interactions and chemical bonding for selecting the dyes used in the industry. Frequently, ink used for the manufacture of ballpoint pens use basic and acid dyes where the production of gel inks rely on the use of pigment dyes as the colorants.

Pen Ink Composition

The selection of analytical methodology greatly depends on the species being examined. The more recent methods that have been used to examine ink have been developed based on the colorants in the ink. Although, ink is a complex mixture and,

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frequently, manufactures do not disclose the ingredients, a generalization can be made about the compounds used in the production of ink. While the specific ink composition depends on the pen type, ink is comprised of colorants (dyes) and a colorless vehicle. The vehicle serves to distribute dye onto paper. Ink patent information is available to the public and has been used to help characterize the general contents that may be used in the various kinds of ink. U.S. Patent 5,769,931 gives insight into the complexity of aqueous-based inks that may be used in a disposable, modern pen. The components include 1) polysaccharide gums; 2) viscosity modifiers; 3) polar, non-aqueous solvents such as alcohols or glycols; 4) water; 5) sequestering agents which improve stability; 6) preservatives; 7) surfactants; 8) corrosion inhibitors; 9) dyes and/or pigments; and 10) pH control agents[6]. LDMS is highly selective for dye detection and the other ink components do not interfere with the direct analysis and generally are not detected in the experiment.

Ink Dye Analysis

Selection of an Analytical Method

The greatest challenge in analyzing dyes is obtaining the structures. Some dye structures are purposely withheld by the manufacturer, yet others are unknown even to the producer. There is not one specific source that reports all the dye structures. Most of the structures collected for this work were found using two resources: *The Sigma-Aldrich Handbook of Stains, Dyes and Indicators* and the CAplus database which is accessible through SciFinder Scholar 2001, an electronic resource provided through Michigan State University. In general, the first step to obtaining dye structures is to establish the names

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of the possible dyes used in the manufacture of ink through patents. All ink patents were acquired from an online database (http://ep.espacenet.com) though the European Patent Office which contains over 30 million documents worldwide. Next, the names are used to search for the structures. Currently, over 250 dyes (names or CI number) that are possibly used in ink have been identified for this research, but only 207 structures were actually found. This poses the difficulty of identifying the dyes contained in a pen ink under investigation. According to the patents, the dyes (specifically anionic, azo dyes) used to manufacture ink appear to be *Acid Dyes* (dye classification). The majority of the dyes (~2300) listed in the Colour Index are classified as *Acid Dyes* (consisting of mostly azo, anthraquione, or triarylamethane dyes), and approximately 40% of them are currently used in production[5].

Many analytical techniques need to be compatible with the volatility and solubility nature of the dyes. Generally, dyes are manufactured as sodium or chloride salts allowing them to be rather soluble in water and ethanol, but prohibit them from being volatile. Techniques such as gas chromatography/mass spectrometry (GC-MS) require volatile samples. Some dyes are insoluble in aqueous media or organic solvents. Most extraction-based methods depend on the dye to be soluble for analysis. Any instrumental method such as high performance liquid chromatography that requires liquid samples are useless if the dyes are not able to dissolve. Many gel inks contain pigment dyes that are insoluble compounds. LDMS is compatible with insoluble dyes since no extraction step is required. Ink samples are analyzed directly from the paper substrate (written entries on a questioned document). Whenever ink is not analyzed directly there

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is a chance that the dye could be altered chemically during the extraction. Direct analysis of ink is the safest means for examination.

Dyes are frequently sold as impure mixtures[1]. The production of impure dyes is not uncommon since dye purification, subsequent to their manufacture, is arduous and expensive. If impurities do not pose a threat to the application, including the manufacture of ink, to which the dye will be used, then dye purification is not highly demanded. Some applications such as the use of laser dyes require dyes to be highly pure. In terms of ink examination, the analytical technique selected for examining dyes needs to be capable of analyzing multi-component systems. Methods such as infrared spectroscopy are typically not suitable for examining more than one constituent at a time. LDMS can easily detect multiple dyes simultaneously.

Methods for Ink Dye Analysis

Early experiments in ink analysis relied heavily on the use of chemical spot tests[7], ion migration tests[8], electrophoresis[9,10], and a variety of extraction-based methods[11-13]. Extraction-based methods are still the most widely used today[11-13]. The analysis of ink can be considered in two categories – methods for ink comparison and methods for relative ink dating. A variety of chromatographic methods have been used for ink comparison including paper chromatography[14], densitometric thin-layer chromatography (TLC) [13,15-17], gas chromatography (GC)[18], and high performance liquid chromatography (HPLC)[19-21]. Additional methods include fourier transform infrared spectroscopy (FTIR)[22,23], microspectrophotometry[24,25], and GC/mass spectrometry (GC-MS)[26]. Most of the recent literature has focused on ink comparisons, which include the use of capillary electrophoresis (CE) combined with UV/Vis and laser induced fluorescence (LIF)

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detection and particle induced x-ray emission (PIXE)[27,28], field desorption mass spectrometry (FDMS)[29], and electrospray ionization mass spectrometry (ESI MS)[30]. High performance liquid chromatography (HPLC)[31] and laser desorption mass spectrometry (LDMS)[32] have been used to examine the dye degradation for the possibility of determining the relative age of a document. Much of the current (1999 – 2002) research in ballpoint pen ink analysis has encompassed mass spectrometry and chromatography which will be evaluated and their utility demonstrated.

Mass Spectrometry

Mass spectrometry (MS) is a highly sensitive and powerful analytical technique that provides elemental and molecular level information such as molecular weight and in some cases, elemental composition that can be used for identifying and characterizing an extensive range of species with molecular weights exceeding 100,000 Daltons (Da). Mass spectrometers separate and allow for the detection of a species (in the form of gas-phase ions) based on the corresponding mass-to-charge (m/z) ratio. The relative abundance of the ions is plotted versus the m/z values yielding a mass spectrum.

Mass spectrometry is practical for a wide range of applications. There are a several mass spectrometry instruments that can be utilized, and the selection of the instrument greatly depends on the analyte being examined. The two most important parts of any MS instrument consist of the ionization source and mass analyzer. The basic design of an MS instrument is shown in Figure 1.1.

Figure 1

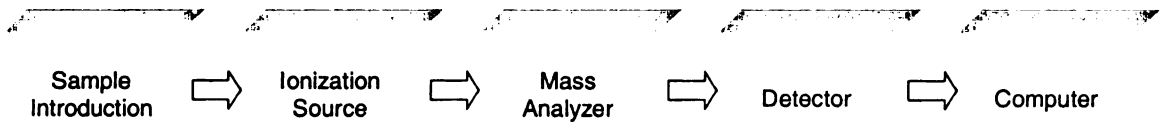

Figure 1.1: Basic schematic of a mass spectrometer

The MS experiment requires a sample to be in the gas phase; however, samples can be initially delivered or **introduced** to the instrument in a solid or liquid form. Ions are generated when the **ionization source** converts the intact species. The **mass analyzer** separates the ions based on their m/z values. The ions are collected and counted by the **detector**. Ultimately, a mass spectrum is produced as the **computer** records the data.

Mass spectrometry has been one of the analytical tools applied to the analysis of inks. Early ink studies used gas chromatography/mass spectrometry (GC-MS)[26] to examine the volatile components. Initially, mass spectrometry methods, including GC-MS which utilizes the ionization method of electron impact (EI), were limited to the analysis of volatile (high vapor pressure) compounds. In order to ionize molecules by EI, samples are required to be in the gas phase. The challenging issue of sample volatility and thermal stability was overcome by the development of desorption/ionization (D/I) techniques which yield gas phase ions from condensed phase analytes. For many years, thermally labile/nonvolatile components were not amenable to analysis by MS, although the possibility was often considered. Mass spectrometric D/I methods encompass field desorption (FD), laser desorption (LD), matrix-assisted laser desorption/ionization (MALDI), secondary-ion mass spectrometry (SIMS), and fast atom bombardment (FAB).

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The early methods of field desorption and laser desorption had limited utility. FD requires the analyte on the sample probe to be heated with a high electric field ($10^7 - 10^8$ V/cm) thus sample degradation is possible[33]. The early use of LD was limited to compounds with molecular weights of approximately 1000 Daltons and below[34]. The basic idea was to focus a pulsed laser such as a CO_2 laser onto a solid target. High laser power results in rapid heating and a portion of the sample can be heated to several hundred degrees in a few nanoseconds. At this point, desorption can occur at rates faster than those for chemical degradation (although some fragmentation has been observed) resulting in desorption of intact molecules. A portion of the sample is not only desorbed, but also ionized. Both FD and LD have been used recently for ink dye analyses[29,32,35-37].

The relatively new method, MALDI, is a D/I method that is related to laser desorption. MALDI is an LD experiment that incorporates the use of a solid matrix for the detection of the analyte. MALDI is an experiment in which an analyte such as a peptide is mixed with a large excess of matrix molecules. These matrix molecules are small, aromatic, organic molecules that have been selected because they efficiently absorb UV light at 337 nm. From an analyte/matrix solution, crystals are grown. When irradiated with light from a pulsed UV laser, matrix and analyte ions are formed, for analytes with molecular weights exceeding 100,000 Daltons. The desorption/ionization method requires a mass analyzer such as time-of-flight (TOF) that is capable of operating with a pulsed ionization source. Significant commercial developments have occurred in the past few years to make TOF MS one of the premiere MS methods.

The LDMS experiments for this research were carried out with an instrument that was designed for the MALDI experiment. In the current design of modern MALDI TOF

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MS instruments, a 100 to 400 well plate is used for sample introduction. The computer controlled sample stage can be moved such that the laser irradiates a selected sample. Thus, MALDI instruments allow for introduction of planar targets with computer control of the area being sampled by the UV laser. For the forensic applications here, the target will be, in most cases, a paper sample with dye on it (a written or printed line). No matrices were necessary for the analysis of the inks presented here. The sample is analyzed directly from the paper since the dye molecules efficiently absorb the UV light. A PerSeptive Biosystems Voyager DE (delayed extraction) TOF mass spectrometer (Farmingham, MA) was used for the analysis of dyes and is shown in Figure 1.2[38]. The instrument utilizes a pulsed nitrogen laser (337 nm, 3 nanosecond pulses at 3 Hz) and a linear time-of-flight mass spectrometer. The user-selected parameters for the LDMS experiments include an accelerating voltage of 20,000 V for detection of positive ions and -15,000 V for the detection of negative ions, an intermediate source grid voltage that is 94% of the accelerating voltage, a guide wire voltage that is opposite in bias and 0.05% in magnitude of the accelerating voltage, and an extraction delay time of 100 ns.

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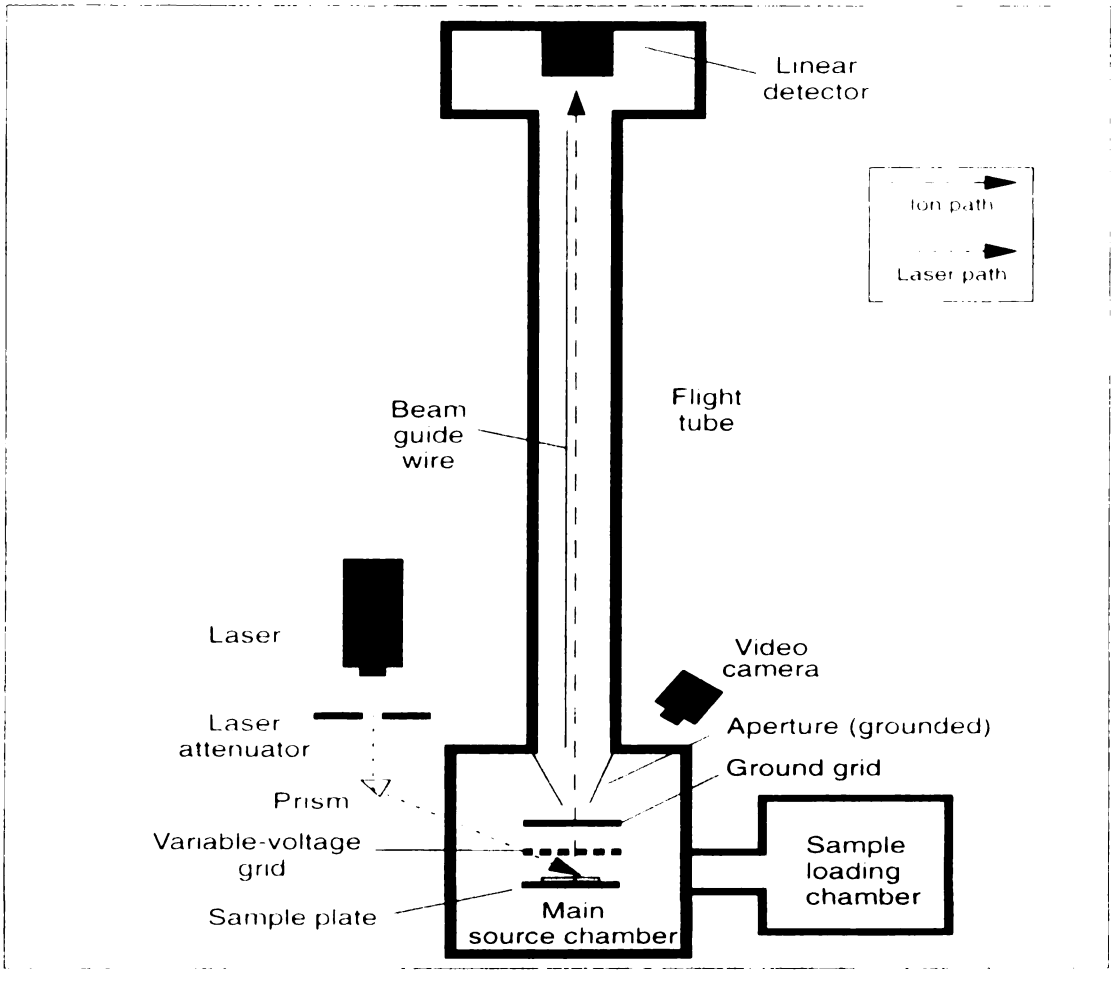

Figure 1.2: Diagram of theVoyager-DE Mass Spectrometer[38]

FAB and SIMS are similar techniques that both use particle bombardment to induce the desorption/ionization of a condensed phase sample. SIMS uses an ion beam consisting of Cs^+, Xe^+, or Ar^+ where FAB makes use of fast Xe atoms[33]. Like MALDI, a FAB experiment utilizes a matrix, usually glycerol, a viscous liquid. Both of these D/I methods have been used to analyze nonvolatile, organic dyes[39-41] and there is current literature on the analysis of colorants on paper by TOF-SIMS[42].

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Electrospray ionization (ESI) MS is another mass spectrometry technique that is capable of detecting non-volatile compounds. ESI was developed about the same time as MALDI for the analysis of high molecular weight, biomolecules, but has also been employed for the detection and characterization of species including dyes[30]. ESI is an attractive method since ions can be formed multiply-charged. Analytes are injected as liquids and pumped continuously through a needle with a flow rate of roughly 1-10 μL/min[43]. Droplets are sprayed from the end of the needle. Using a combination of a drying gas and the applied potential field (3-6 kV) causes the droplets to desolvate and become highly-charged. The coulombic explosion (charge repulsion upon evaporation of the droplets cause them to explode to form smaller droplets) results in the formation of ions that vary in m/z for the same species[43]. The number of charges that a species acquires is analyte dependent. Analytes that have several ionization sites will be highly-charged. ESI is also capable of analyzing species that lack sites of ionization, but usually required an acidic or basic additive in order for the analyte to be detected. The schematic (Figure 1.3) below depicts the ESI apparatus[43].

Recently (2002), ESI MS has been employed by Ng *et al.* for the characterization of ballpoint pen ink dyes[30]. However, the analysis of dyes by ESI has been used since the late 1990's. Most of the investigative work has focused on sulfonated azo dyes and has been carried out by Sullivan *et al.* [44,45]. Some of the dyes examined in this research were the same dyes that were characterized by Ng *et al.*, so their analyses will be discussed further and compared to LDMS.

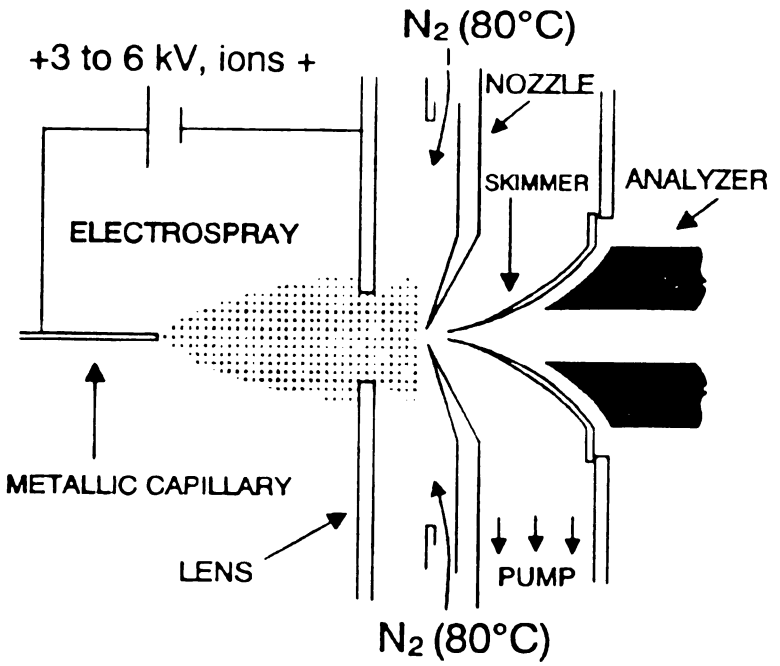

Figure 1.3: Schematic of an electrospray ionization source[43]

Most of the mass spectrometry methods that have been used to analyzed dyes have encompassed D/I methods, however, the limitation of these MS techniques is that multiply-charged dyes are not detectable directly. This fact has made ESI an attractive MS alternative for dye detection. FDMS and LDMS are the most recent D/I techniques that have been utilized for characterizing ink dyes. Recently, the examination of ink dyes has been carried out using ESI MS. The use FDMS, LDMS, and ESI MS for dye analysis will be discussed briefly and compared against one another. In Chapter Two, extensive details on dye characterization will be given for LDMS

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Field Desorption Mass Spectrometry

Field desorption mass spectrometry was used by Sakayanagi *et al.* to identify eight basic dyes in black, blue, and red ballpoint pens inks[29]. The dyes are cationic, possess a single positive charge, hence, the molecular ion was detected in positive FDMS. Once the dyes were determined, various ballpoint pen inks were compared in order to differentiate between manufactures. The m/z ratios obtained from the ballpoint pen inks used in the analysis were summarized. Some of the peaks could not be identified. Basic Violet 3 (Crystal Violet) and Basic Violet 1 (Methyl Violet 2B) are commonly found in both blue and black ballpoint pens inks. Generally, the mass spectra of blue inks contain a peak at m/z 470, which corresponds to Basic Blue 26 (Victoria Blue B). All of the red ballpoint pen inks tested gave a peak at m/z 443, which corresponds to either Basic Red 1 (Rhodamine 6G) or Basic Violet (Rhodamine B) since both of these dyes have the same elemental composition. Sakayanagi *et al.* showed that some of the ballpoint pen inks appear to be identical[29]. In order for the inks to be analyzed, the samples are required to be extracted prior to using FDMS. This may be a problem since aged inks may be hard to extract and pigment dyes are generally insoluble which eliminates inks containing insoluble pigment dyes such as those contained in gel pens using FDMS. Another disadvantage is that FDMS is a destructive method; hence, the samples are destroyed during the analysis. The dyes are extracted from paper samples and deposited on the FD emitter which is used to desorb and ionize the compounds using a high electric field. The heating of the emitter possesses a threat for sample decomposition[46] and dyes may degrade. Positive-ion mode was only reported which does not favor several ink dyes that are anionic salts, however, FD is capable of

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operating in negative ion mode which is required for the detection of anions. LDMS is also capable of operating in both positive and negative ion modes. The solubility of the dyes limits the utility of FDMS where LDMS is capable of analyzing colorants that are not soluble such as Copper Phthalocyanine.

Electrospray Ionization Mass Spectrometry

Electrospray ionization mass spectrometry (ESI MS) was recently used by Ng *et al.* for the characterization of both positively and negatively charged dyes in ballpoint pen inks[30]. ESI was developed about the same time as MALDI and is also capable of analyzing nonvolatile, thermally labile molecules, specifically biomolecules. Generally, ESI is capable of detecting species that are multiply-charged which can be an advantage when analyzing dyes compared to LDMS. Shown in Figure 1.4a is a polysulfonated dye, Acid Blue 9, which contains three sulfonate groups and one iminium group and was detected as both a singly (m/z 769) and doubly-charged (m/z 373) species with negative-ion ESI MS. However, triply and higher-charged dyes were not observed or gave a weak signal in negative-ion mode. Ng *et al.* found that polysulfonated dyes including Acid Blue 92 (Figure 1.4b) and Solvent Brown 20 (Figure 1.4c) which contain three and four sulfonate groups, respectively, could not be successfully detected using ESI[30]. Detection in ESI appears to be highly sensitive to the nature of the species.

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Figure 1.4
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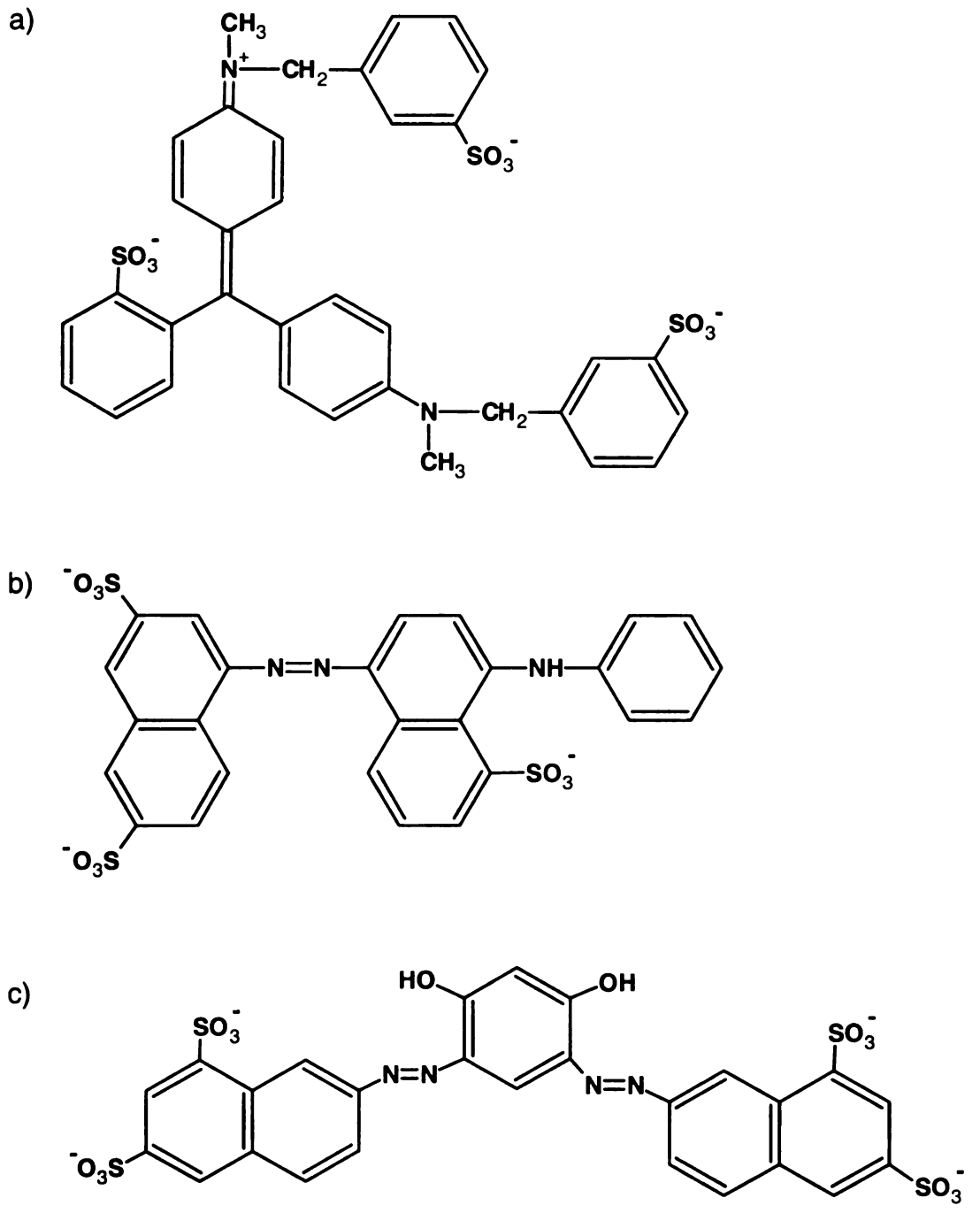


Figure 1.4: Multiply-charged dye structures a) Acid Blue 9, b) Acid Blue 92, and c) Solvent Brown 20

The overall charge state of the two dyes Solvent Orange 3 (Figure 1.5a) and Solvent Yellow 19 (Figure 1.5b) is zero. Solvent Orange 3 is an organic monoazo dye and Solvent Yellow 19 is a chromium (III) complex. In general, the ability to detect dyes depends on their structure. With the exception of the overall charge state, the structures of the two dyes are unrelated, but difficultly in detecting the dyes using ESI MS were apparent. Solvent Orange 3 was protonated and gave rise to a weak signal in the positive-ion ESI mass spectrum where Solvent Yellow 19 can not easily accept a proton under the ESI experimental conditions, and no signal was observed. LDMS can easily detect neutral molecules in both positive and negative-ion modes. In order to make a direct MS comparison of the ability of LD and ESI to detect the two dyes, Solvent Orange 3 and Solvent Yellow 19 should be analyzed using LDMS. However, these dyes were not readily available for purchasing, so Copper (II) Phthalocyanine and Pigment Red 3 were analyzed to make an indirect comparison of the ability of LDMS to detect neutral dyes. Copper (II) Phthalocyanine (Figure 1.5c) is a neutral metal complex dye that is detected without difficulty in both LD modes. Neutral aromatic compounds are detected with ease in LDMS. Pigment Red 3 (Figure 1.5d) is a monoazo dye that gives rise to peaks with m/z values of 308 and 306 in the positive and negative ion LDMS spectra, respectively, as shown in Figures 1.6a and 1.6b. Protonation in LDMS should be structurally favored for Solvent Orange 3 with respect to the amine groups.

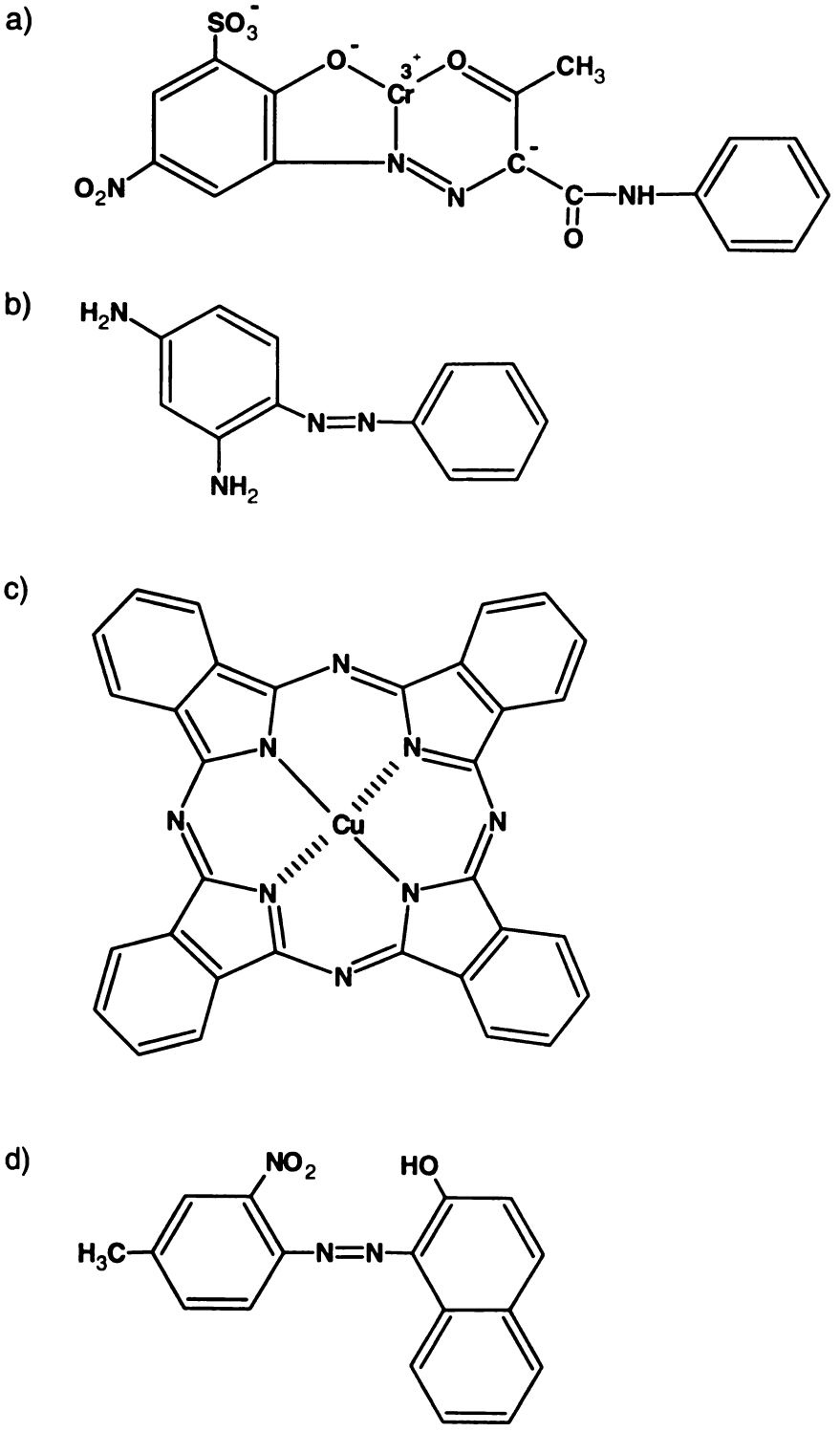


Figure 1.5: Neutral dye structures a) Solvent Yellow 19, b) Solvent Orange 3, c) Copper Phthalocyanine, and d) Pigment Red 3

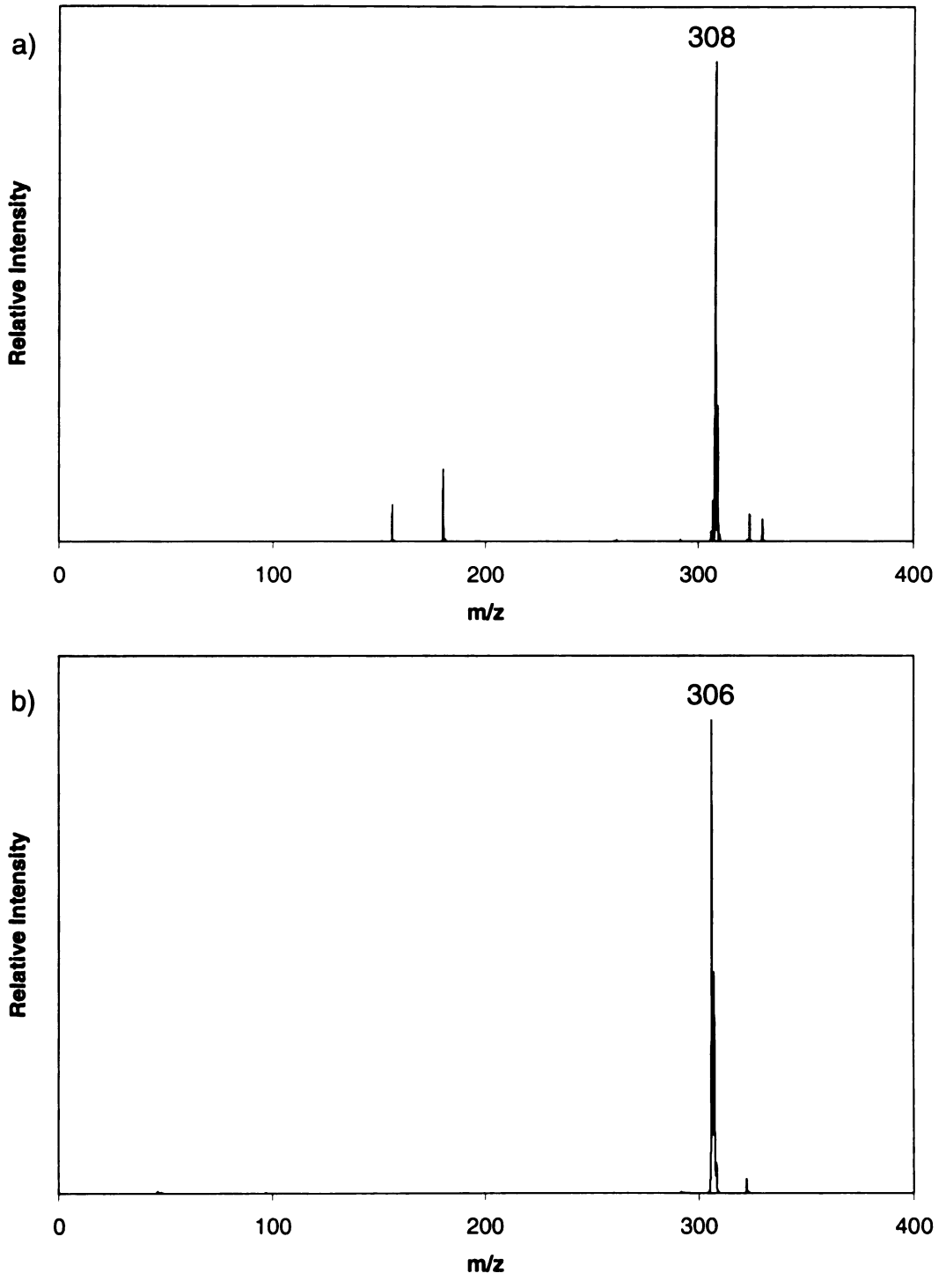


Figure 1.6: LD mass spectra of Pigment Red 3 on paper a) positive-ion and b) negative-ion

In order for ink dyes to be analyzed by ESI MS, the written entries are required to be extracted from the paper (liquid samples are prerequisite for injection). The extractions can usually be directly delivered to the source without additional sample preparation. The necessity to extract the ink dyes may pose a problem if the dyes are insoluble. Also, paper contains several components such as whiting agents that may extract into the solvent and can be detected using ESI. Dyes are not the only components extracted from the ink. Ng *et al.* reported the analysis of the same ink from six different brands of white paper[30]. The difference in paper components did not have a significant effect, but the observed peaks due to the paper were not identified nor were any m/z values reported. Other constituents, specifically a corrosion inhibitor and an antioxidant, in the ink were identified from the negative ion ESI mass spectrum. In general, LDMS is highly selective for dyes. Mass spectral peaks due to paper are not observed when analyzing the ink directly from paper since the spot from the laser is focused within the width of a pen stroke. The size of the laser spot and width of a pen stroke is discussed later. The detection of non-dye ink components are seldom observed in the LDMS experiment since the dyes are the only species that efficiently absorb the lasers radiation. Polyethylene glycol (PEG) is a polymer used in inks as a humectant and has been observed in the mass spectra of gel and liquid inks. Typically, neat polyethylene glycol is not detected in LDMS. The polymer is not capable of being desorbed/ionized without the assistance of another compound such as a matrix as described in matrix-assisted laser desorption/ionization (MALDI). The gel and liquid ink dyes may serve a similar role as a matrix, which aids in the desorption/ionization of an UV non-absorbing species, allowing the polymer to be detected. The paper may also influence the detection of the polymer.

Neat polyethylene glycol with an average molecular weight of 300 was analyzed directly from paper. Surprisingly, the polymer was detected in LDMS directly from the paper substrate and the positive-ion mass spectrum is shown Figure 1.7a. The region of the mass spectrum that contained representative polymer peaks was enhanced as shown in Figure 1.7b. Figure 1.8a illustrates the m/z values that represent the ionization of PEG by the formation of sodium and potassium adducts. The polymer is a mixture of PEG oligomers that differ in the number of repeating units, **n**. The repeating unit of PEG is $-OCH_2CH_2-$ as seen in Figure 1.8b and has a molecular mass of 44 Da. The distribution of the peaks observed in the mass spectrum is typical of a synthetic polymer. Mass spectral peaks that correspond to PEG are separated by 44 amu. The tetramer of ethylene glycol observed in the mass spectrum is the smallest of the PEG polymers and has a molecular weight of 194 Da. The tetramer gives rise to two peaks in the positive ion mass spectrum at m/z values 217 and 233 that correspond to sodium (Na^+) and potassium (K^+) ion adducts, respectively. Paper contains several components and some brighteners used in paper are actually dyes. In fact, some paper contains Copper Phthalocyanine which is a colorant associated with blue gel ink pens. Figure 1.9 demonstrates the detection of PEG in black liquid ink from a Pilot® rollerball pen. Either the paper or the ink components are assisting in the detection of the polymer. Neat PEG is not detected when analyzed from the standard stainless steel sample plate. The D/I of the polymer is *not* by LDMS alone since PEG requires the assistance of another species in order to be desorbed/ionized.

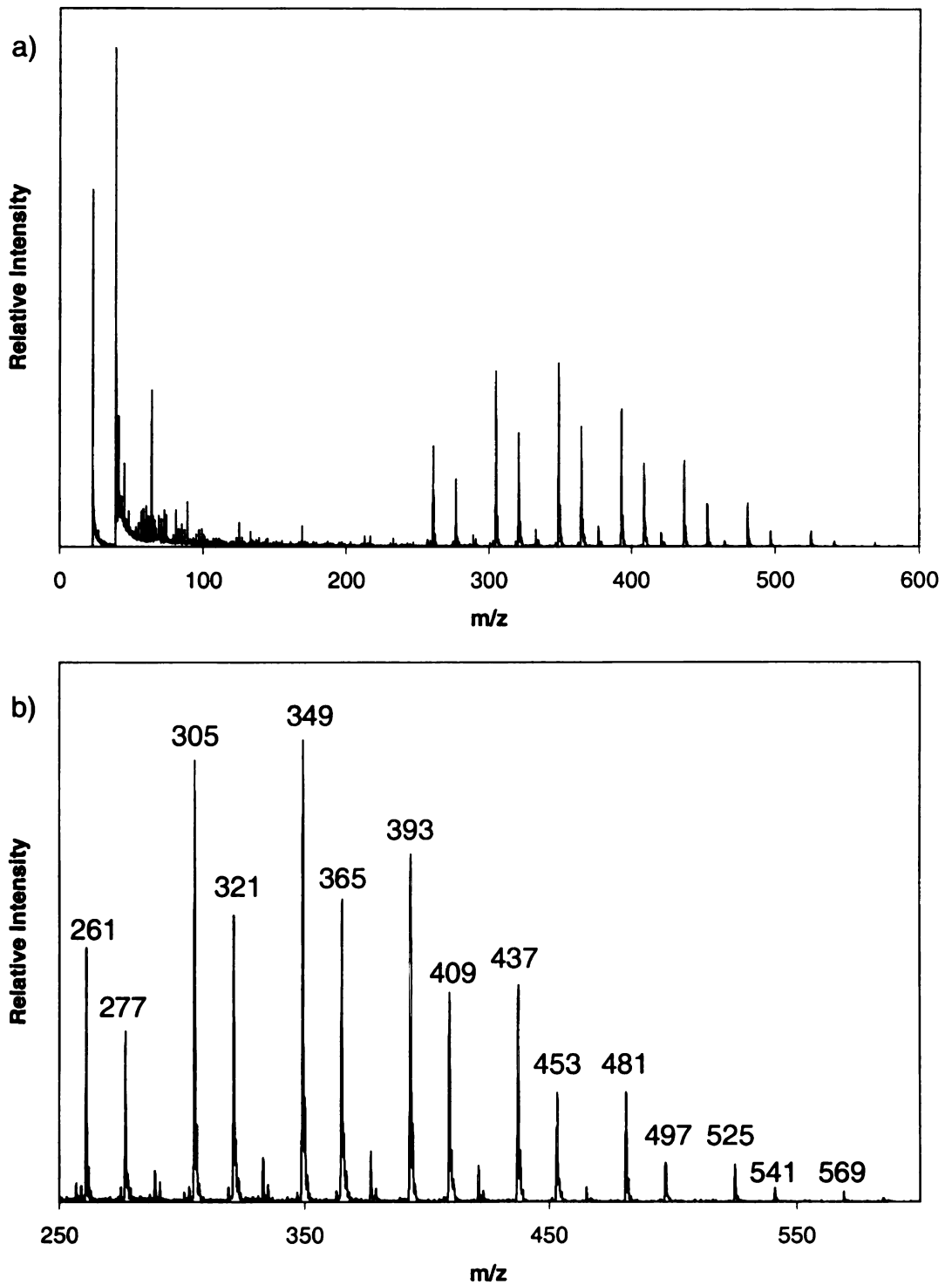


Figure 1.7: Positive-ion LD mass spectra of polyethylene glycol (PEG) on paper a) complete spectrum from m/z 0-600 and b) expanded region from m/z 250-600

a)

n	M	(M + Na^+)	(M + K^+)
4	194	217	233
5	238	261	277
6	282	305	321
7	326	349	365
8	370	393	409
9	414	437	453
10	458	481	497
11	502	525	541
12	546	569	585

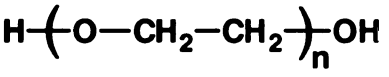
b) 

Figure 1.8: Polyethylene glycol a) m/z values that correspond to the ionization of the polymer (M) by sodium (M + Na^+) and potassium (M + K^+) ion adduction and b) polymer structure (n is the number of repeating units)

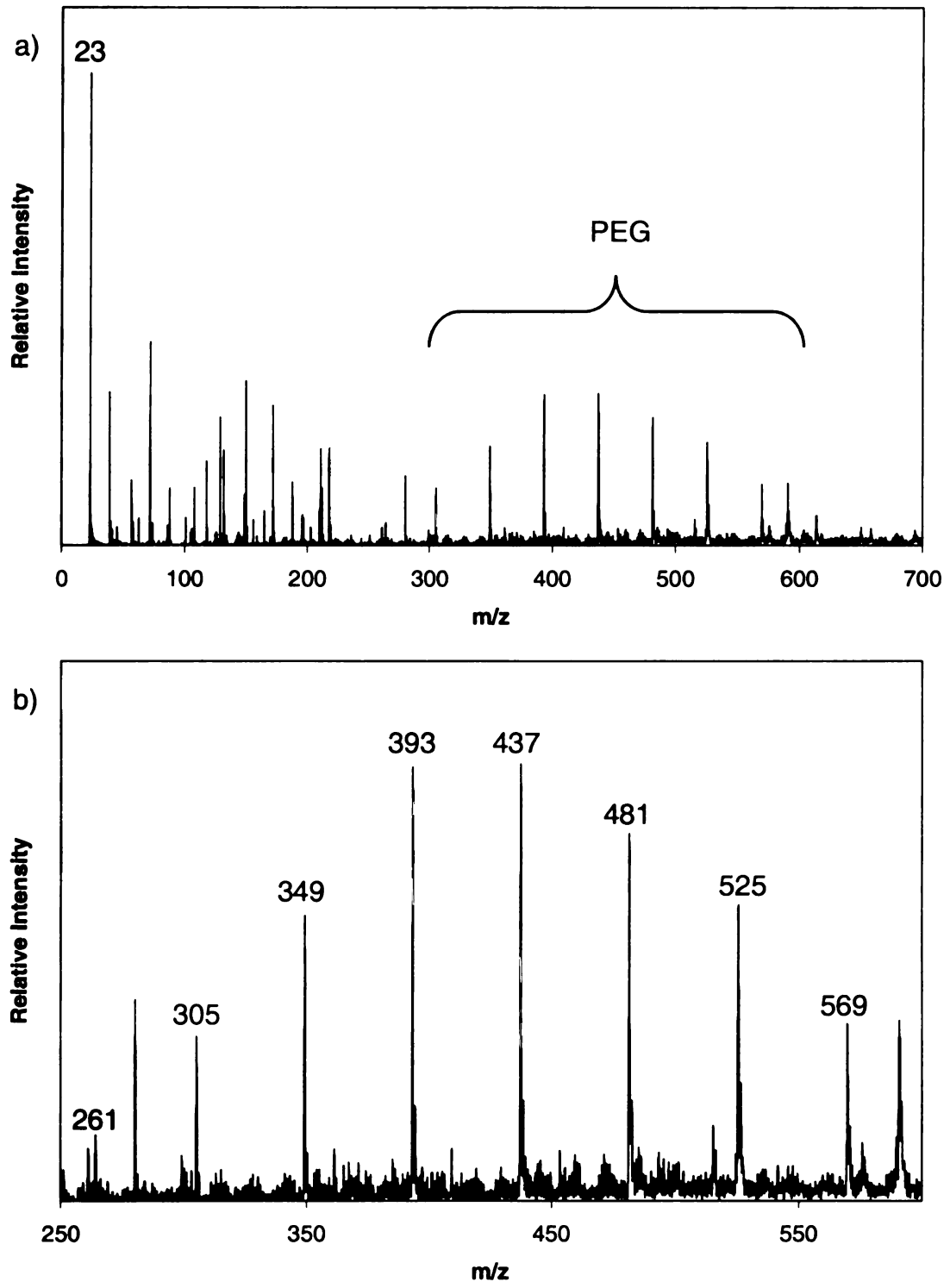


Figure 1.9: Positive-ion LD mass spectra of black liquid from a Pilot® rollerball ink a) complete spectrum from m/z 0-700 and b) expanded region from m/z 250-600

A major drawback to using ESI for the detection of dyes is sample carryover. Samples that are analyzed by ESI leave residue on the walls of the capillary (sample introduced through a capillary) which lead to the contamination of following samples. Carryover is inevitable, but washing of the capillary between samples helps to alleviate the problem. Sample contamination is not a concern in LD since the ink is analyzed directly from the paper. Sample preparation is minimal as described in the experimental section. Extractions can be rather arduous and dilute the sample. Ng *et al.* found that five times more sample was needed to be extracted for dyes for detection in negative-ion versus positive ion modes[30].

Laser Desorption Mass Spectrometry

This project began with a demonstration that UV laser desorption mass (LDMS) spectrometry has the sensitivity necessary to analyze dyes that are present on paper in a written pen line. Demonstrations of impressive sensitivity were made when LD was being developed two decades ago, but at that time IR lasers such as the CO_2 laser were frequently used, and sensitivity is extremely dependent on the ability of the analyte to efficiently absorb the light. Consider a typical ballpoint pen cartridge that contains about 0.6 grams of ink[47]. Written pen lines are typically 0.3-0.4 mm wide. If the ink is 20% dye by weight, and the typical molecular weight of a dye molecule is 400 grams/mole, the ink cartridge would contain approximately 0.3 millimoles of dye. If the ink in a cartridge can write a line approximately 3000 meters long, then there is roughly 0.03 millimoles per square meter, as a molar surface coverage per unit area. In the instrument that will be used, the laser spot size is approximately 0.03 cm^2. In this area there will be roughly 10^{-10} moles – 0.1 nanomoles. With such a small amount of material being

irradiated by the laser, detectable signals for mass spectrometry will only be generated if there is efficient coupling of the energy available at the surface to efficiently desorb and ionize analyte dye molecules.

Even if such small amounts can be studied, there is the problem of using paper as the surface from which the analysis begins. In time-of-flight mass spectrometry, ions are accelerated by a well-defined electric field to a specific kinetic energy. In this particular instrument, the ions are accelerated to kinetic energies of 15-20 thousand electron volts. The time that it takes for these ions to move through a distance of 1 meter (distance of the flight tube) is very accurately measured, and from this, ionic m/z values are determined. In the MALDI experiment, analytes are introduced on a metallic surface on which a potential is applied once the laser has fired. In this proposed research, ions desorb from a non-conducting surface, paper. However, severe deviations in the measured ions' time-of-flight have not been observed from direct paper analyses. UV LDMS is sufficiently sensitive to generate ions from ink on paper since the time-of-flight mass spectrometer adequately resolves/separates the ions.

Chromatography

The general mechanistic concept of chromatography based methods is that components in a mixture are separated while equilibrating between a stationary phase (solid material) and a mobile phase (gas or liquid material). Simple and inexpensive solid-liquid separation methods such as paper chromatography and thin-layer chromatography (TLC) have been applied to the separation of dyes as early as 1950's[48]. A typical TLC experiment consists of a developing tank with a cover, an applicator, a

solvent system, and a TLC plate. More advanced methods for separation of ink dyes have been recently employed such as high performance liquid chromatography (HPLC). The resulting chromatogram is similar to an LD mass spectrum. The analysis of Crystal Violet and corresponding dye homologues have been analyzed by both methods. TLC was sufficient for the analyses carried out in this research. Pertinent information including the number of dyes and the color of the dyes present in each ink was quickly acquired using TLC. Also, extractions of the TLC dye bands (extractions of the individual/separated ink dyes) were frequently performed and subsequently analyzed using LDMS. This step could not be carried out if HPLC was used for separation of dyes.

Thin-Layer Chromatography

Thin-layer chromatography (TLC) is a simple, planer chromatography method that utilizes two phases (1) a liquid, mobile phase (typically, more than one organic solvent is used) and (2) a solid, stationary (such as porous silica) phase for performing separations. The molecules separate based on their interactions with (their affinity for) the stationary and mobile phases. Glass TLC plates coated with silica are inexpensive and are the most commonly used among all types of separation analyses.

TLC has been previously applied to the separation of ink dyes. Several solvent systems (comprises the mobile phase) have been examined to improve the quality of the separations and have been determined specifically for a particular pen type. The ink dyes analyzed here were separated using a three-part mobile phase consisting of 70:35:30 (v:v:v) ethyl acetate:ethanol:water. TLC has several advantages that make the technique valuable for dye separations. The ink is placed directly on the TLC plate and once the

sample spot dries the plate is placed inside the TLC chamber in which the solvent system was given time to equilibrate between the liquid and gas phases. The direct application of the ink does not appear to hinder the separation of the dyes from the ink. The separation of the ink dyes are compared to a standard for comparison. A solution containing the dyes Crystal Violet, Methyl Violet 2B, and tetrapararosaniline was used as the standard (S) and their corresponding TLC bands are labeled S1, S2, and S3. The ink dyes were designated using the letter "D" and are consecutively numbered beginning from the bottom of the TLC chromatogram.

High Performance Liquid Chromatography

The analysis of blue ballpoint pen inks stored under various light conditions were carried out by high performance liquid chromatography by Andrasko *et al.*[31]. Various blue inks were exposed to daylight, extracted from the document, and analyzed by HPLC. In the fresh ink sample, basic dyes, Crystal Violet (CV), Methyl Violet 2B (MV), and tetramethylpararosaniline (TPR), were detected. The only difference between these dyes is the number of methyl groups bonded to the three nitrogen atoms. Andrasko and co-workers reported that as Crystal Violet is exposed to light, the dye decomposes to form CV homologues by the gradual loss of methyl groups[31]. The decomposition products elute before Crystal Violet since the polarity increases with increasing substitution of methyl groups by hydrogen. Andrasko *et al.* demonstrated that Crystal Violet decomposed after three and six days exposed to light[31]. This experiment demonstrated that dyes and decomposition products can be detected using HPLC.

LDMS and Forensic Science Applications

Questioned Document Examination

A questioned document is any material object that contains suspicious character markings[49]. More specifically, questioned document analysis encompasses many types of evidence including typewriting, laser printing, copiers, forgeries and frauds, indented and obliterated writing. Most markings are in handwritten or printed form and the type of analysis which examiners are called upon the most often is handwriting analysis. Traditionally, this type of work has involved determining the author or authenticity of handwriting by careful comparison of known and unknown handwriting samples. More recently, other evidence besides the nature of the handwriting have become the focus of questioned documents involving written entries on paper using an ink pen. Expertise continues to be developed in diverse fields including the chemical characterization of components of the document (paper, ink, watermarks).

The first part of this project consisted of performing a comprehensive analysis of laser desorption mass spectrometry (LDMS) as a tool for the analysis of pen ink dyes on paper. Preliminary work indicated that the technique had great potential in identifications of dyes directly on paper as well as other substrates. The second part of the project was to evaluate sources for inducing dye degradation, initially, for the purpose of an aging study; however, the discovery of using degradation for dye characterization became the main focus and a major theme of this thesis. The process of natural aging of dye molecules on paper has been previously studied and was applied to other ink dyes, but limitations were encountered in this work. Preliminary studies indicated that the mechanism for natural aging of Crystal Violet, a dye commonly found in black and blue

ballpoint pen ink, is straight forward, understandable, and possessed the potential for assisting in dating documents. Dye degradation appears to be unreliable for ink dating; however, examining the photodegradation of dyes is valuable to the forensic community. Dye degradation with subsequent LDMS analysis can be used to characterize ink dyes which can be used as a tool to distinguish between two inks that are macroscopically similar (in terms of the same ink color and same kind of pen ink) and more importantly, contain similar (in terms of physical properties) dyes which is encompassed as the third part of this proposed research. Assaying inks via coupling thin-layer chromatography with LDMS was used to simplify dye identification.

Trace Evidence

The dye characterization method developed in this research is not limited to the examination of questioned documents. The method can be applied to other applications in forensic science that encompass detecting colorants. Such areas of examination include trace evidence and drugs. Colored materials frequently analyzed as trace evidence include automobile paint chips and natural and synthetic fibers. Colorants used in fibers have been examined previously by LDMS by Balko[50].

Instrumental techniques such as microspectrophotometry and fourier transform infrared (FTIR) spectroscopy are frequently used in forensic science to examine paint chips and fibers, however, extracting colorant information from an FTIR spectrum is cumbersome. Paint chips are analyzed as individual layers as well as cross-sections. FTIR analysis of a complex (more than one component) sample results in a fingerprint spectrum and the possibility of using the data to characterize and identify a specific colorant is unlikely. The main peaks that appear in FTIR spectra obtained from

analyzing paint chips are due to the resins or binders in the paint and their corresponding peaks generally overlap and dominant peaks that represent organic pigments, thus, making colorant identification unattainable. Fibers are analyzed by FTIR as well, but the technique is used to determine the type of fiber polymer, not to acquire information on the colorants. Microscopy is the most common method used for analyzing fiber color as well as other physical characteristics of the fiber and microspectrophotometry is an instrumental technique frequently employed strictly to examine the color of the fiber. The technique is also used as a means to compare the color of paint chips. Analysis by microspectrophotometry is problematic when dealing with fibers that contain more than one dye, comparing two fibers that are inherently the same color, and fibers that are black. The visible spectra that are acquired from analyzing fibers and paint are fringerprints and can not be use to identify a specific colorant. A visible spectrum can be thought of as a color spectrum. Two separate fibers that are microscopically similar in color and give rise to similar spectra can be misleading if the fibers in fact contain different dyes or pigments. Microspectrophotometry is not a selective technique for distinguishing dyes or pigments of the same color. Fibers containing more than one colorant may yield spectra that have overlapping spectral features which do not allow for identifying individual colorants and may be similar to the problem with analyzing black fibers using microspectrophotometry. Black fibers are difficult to analyze using microspectrophotometry since the resulting spectrum will be one broad peak that spans the entire range of the visible wavelengths; hence, no distinctions can be made between unknown and known fiber samples that are colored black.

Research Overview

This thesis research has focused on the basic and advanced methods for characterizing ink dyes as a forensic tool for analyzing a questioned document. The following determinations can be drawn when examining the characteristics of ink dyes:

1. Inks on the same or different documents can be differentiated.
2. An ink source of a specific entry can be identified or eliminated from an exemplar.

Advancements in ink dye characterization methods were carried out using a combination of photochemical and analytical methods. This program of research provides new data to demonstrate the above deductions. There were two major approaches taken in this project:

1. A novel method of analysis was employed to help identify and characterize dyes present in writing inks based on chemical and structural information.
2. New methods were explored that encompass artificially aging dyes in writing inks to gain chemical information to aid in dye characterization.

Specifically, this research thesis discusses:

1. The use of laser desorption mass spectrometry for the rapid and direct analysis of dyes (from inks) on paper and the basic MS characterization methods that can be employed when examining the mass spectra.
2. The use of LDMS to study the chemical processes of natural dye degradation and artificial dye degradation induced by incandescent light and UV radiation.

3. The characterization of dyes used in red ballpoint pen ink encompassing the development of new methods for dye characterization to determine the source of the dye (ink) and distinguish between similar pen inks.

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Chapter Two: Direct LDMS and TLC Analysis of Ink Dyes

This chapter discusses the direct methods that were used to examine LD mass spectra for dye characterization. Direct characterization methods involve the examination of an unmodified sample. Analyzing ink dyes by LDMS is accomplished directly from written entries on the paper without any chemical or physical modifications (including dye extractions) to the sample. In a typical GC/MS experiment, a sample mixture is separated using chromatography prior to the analyte detection by mass spectrometry, hence, each mass spectrum acquired from the analysis will contain peaks (peaks due to the molecular and fragment ions) that are associated with one particular analyte. Unlike a mass spectrum obtained from a GC/MS analysis, a mass spectrum collected using LDMS contains peaks that are associated with multiple species that are in the sample mixture and many of the components in the mixture are not detected. However, both MS experiments allow molecular information such as mass and composition (elemental information maybe obtained from isotopic distributions). Dyes are typically manufactured as salts. A salt is an ionic compound that is comprised of a cation and an anion, excluding the hydronium (H^+), hydroxide (OH^-), and oxide (O^{2-}) ions [1]. Many colorants used in ink are dye salts and some are neutral dyes such as pigments. The overall charge state varies among the ionic dyes and can be deduced from the LD mass spectra to characterize and identify neutral or singly-charged colorants. Both positive and negative-ion LD mass spectra were collected for each sample to discern the charge state of dyes prior to the MS analysis. Determining the charge state of the dye will be discussed through the LDMS examination of Copper Phthalocyanine,

Crystal Violet, and Metanil Yellow. Additional information that maybe used for characterization is to examine the LD mass spectra for the presence of ions due to impurities, clusters, and fragments of the analyte. The mass spectral peaks were divided into the following two categories for purposes of characterization: (1) peaks (includes isotopic peaks) that denote the detection of the intact analyte and (2) peaks that are indicative of the analyte indirectly. Acquiring molecular weights, compositions, and charge states can be deduced from examining the mass spectral peaks that are associated with the intact dye. Determining the molecular level information will be demonstrated through the direct LDMS analysis of Copper Phthalocyanine (commonly used in blue gel ink), Crystal Violet and Metanil Yellow (frequent dyes contained in black ballpoint ink). Indirectly characterizing ink dyes indirectly based on impurities, cluster ions, and fragment ions which give rise to peaks in the LD mass spectra will be elucidated by examining the dyes mentioned above.

Experimental

Laser Desorption Mass Spectrometry

LDMS analyses were carried out using a PerSeptive Biosystems Voyager DE (Farmingham, MA) mass spectrometer. The instrument utilizes a pulsed nitrogen laser (337 nm, 3 nanosecond pulses at 3 Hz) and a linear time-of-flight mass spectrometer. The user-selected parameters for the LDMS experiments include an accelerating voltage of 20,000 V for detection of positive ions and -15,000 V for the detection of negative ions, an intermediate source grid voltage that is 94% of the accelerating voltage, a guide wire voltage that is opposite in bias and 0.05% in magnitude of the accelerating voltage,

and an extraction delay time of 100 ns. In some cases, the analysis on the paper substrate required an increased delay time of 200 ns for optimum resolution. Blue ink from a gel pen (Pentel® Hybrid® Gel Grip RXT) containing Copper Phthalocyanine was used as the calibrant for both positive and negative-ion modes.

Single written lines on MSU letterhead paper were analyzed directly. The spot from the nitrogen laser beam was focused on the line within the width of the pen stroke which is approximately 0.3-0.4 mm. Eighty-five pen inks have been analyzed using LDMS; however, the results presented here focus on a few of the analyses. All of the analyses can be found on the world wide web at the following URL: http://poohbah.cem.msu.edu/peninks/pens_main.htm

In order to simulate the analysis of ink on paper, 10,000 pmol/μL of known dye solutions were prepared using 1:1 (v:v) methanol:water, spotted (5 μL) on paper and allowed to dry prior to the LDMS analysis. Squares approximately 2 x 2 mm^2 were cut from the paper and mounted on a disposable, gold MALDI plate using Scotch® double coated tape (3M, St. Paul, MN).

Thin-Layer Chromatography

TLC was carried out using K5F silica gel 150 A TLC plates (Whatman, Ann Arbor, MI) with dimensions of 5 x 10 cm and a stationary phase thickness of 250 um. The solvent system used for the separation of the dyes in the ink consisted of 70:35:30 (v:v:v) ethyl acetate:ethanol:water. Methyl Violet 2B (Aldrich Chemical Co., Milwaukee, WI), purchased as an impure mixture of dyes, included Crystal Violet and tetramethylpararosaniline and was used as the comparison standard. Dyes extracted from TLC bands were analyzed by LDMS. Individual bands were scrapped from the TLC

plate, placed in a centrifudge vial, and extracted with 5-10 μL of 1:1 (v:v) ethanol:water. The vials were vortexed, and the silica was separated from the dye solutions by centrifugation. The dye extracts were dried on paper or the metal plate and subsequently analyzed by LDMS.

LDMS Calibration and Copper Phthalocyanine

Mass Accuracy for Identification

Proper calibration (mass-to-charge ratio calibration) in laser desorption mass spectrometry (time-of-flight) is required to ensure accurate mass measurements for identification purposes. The mass accuracy for small mass molecules (< 1500 Da) in conventional TOF instruments is reliable. Mass accuracy for a given ion is detemined by taking the difference between the true mass and measured mass and dividing the difference by the true mass. Typically, mass accuracy is expressed in parts per million (ppm), so the value is multiplied by 10^6. Low resolution mass spectrometry was used for these dye analyses. Deviations in m/z values are small and were observed in the first decimal place. Isotopically-resolved peaks are achieved in the TOF experiment for low molecular weight molecules which allows accurate assignment of mass-to-charge (m/z) values (the m/z values are presented as whole numbers).

The instrument should be calibrated frequently as the sampling position changes. The sample plate including the paper substrate is not uniform, hence, the distance from the sample to the detector changes as the sampling position moves across the horizontal plane (the sample position varies with the x and y-coordinates). Even small differences (irregularities of the paper's thickness) in the length of the ions' flight path influence the

accuracy of the masses of the ions detected; hence, the calibrant needs to be placed in close proximity to the sample. Also, the calibrant and sample need to be on the same type of substrate. For example, if the written lines on paper are being analyzed, then the calibrant needs to be on paper as well. The thickness of the paper will cause the calibration to be inaccurate.

In order for the mass spectrometer to be properly calibrated, the appropriate calibration standard should be used. When selecting a calibrant, the molecular weight of the standard compound needs to be considered. The mass of the analyte needs to be within the mass range of the calibration. For example, if an unknown dye has a mass of 450 Da, then the calibrant should have a mass higher or near that value. Confirming that a calibration is valid can be accomplished by analyzing samples with known masses. Generally, analytical techniques require more than one standard for a calibration, but using multiple calibrants is not necessary for LDMS. A one point calibration allowed for sufficient mass accuracy for the analyses presented here since the dyes have low molecular weights. The calibrant used for the experiments for this thesis research is Copper Phthalocyanine.

LDMS Ink Dye Analysis

UV laser desorption mass spectrometry (LDMS) has proven to be a powerful technique for the detection of several ink dyes. The detection of Crystal Violet, an organic dye frequently found in black ballpoint pen inks, using LDMS was first demonstrated in 2001[2]. The instrumental method is attractive since a solvent extraction step is not required; hence, the dyes are detected directly from written lines on paper.

Success has been reported with black, blue, and red ballpoint pen ink dyes[2-5]. The direct characterization information that can be extracted from the LDMS mass spectra will be discussed next.

Molecular Information: Mass, Composition, and Charge State

Copper Phthalocyanine is a pigment dye that is frequently encountered in gel pens with blue ink. Copper Phthalocyanine is a stable, neutral dye molecule (M), shown in Figure 2.1a, and has a monoisotopic, molecular mass of 575 Daltons (exact molecular mass of $^{12}C_{32}{}^{1}H_{16}{}^{14}N_{8}{}^{63}Cu$ is 575.07931 Daltons). The molecular masses that are presented here will be rounded to the whole numbers for simplicity since decimal places can be neglected for these experiments. The pigment is easily detected in both positive and negative-ion LDMS modes. The formation of the positive and negative ions of Copper Phthalocyanine has been examined extensively using LDMS. Copper Phthalocyanine is ionized by the addition or loss of an electron giving rise to molecular ions in the negative and positive-ion LD mass spectra, respectively. Shown in Figure 2.2a and 2.2b are the positive and negative-ion LD mass spectra, respectively, of the blue gel ink. In both spectra, the peaks at m/z 575 correspond to the intact desorption/ionization of Copper Phthalocyanine. The mass difference due to the addition or loss of an electron (an electron has a mass of 9.11×10^{-31} kg) is neglected since the change in the mass of the molecule is not significant. As mentioned previously, an electron is responsible for the ionization of the neutral molecule. An electron is donated to a neutral molecule (M) of Copper Phthalocyanine causing the pigment to become negatively-charged ($M^{\cdot -}$) and can be detected in negative-ion LDMS. In positive-ion LDMS, Copper Phthalocyanine becomes positively-charged (M^{+}) when an electron is

removed from the neutral molecule. The loss of an electron is the favored mechanism for the positive ion formation of Copper Phthalocyanine. The most intense isotopic peak for Copper Phthalocyanine has an m/z value of 575 which denotes that electron transfer is the preferred D/I mechanism for the dye in both positive and negative-ion LD modes.

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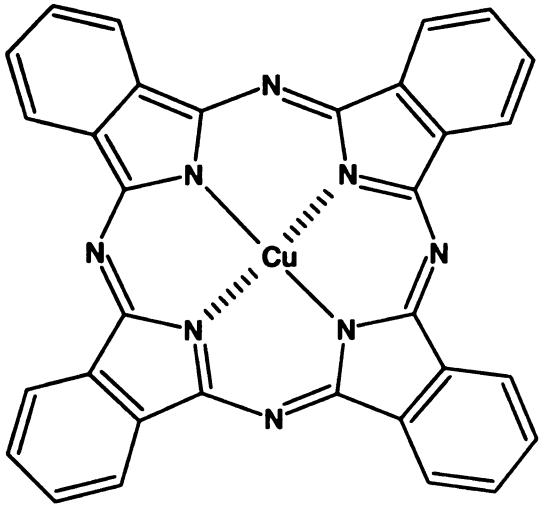

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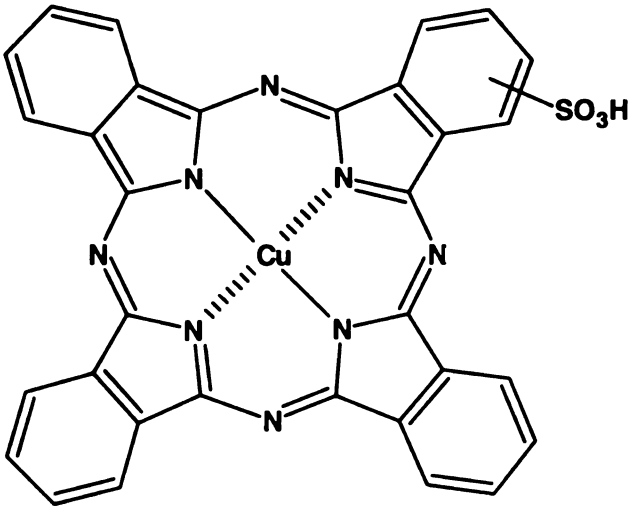

Figure 2.1: Dye structures a) Copper Phthalocyanine and b) monosulfonated Copper Phthalocyanine

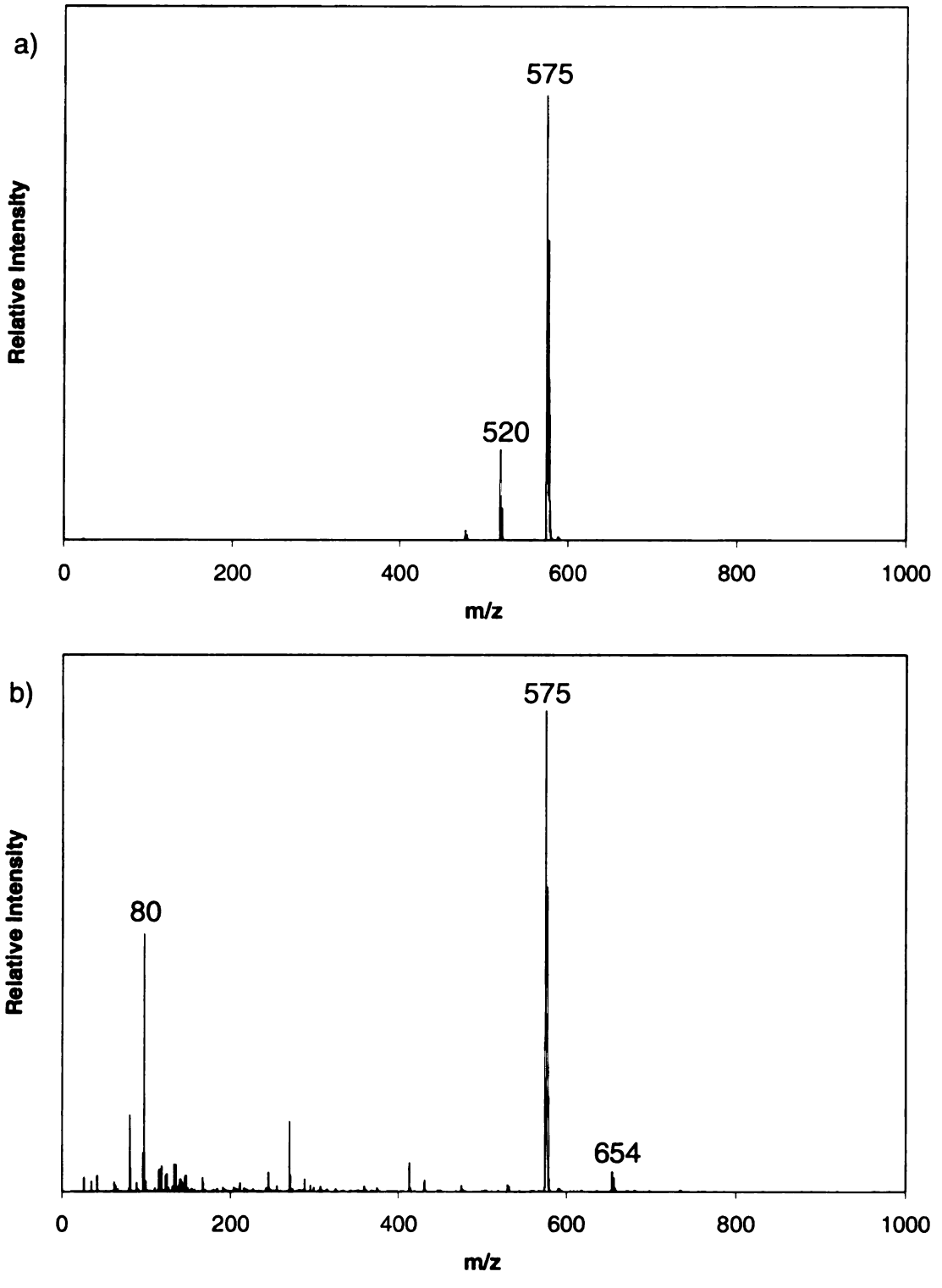

Figure 2.2: LD mass spectra of blue gel ink containing Copper Phthalocyanine a) positive-ion and b) negative-ion

The distribution of the isotopic peaks observed in the LD mass spectra contains information that can be used to characterize Copper Phthalocyanine as well. The isotopic distribution of the peaks depends on the natural abundance of the isotopes (atoms that have the same number of protons but differ in the number of neutrons) that make up the composition of the species. The mass of a neutron is 1.67×10^{-27} kg (~1 Da), so the mass difference between isotopic peaks is due to the number of neutrons of the isotopes. Several atoms have characteristic isotope distributions. The abundance and distribution of isotopes of a dye molecule are specific and can be used to characterize the colorant. Molecular mass and isotopic information are specific for a single molecular formula and can be used to identify a species with the exception of isomers. Copper Phthalocyanine has a unique isotopic distribution as seen in Figure 2.3. The isotopically-resolved peaks of Copper Phthalocyanine obtained experimentally using LDMS and theoretically using an isotopic distribution calculator accessed through the website of Scientific Instrument Services[6] at http://207.97.159.7/cgi-bin/mass10.pl are compared in Figures 2.3a and 2.3b, respectively. The spectrum in Figure 2.3b was recreated by obtaining the theoretical relative peak intensities from the website and plotted versus m/z values of Copper Phthalocyanine. The atoms (C, H, N, and Cu) contained in Copper Phthalocyanine along with the isotopic masses and percent abundances are listed in Table 2.1. The peak at m/z 575 represents $^{12}C_{32}{}^{1}H_{16}{}^{14}N_{8}{}^{63}Cu$, the most abundant combination of atoms. The ^{65}Cu isotope provides the largest contribution to the peak observed at m/z 577. The isotope peak pattern along with the molecular mass are two pieces of information obtained from the mass spectra that are characteristic of ink dyes in general.

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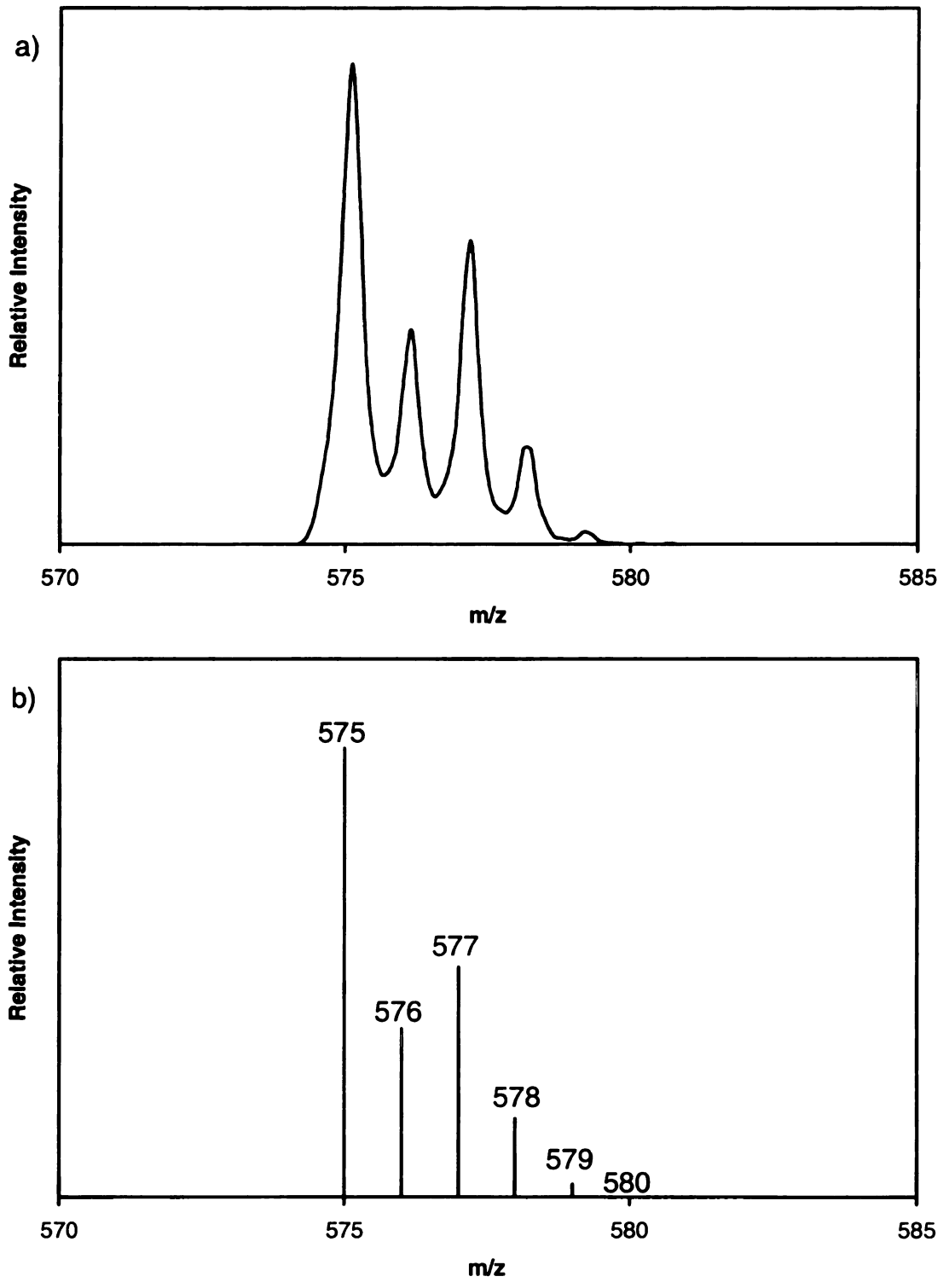

Figure 2.3: The isotopic peak distribution of Copper Phthalocyanine a) experimental isotopic distribution in negative-ion mode b) theoretical isotopic distribution for positive and negative-ion modes

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Atom	Isotopic Mass (u)	Percent Abundance
^{1}H	1.007825	99.9885
^{2}H	2.014102	0.115
^{12}C	12.000000	98.93
^{13}C	13.003355	1.07
^{14}N	14.003074	99.757
^{15}N	15.000109	0.368
^{63}Cu	62.929601	69.17
^{65}Cu	64.927794	30.83

Table 2.1: Isotopic masses and natural abundances for the atoms that comprise Copper Phthalocyanine

Determining the ionization mechanism of a dye in LD is also useful information for characterization. The ionization of the dye is influenced by the charge state of the structure. Charged and neutral dye molecules are ionized differently and can be predicted. The charge state of a dye can be deduced from obtaining the negative and positive-ion LD mass spectra. As stated previously, Copper Phthalocyanine is a neutral molecule. As a general ionization rule in LDMS, neutral dye molecules form ions by electron transfer or proton transfer. The mass (1.67×10^{-27} kg or ~1 Da) of a proton is significant to influence the mass of the protonated or deprotonated molecule. Neutral molecules undergoing proton transfer reactions will have a mass difference of 2 Da between the peaks in the positive and negative-ion LD mass spectra that correspond to the intact analyte. For example, if the neutral molecule (M) has a molecular mass of 450, then the protonated molecule, $(M+H)^+$, will give rise to a peak at m/z 451 and the deprotonated molecule, $(M-H)^-$, will give rise to a molecular ion at m/z 449, hence, there

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is a two mass unit difference between the positive and negative ions of the analyte. The ions formed and detected in LDMS provide important clues concerning the charge distribution of the absorbing dye molecule. LDMS can be used to deduce the charge state (cationic, anionic, or neutral) of the dye. The characteristic charge associated with an unknown dye structure promotes identification. LDMS detects singly-charged or neutral dye molecules and allows a distinction to be made between dyes that are cationic, anionic, and neutral.

Several black ballpoint pen inks from various manufacturers have been analyzed by LDMS. All of the inks contain the same two dyes, Crystal Violet and Metanil Yellow, which have the structures shown in Figures 2.4a and 2.4b, respectively. Analyses of Crystal Violet and Metanil Yellow by LDMS demonstrate the ability of the technique to distinguish between cationic and anionic dyes. Figures 2.5a and 2.5b are representative examples of the positive and negative-ion mass spectra, respectively, that are acquired when black ballpoint ink is examined directly from paper by LDMS. The most abundant peaks in the LD mass spectra correspond to the D/I of the intact dyes that are contained in the ink since the other components in the ink are incapable of efficiently absorbing the UV pulsed laser energy. Energy absorption is necessary to ensure desorption/ionization of the species. The positive and negative-ion LD mass spectra of the black ink each contain one peak that is relatively intense. The positive-ion mass spectrum contains a peak at m/z 372 representing the intact desorption of the cationic component of the dye Crystal Violet $[C_{25}H_{30}N_3]^+$. A peak at m/z 352 in the negative-ion spectrum denotes the D/I of the anionic component of the dye Metanil Yellow $[C_{18}H_{14}N_3O_3S]^-$. Unlike singly-charged colorants, neutral molecules of Copper Phthalocyanine (M) yields both positive

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(M^+) and negative ($M^{\cdot -}$) ions when ink is ablated with the UV laser as discussed previously. In the LD experiment, singly-charged colorants are detected in their native charge state. The negative-ion mass spectrum clearly indicates that Crystal Violet does not form an intact negative ion and Metanil Yellow is undetected in the positive-ion mass spectrum.

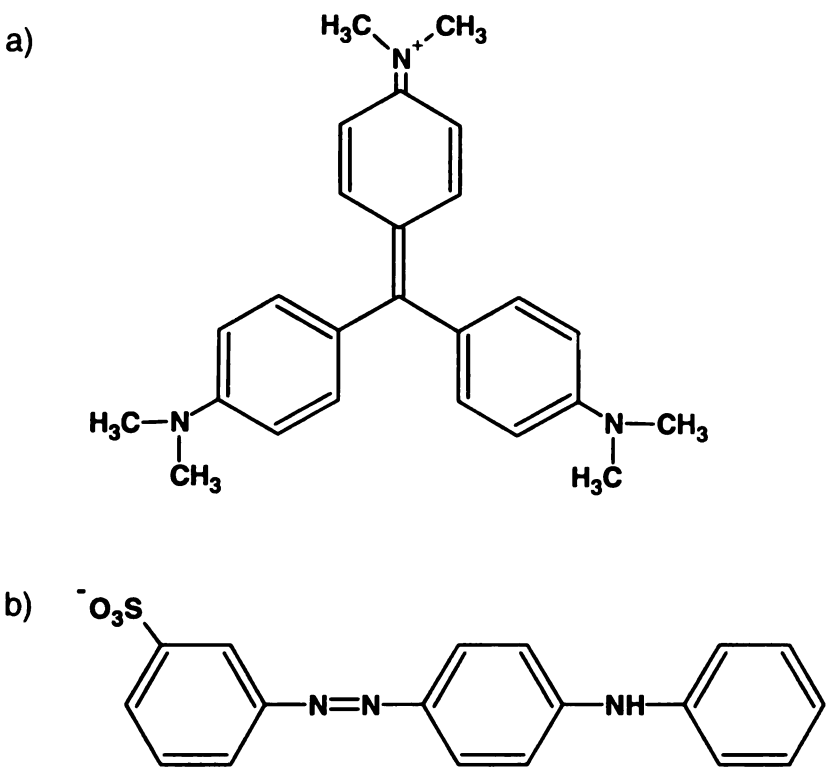

Figure 2.4: Structure of the dyes used in several black ballpoint inks a) Crystal Violet and b) Metanil Yellow

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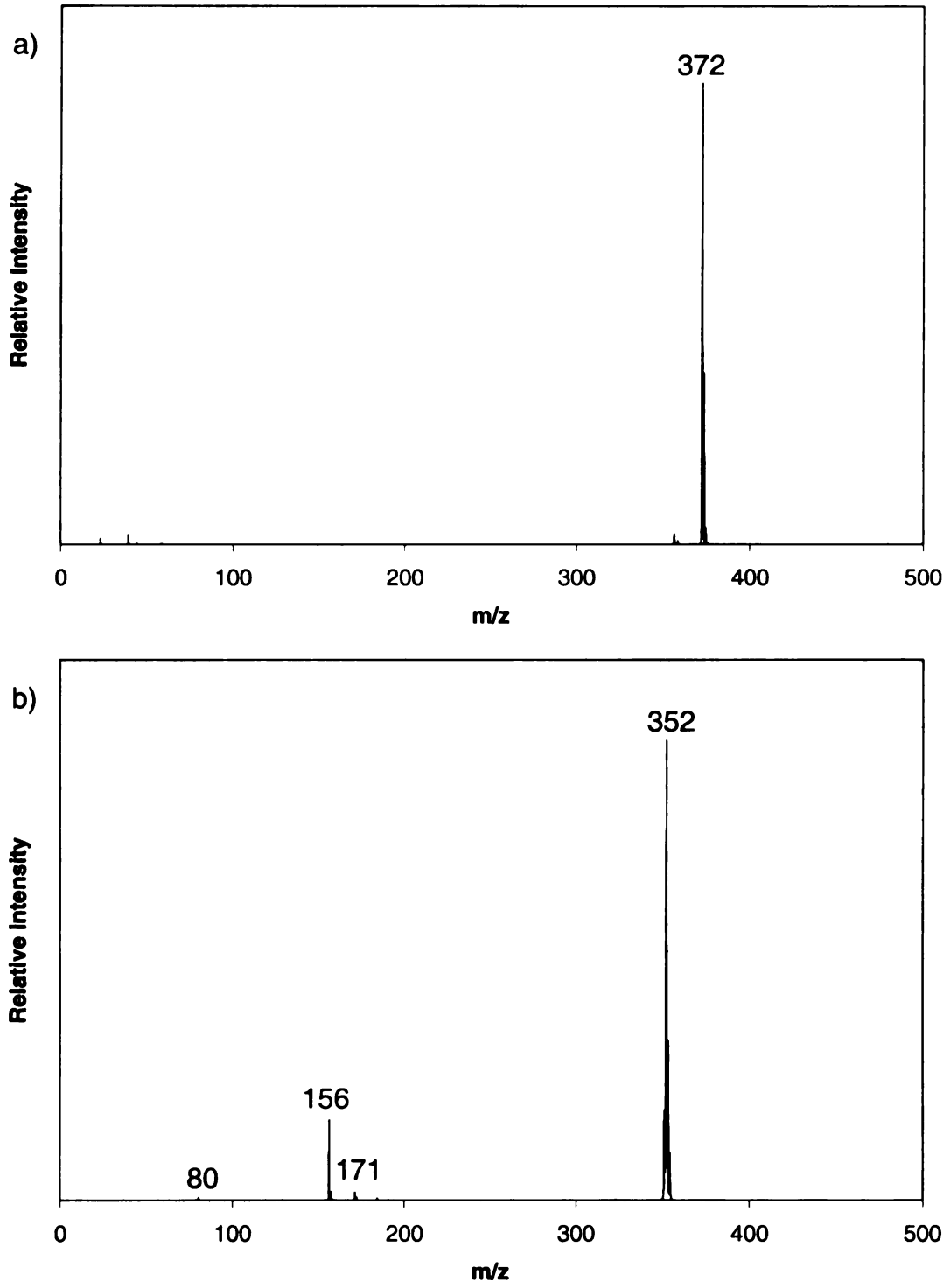

Figure 2.5: LD mass spectra of black ink from Sanford® Supergrip ballpoint pen a) positive-ion and b) negative-ion

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Dye Impurities, Fragment Ions, and Cluster Ions

Singly-charged dyes are detected intact by LDMS, however, there are some instances when additional peaks appear in the mass spectrum of a single commercial dye. The appearance of peaks lower in mass than the peak corresponding to the intact dye may be from two sources, dye impurities or fragment ions, from the analyte. The formation of clusters or presence of impurities may be detected with m/z values higher than that of the intact dye.

Commercial dyes are frequently sold as impure mixtures[7] and Methyl Violet 2B purchased from the Aldrich Chemical Company is an example. The dye mixture is comprised of Crystal Violet, Methyl Violet 2B and tetramethylpararosaniline. In fact, the peak associated with the dye impurity, Crystal Violet, dominates the mass spectrum. As shown in Figure 2.6. The three dyes are cationic and are detected solely in the positive-ion LD mass spectrum. The m/z values of Crystal Violet and Methyl Violet 2B are 372 and 358, respectively. Surprisingly, Crystal Violet is more concentrated than Methyl Violet 2B according to the LD mass spectrum and this observation has also been made by Fales *et al.* [9]. The detection of dye impurities is not uncommon when analyzing ink samples. The peak at m/z 654 in the negative-ion LD mass spectrum of the blue gel ink corresponds to a monosulfonated Copper Phthalocyanine, shown in Figure 2.1b. This dye may be present as an impurity as well. To make certain that the dye is an impurity in the gel ink, the phthalocyanine pigments should be purchased and analyzed directly. Frequently, multiple dyes are used in the manufacture of ink, so both dyes could have been intentionally used.

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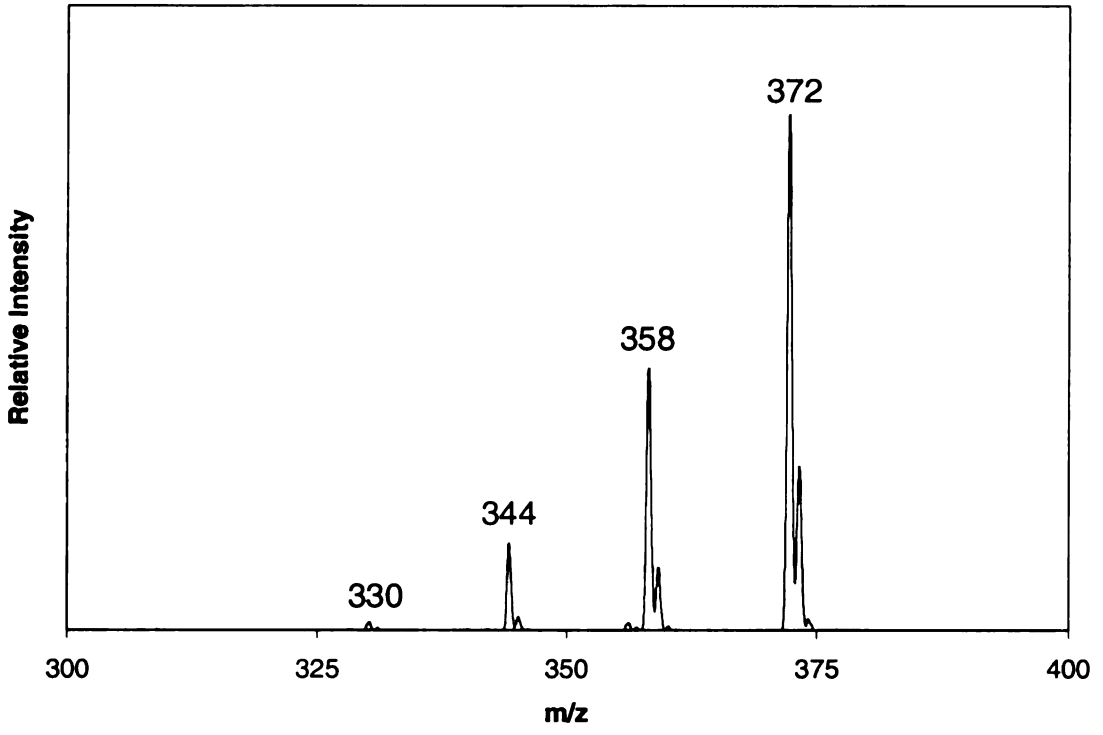

Figure 2.6: Positive-ion LD mass spectrum of Methyl Violet 2B purchased from the Aldrich Chemical Company

Although a dye may be detected intact, peaks can also arise lower in mass compared to the dye that corresponds to prompt fragment ions. Fragment ions are structural pieces that are associated with the intact dye. Fragment ions are formed when the dye molecule breaks apart due to the fact that fragmentation is energetically favored, partially or completely, for that particular species. Fragment ions were identified for the monosulfonated Copper Phthalocyanine complex and Metanil Yellow.

Although, Copper Phthalocyanine (CP) is stable upon D/I of the dye by LDMS, the monosulfonated CP derivative experiences some fragmentation. When a hydrogen atom is replaced by a sulfonic acid (-SO_3H) group a mass increase of 80 Daltons results, and the monosulfonated CP derivative is formed as shown in Figure 2.1b. In Figure 2.2b, the peak at m/z 80 corresponds to sulfite ion ($SO_3^{\cdot -}$) and is evidence that a sulfonated dye

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is present in the ink. Another ion that has an m/z 80 is $HPO_3^{\cdot -}$, however, this functional group has not been observed in dye structures. Examinations of the dye structures in the literature[7,9] lead to this conclusion. Upon laser D/I of the copper phthalocyanine dyes, the monosulfonated metal complex fragments losing the sulfonic acid. The loss of this functionality has also been observed in ESI MS and MALDI MS[10,11]. MALDI and ESI are softer ionization techniques than LD; however, fragment ions can be formed when tandem MS methods are applied. Bruins *et al.* state that m/z 80 denotes $SO_3^{\cdot -}$ and can be used as an indicator that polysulfonated azo dyes are present in the sample[12]. The formation of fragment ions in ESI MS was carried out by implementing collision-induced dissociation (CID)[11,12]. The loss of the metal center has not been observed using MALDI MS nor ESI MS[12] as well as in the experiments performed here with LDMS. Copper appears to be strongly bound to the phthalocyanine. Conneely *et al.* found the metals Cu, Ni and Zn in phthalocyanine complexes are stable, but the analysis of the aluminum phthalocyanine derivative gave rise to a peak at m/z 27 which corresponds to the loss of Al[12]. The peak at m/z 520 in the positive-ion LD mass spectrum of the Copper Phthalocyanine has not been identified; however, the peak is always observed when the pigment is analyzed and has a similar isotope distribution as copper complex.

The low mass peaks (< 300 Da) in the negative-ion LD mass spectrum (Figure 2.5b) of the black ballpoint ink provide some useful information about the structure of Metanil Yellow. When analyzing an ink a complete examination of the LD mass spectrum should be carried out as a general rule. An intriguing aspect of Metanil Yellow is that, although, the dye was detected intact, the colorant is not entirely resistant to prompt fragmentation which can be seen at m/z values below that of the intact dye. In

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order to directly examine Metanil Yellow to avoid possible interference from other ink constituents that in the black ink, the commercial dye was purchased from the Aldrich Chemical Company and was analyzed. Figure 2.7 shows the negative-ion LD mass spectrum of Metanil Yellow analyzed directly from a metal plate. The region of the low mass peaks was enhanced as seen in Figure 2.8. Possible fragment ions are illustrated in Figure 2.9. A Metanil Yellow fragment ion was observed at m/z 156. Other analytical methods including MALDI, TLC, and post source decay (PSD) may be used to make certain that this was a fragment ion of Metanil Yellow rather than an impurity. The peaks at m/z 80, 156, and 171 are associated with fragment ions from the D/I of Metanil Yellow. All three fragment ions (m/z 80, 156, and 171) have been previously observed for the dyestuff, Acid Orange 7[10], as seen in Figure 2.10. The dyestuff was analyzed using ESI tandem MS and MALDI-PSD MS. The structural similarities shared between the Acid Orange 7 and Metanil Yellow result in the formation of the same fragment ions. The peak at m/z 171 corresponds to ions formed from the cleavage of the ketohydrazone single N-N bond and m/z 156 results as ions are formed the homolytical fission of the azo C-N bond[10]. The peak at m/z 80 was previously discussed for the monosulfonated Copper Phthalocyanine complex and corresponds to the loss of sulfonic acid form the structure of Metanil Yellow.

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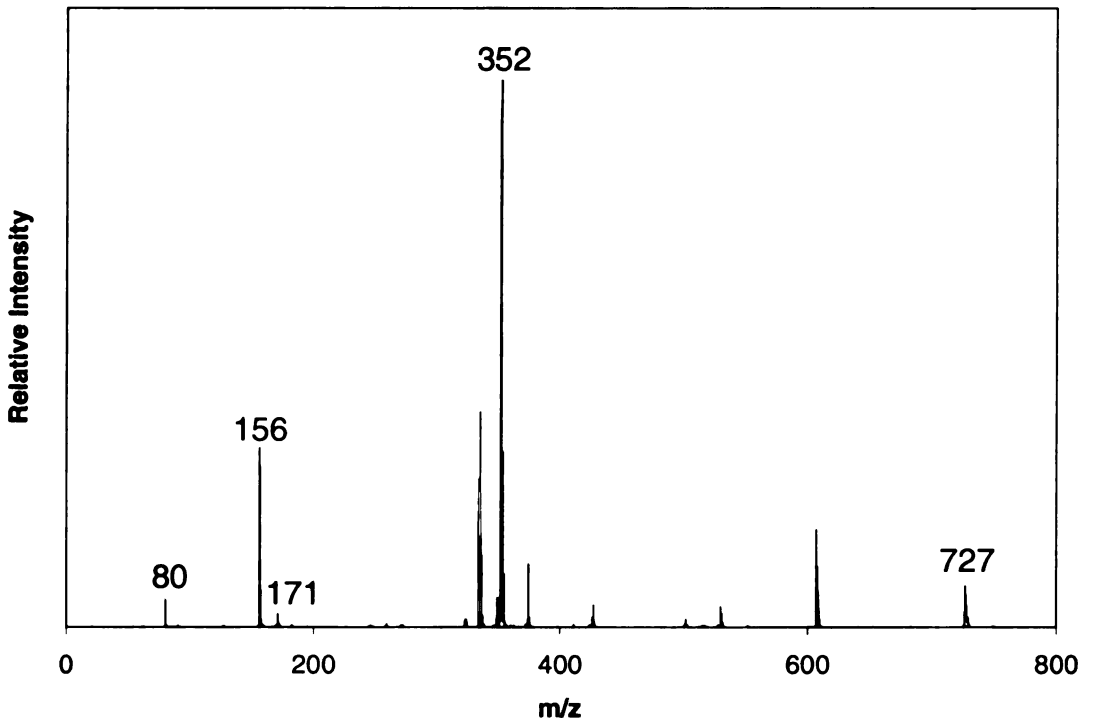

Figure 2.7: The direct analysis of Metanil Yellow from a metal plate by negative-ion LDMS

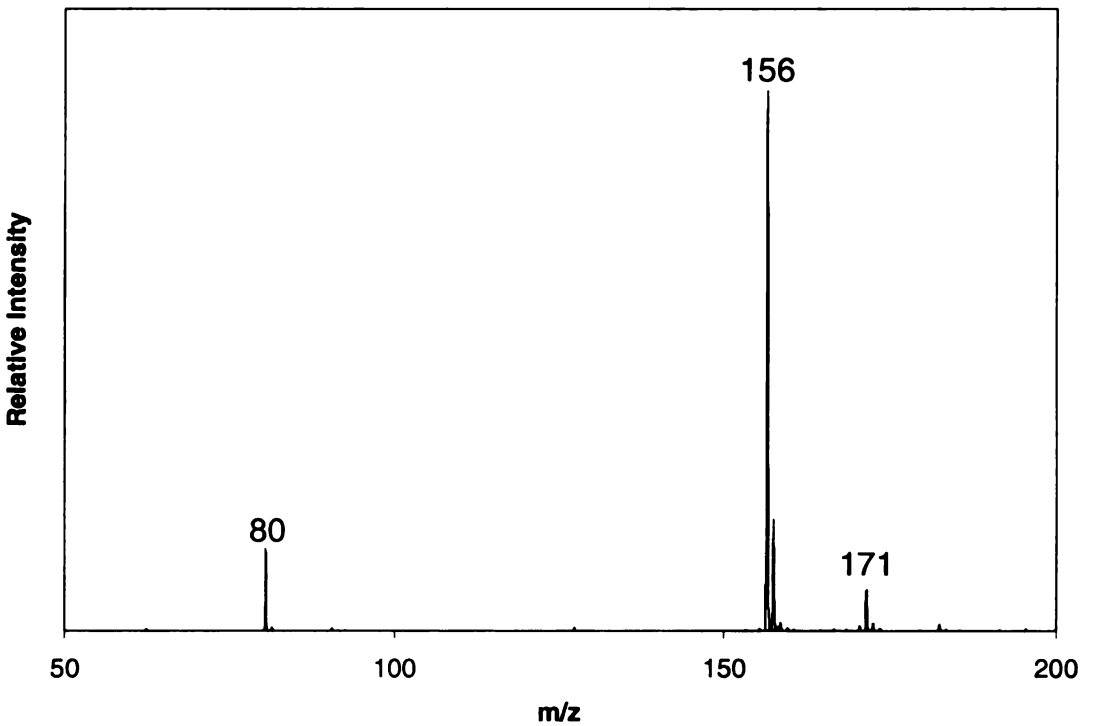

Figure 2.8: Negative-ion LD mass spectrum of Metanil Yellow shows peaks due to fragment ions

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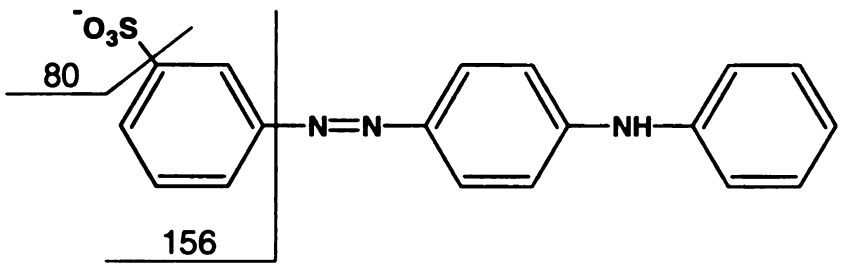

Figure 2.9: Dye structure of Metanil Yellow with labeled values that correspond to possible fragment ions or neutral molecules

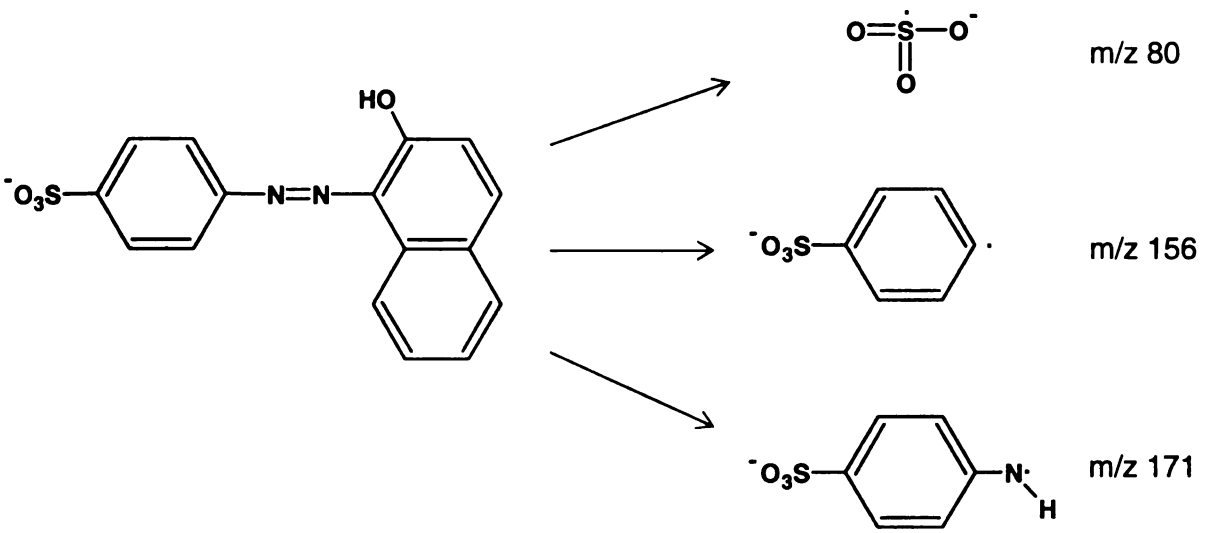

Figure 2.10: Acid Orange 7 (to the left of the arrows) and fragment ions (m/z 80, 156, 171) formed when analyzed by ESI tandem MS and MALDI-PSD MS[11]

To identify the source of low mass peaks, a method that distinguishes between fragment ions and ions representing dye impurities should be applied. Three methods that may be used in combination with LDMS to determine if the peaks are due to fragment ions or dye impurities include TLC, post source decay (PSD), and implementation of a matrix (MALDI). TLC indirectly coupled with LDMS is a simple experiment that can be used to determine the source of the ions. TLC separates species in a mixture and is suitable for the separation of multiply-charged dyes in an ink. A dye present in an ink as an impurity can be separated from other colorants. The individual dyes, including the impurity, can be extracted from the TLC plate and subsequently analyzed by LDMS to confirm that the low mass peaks dyes and not fragment ions. Prompt fragment ions are formed during the desorption/ionization process. Prompt fragments are encountered in other ionization techniques, notably, electron impact (EI) and are generally more extensive in EI than UV LD. Another method to determine if fragment ions were produced is to employ a matrix. Matrices are typically used in LDMS for the detection of analytes that are prone to undergo fragmentation. The method is recognized as matrix-assisted laser desorption/ionization and is used for the analysis of biomolecules. The matrix assists in the D/I of the analyte while reducing prompt fragmentation. A matrix can be added to the ink and the fragment ions should be eliminated or reduced. The addition of the matrix should eliminate or reduce the formation of fragment ions. If the low mass peaks are absent when the ink is analyzed by MALDI, then low mass peaks observed previously in the LD experiment likely represent fragment ions.

The formation of clusters (dimers, trimer, etc.) result from electrostatic interactions between ions and has been observed for ionic dyes examined by LDMS. Desorption/Ionization of Metanil Yellow resulted in the formation of at least one cluster and can be seen in the negative-ion LD mass spectrum in Figure 2.11. The peak at m/z 727 corresponds to a negatively-charged cluster. The peak represents a dimer and consists of two Metanil Yellow ions and one sodium ion [(2 x 352 + 23) = 727]. Multiple clusters may from one dye are easily identified by relative peak intensities. As the number of dye monomers increase, the peak intensity of the cluster decreases. In other words, the peak intensity of a trimer is smaller than the intensity of a dimer, in general, for a given dye. Cluster recognition may be determined by analyzying the commercial dye using TLC. When Metanil Yellow was subjected to TLC, the analysis indicated that no impurity dyes were present in the commercial dye since only one band was present. Based on this additional information, the peak at m/z 727 was confidently assigned to a Metanil Yellow dimer.

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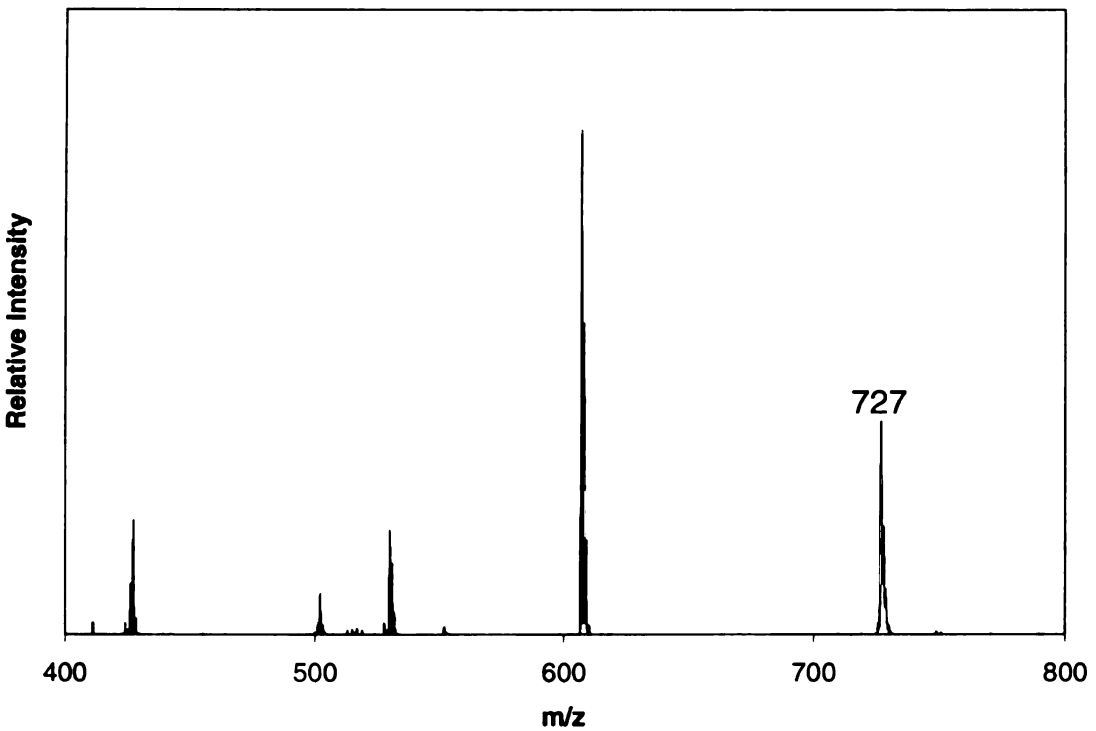

Figure 2.11: Negative-ion LD mass spectra of Metanil Yellow on paper shows peaks due to cluster ions

Coupling TLC with LDMS

Inks are complex mixtures and frequently, contain multiple dyes making the identification by LDMS difficult, at times. TLC can be coupled with LDMS to ease the identification process. The color and the number of dyes present in an ink are determined by TLC. The CI name of the dye is based on its color and the separation of the dyes from an ink by reveals their true color. The ink color can be misleading for determining the color of the dyes in the ink. Frequently, the ink color is not the same color as the dyes and depends on the combination of dyes used. For example, the combination of Crystal Violet and Metanil Yellow are surprisingly used to make black ink for several ballpoint pens as previously stated. When a concentrated, aqueous Crystal Violet solution is dried on paper the spot appears to be black. The addition of Metanil Yellow did not appear to

influence the color of the dye mixture. Despite the use of violet and yellow dyes, the color of the ink is black which can be misleading when trying to identify a dye based on the color of the ink. TLC data, including the number and color of the bands, is acquired to prevent the possibility of misconstruing the data. Knowing the dye color lessens the search for the identification purposes. Indirect coupling of TLC with LDMS first used for the analysis of black ballpoint ink allowed Metanil Yellow to be identified (the identity of Crystal Violet was known). Figure 2.12 shows three distinctly colored bands of a TLC chromatogram upon analyzing black ballpoint ink. The two purple bands correspond to Crystal Violet (D1) and Methyl Violet 2B (D2) and the yellow band represents Metanil Yellow (D3). The bands were scraped, solvent extracted and analyzed by LDMS. The analysis of the extracted dye confirmed that the yellow TLC band denoted the peak at m/z 352 in negative-ion LD mass spectrum, the purple bands, D1 and D2, were correlated with m/z 372 and 358, respectively, in the positive-ion LD mass spectrum. Preliminary experiments of coupling TLC with LDMS directly showed potential. Direct coupling of TLC eliminates the extraction step prior to the LDMS analysis reducing the analysis time. Instead of using a glass TLC support, a plate backed with aluminum was used for the direct analysis. Glass TLC plates are difficult to couple directly with LDMS for two reasons (1) glass is difficult to cut and mount to the sample plate and (2) glass is an insulating material which posses a threat when trying to obtain analyte signal. Figure 2.13 demonstrates that adequate signal can be acquired when the aluminum TLC plate is directly coupled with LDMS. The dyes in black ballpoint ink from a BIC® Cristal pen were separated by TLC and a single purple band which corresponded to Crystal Violet was analyzed directly with LDMS. The positive-ion

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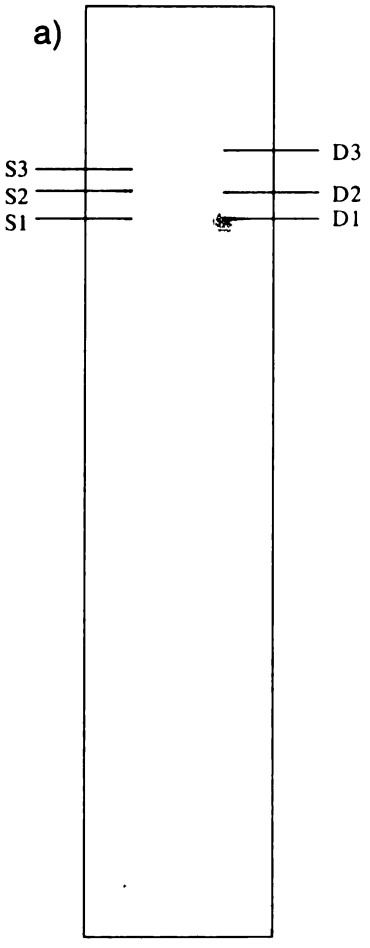
spectrum shows the dye was easily detected showing strong signals with isotopic resolution (results are comparable to detecting Crystal Violet directly from paper). The presence of the lower mass peaks were most likely due to the silica from the TLC plate. Occasionally, a peak at m/z 27 was observed in the positive-ion LD mass spectra. Most likely, this peak is due to the D/I of aluminum metal from the TLC plate. Paper chromatography was also used to separate the ink dyes in black ballpoint ink, but the separation was poorly resolved so further LDMS coupling was not employed.

a)



b)

TLC Band	Dye
S1	Crystal Violet
S2	Methyl Violet 2B
S3	Tetramethylpararosaniline
D1	Crystal Violet
D2	Methyl Violet 2B
D3	Metanil Yellow

Figure 2.12: TLC analysis of black ballpoint ink from BIC® Cristal a) thin-layer chromatogram and b) TLC bands and corresponding dyes

a)

Relative Intensity

b)

Relative Intensity

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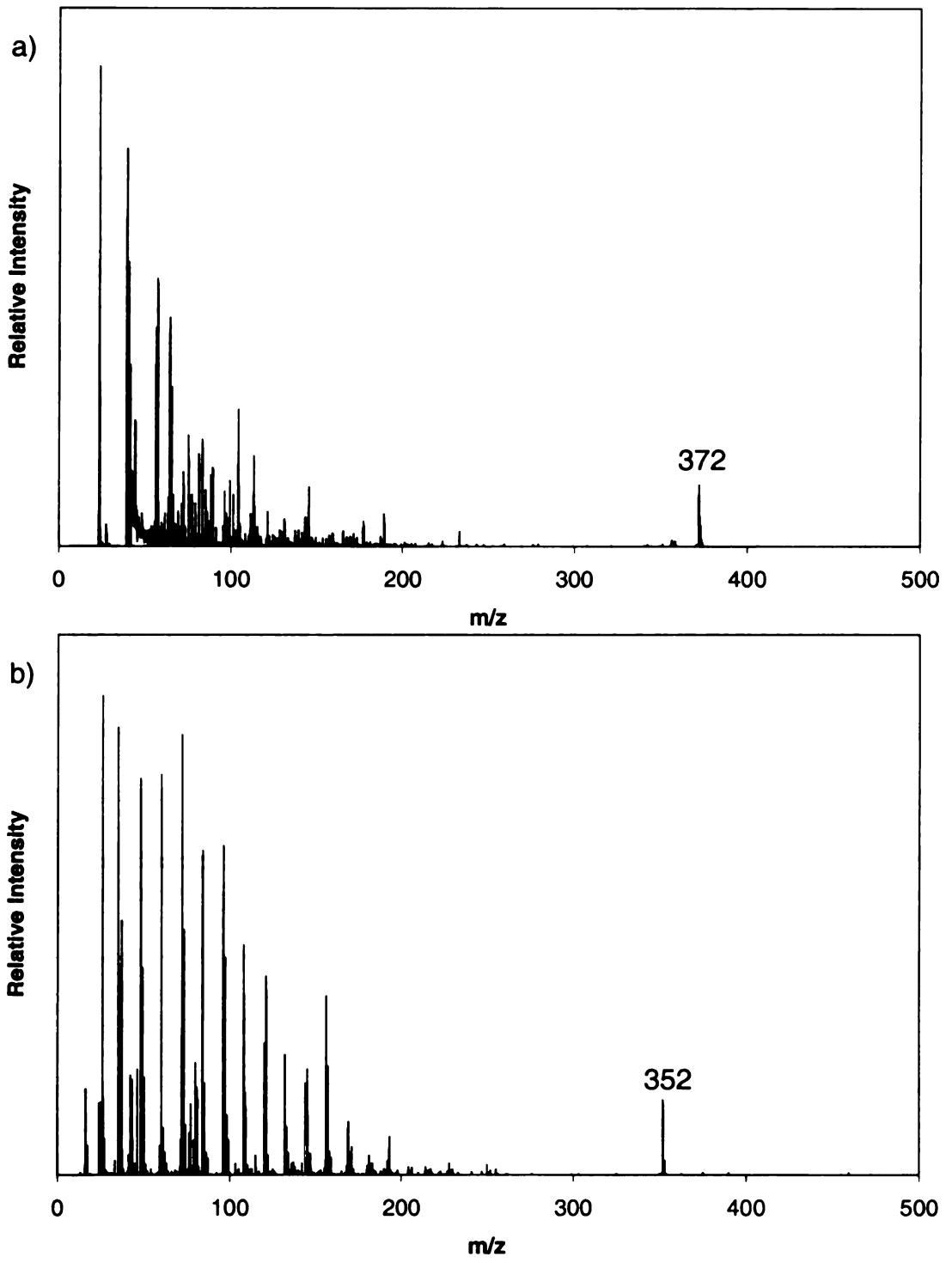

Figure 2.13: Direct coupling of TLC with LDMS: bands are analyzed by LDMS directly from the aluminum TLC plate a) purple band (Crystal Violet) and b) yellow band (Metanil Yellow)

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Chapter Three: Natural and Artificial Dye Degradation

Exploring dye degradation of ink dyes for relative ink dating ultimately lead to the development of using photodegradation and LDMS to characterize ink dyes which is the main focus of this thesis research and is discussed in the following chapter. However, the previous work that has already been accomplished in the area of dye degradation will be briefly discussed. Ink dye degradation was first examined elsewhere for purposes of dating questioned documents. Relative ink dating was carried out previously by examining the degradation of Crystal Violet, a dye commonly used in blue and black ballpoint pen ink[1]. Natural dye degradation and artificial degradation were correlated using an UV accelerated aging plot. There is no ideal method for determining the relative age of a document and the degradation of dyes is no exception. The degradation of Crystal Violet was examined in this research to show that environmental conditions influence the degradation of the dye. There was also a concern that the degradation mechanism of Crystal Violet may not be applicable to other ink dyes, however, several dyes may undergo similar chemical degradation. The assumption was made that any dye with an alkylated amine group would be able to degrade by oxidative dealkylation. Both the natural and artificial degradation of Rhodamine B was studied in this research. Rhodamine B, a dye used in red ink, has structural similarities to Crystal Violet so the degradation mechanism was expected to be similar.

As ink on a document ages, physical and chemical changes take place such as the evaporation of solvents, the hardening of ink resins, and the fading of color (degrading of colorants lead to color changes). Aging characteristics have been associated with the

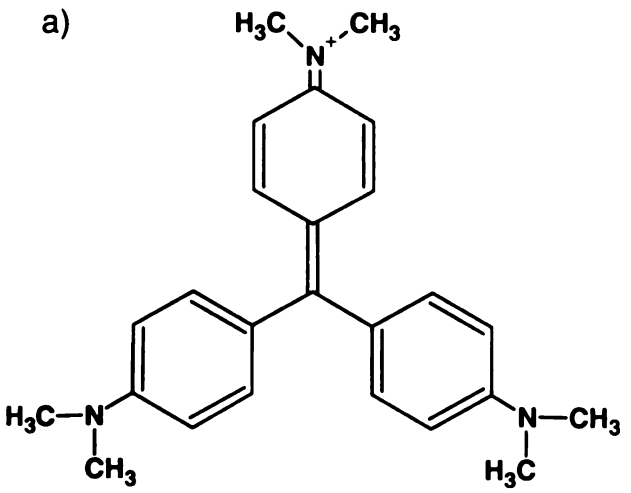
decrease in dye concentration. When the color of the ink fades over time the dye undergoes chemical changes, hence, the colorant degrades resulting in decreased concentration. The physical changes and chemical reactions that take place are influenced by several environmental factors. Part of this research project was to study the processes of natural aging of dye molecules on paper and to determine if this can be exploited in dating handwritten questioned documents, thus, to determine if the degradation of ink dyes can be used for relative ink dating. Preliminary aging studies using LDMS indicated that the mechanisms for natural aging of Crystal Violet, a dye commonly used in ballpoint pens with blue and black ink, was straight forward, understandable, and showed potential for assisting in dating documents. Conditions under which documents are stored were examined to determine the effects on dye degradation. To accurately date a document, environmental conditions need to be held constant, but this is unachievable. Natural light, humidity, and temperature may all play a role in the degradation of ink dyes. Not only do storage conditions render dating impossible, but the natural degradation of dyes over time appears to be limited to a small class of dyes. Several dyes used in the manufacture of ink appear to be chemically stable; hence, they do not form degradation products. Storage conditions as well as dye stability appear to be two limiting factors associated with using dye degradation as a means to date a questioned document.

Oxidative N-Dealkylation

Crystal Violet

Crystal Violet, a common dye in blue and black ballpoint pen ink, is a cationic, triarylmethane dye. Previous work by Grim *et al.* was carried out to demonstrate that Crystal Violet can be used to determine the relative age of a document[1]. Naturally aged samples were analyzed and ink dating curves were formulated based on an accelerated aging study that incorporated the use of ultraviolet light. Ultraviolet radiation was found to mimic the natural aging process of Crystal Violet. The dye was found to degrade naturally and artificially by a process called oxidative demethylation. The structure contains six methyl groups that are chemically modified when the dye degrades. The dye as shown in Figure 3.1a can form a maximum of six degradation products, illustrated in Figure 3.1b, which can be simultaneously detected in LDMS. Oxidative demethylation occurs by the substitution of a methyl group for a hydrogen atom. One methyl group substitution results in a mass difference of 14 Da. Shown in Figure 3.2 is the positive-ion LD mass spectrum of black ballpoint pen ink containing Crystal Violet which has been degraded using UV radiation. In the mass spectrum, the peaks due to Crystal Violet (m/z 372) and the corresponding dye homologues (m/z 358, 344, 330, 316, and 302) are separated by 14 atomic mass units.

a)



b)

n	m/z
6	372
5	358
4	344
3	330
2	316
1	302
0	288

Figure 3.1: Crystal Violet a) dye structure and b) m/z values of the dye and corresponding degradation products (n represents the number of methyl groups bonded to the amine nitrogen atoms)

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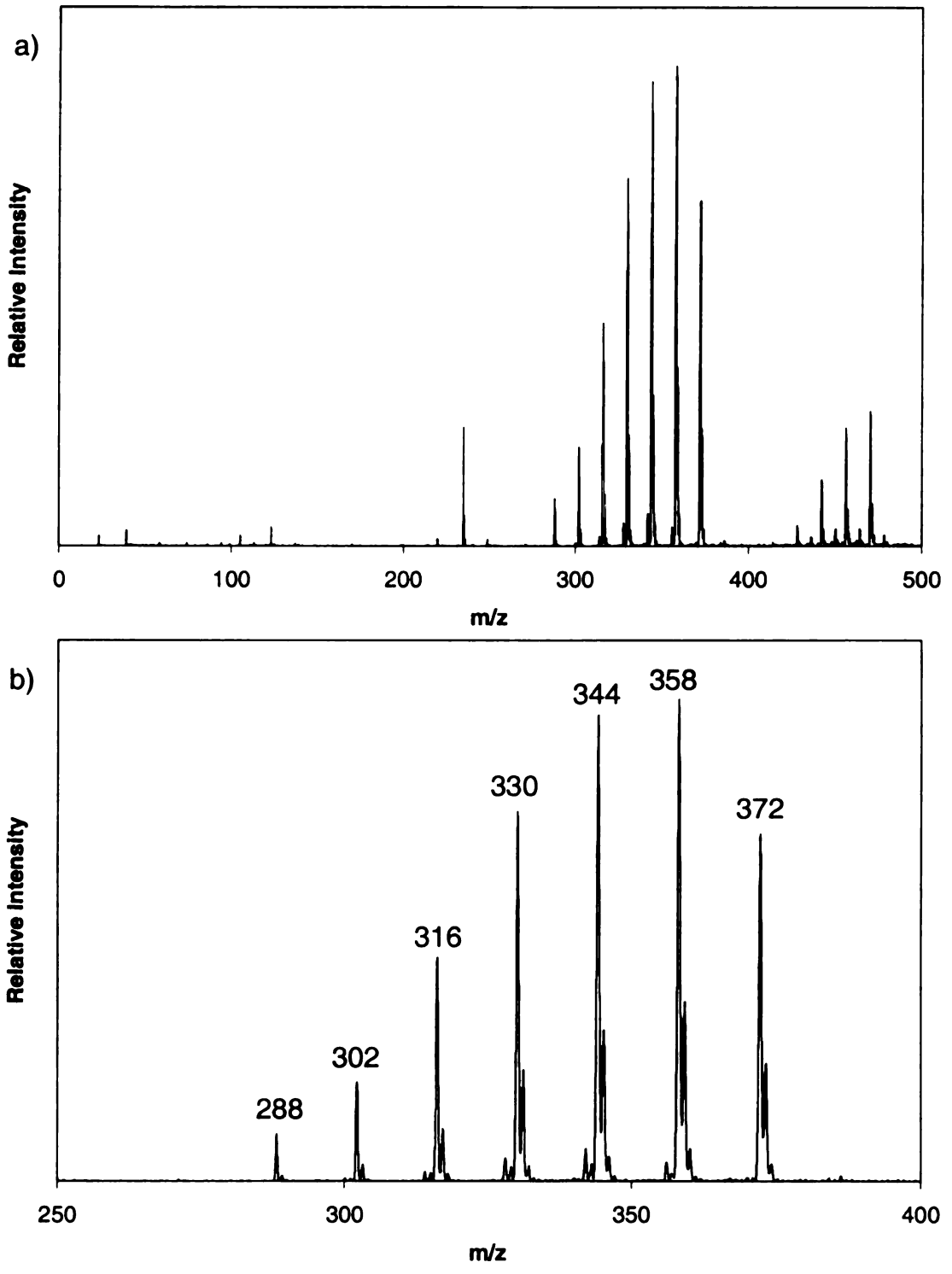
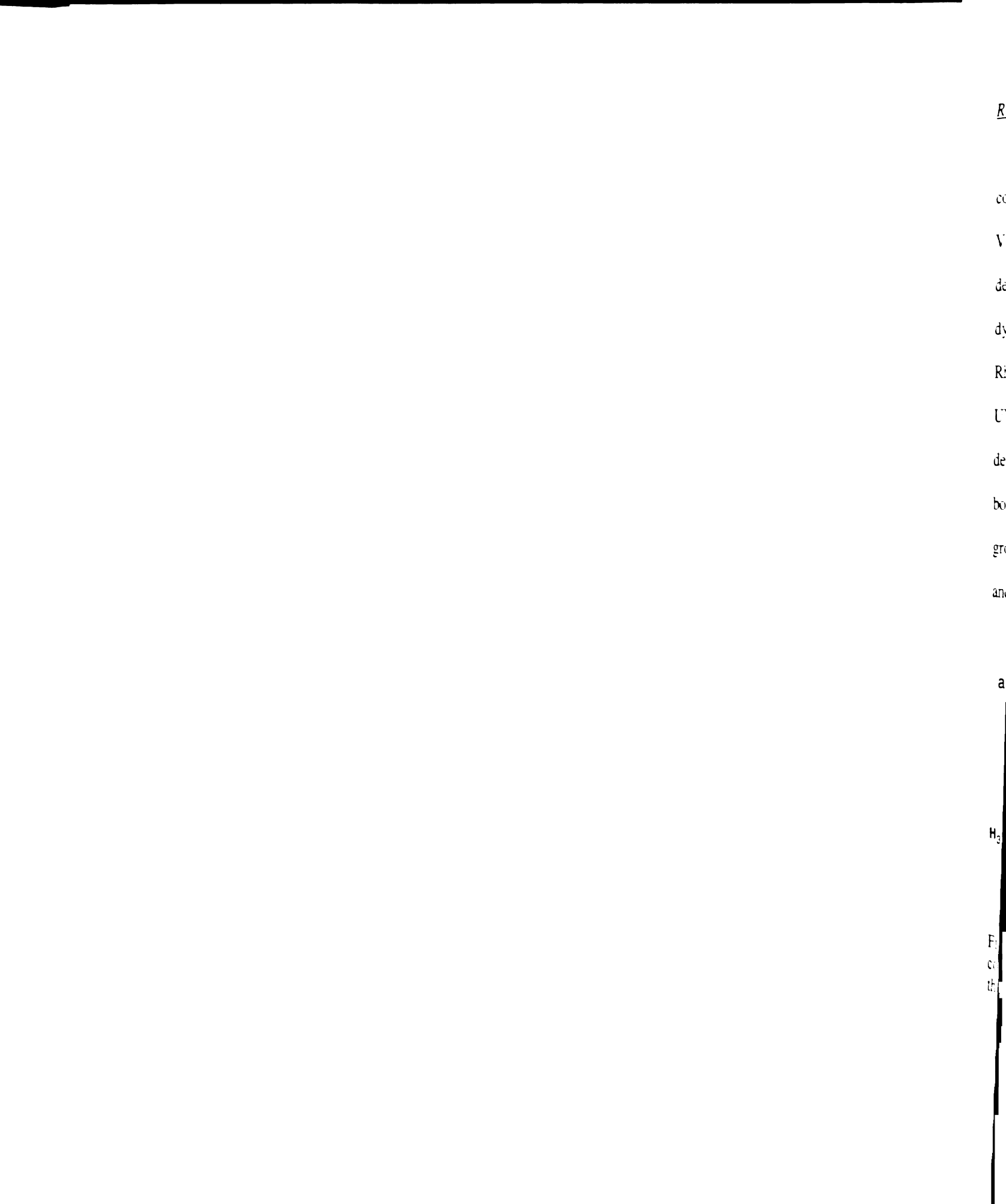


Figure 3.2: Positive-ion LD mass spectra of blue ink from BIC Cristal ballpoint pen degraded with UV radiation a) complete spectrum from m/z 0-500 and b) expanded region from m/z 250-400

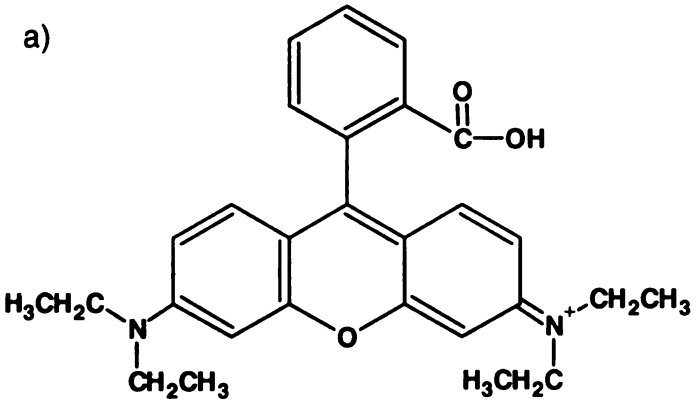
The experiment carried out by Grim *et al.* showed the correlation between natural aging and accelerated UV aging to develop a specific method for relative ink dating[1]. The results suggested that if UV light is used to accelerated dye aging, then 6.25 hours of UV radiation exposure corresponds to a document that is 38 months old, so every hour of irradiation the ink ages approximately 182 days. Grim *et al.* also showed that there was a possibility that Crystal Violet may cease aging after a certain amount of time[1]. The solvent extraction technique is limited to about four years[1]. Positive-ion LD mass spectra were obtained for more than 25 samples which spanned a 50 year period. The samples were not controlled since they differed in ink formulations, type of paper, relative humidity, and other environmental storage conditions. The average molecular weight of the dye plotted as a function of age was used to correlate the age of the document. One would expect that as Crystal Violet ages the number of degradation products increase so the average molecular weight would decrease with increasing age. According to Grim *et al.*, the substantial scatter may be due to the possibility that the dye aging process stops after roughly 15 years[1]. However, the scatter could also be influenced by the uncontrolled variables of the ink samples. An interesting observation made by Grim *et al.* is that the uncontrolled ink samples only reached an average molecular weight of 365 Da and the controlled ink library samples appear to age much more extensively and faster than the uncontrolled samples[1]. This demonstrates that the variables play a substantial role in the aging process for dyes in inks.



Rhodamine B

Rhodamine B is a dye that is frequently and currently used in ballpoint pens that contain red ink. Rhodamine B, shown in Figure 3.3a, is structurally similar to Crystal Violet in terms of containing alkylated amines which allows the dye to undergo oxidative dealkylation. When Rhodamine B is subjected to UV radiation or incandescent light the dye degrades and forms detectable deethylated products that can be detected by LDMS. Rhodamine B forms at least four degradation products which were chemically induced by UV radiation and incandescent light and are illustrated in Figure 3.3b. The four degradation products result from hydrogen atom substitution of two ethyl groups that are bonded to the two nitrogen atoms. A hydrogen atom (mass of 1 Da) replaces an ethyl group (mass of 29 Da) with a maximum of four substitutions. The m/z values of the dye and the degradation products are separated by 28 Da.

a)



b)

n	m/z
4	443
3	415
2	387
1	359
0	331

Figure 3.3: Rhodamine B a) dye structure and a) m/z values of the dye and corresponding degradation products (n represents the number of ethyl groups bonded to the amine nitrogen atoms)

Natural Degradation of Rhodamine B

Rhodamine B was expected to degrade naturally in the same fashion as Crystal Violet. However, examining Rhodamine B for natural degradation demonstrated the dye does not appear to substantially degrade due to oxidative N-dealkylation as compared to Crystal Violet. Eight documents containing red ink with dates ranging from 1960 to 1979 were obtained from the MSU Chemistry archives to study the effects of natural aging of Rhodamine B. Five of the eight samples appeared to contain Rhodamine B, however, two of the samples contained another dye that is higher in concentration than Rhodamine B and the peak at m/z value 429 which is associated with the other dye interfered with the degradation of Rhodamine B, so these samples were not included in the study. The three samples that exclusively contained Rhodamine B were used for the study. Shown in Figures 3.4, 3.5, and 3.6 are the positive-ion LD mass spectra of the three naturally aged samples directly from their paper substrate. The documents were dated 1960, 1965, and 1967. The peak at m/z value 443 represents the intact detection of Rhodamine B. The expected degradation products of Rhodamine B have the corresponding m/z values of 415, 387, 359, and 331 as seen in the mass spectrum of the artificially aged dye. There appears to be limited degradation of Rhodamine B based on the low intensity of the peaks at m/z values 415 and 387. There does not appear to be a trend associated with the natural degradation of Rhodamine B over time based on the examination of the peak intensities of the intact dye and the degradation products. Previous degradation studies with Crystal Violet showed that the peak intensities can be used to determine the extent of the dye degradation which was determined based on the LD spectral interpretation. The formation of the degradation products appears to occur in

a stepwise fashion. The first degradation product formed is the Crystal Violet homologue that has lost one methyl group and each degradation product is formed from the preceding product yielding the characteristic degradation pattern of dyes containing multiple amine groups. The first degradation product formed has an m/z value of 415 which dominates over the other degradation products. Without the formation of the first degradation product the subsequent products could not be formed. Rhodamine B is assumed to follow the same degradation pattern. Surprisingly, the xanthene dye appears to be fairly stable since the mass spectra of the aged inks are quite similar to that of fresh ink containing Rhodamine B. Figure 3.7 shows the positive-ion LD mass spectrum of fresh red ink that contains Rhodamine B.

The natural degradation of Rhodamine B was expected to be similar to that of Crystal Violet and the extent of degradation of Rhodamine B and Crystal Violet was compared. Two documents containing entries written with both red and black ink were examined. The documents were dated 1965 and 1967. The advantage of examining black and red ink from the same document is that the inks are exposed to the same conditions over time. Examining ink samples from the same document for an aging study comparison are more reliable than using ink samples from separate documents. The study confirmed that Crystal Violet degrades more readily than Rhodamine B. The LD mass spectra can be seen in Figure 3.8. The peak intensities of the Crystal Violet degradation product are more prominent than the peaks that correspond to the degradation of Rhodamine B. The lack of natural degradation of Rhodamine B limits the utility of dye degradation in order to determine the relative age of a document.

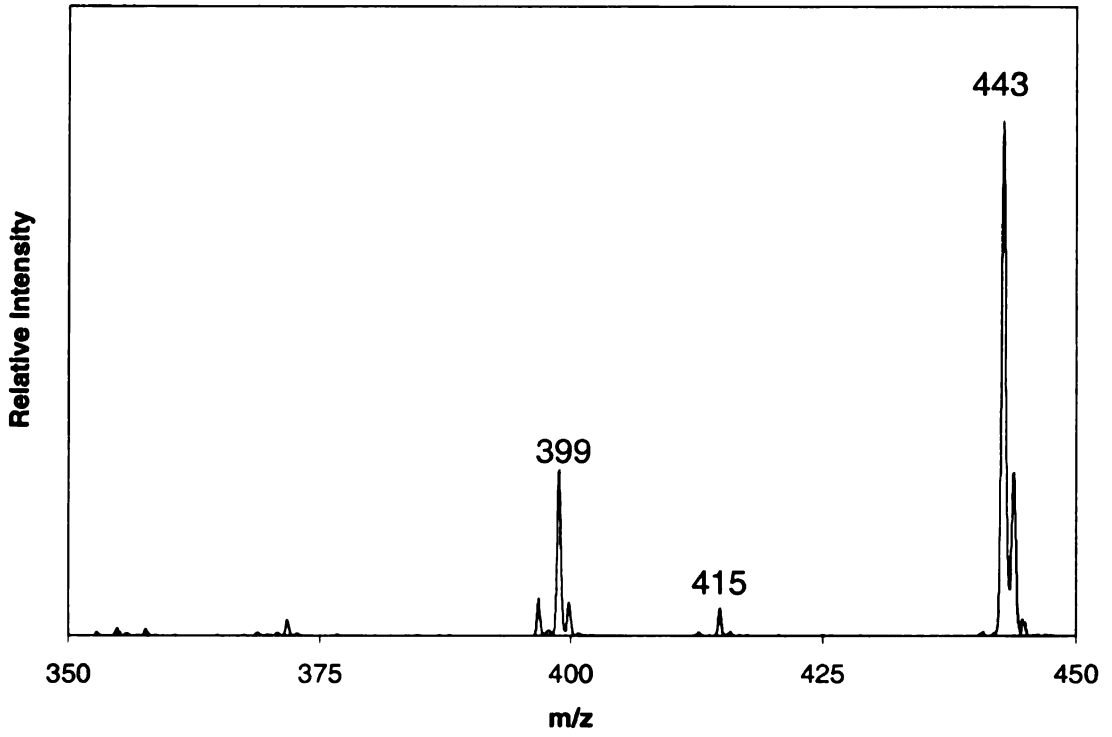


Figure 3.4: Positive-ion LD mass spectrum of red ink on a document dated 1960

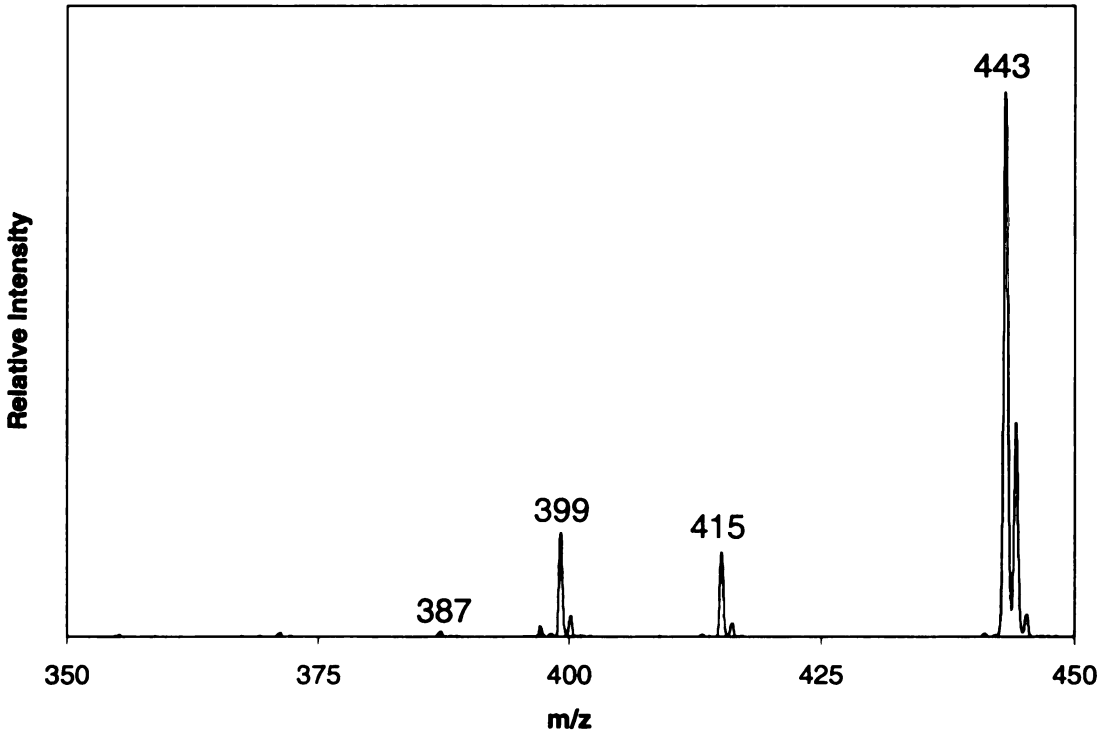


Figure 3.5: Positive-ion LD mass spectrum of red ink on a document dated 1965

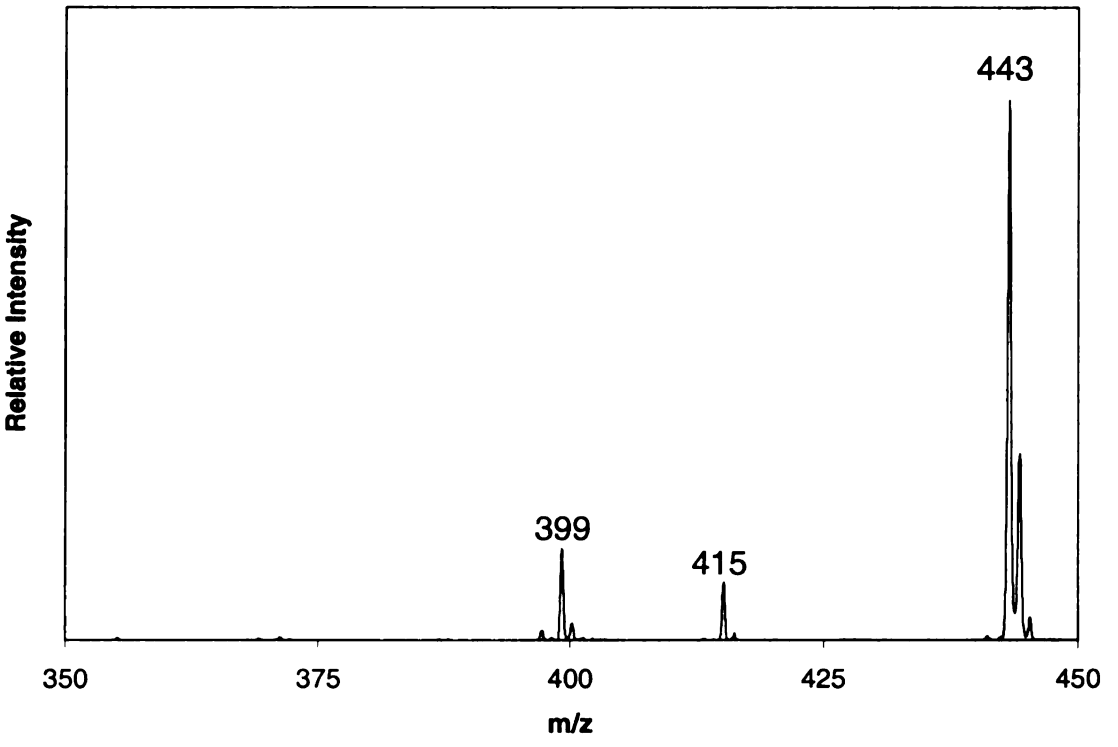


Figure 3.6: Positive-ion LD mass spectrum of red ink on a document dated 1967

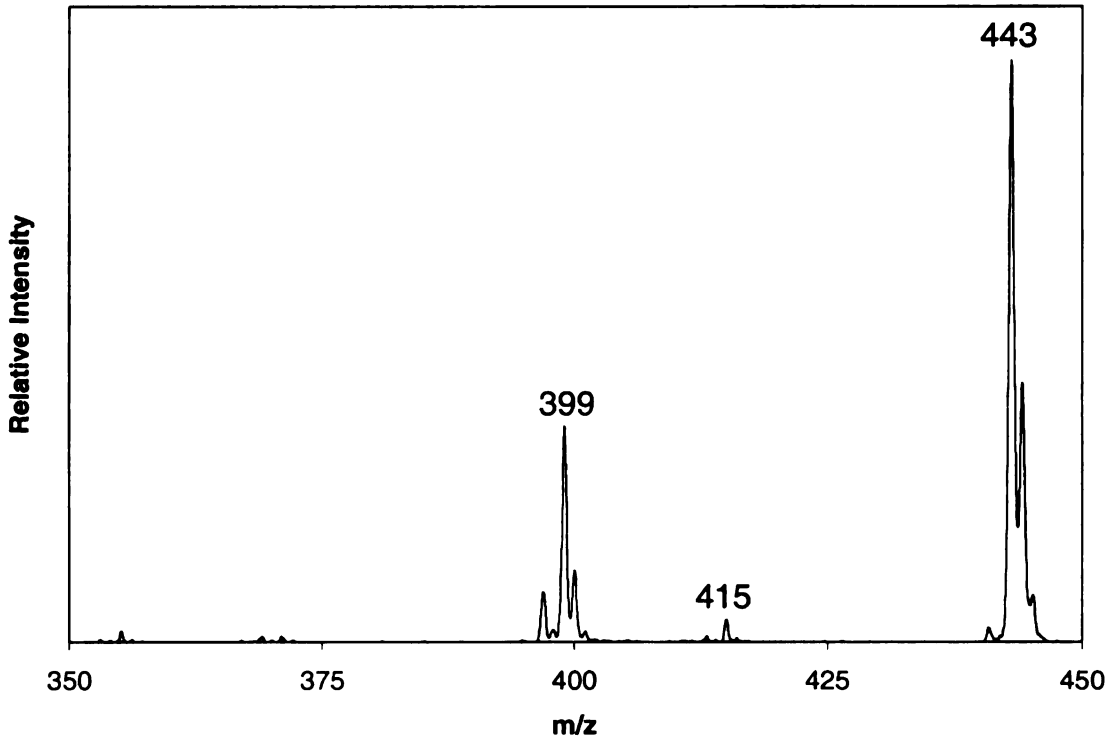


Figure 3.7: Positive-ion LD mass spectrum of red ink freshly written on paper

In conclusion, ink dating based on the degradation of dyes contained in ink is implausible. Although the degradation of ink dyes is unreliable in terms of ink dating, artificial aging can be used to characterize ink dyes. The combined use of light-induced degradation and LDMS can be used to characterize ink dyes. Crystal Violet and Rhodamine B were shown to be degraded through the use of light (UV radiation or incandescent). The degradation pathway and the formation of degradation products are dye specific. Several dyes are light sensitive and can be degraded. The reaction chemistry was applied in conjunction with LDMS for the characterization and identification of ink dyes. To demonstrate the strength of this method the dye characterization was focused on isomeric dyes that are used in red ink. The difficulty in analyzing isomeric dyes in LDMS is that the dyes share the same molecular mass; hence, the mass spectra of the two dyes will contain peaks at the same m/z. Also, the peaks will have the same isotopic distribution. In order to determine which dye is present the dyes need to be structurally modified. One approach to chemically modify the structure is to use light to degrade the dye. In general, dyes that are light sensitive are easily degraded and the degradation products can be detected using LDMS. Rhodamine B and Rhodamine 6G are isomers, but their structural differences cause them to form different degradation products. The characterization method was applied to Rhodamine B and Rhodamine 6G and is discussed in Chapter Four.

References

(1) Grim DM, Siegel JA, Allison J. Evaluation of Laser Desorption Mass Spectrometry and UV Accelerated Aging of Dye on Paper as Tools for the Evaluation of a Questioned Document. *J Forensic Sci.* **2002** (47) 1265-1273

Chapter Four: Photochemistry for Dye Characterization

Direct laser desorption mass spectrometry (LDMS) has recently been demonstrated as a sensitive method for detecting dyes in pen inks on paper[1-4]. In laser desorption, energy is deposited into the ink molecules in 2-3 nanoseconds, quickly raising the temperature of a very small portion of the sample to a point where desorption is favored over degradation. Thus, the ions desorb intact, giving a single mass spectrometric peak (plus isotopic peaks), and providing molecular weight information and possibly elemental composition. Interpreting an LDMS spectrum of a dye may be similar to interpreting an HPLC chromatogram. One dye yields one peak, so structural information is limited. In order to obtain structural information from the LDMS experiment dyes on the paper substrate are degradation photochemically. LDMS analysis following photodegradation yields a set of mass spectral peaks, each representing a degradation product. From the degradation products, and an understanding of possible reactions that may occur, structural insights can be obtained. For example, if Crystal Violet is irradiated with UV radiation, N-demethylation reactions occur and the dye degrades to form Methyl Violet 2B. Replacement of a methyl group by a hydrogen atom results in a net loss of 14 atomic mass units. Crystal Violet can lose up to six methyl groups in this way. Previous work carried out by Grim utilized this chemistry and proposed an approach for determining the age of a document[1]. From an analytical standpoint, the degradation products formed naturally or by accelerated aging are related to the structure. The fact that Crystal Violet forms degradation products showing the loss

of up to six methyl groups provides an important structural context in which the dye could be identified.

Experimental

Laser Desorption Mass Spectrometry

The inks and dyes on paper were analyzed using a PE Biosystems Voyager DE (Farmingham MA) mass spectrometer. The instrument utilizes a pulsed nitrogen laser (337 nm, 3 nanosecond pulses, 3 Hz) and a linear time-of-flight mass spectrometer. The user-selected parameters for the LDMS experiments include an accelerating voltage of 20,000 V for detection of positive ions and -15,000 V for detection of negative ions, an intermediate source grid voltage that is 94% of the accelerating voltage, a guide wire voltage that is opposite in bias, and 0.05% in magnitude of the accelerating voltage, and an extraction delay time of 100 ns. The calibrant used for positive and negative-ion analysis was cesium iodide (CsI) (99.9%; Aldrich, Milwaukee, WI).

Single written lines on MSU letterhead paper were analyzed directly. The nitrogen laser spot can be focused onto a portion of a pen stroke approximately 0.3-0.4 mm wide. The currently available red ballpoint inks analyzed were from BIC® Round Stic and BIC® Cristal pens. The sources of the naturally aged inks are unknown. Incandescent light-induced degradation was performed using a 75 W, 120 V light bulb. A distance of 9 cm between the sample and the light source was maintained.

To parallel the analysis of the inks on paper, aqueous dye solutions were prepared, spotted on paper, and allowed to dry for subsequent LDMS analysis. The dyes used in this work are Rhodamine B (Sigma, St. Louis, MO), Rhodamine 6G (Aldrich,

Milwaukee, WI), Rhodamine 123 hydrate (Aldrich, Milwaukee, WI), and New Fuchsin (Aldrich, Milwaukee, WI). Incandescent degradation was performed using a 75 W, 120 V light bulb.

Thin-Layer Chromatography

TLC was carried out using silica gel 150 Å TLC plates (Whatman, Ann Arbor, MI) with dimensions of 5 x 10 cm and a stationary phase thickness of 250 μm. The solvent system consisted of ethyl acetate:ethanol:water (70:35:30). A dye mixture of Methyl Violet 2B and Crystal Violet (Aldrich Chemical Co., Milwaukee, WI) was used as a standard for comparison. In order to extract the dyes from the TLC plate, the colored bands were scraped from the TLC plate, placed in a centrifuge vial, and 5-10 μL of 1:1 (v/v) ethanol/water were added. The vials were vortexed, and the silica was separated from the dye solutions by centrifugation. The extracts were dried on paper and subsequently analyzed using LDMS.

Dye Characterization by Photodegradation and LDMS

LDMS has been shown as a viable method for detecting dyes in red ballpoint pen inks, however, the discovery of two isomeric dyes was the instigator that lead to the development of a method for characterizing ink dyes. The combination of photodegradation and LDMS for dye identification through a challenging example, which involves the differentiation between two isomeric rhodamine dyes, Rhodamine B and Rhodamine 6G, both of which are used in the manufacturing of red inks[5]. The isomeric, xanthene dyes are cationic and have the molecular formula $[C_{28}H_{31}N_4O_2]^+[Cl]^-$. Shown in Figure 4.1 are the positive-ion LD mass spectra of the isomeric dyes. The detection of

the xanthene dyes is denoted by the peaks at m/z 443. Although, the mass spectrum of Rhodamine B contains additional peaks that can be used to characterize Rhodamine B based on the dye structure, Rhodamine 6G could not be characterized. If an unknown ink sample was analyzed by direct LDMS and produced a spectrum similar to that of Rhodamine B (Figure 4.1a), then the presence of Rhodamine 6G in the ink could not be determined. In order to characterize a pen ink that contains two dyes, they both need to be identified. The challenge to differentiate the ink dyes was overcome by using photochemistry to artificially degrade the dyes and subsequently detecting the degradation by LDMS[4].

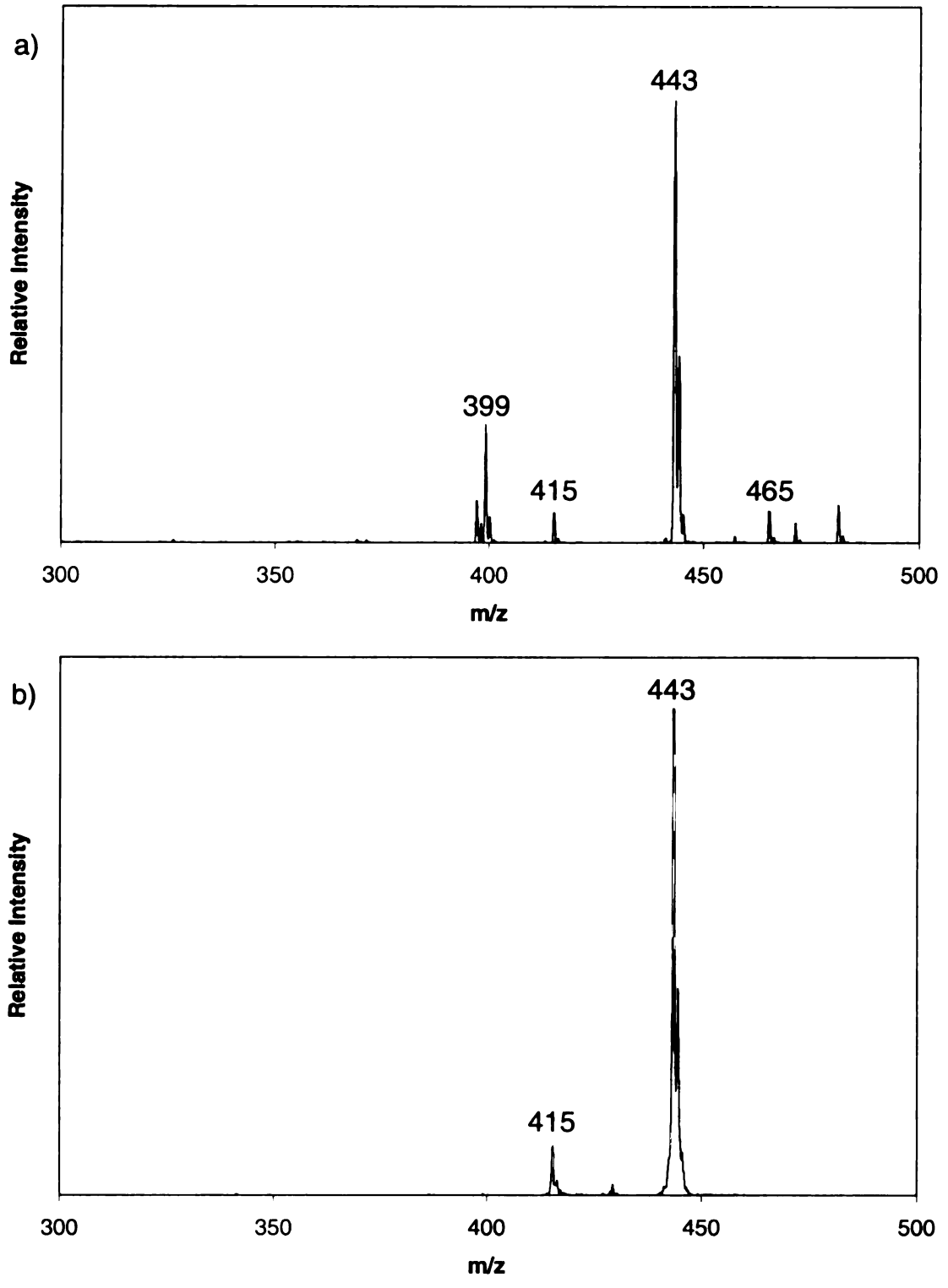
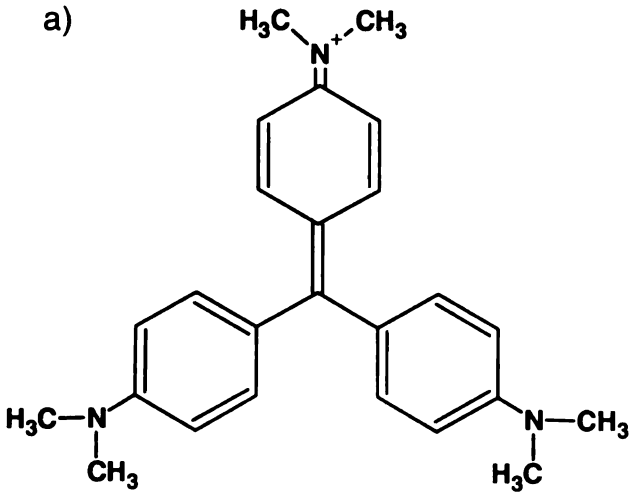


Figure 4.1: Positive-ion LD mass spectra of isomeric, red ink dyes a) Rhodamine B and b) Rhodamine 6G

For this thesis research, we took advantage of the structural similarities shared between the isomeric rhodamine dyes and Crystal Violet and developed an analytical method that involved degrading dyes using incandescent light. The detection of Crystal Violet by direct LDMS has been discussed previously in Chapter Two. Previous work by Grim *et al.* was carried out to demonstrate that Crystal Violet can be used to determine the relative age of a document[3]. Naturally aged ink samples were analyzed and ink dating curves were formulated based on an accelerated aging study that incorporated the use of ultraviolet radiation[3]. Ultraviolet radiation was found to mimic the natural aging process of Crystal Violet. The dye was found to degrade naturally and artificially by a process called oxidative demethylation[3]. The dye structure contains six methyl groups that are chemically modified when the dye degrades. The dye shown in Figure 4.2a can form a maximum of six degradation products, illustrated in Figure 4.2b, which can be simultaneously detected in LDMS. Oxidative demethylation occurs by the substitution of a methyl group for a hydrogen atom. One methyl group substitution results in a mass difference of 14 Da. Shown in Figure 4.3 is the positive-ion LD mass spectrum of blue ballpoint pen ink containing Crystal Violet which has been degraded using UV radiation. In the mass spectrum, the peaks due to Crystal Violet (m/z 372) and the corresponding dye homologues (m/z 358, 344, 330, 316, and 302) are separated by 14 atomic mass units.

a)



b)

n	m/z
6	372
5	358
4	344
3	330
2	316
1	302
0	288

Figure 4.2: Crystal Violet a) dye structure and b) m/z values of the dye and corresponding degradation products (n represents the number of methyl groups bonded to the amine nitrogen atoms)

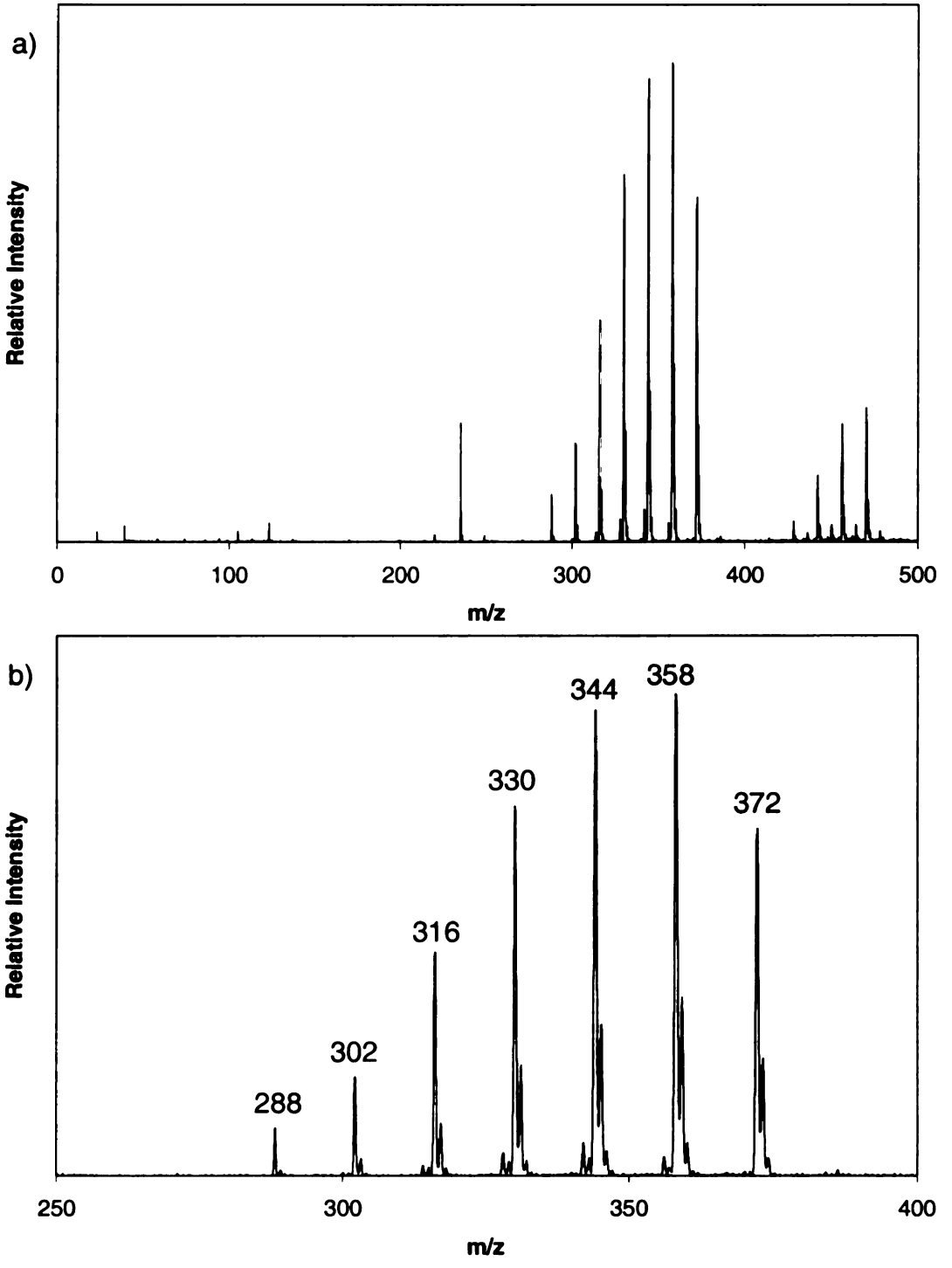
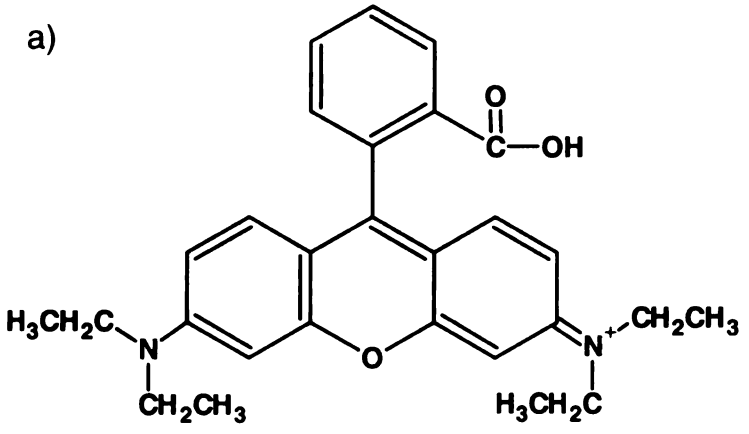


Figure 4.3: Positive-ion LD mass spectra of blue ink from BIC Cristal ballpoint pen degraded with UV radiation a) complete spectrum from m/z 0-500 and b) expanded region from m/z 250-400

The structures of Rhodamine B and Rhodamine 6G are shown in Figures 4.4a and 4.4b, respectively. When the dyes are subjected to UV radiation or incandescent light they degrade and form detectable deethylated products that can be detected by LDMS. Rhodamine B forms four N-deethylated products when chemically induced by incandescent light where two N-deethylated products are form from Rhodamine 6G when the dye is photodegraded and are illustrated in Tables 4.1a and 4.1b, respectively. The degradation products result from a similar degradagtion mechanism as Crystal Violet. As the rhodamine dyes degrade, hydrogen atom substitution of two ethyl groups that are bonded to the two nitrogen atoms results. A hydrogen atom (mass of 1 Da) replaces an ethyl group (mass of 29 Da) with a maximum of four substitutions. The m/z values of the dye and the deethylated degradation products are separated by 28 Da. The deethylated products can be used characterize Rhodamine B, however, the deethyalted products can not be used to discern Rhodamine 6G apart from Rhodamine B if both dyes are analyzed as a mixture. Fortunately, another structural characteristic of Rhodamine 6G can be used to differentiate the dyes which will be discussed later.

a)



b)

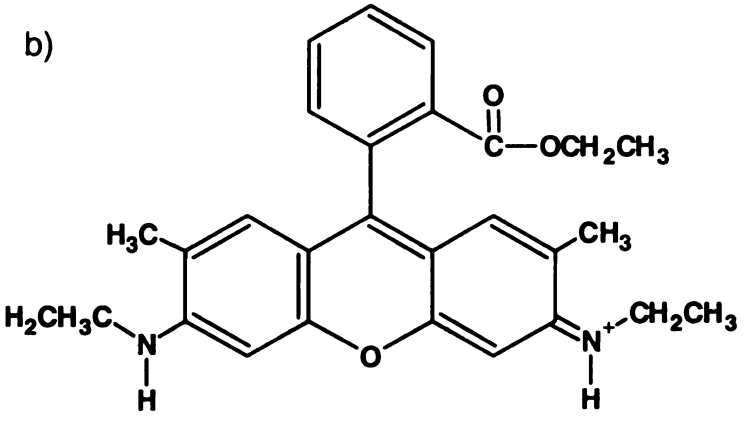


Figure 4.4: Isomeric dye structures a) Rhodamine B and b) Rhodamine 6G

a)

n	m/z
4	443
3	415
2	387
1	359
0	331

b)

n	m/z
2	443
1	415
0	387

Table 4.1: m/z values of the dye and corresponding deethylated degradation products (n represents the number of ethyl groups bonded to the amine nitrogen atoms) for a) Rhodamine B and b) Rhodamine 6G

Isomeric Dye Characterization

According to US Patent 5,993,098[5], two rhodamine dyes, Rhodamine B and Rhodamine 6G, can both be used to manufacture red pen ink. The Sigma-Aldrich Handbook of Stains, Dyes, and Indicators[6] reveals that the two rhodamine dyes are isomeric, cationic dyes and have a molecular mass of 443 Daltons (excludes the mass of the counterion, Cl^-), hence, both dyes yield a peak at m/z 443 in the positive-ion LD mass spectrum. The dyes contain unique functional groups that distinguish them. As previously stated, Rhodamine B contains four ethyl groups, two attached to each nitrogen atom, while Rhodamine 6G contains two ethyl groups, one attached to each nitrogen atom, but these differences do not differentiate the xanthene dyes as a mixture. The appearance of the direct LD mass spectrum would lead one to believe that only Rhodamine B was contained in the ink. The deethylated degradation products overlap in m/z values, so other degradation products need to be used for dye differentiation. However, other structural differences may help to undoubtingly distinguish the dyes in LDMS. Fortunately, the dyes do have other structural differences. Rhodamine B contains a carboxylic acid group where Rhodamine 6G contains an ester functionality, however these groups can not be used as characteristics to identify Rhodamine 6G. Another difference between the dye structures is Rhodamine 6G contains methyl groups ortho to the amine functionalities, on the aromatic rings. The presence of this functional group was used to be able to determine the presence of Rhodamine 6G upon inducing photodegradation of the sample. The direct and indirect (photodegradation) characterization of Rhodamine B and Rhodamine 6G will be discussed in the following two sections.

Rhodamine B

The direct LDMS spectra of the two rhodamine dyes are different in that a low intensity peak at m/z 399 of the spectrum of Rhodamine B (Figure 4.5a) is consistently present. This peak represents a decarbonylated form $(RB\text{-}CO_2)^+$ of Rhodamine B $(RB)^+$. The difference between the intact dye and this species is 44 amu, which corresponds to carbon dioxide. Rhodamine B, a carboxylic acid, may decarbonylate while the analogous peak would not be expected to be seen in the mass spectrum of Rhodamine 6G, which does not contain a free acid group. Rhodamine 6G is commercially available from the Aldrich Chemical Company at a purity of 99% where Rhodamine B, purchased from Sigma, is 95% pure and initially, the peak at m/z 399 was assigned to be a dye impurity. The purity information correlated with the presence of the additional peak at m/z 399 in the mass spectrum of Rhodamine B and the absence of additional peaks in the mass spectrum of Rhodamine 6G. This information and LD mass spectra data could lead us to believe that the peak was due to a dye impurity. However, more recent data suggests that the species is the result of prompt fragmentation. TLC and MALDI MS experiments showed that the peak at m/z 399 corresponds to a fragment ion. Prompt fragment ions were discussed previously in Chapter One and how TLC and MALDI MS can be used to distinguish between dye impurities and fragment ions. The chromatographic separation of Rhodamine B directly yielded one TLC band and upon using indirect TLC coupled to LDMS the band was identified solely as the intact dye. Dye impurities were not observed from the TLC chromatogram. Figure 4.6a and 4.6b shows the positive-ion mass spectra of Rhodamine B analyzed by LDMS and MALDI MS, respectively. Upon using a matrix, the soft ionization reduced the peak intensity which signifies that the species that

corresponds to the peak at m/z 399 is in fact, due to a fragment ion produced during the D/I of Rhodamine B. In any instance, the LDMS spectra of a red dye found in a pen can immediately suggest that the dye is Rhodamine B, if the peak at m/z 399 is present.

The LD mass spectrum in Figure 4.5b was obtained upon photodegrading Rhodamine B for characterizing the dye indirectly through the use of degradation products. Rhodamine forms four deethylated degradation products upon being exposed to incandescent light. These deethylated degradation products of Rhodamine B, which were previously discussed, show the loss of 44 amu as seen with the D/I of the intact dye.

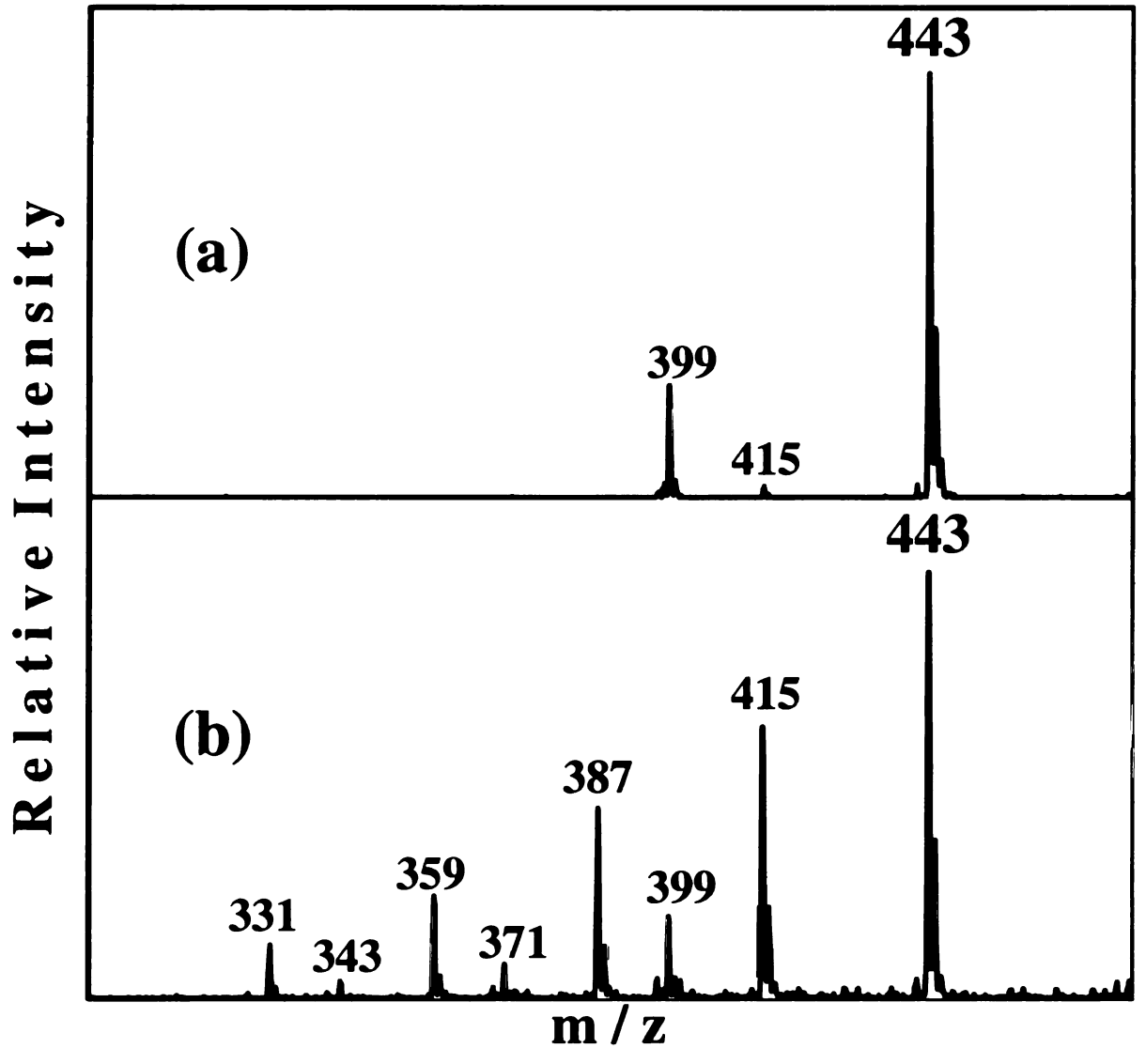


Figure 4.5: Positive-ion LD mass spectra of Rhodamine B (a) no exposure to incandescent light and (b) exposed to incandescent light for 12 hours

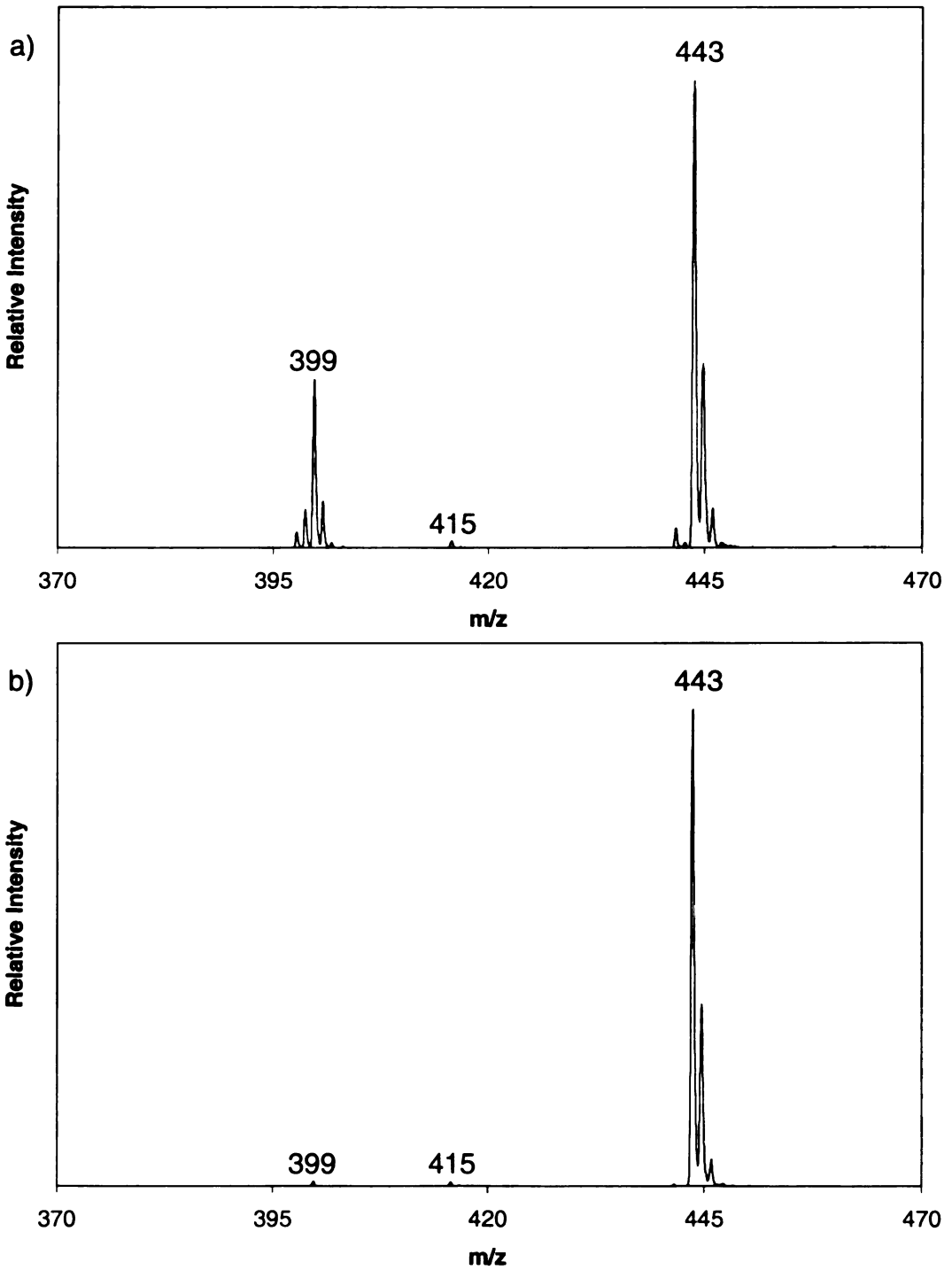


Figure 4.6: Positive-ion mass spectra of Rhodamine B analyzed by a) LDMS and b) MALDI MS

Rhodamine 6G

A peak at m/z 429 is indicative of Rhodamine 6G and is barely, but frequently observed when the dye is analyzed by direct LDMS. Although, the intensity of the peak is relatively small (it is highly likely that the peak may go unnoticed when examining the spectrum) in the LD mass spectrum of the dye (ink) before the sample has been exposed to incandescent light (Figure 4.7a), the peak becomes more intense as the sample is degraded as seen Figure 4.7b. The structure of Rhodamine 6G contains methyl groups ortho to the amine functionalities, on the aromatic rings that are suspected to dissociate from the dye during the D/I process as well as upon photodegrading the sample. Previous work was implemented to determine if the functional group yielded the peak at m/z 429 by examining the photodegradation of two dyes, Rhodamine 123 hydrate and New Fuchsin. The analyses concluded that the methyl group was indicative of the m/z 429 ion. Upon exposing the dye to incandescent light, the formation of the degradation product was proposed to be due to a bimolecular disproportionation reaction which will be discussed later. The induced photodegradation enhanced the detection of this peak. The presence of the peak is characteristic of Rhodamine 6G which helps to discriminate against Rhodamine B which may otherwise have been difficult to discern without. If an ink were to contain both xanthene dyes, then an initial examination of the LD mass spectrum could suggest that only Rhodamine B present due to the lack of peaks that are characteristic of Rhodamine 6G. Without the deethylation of Rhodamine 6G there would be no way to differentiate the dyes using LDMS. The degradation of Rhodamine 6G results in the loss of only two ethyl groups where Rhodamine B shows the loss of four ethyl groups consuming the mass spectrum. The overlapping of the peaks due to

deethylation can not be used directly to differentiate between the two dyes as a mixture. Photodegradation was proven to be highly valuable when distinguishing between isomeric dyes.

LDMS is a versatile and sensitive tool for detecting dyes in a variety of inks. LDMS spectra alone may not provide sufficient information for dye identification. Dyes can be photodegraded directly on paper using incandescent light, and the photoproducts can be analyzed by LDMS, providing structural information. While Rhodamine 6G and Rhodamine B are isomers, the photodegradation/LDMS combination can be used to distinguish between the two dyes. The structural differences between the isomers allow different photodegradation products and photoproducts to be formed and detected.

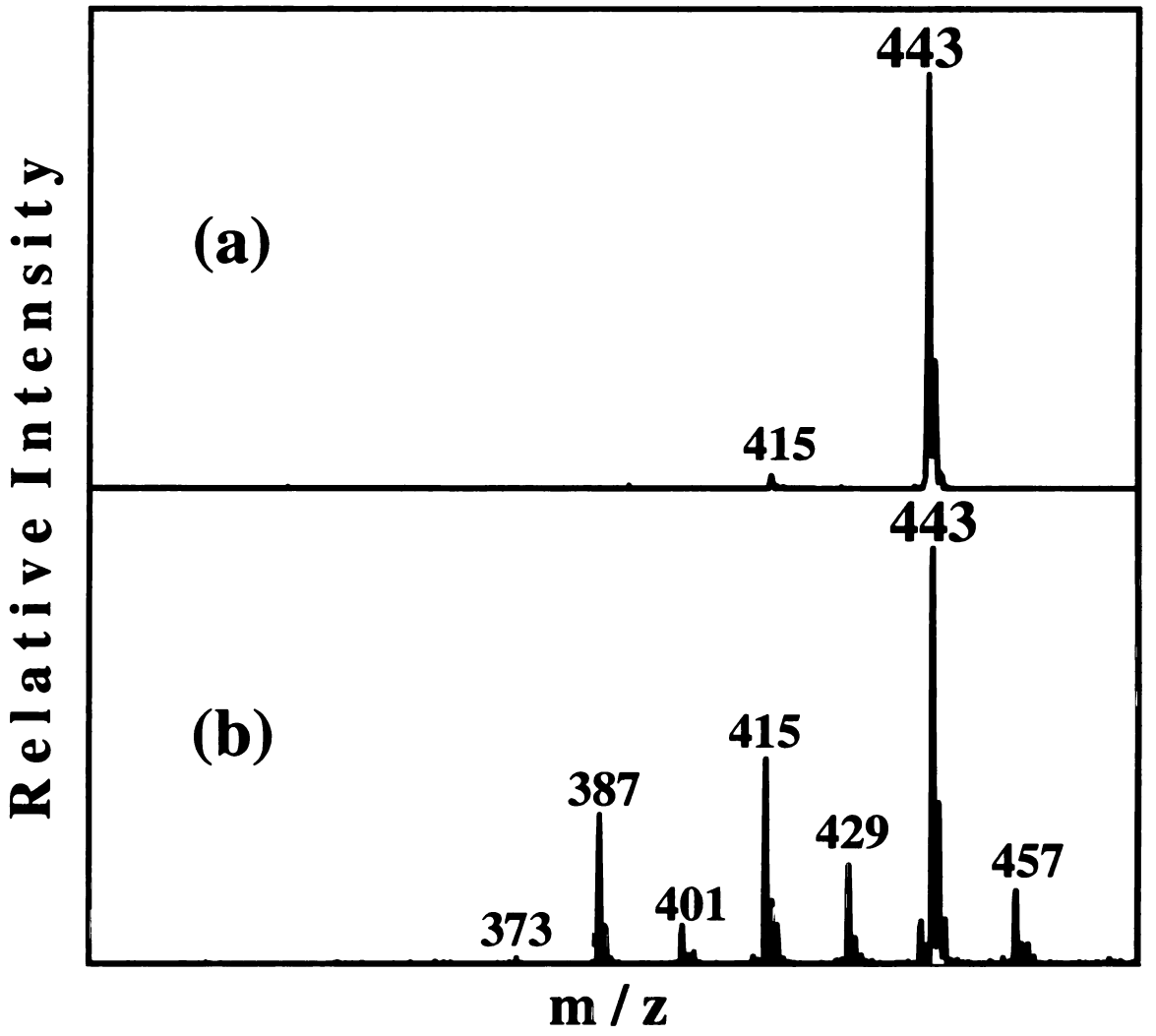


Figure 4.7: Positive-ion LD mass spectra of Rhodamine 6G (a) no exposure to incandescent light and (b) exposed to incandescent light for 12 hours

Red Ballpoint Pen Inks

Several kinds of red ballpoint pens currently available were analyzed. Two specific patterns emerged as a result of analyzing and comparing the LD mass spectra of these red pen inks and the rhodamine dyes. Figure 4.8 shows a positive-ion LDMS spectrum of fresh red ink from a BIC Round Stic® ballpoint pen and represents the majority of the red ink samples from our small set, while Figure 4.9 represents the smaller population of the red ink pens. Figure 4.9a is a positive-ion LDMS spectrum of

fresh red ink from a BIC Cristal® ballpoint pen. LDMS analysis of the smaller population of the red ink pens generated mass spectra that contained predominantly one peak at m/z 443. Most of the red inks, upon positive-ion LDMS examination, yielded mass spectra with two distinct peaks at m/z 443 and 399. The ink samples were photodegraded and compared to Rhodamine B and Rhodamine 6G in order to identify the rhodamine dyes present in the inks. The appearance of the characteristic peaks in the LDMS mass spectra for the rhodamine dyes suggests that the red ink from the BIC Round Stic® pen contains Rhodamine B and the red ink from the BIC Cristal® pen contains Rhodamine 6G.

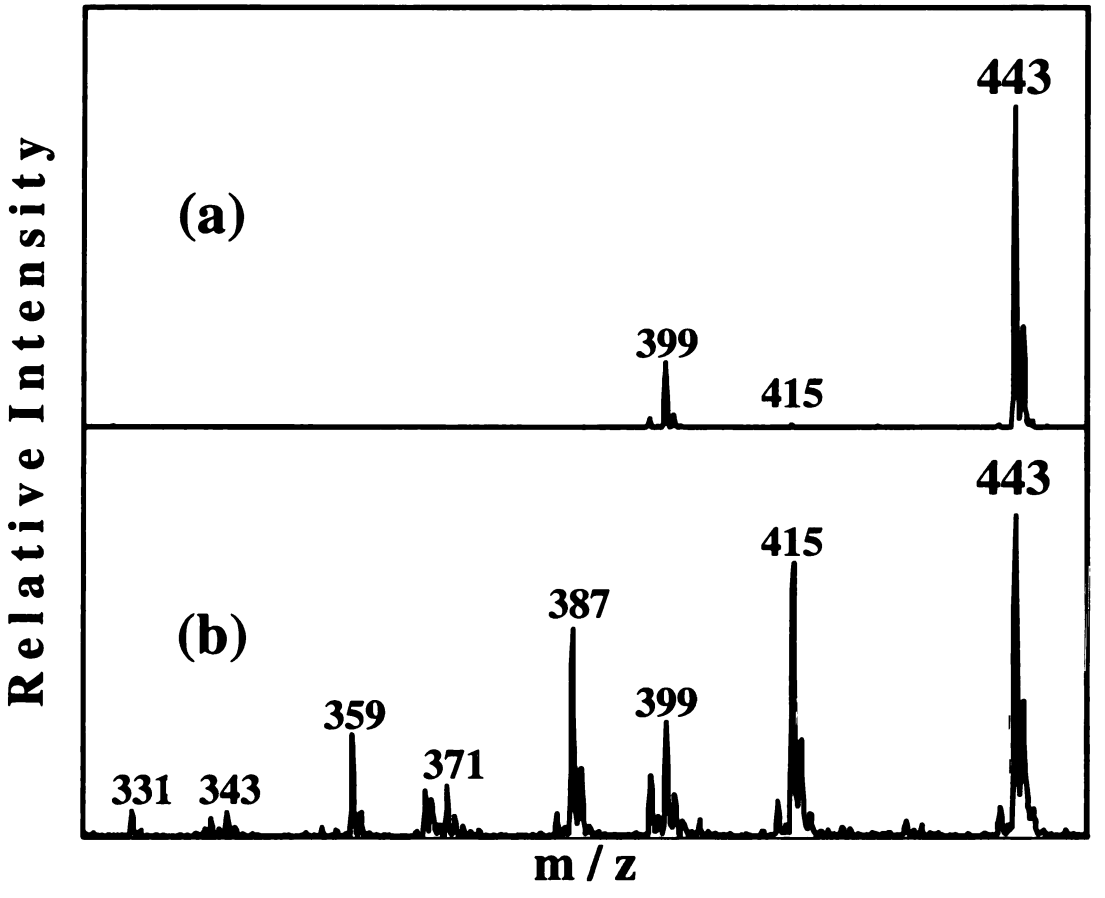


Figure 4.8: Positive-ion LD mass spectra of BIC® Round Stic pen (a) no exposure to incandescent light and (b) exposed to incandescent light for 24 hours

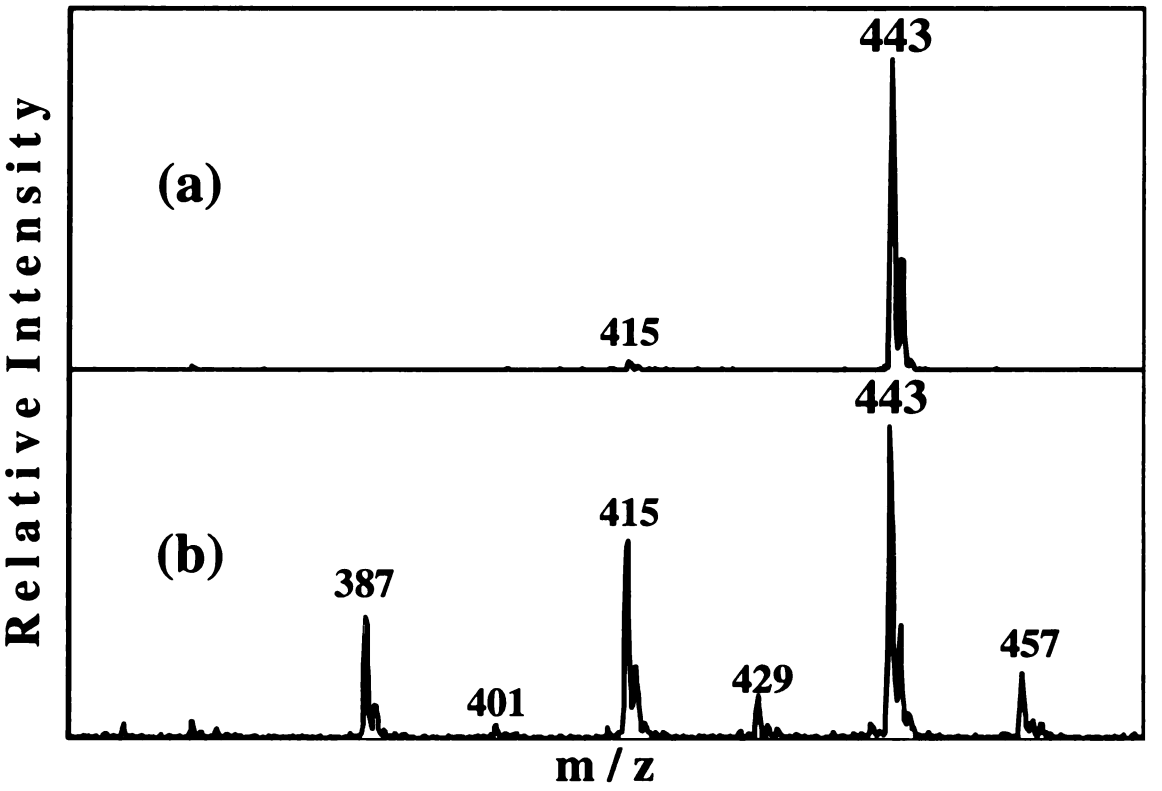


Figure 4.9: Positive-ion LD mass spectra from BIC® Cristal pen (a) no exposure to incandescent light and (b) exposed to incandescent light for 24 hours

Photoproducts of Rhodamine 6G

Photodegrading dyes into smaller molecules using UV and visible light was demonstrated. Rhodamine 6G is the first dye that we have encountered that also forms higher mass molecules photochemically. Specifically, Figure 4.7b shows a peak at m/z 457, representing a photoproduct that is 14 amu higher than the intact dye peak. Peaks higher in mass are not photochemically formed from Rhodamine B, according to the mass spectrum in Figure 4.5b. To investigate the peak at m/z 457, two additional dyes, Rhodamine 123 and New Fuchsin, were selected. The dye structures are shown in Figure 4.10 and Figure 4.11 shows the LDMS spectra of Rhodamine 123, before and after exposure to incandescent light. The peak at m/z 345 corresponds to the intact dye cation. Exposure for 133 hours did not lead to any photoproducts for this small rhodamine dye. Figure 4.12 shows that New Fuchsin also forms both expected photodegradation products and higher mass photoproducts (following exposure to incandescent radiation for 211 hours), as did Rhodamine 6G. In contrast to Rhodamine 6G, New Fuchsin formed several photoproducts, including two representing increases in mass of the intact dye by 14 mass units (m/z 344, 358). The structural features that Rhodamine 6G and New Fuchsin share are methyl groups, ortho to the amine functionalities, on the aromatic rings. In addition, they have less than a full complement of alkyl groups on the nitrogen atoms. Since the formation of higher mass photoproducts occurs for just these dyes alone on paper, one need not consider other components of the ink as possible reactants. While these insights alone do not establish a mechanism, bimolecular disproportionation reactions of dye molecules, such as that shown in Figure 4.13, may contribute to the observed photoproducts.

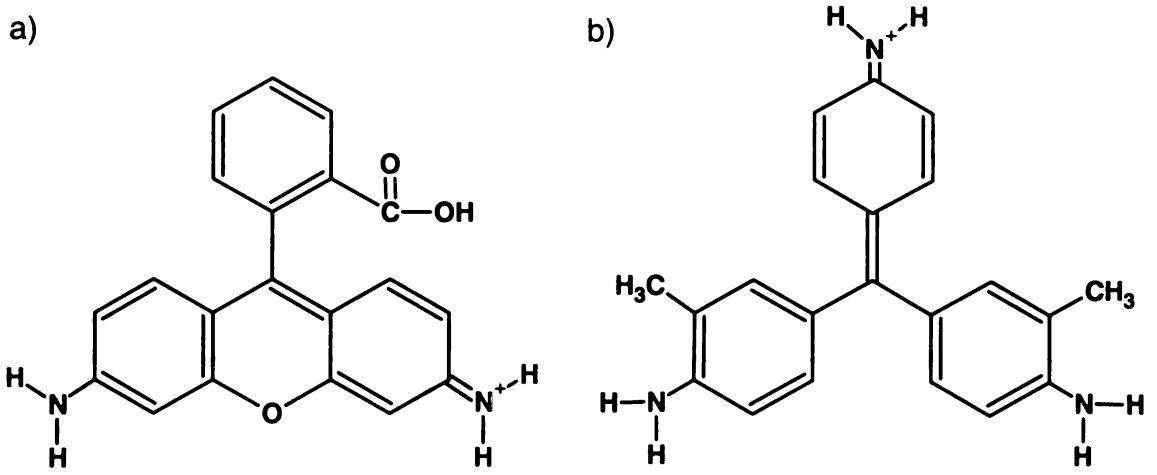


Figure 4.10: Dye structures a) Rhodamine 123 and b) New Fuschin

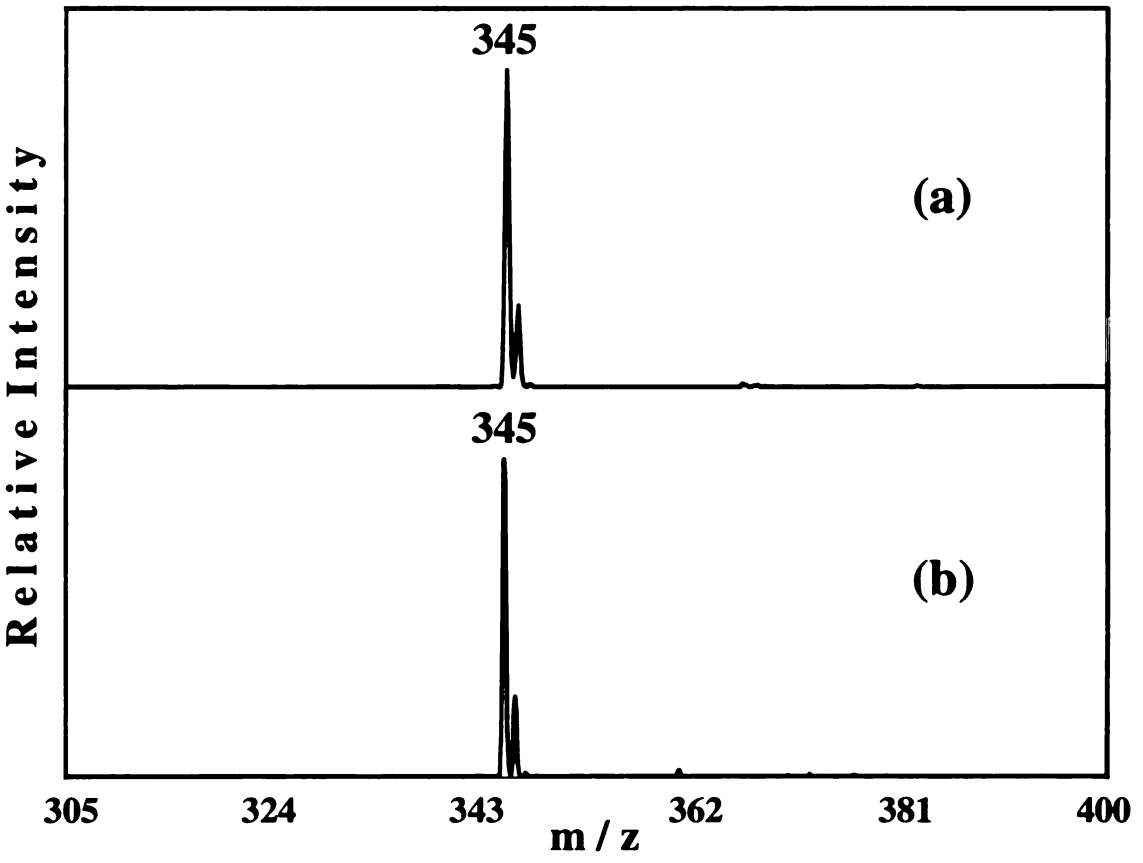


Figure 4.11: Positive-ion LD mass spectra of Rhodamine 123 (a) no exposure to incandescent light and (b) exposed to incandescent light for 133 hours

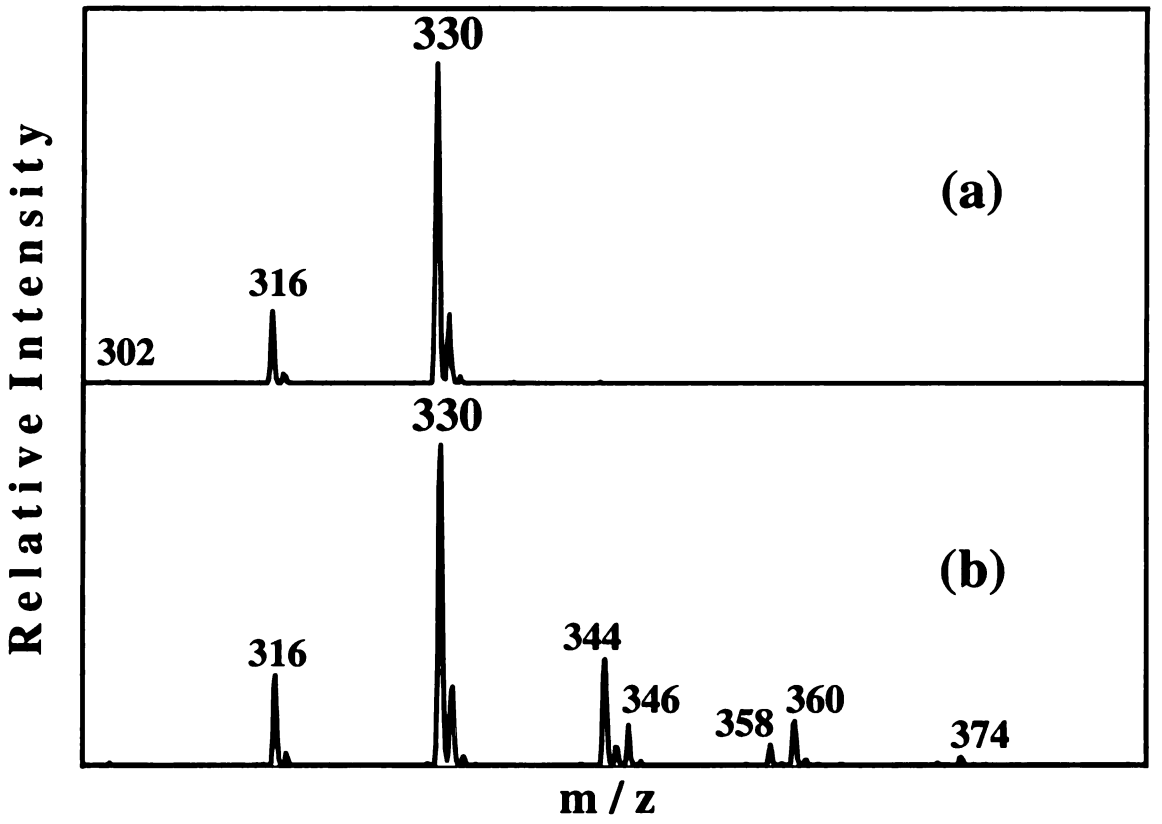


Figure 4.12: Positive-ion LD mass spectra of New Fuschin: (a) no exposure to incandescent light and (b) exposed to incandescent light for 211 hours

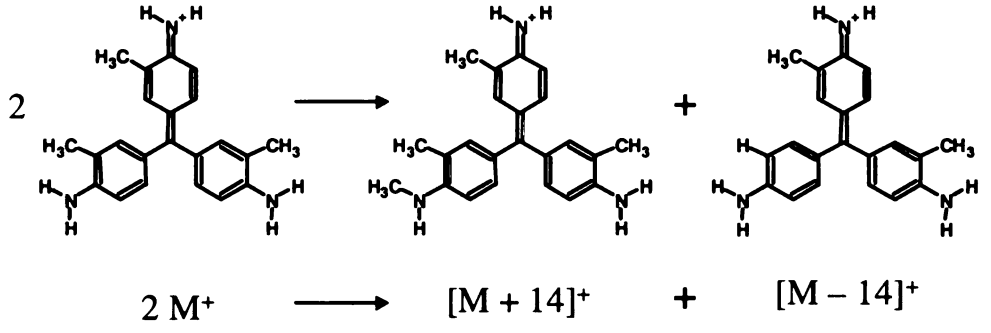


Figure 4.13: Suggested mechanism for the formation of photoproducts

LDMS is a versatile and sensitive tool for detecting dyes in a variety of inks. LDMS spectra alone may not provide sufficient information for dye identification. Dyes can be photodegraded directly on paper using incandescent light, and the photoproducts can be analyzed by LDMS, providing structural information. While Rhodamine 6G and Rhodamine B are isomers, the photodegradation/LDMS combination can be used to distinguish between the two dyes. The structural differences between the isomers allow different photodegradation products and photoproducts to be formed and detected.

The chemistry of dye photodegradation remains to be defined, especially for ink on paper. The degradation of ink on paper is a complex system that may involve reactions with paper components or environmental gases such as water. Work continues to be developed using the photochemistry/LDMS approach for identifying dyes used in inks, and to further define the relationship between photoproducts and dye structure.

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Chapter Five: Conclusions

Web Database

We are currently developing a webpage entitled "Michigan State University Department of Chemistry The Pen and Dye Database." The URL is <http://poohbah.cem.msu.edu/peninks/pens_main.htm>. The webpage is being developed to assist questioned document examiners in the analysis of documents containing inks from commonly available pens. The database contains LD mass spectra, thin-layer chromatograms, and dye information that accrued from this research. Eighty-five pen inks have been analyzed and the results can be accessed through the website. Additional research involving the analysis of multiply-charged ink dyes by MALDI MS can be found on the website.

LDMS and Multiply-Charged Dyes

Laser desorption mass spectrometry (LDMS) has proven to be a useful technique for the analysis/detection of ink dyes directly from the paper substrate. Such dyes include Crystal Violet, Metanil Yellow, Rhodamine B (Basic Violet 10), and Rhodamine 6G (Basic Red 1) which are dyes frequently encountered in blue, black, and red ballpoint pen ink and Copper Phthalocyanine (Pigment Blue 15) which is a common dye found in gel pens with blue ink have been successfully detected using LDMS. In general, when applying LDMS to a larger range of pen inks the identification of dyes through the interpretation of LDMS spectra has been arduous or not possible either due to the complexity of the mass spectra or the lack of information that can be extracted from the

mass spectra. Ideally, each peak present in an LD mass spectrum represents an intact dye molecule, and ink containing more than one dye is frequently encountered. We believe that we have encountered several inks that contain multiply charged dyes based on the LDMS data, TLC data, and patent information.

The LD mass spectra may contain peaks due to prompt fragmentation that can aid in dye identification or the mass spectra may lack any significant peaks. In general, the complex spectra that we have encountered consist of peaks lower in mass (less than 300 Da) than peaks that correspond to the intact dyes. This observation may suggest that an ink contains multiply charged dyes. Fragmentation is likely to be induced when analyzing a species that is multiply charged which yield complex spectra. The energy necessary to desorb the species intact is greater than the energy to induce dye fragmentation, hence, dye fragmentation will prevail giving rise to low mass species. The amount of fragmentation varies depending on the species being analyzed. The lack of peaks in a LD mass spectrum can also suggest that an ink contains multiply-charged dyes. If the multiply charged dyes are sufficiently stable, then fragmentation will not occur during the laser desorption process which will yield mass spectra that contain no peaks.

TLC data has been previously collected which has also given insight into the charge state of the dyes used in ink. Generally, the number of TLC bands gives information about the number of dyes that are present in the ink. Each colored band in on the TLC plate represents a dye. TLC data and the mass spectral data are compared to account for the number of dyes that are contained in the ink. If the TLC and mass spectral data are not in agreement, then one may assume that the ink contains dyes that

are multiply charged. Dye extractions of TLC bands were analyzed directly using LDMS. The absence of peaks in the mass spectra of the TLC extractions aids in the conclusion that multiply charged dyes are present in the ink. Although LD mass spectra of the inks from paper are obtainable, the information is limited, causing the interpretation to be difficult.

Currently to date, 250 dyes found in ballpoint, gel, and liquid ink patents have been cataloged. Most of the dyes encountered in the patents are multiply-charged, specifically polysulfonated azo dyes, and can be found in the various classes of ink such as ballpoint, gel, and liquid. This fact has lead to the assumption that most of the dyes used in the manufacture of pen inks are multiply-charged. Fortunately, structures for the majority of the cataloged dyes have been found which have allowed us to predict their m/z values. The predicted m/z values serve as references when trying to interpret the peaks present in the LD mass spectra. This was unexpected, and introduces several complications to the LDMS analysis.

Singly charged dyes such as Crystal Violet and neutral dyes such as Copper Phthalocyanine are easily desorbed and detected directly from the ink on paper using LDMS, however, LDMS is not a technique that can detect multiply charged species. Currently, LDMS has not been demonstrated as a viable technique for detecting intact multiply charged species, but upon analyzing multiply-charged dyes by LDMS information may still be obtained from the mass spectra. However, multiply-charged dyes can be analyzed using the technique with a modification to the experiment. Matrix-assisted laser desorption/ionization (MALDI) MS has been successfully used for the detection of multiply-charged species including dyes.

MALDI MS and Multiply-Charged Dyes

Ongoing research for the detection of multiply-charged dyes has encompassed developing a method that can be applied to the detection of the ink dyes that are multiply-charged directly from paper. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) has been used previously for the detection of polysulfonated azo dyes[1]. Matrix-assisted laser desorption mass spectrometry (MALDI MS) can be very beneficial when trying to detect intact polyionic dyes. A method used by Sullivan, *et al.*[1] was modified for analyzing multiply-charged dyes on a paper substrate. The MALDI matrix, 2-(4-hydroxyphenylazo)benzoic acid (HABA), is best suited for the analysis of ink from paper and diammonium hydrogen citrate (DAHC) enhances dye detection. The matrix alone is not sufficient to acquire good results. Additives or co-matrices have been used previously and are known to enhance the performance of the MALDI experiment[1-20]. Enhancements include increased analyte sensitivity, improved mass spectral resolution (increased mass accuracy), reduced analyte fragmentation, and increased homogeneity of the sampling. The basis of introducing an additive is to enhance the performance of the MALDI MS experiment by eliminating or reducing the effects of cations such as sodium or potassium ions. In general, dyes are manufactured in the form of sodium salts; therefore, there is a need to suppress the influence of the sodium ions by using an additive. A variety of additives have been used, but diammonium hydrogen citrate (DAHC) was found to have the best performance with the paper substrate. The detection of polyionic dyes using a matrix and additive yields molecular information, such as the molecular mass of the dye, ultimately, leading to the identification of the dye.

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