This is to certify that the
thesis entitled

Detection and Characterization of Multiply-Charged Ink Dyes by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry with 2-(4-Hydroxyphenylazo)-Benzoic Acid and Diammonium Hydrogen Citrate

presented by

Jamie D. Dunn

has been accepted towards fulfillment
of the requirements for the

M.S. degree in Chemistry

John Allen

Major Professor's Signature

5.6.04

Date

DATE DUE	DATE DUE	DATE DUE

DETECTION AND CHARACTERIZATION OF MULTIPLY-CHARGED INK DYES BY MATRIX-ASSISTED LASER DESORPTION/IONIZATION MASS SPECTROMETRY WITH 2-(4-HYDROXYPHENYLAZO)-BENZOIC ACID AND DIAMMONIUM HYDROGEN CITRATE

By

Jamie D. Dunn

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirement
for the degree of

MASTER OF SCIENCE

Department of Chemistry

2007

ABTRACT

DETECTION AND CHARACTERIZATION OF MULTIPLY-CHARGED INK DYES BY MATRIX-ASSISTED LASER DESORPTION/IONIZATION MASS SPECTROMETRY WITH 2-(4-HYDROXYPHENYLAZO)-BENZOIC ACID AND DIAMMONIUM HYDROGEN CITRATE

By

Jamie D. Dunn

Laser desorption mass spectrometry (LDMS) is a technique limited to detecting singly-charged or neutral molecules. Several inks contain dyes that are multiply-charged, hence, LDMS can not be employed directly for their identification. However, the technique can be slightly modified by using a matrix. Matrix-assisted laser desorption/ionization mass spectrometry allows for the detection of multiply-charged species. Polyionic dyes are not sufficiently detected in the MALDI experiment, but can be chemically modified through the use of an additive with a matrix, allowing cation-exchange to occur. We have successfully detected 30 polyionic dyes by MALDI MS with the matrix, HABA, and the additive, DAHC. A method was developed for the detection and characterization of ink dyes that contain multiple charges, specifically, polysulfonated dyes as well as other dyes containing sodium carboxylate and sodium alkoxide groups. Inks containing multiply-charged dyes have been examined in this research by MALDI MS, LDMS, and TLC. Although LDMS does not allow for the direct detection of polyionic dyes, both mass spectrometry techniques contain structural information which was used for characterization purposes. TLC was indirectly coupled with MALDI MS to characterize the dyes in ink.

ACKNOWLEDGEMENTS

I would like to thank Dr. Jay Siegel, Dr. Gary Blanchard and Lisa Dillingham for helping me survive Dr. Allison's retirement, pushing me to complete two theses simultaneously, and allowing me to continue my future work in the department. I would also like to thank Dr. Merlin Bruening and Yingda Xu for giving me the opportunity to work on a new, exciting project. I thank Dr. John Allison for the MS knowledge that I have acquired and can use in future research.

My family has continued to be very supportive of my choices and educational goals and I would like to thank them for being understanding when I could not come home for family gatherings. I also want to thank my friends, Stephanie and Amy Jo, for staying in touch over the years and helping me to forget my life as a graduate student when I would come home to St. Louis.

I would especially like to thank my friend/labmate, Anne Distler, for the great times we shared in the lab and at your condo. Since your graduation, your emails have entertained me as well and have kept me sane during my stay in MI. I also appreciated your knowledge and your help with courses and research.

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.

TABLE OF CONTENTS

List of Tables vi

List of Figures vii

Chapter One: Introduction 1

Dye Classification 1
Dye Application 3
Pen Ink Composition 3
Ink Dye Analysis 4
Selection of an Analytical Method 4
Methods for Ink Dye Analysis 6
Mass Spectrometry 7
Field Desorption MS 14
Electrospray Ionization MS 15
Laser Desorption MS 26
Chromatography 27
Thin-layer Chromatography 28
High Performance Liquid Chromatography 29
LDMS and Forensic Science Applications 30
Questioned Document Examination 30
Trace Evidence 31
Research Overview 34
References 36

Chapter Two: Direct LDMS and TLC Analysis of Ink Dyes 40

LDMS Calibration and Copper Phthalocyanine 43
Mass Accuracy for Identification 43
LDMS Ink Dye Analysis 44
Molecular Information: Mass, Composition, and Charge State 45
Dye Impurities, Fragment Ions, and Cluster Ions 55
Coupling TLC with LDMS 63
References 68

Chapter Three: Direct LDMS Analysis of Ink Dyes: Limitations and Solutions 70

Dye Characterization by Photodegradation and LDMS 71
Isomeric Dye Characterization 79
Rhodamine B 80
Rhodamine 6G 84
References 87

Chapter Four: Detection and Characterization of Multiply-Charged Ink Dyes 88

Determining the Presence of Multiply-Charged Dyes in Pen Ink 92
LDMS 92
TLC 99
Ink Patents 99
Multiply-Charged Ink Dye Detection 100
Cation-Exchange Additives and Matrices 101
Commercial Dyes 104
Highly-Charged Dyes 107
Ink on Paper 108
Multiply-Charged Ink Dye Characterization 122
MALDI MS and TLC 123
Direct LDMS 138
References 139

Chapter Five: Conclusions 142

MALDI MS Limitations and Future Research 142
References 144

LIST OF TABLES

Chapter Two: Direct LDMS and TLC Analysis of Ink Dyes

Table 2.1: Isotopic masses and natural abundances for the atoms that comprise Copper Phthalocyanine 51

Chapter Three: Direct LDMS Analysis of Ink Dyes: Limitations and Solutions

Table 3.1 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 78

Chapter Four: Detection and Characterization of Multiply-Charged Ink Dyes

Table 4.1 Polyionic dyes examined by MALDI MS (HABA and DAHC) on SS plate. Provides m/z values detected in the positive and/or negative-ion mode and functional groups that may contribute to the overall charge on the dye molecule 106

LIST OF FIGURES

Chapter One: Introduction

Figure 1.1: Basic schematic of a mass spectrometer 8

Figure 1.2: Diagram of theVoyager-DE Mass Spectrometer 11

Figure 1.3: Schematic of an ESI source 13

Figure 1.4: Multiply-charged dye structures a) Acid Blue 9, b) Acid Blue 92, and c) Solvent Brown 20 17

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

Figure 1.6: LD mass spectra of Pigment Red 3 on paper a) positive-ion and b) negative-ion 20

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. 23

Figure 1.8: Polyethylene glycol a) m/z values that correspond to the ionization of the polymer by sodium and potassium ion adduction and b) basic polymer structure (n is the number of repeating units) 24

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 25

Chapter Two: Chapter Two: Direct LDMS and TLC Analysis of Ink Dyes

Figure 2.1: Dye structures a) Copper Phthalocyanine and b) monosulfonated Copper Phthalocyanine 47

Figure 2.2: LD mass spectra of blue gel ink containing Copper Phthalocyanine a) positive-ion and b) negative-ion 48

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 50

Figure 2.4: Structure of the dyes used in several black ballpoint inks a) Crystal Violet and b) Metanil Yellow 53

Figure 2.5: LD mass spectra of black ink from Sanford® Supergrip ballpoint pen a) positive-ion and b) negative-ion 54

Figure 2.6: Positive-ion LD mass spectrum of Methyl Violet 2B purchased from the Aldrich Chemical Company 56

Figure 2.7: The direct analysis of Metanil Yellow from a metal plate by negative-ion LDMS 59

Figure 2.8: Negative-ion LD mass spectrum of Metanil Yellow shows peaks due to fragment ions 59

Figure 2.9: Dye structure of Metanil Yellow with labeled values that correspond to possible fragment ions or neutral molecules 60

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] 60

Figure 2.11: Negative-ion LD mass spectra of Metanil Yellow on paper shows peaks due to cluster ions 63

Figure 2.12: TLC analysis of black ballpoint ink from BIC® Cristal a) thin-layer chromatogram and b) TLC bands and corresponding dyes 66

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) 67

Chapter Three: Direct LDMS Analysis of Ink Dyes: Limitations and Solutions

Figure 3.1: Positive-ion LD mass spectra of isomeric, red ink dyes a) Rhodamine B and b) Rhodamine 6G 73

Figure 3.2: 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) 75

Figure 3.3: 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 76

Figure 3.4: Dye structures of a) Rhodamine B and b) Rhodamine 6G 78

Figure 3.5: Positive-ion LD mass spectra of Rhodamine B (a) no exposure to incandescent light and (b) exposed to incandescent light for 12 hours 82

Figure 3.6: Positive-ion mass spectra of Rhodamine B analyzed by a) LDMS and b) MALDI MS 83

Figure 3.7: Positive-ion LD mass spectra of Rhodamine 6G (a) no exposure to incandescent light and (b) exposed to incandescent light for 12 hours 86

Chapter Four: Detection and Characterization of Multiply-Charged Ink Dyes

Figure 4.1: Direct LDMS analysis of liquid, blue ink from a Pilot® Precise V7 rollerball pen on paper a) positive-ion mass spectrum and b) negative-ion mass spectrum 94

Figure 4.2: Structure of multiply-charged ink dyes a) Direct Red 23, b) Acid Blue 93, c) Direct Red 80, d) Acid Yellow 23, e) Solvent Blue 38, f) Acid Black 1, and g) Reactive Black 5 97

Figure 4.3: Negative-ion MALDI MS analysis (HABA and DAHC) of Direct Red 80 on SS plate 108

Figure 4.4: Positive-ion LD mass spectra of blue, liquid ink from Pilot® Precise V7 rollerball pen a) HABA, b) DAHC, and c) HABA and DAHC 110

Figure 4.5: Negative-ion LD mass spectra of blue, liquid ink from a Pilot® Precise V7 rollerball pen a) HABA, b) DAHC, and c) HABA and DAHC 111

Figure 4.6: MALDI MS analysis of Acid Violet 49 on SS plate a) positive-ion mass spectrum and b) negative-ion mass spectrum 114

Figure 4.7: Dye structure of Acid Violet 49 115

Figure 4.8: MALDI MS analysis of Acid Violet 49 on paper a) positive-ion mass spectrum and b) negative-ion mass spectrum 117

Figure 4.9: MALDI MS analysis of liquid, blue ink from Pilot® Precise V7 rollerball pen on paper a) positive-ion mass spectrum and b) negative-ion mass spectrum 119

Figure 4.10: Dye structure of Acid Blue 90 120

Figure 4.11: MALDI MS analysis of Acid Blue 90 on SS plate a) positive-ion mass spectrum and b) negative-ion mass spectrum 121

Figure 4.12: MALDI MS analysis of liquid, blue ink from a Sanford® Uni-ball® Roller pen on SS plate a) positive-ion mass spectrum and b) negative-ion mass spectrum 125

Figure 4.13: Dye structures a) Acid Red 52 and b) Acid Blue 9 127

Figure 4.14: MALDI MS analysis of Acid Red 52 on SS plate a) positive-ion mass spectrum and b) negative-ion mass spectrum 128

Figure 4.15: MALDI MS analysis of Acid Blue 9 on SS plate a) positive-ion mass spectrum and b) negative-ion mass spectrum 129

Figure 4.16: MALDI MS analysis of red, liquid ink from Sanford® Uni-ball® Roller pen on SS plate a) positive-ion mass spectrum and b) negative-ion mass spectrum 132

Figure 4.17: Indirect coupling of TLC with MALDI MS: negative-ion mass spectra of extracted TLC bands a) yellow band, b) orange-red band, and c) red band 133

Figure 4.18: Dye structures of a) Food Yellow 3, b) Acid Red 87, and c) Acid Red 92 134

Figure 4.19: Direct negative-ion LDMS analysis of liquid, red ink from Sanford® Uni-ball® Roller pen a) complete spectrum from m/z 0–100 and b) expanded spectrum from m/z 600–800 135

Figure 4.20: Isotopic peak distributions of Acid Red 87 a) experimental and b) theoretical 136

Figure 4.21: Isotopic peak distributions of Acid Red 92 a) experimental and b) theoretical 137

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 industrial application[1,2]. The chemical composition is the foundation for assigning each dye a unique five-digit CI number. The color and application of a dye is denoted by a CI name. For example, the CI number and name of Crystal Violet are CI 42555 and Basic Violet 3, respectively. The Colour Index recognizes the following 15 application classes: acid dyes, anionic dyes, azoic dyes, basic dyes, direct dyes, disperse dyes, fluorescent brighteners, food dyes, ingrain dyes, mordant dyes, pigment dyes, reactive dyes, solvent dyes, sulfur dyes, and vat dyes[3]. The colorants used in this thesis research encompass multiply-charged (polyionic), specifically, acid and direct dyes. A particular metal complex pigment dye was examined as well and was used as the mass spectrometric calibrant for analyzing the dye used in this research.

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

This research has focused on multiply-charged dyes, specifically, acid or direct dyes that are polysulfonated containing azo groups, used in pen inks. In general, acid dyes are anionic containing one or more hydrogen sulfonic acid ($-SO_3H$) or sodium sulfonic acid ($-SO_3Na$) groups. Other acid dyes contain sodium carboxylate ($RCO_2^- Na^+$) and sodium alkoxide ($RO^- Na^+$) groups which were also studied in this research. Acid dyes comprise 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. Most often, direct dyes are high molecular weight, polyazo compounds. About 30% of direct dyes (~1600) are presently used in industry[5]. Copper Phthalocyanine was studied by LDMS and used as the mass spectrometric calibrant in this research. 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 gel ink. The molecular properties of Copper Phthalocyanine were studied to demonstrate the basic characterization information that can be acquired. Also, the dye served to be an excellent LDMS and MALDI MS 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, 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 and MALDI MS have shown to be 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 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 (CI names or numbers) 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. Also, some dyes are insoluble in aqueous media or organic solvents making extraction-based methods implausible. Currently, many ink analyses require the dye to be soluble. Instrumental methods such as high performance liquid chromatography that require liquid samples are incompatible 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, however, the technique is not cable of detecting multiply-charged dyes. Many dyes that are insoluble are neutral molecules. Dye detection by MALDI MS (incorporates a matrix into the LDMS experiment) does not discriminate against dyes that have multiple charges. ESI MS is another mass spectrometry technique capable of detecting molecules with multiple charges, but recent work showed that the detecting polysulfonated dyes using

electrospray was hindered by a charge state of three or higher. LDMS is used to analyze samples directly from the paper substrate (written entries on a questioned document). In this research, MALDI MS is also used in the same fashion as LDMS; however, an additive and matrix are applied to directly to the ink-paper sample. The additive and matrix are prepared as solutions and the additive is added and dried first, followed by the matrix. Whenever ink is not analyzed directly there is a chance that the dye could be altered chemically. Direct analysis of ink is the safest means for examination, however, in this research, a simple, chemical modification of the dye allows the species to be detected as singly-charged directly from the paper substrate.

Commercial dyes are frequently sold as impure mixtures[1]. The production of impure dyes is common 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 and MALDI MS 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 as 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 included the use of capillary electrophoresis (CE) combined with UV/Vis and laser induced fluorescence (LIF) 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. The current (1999 – 2002) research in ballpoint pen ink analysis has gradually moved toward methods that encompass 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.

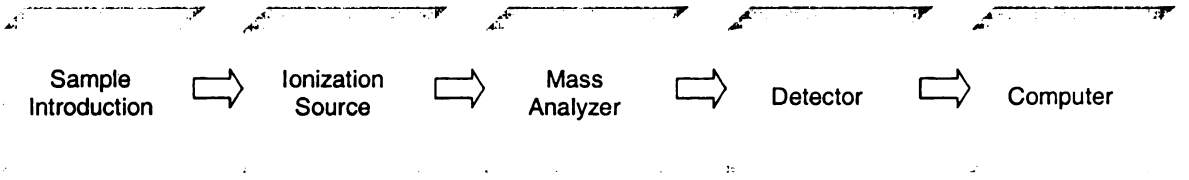


Figure 1.1: Schematic of a basic mass spectrometer

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 electron impact (EI) as the ionization method, 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).

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]. Both methods will be discussed further and compared.

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. Analytes have molecular weights that exceed 100,000 Daltons. From an analyte/matrix solution, crystals are grown. When irradiated with light from a pulsed UV laser, matrix and analyte ions are formed. 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.

Both the LDMS and MALDI MS 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 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). For the detection of multiply-charged dyes, an additive and matrix were introduced to samples. The introduction of the additive and matrix to samples directly on paper was employed to limit the number of analysis steps, prevent sample loss, and reduce the risk of sample contamination. Commercial dyes were also analyzed directly from paper as well as from a stainless steel plate to confirm the identification of the dyes.

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 and MALDI MS 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 extraction delay times ranged from 100 ns to 200 ns.

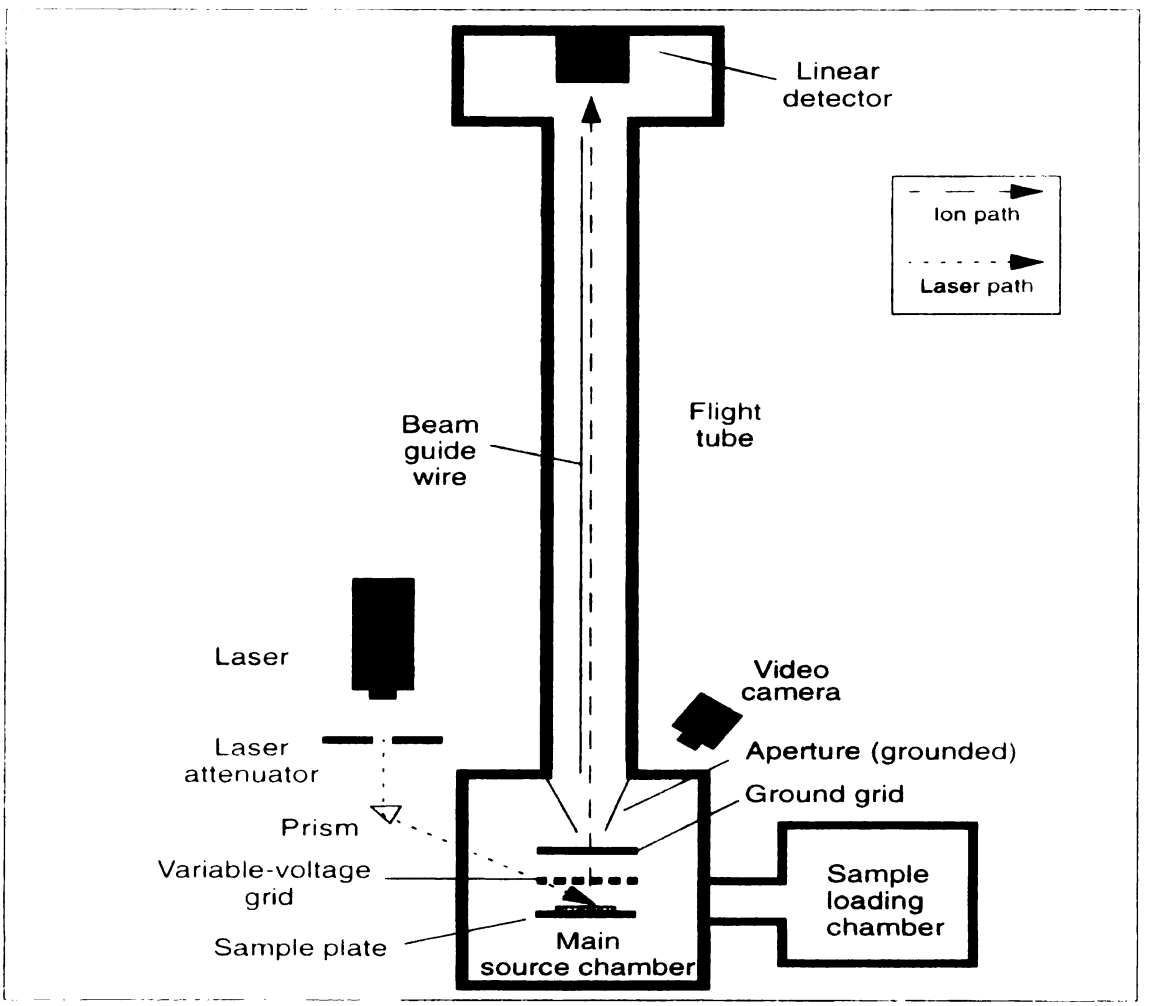


Figure 1.2: Diagram of theVoyager-DE mass spectrometer[38]

FAB and SIMS are similar techniques in that they 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 in 1994, the analysis of inks, dyes, and pigments on paper by TOF-SIMS was employed[42].

Electrospray ionization (ESI) MS is not a D/I technique, but is also 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 over D/I techniques since ions can be formed and detected as multiply-charged species. 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 an 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].

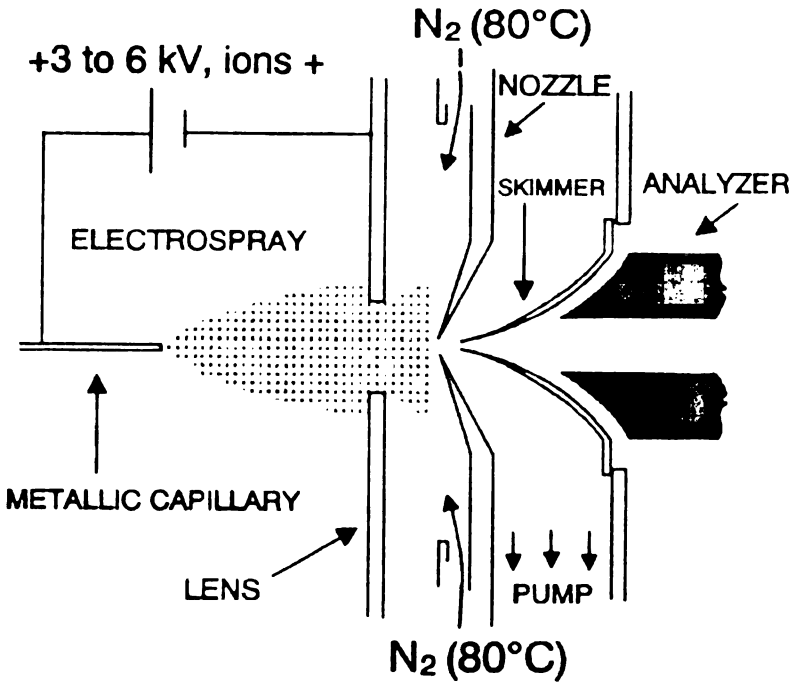


Figure 1.3: Schematic of an electrospray ionization source[43]

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 and MALDI MS.

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 since the technique is a precursor of MALDI MS which was the mass spectrometric method employed for this thesis research. The basic LDMS characterization methods can be applied to MALDI MS. Discussion on the previous use of additives in MALDI MS for enhancing the detection of multiply-charged species will follow in Chapter Four as well as the ink dye analyses carried out for this thesis research.

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 mode. 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 operating in negative-ion mode. LDMS and MALDI MS are 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. MALDI MS employed for the detection of highly-charged dyes. Direct Red 80 has a charge state of -6 (contains 6 sulfonic acid groups) and was detected using MALDI MS in this research.

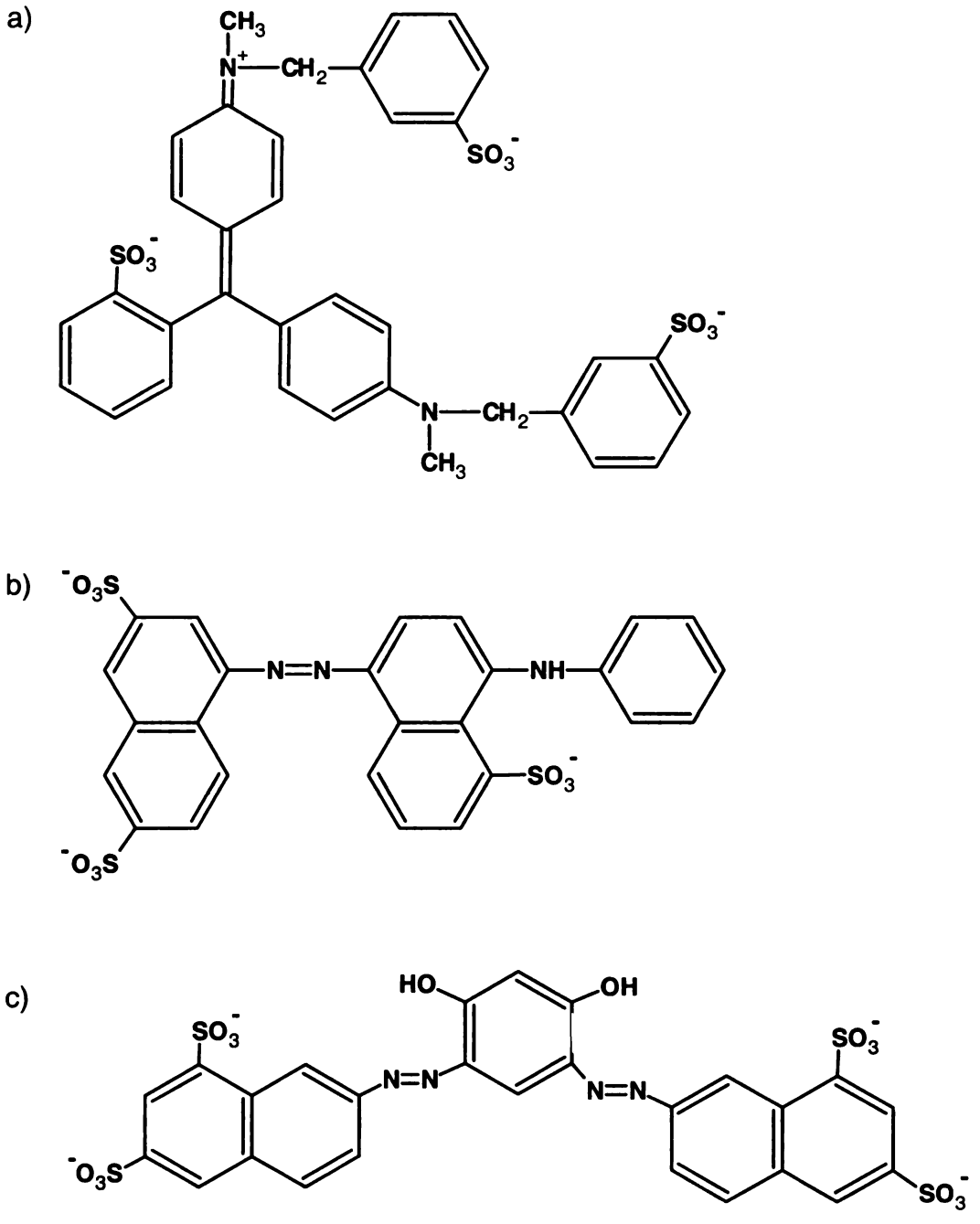


Figure 1.4: Multiply-charged dye structures a) Acid Blue 9, b) Acid Blue 92, and c) Solvent Brown 20

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 a signal was not 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.

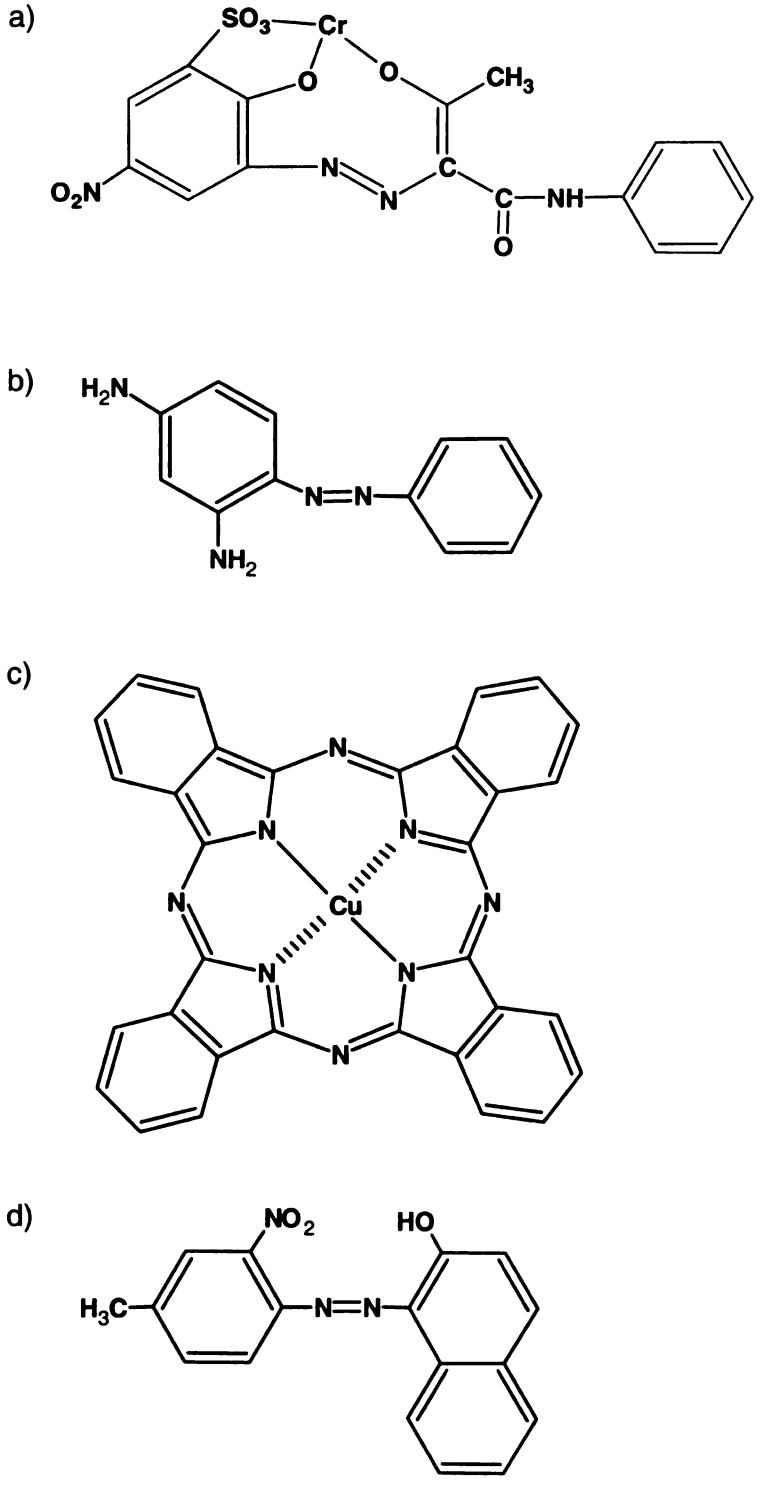


Figure 1.5: Neutral dye structures a) Solvent Yellow 19, b) Solvent Orange 3, c) Copper Phthalocyanine, and d) Pigment Red 3

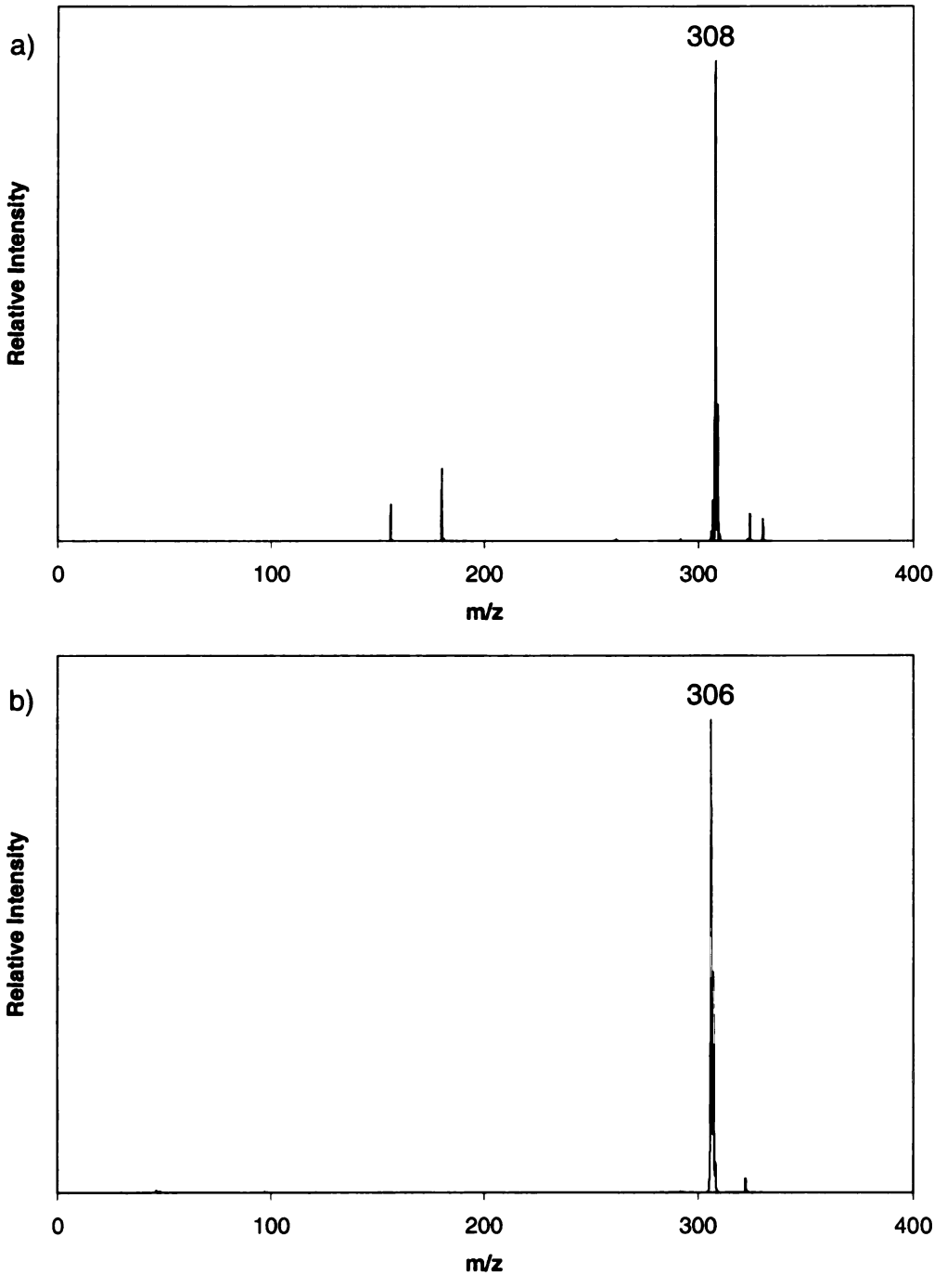


Figure 1.6: LD mass spectra of Pigment Red 3 on paper a) positive-ion and b) negative-ion

In order for ink dyes to be analyzed by ESI MS, the written entries are required to be extracted from the paper (samples in the liquid form is a 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 may not be the only components that get extracted from the ink or paper. 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. However, 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 that is 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 ion (Na^+) and potassium ion (K^+) 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® V-Ball rollerball pen. Either the paper or the ink components are assisting in the detection of the polymer. Neat PEG is not detected when analyzed directly 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.

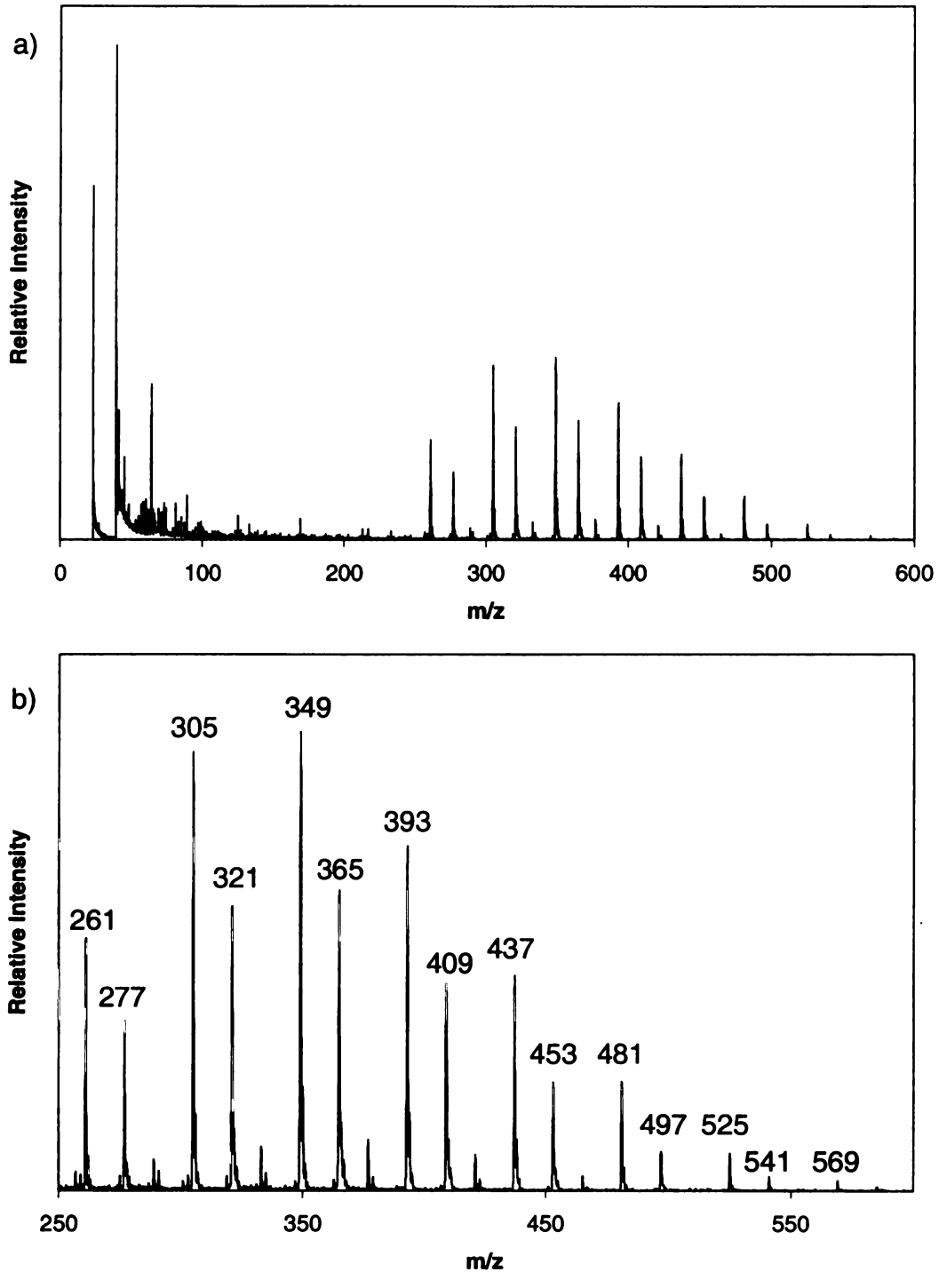


Figure 1.7: Positive-ion LD mass spectra of polyethylene glycol (PEG) on paper a) complete spectrum from m/z 0-600 and b) expanded region from m/z 250-600

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

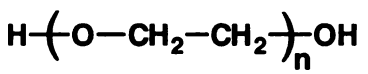
b) 

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

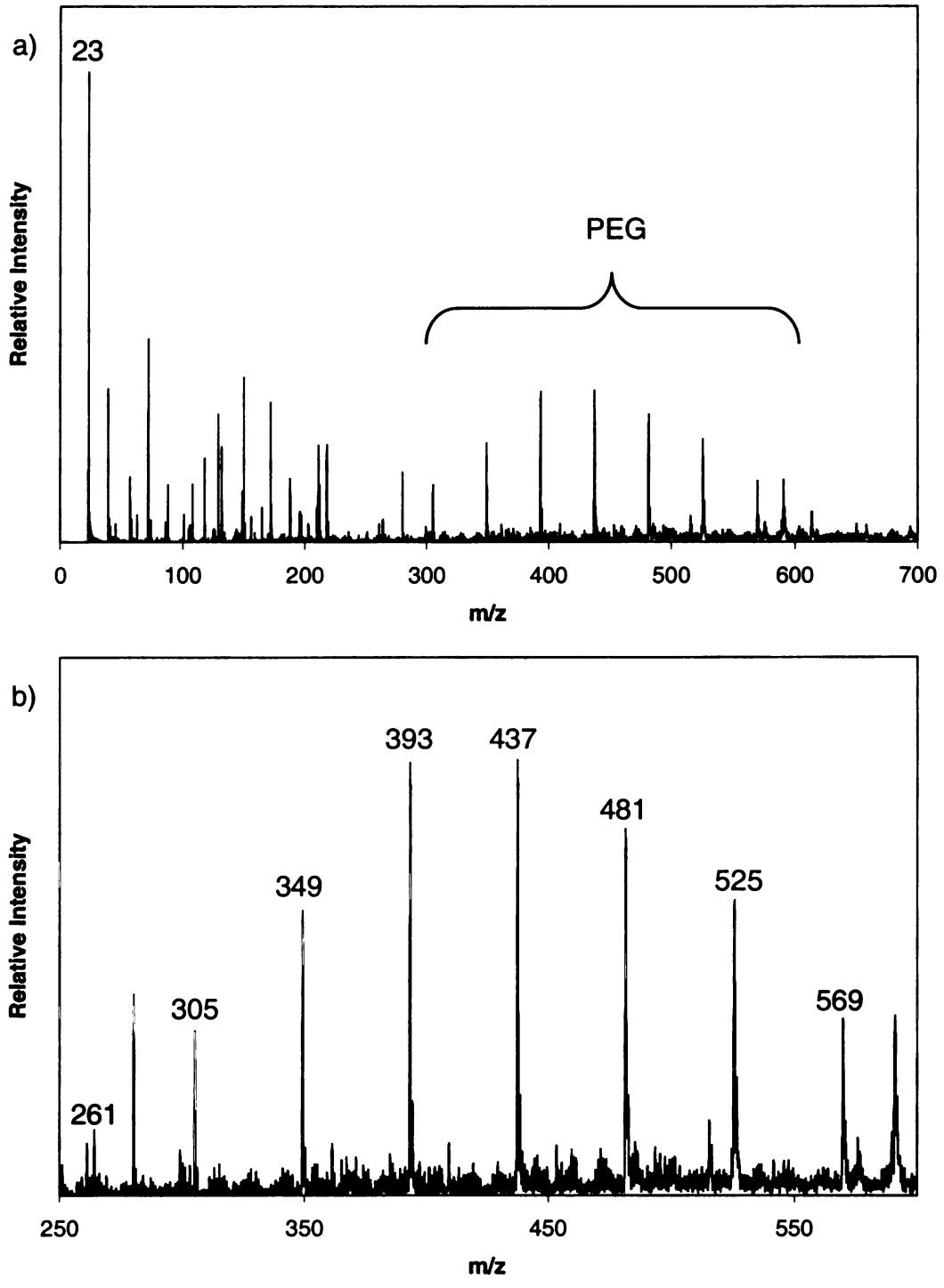


Figure 1.9: Positive-ion LD mass spectra of black, liquid ink from a Pilot® V-Ball rollerball pen a) complete spectrum from m/z 0-700 and b) expanded region from m/z 250-600

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 is roughly 10^{-10} moles – 0.1 nanomoles of dye. 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, the dye needs to be analyzed from paper which may be thought of as potential problem for the D/I of the analyte. The metal suface is an important parameter in the D/I experiment. However, analyses directly for paper have not been problematic. 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 15,000-20,000 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

In 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 appearance of 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 by TLC. Frequently, TLC was indirectly coupled to MS to associate molecular mass with dye color. This involves extracting dyes from the TLC bands (extractions of the individual/separated ink dyes) with subsequent LDMS or MALDI MS analysis. Instruments capable of performing HPLC-MS are commercially available, but this coupling method was not used in this research.

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 plates 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 tetramethylpararosaniline 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 examination of blue ballpoint pen inks stored under various light conditions was 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 dye homologues 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 markings[49]. More specifically, questioned document analysis encompasses many types of evidence including typewriting, laser printing, photocopies, facsimiles, 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 characteristic 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 use of laser desorption mass spectrometry (LDMS) as a tool for the analysis of pen ink dyes on paper has been established. Previous work indicated that the technique has great potential in detecting singly-charged or neutral dye molecules directly on paper as well as other substrates. Direct LDMS ink dye analysis provides basic molecular information (molecular mass, composition, and charge state) that can be used for characterizing dyes; however, the differentiation of isomeric dyes requires more advanced characterization methods. The use of dye photodegradation by incandescent

light and UV radiation with subsequent LDMS analysis proved to be successful. The thorough study of ink dyes using LDMS, TLC, and patent searches lead to the conclusion that LDMS is restricted to singly-charged or neutral dye molecules. Alternative detection methods for analyzing multiply-charged ink dyes (from stainless steel and paper substrates) by LDMS were investigated which is the focus of this thesis research. The use of cation-exchange resins was the first analytical method employed for charge state reduction. The method was simplified by the direct addition of a cation-exchange reagent to the sample. The use of several matrices and cation-exchange additives were explored. A general method that encompasses one matrix and one additive for the analysis of all dyes would be ideal. MALDI MS (with an additive) 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). Assaying inks via coupling thin-layer chromatography with MALDI MS was used to simplify dye identification. The prior use of coupling TLC with LDMS proved to be an excellent method for determining the number of dyes present in one ink and associating dye color (color obtained from thin-layer chromatogram) with molecular mass (mass obtained from the LD mass spectrum) to make dye identification easier.

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. 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. Knowledge of analyzing these kinds of trace evidence was sustained through course work. 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 colored black. The visible spectra that are acquired from analyzing fibers and paint chips 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.

Difficulties arose when Balko tried to detect multiply-charged dyes, specifically, reactive dyes that contained multiple sulfonic acid groups, from the fibers using LDMS directly[50]. Balko stated that the dyes (Reactive Red 11, Reactive Blue 4, and Reactive Yellow 1) were not able to be detected since they are covalently bonded to the molecules that comprise the cotton fabric[50], however, direct LDMS analysis is not capable of detecting multiply-charged species. Most likely the multiple charge distribution prevented the dyes from being detected rather than the interactions between the dye and the fibers. MALDI MS can be applied to the analysis of fiber dyes to ascertain if multiply-charged dyes can be detected from cotton material or other types of fibers.

Research Overview

This thesis research has focused on methods for detecting and characterizing multiply-charged 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 detection and characterization methods were carried out using mass spectrometry. This program of research provides new data to demonstrate the above deductions. The major approach taken in this project:

1. A novel method of analysis was employed to help detect, identify, and characterize multiply-charged dyes present in writing inks based on chemical and structural information.

Specifically, this research thesis discusses:

1. The use of laser desorption mass spectrometry to demonstrate the molecular information that can be attained for the characterization of singly-charged and neutral dye molecules which parallels dye characterization in the MALDI MS analysis.
2. The use of laser desorption mass spectrometry and thin-layer chromatography for determining the presence of multiply-charged dye in an ink.
3. The use of matrix-assisted laser desorption/ionization mass spectrometry with a cation-exchange additive for the analysis of multiply-charged dyes (from inks) on paper directly

4. The basic MS characterization methods that can be employed when examining the MALDI and LD mass spectra of polysulfonated ink dyes.

References

(1) Venkataraman K, editor. The Analytical Chemistry of Synthetic Dyes. John Wiley & Sons, New York, **1977**

(2) Venkataraman K. The Chemistry of Synthetic Dyes. Vol. I, Academic Press Inc., New York, **1952**

(3) Kiernan JA. Classification and Naming of Dyes, Stains and Fluorochromes. *Biotechnic & Histochemistry.* **2001** (76) 261-277

(4) Llewellyn B. Stains File: Structure and Colour in Dyes. **Nov 2002** <http://members.pgonline.com/~bryand/StainsFile/dyes/dyecolor.htm>

(5) Zee, FP van der. "Anaerobic Azo Dye Reduction." Ph.D. Dissertation. Wageningen University, **2002**

(6) Wang A, Chadwick BW, inventors. BIC Corporation, assignee. Ink Composition. US Patent 5,769,931 **Jun 1996**

(7) Crown DA, Conway JA, Kirk PL. Differentiation of Blue Ballpoint Pen Inks Using Chemical Spot Test. *J Crim Law Criminol Police Sci.* **1961** (52) 338-343

(8) Mezger O, Rall M, Heess W. New Methods for Examining Inks. *Z. Angew. Chem.* **1931** (44) 645-651.

(9) Brown C, Kirk PL. Comparison of Writing Inks Using Electrophoresis. *J Crim Law Criminol Police Sci.* **1954** (45) 334-339

(10) MacDonnell HL. Characterization of Fountain Pen Inks by Porous Glass Chromatography and Electrophoresis. *J Crim Law Criminol Police Sci.* **1962** (53) 507

(11) Brunelle RL, Lee H. Determining the Relative Age of Ballpoint Ink Using a Single-Solvent Extraction, Mass-Independent Approach. *J of Forensic Sci.* **1989** (34) 1166-1182

(12) Brunelle RL. A Sequential Multiple Approach to Determining the Relative Age of Writing Inks. *Int J Forensic Doc Examiners.* **1995** (1) 94-98

(13) Cantu AA, Prough RS. On the Relative Aging of Ink – The Solvent Extraction Technique. *J Forensic Sci.* **1987** (32) 1151-1174

(14) Somerford AW, Souder JL. Examination of Fluid Writing Ink by Paper Chromatography. *J Crim Law.* **1952** (43) 124-127

(15) Aginsky VN. Determination of the age of ballpoint pen ink by gas and densitometric thin-layer chromatography. J *Chromatogr.* **1994** (678) 119-125

(16) Aginsky VN. Comparative Examination of Inks by Using Instrumental Thin-Layer Chromatography and Microspectrophotometry. *J Forensic Sci.* **1993** (38) 1111-1130

(17) Brunelle RL, Negri JF, Cantu AA, Lyter AH. Comparison of Typewriter Ribbon Inks by Thin-Layer Chromatography. *J Forensic Sci.* **1977** (22) 807-814

(18) Stewart JL. Ballpoint Ink Age Determination by Volatile Component Comparison – A Preliminary Study. *J Forensic Sci.* **1985** 30 405-411

(19) Colwell LF, Karger BL. Ball Point Pen Examination by High Pressure Liquid Chromatography. *J Asso Off Anal Chemists.* **1977** (60) 613-618

(20) Griffin RME, Kee TG, Adams R. High Performance Liquid Chromagraphic System for the Separation of Basic Dyes. *J Chromatogr.* **1988** (445) 441-448

(21) Lyter AH. Examination of Ball Pen Ink by High Pressure Liquid Chromatography. *J Forensic Sci.* **1982** (27) 154-160

(22) Becker JL, Brunelle RL. Determination of Age of Ballpoint Pen Inks by Fourier Transform Infrared Spectroscopy. Program 36th Annual Meeting of the American Academy of Forensic Sciences, Anaheim, CA, **Feb 1984**

(23) Humecki H. Experiments in Ballpoint Ink Aging Using Infrared Spectroscopy. Proceedings of International Symposium of Non-Handwriting Aspects of Questioned Document Examination, U.S. Government Printing Office, Washington, DC, **1985** 131-135

(24) Zeichner A., et al. Transmission and Reflectance Microspectrophotometry of Inks. *J Forensic Sci.* **1988** (33) 1171-1184

(25) Aginsky, VN. A Microspectrophotometric Method for Dating Ballpoint Inks – A Feasible Study. *J Forensic Sci.* **1995** (40) 475-478

(26) Aginksy VN. Dating and Characterizing Writing, Stamp Pad, and Jet Printer Inks by Gas Chromatography/Mass Spectrometry. *Int J Forensic Doc Examiners.* **1996** (2) 103-110

(27) Vogt C, Vogt J, Becker H, Rohde E. Separation, Comparison, and Identification of Fountain Pen Inks by Capillary Electrophoresis with UV-Visible and Fluorescence Detection by Proton-Induced X-Ray Emission. *J Chromatogr.* **1997** (781) 391-405

(28) Vogt C, Becker A, Vogt, J. Investigation of Ball Point Pen Inks by Capillary Electrophoresis (CE) with UV/Vis Absorbance and Laser Induced Fluorescence Detection and Particle Induced X-Ray Emission (PIXE). *J Forensic Sci.* **1999** (44) 819-831

(29) Sakayanagi M, Komuro J, Konda Y, Watanabe K, Harigaya Y. Analysis of Ballpoint Pen Inks by Field Desorption Mass Spectrometry. *J Forensic Sci.* **1999** (44) 1204-1214

(30) Ng LK, Lafontaine P, Brazeau L. Ballpoint pen inks: Characterization by positive and Negative Ion-Electrospray Ionization Mass Spectrometry for the Forensic Examination of Writing Inks. *J Forensic Sci.* **2002** (47) 1238-1247

(31) Andrasko J. High Pressure Liquid Chromatography Analysis of Ballpoint Pen Inks Stored at Different Light Conditions. *J Forensic Sci.* **2001** (46) 21-30

(32) 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

(33) Watson JT. Introduction to Mass Spectrometry. Lippencott-Raven Publishing, Philadelphia, **1997**

(34) Denoyer E, Van Grienken R, Adams F, Natusch DFS. Laser Microprobe Mass Spectrometry 1: Basic Principles and Performance Characteristics. *Anal Chem.* **1982** (54) 26A-41A

(35) Grim DM, Siegel JA, Allison J. Evaluation of Desorption/Ionization Mass Spectrometric Methods in the Forensic Applications of the Analysis of Inks on Paper. *J Forensic Sci.* **2001** (46) 1411-1420

(36) Grim DM, Siegel JA, Allison J. Does ink age inside of a pen cartridge? *J Forensic Sci.* **2002** (47) 1294-1297

(37) Dunn JD, Siegel JA, Allison J. Photodegradation and Laser Desorption Mass Spectrometry for the Characterization of Dyes Used in Red Pen Inks. *J Forensic Sci.* **2003** (48) 652-657

(38) Voyager Workstation User's Guide version 5, PerSpective Biosystems, **1999**

(39) Monaghan JJ, Barber M, Bordoli RS, Sedgewick RD, Tyler AN. Fast Atom Bombardment Mass Spectra of Involatile Sulphonated Azo Dyestuffs. *Org Mass Spectrom.* **1982** (17) 569-574

(40) Scheifers SM, Verma S, Cooks RG. Characterization of Organic Dyes by Secondary Ion Mass Spectrometry. *Anal Chem.* **1983** (55) 2260-2265

(41) Detter-Hoskin LD, Busch KL. SIMS: Secondary Ion Mass Spectrometry. In: Conners TE, Banerjee, editors. Surface Analysis of Paper. CRC Press, New York **1995** 206-234

(42) Pachuta SJ, Staral JS. Nondestructive Analysis of Colorants on Paper by Time-of-Flight Secondary Ion Mass Spectrometry. *Anal Chem.* **1994** (66) 276-284

(43) De Hoffmann E, Charette J, Stroobant V. Mass Spectrometry Principles and Applications. John Wiley & Sons, Chichester, **1996**

(44) Sullivan AG, Gaskell SJ. The Analysis of Polysulfonated Azo Dyestuffs by Matrix-Assisted Laser Desorption/Ionization and Electrospray Mass Spectrometry. *Rapid Commun Mass Spectrom.* **1997** (11) 803-809

(45) Sullivan AG, Garner R, Gaskell SJ. Structural Analysis of Sulfonated Momoazo Dyestuff Intermediates by Electrospray Tandem Mass Spectrometry and Matrix-Assisted Laser Desorption/Ionization Post-Source Decay Mass Spectrometry. *Rapid Commun Mass Spectrom.* **1998** (12) 1207-1215

(46) Skoog DA, Holler FJ, Nieman TA. Principles of Instrumental Analysis. 5th ed. Saunders College Publishing, Philadelphia, **1998**

(47) Kunjappu JT. Molecular Thinking in Ink Chemistry. Ink World **1999** (5) 50-53

(48) Sherma J, Fried B, editors. Handbook of Thin-Layer Chromatography. 3rd ed. Marcel Dekker, Inc., New York, **2003**

(49) Gialamas DM. Criminalistics. Encyclopedia of Forensic Sciences. Academic Press, **2000** 471-477

(50) Balko LG. "Laser Desorption/Ionization Mass Spectrometric (LD/MS) Analysis of Samples of Forensic Interest: Chemical Characterization of Colorant and Dyes." M.S. Thesis. Michigan State University, **2002**

Chapter 2: 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.

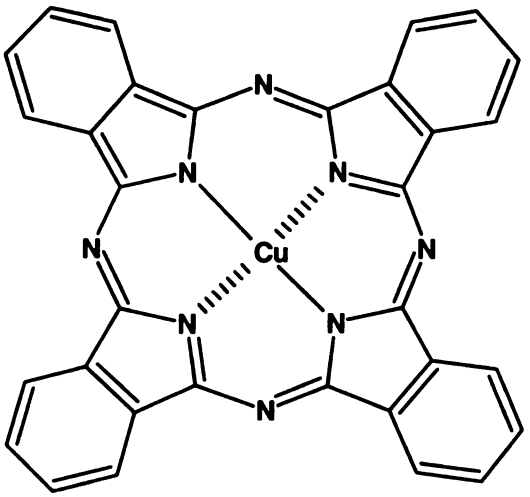
Success has been reported with black, blue, and red ballpoint pen ink dyes[2-5]. The direct characterization information that can be extracted from the LDMS mass spectra will be discussed next.

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.

a)



b)

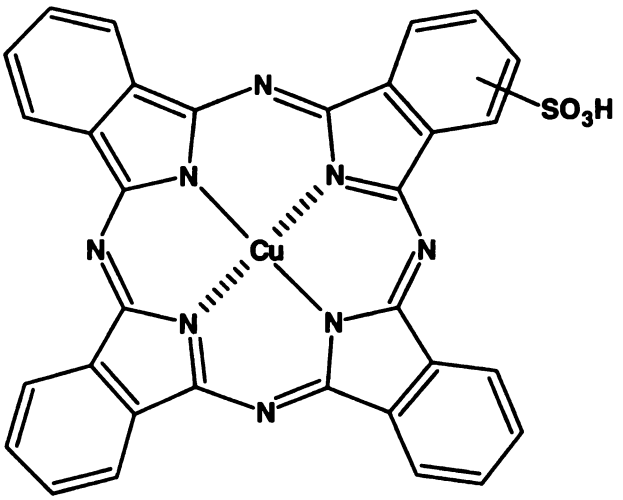


Figure 2.1: Dye structures a) Copper Phthalocyanine and b) monosulfonated Copper Phthalocyanine

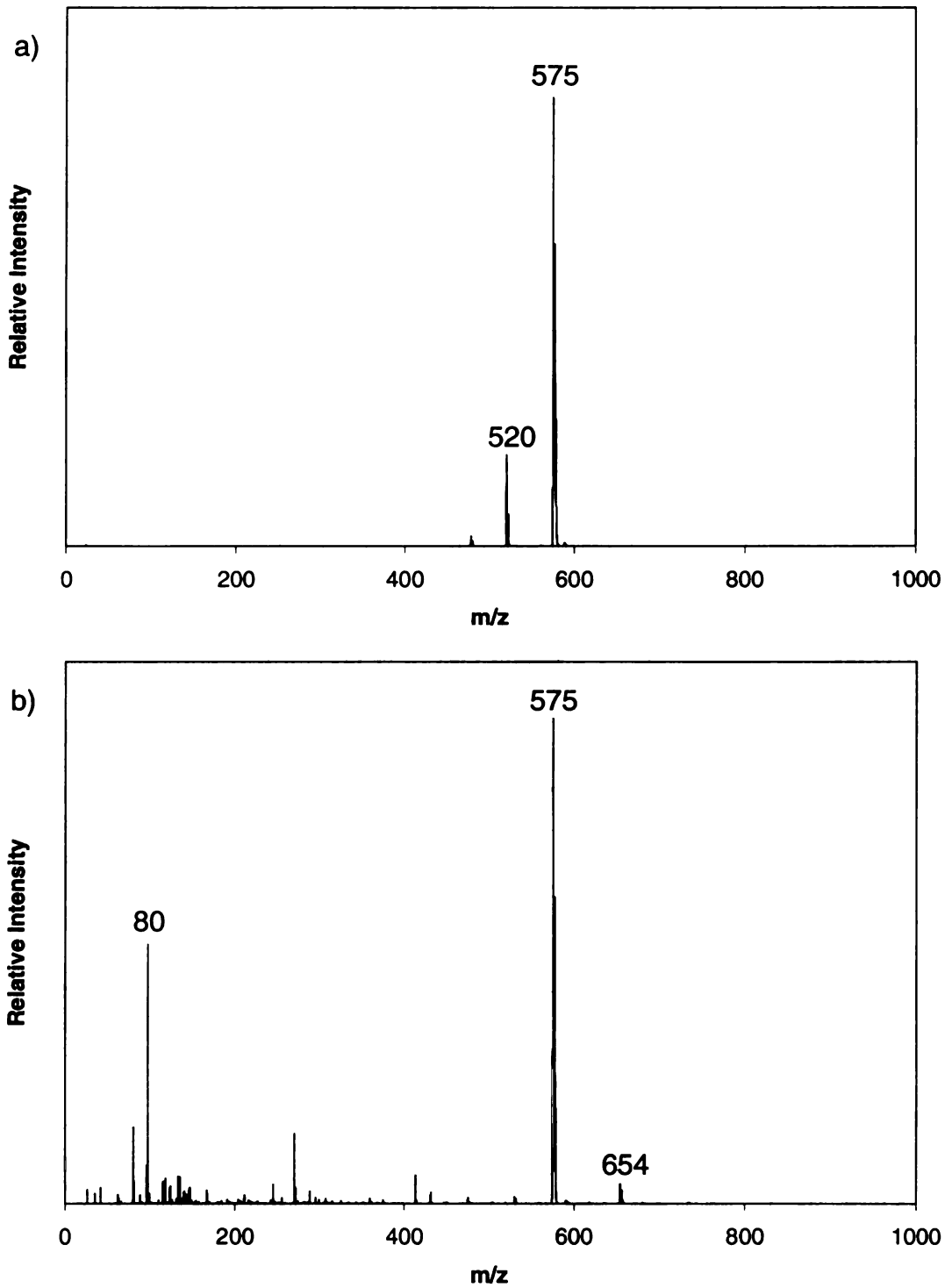


Figure 2.2: LD mass spectra of blue gel ink containing Copper Phthalocyanine a) positive-ion and b) negative-ion

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.

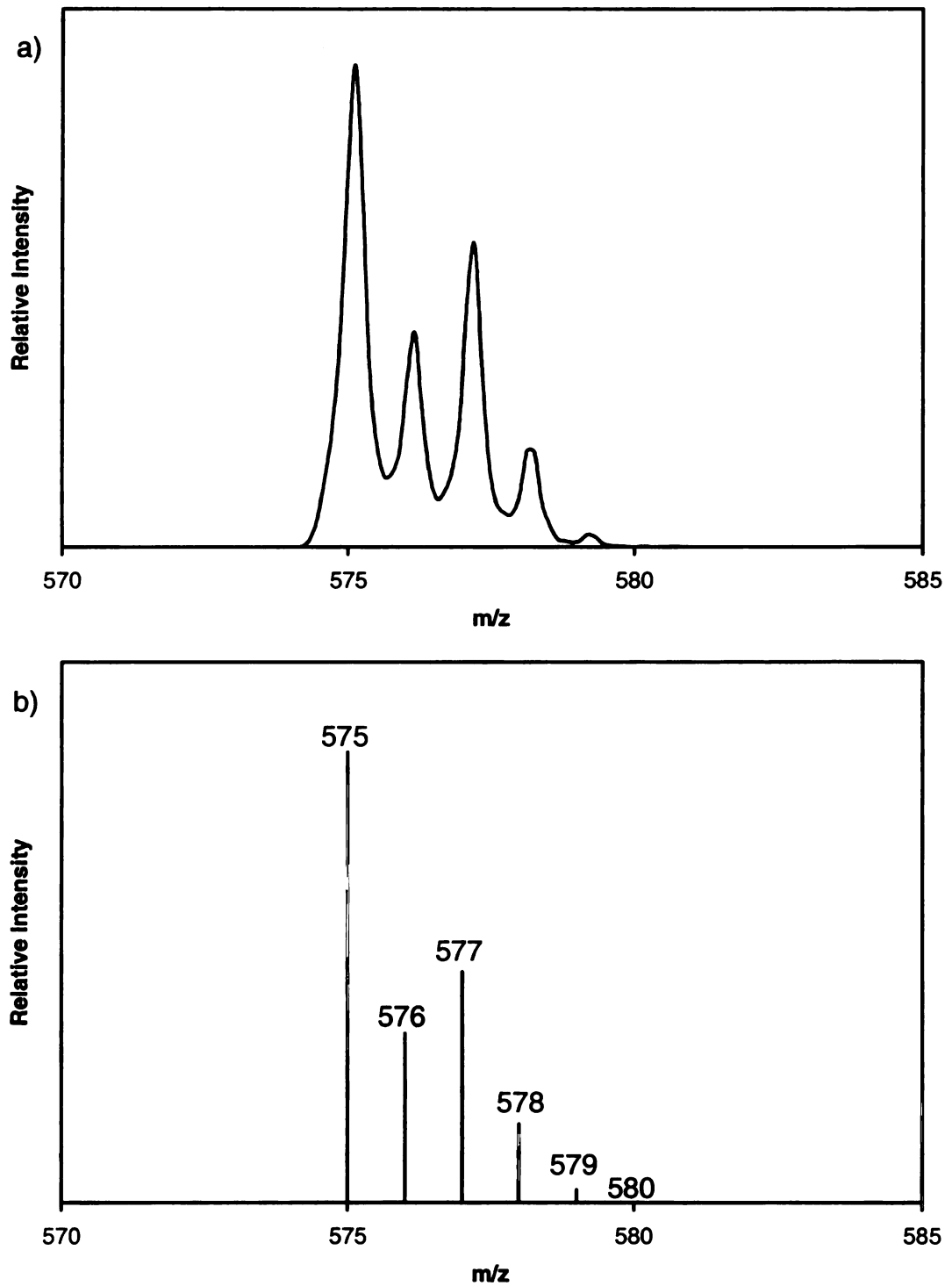


Figure 2.3: The isotopic peak distribution of Copper Phthalocyanine a) experimental isotopic distribution in negative-ion mode b) theoretical isotopic distribution for positive and negative-ion modes

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

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

(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. Desorption/Ionization of multiply-charged dyes directly by LDMS is typically not observed. The charge state needs to be reduced prior to detection and the multiply-charged dyes are detected as singly-charged ion in matrix-assisted laser desorption/ionization MS. The charges associated with the polyionic dye still influence if the species is detected in the positive or negative-ion modes or both.

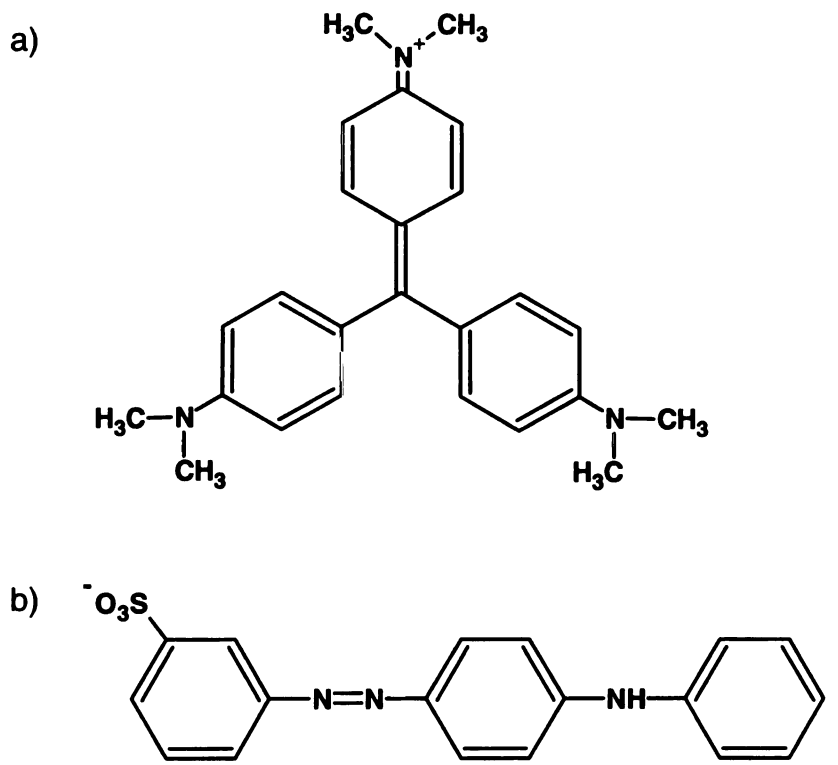


Figure 2.4: Structure of the dyes used in several black ballpoint inks a) Crystal Violet and b) Metanil Yellow

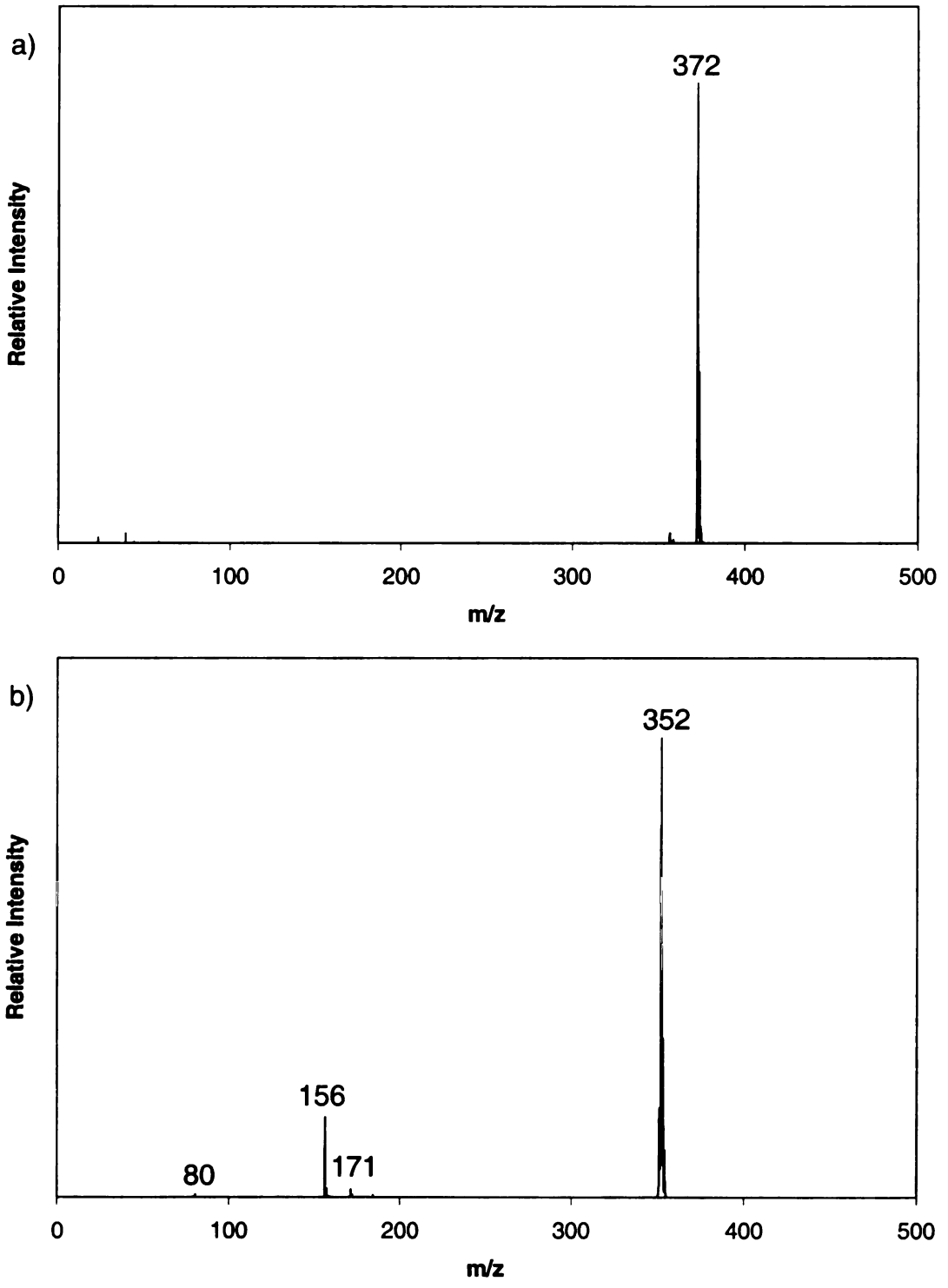


Figure 2.5: LD mass spectra of black ink from Sanford® Supergrip ballpoint pen a) positive-ion and b) negative-ion

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.

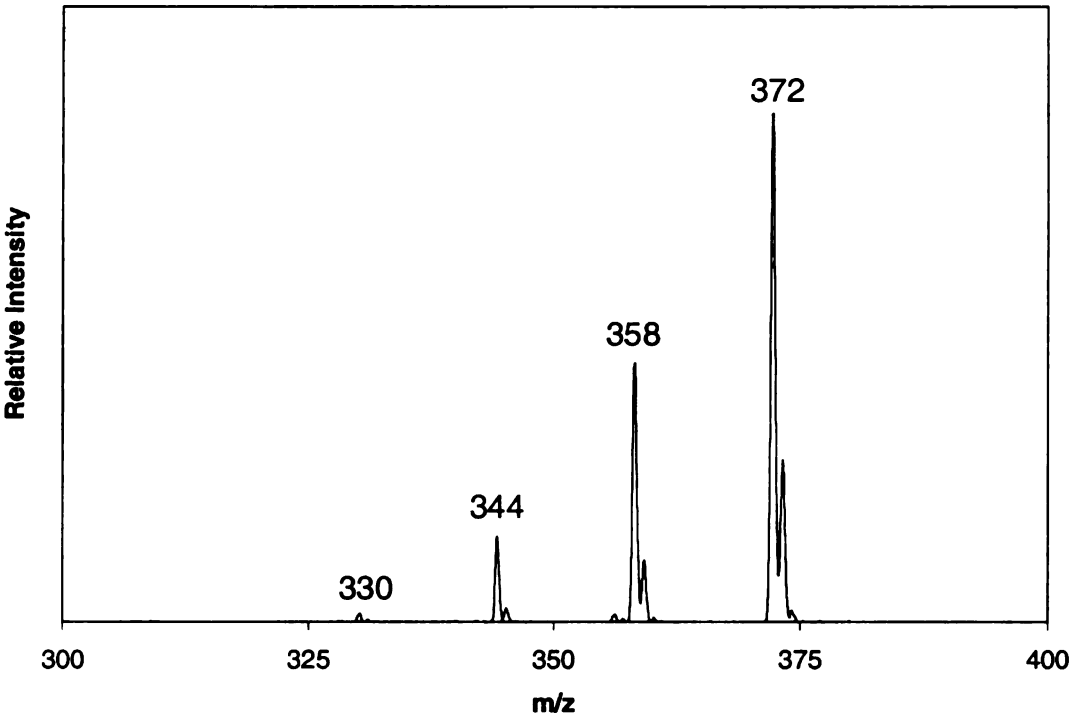


Figure 2.6: Positive-ion LD mass spectrum of Methyl Violet 2B purchased from the Aldrich Chemical Company

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

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

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 2.4 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.

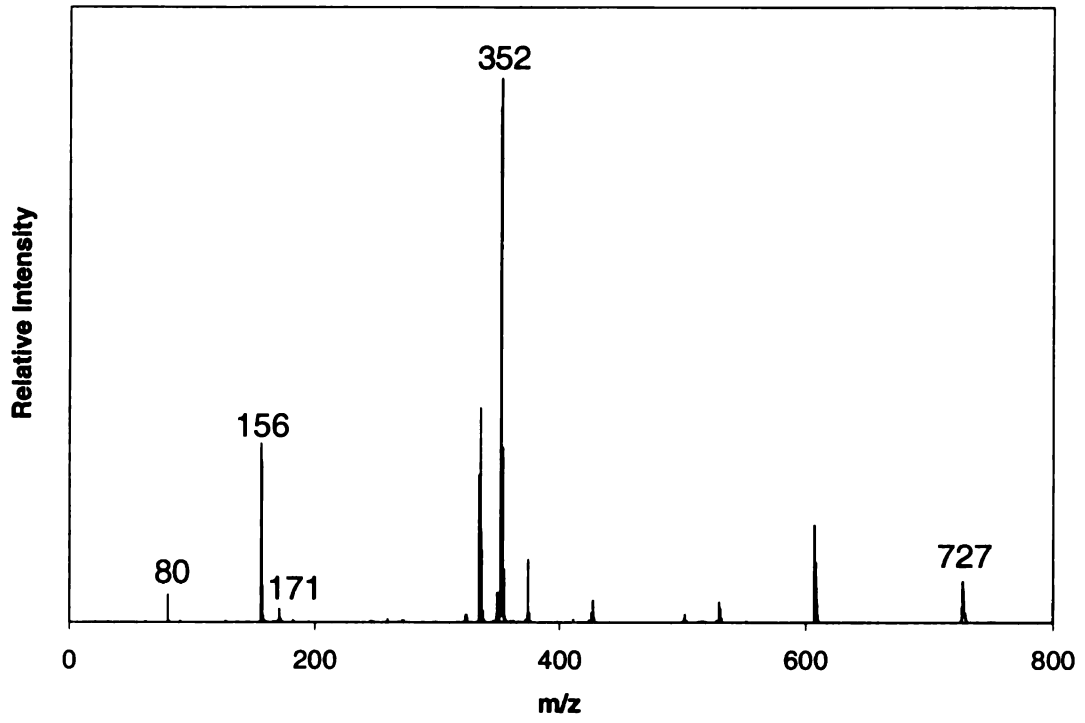


Figure 2.7: The direct analysis of Metanil Yellow from a metal plate by negative-ion LDMS

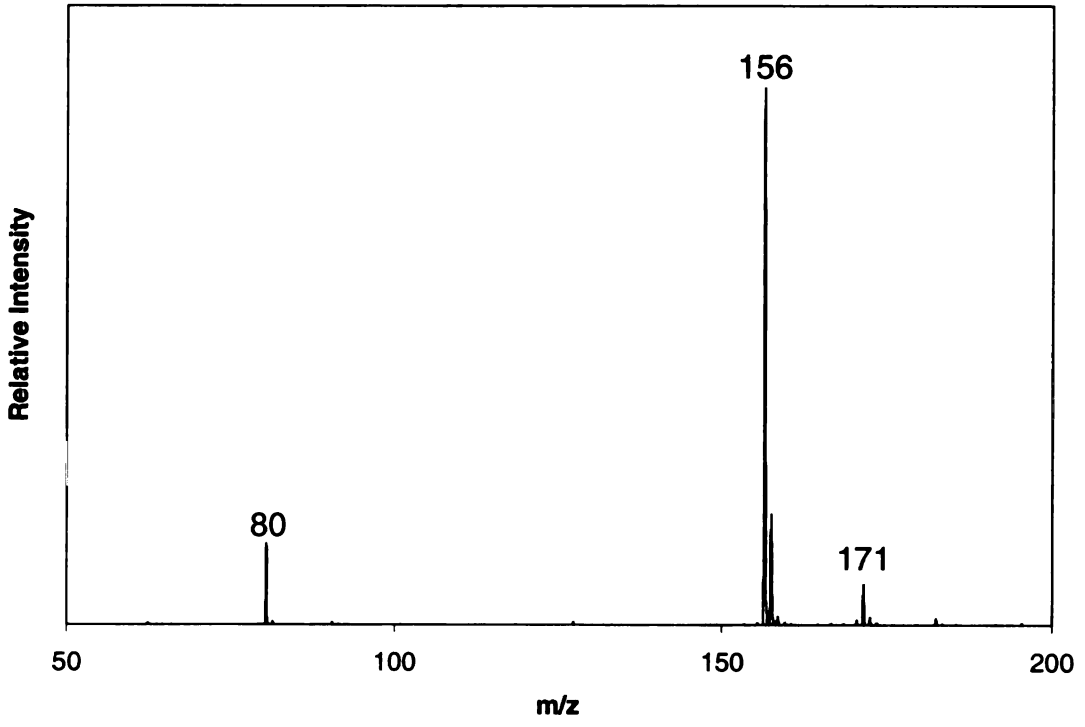


Figure 2.8: Negative-ion LD mass spectrum of Metanil Yellow shows peaks due to fragment ions

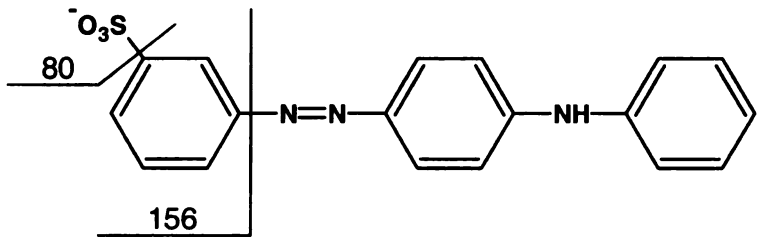


Figure 2.9: Dye structure of Metanil Yellow with labeled values that correspond to possible fragment ions or neutral molecules

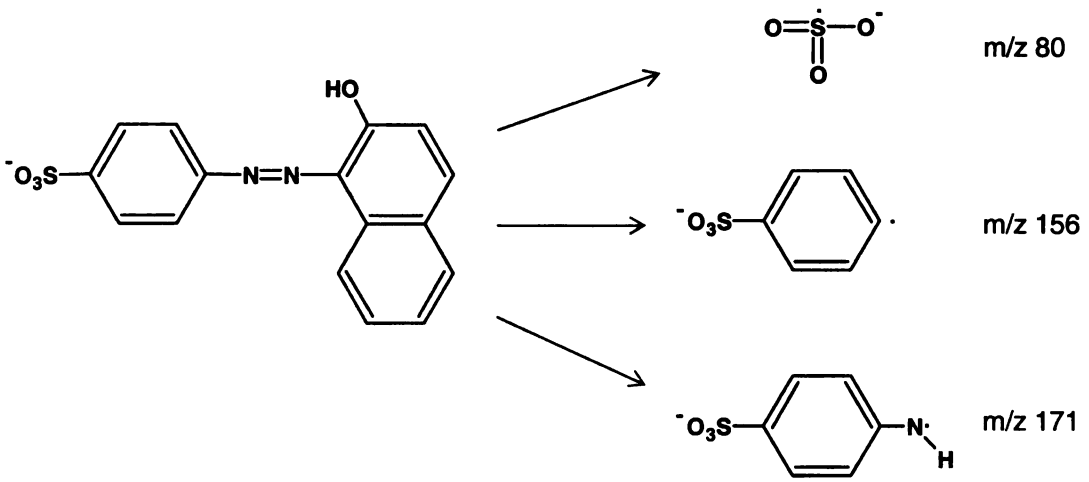


Figure 2.10: Acid Orange 7 (to the left of the arrows) and fragment ions (m/z 80, 156, 171) formed when analyzed by ESI tandem MS and MALDI-PSD MS[11]

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.

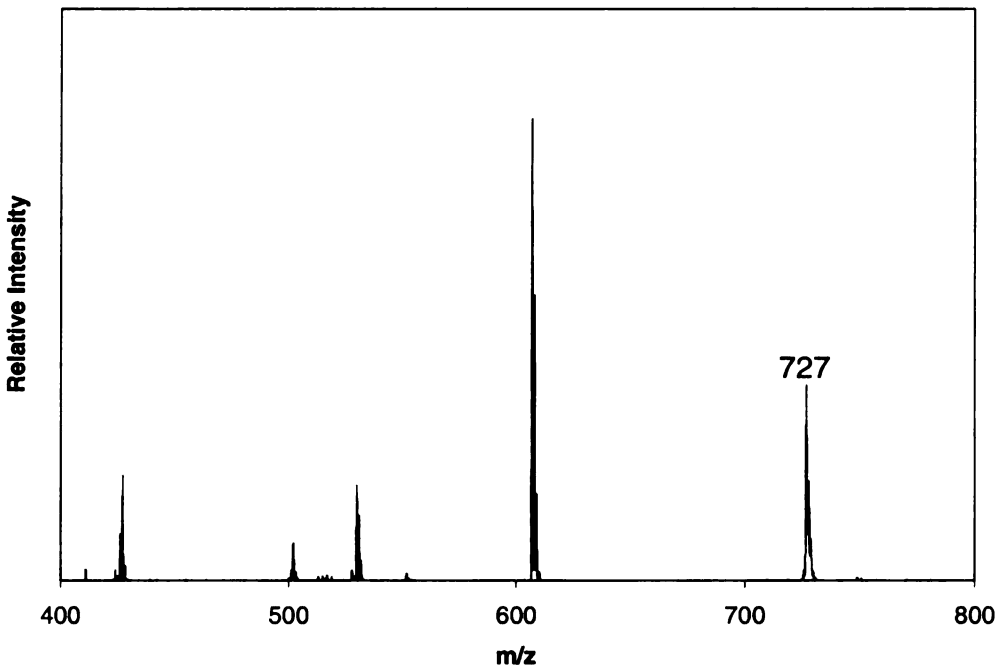


Figure 2.11: Negative-ion LD mass spectra of Metanil Yellow on paper shows peaks due to cluster ions

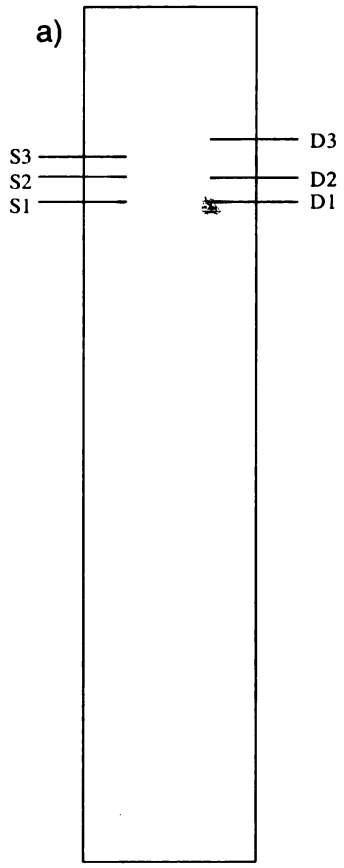
Coupling TLC with LDMS

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

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

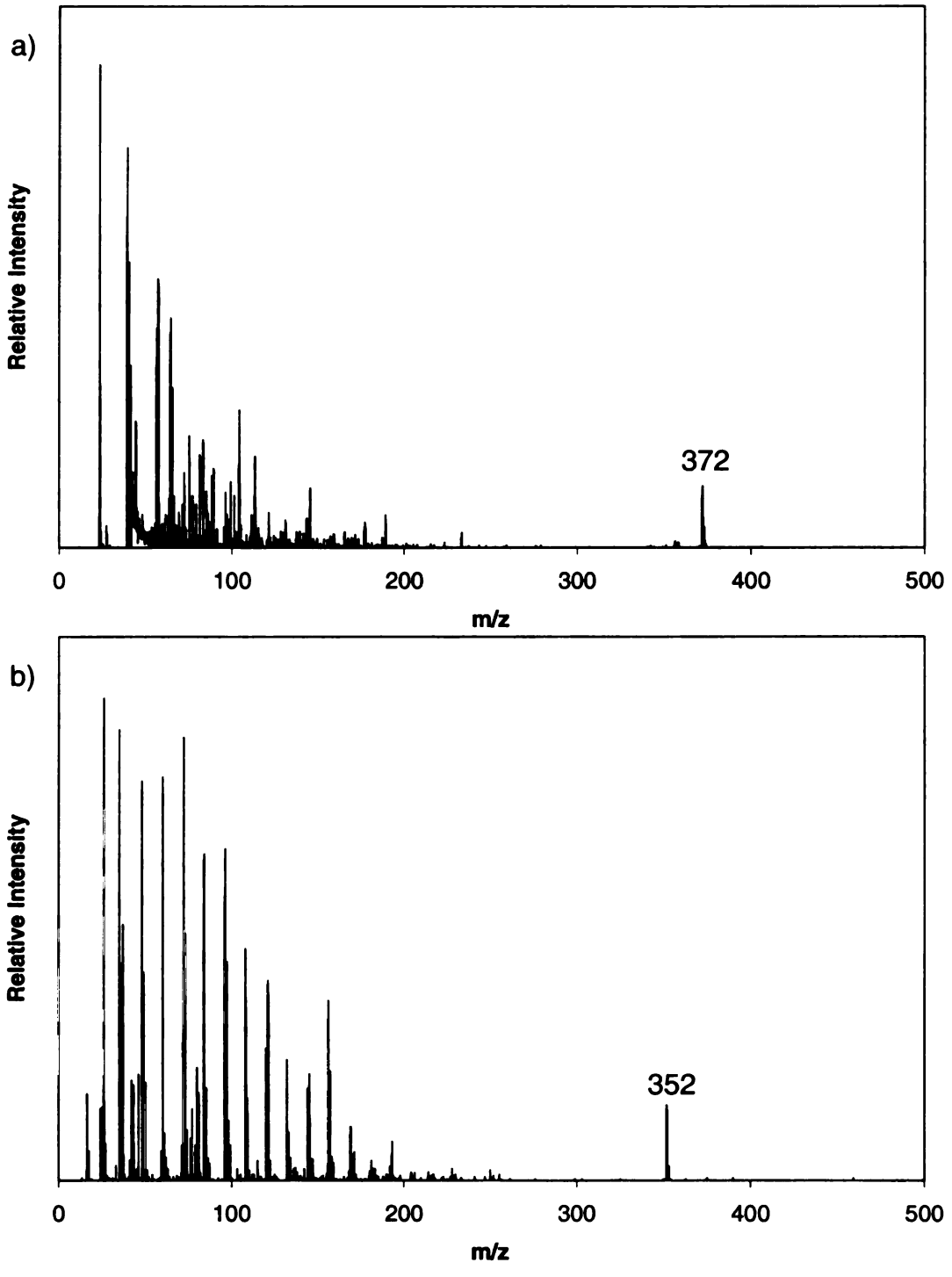


Figure 2.13: Direct coupling of TLC with LDMS: bands are analyzed by LDMS directly from the aluminum TLC plate a) purple band (Crystal Violet) and b) yellow band (Metanil Yellow)

References

(1) Whitten KW, Davis RE, Peck ML. General Chemistry. 5th ed. Saunders College Publishing, Fort Worth, 1996

(2) Grim DM, Siegel JA, Allison J. Evaluation of Desorption/Ionization Mass Spectrometric Methods in the Forensic Applications of the Analysis of Inks on Paper. *J Forensic Sci.* **2001** (46) 1411-1420

(3) Grim DM, Siegel JA, Allison J. Does Ink Age Inside of a Pen Cartridge? *J Forensic Sci.* **2002** (47) 1294-1297

(4) 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

(5) Dunn JD, Siegel JA, Allison J. Photodegradation and Laser Desorption Mass Spectrometry for the Characterization of Dyes Used in Red Pen Inks. *J Forensic Sci.* **2003** (48) 652-657

(6) Manura JJ, Manura DJ. Isotope Distribution Calculator and Mass Spec Plotter. Scientific Instrument Services. 1996-2003 <http://207.97.159.7/cgi-bin/mass10.pl>

(7) Venkataraman K. The Chemistry of Synthetic Dyes. Vol. I, Academic Press Inc., New York, 1952

(8) Fales HM, Pannell LK, Sokoloski EA, Carmeci P. Separation of Methyl Violet 2B by High-Speed Countercurrent Chromatography and Identification by CF-252 Plasma Desorption Mass-Spectrometry. *Anal Chem.* **1985** (57) 376-378

(9) Green FJ. The Sigma-Aldrich Handbook of Stains, Dyes, and Indicators. Aldrich Chemical Company, Inc., Milwaukee, **1990**

(10) Sullivan AG, Garner R, Gaskell SJ. Structural Analysis of Sulfonated Monoazo Dyestuff Intermediates by Electrospray Tandem Mass Spectrometry and Matrix-Assisted Laser Desorption/Ionization Post-Source Decay Mass Spectrometry. *Rapid Commun Mass Spectrom.* **1998** (12) 1207-1215

(11) Conneely A, McClean S, Smyth WF, McMullan G. Study of the Mass Spectrometric Behavior of Phthalocyanine and Azo Dyes Using Electrospray Ionization and Matrix-Assisted Laser Desorption/Ionization. *Rapid Commun Mass Spectrom.* **2001** (15) 2076-2084

(12) Bruins AP, Weidolf LO, Henion JD. Determination of Sulfonated Azo Dyes by Liquid Chromatography/Atmospheric Pressure Ionization Mass Spectrometry. *Anal Chem.* **1987** (59) 2647-2652

Chapter Three: Direct LDMS Analysis of Ink Dyes: Limitations and Solutions

Two limitations were encountered when multiply-charged ink dyes were examined by direct LDMS. One disadvantage is that the mass spectra lack structural information. Unlike an EI MS experiment, little fragmentation, if any, has been observed in the LDMS experiments. This limits the amount of structural information that can be acquired from the LD mass spectra, thus making dye characterization arduous. A second limitation is the ability of multiply-charged dyes to be detected by LDMS. The energy of the laser is insufficient to desorb dyes that contain more than one charge. The latter challenge was overcome by chemically modifying the dye prior to the MS analysis and the results are presented in Chapter Four. Previous research carried out in our laboratory implemented photodegradation to characterize ink dyes[1]. Using photochemistry to characterize multiply-charged dyes is potential research for the future, so the characterization method will be discussed.

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. To parallel inks on paper, aqueous dye solutions were prepared, spotted on paper, and allowed to dry for subsequent LDMS analysis. The dyes analyzed were Rhodamine B (Sigma, St. Louis, MO) and Rhodamine 6G (Aldrich, Milwaukee, WI). Photodegradation was accomplished by using incandescent light (75 W, 120 V light bulb).

Dye Characterization by Photodegradation and LDMS

In the laser desorption experiment, energy is deposited into the ink 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, ionic species desorb intact, giving a single mass spectrometric peak (plus isotopic peaks), and providing molecular weight information and possibly elemental composition. 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 degraded photochemically. LDMS analysis following photodegradation yields a set of new peaks, each representing a degradation product. Structural insights can be obtained from the degradation products and understanding the reactions that occur. For example, if Crystal Violet is irradiated with UV radiation, N-demethylation reactions occur and the dye degrades to form Methyl Violet 2B and extensive degradation leads to the formation of other homologues. Replacement of a methyl group from Crystal Violet 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 manner. Previous work carried out by Grim *et al.* utilized the Crystal Violet degradation

and proposed an approach for determining the age of a document[2]. From an analytical standpoint, the degradation products formed naturally or artificially are related to the dye structure. The fact that Crystal Violet forms a maximum of six degradation products due to demethylation provides an important structural context in which the dye can be identified.

The use of LDMS to detect dyes in red ballpoint inks marked the discovery of two isomeric dyes which instigated the development of a method for characterizing ink dyes by photodegrdation. The combination of photodegradation and LDMS for dye identification was demonstrated through a challenging example, the differentiation between two isomeric xanthene dyes, Rhodamine B and Rhodamine 6G. Both dyes are used in the manufacturing of red ballpoint inks[3] and an ink may contain one or both of the dyes. The xanthene dyes are cationic and have the molecular formula $[C_{28}H_{31}N_4O_2]^+[Cl]^-$. Shown in Figure 3.1 are the positive-ion LD mass spectra of the isomers. The intact detection of each dye is denoted by the peak at m/z 443. Although, the mass spectrum of Rhodamine B contains an additional peak at m/z 399 that can be used to characterize 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 3.1a), then the presence of Rhodamine 6G is uncertain. In order to properly characterize an ink that contains two dyes, both need to be identified. The challenge to differentiate the rhodamine dyes was overcome by using photochemistry to artificially degrade the dyes and subsequently analyze the degradation by LDMS[1].

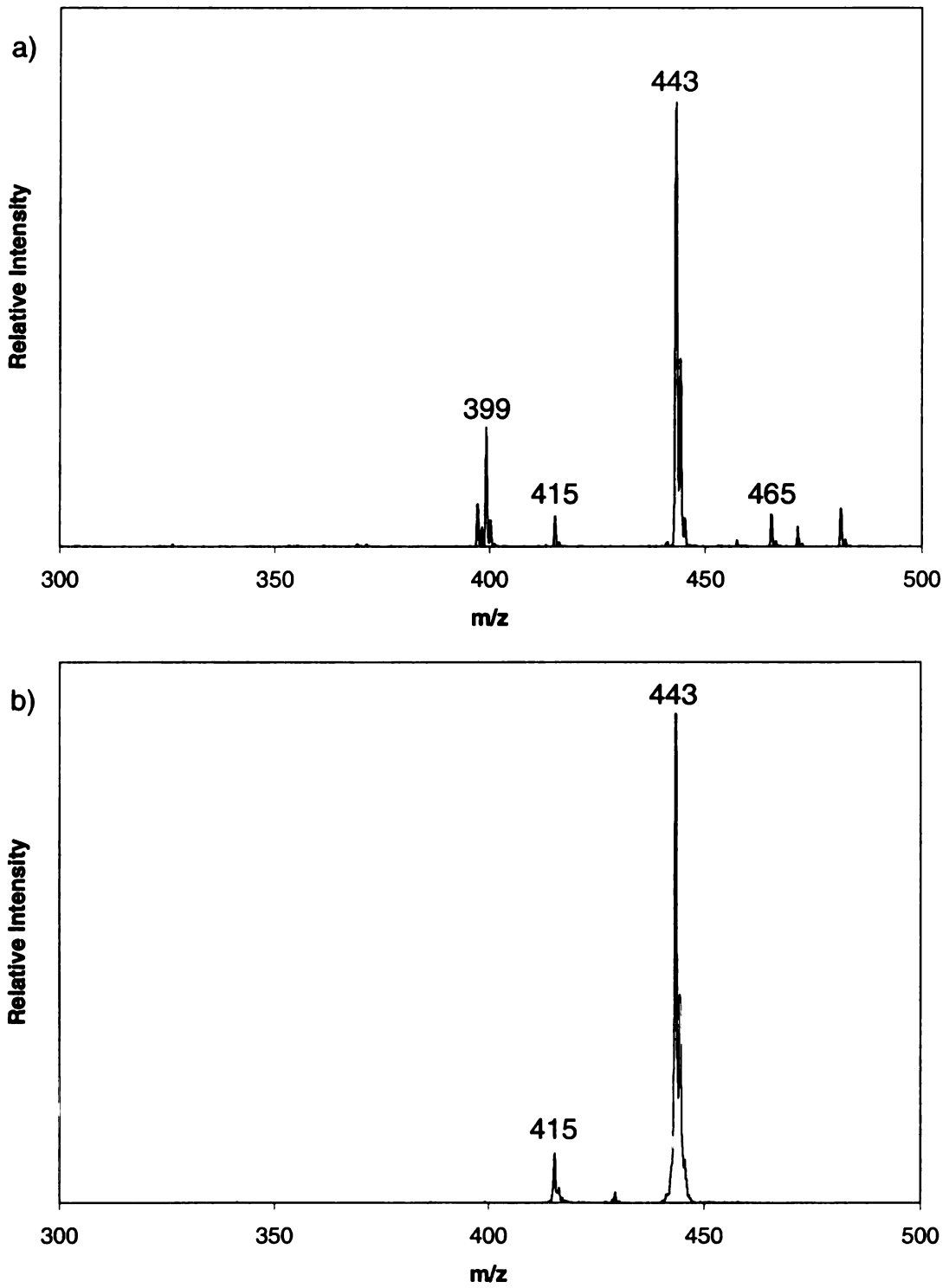
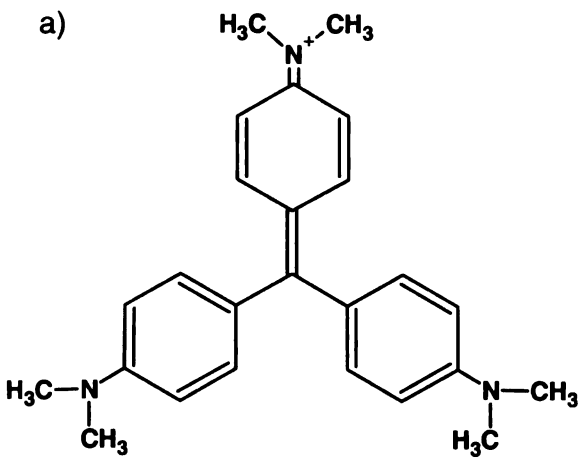


Figure 3.1: Positive-ion LD mass spectra of isomeric, red ink dyes a) Rhodamine B and b) Rhodamine 6G

Structural similarities shared between the isomeric rhodamine dyes and Crystal Violet were used to develop an analytical method that involved degrading dyes using incandescent light. The detection of Crystal Violet by direct LDMS was briefly discussed in Chapter Two. Grim *et al.* analyzed naturally aged ink samples and formulated ink dating curves based on an accelerated aging study that incorporated the use of ultraviolet radiation[2]. Ultraviolet radiation was found to mimic the natural degradation process of Crystal Violet. The dye was found to naturally and artificially degrade by a process called oxidative demethylation[2]. The dye structure contains six methyl groups that are chemically modified when degradation occurs. The dye shown in Figure 3.2a can form a maximum of six degradation products as depicted in Figure 3.2b, which can be simultaneously detected by LDMS. N-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 3.3 is the positive-ion LD mass spectrum of blue ballpoint 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 3.2: 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)

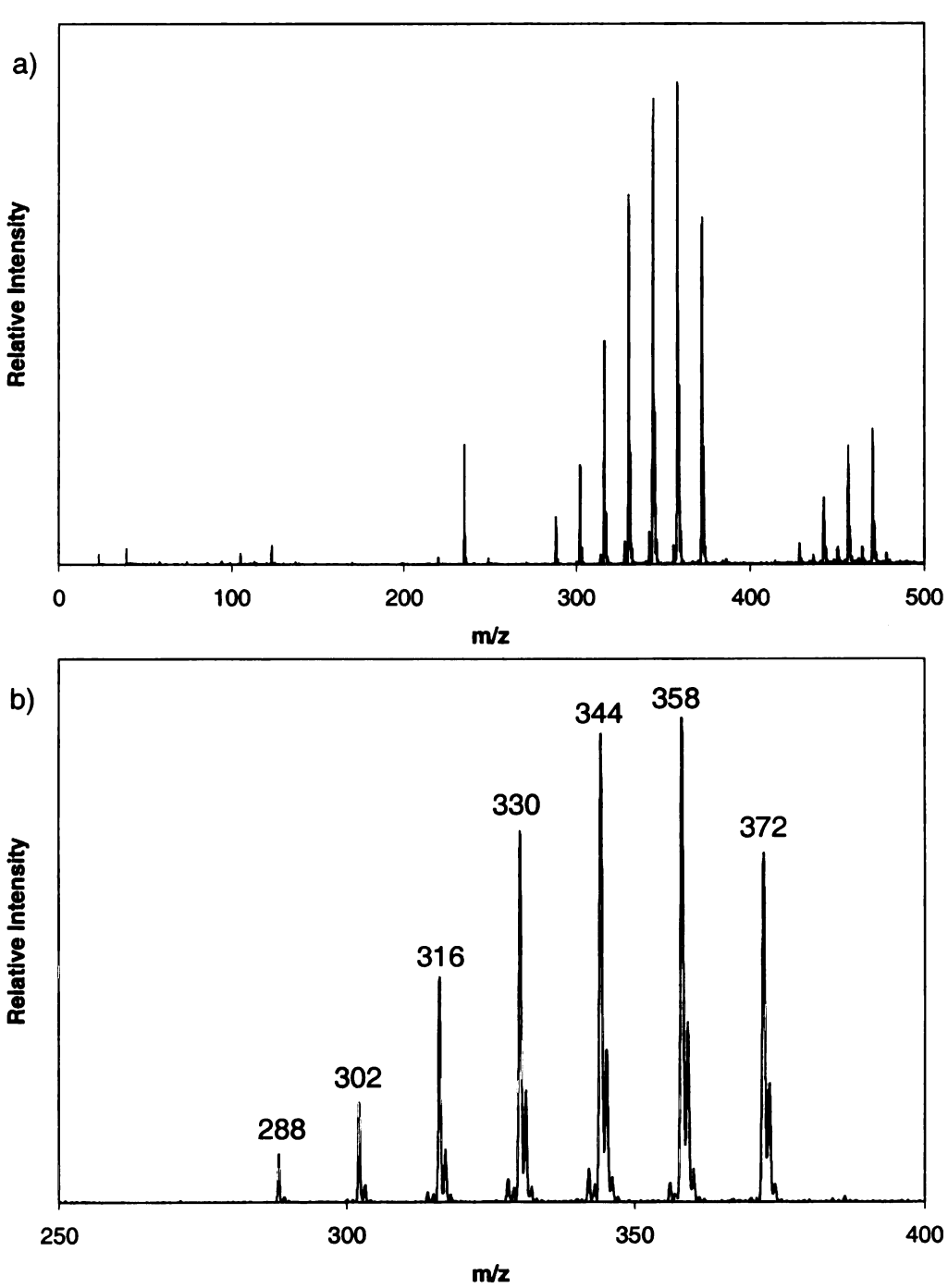


Figure 3.3: Positive-ion LD mass spectra of blue ink from BIC Cristal ballpoint pen degraded with UV radiation a) complete spectrum from m/z 0-500 and b) expanded region from m/z 250-400

The structures of Rhodamine B and Rhodamine 6G are shown in Figures 3.4a and 3.4b, respectively. When the dyes are subjected to UV radiation or incandescent light they degrade and the deethylated products are detectable by LDMS. As the rhodamine dyes degrade, hydrogen atoms replace the ethyl groups bonded to the nitrogen atoms. Rhodamine B forms four N-deethylated products when chemically induced by incandescent light where two N-deethylated products are form from Rhodamine 6G when photodegraded. Tables 3.1a and 3.1b illustrate the m/z values of the deethylated products of Rhodamine B and Rhodamine 6G, respectively. The degradation products result from a similar degradagtion mechanism as Crystal Violet. The m/z values of the dye and the deethylated degradation products are separated by 28 Da. The deethylated products can be used characterize the dyes individually, however, the deethyalted products can not be used to discern Rhodamine 6G apart from Rhodamine B if an ink contains both dyes. Fortunately, another structural characteristic of Rhodamine 6G can be used to differentiate the dyes which will be discussed in the next section.

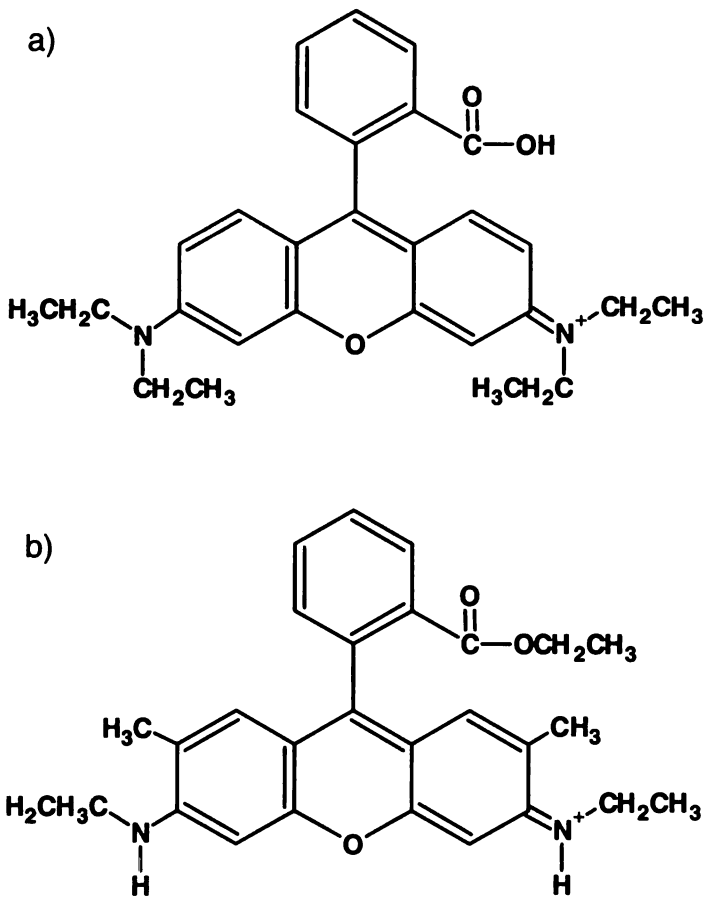


Figure 3.4: Isomeric dye structures a) Rhodamine B and b) Rhodamine 6G

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 3.1: 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[3], two xanthene dyes, Rhodamine B and Rhodamine 6G, can both be used to manufacture red pen ink. The Sigma-Aldrich Handbook of Stains, Dyes, and Indicators[4] revealed that the two rhodamine dyes are cationic isomers with 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 are distinguished by their functional groups. 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 the number of ethyl groups can be used to differentiate the isomeric 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. In this case, the deethylated degradation products overlap in m/z values and can not be used for dye differentiation. However, other structural differences may help to undoubtingly distinguish the dyes in LDMS. Fortunately, the xanthene dyes have additional differences in functional groups. 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 groups. The methyl group was used to characterize Rhodamine 6G as well as to determine the presence of the dye in an ink containing both xanthene dyes. The 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 is consistently present in the mass spectrum of Rhodamine B (Figure 3.5a). The peak represents the decarbonylated form $(RB\text{-}CO_2)^+$ of Rhodamine B $(RB)^+$. The mass difference between the intact dye and this species is 44 amu, which corresponds to carbon dioxide. Rhodamine B, a carboxylic acid, can 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. Initially, the peak at m/z 399 was assigned to be a dye impurity. Rhodamine 6G is commercially available through the Aldrich Chemical Company at a purity of 99% where Rhodamine B available through Sigma is 95% pure. The purity of the dyes appeared to correlate 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 LDMS results 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. The use of TLC and MALDI MS to distinguish between ions due to fragmentation and impure dye mixtures were discussed in Chapter One. The chromatographic separation of Rhodamine B yielded one TLC band and upon using indirect TLC coupled to LDMS the band was identified as the intact dye. Dye impurities were not observed from the TLC chromatogram. Figure 3.6a and 3.6b 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 m/z 399 ion is 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 peaks at m/z 443 and 399 are present.

Although, Rhodamine B can be characterized by direct LDMS, photodegradation can be used to characterize the dye based on the resulting degradation products. The LD mass spectrum in Figure 3.5b was obtained upon photodegrading Rhodamine B. As previously discussed, upon being exposed to incandescent light, Rhodamine B forms four deethylated products. These degradation products of Rhodamine B also show the loss of 44 amu as seen with the D/I of the intact dye.

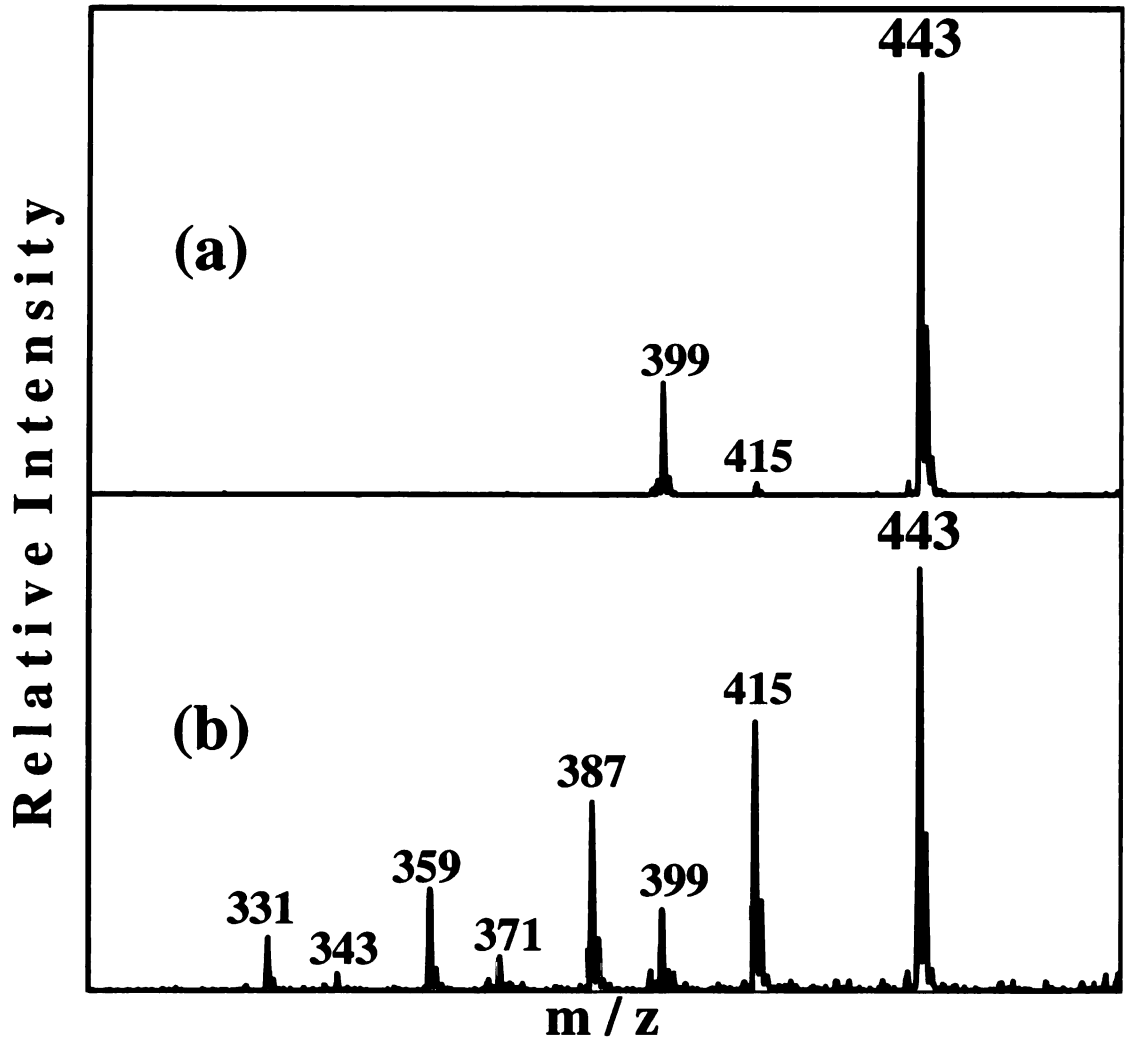


Figure 3.5: Positive-ion LD mass spectra of Rhodamine B (a) no exposure to incandescent light and (b) exposed to incandescent light for 12 hours

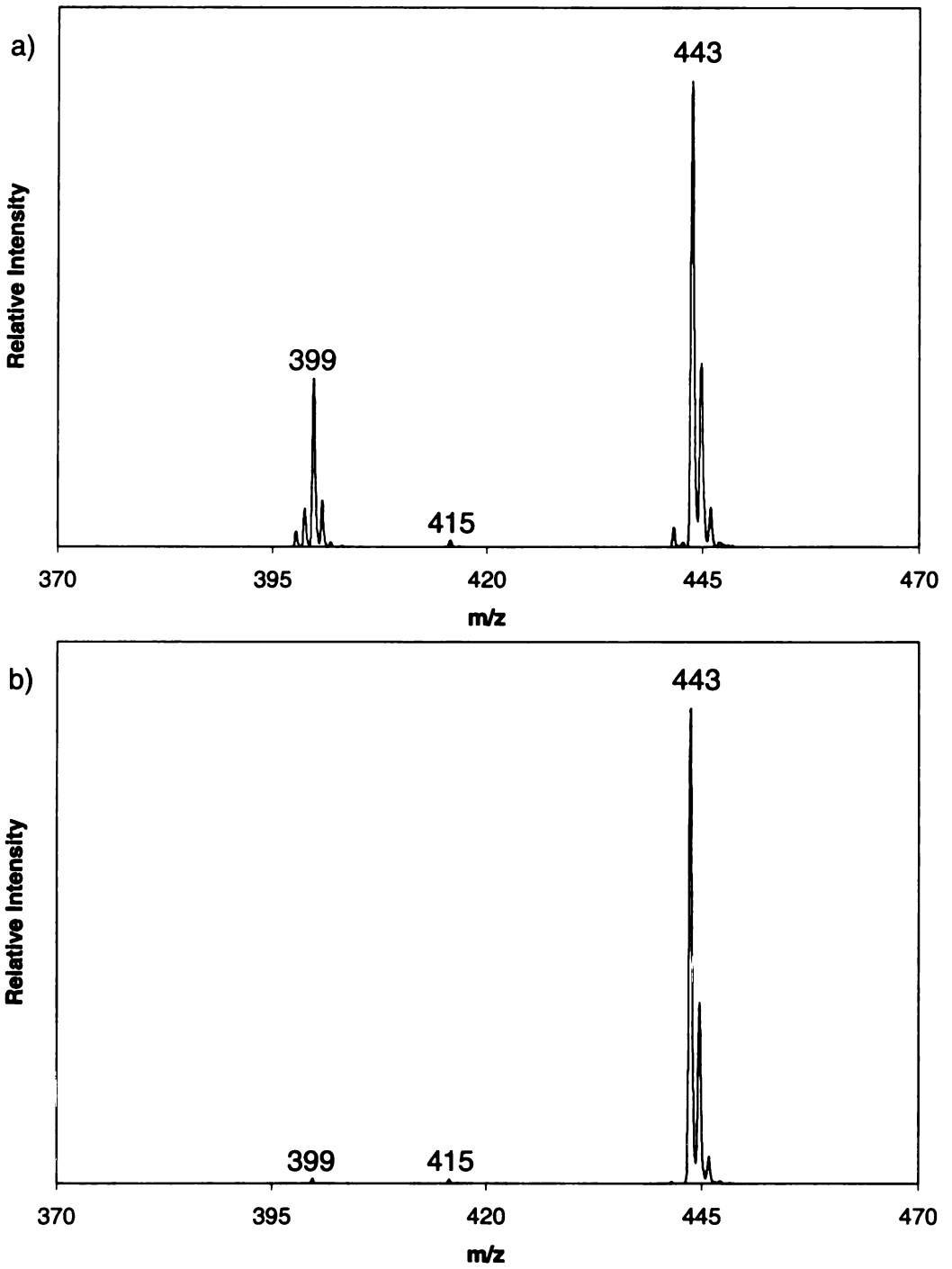


Figure 3.6: Positive-ion mass spectra of Rhodamine B analyzed by a) LDMS and b) MALDI MS

Rhodamine 6G

When Rhodamine 6G is directly analyzed by LDMS a peak at m/z 429 is barely, but frequently observed as shown in Figure 3.7a. The intensity of the peak is relatively small and is likely to be overlooked in the LD mass spectrum of the dye (ink). However, the intensity of the peak increases as the sample was degraded by incandescent light as shown in Figure 3.7b. The structure of Rhodamine 6G contains two methyl groups on the xanthene structure which dissociate from the dye upon photodegrading the sample. A previous study 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[1]. The analysis confirmed the loss of the methyl group from Rhodamine 6G gave rise to m/z 429 ion. The formation of the degradation product was proposed to be due to a bimolecular disproportionation reaction[1]. 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 contained both xanthene dyes, then the initial examination of the LD mass spectrum might suggest that Rhodamine B was the only dye contained in the ink. Without the induced demethylation of Rhodamine 6G there would be no way to differentiate the dyes by LDMS alone. The photodegradation of Rhodamine 6G results, preferentially, in the loss of two ethyl groups where Rhodamine B shows the loss of four ethyl groups consuming the mass spectrum. However, the peaks due to deethylation overlap, hence, N-deethyalation can not be used directly to differentiate between the two dyes as a mixture. Photodegradation with subsequent LDMS analysis 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. Spectra acquired from LDMS 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 allowed different photoproducts to be formed and detected.

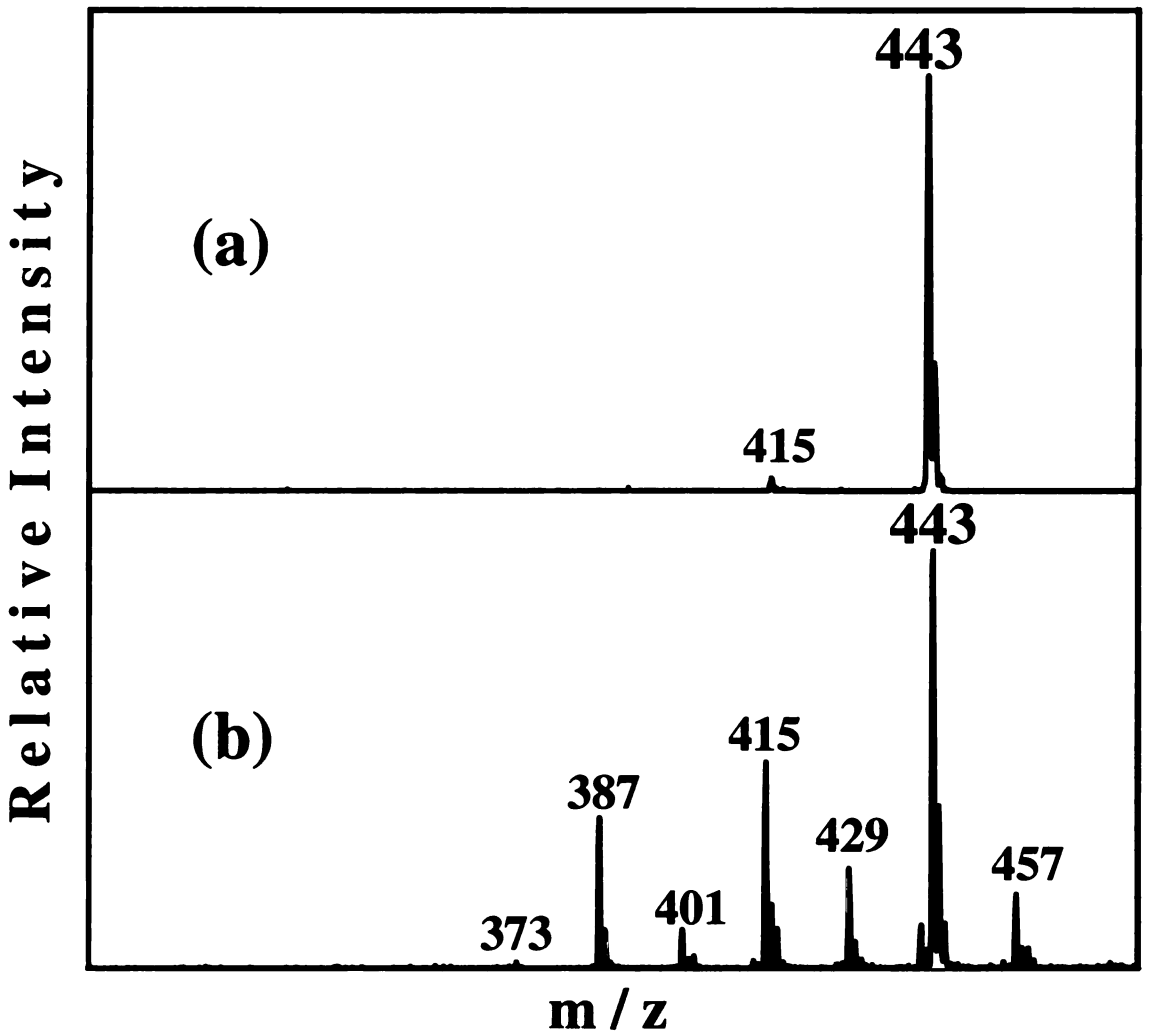


Figure 3.7: Positive-ion LD mass spectra of Rhodamine 6G (a) no exposure to incandescent light and (b) exposed to incandescent light for 12 hours

References

(1) Dunn JD, Siegel JA, Allison J. Photodegradation and Laser Desorption Mass Spectrometry for the Characterization of Dyes Used in Red Pen Inks. *J Forensic Sci.* **2003** (48) 652-657

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

(3) Osada T, inventors. Mitsubishi Pencil Kabushiki Kaisha, assignee. Aqueous Gel Ink-filled Ball Point Pen. US Patent 5,993,098 **Nov 1999**

(4) Green FJ. The Sigma-Aldrich Handbook of Stains, Dyes, and Indicators. Milwaukee: Aldrich Chemical Company, **1990**

Chapter Four: Detection and Characterization of Multiply-Charged Dyes from Pen Ink

We have recently learned that LDMS can not be used to detect several pen ink dyes. Frequently, dyes analyzed directly from ink on paper did not yield substantial peaks in the LDMS experiment. The assumption that we had encountered multiply-charged ink dyes was made certain in this research by MALDI MS. A method was developed for the detection and characterization of ink dyes that contain multiple charges, specifically, polysulfonated dyes as well as other dyes containing sodium carboxylates ($RCO_2^- Na^+$) and sodium alkoxides ($RO^- Na^+$). The method was the focus of this thesis research. The results and discussion presented in this chapter entails (1) the data used to determine the presence of multiply-charged dyes contained in pen inks, (2) the experiments performed to detect multiply-charged dyes, and (3) the molecular information acquired from MALDI and LD mass spectra for characterizing polyionic ink dyes.

Polyionic dyes containing sodium sulfonic acid, sodium carboxylate, sodium alkoxide and iminium chloride groups were detected in this research by MALDI MS. The detection of polysulfonated dyes by MALDI MS has been reported in the literature[1,2]. We have shown that MALDI MS can be used to detect multiply-charged dyes that contain functional groups, besides sodium sulfonic acid ($RSO_3^- Na^+$), that contribute to the overall charge. This research demonstrated that UV MALDI MS can be used to successfully desorb a variety of polyionic dyes, directly from a paper surface, as singly-charged ions, with the appropriate selection of MALDI matrix and additive. The experiment demonstrates the same sensitivity (pmolar amounts of analyte detected) and

the same simplicity as LDMS with the exception of additional sample preparation. The detection of multiply-charged ink dyes by MALDI MS requires only a minor modification, addition of a few microliters of a matrix/analyte solution to the ink-on-paper sample which is allowed to dry, then analyzed as in the LDMS experiments. While LDMS spectra do not show desorption of intact polyionic dyes, fragment ions indicate that multiply-charged dyes are present and allows the dye structures to be predicted.

Experimental

Mass Spectrometry

LDMS and MALDI MS 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. MALDI MS experiments utilized the same parameters except, in some cases, a delay time of 200 ns for optimum resolution was necessary when dyes or ink was analyzed from paper. A gel ink containing Copper Phthalocyanine was used as the calibrant for both positive and negative-ion modes.

Laser Desorption Mass Spectrometry

Single written lines on MSU letterhead paper were analyzed directly. The laser was focused on the ink line within the width of the pen stroke which is approximately 0.3-0.4 mm. Eighty-five pen inks were analyzed by LDMS, however, the results presented here focus on the analysis of three inks. The LD mass spectra of liquid, blue ink from a Pilot® Precise V7 and liquid, red ink from a Sanford® Uni-ball® Roller are shown. Results for liquid, blue ink from a Sanford® Uni-ball® Roller are discussed; however, no LD mass spectra are presented. These particular pens were chosen since the dyes in the inks were undetected by LDMS and were suspected to be polyionic.

In order to simulate the analysis of ink on paper, known dye solutions were prepared with a concentration of 10,000 pmol/μL in 1:1 (v/v) methanol:water, spotted (5 μL) on paper and allowed to dry prior to the LDMS analysis. Several polyionic dyes were purchased from either the Aldrich Chemical Company or the Sigma Chemical Company and are listed in Table 4.1.

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

A smooth surface, 100-well stainless steel (SS) Voyager™ MALDI plate was used for the analyses. Diammonium hydrogen citrate, DAHC, (J.T. Baker & Co., Phillipsburg, NJ) was used as an additive and 2-(4-hydroxyphenylazo)benzoic acid, HABA, (Aldrich Chemical Co., Milwaukee, WI) was used as the matrix for the MALDI MS experiments shown here. The concentrations of the reagents used were 100 mM DAHC in water, 24 mg/mL of HABA (6 mg in 2:2:1 (v/v/v) acetonitrile:water:methanol), and 100 pmol/μL dye solution in 1:1 (v/v) methanol:water. 1 μL aliquot of each solution was spotted in a well on the SS plate in the following order: HABA, DAHC, dye.

For the MALDI MS dye analyses on paper, 5 μL aliquot of 10,000 pmol/μL dye solution in 1:1 (v/v) methanol:water was spotted on MSU letterhead paper and allowed to dry. Squares approximately 2x2 mm^2 were cut from the paper and mounted on a modified MALDI plate using Scotch double coated tape (3M, St. Paul, MN). DAHC was applied first to the paper samples, followed by HABA. Two 1 μL aliquots of DAHC were spotted on the paper samples and each droplet was allowed to dry between aliquots. Three 2 μL aliquots of HABA were placed on top of the paper and each droplet was allowed to dry between aliquots.

For the MALDI MS analyses of ink samples on paper, a single pen stroke was made on MSU letterhead paper and squares approximately 2x2 mm^2 were cut from the paper and mounted on a disposable, gold Voyager™ MALDI plate using Scotch® double coated tape (3M, St. Paul, MN). DAHC was first applied to the paper samples, followed by HABA. Two 1 μL aliquots of DAHC were spotted on the paper samples and were allowed to dry between aliquots. Three 2 μL aliquots of HABA were placed on top of the paper samples and the each droplet was allowed to dry between aliquots. The ink calibrant on paper was mounted adjacent to the samples.

Thin-Layer Chromatography

TLC was carried out using K5F silica gel 150 Å TLC plates (Whatman, Ann Arbor, MI) with dimensions of 5 x 10 cm^2 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) ethyl acetate:ethanol:water. Methyl Violet 2B (Aldrich Chemical Co., Milwaukee, WI) contained Crystal Violet and tetrapararosaniline and a saturated solution was used as a comparison standard. To indirectly couple TLC with MS, individual bands were

scraped from the TLC plate, placed in a centrifuge vial, and 5-10 μL aliquot of 1:1 (v/v) ethanol:water was added to extract the dyes. The vials were vortexed and the silica was separated from the dye solutions by centrifugation.

Determining the Presence of Multiply-Charged Dyes in Pen Ink

When LDMS was applied to 85 pen inks (varied in manufacturer, type and color) the identification through the interpretation of LDMS spectra has been arduous due to the complexity of the mass spectra or the lack of information that can be acquired from the mass spectra. Several inks, available to the public, likely contain dyes that are multiply-charged. The assumption is based on LDMS data, TLC data, and patent information that were accumulated for this research. The presence of polyionic dyes in ink is discussed in the next three sections.

Laser Desorption Mass Spectrometry

The mass spectra in Figure 4.1a and 4.1b show the positive and negative-ion LD mass spectra, respectively, of a Pilot® Percise V7 rollerball pen that contains liquid, blue ink. The molecular information that can be acquired from the mass spectra for dye characterization is minimal. Peaks representing the detection of molecular ions are absent, hence, molecular mass and elemental information of the dye can not be deteremined. However, few peaks that are present correspond to fragment ions from the dye molecule. Most of the dyes (neutral or singly-charged dye molecules) that we have analyzed by LDMS have had m/z values between 300 and 1500. An LD mass spectrum is initially examined for dominant peaks that have m/z values in the range specified above. The majority of the peaks in both LD mass spectra (Figure 4.1) are present at low

mass. In general, the complex mass spectra that have been encountered in this research consist of peaks low in mass (<300 Da). This observation may suggest that an ink contains polyionic dyes. If a dye is tetra-anionic, $[Na]^{4+}$ $[Dye]^{4-}$ and there is no chemical mechanism through which the dye could be desorbed and ionized with a single charge, then the dye will not be detected intact. Dye fragmentation is a likely to occur and may be induced when analyzing a species that is multiply-charged. Each fragment ion formed gives rise to a peak in the mass spectrum. The formation of several fragment ions from one dye may complicate the mass spectrum. The amount of fragmentation varies depending on the species being analyzed. Typically, the mass spectra of multiply-charged dyed contain few peaks, if any. The absence of peaks in a mass spectrum also suggests that an ink contains polyionic dyes. If a multiply-charged dye is structurally stable upon D/I, then the resulting mass spectrum will contain negligible peaks. LDMS and other MS literature (except for electrospray ionization) have certainly shown that ionization mechanisms yield predominantly singly-charged ions. In order to elucidate the information that may be acquired from the LD mass spectra for multiply-charged dye characterization, a variety of commercial dyes containing multiple charges were obtained and analyzed directly by LDMS.

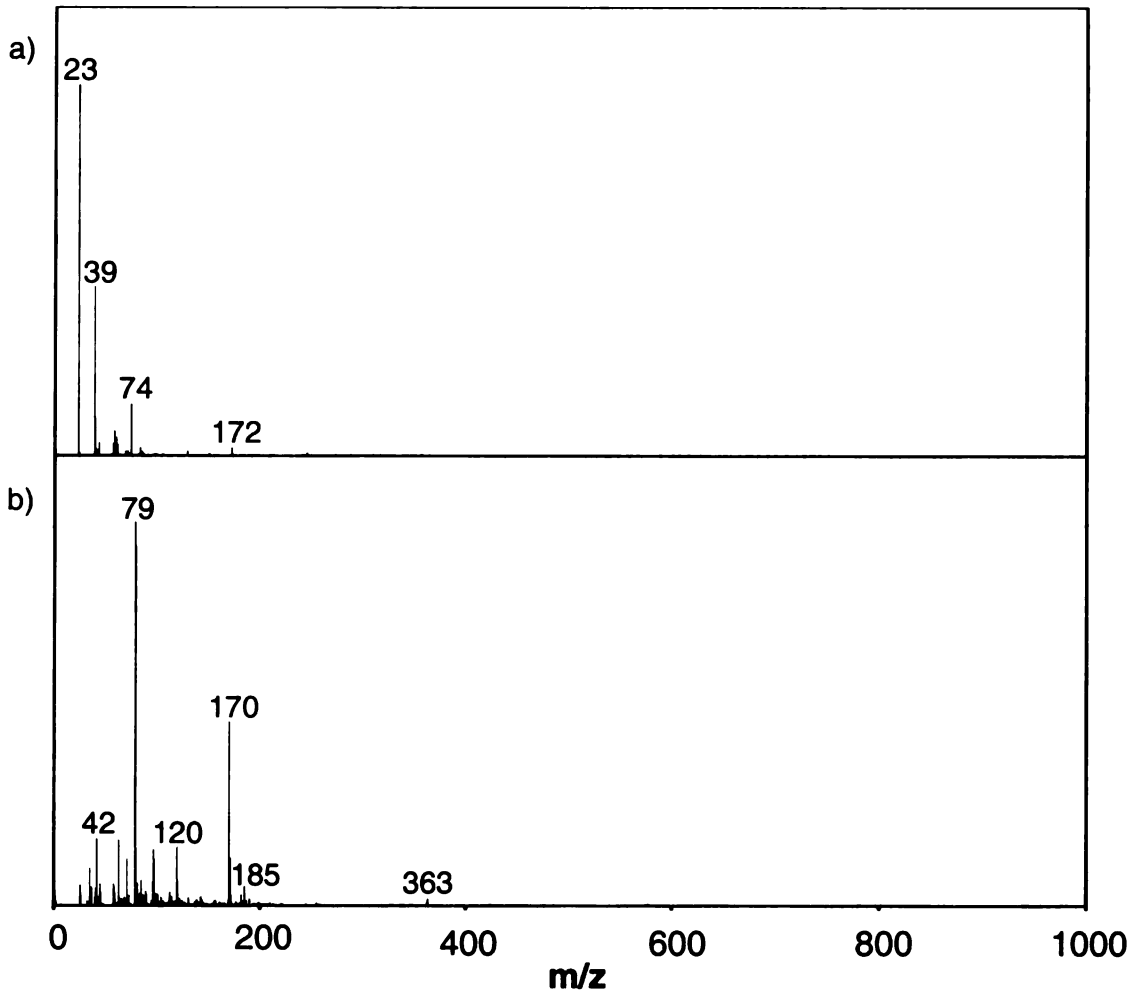


Figure 4.1: Direct LDMS analysis of liquid, blue ink from a Pilot® Precise V7 rollerball pen on paper a) positive-ion mass spectrum and b) negative-ion mass spectrum

Significant peaks due to fragmentation were not observed in the positive-ion LD mass spectrum of the blue rollerball ink. The most abundant peaks in the positive-ion LD mass spectrum (Figure 4.1a) have m/z value of 23 and 39 which correspond to sodium and potassium ions. These alkali metal ions are typical artifacts present in the mass spectra of ink. However, when the dyes are detected intact these peaks are not the most abundant in the mass spectrum. The negative-ion LD mass spectrum (Figure 4.1b) shows an intense peak at m/z value 79 which is frequently seen upon analyzing ink. Once again, the peak intensity of the low mass ion is minimal compared to that of the dye if detected. Currently, the peak has not been identified. Prompt fragmentation is useful information that enables the structure of the dye to be predicted. For example, polysulfonated dyes are known to readily loose sulfonic acid when analyzed by LDMS. The fragment ion that results gives rise to a peak in the negative-ion mass spectrum at m/z 80 which is indicative of a $SO_3^{\cdot -}$. The presence of this peak is an indication that an undetected dye by LDMS contains multiple sulfonic acid groups. Further discussion about the fragmentation of polysulfonated dyes can be found in Chapter Two. Other fragment ions can be used to determine the presence of multiply-charged dyes. For instance, the peak at m/z 170 in the negative-ion LD mass spectrum (Figure 4.1b) appears to correspond to a fragment ion, but may be overlooked since the intensity of the peak is relatively small. An association has been proposed for the fragment ion and is discussed further detail when results are presented for the dye structure. Since fragment ions denote structural pieces of the intact species they aid in dye identification. The LD mass spectra of the liquid, blue ink demonstrates that direct LDMS can not be used to acquire molecular

information for multiply-charged dyes, although, low mass peaks are helpful for determining that a polyionic dye in the ink contains specific functional groups.

In order to make certain that LDMS is incapable of detecting multiply-charge dyes intact, several commercial dyes were purchased and analyzed directly. Dye solutions were prepared with a concentration of 10,000 pmol/μL and 100 pmol/μL in 1:1 (v/v) methanol:water. The dyes were analyzed both on paper and the SS plate directly using LDMS in the positive and negative-ion modes. Analyses were performed with Direct Red 23, Acid Blue 93, Direct Red 80, Acid Yellow 23, Solvent Blue 38, Acid Black 1, and Reactive Black 5 whose structures are shown in Figure 4.2 (structures are illustrated without their counterion, Na^+). The structural characteristic shared among these dyes is the presence of multiple sulfonic acid groups. Each sulfonic acid group contributes -1 to the overall charge of the dye molecule. The number of charges varies among the dyes. The dyes contain other functional groups such has hydroxyl groups that may influence the overall charge state and detection in LDMS. Solvent Blue 38 was the only multiply-charged dye out of the set that was detected intact by LDMS and some fragmentation was observed. The direct analysis of the dye from paper and metal plate were similar. The LD mass spectra of Solvent Blue 38 on the different substrates contained peaks with the same m/z values with similar distributions of relative intensities. Solvent Blue 38 is a Copper Phthalocyanine derivative which differentiates the dye from the other structures. The other dyes were not detected intact on paper, however, Acid Blue 93 (100 pmol/μL concentration only) was detected from the metal substrate. The difference between Acid Blue 93 and the other dyes is that the structure contains a positive charge due to the presence of an iminium group.

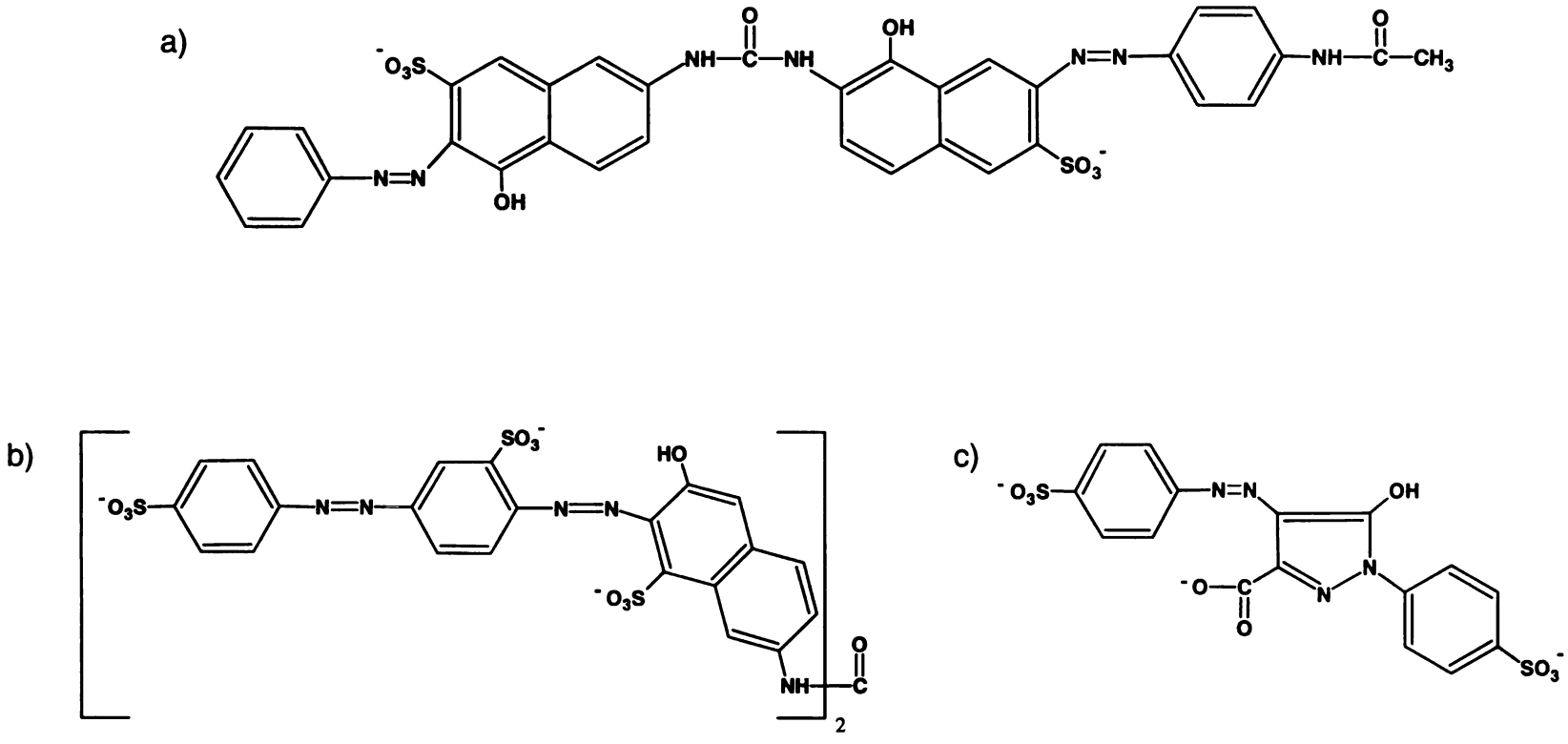


Figure 4.2: Structure of multiply-charged ink dyes a) Direct Red 23, b) Direct Red 80, c) Acid Yellow 23, d) Solvent Blue 38, e) Acid Blue 93, f) Acid Black 1, and g) Reactive Black 5 (continued on page 98)

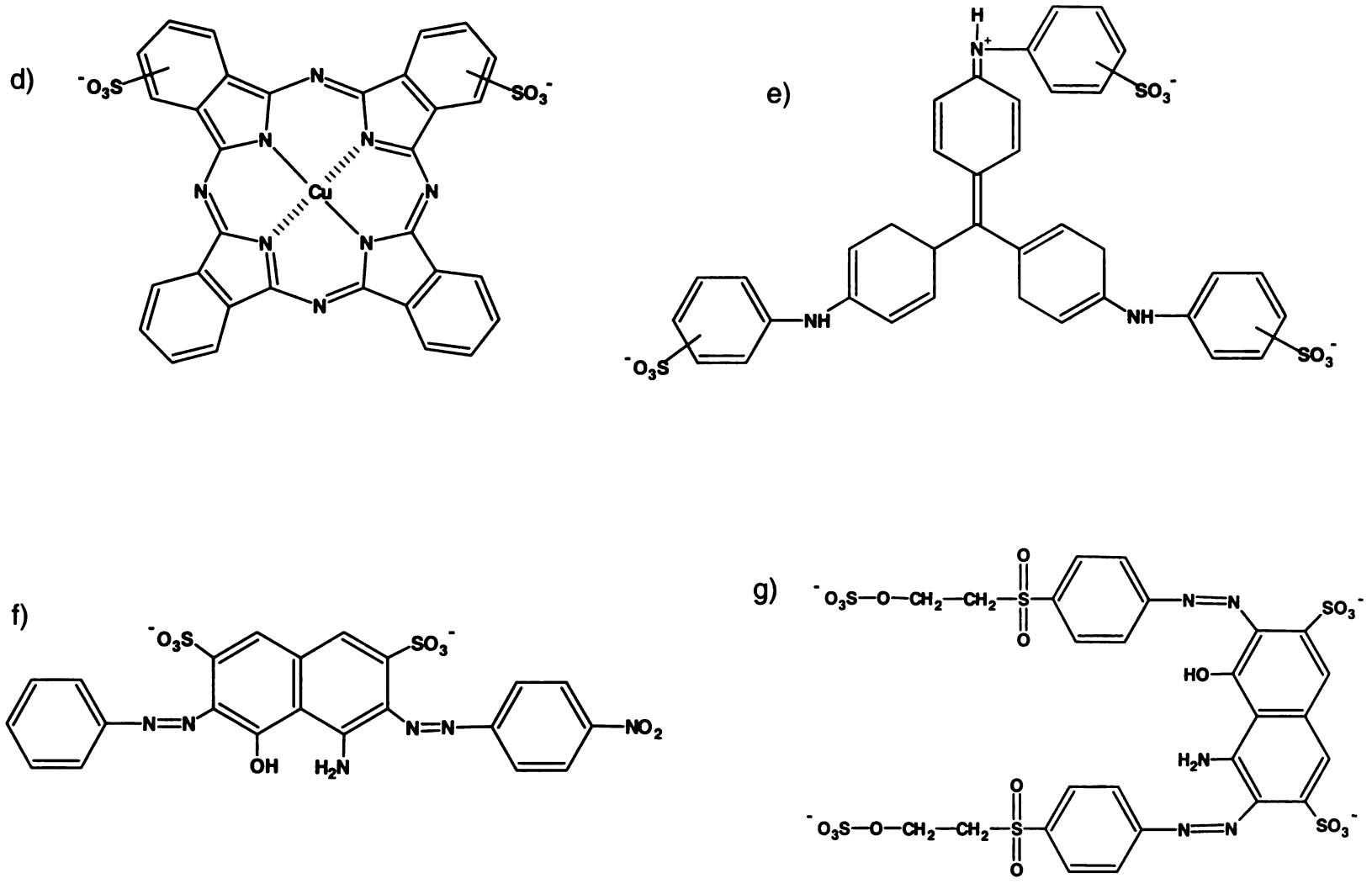


Figure 4.2: Structure of multiply-charged ink dyes a) Direct Red 23, b) Direct Red 80, c) Acid Yellow 23, d) Solvent Blue 38, e) Acid Blue 93, f) Acid Black 1, and g) Reactive Black 5 (continued from page 97)

Thin-Layer Chromatography

TLC data collected has also provided insight into the charge state of the dyes used in inks. Generally, the number and color of the dyes present in the ink are determined by TLC. When an ink is analyzed by TLC each colored band on the chromatogram represents one dye. Each relatively intense peak in the LD mass spectrum represents a single dye as well. The chromatogram and mass spectra, positive and negative-ion, are compared to account for the number of dyes that are contained in an ink. LDMS is incapable of detecting polyionic dyes and if the number of dyes detected in TLC and LDMS are not the same, then the ink most likely contains multiply-charged dyes. Dyes were extracted from the TLC bands and analyzed directly by LDMS. The absence of peaks in the LD mass spectra of the extractions concluded that multiply-charged dyes are present in the inks. TLC data supported the assumption that the liquid, blue rollerball ink contained multiply-charged dyes. The TLC analysis suggested that the ink contained two dyes and the observed color of the bands were blue and purple which were not detected by LDMS. TLC data is acquired and compared to LD mass spectra to account for the presence of polyionic dyes. A discrepancy between the number of TLC bands and the number of peaks observed in the mass spectra signifies that multiply-charged dyes are contained in an ink.

Ink Patents

A review of the patent literature resulted in the identifying more than 250 dyes used for manufacturing pen inks. The dyes used in ballpoint, gel, and liquid ink have been catalogued for this research. U.S. Patent 5,993,098 demonstrates the various dyes that can be used in the manufacture of gel ink[3]. Many of the dyes share a common

feature of containing multiply-charged groups. Thus, the dyes are polyionic. Most of the dyes encountered in the patents are polysulfonated azo dyes, and can be used in various classes of pen ink such as ballpoint, gel, and liquid inks. This finding supported the TLC and LDMS data that multiply-charged dyes are used in the manufacture of inks. The use of polyionic dyes was unexpected, and introduces several complications to the LDMS analysis. Fortunately, structures for the majority of the cataloged dyes have been found, so m/z values have been predicted. The dye catalogue served as a reference for interpreting the peaks present in the LD mass spectra.

Multiply-Charged Ink Dye Detection

The following sections provide the procedure implemented for detecting multiply-charged dyes by MALDI MS. A matrix and additive are both required for enhanced detection of the dye which will be demonstrated. The method was applied to inks that contained dyes that were not detected by LDMS and the analyses were performed from paper. The focus of this research was to find a general MS method that can be applied to the detection of multiply-charged ink dyes directly from paper. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) has been used previously for the detection of polysulfonated azo dyes[1], and we have found that using matrix-assisted laser desorption mass spectrometry (MALDI MS) can be very beneficial when trying to detect intact multiply-charged dyes as previously discussed. We have adapted a method used by Sullivan, *et al.*[1] for the analysis of polyionic dyes on a paper substrate. The MALDI matrix, 2-(4-hydroxyphenylazo)benzoic acid (HABA), is well suited for the analysis of

multiply-charged dyes from ink on paper and diammonium hydrogen citrate (DAHC) is used to further enhance dye detection.

Cation-Exchange Additives and Matrices

Sources were considered for eliminating or reducing the presence of sodium ions which inhibit the desorption/ionization of the multiply-charged dye molecules. DAHC, other amine bases, and cation exchange resins have been used by mass spectrometrists to detect and enhance the mass spectra of multiply charged species[1,4-20]. The detection of multiply-charged dyes first began by using cation-exchange resins. Two types of resinous beads (Bio-Rad AG 50W) were used, one type contained exchangeable protons and the other contained exchangeable ammonium ions. The beads as a solution were mixed with a dilute, aqueous dye solution. The slurry was filtered through a column and the filtrate was collected and subsequently analyzed by MALDI MS. The mass spectra of the analyzed dyes, Direct Red 23 and Acid Blue 93, however, showed only a slight increase in sensitivity from using cation-exchange resins. Resins work by displacing the sodium ions from the dye salt. The resins appeared to be ineffective for the removal of the sodium ions. The order of cation relative selectivity for AG 50W-X8 resin is $NH_4^+ > Na^+ > H^+$, Na^+ should be displaced from the analyte by H^+ since Na^+ has a higher affinity for the resin than the dyes. The poor results attained with the resins lead to examining additives that have been used to isolate the alkali ions from the analyte. When the matrix, α-CHCA (contained 0.1% TFA), was introduced to the sample the sensitivity increased significantly for the dye samples exposed to the resinous bead that contained NH_4^+ exchange resin. 2,5-DHB was also used as a matrix, but the detection of the dyes was not enhanced. However, there was concern that α-CHCA (with 0.1% TFA) alone was

involved in the detection of the dyes and had nothing to do with the cation-exchange resin. At this point, the LDMS experiment turned into an MALDI MS experiment by the introduction of a matrix to the sample.

In the MALDI experiment, picomole amounts of analyte are mixed with an excess of organic matrix molecules which facilitate the absorption of the UV laser light at 337 nm (the wavelength of light emitted from a pulsed N_2 laser). A few microliters of an analyte/matrix solution are deposited on a metal sample plate. Upon rapid solvent evaporation, crystals are formed containing the analyte at a low concentration. When the laser is fired the matrix absorbs the UV radiation assisting in the desorption/ionization of the analyte. Both matrix and analyte ions are detected.

Various MALDI matrices were considered to enhance the detection of the multiply-charged commercial dyes. Matrices that were evaluated included alpha-cyano-4-hydroxycinnamic acid (α-CHCA), 2-5-dihydroxybenzoic acid (2,5-DHB), 3-hydroxypicolinic acid (3-HPA), sinapic acid (SA), 5-methoxysalicylic acid (MSA), and 6-aza-2-thiothymine (ATT). The concentration of the dye solutions was varied to achieve the best sensitivity. Dye concentrations of 5, 10, 100, and 10,000 pmol/μL were examined. Initial results indicated that analyzing10,000 pmol/μL dye solution with α-CHCA on the SS plate provided the best positive and negative-ion MALDI mass spectra., however, peaks in the mass spectra were poorly resolved. Additives or co-matrices have been known to enhance (peak resolution and sensitivity both increased) analyte detection in MALDI MS.

In order to increase resolution, additives were examined with the matrices previously examined. Additives or co-matrices have been used previously and are known

to enhance the performance of the MALDI experiment[4-20]. Enhancements include increased analyte sensitivity, improved peak resolution (increased mass accuracy), reduced analyte fragmentation, and increased sample homogeneity. 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, polyanionic 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. In this research, the additives that were examined included ammonium acetate (AA), diammonium hydrogen citrate (DAHC), and trifluoroacetic acid (TFA).

The common procedure for preparing a solution α-CHCA requires a final solution concentration of 0.1% TFA to increase sample solubility[21]. An increase in sensitivity and resolution was observed in the mass spectra of Direct Red 23 and Acid Blue 93 when TFA was used with α-CHCA. When the concentration of the dye solutions were reduced from 10,000 pmol/μL to 100 pmol/μL the sensitivity increased once again. AA and DAHC have been used previously as additives for reducing the charge state of the phosphate groups on phosphorylated peptides by displacing alkali cations[4]. When an aqueous solution of 1 mM DAHC was used with α-CHCA instead of TFA peaks due to sodium adduction were reduced or eliminated providing a less complicated mass spectrum, hence, chemical noise was reduced. A disadvantage to using α-CHCA is that the matrix yields several peaks in the mass spectrum which may interfere with the detection of the dyes. Matrices are typically used with high molecular weight biomolecules, so the peaks that correspond to the matrix are not a concern in those experiments. However, dyes have lower molecular weights, typically, so a matrix that

yields substantially less peaks was considered for these MS experiments. The MALDI matrix 2-(4-hydroxyphenylazo)-benzoic acid (HABA) with DAHC is well suited for this application and was used as a general method for detecting multiply-charged dyes in this research. The matrix alone does not sufficiently enhance dye detection.

Commercial Dyes

In this research, over 30 multiply-charged dyes (100 pmol/μL was used for all dye concentrations) including the dyes (Direct Red 23, Acid Blue 93, Direct Red 80, Acid Yellow 23, Solvent Blue 38, Acid Black 1, and Reactive Black 5) that were initially examined by direct LDMS were further analyzed by using HABA (24 mg/mL in 2:2:1 ACN:H2O:MeOH) and DAHC (100 mM) from the SS plate. The dyes undetected by LDMS were detected once the matrix and additive were applied, except for Reactive Black 5. However, a negative fragment ion was detected at m/z 742 and corresponds to the loss of two sulfonic acid groups from the dye. The intact dye was expected to give rise to a negative ion with an m/z 902. This peak and the peak observed in the MALDI mass spectrum have a mass difference of 160 amu. Most likely during the D/I process, the dye lost two sulfonic acid groups. The structure of Reactive Black 5 contains two functional groups ($-O_2SCH_2CH_2OSO_3^-\ Na^+$) that the other dyes to not have which may prevent the intact desorption of the dye. Listed in Table 4.1 are the multiply-charged dyes examined in this research by using HABA and DAHC. The m/z values for each dye detected experimentally are listed as well as functional groups that may contribute to the overall charge on the dye molecule in the table. Functional groups that appear to influence the detection of the dye in LDMS include sodium sulfonic acid, sodium alkoxide, and sodium carboxylate. However, the pH of the ink may not necessarily

influence the desorption/ionization process. Hydroxyl goups may also contribute to the charge and most likely depends on the pH of the solution. Some MALDI matrices, such as 2,5-DHB, have multiple hydroxyl groups which do not prohibit detection in LD. Additional studies should be carried out to understand the role of hydroxyl groups on the detection of dyes in LDMS as well as MALDI MS. Although, we have successfully detected several polyionic dyes from the metal plate, the ultimate challenge was to detect the dyes in ink from paper.

CI Name	(+) m/z	(-) m/z	SO_3Na	CO_2Na	ONa	OH	Iminium
Acid Black 1		571	2	0	0	1	0
Acid Black 24	688	686	2	0	0	0	0
Acid Black 26	668	666	2	0	0	1	0
Acid Blue 1	545	543	2	0	0	0	1
Acid Blue 7	669	667	2	0	0	0	1
Acid Blue 9	749	747	3	0	0	0	1
Acid Blue 90	832	830	2	0	0	0	1
Acid Blue 93	756	754	3	0	0	0	1
Acid Green 3	669	667	2	0	0	0	1
Acid Green 16	595	593	2	0	0	0	1
Acid Green 25	579	577	2	0	0	0	0
Acid Red 14		457	2	0	0	1	0
Acid Red 18		537	3	0	0	1	0
Acid Red 37		479	2	0	0	1	0
Acid Red 52	559	557	2	0	0	0	1
Acid Red 87	649	647	0	0	2	0	0
Acid Red 92		785	0	1	1	0	0
Acid Violet 49	712	710	2	0	0	0	1
Acid Yellow 1		313	1	0	0	1	0
Acid Yellow 17		505	2	0	0	1	0
Acid Yellow 42		713	2	0	0	2	0
Direct Black 19	796	794	2	0	0	1	0
Direct Black 22		1016	3	0	0	2	0
Direct Black 38	738	736	2	0	0	1	0
Direct Black 51		556	1	1	0	3	0
Direct Blue 71	942	940	4	0	0	1	0
Direct Blue 106	697	695	2	0	0	0	0
Direct Red 1		582	1	1	0	2	0
Direct Red 4		841	3	0	0	1	0
Direct Red 23		768	2	0	0	2	0
Direct Red 28	653	651	2	0	0	0	0
Direct Red 75		901	4	0	0	2	0
Direct Red 80		1239	6	0	0	2	0
Direct Violet 51	676	674	2	0	0	1	0
Direct Yellow 4		579	2	0	0	2	0
Food Yellow 3	409	407	2	0	0	1	0
Reactive Black 5		742	4	0	0	1	0

Table 4.1: Polyionic dyes examined by MALDI MS (HABA and DAHC) on SS plate. Provides m/z values detected in the positive and/or negative-ion mode and functional groups that may contribute to the overall charge on the dye molecule

Highly-Charged Dyes

An important aspect of the technique of using HABA and DAHC that should be noted is that the method does not appear to be limited based on the number of charges that a dye contains. Most of the colorants that can be used in ink are polysulfonated azo dyes. In this research, over 30 polyionic dyes have been detected successfully directly from paper using HABA and DAHC. The dyes analyzed by the MALDI method were selected from the ink patents and they vary in size or molecular weight and overall charge, but all contain multiple sulfonic acid groups. Dyes containing 2 to 6 sulfonic acid groups have been detected by MALDI MS. We are most certain that dyes containing more than 6 sulfonic acid groups can be detected as well. Investigating dyes that have more than six sulfonic acid groups would be intriguing. As the number of sulfate groups increase the task of detecting the dye becomes more challenging in ESI[22,23] and would be interesting to see if the same phenomena occurs by MALDI mass spectrometry. Mechref *et al.* noticed this phenomenon when trying to detect oligosaccharides[5]. Preliminary results suggested that multiple sulfonic acid groups influence dye detection in MALDI. One particular dye, Direct Red 80, shown in Figure 4.2b, contains six sulfonic acid groups, each contributing -1 to the overall charge on the dye molecule. When the dye was analyzed by MALDI MS the dye was detected at m/z 1239 (Figure 4.3), but the sensitivity was low compared to most of the other dyes that contained fewer multiple sulfonic acid groups. The reduction in sensitivity may be associated with the number of sulfonic acid groups present. However, additional dyes need to be studied to be able to understand the effect the number of charges has on the sensitivity of the experiment. Maintaining isotopic resolution during the analysis of

Direct Red 80 was more difficult than for the other dyes, however, by attenuating the MALDI TOF parameters isotopic resolution can be obtained.

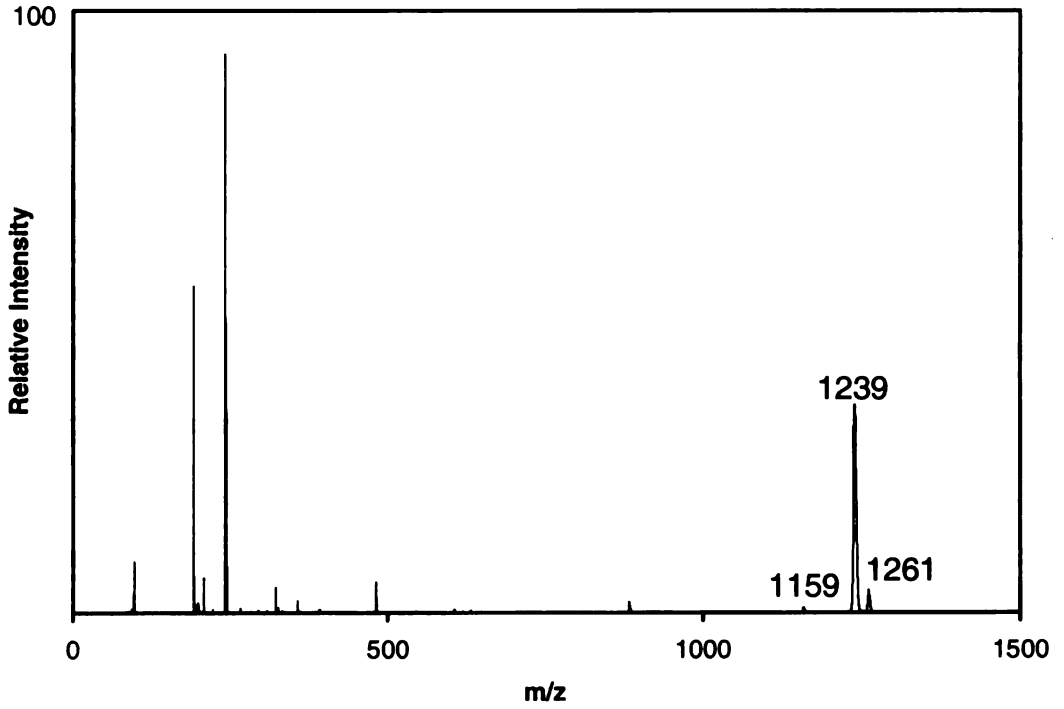


Figure 4.3: Negative-ion MALDI MS analysis of Direct Red 80 (HABA and DAHC) on SS plate

Ink on Paper

We focused on using the matrices α-CHCA and HABA with DAHC for analyzing dyes directly from paper since the two combinations yielded the best results for analyses from the SS plate. Growing matrix crystals of α-CHCA on paper was difficult and dye detection was insufficient. Fortunately, we had great success with HABA. The procedure that was used involved, first, adding a droplet (1 μL) of DAHC to the dye sample on paper, followed by adding increments of the matrix until the appearance of orange crystals were visible on the surface of the dye-paper sample. Typically, three 1

μL aliquots of HABA were used. The soluble dyes are extracted into the aqueous DAHC droplet. The matrix was not added until the sample was dried. Although, the ink sample frequently spreads outside the width of a written line when the additive and matrix solutions are added to the paper, there are no peaks in the positive and negative-ion MALDI mass spectra that arise due to the paper. Paper contains components that could be detected, however, interference from the paper is not a problem since the content of the dye in the ink outweigh the paper components. DAHC and HABA were also examined separately for the detection of dyes on paper and found the reagents must be used simultaneously for the dyes to be sufficiently detected. First, several commercial dyes were examined and then the method was applied to inks. When LDMS failed to detect dyes in an ink, the sample was subjected to analysis by MALDI MS using the method described above.

Dye detection using a matrix and additive yields molecular information, such as the molecular mass of the dye, ultimately, leading to the identification of the dye. The importance of using a matrix and an additive in conjunction for adequate detection will be demonstrated through the examination of the liquid, blue ink from the Pilot® Precise V7 rollerball pen that was previously examined by direct LDMS from paper. Figures 4.4 and 4.5 show the positive and negative-ion LD mass spectra, respectively, of the blue ink using HABA (a), DAHC (b), and HABA and DAHC (c).

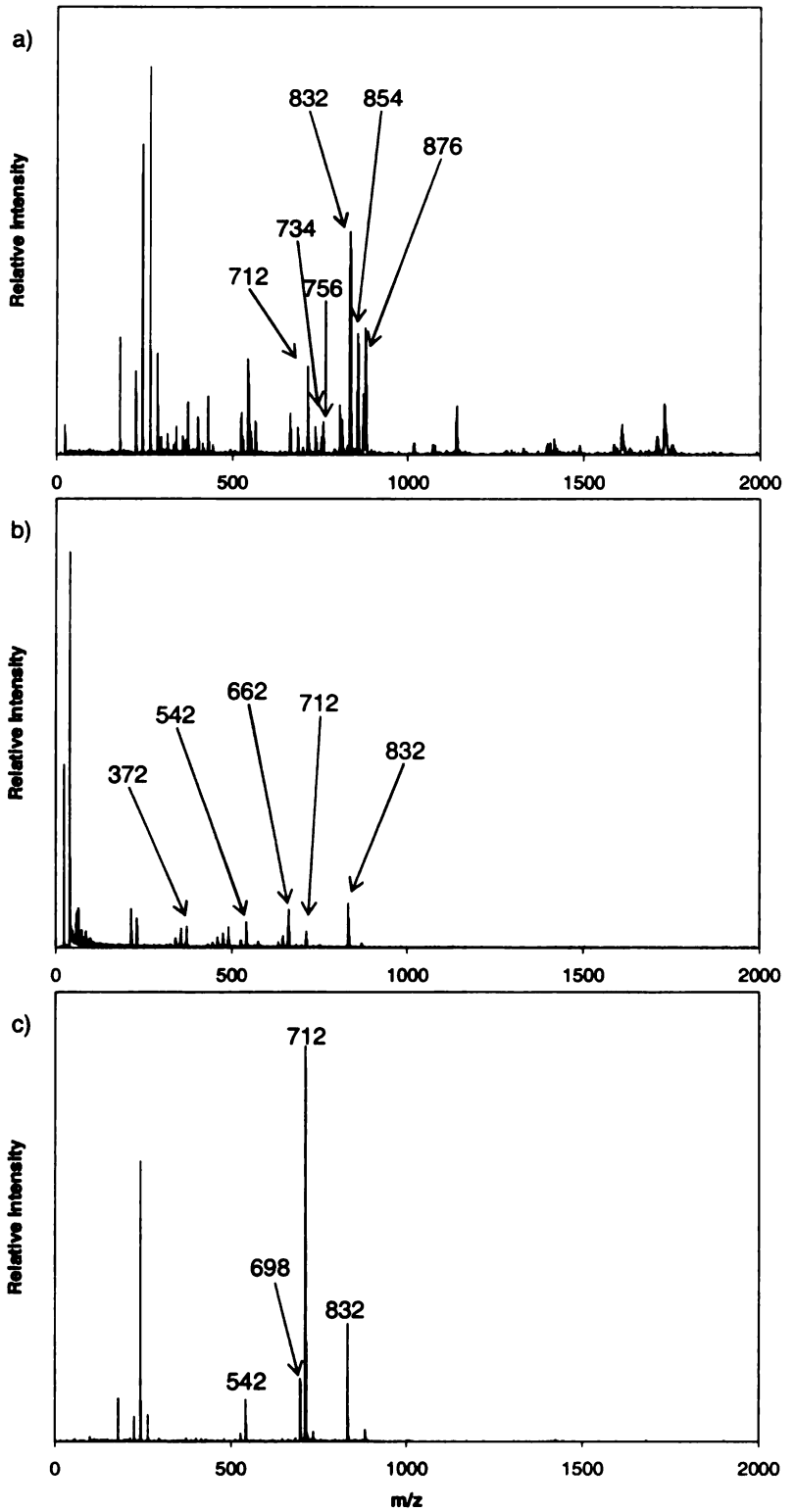


Figure 4.4: Positive-ion LD mass spectra of blue, liquid ink from Pilot® Precise V7 rollerball pen on paper a) HABA, b) DAHC, and c) HABA and DAHC

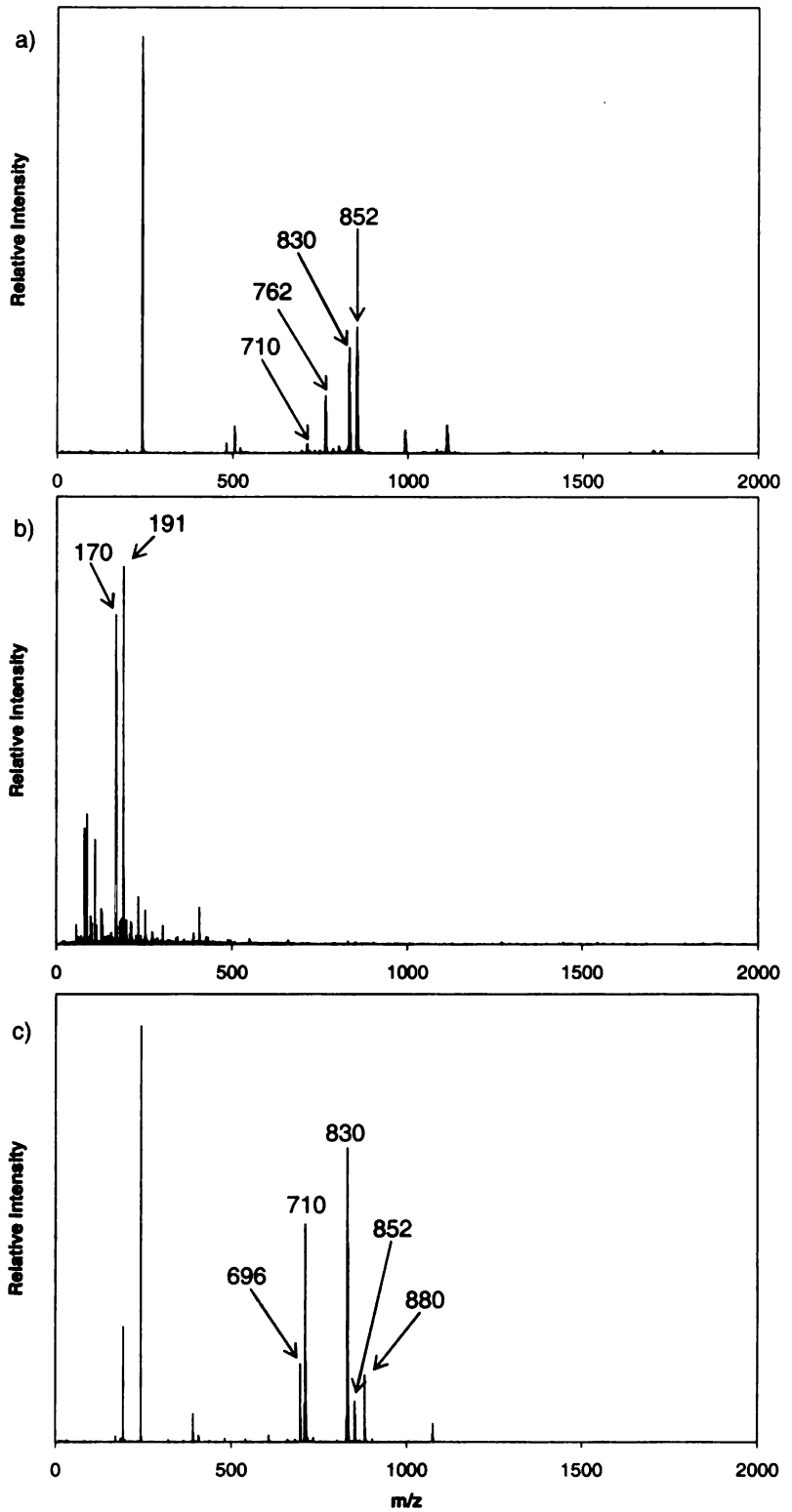


Figure 4.5: Negative-ion LD mass spectra of blue, liquid ink from a Pilot® Precise V7 rollerball pen on paper a) HABA, b) DAHC, and c) HABA and DAHC

The multiply-charged dyes contained in the liquid, blue ink were identified and will be discussed later, however, the m/z values of the dyes are significant for this discussion. In order to understand the importance of using the matrix and additive simultaneously, the m/z value of the intact dyes will be given. Two multiply-charged dyes are contained in the ink. The molecular ions of the first dye have m/z values 712 and 710 in the positive and negative-ion modes, respectively. The positive and negative molecular ions of the second dye are denoted by m/z 832 and 830, respectively. Using HABA alone did detect both dyes, however, the positive-ion mass spectrum contains several peaks, most of which represent sodium adducts (m/z 734, 756, 854, and 876). The presence of these peaks makes identifying the dyes unlikely. When this mass spectrum is compared to the mass spectrum of the experiment that utilized both reagents the peaks due to adducts are absent and peaks that represent the intact dyes are easily identified at m/z 712 and 832. Also, the resolution of the peaks greatly increases when the combination of matrix and additive compared to the using them alone. Isotopic resolution was not observed in the positive and negative-ion mass spectra of the inks analyzed with HABA alone. Accurately, determining the mass of the molecular ion is difficult with poor peak resolution. Although, the use of DAHC alone yielded isotopic resolution, the dyes were not able to be detected in the negative-ion mode. The use of both reagents generated mass spectra with isotopically resolved peaks, enhanced sensitivity, and reduced chemical noise. Additional peaks (m/z 698 and 542 in the positive-ion and m/z 696 in the negative-ion) in the MALDI (with DAHC) mass spectrum are associated with the dyes.

Figure 4.6 demonstrates how using a matrix and additive for the detection of a polyionic dye, Acid Violet 49, directly from the SS plate. Acid Violet 49, shown in Figure 4.7, contains two sulfonic acid groups and one iminium group and has a molecular weight of 734 Da. The loss of the sodium results in the detection of the dye and can be seen in the positive and negative-ion MALDI mass spectra. The replacement of the sodium ion for a proton (protons come from the ammonium ions from DAHC) enables the dye to be detected as shown in Figure 4.6a, the positive-ion MALDI mass spectrum. The D/I of the dye in positive ion mode has a m/z value of 712. The loss of the sodium with no substitution occurs as well and the dye can be detected in the negative-ion mass spectrum shown in Figure 4.6b. The nature of the charge state allows the dye to be detected in both ion modes.

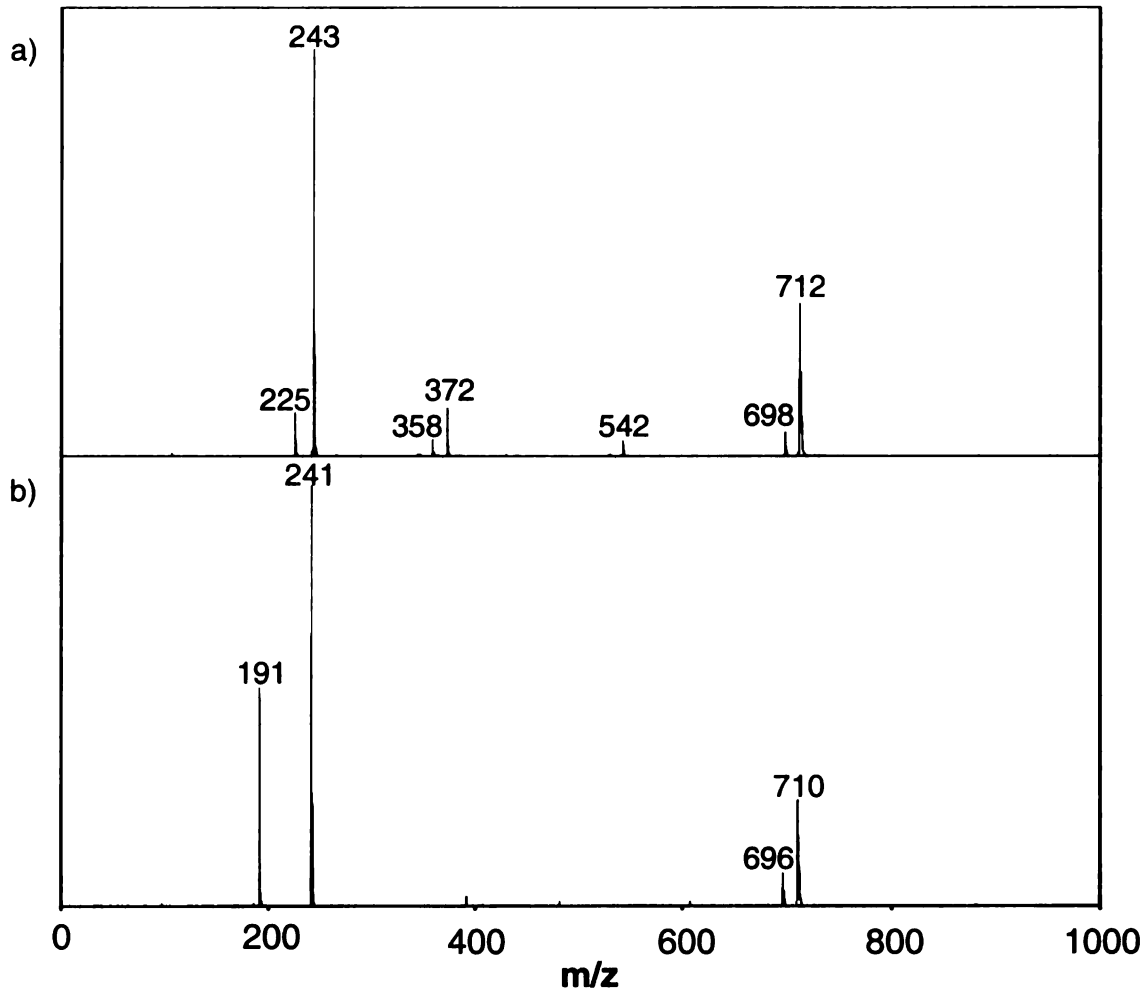


Figure 4.6: MALDI MS analysis of Acid Violet 49 on SS plate a) positive-ion mass spectrum and b) negative-ion mass spectrum

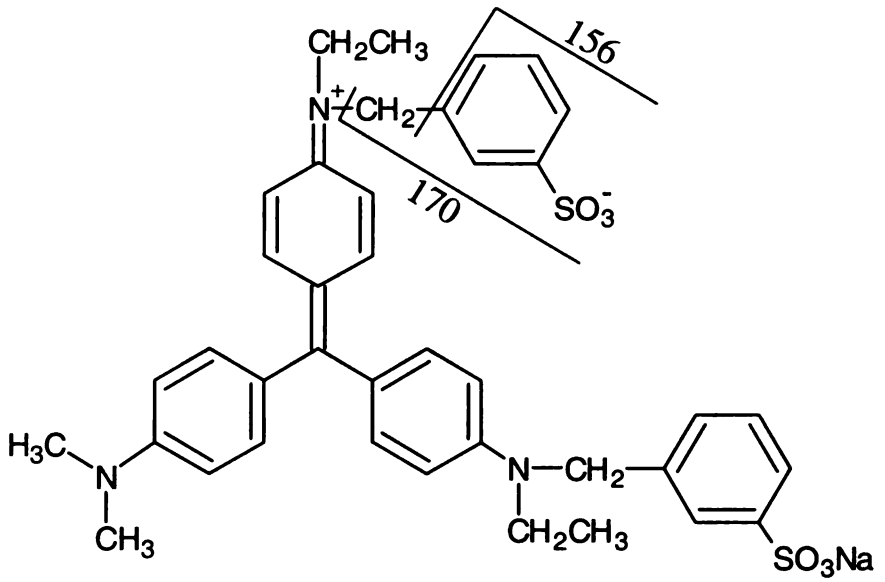


Figure 4.7: Dye structure of Acid Violet 49

As a side note, the peaks with m/z value 243 and 241 in the positive (Figure 4.6a) and negative (Figure 4.6b) ion mass spectra, respectively, correspond to the HABA matrix. HABA is a solid, neutral, organic molecule and has a molecular weight of 242. The matrix molecules are protonated (gains a hydrogen ion) or deprotonated (looses a hydrogen ion) during the desorption/ionization process and is observed in the positive and negative-ion mass spectra, respectively. As previously stated, the matrix is in excess compared to the analyte in the MALDI MS experiment, so the peaks that correspond to the matrix are more abundant than the analyte.

Detecting dyes in a written line directly from a paper substrate is important in the field of question document examination, however, paper is not an ideal sample for the MALDI experiment. MALDI requires the incorporation of the analyte into the matrix crystals. Growing crystals on paper in order to detect the dyes has been a challenge. We have found that HABA with DAHC works well for detecting multiply-charged dyes from

paper. Figure 4.8, demonstrates the success of using HABA and DAHC for the detection of Acid Violet 49 on paper. The mass spectra of the dye on paper and SS are similar with the exception that the peaks that pertain to the dye are more intense in the second set of spectra. Commercial dyes are frequently sold as impure mixtures[24] and this is such the case with Methyl Violet 2B purchased from the the Aldrich Chemical Company as well as Acid Violet 49. The mass spectra of Acid Violet 49 contain additional peaks at m/z value 542 and 372 that correspond to dyes that are similar in structure to Acid Violet 49. These dyes were probably a result of the incomplete synthesis of Acid Violet 49. Actually, the peak at m/z value 372 corresponds to Crystal Violet and the peak at m/z value 542 represents a compound that has a molecular formula $C_{32}H_{35}N_3O_3S$ and is similar to the dye structure in Figure 4.7. The alkyl groups bonded to the nitrogen could be either methyl or ethyl groups, and light, ultraviolet[25] or incandescent[26], induced dye degradation (oxidative dealkylation) can be used to determine which functional groups are present. The three sets of peaks that are present in Figure 4.8a. The subset of peaks are separated by 14 mass units and have been associated with oxidative demethylation[25]. These peaks are characteristics of the dye and these clues can be useful when identifying an unknown dye.

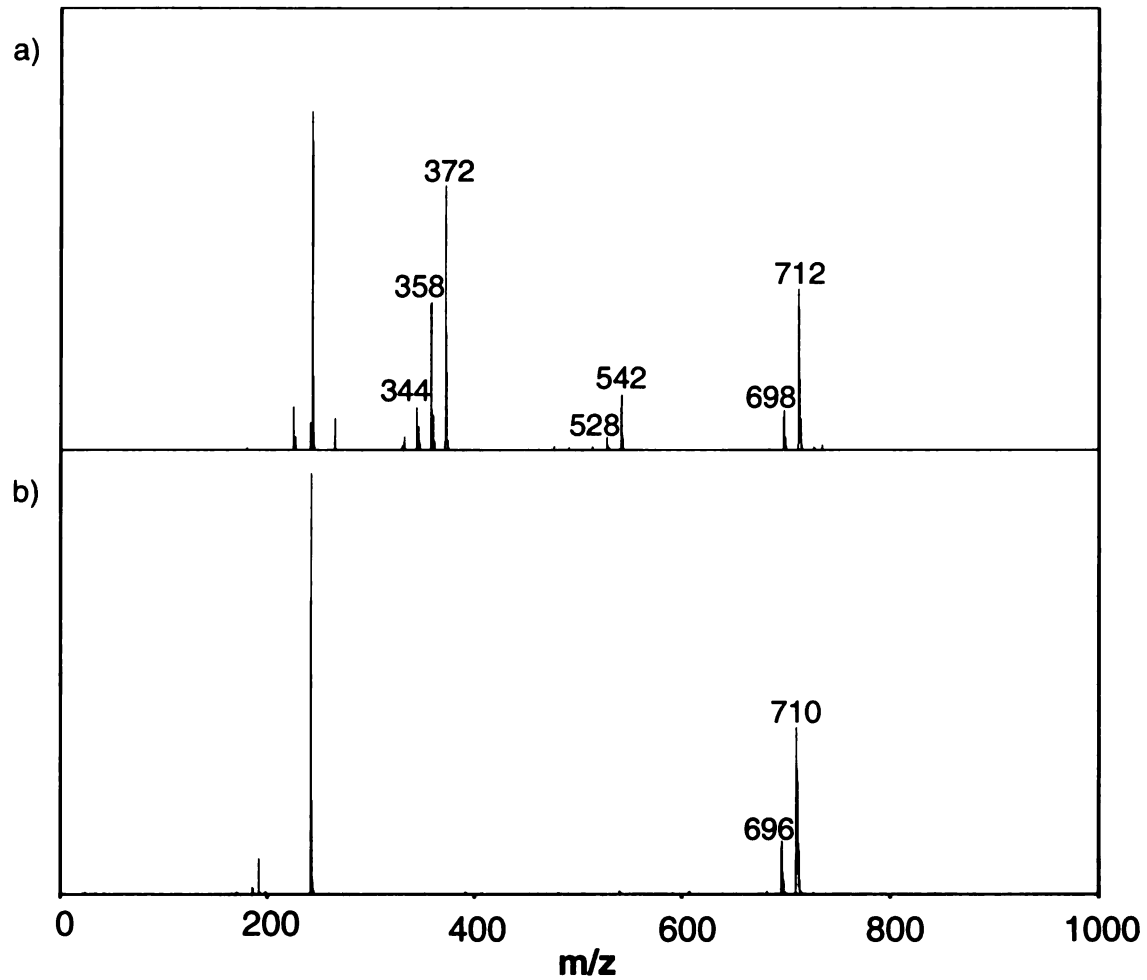


Figure 4.8: MALDI MS analysis of Acid Violet 49 on paper a) positive-ion mass spectrum and b) negative-ion mass spectrum

The Pilot® Precise V7 rollerball pen containing liquid, blue ink was selected to be analyzed by MALDI MS since LDMS was ineffective for detecting the dyes present in the ink. LDMS results were shown in Figure 4.1. The TLC analysis indicated that two dyes (one blue band and one purple band) were present in the ink. Shown in Figure 4.9 are the MALDI mass spectra of the liquid, blue ink on paper. The MALDI analysis of the ink on paper successfully detected two dyes. The mass spectra contain peaks that appear to be associated to dye structures that contain both positive and negative charges.

The peaks at m/z values 712 and 710 in the positive (Figure 4.9a) and negative (Figure 4.9b) ion mass spectra, respectively, correspond to Acid Violet 49. The peaks at m/z values 832 and 830 in the positive and negative ion mass spectra, respectively, have been identified as corresponding to Acid Blue 90, shown in Figure 4.10. MALDI analysis of Acid Blue 90 was carried out directly to ensure the correct peak assignment. Figure 4.11 shows the positive (a) and negative (b) ion MALDI mass spectra of Acid Blue 90 and confirms the dye association. According to the mass spectra (Figure 4.9),, another dye might be contained in ink. The peaks at m/z value 882 and 880 in the positive and negative-ion mass spectra respectively, may correspond to a third dye that has a similar structure to the other two dyes, but no correlation has been made currently.

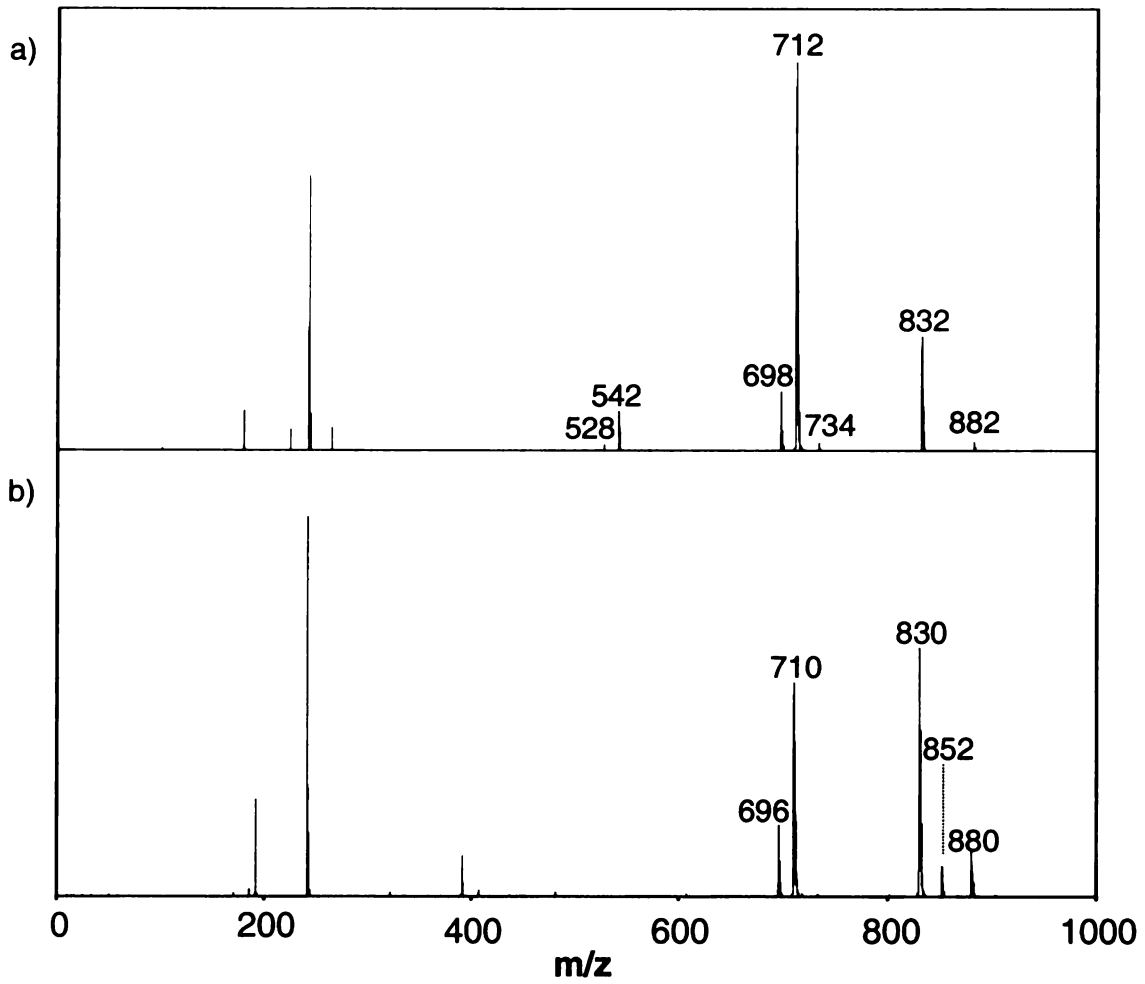


Figure 4.9: MALDI MS analysis of liquid, blue ink from Pilot® Precise V7 rollerball pen on paper a) positive-ion mass spectrum and b) negative-ion mass spectrum

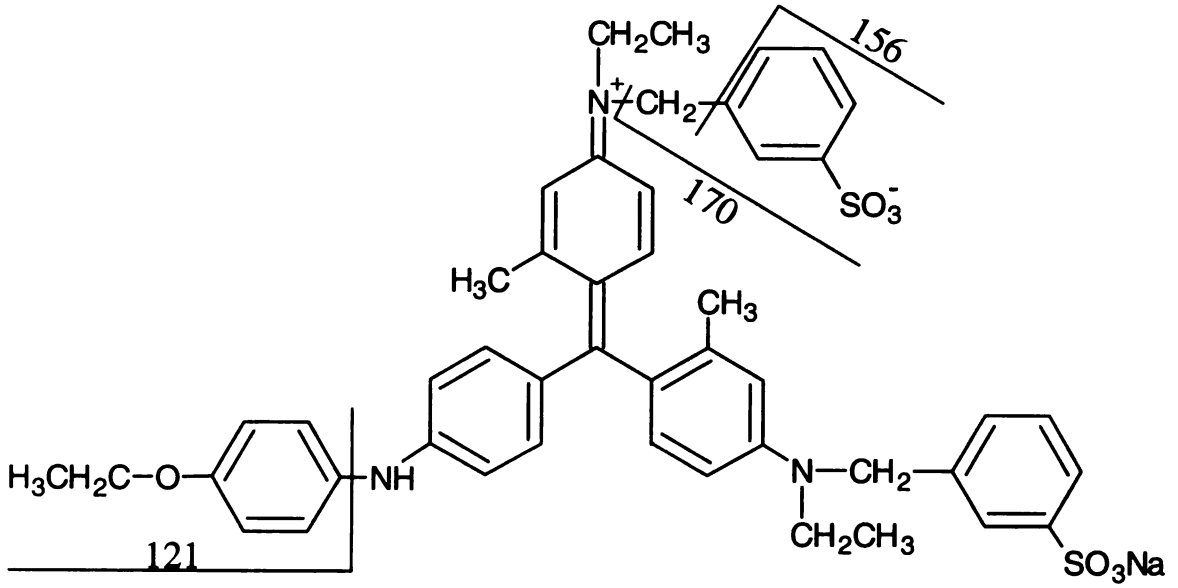


Figure 4.10: Dye structure of Acid Blue 90

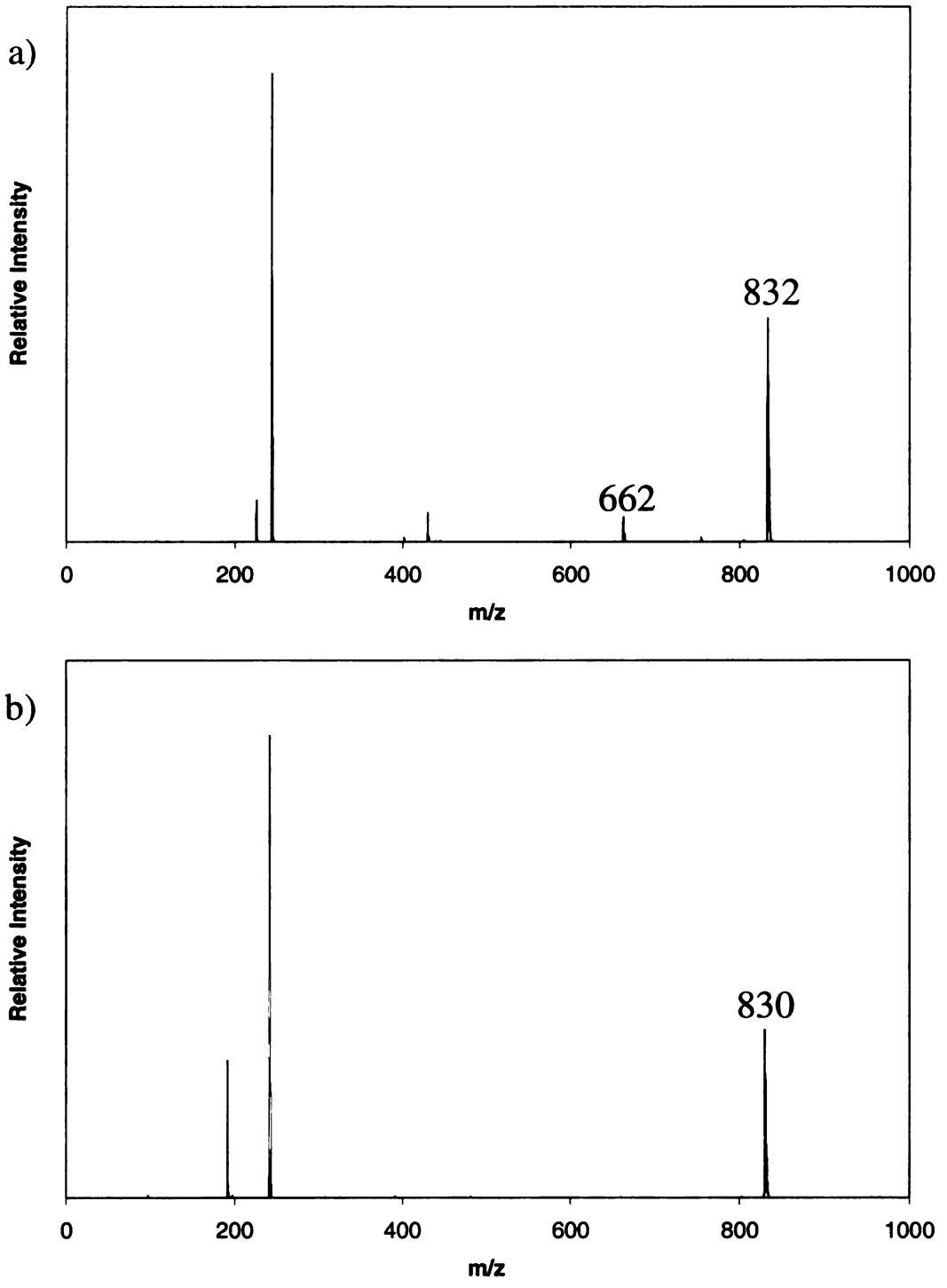


Figure 4.11: MALDI MS analysis of Acid Blue 90 on SS a) positive-ion mass spectrum and b) negative-ion mass spectrum

Multiply-Charged Ink Dye Characterization

The third part of this chapter encompasses the basic characterization information that can be acquired from MALDI and LD mass spectra and thin-layer chromatograms of multiply-charged ink dyes. The previously two chapters described the characterization information that can be acquired from examining ink dyes (neutral and singly-charged dye molecules) directly and indirectly (photodegradation of the sample prior to the MS analysis) by LDMS. MALDI and LD are related ionization techniques, hence, the information that can be deduced from both types of experiments is similar. Molecular information such as mass, composition, and charge state (the reduced charge state in MALDI) can be obtained from MALDI mass spectra in the same manner as LD. The information can be pieced together to identify and characterize the species. Although, MALDI is a softer ionization technique than LD, some fragmentation does occur. The formation of clusters also occurs in MALDI as seen in LD. As discussed previously for LDMS, fragment ions, cluster ions, and dye impurities can be used to characterize ink dyes. These species can be used in to make associations as well from the MALDI experiment. The charge state of a dye molecule, prior to the MS analysis, is more complicated to characterize by MALDI than by LD. In MALDI, the charge state of the dye is reduced by using a cation-exchange additive and a matrix prior to the analysis in order to be able to detect the dye. The multiply-charged dyes are detected as singly-charged species in the MALDI experiment. Acquiring information from the mass spectrum is limited; however, clues do present themselves which can be used to determine the presence of a multiply-charged dye, specifically, a polysulfonated dye. A dye catalogue has been assembled from ink patents for this thesis research which includes

dye structures, molecular compositions, charge states, reduced charged states, m/z values, and molecular masses. The catalogue is used to establish the identities of unknown dyes using the information that is attained from the corresponding MALDI mass spectra. Identifying and characterizing multiply-charged dyes from ink will be demonstrated through the examination of two inks from Sanford® Uni-ball® Roller pens by MALDI MS and TLC.

MALDI MS and TLC

Inks are initially examined by MALDI MS from the SS plate and by TLC to acquire preliminary results. Once dyes have been identified, they are further analyzed from paper. This procedure limits the amount of sample preparation allowing for rapid results and reduces the risk of analyzing ink dyes that are not contained in the catalogue of ink dyes developed for this research. Two pen ink examples will be discussed to demonstrate the use of MALDI MS as well as using TLC indirectly to help identify multiply-charged dyes. The inks examined were Sanford® Uni-ball® Roller pens that contained liquid, blue and red inks.

MALDI MS analysis of ink from the SS plate is a rapid method for acquiring preliminary dye information. Like all of the other MS analyses carried out for this research, a positive and negative-ion mass spectrum is obtained for each ink sample. Figure 4.12 shows the MALDI mass spectra of the liquid, blue ink from a Sanford® Uni-ball® Roller pen. Both spectra contain two relatively intense peaks that are separated by two atomic mass units; hence, the ink appears to contain two dyes. The thin-layer chromatogram also suggested that the ink contained two dyes, one blue and the other red. One might assume that both dyes are neutral molecules; however, the dyes were not

easily detected in LDMS. The dye that is represented by the peaks at m/z 559 and 557 in the positive and negative-ion MALDI mass spectra, respectively, appears to contain an ethylated amine group. The detection of dyes containing alkylated amines such as Crystal Violet and the rhodamine dyes by LDMS were previously discussed. Like the LD mass spectra of Rhodamine B and Rhodamine 6G, the MALDI mass spectra of Sanford® Uni-ball® Roller pen show a peak that is 28 atomic mass units lower than that of the peak of the intact dye. One can assume that the particular dye that is associated with m/z 557 and 559 contains an iminium group or an amine group with at least one ethyl group bonded to the nitrogen. The difference between the mass spectra of this dye and the spectra of Crystal Violet and the rhodamine dyes is that this dye is detected in both D/I modes. Crystal Violet, Rhodamine B, and Rhodamine 6G are cationic dyes that are not detected in the negative-ion mode. Also, this dye was not easily detected in LDMS, unlike the cationic dyes, which suggests that the dye has multiple charges.

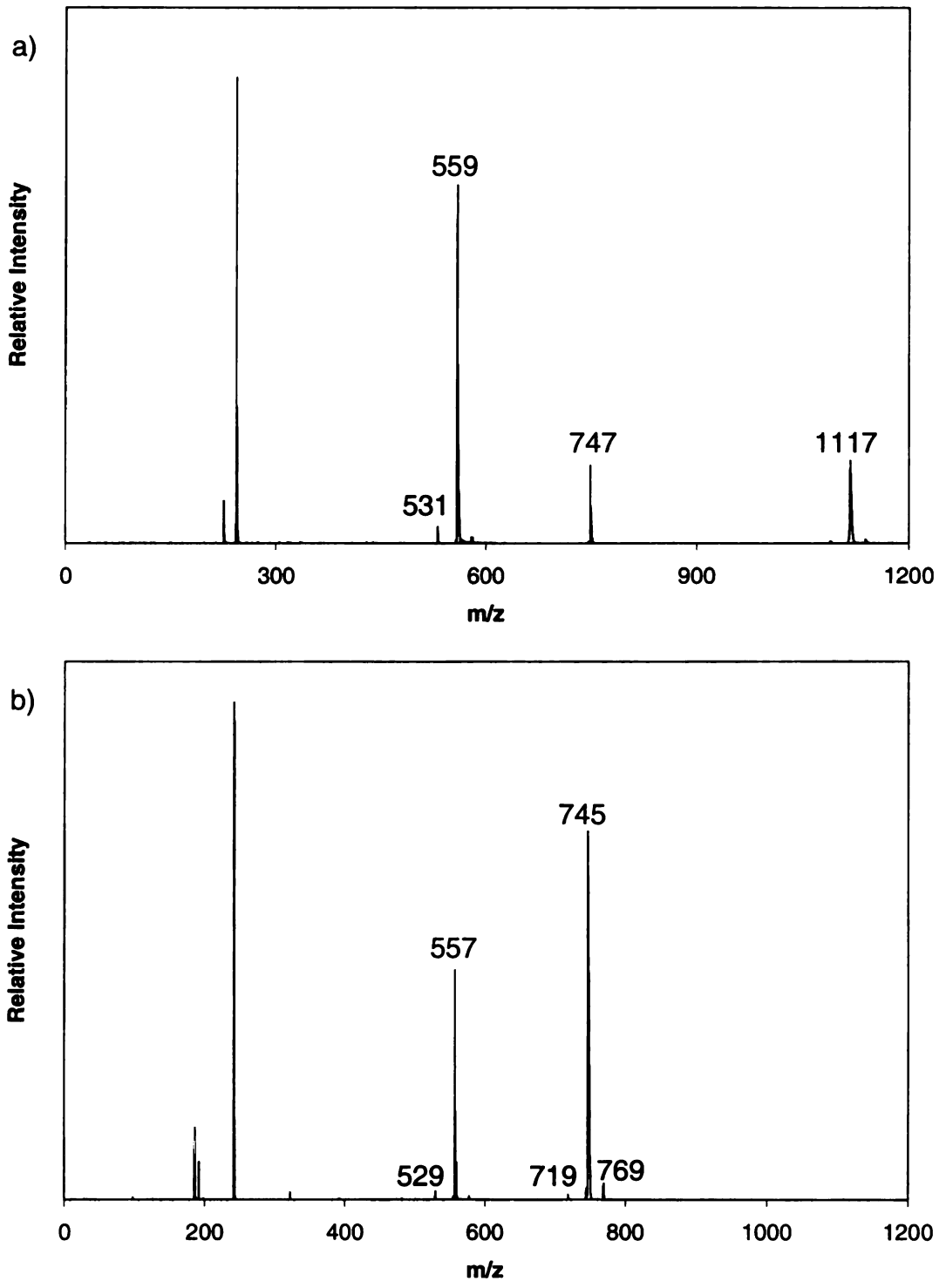


Figure 4.12: MALDI MS analysis of liquid, blue ink from a Sanford® Uni-ball® Roller pen on SS plate) positive-ion mass spectrum and b) negative-ion mass spectrum

One might mistake the peak at m/z 1117, in the positive-ion MALDI mass spectrum, to be another dye present in the ink; however, this peak a dimer. The cluster ion is comprised of two dye molecules plus a proton [2 (558) + 1]. The dye that is represented by the peaks at m/z 747 and 745 in the positive and negative-ion MALDI mass spectra, respectively, also appears to be a multiply charged dye that contains an ethylated nitrogen atom. The peak at m/z 769 in the negative-ion mass spectrum is the result of cation adduction by a sodium ion. Generally, sodium ion adduction for dye analysis suggests the presence of a multiply-charged dye. The loss of an ethyl group from the dye was observed in the negative-ion mass spectrum as well. Coupling TLC indirectly to MALDI MS determined that the red TLC band corresponded to m/z 559 and 557 and the blue TLC band was related to m/z 747 and 745. The MS and TLC data were used to search the dye catalogue and the two dyes were tentatively identified as Sulforhodamine B (Acid Red 52) and Acid Blue 9. The dye structures of the red and blue dyes (the Na^+ counterions were not shown) are illustrated in Figures 4.13a and 4.13b, respectively. The dyes were purchased and analyzed directly to confirm that the correct identifications were made. Figures 4.14 and 4.15 show the MALDI mass spectra of the ink dyes directly. The dyes were accurately identified. Multiply-charged dyes such as Acid Red 52 and Acid Blue 9 which contain both positive and negative charges are detected in positive and negative-ion MALDI MS. Polyionic dyes containing strictly negative charges are detected only in negative-ion MALDI MS, except if the dye is multiply-charged exclusively with sodium alkoxide groups. The means ionization of multiply-detection of polyionic dyes in MALDI MS can be used to help characterize and identify the dye.

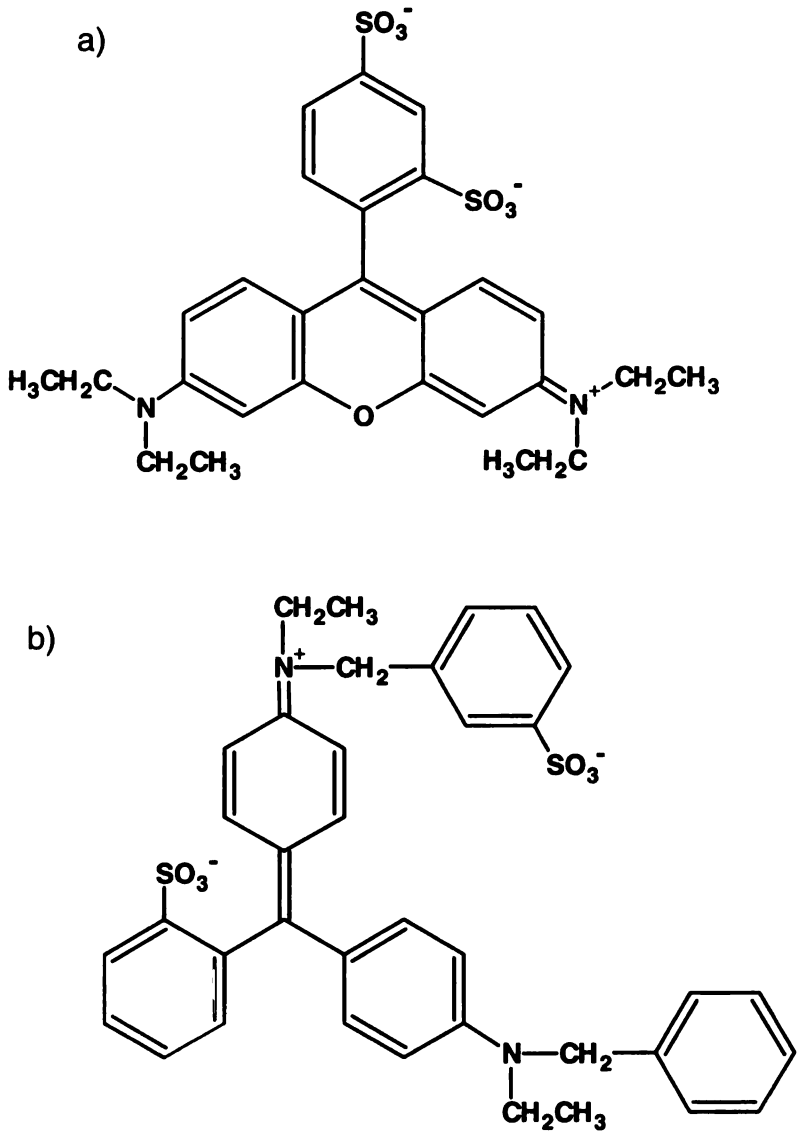


Figure 4.13: Dye structures a) Acid Red 52 and b) Acid Blue 9

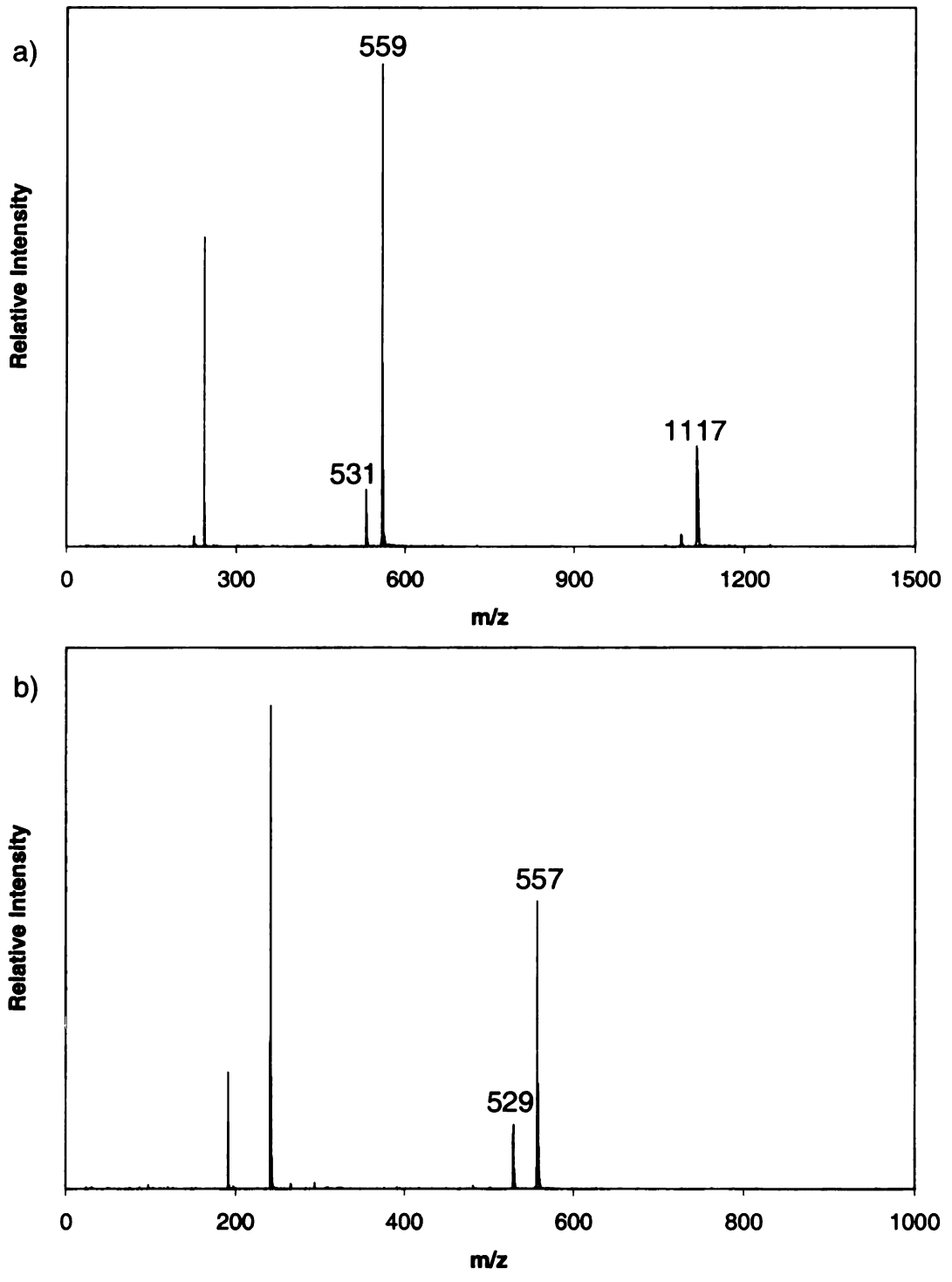


Figure 4.14: MALDI MS analysis of Acid Red 52 on SS plate a) positive-ion mass spectrum and b) negative-ion mass spectrum

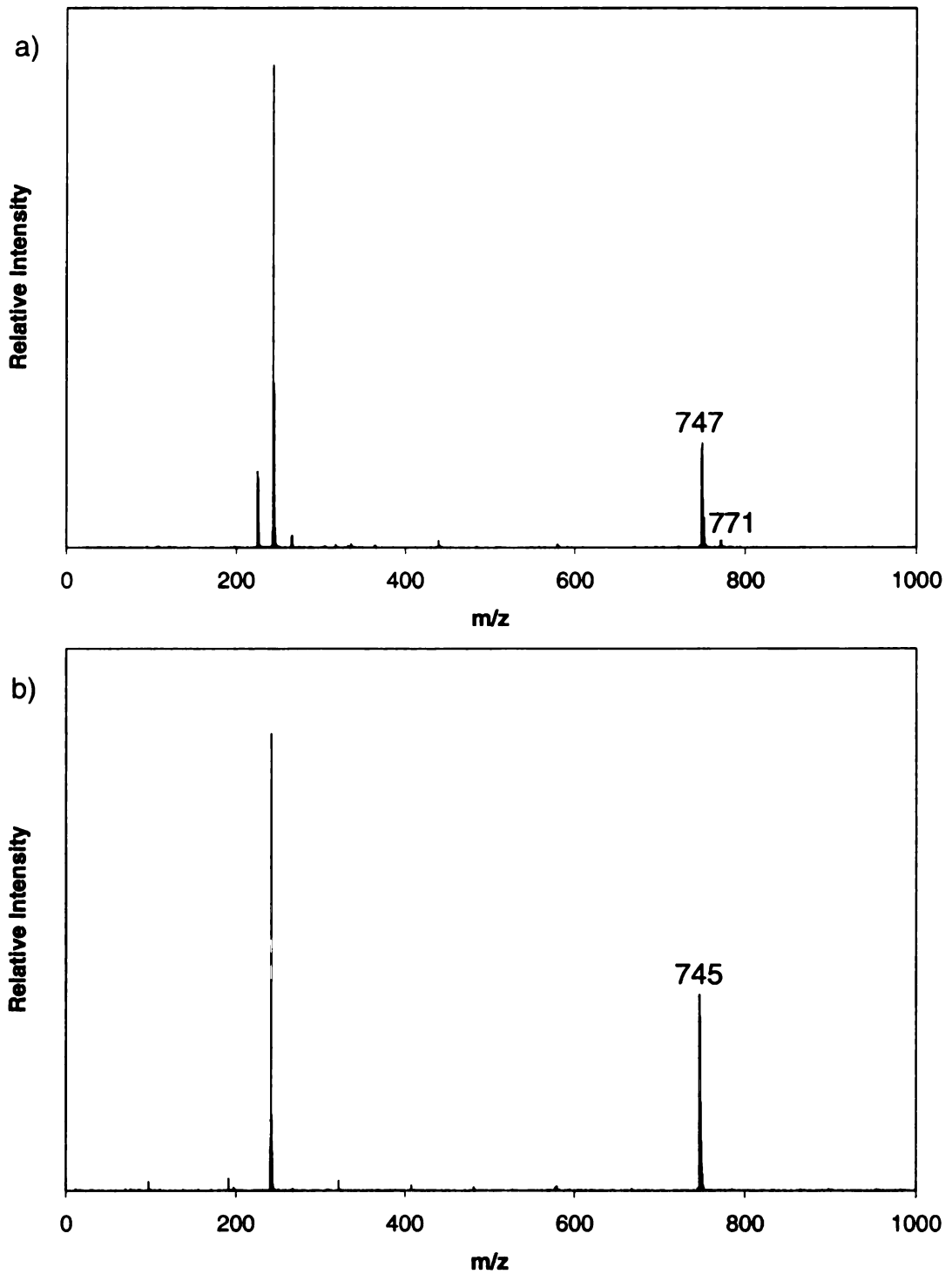


Figure 4.15: MALDI MS analysis of Acid Blue 9 on SS plate a) positive-ion mass spectrum and b) negative-ion mass spectrum

A liquid, red ink was also examined by MALDI MS and the mass spectra obtained can be seen in Figure 4.16. The mass spectra are quite different that the previous blue ink examined. There are several peaks in the negative-mass spectrum (Figure 4.16b) and only one predominant peak in the positive-ion mass spectrum (Figure 4.16a). From an initial examination of the negative-ion mass spectrum, there appears to be at least five dyes that are contained in the ink. However, TLC ink analysis isolated three dyes (yellow, red, red-orange TLC bands). TLC was indirectly coupled to MALDI MS and Figure 3.17 shows the resulting mass spectra. The peak at m/z 407 represents a yellow dye, the peak at 647 denotes the detection of an orange-red dye, and the combination of peaks at m/z 741 and 785 correspond to a red dye. Again using the dye catalogue the dyes were determined to be Food Yellow 3, Acid Red 87, and Acid Red 92 as shown in Figure 4.18a, 4.18b, and 4.18c, respectively. The three dyes are polyanionic; however, the functional groups responsible for the charge distribution are different among the dyes. Sodium sulfonic acid, alkoxide, and carboxylate groups are responsible for the charge state of the dyes. Food Yellow 3 is a disulfonated azo dye.. The peak at m/z 429 is due to the sodium adduction of the dye and demonstrates that the dye is multiply-charged. Food Yellow 3 was detected exclusively in the negative-ion mode as expected since the dye does not contain any positive charges. Acid Red 87 contains two alkoxide groups. Acid Red 92 contains one alkoxide group and one carboxylate group. Neither of these red dyes contains functional groups that carry a positive charge, however, they were detected in positive-ion MALDI MS. Perhaps the alkoxide and carboxylate groups can acquire a positive charge upon D/I. where a dye containing strictly sulfonic acid groups will only be detected in the negative-ion mode. In negative-

ion LDMS, multiple sulfonic acid groups completely inhibit the D/I of Food Yellow 3 where Acid Red 87 and Acid Red 92 are weakly detected. Acid Red 92 is not detected intact. The dye fragments as a result of containing carboxylic acid and is similar to the fragmentation that results when Rhodamine B is examined by LDMS. The decarboxylic acid form of the dye is detected at m/z 741. Even though the laser power was significantly increased for detection in LDMS as compared to the MALDI experiment, the number of ions detected in the LDMS experiment was considerably less. For a typical MS experiment the ion count is about 12,000 and the ion count for this particular LD experiment was less than 800. MALDI MS significantly enhanced the detection of the Food Yellow 3, Acid Red 87, and Acid Red 92. The LD mass spectrum of the red, liquid ink is shown in Figure 4.19. Peaks of low intensity encompass the m/z range of the ink dyes making it difficult to distinguish the dyes from other chemical noise. Some information can be acquired from the negative-ion MALDI mass spectrum to characterize the ink dyes. The acid red dyes give rise to distinct isotope distributions as seen in Figures 4.20 and 4.21. Isotope distributions have been discussed for Copper Phthalocyanine in Chapter Two. The theoretical isotopes of Acid Red 87 and Acid Red 92 were compared with experimental isotopes from the mass spectra to characterize and identify the ink dyes. Another characteristic of Acid Red 92 that can be used to characterize the dye is that the dye contains a carboxylic acid group. As shown previously, Rhodamine B decarbonylated when the xanthene dye was analyzed by positive-ion LDMS. The loss of CO_2 (44 amu) can be observed in the negative-ion MALDI mass spectrum of Acid Red 92.

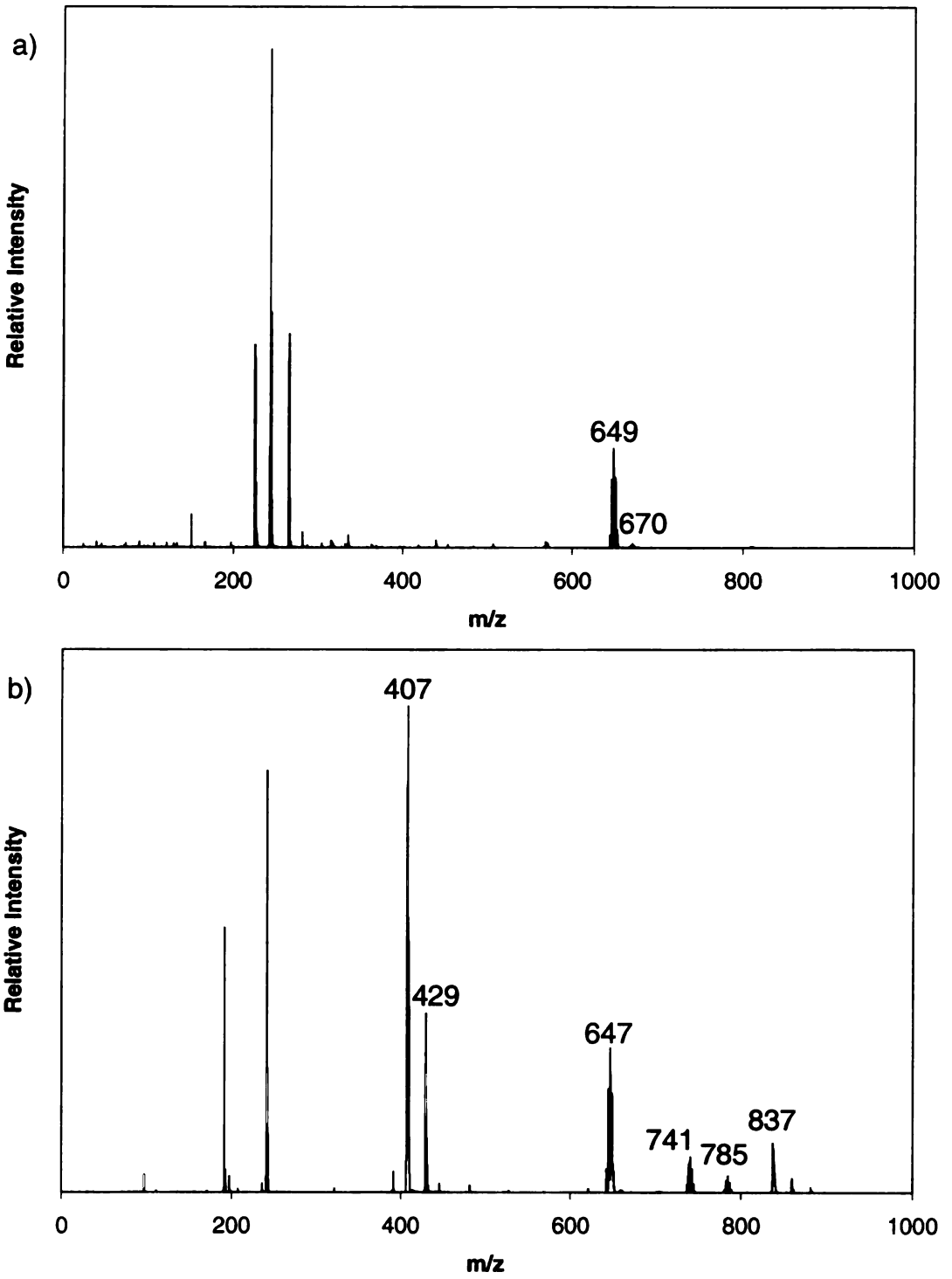


Figure 4.16: MALDI MS analysis of red, liquid ink from Sanford® Uni-ball® Roller pen on SS plate a) positive-ion mass spectrum and b) negative-ion mass spectrum

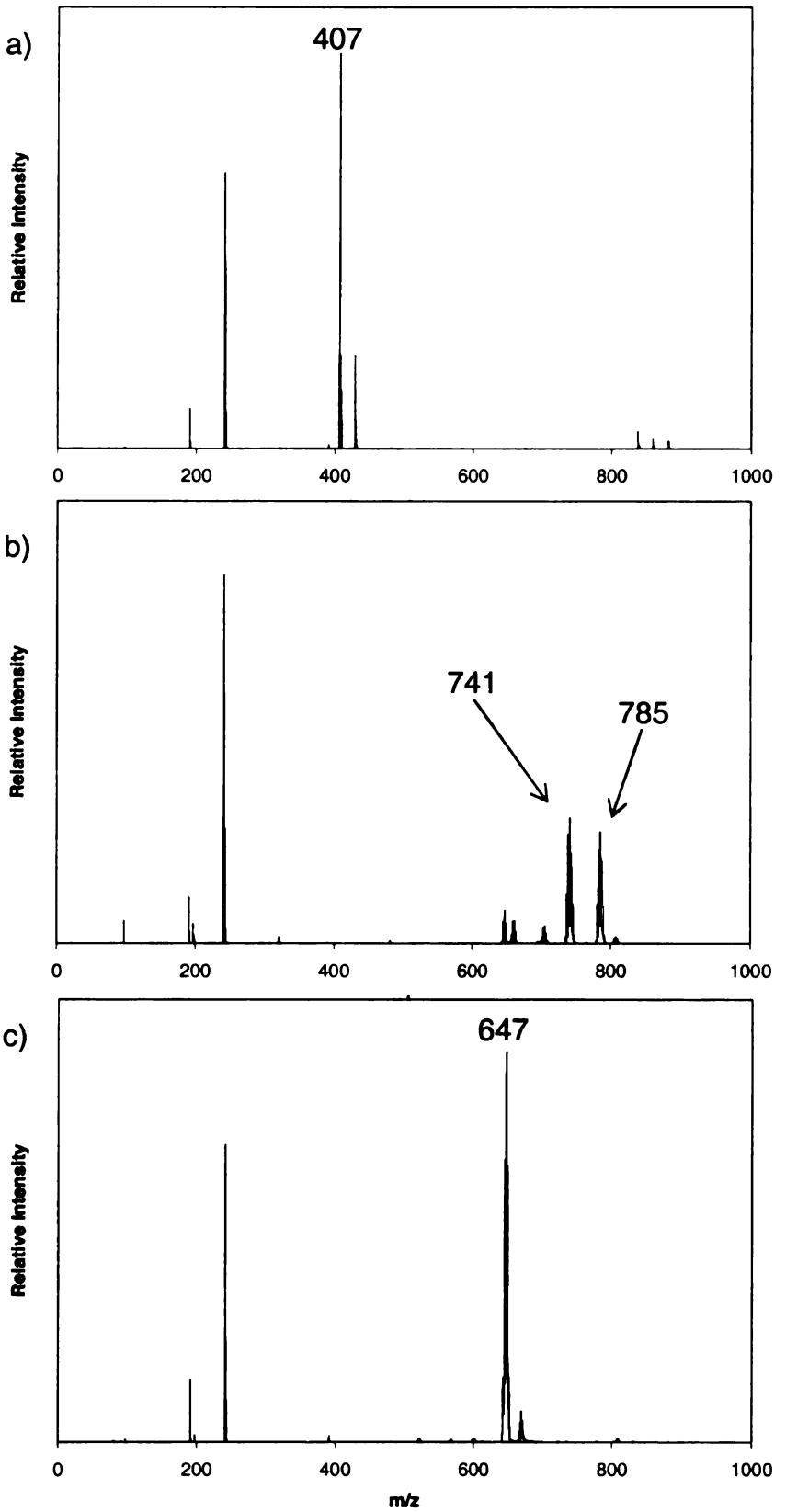
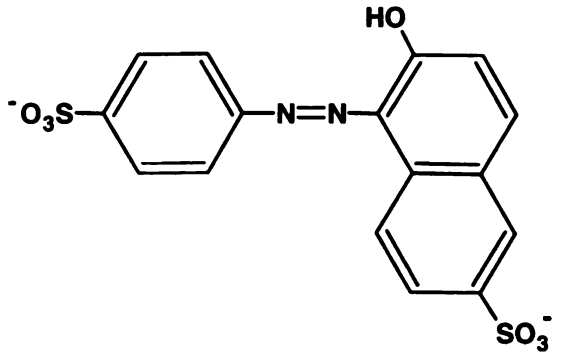
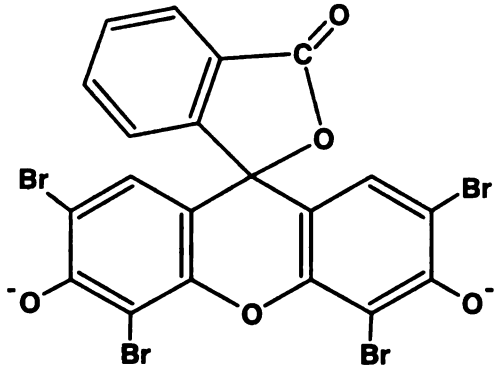


Figure 4.17: Indirect coupling of TLC with MALDI MS: negative-ion mass spectra of extracted TLC bands a) yellow band, b) orange-red band, and c) red band

a)



b)



c)

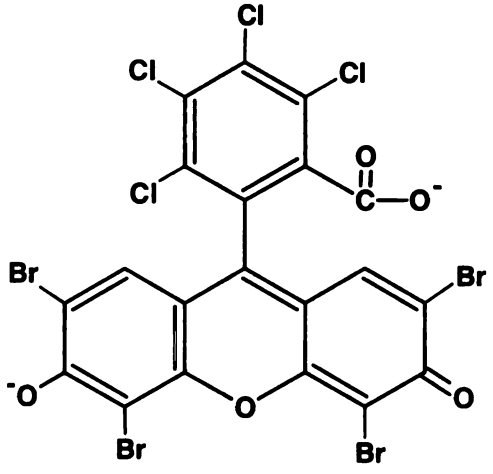


Figure 4.18: Dye structures a) Food Yellow 3, b) Acid Red 87, and c) Acid Red 92

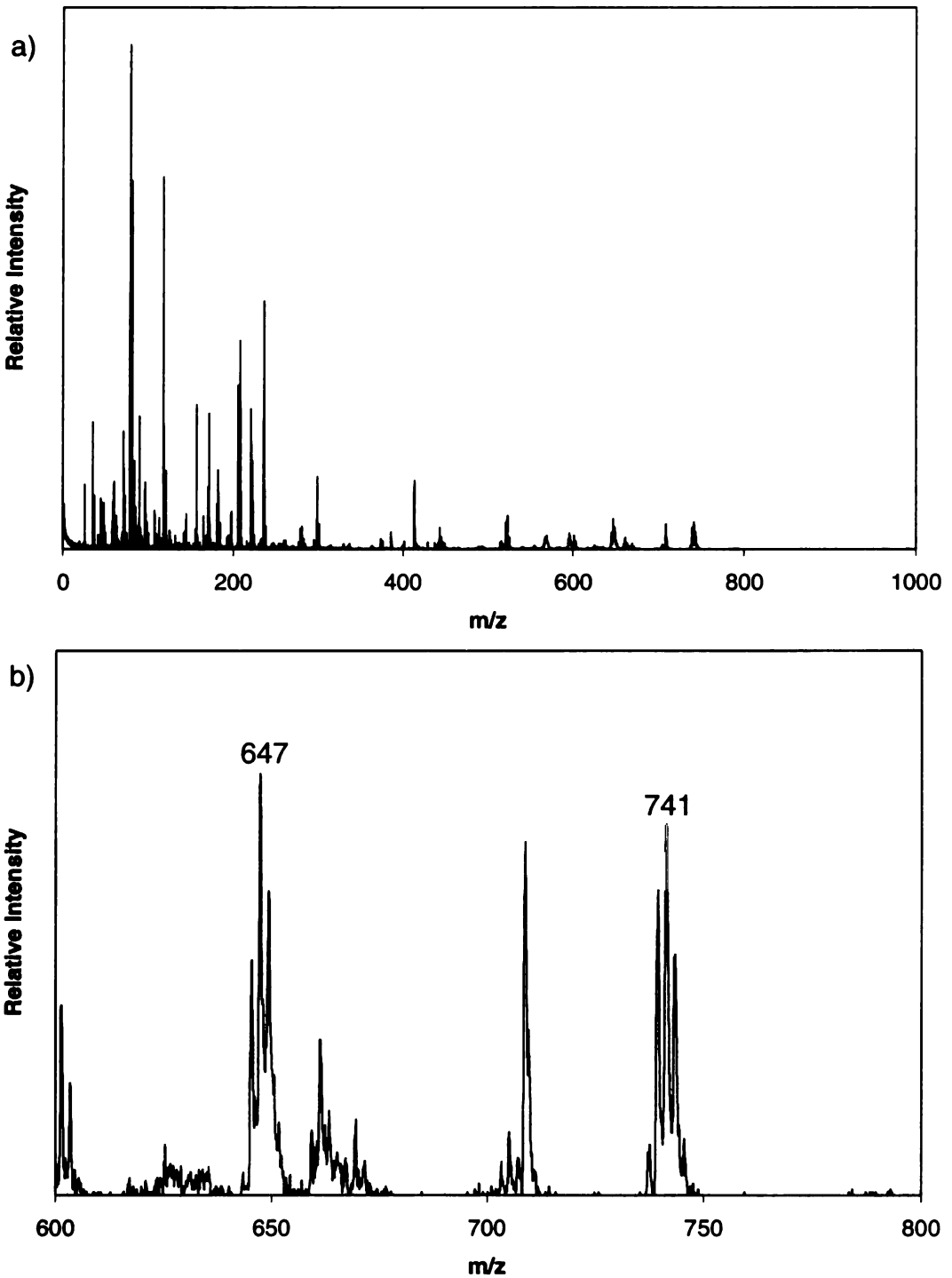


Figure 4.19: Direct negative-ion LDMS analysis of liquid, red ink from Sanford® Uni-ball® Roller pen a) complete spectrum from m/z 0 – 100 and b) expanded spectrum from m/z 600 – 800

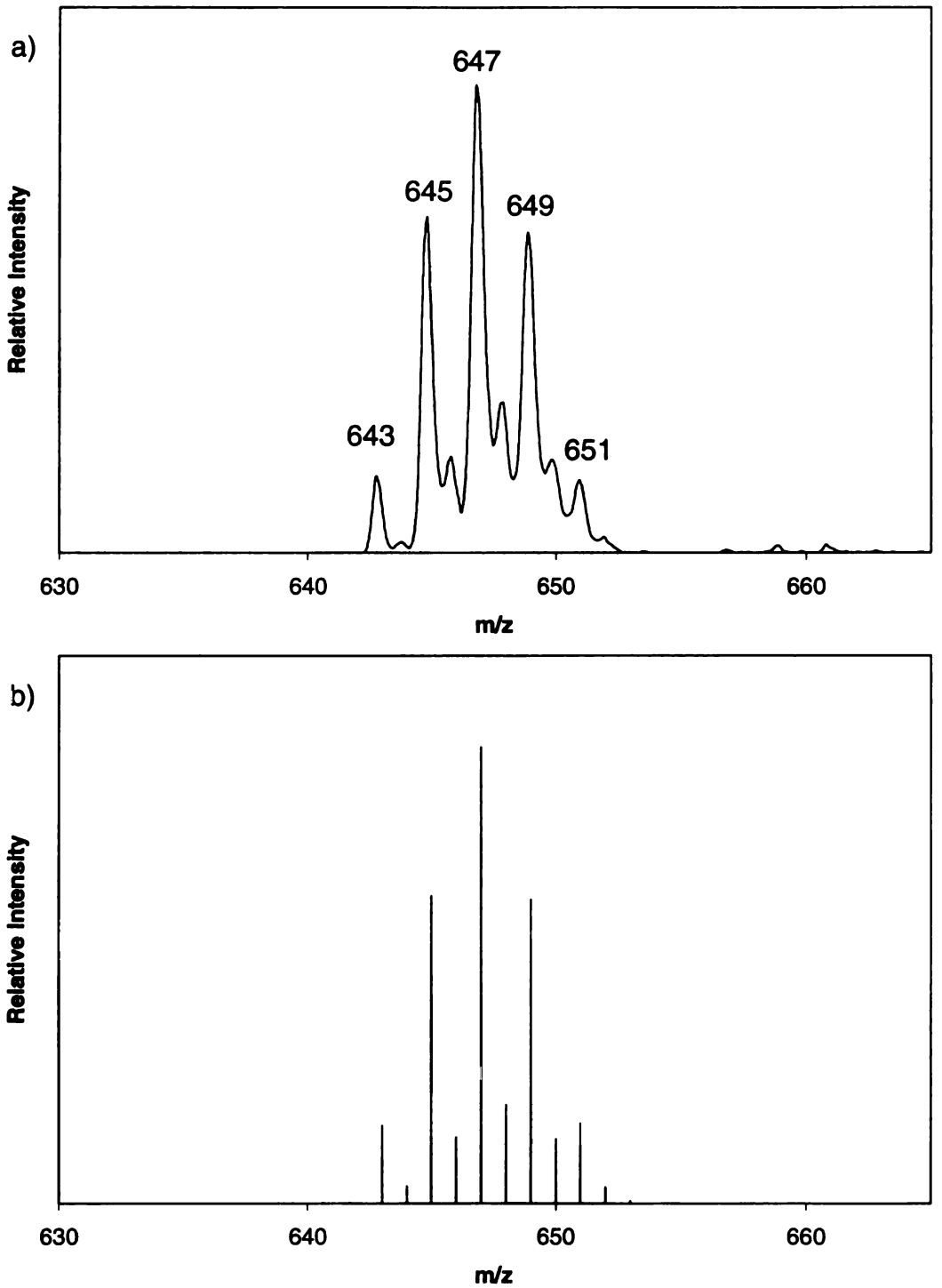


Figure 4.20: Isotopic peak distributions of Acid Red 87 a) experimental and b) theoretical

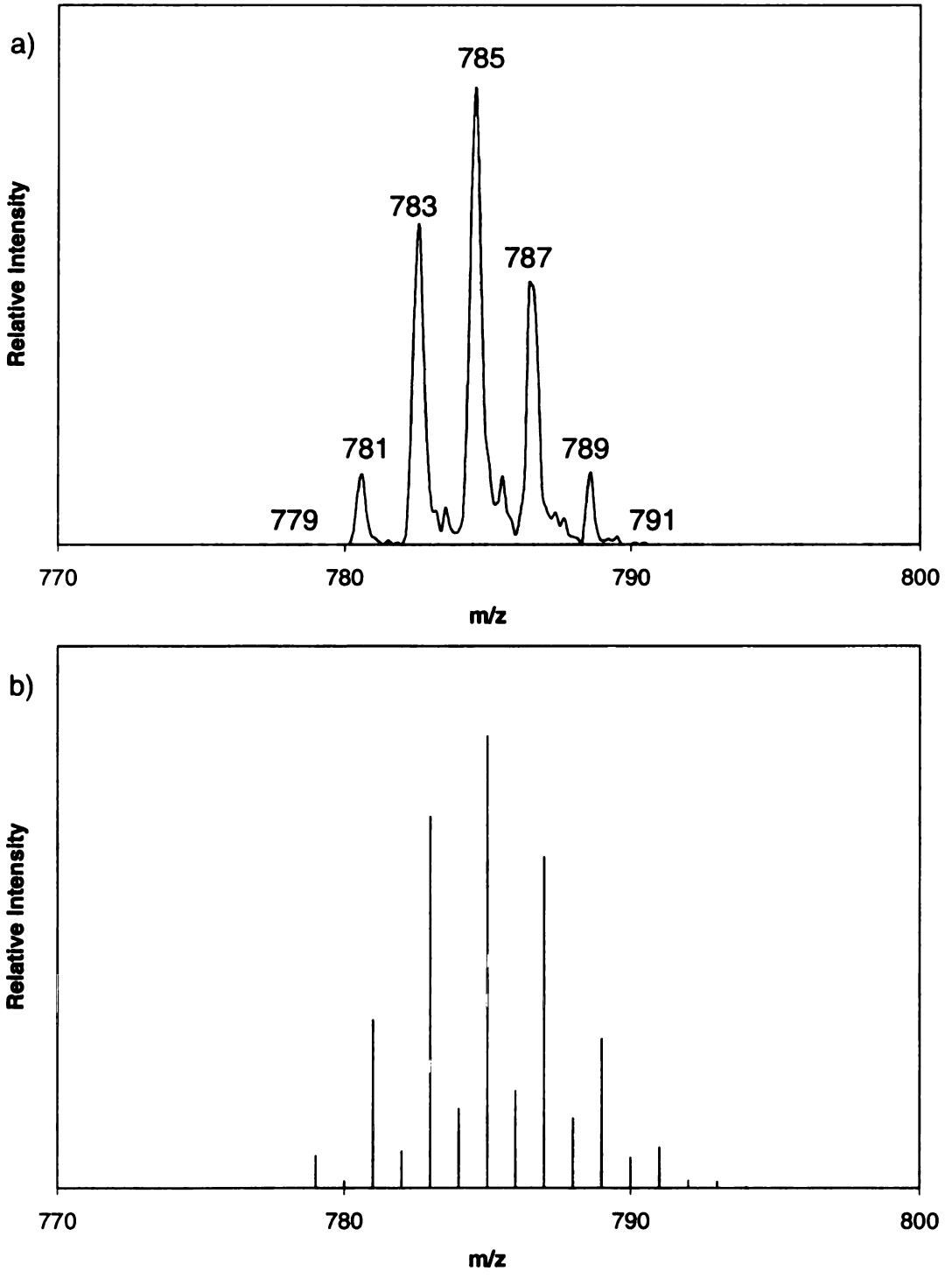


Figure 4.21: Isotopic peak distributions of Acid Red 92 a) experimental and b) theoretical

Direct LDMS Analysis

The peak at m/z 170 in the negative-ion LD mass spectrum (Figure 4.1b) of the liquid, blue ink was classified as corresponding to a fragment ion from Acid Violet 49 and Acid Blue 90. The fragmentation of the structures is illustrated in Figures 4.7 and 4.10. The fragment ion corresponds to $[C_7H_6O_3S]^{\cdot -}$. The dissociation of the C-N bond that lead to the fragment ion is depicted in the dye structures. The dyes were analyzed directly and the negative-ion mass spectra showed the presence of the fragment ion. Without having previous knowledge of the dye structures one would be able to use this fragment ion observed in the LD experiment to assume that the ink contains a dye with the specific functional group ($-CH_2C_6H_4SO_3^- Na^+$). Fragmentation demonstrated that direct LD analyses of ink can be used to make such associations about the multiply-charged dye structures.

References

(1) Sullivan AG, Gaskell SJ. The Analysis of Polysulfonated Azo Dyestuffs by Matrix-Assisted Laser Eesorption/Ionization and Electrospray Mass Spectrometry. *Rapid Commun Mass Spectrom.* **1997** (11) 803-9

(2) Conneely A, McClean S, Smyth WF, McMullan G. Study of the Mass Spectrometric Behavior of Phthalocyanine and Azo Dyes Using Electrospray Ionization and Matrix-Assisted Laser Desorption/Ionization. *Rapid Comm in Mass Spectrom.* **2001** (15) 2076-2084

(3) Osada T, inventors. Mitsubishi Pencil Kabushiki Kaisha, assignee. Aqueous Gel Ink-filled Ball Point Pen. US Patent 5,993,098 **Nov 1999**

(4) Asara JM, Allison J. Enhanced Detection of Phosphopeptides in Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Using Ammonium Salts. *J Am Soc Mass Spectrom.* **1998** 1035-44

(5) Mechref Y, Novotny MV. Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry of Acidic Glycoconjugates Facilitated by the Use of Spermine as a Co-Matrix. *J Am Soc Mass Spectrom.* **1998** 91293-1302

(6) Nordhoff E, Ingendoh A, Cramer R, Overberg A, Stahl B, Karas M, Hillenkamp F. Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry of Nucleic Acids with Wavelengths in the Ultraviolet and Infrared. *Rapid Comm in Mass Spectrom.* **1992** (6) 771-776

(7) Pieles U, Zurcher W, Schar M, Moser HE. Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry: A Powerful Tool for the Mass and Sequence Analysis of Natural and Modified Oligonucleotides. *Nucleic Acids Res.* **1993** (21) 3191-3196

(8) Currie GJ, Yates JR III. Analysis of Oligodeoxynucleotides by Negative-Ion Matrix-Assisted Laser Desorption Mass Spectrometry. *J Am Soc Mass Spectom.* **1993** (4) 995-963

(9) Zhu YF, Chung CN, Taranenko NI, Allman SL, Martin SA, Haff L, Chen CH. The Study of 2,3,4-Trihydroxyacetophenone and 2,4,6-Trihydroxyacetonphenone as Matrices for DNA Detection in Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. *Rapid Comm Mass Sepctrom* **1996** (10) 383-388

(10) Green-Chruch K, Simmons TA, Limbach PA. Chemistry Effects on the Analysis of DNA by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. *In Ultrasensitive Biochemical Diagnostics II* sponsored by SPIE-the International Society for Optical Engineering. **1997** (2985) 88-99

(11) Cheng S, Chan TWD. Use of Ammonium Halides as Co-Matrices for Matrix-Assisted Laser Desorption/Ionization Studies of Oligonucleotides. *Rapid Comm Mass Spectrom.* **1998** (10) 907-910

(12) Zhu YF, Taranenko NI, Allman SL, Martin SA, Haff L, Chen CH. The Effect of Ammonium Salt and Matrix in the Detection of DNA by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. *Rapid Comm Mass Spectrom.* **1996** (10) 1591-1596

(13) Simmons TA, Limbach PA. The use of a Co-Matrix for Improved Analysis of Oligonucleotides by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. *Rapid Comm Mass Spectrom.* **1997** (11) 567-72

(14) Simmons TA, Limbach PA. Influence of Co-Matrix Proton Affinity on Oligonucleotide Ion Stability in Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. *J Am Soc Mass Spectrom.* **1998** (9) 668-75

(15) Leo YC, Cheng SW, Chan TWD. Evaluation of Ammonium Salts as Co-Matrices for Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry of Oligonucleotides. *Rapid Comm Mass Spectrom.* **1998** 12993-8

(16) Vandell VE, Lambach PA. Polyamine Co-Matrices for Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry of Oligonucleotides. *Rapid Comm Mass Spectrom.* **1999** (13) 2014-2021

(17) Distler AM, Allison J. 5-Methoxysalicylic Acid and Spermine: A New Matrix for the Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry of Oligonucleotides. *J Am Soc Mass Spectrom.* **2000** (12) 456-462

(18) Distler AM, Allison J. Improved MALDI-MS Analysis of Oligonucleotides through the Use of Fucose as a Matrix Additive. *Anal Chem.* **2001** (73) 5000-3

(19) Distler AM, Allison J. Additives for the Stabilization of Double-Stranded DNA in UV-MALDI MS. *J Am Soc Mass Spectrom.* **2002** (13)1129-1137

(20) Distler AM, Allison J. Stabilization of Duplex Oligonucleotides for Analysis by Matrix-Assisted laser Desorption/Ionization Mass Spectrometry Using Crystallographic Condensing AgentC (III) Hexamine. *Anal Biochem* **2003** (319)332-334.

(21) Voyager Workstation User's Guide version 5, PerSpective Biosystems, **1999**

(22) Ballantine JA, Games DE, Slater P. The Use of Amine Base to Enhance the Sensitivity of Electrospray Mass Spectrometry Towards Complex Polysulfonated Azo Dyes. *Rapid Comm Mass Spectrom.* **1995** (9) 1403-1410

(23) Ng LK, Lafontaine P, Brazeau L. Ballpoint Pen Inks: Characterization by Positive and Negative Ion-Electrospray Ionization Mass Spectrometry for the Forensic Examination of Writing Inks. *J Forensic Sci.* **2002** (47) 1238-1247

(24) Venkataraman K. The Chemistry of Synthetic Dyes. Vol. I, Academic Press Inc., New York, **1952**

(25) Grim DM, Siegel JA, Allison. Evaluation of Laser Desorption Mass Spectrometry and UV accelerated aging of dyes on paper as tools for the evaluation of a questioned document. *J Forensic Sci.* **2002** (47)1265-1273

(26) Dunn JD, Siegel JA, Allison J. Photodegradation and Laser Desorption Mass Spectrometry for the Characterization of Dyes Used in Red Pen Inks. *J Forensic Sci.* **2003** (48) 652-657

Chapter Five: Conclusions

MALDI MS Limitations and Future Research

Limitations are apparent in analyzing ink dyes by MALDI MS. MALDI, with and without an additive, was used to detect singly-charged and neutral dye molecules as well. Preliminary results indicated that MALDI may suppress the ion signal for dyes that are neutral molecules. Perhaps the matrix and the dye molecules compete for the D/I energy. Additional dyes should be tested to see the influence of the matrix on detection of neutral molecules. Neither the positive nor negative ions of Pigment Red 3 were detected in the MALDI experiment. The dye was detected in the LDMS experiment directly from paper and metal substrates. Both MS techniques should be used when examining ink dyes. The D/I of Copper Phthalocyanine, a neutral pigment dye, was also seen to be influenced by the MALDI experiment. A significant increase in laser power was required for a signal that was comparable to the detection of the neutral dye by LDMS. Another limitation of using MALDI is that some dyes are not soluble in the matrix or additive solution which ultimately inhibits the detection of the dye. The solubility of the dye does not necessarily influence detection in MALDI. Some of the black dyes were barely or not detected, although, most were soluble in the solutions. These dyes should be further examined. Structural similarities among the dyes were limited, so no conclusions could be drawn based on the structures of the molecules. Several black ink from gel or liquid pen were undetectable by MALDI MS. Perhaps, these inks contain carbon black. U.S. Patent describes the composition of a black gel ink[1]. According to the patent, carbon black is used as the colorant.

Characterizing multiply-charged dyes using advanced methods such as photodegradation which was previously used as a characterization method with LDMS can be future research. Some of the multiply-charged dyes contain alkylated amine groups and azo bonds which have been known to photodegrade or thermally degrade, respectively. Once the dyes have been degraded, then MALDI MS can be used to characterize the ink dyes based from the photoproducts. Analyzing multiply-charged ink dyes from other substrates such as fibers can be investigated using the method presented here can also be done in the future. Previously work by Balko showed that polysulfonated dyes in fabric were undetectable by direct LDMS[2]. Applying the method to other forensic evidence would be a great advancement.

References

(1) Takahiro O, inventor. Mitsubishi Pencil Kabushiki Kaisha, assignee. US Patent 5,993,098 Dec **1997**

(2) Balko LG. "Laser Desorption/Ionization Mass Spectrometric (LD/MS) Analysis of Samples of Forensic Interest: Chemical Characterization of Colorant and Dyes." M.S. Thesis. Michigan State University, **2002**