J Forensic Sci, May 2003, Vol. 48, No. 3
Paper ID JFS2002359_483
Available online at: www.astm.org

Jamie D. Dunn,[1,2] *B.S.; Jay A. Siegel,*[2] *Ph.D.; and John Allison,*[1] *Ph.D.*

Photodegradation and Laser Desorption Mass Spectrometry for the Characterization of Dyes Used in Red Pen Inks*

ABSTRACT Photodegradation and laser desorption mass spectrometry (LDMS) is a powerful combination of methods capable of characterizing dyes found in pen inks. Rhodamine dyes in pens that contain red ink were analyzed directly from paper (no extraction step is necessary). Inks exposed to incandescent light form photodegradation products (compounds with lower molecular weights than that of the intact dye) and in some instances, photoproducts (compounds with higher molecular weights than that of the intact dye). The degradation products and photoproducts can be detected with LDMS, and the results can be used for dye identification. Advantages include: (1) the instrumental analysis takes less than a minute; (2) sample preparation is minimal; (3) LDMS is a minimally destructive technique; (4) incandescent light sources are inexpensive, safe to use, and readily available; and (5) isomeric dyes can be distinguished.

KEYWORDS: forensic science, questioned documents, laser desorption mass spectrometry, photodegradation, incandescent light, ink, dyes, rhodamine

It has recently been demonstrated that laser desorption mass spectrometry (LDMS) is a sensitive method for detecting dyes in pen inks on paper (1–5). For example, a commonly used dye in blue pen inks is Crystal Violet (CV), structure I in Fig. 1. When a pulsed ultraviolet (UV) laser is focused onto a pen stroke from a blue pen on paper, Crystal Violet ions are desorbed intact, and can be detected using a time-of-flight mass spectrometer (1–5). In addition to sensitivity, there are two other notable advantages of the method. First, no solvent extraction step is required. Second, while ink is a mixture of components, LDMS analysis shows that the dye molecules are most often the only components in the ink that are detected. Detection requires both absorption of UV radiation and conversion into a gas phase ion. One disadvantage is the efficiency of the desorption step. Energy is deposited into the ink molecules in 2–3 ns, quickly raising the temperature of a very small portion of the sample to a point where desorption is favored over degradation. Thus, the ions desorb intact, giving a single mass spectrometric peak (plus isotopic peaks), and providing molecular weight information and possibly elemental composition. Interpreting an LDMS spectrum of a dye may be similar to interpreting an HPLC chromatogram. One dye yields one peak, so structural information is limited. How can structural information be obtained from the LDMS experiment? We propose to use photodegradation of the dyes on the paper substrate. LDMS analysis following photodegradation will yield a set of mass spectral peaks, each representing a degradation product. From the degradation products, and an understanding of possible reactions that may occur, structural insights can be obtained. For example, if Crystal Violet is irradiated with UV radiation, N-demethylation reactions occur. Crystal Violet degrades to form Methyl Violet 2B, structure II in Fig. 1. Replacement of a methyl group by a hydrogen atom results in a net loss of 14 atomic mass units. Structure I can lose up to six methyl groups in this way. We have taken advantage of this chemistry and proposed an approach for determining the age of a document (4). From an analytical standpoint, the degradation products formed naturally or by accelerated aging are related to the structure. The fact that Crystal Violet forms degradation products showing the loss of up to six methyl groups provides an important structural context in which the dye could be identified.

Another structural aspect in this work is the observation that, if a dye is cationic, LD will only yield positive ions for MS analysis (1–5). If the dye is anionic, it will only yield negative ions. If the dye is neutral, it will form both positive and negative ions in laser desorption (1–5). Such observations provide important clues that lead to identification of the dye in question.

We demonstrate here that LDMS can be used to analyze red pen inks. We used the combination of photodegradation and LDMS for dye identification through a challenging example, which involves the differentiation between two isomeric rhodamine dyes, Rhodamine B and Rhodamine 6G, both of which are used in the manufacturing of red inks. In this work, we focus on the use of incandescent light for photodegradation, as an inexpensive alternative to UV-based methods.

Experimental

Laser Desorption Mass Spectrometry

Instrumentation—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 nanoseconds pulses, 3 Hz) and a linear time-of-flight mass spectrometer. The user-selected parameters for the LDMS experiments

[1] Department of Chemistry, Michigan State University, East Lansing, MI.
[2] School of Criminal Justice, Michigan State University, East Lansing, MI.
* Presented at the American Academy of Forensic Sciences 54th annual conference, February 2002, Atlanta, Georgia.
Received 5 Oct. 2002; and in revised form 20 Dec. 2002; accepted 20 Dec. 2002; published 26 Mar. 2003.

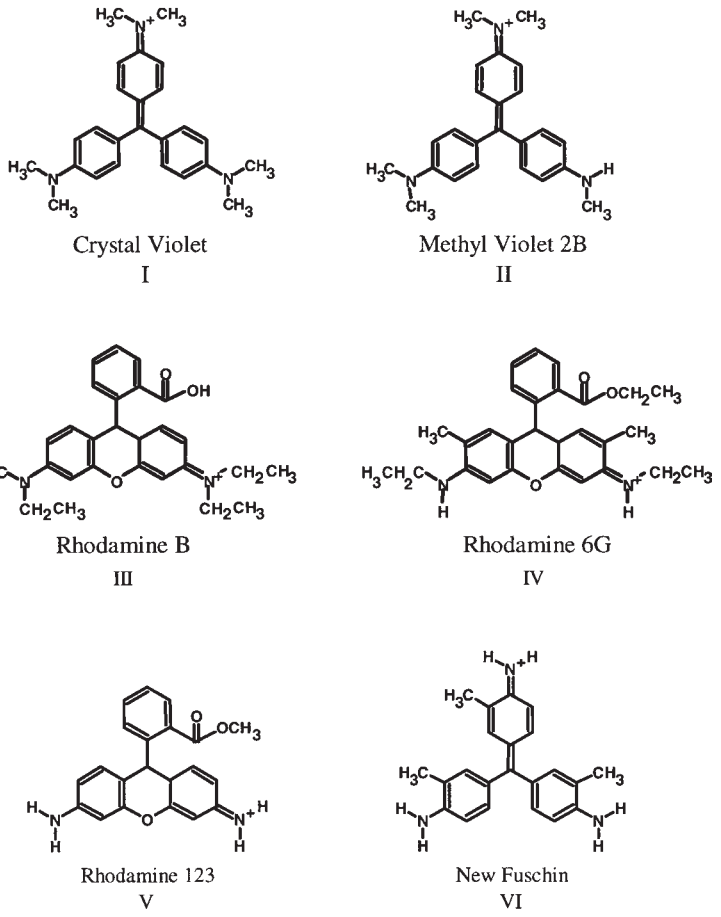

FIG. 1—*Dye structures.*

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. Details of the experiment have been reported previously (1). The calibrant used for positive and negative ion analysis was cesium iodide (CsI) (99.9%; Aldrich, Milwaukee, WI).

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

Analysis of Dyes on Paper—To parallel the analysis of the inks on paper, aqueous dye solutions were prepared, spotted on paper, and allowed to dry for subsequent LDMS analysis. The dyes used in this work are Rhodamine B (Sigma, St. Louis, MO), Rhodamine 6G (Aldrich, Milwaukee, WI), Rhodamine 123 hydrate (Aldrich, Milwaukee, WI), and New Fuchsin (Aldrich, Milwaukee, WI). Incandescent degradation was performed using a 75 W, 120 V light bulb.

Thin-Layer Chromatography—TLC was carried out using silica gel 150 Å TLC plates (Whatman, Ann Arbor, MI) with dimensions of 5 × 10 cm and a stationary phase thickness of 250 μm. The solvent system consisted of ethyl acetate:ethanol:water (70:35:30).

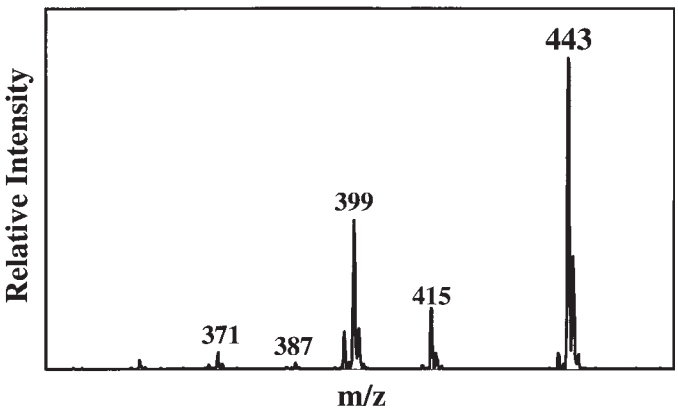

FIG. 2—*Positive ion LD mass spectrum of a red ink used to grade a biology exam from 1967.*

Results and Discussion

Photodegradation and LDMS Analysis of a Red Pen Ink Used to Grade a Biology Exam in 1967

Frequently, teachers use pens with red ink to grade exams. Figure 2 shows the positive ion LD mass spectrum of red ink from a 1967 high school biology exam. The ink was analyzed directly

from the paper using LDMS. The positive ion spectrum contains several peaks. Based on our experiences to date, we would begin an analysis by assuming that the most intense peak, at m/z 443, is representative of an intact dye, and the lower m/z, less intense mass spectral peaks represent the natural degradation products of the dye (1–5). From this mass spectrum, we were able to detect a mixture of at least six components. Each peak represents a component desorbed intact by the pulsed UV radiation. In LDMS, if a dye is neutral, then the dye would be detected in both positive and negative ion modes. Most often, a neutral dye (M) gains a positive charge by protonation, to form $(M+H)^+$, allowing the dye to be detected in positive ion LDMS, and obtains a negative charge by deprotonation, forming $(M-H)^-$ (1–5). Negative ion LDMS was also used to analyze this red ink. The resulting spectrum did not contain any peaks that would signify the presence of a dye that carried a negative charge. The absence of a peak at m/z 441 in negative ion mode lead to the conclusion that the dye present in this ink, represented by the peak at m/z 443, is a cationic dye.

According to US Patent 5,993,098 (6), two rhodamine dyes, Rhodamine B and Rhodamine 6G, can both be used to manufacture red pen ink. *The Sigma-Aldrich Handbook of Stains, Dyes, and Indicators* (7) reveals that the two rhodamine dyes are isomeric, cationic dyes (structures III and IV), and have a molecular mass of 443 Daltons (Da).

Rhodamine dyes have been used in a variety of systems from lasers to pens containing red ink. The family of rhodamine dyes has been produced by BASF since 1887 (8). The two possible dye candidates, Rhodamine 6G and Rhodamine B, could be represented by the peak at m/z 443 in the positive ion mass spectrum in Fig. 2. The dyes contain unique functional groups that distinguish them and will be most relevant to this work. Rhodamine B (III) contains a carboxylic acid group where Rhodamine 6G, (IV), contains an ester group. Also, 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. We will determine which of these two rhodamine dyes was the dye in the pen ink that was used to grade the biology exam in order to demonstrate the use of the photodegradation/LDMS approach. Since LDMS of each would presumably yield only a single peak in the spectrum at m/z 443, LDMS does not provide sufficient structural information about the dyes to determine which dye is present in the ink. Incandescent radiation can be used to induce dye degradation. The photodegradation products can be analyzed using LDMS to provide structural clues to aid in the identification of the dye.

LDMS Analysis of Some Red Ballpoint Pen Inks Currently Available

Several kinds of red ballpoint pens currently available were analyzed to further investigate the unknown red dye that was represented by the peak at m/z 443 in Fig. 2. Two specific patterns emerged as a result of analyzing and comparing the LD mass spectra of these red pen inks. Figure 3*a* shows a positive ion LDMS spectrum of red ink from a BIC Round Stic® ballpoint pen and represents the majority of the red ink samples from our small set, while Fig. 4*a* represents the smaller population of the red ink pens. Figure 4*a* is a positive LDMS spectrum of red ink from a BIC Cristal® ballpoint pen. LDMS analysis of the smaller population of the red ink pens generated mass spectra that contained predominantly one peak at m/z 443. Most of the red inks, upon positive ion LDMS examination, yielded mass spectra with two distinct peaks at m/z 443 and 399. These two peaks have a mass difference of 44 Da and could signify a mixture of dyes, or the peak at m/z 399 could represent a component in the pen ink that is not a colorant. Usually, only the dyes from the pen ink are detected with LDMS. Photodegradation was evaluated as a tool for dye structure determination and the results are presented here. Grim et al. (1) discussed UV photodegradation of Methyl Violet 2B as an approach for accelerated aging. This method is effective for rhodamine dyes as well. Alternative sources of photons for dye degradation were evaluated. Incandescent and fluorescent lights can also be used to induce the degradation of dyes. These two approaches have several advantages over UV irradiation. These light sources are inexpensive, safer to use, and readily available. The results presented here focus on the use of incandescent light as the source for inducing degradation.

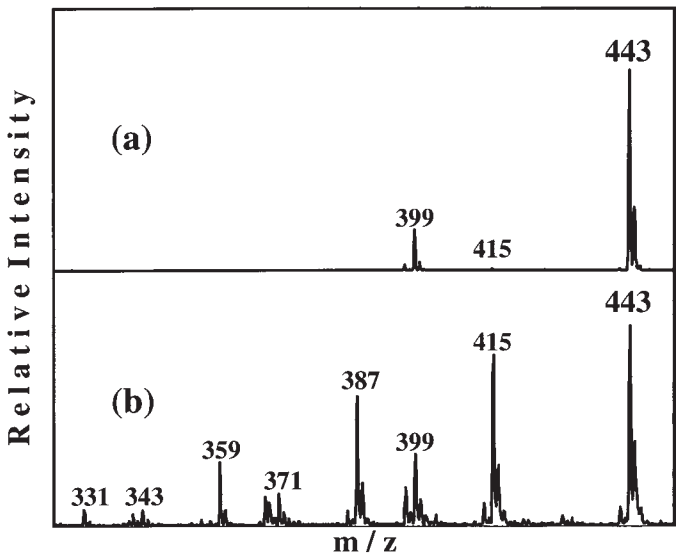

FIG. 3—*Analysis of red ballpoint pen inks currently available: positive ion LD mass spectra of BIC® Round Stic pen: (a) with no exposure to incandescent light and (b) exposed to incandescent light for 24 h.*

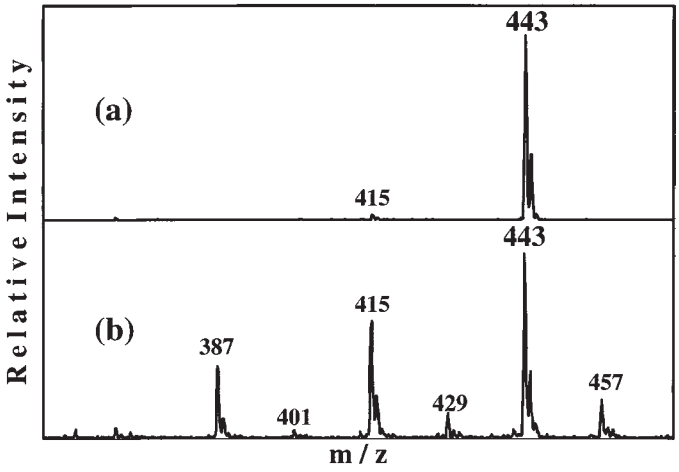

FIG. 4—*Analysis of red ballpoint pen inks currently available: positive ion LD mass spectra from BIC® Cristal pen: (a) with no exposure to incandescent light and (b) exposed to incandescent light for 24 h.*

Incandescent Irradiation as a Tool for Dye Characterization

The red pen ink samples were irradiated with incandescent light using a 75 W light bulb. Figures 3*b* and 4*b* show the positive LD mass spectra of the two representative red ballpoint pen inks, after 24 h of exposure to incandescent irradiation. There are some no-

table differences between the two spectra. The spectrum in Fig. 3*b* contains peaks at m/z 415, 387, 359, and 331, which are all photochemical degradation products from the initial dye, m/z 443. From m/z 443 to m/z 331, pairs of mass spectral peaks are separated by a mass difference of 28 Da, for a total of four losses of 28 mass units. Also, the series of peaks m/z 399 → 371 → 343 show mass differences of 28 Da. The spectrum in Fig. 4*b* also shows the sequential loss of 28 mass units from the intact dye, but there are only two losses of 28 mass units.

The rhodamine dyes are similar to Crystal Violet in that they are cationic dyes that contain alkylated amino groups. The degradation process for the rhodamine dyes should be similar to that of CV degradation, and should occur by oxidative N-deethylation. Oxidative N-deethylation results in substitution of ethyl groups (−29) by hydrogen atoms (+1). Rhodamine B has four possible ethyl groups that all can be replaced by hydrogen atoms which would lead to four degradation products, separated by mass differences of 28 Da. Rhodamine 6G only possesses two ethyl groups that can be lost. Thus, degradation products suggest that some red pen inks contain Rhodamine B and some contain Rhodamine 6G.

Analysis of Pure Rhodamine Dyes on Paper

Analyzing pure dyes will help to establish the identity of unknown dyes, can be used to establish the degradation characteristics of the dye directly, and can provide insight into the mechanism responsible for photodegradation. Photodegradation and LDMS analyses were carried out to examine the degradation of two pure rhodamine dyes directly, to confirm that the rhodamine dyes actually undergo N-deethylation as proposed. Aqueous solutions of Rhodamine B and Rhodamine 6G were prepared with pure rhodamine dyes, spotted on letterhead paper, allowed to dry, and were analyzed by LDMS before exposure to incandescent light. The samples were then subjected to 12 h of incandescent light, and again analyzed by LDMS. The positive ion spectra in Figs. 5*a* and 5*b* are from the analysis of Rhodamine B before and after exposure to incandescent radiation, respectively.

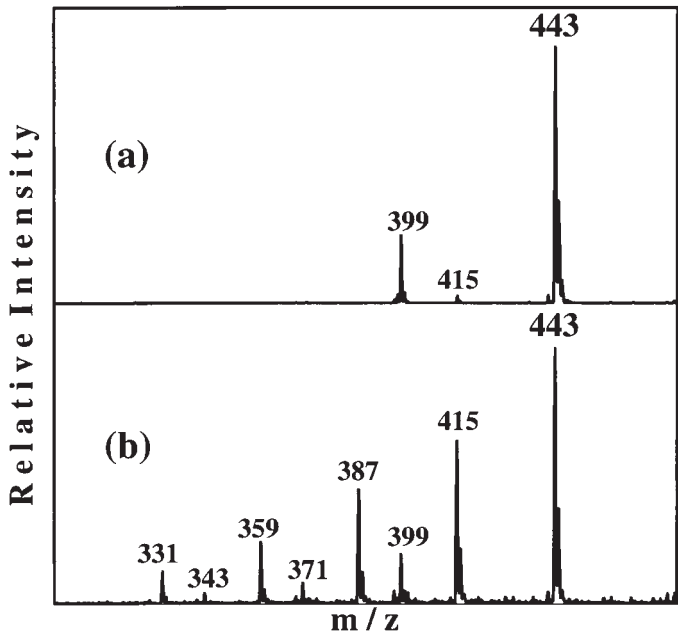

FIG. 5—*Analysis of pure rhodamine dyes: positive ion LD mass spectra of Rhodamine B (a) with no exposure to incandescent light and (b) exposed to incandescent light for 12 h.*

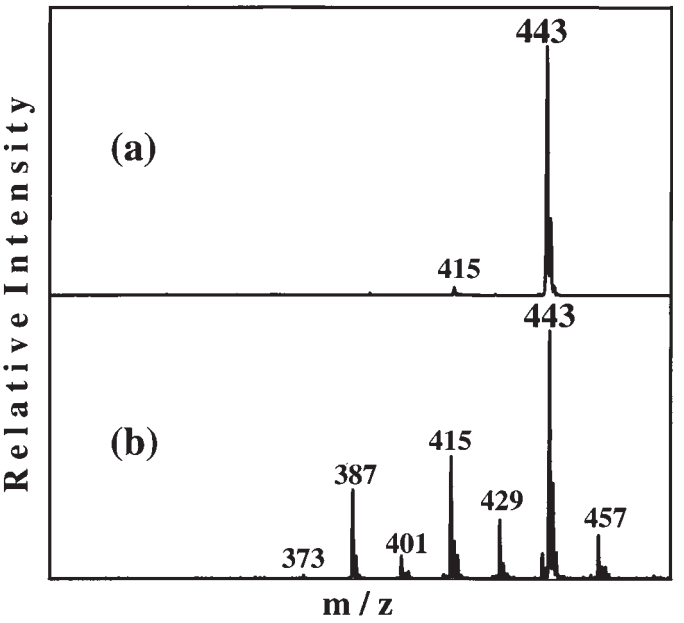

FIG. 6—*Analysis of pure rhodamine dyes: positive ion LD mass spectra of Rhodamine 6G (a) with no exposure to incandescent light and (b) exposed to incandescent light for 12 h.*

These spectra contain the same peaks that were present in the spectra of the red ink from the BIC Round Stic® pen. Figures 6*a* and 6*b* are LDMS spectra obtained from analyzing pure Rhodamine 6G. The spectra in these figures contain the same peaks that were present in the spectra of the red ink from the BIC Cristal® pen. The appearance of the characteristic peaks in the LDMS mass spectra for the rhodamine dyes suggests that the red ink from the BIC Round Stic® pen contains Rhodamine B, the red ink from the BIC Cristal® pen contains Rhodamine 6G, and the pen used to grade the biology exam in 1967 contained Rhodamine B. Further degradation induced by incandescent light of the 1967 red ink sample confirmed the presence of Rhodamine B (spectra not shown). Light-induced dye degradation combined with LDMS provides structural information that can be used to characterize and identify the suspected dyes.

The LDMS spectra of the two rhodamine dyes are different in that the spectrum of Rhodamine B consistently includes a small peak at m/z 399. We believe this peak represents a decarbonylated form of Rhodamine B $(RB\text{-}CO_2)^+$. The difference between the intact dye and this species is 44 Da, which corresponds to carbon dioxide. Rhodamine B, an acid, may decarbonylate while the analogous impurity would not be expected in Rhodamine 6G, which does not contain a free acid group. The compound corresponding to m/z 399 does not form negative ions in LDMS, so it is a cationic dye. It undergoes photochemical N-deethylation, just as the rhodamine dyes do, reflecting a similar structure. In this context it is interesting to note that while Rhodamine 6G is commercially available (Aldrich) at a purity of 99%, Rhodamine B (Sigma) is available at a purity of 95%. This is consistent with an impurity being present, and the impurity being $(RB\text{-}CO_2)^+$. Thus, the LDMS spectra of a red dye found in a pen can immediately suggest that the dye is Rhodamine B, if the impurity peak at m/z 399 is present. Photodegradation can then be used to confirm the dye structure.

Investigating Other Photoproducts of Rhodamine 6G

In our experiences to date, dyes can be photodegraded into smaller molecules using UV and visible light. Rhodamine 6G is the

first dye that we have encountered that also forms higher mass molecules photochemically. Specifically, Fig. 6*b* shows a peak at m/z 457, representing a photoproduct that is 14 amu *higher* than the intact dye peak. Peaks higher in mass are not photochemically formed from Rhodamine B, as seen in the spectrum in Fig. 5*b*. To investigate this, two additional dyes were selected, Rhodamine 123 (V) and New Fuchsin (VI). Figure 7 shows the LDMS spectra of Rhodamine 123, before and after exposure to incandescent light. The peak at m/z 345 corresponds to the intact dye cation. Exposure for 133 h did not lead to any photoproducts for this small rhodamine dye. Figure 8 shows that New Fuchsin also forms both expected photodegradation products and higher mass photoproducts (following exposure to incandescent radiation for 211 h), as did Rhodamine 6G. In contrast to Rhodamine 6G, New Fuchsin formed several photoproducts, including two representing increases in mass of the intact dye by 14 mass units (m/z 344, 358). The structural features that Rhodamine 6G and New Fuchsin share are methyl groups, ortho to the amine functionalities, on the aromatic rings. In addition, they have less than a full complement of alkyl groups on the nitrogen atoms. Since the formation of higher mass photoproducts occurs for just these dyes alone on paper, one need not consider other components of the ink as possible reactants. While these insights alone do not establish a mechanism, bimolecular disproportionation reactions of dye molecules, such as that shown in Fig. 9, may contribute to the observed photoproducts.

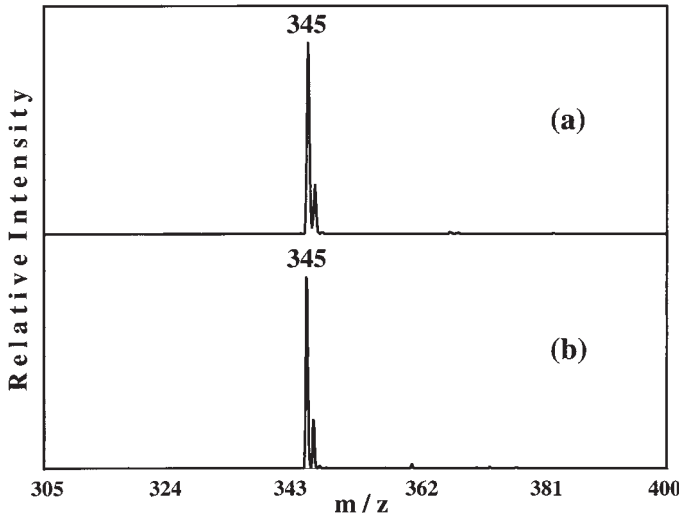

FIG. 7—*LDMS analysis of Rhodamine 123: (a) with no exposure to incandescent light and (b) exposed to incandescent light for 133 h.*

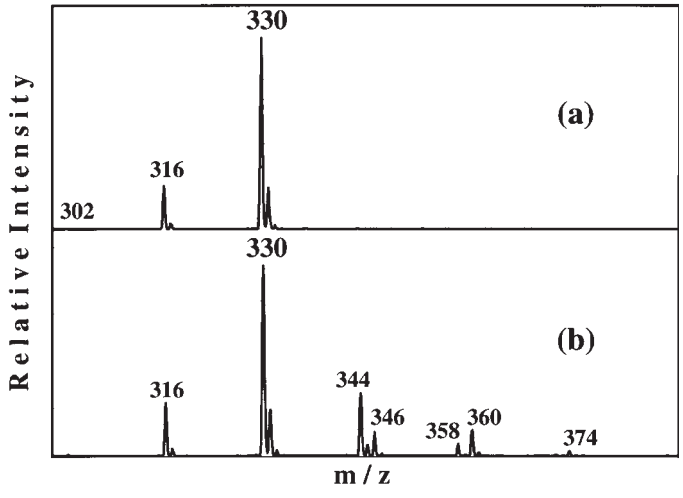

FIG. 8—*LDMS analysis of New Fuschin: (a) with no exposure to incandescent light and (b) exposed to incandescent light for 211 h.*

Ink Batch-to-Batch Dye Variation

From the data presented one might assume that BIC Round Stic® red pens contain Rhodamine B and BIC Cristal® red pens contain Rhodamine 6G. This however, is not the case, since it appears that ink in pens of the same type from different batches have different composition. Six BIC Round Stic® pens were collected from different sources, and the dyes in the inks of each pen were examined by LDMS. There are distinct differences in the LDMS spectra that differentiate among batches of pen inks of the same brand. The spectra of the six pen inks could be generally classified into three categories. Figures 10*a*, 10*b*, and 10*c* show positive LDMS spectra of three BIC Round Stic® ballpoint pens 1, 2, and 3. The spectra show variations in ink dyes used in their manufacture. The peaks in the mass spectra of red ink dyes represent Rhodamine B and Rhodamine 6G, but there appears to be an additional dye, which is used in combination with Rhodamine B. The unknown additional dye will be referred to as Dye 533. Thin-layer chromatography (TLC) was used to identify the color of the additional dye. Ink dye separation by TLC yielded two bands, one band colored red and the other band colored yellow. The red TLC band corresponds to Rhodamine B and the yellow band corresponds to Dye 533. The yellow band was extracted, spotted on letterhead paper, and analyzed. The relative intensity of the peak at m/z 533 compared with the Rhodamine B peak varies between the pen inks as well. The mass spectrum in Fig. 10*a* shows that pen 1 contains predominantly Rhodamine B and a very small amount of Dye 533 compared with Rhodamine B. Figure 10*b* shows the mass spectrum of ink from pen 2 which consists of approximately 54% RB, 13% of the impurity RB-CO_2, and 33% Dye 533 (assuming an equal LDMS response for each dye). Dye 533 appears to be a yellow, cationic dye

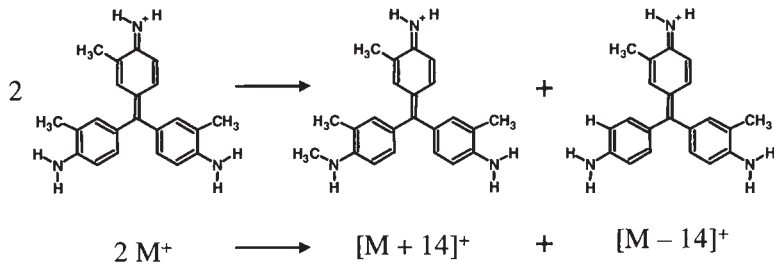

FIG. 9—*Suggested mechanism for the formation of photoproducts.*

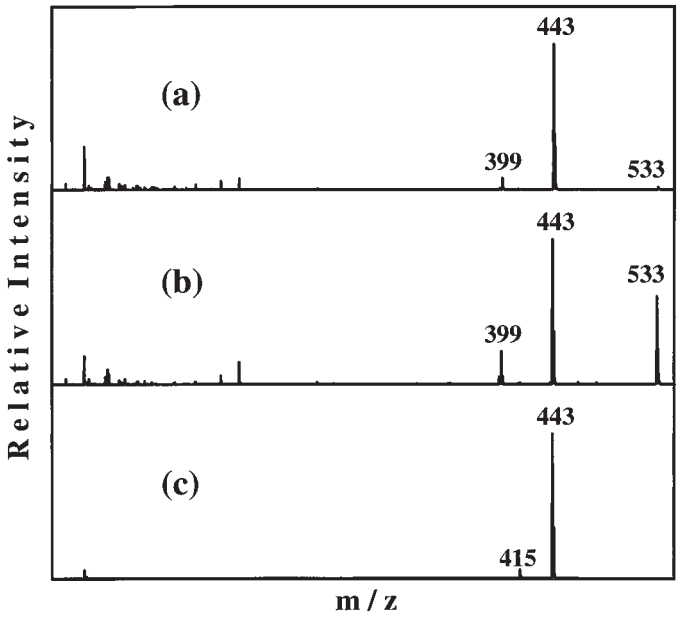

FIG. 10—*LDMS analysis of variations in ink dye composition in BIC® Round Stic red ballpoint pen ink: (a) pen 1, (b) pen 2, and (c) pen 3.*

with a mass of 533 Da, which contains ethylated nitrogen atoms. Photodegradation of the dye on paper was separately carried out using incandescent and UV radiation. The radiation caused the dye to N-deethylate a maximum of four times since four degradation peaks appeared, each separated by 28 mass units. Induced degradation of pure Rhodamine B does not produce a photoproduct with a mass of 533 Da; however, the peak at m/z 533 cannot be completely eliminated as a photoproduct from Rhodamine B, since ink is a complex system. Ink contains other components that could react with Rhodamine B in pen ink to from a product with a mass of 533 Da when photodegraded, if all dyes can be desorbed and ionized with equal efficiency. Additional analysis is required; the purpose of this work is not to evaluate yellow dyes, but to point out that there are other dye components in red ink that can be detected by LDMS.

Conclusion

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

Batch-to-batch variations are found in the dyes used in red pens of a single type from a single manufacturer. From the standpoint of addressing questions such as "Could this writing have come from this pen?" this is certainly a preferable situation increasing the probability of implicating a particular pen.

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

References

1. Grim DM, Siegel JA, Allison J. 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(6):1265–73.
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(6):1411–20.
3. Grim DM, Siegel JA, Allison J. Does ink age inside of a pen cartridge? J Forensic Sci 2002;47(6):1294–7.
4. Grim DM, Dunn JD, Allison J, Siegel JA. Characterizing the degradation of methyl violet from inks on paper using laser desorption mass spectrometry for the analysis of questioned documents. American Academy of Forensic Sciences 54th Annual Conference; 2002 Feb; Atlanta (GA).
5. Dunn JD, Grim DM, Allison J, Siegel JA. Molecular level information on the aging of dyes from ink on paper: what's there and how does it change? American Academy of Forensic Sciences 54th Annual Conference; 2002 Feb; Atlanta (GA).
6. Osada T, inventors. Mitsubishi Pencil Kabushiki Kaisha, assignee. Aqueous gel ink-filled ballpoint pen. US Patent 5,993,098 Nov 1999.
7. Green FJ. The Sigma-Aldrich handbook of stains, dyes, and indicators. Milwaukee: Aldrich Chemical Company, 1990.
8. Ball P. Bright earth: art and the invention of color. New York: Farrar, Straus and Giroux, 2001.

Additional information and reprint requests:
John Allison
335 Chemistry Building
Michigan State University
East Lansing, MI 48824-1322
E-mail: allison@cem.msu.edu