J Forensic Sci, September 2007, Vol. 52, No. 5
doi: 10.1111/j.1556-4029.2007.00535.x
Available online at: www.blackwell-synergy.com

Jamie D. Dunn,[1] *M.S. and John Allison,*[2] *Ph.D.*

The Detection of Multiply Charged Dyes Using Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry for the Forensic Examination of Pen Ink Dyes Directly from Paper

ABSTRACT: Laser desorption mass spectrometry (LDMS) is emerging as a technique for questioned document examination. Its use is limited to detecting ink dyes that are neutral or singly charged. Several inks contain dyes that are multiply charged and LDMS cannot be employed for their identification. We have successfully detected >20 polyionic dyes that can be used in the manufacture of inks using matrix-assisted laser desorption/ionization (MALDI) MS, directly from paper, with the matrix, 2-(4-hydroxyphenylazo)benzoic acid (HABA), and the additive, diammonium hydrogen citrate (DAHC). For example, Acid Violet 49, a charged dye containing one positively-charged site and two negatively charged sulfonate groups, cannot be detected by LDMS, but forms intact, singly charged ions in the MALDI MS experiment. The method described is also useful for identifying multiply charged dye mixtures that are used in modern pen inks.

KEYWORDS: forensic science, questioned documents, laser desorption mass spectrometry, matrix-assisted laser desorption/ionization mass spectrometry, pen, ink, dye, multiply charged dye, additive

In 2001 (1), it was first demonstrated that the organic dye crystal violet, from a blue ballpoint pen ink, could be detected directly from paper using laser desorption mass spectrometry (LDMS). The method is attractive for questioned document examination, as no extraction step is required—molecules are desorbed and ionized directly from the paper surface, and many mass spectra can be generated from the ink in a single pen stroke. LDMS has been used to analyze dyes from blue, black, and red ballpoint pen ink dyes (1–3). For a red pen ink dye such as rhodamine 6G, $[C_{28}H_{31}N_4O_2]^+[Cl]^-$, in which the colorant is cationic, a single peak appears in the positive-ion LDMS spectrum representing the intact, positively-charged species (3). In contrast, when a dye is anionic, such as Solvent Black 29, $[Na^+][C_{32}H_{18}N_6O_8Cr^-]$, a single peak appears in the negative-ion LDMS spectrum, representing the intact negatively-charged colorant (1). In the case of copper phthalocyanine, $C_{32}H_{16}N_8Cu$, a neutral pigment that is commonly found in the blue ink of gel pens, the molecule yields both positive and negative molecular ions (4,5). Thus, the charges of the gas phase ions formed in the LDMS experiment provide important clues about the natural charge state of the colorant.

Laser desorption mass spectrometry was introduced over 30 years ago (6). Since then, lasers have become smaller and less expensive. Substantial developments have been made in the technique of time-of-flight (TOF) mass spectrometry (7), a method that is ideal for use with a pulsed ionization technique such as LD. Commercially available laser-TOF MS instruments are sold to do a more recently developed experiment, matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS). In the MALDI MS experiment, analytes at the picomole level are mixed with an excess of organic "matrix" molecules that absorb UV light at 337 nm (the wavelength of light from a N_2 laser). When a few microliters of an analyte/matrix solution are deposited on the surface of a metal sample introduction plate and allowed to dry, crystals are formed. These matrix crystals, with analyte contained therein at an impurity level, are the MALDI target, which absorbs the laser light. While the matrix absorbs the energy, gas phase ions from both the matrix and the analyte are formed. The MALDI MS experiment, along with electrospray ionization (ESI) MS, have become the methods of choice for a wide range of analytes, from proteins to oligonucleotides (8,9).

While the use of modern MALDI TOF MS instruments for the simpler, matrix-free LDMS analysis of ink colorants is an attractive one, we have recently found that many dyes could not be detected by LDMS. Some dyes, either on paper in the form of a pen stroke, or single components isolated from pen inks using thin layer chromatography (TLC), do not yield molecular ions in the LDMS experiment. In some cases, ions are formed, but they have low m/z values (<200) and could not represent intact dye molecules.

A review of the patent literature resulted in the identification of hundreds of colorants that can be used in pen inks. Many of the dyes used in gel pens and liquid ink pens shared a common feature—they contain multiple charged-groups. The dyes may be polyanionic, or may contain a mixture of positive and negative charged groups. 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 it cannot be detected by LDMS. The energetic limitations in a similar experiment have been discussed by Asara et al. (10). In the LDMS literature and the MS literature, in general (except for ESI MS), ionization and desorption/ionization mechanisms yield predominantly singly charged ions.

[1]Department of Chemistry, Michigan State University, East Lansing MI 48824.
[2]Department of Chemistry, The College of New Jersey, Ewing NJ 08628.

Received 24 Dec. 2006; and in revised form 25 Mar. 2007; accepted 8 April 2007; published 7 Sept. 2007.

We report here that polyionic dyes such as those found in pen inks do not desorb intact in the LDMS experiment, although they may yield fragment ions upon UV laser irradiation. In 1997, the analysis of multiply charged dyes by MALDI MS was reported (11). We show here that MALDI MS can be used to detect polyionic dyes, directly from paper, as singly charged ions with the addition of a MALDI matrix and additive. We show that dyes on paper, that cannot be detected by LDMS, can be detected by MALDI MS. The experiment demonstrates the same simplicity and sensitivity as LDMS, and requires only a minor modification (addition of a few microliters of matrix and analyte solutions to the ink-on-paper sample). While the LDMS spectra do not show desorption of intact multiply charged dyes, LD can generate low mass fragment ions that may suggest that such a multiply charged dye is present (and thus MALDI MS is required), and may provide initial information on the structure of the dye.

Materials and Methods

Mass Spectrometry

Instrumentation—Laser desorption mass spectrometry and MALDI mass spectra were acquired using a PE Biosystems Voyager DE time-of-flight mass spectrometer (Framingham, MA). The instrument utilizes a pulsed nitrogen laser (337 nm, 2 nsec 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 kV for detection of positive ions and −15 kV for the detection of negative ions, an intermediate source grid voltage that was 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 nsec. The MALDI MS experiments utilized the same parameters except for the analyses on the paper substrate, which in some cases, were optimized with a delay time of 200 nsec.

Laser Desorption Mass Spectrometry

Ink on Paper—Single lines were written on MSU letterhead paper, mounted on the sample plate using Scotch double stick tape, and analyzed directly. The nitrogen laser was focused on a pen stroke, which was approximately 0.3–0.4 mm wide. The mass spectra of a black ink from a BIC Cristal® ballpoint pen (BIC USA Inc., Milford, CT) and blue, liquid ink from a Pilot Precise® V7 rollerball pen (Pilot Pen Corporation of America, Trumbull, CT) are presented.

Dyes on Paper—To simulate the analysis of ink on paper, 10 μmol/μL dye solutions were prepared using 1:1 (v/v) methanol/water, spotted (5 μL) on paper, and allowed to dry prior to analysis by LDMS. Results for the analysis of Acid Violet 49 (Sigma, St. Louis, MO) are presented.

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

Dyes on a Sample Plate—A 100-well stainless steel MALDI sample plate was used. Diammonium hydrogen citrate (DAHC; J.T. Baker, Phillipsburg, NJ) was used as an additive and 2-(4-hydroxyphenylazo)benzoic acid (HABA; Aldrich, Milwaukee, WI) was used as the matrix. An aqueous 100-mM DAHC, 24-mg/mL HABA (6 mg in 2:2:1 acetonitrile/water/methanol), and 100-pmol/μL dye (1:1 methanol/water) solutions were used. A 1-μL aliquot of each solution was spotted in a single well in the following order: HABA, DAHC, dye. MALDI mass spectra of a red, liquid ink from a Sanford Uni-ball® Roller rollerball pen (Sanford, Oak Brook, IL) are presented. Ink containing copper phthalocyanine was used as the calibrant for both positive and negative-ion modes.

Dyes on Paper—A 5-μL aliquot of a 10-μmol/μL dye solution (1:1 methanol/water) was spotted on MSU letterhead paper and allowed to dry. Two 1-μL aliquots of 100-mM DAHC were first applied to the paper samples, followed by three 2-μL aliquots of 24-mg/mL HABA solution. Small squares (4 mm^2) were cut from the dye-stained paper and secured onto a modified MALDI plate using Scotch double-coated tape. Ink containing copper phthalocyanine was used as the calibrant for both positive and negative-ion modes. The ink samples were allowed to dry on paper before cutting and mounting them adjacent to the dye-on-paper samples.

Ink on Paper—Single pen strokes were made on MSU letterhead paper and 4-mm^2 squares were cut from the paper and mounted on a modified MALDI plate using Scotch double-coated tape. Two 1-μL aliquots of 100-mM DAHC were first applied to the paper samples, followed by three 2-μL aliquots of 24-mg/mL HABA solution. Ink containing copper phthalocyanine was used as the calibrant for both positive and negative-ion modes. The ink was allowed to dry on paper before cutting and mounting them adjacent to the dye-on-paper samples.

Thin-Layer Chromatography

Thin-Layer Chromatography was carried out using 150-Å K5 F silica gel plates (Whatman, Ann Arbor, MI) with dimensions of 5 × 10 cm and a stationary phase thickness of 250 μm. The mobile phase consisted of 75:35:30 (v/v) ethyl acetate/ethanol/water. A dye mixture of Methyl Violet 2B and Crystal Violet (Aldrich, Milwaukee, WI) was used as a standard for comparison. To extract the dyes from the TLC plate, the colored bands were scraped from the TLC plate, placed in a centrifuge vial, and 5–10 μL of 1:1 (v/v) ethanol/water were added. The vials were vortexed, and the silica was separated from the dye solutions by centrifugation.

Results and Discussion

Laser desorption mass spectrometry has been shown to be an attractive technique for the analysis of a variety of red and blue ballpoint pen inks, by direct laser "sampling" of pen strokes on a questioned document. We have analyzed several black ballpoint pen inks from various manufacturers as well. TLC analysis of a typical black ballpoint pen ink shows the presence of two soluble dyes, one blue and one yellow. The positive- and negative-ion LD mass spectra, acquired when such a black ballpoint pen ink is examined directly from paper, are shown in Figs. 1*a* and 1*b*, respectively. Each spectrum contains one relatively intense peak that represents a dye. The ink contains Crystal Violet (Basic Violet 3) and Metanil Yellow (Acid Yellow 36) whose structures are shown in Figs. 2*a* and 2*b*, respectively. Crystal Violet $[C_{25}H_{30}N_3]^+[Cl]^-$ and Metanil Yellow $[Na]^+[C_{18}H_{14}N_3O_3S]^-$ are manufactured as the chloride and sodium salts, respectively. The positive-ion LD mass spectrum contains a peak at *m/z* 372 representing the desorption of the intact Crystal Violet cations. The peak at *m/z* 352 in the negative-ion mass spectrum represents the intact anions of Metanil Yellow.

In general, the most intense peaks in the LD mass spectra represent the colorants that are present in the ink. The other components in the ink mixture insufficiently absorb the UV photons, which is necessary to ensure desorption/ionization and ultimately, the detection of the species. An intriguing aspect of the negative-ion

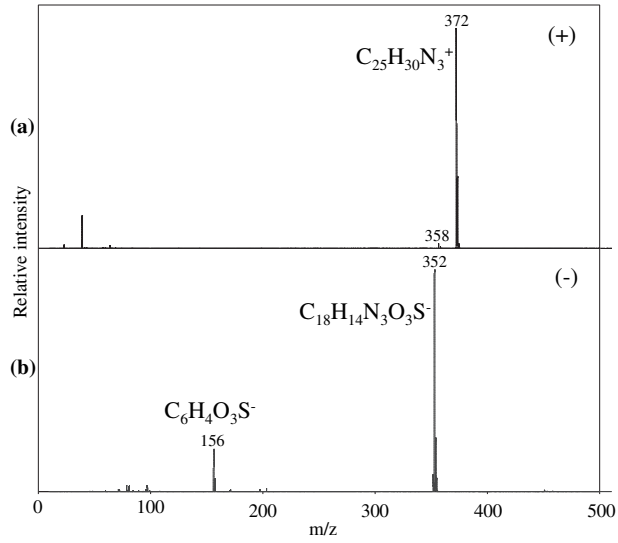

FIG. 1—*(a) Positive and (b) negative-ion LDMS spectra of a black ballpoint pen ink.*

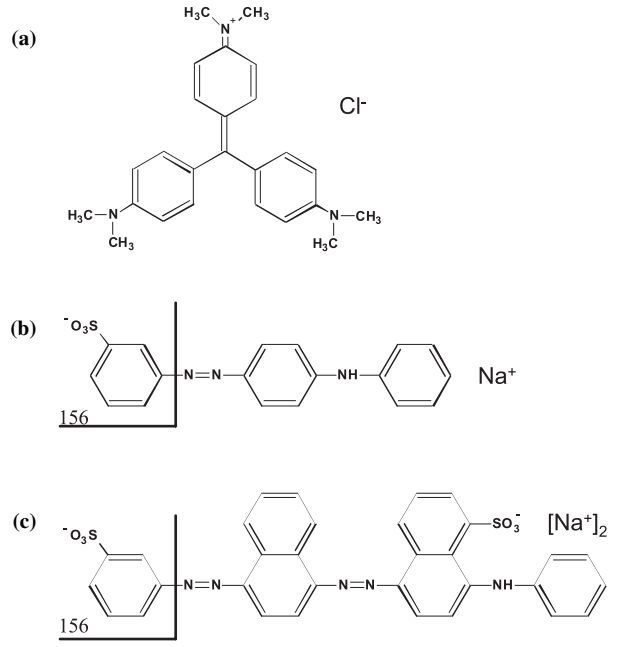

FIG. 2—*Structures of (a) Crystal Violet, (b) Metanil Yellow and (c) Acid Blue 113.*

spectrum is that Metanil Yellow, while detected intact, also fragments. The peak in Fig. 1*b* at *m/z* 156 appears to be a fragment ion of the dye, representing $[C_6H_4SO_3]^-$. The ion is also formed in mass spectrometry experiments as a fragment ion from Acid Blue 113, when the dye is analyzed by capillary zone electrophoresis/tandem mass spectrometry (12). The fragment of the dye structure of Acid Blue 113 is designated in Fig. 2*c*. Metanil Yellow and Acid Blue 113 share a common terminal substructure, which results in the formation of the same fragment ion. Also of note is the small peak at *m/z* 358 in Fig. 1*a*. It is known that Crystal Violet is usually sold as a mixture (1,2). In addition to the structure shown in Fig. 2*a*, there are similar molecules present which may contain less than six methyl groups. Whenever a methyl group on a nitrogen atom is replaced by a H atom, the net mass change is −14 Da. In some samples containing Crystal Violet, a series of peaks separated by 14 Da (372, 358, 344, 330, etc.) can be seen. These are not because of fragmentation of the *m/z* 372 ions, but represent distinct components of the ink.

Not all pen inks respond in LDMS as shown in Fig. 1. As an example of an experiment that did not work, consider the results from the analysis of a blue liquid ink from a Pilot Precise® V7 roller-ball pen. TLC data suggested that two soluble dyes, one blue and one purple, were in the ink. However, the positive- and negative-ion LD mass spectra, Figs. 3*a* and 3*b*, provide minimal information. The most abundant peaks in the positive-ion LD mass spectrum (Fig. 3*a*) have *m/z* values of 23 and 39, which correspond to sodium and potassium ions. These are almost always present in the mass spectra of inks. The negative-ion LD mass spectrum (Fig. 3*b*) contains peaks at *m/z* 79 and 170. While these *m/z* values are too small to represent colorants, they may be in some way related to the dyes present. The LD mass spectra in Fig. 3 demonstrate that LDMS does not always provide direct information on colorants in an ink.

Hundreds of dyes are cited as possible pen ink colorants in the patent literature. Many of them contain multiple charge groups—i.e., the dyes are polyionic. Most of these dyes are polysulfonated azo dyes, and can be found in ballpoint, gel, and liquid inks. While the patent literature suggests these are commonly used, we have not detected such dyes using LDMS. This makes sense, as multiply-charged ions cannot be directly desorbed in the LDMS experiment. They can, however, be detected by MALDI MS. Multiply-charged analytes such as phosphorylated peptides and oligonucleotides have been successfully analyzed by MALDI MS, when additives are used (13–19), which can donate protons to polyanionic analytes, lowering their charge to −1 or +1. Some MALDI MS work has been reported on the analysis of multiply-charged dyes (11).

A number of additives known to assist in such experiments were evaluated, with a variety of common MALDI matrices, to select a matrix/additive combination that would be compatible with polyionic dyes found in pen inks. Initially, these experiments were performed as a standard MALDI MS experiment, in which matrix/analyte crystals are irradiated on a standard stainless steel

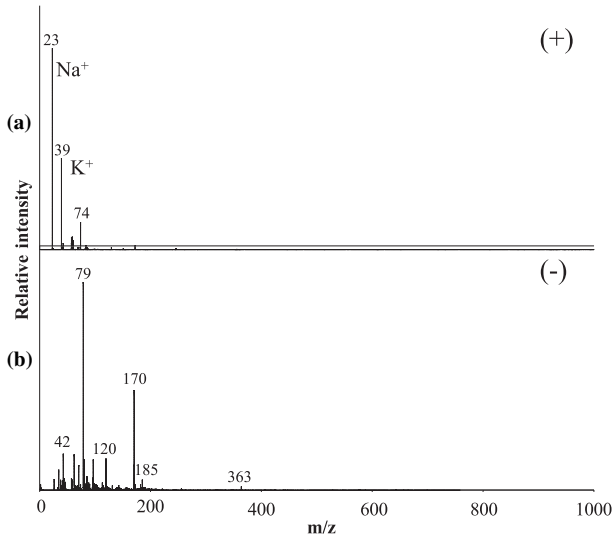

FIG. 3—*(a) Positive and (b) negative-ion LDMS spectra of a blue liquid ink.*

MALDI sample plate. Next the method was extended to determine how it could be used when the dyes were in a pen stroke on paper.

In the MALDI MS experiments, several MALDI matrices with and without additives were investigated for the enhanced detection of available polyionic dyes. The MALDI matrices α-cyano-4-hydroxycinnamic acid (α-CHCA) and 2-(4-hydroxyphenylazo)benzoic acid (HABA), in conjunction with diammonium hydrogen citrate (DAHC), allowed for the detection of multiply charged ink dyes from a stainless steel plate.

Identification of a MALDI matrix/additive system for the analysis of polyionic dyes is a useful method for the analysis of ink samples. One could perform a TLC experiment, extract each band, and analyze each dye by MALDI MS. However, detection of dyes in a written line directly from a paper substrate is preferred in the field of questioned document examination. Paper is certainly not an ideal substrate for a MALDI experiment, since MALDI requires the incorporation of the analyte into matrix crystals. Growing crystals on paper, to detect the dyes, has been a challenge. We focused on α-CHCA and HABA with the DAHC additive for dye analyses directly from paper, as these yielded the best results for analyses on the metal plate. While growing matrix crystals of α-CHCA on paper was found to be rather difficult and insufficient for detecting the dyes, we had success with HABA. The procedure that works well involves adding 1 μL of 100 mM DAHC to the dye sample on paper followed by the matrix. The matrix solution is added to the selected location on the document in 1 μL increments until matrix crystals can be seen on the surface of the dye-paper sample. Typically, three 1 μL aliquots of HABA were sufficient. Only a portion of the soluble dyes is extracted and incorporated into the matrix crystals, but the amount is sufficient to yield a strong ion signal. Best results are obtained when the matrix and additive are added consecutively, without allowing the target to dry between additions. Several commercial dyes on paper were examined first. The method was then applied to actual inks on paper.

Figure 4 demonstrates the success of using HABA and DAHC; it shows the (*a*) positive- and (*b*) negative-ion MALDI mass spectra of Acid Violet 49, a polyionic dye, from paper, using the procedure described above. The charge distribution of Acid Violet 49 is shown in Fig. 5*a*. The dye contains two anionic phenyl sulfonate groups and one cationic iminium group. While the organic moiety

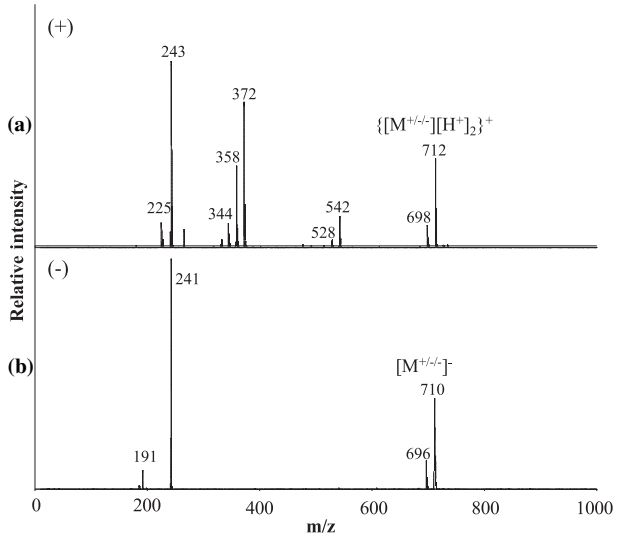

FIG. 4—*(a) Positive and (b) negative-ion MALDI mass spectra of Acid Violet 49 on paper.*

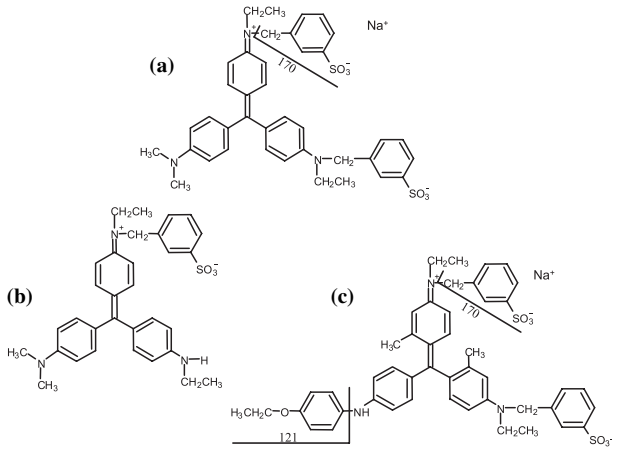

FIG. 5—*Structures of (a) Acid Violet 49, (b) a proposed dye, and (c) Acid Blue 90.*

has a net charge of –1, it carries three charges and thus is a multiply charged dye. It cannot be detected directly by LDMS. Acid Violet 49 (20) is sold as a sodium salt, $[Na]^+[C_{39}H_{40}N_3O_6S_2]^-$; the compound has a molecular weight of 733 Da. To indicate it's charge state, the molecule will be designated as $[Na^+][M^{+/-/-}]$. When the dye undergoes desorption/ionization in the presence of the DAHC, ammonium ions can interact with $-SO_3^-$ groups and donate protons. The dye is detected in cationic form, $\{[M^{+/-/-}][H^+]_2\}^+$ at *m/z* 712, in Fig. 4*a*, and as the $[M^{+/-/-}]^-$ anion, *m/z* 710, in Fig. 4*b*. These are the peaks associated with Acid Violet 49. There are other peaks in the spectrum that appear to represent other components.

Dyes are commonly sold as mixtures, and apparently Acid Violet 49 is no exception. In Fig. 1*a*, the spectrum representing crystal violet indicates that the compound is a mixture of similar compounds. A similar situation appears for Acid Violet 49. The peak at *m/z* 372 corresponds to Crystal Violet and the peak at *m/z* 542 most likely represents the dye proposed in Fig. 5*b*, which is a homologue of Acid Violet 49. All three components have the triaryl methane core (central C with three phenyl groups attached). For each major component of the dye in Fig. 4*a*, with *m/z* values of 712, 542 and 372, there are peaks 14 Da lower, as each component is also in different states of methylation, as was discussed previously for Crystal Violet. Such peaks are characteristics of the dyes and these clues can be useful when identifying an unknown dye.

The differences between the positive and negative-ion spectra of Fig. 4 are important to note. With the charge state $[M^{+/-/-}]^-$, Acid Violet 49 can desorb directly as a negative ion in the MALDI experiment, or can form a positive ion upon addition of two protons from the matrix additive. Thus, the dye is detected in both the positive and negative-ion modes. The Crystal Violet dye is a simple cation $[M^+]$. It (and its demethylated forms) can form gas phase cations but not anions, so they are only detected in the positive-ion mode. Similarly, the compound shown in Fig. 5*b* has a $[M^{+/-}]$ charge structure. Addition of a proton yields the peak at *m/z* 542 in the positive-ion spectrum, but it cannot form a negative ion, so there is no corresponding peak in Fig. 4*b*.

The peaks at *m/z* 243 and 241 in the positive- and negative-ion MALDI mass spectra in Fig. 4 correspond to the HABA matrix. HABA is a solid, neutral, organic molecule and has a molecular weight of 242 Da. The matrix molecules become protonated (gains a hydrogen ion) or deprotonated (loses a hydrogen ion) during the

desorption/ionization process and give rise to these peaks in the mass spectra.

Figure 3 showed LDMS data for a blue pen ink that "did not work." The TLC data showed that at least two dyes were present. To determine if the ink contained multiply-charged dyes, the MALDI matrix HABA and the additive DAHC were desposited on a pen stroke on paper. Shown in Fig. 6 are the positive and negative-ion MALDI mass spectra of the same blue, liquid ink on paper. The MALDI analysis of the ink on paper successfully detected two dyes. The peaks at *m/z* 712 and 710 in the positive- and negative-ion mass spectra, respectively, correspond to Acid Violet 49, as discussed in Fig. 4. Demethylated forms of this dye are again observed 14 Da lower in each spectrum, and the smaller variant detected in the positive-ion spectrum at *m/z* 542 still appears. The peaks at *m/z* values 832 and 830 in the positive- and negative-ion mass spectra, respectively, represent a second component. The *m/z* information, isotopic peaks, dye color, and the fact that it cannot be detected by LDMS but can be by MALDI MS, is consistent with a known multiply-charged dye, Acid Blue 90 (20), shown in Fig. 5*c*. There are a number of noteworthy features of the spectra shown in Fig. 6 concerning this additional component. We have encountered Crystal Violet, with six methyl groups attached to nitrogen atoms (Fig. 2*a*). For more complex variations of Crystal Violet, such as Acid Violet 49, shown in Fig. 5*a*, the number of methyl groups on the *N*'s decreases to two, and for Acid blue 90, Fig. 5*c*, the number is zero. Thus, there are no peaks 14 Da lower than the peak representing the intact dye. As Acid Blue 90 is an $[M^{+/-/-}]$ type of dye, it can also form both a positive and a negative ion, separated in mass by 2 Da. Direct MALDI analysis of Acid Blue 90 (not shown) confirmed that this is the correct assignment.

It appears that another dye is present in the blue, liquid ink. The small peaks in Fig. 6 at *m/z* 882 and 880 in the positive- and negative-ion MALDI mass spectra, respectively, may correspond to a third dye that is similar in charge structure ($[M^{+/-/-}]$) to the other two dyes. No identification has yet been made, however, a possibility can be proposed. Consider the structure shown in Fig. 5*c*. There is one "unoccupied site," one H− on a nitrogen where a larger group could be added. As shown, the $C_2H_5OC_6H_4-$ group has a mass of 121 Da. If the H atom is replaced by a second group of this mass, the *m/z* value of the ion would shift by 120 mass units. Since the 832/712 (positive ions) and 830/710 peaks differ by 120 Da, this may be suggestive of the structure of the additional dye homologue.

The blue ink of Figs. 3 and 6 contains multiply-charged dyes containing aromatic sulfate groups; we note that the peak at *m/z* 170 in the Fig. 3*b*, the negative-ion LD mass spectrum of the liquid blue ink, can now be identified as a fragment ion that could evolve from Acid Violet 49 and/or Acid Blue 90, as shown in Figs. 5*a* and 5*c*, and has the formula of $[C_7H_6O_3S]^-$. LD mass spectrometric analyses of both dyes were performed and the negative-ion mass spectra of both contain a peak representing this 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 suggest that the ink contains a dye with the toluenesulfonate group. The multiply charged dyes could not desorb with the energy available, so bond cleavage occurs to generate a singly charged fragment that is detected. Dye fragmentation demonstrates that LD mass spectra of ink directly from paper can be used to make such associations.

When an ink contains several polyionic dyes, it may be useful to combine data from multiple experiments. One can obtain LDMS spectra, MALDI MS spectra, and perform TLC. Each band can be eluted from the TLC plate and subjected to standard MALDI analysis. This can be a powerful approach, as is presented here for a red liquid ink from a Sanford Uni-ball® rollerball pen.

The positive and negative-ion MALDI mass spectra that were obtained for this red ink, from the stainless steel sample plate, is shown in Fig. 7. The mass spectra are quite different than those shown previously. There are several peaks in the negative-mass spectrum (Fig. 7*b*) and only one predominant peak in the positive-ion mass spectrum (Fig. 7*a*). From an initial examination of the negative-ion mass spectrum, there may be five or more dyes in the ink. TLC analysis yields three bands (yellow, red, and red–orange). Each dye was extracted from the TLC plate and subjected to MALDI MS analysis separately. As the negative-ion spectrum, Fig. 7*b*, was the richest, we focus on the negative-ion spectrum of each separated band. Figure 8 shows the resulting negative-ion mass spectra. The yellow dye forms a negative-ion in the MALDI MS experiment at *m/z* 407. The red dye yields a peak at *m/z* 647.

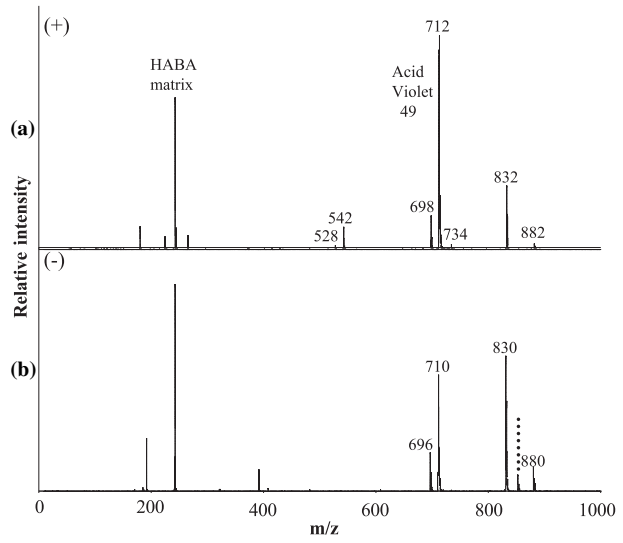

FIG. 6—*(a) Positive and (b) negative-ion MALDI mass spectra of a blue liquid ink.*

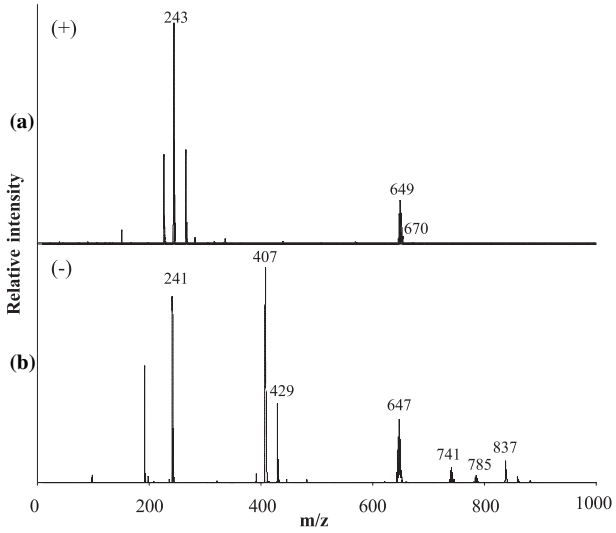

FIG. 7—*(a) Positive and (b) negative-ion MALDI mass spectra of a red liquid ink.*

The red–orange dye is associated with the clusters of peaks in the region of *m/z* 741 and 785.

The data in Fig. 8*a*, when compared with known polyionic dyes that are in use, is consistent with an assignment for this yellow dye as Food Yellow 3. The structure is shown in Fig. 9*a*. This dye anion, of the type $\{M^{-/-}\}$, has two charges and a mass of 406 Da. It appears in the negative-ion spectrum as $[M^{-/-}]H^+$, *m/z* 407, and as $[M^{-/-}]Na^+$, *m/z* 429. Even with the MALDI additive that can donate protons, Figure 7 shows that there is no corresponding positive-ion peak formed. There is a small but real peak at *m/z* 837 both in Fig. 7*b* and in Fig. 8*a*. While these could represent an additional component, it is unlikely that two dyes with MW's of roughly 400 and 800 would migrate to the same location in a TLC experiment. The *m/z* 837 peak may represent a dimer of Food Yellow 3. Each dye carries two negative charges, so if two dyes were combined, the dimer would also require the addition of three positive charges to yield a singly charged anion. The peak at *m/z* 837 could represent $\{[M^{-/-}][M^{-/-}][H^+]_2Na^+\}^-$, with the smaller peaks at higher mass representing dimers with fewer H^+ ions and more Na^+ ions attached, to lower the overall charge.

The red dye is responsible for the peaks in Fig. 7*b* at *m/z* 647, as shown in the spectrum of the TLC-separated component, Fig. 8*c*. There are five major peaks seen, separated by 2 Da, centered at *m/z* 647. They are in a ratio of roughly 1:4:5:4:1. This suggests the presence of halogens, because of their known abundant isotopes. Bromine has two isotopes, ^{79}Br and ^{81}Br, which exist in nature in a 1:1 ratio. If four Bromines are present, the observed set of isotopic peaks would be expected. Considering the mass, color, and number of bromine atoms, a good candidate for the red dye is Acid Red 87, shown as Fig. 9*b*. As in the previously discussed dye, Food Yellow 3, Acid Red 87 is a dye of the type $[M^{-/-}]$. It forms an $[M^{-/-}]H^+$ ion peak (most abundant peak, *m/z* 647) and a smaller peak representing $[M^{-/-}]Na^+$, 22 Da higher. Figure 10 shows an expanded view of the *m/z* 647 peak of Fig. 8*c*, and the theoretical isotope pattern, predicted for the formula of this dye, confirming the assignment.

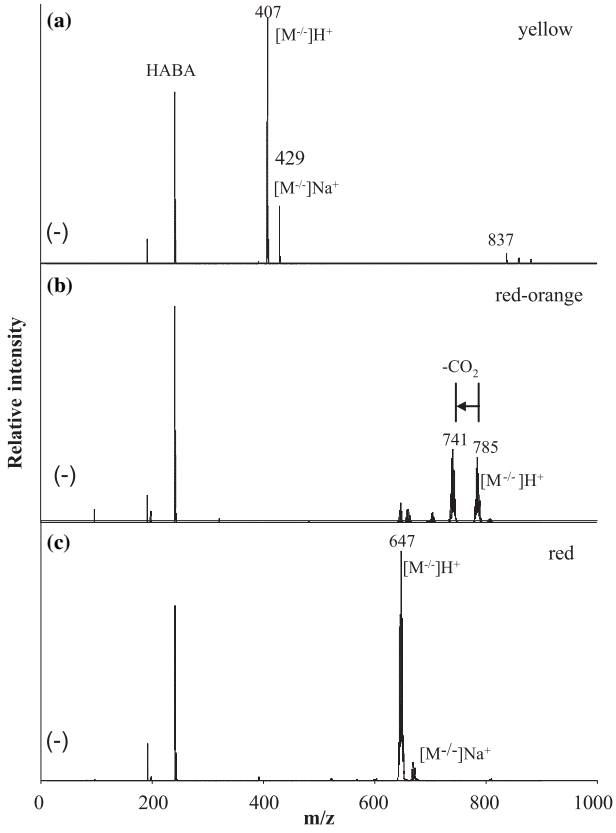

FIG. 8—*Negative-ion MALDI mass spectra of three TLC-separated dyes.*

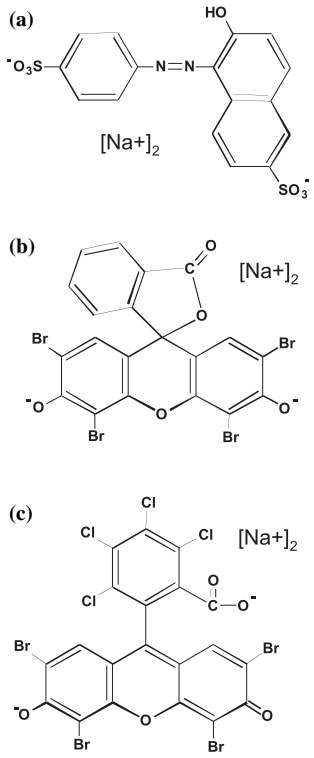

FIG. 9—*Stuctures for (a) Food Yellow 3, (b) Acid Red 87 and (c) Acid Red 92.*

The third dye in this red pen ink, the red–orange dye, also apparently contains halogens. The spectrum, shown in Fig. 8*b*, shows two isotopic clusters of peaks, centered at *m/z* 741 and 785. Both sets of peaks have similar isotopic distributions. The distribution of peaks cannot be associated with multiple bromine atoms, and appears to instead reflect a combination of chlorine and bromine atoms present in the dye. Using an isotope calculator, it was determined that the isotopic pattern was consistent with the presence of four chlorines and four bromines. It is known that this is another polyionic dye. The peaks at *m/z* 741 are 44 Da below those at *m/z* 785. If *m/z* 785 is considered as representing the intact dye, then *m/z* 741 would be a fragment ion. Loss of 44 Da is frequently observed when a carboxylate group, $-COO^-$, is present. With this combined information, a search of commonly used dyes resulted in the identification of the compound Acid Red 92 (20). Its structure is shown in Fig. 9*c*. Thus, the MALDI spectra were rationalized and interpreted.

Conclusion

For questioned document examination, dyes and pigments from ink on paper can be desorbed using a UV laser, and the resulting positive and negative ions can be subsequently analyzed using TOF mass spectrometry. The first combination of lasers and TOF MS was in the LDMS experiment. It was demonstrated that rapid heating because of laser irradiation could desorb a number of molecules intact, but LDMS was never used extensively as an analytical method until MALDI MS was developed. With the promise of biomolecule analysis, the MALDI TOF MS experiment yielded an excellent

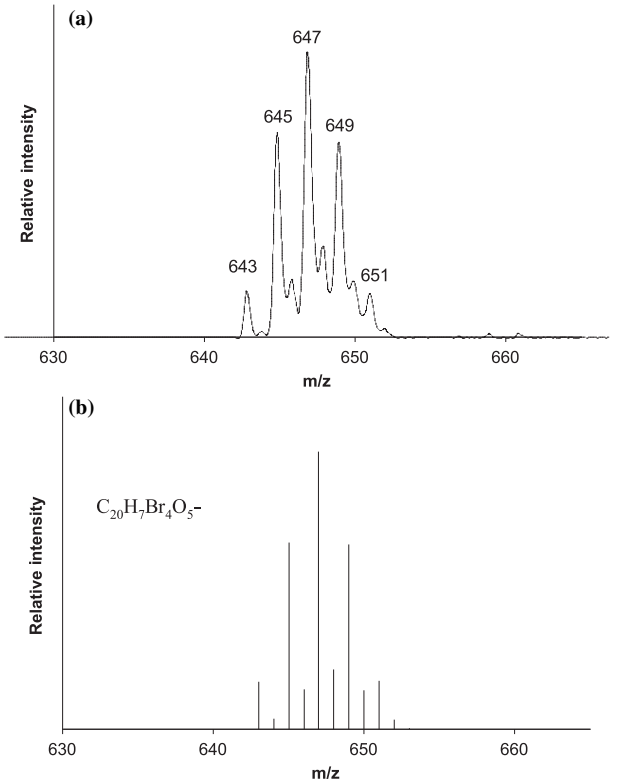

FIG. 10—*Experimental and theoretical isotope distribution patterns for the singly charged anion of Acid Red 87.*

instrument with which one could do the "old" LDMS experiment. We have had much success in using LDMS to analyze colorants; however, there are cases where no useful data can be obtained. We have thus "gone full circle" and have developed an approach for using MALDI MS to expand the arsenal of approaches for identifying colorants in inks on paper. We now have a robust approach to colorant analysis. The first approach is to collect positive- and negative-ion LDMS spectra. In many cases, the dyes will be apparent from these data. When it is known that dyes are present, but no signals are detected, MALDI target crystals can be grown on a pen stroke on paper, incorporating polyionic soluble dyes, and positive/negative MALDI mass spectra can be obtained. We have successfully detected dyes that have contained a maximum of eight sulfate groups.

With LDMS and MALDI MS, we believe that neutral, anionic, cationic, and polyionic dyes, as well as insoluble pigments, can be analyzed. An alternative is to separate the soluble dyes by TLC and analyze each separately. The advantage is that, in this way, one also has a color (or if desired, an optical absorption spectrum) that can be used as information for finding consistent candidate structures. Also, the *inability* to separate colorants by TLC provides important information, indicating that the colorant is an insoluble pigment (such as copper phthalocyanine) rather than a soluble dye.

Acknowledgements

This work was supported under award number 2002-RB-CX-K002 from the Office of Justice Programs, National Institute of Justice, Department of Justice. Points of view in this document are those of the authors and do not necessarily represent the official position of the U.S. Department of Justice.

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Additional information and reprint requests:
John Allison, Ph.D.
Department of Chemistry
The College of New Jersey
P.O. Box 7718
Ewing, NJ 08628
E-mail: allison@tcnj.edu