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Abstract

The objective of the research described here the development and validation of methods for the forensic chemical characterization of dyes extracted from trace evidence fibers. If the chemical composition of dye formulations on trace fibers can be reliably profiled by liquid chromatography, match exclusions can be made with higher reliability, and results will have a solid scientific basis and increased practical significance. Separation and detection of individual dye components provides a qualitative and semi-quantitative fiber dye 'fingerprint' that characterizes the number and relative amounts of dyes present. Chemical identity of extracted dyes can be inferred from liquid chromatography (LC) retention time matching of dye peaks, comparison of UV/visible spectra of the separated dye components, and from molecular weight determination and structural analysis by mass spectrometry. A significant challenge is that such analyses are destructive to the fiber evidence. We have achieved lower limits of detection (low ppb levels) for reliable analysis of single fibers that are 15-25 μm and 0.5 mm, respectively in diameter and length. Larger samples allow multiple for validation or independent analysis

We have developed extraction protocols for (basic) dyes on acrylic, (direct, reactive, and vat) dyes on cotton, (acid) dyes on nylon, and disperse dyes on polyester that are compatible with subsequent liquid chromatographic analysis. Because theses dye types adhere to different polymers by varying mechanisms, methods must be individually designed to disrupt those mechanisms and provide efficient extraction. A single gradient-based ultra-performance UPLC method has been developed for simultaneous separation of basic dyes on acrylic fibers, acidic dyes on nylon fibers, and disperse dyes on polyester fibers; this method avoids using different chromatographic conditions for these dyes and increases sample throughput. Due to cotton's high prevalence in casework, improved extraction methods for the analysis of direct, and reactive dyes on cotton were also developed. We also report the detection of fiber dyes and finishing agents extracted from fibers taken from fabrics subjected to outdoor weathering conditions for up to 12 months.

Microextraction, followed by ultra-performance liquid chromatography, can distinguish similar fibers containing different, but similar, dyes with the combination of retention time matching, UV/visible spectral comparison, and structural analysis by mass spectrometry. The analysis of cotton fibers is challenging because they can be dyed with three different classes of dye, each requiring a different method for extraction and analysis. This work focuses on the chemistries of direct dyes and indigo and their optimum extraction conditions and chromatographic methods. We have successfully extracted direct dyes from cotton fibers as small as 1 mm in length and have quantitated dye amounts by UPLC with UV/visible detection. Analytical figures of merit and validation statistics, including extraction reproducibility, linearity, limits of detection and quantitation, and precision, are reported.

Validation practices for calibration based on forensic and international standards have been employed in this work. Issues that have been addressed with respect to estimation of limits of detection (LOD) include: (a) rational choices of measurement uncertainty are important for valid estimates of LOD ; (b) reporting a range of multiple types of decision limits (limit of detection, minimum consistently detectable amount, limit of detection based on statistical tolerance intervals) provides a stronger statistical basis for specification and control of false positive and false negative detection probabilities; (c) use of Mandel sensitivity, which is independent of the scale in which measurements are expressed, and thus is a useful tool for comparisons of variability between different analytical methods.

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EXECUTIVE SUMMARY

The research objective of this project was to develop and validate analytical chemical methods using ultra performance liquid chromatography (UPLC) for the comprehensive analysis of textile dyes on the four most common types of textile fibers found in practice: acrylic, cotton, polyester, and nylon.

Forensic fiber examinations involve comparison of questioned fibers with one or more known fibers to determine possible associations between victims, suspects, and crime scenes. Initial steps in fiber comparison involve microscopic examination. Polymer type can be established by refractive index measurements or by infrared (IR) spectroscopy and UV/visible or fluorescence micro spectrophotometry may show differences or confirm similarities among questioned and known fibers. However, trace evidence fibers, as well as the dyes that produce color on fibers, are class evidence; because these items mass produced, they may share common characteristics. Establishing a scientific basis for unique matches among questioned and known fibers is difficult.

Trace fiber evidence has been probative in cases ranging from the 1963 JFK assassination,[1] to the Atlanta Child murders[2] of the early 1980s, and the 2002 Washington, DC, sniper case.[3] The fiber examiner typically performs a series of comparisons of the questioned fiber to a known fiber in an attempt to exclude the possibility that a 'questioned' fiber and 'known' fiber could have originated from a common source. If the two fibers can be shown to be substantially different, then the hypothesis that the two fibers originated from a common source can be discounted. The comparison of an 'unknown' to a 'known' fiber is illustrated by the testimony of FBI fiber examiner Paul Stombaugh before the Warren Commission.[1] A tuft of fibers that appeared to match fibers from Oswald's shirt was found caught on a jagged edge of Oswald's rifle stock. Stombaugh testified "there is no doubt in my mind that these fibers could have come from this shirt. There is no way, however, to eliminate the possibility of the fibers having come from another identical shirt." Whenever the hypothesis of a common source for two fibers cannot be rejected, evidence may have probative value, and investigative leads could evolve from the suggested association between victim and suspect. The history of fiber examinations is characterized by a search for increased discrimination to render trace evidence more specific and discriminating. Significance of fiber evidence and discrimination are expanded by combinatorial possibilities of fiber types and dyes.[4-9] If dye formulations on trace fibers can be reliably profiled at trace levels, match exclusions can be made with higher reliability, and "results consistent with" will have increased significance. Separation and detection of individual dye components provides a qualitative and semi-quantitative fiber dye 'fingerprint.' Determining the number and relative amounts of dyes present, and characterizing those dyes at the molecular level by UV/visible absorbance and MS, offers an entirely new level of discrimination. Such information may also open the possibility of tracing specific dye formulations to the dye manufacturer.

Our work has focused on manufactured fibers (nylon, acrylic, and polyester), and the natural fiber, cotton, because of their prevalence in case work.[10,11] Understanding the chemistry and industrial processes involving fibers and dyes is a starting point for design of extraction methods, for developing improved analysis methods, and for correct data interpretation.[4,12] In manufacturing, raw fibers are treated to remove contaminants and lubricants, or to change morphology; fabrics may be bleached; cotton is mercerized to change its morphology and increase dye uptake; nylon and polyester are heat-set to stabilize distortion and to improve

dyeability. Fabrics may be flame-treated to remove surface fuzz, or desized by enzymes to remove weaving aids (*e.g.*, lubricants). Fibers are dyed with processes appropriate for their chemistry (acid dyes, basic dyes, direct dyes, azoic dyes, mordant dyes, sulfur dyes, vat dyes, reactive dyes, disperse dyes, and pigments). Dyes may be loosely associated with fibers (direct dyes on cotton are held by Van der Waals forces and hydrogen bonding), bound by salt linkages (acid dyes on nylon, basic dyes on acrylic), or covalently bonded (reactive dyes on cotton). Dyes may be dispersed through the fiber (*e.g.*, on polyester), mechanically trapped through redox processes (vat dyes on cotton), applied during melt spinning (pigment coloration of nylon, polyolefins, and polyester), or adhered to surfaces with adhesives (pigment dyeing of bedding and apparel fabrics). Fabrics are often finished to impart aesthetic and performance properties, such as stay-press finishing and water-proofing. Preprocessing, dyeing, and finishing all may leave residues on fibers that are useful for discrimination.

Dyes are conjugated molecules, generally consisting of aromatic and/or unsaturated compounds that are either derived from natural sources or are made synthetically. Dyes are often classified according to their application method (*e.g.*, reactive, disperse, and vat) and their chemical constitution (*e.g.*, azo, anthraquinone, metal complex azo). Knowledge of the chemistry of both fibers and dyes is relevant to the extraction of dyes from fibers and to development of appropriate methods of analysis. To extract dye from fiber, the dye's substantivity for the fiber (affinity via intermolecular interactions) must be reduced, then the dye is solvated and transported from the fiber into the extractant. Wiggins[13] summarized solvents for dye extraction. Thin layer chromatography (TLC) of dyes has been widely used in forensic labs.[4-6,13-27] Gaudette[14] mentions that some dyes on 2 mm fibers can be analyzed by TLC, but light-colored fibers may require more than 100× that length. Of 64 fibers listed by polymers, dyes, and color intensities, only 17% of those fibers could be analyzed at 2 mm lengths, 30% at 5 mm, and 61% at 10 mm. For applicability to casework relevant sample sizes, optimizing extraction protocols is critical.

Stefan, *et al*. and Dockery, *et al*. employed experimental design[30-33] to optimize extraction of acid dyes on nylon, basic dyes on acrylic, disperse dyes on polyester, and direct, reactive, and indigo vat dyes on cotton. As an example, with acid dyes on nylon, 10 mixtures of water: pyridine: aqueous ammonia were prepared in duplicate by a laboratory robot, in vials on a 96-well plate. Identical 10-cm fibers were extracted and the absorbance of extracts were measured by a plate reader.[30] In the fitted model, a diagonal ridge of high extraction response runs across the surface from 50:50 pyridine:water to 50 :50 pyridine/ammonia. Of the pure solvents, water gives the best extraction, although the amount of dye extracted is low. Pyridine does not dissolve the dye completely; the solubility of the dye in water is four times higher than that in pyridine. However, pure water is not sufficiently basic to deprotonate the nylon amine end groups and to release acid dyes completely from nylon. Although aqueous ammonia dissolves acid dyes better than does pure pyridine, aqueous ammonia lacks the organic content necessary to fully extract the organic anions of acid dyes. The diagonal ridge runs across the ternary solvent triangle at constant pyridine content of about 45-50%. For extraction of the anthraquinone Acid Blue 45 dye from nylon, the predicted optimum is at a solvent composition of 42% pyridine/58% water. These extraction conditions were confirmed for two other subclasses of acid dyes (azo, and metal complex azo dyes) and produce complete extraction of the tested dyes.[30] All extraction protocols that we have developed can be done in reasonable times (30-60 min, even if done manually).

Liquid chromatography (LC), capillary electrophoresis (CE), and mass spectrometry (MS) are established in applications from drug identification to DNA analysis and forensic toxicology. CE and LC methods offer efficiency, selectivity, short analysis time, low organic solvent consumption, low required sample, and relatively low running costs. CE is well suited for dye analysis because many dyes are ionized, depending on their pK_a and buffer solution pH, however a comprehensive separation method by CE is problematic due to a number of non-ionizable dyes (disperse dyes on polyester, vat dyes on cotton). Modern LC is theoretically superior to CE because dye species need not be ionic or ionizable, and offers mobile and stationary phase tunability. Sirén and Sulkava[34] used CE and UV/visible diode array detection (DAD) for analysis of black dyes from cotton and wool fibers. Xu, *et al.*[35] employed CE to separate reactive, acid, direct, azoic and metal complex dyes extracted from cotton, wool, polyacrylic, polyester, and polyamide fibers; sample stacking was used to improve detection limits. Environmental or industrial applications dominate the dye extract analysis literature.[36-47]

Minor peaks are often observed in separations of dye extracts using any separation technique. These contaminants in the "pure" dyestuffs and side products from incomplete dye synthesis may be signatures of the manufacturing process. Purified component dyes are neither required nor economically feasible on a commercial scale as long as the dyes possess the desired properties. Whether patterns of trace contaminants can be related to manufacturing processes is worth investigating in discussions with industrial manufacturers. However, whether such trace patterns can be reliably used to associate a fiber with a manufacturer is unlikely. The relative amounts of dyes can be correlated with the quantitative dye formulation from the manufacturer might be of forensic significance, if such information were obtainable. Additionally, environmental changes associated with a questioned fiber could affect the quality of such information for comparative purposes.

Ultra-violet (UV) and/or visible detection of dyes from short single fiber lengths can be difficult. One cm of nylon fiber dyed with commercial levels of an acid dye was extracted with 60:40 water:pyridine and the extract was dried down and reconstituted with 190 μL of water prior to CE injection. The absorbance of 3 mAU at the peak maximum produced unreliable spectra. Wheals, *et al*.[49] reported HPLC detection limits of 200 pg/dye, but also found that extracts of short fibers of light shades often yielded insufficient dye. Minor dye components sometimes discriminated fibers even when major components were indistinguishable. Laing, *et al*.[50] analyzed acid dyes by LC with UV/visible detection, but did not show analysis of very short fibers. The target size for forensically relevant fibers derives in part from fiber examinations and population studies reporting that recovered fibers are often as small as 2 mm in length, depending on the degree of dyeing.[50-52] Clearly, methods for the analysis of dyes extracted from fibers require high sensitivity for applicability to forensic casework. Other studies have also reported that UV/visible detection provides neither sufficient sensitivity, nor discrimination, for analysis of trace fiber extracts from structurally-related dyes.[53-58]

LC-MS, and more often LC-MS/MS, is the benchmark analytical approach for chemical quantitation in virtually all biological fluids. Analysis of dye extracted from single fibers of 2-10 mm in length has been achieved by Xu, *et al*.[55] by sample-induced isotachophoresis with micellar electrokinetic capillary chromatography, by Tuinman, *et al*. [56] who directly infused dyes into electrospray MS, and by other researchers with LC or CE coupled to MS.[30-33,48,53-58] Huang, *et al*.[57] demonstrated LC-MS identification of dyes with 22 reference dyes and 10 dyes extracted from fibers. Significantly, this paper showed MS discrimination of dyes that were not reliably

identified by high performance LC (HPLC) with UV/visible detection.[58] Pawlak, *et al*. used HPLC-MS to successfully identify several natural blue dye compounds from a tapestry fiber and concluded that several compounds exhibited complex fragmentation patterns due to chromatographic conditions using electrospray ionization (ESI)-MS.[59] Zhang, *et al*. investigated alternative methods to HCl extraction for six flavonoid and mordant dyes on silk and used HPLC/ESI-MS to identify and quantify extracts for comparison.[60] ESI appears to be the ionization method of choice for most dye classes, however Szostek, *et al*. reported difficulty in ionizing indigotin and brominated indigotin dyes by ESI but demonstrated successful ionization using atmospheric pressure chemical ionization) APCI.[61] Several wool dyes were extracted from historic textiles and analyzed using HPLC and tandem ESI-MS (ion trap MS-MS) by Petroviciu, *et al*. who also noted that most MS literature on dyes focuses on molecular ion identification, and highlighted the importance of the availability of standards to build databases for unambiguous dye identification.[62] The importance of method optimization for fiber extract analysis was highlighted by Rafaëlly, *et al*. who cited the challenges of small extract quantities and commercial availability of dyestuff standards. They concluded that the superior sensitivity and structural elucidation properties of MS were sufficient to overcome low concentrations of extracts.[63]

Conventional HPLC uses 4-5 mm ID columns of 10-25 cm length; injected dyes are diluted by band broadening and relatively large samples are needed. Only a small number of papers in the forensic literature have applied HPLC to dye extracts. Laing, *et al*. used diode array detection for LC analysis of acid dyes.[64] Ultra-performance liquid chromatography (UPLC), introduced commercially in 2004-2005, uses high pressures (>10,000 psi), smaller column particles (<2 µm), short columns (~5 cm), and minimal sample (~100 µL) to obtain high speed, resolution, and sensitivity. UPLC has rapidly become an established, especially in areas requiring sample throughput (speed of analysis) and high resolution. Decreasing column particle size allows for columns to be packed tighter and more uniformly, resulting in reduced band broadening due to eddy diffusion. Smaller particles also provide a shorter pathway into and out of the stationary phase, yielding less band broadening due to mass transfer. Using small particle columns of shorter column length reduces band broadening due to longitudinal diffusion within the column. Together, these three factors produce increased plate count and flatten the van Deemter curve at high flow rates. As a result, UPLC chromatographic peaks are narrower, and flow rates can be effectively doubled over that in HPLC columns by using particles 1-5 µ in size without incurring band broadening and decreased resolution, thus achieving faster separation times. Use of small UPLC particle sizes requires the high pressures to achieve flow through packed columns.

Compared to HPLC separations, UPLC separations produce a narrower, and more concentrated, analyte band allowing for lower limit of detection (LD) by UV/visible analysis. A comparison of detection limits of several food dyes characterized by HPLC and UPLC (both with UV/visible detection shows similar detection limits at first glance, however the required injection volume to achieve these levels by HPLC was 20 µL versus 3 µL for UPLC.[65,66] These results are summarized in Table 1.1.

The HPLC UV/visible literature for dye analysis is abundant with refined analysis techniques, but literature for UPLC-DAD of dyes is underdeveloped at the moment. Achieving comparable limits of detection at lower injection volumes by UPLC suggests the possibility of lowering those limits through higher volume injections and method optimization. UPLC-DAD limits of detection for food dyes were reported by Ji, *et al*. to range from 88 to 21 pg. They also reported

that LD values using UPLC-MS/MS for Tartrazine, Amaranth, Indigo Carmine, Allura Red AC, and Sunset Yellow FCF are high than LDs determined by UV/visible detection.[65]

A search on *Science Direct* (only Elsevier journals) found 1,334 articles on UPLC, most of which involved biomedical and environmental applications; forensic applications of UPLC were targeted mostly in toxicology and drug identification. These applications typically report UPLC/MS analysis times of 30 s to 1-3 min, and detection limits in the range of pg of analyte injected. For example, UPLC (time-of-flight) TOF-MS was used to analyze liver blood from a poisoning case involving Bromo-Dragonfly drug.[67] Another forensic application involved post-mortem analysis of ethyl glucuronide (EtG) as an alcohol metabolite in hair; with an evaporative light scattering detector, levels of EtG at just above 30 pg/mg were detected.[68] A review of applications of LC/MS, including some UPLC discussion, was published by Wood, *et al*.[69]

The development and validation of reliable microextraction protocols followed by sensitive trace analyses by chromatography and mass spectrometry is the subject of the research presented in this report. The main body of the technical report documents accomplishments, methodology, and data from the project. In summary, methodology for the microextraction of basic dyes on acrylic, acid dyes on nylon, disperse dyes on polyester, and reactive dyes, direct dyes, and indigo on cotton textile fibers is reported. The analysis of cotton fibers is challenging because they can be dyed with three different classes of dye, each requiring a different method for extraction and analysis. Reactive dyes present a unique challenge because they are chemically bound to the cellulose structure of the fiber. Release of these dyes from cotton requires breaking of the covalent bond using hot sodium hydroxide. The resulting hydrolysis reactions can also cleave amide bonds and possibly other chemical bonds in the dye molecule. The various structural changes that can take place leads, in many cases, to production of multiple reaction products from a single dye. We demonstrate successful extraction of reactive dyes from single 1 mm cotton fibers with detection limits as low as 3.3 pg. Systematic experiments at varying reaction conditions, with product analysis by MS, were also performed to characterize the degradation of reactive dyes under hydrolysis, and to facilitate interpretation of reactive dye extractions.

Extraction methods developed for basic dyes on acrylic, acid dyes on nylon, and disperse dyes on polyester fibers involve extraction with solvents that do not affect the chemical composition of the polymer, and thus do not impose limitations on use of the fiber for further examinations. With these fibers, IR can be conducted on previously extracted samples if needed. Reactive dye extraction from cotton requires base hydrolysis with sodium hydroxide which can change the chemical form of the dye and modify the fiber polymer chemistry; strongly alkaline solutions are also not compatible with C18 stationary phases for liquid chromatography. Previous research employed 1.5% NaOH which does cause damage. In this work, 0.1875 M sodium hydroxide was employed and the resulting chromatograms showed no evidence of chemical products from cellulose.

Although these processes are destructive to the fiber evidence, the ability to analyze dye extracts from sub-millimeter fiber lengths of single fibers, coupled with detection limits in the hundred picogram range by ultra-performance liquid chromatography (UPLC) with both diode array detection (DAD) and tandem mass spectrometry (MS-MS) makes routine forensic characterization feasible. Microextraction, followed by UPLC, can often distinguish similar fibers containing different, but similar, dyes with the combination of retention time matching, UV/visible spectral comparison, and structural analysis by mass spectrometry. This work focuses on determining the optimum extraction conditions for each dye class and developing

chromatographic methods with suitable resolution and sensitivity for trace analysis. Analysis of fibers as small as 1 mm in length is the target sample size to minimize destruction of fiber evidence. Analytical figures of merit and validation statistics, including extraction reproducibility, measures of calibration performance, linearity, limits of detection and quantitation, and precision, are reported. Modern instrumental analysis of separated dye components can increase the reliability of fiber examinations by providing discriminating information on dye characterization and possibly identification of dyes at the molecular level from trace evidence fibers as small as 0.5 mm.

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TECHNICAL REPORT

A. Comprehensive screening of acid, basic, and disperse dyes extracted from millimeter-length trace evidence fibers by ultra-performance liquid chromatography: methodology and figures of merit

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ABSTRACT

Methodology for the microextraction of basic dyes on acrylic, acid dyes on nylon, and disperse dyes on polyester textile fibers is reported. A single ultra-performance liquid chromatography method suited for qualitative and semi-quantitative analysis of all three dye types has also been developed. Although our approach is destructive to the fiber evidence, the ability to analyze sub-millimeter fiber lengths of single fibers, coupled with limits of detection in the 2-40 ppb range by both UV/visible and tandem mass spectrometric detection make routine forensic characterization feasible.

INTRODUCTION

The ubiquitous nature of textile fibers provides an information-rich evidence source for crime scene investigations, however in cases of similarly dyed fibers current fiber analysis techniques do not provide adequate chemical information for unambiguous match determinations to be made. The standard procedure for analyzing forensic textile fibers involves measurement of physical and optical properties in an attempt to exclude matches of known and questioned fibers, followed by visual color matching and infrared (IR) spectroscopy and UV/visible spectral comparisons.[1] These techniques are efficient, non-destructive, and most often able to discriminate fiber evidence. However, fibers are class evidence and often share properties or characteristics due to common or similar sources, manufacturing methods, or treatments. The conclusion that the questioned fiber exhibits the same physical, optical and chemical properties as the known sample and could have originated from the same source as the known sample or another fiber source composed of fibers with the same properties may not be convincing in the courtroom. Fibers often are multiply dyed. Reliable quantitative extraction of those dyes from a trace evidence fiber can enhance forensic decision-making by distinguishing fibers containing different, but similar, dyes with the combination of retention time matching, UV/visible spectral comparison, and structural analysis by mass spectrometry.

The challenge of analyzing dye extracts from forensic fibers stems from the need to preserve the evidence as much as possible. Extraction of dyes is destructive to the fiber, and recovered trace evidence fibers are often as small as 2 mm in length and contain as little as 2 ng of dye[2], thus very sensitive analysis techniques are needed. Previous research into dye extract analysis has used thin layer chromatography (TLC) as a classification technique, however issues of reproducibility and large required sample size plague the conclusions.[3–13] More advanced techniques have been applied such as capillary electrophoresis (CE), and while CE excels at separating ionized dyes, dye classes such as disperse dyes on polyester and vat dyes on cotton are difficult to ionize.[14–16]

Liquid chromatography has the added benefit of both stationary phase and mobile phase tuning to achieve a separation. Because many dyes have acid or base character, control of the mobile phase pH permits adjustment of retention for improved resolution and tuning of retention times for faster analysis speed. The numerous combinations of mobile and stationary phases offer a universal separation system for small organic molecules such as dyes. HPLC has been previously used to separate mixtures of acid, basic, and disperse dyes.[17,18,20] Where HPLC with UV/visible detection was not sufficient to differentiate between some similarly colored dyes, Huang, *et al*. point out that discrimination at the molecular level by HPLC-MS is usually successful.[17] Dye extracts not discriminated by UV/visible detection were also analyzed using HPLC-MS; success was achieved using 5 mm threads, but not single fibers.[18]

Ultra-performance liquid chromatography (UPLC) has demonstrated its utility for rapid analysis by using high pressure pumps (<15,000 psi) capable of moving samples and mobile phases through columns packed with small diameter (1.7 μm) stationary phase particles. UPLC can achieve very high resolution separations in short analysis times (< 5 min). As a consequence, band broadening is decreased substantially compared to HPLC and the viability of using UV/visible detection for trace analysis increases.

The objective of our present research is to establish comprehensive microextraction techniques coupled with UPLC methodology for the extraction and separation of acid, basic, and disperse dyes from nylon, acrylic, and polyester fibers. Our target fiber size is 1 mm to offset the issue of damaging evidence. We also present performance characteristics for calibration involving these analytical methods and determine limits of detection and quantitation for nine different dyes.

EXPERIMENTAL

Materials. Analytical grade chlorobenzene, glacial acetic acid, pyridine, ammonium hydroxide, ammonium acetate, formic acid, and HPLC/UPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburg, PA). Dyed fabric and textile dye standards were sampled from our collection of production samples, which were donated by textile and dyestuff manufacturers from the southeastern United States. The dyes used in this work are listed in Table 1 using the Color Index nomenclature (Society of Dyers and Colourists, Bradford, UK). The nine dyes selected for this study included three dyes from each of the acid, basic, and disperse classes.

Fiber sample preparation. Prior to extraction analysis, all samples are cut to prescribed lengths. In previous CE analyses for fiber dyes, percent relative standard deviations (RSDs) for peak areas have varied from 10-20%, of most synthetic fibers to as high as 25-35% for cotton.[14-16] We attributed these high RSDs to the inability to cut small fiber sizes reproducibly. Fibers that have intrinsic curled, crinkly, or helical shapes, such as cotton and acrylic fibers, must be stretched lengthwise while cutting. We have designed and machined a device to facilitate reproducible measurement and cutting of fibers down to 5 mm in length (Figure 1). The device has a metal block with a groove within which the fiber can be securely positioned; above the block is a rotating shaft on which multiple razor blades are fixed, spaced in 5 mm distance increments and aligned with cutting slots. Once the fiber is positioned, the operator selects the desired length and can safely rotate the shaft to cut the fiber.

Individual fibers 5-mm in length were cut using the fiber guillotine; 1 mm and 0.5 mm fibers were cut by hand using a table-mounted magnifying glass and scalpel. Each fiber length was cut

in triplicate. Cut fibers were then loaded into Waters Total Recovery® vials for extraction. These vials enable extractions to be performed with low solvent volumes (< 50 μL), enabling concentration of dyes in the resulting extract. When typical low volume vial inserts were employed for extractions, solvent often condensed on the inside vial wall outside the vial insert because of poor sealing between the insert and the top of the vial.

Calibration design. Experimental designs for UPLC-DAD calibration were constructed for all nine dyes based on experiments at 7 levels of dye concentration (0 ppb, 100 ppb, 500 ppb, 1000 ppb, 1500 ppb, 2000 ppb, and 2500 ppb). In this high concentration calibration, a blank sample was measured 15 times as a quality control sample interspersed through the runs, and five replicate samples were analyzed for each sample containing analyte. A lower concentration design was also performed based on five replicate experiments using standard mixtures of the 9 dyes at concentrations 10 ppb, 20 ppb, 30 ppb, 40 ppb, and 50 ppb, with 18 blank injections at zero concentration. For UPLC-MS-MS, calibration designs included standards at 0 ppb, 10 ppb, 200 ppb, 400 ppb, 600 ppb, 800 ppb, and 1000 ppb, with 15 replicate experiments for the blanks and five replicates for samples containing analyte. For each dye peak, QuanLynx™, data management software included with MassLynx™ (Waters Corporation, Milford, MA) was used to integrate peak areas above corrected baselines. For each dye standard at 1000 ppb concentration, the retention time window encompassing the baseline peak width was determined; this window was then employed as the dye peak integration window for all samples, including blanks.

Instrumentation. Dye standards and extracts were separated and detected using a Waters Acquity™ UPLC H-Class equipped with a quaternary solvent pump system and a Waters PDA eλ detector. The column was a 2.1 × 50 mm I.D. 1.7 μm particle size Waters Acquity™ BEH C18 column with a 2.1 × 5 mm I.D. 1.7 μm particle size Waters Acquity UPLC® BEH C18 VanGuard precolumn. The mobile phase gradient was based on mixtures of 50 mM ammonium acetate in water and 0.15% formic acid in methanol as shown in Table 2A. The column temperature was set at 40 °C. The diode array detector scanned the wavelength range from 325 nm to 675 nm at a rate of 40 Hz and 1.2 nm resolution. The sample injection volumes were 10 μL.

Separations with MS characterization were performed using a Waters Acquity UPLC® equipped with a binary solvent system coupled to a Waters Micromass Quattro Premier XE® tandem quadrupole mass spectrometer. For MS compatibility, the mobile phase consisted of 50 mM ammonium acetate in water and 0.15% formic acid in methanol. The mobile phase gradient for MS-MS detection shown in Table 2B differs from that for UPLC-UV/visible detection to compensate for the slower flow rate required for MS. The column was at ambient temperature during runs. Sample injection volumes were 5 μL. The MS-MS transitions, cone voltages, and collision voltages are listed in Table 3.

Extraction of acid dyes on nylon. The optimum solvent conditions for extraction of acid dyes from nylon was previously investigated by Stefan, *et al*., using a mixture of pyridine, ammonium hydroxide, and water.[14] Because the extraction response was robust over the center of the design, here we used equal proportions (33:33:33) of the three components for extractions. Aliquots (100 μL) of the extractant were added to vials containing fibers, and vials were capped and extracted at 100°C for 1 h. After extraction, the vials were uncapped and heated at 90°C to evaporate solvent (*ca*. 30-45 min). Samples were reconstituted in a 50 μL mixture of 50:50 methanol and

50 mM ammonium acetate buffered at pH 4.5, and vortex-mixed to ensure complete solvation of extracted dye.

Extraction of basic dyes on acrylic. Extraction conditions for basic dyes on nylon were also explored by Stefan, *et al.*[15] In the present work, 50:50 mixtures of 88% formic acid and water were employed for all extractions, in agreement with literature-cited values.[6,19,20] Aliquots (100 μL) of extractant were added to the vials containing the fibers and then capped. The extraction was carried out at 100°C for 1 h, and the vials were then uncapped and evaporated at 90°C until dry (*ca.* 30-45 min). The samples were reconstituted in a 50 μL mixture of 50:50 methanol and 50 mM ammonium acetate buffered at pH 4.5. Samples were vortex-mixed to ensure complete solvation of the extracted dye.

Extraction of disperse dyes on polyester. Chlorobenzene was previously reported to extract disperse dyes on polyester.[21] As with acid and basic dyes, aliquots of 100 μL of extractant were added to vials containing the fibers and the extractions were carried out at 100 °C for 1 h. Vials were uncapped and heated to evaporate solvent at 90 °C until dry (*ca.* 30-45 min). The samples were then reconstituted in a 50 μL mixture of 50:50 methanol and 50 mM ammonium acetate buffered at pH 4.5. Samples were vortex-mixed to ensure complete solvation of the extracted dye.

MS-MS Optimization. Electrospray ionization (ESI) and MS-MS transition parameters were tuned by infusing 1000 ppb of each dye into the source with a 0.300 mL/min mobile phase of a 50:50 mixture of 0.05 M ammonium acetate and methanol with 0.15% formic acid by volume. Desolvation temperature was set at 450 °C. Dye standards were infused at 20 μL/min to tune the optimum cone voltage was tuned for maximum ion count, and to adjust collision voltages to maximize fragment ion counts from characteristic mass fragments. Table 3 summarizes the MS-MS parameters employed. Fiber dye extracts were reconstituted in 50 μL volume to provide sufficient volume for such initial tuning and determination of MS conditions and transitions for target ions.

RESULTS AND DISCUSSION

Chromatographic analysis of dyes. A comprehensive separation of all nine dyes was achieved using the mobile phase gradients in Table 2. In developing this gradient mixture, ammonium acetate buffered to pH 4.5 was found to broaden several dye peaks, and Basic Yellow 28 and Acid Yellow 49 dye peaks coeluted. A gradient of ammonium acetate at pH 7 produced two peaks almost baseline-resolved for these dyes. Under these conditions, the Basic Violet 16 baseline peak width decreased from 5.4 s to 4.2 s, and peak height and area increased by *ca.* 20%.

Figure 2 displays the separation, in less than five min, of all nines dyes evaluated for this work. The first (peak 1, Basic Red 46), third (peak 3, Acid Yellow 49), and last dye (peak 9, Disperse Blue 60) produced two peaks. In each case, each pair of peaks exhibited UV/visible spectra identical to those in Table 1. For the last pair of peaks, the absorbance is shifted to longer wavelength compared to many of the other dyes.

Figure 3 shows the chromatograms of dyes extracted from fibers of lengths 5 mm, 1 mm, and 0.5 mm; these extractions were performed in triplicate. All extractions were successful down to 0.5 mm except those involving Acid Yellow 49 and Disperse Blue 60, which were only successful down to 1 mm lengths. A 1 mm fiber extract of Acid Yellow 49 dye appeared to produce

sufficient signal for a 0.5 mm extract to be detectable, but analysis of a 0.5 mm fiber dyed with Acid Yellow 49 failed, either because of difficulty in handling or low dye levels. Pale yellow-colored fibers are difficult to see regardless of length. The stock fabric from which fiber dyed with Disperse Blue 60 were sampled, were lightly colored indicative of low dye loading. As seen below, Disperse Blue 60 has the highest limit of detection (LD) of all nine dyes, and although results suggest that a 0.5 mm extract is above the limit of detection, there is increased noise associated with its absorbance peak.

Validation performance characteristics for dyes. Tables 4 and 5 show UPLC-DAD calibration results for all nine dyes over the selected high (0-2500 ppb) and low (0-50 ppb) concentrations, respectively. Table 6 shows MS-MS calibration results over mid-range (0-600 ppb) concentrations. Figures 12-37 display calibration plots for the nine dyes investigated. All first-order linear calibration models (with intercept and slope parameters) produced coefficients of determination (R^2) of 0.9993 or higher for the wide range calibrations. Calculated signal-to-noise ratios (SNR) were 100 or higher at 100 ppb in the wide range calibrations (based on integrated baseline root-mean-square variation from the MassLynx®). The second calibration (Table 5), performed over a range of standard concentrations that bracket the estimated dye limits of detection, produced lower R^2 values of 0.9913 or higher, except for C.I. Disperse Blue 114 and C.I. Disperse Blue 60 (discussed below). The decrease in linearity over the lower range is expected due to the higher uncertainty—noise and detector linearity have a larger impact on peak shape as concentrations approach the LD—but these results are still excellent. For Disperse Yellow 114, the low-range UPLC-DAD results exhibited higher variability about the calibration line than any other dye; an R^2 of 0.9687 was obtained, but the model exhibited a statistically significant lack of fit ($p < 0.05$).

Table 6 displays calibration results for MS-MS based on 5 replicate standard injections at concentrations 10 ppb, 200 ppb, 400 ppb, 600 ppb, and 6 blank injections. The MS-MS models exhibit heteroscedastic behavior and greater variation at higher concentrations, and consequently these calibrations exhibited lower R^2 values ranging from 0.9371 to 0.9970. Disperse Violet 77 (R^2=0.9700) and Basic Red 46 (R^2=0.9371) showing high residuals, possibly due to the fact that MS ionization conditions were not fully optimized.

Limits of detection are reported in Tables 4-6 are based on three different estimation approaches. Each method calculates the LD or limit of quantitation (LOQ) using

$$LD = (3.3 \times \sigma_b)/S \quad (1)$$

$$LOQ = (10 \times \sigma_b)/S \quad (2)$$

where σ_b is the standard deviation of the blank and S is the slope of the calibration line. The three methods used differ with how σ_b is estimated. LD_1 estimates σ_b using the standard deviation of the integrated blank signals across the width of the actual peak. LD_2 approximates σ_b using the standard deviation of the lowest non-zero concentration calibrator (at 100 ppb for the high concentration DAD calibration; at 10 ppb for the low concentration DAD and MS calibrations). LD_3 estimates σ_b based on the standard error of the *y*-intercept of the calibration model; because the standard error of the *y*-intercept is calculated from the standard deviation of residuals, this may be inflated by the presence of lack fit of the model and by heteroscedasticity (non-constant variability at different concentration levels). There are many ways to calculate LDs; we present these three approaches to indicate a range of reasonable values for the LDs. Most UPLC-

UV/visible LDs calculated using the high concentration calibration design were 10 ppb or lower, which is the equivalent of detecting 100 pg amounts of dye.

LDs of disperse dyes using UV/visible detection are higher than the other dye types. Disperse Blue 60 had the lowest absorbance response of all dyes investigated; the low concentration(10-50 ppb) calibration was not determined because only the 50 ppb standard produced a peak that could be integrated by MassLynx®. LD_1 and LD_3 estimated the limit of detection for Disperse Blue 60 to be 13.50 ppb and 16.80 ppb, respectively. This result illustrates an important point: confirming actual detection for a sample concentration at the estimated LD is required if one plans to operate near the LD. Conducting the low concentration calibration design achieved this requirement for the present study. MS-MS calibration for Disperse Blue 60 produced LDs ranging from 2.88 to 30.50 ppb, depending on the estimation approach. The calibration in Figure 31 exhibits an R^2 of 0.9813; however, the curvature visible in the calibration plot is confirmed by a statistically significant lack of fit ($p < 0.0001$).

Estimated LDs based on the lower concentration calibration designs for all dyes were all less than 4.38 ppb (corresponding to 43.8 pg of dye), except for the Disperse Yellow 114 dye (mentioned above). The calibration for Disperse Yellow 114 had abnormally high variance in the blanks and consequently LD_1 was 12.60 ppb while LD_2 and LD_3 were 1.67 ppb and 2.34 ppb. LDs calculated for ESI-MS-MS by LD_1 and LD_2 were evenly distributed between 0.38 ppb and 10.30 ppb. The high LD_3 values are due to the high amount of variance at 600 ppb for each dye by MS. Optimization of the MS-MS ionization conditions for higher dye concentrations might improve this outcome.

CONCLUSIONS

Guided by previous experience with dye extractions, protocols have been devised for microextraction of acid dyes from nylon, basic dyes from acrylic, and disperse dyes from polyester fibers as small as 0.5 mm in length. Because it is not common to find more than 3-5 dyes on a single fiber, we validated these methods using three dyes from each dye class. The single UPLC method was developed for the separation of the specific acid, basic, and disperse dyes analyzed in this study. Chromatographic conditions used here are a good starting point for the analysis of other dyes from these three dye classes. For different unresolved dyes, gradient timing, composition, and rate of solvent composition change are easily adjusted to optimize separation. UPLC columns with 1.7 µm diameter stationary phase particles achieve fast analysis times, with high pressure, and typical chromatographic analysis times in the range of 3-5 min.

Estimated limits of detection across the broad of three dye classes displayed consistency, notwithstanding a few exceptions. The calibration designs at low concentration produced lower LDs than achieved by UPLC-MS-MS. High concentration range calibrations for UV/visible and MS-MS detection showed lower detection limits than MS-MS in this study. More importantly, both methods are sensitive enough to detect and quantify well under 2 ng of dye on a 2 mm fiber. All but two dye extracts produced detectable dye amounts from 0.5 mm fibers that were only lightly dyed. We have found UPLC with UV/visible detection to yield lower detection limits than UPLC-MS-MS. Further optimization of MS ionization conditions may improve mass spectrometric performance.

Our results demonstrate that minute lengths of trace evidence fibers can be readily analyzed for dye formulation even when only very low dye amounts are present. UPLC with UV/visible detection enabled detection of dyes in extracts from single 1 mm fibers. We have developed

methods for profiling the chemical identity of constituents bound to textile fibers, including dyes, fluorescent brighteners, and finishing agents. By extracting these components from trace evidence fibers, separating them by liquid chromatography, and identifying and quantifying individual dyestuffs, match exclusions may be made based on more discriminating information than possible using visual and spectrophotometric microscopic analysis: comparisons can be based on the identity and relative amounts of dyes present in on the fibers themselves.

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Table 1. List of textile dyes, molecular structures, and UV/visible absorbance spectra.

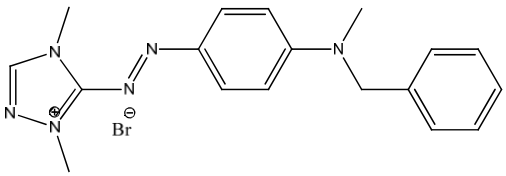
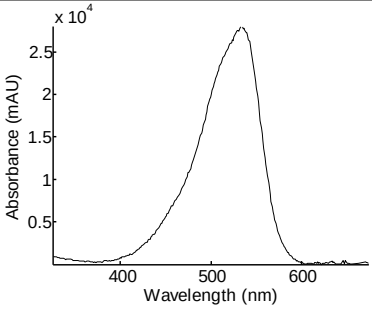
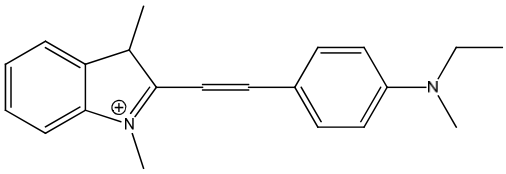
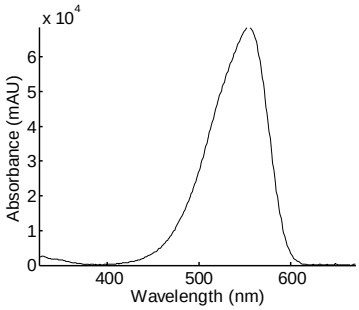
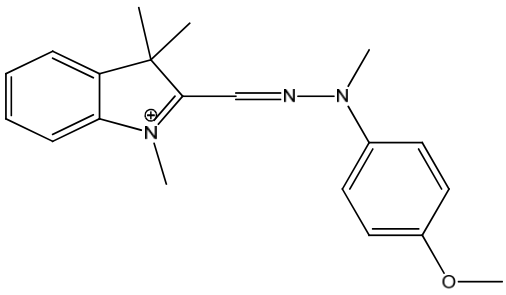
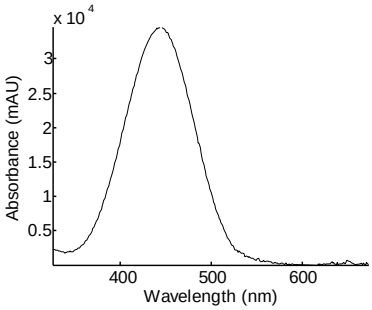
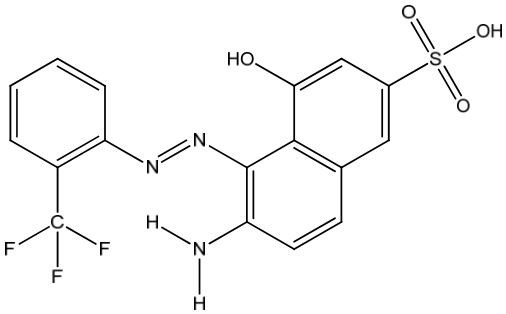
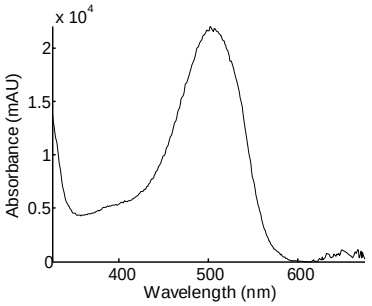
C.I. Name, Formula, Mol. Wt. (g/mol)	Structure	Absorbance Spectrum
Basic Red 46 $C_{18}H_{21}BrN_6$ 401.30		
Basic Violet 16 $C_{23}H_{29}N_2^+$ 333.49		
Basic Yellow 28 $C_{20}H_{24}N_3O^+$ 322.42		
Acid Red 337 $C_{17}H_{12}F_3N_3O_4S$ 411.36		

Table 1. Continued.

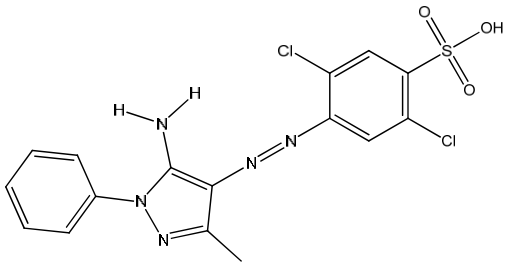
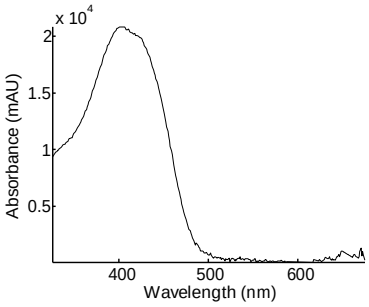
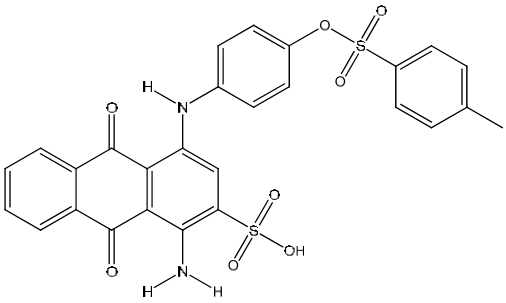
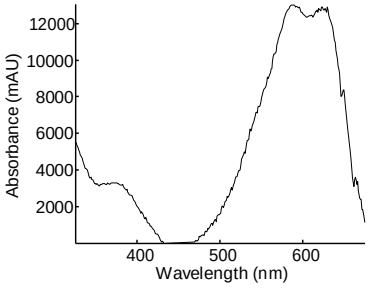
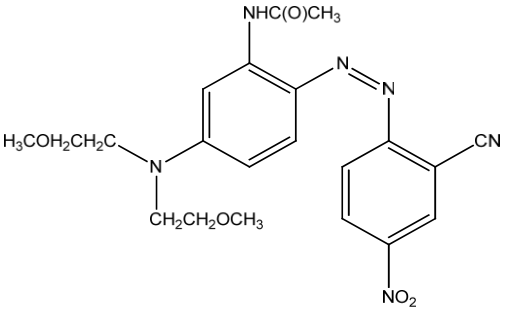
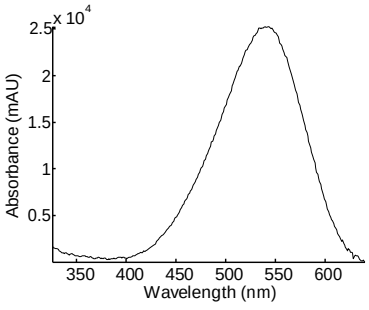
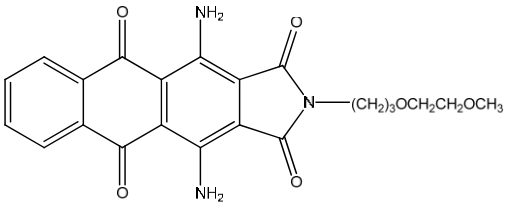
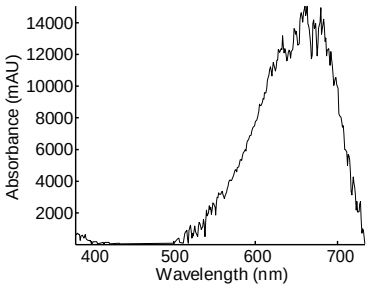
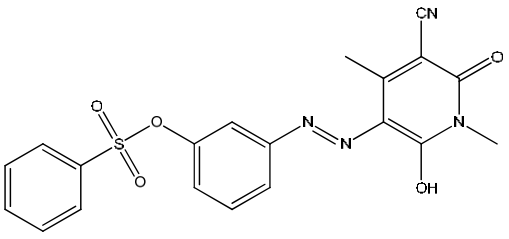
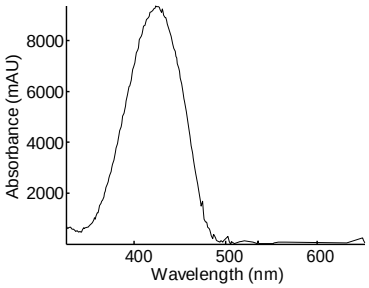
Acid Yellow 49 $C_{16}H_{13}Cl_2N_5O_3S$ 426.28		
Acid Blue 281 $C_{27}H_{20}N_2O_8S_2$ 564.59		
Disperse Violet 77 $C_{21}H_{24}N_6O_5$ 440.45		
Disperse Blue 60 $C_{22}H_{21}N_3O_6$ 423.42		
Disperse Yellow 114 $C_{20}H_{16}N_4O_5S$ 424.43		

Table 2. Mobile phase gradient profiles.

A. UPLC mobile phase gradient for UV/visible detection

Time (min)	Flow rate (mL/min)	Methanol with 0.15 % formic acid (%)	50 mM Ammonium acetate (%)
0.0	0.6	10	90
0.5	0.6	10	90
4.0	0.6	90	10
4.5	0.6	90	10
4.6	0.6	10	90
6.5	0.6	10	90

B. UPLC mobile phase gradient for MS-MS detection

Time (min)	Flow rate (mL/min)	Methanol with 0.15% FA (%)	50 mM Ammonium acetate (%)
0.0	0.3	20	80
0.5	0.3	20	80
4.0	0.3	90	10
4.5	0.3	90	10
4.6	0.3	20	80
6.5	0.3	20	80

Table 3. MS-MS transitions and voltages.

Dye	Transition (m/z)	Cone Voltage (V)	Collision Voltage (V)
Basic Red 46	321-196	30	16
Basic Violet 16	333-319	39	29
Basic Yellow 28	322-136	33	30
Acid Red 337	412-161	45	33
Acid Yellow 49	426-144	40	35
Acid Blue 281	565-409	40	30
Disperse Violet 77	441-221	36	21
Disperse Blue 60	424-348	32	18
Disperse Yellow 114	425-178	47	30

Table 4. High range (0-2500 ppb) UPLC-UV/visible calibration results.

Dye	R^2	LOD_1 (ppb)	LOD_2 (ppb)	LOD_3 (ppb)	LOQ_1 (ppb)	LOQ_2 (ppb)	LOQ_3 (ppb)
C.I. Basic Red 46	0.9996	4.77	5.41	11.80	14.45	16.39	35.76
C.I. Basic Violet 16	0.9999	3.62	1.69	6.32	10.97	5.12	19.15
C.I. Basic Yellow 28	0.9995	6.97	1.07	14.10	21.12	3.24	42.73
C.I. Acid Red 337	0.9998	9.50	2.17	8.90	28.79	6.58	26.97
C.I. Acid Yellow 49	0.9997	3.22	1.96	10.40	9.76	5.94	31.52
C.I. Acid Blue 281	0.9999	4.87	1.56	6.79	14.76	4.73	20.58
C.I. Disperse Violet 77	0.9998	10.10	0.37	9.42	30.61	1.12	28.55
C.I. Disperse Blue 60	0.9993	37.00	13.50	16.80	112.12	40.91	50.91
C.I. Disperse Yellow 114	0.9999	11.30	4.68	7.60	34.24	14.18	23.03

Table 5. Low range (0-50 ppb) UPLC-UV/visible calibration results.

Dye	R^2	LOD_1 (ppb)	LOD_2 (ppb)	LOD_3 (ppb)	LOQ_1 (ppb)	LOQ_2 (ppb)	LOQ_3 (ppb)
C.I. Basic Red 46	0.9914	2.37	3.12	1.21	7.18	9.45	3.67
C.I. Basic Violet 16	0.9952	1.84	2.09	0.91	5.58	6.33	2.76
C.I. Basic Yellow 28	0.9965	2.69	0.93	0.77	8.15	2.82	2.33
C.I. Acid Red 337	0.9957	3.07	1.89	0.86	9.30	5.73	2.61
C.I. Acid Yellow 49	0.9940	4.26	2.59	1.01	12.91	7.85	3.06
C.I. Acid Blue 281	0.9913	4.38	0.41	1.22	13.27	1.24	3.70
C.I. Disperse Violet 77	0.9942	2.23	1.28	0.99	6.76	3.88	3.00
C.I. Disperse Blue 60	–	–	–	–	-	-	-
C.I. Disperse Yellow 114	0.9687	12.60	1.67	2.34	38.18	5.06	7.09

Table 6. MS-MS (0-600 ppb) UPLC-MS-MS calibration results.

Dye	R^2_{MS}	LOD_1 (ppb)	LOD_2 (ppb)	LOD_3 (ppb)	LOQ_1 (ppb)	LOQ_2 (ppb)	LOQ_3 (ppb)
C.I. Basic Red 46	0.9371	1.70	6.44	57.30	5.2	19.5	173.6
C.I. Basic Violet 16	0.9970	0.38	2.55	12.00	1.2	7.7	36.4
C.I. Basic Yellow 28	0.9840	2.34	3.87	28.10	7.1	11.7	85.2
C.I. Acid Red 337	0.9953	10.30	8.08	15.20	31.2	24.5	46.1
C.I. Acid Yellow 49	0.9858	6.41	4.76	26.50	19.4	14.4	80.3
C.I. Acid Blue 281	0.9879	7.09	4.67	27.40	21.5	14.2	83.0
C.I. Disperse Violet 77	0.9700	0.62	1.46	38.80	1.9	4.4	117.6
C.I. Disperse Blue 60	0.9813	4.67	2.88	30.50	14.2	8.7	92.4
C.I. Disperse Yellow 114	0.9833	2.89	7.62	28.80	8.8	23.1	87.3

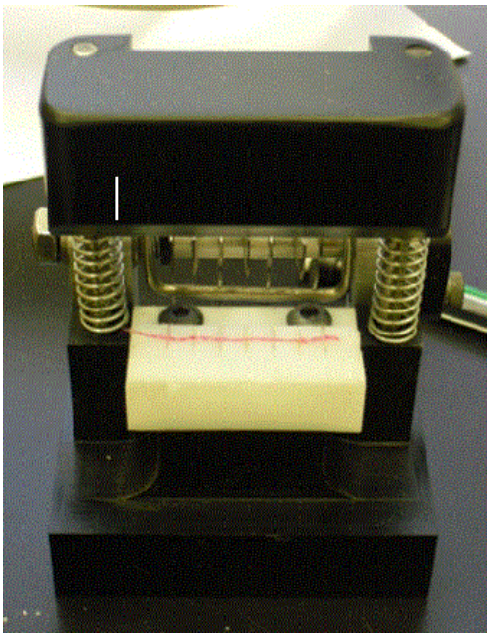


Figure 1. Guillotine device for cutting fibers to 5 mm lengths.

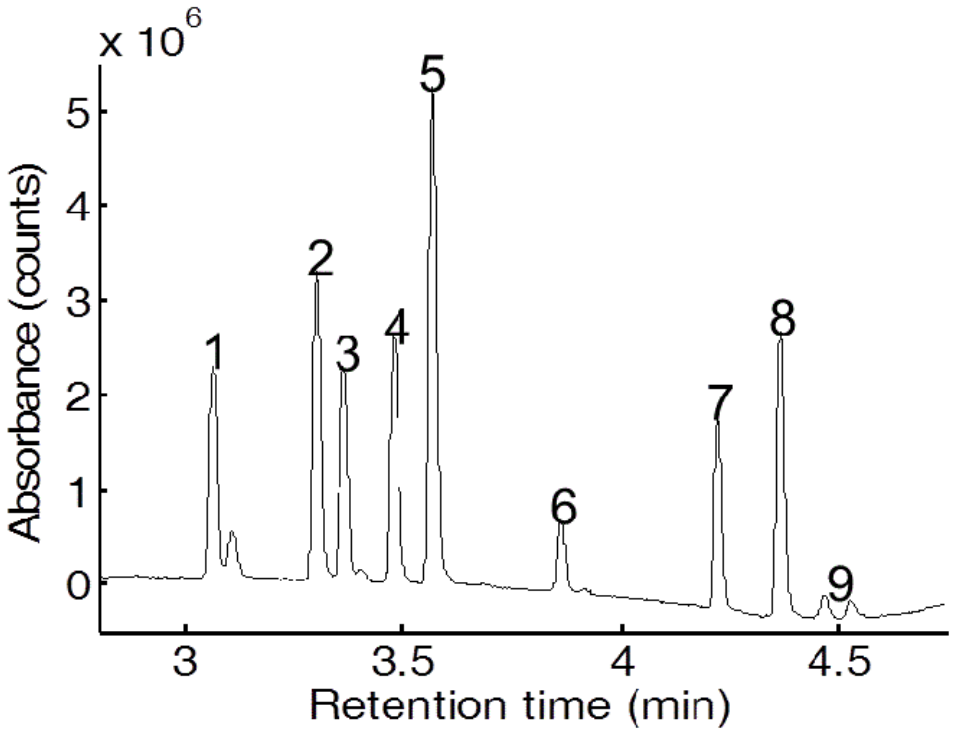


Figure 2. Separation of acid, basic, and disperse dyes at 1 ppm concentration. Peak identification: (1) Basic Red 46 (two peaks); (2) Basic Yellow 28; (3) Acid Yellow 49 (two peaks); (4) Acid Red 337; (5) Basic Violet 16; (6) Disperse Yellow 114; (7) Acid Blue 281; (8) Disperse Violet 77; and, (9) Disperse Blue 60 (two peaks).

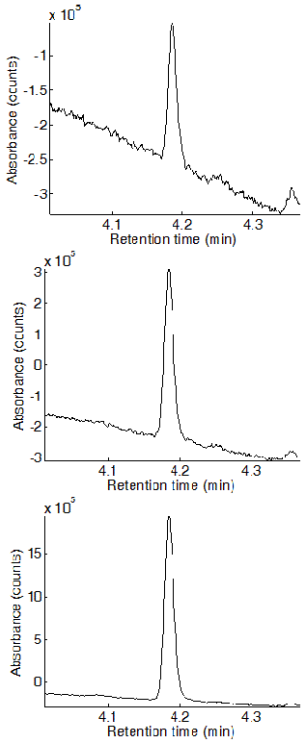


Figure 3. UPLC-DAD chromatograms for Acid Blue 281 extracted from a 0.5 mm fiber (top), 1 mm fiber (middle), and 5 mm fiber (bottom).

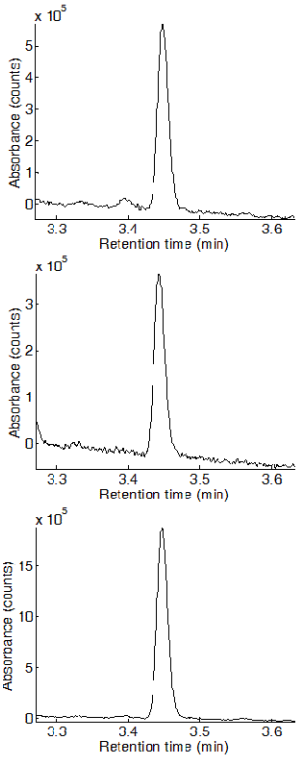


Figure 4. UPLC-DAD chromatograms for Acid Red 337 extracted from a 0.5 mm fiber (top), 1 mm fiber (middle), and 5 mm fiber (bottom).

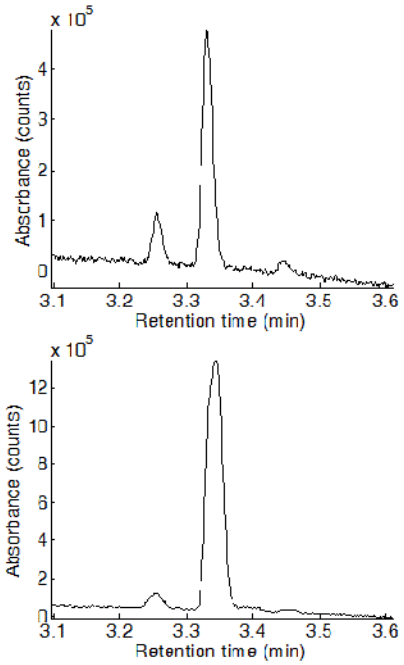


Figure 5. UPLC-DAD chromatograms for Acid Yellow 49 extracted from a 1 mm fiber (top) and a 5 mm fiber (bottom).

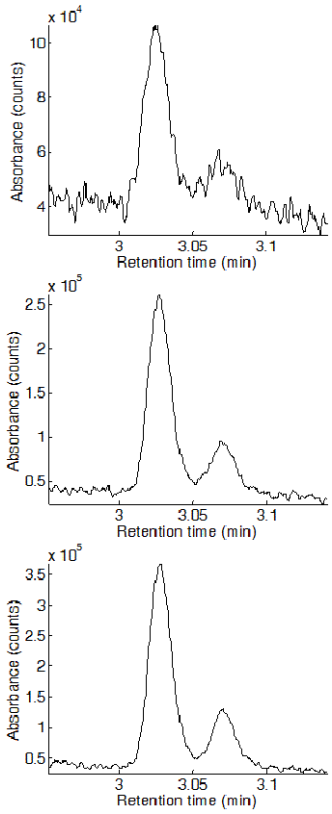


Figure 6. UPLC-DAD chromatograms for Basic Red 46 extracted from a 0.5 mm fiber (top), 1 mm fiber (middle), and 5 mm fiber (bottom).

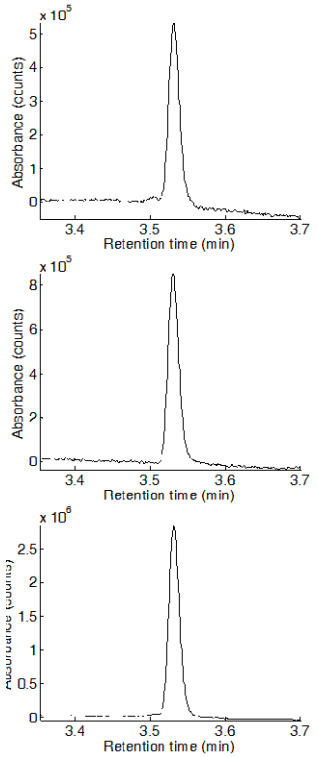


Figure 7. UPLC-DAD chromatograms for Basic Violet 16 extracted from a 0.5 mm fiber (top), 1 mm fiber (middle), and 5 mm fiber (bottom).

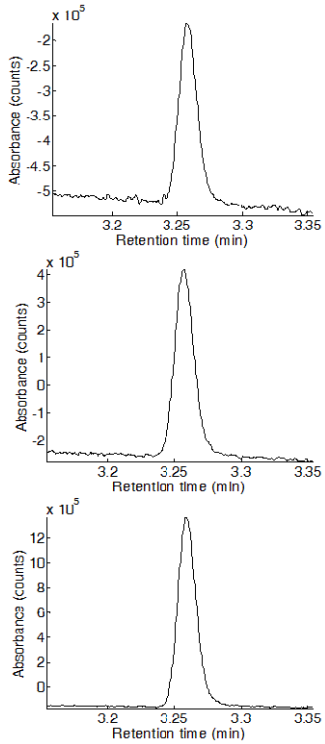


Figure 8. UPLC-DAD chromatograms for Basic Yellow 28 extracted from a 0.5 mm fiber (top), 1 mm fiber (middle), and 5 mm fiber (bottom).

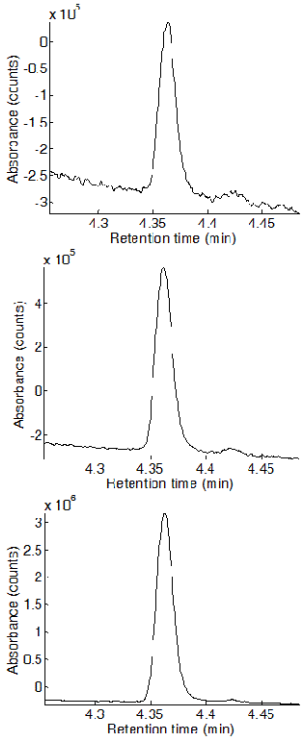


Figure 9. UPLC-DAD chromatograms for Disperse Violet 77 extracted from a 0.5 mm fiber (top), 1 mm fiber (middle), and 5 mm fiber (bottom).

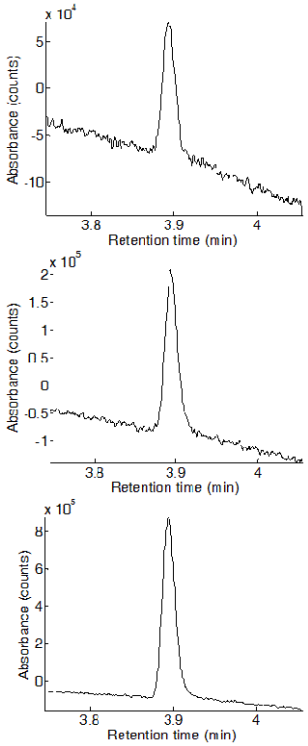


Figure 10. UPLC-DAD chromatograms for Disperse Yellow 114 extracted from a 0.5 mm fiber (top), 1 mm fiber (middle), and 5 mm fiber (bottom).

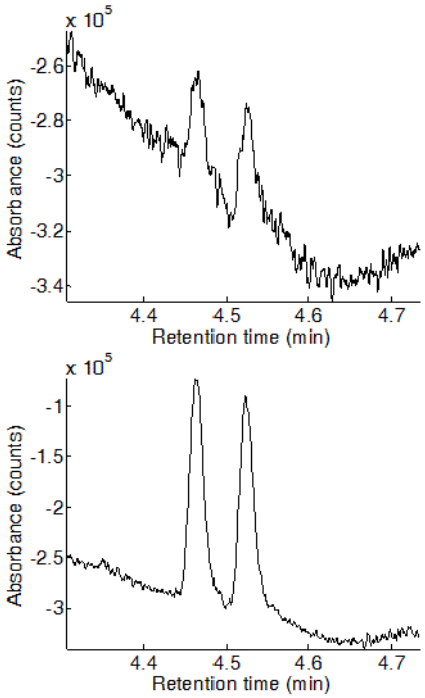


Figure 11. UPLC-DAD chromatograms for Disperse Blue 60 extracted from a 0.5 mm fiber (top), 1 mm fiber (middle), and 5 mm fiber (bottom).

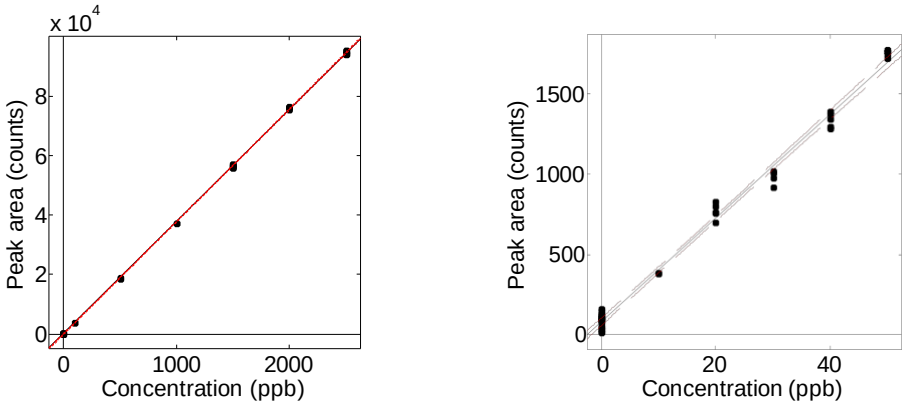


Figure 12. (Right) High and (left) low concentration UPLC-UV/visible calibrations for Acid Blue 281.

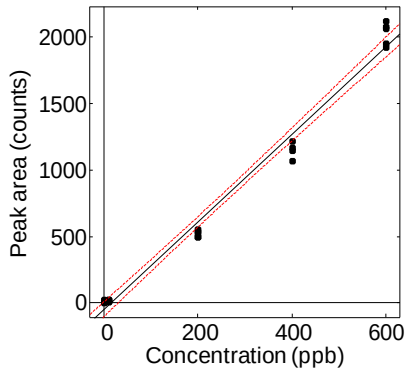


Figure 13. UPLC-MS-MS Calibration plot for Acid Blue 281.

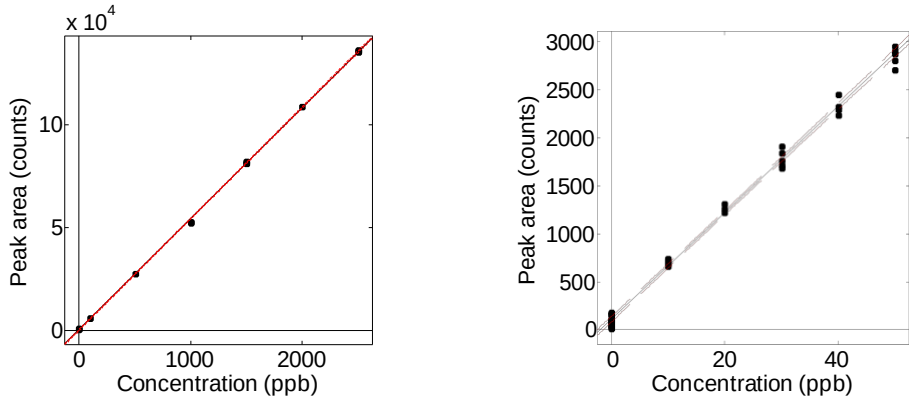


Figure 14. (Right) High and (left) low concentration UPLC-UV/visible calibrations for Acid Red 337.

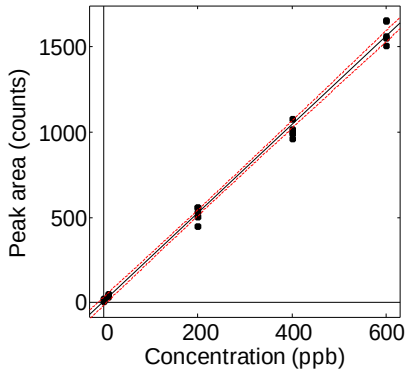


Figure 15. UPLC-MS-MS Calibration plot for Acid Red 337.

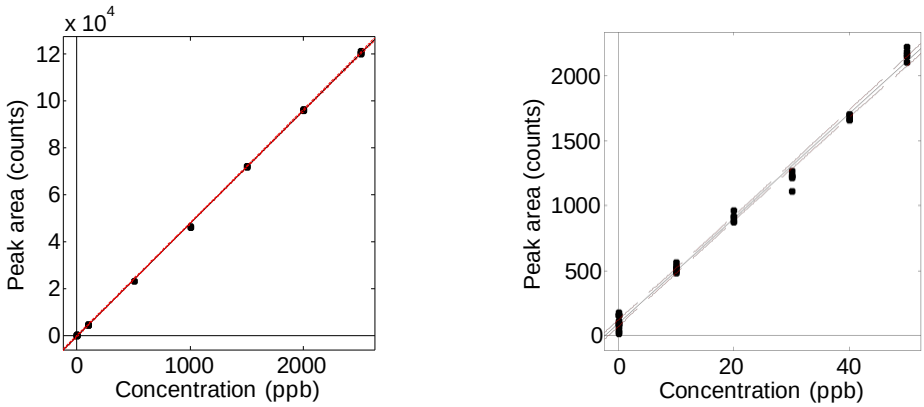


Figure 16. (Right) High and (left) low concentration UPLC-UV/visible calibrations for Acid Yellow 49.

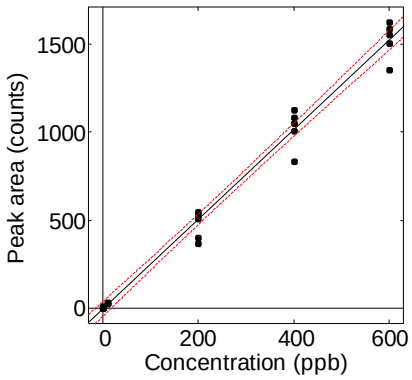


Figure 17. UPLC-MS-MS Calibration plot for Acid Yellow 49.

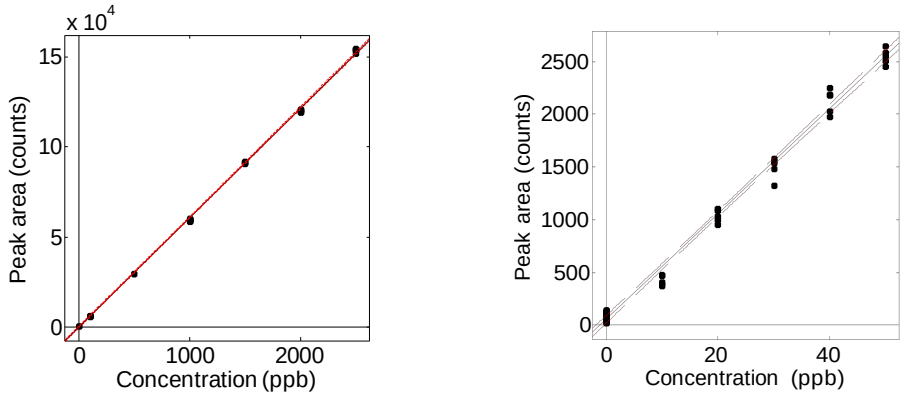


Figure 18. (Right) High and (left) low concentration UPLC-UV/visible calibrations for Basic Red 46.

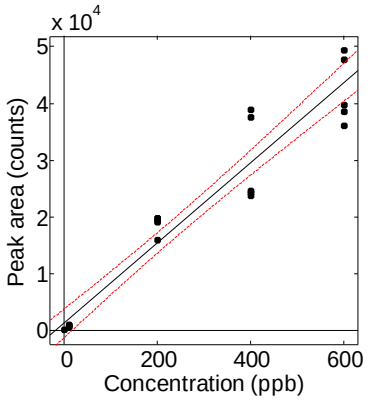


Figure 19. UPLC-MS-MS Calibration plot for Basic Red 46.

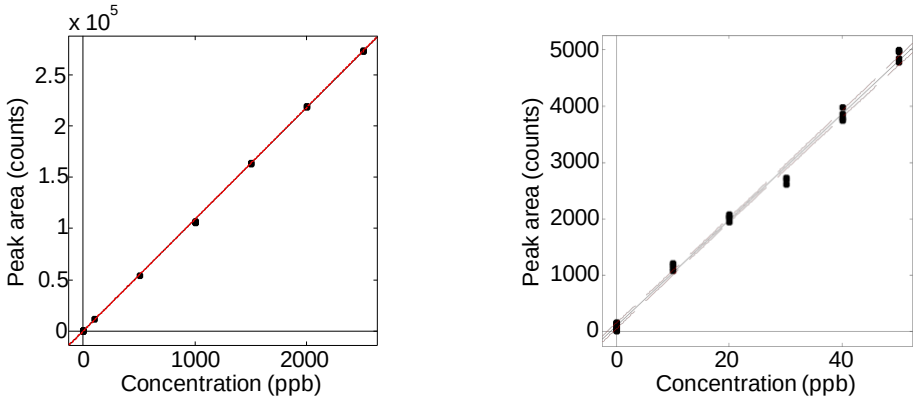


Figure 20. (Right) High and (left) low concentration UPLC-UV/visible calibrations for Basic Violet 16.

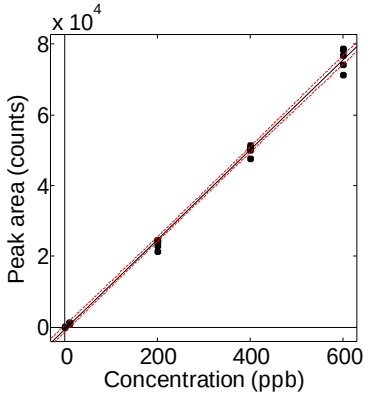


Figure 21. UPLC-MS-MS calibration plot for Basic Violet 16.

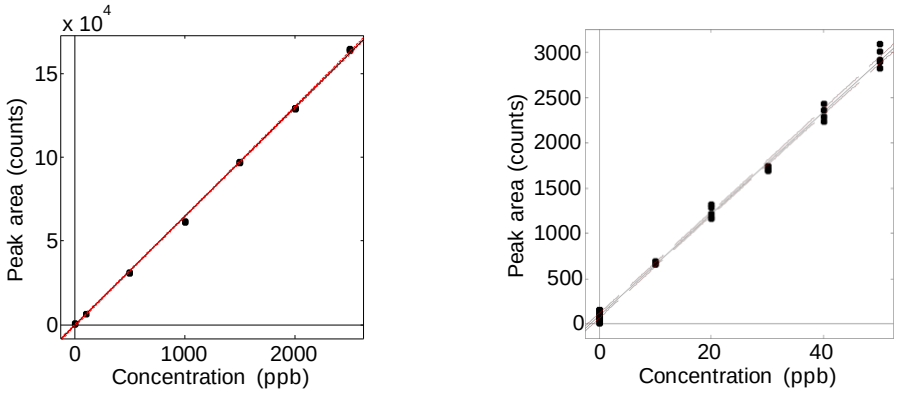


Figure 22. (Right) High and (left) low concentration UPLC-UV/visible calibrations for Basic Yellow 28.

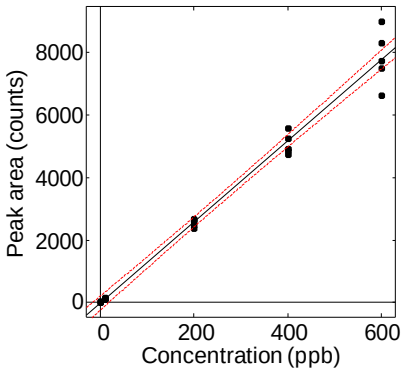


Figure 23. UPLC-MS-MS Calibration plot for Basic Yellow 28.

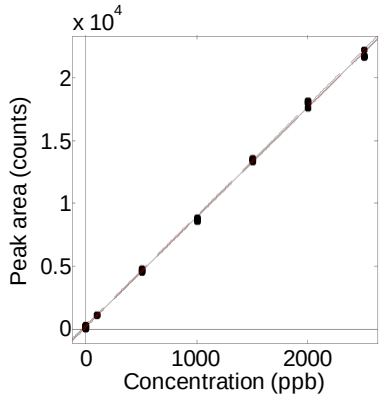


Figure 24. High concentration UPLC-UV/visible calibration for Disperse Blue 60.

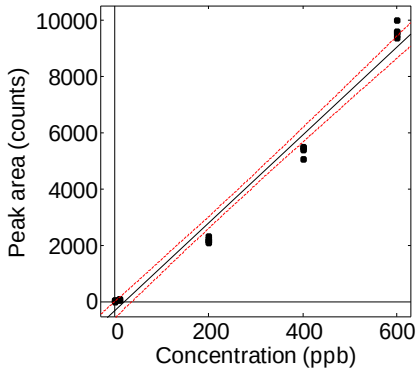


Figure 25. UPLC-MS-MS Calibration plot for Disperse Blue 60.

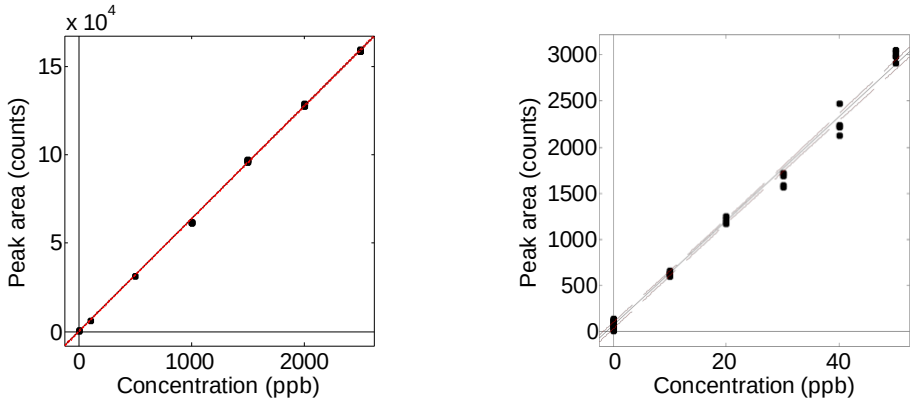


Figure 26. (Right) High and (left) low concentration UPLC-UV/visible calibrations for Disperse Violet 77.

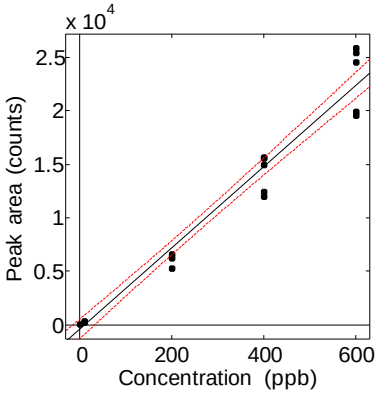


Figure 27. UPLC-MS-MS Calibration plot for Disperse Violet 77.

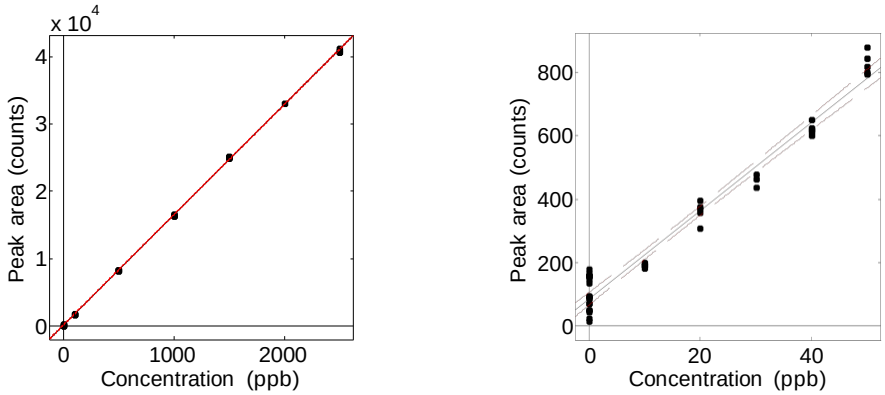


Figure 28. (Right) High and (left) low concentration UPLC-UV/visible calibrations for Disperse Yellow 114.

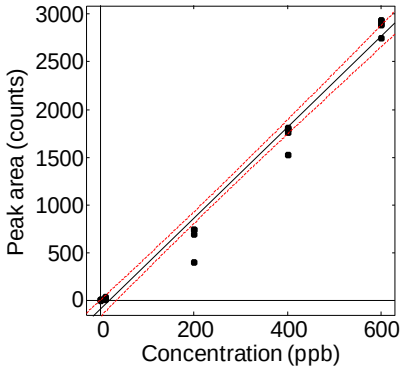


Figure 29. UPLC-MS-MS calibration plot for Disperse Yellow 114.

RMS signal-to-noise ratios of 118.05 and 62.80 for Direct Blue 71 and Indigo, respectively. Limits of detection and quantification of 2.1 pg and 6.2 pg for Direct Blue 721 and Indigo, respectively, support the potential for analysis of even sub-mm length cotton fibers. UPLC-DAD produced detection limits as low as 0.33-1.42 ppb and quantitation limits as low as 1.00-4.30 ppb. Detection and quantification extracts from single of 1-10 mm cotton fibers dyes with Reactive Yellow 160 were also confirmed.

We have also developed methods based on capillary electrophoresis and LC/MS for analysis of fluorescent brighteners and other finishing agents extracted from textile fibers. Trace analysis of dyes and fluorescent brighteners on short single fibers of 1-10 mm length has been accomplished by capillary electrophoresis with diode array detection. These analyses have been done on unweathered and weathered fibers out to one year of environmental exposure.

CONCLUSIONS

1. Discussion of findings

The design of a guillotine for cutting reproducible lengths of fibers down to 5 mm in length has improved reproducibility of fiber analysis results compared to variation see in previous work. Fibers of 0.5 mm and 1 mm still have to be cut by hand using a table-mounted magnifying glass and scalpel, although we are looking at the design of a cutting system for very small fiber lengths. Another minor improvement resulted from use of sample vials designed for high recovery with low solvent volumes (< 50 μL) to enable concentration of dyes in the extract.

The development of a single UPLC method capable of analyzing three dye classes of fibers (acrylic, nylon, and polyester) avoids having to use different columns with multiple chromatographic conditions, and increases sample throughput. The gradient program for UV/visible detection employed mixtures of 50 mM ammonium acetate in water and 0.15% formic acid in methanol. For MS detection, the mobile phase consisted of 50 mM ammonium acetate in water and 0.15% formic acid in methanol, with a gradient that compensates for the slower flow rate required for MS. These experimental conditions are familiar to forensic analysts that perform liquid chromatography and do not require departure from normal protocols.

Reactive dyes are chemically bonded to cotton fiber, and their extraction from cotton is problematic. However, these fiber dyes are important to forensic fiber examiners because they account for approximately 82% of all dyes used in the dyeing of cotton. For reactive dyes, the fiber-dye bond must be broken under stringent hydrolysis conditions that are destructive to the fiber and can cause dye degradation products to be produced. However, despite chemical degradation of the original dye, separation of the resulting product mixture and (ultimately, identification of components by mass spectrometry) can provide forensic profiling to discriminate reactive dyes from one another. Improved understanding of reactive dye degradation can also assist interpretation of extracted components and enable identification of the parent reactive dye.

Analyses of fluorescent brighteners extracted from white fibers offer exciting opportunities for forensic investigators to discriminate essentially colorless fibers. Based on weathering of textile samples acquired under previous funding from the FBI Laboratory, we have characterized using CE and LC/MS changes that occur in in detected amounts of C.I Basic violet 16 and two finishing agents from acrylic fabric weathered by environmental exposure in a hot humid climate

and a hot dry climate for up to one year. Detection of dyes has also been demonstrated on acrylic, nylon, and polyester weathered for one year. This work is ongoing at this time and we expect that paper to be supplemented with more data.

An observation from our prior work is that the microspectrophotometry data may not be sufficient for confident identification of dyes extracted from weathered fibers because of weak UV/visible or fluorescence spectra. Matching of questioned (weathered) samples to known samples may be better enabled from UPLC data; certainly mass spectral analysis can identify dyes even on 12 month-weathered samples. The ability to detect dyestuffs on environmentally weathered fibers adds value for investigators attempting interpretation of environmental effects on fiber evidence and determination of their forensic relevance. We hope that this work will provide trace evidence examiners with greater insight into the possible perturbations observed in casework samples by providing both qualitative and quantitative insights into the effects of environmental exposure. Explanations by trace evidence examiners for observed differences in textile fibers as a result of environmental exposure will be more convincing if accompanied by awareness of possible chemical or physical changes.

As noted in the paper on Mandel sensitivity, this concept offers an advantage over scale dependent approaches such as RSDs and CVs because it normalizes performance relative to the variability of the measurement method. This approach enables calculation of detection limit critical values in a universal way that also provides intuitive and visual understanding of the differences among limit of detection amount, minimal consistently detectable amount, and limit of quantitation. In revising the paper in this report for publication, we will look for potential applications of these ideas for comparison of results from the different analytical methods that we have used.

The chapter on statistical concepts concerning limits of detection, a short summary of our in-house calibration software was provided. This program was written to automate chromatographic data processing with validation in mind at all times. Over the next months we will make further improvements to include; (a) in the case of a straight line model exhibiting lack of fit, the program will be designed to automatically generate the statistical analysis for the next higher- order model, a quadratic equation. This modification is in response to the need of the LC/MS laboratory at the SC State Law Enforcement Division Forensic Laboratory, where non-straight line calibrations are apparently often needed. (b) Analysis of false positive and false negatives outcomes displayed for decision using a receiver operator characteristic curve; (b) a statistical tolerance interval calculation for LOD estimation. (c) estimated uncertainties (e.g., 95% confidence intervals in predicted amount of analyte. (d) a user preference menu that defines critical choices in LOD parameters and other settings to be retained in a profile file for future
use, A major driving force for our development of this software was to automate limits of detection calculations in this NIJ project.

2. Implications for policy and practice

It has been observed that use of trace evidence and testimony of examiners has decreased in recent years, perhaps due to "over-reliance on nuclear DNA, latent print, and mitochodrial DNA evidence."[1] Fiber evidence is also maligned in popular forensic books with phrases such as "hanging by a thread" and "an inexact science," implying fiber comparisons are entirely "subjective."[2,3] Other significant questions raised in the 2009 National Academy of Sciences report[4] include the following:

(a) The Scientific Working Group for Materials Analysis (SWGMAT) has produced guidelines, but no set standards, for the number and quality of characteristics that must correspond in order to conclude that two fibers came from the same manufacturing batch. There have been no studies of fibers (*e.g*., the variability of their characteristics before and after manufacturing) on which to base such a threshold.
(b) There have been no studies that characterize either reliability or error rates in procedures.
(c) There have been no studies to inform judgments about whether environmentally related changes discerned in particular fibers are distinctive enough to reliably individualize their source.

The goal of the forensic fiber examination is comparison of questioned fibers found at a crime scene with one or more known fibers to determine possible associations between victims, suspects, and crime scenes. If a match of questioned to known fibers is not excluded, the possibility of associations between suspect individuals and the crime may provide probative value or investigative leads. Visual comparison of fiber morphology by optical microscopy is can provide discriminating information quickly in the early stages of a fiber examination. For example, acrylic is a synthetic fibers that is manufactured by dissolving polyacrylamide in organic solvent. After extruding the polymer into water and drying, the fiber collapses to a shriveled appearance with crinkled cross-sections ranging from circular to dogbone shapes. Synthetic fibers are often crimped to make them hold together better and to impart bulkiness when made into yarn; the number of crimps per inch might itself be a distinctive signature of such fibers from their manufacturing process. Cotton, a cellulosic natural fiber, has a distinct twisted and non-uniform appearance characteristic of its biological origin.[5] Physical measurements of fiber diameter, and cross-sectional shape, can also contribute to decision- making. Finally, polarized light microscopy can be used to determine optical characteristics such as refractive index, birefringence, and sign of elongation, which can be sufficiently discriminating for rapid identification of generic fiber type.[5, 6] Fibers with different If these methods fail to exclude a match between questioned and known fibers, fluorescence microscopy, UV/visible and/or fluorescence microspectrophotometry, and infrared (IR) spectroscopy are used for chemical structural information, color characterization, and more specific polymer identification. All of these techniques are fast, nondestructive, and enable discrimination of fibers by different and increasingly specific characteristics. Fiber size, shape, color, and polymer identity differentiates most fibers. However, these techniques do not identify dyes. For example, two textile fibers dyed with mixtures of several, possibly different, dyes might be formulated by different manufacturers to achieve a particular (common) color. These fibers could be visually indistinguishable. Visual comparison of shapes of peak, valleys, and rising or falling portions of the UV/visible or fluorescence spectra might reveal subtle differences. However, judgment of the practical significance of these differences is often subjective, and the spectra usually represent unresolved mixtures of several unidentified dyes. Infrared spectra are dominated by the polymer and cannot be used for dye identification. *Modern instrumental analysis of separated dye components can increase the reliability of fiber examinations by providing discriminating information on dye characterization and possibly identification of dyes at the molecular level from trace evidence fibers as small as 0.5 mm.*

Extraction methods developed for basic dyes on acrylic, acid dyes on nylon, and disperse dyes on polyester fibers involve extraction with solvents that do not affect the chemical composition of the polymer, and thus do not impose limitations on use of the fiber for further examinations. With these fibers, IR can be conducted on previously extracted samples if needed. Reactive dye

"Development and Figures of Merit of Microextraction and Ultra-Performance Liquid Chromatography for Forensic Characterization of Dye Profiles on Trace Acrylic, Nylon, Polyester, and Cotton Textile Fibers." 10 August 2013. Scott is presently Senior Chemist at ExxonMobil, Baytown, TX.

Two graduate students have worked on the project during the last year:

Molly R. Burnip, is currently a third year graduate student at USC, and has worked on method development and validation.

Kaylee R. McDonald, is currently a third year graduate student at USC, and has worked on method validation issues.

Undergraduate research students working on this project include:

Molly R. Burnip (B. S., Chemistry, 2012) worked as an undergraduate researcher, 2011-2012. She graduated with Phi Beta Kappa honors in Chemistry. Molly is currently a second year graduate student at USC who has continued to work on the project for her Ph. D. dissertation research.

Nicholas M. Riley (B.S. Chemistry, 2012), was a Fulbright Scholar finalist, partially based on LC/MS dye analysis research. Nick won the highest student leader ship award at USC (Algernon Sydney Sullivan Award, 2012), and graduated with Phi Beta Kappa honors in Chemistry. Nick is a second year chemistry graduate student at the University of Wisconsin, Madison, WI, working on mass spectrometry, proteomics, and quantitative systems biology in the laboratory of Professor Joshua J. Coon.

Andrei Kovaltshuk (now Andrew Green) won a USC Magellan Scholar Award of $3,000 that paid him to work in my laboratory on “Forensic Characterization of Dye Extracts from Millimeter-Length Textile Fibers," during 2011. Andrei graduated with Magna cum Laude honors in Chemistry. Andrei is a second year graduate student in chemistry at the University of California-Riverside, Riverside, CA, working on mass spectrometric instrumentation design.