Anal Bioanal Chem
DOI 10.1007/s00216-009-2876-8

ORIGINAL PAPER

Automated extraction of direct, reactive, and vat dyes from cellulosic fibers for forensic analysis by capillary electrophoresis

C. R. Dockery · A. R. Stefan · A. A. Nieuwland · S. N. Roberson · B. M. Baguley · J. E. Hendrix · S. L. Morgan

Received: 1 February 2009 / Revised: 26 May 2009 / Accepted: 28 May 2009

Abstract Systematic designed experiments were employed to find the optimum conditions for extraction of direct, reactive, and vat dyes from cotton fibers prior to forensic characterization. Automated microextractions were coupled with measurements of extraction efficiencies on a microplate reader UV–visible spectrophotometer to enable rapid screening of extraction efficiency as a function of solvent composition. Solvent extraction conditions were also developed to be compatible with subsequent forensic characterization of extracted dyes by capillary electrophoresis with UV–visible diode array detection. The capillary electrophoresis electrolyte successfully used in this work consists of 5 mM ammonium acetate in 40:60 acetonitrile–water at pH 9.3, with the addition of sodium dithionite reducing agent to facilitate analysis of vat dyes. The ultimate goal of these research efforts is enhanced discrimination of trace fiber evidence by analysis of extracted dyes.

Keywords Forensic analysis · Cotton fibers · Extraction of direct, reactive and vat dyes · Capillary electrophoresis

C. R. Dockery · A. R. Stefan · A. A. Nieuwland · S. N. Roberson · B. M. Baguley · J. E. Hendrix · S. L. Morgan (✉)
Department of Chemistry and Biochemistry,
University of South Carolina,
Columbia, SC 29208, USA
e-mail: morgan@mail.chem.sc.edu

Present Address:
C. R. Dockery
Department of Chemistry and Biochemistry,
Kennesaw State University,
Kennesaw, GA 30144, USA

Present Address:
A. R. Stefan
Polymathic Analytical Labs,
3737 Industrial Blvd.,
Orangeburg, SC 29118, USA

Present Address:
A. A. Nieuwland
Voridian, Hwy. 21 S., Box 1782, Columbia, SC 29202-1782, USA

Present Address:
B. M. Baguley
Washoe Co. Sheriff Forensic Science Division,
911 Parr Blvd.,
Reno, NV 89512, USA

Introduction

The comparison of questioned fibers found at a crime scene with one or more known fibers is central to the forensic examination of fibers. A first step is often visual comparison of fiber morphology by optical microscopy; for example, cotton can be recognized by its distinct morphology [1]. For synthetic and regenerated fibers, identification of generic polymer type can be achieved by measurement of birefringence, sign of elongation, and refractive index with polarized light microscopy [1, 2]. Additional instrumental methods, including infrared and UV–visible microspectrophotometry and thin-layer chromatography, can also provide discriminating information, depending on the fiber and dye types [3]. 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. Our present research involves the potential for extraction of dyes from fibers, and their subsequent analysis using capillary electrophoresis (CE), to provide the identity and relative amounts of dyes present.

Published online: 18 June 2009

Solvent extraction formulations must solvate the dye, reduce substantivity (affinity via intermolecular interactions) of the dye for the fiber, and transport the dye into the bulk solvent. Knowledge of the chemistry of cotton fiber and its possible interactions with the variety dyes that are substantive to cotton via different mechanisms are essential to achieving these goals. Cotton is made of natural cellulose, a polyhydroxyl homopolymer of glucose. Cellulose contains only hydroxyl groups and acetal linkages, and no ionic dye sites are present for dye attachment on the cellulose polymer. Thus, two common approaches to dyeing cotton involve attachment of dyes by hydrogen bonding (direct dyes) or covalent bonding to glucosidic hydroxyls (reactive dyes). Alternatively, colorant can be deposited within the structure of cellulose and converted to an insoluble pigment (vat or sulfur dyes). Three dye classes—direct dyes (33%), reactive dyes (33%), and vat–sulfur dyes (16%)—account for approximately 82% of commercially dyed cotton [4].

Direct dyes typically contain sulfonic acid groups, which make them soluble in water. During dying, the cellulose fiber becomes coated with a molecular layer of the anionic dye. Dye molecules then migrate into the cellulose fiber and are bound via hydrogen bonding to glucosidic hydroxyls. The direct dyes examined in this research are distributed across several classes, representing approximately 97% of all direct dye chemical classes applied commercially to cotton: diazo (49%), polyazo (33%), monoazo (5%), copper-complex azo (5%), and stilbene (5%) [4]. The hydrogen bonding interactions of direct dyes are easily disrupted for extraction by alkaline solvents. Aqueous pyridine has been successfully used at elevated temperatures to extract direct dyes from cotton fiber on the macro-scale. Laing et al. [5] reported efficient extraction of 20 different direct dyes from cotton using pyridine–water (4:3 *v*/*v*). Cheng et al. [6] validated the utility of pyridine–water (4:3 *v*/*v*) by extraction of 13 direct dyed cotton fibers.

Reactive dyes are the most substantive of dyes used on cotton because of their covalent bonding with glucoside hydroxyls. Reactive dyes are frequently preferred over direct dyes for cellulose for their uniformity of application and excellent fastness to laundering and dry cleaning. The reactive dye classes considered in this work include unmetallized azo (66%) and anthraquinone (10%), which together represent 76% of reactive dyes on cotton [4]. Typically, reactive sites on the dye molecule are activated vinyl groups, which react with cellulose through addition of the hydroxyl to the double bond, or activated halogen substituents such as a chlorotriazine or fluorotriazine. Reactive dyes can be hydrolyzed with sodium hydroxide at elevated temperatures to facilitate extraction. Home and Dudley [7] were the first to publish an investigation of solvent systems suitable for extraction of reactive dyes from cotton. The solvents evaluated were sodium sulfide–water–poly(vinylpyrrolidone) (PVP), hydrogen bromide, 60% aqueous sulfuric acid, and 1.5% aqueous sodium hydroxide. Sulfuric acid was used at room temperature, and the other solvents were used at 100ºC. Sodium hydroxide was the only solvent that was reported to give good extraction results, but the extraction time was not mentioned. Extraction of 44 out of 50 reactive dyes was cited as evidence of the suitability of NaOH extraction. Sirén and Sulkava [8] used alkaline hydrolysis followed by CE to analyze black reactive dyes from cotton and wool samples. Complete extraction was reported from bulk cotton treated with 1.5% aqueous NaOH at 100 °C for 20 min. Wool fibers were treated with ammonia–water (4:3 *v*/*v*) at 100 °C for 50 min. Xu et al. [9] extracted reactive dyes from cotton with 1.5% sodium hydroxide in 50% methanol at 100 °C for 80 min and analyzed extracts by micellar electrokinetic capillary chromatography. These authors also report that cotton fibers partially dissolve in concentrated sodium hydroxide at elevated temperatures and that the resulting electroosmotic flow in CE was not reproducible. Because CE using high-ionic-strength solutions tends to produce higher current, sodium or potassium ions must be removed from extracts by cation exchange.

Vat dyes typically do not possess ionic groups and are insoluble in water. To be applied to cotton, vat dyes must first be reduced from the *keto* form to the water-soluble *leuco* form using a reducing agent such as sodium dithionite. The soluble *leuco* dye permeates the cellulose structure during the dyeing process and then is oxidized back to the insoluble *keto* form to be trapped within the cellulose matrix. Extraction of anthraquinone vat dyes (79% of commercial use on cotton [4]) is investigated in this paper. Vat dyes are efficiently extracted from cotton by application of sodium dithionite to reduce them to their water-soluble *leuco* form [5]. A limited number of vat dyes have been extracted with 15% (*w*/*v*) urea in water, followed by heating in 2-chlorophenol. Examination of the dye structures that were extracted by urea suggested that the mechanism only involves reduction of nonanthraquinone carbonyls [6]. However, because sodium dithionite has been reported to extract all vat dyes, this route was selected as our focus.

Recognizing that fiber samples relevant to forensic casework are as small as 2–10 mm in length, the development of extraction-based analysis for such limited size samples must first specify solvent conditions that will extract sufficient dye for subsequent chromatographic, mass spectrometric, and/or spectroscopic analysis. Accordingly, we report here systematic studies designed to optimize solvent conditions for extraction from cotton fibers of representative reactive, direct, and vat dyes. A secondary objective of this work was to demonstrate that the selected extraction solvent conditions for each dye subclass are compatible with subsequent analysis using capillary electrophoresis.

Experimental

All dyed fabrics, along with standard samples of the direct, reactive, and vat dyes used, were donated by dyestuff manufacturers located in the southeastern USA; the dyes selected are in current commercial use. For the purpose of optimizing extraction conditions under macro-scale conditions, 10-cm threads of fiber (yarns consisting of a bundle of twisted fibers) enabled high signal-to-noise in absorbance measurements to determine extraction efficiency. Dyed cotton threads were loaded into a 96-well plate system purchased from Biotech Solutions (Mt. Laurel, NJ, USA). Solvents were added to the threads in 500-μL glass inserts in a 96-well plate using a BioMek 2000 automated liquid-handling workstation (Beckman-Coulter, Fullerton, CA, USA). Extraction solvents were added to the samples by programmed operations for the specific fiber–dye combinations. All chemicals used were of analytical reagent grade. A Teflon liner placed between glass inserts and a plastic lid minimized solvent evaporation during high-temperature extractions. Aluminum plates were also clamped tight with metal clips above and below the 96-well plate system to provide a seal between the Teflon liner and glass inserts.

For the extraction of direct dyes from cotton, a simplex mixture design [10] was employed to study the influence of solvent conditions on extraction efficiency for a representative direct dye, C. I. direct yellow 58. The three solvent components were deionized water (18.2 MΩ from a Millipore Simplicity water purification system, Milford, MA, USA), 29.7% aqueous ammonium hydroxide (Fisher Scientific Co., Fair Lawn, KY, USA), and 100% pyridine (Mallinckrodt, Paris, KY, USA). The ten design points (experiment locations in the mixture space) shown in Table 1 were each replicated, for a total of 20 runs; runs were conducted in a random allocation on the workstation surface. Extractions were carried out at 60 °C for 60 min. The robot was programmed to add the designated amounts of liquid to each of 20 glass inserts in a 96-well sample plate for a total volume per well of 200 μL. Cotton fibers (10 cm of multifilament threads) dyed with diazo, polyazo, monoazo, and copper-complex azo direct dyes were extracted in each well. Following extraction, solvents were evaporated to dryness at 50 °C, and dye residues were reconstituted in 200 μL of deionized water. The extent of extraction was determined by measuring the absorbance at the dye's spectral peak maximum using a SpectraMax M5 UV–visible microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA, USA). A quadratic Scheffé mixture model (including first-order effects for each component, and the three two-factor interactions) was fitted to the absorbance data [10–12]. Design of experiments, modeling, and graphics were carried out using *Design Expert*, v. 7 (Stat-Ease Corp., Inc., Minneapolis, MN, USA).

Table 1 Simplex mixture experimental design for optimization of extraction of direct dyes from cotton fibers using mixtures of pyridine, ammonium hydroxide, and water

Design point	Pyridine (%)	Ammonia (%)	Water (%)
1	100	0	0
2	0	100	0
3	0	0	100
4	0	50	50
5	50	0	50
6	50	50	0
7	67	16	16
8	16	67	16
9	16	16	67
10	33	33	33

Compositions are expressed as volume percent

For the extraction of reactive dyes from cotton, multiple solvent systems using 29.7% aqueous ammonium hydroxide, 1.5% aqueous solutions of sodium hydroxide, and barium hydroxide (Fisher Scientific, Fair Lawn, NJ, USA) were investigated. Following extraction, methods to remove alkaline and alkaline earth cations from the extract were investigated. Cation exchange resin (Dowex® HCR-W2, H^+ form, spherical beads, 16–40 mesh, J.T. Baker, Phillipsburg, NJ, USA) and Waters Oasis HLB 6-cm^3 solid-phase extraction (SPE) cartridges with a vacuum manifold (Waters Corporation, Milford, MA) were used for cation exchange and SPE, respectively. Ammonium bicarbonate (Fisher Scientific) was employed to precipitate barium carbonate.

For extraction of vat dyes, reduction was performed using a sodium dithionite reducing agent prepared according to Laing et al. [5]. Laboratory-grade sodium dithionite (Fisher Scientific) was dissolved in 1,2-dimethoxy ethane (Acros Organics, Fair Lawn, NJ, USA). Derivatization of vat dyes to the oxime form as described by Vogh [13] used hydroxylamine hydrochloride in methanol (Fisher Scientific). Aqueous solutions of polyvinylpyrrolidone K 90 (Fluka Chemie AG, Buchs, Switzerland) were used to strip vat dyes from dithionite extraction solvent.

Anionic dye standards were prepared in deionized water at concentrations of 0.1 mg/mL. Direct, reactive, and vat dyes recovered from extractions (after reconstitution in 200 μL of deionized water) were analyzed by CE with diode array detection using an Agilent (Palo Alto, CA, USA) G1600A CE system equipped with a diode array detector. CE was carried out in a fused silica capillary with an internal diameter of 75 μm and length of 50 cm (42 cm effective length; Polymicro Technologies, Phoenix, AZ, USA). The capillary was conditioned before use by rinsing with 0.5 M ammonium hydroxide for 15 min, with water

for 3 min, and with electrophoresis medium for 15 min. Between sample injections, the capillary was rinsed with NH_4OH for 3 min, with water for 2 min, and with electrophoresis buffer for 3 min. The electrolyte solution consisted of 5 mM ammonium acetate in acetonitrile–water (40:60, *v*/*v*), at pH9.3. We have found that the same buffer can also be used for vat dye analysis, following the addition of 20 mM sodium dithionite. Hydrodynamic injections were done at 50 mbar for 2 s. Separation was performed at 25 °C with an applied voltage of 20 kV. Absorbance was monitored from 190 to 600 nm.

Results and discussion

Direct dyes Absorbance measurements from the total of 20 experiments extracting C. I. yellow 58 dye from cotton, carried out at 60 °C for 60 min, were modeled by the method of least squares to produce a response surface for visualizing the effects of each solvent on the extraction efficiency. A contour plot and three-dimensional surface plot of the predicted extraction response as function of solvent composition is displayed in Fig. 1. The fitted model has factor effects(s) significantly different from zero (p=0.018 for the null hypothesis); the coefficient of determination (R^2) was 0.7136; and lack of fit of the model was not significant (p=0.4616) [10–12]. The pooled relative standard deviation for the replicate absorbance measurements was 16.64% (based on ten degrees of freedom, relative to the highest measured response). This relatively high experimental uncertainty we attribute to the difficulty of cutting cotton threads to reproducible lengths because of their twisted and pliant shape.

The fitted model predicted that highest absorbance response was located at a solvent composition of 34.3% pyridine–65.7% water, with no aqueous ammonia in the solvent mixture. Little difference in the extracted dye amount is seen over the ridge in the surface that is parallel to a constant aqueous pyridine composition of about 35%. The flatness of the response around the optimum conditions also implies a degree of ruggedness: any solvent composition reasonably close to the predicted optimum or along the ridge line will produce similar extraction results. Of the three pure solvents, water provided the best extraction, followed closely by pure ammonia; pure pyridine is not a good extraction solvent for this dye. Extraction surfaces for other direct dyes tested were similar to that shown in Fig. 1. Subsequent extractions of direct dyes were performed using of 35% pyridine–65% water.

Figure 2 shows the capillary electropherogram trace obtained for a mixture of seven direct dyes obtained from manufacturers. Two direct dyes, C.I. direct Black 112 and C.I. direct Blue 71, were found to have two major components, and there are some other extraneous peaks in the electropherogram. Commercial dyes are usually formulated to match a specific color and may consist of mixtures of dyes and may contain isomeric or derivative species or other contaminants. The anions eluted in the expected order of charge/mass ratio, except that C.I. direct Orange 39 eluted after C.I. direct red 84. The excellent separation of all 13 dyes suggests that the electrolyte buffer, consisting of 5 mM ammonium acetate in acetonitrile–water (40:60, *v*/*v*) at pH9.3, is applicable for a diverse range of direct dyes. The same buffer has been also previously been employed by Poiger et al. [14] for the analysis of anionic dyes.

Figure 3 shows capillary electropherograms of a direct dye standard and of the same dye extracted from a 10-cm

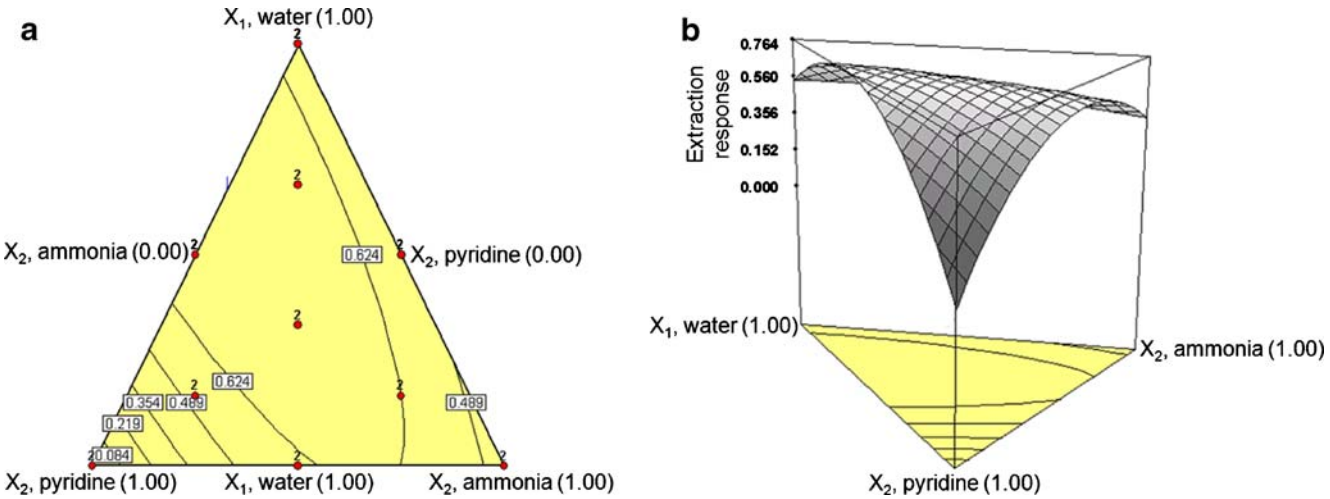

Fig. 1 **a** Contour plot of the fitted response surface for the extraction of direct dye (C. I. direct yellow 58) from cotton using water, pyridine, and aqueous ammonia (60 °C for 60 min). Two replicate experiments were performed at each of ten design points marked by *dots*. **b** Three-dimensional view of the fitted response surface

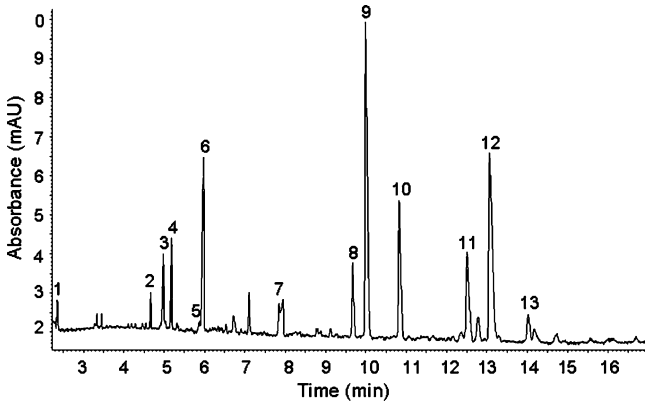

Fig. 2 Electropherogram at 214 nm showing the separation of a mixture of seven direct dyes. Peak identification: (*1*) neutrals; (*2*) C. I. direct black 22; (*3*) C. I. direct black 112, first peak; (*4*) C. I. direct red 84; (*5*) C. I. direct black 112, second peak; (*6*) C. I. direct orange 39; (*7*) C. I. direct yellow 86; (*8*) C. I. direct yellow 58; (*9*) C. I. direct blue 71, first peak; (*10*) C. I. direct blue 71, second peak

dyed cotton fiber using 35% pyridine–65% water. The purity of the dyes is supported by the single major dye peak obtained in the electropherograms (except for a small neutral component peak and some slower migrating minor peaks). The UV–visible spectra of the extracted dye peak (Fig. 3d) matches that of the standard (Fig. 3b), indicating that the chemical nature of the dye has not be altered by the extraction processing. The electropherogram also represents a test of the compatibility of the extraction protocol with subsequent CE analysis. Four sets of triplicate CE analyses of dye standards using the present electrolyte buffer, conducted over a 4-h period, produced a pooled standard deviation of 0.65 min. Thus, migration times, at least over the short term, were reproducible to within a few seconds.

Reactive dyes Reactive dyes are the most resistant to extraction of all dye types due to the covalent bond formed between the dye and cotton fibers. An aqueous solution of a strong base is typically used to remove reactive dyes from the fiber. The alcohol groups on the glucose units in the cellulose backbone of cotton act as a weak acid and are ionized under alkaline conditions. However, as mentioned previously, high Na^+ concentration and high ionic strength are not desirable with CE. The initial attempt to avoid alkaline cations involved a hydrolysis extraction with aqueous ammonium hydroxide in which the ammonium

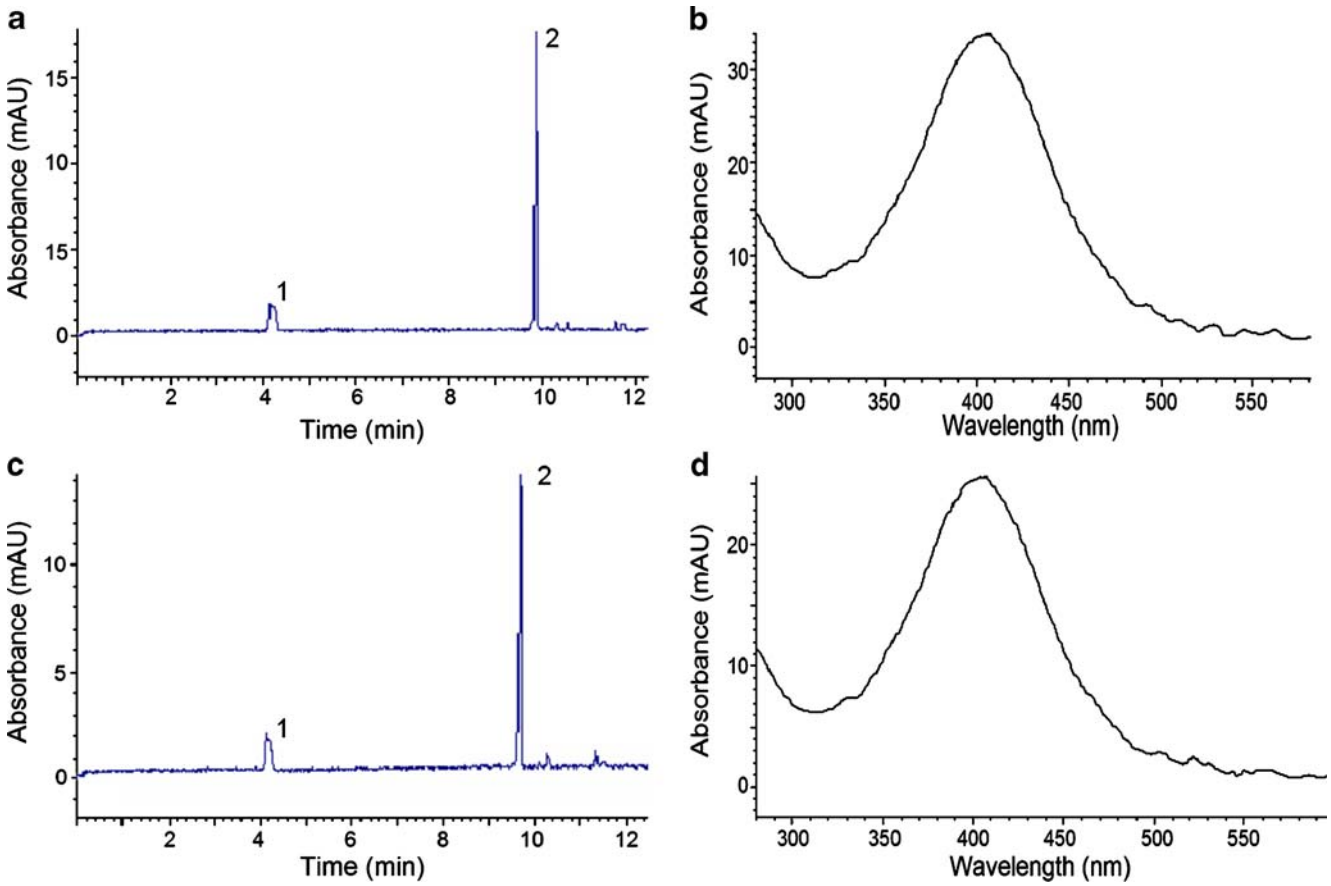

Fig. 3 **a** Electropherogram of a direct dye, C.I. direct yellow 58; **b** UV–visible spectrum of C.I. direct yellow 58 standard; **c** electropherogram of an extract from 10 cm of cotton thread dyed with C.I. direct yellow 58; **d** UV–visible spectrum of peak 2 from extract. Peak identification: (*1*) neutrals; (*2*) C.I. direct yellow 58

counter ion facilitates alkaline hydrolysis of reactive dyes from cellulose and then evaporates. While some reactive dyes could be extracted from the cotton substrate using this approach, extraction times were long (>24 h) and extraction efficiencies low (<50%).

Because the forensic literature reports extraction of reactive dyes from cotton using alkaline hydrolysis of the glucose–dye link in 1.5% aqueous sodium hydroxide [7–9], our attention focused on modifying an alkaline hydrolysis method to be compatible with CE by using purification steps to remove the alkaline counter ion from the extract. Three cleanup methods were investigated: cation exchange resin, precipitation reactions, and SPE.

Alkaline hydrolysis with aqueous sodium hydroxide, followed by a cleanup step with cation exchange resin, removed sodium from the extract. The ion exchange resin, however, introduced numerous undesirable contaminants to the extract solution which could not be easily removed; although the dye peak was the largest in the electropherogram trace, over 25 other peaks compromised the analysis.

The second purification approach used alkaline hydrolysis with aqueous barium hydroxide, followed by ammonium bicarbonate to precipitate barium carbonate ($K_{sp}=2.6\times10^{-9}$), which can be removed by filtration or centrifugation. The remaining aqueous species (NH_4^+ and HCO_3^-) then thermally decompose. However, this procedure also produced excessive contamination; the dye peak was not even the largest in the extract electropherogram.

The third and more successful approach returned to the extraction of reactive dye with alkaline hydrolysis (1.5% aqueous sodium hydroxide) and used solid-phase extraction for extract cleanup. The SPE cartridges used here consist of a syringe body with about 1 cm of a wettable polymer at the bottom to bind nonpolar compounds, much like a C_{18} high-performance liquid chromatography column. After conditioning with 1-mL methanol and equilibration with 1-mL water, the solution of extracted dye was applied, and dyes could be observed to bind to the top of the packing material. Nonbound compounds (including the sodium ion) were removed by rinsing with 1 mL of 5% methanol in water. The presence of sodium in this wash solution, and absence in the remaining extract was confirmed by flame atomic emission spectroscopy. The sample was then eluted from the cartridge with 1 to 2 mL of methanol while observing the color of the packing material to ensure dye removal. Methanol was evaporated and the dye extract reconstituted in 100–200-μL deionized water for CE analysis.

Figure 4 shows an excellent CE separation in reasonable time obtained for a mixture of 12 reactive dye standards using the same electrolyte buffer employed for direct dyes. Ten of the 12 dyes elute in the charge/mass ratio order expected for anions. In addition to the major dye component,

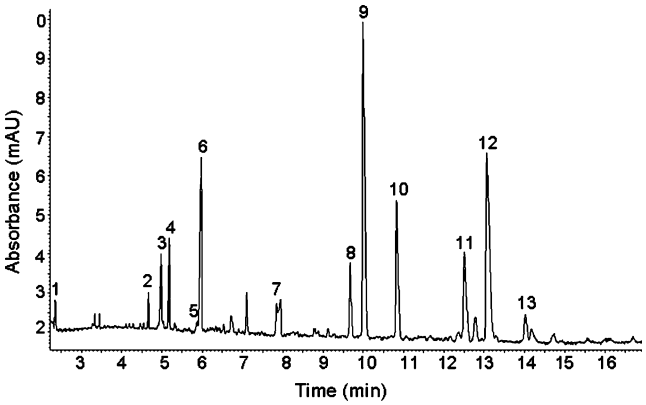

Fig. 4 Electropherogram at 214 nm showing the separation of a mixture of 12 reactive dyes. Peak identification: (*1*) neutrals; (*2*) C.I. reactive blue 21; (*3*) C.I. reactive yellow 160; (*4*) C.I. reactive Orange 72; (*5*) C.I. reactive blue 19; (*6*) C.I. reactive yellow 176; (*7*) C.I. reactive violet 5; (*8*, *9*) C.I. reactive black 5 and C.I. reactive blue 250; (*10*) C.I. reactive red 198; (*11*) C.I. reactive blue 220; (*12*) C.I. reactive red 180; (*13*) C.I. reactive red 239/241

there are several additional minor peaks in the electropherograms of these dyes provided by commercial manufacturers. A representative electropherogram and UV–visible spectrum produced by an extract of cotton dyed with C. I. reactive yellow 176 are shown in Fig. 5. The unidentified faster migrating peak at approximately 3.3 min is probably a

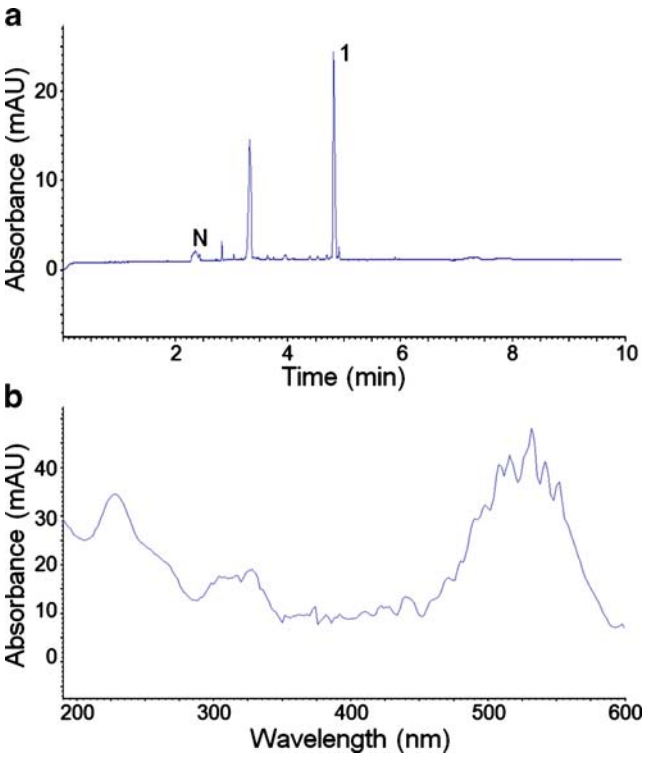

Fig. 5 **a** Electropherogram at 250 nm of an extract from 10 cm of cotton thread dyed with a reactive dye, C.I. reactive yellow 176 (*peak 1*), after treatment with 1.5% aqueous sodium hydroxide and SPE cleanup; **b** UV–visible spectrum of peak 1

contaminant introduced by the sample cleanup because this peak does not appear in the electropherogram of dye standards shown in Fig. 4. The UV–visible spectrum of the extract peak matches that of the dye standard for C.I. reactive yellow 176 (not shown).

Vat dyes Vat dyes are insoluble pigments that are applied to cotton in the reduced water-soluble form. The dyes are then oxidized back to the water-insoluble form once applied to the fiber. Thus, vat dye pigments in the oxidized keto form are insoluble in many aqueous and organic solvents that would be compatible with CE analysis. For instance, vat dyes are soluble in 2,5-hexanedione, but the high viscosity of this solvent precludes subsequent analysis by CE.

In seeking a water-soluble derivative of the vat dyes for aqueous CE, we have experienced only moderate success with the derivatization of the ketones in the quinone backbone of the vat dyes to the water-soluble oxime using hydroxylamine hydrochloride (Fig. 6a) [13]. However, a one-step extraction–derivatization does not completely remove vat dye from cotton fibers, and the oxime derivative is only marginally more soluble than the pigment. Additionally, low derivatization efficiency and derivative stability severely limit usefulness of the oxime method. Another approach we have examined is based on the routine vat dye extraction from faulty industrial dying via reduction to the *leuco* form followed by addition of PVP [15]. The PVP polymer has a higher binding affinity for the dye than does cellulose, effectively dying the PVP preferentially to the cotton. A water-soluble dyed polymeric dispersion of the *leuco* form is produced, followed by conversion to the *keto* form over several hours time. Sheth [16] suggested the possibility of analyzing the dyed PVP by the UV–visible absorbance or fluorescence, but the applicability to microscale extractions has not been investigated; the resulting polymeric matrix and lack of charged species make this method incompatible with CE analysis.

Because removal of vat dye from cotton requires a reducing agent, sodium dithionite can be used to reduce vat dye to the water-insoluble *leuco* form of the dye (Fig. 6b), the cotton fibers were isolated from the extract and the dyes are allowed to air oxidize, regenerating the water-insoluble pigment. Because of insolubility, this solution is not compatible with aqueous CE. However, the oxidized forms of all vat dyes that we have investigated are soluble in solutions consisting of 40:60 acetonitrile–water. Six of the 12 vat dyes in our present collection are negatively ionized and can thus be separated in this anionic CE buffer system. Tetler et al. [17] used a similar electrolyte buffer in their CE–mass spectrometry analysis of anionic vat dyes. The remaining vat dyes require reduction by adding sodium dithionite (by adding the reducing agent to both the analytical sample vials and to the CE buffer, although it may not be necessary to add the reducing agent to the buffer). The low level of sodium dithionite in the buffer does not impact the CE analysis of the other vat dyes nor does it impact CE performance in general. Thus, the same anionic buffer system (5 mM ammonium acetate in acetonitrile–water, 40:60, *v*/*v*, pH9.3) used for the analysis of direct and reactive dyes is also suitable for the separation

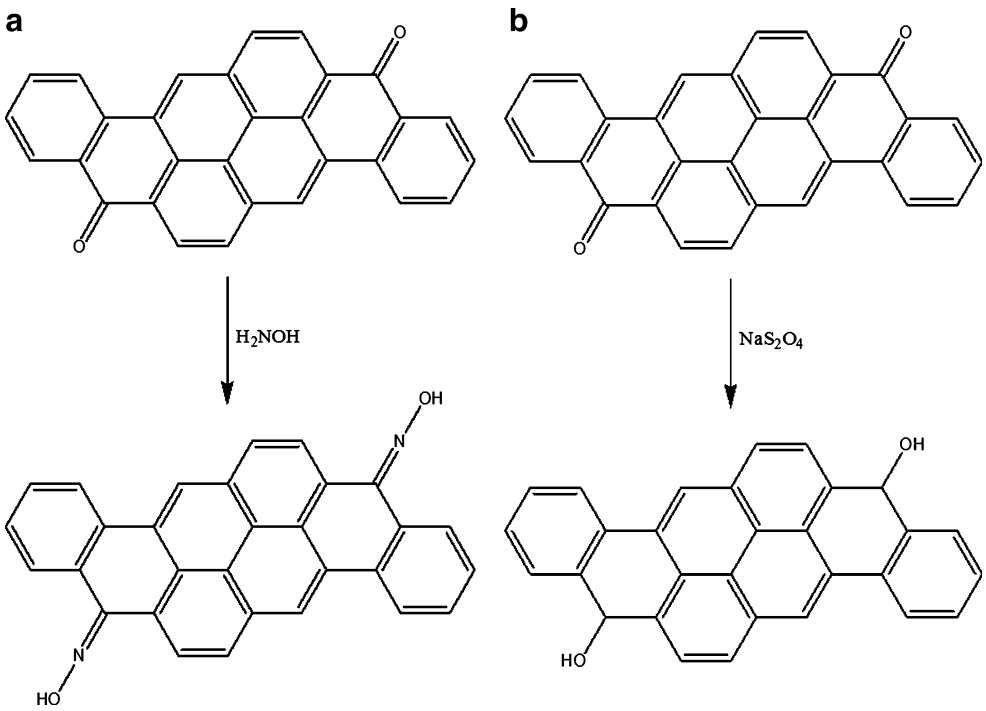

Fig. 6 **a** Derivatization of a vat dye to the oxime with hydroxylamine hydrochloride. **b** Reduction of a vat dye to the water-soluble *leuco* form with sodium dithionite

of vat dyes upon addition of sodium dithionite. Figure 7 displays the CE separation of three vat dye standards using buffer with sodium dithionite added. Note that the small unlabeled peaks are minor components present in the three dyes (and not in the blank). An electropherogram from a standard sample of a vat dye, C.I. Vat Yellow 9, is shown in Fig. 8a; the corresponding electropherogram in Fig. 8b was produced by an extract from a 10-cm thread of cotton dyed with the same dye. The UV–visible spectra of the extracted dye peak is identical to that of the standard.

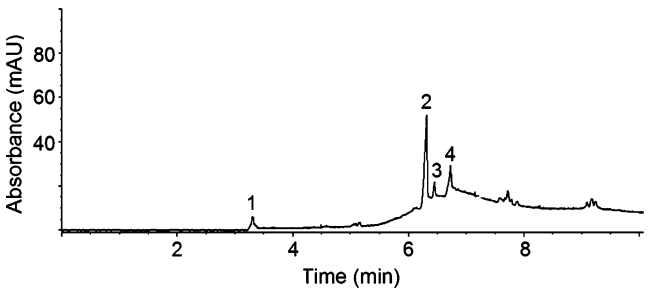

Fig. 7 Electropherogram at 214 nm showing the separation of a mixture of three vat dyes (1 mg/mL). Peak identification: (*1*) neutrals; (*2*) C. I. Vat Yellow 2; (*3*) C. I. Vat Orange 2; and (*4*) C. I. Vat Black 16

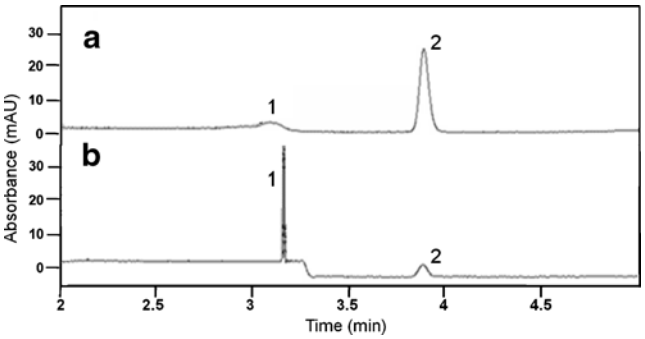

Fig. 8 Electropherograms at 280 nm of: (*A*) a sample of the vat dye, C.I. Vat Yellow 9; (*B*) vat dye extract from 10 cm of cotton thread. Peak identities: (*1*) neutrals; (*2*) C. I. Vat Yellow 9

Conclusions

With the objective of tailoring automated extraction protocols to produce extracts compatible with CE analysis, systematic experiments were employed to find optimum conditions for microextraction of direct dyes from cotton fiber. For this exploratory research, multifiber threads were extracted in order to increase signal-to-noise ratios to a level acceptable for rapid screening of extraction efficiencies on a microplate reader UV–visible spectrophotometer.

The significance of this work is not that entirely new solvent extraction systems have been developed. Previous researchers have employed mixtures of pyridine and water to extract direct dyes from cotton. The extraction response surface for the extraction of C. I. direct yellow 58 predicted optimum extraction at about 35% pyridine–65% water, which is close to previously suggested conditions for direct dyes. However, the shape of the resulting response surface also reveals the effect of changes in solvent composition on the extraction performance. Slight lack of control in the extractant composition when working at the top of the response surface or along the ridge line at an aqueous pyridine composition of about 35% in Fig. 1 does not produce large variations in the amount of dye extracted. However, if an extractant composition that lies on the side of the response surface were used, small variations in solvent composition would propagate to substantial variations in amounts of dye extracted. Thus, working at solvent compositions in the region of the optimum or along the ridge of high response imparts ruggedness to the extraction protocol in routine use.

Following previous literature, reactive dyes were successfully extracted from cotton using a single component solvent of 1.5% aqueous sodium hydroxide. Of the alternative methods studied, this approach coupled with extract cleanup using a reversed-phase solid-phase extraction produced extracts that were relatively free of contaminants. The resultant extract was also demonstrated to be suitable for subsequent CE analysis.

Finally, the chemistry of vat dyes had previously prevented the identification of an extraction solvent suitable for their analysis by aqueous or nonaqueous CE analyses. We have developed a method for CE analysis of vat dyes based on the oxidation/reduction reactions used during industrial dyeing of vat dyes on cotton. The addition of sodium dithionite to the electrolyte solution keeps vat dyes solubilized in the reduced form and enables CE separation of vat dyes. It is noteworthy that all three classes of cotton dyes investigated here (direct, reactive, and vat dyes) can be analyzed by a CE method based on an anionic buffer system; the addition of the reducing agent (sodium dithionite) for extraction of vat dyes does not adversely affect extraction of other dye classes.

There is sufficient variability in chemistry of dyes within each class that some dyes may not extract well under the solvent conditions proposed here. However, the chemical interactions involved in these extraction methods possess sufficient uniqueness that the demonstration of solubility in response to each to these solvents may be indicative of the presence of the respective dyes from the three classes studied. After all, this concept is the basis for classification of dyes from fibers using their response to treatment with various solvents [5, 6]. For example, if dye is extracted with sodium dithionite from a cotton fiber of unknown dying type, a vat dye could be presumed to be present. However, more research is needed to validate a forensically useful

and general protocol for dye identification by such solubility tests. Methods for the extraction of dyes from nylon, acrylic, and polyester fibers, as well as issues involving analysis of single fibers at lengths relevant for forensic casework using diode array and mass spectrometric detection, will be addressed in future work.

Acknowledgments This research was supported under a contract award from the Counterterrorism and Forensic Science Research unit of the Federal Bureau of Investigation's Laboratory Division. Points of view in this document are those of the authors and do not necessarily represent the official position of the Federal Bureau of Investigation.

References

1. Palenik S (1999) In: Robertson J, Grieve M (eds) Forensic examination of fibres, 2nd edn. Taylor & Francis, London, pp 364–378
2. Stoeffler SF (1996) J Forensic Sci 41:297–299
3. Rendle DF, Wiggins KG (1995) Forensic analysis of textile fibre dyes. Rev Prog Color Relat Top 25:29–34
4. Shore J (2002) Colorants and auxiliaries, vol. 1, 2nd edn. Society of Dyers and Colourists, West Yorkshire, England, pp 18–23
5. Laing DK, Dudley RJ, Hartshorne AW, Home JM, Rickard RA, Bennett DC (1991) Forensic Sci Int 50:23–35
6. Cheng J, Wanogho S, Watson ND, Caddy B (1991) J Forensic Sci 31:31–40
7. Home JM, Dudley RJ (1981) Forensic Sci Int 17:71–78
8. Sirén H, Sulkava R (1995) J Chromatogr A 717:149–155
9. Xu X, Leijenhorst H, Van den Hoven P, De Koeijer JA, Logtenberg H (2001) Sci Justice 41:93–105
10. Smith W (2005) Experimental design for formulation. Cambridge University Press, New York
11. Deming SN, Morgan SL (1993) Experimental design: a chemometric approach, 2nd edn. Elsevier Science, Amsterdam
12. Deming SN, Morgan SL (1979) Clin Chem 25:840–855
13. Vogh JW (1971) Anal Chem 43:1618–1623
14. Poiger T, Richardson SD, Baughman GL (2000) J Chromatogr A 886:259–270
15. Nalankilli G (1997) Colourage 44:33–39
16. Sheth GN (1990) Spectrochim Acta A 47:63–68
17. Tetler LW, Cooper PA, Carr CM (2005) Rapid Commun Mass Spectrom 8:179–182