Development of an Analytical Method for Benzidine-Based Dyes

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Benzidine has been an important dye industry intermediate since 1890 (1,2). Over 200 dyes based on benzidine are listed in the Colour Index or are in commercial use. Although the potential of benzidine to cause bladder cancer has been well documented (3,4,5), it was originally believed that when chemically incorporated into a dye, the carcinogenic hazard was removed (6). Recent evidence (7,8,9) has shown that benzidine-based dyes can be reduced to benzidine in living systems and eliminated by the usual benzidine metabolic pathways. Because of this fact, the National Institute for Occupational Safety and Health has recommended that certain benzidine-based dyes be recognized and handled as carcinogens (9).

The only published method available for the determination of personal exposure to benzidine-based dyes utilized the analysis of urine for benzidine and benzidine metabolites (10,11). This method does not allow for quantitation of a daily exposure, since benzidine and its metabolites have been found in the urine of hamsters fed a benzidine-based dye up to 168 hours after a single dosing (12). A method for the determination of personal exposure to azo dyes and diazonium salts has been developed (13), but it is not specific enough to determine an exposure to a benzidine-based dye.

In the development of a sampling and analytical method for benzidine-based dyes, the most important feature was the verification of the benzidine moiety in the dye molecule. Since reduction of some of these dyes in vivo was known to release benzidine, a human carcinogen, the determination of benzidine released by chemical reduction is the most logical approach.

Specificity for a particular dye was not reasonable due to the large number of benzidine-based dyes and the possibility of dye substitution. The method should provide quantitative collection and recovery at the microgram level and be free

from interferences, especially those arising from benzidine congeners, such as o-tolidine (3,3'-dimethylbenzidine) and o-dianisidine (3,3'-dimethyoxybenzidine). Various conditions for reduction of the azo linkages in these dyes were known (14), so cleavage of the benzidine moiety was possible. Also, conditions for the analysis of aromatic amines were well established (12,15,16,17). Preliminary work in our laboratory had shown that benzidine and its congeners could be separated by high pressure liquid chromatograph (HPLC). Based on this reasoning, a method for the determination of benzidine in benzidine-based dyes was developed.

Experimental

Apparatus. The HPLC system used in this study, assembled from modular components manufactured by Waters Associates, consisted of two Model 6000A pumps, a Model 660 solvent programmer, a Model 440 ultraviolet detector with a 280-nm filter and a Waters Model 710A Intelligent Sample Processor. Data were recorded on a Soltec Model B281 dual channel strip chart recorder and integrated by a Hewlett-Packard Model 3354A Laboratory Automation System. The columns used for the analyses were a Waters Associates μ-Bondapak C_{18} and a Waters Model RCM100 Radial Compression Module with a Radial Pak A cartridge. The mobile phase was 60% methanol (Burdick and Jackson) and 40% of an aqueous phosphate buffer. This phosphate buffer was prepared by dissolving 3.390 g (0.025 mole) of KH_2PO_4 (Fisher Scientific Corp.) and 3.530 g (0.025 mole) of Na_2HPO_4 (Matheson Coleman Bell) in 1 L of water (18). A helium purge was maintained in the solvent reservoirs to eliminate dissolved air. The columns were maintained at ambient temperature and run at a 2-mL/min flow rate. Resulting pressure at this flow rate was 500-1900 psi for the Radial Compression Module and 3000 psi for the μ-Bondapak column. A precolumn filter could not be used because the extra dead volume reduced resolution of reduction products.

Millipore 37 mm Mitex (Teflon) filters (5.0 μm) with backup pads and three-piece cassettes were used for the spiked filter studies. A Doerr Model 0272X vacuum pump with a 10-port sampling manifold and 1-L/min critical orifice (Millipore Corporation) was used to pull air through the dye-spiked filter cassettes for the stability studies. Two methods of exposing the cassettes to humidified air were employed. For the 28-day storage study, air was pulled directly through midget impingers filled with a saturated sodium chloride solution. For the 7-day storage study, air was blown through a large saturated sodium chloride-filled impinger and then supplied to a 10-port manifold to which the cassettes were attached. Air was pulled through the cassettes

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using the previously described vacuum pump. With this system the cassettes were always maintained at atmospheric pressure. Relative humidity generated by either of these techniques was approximately 75% (19).

Visible-spectrum studies of the course of the dye reduction were performed on a Beckmann Model 25 Ultraviolet-Visible Spectrophotometer scanning the region of 750-350 nm.

Reagents. The dyes used in this study (see Figure 1) were obtained from the following sources: Congo Red (Colour Index (C.I.) Direct Red 28, C.I. No. 22120) A. D. Mackay Inc.; Direct Black GX (C.I. Direct Black 38, C.I. No. 30235), Direct F. Blue 2B 250% (C.I. Direct Blue 6, C.I. No. 22610), Direct Brown BRL 200% (C.I. Direct Brown 95, C.I. No. 30145) Fabricolor Inc.; Evans Blue (C.I. Direct Blue 53, C.I. No. 23860), Benzo Azurine G (C.I. Direct Blue 8, C.I. No. 24140) Pfaltz and Bauer Inc. Benzidine was obtained from Sigma Chemical Co., o-tolidine from Fisher Scientific Corp. and 3,3'-dimethoxybenzidine (o-dianisidine) from Eastman Kodak Company. Aniline, p-aminophenol, p-phenylenediamine and p-nitroaniline used in the interference study were obtained from Chem Service Inc.

The phosphate buffer solution used for reduction of the dyes was prepared by dissolving 1.179 g (0.0087 mole) of KH_2PO_4 and 4.300 g (0.0303 mole) of Na_2HPO_4 in water to make 100 mL of solution. For the reduction of the dyes, a solution was prepared which contained 10 mg of sodium hydrosulfite (Fisher Scientific) in 1 mL of the above buffer solution (0.087 M KH_2PO_4 - 0.303 M Na_2HPO_4). This sodium hydrosulfite containing solution was prepared immediately before addition to the desorbed dye to prevent decomposition of the sodium hydrosulfite. All solutions, including the HPLC aqueous phase, were filtered through a 0.22-μm cellulose ester membrane filter before use to prevent plugging of the HPLC system during analysis.

Procedure. A primary standard solution of benzidine in methanol was prepared. Standards of lower concentrations were prepared by dilution of the primary standard. Typical liquid chromatograph calibration curves were linear in the region from 0.38 to 30.6 μg/mL. The standards were stable for several months when stored in the dark. Aqueous solutions of known dye-formulation concentration were prepared.

Filter samples for the stability and repeatability studies were prepared by spiking the filter with a known volume of the dye solution using fixed volume pipettes or volumetric syringes. The filters were then dried in a dessicator filled with anhydrous calcium sulfate and phosphorous pentoxide. The dessicator provided more rapid and uniform drying of the

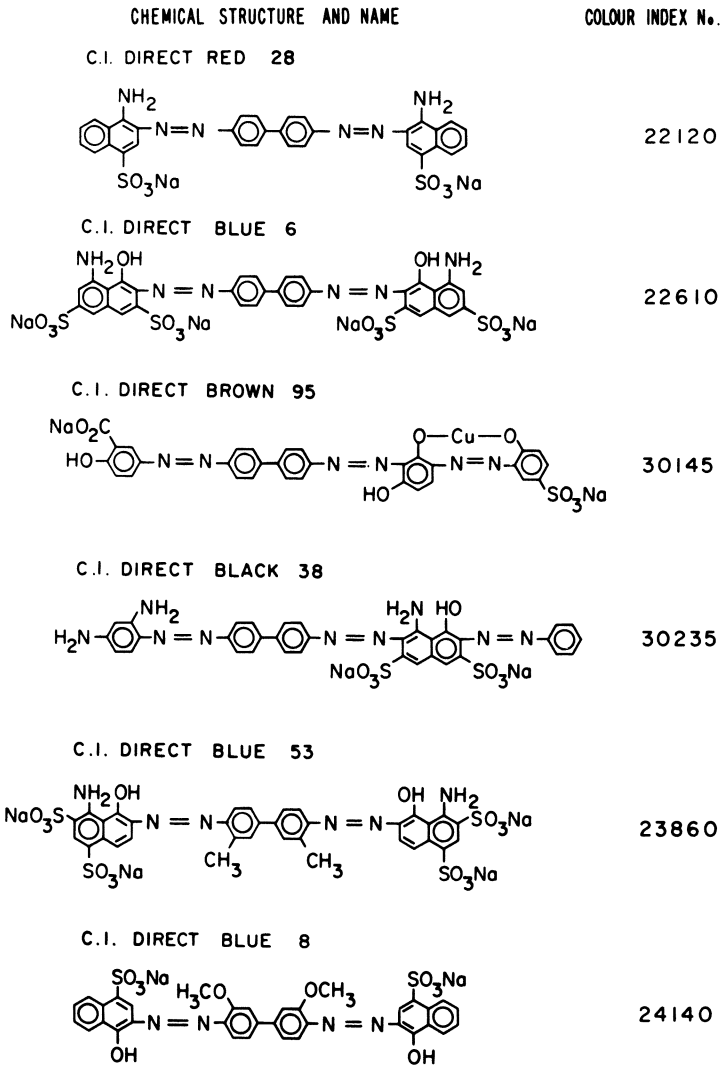

Figure 1. Structures and names of dyes used in this study

filters than air drying. The filters were then stored at room temperature in a filter shipping case until reduction and analysis.

The spiked filters were placed in a 50-mL beaker with the spiked side up and 1 mL of water was added. The beaker was shaken so that all of the filter area had been washed by the water. One mL of the reduction-buffer solution without the sodium hydrosulfite was added to the filter and water. The beaker was shaken a second time. The filter was then turned over (spiked side down) and the beaker placed in an ultrasonic bath for 15 minutes. At the end of this period, the solution in the beaker was colored. A 1-mL aliquot of this solution was transferred to a 4-mL vial. One mL of a freshly prepared solution of 100-mg sodium hydrosulfite in 10-mL phosphate reduction buffer was then added to the vial. The vial was then capped and shaken several times during the course of an hour. During this time, the original color of the solution disappeared or changed to a different color, depending on the dye present. This solution was then injected into the liquid chromatograph. A 10-μL aliquot was used, giving a measurement limit of 0.38 ng benzidine/μL. The analytical reproducibility at this limit was 10% coefficient of variation (CV).

Results and Discussion

Initial work on the reduction reaction involved a study of the completeness of the reaction and verification of the reduction product, benzidine. The presence of benzidine in the reduced dye sample was confirmed by gas chromatographic/mass spectrometric analysis. In order to determine the completeness of the dye-reduction reaction, the reduction of C.I. Direct Black 38, C.I. Direct Brown 95 and C.I. Direct Blue 6 were studied individually in the visible spectrum. A baseline was recorded using the phosphate reduction buffer in both cells. Subsequent additions of known amounts of dye and scanning allowed absorption maxima and molar absorptivity to be determined. Then 3 mg of sodium hydrosulfite was added to the dye-containing cell. The concentration of dye remaining after reduction was calculated using Beer's Law. The remaining dye varied from 0 to 6% of the original amount of dye added (Table I). Reduction was complete within 30 minutes.

In the initial phases of the analytical method development a Waters Associates C_{18} μ-Bondapak column was used. With certain dyes, such as C.I. Direct Black 38, aniline is used as a terminal group (Figure 1). This column caused the benzidine peak of the reduced dye to obscure a peak due to aniline. If these two compounds were not resolved, the method could not differentiate between benzidine-based and aniline-based dyes. This problem was removed by use of the Waters Radial Compression Module Model RCM 100 with a Radial Pak A cartridge.

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Table I
Visible Spectrum Studies of Benzidine-Based Dyes

Dye	Molar Absorptivity	Concentration (μg/mL) Before Reduction	Concentration (μg/mL) After Reduction
C.I. Direct Blue 6	26.5	9.1	0.1
C.I. Direct Brown 95	23.8	18.1	1.1
C.I. Direct Black 38	19.0	15.9	0.0

This system resolved the aniline peak (retention time (rt) = 2.67 min) from the benzidine peak (rt = 2.27 min) as can be seen in Figure 2. Other potential interferences were selected for study by looking at the expected fragments from the reduction of various dyes. Reduced dye samples were spiked with aniline (rt = 2.67 min), p-aminophenol (rt = 1.97 min), p-phenylenediamine (rt = 1.93 min) and p-nitroaniline (rt = 3.16 min). None of these materials interfered with the detection of the benzidine peak. To determine if other types of dyes might interfere with the analysis, two sets of filters were spiked at low and high levels separately with C.I. Direct Red 28 (13.7 μg and 137 μg), C.I. Direct Blue 53 formulation (o-tolidine-based) (21.2 μg and 212 μg) and C.I. Direct Blue 8 formulation (o-dianisidine-based)(23.3 μg and 233 μg). Results from the analyses showed there were no interferences from the other dyes present (Figure 3 and Table II). The coefficient of variation for the average analytical method recovery (CV_{AMR}) has been defined by the following equation (20) to account for the propagation of error resulting from the division of the amount of benzidine found on the filter by the amount in the liquid sample:

$$CV_{AMR} = [(CV_L)^2 + (CV_F)^2)]^{1/2}$$

where: CV_L = coefficient of variation for the liquid samples.
CV_F = coefficient of variation for the filter samples.

Three of the dyes (C.I. Direct Blue 8, C.I. Direct Black 38 and C.I. Direct Brown 95) had been analyzed for residual benzidine content during previous work (21). None of the dyes contained sufficient quantities of residual benzidine to require a correction to be made to the total amount of benzidine found in the reduced dye samples.

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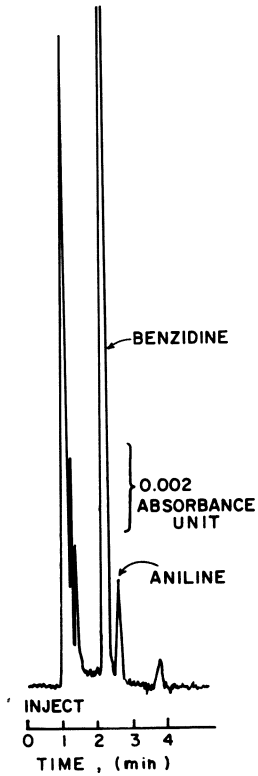

Figure 2. Chromatogram of a 10-μL injection of reduction products of C.I. Direct Black 38 at the 124.0-μg level showing resolution of benzidine (retention time = 2.27 min) and aniline (retention time = 2.67 min). Chromatographic conditions: Radial Pak A; 2 mL/min; 60% methanol/40% phosphate buffer; ambient temperature.

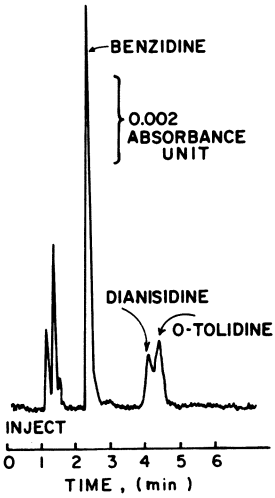

Figure 3. Chromatogram of a 10-μL injection of reduction products of a mixture of C.I. Direct Red 28 (28.8 μg), C.I. Direct Blue 53 (28.6 μg) and C.I. Direct Blue 8 (34.4 μg) showing resolution of benzidine (retention time = 2.33 min), o-dianisidine (retention time = 4.02 min), and o-tolidine (retention time = 4.27 min). Chromatographic conditions as in Figure 2.

Since no chemically pure dyes (greater than 95% purity) were available, recovery studies were done by reducing and analyzing a liquid sample of the dye along with the spiked filter. These liquid samples were prepared by adding a quantity of dye solution (2-200 μL) equivalent to one-half the amount present on the spiked filter to 1 mL of the reduction buffer solution. To this solution 10 mg of sodium hydrosulfite in 1 mL of the reduction buffer was added. The sample was then analyzed in the same manner as the spiked filter sample. The amount of dye contained in the filter and liquid spiked samples was the same, but concentration did vary. However, volume variations became greater than 9% (200 μL addition to 2 mL of solution) on only the most concentrated samples. Since total sample amount was the same for filter and liquid samples, any differences in sample amounts would be due to recovery losses.

Table II
Recovery of Free Benzidine from Filter Samples Containing C.I. Direct Red 28, C.I. Direct Blue 53, and C.I. Direct Blue 8

Loading of C.I. Direct Red 28 (μg)	Average Recovery (%)[a]	Coefficient of Variation (%)
13.7	98.0	4.3
136.7	106.0	2.6

[a]Averages of six samples.

Based on the analyses of these liquid samples an approximation of amount of the benzidine-containing dye compound in a particular dye formulation could be made using molecular weight calculations. For 30 samples of C.I. Direct Red 28 formulation (molecular weight (M_r) = 696) at the 15-μg level, the benzidine-containing compound was found to compose 91.2% of the formulation (CV = 1.1%). With the other dyes the following compositions were found: C.I. Direct Blue 6 formulation (M_r = 932), 30.7% benzidine-containing compound (23 samples at the 48.8 - 52.0-μg level) CV = 8.5%; C.I. Direct Black 38 formulation (M_r = 781), 41.6% benzidine-containing compound (18 samples at the 14.92-μg level) CV = 8.5%; C.I. Direct Brown 95 formulation (M_r = 759), 39.4% benzidine-containing compound (24 samples at the 30.8-μg level) CV = 16.7%.

An examination of the side-by-side analyses of liquid and filter samples should be able to detect any day-to-day variations in analyses. When the raw data from the liquid samples was studied, there appeared to be such a day-to-day variation in the results. However, analysis of variance

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indicated no variation at the 0.05 level of significance. To further investigate any possible sources of variation, the method was subjected to a ruggedness test as described by Youden (22), using benzidine concentration as the response variable. The HPLC mobile phase buffer and reduction buffer were prepared with 10% additional disodium hydrogen phosphate and 10% additional potassium dihydrogen phosphate for the high levels in the test. Sodium hydrosulfite concentration in the reduction buffer was also increased by 10%. The mobile phase makeup was changed to 35% buffer/65% methanol for the high level. Flow rate was increased to 2.2 mL. An old and new column were used to evaluate column backpressure. Ten μL of sample solution were removed before addition of the reductant solution to simulate volume variation caused by use of out of calibration pipettes. The major contributions to the variation resulted from HPLC buffer concentration, column backpressure and mobile phase composition. According to instrument specifications, mobile phase makeup was controlled to within ± 2% of set point so that any variation should have been randomized during the testing. During later work it was found that ambient temperature variations (± 5° C) caused changes in volumes delivered by the pumping system. This volume change amounted to as much as 0.2 mL/min. This problem was solved by premixing the mobile phase. Column backpressure could also have been a contributing factor to the variation in volume delivery since solvent compressibility is affected greatly by temperature at lower pressures (23). Based on this ruggedness test, HPLC mobile-phase buffer concentration could be a major source of variation. To minimize this variation in the method, care should be used when preparing this buffer.

The analysis method was evaluated with four different benzidine-based dye formulations (Figure 1). The results of a repeatability study utilizing spiked samples are shown in Table III. Spiking levels were arbitrarily chosen but do reflect the lower limit of the method.

All coefficients of variation passed the Bartlett's test (24) for homogenity at the 1% significance level, except C.I. Direct Black 38. With the lowest level of C.I. Direct Black 38 excluded, the coefficients of variation for the remaining two levels were homogeneous. The pooled analytical coefficients of variation (CV_1) for each dye are as follows: C.I. Direct Red 28, 0.055; C.I. Direct Blue 6, 0.083; C.I. Direct Brown 95, 0.108; C.I. Direct Black 38, 0.058 (2 levels only).

To investigate the storage stability of these dyes, a 28-day storage study was undertaken. Filters were spiked and dried by the procedure described in the experimental section. Sixty liters of humidified air (75% relative humidity) were drawn through each spiked filter before storage, using the previously described vacuum pump system. During the

Table III
Recovery of Benzidine from Benzidine-Based Dye Filter Samples After One-Day Storage

Dye	Loading of Dye (μg)	Average Recovery (%)[a]	Coefficient of Variation (%)
C.I. Direct Red 28	13.7	94.4	7.2
	27.3	101.0	5.6
	273.4	109.0	2.5
C.I. Direct Blue 6	15.0	100.0	6.6
	30.0	98.9	11.7
	300.0	108.0	4.8
C.I. Direct Brown 95	12.1	78.5	9.2
	24.3	96.4	14.8
	242.6	110.0	6.6
C.I. Direct Black 38	6.2	78.1[b]	16.6
	12.4	91.6	7.9
	124.0	102.0	2.2

[a]Based on six samples. [b]Based on five samples.

humidification step utilizing the midget impingers, several of the samples were acidentally wetted with the sodium chloride solution. This was not noticed until the samples were prepared for analysis. These contaminated samples were not included in the data analysis. Also, occasional plugging of an impinger with salt was noted. This resulted in a partial vacuum being created in the cassette. When the impinger was unplugged, the rush of air might possibly have dislodged a portion of the dye from the filter causing greater variation in the analysis. When several of the cassettes were opened for analysis, the spiked dye spot seemed to be lying loose on the filter. These samples also were not included in the data analysis. A pump malfunction in the HPLC system invalidated the day-14 analyses for C.I. Direct Blue 6, C.I. Direct Brown 95 and C.I. Direct Black 38. The remaining data from the storage study is shown in Table IV.

The variability of the recoveries was subjected to analysis of variance at the 0.05 level of significance. Significant differences were observed with the recoveries from C.I. Direct Red 28. Duncan's multiple range test indicated that the recovery on day 1 was significantly different from the other three analyses at days 7, 14 and 28. Analyses at days 7, 14, and 28 were not significantly different. With C.I. Direct Blue 6, C.I. Direct Black 38 and C.I. Direct Brown 95 there were no differences between recoveries at days 1, 7, and 28 for each dye. However, the analytical coefficients of variation became larger on day 28 for these three dyes.

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Table IV
Evaluation of the Stability of Filter Samples Containing Benzidine-Based Dyes Stored Up to Twenty-Eight Days

Dye	Loading (μg)	Average Recovery[a] (%) Day 1	Day 7	Day 14	Day 28
C.I. Direct Red 28	13.7	101.0[b] (± 8.7)	83.8[c] (± 16.0)	86.4[d] (± 9.3)	88.8[c] (± 21.1)
C.I. Direct Blue 6	15.0	103.0[b] (± 12.7)	105.0 (± 7.0)	--[e]	108.0 (± 12.5)
C.I. Direct Brown 95	12.1	71.4 (± 7.0)	73.9 (± 8.6)	--[e]	66.0 (± 18.4)
C.I. Direct Black 38	6.2	78.1[b] (± 16.1)	68.8 (± 8.6)	--[e]	69.5 (± 21.7)

[a]Based on six samples. 95% confidence limit contained in parentheses. [b]Based on five samples. [c]Based on three samples. [d]Based on four samples. [e]Analyses lost due to equipment malfunction.

The repeatability and stability studies were done at concentration levels which preliminary evidence on liquid samples had shown to be reproducible within 10% CV. However, this was not the case for three of the dyes (C.I. Direct Red 28, C.I. Direct Brown 95, and C.I. Direct Black 38). In this situation, the lowest analytically quantifiable limit (LAQL) had to be redefined. From the results of the repeatability study, the second highest level was the logical choice for this redefined LAQL. A second abbreviated stability study was conducted with C.I. Direct Red 28, C.I. Direct Black 38, and C.I. Direct Brown 95 at this new LAQL. Because of the problems encountered with the midget impinger humidification system, changes were made to incorporate one large impinger and supply the humidified air to the filter cassettes at atmospheric and not reduced pressure. Again, 60 L of humidified air were pulled through each cassette. The results of this study are shown in Table V. Recoveries of C.I. Direct Black 38 and C.I. Direct Brown 95 were approximately 90%. There were no significant differences between recoveries of the first day's analyses and the last day's analyses at the 0.05 level of significance.

Although the LAQL varied with each dye, the second stability study indicated that the LAQL should be in the range of 25-30-μg dye per sample for at least two of the dyes. Since the method did not differentiate between benzidine-based dyes, one LAQL was necessary for the method. By utilizing the 30-μg level as the LAQL, reduction and analysis in the working

Table V
Evaluation of the Stability of Filter Samples Containing Benzidine-Based Dyes Stored Up to Seven Days

Dye	Loading (μg)	Average Recovery[a] (%) Day 1	Day 7
C.I. Direct Red 28	28.8	97.8 (± 6.3)	95.9 (± 6.6)
C.I. Direct Blue 6	15.0	103.0 (± 12.7[b])	105.0 (± 7.0)
C.I. Direct Brown 95	26.6	90.4 (± 5.7)	91.2 (± 6.3)
C.I. Direct Black 38	12.3	88.5 (± 10.0)	85.6 (± 11.5)

[a]Based on six samples. 95% confidence intervals contained in parentheses. [b]Based on five samples.

range of each of the four dyes evaluated is assured.

To minimize loss by decomposition, samples should be analyzed as soon as possible after collection, preferably within seven days. The samples should be stored in a dark environment while awaiting analysis to prevent photosensitive compounds from degrading and changing sample composition.

Since the method does not distinguish between various benzidine-based dyes and analytically pure dyes are not easily available, recovery correction factors cannot be used. This will cause the results to be equal to or below the true level of dye exposure.

This method has been used to analyze both symmetrical (C.I. Direct Red 28 and C.I. Direct Blue 6) and unsymmetrical (C.I. Direct Black 38 and C.I. Direct Brown 95) benzidine-based dyes. Based on this work, the application of the method to other benzidine-based dyes should be straightforward. When field samples are submitted for benzidine-based dye analysis, bulk samples of the dyes present in the sample also should be submitted. With these bulk samples, the analyst should be able to determine if this method is applicable to the various dyes submitted and if any interferences are present. The method presently has not been tested on field samples. An existing sampling method (13) for azo dyes and diazonium salts should be directly applicable to this method with a change from a cellulose ester to a Teflon filter. This change is necessary to insure quantitative recovery of the sample from the filter.

In summary, a method for the identification and quantification of benzidine-based dye containing samples has been developed. This method could be expanded to include samples taken from media other than air with minor modification, for instance, in assaying benzidine-based dye formulations at the microgram level.

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Abstract

A method for the determination of personal exposure to benzidine-based dyes has been developed. This procedure involved the reduction of benzidine-based dye filter samples to free benzidine with neutral buffered sodium hydrosulfite solution. The benzidine-containing reduction solution was then analyzed by high performance liquid chromatography. The reduction was found to be quantitative by visible-spectrum analysis. This reduction and analysis method was evaluated with four benzidine-based dyes over the range from 12 to 300 micrograms per sample. Precision for the reduction and analysis of the four dyes falls within 11% coefficient of variation. This method can differentiate between benzidine- and benzidine congener-based dyes. Results are reported in terms of free benzidine.

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