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DETERMINATION OF UNSULFONATED AROMATIC AMINES IN FD&C YELLOW NO. 6 BY THE DIAZOTIZATION AND COUPLING PROCEDURE FOLLOWED BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Data are presented for the determination of parts-per-billion (10^9) levels of aniline, benzidine, 4-aminobiphenyl (4-ABP) and 4-aminoazobenzene in the regulated color additive FD&C Yellow No. 6. The determination involves chloroform extraction of the amines from the color additive, followed by diazotization and coupling with the disodium salt of 3-hydroxy-2,7-naphthalenedisulfonic acid (R-salt). The coupling products are then analyzed by reversed-phase high-performance liquid chromatography. An interference discovered during the determination of 4-ABP required the use of 4,5-dihydro-5-oxo-1-(4-sulfophenyl)-1H-pyrazole-3-carboxylic acid (pyrazolone-T) as an alternative coupling agent. The identity of each coupling product is confirmed by obtaining a UV–visible spectrum of the eluting solute. The liquid chromatograph is calibrated in the presence of the color additive by using the external standard method.

INTRODUCTION

FD&C Yellow No. 6 is a synthetic, water-soluble, monoazo color additive allowed by the Food and Drug Administration (FDA) (Washington, DC, U.S.A.) for use in foods, drugs and cosmetics[1]. The color additive is composed principally of the disodium salt of 6-hydroxy-5-[(4-sulfophenyl)azo]-2-naphthalenesulfonic acid. Each batch of this color manufactured for use in foods, drugs or cosmetics in the United States must be analyzed and approved by the FDA. More than one million pounds of FD&C Yellow No. 6 were certified in 1983[2].

The color additive is synthesized by coupling diazotized sulfanilic acid (4-aminobenzenesulfonic acid) with Schaeffer's salt (the sodium salt of 6-hydroxy-2-naphthalenesulfonic acid) as shown in Fig. 1. Impurities in the intermediates and reagents used in the preparation and isolation of the color during manufacture and side reactions occurring during synthesis lead to the contamination of the additive with a number of impurities.

FD&C Yellow No. 6 usually contains small amounts of sulfanilic acid as a

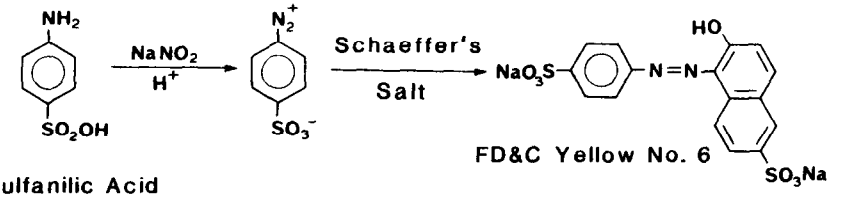

Fig. 1. Manufacturing procedure for synthesis of FD&C Yellow No. 6.

consequence of its use as an intermediate in the preparation of the color additive. Technical grade sulfanilic acid typically contains 0.1–1.2% aniline[3], and other unsulfonated aromatic amines may be present in the sulfanilic acid as a consequence of the aniline contamination. For example, benzidine has been shown to be an oxidation product of aniline[4], and benzidine levels of 5–50 ppm have been found in technical grade sulfanilic acid[5]. Reaction between diazotized aniline and aniline forms 1,3-diphenyltriazene, which can rearrange to form 4-aminoazobenzene (4-AAB)[6]. Thermal[7] or photochemical[8] decomposition of 1,3-diphenyltriazene can lead to the formation of 4-aminobiphenyl (4-ABP).

Recently developed methodology capable of determining unsulfonated aromatic amines in D&C Red No. 33[9] and FD&C Yellow No. 5[10] has been applied to the determination of aniline, benzidine, 4-AAB and 4-ABP at the parts-per-billion level (ppb*) in FD&C Yellow No. 6. The procedure is based on extraction of the amines from the color with chloroform, followed by diazotization and coupling of the diazonium salts with the disodium salt of 3-hydroxy-2,7-naphthalenedisulfonic acid (R-salt) or, in some cases, with 4,5-dihydro-5-oxo-1-(4-sulfophenyl)-1H-pyrazole-3-carboxylic acid (pyrazolone-T). The coupling products are then separated by high-performance liquid chromatographic (HPLC) analysis and quantitated by measurement at 254 and 510 nm. The presence of the amines was confirmed in some samples by obtaining UV-visible spectra of the amine coupling products as they eluted from the HPLC column. The reproducibility of the method was tested by performing multiple analyses on several samples.

EXPERIMENTAL

Apparatus

Liquid chromatograph. An Altex Model 322 MP gradient chromatograph (Model 110A pumps and Model 420 controller) was used with a Rheodyne Model 7125 loop injector fitted with a 250-μl loop.

Detectors. A Waters Assoc. Model 440 dual-channel UV detector was set at 254 nm on both channels, and an Altex Model 155-10 variable-wavelength detector was set at 510 nm.

Integrators. A Shimadzu C-R1A integrator/printer-plotter was connected to the 2-V output of one of the 254-nm channels through a recorder attenuator set to provide sensitivity of 0.01 a.u.f.s.; a Varian Vista 401 dual-channel data system was connected to a 510-nm detector with sensitivity set at 0.01 a.u.f.s. (maximum sensi-

* Throughout the article the American billion (10^9) is meant.

tivity of detector). The 254-nm chromatograms were recorded on the second channel of the Vista data system, using the other 254-nm signal from the Waters Assoc. detector. The data system was programmed to simultaneously plot the 254- and 510-nm signals.

HPLC column. A Microsorb (3 μm) C_{18} column, 10 cm × 4.6 mm I.D., was obtained from Rainin Instrument (Woburn, MA, U.S.A.).

Diode-array spectrophotometer. A Hewlett-Packard Model 8450A spectrophotometer was fitted with an HPLC flow cell [Model 178.32, Z = 15, 10-mm pathlength, 8-μl cell volume (Hellma Cells, Jamaica, NY, U.S.A.)].

Extraction columns. Extrelut QE disposable columns, 15 × 4 cm I.D. (EM Science, Gibbstown, NJ, U.S.A.), were used for some extractions.

Reagents

All solvents were distilled-in-glass (Burdick & Jackson Labs., Muskegon, MI, U.S.A.). All other chemicals were reagent grade.

Dissolving solutions. (1) A 1-g portion of sodium chloride was dissolved in 100 ml of 0.01 *M* sodium hydroxide. (2) A 2-g portion of sodium chloride was dissolved in 100 ml of 0.02 *M* sodium hydroxide.

Diazotization and coupling solutions. (1) A 0.1-g portion of sodium nitrite was dissolved in 100 ml of water. (2) A 0.5-g portion of reagent grade R-salt plus a 0.6-g portion of anhydrous sodium carbonate was dissolved in 100 ml of water. (3) A 0.5-g portion of recrystallized pyrazolone-T hydrochloride plus a 1-g portion of anhydrous sodium carbonate was dissolved in 100 ml of water.

HPLC dissolving solution. A 1.5-g portion of sodium dihydrogen phosphate was dissolved in 100 ml of water.

HPLC eluents. (1) Eluent A was prepared by dissolving 1.5 g of ammonium acetate in acetonitrile–water (0.5:99.5). Water was purified by using a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). (2) Eluent B was acetonitrile.

Standard stock solutions. (1) A 106.9-mg portion of analytical reagent grade aniline (Mallinckrodt Chemical Works, St. Louis, MO, U.S.A.) was dissolved in 100 ml of 95% ethanol. The purity was established from the absorbance of diluted aliquots in water (1-cm cell) at 229 nm [molar absorptivity of aniline in water = 7900 (ref. 11)]. (2) A 10.39-mg portion of 4-AAB (recrystallized product obtained from FDA supplies) was dissolved in 100 ml of methanol. (3) A 36.2-mg portion of benzidine dihydrochloride (ICN K&K Labs., Plainview, NY, U.S.A.), calculated as the free amine, was dissolved in 100 ml of methanol. (4) A 57.4-mg portion of 4-ABP (Aldrich, Milwaukee, WI, U.S.A.) was dissolved in 100 ml of methanol. The purities of 2, 3 and 4 were established by elemental analysis. All four standard stock solutions were refrigerated during storage. Fresh dilutions in water were prepared from the stock solutions before the calibration analyses were performed.

Extraction method A

A 5-g (± 0.01 g) portion of color was weighed and placed in a 250-ml separatory funnel containing 100 ml of 1% (w/v) sodium chloride in 0.01 *M* sodium hydroxide. The mixture was swirled and extracted (60 sec of vigorous shaking) with 25 ml of chloroform. The layers were allowed to separate, and the chloroform was drained through a washed (25 ml of chloroform) glass wool pledget placed in the

constriction of a funnel into a 200-ml round-bottom flask. The aqueous solution was extracted with two additional 25-ml portions of chloroform (30 sec of vigorous shaking), which were also drained into the round-bottom flask. A 5-ml portion of 0.005 *M* sulfuric acid was added to the chloroform extract, and the chloroform was removed under aspirator vacuum (*ca.* 11 mm) on a rotary evaporator (45°C). Care was taken to ensure that no chloroform droplets remained in the aqueous layer and that the flask was removed from the vacuum as soon as all of the chloroform was gone. Residual chloroform vapors were gently driven from the flask with a 2-min air purge.

Extraction method B

A 10-g (± 0.01 g) portion of color was weighed into a beaker and dissolved in approximately 70 ml of warm water containing five drops of 1 *M* sodium hydroxide. The solution was quantitatively transferred to a 100-ml volumetric flask and brought to volume with water. A 50-ml aliquot was transferred to a dry Extrelut QE column, and a 200-ml round-bottom flask was placed under the column. A 25-ml portion of chloroform was poured onto the top of the column. A second 25-ml portion of chloroform was added after the first portion had drained into the column. Two more 25-ml portions of chloroform were passed through the column to make a total of 100 ml of chloroform. The chloroform extract was treated as described in Extraction method A after the addition of a 5-ml portion of 0.005 *M* sulfuric acid.

Diazotization and coupling

The 5-ml portion of 0.005 *M* sulfuric acid containing the extracted amines was chilled for 5 min in an ice bath. A 1-ml portion of sodium nitrite diazotization solution (1 mg/ml) was added, and the solution was carefully swirled to wash the walls of the flask. After the solution was chilled for 15 min, 1 ml of coupling solution (R-salt or pyrazolone-T) was added to the flask, and the contents were carefully swirled to wash the walls of the flask and chilled for 15 min. The contents of the flask were taken to dryness under aspirator vacuum (*ca.* 11 mm) at 45°C, and a gentle air purge for 2 min was used to completely dry the residue.

HPLC analysis

The HPLC column was equilibrated with 100% eluant A for 10 min at a flow-rate of 1 ml/min. During equilibration the contents of the flask were dissolved in 5 ml of 1.5% (w/v) sodium dihydrogen phosphate in water. The injection syringe (3 ml) was rinsed with two 0.25-ml portions of the solution, and then 2 ml of the solution was drawn into the syringe, the bubbles were removed, and the injection loop was flushed with the entire 2 ml. At the end of the equilibration, the coupling product was injected onto the column, and a linear gradient from 0 to 40% eluant B in 30 min was initiated. At completion of the gradient, the column was flushed with 100% eluant B for 5 min, and the column was equilibrated with 100% eluant A to begin the next analysis.

Calibration and calculations

HPLC calibration data were obtained in the color matrix by using both extraction techniques. For extraction method A, a 5-g portion of FD&C Yellow No. 6 (containing no significant amounts of the analytes) was added to a separatory

funnel containing 50 ml of 2% (w/v) sodium chloride in 0.02 *M* sodium hydroxide. Aliquots of standard solutions of the four amines were added along with sufficient water to bring the volume in the separatory funnel to 100 ml. The HPLC analysis was performed as described. For extraction method B, aliquots of standard solutions of the amines were added to a 100-ml volumetric flask, followed by a 10-g portion of FD&C Yellow No. 6 dissolved in water (containing five drops of 1 *M* sodium hydroxide) and sufficient water to bring the contents to volume. The HPLC analysis was performed as described.

Peak heights were measured by the integrator for each amine coupling product at both 510 and 254 nm. At least four different levels for each amine were analyzed, and the data were evaluated statistically to calculate the regression equation for each amine and determine the limits of determination[12]. The levels of amines in commercial FD&C Yellow No. 6 (254 and 510 nm) were calculated from the regression equations.

UV–visible spectra

The UV–visible spectra of the amine coupling products were obtained as they eluted from the HPLC column by using a diode-array spectrophotometer fitted with an HPLC flow cell. A wavelength range of 240–650 nm was selected and a reference baseline (balance) of the HPLC eluate was measured at the end of the equilibration period. However, if the baseline was free of interfering responses, it was measured in the vicinity of the peak of interest. When the HPLC detector indicated that a solute of interest was eluting, the spectrophotometer was instructed to store a 1-sec spectrum every 2 sec until five spectra of the component were stored in the memory. Each spectrum was examined and the one providing the best signal-to-noise ratio was plotted.

RESULTS AND DISCUSSION

The method reported for the determination of aromatic amines in D&C Red No. 33[9] was modified for their determination in FD&C Yellow No. 6. Since FD&C Yellow No. 6 is more water-soluble than D&C Red No. 33, the amount of color extracted was increased to 5 g, which resulted in a five-fold decrease in the limit of determination. The aromatic amine 2-aminobiphenyl, which was found in commercial D&C Red No. 33 at levels often exceeding those of 4-ABP, was not included in this study.

The extraction technique employed in the analysis of D&C Red No. 33 (extraction method A) worked well for the majority of FD&C Yellow No. 6 samples. However, for several colors significant amounts of FD&C Yellow No. 6 partitioned into the chloroform extract. It is speculated that a wetting agent was added by some manufacturers during the manufacture of the color additive, which caused the FD&C Yellow No. 6 to partition into the organic layer. The presence of large amounts of FD&C Yellow No. 6 in the extract produced interfering peaks in the chromatograms of the amine coupling products. Therefore, it was necessary to use an extraction technique that avoids physical shaking for these problem samples. Hunziker and Miserez[13] successfully employed columns packed with diatomaceous earth to extract aromatic amines from synthetic color additives. Placing an aqueous, alkaline solution

of FD&C Yellow No. 6 onto the column and then extracting the aromatic amines by passing chloroform through the column allows the unsulfonated compounds to elute along with the chloroform, while the aqueous solvent, the FD&C Yellow No. 6 and the particulate matter remain on the column. Five of the 34 colors analyzed during the study contained sufficient FD&C Yellow No. 6 in the chloroform extract to interfere with the HPLC determination. These samples were reanalyzed by using the column extraction procedure.

HPLC calibration data were obtained by using both methods of extraction, and the two sets of data were treated separately. The external standard method was used for calibration. The liquid chromatograph was calibrated in the presence of the color to compensate for any matrix effects that might alter the recovery of the analytes from the sample. The absolute recovery, *i.e.*, the amount of analyte that is extracted from the sample into the chloroform, is estimated to be 75% of the actual amount present on the basis of a comparison with the analytical response obtained by direct analysis. Peak height measurements were used for calibration and for analysis since this technique yielded better results in our laboratories than measurement of peak areas. For the calibration analyses by extraction method A, separate weighings of color were spiked with the four aromatic amines at nine or ten different levels. For the calibration analyses by extraction method B (column extraction), only four levels were used. The FD&C Yellow No. 6 used for calibration had previously been found to contain low levels of the analytes of interest and to be free of any interfering compounds. The spiking levels for the aromatic amines ranged from 2.3 to 23.2 ppb for benzidine, from 34.2 to 513.6 ppb for aniline, from 4.6 to 23.0 ppb for 4-ABP and from 4.2 to 41.6 ppb for 4-AAB.

The calibration data were evaluated statistically to generate the regression equation and to evaluate the performance of the method. The data for extraction method A (calibration set A) were collected over a 7-week period, whereas the data for extraction method B (calibration set B) were obtained in 2 weeks. The summaries of the statistical evaluations of the calibration data at both 254 and 510 nm are presented in Tables I and II. The Shimadzu integrator was used for the quantitation at 254 nm. All of the correlation coefficients are greater than 0.99, indicating a linear relationship between peak height and concentration. The calculated limits of determination (X_{LD}) reflect the range of levels used for a particular set of calibration data, but not necessarily the lowest level for which adequate quantitation can be achieved. For example, calibration data were obtained for aniline levels that ranged from 34 to 513 ppb (extraction method A), and the calculated X_{LD} values, 47.7 and 54.5 ppb, reflect this range. The reproducibility of the method at levels below the X_{LD} values suggests that adequate quantitation can be achieved at these lower levels. The recovery for the method was estimated from the calibration data by using the observed instrument response for a known x value to obtain a corresponding calculated x value (ppb). The ratio of the two values, expressed as a percentage, is the estimated recovery. The range of recoveries for a given calibration using extraction method A is shown in Table I. Similar values were obtained with the calibration data for extraction method B.

Only four calibration points were obtained for calibration set B (Table II). The ranges of the levels are about the same for both sets of calibration data; however, the limits of determination for three of the four analytes in set B are much higher than

TABLE I

STATISTICAL EVALUATION OF CALIBRATION SET A FOR DETERMINATION OF AROMATIC AMINES IN FD&C YELLOW NO. 6

Amine	*n**	*Calibration range (ppb)*	*Wavelength (nm)*	*r***	*X_{LD}*** (ppb)*	*C.V. (%)*	*Recovery range (%)*
Benzidine	10	2.3–23.2	510	0.9964	4.1	8.7	88.2–125.8
			254	0.9927	5.8	13.1	87.8–122.7
Aniline	9	34.2–513.6	510	0.9991	47.7	4.2	95.7–115.4
			254	0.9988	54.5	4.8	96.1–115.6
4-ABP	10	4.6–23.0	510	0.9909	5.9	7.3	92.4–109.5
			254	0.9927	5.3	6.8	91.0–107.7
4-AAB	10	4.2–41.6	510	0.9940	9.8	7.3	87.1–122.2
			254	0.9950	9.0	6.7	87.0–123.7

* Number of calibration points.
** Correlation coefficient.
*** Limit of determination at the 99% confidence level.

in set A. Although the calibration data for benzidine, 4-ABP and 4-AAB in set B show a linear relationship, the calculated X_{LD} values are higher because of the lower number of calibration points.

The coefficients of variation (C.V.), which are the lowest for aniline in both calibration sets, reflect the higher concentration levels for aniline. The C.V. values for the other analytes are quite good in view of the lower calibration levels. In most cases, the C.V. values for a particular analyte differ only slightly for the two detection wavelengths, suggesting that both detection wavelengths are equally suitable for quantitation.

A total of 34 certified samples of FD&C Yellow No. 6 were surveyed for aromatic amines. The samples were selected so that all of the manufacturers of the color additive were represented by at least one batch of color. The manufacturers

TABLE II

STATISTICAL EVALUATION OF CALIBRATION SET B FOR DETERMINATION OF AROMATIC AMINES IN FD&C YELLOW NO. 6

Amine	*n**	*Calibration range (ppb)*	*Wavelength (nm)*	*r***	*X_{LD}*** (ppb)*	*C.V. (%)*
Benzidine	4	3.5–17.4	510	0.9955	12.5	7.6
			254	0.9959	11.9	7.6
Aniline	4	34.2–384.8	510	0.9999	44.4	1.5
			254	0.9999	56.8	1.9
4-ABP	4	5.5–20.7	510	0.9924	19.8	8.5
			254	0.9962	13.0	6.2
4-AAB	4	8.3–41.6	510	0.9971	23.8	6.4
			254	0.9958	28.6	7.8

*, **, *** See footnotes to Table I.

TABLE III

DETERMINATION OF AROMATIC AMINES IN COMMERCIAL FD&C YELLOW NO. 6 WITH QUANTITATION AT 510 AND 254 nm*

Sample	*Aniline (ppb)*		*4-ABP (ppb)*		*4-AAB (ppb)*	
	510	*254*	*510*	*254*	*510*	*254*
A	117.8	118.0	1.2	1.4	–**	2.8
B	–***,§	147.1	1.8	1.5	–	–
C	181.5	173.9	0.6	0.7	3.9	3.7
D	356.2	337.4	10.3	4.0	1098.9§§	1075.7§§
E	421.8	417.2	0.8	3.4	3.5	6.9
F	12.7	11.3	–	1.5	–	1.4
G	35.0	32.6	5.5	4.6	1.9	2.3
H	50.9	49.9	1.6	1.6	37.5	36.7
I	139.3	136.4	0.1	–	0.8	1.5
J	96.1	95.6	8.0	6.7	–	–
K	29.5	30.1	0.3	0.7	1.5	–
L	245.9	246.2	–§,§§§	7.9	14.1	13.9
M	35.5	36.8	2.1	1.5	1.9	1.7
N	25.9	25.6	–	–	1.9	6.8
O	108.0	108.6	–	–	4.3	4.4
P	28.9	27.8	–	0.3	–	–
Q	95.3	93.7	–	–	–	–
R	60.3	61.9	13.3	11.9	–	0.8
S	37.4	36.8	–	0.9	1.4	1.5
T	160.8	163.0	3.6	3.4	0.7	0.8
U	151.8	154.8	14.7	12.5	7.3	6.9
V	14.7	13.3	0.4	0.6	–	–
W	12.7	11.3	0.1	0.3	0.6	1.1
X	28.0	25.8	0.1	–	0.6	1.1
Y	37.3	35.4	8.7	6.9	4.9	4.5
Z	84.0	80.6	3.1	2.5	0.8	–
AA	267.6	259.0	14.3	12.7	7.9	7.6
BB	89.3	85.8	5.0	4.0	4.4	3.6
CC	13.7	11.5	0.1	1.1	–	–
DD	19.3	18.0	1.4	1.6	0.9	0.5
EE	5.9	4.8	–	–	110.2§§	110.5§§
FF	6.5	5.3	0.2	0.4	1.3	1.6
GG†	221.2	218.9	23.0	19.7	22.5	21.5
HH†	37.5	35.7	1.2	1.1	1.0	0.6
Average	97.8	97.4	3.7	3.3	3.9	4.0

* Samples with quantitative results at only one wavelength were not counted as positive, although the values shown were used in the calculation of the averages.

** Dashes indicate a lack of response at the retention time of interest, unless otherwise footnoted. For each dash a value of zero was used in the calculation of the average, with exceptions footnoted.

*** Interfering component coeluted with aniline, making quantitation at 510 nm impossible.

§ Not included in calculation of average (average based on analysis of 33 samples).

§§ Not included in calculation of average (average based on analysis of 32 samples).

§§§ 510 nm detector disconnected during analysis of this sample.

† Pharmacology sample.

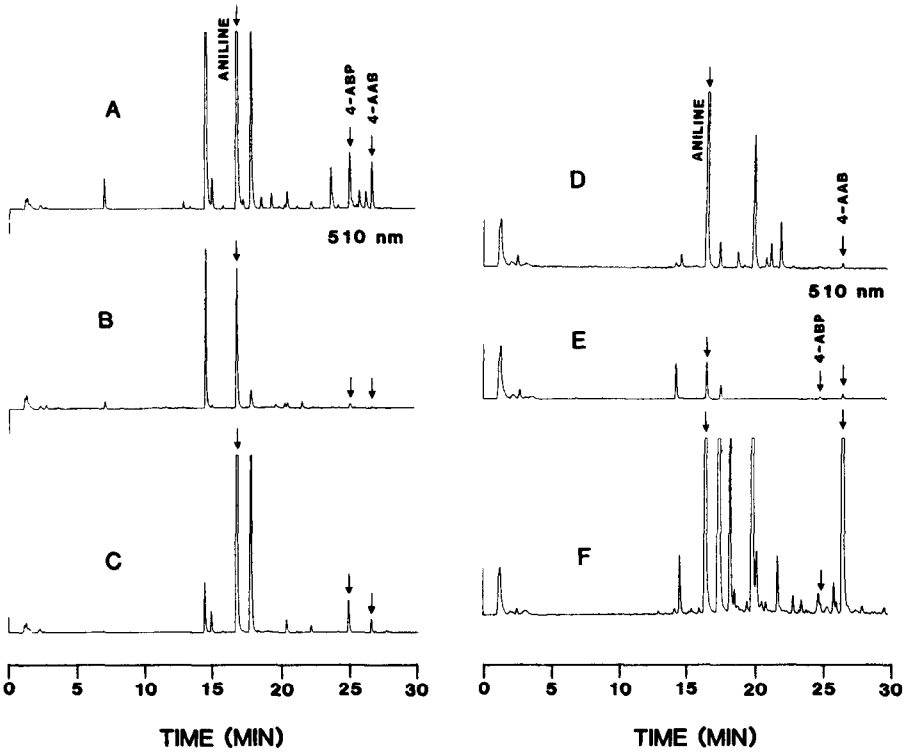

Fig. 2. HPLC chromatograms from analysis of samples of commercial FD&C Yellow No. 6 with R-salt used as the coupling agent: A = sample GG; B = sample HH; C = sample U; D = sample C; E = sample FF; F = sample D.

that produce the largest volumes of FD&C Yellow No. 6 were represented by as many as eight samples. Most of the colors were certified in 1982 and 1983. The two pharmacology samples used in the animal feeding studies of FD&C Yellow No. 6 were included in the survey. The results of the analyses of the color additive are presented in Table III. Representative chromatograms obtained from the analysis of six samples of the commercial color additive are shown in Fig. 2. Chromatograms A and B in Fig. 2 were obtained from analyses of pharmacology samples GG and HH, respectively.

Aniline was found in all 34 of the samples surveyed at an average level of 97.6 ppb with a range of 5.4–419.5 ppb 4-AAB was found in 23 samples; the average level for all samples except D and EE was 4.0 ppb with a range of none found (NF)–37.1 ppb (excluding results for samples D and EE, 1087.3 and 110.4 ppb, respectively).

Sixteen samples had integrator responses for benzidine when they were first analyzed, and six of these 16 samples had responses at both wavelengths used for quantitation. Since the benzidine–R-salt coupling product absorbs at 610 nm, whereas the other coupling products do not, the presence of benzidine in a sample can be confirmed by changing the wavelength of measurement to 610 nm. When samples with analytical responses at 254 and 510 nm at the retention time of benzidine were rechromatographed with measurement at 610 nm, no responses were observed at the

TABLE IV

MULTIPLE ANALYSES OF COMMERCIAL FD&C YELLOW NO. 6 FOR ANILINE, 4-ABP AND 4-AAB WITH QUANTITATION AT 510 AND 254 nm*

Sample	*Aniline (ppb)*		*4-ABP (ppb)*		*4-AAB (ppb)*	
	510	*254*	*510*	*254*	*510*	*254*
DD	18.7	17.5	1.1	1.5	0.6	ND**
	19.3	17.8	1.5	1.6	1.2	ND
	19.3	17.7	1.5	1.8	1.0	1.1
	18.8	18.0	1.3	1.5	1.2	1.2
	20.4	19.0	1.4	1.6	0.7	ND
Average	19.3	18.0	1.4	1.6	0.9	0.5
C.V. (%)	3.5	3.3	12.3	7.7	29.7	137.1
FF***	6.2	5.3	0.4	0.5	ND	1.2
	6.3	5.1	ND	0.5	1.4	1.2
	6.5	4.8	ND	0.4	1.4	1.2
	6.6	5.4	0.4	0.4	3.6	3.2
	6.3	5.4	ND	0.4	ND	1.8
	5.8	4.8	ND	0.5	1.4	1.6
	8.4	7.8	ND	0.6	ND	0.8
	6.6	5.0	0.6	0.6	2.3	2.0
	4.5	2.8	ND	ND		
	7.9	6.5	0.4	0.4		
Average	6.5	5.3	0.2	0.4	1.3	1.6
C.V. (%)	16.4	24.1	133.0	39.6	101.0	45.8
U	148.3	147.2	14.6	12.0	6.2	5.8
	148.6	157.5	14.8	12.2	6.8	6.5
	156.3	157.4	15.4	13.0	7.4	7.1
	152.4	158.5	15.4	13.2	8.9	8.3
	153.4	153.3	13.5	11.9	7.4	6.6
Average	151.8	154.8	14.7	12.5	7.3	6.9
C.V. (%)	2.2	3.0	5.3	4.8	13.7	13.5
GG	206.8	195.1	21.1	17.2	21.3	19.7
	220.5	226.3	22.6	20.6	24.9	24.3
	236.3	235.4	25.4	21.4	21.4	20.4
Average	221.2	218.9	23.0	19.7	22.5	21.5
C.V. (%)	6.7	9.6	9.5	11.3	9.1	11.5
HH	34.3	31.2	0.9	0.9	0.8	ND
	38.8	37.8	1.0	0.9	0.9	0.9
	39.3	38.2	1.7	1.4	1.2	0.9
Average	37.5	35.7	1.2	1.1	1.0	0.6
C.V. (%)	7.3	11.0	36.3	27.1	21.5	86.6

* Extraction method A was used for all of these determinations.

** ND = None detected.

*** For sample FF, two 4-AAB determinations were omitted because of reagent contamination with the 4-AAB standard solution.

retention time of benzidine, even at settings of maximum sensitivity. Therefore, the presence of benzidine was not confirmed for the samples analyzed in the survey.

Nearly all of the samples analyzed in the survey produced an HPLC response for the 4-ABP–R-salt coupling product. The average level found was 3.5 ppb with a range of NF–21.4 ppb. However, the UV–visible absorption spectrum of the HPLC eluate suggested that an interfering substance was present in some samples and that this interference masked the actual levels of 4-ABP present in those color additive samples. This problem was addressed by using a different coupling agent to change the chromatographic properties of the coupling product and thereby remove the interference. The results are discussed below in greater detail.

The reproducibility of the method was investigated by performing multiple analyses of several different samples of FD&C Yellow No. 6. The results are shown in Table IV for samples DD, FF, U, GG and HH. The calculated C.V. values suggest good reproducibility even at levels below the method limit of determination. For example, the results of repetitive aniline determinations for sample DD (Table IV) demonstrate good precision at the 19-ppb level (C.V. = 3.5%), although the calculated X_{LD} value for aniline at 510 nm is 47.7 ppb. In this sample the C.V. values are low even at 4-ABP levels of 1–2 ppb. However, it should be noted that the multiple analyses for sample DD were performed in a single day. The good reproducibility can be accounted for in part by the short interval for data collection.

The multiple determinations for sample FF (Table IV) were performed over an 8-week period. Consequently, a C.V. value of 16.4% for the aniline determinations is quite good, especially at the 6-ppb level.

Sample U, which contained significant amounts of aniline, 4-ABP and 4-AAB, was subjected to five separate amine determinations performed over a 7-week period. The C.V. values (Table IV) were low for both aniline and 4-ABP, indicating good precision at those levels. Although the C.V. values for 4-AAB were higher, they indicate adequate reproducibility.

The two pharmacology samples (GG and HH) were each analyzed three times over a 2-week period. Representative chromatograms of GG and HH obtained with the 510-nm detector are shown in Fig. 2, chromatograms A and B, respectively. The results of multiple analyses for samples GG and HH are presented in Table IV. The C.V. values indicate good or adequate precision at the levels tested. At the 1-ppb levels of 4-ABP and 4-AAB, the C.V. values were the highest, indicating the greatest variation at the lowest levels.

The identities of the aromatic amine responses from the analysis of FD&C Yellow No. 6 were verified by using a rapid-scan diode-array spectrophotometer with an HPLC flow cell installed in the cuvette holder to obtain UV–visible spectra of the coupling products as eluting HPLC solutes. A reference spectrum for each aromatic amine coupling product was first obtained under the conditions of analysis. The reference spectrum was then compared with the spectrum for the corresponding component obtained during the analysis of a sample of commercial FD&C Yellow No. 6.

The spectra of the aniline coupling products obtained with this technique during the analysis of samples D, U and GG were examined and compared with the spectrum of an authentic material obtained in the same manner. In all cases, the spectrum obtained was identical to that of the reference material, confirming the

identity of the response for these samples. In a similar manner, the spectra of the 4-AAB coupling products obtained for samples D and GG were examined, and the spectra were found to be identical to that of the reference material.

As noted above, attempts to confirm the identity of the 4-ABP responses obtained during HPLC analysis produced ambiguous results. A similar problem was encountered during the analysis of FD&C Yellow No. 5 for aromatic amines[10]. However, in the case of FD&C Yellow No. 6, the data clearly indicated that 4-ABP was present at significant levels. This is illustrated in Fig. 3, which shows the absorption spectra of the 4-ABP eluates from samples GG, AA, R and Y. The spectra clearly show that these samples contain a component that interferes with the determination of 4-ABP. This interference has an absorption spectrum that is very similar to the spectrum of the 4-ABP–R-salt coupling product and produces a response on both the 254- and 510-nm detectors.

An attempt was made to obtain a better estimate of the levels of 4-ABP present in FD&C Yellow No. 6 by using pyrazolone-T in place of R-salt as the coupling agent. This technique was used in resolving the similar interference encountered during the determination of 4-ABP in FD&C Yellow No. 5[10]. Most of the samples that were found to contain the highest levels of 4-ABP when R-salt was used as the coupling agent (samples D, L, R, Y, AA and GG) were reanalyzed with pyrazalone-T as the coupling agent. Pharmacology sample HH was also reanalyzed. The wavelengths of measurement were 254 and 460 nm (460 nm is the absorption maximum for the 4-ABP–pyrazolone-T coupling product). The results of these analyses are shown in Table V. Representative chromatograms are shown in Fig. 4. The liquid chromatograph was calibrated with a 4-ABP level of 11.3 ppb by using duplicate analyses with extraction method A (shakeout) and a single analysis with extraction method B (column extraction).

The values obtained by using pyrazolone-T as the coupling agent are lower

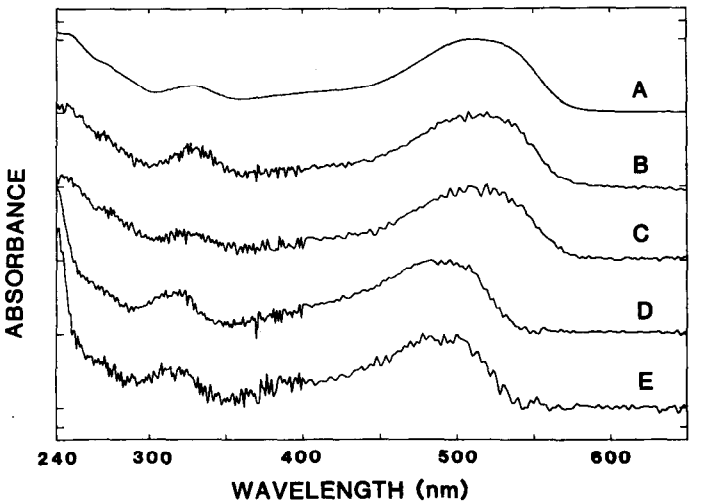

Fig. 3. UV–visible spectral scan of the 4-ABP–R-salt coupling product obtained during analysis of samples of commercial FD&C Yellow No. 6: A = authentic product; B = sample GG; C = sample AA; D = sample R; E = sample Y.

TABLE V

DETERMINATION OF 4-ABP IN COMMERCIAL FD&C YELLOW NO. 6 WITH PYRAZOLONE-T AS THE COUPLING AGENT AND QUANTITATION AT 254 AND 460 nm

Sample	*4-ABP*	
	254	*460*
L	7.0	5.8
	7.7	7.0
AA	10.4	11.4
	10.0	10.2
R	ND	1.3
Y	0.8	1.1
D*	4.0	4.2
GG**	17.0	17.3
HH**	ND***	ND

* Extraction method B (column extraction).
** Pharmacology sample.
*** ND = None detected.

than those obtained with R-salt. These differences reflect the level of error arising from the interfering component encountered when the R-salt reagent was used. The scan of the HPLC eluates with the diode-array spectrophotometer confirmed the identity of the peak as the 4-ABP–pyrazolone-T coupling product. On the basis of these results, the values reported in Table III for 4-ABP, which were obtained with R-salt as the coupling agent, should be viewed as upper limits because of the possibility of interference.

The analysis of several samples produced chromatograms with numerous HPLC responses besides those of the four aromatic amines. The diode-array spectrophotometer was used to obtain UV–visible spectra for several of these components. All of the components scanned had spectra that closely resembled those of the amine coupling products. The variability of the composition of the FD&C Yellow No. 6 chloroform extractables is shown by the HPLC chromatograms in Fig. 2. Some of the large extraneous peaks, such as the one appearing approximately 1 min after the aniline peak (Fig. 2), were significant responses in many sample chromatograms. The number and size of these additional responses varied from sample to sample.

Besides the variability in the HPLC chromatograms of the amine coupling products, several other differences were noted among the certified samples surveyed during various steps of the determination. For example, 13 of the 34 samples formed emulsions during the chloroform extraction step. In most cases these emulsions were successfully broken by stirring the mixture with a 9-in. transfer pipet. However, some emulsions were very difficult to break.

In 15 of the survey samples the chloroform extracts were colored, ranging from very pale yellow to deep orange. The deeply colored extracts were found to be contaminated with large amounts of the color additive and were reanalyzed by extraction method B. For most of the samples that had colored chloroform extracts, a residue was found on the sides of the round-bottom flask after the chloroform was removed. The material, which remained insoluble throughout the diazotization, coupling and

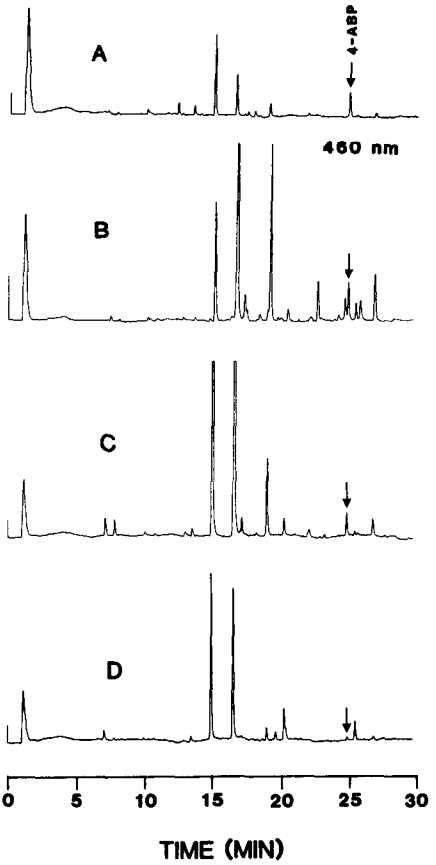

Fig. 4. HPLC chromatograms obtained from analysis of samples of commercial FD&C Yellow No. 6 with pyrazolone-T used as the coupling agent: A = sample FF fortified with 11.3 ppb 4-ABP; B = sample GG; C = sample AA; D = sample R.

HPLC steps, could be removed by rinsing the flask with acetone or ethanol. The water-insoluble portion of the extract, which consists in part of unsulfonated FD&C Yellow No. 6, was collected and saved for future investigations.

CONCLUSIONS

The presence of aniline, 4-ABP and 4-AAB in extracts of the color additive FD&C Yellow No. 6 has been confirmed by HPLC analysis and UV–visible spectrophotometry. Benzidine was not identified in any of the samples of the commercial color additive that were analyzed. The method employed allows quantitation at levels greater than or equal to 5 ppb. Amine levels can generally be estimated in amounts as low as 1 ppb. Good reproducibility of the method has been demonstrated.

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