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DETERMINATION OF THE LOWER SULFONATED SUBSIDIARY COLORS IN FD&C YELLOW NO. 6 BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

JOHN E. BAILEY, Jr.
Division of Color Technology, Food and Drug Administration, Washington, DC 20204 (U.S.A.)
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SUMMARY

Data are presented for the determination of 5-(phenylazo)-6-hydroxynaphthalene-2-sulfonic acid (ANSC) and 4-[(2-hydroxynaphthalene-l-yl)azo]benzenesulfonic acid (BNSC) in FD&C Yellow No. 6 by reversed-phase high-performance liquid chromatography (RP-HPLC). The method employs gradient elution with a water–tetrahydrofuran solvent system buffered with ammonium acetate and quantitation in the UV (254 nm) and visible (490 nm) regions. The external calibration method was used with twelve data points for each analyte. Each analysis was performed in duplicate. In analyses of commercial FD&C Yellow No. 6, ANSC was found at levels ranging from none to 0.52%, and BNSC was found at levels ranging from none to 0.73%. The identities of the analytes were confirmed by using a rapid-scan diode-array spectrophotometer to obtain the electronic absorption spectra during RP-HPLC analysis.

INTRODUCTION

FD&C Yellow No. 6 (Sunset Yellow FCF, Colour Index No. 15985) is one of seven synthetic color additives permitted for use in foods in the U.S.A.[1]. As one of the major colors used in foods, it accounted for 24% of the 6.4 million pounds of food colors certified by the Food and Drug Administration (FDA) in the fiscal year 1984.

FD&C Yellow No. 6 is manufactured by coupling diazotized sulfanilic acid (4-aminobenzenesulfonic acid) with Schaeffer's salt (6-hydroxynaphthalene-2-sulfonic acid) as shown in Fig. 1. As with any chemical reaction, the product that is formed

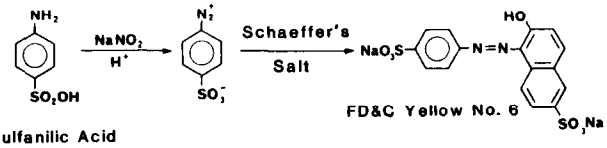

Fig. 1. Synthesis of FD&C Yellow No. 6.

contains impurities since the reactants themselves are usually impure and side reactions also occur. In the case of FD&C Yellow No. 6, the impurities are of three types: uncombined intermediates, which do not impart color to the product; subsidiary colors, which are colored compounds that are structurally related to the product; and other substances which may arise from contamination of the intermediates or other reagents used during synthesis. Additional components of the color include moisture and inorganic salts. The nature and amounts of these impurities are regulated by specifications that are enforced by batch analysis during color additive certification[1].

Included in the impurities that are likely to be found in FD&C Yellow No. 6 are 5-(phenylazo)-6-hydroxynaphthalene-2-sulfonic acid (ANSC) and 4-[2-hydroxynaphthalene-l-yl)azo]benzenesulfonic acid (BNSC), which are shown in Figs. 2 and 3, respectively. ANSC arises when aniline, which is present as a contaminant in sulfanilic acid[2,3], is diazotized and coupled with Schaeffer's salt. BNSC arises when 2-naphthol, which is a contaminant in Schaeffer's salt[4], is coupled with diazotized sulfanilic acid. These two impurities together are called the lower sulfonated subsidiary colors. The current specifications limit the total amount of subsidiary color, including the lower sulfonated subsidiary colors, permitted in FD&C Yellow No. 6 to 5% by weight.

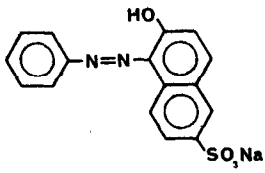

Fig. 2. Lower sulfonated subsidiary color (ANSC) formed from the coupling of aniline with Schaeffer's salt.

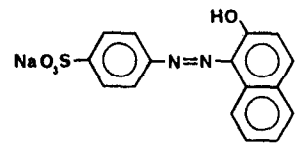

Fig. 3. Lower sulfonated subsidiary color (BNSC) formed from the coupling of diazotized sulfanilic acid with 2-naphthol.

These regulatory specifications will be revised to ensure that the manufactured batches of the color approximate the composition of the material used in the animal feeding studies that were performed to establish safe levels of exposure. The levels of ANSC and BNSC in batches of the commercial color additive need to be determined to establish these regulatory specifications and to provide a basis for assessing the potential risk associated with the use of the color.

The evaluation of potential risk associated with the use of color additives must include an assessment of the potential exposure to the metabolites of the lower sulfonated subsidiary colors. The metabolism of azo dyes results in the reduction and cleavage of the azo bond[5]. The metabolism of ANSC releases aniline, whereas the metabolism of BNSC produces 1-amino-2-hydroxynaphthalene. These metabolites, unlike the parent compounds, are unsulfonated and, therefore, are biologically available.

The lower sulfonated subsidiary colors in FD&C Yellow No. 6 have been determined previously. Bell[6] reported a thin-layer chromatographic (TLC) procedure for the determination of the higher and lower sulfonated subsidiary colors. The procedure was developed with BNSC only, and results were not reported for batch

analysis of commercial FD&C Yellow No. 6. Cox *et al.*[7] employed reversed-phase high-performance liquid chromatography (RP-HPLC) for the determination of intermediates, subsidiary colors and two reaction by-products in FD&C Yellow No. 6. In their evaluation of the TLC procedure, Cox *et al.*[7] found that ANSC and BNSC were not separated from one another. The RP-HPLC procedure, which used a buffered water–methanol solvent system, also did not separate the two lower sulfonated subsidiary colors. The liquid chromatograph was calibrated by using ANSC, and the values reported for the lower sulfonated subsidiary colors were the combined levels of ANSC and BNSC, calculated as ANSC. ANSC was considered the subsidiary color most likely to occur in commercial FD&C Yellow No. 6, and the expected error due to the presence of BNSC was considered small since the two subsidiary colors have similar absorption spectra and absorptivities.

This paper describes the use of gradient elution HPLC for the separation and quantitation of ANSC and BNSC in batches of commercial FD&C Yellow No. 6. A buffered water–tetrahydrofuran (THF) solvent system is used with quantitation in the UV (254 nm) and visible (490 nm) regions. Results are presented for calibration and determination in samples of commercial FD&C Yellow No. 6.

EXPERIMENTAL

HPLC instrumentation

HPLC separations were performed on a Varian (Palo Alto, CA, U.S.A.) Model 5060 gradient liquid chromatograph with a Varian Model 8055 autosampler. Detection was with a Waters Assoc. (Milford, MA, U.S.A.) Model 440 detector at 254 nm and an Altex (Berkeley, CA, U.S.A.) Model 155-10 variable-wavelength detector set at 490 nm. A Rheodyne (Cotati, CA, U.S.A.) Model 7010 loop injector with a 20-μl loop was used. A Spectra-Physics (San Jose, CA, U.S.A.) Model 4200 dual channel integrator/printer-plotter was used for data collection and treatment. (The chromatograms shown in Figs. 4 and 5 were recorded on a Shimadzu (Columbia, MD, U.S.A.) C-R1A printer-plotter for presentation purposes only.) The chromatograms were recorded at 5 mm/min. The separations were made by using a Waters Assoc. NovaPak C_{18} column, 5-μm particle size, 15 cm × 3.9 mm I.D. (Cat. No. 086344).

Reagents

All chemicals were analytical reagent grade. HPLC grade solvents were used for the preparation of mobile phases. HPLC solvent A was prepared by placing 1.5 g of ammonium acetate and 0.5 ml of THF in a 100-ml volumetric flask and diluting to volume with purified water (Millipore/Continental Water Systems, Bedford, MA, U.S.A.); solvent B was prepared in a similar manner by placing 1.5 g of ammonium acetate and 50 ml of THF in a 100-ml volumetric flask and diluting to volume with purified water.

Standards

ANSC and BNSC were available as reference materials from previous investigations[8]. The ANSC reference compound was in the acid form, whereas the BNSC compound was the sodium salt. The purity was checked spectrophotometrically and stock solutions were prepared by dissolving weighed portions of the lower sulfonated

subsidiary colors in water. The weights were corrected for percent purity and, in the case of ANSC, the weight was converted to the equivalent weight of the sodium salt.

Sample preparation

Each color was prepared for analysis by dissolving a 1-g portion in 100 ml of distilled water. The mixture was then shaken to dissolve all of the solids. The resulting solution was found to be stable for at least several months.

HPLC analysis

The HPLC column was equilibrated with 100% solvent A for 10 min at a flow-rate of 1 ml/min. At the end of the equilibration time, the autosampler loaded the injector loop, injected the solution of the color onto the column and initiated the solvent gradient (linear) of 0 to 60% solvent B in solvent A over 25 min. The solvent composition was held at 60% solvent B in solvent A for 5 min, and then the column was re-equilibrated with 100% solvent A.

Measurements and calculations

The liquid chromatograph was calibrated by performing the determination with a color that had been spiked with the two lower sulfonated subsidiary colors. An FD&C Yellow No. 6 sample that was found to contain relatively low levels of the contaminants was used for the calibration. A 1-g portion of the color was added to a 100-ml volumetric flask, followed by portions of the analyte stock solutions and then sufficient water to dilute the contents to volume. The HPLC response for each component was measured for twelve different concentration levels. After correction for the color blank, the results were treated statistically to calculate the regression equation[9]. The levels of ANSC and BNSC in samples of commercial FD&C Yellow No. 6 were determined from the regression equation. Each analysis was performed in duplicate by repeating the injection.

UV–VIS spectra of eluting solutes

The technique for obtaining the electronic absorption spectra of eluting analytes has been described previously[10].

RESULTS AND DISCUSSION

HPLC analysis

Attempts to separate ANSC and BNSC by RP-HPLC analysis with buffered water–methanol[7] and buffered water–acetonitrile[11] solvent systems were not successful. HPLC analyses were performed with a wide variety of C_8 and C_{18} columns and both isocratic and gradient elution. However, when methanol or acetonitrile was replaced by THF, ANSC and BNSC were completely separated from each other. Fig. 4 shows the HPLC chromatograms obtained at 490 nm for a solution of FD&C Yellow No. 6 with and without added analytes. The use of THF in the solvent system required changing the gradient program to compensate for the greater eluting strength of THF relative to that of methanol or acetonitrile. Thus, a final gradient composition of 60% solvent B in solvent A with THF as the organic modifier eluted the lower sulfonated subsidiary colors at approximately the same retention time as

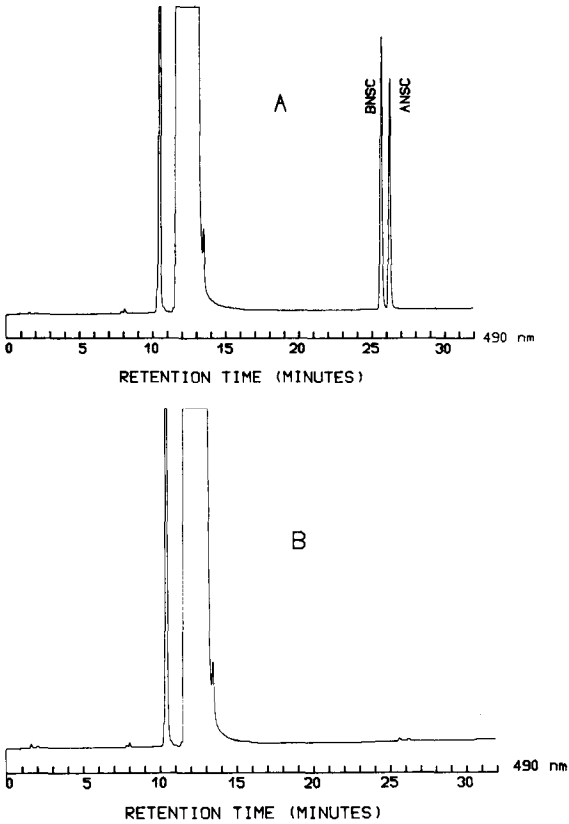

Fig. 4. HPLC chromatograms obtained at 490 nm for (A) color blank fortified with ANSC and BNSC and (B) unfortified color blank.

a final gradient composition of 75% solvent B in solvent A with methanol or acetonitrile solvents of similar composition (1.5% ammonium acetate in a 50:50 mixture of organic modifier and water). THF also produced a much higher back pressure than that observed with acetonitrile or methanol. THF has a greater tendency to form UV-absorbing impurities and, as a result, the baseline for the color blank obtained at 254 nm tended to drift during gradient elution. Although this problem is

TABLE I

REGRESSION ANALYSIS OF CALIBRATION DATA FOR LOWER SULFONATED SUBSIDIARY COLORS IN FD&C YELLOW NO. 6

Component	*No. of data points*	*Calibration range (%)*	*Wavelength (nm)*	*Regression equation*	r^*
BNSC	12	0.010–0.49	254	$y = 8932059x + 4640$	1.0000
			490	$y = 1789972x - 3207$	0.9999
ANSC	12	0.011–0.566	254	$y = 11148312x + 9664$	1.0000
			490	$y = 1290711x - 716$	1.0000

* r = Correlation coefficient.

TABLE II

DETERMINATION OF BNSC AND ANSC IN FD&C YELLOW NO. 6

Each result is the average of duplicate determinations. Mfr. = manufacturer, Av. = average, LS = lower sulfonated subsidiary color, NA = not applicable, and NF = none found.

Sample	*Mfr.*	*This method*						*Total LS (%)*		
		BNSC (%)			*ANSC (%)*			*This method*	*TLC method*[6]	*HPLC method*[7]
		254 nm	*490 nm*	*Av.*	*254 nm*	*490 nm*	*Av.*			
1*	AA3003	0.068	0.070	0.069	0.224	0.221	0.222	0.291	0.20	0.24
2*	AA8634	0.027	0.028	0.028	0.180	0.176	0.178	0.206	0.20	0.14
3	A	0.008	0.008	0.008	0.286	0.283	0.284	0.292	0.40	NA
4		0.018	0.018	0.018	0.520	0.521	0.521	0.539	0.40	NA
5		0.264	0.258	0.261	0.394	0.393	0.394	0.655	0.80	NA
6		0.555	0.600	0.578	0.196	0.193	0.193	0.771	0.80	NA
7		0.720	0.736	0.728	0.372	0.362	0.367	1.095	1.10	NA
8		0.004	0.005	0.004	0.388	0.388	0.388	0.392	0.40	NA
9		0.032	0.031	0.032	0.479	0.480	0.480	0.512	0.60	NA
10	B	0.077	0.076	0.076	0.008	0.008	0.008	0.084	0.20	NA
11		0.058	0.060	0.059	NF	NF	NF	0.059	0.09	NA
12		0.008	0.008	0.008	0.215	0.212	0.214	0.222	0.20	NA
13		0.660	0.664	0.662	0.054	0.048	0.051	0.713	0.80	NA

14	C	0.044	0.043	0.044	0.116	0.116	0.116	0.160	0.30	NA
15		0.020	0.022	0.021	0.366	0.366	0.366	0.387	0.40	NA
16		0.028	0.028	0.028	0.191	0.192	0.192	0.220	0.30	NA
17	D	NF	0.002	0.002	0.279	0.278	0.278	0.280	0.30	NA
18		NF	0.003	0.003	0.064	0.064	0.064	0.067	0.06	NA
19		0.003	0.004	0.004	0.077	0.076	0.076	0.080	0.08	NA
20	E	0.082	0.079	0.080	0.214	0.212	0.213	0.293	0.40	NA
21		0.048	0.044	0.046	0.179	0.174	0.176	0.222	0.20	NA
22	F	0.079	0.072	0.076	0.008	0.008	0.008	0.082	0.20	NA
23		0.078	0.077	0.078	0.004	0.004	0.004	0.082	0.07	NA
24		0.058	0.056	0.057	0.006	0.006	0.006	0.063	0.10	NA
25	G	0.042	0.042	0.042	0.005	0.005	0.005	0.047	0.10	NA
26	H	0.070	0.068	0.069	0.046	0.045	0.046	0.115	0.02	NA
27		0.070	0.067	0.068	0.064	0.063	0.064	0.132	0.20	NA
28	I	0.050	0.050	0.050	0.002	0.002	0.002	0.052	0.07	NA
29	J	0.046	0.046	0.046	0.032	0.032	0.032	0.078	0.40	NA
30	K	0.222	0.220	0.221	0.016	0.015	0.015	0.236	0.50	NA
31	L	0.426	0.418	0.422	0.046	0.041	0.044	0.466	0.60	NA
Overall average				0.125			0.162	0.287	0.338	
Range				0.002–0.728			NF–0.521	0.047–1.095	0.02–1.10	

* Pharmacology sample.

more severe than that encountered with other organic modifiers, the data system was easily programmed to compensate for the change in baseline, to detect the analyte signals and to accurately report their areas.

Calibration

The liquid chromatograph was calibrated according to the external standard procedure by analyzing separate weighings of commercial FD&C Yellow No. 6 spiked with ANSC and BNSC. A total of twelve data points were obtained for each lower sulfonated subsidiary color, ranging from 0.010 to 0.49% by weight for BNSC and from 0.011 to 0.566% by weight for ANSC. The calibration analyses were interspersed with color analyses during the entire course of the study. The analyte responses, corrected for the analyte levels present in the color blank, were treated statistically to generate the regression equation and to evaluate the performance of the method[9]. The results of the statistical analysis are shown in Table I. A linear relationship was obtained for the data points, and the intercepts were all close to zero.

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. The ratio of the two values, expressed as a percentage, is the estimated recovery. The range and average of the recoveries for each calibration set are as follows: for BNSC at 254 nm, 97.4–107.7%, 100.4%; for BNSC at 490 nm, 98.4–123.3%, 102.7%; for ANSC at 254 nm, 98.4–108.6%, 100.6%; for ANSC at 490 nm, 99.3–104.5%, 100.4%.

Analysis of commercial FD&C Yellow No. 6

A total of 31 certified samples of commercial FD&C Yellow No. 6 were analyzed for ANSC and BNSC. The samples were selected so that all of the domestic and foreign manufacturers of the color additive were represented by at least one batch of color. The primary manufacturers were represented by as many as seven batches of color. Manufacturers A–E are domestic, I–J are European and K and L are Japanese. Included in the survey are the two samples (entries 1 and 2 in Table II) from the animal feeding studies that were used by the FDA to evaluate the toxicity of FD&C Yellow No. 6. Batch AA3003 was used for the low dose feeding levels (0.75, 1.5 and 3%), and batch AA8634 was used for the high dose feeding level (5%). The results of the analyses for BNSC and ANSC, calculated at 254 and 490 nm, are shown in Table II. Fig. 5 shows the HPLC chromatograms obtained from the analysis of samples 1, 2, 15, 6, 30 and 20. Each sample was analyzed in duplicate, and the results were averaged. The levels of BNSC found in commercial FD&C Yellow No. 6 ranged from 0.002 to 0.728% and averaged 0.125%; the levels of ANSC ranged from 0.002 to 0.521% and averaged 0.162%. On the basis of these results, there does not seem to be a tendency for one lower sulfonated subsidiary color to predominate over the other. However, for some individual manufacturers, there does seem to be a consistent pattern in which one lower sulfonated subsidiary color predominates over the other. This consistency of composition would, of course, parallel the composition of the intermediates used to manufacture the color. However, the sources of the intermediates may vary widely, depending on availability and price. Some manufacturers produce their own intermediates but will, if necessary, purchase the

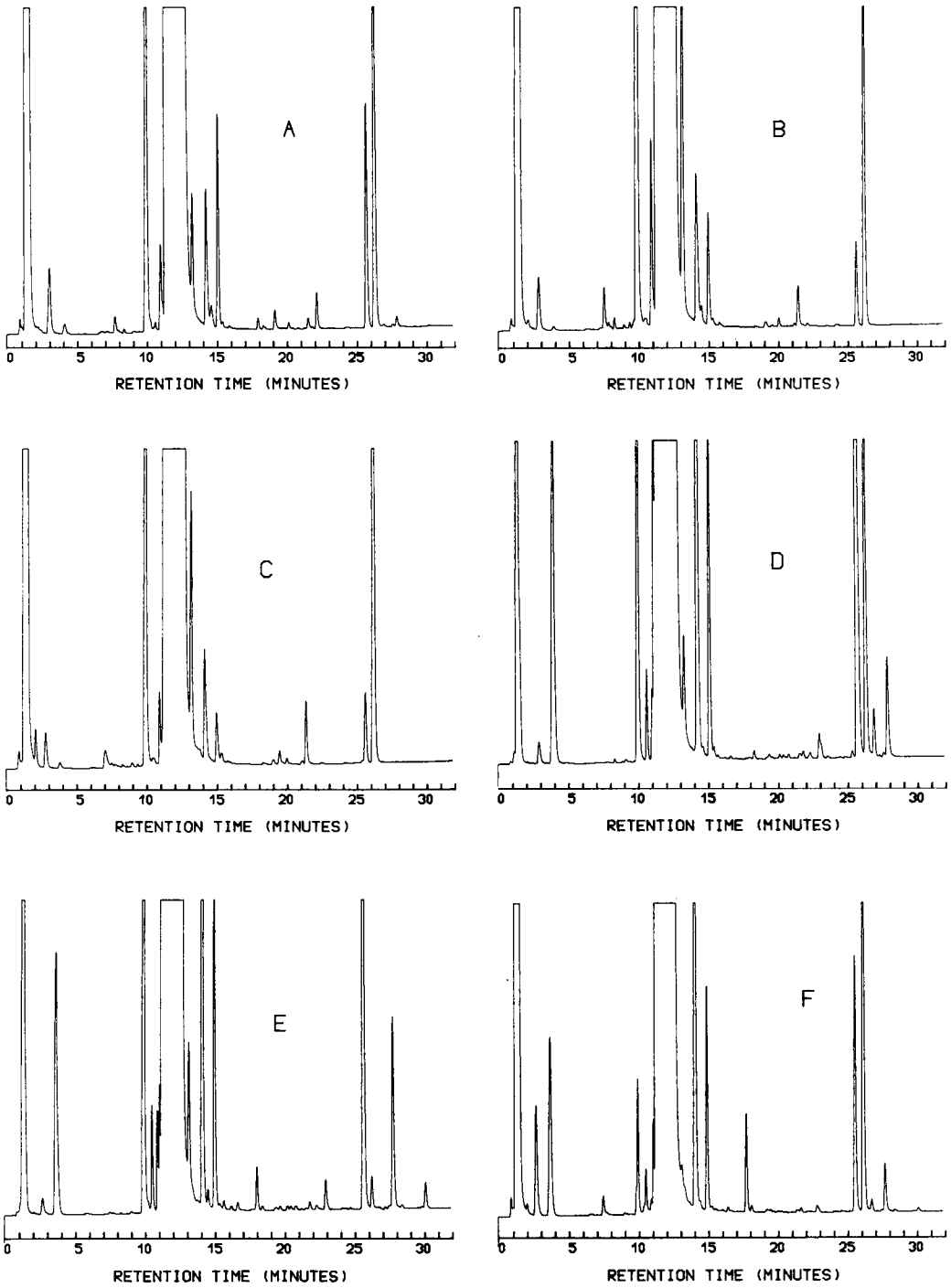

Fig. 5. HPLC chromatograms for (A) sample 1, (B) sample 2, (C) sample 15, (D) sample 6, (E) sample 30 and (F) sample 20. (Chromatograms were recorded at 490 nm.)

chemicals from an outside source. In general, the non-domestic color manufacturers seem to produce a color that contains lower levels of these lower sulfonated subsidiary colors, especially ANSC.

Table II also shows the averages of the analytical results obtained at 254 and 490 nm for ANSC and BNSC as well as the sums of these averages. The sums of the ANSC and BNSC levels can be correlated with the values for the lower sulfonated subsidiary colors obtained by using the TLC[6] and HPLC[7] methods. The composite values are compared in Table II with the results obtained for color certification analyses[12] in which the TLC procedure[6] was used. Analytical results for the lower sulfonated subsidiary colors obtained with the HPLC method[7] were available only for the two pharmacology samples. These values are also shown in Table II. In general, the values obtained by using the TLC[6] and HPLC[7] procedures agree with the levels of lower sulfonated subsidiary colors found in this study.

In order to characterize the HPLC responses obtained during analysis, a diode-array UV–VIS spectrophotometer was used to obtain the electronic absorption spectrum of each component. The spectra thus obtained were then compared with the spectra of authentic ANSC and BNSC obtained in a similar manner to confirm the identity of each contaminant. In all cases, the spectra obtained for the analyzed samples were identical to the spectra of the standards obtained in a similar manner, thus confirming the identities of the responses.

Also of interest in this study was the presence of colored components other than BNSC and ANSC that were discovered during analysis of some of the FD&C Yellow No. 6 samples. In some cases, the responses for these unidentified constituents exceeded those for the lower sulfonated subsidiary colors (*e.g.*, as shown in Fig. 5, chromatogram E). Analysis of reference materials for the isomeric lower sulfonated subsidiary colors that are formed when orthanilic and metanilic acid are coupled with 2-naphthol did not produce responses with retention times that matched those of the unidentified components. It is possible that one of these unidentified components may be the product that is formed when sulfanilic acid couples with 1-naphthol. Investigation of this possibility depends on the acquisition of an adequate reference material. Characterization of these unidentified components is planned for future studies.

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