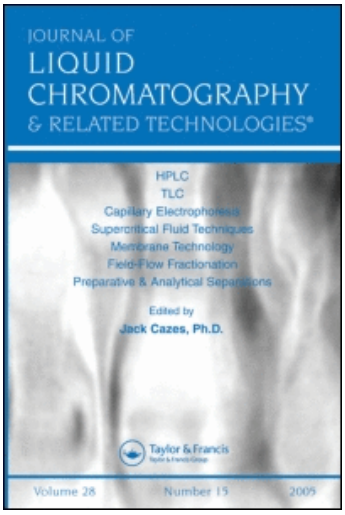

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DETERMINATION OF INTERMEDIATES AND SUBSIDIARY COLORS IN THE COLOR ADDITIVE FD&C RED NO. 4 (PONCEAU SX) USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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□ *Specifications in the U.S. Code of Federal Regulations for the color additive FD&C Red No. 4 (Color Index 14700) limit the levels of the intermediates, 5-amino-2,4-dimethyl-1-benzenesulfonic acid (AMBSA) and 4-hydroxy-1-naphthalenesulfonic acid (HNSA), and of subsidiary colors. The present study reports the development of a high-performance liquid chromatography (HPLC) method for the quantitative determination of these intermediates and subsidiary colors in one analysis. The most commonly-found subsidiary color, 3-[(2,4-dimethyl-6-sulfophenyl)azo]-4-hydroxy-1-naphthalenesulfonic acid (6SSC), is determined and is used as a secondary reference material to determine the quantities of other subsidiary colors present. High-speed countercurrent chromatography was used to obtain purified HNSA and 6SSC for use as reference materials. AMBSA, HNSA, and 6SSC were quantified by using five-point calibration curves with data points that ranged from 0.028–0.234%, 0.031–0.252%, and 0.113–2.036% by weight, respectively. The HPLC method is rapid (50 min for the total analysis cycle) and simple to implement. It was applied to the analysis of test portions from 23 lots of FD&C Red No. 4 submitted to the U.S. Food and Drug Administration (FDA) for certification and was later implemented by the FDA for routine batch certification of FD&C Red No. 4.*

Keywords 3-[(2,4-dimethyl-6-sulfophenyl)azo]-4-hydroxy-1-naphthalenesulfonic acid, 4-hydroxy-1-naphthalenesulfonic acid, 5-amino-2,4-dimethyl-1-benzenesulfonic acid, C.I. 14700, FD&C Red No. 4, High-speed countercurrent chromatography (HSCCC), HPLC, Ponceau SX

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INTRODUCTION

FD&C Red No. 4 (R4, Ponceau SX, Color Index No. 14700, mainly the disodium salt of 3-[(2,4-dimethyl-5-sulfophenyl)azo]-4-hydroxy-1-naphthalenesulfonic acid, 1 in Figure 1) is a color additive listed in the U.S. Code of Federal Regulations (CFR)[1] for use in externally applied drugs and cosmetics. It is manufactured following the same two-step procedure that was used in 1886 for preparing the dye Ponceau:[2] diazotization of 5-amino-2,4-dimethyl-1-benzenesulfonic acid (AMBSA), followed by coupling of the diazotized AMBSA with 4-hydroxy-1-naphthalenesulfonic acid (HNSA) (Figure 1).

Unreacted intermediates and reaction byproducts such as subsidiary colors may be carried over as impurities into the final dye product. Subsidiary colors are positional isomers of the main dye component of FD&C Red No. 4 or are related dyes with higher or lower numbers of substituent groups. The most commonly-found subsidiary color is 3-[(2,4-dimethyl-6-sulfophenyl)azo]-4-hydroxy-1-naphthalenesulfonic acid (6SSC) (Figure 1). In order to be used for coloring products marketed in the U.S., FD&C Red No. 4 must be batch-certified by the U.S. Food and Drug Administration (FDA) to ensure compliance with limiting specifications described in the CFR.Specifications are "not more than 0.2%" for each of the two intermediates, AMBSA and HNSA, and "not more than 2%" for "subsidiary colors."[1]

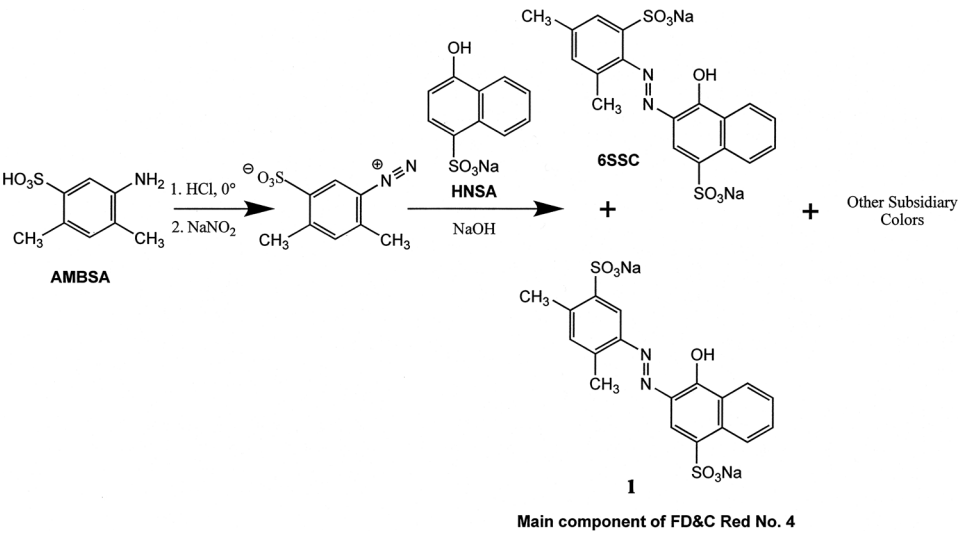

FIGURE 1 Preparation of FD&C Red No. 4 by diazotization of 5-amino-2,4-dimethyl-1-benzenesulfonic acid (AMBSA) followed by coupling the diazotized AMBSA with 4-hydroxy-1-naphthalenesulfonic acid (HNSA).

Prior to FDA implementation of the method reported here, the two intermediates were determined by a labor-intensive procedure that included separation by gravity-elution column chromatography and quantification by UV spectrophotometry.[3,4] Similarly, the subsidiary colors were determined by a multi-step procedure that involved streaking the dye solution on a semipreparative silica gel thin-layer chromatography (TLC) plate, developing and drying the plate, removing the subsidiary color band(s) by scraping, extracting the subsidiary color(s) from the silica gel, and, finally, quantifying by visible spectrophotometry.[3,4] In the quest to simplify and modernize the methods used to analyze components of color additives submitted for FDA certification, the present study reports the development of a rapid and reliable high-performance liquid chromatography (HPLC) method for the separation and quantification in one analysis of the CFR-specified intermediates and subsidiary colors in FD&C Red No. 4. The method involves purified AMBSA and HNSA as reference materials for quantification. Because the visible absorption spectra of the subsidiary colors found in FD&C Red No. 4 are closely related to the spectrum of 6SSC, the total amount of subsidiary colors is quantified by using purified 6SSC as a secondary reference material.

EXPERIMENTAL

Materials

The samples of R4 used in this study originated from commercial lots submitted to the FDA for certification during 2001–2007. n-Butanol (A.C.S.-reagent grade) and methanol and water (both HPLC-solvent grade) were from J.T. Baker (Phillipsburg, NJ, USA). Trifluoroacetic acid (≥98%) was from Fluka (Buchs, Switzerland) and ammonium acetate (HPLC grade) was from Fisher Scientific (Fair Lawn, NJ, USA). The 5-amino-2,4-dimethyl-1-benzenesulfonic acid (AMBSA) used as a reference material was obtained from FDA's Color Certification Branch (prepared by J.E. Bailey, 1975). Its purity (∼99%) was confirmed by elemental analysis (Complete Analysis Laboratories, Parsippany, NJ, USA) and HPLC at $\lambda = 240$ nm. The sodium salt of 4-hydroxy-1-naphthalenesulfonic acid (HNSA) (∼99% pure by elemental analysis and HPLC at $\lambda = 240$ nm) used as a reference material was obtained by purification of a commercially-available material of "Practical" grade (∼80% pure) using high-speed countercurrent chromatography.[5] The 3-[(2,4-dimethyl-6-sulfophenyl)azo]-4-hydroxy-1-naphthalenesulfonic acid (6SSC) (∼96.2% pure by HPLC at $\lambda = 254$ and 485 nm and by elemental analysis) used as a reference material was obtained by further purifying a material of ∼89–90% purity (Color

Certification Branch[6,7]) using high-speed countercurrent chromatography (HSCCC) as described in the following section.

Purification of 6SSC by High-Speed Countercurrent Chromatography

Instrumentation

The purification was performed with a J-type HSCCC system (Model CCC-1000, Pharma-Tech Research, Baltimore, MD, USA) that consisted of a column (three Ito multilayer coils connected in series, made of 1.6 mm I.D. Tefzel tubing with a total capacity of ~325 mL) mounted on a rotating frame, a rotation-speed controller, and a SSI Model 300 LC pump (Scientific Systems, State College, PA, USA). The column effluent was monitored with a UV detector, model UVicord SII, with a 275-nm UV lamp (Pharmacia LKB, Uppsala, Sweden) and a chart recorder (Kipp & Zonen, Delft, The Netherlands). The effluent was collected using a Foxy fraction collector (Isco, Lincoln, NE, USA).

Purification Procedure

The HSCCC purification was performed following the general directions described previously.[8,9] The two-phase solvent system used for purification of the sulfonated intermediate HNSA[5] was also appropriate for the purification of 6SSC. The system consisted of n-butanol-water (500 mL:500 mL) to which 2 mL of trifluoroacetic acid (0.2%, v/v) was added. The 6SSC (~89–90% pure) had a partition coefficient ($K_{\text{upper phase}}/K_{\text{lower phase}}$) of 1.29 in this solvent system. After equilibration in a separatory funnel, the two phases were separated before use, resulting in 550 mL of organic upper phase (UP) and 450 mL of aqueous lower phase (LP). The organic UP was used as the stationary phase and the aqueous LP was used as the mobile phase. The purification was initiated by filling the entire column with the stationary phase using the LC pump, and then loading the 6SSC sample (554 mg) dissolved in a mixture of UP and LP (9 mL:9 mL) using a syringe. The mobile phase was then pumped into the column at 2 mL/min while the column was rotated at 850 rpm in head-to-tail elution mode. The effluent was monitored with a UV detector at 275 nm (the recorder was set at 20 min/cm) and collected in fractions (4 mL/tube) using the fraction collector. The solvent front emerged at the 42nd tube, and the retention of the stationary phase was 36.9% after elution of 140 fractions. Based on HPLC analyses, fractions 93–102 and 103–114 were united into two respective 100-mL flasks and brought to dryness, resulting in 61.8 mg and 70.8 mg of 6SSC (free acid), ~95.0% and ~96.2% pure, respectively, based on HPLC at 254 and 485 nm and on elemental analysis.

Analytical HPLC

Analytical reversed-phase HPLC was performed with a Waters Alliance 2690 separation module (Waters, Milford, MA, USA). The eluents were (A) 0.1 M ammonium acetate in water/methanol (95:5, v/v) and (B) methanol. The column (Hypersil MOS-1, RPC-8 column, 5 μm particle size, 120 Å pore size, 250 × 4.6 mm I.D., Keystone Scientific, Bellefonte, PA, USA) was eluted by using a linear gradient of 10–100% of B in 30 min and 100% of B for 10 min. The column was re-equilibrated with 10% of B for 10 min. The effluent was monitored with a Waters 996 photodiode array detector set at 240 and 485 nm. Other conditions included: injection volume, 20 μL; column temperature, 25°C; and flow-rate, 1 mL/min.

Test solutions of R4 samples were prepared for HPLC analysis by dissolving approximately 100 mg of dye in a solution of water and methanol (7 mL:3 mL). A portion (~1.5 mL) of the test solution was filtered through a 0.45-μm pore-size syringeless AUTOVIAL 5 filter device (Whatman, Sanford, ME, USA) prior to chromatography.

Method Validation

Using the aforementioned reference materials, the amounts of AMBSA, HNSA, and 6SSC in test portions of R4 were determined using five-point calibration curves. The curves were obtained by an external-standard procedure that involved analyzing separate similar test portions of an R4 sample used as a matrix that was fortified with the analytes. Each determination was performed in duplicate (two different aliquots at each calibration point). The R4 sample used as the matrix (Sample 12 in Table 1) had been previously found by HPLC to contain very small amounts of the analytes. For the calculation of the calibration curves, the contribution of the matrix was subtracted. The data points (*w*/*w*) ranged from 0.028–0.234% for AMBSA, 0.031–0.252% for HNSA, and 0.113–2.036% for 6SSC. The instrument response was linear over these ranges (see Figure 2) and the limit of detection (LOD), based on the calibration data, was 0.020% for AMBSA, 0.015% for HNSA, and 0.138% for 6SSC. The total subsidiary color content of an R4 test sample was determined by adding the HPLC-generated peak areas of all the components that absorbed at 485 nm, except the main dye component, and then quantifying by using the calibration curve obtained at 485 nm for 6SSC. The precision of the method in terms of relative standard deviation (RSD), determined by analyzing five vials of the same sample solution (Sample 11 in Table 1), was 0.2% for AMBSA, 0.04% for HNSA, 0.00% for 6SSC, and 3.20% for total subsidiary colors.

TABLE 1 Intermediates and Subsidiary Colors (SC) Determined in Certified Lots of FD&C Red No. 4[a]

Sample No.	Manufacturer	AMBSA Found by HPLC[b] at 240 nm (%)	HNSA Found by HPLC[b] at 240 nm (%)	6SSC Found by HPLC[b] at 485 nm (%)	Total SC Found by HPLC[b] at 485 nm (%)
1[c]	AA1650	ND[d]	0.04	ND	0.94
2	A	0.02	0.10	ND	0.52
3	B	0.03	ND	ND	0.48
4	B	ND	ND	ND	0.23
5	C	ND	ND	ND	ND
6	C	ND	0.12	ND	0.32
7	C	ND	0.02	0.45	0.57
8	C	0.02	0.02	0.48	0.58
9	C	ND	0.06	0.52	0.68
10	D	0.04	ND	ND	0.43
11	D	0.04	0.06	ND	0.52
12	E	0.02	ND	ND	0.82
13	E	ND	0.07	ND	0.56
14	E	ND	ND	ND	1.06
15	E	ND	ND	ND	0.22
16	E	ND	ND	ND	0.29
17	F	ND	0.13	ND	0.74
18	F	ND	0.05	ND	0.29
19	F	0.02	ND	0.30	2.26
20	F	ND	0.05	0.51	0.87
21	F	ND	0.06	0.57	1.03
22	F	0.02	0.08	0.81	1.29
23	F	ND	0.11	ND	0.65

[a]Color lots certified during 2001–2007.
[b]Average of duplicate analyses.
[c]From toxicology test lot.
[d]Not detected (<0.020% AMBSA, <0.015% HNSA, <0.138% 6SSC).

RESULTS AND DISCUSSION

Chromatograms of the R4 sample used as the matrix (Sample 12 in Table 1) and the matrix fortified with 0.055% (*w*/*w*) AMBSA, 0.063% (*w*/*w*) HNSA, and 0.283% (*w*/*w*) 6SSC are shown in Figure 3. Table 1 shows the determined values of the intermediates AMBSA and HNSA, the subsidiary color 6SSC, and the total subsidiary color content of 23 certified lots of FD&C Red No. 4. These lots represent domestic and foreign manufacturers that requested certification for this color additive during the years 2001–2007. Figure 4 shows HPLC chromatograms of three test portions (Samples 9, 16, and 23 in Table 1) at 240 nm and 485 nm, the respective absorption wavelengths used to quantify the intermediates and subsidiary colors. The study includes one lot of R4 (Sample 1 in Table 1**,** "toxicology test lot") that was used in the animal-feeding studies upon which FDA based its safety evaluation for listing this color additive.

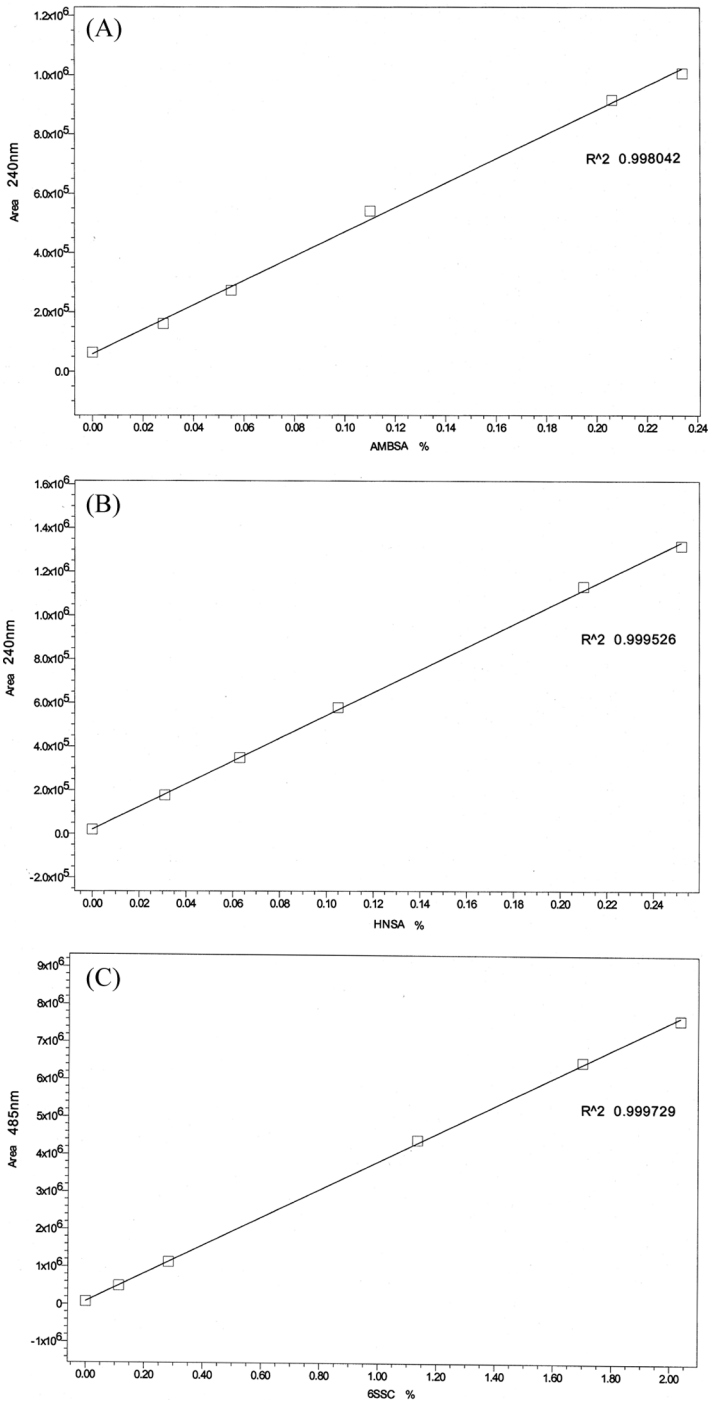

FIGURE 2 Standard calibration curves for quantitative determination of (A) AMBSA, (B) HNSA, and (C) 6SSC in the color additive FD&C Red No. 4 using HPLC.

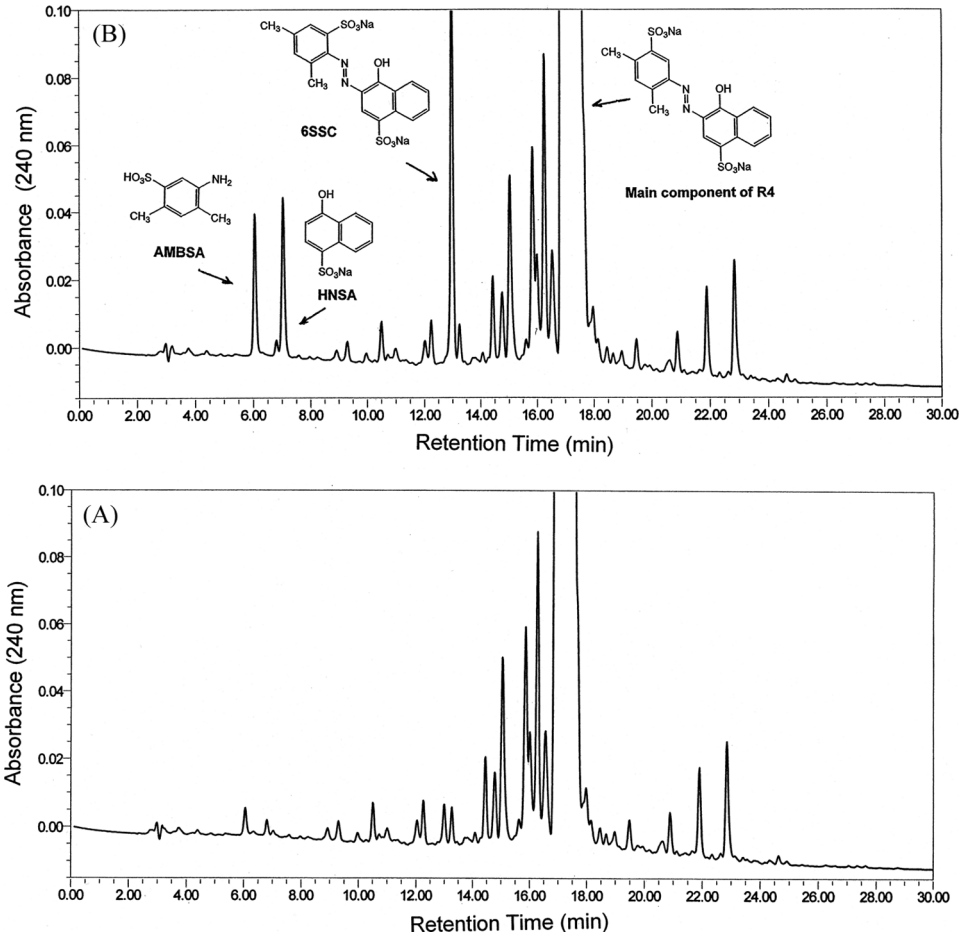

FIGURE 3 HPLC chromatograms of (A) FD&C Red No. 4 sample used as the matrix (Sample 12 in Table 1) and (B) FD&C Red No. 4 matrix fortified with 0.055% (*w*/*w*) AMBSA, 0.063% (*w*/*w*) HNSA, and 0.283% (*w*/*w*) 6SSC.

The results obtained by the newly-developed HPLC method for the three analytes (Table 1) were compared with those obtained by the previously-used combination of gravity-elution column chromatography/spectrophotometry and TLC/spectrophotometry methods (not shown). While there is overall agreement between the two sets of data, the high-resolution separation capability of the HPLC analysis yields more accurate results since it eliminates artificially-enhanced spectrophotometric readings caused by coelution of impurities that absorb in the same region. The new HPLC method has several other advantages over the other methods. Estimation of the total subsidiary color content is conveniently

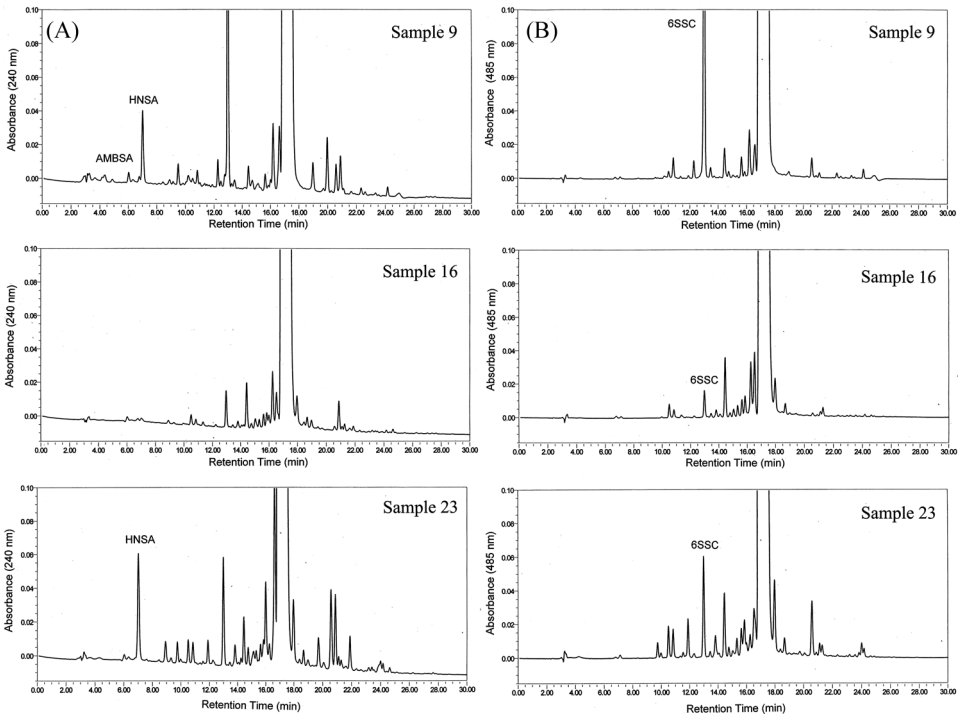

FIGURE 4 HPLC chromatograms of certified lots of FD&C Red No. 4 (Samples 9, 16, and 23, respectively, in Table 1) at (A) 240 nm and (B) 485 nm.

accomplished by using the calibration curve obtained for the subsidiary color 6SSC. The present method is much less labor-intensive, uses less solvent, and requires much less time for each analysis. The total time required to analyze both the intermediates and subsidiary colors in FD&C Red No. 4 is 30 min (or 50 min if the HPLC system is re-equilibrated for a second analysis). In contrast, the gravity-elution column chromatography/spectrophotometric method (for intermediates) combined with the TLC/spectrophotometric method (for subsidiary colors) requires up to 6 hr to analyze one test portion.

CONCLUSION

The HPLC method presented here for the determination of intermediates and subsidiary colors in FD&C Red No. 4 is applicable for use in routine batch-certification analysis. The method replaces the combined use of gravity-elution column chromatography/spectrophotometry and thin-layer chromatography/spectrophotometry, thus significantly reducing analysis

time. It is also more accurate, simpler to implement, and generates less waste solvent.

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