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Subsidiary colors in D&C Red No. 34 and its lakes: Synthesis, structural characterization, and analysis by ultra-performance liquid chromatography

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ABSTRACT

D&C Red No. 34 is the calcium salt of 3-hydroxy-4-[(1-sulfo-2-naphthalenyl)azo]-2-naphthalenecarboxylic acid. Its lakes are insoluble pigments formed by precipitating the dye anion onto an insoluble substratum, typically rosin. Subsidiary colors are colored impurities that are positional isomers of the dye anion or have higher or lower numbers of substituent groups. D&C Red No. 34 and its lakes are color additives listed in the U.S. Code of Federal Regulations (CFR) for use in externally applied drugs and cosmetics. These color additives are required to be batch certified by the U.S. Food and Drug Administration (FDA) to ensure compliance with the CFR requirements, including a limit of not more than four percent subsidiary colors in the straight color. There is no specified limit for subsidiary colors in the lakes, but the impurities may be analyzed for compliance with good manufacturing practices. In this paper, the syntheses of seven D&C Red No. 34 subsidiary colors are reported. Various combinations of impurities in the two intermediate starting materials for the dye, 2-amino-1-naphthalenesulfonic acid and 3-hydroxy-2-naphthalenecarboxylic acid, were used to synthesize the new compounds, which were purified by recrystallization and fully characterized by elemental analysis, ultra-performance liquid chromatography (UPLC), nuclear magnetic resonance spectroscopy, infrared spectroscopy, and visible spectrophotometry. The new compounds were used as reference materials in UPLC analyses of test samples from 33 certified lots of D&C Red No. 34 and its lakes. FDA is currently using these new reference materials for batch certification of the color additives.

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1. Introduction

D&C Red No. 34 is the calcium salt of 3-hydroxy-4-[(1-sulfo-2-naphthalenyl)azo]-2-naphthalenecarboxylic acid (R34, Colour Index (C.I.) 15,880:1, CAS No. 6417-83-0, C.I. Pigment Red 63:1). The R34 dye anion is prepared by a two-step reaction: diazotization of 2-amino-1-naphthalenesulfonic acid followed by an azo coupling reaction with 3-hydroxy-2-naphthalenecarboxylic acid, as shown in Fig. 1 (**1**) [1,2]. R34 lakes are insoluble pigments formed by extending the dye ("straight color") onto an insoluble substratum with a precipitating cation. Typically, rosin is used as the substratum and calcium chloride is used as the precipitant. The R34 straight color exhibits limited solubility in water so its lakes are prepared by synthesizing the dye anion "in situ" during the laking process [3,4].

R34 and its lakes are color additives listed in the U.S. Code of Federal Regulations (CFR) for use in externally applied drugs and cosmetics [5,6]. The U.S. Food and Drug Administration (FDA) routinely analyzes R34 and its lakes as part of its batch certification program. FDA batch certification ensures that these color additives comply with published specifications and other requirements, including a limit of not more than four percent subsidiary colors in the straight color [5]. There is no specified limit for subsidiary colors in R34 lakes, but the impurities may be analyzed for compliance with good manufacturing practices [6].

In commercially prepared R34 straight colors and lakes, unreacted intermediate starting materials and subsidiary colors may be present as impurities [1]. The unreacted intermediates result from incomplete diazotization of 2-amino-1-naphthalenesulfonic acid or an excess of 3-hydroxy-2-naphthalenecarboxylic acid. In

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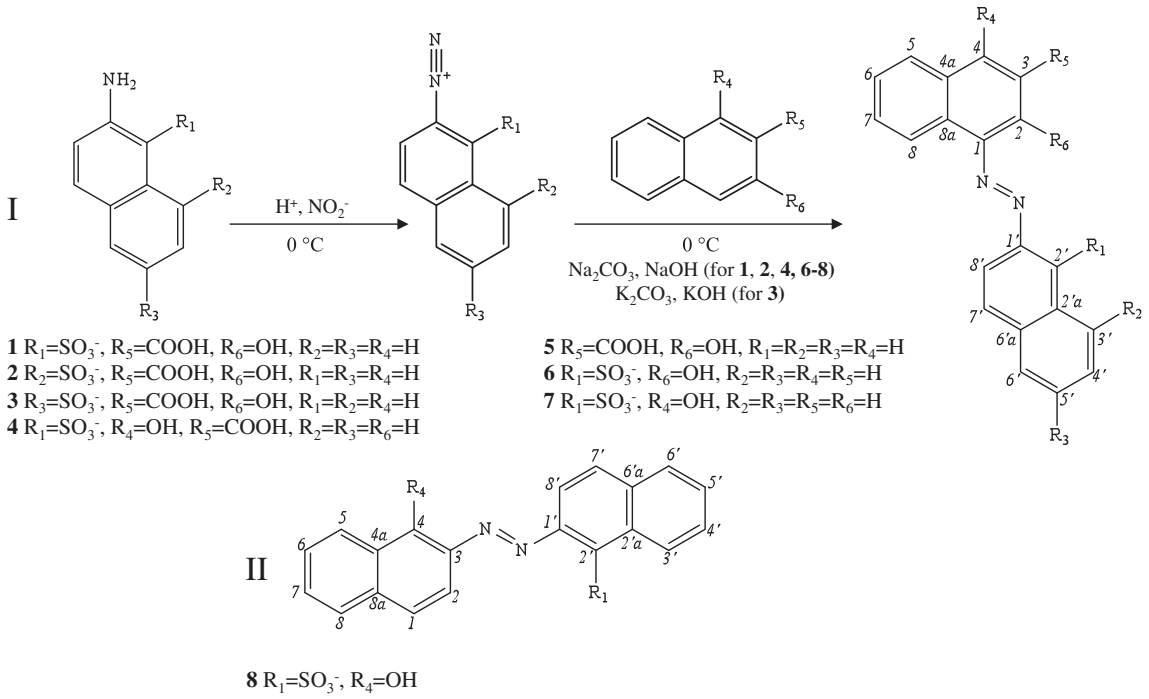

Fig. 1. (I) The structures, azo coupling reaction scheme, and ^{1}H and ^{13}C NMR chemical shift numbering for D&C Red No. 34 (**1**) and seven subsidiary colors (**2**–**7**). (II) The structure and chemical shift numbering for **8**.

general, subsidiary colors are positional isomers of the R34 dye anion or are related compounds containing greater or fewer substituent groups. Subsidiary colors arise due to side reactions of reactive impurities that may be present in either starting material. For example, monosulfonated reactive impurities such as 7-amino-1-naphthalenesulfonic acid and 6-amino-2-naphthalenesulfonic acid are commonly present in small amounts in 2-amino-1-naphthalenesulfonic acid due to sulfonation side reactions [7]. Likewise, reactive impurities such as α- or β-naphthol are commonly present in 3-hydroxy-2-naphthalenecarboxylic acid [8]. Subsidiary colors in R34 can form by two different pathways involving these reactive impurities. Either an impurity in 2-amino-1-naphthalenesulfonic acid can undergo diazotization and coupling with 3-hydroxy-2-naphthalenecarboxylic acid or an impurity in 3-hydroxy-2-naphthalenecarboxylic acid can undergo coupling with diazotized 2-amino-1-naphthalenesulfonic acid. The reactive impurities in both of the starting materials also can undergo diazotization and coupling reactions with each other. However, those subsidiary colors are generally not observed in R34 samples and, therefore, are not considered here.

The subsidiary colors reported in this paper are 3-hydroxy-4-[(8-sulfo-2-naphthalenyl)azo]-2-naphthalenecarboxylic acid (**2**), 3-hydroxy-4-[(6-sulfo-2-naphthalenyl)azo]-2-naphthalene carboxylic acid (**3**), 1-hydroxy-4-[(1-sulfo-2-naphthalenyl)azo]-2-naphthalenecarboxylic acid (**4**), 3-hydroxy-4-[(2-naphthalenyl) azo]-2-naphthalenecarboxylic acid (**5**), 2-[(2-hydroxy-1-naphthalenyl)azo]-1-naphthalenesulfonic acid (**6**), 2-[(4-hydroxy-1-napthalenyl)azo)]-1-naphthalenesulfonic acid (**7**), and 2-[(1-hydroxy-2-naphthalenyl)azo]-1-naphthalenesulfonic acid (**8**). Subsidiary color **6** (C.I. 15,630, CAS No. 1248-18-6) was formerly certifiable as D&C Red No. 10 (the sodium salt) and D&C Red No. 11 (the calcium salt). The R34 subsidiary color structures are shown Fig. 1, and Table 1 lists the starting materials used in their syntheses.

In the past, FDA determined the subsidiary colors in R34 straight colors by thin-layer chromatography (TLC). The TLC method could take up to 8 h to complete and did not completely separate the subsidiary colors from the main R34 color band. High-performance liquid chromatography (HPLC) [9–14] and more recently ultra-performance liquid chromatography (UPLC) [15] have been successfully applied to the determination of impurities in color additives and color additives in products. We recently reported a UPLC method for the determination of the intermediates and subsidiary colors in R34 and its lakes [16]. As part of the UPLC method development, it was essential to obtain purified reference materials for the subsidiary colors. Herein we report the synthesis, isolation, and purification of seven R34 subsidiary colors as well as the structural characterization of each compound by elemental analysis, nuclear magnetic resonance (NMR) spectroscopy, infrared

Table 1
D&C Red No. 34 subsidiary colors and impurities and intermediates used in their syntheses.

Compound	Impurity	Intermediate
3-hydroxy-4-[(8-sulfo-2- naphthalenyl)azo]-2- naphthalenecarboxylic acid (**2**)	7-amino-1-naphthalenesulfonic acid	3-hydroxy-2-naphthalenecarboxylic acid
3-hydroxy-4-[(6-sulfo-2- naphthalenyl)azo] -2- naphthalenecarboxylic acid (**3**)	6-amino-2-naphthalenesulfonic acid	3-hydroxy-2-naphthalenecarboxylic acid
1 -hydroxy-4-[(1 -sulfo-2- naphthalenyl)azo]-2- naphthalenecarboxylic acid (**4**)	1-hydroxy-2-naphthalenecarboxylic acid	2-amino-1-naphthalenesulfonic acid
3-hydroxy-4-[(2- naphthalenyl)azo]-2- naphthalenecarboxylic acid (**5**)	2-naphthylamine	3-hydroxy-2-naphthalenecarboxylic acid
2-[(2-hydroxy-1- naphthalenyl)azo] -1- naphthalenesulfonic acid (**6**)	2-naphthol	2-amino-1-naphthalenesulfonic acid
2-[(4-hydroxy-1- naphthalenyl)azo] -1- naphthalenesulfonic acid (**7**)	1-naphthol	2-amino-1-naphthalenesulfonic acid
2-[(1-hydroxy-2- naphthalenyl)azo] -1- naphthalenesulfonic acid (**8**)	1-hydroxy-2-naphthalenecarboxylic acid	2-amino-1-naphthalenesulfonic acid

(IR) spectroscopy, and visible spectrophotometry. We also report results from UPLC determinations of subsidiary colors in 33 certified lots of R34 dyes using the new reference materials.

2. Results and discussion

2.1. Synthesis of subsidiary colors

Seven subsidiary colors related to the R34 dye anion were synthesized from starting materials representative of impurities in either 2-amino-1-naphthalenesulfonic acid or 3-hydroxy-2-naphthalenecarboxylic acid. The compounds were prepared as sodium or potassium salts following the two-step sequence described in Fig. 1, **I**. In general, the compounds were obtained via diazotization of a naphthylamine derivative, and the resulting diazonium salt was coupled with a corresponding naphthol derivative: see Table 1. The sodium salt of R34 (R34-Na), compound **1**, was also synthesized for NMR and visible spectrophotometric characterization since R34 is insoluble in water.

Diazotization of 2-amino-1-naphthalenesulfonic acid followed by coupling with 3-hydroxy-2-naphthalenecarboxylic acid gave compound **1**. Diazotization of 7-amino-1-naphthalenesulfonic acid and 6-amino-2-naphthalenesulfonic acid followed by coupling with 3-hydroxy-2-naphthoic acid gave compounds **2** and **3**, respectively. Similarly, diazotization of 2-amino-1-naphthalenesulfonic acid followed by coupling with 2-naphthol gave compound **6**. All three subsidiary colors were obtained as pure compounds.

Compound **4** was produced when diazotized 2-amino-1-naphthalenesulfonic acid was reacted with 1-hydroxy-2-naphthoic acid. However, during the preparation of compound **4**, a trace amount of compound **8** was also formed (<2% determined by UPLC). In the ^{1}H NMR spectrum of the crude mixture, only the characteristic patterns of compound **4** were observed. However, after isolating compound **8** as a pure compound, its structure was elucidated by NMR. The results show that compound **8** is apparently produced in a side reaction by *ortho* coupling (at the alpha position relative to the hydroxyl group) with 1-hydroxy-2-naphthoic acid, resulting in a decarboxylated product. The preparation of compound **7**, from the reaction of diazotized 2-amino-1-naphthalenesulfonic acid with 1-naphthol, similarly resulted in a trace amount of compound **8** which is also produced as a result of an *ortho* coupling. Similar types of side reactions have been reported by Bourne et al. in the diazo coupling reaction of 1-naphthol and 4-aminobenzene sulfonic acid (sulfanilic acid) [17]. Bourne reported that the reaction of diazotized sulfanilic acid with 1-naphthol proceeded by two parallel reactions that gave primarily the monoazo dyes coupled at *para*- and *ortho*- positions and then, by subsequent secondary coupling reactions of the monoazo dyes, gave bisazo dyes. (Bisazo products were not detected by UPLC in compounds **4** and **7**).

Compound **5**, was not synthesized due to the toxicity of the starting material (2-naphthylamine) [18]. Instead, an archived batch that was a historically synthesized FDA sample was recrystallized and used as the reference material.

Compounds **1**–**8** were obtained in 45–97% yield and purified by recrystallizing from hot water. Compounds **1**, **2**, **4**, and **6**–**8** were isolated as sodium salts, while compound **3** was isolated as potassium salt because the sodium salt of **3** was very soluble and formed an oily residue, making it difficult to crystallize. Compound **5** was isolated with neutral charge, and the absence of any cations was confirmed by elemental analysis. The purity and chemical identity of the compounds were established by elemental analysis and UPLC. NMR and IR spectroscopy were used to confirm the structural identity of each compound.

2.2. NMR spectroscopy

Compounds **1**–**8** were initially characterized by ^{1}H and ^{13}C NMR spectroscopy. Two-dimensional NMR experiments including correlation spectroscopy (COSY), nuclear Overhauser effect spectroscopy (NOESY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) permitted complete assignments of the ^{1}H and ^{13}C NMR chemical shifts. The results are given in Tables 2 and 3.

For the most part, well-resolved ^{1}H multiplets and signal integrals were observed. COSY spectra were used to identify hydrogen spin systems, while NOESY spectra were used to establish the

Table 2
^{1}H NMR assignments for compounds **1**–**8**, chemical shifts δ (ppm) obtained in DMSO-d6, multiplicity, and coupling constants (^{3}JHH and ^{4}JHH in Hz).

Position	Chemical shifts, *multiplicity* (Coupling constants)							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1								7.02, *d* (9.5)
2				8.38, *s*			7.77, *d* (10.2)	7.12, *d* (9.5)
3						6.63, *d* (9.7)	6.77, *d* (10.2)	
4	8.68, *s*	8.63, *s*	8.66, *s*		8.49, *s*	7.82, *d* (9.7)		
5	7.90, *dd* (7.5,1.2)	7.95, *dd* (7.5, 1.2)	7.98, *dd* (7.7, 1.0)	8.33, *dd* (7.3, 1.3)	7.90, *dd* (7.9, 1.4)	7.65, *dd* (7.3, 1.2)	8.53, *dd* (7.8, 1.0)	8.33, *dd* (7.4, 1.0)
6	7.48, *td* (7.5, 1.0)	7.52, *td* (7.5, 1.0)	7.54, *td* (7.7, 1.0)	7.47, *td* (7.3, 1.2)	7.32, *ddd* (7.9, 6.9, 1.1)	7.41, *td* (7.3, 1.0)	7.76, *ddd* (7.8, 7.5, 1.4)	7.50, *td* (7.4, 1.0)
7	7.73, *ddd* (7.7, 7.5, 1.2)	7.73, *td* (7.5, 1.2)	7.77, *td* (7.7, 1.0)	7.63, *td* (7.3, 1.3)	7.55, *ddd* (8.3, 6.9, 1.4)	7.57, *td* (7.3, 1.2)	7.56, *ddd* (8.0, 7.5, 1.0)	7.70, *td* (7.4, 1.0)
8	8.43, *dd* (7.7, 1.0)	8.56, *dd* (7.5, 1.0)	8.61, *dd* (7.7, 1.0)	8.91, *dd* (7.3, 1.2)	8.80, *dd* (8.3, 1.1)	8.42, *dd* (7.3, 1.0)	8.05, *dd* (8.0, 1.4)	7.64, *dd* (7.4, 1.0)
2′		8.61, *d* (1.4)	8.21, d (1.4)		8.47, *d* (1.7)			
3′	9.06, *dd* (8.6, 1.2)		8.17, *d* (9.0)	9.31, *dd* (7.4, 1.5)	8.15, *dd* (6.8, 2.6)	9.10, *dd* (7.4, 1.0)	8.88, *dd* (8.6, 1.0)	9.10, *dd* (7.3, 1.0)
4′	7.57, *ddd* (8.6, 6.8, 1.6)	8.25, *dd* (7.7, 0.6)	8.16, *dd* (9.0, 1.8)	7.53, *ddd* (7.4, 6.8, 1.6)	7.60, *ddd* (6.8, 6.6, 2.7)	7.53, *ddd* (7.4, 6.8, 1.5)	7.51, *ddd* (8.6, 7.5, 1.5)	7.54, *ddd* (7.4, 7.3, 1.0)
5′	7.51, *ddd* (7.7, 6.8, 1.2)	7.71, *t* (7.7)		7.49, *ddd* (7, 6.8, 1.5)	7.59, *td* (6.6, 2.6)	7.46, *ddd* (7.0, 6.8, 1.0)	7.39, *ddd* (7.5, 7.3, 1.0)	7.46, *td* (7.4, 1.0)
6′	7.88, *dd* (7.7, 1.6)	7.97, *dd* (7.7, 0.6)	8.41, *d* (1.8)	7.89, *dd* (7, 1.6)	8.00, *dd* (6.6, 2.7)	7.87, *dd* (7.0, 1.5)	7.84, *dd* (7.3, 1.5)	7.86, *dd* (7.4, 1.0)
7′	8.05, *d* (9.2)	8.05, *d* (8.5)	8.02, *d* (8.5)	7.93, *d* (8.6)	8.06, *d* (8.8)	8.03, *d* (8.8)	7.99, *d* (9.1)	7.99, *d* (9.2)
8′	8.40, *d* (9.2)	7.88, *dd* (8.5, 1.4)	7.79, *dd* (8.5, 1.4)	7.33, *d* (8.6)	8.07, *dd* (8.8, 1.7)	8.41, *d* (8.8)	8.24, *d* (9.1)	8.30, *d* (9.2)

Table 3
^{13}C NMR assignments for compounds **1**–**8** and their chemical shifts δ (ppm) obtained in DMSO-d6.

Position	Chemical shifts							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1	130.07	130.68	129.57	135.84	131.80	129.90	135.60	121.60
2	165.80	165.60	165.56	118.62	157.72	177.13	124.96	129.17
3	123.66	124.85	124.66	109.81	123.37	126.94	128.48	130.90
4	148.17	145.31	145.34	170.52	134.54	141.53	183.84	176.60
4a	126.23	126.20	126.06	127.05	125.33	128.02	129.57	132.94
5	131.87	131.01	130.98	124.05	129.58	129.08	122.91	127.04
6	127.32	126.88	126.62	124.17	123.03	126.37	132.40	126.66
7	132.73	131.84	131.76	128.55	129.15	129.32	127.80	133.16
8	122.08	121.67	121.61	122.88	122.44	121.82	125.38	127.76
8a	135.87	134.28	134.50	134.15	130.86	133.94	131.89	137.00
C=O	175.58	171.47	171.60	171.58	170.27			
1′	136.57	146.97	146.44	149.05	151.53	137.33	136.75	136.59
2′	131.03	116.54	124.30	136.68	125.40	129.67	129.95	131.28
2′a	130.50	123.43	131.69	130.67	133.54	130.62	130.67	130.57
3′	128.73	138.56	130.78	128.73	129.21	128.46	127.26	128.36
4′	127.20	114.34	116.93	125.76	126.95	126.46	126.79	126.52
5′	126.54	127.19	141.00	125.28	127.17	125.19	124.18	125.18
6′	128.42	128.11	117.53	127.63	127.96	127.84	128.19	127.86
6′a	132.41	133.67	133.07	133.11	133.76	131.35	124.56	129.06
7′	132.16	128.84	127.98	129.90	129.15	131.15	131.67	131.09
8′	115.98	124.97	125.40	118.17	117.17	115.85	114.42	115.40

spatial proximities of adjacent spin systems. HSQC spectra identified ^{1}H and ^{13}C spin pairs, and HMBC spectra permitted assignment of all of the quaternary carbons. In this manner, all of the ^{1}H and ^{13}C chemical shifts could be assigned unambiguously, with the sole exception of the primed-ring carbon and hydrogen atoms of compound **3**. This is due to the pseudo-symmetry of the primed-ring hydrogen atoms (7′, 8′, 2′ and 3′, 4′, 6′; each 3-spin system has outer hydrogen atoms arrayed *ortho* and *meta* to the central hydrogen) and because the chemical shifts of the azo- and sulfonate-bearing carbon atoms are only 5.44 ppm apart. The ACD/NMR Predictor program (v.12.00 by Advanced Chemistry Development, Inc) suggested ^{13}C chemical shifts for the sulfonate- and azo-carbons of 143.82 and 149.59 ppm, respectively (5.77 apart) [19]. Thus, the observed shifts of 141.00 and 146.44 ppm were assigned to C-5′ and C-1′, respectively.

The ^{1}H, ^{13}C, and two-dimensional NMR characterization confirmed the accuracy of the predicted structures for all of the compounds. The NMR results show that compounds **2**–**8** are either positional isomers of the R34 dye anion or are missing one functional group. R34 has three functional groups, −OH at C2, $-COO^-$ at C3, and $-SO_3^-$ at C2′. Compounds **2**–**4** contain all three functional groups in different positions relative to R34 and, therefore, are isomers of R34. Compounds **5**–**8** each contain two of the three functional groups. All seven subsidiary colors contain an −OH group, compounds **2**, **3**, **5**, and **6** at C2 and compounds **4**, **7**, and **8** at C4. Compounds **2**–**4** contain a $-COO^-$ group at C3 and an $-SO_3^-$ group at C3′, C5′, and C2′, respectively. Compound **5** is missing an $-SO_3^-$ group and contains a $-COO^-$ group at C3. Compounds **6**, **7** and **8** are missing the $-COO^-$ group and contain $-SO_3^-$ groups at C2′. In compounds **1**–**7**, the azo group connects C1 and C1′, whereas in compound **8** the azo group connects C3 and C1′.

2.3. IR spectroscopy

Infrared spectroscopy is a widely used technique for the determination of molecular structure, particularly functional groups, and for the identification of compounds. IR spectra for R34-Na (**1**) and the seven subsidiary colors **2**–**8** were obtained and compared. The various characteristic frequencies were used to evaluate the extent of frequency shifts that might have occurred due to changes in the local environment of a functional group; see Table 4. The phenolic hydroxyl group gave rise to a broad and prominent band covering the region 3600–3200 cm^{-1}, which appeared to be hydrogen bonded. The aromatic = CH stretch vibrations were observed near 3100–3030 cm^{-1}. The IR spectrum in the range from 1700 to 700 cm^{-1} was relatively complex; the analytically useful group vibrations were intermixed with those characteristic of aromatic rings. The C=C stretch vibrations of the aromatic rings were observed at 1625–1600 cm^{-1}, 1598–1500 cm^{-1}, and 1490–1420 cm^{-1}. The aromatic ring = C–H in-plane bending and out-of-plane bending vibrations were observed in the regions 1200–1100 cm^{-1} and 905–870 cm^{-1}, respectively. The antisymmetric and symmetric stretch bands for the compounds with carboxylate salts **1**, **2**, **4** and **5** were located at 1580–1550 cm^{-1} and 1400–1390 cm^{-1}, respectively. In the IR spectrum observed for compound **3**, the conjugation of C=O in COOH with the aromatic ring was also observed at 1721 cm^{-1}. For compounds **1**–**4** and **6**–**8**, the sulfonic acid salts, SO_3^- stretching vibrations were observed in the range of 1260–1255 cm^{-1}. The naphthalene rings substitution patterns could also be examined. The vibration bands for four adjacent hydrogen and two adjacent hydrogen atoms were observed at 790–700 cm^{-1} and 860–800 cm^{-1}, respectively. The observed spectra were consistent with compounds **1**, **4**, and **5**–**8**

Table 4
IR frequencies for compounds **1**–**8**.[a]

Compounds	−OH	Aromatic ring = CH	O=C-O-	Aromatic ring C=C	R–SO3- salt	Aromatic ring in-plane H bending	Aromatic ring out-of-plane H bending	Two adjacent H in aromatic ring	Four adjacent H in aromatic ring
1	3455m		1568sh 1555m	1622m 1603m 1473s 1448s 1422m	1259m	1142m 1056m 1011m	878w	868w 834w 811w	774w 751w
2	3481m 3426m	3058w	1564s 1555s	1660sh 1618m 1598m 1492m 1450m	1258m	1199s 1124m 1050m 1003m	883w	869w 831m	752m 737w
3	3439m	3055w	1721m 1556s	1613m 1596m 1495s 1452m 1433m	1255m	1192s 1123m 1035s 1016m	895w 876w	813w	756w 743w
4	3441m	3066w	1576s	1622s 1603s 1576s 1528m 1502vs 1490sh 1444m 1426m	1258m	1175m 1161m 1072m 1046m 1028m	876w	824w 814w 798w	761w 751sh
5	3439m	3059w	1555s 1397m	1613m 1593m 1496s 1451sh 1442m 1422m		1162m 1146m 1014m	904w 875w	862w 814w	762sh 752w
6	3557m 3493m	3097w 3044w		1473s 1448m 1424m	1255m	1140m 1059m	871w	852w 812w 786w	775w
7	3439m	3064w		1620s 1606m 1597s 1579m 1502s 1488m 1477m 1442m 1412m	1260m	1186m 1168m 1161m 1126m 1057m 1047m 1018m	880w	852w 838w 812m	757m 748m
8	3447m	3051w		1480s 1464sh 1426m	1249m	1054m	879w	818w 792w	772w 747w

[a] IR band intensity: s, strong; m, medium; w, weak; sh, shoulder.

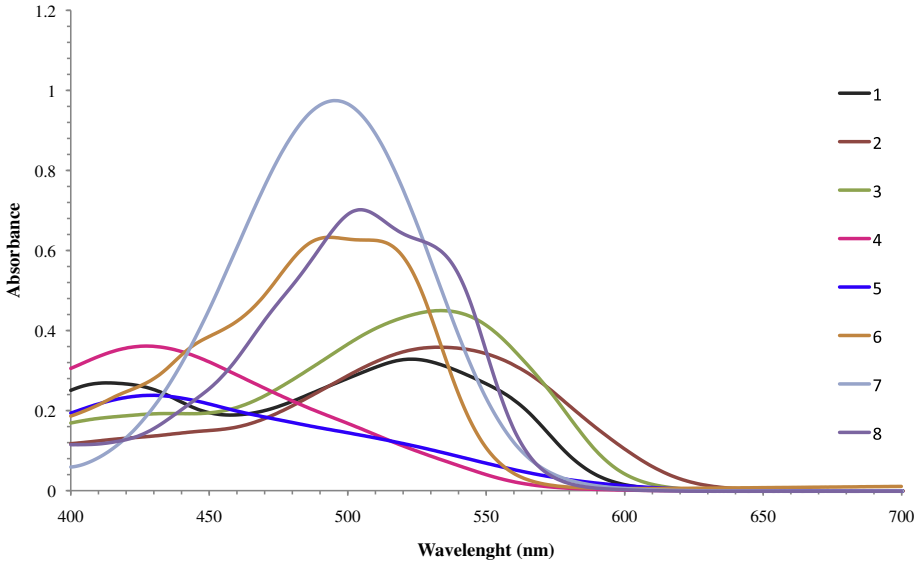

Fig. 2. Visible absorption spectra of observed for compounds **1**–**8** in DMSO.

having two sets of adjacent hydrogen atoms, in both primed and unprimed naphthalene rings, and compounds **2** and **3** having only one set of four adjacent hydrogen atoms in the unprimed naphthalene ring. All of these results are consistent with those obtained by NMR.

2.4. Visible spectrophotometry

The visible absorption spectra of compounds **1**–**8** in DMSO (0.02 mg/mL) are shown in Fig. 2 and selected spectral data are summarized in Table 5. The absorption spectra exhibit significant differences in absorptivity values and absorption maxima (λ_{max}) due to the differences in functional group substitution. Compounds **1**–**3** have a reddish color, compounds **4** and **5** have a yellowish color, and compounds **6**–**8** have an orange color. Compounds **1**–**3** each have a λ_{max} at 520–535 nm, compounds **6**–**8** each have a λ_{max} at 485–510 nm, and compounds **4** and **5** each have a λ_{max} at 420–430 nm. The absorptivity values for compounds **6**, **7**, and **8** are relatively highest, followed by those for compounds **1**–**4**. Compound **5** has the lowest absorptivity value (Table 6).

2.5. UPLC analyses

Survey UPLC analyses were conducted of samples from 33 certified lots of R34 straight colors and lakes using the seven subsidiary colors as reference materials. Thirty-two samples were from lots produced by five foreign and domestic manufacturers that were certified by FDA between 2005 and 2011. Sample 33 was from a lot produced by a domestic manufacturer that was used for toxicological testing in support of the color additive listing (the toxicology test lot). The results of these analyses are summarized in Table 5. Samples 4, 22, 29, 30, 31, and 33 are straight colors and were found to contain relatively higher amounts (0.598–2.97%) of the subsidiary colors compared to the R34 lakes, for which the subsidiary colors fell in the range 0.0805–1.97%. Compounds **3** and **6** were the most commonly found subsidiary colors and all samples contained both of those compounds except samples 2, 10, and 31. The three exceptions contained only one subsidiary color; sample 2 contained compound **3** while samples 2 and 31 each contained compound **6**. In addition, eleven of the samples contained compound **2**. Within these eleven, samples 27 and 32 also contained compounds **7** and **5**, respectively. Compound **4** was not found in any of the 33 samples. Compound **8** is a side reaction impurity in compounds **4** and **7** and as expected also was not found in any of the samples. Results for total subsidiary colors determined by UPLC in the straight colors are consistently lower than the results previously obtained by TLC (the lakes were not analyzed by TLC). The higher TLC results are attributed to the incomplete separation of the individual subsidiary colors from the main R34 color band and, therefore, we consider the UPLC results to be more accurate.

Table 5
Absorption maxima (λ_{max}) and absorptivity values (ε) for compounds **1**–**8** obtained in DMSO.

D&C Red No. 34 and subsidiary colors	λ_{max} (nm)	ε (L/mg•cm)
1	523	0.0319
2	533	0.0345
3	533	0.0420
4	427	0.0355
5	430	0.0240
6	493	0.0618
7	495	0.0957
8	504	0.0696

3. Experimental

3.1. Materials

All chemicals were used as purchased without further purification. 1-Hydroxy-2-naphthalenecarboxylic acid, 3-hydroxy-2-naphthalenecarboxylic acid, and 2-amino-1-naphthalenesulfonic acid were purchased from Aldrich Chemical Co. 2-Naphthol, 6-amino-2-naphthalenesufonic acid, and 7-amino-1-naphthalenesulfonic acid were purchased from Fluka, TCI America, and 3B Scientific Corporation, respectively. 1-Naphthol was purchased from TCI America. Water was purchased from Thermo-Fisher Scientific and methanol was purchased from J.T. Baker. Dimethyl sulfoxide (DMSO) and DMSO-d_6 were purchased from Thermo-Fisher Scientific and Isotec Inc., respectively. All solvents were HPLC grade. Samples of the D&C Red No. 34 lots analyzed in

Table 6
UPLC determinations of subsidiary colors in certified lots of D&C Red No. 34 straight colors and lakes using compounds **2**–**8** as reference materials.

Sample number	Manufacturer	Color additive[a]	Total subsidiary colors found (%)[b]	**2**	**3**	**5**	**6**	**7**
1	A	R34L	0.445		0.443		0.00201	
2	A	R34L	1.40		1.40			
3	B	R34L	0.437		0.435		0.00198	
4	C	R34	2.43		2.04		0.392	
5	A	R34L	1.35		1.28		0.0681	
6	A	R34L	1.47		1.40		0.0636	
7	B	R34L	0.692		0.382		0.310	
8	B	R34L	0.568		0.255		0.312	
9	B	R34L	0.343		0.0196		0.324	
10	B	R34L	0.289				0.289	
11	F	R34L	1.33		1.29		0.0427	
12	B	R34L	0.345	0.001710	0.0253		0.318	
13	B	R34L	0.484		0.360		0.0124	
14	A	R34L	0.803		0.764		0.0388	
15	A	R34L	1.58		1.55		0.0271	
16	A	R34L	1.54		1.48		0.0542	
17	A	R34L	1.77		1.69		0.0836	
18	A	R34L	1.77		1.68		0.0898	
19	A	R34L	1.80		1.71		0.0886	
20	B	R34L	0.980	0.0100	0.0674		0.902	
21	B	R34L	1.53		0.0552		1.47	
22	C	R34	2.97	0.110	1.89		0.971	
23	B	R34L	0.600	0.105	0.0582		0.437	
24	B	R34L	0.622	0.119	0.0537		0.449	
25	B	R34L	0.678	0.0963	0.0537		0.528	
26	B	R34L	0.687		0.369		0.318	
27	A	R34L	1.44	0.143	1.26		0.00777	0.0238
28	B	R34L	0.643	0.0795	0.0327		0.531	
29	C	R34	0.598	0.0514	0.169		0.377	
30	D	R34	2.40	0.0673	1.92		0.411	
31	E	R34	0.0805				0.0805	
32	G	R34L	0.796	0.0738	0.0265	0.204	0.492	
33	T[c]	R34	2.57		1.73		0.842	

[a] Lots certified during 2005–2011, R34 - straight colors and R34L – R34 lakes (67–81% straight color).
[b] All values are averages of duplicate analyses; note that compounds **4** and **8** were not found in any lots.
[c] Toxicology test lot.

this study were obtained from FDA's Office of Cosmetics and Colors. Galbraith Laboratories, Inc., performed the elemental analyses of compounds **1**–**8**.

3.2. Physical measurements

NMR solutions were prepared by dissolving 18–20 mg of each purified compound in 1 mL of DMSO-d_6. ^{1}H, ^{13}C, DEPT, COSY, NOESY, and HSQC spectra were recorded on a Bruker AV-400 spectrometer and HMBC spectra were recorded on a Bruker AV-600 spectrometer. Peak positions of the residual CD_2H signal (δ 2.50) and the $^{13}CD_3$ signal (δ 39.51) were used as secondary references relative to internal $(CH_3)_4Si$ at 0 ppm. Chemical shifts (δ) and coupling constants (J) are reported in parts per million (ppm) and hertz (Hz), respectively. Experiments were performed using standard Bruker software.

^{1}H NMR spectra were obtained with average spectral widths of 18 ppm; 32,768 experimental points (zero-filled to 65,536); 30° pulses; 2.28-s acquisition times; and 1.5-s relaxation delay times. ^{13}C and DEPT NMR spectra were acquired with average spectral widths of 240 ppm; 65,536 experimental points; 30° (^{13}C) and 135° (final DEPT) pulses; 1.37-s acquisition times; and 2-s relaxation delay times.

COSY NMR spectra were determined with average spectral widths of 4000 Hz in each domain and with 2048 data points in the F_2 dimension. ^{1}H pulse widths of 11.5 μs (90°) and 1.5-s relaxation delay times were used to acquire 256 incremented proton NMR spectra of 8 scans each. Free-induction decays were processed as 1024 × 1024 matrices with appropriate zero filling in t_1 and sine bell weighting. Phase-sensitive NOESY NMR spectra were determined using the same spectral windows and data points as for COSY. ^{1}H pulse widths of 11.5 μs (90°) and 2.5-s relaxation delay times were used to acquire 256 incremented proton NMR spectra of 28 scans each. Mixing times of 0.6 s were employed. Free-induction decays were processed as 1024 × 1024 matrices with appropriate zero filling in t_1 and squared sine bell weighting.

HSQC NMR spectra were obtained with average spectral widths of 24,000 and 4000 Hz in the carbon and proton dimensions, respectively, and with 2048 data points in the F_2 dimension; 256 incremented ^{1}H spectra of 24 scans each were acquired by using 11.5-μs (90°) ^{1}H pulse widths and 2.2-s relaxation delay times. Free-induction decays in both dimensions were processed as 2048 × 1024 matrices with appropriate zero filling in t_1 and squared sine bell weighting. The value $^1J(CH) = 145$ Hz was used for calculating the delay Δ, which is equal to 3.45 ms.

HMBC NMR spectra were recorded with average spectral widths of 14,000 and 2100 Hz in the carbon and proton dimensions, respectively, and with 2048 data points in the ^{1}H dimension; 256 incremented ^{1}H spectra of 40 scans each were acquired by using 10.3-μs (90°) ^{1}H pulse widths and 2.4-s relaxation delay times. Free-induction decays in both dimensions were processed as 2048 × 1024 matrices with appropriate zero filling in t_1 and sine bell weighting. The value $^nJ(CH) = 9$ Hz was used for calculating the delay Δ_{LR}, which is equal to *ca.* 55 ms, and $^1J(CH) = 155$ Hz was used for Δ in the J-filter.

Fourier transform infrared (FT-IR) spectroscopic measurements were carried out on an Agilent (formerly Varian) FTS 7000e IR spectrometer operating with Resolution Pro 4.0 software in the

transmission mode. The optical bench included a Michelson interferometer with an air bearing moving mirror, a potassium bromide substrate beam splitter, and a linearized mercury cadmium telluride (MCT) detector operating at liquid nitrogen temperature. The spectrometer performance met the following criteria: In the absence of a test sample, a 3 min data collection at 4 cm^{-1} resolution yielded between 2200 and 2000 cm^{-1} a peak-to-peak noise level <0.0005 absorbance unit (AU) for absorption spectra. Test samples (~2 mg) were measured as potassium bromide pellets (~200 mg). Test sample single-beam spectra were ratioed against that of an open beam (air). FTIR spectra were collected over the wavenumber range of 4000 cm^{-1}–700 cm^{-1} at a resolution of 4 cm^{-1}. To enhance the signal-to-noise ratio, 256 scans were co-added and signal averaged.

Visible absorption spectra were obtained with a double beam PerkinElmer Lambda 25 UV–visible spectrophotometer over a wavelength range 300–750 nm. The spectra were recorded in DMSO at room temperature (22 °C). The λ_{max} values are those selected by the spectrophotometer software.

UPLC analyses were conducted using the method described previously.[16] The subsidiary color peaks in the chromatograms were determined at 485 nm and identified from the retention times and photodiode array spectra. The subsidiary color(s) present in the samples were quantified by using a calibration curve generated by analyzing separate standard solutions spiked with the subsidiary color reference materials (**2**–**8**) prepared in the absence of the R34 matrix. The calibration solutions contained 0–2.01% by weight of each subsidiary color and the instrument response was linear over this range. The UPLC system consisted of an Acquity Separation Module, a photodiode array detector (monitored at 485 nm for the subsidiary colors), an Aquity UPLC BEH C18 column (50 × 2.1 mm id, 1.7 μm particle size), a guard column, and a column heater set at 25 °C (all from Waters Corp., Milton, MA). Eluant A was 20 mM aqueous ammonium acetate and eluant B was acetonitrile. The gradient consisted of linear segments of 0–25% B in 2.5 min, hold at 25% B for 5 min, 25%–35% B in 1.67 min, and hold at 35% B for 5 min. The column was re-equilibrated with 0% B for 5 min. The injection volume was 1 μL. The flow rate was 0.25 mL/min. The apparatus was controlled and the data were collected and analyzed using Empower 2 software.

3.3. Synthesis and characterization of compounds 1–8

3.3.1. 3-Hydroxy-4-[(1-sulfo-2-naphthalenyl)azo]-2-naphthalenecarboxylic acid (1)

2-Amino-1-naphthalenesulfonic acid (0.025 mol) was dissolved in 50 mL of water by adding 3 mL of 30% NaOH solution. Concentrated HCl (30 mL) was added to this solution, which was then cooled to ~0 °C. An aqueous solution of $NaNO_2$ (10 mL, 0.033 mol) was added dropwise. The mixture was vigorously stirred at ~0 °C for 30 min and checked for excess HNO_2 (I_2-starch test). An aqueous solution of 10% sulfamic acid (4 mL) was added to eliminate excess HNO_2. The light brown diazonium salt was filtered and repeatedly washed with ice water and the wet precipitate was immediately used in the following coupling reaction. 3-Hydroxy-2-naphthalenecarboxylic acid (0.025 mol) and sodium carbonate (0.027 mol) were added to a 150 mL solution of 2:1 water:ethanol (v/v) and cooled to ~0 °C. The wet diazonium salt was then added in small portions with vigorous stirring. A red solution formed followed by precipitation of the dye. The product was filtered, dried, and recrystallized twice from hot water.

Yield: 10.41 g (89%). Anal. Calc. for $Na_2C_{21}H_{12}O_6N_2S \cdot H_2O$: C 52.1, H 2.91, N 5.78, S 6.62. Found: C 53.5, H 2.81, N 6.02, S 6.73. λ_{max} (ε) in DMSO: 523 nm (0.0319 L mg^{-1} cm^{-1}).

3.3.2. 3-Hydroxy-4-[(8-sulfo-2-naphthalenyl)azo]-2-naphthalenecarboxylic acid (2)

7-Amino-1-naphthalenesulfonic acid (0.017 mol) was dissolved in 50 mL of water by adding 3 mL of 30% NaOH solution. Concentrated HCl (30 mL) was added to this solution, which was then cooled to ~0 °C. An aqueous solution of $NaNO_2$ (10 mL, 0.019 mol) was added dropwise. The mixture was vigorously stirred at ~0 °C for 30 min and checked for excess HNO_2 (I_2-starch test). An aqueous solution of 10% sulfamic acid (3 mL) was added to eliminate excess HNO_2. The light brown diazonium salt was filtered and repeatedly washed with ice water and the wet precipitate was immediately used in the following coupling reaction. 3-Hydroxy-2-naphthalenecarboxylic acid (0.017 mol) and sodium carbonate (0.028 mol) were added to a 150 mL solution of 2:1 water:ethanol (v/v) and cooled to ~0 °C. The wet diazonium salt was then added in small portions with vigorous stirring. A red solution formed followed by precipitation of the dye anion. The product was filtered, dried, and recrystallized twice from hot water.

Yield: 6.84 g (85%). Anal Calc. for $Na_2C_{21}H_{12}O_6N_2S$: C 54.08, H 2.59, N 6.01, S 6.88. Found: 54.01, H 2.45, N 5.98, S 6.88, Na 9.54. λ_{max} (ε) in DMSO: 533 nm (0.0345 L mg^{-1} cm^{-1}).

3.3.3. 3-Hydroxy-4-[(6-sulfo-2-naphthalenyl)azo]-2-naphthalenecarboxylic acid (3)

6-Amino-2-naphthalenesulfonic acid (0.025 mol) was dissolved in 50 mL of water by adding 2.16 g of KOH. Concentrated HCl (20 mL) was added to this solution, which was then cooled to ~0 °C. An aqueous solution of $NaNO_2$ (10 mL, 0.029 mol) was added dropwise. The mixture was vigorously stirred at ~0 °C for 30 min and checked for excess HNO_2 (I_2-starch test). An aqueous solution of 10% sulfamic acid (6 mL) was added to eliminate excess HNO_2. The light brown diazonium salt was filtered and repeatedly washed with ice water and the wet precipitate was immediately used in the following coupling reaction. 3-Hydroxy-2-naphthalenecarboxylic acid (0.024 mol) and potassium carbonate (0.035 mol) were added to a 150 mL solution of 2:1 water:ethanol (v/v) and cooled to ~0 °C. The wet diazonium salt was then added in small portions with vigorous stirring. A red solution formed immediately followed by precipitation of the dye anion. The product was filtered, dried, and recrystallized twice from hot water.

Yield: 10.0 g (87%). UV $\lambda_{max}(\varepsilon)$ in DMSO: 533 nm (0.0420 L mg^{-1} cm^{-1}).

Anal Calc for $KC_{21}H_{13}O_6N_2S$: C 52.82, H 2.95, N 5.87, S 6.72, K 8.19. Found: C 53.10, H 2.87, N 5.98, S 6.60, K 8.01.

3.3.4. 1-Hydroxy-4-[(1-sulfo-2-naphthalenyl)azo]-2-naphthalenecarboxylic acid (4)

2-Amino-1-naphthalenesulfonic acid (0.050) was dissolved in 100 mL of water by adding 6 mL of 30% NaOH solution. Concentrated HCl (30 mL) was added to this solution, which was then cooled to ~0 °C. An aqueous solution of $NaNO_2$ (10 mL, 0.061 mol) was added dropwise. The mixture was vigorously stirred at ~0 °C for 30 min and checked for excess HNO_2 (I_2-starch test). An aqueous solution of 10% sulfamic acid (8 mL) was added to eliminate excess HNO_2. The yellow diazonium salt was filtered and repeatedly washed with ice water and the wet precipitate was immediately used in the following coupling reaction. 1-Hydroxy-2-naphthalenecarboxylic acid (0.050 mol) and sodium carbonate (0.060 mol) were added to a 500 mL solution of 4:1 water:ethanol (v/v) and cooled to ~0 °C. The wet diazonium salt was then added in small portions with vigorous stirring. A red solution formed immediately followed by precipitation of the dye anion. The product was filtered, dried, and recrystallized twice from hot water.

Yield: 21.6 g (97%). UV $\lambda_{max}(\varepsilon)$ in DMSO: 427 nm (0.0355 L mg^{-1} cm^{-1}).

Anal Calc. for $Na_2C_{21}H_{12}O_6N_2S$: C 54.08, H 2.59, N 6.01, S 6.88. Found: C 51.37, H 2.76, N 5.78, S 6.85.

3.3.5. 3-Hydroxy-4-[(2-naphthalenyl)azo]-2-naphthalenecarboxylic acid (5)

A previously synthesized FDA sample (produced from 2-naphthylamine and 3-hydroxy-2-naphthalenecarboxylic acid as shown in Fig. 1) was recrystallized from hot water as follows. 2.05 g was dissolved in 3 L of hot water at pH 11 (adjusted with 30% NaOH solution). A precipitate formed immediately after the pH was adjusted to 7 with concentrated HCl. The precipitate was filtered, washed with water, and allowed to air-dry overnight.

Yield: 0.93 g (45%). UV $\lambda_{max}(\varepsilon)$ in DMSO: 430 nm (0.0240 L mg^{-1} cm^{-1}).

Anal Calc. for $C_{21}H_{14}O_3N_2$: C 73.67, H 4.12, N 8.18. Found: C 72.88, H 3.96, N 8.04.

3.3.6. 2-[(2-hydroxy-1-naphthalenyl)azo]-1-naphthalenesulfonic acid (6)

2-Amino-1-naphthalenesulfonic acid (0.025) was dissolved in 50 mL of water by adding 5 mL of 30% NaOH solution. Concentrated HCl (20 mL) was added to this solution, which was then cooled to ~0 °C. An aqueous solution of $NaNO_2$ (10 mL, 0.030 mol) was added dropwise. The mixture was vigorously stirred at ~0 °C for 30 min and checked for excess HNO_2 (I_2-starch test). An aqueous solution of 10% sulfamic acid (3 mL) was added to eliminate excess HNO_2. The yellow diazonium salt was filtered and repeatedly washed with ice-cold water and the wet precipitate was immediately used in the following coupling reaction. 2-naphthol (0.025 mol) and sodium carbonate (0.032 mol) were added to a 250 mL solution of 4:1 water:ethanol (v/v) and cooled to ~0 °C. The wet diazonium salt was then added in small portions with vigorous stirring. A red solution formed immediately followed by precipitation of the dye anion. The product was filtered, dried, and recrystallized twice from hot water.

Yield: 9.34 g (89%). UV $\lambda_{max}(\varepsilon)$ in DMSO: 493 nm (0.0618 L mg^{-1} cm^{-1}).

Anal Calc. for $NaC_{20}H_{15}O_5N_2S \cdot H_2O$: C 57.41, H 3.61, N 6.70, S 7.66. Found: C 56.64, H 3.66, N 6.69, S 7.81.

3.3.7. 2-[(4-hydroxy-1-naphthalenyl)azo]-1-naphthalenesulfonic acid (7)

2-Amino-1-naphthalenesulfonic acid (0.050) was dissolved in 150 mL of water by adding 6 mL of 30% NaOH solution. Concentrated HCl (30 mL) was added to this solution, which was then cooled to ~0 °C. An aqueous solution of $NaNO_2$ (10 mL, 0.051 mol) was added dropwise. The mixture was vigorously stirred at ~0 °C for 30 min and checked for excess HNO_2 (I_2-starch test). An aqueous solution of 10% sulfamic acid (4 mL) was added to eliminate excess HNO_2. The yellow diazonium salt was filtered and repeatedly washed with ice-cold water and the wet precipitate was immediately used in the following coupling reaction. 1-naphthol (0.049 mol) and sodium carbonate (0.074 mol) were added to a 250 mL solution of 4:1 water:-ethanol (v/v) and cooled to ~0 °C. The wet diazonium salt was then added in small portions with vigorous stirring. A red solution formed immediately followed by precipitation of the dye anion. The product was filtered, dried, and recrystallized twice from hot water.

Yield: 10.1 (50%). UV $\lambda_{max}(\varepsilon)$ in DMSO: 495 nm (0.0957 L mg^{-1} cm^{-1}).

Anal Calc. for $NaC_{20}H_{13}O_4N_2S$: C 60.00, H 3.27, N 7.00, S 8.01. Found: C 59.11, H 3.25, N 6.78, S 8.02.

3.3.8. 2-[(1-hydroxy-2-naphthalenyl)azo]-1-naphthalenesulfonic acid (8)

The compound was serendipitously isolated as a pure compound from the synthesis of compound **4**. 2-Amino-1-naphthalenesulfonic acid (0.029 mol) was added in 50 mL of water. Concentrated HCl (6.5 mL) was added to this mixture, which was cooled to ~0 °C. An aqueous solution of $NaNO_2$ (10 mL, 0.029 mol) was added dropwise. The mixture was vigorously stirred at ~0 °C for 30 min and checked for excess HNO_2 (I_2-starch test). An aqueous solution of 10% sulfamic acid (2 mL) was added to eliminate excess HNO_2. The solution containing the diazonium salt was kept cold for the subsequent coupling reaction. 1-Hydroxy-2-naphthoic acid (0.025 mol) and sodium carbonate (0.035 mol) were added to a cooled solution (~0 °C) of solution of 200 mL of water and 25 mL of 30% NaOH. The solution containing the yellow diazonium salt was then added dropwise to the cold 1-hydroxy-2-naphthalenecarboxylic acid solution with vigorous stirring. A red solution formed immediately followed by precipitation of the dye anion. The product was filtered, dried, and recrystallized twice from hot water.

Yield: 1.39 (12%). UV $\lambda_{max}(\varepsilon)$ in DMSO: 504 nm (0.0696 L mg^{-1} cm^{-1}).

Anal Calc. for $NaC_{20}H_{13}O_4N_2S$: C 60.00, H 3.27, N 7.00, S 8.01. Found: C 59.44, H 3.59, N 6.85, S 8.14.

4. Conclusion

Seven subsidiary colors related to the major dye component of R34 were successfully synthesized and characterized. The compounds were synthesized from various combinations of starting materials representative of impurities in either of the intermediate starting materials, 2-amino-1-naphthalenesulfonic acid and 3-hydroxy-2-naphthalenecarboxylic acid, using azo coupling reactions, and were purified by recrystallization. The compounds were fully characterized by elemental analysis, UPLC, NMR spectroscopy, IR spectroscopy, and visible spectrophotometry. They were used as reference materials in a UPLC survey of 33 certified lots of R34 and R34 lakes. FDA is currently using the new compounds as reference materials in the batch certification of R34 straight colors and lakes.

Appendix A. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.dyepig.2012.05.001.

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