Published in final edited form as:

J Chromatogr A. 2015 February 6; 0: 120–129. doi:10.1016/j.chroma.2014.12.072.

Preparative separation and identification of novel subsidiary colors of the color additive D&C Red No. 33 (Acid Red 33) using spiral high-speed counter-current chromatography[☆]

Adrian Weisz^{a,*}, Clark D. Ridge^b, Eugene P. Mazzola^b, and Yoichiro Ito^c

Adrian Weisz: adrian.weisz@fda.hhs.gov

^aOffice of Cosmetics and Colors, Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, College Park, MD 20740, USA

^bOffice of Regulatory Science, Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, College Park, MD 20740, USA

^cBioseparation Technology Laboratory, Biochemistry and Biophysics Center, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA

Abstract

Three low-level subsidiary color impurities (**A**, **B**, and **C**) often present in batches of the color additive D&C Red No. 33 (R33, Acid Red 33, Colour Index No. 17200) were separated from a portion of R33 by spiral high-speed counter-current chromatography (HSCCC). The separation involved use of a very polar solvent system, 1-BuOH/5 mM aq. (NH₄)₂SO₄. Addition of ammonium sulfate to the lower phase forced partition of the components into the upper phase, thereby eliminating the need to add a hydrophobic counterion as was previously required for separations of components from sulfonated dyes. The very polar solvent system used would not have been retained in a conventional multi-layer coil HSCCC instrument, but the spiral configuration enabled retention of the stationary phase, and thus, the separation was possible. A 1 g portion of R33 enriched in **A**, **B**, and **C** was separated using the upper phase of the solvent system as the mobile phase. The retention of the stationary phase was 38.1%, and the separation resulted in 4.8 mg of **A** of >90% purity, 18.3 mg of **B** of >85% purity, and 91 mg of **C** of 65–72% purity. A second separation of a portion of the **C** mixture resulted in 7 mg of **C** of >94% purity. The separated impurities were identified by high-resolution mass spectrometry and NMR spectroscopic techniques as follows: 5-amino-3-biphenyl-3-ylazo-4-hydroxy-naphthalene-2,7-disulfonic acid, **A**; 5-amino-4-hydroxy-6-phenyl-3-phenylazo-naphthalene-2,7-disulfonic acid, **B**; and 5-amino-4-hydroxy-3,6-bis-phenylazo-naphthalene-2,7-disulfonic acid, **C**. The isomers **A** and **B** are compounds reported for the first time. Application of the spiral HSCCC method resulted in the additional benefit of yielding 930 mg of the main component of R33, 5-amino-4-hydroxy-3-phenylazo-naphthalene-2,7-disulfonic acid, of >97% purity.

[☆]Presented in part at the 8th International Conference on Countercurrent Chromatography, London, United Kingdom, 23–25 July 2014.

*Corresponding author. Tel.: +1 2404021145; fax: +1 3014362961.

Keywords

D&C Red No. 33; Spiral high-speed counter-current chromatography; NMR spectra of dye impurities; 5-Amino-3-biphenyl-3-ylazo-4-hydroxy-naphthalene-2; 7-disulfonic acid; 5-Amino-4-hydroxy-6-phenyl-3-phenylazo-naphthalene-2; 7-disulfonic acid; 5-Amino-4-hydroxy-3; 6-bis-phenylazo-naphthalene-2; 7-disulfonic acid

1 Introduction

D&C Red No. 33 (Acid Red 33, Colour Index No. 17200) is a color additive permitted in the United States for coloring ingested drugs, externally applied drugs and cosmetics, mouthwashes, and dentifrices [1]. Under different names, the dye is also used in cosmetics in the European Union, Japan, and other countries [2]. It is manufactured by coupling diazotized aniline with 5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid (commonly known as H-acid) in alkaline conditions [1]. The reaction produces the main component, the disodium salt of 5-amino-4-hydroxy-3-(phenylazo)-2,7-naphthalenedisulfonic acid (CAS Reg. No. 3567-66-6, **1** in Fig. 1), accompanied by a host of synthetic by-products found in various amounts in the final product.

D&C Red No. 33 is batch-certified by the U.S. Food and Drug Administration (FDA) to ensure compliance with limits on levels of impurities specified in the Code of Federal Regulations (CFR) [1], such as not more than 0.3% for the intermediate H-acid and not more than 3% for the subsidiary color 4,5-dihydroxy-3-(phenylazo)-2,7-naphthalenedisulfonic acid disodium salt (commonly known as Chromotrope 2R). Several other unspecified impurities may occur in the dye that are detectable by reversed-phase high-performance liquid chromatography (HPLC). Some of these are more hydrophobic than the main component **1**, eluting as a group 6–7 min later, as indicated by the X region in the HPLC chromatogram shown in Fig. 2. In a previous study, this group of impurities was collected in a “purple-violet” fraction without being further researched because the aim of that work was to obtain purified **1** by preparative HPLC [3]. Similarly, the author of an even earlier study found “very small amounts of an unidentified blue color” in samples certifiable as D&C Red No. 33 [4]. These impurities are of interest because they are often observed in the HPLC chromatograms of batches of D&C Red No. 33 submitted for certification, and their identities are not known.

As part of FDA’s ongoing efforts to further describe the composition of synthetic dyes used as color additives in foods, drugs, cosmetics, and medical devices [5–8], the current study presents a method for the preparative separation of three hydrophobic components (**A**, **B**, and **C** in Fig. 2) from the main component **1** and from each other using spiral high-speed counter-current chromatography (HSCCC) [9]. The chemical identification and characterization of the separated components are also presented.

Conventional HSCCC [10–12] is a liquid–liquid partition technique in which one of the liquid phases (the stationary phase) is retained in the multi-layered coil column while the other liquid phase (the mobile phase) is pumped through the rotating column. Conventional HSCCC in both its standard elution mode and pH-zone-refining CCC elution mode [13] has

been applied successfully to the preparative separation and/or purification of components from various synthetic dyes such as those of the xanthene [14–19], triphenylmethane [20], quinoline [21,22], azo [23–25], pyrene [26], and indigo [24] types. The technique has also been used for purifying dye intermediates [27] and reaction by-products [28] and for separating components of natural colorants [29–34]. A drawback of the multi-layered coil column is its poor capacity for retaining polar solvent systems. Highly polar compounds, such as sulfonated dyes, partition mostly into the lower aqueous phase of commonly used organic/aqueous two-phase solvent systems. To facilitate partitioning of such compounds into the upper organic phase and thus their separation by HSCCC, a ligand (hydrophobic counterion) was added in previous studies [21,22,26]. Use of a ligand would not have been necessary if a more polar solvent system could have been used.

Recently, an alternative to the multi-layered coil HSCCC column was developed that is capable of retaining the stationary phase even of highly polar two-phase solvent systems [35–37]. That alternative consists of a spiral-tube assembly column in which the inert plastic tube is coiled into a rigid spiral frame [9]. This HSCCC variant, called spiral HSCCC, has been applied to the separation of peptides and proteins [9,38,39] and to the separation of gram quantities of a highly polar polysulfonated color additive without using a hydrophobic counterion in the stationary phase [40].

2 Experimental

2.1 Materials

The portion of D&C Red No. 33 used in this study originated from a sample of the test batch (ZB1034) used in the toxicology studies upon which the FDA based its safety evaluation of D&C Red No. 33. Methanol (Fisher Scientific, Fair Lawn, NJ, USA), ammonium acetate (NH_4OAc , Fisher), and water were of chromatography grade. Ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$, 99.0%, Sigma-Aldrich, St. Louis, MO, USA), 1-butanol (1-BuOH, 99.9%, Sigma-Aldrich), ethyl acetate (EtOAc , 99.8%, Sigma-Aldrich), and Chromotrope 2R (Sigma-Aldrich) were also used.

2.2 Spiral high-speed counter-current chromatography

2.2.1 Instrumentation—The separations were performed with a type-J planetary-motion centrifuge high-speed CCC system (Model CCC-1000, Pharma-Tech Research, Baltimore, MD, USA). The column was a set of three custom-designed spiral-tube assemblies (CC Biotech LLC, Rockville, MD, USA) connected in series around the rotary frame of the centrifuge at a distance of 7.5 cm from its central axis. Each spiral-tube assembly consisted of (i) a spiral-tube support (STS) rotor with four interwoven spiral grooves about 5 cm deep, connected in series through radial grooves, and (ii) a piece of polytetrafluoroethylene (PTFE) tubing of 1.6 mm i.d. (Zeus Industrial Products, Orangeburg, SC, USA) inserted into the spiral grooves. Photographs of the instrument and a similar STS rotor have been previously published [9,24].

Each of the three STS rotors (16 cm in diameter and 6 cm in length) was manufactured by the laser-sintering technique and contained approximately 12 spiral layers of PTFE tubing with a capacity of approximately 100 ml. Thus, the total column volume was approximately

300 ml. During the column preparation, the wound tubing was compressed several times with a special tool (CC Biotech LLC) with four protrusions that fit into the radial grooves. This process of tightening the tubing layers increased the number of spiral layers accommodated in the STS rotor. Each spiral tube assembly was then embedded in melted beeswax (Brushy Mountain Bee Farm, Moravian Falls, NC, USA) to protect the tubing from damage caused by centrifugal-force vibrations. The final step in forming the spiral HSCCC column was to connect the three spiral tube assemblies to each other through flow tubes of 0.85 mm ID PTFE tubing (SW 20, Zeus Industrial Products).

The instrument was connected to a pump (Waters 600F, Waters Corp., Milford, MA, USA) and a speed controller (Pharma-Tech) that regulated the rotation speed of the column. The following equipment was added to this system: (a) a right-angle flow-switching valve (Upchurch Scientific, Oak Harbor, WA, USA) to facilitate introduction of the stationary phase, sample solution, and mobile phase into the column without introducing air into the system; (b) a UV detector with 254-nm lamp (model Uvicord SII, Pharmacia LKB, Uppsala, Sweden); (c) a chart recorder (Kipp & Zonen, Delft, The Netherlands) for monitoring the effluent; and (d) a Foxy fraction collector (Isco, Lincoln, NE, USA).

2.2.2 Partition coefficient measurements—Two milliliters of 1-BuOH were placed in each of 4 test tubes to which were added 2 ml of 1 mM, 5 mM, 10 mM, and 20 mM aq. $(\text{NH}_4)_2\text{SO}_4$, respectively. 200 μl from a stock solution of 1.2 mg D&C Red No. 33/ml H_2O was then added to each of these two-phase solutions. The process was repeated with another set of 4 test tubes, except that instead of adding D&C Red No. 33, 200 μl from a stock solution of 1.2 mg Chromotrope 2R/ml H_2O was added. After mixing and centrifugation to separate the two phases in the test tubes, aliquots from the upper phase (UP) and lower phase (LP) of each test tube were analyzed by HPLC. The partition coefficients were obtained by dividing the respective HPLC peak areas obtained for the analytes **1**, Chromotrope 2R, and **C** (retention times 10.4 min, 11.7 min, and 17.6 min, respectively in Fig. 2) in the UP by those obtained for them in the LP. The results are summarized in Table 1.

2.2.3 Enrichment of the original dye sample in hydrophobic components—The study portion of D&C Red No. 33 was enriched in the hydrophobic components by liquid–liquid extraction using the two-phase solvent system 1-BuOH/5 mM aq. $(\text{NH}_4)_2\text{SO}_4$ (1:1). A 25.06 g portion of the dye was placed in an 1 l Erlenmeyer flask and dissolved by adding 800 ml of the LP and then agitating. The aqueous solution was transferred into a 2l separatory funnel and extracted with the UP (1×400 ml, then 3×250 ml). The dye was partitioned into the two phases by vigorous agitation. Due to the very dark color of the solution, the two phases in the separatory funnel were identified by illuminating with a powerful flashlight (Lightstar 300, TerraLUX, Dallas, TX, USA) from behind the separatory funnel. The two phases were separated and the UP extracts were combined. The extract solvent was removed by rotary evaporation (21 Torr, 55–60 °C), and the resulting dry product weighed 1.195 g. The percentages of the hydrophobic components in the initial and enriched samples are shown in Table 2.

2.2.4 Separation procedure

General procedure: In all of the separations performed, the two-phase solvent system was equilibrated in a separatory funnel, and the two phases were separated before use. The separation was initiated by filling the column with the stationary phase (LP for one set of separations and UP for another set of separations) using the pump. Next, the sample dissolved in a mixture of UP and LP was loaded using pressurized air or a syringe. The mobile phase was then pumped into the column while the column was rotated at ~700 rpm. The effluent was monitored at 254 nm and collected in fractions of 3 or 4 ml/tube. The attenuation of the recorder was set at 1 V or 2 V and the chart speed at 20 min/cm. The backpressure at the pump outlet during the separations was 120–150 psi. After the separation was completed, the column contents were collected using pressurized air, the volume of the stationary phase was measured, and the retention of the stationary phase was calculated.

2.2.4.1 Spiral HSCCC separation of the D&C Red No. 33 main component 1 from the hydrophobic components (Fig. 3): The solvent system consisted of 1-BuOH/5 mM aq. $(\text{NH}_4)_2\text{SO}_4$ (500 ml:500 ml). The LP was used as the stationary phase and the UP as the mobile phase in tail-to-head elution mode. The sample solution was prepared as follows: ~1.0 g of dye enriched in the hydrophobic components (see Section 2.2.3) was dissolved in 40 ml of LP and 10 ml of UP and loaded into the column using compressed air (80 psi). Other conditions included: flow rate, 2 ml/min; fraction volume, 4 ml; attenuation of the recorder, 1 V until elution of the solvent front (SF, fraction 41) and 2 V for the rest of the separation.

2.2.4.2 Spiral HSCCC separation of the D&C Red No. 33 hydrophobic components from each other (Fig. 4): The solvent system consisted of 1-BuOH/ H_2O (500 ml:500 ml). The UP was used as the stationary phase and the LP as the mobile phase in head-to-tail elution mode. The sample solution, ~0.5 g of pooled fractions containing the hydrophobic components (collected in the separation described in Section 2.2.4.1), was dissolved in 10 ml of LP and 10 ml of UP and loaded into the column by syringe. Other conditions included: flow rate, 2 ml/min; fraction volume, 3 ml; attenuation of the recorder, 2 V.

2.3 Analytical HPLC

HPLC analyses were performed with a Waters Alliance 2690 Separation Module (Waters, Milford, MA, USA). The eluents were 0.025 M aq. NH_4OAc and methanol. The column (MOS-1 Hypersil, 5 μm , 250 mm $\times$ 4.60 mm i.d., ThermoFisher Scientific, Waltham, MA, USA) was eluted isocratically at 10% methanol for 3 min, followed by two consecutive linear gradients of 10–75% methanol in 16 min and of 75–100% methanol in 1 min, respectively, then 100% methanol for 5 min. The effluent was monitored with a Waters 2998 photodiode array detector set at 254 nm. Other conditions included: flow-rate, 1 ml/min; column temperature, 25 $^\circ\text{C}$; injection volume, 20 μl .

An aliquot (~100–200 μl) from the spiral HSCCC collected fractions was diluted with 1 ml of methanol prior to HPLC analysis.

2.4 Liquid chromatography–mass spectrometry

High-resolution mass spectra (MS) of the spiral HSCCC-separated D&C Red No. 33 main component **1** and three impurities **A**, **B**, and **C** were obtained on an Agilent 6520 Q-TOF LC/MS system (Agilent Technologies, Santa Clara, CA, USA) equipped with Agilent MassHunter Workstation software for data acquisition and data analysis. The LC conditions were described in Section 2.3. The isolated compounds were dissolved in water (20 ng/μl) and analyzed in the positive electrospray ionization (ESI) mode. The high-resolution MS measurements of the quasi-molecular ions (M+H)⁺ of the four compounds are as follows: **1**, m/z 424.02811, which matches the calculated mass of 424.02677 for its protonated form, C₁₆H₁₃N₃O₇S₂; **A**, m/z 500.06026, which matches the calculated mass of 500.058621 for its protonated form, C₂₂H₁₇N₃O₇S₂; **B**, m/z 500.05942, which matches the calculated mass of 500.058621 for its protonated form, C₂₂H₁₇N₃O₇S₂; **C**, m/z 528.06571, which matches the calculated mass of 528.064769 for its protonated form, C₂₂H₁₇N₅O₇S₂.

2.5 Nuclear magnetic resonance spectroscopy

The ¹H NMR, ¹³C NMR, COSY, HSQC, and HMBC spectra of the D&C Red No. 33 main component **1** and the three impurities **A**, **B**, and **C** were determined using an Agilent NMR spectrometer operating at 600 MHz. Approximately 10 mg portions of each of the isolated compounds were dissolved in 600 μl of D₂O. The acquisition time in the directly detected dimension for all 2D spectra was 0.450 s per scan. A 0.7-s relaxation delay was used between all scans. The COSY spectra had a spectral width of 4800 Hz in each dimension and used 256 increments in the indirect dimension with 8 scans per increment for a total acquisition time of 41 min for each spectrum. The HSQC and HMBC spectra each had spectral widths of 9600 Hz in the ¹H dimension. The HSQC had a spectral width of 30,170 Hz in the ¹³C dimension while the HMBC had a wider 36,200 Hz ¹³C window. The HSQC used 128 increments with 4 scans for a total time of 21 min. The HMBC used 256 increments in the ¹³C dimension with 16 scans per increment for a total acquisition time of 2 h 50 min. All spectra were processed with appropriate zero filling and signal weighting. ¹³C and ¹H chemical shift data obtained, HMBC connectivities, and position assignments were tabulated for the four compounds as follows: **1**, 5-amino-4-hydroxy-3-phenylazo-naphthalene-2,7-disulfonic acid, Table S1S in the supplementary material and Fig. 5; **A**, 5-amino-3-bipheny-3-ylazo-4-hydroxy-naphthalene-2,7-disulfonic acid, Table S2S in the supplementary material and Fig. 6; **B**, 5-amino-4-hydroxy-6-phenyl-3-phenylazo-naphthalene-2,7-disulfonic acid, Table 3S in the supplementary material and Fig. 7; **C**, 5-amino-4-hydroxy-3,6-bis-phenylazo-naphthalene-2,7-disulfonic acid, Table S4S in the supplementary material and Fig. 8.

3 Results and discussion

The reversed-phase HPLC analysis of the D&C Red No. 33 sample chosen for the present work (Fig. 2a) shows that the main component **1** (RT ~10.4 min) is accompanied by various impurities including **A** (RT ~15.7 min), **B** (RT ~15.9 min), and **C** (RT ~17.6 min), which are the focus of this work.

Since the concentration levels of **A**, **B**, and **C** in the original sample were very small (0.22, 0.76, and 0.85%, respectively, Table 2), a preliminary step was taken to enrich the sample of D&C Red No. 33 in these components in order to obtain meaningful quantities for their identification. Sulfonated dyes such as D&C Red No. 33 partition almost exclusively in the lower aqueous phase of a conventional or even of a polar solvent system like 1-BuOH/H₂O. Addition of (NH₄)₂SO₄ was proposed [41] and implemented [40,42] in the use of spiral HSCCC to force partition of polar components into the upper organic phase by the salting-out process. Table 1 shows partition-coefficient measurements by HPLC at 530 nm of components of D&C Red No. 33 in the solvent system 1-BuOH/H₂O to which varying amounts of (NH₄)₂SO₄ were added. The components varied in polarity, with **1** being the most polar, Chromotrope 2R (RT ~11.7 min) of intermediate polarity, and **C** being the least polar of the analyzed components. At a concentration of 5 mM (NH₄)₂SO₄, component **C** has a partition coefficient of 1.1 (close to the ideal $K_{UP/LP} = 1$ for HSCCC separations), and almost 7 times more of it partitions into the upper phase of the solvent system than does **1** (Table 1). This solvent system (1-BuOH/5 mM aq. (NH₄)₂SO₄) was used to enrich the sample of D&C Red No. 33 in the hydrophobic components (Section 2.2.3). Fig. 2b shows the HPLC chromatogram of the D&C Red No. 33 sample enriched in the impurities **A**, **B**, and **C**. Table 2 shows the percentages of **A**, **B**, and **C** in the enriched D&C Red No. 33 sample as compared to the percentage of each in the original sample.

Since ~ 85% of the enriched sample was still the more polar main component **1**, our approach was to first separate it from the minor hydrophobic components **A**, **B**, and **C**, and then to separate them from each other. The method of choice was spiral HSCCC due to the high polarity of the solvent system 1-BuOH/5 mM aq. (NH₄)₂SO₄. Such polar solvent systems are not well-retained in the conventional multi-layered coil column in which the retention of the stationary phase almost entirely depends on the Archimedean screw effect. The spiral column assemblies can additionally use the radially acting centrifugal force to retain the stationary phase as was described when this new column design was introduced in the spiral-disk assembly format in 2003 [43] and in the improved spiral-tube assembly format in 2009 [36].

The chromatogram obtained for the separation of ~1 g of an enriched portion of D&C Red No. 33 by spiral HSCCC is shown in Fig. 3a. The organic upper phase of the solvent system 1-BuOH/5 mM aq. (NH₄)₂SO₄ was used as the mobile phase, and the aqueous lower phase was used as the stationary phase. Using the organic upper phase as the mobile phase insured that the hydrophobic components **A**, **B**, and **C** would elute before the polar main component **1**. Based on HPLC analyses, the eluates collected in the hatched areas in Fig. 3a contained the hydrophobic components. The pooled blue-colored fractions 72–142, 91 mg, contained mainly **C**, concentrated from ~7% in the enriched starting material to ~65–72% (by HPLC at 254 nm), accompanied by small amounts of **A** and **B** (Fig. 3b). The two magenta-colored fractions 145–146, 4.8 mg, contained **A** at >90% purity (Fig. 3c). The pooled lighter magenta-colored fractions 154–180, 18.3 mg, contained **B** at >85% purity (Fig. 3d).

The main component, **1**, eluted in the red-colored fractions 185–211 (at which point the separation was stopped) and was also found in the column contents, for a total of 930 mg at >97% purity (Figs. 3e and 5). The retention of the stationary phase (an indicator of the

resolution of the separation), calculated after the separation was completed, was 38.1%. It is noteworthy that in a similar separation using a multilayer-coil-equipped countercurrent chromatograph (not shown here), the retention of the stationary phase after the separation was less than half of that amount, at only 18.4%.

To obtain **C** at a higher purity, a portion of the mixture shown in Fig. 3b (~50 mg, also containing a small amount of $(\text{NH}_4)_2\text{SO}_4$ from the original separation) was subjected to a second separation by spiral HSCCC. Fig. 4a shows the chromatogram obtained for this separation for which the lower phase of the solvent system 1-BuOH/H₂O was used as the mobile phase. The aqueous phase was used as the mobile phase, so traces of $(\text{NH}_4)_2\text{SO}_4$ that were left in the sample from the original separation would elute at the solvent front. The retention of the stationary phase in this case was 60.0%. The hatched fractions 72–82, 7 mg, contained **C** at 92–94% purity (Fig. 4b).

The purity of the separated compounds was sufficient for their identification and characterization by HPLC, UV–visible spectrophotometry, high-resolution MS, and various ¹H and ¹³C NMR spectroscopic techniques. Details of the structural determinations and assignments of the 1D and 2D NMR spectra of **1**, **A**, **B**, and **C** can be found in the supplementary material associated with this article (Tables 1S–4S).

Impurity **A** was identified as the compound 5-amino-3-biphenyl-3-ylazo-4-hydroxy-naphthalene-2,7-disulfonic acid. Its structure, ¹H NMR spectrum and COSY assignments, and UV–visible spectrum are shown in Fig. 6. A possible pathway for **A**'s formation during the synthesis of the dye is the reaction of **H** acid with a minor amount of diazotized 3-aminobiphenyl instead of with diazotized aniline. While **A** is a newly identified compound, its 3-biphenyl-4-ylazo positional isomer has been previously identified as an impurity in Niagara blue 2B [44], a biological stain chemically related to D&C Red No. 33.

Impurity **B** was identified as the compound 5-amino-4-hydroxy-6-phenyl-3-phenylazo-naphthalene-2,7-disulfonic acid. Its structure, ¹H NMR spectrum and COSY assignments, and UV–visible spectrum are shown in Fig. 7. **B**, an isomer of **A** that is also a compound not reported previously, might be formed by arylation of **1** when a second molecule of diazotized aniline undergoes a Gomberg–Bachmann reaction under the alkaline conditions of the manufacturing of D&C Red No. 33 [45].

The blue impurity **C** was identified as the compound 5-amino-4-hydroxy-3,6-bis-phenylazo-naphthalene-2,7-disulfonic acid. Its structure, ¹H NMR spectrum and COSY assignments, and UV–vis spectrum are shown in Fig. 8. **C** is produced when a second molecule of diazotized aniline reacts with **H**-acid, and its preparation has been reported [46,47].

An important point to be noted is the discrepancy between part of the formal names of the four compounds and the nature of their “phenylazo” groups. Inspection of their structures reveals that at low to moderately basic pH values, the 3-phenylazo-4-hydroxy systems exist not as 4-hydroxy-3-phenylazo groups but rather as 4-keto-hydrazones. These groups exhibit a “hydrazone-azo/acid-base” equilibrium, and the phenylazo structures become predominant only at relatively high pH values [48,49]. However, the 6-phenylazo group of compound **C**

occurs as depicted in Fig. 8. This structural difference can be recognized by the considerable difference in NMR chemical shift values between C-3 (d152) and C-6 (d129) of compound C.

4 Conclusions

A spiral HSCCC method was developed for the preparative separation of three unidentified hydrophobic impurities (**A**, **B**, and **C**) often observed in samples of the color additive D&C Red No. 33 submitted to FDA for batch certification. The separated compounds were characterized by high-resolution mass spectrometry and NMR spectroscopic techniques and found to be subsidiary colors of the dye. Their identities are as follows: 5-amino-3-biphenyl-3-ylazo-4-hydroxy-naphthalene-2,7-disulfonic acid, **A**; 5-amino-4-hydroxy-6-phenyl-3-phenylazo-naphthalene-2,7-disulfonic acid, **B**; and 5-amino-4-hydroxy-3,6-bis-phenylazo-naphthalene-2,7-disulfonic acid, **C**. The two isomers **A** and **B** are compounds reported for the first time. Application of the spiral HSCCC method resulted in the additional benefit of purifying the main component of D&C Red No. 33, 5-amino-4-hydroxy-3-phenylazo-naphthalene-2,7-disulfonic acid, to >97% purity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Code of Federal Regulations, 21 CFR 74.1333, 74.2333, D&C Red No. 33. U.S. Government Printing Office; Washington, DC: 2013.
2. Rosholt, AP., editor. CTFA International Color Handbook. 4. The Cosmetic, Toiletry, and Fragrance Association; Washington, DC: 2007. p. 698-699.
3. Bailey JE Jr. Purification of D&C Red No. 33 by preparative high performance liquid chromatography. *J Chromatogr.* 1984; 314:379-385. [PubMed: 6526888]
4. Sclar RN. Studies on coal-tar colors. XIII. D&C Red No. 33. *J Assoc Off Agric Chem.* 1953; 36:930-936.
5. Andrzejewski D, Weisz A. Rapid quantification of hexachlorobenzene in the color additives D&C Red Nos. 27 and 28 (phloxine B) using solid-phase microextraction and gas chromatography-mass spectrometry. *J Chromatogr A.* 1999; 863:37-46. [PubMed: 10591462]
6. Weisz A, Andrzejewski D. Identification of 2-bromo-3,4,5,6-tetrachloroaniline and its quantification in the color additives D&C Red Nos. 27 and 28 (phloxine B) using solid-phase microextraction and gas chromatography-mass spectrometry. *J Chromatogr A.* 2003; 1005:143-153. [PubMed: 12924789]
7. Weisz A, Andrzejewski D, Rasooly IR. Determination of 2,4,6-tribromoaniline in the color additives D&C Red Nos. 21 and 22 (Eosin Y) using solid-phase microextraction and gas chromatography-mass spectrometry. *J Chromatogr A.* 2004; 1057:185-191. [PubMed: 15584238]
8. Weisz A, Wright PR, Andrzejewski D, Meyers MB, Glaze K, Mazzola EP. Identification of the decarboxylated analog of tetrabromotetrachloro-fluorescein and its quantification in the color additives D&C Red Nos. 27 and 28 (phloxine B) using high-performance liquid chromatography. *J Chromatogr A.* 2006; 1113:186-190. [PubMed: 16494889]
9. Ito Y, Knight M, Finn TM. Spiral countercurrent chromatography. *J Chromatogr Sci.* 2013; 51:726-738. [PubMed: 23833207]
10. Ito, Y. Principle, apparatus, and methodology of high-speed countercurrent chromatography. In: Ito, Y.; Conway, WD., editors. *High-Speed Countercurrent Chromatography. Chemical Analysis.* Vol. 132. Wiley; New York, NY: 1996. p. 3-44.

11. Ito Y. Golden rules and pitfalls in selecting optimum conditions for high-speed counter-current chromatography. *J Chromatogr A*. 2005; 1065:145–168. [PubMed: 15782961]
12. Conway, WD. Countercurrent Chromatography: Apparatus, Theory & Applications. VCH; New York: 1990.
13. Ito Y. pH-Zone-refining counter-current chromatography: Origin, mechanism, procedure and applications. *J Chromatogr A*. 2013; 1271:71–85. [PubMed: 23219480]
14. Weisz A, Andrzejewski D, Highet RJ, Ito Y. Preparative separation of components of the color additive FD&C Red No. 3 (erythrosine) by pH-zone-refining counter-current chromatography. *J Chromatogr A*. 1994; 658:505–510.
15. Weisz A, Andrzejewski D, Ito Y. Preparative separation of components of the color additive D&C Red No. 28 (phloxine B) by pH-zone-refining counter-current chromatography. *J Chromatogr A*. 1994; 678:77–84. [PubMed: 7921192]
16. Weisz A, Scher AL, Shinomiya K, Fales HM, Ito Y. A new preparative-scale purification technique: pH-zone-refining countercurrent chromatography. *J Am Chem Soc*. 1994; 116:704–708.
17. Weisz, A.; Andrzejewski, D.; Shinomiya, K.; Ito, Y. Preparative separation of components of commercial 4,5,6,7-tetrachlorofluorescein by pH-zone-refining countercurrent chromatography. In: Conway, WD.; Petroski, RJ., editors. *Modern Countercurrent Chromatography*, ACS Symposium Series. Vol. 593. American Chemical Society; Washington, DC: 1995. p. 203-217.
18. Weisz, A. Separation and purification of dyes by conventional high-speed countercurrent chromatography and pH-zone-refining countercurrent chromatography. In: Ito, Y.; Conway, WD., editors. *High-Speed Countercurrent Chromatography*. Chemical Analysis. Vol. 132. Wiley; New York, NY: 1996. p. 337p. 384
19. Oka H, Suzuki M, Harada KI, Iwaya M, Fujii K, Goto T, Ito Y, Matsumoto H, Ito Y. Purification of food color Red No. 106 (acid red) using pH-zone-refining counter-current chromatography. *J Chromatogr A*. 2002; 946:157–162. [PubMed: 11873964]
20. Fales HM, Pannel LK, Sokoloski EA, Carmeci P. Separation of Methyl Violet 2B by high-speed countercurrent chromatography and identification by californium-252 plasma desorption mass spectrometry. *Anal Chem*. 1985; 57:376–378.
21. Weisz A, Mazzola EP, Matusik JE, Ito Y. Preparative separation of isomeric 2-(2-quinolinyl)-1H-indene-1, 3(2H)-dione monosulfonic acids of the color additive D&C Yellow No. 10 (Quinoline Yellow) by pH-zone-refining counter-current chromatography. *J Chromatogr A*. 2001; 923(1–2): 87–96. [PubMed: 11510564]
22. Weisz A, Mazzola EP, Ito Y. Preparative separation of di- and trisulfonated components of Quinoline Yellow using affinity-ligand pH-zone-refining counter-current chromatography. *J Chromatogr A*. 2009; 1216:4161–4168. [PubMed: 19281993]
23. Freeman HS, Hao Z, McIntosh SA, Mills KP. Purification of some water soluble azo dyes by high-speed countercurrent chromatography. *J Liq Chromatogr*. 1988; 11:251–266.
24. Weisz A, Ito Y. Performance comparison of three types of high-speed counter-current chromatographs for the separation of components of hydrophilic and hydrophobic color additives. *J Chromatogr A*. 2011; 1218:6156–6164. [PubMed: 21215406]
25. Vu NT, Rickard JD, Sullivan MP, Richfield-Fratz N, Weisz A. Determination of intermediates and subsidiary colors in the color additive FD&C Red No. 4 (Ponceau SX) using high-performance liquid chromatography. *J Liq Chromatogr Relat Technol*. 2011; 34:106–115.
26. Weisz A, Mazzola EP, Ito Y. Preparative separation of 1, 3,6-pyrenetrisulfonic acid trisodium salt from the color additive D&C Green No. 8 (pyranine) by pH-zone-refining counter-current chromatography. *J Chromatogr A*. 2011; 1218:8249–8254. [PubMed: 21982993]
27. Weisz A, Ito Y. Preparative purification of 4-hydroxy-1-naphthalenesulfonic acid sodium salt by high-speed counter-current chromatography. *J Chromatogr A*. 2008; 1198–1199:232–234. [PubMed: 22422014]
28. Weisz A, Mazzola EP, Murphy CM, Ito Y. Preparative separation of isomeric sulfophthalic acids by conventional and pH-zone-refining counter-current chromatography. *J Chromatogr A*. 2002; 966:111–118. [PubMed: 12214685]

29. Oka, H.; Ikai, Y.; Yamada, S.; Hayakawa, J.; Harada, K-I.; Suzuki, M.; Nakazawa, H.; Ito, Y. Separation of Gardenia Yellow components by high-speed countercurrent chromatography. In: Conway, WD.; Petroski, RJ., editors. *Modern Countercurrent Chromatography*, ACS Symposium Series. Vol. 593. American Chemical Society; Washington, DC: 1995. p. 92-106.
30. Degenhardt, A.; Knapp, H.; Winterhalter, P. Separation of natural food colorants by high-speed countercurrent chromatography. In: Ames, JM.; Hofmann, T., editors. *Chemistry and Physiology of Selected Food Colorants*, ACS Symposium Series. Vol. 775. American Chemical Society; Washington, DC: 2001. p. 22-42.
31. Schwartz M, Hillebrand S, Habben S, Degenhardt A, Winterhalter P. Application of high-speed countercurrent chromatography to the large-scale isolation of anthocyanins. *Biochem Eng J*. 2003; 14:179–189.
32. Vidal S, Francis L, Noble A, Kwiatkowski M, Cheynier V, Waters E. Taste and mouth-feel properties of different types of tannin-like polyphenolic compounds and anthocyanins in wine. *Anal Chim Acta*. 2004; 513:57–65.
33. Aman R, Carle R, Conrad J, Beifuss U, Schieber A. Isolation of carotenoids from plant materials and dietary supplements by high-speed counter-current chromatography. *J Chromatogr A*. 2005; 1074:99–105. [PubMed: 15941044]
34. Winterhalter P. Application of countercurrent chromatography (CCC) to the analysis of natural pigments. *Trends Food Sci Technol*. 2007; 18:507–513.
35. Ito Y, Clary R, Powell J, Knight M, Finn TM. Spiral tube support for high-speed countercurrent chromatography. *J Liq Chromatogr Relat Technol*. 2008; 31:1346–1357. [PubMed: 19343107]
36. Ito Y, Clary R, Powell J, Knight M, Finn TM. Improved spiral tube assembly for high-speed counter-current chromatography. *J Chromatogr A*. 2009; 1216:4193–4200. [PubMed: 19062024]
37. Cao X, Pei H, Huo L, Hu G, Ito Y. Development and evaluation of a spiral tube column for counter-current chromatography. *J Sep Sci*. 2011; 34:2611–2617. [PubMed: 21898798]
38. Knight M, Finn TM, Zehmer J, Clayton A, Pilon A. Spiral counter-current chromatography of small molecules, peptides and proteins using the spiral tubing support rotor. *J Chromatogr A*. 2011; 1218:6148–6155. [PubMed: 21741049]
39. Baldermann S, Mulyadi AN, Yang Z, Murata A, Fleischmann P, Winterhalter P, Knight M, Finn TM, Watanabe N. Application of centrifugal precipitation chromatography and high-speed counter-current chromatography equipped with a spiral tubing support rotor for the isolation and partial characterization of carotenoid cleavage-like enzymes in *Enteromorpha compressa* (L.) Nees. *J Sep Sci*. 2011; 34:2759–2764. [PubMed: 21898817]
40. Weisz A, Ridge CD, Roque JA, Mazzola EP, Ito Y. Preparative separation of two subsidiary colors of FD&C Yellow No. 5 (Tartrazine) using spiral high-speed counter-current chromatography. *J Chromatogr A*. 2014; 1343:91–100. [PubMed: 24755184]
41. Zeng Y, Liu G, Ma Y, Chen X, Ito Y. Organic-high ionic strength aqueous two-phase solvent system series for separation of ultra-polar compounds by spiral counter-current chromatography. *J Chromatogr A*. 2011; 1218:8715–8717. [PubMed: 22033108]
42. Zeng Y, Liu G, Ma Y, Chen X, Ito Y. Organic-high ionic strength aqueous solvent systems for spiral counter-current chromatography: graphic optimization of partition coefficient. *J Liq Chromatogr Relat Technol*. 2013; 36:504–512. [PubMed: 23467197]
43. Ito Y, Yang F, Fitze PE, Sullivan JV. Spiral disk assembly for HSCCC: column design and basic studies on chromatographic resolution and stationary phase retention. *J Liq Chromatogr Relat Technol*. 2003; 26:1355–1372.
44. Lloyd JB, Field FE. Red impurity in trypan blue. *Experientia*. 1970; 26:868–869. [PubMed: 5452020]
45. Smith, MB.; March, J. *March's Advanced Organic Chemistry*. Wiley; 2001.
46. Morris RJ, Jensen FR, Lusebrink TR. The relation between the absorption spectra and the chemical constitution of dyes. Some effects of polar groups, insulating links and steric noncoplanarity on the absorption spectra of unsym. trisazo benzidine dyes. *J Org Chem*. 1954; 19:1306–1315.
47. Bulearca M, Sebe I. Reactive dyes with α,β -chlorohydrinic structure in the molecule (II). *Rev Chim (Bucharest, Romania)*. 2009; 60:820–825.

48. Bell SJ, Mazzola EP, Coxon B. Nitrogen-15 NMR investigation of azo-hydrazone acid-base equilibria of FD + C Yellow No. 5 (tartrazine) and two analogs. *Dyes & Pigments*. 1989; 11:93–99.
49. Mazzola EP, Bell SJ, Turujman SA, Barrows JN, Bailey JE Jr, McMahon JB, Germann TK, Reynolds WF. Nuclear magnetic resonance investigations of azo-hydrazone, acid-base equilibria of FD&C yellow no 5 (tartrazine). *Can J Chem*. 1999; 77:1941–1945.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.chroma.2014.12.072>.

Highlights

- Minor components of D&C Red No. 33 enriched by biphasic extraction.
- Spiral HSCCC separation of novel subsidiary colors.
- Identification by MS and NMR techniques of each component.

Fig. 1.
Preparation of D&C Red No. 33 by coupling diazotized aniline with H-acid in alkaline conditions.

Fig. 2. HPLC analyses of (a) a portion of D&C Red No. 33 containing CFR-specified (H-acid and Chromotrope 2R) and unspecified (**A**, **B**, and **C**) impurities, and (b) a sample of D&C Red No. 33 enriched in the impurities **A**, **B**, and **C**. The concentration of the solutions analyzed were 0.5 mg/ml water.

Fig. 3.

(a) Spiral HSCCC elution profile obtained for the separation of 1 g of D&C Red No. 33 enriched in hydrophobic impurities **A**, **B**, and **C**; the upper phase of the solvent system 1-BuOH/5 mM aq. $(\text{NH}_4)_2\text{SO}_4$ was used as the mobile phase; (b), (c), (d), and (e) are the HPLC chromatograms of pooled fractions containing the separated components along with their respective corresponding colors.

Fig. 4.

(a) Spiral HSCCC elution profile obtained for the separation of compound **C** from a ~50 mg portion of the mixture shown in Fig. 3b; the lower phase of the solvent system 1-BuOH/H₂O was used as the mobile phase; (b) HPLC of pooled fractions 72–82.

Fig. 5. D&C Red No. 33 main component **1**: (a) HPLC and UV-vis spectrum; (b) ¹H NMR spectrum with COSY assignments.

Fig. 6. D&C Red No. 33 impurity A: (a) HPLC and UV-vis spectrum; (b) ^1H NMR spectrum with COSY assignments.

Fig. 7. D&C Red No. 33 impurity **B**: (a) HPLC and UV-vis spectrum; (b) ^1H NMR spectrum with COSY assignments.

Fig. 8.
D&C Red No. 33 impurity C: (a) HPLC and UV-vis spectrum; (b) ^1H NMR spectrum with COSY assignments.

Table 1

Partition coefficient measurements of components of D&C Red No. 33 in the solvent system 1-BuOH/aq (NH₄)₂SO₄ at 530 nm by HPLC.

(NH ₄) ₂ SO ₄ concentration (mM)	K _{UP/LP}			$\frac{K_C}{K_1}$	$\frac{K_C}{K_{\text{Chromotrope 2R}}}$
	1	Chromotrope 2R	C (RT 17.6')		
1	0.064	0.156	0.225	3.52	1.44
5	0.166	0.215	1.107	6.67	5.15
10	1.494	0.330	1.734	1.16	5.25
20	1.690	0.469	3.503	2.07	7.47

Table 2

Enrichment of D&C Red No. 33 in the hydrophobic impurities **A**, **B**, and **C** by partitioning into the upper phase of the solvent system 1-BuOH/5 mM aq (NH₄)₂SO₄.

Component (HPLC retention time)	% of component in original D&C Red No. 33 sample	% of component in enriched D&C Red No. 33 sample
A (15.7')	0.22	1.59
B (15.9')	0.76	2.47
C (17.6')	0.85	7.30