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Preparative separation of two subsidiary colors of FD&C Yellow No. 5 (Tartrazine) using spiral high-speed counter-current chromatography

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Abstract

Specifications in the U.S. Code of Federal Regulations for the color additive FD&C Yellow No. 5 (Colour Index No. 19140) limit the level of the tetrasodium salt of 4-[(4',5'-disulfo[1,1'-biphenyl]-2-yl)hydrazono]-4,5-dihydro-5-oxo-1-(4-sulfophenyl)-1*H*-pyrazole-3-carboxylic acid and that of the trisodium salt of 4,4'-[4,5-dihydro-5-oxo-4-[(4-sulfophenyl)hydrazono]-1*H*-pyrazol-1,3-diyl]bis[benzenesulfonic acid], which are subsidiary colors abbreviated as Pk5 and Pk7, respectively. Small amounts of Pk5 and Pk7 are needed by the U.S. Food and Drug Administration for confirmatory analyses and for development of analytical methods. The present study describes the use of spiral high-speed counter-current chromatography (HSCCC) with the recently introduced highly polar organic/high-ionic strength aqueous solvent systems to separate Pk5 and Pk7 from a sample of FD&C Yellow No. 5 containing ~3.5% Pk5 and ~0.7% Pk7. Multiple ~1.0 g portions of FD&C Yellow No. 5 (totaling 6.4 g dye) were separated, using the upper phase of the solvent system 1-BuOH/EtOH_{abs}/saturated ammonium sulfate/water, 1.7:0.3:1:1, v/v/v/v, as the mobile phase. After applying a specially developed method for removing the ammonium sulfate from the HSCCC-collected fractions, these separations resulted in an enriched mixture (~160 mg) of Pk5 and Pk7 (~46% and ~21%, respectively). Separation of the enriched mixture, this time using the lower phase of that solvent system as the mobile phase, resulted in ~61 mg of Pk5 collected in fractions whose purity ranged from 88.0% to 92.7% (by HPLC at 254 nm). Pk7 (20.7 mg, ~83% purity) was recovered from the upper phase of the column content. Application of this procedure also resulted in purifying the major component of FD&C Yellow No. 5 to >99% purity. The separated compounds were characterized by high-resolution mass spectrometry and several ¹H and ¹³C nuclear magnetic resonance spectroscopic techniques (COSY, NOESY, HSQC, and HMBC).

1. Introduction

FD&C Yellow No. 5 (Tartrazine, Colour Index No. 19140) is a color additive permitted in the United States for coloring foods (including dietary supplements), ingested and externally applied drugs, and cosmetics [1]. It is currently manufactured using a procedure described in the mid-1880s by J.H. Ziegler [2–4] that involves the following steps: condensing phenylhydrazine-*p*-sulfonic acid, **1**, with various oxalacetic acid derivatives (*e.g.*, the diethyl ester, **2**); coupling the product, ethyl ester pyrazolone-T, **3**, with diazotized sulfanilic acid; and hydrolyzing the ester with sodium hydroxide (Figure 1A). The reaction produces mainly the trisodium salt of 4,5-dihydro-5-oxo-1-(4-sulfophenyl)-4-[4-sulfophenyl-azo]-1H-pyrazole-3-carboxylic acid, **Y5** in Figure 1A (CAS Reg No. 1934-21-0), accompanied by a variety of organic impurities, which include intermediates, side-reaction products, and subsidiary colors.

FD&C Yellow No. 5 is batch-certified by the U.S. Food and Drug Administration (FDA) to ensure compliance with specifications in the Code of Federal Regulations (CFR) [1]. Among the specifications is a limit of 1% for each of two polysulfonated subsidiary colors: the tetrasodium salt of 4-[(4',5-disulfo[1,1'-biphenyl]-2-yl)hydrazono]-4,5-dihydro-5-oxo-1-(4-sulfophenyl)-1H-pyrazole-3-carboxylic acid; and the trisodium salt of 4,4'-[4,5-dihydro-5-oxo-4-[(4-sulfophenyl)hydrazono]-1H-pyrazol-1,3-diyl]bis[benzenesulfonic acid] [1]. These compounds were identified and characterized by previous investigators who assigned them the abbreviations Pk5 and Pk7, respectively, based on their high-performance liquid chromatography (HPLC) peak locations [5,6]. The structures of Pk5 and Pk7 are shown in Figure 1B.

Small amounts of Pk5 and Pk7 are needed by the FDA for confirmatory analyses and for development of analytical methods. Purified forms of these subsidiary colors are not available commercially. In the past, purified Pk5 was obtained in sufficient amounts for its characterization using a procedure that involved preparative HPLC to obtain mixtures of FD&C Yellow No. 5 enriched in Pk5, followed by multiple semi-preparative HPLC separations with various enriching steps in between [5]. Previously, purified Pk7 was obtained from samples of FD&C Yellow No. 5 and from synthetic mixtures using multiple preparative HPLC separations [6].

In the present study, high-speed counter-current chromatography (HSCCC) [7–9] was chosen to separate Pk5 and Pk7 from a sample of FD&C Yellow No. 5. HSCCC in standard-elution mode and pH-zone-refining CCC elution mode [10] has been successfully applied to the preparative separation and/or purification of synthetic dye components [11–19], purification of dye intermediates [20], and separation of natural colorants [21–25]. Conventional HSCCC is a liquid-liquid partition technique in which one of the liquid phases (the stationary phase) is retained in a multi-layered coil column by a combination of centrifugal force and the Archimedean screw effect produced by the planetary motion of the column, while the other liquid phase (the mobile phase) is pumped through the rotating column. The multi-layered coil provides efficient mixing of the two liquid phases, resulting in good peak resolution. The commonly used organic/aqueous two-phase solvent systems accommodate separations of hydrophobic to moderately polar compounds. However, due to

the low partition coefficient values of highly polar compounds, such as catecholamines [26] and dyes containing sulfonic acid [17, 19, 27], their separations require addition of a ligand (hydrophobic counterion) to facilitate their partitioning into the organic phase. Use of a more polar solvent system would enhance the partition coefficient of polar compounds and avoid the need to use a ligand. However, such polar solvent systems are not well-retained in the conventional multi-layered coil column. To resolve this problem, a recently developed spiral-tube assembly made of a piece of inert plastic tube coiled in a rigid spiral-tube support [28], which has the capability of retaining highly polar two-phase solvent systems, has been introduced as an alternative column for HSCCC [29,30]. This HSCCC variant, called spiral HSCCC for its use of spiral-tube support coils, was effectively implemented for separating small molecules, peptides, and proteins [31,32]. The present study describes the use of spiral HSCCC with the recently introduced highly polar organic/high-ionic strength aqueous solvent systems [33,34] to separate Pk5 and Pk7 from a sample of FD&C Yellow No. 5 containing ~3.5% Pk5 and ~0.7% Pk7.

2. Experimental

2.1. Materials

The samples of FD&C Yellow 5 used in this study originated from batches submitted to the FDA for certification. Methanol (Fisher Scientific, Fair Lawn, NJ, USA), ethanol (EtOH_{abs}, 200 Proof, Sigma-Aldrich, St. Louis, MO, USA), ammonium acetate (NH₄OAc, Fisher), and water were of chromatography grade. Ammonium sulfate ((NH₄)₂SO₄, 99.0%, Sigma-Aldrich), sulfuric acid (H₂SO₄, 95.0–98.0%, Sigma-Aldrich), ammonium hydroxide (NH₄OH, 28–30%, J.P. Baker, Phillipsburg, NJ, USA), all A.C.S. reagent grade, and 1-butanol (1-BuOH, 99.9%, Sigma-Aldrich) were also used.

2.2. Spiral high-speed counter-current chromatography

2.2.1. Instrumentation—Separations were performed with a commercial J-type high-speed CCC system (Model CCC-1000, Pharma-Tech Research, Baltimore, MD, USA), which was fitted with three prototype spiral-tube assemblies (CC Biotech LLC, Rockville, MD, USA) connected in series for use as the column. Each spiral-tube assembly consisted of a spiral-tube support (STS) rotor wound with 1.6 mm i.d. polytetrafluoroethylene (PTFE) tubing. Adhesive tape was wrapped around each STS rotor to equalize their weights and balance the centrifuge system. The total capacity of the column containing the three spiral-tube assemblies was originally ~240 ml but was only ~180 ml at the end of the experiments due to several repairs made in between the experiments. The column was mounted on a rotating frame (centrifuge). Photographs of the instrument and an STS rotor have been previously published [35,36].

The instrument was connected to a speed controller and one of two pumps (Shimadzu LC–10ADVP, Shimadzu, Kyoto, Japan, for some of the experiments, and Waters 600F, Waters Corp., Milford, MA, USA, for others). 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, model Uvicord SII with a

254-nm UV lamp (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. Graphic selection of the organic/high-ionic strength aqueous solvent system

The appropriate solvent systems for the separation of Pk5, Pk7, and Y5 were found by applying the two-step partition coefficient measurement method described previously [33]. That method was specifically developed for separation of highly polar mixtures and makes use of 21 solvent systems (numbered from 0 to 20) that consist of saturated aqueous $(\text{NH}_4)_2\text{SO}_4$ (AS) with 1-BuOH and EtOH_{abs} in various ratios.

According to the method, the first step was to measure the partition coefficient, $K_{\text{upper phase}/\text{lower phase}}$ ($K_{\text{UP/LP}}$), values for Pk5, Pk7, and Y5 by HPLC using Solvent System No. 10 (1-BuOH/ EtOH_{abs} /AS/water, 1:1:1:1, v/v/v/v). A small amount (~2 mg) of FD&C Yellow No. 5 was placed in a test tube that contained 2 ml of UP and 2 ml of LP of Solvent System No. 10. The mixture was agitated and allowed to settle; 1 ml of UP and 1 ml of LP were then removed and placed in separate 20-ml vials, each of which contained 2 ml of methanol. The solvent in the UP vial was evaporated (Buchi Rotavapor R-124, Flawil, Switzerland). The LP mixture, from which $(\text{NH}_4)_2\text{SO}_4$ precipitated upon contact with the methanol, was filtered through an Autovial 5 syringeless filter of 0.45- μm pore size (Whatman, Sanford ME, USA), and the solvent was eliminated by rotary evaporation. The residue in each vial was re-dissolved in 1 ml methanol/water (1:1, v/v), and an aliquot was analyzed by HPLC. $K_{\text{UP/LP}}$ values for Pk5, Pk7, and Y5 were obtained by dividing the UP area of each analyte at 254 nm by its LP area at that wavelength, and they are shown in Table 1.

Since the average of the $K_{\text{UP/LP}}$ values for the analytes was greater than 1 using Solvent System No. 10, the second step of the method was to measure $K_{\text{UP/LP}}$ using Solvent System No. 0 (1-BuOH/ EtOH_{abs} /AS/water, 2:0:1:1, v/v/v/v). This second set of values is also shown in Table 1. The log $K_{\text{UP/LP}}$ values calculated from the two measurements were plotted on a graph, and the two log values obtained for each analyte were connected by a straight line (Figure 2). Drawing a vertical line that intersects all three log K lines where they are farthest apart from each other (Figure 2) suggested that Solvent System Nos. 3 and 4 (1-BuOH/ EtOH_{abs} /AS/water, 1.7:0.3:1:1, v/v/v/v and 1-BuOH/ EtOH_{abs} /AS/water, 1.6:0.4:1:1 [33]) would yield the separation of the three analytes with the best resolution [33].

An alternative method was used later in the study to avoid precipitation of $(\text{NH}_4)_2\text{SO}_4$ and eliminate the filtration step in preparing the solutions for the partition coefficient measurements. A small amount (~2 mg) of FD&C Yellow No. 5 was placed in a test tube that contained 2 ml of UP and 2 ml of LP of Solvent System No. 10. The mixture was agitated, allowed to settle, and then 400 μl of each phase was removed and placed in separate test tubes, each containing 2 ml of a mixture of methanol/water, 1:1, v/v. An aliquot from each diluted phase was analyzed by HPLC. The procedure was repeated with Solvent System No. 0.

2.2.3. Preparation of larger quantities of organic/high-ionic strength aqueous solvent systems

The highly polar two-phase solvent systems, Nos. 3 and 4 used in this study were prepared in a 2-liter separatory funnel as described recently [33], except that the saturated ammonium sulfate solution component was prepared slightly differently. The AS was prepared by adding 1000 ml of water to 800 g of $(\text{NH}_4)_2\text{SO}_4$, thoroughly mixing, and allowing the solution to return to room temperature. The clear supernatant was then used to prepare Solvent Systems Nos. 3 and 4, which consisted of 1-BuOH-EtOH_{abs}-AS-water in ratios of 1.7:0.3:1:1 and 1.6:0.4:1:1, v/v/v/v, respectively. H_2SO_4 at a concentration of 2.5% (v/v) was added to each solvent system. When H_2SO_4 was added, the two phases separated instantaneously.

2.2.4. Partition coefficient measurements for determining the appropriate concentration of the sample solution

The dependence of the partition coefficient on the concentration of the sample solution was determined as follows: (i) a stock solution of the dye was prepared by dissolving 100 mg of FD&C Yellow No. 5 in 1 ml of water; (ii) 4 ml (2 ml of UP and 2 ml of LP) of Solvent System No. 3 was placed in each of six test tubes; (iii) 12.5, 25, 50, 100, 150, and 200 μl of FD&C Yellow No. 5 stock solution were added, respectively, to each of the test tubes to yield corresponding solutions containing 0.31, 0.63, 1.25, 2.50, 3.75, and 5.00 mg/ml FD&C Yellow No. 5; and (iv) for each solution, the $K_{\text{UP/LP}}$ was obtained for Pk5, Pk7, and Y5 by the HPLC method described in section 2.2.2. The relation between the concentration of each of the respective analytes and its partition coefficient at 254 nm is shown by the graph in Figure 3A. The same sequence was followed for obtaining the relation between the concentration of each of the analytes and its partition coefficient in Solvent System No. 3 to which 2.5% H_2SO_4 was added (Fig. 3B).

2.2.5. Separation procedure—The highly polar two-phase solvent systems used in the present work were Solvent System Nos. 3 and 4, described above, to which H_2SO_4 (2.5%, v/v) was added. After equilibration in a separatory funnel, the organic UP was used as the stationary phase, and the aqueous LP was used as the mobile phase for some separations, while the roles were reversed for others. Each separation was initiated by filling the entire column with the stationary phase using the LC pump. Next, a syringe was used to load the FD&C Yellow No. 5 sample dissolved in a mixture of UP and LP (~0.3 g in 3 ml:3 ml, and 1.0 g in 35 ml:5 ml) to which H_2SO_4 was added to cause the mixture of dyes to partition mostly in the UP (~5%, v/v). The mobile phase was then pumped into the column (3 ml/min and 1 ml/min, respectively) while the column was rotated at ~750 rpm in the tail-to-head elution mode (when LP was used as the mobile phase) or the head-to-tail elution mode (when UP was used as the mobile phase). The effluent was monitored with the UV detector at 254 nm and collected in fractions (3 ml, 4 ml, or 6 ml/tube) using the fraction collector. The range of the recorder was set at 2V and the chart speed at 20 min/cm. The backpressure at the pump outlet during the separation was ~190 psi. The retention of the stationary phase, calculated after the separation, was ~66% when the LP was used as the mobile phase and the sample size was ~0.3 g. The retention was 25.5% to 35.3% when the UP was used as the mobile phase and the sample size was ~0.8 g to 1.0 g.

2.2.6. Elimination of $(\text{NH}_4)_2\text{SO}_4$ and trace H_2SO_4 from the HSCCC-collected fractions—Based on the HSCCC chromatogram and HPLC analyses, the fractions of interest were combined and the solvent eliminated by rotary evaporation. Cold methanol was added to the residue, and the precipitated $(\text{NH}_4)_2\text{SO}_4$ was removed by filtration on a cold Bchner funnel. The precipitate was washed with 2–3 ml of cold methanol, and the methanolic filtrate was dried using a rotary evaporator. The residue (usually red-orange and still containing a small amount of $(\text{NH}_4)_2\text{SO}_4$ and traces of H_2SO_4) was re-dissolved in 2–3 ml of water and loaded onto a Supelclean LC-18 3 ml-tube (Supelco, Bellefonte, PA, USA) conditioned with 2 ml of methanol followed by 2 ml of water. The remaining $(\text{NH}_4)_2\text{SO}_4$ and trace H_2SO_4 were flushed from the tube with 2–3 ml of water, and the pure analyte was then eluted with 3–5 ml of NH_4OH (~3% NH_3 in water) and collected after evaporation of the eluent (rotary evaporator).

2.3. Analytical HPLC

HPLC analyses were performed with a Waters Alliance 2690 Separation Module (Waters, Milford, MA, USA). The eluents were 0.2 M aqueous NH_4OAc and methanol. The column (Exterra C18, 5 μm , 250×4.60 mm i.d., Waters, Waltham, MA, USA) was eluted by using consecutive linear gradients of 0–10% methanol in 5 min, 10–25% methanol in 10 min, and 25–40% methanol in 20 min, followed by 100% methanol for 6 min. The effluent was monitored with a Waters 996 photodiode array detector set at 254 nm. Other conditions included: flow-rate, 1 ml/min; column temperature, 25°C; injection volume, 20 μL .

An aliquot (~100 μL) from the HSCCC-collected fractions was diluted with 2 ml of methanol. Prior to chromatography, the precipitated $(\text{NH}_4)_2\text{SO}_4$ was removed by filtration through a syringeless Autovial 5 filter device (Whatman, Sanford, ME, USA) with a pore size of 0.45 μm . Though a small amount of $(\text{NH}_4)_2\text{SO}_4$ remained dissolved in the methanolic solution, it did not affect the HPLC analysis.

2.4. Liquid chromatography-mass spectrometry

The high-resolution mass spectra of the HSCCC-separated FD&C Yellow No. 5 components 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 dyes were dissolved in water (20 ng/ μL) and analyzed in positive- and negative electrospray ionization (ESI) modes. The high-resolution measurements of the quasi-molecular ions $(\text{M}+\text{H})^+$ of the isolated components were as follows: **Pk5** m/z 625.00099, which matched the calculated mass of 625.000517 for $\text{C}_{22}\text{H}_{17}\text{N}_4\text{O}_{12}\text{S}_3$, the protonated form of Pk5; **Pk7** m/z 581.01053, which matched the calculated mass of 581.010687 for $\text{C}_{21}\text{H}_{17}\text{N}_4\text{O}_{10}\text{S}_3$, the protonated form of Pk7; **Y5** m/z 469.01254, which matched the calculated mass of 469.012400 for $\text{C}_{16}\text{H}_{13}\text{N}_4\text{O}_9\text{S}_2$, the protonated form of Y5.

2.5. Nuclear magnetic resonance (NMR) spectroscopy

The ^1H NMR, ^{13}C NMR, COSY, NOESY, HSQC, and HMBC spectra of Pk5, Pk7, and Y5 were determined on an Agilent NMR spectrometer operating at 600 MHz. Approximately 10-mg portions of each of the separated compounds were dissolved in 600 μL of D_2O . The

acquisition time in the directly detected dimension for all 2D spectra was 0.150 s per scan. A 1.0-s relaxation delay was used between all scans. The COSY spectra had a spectral width of 8120 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. For the NOESY spectrum, the sweep width was 6280 Hz in both dimensions with 200 increments in the indirect dimension. It had 16 scans per increment and had an NOE mixing time of 0.5 s. Total acquisition time for the NOESY spectrum was 3 h. The HSQC and HMBC spectra each had sweep widths of 9600 Hz in the ^1H dimension. In the ^{13}C dimension the HSQC spectrum had a sweep width of 30170 Hz and the HMBC spectrum had a sweep width of 36200 Hz. Both used 256 increments in the ^{13}C dimension with 16 scans per increment for a total acquisition time of 2 h 50 min. A larger number of scans was used in the HMBC determination than usually considered necessary for samples of this concentration in order to ensure detection of the signals that were broadened due to known exchange processes. All spectra were processed with appropriate zero filling and signal weighting. The obtained ^{13}C and ^1H chemical shift data, HMBC connectivities, and position assignments were tabulated for each of the isolated sulfonated dyes: **Pk5**, 4-[(4',5-disulfo[1,1'-biphenyl]-2-yl)hydrazono]-4,5-dihydro-5-oxo-1-(4-sulfophenyl)-1*H*-pyrazole-3-carboxylic acid, Table 2 and Fig. 4; **Pk7**, 4,4'-[4,5-dihydro-5-oxo-4-[(4-sulfophenyl)hydrazono]-1*H*-pyrazol-1,3-diyl]bis[benzenesulfonic acid], Table 3 and Fig. 5; **Y5**, 4,5-dihydro-5-oxo-1-(4-sulfophenyl)-4-[4-sulfophenyl-azo]-1*H*-pyrazole-3-carboxylic acid, Table 4 and Fig. 6.

3. Results and discussion

All batches of dye submitted for certification as FD&C Yellow No. 5 contain Pk5 and Pk7 in varying quantities. For the present study, a batch of dye that contained ~ 3.5% Pk5 and ~ 0.7% Pk7 was chosen, and its HPLC chromatogram is shown in Fig. 7A.

Due to the high polarity of the compounds to be separated, spiral HSCCC was used in combination with an organic/high-ionic strength aqueous two-phase solvent system [33]. The configuration of the spiral-tube assembly coil is unique in its capability for retaining the stationary phase of highly polar two-phase solvent systems, thus permitting separation of very polar compounds, such as polysulfonated dyes, without the addition of a ligand (hydrophobic counterion, *e.g.*, dodecylamine) [17,19,27]. Two solvent systems, No. 3, 1-BuOH/EtOH_{abs}/AS/H₂O (1.7:0.3:1:1, v/v/v/v), and No. 4, 1-BuOH/EtOH_{abs}/AS/H₂O (1.6:0.4:1:1, v/v/v/v), were chosen for the separations described here by applying a recently published method – graphic optimization of the partition coefficients [33] – to the information presented in Fig. 2. Specifically, the systems were chosen in such a way as to balance the separation time (progressively increasing as the solvent system numbers increase) and the separation resolution (reflected in the distances between the respective intersections of the analytes' log K lines with the vertical line in the graph). The log K line for Y5 crosses the log K=0 line between Solvent System No. 3 and Solvent System No. 4, although much closer to No. 3. (Fig.2). This indicates that either of these two systems would be suited for the separation of this analyte, but No. 3 would be preferable. Y5 has a K value slightly less than 1 for Solvent System No. 3, which means its elution occurs after approximately that of one column volume of eluent. The log K lines for Pk5 and Pk7 are well-distanced from each other and from the Y5 line at Solvent System No. 3 (Fig. 2),

thereby predicting a separation with high resolution. Choosing a nearby solvent system such as No. 2 or No. 5 would be appropriate with respect to the resolution, but a system numbered less than 3 would elute the sample too fast, and one numbered greater than 4 would prolong the separation time.

Figure 3 shows that there is a non-linear isothermal relationship between the partition coefficient of the FD&C Yellow No. 5 analytes (in the mixture) and their respective levels of concentration in Solvent System No. 3. The more concentrated the analytes in the aqueous mobile phase, the faster they will elute, resulting in poor separation. To circumvent this obstacle, 2.5% H₂SO₄ v/v was added (after optimization) to the solvent system to lower the concentration of the analytes in the aqueous mobile phase by prolonging their retention in the organic stationary phase, thereby improving the separation of the analytes from each other (Fig. 3).

Fig. 7B shows the elution profile of the spiral HSCCC separation of 0.3 g of FD&C Yellow No. 5 for which the mobile phase was the LP of Solvent System No. 4 with 2.5% H₂SO₄ v/v added. The retention of the stationary phase (a measure of the resolution of the separation), calculated after the separation was completed, was 66.4%. Based on HPLC analyses, the eluates collected in the hatched areas in Figure 7B contained the separated compounds of interest. Fractions 114–118 contained Pk5 (of >90% purity in fractions 114–116, and of >95% purity in fractions 117–118, by HPLC at 254 nm); fractions 127–137 contained Y5 (of ~99% purity); and fractions 119–124 contained a mixture enriched in Pk7 (over 25%). In a second step using the same solvent system, Pk7 (of >88.3% purity) was separated from the other components in fractions 119–124 by initially using the aqueous lower phase as the mobile phase and then switching to use the organic upper phase as the mobile phase, a modification named elution-extrusion CCC [37] (Fig. 7C). Due to the very small amount of Pk5 and Pk7 in the original sample, the separation of 0.3 g resulted in only <3 mg of Pk5 and <1 mg of Pk7, which was not enough for our purpose.

The nonlinear isothermal dependence of the partition coefficients of the analytes on the concentration of the sample solution (Fig. 3) did not permit separation of a larger quantity of sample using the LP as the mobile phase given the fixed volume of the column. To obtain a larger quantity of the analytes, a different approach was taken. Fig. 3 shows that at low concentrations, Y5 is partitioned mostly in the UP. Thus, by changing the mobile phase from the aqueous LP to the organic UP of the solvent system, Y5 would elute in the first part of the separation followed by fractions enriched with the analytes in a mixture that contains a small amount of remaining Y5. Under these conditions, even concentrated sample solutions could be loaded because early in the course of the separation, the organic mobile phase would strip the Y5 from the sample and dilute it over the length of the column, partitioning it into the organic mobile phase. It thus seemed feasible to obtain larger quantities of separated Pk5 and Pk7 by collecting the later-eluted fractions that contained enriched mixtures of Pk5 and Pk7 and then separating Pk5 from Pk7 by applying the above-described method that uses the aqueous LP as the mobile phase.

Applying this latter approach, seven separations were performed, six of which involved ~1.0 g and one involved 0.4 g of FD&C Yellow No. 5. Due to the larger sample size and the use

of the organic phase as the mobile phase, the retention of the stationary phase in these experiments varied between ~25.5% and ~35.3%. A typical spiral HSCCC elution profile of such a separation is shown in Fig. 8. The fractions containing Pk5 and Pk7 from all these separations were combined, resulting in 2.07 g of enriched mixture (after partial removal of the $(\text{NH}_4)_2\text{SO}_4$), which was once more subjected to separation using the UP as the mobile phase to eliminate the remaining Y5. The resulting enriched mixture (~160 mg, after removal of the $(\text{NH}_4)_2\text{SO}_4$) consisted of ~46% Pk5, ~21% Pk7 and ~28% Y5 (Fig. 8). It was dissolved in 5 ml UP and 3.5 ml LP to which 200 μl H_2SO_4 was added in order to force the dyes into the UP. Pk5 and Pk7 were separated by using the original procedure in which the aqueous LP was used as the mobile phase (1 ml/min, 3 ml/fraction collected). Pk5 was collected in fractions 155–229. After removal of the $(\text{NH}_4)_2\text{SO}_4$, the first eluted fractions (Fr. 155–160) contained Pk5, ~3 mg, of >98% purity. The remaining fractions (Fr. 161–229) contained Pk5, 61.1 mg, of a purity that ranged from 88.0% to 92.7% (by HPLC at 254 nm, Fig. 4). The column content was pushed out with pressurized air, and the retention of the stationary phase after the separation was calculated as 67.4%. The organic phase of the column content contained Pk7 and some Y5. After elimination of the solvent by rotary evaporation, the residue was dissolved in water and passed through a Supelclean LC-18 cartridge. Elution with ~3% NH_3 in water resulted in two bands: the first, yellow, contained mostly Y5; and the second, dark brown, contained mostly Pk7 (20.7 mg, of ~83% purity, Fig. 5). To facilitate the NMR characterization of Pk5 and Pk7, a small amount of the separated Y5 (~40 mg) was also analyzed by NMR after being desalted (Fig. 6).

The separated components were identified by HPLC and characterized by UV-visible spectra, high-resolution MS techniques in both positive and negative modes, and by various ^1H and ^{13}C NMR spectroscopic techniques. Pk5 and Y5 have nearly identical UV-visible spectra but slightly different HPLC retention times (Figs. 4 and 6), while both the UV-visible spectrum and retention time of Pk7 are unique (Fig. 5). The high-resolution MS analyses confirmed the elemental composition of the separated Pk5, Pk7, and Y5.

Several ^1H and ^{13}C NMR spectroscopic techniques (COSY, NOESY, HSQC, and HMBC) were used to characterize Pk5 and Pk7. Although the structures of these two common manufacturing impurities of FD&C Yellow No. 5 have been known since the 1980s [5,6], the assignment of chemical shifts in their ^1H and ^{13}C NMR spectra is not straightforward due to the presence of two sulfophenyl groups in Pk5 and three in Pk7. However, certain carbons in the sulfophenyl rings can be identified by the NMR signal broadening that the main component, Y5, and Pk5 and Pk7 exhibit due to their hydrazone-azo acid-base equilibrium [38]. Specifically, hydrogens and carbons at positions 3, 5, 6, 1', 2'/6', 1'', and 2''/6'' have appreciably different chemical shifts in their hydrazone and azo-anionic forms, and the resulting line broadening becomes apparent when the solution pH approaches the $\text{p}K_a$ (Table 4 and [39]).

Methine carbons of the tri-substituted C-ring of Pk5 (Table 2) are easily identified, and C-1'' (δ 142.2) can be readily differentiated from C-4'' (δ 140.9) by its substantial line broadening. C-1' (δ 140.3) of the A-ring is, likewise, distinguished from C-4' (δ 140.9) on the basis of its smaller, but still obvious, signal broadening. H-2'/6' (δ 7.90) and H-3'/5' (δ 7.86) are then differentiated by their HMBC correlations to C-4' and C-1', respectively. C-1'' (δ 140.0) is

easily distinguished from C-4" (δ 143.3) of the D-ring by its HMBC connectivity to H-3" (δ 7.77) in the C-ring. Similarly, H-2"/6" (δ 7.64) are readily differentiated from H-3"/5" (δ 7.91) by their HMBC correlations to C-2" (δ 132.2) in the C-ring. In addition, a NOESY correlation between H-3" and H-2"/6" demonstrates that the C- and D-rings are connected at C-2" and C-1".

The quaternary carbons, C-1" (δ 144.3) and C-1' (δ 140.0), of the C- and A-rings, respectively, of Pk7 (Table 3) are distinguished from C-4" (δ 140.9) and C-4' (δ 140.6) on the basis of differential line broadening in the same manner as seen previously for Pk5. H-3"/5" (δ 7.78) and H-3'/5' (δ 7.85) are then identified by their HMBC connectivities to C-1" and C-1', respectively. In addition, H-2"/6" (δ 7.84) are differentiated from H-3"/5" (δ 8.09) by their HMBC correlations to C-3 (δ 132.8) of the B-ring. Finally, C-1" (δ 144.6) and C-4" (δ 147.6) are identified by means of strong and weak, respectively, HMBC connectivities to H-3"/5".

Conclusion

A preparative method based on spiral HSCCC and use of a highly polar organic/high-ionic strength aqueous solvent system was developed to separate the closely related minor components Pk5 (~3.5%) and Pk7 (~0.7%) from a sample of FD&C Yellow No. 5. The non-linear isothermal dependence of the partition coefficient of the analytes on the concentration of the sample solution prevented separation of large quantities of dye using the lower phase of the solvent system as the mobile phase. Useful quantities of Pk5 and Pk7 were obtained by eliminating the major component of the sample, Y5, through use of the acidified upper phase as the mobile phase to enrich the product in Pk5 and Pk7, and by then separating them from the enriched product by using the lower phase as the mobile phase. Application of this method also resulted in purifying the major component of FD&C Yellow No. 5 to ~99% purity. The present work is the first reported HSCCC separation of gram quantities of a highly polar polysulfonated dye without using a hydrophobic counterion (*e.g.*, dodecylamine) in the stationary phase [17,19]. This was possible due to the higher retention of a highly polar solvent system enabled by the spiral-tube support configuration of the HSCCC column. The study also includes the first reported in-depth structural analysis of Pk5 and Pk7 using COSY, NOESY, HSQC, and HMBC NMR spectroscopic techniques.

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Figure 1.

(A) Method of preparing FD&C Yellow No. 5 by condensing phenylhydrazine-*p*-sulfonic acid, 1, with oxalacetic acid diethyl ester, 2; (B) The structures of Pk5 and Pk7, two subsidiary colors commonly found in FD&C Yellow No. 5.

Figure 2.

Graphic selection of the organic/high-ionic strength aqueous two-phase solvent system No. 3 for the separation of Pk5, Pk7, and Y5 from a sample of FD&C Yellow No. 5 by the 2-step partition coefficient measurement method (for details, see section 2.2.2. and [33]).

Figure 3.

Partition coefficient measurements at various concentrations of FD&C Yellow No. 5 in organic/high-ionic strength aqueous Solvent System No. 3 (1-BuOH/EtOH_{abs}/(NH₄)₂SO₄ sat. aq. solution/H₂O, 1.7:0.3:1:1, v/v/v/v). (A) No acid added; (B) 2.5% v/v H₂SO₄ added.

Figure 4. Characterization of the HSCCC-separated Pk5. (A) HPLC analysis and UV-visible spectrum; (B) ^1H NMR spectrum and COSY assignments.

Figure 5. Characterization of the HSCCC-separated Pk7. (A) HPLC analysis and UV-visible spectrum; (B) ^1H NMR spectrum and COSY assignments.

Figure 6. Characterization of the HSCCC-separated Y5. (A) HPLC analysis and UV-visible spectrum; (B) ^1H NMR spectrum and COSY assignments.

Figure 7.

Separation of Pk5, Pk7, and Y5 from a 0.3 g sample of FD&C Yellow No. 5 by spiral HSCCC. (A) Reversed-phase HPLC chromatogram of the original mixture (10 mg/ml in water); (B) Elution profile of the spiral HSCCC separation and the analytical HPLC chromatograms of the fractions containing the analytes; (C) Elution-extrusion spiral HSCCC chromatogram of the fractions in Fig. 7B that were enriched in Pk7, and analytical HPLC chromatogram of the fractions containing the separated Pk7.

Figure 8. Separation of Y5 from an enriched mixture of Pk5 and Pk7 (~0.8 g) by spiral HSCCC using the organic UP as the mobile phase.

Table 1

Partition coefficient measurements for Pk5, Pk7, and Y5 in highly-polar two-phase solvent systems

Solvent system	Pk5	Pk7	Y5
UP ^b	542823	396429	6.6E+07
LP ^c	86479	4507	5343780
K _{UP/LP}	6.28	87.96	12.44
Log K	0.798	1.944	1.095
UP ^b	27980	126647	1.1E+07
LP ^c	350242	109615	3.7E+07
K _{UP/LP}	0.08	1.16	0.29
Log K	-1.097	0.065	-0.538

^a 1-BuOH/EtOH_{abs}/(NH₄)₂SO₄ sat. aq. solution/H₂O (1:1:1:1, v/v/v/v) [33]^b HPLC area of the analyte in the upper phase at 254 nm^c HPLC area of the analyte in the lower phase at 254 nm^d 1-BuOH/EtOH_{abs}/(NH₄)₂SO₄ sat. aq. solution/H₂O (2:0:1:1, v/v/v/v) [33]

Table 2

^{13}C and ^1H NMR chemical shift data^a of Pk5 [in ppm relative to $(\text{CH}_3)_4\text{Si}$ in D_2O].

Position	$\delta^{13}\text{C}$	$\delta^1\text{H}$	HMBC Connectivities
3	(not observed)	---	(none)
4	(not observed)	---	(none)
5	164.3	---	(none)
6	166.8	---	(none)
1'	140.3	---	$\delta 7.86$
2'/6'	121.4	7.90 d (8.4)	$\delta 7.90$
3'/5'	127.4	7.86 d (8.4)	$\delta 7.86$
4'	140.9	---	$\delta 7.90$
1''	142.2	---	$\delta 7.77, \delta 7.89, \delta 8.08$
2''	132.2	---	$\delta 7.64, \delta 8.08$
3''	129.2	7.77 d (1.8)	$\delta 7.89, \delta 8.08$
4''	140.9	---	$\delta 7.77, \delta 8.08$
5''	127.6	7.89 dd (8.4, 1.8)	$\delta 7.77, \delta 8.08$
6''	118.8	8.08 d (8.4)	(none)
1'''	140.0	---	$\delta 7.77, \delta 7.91$
2'''/6'''	131.1	7.64 d (8.4)	$\delta 7.64$
3'''/5'''	127.1	7.91 d (8.4)	$\delta 7.91$
4'''	143.3	---	$\delta 7.64$

^aChemical shifts referenced to dioxane at 3.70 ppm (^1H) and 67.4 ppm (^{13}C); coupling constants are in parentheses.

Table 3

^{13}C and ^1H NMR chemical shift data^a of Pk7 [in ppm relative to $(\text{CH}_3)_4\text{Si}$ in D_2O].

Position	$\delta^{13}\text{C}$	$\delta^1\text{H}$	HMBC Connectivities
3	132.8	---	$\delta 7.84$
4	127.4	---	(none)
5	158.0	---	(none)
1'	140.0	---	$\delta 7.85$
2'/6'	120.4	7.87 d (8.8)	$\delta 7.87$
3'/5'	127.5	7.85 d (8.8)	$\delta 7.85$
4'	140.6	---	$\delta 7.87$
1''	144.3	---	$\delta 7.78$
2''/6''	117.8	7.44 d (8.6)	$\delta 7.44$
3''/5''	128.0	7.78 d (8.6)	$\delta 7.78$
4''	140.9	---	$\delta 7.44$
1'''	144.6	---	$\delta 8.09$
2'''/6'''	126.7	7.84 d (8.4)	$\delta 7.84, \delta 8.09$
3'''/5'''	128.8	8.09 d (8.4)	$\delta 7.84, \delta 8.09$
4'''	147.6	---	$\delta 8.09$

^a Chemical shifts referenced to dioxane at 3.70 ppm (^1H) and 67.4 ppm (^{13}C); coupling constants are in parentheses.

Table 4

^{13}C and ^1H NMR chemical shift data^a of Y5 [in ppm relative to $(\text{CH}_3)_4\text{Si}$ in D_2O].

Position	$\delta^{13}\text{C}$	$\delta^1\text{H}$	HMBC Connectivities
3	141.4	---	(none)
4	126.7	---	(none)
5	164.6	---	(none)
6	166.9		(none)
1'	140.4	---	$\delta 7.96$
2'/6'	121.6	7.89 d (8.8)	$\delta 7.89$
3'/5'	1275	7.96 d (8.8)	$\delta 7.96$
4'	140.8	---	$\delta 7.89$
1''	144.3	---	$\delta 7.85$
2''/6''	117.9	7.67 d (8.7)	$\delta 7.67$
3''/5''	128.1	7.85 d (8.7)	$\delta 7.85$
4''	141.0	---	$\delta 7.67$

^aChemical shifts referenced to dioxane at 3.70 ppm (^1H) and 67.4 ppm (^{13}C); coupling constants are in parentheses.