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Preparative separation of di- and trisulfonated components of Quinoline Yellow using affinity-ligand pH-zone-refining counter-current chromatography

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Abstract

Four positionally isomeric 2-(2-quinoliny)-1H-indene-1,3(2H)-dionedisulfonic acids (SA) and one triSA, components of the color additive Quinoline Yellow (QY, Color Index No. 47005), were isolated from the dye mixture by affinity-ligand pH-zone-refining counter-current chromatography (CCC) through complementary use of ion-exchange and ion-pair reagents as the ligand. The added ligands facilitated the partitioning of the very polar polysulfonated components into the organic stationary phase of the two-phase solvent systems that consisted of isoamyl alcohol-methyl *tert*-butyl ether-acetonitrile-water (3:5:1:7), (3:4:1:7) or (3:1:1:5). Thus, separation of a 5 g portion of QY using sulfuric acid as the retainer and dodecylamine as the ligand (an ion-exchange reagent, 20% in the stationary phase), resulted in 1.21 g of 6',5-diSA and 1.69 g of 6',8',5-triSA, both of over 99% purity. A minor component, 8',4-diSA, not previously reported was also obtained (4.8 mg of over 94% purity) through a similar separation of a different batch of QY using hydrochloric acid as the retainer and 10% dodecylamine as the ligand in the stationary phase. Two components that co-eluted (0.55 g) in the 5 g separation were separated when trifluoroacetic acid was used as the retainer and tetrabutylammonium hydroxide (an ion-pair reagent) as the ligand. The separation resulted in 20.7 mg of 6',4-diSA, not previously reported, and 111.8 mg of 8',5-diSA, both of over 98% purity. The isolated compounds were characterized by high-resolution mass spectrometry and proton nuclear magnetic resonance with correlated spectroscopy assignments.

Keywords

Counter-current chromatography; pH-zone-refining CCC; Quinoline Yellow; D&C Yellow No. 10; Sulfonic acids; Disulfonic acids; Trisulfonic acid; Isomers; Ligand; Dyes; NMR

1. Introduction

D&C Yellow No. 10 [Quinoline Yellow (QY), Colour Index No. 47005] is a US-certified color additive listed for use in drugs and cosmetics [1]. QY is currently prepared as was described more than a hundred years ago [2] (Fig. 1): 2-methylquinoline (quinaldine), **1**, is condensed with phthalic anhydride, **2**, using zinc chloride as the catalyst, at 190° to 210°C; the condensation product, 2-(2-quinolinyl)-1H-indene-1,3(2H)-dione, **3**, that can exist in three tautomeric forms, is then sulfonated with fuming sulfuric acid and the products are isolated as sodium salts. Depending on the degree of sulfonation, mono-, di-, and trisulfonated components may be formed (Fig. 1).

D&C Yellow No. 10 consists primarily of a mixture of the sodium salts of the monosulfonic acid isomers of 2-(2-quinolinyl)-1H-indene-1,3(2H)-dione with up to 15% of the disodium salts of the disulfonated positional isomers [1] (Fig. 2a). A variant form of QY, used for coloring foods in Europe (E 104) and for drugs and cosmetics in Japan (Yellow 203) and other countries, contains mostly a mixture of the di- and trisulfonated isomers (Fig. 2b and c, respectively) [3-5]. Even though that alternate form of QY is not certifiable in the USA, separation and purification of its di- and trisulfonated components, which are not commercially available, are of interest. The isolated components are needed as reference materials when developing high-performance liquid and thin-layer chromatographic methods of analysis for US Food and Drug Administration (FDA) batch certification of D&C Yellow No. 10.

Di- and trisulfonated components of QY were obtained in the past either synthetically by directly sulfonating 2-(2-quinolinyl)-1H-indene-1,3(2H)-dione [6], or by isolating them from samples of commercial color additives [5,7,8]. In one case of the latter approach, disulfonated components of D&C Yellow No. 10 were isolated in two different ways, by applying countercurrent distribution and by using fractional crystallization of the potassium salts of the color additive [7]. In another case, 200 mg of a trisulfonated compound and 50 to 100 mg of three disulfonated components were isolated by use of preparative HPLC [5]. More recently, high-speed counter-current chromatography (HSCCC) was implemented for the separation of 25 mg of QY [8]. That procedure, which required 40 h to complete, resulted in 2.6 mg of one disulfonated component and 1.3 mg of another.

The present study involves the separation of di- and trisulfonated components from gram quantities of QY using a modified HSCCC technique, pH-zone-refining CCC, which was developed in the mid-1990s as a novel preparative-scale separation technique [9-11]. pH-Zone-refining CCC is among the liquid-liquid partition chromatographic techniques that do not use a solid support. It enables separation, with high resolution, of ionic or ionizable compounds such as organic acids and bases according to their pK_a values and hydrophobicities. The separation procedure and mechanism of separation by this method as well as many applications of the technique have been reviewed [10-14]. Carboxylic acid containing compounds are among the most common targets for pH-zone-refining CCC. For example, preparative separations have been reported for compounds containing isomeric and stereoisomeric mono- and dicarboxylic acids [15-17], amino acids and peptides [12] and monocarboxylated fluorescein dyes [18]. Originally, sulfonated dyes, which are much more hydrophilic compounds than the carboxylic acid dyes, were considered not amenable to separation by pH-zone-refining CCC because of their very low pK_a values that prevent their partitioning into the organic phase of a conventional two-phase solvent system. Later, however, it was shown that the addition of a ligand (ion exchanger) to both the sample solution and the organic stationary phase enables separation of sulfonated dyes by affinity-ligand pH-zone-refining CCC because the ligand facilitates their partitioning into the organic stationary phase [12,19,20]. Similarly, other very hydrophilic compounds, depolymerized fucans [21] and naphthalenesulfonic acids

[22], were fractionated or separated, respectively, using a ligand (the anion exchanger Amberlite LA2) in the organic stationary phase.

In an earlier work [20], the addition of a ligand (the ion exchanger 5% dodecylamine) to both the sample solution and the organic stationary phase enabled pH-zone-refining CCC preparative separation of the monosulfonated positional isomers of the D&C Yellow No. 10 form of QY. Under the same conditions, however, the more polar di- and trisulfonated components eluted together in the earlier fractions. The current study, part of which was previously reported [19], describes the conditions that permitted separation of the di- and trisulfonated components of QY using affinity-ligand pH-zone-refining CCC with an ion exchanger or an ion-pairing reagent as the ligand. The isolated di- and trisulfonated dyes, designated as diSA and triSA, are characterized by high-resolution mass spectrometry, ^1H nuclear magnetic resonance (NMR) spectroscopy, and ^1H - ^1H NMR correlated spectroscopy (COSY).

2. Experimental

2.1. Materials

The samples of QY used in this study were each obtained by one of three means: (i) submitted to the FDA for batch certification as D&C Yellow No. 10; (ii) purchased (European QY: Fluka, Buchs, Switzerland; Japanese QY: Namizirushi Colors, Kishi Kasei, Tokyo-Yokohama, Japan); and (iii) donated (Y203) by Dr. Hisao Oka, then at Aichi Prefectural Institute of Public Health, Nagoya, Japan. The HPLC analyses of the European and Japanese QY used for the CCC separations are shown in Figs. 2b and 2c. Acetonitrile (ACN), methanol (MeOH), water, and ammonium acetate (NH_4OAc) were of chromatography grade. *iso*-Amyl alcohol (iAA, 98.8%, J.T. Baker, Phillipsburg, NJ, USA) and sulfuric acid (H_2SO_4 , 95.8%, Mallinckrodt, St. Louis, MO, USA) were ACS reagent grade. Methyl *tert*-butyl ether (MtBE, >99.5%, Fluka), trifluoroacetic acid (TFA, Sigma, St. Louis, MO, USA), ammonium hydroxide (>25% NH_3 in water, Fluka), dodecylamine (DA, >98%, Fluka), tetrabutylammonium hydroxide (TBAOH, Sigma, 40% w/w solution in water), and $[\text{D}_6]\text{H}_2$ dimethyl sulfoxide (DMSO- d_6 , >99.8%, Aldrich, Milwaukee, WI, USA) were used as received.

2.2. pH-Zone-refining CCC

2.2.1. Instrumentation—The separations were performed using a high-speed CCC centrifuge (P.C. Inc., Potomac, MD, USA) that holds an Ito multilayer-coil separation column and a counterweight whose centers revolve around the centrifugal axis at a distance of 10 cm. A multilayer column was constructed by one of the authors (Y.I.) from Tefzel tubing (1.6 mm I.D., with a total capacity of ~ 315 ml). The β value (a geometrical parameter) ranged from 0.5 at the internal terminal to 0.85 at the external terminal. The instrument was connected to a speed controller, an LC pump, a UV detector (254 nm) with a potentiometric recorder, and a fraction collector. The pH of the effluent in some of the separations was monitored with an on-line pH detector [23].

2.2.2. Affinity-ligand separation procedure—Detailed method development for performing affinity-ligand pH-zone-refining CCC in the ion-exchange and ion-pairing modes was described elsewhere [19].

2.2.2.1. Using an ion exchanger as the ligand (Fig. 3): 2.2.2.1.1. Affinity-ligand pH-zone-refining CCC of 1 g Y203: The two-phase solvent system consisted of isoamyl alcohol-MtBE-acetonitrile-water (3:5:1:7, v/v). To a portion of the upper organic phase (stationary phase), 20% dodecylamine (DA) was added as the ligand, which adjusted the pH of the solution to 11.7. The sample solution was prepared as follows: the sample was dissolved in 20 ml lower

aqueous phase and mixed with 20 ml of ligand-containing stationary phase. The sulfonated dyes were brought into the upper phase of the sample solution by adding 1.84 g (18 mmol) H_2SO_4 . The pH of the sample solution became ~ 0.8 . The separation was initiated by completely filling the column with ligand-free stationary phase using the LC pump. Approximately 150 ml of ligand-containing stationary phase was pumped into the column, thereby displacing part of the column contents. Then the sample solution was loaded into the column through the sample-injection valve with pressurized nitrogen (60-80 psi). After the sample solution was loaded into the column, the mobile phase, the lower aqueous phase to which NH_3 was added as eluter (113 mM solution of pH 11.2), was pumped in the head-to-tail direction through the rotating column (800 rpm) at 3 ml/min. The column effluent was monitored with a UV detector at 254 nm. Fractions of 6 ml each were collected using a fraction collector. The solvent front (first fraction containing mobile phase) emerged at fraction 35.

Reconstructed pH-zone-refining CCC elution profile was produced by HPLC analysis of a constant amount from each CCC collected fraction (25 μl diluted with 1.5 ml water and 0.5 ml MeOH). The area of the HPLC peak obtained at 254 nm was plotted and shows the relative quantities of the compounds recovered.

2.2.2.1.2. Affinity-ligand pH-zone-refining CCC of 5 g Y203: The two-phase solvent system consisted of isoamyl alcohol- *Mt*BE-acetonitrile-water (3:5:1:7, v/v). To a portion of the upper organic phase (stationary phase), 20% dodecylamine (DA) was added as the ligand, which adjusted the pH of the solution to 11.7. The sample solution was prepared as follows: the sample was dissolved in 60 ml lower aqueous phase and mixed with 60 ml of ligand-containing stationary phase. The sulfonated dyes were brought into the upper phase of the sample solution by adding 4.05 g (39.6 mmol) H_2SO_4 . The pH of the sample solution became ~ 0.8 . The separation was initiated by completely filling the column with ligand-free stationary phase using the LC pump. Approximately 100 ml of ligand-containing stationary phase was pumped into the column, thereby displacing part of the column contents. Then the sample solution was loaded into the column through the sample-injection valve with pressurized nitrogen (60-80 psi). After the sample solution was loaded into the column, the mobile phase, the lower aqueous phase to which NH_3 was added as eluter (163 mM solution of pH 11.7), was pumped in the head-to-tail direction through the rotating column (800 rpm) at 3 ml/min. The column effluent was monitored with a UV detector at 254 nm. Fractions of 6 ml each were collected using a fraction collector. The solvent front emerged at fraction 4. During this 5 g separation, carryover of the stationary phase occurred. After the 28th fraction, the flow rate was reduced to 0.6 ml/min for 12 hours, at which point the flow was returned to 3 ml/min and no more carryover of the stationary phase was observed. The level of retention of the stationary phase was determined after the separation by pushing the column contents out (with pressurized nitrogen) and measuring the volume of stationary phase relative to the total column volume [14]. Reconstructed pH-zone-refining CCC elution profile was produced as described above for the 1 g separation.

2.2.2.2. Using an ion-pairing reagent as the ligand (Fig. 4): The two-phase solvent system used for the separation of the two components that co-eluted in the 5 g Y203 separation (Fig. 3) consisted of isoamyl alcohol- *Mt*BE-acetonitrile-water (3:4:1:7, v/v). The column was filled with the upper phase which was used as the stationary phase. The sample solution was prepared as follows: 0.55 g of the mixture obtained by unifying fractions 220-246 from the above separation (Fig. 3) was dissolved in 15 ml of lower aqueous phase; to this solution, 15 ml of upper phase which contained the ion-pairing reagent (2 g of aqueous tetrabutylammonium hydroxide, 3.08 mmol) was added with mixing. The dyes partitioned mostly in the upper phase and the pH of the sample solution became ~ 12.8 . The sample solution was loaded into the column and the mobile phase, consisting of the lower aqueous phase to which was added trifluoroacetic acid (TFA), yielding a solution of 80.7 mM in TFA and of pH 1.6, was pumped

in the head-to tail direction through the rotating column as described above. In this case, fractions of 3 ml each were collected. The solvent front emerged at fraction 18. Reconstructed pH-zone-refining CCC elution profiles were produced as described above.

2.3. Analytical HPLC

The HPLC analyses were performed with a Waters Alliance 2690 separation module (Waters, Milford, MA, USA). The eluents were (A) 0.1M NH₄OAc in water/methanol (95:5, v/v) and (B) methanol. The column (Hypersil MOS-1 RPC-8, 5 µm particle size, 250 mm × 4.6 mm I.D., Keystone Sci., Bellefonte, PA, USA) was eluted by using a linear gradient of 10-100% methanol in 30 min. The effluent was monitored with a Waters 996 photodiode array detector set at 415 nm and 254 nm. Other conditions included: flow-rate, 1 ml/min; column temperature, 25°C; injection volume, 20 µl.

An aliquot (25 µl) from the pH-zone-refining CCC-collected fractions was diluted with 2 ml of water/methanol (75:25, v/v). The solution was filtered through a Uniprep 0.45-µm glass microfiber syringeless filter unit (Whatman, Clifton, NJ, USA) prior to chromatography. The concentration of the analyzed samples in Fig. 2 was 0.1 mg/ml.

2.4. Liquid chromatography–mass spectrometry

The high-resolution mass spectra of the CCC-separated QY 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 electrospray ionization (ESI) mode. The high-resolution measurements of the quasi molecular ions (M+H)⁺ of the isolated components were as follows: **6',4-diSA** *m/z* 434.00124, **6',5-diSA** *m/z* 434.00109, **8',4-diSA** *m/z* 434.00076, **8',5-diSA** *m/z* 434.00096, all of which matched the calculated mass for C₁₈H₁₂N₁O₈S₂ of 433.99988, the protonated quinophthalone substituted with two sulfonic acid groups; and **6',8',5-triSA** *m/z* 513.95744, which matched the calculated mass for C₁₈H₁₂N₁O₁₁S₃ of 513.9567, the protonated quinophthalone substituted with three sulfonic acid groups.

2.5. ¹H Nuclear magnetic resonance (NMR) spectroscopy

The ¹H NMR and COSY spectra of 6',5-, 8',5-, 6',4-, and 8',4-diSA and 6',8',5-triSA were determined on a Varian VXR NMR spectrometer operating at 400 MHz. Approximately 3 mg of the purified compounds were dissolved in 140 µl of DMSO-*d*₆. The following signals were obtained and assigned for each of the isolated sulfonated isomers: **6',5-diSA**, [2-(2-quinolinyl)-1*H*-indene-1,3(2*H*)-dione-6',5-disulfonic acid, (Fig. 5)], 8.52 (9.3 d, H-4'), 8.48 (9.3 d, H-3'), 8.18 (1.75 d, H-5'), 8.0 (8.5, 1.75 dd, H-7'), 7.92 (8.5 d, H-8'), 7.89 (8.1.5 dd, H-6), 7.8 (1.5,0.7 dd, H-4), and 7.6 (8,0.7 dd, H-7); **6',8',5-triSA**, [2-(2-quinolinyl)-1*H*-indene-1,3(2*H*)-dione-6',8',5-trisulfonic acid, (Fig. 5)], 8.7 (9.3 d, H-3'), 8.52 (9.3 d, H-4'), 8.36 (1.75 d, H-5'), 8.14 (1.75 d, H-7'), 7.9 (8.1.5 dd, H-6), 7.88 (1.5 d, H-4a), 7.8 (1.5 d, H-4b), 7.68 (8 d, H-7a), and 7.62 (8 d, H-7b); **8',4-diSA**, [2-(2-quinolinyl)-1*H*-indene-1,3(2*H*)-dione-8',4-disulfonic acid, (Fig. 6)], 8.7 (9.3,1.7 dd, H-3'), 8.49 (9.3 d, H-4'), 8.14 (7.7,1.75 dd, H-7'), 8.0 (8.1.75 dd, H-5), 7.99 (7.7,1.5 dd, H-5'), 7.69 (8.1.75 dd, H-7), 7.65 (8 t, H-6), and 7.55 (7.7 t, H-6'); **6',4-diSA**, [2-(2-quinolinyl)-1*H*-indene-1,3(2*H*)-dione-6',4-disulfonic acid, (Fig. 7)], 8.53 (9.3 d, H-3'), 8.49 (9.3 d, H-4'), 8.15 (1.75 d, H-5'), 7.96 (8.5,1.75 dd, H-7'), 7.95 (8.1.5 dd, H-5), 7.94 (8.5 d, H-8'), 7.59 (8 t, H-7), and 7.58 (8 d, H-6); **8',5-diSA**, [2-(2-quinolinyl)-1*H*-indene-1,3(2*H*)-dione-8',5-disulfonic acid, (Fig. 7)], 8.7 (9.3 d, H-4'), 8.48 (9.3,4 dd, H-3'), 8.1 (7.7,1.75 dd, H-7'), 7.96 (7.7, 1.75 dd, H-5'), 7.92 (1.5 d, H-4a), 7.88 (8, d, H-6), 7.8 (1.5 d, H-4b), and 7.64 (8 d, H-7a), 7.59 (8 d, H-7b), and 7.52 (7.7 td, H-6').

3. Results and discussion

Sulfonated dyes tend to partition almost exclusively in the aqueous phase of a conventional two-phase solvent system, even if the aqueous phase is made highly acidic. Addition of a hydrophobic ligand facilitates the retention of the dyes in the organic stationary phase. For the separation of sulfonated dyes by affinity-ligand pH-zone-refining CCC, two kinds of ligands have been used successfully: ion exchange reagents also called hydrophobic counterions [14] (i.e., dodecylamine and tridodecylamine), which are always retained in the organic stationary phase; and ion-pairing reagents (i.e., tetrabutylammonium hydroxide), which partition into either phase. The recommended steps for selecting an appropriate two-phase solvent system for the separation of sulfonated dyes by affinity-ligand pH-zone-refining CCC in the ion-exchange and ion-pairing modes have been reported [19] and were followed in the present study. Addition of an ion-exchange reagent (dodecylamine, 5%) to both sample solution and organic stationary phase enabled separation of the monosulfonated components of QY [20]. When those conditions were implemented in the present work, however, the di- and trisulfonated components could not be separated. To separate those more polar components of QY, a higher concentration of ion-exchange reagent was needed as well as complementary use of the ion-exchange and ion-pairing separation modes.

3.1. Ion-exchange mode

Fig. 3 shows reconstructed pH-zone-refining CCC elution profiles of separations of 1 g- and 5 g-sample portions of Y203 in which the ion-exchange reagent concentration in both sample solution and stationary phase was raised to 20%. The retention of the stationary phase measured after the 1 g separation was 18%. Applying the same conditions, it was observed that for the 5 g separation the retention of the stationary phase would have been 0% due to the carryover caused by the high sample load. To eliminate the carryover of the stationary phase due to lower interfacial tension in the mixing area during a 5 g separation, and to retain the stationary phase in the column, two approaches were applied. In the first approach, at the point in the separation when the column contained ~50% mobile phase and 50% stationary phase, the flow rate of the mobile phase was reduced to one-fifth of the original flow rate (i.e., 0.6 ml/min instead of 3 ml/min) for 12 hours (overnight), and then the flow rate was increased to its original value. This procedure resulted in a satisfactory retention of the stationary phase of 36.1%.

The second approach (applied to a different 5 g separation, see below) was to stop pumping the mobile phase while continuing to rotate the column. This procedure produced a countercurrent movement of the two phases along the column (lengthwise) due to the Archimedean screw effect [24], which brought the stationary phase closer to the head of the column. After a certain period of just rotation (overnight for the 5 g separation, but generally 2-3 hours was found to be sufficient for smaller sample separations), no more carryover of the stationary phase was observed when the flow rate of the mobile phase was increased to its original flow rate, and a satisfactory retention of the stationary phase was obtained. One reason that no more carryover of the stationary phase occurred after the rotation without pumping is that the sample concentration became greatly reduced, which in turn stabilized the two-phase solvent separation.

Since the loss of stationary phase was avoided by these methods, a significant separation was obtained for two major components: 6',5-diSA (1.21 g of over 99% purity by HPLC) and 6', 8',5-triSA (1.69 g of over 99% purity by HPLC). Their ¹H NMR characterization is shown in Fig. 5. Two other components (0.55 g) eluted together as shown in Fig. 3. A similar procedure was applied to a 5 g sample consisting of a mixture of QY of European and Japanese origin, with the differences being the ratio of the solvents used (3:1:1:5, v/v), use of HCl in the sample solution and use of only 10% DA in the stationary phase. The retention of the stationary phase after the separation in this case was 14.1%. As a result, an additional minor disulfonic acid, 8',

4-diSA (4.8 mg of over 94% purity by HPLC) not previously reported was obtained. Its ^1H NMR characterization is shown in Fig. 6.

The mechanism of separation of the di- and trisulfonated components using an ion exchanger as the ligand is similar to the mechanism proposed for the separation of the monosulfonated components described earlier [20].

3.2. Ion-pairing mode

By using an ion-pairing reagent (tetrabutylammonium hydroxide) as the ligand in the sample solution, the pH-zone-refining CCC separation of the two components that eluted together (0.55 g mixture) in Fig. 3 were well-separated, as shown in Fig. 4. The retention of the stationary phase after separation was 75%. Compound 6',4-diSA (Fractions 31 and 32, 20.7 mg of over 98% purity by HPLC) is a newly-identified disulfonated positional isomer of QY. Its ^1H NMR characterization is shown in Fig. 7. It is noteworthy that 6',5-diSA and 6',4-diSA completely overlap and elute together under the HPLC conditions used, but are well separated by complementary use of ion-exchange and ion-pairing modes of affinity-ligand pH-zone-refining CCC (Fig. 2, Fig. 5 and Fig. 7). The second separated component of the mixture (fractions 70 -120, 111.8 mg of over 98% purity by HPLC) was identified as 8',5-diSA, a common component of the European and Japanese versions of QY whose ^1H NMR characterization is shown in Fig. 7.

In addition to characterization by ^1H NMR spectroscopy, the isolated di- and trisulfonated dyes were characterized by high-resolution mass spectra. The HRMS provided the elemental composition of the isolated isomers. Structural assignment of the QY disulfonated positional isomers by various mass spectrometric techniques was previously reported [25,26].

Assignments of most of the proton NMR signals in the di- and trisulfonated compounds were relatively straightforward. Ambiguities arose with (i) the 3',4'-protons in all five compounds, (ii) differentiating terminal protons for contiguous 3-spin systems, *e.g.* both 5'/7' and 5/7 in 8',4-diSA, and (iii) 5',7' in 6',8',5-triSA. However, COSY spectra revealed *peri* (H-4',5') and/or *zigzag* (H-4',8') coupling in each of the five compounds. The 5/7-protons could be differentiated in 6',4- and 8',4-diSA by virtue of the stronger *ortho*-deshielding effect of the 4-sulfonate group on the 5-protons. These assignments are consistent with those of two previous investigations [3,8] but at variance with certain designations of two others, *viz.* interchanging the assignments of the 3'/4' protons in 8',5-diSA [4] and both the 3'/4' and 5'/7' protons in 6',8',5-triSA [5]. However, no discussion is provided for the NMR spectral assignments in reference [5].

Interestingly, two sets of signals are observed for protons 4 and 7 in 8',5-diSA (similar to [8]) and also in 6',8',5-triSA. Our spectra (and presumably those of [8]) were recorded at room temperature while those in [4] and [5] were obtained at 80° and 60° C, respectively, where no additional signal multiplicities were observed. Restricted rotation [8] about the 2',2-bond and slower tautomeric interconversion would be expected to occur at lower temperatures and give rise to *cis* and *trans* sets of protons at C-4 and C-7. Moreover, "extra" couplings were observed for the 3'-protons in 8',4-diSA and 8',5-diSA. However, these protons exhibit only *ortho*-couplings to H-4' in their COSY spectra. The additional splittings are likely due to NH couplings in tautomers in which the 1'-nitrogen is protonated.

4. Conclusions

It was shown that by optimizing the conditions for pH-zone-refining CCC (*e.g.*, using a higher concentration of ligand in the stationary phase and taking advantage of the effect of the Archimedean screw), multi-gram mixtures of very polar compounds, such as the polysulfonated components of Quinoline Yellow can be separated and/or purified.

Complementary use of ligands (ion-exchange and ion-pair reagents) in affinity-ligand pH-zone-refining CCC accomplished the difficult task of separating closely-related disulfonated positional isomers components of Quinoline Yellow. No other preparative-scale chromatographic separation of multi-gram quantities of these dyes has been previously reported.

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Figure 1.

Preparation of Quinoline Yellow by condensing quinaldine, **1**, with phthalic anhydride, **2**, and sulfonating the condensation product **3**. The degree of sulfonation will determine the content in mono-, di- and trisulfonated components of the final product.

Figure 2.
HPLC analyses of sample portions of Quinoline Yellow, (a) US-certified as D&C Yellow No. 10, (b) E104 (Europe), (c) Yellow No. 203 (Japan).

Figure 3. Reconstructed pH-zone-refining CCC elution profiles of separations of 1 g and 5 g sample portions of Yellow No. 203 by affinity-ligand pH-zone-refining CCC in the ion exchange mode.

Figure 4.

Reconstructed pH-zone-refining CCC elution profile of the separation by affinity-ligand pH-zone-refining CCC in the ion-pairing mode of the two components (0.55 g mixture) of Yellow No. 203 that elute together in the separation shown in Figure 3.

Figure 5.
 ^1H NMR spectra and COSY assignments of 6',5-diSA and 6',8',5-triSA.

Figure 6.
(a) HPLC and (b) ^1H NMR spectrum with COSY assignments of 8',4-diSA.

Figure 7.
 ^1H NMR spectra and COSY assignments of 6',4-diSA and 8',5-diSA.