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# 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☆

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ABSTRACT

In developing analytical methods for batch certification of the color additive D&C Green No. 8 (G8), the U.S. Food and Drug Administration needed the trisodium salt of 1,3,6-pyrenetrisulfonic acid (P3S) for use as a reference material. Since P3S was not commercially available, preparative quantities of it were separated from portions of a sample of G8 that contained ~3.5% P3S. The separations were performed by pH-zone-refining counter-current chromatography using dodecylamine (DA) as the hydrophobic counterion. The added DA enabled partitioning of the polysulfonated components into the organic stationary phase of the two-phase solvent system used, 1-butanol–water (1:1). Thus, a typical separation that involved 20.3 g of G8, using sulfuric acid as the retainer acid and 20% DA in the stationary phase and 0.1 M sodium hydroxide as the mobile phase, resulted in ~0.58 g of P3S of greater than 99% purity. The identification and characterization of the separated P3S were performed by elemental analyses, proton nuclear magnetic resonance, high-resolution mass spectrometry, ultra-violet spectra, and high-performance liquid chromatography.

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## 1. Introduction

D&C Green No. 8 (G8, pyranine, Color Index No. 59040, mainly the trisodium salt of 8-hydroxy-1,3,6-pyrenetrisulfonic acid, HP3S) is a color additive used in externally applied drugs and cosmetics in the U.S. [1]. It is manufactured by sulfonating pyrene to form the tetrasulfonic acid compound, P4S, followed by hydrolyzing one of the sulfonic acids to yield the monohydroxytrisulfonate, which is isolated as the trisodium salt, HP3S [2] (Fig. 1). During manufacture, various contaminants may be produced, including 1,3,6-pyrenetrisulfonate, P3S. G8 is subject to batch certification by the U.S. Food and Drug Administration (FDA) to ensure compliance with certain chemical specifications including "not more than 6 percent" of P3S [1]. To develop analytical methods for batch certification of G8, purified P3S is required as a reference material but is not commercially available. In the past, small amounts of P3S were donated by a color additive manufacturer for use as a standard for the development of thin-layer chromatography methods for its determination in G8 [3,4].

The present study describes the separation of preparative quantities of P3S from a sample of G8 that contained ~3.5% P3S, using pH-zone-refining counter-current chromatography [5–7]. This liquid–liquid chromatographic technique enables the separation of organic acids and bases according to their p$K_a$ values and hydrophobicities without using a solid support. The separation procedure and mechanism of separation by this method as well as many applications of this technique have been reviewed [8–10]. Compounds containing one ore more carboxylic acid groups are common targets for pH-zone-refining CCC. Preparative separations have been reported for compounds containing isomeric and stereoisomeric mono- and dicarboxylic acids [11–13], amino acids and peptides [8] and monocarboxylated both natural products [14–16] and fluorescein dyes [17]. Separations of compounds containing sulfonic acids using conventional pH-zone-refining CCC are less common [12,18]. Dyes containing sulfonic acid groups are more hydrophilic than the carboxylic acid dyes and their very low p$K_a$ values prevent their partitioning into the organic phase of a conventional two-phase solvent system. It was shown that the addition of a ligand (an ion-exchanger, e.g., dodecylamine, or an ion-pair reagent, e.g., tetrabutylammonium hydroxide) to both the sample

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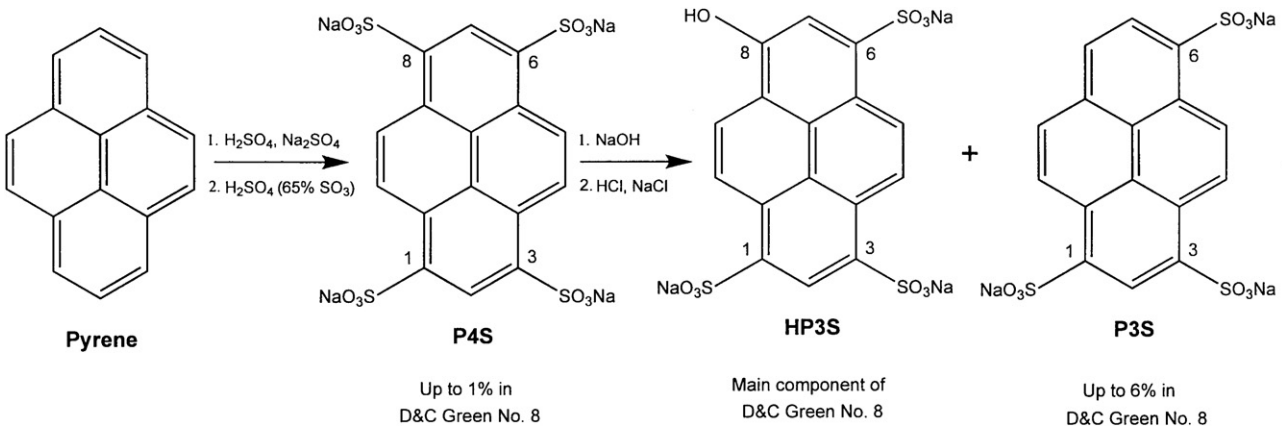


**Fig. 1.** Preparation of D&C Green No. 8 by sulfonation of pyrene.

solution and the organic stationary phase enables separation of sulfonated dyes by pH-zone-refining CCC because the ligand facilitates their partitioning into the organic stationary phase [8,19–21]. For the separation of the components of G8, use was made of dodecylamine as an ion-exchanger because it permanently stays in the stationary phase to act as the counterion for the sulfonic acid-substituted dye components. Other very hydrophilic compounds, depolymerized fucans [22] and naphthalenesulfonic acids [23], were fractionated or separated, respectively, using a ligand (the anion exchanger Amberlite LA2) in the organic stationary phase.

The present work describes the conditions that permitted separation by pH-zone-refining CCC of P3S from a mixture of closely related, very polar components in which the quantity of P3S is far outweighed by the quantity of HP3S. The separated P3S and HP3S are characterized by high-resolution mass spectrometry, $^1$H nuclear magnetic resonance (NMR) spectroscopy, and $^1$H–$^1$H NMR correlated spectroscopy (COSY), UV–vis spectrophotometry, and high-performance liquid chromatography.

## 2. Experimental

### 2.1. Materials

The samples of G8 used in this study were from batches submitted to the FDA for certification. Methanol, water, and ammonium acetate ($NH_4OAc$) were of chromatography grade. Dodecylamine (DA, 98%) and 1-butanol were from Sigma–Aldrich, St. Louis, MO, USA. Sulfuric acid ($H_2SO_4$, 95.9%) was from Fisher Scientific, Fair Lawn, NJ, USA, and sodium hydroxide (NaOH, A.C.S. reagent grade) was from J.T. Baker, Phillipsburg, NJ, USA.

### 2.2. pH-zone-refining CCC

#### 2.2.1. Instrumentation

The separations were performed with a commercial J-type high-speed CCC system (Model CCC-1000, Pharma-Tech Research, Baltimore, MD, USA) that consisted of (i) a column (three Ito-multilayer coils, connected in series, made of 2.6-mm i.d. PTFE tubing, with a total capacity of 800 ml) mounted on a rotating frame (centrifuge), (ii) a rotation-speed controller, and (iii) an LC-10AD$_{VP}$ pump (Shimadzu, Kyoto, Japan). The column effluent was monitored with a UV detector, model UVicord SII, with a 254-nm UV lamp (Pharmacia LKB, Uppsala, Sweden) and a chart recorder (Kipp & Zonen, Delft, The Netherlands). The effluent was collected using a Foxy fraction collector (Isco, Lincoln, NE, USA). The pH of the eluted fractions was measured manually using an Accumet AP61 pH meter (Fisher Scientific, Fair Lawn, NJ, USA).

#### 2.2.2. Separation procedure

The pH-zone-refining CCC separations were performed following the general procedures described previously [10,19]. The two-phase solvent system consisted of 1-butanol–water (1:1, v/v). The solvent system was equilibrated in a separatory funnel and the two phases were separated before use. To a portion of the upper organic phase (stationary phase), 20% (v/v) DA was added as the ligand and then 0.5% (v/v) conc. $H_2SO_4$ was added to the mixture. The sample solution was prepared as follows: the sample (20.3 g of G8 that contained ~3.5% P3S) was dissolved in 50 ml of lower aqueous phase followed by addition of 50 ml of stationary phase (that contained the DA and $H_2SO_4$). The sulfonated dyes were brought into the upper phase of the sample solution by adding to it another

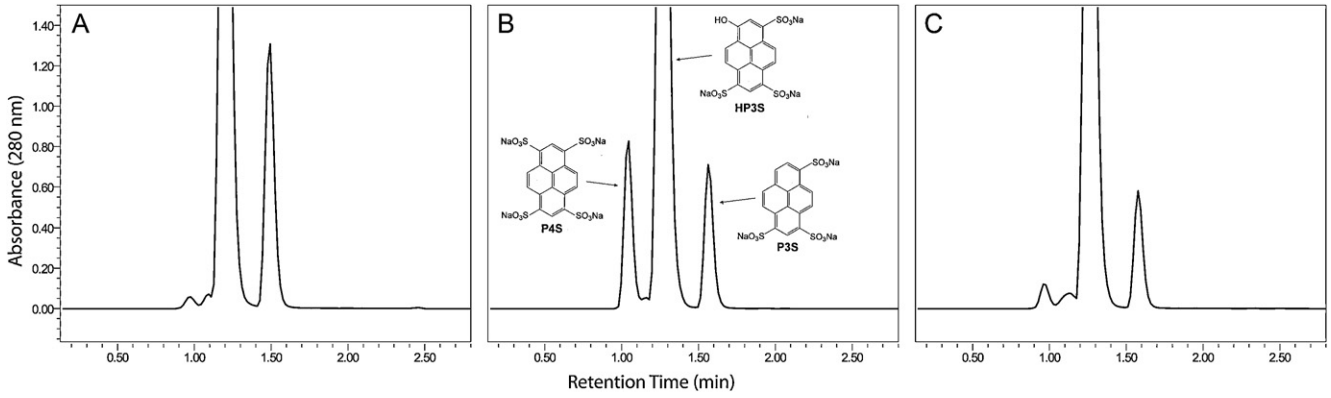


**Fig. 2.** Analytical HPLC chromatograms of sample portions of commercial D&C Green No. 8 obtained from three different sources.