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Preparative separation of components of the color additive FD&C Red No. 3 (erythrosine) by pH-zone-refining counter-current chromatography

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ABSTRACT

The components in 3 g of the commercial color additive FD&C Red No. 3 were separated by pH-zone-refining counter-current chromatography. The main component, 2',4',5',7'-tetraiodofluorescein, and two positional isomeric lower-iodinated subsidiary colors, 2',4',5'-triiodofluorescein and 2',4',7'-triiodofluorescein, were isolated and characterized by ^{1}H NMR and chemical ionization mass spectrometry.

INTRODUCTION

FD&C Red No. 3 (Colour Index No. 45430) is a xanthene color additive that is listed in the US Code of Federal Regulations (CFR) for use in food, drugs and cosmetics [1]. It is subject to batch certification by the US Food and Drug Administration (FDA) to ensure compliance with CFR specifications. The predominant component of FD&C Red No. 3 is the disodium salt of 2',4',5',7'-tetraiodofluorescein (**1**). Subsidiary colors, which are lower-iodinated fluoresceins, are limited to not more than 10% [1]. Under the name erythrosine B this dye is widely used as a biological stain [2,3]. FD&C Red No. 3 is synthetically prepared by the iodination of fluorescein, followed by alkaline hydrolysis of the reaction products (Fig. 1).

For FDA's batch certification of FD&C Red No. 3, pure **1** and pure lower-iodinated subsidiary colors are needed for use as reference materials. Analytical-scale [4–8] and semi-preparative-scale [9] separations of components of FD&C Red No. 3 have been previously reported. Two preparative-scale methods for the separation and purification of **1** from the commercial dye have also been reported [10,11]. One of these methods [10] involves the use of gel chromatography to separate relatively small quantities (10–30 mg) of commercial dye. The other method [11] accomplishes preparative separation of **1** from commercial erythrosine by conversion of the dye to a diacetate derivative,

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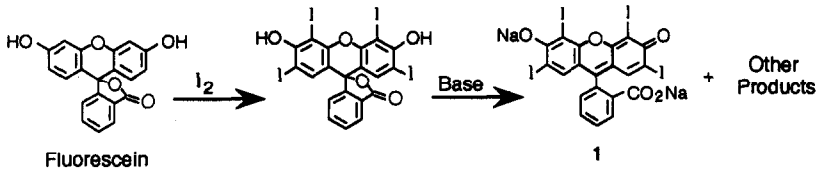

Fig. 1. Preparation of FD&C Red No. 3 (1 is the main component of this color additive).

followed by multiple recrystallizations. The diacetate is then converted to the lactone form of **1**. No preparative-scale method for the separation of lower-iodinated subsidiary colors of FD&C Red No. 3 was reported in the literature.

In the present study, a novel counter-current chromatographic (CCC) technique, pH-zone-refining CCC [12–14], was chosen for preparative separation of components of FD&C Red No. 3. pH-Zone-refining CCC allows the separation, with high resolution, of multigram quantities of organic acids. The method requires the addition of an acid, such as trifluoroacetic acid (TFA), to the solution of the organic acids or the stationary phase, followed by isocratic elution with a basic mobile phase. The acids elute as well-resolved rectangular peaks, in the order of their pK_a values and hydrophobicities [12,13]. In the present work, pH-zone-refining CCC was used to separate **1** and two of its lower-iodinated subsidiary colors from 3 g of commercial FD&C Red No. 3.

EXPERIMENTAL

Materials

The FD&C Red No. 3 used in this study originated from a commercial lot submitted to FDA for batch certification. Ammonium acetate (NH_4OAc), methanol, water and acetonitrile were chromatography grade. Diethyl ether (anhydrous) and hydrochloric acid (36.5–38.0%) were ACS reagent grade. Sodium sulfate anhydrous (granular) was analytical-reagent grade. Ammonium hydroxide (28–30% in water, Fisher Scientific, Pittsburgh, PA, USA), TFA (Sigma, St. Louis, MO, USA), deuterium oxide (^2H_2O, 99.9%, MSD Isotopes, Montreal, Canada) and sodium deuteroxide (NaO^2H, 99.9%, *ca.* 40% in ^2H_2O, Fluka, Buchs, Switzerland) were used as received.

pH-Zone-refining counter-current chromatography

The separation was performed using a commercial high-speed CCC centrifuge (P.C., Potomac, MD, USA) that holds an Ito multilayer-coil separation column and a counterweight whose centers revolve 10 cm around the centrifugal axis. A multilayer column was constructed by one of us (Y.I.) from polytetrafluoroethylene tubing (*ca.* 165 m × 1.6 mm I.D., with a total capacity of approximately 325 ml). The β value (a centrifugal parameter) [15] ranged from 0.5 at the internal terminal to 0.85 at the external terminal. The column consisted of 16 coiled layers. (Similar columns are commercially available from P.C.; Pharma-Tech Research Corp., Baltimore, MD, USA; and Shimadzu, Kyoto, Japan.)

The two-phase solvent system consisted of diethyl ether–acetonitrile–0.01 *M* aqueous NH_4OAc (4:1:5). The solvent system was thoroughly equilibrated in a separatory funnel and the two phases were separated shortly before use. The lower phase was adjusted to pH 7.53 with NH_4OH. TFA (400 μl) was added to the upper phase (500 ml).

For the preparative separation, 3 g of FD&C Red No. 3 was suspended in 40 ml of the solvent system (20 ml of the lower phase and 20 ml of the unacidified upper phase).The separation was initiated by filling the entire column with the stationary (upper) phase by using a metering pump (Accu-Flo pump; Beckman, Palo Alto, CA, USA) and then loading the suspension of the color additive into the column by syringe. The mobile (lower) phase was then pumped into the column at 3 ml/min while the column was

rotated at 800 rpm in forward mode. The column effluent was monitored with a UV detector (Uvicord S; LKB, Stockholm, Sweden) at 206 nm, to which was attached an LKB 6-channel strip-chart recorder at a chart speed of 1 cm/20 min with a full-scale response of 2 absorbance units. Fractions (6 ml) were collected using a fraction collector (Ultrorac, LKB). The pH of each eluted fraction was measured with a pH meter (Accumet 1001, Fisher Scientific). The separated fractions were analyzed by analytical reversed-phase high-performance liquid chromatography (RP-HPLC).

Analytical RP-HPLC

The system used was previously described [16,17]. It consisted of a Model 8800 ternary pump, Model 8500 dynamic mixer, Model 8780 autosampler and Model 4270 integrator (all Spectra-Physics, San Jose, CA, USA), and a Model 490 dual-wavelength UV–Vis detector set at 254 and 520 nm (Waters Assoc., Milford, MA, USA). The autosampler was equipped with a Model 7010 injector (Rheodyne, Cotati, CA, USA) with a 20-μl sample loop. A Hypersil MOS-1 RPC-8 column (5 μm particle size, 250 × 4.6 mm I.D., Keystone Scientific, Bellefonte, PA, USA) was used throughout.

The eluents used were 0.1 *M* aqueous NH_4OAc and methanol. The column was eluted using consecutive linear gradients of 25 to 90% methanol in 25 min, 90 to 100% methanol in 5 min, and 100% methanol for 5 min. The column was re-equilibrated with 25% methanol for 15 min. Other conditions were injection volume, 20 μl; full scale response, 0.128 absorbance units; and flow-rate, 1 ml/min.

An aliquot of each selected fraction from the pH-zone-refining CCC separation was diluted with approximately 2 ml of methanol–water (50:50, v/v). The solution was filtered through a Uniprep 0.45-μm glass microfiber syringeless filter unit (Whatman, Clifton, NJ, USA) prior to chromatography.

Isolation of iodinated fluoresceins from pH-zone-refining CCC fractions

The iodinated fluorescein dyes were isolated in the lactone form, as previously described for the brominated tetrachlorofluoresceins [17]. Fractions with the same pH values and RP-HPLC retention times were combined and concentrated to *ca.* 5 ml on a rotary evaporator at *ca.* 30 Torr (1 Torr = 133.322 Pa) and 50°C. The residue was acidified with 20–40 ml of 10% HCl and the precipitated lactones were extracted into ethyl acetate. The organic layer was washed twice (10 or 20 ml water) and dried (anhydrous Na_2SO_4), and the solvent was evaporated.

Mass spectrometry

The negative ion chemical ionization (NICI) mass spectra were obtained on a Finnigan Mat TSQ-46 quadrupole mass spectrometer interfaced to an INCOS 2300 data system. The instrument was operated at a source temperature of 100°C, ionization energy of 70 eV, emission current of 0.35 mA, 0.25 Torr methane and preamplifier setting of 10^{-8} A/V, and was scanned from *m/z* 100 to 900 in 1.0 s. The iodofluoresceins (lactone form) were dissolved in methanol and were introduced into the mass spectrometer via the direct chemical ionization probe at a probe heating rate of 20 mA/s. Fragmentation patterns are given for [*m/z* (relative intensity)]: 2′,4′,5′,7′-tetraiodofluorescein: 836 (100.0%, $M^{-\cdot}$), 709 [31%, $(M-I)^-$]; 2′,4′,7′-triiodofluorescein: 710 (100.0%, $M^{-\cdot}$); and 2′,4′,5′-triiodofluorescein: 710 (100.0%, $M^{-\cdot}$).

^1H Nuclear magnetic resonance

^1H NMR spectra were obtained on a Varian XL 300 Fourier transform NMR spectrometer at 300 MHz. Typical concentrations consisted of 4 mg of separated component in the lactone form, dissolved in 0.5 ml of 0.5% NaO^2H in ^2H_2O. The following signals were obtained and assigned for each of the three isolated components: 2′,4′,5′,7′-Tetraiodofluorescein: 7.72 ppm (d, H-a); 7.61 ppm (s, 2H-b,c); 7.56 ppm (t), 7.46 ppm (t), 2H-d,e; 6.86 ppm (d, H-f). 2′,4′,7′-Triiodofluorescein: 7.74 ppm (d,d, H-a); 7.61 ppm (s, 2H-b,c); 7.60 ppm, 7.53 ppm (m, 2H-d,e); 7.03 ppm (d, H-f); 6.59 ppm (s, H-g). 2′,4′,5′-Triiodofluorescein: 7.72 ppm (d,d, H-a); 7.62 ppm (s, H-b); 7.55 ppm (t,d), 7.46 ppm (t,d), 2H-c,d;

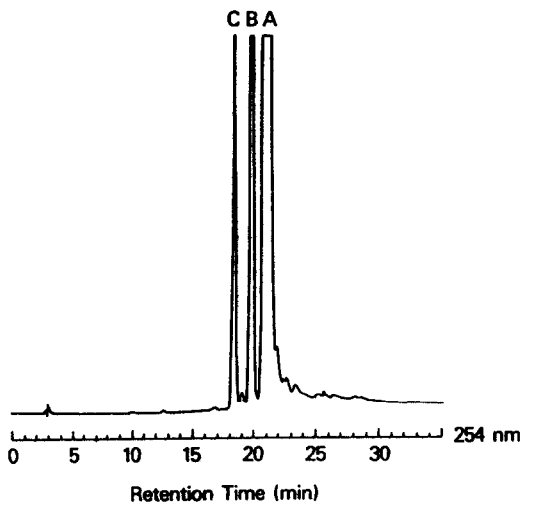

Fig. 2. Analytical RP-HPLC of the commercial lot of FD&C Red No. 3 used in this study.

6.97 ppm (d, H-e); 6.96 ppm (d,d, H-f); 6.59 ppm (d, H-g).

RESULTS AND DISCUSSION

Analytical RP-HPLC of the FD&C Red No. 3 used in the present work gave three peaks (Fig. 2); 3 g of this mixture were used for the preparative separation by pH-zone-refining CCC. The counter-current chromatogram of the separation is shown in Fig. 3. The solvent front (first fraction containing mobile phase) emerged at fraction 13. The retention of the stationary phase, calculated after the separation, was 66.7% of the total column capacity. The chromatogram has a broad rectangular shape as previously obtained for pH-zone-refining CCC [12–14]. The three broad absorbance plateaus (solid line) correspond to the three pH plateaus (dotted line). Each plateau represents elution of a pure compound. The eluates corresponding to

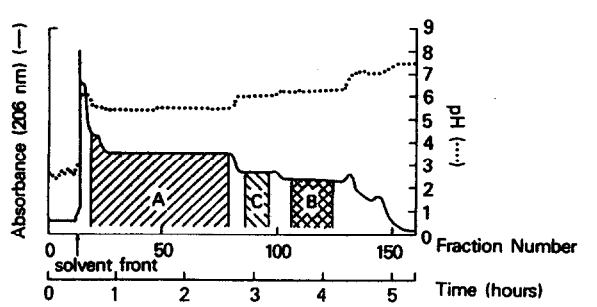

Fig. 3. pH-Zone-refining counter-current chromatogram for the separation of the components in 3 g of FD&C Red No. 3. A, B and C are the eluted fractions containing pure compounds corresponding to peaks A, B and C in Fig. 2.

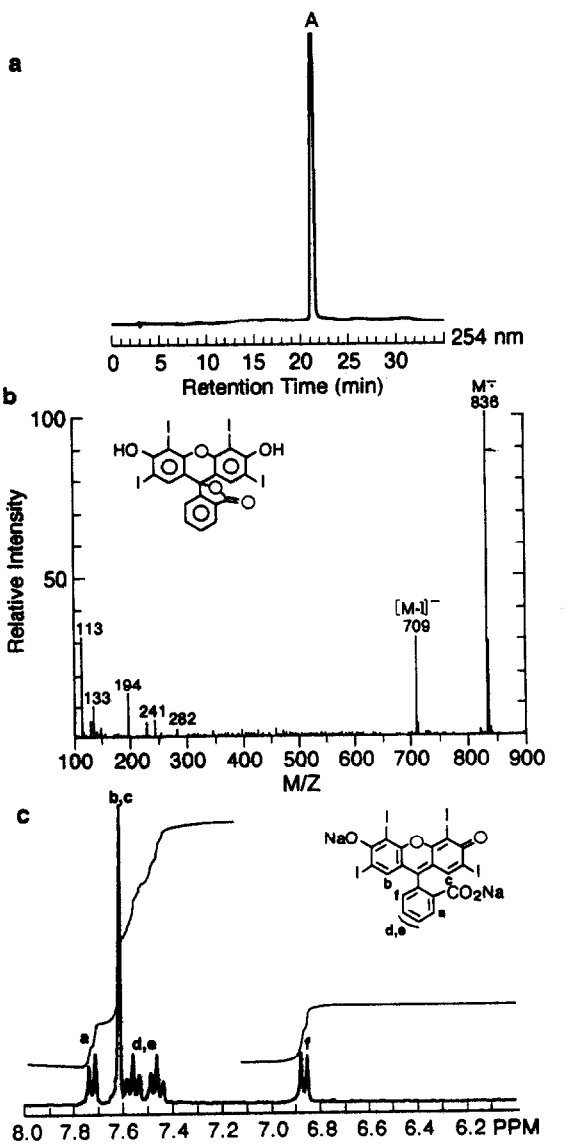

Fig. 4. Characterization of the compound contained in fractions 25–79 of the pH-zone-refining CCC separation (see Fig. 3). (a) Analytical RP-HPLC of the combined CCC fractions 25–79, (b) negative ion chemical ionization (methane) mass spectrum of fractions 25–79, (c) ^{1}H NMR spectrum of fractions 25–79 (in $NaO^2H/^2H_2O$, 300 MHz).

these plateaus were collected in fractions 25–79, 86–96 and 106–124.

Fractions 25–79 contained a single component whose RP-HPLC peak (Fig. 4a) corresponded to peak A in Fig. 2. This component was isolated as the lactone (400 mg) and identified, by CI mass spectrometry (MS) and ^{1}H NMR, as 2',4',5',7'-tetraiodofluorescein (Fig. 4b and c). It should be noted that the response before the first absorbance plateau (Fig. 3, fractions 15–18) decreases in intensity until its level stabilizes (at fraction 25). The eluate corresponding to these fractions

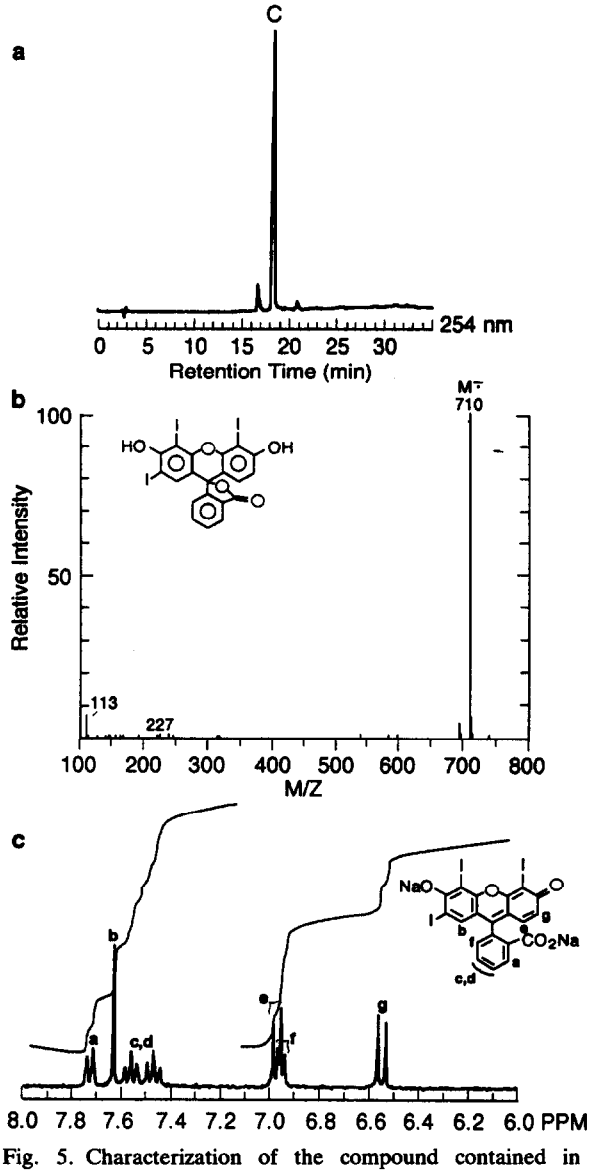

Fig. 5. Characterization of the compound contained in fractions 86–96 of the pH-zone-refining CCC separation (see Fig. 3). (a) Analytical RP-HPLC of the combined fractions 86–96, (b) negative ion chemical ionization (methane) mass spectrum of fractions 86–96, (c) ^{1}H NMR spectrum of fractions 86–96 (in $NaO^2H/^2H_2O$, 300 MHz).

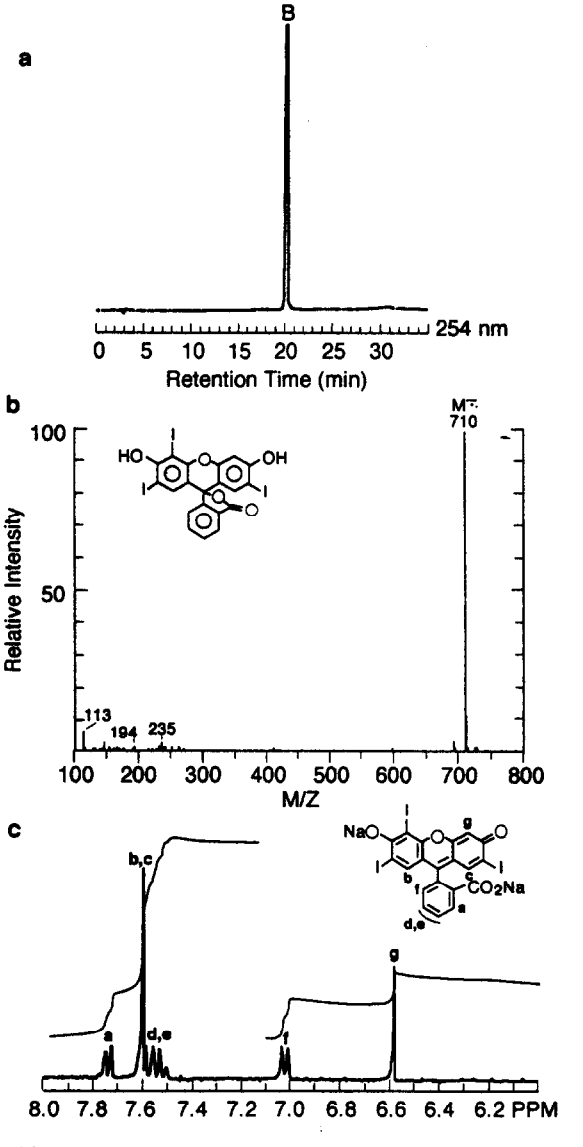

Fig. 6. Characterization of the compound contained in fractions 106–124 of the pH-zone-refining CCC separation (see Fig. 3). (a) Analytical RP-HPLC of the combined fractions 106–124, (b) negative ion chemical ionization (methane) mass spectrum of fractions 106–124, (c) ^{1}H NMR spectrum of fractions 106–124 (in $NaO^2H/^2H_2O$, 300 MHz).

consisted of a suspension that contained **1** slightly contaminated with other impurities. By optimizing the quantity of retainer acid (TFA) added to the stationary phase in future separations, the recovery of pure **1** may be improved.

Fractions 86–96 contained a single component whose RP-HPLC peak (Fig. 5a) corresponded to peak C in Fig. 2. The compound was isolated as the lactone (22 mg) and identified, by CI-MS and ^{1}H NMR, as 2′,4′,5′-triiodofluorescein (Fig. 5b and c).

Fractions 106–124 contained a single component whose RP-HPLC peak (Fig. 6a) corresponded to peak B in Fig. 2. The compound was isolated as the lactone (52 mg) and identified, by CI-MS and ^{1}H NMR, as 2′,4′,7′-triiodofluorescein (Fig. 6b and c).

CONCLUSIONS

This work demonstrates that pH-zone-refining CCC is an effective method for the preparative-scale separation and purification of acidic components of FD&C Red No. 3, including the two isomeric triiodofluorescein subsidiary colors (2′,4′,5′- and 2′,4′,7′-triiodofluorescein). Most previous references in the literature to those two subsidiary colors do not describe how the compounds were obtained or isolated. The one case that describes the preparation and isolation of a triiodofluorescein [18] does not indicate whether the isolated compound represents a single isomer or both triiodo isomers. The present study, together with previous work [12,14,19,20], demonstrates that pH-zone-refining CCC is an effective technique for the preparative-scale separation and purification of acidic components of xanthene dye mixtures.

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