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Journal of Chromatography A, 678 (1994) 77–84

JOURNAL OF CHROMATOGRAPHY A

Preparative separation of components of the color additive D&C Red No. 28 (phloxine B) by pH-zone-refining counter-current chromatography☆

Adrian Weisz[a,*], Denis Andrzejewski[b], Yoichiro Ito[c]

[a]*Office of Cosmetics and Colors, US Food and Drug Administration, Washington, DC 20204, USA*
[b]*Office of Scientific Analysis and Support, US Food and Drug Administration, Washington, DC 20204, USA*
[c]*Laboratory of Biophysical Chemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA*

(Received March 3rd, 1994)

Abstract

A pH-zone-refining counter-current chromatographic method was developed for the preparative (multigram) separation and purification of components of the commercial color additive D&C Red No. 28 (phloxine B). The chromatography of 3 and 6 g of color additive yielded 1.07 and 4.06 g, respectively, of pure 2',4',5',7'-tetrabromo-4,5,6,7-tetrachlorofluorescein, the principal component of D&C Red No. 28. The importance of the quantity of retainer acid (trifluoroacetic acid) relative to the amount of salt in the color additive is discussed.

1. Introduction

D&C Red No. 28 (Colour Index No. 45410) is a xanthene color additive used in drugs and cosmetics in the USA. It is identified as principally **1** (the disodium salt of 2',4',5',7'-tetrabromo-4,5,6,7-tetrachlorofluorescein), and may contain $\leq 4\%$ of lower-halogenated subsidiary colors (including **2**) and $\leq 2\%$ of the ethyl ester **3** [1]. Under the name phloxine B, this dye is used as a biological stain [2,3]. D&C Red No. 28 is manufactured by bromination of 4,5,6,7-tetrachlorofluorescein (TCF), followed by alkaline hydrolysis of the reaction product (Fig. 1).

D&C Red No. 28 is subject to batch certification by the US Food and Drug Administration (FDA) [1] before it may be used for coloring drugs or cosmetics. For FDA's color additive certification program, pure **1** and pure lower-brominated subsidiary colors are needed for use as reference materials. Pure xanthene dyes are also desirable for use as biological stains. Their use would allow comparison of specimens stained in different laboratories [4–6] and promote standardization of biological stains [4,7].

Several methods for preparative-scale separation and purification of phloxine B were reported [8–10]. One of these uses acid precipitation [8] and does not separate the lower-halogenated isomers from **1**. The other methods use

* Corresponding author.

☆ Presented at the *10th International Symposium on Preparative Chromatography, Arlington, VA, June 14–16, 1993.*

0021-9673/94/$07.00
SSDI 0021-9673(94)00441-B

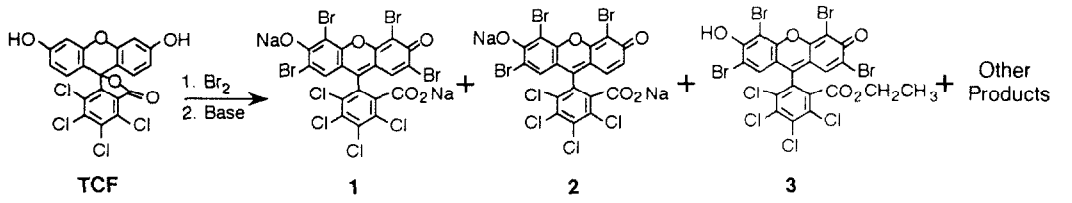

Fig. 1. Bromination of 4,5,6,7-tetrachlorofluorescein, followed by alkaline hydrolysis of the reaction product.

gel chromatography [9] and conventional high-speed counter-current chromatography (HSCCC) [10], and separate relatively small quantities (10–30 and 50 mg, respectively) of dye in each trial.

In the present study, a modified HSCCC technique, pH-zone-refining CCC [11–15], was used for the separation of multigram quantities of **1** from D&C Red No. 28. pH-Zone-refining CCC allows the separation, with high resolution, of the components of multigram mixtures of organic acids. The method requires the addition of an acid, such as trifluoroacetic acid (TFA), to the sample solution or 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 [11–14]. Although UV detection may not indicate that separations have occurred, giving one broad rectangular peak, monitoring of the eluted fractions with a pH meter results in a series of plateaus that correspond to the separated components. An increase in sample size results in lengthening each plateau without changing the overall elution profile [11–14]. In this work, pH-zone-refining CCC was used to separate components from 3- and 6-g portions of commercial D&C Red No. 28.

2. Experimental

2.1. Materials

Two lots of D&C Red No. 28 were selected from samples of commercial lots submitted to FDA for batch certification. Ammonium acetate (NH_4OAc), methanol, water and acetonitrile were chromatography grade. Diethyl ether (anhydrous), hydrochloric acid (36.5–38.0% HCl) and ammonium hydroxide (28–30% NH_3) were ACS-reagent grade. Anhydrous sodium sulfate (granular) was analytical-reagent grade. TFA (Sigma, St. Louis, MO, USA), deuterium oxide (99.9% ^2H, MSD Isotopes, Montreal, Canada) and sodium deuteroxide (99.9% ^2H, ca. 40% NaO^2H in ^2H_2O; Fluka, Buchs, Switzerland) were used as received.

2.2. pH-Zone-refining CCC

The separations were performed using a commercial high-speed CCC centrifuge (P.C. Inc., Potomac, MD, USA) that holds an Ito multilayer-coil separation column and a counterweight whose centers revolve 10 cm around the centrifugal axis. The 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) [16] 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. Inc.; Pharma-Tech Research Corp., Baltimore, MD, USA, and Shimadzu, Kyoto, Japan.)

The two-phase solvent system, used for both separations (experiments I and II) described below, 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 basic aqueous eluent was prepared by addition of concentrated NH_4OH to the lower (mobile) phase. TFA was added either to the upper organic (stationary) phase (experiment I) or to the sample solution (experiment II). The sample preparation is described under Results and discussion.

The separation was initiated by using a metering pump (Beckman Accu-Flo pump; Beckman, Palo Alto, CA, USA) to fill the entire column with the stationary (upper) phase, and then the suspension of the color additive was loaded into the column by syringe. The mobile (lower) phase was pumped into the column at 3 ml/min while the column was rotated at 800 rpm in the 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 set at a chart speed of 1 cm/20 min and 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, Pittsburgh, PA, USA). The separated fractions were analyzed by analytical reversed-phase high-performance liquid chromatography (RP-HPLC).

2.3. Analytical RP-HPLC

The system used was previously described [10,17]. It consisted of a Model 8800 ternary pump, Model 8500 dynamic mixer, Model 8780 autosampler, Model 4270 integrator (all Spectra-Physics, San Jose, CA, USA) and a Model 490 multiwavelength 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 were 0.1 *M* aqueous NH_4OAc and methanol. The column was eluted by 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.

2.4. Isolation of the halogenated fluoresceins from pH-zone-refining CCC fractions

The halogenated fluoresceins were isolated in the lactone form, as previously described [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), dried (anhydrous Na_2SO_4), and the solvent was evaporated.

Recoveries of the main component **1** were calculated from the amounts of **1** in the dyes as determined during certification analyses by the FDA (94.5% in the lot used for experiment I and 89.5% in the lot used for experiment II).

2.5. Mass spectrometry

Positive ion chemical ionization (PICI) 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 fluoresceins (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. The protonated molecular ions [m/z (relative intensity)] for 2′,4′,5′,7′-tetrabromo-4,5,6,7-tetrachlorofluorescein and 2′,4′,5′-tribromo-4,5,6,7-tetrachlorofluorescein (corresponding to A and B, respectively, in Figs. 2 and 3) were 783/785/787/789/791 (40.65:82.49:100.0:87.99:47.38, MH^+) (Fig. 4b) and 703/705/707/709/711 (15.78:63.43:

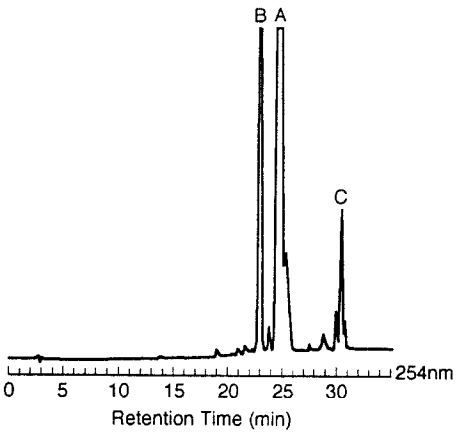

Fig. 2. Analytical RP-HPLC of the commercial lot of color additive D&C Red No. 28 used in experiment I.

100.0:91.05:47.49, MH^+) (Fig. 5b), which are similar to values previously reported for this compound [17].

2.6. ^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. 2′,4′,5′,7′-Tetrabromo-4,5,6,7-tetrachlorofluorescein (Fig. 4c): 7.38 ppm (s, 2H-a). 2′,4′,5′-Tribromo-4,5,6,7-tetrachlorofluorescein (Fig. 5c): 7.40 ppm (s, H-a), 7.00 ppm (d, H-b), 6.70 ppm (d, H-c). The NMR spectrum of 2′,4′,5′-tribromo-4,5,6,7-tetrachlorofluorescein is similar to that previously reported for this compound [17].

3. Results and discussion

3.1. Experiment I: Separation of components in 3 g of commercial D&C Red No. 28

Analytical RP-HPLC of D&C Red No. 28 gave three main 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 two-phase solvent system used consisted

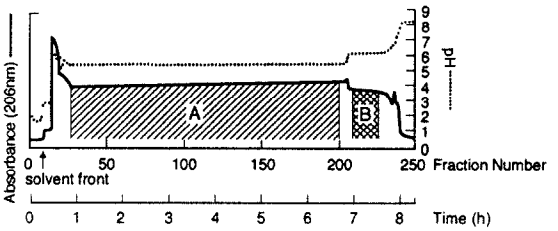

Fig. 3. pH-Zone-refining counter-current chromatogram of the separation of components in 3 g of the D&C Red No. 28 used to obtain Fig. 2. Hatched areas A and B represent fractions containing single components corresponding to peaks A and B, respectively, in Fig. 2.

of diethyl ether–acetonitrile–0.01 *M* NH_4OAc (4:1:5, v/v/v). The pH of the lower (mobile) phase was adjusted to 8.1 by addition of ammonium hydroxide. TFA (600 μl) was added to the upper, stationary phase (500 ml). The sample mixture was prepared by mixing 3 g of dye in a solvent consisting of 20 ml of the lower phase and 10 ml of the upper phase. The solvent front (first fraction containing mobile phase) emerged at fraction 8. The resulting chromatogram has a broad rectangular shape (Fig. 3) characteristic of pH-zone-refining CCC [11–15]. The two absorbance plateaus (solid line) correspond to the two pH plateaus (dotted line). Each pH plateau represents elution of a pure compound. A decrease in absorbance occurs before the first plateau (fractions 18–25). These fractions contained the main component, **1**, as a suspension, slightly contaminated with other impurities. The fractions corresponding to the pH plateaus (fractions 27–200 and 209–225) contained single components whose RP-HPLC peaks (Figs. 4a and 5a) corresponded to peaks A and B, respectively, in Fig. 2. The compounds were isolated in the lactone form (1.07 g and 61 mg, respectively) and identified by CI-MS (Figs. 4b and 5b) and ^1H NMR (Figs. 4c and 5c) as 2′,4′,5′,7′-tetrabromo-4,5,6,7-tetrachlorofluorescein and 2′,4′,5′-tribromo-4,5,6,7-tetrachlorofluorescein, respectively. The least polar component of the mixture, which corresponds to peak C in Fig. 2, remained in the stationary phase in the column in a relatively purified form, as shown by RP-HPLC in Fig. 6. It was tentatively identified as the ethyl ester, **3**, on the basis of an analytical RP-HPLC

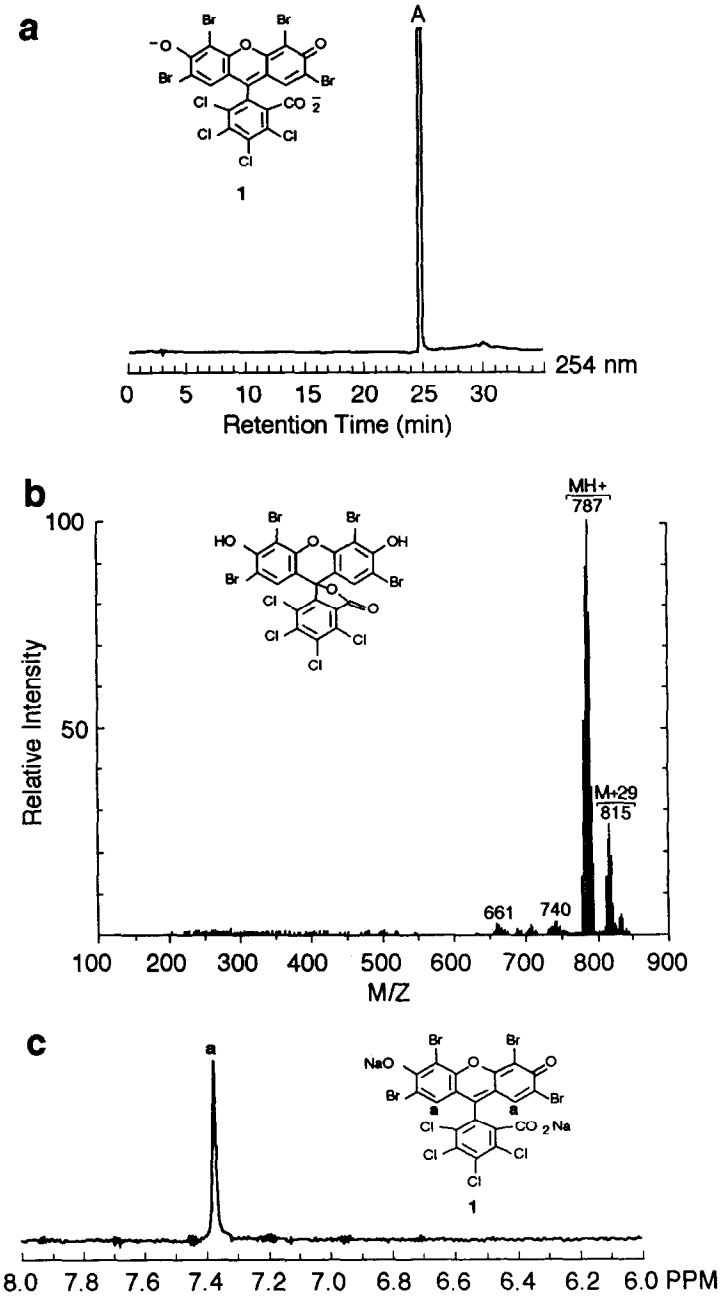

Fig. 4. Characterization of the compound contained in fractions 27–200 of the pH-zone-refining CCC separation in Fig. 3. (a) Analytical RP-HPLC of the combined fractions 27–200, (b) PICI (methane) mass spectrum and (c) ^{1}H NMR spectrum (in NaO^2H–^2H_2O, 300 MHz).

retention time similar to that of the compound which was synthesized according to a procedure for preparing the ethyl ester of Rose Bengal [18].

The ca. 39% recovery of the pure main component, **1**, in this experiment is considerably less than would be expected on the basis of previous

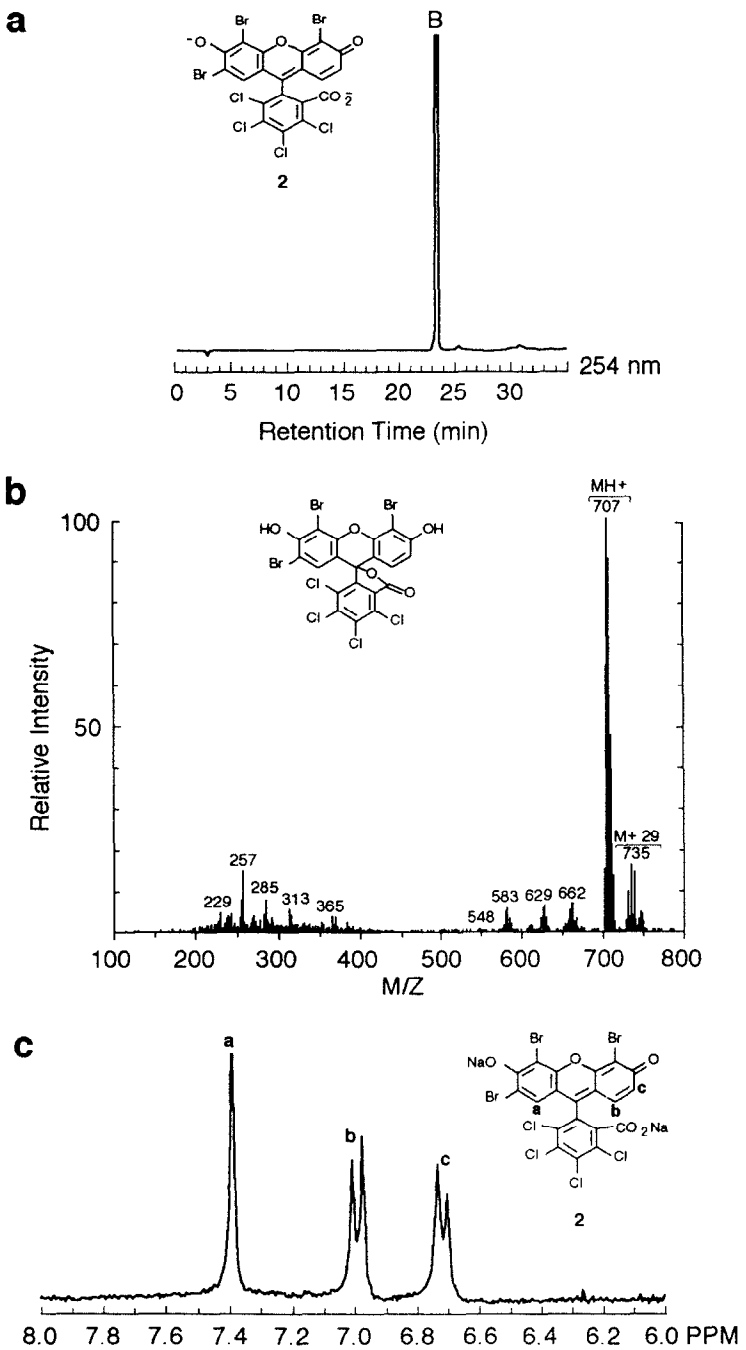

Fig. 5. Characterization of the compound contained in fractions 209–225 of the pH-zone-refining CCC separation in Fig. 3. (a) Analytical RP-HPLC of the combined fractions 209–225, (b) PICI (methane) mass spectrum and (c) ^{1}H NMR spectrum (in $NaO^{2}H$–$^{2}H_2O$, 300 MHz).

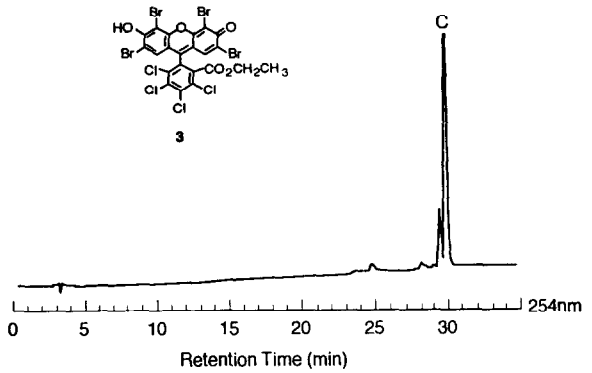

Fig. 6. Analytical RP-HPLC of the column content after the pH-zone-refining CCC separation of D&C Red No. 28 shown in Fig. 2.

experience with pH-zone-refining CCC [11–15]. This lower yield is apparently due to the elution of the suspension in fractions 18–25. It is thought that the elution of the suspension was caused by an insufficient amount of retainer acid (TFA) in the column. The retention of the stationary phase, calculated after the separation, was 61.3% of the total column capacity. This implies that 38.7% of the stationary phase and the corresponding amount of TFA (230 μl) were lost from the column before the separation. The remaining quantity of TFA in the column (0.0050 mol) was not enough to acidify the sodium salt of the dye present in the color additive (0.0072 equivalents). The part of the color additive that remained in the sodium salt form was not retained in the stationary phase and consequently was eluted as a suspension with the mobile phase. It appears that the recovery of pure **1** would have been higher if enough TFA had been added to ensure that the retained stationary phase would acidify all the dye in the sample solution. In the following experiment the loss of retainer acid from the column was circumvented by the addition of the retainer acid to the sample solution. Thus, the components in 6 g of D&C Red No. 28 were separated by the addition of TFA to the sample solution in sufficient quantity to convert all the dye to the acid form.

3.2. Experiment II: Separation of components in 6 g of commercial D&C Red No. 28

Analytical RP-HPLC of the D&C Red No. 28 used in this separation gave four peaks (Fig. 7); 6 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. 8. The two-phase solvent system used consisted of diethyl ether–acetonitrile–0.01 *M* NH_4OAc (4:1:5, v/v/v). The pH of the lower (mobile) phase was adjusted to 9.2 by addition of ammonium hydroxide. The sample mixture was prepared by mixing 6 g of dye in a solvent consisting of 20 ml of the lower phase and 30 ml of the upper phase. TFA (1.2 ml) was added to the sample solution. The solvent front (first fraction containing mobile phase) emerged at fraction 7. The retention of the stationary phase, calculated after the separation, was 54.7% of the total column capacity. The resulting chromatogram has a broad rectangular shape (Fig. 8a) that includes two pH plateaus (dotted line): a short and a long one (fractions 45–47 and 55–215, respectively). In this case, no suspension was eluted and no decrease occurred in the absorbance intensity before the elution of the main component. Fractions 55–215 contained a

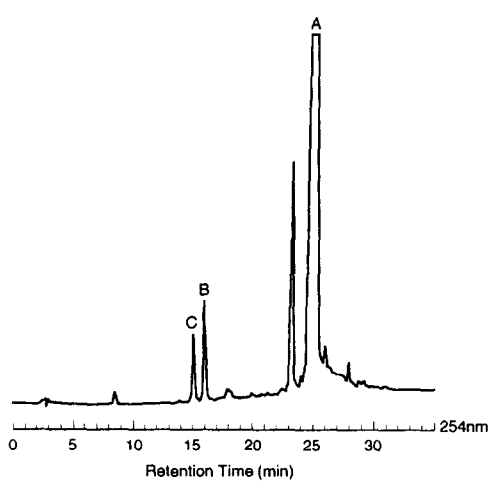

Fig. 7. Analytical RP-HPLC of the commercial lot of color additive D&C Red No. 28 used in experiment II.

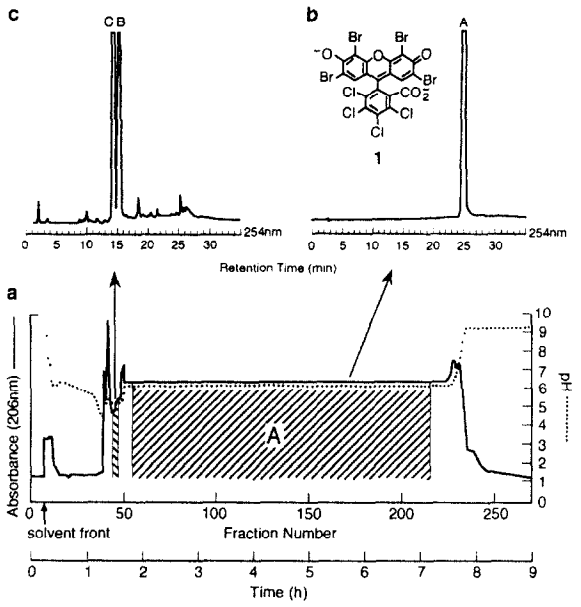

Fig. 8. (a) pH-Zone-refining counter-current chromatogram of the separation of components in 6 g of the D&C Red No. 28 used to obtain Fig. 7, (b) analytical RP-HPLC of the combined fractions 55–215 and (c) analytical RP-HPLC of the combined fractions 45–47.

single component whose RP-HPLC peak (Fig. 8b) corresponded to peak A in Fig. 7. The compound was isolated as the lactone (4.06 g) and identified by CI-MS and ^{1}H NMR as 2′,4′,5′,7′-tetrabromo-4,5,6,7-tetrachlorofluorescein. The ca. 80% recovery of pure **1** was improved in this separation by avoiding elution of the contaminated suspension through the presence of sufficient retainer acid (0.016 mol TFA) to acidify the sodium salt of the dye (0.014 equivalents). The two components corresponding to peaks B and C in Fig. 7 eluted in a very concentrated form in fractions 45–47 (Fig. 8c). On the basis of preliminary studies, it appears that these two acidic components have very closely related structures [19].

4. Conclusions

This study and previous work [11,15,20–22] demonstrate that pH-zone-refining CCC is an effective method for the separation and purification of multigram quantities of acidic components of hydroxyxanthene dye mixtures.

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