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Complementary use of counter-current chromatography and preparative reversed-phase high-performance liquid chromatography in the separation of a synthetic mixture of brominated tetrachlorofluoresceins☆

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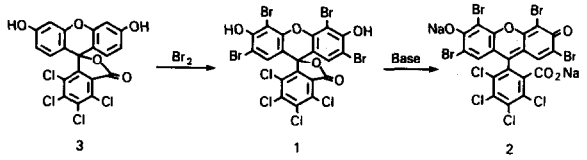
ABSTRACT

A synthetically prepared mixture of brominated 4,5,6,7-tetrachlorofluoresceins was separated by a combination of preparative reversed-phase high-performance liquid chromatography and high-speed counter-current chromatography. Two new lower-brominated subsidiary colors of D&C Red Nos. 27 and 28 (phloxine B), 4′,5′-dibromo-4,5,6,7-tetrachlorofluorescein and 2′,4′,5′-tribromo-4,5,6,7-tetrachlorofluorescein, were isolated and characterized by ^{1}H NMR and chemical ionization mass spectrometry.

INTRODUCTION

D&C Red No. 27 (mainly 2′,4′,5′,7′-tetrabromo-4,5,6,7-tetrachlorofluorescein, **1**, Colour Index No. 45410:1) and its disodium salt, D&C Red No. 28 (mainly phloxine B, **2**, Colour Index No. 45410), are xanthene color additives that are listed in the US Code of Federal Regulations (CFR) for use in drugs and cosmetics [1]. D&C Red No. 27 is manufactured by bromination of 4,5,6,7-tetrachlorofluorescein, **3**, and D&C Red No. 28 is manufactured by alkaline hydrolysis of **1** [1], as shown in Fig. 1. Among the impurities expected to be present in the color additives are lower-brominated subsidiary



3 1 2

Fig. 1. Manufacture of D&C Red Nos. 27 (**1**) and 28 (**2**) from 4,5,6,7-tetrachlorofluorescein (**3**).

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colors of **1** and **2** that are formed by substitution of **3** with fewer than four bromine atoms. Before they may be used as color additives, **1** and **2** are subject to batch certification by the US Food and Drug Administration (FDA) to assure compliance with the specifications set forth in the CFR. The current specifications for D&C Red Nos. 27 and 28 limit the total amount of lower-halogenated subsidiary colors, including the lower-brominated subsidiary colors, to $\leqslant 4\%$ (w/w) [1]. In the development and validation of a reversed-phase high-performance liquid chromatographic (RP-HPLC) method for the analysis of **1** and **2** that are submitted to the FDA for certification, pure lower-brominated subsidiary colors were needed as reference materials. An attempt to prepare 4′,5′-dibromotetrachlorofluorescein by partial bromination of **3** yielded a complex mixture shown by analytical RP-HPLC to contain four main components. This mixture was separated by preparative RP-HPLC into fractions containing pure 4′,5′-dibromotetrachlorofluorescein and other fractions containing multiple components. Fractions containing a mixture that included one of the major products were combined and further separated by high-speed counter-current chromatography (HSCCC) [2,3]. HSCCC was chosen for this separation because it was previously used successfully to purify the tetrabrominated analogues, **1** and **2** [4].

This paper describes the application of HSCCC as a complement to preparative RP-HPLC in the separation of a complex mixture containing various brominated tetrachlorofluoresceins.

EXPERIMENTAL

Materials

Ammonium acetate (NH_4OAc), methanol, water, ethyl acetate and *n*-butanol were chromatography grade. Deuterium oxide (99.9%, MSD Isotopes, Montreal, Canada), sodium deuteroxide (99.9%, *ca.* 40% in ^2H_2O, Fluka, Buchs, Switzerland), bromine (99.5%, Aldrich, Milwaukee, WI, USA), and 4,5,6,7-tetrachlorofluorescein (Thomasset Colors, now Hilton Davis Co., Cincinnati, OH, USA) were used as received.

Synthesis of the brominated tetrachlorofluorescein mixture

A crude mixture of brominated tetrachlorofluoresceins was prepared by brominating tetrachlorofluorescein following the preparation of 4′,5′-dibromofluorescein [5]. Thus, a *ca.* 5°C solution of sodium hypobromite (from 3.84 g, 24 mmol of bromine in 50 ml of 1 *M* NaOH) was added to a stirred *ca.* 5°C solution (cooled in an ice–water bath) of the disodium salt of tetrachlorofluorescein (from 4.7 g, 10 mmol of tetrachlorofluorescein and 100 ml of 0.4 *M* NaOH) over 10 min. After 10 min of additional stirring, the solution was acidified with 3 *M* HCl until no further precipitate formed. The precipitate was filtered, washed (5 × 30 ml water), and dried (130 mmHg, 100°C, 5 h), yielding an orange powder (7.25 g).

Preparative RP-HPLC

Preparative RP-HPLC was performed using a Waters Delta Prep 3000 system and a Waters Delta-Pak C_{18} preparative cartridge column (15 μm particle size, 100 Å pore size, 300 × 47 mm I.D.) in a Waters 1000 PrepPAK module. Detection was at 254 nm with a Linear UVIS 204 detector and a variable-pathlength preparative flow cell adjusted to 0.05 mm. The eluents, A = water, B = methanol and C = 1.00 *M* aqueous NH_4OAc, were sparged with helium. The column was washed with methanol and equilibrated at 40 ml/min with A–B–C (55:40:5) for 6 min and then with A–B–C (45:50:5) for 5 min. A solution of the diammonium salt prepared from 2 g of the crude brominated tetrachlorofluoresceins in 74 ml of water, 5 ml of 5% NH_4OH, 1.5 g of NH_4OAc, and 31 ml of methanol was syringe-filtered through a Millipore Millex-HV 0.45-μm pore size, 25-mm diameter filter unit. The filtrate was pumped onto the column at 15 ml/min and the column was eluted at 40 ml/min with a 90-min linear gradient from A–B–C (45:50:5) to B–C (95:5). Over 46 fractions were collected with volumes of *ca.* 20 ml when there were major peaks, *ca.* 50 ml when there were weaker and tailing peaks, and *ca.* 100 ml or more when there was little detector response. Diluted aliquots of these fractions were analyzed by analytical RP-HPLC.

Analytical RP-HPLC

The system 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

546 nm (Waters Assoc., Milford, MA, USA). The autosampler was equipped with a Model 7010 injector (Rheodyne, Cotati, CA, USA) with a 200-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.

Eluents 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, and flow-rate 1 ml/min.

An aliquot of each desired fraction from the preparative RP-HPLC and HSCCC separations was diluted with approximately 3 ml of methanol–0.1 *M* aqueous NH_4OAc (50:50, v/v). The solution was filtered through a LID/X glass microfiber syringeless filter unit AQOR.45 (Genex, Gaithersburg, MD, USA) prior to chromatography.

Isolation of fluorescein dyes from preparative RP-HPLC and HSCCC fractions

The dyes were isolated in the lactone form as the following example shows. Fractions 30–33 from the preparative RP-HPLC separation were combined and concentrated to *ca.* 1 ml on a rotary evaporator at 25 and 30 mmHg at *ca.* 50°C. The residue was acidified with 10–15 ml of 10% HCl and the precipitated lactones were extracted into ethyl acetate. The organic layer was washed (2 × 10 ml water) and dried (anhydrous Na_2SO_4), and the solvent was evaporated, yielding *ca.* 100 mg orange solid.

Preparative high-speed counter-current chromatography

The commercial high-speed counter-current chromatograph (P.C. Inc., Potomac, MD, USA) [6] holds an Ito multilayer-coil separation column and a counterweight whose centers revolve 10 cm around the centrifugal axis. The column consists of approximately 150 m × 1.6 mm I.D. polytetrafluoroethylene tubing with a total capacity of approximately 300 ml. The β value [7] ranges from 0.5 at the internal terminal to 0.85 at the external terminal.

The two-phase solvent system, composed of ethyl acetate–*n*-butanol–(0.01 *M* aqueous NH_4OAc adjusted to pH 8.6 with ammonium hydroxide) (1:1:2), was selected on the basis of previous experience with HSCCC purification of **2** [4]. The solvent system was thoroughly equilibrated in a separatory funnel and the two phases were separated shortly before use.

The column was completely filled with the stationary (upper) phase by using a metering pump (Milton Roy minipump; LDC Analytical, Riviera Beach, FL, USA). The lactone mixture (*ca.* 100 mg, isolated from fractions 30–33 of the preparative RP-HPLC separation as described above) was dissolved in a mixture of 10 ml solvent system (5 ml each of the upper and lower phases) and 100 μl of 30% ammonium hydroxide. This solution was injected into the column by syringe. The mobile (lower) phase was then pumped into the column at 2 ml/min while the column was rotated at 800 rpm. The column effluent was monitored with a UV detector (Uvicord S; LKB Instruments, Stockholm, Sweden) at 280 nm, and 4-ml fractions were collected (Ultrorac, LKB Instruments).

Mass spectrometry

The positive ion chemical ionization (PICI) mass spectra were obtained on a Finnigan Mat TSQ-46 instrument interfaced to an INCOS 2300 data system with TSQ software (Revision D). The spectrometer had a source temperature of 100°C, emission of 0.35 mA at 70 eV, 0.25 Torr methane, pre-

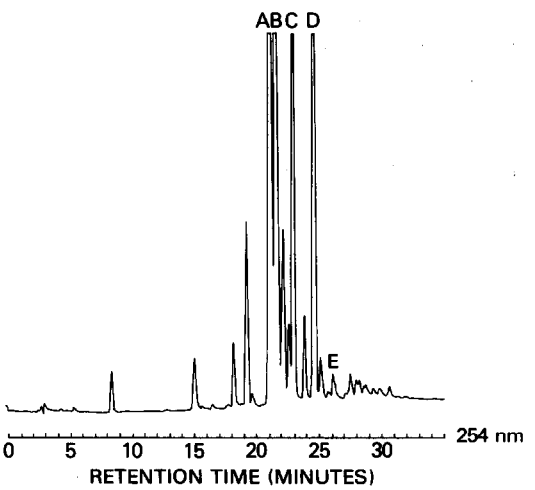

Fig. 2. Analytical RP-HPLC of the crude brominated tetrachlorofluorescein. See text.

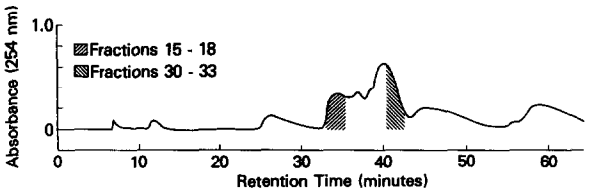

Fig. 3. Overloaded preparative RP-HPLC separation of the crude brominated tetrachlorofluorescein.

amplifier setting of 10^{-8} A/V, and conversion dynode of -5 kV, and was scanned from *m/z* 65 to 765 in 1.0 sec. The fluoresceins (lactone form, obtained as described above) were dissolved in either ethyl acetate or methanol and were introduced via the direct chemical ionization (DCI) probe at a heating rate of 20 mA/s. Fragmentation patterns *m/z* (relative intensity): peak A (Fig. 4b): 625 (26.2, MH^+), 580 [2.6, $(M-CO_2)^+\cdot$], 545 [0.7, $(M-CO_2-Cl)^+$]; peak B (Fig. 8b): 469 (74.1, MH^+), 424 [11.8, $(M-CO_2)^+\cdot$], 389 [5.6, $(M-CO_2-Cl)^+$]; peak C (Fig. 7b): 703 (12.4, MH^+), 658 [1.1, $(M-CO_2)^+\cdot$], 623 [0.4, $(M-CO_2-Cl)^+$].

^1H Nuclear magnetic resonance

^1H NMR spectra were obtained on a Varian XL 200 Fourier transform NMR spectrometer at 200 MHz. Typical concentrations were 4 mg compound (lactone form, see above) in 0.5 ml of 0.5% NaO^2H in ^2H_2O. Peak A (Fig. 4c): 7.19 ppm (d, H-a), 6.81 ppm (d, H-b). Peak B (Fig. 8c): 7.18 ppm (d, H-a), 6.72 ppm (d, H-b), 6.68 ppm (s, H-c). Peak C (Fig. 7c) 7.50 ppm (s, H-a), 7.10 ppm (d, H-b), 6.80 ppm (d, H-c).

RESULTS AND DISCUSSION

In adapting the published method for the preparation of 4′,5′-dibromofluorescein by bromination of fluorescein [5], it was expected that 4′,5′-dibromotetrachlorofluorescein would be the single major product of bromination of 4,5,6,7-tetrachlorofluorescein. Instead, the reaction resulted in a complex mixture as shown by analytical RP-HPLC (Fig. 2). B and D, two of the four major peaks, had retention times matching those of the unbrominated and tetrabrominated dyes, **3** and **2** (Fig. 1), respectively. Because the two other major peaks were probably

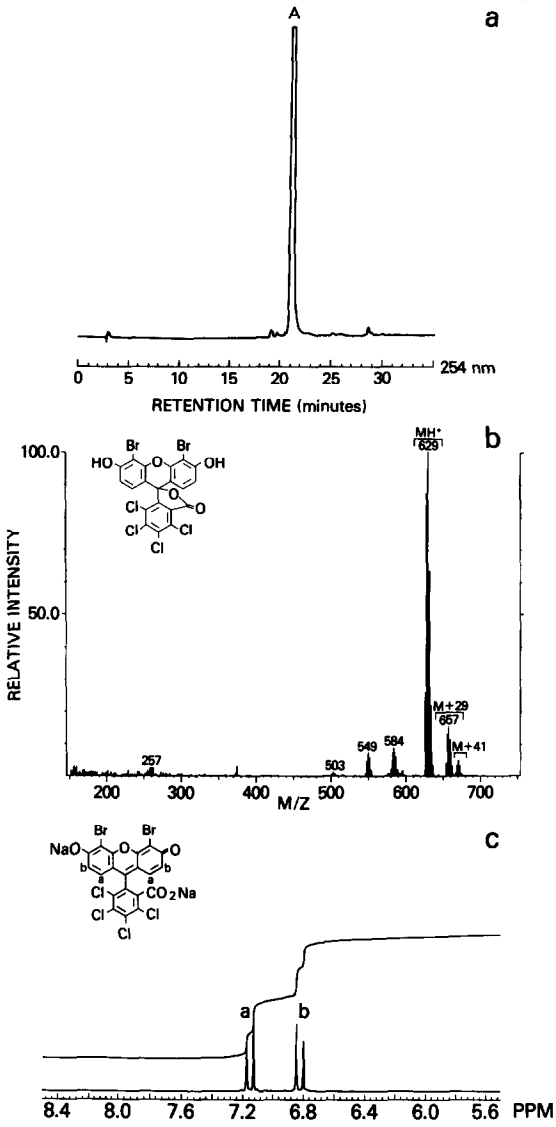

Fig. 4. Characterization of the compound contained in fractions 15–18 of the preparative RP-HPLC separation (see Fig. 3). (a) Analytical RP-HPLC of fraction 17, (b) positive ion chemical ionization (methane) mass spectrum of fraction 17, (c) ^1H NMR spectrum of fraction 17 (in $NaO^2H/^2H_2O$, 200 MHz).

lower-brominated subsidiary colors of D&C Red Nos. 27 and 28, separation of 2 g of the mixture by preparative RP-HPLC was attempted. This overloaded separation yielded several peaks (Fig. 3), and the eluate corresponding to two of these peaks was collected in fractions 15–18 and 30–33.

Fractions 15–18 contained a single component

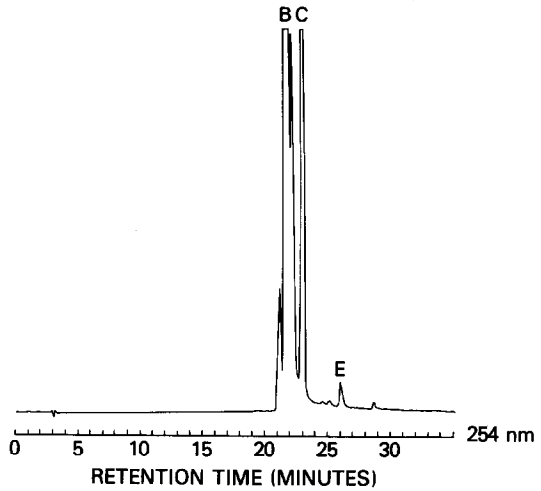

Fig. 5. Analytical RP-HPLC of combined fractions 30–33 (see Fig. 3).

whose HPLC peak (Fig. 4a) corresponded to peak A in Fig. 2. This component was isolated as the lactone (94 mg) and identified, by MS and ^{1}H NMR, as 4′,5′-dibromotetrachlorofluorescein (Fig. 4b and c).

The analytical RP-HPLC of fractions 30–33 (Fig. 5) produced two main peaks that corresponded to peaks B and C in Fig. 2. To separate this mixture (approximately 100 mg), the counter-current chromatographic method developed previously to purify the tetrabrominated dye [4] was chosen over the

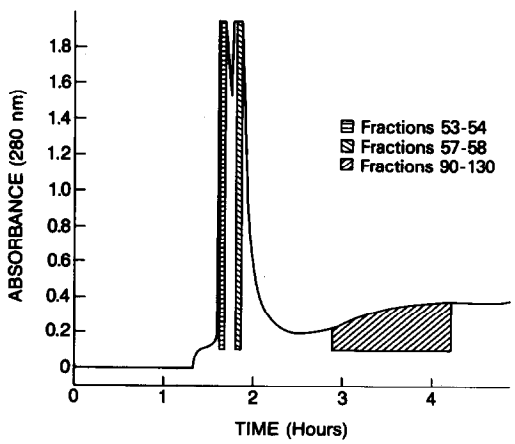

Fig. 6. HSCCC separation of combined fractions 30–33 (see Fig. 3).

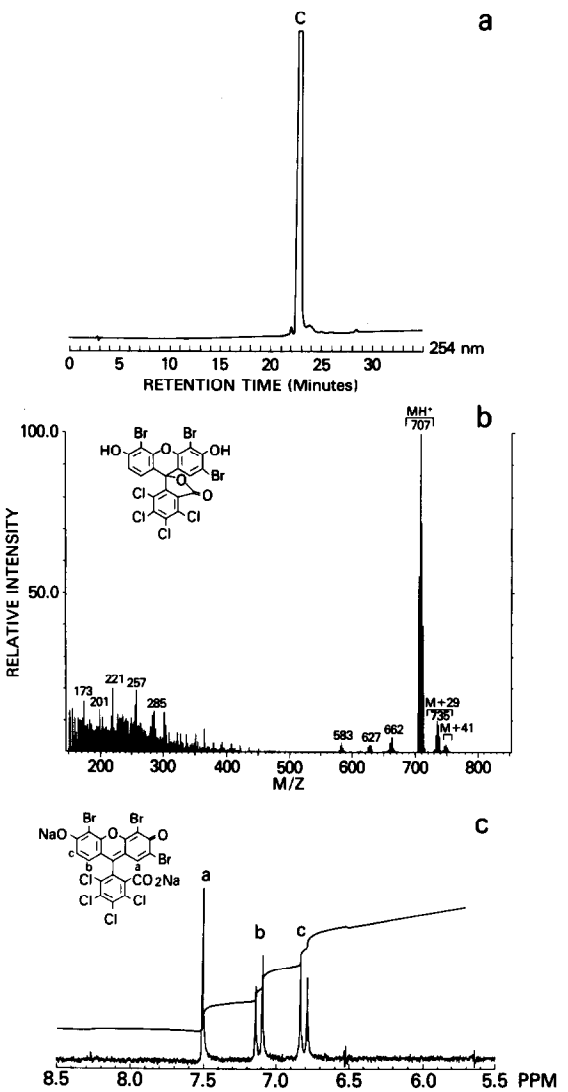

Fig. 7. Characterization of the compound contained in fractions 57–58 of the HSCCC separation (see Fig. 6). (a) Analytical RP-HPLC of HSCCC fraction 58, (b) positive ion chemical ionization (methane) mass spectrum of fraction 58, (c) ^{1}H NMR spectrum of fraction 58 (in $NaO^2H/^2H_2O$, 200 MHz).

feasible alternative of using the preparative HPLC procedure with the lower load. The HSCCC separation of the mixture resulted in three peaks, and the corresponding eluate was collected in fractions 57–58, 90–130, and 53–54 (Fig. 6).

HSCCC fractions 57–58 contained a single component whose HPLC peak (Fig. 7a) corresponded

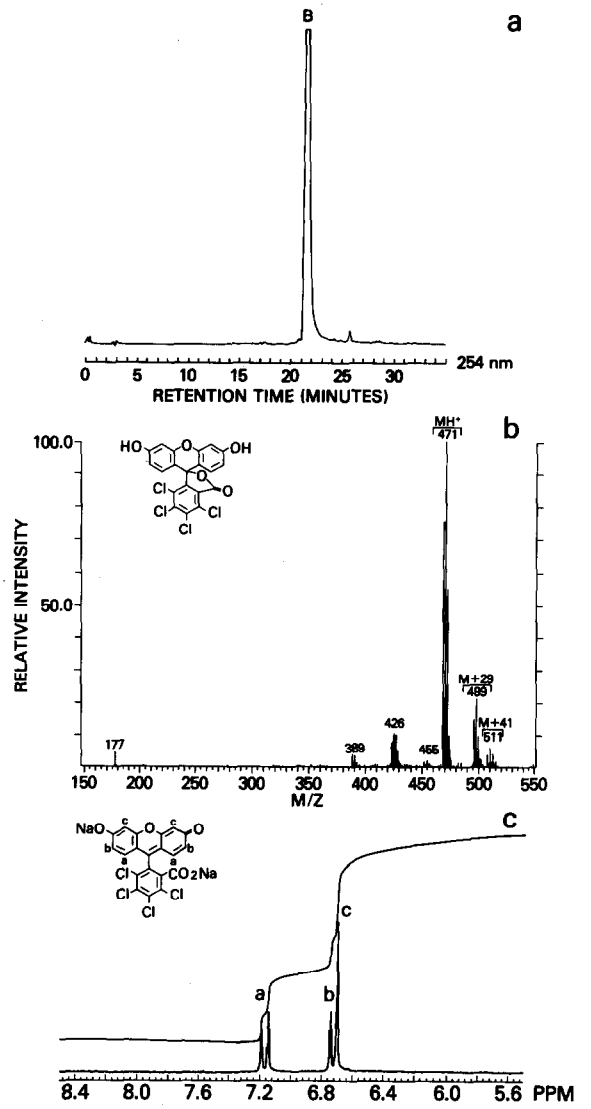

Fig. 8. Characterization of the compound contained in fractions 90–130 of the HSCCC separation (see Fig. 6). (a) Analytical RP-HPLC of combined fractions 90–130, (b) positive ion chemical ionization (methane) mass spectrum of combined fractions 90–130, (c) ^1H NMR spectrum of combined fractions 90–130 (in $NaO^2H/^2H_2O$, 200 MHz).

to peak C in Fig. 2. The compound was isolated as the lactone (20 mg) and identified by MS and ^1H NMR as 2′,4′,5′-tribromotetrachlorofluorescein (Fig. 7b and c).

HSCCC fractions 90–130 contained one compound whose HPLC peak (Fig. 8a) corresponded to peak B in Fig. 2. The compound was isolated as the lactone (12 mg) and identified by MS and ^1H NMR as the unbrominated starting material, 4,5,6,7-tetrachlorofluorescein (Fig. 8b-c).

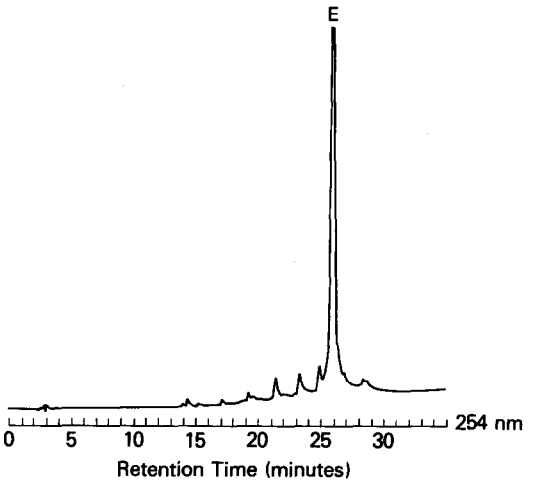

Fig. 9. Analytical RP-HPLC of HSCCC fraction 53 (see Fig. 6).

HSCCC fractions 53–54 contained a major component, whose HPLC peak (Fig. 9) corresponded to the minor peak E in Fig. 2, and several minor components. To date, peak E remains unidentified.

CONCLUSIONS

This work demonstrates that HSCCC can be used in combination with other chromatographic techniques to successfully separate complex mixtures. The use of silica gel HPLC followed by HSCCC [8] and the use of HSCCC followed by RP-HPLC [9], both on an analytical scale, to substantially improve purification of various compounds has been previously reported. To our knowledge, this work represents the first reported coupling of HSCCC and RP-HPLC for purification on a preparative scale. Two new lower-brominated subsidiary colors of D&C Red Nos. 27 and 28 (phloxine B), 4′,5′-dibromo-4,5,6,7-tetrachlorofluorescein and 2′,4′,5′-tribromo-4,5,6,7-tetrachlorofluorescein, were isolated and characterized by ^1H NMR and chemical ionization MS.

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