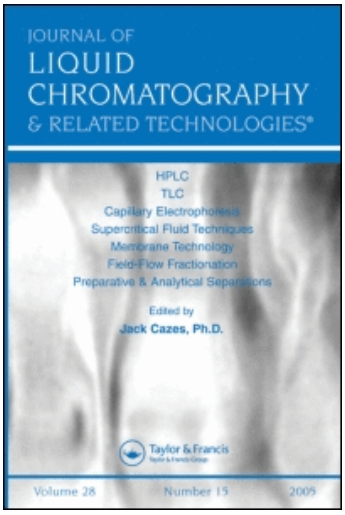

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SEPARATION OF A NEWLY IDENTIFIED CONTAMINANT FROM COMMERCIAL 4,5,6,7-TETRACHLOROFLUORESCEIN BY pH-ZONE-REFINING COUNTERCURRENT CHROMATOGRAPHY†

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ABSTRACT

A 5-g sample of commercial 4,5,6,7-tetrachlorofluorescein (TCF) was subjected to pH-zone-refining countercurrent chromatography to separate an unidentified contaminant present in all TCF batches obtained from four different suppliers. The separated contaminant (44 mg) was characterized by ^{1}H- and ^{13}C nuclear magnetic resonance spectrometry and negative ion chemical ionization mass spectrometry as a new seven-membered lactone TCF isomer with the structure 11,12,13,14-tetrachloro-3,14b-dihydroxy-[2]-benzoxepino- [3,4,5-*kl*]-xanthene-10(14b*H*)-one.

INTRODUCTION

The compound, 4,5,6,7-tetrachlorofluorescein (TCF), **1**, is a dye of the hydroxyxanthene class. TCF is an intermediate in the preparation of the more highly halogenated dyes, such as the U.S.-certified color additives D&C Red No. 27 (R27, mainly 2′,4′,5′,7′-tetrabromo-4,5,6,7-tetrachlorofluorescein, **2**) and its disodium salt, D&C Red No. 28 (R28, Phloxine B, **3**) and the widely used biological stain Rose Bengal, **4** (Figure 1). Commercial TCF was found to contain many impurities, several of which were recently separated by pH-zone-refining countercurrent chromatography (CCC).[1] The impurities can be carried over to the color additives during the manufacturing process, thus reducing the probability of their complying with FDA certification specifications. pH-Zone-refining CCC was used extensively for the separation and purification of other acidic dyes of the hydroxyxanthene type.[2] In the present study, pH-zone-refining CCC was successfully applied to the separation of a less polar isomer of TCF that was present in all batches of TCF obtained from several different suppliers.

EXPERIMENTAL

Materials

The samples of commercial batches of TCF used in this study were from four American suppliers (Aldrich, Milwaukee, WI; K&K Lab., Inc., Plainview, N.Y.; Tomasset Colors, now Hilton-Davis Co., Cincinnati, OH; and ICN Biomedicals, Inc., Irvine, CA). Ammonium acetate, methanol, water, ethyl acetate and acetonitrile were chromatography grade. Hydrochloric acid (36.5-38.0%) was ACS reagent grade. Methyl *tert*-butyl ether (Fluka, Buchs, Switzerland), ammonium hydroxide (>25% NH_3 in water, Fisher Sci., Pittsburgh, PA), trifluoroacetic acid (TFA, Sigma Chem., St. Louis, MO), and tetradeuteromethanol ($C^2H_3O^2H$, 99.9%, MSD Isotopes, Montreal, Canada) were used as received.

Instrumentation and pH-Zone-Refining CCC Procedure

The separation presented here was performed with a Model CCC-1000 high-speed countercurrent chromatograph (Pharma-Tech Research Corp., Baltimore, MD) that consisted of a column mounted on a rotating frame, a speed controller and an LC pump. To this basic system, several improvements

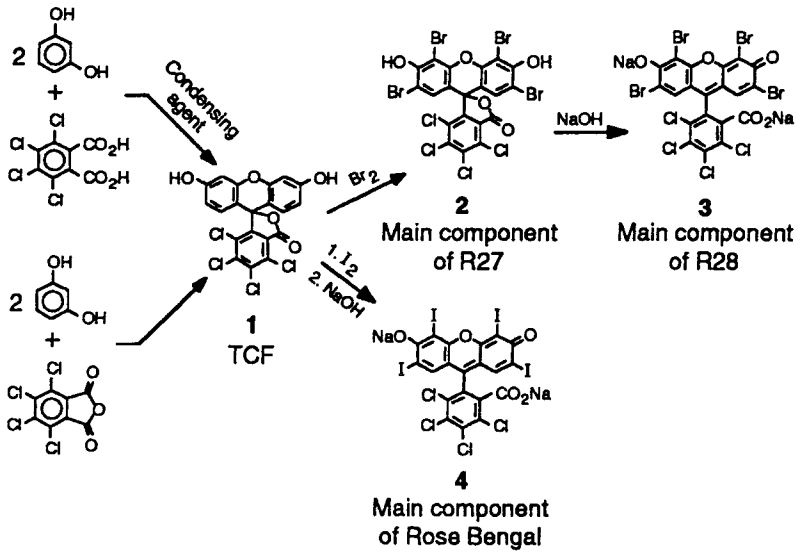


Figure 1. Preparation of 4,5,6,7-tetrachlorofluorescein (TCF) and higher halogenated dyes.

were made (e.g., continuous pH monitoring, computerized data acquisition) to facilitate chromatography[3]. The column consisted of three multilayer coils connected in series made of 1.6 mm I.D. Tefzel® tubing with total capacity of ~325 mL.

The two-phase solvent system used for the present separation consisted of methyl *tert*-butyl ether/acetonitrile/water (4:1:5). The solvent system was equilibrated in a separatory funnel, and the two phases were separated before use. The upper (organic) phase was acidified with TFA, and the lower (aqueous) phase was made basic by addition of ammonium hydroxide. The acidic organic phase was used as the stationary phase, and the basic aqueous phase was used as the mobile phase. The separation was initiated by filling the entire column with the stationary phase using the LC pump, and then loading the sample, dissolved in a minimum volume of the stationary phase, into the column by syringe. The mobile phase was then pumped into the column at 3 mL/min while the column was rotated at 1000 rpm. The column effluent was monitored (pH, UV-scanning from 220 to 450 nm) and a fraction collector was used to obtain 6 mL fractions. The fractions collected were analyzed by analytical HPLC.

High-Performance Liquid Chromatography

The analytical RP-HPLC analyses were performed with a Spectra-Physics system described previously.[4] The UV-Vis detector was set at 254 nm. The eluents were 0.1M aqueous NH_4OAc and methanol. The column (Hypersil MOS-1 RPC-8, 5-μm particle size, 250 x 4.6 mm I.D., Keystone Sci., Bellefonte, PA) was eluted by using consecutive linear gradients of 25-90% methanol in 25 min, 90-100% methanol in 5 min, and 100% methanol for 5 min. The column was re-equilibrated with 25% methanol for 15 min. Other conditions included injection volume, 20 μL; full scale response, 0.128 absorbance units; and flow-rate, 1 mL/min.

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 120°C, ionization energy of 70 eV, emission current of 0.35 mA, 0.4 Torr methane and preamplifier setting of 10^{-8} A/V, and was scanned from *m/z* 60-760 in 1.0 s. The purified TCF (lactone form) and its isomer (TCF isomer) were dissolved in ethyl acetate and were introduced into the mass spectrometer via the direct chemical ionization probe at a probe heating rate of 50 mA/s. Fragmentation patterns are given for [*m/z* relative intensity] the most intense ion in the molecular and fragment ions clusters: TCF-470 (14.8%, M^-), 426 [100%, $(M\text{-}CO_2)^-$], 390 [26.5%, $(M\text{-}CO_2\text{-}HCl)^-$]; TCF isomer- 470 (100%, M^-) and 388 [97%, $(M\text{-}CO_2\text{-}HCl)^-$].

Nuclear Magnetic Resonance

^{1}H-NMR and ^{13}C-NMR spectra were obtained on a Varian XL 300 Fourier transform NMR spectrometer at 300 MHz. For the analysis, 4 mg of the isolated compound was dissolved in 0.5 mL of $C^2H_3O^2H$. The following signals were obtained and assigned for the separated contaminant: 7.29 ppm (t, H-a); 6.74 ppm (d,d, H-b); 6.72 ppm (d, H-c); 6.64 ppm (d, H-d); 6.58 ppm (d,d, H-e); 6.49 ppm (d,d, H-f). The two-dimensional correlation spectrum was obtained using the pulse sequence provided with the spectrometer. The C-13 spectrum at 75 MHz was obtained with a 300 acquisition pulse and 4 second pause between acquisitions: 166.8, 161.2, 158.6, 154.0, 153.3, 152.0, 139.8, 135.6, 132.6, 131.0, 129.2, 128.5, 126.5, 113.5, 111.0, 108.7, 108.4, 104.3, 103.1, 81.5 ppm.

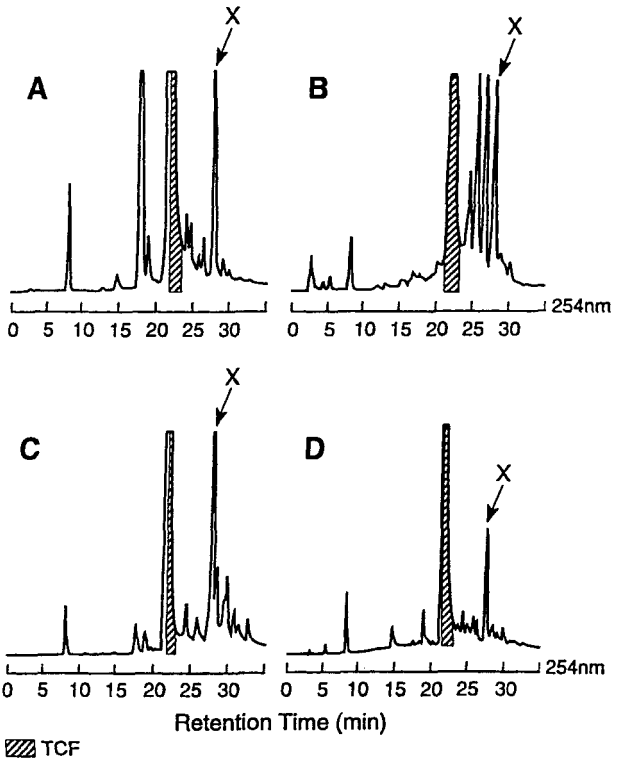


Figure 2. HPLC analyses of commercial batches of TCF obtained from four different sources.

RESULTS AND DISCUSSION

HPLC analysis of commercial batches of TCF obtained from four different sources showed that all had in common a major contaminant assigned as X in Figure 2. Since contaminant X can be carried over to R27 and R28 during the manufacturing process (Figure 1), it was of interest to isolate and identify it.

The pH profile of the eluent obtained for the separation of 5 g of TCF by pH-zone-refining CCC is shown in Figure 2B. The two-phase solvent system for this separation consisted of methyl *tert*-butyl ether/acetonitrile/water (4:1:5, 600 mL: 150 mL: 750 mL). After equilibration, the separated phases were degassed by sonication for 2-3 min. To 500 mL of upper phase (total upper phase was 625 mL) was added 200 μL (0.31 g, 2.7 mmol) of TFA, yielding a solution 5.4 mM in TFA with pH ca. 2.55. The acidified upper phase was

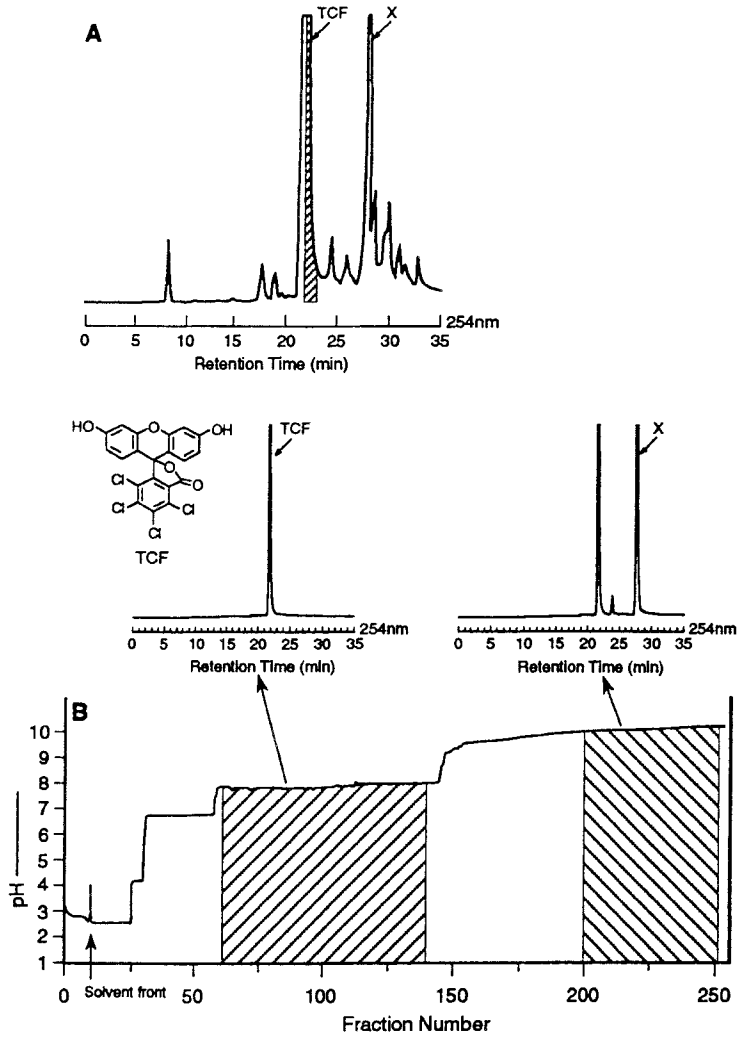


Figure 3. Separation of 5 g of commercial TCF by pH-zone-refining countercurrent chromatography (CCC). (**A**) HPLC of the sample (**B**) pH profile of the fractions collected during the CCC separation and HPLC of the fractions that eluted in the hatched areas.

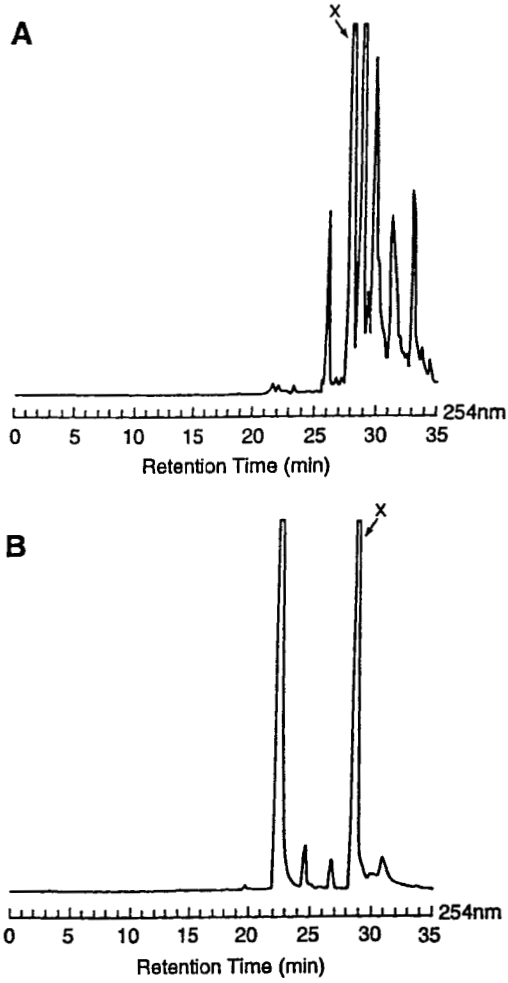
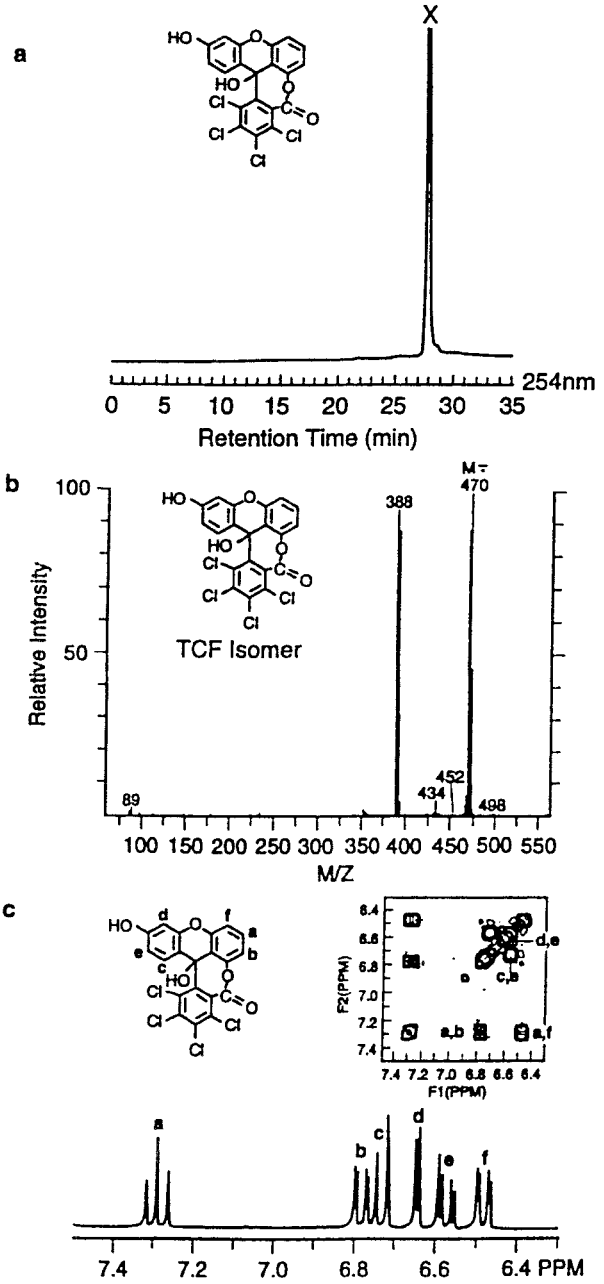


Figure 4. HPLC analysis of the column content after the pH-zone-refining CCC separation in Fig. 3. (**A**) upper phase (stationary phase), (**B**) lower phase (mobile phase).

used as the stationary phase. To 850 mL of the lower phase (total lower phase was 860 mL) was added 1 mL (0.87 g, ca. 13.2 mmol) of ammonium hydroxide, >25% NH_3 in water yielding a solution 15.5 mM in NH_3 with pH ca. 10.7. The basified lower phase was used as the mobile phase. The same solution was prepared twice in order to have a sufficient quantity of mobile



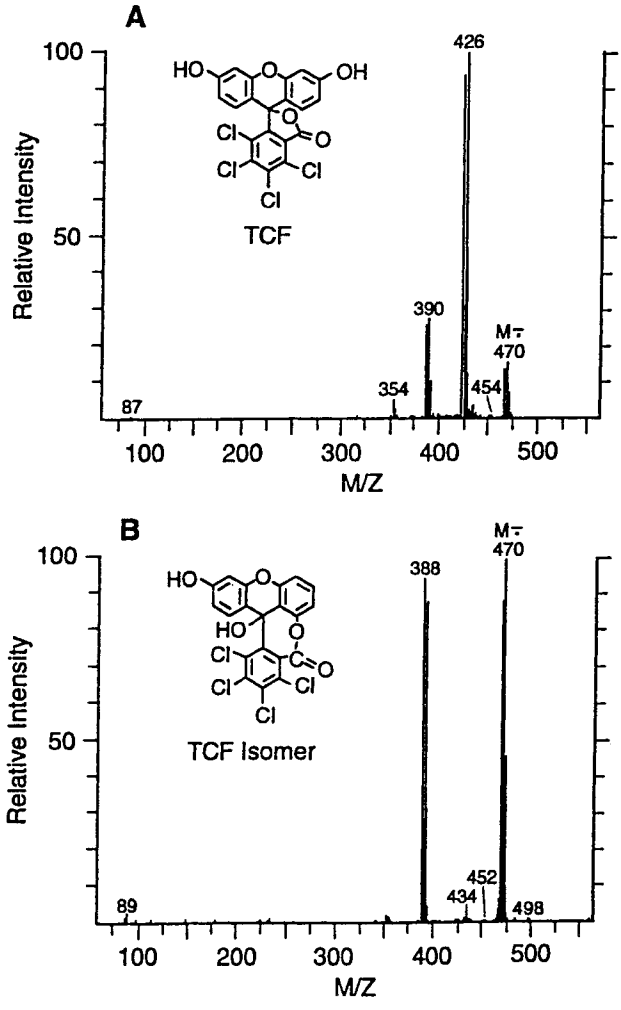


Figure 6. Negative ion chemical ionization mass spectrum of (**A**) TCF and (**B**) TCF isomer (contaminant X).

Figure 5 (left). Characterization of contaminant X separated from commercial TCF. (a) HPLC analysis, (b) negative ion chemical ionization mass spectrum, (c) ^{1}H NMR spectrum with COSY assignments.

phase for the separation. The sample solution was made of 5 g TCF (HPLC of the sample shown in Figure 3A) partly dissolved and partly suspended in 110 mL of acidified stationary phase. The sample solution and 4 mL more of stationary phase used to wash the syringe were injected into the column. Other experimental details are given in Experimental. The separation was completed in ca. 8 hours. The solvent front (first fraction containing mobile phase) emerged at fraction 11. The retention of the stationary phase, measured after the separation, was 60%. TCF eluted with a pH plateau and it was collected in fractions 64-141 (hatched area on the left in Figure 3B). The TCF isolated in lactone form from these combined fractions (2.75 g) was over 99% pure (see HPLC in Fig. 3B).

The separation was continued after the elution of the acidic component of the mixture (TCF) to collect the less polar contaminants. The eluent collected from fractions 200 to 253 (the hatched area on the right in Fig. 3B), at which the separation was stopped, contained a dark liquid whose HPLC peaks (Fig. 3B) corresponded to the retention times of TCF and contaminant X, respectively, in the approximate ratio of 1:1. When the column content was collected, and the two phases were analyzed by HPLC, it was found that the lower phase (mobile phase) contained the same composition as fractions 200-253 (Fig. 4B). Several minor, less polar, contaminants of the original mixture were concentrated in the upper phase of the column content (Figure 4A). To isolate contaminant X, fractions 200 to 253 were combined and freeze dried. To the powder thus obtained, ammonium hydroxide (5% NH_3 in water) was added and the mixture was extracted with ethyl acetate. The solvent was eliminated (rotary evaporator) and the residue was recrystallized from methanol/water. The total recrystallized material obtained was 44 mg. HPLC analysis of the material resulted in a single peak corresponding to the retention time of contaminant X (Figure 5a). The following structure, 11,12,13,14-tetrachloro-3,14b-dihydroxy-[2]benzoxepino-[3,4,5-*kl*]xanthene-10 (14b*H*)-one, has been proposed for this contaminant on the basis of mass spectral, ^{13}C and ^{1}H NMR analysis with COSY (correlated spectroscopy) assignments (Figure 5b,c). Contaminant X is a seven-membered lactone isomer of TCF that is less readily hydrolyzed in basic conditions than TCF. It is noteworthy that the different mass spectral behavior of the two isomers make mass spectrometry a reliable tool for their differentiation (Figure 6).

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