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Isolation of 4′-bromo-4,5,6,7-tetrachlorofluorescein from a synthetic mixture by pH-zone-refining counter-current chromatography with continuous pH monitoring[1]

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Abstract

A synthetic mixture containing mainly 4′-bromo-4,5,6,7-tetrachlorofluorescein (4′BrTCF, ca. 25% by high-performance liquid chromatography) was prepared by direct bromination of tetrachlorofluorescein in ethanol. The 4′BrTCF was isolated and purified by pH-zone-refining counter-current chromatography (CCC), using two different two-phase solvent systems: diethyl ether–acetonitrile–water (4:1:5, v/v) and *tert.*-butyl methyl ether–water (1:1, v/v). For each system, the upper organic phase was acidified and used as the stationary phase and the lower aqueous phase was made basic and used as the mobile phase. pH-Zone-refining CCC of two 5-g batches of the crude mixture yielded 1.65 g of 4′BrTCF that was ca. 95% pure by HPLC. For these separations, a commercial counter-current chromatograph was fitted with a pH electrode in a flow cell to record the pH of the effluent 'on-line,' thereby replacing the tedious and time-consuming manual measurement of the pH of each fraction. Additionally, UV spectra of the effluent were continuously monitored and stored by using a computerized scanning UV–Vis detector equipped with an adjustable short-pathlength flow cell.

Keywords: Counter-current chromatography; Preparative chromatography; Color additives; Bromotetrachlorofluorescein

1. Introduction

We recently described a new preparative-scale separation technique, i.e. pH-zone-refining counter-current chromatography (pH-zone-refining CCC) [1–3]. In this technique, a multigram mixture of acids (or bases) is isocratically eluted with aqueous base (or acid) through an acidified (basified) organic stationary phase in a CCC column. As the components of the mixture of acids are eluted from the column, the pH of the effluent increases in steps, with coinciding absorbance and pH plateaus showing elution of single major components [1]. Use of an organic mobile phase and an aqueous stationary phase was also described [3].

Typically, the pH of each fraction has been measured manually, and the chromatogram has been recorded at a specific UV wavelength (usually 206, 254 or 280 nm) with a fixed-pathlength flow cell. In the present study, three technical improvements were made to facilitate chromatographic separation and to

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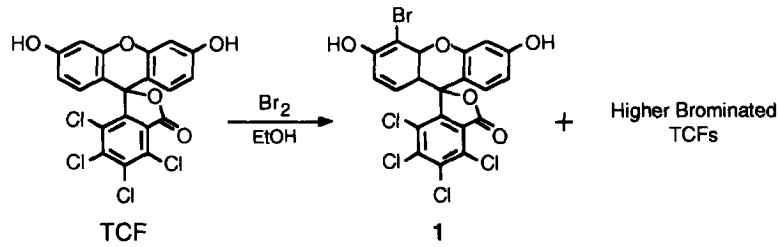

Fig. 1. Synthesis of crude 4′BrTCF.

obtain and record more chromatographic data: (i) the effluent pH was recorded 'on-line' by using a pH electrode in a flow cell, (ii) UV spectra of the effluent were continuously monitored and stored by using a computerized scanning UV–Vis detector, and (iii) the UV–Vis detector was equipped with an adjustable short-pathlength flow cell. This improved CCC system was used for the preparative (multigram) isolation and purification of 4′-bromo-4,5,6,7-tetrachlorofluorescein (4′BrTCF, **1**, Fig. 1) from a crude synthetic mixture. The isolation of 4′BrTCF on a smaller scale without these improvements was described previously [4]. 4′BrTCF is a lower-brominated subsidiary color of the U.S. certified color additives D&C Red No. 27 (mainly 2′,4′,5′,7′-tetrabromo-4,5,6,7-tetrachlorofluorescein, Colour Index No. 45410:1) and its disodium salt D&C Red No. 28 (phloxine B, Colour Index No. 45410). Pure **1** and other purified lower-brominated subsidiary colors are needed for use as reference materials in the Food and Drug Administration's color additive certification program. pH-Zone-refining CCC was previously used successfully to separate tribromo- and tetrabromotetrachlorofluoresceins from D&C Red No. 28 [5].

2. Experimental

2.1. Materials

Ammonium acetate (NH_4OAc), methanol, water and acetonitrile were chromatography grade. Ethyl acetate, diethyl ether (anhydrous), sodium hydroxide, hydrochloric acid (36.5–38.0% HCl) and anhydrous sodium sulfate (granular) were ACS-reagent grade. Ammonium hydroxide (>25% NH_3, Fluka, Buchs, Switzerland), *tert.*-butyl methyl ether (>99.5%, Fluka), trifluoroacetic acid (TFA, >98%, Fluka), pH 4 and 7 buffer solutions (Fisher Scientific, Fair Lawn, NJ, USA), 4,5,6,7-tetrachlorofluorescein (TCF, dye content ca. 90%, Aldrich, Milwaukee, WI, USA), bromine (99.5%, Aldrich), ethanol (200 proof, Warner-Graham, Cockeysville, MD, USA), deuterium oxide (99.9% ^2H, MSD Isotopes, Montreal, Canada) and sodium deuteroxide (99.9% ^2H, ca. 40% in ^2H_2O, Fluka) were used as received.

2.2. Synthesis of the mixture containing mainly 4′BrTCF

The filtrate from a solution of TCF (20.2 g, ca. 38 mmol) in 3.8 l of absolute ethanol was stirred at ambient temperature with Br_2 (2.8 ml, 53 mmol) for 3 h. The solvent was removed by rotary evaporation (45–50°C), yielding a brown powder (29.5 g). Two 5-g batches from this crude mixture were subjected to pH-zone-refining CCC.

2.3. pH-Zone-refining CCC

2.3.1. Instrumentation

The separations were performed using a commercial high-speed CCC system (Model CCC-1000, Pharma-Tech Research, Baltimore, MD, USA). The basic system consisted of a tubular Tefzel column (three multilayer coils connected in series with a total column volume of ca. 325 ml) mounted on a rotating frame (centrifuge), a speed controller and a Model 300 LC pump (Scientific Systems, State College, PA, USA). To this system we added: (a) a right-angle flow-switching valve (Upchurch Scientific, Oak Harbor, WA, USA) to syringe-inject the sample solution into the column; (b) a Model 450C

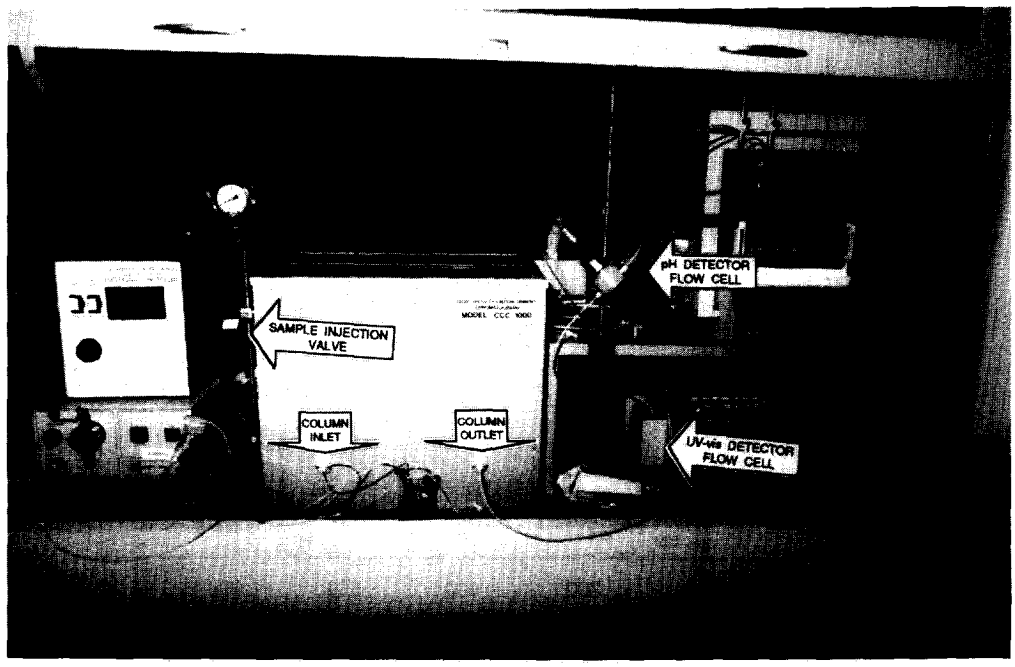

Fig. 2. Commercial CCC system with added on-line pH and UV–Vis monitoring.

combination pH electrode in a Model FC49K Kynar flow cell (both from Sensorex, Stanton, CA, USA), a Model SA 720 pH meter with recorder output (Orion, Boston, MA, USA) and a chart recorder (Model 3314, Soltec, Sun Valley, CA, USA) for plotting the effluent pH 'on line'; (c) a computerized scanning UV–Vis detector with a preparative flow cell with a pathlength nominally adjustable from ca. 0.01 to 3.00 mm (Spectra FOCUS, Thermo Separation Products, Piscataway, NJ, USA) for monitoring and storing spectra of the effluent; and (d) a Foxy fraction collector (Isco, Lincoln, NE, USA). The CCC system with the additions described above is shown in Fig. 2.

2.3.2. Separation procedure

The two-phase solvent system used for the isolation of 4′BrTCF from the crude mixture was diethyl ether–acetonitrile–water (4:1:5, v/v). The two-phase solvent system used for the purification of the isolated 4′BrTCF was *tert.*-butyl methyl ether–water (1:1, v/v). The solvent system used was equilibrated in a separatory funnel, and the two phases were separated shortly before use. TFA was added to the upper (organic) phase, and ammonium hydroxide or sodium hydroxide was added to the lower (aqueous) phase. The acidic organic phase was used as the stationary phase, and the basic aqueous phase was used as the mobile phase.

The following steps were taken prior to separation: (a) the column was filled with the stationary phase by using the LC pump; (b) the sample mixture (preparation of the sample solution is described under Results and Discussion) was loaded into the column by using a syringe and the switching valve; (c) the pH meter, the electrode in the flow cell and the chart recorder were calibrated with pH 7 and pH 4 buffers (recorder zero and variable range were set for a full chart scale of pH 1 to pH 11); (d) the UV–Vis flow cell was filled with mobile phase; (e) the computerized UV–Vis detector was set for data acquisition (spectra saved on the computer ca. 5 times per second); (f) the column was rotated at 1000 rpm; (g) the column outlet was connected to the UV–Vis detector flow cell (further connected to the pH flow cell and fraction collector, Fig. 2). At this stage, elution with basic mobile phase (3 ml/min), recording of the effluent pH (chart speed 8 cm/h), fraction collection

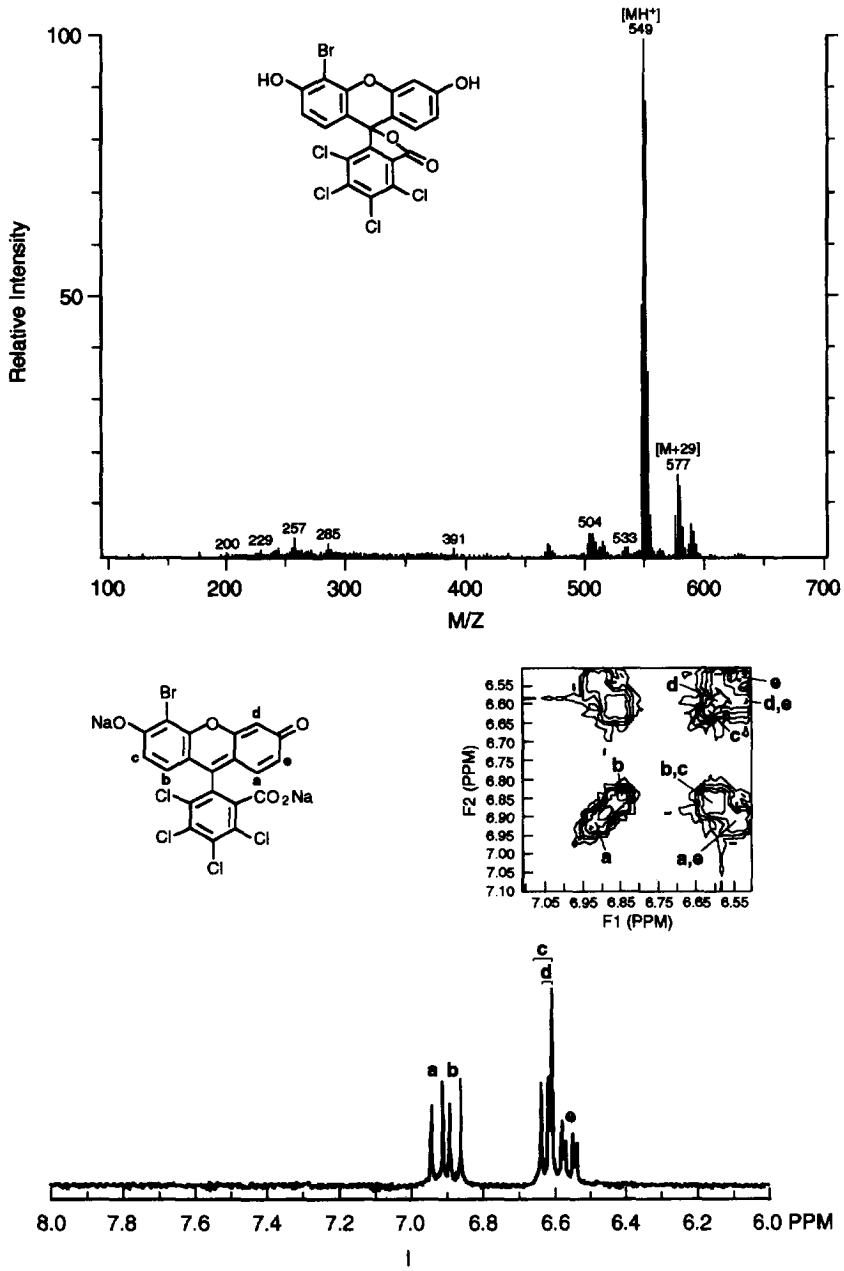

Fig. 3. CI mass spectrum of 4'BrTCF and ^{1}H NMR spectrum with COSY assignments.

(3 ml/fraction) and scanning UV detection (from 200 to 450 nm, variable-pathlength cell set at 0.05 mm) were simultaneously begun. The choice of pathlength was based on prior pH-zone-refining CCC separations of related fluorescein color additives [1,2,4,5].

The collected fractions were analyzed by analytical high-performance liquid chromatography

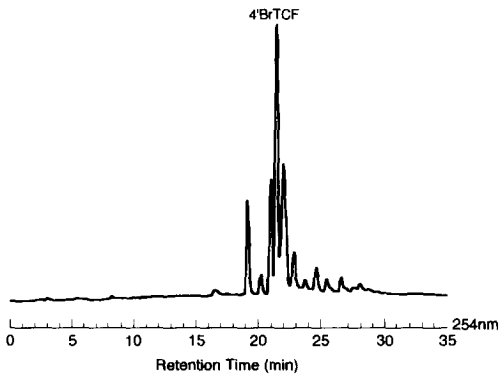

Fig. 4. RP-HPLC of the crude synthetic mixture containing mainly 4'BrTCF.

(HPLC). The HPLC system and conditions used were previously described [6,7].

2.4. Isolation of 4'BrTCF from pH-zone-refining CCC fractions

The method used to isolate 4'BrTCF in the lactone form was previously described for the isolation of other halogenated fluoresceins [7]. Briefly, fractions with the same pH values that contained over 85 area % of 4'BrTCF by HPLC (retention times corresponded to that of a previously obtained standard [4]) were combined by using either dilute aqueous ammonia (batch 1) or methanol (batch 2) as wash. For batch 1, the combined CCC fractions were acidified with 10% HCl and 4'BrTCF was precipitated as the lactone. The precipitate was suction-filtered, washed with water, and vacuum-dried at 50°C. For batch 2, the combined CCC fractions were concentrated to ca. 5 ml on a rotary evaporator (ca. 4 kPa and 50°C). The residue was acidified with 30–40 ml of 10% HCl, and the precipitated lactone was extracted into ethyl acetate. The ethyl acetate solution was washed (2×20 ml water) and dried (anhydrous sodium sulfate), and the solvent was evaporated.

2.5. Mass spectrometry (MS)

The mass spectrum of 4'BrTCF was obtained by positive ion chemical ionization (PICI) on a Finnigan Mat TSQ-46 quadrupole mass spectrometer. The instrument was operated at a source temperature of 100°C, ionizing energy of 70 eV, emission current of 0.35 mA and 0.25 Torr (ca. 33 Pa) methane and was scanned from m/z 65 to 865 in 1.5 s. The compound, in lactone form, was dissolved in ethyl acetate and was introduced into the mass spectrometer via the direct CI probe at a probe heating rate of 20 mA/s. The protonated molecular ion [m/z (relative intensity)] for 4'BrTCF (Fig. 3) was 549 (100).

2.6. ^1H Nuclear magnetic resonance (NMR) spectrometry

The ^1H NMR spectrum and COSY (correlated spectroscopy) assignments of 4'BrTCF were obtained on a Varian XL Fourier transform NMR spectrometer at 300 MHz. The purified compound, 4 mg in lactone form, was dissolved in 0.5 ml of 0.5% NaO^2H in ^2H_2O. The following signals were obtained and assigned (Fig. 3): 6.93 ppm (d, H-a), 6.88 ppm (d, H-b), 6.62 ppm (d, H-c), 6.61 ppm (d, H-d) and 6.56 ppm (dd, H-e).

3. Results and discussion

HPLC analysis of the crude synthetic mixture of brominated fluoresceins (Fig. 4) showed that 4'BrTCF was the major component (ca. 25 area %). The pH-zone-refining counter-current chromatogram obtained for the separation of 5 g of this mixture (batch 1) is shown in Fig. 5a. The two-phase solvent system for this separation consisted of diethyl ether–acetonitrile–water (4:1:5, v/v). After equilibration, the separated phases were degassed by sonication for 2–3 min. To 500 ml of the upper phase was added 1 ml (1.41 g, 12.4 mmol) of TFA, yielding a solution 24.7 mM in TFA with pH ca. 2.3. The acidified upper phase was used as the stationary phase. To 850 ml of the lower phase was added 4.42 g of 50% aqueous NaOH (55 mmol), yielding a solution 65 mM in NaOH with pH ca. 12.7. The basified lower phase was used as the mobile phase. The sample solution was prepared by dissolving 5 g of crude monobrominated TCF mixture in 60 ml of acidic stationary phase and 10 ml of unbasified lower phase. The sample solution and 2 ml more of stationary phase used to wash the syringe were

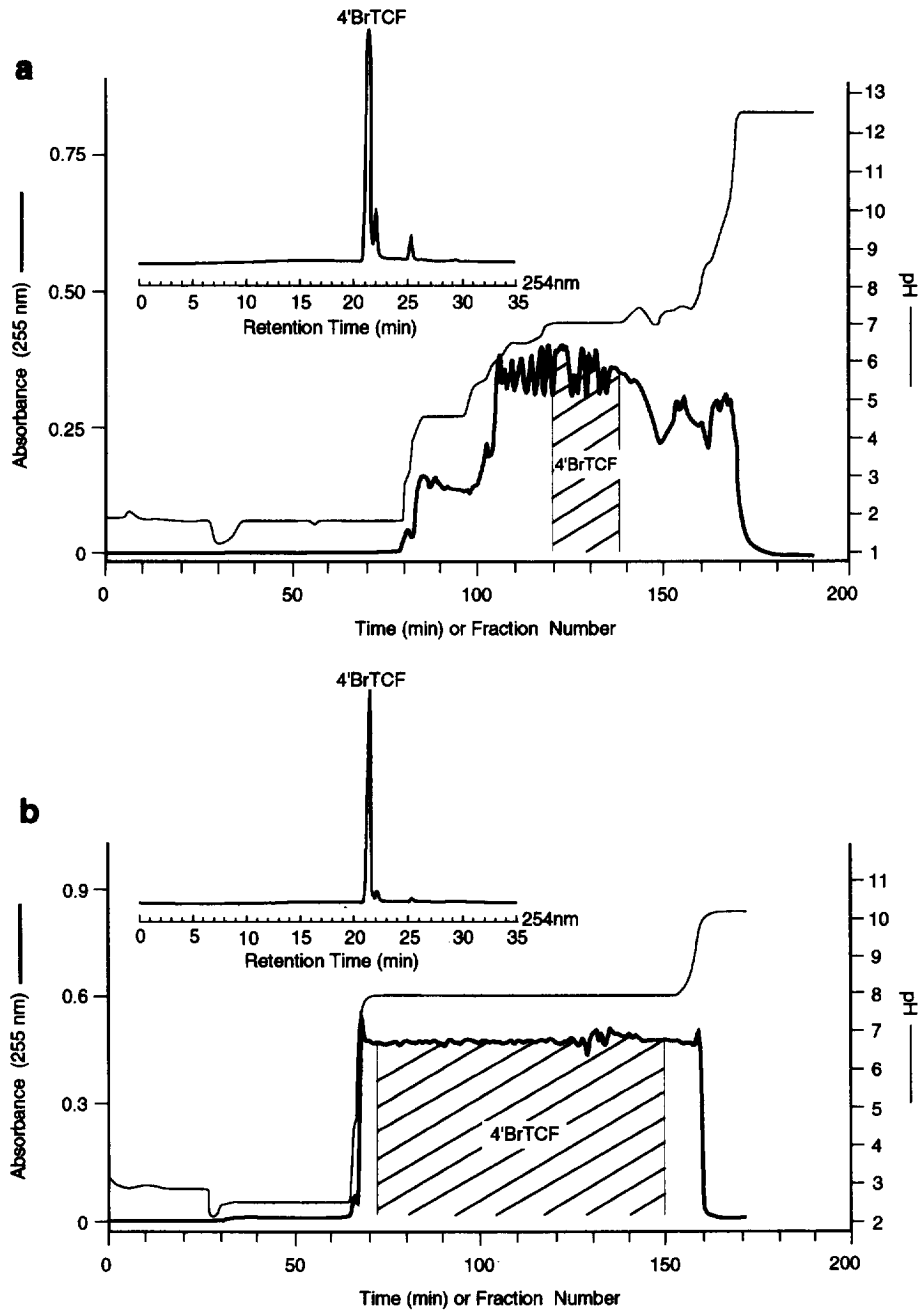

Fig. 5. pH-Zone-refining CCC with on-line UV and pH monitoring of (a) crude (ca. 25 area % 4'BrTCF, 5 g) and (b) partially purified (ca. 90 area % 4'BrTCF, 2.4 g) mixtures. HPLC of (a) the isolated and (b) the purified 4'BrTCF obtained from the combined fractions shown by the hatched areas.

injected into the column. Other experimental details are given in Section 2.3.2. The separation was completed in 3 h. The solvent front (first fraction containing mobile phase) emerged at fraction 27. The retention of the stationary phase measured after the separation was 31%. 4′BrTCF eluted as a broad peak with a pH plateau (fractions 121–138, hatched area in Fig. 5a). The product isolated in the lactone form from these combined fractions (1.28 g) contained ca. 90 area % 4′BrTCF (see high-performance liquid chromatogram in Fig. 5a). This product and a second batch of ca. 90 area % product (similarly isolated from another 5-g portion of crude mixture) were combined (total 2.4 g) for further purification.

The isolated 4′BrTCF was then purified by pH-zone-refining CCC with a different solvent system, *tert.*-butyl methyl ether–water (1:1, v/v). To 500 ml of the upper phase was added 0.35 ml (0.51 g, 4.5 mmol) of TFA, yielding a stationary phase 8.9 m*M* in TFA with pH ca. 2.8. To 675 ml of the lower phase was added 1.4 ml (1.15 g, ca. 17.5 mmol) of ammonium hydroxide, >25% NH_3 in water, yielding a mobile phase ca. 26 m*M* in NH_3 with pH ca. 10.7. The sample solution was prepared by dissolving 2.4 g of the ca. 90 area % product in 10 ml of acidified stationary phase and 3 ml of unbasified lower phase. All other experimental conditions were similar to those of the previous separation. The pH-zone-refining counter-current chromatogram of this separation is shown in Fig. 5b. The separation was completed in less than 3 h. The solvent front emerged at fraction 28. The retention of the stationary phase measured after the separation was 52.3%. The purified compound eluted as a broad rectangular absorbance peak that corresponded to a long pH plateau (fractions 72–157, Fig. 5b). Fractions 72–150 that contained greater than 92.5% 4′BrTCF by HPLC (hatched area in Fig. 5b) were combined. The 4′BrTCF was isolated in the lactone form (1.63 g, ca. 95% pure by HPLC, Fig. 5b) and was identified by ^1H NMR spectrometry and CIMS (see Fig. 3).

For these separations, a flow cell containing a pH electrode was inserted between the detector and the fraction collector, thus permitting 'on-line' measurement of the effluent pH. Previously, the pH of each fraction had been manually recorded, a tedious and time-consuming process. The counter-current chromatograms with the overlaid 'on-line' pH curves (thin line) of the above separations are shown in Fig. 5.

4. Conclusions

Monobrominated TCF, 4′BrTCF, has been prepared and purified. 4′,5′-Di-, 2′,4′,5′-tri- and 2′,4′,5′,7′-tetrabromo-TCF were previously obtained [5,7].

This work shows that the pH of the effluent (at times biphasic) can be effectively measured on-line in these systems, thereby eliminating the need for tedious manual pH measurements of multiple fractions. On-line monitoring of pH indicates directly (1) when separated components elute, even if UV–Vis monitoring shows an extended plateau and (2) when chromatography is completed (i.e., the pH of the effluent reaches the original pH of the basic mobile phase). These indications are especially useful for the separation of compounds that do not absorb in the UV–Vis region. The continuous storage of spectra and easy retrieval of chromatograms at different wavelengths permit rescaling and choosing an optimum wavelength for post-run display. The adjustable pathlength detector cell allows chromatograms to be on-scale even for a highly concentrated effluent.

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