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Preparation of two novel monobrominated 2-(2′,4′-dihydroxybenzoyl)-3,4,5,6-tetrachlorobenzoic acids and their separation from crude synthetic mixtures using vortex counter-current chromatography

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2-(2′,4′-Dihydroxy-5′-bromobenzoyl)-3,4,5,6-tetrachlorobenzoic acid

ABSTRACT

The present work describes the preparation of two compounds considered to be likely precursors of an impurity present in samples of the color additives D&C Red No. 27 (Color Index 45410:1) and D&C Red No. 28 (Color Index 45410, phloxine B) submitted to the U.S. Food and Drug Administration for batch certification. The two compounds, 2-(2′,4′-dihydroxy-3′-bromobenzoyl)-3,4,5,6-tetrachlorobenzoic acid (3BrHBBA) and its 5′-brominated positional isomer (5BrHBBA), both not reported previously, were separated from synthetic mixtures by vortex counter-current chromatography (VCCC). 3BrHBBA was prepared by chemoselective ortho-bromination of the dihydroxybenzoyl moiety. Two portions of the obtained synthetic mixture, 200 mg and 210 mg, respectively, were separated by VCCC using two two-phase solvent systems that consisted of hexane–ethyl acetate–methanol–aqueous 0.2% trifluoroacetic acid (TFA) in the volume ratios of 8:2:5:5 and 7:3:5:5, respectively. These separations produced 35 mg and 78 mg of 3BrHBBA, respectively, each product of over 98% purity by HPLC at 254 nm. 5BrHBBA was prepared by monobromination of the dihydroxybenzoyl moiety in the presence of glacial acetic acid. To separate the obtained synthetic mixture, VCCC was performed in the pH-zone-refining mode with a solvent system consisting of hexane–ethyl acetate–methanol–water (6:4:5:5, v/v) and with TFA used as the retainer acid and aqueous ammonia as the eluent base. Separation of a 1-g mixture under these conditions resulted in 142 mg of 5BrHBBA of ~99% purity by HPLC at 254 nm. The isolated compounds were characterized by high-resolution mass spectrometry and proton nuclear magnetic resonance spectroscopy.

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1. Introduction

D&C Red No. 27 (R27, mainly 2′,4′,5′,7′-tetrabromo-4,5,6,7-tetrachlorofluorescein, 1, Color Index 45410:1) and its disodium salt, D&C Red No. 28 (R28, mainly 2, Color Index 45410, phloxine B) are color additives permitted for use in drugs and cosmetics in the U.S. [1]. Their manufacture involves several steps: condensation of two equivalents of resorcinol with either one equivalent of tetrachlorophthalic anhydride [2] or one equivalent of tetrachlorophthalic acid in the presence of a condensing agent [3] to obtain the intermediate 4,5,6,7-tetrachlorofluorescein (TCF in Fig. 1); bromination of TCF to yield the main component of R27; and alkaline hydrolysis of R27 with sodium hydroxide to obtain the main component of R28 (Fig. 1). In addition to the main components, various impurities may be produced during the manufacturing process. R27 and R28 are batch-certified by the U.S. Food and Drug Administration (FDA) to ensure compliance with specifications in the Code of Federal Regulations (CFR) [1] that limit the level of each regulated impurity in those color additives. The impurities may be unreacted starting materials, e.g., "tetrachlorophthalic acid" (TCPA in Fig. 1), specified at "not more than 1.2%"; reaction byproducts, e.g., "2,3,4,5-tetrachloro-6-(3,5-dibromo-2,4-dihydroxybenzoyl) benzoic acid" (3,5diBrHBBA in Fig. 2), specified at "not more than 0.7%"; or subsidiary colors, e.g., "lower halogenated subsidiary colors" [4–8], specified at "not more than 4%." The application of new technologies has enabled identification and quantification of R27 and R28 impurities that are not specified in the CFR [9–12] but may be good manufacturing practice [1] violations.

As a step in the process of identifying an additional R27 and R28 impurity that is not specified in the CFR, the current work presents the synthesis and purification of two possible precursors

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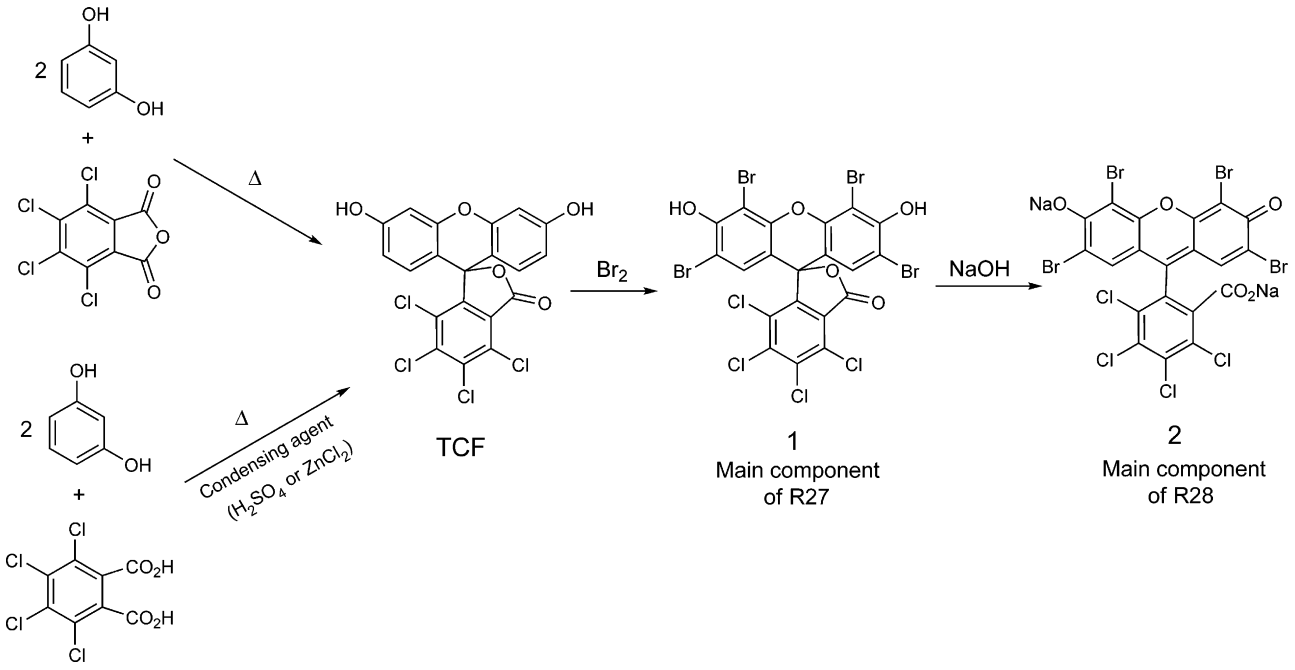

Fig. 1. Preparation of D&C Red Nos. 27 and 28 by condensation of 3,4,5,6-tetrachlorophthalic anhydride (or acid) with resorcinol.

of that impurity. The two compounds, 2-(2′,4′-dihydroxy-3′-bromobenzoyl)-3,4,5,6-tetrachlorobenzoic acid (3BrHBBA in Fig. 2) and its 5′-brominated positional isomer (5BrHBBA in Fig. 2), have not been reported previously and are lower brominated homologues of the dibrominated impurity specified in the CFR (3,5diBrHBBA in Fig. 2). The separation of 3BrHBBA and 5BrHBBA from their respective synthetic mixtures was performed by a newly developed technique named vortex counter-current chromatography (VCCC) [13–15].

Counter-current chromatography (CCC), developed more than four decades ago [16,17], is a liquid–liquid partition chromatographic technique that does not use a solid support. Subsequent development of the technique resulted in high-speed counter-current chromatography (HSCCC) that uses centrifugal force in a

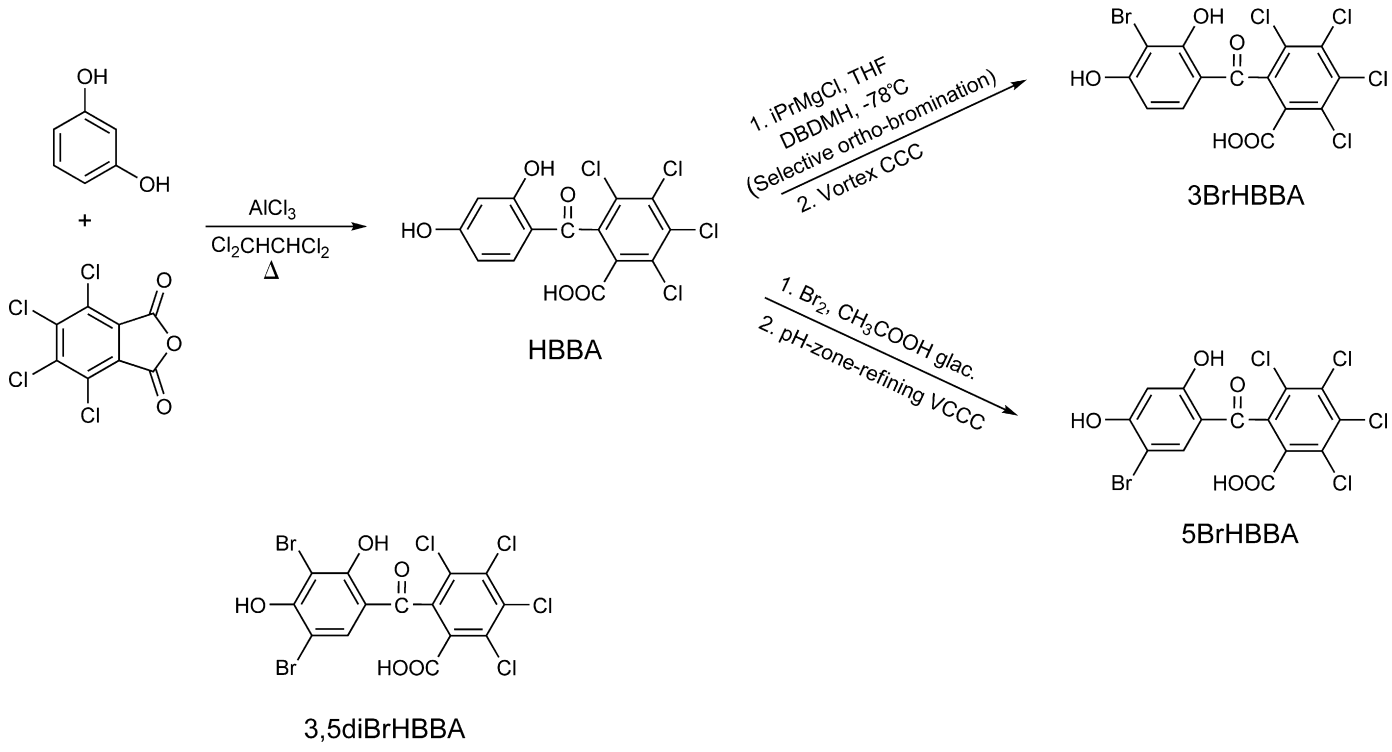

Fig. 2. Synthetic pathways for the preparation of 3BrHBBA and 5BrHBBA, and the structure of 3,5diBrHBBA.

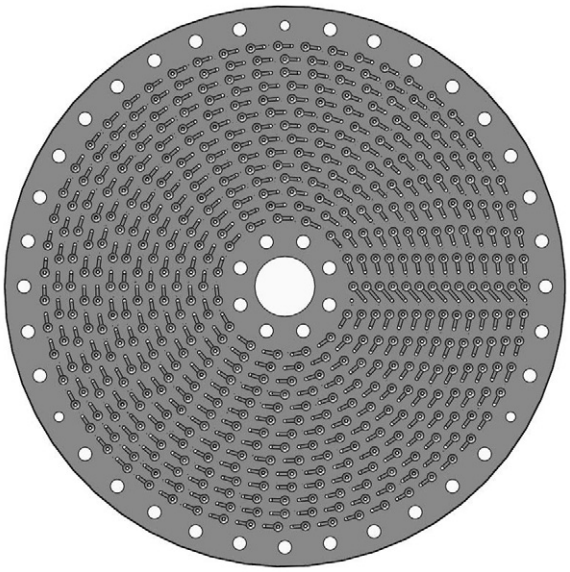

Fig. 3. Top view of improved VCCC column (for details see text).

planetary motion and the Archimedean Screw effect to retain one of the liquid phases (stationary phase) in an Ito multilayered-coil column while the second liquid phase (mobile phase) is pumped through the rotating column. The principles of HSCCC [18–20], instructions on how to perform HSCCC, and its many applications for the separation of synthetic and natural products have been discussed [21–26]. In the conventional mode of operation, HSCCC has been applied to the separation of up to several hundred milligrams of sample. A modified HSCCC technique, known as pH-zone-refining CCC [27–31], permits the separation of multigram quantities of ionic or ionizable compounds, such as organic acids and bases, according to their pK_a values and hydrophobicity. Both conventional HSCCC and pH-zone-refining CCC have been applied to the separation of synthetic dye components [32–37]. VCCC differs from HSCCC in that the separation does not take place in an Ito multilayered-coil column, but rather in a column that consists of a high-density polyethylene disk with a set of connected perpendicular cylindrical holes [13]. VCCC also differs from HSCCC in the manner of column rotation: although both types of columns revolve around the central axis of the centrifuge at the same rate and in the same direction as the centrifuge itself moves, the VCCC column rotates around its axis in the *opposite direction* to the rotation of the centrifuge, while the HSCCC column rotates around its axis in the *same direction* as that of the rotation of the centrifuge. Among the advantages of VCCC over the HSCCC technique, are the following: (i) due to the perpendicular arrangement of the cylindrical holes, solute band spreading is minimized along the length of the column and (ii) the Archimedean Screw effect is eliminated, thus producing a very low column pressure that avoids the risk of solvent leakage [13].

Synthetic mixtures of 3BrHBBA and 5BrHBBA, which contained other closely related components, were considered to be appropriate candidates for the first practical application of VCCC. Three separations are described in this paper, two performed in the conventional CCC mode and the third in the pH-zone-refining CCC mode. All of the separations were achieved using the most recently designed preparative VCCC column, which has 3 mm i.d. threaded cylindrical holes [15] (Fig. 3) and which was previously used only in test sample separation experiments [15].

2. Experimental

2.1. Materials

Methanol (MeOH), water, ammonium acetate (NH_4OAc), diethyl ether, ethyl acetate, and acetone were of chromatography grade. Ammonium chloride (NH_4Cl, Fisher Scientific, Fair Lawn, NJ, USA), ammonium hydroxide (>25% NH_3 in water, Fluka, Buchs, Switzerland), trifluoroacetic acid (TFA, Sigma–Aldrich, St. Louis, MO, USA), glacial acetic acid (A.C.S. reagent, Acros Organics, Fair Lawn, NJ, USA), sodium chloride (NaCl, >99.5%, Fluka), isopropyl magnesium chloride (i-PrMgCl, 2.0 M in diethyl ether, Sigma–Aldrich), 1,3-dibromo-5,5-dimethylhydantoin (DBDMH, Sigma–Aldrich), tetrahydrofuran (THF, anhydrous, ≥99.9%, Sigma–Aldrich) and bromine (99.9%, Sigma–Aldrich) were used as received.

2.2. Synthesis of 3BrHBBA

The synthetic mixture of 3BrHBBA used in this work was prepared by following the two-step sequence shown in Fig. 2. 2-(2′,4′-Dihydroxybenzoyl)-3,4,5,6-tetrachlorobenzoic acid (HBBA) was prepared previously [9] according to the method of Ullman and Schmidt [38], using resorcinol instead of phenol as the starting material. Chemoselective ortho-bromination of HBBA was performed based on the procedure of Kwak et al. [39], using HBBA instead of eugenol as the starting material. Thus, a 50-ml three-necked, pear-shaped flask that was equipped with an argon inlet system, a rubber septum, and a magnetic stirbar was charged with HBBA (1.20 g, 3.03 mmol) and anhydrous THF (17 ml). The flask was placed in a low-form cylindrical Dewar and cooled to −78 °C (dry ice-acetone bath) while the mixture was magnetically stirred under the argon atmosphere. To the cold mixture, i-PrMgCl (2.0 M solution in diethyl ether, 2 ml, 3.97 mmol) was added drop-wise via syringe through the rubber septum. The mixture was stirred for 30 min at −78 °C, then a solution of DBDMH (0.29 g, 1.03 mmol) in anhydrous THF (5 ml) was added in small portions via syringe. The resulting orange solution was stirred at −78 °C for 3 h. The reaction was stopped by adding saturated aqueous NH_4Cl solution (20 ml). After 15 min of additional magnetic stirring, the mixture was transferred to a 125-ml separatory funnel and extracted with diethyl ether (2 × 50 ml) and then with ethyl acetate (50 ml). The combined organic phases were washed with water and then with saturated aqueous NaCl solution, dried over Na_2SO_4, and concentrated by rotary evaporation (20 mm Hg). The obtained light-brown sticky material was re-dissolved in MeOH (10–15 ml) and re-concentrated (rotary evaporator). The resulting crude material (0.41 g, light-brown powder), composed mainly of 3BrHBBA (∼78.0%) and HBBA (∼12.3%) by HPLC at 254 nm (Fig. 4A), was subjected to VCCC as described in Section 2.4.2.

2.3. Synthesis of 5BrHBBA

The synthetic mixture of 5BrHBBA used in this work was prepared by bromination of HBBA, using half as much bromine as that reported for the preparation of the dibrominated compound 3,5diBrHBBA [40]. Thus, HBBA (2.04 g, 5.18 mmol) and hot glacial acetic acid (15 ml) were placed in a 200-ml round-bottomed flask equipped with a magnetic stirbar. The mixture was magnetically stirred (not all of the HBBA dissolved) and cooled to below room temperature (18–20 °C). To the cooled mixture, bromine (0.83 g, 0.27 ml, 5.18 mmol) was added drop-wise while stirring. The mixture became a clear light-brown solution, which was stirred for another hour. Elimination of the solvent (rotary evaporator) resulted in a sticky light-brown solid (2.20 g) that contained mainly 5BrHBBA (∼73.9%) and 3,5diBrHBBA (∼20.2%) by HPLC at 254 nm

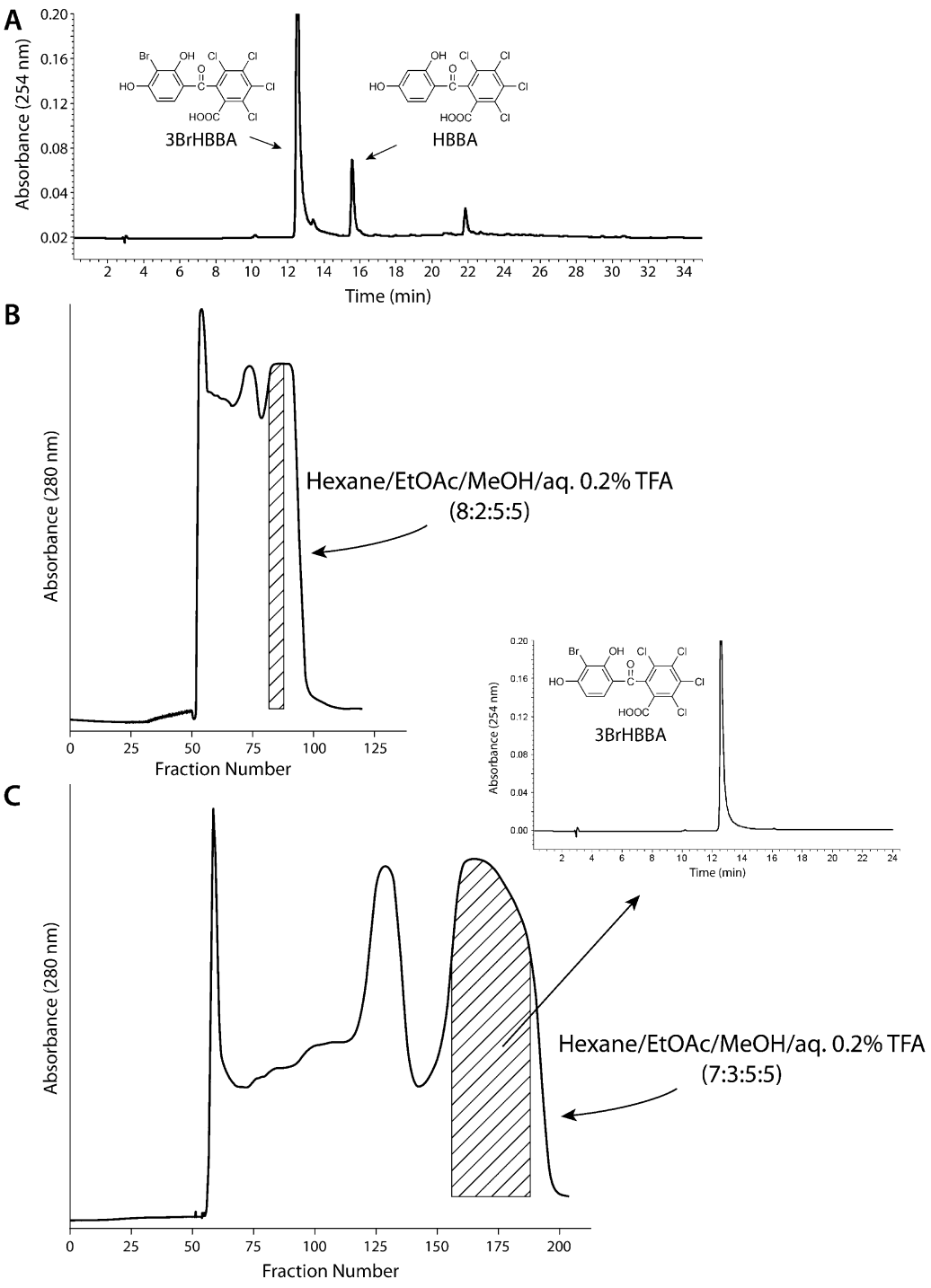

Fig. 4. Separation by VCCC of 3BrHBBA from a synthetic mixture. (A) HPLC analysis of the synthetic mixture. (B) Chromatogram of the VCCC separation of 200 mg mixture using a less polar solvent system. (C) Chromatogram of the VCCC separation of 210 mg mixture using a more polar solvent system and analytical HPLC chromatogram of the separated 3BrHBBA.

(Fig. 5A). This mixture was separated by pH-zone-refining VCCC as described in Section 2.4.2.

2.4. Vortex counter-current chromatography

2.4.1. Instrumentation

The VCCC separations were performed with a prototype instrument built in the NIH Machine Shop according to the specifications of one of the present authors (Y. Ito). Its design and functioning have been presented previously [13]. The instrument was equipped with an improved VCCC column described elsewhere [15] and shown in Fig. 3. The apparatus holds the VCCC column and its counterweight in opposite positions 10 cm from the central axis of the centrifuge. As described above, the VCCC column revolves around the central axis of the centrifuge at the same rate and in the same direction as the centrifuge itself moves, but the VCCC column also rotates around its own axis in the opposite direction to that of the rotation of the centrifuge.

The separation column was fabricated from a high-density polyethylene disk of 16 cm in diameter and 5 cm in height by

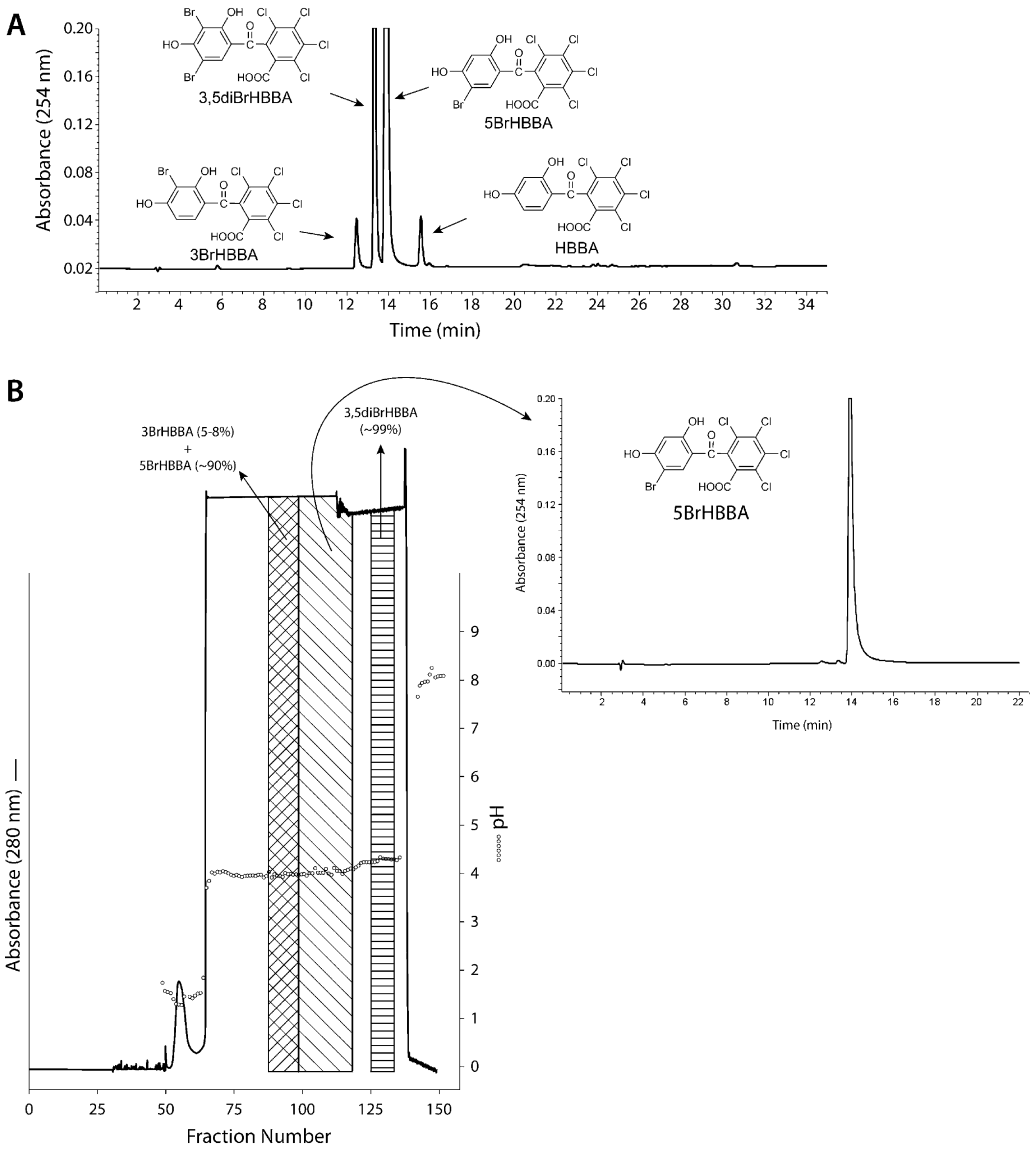

Fig. 5. Separation of a 1-g portion of 5BrHBBA synthetic mixture by pH-zone-refining VCCC. (A) HPLC analysis of the synthetic mixture. (B) Chromatogram of the pH-zone-refining VCCC separation and analytical HPLC chromatogram of the separated 5BrHBBA.

making multiple holes on each side with a 3 mm-diameter drill, as shown in Fig. 3. These cylindrical partition holes (996 in total) were connected in series in such a way that the exit (central opening at 0.75 mm i.d.) of each top cylinder was connected to the inlet (side opening of 1 mm i.d.) of the bottom cylinder. All holes were threaded with a pair of 6–40 taps to form right-handed threads in the upper holes and left-handed threads in the lower holes [14]. This threading pattern doubled the inner wall surface, thereby enhancing the mass-transfer partition capability over that of a previously designed VCCC column. The threaded column had a total length of about 48 m and a capacity of 364 ml.

The VCCC column was mounted on the rotating frame (centrifuge) whose rotation was monitored with a rotation-speed controller (Speedmaster, Leeson Electric Corporation, Grafton, WI). The solvent was pumped with a Waters model 510 HPLC pump (Boston, MA, USA) while the column effluent was monitored with a UV detector containing a 280-nm UV lamp (LKB UVicord SII, Bromma, Sweden) and a strip-chart recorder (Millipore, Boston, MA, USA). The fractions were collected using an LKB model 7000 Ultrorac fraction collector. The melting points of the separated 3BrHBBA and 5BrHBBA were measured with an Electrothermal MEL-TEMP 3.0 (Barnstead, Dubuque, IA, USA).

2.4.2. Separation procedure

The two conventional VCCC separations described here were performed following the general directions indicated for HSCCC [19,21], using a two-phase solvent system composed of hexane–ethyl acetate–methanol–aqueous 0.2% TFA at the volume ratios of 8:2:5:5 and 7:3:5:5, respectively. The solvent system was equilibrated in a separatory funnel, and the two phases were separated before use. The organic upper phase (UP) was used as the stationary phase, and the aqueous lower phase (LP) was used as the mobile phase. Each separation was initiated by filling the entire column with the stationary phase using the LC pump and then injecting the synthetic sample solution. The first sample solution (involving the 8:2:5:5, v/v solvent system) was prepared by dissolving 200 mg of synthesized 3BrHBBA in a 10-ml mixture of UP and LP (1:4). Similarly, the other sample solution was prepared by dissolving 210 mg of synthesized 3BrHBBA in a 10-ml UP-LP mixture (1:1). The mobile phase was then pumped into the column

inlet at a flow rate of 1 ml/min while the column was rotated at 1000 rpm in tail-to-head elution mode. The effluent from the column outlet was continuously monitored with the UV detector at 280 nm (the recorder was set at 50 min/cm) and collected in fractions (4 ml/tube) using the fraction collector. The first 200 ml of the effluent were collected in a graduated cylinder to estimate the retention of the stationary phase. The collected fractions were analyzed by HPLC.

The pH-zone-refining VCCC separation was performed following the previously established procedures for standard pH-zone-refining CCC of compounds containing a carboxylic acid group [21,41]. A two-phase solvent system composed of hexane–ethyl acetate–methanol–water (6:4:5:5, v/v) was equilibrated in a separatory funnel, and the two phases were separated shortly before use. The organic upper phase (UP) was acidified with TFA (retainer) at a concentration of 10 mM (pH 1.23) and was used as the stationary phase. The aqueous lower phase (LP) was made basic with ammonium hydroxide (eluter) at a concentration of 10 mM (pH 9.96) and was used as the mobile phase. The sample solution consisted of 1 g of the 5BrHBBA synthetic mixture dissolved in a 20-ml UP-LP mixture (1:1). A 150 μl portion of TFA was added to the sample solution to bring most of the sample into the upper phase. The pH of the sample solution became 1.6. The separation was then carried out as described above for conventional VCCC. After separation, the pH of each collected fraction was measured manually, using an Accumet AP61 pH meter (Fisher Scientific, Fair Lawn, NJ, USA), to trace the pH curve on the chromatogram. The collected fractions were analyzed by HPLC.

2.5. Analytical HPLC

The HPLC analyses were performed with a Waters Alliance 2690 Separation Module (Waters, Milford, MA, USA). The eluents were (A) 0.1 M NH_4OAc in water/methanol (95:5, v/v) and (B) methanol. The column (Hypersil C-8 MOS 5 μm particle size, 250 mm × 4.6 mm i.d., Phenomenex, Torrance, CA, USA) was eluted by using consecutive linear gradients of 25–90% methanol in 25 min and 90–100% methanol in 5 min, followed by 100% methanol for 5 min. The effluent was monitored with a Waters 996 photodiode array detector set at 254 nm. Other conditions included: flow-rate, 1 ml/min; column temperature, 37 °C; injection volume, 20 μl.

An aliquot (100–200 μl) from the vortex CCC-collected fractions was diluted with 2 ml of water/methanol (3:1, v/v). The solution was filtered through a Uniprep 0.45-μm glass microfiber syringeless filter unit (Whatman, Clifton, NJ, USA) prior to chromatography. The concentration of the samples analyzed in Figs. 4C and 5B was 0.2 mg/ml.

2.6. Liquid chromatography–mass spectrometry

The high-resolution mass spectra of the VCCC-separated 3BrHBBA and 5BrHBBA were obtained on an Agilent 6520 Q-TOF LC/MS system (Agilent Technologies, Santa Clara, CA, USA) equipped with Agilent MassHunter Workstation software for data acquisition and data analysis. The samples were dissolved in methanol (20 ng/μl) and analyzed in the negative electrospray ionization (ESI) mode. The high-resolution measurements of the monoisotopic ion mass of the pseudomolecular ions $[M-H]^-$ of the separated components were as follows: 3BrHBBA, *m/z* 470.80197; and 5BrHBBA, *m/z* 470.80374. These values matched the calculated mass of 470.799623 for $^{12}C_{14}{}^{1}H_4{}^{16}O_5{}^{79}Br_1{}^{35}Cl_4$, the deprotonated monobrominated 2-(2′,4′-dihydroxybenzoyl)-3,4,5,6-tetrachlorobenzoic acid.

2.7. ^1H nuclear magnetic resonance (NMR) spectroscopy

The ^1H NMR spectra of the VCCC-separated 3BrHBBA and 5BrHBBA were obtained on a Bruker AM-400 NMR spectrometer operating at 400 MHz. Approximately 3.8 mg portions of each of the isolated compounds were dissolved in 400 μl of CD_3OD. The following signals were obtained and assigned for each of the compounds: 3BrHBBA (Fig. 6C), 7.13 (d, J = 9 Hz, H-6′), 6.47 (d, J = 9 Hz, H-5′); 5BrHBBA (Fig. 7C), 7.36 (s, H-6′), 6.45 (s, H-3′).

3. Results and discussion

Bromination of HBBA using Br_2 and hot glacial acetic acid to prepare dibrominated 3,5diBrHBBA was previously reported [40]. Using the same method but with half the amount of Br_2, a mixture that consisted of the monobrominated 5BrHBBA (∼74%) and the dibrominated compound (∼20%) was obtained in the present work. That method did not produce any ortho-brominated 3BrHBBA, corroborating the well-documented challenge of obtaining ortho-brominated phenols [42]. Therefore, an alternative approach was implemented following the method described recently [39] of using DBDMH instead of Br_2 to selectively ortho-brominate HBBA in position 3′. A mixture was thus obtained that consisted mostly of 3BrHBBA (∼78%) and some unreacted starting material (∼12% HBBA). The synthetic pathways for the preparation of 3BrHBBA and 5BrHBBA are shown in Fig. 2.

Conventional HSCCC involves separation of up to several hundred milligrams of sample while pH-zone-refining CCC is best suited for separations of gram quantities of sample. The separations of 3BrHBBA were performed in the conventional HSCCC mode since they involved samples of ∼200 mg synthetic mixtures. Due to the higher amount of synthetic mixture available, 1-g, the separation of 5BrHBBA was performed in the pH-zone-refining mode.

Fig. 4A shows the analytical HPLC of the 3BrHBBA synthetic mixture. Fig. 4B shows the elution profile of the VCCC separation of a 200 mg portion of that mixture using the solvent system hexane–ethyl acetate–methanol–aqueous 0.2% TFA (8:2:5:5, v/v). The separation was completed in ∼6.5 h. The solvent front (first fraction containing mobile phase) emerged at fraction 50, and the retention of the stationary phase was ∼44% of the total column volume. The eluates collected in fractions 82–87 (hatched area in Fig. 4B) contained 3BrHBBA. The 3BrHBBA isolated from these combined fractions (35 mg, white powder, m.p. 242–244 °C with decomposition) was of ∼99% purity by HPLC at 254 nm (not shown). The eluates collected in fractions abutting the hatched area, fractions 79–81 and 88–90, contained 3BrHBBA of 94–95% purity.

A similar VCCC separation of a second portion (210 mg) of the 3BrHBBA synthetic mixture was performed using the same solvents but in a ratio (7:3:5:5, v/v) that produced a more polar solvent system. The UV profile of the eluate obtained from that separation is shown in Fig. 4C. The separation was completed in ∼13 h. Notably, while emergence of the solvent front (fraction 48) and retention of the stationary phase (∼49%) are comparable to the corresponding results of the first separation, the duration of the second separation was twice as long (∼13 h) as that of the first. The eluates collected in fractions 155–182 (hatched area in Fig. 4C) contained 3BrHBBA. The 3BrHBBA isolated from these combined fractions (78 mg) was of ∼98% purity by HPLC at 254 nm (Fig. 4C). In this second separation, due to the more polar stationary (upper) phase, all the components of the mixture were retained longer in the column, resulting in a higher peak resolution. If an even higher peak resolution had been necessary, it probably could have been accomplished by adjusting the solvent ratio (e.g., 6:4:5:5 v/v) to produce an even more

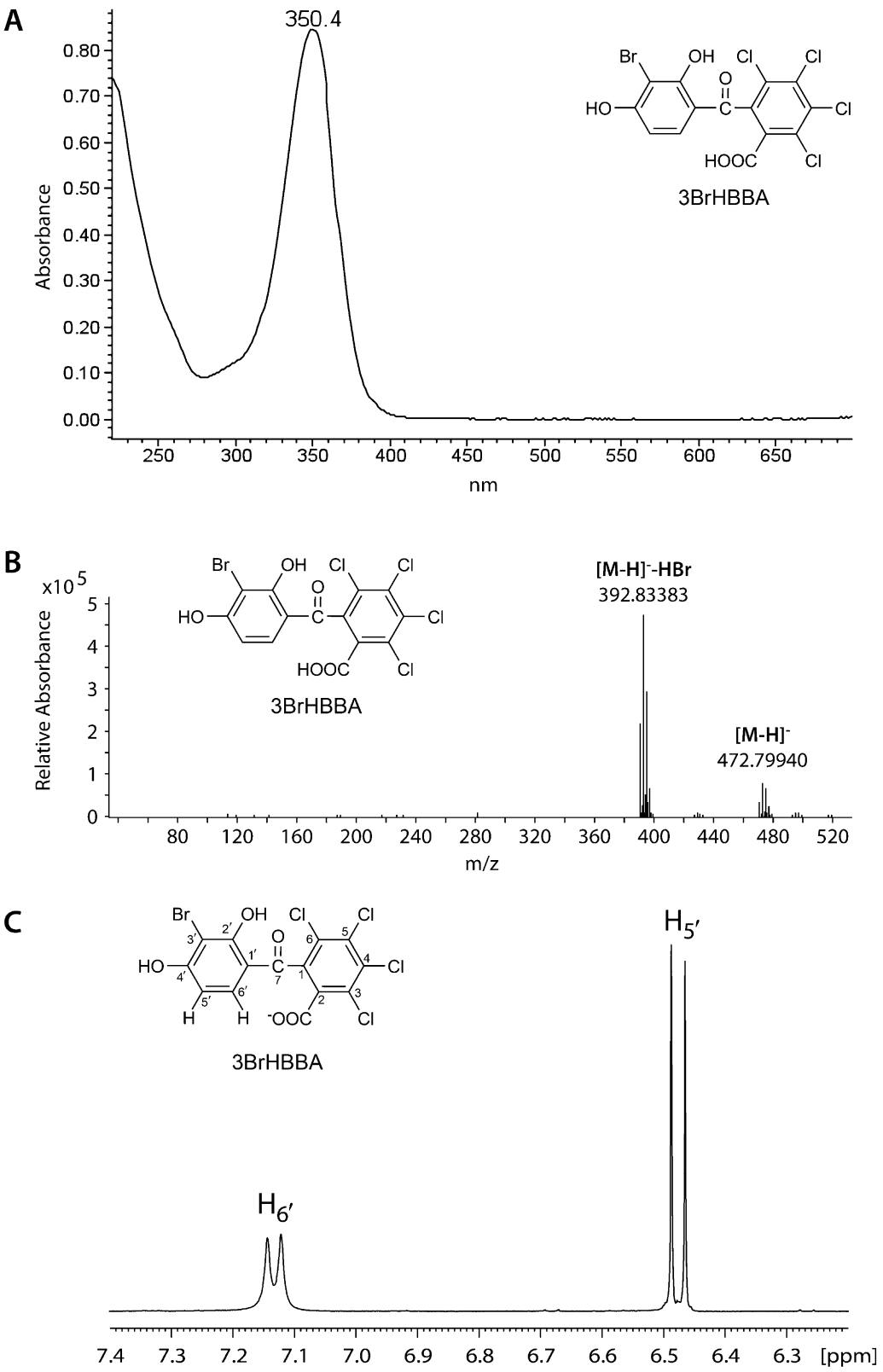

Fig. 6. Characterization of the separated 3BrHBBA: (A) UV spectrum in water/methanol; (B) negative ion ESI high-resolution mass spectrum; and (C) ^{1}H NMR spectrum (CD_3OD, 400 MHz).

polar system. However, such a change would have considerably lengthened the already extended duration of the separation.

Fig. 5A shows the analytical HPLC of the 5BrHBBA synthetic mixture. The pH-zone-refining VCCC elution profile of the separation of a 1-g portion of the mixture is shown in Fig. 5B. The solvent front emerged at fraction 50, and the retention of the stationary phase was ~45% of the total column volume. The obtained UV pH-zone-refining VCCC chromatogram (Fig. 5B) consists of one major

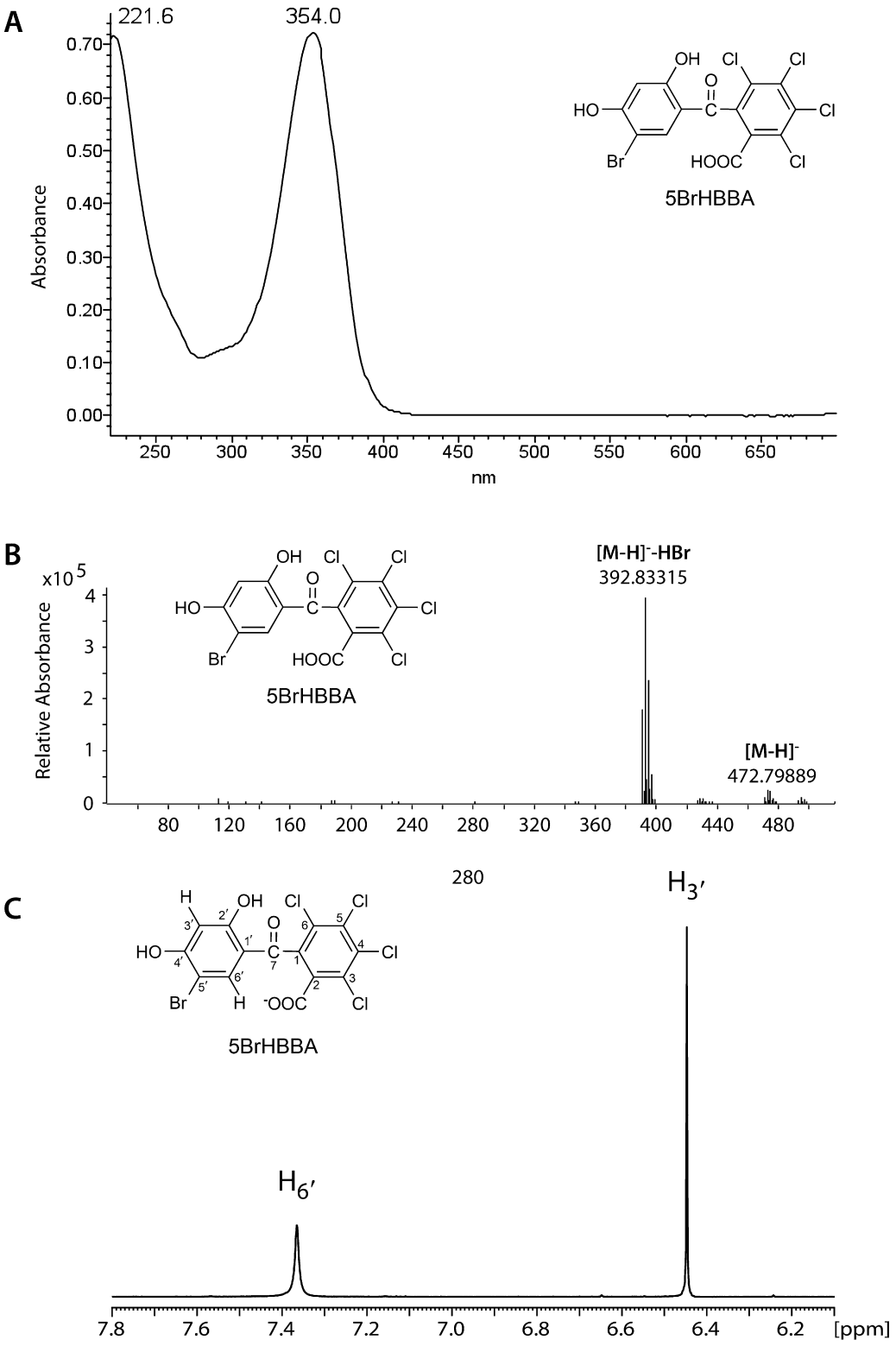

Fig. 7. Characterization of the separated 5BrHBBA: (A) UV spectrum in water/methanol; (B) negative ion ESI high-resolution mass spectrum; and (C) ^{1}H NMR spectrum (CD_3OD, 400 MHz).

broad rectangular peak and one narrower rectangular peak. The two absorbance plateaus correspond to the two pH plateaus. The eluates corresponding to the major broad peak contained 5BrHBBA with two levels of purity. The 5BrHBBA isolated from the combined fractions 101–115 (142 mg, white powder, m.p. 250–252 °C with decomposition) was of ~99% purity by HPLC at 254 nm (Fig. 5B). Fractions 88–100 contained 5BrHBBA of ~90% purity. The eluates corresponding to the narrow peak (fractions 125–130) contained 3,5diBrHBBA of ~99% purity by HPLC at 254 nm (not shown).

The separated 3BrHBBA and 5BrHBBA were characterized by UV, negative ESI high-resolution mass-spectrometry, and ^{1}H NMR (Figs. 6 and 7, respectively). It is noticeable in Figs. 6C and 7C that

H-6′ for both compounds is considerably broadened, relative to H-3′ and H-5′, and this is due to restricted rotation of the two aryl groups. For both positional isomers, the 6-Cl and 2-COOH groups cannot rotate past H-6′. Instead, there is a relatively slow (on the proton NMR chemical-shift time scale) librational motion about the C-1/C-7 bond, which leads to signal broadening in both cases [43].

4. Conclusion

This study demonstrates the first practical application of VCCC, previously used only in test sample separation experiments [15]. The work involved the use of the most recently designed preparative VCCC column, in both the conventional CCC and the pH-zone-refining CCC modes, for the successful separation of two novel monobrominated 2-(2′,4′-dihydroxybenzoyl)-3,4,5,6-tetrachlorobenzoic acids from synthetic mixtures. The availability of these monobrominated positional isomers will permit further investigation of an impurity present in the color additives D&C Red No. 27 and D&C Red No. 28.

References

[1] Code of Federal Regulations, 21 CFR 74.1327, 74.1328, 74.2327, and 74.2328, U.S. Government Printing Office, Washington, DC, 2011.
[2] C. Graebe, Justus Liebigs Ann. Chem. 238 (1887) 333.
[3] W.R. Orndorff, E.F. Hitch, J. Am. Chem. Soc. 36 (1914) 681.
[4] A.B. Leatherman, J.E. Bailey, S.J. Bell, P.M. Watlington, E.A. Cox, C. Graichen, M. Singh, in: K. Venkataraman (Ed.), The Analytical Chemistry of Synthetic Dyes, Wiley, New York, 1977, p. 465.
[5] A. Weisz, A.L. Scher, Y. Ito, J. Chromatogr. A 732 (1996) 283.
[6] A. Weisz, A.L. Scher, D. Andrzejewski, Y. Shibusawa, Y. Ito, J. Chromatogr. 607 (1992) 47.
[7] A. Weisz, D. Andrzejewski, Y. Ito, J. Chromatogr. A 678 (1994) 77.
[8] P.R. Wright, N. Richfield-Fratz, A. Rasooly, A. Weisz, J. Planar Chromatogr. 10 (1997) 157.
[9] A. Weisz, Dyes Pigments 35 (1997) 101.
[10] D. Andrzejewski, A. Weisz, J. Chromatogr. A 863 (1999) 37.
[11] A. Weisz, D. Andrzejewski, J. Chromatogr. A 1005 (2003) 143.
[12] A. Weisz, P.R. Wright, D. Andrzejewski, M.B. Meyers, K. Glaze, E.J. Mazzola, J. Chromatogr. A 1113 (2006) 186.
[13] Y. Ito, Z. Ma, R. Clary, J. Powell, M. Knight, T.M. Finn, J. Chromatogr. A 1218 (2011) 6165.
[14] Y. Ito, Z. Ma, R. Clary, J. Powell, M. Knight, T.M. Finn, J. Chromatogr. A 1218 (2011) 4065.
[15] Y. Ito, R. Clary, J. Witten, Y. Zeng, Chromatographia, submitted.
[16] Y. Ito, R.L. Bowman, Science 167 (1970) 281.
[17] Y. Ito, R.L. Bowman, J. Chromatogr. Sci. 8 (1970) 315.
[18] Y. Ito, CRC Crit. Rev. Anal. Chem. 17 (1986) 65.
[19] Y. Ito, in: Y. Ito, W.D. Conway (Eds.), High-Speed Countercurrent Chromatography, Chemical Analysis, vol. 132, Wiley, New York, 1996, p. 3 (Chapter 1).
[20] W.D. Conway, Countercurrent Chromatography. Apparatus. Theory & Applications, VCH, New York, 1990.
[21] Y. Ito, J. Chromatogr. A 1065 (2005) 145, and references cited therein.
[22] Y. Ito, W.D. Conway (Eds.), High-Speed Countercurrent Chromatography, Chemical Analysis, vol. 132, Wiley, New York, 1996, p. 3 (Chapter 1).
[23] A. Berthod (Ed.), Countercurrent Chromatography, the Support-Free Liquid Stationary Phase, Wilson & Wilson's Comprehensive Analytical Chemistry, vol. 38, Elsevier, Amsterdam, 2002.
[24] F. das Neves Costa, G.G. Leitao, J. Sep. Sci. 33 (2010) 336.
[25] L. Fang, Y. Liu, B. Yang, X. Wang, L. Huang, J. Sep. Sci. 34 (2011) 2545.
[26] M.R. Almeida, G.G. Leitão, B.V. Silva, J.P. Barbosa, A.C. Pinto, J. Braz. Chem. Soc. 21 (2010) 764.
[27] A. Weisz, A.L. Scher, K. Shinomiya, H.M. Fales, Y. Ito, J. Am. Chem. Soc. 116 (1994) 704.
[28] Y. Ito, K. Shinomiya, H.M. Fales, A. Weisz, A.L. Scher, in: W.D. Conway, R.J. Petroski (Eds.), Modern Countercurrent Chromatography, American Chemical Society, Washington, DC, 1995, p. 156.
[29] Y. Ito, in: Y. Ito, W.D. Conway (Eds.), High-Speed Countercurrent Chromatography, Chemical Analysis, vol. 132, Wiley, New York, 1996, p. 121 (Chapter 6).
[30] Y. Ito, Y. Ma, J. Chromatogr. A 753 (1996) 1, and references cited therein.
[31] B. Billardello, A. Berthod, in: A. Berthod (Ed.), Countercurrent Chromatography, the Support-Free Liquid Stationary Phase, Wilson & Wilson's Comprehensive Analytical Chemistry, vol. 38, Elsevier, Amsterdam, 2002, p. 177.
[32] A. Weisz, Y. Ito, J. Chromatogr. A 1198–1199 (2008) 232.
[33] N.T. Vu, J.D. Rickard, M.P. Sullivan, N. Richfield-Fratz, A. Weisz, J. Liq. Chromatogr. Related Technol. 34 (2011) 106.
[34] A. Weisz, Y. Ito, J. Chromatogr. A 1218 (2011) 6156.
[35] A. Weisz, D. Andrzejewski, R.J. Highet, Y. Ito, J. Liq. Chromatogr. Related Technol. 21 (1998) 183.
[36] A. Weisz, E.P. Mazzola, Y. Ito, J. Chromatogr. A 1216 (2009) 4161.
[37] A. Weisz, E.P. Mazzola, Y. Ito, J. Chromatogr. A 1218 (2011) 8249.
[38] F. Ullman, W. Schmidt, Berichte 52 (1919) 2098.
[39] J.-H. Kwak, J.-K. In, M.-S. Lee, E.-H. Choi, H. Lee, J.T. Hong, Y.-P. Yun, S.J. Lee, S.-Y. Seo, Y.-G. Suh, J.-K. Jung, Arch. Pharm. Res. 31 (2008) 1559.
[40] W.R. Orndorff, W.A. Adamson, J. Am. Chem. Soc. 40 (1918) 1235.
[41] A. Weisz, Y. Ito, in: I.D. Wilson, E.R. Adlard, M. Cooke, C.F. Poole (Eds.), Encyclopedia of Separation Science, vol. 6III, Academic Press, London, 2000, p. 2588.
[42] D.E. Pearson, R.D. Wysong, C.V. Breder, J. Org. Chem. 32 (1967) 2358.
[43] G. Binsch, H. Kessler, Angew. Chem. Int. Ed. Engl. 19 (1980) 411.