A New Preparative-Scale Purification Technique: pH-Zone-Refining Countercurrent Chromatography

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Received May 6, 1993®

Abstract: A novel countercurrent chromatographic technique is described for the preparative separation—with excellent resolution—of up to multigram quantities of organic acids. This separation is accomplished by addition of a simple acid (e.g., trifluoroacetic acid) to the sample solution or stationary phase, followed by isocratic elution with a basic mobile phase. The acids elute as well-resolved rectangularly shaped peaks. This technique should find widespread application in any organic chemistry laboratory for the separation of crude mixtures and for the purification of isolated acids.

Introduction

Recently, we called attention to the ability of a short-chain organic acid (bromoacetic acid) to unusually sharpen peaks during high-speed countercurrent chromatography (CCC) of bromoacetyl triiodothyronine.[1] We showed that the bromoacetic acid retards and sharpens the peak for the thyronine derivative by delaying its elution until bromoacetic acid elutes and the pH rises sharply. In that paper, we suggested using the peak-sharpening phenomenon to collect eluting compounds in fewer fractions and with increased detectability. Here, we wish to focus on the remarkable separation and purification of multigram quantities of carboxylic acids that can be achieved by addition of an appropriately chosen acid to the sample solution and/or the stationary phase, followed by elution with a basic mobile phase.

Results and Discussion

In pursuing the preparative aspects of this phenomenon, we found that when the sample amount is increased, a sequence of rectangular peaks with almost no overlap is produced. The formation of rectangular peaks with sharp sides is demonstrated in a model separation of a mixture of *N*-(2,4-dinitrophenyl)-amino acids (DNP-amino acids). Three DNP-amino acids were eluted from an organic stationary phase containing trifluoroacetic acid (TFA) by using a basic aqueous mobile phase. The resulting chromatogram (Figure 1A, solid line) contained three consecutive absorbance plateaus of nearly the same height. As one test of purity, the partition coefficient of each fraction was measured in a reference solvent system, and the resulting graph (···) showed three plateaus whose lengths coincided with those of the absorbance plateaus. The partition coefficients represented by these plateaus were identical to the partition coefficients of the pure DNP-amino acids in the same solvent system. Thus the absorbance plateaus were the tops of three abutting rectangular peaks each containing a single pure DNP-amino acid. The sharp transitions in absorbance and partition coefficients between the peaks indicate minimal overlap. Transitions in the pH (– –) of the eluent also coincide with these transitions. The true rectangular shape of the peaks and the sharpness of the separation

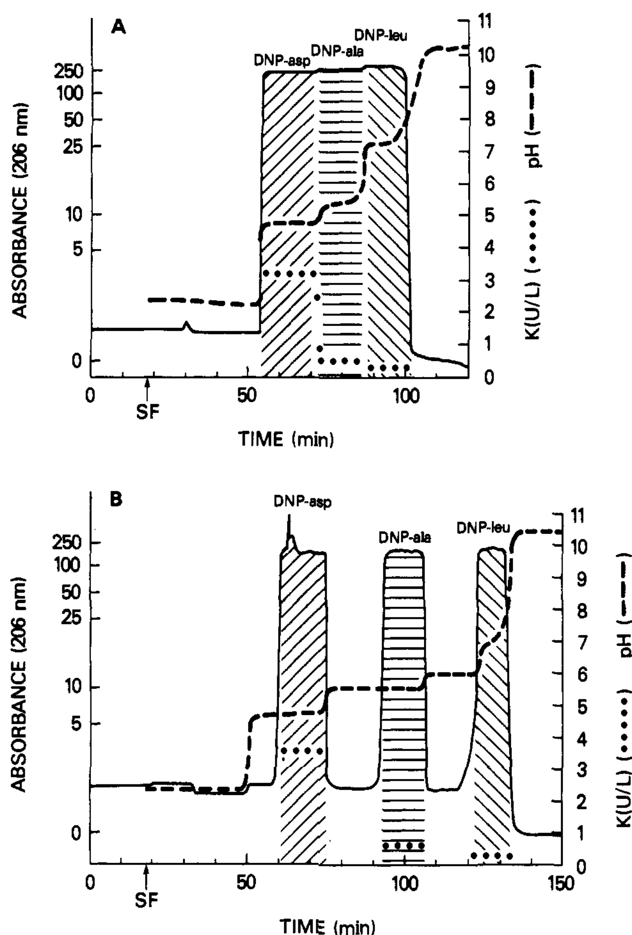

Figure 1. Model separations of a mixture of three DNP-amino acids by pH-zone-refining CCC. (A) DNP-L-aspartic acid, DNP-L-alanine, and DNP-L-leucine (100 mg each). (B) sample as in (A) plus acetic, propionic, and butyric acids (0.04% each, volume/volume) added to stationary phase. The continuous absorbance (solid line), pH (– –), and partition coefficient (···) of each fraction in a standard solvent system are shown. SF, solvent front.

are dramatically emphasized by addition of "spacer" acids to the system. Thus, after addition of TFA and acetic, propionic, and butyric acids to the stationary phase, CCC of the same mixture again produced three rectangular peaks for the DNP-amino acids,

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® Abstract published in *Advance ACS Abstracts*, December 15, 1993.
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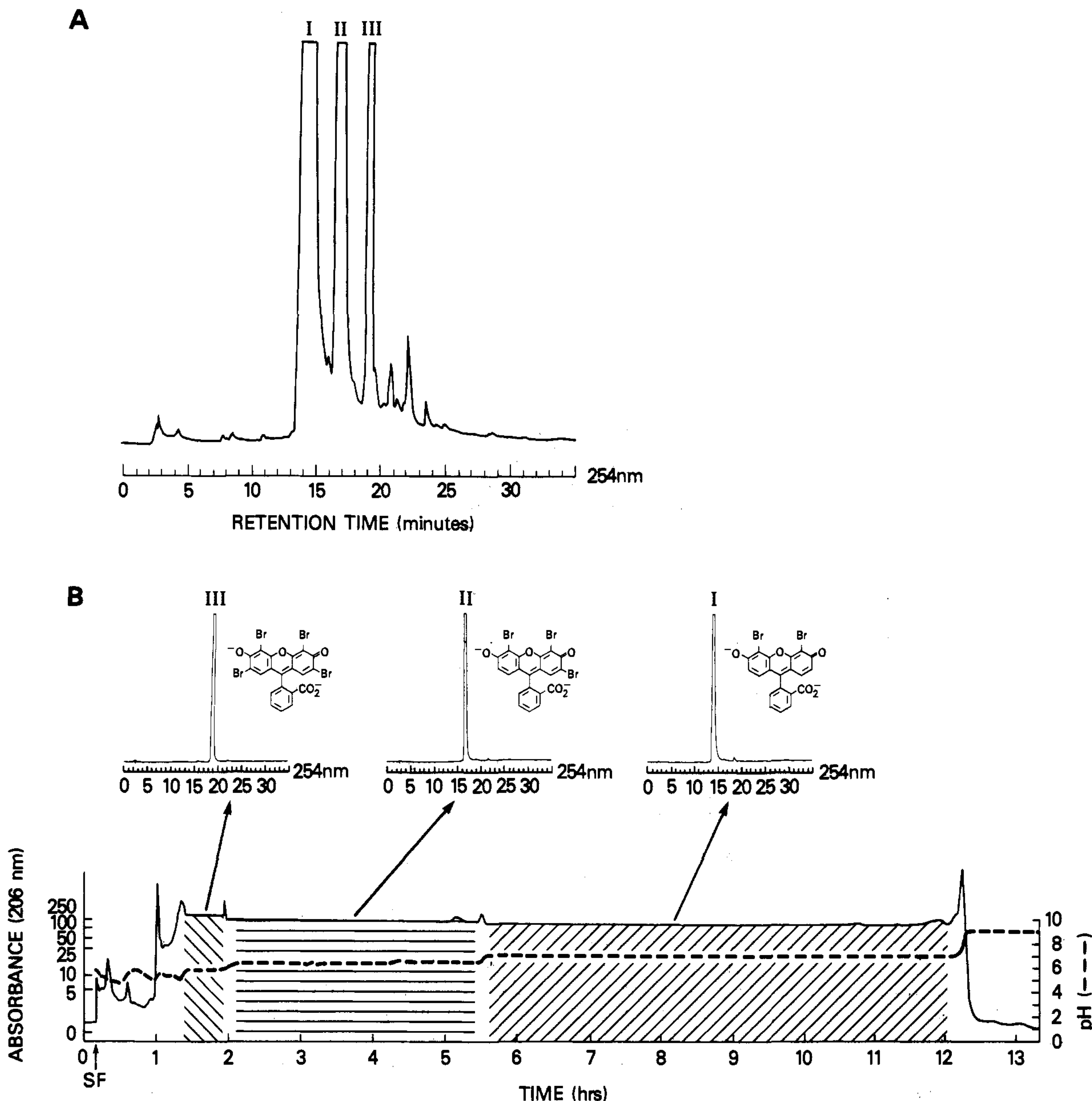

Figure 2. Separation of 5 g of D&C Orange No. 5 by pH-zone-refining CCC. (A) RP-HPLC analysis of the D&C Orange No. 5 used in the present study. I, II, and III are the major components of this brominated hydroxyxanthene color additive. (B) pH-zone-refining countercurrent chromatogram of 5 g of D&C Orange No. 5 and RP-HPLC chromatograms of the dyes isolated from the combined fractions from each hatched region. The continuous absorbance (solid line) and the pH (– –) of each fraction are shown.

peaks that were clearly separated by valleys containing (undetected) propionic and butyric acids (Figure 1B). The spacer acids for this experiment were chosen on the basis of preliminary studies described in the Experimental Section.

In a more practical application, the technique was used to isolate pure components from all of the hydroxyxanthene dyes that are permitted for coloring food, drugs, or cosmetics in the United States. The hydroxyxanthene dyes investigated were 4,5,6,7-tetrachlorofluorescein, D&C Orange No. 5 (mainly 4′,5′-dibromofluorescein), D&C Red No. 22 (eosin Y, mainly the disodium salt of 2′,4′,5′,7′-tetrabromofluorescein), D&C Orange No. 10 (mainly 4′,5′-diiodofluorescein), FD&C Red No. 3 (erythrosine, mainly 2′,4′,5′,7′-tetraiodofluorescein), D&C Red No. 27 (mainly 2′,4′,5′,7′-tetrabromo-4,5,6,7-tetrachlorofluorescein), and D&C Red No. 28 (phloxine B, the disodium salt of D&C Red No. 27).

As one example, the separation of the color additive D&C Orange No. 5 is described below. D&C Orange No. 5 is identified as a mixture containing mainly three hydroxyxanthene dyes: 50–60% of 4′,5′-dibromofluorescein; 30–40% of 2′,4′,5′-tribromofluorescein; and ≤10% of 2′,4′,5′,7′-tetrabromofluorescein[2] (peaks I, II, and III in Figure 2A). The pure di-, tri-, and tetrabromofluoresceins are needed as reference materials in the Food and Drug Administration's (FDA) analyses of D&C Orange No. 5 for certification purposes.

The pH-zone-refining CCC chromatogram of a *suspension* containing 5 g of D&C Orange No. 5 is shown in Figure 2B. The chromatogram has a broad rectangular shape comparable to the chromatogram of the DNP-amino acids (Figure 1A). The three broad absorbance plateaus (solid line) again correspond to the three pH plateaus (– –). Each plateau represents elution of a pure compound as illustrated by the associated reversed-phase high-performance liquid chromatography (RP-HPLC) chromatograms of aliquots of the combined fractions from the three hatched regions. Each pH transition on the dashed line again corresponds to an absorbance transition. Impurities were concentrated in the few fractions corresponding to these transition zones (HPLC analysis not shown). The sequence of elution of

(2) *Code of Federal Regulations*, Title 21, Section 74.1255; U. S. Government Printing Office: Washington, DC, 1993.

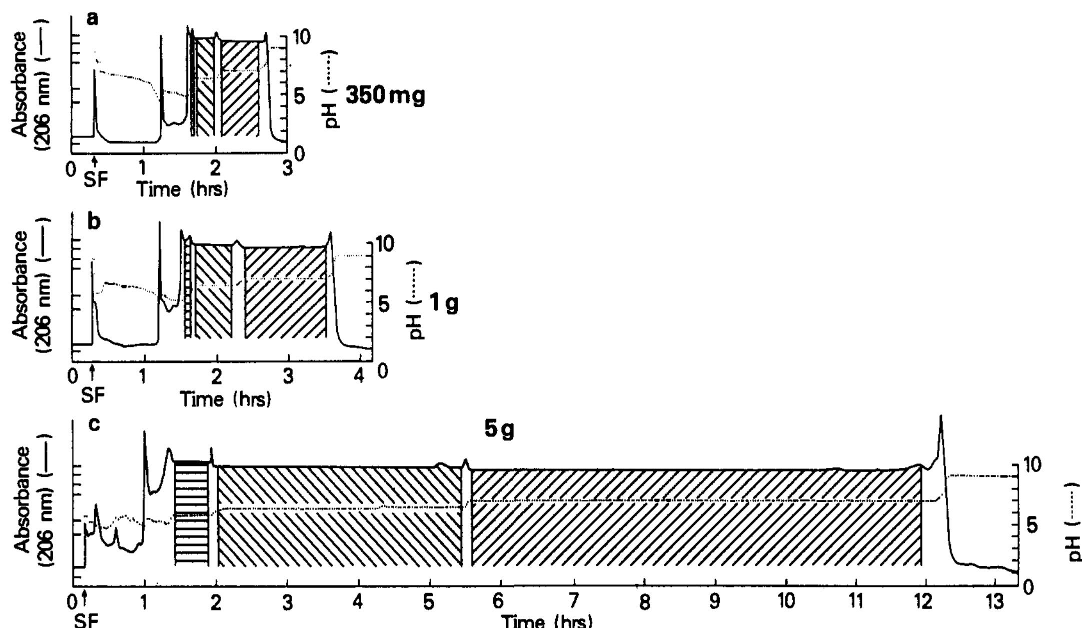

Figure 3. pH-zone-refining CCC separations of 0.35, 1, and 5 g of D&C Orange No. 5.

Table 1. pH-Zone-Refining Countercurrent Chromatography of D&C Orange No. 5[a]

sample size (mg)	recovery[b] (%) I	II	III
350	77.2	87.3	42
5000	82.0	90.3	77

[a] FDA certified batch. Recoveries were calculated based on the amounts of I, II, and III in the dye as determined for certification by FDA (56.6, 33.7, and 6.8%, respectively). [b] Isolated as lactones[12] and identified by chemical-ionization mass spectrometry and by ^{1}H nuclear magnetic resonance.

the three main components in this case is in order of their decreasing acid dissociation constant, pK_{a2}: III < II < I. For 2′,4′,5′,7′-tetrabromofluorescein (III), pK_{a1} = 3.25 and pK_{a2} = 3.80.[3] For 2′,4′,5′-tribromofluorescein (II), pK_{a1} = 3.64 and pK_{a2} = 4.34, and for 4′,5′-dibromofluorescein (I), pK_{a1} = 3.55 and pK_{a2} = 4.8 (D. Fompeydie, Faculté des Sciences Pharmaceutique et Biologiques, Paris, France, personal communication, 1992). The pH-zone-refining CCC separations of 0.35, 1, and 5 g of D&C Orange No. 5 resulted in proportionate increases in peak width and pH plateau length, but the small degree of overlap between the peaks was maintained (Figure 3). We especially wish to emphasize the excellent recoveries of the isolated dyes as shown in Table 1.

pH-Zone-refining CCC has several valuable features. The separation of a 5-g sample with minimal overlap was achieved on a column previously recommended for the separation of up to 0.5 g of sample. Impurities are concentrated in the few fractions that elute just before and after the main components, a feature that the technique has in common with zone-refining, where the impurities are concentrated in front of the main component.[4] As in the latter technique, this effect may itself find utility in isolation and analysis of important trace components.

As shown above, the technique permits loading the sample as a suspension into the separation column. Thus, even mixtures of acidic compounds that are only partially soluble in the solvent system can be efficiently separated. It is important to stress that when more sample is introduced, the only effect is to widen the plateau regions that contain pure compounds (Figure 3). Furthermore, the elution time may be shortened by increasing the concentration of base in the mobile phase. The rectangular peak shape is inherent to the present method and provides an advantage, especially for scale-up, over the Gaussian or skewed peak shapes obtained from conventional chromatographic separations. In the case of two closely related compounds, the sharp rise and fall of the peak edges permit separation with minimal overlap. The formation of a train of rectangular peaks, each traveling at the same (isotachic) velocity, and the enrichment of trace components are features that also occur in displacement chromatography[5,6] and isotachophoresis.[7]

The mechanism of the efficient separations achieved by CCC is fairly complex because of the hydrodynamic nature of the system. A full theoretical treatment is in preparation and will be presented elsewhere.[8,9] A simple view, however, is to recall that in CCC, as in all chromatographic systems, *separation* is achieved through multiple phase transfers that magnify small differences in the partitioning of the analytes. In pH-zone-refining CCC the analytes are carboxylic acids whose salts partition mainly into the aqueous phase and whose acid forms partition mainly into the organic phase. As one salt component encounters the front containing an acid of lower pK_a and hydrophobicity (intrinsic partition coefficient, $\delta_C = [HA_{org}]/[HA_{aq}]$, of the acid HA itself in the two-phase solvent system), its ionization is repressed and it enters the stationary organic phase. As a result, the strongest and least hydrophobic acid elutes first followed by acids with increasing pK_as and hydrophobicities. In the pH-zone-refining CCC separation of a mixture of acids, a stronger acid will elute before a weaker acid if the hydrophobicities of the acids are the same. Similarly, the least hydrophobic acid in the mixture will elute first if the pK_as are the same. If both the pK_as and the hydrophobicities of the acids in the mixture differ, then the order of elution will depend on a combination of their δ_Cs and pK_as. The critical factor here is that the analyte acids themselves control the pH of the system in a dynamic fashion. It is this feature that distinguishes the present method from simple pH-gradient elution

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(9) Scher, A. L.; Weisz, A.; Ito, Y. *Countercurrent Chromatography Symposium*, ACS National Meeting, August 22–27, 1993; Chicago, IL.

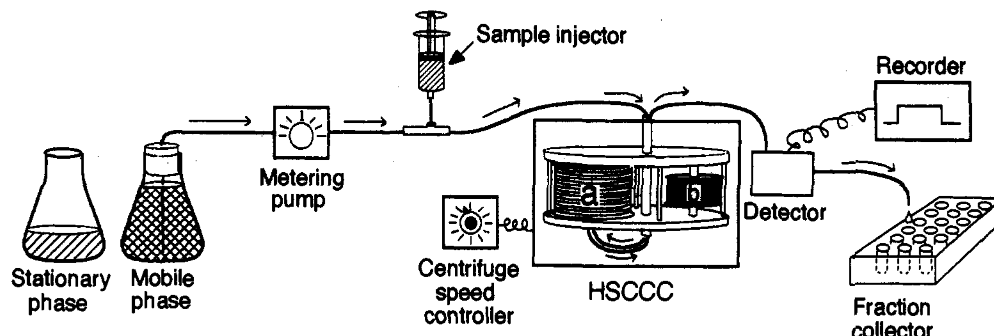

Figure 4. Schematic presentation of the high-speed countercurrent chromatograph (HSCCC) used in the present study: (a) Ito multilayer-coil separation column and (b) counterweight.

in which the pH and consequent ionization of the components are controlled by the buffer used for elution.

Several similarities and differences between displacement chromatography and pH-zone-refining CCC are given below. In displacement chromatography, the "displacer" that is added to the mobile phase acts behind the sample band and forces the sample components, adsorbed onto the stationary phase into the mobile phase. On the other hand, in pH-zone-refining CCC, the "retainer acid" that is added to the stationary phase or sample solution acts in front of the sample band and transfers the solutes from the mobile phase to the stationary phase. Both the function and the site of activity of the reagents ("displacer" and "retainer acid") are reversed in these two techniques, but their actions produce similar effects (e.g., a train of rectangularly shaped peaks). Other differences include the factors that determine the order of elution of the sample components; in displacement chromatography, the order of elution is determined by the increasing affinity of the components for the stationary phase, while in pH-zone-refining CCC, the elution order is governed strictly by the pK_as and hydrophobicities of the components.

The dependency of this technique on pK_a differences should allow separation of acids with small structural differences, e.g., equatorial and axial carboxyl groups in cyclohexanecarboxylic acids that differ in their acidity by approximately 0.5 pK units.[10] Studies are in progress on applying pH-zone-refining CCC to the separation of such stereoisomers (C. Denekamp and A. Mandelbaum, Technion-Israel Institute of Technology, Haifa, Israel; A. Weisz, U. S. Food and Drug Administration, Washington, DC; and Y. Ito, National Institutes of Health, Bethesda, MD; unpublished results, 1993).

We are currently investigating the limits on sample amount and volume, and the effects of acid and base concentrations, and are developing a theoretical treatment. It has not escaped our attention that the technique should also be applicable to the separation of organic bases and other equilibrating systems.

Experimental Section

Equipment. 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. A schematic presentation of the apparatus is shown in Figure 4. A 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)[11] 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., Potomac, MD, USA; Pharma-Tech Research Corp., Baltimore, MD, USA; and Shimadzu, Kyoto, Japan.)

pH-Zone-Refining CCC of DNP-Amino Acids. The solvent system used, methyl *tert*-butyl ether/acetonitrile/water (4:1:5 by volume), was equilibrated in a separatory funnel at room temperature, and the resulting two phases were separated. The mainly organic upper phase that was acidified either with TFA (0.04% volume/volume) or with TFA and acetic, propionic, and butyric acids (each, 0.04% volume/volume) was used as the stationary phase. The lower phase that was made basic with concentrated ammonium hydroxide (0.1% volume/volume), pH 10.8, was used as the mobile phase. The CCC column was filled with the acidic stationary phase by using a metering pump (Beckman Accu-Flo pump; Beckman, Palo Alto, CA, USA). A mixture of 100 mg each of DNP-L-aspartic acid, DNP-L-alanine, and DNP-L-leucine in 3 mL each of stationary phase and neutral lower phase (without added ammonium hydroxide) was loaded into the column by syringe. The mobile phase was then pumped into the column at 3 mL/min, while the column was rotated at 800 rpm in forward mode. The absorbance of the eluate was continuously monitored using an LKB Uvicord S (LKB Instruments, Stockholm, Sweden) at 206 nm, and 3-mL fractions were collected. The pH of each eluted fraction was measured with a pH meter (Accumet 1001, Fisher Scientific, Pittsburgh, PA, USA). Partition coefficients for the collected fractions were measured as follows: An aliquot of each fraction was brought to dryness, and the residue was redissolved in a two-phase solvent system composed of chloroform/acetic acid/0.1 M hydrochloric acid (2:2:1 by volume). Each phase was diluted with methanol, and its absorbance was measured at 430 nm with a PM6 Zeiss spectrophotometer.

The spacer acids were chosen on the basis of preliminary studies described here. In early work, aliphatic acids including TFA were chromatographed individually and together, and the order of elution was assigned on the basis of the pH of the plateaus. A mixture of preparative amounts of the aliphatic acids and analytical amounts of seven DNP-amino acids were chromatographed, resulting in a chromatogram with three peaks containing the seven DNP-amino acids that were separated by the aliphatic acid plateaus. Three DNP-amino acids—each expected, on the basis of earlier trials, to be in each of the three DNP-amino acid peaks—were chosen for the experiments (Figure 1 (parts A and B)).

pH-Zone-Refining CCC of D & C Orange No. 5. The two-phase solvent system used consisted of diethyl ether/acetonitrile/0.01 M aqueous ammonium acetate adjusted to pH 9 with ammonium hydroxide (4:1:5 by volume). The solvent system was equilibrated, and the two phases were separated shortly before use. The sample mixture was prepared by distributing 5 g of certified D&C Orange No. 5 between 40 mL each of the upper and lower phases. Trifluoroacetic acid, 200 μL, was added to the sample solution. Following the addition of the acid, part of the dye precipitated. After the dye mixture was sonicated for approximately 1 min, the 80-mL sample suspension was introduced into the column by repeated injections using a 20-mL glass syringe. All other experimental conditions were as described above for the separations of the DNP-amino acids. Most of the eluted fractions were analyzed by analytical RP-HPLC. The RP-HPLC conditions were described previously.[12] The brominated fluorescein dyes were isolated from the pH-zone-refining CCC fractions in the lactone form, as previously described for the brominated tetrachlorofluoresceins.[12]

Mass Spectrometry. The positive ion chemical ionization mass spectra (methane) were obtained as previously described.[12] Fragmentation patterns m/z (rel intensity) were as follows: 4′,5′-dibromofluorescein (I)

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489/491/493 (62.9:100:61.8, MH^+), 517/519/521 [12.6:23.4:11.9, $(M + C_2H_5)^+$], 529/531/533 [4.1:9.0:4.4, $(M + C_3H_5)^+$]; 2',4',5'-tribromofluorescein (II) 567/569/571/573 (36.7:100:88:35.4, MH^+); 2',4',5',7'-tetrabromofluorescein (III) 645/647/649/651/653 (21.6:78.1:100:65.7:17.8, MH^+).

^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. The following signals were obtained and assigned for each of the three isolated components: 4',5'-dibromofluorescein (I) 6.72 ppm (d, 2H), 7.16 ppm (d, 2H), 7.44 ppm (d, 1H), 7.72 ppm (m, 2H), 7.89 ppm (d, 1H); 2',4',5'-tribromofluorescein (II) 6.75 ppm (d, 1H), 7.16 ppm (d, 1H), 7.33 ppm (d, 1H), 7.55 ppm (s, 1H), 7.73 ppm (m, 2H), 7.9 ppm (d, 1H); 2',4',5',7'-tetrabromofluorescein (III) 7.28 ppm (d, 1H), 7.56 ppm (s, 2H), 7.73 ppm (m, 2H), 7.9 ppm (d, 1H).