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Review

pH-Zone-refining countercurrent chromatography

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Abstract

A recently developed preparative technique, pH-zone-refining countercurrent chromatography (CCC), separates organic acids and bases according to their pK_a and hydrophobicity. The hydrodynamic mechanism of pH-zone-refining CCC is illustrated in two elution modes along with a simple mathematical analysis. Separations include acidic and basic derivatives of amino acids and peptides, hydroxyxanthene dyes, alkaloids, indole auxins, and structural and geometrical isomers. In addition, recently developed affinity ligand separations of enantiomers, polar catecholamines, and free peptides are also described. Technical guidance is provided for interested users so that they can conduct a systematic search for the optimum solvent system and experimental conditions. Advantages as well as limitations of the present technique are discussed.

Keywords: Reviews; Countercurrent chromatography; pH-Zone-refining countercurrent chromatography; Amino acids; Peptides; Xanthenes; Alkaloids; Catecholamines; Proteins

Contents

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1. Introduction

Chromatographic partitioning of solutes between two immiscible solvent phases is a good method for the separation and purification of natural products. By eliminating various complications that sometimes arise from the interaction between solute molecules and the solid support matrix present in most other chromatographic methods, this technique can purify substances with a high recovery and reproducibility. In the 1950s, countercurrent distribution was frequently used for the separation of natural products [1]. However, due to its inherent disadvantages such as the time-consuming operation and bulky, fragile instrumentation, the method was overwhelmed by liquid chromatography during 1960s.

In 1970, a new liquid–liquid partition method was introduced to perform countercurrent separation in a continuous mode. The method is named "countercurrent chromatography (CCC)", since it combines the merits of countercurrent distribution and liquid chromatography [2–6]. The successively introduced preparative partition method called droplet CCC used unit gravity to move the droplets of the mobile phase through the column of the stationary phase in a tubular space [7–9]. Although this simple CCC system produced a high partition efficiency of 900 theoretical plates for a DNP (dinitrophenyl)-amino acid separation, it required a long separation time of 70 h. Continuous development of the CCC technology led to more efficient centrifugal CCC schemes that utilize a centrifugal force field created by a planetary motion of the coil [10–13]. Various complications such as leakage of the solvent through the conventional rotary seal in the continuous flow centrifuge system was eliminated by the use of a seal-free flow-through device in these coil planet centrifuge systems. Using these centrifuge schemes, a series of studies established an ideal combination of the mode of planetary motion and the geometry of coiled columns on the holder. In the early 1980s, an efficient scheme called "high-speed CCC (HSCCC)" was introduced that utilizes the combination of a coaxial coil arrangement and a particular mode of synchronous planetary motion [6,14–17]. This new CCC system fulfils two important requirements for CCC, efficient phase mixing and reliable retention of the stationary phase in the column. This remedies conflicts in other CCC systems, enabling high efficiency separations to be run in a short period of time. Since the middle 1980s the method has been widely used for separation and purification of natural and synthetic products [6].

pH-Zone-refining CCC [18–20], recently developed in our laboratory further, extends the preparative capability of HSCCC. The sample loading capacity is increased over ten times, and fractions are highly concentrated. This new preparative method originated from the observation of an unusually sharp product peak in the course of purification of a bromoacetylthyronine analog as described below.

2. Development toward pH-zone-refining CCC

In HSCCC, isocratic elution usually produces symmetrical peaks where the peak width increases with retention time, as observed in many other chromatographic methods. In the course of purification of $BrAcT_3$ (N-bromoacetyl-3,3′,5-triiodo-L-thyronine), we observed that the product formed an unusually sharp peak corresponding to over 2000 theoretical plates while the preceding impurity peak showed a normal width of about 500 plates (Fig. 1left) [21].

The cause of this strange phenomenon was investigated using a two-phase solvent system composed of hexane, ethyl acetate, methanol and 15 m*M* ammonium acetate buffer at pH 4 [22]. When the volume ratio was shifted to a slightly more polar solution from 5:5:5:5 to 4:5:4:5, the product peak became much broader and skewed whereas the preceding impurity peak was sharpened as shown in Fig. 1 (right panel). This indicated that the sharp peak is not particularly related to the chemical nature of the product, but is caused by some unknown agent present in the sample solution.

After various trials such as testing the blank sample solution (the reaction mixture without adding T_3), the cause was finally found when the collected

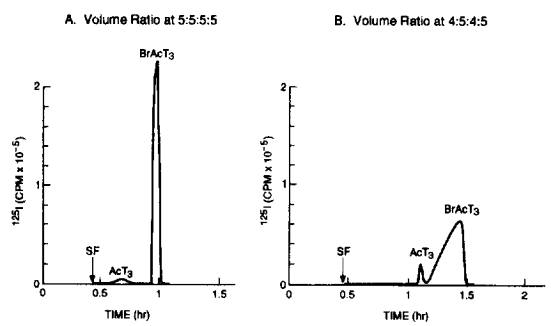

Fig. 1. Chromatograms of $BrAcT_3$ obtained with a standard two-phase solvent system with a slight difference in volume ratio but otherwise identical conditions. Experimental conditions: apparatus, multilayer CPC with semianalytical column of 1.6 mm ID and 320 ml capacity; sample, crude bromoacetylation mixture and a minute amount of [^{125}I]T_3 in 4 ml of solvent of equal amount of each phase; mobile phase: aqueous phase (pH 5.2), flow-rate 3 ml/min, speed 800 rpm; detection ^{125}I radioactivity; retention of stationary phase, 69.8% (A) and 64.7%; SF, solvent front. Note that the peak profile of $BrAcT_3$ and AcT_3 was reversed by changing the solvent ratio.

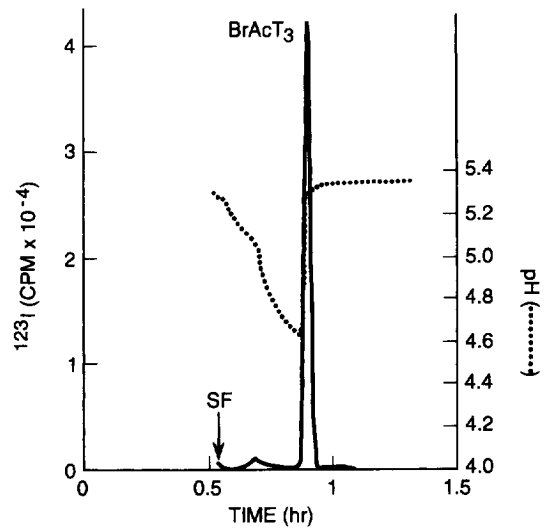

Fig. 2. Disclosure of the cause of sharp peak formation by manual pH measurement to find the cause of the sharp peak. Sample: CCC purified $BrAcT_3$ (ca. 0.1 mmol) + blank bromoacetylation mixture. For other conditions see Fig. 1. The elution of the sharp peak coincides with the abrupt rise of the pH suggesting tha the acid in the sample solution is causing the peak sharpening.

fractions were manually analyzed by a pH meter. As shown in Fig. 2, the pH curve showed a gradual decline after the solvent front followed by an abrupt rise which coincided with the elution of the sharp product peak. Mass spectrometric (MS) analysis of the sample solution showed the presence of bromoacetic acid, the reaction by-product of $BrAcT_3$ synthesis. Today we would refer to the bromoacetic acid as a "retainer acid" since it retains the analyte longer in the column. More recently, we have found that many other organic acids such as TFA (trifluoroacetic acid), acetic acid, propionic acid, *n*-butyric acid, etc. can be individually or collectively used as a retainer. Further studies have shown that a similar effect is produced by introducing the retainer acid in the stationary phase as well as in the sample solution. In this case the pH curve forms a low flat zone starting at the solvent front which is maintained until the elution of the analyte.

Fig. 3 shows the underlying mechanism of the sharp peak formation [22]. A portion of the separation column shows the organic stationary phase in the upper half and the aqueous mobile phase in the lower half. Due to its nonlinear isotherm, the retainer

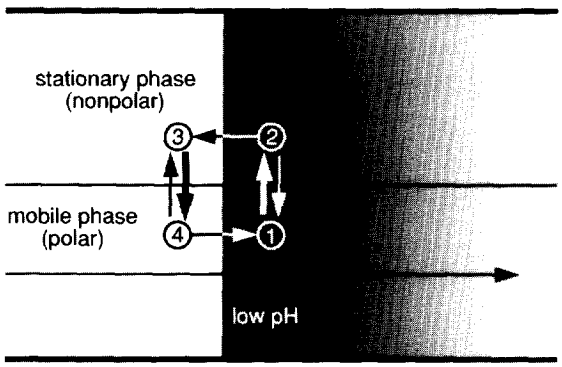

Fig. 3. Schematic illustration of the peak sharpening process in the separation column. A portion of the column contains nonpolar stationary phase in the upper half and the polar mobile phase in the lower half where the solute molecules circulate at the sharp edge of the low pH region (shaded). The acid analyte circles around the sharp retainer border by repeating protonation and deprotonation as described in Section 2.

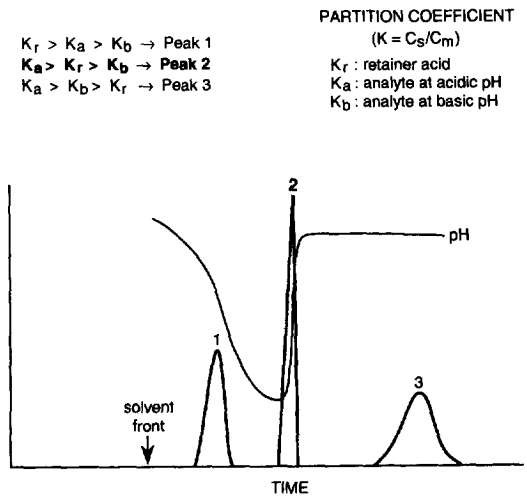

Fig. 4. General requirement of sharp peak formation. Peak 1 is obtained when both K_a and K_b are smaller than K_r while peak 3 is obtained when both K_a and K_b are greater than K_r. Sharp peak 2 is formed when K_r falls between K_a and K_b, as indicated above.

acid forms a sharp trailing border which moves through the column at a rate lower than that of the mobile phase. When the acid analyte is present in the mobile phase at position 1 it becomes a hydrophobic protonated form due to low pH and subsequently partitions into the organic stationary phase at position 2. As the sharp retainer border moves forward, the analyte is exposed to a higher pH at position 3 where it is deprotonated and transferred to the lower aqueous phase at position 4. In the aqueous mobile phase the analyte quickly migrates through the sharp retainer border to repeat the above cycle. Consequently, the analyte is always confined in a narrow region around the sharp retainer border and elutes as a sharp peak together with the sharp retainer acid border.

In order to trap the analyte peak around the sharp retainer border, a certain requirement must be satisfied. In Fig. 4, K_r stands for the partition coefficient (solute concentration in the stationary phase divided by that in the mobile phase) of the retainer acid, and K_a and K_b for those of the analyte at acidic and basic conditions, respectively. If K_r is greater than K_a and K_b, the analyte elutes earlier than the retainer border forming a broad peak (peak 1). If K_r is smaller than K_a and K_b, the analyte elutes after the retainer border again with a broad peak (peak 3). Peak sharpening takes place only when K_r falls between K_a and K_b (peak 2).

It was found that the present method allows the use of multiple retainer acids as spacers to separate sharp analyte peaks at their borders. Fig. 5 shows a separation of three DNP-amino acids by the spacer acids. The separation was performed with a two-phase solvent system composed of methyl-*tert.*-butyl ether–water where TFA and three spacer acids were added to the upper organic stationary phase and ammonia (eluter base) to the lower aqueous mobile phase. Polar DNP-aspartic acid (DNP-asp) was eluted between acetic acid and propionic acid, DNP-alanine (DNP-ala) between propionic acid and *n*-butyric acid, and hydrophobic DNP-leucine (DNP-leu) after *n*-butyric acid [20].

This pH-peak-focusing method has useful applications such as concentration and detection of minor components and improvement of analytical separations by shifting the retention time of the analyte away from nonionic impurities. However, the most important application is found in preparative-scale separations. When the sample size of DNP-amino

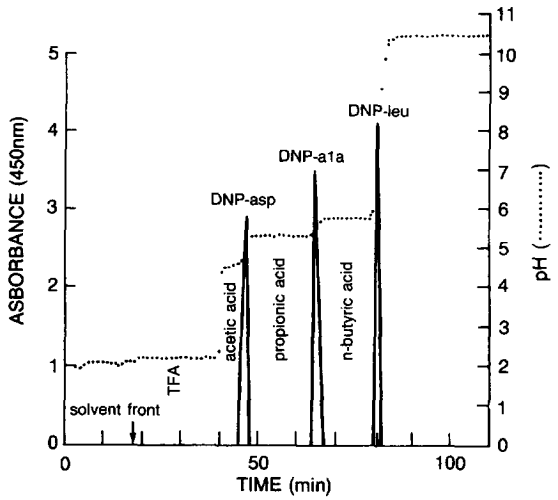

Fig. 5. Separation of DNP-amino acids by spacer acids. Three spacer acids introduced in the stationary phase form pH-zones to isolate sharp analyte peaks at their boundaries. Experimental conditions: apparatus, multilayer coil planet centrifuge equipped with a semipreparative column (1.6 mm I.D. and 315 ml capacity); solvent system, methyl-*tert.*-butyl ether–acetonitrile–water (4:1:5); retainer acids, TFA, acetic acid, propionic acid, and *n*-butyric acid, each 0.4 μl/ml in the organic stationary phase; eluter base, 0.1% ammonia in the aqueous mobile phase (pH 10.77); sample, DNP-L-aspartic acid, DNP-L-alanine and DNP-L-leucine each 1 mg; flow-rate, 3 ml/min; revolution, 800 rpm; retention of the stationary phase = 81.0%.

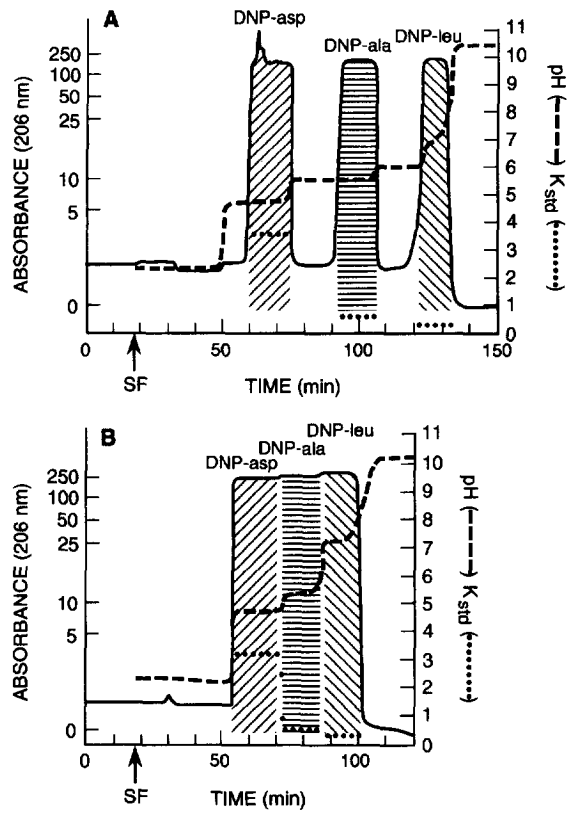

Fig. 6. pH-Zone-refining CCC of DNP-amino acids with (A) and without (B) spacer acid in the stationary phase using the reverse displacement operation. (A) Rectangular peaks of three DNP amino acids were widely separated from each other by the spacer acids (acetic acid, propionic acid and *n*-butyric acid). (B) Elimination of the spacer acids resulted in fusion of the rectangular peaks with minimum overlapping as demonstrated by associated pH values and partition coefficient values (K_{std}). Experimental conditions are identical to those in Fig. 5, except that the sample size was increased to 100 mg for each component. The K_{std} values were obtained by partitioning an aliquot of each fraction to the standard solvent system composed of chloroform–acetic acid–0.1 *M* HCl (2:2:1). SF = solvent front.

acids in the above separation is increased by 100 fold, each from 1 mg to 100 mg, under otherwise identical conditions, the upper chromatogram shown in Fig. 6 is obtained [18,20]. Each component forms a highly concentrated rectangular peak associated with its specific pH as shown by the dotted line. The elimination of the three spacer acids results in fusion of these three peaks while preserving their original rectangular shapes as demonstrated by well defined pH-zones and K_{std} values measured with a standard solvent system as shown in the lower chromatogram. Fig. 7 shows a similar separation obtained by using the organic phase as the mobile phase [23]. In this case the elution order of the three analytes and the trend of the pH curve (dotted line) are reversed.

This new CCC method produces characteristic pH-zones according to pK_a and hydrophobicity of analytes and therefore it was named "pH-zone-refining CCC" [18–20]. The method shares many common features with displacement chromatography and provides some advantages over the conventional CCC technique such as increased sample loading capacity, high concentration of fractions, concentration and detection of minor components, and detection and precise localization of rectangular

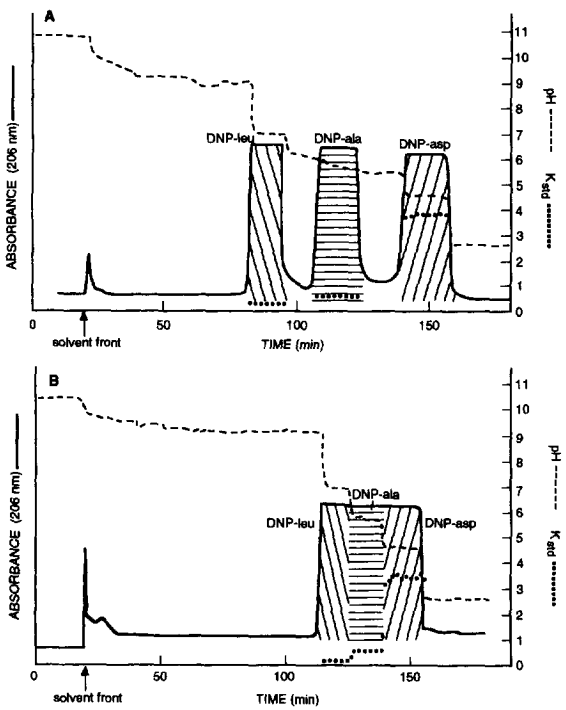

Fig. 7. pH-Zone-refining CCC of DNP-amino acids with (A) and without (B) spacer acid in the stationary phase using the normal displacement operation. (A) Rectangular peaks of three DNP amino acids were widely separated from each other by the spacer acids (*n*-butyric acid, propionic acid and acetic acid). (B) Elimination of the spacer acids resulted in fusion of the rectangular peaks with minimum overlapping as demonstrated by associated pH values and partition coefficient values (K_{std}). Experimental conditions are similar to Fig. 6 except that the organic phase was used as the mobile phase and the aqueous phase as the stationary phase. Note that the eluting order of the three DNP-amino acids were reversed and the pH curve showed an opposite downward trend.

major peaks by monitoring the effluent pH even though the analytes have no chromophore.

3. Theoretical considerations on pH-zone-refining CCC

3.1. Mechanism of pH-zone-refining CCC

A discussion of the mechanism of the present technique may be divided into two parts depending on whether the aqueous or organic phase is mobile. As suggested by their terms (see Table 1), the normal displacement mode separates analytes in a manner similar to displacement chromatography, i.e. a component of the organic mobile phase ("eluter") displaces the analytes from the stationary phase toward the mobile phase at the back of the solute zones. Because of this action, "eluter" in the normal displacement mode was formally called "displacer". In the reverse displacement mode, the above process is reversed: a component in the organic stationary phase ("retainer") transfers the analytes from the mobile phase into the stationary phase at the front of the solute zones. The elution profiles resulting from these two elution modes are quite similar except that the elution order of the analytes is reversed.

The mechanism in both elution modes can be demonstrated by the following model experiments [19,20,23]. Fig. 8 shows preparation of solvent phases to initiate the experiment. About equal volumes of ether and water is equilibrated in a separatory funnel and the two phases separated. A retainer acid such as TFA is added to the upper organic phase while an eluter base such as ammonia is added to the lower aqueous phase. As explained earlier, the reverse displacement mode uses the organic phase as the stationary phase and the aqueous phase as the mobile phase, and vice versa in the normal displacement mode. In each experiment the column is first entirely filled with the stationary phase. This is followed by injection of sample solution containing three acidic analytes. The mobile phase then is pumped into the column and the column rotated at a desired rate.

3.1.1. Reverse displacement mode

Fig. 9 illustrates the mechanism of reverse displacement pH-zone-refining CCC where the aqueous phase is used as the mobile phase [19,20]. In the upper diagram, a portion of the separation column shows the organic stationary phase (shaded) in the upper half and the aqueous mobile phase in the lower half. Due to its nonlinear isotherm, the retainer acid, TFA, forms a sharp trailing border that moves through the column space occupied by the mobile phase at a rate considerably lower than that of the mobile phase. Three analytes, S_1, S_2 and S_3, competitively form solute zones behind the sharp TFA border according to their pK_a and hydrophobicity. Among these, S_1 with the lowest pK_a and hydrophobicity is located immediately behind the TFA

Table 1
Comparison between pH-zone-refining CCC and displacement chromatography

	pH-Zone-refining CCC		Displacement chromatography
	Reverse-displacent mode	Normal-displacement mode	
Key reagents	Retainer	Eluter	Displacer
Solute transfer	MP[b]→SP[c]	SP→MP	SP→MP
Acting location	Front of solute bands	Back of solute bands	Back of solute bands
Solute bands			
Form	A train of individual solute bands with minimum overlapping		
Traveling rate	All move together at the same rate as that of the key reagent		
K[a] value in	Same as and determined by that of the key reagent		
Impurities	Concentrated at the boundaries of the solute bands		
Peak profile	A train of rectangular peaks associated with sharp impurity peaks at their boundaries		
Solute concentration in mobile phase is determined by	Concentration of counterions in aqueous phase	Concentration of counterions in aqueous phase and K value	Solute affinity to stationary phase
Elution order is determined by	Solute pK_a and hydrophobicity	Solute pK_a and hydrophobicity	Solute affinity to stationary phase

[a] K: Partition coefficient expressed by solute concentration in the stationary phase divided by that in the mobile phase.
[b] MP: Mobile phase.
[c] SP: stationary phase.

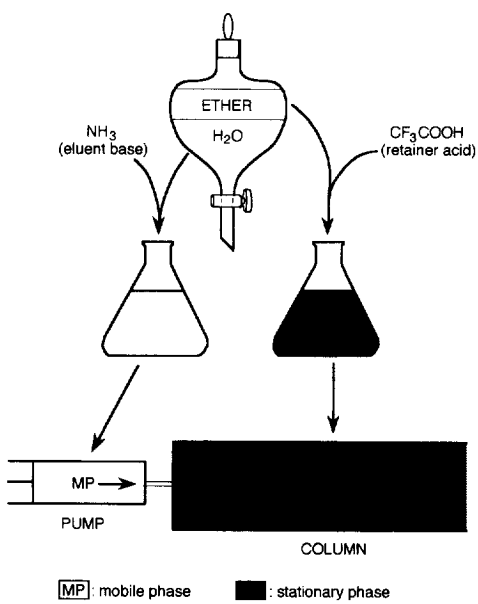

Fig. 8. Model experiment for reverse displacement pH-zone-refining CCC for separation of carboxylic acids: preparation of solvent phases

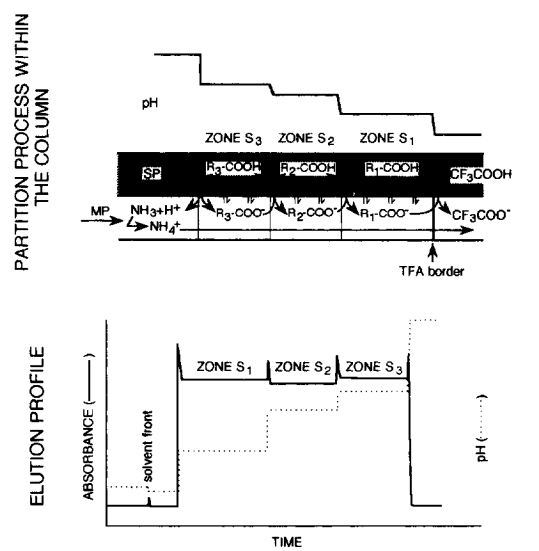

Fig. 9. Model experiment for reverse displacement pH-zone-refining CCC for separation of carboxylic acids: partition process within the column and elution profile.

border, while S_3 with the highest pK_a and hydrophobicity is located at the end of the solute zones where it forms a sharp trailing border.

As indicated by curved arrows, proton transfer takes places at each zone boundary governed by the difference in pH between the neighboring zones. The loss of the solute from the mobile phase to the stationary phase at the zone front is compensated by its return at the back of each zone while ammonium ion in the aqueous phase serves as counterion for all species. After equilibrium is reached, the three solute zones move at the same rate as that of the TFA border, while constantly maintaining their width and pH. Charged minor components present in each zone are efficiently eliminated either forward or backward according to their partition coefficients (pK_a and hydrophobicity) and eventually accumulate at the zone boundaries. Consequently the three analytes elute as a train of rectangular peaks with sharp impurity peaks at their narrow boundaries as illustrated in the lower diagram (Fig. 9).

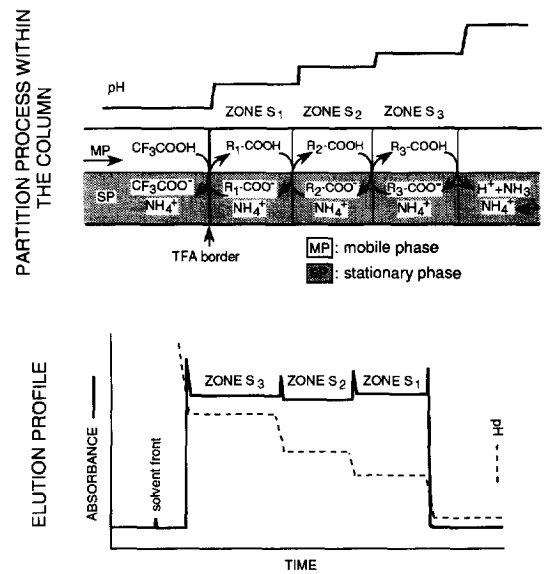

Fig. 10. Model experiment for normal displacement pH-zone-refining CCC for separation of carboxylic acids: partition process within the column and elution profile. Preparation of the solvent phases was performed as in Fig. 6 except that the acidified upper organic phase was used as the mobile phase and basified aqueous phase as the stationary phase.

3.1.2. Normal displacement mode

Fig. 10 similarly illustrates the mechanism of the normal displacement mode of pH-zone-refining CCC [23]. In this elution mode, the organic mobile phase occupies the upper half and the aqueous stationary phase (shaded) the lower half. Here again, TFA or the eluter acid (which corresponds to the retainer in the reverse displacement mode) forms a sharp trailing border which moves through the column at a rate lower than that of the mobile phase. Three analytes, S_1, S_2 and S_3, form solute zones in front of the sharp TFA border according to their pK_a and hydrophobicity. In this case, S_1 with lowest pK_a and hydrophobicity is found immediately after the TFA border followed by S_2 and then S_3, the order of the zone arrangement being reversed from that observed in the reverse displacement mode.

Proton transfer takes place at each zone boundary according to the difference in pH between the two neighboring zones and this causes exchange of the solute between the two phases. As TFA moves through the column by gradually displacing S_1 from the preceding S_1 zone, each solute zone advances similarly by displacing the preceding zone at its front border. This process closely resembles that observed in displacement chromatography. After equilibrium is established, each solute zone moves at the same rate as that of the TFA border while maintaining its width and pH. Charged impurities present in each zone are eliminated according to their partition coefficient and accumulate at the zone boundaries as in the reverse displacement mode. Consequently, the three analytes are eluted as a train of rectangular peaks associated with sharp impurity peaks at their zone boundaries as shown in the lower diagram. In this normal displacement mode, the eluting orders of both analytes and pH-zones are completely opposite to those observed in the reverse displacement mode (Fig. 9).

3.1.3. pH-Zone-refining CCC vs. displacement chromatography

As described above, pH-zone-refining CCC closely resembles displacement chromatography [24,25] in many aspects, including formation of highly concentrated rectangular peaks, concentration of minor components at the boundaries of the major peaks, and isotachic movement of all solute zones. However, there are important differences between these two techniques. For instance, in pH-zone-refining CCC a retainer (reverse displacement mode) or

an eluter (normal displacement mode) transfers the analytes from the stationary phase to the mobile phase by a process of deprotonation or protonation, which lowers the partition coefficient of the analytes. In displacement chromatography, the displacer literally displaces the analytes by competitive binding onto the solid support.

Another important difference between these two methods is the concentration of the analyte in the mobile phase. In displacement chromatography, concentration of the analyte is determined by its adsorptivity onto the solid support. Consequently, the earlier eluting analyte shows a lower concentration than the later eluting analytes. In pH-zone-refining CCC, on the other hand, the analyte concentration is mainly determined by the molar concentration of the counterion in the aqueous phase, and therefore all monovalent analytes are eluted at the similar molar concentrations in the aqueous mobile phase. Similarities and differences between these two techniques are summarized in Table 1 [20].

3.2. Mathematical analysis

A simple mathematical model has been proposed for the reverse displacement mode of pH-zone-refining CCC [19,20].

In this model experiment, about equal volumes of methyl-*tert.*-butyl ether and distilled water are thoroughly equilibrated in a separatory funnel at room temperature. After the two phases are separated, a retainer acid (TFA) is added to the upper organic phase at a concentration of C_R while an eluter base (NH_3) is added to the lower aqueous phase at a concentration of C_E. The column is then completely filled with the acidified organic (stationary) phase and the basified aqueous (mobile) phase is pumped into the column at a flow-rate of u_m (ml/s) while the column is rotated at the desired rate. During the elution, the effluent pH is continuously monitored by a flow-through pH meter. As the mobile phase moves through the column by partially displacing the excess stationary phase, it extracts TFA from the retained stationary phase. This causes a depletion of TFA from the retained stationary phase and forms a sharp trailing boundary of TFA across the column due to the nonlinear isotherm. The travelling rate of this TFA boundary through the column becomes constant as soon as the TFA concentration in the mobile phase front reaches the partition equilibrium against that in the stationary phase (C_R). At this point, the concentration of TFA in the mobile phase is expressed as $C_r = C_R/K_r$, where K_r is the partition coefficient of TFA at this equilibrium state.

3.2.1. Retention volume and moving rate of the retainer border

Fig. 11 schematically illustrates the longitudinal cross section through a portion of the equilibrated separation column which arbitrarily shows the organic stationary phase in the upper half and the aqueous mobile phase in the lower half. The sharp TFA border is formed across the column which moves at a constant rate (u ml/s through the column space occupied by the mobile phase) lower than that of the mobile phase front (u_m ml/s).

Using the retention volume (V_r) and the moving rate (u) of the sharp TFA border, the following equations may be written:

$$C_R V_s = C_r(V_r - V_m) \quad (1)$$

$$V_m/u = V_r/u_m \quad (2)$$

where V_s and V_m indicate the volumes of stationary and mobile phases which remain within the column after the solvent front is eluted. Eq. (1) shows that the net amount of the retainer acid in the stationary phase retained in the column is equal to the amount eluted from the column. In Eq. (2) the left term indicates the time required for the sharp TFA border to travel through the entire length of the column. This equals the retention time of the TFA border in the chromatogram indicated in the right term.

Eqs. (1,2) lead to the following equations:

$$V_r = K_r V_s + V_m \quad (3)$$

$$u = u_m/[(V_s/V_m)K_r + 1] \quad (4)$$

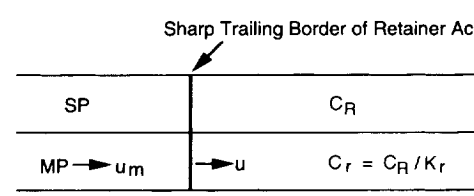

Fig. 11. Portion of separation column showing a sharp trailing border of the retainer acid.

$$K_r = [(u_m/u) - 1](V_m/V_s). \quad (5)$$

Here, V_r indicates the retention time of the sharp TFA trailing border, not the peak maximum of the elution peak with a normal distribution, and K_r is determined by the initial TFA concentration (C_R) in the stationary phase.

3.2.2. Formation of solute zone behind the retainer border

In the second experiment the column is similarly filled with the acidified organic stationary phase. This is followed by injection of the sample solution containing a relatively large amount of an acidic solute with a pK_a and hydrophobicity higher than that of TFA. The mobile phase containing an eluter base (NH_3) at concentration C_E is then eluted through the column at a flow-rate of u_m (ml/s). This results again in a formation of the sharp trailing TFA border which travels at a uniform rate of u (ml/s).

As explained earlier, solute molecules undergo a partition cycle around the sharp retainer border by repeated protonation and deprotonation caused by the large difference in pH level between each side of the retainer border (Fig. 3). As this process continues with an abundant supply of solute from the mobile phase, the solute concentration around the retainer border gradually increases. This elevated solute concentration leads to a lowered pH which in turn raises the solute partition coefficient behind the retainer acid border until the partition equilibrium is established between the two phases.

In order to facilitate the mathematical treatment of the above hydrodynamic process, the TFA trailing border is set stationary as indicated in Fig. 12. Both mobile and stationary phases move countercurrent to each other by passing through the TFA border at the relative rate of ($u_m - u$ ml/s) and ($-uV_s/V_m$ ml/s) as indicated by a pair of arrows across the TFA border in the diagram. Now, compare the partition processes taking place on each side of the TFA border under a steady supply of the solute from the mobile phase. On the right side, where the pH is low, all solute present in the flowing mobile phase is immediately protonated into the hydrophobic nonionic form. It is then quickly transferred into the stationary phase and sent back to the left side of the TFA border. On the left side of the TFA border where the pH is initially

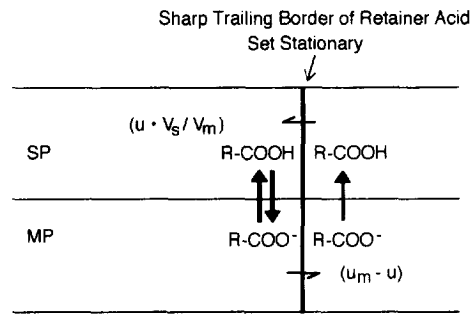

Fig. 12. Portion of the separation column showing analyte movement around a retainer border which is set stationary.

high, the solute is mostly distributed to the mobile phase. With a continuous supply of the solute from the mobile phase however, the solute concentration on the left side of the retainer border starts to rise. The resulting high concentration of solute causes a decrease in pH which in turn increases the partition coefficient of the solute (due to its nonlinear isotherm) until a steady state partition equilibrium is established between the two phases. These solute movements are indicated by the thick arrows across the interface in the diagram. At this partition equilibrium, the rate of the solute transfer from the mobile phase to the stationary phase on the right side of the TFA border is given by the solute concentration in the mobile phase (C_m) multiplied by the travelling rate of the mobile phase through the TFA border ($u_m - u$). The amount of the solute molecules transferred from the mobile phase is then uniformly distributed in the stationary phase at the concentration (C_s) that is determined by the relative flow-rate of the stationary phase through the trailing TFA border (uV_s/V_m). Therefore, at a given moment

$$C_m(u_m - u) = C_s uV_s/V_m \quad (6)$$

From Eq. (6), the solute partition coefficient (K_s) is obtained, i.e.,

$$K_s = C_s/C_m = [(u_m/u) - 1](V_m/V_s) \quad (7)$$

Comparison between Eqs. (5,7) implies the important fact that the solute partition coefficient (K_s) on the left side of the retainer border is equal to that of the retainer acid itself (K_r) on the right side of the retainer border, i.e., $K_s = K_r$. This clearly indicates

that the ratio of the solute concentration between the stationary and mobile phases in the equilibrated solute zone is equal to and determined by that of the retainer acid. Once this partition equilibrium is reached, further supply of the solute through the mobile phase results in the development of the solute zone behind the sharp retainer acid border where the width of the solute zone increases proportionally to the amount of the solute loaded into the column.

Fig. 13 shows the simplified distribution equilibrium of TFA (CF_3COOH) and solute S (RCOOH) between the stationary organic phase and the flowing aqueous phase on an assumption that the concentration of ionized components in the organic phase is negligible. The pH of the mobile phase in the solute zone (zone S) on the left side of the sharp TFA border is given from the following three equations [20]:

$$K_{D-s} = [RCOOH_{org}]/[RCOOH_{aq}] \quad (8)$$

$$K_s = [RCOOH_{org}]/([RCOOH_{aq}] + [RCOO^-{}_{aq}]) \quad (9)$$

$$K_{a-s} = [RCOO^-{}_{aq}][H^+_{aq}]/[RCOOH_{aq}] \quad (10)$$

where K_{D-s} is the partition ratio of solute S (RCOOH) and K_{a-s}, the dissociation constant of the solute. These equations are reduced to

$$pH_{z-s} = pK_a + \log\{(K_{D-s}/K_s) - 1\} \quad (11)$$

where pH_{z-s} is pH of the mobile phase in the solute zone. As shown in the above equation, the pH of the solute zone is determined by the pK_a and hydrophobicity (K_{D-s}) of the solute as well as its partition coefficient (K_s). Since $K_s = K_r$, pH_{z-s} can be computed using Eq. (16) described below.

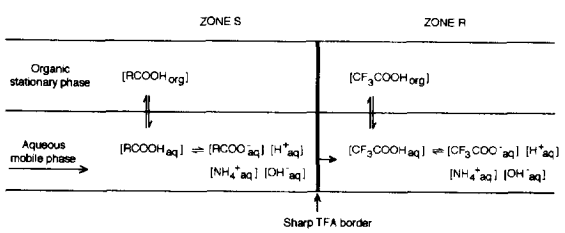

Fig. 13. Distribution equilibrium of retainer acid (zone R) and solute S (zone S) on respective sides of the sharp TFA border in the separation column.

The distribution equilibrium of TFA (CF_3COOH) between the stationary organic phase and the flowing aqueous phase in front of its sharp trailing border is shown in zone R in Fig. 13. From this diagram the partition coefficient of TFA (K_r) is obtained by solving the following four simultaneous equations [20]:

$$K_r = [CF_3COOH_{org}]/([CF_3COOH_{aq}] + [CF_3COO^-_{aq}]) = C_R/([CF_3COOH_{aq}] + [CF_3COO^-_{aq}]) \quad (12)$$

$$K_{D-r} = [CF_3COOH_{org}]/[CF_3COOH_{aq}] = C_R/[CF_3COOH_{aq}] \quad (13)$$

$$[CF_3COO^-_{aq}][H^-_{aq}]/[CF_3COOH_{aq}] = K_{a-r} \quad (14)$$

$$[CF_3COO^-_{aq}] + [OH^-_{aq}] = [H^-_{aq}] + [NH^-_{4\,aq}] \quad (15)$$

where K_{D-r} indicates the partition ratio of TFA; C_E, the concentration of eluter base; and K_{a-r}, the dissociation constant of TFA.

From Eqs. (12–15), the partition coefficient of the retainer acid is given by the following equation [20]:

$$K_r = \{(C_E/2C_R) + 1/K_{D-r} + [(C_E/2C_R)^2 + K_{a-r}/C_R K_{D-r}]^{1/2}\}^{-1} \quad (16)$$

which indicates that K_r becomes greater as C_R increases and/or C_E decreases.

3.2.3. Multiple solute zones behind the retainer border

When two or more different solutes are introduced in large quantities, the solute (S_1) with the lowest pK_a and hydrophobicity to yield the lowest pH at the equilibrium concentration drives out all other components and establishes its equilibrium zone immediately behind the retainer acid. As previously analyzed, the partition coefficient of solute S_1 within the equilibrium zone is equal to that of the preceding retainer acid, i.e. $C_{s-1}/C_{m-1} = K_r$. As does the retainer acid, S_1 forms a sharp trailing border which is then followed by the second solute (S_2) with the second lowest pK_a and hydrophobicity which produces the next higher pH at the equilibrium concentration.

Similarly, the partition coefficient of solute S_2 within the equilibrium zone is equal to those of the preceding zones, i.e. $C_{s-2}/C_{m-2} = C_{s-1}/C_{m-1} = K_r$. The sharp trailing boundary of this S_2 zone is again followed by the third solute (S_3) with the next higher pK_a and next higher hydrophobicity, and so forth. Consequently the solutes form a train of equilibrium zones which are arranged in an increasing order of pK_a and hydrophobicity behind the sharp border of the retainer acid as shown in Fig. 14. As mentioned earlier, the partition coefficient of the solute in each zone is equal to that of the retainer acid, therefore,

$$K_r = C_{s-1}/C_{m-1} = C_{s-2}/C_{m-2} = C_{s-3}/C_{m-3} = \cdots \quad (17)$$

$$pH_{z-r} < pH_{z-1} < pH_{z-2} < pH_{z-3} < \cdots \quad (18)$$

The sharp trailing border of the retainer acid and the following solute zones move through the column at the same rate and elute in this order to form a succession of pH-zones each corresponding to a single solute with a specific pK_a and a characteristic hydrophobicity (K_{D-s}).

Fig. 15 elucidates the relationship between the mobile phase pH and solute partition coefficient (K) within the column (left) and the profile of pH-zones eluted (right) [19,20]. These curves may be drawn from Eq. (11) by inserting K_{D-s} and pK_a of the solutes. If these parameters are not available, each curve can be experimentally obtained by equilibrating increasing amounts of the solute with the solvent system (containing the eluter base but no retainer acid), and measuring both pH of the lower aqueous phase and the solute concentration in the upper and lower phases. Then, the diagram is constructed by

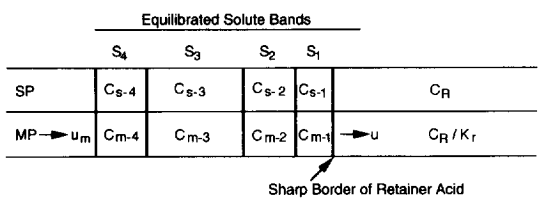

Fig. 14. Portion of the separation column showing a train of equilibrated solute zones.

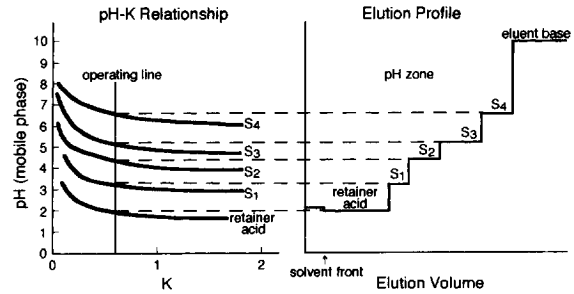

Fig. 15. Relationship between pH/K curves and eluted pH-zones.

plotting the pH on the ordinate against K (the solute concentration in the upper organic phase divided by that of the lower aqueous phase) on the abscissa. Due to the nonlinear isotherm, K increases with increasing amounts of solute which causes a decrease in pH. In Fig. 15 (left) five pH/K curves are arranged from the top to bottom in the order of decreasing pK_a of the solutes where the lowest curve represents that of the retainer acid with the lowest pK_a. The vertical line drawn through the critical K value (K_r), called the operating line, intersects each pH curve and determines the pH level in the corresponding solute zone eluted as shown in the right diagram.

The pH/K diagram is useful for predicting the experimental results including the order of solute elution, the pH level of each solute zone, and feasibility of the separation. A good separation is expected from a set of pH/K curves which show even distribution of each curve with a great distance apart.

The elution curve and pH-zones in a given set of samples may be predicted by a computer simulation program based on the countercurrent distribution method [26]. The simulated chromatograms for separations of organic acid by the reverse displacement mode contain flat-top peaks with steep sides and are consistent with experimental chromatograms. The results also confirmed the validity of Eq. (11) that the separation depends mainly on differences in the pK_a and K_D of the acid analytes. According to the analysis, mixtures of acids with $\Delta p(K_a/K_D) = 1$ are theoretically separated with recoveries of 99% of each acids with ≥99% purity, even on a low-efficiency column of 275 theoretical plates. These

simulations are valuable for predicting and optimizing pH-zone-refining CCC separations.

4. Application of pH-zone-refining CCC

As discussed earlier, the method shares many unique features with displacement chromatography and provides a number of advantages over the conventional CCC technique. Consequently, the method opens a rich domain of applications in CCC. Below, various applications of pH-zone-refining CCC are presented under two subheadings, standard separations and affinity ligand separations. The standard separations require a set of two agents, e.g. a retainer agent in the organic phase and an eluter agent in the aqueous phase for the reverse displacement operation. The separation of certain groups of compounds, however, requires an additional agent, a ligand, in the stationary phase. These groups include enantiomers, highly polar analytes such as catecholamines and sulfonated dyes, and zwitterions such as amino acids and peptides. Samples, solvent systems, and essential ingredients such as retainers, eluters and ligands used in various applications for standard and affinity ligand pH-zone-refining CCC techniques are summarized in Table 2 and Table 3, respectively.

These separations were performed in our laboratory using a commercial model of the HSCCC centrifuge (Ito Multilayer Coil Separator/Extractor, P.C. Inc., Potomac, MD, USA) (comparable instruments are also available from Pharma-Tech Research Corporation, Baltimore, MD, USA and Shimadzu Corporation, Kyoto, Japan). Fig. 16 diagrammatically illustrates our elution system. The HSCCC centrifuge carries a multilayer coil separation column (a) and a counterweight (b) at symmetrical positions at 10 cm from the central axis of the apparatus. The column holder undergoes a planetary motion which provides both phase mixing and retention of the stationary phase in the column. This planetary motion also untwists the flow tubes to allow continuous elution through the rotating column without the use of rotary seals. In each separation, the column is first completely filled with the stationary phase using a metering pump followed by injection of the sample solution. Then the mobile phase is pumped into the column while the column is rotated at 800 rpm (72 *g*). The effluent is continuously monitored and collected into test tubes for further analysis.

4.1. Standard separations

4.1.1. Amino acid derivatives

Among various amino acid derivatives, DNP-amino acids have been most extensively used as a standard set of samples to demonstrate the partition efficiency of CCC systems, mainly because of their potent yellow color which facilitates the observation of their partition behavior in the test tube as well as the location of solute peaks in the collected fractions.

Three chromatograms of DNP-amino acids in Fig. 17 demonstrate the capability of pH-zone-refining CCC [19]. All separations were performed in the reverse displacement mode with the same solvent system composed of methyl-*tert.*-butyl ether–acetonitrile–water at a volume ratio of 4:1:5 where a 200-μl quantity of TFA (retainer acid) was added to the sample solution, and 0.1% (v/v) aqueous ammonia (28%) (eluter base) to the aqueous mobile phase to raise the pH to 10.5.

The top chromatogram was obtained from 6 mg of the sample mixture consisting of six different DNP-amino acids. All components were eluted together as a sharp single peak without any visible evidence of separation. When the sample size was increased 100 times, i.e. from 6 mg to 600 mg, the UV tracing at 206 nm produced a highly concentrated rectangular peak which was divided into six flat pH-zones (dotted line) as shown in the middle chromatogram. The partition coefficient of fractions measured with a standard solvent system composed of chloroform–acetic acid–0.1 *M* HCl revealed that each pH-zone corresponds to one species as indicated in the chromatogram. The bottom chromatogram illustrates the separation of 500 mg each of DNP-glutamic acid and DNP-valine under similar conditions. Each component formed a long plateau associated with its specific pH where the length of each plateau increased in proportion to the applied sample size. A sharp transition between the two plateaus indicates minimum overlap between the two peaks. A gradual decline of pH curves in both pH-zones was apparently caused by a steady increase of the travelling rate of the retainer acid border and the following solute

Table 2
Samples and solvent systems applied to standard pH-zone-refining CCC

Sample[a]	Solvent systems[b] (volume ratio)	Key reagents[c]		References
		Retainer	Eluter	
DNP-amino acids				
(up to 1 g)	MBE/AcN/H_2O (4:1:5)	TFA (200 μl/SS)	NH_3 (0.1%/MP)	[19]
	MBE/AcN/H_2O (4:1:5)	TFA (0.04%/SP)	NH_3 (0.1%/MP)	[18]
	MBE/AcN/H_2O (4:1:5)	TFA spacer acids (each 0.04%/SP)	NH_3 (0.1%/MP)	[18]
	MBE/H_2O (DPCCC)	NH_3 (22 m*M*/SP)	TFA (10.8 m*M*/MP)	[23]
	MBE/H_2O (DPCCC)	NH_3 (44 m*M*/SP)	TFA (10.8 m*M*/MP)	[23]
	MBE/H_2O (DPCCC)	NH_3 (22 m*M*/SP)	TFA (10.8 m*M*/MP) (spacer acids/MP or SS)	[23]
Proline (OBzl) (1 g)	MBE/H_2O	TEA (10 m*M*/SP)	HCl (10 m*M*/MP)	[19]
Amino acid (OBzl) (0.7 g)	MBE/H_2O	TEA (10 m*M*/SP)	HCl (10 m*M*/MP)	[27]
Amino acid (OBzl) (10 g)	MBE/H_2O	TEA (5 m*M*/SP)	HCl (20 m*M*/MP)	[27]
CBZ-dipeptides (0.8 g)	MBE/AcN/H_2O (2:2:3)	TFA (16 m*M*/SP)	NH_3 (5.5 m*M*/MP)	[28]
CBZ-dipeptides (3 g)	MBE/AcN/H_2O (2:2:3)	TFA (16 m*M*/SP)	NH_3 (5.5 m*M*/MP)	[28]
CBZ-tripeptides (0.8 g)	*n*-BuOH/MBE/AcN/H_2O (2:2:1:5)	TFA (16 m*M*/SP)	NH_3 (2.7 m*M*/MP)	[28]
Dipeptide-βNA (0.3 g)	MBE/AcN/H_2O (2:2:3)	TEA (5 m*M*/SP)	HCl (5 m*M*/MP)	[28]
Indole auxins (1.6 g)	MBE/H_2O	TFA (0.04%/SP)	NH_3 (0.1%/*M*P)	[19]
TCF (0.01–1 g)	DEE/AcN/10 m*M* $AcONH_4$ (4:1:5)	TFA (200 μl/SS)	MP	[29,32,33]
Red #3 (0.5 g)	DEE/AcN/10 m*M* $AcONH_4$ (4:1:5)	TFA (200 μl/SS)	MP	[29,31]
Orange #5 (0.01–5 g)	DEE/AcN/10 m*M* $AcONH_4$ (4:1:5)	TFA (200 μl/SS)	MP	[18,19,29]
Orange #10 (0.35 g)	DEE/AcN/10 m*M* $AcONH_4$ (4:1:5)	TFA (200 μl/SS)	MP	[29]
Red #28 (0.1–6 g)	DEE/AcN/10 m*M* $AcONH_4$ (4:1:5)	TFA (200 μl/SS)	MP	[29]
Eosin YS (0.3 g)	DEE/AcN/10 m*M* $AcONH_4$ (4:1:5)	TFA (200 μl/SS)	MP	[29]
Amaryllis alkaloids	MBE/H_2O	TEA (5 m*M*/SP)	HCl (5 m*M*/MP)	[20,40]
(3 g)	MBE/H_2O (DPCCC)	HCl (10 m*M*/SP)	TEA (10 m*M*/MP)	[20,40]
Vinca alkaloids	MBE/H_2O (DPCCC)	HCl (5 m*M*/SP)	TEA (5 m*M*/MP)	[20]
Structural isomers (15 g)	MBE/AcN/H_2O (4:1:5)	TFA (0.32%/SP)	NH_3 (0.8%/MP)	[20,42]
Stereoisomers (0.4 g)	Hex/EtOAc/MeOH/H_2O (1:1:1:1)	TFA, octanoic acid (each 0.04%/SP)	NH_3 (0.025%/MP)	[19,43]
Fish oil (0.5 g)	Hex/EtOH/H_2O (5:4:1)	TFA (10 m*M*/SP)	NH_3 (0.1%/MP)	[53]

[a] DNP: dinitrophenyl. CBZ: carbobenzoxy. OBzl: benzylesters. βNA: naphthyl amide. TCF: tetrachlorofluorescein. Amaryllis alkaloids: crinine, powelline and crinamidine. Vinca alkaloids: vincamine and vincine. Structural isomers: 2- and 6-nitro-3-acetamido-4-chlorobenzoic acid. Stereoisomers: 4-methoxymethyl-1-methyl-cyclohexane carboxylic acid. Fish oil: mixture of docosahexaenoic acid and eicosapentaenoic acid.

[b] The upper organic phase was used as the stationary phase (SP) and the lower aqueous phase, the mobile phase (MP) except in DPCCC where the above relationship is reversed. MBE: methyl-*tert.*-butyl ether. AcN: acetonitrile. BuOH: butanol. Hex: hexane. EtOAc: ethyl acetate. MeOH: methanol. $AcONH_4$: ammonium acetate. DEE: diethyl ether. DPCCC: displacement mode.

[c] TFA: trifluoroacetic acid. AcOH: acetic acid. SP: in stationary phase. MP: in mobile phase. SS: in sample solution. TEA: triethylamine.

zones through the column, since the retainer acid was added exclusively to the sample solution.

Fig. 18 shows the separation of seven DNP-amino acids obtained by the normal displacement mode of pH-zone-refining CCC [23]. As described earlier, the order of the elution became opposite to that of the reverse displacement mode (Fig. 17, middle chromatogram). Because the organic phase was mobile, the pH values of the effluent became somewhat erratic but followed, as expected, the reversed downward trend. The preparative separation of three DNP-amino acids was successfully carried out by the normal displacement mode of pH-zone-refining CCC where DNP-leu, DNP-ala and DNP-asp, each 0.5 g, were resolved in 6 h [23].

pH-Zone-refining CCC can be equally well ap-

Table 3
Samples and solvent systems applied to ligand affinity pH-zone-refining CCC

Sample[a]	Solvent systems[b] (volume ratio)	Key reagents[c]			References
		Retainer	Eluter	Ligand	
(±)-DNB-leucine (2 g)	MBE/H_2O	TFA (40 m*M*/SP)	NH_3 (20 m*M*/MP)	DPA (40 m*M*/SP)	[20,45]
(±)-DNB-valine (2 g)	MBE/H_2O	TFA (40 m*M*/SP)	NH_3 (20 m*M*/MP)	DPA (40 m*M*/SP)	[53]
Catecholamines (3 g)	MBE/H_2O	NH_4OAc (200 m*M*/SP)	HCl (50 m*M*/MP)	DEHPA (20%/SP)	[46]
Dipeptides (1 g)	MBE/AcN/50 m*M* HCl (4:1:5) (SP) MBE/AcN/H_2O (4:1:5) (MP)	TEA (20 m*M*/SP)	HCl (20 m*M*/MP)	DEHPA (10%/SP)	[46]
Dipeptides (1 g)	MBE/*n*-BuOH/AcN/50 m*M* HCl (2:2:1:5) (SP) MBE/*n*-BuOH/AcN/H_2O (2:2:1:5) (MP)	TEA (20 m*M*/SP)	HCl (20 m*M*/MP)	DEHPA (30%/SP)	[46]
Bacitracins (5 g)	MBE/50 m*M* HCl (1:1) (SP) MBE/H_2O (MP)	TEA (40 m*M*/SP)	HCl (20 m*M*/MP)	DEHPA (10%/SP)	[46]
FD&C Yellow No. 6 (2 g)	MBE/AcN/H_2O (2:2:3)	H_2SO_4 (0.2%/SP)	NH_3 (0.4%/MP)	TDA (5%/SP)	[50]

[a] DNB: dinitrobenzoyl.
[b] MBE: methyl-*tert.*-butyl ether. AcN: acetonitrile. BuOH: butanol.
[c] TFA: trifluoroacetic acid. NH_4OAc: ammonium acetate. TEA: triethylamine. DPA: N-dodecanoyl-L-proline-3,5-dimethylanilide. DEHPA: di-(2-ethylhexyl) phosphoric acid. TDA: tridodecylamine. SP: organic stationary phase; MP: aqueous mobile phase.

plied to the separation of basic compounds using a retainer base such as triethylamine and an eluter acid such as hydrochloric acid (HCl). This capability was first demonstrated by the separation of amino acid benzyl esters [27]. Fig. 19a shows a chromatogram of a set of amino acid benzyl esters using a two-phase solvent system composed of methyl-*tert.*-butyl ether and water where triethylamine (10 m*M*) was added to the organic stationary phase and HCl (10 m*M*) to the aqueous mobile phase. Seven components were well resolved in 3 h. Some irregularities of the pH-zone pH was caused by carry-over of the stationary phase due to precipitate formation in the separation column. This complication, however, did not impair the separation as demonstrated by the standard K values (x-line in the chromatogram) and the TLC (thin layer chromatography) analysis in Fig. 19b. The preparative separations of three amino acid benzyl esters are shown in Fig. 20 where three sample sizes of 0.6 (A), 3 g (B) and 6 g (C) were separated with the same solvent system composed of methyl-*tert.*-butyl ether/water where 5 m*M* triethylamine was added to the organic stationary phase and 20 m*M* HCl to the aqueous mobile phase. Comparison of these three chromatograms clearly shows that the increase of the sample size results in a proportional increase of the peak width whereas the width of the mixing zones remains the same as indicated by the sharp transition of the standard K values between the peaks (x-line). This demonstrates a great potential of the present technique for preparative-scale separations.

Using these amino acid derivatives, a series of experiments was performed to investigate the effects of the concentration of the retainer and eluter on the analyte concentration in both reverse displacement and normal displacement mode operation.

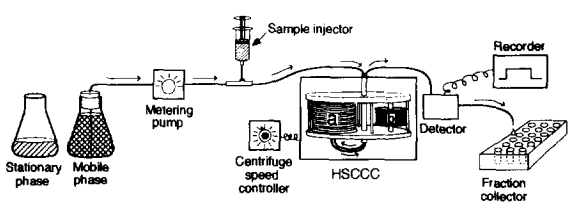

Fig. 16. Schematic presentation of high-speed countercurrent chromatograph (HSCCC) used in the present study: (a) multilayer-coil separation column and (b) counterweight.

Fig. 21 shows the effect of molar concentrations of the eluter acid (A) and retainer base (B) on the analyte concentration in the mobile phase in the reverse displacement mode operation. In Fig. 21A, the molar concentrations of three amino acid benzyl esters were plotted against those of HCl (eluter acid) in the mobile phase. As the HCl concentration is increased from 5 to 40 m*M*, all analytes increase their concentration at a nearly 1:1 molar ratio, suggesting that the eluter acid acts as the counterion to effectively determine the analyte concentration in the mobile phase. On the other hand, increased HCl concentration causes a sharp decrease of the partition coefficient (K) of the retainer base resulting in the reduced retention time of the analyte peaks.

Fig. 21B similarly shows the effects of the retainer base (triethylamine) concentration in the stationary phase, while the concentration of the eluter acid (HCl) in the mobile phase was fixed at 10 m*M*. In this case, the concentration of all analytes were almost unaltered with the increased triethylamine concentration by maintaining a near 1:1 molar ratio to the eluter acid (10 m*M*) that serves as the counterion mentioned above. Increasing the concentration of the retainer base, however, causes an increase in the partition coefficient (K), enhancing the retention of the analytes. As described earlier, the partition coefficient (K) of the retainer is equal to those for all analytes within the succeeding pH-zones. Therefore the above finding indicates that an increased retainer concentration increases the analyte concentration in the stationary phase while the analyte concentration in the mobile phase remains unchanged. In short, the eluter acid determines the analyte concentration in the mobile phase while the retainer base modifies that in the stationary phase. The retention time of the analyte is counterbalanced by concentrations between the retainer and eluter. A short retention time may result in incomplete resolution between the early-eluting zones. On the other hand, an excessively long retention time may cause decomposition of the analyte which follows immediately behind the retainer base by hydrolysis of these benzyl esters by the exposure to high pH. However, this complication may be eliminated by the use of a suitable spacer which occupies the column space between the retainer base and analytes.

Similar experiments were performed using three

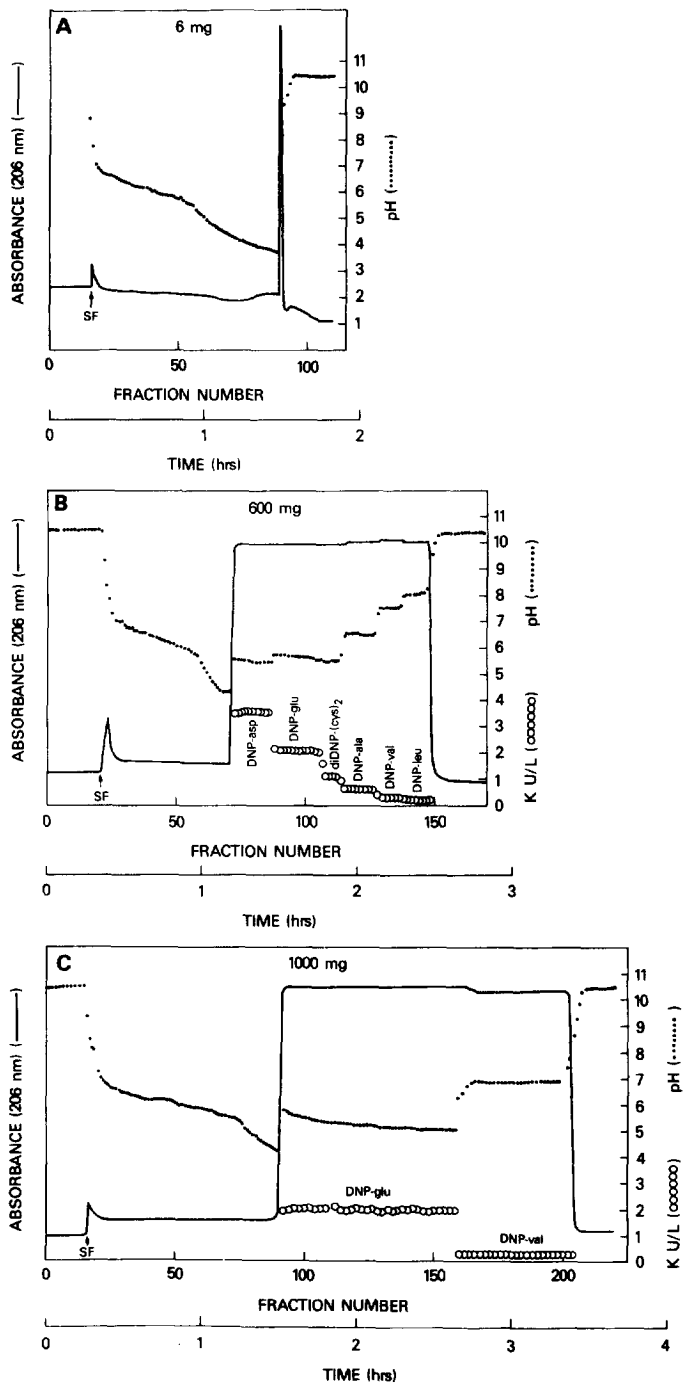

Fig. 17. Chromatogram of DNP-amino acids. Top: separation of six different DNP-amino acids in a small amount (6 mg) of sample mixture. Middle: separation of six different DNP-amino acids in a large amount (600 mg) of the sample mixture. Bottom: separation of DNP-L-glutamic acid and DNP-L-valine (500 mg of each). SF = solvent front. Experimental conditions: apparatus, multilayer coil planet centrifuge with 10 cm revolution radius; separation column, 16 coiled layers of 1.6 mm ID PTFE tubing with 320 ml capacity; solvent system, methyl-*tert.*-butyl ether–acetonitrile–water (4:1:5) where 0.1% aqueous NH_3 (ca. 14 m*M*) was added to the aqueous mobile phase (pH 10.5) and 200 μl TFA was added to the sample solution; detection, 206 nm; flow-rate, 3 ml/min; revolution, 800 rpm.

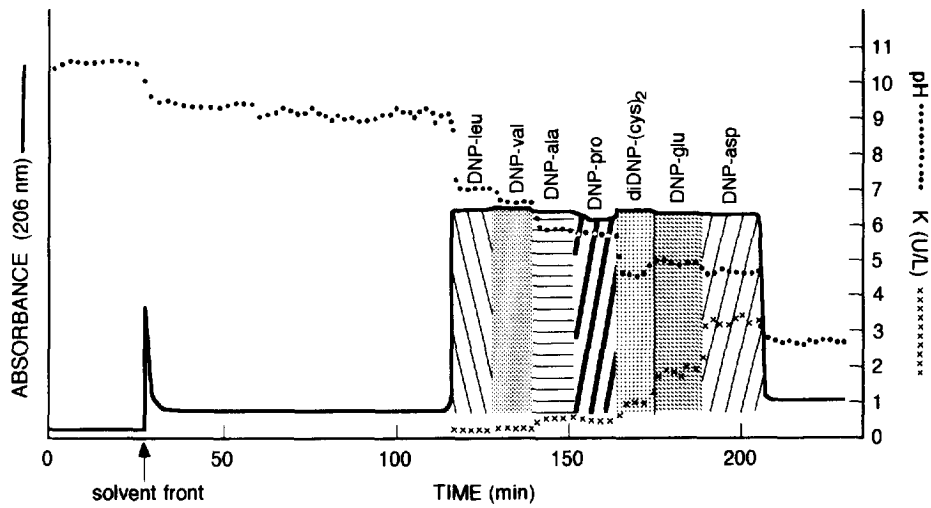

Fig. 18. Separation of seven DNP-amino acids by pH-zone-refining CCC using the displacement operation. An abrupt change of the pH at each peak boundary suggests minimum overlap between neighboring zones while the partition coefficient values (K_{std}) indicate that each mixing zone is no more than several milliliters. Experimental conditions: apparatus, multilayer coil HSCCC centrifuge with 10 cm revolution radius; separation column, 1.6 mm ID PTFE multilayer coil with 325 ml capacity; sample, seven DNP-amino acids as indicated in the chromatogram, each 100 mg dissolved in 10 ml of solvent consisting of 8 ml lower phase and 2 ml methyl-*tert.*-butyl ether; solvent system, methyl-*tert.*-butyl ether–water, 22 m*M* NH_3 in aqueous stationary phase (pH 10.53) and 10.8 m*M* TFA in organic mobile phase (pH 2.68); flow-rate, 3.3 ml/min, tail to head elution mode; detection, 206 nm; revolution, 600 rpm; stationary phase retention, 76.2%.

DNP-amino acids in the normal displacement operation. Fig. 22 shows the effects of the eluter (TFA) and retainer (NH_3) on the concentration of the analytes and their partition coefficient. The effects of eluter acid (TFA) concentration is shown in Fig. 22A where concentrations of three analytes (DNP-leu, DNP-ala and DNP-asp) are plotted against those of the eluter acid (TFA) in the organic mobile phase. Concentrations of DNP-leu and DNP-ala are almost identical while that of divalent DNP-asp is much less than those of the above two monovalent acids. Fig. 22B similarly shows the effects of retainer (NH_3) concentration in the aqueous stationary phase. Concentrations of all analytes sharply rise with increased NH_3 concentration up to 22 m*M* where they gradually saturate. Here again, the concentration of divalent DNP-asp is much lower than those of other two monovalent acids. These results indicate that the concentration of each analyte ion in the stationary phase is mainly determined by the concentration of its ammonium counterion. The above results also suggest that the concentration of divalent acid may become one half of the concentration of the monovalent acids to yield a 1:1 charge ratio to the ammonium counterion. The actual plots of the divalent acid (DNP-asp) however, fall about 2/3 of those of the monovalent acids as shown by the thin lines. Similar results are also obtained in other divalent amino acid derivatives such as DNP-glu and diDNP-$(cys)_2$ indicating that the concentration of these divalent acids in the stationary phase can significantly exceed the 1:1 charge ratio to that of the ammonium counterion due to their relatively high polarities. However, the possibility of coeluting a mono- and di-salt mixture is not ruled out.

4.1.2. Peptide derivatives

Similar to the amino acid derivatives above described, peptides can be easily fractionated by pH-zone-refining CCC, if either amino or carboxylic terminal is blocked. Fig. 23 shows separations of a set of Z or CBZ (carbobenzyloxy)-dipeptides by pH-zone-refining CCC using a two-phase solvent system composed of methyl-*tert.*-butyl ether–acetonitrile–water (2:2:3, v/v) with 16 m*M* TFA in the organic stationary phase and 5.5 m*M* ammonia in the aqueous mobile phase [20,28]. Eight components, each 100 mg, were well resolved within 4 h. A

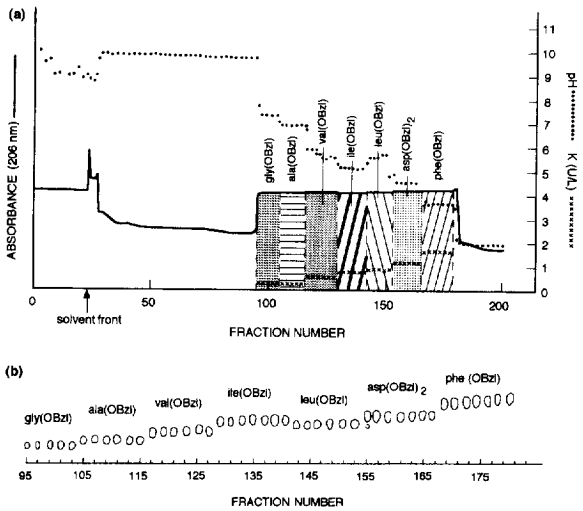

Fig. 19. Separation of seven amino acid benzyl esters by pH-zone-refining CCC. (a) Elution profile. Experimental conditions: apparatus, HSCCC centrifuge with 10 cm revolution radius; column, semipreparative multilayer coil, 160 m×1.6 mm ID, 325 ml capacity; solvent system, methyl-*tert.*-butyl ether–water, 0 m*M* triethylamine in upper organic stationary phase and 10 m*M* HCl in lower aqueous phase; sample, a mixture of seven amino acid benzyl esters as indicated in the chromatogram, each 100 mg dissolved in 20 ml solvent; flow-rate, 3 ml/min; detection, 206 nm; revolution, 800 rpm; retention of stationary phase, 71.2%. (b) TLC analysis of peak fractions. TLC plate: Kieselgel 60 F254. EM separations: solvent, chloroform–methanol–32% acetic acid (16:4:1, v/v).

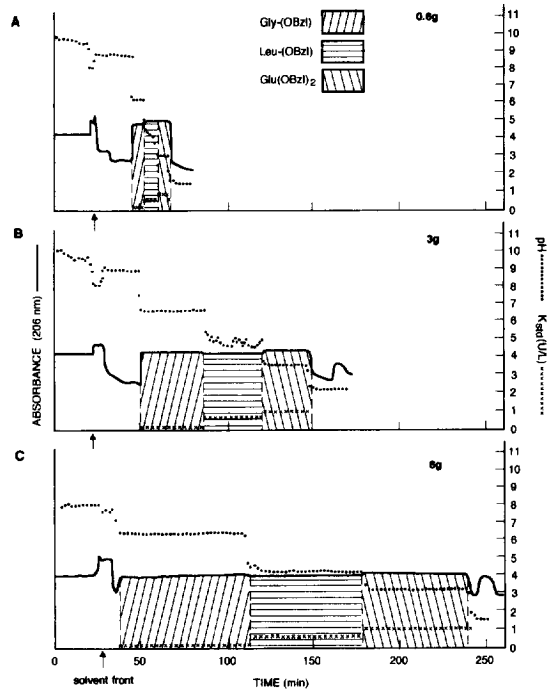

Fig. 20. Separation of three amino acid benzyl esters by pH-zone-refining CCC. Experimental conditions: apparatus and column as described in Fig. 19; solvent system, methyl-*tert.*-butyl ether–water, 5 m*M* triethylamine in organic stationary phase and 20 m*M* HCl in aqueous mobile phase; sample, equal amounts of gly(OBzl)·Tos, leu(OBzl)·Tos and glu(OBzl)·Tos, each 0.2 g (A), 1 g (B) and 2 g (C); flow-rate, 3 ml/min; detection, 206 nm; revolution, 800 rpm; retention of stationary phase, 76.5% (A), 63.3% (B) and 77.8% (C).

gram-quantity separation of three components, Z-gly-gly, Z-gly-ala and Z-gly-leu, was also successfully performed with a similar solvent system. A chromatogram of CBZ-tripeptides is shown in Fig. 24 where only the terminal amino acids are different [20,28]. The separation was performed with a slightly more polar solvent system composed of methyl-*tert.*-butyl ether–*n*-butanol–acetonitrile–water (2:2:1:5) where 16 m*M* TFA in the organic stationary phase and 2.7 m*M* ammonia in the aqueous mobile phase. Five components each 100 mg were well resolved in 4 h (Fig. 24). A set of basic β-naphthylamide derivatives of dipeptides was separated with a solvent system composed of methyl-*tert.*-butyl ether–acetonitrile–water (2:2:3, v/v) with 5 m*M* triethylamine in the organic stationary phase and 5 m*M* HCl in the aqueous mobile phase. Three components each 100 mg were resolved within 2 h (Fig. 25) [20,28].

A series of studies was performed on the effects of hydrophobicity of the solvent systems on the separation of these peptide derivatives. The results indicated that the peak resolution of these derivatives is generally improved by the use of a polar solvent system such as methyl-*tert.*-butyl ether–*n*-butanol–acetonitrile–water (2:2:1:5, v/v) [28].

4.1.3. Xanthene dyes

Purification of dyes by pH-zone-refining CCC is so far limited to hydroxyxanthene dyes which have been extensively reviewed by Weisz [29]. These dyes, including D&C Orange No. 5 [18,19,29], D&C Red No. 22 (Eosin Y) [29], D&C Red No. 28 (phloxine B) [29,30], FD&C Red No. 3 (erythrosine) [29,31], Rose Bengal [29], 4,5,6,7-tetrachlorofluorescein [29,32,33], etc., are difficult to purify by the

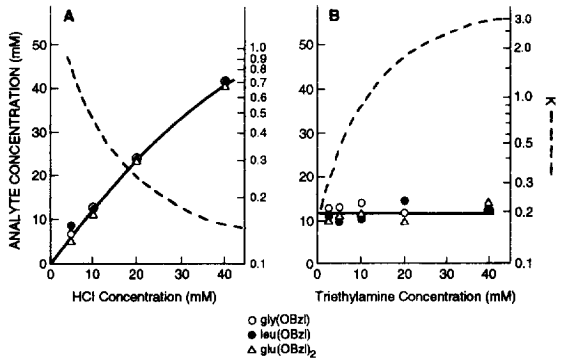

Fig. 21. Effects of eluter acid (A) and retainer base (B) on analyte concentration in the mobile phase. (A) Analyte concentration in the mobile phase is plotted against the concentration of the eluter acid from 5 to 40 m*M* while the concentration of the retainer base was fixed at 10 m*M*. (B) Analyte concentration in the mobile phase is plotted against the concentration of the retainer acid from 2.5 to 40 m*M* while the eluter acid concentration is fixed at 10 m*M*. Experimental conditions: apparatus and column, see Fig. 19; solvent system, methyl-*tert.*-butyl ether–water, triethylamine in organic stationary phase and HCl in aqueous mobile phase at the indicated concentration; sample, amino acid benzyl esters as indicated, each 0.5 m*M* in 10 ml solvent (5 ml stationary phase and 5 ml water) and adding 200 μl of TFA; flow-rate, 3 ml/min; detection, 206 nm; revolution, 800 rpm (600 rpm until 60 ml of the mobile phase eluted).

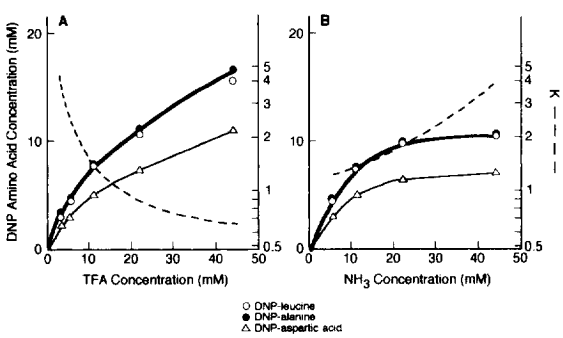

Fig. 22. Effects of eluter (TFA) (A) and retainer (NH_3) (B) on the concentration of DNP-amino acids and the partition coefficient (K) for TFA. Experimental conditions: apparatus and column, see Fig. 19; solvent system, methyl-*tert.*-butyl ether–water, (A) NH_3 11 m*M* in aqueous stationary phase and TFA 2.7–43 m*M* in organic mobile phase, (B) TFA 10.8 m*M* in organic mobile phase and NH_3 5.5–44 m*M* in aqueous stationary phase; sample, DNP-leu (open circle), DNP-ala (solid circle) and DNP-asp (triangle), each 100 mg in 5 ml of solvent consisting of 4 ml aqueous phase and 1 ml methyl-*tert.*-butyl ether; flow-rate, 3.3 ml/min, tail to head elution mode; detection, 206 nm; revolution, 600 rpm.

conventional separation methods. The literature documents the presence of contaminants that vary in type and level among batches of these dyes [34,35]. Excess levels of specified contaminant may result in the failure of a batch of color additives to meet certification requirements established by the U.S. Food and Drug Administration [36]. The presence of contaminants in the dyes can also cause variation in their staining properties and this may become the main cause of anomalous histochemical staining [37–40].

Fig. 26 shows a chromatogram of 5 g of D&C Orange No. 5 by pH-zone-refining CCC [18]. Three main components were eluted as a train of rectangular peaks associated with sharp impurity peaks at their narrow boundaries. The pH measurement of collected fractions revealed that three flat zones each correspond to pure components as indicated by HPLC analysis.

Three chromatograms in Fig. 27 show separations of the same sample in amount of 350 mg, 1 g, and 5 g which is also shown in Fig. 26. In each chromatogram the shaded area represents fractions containing pure components. As clearly shown in this diagram, increasing the sample size results in a proportional increase of the peak width, while the width of the mixing zones containing impurities remains the same. Since the impurities are in a narrow zone at the beginning and end, increasing the sample size gives a wider central zone, producing a higher percentage of pure compounds.

Fig. 28, Fig. 29 and Fig. 30 illustrate chromatograms of three other hydroxyxanthene dyes produced by pH-zone-refining CCC [29]. Here, it is interesting to note that in all these separations the elution order for these components is reversed between CCC and HPLC: in CCC heavily halogenated compounds elute first followed by less halogenated ones. This order is completely reversed in HPLC separation. As described earlier, the elution order in pH-zone-refining CCC is determined by the zone-pH which is in turn determined by both pK_a and hydrophobicity of the analytes (see Eq. 11) [19,20]. The heavily halogenated hydroxyxanthene dyes have extremely low pK_a which overshadows the effect of its increased hydrophobicity. Consequently, in each set of separations, the most heavily halogenated compound

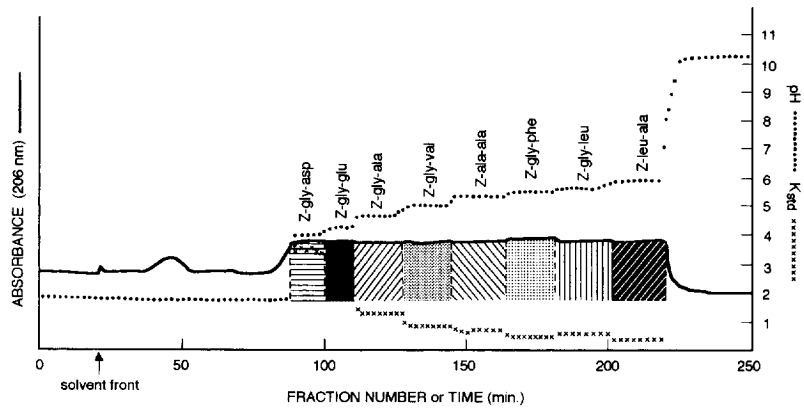

Fig. 23. Separation of eight CBZ (Z)-dipeptides by pH-zone-refining CCC. Experimental conditions: apparatus and column, see Fig. 19; solvent system, methyl-*tert.*-butyl ether–acetonitrile–water (2:2:3, v/v), 16 m*M* TFA in organic stationary phase (pH 1.83) and 5.5 m*M* NH_3 in aqueous mobile phase (pH 10.62); sample, eight CBZ-dipeptides as indicated in the chromatogram, each 100 mg dissolved in 50 ml solvent (25 ml each phase); flow-rate, 3.3 ml/min, head to tail elution mode; detection, 206 nm; revolution, 800 rpm (first 66 ml eluted at 600 rpm to prevent the carryover of the stationary phase); stationary phase retention, 65.1%.

elutes first in pH-zone-refining CCC while it elutes last in HPLC.

4.1.4. Alkaloids

Many biologically active alkaloids may be effectively separated by pH-zone-refining CCC using triethylamine in the organic phase and HCl in the aqueous phase. Fig. 31 shows chromatograms of alkaloids from a crude amaryllis extract [20,41]. The separation was performed with a two-phase solvent system composed of methyl-*tert.*-butyl ether/water. The upper chromatogram was obtained by eluting with an aqueous phase (reverse displacement mode) and the lower chromatogram by eluting with an organic phase (normal displacement mode). In both elution modes three components were well resolved. The sample size was 3 g in each separation. Elution

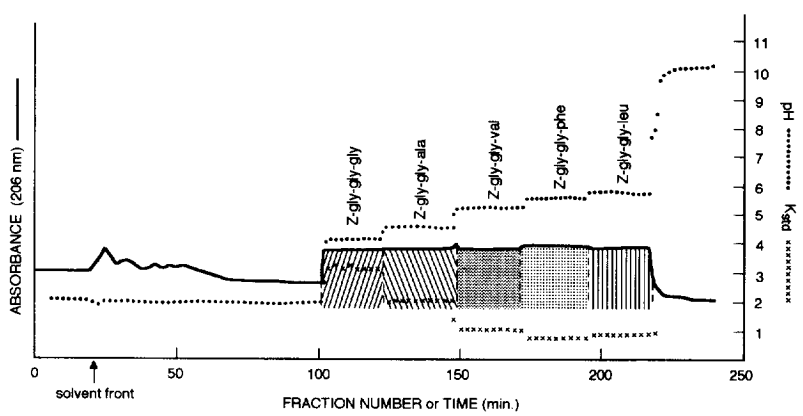

Fig. 24. Separation of five CBZ-tripeptides differing in the terminal amino acid residues. Experimental conditions: apparatus and column, see Fig. 19; solvent system, methyl-*tert.*-butyl ether–*n*-butanol–acetonitrile–water (2:2:1:5, v/v), 16 m*M* TFA in organic stationary phase (pH 2.00), 2.7 m*M* NH_3 in aqueous mobile phase (pH 10.35); sample, five CBZ(Z)-tripeptides as indicated in the chromatogram, each 100 mg, dissolved in 50 ml of solvent (25 ml each phase); flow-rate, 3.3 ml/min, head to tail elution mode; detection, 206 nm; revolution, 800 rpm (600 rpm for first 66 ml elution); stationary phase retention, 59.4%.

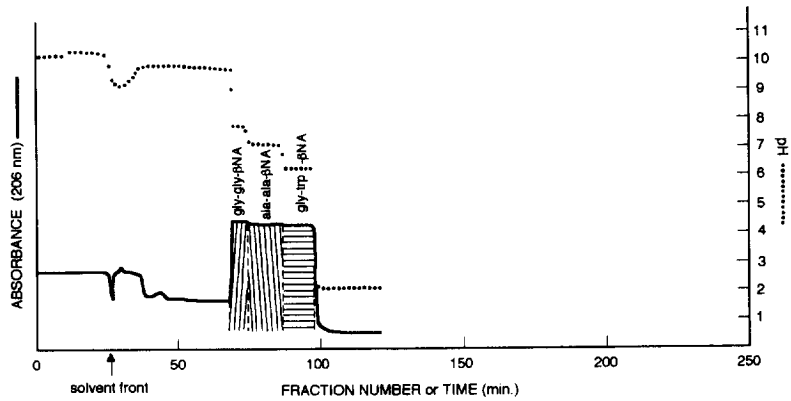

Fig. 25. Separation of three dipeptide βNA derivatives by pH-zone-refining CCC. Experimental conditions: apparatus and column, see Fig. 19; solvent system, methyl-*tert.*-butyl ether–acetonitrile–water (2:2:3, v/v), 5 m*M* triethylamine in organic stationary phase (pH 10.18). 5 m*M* HCl in aqueous mobile phase (pH 2.08); sample, three dipeptide βNA derivatives as indicated in the chromatogram, each 100 mg, dissolved in 30 ml of solvent (15 ml each phase); flow-rate, 3.3 ml/min, head to tail elution mode; detection, 206 nm; revolution, 800 rpm (600 rpm for first 66 ml elution); stationary phase retention, 67.0%.

with the organic phase yields a free base in an organic solvent which is easily evaporated. For unstable alkaloids, however, aqueous phase elution may be preferred because the sample is collected as the salt form which is often more stable.

Fig. 32 shows a chromatogram obtained from a crude extract of *Vinca minor* by the normal displacement mode of pH-zone-refining CCC [20]. Two major components, vincine and vincamine, were separated and each eluted as a free base in about 2 h. The pH curve shows an irregularity due to the organic mobile phase.

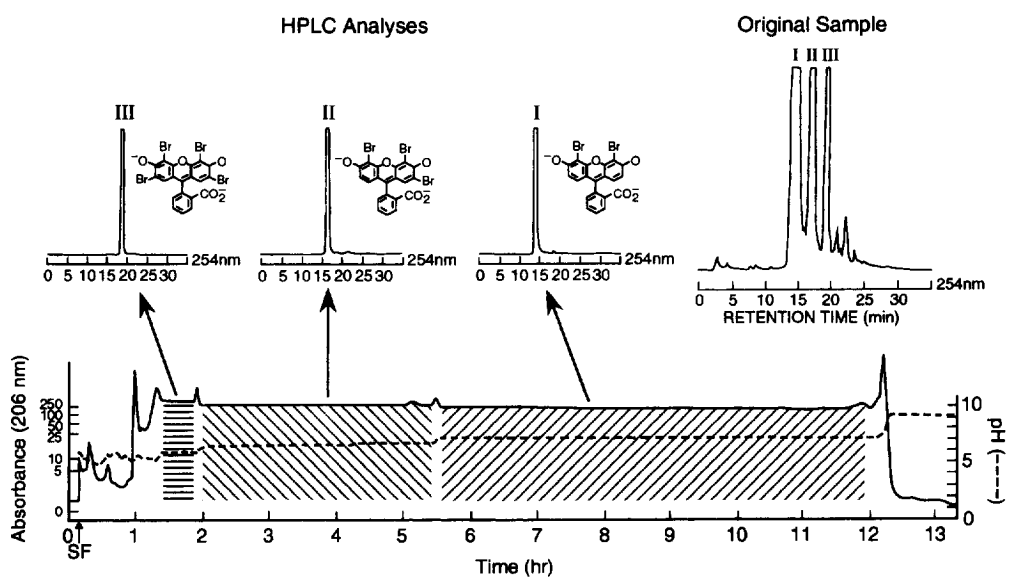

Fig. 26. Separation of 5 g of D&C Orange No. 5 by pH-zone-refining CCC. Experimental conditions: apparatus and column, see Fig. 19; solvent system, diethyl ether–acetonitrile–0.01 *M* aqueous ammonium acetate (pH 9 by ammonia) (4:1:5, v/v), lower aqueous phase mobile; sample, 5 g of D&C Orange No. 5 dissolved in 40 ml solvent (20 ml each phase), TFA 200 μg added to the sample solution as retainer; flow-rate, 3 ml/min; detection, 206 nm; revolution, 800 rpm: SF, solvent front.

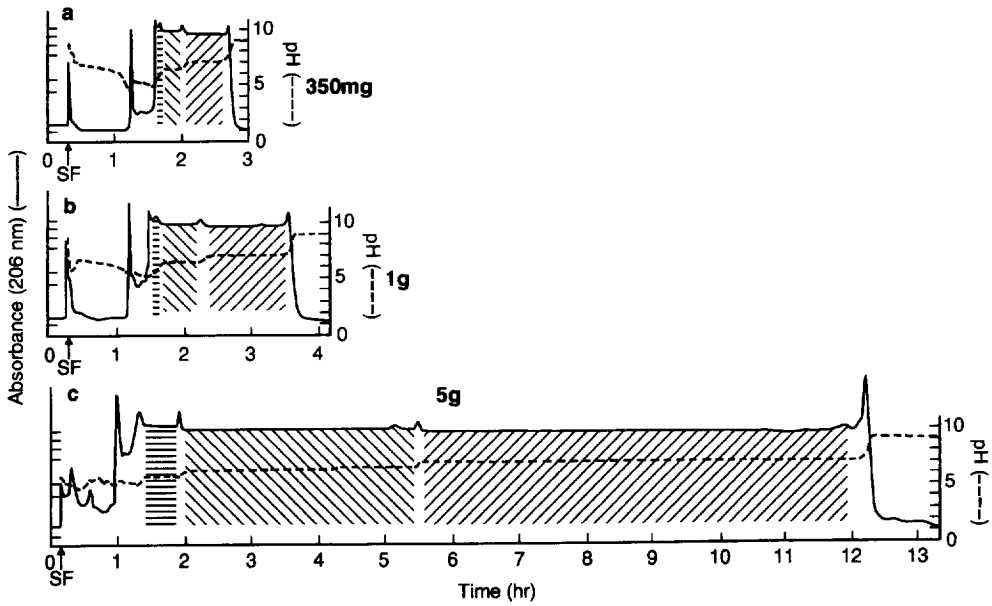

Fig. 27. pH-Zone-refining CCC of D&C Orange No. 5 (a) 350 mg, (b) 1 g, (c) 5 g. For experimental conditions, see Fig. 24.

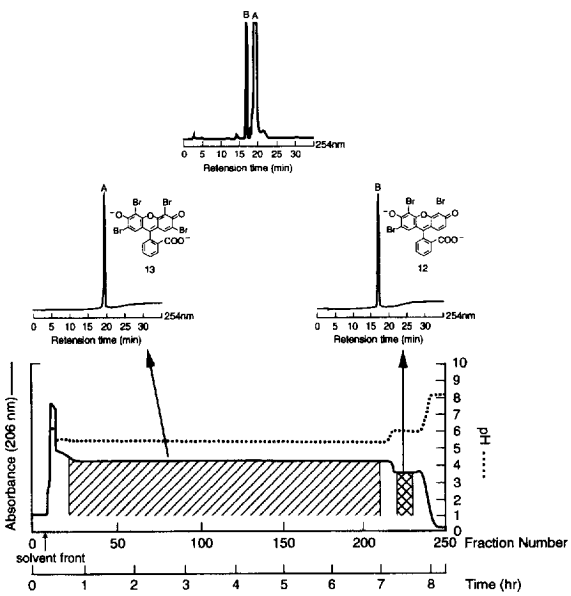

Fig. 28. Separation of 5 g of D&C Red No. 22 by pH-zone-refining CCC. Experimental conditions: apparatus and column, see Fig. 19; solvent system, diethyl ether–acetonitrile–0.01 *M* ammonium acetate (4:1:5, v/v), 0.01% NH_3 in aqueous mobile phase (pH 8.35) and 800 μl TFA in organic stationary phase; sample, 5 g of D&C Red No. 22 in 30 ml of solvent (15 ml each phase); flow-rate, 3 ml/min, head to tail elution mode; detection, 206 nm; revolution, 800 rpm.

4.1.5. Miscellaneous separations

Fig. 33 shows the separation of indole auxins by pH-zone-refining CCC [19]. A synthetic mixture of three components was separated in a train of rectangular peaks. The pH measurement of collected fractions revealed three flat pH-zones each corresponding to one species as indicated in the chromatogram.

The present method can be very useful for purification of structural isomers from a crude synthetic reaction. Fig. 34 shows a separation of 2- and 6-nitro-3-acetamido-4-chlorobenzoic acids (compounds I and II, respectively) by pH-zone-refining CCC [20,42]. A 15-g amount of sample mixture was resolved in two peaks and its mixing zone in 8 h. The mixing zone containing 90% of I and 10% of II may be rechromatographed under the same conditions to improve the yield of products. The applied experimental conditions are indicated in the figure caption.

One of the advantages of the present method is that compounds with no chromophore can be conveniently monitored by pH alone: the fractions of major components are located in their flat pH-zones and those of minor components at their boundaries. This potential is demonstrated in separation of

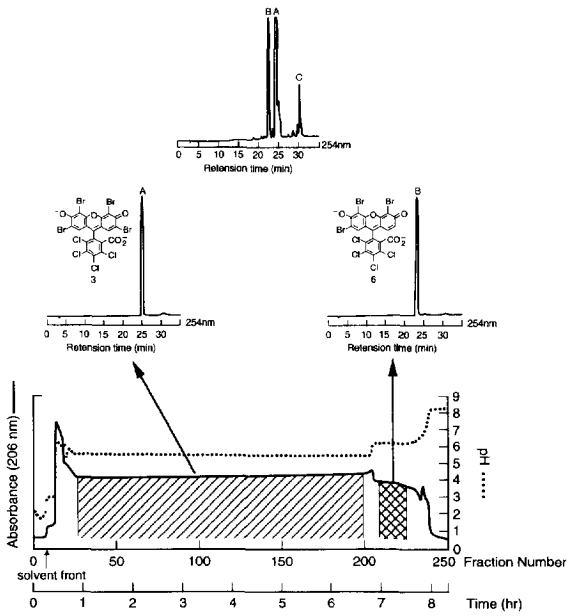

Fig. 29. Separation of 3 g of D&C Red No. 28 by pH-zone-refining CCC. Experimental conditions: apparatus and column, see Fig. 19; solvent system, diethyl ether–acetonitrile–0.01 *M* ammonium acetate (4:1:5, v/v), 0.01% NH_3 in aqueous mobile phase (pH 8.12) and 600 μl TFA/500 ml of organic stationary phase; sample, 3 g of D&C Red No. 28 in 30 ml of solvent (20 ml lower phase and 10 ml upper phase); flow-rate, 3 ml/min, head to tail elution mode; detection, 206 nm; revolution, 800 rpm.

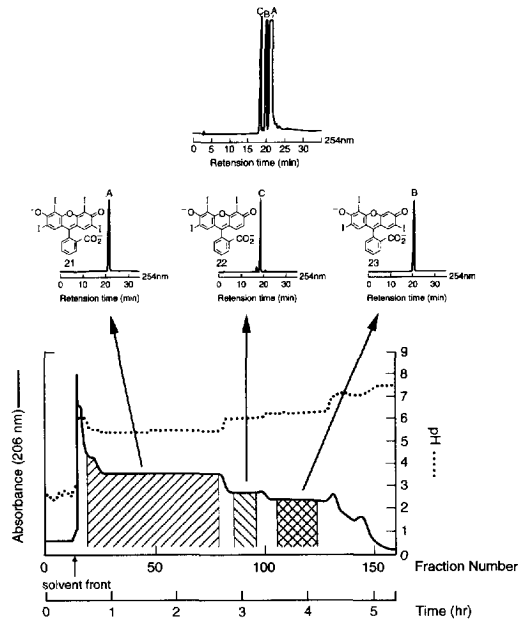

Fig. 30. Separation of 3 g of D&C Red No. 3 by pH-zone-refining CCC. Experimental conditions: apparatus and column, see Fig. 19; solvent system, diethyl ether–acetonitrile–0.01 *M* ammonium acetate (4:1:5, v/v), pH of aqueous mobile phase was adjusted by NH_3 at pH 7.53 and 400 μl TFA/500 ml of organic stationary phase; sample, 3 g of D&C Red No. 3 in 40 ml of solvent (20 ml lower phase and 20 ml of unacidified upper phase); flow-rate, 3 ml/min, head to tail elution mode; detection, 206 nm; revolution, 800 rpm.

stereoisomers, 1-methyl-4-methoxymethylcyclohexane-carboxylic acids, using octanoic acid as a spacer [19,43]. As shown in Fig. 35, the two isomers were eluted after the octanoic acid each forming a pH-zone with a relatively narrow mixing zone. The collected fractions were analyzed by gas chromatography (GC)–MS as indicated in the diagram.

4.2. Affinity ligand separation

4.2.1. Enantiomers

Chiral separations by HPLC requires a special ligand (chiral selector) which is bound onto the solid stationary phase. In CCC which uses no solid support in the column, the chiral selector is simply dissolved in the liquid stationary phase to carry out the separation by either conventional or pH-zone-refining CCC technique.

Fig. 36 shows analytical chromatograms of four racemic pairs of DNB (dinitrobenzoyl)-amino acids obtained by the conventional HSCCC technique. The upper chromatogram was produced from a ligand-free solvent system resulting in poor peak resolution. The lower chromatogram was obtained with the stationary phase containing a chiral selector, DPA (N-dodecanoyl-L-proline-3,5-dimethylanilide) [44]. Under otherwise identical conditions, the racemic mixture was resolved into seven peaks with one overlapping peak. The same ligand was successfully used to separate a gram quantity of enantiomers.

The chromatogram in Fig. 37a was obtained from 2 g of a DNB-leucine racemate using DPA as an affinity ligand [20,45]. The racemic mixture was resolved in highly concentrated rectangular peaks with minimum overlap. The fractions were analyzed by analytical CCC (indicated in the diagram) and also with CD and optical rotation instruments. Fig. 37b shows a similar separation of DNB-valine racemate. This technique should be very useful in the

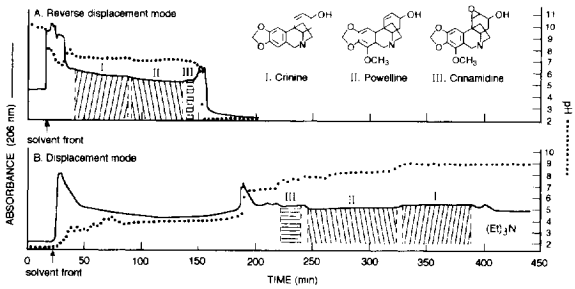

Fig. 31. Chromatograms of crude alkaloid extract of *Crinum moorei* obtained by reverse displacement mode (A) and normal displacement mode (B) of pH-zone-refining CCC. Experimental conditions: apparatus, HSCCC centrifuge equipped with a multilayer coil of 1.6 mm ID and about 300 ml capacity; solvent system, methyl-*tert.*-butyl ether–water; stationary phase, (A) organic phase (5 m*M* triethylamine) and (B) aqueous phase (10 m*M* HCl); mobile phase, (A) aqueous phase (5 m*M* HCl) and (B) organic phase (10 m*M* triethylamine); flow-rate, 3.3 ml/min; sample, crude alkaloid extract of *Crinum moorei*, 3 g dissolved in 30 ml of each phase; revolution, (A) 800 rpm (600 rpm until 66 ml of mobile phase was eluted) and (B) 600 rpm throughout.

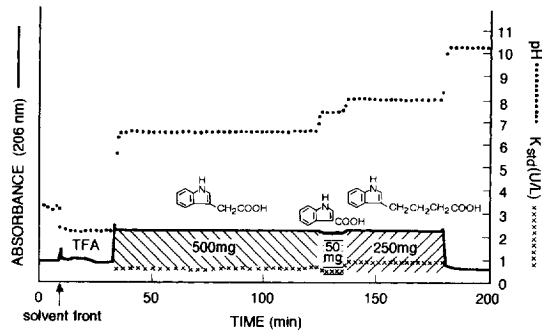

Fig. 33. Separation of three indole auxins by pH-zone-retaining CCC using the reverse displacement mode. Experimental conditions: apparatus and column, see Fig. 30; solvent system, methyl-*tert.*-butyl ether–water, TFA (0.04%, v/v) in organic stationary phase (pH 2.5) and NH_3 (0.05%, v/v) in aqueous mobile phase (pH 10.4); flow-rate, 3 ml/min, head to tail elution mode; sample, a mixture of three indole auxins, indole-3-acetic acid (500 mg), indole-3-carboxylic acid (50 mg) and indole-3-butyric acid (250 mg) in 20 ml of mobile phase; detection, 206 nm; revolution, 800 rpm.

pharmaceutical industry where 20% of modern drugs are now produced in chiral forms.

4.2.2. Catecholamines

Catecholamines containing two or more hydroxyl groups strongly favor partition into the aqueous phase even in a polar butanol two-phase solvent system. However, the use of a ligand such as DEHPA [di-(2-ethylhexyl)phosphoric acid] in the organic stationary phase radically improves their partition behavior. Fig. 38 shows the separation of three polar catecholamines and two related com-

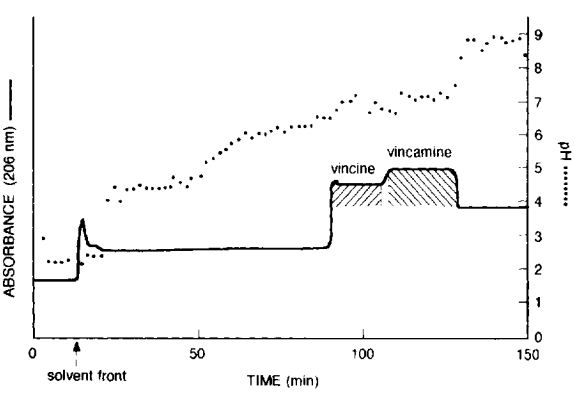

Fig. 32. Separation of alkaloids from *Vinca minor* by pH-zone-refining CCC using the normal displacement mode. Experimental conditions: apparatus and column, see Fig. 30; solvent system, methyl-*tert.*-butyl ether–water, 5 m*M* triethylamine in organic mobile phase and 5 m*M* HCl in aqueous stationary phase; flow-rate, 3.3 ml/min, tail to head elution mode; sample, crude alkaloid extract of *Vinca minor*, 300 mg dissolved in 30 ml of solvent system (equal volumes of each phase); revolution, 800 rpm; retention of stationary phase, 90.4%.

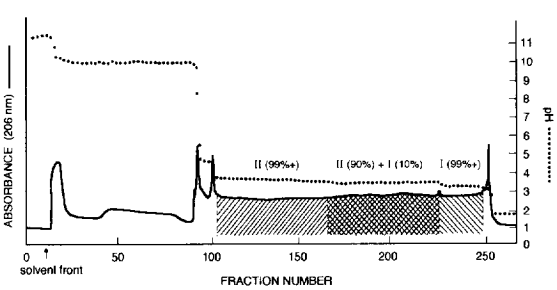

Fig. 34. Chromatogram of isomeric mononitro-derivatives of 2- and 6-nitro-3-acetamido-4-chlorobenzoic acid by pH-zone-refining CCC using the normal displacement mode. Experimental conditions: apparatus and column, HSCCC centrifuge with a multilayer coil of 1.6 mm ID and 325 ml capacity; solvent system, methyl-*tert.*-butyl ether–acetonitrile–water (4:1:5); stationary phase aqueous phase, 0.8% aqueous ammonia; mobile phase organic phase, 0.32% TFA; flow-rate, 3.3 ml/min; sample, nitration product of 3-acetamido-4-chlorobenzoic acid (15 g) dissolved in 100 ml in equal volumes of each phase; detection, 206 nm; revolution, 800 rpm (600 rpm until 66 ml of mobile phase was eluted).

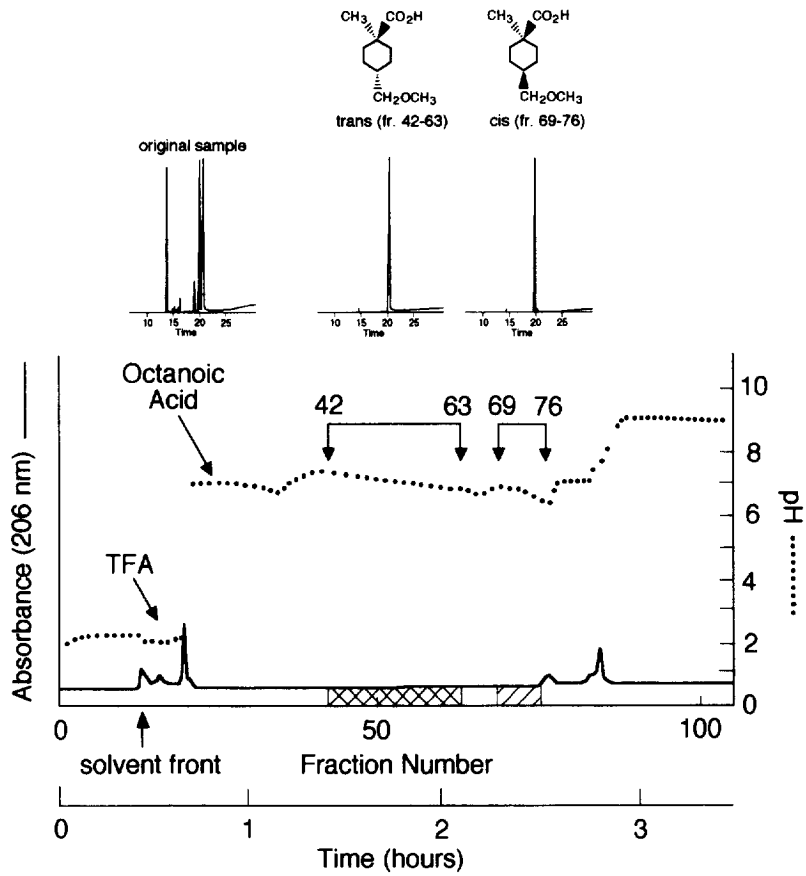

Fig. 35. Separation of *trans*- and *cis*-stereoisomers of 1-methyl-4-methoxymethylcyclohexane-carboxylic acids by pH-zone-refining CCC using octanoic acid as a spacer. Experimental conditions: apparatus and column, HSCCC centrifuge with a multilayer coil of 1.6 mm ID and 325 ml capacity; solvent system, methyl-*tert*.-butyl ether–acetonitrile–water (4:1:5), 0.32% TFA in organic stationary phase (pH 1.74), and 0.8% aqueous ammonia in aqueous mobile phase (pH 11.2); flow-rate, 3 ml/min, head to tail elution mode; sample, 400 mg of a synthetic mixture of *cis* and *trans* isomers dissolved in 3 ml of solvent (2.5 ml of unacidified upper phase and 0.5 ml of unbacified lower phase); detection, 206 nm; revolution, 800 rpm.

pounds by the conventional HSCCC technique [44]. The left chromatogram (A) was obtained from a ligand-free solvent system. All components were eluted as a single peak at the solvent front. Introducing the above ligand in the stationary phase resulted in an excellent resolution of all components as shown in the right chromatogram (B).

pH-Zone-refining CCC was successfully performed using the same ligand as shown in Fig. 39 [46]. Six components including four polar catecholamines and two related compounds, each 100 mg, were well resolved in 3 h.

4.2.3. Peptides

The above ligand is also effectively used for the separation of free peptides. Fig. 40 shows chromatograms of four isomeric pairs of dipeptides by the conventional HSCCC technique [44]. The left chromatogram was obtained from a ligand-free solvent system. All components were eluted as a single peak at the solvent front. The use of 10% DEHPA in the stationary phase resulted in the separation of four isomeric groups where each isomeric pair was further resolved to various degrees as shown in the right chromatogram.

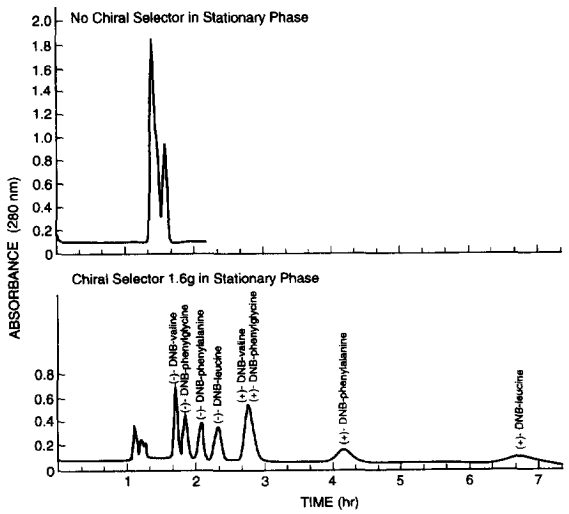

Fig. 36. Separation of four pairs of (±)-DNB-amino acids by the standard analytical HSCCC technique with and without ligand in the stationary phase. Upper chromatogram was obtained from a ligand-free stationary phase and the lower chromatogram from a stationary phase containing 1 g of DPA (N-dodecanoyl-L-proline-3,5-dimethylanilide) as a ligand. Experimental conditions: apparatus and columns, analytical HSCCC centrifuge with a set of three multilayer coils each consisting of eleven layers of 0.85 mm ID PTFE tube with a total capacity of ca 60 ml; solvent system, hexane–ethyl acetate–methanol/10 m*M* HCl (8:2:5:5) without ligand (upper chromatogram) and a ligand DPA 1.6 g in the stationary phase (lower chromatogram); flow-rate, 1 ml/min, head to tail elution mode; sample, 10 mg each of (±)-DNB-amino acids indicated in the chromatogram dissolved in 2 ml of solvent (1 ml each phase); detection, 280 nm; revolution, 1000 rpm (ca. 84 *g*).

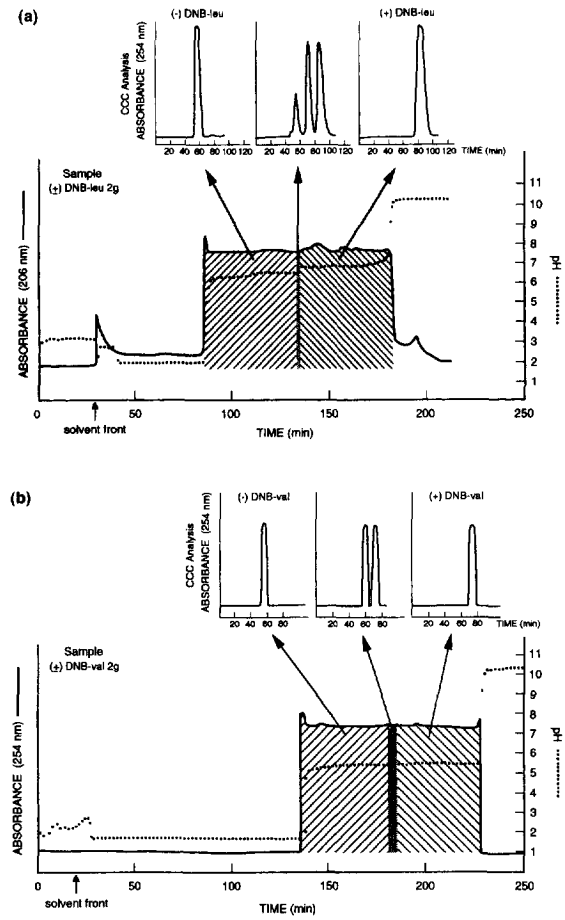

Fig. 37. Chiral separation of (±)-DNB-leucine (a) and (±)-DNB-valine (b) by pH-zone-refining CCC using DPA as a ligand. CCC conditions: apparatus and column, a HSCCC centrifuge with a semipreparative multilayer coil of 1.6 mm ID PTFE tubing with 330 ml capacity; solvent system, methyl-*tert*.-butyl ether–water. TFA (40 m*M*) + ligand DPA (40 m*M*) in organic stationary phase and ammonia (20 m*M*) in aqueous mobile phase; sample, (a) (±)-DNB-leucine and (b) (±)-DNB-valine, each 2 g; flow-rate, 3 ml/min, head to tail elution mode; detection, 206 nm; revolution, 800 rpm. Analytical CCC was carried out using the same column by the standard CCC technique under the following conditions: solvent system, hexane–ethyl acetate–methanol–10 m*M* HCl (8:2:5:5), organic stationary phase containing DPA (20 m*M*); flow-rate, 3 ml/min, head to tail elution mode; revolution, 800 rpm.

For pH-zone-refining CCC of free peptides, a series of experiments was performed using DEHPA as a ligand in the stationary phase [47]. A set of three dipeptides with a broad range in hydrophobicity was separated with a solvent system composed of methyl-*tert*.-butyl ether, acetonitrile and water at a volume ratio of 4:1:5 where triethylamine and various amount of the ligand were added to the organic stationary phase and HCl to the aqueous mobile phase. The results are shown in the left three chromatograms in Fig. 41. At a 10% ligand concentration, the second and third peaks were fused together while the polar tyrosyl–glycine peak was isolated and eluted earlier. Increasing the ligand concentration to 20–30% resulted in fusion of the first and second peaks while the hydrophobic tyrosyl–leucine peak was isolated and eluted much later. Increasing the polarity of the solvent system by modifying the phase composition improved the

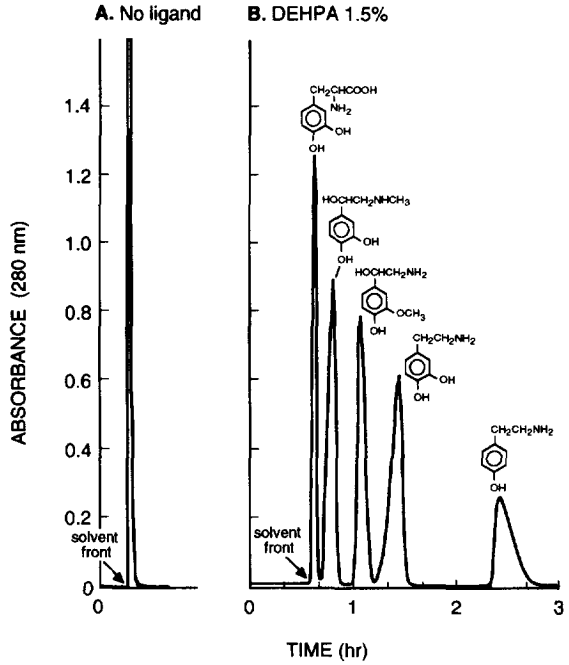

Fig. 38. Separation of three catecholamines and two related compounds by the affinity HSCCC without ligand (A) and 1.5% DEHPA in the organic stationary phase. Experiment conditions: apparatus and column, a HSCCC centrifuge equipped with a semipreparative multilayer coil of 1.6 mm ID Tefzel tubing with a total capacity of 325 ml; solvent system, methyl-*tert.*-butyl ether–water, ammonium acetate (0.1 *M*) in organic stationary phase and HCl (0.05 *M*) in aqueous mobile phase (A) and 1.5% DEHPA added to the organic stationary phase (B); sample, 5 mg each of three catecholamines and two related compounds indicated in the diagram; flow-rate, 3.3 ml/min, head to tail elution mode; detection, 280 nm; revolution, 800 rpm.

sharpness of the fused first and second peaks as shown in the right chromatogram. The results of these preliminary studies indicated that both ligand concentration and solvent composition should be adjusted according the hydrophobicity of the peptides. Under optimized conditions, pH-zone-refining CCC of dipeptides was successful. As shown in Fig. 42, both polar and nonpolar groups of dipeptides, each consisting of two isomeric pairs, were well resolved in 3–4 h.

Fig. 43 shows a preliminary result of a separation of related natural products [47]. Five grams of bacitracin complex consisting of multiple components were subjected to pH-zone-refining CCC under optimized conditions similar to those applied to the hydrophobic dipeptides. The UV tracing at 280 nm shows multiple peaks while the pH curve yielded flat zones at around pH 2. As indicated by HPLC analysis, two major components, bacitracins A and F, were isolated in peaks 3 and 5, respectively.

4.2.4. Proteins

Proteins may be separated with a suitable ligand in the stationary phase. However, the use of the usual organic solvents should be avoided since they would often denature the proteins. This problem can be solved by the use of aqueous–aqueous polymer phase systems composed of polyethylene glycol (PEG) and dextran [48] which do not appear to cause denaturation. In order to retain the target protein in the PEG-rich stationary phase, a PEG–ligand complex which can specifically bind to the protein is introduced in the solvent system [49]. Preliminary studies are currently underway in our laboratory to purify recombinant 3-oxo-Δ^5-steroid isomerase from *Escherichia coli* lysate using a PEG–estradiol derivative as a ligand in the polymer phase system.

4.2.5. Sulfonated compounds

In the past, pH-zone-refining CCC of acidic compounds was limited to carboxylic acids. These compounds were universally separated by adding retainer TFA to the organic stationary phase and eluter ammonia to the aqueous mobile phase. The separation of strongly acidic compounds such as sulfonated dyes, however, requires a basic ligand such as tridodecylamine in the acidified organic stationary phase. Preliminary experiments were performed using a solvent system composed of methyl-*tert.*-butyl ether, acetonitrile, and water at a volume ratio of 2:2:3. Tridodecylamine was added to the acidified organic stationary phase and the sample was eluted with the mobile phase containing ammonia. The results obtained from the separation of FD&C Yellow No. 6 is shown in Fig. 44 which shows HPLC analysis of the original sample (A) and the pH-zone-refining CCC separation together with HPLC analysis of fractions (B). The elution curve produced a characteristic rectangular peak associated with two pH-zones [50]. Hydrophobic impurities (d and e) are concentrated and eluted after the main peak [Fraction (Fr.) 106 and Fr. 113]. HPLC analysis of fractions corresponding to the main peak (Frs.

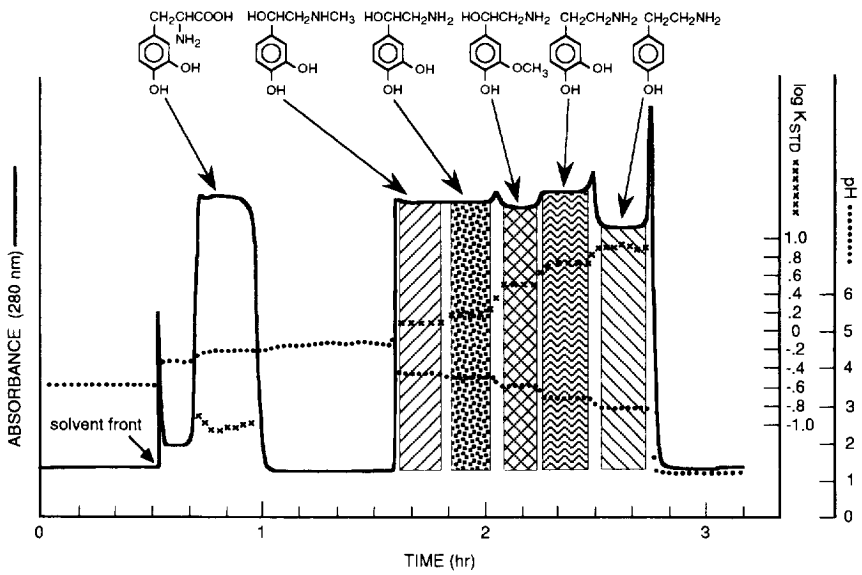

Fig. 39. Separation of catecholamines and related compounds by pH-zone-refining CCC using a ligand in the stationary phase. Experimental conditions: apparatus and column, see Fig. 36; solvent system, methyl-*tert.*-butyl ether–water, 200 m*M* ammonium acetate and 20% (v/v) DEHPA in organic stationary phase and 50 m*M* HCl in aqueous mobile phase; sample, six analytes as indicated in the chromatogram, each 500 mg dissolved in 40 ml of aqueous phase to which 4 ml of conc. HCl was added; flow-rate, 3.3 ml/min, head to tail elution mode; detection, 280 nm; revolution, 800 rpm; retention of stationary phase, 70%.

65–78, 81 and 84–100) and the earlier peak (Fr. 48) revealed that all fractions consist of the same component. One explanation is that these fractions contain free acid (Fr. 48), monoammonium salt (Fr. 81) and diammonium salt (Frs. 84–100). This possibility is currently under investigation.

5. Technical guidance

5.1. Samples and sample solutions

Successful application of pH-zone-refining CCC requires the conditions where target analyte(s) must be ionic and stable at a wide range of pH, preferably from 1 to 10. In addition there must be at least 0.1 mmol or ideally over 1 mmol of each species in the sample mixture. Otherwise the mixing zone would occupy a substantial portion of the solute zone reducing the yield of pure fractions.

The sample solution is prepared by dissolving a desired amount of sample in the stationary phase containing the retainer and adding a lessor amount of the mobile phase free of the eluter. Although it is ideal to completely dissolve the sample, a sample solution containing undissolved particles may be introduced into the column after making a fine homogeneous suspension by sonicating for several minutes. In pH-zone-refining CCC, the analyte concentration in the mobile phase is largely determined by the molar concentration of counterions and therefore the analyte concentration in the eluted fraction is irrelevant to the initial concentration in the sample solution. Application of highly concentrated sample solution tends to modify the solvent composition and interfacial tension which may lead to a loss of the stationary phase from the column. If the settling time of the sample solution is prolonged, further dilution of the sample is recommended. Our experiments have shown that the sample volume can be as large as 200 ml for a separation column of a 320 ml capacity, provided that the retention of the stationary phase is over 70%. In the sample solution (consisting of two phases), the target analyte should be almost entirely distributed into the stationary phase. If this is not the case, it is most likely that the sample contains a large amount of salt which alters the pH of the sample solution. If this pH shift occurs, retainer

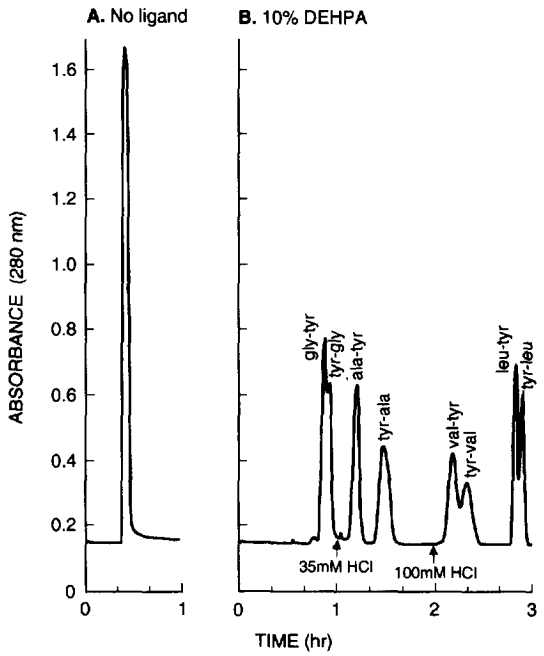

Fig. 40. Separation of four isomeric pairs of dipeptides by the standard HSCCC technique using (A) ligand-free stationary phase and (B) an affinity ligand (DEHPA) in the stationary phase. Experimental conditions: apparatus and column, a HSCCC centrifuge equipped with a multilayer coil of 1.6 mm ID Tefzel tubing with 325 ml capacity; solvent system, methyl-*tert.*-butyl ether–0.1 *M* KH_2PO_4 (1:1), 10% DEHPA added to the organic stationary phase; elution, HCl at 0.035 *M* added stepwise to mobile phase after 1 h and at 0.1 *M* after 2 h to shorten the separation time; flow-rate, 3.3 ml/min; sample, 5 mg each of eight dipeptides indicated in the chromatogram; detection, 280 nm; revolution, 800 rpm.

should be added to the sample solution to bring the pH into a proper range. Therefore, it is recommended to measure the pH of the sample solution routinely before application to the column.

5.2. Solvent systems

As indicated in Tables 2 and 3, the majority of samples can be successfully separated with a solvent system composed of ether (diethyl or methyl-*tert.*-butyl)/acetonitrile/water by adjusting the volume ratio from 1:0:1 to 2:2:3. For separation of polar compounds such as peptides, *n*-butanol is partially substituted for ether in the above solvent system. For hydrophobic analytes a less polar quaternary system composed of hexane–ethyl acetate–methanol–water is used by varying the volume ratio from 5:5:5:5 to 10:0:5:5 as applied to the standard HSCCC technique [5,51]. Table 4 lists these solvent systems in the order of their polarity. The systematic search of a solvent system suitable for pH-zone-refining CCC may begin with a methyl-*tert.*-butyl ether–water system for an acid analyte as follows:

1. A 2 ml volume each of the ether and 0.1% NH_4OH (eluter) (ca. 12 m*M*, pH 10) is delivered into a test tube (13×100 mm).
2. Add a small amount of the sample (so that no significant change is made in pH), apply a stopper and vortex several times to equilibrate the contents.
3. Measure the analyte concentration in the upper and the lower phases and obtain the partition coefficient (K) by dividing the analyte concentration in the upper phase by that in the lower phase.
4. If $K_{base} \ll 1$, add retainer TFA (ca. 20 m*M*) to the contents to bring the pH to around 2, and reequilibrate the contents by vortexing.
5. Using procedure 3, obtain K. If $K_{acid} \gg 1$, the solvent composition is suitable for separation.
6. If K_{base} is not small enough, repeat the whole procedure using a less polar solvent system (see Table 4).
7. If K_{acid} is not large enough, repeat the whole procedure using a more polar solvent system (see Table 4).

Table 4
Systematic search for the solvent system

Solvent 1	Hexane/EtOAc/MeOH/H_2O	
	10:0:5:5	Hydrophobic
	9:1:5:5	
	8:2:5:5	
	7:3:5:5	
	6:4:5:5	
	5:5:5:5	
Solvent 2	MBE/*n*-BuOH/AcN/H_2O	
	1:0:0:1	
	4:0:1:5	
	6:0:3:8	
	2:0:2:3	
	4:2:3:8	
	2:2:1:5	Hydrophilic

EtOAc: ethyl acetate. MeOH: methanol. MBE: methyl-*tert.*-butyl ether. BuOH: butanol. AcN: acetonitrile.

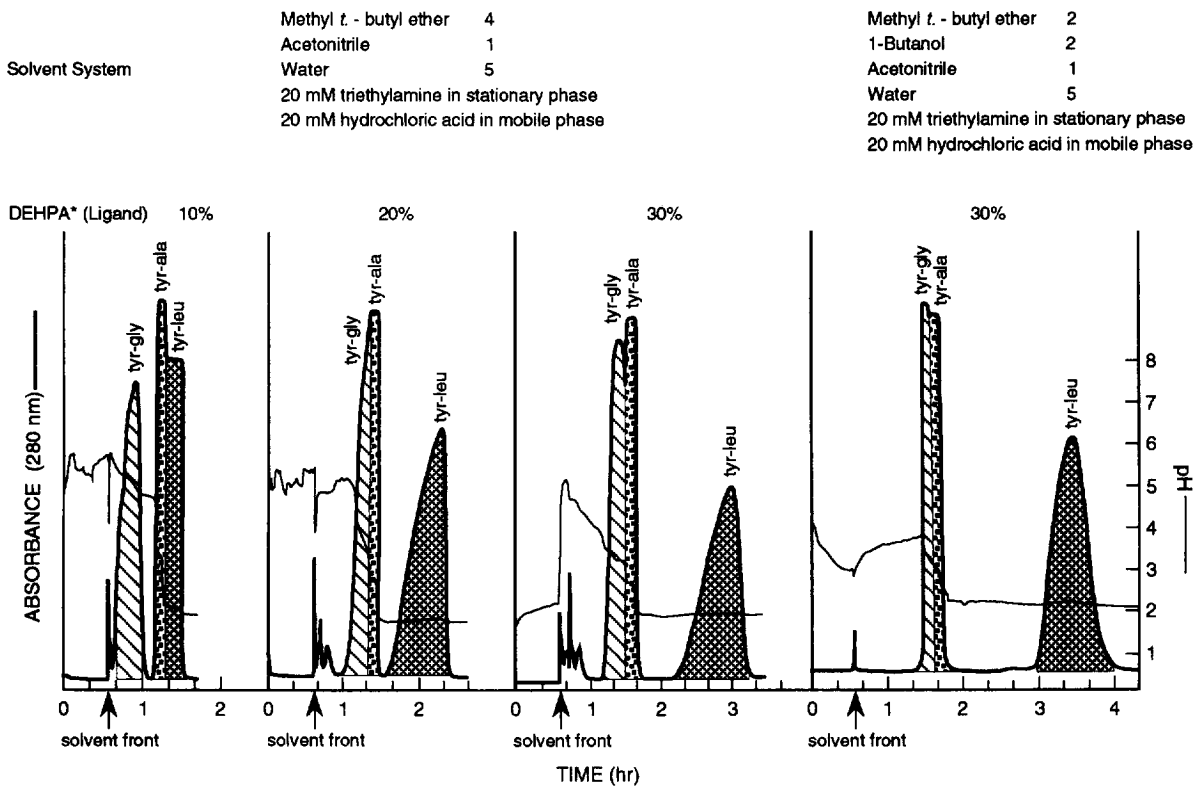

Fig. 41. Separation of three dipeptides by pH-zone-refining CCC using DEHPA in the stationary phase. Experimental conditions: apparatus and column, see Fig. 38; solvent system, as indicated in the diagram; sample, three dipeptides indicated in the chromatogram, each 100 mg; flow-rate, 3 ml/min, head to tail elution mode; detection, 280 nm; revolution, 800 rpm.

For a basic analyte, substitute HCl for ammonia in procedure 1 to test $K_{acid} \ll 1$, and substitute triethylamine for TFA in procedure 4 to test $K_{base} \gg 1$.

For a zwitterionic compound or a highly polar analyte, the use of a ligand is required to retain the analyte in the stationary phase. As shown in Table 3, di-(2-ethylhexyl)phosphoric acid was effectively used for the separation of peptides and catecholamines while tridodecylamine was used for the separation of sulfonated dyes. For chiral separations, a suitable chiral selector is dissolved in the stationary phase: the higher the selector concentration, the greater is the peak resolution [45,52].

All these ligands are dissolved in the stationary phase at the optimum concentration determined by the preliminary study. One important requirement of these ligands is that they must be almost unilaterally distributed in the stationary phase. A small leakage of the ligands into the mobile phase may be eliminated by placing a ligand-free stationary phase at the end of the column as described in the separation procedure below.

The use of a ligand such as di-(2-ethylhexyl)phosphoric acid requires that the solvent pH be kept below a subacidic range to prevent intensive emulsification of the two solvent phases in the column.

5.3. Optimization of experimental conditions

After the solvent composition is determined, a proper amount of the retainer (eluter) is added to the organic phase and the eluter (retainer) to the aqueous phase for the reverse displacement mode (normal displacement mode) operation. In reverse displacement mode about equal molar concentrations of retainer and eluter such as each at 10–20 m*M* produces a satisfactory separation in most cases. The

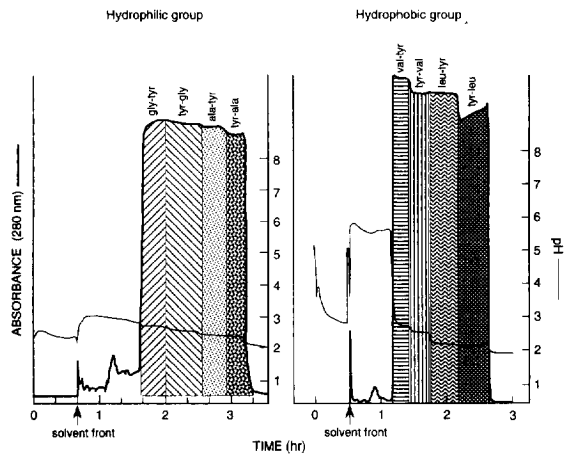

Fig. 42. Separation of dipeptides by pH-zone-refining CCC. Hydrophobic (right) and hydrophilic (left) groups of dipeptides each consisting of two isomeric pairs were separated under the optimized conditions. Experimental conditions: apparatus and columns, see Fig. 38; solvent systems, (hydrophobic group) methyl-*tert.*-butyl ether–acetonitrile–water (4:1:5), 20 m*M* triethylamine and 10% DEHPA in organic stationary phase and 20 m*M* HCl in aqueous mobile phase, (hydrophilic group) methyl-*tert.*-butyl ether–*n*-butanol–acetonitrile–water (2:2:1:5) 20 m*M* triethylamine and 30% DEHPA in organic stationary phase and 20 m*M* HCl in aqueous mobile phase; flow-rate, 3 ml/min, head to tail elution mode; sample, dipeptides indicated in the chromatogram, total amount of 1 g for each group; detection, 280 nm; revolution, 800 rpm.

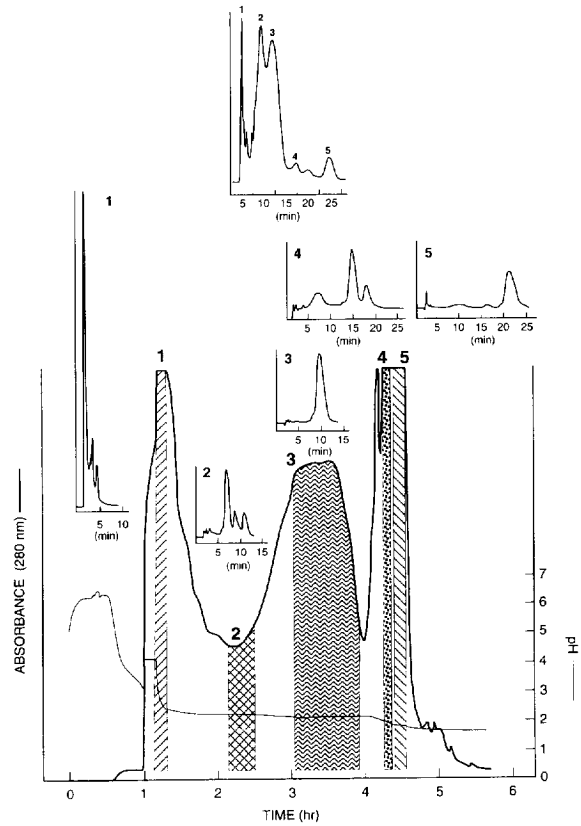

Fig. 43. Preparative separation of bacitracin complex by pH-zone-refining CCC. Experimental conditions: apparatus and column, see Fig. 38; solvent system, methyl-*tert.*-butyl ether–acetonitrile–water (4:1:5), 40 m*M* triethylamine and 10% DEHPA in organic stationary phase, and 20 m*M* HCl in aqueous mobile phase; flow-rate, 3 ml/min; sample, 5 g of bacitracin dissolved in 40 ml of solvent (20 ml each phase); detection, 280 nm; revolution, 800 rpm.

concentration of eluter in the mobile phase mainly determines the concentration of analyte in the eluted fractions (see Fig. 21). Thus, increasing the eluter concentration results in a higher concentration and a shorter retention time of the analyte. On the other hand, the concentration of retainer in the stationary phase alters the concentration of analyte in the stationary phase within the column. Increasing the retainer concentration raises analyte concentration in the stationary phase associated with long retention time (or increased K value).

In general, a large amount of sample can be effectively separated by applying high concentrations of both retainer and eluter, e.g., 40 m*M* each. However, use of this high retainer concentration often induces carryover of the stationary phase. This is apparently caused by precipitation of the analyte due to its excessively high concentration in the stationary phase. This complication may be avoided by reducing the relative concentration of the retainer in the stationary phase.

In the normal displacement mode operation, the retainer in the aqueous stationary phase serves as the counterion for the retained analytes to determine their concentration in the stationary phase. The eluter in the organic mobile phase, on the other hand, modifies the partition coefficient of analytes in such a way that increasing the eluter concentration shortens the retention time and increases the analyte concentration in the mobile phase (see Fig. 22A).

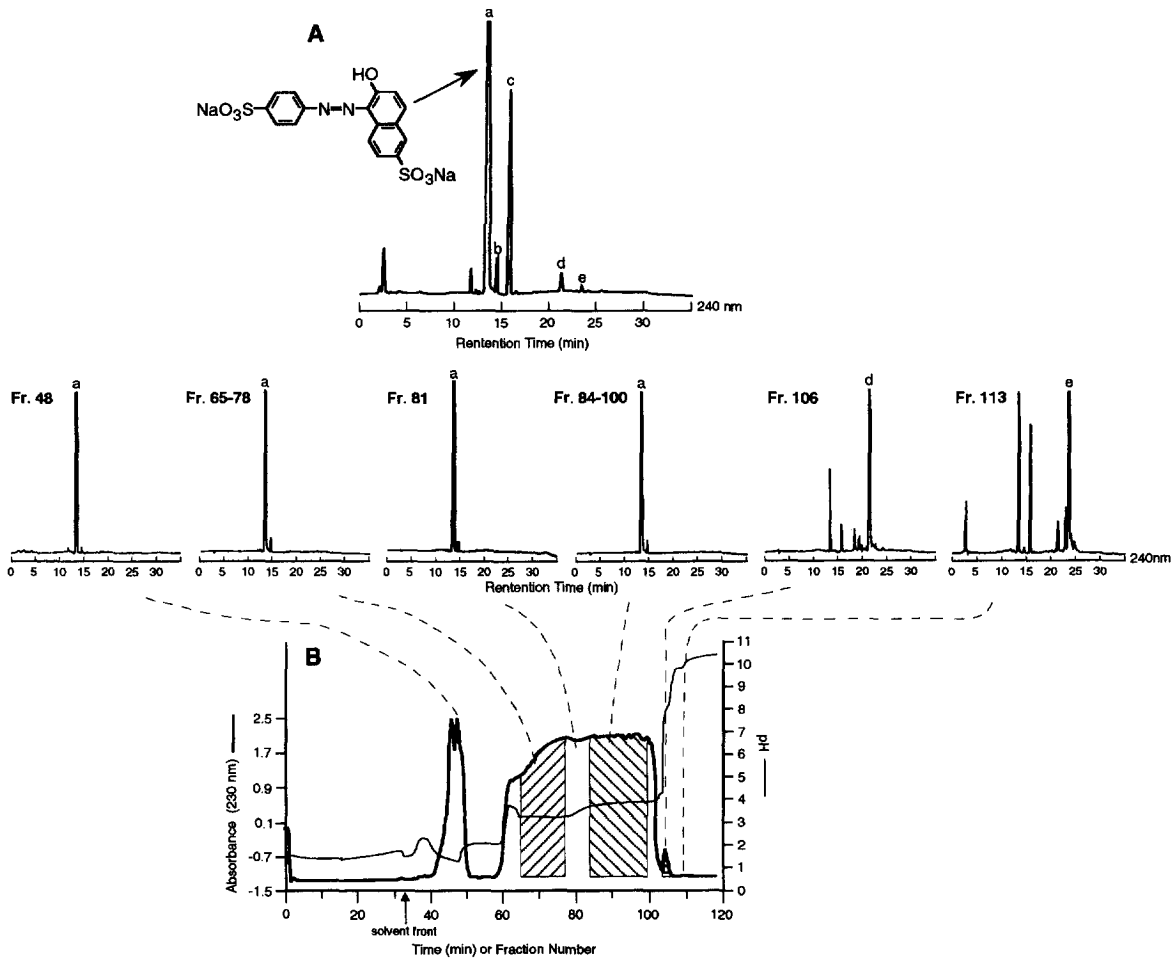

Fig. 44. Separation of FD&C Yellow No. 6 by pH-zone-refining CCC. Experimental conditions: apparatus, HSCCC centrifuge equipped with 3 multilayer coils, 1.6 mm ID and 325 ml capacity (Pharma-Tech Research Corporation, Baltimore, MD, USA); solvent system, methyl-*tert.*-butyl ether–acetonitrile–water (2:2:3); stationary phase, upper organic phase containing 0.2% sulfuric acid and 5% tridodecylamine; mobile phase, lower aqueous phase containing 0.4% ammonia (pH ca. 10.8); sample, 2 g FD&C Yellow No.6 in 14 ml solvent (4 ml stationary phase and 10 ml lower phase), pH 1.0 for upper phase and 1.7 for lower phase; revolution, 1000 rpm; flow-rate, 3 ml/min; retention of stationary phase, ca. 50%.

Usually, 10–20 m*M* each of the eluter and retainer in the respective phases produces a satisfactory result.

5.4. Separation procedure

The separation is initiated by filling the column with the stationary phase. For standard pH-zone-refining CCC (no ligand), the column is first completely filled with the acidified or basified stationary phase. This is followed by injection of sample solution through the sample port. Then the mobile phase containing the eluter is pumped into the column while the apparatus is rotated at an optimal rate (usually 800 rpm). The effluent is continuously monitored through a UV detector and then a pH monitor cell [54] (alternatively, the pH of the collected fractions is manually determined by a pH meter) and collected into test tubes using a fraction collector.

In pH-zone-refining CCC using a ligand in the

stationary phase, the above procedure results in a steady leakage of a small amount of the ligand which contaminates collected fractions. However, this complication can be overcome by placing a small amount of ligand-free stationary phase at the end of the column. This is achieved by first filling the column partially with a ligand-free stationary phase followed by a measured volume of the stationary phase containing the ligand. Thus, the ligand-free stationary phase retained at the end portion of the column serves as an absorbent for the ligand.

6. Advantages and limitations of pH-zone-refining CCC

As mentioned earlier, pH-zone-refining CCC shares many unique features with displacement chromatography. However, comparison between these two chromatographic methods shows that pH-zone-refining CCC has some important advantages over displacement chromatography. Displacement chromatography, which was introduced by Tiselius in the 1940s [24] and revived by Horváth et al. in the 1980s [25], is still not widely utilized, probably because it requires considerable effort to set up optimized experimental conditions especially in the choice of a proper displacer. In contrast pH-zone-refining CCC can be successfully carried out in many cases with a few sets of two-phase solvent systems where TFA and ammonia are almost universally used for the separation of organic acids, and triethylamine and HCl for the separation of organic bases. As described earlier in Section 5 in this article, the optimization of the experimental condition for pH-zone-refining CCC is even easier than that required for the conventional CCC technique.

One obvious disadvantage of pH-zone-refining CCC is that the analytes should be ionic, whereas displacement chromatography covers a broad spectrum of samples including both ionic and non-ionic compounds. In principle, however, non-ionizable analytes can be separated by modifying the present method using a suitable affinity-ligand in the stationary phase and a displacer in the mobile phase. In this case the method may be called "displacement countercurrent chromatography".

Various examples of application described in this review article elucidate the advantages of the present method over the conventional CCC technique. These include:

1. Sample loading capacity is increased over 10 times for a given column.
2. Fractions are highly concentrated.
3. Increase in sample size produces a higher percentage of pure fractions.
4. Minor components are concentrated and detected at the boundaries of the major peaks.
5. Samples with no chromophore can be effectively monitored by pH.

On the other hand, pH-zone-refining CCC has the following few limitations: the analytes should be ionizable with the difference in pK_a being greater than 0.2. In addition, the sample size should be at least 0.1 mmol and preferably over 1 mmol for each species. The second limitation, however, will be overcome if an analytical CCC system is developed for pH-zone-refining CCC.

The above features of pH-zone-refining CCC suggests that the method possesses great potential for preparative-scale separations in both research laboratories and industrial firms.

7. List of symbols

V_m	Volume of the mobile phase (MP) in the column (ml)
V_s	Volume of the stationary phase (SP) in the column (ml)
u_m	Flow-rate of MP (ml/s)
u	Travelling rate of the trailing border of the retainer acid through MP (ml/s)
V_r	Volume of MP required to elute the retainer acid completely from the column (ml)
K_r	Partition coefficient of the retainer acid in the equilibrium zone
K_s	Partition coefficient of solute S in the equilibrium zone
$K_{D\text{-}r}$	Partition ratio of the retainer acid
$K_{D\text{-}s}$	Partition ratio of solute S
$K_{a\text{-}r}$	Dissociation constant of the retainer acid
$K_{a\text{-}s}$	Dissociation constant of solute S

$pH_{z\text{-}r}$ pH of MP in the equilibrium zone of the retainer acid

$pH_{z\text{-}s}$ pH of MP in the equilibrium zone of solute S

C_r Molar concentration of the retainer acid in MP

C_R Molar concentration of the retainer acid in SP

C_E Molar concentration of the eluter base in MP

C_m Molar concentration of solute S in MP in the equilibrium zone

C_s Molar concentration of solute S in SP in the equilibrium zone

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