Anal. Chem. **2004,** *76,* 6179−6186

Articles

Anion-Exchange Displacement Centrifugal Partition Chromatography

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Ion-exchange displacement chromatography has been adapted to centrifugal partition chromatography. The use of an ionic liquid, benzalkonium chloride, as a strong anion-exchanger has proven to be efficient for the preparative separation of phenolic acid regioisomers. Multigram quantities of a mixture of three hydroxycinnamic acid isomers were separated using iodide as a displacer. The displacement process was characterized by a trapezoidal profile of analyte concentration in the eluate with narrow transition zones. By taking advantage of the partition rules involved in support-free liquid−liquid chromatography, a numerical separation model is proposed as a tool for preliminary process validation and further optimization.

The concept of countercurrent chromatography (CCC) is now widely used to refer to a solid support-free liquid−liquid chromatography which uses two immiscible solvents (or solutions) prepared from an equilibrated liquid−liquid biphasic system. Since its invention by Ito in the late 1960s, many applications and developments have been proposed.[1−3] Centrifugal partition chromatography (CPC) is one of the few technical solutions to the challenge of maintaining a liquid phase stationary while another one is pumped through it. It is based on partition cells radially engraved in a disk connected to each other by capillary ducts (Figure 1).

The centrifugal force field resulting from disk rotation causes decantation in each cell, thus allowing for a continuous process. One of the most striking aspects of this concept is the ability the user has to customize the mobile and stationary phases to obtain a wide range of chromatographic behaviors. Consequently, all of the development modes used in liquid chromatography can be applied to CCC and CPC, especially the displacement mode. The latter was proposed for the first time by Tiselius in 1943 and is mainly used today for preparative separations in the biotechnology industry.[4,5]

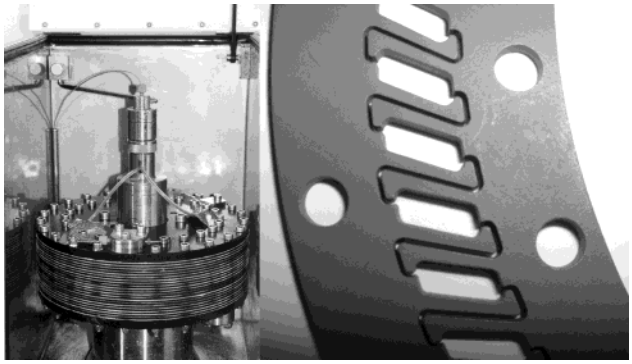

Figure 1. Kromaton Technologies FCPC 200 mL rotor (left) and partition disk (right). Note the connecting ducts centered on the bottom and the top of each cell. The upper and lower cell walls consist of the interdisk Teflon gaskets.

Basically, displacement chromatography in CCC can be performed by dissolving a displacer in the mobile phase and a retainer or an ion-exchanger in the stationary phase.[6] By adding an acid or a base in the stationary phase as a retainer, Ito introduced the pH-zone-refining mode.[7] For the first time in CCC and CPC, isotachic rectangularly shaped blocks of analytes separated by steep boundaries, the so-called shock layers, were observed.[8] This protocol is restricted to solutes showing a dramatic

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10.1021/ac049499w CCC: $27.50
Published on Web 09/24/2004

difference in polarity and, therefore, in solubility between their neutral and ionized forms. These limitations exclude the application of pH-zone-refining to ionized or strictly water-soluble molecules. The addition of a complexing agent, for example, DEHPA (diethylhexylphosphoric acid), in the stationary phase to selectively extract inorganic ions has opened new ion-based perspectives.[9] Selectors can be dissolved into the stationary phase to modulate the partition of the analytes or the selectivity of the solvent system.[7,10−13] Ion-exchange in CPC has been introduced by Chevolot et al. and applied to the purification of polysulfated polysaccharides (fucans and heparins) and naphthalen sulfonic acids isomers using the lipophilic secondary amine Amberlite LA2 (*N*-lauryl-*N*-trialkylmethylamine) as a weak anion-exchanger.[14−16] In this protocol, initial conditions are chosen so that the amine is in its protonated form $LA2H^+$. The anions are extracted in the stationary phase as lipophilic ion pairs. Then a basic aqueous mobile phase is pumped through the stationary phase and generates the displacement process by neutralizing $LA2H^+$.

The limitations of pH-zone-refining and the preparative aspect of both CCC and CPC techniques[17,18] and displacement mode[4,5] motivated us to explore new possibilities in ion-exchange displacement-mode CPC. Moreover, scale-up from laboratory to production in CCC techniques is linear and does not depend on random fluctuations due to imperfect packing of oversized columns.[19,20] In this context, we propose to use benzalkonium chloride as a strong anion-exchanger to separate ionized or ionizable compounds. The sample model is a synthetic mixture of hydroxycinnamic acid isomers, chosen as typical aromatic carboxylic acids.[21] A modeling software was developed in order to evaluate the influence of different parameters on the chromatographic performance by generating various simulated separations. This approach led us to a better understanding of the chromatographic process and should result in a ready-to-use optimization tool.

EXPERIMENTAL SECTION

Materials. Benzalkonium chloride (mixture of isomers; C_8−C_{10}, <5%; C_{12}, 60−70%; C_{14}, 30−40%; C_{16}−C_{18}, <5%); sodium iodide; trifluoroacetic acid; and *ortho*-, *meta*-, and *para*-hydroxycinnamic acids were purchased from Acros Organics (Noisy le Grand, France). Organic solvents and sodium hydroxide were from Carlo Erba (Rodano, Italy). Water was purified by deionization and inverse osmosis.

Apparatus. The separations were performed on a FCPC Kromaton Technologies apparatus (Angers, France) using a rotor of 20 circular partition disks (1320 partition cells; column capacity, 200 mL; see Figure 1). Rotation speed can be adjusted from 200 to 2000 rpm, producing a centrifugal force field in the partition cell of ∼120*g* at 1000 rpm and 480*g* at 2000 rpm. The solvents were pumped by a Dionex P580HPG 4-way binary high-pressure gradient pump (Sunnyvale, CA). The samples were introduced into the CPC column through a Techlab Economy 2/ED pump (Erkerode, Germany). The effluent was monitored with a Dionex UVD 170S detector equipped with a preparative flow cell (6-μL internal volume, path length of 2 mm). Fractions were collected by a Pharmacia Superfrac collector (Uppsala, Sweden). The apparent pH of solutions was measured by a Fisher Bioblock electrode (Illkich, France) connected to a Radiometer pH meter, type PHM240 (Copenhagen, Denmark). All experiments were conducted at room temperature (22 ± 1 °C).

Fraction analysis and quantification were performed on a customized Dionex Summit HPLC system equipped with a P580 pump, an ASI-100 automated injector, a STH column oven, a UVD340S diode array detector, and a C18 Uptisphere 5HDO-25QS (250 × 4.6 mm i.d., 5-μm particle size) column (Interchrom, Montluçon, France). The mobile phases were prepared from HPLC-grade acetonitrile and water acidified by TFA at concentration of 200 μL/L. The acetonitrile/water gradient was set as follows: from 20/80 to 40/60 in 13 min, then to 100/0 in 3 min; stable for 4 min; finally, to 20/80 in 5 min; and stable for 5 min. Flow rate was 1 mL/min. The linear calibration curve was plotted at 288 nm, from 0 to 100 mg/L, with five levels of 5 points each. All correlation coefficients were higher than 99.97%. The temperature of the column oven was set to 25 °C. All chromatographic data management was ensured by the Chromeleon software in its 6.0.1 version (Dionex, U.S.A.). Fraction evaporation under simultaneous vacuum and centrifugation was performed in a Jouan RC10.22 centrifugal evaporator (Jouan Inc, Wincester, VA).

Solvent System Screening. The distribution constant of benzalkonium chloride in biphasic systems was measured as follows: a 0.1 M stock (36 g L^{-1}) solution of benzalkonium chloride in dichloromethane was prepared considering a mean molecular weight of 360 g mol^{-1}. A 100-μL aliquot of this solution was transferred to a vial. After solvent evaporation, 2.5 mL of each phase of the previously equilibrated system was pipetted into the vial to dissolve the benzalkonium salt with vigorous agitation. The mixture was centrifuged for 15 min at 1600*g* if necessary. A 1-mL portion of each phase was transferred to another vial, and the solvent was removed under vacuum and centrifugation at 55 °C. The residue was dissolved in 1 mL of acetonitrile and directly injected into the PDA detector through a 250-μL loop, allowing for an ultrafast analysis. Ratios of peak areas were interpreted as distribution constants. Repeatability of the method was assessed with five analyses of five samples, showing a standard deviation of 8% around the mean value. For chlorinated systems, values were

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verified by manual UV measurement using the conjugate-phase method.[22]

Preparation of Solvent Systems. Biphasic systems were prepared by mixing the solvents in a separatory funnel, shaking them vigorously, and allowing them to settle until the phases became limpid. After phase separation, the ion-exchanger was added to 250 mL of organic phase. The mobile phase was prepared by dissolving the adequate amount of NaI in the aqueous phase.

Preparation of Sample Solutions. Each acid in its protonated form was weighed and dissolved in 10 mL of water, then NaOH was added so that the pH was close to 7. Two different procedures were used to prepare the sample, the benzalkonium ion pairs being generated either before or after injection. In the first case, the resulting aqueous solution was extracted several times with 10 mL of a chloroform solution of 0.1 M benzalkonium chloride until complete extraction of the analytes. The ion-pair-containing organic phases were pooled and evaporated under vacuum. This sample was dissolved in 10 mL of stationary phase and injected before displacement by the NaI-containing aqueous mobile phase. In the second procedure, the sample salt solution was lyophilized and dissolved in 10 mL of aqueous NaI-free mobile phase equilibrated with benzalkonium-containing organic phase. To allow the sample acids to transfer to the stationary organic phase as benzalkonium ion pairs, 200 mL of the NaI-free mobile phase was pumped in after sample injection.

CPC Experimental Conditions. The CPC column was washed and filled with methanol when not in use. Benzalkonium chloride was dissolved in the heavy organic phase, and all experiments were carried out by pumping the aqueous phase in the ascending mode. For each retainer concentration, a blank experiment without any sample injection and under otherwise identical experimental conditions was necessary to determine the mobile and stationary phase volume at hydrodynamic steady state. These values were used to calculate the required amount of benzalkonium chloride to be dissolved in the stationary phase in order to obtain the desired column capacity. Before any experiment, water was pumped in the rotating column, followed by the injection of two column volumes of the stationary phase in ascending mode, at 20 mL/min and at 500 rpm. When the sample was a mixture of ion pairs in the organic phase, it was injected in the ascending mode at 5 mL/min, then the displacement mobile phase was pumped at 1100 rpm, and the fractions were collected. When the sample was a mixture of sodium salts in the aqueous phase, it was injected at 2 mL/min at 1100 rpm. In this latter case, the addition of a small amount of the conjugate phase to the sample is necessary to restore saturation of the aqueous phase.[23] Then 200 mL of NaI-free mobile phase was pumped at 5 mL/min in order to allow extraction of the acids into the stationary phase. The outgoing phase corresponding to this step did not contain any acid. The displacement mobile phase was then pumped at 5 mL/min, and the fractions were collected. The outgoing aqueous phase was monitored by the on-line UV detector. In each case, the displaced volume of stationary phase corresponded to the void volume (V_m). The remaining retained stationary phase (V_s) allowed the calculation of the effective retainer amount in the column. For the cell content analysis immediately after injection, column content was pumped out by performing a dual-mode experiment: stationary phase was pumped into the column in descending mode, and fractions corresponding to the head of the column were collected.

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RESULTS AND DISCUSSION

Modeling. A numerical model of the chromatographic process has been proposed as a tool for column content and effluent composition prediction. The concentration of the chemical species can be evaluated assuming the following hypotheses for the chromatographic process.

(i). The column is divided into a limited number of column segments that mimic chromatographic theoretical plates.

(ii). Each cell is divided in two parts, volume V_{mob} and V_{stat}, corresponding to the aqueous mobile and exchanger-containing organic stationary phases. The ratio V_{stat}/V_{mob} is noted v.

(iii). Initially, a small number of cells at column input contains both stationary phase and the benzalkonium acid ion pairs.

(iv). The continuous chromatographic process is modeled as a series of "matter transfer"−"chemical equilibration" cycles.[15,24] Each cycle involves the injection of a fresh mobile phase volume, V_{mob}, containing the displacer (Na^+, I^-) at the inlet of the column, the transfer of each cell mobile phase content into the following cell, the collection of the last cell mobile phase volume, and the reequilibration of each cell. The model postulates that V_{stat} and V_{mob} are constant and that the equilibriums are completely reached in each cell.

(v). The strong anion-exchanger is benzalkonium cation (Bz^+), and Cl^- is the counterion (or the carrier). Benzalkonium chloride is a lipophilic quaternary ammonium salt. In the numerical model, it is assumed to exclusively dwell in the organic stationary phase. The analytes, though weak acids, HA_i ($1 \le i \le n$), are present in the mobile phase in their fully deprotonated form A_i^- and present in the stationary phase as their ion pair form Bz^+, A_i^-.

In the case of a sample constituted of a mixture of two acids, the chemical equilibriums involve the following species in the mobile phase: Na^+ (nonreactive ion), Cl^-, I^-, A_1^- and A_2^-. A supplementary species C^{2-} resulting from the association of two molecules of acid is introduced to account for a putative π-stacking phenomenon between phenolic solutes in the aqueous phase. The autoassociation (between A_i^- molecules or A_2^- molecules) was not taken into account because it does not lead to any coelution between A_i^- and A_2^- species. In the stationary phase, the ion pairs with benzalkonium are Bz^+Cl^-, Bz^+I^-, $Bz^+A_i^-$ and $Bz^+A_2^-$. Nine equations involving species concentration need to be solved to totally determine the state of such a system at equilibrium. Five equations are those describing the I^-, Cl^-, A_i^-, A_2^-, and Bz^+ species mass balance. The overlined symbols are used for concentrations and species in the organic phase. Thus, if n_{I^-} is the total number of I^- ions in a cell, then

$$n_{I^-} = V_{mob}[I^-] + V_{stat}\overline{[Bz^+, I^-]} \quad (1)$$

Defining virtual concentration $c_{I^-} = n_{I^-}/V_{mob}$, we get

$$c_{I^-} = [I^-] + v\overline{[Bz^+, I^-]} \quad (2)$$

In the same way, one gets the other mass-balance equations:

$$c_{Cl^-} = [Cl^-] + \nu\overline{[Bz^+, Cl^-]} \quad (3)$$

$$c_{A_1^-} = [A_1^-] + \nu\overline{[Bz^+, A_1^-]} + [C] \quad (4)$$

$$c_{A_2^-} = [A_2^-] + \nu\overline{[Bz^+, A_2^-]} + [C] \quad (5)$$

$$c_{Bz^+} = \nu\overline{[Bz^+, I^-]} + \nu\overline{[Bz^+, Cl^-]} + \nu\overline{[Bz^+, A_1^-]} + \nu\overline{[Bz^+, A_2^-]} \quad (6)$$

The last four equations arise from the application of the mass action law to the ion-exchange equilibriums and are characterized by their selectivity coefficient.

$$I^- + (Bz^+, Cl^-) \rightleftharpoons Cl^- + (Bz^+, I^-) \quad (7)$$

$$K_{I^-/Cl^-} = \frac{[Cl^-]\overline{[Bz^+, I^-]}}{[I^-]\overline{[Bz^+, Cl^-]}} \quad (8)$$

$$I^- + (Bz^+, A_1^-) \rightleftharpoons A_1^- + (Bz^+, I^-) \quad (9)$$

$$K_{I^-/A_1^-} = \frac{[A_1^-]\overline{[Bz^+, I^-]}}{[I^-]\overline{[Bz^+, A_1^-]}} \quad (10)$$

$$I^- + (Bz^+, A_2^-) \rightleftharpoons A_2^- + (Bz^+, I^-) \quad (11)$$

$$K_{I^-/A_2^-} = \frac{[A_2^-]\overline{[Bz^+, I^-]}}{[I^-]\overline{[Bz^+, A_2^-]}} \quad (12)$$

These equations are built taking iodide ions as reference (i.e., $K_{I^-/I^-} = 1$). The ion pair (Bz^+, I^-) is much more stable than (Bz^+, Cl^-), and therefore, $K_{I^-/Cl^-} \gg 1$. The association between A_i^- and A_2^- in the pair C^{2-} in the aqueous phase

$$A_1^- + A_2^- \rightleftharpoons C^{2-} \quad (13)$$

is governed by

$$K_{C^{2-}} = \frac{[C^{2-}]}{[A_1^-][A_2^-]} \quad (14)$$

giving the polynomial equilibrium equations

$$K_{C^{2-}}[A_1^-][A_2^-] = [C^{2-}] \quad (15)$$

$$K_{I^-/Cl^-}[I^-]\overline{[Bz^+, Cl^-]} = [Cl^-]\overline{[Bz^+, I^-]} \quad (16)$$

$$K_{I^-/A_1^-}[I^-]\overline{[Bz^+, A_1^-]} = [A_1^-]\overline{[Bz^+, I^-]} \quad (17)$$

$$K_{I^-/A_2^-}[I^-]\overline{[Bz^+, A_2^-]} = [A_2^-]\overline{[Bz^+, I^-]} \quad (18)$$

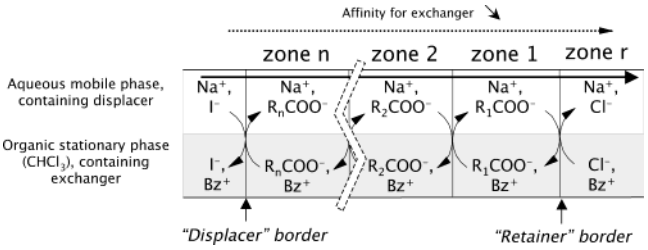

Figure 2. Isotachic train in the displacement mode.

This kind of equation system can be solved either with an iterative method[25] or with a polynomial equation solver. We used a mixed approach, favoring the iterative method and using the other one if the first failed due to an inadequate set of initial concentration values.[26]

The computer code for general nonlinear equation system resolution by an iterative method was borrowed from the publicly available Scilab source code.[27] The SYNAPS library was used to specifically solve polynomial equation sets by means of symbolic computations.[26] The numerical model itself is easily extended to any number of analytes and their possible associated forms in the aqueous phase.

The chromatographic process involved in displacement mode differs from elution in several aspects: mainly, the analytes progress as an isotachic train. In each cell, the analyte that shows the greatest affinity for the retainer in the organic stationary phase excludes by competition the analytes with lower affinity and so acts as a displacer by forcing them to solubilize in the aqueous mobile phase and to progress in the column. The shock layer is the overlapping region between two separated products, each of them showing a steep concentration drop or rise at column outlet. The sharp front of the analyte train is formed by competition with the carrier anion (chloride) on the exchanger, whereas the end of the analyte train is maintained as a shock layer by iodide, the displacer. Once the analytes are separated by mutual exclusion, they progress in the column as neighboring segments (Figure 2).

The acid concentration in the mobile phase being the same as the displacer, the separation time decreases when the displacer concentration increases, so the higher the NaI concentration, the faster the separation. Inversely, the benzalkonium chloride concentration in the stationary phase determines column capacity. Every benzalkonium cation must have formed an ion pair with iodine to allow the acids to flow out from the column, so the higher the benzalkonium concentration, the longer the separation.

In this development mode, the separation relies only on the association constant ratio of the different anions with the exchanger. Acids never protonate, so pK_a values can be neglected,

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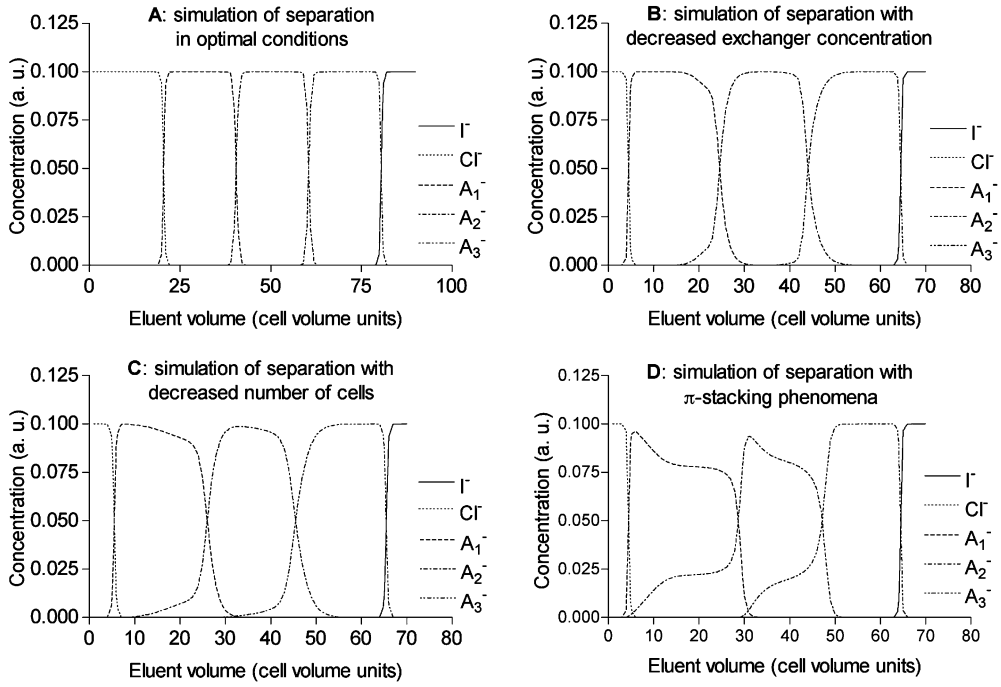

Figure 3. Simulated separation of three analytes A_1^-, A_2^-, A_3^- with Cl^- as retainer, and I^- as displacer. For all simulations, $K_{I^-/Cl^-} = 10^6$, $K_{I^-/A_1^-} = 10^4$, $K_{I^-/A_2^-} = 10^3$, $K_{I^-/A_3^-} = 10^2$, $K_{C^{2-}} = 40$, $K_{I^-/I^-} = 1$. Each analyte at initial concentration $n/V_{mob} = 2.0$ (arbitrary units, a.u.) is injected in the first five cells. The ratio $v = V_{stat}/V_{mob}$ is equal to 1, and the displacer concentration is fixed at 0.1 au. Simulation A shows an ideal separation (40 cells, retainer concentration 0.05 au) with steep evolution of chemical species content in the shock layers. If column capacity is lowered by reduction of the retainer concentration to 0.01 a.u. (B), the shock layers become wider. A similar effect is obtained by reduction of the number of cells to 15 (C). The formation of p-stacked ion pairs in the aqueous phase ($K = 40$ for all) can clearly deteriorate the quality of the separation process (D, retainer concentration 0.01 au, 40 cells).

unlike in pH-zone-refining.[7] The elution order can be predicted from the relative association constant values of analytes. Graphical representations of separations under optimal and critical conditions (low exchanger concentration, short column, π-stacking phenomenon) have been drawn with the modeling tool (Figure 3). Even though this simulation represents only a rough model of what really happens inside of the column, the general features of displacement chromatography are clearly visible; the analytes flow out as blocks at constant concentration equal to that of the displacer.

Exchanger/Displacer Screening. Several tetrasubstituted ammonium ions have been evaluated or used for metallic ion extraction and biotechnology applications.[28,29] We chose benzalkonium chloride as a strong cationic exchanger for this preliminary work mainly because it bears a chromophore, rendering it easily detectable by UV spectrometry. The chosen material was a commercial mixture containing mainly C_{12} and C_{14} side chain isomers, showing a homogeneous chromatographic behavior in HPLC. Iodide was chosen as the anionic displacer as its sodium salt. It is highly water-soluble, shows a greater affinity for quaternary ammonium than many other counterions, and gives an extremely lipophilic ion pair when bound to the ammonium. Other organic or inorganic anions, such as citrate, sulfate, oxalate, and nitrate,[30,31] are known to have better affinities for quaternary ammoniums in anion-exchange resins, but in our case, they did not show any affinity for the benzalkonium chloride dissolved in an organic phase.

Biphasic System Screening. The requirements for a biphasic system that is suitable for ion-exchange liquid−liquid chromatography are somewhat paradoxical: the exchanger should theoretically solely reside in the stationary phase, while the free anionic feed solutes should prefer the mobile phase. Chlorinated solvents (chloroform, dichloromethane) are the most efficient solvents to dissolve ammonium salts.[32] Unfortunately, chlorinated solvent biphasic systems are toxic and responsible for high backpressure, mainly due to the density difference between the two phases. Moreover, dichloromethane is not very compatible with PEEK tubing. Therefore several biphasic systems were considered. The most salient data are summarized in Table 1. Pentane, heptane, toluene, and methyl *tert*-butyl ether showed poor affinity for benzalkonium chloride, even when mixed with a high amount of a linear alcohol. Alkyl acetates showed low affinity and formed steady emulsions. Methyl isobutyl ether showed low affinity and oxidizing effect on the iodine displacer. Fluorous solvents (HFE 7100 and trifluorotoluene) showed no affinity for the quaternary ammonium. In ternary systems, the third solvent was an alkanol preferring the organic phase: 1-butanol, 1-pentanol, or 1-hexanol. The system chloroform/1-butanol/water showed a distribution constant of nearly 70 for benzalkonium chloride with satisfactory decantation and was therefore chosen.

Sample Injection Mode. Two sample injection modes were tested. First, ionized acids in their benzalkonium salt form were

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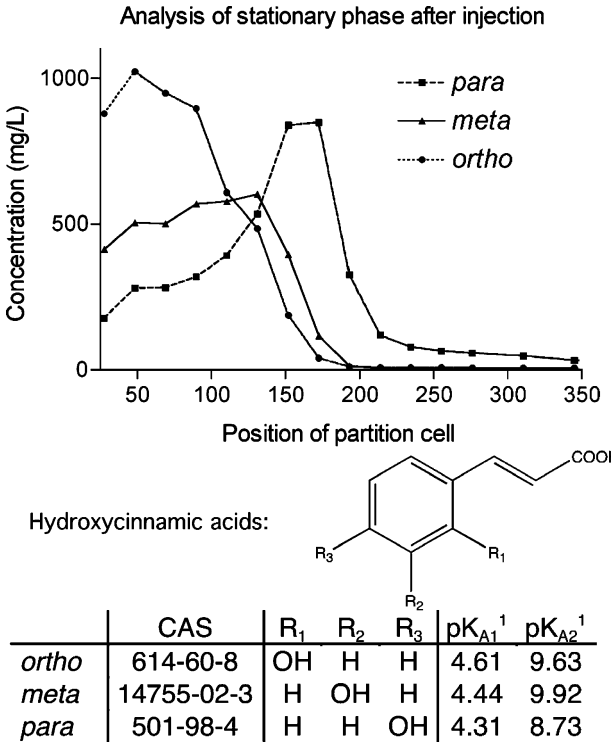
Table 1. K_D Measurements for Benzalkonium Chloride in Different Biphasic Systems

solvent system (v/v/v)	$K_{D(org/aq)}$[a]	solvent system (v/v/v)	$K_{D(org/aq)}$
1-butanol/water 50/50	12	toluene/water 50/50	0.01
1-pentanol/water 50/50	44	ethyl acetate/water 50/50	0.25
pentane/water 50/50	0.01	propyl acetate/water 50/50	0.1
heptane/water 50/50	0.01	butyl acetate/water 50/50	0.1
MiBK/water	0.4	chloroform/water 50/50	27
MtBE/water 50/50	0.02	chloroform/1-butanol/water 45/10/45	67
HFE 7100/water 50/50	0.01	chloroform/pentanol/water 45/10/45	68
HFE 7100/methanol 50/50	0.02		

[a] $K_{D(org/aq)}$, distribution constant of benzalkonium chloride at concentration of 0.01 M.

manually formed in a separatory funnel before injection. This led to a high concentration of benzalkonium in the sample and, therefore, to a long experiment time. The second method consisted of directly injecting the ionized acids in their sodium salt form in the column and allowing them to be extracted in the stationary phase according to their affinity for benzalkonium. This injection mode is faster, easier, and more tunable and gave better separations. The three isomers of hydroxycinnamic acid are extracted according to their association constant with benzalkonium. Analysis of the first cell contents after sample extraction shows a prefractionation of the sample (Figure 4).

Chromatographic Parameters. *(A) Rotation Speed and Flow Rate.* In solid-support displacement chromatography, the flow rate affects the shock-layer thickness: if the flow rate is large compared to the axial diffusion coefficient, increasing the flow rate increases the shock layer thickness.[33] In liquid−liquid chromatography and especially CPC, rotation speed and flow rate are of great importance. The higher the flow rate and rotation speed, the better the mobile phase dispersion. Studies on the relationship between flow pattern and separation efficiency have established that on model separations the number of theoretical plates increases with the mobile phase dispersion.[34−36] As a rule, the highest rotation speed is recommended, the main limitating factor being the pressure drop, which in first approximation, quadratically increases with rotation speed.[37] Regarding flow rate, experimental separations and theoretical considerations suggest some limitations to this relationship, mainly related to the settling of the emulsion in the partition cell. First, depending on the biphasic system, the dispersion may be so important that droplet coalescence does not properly occur in the partition cells, leading to bleeding of the stationary phase out of the column. The retention of the stationary phase, expressed as the stationary phase volume remaining in the column Sf, decreases with increased flow rate.[36] On the other hand, one must consider that mass transfer kinetics is not instantaneous. This is due in part to the intrinsic mass transfer parameters of the solutes, particularly in the case of ion-pair extraction or exchange.[38−40] A flow rate increase can degrade efficiency.[41] These limitations have been observed in CCC and CPC in the elution mode and also in the present work: with highly concentrated samples (3 × 1 g), it was necessary to work with a reduced flow rate, that is, 2 mL/min, whereas 5 mL/min was used



	CAS	R_1	R_2	R_3	pK_{A1}[1]	pK_{A2}[1]
ortho	614-60-8	OH	H	H	4.61	9.63
meta	14755-02-3	H	OH	H	4.44	9.92
para	501-98-4	H	H	OH	4.31	8.73

Figure 4. Stationary-phase content after injection of 25 mg of each hydroxycinnamic acid (sodium salt form) in 5 mL aqueous phase. Experimental conditions: apparatus, FCPC equipped with a 200 mL column; solvent system, chloroform/*n*-butanol/water (45:10:45); rotation speed, 1100 rpm, $P = 75$ bar; stationary phase, benzalkonium chloride 15 mM in organic phase; mobile phase, conjugate aqueous phase without displacer; mobile phase, for injection, aqueous phase in ascending mode, 1 mL/min; for dual-mode, organic phase in descending mode, 1 mL/min; HPLC detection and quantification.[1] Values from Beilstein and CAS databases.

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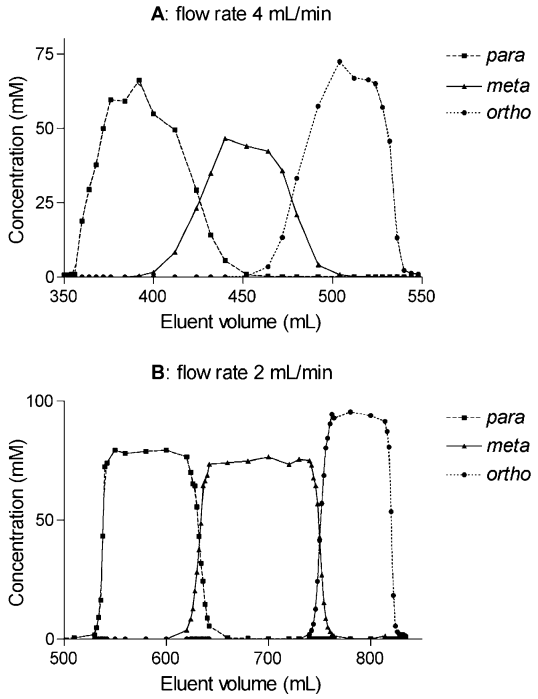

Figure 5. Separation of three hydroxycinnamic acids by ion-exchange displacement CPC (A) at 4 mL/min and (B) at 2 mL/min. Experimental conditions: sample, 1 g each of *ortho*-, *meta*-, and *para*-hydroxycinnamic acids in basic form. Injection volume: (A) 6 mL of mobile phase, pH = 8.26; (B) 20 mL of mobile phase, pH = 7.63. Exchanger/displacer concentration: (A) benzalkonium chloride in stationary phase, 672 mM; sodium iodide concentration in mobile phase, 60 mM; pH 6.66; (B) benzalkonium chloride in stationary phase, 610 mM; sodium iodide concentration in mobile phase, 60 mM; pH 7.07. Rotation speed: (A) 1500 rpm, $P = 70$ bar; (B) 1400 rpm, $P = 68$ bar. Detection and quantification by HPLC.

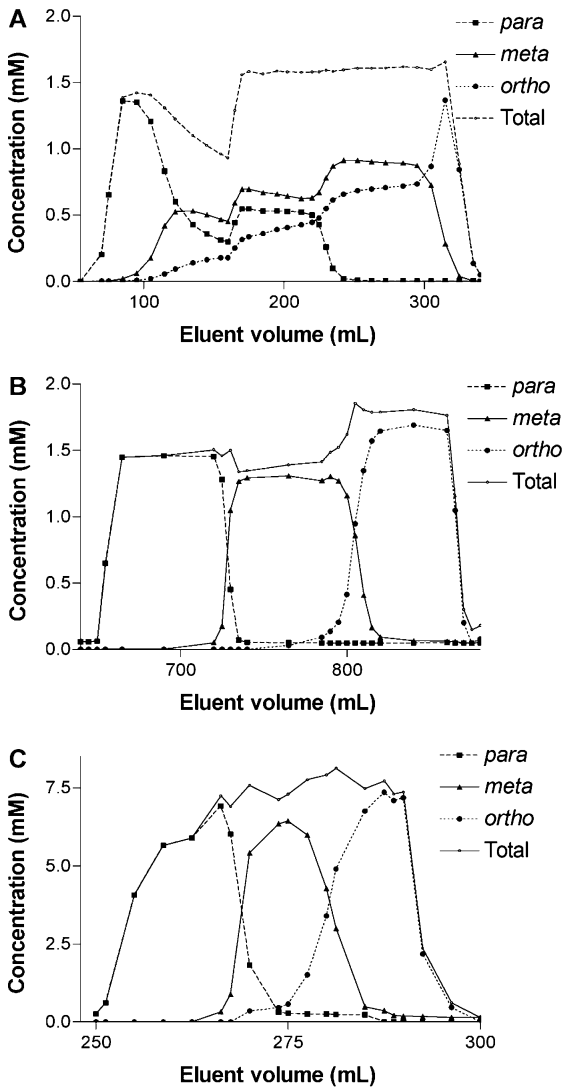

Figure 6. Separation of three hydroxycinnamic acids by ion-exchange displacement CPC. Experimental conditions: sample, 25 mg each of *ortho*-, *meta*-, and *para*-hydroxycinnamic acids in basic form; injection volume, 5 mL of aqueous mobile phase and some drops of organic phase, pH = 7; rotation speed, 1100 rpm; flow-rate, 5 mL/min; $V_m = 50$ mL; $P = 78$ bar. Exchanger/displacer concentration: (A) benzalkonium chloride in stationary phase, 3 mM; sodium iodide concentration in mobile phase, 1.5 mM; (B) benzalkonium chloride in stationary phase, 12.3 mM; sodium iodide concentration in mobile phase, 1.5 mM; (C) benzalkonium chloride in stationary phase, 15 mM; sodium iodide concentration in mobile phase, 7.5 mM. Ion-exchanger/analytes ratio: (A) 1, (B) 4, and (C) 5. Ion-exchanger/displacer ratio: (A) 2, (B) 8.2, (C) 2. Detection and quantification by HPLC.

for smaller samples (3 × 100 mg) (Figure 5). The greater difference between the eluent volume at 4 and 2 mL/min is due to the much higher stationary phase retention at 2 mL/min. The bleeding of stationary phase at 4 mL/min decreases the global column capacity.

(B) Exchanger and Displacer Concentrations. Both exchanger and displacer concentrations can be tuned in CPC, rendering experimental optimization more flexible. The exchanger concentration directly determines the capacity of the system and can also be related to particle size[41] or column length in solid-support ion-exchange chromatography.[42] The exchanger concentration must be high enough to extract the sample in the organic phase in the first partition cells and to further enable just enough chromatographic development so that the isotachic train forms and the analyte block shape sharpens.[33] Increasing the exchanger concentration allows the injection of larger amounts of sample. The upper limitations for the exchanger concentration are, on one hand, the retention time, which increases with the exchanger concentration, and on the other hand, the high viscosity of a saturated phase. One can express the relative amount of exchanger and sample by their molar ratio. The amount of sample is known, and the amount of exchanger must be calculated by considering the stationary phase retention at the equilibrium, V_s.

The characteristic results of inadequate and optimum conditions concerning exchanger concentration are showed in Figure 6A and B. The effect of displacer concentration modulation, that is, the iodide ions, is well-known in displacement chromatography. It determines outlet solute concentration and, consequently, the global separation duration and also the shock layer thickness.[33] A low displacer concentration increases the yields of separated products, but in large elution volumes. Increasing the displacer concentration reduces the purification yields, but an excessive displacer concentration causes non-Gaussian peaklike profiles

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showing large overlapping zones (Figure 6C). In the case of three hydroxycinnamic acids, the optimum ratios were found to be

$$\frac{\text{ion} - \text{exchanger}}{\text{analytes}} = 4$$

and

$$\frac{[\text{ion} - \text{exchanger}]}{[\text{displacer}]} = 10$$

CONCLUSION

Benzalkonium chloride can be used as a strong anion-exchanger in displacement-mode CPC and has proven to be highly efficient when applied to the separation of phenolic acid analogues. The true displacement process is confirmed by the characteristic trapezoidal profile of analyte concentrations. Unlike pH-zone refining, another displacement mode used in CCC and CPC, this type of chromatography does not involve any change of solute ionization state and can be applied to the purification of ionized molecules. As already demonstrated with the weak anion exchanger Amberlite LA2, this liquid−liquid version of displacement ion-exchange chromatography appears to be well-suited to preparative applications, since the liquid nature of the stationary phase both enhances the preparative aspect of the method and allows the fine-tuning of column specifications.[14] The modeling of the process has been made with basic equations involved in liquid−liquid partitioning as a starting point, thus allowing for reliable simulations. A large variety of ion-exchangers should fit with this elution mode, providing they fulfill the strict partitioning requirements. The results that are presented here open new perspectives for preparative separations of bioactive natural products using counter current chromatography.

ACKNOWLEDGMENT

We thank Dr. Karen Plé for linguistic improvement.

Received for review March 31, 2004. Accepted July 6, 2004.

AC049499W