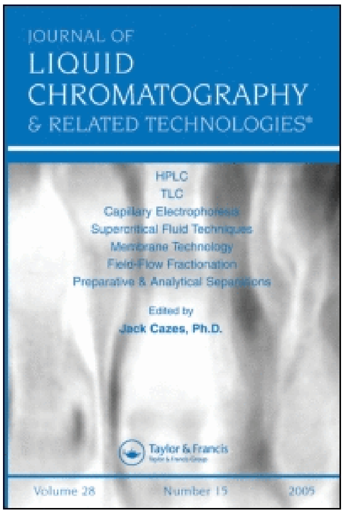

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R. Pennanec[a]; C. Viron[b]; S. Blanchard[a]; M. Lafosse[a]
[a] Institut de Chimie Organique et Analytique, Orléans, Cedex 2, France [b] Laboratoires Christian Dior, Saint Jean de Braye Cedex, France

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FUNDAMENTAL INVESTIGATIONS AND TRENDS IN CCC

ORIGINAL USES OF THE pH-ZONE REFINING PRINCIPLE: ADAPTATION TO SYNTHESIS IMPERATIVES AND TO IONIC COMPOUNDS

R. Pennanec,[1,*] C. Viron,[2] S. Blanchard,[1] and M. Lafosse[1]

[1] Institut de Chimie Organique et Analytique, CNRS
UPRES A 6005, UFR Sciences, BP 6759, F-45067 Orléans,
Cedex 2, France
[2] Laboratoire Actifs, Biologie, & Cosmétique, Laboratoires
Christian Dior, F-45804 Saint Jean de Braye Cedex, France

ABSTRACT

Countercurrent chromatography is a powerful separation technique based on the partition of solutes between two immiscible liquid phases. The first one, maintained in the column by a centrifugal force, acts as the stationary phase; the second phase, circulating through the apparatus, elutes compounds according to their partition coefficients. The total liquid nature of this process allows the addition of auxiliary components in both phases, increasing the separation power of countercurrent chromatography. In 1991, Y. Ito introduced pH-Zone refining systems in which the stationary phase contains a retainer and the mobile one an eluter.

The acid-base system that acts as the retainer-eluter pair interacts with solutes and enables a separation based on acidic constant

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*Corresponding author.

(pKa) base. First, this method was successfully used to separate and purify two poly-aza heterocyclic isomers, obtained by hetarynic dimerization and which are precursors of antitumor agents.

Because of several imperatives, a new system, adapted from the classical one (eluter-retainer), was developed to respect our limitations. Secondly, ionic solutes such as sulfonic acid compounds cannot be studied by such a system, based on the influence of the acidic constant. Therefore, the retainer is replaced by a cationic liquid resin with very strong hydrophobic properties. Added to the organic stationary phase, this resin gives the stationary phase an ion-exchange behavior, guided by the eluter contained in the mobile phase. Applied to ionic solutes, the study of this new system highlights the main parameters of the separation.

INTRODUCTION

Countercurrent chromatography (CCC)[1,2,3,8] is an analytical, semi-preparative, and preparative technique which is based on the partition of compounds between two immiscible liquid phases. One of these phases is maintained stationary by centrifugal forces, the second one, pumped through the stationary phase, is the mobile phase.

Since no solid support is involved in this liquid process, there is no irreversible adsorption. Moreover, the stationary phase ratio (60 to 80% of the total internal volume of the column) is much higher than in HPLC (typically 5%); therefore, CCC is a good preparative method. For compounds which are ionizable in medium pH conditions, pH-Zone refining is one of the most important advances[4,5] in countercurrent chromatography. It is based on equilibrium between the protonated and non-protonated forms of compounds (see Figure 1). In the case of nitrogenous compounds, the protonated form is an ionic form, present mainly in the aqueous phase; in contrast, the neutral non-protonated one is partitioned in the organic phase. In a practical approach, a base (for example triethylamin) added in the organic stationary phase acts as "retainer" and increases the retention of nitrogenous compounds.

Moreover, an acid (for example hydrochloric acid) in the aqueous mobile phase as "eluter" increases their elution. The first aim of this paper was to investigate this method to separate and purify two poly-aza heterocyclic isomers obtained by hetarynic dimerization,[6] and which are precursors of antitumor agents. Since the method by pH-Zone Refining is based on the pKa of solutes, it is not suitable for ionic compounds, or compounds with extreme pKa; therefore, ion-pair displacement has been developed.[7,8] A highly hydrophobic anion-exchange resin (Amberlite LA2) is added to the organic stationary phase in a

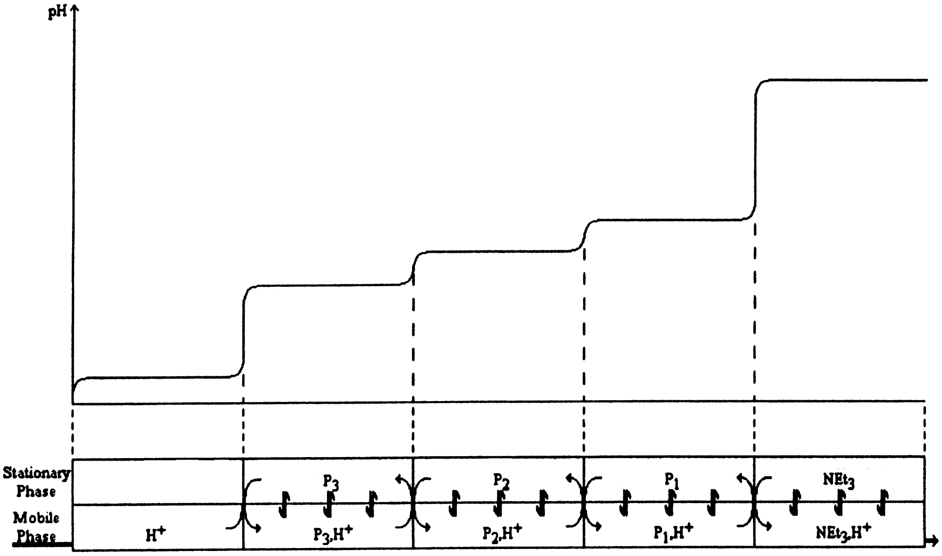

Figure 1. pH-Zone refining principle illustration for nitrogenous compounds (P_i = solutes, NEt_3 = retainer, H^+ = eluter).

two-phase solvent system. The nitrogenous resin has to be protonated (cationic form) and stays in the organic phase. On the contrary, the contact with a basic aqueous phase neutralizes the resin.

As shown in Figure 2, the principle of ion-pair displacement CCC is very similar to the pH-Zone refining system: the activated resin partitions anions in the organic stationary phase, and acts as a retainer. In a final step, a basic aqueous solution de-activates the resin, playing the role of eluter. Consequently, this displacement process leads to a step-elution of compounds according to their affinity for the resin and their hydrophobicity. In the second part of this paper, the two-phase system (water/methyl *ter*-butyl ether) used in pH Refining CCC is studied to separate sulfonic acidic compounds by adding Amberlite LA2 and sodium hydroxide as retainer and eluter, respectively.

EXPERIMENTAL

Reagents

Methyl t-butyl Ether (MtBE) is analytical grade, purchased from J. T. Baker (Deventer, Holland). Deionized water was produced by an Elga UHQ unit.

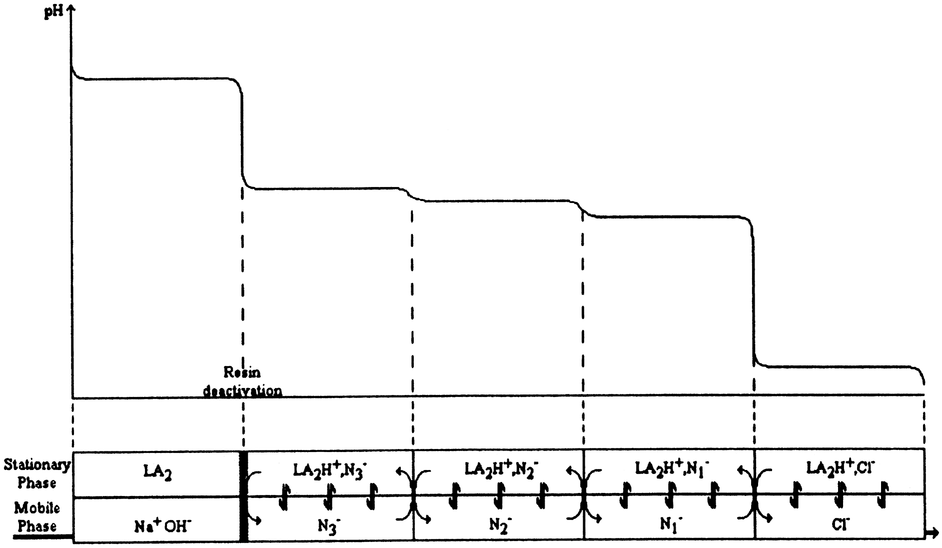

Figure 2. Ion-pair displacement countercurrent chromatography principle (Ni = solutes, LA2 = Amberlite resin, NaOH = eluter).

Amberlite LA2 was purchased from Fluka (Buchs, Switzerland) as a 2.2-2.3 meq/mL solution. Other reagents (Sodium hydroxide, hydrochloric acid, etc.) were purchased from Sigma-Aldrich (St Louis, MO, USA).

CPC Apparatus

Analyses were mainly performed on a hydrostatic mode apparatus: the Sanki CPC model LLB-M (Sanki Engineering, Kyoto, Japan) with 230 mL capacity. A TSP 100 isocratic pump (Les Ulis, France) was chosen to deliver liquid phases at a flow-rate of 4 mL/min.

A triple monitoring system was performed: an isopropanol make-up (1mL/mn) was made at the outlet column. The effluent was split: the low ratio (1/10) was directed to evaporative light scattering detection (ELSD) model Sedex 45 (Sedere, Alfortville, France), and the high ratio (9/10) to a Kratos Spectroflow UV detector model 783 (9/10), then an online pH-detection (Beckman ϕ10 pHmeter) set before a fraction collector (ISCO, Retriever II). ELSD is a universal detector which enables better monitoring than UV detection of the CPC effluent for all the species (solutes or additives) without chromophores,[9] and enables one to use highly UV-absorbing solvents.

HPLC Apparatus

CPC-collected fractions were analysed by HPLC using a Merck D-7000 pump (Merck, Darmstadt, Germany) at a flow rate of 1 mL/min. Detection was performed by a Varian UV-detector (214nm).

Fractions from a pH-Zone refining system were analysed on an Astec NH_{2p} column (250 x 4.6 mm i.d.) to determine the quantity of each poly-aza heterocyclic compound. (Figure 3). The eluent was a heptane / dichloromethane (90:10) mixture in isocratic elution. Fractions from the ion-pair development system were analyzed on a Hypersil SAX column (250 x 4.6 mm i.d.) with isocratic elution water / methanol (50:50), 50 mM Na_2SO_4.

pH-Zone Refining CPC General Procedure

A CPC column is first filled (ascending mode, 200 rpm) with stationary phase containing the retainer. The rotational speed is increased to 800 rpm and the elution mode is chosen (ascending or descending, depending on the nature of mobile and stationary phases), the sample is injected, and pushed inside the apparatus by the mobile phase (4 mL/min.) containing the eluter.

The rotational speed and mobile phase flow were chosen to generate a pressure below the CPC rotating seal limit pressure (60 bar). The sample, a mixture of each isomer from the synthesis, is dissolved in a 1:1 stationary-mobile phase mixture.

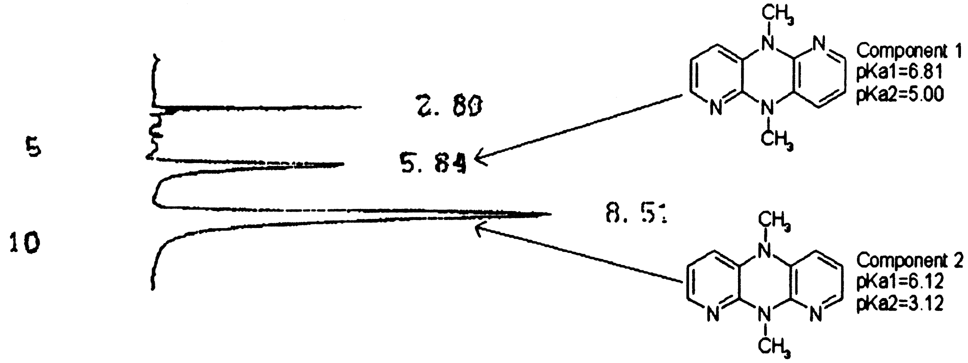

Figure 3. HPLC separation of the two nitrogenous compounds. Conditions: Astec NH_{2p} column 250 x 4.6 i.d., flow rate = 1mL/min., mobile phase : 90 % heptane / 10 % CH_2Cl_2.

Ion-Pair Displacement CCC General Procedure

The liquid resin (Amberlite LA2) is dissolved in the methyl *ter*-butyl ether (MtBE). An aqueous hydrochloric solution is then added in very large excess, in order to activate the resin. The upper organic layer constitutes the stationary phase. The mobile phase is a sodium hydroxide aqueous solution.

As in the pH-zone refining system, the two-phase sample mixture is injected inside the column filled with the stationary phase, pushed by the mobile phase. Filling procedures are identical.

RESULTS

pH-Zone Refining Separation of Poly-Aza Heterocyclic Compounds

The aim is to obtain a large amount of each isomer from a crude mixture produced by the hetarynic coupling reaction. As each isomer has to be used thereafter in further syntheses, the purity degree has to be high and contamination must be avoided by the purification method. Some papers[10] have shown that a pH-zone refining system could be a good method to resolve isomer mixtures having different acidic constants. A computational estimation of pKa of the poly-aza heterocyclic isomers confirms the feasibility of the pH-zone refining separation.

Firstly, the organic layer was chosen as stationary phase. In order to increase the retention of components 1 and 2, 20 mM of triethylamine (as retainer) was added to the organic phase. To increase the elution, 5 mM of hydrochloric acid (as eluter) was added to the aqueous mobile phase. The large ratio of retainer/eluter quantities explains the strong retention of components shown in Figure 4a. The compounds were eluted in about 200 minutes, in a single square peak characteristic of this method. HPLC analysis of each fraction (1 fraction per minute) permits evaluation of the purification, as illustrated in Figure 4b.

In accordance with the pH-Zone refining principle, the component having the highest pKa (component 1, Figure 3) was eluted first. Unfortunately, overlap was too large for a suitable isolation. Moreover, compounds were eluted in the aqueous phase and in presence of hydrochloric acid; therefore, the system was inverted, i.e. the mobile phase is the organic one with triethylamine as eluter. But in the following synthesis using the compound purified by CCC, the presence of triethylamin is unsuitable; we decided, therefore, to suppress this eluter. In spite of these changes, a square peak was observed as shown in Figure 5a, and the elution time was shorter than in the previous process. The analysis of collected fractions is made by HPLC (Figure 5b): the weak overlap allows preparative separation with a good recovery to be envisaged.

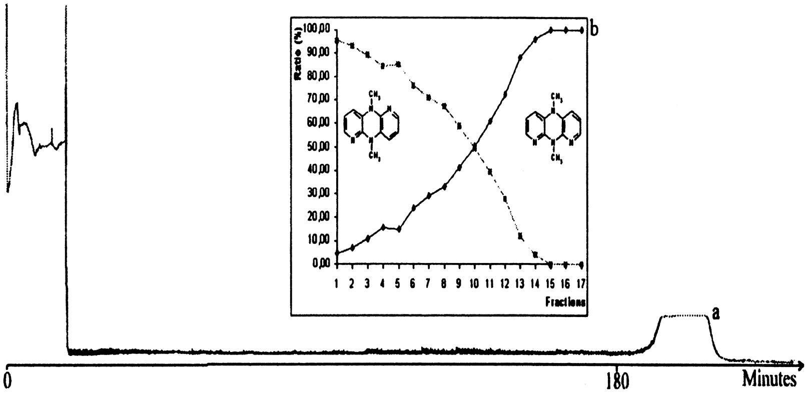

Figure 4. a) pH-Zone refining CPC of the reactional mixture. Conditions: mobile phase: water + 5mM HCl, stationary phase: MtBE + 20 mM Et_3N. Flow = 4 mL/min. Rotational speed: 700 rpm. ELSD detection. b) Representation of HPLC analysis of the CPC square peak.

Ion-Pair Displacement CPC of Sulfonic Compounds

As the aim was to purify various sulfonic compounds by CPC as an alternative to other expensive preparative methods, we have preferred to test the method, introduced in 1998 by Chevolot et al,[7] by studying this resin in a CPC system with two standards: 2-Naphthalen Sulfonic Acid and 1,5-Naphthalen di-Sulfonic Acid (Figure 6). The same two-phase system (water-MtBE) was kept because it provides high and reproducible stationary phase retention.

As Chevolot used methyl isobutyl ketone[7] instead of MtBE, several assays of the behaviour of this resin in the water-MtBE system had to be done. Firstly, it was necessary to verify the activity as retainer of the activated resin for the two standards, by estimating the partition of sulfonic acids as a function of protonation of the resin. Therefore, relatively rapid assays were performed: in a test tube. Sulfonic acids were dissolved in the aqueous phase and this phase was added and mixed to MtBE containing either the protonated resin or the non-protonated one, followed by HPLC measurement of the sulfonic acid quantity in aqueous layer. When the resin was non-protonated, the peak height intensity was unchanged showing that sulfonic acids stay completely in the aqueous phase, as shown in Figure 6. On the other hand, when the resin was protonated, the sulfonic acids were completely transferred into the organic layer. As shown, the protonation of the resin dramatically changes the partition direction and strongly increases the partition coefficient of these ionic compounds in the organic phase.

The second point of verification was the reproducibility of the system. The test consisted in three identical operations. Three different two-phase systems

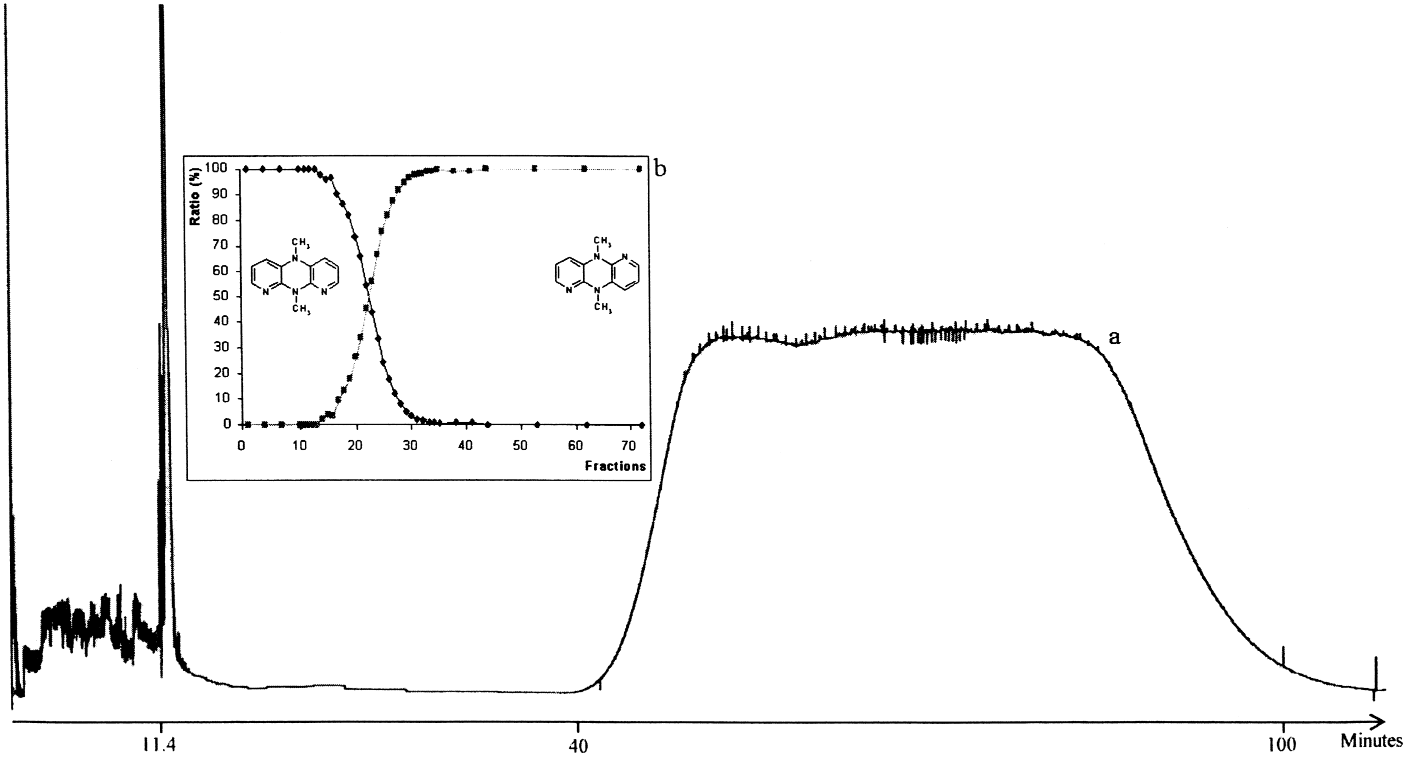

Figure 5. a) pH-Zone refining CPC of the reactional mixture. Conditions: Mobile phase: MtBE, stationary phase: water + 0.1 % HCl. Flow = 4 mL/min. Rotational speed: 700 rpm. ELSD detection. b) Representation of HPLC analysis of the CPC square peak.

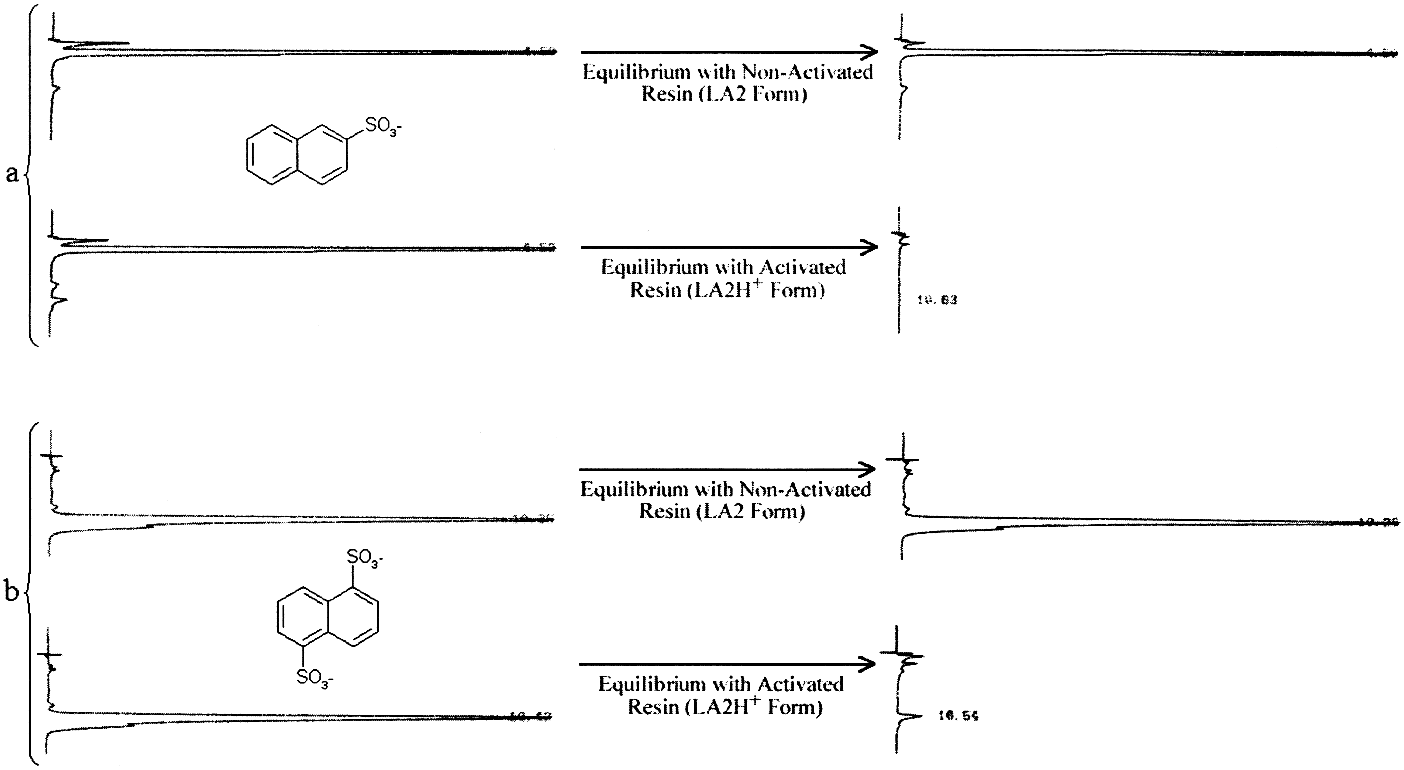

Figure 6. Preliminary HPLC studies on the activity of the resin for: a) 2 naphtalen sulfonic, b) 1,5 naphtalen di-sulfonic acids. Conditions: Hypersil SAX 250 x 4.6 i.d. column, flow rate = 1mL/min., mobile phase: 50% Methanol / 50% water + 50mM Na_2SO_4.

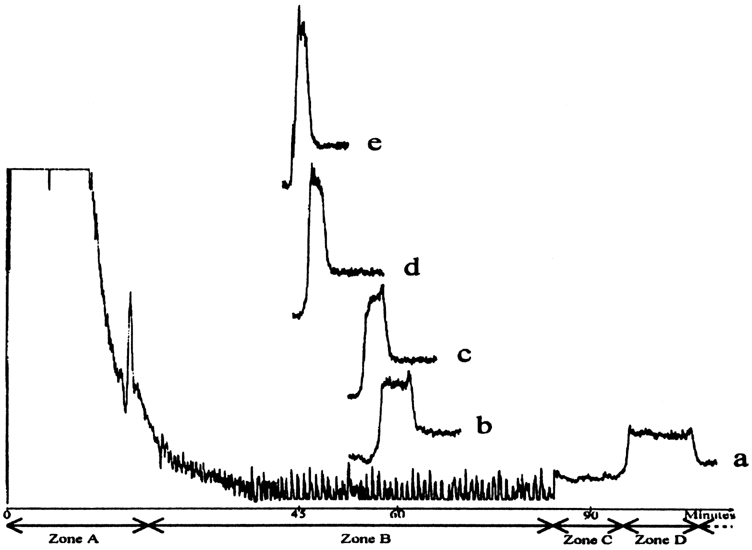

Figure 7. Separation evolution function of sodium hydroxide concentration: CPC analysis: 700 rpm, flow = 4 mL/min. DEDL detection. Stationary phase: MtBE with 2% activated Amberlite LA2. Mobile phase: water with a) 10mM NaOH, b) 20mM NaOH, c) 30mM NaOH, d) 40mM NaOH, e) 50mM NaOH.

were prepared. A quantity of LA2 resin was dissolved in a binary mixture of water-MtBE (1:1), and hydrochloric acid was added to protonate the resin. This system was reproduced three times in a similar way. The same acid quantity was used to protonate LA2 resin.

The same quantity (100 mg in 4 mL) of 2-Naphthalen Sulfonic acid was injected in each two-phase system. Stationary phase retention was the same (70%) for the three experiments. Consequently, the retention time of sulfonic acid was approximately similar (48.5 min +-3%), showing a good reproducibility of the experiment in this system.

The last study consisted in evaluating the influence of the quantity of the NaOH (eluter) on the elution of sulfonic acid (retention time, peak width, and height). Five solutions of 10, 20, 30, 40, and 50 mmol/L of NaOH were prepared for various aqueous mobile phases. Organic phase was the same and consisted of 2% protonated Amberlite LA2 in MtBE and the sample was 100 mg of 2-Naphtalen sulfonic acid in 4 mL solution of water/MtBE (1:3) mixture. With a 700 rpm rotational speed and a 4 mL/min flow-rate, stationary phase retention was 70% for the five experiments, confirming good reproducibility due to the binary mixture, water/MtBE. Figure 7a shows the profile of the elution for 10 mM of NaOH. The profile can be described in 4 parts, named A, B,C, and D. A high signal (zone A) is due to the exit of Amberlite when the stationary phase is flushed by the mobile phase. The pH of the effluent is approximately 2. The small peak after this elution corresponds to the end of the formation of the void volume. Then, zone B at pH 3-4 corresponds to the exit of the aqueous mobile phase with a baseline noise, created by a bleeding of stationary phase in droplets containing Amberlite detected by ELSD. Zone C at pH 4 corresponds to a lowering of this bleeding. This cannot yet be explained but it constitutes a good indicator of the imminent exit of the solutes. The exit of solute is in zone D at pH 6-7, in a square peak before the exit of NaOH at pH 11-12.

Figure 7 shows the different zone Ds as a function of NaOH concentration. The retention of sulfonic acid decreases rapidly as the NaOH increases; the width decreases while the height increases, Figure 8.

Separation of the Sulfonic Acid Mixture

The influence of parameters being known, we have chosen the system 2% of resin with 25 mM of NaOH to separate the mixture of mono and di-anions. The sample injected was composed of 100 mg of each sulfonic acid. Figure 9a shows the presence of two square peaks weakly separated with the same zones as those observed in Figure 7. Each fraction was analysed by HPLC and Figure 9b shows that each sulfonic acid can be obtained pure with an overlap on 4 fractions. The role of partition coefficient clearly appears here; the di-anion, supposed to interact strongly and solely with the resin, is eluted before the mono-anion, showing that ion-pair mechanism is not the only parameter regulating the component retention.

A smaller quantity of the NaOH eluter (Figure 10) increases the retention and the width of square peaks, as previously, with an overlap similar to Figure 9, whereas, a higher quantity of LA2 resin retainer (5%) with a 25 mM of NaOH, strongly increases the retention time (Figure 11) with square peaks in a high baseline noise caused by a significant bleeding of the stationary phase. Consequently, a suitable proportion between retainer and eluter must be reached in order to optimise the stability and efficiency of the system.

The experimental conditions of Figure 9 (2% of Amberlite with 25 mM of NaOH) were chosen and tested with a larger sample size (300 mg of each sulfonic acid) for a preparative scale separation. A good separation was obtained, as shown in Figure 12. Each fraction was analysed and quantified by HPLC, and separation yield estimated in Table 1 gives a good recovery with a weak overlap.

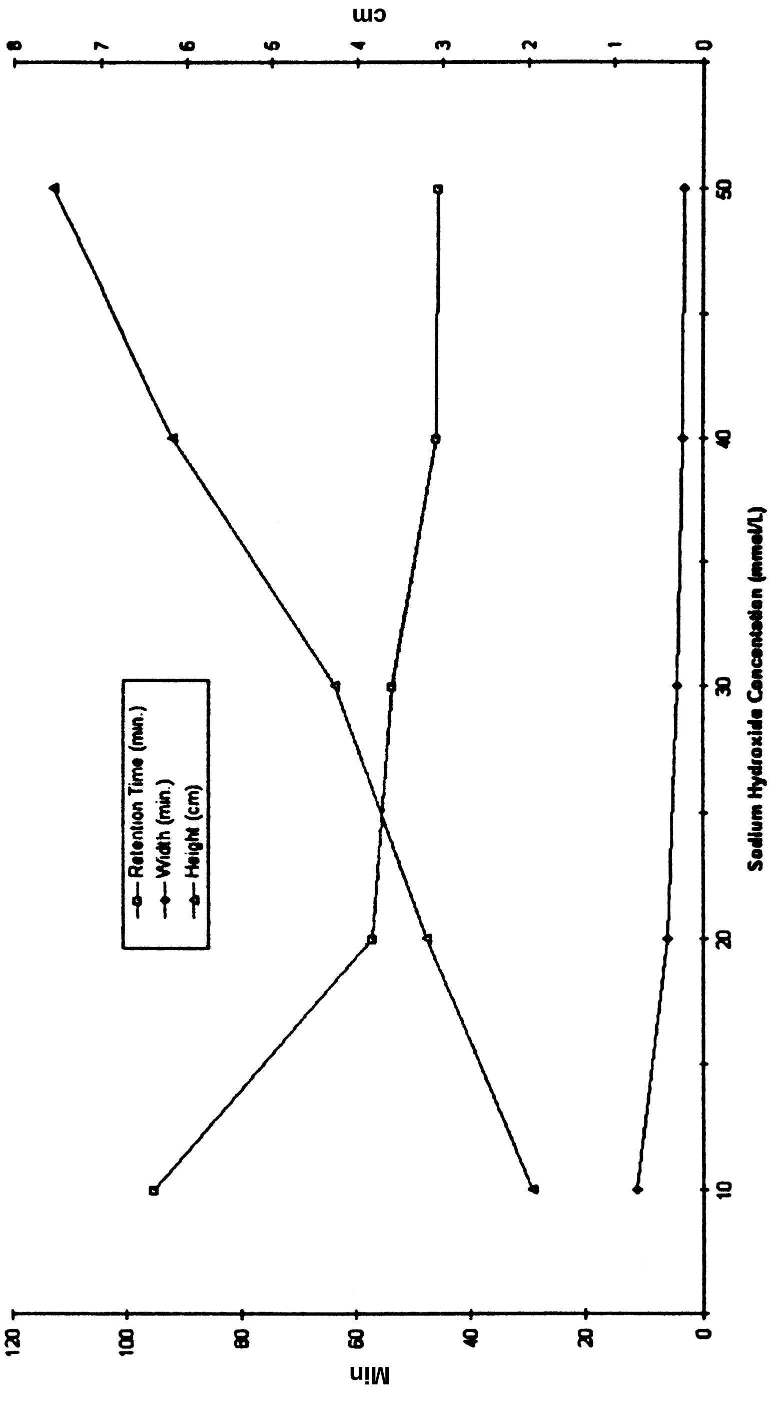

Figure 8. Evolution of retention time, width and height of the square peak with sodium hydroxide concentration.

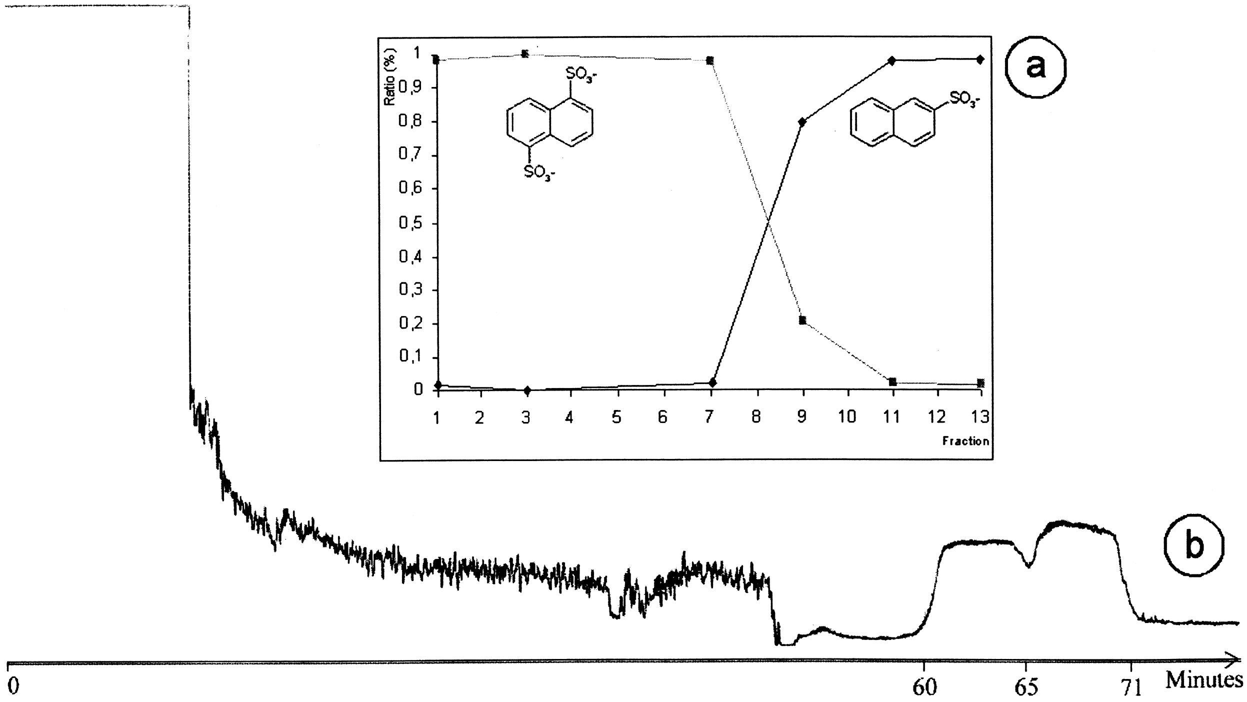

Figure 9. a) CPC of sulfonic acid mixture: flow = 4mL/min., 800 rpm, mobile phase: water + 25 mM NaOH, stationary phase: MtBE + 2% LA2. b) HPLC studies on collected fraction (1 fraction per minute).

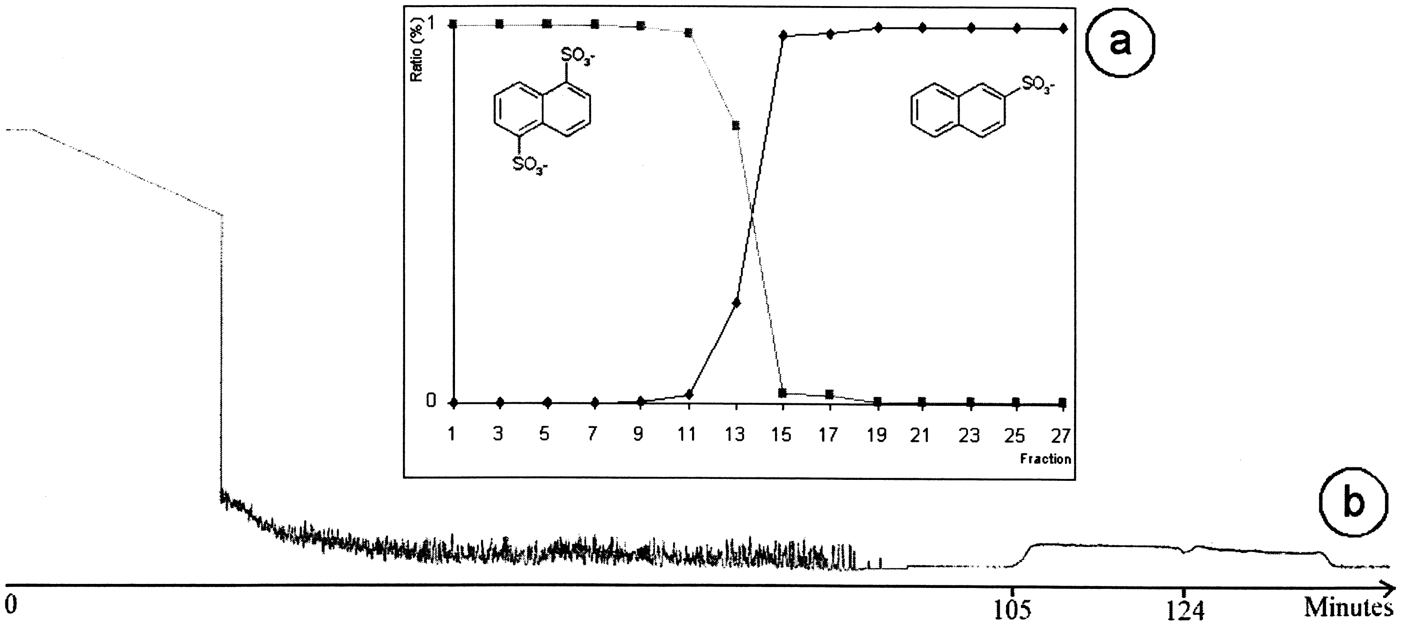

Figure 10. a) CPC of sulfonic acid mixture: flow = 4mL/min., 800 rpm, mobile phase: water + 10 mM NaOH, stationary phase: MtBE + 2% LA2. b) HPLC studies on collected fraction (1 fraction per minute).

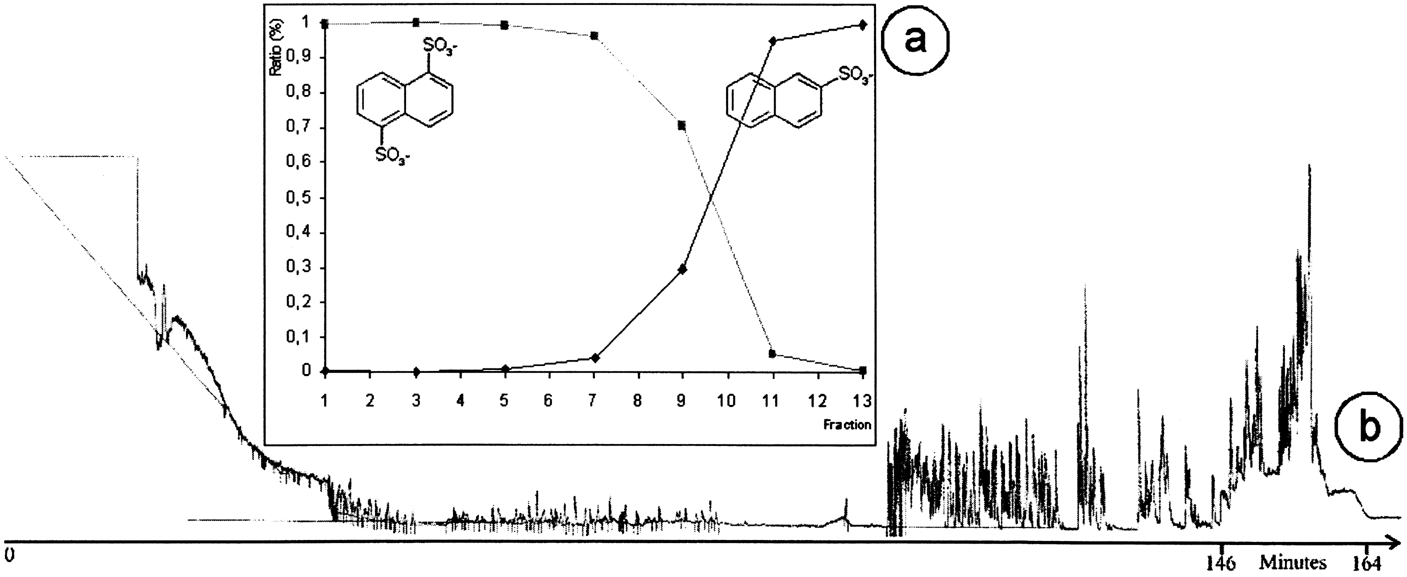

Figure 11. a) CPC of sulfonic acid mixture: flow = 4mL/min., 800 rpm, mobile phase: water + 25 mM NaOH, stationary phase: MtBE + 5% LA2. b) HPLC studies on collected fraction (1 fraction per minute).

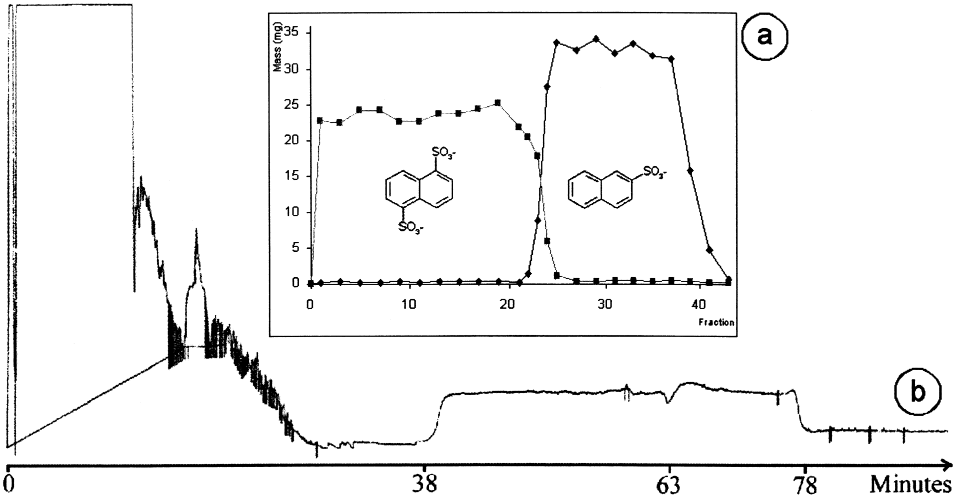

Figure 12. a) CPC preparative separation of sulfonic acid mixture: flow = 4mL/min., 800 rpm, mobile phase: water + 25 mM NaOH, stationary phase: MtBE + 2% LA2. b) HPLC studies on collected fraction: Quantification of each compound in each collected fraction (1 fraction per minute).

Table 1. Purity and Yield Estimation of Large Scale Naphtalen Sulfonic Acids Mixture

Collected Fractions	2-Naphtalen Sulfonic Acid Quantity	1,5-Naphtalen Sulfonic Acid Quantity	Purity of Main Compound	Separation Yield
01→21	2.6 mg	266.9 mg	99.0 %	89.0 %
27→41	209.0 mg	2.9 mg	98.6 %	69.6 %

CONCLUSION

CPC applied to ionizable compounds by the pH-Zone refining system and to ionic compounds by ion-pair displacement appears to be a very versatile technique. The binary mixture water-MtBE appears to be a basic two-phase system for both techniques by choosing the best retainer and eluter. We have demonstrated the interest for purifying synthetic organic chemicals, and principally, for separating isomers. The ion-pair displacement study has shown that the mechanism is not solely by ionic interactions, and that for our future work on various sulfonic acids, we will have to take into account the partition phenomenon.

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