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Enantiomer Separation by Countercurrent Chromatography Using Cinchona Alkaloid Derivatives as Chiral Selectors

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Cinchona-derived anion-exchange-type chiral selectors have been adapted and employed in countercurrent chromatography (CCC) for the separation of enantiomers of N-derivatized amino acids and 2-aryloxypropionic acids. The accurate optimization of the enantioseparation in terms of solvent system composition, pH values, ionic strength, and CCC operating conditions was performed. A wide range of solvent mixtures was evaluated. Successful resolutions were achieved in systems such as ammonium acetate buffer/*tert*-amyl alcohol/methanol/heptane and especially ammonium acetate buffer/methyl isobutyl ketone or diisopropyl ether. Up to 300 mg (0.92 mmol) of *N*-(3,5-dinitrobenzoyl)-(±)-leucine was totally resolved in a single run using a 10 mM concentration of chiral selector in 122 mL of stationary phase. This amount could be increased up to 900 mg (2.77 mmol) when pH-zone-refining mode was applied. The results here presented account for the high potential of CCC as a preparative enantiomer separation technique.

The search for novel either broadly or dedicatedly applicable chiral selectors (CSs) and the development of separation techniques for the resolution of enantiomers is an open field still attracting the creativity and efforts of researchers all over the world.[1] The large number of publications and books about the subject is a consequence of the well-known interest in chirality and its scientific and economic impact on pharmaceutical and biological sciences. Chiral selectors were primarily found in nature, although with time, sophisticated modifications and fully synthetic compounds have demonstrated to be very efficient in the recognition of enantiomers.

Among the natural building blocks, oligo- and polysaccharides, proteins, peptides, and amino acids, alkaloids, and antibiotics are the most commonly used scaffolds for chiral selectors.[2,3] In this context, the applications of alkaloids as CSs have also been extensively reported in the literature.[4−6] Concretely, Lindner and co-workers described the ability of cinchona derivatives, especially quinine (QN) and quinidine (QD), as efficient CSs in anion-exchange mode.[7] In a first approach, this type of selectors was tested in HPLC,[8,9] although these selectors high potential to form very stable complexes with chiral acidic compounds has allowed their efficient use as CSs also in capillary electrophoresis (CE),[10,11] capillary electrochromatography (CEC),[12] supercritical fluid chromatography (SFC), and extraction experiments,[13,14] among others.

Exhaustive investigations have been performed in order to understand the influence of every substituent and functionality to enhance the chiral recognition abilities of this type of selectors[8,15−17] (See general structure in Figure 1.). On one hand, the possibility of protonating the quinuclidine nitrogen of their molecule makes them especially suitable for the resolution of acidic compounds, such as amino acid derivatives and other chiral acidic substances due to ion pair formation. On the other hand, the derivatization of the hydroxy group to a carbamate function in the O^9-position, especially bearing bulky substituents, such as *tert*-butyl or adamantyl, was leading to the highest enantioselectivity values. Selectivity factors of ∼30 were obtained at 25 °C in HPLC for the resolution of the enantiomers of *N*-(3,5-dinitroben-

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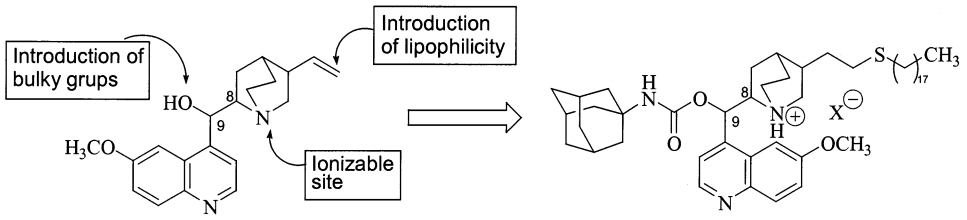

Figure 1. Chemical structure of the quinine (QN)- and quinidine (QD)-derived chiral selectors.

zoyl)-(±)-leucine (DNB-Leu) with some of these carbamate derivatives.[15,18]

Furthermore, this remarkable stereoselectivity observed when the chiral selector was covalently bonded to the chromatographic matrix was also displayed in solution: carbamoyl-type QN and QD derivatives were able to produce stereoselective enrichments of acidic analytes in liquid−liquid extraction experiments.[13] These experimental data, obtained from a single equilibration stage between the two immiscible phases, served as promising signs for an interesting application of multiple liquid−liquid extraction equilibria as revealed in the countercurrent chromatography (CCC) concept.

CCC and its particular modalities, centrifugal partition chromatography (CPC) among them, are techniques with a clear preparative potential and focus. Their successful applications in the purification of natural products have been sufficiently reported.[19−21] In contrast, the examples devoted to the separation of enantiomers are still limited, although they have shown that the general principle of these techniques, the partition phenomenon between two immiscible liquid phases, which controls the retention of the analytes,[19,22,23] is perfectly applicable also to chiral resolutions by addition of an appropriate chiral selector to the system. The ideal solvent system, one of the key points in the success of the separation, should meet certain requirements. Thus, the leakage of CS from one phase to the other (e.g., from the stationary to the mobile phase) should be avoided while promoting the desired partition of the analyte enantiomers between the two liquid phases. Moreover, the solubility of the CS in the stationary phase has to be sufficient to bring about the separation and the elution of the analytes in a reasonable amount and time for preparative purposes.

In these aspects lies the main appeal of the CCC techniques: solvent mixtures with low cost constitute the stationary and the mobile phases, and their properties have the particularity of being mainly modulated thanks to changes in the solvent system compositions. Solvents can be easily changed and removed and no irreversible adsorption of the sample in the stationary phase is possible; hence, a wide range of solvent mixtures can be chosen to perform the fine-tunable chromatographic process.

However, as mentioned before, there are not many examples described of enantiomer separations using this technique.[23−34] The difficulty of finding a suitable CS (being highly selective for the given racemic compounds) and the appropriate system of solvents, which might not suppress this selectivity and keep the capacity to elute the chiral solutes of interest, can presumably be the main reasons for the scarcity of applications in this field. The CSs employed until present were in general those directly used also in liquid chromatography or in other separation techniques. Very long retention times and rather poor resolutions were common features of the first attempts. However, this has been in general overcome in the most recent applications thanks to the improvements in the instrumentation and to further optimizations of the different parameters involved in the overall separation process.[23,29,30,32,34] As a consequence, we conceptually designed modified cinchona-derived selectors in such a way to bear an adamantylcarbamate function to enhance its chiral recognition ability and to increase also the overall lipophilicity by introducing an octadecylthia moiety to avoid the selector distribution into the mobile phase (see Figure 1). In the present article, its use in the context of a consequent search of suitable immiscible liquid−liquid solvent system(s) and the optimization of the resolutions of several enantiomeric pairs (Figure 2) were brought about in

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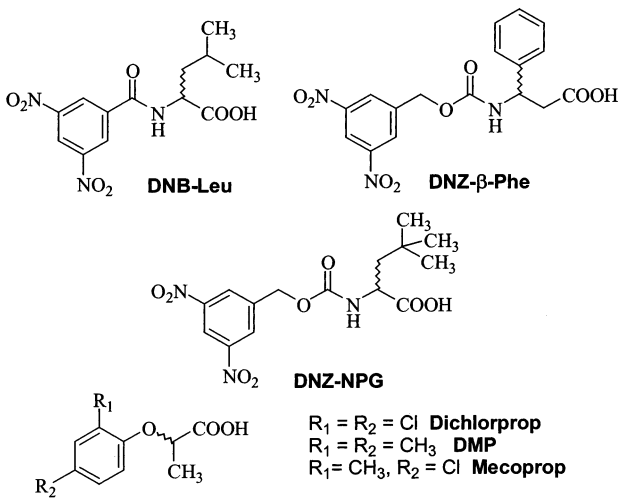

Figure 2. Chemical structures of the racemic compounds tested.

CCC and will be discussed in detail. This also includes the necessary preliminary screening experiments.

EXPERIMENTAL SECTION

Materials. 2,4-Dimethylphenol, 2-bromopropionic acid, and glacial acetic acid were purchased from Aldrich (Steinheim, Germany). Racemic leucine (Leu) and neopentylglycine (NPG) were supplied by Fluka (Buchs, Switzerland). Dichlorprop was a gift from Rhône-Poulenc (Lyon, France), and mecoprop was purchased from Lancaster (Eastgate, England).

The synthesis of the CSs was described elsewhere.[35] Racemic *β*-phenylalanine (*β*-Phe) was prepared following a literature procedure.[36] N-Acylated amino acids were available from prior studies[9,37] or were prepared following well-established protocols. 2-(2,4-Dimethylphenoxy)propionic acid (DMP) was synthesized from 2,4-dimethylphenol by alkylation with 2-bromopropionic acid in aqueous basic media.

Mobile phases for chromatography were prepared from analytical reagent-grade ammonium acetate from Merck and HPLC-grade water, together with HPLC solvents. Trifluoroacetic acid (TFA) and ammonia used for the pH-zone-refining mode were supplied by Panreac Química S.A. (Montcada i Reixac, Spain). The HPLC column used to control the CCC fractions contained a *tert*-butylcarbamoylated quinine as chiral selector.[15]

Apparatus. An HPCPC model LLB-M (EverSeiko, Tokyo, Japan) was used. It is a bench CPC (30 × 45 × 45 cm) with a stacked circular partition disk rotor (2136 channels, 220 mL of internal volume). The CPC was connected to a conventional HPLC system HP 1100 (pump, autosampler, UV detector, and chromatography data station software) from Hewlett-Packard (Figure 3). A manual Rheodyne injector provided with a 2.4-mL loop was used. Commonly, 2.4 mL of racemate solution was injected, but when larger amounts of sample were analyzed, they were introduced directly into the system through the pump.

The pH of the mobile phases was measured with a Crison pH meter, model Basic 20 (Alella, Spain). Fractions of 4.5 mL were collected and analyzed individually during the whole elution of the analytes in order to get a profile of the enantiomeric excesses of the peaks. This control of the CCC-collected fractions and the extraction experiments was performed on the same HPLC system, changing the CPC device for the appropriate HPLC chiral column for the resolution of the considered enantiomers. The homemade HPLC column (150 × 4.6 mm i.d.) contained a *tert*-butylcarbamoylated-QN chiral selector (these columns are now commercially available from Bischoff, Leonfelder, Germany or Iris Technologies, Lawrence, KS). The mobile phases were mixtures of methanol and 1.0 M ammonium acetate adjusted to an apparent pH (pH_a) of 6.0 by adding glacial acetic acid. The ratio methanol/buffer depended on the racemic solutes analyzed; it was: 95:5 for DNB-Leu, 80:20 for *N*-(3,5-dinitrobenzyloxycarbonyl)-(±)-neopentylglycine (DNZ-NPG) and *N*-(3,5-dinitrobenzyloxycarbonyl)-(±)-*β*-phenylalanine (DNZ-*β*-Phe), and 30:70 for the 2-aryloxypropionic acids. Flow rate was always 1 mL/min, and the UV detection was at 254 nm for the amino acid derivatives, 230 nm for dichlorprop, and 276 nm for DMP and mecoprop. Temperature was kept constant at 25 °C during the CCC experiments and the HPLC runs.

Stereoselective Extraction Screening Conditions. In a small test tube with a screw cap, 100 *μ*L of a 100 mM solution of racemic DNB-Leu in methanol (10 *μ*mol) were pipetted. Then, 100 *μ*L of a 100 mM solution of the CS in dichloromethane (10 *μ*mol) was added, and the solvents were removed under vacuum at 30 °C.

The solvent systems to be tested were prepared in different flasks and equilibrated before use by stirring vigorously during 1 min. An aliquot of 2 mL of the upper phase and an equal volume from the lower phase were added to the test tube. The vial was closed and vortexed vigorously in a water bath at 25 °C for 5 min. The phases were allowed to separate, and 1 mL of the aqueous lower phase was transferred to another vial. Most of the solvent was removed by gentle heating, and the sample was dried under vacuum at 60 °C. The residue was dissolved in 2 mL of methanol and directly injected into the HPLC system, where the enantiomeric excess (ee) was evaluated using an appropriate cinchona-derived column.[15]

To check the possible selector leaching into the mobile phase, the aqueous component was investigated by thin-layer chromatography (TLC) developing with chloroform/methanol mixtures (10:1).

CCC Experimental Conditions. The CS was dissolved in the liquid phase with less density (stationary phase), and therefore, in all the cases the mobile phase was pumped through in descending mode. The CCC column was kept in methanol when not in use after exhaustive cleaning with the suitable solvents. Thus, before starting any experiment, water was pumped through to eliminate methanol traces. As schematized in Figure 3, several preparation steps were performed before the injection of the samples and they were different depending on the operational mode. In *classical CCC mode*, water from the column was completely displaced with the stationary phase in descending mode. Centrifugal rotation was then applied, and the mobile phase was pumped until no bleeding of stationary phase was observed in the eluate. The displaced volume of stationary phase corresponded to the void volume and allowed the calculation by difference of the retained stationary phase (V_{st}) and, therefore,

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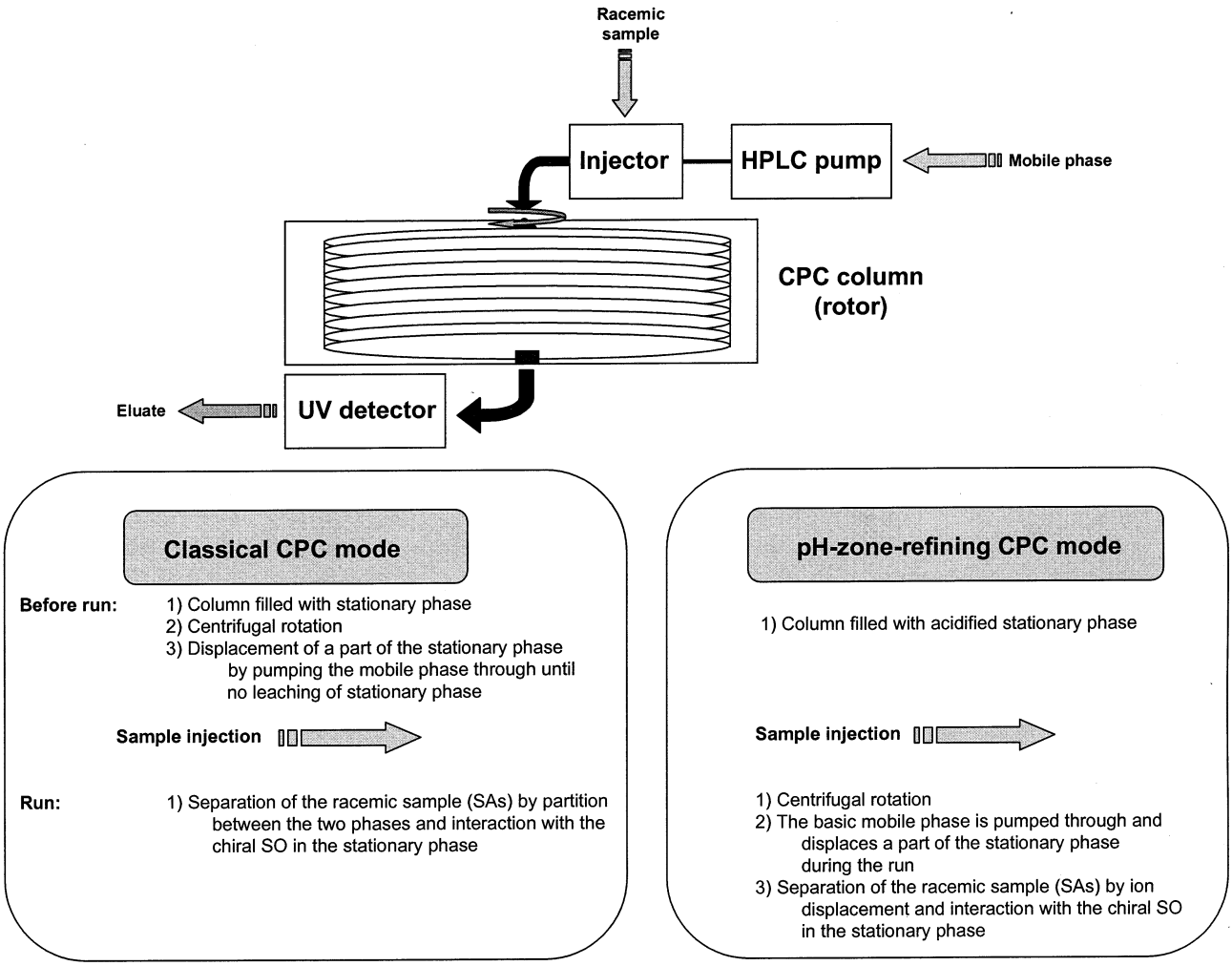

Figure 3. Separation system and operational scheme.

the amount of CS present in the CCC column. Injection of the enantiomeric pairs to be separated, dissolved in stationary phase in order not to disturb the equilibrium conditions, was then performed. In *pH-zone-refining CCC mode*, the column was filled analogously with the acidified stationary phase. However, injection of the analytes was performed before centrifugal rotation was applied and the basic mobile phase started to be pumped through. In the eluate, the first part of the displaced stationary phase appeared and afterward the collection of fractions was undertaken. The apparent pH and the enantiomer content were determined for every fraction. The volume of nondisplaced chiral stationary phase or V_{st} was also used to calculate the amount of selector present in the CCC column during the separation process.

Evaluation of the Recovery of Solutes and Selectors after the CCC Runs. The collected mobile-phase fractions (standard 4.5 mL) containing the separated enantiomers were acidified with a small volume of concentrated hydrochloric acid and extracted three times with ethyl acetate. The combined organic layers were dried with anhydrous sodium sulfate and filtered, and the solvent was evaporated under vacuum. The residue was weighed and found to be 90−95% of the injected amount of racemic solute by summing up the yield of the individual fractions.

The QN and QD derivatives dissolved in the stationary phase were recovered from the organic phase by successively washing it with a 5% (w/v) $NaHCO_3$ solution (× 3), 0.25 M ammonium acetate solution (× 1), and water (× 2). The remaining organic layer was then dried with anhydrous sodium sulfate and filtered, and the solvent was evaporated under vacuum. When an alcohol was present in the stationary phase, a partial evaporation of this component was performed previous to the above-described treatment. The selector was dried overnight at 60 °C under reduced pressure, resulting in ∼95% recovery.

RESULTS AND DISCUSSION

Screening of Conditions. Several stages were planned in the development of the complete application of CCC to the separation of enantiomers. First, an initial procedure was designed to assess the potential enantioselectivity of the CSs in different solvent systems by means of extraction experiments. Moreover, important features of the solvent systems, such as ready separation of the two immiscible liquid phases, were critical to evaluate. In a second stage, blank and control CCC runs without the selector dissolved in the stationary phase helped to establish the operating final CCC conditions, which were later applied also in the presence of the CSs. Some of the best resolutions were consequently scaled up.

Extraction Experiments. Liquid−liquid extraction experiments were necessary to find suitable systems of solvents, which had to meet the following requirements. First, the CS should be

soluble in only one of the two phases (in our case, in the stationary phase). For a two-phase aqueous/organic system, the selector should be either highly lipophilic to remain entirely in the organic phase or highly hydrophilic (ionic), if the aqueous phase is to be used as the stationary phase. The CSs considered in the present study were designed to be lipophilic even in the presence of a positively charged amino group at the working pH. Therefore, the organic phase will act as stationary phase in all the solvent systems tested. Second, the racemic mixtures (see Figure 2) should be readily soluble in both phases, whereby in the present case the solubility of the ion-pair enantiomer−CS associate in the lipophilic phase needs to be considered. The optimum distribution coefficient is ∼1. This is approximately the same solute concentration (without specifying the individual enantiomers) in both phases after extraction. High solute distribution in the stationary phase will lead to long retention times. On the contrary, higher concentrations of compound to be separated in the mobile phase will reduce the chromatographic efficiency of enantioseparation due to the reduced interaction between enantiomers and CS, this latter being mainly in the stationary phase, due to the ion-pair-type extraction.

A wide range of binary, ternary, and quaternary solvent systems was evaluated. First, their readiness for forming nonstable emulsions was taken into account. The solvent systems that separated rapidly were subsequently tested with the CS, to detect any possible distribution between both phases. Initial extraction experiments were performed using the lipophilized CSs, and afterward, DNB-Leu was added to the same mixtures as a racemate. The molar CS/racemate ratio was 1 for all the experiments. The CS concentration in the extraction experiments was similar to that used later for the CCC stationary phase, because in some cases, it is actually the CS that causes emulsification of the system. The solvent systems that still showed ready separation in the presence of the CS were candidates for further determinations. The enantioselective partition of DNB-Leu between the two phases was determined by HPLC.

On the basis of previous results performed by liquid−liquid extraction and liquid membranes,[13] it was expected that a ternary system consisting of an ammonium acetate buffer, an alcohol, and a hydrocarbon might yield good enantioselectivity and liquid−liquid (CCC-type) chromatographic behavior. However, the systems containing 1-decanol and an hydrocarbon, such as isooctane or pentadecane, were not appropriate, as their high boiling points were not suitable for the recovery of the CS after CCC runs. Thus, other alcohols in combination with heptane were tested. By far the highest enantioselectivities were observed with systems containing an alcohol of medium lipophilicity (1-butanol or *tert*-amyl alcohol) in combination with heptane. There were also some other binary and ternary systems with methyl isobutyl ketone (MIBK), diisopropyl ether (DIPE), or methyl *tert*-butyl ether (MTBE) that gave rise to good enantioselectivities.

Concerning the CSs, *O*[9]-(1-adamantylcarbamoyl)-QN and QD derivatives had previously demonstrated the ability to recognize the enantiomers of amino acid derivatives in HPLC with even higher α values than the corresponding *tert*-butyl analogues.[16] In the present case, the adamantyl-derived selectors were made more lipophilic by addition of an octadecylthia group to the vinyl moiety (**1** and **2**, Figure 1). Other approaches, such as immobilization onto polysiloxane, were also investigated. However, exploratory studies revealed a pronounced tendency of the polysiloxane derivatives to form stable emulsions, whereas the use of the corresponding nonpolymerized but lipophilized CS assured a rapid phase separation for the same solvent systems.

CCC Runs. A CCC run without the addition of CS was useful to estimate nonstereoselective retention by injecting the racemic solutes that should later be separated into the enantiomers. Moreover, this control experiment was performed to check how the two-phase solvent system behaves under real operating CCC conditions as outlined in Figure 3. Then, the QN- or QD-derived CS was introduced and the CCC runs were undertaken. Although selectors **1** and **2** are in fact diastereomers, as is usual for QN and QD derivatives acting as enantiodiscriminating agents, their enantioselectivity was complementary and the expected inversion of the elution order of enantiomers was always observed. As QN-CS **1** is an oil and QD-CS **2** a solid, both selectors were tested in order to check their behavior in solution and their slightly different chromatographic properties.

Three main types of solvent systems were investigated: (A) quaternary solvent systems; (B) solvent systems containing MIBK; (C) solvent systems containing DIPE.

(A) Quaternary Solvent Systems. Based on the above-discussed extraction results, several CCC runs were performed with the system ammonium acetate buffer 0.1 M pH 6.0/*tert*-amyl alcohol/MeOH/heptane (10/5/1/5) containing 10.6 mM CS. This system was rather stable at a rotational speed (ω) of 1000 rpm and a flow rate of 3 mL/min. Analytical amounts of the racemic mixtures (1−10 mg) were injected (see Figure 2). While baseline separations were observed for DNB-Leu and DNZ-NPG, only a partial resolution was achieved for DNZ-*β*-Phe (Figure 4). Therefore, several variations of the ionic strength and pH of the running buffer, proportions of the different solvents, concentration, and QN- or QD-type CSs (see Figure 1) were investigated. Although some improvement in the separation was obtained in a system of ammonium acetate buffer 0.25 M pH 7.0/*tert*-amyl alcohol/MeOH/heptane (10/3/1/7) containing 10.6 mM QN selector **1**, no complete resolution was possible for DNZ-*β*-Phe.

Once a successful analytical-scale enantioseparation was achieved, a first upscaling procedure was performed to determine the maximum amount of injectable solutes that still can be near-baseline separated. This is referred in a first attempt as the maximum throughput of analyte in this solvent system in a single run. However, it should be taken into account that the detector reached signal saturation at ∼3000 mAU. Thus, for semipreparative separations (10−100 mg), peaks would be much higher and cannot be recorded that way. Nevertheless, separation could still be observed by HPLC analysis of individual fractions, thus reconstructing an elution profile. The off-line HPLC results were in any case conclusive in order to know the exact composition (ee) of the different collected fractions.

A summary of the results from the first upscaled CCC runs is listed in Table 1. In general, different α values were obtained in CCC and HPLC for a given pair of selector/racemate. However, it has to be taken into account that conditions are not directly extrapolable from one technique to the other. For DNB-Leu and DNZ-NPG, enantioselectivity factors were lower in CCC, whereas for DNZ-*β*-Phe, α values slightly increased.

Nevertheless, enantioselectivity might be affected either by the fixation of the CS to a chromatographic support or by the

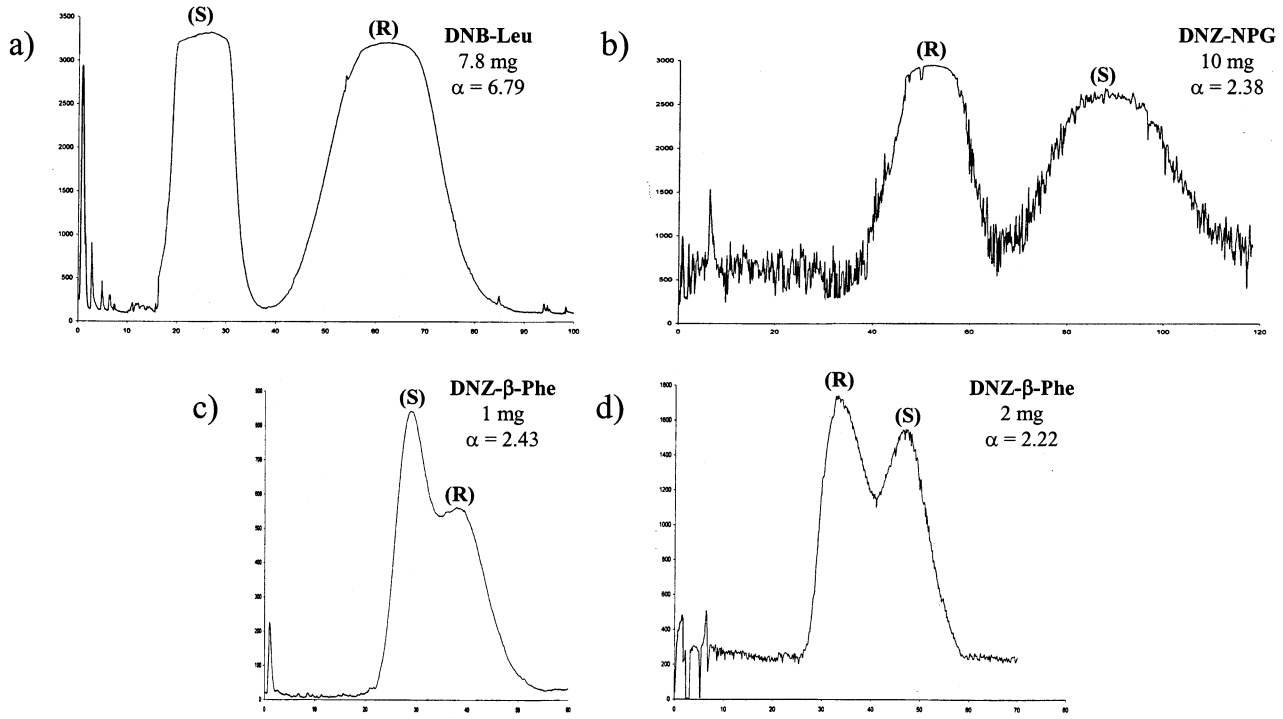

Figure 4. CCC chromatograms. Flow rate 3 mL/min; $\omega = 1000$ rpm; descending mode; λ 240 nm. Solvent systems: (a, c) ammonium acetate buffer 0.1 M pH 6.0/*tert*-amyl alcohol/MeOH/heptane (10/5/1/5), 10.6 mM QD selector **2**, $P = 70$ bar; (b, d) ammonium acetate buffer 0.25 M pH 7.0/*tert*-amyl alcohol/MeOH/heptane (10/3/1/7), 10.6 mM QN selector **1**, $P = 79$ bar.

Table 1. CCC Separations Using the Quaternary System Ammonium AcetatBuffer/*tert*-Amyl Alcohol/MeOH/Heptane[a]

	m_{rac} (mg)	$r_{CS/rac}$	t_1 (min)	t_2 (min)	k_1	k_2	α	eo	conditions[b]
DNZ-NPG	2.0	359	36	62	0.8	2.1	2.69	*S*	A
($\alpha_{HPLC} = 3.0$)[c]	10	66	51	93	1.5	3.6	2.38	*R*	B
	50	13	49	92	1.4	3.6	2.53	*R*	B
	100	6.6	70	143	2.5	6.2	2.48	*S*	A
DNB-Leu	2.0	300	25	57	0.2	1.8	7.60	*S*	A
($\alpha_{HPLC} = 15$)[c]	7.8	75	27	65	0.3	2.2	6.79	*S*	A
	100	5.9	36	145	0.8	6.2	7.71	*R*	B
DNZ-*β*-Phe	1.0	720	31	48	0.6	1.4	2.43	*S*	A
($\alpha_{HPLC} = 1.6$)[c]	2.0	350	34	50	0.7	1.5	2.22	*R*	B
	12.5	56	37	59	0.9	2.0	2.28	*R*	B

[a] m_{rac}, mass of racemate injected; $r_{CS/rac}$, molar ratio CS/racemate; k_1 and k_2, retention factors of the enantiomers. eo, elution order, configuration of the first-eluted enantiomer. [b] Flow rate, 3 mL/min; $\omega = 1000$ rpm; descending mode; λ 240 nm. (A) Ammonium acetate buffer 0.1 M pH 6.0/*tert*-amyl alcohol/MeOH/heptane (10/5/1/5), 10.6 mM QD-CS **2**. (B) Ammonium acetate buffer 0.25 M pH 7.0/*tert*-amyl alcohol/MeOH/heptane (10/3/1/7), 10.6 mM QN-CS **1**. [c] α_{HPLC} values were obtained with a column containing the adamantylcarbamate-QN-CS bonded to silica gel using mixtures of ammonium acetate buffer/methanol as mobile phase.[35]

differences in solvents used in both techniques affecting its solvatation and conformational equilibria. In HPLC, the CS−enantiomer association takes place at the interphase between the mobile and the stationary phases. In CCC, the racemic solute must undergo a phase transition from the mobile to the stationary phase to form an ion-pair with the CS. Generally, for the same volume of stationary phase and CS concentration, resolution decreased at higher solute amounts injected and loaded on, as is usual in HPLC, but to a much lesser extent than expected for the CCC technique. In Figure 5, the chromatogram corresponding to the enantioseparation of 50 mg of DNZ-NPG is shown. During elution, peaks were fractionated and subsequently analyzed by HPLC. There was only a small mixed zone, and essentially pure enantiomers were eluted around the maximums of the chromatographic peaks.

Despite these promising results, some drawbacks were observed. The solvent system was optimized to yield the highest enantioselectivity. However, solubility of the racemic mixtures was relatively low, retention times were long, and CS/racemate ratios were usually high, but in the same order of the ones commonly found in HPLC. These features implied a limited loading capacity for the system used.

(B) Solvent Systems Containing MIBK. After these observations, we decided to switch to other more promising solvent systems, which originated lower enantioselectivity values in the first screenings but offered other features. Secondary solvent systems, constituted of ammonium acetate buffer and MIBK, MTBE, or DIPE were under investigation. Some of these organic solvents (MTBE, DIPE) showed the same reduced solubility for amino acid derivatives, but this problem was overcome by using MIBK. Exploratory tests in tube with these binary systems demonstrated that the best enantioselectivity values were achieved with MIBK for DNB-Leu.

Table 2. CCC Separations in Buffer/MIBK Solvent Systems[a]

				QN-CS **1**					QD-CS **2**				
racemate	pH	m_{rac} (mg)	$r_{CS/rac}$	t_1 (min)	t_2 (min)	k_1	k_2	α	t_1 (min)	t_2 (min)	k_1	k_2	α
DNB-Leu	6.85	150	3	211	282	11.1	15.1	1.36	222	297	12.4	16.9	1.37
	8.0	150	3	71	116	3.1	5.6	1.84	83	158	4.42	9.33	2.11
	8.0[b]	150	2.6[c]	83	157	2.7	6.0	2.20					
DNZ-NPG	6.85	170	3	*d*					*d*				
	8.0	170	3	170	200	8.7	10.4	1.20	175	232	10.4	14.1	1.36
	8.0[b]	170	2.6[c]	191	227	7.5	9.1	1.21					
DNZ-β-Phe	6.85	179	3	*d*					*d*				
	8.0	179	3	190	230	9.9	12.1	1.23	210	255	12.7	15.6	1.23
	8.0[b]	179	2.6[c]	188	215	7.4	8.6	1.16					

[a] m_{rac}, mass of racemate corresponding to 0.46 mmol; $r_{CS/rac}$, molar ratio CS/racemate; k_1 and k_2, retention factors of the enantiomers. Flow rate, 3 mL/min; ω = 1200 rpm; descending mode; λ 230 nm. Ammonium acetate buffer 0.1 M/MIBK (1:1), 10 mM CS (V_{st} =138 mL), except as noted. [b] Ammonium acetate buffer 0.1 M/acetone/MIBK (2:1:2), V_{st} = 122 mL. [c] The molar ratio CS/racemate is reduced as a result of the smaller stationary-phase volume retained in the CCC column for this solvent system. [d] The racemates were retained in the CCC column in these conditions.

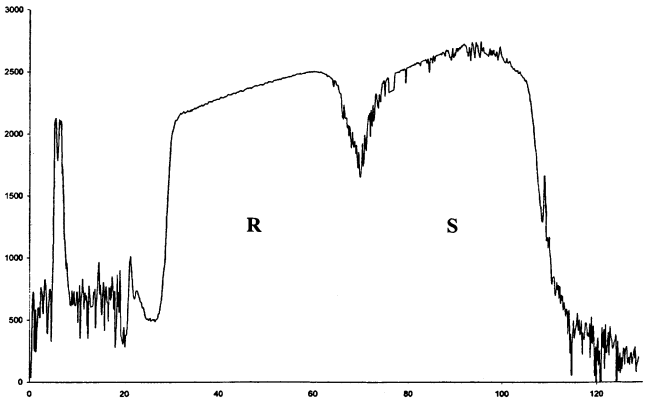

Figure 5. Resolution of 50 mg of DNZ-NPG. Solvent system: ammonium acetate buffer 0.25 M pH 7.0/*tert*-amyl alcohol/MeOH/heptane (10/3/1/7), 10.6 mM QN selector **1**. Flow rate 3 mL/min; ω = 1000 rpm; descending mode; λ 240 nm, P = 79 bar. Vertical axis, arbitrary absorbance units. Horizontal axis, time (min).

In Table 2, results corresponding to the separation of amino acid derivatives using MIBK are presented. It should be noted that the molar ratios of CS/racemate were highly reduced thanks to the increased solubility of the solutes. Modulation of the pH values of the buffer was, as expected, a key point to control the elution of the analytes. Higher pHs were led to shorter retention times but lower enantioselectivity.[38] However, total resolutions were possible for all amino acid derivatives at pH 8.0 with QN-CS **1**. At pH 6.85, DNZ-NPG and DNZ-β-Phe remained retained in the CCC column.

In contrast to the total resolution achieved in the same conditions using QN-CS **1**, only a partial resolution was attained for DNZ-NPG and DNZ-β-Phe using QD-CS **2**. Overloading as a cause for that observation was discarded as comparable results in terms of resolution were achieved when half the amount of racemic solute was injected. A slower kinetics observed in all cases when CS **2** was used may be the cause for this partial resolution.

(38) Franco, P.; Oberleitner, W. R.; Maier, N. M.; Lindner, W.; Minguillón, C. Enantiomer separation by centrifugal partition chromatography (CPC) using cinchona alkaloid derivatives as chiral selectors. Poster presented at the 12th Symposium on Chiral Discrimination (Chirality 2000), Chamonix, France, September 2000.

Regarding the 2-aryloxypropionic acids tested, only a slight enantiomeric enrichment was observed for mecoprop at pH 6.85 (data not shown).

In the search for improved conditions, a third solvent was introduced in the system. Thus, using 0.1 M ammonium acetate buffer, pH 8.0/acetone/MIBK (2/1/2) containing a concentration of 10 mM QN selector **1**, retention times were slightly longer than those obtained with the binary system at the same pH, even though the volume of stationary phase retained in the CCC system was smaller. This ternary system was also less stable than the binary one previously described. Some leaching of the stationary phase was observed during operation. Nevertheless, enantioselectivity increased for DNB-Leu, the only racemic mixture whose baseline resolution was achieved. Saturation of the CS was attained when 450 mg (1.38 mmol) of this solute was injected under these conditions (molar ratio CS/racemate, 0.88) (Figure 6).

(C) Solvent Systems Containing DIPE. As already mentioned, no remarkable separation was observed for any of the aryloxypropionic acid derivatives tested in systems containing MIBK. In contrast, some discrimination for the enantiomers of these compounds was detected when DIPE was used in a binary system (Table 3). In this case, an increase of the ionic strength of the buffer solution was needed to avoid emulsification. As these compounds were in general terms less retained, the decrease of the pH of the buffer was leading to better chiral recognition, though insufficient for practical purposes.

Unfortunately, amino acid derivatives were not sufficiently soluble under these conditions. This last drawback was partially overcome by including 2-propanol in the binary mixture. Although comparable or slightly better α values could be observed for the amino acid derivatives in these DIPE systems than with the ones containing MIBK, they turned out to be not suitable to carry out a preparative separation due to the limited solubility of these racemates in this ether.

Ion-Exchange Displacement Chromatography (pH-Zone-Refining Mode). To improve the preparative potential of CCC for the separation of enantiomers, and taking into account the ionizable character of the studied solutes and CSs, pH zone refining was applied. This chromatographic mode in CCC is a displacement type chromatography introduced by Ito et al.,[39] and

(39) Weisz, A.; Seher, A. L.; Shinomiya, K.; Fales, M.; Ito, Y. *J. Am. Chem. Soc.* **1994**, *116*, 704−708.

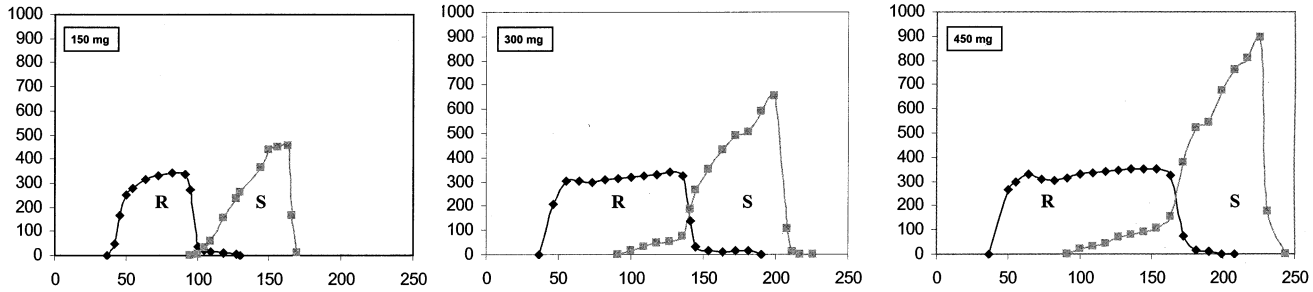

Figure 6. Elution profiles corresponding to the separation of increasing amounts of DNB-Leu injected. Solvent system: ammonium acetate buffer 0.1 M pH 8.0/acetone/MIBK (2/1/2), 10 mM QN selector **1**. Flow rate 3 mL/min; $\omega = 1300$ rpm; descending mode, $P = 76$ bar. Vertical axis, arbitrary absorbance units. Horizontal axis, time (min).

Table 3. CCC Separations in Buffer/DIPE Solvent Systems[a]

				QN-CS **1**					QD-CS **2**				
racemate	pH	m_{rac} (mg)	$r_{CS/rac}$	t_1 (min)	t_2 (min)	k_1	k_2	α	t_1 (min)	t_2 (min)	k_1	k_2	α
DNB-Leu	6.85	50	9	75	142	3.8	8.1	2.15	80	151	3.8	8.0	2.11
DNZ-NPG	6.85	170	3	173	235	10.1	14.0	1.39	175	244	9.5	13.6	1.44
DNZ-β-Phe	6.85	57.5	10	85	115	4.4	6.3	1.44	98	143	4.9	7.6	1.55
dichlorprop	6.85	108.5	3	124	133	6.9	7.5	1.08	113	136	5.8	7.1	1.23
	6.85[b]	108.5	3.5	68	71	5.8	6.1	1.05	59	66	2.6	3.0	1.18
	6.0[b]	108.5	3	87	92	3.8	4.0	1.06	75	84	3.1	3.6	1.16
DMP	6.85	89.5	3	114		6.3		1.00	113		5.8		1.00
	6.85[b]	89.5	3.5	64		5.4		1.00	66	67	2.9	3.0	1.02
	6.0[b]	89.5	3	86	88	3.7	3.8	1.02	79	82	3.3	3.5	1.05

[a] m_{rac}, mass of racemate corresponding to 0.46 mmol, except for DNB-Leu and DNZ-β-Phe (0.15 mmol), where solubility was limited. $r_{CS/rac}$, molar ratio CS/racemate; k_1 and k_2, retention factors of the enantiomers. Flow rate, 3 mL/min; $\omega = 1000$ rpm; descending mode; λ 230 nm. Ammonium acetate buffer 0.1 M/2-PrOH/DIPE (60:19:21), 10 mM CS ($V_{st} = 143$ mL). [b] Ammonium acetate buffer 0.4 M/DIPE (1:1), 10 mM CS ($V_{st} = 159$ mL at pH 6.85 and 135 mL at pH 6.0).

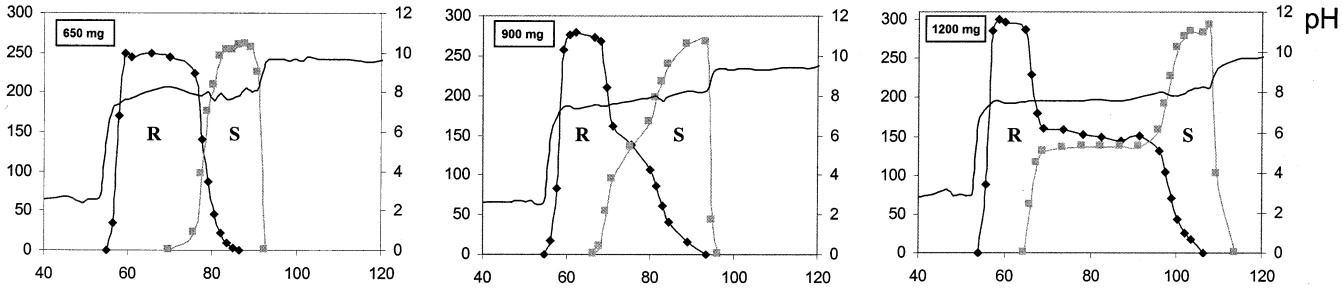

Figure 7. Elution profiles corresponding to the separation of increasing amounts of DNB-Leu by pH-zone-refining CCC. Stationary phase, MIBK containing TFA (10 mM) and QN selector **1** (10 mM); mobile phase, water containing ammonia (20 mM). Flow rate 3 mL/min; $\omega = 1200$ rpm; descending mode, $V_{st} = 166-170$ mL, $P = 72$ bar. Vertical left axis, arbitrary absorbance units. Vertical right axis, pH. Horizontal axis, time (min).

it has been successful in the resolution of ionizable compounds.[40] The products to be separated elute in blocks according to their pK_a values and partition coefficients. In the chiral field, its principal usefulness has been demonstrated for the separation of DNB-Leu with a proline-derived CS.[23,41]

In the present investigation, the system water/MIBK was chosen to perform the experiments, using TFA as retainer in the organic stationary phase and ammonia as displacer in the aqueous mobile phase. The separation was brought about by filling the entire CCC column with the acidified organic phase containing the CS, followed by introduction of the racemic sample dissolved in the stationary phase. The mobile phase was then pumped through the column while the apparatus was running at 1200 rpm. Along the column, the NH_3 border determined the elution in zones of the two enantiomers, as a function of the different pK_a values of their complexes with the CS.

Increasing amounts of DNB-Leu, up to 1200 mg (3.70 mmol), were injected in the presence of QN-derived CS **1** in the stationary phase. The resulting enantiomer separation is shown in Figure 7. Saturation of the system was attained at ~900 mg of racemate (2.77 mmol, molar ratio CS/racemate, 0.60), whereas there was 450 mg (1.38 mmol) when the classical CCC mode was applied.

With the aim of enhancing the incipient separation observed for dichlorprop using the binary system buffer/DIPE in conventional CCC, the system water/2-propanol/DIPE was also investigated in the displacement mode. Although the separation was somehow improved in these conditions, only a partial resolution was achieved (Figure 8).

(40) Foucault, A. P.; Chevolot, L. *J. Chromatogr., A* **1998**, *808*, 3−22.
(41) Ma, Y.; Ito, Y.; Foucault, A. *J. Chromatogr., A* **1995**, *704*, 75−81.

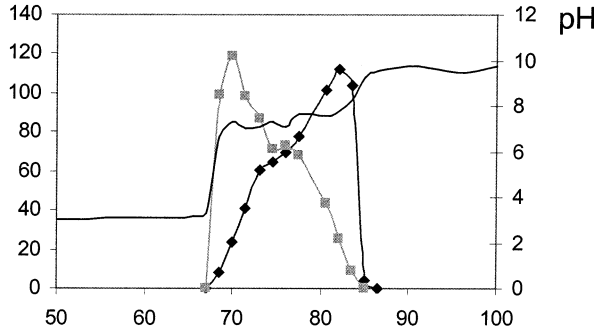

Figure 8. Elution profile corresponding to the separation of 108.5 mg (0.46 mmol) of dichlorprop by pH-zone-refining CCC. Solvent system, DIPE/2-PrOH/water (60/19/21). Stationary phase contained TFA (10 mM) and QN selector **1** (10 mM); mobile phase, contained ammonia (20 mM). Flow rate 3 mL/min; ω = 1200 rpm; descending mode, V_{st} = 164 mL, P = 64 bar. Vertical left axis, arbitrary absorbance units. Vertical right axis, pH. Horizontal axis, time (min).

Comparison of Operational CCC Modes: Loading Limits. The limiting loadability in pH zone refining for DNB-Leu (Figure 7), corresponding to a CS/racemate molar ratio of 0.6, implies that 2 equiv of CS is able to resolve 3 equiv of racemate. This apparently incongruous result led us to the necessity of further understanding the underlying mechanism of this displacement chromatographic mode. In HPLC or classical CCC, applied to the separation of enantiomers, one might expect that at the saturation limit the molar ratio CS/racemate has to be 1:1. Thus, if we check the results presented above (see Figure 6), when 300 mg of DNB-Leu was separated, the CS/racemate molar ratio was 1.33; that is, 4 equiv of CS resolved ~3 equiv of analyte. The attempt of achieving 450 mg of racemic mixture in a single run (CS/racemate molar ratio, 0.88) was unsuccessful as 9 equiv of CS was not enough to separate 10 equiv of racemate. Saturation was attained. However, this was not the case in the displacement mode.

In pH zone refining, the partition of the analyte into the mobile phase has been drastically reduced at the beginning of the chromatographic process. All the sample remains in the stationary phase in equilibrium between the free acidic and the associated form with the CS. When the pH starts changing and reaches the pK_a value of the analyte enantiomers, the less interacting enantiomer starts to distribute to the mobile phase, while the other one, which is bonded with more affinity to the CS, stays in the stationary phase. In contrast to what we have in classical chromatography, in this displacement mode, the more retained enantiomer would not pass to the mobile phase until all the less retained counterpart has abandoned the stationary phase. Two conditions limit this phenomenon. The number of molecules of the more retained enantiomer has to be lower or equal to the number of molecules of CS, and the difference in association constants between each one of the enantiomers and the CS has to be high enough.

Consequently, for a racemic mixture, the limit of loading capacity in pH zone refining would be a 2:1 CS/racemate ratio. The experimental results are in good agreement with this rationale. As already mentioned, total resolution of 3 equiv of DNB-Leu was performed in the presence of 2 equiv of CS, whereas saturation was reached when 1200 mg of racemic solute was injected (see Figure 7), as the CS/racemate molar ratio was 5:11.

Furthermore, this rationale can be extended to nonracemic mixtures. In this case, if the mixture is enriched in the less retained enantiomer, the amount of sample can be even larger, as the limiting factor would be the amount of the more interacting enantiomer. However, when the nonracemic mixture would be enriched in the more retained counterpart, less sample might be injected. At this point, the availability of CSs having similar physicochemical properties but opposite chiral recognition abilities, as is the case for QN and QD derivatives, becomes advantageous.

The application of pH zone refining has demonstrated an improvement in the loading capacity of the technique. However, it is interesting to point out an important difference in operational terms between this displacement chromatography and the classical CCC mode. The separations undertaken by pH zone refining can be only performed by single-run processes. That is, the stationary phase containing the CS can only operate once, as the initial conditions change during the run (see Figure 3). Therefore, the column should be emptied and later refilled with newly acidified organic phase. In contrast, the classical CCC mode maintains the stationary phase unmodified into the column and consecutive injected samples can be resolved, as in HPLC. The convenience of using one or the other mode must be investigated for every case, to take the maximum profit of the planned runs.

CONCLUSIONS

The combination of QN- or QD-derived CSs and CCC provides a very efficient tool for the resolution of certain amino acid derivatives at preparative scale. The appropriate chemical modification of the CSs, starting from the naturally available alkaloids, was needed to ensure the correct distribution in the chromatographic solvent systems. The introduction of a bulky adamantylcarbamate functionality together with a lipophilic side chain resulted in a suitable compromise to enhance chiral recognition, while keeping appropriate physicochemical properties such as the CS staying stable in the organic phase during the CCC run. For comparative reasons, all experiments were performed with 10 mM concentrations of the CSs; however, this value is below the solubility limit of these compounds in the solvent systems tested, and therefore, higher loading capacities could be envisaged. The results thus obtained confirm the high potential of the technique for preparative enantioseparations, especially in the pH-zone-refining mode, and open up new possibilities in the search of other applicable chiral selectors.

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