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Preparative enantioseparation of (±)-*N*-(3,4-*cis*-3-decyl-1,2,3,4-tetrahydrophenanthren-4-yl)-3,5-dinitrobenzamide by centrifugal partition chromatography

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ABSTRACT

The racemic compound (±)-*N*-(3,4-*cis*-3-decyl-1,2,3,4-tetrahydrophenanthren-4-yl)-3,5-dinitrobenzamide ((±)-**1**), an analogue of increased lipophilicity of the chiral selector (CS) contained in the Whelk-O® HPLC chiral stationary phase (CSP) has been resolved into its enantiomers by applying centrifugal partition chromatography (CPC). Considering the known enantioselectivity of the Whelk-O® CS for naproxen, and the reciprocity concept in enantioseparation, (*S*)-naproxen related compounds were tested as CSs. In the search for an adequate solvent system, the partition behaviour of the two solutes, CS and racemate, has been studied using several biphasic solvent mixtures. The optimal CS concentration and sample loading capacity were determined in the chosen solvent system. The search for an appropriate CS and solvent system, the scale-up and optimization of the enantiomer separation by CPC, as well as the rationale for the unexpected elution order of enantiomers, are here described. The comparison of the preparative CPC separation achieved with that in HPLC, using a CSP containing an analogous CS, resulted favourable to the former in terms of loading capacity, solvent consumption and throughput.

1. Introduction

Nowadays, the significance of the enantiomeric purity in the chiral active ingredients used in drug manufacture is well recognized. This has generated the need to have available methodologies for the analytical control of the enantiomer composition on samples and/or for the production of isolated enantiomers. Although diverse procedures exist [1], chromatographic techniques are amongst the most used to resolve mixtures of enantiomers at a preparative level [2].

The increasingly growing application of simulated moving bed chromatography (SMB) as an enantioselective process, has demonstrated the power of this technique at a preparative scale [3]. Nevertheless, one of the main disadvantages of SMB is the considerable investment in equipment required. Countercurrent chromatography (CCC) [4] can be considered among the possible alternatives easily adapted to be run in a continuous mode. CCC, which include modalities such centrifugal partition chromatography (CPC) [5], a separation technique in which the mobile and stationary phases are immiscible liquids, is especially suitable for preparative operation [6]. Nevertheless, the extent of the CCC applicability to enantioselective separations is dependent on the availability of chiral selectors (CSs), experimental conditions and technical devices, which make the separation of a broad range of enantiomeric compounds feasible at a competitive level.

The resolution of enantiomers by CCC involves the addition of a suitable CS to one of the phases of the biphasic solvent system involved. The phase containing the CS is used as the stationary phase in the chromatographic system. To be applicable to preparative purposes the CS must be fairly soluble in these conditions as loadability and also enantioselectivity, are dependent on the amount of CS involved in the separation [7–9]. In addition, the CS should maintain its enantiorecognition capacity in the liquid phase. The CSs used up to now in CCC for chiral separations came from other techniques, mainly HPLC, and have been recently reviewed [9].

Among the number of chiral stationary phases (CSPs) for HPLC enantioseparation existing on the market, Whelk-O® CSP, commercialized by Regis Technologies, has shown an outstanding applicability. This CSP, which contains a low molecular weight CS, was originally designed by Pirkle and Welch for the chiral separation of underivatized non-steroidal anti-inflammatory drugs

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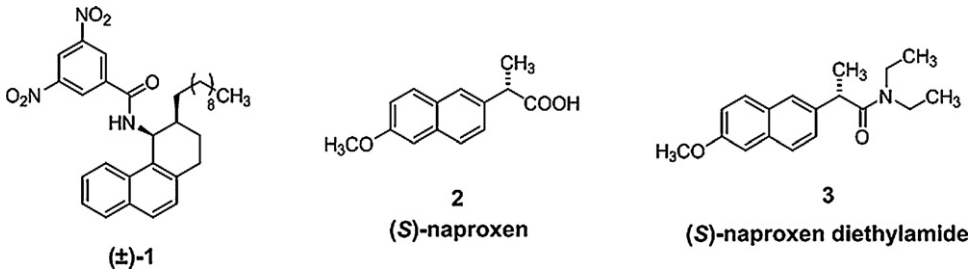

Fig. 1. Structures of the analyte (±)-**1**, and the CSs **2** and **3**.

(NSAID) such as naproxen [10,11]. Later it has been applied to the separation of a wide variety of racemates [12]. Nowadays, Whelk-O® CSP has become one of the CSPs of reference included in most screening processes addressed to the search of adequate conditions for enantioselective separation in drug discovery [13,14].

In the research for new CSs applicable to CCC/CPC enantioseparations, the CS contained in Whelk-O® CSP was considered to be a good candidate. Therefore the synthesis of **1** (Fig. 1), an analogue of the CS in the Whelk-O® CSP, was undertaken following a procedure similar to that reported in the literature for this latter [11]. To retain the CS in the organic phase of an organic/aqueous solvent system during CCC/CPC operation, the introduction of an aliphatic chain in the 3rd position of the phenanthrene backbone was considered. The synthetic path followed led to racemic (±)-**1**. Therefore, a final enantioseparation step was required to obtain the enantiomerically pure compound. Due to the advantages that CCC/CPC offers for preparative operation, this was chosen as a technique to perform the separation of the enantiomers. In order to find a suitable CS for the separation of (±)-**1** enantiomers the principle of reciprocity in enantiomer recognition was applied. (*S*)-Naproxen and derivatives were considered for this purpose.

Herein we describe the separation of (±)-**1** enantiomers by centrifugal partition chromatography (CPC). The search for an adequate CS and solvent system for this separation together with the optimization of conditions, in terms of CS concentration and loadability of the resulting chromatographic system, will be discussed.

2. Experimental

2.1. Reagents and apparatus

HPLC-grade solvents were used in the preparation of liquid phases for CPC and HPLC. All chemicals and solvents needed were purchased from Aldrich (Steinheim, Germany), Fluka (Buchs, Switzerland) or Panreac (Barcelona, Spain).

NMR spectra were recorded on a Varian Mercury 400 (400 MHz) using $CDCl_3$ as a solvent. Spectra were completely assigned thanks to the concomitant registering of 2D NMR (COSY and HSQC) spectra. The purification of the different synthetic intermediates was carried out on an automated flash chromatography system, Combyflash® Rf (Teledyne Isco Lincoln USA) using a chromatographic silica gel RediSep® Rf 40 g flash column.

The CPC experiments were performed in a HPCPC model LLB-M (EverSeiko, Tokyo, Japan). This device consists of a bench centrifuge (30 cm × 45 cm × 45 cm) with a stacked circular partition disk rotor (2136 partition channels; 190 mL experimentally measured internal volume). This apparatus was connected between a manual injector (Rheodyne), equipped with a 2.4 mL loop, and the detector of a conventional HPLC system (pump, autosampler, UV detector and chromatographic data station) HP 1100 (Agilent Technologies, Palo Alto, CA, USA). The elution of the analytes was directly monitored by UV detection (254 nm). Additionally the fractions of the eluate (3 mL) collected throughout the experiment (Gilson FC 203B fraction collector) were analysed by enantioselective HPLC to individually determine the enantiomeric composition during elution. Elution profiles were constructed from the data obtained. The analytical control of the fractions was performed on the same HPLC system using the autosampler and substituting the CPC device for the chiral HPLC column. Temperature was maintained at 25 °C during CPC and HPLC runs.

2.2. Synthesis of racemic (±)-*1*

The procedure used was analogous to that reported by Pirkle et al. [10,11] for the synthesis of the CS contained in the Whelk-O® CSP. Nevertheless, in the present case a decyl chain is introduced by alkylation in the α-position of the intermediate ketone **4** (Fig. 2). Experimental details are given in the supporting information.

2.3. N,N-Diethyl-(S)-naproxenamide (*3*)

CS **3** was prepared from (*S*)-naproxen. The enantiomeric purity of **3** was corroborated by HPLC on a (*S*,*S*)-Whelk-O® CSP using methanol/water/acetic acid (80:20:0.1) as a mobile phase. A single peak at $k'_1 = 2.14$ was detected. When the same reaction was performed using racemic naproxen as a starting product a second peak at $k'_2 = 4.79$ ($\alpha = 2.24$, $R_s = 8.27$) was also observed in the same chromatographic conditions. According to the description of the elution order of naproxenamides in (*S*,*S*)-Whelk-O® CSP [15], the first eluted peak corresponds to the (*S*)-naproxenamide isomer.

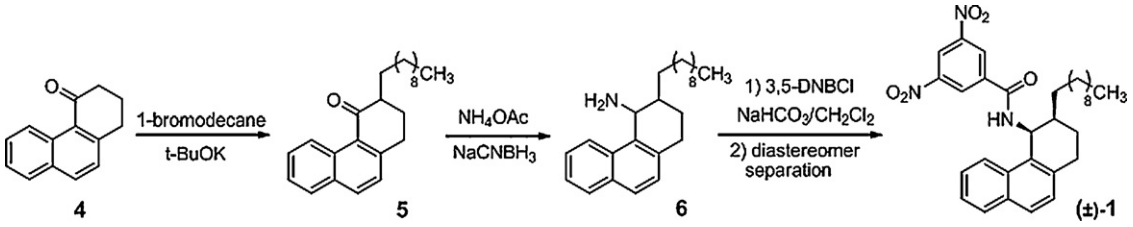

Fig. 2. Synthetic path for the preparation of racemate (±)-**1**.

Table 1
Solvent systems tested.

	Composition of the solvent system[a]	CS	Behaviour of CS	Behaviour of (±)-**1**
Binary solvent systems				
1	MTBE/NH_4OH 10 mM aqueous solution	2	L	U
2	MIBK/NH_4OH 10 mM aqueous solution	2	L	U
Ternary solvent systems[b]				
3	MTBE/ACN/water (20:30:50)	3	L	U
4	MIBK/acetone/water (40:20:40)	3	P	-
5	Heptane/n-butanol/ACN (50:18:32)	3	P	-
6	Heptane/n-butanol/ACN (30:16:54)	3	P	-
7	Heptane/n-butanol/ACN (80:6:14)	3	P	-
Quaternary solvent systems[c,d]				
8	Heptane/EtOAc/MeOH/water (4:1:4:1, U)	2	L	U
		3	L	U
9	Heptane/EtOAc/MeOH/water (6:1:6:1, W)	2	L	P
		3	L	U
10	Heptane/EtOAc/MeOH/water (9:1:9:1, X)	2	L	P
		3	L	P
11	Heptane/EtOAc/MeOH/water (19:1:19:1, Y)	2	P	–
		3	P	–

L, compound retained in the Lower phase of the system; U, compound retained in the Upper phase; P, Partition of compound in the two phases. Shaded cells indicate the compositions providing retention of the CS in one of the phases and partition of the racemate. MTBE, methyl tert-butyl ether; MIBK, methyl isobutyl ketone; ACN, acetonitrile; EtOAc, ethyl acetate; MeOH, methanol.

[a] v/v ratios indicated in parentheses.
[b] Compositions calculated on ternary diagrams [5].
[c] Water was substituted by a 10 mM NH_4OH solution for chiral selector **2**.
[d] Arizona solvent system family [18]. The letter in parentheses designates the composition described.

2.4. Selection of solvent system for the CPC experiments

The qualitative distribution of CSs and racemate in diverse biphasic solvent mixtures was tested by means of liquid–liquid extraction experiments to determine the most adequate solvent system to perform the CPC runs. A 100 mM solution of (±)-**1** (50 μL, 5 μmol) in ethyl acetate and 50 μL of a 100 mM solution of the CS (**2** or **3**) (5 μmol) in methanol were dispensed into test tubes with screw caps. After removal of the solvent under vacuum, an aliquot of 1 mL of the upper phase and an equal volume from the lower phase of the previously prepared solvent mixtures (Table 1) were added. The vials were closed and vortexed vigorously in a water bath at 25 °C for 5 min. The distribution of racemate and CS in the two phases was analysed by TLC (hexane/ethyl acetate 2:8 for CS **2** and 6:4 for CS **3**). All the solvent systems tested produced the partition of racemate (±)-**1**. The solvent systems that retained the CS in the lower aqueous phase were considered for the development of the CPC experiments.

2.5. Preparation of solvent systems and CPC experimental conditions

The quaternary biphasic solvent systems prepared for the separation of the enantiomers consisted of mixtures of heptane/ethyl acetate/methanol/10 mM aqueous NH_4OH (4:1:4:1) and (9:1:9:1), in the case of CS **2**, and heptane/ethyl acetate/methanol/water (9:1:9:1) for **3**. All mixtures were shaken in a separatory funnel and allowed to equilibrate for 16 h. Each phase was filtered and degassed separately before use.

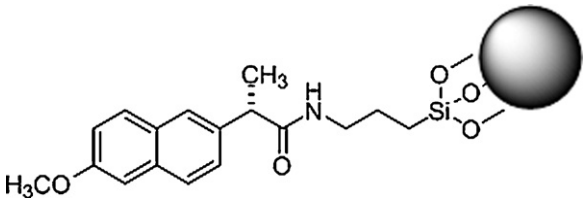

Fig. 3. Structure of the CSP used in the enantioselective HPLC analysis.

The lower phase, with a higher aqueous content, was used as stationary phase. Therefore, the elution was performed in ascending mode. The flow rate of the upper mobile phase was set at 3 mL/min and the rotational speed of the centrifuge at 1100 rpm. In these conditions the retention of the stationary phase in the centrifuge was 60%. The amount of CS involved in the separation was calculated from V_s value (115 mL). Samples were injected dissolved in the mobile phase once the system had reached a steady state.

2.6. Enantioselective HPLC analysis of fractions

Elution from the CPC system was directly monitored by UV detection at 254 nm. Nevertheless, the eluate was collected in fractions and their enantiomeric content was determined by enantioselective HPLC. A previously described CSP [16] containing a (*S*)-naproxen derivative as CS (Fig. 3) was used. The mobile phase was constituted by a mixture of heptane/2-propanol (80:20) (column dimensions: 100 mm × 4.6 mm i.d.) ((±)-**1**: $k'_1 = 6.34$, $\alpha = 1.5$, $R_s = 3.15$; CS **3**: $k' = 0.40$). The samples were prepared by diluting 250 μL of the eluate (containing mainly heptane and ethyl acetate) with 750 μL of the mobile phase (heptane/2-propanol 80:20). Elution profiles for the two enantiomers were constructed with the results obtained. All runs were performed at least twice.

2.7. Recovery of (±)-*1* enantiomers and CS *3* after CPC runs

Once the enantiomeric excesses (ee) of the samples recovered were determined, the fractions with an enantiomeric purity over 85% (% ee higher than 70%) were combined and the solvent was removed by rotary evaporation. The ee of the two enriched fractions was increased over 99% in a second CPC separation. The small amount of CS **3** that accompanies mainly the first eluted enantiomer of **1** in the CPC runs was easily removed by flash normal phase chromatography (hexane/ethyl acetate – 7:3).

Once the elution of the analytes was complete, the stationary phase was displaced from the stopped centrifuge by pumping the mobile phase in descending mode. The solvent of the solution containing CS **3** was eliminated by rotary evaporation. The resulting

residue was dried under vacuum. A recovery of 85% of CS **3** was determined.

2.8. Quantitative evaluation of CS and elution order of enantiomers in the eluate

A calibration curve was determined for **3** injecting known amounts of this compound on the (*S*)-naproxen-derived CSP for HPLC. For this purpose stock solutions of **3** at various concentrations were prepared. The curve obtained allowed us to quantify the amount of compound **3** accompanying mainly (*R*,*R*)-**1**.

The quantitative distribution of enantiomers and CS in the biphasic solvent system used in the CPC separation (heptane/ethyl acetate/methanol/water (9:1:9:1)) was examined in test tubes. Three independent determinations, racemate (±)-**1**, CS **3** and the two compounds simultaneously, were performed. In order to keep the experimental conditions as close as possible to CPC experiments a 100 mM concentration of CS **3** in the lower phase was used and 4 mg of (±)-**1** were added to 2 mL (1 mL upper phase + 1 mL lower phase) of the biphasic mixture. The distribution of racemate and CS in the two phases was determined by enantioselective HPLC using a previously described CSP (Fig. 3) [16] and conditions. Samples were prepared by diluting 250 μL of either the upper or the lower phase in 750 μL of 2-propanol. Distribution experiments were performed in duplicate.

3. Results and discussion

Most CSs used in CCC/CPC enantioseparations come from their application to chiral HPLC [17]. However, these CSs have to be adapted to the liquid–liquid conditions. As there is no solid support, the CS has to be retained in one of the liquid phases involved in the process by adapting its physicochemical properties. Considering that most biphasic liquid systems consist of a lipophilic organic phase and a polar aqueous phase, the properties of the intended CS have to be as close as possible to one of these two extreme situations, which will provide the adequate solubility and the lack of partition required for an ideal CS. Given the already lipophilic nature of the CS contained in the Whelk-O® CSPs, the additional increase in lipophilicity by the introduction of an aliphatic chain on the fundamental backbone of the molecule was considered. This aliphatic chain was introduced on the α position of ketone **4** (Fig. 2) by alkylation on basic conditions. The resulting alkylated ketone **5** was converted into the amine **6** by reductive amination. The generation of a second stereogenic center on the molecule in this reaction originates a mixture of stereoisomers in which the desired *cis* diastereomers are the major compounds. Nevertheless, the separation of stereisomeric mixture was only undertaken on the final amide mixture after the acylation reaction. Thus, firstly *cis* diastereomers were separated from the minor *trans* diastereomers by conventional flash chromatography and finally the enantioseparation of the *cis* (±)-**1** racemic compound was undertaken. Although the enantioseparation by HPLC of similar racemates has been described [11], due to the advantages that CCC/CPC provides for preparative operation, and given our interest in the application of this technique to enantioseparation, this was the procedure applied.

3.1. Selection of suitable chiral selector and solvent system

Considering the so-called reciprocity principle in enantiomer recognition, and taking into account that Pirkle et al. developed the Whelk-O® CSP on the basis of (±)-naproxen enantioseparation, commercially available (*S*)-naproxen (**2**) was the first tentative candidate as CS in the separation of (±)-**1**. In order to select the appropriate solvent system liquid–liquid extraction experiments were performed with **2** and (±)-**1**. Binary and quaternary solvent systems were tested (Table 1). Due to the acidic character of **2** ((*S*)-naproxen, p*Ka*: 4.5), water was substituted by an ammonia solution to ensure the retention of the CS in the aqueous phase, which was intended to act as a stationary phase. Although the CS was successfully retained in the aqueous phase as expected, no partition of (±)-**1** was detected in the binary solvent mixtures tested. Given the extreme polarities of the two solutes, a more flexible solvent system was considered. The quaternary solvent system family, composed of mixtures of heptane, ethyl acetate, methanol and water (Arizona family [18]) permits a finely tuned polarity. Partition of the racemate, while maintaining retention of CS **2** in the lower phase of the system, was attained for solvent systems W and X (entries 9 and 10 in Table 1). Unfortunately, attempts to resolve the enantiomers of (±)-**1** by using **2** as CS were unsuccessful. This negative result was attributed to the solvation of the anionic CS in the aqueous media which may hinder association with the neutral analyte. To avoid this ionization effect, the derivatization of the acidic position of (*S*)-naproxen with diethylamine was performed.

Various compositions of ternary mixtures (Table 1), covering a wide range of polarities and selectivities, were tested in the search of a solvent system adequate for the resulting amide **3**. Although in the most polar the racemate did not partition as required, in most cases it was CS **3** that partitioned in the two phases. The quaternary mixture of heptane, ethyl acetate, methanol and water in a ratio of 9:1:9:1 (system X) was the one that showed the best compromise between the retention of CS **3** and partition of the analyte (±)-**1**. Moreover, this system allowed a high solubility of **3** in the more polar lower phase.

A first attempt in the CPC separation of (±)-**1** was undertaken using solvent system X setting the CS **3** concentration at 50 mM. The enantioselective analysis of the eluate fractions allowed us to follow the elution of enantiomers separately. A slight leak of the CS to the mobile phase was detected. Nevertheless, it did not interfere with the determination of the enantiomeric content of the eluted fractions due to the different retention times observed for enantiomers and CS in the analytical chiral HPLC conditions used. Although the amount of racemate injected (100 mg, 0.2 mmol, molar ratio CS/analyte: 36.8) was far from the theoretical loadability limit for a highly enantioselective CS (molar ratio CS/analyte: 1) [8], only a partial separation of (±)-**1** enantiomers was obtained. A value of 1.13 was calculated for selectivity (α) on the elution profile.

3.2. Optimization of CPC preparative separation conditions: increasing CS concentration and loading capacity

Given the known relationship between concentration of CS in the stationary phase and enantioselectivity value [7–9], the amount of CS contained in the stationary phase was consecutively increased from 50 mM, used in the first separation, up to 75 and 100 mM. The enantioselectivity values for these two later runs in the resolution of 100 mg of (±)-**1** were calculated to be of 1.27 and 1.28, respectively. The slight increase produced from 75 to 100 mM is an indication that the maximum enantioselectivity value for the separation of (±)-**1** enantiomers using CS **3** in the chosen solvent system is almost attained [9] (Fig. 4). Simultaneously, although a partial separation is obtained in all instances, the resolution value increases also with increasing CS concentration. Therefore, given that the final aim of the study is the preparative separation of (±)-**1** enantiomers and considering the relationship between CS concentration and loading capacity of the chromatographic system [9], 100 mM was the concentration of CS used in the subsequent runs.

Once the CS concentration was optimized from the point of view of the enantioselectivity value, the best conditions to conduct repeated runs, in terms of sample loading *versus* yield and enantiomeric purity of the recovered enantiomers, were determined.

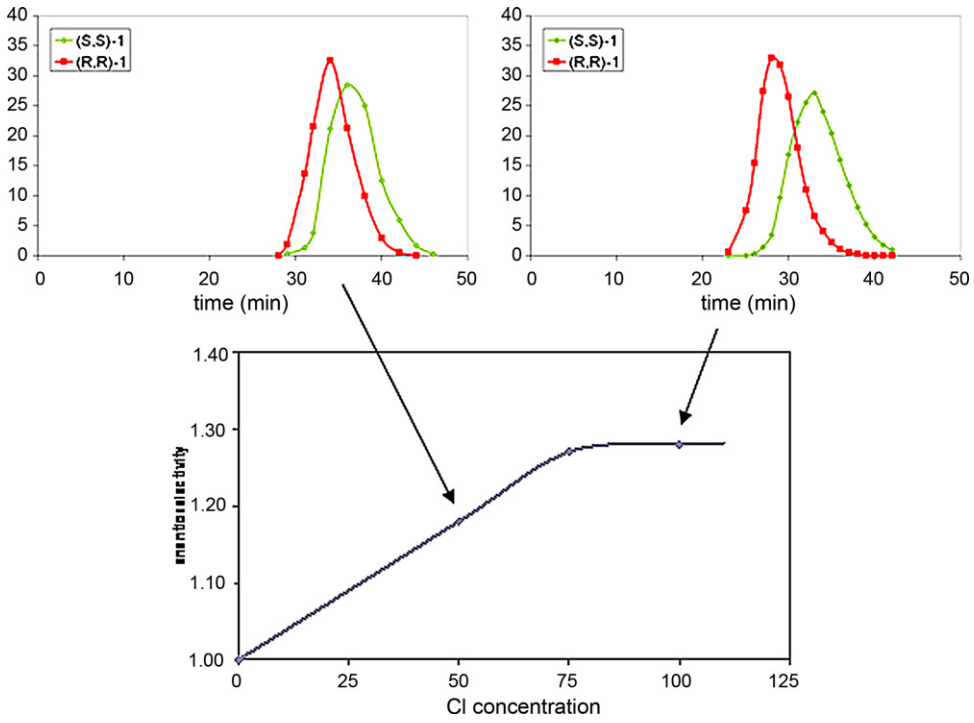

Fig. 4. Dependence of enantioselectivity on CS concentration.

Table 2
Yield and purity of recovered enantiomers in CPC runs at increasing amounts of racemate injected (CS concentration: 100 mM).

Racemate injected (mg)	$r_{CS/rac}$ [a]	α	(*R*,*R*)-**1** [b] (mg (%))	ee (*R*,*R*)-**1**	(*S*,*S*)-**1** [b] (mg (%))	ee (*S*,*S*)-**1**
200	27.10	1.34	70 (35%)	89%	60 (30%)	85%
300	19.10	1.28	90 (30%)	83%	60 (20%)	80%
400	12.20	1.21	116 (29%)	82%	76 (19%)	78%
500	11.80	1.21	150 (30%)	84%	100 (20%)	78%
650	10.90	1.21	169 (26%)	78%	78 (12%)	74%
900	6.90	1.23	135 (15%)	74%	99 (11%)	70%

[a] $r_{CS/rac}$, molar ratio CS/racemate involved in the separation.
[b] Amount of enantiomer recovered and percentage in respect to the injected amount. Fractions containing an enantiomeric excess (ee) over 70% were collected.

The progressive injection of increasing amounts of (±)-**1** (from 200 to 900 mg) in the chosen solvent system and CS concentration produced only a slight decrease of enantioselectivity and resolution values (Fig. 5). Considering the partial separation obtained the *peak-shaving* technique was applied. Fractions showing an enantiomeric purity over 85% (70% ee) for any of the two enantiomers were joined (Fig. 6). Yield and % ee were notably affected by the increase in the amount of sample injected (Table 2). The successive injection of amounts in the order of 450–500 mg was chosen to provide the best outcome in terms of yield and % ee. At these conditions, amounts in the order of 30% of the injected sample for the first eluted enan-

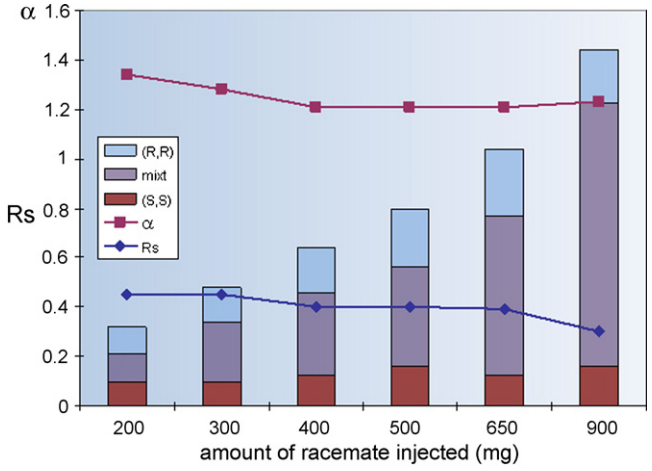

Fig. 5. Dependence of enantioselectivity and resolution on the amount of sample injected. The yield obtained for each enantiomer on a single run is indicated.

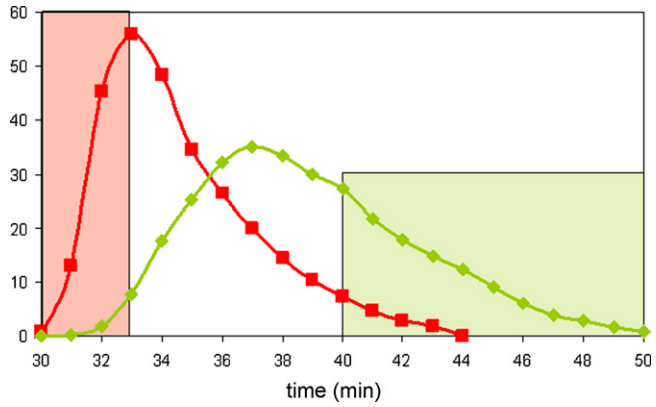

Fig. 6. Eluting profile corresponding to the separation of 400 mg of (±)-**1**. The shaded areas correspond to the regions in which the enantiomers were recovered (enantiomeric purity over 85%).

Table 3
Partition ratios (K_D).

	(*S*,*S*)-**1**	(*R*,*R*)-**1**	(±)-**1**	CS **3**
Isolated compounds[a]	0.78	0.76	0.77	7.62
Simulating CPC conditions[b]	0.49	0.31	0.39	3.75

[a] Partition ratios for each individual compound determined in the heptane/ethyl acetate/MeOH/water (9:1:9:1) solvent system.
[b] Partition ratios determined in conditions similar to those in the CPC experiment (CS concentration: 100 mM).

tiomer (84% ee) and a 20% of the second eluting enantiomer (78% ee) were recovered. The remaining 50% of the sample was re-injected together with more racemate.

A second run performed under the same conditions, in which the enantiomers recovered during a first cycle were re-injected, led to an increase in enantiomeric purity over 99% (98% ee) for the first eluting enantiomer and over 98% (96% ee) for the second eluting enantiomer. This procedure make feasible to obtain the desired compound (*R*,*R*)-**1** with the adequate enantiomeric purity.

3.3. Studying the inversion of elution order for (±)-*1* enantiomers by CPC respect to HPLC

Although not previously observed during the qualitative selection of the adequate solvent system in test tubes, a certain partition of CS **3** to the eluate was detected during the consecutive CPC runs. The concentration of CS **3** in the eluate was not constant during the elution. Instead, a maximum corresponding to the maximum elution of the first eluting enantiomer was observed. Also, the increase in the amount of racemate injected produced a simultaneous increase in the leak of CS to the eluate (Fig. 7). The molar ratio analyte/CS eluting at the maximum elution time for the first eluting enantiomer was determined to be close to 1, independently of the amount of analyte injected.

Given that the pair of Whelk-O® CS/naproxen-derived enantiomers has been the object of extensive studies [19,20], the absolute configuration of the isomers that originate the more stable adsorbates is known. According to the literature [15], in compounds similar to (±)-**1** the (*R*,*R*) isomer is the enantiomer establishing a stronger interaction with amides derived from (*S*)-naproxen. Therefore the most retained enantiomer in HPLC using a CSP derived from (*S*)-naproxen, is the (*R*,*R*)-**1** isomer. Nevertheless, when the fractions collected from the CPC separations were analysed by enantioselective HPLC on the (*S*)-naproxen-derived CSP, a reversed elution order of enantiomers was observed. Thus, the more retained isomer in HPLC resulted in the less retained enantiomer in CPC. This observation, together with the leak of CS **3** observed accompanying this isomer, was attributed to the elution of the enantiomer in the form of the more stable adsorbate with the CS [(*R*,*R*)-**1**·(*S*)-**3**]. The elution of the more lipophilic adsorbate instead of the free enantiomer may be favoured by the highly lipophilic nature of the mobile phase. Although not frequent, a similar behaviour has previously been described for the separation of (±)-kynurenine in an aqueous two phase solvent system (ATPS) using bovine serum albumin as CS [21].

To confirm this hypothesis, the partition ratio of (±)-**1** in the presence of CS **3**, at the same concentration used in the CPC experiment, was determined in comparison to the value obtained using the same solvent system in the absence of CS (Table 3). The partition ratio, K_D, in countercurrent chromatography is defined as the ratio of the solute concentration in the stationary phase over the solute concentration in the mobile phase [22]. Given that usually the CS in CPC enantioseparations acts by producing retention of the analyte in the stationary phase, often the more lipophilic phase of the solvent system, the partition ratio determined in the presence of the CS is higher than in the absence of this compound. Nevertheless, in the particular case of (±)-**1**, the more lipophilic phase of the solvent system is the mobile phase. However, K_D values are lower in the presence of the CS, in spite of being this latter located preferentially in the more polar stationary phase. Therefore, the CS is not even retaining the analyte in the stationary phase but promoting its elution to the mobile phase. Moreover, the partition ratio for the CS is also lower in the presence of the analyte, indicating the tendency for the CS to elute to the mobile phase when the analyte is present. Additionally, the partition ratio for the (*R*,*R*)-**1**, enantiomer, which forms the most stable adsorbate with the CS, is more affected by this phenomenon that its (*S*,*S*)-**1** counterpart.

The separation of CS **3** contained in the collected fractions of (*R*,*R*)-**1** was easily performed using conventional methods.

3.4. Comments on CPC and HPLC in the preparative separation of (±)-*1* enantiomers

Although the effect of differences in conditions (solvents used) and technical factors for the two techniques should not be overlooked, an attempt has been made to evaluate CPC in comparison with HPLC in the separation of (±)-**1** enantiomers. Two CSP for HPLC were used. Some of the advantages of the CPC technique for preparative operation can be illustrated by the results obtained (Table 4).

Given the availability in our laboratory of a CSP containing a (*S*)-naproxen-derived CS (Fig. 3) [16], and in spite of the analytical dimensions of the column (300 mm × 4.6 mm i.d.), a simple loading study was performed. Heptane/2-propanol (80:20) was used as mobile phase in the HPLC separation and increasing amounts of (±)-**1** were injected until "touching-bands" resolution (R_s = 1) was attained. The requirements on CS for a given amount of racemate processed are highly favourable to the CPC separation, which allows a better use of this compound. This implies less synthesis work in the preparation of the stationary phase for CPC. Also, the amount of solvent needed is considerably lower for the CPC separation, even considering that in these conditions a double run is required to increase the enantiomeric excess of the recovered enantiomers over 98%.

In contrast, limitations are also made evident for the CPC separation. Thus, HPLC permits a high enantiomeric excess in a single operation while, as mentioned, a second purification is required in the CPC conditions used. This is due to the inherent relatively low efficiency of the technique regarding that of HPLC. In this regard, the absence of solid support provides CCC/CPC with additional tools to improve resolution in a given separation. Among them, multiple dual-mode CCC has recently been demonstrated to improve the resolution in the separation of (±)-**1** enantiomers over 1.51, which corresponds to the complete separation of the isomers [23] in one run.

A second CSP containing a 3,5-dinitrobenzoyl derivative of L-phenylalanine as CS [16] showed a slightly lower enantioselectivity value for the separation of (±)-**1** enantiomers (α: 1.85, heptane/2-PrOH 90:10) than the (*S*)-naproxen-derived CSP. Considering the availability of a semipreparative column (300 mm × 10 mm i.d.) containing this CSP, the loading capacity for (±)-**1** was also tested. This column allowed us to reduce substantially the time required for the separation of a given amount of racemate with respect to the former analytical column. However, solvent consumption continues to be more than one order of magnitude higher than that calculated for the CPC separation and the relative amount of CS required is still far from the low amount needed in CPC.

The described preparative HPLC separation of a compound analogous to (±)-**1** [24] on a (*S*)-naproxen-derived CSP [25] allows us

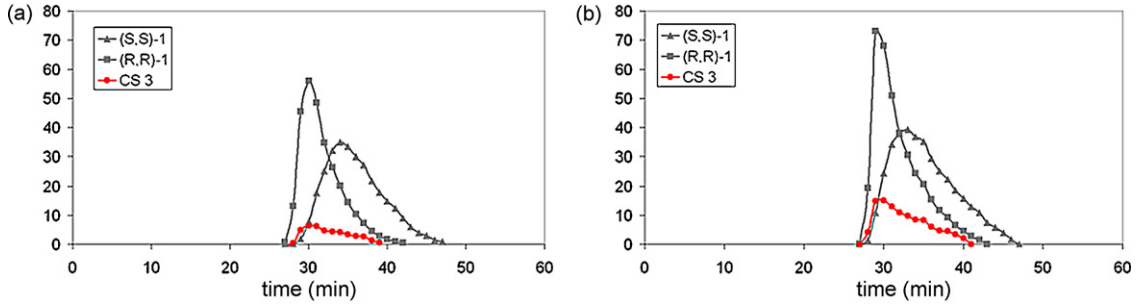

Fig. 7. Elution profile of the CS **3** during the separation of (±)-**1** enantiomers. Amount of racemate injected: (a) 400 mg; (b) 650 mg.

Table 4
Separation of (±)-**1** enantiomers on CPC and HPLC.

	CPC	HPLC analytical	HPLC semipreparative
Stationary phase	MeOH/water 90:10	Modified silica	Modified silica
Size of the column	140 mL[a]	300 mm × 4.6 mm i.d.	300 mm × 10 mm i.d.
Chiral selector	**3** (*N*,*N*-Diethyl-(*S*)-naproxenamide)	*N*-Silylpropyl-(*S*)-naproxenamide	*N*-(3,5-Dinitrobenzoyl)-L-phenylalaninamide
Mobile phase	Heptane/EtOAc (90:10)	Heptane/2-PrOH (80:20)	Heptane/2-PrOH (90:10)
CS in SP (mmol)	14.0	1.5	5.9
Loading capacity (mg)	500.0	1.0	10.0
$r_{CS/rac}$	14.9	807.3	315.3
Flow (mL/min)	3.0	1.0	8.0
Solvent consumption per gram of racemate (L)	0.7	20.0	48.0
Time required per gram of racemate (h)	4.0	333.0	100.0
Enantiomeric excess (*R*,*R*)/(*S*,*S*)	98/96	98/99	95/97

MeOH, methanol; EtOAc, ethyl acetate; 2-PrOH, 2-propanol.
[a] Volume of the stationary phase retained in the column (190 mL, total volume of the column).

to corroborate the observations. A molar ratio CS/racemate in the order of 35 mol/mol can be calculated for the described separation. This value, although considerably lower than those calculated from the HPLC experiments performed in our study, is still more than twice the amount calculated for the CPC separation. Unfortunately, run time is not given for the described separation although a flow rate of 35 mL/min is indicated. Considering this flow rate, the HPLC separation described would only be favourable to the CPC separation in terms of solvent consumption if the elution of the two enantiomers was shorter than 15 min.

4. Conclusions

In this study the separation of (±)-*N*-(3,4-*cis*-3-decyl-1,2,3,4-tetrahydrophenanthren-4-yl)-3,5-dinitrobenzamide, (±)-**1**, enantiomers by CPC is described. The separation was optimized in terms of CS concentration and loadability/yield of the resulting enantiomers. In spite of the partial separation produced and the slight leak of CS, the lower ratio CS/racemate and the lower solvent and time consumption makes CPC separation feasible and an alternative to HPLC. The inversion of the eluting order of enantiomers in CPC experiments respect HPLC using the analogous chiral moiety as CS has also been studied. This inversion is the result of the partition of the most stable adsorbate CS-enantiomer between the two phases due to the higher lipophilicity of this complex over that of the free enantiomers of **1**.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2009.12.023.

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