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Synthesis and evaluation of a chiral stationary phase based on quinine: Enantioresolution of dinitrophenyl derivatives of α-amino acids

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ABSTRACT

The natural alkaloid quinine (QN) was immobilized on porous silica particles, and part of the material was subsequently endcapped with n-hexyl hydrocarbon chains. Two synthetic strategies for silanization of the support were first compared. These columns were thoroughly evaluated in order to study the influence of endcapping in the enantiorecognition features. Enantioseparations of twenty N-derivatized 2,4-dinitrophenyl α-amino acids (DNP-amino acids) were studied by changing mobile phase pH, buffer concentration, type of organic solvent in the mobile phase, and column temperature. Maximum retention factors were observed at pH ≈6, at this intermediate pH the tertiary amine of the quinine is protonated to a high degree and therefore available for strong electrostatic interactions with unprotonated anionic DNP-amino acids. The enantioselectivity factors, however, increased as the pH did in the range between 5 and 7. The increase in ionic strength had influence on retention, but not on enantioselectivity, allowing the use of this variable for optimization of retention factors. Finally, the thermodynamic transfer parameters of the enantiomers from the mobile to both CSPs (with and without endcapping, QN-CSP(EC) and QN-CSP, respectively) were estimated from van't Hoff plots within the range of 10–40 °C. Thus, the differences in the transfer enthalpy, $\Delta(\Delta H^\circ)$, and transfer entropy, $\Delta(\Delta S^\circ)$, enabled an investigation of the origin of the differences in interaction energies.

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1. Introduction

The introduction of chromatographic methods based on chiral stationary phases (CSPs) has become one of the most frequently used approaches to chiral resolution, mainly owing to the intrinsic simplicity of CSPs and the straightforward extension of such phases to semi-preparative and preparative scales.

Several natural chiral compounds have been used as raw material to develop a broad variety of optically active stationary phases. Natural products constitute an attractive source of resolving agents for the development of highly efficient and selective CSPs that would be subsequently transferred to a preparative scale at lower costs.

Cinchona alkaloids are, in fact, natural and accessible chiral products widely known for their therapeutic properties. These compounds have also numerous technological applications, one of the most widespread related to their chemical structure that allows them to induce asymmetric synthesis reactions. Numerous review papers have discussed in detail the use of these alkaloids in homogeneous and heterogeneous chiral catalysis [1,2]. These compounds have also been used as resolving chiral bases for crystallization of chiral acids [3,4].

In chromatographic methods, the use of these alkaloids as chiral selectors (CSs) was early tested. First, quinine acetate was used as ion-pair reagent dissolved in dichloromethane with up to 1% (v/v) n-pentanol in combination with a diol column. This system was useful to separate enantiomers of carboxylic and sulphonic acids with enantioselectivity factors ranging between 1.30 and 1.50 [5]. Then, natural quinine (QN) and acetylquinine were fixed to the solid silica surface, and the chiral column was successfully employed under normal phase conditions [6–10].

Wolfgang Lindner and co-workers developed a group of Pirkle-type chiral columns for anion-exchange conditions based on several cinchona derivatives, mainly carbamates of quinine and quinidine [11–15]. These CSPs were successfully used for the direct resolution of chiral anions and for several amino acid derivatives, such as N-3,5-dinitrobenzoyl, N-2,4-dinitrophenyl (DNP), N-3,5-dinitrobenzyloxycarbonyl, benzoyl [14,15] and fluorenylmethoxycarbonyl [16] derivatives. Enantioseparations were possible due to electrostatic interactions along with additional intermolecular interactions, including hydrogen bonding, dipole–dipole, π–π, hydrophobic interactions and steric hindrance. In spite of the numerous cinchona derivatives that have been developed, some of which are commercially available [17], to the best of our knowledge, the native (non-derivatized) alkaloids have not

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been used in a systematic study under different ion-exchange conditions. In capillary-electrophoretic separation of acidic solutes, QN was also used as additive to buffer solution in [18].

In this study, QN was immobilized on porous mercaptopropyl silica particles through the vinylic double bond. A modification of the initial silanization reaction was introduced in order to increase the CS density on the solid surface. The unreacted –SH groups were then endcapped on about half of the synthesis product, and both materials were packed into analytical columns. These columns were used to separate the enantiomers of N-derivatized α-amino acids. Both phases were critically compared in their retention and enantiorecognition abilities.

In order to optimize the chromatographic conditions, the following mobile-phase parameters were evaluated: mobile-phase pH, total buffer concentration, type of organic solvent (methanol or acetonitrile) and column temperature. The thermodynamic parameters for the transfer between mobile and stationary phases were estimated. The knowledge of the dependence between enantioselectivity and temperature for a particular racemate in the CSP–eluent system facilitates the proper choice of temperature for optimizing a given separation.

2. Experimental

2.1. Chemicals

All the reagents used were reagent-grade or higher quality. Spherical silica particles (Nucleosil 100-5 and 120-5), with 5-μm average diameter, were purchased from Macherey-Nagel (GmbH & Co., Düren, Germany). These two silica materials have different pore size and specific surface area; their physical properties are reported in Table 1. Anhydrous quinine (98%) was provided by Fluka (Buchs, Switzerland), 2,2′-azoisobutyronitrile (AIBN), 1-hexene, 2,4-dinitrofluorbenzene and (3-mercaptopropyl)-trimethoxysilane (95%) were obtained from Aldrich (St. Louis, MO, US). Racemic amino acids were from Sigma (St. Louis, MO, US) or from BDH (BDH Ltd., UK). HPLC-grade methanol (MeOH) and acetonitrile (MeCN) were purchased from Mallinckrodt (Mallinckrodt Baker Inc., Phillipsburg, NJ, US). Water was purified by means of a Milli-Q Purification System (Simplicity, Millipore, Massachusetts, US).

2.2. Synthesis of the chiral stationary phase (CSP)

2.2.1. Synthesis of 3-mercaptopropylsilica gel

The following procedure (synthesis 1) was used for one of the CSPs based on QN. Nucleosil 120-5 (3 g) silica was suspended in 75 ml of freshly distilled toluene under anhydrous conditions. (3-Mercaptopropyl)-trimethoxysilane (5.10 ml, 26.7 mmol) dissolved in 5.1 ml of freshly distilled pyridine was added dropwise under a stream of nitrogen. The slurry was stirred and refluxed under a nitrogen atmosphere for 36 h. After sedimentation, the solid was washed successively with toluene, diethyl ether, and n-hexane and finally dried under vacuum. The silanization efficiency was estimated from the results of elemental analysis (see Table 1).

For the other procedure (synthesis 2), Nucleosil 100-5 silica was used. The modification consisted in the addition of 750 ppm of water to 75 ml of distilled toluene before the suspension of the silica particles. The remaining procedure was followed in the same way as above. Elemental analysis was conducted after carrying out this protocol.

2.2.2. Synthesis of alkaloid-based CSP

The 3-mercaptopropylsilica (about 3.35 g) was suspended in 50 ml of previously dried chloroform with QN (1.2 mmol) and 55 mg AIBN. The slurry was stirred and refluxed under nitrogen for 35 h. The solid was washed with a sequence of chloroform, methanol, acetonitrile, again methanol and diethyl ether, and finally dried over P_2O_5. The CS coverage was calculated on the basis of nitrogen content.

2.2.3. Endcapping reaction

A portion of about 1.75 g of QN-CSP was treated with 1 ml of 1-hexene and 100 mg AIBN in 50 ml of dried chloroform. The slurry was stirred and refluxed under nitrogen for 15 h. The solid was washed (chloroform, methanol and diethyl ether) and dried under vacuum. The calculated coverage of hexyl groups after the endcapping reaction was 0.107 mmol/g of material. The H^1–C^{13} NMR spectra of these CSPs, obtained at the Instituto de Ciencia de Materiales, University of Zaragoza, Spain, confirmed the covalent linkage between the cinchona alkaloid and the silica particles.

These CSPs were downward-packed (Alltech packer, model 1666) into 5.0 × 0.46-cm analytical columns by a conventional slurry technique at 5000 psi.

2.3. Mobile phases

The mobile phase consisted of aqueous NH_4OAc buffer mixed with the organic solvent (either MeOH or MeCN). The pH of these buffer solutions was measured with a glass electrode (Methrom) connected to a Fisher Scientific Accumet AR pH-meter after calibration with aqueous standard buffer solutions ($^{s}_{w}$pH by following the nomenclature suggested by IUPAC [19]). After the mixing of the aqueous and organic phases, the $^{s}_{w}$pH of the mobile phase was readjusted with either hydrochloric acid or sodium hydroxide to a fixed $^{s}_{w}$pH value between 5.0 and 7.0. In addition, three buffer concentrations between 0.05 and 0.15 M, in 72% (v/v) MeOH were systematically studied.

2.4. Apparatus

Chromatographic studies were performed on an HP 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA) equipped with vacuum degasser, binary pump, autosampler, thermostated-column device, DAD detector and computer-based HP Chemstation. The mobile phase flow-rate was set to 1 ml/min.

The protocol followed for the synthesis of the DNP-compounds was from the literature [20]. The sample injection volume was 5 μl, and the derivatized α-amino acids were detected at 365 nm. The hold-up times were estimated by injection of KBr with detection at 210 nm. Under all the conditions, retention times were taken at the peak maxima.

3. Results and discussion

The structures of the chiral stationary phases prepared in this study consist in QN linked to mercaptopropylsilica, both without and with a final endcapping reaction with 1-hexene. These two phases were physically characterized and then packed into 5-cm columns. All the relevant chromatographic parameters were systematically evaluated for optimization of the enantioresolution of DNP-amino acids.

3.1. Considerations concerning the CSP synthesis

The efficiency of the surface coverage reaction should be as high as possible due to the strong dependence between enantioseparation factors and CS concentration. Recently, the replacement of organic-liquid solvents with supercritical carbon dioxide for the synthesis of mercaptopropyl-bonded silica has lead to a notable improvement in solid-support coverage [21,22].

Table 1
Elemental analysis of QN-based CSPs. Estimation of the CS content.

Silica-pore size(Å)	Surface area (m^2/g)	Synthesis model	Elemental analysis	Loading of SH groups (yield of silanization)	Loading of CS (yield of CS addition/added)
120	200	SM1	7.69% C 0.63% N 1.79% S	0.603 mmol SH/g silica	0.298 mmol QN/g silica
			1.12% H	(37.7%)	(49.4%)
100	350	SM2	11.27% C 0.91% N 3.45% S	1.258 mmol SH/g silica	0.431 mmol QN/g silica
			1.83% H	(44.9%)	(34.2%)

In most of the studies about silica-surface modification in a liquid medium, extreme caution has been taken to prevent moisture during the silanization reaction [6,11]. By contrast, Engelhardt et al. [23] described that higher yields of silica functionalization through a reaction with alkoxysilanes were possible when the silica surface had been previously activated by the formation of a water monolayer. In this study, the anhydrous conditions during the synthesis were compared with a procedure in which a controlled amount of water was previously added.

Table 1 reports the results of this comparison. Under strict anhydrous conditions (protocol synthesis 1), 3.0 μmol/m^2 of mercaptopropylsilyl groups (estimated from sulfur content in the elemental analysis) were incorporated. This is considered a reasonable silanization efficiency [24–26]. An increment of 20% in the density of –SH groups incorporated to the solid was achieved by following the protocol 2. This increment was higher than expected on the basis of the available areas of the two starting material.

The surface CS densities obtained after the second reaction were comparable to and even higher than those reported in the literature. Maier et al. [13] synthesized four CSPs fixing t-butyl carbamoyl derivatives of quinine, quinidine, cinchonine and cinchonidine obtaining coverages of 0.29, 0.25, 0.31 and 0.20 mmol CS/g for a silica surface area of 308 m^2/g. In other studies, the same authors reported CS concentrations ranging from 0.13 to 0.35 mmol CS/g silica [11,27]. Subsequently, the authors reported a selector concentration of 0.32 mmol CS/g of silica for a chiral phase based specifically on QN [28]. Salvadori's group [6], who followed a slightly different synthesis procedure, estimated a value for the coverage of QN and a derivative thereof of 0.39 mmol CS/g of silica. Their assessments were based on the hydrolysis of the CSP and the spectroscopic measurement of the QN released.

3.2. Considerations concerning retention: influence of the mobile-phase conditions

Since previous studies had suggested that quinine and quinidine were relatively labile upon warming with acetic acid at 70–80 °C for 48 h [29], we checked the stability of the stationary phases over a period of time. Two racemates, DNP-Phe and DNP-Ile, were injected repeatedly under constant mobile-phase-pH and column conditions. These experiments indicated that the variation in retention times of these solutes in these columns was reasonable over more than 8000 column volumes, and although the columns have been extensively used with relatively aggressive mobile phases no deterioration in selectivity factors occurred over several months.

These CSPs can be classified as WAXs, and thus the buffer concentration, eluent pH, type and content of organic solvent can be used to control solute retention and selectivity. We summarized the retention factors, enantioselectivities and resolution factors of all the solutes in both columns in Table 2. These data correspond to chromatographic runs carried out at 20 °C and at two pHs: 6.3 and 7.0. When several amino acids were also run as pure enantiomers to evaluate the order of elution, the L-amino acid eluted before the D-enantiomer.

Fig. 1 depicts the dependence of retention of some DNP-amino acids on these QN-based columns on the mobile phase pH. The retention factors of all derivatives have a maximum at $^{s}_{w}$pH about 6.0–6.5 in the mobile phase with 72% (v/v) MeOH. The decreased retention at pHs higher than 7 results from the loss of the proton in the quinuclidinic nitrogen of the alkaloid. The pK_a corresponding to the quinuclidinic nitrogen in 72% MeOH decrease to about 7.7 respect to the value in water [30,31]; that is, at $^{s}_{w}$pH = 7.05, about a 20% of the quinine is unprotonated.

Decreased retention at $^{s}_{w}$pH 5.0–5.5 is attributed to the acid–base properties of the solutes. Typical values of pK_{a1} in water for native amino acids (carboxylic groups) are between 2.0 and 2.7. After mixing with 72% (v/v) MeOH the pK_{a1} increases about 1.05 units [32]. Thus, at the working pH, all the carboxylic groups are charged. The inclusion of the DNP-group to amino acids converts the primary aliphatic N into an N-substituted (much less basic) aniline. Although the pK_a's of these specific derivatives are unavailable, the pK_a values of similar structures, (for instance, N-methylaniline and N-ethylaniline) are, respectively, 4.85 and 5.12 in water [30]; and these values will be still lower as a result of the two nitro groups attached to the aromatic ring. These data would indicate that although the DNP-amino acids persist as anions at $^{s}_{w}$pH close to 6, a fraction of those derivatives become zwitterions at $^{s}_{w}$pH close to 5 and will accordingly be less strongly retained by the protonated QN-CSP.

In addition to electrostatic attraction, retention is strongly dependent on the hydrophobicity and aromaticity of the molecules. A very high retention was observed for lysine and tyrosine derivatives. DNP-reactions involving an amino acid with ε-amino groups (*e.g.*, lysine), a phenolic hydroxyl (*e.g.*, tyrosine) or an imidazol group (*e.g.*, histidine) usually yield derivatives containing two DNP moieties [33,34]. The impressive increase in retention observed for lysine and tyrosine derivatives (but not for histidine) account for the magnitude of the hydrophobic and/or π–π interactions between the quinoleic group of the QN-CSP and the DNP_2-Lys and DNP_2-Tyr derivatives.

The influence of eluent pH on enantioselectivity can be seen in Fig. 2. The enantioseparation of all the DNP-amino acids systematically decreases with decreasing pH. The same pattern was observed at the other temperatures as well as with the column without endcapping.

In order to study the influence of the ionic strength, the chiral separation of several DNP-amino acids was assessed at three different total buffer concentrations (C_{tot}). The mobile phase was a mixture of 72% MeOH and 28% ammonium acetate buffer at $^{s}_{w}$pH = 6.03, where the QN has full exchange capacity; and the measurements were carried out at two temperatures. As expected in systems with retention mechanism based primarily on anion-exchange, retention decreases when the buffer concentration is increased. The enantioselectivity, however, was practically not affected by the ionic strength. Thus, within reasonable limits, the

Table 2
Retention factor of first-eluting stereoisomer (k_1), enantioselectivity (α), and resolution (R_s) for DNP-amino acids at 20 °C over QN-CSP and QN-CSP(EC) at two different $^{s}_{w}$pH values.

DNP-amino acid	QN-CSP						QN-CSP(EC)					
	6.30			7.00			6.30			7.00		
	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s
Pro	9.84	1.26	1.6	6.30	1.26	1.6	14.26	1.25	2.0	9.54	1.25	1.9
Ser	9.64	1.28	1.8	6.11	1.29	1.9	13.99	1.29	2.3	9.01	1.29	2.3
Val	11.17	1.28	1.8	8.92	1.30	1.7	18.61	1.28	2.3	12.20	1.29	2.3
Ala	12.47	1.18	1.2	7.93	1.18	1.3	19.43	1.18	1.5	12.53	1.18	1.5
Leu	11.89	1.29	1.8	7.52	1.28	1.7	13.91	1.29	1.7	13.93	1.31	2.4
Phe	22.31	1.34	2.0	13.78	1.35	2.0	24.72	1.30	1.8	24.89	1.32	2.3
Trp	39.18	1.48	2.4	23.48	1.49	2.5	50.27	1.46	2.1	45.02	1.49	3.3
Met	19.48	1.20	1.2	11.81	1.20	1.3	29.72	1.19	1.5	19.76	1.20	1.7
Nor	15.56	1.22	1.4	9.76	1.22	1.5	27.36	1.22	1.8	18.09	1.31	1.8
Abu	12.26	1.22	1.4	7.70	1.22	1.4	24.58	1.21	1.8	12.90	1.22	1.8
Thr	7.84	1.29	1.9	5.05	1.30	1.8	14.69	1.30	2.4	7.63	1.30	2.3
Arg	2.61	1.15	< 1	2.43	1.17	< 1	2.52	1.14	< 1	2.11	1.15	< 1
Ile	12.57	1.21	1.2	10.63	1.22	1.3	18.19	1.20	1.6	16.51	1.21	1.7
Cys	13.43	1.14	< 1	-	-	-	15.44	1.13	0.6	10.62	1.14	0.6
His	7.37	1.14	1.1	6.73	1.15	1.1	38.17	1.17	1.3	24.28	1.17	1.3
Asp	8.44	1.09	0.6	6.91	1.11	0.6	10.70	1.11	0.8	7.12	1.12	0.8
Asn	8.56	1.09	0.6	7.01	1.11	0.6	10.73	1.12	0.8	6.99	1.08	0.9
Lys	103.22	1.18	0.9	81.17	1.19	0.7	141.62	1.16	1.3	104.96	1.16	1.3
Tyr	97.51	1.16	0.8	76.98	1.16	0.7	135.79	1.14	0.8	87.20	1.15	1.1
Glu	52.96	1.00	0.0	61.00	1.00	0.0	93.34	1.00	0.0	75.67	1.00	0.0

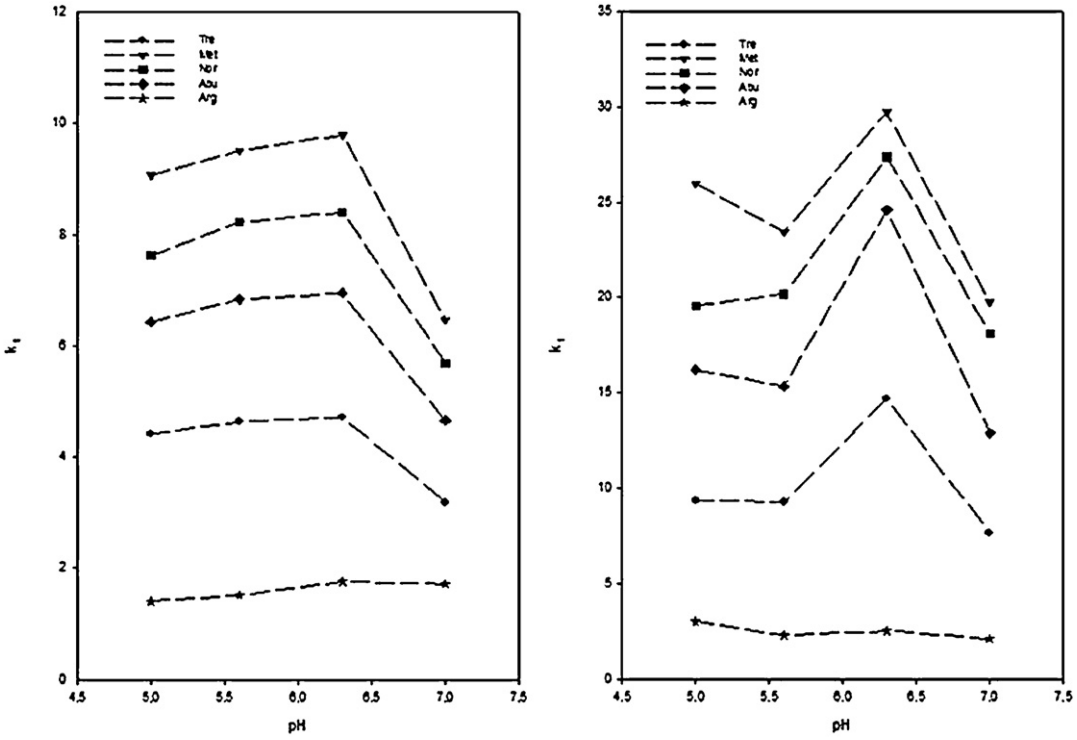

Fig. 1. Retention factors for DNP-amino acids as a function of $^{s}_{w}$pH. QN-CSP column, 40 °C (left). QN-CSP(EC) column, 20 °C (right). Chromatographic conditions: mobile phase 72% (v/v) MeOH, 28% buffer. Flow 1 ml/min.

Table 3
Retention factor of the first-eluting enantiomer (k_1) and enantioselectivity (α) of DNP-amino acids on QN-CSP at $^{s}_{w}$pH = 6.03 and different ionic strengths of the mobile phase at 20 °C and 40 °C.

DNP-amino acid	0.05 M				0.10 M				0.15 M	
	20 °C		40 °C		20 °C		40 °C		40 °C	
	k_1	α	k_1	α	k_1	α	k_1	α	k_1	α
Pro	40.12	1.27	24.26	1.23	26.23	1.27	16.25	1.22	9.97	1.22
Ser	38.40	1.30	21.77	1.22	25.40	1.30	14.72	1.22	8.98	1.22
Ala	42.63	1.30	24.97	1.24	27.91	1.29	16.64	1.24	10.51	1.23
Met	–	–	34.40	1.16	–	–	25.50	1.17	16.09	1.17
Arg	3.29	1.15	1.97	1.14	3.20	1.15	1.90	1.14	1.75	1.18

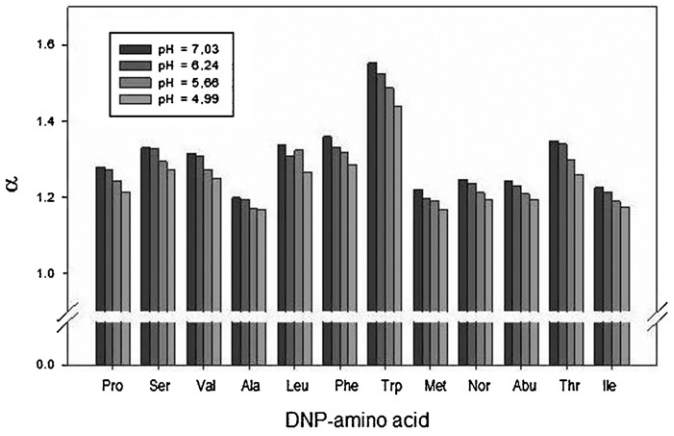

Fig. 2. Enantioseparation factors of racemic DNP-amino acids as a function of ${}^{s}_{w}$pH. Column QN-CSP(EC). Chromatographic conditions: mobile phase 72% (v/v) MeOH, 28% buffer. Flow 1 ml/min. Column temperature: 10 °C.

retention can be optimized by changing the ionic strength without any consequence with respect to enantioselectivity (Table 3).

Considering the type of the organic solvent used in the mobile phase for the DNP-derivatives, we recorded the chromatograms of several solutes for isoeluotropic mobile phases at the same ${}^{s}_{w}$pH based on either MeOH or MeCN. The use of MeCN gave better peak shapes in terms of symmetry whereas MeOH systematically led to a higher enantioselectivity (Table 4). It can be inferred that the π–π interactions would be weakened in the (70:30) MeCN/buffer mixture, which has a dielectric constant about 20% larger than the (80:20) MeOH/buffer mixture, decreasing the subtle differences that makes enantioseparation possible.

3.3. Comparison between QN-CSP and QN-CSP(EC)

The influence of endcapping on the enantiorecognition features of this type of column has been barely studied. Pirkle and Readnour [35] investigated the influence of endcapping in the enantiorecognition ability of an alanine-based CSP under normal phase conditions, using a mixture of n-hexane and 2-propanol as eluent. They concluded that the enantioselectivities in both columns only changed when the column contained a low surface density of CS (between 0.036 and 0.083 mmol/g).

We performed a systematic comparison between both columns. Fig. 3 shows the separation of DNP-Ala and DNP-Ser from the QN-based columns both with and without endcapping treatment. The increase in hydrophobicity of the CSP caused by the hexyl residues linked to the surface led to higher retention.

Fig. 4 compares the retention factors of the first eluted enantiomer of the DNP-amino acids on both columns at ${}^{s}_{w}$pH = 6.24 and 7.03 and at four temperatures. A strong correlation between the retentions in both columns, which is apparently independent of temperature, is clearly observed. Larger retentions were measured in the more hydrophobic column. At ${}^{s}_{w}$pH = 7.03 and at the higher temperatures, Arg and Cys become the exception: they had retention factors about 10–15% greater with the more polar CSP.

When the enantioseparation factors obtained with both columns were compared by a Student *t*-test of the paired observations, any significant difference (at 95% of confidence) between both α-values under any condition was observed.

The retention of a given chiral solute in a CSP will be determined by two contributions: the specific interaction between each enantiomer with the chiral moiety and the nonspecific one with the solid surface that is equal for both enantiomers. In such a circumstance, when the non-stereoselective retention dominates, the *experimental* enantioseparation is clearly smaller than

Table 4
Retention factor of first-eluting stereoisomer (k_1), enantioselectivity (α), resolution (R_s), and assymmetry factor (A) of both enantiomers for DNP-amino acids at 20 °C, with isoeluotropic mobile phases based on MeOH and MeCN on QN-CSP.

DNP-amino acid	MeOH 20% buffer 100 mM ${}^{s}_{w}$pH = 5.50				MeCN 30% buffer 100 mM ${}^{s}_{w}$pH = 5.50			
	k_1	α	R_s	A	k_1	α	R_s	A
Pro	6.82	1.25	1.5	1.9-1.6	5.93	1.19	<1	1.0–1.4
Nor	8.90	1.23	1.3	1.0-1.0	11.25	1.16	1.3	1.3–1.4
Phe	13.08	1.30	2.1	1.8-1.9	13.48	1.22	1.8	1.3–1.5
Trp	24.36	1.42	2.6	1.8-1.8	18.60	1.31	2.4	1.5–1.4
Ala	8.55	1.00	–	2.3	7.88	1.00	–	1.8

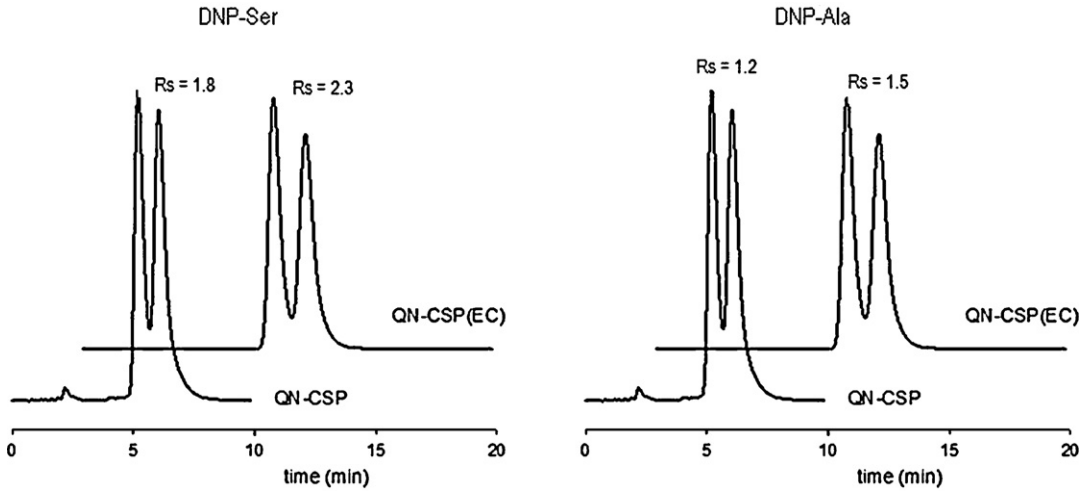

Fig. 3. Chromatograms of DNP-Ala and DNP-Ser obtained in QN-based columns. Chromatographic conditions: mobile phase 72% MeOH, 28% buffer ${}^{s}_{w}$pH = 6.30. Flow 1 ml/min. Column temperature: 20 °C, detection 365 nm.

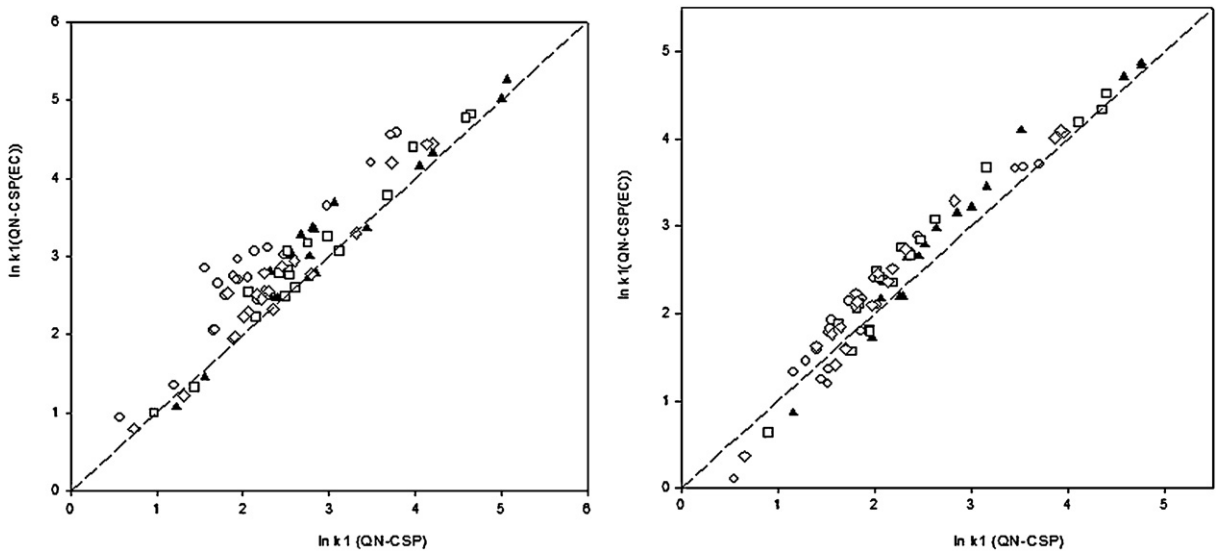

Fig. 4. Comparison between retention factors obtained in quinine-based chiral columns without and with endcapping for DNP-amino acids. Mobile phase: 72% (v/v) MeOH, 28% buffer. $^{s}_{w}$pH = 6.24 (left), $^{s}_{w}$pH = 7.03 (right). Column temperature: (▲) 10 °C; (□) 20 °C; (◯) 30 °C; (◊) 40 °C.

the *true* enantioselectivity. If the introduction of hydrocarbon chains leads to a more retentive stationary phase, this difference should be attributed *a priori* to hydrophobic, non-enantioselective interactions, and thus, the experimental enantioselective factors should decrease relative to the corresponding parameters for the untreated CSP. On the basis of these theoretical considerations, the incremented retention of DNP-amino acids in the endcapped column without any change in enantioselectivity is surprising.

On the other hand, slightly higher resolution factors were achieved with the end-capped column due to improved plate counts systematically obtained with the end-capped column.

3.4. Influence of temperature

The enantioseparations of the DNP-amino acids in both columns were measured in the $^{s}_{w}$pH range of 5.0–7.0 over the temperature range of 10–40 °C. Fig. 5 shows plots of $\ln k_i$ (and $\ln \alpha$) of five DNP-amino acids of different polarities vs. $1/T$ as examples of the thermal behavior of these compounds. The plots were satisfactorily linear within this narrow temperature interval, and the average standard deviations of the data fits were mostly smaller than 0.05. The *experimental* thermodynamic transfer quantities of the enantiomers to the stationary phase were obtained from:

$$\Delta H_i^\circ = \frac{-R\,\partial \ln k_i}{1/T} \quad (1)$$

$$\Delta(H^\circ) = \frac{-R\,\partial \ln \alpha}{\partial(1/T)} \quad (2)$$

$$\Delta(\Delta S^\circ) = \frac{\Delta(\Delta H^\circ) - \Delta(\Delta G^\circ)}{T} \quad (3)$$

where $\Delta(\Delta H^\circ)$, $\Delta(\Delta S^\circ)$ and $\Delta(\Delta G^\circ)$ represent the molar differential enthalpy, entropy and Gibbs free energy of transfer from the mobile to the stationary phase, respectively, and ΔH_i° represents the molar enthalpy for the transfer of the *i*-enantiomer between phases. We must consider, however, that since the non-enantioselective interactions also contributed to retention and were no discounted, the thermodynamic functions estimated from Eqs. (1)–(3) are only apparent values so that the quantities reported here should therefore be regarded as rough estimations of the true thermodynamic parameters. Nonetheless, because the conclusions made from these quantities are based on the observations of a large

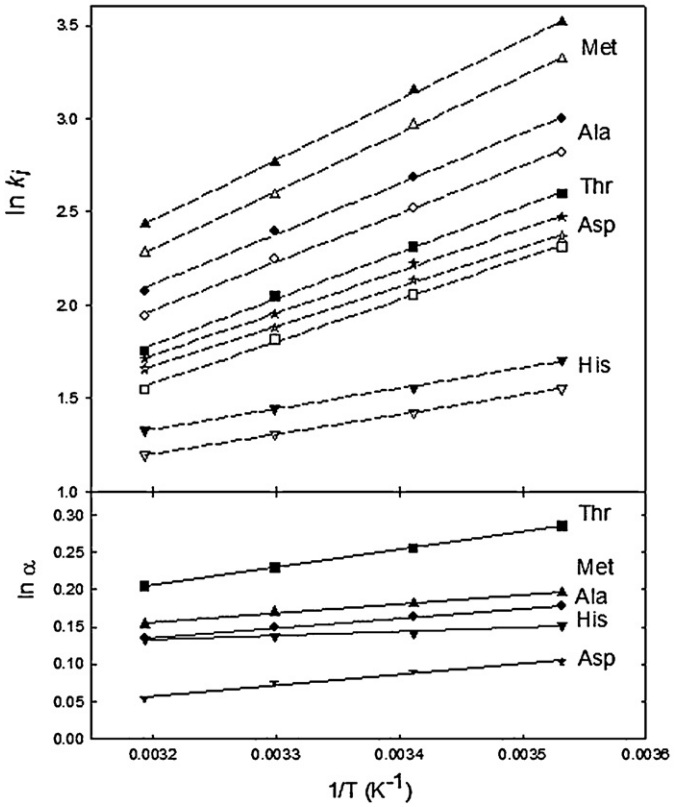

Fig. 5. Plots of $\ln k_i$ (top) and $\ln \alpha$ (down) against $1/T$ for DNP-amino acids. Column: QN-CSP. Mobile phase: 72% (v/v) MeOH, 28% buffer $^{s}_{w}$pH = 6.24.

number of systems, even though their individual values are not exact, the overall data show clear trends.

The results, gathered in Table 5, indicate that most solutes had negative enthalpies and entropies of transfer, and only His and Lys had statistically null values. The thermodynamic differences $\Delta(\Delta H^\circ)$ were plotted against $T\Delta(\Delta S^\circ)$ at 298 K (Fig. 6). The enantioseparations in these systems were dominated by the enthalpic differences although these differences were correlated with the entropic contributions to the Gibbs free energy. The correlation

Table 5
Thermodynamic-transfer functions for equilibrium between phases of DNP-amino acids on QN-CSP at ${}^{s}_{w}\mathrm{pH} = 7.03$.

DNP-aminoacid	QN-CSP					QN-CSP(EC)				
	ΔH_1°[a] (kJ mol^{-1})	$\Delta(\Delta H^{\circ})$ (kJ mol^{-1})	$\Delta(\Delta G^{\circ})$[c] (kJ mol^{-1})	$-T\Delta(\Delta S^{\circ})$[d] (kJ mol^{-1})	$RT\ln k_1$ (kJ mol^{-1})	ΔH_1°[b] (kJ mol^{-1})	$\Delta(\Delta H^{\circ})$ (kJ mol^{-1})	$\Delta(\Delta G^{\circ})$[c] (kJ mol^{-1})	$-T\Delta(\Delta S^{\circ})$[d] (kJ mol^{-1})	$RT\ln k_1$ (kJ mol^{-1})
Pro	−16.8 (±0.6)[b]	−1.37 (±0.03)[a]	−0.568	0.80	4.84	−15 (±1)[a]	−1.21 (±0.01)[a]	−0.557	0.66	5.78
Ser	−20.0 (±0.2)	−2.24 (±0.02)	−0.616	1.62	4.41	−18 (±2)	−2.09 (±0.04)	−0.628	1.46	5.79
Val	−15 (±1)	−1.70 (±0.03)	−0.631	1.07	5.34	−17 (±2)	−1.50 (±0.04)	−0.622	0.88	6.48
Ala	−20.9 (±0.1)	−1.24 (±0.03)	−0.409	0.83	5.05	−18 (±3)	−1.09 (±0.03)	−0.408	0.68	6.60
Leu	−23 (±3	−2.2 (±0.4)	−0.606	1.58	4.92	−17 (±2)	−1.77 (±0.07)	−0.659	1.11	6.82
Phe	−28 (±2)	−2.40 (±0.07)	−0.729	1.67	6.39	−22 (±2)	−1.93 (±0.03)	−0.676	1.26	8.42
Trp	−26.1 (±0.9)	−3.1 (±0.1)	−0.976	2.14	7.69	−26 (±2)	−2.85 (±0.01)	−0.970	1.88	9.99
Met	−27 (±2)	−1.15 (±0.07)	−0.452	0.69	6.02	−22 (±2)	−1.09 (±0.01)	−0.448	0.64	7.85
Nor	−26 (±3)	−1.36 (±0.05)	−0.494	0.86	5.55	−19 (±1)	−1.11 (±0.09)	−0.505	0.60	7.68
Abu	−19.3 (±0.7)	−1.25 (±0.04)	−0.484	0.76	4.97	−16 (±1)	−1.29 (±0.03)	−0.483	0.81	6.46
Thr	−22 (±2)	−2.21 (±0.04)	−0.690	1.57	3.95	−16 (±2)	−2.09 (±0.05)	−0.657	1.43	5.29
Arg	−15 (±2)	−1.17 (±0.04)	−0.380	0.79	2.16	−12 (±2)	−1.4 (±0.2)	–	–	2.11
Ile	−15 (±2)	−1.39 (±0.08)	−0.485	0.90	5.76	−17 (±1)	−1.18 (±0.06)	−0.459	0.73	7.25
His	−12.7 (±0.6)	−1.2 (±0.2)	−0.491	0.73	4.29	−7 (±2)	−1.0 (±0.2)	−0.407	0.56	4.20
Asp	−18 (±1)	−0.5 (±0.5)	−0.249	0.29	4.71	−19 (±3)	−1.4 (±0.1)	−0.276	1.17	5.35
Asn	−19 (±1)	−0.6 (±0.5)	−0.249	0.33	4.75	−16 (±3)	−1.4 (±0.8)	−0.191	1.21	5.35
Lys	−27 (±2)	−0.4 (±0.3)	−0.414	−0.05	10.72	−25 (±1)	−0.7 (±0.2)	−0.371	0.35	11.82
Tyr	−32 (±1)	−0.8 (±0.1)	−0.370	0.35	10.59	−25 (±4)	−0.6 (±0.1)	−0.334	0.20	11.89
Glu	−25 (±2)	–	–	–	10.02	−21 (±4)	–	–	–	11.50

[a] Subscript 1 refers to the first-eluting enantiomer.
[b] Brackets: standard deviation of $\Delta(\Delta H^{\circ})$ and of ΔH_1°, estimated from the slope standard deviations.
[c] Calculated of $-RT\ln\alpha$ (20 °C).
[d] Calculated as $\Delta(\Delta G^{\circ}) - \Delta(\Delta H^{\circ})$.

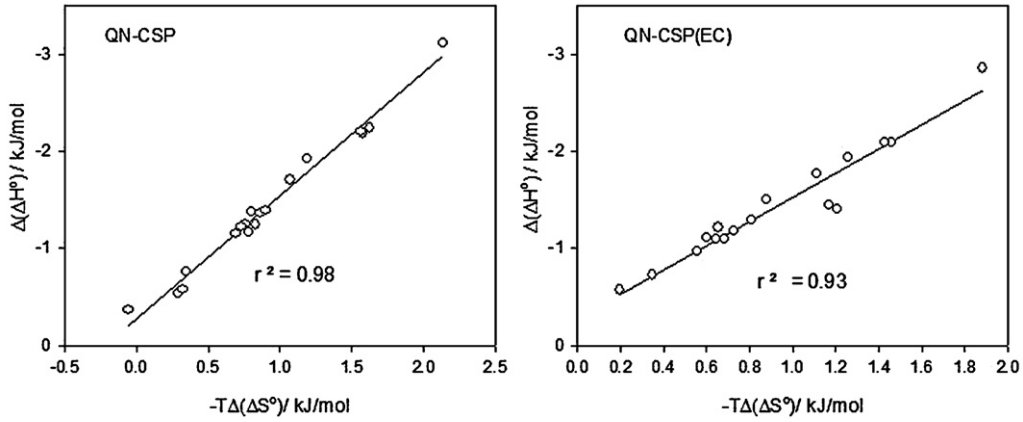

Fig. 6. Plots of $\Delta(\Delta H^{\circ})$ vs. $T\Delta(\Delta S^{\circ})$ for DNP-amino acids measured in the QN-based columns QN-CSP (left) and QN-CSP(EC) (right). Mobile phase conditions as in Fig. 5.

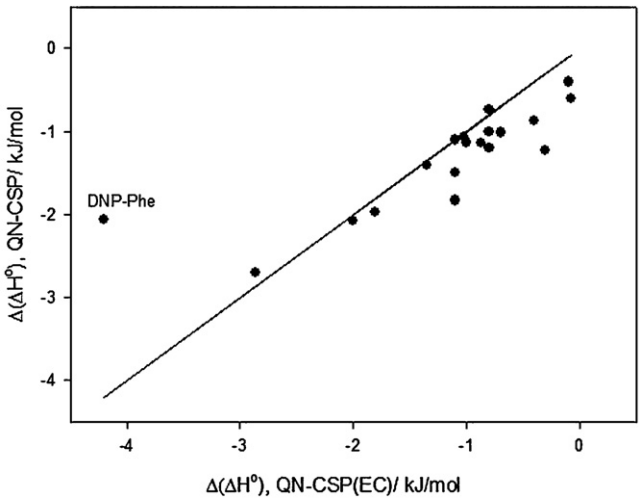

Fig. 7. Comparison of enthalpic differences for the same solutes obtained on both chiral columns.

coefficients between both parameters are indicated in the plots. Similar results can be obtained at other eluent pHs.

$\Delta(\Delta H^{\circ})$ is a measure of the differential enantioselective interactions between each enantiomer and the CS. The more retained solute of the pair has the more negative ΔH° of the two, indicating a higher magnitude for the attractive interactions with the chiral adsorption site. The formation of a stronger, tighter complex with the CS through a larger number of interaction points [36,37] can explain the negative $\Delta(\Delta S^{\circ})$. That the interactions between the solute and components of the mobile phase are nonchiral and therefore do not contribute to the enantioseparative process is an implicit consideration; nevertheless, evidence has been reported demonstrating that the solvent sometimes can play a fundamental role that affects the enantioselectivity [38,39]. As part of the retention process, molecules of the solvent and/or other species sorbed to the stationary phase likely become displaced by the solute. The release of different amounts of these achiral species by the two enantiomers could give a positive contribution to $\Delta(\Delta S^{\circ})$. In the end, the experimental values reflect the net balance of the entire enantiorecognition process.

We discussed above that statistically equal enantioseparation factors were obtained with the endcapped and the more polar QN-based column in spite of the differences in retention. Fig. 7 compares the enthalpic differences for these solutes, $\Delta(\Delta H^{\circ})$, measured with both columns. With the exception of DNP-Phe, the other solutes exhibited lower $\Delta(\Delta H^{\circ})$ values in the endcapped column, indicating that a greater difference in interaction (enantioselective) energies are involved with the more polar CSP. As stated above, the endcapping treatment provided non-enantioselective interaction sites that increase retention, but these hexyl groups did not improve the enantiorecognition process.

Finally, even though, the efficiencies improved as the temperature was raised, this beneficial effect was offset by a decrease in selectivity. As a consequence of these two opposing effects, the resolution usually passed through a maximum at around 20 °C.

4. Conclusions

The main conclusions obtained from this study are the following:

1. A stable QN-based chiral column has been developed. Two sylanization reaction schemes, differing in the silica water content, have been compared. We found that the existence of a water monolayer on the surface has a positive influence on the yields of the mercaptopropylsilica obtained.
2. At the working $^{S}_{W}$pH (between 5.0 and 7.0), the retention of acidic analytes (anionic DNP-amino acids) is predominantly controlled by ion pairing mechanism. The hydrophobicity of the solutes, however, can also be considered a consequential feature to explain retention. All the mobile-phase variables have been assessed.
3. A comparison between an endcapped column and the one without n-hexyl chains revealed that endcapping increased solute retention through hydrophobicity, in the absence of any effect on the enantioselectivity factors.
4. Good linear relationships exist between $\ln k_i$ and the reciprocal of absolute temperature. These DNP-amino acids had the expected behavior, *i.e.*, enantioselectivity decreased as temperature was raised, indicating that the differential enthalpy of interaction of the enantiomorphs with the CSP dominated the enantiorecognition process. These differences in $\Delta(\Delta H^{\circ})$ are higher for the non-endcapped CSP, which explains that the experimental enantioselectivity factors are statistically similar in both columns.

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