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Chiral ligand-exchange chromatography with *Cinchona* alkaloids. Exploring experimental conditions for enantioseparation of α-amino acids

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ABSTRACT

The natural *Cinchona* alkaloid quinidine as chiral selector in chiral ligand-exchange chromatography was systematically studied. Chromatographic conditions for enantioseparation of twenty α-amino acids were first time studied by changing mobile phase parameters such as pH, concentration of organic solvent, type of salt, ligand to metal ratio and also column temperature. Maximum retention and enantioselectivity factors were observed at the region close to pH = 8, since the tertiary amine (the quinuclidinic nitrogen) of the quinidine is protonated only in a small degree, and therefore is available for the chelate formation. Additionally at this pH value there is no other competing ligand for complex the metallic cation. The thermodynamic transfer parameters of the enantiomers from the mobile to the stationary phase from van't Hoff plots within the range of 10–35 °C were estimated. Thus, the differences in the transfer enthalpy Δ(ΔH), and transfer entropy Δ(ΔS) enabled an investigation of the origin of the differences in interaction energies Δ(ΔG). Finally, the molecular geometry of the formed diastereomeric complexes was modelled and energetic differences between both compounds were calculated by a semi empirical method.

1. Introduction

Chiral recognition and enantiomer distinction are fundamental phenomena in nature and chemical systems. They have impact in many chemical fields dealing with bioactive compounds, such as drug discovery, research and development of pharmaceuticals, agrochemicals, food additives, fragrances and pollutants. Enantioseparation represents an important field in analytical science and therefore, the availability of strategies enabling the racemic resolution is a continuously challenging task. In the last decades, a large number of publications about enantioseparation by chromatographic and electrophoretic techniques appeared in literature. Initially, gas chromatography (GC) and high performance liquid chromatography (HPLC) were used for this purpose, but then capillary electrophoresis (CE) was included.

The biological and pharmacological properties of amino acids strongly depend on their stereochemistry and hence amino acids enantiomeric purity is of utmost importance. Several approaches for resolving underivatized amino acids have been successfully applied [1]. Chiral ligand-exchange chromatography (CLEC), firstly proposed by Davankov in the early seventies [2], still represents the elective choice since it does not require any prior sample handling. CLEC consists in the reversible coordination of chelating analyte species from the mobile phase into the coordination sphere of a metal ion that is immobilized by complexation with a chelating chiral selector, forming mixed ternary selector/metal ion/solute complexes. Depending on the steric and functional properties of the analytes, these diastereomeric ternary chelates show different rates of formation and/or thermodynamic stabilities, giving rise to different retention times for the corresponding solute enantiomers. During the chromatographic process, the coordinated ligands are reversibly replaced by other ligands from the mobile phase. Cu(II) is the chelating metal ion of first choice, while Zn(II) and Ni(II) may be proper alternatives [3]. Frequently employed CLEC type selectors include cyclic amino acids such as proline [4] and hydroxyproline [5] as well as sulfur containing amino acids derived from cysteine [6] and penicillamine [7], and also amino alcohols [8].

Applicability of CLEC relies on the presence of metal-chelating functionalities in both the chiral selector (an enantiomeric pure compound) and the analyte. *Cinchona* alkaloids are well known chiral auxiliaries for promotion enantioselective transformations in catalytic processes [9]. Their chemical structure consists of a conjugated heterocyclic quinoline ring linked to a rigid bicyclo heterocyclic aliphatic quinuclidine ring through a carbon atom, C9, linked to a hydroxyl group. In this family of molecules, only C8 and C9 may vary in their configuration resulting

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in the pseudo-enantiomeric quinine and quinidine. *Cinchona* alkaloids especially quinine (QN) and quinidine (QD), became popular in liquid chromatography more recently, when Lindner and co-workers have extensively studied several derivatives, mainly the carbamates as selectors incorporated to chiral stationary phases employed in ion-exchange mode [3]. Although *Cinchona* alkaloids have high potential to form complexes with chiral acidic compounds, they were first time reported as chiral selectors in CLEC recently in our previous work [10]. Now we propose to replace QN by QD as chiral selector and also to extend the study to other transition cations. The liquid-liquid extraction of *Cinchona* alkaloids through the formation of mixed complexes with optically active usnic acids mediated by divalent cations such as Cu(II), Co(II) and Zn(II) [11] has been described.

In this work, chiral separations of α-amino acids using a CLEC system with QD as chiral ligand and a conventional non-chiral octadecylsilica (ODS) column were achieved. To the best of our knowledge, no precedents exist for the systematic study of the influence of the experimental variables over the retention and enantioselectivity for this chromatographic system. In order to study the chemical stability and viability of the aforementioned metallic complexes, the semi-empirical PM6-DH+ method to model the complex geometries and to obtain the most stable structures of intermediate coordination complexes presumably mediating the enantioseparations has been employed.

2. Experimental

2.1. Chemicals

The chemicals used were reagent-grade or better. The amino acids (both racemic and pure enantiomers) were purchased from Sigma (St. Louis, MO, US) or from BDH (BDH Ltd., UK); QD was from Fluka (Buchs, Switzerland); the cupric acetate, cupric nitrate, cupric sulfate, cobalt acetate and zinc acetate were from Baker (J.T. Baker Chemical Co., Phillipsburg, NJ, US) and the HPLC-grade methanol (MeOH) from Mallinckrodt (Mallinckrodt Baker Inc., Phillipsburg, NJ, US). Water was purified by means of a Milli-Q Purification System (Simplicity, Millipore, Massachusetts, MA, US).

Mobile phase solutions were filtered through a 0.22 μm Millipore filter and degassed with 10 min sonication before use. Amino acid solutions (7 mg/mL) were prepared in filtered mobile phase and sonicated until completely dissolved.

2.2. Instrumentation

The HPLC experiments were carried out on an Agilent liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with 1100 vacuum degasser, binary pump and column thermostat, 1260 Infinity Autosampler and 1290 Infinity Diode Array Detector. Data acquisition was done by the Open LAB Chromatography Data System (CDS) software (ChemStation C.01.03).

A commercial Eclipse XDB-C18 (Agilent, USA) analytical column (75 mm × 4.6 mm; 3.5 μm) was employed.

2.3. Mobile phase preparation and column equilibration

The mobile phase preparation was described in detail previously [10]. Briefly, the weighed alkaloid (QD) and the metallic salt (in turn $Cu(CH_3COO)_2$, $Cu(NO_3)_2$, $CuSO_4$, $Co(CH_3COO)_2$, $Ni(CH_3COO)_2$ or $Zn(CH_3COO)_2$) were dissolved into a mixture of 20% (v/v) MeOH and 80% (v/v) aqueous 0.1 M NH_40AC/NH_3 buffer or in a 10% (v/v) MeOH and 90% (v/v) 0.1 M aqueous buffer to reach a final concentration of 0.5 mM for the studied divalent cation and 0.5 or 1 mM for QD. The mobile phase $^{s}_{w}$pH was readjusted with either hydrochloric acid or sodium hydroxide to the desired pH value (8.00 or 9.00).

To equilibrate the ODS column, the filtered mobile phase was run at 0.1 mL/min, in an open cycle, until the detector response proved stable (approximately 200 column volumes). The mobile phase flow rate for analysis was set to 0.5 mL/min. After running all the analyses with each mobile phase, the analytical column was always cleaned with a 30:70 (v/v) MeOH: H_2O mixture and then reconditioned by flowing the new mobile phase through the column. These procedures allowed the restoring of the column for the next appropriate ODS surface coating.

The native α-amino acids were detected at 254 nm. KBr detected at 210 nm was used for unretained marker in all analysis. The retention times were taken at maxima of the peaks. The elution order within a racemic pair was determined (when it was possible) by the injection of each pure enantiomer.

2.4. The PM6-DH + computational method

The accuracy of semi empirical quantum method PM6 in predicting formation heats for compounds of interest in biochemistry is somewhat superior to Hartree-Fock (HF) or B3LYP DFT methods, using the 6-31G(d) basis-set. For a representative set of compounds, PM6 gave an average unsigned error (AUE) of 4.4 kcal mol^{-1}; for the same set HF and B3LYP had AUE of 7.4 and 5.2 kcal mol^{-1}, respectively [12]. Hobza et al. have introduced an extension of the semi empirical PM6 method in two directions [13]. The first one includes an empirical correction to the dispersion energy that improves the description of complexes controlled by the dispersion energy. The second one introduces an additional electrostatic term that improves the description of hydrogen-bonded complexes. The resulting method, i.e. the PM6 with corrections for dispersion and hydrogen bonding, was labeled PM6-DH+. This method provides stabilization energies that agree very closely with the benchmark values obtained by much more expensive methods. For the purpose of verifying the stability of this type of complexes, a molecular dynamic simulation during 15 ps with a time step of 0.5 fs for one of the complexes at 900 K, keeping the temperature constant by coupling the system to a Berendsen [14] thermostat with a bath relaxation time of 0.5 ps was performed.

3. Results and discussion

3.1. Chiral separation mechanism

When chiral mobile phase additives are used to regulate analyte retention in reversed phase HPLC, often results in the formation of diastereomeric ion pairs which can be easily separated on conventional reversed phase columns. During passage of a racemic analyte through the HPLC column, diastereomeric mixed-ligand complexes can be formed by a displacement mechanism. Since for the basic concept of CLEC, a transition metal will complex with electron-rich ligands, the complexing between divalent cations and *Cinchona* alkaloids is feasible due to the presence of a hydroxyl and a tertiary amino group situated on adjacent carbon atoms. The difference in the three-dimensional structures between both complexes would potentially lead to the desired enantioseparation. The enantioselectivity depends on the differences in the relative stabilities of that complexes, the energy and their affinity for the stationary phase, thereby resulting in different chromatographic retention.

3.2. Chiral separation conditions

In order to optimize the separation conditions for racemic α-amino acids by using CLEC, some effective factors such as organic modifier content, concentration of chiral ligand, kind of salt (either metallic cation and counter-ion), mobile phase pH and column temperature were investigated.

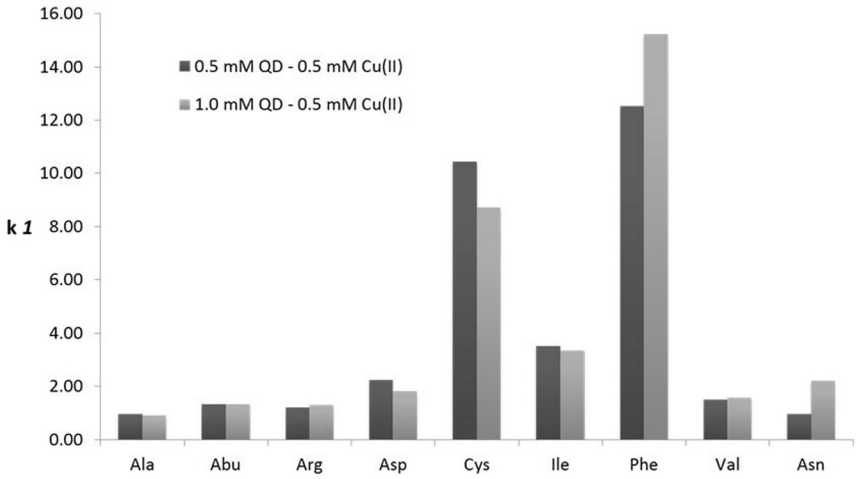

Fig. 1. Retention factors (k_1) for α-amino acids chromatographed with mobile phases containing 10% (v/v) of MeOH and variable amounts of QD at pH = 8.00.

3.2.1. Amount of organic modifier

Amino acid retention decreased by the addition of organic solvent to the buffered eluent. Retention reduction was dependent on the amino acid hydrophobicity, decreasing about 20% average as the amount of methanol increases 10% (v/v). Simultaneously, enantioselectivity factor values diminished in about 7%. Thus, the organic modifier strongly affects all the hydrophobic interactions, and in particular, those between the analytes and the ODS support. Such interactions are important sources of retention especially for hydrophobic amino acids. The use of a low percentage of organic solvent can be useful to drastically reduce the analysis time for the most retained samples. However, this parameter should be used with special care because the column stability could be compromised.

3.2.2. Ligand concentration

There are many works dealing with this type of separations using different metallic cation to chiral ligand ratios. Karger et al. [15] have separated derivatized amino acids with an equimolar (0.8 mM) chelate formed between Zn(II) and a tridentate ligand in the mobile phase. Gil-Av et al. [16] have separated underivatized amino acids with a 1:2 Cu(II):proline chelate. The influence of the metal chelate concentration in the mobile phase was studied by evaluating the chromatographic response using mobile phases with 1:1 and 1:2 Cu(II):QD ratios. Fig. 1 shows that the retention factor of amino acids are not changed significantly (except for Cys and Phe) when QD concentration in the mobile phase is changed, but enantioselectivity vanished for all amino acids when QD concentration duplicates the Cu(II) one (data not shown). An increase in ligand concentration could result in the formation of more binary complexes that can be partitioned into the stationary phase and could cause an increase in k values. When ligand concentration is increased more than the equimolar concentration with metal, the k values level off, suggesting that the stationary phase is saturated and as a result, the separation of the amino acids become maximal, stating that the determining factor is then, the complexes dissociation degree. Considering also the limited alkaloid solubility, mainly with low amount of organic modifier, all the following experiments were carried out with 1:1 Cu(II):QD ratio (0.5 mM of each one).

3.2.3. Copper counter-ion

Only a very limited consideration has been reserved to the effect exerted by the metal(II) salt counter-ion on the enantioseparation achievement [17–21]. However, it is fully known that the counter-ion has a crucial effect in several complexation events. Data reported in Fig. 2 show that the anion of the Cu(II) salt employed in the eluent has an important influence on either retention and enantioselectivity. Mobile phases containing 10% (v/v) MeOH and pH adjusted to 8.00 were prepared by weighing the needed amount of copper salt ($Cu(NO_3)_2$, $Cu(CH_3COO)_2$ or $CuSO_4$) to obtain a 0.5 mM solution. Higher k and α values were found using copper acetate. Column efficiency remains practically the same with all the salts. The unequal physicochemical properties of acetate and nitrate anions are extraordinarily amplified inside the complex multi-interaction environment of CLEC. When $CuSO_4$ was used, no separation was possible for any of the studied analytes.

3.2.4. Metallic cation

A main component of the complexation process occurring between the alkaloid QD and the amino acids is the central cation. From a purely electrostatic standpoint, it follows that a major driving force in the

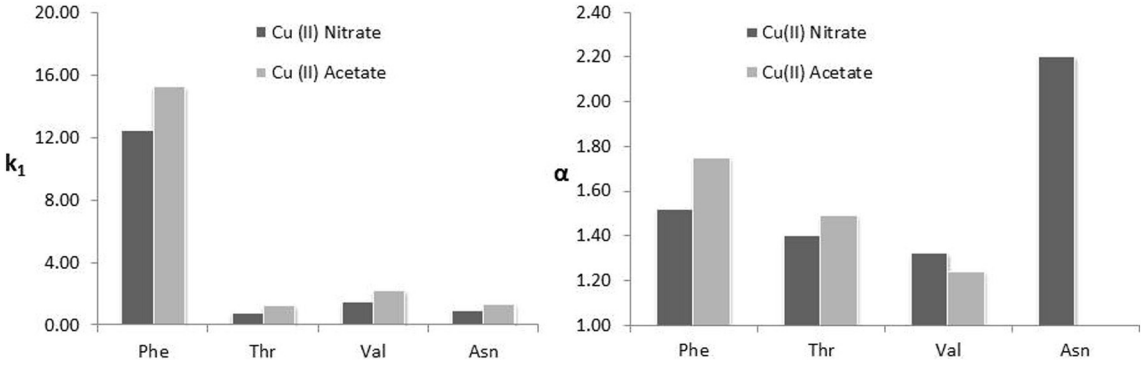

Fig. 2. Retention (left) and enantioselectivity (right) factors for α-amino acids eluted with mobile phases prepared with different copper counter-ion.

Table 1
Retention (k_1), enantioselectivity (α) and resolution (Rs) factors for racemic α-amino acids eluted with mobile phases buffered at pH = 8.00 containing 10% (v/v) MeOH, 0.5 mM QD and 0.5 mM of the corresponding metallic cation. Flow rate 0.5 mL/min; detection at 254 nm.

Amino acid	Cu(II)			Co(II)			Ni(II)		
	k_1	α	Rs	k_1	α	Rs	k_1	α	Rs
Abu	1.82	a.p.	<1	1.90	1.00		3.01	1.00	
Arg	3.07	1.00		3.59	1.00		0.72	1.21	1.01
Phe	15.28	1.75	1.20	18.44	1.60	6.73	1.84	1.42	1.91
Ile	4.53	a.p.	<1	4.83	1.06	<1	3.09	1.00	
Leu	4.22	1.33	<1						
Met	4.14	a.p.	<1	3.99	1.00		0.18	1.00	
Nor	5.22	1.66	1.10	6.51	1.47	1.69	3.11	1.00	
Orn	4.35	1.00		5.15	1.17	<1	0.77	1.10	<1
Ser	2.03	a.p.	<1	1.60	1.00		3.20	1.00	
Thr	1.24	1.49	<1	1.56	1.04	<1	1.83	1.00	
Trp	10.03	2.20	1.20				6.09	1.00	
Val	2.20	1.24	<1	2.55	1.11	<1	0.75	1.00	

a.p. asymmetric peak.

complexation process would then arise from the acidity or charge-to-radius ratio of the metal ion. Acidity is only one of many metal ion properties that will influence the structural and functional requirements of the solute necessary for specific complexation and chiral recognition. Other metal ions were therefore examined as to their influence on the retention and chiral separation of amino acids. In particular, Co(II) and Zn(II) were selected due to their known complexation capacity, as occurs with Cu(II), with *Cinchona* alkaloids [11] and Ni(II), because its intermediate acidic properties are between those for Co(II) and Cu(II). Mobile phases were prepared dissolving the needed amount of the acetate salt of each divalent cation into a hydro organic mixture containing 10% (v/v) MeOH and the pH was adjusted to 8.00. Table 1 shows the retention, enantioselectivity and resolution factors for the amino acids that could be separated. Asp, Ala, Lys, Pro and Tyr were not enantioresolved under any chromatographic condition. Similar results were obtained except for Tyr when QN was used as chiral selector [10]. Cu(II) is the cation which gives the best racemic resolution capacity, followed by Co(II). Interestingly Arg was only separated by mobile phases containing Ni(II) whereas no separation of any analyte was observed with Zn(II).

3.2.5. Eluent pH

Another critical factor for the enantioseparation is the mobile phase pH. In a previous work [10] we studied the influence of mobile phase pH between 6.50 and 9.00 over retention and enantioselectivity for amino acids separated by CLEC with QN as chiral selector. At the lower pH limit, the retention of all the amino acids decreased drastically due to the protonation of the quinuclidinic nitrogen of the alkaloid, and an intermediate pH ≈ 8.0–8.5 was most appropriate. The pK_a value of the quinuclidinic nitrogen in water for QD is 10.00 [22], whereas in 20% (v/v) MeOH can be estimated to decrease to about 9.75 [23]. Therefore, at pH 8.00 the ratio [QD]/[QDH+] is 0.018, that is, <2% of the alkaloid molecules are unprotonated. This ratio increases to about 20% at pH = 9.00, and as a consequence, the eluent pH increases which should enhance the formation of the ternary complexes. However at this alkaline pH close to the silica-based column limit, the formation of Cu(II)/NH_3 complexes (NH_3 is present in high concentration in the buffer solution) is a competitive equilibrium. In aqueous solution the distribution function of the free metal, β_0, is about 940 times higher at pH = 8.00 than at pH = 9.00, and thus, there is a very small amount of free Cu(II) suitable for the QD chelation. As an example, Fig. 3 shows the comparative chromatograms for D,L-Phe and D,L-Nor eluted with the same mobile phase adjusted to pH 8.00 (left) and 9.00 (right). Retention and essentially enantioselectivity were higher at pH = 8.00 for all the amino acids tested.

3.2.6. Temperature effect

In an attempt to understand the thermodynamics of enantiorecognition with the employed CLEC system, the temperature dependence of retention and enantioseparation of amino acids was investigated from 10 to 35 °C. Fig. 4 shows the van't Hoff plots (top) and ln α vs 1/T (down) for seven amino acids with different polarities as typical examples of the thermal behavior of these compounds. It can be observed that the retention and enantioseparation of all enantiomeric pairs decreased with increasing temperature. The plots were satisfactorily linear within this narrow temperature range and relatively low

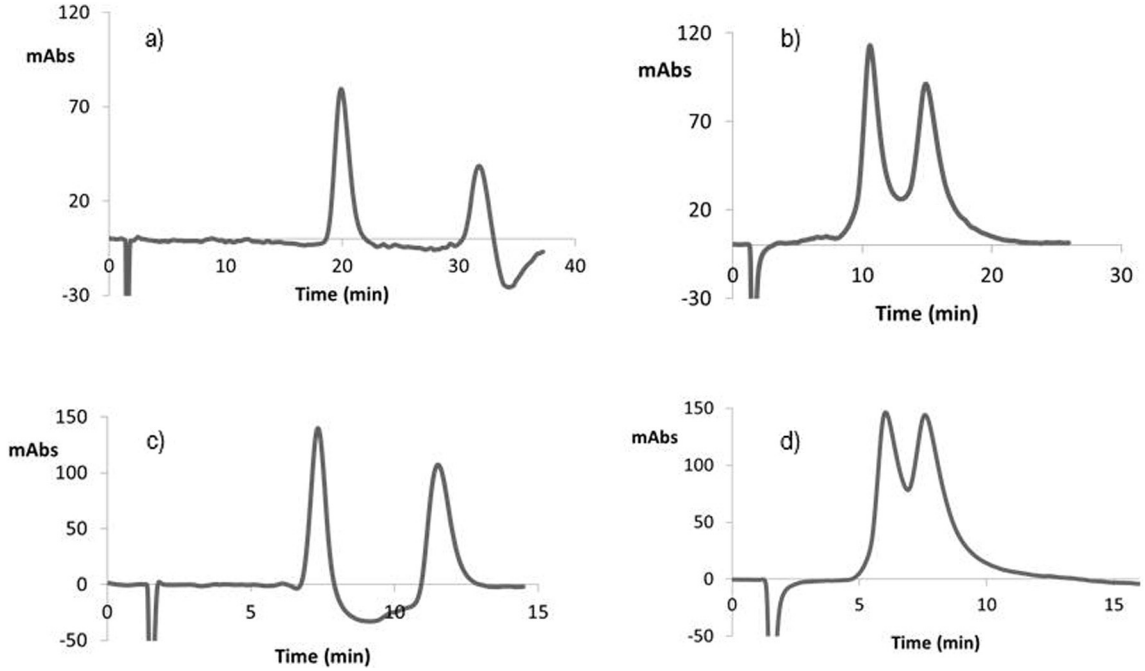

Fig. 3. Comparative chromatograms for D,L-Phe (top) and D,L-Nor (down). Mobile phase: 0.5 mM Cu(II) acetate, 0.5 mM QD, 20% (v/v) MeOH, pH adjusted to 8.00 (left) and 9.00 (right).

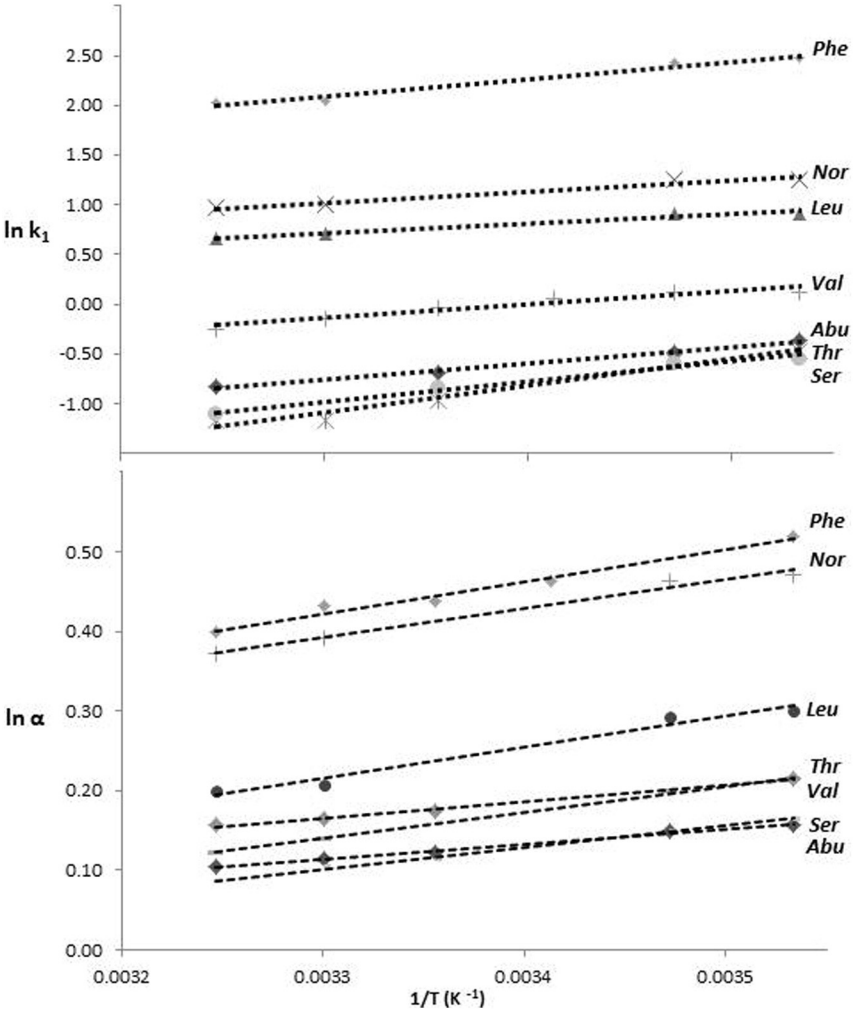

Fig. 4. van't Hoff plots (top) and enantioselectivity dependence (down) with temperature for Phe, Nor, Leu, Val, Abu, Thr and Ser. Mobile phase: 0.5 mM Cu(II) acetate, 0.5 mM QD, 20% (v/v) MeOH, pH = 8.00.

temperature will facilitate obtaining better separations. As an example of the observed general tendency, Fig. 5 shows a sequence of chromatograms for D,L-Thr obtained in all the studied temperature range.

3.2.7. Thermodynamics of enantioseparation

The retention behavior and thermodynamic parameters determined in this study were used to estimate the enthalpy, entropy and Gibbs free energy of association between the two enantiomers and the ODS stationary phase. Data obtained from retention and separation at temperatures ranging from 10 to 35 °C were processed using the van't Hoff equation to estimate the thermodynamic properties of the separation.

$$\ln k_i = -(\Delta H/RT) + \Delta S/R + \ln\varphi \quad (1)$$

where R, T, and φ are the universal gas constant, the absolute temperature, and the phase volume ratio, respectively. ΔH and ΔS are the molar enthalpy and entropy of transference between phases, respectively. Since the definition for the enantioselectivity factor $\alpha = k_2 / k_1$ (where 1 and 2 refer to the elution order of the enantiomeric pair), and the Gibbs-Helmholtz relation, then

$$\ln\alpha = -(\Delta(\Delta H)/RT) + \Delta(\Delta S)/R \quad (2)$$

where Δ(ΔH) and Δ(ΔS) are the difference between enantiomers for the enthalpy and entropy, respectively. These parameters are usually temperature independent, and then they can be calculated form the slopes and intercepts of the linear plot of above equation. The results are listed in Table 2.

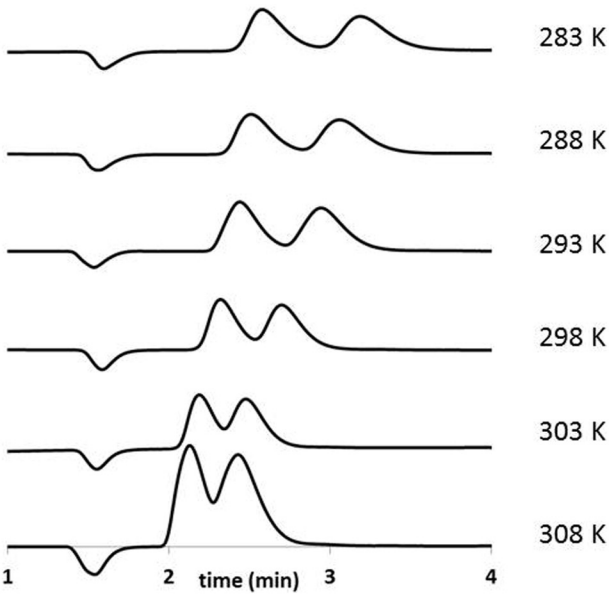

Fig. 5. Comparative chromatograms for D,L-Thr at different temperatures. Mobile phase as in Fig. 4.

Table 2
Thermodynamic properties for amino acids transference equilibrium between mobile and stationary phases.

Amino acid	ΔH (kJ/mol)	Δ(ΔH) (kJ/mol)	Δ(ΔG) (kJ/mol)	−TΔ(ΔS) (kJ/mol)	Δ(ΔS) (kJ/mol)
Asp	−31.2	–	–	–	–
Abu	−13.4	−1.6	−0.4	1.2	−0.0042
Ala	−21.6	–	–	–	–
Arg	−21.5	–	–	–	–
Asn	−24.3	–	–	–	–
Phe	−15.0	−3.3	−1.1	2.2	−0.0075
Ile	−5.9	−4.6	−0.5	4.1	−0.0138
Leu	−7.9	−3.3	−0.7	2.6	−0.0087
Lys	−21.1	–	–	–	–
Met	−14.8	−1.2	−0.3	0.9	−0.0030
Nor	−9.2	−3.0	−1.1	1.9	−0.0065
Orn	−16.0	–	–	–	–
Pro	−17.3	−0.5	−0.3	0.2	−0.0580
Ser	−25.3	−2.3	−0.3	1.9	−0.0066
t-Leu	−12.5	−0.6	−0.4	0.2	−0.0413
Thr	−16.8	−2.7	−0.5	2.3	−0.0078
Val	−13.6	−1.7	−0.5	1.2	−0.0041

Since the nonenantioselective interactions also contribute to retention and they were not discounted, the thermodynamic functions estimated before are only apparent values. Therefore, the reported quantities should be regarded as rough estimations of the true thermodynamic parameters. Even though, since those are approximated values, the discussion here is based on the observed trends, which correspond to the parameters obtained for a large number of systems. Table 2 summarizes the apparent thermodynamic functions for the transfer of these solutes between both phases. The results indicate that all solutes have negative enthalpies and entropies of transfer. The enantioseparations in these systems were dominated by the enthalpic differences, whereas the entropic contributions to the Gibbs free energy were systematically correlated with the enthalpic differences.

3.2.8. Molecular modelling

The chemical structure of the alkaloid QD was optimized at PM6-DH+ level of theory with the software package MOPAC [24] taking into account the dielectric constant fixed at 70.9 [25] through a polarizable continuum method (PCM) available in this software package. For this purpose the most stable conformation found by Urakawa et al. [26] named *Open 3* was used as starting point for the structure of the alkaloid.

A set of ternary complexes formed between the alkaloid, the divalent cobalt ion and the amino acid Val were modelled using the free molecule and visualizer editor Avogadro [27]. The starting structures were built in both configurations for the D- and L-amino acid and two possible coordination for the cobalt atom were set, with both nitrogen atoms pointing at cis or trans configurations. The complex molecules were labeled in agreement with the corresponding alkaloid and its configurations with the nitrogen atom position either adjacent or opposite were considered. From all the potential structures for the complexes formed by QD, Co(II) and D,L-Val, convergence to a chemically stable structures were obtained for only four of them, having square planar coordination around the central cation. In Fig. 6 are displayed the relative energies from the most stable of four possible configurations.

4. Conclusions

Quinidine, employed as chiral selector in the mobile phase under a CLEC mode for the enantioseparation of several α-amino acids through the diastereomeric complexes formed with Cu(II), Co(II) and Ni(II) ions has been successfully employed. In this approach the retention times and enantioselectivity factors can be easily modulated by controlling some of the mobile phase parameters: pH, methanol content, type of

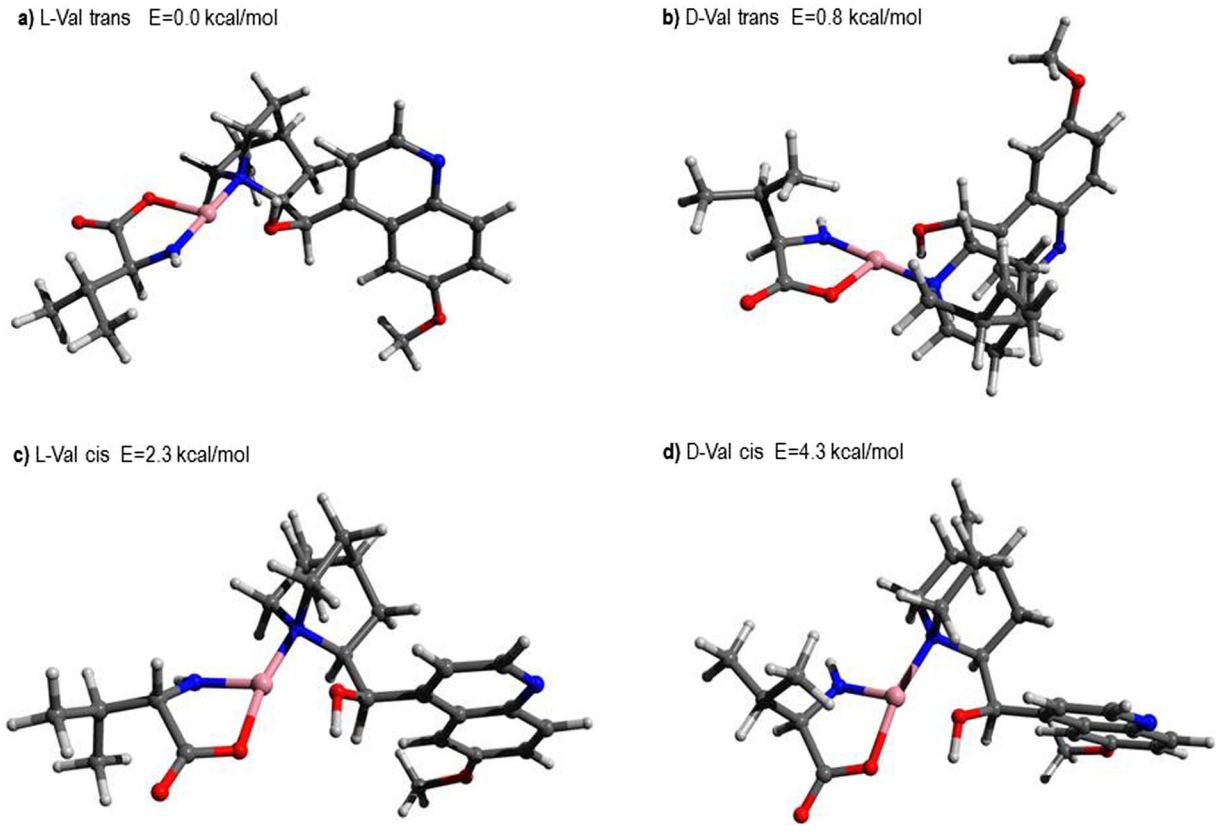

Fig. 6. Proposed structure for the complexes formed between D,L-Val and QD mediated by Co(II) and their relative energies. Each color corresponds to an atom: pink for cobalt, gray for carbon, blue for nitrogen, red for oxygen and white for hydrogen. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

salt, ligand to metal ratio and also, the column temperature. By using the extended semi empirical PM6-DH+ method, the diastereomeric complex geometry was theoretically modelled and, considering the solvent effects, the energetic differences between the diastereomeric forms of a given amino acid (Val) with the QD molecule were calculated.

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