Trends in Chromatography

Original Communication

Ligand-exchange enantioresolution of dihydroisoxazole amino acid derivatives acting as glutamatergic modulators

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ABSTRACT

Four dihydroisoxazole prolines (**1-4**) and four dihydroisoxazole cyclopentane (**5-8**) derivatives, evaluated as glutamatergic modulators, were submitted to chiral ligand-exchange chromatography (CLEC) analysis. A chiral stationary phase (CSP) obtained through the coating of C-18 chains with S-trityl-(*R*)-cysteine [(*R*)-STC] failed the enantioseparation of compounds **3**, **4**, **7** and **8**. On the other hand, a chiral mobile phase (CMP) system from O-benzyl-(*S*)-serine [(*S*)-OBS] coupled with Cu(II) nitrate as the metal source into the eluent, produced an excellent enantioseparation of all compounds. Worthy to be mentioned is the results achieved for compound **3**: α = 7.26 and R_S = 25.75. The established CMP-CLEC method was fully validated with the enantiomeric couple **2** as model compound. In all the cases, very good precision (RSD% values ranging from 1.77 to 4.09% in the long-period) and accuracy (Recovery% values ranging from 97.58 to 99.92% in the long-period) values along with appreciably low LOD (25.61 μg mL^{-1}, and 24.79 μg mL^{-1}, for the first and second eluted enantiomer, respectively) and LOQ (77.62 μg mL^{-1}, and 75.08 μg mL^{-1}, for the first and second eluted enantiomer, respectively) values turned out. The statistical relevance of the developed CMP-CLEC method allowed us to successfully undertake the semi-preparative scale enantioisolation of compounds **7** and **8**. As a result of a quantitative structure-property relationship (QSPR) study, the van der Waals energy was found to be the most suitable descriptor for explaining the retention behaviour with the (*S*)-OBS-based system, outlining the prominent role of hydrophobic and dispersive interactions in this CMP-CLEC environment.

KEYWORDS: glutamatergic modulators, chiral ligand exchange chromatography, chiral mobile phase, chiral stationary phase, semi-preparative scale-up, quantitative structure-property relationship study

ABBREVIATIONS

AcOH	:	Acetic acid
CLEC	:	Chiral ligand exchange Chromatography
CMP	:	Chiral mobile phase
CSP	:	Chiral stationary phase
ELSD	:	Evaporative light scattering detector
E_vdw	:	Van der Waals Energy
MeCN	:	Acetonitrile
MeOH	:	Methanol
MLR	:	Multiple linear regression
QM	:	Quantum mechanical
QSPR	:	Quantitative structure-property relationship study
(*S*)-OBS	:	O-benzyl-(*S*)-serine
(*R*)-STC	:	S-trityl-(*R*)-cysteine
TFA	:	Trifluoroacetic acid

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1. INTRODUCTION

Dihydroisoxazole amino acid derivatives **1-8** [1-6] (Fig. 1) are selective glutamate receptor ligands designed by locking the amino acidic chain of the natural ligand into a conformationally constrained cyclic derivative. In derivatives **3** [1, 2], **4** [1, 2], **7** [3] and **8** [3], the 3-hydroxyisoxazoline nucleus was used as a bioisostere of the distal carboxylate. Depending on the different conformation freezed in the bicyclic structure, these derivatives act as AMPA/KA agonists (compounds **1** and **2**) [1], NMDA agonists (compound **8**) [3], NMDA antagonists (compound **6**) [4-6], metabotropic receptor ligands (compound **5**) [4-6], or inhibitors of the excitatory amino acid transporters (EAATs), the proteins involved in Glu re-uptake (compounds **3** and **4**) [2]. Notably, the interaction with the macromolecular target is often highly enantioselective [7], thus the availability of methods enabling the resolution of the racemic mixture is desirable. In these settings, suitable enantioselective chromatographic methods not only allow evaluating the outcome of asymmetric synthesis procedures but, when applied at a preparative level, can represent a valid alternative to the traditional synthetic approach, especially at the first stage of drug development, that is when only small amounts of both enantiomers are required to be tested [8].

Four main liquid chromatographic approaches are recognized to be especially effective to get the enantioseparation of free underivatized amino acids. The relative analytical protocols are based on: chiral ligand-exchange chromatography (CLEC) methods [9-12]; teicoplanin macrocyclic antibiotic stationary phases [13]; chiral crown ether stationary phases (especially for amino acids carrying a primary amino group) [14]; and α- or β-cyclodextrin stationary phases (almost exclusively for aromatic amino acids) [15]. More to the point, the use of ion-exchange-based chiral stationary phases (CSPs) is often noticeably profitable for the enantioseparation of underivatized ionic compounds endowed with peculiar physico-chemical features [16, 17].

Except for compounds **7** and **8**, which were exclusively synthesized as racemates, a teicoplanin-based chiral stationary phase (Teicoplanin-CSP) was successfully used in previous studies by the authors [1, 2, 5], to assess the enantiomeric excess of the underivatized amino acids **1-6**. The absence of relevant chromophoric moieties in their molecular structure (Fig. 1) required to couple the HPLC apparatus with a "mass-sensitive" evaporative light scattering detector (ELSD).

Due to the peculiar chelating feature of compounds **1-8**, we deemed the CLEC strategy to be representing a valuable alternative to the previously employed glycopeptide-type CSP.

Besides the possibility to get underivatized amino acids enantioseparated [9-12], CLEC methodology offers, over the others, a number of additional advantages: the possibility to detect UV transparent molecules through the generation of UV/vis-active metal complexes, and hence the employment of common UV-vis detectors; the use of commercially available and cost-effective chiral enantiodiscriminating agents; the employment of rather inexpensive RP columns associated with their successive easy restore; and the easy analytical to semi-preparative scale-up, which could be particularly profitable to get isolated **7** and **8** enantiomers. Moreover, due to the frequent exclusive use of water eluent systems, the "eco-friendly" character of the whole CLEC process is worth to be mentioned as well [9-12].

2. MATERIALS AND METHODS

2.1. Chemicals

The Dowex 50W-X2-200 and Dowex 1X8-200 ion-exchange resins, glacial acetic acid (AcOH), acetonitrile (MeCN), methanol (MeOH), trifluoroacetic acid (TFA), Cu(II) nitrate pentahemihydrate, sodium nitrite ($NaNO_2$), racemic proline (rac-Pro) and the chiral selectors O-benzyl-(*S*)-serine [(*S*)-OBS)] and S-trityl-(*R*)-cysteine [(*R*)-STC)] were purchased from Sigma–Aldrich (Milano, Italy). Ammonia solution (NH_4OH, 30%), was purchased from Carlo Erba. HPLC-grade water was obtained from a New Human Power I Scholar water purification system (Human Corporation, Seoul, Korea). All the analytes investigated were synthesized according to the procedure described in Refs. [1-6].

2.2. Instrumentation

The analytical HPLC measurements were made on a Shimadzu (Kyoto, Japan) LC-Workstation

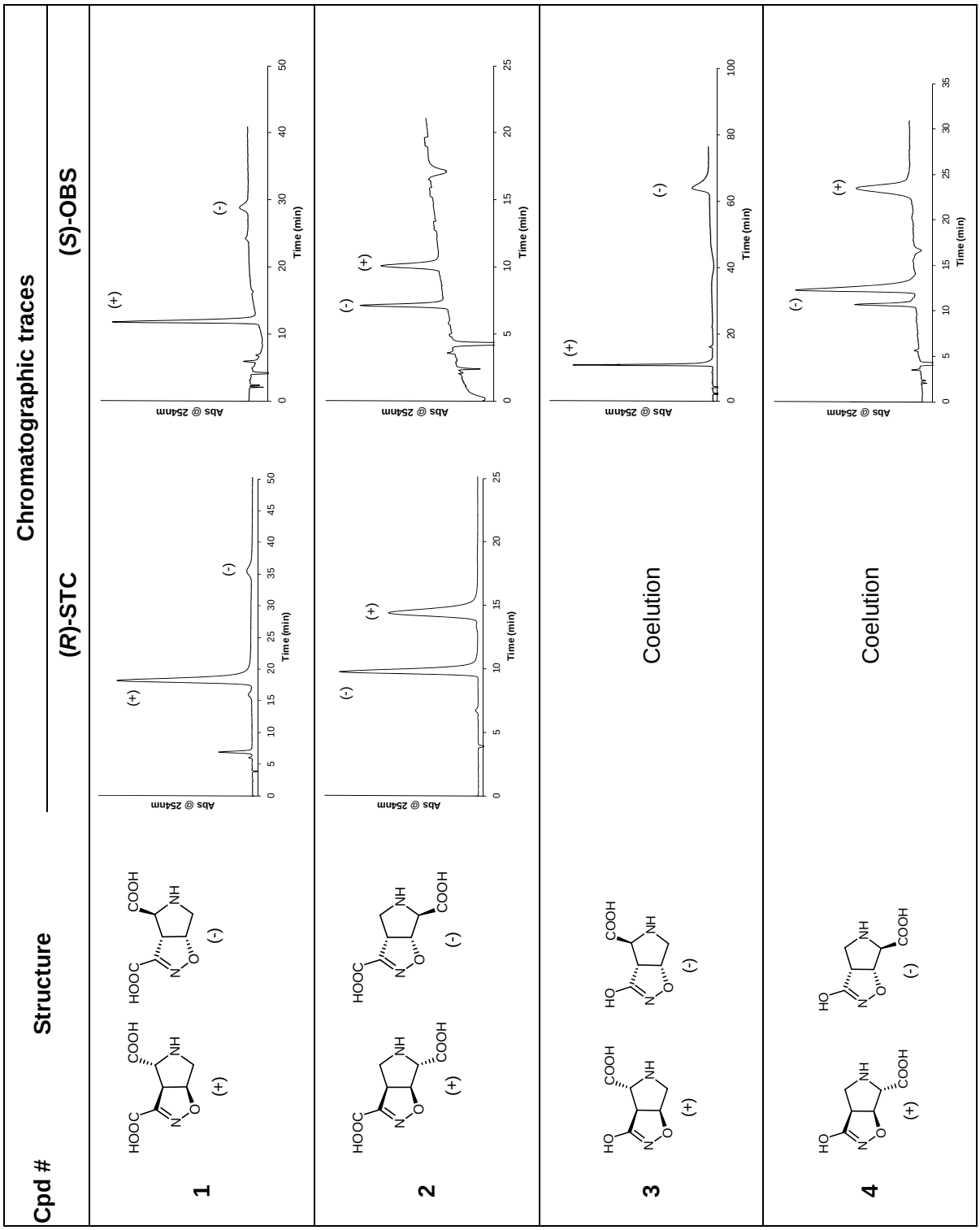
Cpd #
Structure
Chromatographic traces
(R)-STC
(S)-OBS
1
2
3
4
Coelution
Coelution
Abs @ 254nm
Time (min)

Fig. 1

Fig. 1 continued..

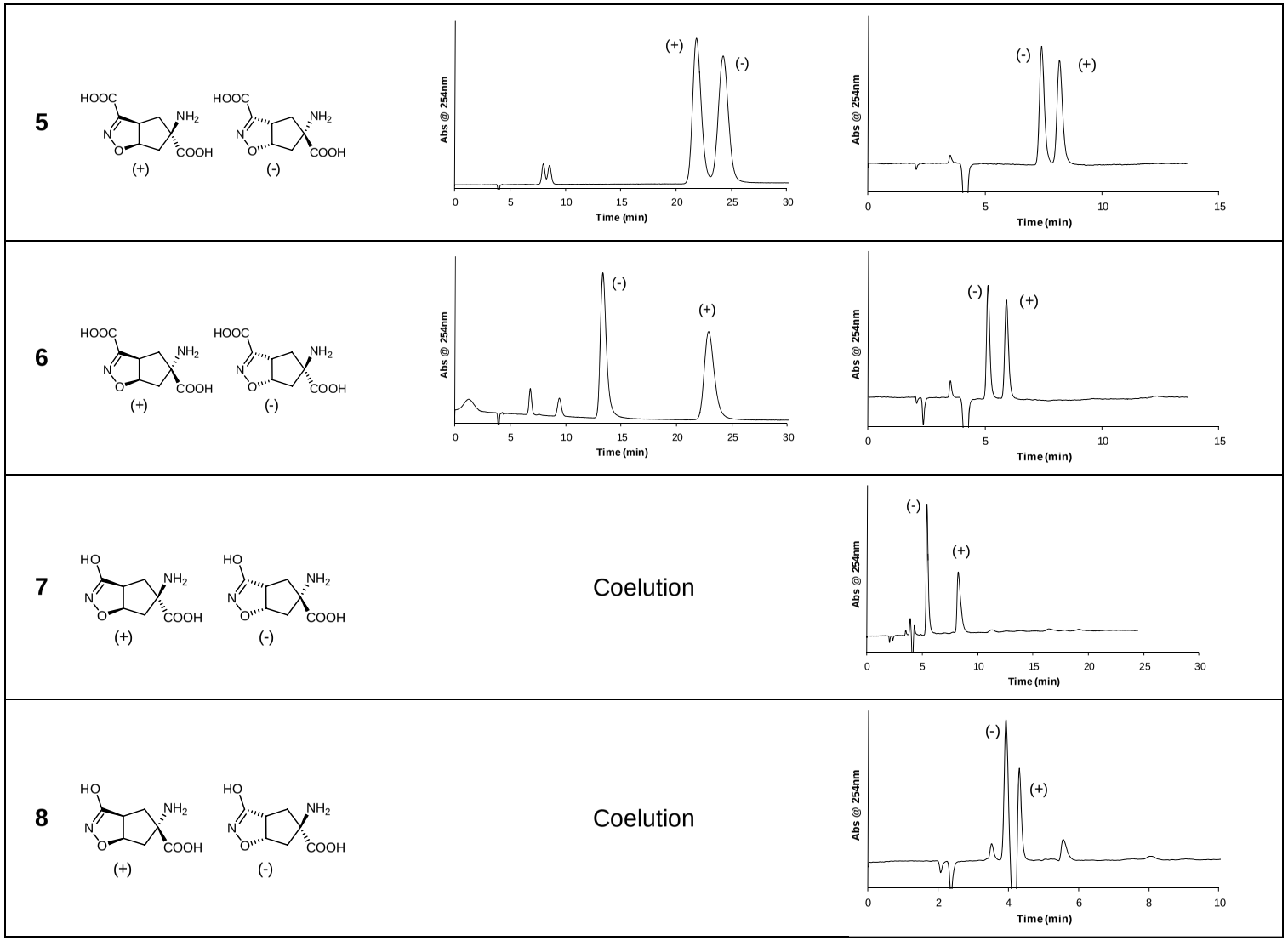

Fig. 1. Structure of the investigated compounds and chromatographic traces obtained with the two employed CLEC systems.

Class LC-10 equipped with a CBM-10A system controller, two LC-10AD high-pressure binary gradient delivery systems, an SPD-10A variable-wavelength UV-visible detector and a Rheodyne 7725i injector (Rheodyne, Cotati, CA, USA) with a 20 μL stainless-steel loop. A GraceSmart RP-18 column (Grace, Sedriano, Italy) 250 x 4.6 mm i.d., 5 μm, 100 Å was used as the analytical column. The column temperature was controlled through a Grace (Sedriano, Italy) heather/chiller (Model 7956R) thermostat. The semi-preparative HPLC system consisted of a Shimadzu LC-8A pump, a SPD-10Avp variable wavelength UV–visible detector, and a Rheodyne 7725i injector with a 2-mL stainless-steel loop. The chromatographic profile was obtained with Class VP (Shimadzu, ver. 4.3) software. For semi-preparative-scale separations, a LiChrospher 100 RP-18 column (Merck, 250 mm x 25.0 mm i.d., 5 μm, 100 Å) was utilized.

2.3 Dynamic coating with (*R*)-STC and column evaluation

A conventional RP-18 analytical column was dynamically coated with (*R*)-STC units. The chiral selector (250 mg) was solubilized into a water/MeOH solution (250 mL, 50:50, v/v), carefully filtered through a 0.22 mm Millipore filter and degassed with 10 min sonication. The optimal adsorption of the selector was achieved by recycling the prepared solution for 5 days at 0.5 mL min^{-1}. With this procedure, approximately 0.05 g of the selector were established to be hydrophobically bonded to the RP-18 sorbent surface. After washing with water/MeOH solution (50 mL, 98:2, v/v) in order to displace the excess of chiral discriminating agent and MeOH, a Cu(II) nitrate solution was flowed through the column and used as the mobile phase after 2 h of equilibration. The $NaNO_2$ injection peak was used as completely unretained marker in all analyses to calculate the t_0 value. Column performance was assessed by periodic injection of racemic proline (rac-Pro). The dynamic CSP used in this study was found to be stable and uniformly effective in the chiral separation of amino acids for at least 30 days.

2.4. Mobile phase preparation

2.4.1. Chiral selector: (*R*)-STC

The mobile phase for the analytical runs was prepared by dissolving Cu(II) nitrate (1.0 mmol L^{-1}) in HPLC-grade water. The resulting solution was filtered through a 0.22 μm Millipore filter and degassed by sonication for 20 min. The sample solutions were prepared at concentrations between approximately 0.1 and 0.5 mg mL^{-1} in filtered mobile phase components and sonicated until completely dissolved. The UV detection wavelengths were set at 254 and 210 nm, and the flow rate was 1.0 mL min^{-1}.

2.4.2. Chiral selector: (*S*)-OBS

The mobile phase for the analytical runs was prepared by dissolving Cu(II) nitrate (1.0 mmol L^{-1}) and (*S*)-OBS (2.0 mmol L^{-1}) separately in HPLC-grade water. The resulting solution was then mixed, filtered through a 0.22 μm Millipore filter and degassed by sonication for 20 min. The analytical column was then conditioned by recycling the selected mobile phase for at least 24 h with a flow rate of 1.0 mL min^{-1}, then an equal fresh solution was used as the mobile phase for the analyses. The UV detection wavelengths were set at 254 and 210 nm. The sample solutions for the analytical runs were prepared at concentrations between approximately 0.1 and 0.5 mg mL^{-1} in filtered mobile phase components and sonicated until completely dissolved.

The same mobile phase composition was employed to run the analyses of compounds **7** and **8** at a semi-preparative scale level. The mobile phase was prepared in the same way as for the analytical-scale analyses. For the semi-preparative application the column was equilibrated by recycling a 3.0 mM (*S*)-OBS, 1.5 mM Cu(II) nitrate solution for 24 h. The UV detection wavelength was set at 254 nm. A 5.0 mL min^{-1} flow rate was selected with the objective of achieving good chromatographic performance (mainly in terms of resolution factor, R_S) within reasonable analysis time.

2.5. Cation-exchange chromatography

A cation-exchange chromatography was employed for copper removal. A 300 mm×10 mm i.d. column was packed with Dowex 50W-X2-200 hydrogen, strongly acidic cation-exchange resin and subsequently washed with water until neutral pH. The fractions previously collected during the enantiomer separation process were concentrated and few drops of concentrated hydrochloric acid

were added until pH 1.0 was reached. At this pH value, the ternary complex is unstable and the solution loses its blue colour. The chromatography of the copper-analyte-selector mixture thus obtained was performed by washing the resin with water until neutral pH and eluting with 5% NH_4OH solution. The fractions obtained were checked by TLC and appropriately collected to recover the eluted enantiomer/chiral selector mixtures, free from any traces of copper ion.

2.6. Reversed-phase separation of enantiomer analyte from (*S*)-OBS

Semi-preparative scale RP analyses were successfully carried out to achieve the separation of the chiral selector (*S*)-OBS from enantiomers of compounds **7** and **8**. A water/MeCN/TFA (95:5:0.1, v/v/v) mobile phase was used with the RP-18 stationary phase previously employed for the semi-preparative scale CLEC application. Before being used, the stationary phase was carefully washed with a water/MeOH (50:50, v/v) solution to remove (*S*)-OBS and Cu(II) nitrate. Due to the absence of relevant chromophoric groups in the molecular structure of the investigated compounds (**7** and **8**), a proper fractionation of the eluate was required. TLC and analytical scale CLEC analyses were realized to monitor the fractions which were, successively, collected and dried.

2.7. Computational methods

The structure of the ternary complexes composed of the selector, Cu(II), and the analyte enantiomer were designed using Maestro 9.0 (Schrödinger ver. 2009, New York, USA) [18] and geometrically minimized using MacroModel 9.7 (Schrödinger ver. 2009) [19] and the OPLS-2005 force-field [20]. To assess the final energy and geometry more accurately, each resulting complex was further optimized in the gas phase using quantum mechanical (QM) calculations with Jaguar 7.6 (Schrödinger ver. 2009) [21], using the DFT-B3LYP level of theory and the 6-31G** basis-set with the approximation of the self-consistent field (SCF) set at the ultra-fine accuracy level. During all these calculations, a formal charge of +2 and a spin multiplicity of 2 were assigned to each complex. The resulting QM optimized conformation was instrumental for the calculation of 31 3D-descriptors included in the MOE software version 2008.10 [22]. In particular, the default all-atom MMFF94x force field [23] was used for the computation of the potential energy descriptors.

The following Multiple Linear Regression (MLR) statistical analyses were carried out using the statistical package XLSTAT2010.4 [24].

3. RESULTS AND DISCUSSION

Differently from other methods, CLEC has been successfully exploited with both the main approaches of chiral liquid chromatography: the one based on the use of CSPs by employing chiral selectors covalently immobilized onto a solid support (bonded-CSPs, B-CSPs) [25] or physically adsorbed onto conventional packing materials (coated-CSPs, C-CSPs) [10, 25, 26]; and the other relying upon the addition of chiral additives to the mobile phase (chiral mobile phases, CMPs) [9, 11, 12, 27].

It is well known that, depending on the fixed analysis conditions, a different number of complexation equilibria can take place in CLEC-CMP systems, especially when the chiral selector partitions between the two chromatographic phases [28, 29]. Accordingly, cases are described in which more than the two peaks corresponding to the transient diastereomeric complexes are sufficiently stabilized so as to impair the goodness of the overall CLEC analysis and make the rationalization of the enantiorecognition event quite complicated [30, 31].

Conversely, in CLEC-CSP systems a reduced number of equilibria materializes, thus improving the quality of the chromatographic trace [28, 29]. In this case, indeed, the enantiorecognition mechanism exclusively relies upon the different participation of the two enantiomers in the formation of the corresponding diastereomeric complex [28, 29].

Based on these assumptions, we privileged a C-CSP medium obtained from the dynamic coating of C18 chains with S-trityl-(*R*)-cysteine [(*R*)-STC] units [10] (Fig. 2a) to attempt the enantioseparation of compounds **1-8**.

Previous studies demonstrated relevant enantiodiscriminating properties by the (*R*)-STC-based C-CSP system towards natural and unnatural underivatized amino acids [10, 26]. Moreover, the

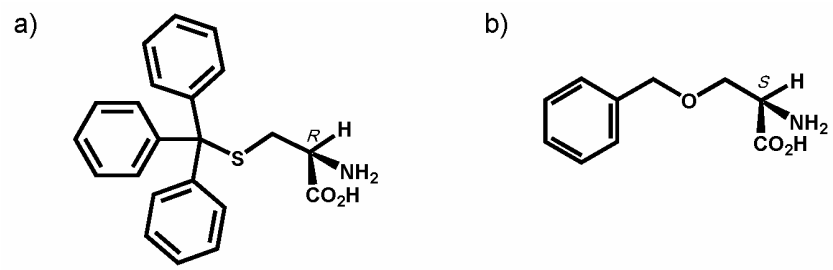

Fig. 2 Structures of a) S-trityl-(*R*)-cysteine [(*R*)-STC] and b) O-benzyl-(*S*)-serine [(*S*)-OBS].

profitable stability of such a CLEC environment (more than 30 days of repeated analysis) was the result of the concurrent presence of a sulphur atom and a trityl region in the chiral selector structure. A 1.0 mM $Cu(NO_3)_2$ aqueous solution was chosen as the mobile phase, due to the recognized scarce interference of nitrate anions in the ternary metal complex formation [32, 33]. The C-CSP device proved to be unable in enantioseparating the compounds endowed with an hydroxyl functionality on the dihydroisoxazole ring, that is the enantiomeric couples **3**, **4**, **7** and **8** (Fig. 1). Therefore, not sufficient differences in free energy during the diastereomeric complexes formation can be invoked to explain the lack of separation between the enantiomers of such compounds [34]. Conversely, the cysteine based selector proved to be especially effective in the enantiorecognition of compounds **1**, **2**, **5** and **6** (Fig. 1).

The partial success provided by the C-CSP from (*R*)-STC units prompted us to explore an alternative and conceptually different CLEC approach, based on the use of a CLEC-CMP system from O-benzyl-(*S*)-serine [(*S*)-OBS] [9-12] (Fig. 2b). In previous works [9-12] (*S*)-OBS containing eluents showed a high enantioresolving ability towards acidic amino acids and conformationally constrained cyclic amino acid derivatives. Very profitably, by running the analyses with 1.0 mM (*S*)-OBS and 0.5 mM $Cu(NO_3)_2$ eluent system, an excellent chromatographic performance was achieved for all the submitted racemates, without the occurrence of additional interference peaks on the chromatographic traces. For the above reason, Cu(II) nitrate was maintained as cupric source into the eluent. In most cases, the selected CLEC-CMP system afforded remarkably better results for the dihydroisoxazole proline (**1-4**) derivatives than for those compounds carrying the dihydroisoxazole cyclopentane nucleus (**5-8**) (Fig. 1) (Table 1). Accordingly, as far as the α values are concerned, the experimental evidence can be due to a higher difference in the thermodynamic stability of diastereomeric ternary complexes carrying **1-4** derivatives along with a more profitable partitioning between the two chromatographic phases of the diastereomeric complexes carrying the proline derivatives [35, 36]. Moreover, more profitable mass transfer kinetics can be inferred to elucidate the higher R_S values computed for compounds **1-4** with respect to **5-8** derivatives. Worth mentioning, in particular, is the result achieved for compound **3** (α = 7.08 and R_S = 21.58) (Table 1).

Interestingly, for compound **5** (Fig. 1), the two CLEC media produced an opposite enantiorecognition mechanism as evident from the enantiomeric elution order reported in Fig. 1 and Table 1. With both (*R*)-STC and (*S*)-OBS, an inversion of the enantiomeric elution order turned out for the two regioisomeric couples **1** and **2**. The same behavior was experienced by the enantiomers of compounds **3** and **4** when eluted with the (*S*)-OBS-based system. A different mechanism of chiral recognition also interested the structurally related compounds **5** and **6** when eluted with (*R*)-STC-based system, while the enantiomers of all the four dihydroisoxazole cyclopentane derivatives (**5-8**) were eluted with the same order in the analysis with the (*S*)-OBS containing eluent phase (Fig. 1, Table 1).

The presence of the hydrophobic benzyl portion in (*S*)-OBS can account for its concurrent presence both in the mobile and stationary phase [11, 36]. Therefore, the CLEC system based on (*S*)-OBS

Table 1. Selected chromatographic data (retention factor k, separation factor α, and resolution factor R_S values) obtained in the analysis of the investigated compounds **1-8** with the two employed CLEC systems.

Cpd #	(*R*)-STC			(*S*)-OBS		
	k_1	α	R_S	k_1	α	R_S
1	7.46 (+)	2.08	9.43	4.19 (+)	3.05	12.53
2	3.54 (-)	1.61	5.23	2.31 (-)	1.59	5.86
3		coelution		4.06 (+)	7.08	21.58
4		coelution		4.70 (-)	2.10	9.03
5	9.14 (+)	1.12	1.55	2.44 (-)	1.14	1.89
6	5.2 (-)	1.85	7.27	1.38 (-)	1.27	2.88
7		coelution		1.52 (-)	1.86	6.20
8		coelution		0.82 (-)	1.21	1.93

units is far from being a "pure" CMP environment but it rather represents an intermediate situation between this extreme case and that typical of CSP media. The same enantiomeric elution order observed for compounds **1**, **2** and **6** with the two investigated CLEC systems could be explained assuming a dominant effect by the stratified (*S*)-OBS molecules over those in the mobile phase. An opposite behavior could be instead inferred to elucidate the reversed enantiomeric elution order experienced by compound **5**. However, peculiar partitioning equilibria of the transient diastereomeric ternary complexes can not be ruled out in the analysis with the CMP system.

3.1. Mechanism of diastereomeric retention

With the aim of identifying relevant factors governing the sample retention in the (*S*)-OBS-based system, a quantitative structure-property relationship (QSPR) study was performed. The ternary complexes composed of the selector, Cu(II), and the analyte enantiomer were optimized in the gas phase using quantum mechanical (QM) calculations (see section 2.7 for methodological details). For each complex, the resulting QM optimized conformation was instrumental for the calculation of 31 3D-descriptors. The multiple linear regression (MLR) statistic approach was useful to identify a statistically significant correlation ($R^2 = 0.6090$) between the experimental logarithmic diastereomeric retention factor values (log k) and the van der Waals Energy (E_vdw) component of the MMFF94x force field [23]. Since the disposition of the analyte points in the graph highlighted a double grouping according to the enantiomeric elution order established on the basis of the sign of the corresponding optical rotation value, two distinct correlations were then considered. For the enantiomers of compounds **7** and **8**, the assignment of their absolute configurations was based on that previously established for the enantiomers of compounds **5** and **6** [5]. The re-calculated coefficients of regression for each subset (separately encompassing the log k_1 and log k_2 values, where the subscripts refer to the diastereomeric elution order) increased considerably: R^2 (log k_1-subset) = 0.8347 and R^2 (log k_2-subset) = 0.7363 (Fig. 3). This result is a further indication of the relevant (but not exclusive) role of hydrophobic and dispersive interactions [35, 36] in the employed CMP-CLEC environments.

Therefore, as a matter of fact, enantioseparation improves upon an increase of the difference of the E_vdw of the diastereomeric ternary complexes. The evidently lower correlation observed for the set of log k_2 values clearly indicates that other factors besides E_vdw contribute to rule the diastereomeric retention behaviour in the investigated CLEC-CMP system.

3.2. Method validation

The established CLEC-CMP method was validated by using the enantiomeric couple of **2** as a model. The validated analytical method was then fruitfully adopted for the semi-preparative scale isolation of the enantiomers of couples **7** and **8**.

3.2.1. Selectivity

Aimed at identifying the presence of interference peaks within the investigated analysis time, three chromatograms of the selected solvent blank (that is the mobile phase) were consecutively run. The peaks obtained (with very small areas in arbitrary units) did not overlap those corresponding to the submitted species. In addition, appreciable resolution factor (R_S) values between the two enantiomeric peaks of compound **2** (R_S = 5.86, Table 1) were achieved with the selected eluent system.

3.2.2. Linearity

Independent sets of analyses were carried out on the two enantiomers of compound **2**. Five calibration standards (83, 165, 250, 333, 415 μg mL^{-1}) having concentration values uniformly covering the ranges of values specified in Table 2 were prepared. All the standard solutions were run in triplicate. The calibration curves were then constructed by plotting the concentration value of each enantiomeric solution (as independent variable) against the corresponding peak area value (in arbitrary units). The mathematical models achieved for the enantiomers of compound **2** were characterized by a satisfactory and comparable linearity within the investigated concentration range (Table 2).

3.2.3. LOD and LOQ

By utilizing the mathematical models (regression equations, Eqs. 1 and 2) reported in Table 2, appreciable LOD and LOQ values were obtained for the enantiomers of compound **2**. The LOD and

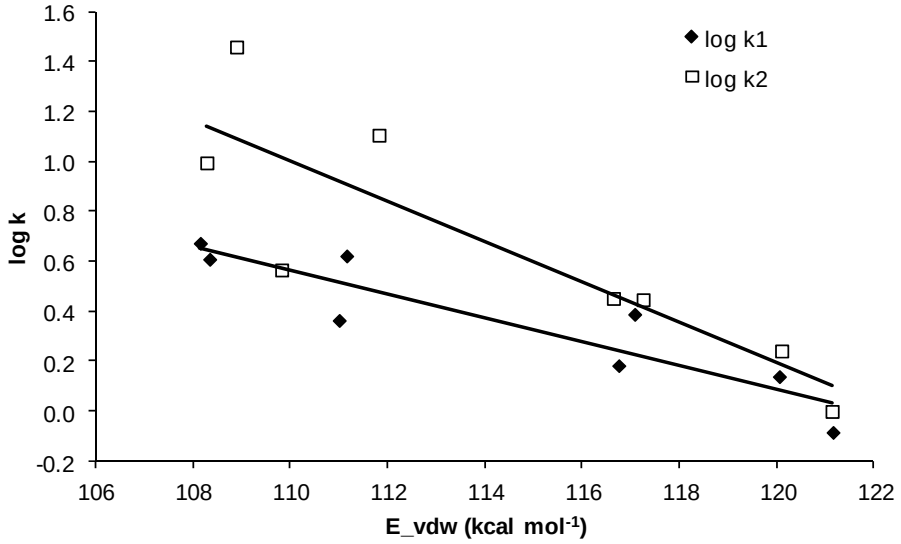

Fig. 3. Plot of the enantiomeric logarithmic retention factor values (log k) versus the energy of van der Waals (E_vdw) values of the corresponding ternary complexes in which the enantiomers are included.

Table 2. Calibration data for compound **2**: regression equations, correlation coefficient (R^2) values, explored linearity range, LOD and LOQ values.

Eluted enantiomer	Regression Equation	Eq. #	R^2	Linearity range (μg mL^{-1})	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
1st	y = 13915.20 (±391.93)x + 39955.99 (±108009.26)	1	0.9898	83-415	25.61	77.62
2nd	y = 14743.71 (± 401.67)x - 21403.90 (± 110692.46)	2	0.9904	83-415	24.78	75.08

LOQ values were derived from the following Eq. 3 and Eq. 4:

$$C_{LOD} = 3.3\frac{\sigma_y}{b} \quad (3)$$

$$C_{LOQ} = 10\frac{\sigma_y}{b} \quad (4)$$

Where C_{LOD} and C_{LOQ} refer to the sample concentration corresponding to the LOD and LOQ, respectively, and *b* is the slope of the relative calibration equation (Table 2).

3.2.4. Intra-day and inter-day precision

By employing the relative calibration curves as formalized by the equation reported in Table 2 the intra-day precision was evaluated for each enantiomer of compound **2**. An external set composed of two control solutions, whose theoretical concentration values are reported in Table 3, was run in triplicate (n = 3) within a period of 6 hours. The procedure was repeated for a period of three consecutive days. The previously obtained mathematical models (Eqs. 1 and 2, Table 2) were then used to calculate the concentration of the control solutions (mean observed concentrations, Table 3). The intra-day precision was evaluated in terms of relative standard deviation (RSD%) among the concentration values obtained from consecutive injections. For each control solution, the variation within replicate injections performed in a time-frame of three consecutive days (n = 9) was employed to determine the inter-day precision (Table 4). As evident from data shown in Table 3, the adopted CLEC-CMP method revealed to be suitable to assure appreciable precision values in the short period. Indeed, the variation range of RSD% values observed during the consecutive three days of analysis varied between 1.65-3.03% for day 1, 1.57-5.32% for day 2, 0.36-4.24% for day 3.

The relevant statistical quality of the method was also observed in the variation of RSD% values obtained during the long-period of analysis (ranging from 1.77 to 4.09%, Table 4).

3.2.5. Intra-day and inter-day accuracy

The Recovery percentage approach was selected to estimate the accuracy of the established CLEC-CMP method. For each determination, the Recovery% value was calculated by employing the following equation (5):

$$Recovery\% = \frac{C_{measured}}{C_{theoretical}} \quad (5)$$

Table 3. Statistical analysis for compound **2** in the short period (intra-day precision and accuracy values).

Eluted enantiomer	Solution	Theoretical concentration (μg mL^{-1})	Day	Mean observed concentration (μg mL^{-1})	n[a)]	Precision (RSD%)	Accuracy (Recovery %)
1st	1	125	1	123.22	3	2.36	98.57
			2	126.16		1.57	100.92
			3	125.32		0.36	100.26
	2	375	1	360.90	3	2.54	96.24
			2	377.56		3.04	100.68
			3	375.20		2.30	100.05
2nd	1	125	1	120.22	3	1.65	96.17
			2	123.09		5.32	98.47
			3	126.12		4.24	100.90
	2	375	1	358.12	3	3.03	95.50
			2	371.62		4.09	99.10
			3	367.98		3.14	98.13

[a)]Number of replicates

Table 4. Statistical analysis for compound **2** in the long period (inter-day precision and accuracy values).

Eluted enantiomer	Solution	Theoretical concentration (μg mL^{-1})	Mean observed concentration (mg mL^{-1})	n[a)]	Precision (RSD%)	Accuracy (Recovery %)
1st	1	125	124.90	9	1.77	99.92
	2	375	371.22		3.11	98.99
2nd	1	125	123.14	9	4.09	98.51
	2	375	365.91		3.43	97.58

[a)]Number of replicates

where $C_{measured}$ represents the enantiomer concentration as calculated through the corresponding regression equation reported in Table 3 (mean observed concentration), while $C_{theoretical}$ represents the concentration value of the utilized external set solution (theoretical concentration).

By analogy with the estimation of short- and long-term precision, the two external control solutions were also used to estimate the intra-day and inter-day accuracy. Accordingly, while the former was determined by considering the three runs for each control solution within a single day (n = 3), the latter was evaluated by taking into account the three consecutive days of analysis (n = 9).

The percentage Recovery varied between 95.50-98.57% for day 1, 98.47-100.92% for day 2, and 98.13-100.90% for day 3. A good accuracy was also estimated in the long-period, ranging the recovery% values between 98.51-99.92% (Table 4).

3.3. Semi-preparative enantioseparation of compounds 7 and 8

The semi-preparative HPLC enantioseparation approaches have gained specific attention in the recent years, as representing an effective alternative to often complex and/or expensive enantioselective synthetic procedures [8]. As a result of the easy analytical to semi-preparative scale-up of CLEC procedures, during the last decades semi-preparative CLEC protocols were successfully pursued to fulfil the enantioisolation of compounds endowed with one or more asymmetric centres, either in CSP [10] or in CMP [9, 37] systems. Indeed, the wide commercial availability of chiral compounds endowed with chelating features makes easier the appropriate selection of the chiral recognition agent.

The possibility to realize the enantioisolations of underivatized species is one of the most attracting features of the CLEC methods. This advantage is particularly significant when only scanty amounts of sample are available, which limits labelling procedures.

A limiting step of semi-preparative CLEC applications is represented by the necessity to remove the Cu(II) ions after the enantioseparation process, although this can be considered a marginal disadvantage since several strategies were and are still being proposed with this respect [37, 38].

A further unattractive feature concerns the separation of the chiral selector from the enantiomer analyte when CLEC-CMP runs are performed. However, the possibility to easily restore the RP stationary phase by applying simple cleaning procedures, allows its successive utilization in the canonical way to accomplish this step.

Recently [9], a CLEC-CMP system based on (*S*)-OBS as the chiral selector and $Cu(NO_3)_2$ as the source of cupric cations into the eluent, has been successfully employed for achieving the semi-preparative enantioseparation of one of the four glutamatergic modulators 1-aminospiro[2.2] pentyl-1,4-dicarboxylic acids (ASPED) [39], that is the enantiomeric couple identified as ASPED-C.

Based on the profitable achievement, a similar application was attempted on compounds **7** and **8**, these being only available as racemates. Isolation

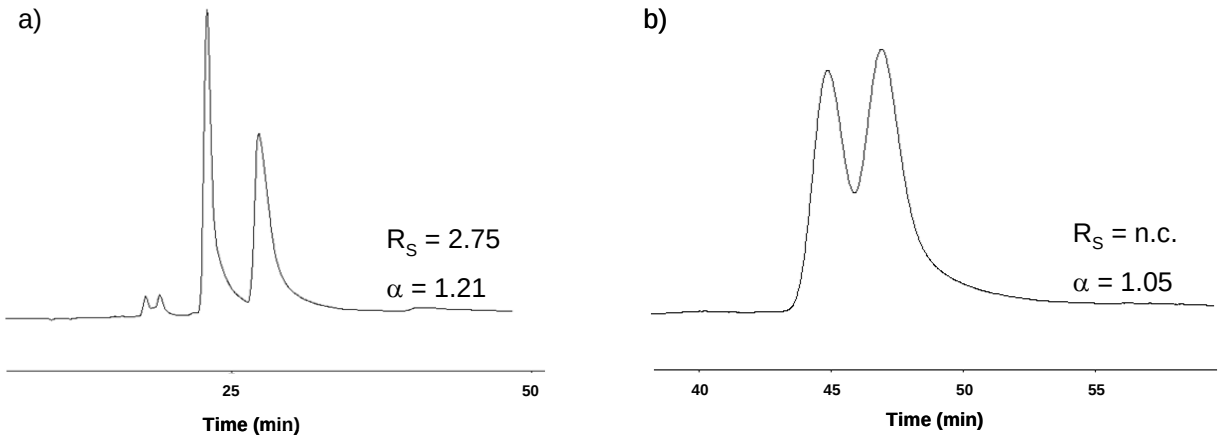

Fig. 4. Semi-preparative enantioseparation of a) compound **7** and b) compound **8**. [n.c.: not calculated by the Class VP (Shimadzu, ver. 4.3) software.]

of enantiomers of **7** and **8** would enable both their physicochemical characterization and the individual biological evaluation. However, for the present study, the semi-preparative separation was performed with the exclusive objective of establishing the enantiomeric elution order through the evaluation of their individual optical rotation sign.

The validated CLEC-CMP analytical method was fruitfully adopted for the semi-preparative scale isolation of **7** and **8** enantiomers. After a suitable flow-rate being identified, "loadability" of the racemate was evaluated, representing the loading capacity a critical factor in semi-preparative separations [40]. A series of attempts was performed with the aim of determining the effect of increasing sample load on the chromatographic performance. The racemates resulted completely soluble in the range of investigated concentrations. For both species, the extent of the peaks overlap was observed to increase by increasing the amount of sample injected. Moreover, the α values remained substantially unaltered, while the R_S factor values experienced a progressive lowering as the load was increased.

A concentration equal to 2.0 mg/2.0 mL (that is the loop capacity) was selected to perform the semi-preparative runs. The corresponding α and R_S factor values are reported in Fig. 4: although the enantiomeric resolution was kept for compound **7**, a worsening of the chromatographic performance was instead observed for compound **8**.

Each of the four eluates, all in the form of diastereomeric complex, was collected and carefully dried. Then, a cation-exchange resin efficiently allowed the Cu(II) nitrate removal from the mixture. The fractions containing the enantiomers and the (*S*)-OBS were collected and evaporated to furnish, in both cases, a solid residue which was submitted to an anion-exchange chromatography according to a previously established procedure [9, 37]. However, in contrast with the previous study, this procedure proved to be ineffective due to the lack of a second carboxylate function in the selectand.

Therefore, a RP chromatographic approach was applied to achieve the isolate enantiomer analytes from (*S*)-OBS. As a result, few milligrams of the two enantiomers for each compound were obtained in the suitable form to be used for optical rotation studies. With this procedure, the elution order k(-) < k(+) was revealed for both **7** and **8** enantiomeric couples.

4. CONCLUSIONS

CLEC demonstrated to be a valid alternative to the previously employed teicoplanin-based CSP for the enantioseparation of compounds **1-8**.

A CMP system based on (*S*)-OBS allowed the enantioisolation of all the enantiomeric couples, while the C-CSP from (*R*)-STC was unsuited to distinguish the enantiomers of **3**, **4**, **7** and **8**.

Particularly relevant is the performance produced by the CMP system in the enantioseparation of compound **3**: α = 7.08, R_S = 21.58.

The outstanding ability of the employed CMP medium, along with the appreciable statistical quality of the established analytical-scale method, facilitated the semi-preparative scale enantioisolation of **7** and **8** with a better result on the former ($\alpha = 1.21$, $R_S = 2.75$).

Finally, the availability of the optical rotation of all the enantiomers allowed us to confirm, with a molecular modeling approach, that retention process in CLEC-CMP domains is significantly ruled by hydrophobic and dispersive interactions, encoded in this study by the van der Waals-energy descriptor.

REFERENCES

1. Conti, P., De Amici, M., De Sarro, G., Rizzo, M., Stensbøl, T. B., Bräuner-Osborne, H., Madsen, U., Toma, L. and De Micheli, C. 1999, J. Med. Chem., 42, 4099.
2. Pinto, A., Conti, P., De Amici, M., Tamborini, L., Grazioso, G., Colleoni, S., Mennini, T., Gobbi, M. and De Micheli, C. 2008, Tetrahedron: Asymmetry, 19, 867.
3. Conti, P., De Amici, M., Bräuner-Osborne, H., Madsen, U., Toma, L. and De Micheli, C. 2002, Il Farmaco, 57, 889.
4. Conti, P., De Amici, M., Joppolo di Ventimiglia, S., Stensbøl, T. B., Madsen, U., Bräuner-Osborne, H., Russo, E., De Sarro, G., Bruno, G. and De Micheli, C. 2003, J. Med. Chem., 46, 3102.
5. Roda, G., Conti, P., De Amici, M., He, J., Polavarapu, P. L. and De Micheli, C. 2004, Tetrahedron: Asymmetry, 15, 3079.
6. Conti, P., De Amici, M., Grazioso, G., Roda, G., Pinto, A., Hansen, K. B., Nielsen, B., Madsen, U., Bräuner-Osborne, H., Eqebjerg, J., Vestri, V., Pellegrini-Giampietro, D. E., Sibille, P., Acher, F. C. and De Micheli, C. 2005, J. Med. Chem., 48, 6315.
7. Maier, N. M. and Lindner, W. 2006, Chirality in Drug Research, Francotte, E. and Lindner, W. (Eds.), Wiley-VHC, Weinheim, 189.
8. Francotte, E. 2001, J. Chromatogr. A, 906, 379.
9. Natalini, B., Sardella, R., Giacchè, N., Camaioni, E., Marinozzi, M., Macchiarulo, A. and Pellicciari, R. 2010, Anal. Bioanal. Chem., 397, 1997.
10. Natalini, B., Sardella, R., Macchiarulo, A. and Pellicciari, R. 2008, J. Sep. Sci., 31, 696.
11. Natalini, B., Sardella, R. and Pellicciari, R. 2005, Curr. Anal. Chem., 1, 85.
12. Sardella, R., Macchiarulo, A., Carotti, A., García Rubiño, M. E. and Natalini, B. 2012, J. Chromatogr. A, 1269, 316.
13. Berthod, A., Liu, Y., Bagwill, C. and Armstrong, D. W. 1996, J. Chromatogr. A, 731, 123.
14. Hyun, M. H., Han, S. C., Lipshutz, B. H., Shin, Y. -J. and Welch, C. J. 2001, J. Chromatogr. A, 910, 359.
15. Shpigun, O. A., Shapovalova, E. N., Ananieva, I. A. and Pirogov, A.V. 2002, J. Chromatogr. A, 979, 191.
16. Sardella, R., Lämmerhofer, M., Natalini, B. and Lindner, W. 2006, Chirality, 20, 571.
17. Sardella, R., Lämmerhofer, M., Natalini, B. and Lindner, W. 2008, J. Sep. Sci., 31, 702.
18. Maestro, Version 9.0, 2009, Schrödinger, LLC, New York, NY.
19. MacroModel, Version 9.7, 2009, Schrödinger, LLC, New York, NY.
20. Kaminski, G. A., Friesner, R. A., Tirado-Rives, J. and Jorgensen, W. L. 2001, J. Phys. Chem. B, 105, 6474.
21. Jaguar, Version 7.6, 2009, Schrödinger, LLC, New York, NY,.
22. Molecular Operating Environment (MOE), Version 2008.10, Chemical Computing Group, Inc., Montreal, 2008.
23. Weiner, S. J., Kollman, P. A., Case, D. A.U., Singh, C., Ghio, C., Alagona, G., Profeta, S. and Weiner, P. 1984, J. Am. Chem. Soc., 106, 765.
24. XLSTAT2010.4, Addinsoft SARL, NY, USA.
25. Natalini, B. and Sardella, R. 2012, Chirality, Tranter, G. E. (Ed.), Elsevier, Amsterdam, 115.
26. Natalini, B., Sardella, R., Macchiarulo, A. and Pellicciari, R. 2008, J. Chromatogr. B, 875, 108.
27. Natalini, B., Marinozzi, M., Sardella, Macchiarulo, A. and Pellicciari, R. 2004, J. Chromatogr. A, 1033, 363.
28. Natalini, B., Sardella, R., Macchiarulo, A., Marinozzi, M., Camaioni, E. and Pellicciari, R. 2011, Adv. Chromatogr., Vol. 49, Grushka, E. and Grinberg, N. (Eds.), Taylor and Francis, Boca Raton, 71.

29. Davankov, V. A., Kurganov', A. A. and Ponomareva, T. M. 1988, J. Chromatogr., 452, 309.
30. Kurganov', A. A. 2001, J. Chromatogr. A, 906, 51.
31. Davankov, V. A. 1994, J. Chromatogr. A, 666, 55.
32. Diniz, V. and Volesky, B. 2005, Water Res., 39, 2229.
33. Natalini, B., Sardella, R., Carbone, G., Macchiarulo, A. and Pellicciari, R. 2009, Anal. Chim. Acta, 638, 225.
34. Pirkle, W. and Pochapsky, T. C. 1989, Chem. Rev., 89, 347.
35. Galaverna, G., Corradini, R., Dallavalle, G., Dossena, A. and Marchelli, R. 2001, J. Chromatogr. A, 922, 151.
36. Natalini, B., Giacchè, N., Sardella, R., Macchiarulo, A. and Pellicciari, R. 2010, J. Chromatogr. A, 1217, 7523.
37. Natalini, B., Marinozzi, M., Bade, K., Sardella, R., Thomsen, C. and Pellicciari, R. 2004, Chirality, 16, 314.
38. Nowshuddin, S. and Reddy, A. R. 2006, Tetrahedron Lett., 47, 5159.
39. Pellicciari, R., Marinozzi, M., Camaioni, E., del Carmen Nùnez, M., Costantino, G., Gasparini, F., Giorgi, G., Macchiarulo, A. and Subramanian, N. 2002, J. Org. Chem., 67, 5497.
40. Kurganov', A. A., Davankov, V. A., Eisenbeiss, F. and Kinkel, J. 1994, J. Chromatogr. A, 666, 99.