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## Research Article

# Direct chiral resolution of underivatized amino acids on a reversed-phase stationary phase dynamically modified with the ion-exchanger *N*<sup>+</sup>-decyl-L-spinacine

Increasing attention has been devoted in the last decades to chiral chromatography, principally to the HPLC techniques using a chiral stationary phase. Many chiral HPLC columns are commercially available, but, unfortunately, they are most often rather expensive. A cheap alternative to the commercial chiral columns is the dynamic-coating procedure of a standard achiral stationary phase with a chiral selector containing both a chiral domain and a chain or a group able to tightly (but noncovalently) bind the achiral support. This is the case of *N*<sup>+</sup>-decyl-L-spinacine, already successfully employed to dynamically cover a reversed-phase column to separate racemic mixtures of amino acids through the ligand-exchange mechanism. In the present work, the same chiral selector is employed to separate racemic mixtures of amino acids and oligopeptides, in the absence of metal ions: no coordination complex is formed, but only electrostatic and weak nonbonding interactions between the chiral phase and the analytes are responsible for the observed enantioselectivity. The new method is simpler than the previous one, very effective in the case of aromatic amino acids and oligopeptides and also suitable for preparative purposes.

**Keywords:** Amino acid enantiomers / Chiral separation / Dynamic column coating / High performance liquid chromatography / Oligopeptides / Spinacine  
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## 1 Introduction

The enantiomer resolution of racemic mixtures is one of the most interesting and growing fields in chromatography [1], especially for the determination of the optical purity of bioactive molecules and new drugs and to obtain enantiomerically (or stereochemically) enriched compounds at a preparative (or semipreparative) level. Two main strategies have been developed for the HPLC separation of enantiomeric mixtures: an indirect and a direct method. The indirect approach includes conversion of enantiomers into diastereomers, using a chiral derivatizing reagent, before the HPLC analysis [2]. The high optical and chemical purity requested for the derivatizing agent and the need for a simple, fast, and quantitative derivatization

reaction, reduce the interest and applicability of the indirect methods.

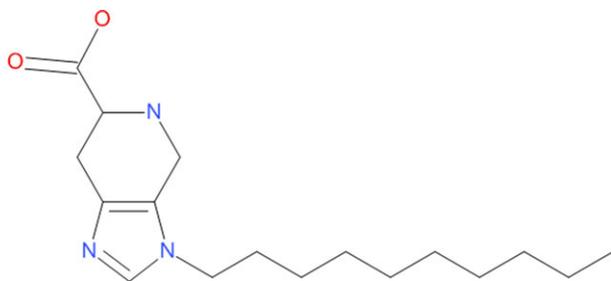
The direct approach requires a chiral phase, either mobile, or stationary, by which the underivatized enantiomers can be recognized. In the former case a chiral mobile phase additive (CMPA), such as a cyclodextrin, BSA or a metal chelate acting as a ligand exchanger [3], is employed. However, the high consumption of the enantiomerically pure CMPA and the reduced suitability of these methods for preparative purposes [4] make the direct methods based on a chiral mobile phase of low practical utility.

Many types of chiral selectors have been used to date in the design of chiral stationary phases (CSPs); the topic has been recently reviewed by various authors [5–10]. The use of a CSP avoids the analyte derivatization; the amount of chiral selector necessary to (permanently or dynamically) derivatize the stationary phase is relatively low and its enantiomeric purity can be lower than 100% since it influences the column selectivity and not the quantitative analysis. CSPs are suitable for both analytical and preparative purposes.

In this context, a particular attention has been focusing in the last decades on amino acid enantioseparation [3, 11], due to the fundamental importance of these molecules in biochemistry as building blocks of proteins, in many

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**Abbreviations:** CMPA, chiral mobile phase additive; CSP, chiral stationary phases; CLEC, chiral ligand-exchange chromatography



Scheme 1. The chiral selector Spi( $\tau$ -dec).

subdomains of pharmacology as well as microbiology. Besides the fact that enantiomeric pure products are required for artificial peptide synthesis, amino acid chiral determination is of great importance in structure determination of many microbial products where the presence of D- and/or nonproteinogenic amino acids is common [12–14].

It is doubtless that the separation of amino acids in their native form using the direct procedure is advantageous from many points of view [3, 11, 12]. The most common chiral selectors working as active sites in CSPs employed for the optical recognition of amino acids are the following: ligand exchangers, requiring the presence of a complexing metal ion [15–23]; crown ethers [24–31]; ion exchangers [32–35]; polysaccharides [36–42]; cyclodextrins [28, 43, 44]; macrocyclic glycopeptide antibiotics (e.g. avoparcin, teicoplanin, vancomycin, or ristocetin) [6, 28, 36, 45–53].

Although many chiral columns are commercially available, there is a continuous demand for new chiral stationary phases to meet the changing needs of new analyses. The dynamic coating of an RP-HPLC stationary phase with a chiral selector represents an uncomplicated and inexpensive alternative to covalently bonded phases for preparing new chiral stationary phases [16, 18, 20, 45, 54]. This method presents the great advantage that a commercial (and relatively inexpensive) achiral column, e.g. an RP-C<sub>18</sub> column, can be used to support the chiral selector. The synthesis of the chiral selector is rather simple, the column-preparation procedure is quick and easy and chiral columns with different retention and/or selectivity can be readily prepared from the same starting materials, simply using different column-coating conditions.

The chiral selector *N*<sup>τ</sup>-decyl-1-spinacine (Spi( $\tau$ -dec)) (Scheme 1), has been used in the past to prepare chiral stationary phases for chiral ligand-exchange chromatography (CLEC), by means of the dynamic column coating procedure [19, 55]. Spi( $\tau$ -dec) proved to be a good chiral selector for underivatized amino acid enantiomers, when a buffered mobile phase, containing the Cu<sup>2+</sup> ions, was used (CLEC mechanism). This molecule forms a very stable chiral layer on an RP-C<sub>18</sub> support, if an aqueous mobile phase is employed; on the other hand, it can be easily washed away by a pure organic solvent. The loading and the structure of the chiral coverage can be finely tuned by means of the column preparation conditions thus modulating the retention and enantioselectivity capabilities.

To simplify the chiral system as much as possible, considering that once Spi( $\tau$ -dec) is adsorbed on the RP-C<sub>18</sub> support the stationary phase is already chiral, we investigated the enantioselective capabilities of this CSPs toward racemic mixtures of amino acids, using aqueous buffers as mobile phases, without the addition of complexing metal ions.

## 2 Materials and methods

### 2.1 Materials

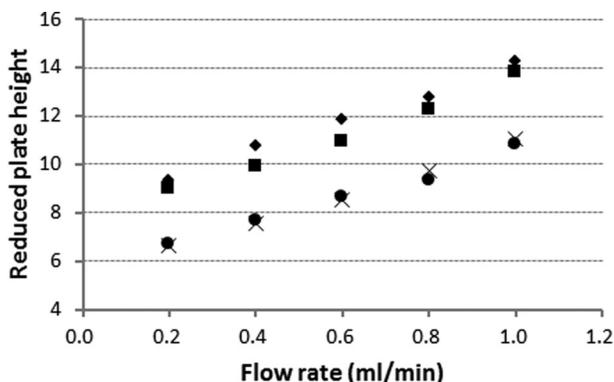
HPLC-grade methanol (MeOH, Sigma–Aldrich) and tetradistilled water were used as solvents. Potassium phosphate and acetic acid used in mobile phase preparation (Carlo Erba) as well as amino acids and oligopeptides (Sigma–Aldrich) were high purity products. The samples were injected as 0.01 – 0.1% aqueous solutions. Eluent pH was adjusted by addition of suitable amounts of standard KOH or HClO<sub>4</sub> solutions, under potentiometric control. The synthesis and characterization of the chiral selector Spi( $\tau$ -dec) are described elsewhere [55].

### 2.2 Chromatographic equipment

HPLC measurements were made with a Waters Model 600 multisolvent delivery system, equipped with a Rheodyne Model 7010 injection valve (20  $\mu$ L sample loop), and a Waters Model 996 photodiode-array detector (8  $\mu$ L cell), coupled with a personal computer driven by Waters software. The wavelength range employed for detection was 200–300 nm.

### 2.3 Preparation of the chiral Column covered with Spi( $\tau$ -dec)

A commercial Symmetry<sup>®</sup>-C<sub>18</sub> (Waters) stainless-steel analytical column (4.6  $\times$  250 mm, 5  $\mu$ m) was employed [19]. The column dead volume, measured by using KNO<sub>3</sub> as a marker, was 1.93 mL. A solution of Spi( $\tau$ -dec) 0.9% w/v in MeOH/H<sub>2</sub>O 60:40, buffered at pH\* = 7.2 with phosphate buffer 10<sup>-2</sup> M, was run through the column (previously equilibrated with the solvent and thermostatted at 30°C), at 0.2 mL/min, in frontal mode [56], until the breakthrough step was observed and the detector response proved stable. The chiral-selector load, calculated from the Spi( $\tau$ -dec) breakthrough time, graphically measured, and corrected for the column dead volume, was 0.63  $\mu$ mol. The modified column, after washing with aqueous phosphate buffer 10<sup>-2</sup> M (pH = 6), was then equilibrated with the mobile phase and it was ready for the use. The chiral phase, checked through the injection of a standard racemic mixture, proved stable for several weeks and reproducible among different preparations (under the same experimental conditions).



**Figure 1.** Efficiency of the Symmetry column, covered with Spi( $\tau$ -Dec), for D-leucine ( $\blacklozenge$ ,  $k = 1.5$ ), L-leucine ( $\blacksquare$ ,  $k = 1.6$ ), D-tyrosine ( $\blacktriangle$ ,  $k = 2.9$ ), and L-tyrosine ( $\times$ ,  $k = 3.1$ ), at variable flow rate. Eluent: phosphate buffer 0.01 M, pH = 6; 20°C.

## 2.4 Chromatographic measurements

Potassium nitrate was used as column void volume marker (on the unmodified column). Column efficiency was calculated as reduced plate height ( $h$ ) from the equation:

$$h = 0.18 L (w_h/t_R)^2/d_p$$

where  $L$  is the column length,  $w_h$  is the peak width measured at half peak height and  $d_p$  is the stationary phase particle diameter;  $h$  is a pure number. Enantioselectivity ( $\alpha$ ) between two peaks is computed as the ratio of the corresponding retention factors ( $k$ ). The elution order within a racemic sample was checked *via* the injection of single enantiomers, when available. Peak asymmetry factor,  $A_s$ , is the ratio between the right half width at 1/10 of peak height and the corresponding left half width.

## 3 Results and discussion

### 3.1 Column efficiency

Two racemic mixtures (L/D-Tyr and L/D-Leu) were employed to study the dependence of the chromatographic efficiency on the eluent flow rate (Fig. 1). For both the aliphatic and the aromatic sample, the plate height linearly increases with the mobile phase velocity: it is evident from the picture that the minimum of the Van Deemter plot should be located at a flow rate lower than 0.2 mL/min. The column efficiency improves with solute retention, as it can be deduced comparing the behavior of L/D-Leu ( $k \approx 1.5$ ) with that of L/D-Tyr ( $k \approx 3$ ). No significant difference in efficiency can be observed within each enantiomeric couple. The efficiency slightly improves if the column temperature is lowered (data at 10°C are reported as Supporting Information); this could be at least partially ascribed to the corresponding increase in retention.

**Table 1.** Retention and selectivity of three racemic amino acids on the symmetry column covered with Spi( $\tau$ -Dec) as a function of the column temperature

		Trp	Leu	Tyr
$T = 40^\circ\text{C}$	$k_L$	20.6	1.7	2.2
	$k_D$	24.4		
	$\alpha$	1.18	1.00	1.00
$T = 30^\circ\text{C}$	$k_L$	26.3	1.7	2.5
	$k_D$	32.5		2.4
	$\alpha$	1.24	1.00	1.04
$T = 20^\circ\text{C}$	$k_L$	33.1	1.6	3.1
	$k_D$	43.5	1.5	2.9
	$\alpha$	1.32	1.06	1.07
$T = 10^\circ\text{C}$	$k_L$	46.9	1.6	4.2
	$k_D$	65.9	1.5	3.9
	$\alpha$	1.41	1.07	1.07

Eluent: aqueous phosphate buffer 0.01 M, pH = 6; flow rate = 1 mL/min.

### 3.2 Column temperature

A decrease in column temperature slows down the chromatographic run of aromatic amino acids: retention times of both enantiomers significantly increase when the temperature is lowered from 40 to 10°C (Table 1); the effect is more marked for the most retained enantiomer, leading to an improved selectivity at the lowest temperatures. The effect on retention is instead almost negligible for the aliphatic sample (Leu) although the selectivity slightly improves at low temperature.

### 3.3 Eluent pH

Neither the buffer pH nor its chemical composition proved to have a significant influence on both the retention and the selectivity of aromatic and aliphatic amino acids (Table 2). In fact, these samples remain always zwitterionic in the whole explored pH range, since they possess a carboxylic group with a  $pK_a$  close to 2.5 and an amine group with a  $pK_a$  value around 9.5 [57], the former unprotonated and negatively charged and the latter protonated and positively charged. The same holds true for Spinacine, the active site of the chiral selector [58]. The effect on retention is instead remarkable when the amino acid bears an additional acid (Glu) or basic (His and Lys) side group. The side carboxylic group of glutamic acid is characterized by a  $pK_a$  value of about 4.1 [57]: this means that, a pH higher than 6, the amino acid is negatively charged, while at pH 4.5 about half of it is in its neutral and zwitterionic form. In this last form Glu can interact more strongly with the stationary phase. On the contrary, His and Lys hold an additional side basic group with a  $pK_a$  value of about 6.5 and 10.5 [57], respectively. Therefore, they are positively charged at acidic pH while they release a proton (and their charge) when pH

**Table 2.** Retention and selectivity of some racemic amino acids on the symmetry column covered with Spi( $\tau$ -Dec) as a function of the eluent pH

pH	4.5		6.0		7.5	
	<i>k</i>	$\alpha$	<i>k</i>	$\alpha$	<i>k</i>	$\alpha$
L-Trp	26.5		26.3		26.2	
D-Trp	33.2	1.26	32.5	1.24	31.4	1.20
D-Phe	5.2		5.1		5.1	
L-Phe	5.3	1.02	5.3	1.03	5.3	1.03
D- <i>n</i> Leu	1.8		1.7		1.7	
L- <i>n</i> Leu	1.9	1.04	1.8	1.05	1.8	1.05
L/D-Ala	0.4	1.00	0.4	1.00	0.4	1.00
L/D-Pro	0.5	1.00	0.5	1.00	0.5	1.00
L/D-Met	1.2	1.00	1.1	1.00	1.1	1.00
L/D-Lys	0.0	1.00	1.0	1.00	2.2	1.00
L/D-His	0.2	1.00	0.8	1.00	0.6	1.00
L/D-Glu	1.1	1.00	0.2	1.00	0.1	1.00

Eluent: aqueous buffer 0.01 M (acetate for pH 4.5 and phosphate for pH 6.0 and 7.4); flow rate = 1 mL/min, 30°C.

**Table 3.** Chromatographic parameters (retention factor, selectivity, reduced plate height, and asymmetry) for racemic tryptophan on the symmetry column covered with Spi( $\tau$ -Dec) as a function of the buffer concentration in the mobile phase

Buffer concentration	$10^{-4}$ M				$10^{-3}$ M			
	<i>k</i>	$\alpha$	<i>h</i>	<i>A<sub>s</sub></i>	<i>k</i>	$\alpha$	<i>h</i>	<i>A<sub>s</sub></i>
L-Trp	24.3	1.22	6.2	1.9	24.5	1.22	7.2	2.0
D-Trp	29.7		6.1	2.0	29.9		7.2	1.8
Buffer concentration	$10^{-2}$ M				$5 \cdot 10^{-2}$ M			
	<i>k</i>	$\alpha$	<i>h</i>	<i>A<sub>s</sub></i>	<i>k</i>	$\alpha$	<i>h</i>	<i>A<sub>s</sub></i>
L-Trp	26.3	1.24	4.4	1.2	24.9	1.21	7.5	1.4
D-Trp	32.5		4.3	1.4	30.0		6.7	1.3

Eluent: phosphate buffer at pH 6.0; flow rate = 1 mL/min, 30°C.

increases. This behavior can explain the significantly higher retention at the highest pH values.

### 3.4 Buffer concentration

In principle, the concentration of the buffer used in the mobile phase should influence the retention of amino acids due to the change of both the ionic strength and the buffering power. The latter is especially important since in the chromatographic band, flowing along the column, the sample concentration can be rather high, locally influencing the pH value and thus the behavior of the sample itself.

However, data reported in Table 3 show that the effect of the variation of the buffer concentration (in the range  $10^{-4}$ – $5 \cdot 10^{-2}$  M) on retention and selectivity of the couple L/D-Trp is rather small. The most retained enantiomer generally

**Table 4.** Retention and selectivity of some racemic aromatic amino acids on the symmetry column covered with Spi( $\tau$ -Dec)

Sample	<i>k</i> <sub>1</sub>	<i>k</i> <sub>2</sub>	$\alpha$
L/D-Trp	15.2	20.0	1.32
L/D- $\alpha$ -methyl-Trp	17.1	26.0	1.52
L/D-5-methyl-Trp	50.2	65.6	1.31
L/D-6-methyl-Trp	49.7	72.1	1.45

Eluent: Acetonitrile/aqueous phosphate buffer 0.01 M (pH 6.0) 5:95; flow rate = 1 mL/min, 10°C.

**Table 5.** Retention and selectivity of some racemic amino acids and oligopeptides on the symmetry column covered with Spi( $\tau$ -Dec)

Sample	<i>k</i> <sub>1</sub>	<i>k</i> <sub>2</sub>	$\alpha$
L/D-Trp	46.85	65.89	1.41
L/D-DOPA	3.16	3.40	1.08
L/D-Nle	1.64	1.72	1.05
L/D-Leu	1.46	1.56	1.07
L/D- <i>tert</i> -Leu	1.56	1.65	1.06
L/D-Ile	1.35	1.44	1.07
L/D- <i>allo</i> -Ile	1.26	1.36	1.08
L/D-Tyr	3.89	4.17	1.07
L/D- <i>m</i> -Tyr	7.02	7.44	1.06
Gly-L/D-Phe	11.25	11.58	1.03
Gly-L/D-Leu	2.79	3.22	1.15
L/D-Leu-Gly	3.04	3.53	1.16
Gly-L/D-Met	1.76	1.96	1.11
Gly-L/D-Leu-L/D-Ala	7.97 <sup>a)</sup> 16.28 <sup>b)</sup>	8.63 <sup>a)</sup> 19.15 <sup>b)</sup>	1.08

a) Values corresponding to the first and second peak, corresponding to the least retained couple of the four stereoisomers.

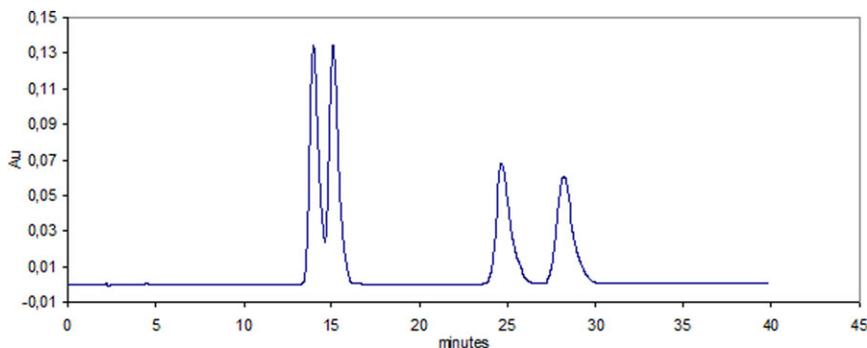
b) Values corresponding to the third and fourth peak, corresponding to the most retained couple of the four stereoisomers.

Eluent: aqueous phosphate buffer 0.01 M (pH 6.0); flow rate = 1 mL/min, 10°C.

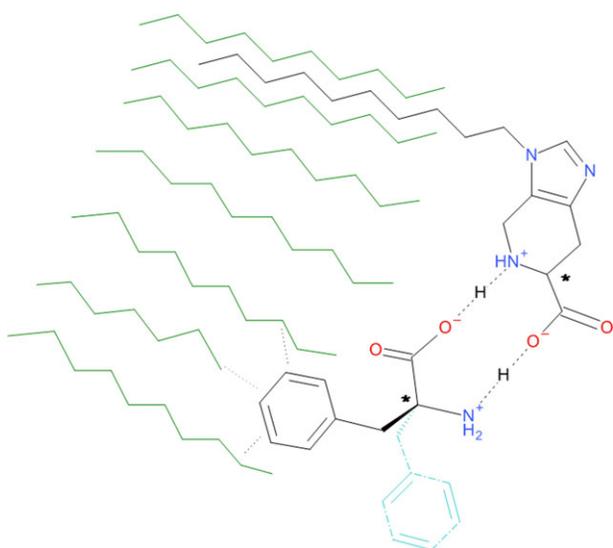
shows the best efficiency, while the asymmetry is always very similar for the two enantiomers. The trend of efficiency as a function of the buffer concentration is not easy to interpret: the best value is obtained at a concentration of  $10^{-2}$  M.

### 3.5 Organic modifier

The addition of a low amount (5%) of acetonitrile to the aqueous buffer used as mobile phase significantly speeds up the elution of aromatic samples (see Trp retention in Tables 4 and 5) and it proved very useful to elute some alkyl derivatives of Trp with acceptable retention times (Table 4); the corresponding enantioselectivity was still very good. However, the effects of type and percentage of the organic modifier in the eluent were not further investigated to preserve the chiral coverage of the column.



**Figure 2.** Separation of the four stereoisomers of Gly-L/D-Leu-L/D-Ala on the Symmetry column covered with Spi( $\tau$ -Dec). Eluent: aqueous phosphate buffer 0.01 M (pH 6.0); flow rate = 1 mL/min, 30°C.



**Scheme 2.** Structural hypothesis of the retention mechanism of L/D-Phe on the chiral stationary phase containing the selector Spi( $\tau$ -dec), showing the interaction points governing the enantioselectivity.

### 3.6 Some applications

The potentialities of the chiral stationary phase covered with Spi( $\tau$ -Dec) have been further exploited on a number of racemic mixtures of amino acids and oligopeptides and the results are shown in Table 5 and Fig. 2. Additional data and chromatograms, obtained at the flow rate of 0.2 mL/min, are reported as Supporting Information.

### 3.7 Enantioselective mechanism

In the absence of complexing metal ions, the high retention and the good resolution of aromatic and bulky aliphatic amino acids can be ascribed to both electrostatic and hydrophobic contributions, as exemplified in Scheme 2. At neutral pH, both the chiral selector and the amino acid enantiomers are zwitterionic, with the deprotonated and negatively charged carboxylic group and the protonated and positively charged amino group. These four groups

can interact through the formation of two hydrogen bonds, forming a macrocycle. The third point of interaction, which both strengthens retention and causes the enantioselectivity could be the hydrophobic interaction of the side (aliphatic or aromatic) group with the octadecyl chains of the support. Unfortunately, no interaction is possible between the PR-C<sub>18</sub> phase and the side chains of polar amino acids and no chiral discrimination is observed. It is worth noting that the elution order for L/D-Trp (L enantiomer first) is contrary to that of the other aromatic and aliphatic amino acids. Since the retention of Trp (and its derivatives), having a big indolic side group, is much higher than that of the other amino acids, it can be suggested that the retention of Trp is mainly due to the hydrophobic interaction with the RP-C<sub>18</sub> support, with the polar contacts between the carboxylic and aminic groups playing a role only in the chiral selection. This hypothesis is somewhat supported by the fact that acceptably short analysis times have been achieved for Trp derivatives only in the presence of a small amount of organic modifier or increasing the column temperature. Finally, even in the case of oligopeptides the same interactions with the stationary phase described above should come into play, although it is more difficult to describe in detail an interaction model that accurately reflects the more complicated structure of these samples.

## 4 Conclusions

The chiral chromatographic column described in the present paper, rather cheap and easy to prepare, proved very effective in the enantioseparation of underivatized aliphatic or aromatic amino acids and oligopeptides. The dynamic column coating procedure was confirmed as a powerful tool for the preparation of new chiral columns, with the desired characteristics of retention and selectivity, by simply varying the chiral selector structure and the preparation conditions. In addition, it is possible to wash and renew completely the stationary phase with a quick and cheap procedure, when necessary; the same is not possible when the chiral selector is chemically bound to the support.

The chiral discrimination method described here is simpler than that previously reported for the same chiral selector, based on the ligand-exchange mechanism [19],

because a pure aqueous buffer can be employed as mobile phase, without the addition of complexing metal ions. In addition, the retention times are much shorter for the aromatic and aliphatic amino acids as well as for oligopeptides, still maintaining a good enantioseparation. No resolution was instead achieved for amino acids with a polar side chain, for which the CLEC method proved superior. We can conclude that the two methods, using the same chiral column but coupled with different mobile phases, are complementary and easily interchangeable. The absence of metal ions, necessary for traditional CLEC protocols, makes the proposed system especially suitable for preparative scale applications. On the other hand, the absence of metal complexes reduces the detectability of compounds that lack relevant chromophoric moieties and therefore the here proposed method cannot be easily applied to trace analysis.

The number of possible chiral selectors which can be designed and synthesized for the dynamic coating procedure, each of them with different capabilities of enantio-discrimination, is practically infinite. It is not irrational to imagine that a chromatographic lab, normally using commercial reversed-phase columns, could be also equipped with a series of chiral selectors to use at the need for any possible chiral analysis, without the necessity to buy several (expensive) chiral columns.

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*The authors have declared no conflict of interest.*

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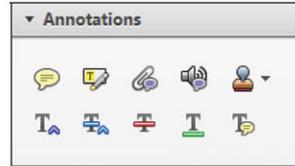
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standard framework for the analysis of microeconomics. Nevertheless, it also led to the emergence of a new paradigm of strategic behavior. The number of competitors is that the structure of the industry is a key determinant of the main components of the cost structure. At the industry level, are exogenous variables important? Works on entry by entrants (M henceforth)<sup>1</sup> we open the 'black b



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there is no room for extra profits as mark-ups are zero and the number of firms (n) values are not determined by market clearing. Blanchard and Kiyotaki (1987), in a perfect competition in general equilibrium model, derive the effects of aggregate demand and supply shocks in a classical framework assuming monopolistic competition and an exogenous number of firms.

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dynamic responses of mark-ups are consistent with the VAR evidence.

standard framework for the analysis of microeconomics. Nevertheless, it also led to the emergence of a new paradigm of strategic behavior. The number of competitors is that the structure of the industry is a key determinant of the main components of the cost structure. At the industry level, are exogenous variables important? Works on entry by entrants (M henceforth)<sup>1</sup> we open the 'black b



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standard framework for the analysis of microeconomics. Nevertheless, it also led to the emergence of a new paradigm of strategic behavior. The number of competitors is that the structure of the industry is a key determinant of the main components of the cost structure. At the industry level, are exogenous variables important? Works on entry by entrants (M henceforth)<sup>1</sup> we open the 'black b



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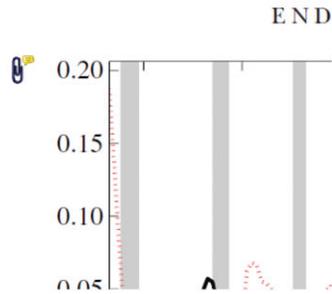
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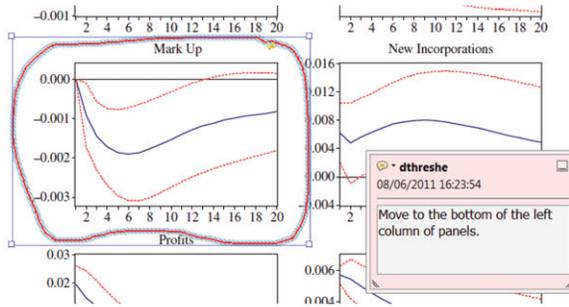


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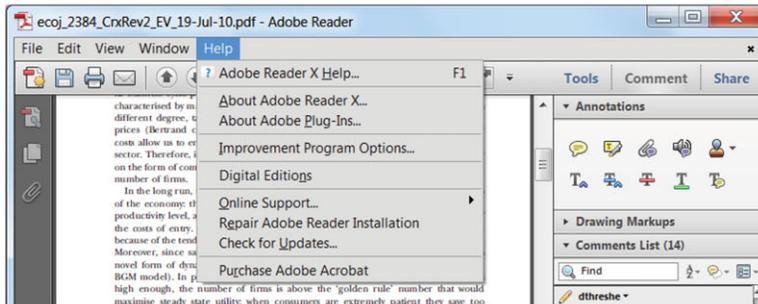
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