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1 **Enantioseparations of polyhalogenated 4,4'-bipyridines on**
2 **polysaccharide-based chiral stationary phases and**
3 **molecular dynamics simulations of selector-selectand**
4 **interactions**

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26
27 **Keywords:** Bipyridines / electrostatic potential / enantiomer elution order / molecular
28 dynamics / polysaccharide-based chiral stationary phases

29
30 **Abbreviations:** **CSP**, chiral stationary phase; **DFT**, density functional theory; **EEO**,
31 enantiomer elution order; **ESH**, explicit σ -hole; **MD**, molecular dynamics; **MP**, mobile
32 phase; **NP**, normal phase; **TCIBP**, 3,3',5,5'-TetraChloro-2-Iodo-4,4'-BiPyridyl; **XB**,
33 halogen bond

34 **Abstract**

35 2'-(4-Pyridyl)- and 2'-(4-hydroxyphenyl)-TCIBPs (TCIBP = 3,3',5,5'-tetrachloro-2-iodo-
36 4,4'-bipyridyl) are chiral compounds that showed interesting inhibition activity against
37 transthyretin fibrillation *in vitro*. We became interested in their enantioseparation since
38 we noticed that the *M*-stereoisomer is more effective than the *P*-enantiomer. Based
39 thereon, we recently reported the enantioseparation of 2'-substituted TCIBP
40 derivatives with amylose-based chiral columns. Following this study, herein we
41 describe the comparative enantioseparation of both 2'-(4-pyridyl)- and 2'-(4-
42 hydroxyphenyl)-TCIBPs on four cellulose phenylcarbamate-based chiral columns
43 aiming to explore the effect of the polymer backbone, as well as the nature and position
44 of substituents on the side groups/moieties on the enantioseparability of these
45 compounds. In the frame of this project, the impact of subtle variations of analyte and
46 polysaccharide structures, and mobile phase (MP) polarity on retention and selectivity
47 was evaluated. The effect of temperature on retention and selectivity was also
48 considered, and overall thermodynamic parameters associated with the analyte
49 adsorption onto the CSP surface were derived from van't Hoff plots. Interesting cases
50 of enantiomer elution order (EEO) reversal were observed. In particular, the EEO was
51 shown to be dependent on polysaccharide backbone, the elution sequence of the two
52 analytes being *P-M* and *M-P* on cellulose and amylose *tris*(3,5-
53 dimethylphenylcarbamate), respectively. In this regard, a theoretical investigation
54 based on molecular dynamics (MD) simulations was performed by using amylose and
55 cellulose *tris*(3,5-dimethylphenylcarbamate) nonamers as virtual models of the
56 polysaccharide-based selectors. This exploration at the molecular level shed light on
57 the origin of the enantiodiscrimination processes.

58 **1 Introduction**

59 In chiral chromatography, the basic components of the recognition process are chiral
60 analyte, chiral stationary phase (CSP), and mobile phase (MP) [1]. In this molecular
61 environment, the chromatographic separation process originates from consecutive
62 single adsorption and desorption steps occurring on the CSP surface as the analyte
63 moves along the column [2,3]. Intermolecular noncovalent interactions play a pivotal
64 role in this process, and hydrogen bonds (HBs), halogen bonds (XBs), dipole-dipole,

65 π - π stacking, steric repulsive, and van der Waals interactions underlie the adsorption
66 process and the formation of transient diastereomeric assemblies between the chiral
67 selector and the enantiomer pair [4]. The overall stereoselective contact between
68 chiral selector and enantiomer originates from the sum of single noncovalent
69 interactions, which is defined as steric, electrostatic or hydrophobic depending on the
70 structural and electronic features of the interacting partners. MP polarity impacts the
71 overall process, affecting electron density distribution and associated electrostatic
72 potential (V) of the recognition sites [4,5] and, consequently, noncovalent interaction
73 strength. A CSP represents a diffuse chirotopic environment. Indeed, as stated by
74 Hirschmann and Hanson, chirality “*is an all-pervasive property, as it affects all parts*
75 *of a chiral structure*” [6]. In the same perspective, Mislow and Siegel defined chirotopic
76 “*any atom, and, by extension, any point or segment of the molecular model [...] that*
77 *resides within a chiral environment*” [7]. On this basis, all sites of a CSP are in principle
78 potentially able to participate in enantioselective contacts, contributing to enantiomer
79 discrimination. This concept is particularly true for CSP with high density of chiral
80 elements such as polysaccharide-based CSPs. Indeed, in addition to the presence of
81 a large number of chiral centers, these polymeric CSPs are characterized by
82 conformational chirality dependent on the helical twist generated by the specific
83 glycosidic β - and α -1,4-linkages in cellulose and amylose chain, respectively [8]. Thus,
84 a number of noncovalent interactions can potentially occur into the polymeric groove
85 but, actually, only some of them act to recognize the enantiomers of a given chiral
86 analyte, depending on its particular structure, size and shape, the sum of geometry
87 and electronic distribution. Given this context, subtle variations of analyte and CSP
88 structures, and MP polarity may deeply impact retention and enantioseparation on
89 polysaccharide carbamate-based CSPs. For this reason, with the aim to detect
90 noncovalent interactions and recognition patterns, molecular design can be fruitfully
91 used to obtain specific structures suitable for recognition studies in liquid-phase
92 environment. In this frame, we recently demonstrated that the structure of the 2'-
93 substituent has a pivotal impact on the enantioseparation of 2'-substituted TCIBPs
94 (TCIBP = 3,3',5,5'-tetrachloro-2-iodo-4,4'-bipyridyl) (Fig. 1) on amylose-based CSPs
95 [3]. Following this study, we report herein the comparative enantioseparation of both
96 2'-(4-pyridyl)- (**1**) and 2'-(4-hydroxyphenyl)-TCIBPs (**2**) on four cellulose
97 phenylcarbamate-based CSPs aiming to explore the effect of the polymer backbone,
98 as well as the nature and position of substituents on the side groups/moieties on

99 enantioseparability of these compounds. This issue is of interest because recently
100 compounds **1** and **2** showed relevant inhibition activity against transthyretin fibrillation
101 *in vitro*, the *M*-enantiomer being more effective than the *P*-enantiomer [9]. In the frame
102 of this study, the impact of subtle variations of analyte and cellulose-based CSP
103 structures, and MP polarity on retention and selectivity was evaluated. The effect of
104 temperature on retention and selectivity was also considered, and overall
105 thermodynamic parameters associated with the analyte adsorption onto the CSP
106 surface were derived from van't Hoff plots. Finally, a theoretical investigation based
107 on molecular dynamics (MD) simulations [10] was performed by using amylose and
108 cellulose *tris*(3,5-dimethylphenylcarbamate) (A-3,5diMe and C-3,5diMe) nonamers, as
109 virtual models of the polysaccharide-based selectors, with the aim of exploring the
110 origin of the enantiodiscrimination processes at the molecular level.

111 **2 Materials and methods**

112 **2.1 Chemicals**

113 Compounds **1** and **2** were synthesized, purified and characterized as reported [9].

114 **2.2 Chromatography**

115 An Agilent Technologies (Waldbronn, Germany) 1100 Series HPLC system (high-
116 pressure binary gradient system equipped with a diode-array detector operating at
117 multiple wavelengths (220, 254, 280, 360 nm), and a 20 μ l loop) was employed. Data
118 acquisition and analyses were carried out with Agilent Technologies ChemStation
119 Version B.04.03 chromatographic data software. The UV absorbance is reported as
120 milliabsorbance units (mAU). Lux Cellulose-1 (coated) (cellulose *tris*(3,5-
121 dimethylphenylcarbamate) (C-3,5diMe)), Lux Cellulose-2 (coated) (cellulose *tris*(3-
122 chloro-4-methylphenylcarbamate) (C-3Cl,4Me)), Lux Cellulose-4 (coated) (cellulose
123 *tris*(4-chloro-3-methylphenylcarbamate) (C-4Cl,3Me)), and Lux i-Cellulose-5
124 (immobilized) (cellulose *tris*(3,5-dichlorophenylcarbamate) (C-3,5diCl)) were used as
125 chiral columns (5 μ m, 250 \times 4.6 mm) (Phenomenex Inc., Torrance, CA, USA). HPLC
126 grade *n*-hexane (Hex), isopropanol (IPA), and methanol (MeOH) were purchased from
127 Sigma-Aldrich (Taufkirchen, Germany). Analyses were performed in isocratic mode at
128 25 °C. The flow rate (*FR*) was set at 0.8 ml/min. Dead time (t_0) was measured by
129 injection of tri-*tert*butylbenzene (Sigma-Aldrich) as a non-retained compound [11]. The

130 enantiomer elution order (EEO) was determined by injecting enantiomers of known
131 absolute configuration [9]. The van't Hoff experiments were conducted at 5, 10, 15,
132 20, 25, 30, 35, 40, and 45 °C by using a thermostat jacket equipped with a RE104
133 LAUDA circulating water-bath (Lauda, Königshofen, Germany). When the temperature
134 was changed, the column was allowed to equilibrate for 1 h before injecting the
135 sample. Thermodynamic parameters were derived from the slopes and the intercepts
136 of the van't Hoff plots (see Supporting Information for details) by linear regression
137 analysis. Statgraphics Centurion XVI (Statpoint Technologies, Inc., Warrenton, VA,
138 USA) was used for all linear regression analyses.

139 **2.3 Computational**

140 The 3D structures of compounds **1** and **2** and methyl 3,5-dimethylphenylcarbamate,
141 methyl 3-chloro-4-methylphenylcarbamate, methyl 4-chloro-3-
142 methylphenylcarbamate and methyl 3,5-dichlorophenylcarbamate, as frameworks
143 representing the CSP side chains, were prepared using the build function, and model
144 kits and tools provided by Spartan '10 Version 1.1.0 (Wavefunction Inc., Irvine, CA,
145 USA) [12] for building and editing organic molecules. On this basis, molecular
146 structures were generated and their refinement was performed by a MMFF procedure.
147 Then, each structure was submitted to a conformational systematic search using
148 MMFF, spanning all shapes accessible to the molecule without regard to energy. After
149 the elimination of duplicates and high-energy conformers, a set of energetically
150 accessible conformers was selected. For each conformer, geometry optimization was
151 performed employing the DFT method with the B3LYP functional and the 6-311G*
152 basis set, and finally the respective Boltzmann distribution was constructed. Geometry
153 optimization and computation of electrostatic potential isosurfaces (V_s) and related
154 parameters (V_s extrema, $V_{s,max}$ and $V_{s,min}$ values, given in au) were performed by
155 using Gaussian 09 (DFT, B3LYP, 6-311G*) (Wallingford, CT 06492, USA) [13]. Search
156 for the exact location of such $V_{s,max}$ and $V_{s,min}$ was made through the Multiwfn code
157 [14] and through its module enabling quantitative analyses of molecular surfaces [15].
158 The AMBER18 Antechamber toolkit (University of California, San Francisco, USA) [16]
159 was used to assign the generalized Amber Force Field (GAFF) atom type and the
160 AM1-BCC type of charge to 4,4'-bipyridines **1** and **2**. The Gaussian 09 program (DFT,
161 B3LYP, 3-21G*) [13] was used for the *ab initio* geometry optimization calculation of
162 the monomeric units of β -D- and α -D-glucose-1,4-dimethoxy-*tris*(3,5-

163 dimethylphenylcarbamate). The optimized structures were used to build nonamers (9-
164 mer) of C-3,5diMe and A-3,5diMe, respectively [17]. C-3,5diMe was characterized by
165 a left-handed threefold (3/2) helix according to the structure reported by Vogt and
166 Zugenmaier [18], setting the dihedral angles of the units, defined by $H_1-C_1-O-C_4(\Phi)$
167 and $H_4'-C_4'-O-C_1(\varphi)$ to 60° and 0° (Supporting Information, Fig. S1A). A-3,5diMe was
168 characterized by a 4/3 left-handed helical structure according to the structure reported
169 by Okamoto and co-authors [19,20], setting the dihedral angles of the units, defined
170 by $H_1-C_1-O-C_4(\Phi)$ and $H_4'-C_4'-O-C_1(\varphi)$ to -68.5° and -42.0° (Fig. S1B). The terminal
171 residues of the polymers were closed with methoxyl groups. The polymer structures
172 were energy-minimized using the GAFF force-fields with AM1-BCC charges assigned
173 with the Antechamber toolkit. The atoms of the backbone were fixed in their positions
174 during the simulations by assigning a force constant of 20 kcal/mol so that, starting
175 from the setting initial values, the applied restriction restrained the rotation of backbone
176 dihedral angles of residues 2-8 (Fig. S2). The energies and the structure of the
177 polymers were first prepared using 100 ns MD simulations (see Supporting Information
178 for details about MD stages) with Hex/IPA 90:10 as medium. This structure was used
179 in the final MD simulations. The AMBER18 software [16] was used to carry out 100 ns
180 MD simulations. The initial positions of each enantiomer were determined by
181 molecular docking (see Supporting Information for details). Solvent effect was taken
182 into account by means of the explicit periodic solvent box (Hex:IPA 90:10). In this
183 regard, the polysaccharide-analytes complexes were prepared for MD runs by
184 solvating the system with an octahedral box with a 10 \AA radius polysaccharide cutoff
185 by using Packmol-*memgen* [21,22] and an in-house script to manage solvent mixtures.
186 100 ns of the trajectories from each case were considered for statistical analysis. The
187 Chimera software (UCSF, San Francisco, USA) was used for visualization and
188 analysis of the MD trajectories [23]. Interaction energies between the polysaccharide
189 nonamer and the enantiomer were calculated, which include van der Waals (vdW) and
190 electrostatic (el) energies.

191 **3 Results and discussion**

192 **3.1 Electrostatic potential analysis of analytes and chiral selectors**

193 For compounds **1** and **2**, the electrostatic potential maxima ($V_{S,max}$, Fig. 2, pale blue
194 points) and minima ($V_{S,min}$, Fig. 2, red points) values were computed in order to inspect
195 the electron charge density distribution on the main electron-poor (electrophile, Lewis
196 acid) and electron-rich (nucleophile, Lewis base) recognition sites, respectively (Fig.
197 2A,B). Recently, V analysis has been fruitfully used to gain insights on selector/analyte
198 contacts by evaluating the electron charge density on molecular regions involved in
199 noncovalent interactions [5,24,25]. In compounds **1** and **2**, the distinctive substituents
200 located at 2'-position are a 4-pyridyl ring in **1** and a 4-hydroxyphenyl group in **2**.
201 Moreover, both compounds contain a common 3,3',5,5'-tetrachlorinated motif which
202 represents a symmetric hydrophobic region surrounding the chiral axis. Another
203 hydrophobic region is present at the 2-position where an iodine atom is located as
204 substituent. This halogen may act as halogen bond (XB) donor interacting through its
205 electrophilic σ -hole with the nucleophilic regions of the CSP (Fig. 2C). Higher
206 polarization was induced by the 4-pyridyl substituent (**1**: $V_{S,max} = 0.0535$ au) at the
207 position 2' of the 4,4'-bipyridinyl scaffold compared to the 4-hydroxyphenyl substituent
208 (**2**: $V_{S,max} = 0.0496$ au). In our previous studies, we demonstrated by chromatographic
209 and computational analyses that the carbonyl oxygens of C-3,5diMe and A-3,5diMe
210 are able, as Lewis bases, to form XBs with the electrophilic σ -hole regions of halogen
211 substituents bound to the 4,4'-bipyridine rings [17]. HB sites are located on the
212 aromatic substituents in 2'-position, a nitrogen as HB acceptor ($V_{S,min} = -0.0658$ au)
213 and a hydroxyl group as HB acceptor/donor ($V_{S,min} = -0.0421$ au; $V_{S,max} = 0.1128$ au),
214 respectively. As the OH group in **2** is free to rotate around the C-O bond, the
215 directionality of the HB sites on the OH may change, in principle making compound **2**
216 more adaptable to the CSP chiral cavity than **1**. In the latter case, the rotation of the
217 4-pyridyl substituent does not change the directionality of the HB involving the pyridyl
218 nitrogen.

219 Chiral columns based on C-3,5diMe, C-3Cl,4Me, C-4Cl,3Me, and C-3,5diCl were
220 selected for this study in order to evaluate the impact of aryl chlorination on their
221 enantioseparation performances. All columns contain selectors based on the same
222 cellulose backbone which is derivatized with distinctive side chains determining the
223 stereoelectronic properties of each selector [8]. The effect of introducing chlorine in

224 the CSP structure is to modify the electron charge density distribution on the side chain
 225 moieties, thus the electron charge density on both C=O and phenyl ring decreases (π -
 226 acidity increases), whereas the acidity of the N-H increases [26]. This trend has been
 227 confirmed by calculating $V_{S,max}$ and $V_{S,min}$ values on pivotal regions of the side chains
 228 of C-3,5diMe, C-3Cl,4Me, C-4Cl,3Me, and C-3,5diCl (Table 1).

229 **Table 1.** Cellulose carbamate-based CSPs/columns used in the study, and $V_{S,max}$ and $V_{S,min}$
 230 values associated with the main recognition sites (carbamate N-H and C=O)

Column ^{a)}	Ar (R',R''-C ₆ H ₄)	Abbreviation	$V_{S,min}$ C=O (au) ^{b)}	$V_{S,max}$ N-H (au) ^{b)}
Cellulose-1	3,5-dimethyl	C-3,5diMe	-0.0630	0.0827
Cellulose-2	3-chloro-4-methyl	C-3Cl,4Me	-0.0576	0.0902
Cellulose-4	4-chloro-3-methyl	C-4Cl,3Me	-0.0578	0.0910
i-Cellulose-5	3,5-dichloro	C-3,5diCl	-0.0532	0.0987

231 ^{a)} Lux series columns (Phenomenex Inc., Torrance, CA, USA). ^{b)} V_S values calculated at
 232 DFT/B3LYP/6-311G* level, $V_{S,max}$ (Fig. 2C, maxima a) and $V_{S,min}$ (Fig. 2C, minima b)

233
 234 It is known that the introduction of chlorine increases the fraction of free N-H groups
 235 [27], whereas the fraction of N-H involved in intramolecular HBs, contributing to
 236 maintain the high-ordered structure of the CSP, decreases. This could produce for the
 237 chlorinated CSPs a wider cavity available for the enantiomers with respect to the
 238 dimethylated selector, the overall enantioseparation resulting from the balance of
 239 carbamate polarity and intramolecular HB ability [27].

240 3.2 Chromatographic screening

241 The enantioseparability of TCIBPs **1** and **2** was tested on coated C-3,5diMe, C-
 242 3Cl,4Me, C-4Cl,3Me, and immobilized C-3,5diCl columns by using Hex/IPA 90:10 as
 243 MP. A comparison between the behaviours of the four columns is reported in Figure 3
 244 (see Supporting Information, Table S2 for numerical data).

245 Good selectivity was achieved for the enantioseparation of **1** on C-3,5diMe ($\alpha = 2.82$)
 246 exclusively, whereas lower selectivity values ranging from 1.07 to 1.15 were obtained
 247 in other cases. No enantioseparation was observed for **1** and **2** on C-3Cl,4Me and C-
 248 4Cl,3Me, respectively. Retention of both enantiomers was higher for **1** (average $k_1 =$
 249 4.9; average $k_2 = 6.4$) compared to **2** (average $k_1 = 2.1$; average $k_2 = 2.3$) in almost all
 250 cases. The first eluted enantiomer of **2** showed higher retention only on C-3,5diMe (k_1
 251 (**2**) = 2.94 vs k_2 (**1**) = 2.67). Given the presence of a HB acceptor (pyridyl nitrogen) as
 252 a distinctive recognition site, for compound **1** retention of the first eluted enantiomer
 253 tended to increase as the HB donor ability of the selector amidic N-H also increased
 254 (towards more positive $V_{S,max}$ values moving from C-3,5diMe to C-3,5diCl). The

255 opposite trend was observed for compound **2** due to the presence of a HB donor (OH
256 hydrogen) as distinctive recognition site. In this case, retention of both enantiomers
257 increased as the N-H $V_{S,max}$ values and the electron charge density on the carbamate
258 C=O decreased and increased (towards more negative $V_{S,min}$ values moving from C-
259 3,5diCl to C-3,5diMe), respectively. As a particular case, retention of the second eluted
260 enantiomer of compound **1** increased moving from C-3,5diMe ($k_2 = 7.54$) to C-3,5diCl
261 ($k_2 = 8.26$), whereas the two chloromethyl substituted C-3Cl,4Me and C-4Cl,3Me
262 provided lower k_2 values (4.78 and 5.15, respectively). On the other hand, EEO
263 reversal was observed on C-3Cl,4Me and C-4Cl,3Me (*M-P*) for both compounds
264 compared to the 3,5-disubstituted C-3,5diMe and C-3,5diCl (*P-M*), this evidence
265 revealing the occurrence of a different adsorption mechanism [3,28-31] It is worth
266 noting that EEO is also a key factor for the method development [32]. Indeed, as chiral
267 separation methods are optimized for optical purity control of a chiral analyte, the
268 possibility to modify the EEO may be advantageous in order to have the polluting
269 enantiomer eluted first [28,31,32].

270 The addition of 5% MeOH to the MP was detrimental for retention and selectivity in
271 almost all cases (Supporting Information, Table S3 and Fig. S3). However, for
272 compound **1** on the C-3,5diMe the use of the mixture Hex/IPA/MeOH 90:5:5
273 contributed to reduce elution time (k_1 , -12%; k_2 , -52%) keeping selectivity value
274 acceptable ($\alpha = 1.54$) (Fig. S3A,B). In this regard, it is worth noting that the addition of
275 MeOH to the MP impacted retention of the second eluted enantiomer of compound **1**
276 more on the C-3,5diMe (k_2 , -52%) compared to the other chlorinated C-3Cl,4Me, C-
277 4Cl,3Me, and C-3,5diCl (k_2 , -17.6%, -32.6%, -34.4%, respectively). This suggested
278 that a second key interaction involving the carbamate C=O possibly affected by 5%
279 MeOH addition may participate in chiral recognition. In this regard, the involvement of
280 a XB between the 2-iodine of compound **1** as XB donor and the carbonyl of the CSP
281 as XB acceptor could be envisaged, the $V_{S,max}$ value on 2-iodine being higher for **1**
282 (0.0535 au) compared to **2** (0.0496 au).

283 This chromatographic results confirmed previous observations showing that the
284 anisotropic properties of chiral substituted 4,4'-bipyridines strongly depend on the
285 stereoelectronic features of the 2,2',3,3',5,5'-substituents bore by the orthogonal
286 heteroaromatic rings, as a consequence of the atropisomeric motif [33,34]. For **1** and
287 **2**, it was expected that the enantiodiscrimination degree should be related to the
288 strength of noncovalent interactions involving both 2- and 2'- positions, due to the

289 symmetry of the 3,3',5,5'-tetrachloro pattern. On the other hand, the direct contribution
290 to retention and selectivity of the 4,4'-bipyridine core was shown to be low, in particular
291 due to the weakness of the pyridine nitrogens as HB acceptors ($-0.0490 \text{ au} \leq V_{S,\text{min}} \leq$
292 -0.0426 au). However, in compound **1** three electron-withdrawing heteroaromatic
293 substructures polarized iodine, thus contributing to its capability to exert XB.

294 **3.2.1 Effect of temperature on enantioseparation**

295 With the aim to explore the impact of temperature on enantioseparation, and compare
296 the thermodynamic profiles of the cellulose-based CSPs as derived from van't Hoff
297 analysis (see Supporting Information for details on van't Hoff and thermodynamic
298 equations), retention and selectivity of compounds **1** and **2** on the four cellulose-based
299 CSPs were determined at different temperatures from 5 to 45°C in 5°C increments
300 (Supporting Information, Tables S4-S7) using Hex/IPa 90:10 as MP. Several papers
301 have dealt with theory of adsorption phenomena in chromatography, and with methods
302 for profiling temperature dependence of retention and selectivity and thermodynamic
303 quantities associated with the adsorption of analytes on the CSP surface [35,36].
304 Some studies stressed that thermodynamic quantities derived from the classical van't
305 Hoff equation are macroscopic entities which do not account for surface heterogeneity
306 of the CSPs that determines individually achiral and chiral features of
307 enantioseparation [37]. On the other hand, thermodynamic parameters depend on
308 analyte, MP and the diffuse chiral (chirotopic) environment profile of the CSP.
309 Therefore, the nature of the analyte/CSP contact can be explored on the basis of
310 thermodynamic considerations, and useful information can emerge by comparison of
311 thermodynamic data of analogue analyte/CSP pairs as subtle variations of the
312 chromatographic system occur. In addition, temperature is a useful variable to
313 optimize enantioseparation [3,38,39].

314 The thermodynamic quantities derived from van't Hoff plots (Fig. 4) are reported in
315 Table S8 (Supplementary information). On this basis, the following remarks emerged:

316 i) compounds **1** and **2** showed different thermodynamic profiles, and the temperature
317 dependence pattern was observed to be a function of the 2'-substituent structure and
318 of the CSP type;

319 ii) for compounds **1** and **2** the enantioseparations were enthalpy-driven on the 3,5-
320 disubstituted CSPs because the temperature range was below the calculated T_{ISO} ,
321 and the thermodynamic ratio $Q = \Delta\Delta H / (298 \times \Delta\Delta S) > 1$ [40] ($157^\circ\text{C} \leq T_{ISO} \leq 587^\circ\text{C}$;

322 $1.44 \leq Q \leq 2.85$) (Fig. 4A,B,G,H). On the contrary, the enantioseparations were shown
323 to be entropy-driven on the 3,4-disubstituted CSPs in almost all cases (Fig. 4C,E,F) ($-$
324 $70^{\circ}\text{C} \leq T_{ISO} \leq 15^{\circ}\text{C}$; $0.68 \leq Q \leq 0.97$). These different thermodynamic profiles could
325 explain the EEO reversal from *P-M* to *M-P* observed as the substitution pattern of the
326 CSP phenyl rings changes from the 3,5- to 3,4-disubstitution;

327 iii) for compound **1** partial separation was observed on C-3Cl,4Me in the range 30-
328 45°C ($1.017 \leq \alpha \leq 1.035$) (Supporting Information, Fig. S4A), whereas for compound
329 **2** on the C-4Cl,3Me very low enantioseparation was detectable at 45°C exclusively (α
330 $= 1.018$) (Fig. S4B);

331 iv) in the case of compound **2** enantioseparation on C-3Cl,4Me, the thermodynamic
332 profiles revealed the presence of two concurrent mechanisms in the range $5-45^{\circ}\text{C}$, an
333 entropy controlled ($T_{ISO} = -55^{\circ}\text{C}$, $Q = 0.73$) at low temperature and an enthalpy
334 controlled mechanism ($T_{ISO} = 97^{\circ}\text{C}$, $Q = 1.24$) at higher temperature. The two
335 mechanisms coalesced between 30 and 20°C , providing at 25°C the best value of
336 selectivity ($\alpha = 1.07$), and a concave profile for the plot $\ln \alpha = f(1/T)$ (Supporting
337 Information, Fig. S5);

338 v) on this basis, enantioseparation of compounds **1** and **2** could in some cases be
339 optimized by varying the temperature. For compound **1** on C-3,5diMe, elution time
340 could be reduced at 45°C maintaining good selectivity ($\alpha_{25^{\circ}\text{C} \rightarrow 45^{\circ}\text{C}} = 2.83 \rightarrow 2.32$). In
341 the other cases, enantioselectivity was almost independent of the temperature
342 variation (Fig. S5). However, for **1** on C-4Cl,3Me, the enantioseparation under entropic
343 control could be optimized at 45°C ($\alpha_{25^{\circ}\text{C} \rightarrow 45^{\circ}\text{C}} = 1.11 \rightarrow 1.13$). For both compounds **1**
344 and **2** on C-3,5diCl, enantioseparation could be optimized under enthalpic conditions
345 at 5°C ($\alpha_{25^{\circ}\text{C} \rightarrow 5^{\circ}\text{C}}$ (**1**) $= 1.10 \rightarrow 1.12$; $\alpha_{25^{\circ}\text{C} \rightarrow 5^{\circ}\text{C}}$ (**2**) $= 1.14 \rightarrow 1.16$).

346 **4 Molecular dynamics simulations**

347 As depicted in Figure 5, C-3,5diMe and A-3,5diMe [3] showed complementary
348 enantioseparation ability towards compounds **1** and **2**. Indeed, compound **1** (Fig. 5A)
349 ($\alpha = 2.82$) was enantioseparated on the C-3,5diMe better than compound **2** (Fig. 5C)
350 ($\alpha = 1.14$), whereas **2** (Fig. 5D) ($\alpha = 1.26$) was enantioseparated on the A-3,5diMe with
351 selectivity higher than compound **1** (Fig. 5B) ($\alpha = 1.04$). A backbone-dependent
352 reversal of EEO was also observed, the elution sequence being *P-M* and *M-P* on C-

353 3,5diMe and A-3,5diMe, respectively. In addition, thermodynamic analysis evidenced
 354 an enthalpic contribution to free energy difference ($\Delta\Delta G^\circ$) associated to the
 355 enantioseparations higher for C-3,5diMe ($Q = 1.44, 1.60$) compared to A-3,5diMe (Q
 356 $= 1.04, 1.08$). The enthalpic contribution to enantioseparation was higher for
 357 compound **2** compared to **1**, the difference being more pronounced on C-3,5-diMe
 358 ($\Delta Q_{1,2} = 0.16$) compared to the amylose-based selector ($\Delta Q_{1,2} = 0.02$).

359 On this basis, with the aim to explore the molecular basis of these chromatographic
 360 behaviors, a theoretical investigation based on MD simulations was performed by
 361 using C-3,5diMe and A-3,5diMe nonamers as virtual models of the polysaccharide-
 362 based selectors.

363 The 100 ns MD simulations in the AMBER force field [41] were performed by using the
 364 mixture Hex/IPA 90:10 as a virtual solvent in accord with the experimental conditions
 365 used in the chromatographic studies. With the aim to confirm the hypothesis that a XB
 366 involving the 2-iodine substituent of the enantiomer (*M*)-**1** could contribute to the high
 367 adsorption on C-3,5diMe ($t_R = 30.25$ min), the explicit σ -hole (ESH) concept [42,43]
 368 was used to model the electrophilic electron charge density depletion on the iodine
 369 atom [17] (see Supporting Information for details). For both analytes, the simulations
 370 were performed with and without ESH in order to also evaluate the MD results when
 371 the electrophilic character of iodine is suppressed. The total interaction energies
 372 calculated for (*M*)- and (*P*)-enantiomers of **1** and **2** in their complexes with each of the
 373 polysaccharide nonamer are summarized in Table 2.

374 **Table 2.** Binding energies (E_{int}) (kcal/mol) and component contributions (E_{el} , E_{vdW}) for the
 375 association of (*M*)-**1**, (*P*)-**1**, (*M*)-**2**, and (*P*)-**2** with C-3,5diMe ($EEO_{exp} = P-M$) and A-3,5diMe
 376 ($EEO_{exp} = M-P$)

TCIBP	C-3,5diMe				A-3,5diMe			
	EEO_{calc}	E_{int}	E_{el}	E_{vdW}	EEO_{calc}	E_{int}	E_{el}	E_{vdW}
1	<i>P</i>	-30.63	-3.43	-27.20	<i>M</i>	-32.16	-6.06	-26.10
	<i>M</i> *	-33.23	-12.08	-21.15	<i>P</i>	-35.48	-6.03	-29.45
2	<i>P</i>	-31.76	-3.55	-28.21	<i>M</i>	-38.30	-9.49	-28.81
	<i>M</i>	-36.29	-13.78	-22.51	<i>P</i>	-41.25	-8.29	-32.96

377 * Explicit σ -hole was introduced on 2-iodine of (*M*)-**1**

378 The reported energies are mean values which were calculated from 5000 complexes
 379 obtained by snapshots taken every 20 ps from the 100 ns MD trajectories. The
 380 interaction energy (E_{int}) between enantiomer and selector is calculated on the basis of
 381 the energies of the selector-enantiomer complex, the selector and the enantiomer (eq.
 382 1)

383 $E_{\text{int}} = E_{\text{total}} - E_{\text{enantiomer}} - E_{\text{polysaccharide-based selector}}$ (1)

384 where the E_{int} term derived from the contributions of the van der Waals (vdW) and the
385 electrostatic (el) interaction terms (eq. 2).

386 $E_{\text{int}} = E_{\text{el}} + E_{\text{vdW}}$ (2)

387 In Figure 6, representative snapshots and noncovalent interactions from the simulated
388 MD trajectories of **1** and **2** complexes with C-3,5diMe (**A-D**) and A-3,5diMe (**E-H**) are
389 depicted. The following remarks emerged:

390 i) in accord with a previous observation [17], MD simulation provided a more compact
391 structure for A-3,5diMe nonamer (with smaller cavities) compared to the C-3,5diMe;

392 ii) coherently, in all simulations involving the A-3,5diMe nonamers, the bulky iodine
393 substituent protruded out of the polymer groove (Fig. 6F,G,H) or was oriented towards
394 the void inside the groove (Fig. 6E), thus no XB was detected even if the ESH was
395 introduced on the iodine. This finding is in accord with our previous observations on
396 the detrimental effect of the compact structure of A-3,5diMe on XBs involving iodine
397 [17];

398 iii) analogously, in all cellulose-based complexes involving the (*P*)-**1**, (*P*)-**2**, and (*M*)-**2**
399 enantiomers, modelled either with and without ESH, the iodine was oriented outside
400 the polymer (Fig. 6A,C,D);

401 iv) otherwise, a XB between the 2-iodine and the carbamate C=O was detected in the
402 complex (*M*)-**1** / C-3,5diMe as the ESH was introduced on the iodine of the analyte. In
403 this case, the calculated EEO (Table 2) is fully consistent with the experimental elution
404 sequence. On the contrary, the simulation performed without ESH correction provided
405 a theoretical EEO not consistent with experimental EEO showing that the electrophilic
406 feature of iodine has a pivotal role in the enantiodiscrimination. On this basis, the high
407 retention of the enantiomer (*M*)-**1** was related to a four-component noncovalent
408 interaction pattern consisting of one HB, two π - π interactions and a XB (Fig. 6B);

409 v) for each MD simulations, the E_{vdW} component was found to be the major contribution
410 to the interaction energy. Indeed, in all cases, hydrophobic contacts between the
411 haloaromatic scaffold of the analyte and the surface of the polymer appeared to govern
412 analyte / selector association along with distinctive HBs and π - π stacking interactions;

413 vi) the fit of both enantiomers (*P*) on the C-3,5-diMe was very similar in accord with
414 the close chromatographic retention values observed for the two *P*-enantiomers (t_R (**1**)
415 = 13.00 min, t_R (**2**) = 14.00 min);

416 vii) in both **2** / A-3,5diMe complexes, each enantiomer is bound to the polysaccharide
417 surface with the 4-hydroxyphenyl part protruding deeply inside the groove, and with
418 the hydroxyl group engaged in HBs with carbamate sites, while buried into the
419 hydrophobic environment generated by the nonpolar regions of the polymer. This
420 profile is consistent with the high retention of both enantiomers of **2** on A-3,5diMe (t_R
421 (*M*) = 26.31 min, t_R (*P*) = 32.24 min) [3];

422 viii) finally, it is interesting to note that on A-3,5diMe the 2'-(4-pyridyl) substituent was
423 found in the external part of the surface (Fig. 6E;F), whereas the 2'-(4-hydroxyphenyl)
424 substituent penetrated into the groove of the CSP, being blocked inside by HB
425 interactions. Indeed, in this case, the hydroxyl group (and its associated recognition
426 sites) is free to rotate around the C-O bond, protruding inside the chiral cavity as a
427 molecular drill and making the analyte more adaptable compared to compound **1**.

428 **5 Concluding remarks**

429 The enantioseparation of TCIBPs **1** and **2** on 3,5-disubstituted (C-3,5diMe and C-
430 3,5diCl) and 3,4-disubstituted (C-3Cl,4Me and C-4Cl,3Me) cellulose-based CSPs and
431 related recognition mechanisms were explored through a multidisciplinary approach
432 based on chromatographic and thermodynamic analysis, electrostatic potential
433 analysis and MD simulations. Under NP elution conditions, lower selectivities were
434 obtained in almost all cases compared to amylose-based selectors, which we had
435 used in a previous study. The enantioseparation of 2'-(4-pyridyl)-TCIBP on C-3,5diMe
436 represented an exception, and good selectivity could be obtained by using Hex/IPA
437 90:10 as MP (α = 2.82). Under these elution conditions, the analysis time was rather
438 long (> 30 minutes). However, good selectivities could be obtained with shorter elution
439 time by adding 5% MeOH to the MP (Hex/IPA/MeOH 90:5:5) (t < 20 min; α = 1.54) or
440 by increasing elution temperature to 45°C (t < 22 min; α = 2.32).

441 EEO reversals dependent on the substitution pattern of the phenyl group of the CSPs
442 were observed, the elution sequence being *P-M* and *M-P* on the 3,5- and 3,4-
443 disubstituted CSPs, respectively, for both analytes. In particular, temperature-
444 dependent enantioseparations performed in the range 5-45 °C allowed for identifying

445 enthalpy- ($T_{ISO} \geq 157^{\circ}\text{C}$, $Q > 1$) and entropy-controlled ($T_{ISO} \leq 15^{\circ}\text{C}$, $Q < 1$) profiles for
446 3,5- and 3,4-disubstituted CSPs, respectively.

447 The molecular bases of the complementary enantioseparation profiles and the
448 backbone-dependent EEO reversal obtained for **1** and **2** on C-3,5diMe (*P-M*) and A-
449 3,5diMe (*M-P*) were explored by MD simulations. Interaction energies calculated from
450 100 ns MD trajectories provided EEOs which were fully consistent with the
451 experimental elution sequences. Analysis of calculated energies, analyte / selector
452 complexes and noncovalent interaction patterns evidenced a) the more compact
453 structure of the amylose-based polymer compared to the cellulose-based
454 polysaccharide, and its capability to envelop the analytes which are able to penetrate
455 into the cavity, b) the dominant contribution of van der Waals interactions to the overall
456 analyte / selector binding, c) the pivotal role of the distinctive HB sites at the 2'-position
457 of the TCIBP scaffold, inducing diverse adsorption mechanisms due to distinctive
458 electronic and steric properties, d) the noncovalent interaction pattern causing the high
459 adsorption of the enantiomer (*M*)-**1** on C-3,5diMe, and finally e) the contribution of a
460 XB interaction to the adsorption of (*M*)-**1** on C-3,5diMe.

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464 **Conflict of interest**

465 The authors have declared no conflict of interest.

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564 **Supporting information**

565 **Supporting information file:** Additional HPLC, thermodynamic and computational
566 data.

567

568 **FIGURE CAPTIONS**

569 **Figure 1.** Structures of chiral 4,4'-bipyridines **1** and **2** and cellulose-based chiral
570 stationary phases.

571 **Figure 2.** Maps of potential recognition sites of compounds **1** (**A**) and **2** (**B**), and of
572 carbamate side chain of C-3,5diMe (**C**) described in terms of $V_{S,max}$ (pale blue) and
573 $V_{S,min}$ (red) (values are reported in au) representation. For values associated to the V
574 extrema **a** and **b** see Table 1.

575 **Figure 3.** Correlation between retention range (k values) on four cellulose-based CSPs
576 for compounds **1** and **2** and $V_{S,max}$ and $V_{S,min}$ values calculated on each cellulose
577 carbamate recognition site (N-H and C=O).

578 **Figure 4.** In k_M and In k_P vs $1/T$ plots for the enantioseparation of **1** and **2** on C-3,5diMe,
579 C-3Cl,4Me, C-4Cl,3Me, and C-3,5diCl (Hex/IPA 90:10, $FR = 0.8$ ml/min, temperature
580 range 278.15-318.15 K).

581 **Figure 5.** Chromatograms of enantioseparations of compounds **1** and **2** on C-3,5-diMe
582 (**A** and **C**, respectively) and A-3,5-diMe (**B** and **D**, respectively) [3], MP = Hex/IPA
583 90:10, $FR = 0.8$ ml/min, $T = 25^\circ\text{C}$.

584 **Figure 6.** Representative snapshots and noncovalent interactions from the simulated
585 MD trajectories of **1** and **2** complexes with C-3,5diMe (**A-D**) and A-3,5diMe (**E-H**).

586

587 **TABLE CAPTIONS**

588 **Table 1.** Cellulose carbamate-based CSPs/columns used in the study, and $V_{S,max}$ and
589 $V_{S,min}$ values associated with the main recognition sites (carbamate N-H and C=O)

590 **Table 2.** Binding energies (E_{int}) (kcal/mol) and component contributions (E_{el} , E_{vdW}) for
591 the association of (*M*)-**1**, (*P*)-**1**, (*M*)-**2**, and (*P*)-**2** with C-3,5diMe ($EEO_{exp} = P-M$) and
592 A-3,5diMe ($EEO_{exp} = M-P$)

593