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3 **Comparative enantioseparation of planar chiral ferrocenes**
4 **on polysaccharide-based chiral stationary phases**

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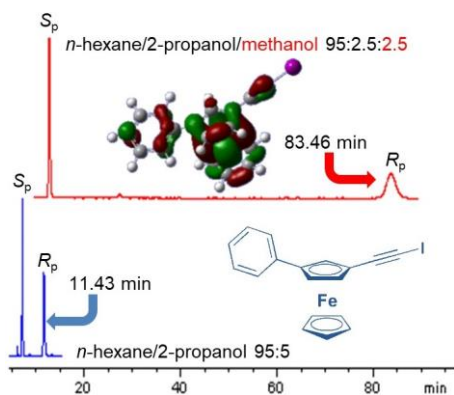
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37 *Graphical abstract* - High-performance liquid chromatography (HPLC) enantioseparations of ten 1,2-
38 and 1,3-disubstituted planar chiral ferrocenes on five polysaccharide-based chiral stationary phases
39 (CSPs) were comparatively explored under multimodal elution conditions. The presence of π -extended
40 systems in the analyte structure was shown to impact affinity of the most retained enantiomer toward
41 amylose-based selectors, observing retention times higher than 80 min with methanol-containing mobile
42 phases (MPs).



43

44

45 *ABSTRACT* - Planar chiral ferrocenes are well-known compounds which have attracted interest for
46 application in synthesis, catalysis, material science, and medicinal chemistry for several decades. In spite
47 of the fact that asymmetric synthesis procedures for obtaining enantiomerically enriched ferrocenes are
48 available, sometimes the accessible enantiomeric excess of the chiral products is unsatisfactory. In such
49 cases and for resolution of racemic chiral ferrocenes, enantioselective high-performance liquid
50 chromatography (HPLC) on polysaccharide-based chiral stationary phases (CSPs) has been used in few
51 literature reports. However, while moderate/high enantioselectivities have been obtained for planar
52 chiral ferrocenes bearing polar substituents, the enantioseparation of derivatives containing halogens or
53 exclusively alkyl groups remains rather challenging. In this study, the enantioseparation of ten planar
54 chiral 1,2- and 1,3-disubstituted ferrocenes was explored by using five polysaccharide-based CSPs under
55 multimodal elution conditions. Baseline enantioseparations were achieved for nine analytes with
56 separation factors (α) ranging from 1.20 to 2.92. The presence of π -extended systems in the analyte
57 structure was shown to impact affinity of the most retained enantiomer toward amylose-based selectors,
58 observing retention times higher than 80 min with methanol-containing mobile phases (MPs).
59 Electrostatic potential (V) analysis and molecular dynamics (MD) simulations were used in order to
60 inspect interaction modes at the molecular level.

61

62 1. INTRODUCTION

63 Ferrocene ($[\text{Fe}(\eta^5\text{-C}_5\text{H}_5)_2]$) is an iconic framework in organometallic chemistry,¹ which, in 1952, was
64 characterized as the first sandwich compound by Woodward and co-authors.² Structurally, ferrocene is a
65 rigid three-dimensional unit wherein iron is located between two cyclopentadienyl (Cp) rings. Owing to
66 its stability, and the possibility of functionalization at the cyclopentadienyl rings,³⁻⁷ ferrocene derivatives
67 have over time found application in several fields such as catalysis,⁸⁻¹⁰ medicinal chemistry,¹¹ and
68 material chemistry.¹²⁻¹⁴ Moreover, the ferrocenium/ferrocene system (Fc/Fc^+) is a versatile redox couple
69 which have proven to be suitable for the preparation of switchable functional systems.^{1,15,16}

70 Due to their versatility to access molecular wires, and responsive switchable systems and materials,¹⁷⁻
71 ¹⁹ particular attention has been devoted to the so-called “ π -extended ferrocenyl frameworks”, which are
72 featured by delocalized orbital networks based on the combination of a ferrocene unit and a π -conjugated
73 extended cloud, such as the ethynyl group.^{13,20} Planar chiral aryl ethynyl ferrocenes have been used as
74 intermediates for the preparation of helical ferrocenes showing peculiar chiroptical properties with very
75 high optical value and huge intensity of the CD signals.²¹

76 Recently, our groups developed the design, synthesis, and characterization of compounds **1-8** as new
77 chiral iodoethynyl ferrocenes showing three key features (Figure 1):⁶ *a*) planar chirality due to the
78 presence of two different substituents on the same Cp ring, *b*) a π -extended ethynyl ferrocenyl moiety,
79 with the possibility to further extend the π -conjugation to the second Cp-substituent depending on its
80 electronic features (Figure 2a,b), and *c*) an iodine substituent as an electrophilic σ -hole donor activated
81 by $\text{C}\equiv\text{C}$ group (Figure 2d).^{22,23} In this regard, it is worth mentioning that halogen bonds (XBs) were
82 identified in the crystal packing of compounds **1**, **3-5**, and **7** with both lengths and angles falling within
83 the range of values typically observed for this type of σ -hole bonds.⁶

84 These features also make compounds **1-8** suitable as test probes to explore the impact of both π -
85 extended clouds and electrophilic σ -holes in enantioselective processes occurring in HPLC environment,
86 with the aim to profile noncovalent interactions involved in the enantioselective recognition. In previous

87 studies, we demonstrated that σ -hole interactions, such as XBs and chalcogen bonds (ChBs), which
88 involve electrophilic regions of electron charge density depletion (σ -holes), may participate in the
89 enantioselective interaction between polysaccharide-based selectors and selectands containing
90 electrophilic σ -holes.²⁴⁻²⁷

91 On this basis, we explored the enantioseparation of chiral ferrocenes **1-8** along with **9** and **10**, as
92 reference compounds for comparison, by using five polysaccharide carbamate-based chiral stationary
93 phases (CSPs) (Supporting Information, Table S1) under multimodal elution conditions. The
94 experiments were integrated with computational analysis by using electrostatic potential (V) calculations
95 and molecular dynamics (MD) simulations in order to disclose the mechanistic features of these
96 enantioseparations.

97 The second aim of this study was to identify suitable methods for baseline enantioseparation of chiral
98 ferrocenes containing small or nonpolar groups. Indeed, while moderate/high enantioselectivities have
99 been obtained under normal-phase (NP) elution conditions for planar chiral ferrocenes bearing hydroxyl,
100 carbonyl, and carboxyl groups as substituents,^{21,28-30} the enantioseparations reported for derivatives
101 containing small groups, halogens or exclusively alkyl groups are rather unsatisfying.^{31,32} It is worth
102 mentioning that few papers have reported systematic studies on enantioseparation of organometallic
103 compounds on polysaccharide-based CSPs so far.^{28,31-39}

104 **2. MATERIALS AND METHODS**

105 **2.1. Chemicals and reagents**

106 Compounds **1-10** were prepared and characterized as previously reported.^{6,40} HPLC grade *n*-hexane
107 (Hex), methanol (MeOH), isopropyl alcohol (IPA), acetonitrile (ACN) and water were purchased from
108 Sigma-Aldrich (Taufkirchen, Germany).

109 **2.2. Chromatography**

110 An Agilent Technologies (Waldbronn, Germany) 1100 Series HPLC system (high-pressure binary
111 gradient system equipped with a diode-array detector operating at multiple wavelengths (220, 254, 280,

112 360 nm), a programmable autosampler with a 20 μ l loop, and a thermostated column compartment) was
113 employed. Data acquisition and analyses were carried out with Agilent Technologies ChemStation
114 Version B.04.03 chromatographic data software. The UV absorbance is reported as milliabsorbance
115 units (mAU). Lux Cellulose-1 (C-1) (cellulose *tris*(3,5-dimethylphenylcarbamate), CDMPC), Lux i-
116 Cellulose-5 (iC-5) (cellulose *tris*(3,5-dichlorophenylcarbamate), CDCPC), Lux Amylose-1 (A-1) and
117 Lux i-Amylose-1 (iA-1) (amylose *tris*(3,5-dimethylphenylcarbamate), ADMPC), and Lux i-Amylose-3
118 (iA-3) (amylose *tris*(3-chloro-5-methylphenylcarbamate), ACMPC) (5 μ m) (Phenomenex Inc., Torrance,
119 CA, USA) were used as chiral columns (250 \times 4.6 mm) (Table S1). Analyses were performed in
120 isocratic mode at 25°C. The flow rate (*FR*) was set at 0.8 ml/min. For compounds **1**, **3-6**, **7**, and **8**, the
121 enantiomer elution order (EEO) was determined by injecting enantiomers of known absolute
122 configuration prepared by asymmetric syntheses.⁶ For compounds **9** and **10**, the relative EEO was
123 assigned by injecting pure enantiomers of unknown absolute configuration which are denoted as X_9 , Y_9
124 and X_{10} , Y_{10} .

125 **2.3. Computational**

126 V extrema calculated on the molecular electron density isosurfaces (maxima and minima) ($V_{S,max}$ and
127 $V_{S,min}$) (au, electrons/bohr) were computed by using Gaussian 09 (Wallingford, CT 06492, USA),⁴¹ at the
128 density functional theory (DFT) level of theory, using the B3LYP functional and the def2TZVPP basis
129 set. Search for the exact location of $V_{S,max}$ and $V_{S,min}$ was made through the Multiwfn code⁴² and through
130 its module enabling quantitative analyses of molecular surfaces (isovalue 0.002 au).⁴³ In our
131 computations, .wfn files were obtained through the Gaussian 09 package. Details for molecular
132 dynamics (MD) are reported in the Supporting Information file.

133 **3. RESULTS AND DISCUSSION**

134 *3.1. Chromatographic screening*

135 In the frame of this study, analytes, CSP and mobile phase (MP) were considered as experimental
136 variables, examined individually, and their influence on retention and selectivity was examined through

137 the evaluation of retention and separation factors (k and α), on the basis of the following preliminary
138 remarks:

139 a) within the 1,2-disubstituted **1-5** and 1,3-disubstituted **6-8** series, the influence of the distinctive
140 functional group on enantioseparation was evaluated. As shown in Figure 2, different substituents on the
141 Cp ring may determine different degrees of delocalization across the entire system. For instance, for
142 compound **4** (Ph) and **5** (2-Naphth) (Figure 2b,c) the delocalization is across the entire molecule,
143 whereas for compound **3**, the calculated HOMO does not extend on the second substituent (Me) (Figure
144 2a). The impact of the triple bond on the enantioseparation was examined, and for this purpose
145 compounds **9** and **10** served as reference systems for comparison. The effect of 1,2- and 1,3-
146 disubstitution on the enantioseparation was also considered. The local electron charge density of specific
147 molecular regions of the analytes was inspected in terms of calculated V_s (Supporting Information, Table
148 S2), positive and negative V_s values being associated with electrophilic and nucleophilic regions;

149 b) the performances of different polysaccharide selectors were evaluated under multimodal elution
150 conditions, and compared in terms of polysaccharide backbone (cellulose-based C-1 *versus* amylose-
151 based A-1), and type of carbamate pendant groups (methylated A-1, iA-1, C-1, chlorinated iC-5,
152 methylated and chlorinated iA-3). In this regard, the electronic properties of the carbamate moiety,
153 which are tuned by methyl and chlorine substituents located on the phenyl ring of the carbamate pendant
154 groups, were determined by DFT calculations (Table 1). The impact of the anchoring technique
155 (immobilization *versus* coating)^{44,45} was also considered by comparing the performances of amylose
156 *tris*(3,5-dimethylphenylcarbamate) (ADMPC)-based columns (A-1 *versus* iA-1);

157 c) the effect of MP on the enantioseparations was evaluated comparatively by using *n*-hexanic
158 mixtures (Hex/IPA 95:5 (A) and Hex/IPA/MeOH 95:2.5:2.5 (B) v/v), polar organic (PO) conditions
159 (MeOH 100% (C)), and aqueous-organic mixtures (MeOH/water 95:5 (D) and 90:10 v/v). In
160 particular, the comparative use of A-E, as MPs, allowed for evaluating the effect of increasing
161 hydrophobicity of the medium. The introduction in the MP of 2.5% MeOH (B) allows modulation of the

162 binding between analyte and polysaccharide-based selector by favouring a better penetration of the
163 analyte into the groove, and tuning hydrophobic *versus* hydrogen bond (HB) interactions,^{46,47} while
164 keeping intact the high-ordered three-dimensional structure of the polysaccharide. Otherwise, the use of
165 pure MeOH (C), as a MP, impacts intramolecular HBs determining the high-ordered structure of the
166 polysaccharide, thus producing a huge effect within the polysaccharide structure.⁴⁶⁻⁴⁹ In particular, the
167 hydroxyl groups of MeOH molecules strongly interact, as HB donors/acceptors, with C=O and N-H
168 groups of the polymer, competing with intramolecular HBs within the selector, and with selector-
169 selectand intermolecular HBs.^{46,50} Indeed, with MeOH, hydrophobic interactions tend to be more
170 favoured compared to HBs, and the addition of water was expected to enhance hydrophobic interactions
171 and increase capacity factors in accordance with a typical reversed-phase (RP) system.⁵¹

172 On this basis, twenty-five chromatographic systems generated by the combination of C-1, iC-5, A-1,
173 iA-1, and iA-3 with the mixtures A-E, as MPs, were evaluated and characterized by k (Supporting
174 Information, Figure S1) and α (Figure S2) values toward ferrocenes **1-10** (Tables S3-S12). Baseline
175 enantioseparations were obtained for compounds **1-8** and **10** with α values ranging from 1.10 to 11.41,
176 whereas partial enantioseparation ($\alpha = 1.07$) was obtained only for **9**, with the system C-1/A. For
177 compounds **1**, **3**, and **10**, baseline enantioseparations were achieved under NP elution conditions and
178 with aqueous-MeOH mixtures as MPs, but MeOH showed to exert a detrimental effect on the
179 enantioseparation in these cases. Compounds **2**, and **4-8** were baseline enantioseparated under all three
180 elution modes, namely *n*-hexanic mixtures, PO and aqueous-organic elution conditions.
181 Chromatographic conditions and parameters of the best enantioseparations obtained for compounds **1-8**
182 and **10**, as a suitable compromise between a sufficiently high enantioselectivity value, a baseline
183 resolution, and a run time as short as possible, are reported in Table 2 (see Supporting Information,
184 Figure S3 for chromatographic traces). For compounds **3-5**, **7**, and **8** optimized enantioseparations were
185 obtained by using chlorinated chiral columns (iA-3 or iC-5) with selectivity factors ranging from 1.46 to
186 2.92. Otherwise, for compounds **1**, **2**, **6**, and **10**, optimized enantioseparation methods were developed

187 by using coated methylated chiral columns (C-1 or A-1) ($1.19 \leq \alpha \leq 2.13$). In the series of optimized
188 enantioseparations, methanol-containing MPs were successfully used for compounds **1-4**, **6**, and **10**, and
189 NP elution conditions for **5**, **7**, and **8**.

190 3.2. Impact of analyte structure on enantioseparation

191 With the aim to evaluate the impact of the structural features of each analyte on its enantioseparability,
192 the rate of baseline enantioseparations (*rbs*) was determined from the wealth of chromatographic results
193 (Figure 3). In the frame of the twenty-five chromatographic systems explored in this study, *rbs* increased
194 following the order **7**, **8** (64%, 62%) > **2** (32%) > **4,5** (28%) > **6** (16%) > **3,10** (12%) > **1** (8%) > **9** (0%).
195 From this trend, some remarks emerged:

196 a) 1,3-disubstituted ferrocenes showed high enantioseparability compared to the 1,2-disubstituted
197 analogues (**7** > **4**, **8** > **5** and **6** > **1**). Indeed, average retention factors (Figure S4a) and selectivity factors
198 (Figure S4b) of **6**, **7**, and **8** ($1.56 \leq k \leq 11.88$; $1.05 \leq \alpha \leq 3.45$) were, in general, higher than those of **1**, **4**,
199 and **5** ($0.65 \leq k \leq 1.62$; $1.05 \leq \alpha \leq 1.32$), respectively. This behaviour could be caused by the fact that in
200 the 1,3-pattern both substituents are accessible to the selector, whereas the substituents are sterically
201 constrained in 1,2-substituted derivatives. On the other hand, no relevant difference in terms of V_S values
202 (Table S2), justifying the different *rbs*, was observed between 1,2- and 1,3-substituted derivatives;

203 b) the ethynyl framework represents a key structural element given that the enantioseparability
204 decreased moving from **1** and **2** (*rbs*: 8% and 32%) to **9** and **10** (*rbs*: 0% and 12%), respectively. In
205 terms of V_S values associated to nucleophilic and electrophilic region, it is worth mentioning that in **1**
206 and **2** the ethynyl group contributes to increase the electrophilic character of the iodine ($V_{S,\max}(\text{I}) =$
207 0.0721 au (**1**) and 0.0755 au (**2**)), compared to the analogues **9** and **10** ($V_{S,\max}(\text{I}) = 0.0465$ au (**9**) and
208 0.0529 au (**10**)), respectively. Interestingly, both *rbs* and positive $V_{S,\max}$ on iodine decreases along the
209 bromiodo-substituted series **6** (*rbs* 16%, $V_{S,\max}(\text{I}) = 0.0737$ au), **1** (*rbs* 8%, $V_{S,\max}(\text{I}) = 0.0721$ au), **9**
210 (*rbs* 0%, $V_{S,\max}(\text{I}) = 0.0465$ au). In addition, compound **2** presents higher electron density on the $\text{C}\equiv\text{N}$

211 group ($V_{S,\min}(\text{N}) = -0.0721$ au) and a more extended π -system compared to the analogue **10** ($V_{S,\min}(\text{N}) =$
212 -0.0714 au);

213 c) the presence of a π -extended system involving the second substituent of the iodoethynyl ferrocene
214 impacts both retention and selectivity. Indeed, compounds **2**, **4**, **5**, **7**, and **8** showed average retention for
215 both first and second eluted enantiomer, average selectivity and *rb*s ($0.76 \leq k \leq 11.88$; $1.20 \leq \alpha \leq 3.45$;
216 $28\% \leq rbs \leq 64\%$) higher compared to compounds **1**, **3**, **6**, **9**, and **10** ($0.54 \leq k \leq 1.64$; $1.01 \leq \alpha \leq 1.11$;
217 $0\% \leq rbs \leq 16\%$);

218 d) the EEO was determined for each enantioseparation, and the assignments are summarized in Table
219 S5 (Supporting Information). *S-R* was the most frequent EEO, and only compound **2** retained its EEO in
220 all chromatographic systems. Several cases of reversal of EEO dependent on analyte structure, selector
221 structure, and MP type could be observed, but in general they are not easy to explain. Compounds
222 containing π -extended systems provided interesting cases of EEO. For instance, on C-1/A and C-1/B all
223 compounds showed *S-R* as EEO with the exception of **7** and **8** (*R-S*). Interestingly, by changing the *n*-
224 hexanic mixtures to pure MeOH with the same chiral column (C-1/C), only compound **8** retained the *R-S*
225 sequence as EEO, whereas compound **7** showed MP-dependent EEO reversal to *S-R*, despite the
226 structural similarity of the two analytes. Compound **5** showed the *S-R* elution order on all amylose-based
227 selectors with mixture A, but adding 2.5% MeOH provided EEO reversal to *R-S* on the same selectors.
228 Again, only for compound **5**, a reversal of elution order was observed on immobilized iA-1 (*R-S*)
229 compared to the coated A-1 (*S-R*) by using MeOH or the aqueous mixture D as MPs. The same
230 phenomenon was not observed with *n*-hexanic mixtures (mixtures A and B).

231 3.3 Impact of selector structure on enantioseparation

232 Among the chiral columns used in this study, better results were obtained on the A-1 and iA-3, each
233 column providing baseline enantioseparations for seven compounds. C-1 was also able to
234 enantioseparate six compounds, whereas iC-5 and iA-1 provided baseline enantioseparations only for
235 compounds **2**, **4**, **5**, **10** and **7**, **8**, respectively (Supporting Information, Figure S6). Compounds **2** and **10**

236 containing the C≡N group as a strong HB acceptor showed very high retention on the iC-5 ($5.39 \leq k \leq$
237 6.29) under NP conditions, but moderate selectivity on this chiral column ($\alpha = 1.11$ (**2**), 1.15 (**10**)). Thus,
238 the acidic amidic hydrogen of the CDCPC strongly contributes to determine the affinity of the analyte
239 toward the selector participating in the C≡N⋯H-N HB, but less to enantioselectivity.

240 Among all compounds, 1,3-disubstituted **7** and **8** showed an interesting behaviour with very high
241 affinity for the amylose-based selectors by using methanol-containing MPs (Tables S9 and S10). The
242 same behaviour was not observed for the 1,2-analogues **4** and **5**.

243 *3.4 Enantioseparation of compounds 7 and 8: CDMPC vs ADMPC*

244 As shown in Figure 4, 1,3-disubstituted ferrocenes **7** and **8** showed a complementary behaviour on C-
245 1 as the MP changes, and in both cases two mechanisms seem to control enantioseparation on this
246 column depending on MP polarity:

247 *i)* a mechanism based on polar interactions occurring with mixture A, which proved to be more
248 effective, in terms of selectivity, for compound **8** (R = naphthyl) ($\alpha = 1.25$) compared to derivative **7** (R
249 = Ph) ($\alpha = 1.08$). The addition of methanol to mixture A, or the use of pure MeOH as a MP, appeared to
250 be detrimental for the enantioseparation in both cases;

251 *ii)* a mechanism occurring under hydrophobic conditions when increasing amounts of water (5% and
252 10%) were added to the MP consisting of pure methanol. In these cases, the separation system behaves
253 as a typical reversed-phase system, and selectivity factors increased with increasing amount of water
254 additive [52]. Under these conditions, compound **7** ($\alpha = 1.19$) was enantioseparated better than
255 compound **8** ($\alpha = 1.09$).

256 Interestingly, for compound **7** the transition between the two mechanisms is associated with a MP-
257 dependent reversal of EEO, which is *R-S* with A and B as MPs, and *S-R* with pure methanol and aqueous
258 mixtures D and E. Otherwise, the naphthyl derivative **8** retained the same *R-S* elution sequence with all
259 MPs (A-E).

260 The features of the enantioseparations of compounds **7** and **8** on A-1 were shown to be completely
261 different (Figure 4). In these cases, with mixture A the phenyl derivative **7** was enantioseparated better
262 ($\alpha = 2.27$) than compound **8** ($\alpha = 1.29$), showing an opposite trend compared to the enantioseparations
263 on CDMPC, also in terms of EEO (CDMPC: *R-S*; ADMPC: *S-R*). On the amylose-based selector, the
264 use of methanol-containing MPs such as B and C increased retention and selectivity producing large
265 enantioseparation for both compounds **7** ($\alpha_B = 8.87$; $\alpha_C = 11.41$) and **8** ($\alpha_B = 4.50$; $\alpha_C = 8.92$). In
266 particular, for compound **7**, the addition of only 2.5% MeOH to the binary mixture A caused the
267 increase of the retention time of the second eluted (*R*)-enantiomer from 11.43 min to 83.46 min, and a
268 further increase to 96.25 min by using pure MeOH as MP. The effect occurred in less degree for
269 compound **8**, the retention time of the second eluted enantiomer changing from 14.08 min to 24.38 and
270 46.6 min with A, B, and C as MPs, respectively.

271 The application of van't Hoff analysis allowed for identifying different thermodynamic profiles for
272 compound **7** and **8** as the cause of the different selectivity observed by changing the ADMPC to
273 CDMPC. Thus, retention and selectivity of compounds **7** and **8** on C-1 and A-1 with hex/IPA 95:5 v/v as
274 a MP, were determined at different temperatures from 5 to 45 °C in 5 °C increments (Supplementary
275 material, Table S13). The thermodynamic quantities derived from van't Hoff plots (Figure S7) are
276 reported in Table S14 (Supplementary material). The enantioseparations of both analytes on C-1 were
277 shown to be enthalpy-driven with $T_{iso} = 147^\circ\text{C}$ and 85°C for **7** and **8**, respectively. Otherwise, the
278 enantioseparation of **8** on A-1 is entropy-driven ($|T\Delta\Delta S^\circ| > |\Delta\Delta H^\circ|$) and a $T_{iso} = -37^\circ\text{C}$ was calculated in
279 this case. An enthalpy-driven process also occurred ($|T\Delta\Delta S^\circ| < |\Delta\Delta H^\circ|$) for the enantioseparation of **7** on
280 A-1, where the difference between the free energies associated to the transfer of the enantiomers from
281 the MP to the selector surfaces is essentially due to a negative enthalpy contribution (-281.3 cal/mol),
282 whereas the entropy term is positive and close to zero (0.78 cal·K⁻¹·mol⁻¹). Indeed, the
283 enantioselectivity is almost independent of the temperature variation. Moving from C-1 to A-1, an
284 increase of the entropy contribution to enantioselection could be determined by comparing the values of

285 the thermodynamic ratio $Q = \Delta\Delta H^\circ / (298 \times \Delta\Delta S^\circ)$: **7**, 1.41 (C-1) \rightarrow 1.21 (A-1); **8**, 1.20 (C-1) \rightarrow 0.79 (A-
286 1).

287 Large separations for compounds **7** and **8** were also observed on iA-1 (Supporting Information,
288 Figures S8 and S9) and iA-3 (Figures S10 and S11), but not with cellulose-based selectors, showing that
289 the high affinity of the second eluted enantiomers of these compounds toward the selectors is mainly
290 determined by the features of backbone with methanol-containing MP, thus occurring under
291 hydrophobic conditions. With the aim to confirm this feature, we explored the impact of adding polar
292 and nonpolar components to the MP on the retention of the second (*R*)-enantiomer of compounds **7** and
293 **8** by using iA-1 (Figures S8 and S9). Considering that solvents favour and disfavour hydrophobic and
294 polar interactions, respectively, following the order acetonitrile (ACN) < IPA < MeOH < water,
295 changing the aqueous mixture D to MeOH/IPA and MeOH/acetonitrile (ACN) as MPs caused a decrease
296 in the retention of the (*R*)-enantiomer of **7** decreased from 113.10 min to 23.28 and 18.38 min,
297 respectively. Otherwise, by adding 5% water to pure ACN, retention time of the (*R*)-enantiomer
298 increased from 15.12 min to 26.89 min. On this basis, by changing MeOH to ACN in mixture D a drop
299 of retention time of the (*R*)-enantiomer from 113.10 min to 26.89 was observed. On the other hand, the
300 retention factors of the first eluted (*S*)-enantiomer ($0.56 \leq k_2 \leq 3.50$) showed to be less influenced by the
301 polarity of the MP compared to the (*R*)-enantiomer ($2.56 \leq k_2 \leq 27.79$). A similar trend was obtained for
302 compound **8**. The evaluation of the ‘dance’ of the second peak on the time scale under multimodal
303 conditions, allowed to confirm that the high affinity of the analyte toward amylose-based selectors
304 actually originates from hydrophobic conditions.

305 With the aim to explore the mechanism at the molecular level, a MD simulation was performed by
306 using the (*R*)-enantiomer of compound **7** as selectand, an ADMPC nonamer as selector, and MeOH as a
307 virtual solvent (Fig. 5). The modelling of the experimental enantioseparation confirmed that the (*R*)-
308 enantiomer of compound **7** penetrates deeply in the groove of the selector (Fig. 5a). The selectand
309 remained blocked during 100 ns of MD and confined in a hydrophobic cavity which appeared to be

310 profiled by six aromatic rings of the selector (Fig. 5b). Interestingly, a HB between the π -ethynyl cloud
311 and the amidic hydrogen of the selector ($d = 2.347 \text{ \AA}$) was observed to contribute to the binding of the
312 (*R*)-enantiomer into the polymer groove, confirming the pivotal role of this structural element on
313 enantioseparation.

314 **4. CONCLUSION**

315 In this study, the enantioseparation of ferrocenes **1-10** has been explored systematically and, as a
316 result, methods for baseline enantioseparations were successfully developed for nine compounds with
317 selectivity factors ranging from 1.20 to 2.92. Otherwise, 1-bromo-2-iodo-ferrocene (**9**) could be only
318 partially enantioseparated, confirming that the enantioseparation of small nonpolar planar chiral
319 ferrocenes remains rather challenging. In most cases, amylose-based CSPs provided better
320 enantioseparation performances compared to cellulose-based ones. Due to the hydrophobic feature of the
321 ferrocenes used in this study as analytes, aqueous methanol-containing MPs allowed for improving
322 enantioseparation performances in several cases.

323 The impact of π -extended clouds on the enantioseparations was clearly demonstrated, compounds **7**
324 and **8** providing interesting trends and behaviours as both selector and MP features changes.
325 Interestingly, the addition of small (2.5%) percentages of methanol to the Hex/IPA 95:5 increased
326 significantly the affinity of the second eluted enantiomers of compounds **7** and **8**, producing large
327 enantioseparations on amylose-based selectors ranging from 4.69 to 11.41, and from 4.50 to 8.92,
328 respectively.

329 MD simulations disclosed *a*) the confinement of the analyte in a hydrophobic cavity deeply inside the
330 ADMPC groove and *b*) the stabilization of the selector-selectand complex exerted by a HB between the
331 ethynyl π -cloud and the amidic hydrogen of the selector, as the molecular basis underlying the high
332 affinity of analytes **7** and **8** toward amylose-based selectors.

333

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499

500 **FIGURE CAPTIONS**

501 **FIGURE 1** Structures and numbering of chiral ferrocenes **1-10**

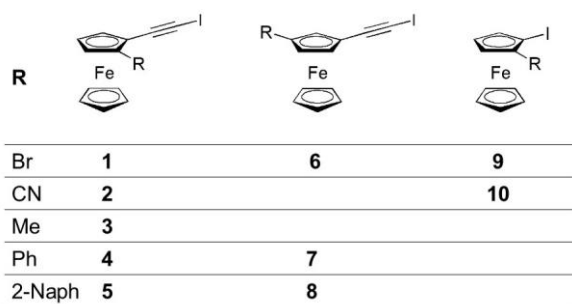
502 **FIGURE 2** The highest occupied molecular orbitals (HOMO) (contour value = 0.02 au) for compounds
503 **3-5** (a-c), and electrostatic potential isosurface (V_S) (isovalue 0.002 au) of compound **6** (d)
504 (DFT/B3LYP/def2TZVPP; color legend: minimum and maximum V_S values are depicted in red and blue,
505 respectively, and colors in between (orange, yellow, green) depict intermediate values)

506 **FIGURE 3** Rate of baseline enantioseparations of compounds **1-8** and **10** under multimodal elution
507 conditions

508 **FIGURE 4** Comparison of the enantioseparation traces of compounds **7** and **8** on C-1 and A-1 under
509 multimodal elution conditions

510 **FIGURE 5** Representative snapshot from the simulated MD trajectories (100 ns) of (*R*)-**7** complex with
511 ADMPC (solvent box, MeOH): a) electron density surface (legend colours: green, aromatic ring; red,
512 C=O; blue, N-H; grey, Ph + Cp + C≡C of (*R*)-**7**; magenta, iodine), b) tube model of the (*R*)-**7**/ADMPC
513 complex (legend colours: orange, Ph + Cp + Fe + C≡C of (*R*)-**7**; magenta, iodine; green, aromatic rings
514 featuring and delimiting the hydrophobic binding cavity of ADMPC)

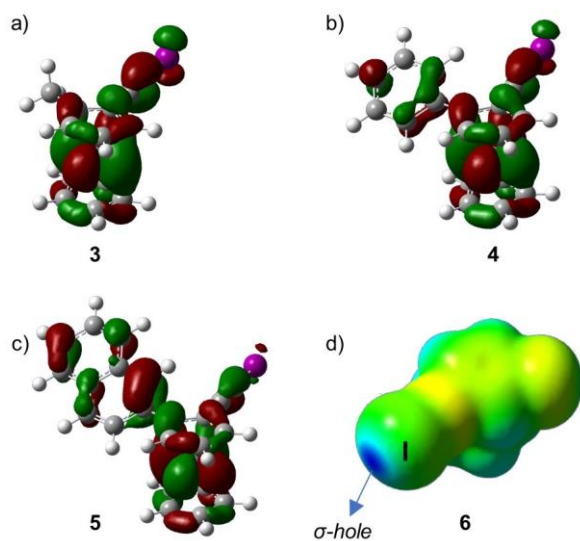
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517 **FIGURE 1** Structures and numbering of chiral ferrocenes **1-10**

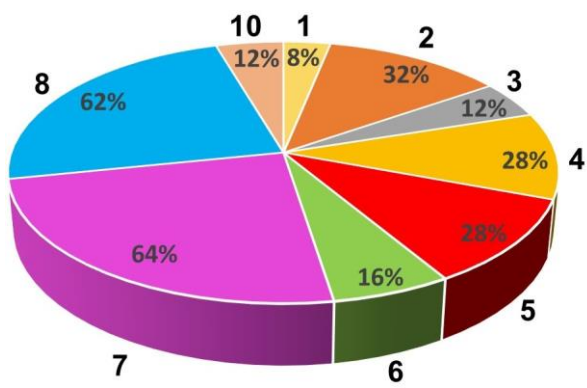
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520 **FIGURE 2** The highest occupied molecular orbitals (HOMO) (contour value = 0.02 au) for compounds **3-5** (a-c), and
 521 electrostatic potential isosurface (V_S) (isovalue 0.002 au) of compound **6** (d) (DFT/B3LYP/def2TZVPP; color legend:
 522 minimum and maximum V_S values are depicted in red and blue, respectively, and colors in between (orange, yellow, green)
 523 depict intermediate values)

524



525

526 **FIGURE 3** Rate of baseline enantioseparations of compounds **1-8** and **10** under multimodal elution conditions

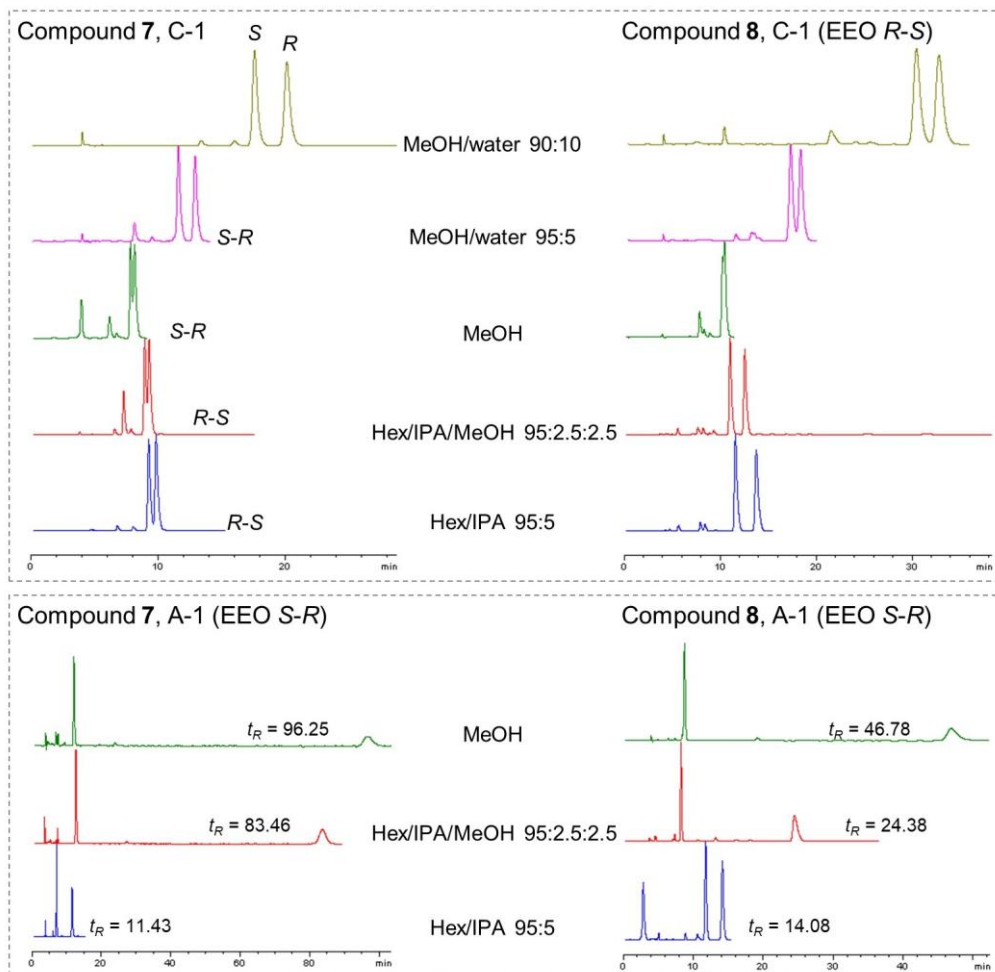
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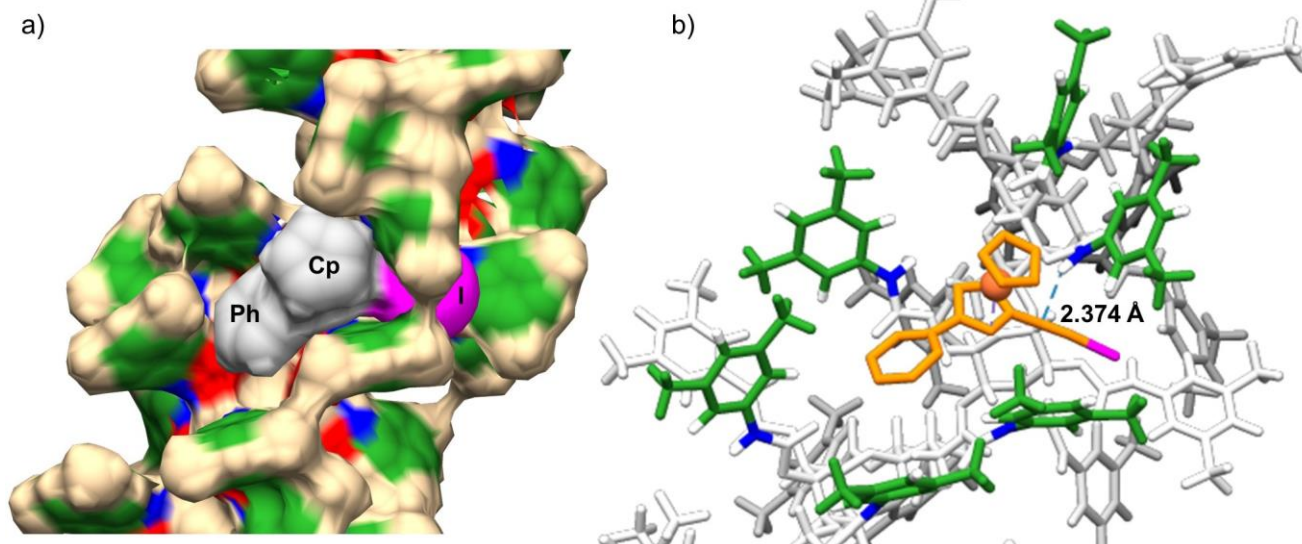


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533 **FIGURE 4** Comparison of the enantioseparation traces of compounds **7** and **8** on C-1 and A-1 under multimodal elution
534 conditions

535

536



537

538 **FIGURE 5** Representative snapshot from the simulated MD trajectories (100 ns) of (*R*)-**7** complex with ADMPC (solvent
 539 box, MeOH): a) electron density surface (legend colours: green, aromatic ring; red, C=O; blue, N-H; grey, Ph + Cp + C≡C of
 540 (*R*)-**7**; magenta, iodine), b) tube model of the (*R*)-**7**/ADMPC complex (legend colours: orange, Ph + Cp + Fe + C≡C of (*R*)-**7**;
 541 magenta, iodine; green, aromatic rings featuring and delimiting the hydrophobic binding cavity of ADMPC)

542

543 **TABLE 1** $V_{S,max}$ and $V_{S,min}$ values (a.u.) associated with the main recognition sites (carbamate N–H, C=O, and Ar) of
 544 cellulose- and amylose-based selectors used in the study (DFT/B3LYP/def2TZVPP)

Chiral selector	Side chain	$V_{S,max}$ (N–H)	$V_{S,min}$ (C=O)	$V_{S,min}$ (Ar)
CDMPC/ADMPC	3,5-dimethylphenylcarbamate	0.0843	-0.0625	-0.0271
CDCPC	3,5-dichlorophenylcarbamate	0.0990	-0.0536	-0.0066
ACMPC	3-chloro-5-methylphenylcarbamate	0.0914	-0.0595	-0.0163

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546

547 **TABLE 2** Chromatographic parameters (t , k , α) for optimized baseline enantioseparations of *rac*-**1-8** and **10** on
 548 polysaccharide carbamate-based chiral columns under multimodal elution conditions

Compound	Column	MP	t_1 [min]	t_2 [min]	k_1	k_2	α	EEO
1	A-1	E	7.42	8.11	0.88	1.05	1.20	<i>S-R</i>
2	A-1	D	4.80	5.80	0.23	0.49	2.13	<i>S-R</i>
3	iA-3	E	6.79	8.09	0.71	1.03	1.46	<i>S-R</i>
4	iA-3	C	4.79	5.63	0.22	0.44	1.97	<i>R-S</i>
5	iC-5	A	7.22	9.29	0.95	1.51	1.59	<i>S-R</i>
6	A-1	C	7.91	8.68	1.05	1.25	1.19	<i>R-S</i>
7	iA-3	A	6.16	11.03	0.70	2.05	2.92	<i>S-R</i>
8	iA-3	A	7.34	10.12	1.03	1.80	1.75	<i>S-R</i>
10	C-1	D	6.18	7.60	0.55	0.90	1.65	<i>Y-X</i>

549 Abbreviation: EEO, enantiomer elution order; the notation *X* or *Y* is reported for unknown absolute configuration; C-1, Lux
 550 Cellulose 1; iC-5, Lux i-Cellulose-5; A-1, Lux Amylose-1; iA-1, Lux i-Amylose-1; iA-3, Lux i-Amylose-3; A, Hex/IPA 95:5
 551 v/v; B, Hex/IPA/MeOH 95:2.5:2.5 v/v/v; C, MeOH 100%; D, MeOH/water 95:5 v/v; E, MeOH:water 90:10 v/v

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553