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Halogen bond in high-performance liquid chromatography enantioseparations: description, features and modelling

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ABSTRACT

Halogen bond (XB)-driven enantioseparations involve halogen-centred regions of electronic charge depletion (σ-hole) as electrophilic recognition sites. The knowledge in this field is still in its infancy. Indeed, although the influence of halogens on enantioseparation have been often considered, only recently the function of electrophilic halogens (Cl, Br, I) as enantioseparations 'drivers' has been demonstrated by our groups. Further to these studies, in this paper we focus on some unexplored issues. First, as XB-driven chiral recognition mechanisms are at an early stage of comprehension, a theoretical investigation based on a series of 32 molecular dynamic (MD) simulations was performed by using polyhalogenated 4,4'-bipyridines and polysaccharide-based polymers as ligands and receptors, respectively. Enantiomer elution orders (EEOs) were derived from calculations and the theoretical model accounted for some analyte- and chiral stationary phase (CSP)-dependent experimental EEO inversions. Then, the function of halogen-centred σ-holes in competitive systems, presenting also

hydrogen bond (HB) centres as recognition sites, was considered. In this regard, Pirkle's enantioseparations of halogenated compounds performed on WhelkO-1 were theoretically re-examined and electrostatic potentials (EPs) associated with both σ-holes on halogens and HB centres were computed and compared. Then, the enantioseparation of halogenated 2-nitro-1-arylethanols was performed on cellulose tris(3,5-dimethylphenylcarbamate) (CDMPC) and the influence of halogen substituents on the chromatographic results was evaluated by correlating theoretical and experimental data.

Keywords: Alcohols; Bipyridines; Electrostatic potential; Halogen bond; Molecular Dynamic; Polysaccharide-based chiral stationary phases

1. Introduction

Halogen atoms (F, Cl, Br, I) involved in covalent bonds present anisotropic distribution of the electron density (Fig. 1A) [1,2]. Indeed, two regions with opposite properties can be distinguished: i) a region of higher electron density, which forms a belt orthogonal to the corresponding covalent bond, usually characterized by negative electrostatic potential (EP), and ii) a region of lower electron density (the so-called 'σ-hole' located on unpopulated σ* orbitals) where the EP is generally positive.

On this basis, covalently bound halogens, in particular the heavier Br and I, can function as electrophile sites with properties of Lewis acids (Fig. 1B). In this perspective, the halogen bond (XB) is defined as the attractive interaction between the positive σ-hole region, which serves as a XB donor (XBD) and an electron-rich nucleophile site as a XB acceptor (XBA), with properties of Lewis base [3].

Although the first XB-based complex ($I_2 \cdots NH_3$) dates back to 1814 [4], it is only in the last two decades that the interest in XB has grown exponentially in several fields [5]. To date, several studies performed *in silico* [6], in the solid state [7], and in solution [8] have contributed to make XB a versatile tool for molecular engineering [9], catalysis [10], biochemistry [11], analytical chemistry [12-14] and molecular recognition [15].

Currently, XB application in chiral chromatography is in its infancy. In fact the first systematic observations can be traced back to 1996, when Pirkle and co-workers highlighted an unexpected halogen effect on the enantioseparation of halogenated amide derivatives of 1-phenylethylamine [16] and 5-methyl-5-phenylhydantoins [17]. Nevertheless, in these studies and in other successive investigations [18,19] halogen-dependent effects on enantioseparation were never explicitly related to the XB. The reason was likely due to the role which usually was ascribed to halogens in chiral recognition. Indeed, for a long time F, Cl, Br and I as substituents were merely considered Lewis bases in the perspective of an isotropic distribution of the electron density [19]. On this basis, the effects of halogens on enantioseparation were above all related to their properties as hydrogen bond (HB) acceptor.

Furthermore, halogens have been used due to their ability to tune the electronic properties and, consequently, the interaction capability of close sites. Moreover, they have been also considered to be involved in repulsive interactions due to their size. Finally, halogens increase the hydrophobic properties of the structures bearing them.

Recently, our groups have systematically investigated halogen-dependent enantioseparations on polysaccharide-based chiral stationary phases (CSPs), demonstrating that XBs can actually drive enantioseparations [20-22]. Further to these studies, XB has been recognized as an electrostatic interaction which can work in HPLC environment [23,24]. This perspective broadens the versatility of halogens which, therefore, can participate in intermolecular interactions and control molecular recognition serving either as Lewis bases through the negative belt or as Lewis acids through regions of electronic charge depletion (σ-hole). Although so far XB in chiral chromatography was still poorly reported, the XB concept has gradually become more familiar also in this field [25,26].

Computational tools and studies *in silico* have greatly contributed to the understanding of σ-hole-based interactions. In particular, EPs have been widely used as an indicator of the anisotropy of the molecular charge distribution. Indeed, EP analysis allows to achieve detailed information of σ-hole depth and size and it can rationalize the XB preference in competitive systems [27].

On the other hand, computational techniques have been also used as tool in chiral chromatography to predict retention, selectivity and enantiomer elution order (EEO) with the aim to understand and rationalize recognition mechanisms [23,28,29]. In this field, EPs and related EP surfaces (EPSs), where values of the EPs are mapped onto an isovalue electron density surface, contributed to investigate in detail the shape, which is the sum of geometry and electronic distribution, of both analyte and selector [30-32]. Furthermore, chromatography discrimination is a dynamic process based on reiterative adsorption-desorption steps involving selector surface, cavities and groove. In this perspective,

molecular dynamic (MD) simulations proved to be extremely versatile, in particular for studying processes where solvent effects have remarkable influence on driving interactions [33-35].

On this basis, some unexplored issues concerning XB-enantioseparations will be addressed herein by using both MD simulations and EP analysis as computational tools. First, with the aim to examine in depth XB-driven enantioseparations which are still at an early stage of comprehension and realization, a theoretical investigation based on a series of 32 MD simulations was performed. In this study, polyhalogenated 4,4'-bipyridines **1**-**8** (Fig. 2A) [36,37], and cellulose (CDMPC) and amylose (ADMPC) tris(3,5-dimethylphenylcarbamate) were used as ligands and polymeric receptors, respectively. Then, a systematic study aiming to identify the role of halogen-centred σ-holes in competitive systems, presenting also hydrogen bond (HB) centres as recognition sites, was carried out. For this purpose, first Pirkle's enantioseparations of halogenated amide derivatives of 1-phenylethylamine performed on WhelkO-1 [16] were theoretically re-examined and electrostatic potentials (EPs) associated with both σ-holes on halogens and HB centres were computed and compared. Then, the enantioseparation of 2-nitro-1-haloarylethanols **9**-**14** was performed on CDMPC under normal phase (NP) elution conditions, using compound **15** as a term of comparison (Fig. 2B). In these contexts, the influence of halogen substituents on the chromatographic outcomes was evaluated by correlating theoretical and experimental data.

2. Experimental

2.1. Chemicals

Compounds **1**-**8** were obtained as described in the literature [36]. Compounds **9**-**15** were prepared according with a literature procedure [38]. Synthesis details and ^{1}H and ^{13}C NMR spectra are available in the Supplementary data.

2.2. Chromatography

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

360 nm), a programmable autosampler with a 20 μl loop, and a thermostatted column compartment) was employed for both analytical and multimilligram separations. Data acquisition and analyses were carried out with Agilent Technologies ChemStation Version B.04.03 chromatographic data software. The UV absorbance is reported as milliabsorbance units (mAU). Lux Cellulose-1 (cellulose tris-3,5-dimethylphenylcarbamate; 5 μm) (Phenomenex, USA) was used as chiral column (250 × 4.6 mm). HPLC grade *n*-hexane (Hex) and 2-propanol (IPA) were purchased from Sigma-Aldrich (Taufkirchen, Germany). The retention factor (k) was determined as $k = (t_R\text{-}t_0)/t_0$, where t_R is the retention time for the eluted enantiomer; k_1 is the retention factor of the first-eluted enantiomer. The separation factor (α) was calculated as $\alpha = k_2/k_1$. Dead time (t_0) was measured by injection of tri-tert-butylbenzene (Sigma-Aldrich, Taufkirchen, Germany) as a non-retained compound [39]. Analyses were performed in isocratic mode at 25°C. The flow rate (*FR*) was set at 0.8 ml/min. Chromatographic parameters for compound **1**-**8** and **16**-**19** on CDMPC / ADMPC and WhelkO-1, respectively, were obtained as described in the literature [16,21,22]. The experimental enantiomer elution orders (EEOs) of compounds **1**-**8** [22] and **9**, **10**, **12**-**15** [38,40-43] were assigned as reported.

2.3. Computationals

2.3.1. Molecular property and electrostatic potential calculations

Conformational search was performed through molecular mechanics, using the MMFF94 force field and the Spartan '10 Version 1.1.0 (Wavefunction Inc., Irvine, CA) program [44]. Geometry optimization and computation of molecular properties, EPSs and related parameters, (EP extrema, maxima (max EP) and minima (min EP) values, given in kJ/mol; area and volume are given in $Å^2$ and $Å^3$, respectively) were performed and graphically generated (Spartan '10 Version 1.1.0) employing the density functional theory (DFT) method with the B3LYP functional and the 6-311G* basis set (available for elements H-Ca, Ga-Kr and I). Calculations were performed in the vacuum, thus the solvent effect was not considered. The EP describes the value of the electrostatic potential onto an electron density surface and

it was used as an indicator of the charge distribution on the molecules. The surface mapped values of the EP as derived from Spartan '10, used the default values of the program (isovalue: 0.002, high resolution). On EPS, colours towards red depict negative potential, while colours towards blue depict positive potential and colours in between (orange, yellow, green) depict intermediate values of potential. Polar surface area (EPS polar area) is defined as the area due to nitrogen and oxygen and any hydrogen attached to nitrogen and oxygen. Statgraphics Centurion XVI (Statpoint Technologies, Inc., Warrenton, VA, USA) was used for all linear regression analyses.

2.3.2. MDs simulations

For the MD simulations, 4,4'-bipyridines **1**-**8** were constructed by using the standard bond lengths and angles from the fragment database of GaussView 5.0 and optimized with Gaussian 09 (DFT, B3LYP, 3-21G*) (Wallingford, CT 06492, USA) [45,46]. The explicit σ-hole (ESH) was used, as previously described [21,22,47,48], in order to account for charge anisotropy of the electrostatic potential on top of the halogen atoms. On this basis, a massless dummy atom connected to I, Cl and Br was introduced manually, by using distance and charge values as described by Hobza and co-workers [48]. The parameters used for Cl, Br, I were 1.0, 1.3, 1.6 Å, and 0.1, 0.2, 0.3 units of positive charge for the extra point (ExP), respectively (Table S1). The AMBER14 Antechamber toolkit (University of California, San Francisco, USA) [49] was used to assign the generalized Amber Force Field (GAFF) atom type and the AM1-BCC type of charge to 4,4'-bipyridines **1**-**8**.

The Gaussian 09 program (DFT, B3LYP, 3-21G*) [45] was used for the *ab initio* geometry optimization calculation of a monomeric unit of β-D- and α-D-glucose-1,4-dimethoxy-tris(3,5-dimethylphenylcarbamate). The optimized structures were used to build nonamers (9-mer) of CDMPC and ADMPC, respectively [21,22]. CDMPC was characterized by a left-handed threefold (3/2) helix according with the structure reported by Vogt and Zugenmaier [50], setting the dihedral angles of the units, defined by H_1-C_1-O-$C_{4'}$ (ϕ) and $H_{4'}$-$C_{4'}$-O-C_1 (φ) to 60° and 0° (Fig. S1-A). ADMPC was

characterized by a 4/3 left-handed helical structure according with the structure reported by Okamoto and co-workers [51,52], setting the dihedral angles of the units, defined by H_1-C_1-O-$C_{4'}$ (ϕ) and $H_{4'}$-$C_{4'}$-O-C_1 (φ) to -68.5° and -42.0° (Fig. S1-B). The terminal residues of the biopolymers were closed with methoxyl groups. The polymer structures were energy-minimized using the GAFF force-fields with AM1-BCC charges assigned with the Antechamber toolkit. The atoms of the terminal methoxyls, closing the polymer backbone, were fixed in their positions during the simulations by assigning a force constant of 200 kcal/mol so that, starting from the setting initial values, the backbone dihedral angles of residues 2-8 could rotate moderately on the basis of the applied restriction. The energies and the structure of the biopolymer were first minimized using 2 ns of MD simulations. This structure was used in the final MD simulations.

The AMBER14 software (University of California, San Francisco, USA) [49] was used to carry out the MD simulations. Hex or MeOH solvent effects were taken into account by means of the explicit periodic solvent box. In this regard, the complexes polysaccharide-analytes were prepared for MD runs by solvating the system with an octahedral box with a 10 Å radius polysaccharide cutoff. 2200 and 350 molecules were added approximately for MeOH and Hex, respectively. The Chimera software (UCSF, San Francisco, USA) was used for visualization and analysis of the MD trajectories [53].

All MD data were examined in depth over 10 ns. With this aim, following a procedure recently applied to MD results from compounds **4** [22], six halogens n-X (n = 2, 2', 3, 3', 5,5'), as molecular descriptors on the 4,4'-bipyridines **1**-**8**, and 126 (14 x 9) descriptors At_m on each 9-mer of CDMPC and ADMPC (14/monomer) were selected and the overall distances $r_{n\text{-}X\cdots Atm}$, measured in the course of MD time (10 ns), were statistically analysed. In Figure 3, the 14 descriptors / monomer are indicated in bold along with the carbonyls $CO_{(2)}$, $CO_{(3)}$, and $CO_{(6)}$ which are considered the main XBAs on the polymers. Considering that the overall number of extracted distances was 6 x 14 x 9 x 5000 = 3780000, a cutoff of 6 Å was applied, not considering longer distances. Moreover, the contribution of each site on both

187 analyte and polysaccharide were calculated from the equation $D(r) = 100/r^3$ where r is the distance
188 between analyte and polysaccharide descriptors, assigning arbitrarily $D(r) = 100$ for $r = 1$ Å.

189 **3. Results and discussion**

190 In our previous studies, we used thirty-four 2,2',3,3',5,5'-hexahalogenated 4,4'-bipyridines as probes
191 to detect XB interactions in HPLC environment [21,22]. These analytes can be considered 'ideal'
192 structures in this field because halogens are electronically activated as XBDs by the electron-poor
193 heteroaromatic scaffold. Furthermore, they do not contain other chemical descriptors as competitive
194 electrophiles; consequently, they serve as benchmark XBDs. On this basis, we demonstrated that XBs
195 can occur in HPLC environment between the σ-holes of halogenated analytes and the carbonyls located
196 in the carbamate moieties of CDMPC and ADMPC (Fig. 4), with a level of efficacy dependent on the
197 substitution pattern. Moreover, the enantioseparation of this series on both CDMPC and ADMPC is
198 solvent-dependent. Indeed, it is driven by XB interactions under NP elution conditions, whereas MeOH
199 proved to oppose to XB formation as a competitive electrophile.

200 *3.1. Molecular dynamics of polyhalogenated 4,4'-bipyridines **1**-**8** on cellulose and amylose tris(3,5-*
201 *dimethylphenylcarbamate)*

202 Among all 4,4'-bipyridines, we selected the chromatographic outcomes of compounds **1**-**8** as an
203 experimental base [21,22] to perform MD simulations and evaluate the ability of these calculations to
204 predict the EEOs. Indeed, since interesting cases of EEO inversion had been observed for **1**-**8**, this series
205 fit our purposes. Thus, MD calculations were performed to simulate the interaction mode of the series **1**-
206 **8** with both CDMPC and ADMPC (32 simulations), using *n*-hexane as a solvent in accord with the
207 privileged HPLC environment. In this study, the explicit σ-hole (ESH) concept [21,22,47,48] was used
208 to model the XB in polysaccharide-polymer complexes. For compound **6**, an additional simulation was
209 also performed without ESH to evaluate the MD results when the electrophilic character of the halogens
210 is suppressed. Furthermore, for the same compound, a simulation was performed by introducing MeOH

in the solvent box with the aim to explore the effect of MeOH, as a competitive electrophile, on the complex polymer/enantiomer.

In accord with the literature [52,54], the constructed CDMPC was found to stay in an elongated conformation, whereas ADMPC forms a more compact structure (Fig. S1). CDMPC cavities are slightly bigger than for ADMPC, which presents stronger intramolecular HBs. Coherently, for the CDMPC the root mean square deviation (RMSD) profiles showed fluctuations slightly higher than ADMPC over 10 ns of MD (Fig. 4 and Fig S2). In general, narrow fluctuations were observed for the 'host' enantiomer, whereas a wider fluctuation was observed for the complex polymer/enantiomer because, at this level, hydrogen bonds and π–π interactions contribute to stabilize the overall polymeric structure.

Table 1 shows the parameters associated with X···O=C and X···π (3,5-dimethylphenyl moiety) contacts found in the MD runs over 10 ns (ESH, solvent box: *n*-hexane), considering the carbonyls $CO_{(2)}$, $CO_{(3)}$, and $CO_{(6)}$ and the 3,5-dimethylphenyl rings (Fig. 3) as XBAs.

The geometrical parameters analyzed were i) the distance (d) between halogens and XBA centres, ii) the angle formed by aromatic carbon, halogen, and oxygen atom (C- X···O, reference value 180° [48]), and iii) the angle formed by halogens, carbonyl oxygen and carbonyl carbon (X···O=C, reference value 120° [48]). In particular, as reported [55], any distance shorter than the sum of the van der Waals radii of oxygen and halogen may be considered as an implication of XB. For compounds **1**-**8**, 35 X···O and 10 X···π contacts were found, with a clear prevalence of I···O contacts (32). In particular, the iodinated analogues **3**-**8** showed the distribution of I···O distances clustering around 2.88-3.43 Å, corresponding to about 82.3-98% of the sum of the van der Waals radii (3.5 Å) [56]. Moreover, the corresponding C-I···O angles ranged from 160° to 179° in almost all cases and only for the CDMPC-complexes involving the enantiomers (*P*) of compounds **4**, **5** and **8** the lower values of 147°, 159° and 155° were observed, respectively. It is worth noting that, in general, angles ranging from 160° to 180° are considered acceptable to decide if the interaction corresponds to a XB [57]. On the contrary, the I···O=C angles

showed a wider distribution with ten values clustering around 121-127° (close to the reference value 120°), five values clustering around 95-104° (< 120°) and seventeen values around 133-168° (> 120°). Interestingly, both the number of interactions observed and the corresponding penetration parameters tended to reflect the order I > Br > Cl, in agreement with the experimental outcomes.

The EEOs assigned on this basis were in agreement with the experimental elution order in 18 simulations out of 32 (Table 1, bold lines), with an overall success rate of 56.2%. It is worth noting that the rate increases to 75% considering the CDMPC exclusively, whereas it decreases to 37.5% for ADMPC. This observation could be related to the fact that on ADMPC other entropy-driven forces had been found to control enantiorecognition along XB [22]. Consequently, it was likely that a model based exclusively on XB interactions would not adequately describe XB-driven enantioseparations on the amylose-based CSP. Indeed, on ADMPC the EEOs could not be unambiguously assigned for the enantiomers of **3** and **8**, whereas the calculated EEO was inverted with respect to the experimental EEO for **1**, **5** and **7**. On the contrary, on CDMPC the EEO could not be assigned only for **1** and the formal EEO inversion was observed only for **7**. In particular, the unpredictable behaviour of **1** was expected considering its poor ability as XBD.

Focusing on the simulations involving hexaiodinated compound **3** with CDMPC, in accord with the experimental EEO (*M*-*P*), shorter contacts I···O (2.99, 3.12 Å) were observed for the complex CDMPC-(*P*) compared to the complex CDMPC-(*M*) (3.18 Å). In Figure 5, the occupancy graphs associated to the complexes CDMPC-(*M*) and CDMPC-(*P*) are reported. The occupancy analysis allowed to evaluate which regions of space are highly populated by the analyte over 10 ns MD. By comparing the two graphs, the (*P*)-enantiomer showed a clear tendency to move toward the inner part of the polymer compared to the (*M*)-enantiomer. The same tendency could be clearly observed in the occupancy graphs representing the regions populated by iodine substituents exclusively (Fig. S3).

With the aim to gain further information, the overall contacts occurring in the course of MDs were examined. Indeed, taking into account the dynamic feature of the enantioseparation event, we analysed

statistically the distances between each of the six halogens on 4,4'-bipyridine **1**-**8** and 14 points (N, O, H) located on each monomer of the CDMPC and ADMPC nonamers (Fig. 3). The contact distances were acquired (5000 steps) during the MD time (10 ns). All collected values within 6 Å were extracted (Supplementary data) to evaluate the sites involved in close contacts, without considering the type of interactions. The Σ D(*r*) value, calculated from all distances extracted by applying the 6 Å cutoff, as well as the values calculated for each single sites are reported in Tables S2-S9. In Table S10, the results of two additional MDs, performed using compound **6** without ESH and with MeOH, respectively, are summarized. From the calculated distribution values associated with the recognition sites, the following observation emerged:

i) by comparing the Σ D(*r*) value of each couple of enantiomers, the calculated EEOs for compounds **1**-**8** were derived, which showed to be in agreement with the experimental values in 18 simulations out of 32 (56.2%) as previously observed. Also in this case, the success rate of the prediction rises to 62.5% for CDMPC and decreases for amylose (50%). Interestingly, moving from the first approach, based on XBs exclusively (Table 1), to the second one which considers other types of interactions instead, the EEO prediction success rate decreased for CDMPC (from 75% to 62.5%), whereas it increased for ADMPC (from 37.5% to 50%) (Table 2);

ii) in almost all cases, the carbonyls $CO_{(3)}$ and $CO_{(6)}$ proved to be the most frequent recognition sites on the polymers, with percentages clustering around 26.0-60.7% and 26-50%, respectively. These results are in agreement with a recent MD study, where the privileged involvement of the frameworks at C6 and C3 as recognition sites was reported for polysaccharide-based CSPs [58]. In this regard, the frequency distribution graphs derived from the MD results obtained for (*M*)-**1** and (*M*)-**3** are reported and compared in Figure 6. As expected, for **3**, a good XBD, the carbonyls of the carbamate frameworks at C_3 and C_6 are the most frequent recognition sites (Fig. 6C), whereas for **1**, a poor XBD, a wider distribution

is observed without any specific involvement of the carbonyl sites (Fig. 6A). Moreover, as expected, iodine (Fig. 6D) is more frequent as a recognition site than chlorine (Fig. 6B);

iii) the analysis of the calculated distribution values allowed to rationalize recognition mechanisms. For example, for the complex CDMPC/(*P*)-**8** (Table S9) the distribution values for 3-I, 5-Br and 5'-I were 42.7%, 11.2% and 29.4%, respectively. Differently, for CDMPC/(*M*)-**8** the corresponding values changed to 40.6%, 0% and 43.4%, respectively. On this basis, 3-I behaves as a non-enantioselective interaction site as its involvement is almost the same in the two enantiomers (42.7 *vs* 40.6%). On the contrary, for iodine at position 5' the distribution increases from 29.4% (*P*) to 43.4% (*M*). In the meantime, the distribution value of 5-Br decreased from 22.3% to zero. On this basis the involvement of both 5-Br and 5'-I as recognition sites appeared to be enantioselective;

iv) for compound **6**, the difference between the Σ D(*r*) values of the two complexes CDMPC/(*M*)-**6** and CDMPC/(*P*)-**6** was 90901, when the MDs were performed by applying the ESH correction and *n*-hexane in the solvent box (Table S7). Interestingly, the corresponding result without ESH correction, namely not treating the halogens as electrophiles, decreased the difference between the two complexes to 4945 (Table S10). Analogously, the Σ D(*r*) difference collapsed to 3968 by changing *n*-hexane to MeOH in the solvent box. In the meantime, as expected, the distribution values associated with $CO_{(6)}$ also decreased dramatically ($CO_{(6)}$: ***1.*** ESH, *n*-hexane, CDMPC/(*P*)-**6** 156546.85, CDMPC/(*M*)-**6** 164025.57; ***2.*** ESH, MeOH, CDMPC/(*P*)-**6** 2715.22, CDMPC/(*M*)-**6** 6486.82; ***3.*** without ESH, *n*-hexane, CDMPC/(*P*)-**6** 1918.95, CDMPC/(*M*)-**6** 17117.76), showing less involvement of this site when either the halogens do not behave as electrophile (without ESH) or the carbonyl is not available as acceptor (MeOH).

3.2. Influence of halogen substituents in competitive systems

On one hand, exploiting halogenated 4,4'-bipyridines as 'ideal' probes allowed to explore features and behaviour of XB in HPLC environment. On the other hand, more 'real' halogenated probes needed

to examine in depth the role of XB in competitive systems, evaluating the influence of halogen-centred σ-hole on recognition processes driven by other noncovalent interactions, such as HBs or π-π interactions.

3.2.1. Influence of halogen substituents in the enantioseparations of amide derivatives of 1-phenylethylamine: a re-examination

First, we applied the EP analysis to re-examine the chromatographic outcomes reported by Pirkle and co-workers [16] for the enantioseparation of amide derivatives of 1-phenylethylamine **16**, **17** and **18** using (3*R*,4*S*)-Whelk-O1 as CSP (Table 3). These compounds bear Cl, Br and I, respectively, as distinctive substituent at the *para* position of the phenyl ring. In addition, these structures contain a carbonyl as HB acceptor (HBA) and the amidic NH as HB donor (HBD) along with an aromatic ring as π-basic site [16]. Consequently, on (3*R*,4*S*)-Whelk-O1, HBs between the two amide moieties on both analyte and selector as well as π-π interaction driven by the π-acid dinitrophenyl group were expected to drive enantioseparation. In this perspective, it was likely that electron-withdrawing halogens could reduce the π-basicity of the aromatic ring on analyte with detrimental effect on the enantioseparation [59]. On the contrary, as shown in Table 3, for this series of compounds retention and selectivity increased as the polarizability of the distinctive halogen, iodine producing the highest value of selectivity [60]. With the aim to –gain an insight into the electrophilic function of the halogen substituents in this series of enantioseparations, we calculated the EPs associated with both HB sites and regions of electronic charge depletion on halogens (σ-hole). First, we observed that the introduction of halogens on the aromatic ring (series **16**-**18**) reduced the electron density on the carbonyl and, consequently, its effectiveness as HBA. Meanwhile, the π-basicity of the aromatic ring also decreased changing the min EP associated with the aromatic π-cloud from -72.4 kJ/mol (**19**) to values clustering around -45 kJ/mol (**16**-**18**). On the other hand, the EWG tendency of halogens increased the acidity of the amidic hydrogen which behaved as a better HBD. Nevertheless, the decrease of the EWG effect on

passing from **16** to **17** and **18** did not produce a corresponding decrease of retention or enantioselectivity. The positive EP on halogen increased as both retention and enantioselectivity instead, following the order **16** (*p*-Cl) < **17** (*p*-Br) < **18** (*p*-I). Consequently, EP analysis revealed that an additional XB between the halogen and the carbonyl group of the amide moiety of the CSP could reasonably explain the chromatographic outcomes, stabilizing the analyte in the CSP cleft.

3.2.2. Influence of halogen substituents in the enantioseparations of 2-nitro-1-halophenylethanols

On the basis of the previous observations, we focused our interest on two series of 2-nitro-1-halophenylethanols, **9**-**11** and **12**-**14**, as competitive systems, containing both halogens and HB sites on the same scaffold (Table 4). In particular, compounds **9**-**11** bear the halogen substituent at the *para* position of the phenyl ring, while in the series **12**-**14** the distinctive halogen is located at the *ortho* position, close to the HB sites. On one hand it could be envisaged that the nitro group increased the positive EP on halogens. Indeed, as proved by the max EP values calculated for the halogen substituents, the σ-holes depth for the series **9**-**11** was lower compared to the series **12**-**14**, which bear the nitro group closer to the halogen substituent. On the other hand the halogens could influence the chromatographic behaviour depending on their position with respect to the HB sites centred on the hydroxyl and nitro groups. Indeed, taking the EP values associated with the HB sites of compound **15** as a reference, a halogen effect on HB site electron density, dependent on the substitution position, could be observed.

On this basis, CDMPC having demonstrated its property as a good XBA under NP elution conditions [22], the chromatographic behaviour of the series **9**-1**1** and **12**-**14** was explored at 25°C on CDMPC using the mixture Hex/IPA 90:10 as MP (*FR* = 0.8 ml/min). Furthermore, these compounds had already proven to be enantioseparable on polysaccharide-based CSPs [38, 40-43], consequently they appeared suitable for our purposes. It is worth noting that only the enantioseparation of the 2-nitro-1-(4'-iodophenyl)ethanol **11** has been unexplored so far.

The chromatographic profiles obtained for compounds **9**-**14** on CDMPC are reported in Figure 7 and the corresponding chromatographic parameters are listed in Table 4. The enantioseparation of the 2-nitro-1-phenylethanol **15** was considered as a term of comparison.

Compounds **9**-**11** showed higher retention and enantioseparation compared to the reference compound **15**. Only for **9** a slightly lower retention was observed. Both retention and selectivity increased following the order **9** < 1**0** < **11**. The introduction of the EWG halogen at position *para* of the phenyl ring decreases the electron density on both hydroxyl (from -119.5 kJ/mol to values around -106 kJ/mol) and nitro oxygens (from -152.2 kJ/mol to values around -143 kJ/mol), which, consequently, reduce their HBA ability. Meanwhile, the acidity of the hydroxyl hydrogen increases and the EP values in this case rises from 250.4 kJ/mol, calculated for **15**, to values clustering around 266.3-269.5 kJ/mol. Nevertheless, it seemed there was no clear correlation between the variation of HB ability and the chromatographic behaviour trend. On the other hand, it was evident that the values of retention and selectivity increased as the halogen centred σ-hole depth following the order Cl (42.6 kJ/mol) < Br (76.9 kJ/mol) < I (107.3 kJ/mol).

In the series **12**-**14** the introduction of a halogen substituent close to the HB sites has a detrimental effect on their functions. Indeed, only the electron density on the hydroxyl oxygen increases with EP values changing from -119.5 kJ/mol (**15**) to -137.3-140 kJ/mol (**12**-**14**). On the contrary, the electron density on the nitro oxygen as well as the acidity of the hydroxyl hydrogen decreases as their ability as HB acceptor and donor, respectively. Consequently, retention and selectivity collapsed compared to the series **9**-**11**. Nevertheless, also in this case, the chromatographic parameters showed to increase as the σ-hole depth on halogen following the order **12** (55.3 kJ/mol) < **13**(88.1 kJ/mol) < **14** (115.2 kJ/mol).

On the basis of both structural features and chromatographic outcomes of the 2-nitro-1-halophenylethanols, a cooperative function of both HB and XB could be envisaged to drive enantiorecognition [61]. This hypothesis was evaluated by linear regression and the correlation extent

between ln k_1, ln k_2, as dependent variables, and six molecular descriptors (Table S11) was explored. Among dipole moment, logP, EPS volume, EPS area, EPS polar area (EPS_{pa}) and max EP on halogens, as independent variables, only EPS_{pa} showed P-values < 0.05 (Table S12). In this case, the result of fitting linear regression models to describe the relationships between ln k_1 / ln k_2 and EPS_{pa} indicated a statistically significant relationship (ln k_1 = f(EPS_{pa}): P-value = 0.01, r^2 = 0.8258; ln k_2 = f(EPS_{pa}): P-value = 0.008, r^2 = 0.8540).

Interestingly, the results of fitting a multiple linear regression model to describe the relationship between ln k_2 and both EPS_{pa} and max EP (X), as independent variables, indicated a statistical significant relationship with P-values < 0.05, as reported in Figure 8. The same good correlation was not obtained for ln k_1, pointing out the potential role of XB as secondary stereoselective interaction acting in cooperation with HB. In particular, when ln k_1 was considered as f(ES_{Ppa}, max EP (X)), the P-value associated with max EP (X) was shown to be greater to 0.05 (0.0614), so that the term was not statistically significant.

4. Conclusions

In the first part of this paper, we described the results of a computational study based on MD simulations of eight halogenated 4,4'-bipyridines, as 'ideal' probes, on both CDMPC and ADMPC. The theoretical model allowed to derive EEOs which were in accord with the experimental values with a success rate of 75% on CDPMC. In the second part, the chromatographic behaviour of 'real' probes was explored highlighting that XB as a secondary stereoselective interaction could also cooperate in enantiorecognition processes driven by HB or π-π interactions.

Although halogen bond has just made its debut in HPLC environment, interesting prospects can be envisaged in the near future for this noncovalent interaction as a powerful tool for the recognition of halogenated enantiomers due to growing interest around halogenated compounds containing heavier halogens (Br, I) in several applicative fields. On the other hand, the application of the reciprocity principle [62] paves the way to the design of a new generation of CSPs functioning by XBs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:

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FIGURE CAPTIONS

Fig. 1. Description of (A) halogen electron density anisotropy (on CF_3I as a representative model) and (B) halogen bond.

Fig. 2. Structures of polyhalogenated 4,4'-bipyridines **1**-**8** (A) and 2-nitro-1-arylethanols **9**-**15** (B).

Fig. 3. Location of 14 descriptors/ monomer and carbonyls $CO_{(2)}$, $CO_{(3)}$, and $CO_{(6)}$ on CDMPC and ADMPC.

Fig. 4. Root mean square deviations (RMSDs) (values on the y-axis are reported in Å) of all atoms (polymer + ligand) and single enantiomers calculated over 10 ns (values on the x-axis are reported as

step, 1 step = 2 ps) for compounds **3** and **8** (ESH, Hex). Legend: complex polymer/(*M*)-enantiomer (blue), complex polymer/(*P*)-enantiomer (green), (*M*)-enantiomer (orange), (*P*)-enantiomer (yellow).

Fig. 5. MD simulations of CDMPC/**3** complexes (10 ns), comparison of occupancy graphs: CDMPC/(*M*)-**3** (A), CDMPC/(*P*)-**3** (B).

Fig. 6. Distribution of the interaction sites in the course of MD simulations (10 ns) of (*M*)-**1** (graphs A, B) and (*M*)-**3** (graphs C, D): $CO_{(3)}$ (■), $CO_{(6)}$ (■) (graphs A, C); 3'-Cl (■), 2-Cl (■), 3-Cl (■) (graph B); 5'-I (■), 2-I (■) (graph D).

Fig. 7. Comparative enantioseparation of 2-nitro-1-halophenylethanols **9**-**14** on CDMPC (hex/IPA 90:10, *FR* = 0.8 ml/min, T = 25°C).

Fig. 8. Linear regression analysis describing the relationships between ln k_2 (dependent variable) and EPS_{pa} and max EP (X) as independent variables.