

Cinchona-based zwitterionic stationary phases: Exploring retention and enantioseparation mechanisms in supercritical fluid chromatography with a fragmentation approach

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Adrien Raimbault, Cam Mai Anh Ma, Martina Ferri, Stefanie Bäurer, Pascal Bonnet, et al.. Cinchonabased zwitterionic stationary phases: Exploring retention and enantioseparation mechanisms in supercritical fluid chromatography with a fragmentation approach. Journal of Chromatography A, 2020, 1612, pp.460689. 10.1016/j.chroma.2019.460689 . hal-02904771

HAL Id: hal-02904771 https://hal.science/hal-02904771

Submitted on 7 Mar 2022

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Version of Record: https://www.sciencedirect.com/science/article/pii/S0021967319311185 Manuscript_ae85be7fdce23f091dd75c65c6a86a0e

1	Cinchona-based zwitterionic stationary phases: Exploring retention									
2	and enantioseparation mechanisms in supercritical fluid									
3	chromatography with a fragmentation approach									
4										
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20	Abstract									
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22	Chiralpak ZWIX(+) and ZWIX(-), are brush-type bonded-silica chiral stationary phases									
23	(CSPs), based on complex diastereomeric Cinchona alkaloids derivatives bearing both a									
24	positive and a negative charge. In the present study, we aimed to improve the understanding									
25	of retention and enantioseparation mechanisms of these CSPs employed in supercritical fluid									
26	chromatography (SFC). For this purpose, 9 other stationary phases were used as									
27	comparison systems: two of them are commercially available and bear only a positive charge									
28	(Chiralpak QN-AX and QD-AX) and the 7 others were designed purposely to be structurally									
29	similar to the parent ZWIX phases, but miss some portion of the complex ligand. First, cluster									
30	analysis was employed to identify similar and dissimilar behavior among the 11 stationary									
31	phases, where ionic interactions appeared to dominate the observed differences. Secondly,									
32	the stationary phases were characterized with linear solvation energy relationships (LSER)									
33	based on the SFC analysis of 161 achiral analytes and a modified version of the solvation									
34	parameter model to include ionic interactions. This served to compare the interaction									

35 capabilities for the 11 stationary phases and showed in particular the contribution of

attractive and repulsive ionic interactions. Then the ZWIX phases were characterized for their 36 enantioseparation capabilities with a set of 58 racemic probes. Discriminant analysis was 37 38 applied to explore the molecular structural features that are useful to successful 39 enantioseparation on the ZWIX phases. In particular, it appeared that the presence of 40 positive charges in the analyte is causing increased retention but is not necessarily a favorable feature to enantiorecognition. On the opposite, the presence of negative charges in 41 42 the analyte favors early elution and enantiorecognition. Finally, a smaller set of 30 pairs of enantiomers, selected by their structural diversity and different enantioseparation values on 43 the ZWIX phases, were analyzed on all chiral phases to observe the contribution of each 44 45 structural fragment of the chiral ligand on enantioselectivity. Molecular modelling of the 46 ligands also helped in understanding the three-dimensional arrangement of each ligand, notably the intra-molecular hydrogen bonding or the possible contribution of ionic 47 interactions. In the end, each structural element in the ZWIX phases appeared to be a 48 significant contributor to successful enantioresolution, whether they contribute as direct 49 interaction groups (ion-exchange functions) or as steric constraints to orientate the 50 51 interacting groups towards the analytes. 52

- 53
- 54 **Keywords:** Chiral stationary phases; Cinchona-based chiral zwitterionic ion exchanger;
- 55 Enantioseparation mechanisms; Quantitative structure-retention relationships (QSRR);
- 56 Solvation parameter model; Supercritical fluid chromatography

57 **1.** Introduction

58

59 Cinchona-based chiral stationary phases were introduced in the late 1980s in form of free 60 quinine bonded to silica [1] and as carbamate derivatives in 1996 by Lämmerhofer and Lindner [2]. They are brush-type enantioselective stationary phases where the ligand is 61 62 derived from guinine and guinidine diastereomers bonded on silica gel. Recently, similar 63 phases bonded so superficially porous silica were proposed by Armstrong and co-workers [3]. Because quinine and quinidine both possess a tertiary amine function (quinuclidine ring), 64 which should be protonated in most liquid and supercritical fluid mobile phases, a quinine- or 65 66 quinidine-based stationary phase should act as an anion-exchanger. One such phase is presented in Figure 1 (QN). Two commercial stationary phases are available, which were 67 68 derived from quinine- and quinidine-bonded silica, with a carbamate function modifying the hydroxyl group and retaining the anion-exchange capabilities: Chiralpak QN-AX and QD-AX 69 70 (Figure 1). These stationary phases are mostly employed in HPLC, where they have 71 demonstrated excellent enantioselective properties for a broad range of chiral acids [2,4]. 72 Further modification of the carbamate function to include a chiral sulfonic acid group yielded 73 two zwitterionic stationary phases, commercially available as Chiralpak ZWIX(+) and ZWIX(-) 74 [5,6]. These phases were notably demonstrated to be particularly useful for the enantioseparation of free or derivatized amino acids and oligopeptides by HPLC [6-13]. 75 Cinchona alkaloids and their derivatives were also employed as chiral selectors in capillary 76 77 electrophoresis [14,15] and capillary electrochromatography [16,17], where the high efficiency values usually yield excellent resolution. 78 79 Supercritical fluid chromatography (SFC) is known to be most effective for enantioselective 80 separations [18,19], as the resolution per unit time is often advantageous compared to HPLC 81 separations, making it suitable for high-throughput enantioselective analysis. In addition, the 82 economic and ecological aspects related to the use of pressurized carbon dioxide as 83 principal component of the mobile phase render it an attractive method for the industry, both at the analytical and preparative scales. The Cinchona-based chiral phases were only rarely 84 employed in SFC. One possible reason is the wrong perception that ionic species cannot 85 exist in the apparently non-polar SFC mobile phase. However, it was proven in several 86 87 studies that ionic species exist in carbon dioxide – organic solvent mixtures and can even be resolved [20,21] and that ionic interactions exist, which are measurable [4,22,23] and can be 88 modified when acidic and basic additives are introduced in the co-solvent [24]. Thus a few 89 90 papers relate the use of ZWIX phases to resolve N-protected amino acids [25,26] or indoletype analytes [27]. Recently, we have demonstrated that elution and enantioresolution of a 91

92 wide range of free natural amino acids were possible with a wide elution gradient running93 from SFC to HPLC conditions [28].

94

Retention and enantioseparation mechanisms on the **ZWIX** phases were explored mostly in 95 96 liquid phase by the group of Lindner on the one hand [9,29], Ilisz and Péter on the other hand [30], but only little was done in SFC conditions. Pell and Lindner showed that the ion-97 exchange mechanism existed on QN-AX and QD-AX phases in SFC conditions, thanks to 98 99 the presence of methoxylcarbonic acid formed by the reaction of CO₂ and acting as counterion in the anion-exchange process [4]. This ion-exchange mechanism could be tuned 100 101 with the introduction of acidic or basic additives in the mobile phase. For these stationary 102 phases, the same chiral recognition mechanism was observed between HPLC and SFC 103 experiments. More recently, Wolrab et al. observed major differences between HPLC and SFC separations on the ZWIX phases and proceeded to explain the differences [31]. A major 104 conclusion from this work is that mechanistic investigations done on the ZWIX phases in 105 HPLC conditions may not all be transferable to SFC conditions. Naturally, the components of 106 107 the mobile phase can have a significant contribution to the enantiorecognition as they define 108 the environment in which the process occurs [6,30,32], may have different solvation 109 properties towards the chiral analytes and the chiral stationary phase, which is sometimes resulting in different conformations of the stationary phase. 110

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To better understand the behavior of Chiralpak ZWIX(+) and ZWIX(-) in SFC, a fragment-112 based approach to deciphering the complex enantioselective ligands was selected: nine 113 114 other (commercial or in-house) stationary phases were used as comparison. All nine phases had some similarity to the ZWIX phases, each one having one less fragment than the parent 115 ligand. The purpose was to understand the contribution of each functional group in the 116 117 retention mechanisms and appreciate how each functional group was determinant in the enantioseparation process. For this purpose, the eleven columns were first characterized by 118 means of quantitative structure-retention relationships (QSRR) with a modified version of the 119 120 solvation parameter model, to include ionic interactions, based on the analysis of 161 achiral 121 analytes. Cluster analysis and the QSRR models served to compare the columns. Secondly, 122 based on the analysis of 58 racemic probes, discriminant analysis served to identify the 123 molecular features that are most favorable to successful enantioseparation on the ZWIX 124 phases. Finally, 30 racemates with structural diversity were analyzed to explore the differences in enantioresolution. Molecular modelling assisted in understanding the observed 125 126 differences.

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128 129

2. Material and methods

130 2.1 Stationary phases

All experiments were carried out on the eleven columns. Four columns are commercially available, named Chiralpak **ZWIX(+)**, Chiralpak **ZWIX(-)**, Chiralpak **QN-AX** and Chiralpak **QD-AX**, and were kindly provided by Chiral Technologies Europe (Illkirch, France) with ligands bonded on 3 µm particles. The seven other ligands were prepared in-house, bonded on 3 µm Daisogel silica, 120 Å (Osaka Soda) and packed. Their synthesis and liquid-phase characterization was described in a previous study [33]. All columns had the same dimensions of 150 x 3 mm.

- 138
- 139 2.2 Chemicals and Solvents

161 achiral (Table S1) and 66 chiral molecules (Table S2) in their racemic form were 140 purchased from Sigma-Aldrich (Sigma Aldrich Chimie, France). The achiral analytes were 141 selected to provide a diverse representation of chemical functions to obtain meaningful 142 143 retention models with the solvation parameter model. The chiral probes in Table S2 were selected to obtain meaningful information from the discriminant analyses with different 144 structures and shapes. These sets were fully described in previous papers [34,35]. The 145 reduced set of chiral analytes (Table S2, in bold) was selected as a subset of the wider set 146 147 maintaining structural diversity but also included an addition of 8 free amino acids. As the 148 individual enantiomers were not available in most cases, the elution order was not assessed. 149 Solutions of all analytes were prepared at about 1 mg/mL in methanol (MeOH). The HPLC-150 grade methanol used for analyte dissolution and as mobile phase co-solvent was supplied by VWR (Fontenay-sous-Bois, France). Ultra-pure water was supplied by an Elga UHQ system 151 from Veolia (Wissous, France). CO₂, with a purity of 99.995 %, was delivered by Air Liquide 152 (Paris, France). Ammonium formate was provided by Sigma Aldrich (Sigma Aldrich Chimie, 153 154 France).

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- 156

2.3 Instruments and operating conditions

The supercritical fluid chromatography system was an ACQUITY Ultra Performance 157 Convergence Chromatography[™] (UPC^{2®}) from Waters Corporation (Millford, MA, USA). It 158 was equipped with a binary solvent delivery pump compatible with mobile phase flow rates 159 up to 4 mL/min and pressures up to 414 bar, an autosampler that included partial loop 160 volume injection system, a back-pressure regulator, 2-position column oven compatible with 161 150 mm length columns and a photodiode-array (PDA) detector. Empower[®] 3 was used for 162 163 integration of peaks for column efficiency measurements. An ACQUITY QDa® single-164 guadrupole mass detector with electrospray ionization source was also used for amino acids detection. An isocratic solvent manager was used as a make-up pump and was positioned
before the mass detector. The main flow stream was then split by the on-board flow-splitter
assembly.

168

169 All analyses (chiral and achiral sets) were performed in isocratic conditions (CO₂-MeOH 170 90:10 (v/v), 25°C, 150 bar, 3 mL/min), apart from the analyses of amino acids for which a 171 wide gradient elution (10 to 100% co-solvent) was done with methanol containing 5% H₂O 172 and 50 mM ammonium formate, according to previously developed method [28]. In that, 173 case, the flow rate was reduced to 0.5 mL/min to avoid reaching the upper pressure limit of the pumping system. The UV detection wavelength was 210 nm while single-ion recording 174 was used for MS detection of amino acids according to the mass of the protonated molecular 175 176 ion. 177 2.4 Data analysis 178 179 180 Abraham descriptors (Tables S1 and S2) were extracted from an in-house database based 181 on all existing literature on the solvation parameter model. Additional structural descriptors 182 were computed as previously described [22,35]. 183 Multivariate data analyses (hierarchical cluster analysis (HCA), multiple linear regression and discriminant analysis (DA)) were done with XLSTAT 19.03 software (Addinsoft, New York, 184 NY, USA). The retention data (log k values) for achiral analytes were normalized (centred 185 and reduced to adjust mean value on 0 and standard deviation on 1) prior to HCA 186 calculation, to ensure that equal significance would be associated to each analyte. HCA was 187 based on Ward aggregation method with Euclidean distance. 188 The quality of QSRR fits was estimated using the adjusted determination coefficient (R^2_{adj}), 189 standard error in the estimate (SE) and Fisher F statistic. The statistical significance of 190 191 individual coefficients was evaluated with the 95% confidence intervals. 192 The linear solvation energy relationship (LSER) equation used in this work is based on the 193 five Abraham descriptors and two additional descriptors to take ionic interactions into 194 account: 195 $\log k = c + eE + sS + aA + bB + vV + dD^{-} + d^{+}D^{+}$ 196 (1) 197 In this equation, capital letters represent the solute descriptors, related to particular 198 199 interaction properties, each molecule has its own molecular descriptors. While lower case 200 letters represent the system constants, related to the complementary effect of the two

201 phases (stationary and mobile). *c* is the model intercept term and is dominated by the phase

ratio. *E* is the excess molar refraction (calculated from the refractive index of the molecule) 202 203 and models polarizability contributions from n and π electrons; S quantifies the presence of dipoles and polarizability; A and B are the solute overall hydrogen-bond acidity and basicity; 204 V is the McGowan characteristic volume in units of cm³ mol⁻¹/100; D and D^+ are the solute 205 negative and positive charges respectively, calculated with the pK_a and apparent pH of the 206 207 mobile phase. The five first descriptors are known as Abraham descriptors and were used in 208 many occasions to characterize chromatographic systems, while the latter two were introduced more recently to characterize hydrophilic interaction liquid chromatography 209 210 (HILIC) systems [36], and were later demonstrated to be suitable to describe SFC systems, provided we admit to some approximations [22,23]. The system constants (e, s, a, b, v, d, 211 a^{+}), obtained through a multilinear regression of the retention data for a certain number of 212 213 solutes with known descriptors, reflect the magnitude of difference for that particular property between the mobile and stationary phases. Thus, if a particular coefficient is numerically 214 215 large, then any solute having the complementary property will interact very strongly with 216 either the mobile phase (if the coefficient is negative) or the stationary phase (if the 217 coefficient is positive). The system constants facilitate the comparison of the separation characteristics of different stationary phases and enable the identification of stationary 218 219 phases of similar or dissimilar selectivity. 220 In previous works, we have demonstrated that two other structural features that contributed little to retention were however significant to describe enantioseparation mechanisms on 221 222 polysaccharide or macrocylic glycopeptide stationary phases: flexibility (F) and globularity or 223 sphericity (G) [35,37–39]. In the present work, the F and G descriptors were first introduced in Equation (1) but appeared to be insignificant to explain retention. They were however 224

retained for the evaluation of chiral separations through discriminant analysis. The quality of
 discriminant analysis was estimated based on ROC (receiver operating characteristics)
 curves (visible in Figure S1) and confusion matrices.

228

229 2.5 Molecular modelling

230

231 The 3D structures of each enantioselective ligand presented in Figure 1 were prepared from 232 2D coordinates. The 3D conformations were generated using the Structure Preparation 233 function and hydrogen atoms were added using Protonate3D function in MOE2014.09 234 (Chemical Computing Group Inc., Montreal, Canada). Then the structures were submitted to conformational search using LowModeMD with default parameters. The MMFF94x force field 235 236 with Born solvation was used and the lowest energy conformer was retained for comparison 237 of the ligand conformations. The structures were observed and copied from Discovery Studio 4.0 Visualizer (Accelery, San Diego, California, USA). 238

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240	3. Results and discussion
241	
242	3.1 Description of the stationary phases
243	
244	The chiral selectors in the eleven stationary phases compared in this study are presented in
245	Figure 1.
246	As can be seen in Figure 1, apart from the above-cited ZWIX(+), ZWIX(-), QN, QN-AX and
247	QD-AX, six other fragmented ligands were designed. First, removal of the quinoline group
248	from the ZWIX(+) and ZWIX(-) chiral selectors yielded QCISS and QCDRR ligands,
249	respectively. The steric constriction of the sulfonic group was further removed to yield
250	QCITAU and QCDTAU, based on quincorine (QCI) / quincoridine (QCD) and taurine (TAU).
251	Finally, the cationic quincoridine group was removed from QCDRR and QCDTAU to obtain
252	the strong cation-exchangers 2-aminocyclohexanesulfonic acid-derived SCXSS and taurine-
253	derived SCXTAU . The ligand coverage on the stationary phase surface was adjusted to ca.
254	200 μ mol/g (within ± 30 μ mol/g) for all stationary phases [33].
255	
256	All these phases (the seven in-house and the Chiralpak QN-AX and QD-AX) were highly
257	similar to the ZWIX phases, each one having only some portion of the structure less than the
258	original ligand. The purpose was to understand the involvement of each functional group in
259	the retention and enantioseparation mechanisms and measure how each functional group
260	was determinant in the enantioseparation process. Due to the large number of stationary
261	phases and the number of analytes included to obtain most accurate and representative
262	models, only one mobile phase composition was employed, although acidic and basic
263	additives should clearly have an effect on enantioselective capabilities of these phases [31].
264	
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266	3.2 Multivariate data analysis: Hierarchical cluster analysis
267	
268	Hierarchical cluster analysis (HCA) is a useful method to group chromatographic columns
269	according to the similarity in their retention behaviour. Because the retention data were
270	normalized, the similarity will reflect elution order of the analytes, rather than overall
271	retention. The result are presented in a dendrogram (Figure 2), where the columns that are
272	linked with a short horizontal line are most similar and have close chromatographic
273	selectivity, and the columns that are linked with a long horizontal line are most dissimilar and
274	exhibit different chromatographic selectivity. In Figure 2, three principal clusters appear:

275	- In the upper part of the figure, the ZWIX(+) and ZWIX(-) phases are grouped with the
276	four other zwitterionic phases QCITAU, QCISS, QCDRR and QCDTAU.
277	- In the middle of the figure, the three phases possessing only a cationic charge are
278	grouped (QN, QN-AX and QD-AX).
279	- In the lower part of the figure, the two phases possessing only an anionic charge are
280	grouped (SCXSS and SCXTAU).
281	Clearly, the clustering is mostly based on ionic interactions, while the presence of quinoline
282	and the hexyl ring on the sulfonic group are much less significant in terms of retention
283	behaviour. Quite logically, diastereomeric or pseudoenantiomeric phases are most similar
284	(linked with the smallest lines): ZWIX(+) and ZWIX(-); QN-AX and QD-AX; QCISS and
285	QCDRR; QCITAU and QCDTAU.
286	While the dendrogram is a simple way to compare columns, it is not highly informative.
287	Therefore, linear solvation energy relationships were calculated for each column to explore
288	interaction differences more precisely.
289	
290	
291	3.3 Retention mechanisms examined with achiral compounds
292	
293	161 achiral molecules (Table S1) were analyzed on the eleven columns studied in order to
294	model their retentive properties. A modified version of the LSER model (Equation (1)) was
295	used with the five Abraham descriptors and the addition of two descriptors taking ionic
296	interactions into account. The model therefore contains a total of seven different descriptors.
297	The results are presented in Table 1 and Figure 3. Considering that no retention prediction
298	was desired (only interpretation of interaction terms), the statistics were considered as
299	reasonably good.
300	Retention mechanisms on all eleven phases are dominated by hydrogen bonding with proton
301	donors (a terms) and by interactions with π and n electrons (e) that must result principally
302	from π -stacking with aromatic analytes. Hydrogen-bonding with proton acceptors (<i>b</i> term),
303	the contribution of molecular volume (v) and ionic interactions (d and d ⁺ terms) are
304	significant on certain columns and are clearly discriminant. Dipole-dipole interactions (s)
305	contribute moderately and offer little discrimination between the stationary phases examined.
306	
307	Examining the models obtained on the ZWIX phases, a first, quite surprising observation is
308	that the ionic interaction terms (d and d^{\dagger}) are rather small although significant ionic
309	interactions were expected. The d^+ term is slightly larger than the d^- term, in accordance with
310	previous reports that the surface of ZWIX stationary phases is slightly acidic [40]. Besides,
311	the four other zwitterionic phases (QCISS, QCDRR, QCITAU and QCDTAU) also have

rather small ionic interaction terms. This would tend to indicate that the cationic and anionic 312 groups in the ligands must be associated to each other, thereby reducing their availability for 313 314 interaction with ionic analytes. Indeed, the d term, reflecting retention of anionic compounds, 315 is large and positive on the anion-exchange stationary phases (QN-AX, QD-AX and QN) while the d^{+} term, reflecting retention of cationic compounds, is large and positive on the 316 cation-exchange stationary phases (SCXSS and SCXTAU). In addition, the d⁺ term, 317 318 reflecting retention of cationic compounds, is reduced on the anion-exchange stationary phases while the d term, reflecting retention of anionic compounds, is large and negative on 319 320 the cation-exchange stationary phases, probably due to electrostatic repulsion interactions. 321 In the case of simple ion-exchangers (not zwitterionic), the charges on the ligands are thus 322 more freely available to interact with ionic analytes than on the ZWIX phases. On the contrary, the phases possessing a single ionic function, whether it is cationic or anionic, 323 display strong ionic interactions. This is consistent with previous observations in HPLC that 324 the sulfonic group "mostly act as intramolecular counterions leading to reduced run times 325 compared to parent anion exchangers" [5]. Another interesting explanation for the moderate 326 values of d and d⁺ terms may be found in the electrostatic attraction-repulsion (push-and-327 pull) mechanism recently proposed by Mimini et al. [41]. Basically any anionic analyte should 328 329 be both attracted by the cationic function of the stationary phase and repulsed by the anionic 330 function, and vice-versa for an anionic analyte. This combined effect of attraction and repulsion could explain the overall moderate retention of ionic species on the **ZWIX** phases. 331 In other words, the two ionic functions in the chiral ligand partly cancel each other. 332 Another interesting feature is that the v term, reflecting the effect of molecular volume on 333 334 retention, is negative on all stationary phases but most significant on the **ZWIX** phases. The negative terms indicate that an increase in hydrocarbon volume is causing decreased 335 336 retention on all columns, as usually observed on polar stationary phases in SFC [42], related 337 to a normal-phase retention mode. The most significant negative value of v term on ZWIX 338 phases can be related to the difficulty for large molecules to insert in the stationary phase, 339 due to the bulky ligand leaving little space for insertion, or due to the cohesiveness of the 340 stationary phase resulting from ionic and hydrogen-bonding interactions between them. Only 341 the strong cation exchanger **SCXTAU** had comparably large negative v term, which can be 342 explained by the absence of hydrophobic ligand portion in this stationary phase that could 343 contribute to positive dispersive interactions to compensate for the phase cohesiveness. 344 Compared to ZWIX phases, the four other zwitterionic columns QCISS, QCDRR, QCITAU 345

and QCDTAU are lacking a quinoline group, and two of them are lacking a butyl linker that is
 constraining the sulfonic group through a cyclohexyl function.

- A small increase is observed for the interactions with anionic species (d), and a small
- 349 decrease for the interactions with cationic species (d^{+}) for the phases that do not possess a
- quinoline group, which may be attributed to easier access of the ionic analytes to the cationic
- 351 charge borne by the protonated nitrogen atom, when the bulky quinolic group is absent,
- 352 causing increased attraction of anions and increased repulsion of cations. Other terms were
- 353 mostly similar on all zwitterionic phases.
- The three phases **QN-AX**, **QD-AX** and **QN** are similar to the **ZWIX** phases apart from the
- 355 sulfonic group. As mentioned above, the most significant differences observed are in the
- ionic interactions and *v* term. On a practical note, this will reflect on the retention of acidic
- 357 species that should comparatively be more retained on the anion-exchange stationary
- 358 phases than on the zwitterionic phases, as appears in Figure 4a. In this figure, it appears that
- the retention of Chiralpak **ZWIX(+)** and Chiralpak **QN-AX** are most similar, apart from the
- 360 species that should bear a negative charge in the present operating conditions. Other small
- 361 differences are noticeable in Figure 3: a small decrease of hydrogen bonding with proton
- donors and acceptors (*a* and *b* terms respectively) and interactions with dipoles and
- polarizable species (*s* and *e* terms respectively) are observed for the 3 phases possessing
 no sulfonic group when compared to the **ZWIX** phases.
- 365 The simple cation-exchangers **SCXSS** and **SCXTAU** are the most different from the ZWIX
- 366 phases, not only due to a different pattern of ionic interactions, but also with significantly
- 367 weaker hydrogen bonding interactions with proton donors (*a* term) and significantly stronger
- 368 hydrogen bonding with proton acceptors (*b* term). Overall, this reflects in generally weaker
- 369 retention on the cation-exchange phases compared to the zwitterionic phases, as appears in
- Figure 4b, where most points fall below the first bisector. However, when a trend line is
- 371 plotted across hydrophobic analytes (black diamonds), although some dispersion appears for
- 372 polar non-ionic analytes (open circles), the lower retention of acidic anionic compounds (red
- triangles) and the stronger retention of basic cationic analytes (blue squares) are evident.
- 374

375 Finally, based on the LSER models, the eleven phases were plotted in the spider diagram reflecting the overall selectivity of the phases (Figure 5). This figure was previously 376 377 developed to allow easy comparison of a large number of stationary phases based on the system constants from LSER characterization [23,43]. Quite simply, the figure is a projection 378 379 of the seven-dimension selectivity space defined by the seven system constants in Eq. (1) on 380 a plane. Most hydrophobic stationary phases are situated on the left of the figure and most 381 polar stationary phases are located on the right. From top to bottom, hydrogen bonding and ionic interactions contribute significantly to scattering the stationary phases. Bubble size is 382 related to the overall strength of interactions (length of the solvation vector). To better 383 384 appreciate the diversity of selectivities provided by the eleven phases, 36 other stationary

phases previously characterized with the same protocol were also plotted. The full list of thecolumns relating to their number in the figure is presented in Table S3.

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388 A first observation is that all eleven phases are grouped on the right of the figure, with polar 389 stationary phases, but they are clearly scattered along the vertical axis, as a result of their 390 different ionic interactions: the anion-exchange stationary phases are grouped at the bottom 391 of the figure, the cation-exchange phases are situated in the upper part of the figure, and the zwitterionic phases are in-between. Secondly, all eleven phases are singular (not 392 superimposed with any other in the figure) but have some similarity to other phases. The 393 394 ZWIX phases are not too far from the achiral zwitterionic phases based on sulfobetaine 395 ligand (# 22 and 23). They are also close to a stationary phase bonded with amino-396 anthracene ligand, probably due to the similarity to the guinoline group. The cation-exchange 397 phases are not too far from the bare silica phases (# 31-36) which can also interact with 398 cationic analytes through their silanol groups. Finally, the phases possessing amine or pyridine groups in their bonded ligands (# 21, 24 and 25) are all at the bottom of the figure, 399 400 close to the zwitterionic and anion-exchange phases characterized in this work.

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3.4 Enantioseparation mechanism examined with discriminant analysis

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405 In previous studies on polysaccharide or macrocyclic glycopeptide enantioselective 406 stationary phases, we have demonstrated that discriminant analysis was a useful tool to 407 understand the molecular features that contribute to favorable enantiorecognition [37–39]. 408 Basically, discriminant analysis is applied to a diverse selection of racemates (58 in the present case) analyzed in a single chromatographic system (one stationary phase and one 409 410 mobile phase composition). The probe molecules are characterized by molecular descriptors and by the chromatographic result that can be simplified in two categories: separated 411 412 (separation factor > 1) or not separated (separation factor = 1). However, we had also 413 observed that applying this strategy to the whole set of racemates was often unsuccessful 414 because not all racemates that were resolved had been discriminated for the same reasons. Thus a discriminant analysis including the whole set of analytes usually results in poor 415 statistics, indicating that the models obtained are not relevant. To improve the significance of 416 417 the results, a more efficient strategy was defined. First, the experimental retention of the two 418 enantiomers is compared to the theoretical retention predicted with the solvation parameter model (Eq. (1)). In most cases, the two enantiomers both elute earlier than the prediction or 419 420 they both elute later than the prediction. A first discriminant analysis is then carried out to 421 observe the features that contribute to early elution or late elution of the enantiomers. At this

422 stage, it is useful to introduce two additional descriptors that are helpful to describe

enantiorecognition processes: flexibility (F) and globularity (G). The other descriptors are thesame seven used in the LSER models above.

425 The result of this discriminant analysis can be observed for Chiralpak **ZWIX(-)** in Figure 6a. It 426 appears that late-eluting analytes are mostly flexible molecules (positive F term) and cationic 427 molecules (positive D⁺ term). Flexible molecules have more ways to adapt to a complex, 428 rather rigid ligand, which should be favorable to increased possibilities of interactions between a multi-functional analyte and the multi-functional ligand. The favorable contribution 429 of positive charges may be related to better accessibility of the anionic sulfonic group than 430 431 the cationic quinuclidine causing stronger retention for cations. Conversely, anionic 432 compounds seem to be repulsed as the presence of negative charges is favorable to early

433 elution (negative D⁻ term). This may be observed on a molecular model of the Chiralpak

ZWIX(-) ligand in Figure 7, where the quinuclidine group, being closer to the silica surface,
 should be most difficult to interact with than the pending sulfonic group.

436 Secondly, discriminant analysis based on the separated / non separated classes is applied

437 independently on the 32 early-eluting (Figure 6b) and 26 late-eluting racemates (Figure 6c).

438 Comparing the two figures, it appears that the structural features that contribute favorably to

enantiorecognition are not all identical between early-eluting and late-eluting racemates.

440 More precisely, the same three features (acidic character A, globularity G and negative

441 charges D⁻) appear to be favorable but not in the same proportions. For early-eluting

analytes, which should be rather rigid and not cationic, the presence of a negative charge is

the most favorable feature (large positive D⁻ term), followed by proton-donor capability

444 (positive A term). We may suppose that the ability to interact favorably with the cationic

group (quinuclidine function) and with the quinoline and/or carbamate functions must

446 contribute favorably to enantiorecognition. Late-eluting molecules are mostly cationic and

flexible, but these features are not favorable to enantiorecognition for this group (negative Fand D⁺ terms).

In both cases (early and late-eluting), globularity is highly significant (positive G term) while
 small molecular volume is also a favorable feature (negative V term). This indicates that

451 small spherical molecules are generally better resolved with this chiral selector. The chiral

452 selector is rather constrained with three rather rigid portions: the quinoline ring, the

453 quinuclidine group and the cyclohexanesulfonic function. The three of them define a small

454 space for chiral molecules to fit in. A small spherical molecule should better adapt to this

455 defined space than a large and/or non-spherical (planar or linear) molecule.

456 Very similar results were obtained in all cases on Chiralpak **ZWIX(+)**.

457

A subset of the 58 racemates was selected for structural diversity: 22 molecules (written in 458 bold in Table S2) representing different analyte classes and with different separation features 459 on the ZWIX phases (resolved or nor resolved) were chosen. Additionally, 8 free amino acids 460 461 that were resolved on the **ZWIX** phases were included in this final test set. The 30 pairs of 462 enantiomers were analysed on the ten chiral phases (thus excluding the non-chiral SCXTAU). Some representative examples are shown in Table 2. Only racemates that could 463 464 be resolved on one stationary phase at least are presented in this table because the racemates that cannot be (at least partially) resolved on any stationary phase provide little 465 information on enantiorecognition mechanisms. 466 467 468 One example of the contribution of shape is that of benzodiazepines. For instance,

Oxazepam is a large and rather flat molecule. Because of its rigidness, it was eluted rather 469 earlier than predicted but its flatness is probably responsible for the lack of enantioresolution 470 471 on the ZWIX phases. Some enantioselectivity was however observed on the QN-AX and **QD-AX** phases. Molecular modelling allows observing the different three-dimensional 472 orientation of each CSP ligand: the folding of each ligand is highly dependent on intra-473 474 molecular interactions and steric constraints. Comparing the structures of the ligands (see 475 superimposed ligands of ZWIX(-) and QD-AX in Figure 7), it appears that the structure of QN-AX and QD-AX should be more open, less sterically constrained than the structure of 476 **ZWIX** ligands. The replacement of the large cyclohexylsulfonic group by a smaller t-butyl 477 478 group should make it easier for a large molecule to adapt to the chiral selector.

- 480 On the contrary, 5-methyl-5-phenylhydantoin is a small and rather compact (spherical)
- 481 molecule that was well resolved on the **ZWIX** phases, as can be seen in the chromatograms
- in Figure 8a. Resolution was also possible on the anion-exchange phases (**QN-AX**, **QD-AX**
- and **QN**), and it was also possible on the two phases lacking the quinoline group but
- retaining the cyclohexyl group (**QCISS** and **QCDRR**). However, enantioseparation was lost
- 485 when the steric constraint on the sulfonic group was not present (**QCITAU** and **QCDTAU**).
- 486 Clearly, the orientation of the sulfonic group, forced by the presence of the cyclohexyl group,
- 487 is favorable to enantiorecognition. This observation can be contrasted to previous
- observations in the liquid phase that "the *trans*-2-aminocyclohexanesulfonic acid moiety... is
- 489 truly beneficial but not essential for the observed enantioselective properties of the
- 490 zwitterionic chiral stationary phases" [5].
- Acidic compounds and proton-rich compounds were generally well resolved on **ZWIX** phases
 and anion exchangers, while no resolution could be obtained on the other columns. Only the
 anion-exchange phases were also favorable to resolution of acids.
- 494 Enantiorecognition was observed for all amino acids on the **ZWIX** phases as a result of the 495 double ion-pairing with the zwitterionic analytes [30] but was lost on most of the others, apart
- 496 from the other zwitterionic phases where some enantioselectivity may be observed, even if
- the peak shapes were poor. An example can be seen in Figure 8b with the chromatograms
- 498 of Alanine. The zwitterionic character thus seems necessary but not sufficient to ensure
- enantioresolution of zwitterionic analytes. Comparing the ligand structures in Figure 7, the
- 500 **QCISS** ligand should be much more flexible than the **ZWIX(+)** ligand as the large quinoline
- 501 group is not present to force the conformation in one direction. Flexibility in a chiral selector
- is generally recognized as an unfavorable feature.
- 503 The simplest ligand in **SCXSS** was totally incapable of resolving any of the pairs of
- enantiomers in this set. Clearly, the simple chirality borne by the cyclohexylsulfonic ligand isnot sufficient to discriminate the enantiomers.
- 506 Overall, among the 22 pairs of enantiomers other than amino acids, 7 are separated on the
- 507 ZWIX phases, whereas 8 pairs of enantiomers were separated on the QN-AX and QD-AX
- 508 phases, 6 pairs on the **QN** phase and only 2 on the four other zwitterionic phases.
- 509 All structural features in the **ZWIX** phases then seem to play a significant role in the multi-
- 510 modal enantioseparation mechanism. This synergistic effect that was desired in the
- 511 conception of the **ZWIX** ligands [5] is then proven to be completely true.
- 512
- 513
- 514

4. Conclusion 515

The mechanisms for retention and enantioseparation of the Chiralpak ZWIX(+/-) columns 516 517 were studied with the analysis of 161 achiral compounds and 66 pairs of enantiomers. The 518 joint presence of a cationic and anionic charge in the **ZWIX** phases appears to be reducing the overall strength of ionic interactions, compared to simple anion-exchange or cation-519 exchange phases, probably due to intra-molecular ionic interactions, or ionic interactions 520 between proximate ligands. The influence of the sterically constrained sulfonic group and the 521 quinoline group were shown to be highly significant to explain the successful 522 enantiorecognition in the **ZWIX** phases. Basically, all structural elements of the **ZWIX** ligands 523 appeared to be significant contributors to enantioselectivity. ZWIX phases also appeared to 524 favour small and spherical molecules, which should better fit in the chiral selector cavity, as 525 large and/or flat molecules remained unresolved. 526 527 528

Acknowledgments 529

- 530 Adrien Raimbault is grateful for a PhD grant received from the Ministry of Higher Education
- and Research. Caroline West is grateful for the support received by the Institut Universitaire 531
- 532 de France (IUF), of which she is a Junior Member. Pilar Franco (Chiral Technologies Europe)
- 533 is warmly acknowledged for the gift of columns and for interesting discussions.
- 534

Compliance with ethical standards 535

The authors declare they have no conflict of interest. 536

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- 688
- 689

- 690 Figure captions
- Figure 1. Structures of the chiral selectors in the 11 stationary phases compared.
- 693

Figure 2. Hierarchical cluster analysis on the normalized retention data (normalized log k

- values) measured for the 161 analytes in Table S1 on the 11 stationary phases in Figure 1.
- 696 Retention dissimilarity in the abscissa reflects Euclidean distance between the stationary
- 697 phases. Chromatographic conditions: CO_2 -methanol 90:10 (v/v), 25°C, 15 MPa, 3 mL/min.
- 698

Figure 3. Coefficients of the normalized LSER models calculated with Equation (1) and the
retention data measured for the 161 achiral analytes in Table S1 for the eleven stationary
phases described in Figure 1. Chromatographic conditions: CO₂-methanol 90:10 (v/v), 25°C,
MPa, 3 mL/min.

703

704 Figure 4. Plots of logarithms of retention factors for the analytes in Table S1 on the 705 Chiralpak **ZWIX** columns compared to (a) an anion-exchange stationary phase (Chiralpak 706 QN-AX) and (b) a cation-exchange stationary phase (the in-house prepared SCXSS). Black 707 diamonds are hydrophobic analytes, open circles are polar non-ionizable analytes, red triangles are acidic compounds bearing a negative charge, blue squares are basic 708 709 compounds bearing a positive charge. The interrupted line is the first bisector indicating 710 identical retention values. The continuous grey line in (b) is showing the trend line between hydrophobic analytes. Chromatographic conditions: CO₂-methanol 90:10 (v/v), 25°C, 15 711 712 MPa, 3 mL/min.

713

Figure 5. Spider diagram based on the LSER models in Table 1 to observe the overall
selectivity of the eleven columns in Figure 1. The 36 numbered columns are identified in
Table S3. Chromatographic conditions: CO₂-methanol 90:10 (v/v), 25°C, 15 MPa, 3 mL/min.

Figure 6. Selected discriminant analyses between (a) early-eluting and late-eluting
racemates, (b) early eluting separated and non-separated racemates and (c) late-eluting
separated and non-separated racemates. Conditions: Chiralpak ZWIX(-),CO₂-methanol
90:10 (v/v), 25°C, 15 MPa, 3 mL/min, 58 racemates in Table S2. Negative features are
common to the analytes in the left group; positive features are common to the analytes in the
right group; zero features are non-discriminant.

724

Figure 7. Molecular modelling of the ligands and superimposition of them for useful

726 comparison.

727

- **Figure 8.** Chromatograms of (a) 5-Methyl-5-phenylhydantoin and (b) Alanine. Analytical
- conditions: Chiralpak ZWIX(+) (blue), Chiralpak QN-AX (red), QN (orange), QCISS (light
- green), QCITAU (dark green), SCXSS (black). Conditions (a) CO_2 -MeOH 90:10 (v/v), 25 °C,
- 150 bar, 3 mL/min, UV detection (210nm); (b) gradient elution from 10% to 100% co-solvent
- (methanol containing 50 mM of ammonium formate and 5 % water), 25 °C, 150 bar, 0.5
- 733 mL/min, ESI(+)-MS detection m/z = 90.

















Figure 6 (continued)







Table 1. system constants and statistics for the 11 columns tested obtained with Eq.(2)

n is the number of solutes finally retained in the multiple linear regression;

 R^2 is the determination coefficient; SE in the standard error in the estimate

Column	С	е	S	а	b	V	ď	d+	n	R^2_{adj}	SE
ZWIX(+)	-1.004	0.569	0.291	1.283	0.167	-0.241	0.046	0.190	126	0.876	0.190
	0.081	0.056	0.080	0.067	0.084	0077	0.058	0.086			
ZWIX(-)	-0.916	0.537	0.283	1.333	0.217	-0.240	0.049	0.176	127	0.899	0.163
	0.068	0.048	0.069	0.059	0.072	0.066	0.053	0.068			
QN-AX	-0.916	0.493	0.179	1.477	-0.055	-0.093	1.154	0.036	145	0.944	0.153
	0.059	0.039	0.060	0.054	0.067	0.036	0.067	0.063			
QD-AX	-0.873	0.482	0.187	1.396	-0.022	-0.089	1.073	0.024	143	0.941	0.155
	0.061	0.040	0.061	0.055	0.069	0.037	0.065	0.064			
QN	-1.132	0.484	0.119	1.229	0.081	0.009	1.205	0.108	150	0.930	0.159
	0.058	0.040	0.059	0.055	0.068	0.037	0.070	0.063			
QCISS	-1.154	0.527	0.141	1.508	0.218	-0.082	-0.094	0.038	150	0.930	0.151
	0.058	0.039	0.060	0.051	0.067	0.036	0.048	0.065			
QCDRR	-0.842	0.481	0.034	1.359	0.297	-0.074	0.260	-0.039	147	0.949	0.122
	0.047	0.032	0.049	0.042	0.056	0.029	0.044	0.053			
QCITAU	-1.079	0.518	0.236	1.438	0.174	-0.142	0.309	-0.044	148	0.935	0.158
	0.058	0.041	0.061	0.053	0.071	0.037	0.050	0.066			
QCDTAU	-1.014	0.490	0.184	1.339	0.185	-0.137	0.212	0.018	147	0.939	0.139
	0.052	0.036	0.054	0.047	0.062	0.033	0.044	0.058			
SCXRR	-1.482	0.554	0.144	0.928	0.658	-0.191	-0.260	1.799	145	0.851	0.215
	0.093	0.058	0.081	0.072	0.086	0.057	0.063	0.163			
SCXTAU	-1.374	0.566	0.242	0.997	0.657	-0.446	-0.202	1.531	134	0.874	0.217
	0.103	0.059	0.084	0.076	0.094	0.063	0.065	0.126			

	ZWIX+	ZWIX-	QN-AX	QD-AX	QN	QCISS	QCDRR	QCITAU	QCDTAU	SCXSS
Oxazepam	×	×	\checkmark	\checkmark	\checkmark	×	×	×	×	×
Mandelic acid, ethyl ester	\checkmark	\checkmark	✓	~	×	\checkmark	\checkmark	✓	\checkmark	×
5-methyl-5- phenylhydantoin	\checkmark	\checkmark	✓	~	✓	\checkmark	\checkmark	×	×	×
Suprofen	\checkmark	\checkmark	\checkmark	\checkmark	×	×	×	×	×	×
Warfarin	\checkmark	\checkmark	\checkmark	\checkmark	×	×	×	×	×	×
Alanine	\checkmark	\checkmark	×	×	×	×	×	×	×	×
Glutamine	✓	✓	×	×	×	×	×	×	×	×

Table 2. Examples of enantioseparations for selected racemates on the ten chiral columns studied.