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Adrien Raimbault, Cam Mai Anh Ma, Martina Ferri, Stefanie Bäurer, Pascal Bonnet, et al.. Cinchona-based 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-02904771v1>

Submitted on 7 Mar 2022

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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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18
19
20 **Abstract**

21
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

36 attractive and repulsive ionic interactions. Then the ZWIX phases were characterized for their
37 enantioseparation capabilities with a set of 58 racemic probes. Discriminant analysis was
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
41 favorable feature to enantio-recognition. On the opposite, the presence of negative charges in
42 the analyte favors early elution and enantio-recognition. Finally, a smaller set of 30 pairs of
43 enantiomers, selected by their structural diversity and different enantioseparation values on
44 the ZWIX phases, were analyzed on all chiral phases to observe the contribution of each
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,
47 notably the intra-molecular hydrogen bonding or the possible contribution of ionic
48 interactions. In the end, each structural element in the ZWIX phases appeared to be a
49 significant contributor to successful enantio-resolution, whether they contribute as direct
50 interaction groups (ion-exchange functions) or as steric constraints to orientate the
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
61 Lindner [2]. They are brush-type enantioselective stationary phases where the ligand is
62 derived from quinine and quinidine diastereomers bonded on silica gel. Recently, similar
63 phases bonded so superficially porous silica were proposed by Armstrong and co-workers
64 [3]. Because quinine and quinidine both possess a tertiary amine function (quinuclidine ring),
65 which should be protonated in most liquid and supercritical fluid mobile phases, a quinine- or
66 quinidine-based stationary phase should act as an anion-exchanger. One such phase is
67 presented in Figure 1 (**QN**). Two commercial stationary phases are available, which were
68 derived from quinine- and quinidine-bonded silica, with a carbamate function modifying the
69 hydroxyl group and retaining the anion-exchange capabilities: Chiralpak **QN-AX** and **QD-AX**
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
75 enantioseparation of free or derivatized amino acids and oligopeptides by HPLC [6–13].
76 *Cinchona* alkaloids and their derivatives were also employed as chiral selectors in capillary
77 electrophoresis [14,15] and capillary electrochromatography [16,17], where the high
78 efficiency values usually yield excellent resolution.

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
84 at the analytical and preparative scales. The *Cinchona*-based chiral phases were only rarely
85 employed in SFC. One possible reason is the wrong perception that ionic species cannot
86 exist in the apparently non-polar SFC mobile phase. However, it was proven in several
87 studies that ionic species exist in carbon dioxide – organic solvent mixtures and can even be
88 resolved [20,21] and that ionic interactions exist, which are measurable [4,22,23] and can be
89 modified when acidic and basic additives are introduced in the co-solvent [24]. Thus a few
90 papers relate the use of **ZWIX** phases to resolve N-protected amino acids [25,26] or indole-
91 type analytes [27]. Recently, we have demonstrated that elution and enantioresolution of a

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

94
95 Retention and enantioseparation mechanisms on the **ZWIX** phases were explored mostly in
96 liquid phase by the group of Lindner on the one hand [9,29], Ilisz and Péter on the other hand
97 [30], but only little was done in SFC conditions. Pell and Lindner showed that the ion-
98 exchange mechanism existed on **QN-AX** and **QD-AX** phases in SFC conditions, thanks to
99 the presence of methoxycarbonic acid formed by the reaction of CO₂ and acting as
100 counterion in the anion-exchange process [4]. This ion-exchange mechanism could be tuned
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
104 SFC separations on the **ZWIX** phases and proceeded to explain the differences [31]. A major
105 conclusion from this work is that mechanistic investigations done on the **ZWIX** phases in
106 HPLC conditions may not all be transferable to SFC conditions. Naturally, the components of
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
110 resulting in different conformations of the stationary phase.

111
112 To better understand the behavior of Chiralpak **ZWIX(+)** and **ZWIX(-)** in SFC, a fragment-
113 based approach to deciphering the complex enantioselective ligands was selected: nine
114 other (commercial or in-house) stationary phases were used as comparison. All nine phases
115 had some similarity to the **ZWIX** phases, each one having one less fragment than the parent
116 ligand. The purpose was to understand the contribution of each functional group in the
117 retention mechanisms and appreciate how each functional group was determinant in the
118 enantioseparation process. For this purpose, the eleven columns were first characterized by
119 means of quantitative structure-retention relationships (QSRR) with a modified version of the
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
125 differences in enantioresolution. Molecular modelling assisted in understanding the observed
126 differences.

127

128 2. Material and methods

129

130 2.1 Stationary phases

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

138

139 2.2 Chemicals and Solvents

140 161 achiral (Table S1) and 66 chiral molecules (Table S2) in their racemic form were
141 purchased from Sigma-Aldrich (Sigma Aldrich Chimie, France). The achiral analytes were
142 selected to provide a diverse representation of chemical functions to obtain meaningful
143 retention models with the solvation parameter model. The chiral probes in Table S2 were
144 selected to obtain meaningful information from the discriminant analyses with different
145 structures and shapes. These sets were fully described in previous papers [34,35]. The
146 reduced set of chiral analytes (Table S2, in bold) was selected as a subset of the wider set
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
151 VWR (Fontenay-sous-Bois, France). Ultra-pure water was supplied by an Elga UHQ system
152 from Veolia (Wissous, France). CO₂, with a purity of 99.995 %, was delivered by Air Liquide
153 (Paris, France). Ammonium formate was provided by Sigma Aldrich (Sigma Aldrich Chimie,
154 France).

155

156 2.3 Instruments and operating conditions

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

165 detection. An isocratic solvent manager was used as a make-up pump and was positioned
166 before the mass detector. The main flow stream was then split by the on-board flow-splitter
167 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
174 the pumping system. The UV detection wavelength was 210 nm while single-ion recording
175 was used for MS detection of amino acids according to the mass of the protonated molecular
176 ion.

177 178 2.4 Data analysis

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
184 discriminant analysis (DA)) were done with XLSTAT 19.03 software (Addinsoft, New York,
185 NY, USA). The retention data (log *k* values) for achiral analytes were normalized (centred
186 and reduced to adjust mean value on 0 and standard deviation on 1) prior to HCA
187 calculation, to ensure that equal significance would be associated to each analyte. HCA was
188 based on Ward aggregation method with Euclidean distance.

189 The quality of QSRR fits was estimated using the adjusted determination coefficient (R^2_{adj}),
190 standard error in the estimate (*SE*) and Fisher *F* statistic. The statistical significance of
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:

$$195
196 \log k = c + eE + sS + aA + bB + vV + d^-D^- + d^+D^+ \quad (1)$$

197
198 In this equation, capital letters represent the solute descriptors, related to particular
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

202 ratio. E is the excess molar refraction (calculated from the refractive index of the molecule)
203 and models polarizability contributions from n and π electrons; S quantifies the presence of
204 dipoles and polarizability; A and B are the solute overall hydrogen-bond acidity and basicity;
205 V is the McGowan characteristic volume in units of $\text{cm}^3 \text{mol}^{-1}/100$; D^- and D^+ are the solute
206 negative and positive charges respectively, calculated with the pK_a and apparent pH of the
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
209 introduced more recently to characterize hydrophilic interaction liquid chromatography
210 (HILIC) systems [36], and were later demonstrated to be suitable to describe SFC systems,
211 provided we admit to some approximations [22,23]. The system constants (e , s , a , b , v , d^- ,
212 d^+), obtained through a multilinear regression of the retention data for a certain number of
213 solutes with known descriptors, reflect the magnitude of difference for that particular property
214 between the mobile and stationary phases. Thus, if a particular coefficient is numerically
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
218 characteristics of different stationary phases and enable the identification of stationary
219 phases of similar or dissimilar selectivity.

220 In previous works, we have demonstrated that two other structural features that contributed
221 little to retention were however significant to describe enantioseparation mechanisms on
222 polysaccharide or macrocyclic 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
224 in Equation (1) but appeared to be insignificant to explain retention. They were however
225 retained for the evaluation of chiral separations through discriminant analysis. The quality of
226 discriminant analysis was estimated based on ROC (receiver operating characteristics)
227 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
235 conformational search using LowModeMD with default parameters. The MMFF94x force field
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
238 4.0 Visualizer (Accelrys, San Diego, California, USA).

239

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 **QCRRR** ligands,
249 respectively. The steric constriction of the sulfonic group was further removed to yield
250 **QCITAU** and **QCDAU**, based on quincorine (QCI) / quincoridine (QCD) and taurine (TAU).
251 Finally, the cationic quincoridine group was removed from **QCRRR** and **QCDAU** 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 $\mu\text{mol/g}$ (within $\pm 30 \mu\text{mol/g}$) 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

265

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**, **QCDDR** and **QCDDTAU**.
- 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 **QCDDR**; **QCITAU** and **QCDDTAU**.

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 (*b* 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*⁺) 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**, **QCDDR**, **QCITAU** and **QCDDTAU**) also have

312 rather small ionic interaction terms. This would tend to indicate that the cationic and anionic
313 groups in the ligands must be associated to each other, thereby reducing their availability for
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**)
316 while the d^+ term, reflecting retention of cationic compounds, is large and positive on the
317 cation-exchange stationary phases (**SCXSS** and **SCXTAU**). In addition, the d^+ term,
318 reflecting retention of cationic compounds, is reduced on the anion-exchange stationary
319 phases while the d' term, reflecting retention of anionic compounds, is large and negative on
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
323 contrary, the phases possessing a single ionic function, whether it is cationic or anionic,
324 display strong ionic interactions. This is consistent with previous observations in HPLC that
325 the sulfonic group “mostly act as intramolecular counterions leading to reduced run times
326 compared to parent anion exchangers” [5]. Another interesting explanation for the moderate
327 values of d' and d^+ terms may be found in the electrostatic attraction-repulsion (push-and-
328 pull) mechanism recently proposed by Mimini *et al.* [41]. Basically any anionic analyte should
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
331 repulsion could explain the overall moderate retention of ionic species on the **ZWIX** phases.
332 In other words, the two ionic functions in the chiral ligand partly cancel each other.
333 Another interesting feature is that the v term, reflecting the effect of molecular volume on
334 retention, is negative on all stationary phases but most significant on the **ZWIX** phases. The
335 negative terms indicate that an increase in hydrocarbon volume is causing decreased
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
345 Compared to **ZWIX** phases, the four other zwitterionic columns **QCISS**, **QCRRR**, **QCITAU**
346 and **QCDAU** are lacking a quinoline group, and two of them are lacking a butyl linker that is
347 constraining the sulfonic group through a cyclohexyl function.

348 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
350 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.

354 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
356 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
359 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
362 donors and acceptors (a and b terms respectively) and interactions with dipoles and
363 polarizable species (s and e terms respectively) are observed for the 3 phases possessing
364 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
370 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
373 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
376 reflecting the overall selectivity of the phases (Figure 5). This figure was previously
377 developed to allow easy comparison of a large number of stationary phases based on the
378 system constants from LSER characterization [23,43]. Quite simply, the figure is a projection
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
382 ionic interactions contribute significantly to scattering the stationary phases. Bubble size is
383 related to the overall strength of interactions (length of the solvation vector). To better
384 appreciate the diversity of selectivities provided by the eleven phases, 36 other stationary

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

387

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
392 zwitterionic phases are in-between. Secondly, all eleven phases are singular (not
393 superimposed with any other in the figure) but have some similarity to other phases. The
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 quinoline 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
399 pyridine groups in their bonded ligands (# 21, 24 and 25) are all at the bottom of the figure,
400 close to the zwitterionic and anion-exchange phases characterized in this work.

401

402

403 3.4 Enantioseparation mechanism examined with discriminant analysis

404

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 enantio recognition [37–39].
408 Basically, discriminant analysis is applied to a diverse selection of racemates (58 in the
409 present case) analyzed in a single chromatographic system (one stationary phase and one
410 mobile phase composition). The probe molecules are characterized by molecular descriptors
411 and by the chromatographic result that can be simplified in two categories: separated
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.
415 Thus a discriminant analysis including the whole set of analytes usually results in poor
416 statistics, indicating that the models obtained are not relevant. To improve the significance of
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
419 model (Eq. (1)). In most cases, the two enantiomers both elute earlier than the prediction or
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
423 enantiorecognition processes: flexibility (F) and globularity (G). The other descriptors are the
424 same 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
429 between a multi-functional analyte and the multi-functional ligand. The favorable contribution
430 of positive charges may be related to better accessibility of the anionic sulfonic group than
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
434 **ZWIX(-)** ligand in Figure 7, where the quinuclidine group, being closer to the silica surface,
435 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
439 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
442 analytes, which should be rather rigid and not cationic, the presence of a negative charge is
443 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
445 group (quinuclidine function) and with the quinoline and/or carbamate functions must
446 contribute favorably to enantiorecognition. Late-eluting molecules are mostly cationic and
447 flexible, but these features are not favorable to enantiorecognition for this group (negative F
448 and D^+ terms).

449 In both cases (early and late-eluting), globularity is highly significant (positive G term) while
450 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

458 A subset of the 58 racemates was selected for structural diversity: 22 molecules (written in
459 bold in Table S2) representing different analyte classes and with different separation features
460 on the **ZWIX** phases (resolved or nor resolved) were chosen. Additionally, 8 free amino acids
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
463 **SCXTAU**). Some representative examples are shown in Table 2. Only racemates that could
464 be resolved on one stationary phase at least are presented in this table because the
465 racemates that cannot be (at least partially) resolved on any stationary phase provide little
466 information on enantiorecognition mechanisms.

467
468 One example of the contribution of shape is that of benzodiazepines. For instance,
469 Oxazepam is a large and rather flat molecule. Because of its rigidity, it was eluted rather
470 earlier than predicted but its flatness is probably responsible for the lack of enantioresolution
471 on the **ZWIX** phases. Some enantioselectivity was however observed on the **QN-AX** and
472 **QD-AX** phases. Molecular modelling allows observing the different three-dimensional
473 orientation of each CSP ligand: the folding of each ligand is highly dependent on intra-
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
476 **QN-AX** and **QD-AX** should be more open, less sterically constrained than the structure of
477 **ZWIX** ligands. The replacement of the large cyclohexylsulfonic group by a smaller t-butyl
478 group should make it easier for a large molecule to adapt to the chiral selector.

479
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
482 in Figure 8a. Resolution was also possible on the anion-exchange phases (**QN-AX**, **QD-AX**
483 and **QN**), and it was also possible on the two phases lacking the quinoline group but
484 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 **QCDAU**).
486 Clearly, the orientation of the sulfonic group, forced by the presence of the cyclohexyl group,
487 is favorable to enantioselectivity. This observation can be contrasted to previous
488 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].
491 Acidic compounds and proton-rich compounds were generally well resolved on **ZWIX** phases
492 and anion exchangers, while no resolution could be obtained on the other columns. Only the
493 anion-exchange phases were also favorable to resolution of acids.
494 Enantioselectivity 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
497 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
499 enantioselectivity 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
502 is generally recognized as an unfavorable feature.
503 The simplest ligand in **SCXSS** was totally incapable of resolving any of the pairs of
504 enantiomers in this set. Clearly, the simple chirality borne by the cyclohexylsulfonic ligand is
505 not 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 enantioselectivity 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

515 **4. Conclusion**

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

527

528

529 **Acknowledgments**

530 Adrien Raimbault is grateful for a PhD grant received from the Ministry of Higher Education
531 and Research. Caroline West is grateful for the support received by the Institut Universitaire
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

535 **Compliance with ethical standards**

536 The authors declare they have no conflict of interest.

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- 688
- 689

690 **Figure captions**

691

692 **Figure 1.** Structures of the chiral selectors in the 11 stationary phases compared.

693

694 **Figure 2.** Hierarchical cluster analysis on the normalized retention data (normalized log k
695 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: CO₂-methanol 90:10 (v/v), 25°C, 15 MPa, 3 mL/min.

698

699 **Figure 3.** Coefficients of the normalized LSER models calculated with Equation (1) and the
700 retention data measured for the 161 achiral analytes in Table S1 for the eleven stationary
701 phases described in Figure 1. Chromatographic conditions: CO₂-methanol 90:10 (v/v), 25°C,
702 15 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
708 triangles are acidic compounds bearing a negative charge, blue squares are basic
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
711 hydrophobic analytes. Chromatographic conditions: CO₂-methanol 90:10 (v/v), 25°C, 15
712 MPa, 3 mL/min.

713

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

717

718 **Figure 6.** Selected discriminant analyses between (a) early-eluting and late-eluting
719 racemates, (b) early eluting separated and non-separated racemates and (c) late-eluting
720 separated and non-separated racemates. Conditions: Chiralpak ZWIX(-), CO₂-methanol
721 90:10 (v/v), 25°C, 15 MPa, 3 mL/min, 58 racemates in Table S2. Negative features are
722 common to the analytes in the left group; positive features are common to the analytes in the
723 right group; zero features are non-discriminant.

724

725 **Figure 7.** Molecular modelling of the ligands and superimposition of them for useful
726 comparison.

727

728 **Figure 8.** Chromatograms of (a) 5-Methyl-5-phenylhydantoin and (b) Alanine. Analytical
729 conditions: Chiralpak ZWIX(+) (blue), Chiralpak QN-AX (red), QN (orange), QCISS (light
730 green), QCITAU (dark green), SCXSS (black). Conditions (a) CO₂-MeOH 90:10 (v/v), 25 °C,
731 150 bar, 3 mL/min, UV detection (210nm); (b) gradient elution from 10% to 100% co-solvent
732 (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.

734

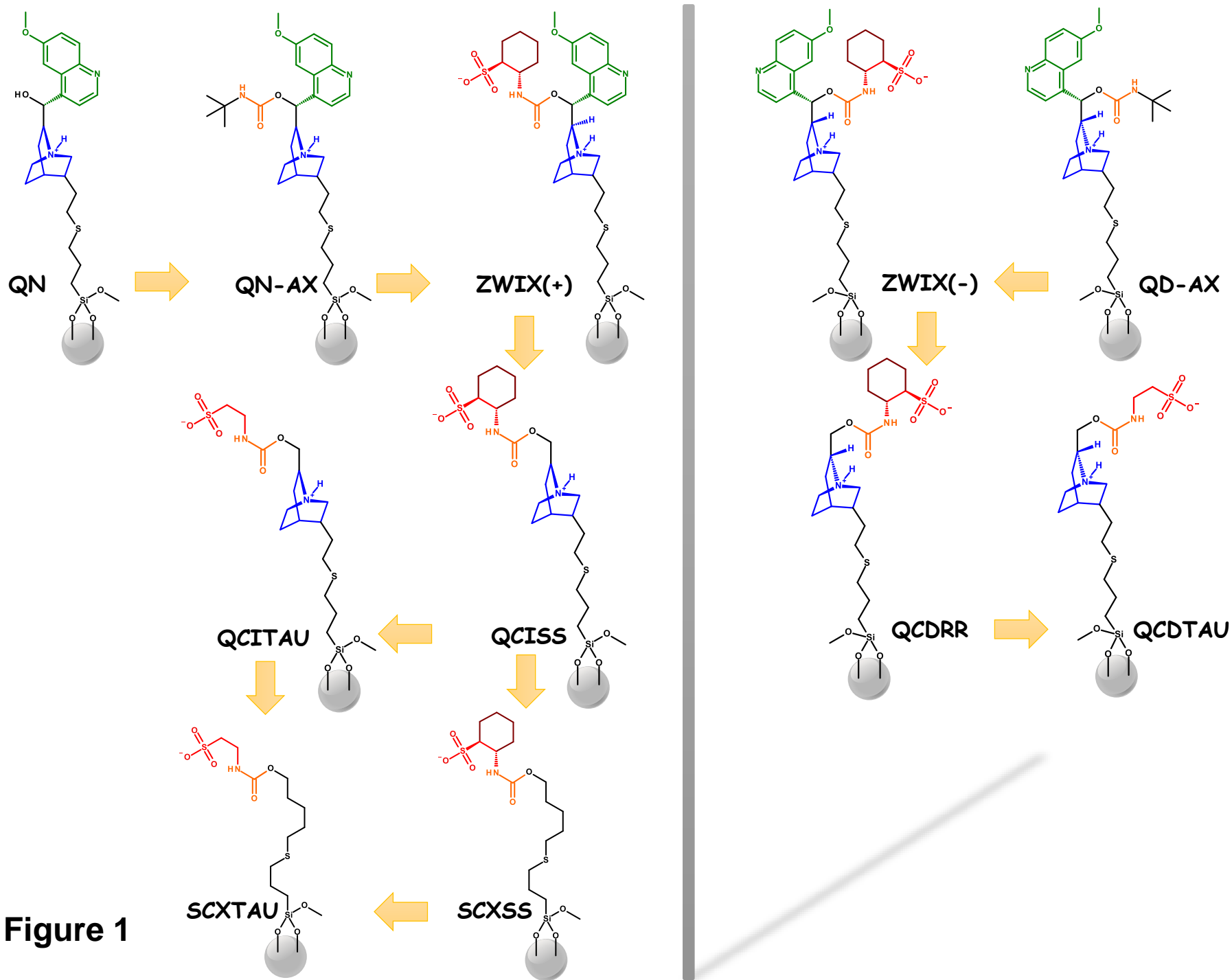


Figure 1

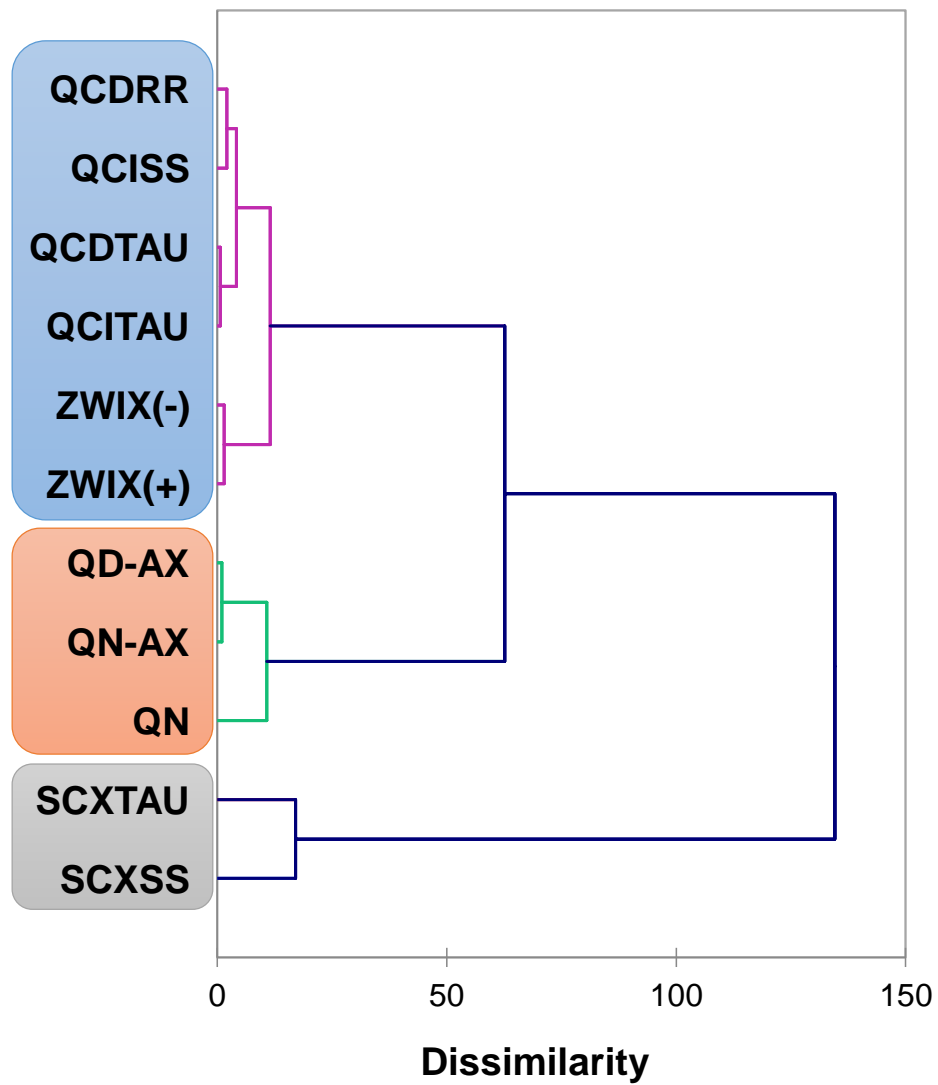


Figure 2

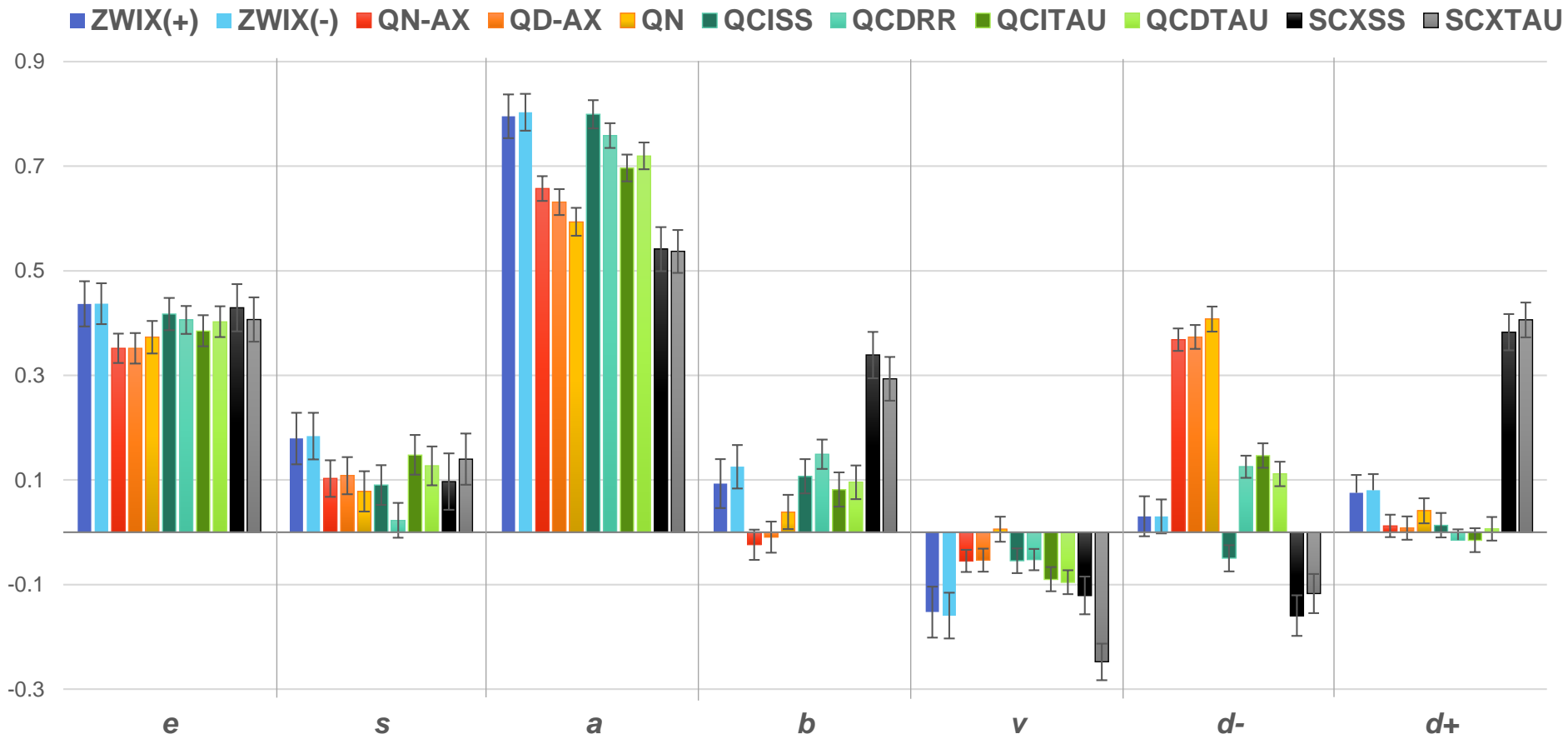


Figure 3

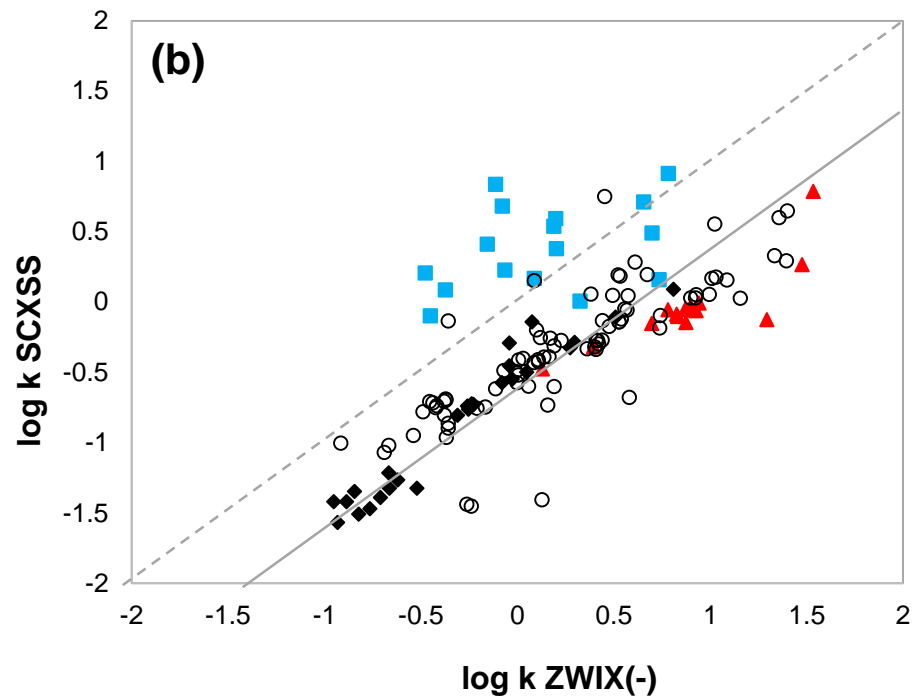
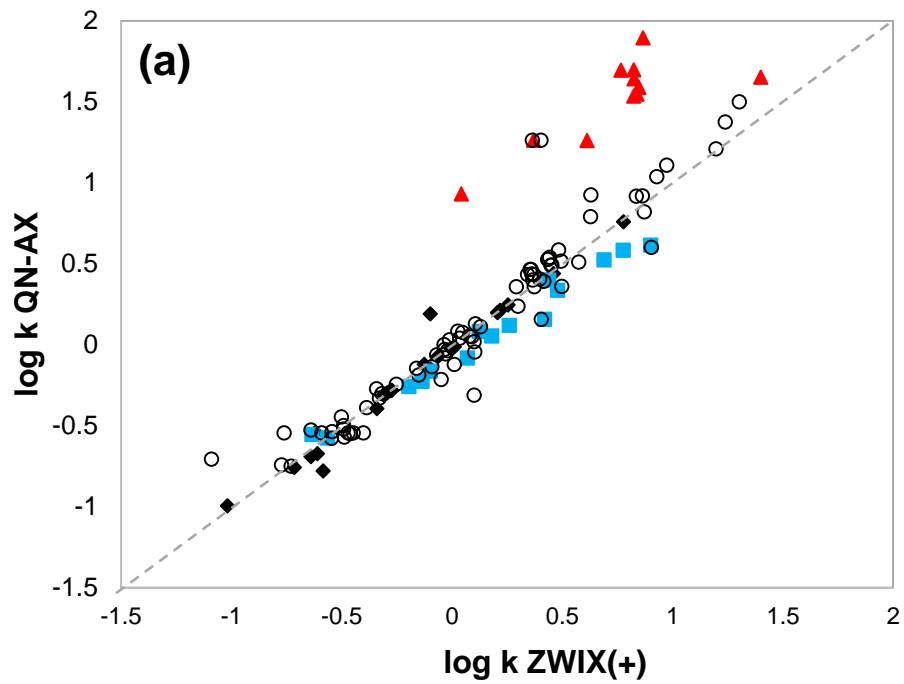


Figure 4

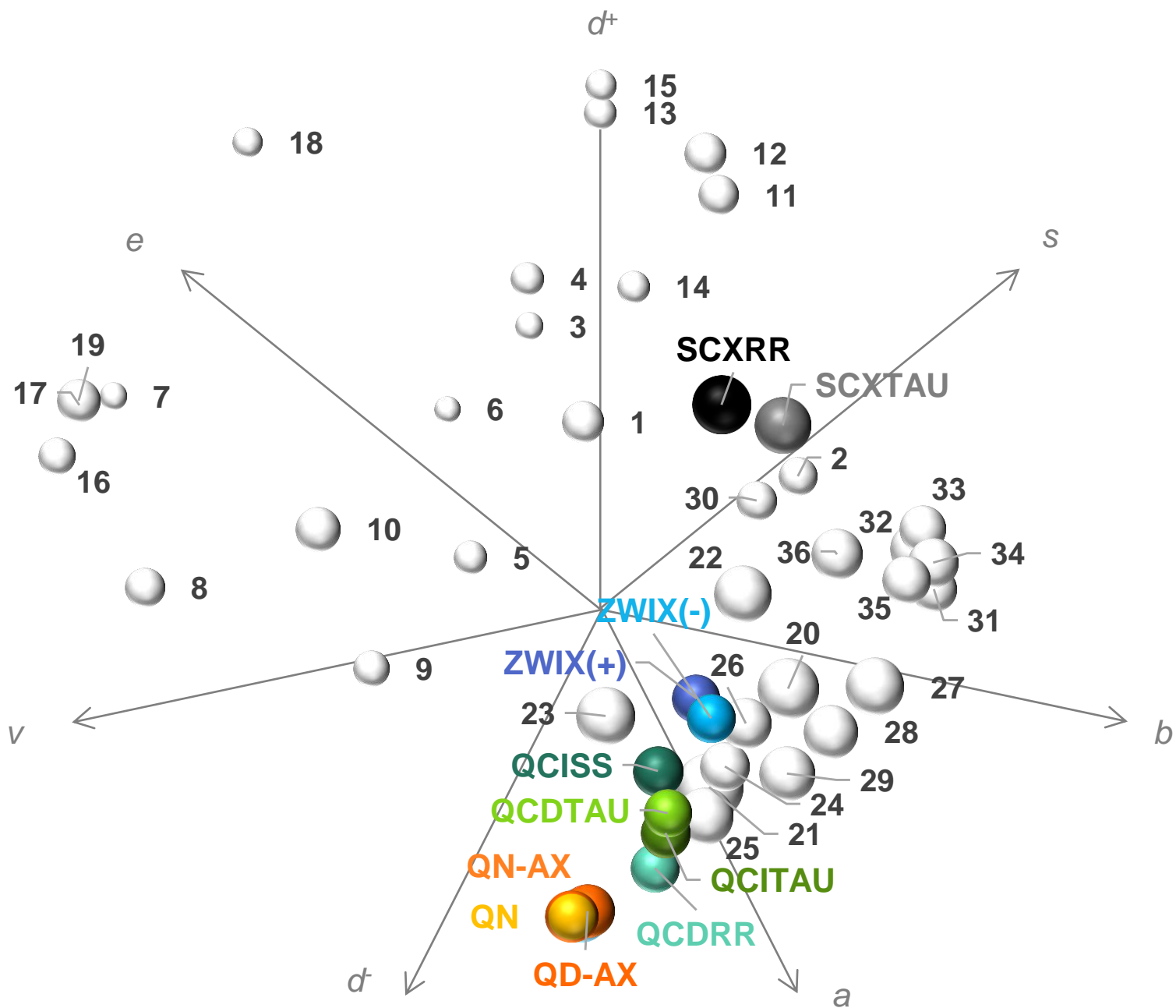


Figure 5

(a) Chiralpak ZWIX(-)
Eluted before or after prediction

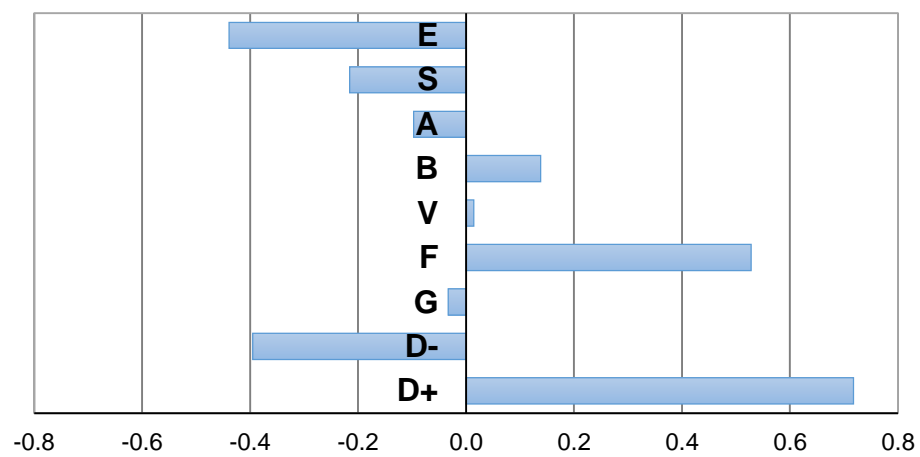
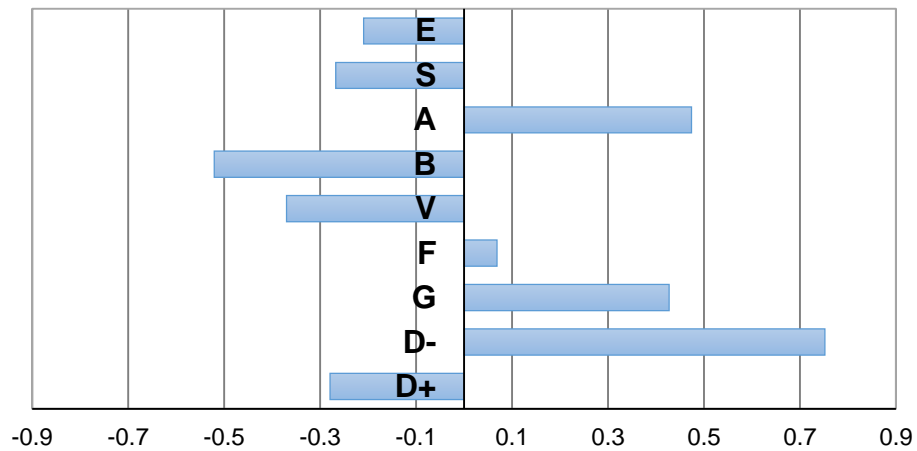


Figure 6

(b) Chiralpak ZWIX(-) - Early eluting
Non-separated Separated



(c) Chiralpak ZWIX(-) - Late eluting
Non-separated Separated

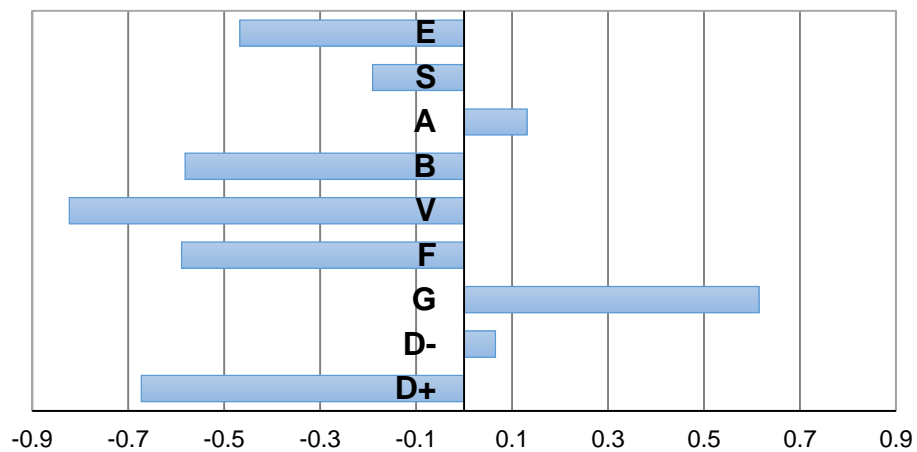
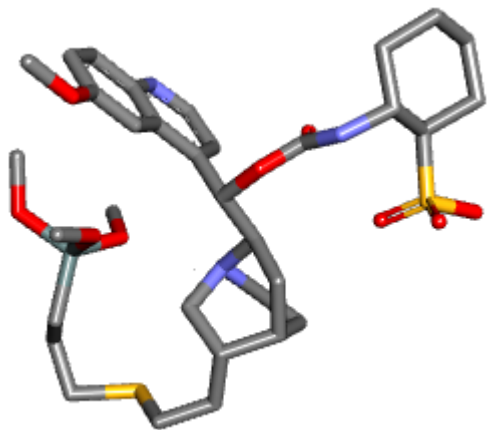
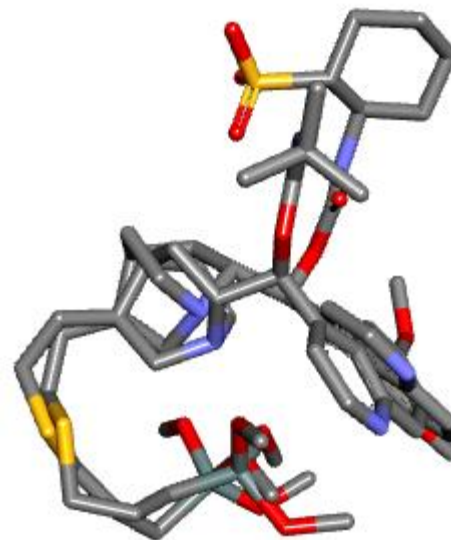


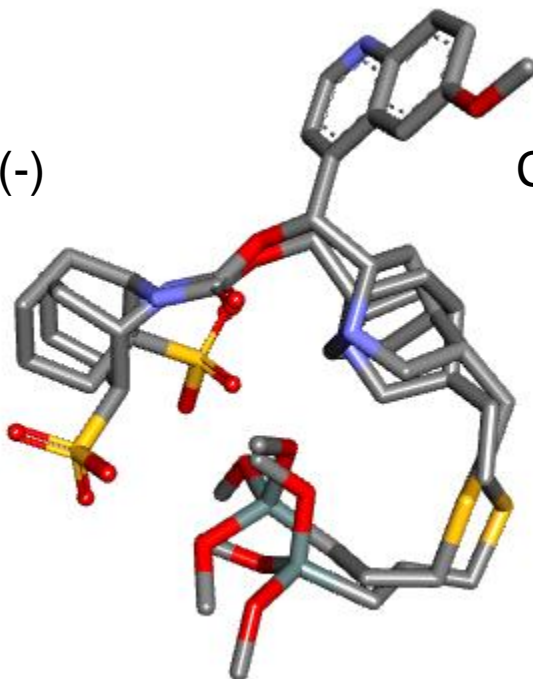
Figure 6 (continued)



Chiralpak ZWIX(-)



Chiralpak ZWIX(-) and QD-AX



Chiralpak ZWIX(+) and QCISS

Figure 7

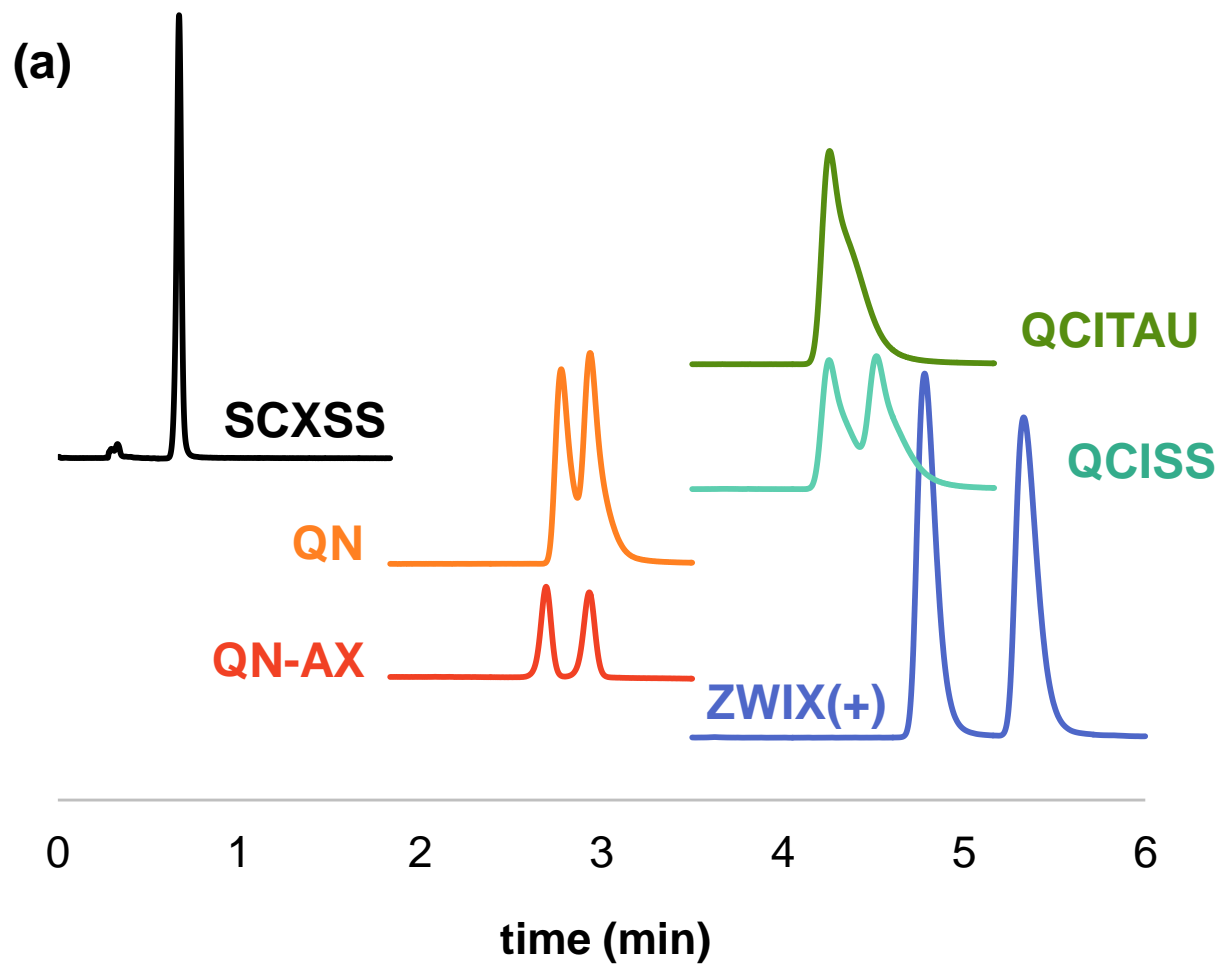


Figure 8

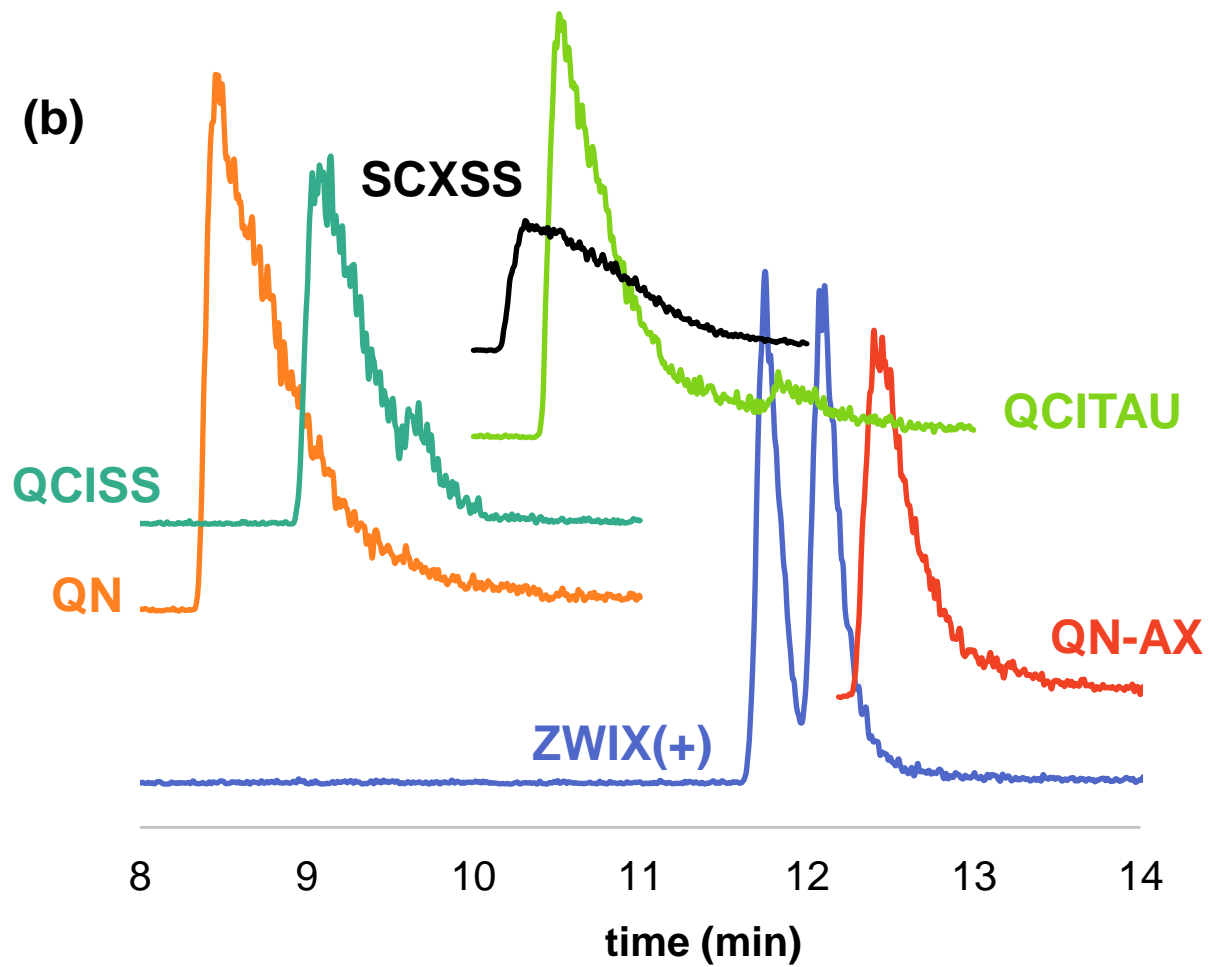


Figure 8

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;

R² is the determination coefficient; SE in the standard error in the estimate

| Column | c | e | s | a | b | v | d⁻ | d⁺ | n | R²_{adj} | SE |
|----------------|------------------------|-----------------------|-----------------------|-----------------------|------------------------|------------------------|------------------------|------------------------|----------|------------------------------------|-----------|
| ZWIX(+) | -1.004 <i>0.081</i> | 0.569 <i>0.056</i> | 0.291 <i>0.080</i> | 1.283 <i>0.067</i> | 0.167 <i>0.084</i> | -0.241 <i>0.077</i> | 0.046 <i>0.058</i> | 0.190 <i>0.086</i> | 126 | 0.876 | 0.190 |
| ZWIX(-) | -0.916 <i>0.068</i> | 0.537 <i>0.048</i> | 0.283 <i>0.069</i> | 1.333 <i>0.059</i> | 0.217 <i>0.072</i> | -0.240 <i>0.066</i> | 0.049 <i>0.053</i> | 0.176 <i>0.068</i> | 127 | 0.899 | 0.163 |
| QN-AX | -0.916 <i>0.059</i> | 0.493 <i>0.039</i> | 0.179 <i>0.060</i> | 1.477 <i>0.054</i> | -0.055 <i>0.067</i> | -0.093 <i>0.036</i> | 1.154 <i>0.067</i> | 0.036 <i>0.063</i> | 145 | 0.944 | 0.153 |
| QD-AX | -0.873 <i>0.061</i> | 0.482 <i>0.040</i> | 0.187 <i>0.061</i> | 1.396 <i>0.055</i> | -0.022 <i>0.069</i> | -0.089 <i>0.037</i> | 1.073 <i>0.065</i> | 0.024 <i>0.064</i> | 143 | 0.941 | 0.155 |
| QN | -1.132 <i>0.058</i> | 0.484 <i>0.040</i> | 0.119 <i>0.059</i> | 1.229 <i>0.055</i> | 0.081 <i>0.068</i> | 0.009 <i>0.037</i> | 1.205 <i>0.070</i> | 0.108 <i>0.063</i> | 150 | 0.930 | 0.159 |
| QCISS | -1.154 <i>0.058</i> | 0.527 <i>0.039</i> | 0.141 <i>0.060</i> | 1.508 <i>0.051</i> | 0.218 <i>0.067</i> | -0.082 <i>0.036</i> | -0.094 <i>0.048</i> | 0.038 <i>0.065</i> | 150 | 0.930 | 0.151 |
| QCDDR | -0.842 <i>0.047</i> | 0.481 <i>0.032</i> | 0.034 <i>0.049</i> | 1.359 <i>0.042</i> | 0.297 <i>0.056</i> | -0.074 <i>0.029</i> | 0.260 <i>0.044</i> | -0.039 <i>0.053</i> | 147 | 0.949 | 0.122 |
| QCITAU | -1.079 <i>0.058</i> | 0.518 <i>0.041</i> | 0.236 <i>0.061</i> | 1.438 <i>0.053</i> | 0.174 <i>0.071</i> | -0.142 <i>0.037</i> | 0.309 <i>0.050</i> | -0.044 <i>0.066</i> | 148 | 0.935 | 0.158 |
| QCDAU | -1.014 <i>0.052</i> | 0.490 <i>0.036</i> | 0.184 <i>0.054</i> | 1.339 <i>0.047</i> | 0.185 <i>0.062</i> | -0.137 <i>0.033</i> | 0.212 <i>0.044</i> | 0.018 <i>0.058</i> | 147 | 0.939 | 0.139 |
| SCXRR | -1.482 <i>0.093</i> | 0.554 <i>0.058</i> | 0.144 <i>0.081</i> | 0.928 <i>0.072</i> | 0.658 <i>0.086</i> | -0.191 <i>0.057</i> | -0.260 <i>0.063</i> | 1.799 <i>0.163</i> | 145 | 0.851 | 0.215 |
| SCXTAU | -1.374 <i>0.103</i> | 0.566 <i>0.059</i> | 0.242 <i>0.084</i> | 0.997 <i>0.076</i> | 0.657 <i>0.094</i> | -0.446 <i>0.063</i> | -0.202 <i>0.065</i> | 1.531 <i>0.126</i> | 134 | 0.874 | 0.217 |

