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1 **Effects of high concentrations of mobile phase additives on retention and separation**
2 **mechanisms on a teicoplanin aglycone stationary phase in supercritical fluid**
3 **chromatography**

4

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11

12 **Abstract**

13

14 The objective of this study is to understand the behavior of a peptide in a medium containing
15 supercritical carbon dioxide mixed with an alcohol (methanol) and acidic or basic additives in
16 uncommonly high concentrations. Chirobiotic TAG is a chromatographic column made of
17 silica bonded with a macrocyclic peptide, teicoplanin aglycone. With this stationary phase,
18 two additives (trifluoroacetic acid and isopropylamine) were tested under extreme
19 concentration conditions to observe the behavior of this peptide. Indeed, concentrations
20 exceeding 1 M in the methanol co-solvent (>0.1 M overall concentration in the CO₂-methanol
21 mixture) were used whereas usual additive concentrations employed in supercritical fluid
22 chromatography (SFC) rarely exceed 50 mM in the co-solvent. One purpose was to modify
23 the apparent pH of the fluid, which is normally slightly acidic (around 5) and consequently
24 possibly changing the ionization state of the peptide. Firstly, the effect of acidic and basic
25 additives on the polarity and the apparent pH were evaluated with the help of color
26 indicators. This served to assess the ionization state of the peptide under the selected
27 operating conditions. Secondly, 54 achiral and 24 chiral molecules were injected in the
28 chromatographic column at different levels of additives. The achiral species served at
29 establishing retention models based on linear solvation energy relationships (LSER), while
30 the chiral species were examined for their enantioresolution. From the LSER equations and
31 observation of chromatograms, it appeared that specific interactions between the peptide-
32 based stationary phase and the analytes evolved when increasing the concentration of
33 additives, particularly hydrogen bonds and ionic interactions. A bare silica stationary phase
34 (Acquity BEH) served as reference to deconvolute the contributions of silica support and
35 bonded peptide. This study, with these extreme conditions of mobile phase, could be useful
36 to understand the behavior of such peptides in SFC mobile phases and also improve the
37 knowledge of the effects of additives in SFC, which should be helpful in the future prospect of
38 analyzing large biomolecules in SFC.

39

40 **Keywords**

41 Additives; Linear solvation energy relationships; Mobile phases; Peptides; Solvation
42 parameter model; Supercritical fluid chromatography

43

44

1. Introduction

Supercritical Fluid Chromatography (SFC) has faced a renewed interest in recent years [1,2], thanks to the introduction of modern analytical systems [3] and because it is considered as a green analytical technique with a low consumption of solvents, thereby producing little waste. Indeed, the main solvent employed as mobile phase is pressurized carbon dioxide (CO₂) which is easily renewable and has a low toxicity. SFC was previously mainly used for chiral applications [4–7], especially at the preparative scale in the pharmaceutical industry. Currently, more and more achiral and analytical-scale applications are being developed with this technique [1]. In particular the analysis of biomolecules is raising expectations. While several recent papers have demonstrated the feasibility of eluting small polar biomolecules like nucleotides or nucleosides [8–12], the elution of large biomolecules was only rarely shown. Lipophilic peptides and proteins were analyzed with SFC in a few occasions [13–15]. Hydrophilic peptides and proteins were explored recently with SFC [16,17] or enhanced-fluidity liquid chromatography (EFLC) in the hydrophilic interaction liquid chromatographic mode (HILIC) [18]. EFLC typically employs a larger portion of liquid solvent than is usually done in SFC, and this liquid solvent often contains a significant percentage of water. The question of peptides and proteins solubility and stability in a typical SFC mobile phase, where the portion of liquid solvent remains moderate (typically inferior to 50%), is thus still vacant [17].

This work aims at understanding the behavior of free (non-derivatized) peptides when they are placed in typical SFC mobile phases, i.e. a mobile phase made of a pressurized CO₂-alcohol mixture. In addition, the effect of acidic and basic additives introduced in the methanol co-solvent is of interest. While the effects of additives in SFC mobile phases have long been explored [19–23], they are still not fully understood. It is generally accepted that they adsorb on the stationary phase (like any other mobile phase component), thereby changing the surface in contact with analytes [24], may suppress ionization [20] and/or form ion pairs with ionic or ionizable analytes [16,25]. Typical concentrations used in current SFC practice range in the 1-50 mM or 0.1-1% in the alcohol co-solvent. The behavior of a CO₂-alcohol mixture with higher concentrations of additive has only rarely been explored in SFC. A recent study from Speybrouck and co-workers explored the effects of large concentrations of additive (10% isopropylamine) on enantioresolution of drug-like molecules with polysaccharide stationary phases [26]. Unusual effects were observed like reversals of elution order of the enantiomers, suggesting that the retention mechanism had been deeply modified.

There may be two different ways to study the behavior of a molecule in a fluid with chromatography. The first method is to directly inject this molecule on any column with

82 different mobile phases to control the evolution of its retention under several conditions. The
83 second approach is to study this molecule as a stationary phase (coated or bonded on a
84 support) and to inject known molecules in varied conditions, to observe how the interactions
85 that the target molecule (as stationary phase) is able to do vary with the operating conditions.
86 The latter option was selected in the present study, with the use of a commercial
87 chromatographic column packed with silica particles bonded with a macrocyclic peptide. The
88 chosen column was Chirobiotic TAG based on teicoplanin aglycone, whose structure is
89 presented in Fig.1. It is a complex macrocyclic peptide possessing four cavities within its
90 structure. This stationary phase is normally employed as a chiral selector, where each of
91 these four cavities may participate in the enantiorecognition process. Note that the structure
92 presented in Figure 1 is not the exact structure of the stationary phase, as the TAG molecule
93 is bonded to silica gel, probably through the amine function [27], and perhaps also through
94 the phenolic groups [28]. Teicoplanin, which has 3 additional sugar units relative to the
95 aglycone molecule, is an antibiotic produced by the fermentation of *Actinoplanes*
96 *teicomyceticus*. While teicoplanin-bonded silica was first introduced as a chiral selector [29],
97 it was later modified to remove the glycosyl groups and obtain different enantiorecognition
98 capabilities [30]. This column was already cited in many studies in HPLC, notably for
99 pharmaceutical applications [31–33], and sometimes in SFC [34–37]. In addition, although it
100 is an enantioselective stationary phase, it may also be useful to the analysis of achiral
101 molecules [38].

102 The TAG structure comprises two free ionizable functions: a carboxylic acid function and a
103 primary amine function. The latter may however serve as an attachment to the silica support
104 and may then be converted to a urea function. However, the three-step bonding protocol
105 employed to prepare the stationary phase [30] also involves modifying the silica surface with
106 amino-propyl groups prior to bonding of the peptide. It is therefore likely that amine groups
107 also remain on the silica surface, along with possible residual silanol groups. All these
108 ionizable functions will have different ionization states depending on the mobile phase pH
109 and would therefore contribute differently to the interaction capabilities. This in turn would
110 affect retention and possibly enantiorecognition of the analytes injected in this column.
111 Similarly, the acid and amine ionizable functions would be present in all linear and non-
112 derivatized peptides as well as in free amino acids, and their ionization state would have a
113 significant influence on their solubility in the mobile phase fluid and on their retention
114 properties in any stationary phase.

115 It is likely that large concentrations of acidic or basic additives in SFC could affect the
116 ionization state of biomolecules like peptides, thereby impacting their solubility in the CO₂-
117 alcohol mobile phase and their interaction properties, whether they are immobilized on a
118 stationary phase or injected as analytes. One objective of the present paper was thus to

119 assess whether the ionization state of a peptide could be manipulated through the
120 concentration of acidic or basic additives.

121 Two additives were tested: trifluoroacetic acid (TFA) and isopropylamine (IPA) at different
122 concentrations in methanol mixed to 90% pressurized CO₂. These additive concentrations
123 were pushed to the extreme to go above 1 M in methanol, representing an overall
124 concentration greater than 100 mM in the mobile phase. Mathematical models based on
125 Linear Solvation Energy Relationships (LSER) [39,40] were used to model the possible
126 interactions between probe molecules and the peptide stationary phase to better understand
127 the evolution of these interactions depending on the nature and concentration of additives in
128 the mobile phase. A modified version of the solvation parameter model, comprising five
129 Abraham descriptors [41], two additional descriptors to account for ionic interactions [42], and
130 two more descriptors that were previously developed to characterize enantioselective
131 stationary phases [43], was employed. These models were made following the injection of 54
132 achiral molecules under 11 different mobile phase conditions. 24 racemates were also
133 analyzed in order to see the evolution of enantioseparation with the presence of additives. A
134 silica stationary phase (Acquity BEH) served as reference material to deconvolute the effects
135 related to the silica support or the bonded peptide.

136

137

138 **2. Material and Methods**

139

140 **2.1. Chemicals and Solvents**

141 Nile Red, Thymol Blue, Bromocresol Green, Methyl Red, Bromocresol Purple and
142 Bromothymol Blue were provided by Sigma-Aldrich (Sigma Aldrich Chimie, France). Their
143 structures and UV-visible spectra can be observed in supplementary material (Fig. S1 and
144 S2). Solutions were prepared in methanol. The colors and aqueous pK_a values associated to
145 each acid-base pair are presented in Fig. 2.

146 54 achiral and 24 chiral molecules were purchased from Sigma Aldrich. The complete list of
147 analytes together with molecular descriptors can be found in electronic supplementary
148 information (Tables S1 for achiral probes and S2 for chiral probes). Although the set may be
149 considered rather small, the achiral molecules were selected so as to provide a good
150 distribution of properties to achieve meaningful LSER description, according to the
151 recommendations of Vitha and Carr [44]. As a rule of thumb dictates that a minimum of three
152 analytes per variable should be employed, 54 adequately chosen analytes should be largely
153 sufficient to account for the effects of 9 variables. Descriptors for the chiral molecules are
154 also indicated for information although they did not serve to establish models but only to
155 assess the structural variance among this set. Solutions of all analytes were prepared in

156 methanol. Ultra-pure water was supplied by an Elga UHQ system from Veolia (Wissous,
157 France). The HPLC-grade methanol used as the mobile phase was supplied by VWR
158 (Fontenay-sous-Bois, France). CO₂, with a purity of 99.995 %, was delivered by Air Liquide
159 (Paris, France). Isopropylamine (IPA) and trifluoroacetic acid (TFA) were provided by Sigma
160 Aldrich (Sigma Aldrich Chimie, France).

161

162 2.2 Instruments and stationary phases

163 All LSER experiments were carried out on two columns: Chirobiotic TAG column (150 x 4.6
164 mm; 5 μm), provided by Merck and Acquity UPC² BEH (100 x 3.0 mm; 1.7 μm) provided by
165 Waters, that served for comparison purposes. The charge state of teicoplanin aglycon
166 depending on mobile phase pH was calculated based on aqueous pK_a values determined
167 with Chemicalize program (<http://www.chemicalize.org/>).

168 The supercritical fluid chromatography system used was an ACQUITY Ultra Performance
169 Convergence Chromatography™ (UPC²®) from Waters Corporation (Millford, MA, USA). It
170 was equipped with a binary solvent delivery pump compatible with mobile phase flow rates
171 up to 4 mL/min and pressures up to 414 bar, an autosampler that included partial loop
172 volume injection system, a back-pressure regulator, a column oven compatible with 150 mm
173 length columns and a photodiode-array (PDA) detector. Empower® 3 (Waters Corporation)
174 was used to process the chromatograms.

175

176 2.3 Analytical methods

177 The mobile phase was a mixture of 90% CO₂ and 10% MeOH, comprising additive or not.
178 Two additives commonly employed in chiral SFC, an acidic one (trifluoroacetic acid) and a
179 basic one (isopropylamine), were selected. 11 mobile phase compositions were tested: no
180 additive; 20 mM, 122 mM, 366 mM and 610 mM trifluoroacetic acid in methanol; and 20 mM,
181 61 mM, 122 mM, 366 mM, 610 mM and 1220 mM isopropylamine in methanol. As a note of
182 caution, it is worth saying that a very thorough rinsing of the system is absolutely necessary
183 after operation, as such high concentrations of additives tend to leave traces in the system
184 for a long while (especially isopropylamine). Obviously, the adsorption on the stationary
185 phase is also quite extensive thus the columns must be rinsed for a long time to restore the
186 initial state. The 54 achiral and 24 chiral molecules (Tables S1 and S2) were injected with
187 each of these mobile phase conditions. The flow rate was 3.0 mL/min for the Chirobiotic TAG
188 column, and 1 mL/min for the BEH column, to allow for comparable internal pressure in the
189 two columns and avoid reaching the pump upper pressure limit. The backpressure was
190 maintained at 15 MPa and the column oven was heated to 25 °C. The injection volume was
191 set at 2 μL. The dead time and retention times of all compounds were recorded to calculate
192 the log *k* value, required to achieve the LSER models.

193

194 2.4 Evaluation of polarity and apparent pH

195 Measurements of UV-visible spectra in the carbon dioxide – methanol – additive
196 compositions were done as previously described [45]. Briefly, solutions of the color indicators
197 (Nile Red polarity indicator and pH indicators) were injected in the Acquity BEH column to
198 ensure that the analyte would have time to be desolvated from the dilution solvent plug
199 (methanol), and would thus reflect the mobile phase polarity and acidity, with operating
200 conditions identical to the LSER experiments described above. UV-visible spectra were
201 recorded with the diode-array detector between 300 and 700 nm with 1.2 nm intervals. The
202 spectra for pH indicators were first recalculated for 1 nm intervals, then they were normalized
203 and compared to UV-visible spectra measured in aqueous phases with controlled pH values
204 through principal component analyses (PCA). PCA were computed with XLStat 2015.2.02
205 software (Addinsoft, New York, NY). The reader is referred to previous works for full details
206 on this procedure [45].

207

208 2.5 LSER model

209 The LSER equation usually employed in chromatography, based on Abraham descriptors, is:

210

$$211 \log k = c + eE + sS + aA + bB + vV \quad (1)$$

212

213 In this equation, capital letters represent the solute descriptors, related to particular
214 interaction properties, each molecule has its own molecular descriptors. While lower case
215 letters represent the system constants, related to the complementary effect of the two
216 phases (stationary and mobile). c is the model intercept term and is dominated by the phase
217 ratio. E is the excess molar refraction (calculated from the refractive index of the molecule)
218 and models polarizability contributions from n and π electrons; S is the solute
219 dipolarity/polarizability; A and B are the solute overall hydrogen-bond acidity and basicity; V
220 is the McGowan characteristic volume in units of $\text{cm}^3 \text{mol}^{-1}/100$. The system constants (e , s ,
221 a , b , v), obtained through a multilinear regression of the retention data for a certain number
222 of solutes with known descriptors, reflect the magnitude of difference for that particular
223 property between the mobile and stationary phases.

224 However, in previous works we have had many occasions to point out that the lack of terms
225 to account for ionic interactions in Eq. (1) is a problem when dealing with ionizable analytes.
226 To take account of electrostatic interactions with anionic and cationic species, we have
227 previously proposed to extend Eq. (1) with two additional terms [42], as follows:

228

$$229 \log k = c + eE + sS + aA + bB + vV + d^-D^- + d^+D^+ \quad (2)$$

230

231 where D^- represents the negative charge carried by anionic and zwitterionic species, and D^+
232 represents the positive charge carried by cationic and zwitterionic species. They are
233 calculated with the apparent pH (that may be different from effective pH^*) of the mobile
234 phase and the pK_a values of the analyte. For neutral species, D^- and D^+ are zero so that Eq.
235 (2) reverts to Eq. (1). We had initially designed Eq. (2) for hydrophilic interaction liquid
236 chromatography (HILIC) [42,46,47], but they were also applied to other HPLC systems
237 [48,49]. We have recently showed that it is also well suited to the description of SFC systems
238 [24,50,51], provided we admit to some approximations (aqueous pK_a values and apparent pH
239 equal to 5).

240 Finally, two more descriptors (F and G), previously introduced to characterize
241 enantioselective stationary phases [43,52], are needed to provide information related to the
242 3D behavior of the probe molecules interacting with chiral selectors. As described in previous
243 works, the F descriptor reflects the flexibility of the molecule (fraction of rotatable bonds),
244 which is important for chiral recognition because flexible molecules have more conformers,
245 thus more ways for intermolecular interactions, which is generally detrimental to
246 enantioresolution. The G descriptor accounts for globularity (or sphericity) and can be related
247 to steric impedance to insertion into the stationary phase but also to the possibility for a small
248 molecule to better fit on the walls of a spherical cavity thus establish several interactions at a
249 time. While F and G are often found to contribute very little to retention mechanisms, they are
250 both highly significant to enantiorecognition. The full LSER equation employed in the present
251 work is then:

252

$$253 \quad \log k = c + eE + sS + aA + bB + vV + fF + gG + d^-D^- + d^+D^+ \quad (3)$$

254

255 This 9-term equation was never employed before and should allow for a most complete
256 assessment of interactions with the complex, enantioselective and ionizable stationary
257 phase.

258

259 Multiple linear regressions (MLR) were performed using XLStat 2015.2.02 software. The
260 quality of the MLR fits was estimated using the adjusted determination coefficient (R^2_{adj}),
261 standard error in the estimate (*RMSE*) and Fisher *F* statistic. The statistical significance of
262 individual coefficients was evaluated with the 95% confidence intervals.

263 Because the selection of probe molecules is rather small (54 achiral molecules for 9
264 independent variables), the statistical quality of the models is moderate (R^2_{adj} values ranging
265 from 0.77 to 0.93). However, no prediction capability was desired, only some understanding

266 of the interactions contributing to retention and their variation with mobile phase composition.
267 For this reason, only the terms that vary significantly will be discussed in the following.

268

269

270 **3. Results & Discussion**

271

272 3.1 Evaluation of changes in polarity with a solvatochromic dye

273

274 Nile Red was selected as a solvatochromic probe to assess possible changes in the polarity
275 of the mobile phase when large concentrations of additives were present. Its structure and
276 UV-visible spectra are visible in electronic supplementary information (Fig. S1). UV-visible
277 spectra of Nile Red were recorded in SFC conditions with all levels of acidic and basic
278 additives as described in methods section. Based on the first derivative of smoothed spectra,
279 the wavelength at maximum absorbance was measured, from which the transition energy
280 E_{NR} was calculated using the Planck-Einstein equation. As appears in the spectra (Fig. S1),
281 large concentrations of additives yielded very small shifts in the absorbance maximum.
282 Namely, large concentrations of trifluoroacetic acid (TFA) caused a small bathochromic shift
283 (E_{NR} decreases from 55.9 to 55.8 kcal.mol⁻¹), which is comparable to the polarity increase
284 that would result from increasing the percentage of methanol co-solvent from 10 to 11% [45].
285 Conversely, large concentrations of isopropylamine (IPA) caused a small hypsochromic shift
286 (E_{NR} increases from 55.9 to 56.0 kcal.mol⁻¹), which is comparable to the polarity decrease
287 that would result from decreasing the percentage of methanol co-solvent from 10 to 9%.
288 Thus the two additives have opposite effects on polarity: TFA slightly increases the polarity in
289 the cybotactic region (solvation sphere around the analyte) while IPA slightly decreases it.
290 This conclusion is not so surprising considering the structures of the two molecules,
291 compared to CO₂ and methanol, as TFA possesses a high proportion of heteroatoms with
292 significant electronegativity (O and F) while IPA possesses a larger portion of hydrocarbon
293 moieties. In the following, we shall consider these changes as negligible.

294

295 3.2 Evaluation of the ionization state of teicoplanin aglycone

296

297 In previous works, we had demonstrated how a selection of color indicators of pH could be
298 used to obtain some information on the apparent pH of the SFC mobile phases [45]. In
299 particular, we had shown that additives in usual concentrations could modify the apparent pH
300 of the fluid. Indeed, mixtures of carbon dioxide and alcohols comprising no additives are
301 slightly acidic, with an apparent pH close to 4.5-5, due to the formation of alkyloxycarbonic
302 acid. In usual concentrations, acidic additives can reduce the apparent pH down to 1-2 while

303 basic additives would cause only a moderate increase (5-6). As mentioned in the
304 introduction, typical concentrations used in current SFC practice range in the 1-50 mM or
305 0.1-1% in the alcohol co-solvent. At such low concentrations, a basic additive is not
306 susceptible to compensate for the large concentration of alkyloxycarbonic acid, possibly
307 explaining why the increase in apparent pH is rather small. However, higher concentrations
308 of basic additive may create a really basic environment for the analytes and stationary
309 phase. With these further experiments, we wished to verify this point and the extent of
310 apparent pH increase.

311
312 In Fig. 2, the selection of five color indicators of pH (one of which has two pK_a values) with
313 their aqueous pK_a values can be seen. As detailed in section 2.4 above, UV-visible spectra
314 were measured for each of the color indicators of pH with the diode-array detector of the
315 SFC system. The spectra were then compared to spectra measured in aqueous phases of
316 controlled pH. All spectra can be observed in electronic supplementary information (Fig. S2).
317 A simple comparison of spectra is not sufficient. As was demonstrated in our previous study,
318 the use of principal component analysis (PCA) to compare all spectral data is most
319 informative to obtain a better estimate of apparent pH. In the present case, PCA were
320 computed for each color indicator individually, and also with combinations of color indicators
321 that proved the most informative. One interesting combination can be observed in electronic
322 supplementary information (Fig. S3) with a PCA score plot computed with the spectral data
323 measured for Thymol Blue, Bromocresol Green, Methyl Red and Bromocresol Purple. On
324 this plot, the first principal component (PC1) is essentially a pH axis between 2 and 8, while
325 the second principal component (PC2) is a pH axis in the ranges 1-2 and 8-10. Based on the
326 examination of all spectra and PCA plots, the estimated apparent pH values can be observed
327 in Fig. 2.

328 We had demonstrated before that the mobile phase comprising no additive has an apparent
329 pH close to 5, or somewhat smaller depending on the proportion of methanol (around 4.5-5).
330 When large concentrations of TFA are introduced, the apparent pH is strongly decreased,
331 around 1-2. Indeed, all spectra in these conditions show the acidic form for each of the five
332 color indicators. When large concentrations of IPA are introduced, the apparent pH is slowly
333 increasing and reaches a value of about 6. This is demonstrated by the spectra of
334 Bromocresol Purple, where the absorption of the basic form is observed at the largest
335 concentrations of IPA, but still in equilibrium with a significant portion of the acidic form. The
336 apparent pH is therefore never above the pK_a value of Bromocresol Purple (6.3).
337 Furthermore, the basic forms of Bromothymol Blue and Thymol Blue are never observed
338 (pK_a values 7.1 and 8.9).

339 It must be mentioned again here that all these values are estimates of apparent pH, based
340 on aqueous pK_a values, which are obviously not identical in pressurized CO_2 -methanol
341 environment.

342
343 The structure of teicoplanin aglycon was processed with the Chemicalize program to
344 compute aqueous pK_a values for each of the ionizable function (carboxylic acid, amine,
345 phenolic and amide groups). Based on these values, a curve of charge state vs. mobile
346 phase pH was computed and can be seen in Fig. 3. On this figure, the pK_a values of the
347 principal ionizable functions is pointed with arrows, and the estimated pH domains are
348 presented. Not all of these ionizable functions may be available, as the amine [27] or phenol
349 groups [28] may both have served as attachment points to the supporting silica gel. The
350 availability of the amine function is clearly a matter of debate as some authors report bonding
351 via the amine through a urea attachment [27], while other bonding protocols have maintained
352 a quaternary ammonium function as the binding site [53]. Besides, several papers have
353 demonstrated that interaction with the protonated amine function was significant to
354 enantioselectivity [54], suggesting that the amine was still free in the bonded phase. Our
355 own works [55] demonstrate that interactions with anionic compounds is significant to
356 successful enantioseparation. This may result from positive interactions with an amine group,
357 but whether this group is present in the peptide ligand or in the aminopropyl ligands cannot
358 be told. Both options shall be considered in the following. According to these estimations, we
359 can however suppose that:

- 360 (i) in the CO_2 -methanol mobile phase comprising no additive, both the acid and
361 amine functions should be ionized, thus the net charge for the ligand would be
362 zero. If the amine function is unavailable, the net charge is -1.
363 (ii) when IPA is introduced, deprotonation of a free amine function would not occur,
364 thus the net charge would still be the same;
365 (iii) when TFA is introduced, the acid function should protonate, thus the net charge
366 would be +1, or zero in case the amine function is not free.

367 These observations should be kept in mind when interpreting retention and separation
368 behavior, as will be done below.

369

370 3.3 LSER characterization of the effects of additives

371

372 In a previous paper, we had characterized three macrocyclic peptide stationary phases
373 (Chriobiotic V, T and TAG based respectively on vancomycin, teicoplanin and teicoplanin
374 aglycon) [55]. LSER methodology was employed, based on Eq. (2), to describe the retention

375 mechanisms in a carbon dioxide-methanol environment, without additives. In the present
376 paper, a most complete LSER model, based on Eq. (3), is employed, with the intention to
377 describe the effects of additives. The full LSER models obtained from Eq. (3) can be
378 observed in supporting information, in histograms (Figure S1a for the TAG column and
379 Figure S1b for the silica column) or in the tables reporting the statistical features of each
380 model (Tables S3 and S4).

381 To some extent, the mobile phase employed here (carbon dioxide – methanol, with or
382 without an additive) should be comparable to polar organic and polar ionic modes where only
383 organic solvents (methanol and/or acetonitrile) are used as eluents, together with additives to
384 adjust the acidity in the polar ionic mode.

385 On both columns, the most significant variations were observed for the a , b , d and d' terms,
386 related to hydrogen bonding and ionic interactions. Thus only these four selected system
387 constants will be discussed and are represented in Figure 4a (Chirobiotic TAG) and 4b
388 (Acquity BEH). On the figures, the evolution of the system constants with increasing
389 apparent mobile phase pH is represented from left to right, corresponding on the left to the
390 highest concentration of TFA (610 mM in methanol co-solvent) and on the right to the highest
391 concentration of IPA (1220 mM in methanol).

392

393

394 3.3.1 Evolution of retention with IPA concentration

395 *Hydrogen bonds*

396 With both stationary phases, when IPA concentration increases, a significant variation in the
397 a and b system constants related to hydrogen bonding is observed, with opposite trends for a
398 and b . Indeed, an increase in the a term (related to retention of proton donors) and a
399 decrease in the b term (related to retention of proton acceptors) are observed when the
400 concentration of IPA increases from no additive to 1220 mM IPA in the methanol co-solvent.

401 Moreover, the trends of a and b variations are nearly collinear (they vary with the same
402 slopes). This suggests that the reason for the variation is identical on both columns. In the
403 LSER equation, each system constant is related to the difference in the interactions between
404 analyte and stationary phase on the one hand, analyte and mobile phase on the other hand.
405 As a result, the increase in one system constant may be due to (i) increase of analyte –
406 stationary phase interactions or (ii) decrease of analyte – mobile phase interactions.
407 Conversely, the decrease in one system constant may be due to (i) decrease of analyte –
408 stationary phase interactions or (ii) increase of analyte – mobile phase interactions.

409 Considering previous observations on a large variety of stationary phases [24], the increase
410 in a term on both stationary phases may be explained by an adsorption phenomenon of the
411 additive on the surface of the stationary phase. Indeed, as should be the case for any other

412 component of the mobile phase, the additive gets adsorbed at the surface of the stationary
413 phase, which modifies the possible interactions between the analytes and stationary phase.
414 The higher the concentration of additive in the mobile phase, the greater the quantity of
415 additive adsorbed on the stationary phase. In this case, because isopropylamine is basic,
416 hydrogen-bonding interactions with proton donors (*a* term) increase.
417 Basic analytes do not interact favorably with isopropylamine thus the adsorbed additive
418 should not cause particularly favorable interactions with electron donors (*b* term). On the
419 opposite, the adsorption of isopropylamine is increasingly shielding proton-donor interaction
420 sites of the stationary phases. Additionally, isopropylamine likely acts as a competitor for
421 adsorption of basic analytes on the stationary phases. As a result of both effects (adsorption
422 and increasing elution strength towards basic compounds), the retention of basic compounds
423 reduces when IPA concentration increases (*b* term decreases). This is illustrated in Fig. 5
424 where it appears that the retention time of the two enantiomers of 5-methyl-5-
425 phenylhydantoin, an electron-donor chiral compound, significantly decreases with increasing
426 IPA concentration. However, the peak shapes are improved and good resolution is
427 maintained in that case.

428

429 *Ionic interactions*

430 The d' and d'' terms are related to interactions with anionic and cationic species respectively.
431 Again their variation on both columns is most similar, indicating that the effects observed
432 probably have the same causes.

433 The d' term varies significantly in a non-monotonous manner. First, it is near zero on both
434 stationary phases when no additive is present. Anionic compounds may be partly repulsed
435 by the stationary phases. Indeed, at the apparent pH obtained with such a mobile phase
436 (around 5), some accessible residual silanol groups and the carboxylic acid function of the
437 teicoplanin aglycone may be partly deprotonated. Therefore, there would be some repulsion
438 between the negative charges of the analytes and the negative charges on the stationary
439 phases. Thus negatively charged species experience rather low retention in these conditions.
440 The d' term increases strongly when IPA is first introduced in the mobile phase (20 mM in
441 methanol co-solvent). This is again consistent with adsorption of isopropylamine on the
442 stationary phase as anionic compounds are expected to interact favorably with adsorbed
443 isopropylamine molecules (which may be in the neutral or protonated form).

444 Further increases in IPA however see the d' term reach a maximum then a subsequent
445 decrease. Considering the above observations on ionization state of teicoplanin aglycone,
446 deprotonation of free amine functions (whether from the peptide or the aminopropyl groups)
447 should not contribute to this effect. However, the decrease in retention when adding high
448 concentrations of IPA can be explained by two phenomena. First, at high IPA concentrations,

449 the stationary phase is saturated with additive and then more and more free IPA is found in
450 the mobile phase, creating ion pairs with the anionic analytes, which increases favorable
451 interactions with the mobile phase, resulting in a decrease in the overall value of the d'
452 constant. Second, we may infer that, at such high base concentration, the methoxycarbonic
453 acid present in the mobile phase due to the reaction of carbon dioxide with methanol may be
454 titrated by isopropylamine, and the mobile phase apparent pH is now somewhat increasing
455 towards more neutral values (up to 6).

456 We had previously demonstrated a similar reversal of dominant behavior – with a dominant
457 effect of adsorption at low concentrations and dominant effect of mobile phase properties at
458 high concentrations – when examining the effects of the nature of the co-solvent with porous
459 graphitic carbon stationary phases [56].

460 The trend in d' term is illustrated by the chromatograms in Fig. 6 of an acidic compound,
461 aspirin (aqueous $pK_a = 3.41$), which, at an apparent pH greater of about 6, should be
462 predominantly in its deprotonated form. It can be observed that the low concentrations of IPA
463 cause a sharp increase in retention compared to the “no additive” mobile phase composition,
464 while further increases of IPA concentration cause retention decrease.

465
466 Concerning the retention of cationic species (d^+ term), comparing the “no additive”
467 composition and 20 mM IPA in methanol, the first introduction of small concentrations of IPA
468 in mobile phase causes a significant decrease in the d^+ term from positive values to near
469 zero on the TAG column, indicating that cationic species are much less retained when IPA is
470 present in mobile phase. Further increases in IPA concentration cause no significant
471 changes. On the silica column, the introduction of IPA in the mobile phase is mostly
472 insignificant. Therefore, this is the only case where the introduction of IPA causes different
473 variations between the two columns. We may attribute these differences to the different
474 interaction capability of the “bare” stationary phases (uncovered with additive): on the silica
475 surface, only silanol groups (neutral or ionized) should interact with cationic species while on
476 the teicoplanin aglycone stationary phase, the deprotonated carboxyl group should also
477 contribute to interaction with cationic species.

478

479 3.3.2 Effect of IPA on enantioseparations

480 From a chirality point of view, overall, a loss of enantioseparation is observed when adding
481 high concentrations of IPA for most of the compounds that were resolved when no additive
482 was used. This is in contradiction with previous observations from Phinney and Sander,
483 where the introduction of isopropylamine in the SFC mobile phase was seen to slightly
484 diminish separation factors on a vancomycin stationary phase, without loss of separation

485 [23]. However, the enantiorecognition site may be significantly different between vancomycin
486 and teicoplanin aglycone.

487 An example of chromatograms is presented in Fig. 7 where the ketoprofen enantiomers
488 show some debut separation when the mobile phase does not contain any additive, but even
489 the smallest concentrations of IPA cause a co-elution of the enantiomers. This molecule
490 should also be partly anionic at the apparent pH of the mobile phase (aqueous $pK_a = 4.45$),
491 that is to say that this molecule has a non-zero D^+ descriptor value, and behaves according to
492 the variations of d' term, with a sharp increase of retention at low IPA concentrations,
493 followed by a decrease in retention when IPA concentration increases.

494
495 A single exception was observed with dichlorprop, for which the separation factor improved
496 with IPA compared to no additive (for instance, $\alpha = 1.65$ with 120 mM IPA compared to 1.43
497 without additive). In all other cases examined, separation factors decreased when IPA was
498 used. The loss of enantioresolution can have two possible origins. Firstly, the chiral cavities
499 may be rendered inaccessible to the enantiomers by the thick layer of adsorbed additive.
500 Secondly, ionizable analytes for which the pK_a values are close to the apparent pH may
501 change protonation state, thereby affecting the way they interact with the stationary phase.
502 The ionizable functions are known to contribute significantly to enantiorecognition in this
503 stationary phase. This can however not contribute to changes for non-ionizable species like
504 methylphenylsulfoxide or chlormezanone.

505
506 Interestingly, a recent paper from Pokrovskiy [57] showed that the manipulation of ionic
507 interactions through the selection and concentration of additives in the SFC mobile phase
508 was significant to enantioresolution on another antibiotic stationary phase, erythromycin.

509

510 3.3.3 Evolution of retention with TFA concentration

511 On both columns, the only significant effect of TFA introduction is observed with the d^+ term,
512 namely, increasing the concentration of TFA causes a sharp increase of d^+ values, indicating
513 that cationic species are more retained when TFA is present in the mobile phase. Without an
514 additive, the d^+ term was already positive on both columns due to interactions between
515 cationic analytes and the silanol groups or carboxyl function of the teicoplanin aglycone. At
516 the largest concentration of TFA tested (610 mM in methanol), the d^+ term is the most
517 significant contributor to retention.

518 This is perhaps counter-intuitive as one might expect TFA to act as an ion-pairing agent to
519 favor the elution of protonated basic compounds. Besides, as the carboxylic acid function of
520 the teicoplanin aglycone should protonate, strong coulombic interactions between the
521 stationary phase and cationic analytes were expected to decrease. The opposite behavior

522 observed is most likely related to the same phenomenon as described for IPA: TFA adsorbs
523 on the stationary phase and creates a layer on top of the surface and ligands. Because TFA
524 interacts strongly with cationic species, the interactions between analyte and stationary
525 phase strongly increase. Contrary to the observations with IPA, no reversal of trend was
526 observed at the largest concentrations on the TAG column (although we may suspect some
527 of it on the BEH column). But then it was not possible to increase the concentration as high
528 as in the IPA experiments due to solubility issues, thus if a reversal should occur at higher
529 TFA concentrations, it was impossible to verify.

530 An example is given in Fig. 8 with the chromatograms of zopiclone, where the retention is
531 seen to increase continuously with the concentration of TFA.

532

533 3.3.4 Effect of TFA on enantioseparations

534

535 Similarly to the effect of IPA, TFA had a negative influence on separation factors. Apart from
536 one exception, separation factors decreased when TFA was introduced, compared to no
537 additive. In one case, 5-methyl-5-phenylhydantoin, for which the separation factor was
538 already very large without an additive ($\alpha = 3.07$), it increased further with the introduction of
539 TFA ($\alpha = 3.30$ with 120 mM TFA).

540 The loss of enantioselectivity when an acidic additive is introduced may again have different
541 origins, including obstruction of the chiral cavities by the adsorption of a large amount of
542 TFA, varying charge state of ionizable analytes, or the protonation of the carboxylic group of
543 the stationary phase, which may be deleterious to enantioselectivity.

544

545 **Conclusion**

546 In this paper, we have evaluated how large concentrations of acidic and basic additives
547 influenced the retention and enantioseparation behavior of a complex macrocyclic peptide-
548 bonded stationary phase. It was first demonstrated that these highly concentrated additives
549 had negligible influence on the mobile phase polarity. The influence on mobile phase acidity
550 was however more significant, with TFA inducing strong decrease in the apparent pH (from
551 4.5-5 to 1-2), and IPA inducing some moderate increase (up to 6). This could cause changes
552 in the ionization state of the carboxylic acid function of teicoplanin aglycone, but should have
553 limited effect on the ionization state of free amine functions. Therefore, the probable
554 unavailability of the amine function in teicoplanin aglycone could not be assessed through
555 these means.

556 Considering these elements, the retention and enantioseparation behavior were examined
557 with the help of LSER methodology, with a most complete description achieved with nine
558 molecular descriptors. Based on the comparison to the effects observed on a bare silica

559 phase, many effects could be explained by the adsorption of additives on the stationary
560 phase. With the high concentrations that could be achieved for isopropylamine, it was
561 observed that the primary effect of additives on retention mechanisms is through adsorption
562 on the stationary phase, then when even higher concentrations are used, the effects in the
563 mobile phase dominate the retention mechanisms. With trifluoroacetic acid, only adsorption
564 effects were observed, but then it was not possible to increase its concentration as high as
565 that of isopropylamine, due to solubility issues.

566 These results should be helpful in further understanding the effects of mobile phase additives
567 in SFC, but also to understand the behavior of large biomolecules analyzed in SFC.

568

569

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577

578 **Compliance with ethical standards**

579 The authors declare they have no conflict of interest.

580 **References**

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759

760 **Figure captions**

761

762 **Fig. 1:** Structure of Teicoplanin Aglycone (TAG), the chiral selector in the stationary phase
763 examined.

764

765 **Fig. 2:** Evaluation of apparent pH when large concentrations of acidic and basic additives are
766 employed. TFA is trifluoroacetic acid, IPA is isopropylamine.

767

768 **Fig. 3:** Ionization states of teicoplanin aglycon depending on mobile phase pH, according to
769 aqueous pK_a values

770

771 **Fig. 4:** Selected system constants from the LSER models of (a) the teicoplanin aglycone
772 stationary phase (Chirobiotic TAG) and (b) a bare silica stationary phase (Acquity BEH),
773 retaining only the 4 system constants that vary significantly with a change in the nature or
774 concentration of the additive (TFA or IPA). Mobile phase: carbon dioxide – methanol (with or
775 without additive according to legend, from left to right) 90:10 (v/v), 25°C, 15 MPa, 3 mL/min
776 on TAG or 1 mL/min on BEH.

777

778 **Fig. 5:** Chromatograms of *rac*-5-methyl-5-phenylhydantoin on the Chirobiotic TAG column
779 with increasing concentrations of IPA from bottom to top: without additive (gray), IPA 61 mM
780 (orange) and IPA 610 mM (brown). The inserted histograms show the related variation of
781 LSER *b* term when IPA concentration increases.

782

783 **Fig. 6:** Chromatograms of aspirin on the Chirobiotic TAG column with increasing
784 concentrations of IPA: no additive (gray), IPA 122 mM (light orange), IPA 366 mM (dark
785 orange) and IPA 1220 mM (brown). The inserted histograms show the variation of related
786 LSER *d'* term when IPA concentration increases.

787

788 **Fig. 7:** Chromatograms of *rac*-ketoprofen on the Chirobiotic TAG column with increasing
789 concentrations of IPA: no additive (gray), IPA 61 mM (yellow), IPA 122 mM (orange), IPA
790 610 mM (light brown), IPA 1220 mM (dark brown). The inserted histograms show the
791 variation of related LSER *d'* term when IPA concentration increases.

792

793 **Fig. 8:** Chromatograms of zopiclone on the Chirobiotic TAG column with increasing
794 concentrations of TFA: no additive (gray), TFA 20 mM (light blue), TFA 122 mM (blue), TFA
795 610 mM (dark blue). The inserted histograms show the variation of related LSER *d'* term
796 when TFA concentration varies.

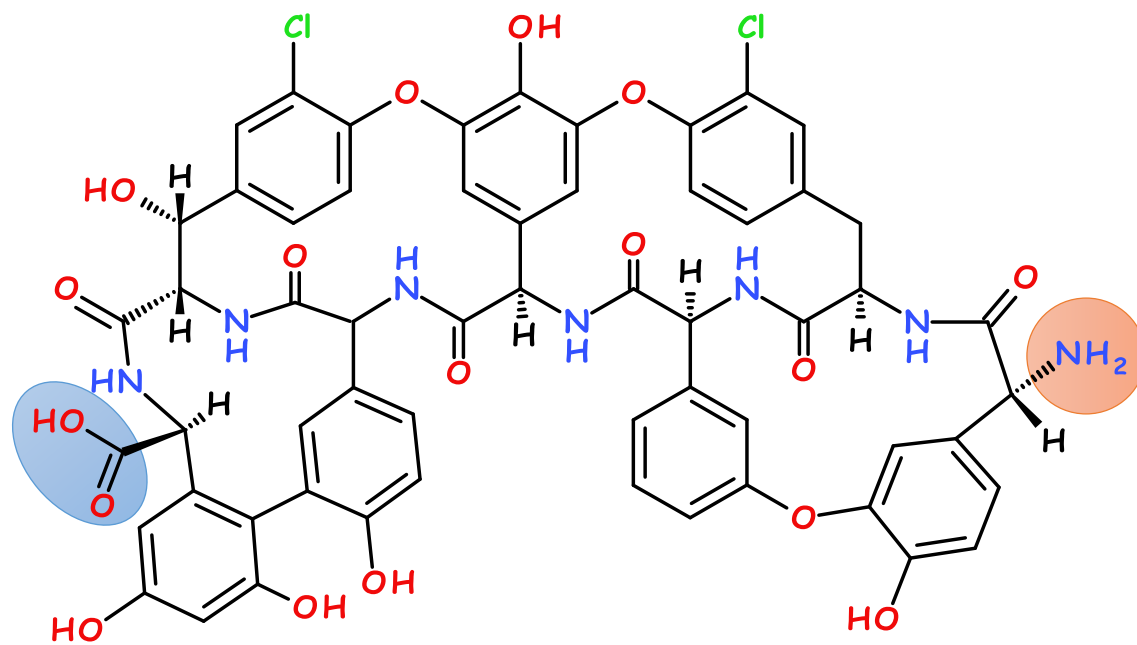


Fig. 1: Structure of Teicoplanin Aglycon (TAG), the chiral selector in the stationary phase examined.

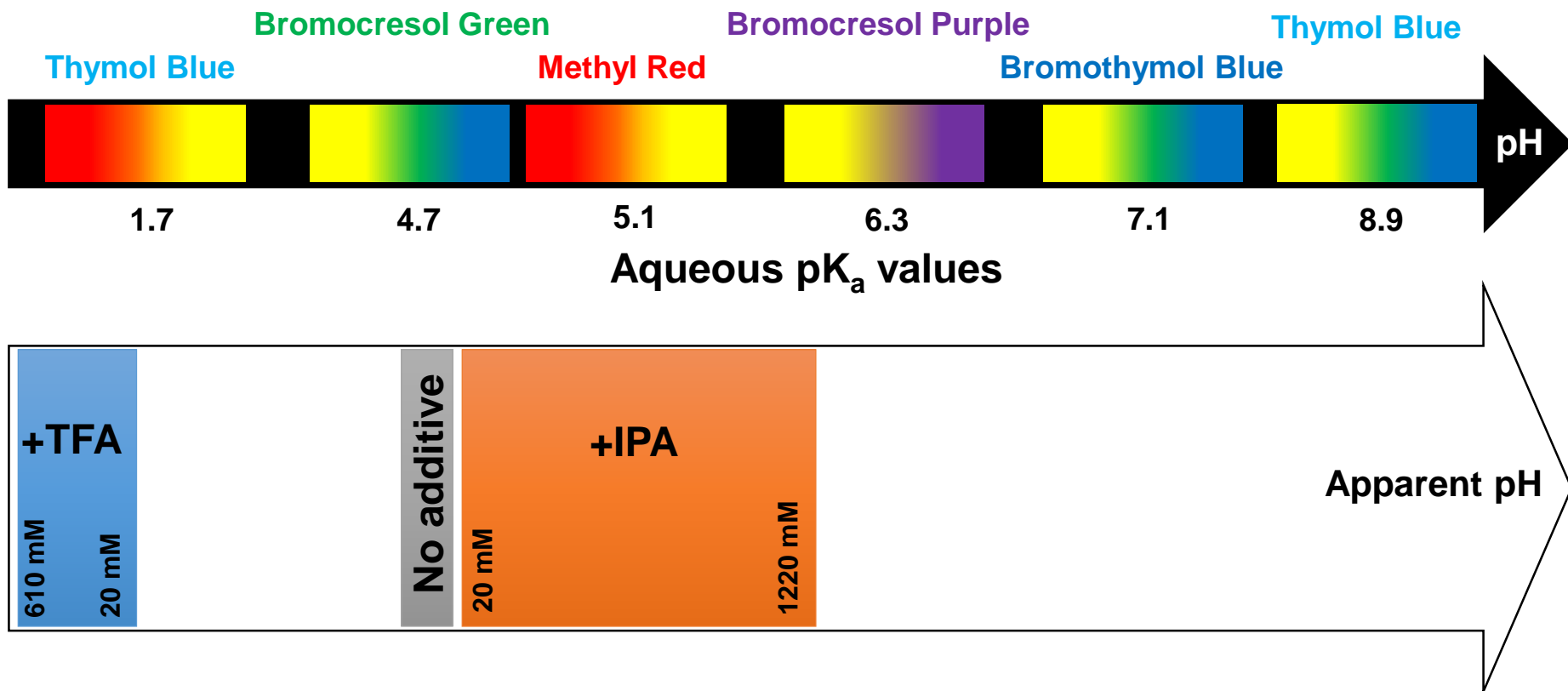


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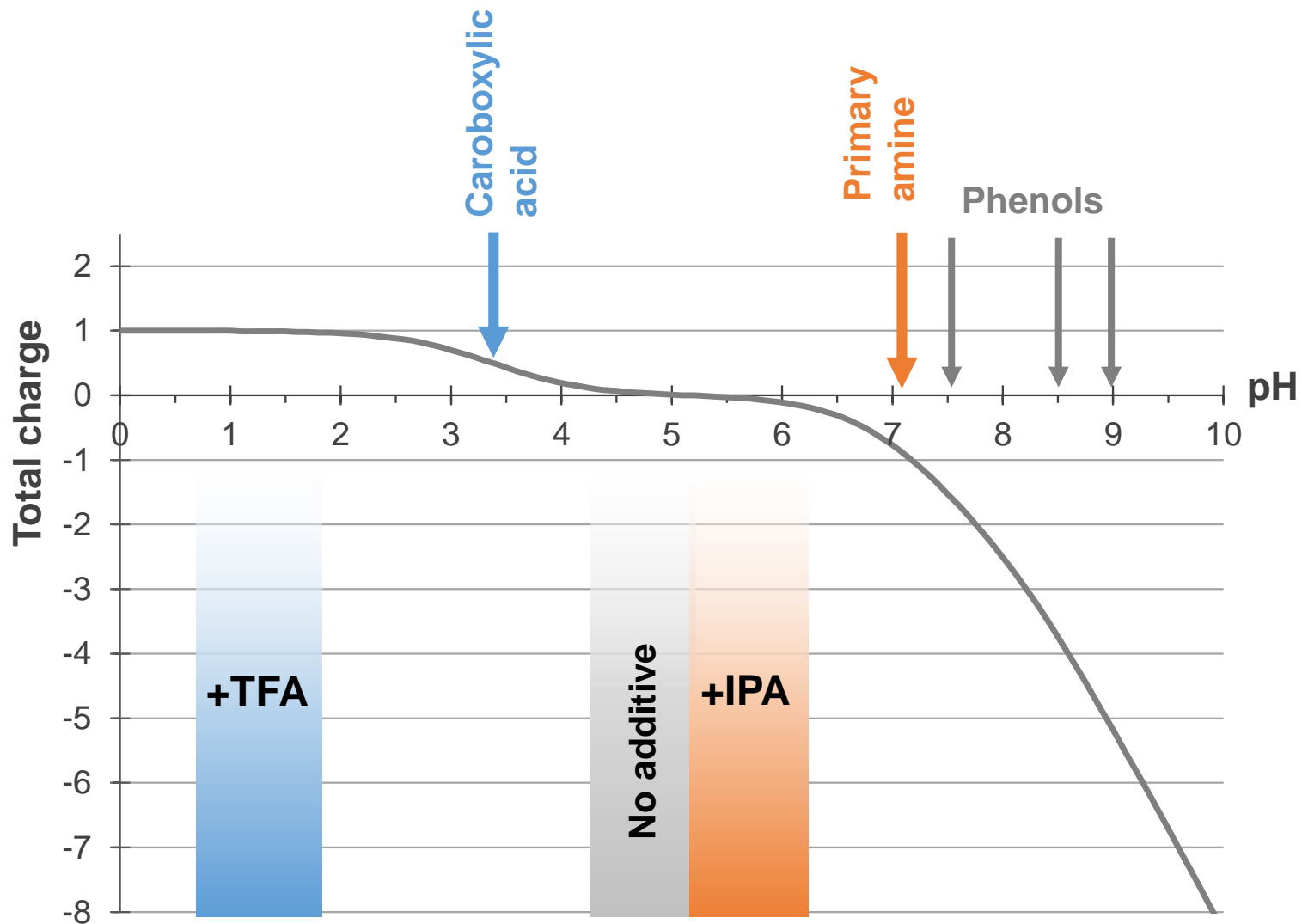


Fig. 3: Ionization states of teicoplanin aglycon depending on mobile phase pH according to aqueous pK_a values

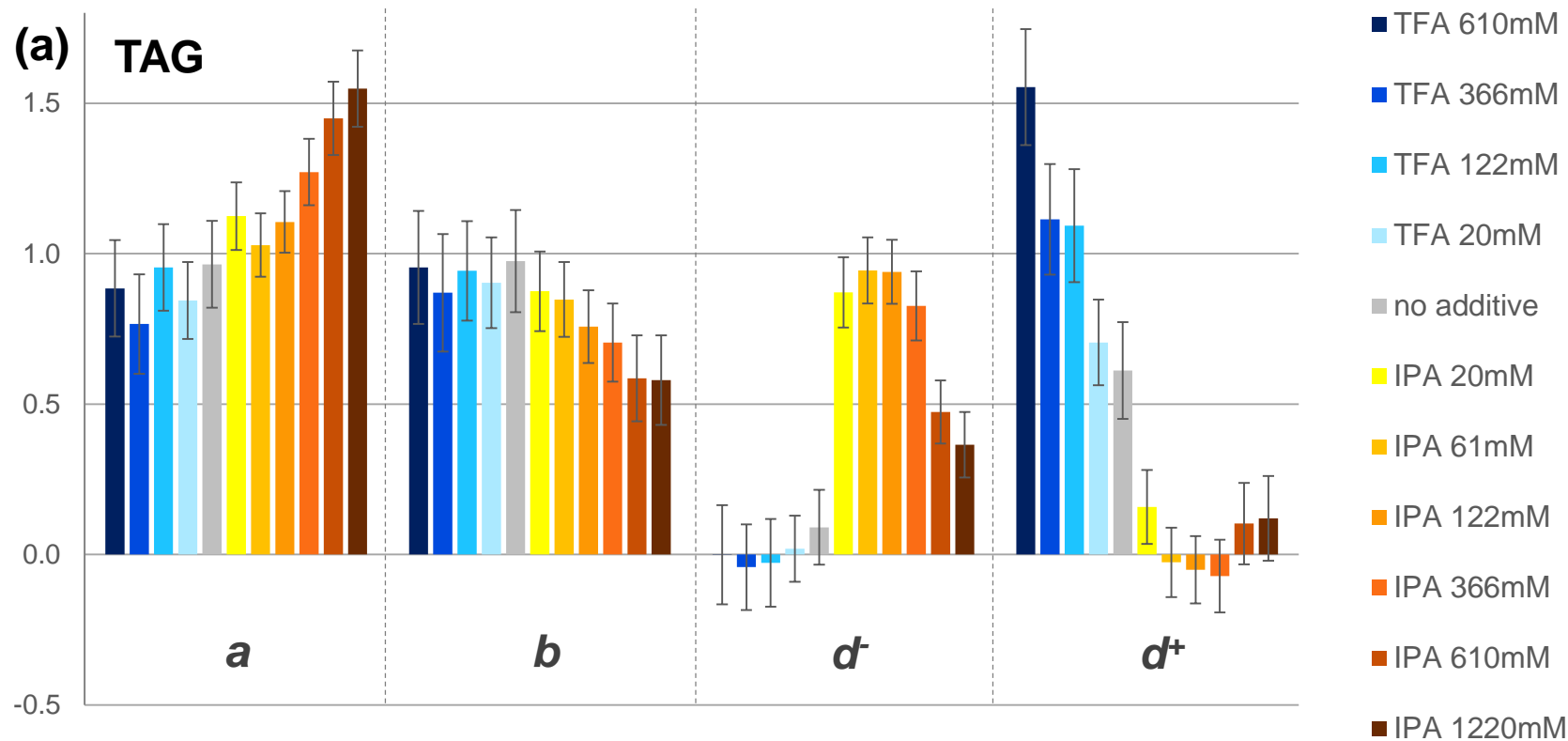


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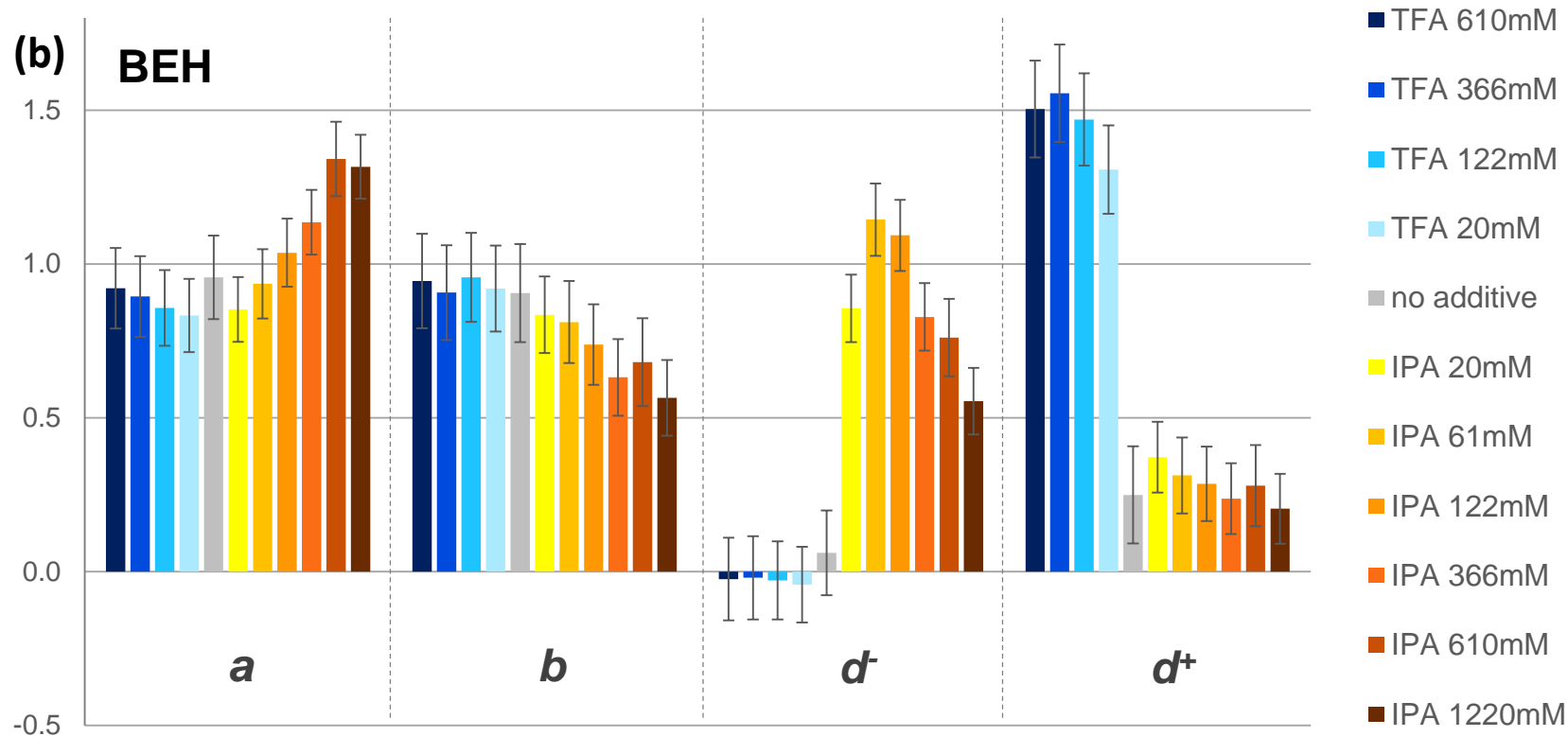


Fig. 4 (continued): Selected system constants from the LSER models of (a) the teicoplanin aglycone stationary phase (Chirobiotic TAG) and (b) a bare silica stationary phase (Acquity BEH), retaining only the 4 system constants that vary significantly with a change in the nature or concentration of the additive (TFA or IPA). Mobile phase: carbon dioxide – methanol (with or without additive according to legend, from left to right) 90:10 (v/v), 25°C, 15 MPa, 3 mL/min on TAG or 1 mL/min on BEH.

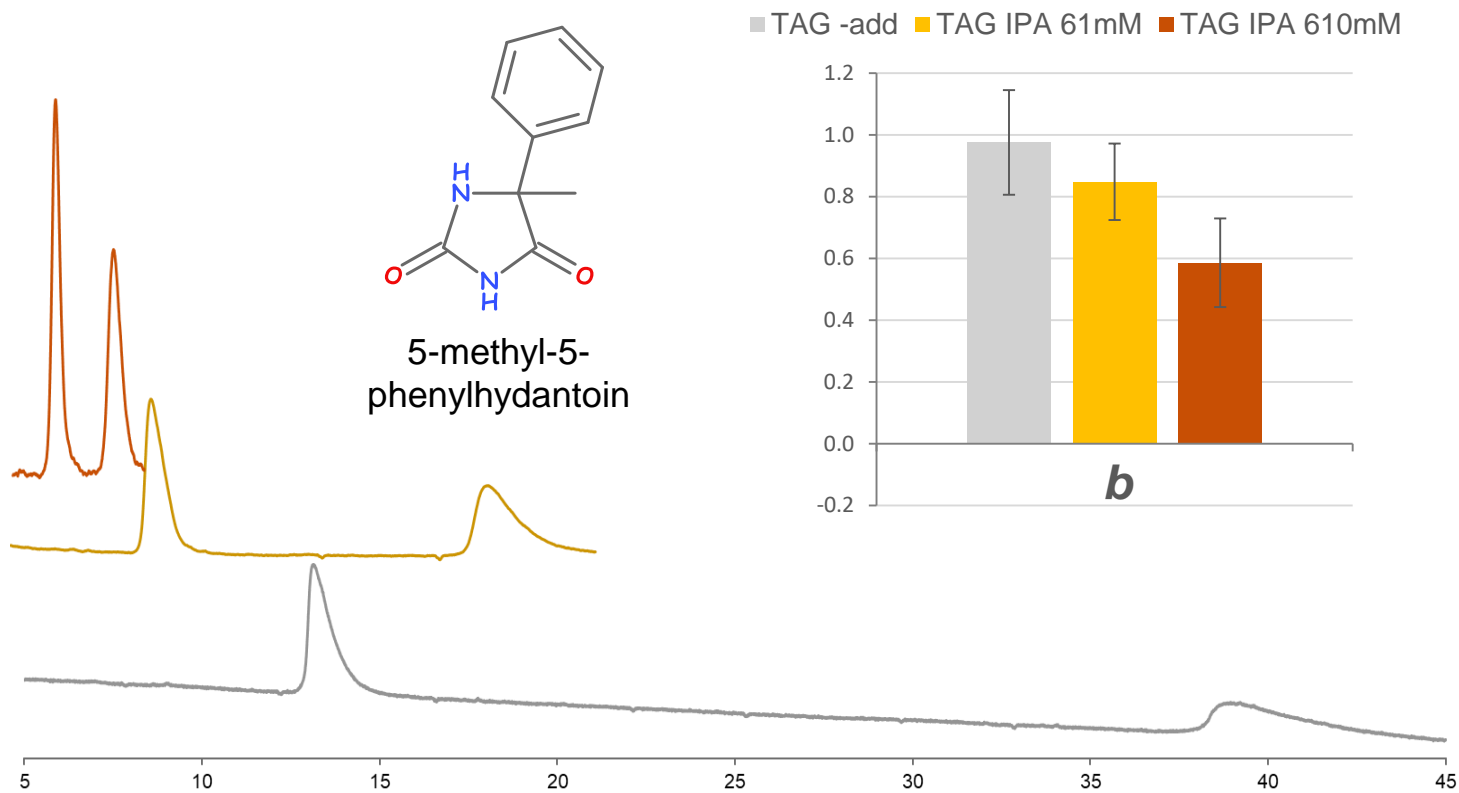


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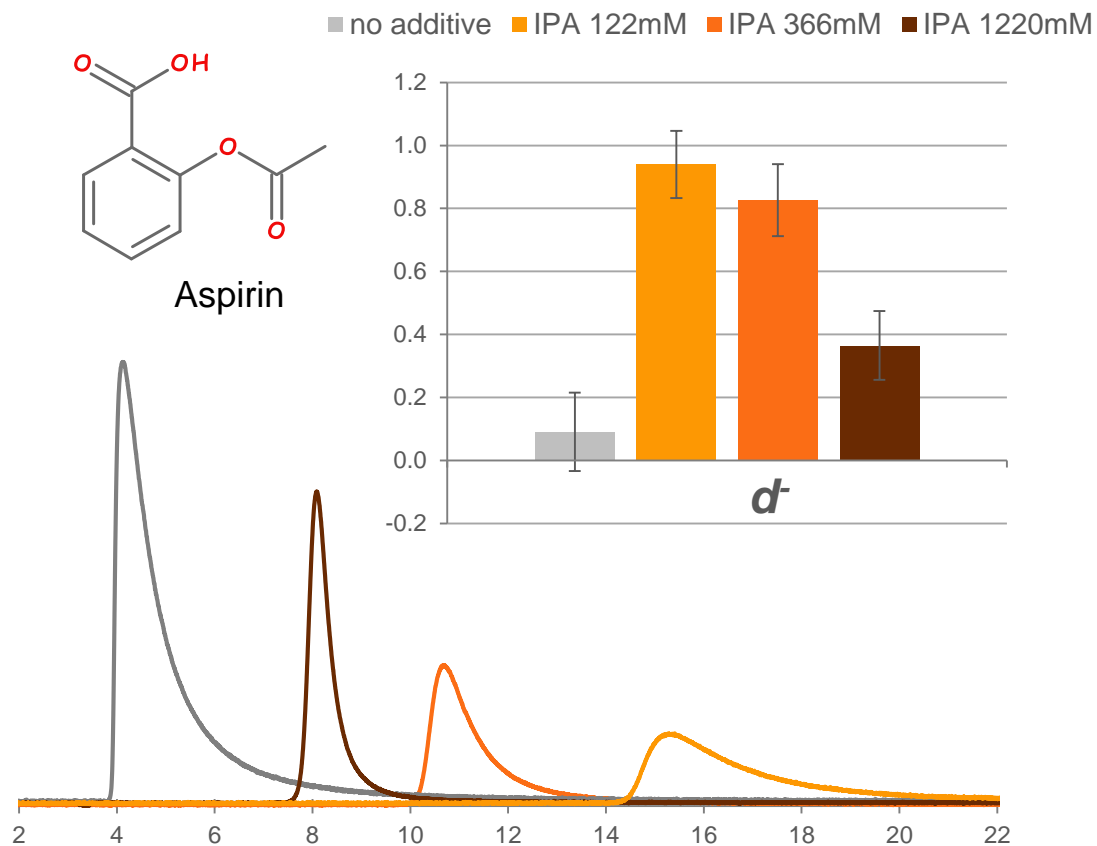
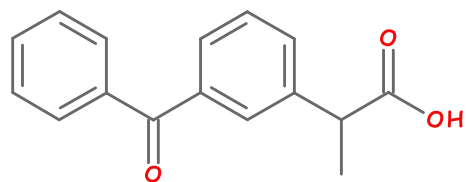


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Ketoprofen

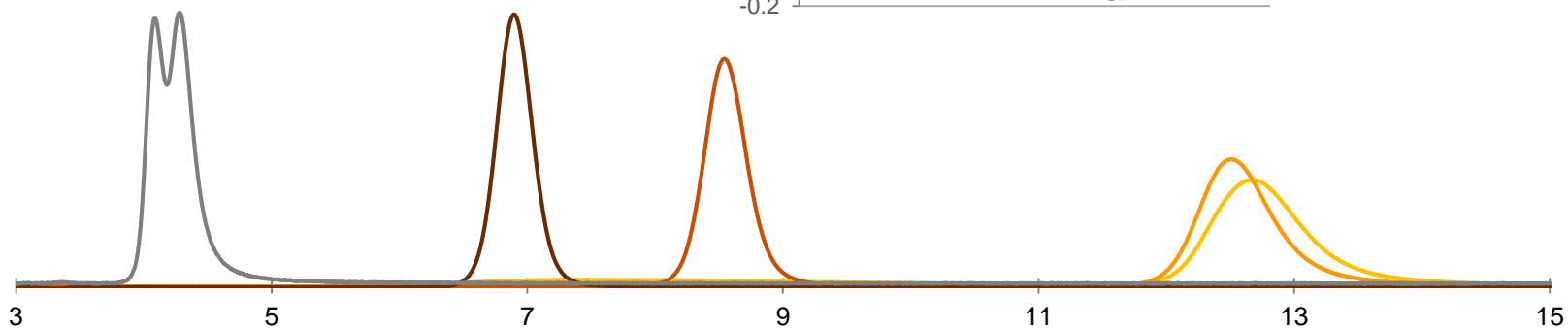
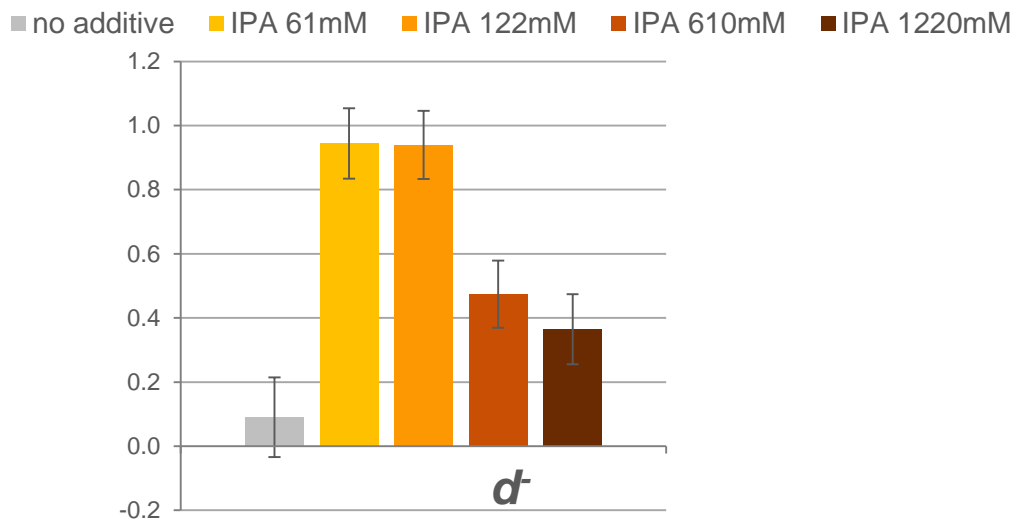


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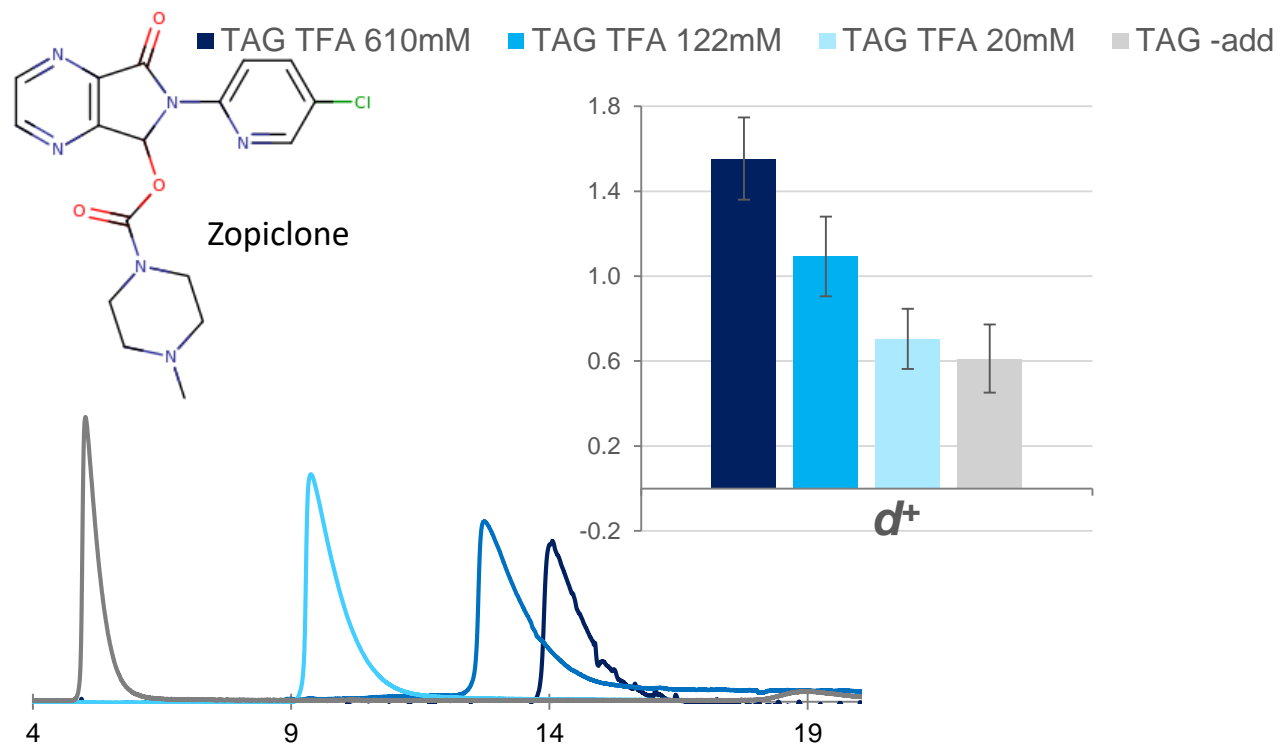


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