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Characterization of three macrocyclic glycopeptide stationary phases in supercritical fluid chromatography

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► **To cite this version:**

Syame Khater, Caroline West. Characterization of three macrocyclic glycopeptide stationary phases in supercritical fluid chromatography. *Journal of Chromatography A*, 2019, 1604, pp.460485 -. 10.1016/j.chroma.2019.460485 . hal-03487765

HAL Id: hal-03487765

<https://hal.science/hal-03487765v1>

Submitted on 21 Dec 2021

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Abstract

Macrocyclic glycopeptides have been used as chromatographic stationary phases for over twenty years, particularly for their ability to separate enantiomers. While they are mostly used with buffered aqueous liquid mobile phases, they can also be used in supercritical fluid chromatography (SFC) with mobile phases comprising pressurized carbon dioxide and a co-solvent (like methanol), possibly comprising acidic or basic additives.

In the present study, we compared three macrocyclic glycopeptide stationary phases (Chirobiotic V2, Chirobiotic T and Chirobiotic TAG) in SFC with carbon dioxide – methanol (90:10) containing no additives. First, the interactions contributing to retention are evaluated with a modified version of the solvation parameter model, comprising five Abraham descriptors (E, S, A, B, V) and two additional descriptors to take account of interactions with ionizable species (D^- and D^+). Linear solvation energy relationships (LSER) are established based on the retention of 145 achiral analytes.

Secondly, the contributions of interactions to enantioseparations are discussed, based on the analysis of 67 racemates. The individual success rate on each phase was observed to be moderate, especially as these phases are known to be more efficient when acidic or basic additives are employed. Chirobiotic TAG proved more successful than the other two phases. Discriminant analyses were computed to gain some insight on retention mechanisms, but only Chirobiotic TAG provided interpretable results.

Finally, the effects of a small proportion of acidic or basic additive on enantioseparation with Chirobiotic T stationary phase are briefly discussed.

Keywords: chiral stationary phases; ionic interactions; macrocyclic glycopeptides; solvation parameter model; supercritical fluid chromatography.

48 **1. Introduction**

49

50 Macrocyclic glycopeptides are a class of antibiotics that were found to provide
51 interesting chiral selectors for chromatography 25 years ago by the group of
52 Armstrong [1]. As they are natural molecules produced by bacterial fermentation,
53 several different molecules covalently linked to silica gel (formerly principally 5- μm
54 fully porous particles) were explored as possible enantioselective stationary phases,
55 among which the most frequently cited are ristocetin (R), vancomycin (V) and
56 teicoplanin (T) (Figure 1). The latter was also modified to remove the three glycosidic
57 groups and produce another chiral selector, teicoplanin aglycon (TAG) that provides
58 significantly different enantioselectivity from glycosylated teicoplanin [2]. The sugar
59 units may indeed hinder the access to some chiral recognition sites (for amino acids,
60 for instance), while they seem to favour enantioselectivity in other cases. These
61 stationary phases are marketed under the trade name Chirobiotic®. Other, non-
62 commercialized antibiotics were explored in different publications.

63 Following the general trends observed in high-performance liquid chromatography,
64 similar phases based on sub-2 μm fully porous particles [3,4] or sub-3 μm
65 superficially porous particles [5–7] were recently introduced. The benefit of both is
66 the significantly increased efficiency, with the extra advantage of low pressure drops
67 when superficially porous particles are employed.

68 All these stationary phases are mostly employed in high-performance liquid
69 chromatography (HPLC). As they are multimodal [8], they may be employed in
70 normal-phase [9,10], reversed-phase [1,9,10], polar organic [4,10] or polar ionic
71 mode [7,11]. Besides, they have also been applied previously in capillary
72 electrophoresis (CE) [12,13], capillary electrochromatography [14], thin-layer
73 chromatography (TLC), enhanced-fluidity liquid chromatography (EFLC) both in the
74 normal-phase and reversed-phase modes [15] and supercritical fluid chromatography
75 (SFC) [4,15–18].

76 While other fluids have been employed in the past, SFC is (now) essentially based
77 on the use of pressurized carbon dioxide as the principal component of the mobile
78 phase, together with a co-solvent (mostly short-chain alcohols). Because of the
79 attributes of carbon dioxide (favourable health and safety features, low price, easy-to-
80 remove after preparative separations) and the inherent advantages of the technique

81 (low-viscosity fluid and high diffusivities providing high efficiencies even at high flow
82 rates), SFC was always a favourite to achieve enantioseparations combining high
83 resolution to short analysis time. While the largest part of enantioselective SFC is
84 conducted on polysaccharide-type stationary phases due to their broad applicability
85 and high loadability at preparative scale, macrocyclic antibiotic stationary phases still
86 hold a place there, thanks to their complementarity to polysaccharides, making them
87 particularly fit for specific applications like amino-acids or amines. Indeed, while
88 polysaccharide phases are most effective with neutral analytes, antibiotics, bearing
89 ionizable groups (carboxylic acids and amines), are particularly useful in the
90 enantioseparation of ionizable species. They are of course also capable to resolve
91 some neutral species.

92
93 Because enantioseparations (in gas, liquid or supercritical fluid) are still largely
94 achieved through trial-and-error processes, it is desirable to improve the
95 understanding of the mechanisms of retention and enantiorecognition. It was
96 previously demonstrated how some understanding of retention mechanism on chiral
97 stationary phases (CSP) could be gained from the use of linear solvation energy
98 relationships (LSER) [9,19–22]. With a modified version of the solvation parameter
99 model, comprising five Abraham descriptors (E, S, A, B, V) [23] and two additional
100 descriptors that were introduced to bring complementary information related to
101 analyte flexibility (F) and sphericity (or globularity, G) [21,22], we have previously
102 examined polysaccharide CSP in normal-phase HPLC and SFC [24–26]. While F and
103 G descriptors usually had little or no contribution to retention, they were very
104 significant to enantiorecognition. However, this model was developed solely for
105 neutral molecules. This is a limitation when macrocyclic glycopeptide CSP are
106 concerned, because they carry ionizable functions (free acid and amine functions). In
107 HPLC, these CSP are mostly employed with organic solvents (acetonitrile or
108 methanol) combined to aqueous buffers. The variation of buffer pH is causing
109 changes in the contributions of the acid and base functions, which are significantly
110 affecting the enantiorecognition capabilities of the stationary phases. Therefore, a
111 useful retention model on these CSP should take account of interactions occurring
112 for ionizable species.

113 We have previously demonstrated that another modified version of the solvation
114 parameter model, comprising five Abraham descriptors (E, S, A, B, V) and two

115 additional descriptors to take account of ionic interactions with anions and cations (D^-
116 , D^+) was useful to characterize retention mechanisms on a variety of achiral
117 stationary phases in hydrophilic interaction liquid chromatography (HILIC) [27,28],
118 mixed-mode HPLC [29] or SFC [30–32]:

119
120
$$\log k = c + eE + sS + aA + bB + vV + d^-D^- + d^+D^+ \quad (1)$$

121
122 In this equation, capital letters represent the solute descriptors, related to particular
123 interaction properties, while lower case letters represent the system constants,
124 related to the complementary effect of the phases on these interactions. c is the
125 model intercept term and is dominated by the phase ratio. E is the excess molar
126 refraction (calculated from the refractive index of the molecule) and models
127 polarizability contributions from n and π electrons; S is the solute dipolarity /
128 polarizability; A and B are the solute overall hydrogen-bond acidity and basicity; V is
129 the McGowan characteristic volume in units of $\text{cm}^3 \text{mol}^{-1}/100$; D^- represents the
130 negative charge carried by anionic and zwitterionic species, and D^+ represents the
131 positive charge carried by cationic and zwitterionic species (see previous works for
132 further details on the calculation of D terms [27,30]). D^- and D^+ can also represent
133 partial charges on ionizable species, as they depend on the mobile phase pH.

134
135 In the present paper, the modified solvation parameter model (Eq. (1)) is employed to
136 characterize three macrocyclic (glyco)peptide CSP based on vancomycin, teicoplanin
137 and teicoplanin aglycone in supercritical fluid chromatography. Secondly, the
138 enantioseparation capability of the three phases is compared, based on the analysis
139 of 67 model racemates. Finally, the effects of introducing a small concentration of
140 acidic or basic additive in the mobile phase are observed on the teicoplanin
141 stationary phase.

142
143

144 **3. Material and methods**

145

146 **3.1. Stationary phases**

147

148 The three stationary phases used in this study are commercially available from
149 Sigma-Aldrich: Astec Chirobiotic T, TAG and V2 phases were all packed in 150 x 4.6
150 mm columns, with fully porous 5 μm silica particles. The charge state depending on
151 mobile phase pH was calculated based on aqueous pK_a values determined with
152 Chemicalize program (<http://www.chemicalize.org/>).

153

154 **3.2. Chemicals**

155

156 The solvent used was HPLC-grade methanol (MeOH) provided by VWR (Fontenay-
157 sous-Bois, France). Carbon dioxide of industrial grade 99.5% was provided by Air
158 Liquide (France). Diethylamine was obtained from Sigma-Aldrich (Saint-Quentin
159 Fallavier, France); formic acid was provided by VWR. Solutions of all test compounds
160 were prepared in MeOH. 150 achiral test compounds (Table S1), comprising 115
161 analytes that should be charge-neutral in the present operating conditions, and 35
162 species that may be partly or completely ionized (14 anionic and 21 cationic form),
163 were obtained from a range of suppliers. 67 chiral racemic test compounds (Table
164 S2) were used to assess the complementarity of the three phases in terms of
165 enantioseparation and served to evaluate the contributions of molecular features to
166 enantiorecognition. Among those, 45 analytes should be charge-neutral in the
167 present operating conditions, and 22 species may be partly or completely ionized (15
168 anionic and 7 cationic form).

169 The Abraham solute descriptors (E, S, A, B, V) used for LSERs with Eq. (1) were
170 extracted from an in-house database established from all available literature on the
171 solvation parameter model (Tables S1 and S2). The two additional descriptors for
172 charges (D^- and D^+) were calculated based on aqueous pK_a values determined with
173 Chemicalize program and apparent pH 5. The additional descriptors for flexibility (F)
174 and globularity (G) were computed as previously described [21,22]. The series of
175 test-compounds has been selected by observing the requirements of a good LSER
176 analysis [33]. The compounds were chosen so as to provide a uniform distribution of
177 each descriptor within a wide enough space and absence of cross-correlation
178 among the descriptors was checked.

179

180 **3.3. Chromatographic system and conditions**

181

182 Chromatographic separations were carried out using an ACQUITY Ultra Performance
183 Convergence Chromatography™ (UPC²) system from Waters (Millford, MA, USA).
184 The system was equipped with a binary solvent delivery pump compatible with
185 mobile phase flow rates up to 4 mL/min and pressures up to 414 bar, an
186 autosampler, a backpressure regulator, a column oven compatible with 150 mm
187 length columns, and a photodiode-array (PDA) detector. Chromatograms were
188 recorded with Empower® 3 software.

189 The detection wavelength was 210 nm.

190 The mobile phase used in this study is always CO₂-MeOH (v/v) with or without an
191 additive. When an additive was present, it was introduced at 0.1% in the methanol
192 co-solvent. Flow rate was 3 mL min⁻¹. The oven temperature was set at 25°C and the
193 outlet pressure was maintained at 15 MPa. Strictly speaking, the fluid is thus not
194 “supercritical” but could be called “subcritical”. No distinction will be made in the
195 following.

196

197 **3.4. Data analysis**

198

199 Multiple linear regressions (MLR) and discriminant analysis (DA) were performed
200 using XLStat 2015.2.02 software (Addinsoft, New York, NY). The quality of the MLR
201 fits was estimated using the adjusted determination coefficient (R^2), standard error in
202 the estimate (SE) and Fisher F statistic. The statistical significance of individual
203 coefficients was evaluated with the 95% confidence intervals.

204 The quality of DA was estimated based on ROC (receiver operating characteristics)
205 curves and confusion matrices.

206

207 **4. Results and discussion**

208

209 The three macrocyclic (glyco)peptides employed in this study are presented in Figure
210 1.

211 Vancomycin (Chirobiotic V2) possesses 3 fused rings (some of which may be
212 opened during the bonding process [1]), 18 stereogenic centres, one pendant
213 disaccharide group, one free acid function (highlighted with an orange ellipse in
214 Figure 1) and two free basic functions: one secondary amine function on a peptide

215 lateral chain and one primary amine function in the glycosidic groups (highlighted
216 with green ellipses in Figure 1).

217 Teicoplanin (Chirobiotic T) possesses 4 fused rings, 23 stereogenic centres, 3
218 attached glycosidic groups (among which one has a hydrophobic acyl chain), one
219 free acidic group and one free primary amine group. Finally, as indicated by the
220 name, teicoplanin aglycon (Chirobiotic TAG) is the same as teicoplanin, minus the
221 three glycosidic groups, which is reducing the number of stereogenic centres to 8.
222 Not represented on this figure with planar structures, the three-dimensional shape
223 should be somewhat folded, in a basket shape [13]. At least, this is the natural shape
224 of these molecules prior to bonding. Bonding may occur through the amine functions
225 (better nucleophile) [34], thereby forming urea functions, but also through the
226 phenolic groups [1,2] and forming carbamate functions. Availability of the amine
227 function is thus improbable. In addition, there is no saying what the exact three-
228 dimensional shape is when they are bonded to silica, especially in a pressurized
229 carbon dioxide – methanol environment, as there is evidence that the
230 chromatographic behaviour of macrocyclic peptides is highly dependent on the
231 environment [10,16,35]. Significant changes in conformation may occur, depending
232 on mobile phase composition. Nevertheless, these figures illustrate the variety of
233 possible interactions that may occur between such complex structures and analytes.

234

235 ***4.1. Interactions contributing to retention***

236

237 The results of LSER characterization are presented in Figure 2, with complete data
238 and statistics available in Table 1. The statistics were reasonably good, with R^2_{adj}
239 values ranging from 0.87 to 0.89 and standard deviation in the estimate of about 0.21
240 for all three columns. These results are clearly not as good as is usually observed
241 when the solvation parameter model is applied in gas chromatography or reversed-
242 phase liquid chromatography but, considering the complexity of the system used
243 here, they should be good enough to interpret the contributions of interactions to
244 retention.

245

246 A first general observation is that the three phases share the same pattern of
247 interactions with positive e , s , a , b and d^+ terms, and negative v and d^- terms. This

248 pattern is relevant of normal-phase mode of retention, as is usually observed when
249 polar stationary phases are used in SFC, with:

- 250 - polar interaction terms being positive, meaning that polar molecules interact
251 favourably with the stationary phase;
- 252 - v term being negative, indicating that large molecular volume is unfavourable
253 to insertion in the stationary phase, as may be expected from the size of the
254 fused rings. It is however impossible to judge whether the analytes are
255 included in the macrocycles or not.

256 The most significant contributions are those of hydrogen bonding interactions (a and
257 b). Hydrogen bonding should occur mostly through the numerous peptide functions,
258 phenolic functions (unless they are involved in covalent bonding to the silica surface)
259 and hydroxyl functions from the glycosidic groups. Judging that no significant
260 difference is observed in these terms between the teicoplanin and teicoplanin
261 aglycone phases, we may conclude that the glycosidic groups are however not the
262 major contributors to the measured hydrogen bonding interactions. This is also
263 consistent with previous observations in HPLC indicating that the overall polarity of
264 teicoplanin and teicoplanin aglycone stationary phases was rather close [2].

265 Hydrogen bonding with proton donor analytes (a term) is larger on the vancomycin
266 phase than on the two teicoplanin phases, indicating that proton donors should be
267 more strongly retained on this stationary phase. On the opposite, vancomycin has
268 lower contribution of hydrogen bonding with proton acceptor analytes (b term) than
269 the teicoplanin phases. While the contribution of proton-donor and proton-acceptor
270 hydrogen bonding on the teicoplanin phases is mostly the same (identical values of a
271 and b terms), the contribution of b -type interactions is twice less than a -type
272 interactions in vancomycin. The presence of urea functions resulting from the
273 attachment of two free amine functions in vancomycin, compared to only one free
274 amine function in teicoplanin phases may be responsible for stronger interactions
275 with proton-donor analytes (a term) in the former. Different accessibility to the silica
276 gel underneath the macrocyclic peptides may also explain the differences observed,
277 but this is impossible to assess. Finally, in the case of Chirobiotic V2 and TAG, the
278 three-step bonding procedure involves modification of the supporting silica gel with
279 amino-propyl groups [2]. The pendant amine groups which may remain after bonding
280 of the peptides may also contribute to hydrogen bonding with the analytes. In the
281 case of Chirobiotic T, the two-step bonding procedure involving 3-(triethoxysilyl)-

282 propyl-isocyanate should leave no amine functions but perhaps some unreacted
283 isocyanate functions.

284

285 The contributions of e and s terms are also positive, although less significant than
286 hydrogen bonding. Positive e term may result principally from π - π interactions
287 between the aromatic probe solutes and the numerous aromatic rings in the
288 macrocycles. Positive s term results from dipole-dipole interactions with all polar
289 functions in the stationary phase.

290

291 In the present operating conditions, the apparent pH of the mobile phase should be
292 close to 5 [36]. The aqueous pK_a values of acid and basic functions were indicated to
293 be respectively approximately 2.9, 10.4 and 11.7 on vancomycin; 2.8 and 11.0 on
294 teicoplanin; 2.7 and 11.1 on teicoplanin aglycon [37]. Computation of pK_a values and
295 charge state in the free (non-bonded) peptides was achieved here with Chemicalize
296 program. The curves of charge state vs. pH are presented in Figure 3. A number of
297 uncertainties are attached to these curves as the pK_a values in pressurized carbon
298 dioxide – methanol mixture may differ from aqueous pK_a values, and because the full
299 availability of all ionizable functions is unknown. For instance, while it is likely that the
300 amine functions served as primary attachment points (most reactive nucleophile),
301 additional linkages may exist through the phenolic groups. In the case of
302 vancomycin, only one amine function out of two may be bonded to the silica surface.
303 However, it seems reasonable to assume that both the carboxylic acid and the free
304 amine function (possibly remaining in the case of vancomycin) would be partially or
305 totally ionized.

306 The anionic acid function is then expected to participate in positive ionic interactions
307 with cationic analytes, but also to repulsive interactions with anionic analytes.

308 Conversely, cationic amine functions (provided free amines remain) are expected to
309 participate in positive ionic interactions with anionic analytes and repulsive
310 interactions with cationic analytes. Here again, accessible ionizable groups from the
311 supporting silica may also contribute to the interactions with ionizable species: (i)
312 residual silanol groups, whether they are ionized or neutral, may contribute to
313 attraction of cationic species and repulsion of anionic species; (ii) amino-propyl
314 groups bonded to the silica surface prior to attachment of the peptide (in the case of

315 Chirobiotic V2 and TAG), may contribute to attraction of anionic species and
316 repulsion of cationic species.

317 On all three columns, the d' term is negative, and the d^+ term is positive. This
318 suggests that the acid function would be easily accessible, thereby contributing to
319 overall positive retention of cations and repulsion of anions. The possibly free amine
320 function on vancomycin phase may explain more repulsion of cations than in the
321 teicoplanin phases (significantly lower d^+ term, nearly not significant), but is
322 apparently not contributing to favourable interactions with anions (no change in d'
323 term).

324 Finally, accessibility to the silica surface and a large number of silanol groups
325 (compared to the small number of acidic and basic functions of the ligands) may also
326 be a contributor to positive interactions with cations and repulsion of anions.
327

328 To some extent, the mobile phase employed here (carbon dioxide – methanol,
329 comprising no additive) should be comparable to normal-phase mode with heptane-
330 ethanol mobile phase (data in Table 1). LSER models reported in the literature for the
331 three stationary phases in heptane-ethanol 90:10 mobile phase [9,19,38] are
332 presented in Table 1 with the results obtained in the present conditions (carbon
333 dioxide-methanol 90:10). The reported models relate only the five Abraham
334 descriptors so only those shall be compared. As often observed in normal-phase
335 HPLC whatever the stationary phase, the e coefficient was zero in the reported LSER
336 models. However, the s term is larger in the NPLC models than in the SFC models,
337 while the e and s terms are known to co-vary to some extent. The relative strength of
338 hydrogen bonding interactions is also significantly affected. As pointed out above, the
339 a and b terms had comparable values for the teicoplanin and teicoplanin aglycone
340 stationary phases in SFC, while the NPLC data show much larger b values than a
341 values, indicating that proton donors are less retained in NPLC than SFC, while
342 electron donors are much more retained in NPLC. Comparable variations in e , s , a
343 and b terms were previously noted on polysaccharide CSP when comparing the
344 same mobile phase compositions in SFC and NPLC [26]. Finally, the v term is
345 negative in both SFC and NPLC modes, as is normally observed in normal-phase
346 chromatographic modes. We may have expected to observe higher v terms on
347 teicoplanin than the other two, due to the presence of a long hydrophobic chain in
348 teicoplanin, but no such thing is observed.

349 LSER models were also reported on macrocyclic peptide stationary phases in
350 reversed-phase conditions [9,19,39], or in polar ionic mode [20], which show no
351 relation to the models obtained in the present paper.

352

353 **4.2. Enantioseparation capabilities**

354

355 Based on the analysis of the 67 racemates presented in Table S2, we examined the
356 complementarity between the three phases with Venn diagrams (Figure 4). First of
357 all, it is important to notice that no additives were employed in the mobile phase,
358 which is rather uncommon for peptide stationary phases and not very favourable to
359 their enantiorecognition capabilities. These observations must then not be
360 considered as generally applicable to chiral SFC. In other operating conditions
361 including acidic or basic additives, some of the enantiomeric pairs that were
362 unresolved in this work may well be resolved [18,40]. Clearly, our intention in this
363 paper was mostly placed on understanding the features of the three stationary
364 phases than on the achievement of high success rates.

365 The first observation is that each CSP provided enantiorecognition only for a
366 relatively small portion of the pairs of enantiomers *explored in the present conditions*:
367 the least successful was the vancomycin selector (12% success rate) and the most
368 successful was the teicoplanin aglycone (37%), with the teicoplanin selector
369 providing an intermediate performance (22%). The teicoplanin aglycone was
370 previously found to be more selective towards acidic compounds than the glycosidic
371 form [2]. The superiority of the aglycone form over the glycosidic form in SFC was
372 previously noted by other authors in liquid-phase or supercritical conditions
373 [18,41,42]. Note that “success” qualified any sign that some enantiorecognition
374 occurred, in other words whenever the separation factor was strictly superior to 1,
375 whatever the resolution. If a more constraining criterion had been used, like
376 resolution > 1, the “success rate” would have been even lower (and not at all
377 informative).

378 The second observation is that a significant portion of racemates could be separated
379 by two or three CSP. The teicoplanin aglycone was again the most interesting, as it
380 provided a larger number of unique separations. With all three columns combined,
381 about a half of the racemates in Table S2 could be separated with these operating

382 conditions, which are, again, not the most favourable to operating peptide stationary
383 phases.

384 The generally superior capabilities of teicoplanin aglycone may also be observed in
385 Figure 5, where the enantioselectivity is compared for a selection of racemates
386 between the glycosidic and aglycone forms. Apart from a sole case when teicoplanin
387 provided clearly superior enantioselectivity (2,3-epoxypropylbenzene), teicoplanin
388 aglycone was generally found the best.

389
390 As we had occasions to point out in previous studies on polysaccharide CSP [24,25],
391 the interactions contributing to retention may not necessarily be favourable to
392 enantioselectivity and vice-versa. It is therefore necessary to deconvolute these
393 effects. We had previously demonstrated how discriminant analysis could be useful
394 to understand the contributions of different molecular features to enantioseparation
395 [22,24,25]. The method is simply based on classifying the racemates into classes
396 indicating whether or not there are separated, without reference to separation factors.
397 This way, the analysis is not dependent on mixed contributions of enantioselective
398 and non-enantioselective interactions resulting from measurements done in linear
399 conditions [35]. To achieve most significant results, it is best to have a large group of
400 analytes with structural diversity, so as to obtain a sufficient number of analytes in
401 each class (separated or non-separated). Because the number of resolved
402 racemates was rather low in the case of the vancomycin and teicoplanin phases with
403 the present operating conditions, the results of discriminant analyses could not be
404 relied upon. Indeed, the confusion matrices indicated that the information retrieved
405 was not interpretable. Only the results for teicoplanin aglycon are presented in Figure
406 6. In this case, meaningful statistics were obtained: the AUC (area under the curve)
407 for ROC curve was 0.87, and the confusion matrix indicated overall correctly
408 assigned objects as high as 84%.

409 Figure 6 is showing the contributions of structural features to enantioseparation on
410 the teicoplanin aglycon phase. Based on this statistical analysis, we may observe
411 that the most significant contribution is related to negative charges on the analyte as
412 the D^- term is large and positive. Other significant contributions indicate that large
413 volume is deleterious to enantioseparation (negative V term), as are aromaticity and
414 electron-donating properties (negative E and B terms). The presence of dipoles is
415 favourable to enantioseparation (positive S terms). No conclusion can be drawn on

416 other structural features (near-zero values), either because these features would
417 have no influence on enantioseparation or because the structural variance in this set
418 does not reflect their contributions.

419 The significant positive contribution of negative charge (D^-) is in accordance with
420 many previous reports that anionic forms of acidic compounds are better resolved
421 than neutral forms [43]. Interestingly, recent work from Pokrovskiy et al. [44] also
422 demonstrated significant contribution of ionic interactions to enantioseparation on an
423 erythromycin stationary phase in SFC, but in that case interaction between the
424 carboxylate group of the CSP and a basic (possibly protonated) analyte was
425 evidenced.

426 The negative (B term) and moderate (A term) contributions of hydrogen bonding may
427 come as a surprise, as they are generally considered to contribute significantly to
428 enantioselectivity [43]. However, these observations were all based on HPLC
429 experiments. The chemical environment of the antibiotic is surely significantly
430 affecting the interaction capabilities, thus different contributions of interactions in
431 carbon dioxide-methanol mobile phases are not surprising. As pointed out by Ilisz et
432 al. [43], “there is no generally valid conception for chiral recognition of racemic
433 compounds on macrocyclic glycopeptide-based CSPs. There are probably several
434 mechanisms [...] depending on the nature of the analyte and the mode of
435 chromatography”.

436 The negative influence of aromaticity (negative E term) may also be surprising as π -
437 π interactions are normally considered to be beneficial to enantioselectivity on these
438 stationary phases. As the discriminant analysis is a statistical approach, it does not
439 mean that any aromatic ring would be deleterious to enantioselectivity. For instance,
440 the enantiomers of (2,3-epoxypropyl)-benzene were well resolved, as appears in
441 Figure 5. However, Chen and Ward report some negative contribution of π - π
442 interactions when the aromatic ring is distant from the chiral centre [45]. As the E
443 descriptor is taking account of all π electrons in the analyte, whatever their position
444 relative to the chiral centre, opposite effects cannot be deconvoluted with this
445 methodology. Chen and Ward had also demonstrated that chiral recognition in
446 teicoplanin stationary phases were highly sensitive to the size of the analyte in the
447 polar organic mode, which is in accordance with the observed negative contribution
448 of V term in the present work. Steric repulsion was also significant to explain the
449 enantioseparation of chiral sulfoxides on teicoplanin [41]. As teicoplanin aglycon

450 should be rather rigid (especially as no pending sugar units may be moving there, as
451 is the case with vancomycin and teicoplanin), the negative contribution of size (V
452 term) was not unexpected: the chiral selector cannot conformationally adapt to large
453 molecules.

454 Previous works employing Abraham descriptors to obtain some information on the
455 chiral recognition mechanism on Chirobiotic T in normal-phase HPLC mode also
456 showed negative influence of basic character (b term) and positive influence of
457 dipole-dipole interactions (s term), but the contribution of molecular volume was
458 found to be positive (v term). As these observations were based on a single pair of
459 enantiomers, they may not be conclusive.

460

461

462 **4.3. Effect of an acidic or basic additive on enantioseparation**

463

464 Because the macrocyclic peptide CSP are known to behave differently when pH of the
465 mobile phase is varied [10], the influence of one acidic and one basic additive on
466 enantioseparation with Chirobiotic T was examined. In a previous study conducted in
467 SFC on ristocetin stationary phase, selectivity was found to be unchanged or
468 increased with acetic acid, whereas trimethylamine had the opposite effect [16]. On a
469 vancomycin stationary phase, basic additives were found to slightly reduce selectivity
470 [17].

471 In the present work, in most cases, when some enantioseparation was observed
472 without an additive, the separation factor appeared to reduce with formic acid, and
473 reduce even more with diethylamine. In many cases, enantioseparation was
474 completely lost when the basic additive was employed. Selected cases are presented
475 in Figure 7. 2-Phenylbutyric acid is a significant exception, showing a much larger
476 enantioseparation with diethylamine than in the other two conditions. It may be
477 surprising to observe that both acidic and basic additive cause a loss of
478 enantioselectivity.

479

480 These observations are however very interesting and should prompt further
481 examination of additives influence on the retention and separation mechanisms with
482 these CSP.

483

484 **5. Conclusions**

485

486 Three stationary phases based on macrocyclic (glyco)peptides were compared using
487 a large number of achiral and chiral analytes in supercritical fluid chromatography
488 with carbon dioxide – methanol mobile phases. Chirobiotic TAG, based on
489 teicoplanin aglycone, proved to be superior to the glycosylated version and to
490 vancomycin as a chiral selector. Chemometric methods were applied to gain some
491 insights in the retention mechanisms (LSER methodology) and separation
492 mechanisms (discriminant analysis). The introduction of interaction terms related to
493 the presence of charges (D^- and D^+) was essential to achieve a complete description
494 of these ionizable stationary phases. The effects of acidic and basic additives were
495 only lightly observed in this paper and shall be explored in more details in further
496 works.

497

498

499 **Acknowledgment**

500 Dave Bell (formerly at Sigma Aldrich) is acknowledged for the kind gift of columns for
501 this study. We also thank Waters for the support received through the Centers of
502 Innovation (COI). CW also thanks the Institut Universitaire de France (IUF), of which
503 she is a junior member, for the support received.

504

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506

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- 661
- 662

663 **Figure Caption**

664

665 **Figure 1.** Structures of the macrocyclic glycopeptides in the three stationary phases
666 examined here. The peptides are bonded to silica particles. Orange and green
667 ellipses highlight the free acidic and basic functions in each antibiotic selector.

668

669 **Figure 2.** Histograms representing LSER system constants calculated with the
670 retention data measured for the 150 analytes in Table S1 and Eq. (1).
671 Chromatographic conditions: CO₂-solvent 90:10 (v/v), 25°C, 15 MPa, 3 mL/min. From
672 left to right, Chirobiotic V2 (orange), Chirobiotic T (light blue) and Chirobiotic TAG
673 (dark blue). Error bars indicate the 95% confidence limits.

674

675 **Figure 3.** Total charge of vancomycin and teicoplanin as a function of mobile phase
676 pH, as calculated by Chemicalize.

677

678 **Figure 4.** Venn diagram showing the complementarity of the three stationary phases
679 for the enantioseparation of the 67 chiral analytes in Table S2. NB: “success” is
680 defined as separation factor > 1.

681

682 **Figure 5.** Histograms representing the logarithm of separation factors for selected
683 racemates analyzed with Chirobiotic T (light blue) and Chirobiotic TAG (dark blue).
684 Other conditions as in Figure 2.

685

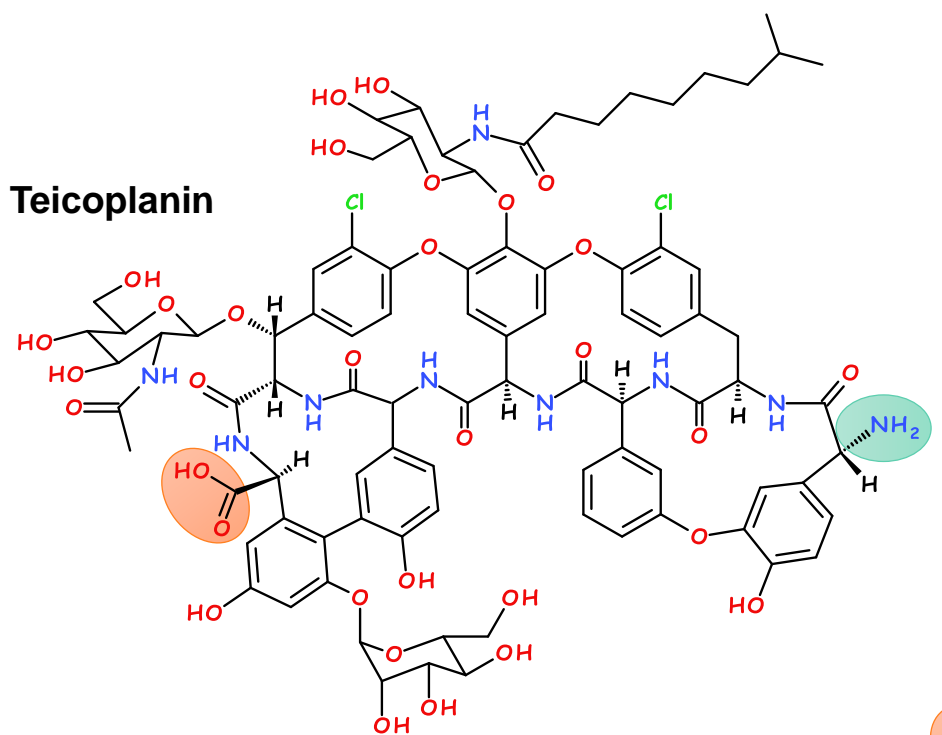
686 **Figure 6.** Discriminant analysis between non-separated (left) and separated (right)
687 racemates with Chirobiotic TAG using nine molecular descriptors as variables and
688 the 67 racemates in Table S2. Other conditions as in Figure 2. Negative features are
689 common to the racemates that were not separated on this stationary phase, positive
690 features are common to separated racemates.

691

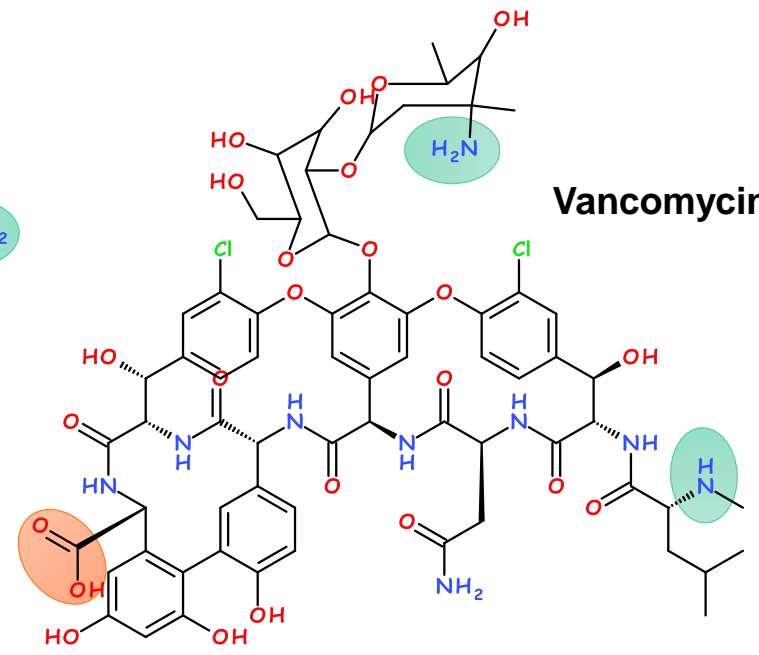
692 **Figure 7.** Histograms representing the logarithm of separation factors for selected
693 racemates analyzed with Chirobiotic T and different composition of mobile phase: no
694 additive (blue), 0.1% formic acid (red) or 0.1% diethylamine (green). Other conditions
695 as in Figure 2.

696

Teicoplanin



Vancomycin



Teicoplanin aglycon

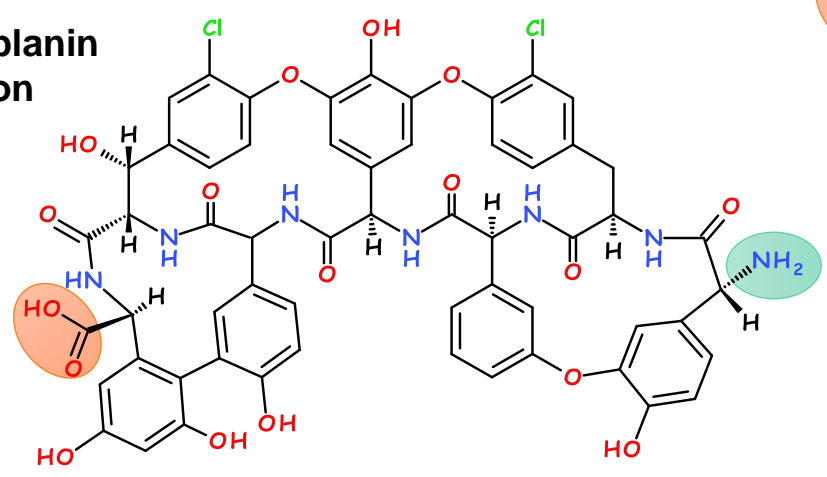


Figure 1.

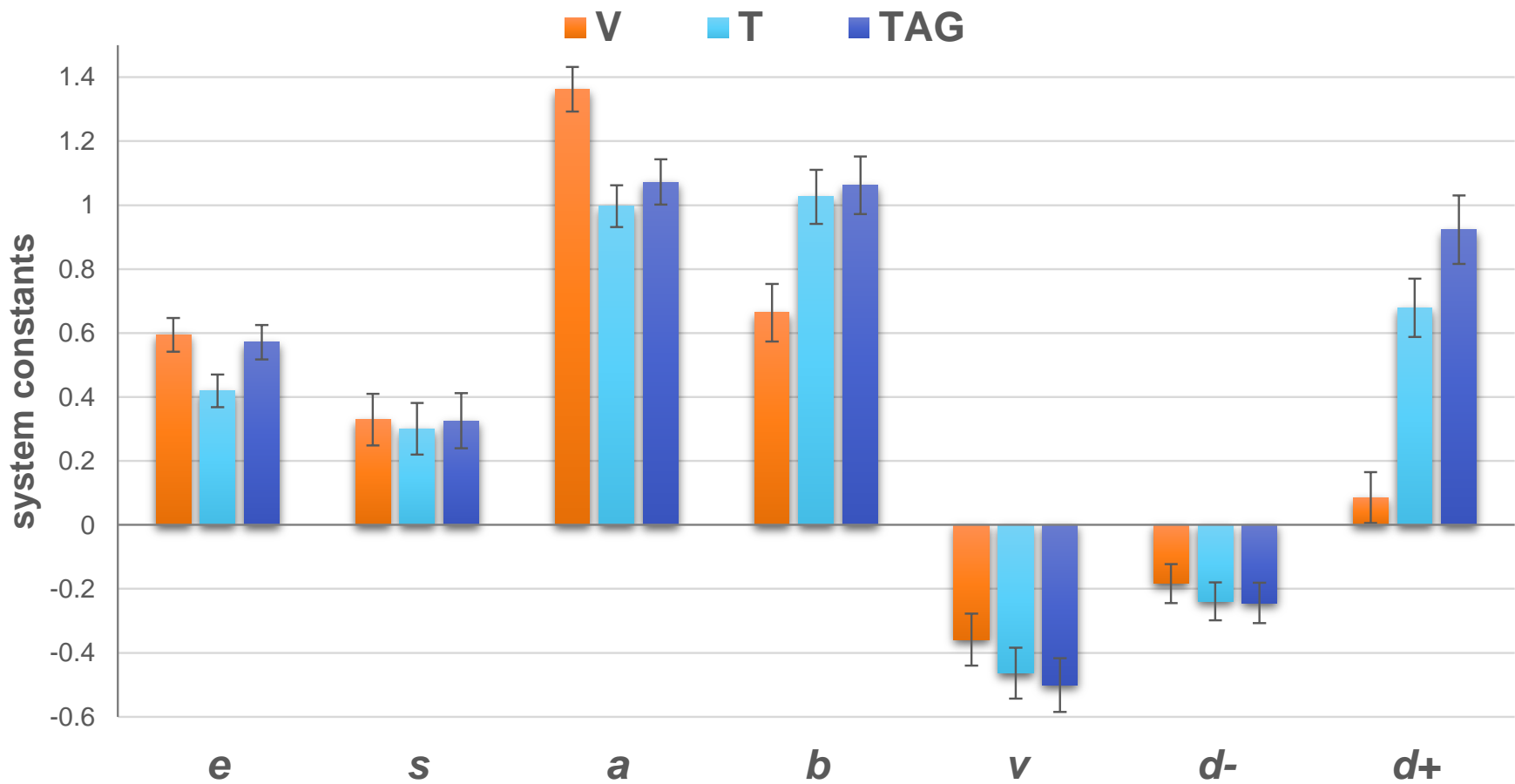


Figure 2.

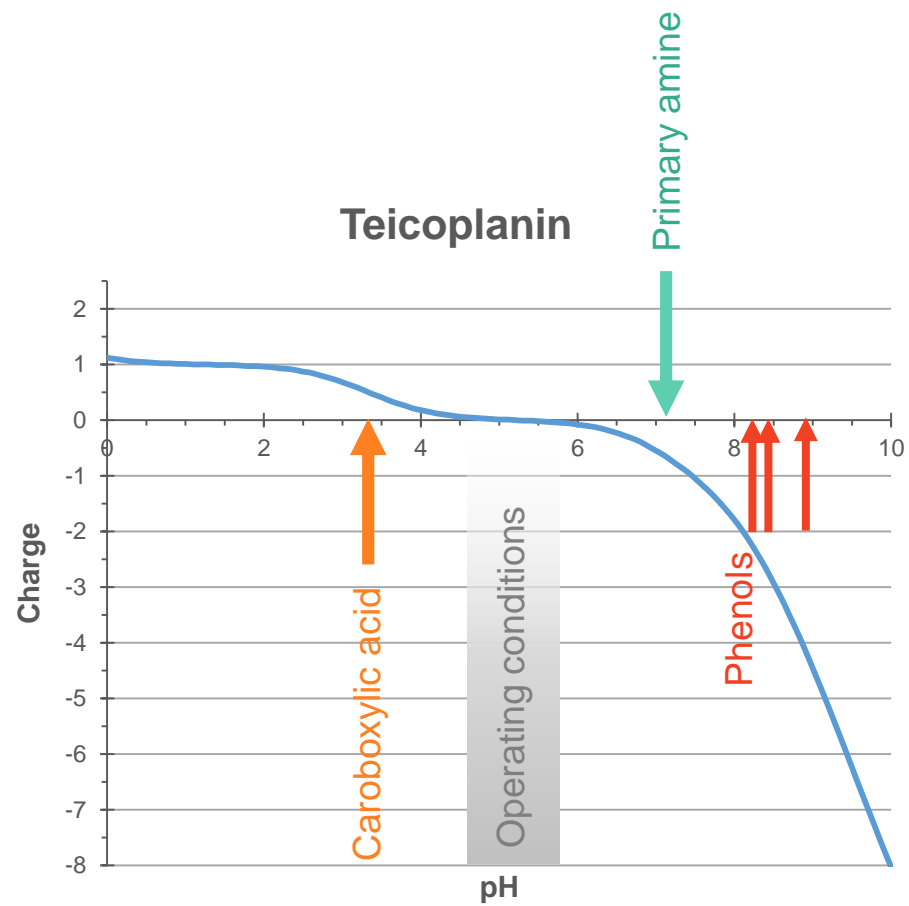
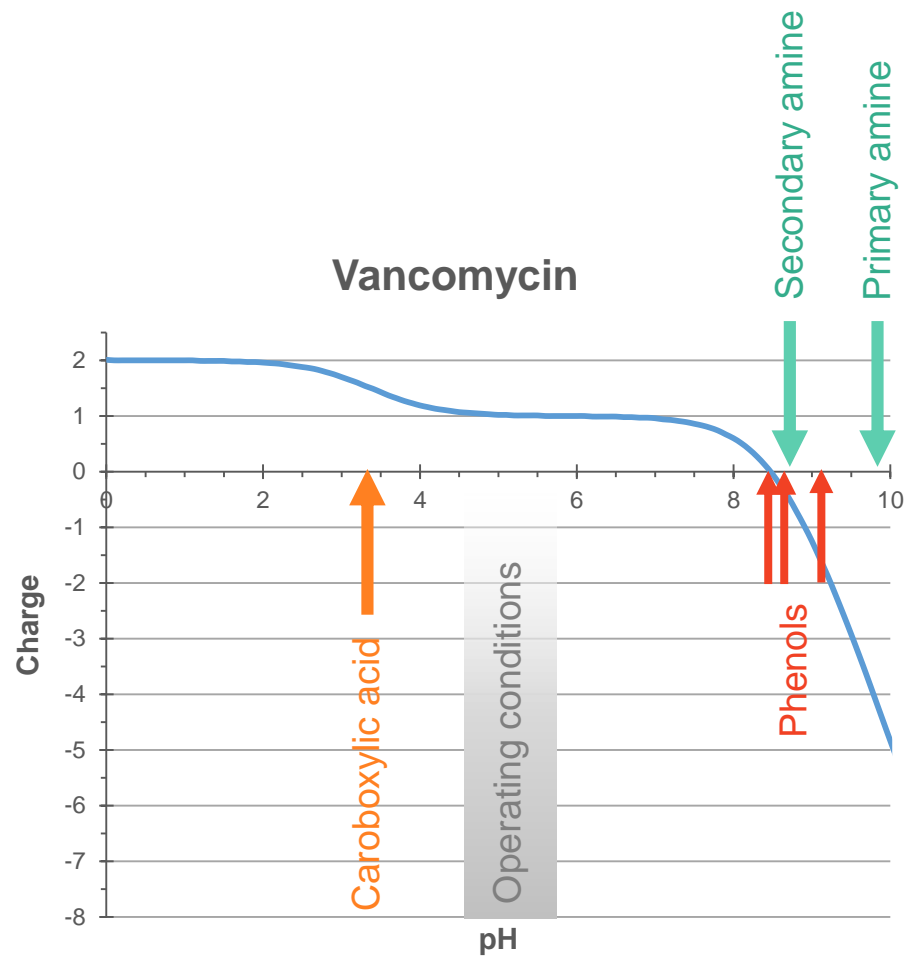


Figure 3.

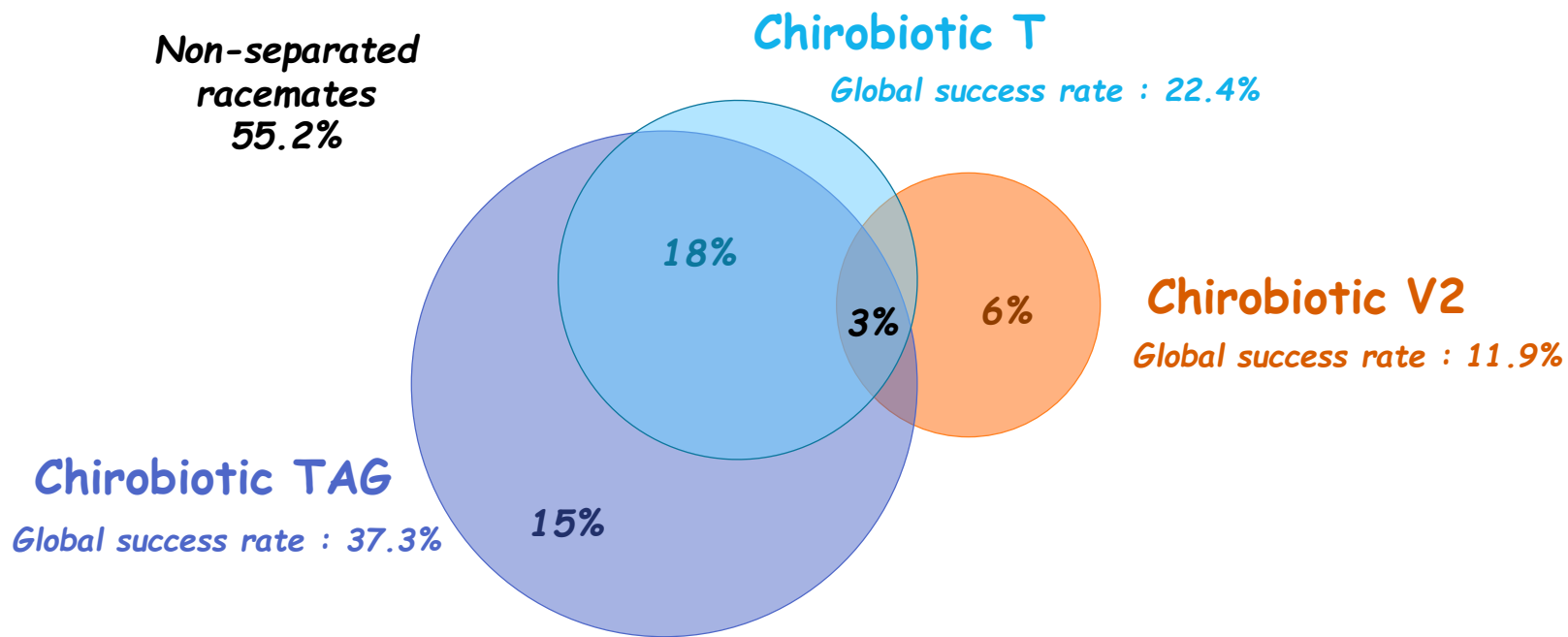


Figure 4.

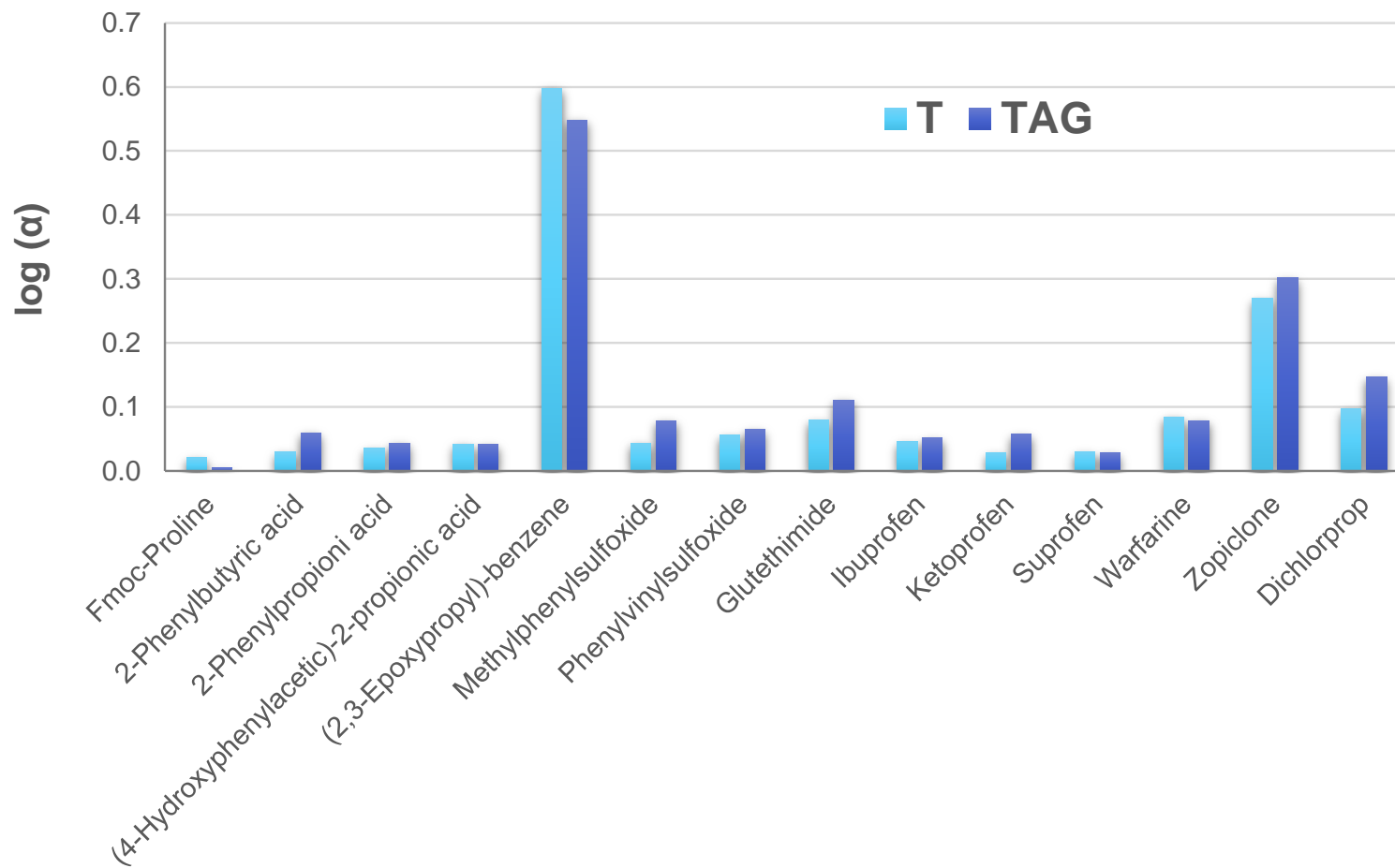


Figure 5.

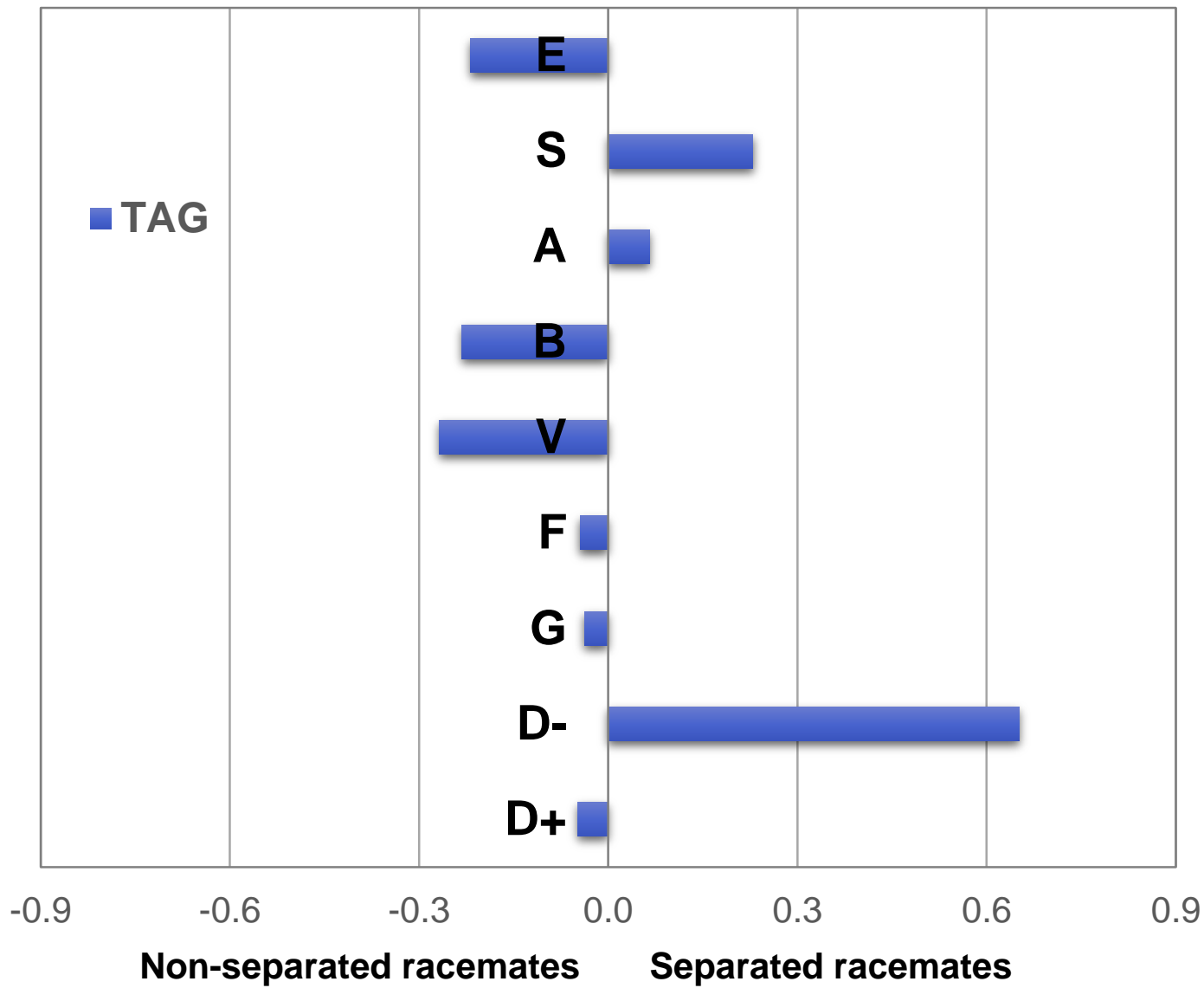


Figure 6.

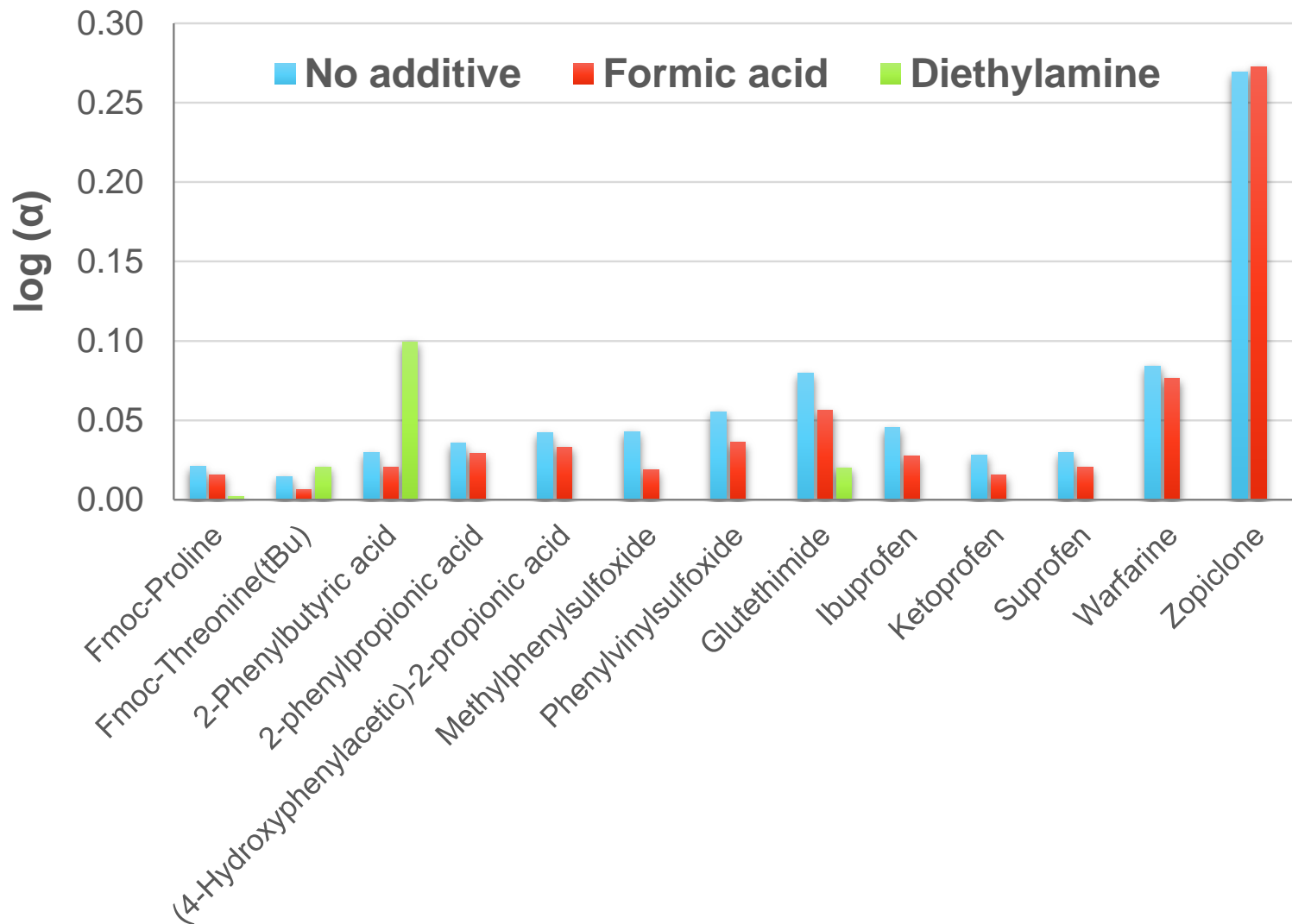


Figure 7.

