

# Characterization of three macrocyclic glycopeptide stationary phases in supercritical fluid chromatography

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1	Characterization of three n	nacrocyclic glycopeptide stationary phases in					
2	supercr	itical fluid chromatography					
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15

## 16 Abstract

17

18 Macrocyclic glycopeptides have been used as chromatographic stationary phases for 19 over twenty years, particularly for their ability to separate enantiomers. While they are 20 mostly used with buffered aqueous liquid mobile phases, they can also be used in 21 supercritical fluid chromatography (SFC) with mobile phases comprising pressurized 22 carbon dioxide and a co-solvent (like methanol), possibly comprising acidic or basic 23 additives. 24 In the present study, we compared three macrocyclic glycopeptide stationary phases 25 (Chirobiotic V2, Chirobiotic T and Chirobiotic TAG) in SFC with carbon dioxide -26 methanol (90:10) containing no additives. First, the interactions contributing to 27 retention are evaluated with a modified version of the solvation parameter model, 28 comprising five Abraham descriptors (E, S, A, B, V) and two additional descriptors to 29 take account of interactions with ionizable species (D<sup>-</sup> and D<sup>+</sup>). Linear solvation 30 energy relationships (LSER) are established based on the retention of 145 achiral 31 analytes. Secondly, the contributions of interactions to enantioseparations are discussed, 32 33 based on the analysis of 67 racemates. The individual success rate on each phase 34 was observed to be moderate, especially as these phases are known to be more 35 efficient when acidic or basic additives are employed. Chirobiotic TAG proved more 36 successful than the other two phases. Discriminant analyses were computed to gain 37 some insight on retention mechanisms, but only Chirobiotic TAG provided 38 interpretable results. 39 Finally, the effects of a small proportion of acidic or basic additive on 40 enantioseparation with Chirobiotic T stationary phase are briefly discussed. 41 42 43 **Keywords:** chiral stationary phases; ionic interactions; macrocyclic glycopeptides; 44 solvation parameter model; supercritical fluid chromatography. 45 46 47

### 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, for instance), while they seem to favour enantiorecognition in other cases. These 60 stationary phases are marketed under the trade name Chirobiotic®. Other, non-61 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 superficially porous particles [5–7] were recently introduced. The benefit of both is 65 66 the significantly increased efficiency, with the extra advantage of low pressure drops when superficially porous particles are employed. 67 68 All these stationary phases are mostly employed in high-performance liquid chromatography (HPLC). As they are multimodal [8], they may be employed in 69 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]. While other fluids have been employed in the past, SFC is (now) essentially based 76 on the use of pressurized carbon dioxide as the principal component of the mobile 77 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 polysaccharide phases are most effective with neutral analytes, antibiotics, bearing 88 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 previously demonstrated how some understanding of retention mechanism on chiral 96 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. We have previously demonstrated that another modified version of the solvation 113

114 parameter model, comprising five Abraham descriptors (E, S, A, B, V) and two

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

119

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

121

In this equation, capital letters represent the solute descriptors, related to particular
interaction properties, while lower case letters represent the system constants,
related to the complementary effect of the phases on these interactions. *c* is the
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  $\pi$  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 cm<sup>3</sup> mol<sup>-1</sup>/100; D<sup>-</sup> represents the

130 negative charge carried by anionic and zwitterionic species, and D<sup>+</sup> 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

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

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143

144 **3. Material and methods** 

145

146 3.1. Stationary phases

- 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<sub>a</sub> 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
- were prepared in MeOH. 150 achiral test compounds (Table S1), comprising 115
- analytes that should be charge-neutral in the present operating conditions, and 35
- species that may be partly or completely ionized (14 anionic and 21 cationic form),
- 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
- 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
- solvation parameter model (Tables S1 and S2). The two additional descriptors for
- 172 charges (D<sup>-</sup> and D<sup>+</sup>) were calculated based on aqueous pKa values determined with
- 173 Chemicalize program and apparent pH 5. The additional descriptors for flexibility (F)
- and globularity (G) were computed as previously described [21,22]. The series of
- test-compounds has been selected by observing the requirements of a good LSER
- 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
- among the descriptors was checked.
- 179

#### 180 **3.3.** Chromatographic system and conditions

- 182 Chromatographic separations were carried out using an ACQUITY Ultra Performance
- 183 Convergence Chromatography<sup>TM</sup> (UPC<sup>2</sup>) system from Waters (Millford, MA, USA).
- 184 The system was equipped with a binary solvent delivery pump compatible with
- mobile phase flow rates up to 4 mL/min and pressures up to 414 bar, an
- 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 CO<sub>2</sub>-MeOH (v/v) with or without an
- additive. When an additive was present, it was introduced at 0.1% in the methanol
- 192 co-solvent. Flow rate was 3 mL min<sup>-1</sup>. 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
- 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<sup>2</sup>), standard error in
- 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
- The three macrocyclic (glyco)peptides employed in this study are presented in Figure1.
- 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
- 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 (highlighted216 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 of these molecules prior to bonding. Bonding may occur through the amine functions 224 225 (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

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

- pattern is relevant of normal-phase mode of retention, as is usually observed whenpolar stationary phases are used in SFC, with:
- polar interaction terms being positive, meaning that polar molecules interact
   favourably with the stationary phase;
- v term being negative, indicating that large molecular volume is unfavourable
   to insertion in the stationary phase, as may be expected from the size of the
   fused rings. It is however impossible to judge whether the analytes are
   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)-

propyl-isocyanate should leave no amine functions but perhaps some unreactedisocyanate functions.

284

The contributions of *e* and *s* terms are also positive, although less significant than hydrogen bonding. Positive *e* term may result principally from  $\pi$ - $\pi$  interactions between the aromatic probe solutes and the numerous aromatic rings in the macrocycles. Positive *s* term results from dipole-dipole interactions with all polar 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 pKa 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 pKa 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 pKa values in pressurized carbon 298 dioxide – methanol mixture may differ from aqueous pK<sub>a</sub> 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

interactions with cationic analytes. Here again, accessible ionizable groups from the

311 supporting silica may also contribute to the interactions with ionizable species: (i)

residual silanol groups, whether they are ionized or neutral, may contribute to

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  $d^2$
- 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
- 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
- dioxide-methanol 90:10). The reported models relate only the five Abraham
- 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
- 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*
- and *b* terms were previously noted on polysaccharide CSP when comparing the
- same mobile phase compositions in SFC and NPLC [26]. Finally, the *v* term is
- 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
- teicoplanin than the other two, due to the presence of a long hydrophobic chain in
- teicoplanin, but no such thing is observed.

- LSER models were also reported on macrocyclic peptide stationary phases in reversed-phase conditions [9,19,39], or in polar ionic mode [20], which show no 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 complementarity between the three phases with Venn diagrams (Figure 4). First of 356 357 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

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

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

resolution > 1, the "success rate" would have been even lower (and not at all

informative).

378 The second observation is that a significant portion of racemates could be separated

by two or three CSP. The teicoplanin aglycone was again the most interesting, as it

380 provided a larger number or unique separations. With all three columns combined,

about a half of the racemates in Table S2 could be separated with these operating

conditions, which are, again, not the most favourable to operating peptide stationaryphases.

The generally superior capabilities of teicoplanin aglycone may also be observed in
Figure 5, where the enantioselectivity is compared for a selection of racemates
between the glycosidic and aglycone forms. Apart from a sole case when teicoplanin
provided clearly superior enantioselectivity (2,3-epoxypropylbenzene), teicoplanin
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 the present operating conditions, the results of discriminant analyses could not be 403 404 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%.

Figure 6 is showing the contributions of structural features to enantioseparation on the teicoplanin aglycon phase. Based on this statistical analysis, we may observe that the most significant contribution is related to negative charges on the analyte as the D<sup>-</sup> term is large and positive. Other significant contributions indicate that large volume is deleterious to enantioseparation (negative V term), as are aromaticity and electron-donating properties (negative E and B terms). The presence of dipoles is 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<sup>-</sup>) 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 erymomycin stationary phase in SFC, but in that case interaction between the
- 424 carboxylate group of the CSP and a basic (possibly protonated) analyte was425 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 enantiorecognition [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  $\pi$ -437  $\pi$  interactions are normally considered to be beneficial to enantiorecognition 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 enantioresolution. For instance, 440 the enantiomers of (2,3-epoxylpropyl)-benzene were well resolved, as appears in 441 Figure 5. However, Chen and Ward report some negative contribution of  $\pi$ - $\pi$ 442 interactions when the aromatic ring is distant from the chiral centre [45]. As the E 443 descriptor is taking account of all  $\pi$  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 of V term in the present work. Steric repulsion was also significant to explain the 448 449 enantioseparation of chiral sulfoxides on teicoplanin [41]. As teicoplanin aglycon

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

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

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

these CSP.

#### 484 **5. Conclusions**

485

486 Three stationary phases based on macrocylic (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<sup>-</sup> and D<sup>+</sup>) 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

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- 661

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 antiobiotic 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	racemated 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.



Figure 1.



#### **TAG T**

Figure 2.



Figure 3.



# Figure 4.



Figure 5.



Figure 6.



Figure 7.

**Table 1.** LSER system parameters for the three stationary phases studied, in the present SFC conditionsand in normal-phase HPLC conditions (from ref. [9,19,38]).

Stationary phase	Mobile phase	c	е	S	а	b	V	ď	d+	R <sup>2</sup>	SE	F	n
Vancomycin	SFC	-1.37	0.59	0.33	1.36	0.66	-0.36	-0.18	0.09	0.89	0.22	162	145
	CO <sub>2</sub> -MeOH 90:10	0.08	0.05	0.08	0.07	0.09	0.08	0.06	0.08				
	NPLC	-0.61	0.00	0.87	0.61	1.58	-0.91			0.99	0.09	789	39
	HPT-EtOH 90:10	0.06		0.06	0.04	0.07	0.04						
Teicoplanin	SFC	-1.00	0.42	0.30	1.00	1.03	-0.46	-0.24	0.68	0.89	0.21	154	145
	CO <sub>2</sub> -MeOH 90:10	0.08	0.05	0.08	0.07	0.09	0.08	0.06	0.09				
	NPLC	-0.51	0.00	0.66	0.54	1.80	-0.98			0.98	0.11	506	46
	HPT-EtOH 90:10	0.08		0.06	0.05	0.08	0.06						
Teicoplanin	SFC	-1.00	0.57	0.33	1.07	1.06	-0.50	-0.24	0.92	0.88	0.22	139	142
aglycon	CO <sub>2</sub> -MeOH 90:10	0.09	0.05	0.09	0.07	0.09	0.08	0.06	0.11				
	NPLC	-0.49	0.00	0.77	0.47	2.00	-1.01			0.98	0.11	568	45
	HPT-EtOH 90:10												