New perspectives in high efficient and ultrafast chiral liquid chromatography through zwitterionic teicoplanin-based 2-micron superficially porous particles

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Abstract

With the aim of pushing forward the limits of high efficient and ultrafast chiral liquid chromatography, a new Chiral Stationary Phase (CSP) has been prepared by covalently bonding the teicoplanin selector on 2.0 μm Superficially Porous Particles (SPPs). An already validated bonding

protocol, which permits to achieve teicoplanin-based CSPs exhibiting zwitterionic behaviour, has been employed to prepare not only the 2.0 µm version of the CSP but also two other analogous CSPs based, respectively, on 2.7 µm SPPs and 1.9 µm Fully Porous Particles (FPPs). The kinetic performance of these CSPs has been compared through the analysis of both van Deemter curves and kinetic plots by employing in-house packed columns of 4.6 mm internal diameter and different lengths (20, 50 and 100 mm). In particular on the columns packed with 2.0 µm SPPs, extremely large efficiencies were observed for both achiral (>310,000 theoretical plates/meter, N/m; h_r: 1.61) and chiral compounds (>290,000 N/m; h_r: 1.72) in HILIC conditions. Thanks to their efficiency and enantioselectivity, these CSPs were successfully employed in ultrafast chiral separations. As an example, the enantiomers of haloxyfop were baseline resolved in about 3 seconds, with a resolution higher than 2.0, (flow rate: 8 mL/min) on a 2 cm long column packed with the 2.0 µm chiral SPPs.

1. Introduction

In the last decades, the technological progress and the continuous research of higher and higher column efficiency has led, on the one hand, to the development of stationary phases made of sub 2-µm fully porous particles (FPPs) and, since their introduction into the market in 2007 [1], to the employment of so-called second-generation superficially porous particles (SPPs, even referred to as core-shell or pellicular particles). Second-generation SPPs are made of a solid core surrounded by a porous layer which occupies about 75% of the overall particle volume. They have a particle diameter generally either 2.6 or 2.7 µm, depending on manufacturer [1-7]. In both approaches, the rationale is to decrease the contribution to band broadening due to intraparticle dispersion. As a consequence in achiral reversed-phase (RP) liquid chromatography, nowadays, the efficiency of modern chromatographic columns (be they packed with sub 2-µm FPPs or second-generation SPPs) easily reaches 300,000-350,000 theoretical plates per meter (N/m).

As a matter of fact, this extraordinary improvement of performance has instead only partially touched the field of chiral separations. Essentially up to 2010 [8], CSPs were prepared on FPPs with particle diameter of 3-5 μm. Improvements in the preparation of high efficient CSPs have lagged behind due to (i) the difficulty to adapt traditional techniques of surface modification to the preparation of small particles; (ii) the tendency of small particles to aggregate during chemical modification with consequent inefficient/poor packing; (iii) the low mechanical resistance and long-term stability of particles functionalized with chiral selector at the high flow rates/high pressure required to drive the flow through the packed bed in ultra-high performance liquid chromatography (UHPLC); (iv) the lack of fundamental studies of mass transfer in CSPs [9].

Starting from 2010, Gasparrini and coworkers firstly reported on the use of brush-type CSPs prepared on sub-2μm FPPs for the high efficient separation of enantiomers in the second-time scale [8,10-12]. In 2011, thanks to the work by Lindner and coworkers, the first example on the use of second-generation SPPs for the preparation of a weak anion-exchanger CSP was presented [13]. The first work aimed at evaluating the kinetic performance of chiral SPPs and FPPs dates 2012, when Chankvetadze et al. compared polysaccharide-based CSPs prepared on both kinds of particles [14]. The conclusion of this work was that columns packed with SPPs exhibited not only higher enantioselectivity (at comparable selector loading), but also both better kinetic performance at high flow-rate and larger enantioresolution than those made of FPPs. The systematic study of the performance of chiral SPPs and FPPs has been performed by Armstrong and coworkers who characterized many different types of CSPs (such as, cyclodextrins, cyclofructan-6, macrocyclic antibiotics, etc.) prepared on both supports [15-20]. In agreement with the conclusions drawn by Chankvetadze et al. [14], in these studies chiral SPPs were found to be more efficient from a kinetic viewpoint and thus more suitable for the transition to ultrafast chiral separations than their FPP counterparts. In principle the same basic concepts for which achiral hydrophobic SPPs outperform the fully porous ones (incidentally, a better packing quality, a reduced longitudinal diffusion and a

74 smaller solid liquid mass transfer resistance), have been considered at the base of the better
75 behavior of chiral SPPs.
76 More recently, some of the authors of this work, partially challenged this vision [21]. Basically,
77 they tackled the idea that chiral SPPs must always be kinetically more performant than fully porous
78 ones. Unexpected results were indeed found by comparing the behavior of brush-type Whelk-O1
79 CSPs made on 2.6 µm SPPs, on the one hand, with that of both 1.8 and 2.5 µm FPPs, on the other
80 [21]. In their study, Ismail et al. found the columns packed with FPPs to exhibit better performance
81 than those made of SPPs, especially for the second eluted enantiomer. Following these authors,
82 therefore, to assess the superiority of either particle type requires a deeper investigation of the effect
83 of several factors on the chromatographic performance. In particular, Ismail et al. mention the need
84 to carefully investigate the effect of chiral selector surface density on the adsorption/desorption
85 kinetics, on the other [8,21]. The latter observation seems particularly important since, as reported
86 by many authors [15,18,21], significant differences in the surface density of chiral selectors were
87 observed during functionalization of SPPs and FPPs even if the same experimental protocol was
88 employed in both cases. Nevertheless, this effect has never been systematically investigated.

89 In this work, a deep evaluation of the kinetic and thermodynamic performance of three columns
90 prepared respectively on 2.0 µm Halo® SPPs (here referred to as UHPC-SPP-Halo-Tzwitt 2.0), 2.7
91 µm Halo® SPPs (UHPC-SPP-Halo-Tzwitt 2.7) and 1.9 µm monodispersed Titan® FPPs (UHPC-
92 FPP-Titan-Tzwitt 1.9) [22] is presented. In all cases, the teicoplanin selector was bonded to the
93 particle so to guarantee to the CSP a zwitterionic character. The comparison between the three
94 columns is based on the evaluation of both van Deemter curves and kinetic plots.

95 **2. Theory**

96 The efficiency of a column is usually evaluated through the well-known van Deemter equation (1),
97 which correlates the plate height, H, to the interstitial velocity μ_{int} (i.e., the velocity of the fluid

98 truly moving inside the column). In its basic formulation [23,24], the van Deemter equation is
99 written as:

100 $$H = A + {}^{B}/_{\mu_{int}} + C\mu_{int} \quad (1)$$

101 where A represents the eddy dispersion, B the longitudinal diffusion and C the solid-liquid mass
102 transfer resistance. The interstitial velocity is defined by:

103 $$\mu_{int} = \frac{\Phi}{\pi r^2 \varepsilon_e} \quad (2)$$

104 being Φ the flow rate, r the column radius and ε_e the external porosity ($\varepsilon_e = \frac{V_e}{V_{col}}$, with V_e the
105 interstitial volume and V_{col} the geometrical volume of the column).
106 In addition to van Deemter plots, the kinetic performance of columns can be also evaluated through
107 the kinetic plots. They provide the highest plate number, N, achievable in the shortest time possible
108 while working at the maximum pressure of the system, ΔP_{max}[25]. Hold up time, t_0, versus N plots
109 can be used to quickly estimate which column offers the fastest separation for a fixed efficiency or
110 the highest N value that can be obtained in a given analysis time. Other forms of kinetic plots can
111 be used to correlate either the column length, L, or the retention time, t_R, to N.
112 The following equations are employed for the conversion of experimentally determined linear
113 velocity μ_0:

114 $$\mu_0 = \frac{L}{t_0} \quad (3)$$

115 and H values in kinetic plots:

116 $$N = \frac{\Delta P_{max}}{\eta}\left[\frac{K_0}{\mu_0 H}\right] \quad (4)$$

117 $$t_0 = \frac{\Delta P_{max}}{\eta}\left[\frac{K_0}{\mu_0^2}\right] \quad (5)$$

118 $$t_R = \frac{t_0}{(k'+1)} \quad (6)$$

being η the viscosity of the mobile phase, K_0 the column permeability and k' the retention factor. ΔP_{max} values were set at 600 bar for the UHPC-SPP-Halo-Tzwitt 2.7 column and to 1000 bar for both the UHPC-SPP-Halo-Tzwitt 2.0 and UHPC-FPP-Titan-Tzwitt 1.9 columns.

3. Experimental

3.1 Materials and chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, Mo, USA). HPLC gradient grade solvents were filtered before use on 0.2 μm Omnipore filters (Merck Millipore, Darmstadt, Germany). Chiral samples were from Sigma-Aldrich (St. Louis, Mo, USA). Titan® monodispersed silica (pore size 120 Å, particle size 1.9 μm and specific surface area 282 $m^2\ g^{-1}$), Halo® 2.0 and 2.7 μm (90Å, 125 and 123 m^2/g, respectively) and teicoplanin selector were provided by Merck Sigma–Aldrich (St. Louis, MO, USA). Empty stainless steel columns, 20, 50 and 100 mm × 4.6 mm (L × I.D.), were from IsoBar Systems by Idex (Wertheim-Mondfeld, Germany).

3.2 Instruments

The UHPLC chromatographic system used for all achiral tests in HILIC was an UltiMate 3000 RS system (Thermo Fisher Dionex Sunnyvale, California), equipped with a dual gradient RS pump, an in-line split loop Well Plate Sampler, a thermostatted RS Column Ventilated Compartment (temperature range 5-110 °C) and a diode array detector (Vanquish detector) with a low dispersion 2.0 μL flow cell. In addition, a second DAD detector with a 2.5 μL flow cell was employed for flow-rates higher than 4.0 mL/min. Vanquish and DAD detector, both, were set at a filter time constant of 0.002 s, a data collection rate of 100 Hz and a response time of 0.04 s. Inlet and outlet viper tubes (2×350 mm×0.10 mm I.D.) were employed. Data acquisition and processing were performed with Chromeleon 6.8 software from Thermo Fisher. The extra-column peak variance (calculated through peak moments) was 3.94 μL^2 at a flow-rate of 1.0 mL/min. Data acquisition, data handling and instrument control were performed by Chromeleon software.

144 **3.3 Preparation of chiral stationary phases.**

145 All columns were packed with CSPs synthesized according to the same Supelco proprietary
146 bonding protocol immobilizing teicoplanin selector onto SPP-Halo 2.0 µm and 2.7 µm and
147 monodispersed FPP-Titan-120 1.9 µm silica particles, leading to the zwitterionic chiral stationary
148 phases named UHPC-SPP-Halo-Tzwitt 2.0, UHPC-SPP-Halo-Tzwitt 2.7 UHPC-FPP-Titan-Tzwitt
149 1.9, respectively. UHPC-FPP-Titan-Tzwit 1.9 µm silica particles were prepared by adjusting the
150 synthetic procedure (medium density selector) in order to achieve comparable retention as the SPP
151 CSPs. All CSPs were slurry packed with a pneumatically driven Haskel pump (roughly ΔP_{max} 950
152 bar) into stainless steel columns. Elemental analysis (C, H, N) of the different CSPs were used to
153 extract values of selector loading and surface coverage. UHPC-SPP-Halo-Tzwitt 2.0 particles: 5.75
154 %C, 0.74 %H and 0.63 %N, corresponding to 56 µmol of selector per gram of silica and to 0.45
155 µmol of selector per m^2 (based on N); UHPC-SPP-Halo-Tzwitt 2.7 particles: 6.05 %C, 0.79 %H
156 and 0.66 %N, corresponding to 59 µmol of selector per g of silica and to 0.47 µmol of selector per
157 m^2 (based on N); UHPC-FPP-Titan-Tzwitt 1.9 particles: 8.19 %C, 1.06 %H and 0.92 %N,
158 corresponding to 85 µmol of selector per g of silica and to 0.27 µmol of selector per m^2 (based on
159 N). As expected, the two SPP CSPs showed a lower loading (µmol/g) of selector but a higher
160 surface density of teicoplanin (µmol/m^2) in comparison with the 1.9 µm FPPs.

161 **3.4 Methodology**

162 All separations were performed in Hydrophilic Interaction Liquid Chromatography (HILIC)
163 conditions by using a mobile phase made by ACN/H_2O 85:15 + 20 mM $HCOONH_4$ (${}^{s}pH$= 7.5).
164 Injected volumes were 0.5-1.0 µL. For data evaluation, the values of resolution (R_s) and efficiency
165 (N/m) were calculated according to the European Pharmacopeia using peak width at half height
166 ($w_{0.5}$). Hold up time was estimated by injection of naphthalene. All data were processed with
167 Origin 8.0.

168

169 **3.4.1 Pycnometry measurement**

Thermodynamic hold-up volume (V_0^{pyc}) was determined by static pycnometry:

$$V_0^{pyc} = \frac{w_{CHCl3} - w_{THF}}{\rho_{CHCl3} - \rho_{THF}} \quad (7)$$

where w and ρ are the mass of the column and the solvent density, respectively[26,27].

3.4.2 ISEC measurement

Inverse size exclusion chromatography (ISEC) was performed to determine both external, ε_e, and particle porosity, ε_p. A wide range of polystyrene standards (molecular weight between 500 and 3.6 × 106 Dalton) was injected into the columns, using neat THF as the mobile phase [28,29]. Total porosity, ε_t, was calculated as the ratio between V_0^{pyc} and V_{col}. Results of ISEC measurements are reported in Table 1S.

4. Results and Discussion

4.1 Physical and geometric characterization of columns

In order to have a complete characterization of columns, the specific and the column permeability (K_{sf} and K_0, respectively) were calculated from the linear velocity vs. ΔP_{col} linear plots (Fig.1). For this study, 100×4.6 mm (L×I.D.) columns were employed. As known, the linear velocity μ_0 and pressure drop ΔP_{col} are correlated by the Darcy's law with the equation: $K_0 = \frac{\mu_0 \eta L}{\Delta P}$ [30]. The column permeability (K_0) was 0.499, 0.500 and 1.00 × 10^{-14} m^2 for the UHPC-Halo-SPP-Tzwitt 2.0, the UHPC-Titan-FPP-Tzwitt 1.9 and the UHPC-Halo-SPP-Tzwitt 2.7 column, respectively (Fig. 1A). As expected, the column packed with 2.7 µm SPPs showed the highest K_0 value due to the larger particle diameter. This means that this column generates, at the same flow rate, a lower back-pressure (almost twice smaller) than the other two (Fig. 1B). Finally, the total porosity (ε_t) of columns was calculated through ISEC analysis. The two columns packed with SPPs, as expected, exhibited very similar ε_t values, consistently lower than the UHPC-Titan-FPP-Tzwitt 1.9 column. These data are summarized into Table 1S.

195

196 **4.2 van Deemter analysis on achiral samples**

197 All analysis, including van Deemter plots, were made on a Dionex Ultimate 3000RS with flow rates
198 ranging from 0.2 mL/min up to 4.0 mL/min with a maximum operating pressure of 550 bar. This
199 wide range of flow rates has permitted to achieve a complete view on the kinetic performance of the
200 whole set of columns. The first evaluation was made using a mixture of the achiral solutes
201 naphthalene (hold-up volume marker), thiourea, uracil and adenosine. In this work, all H values
202 were not corrected for the extra-column variance since its contribution to band broadening was
203 found to be negligible. van Deemter plots have been expressed as H as a function of μ_{inter} (Fig. 2),
204 μ_0 (Fig. 1S) or flow rate depending on need; μ_{inter}, which takes into account the external porosity
205 of each column, was used to have a correct comparison between columns packed with SPP and FPP
206 CSPs. In Fig. 2A, the van Deemter plots of thiourea (k': 0.62-0.65) on the three columns are shown.
207 Clearly, the column packed with UHPC-SPP-Halo-Tzwitt 2.0 CSP provides the best efficiency with
208 more than 311,000 N/m, corresponding to a plate height H = 3.21 µm (h_r: 1.60) at a flow rate of 1.5
209 mL/min (μ_{inter} = 3.63 mm/s). Also the column packed with UHPC-FPP-Titan-Tzwitt 1.9 CSP
210 showed remarkable efficiency, with 277,000 N/m (H = 3.61 µm, h_r: 1.90) at a flow rate of 1.4
211 mL/min (μ_{inter} = 3.32 mm/s). The lowest efficiency was observed on the column packed with
212 UHPC-SPP-Halo-Tzwitt 2.7 CSP, with less than 250,000 N/m (H = 4.03 µm, h_r: 1.49) at a flow rate
213 of 1.1 mL/min (μ_{inter} = 2.60 mm/s) (Table 1). Moreover, looking at the shape of plots, it can be
214 observed that the C-branch of the van Deemter curve for the 2.0µm SPP column is lower than both
215 those of the 1.9µm FPP and the 2.7µm SPP columns. This characteristic is of fundamental
216 importance for UHPLC separations since a flatter C-branch permits to increase the flow rate
217 without significant loss of efficiency. Indeed, moving from the optimal flow rate to 3.0 mL/min,
218 efficiency drops were 4.7%, 25% and 15% respectively for the UHPC-SPP-Halo-Tzwitt 2.0, the
219 UHPC-SPP-Halo-Tzwitt 2.7 and UHPC-FPP-Titan-Tzwitt 1.9 columns. The van Deemter curves on

220 the three columns for a test probe with a moderate retention (uracil, k': 0.99-1.03) are reported in
221 Fig. 2B and 1S A and 1S C. Also in this case, at a flow rate of 1.2 mL/min, 311,000 N/m were
222 recorded on the UHPC-SPP-Halo-Tzwitt 2.0 column, resulting in significantly higher efficiency
223 than on both the UHPC-SPP-Halo-Tzwitt 2.7 column (30%) and the UHPC-FPP-Titan-Tzwitt 1.9
224 one (15%), respectively.
225 Considering a molecule with an higher retention factor, such as adenosine (k': 1.82-2.07) (see
226 Fig.1S B and D), the gap in terms of optimal flow rate becomes even larger in favor of the UHPC-
227 SPP-Halo-Tzwitt 2.0 column, which shows an optimal flow rate respectively 1.5 and 2.4 times
228 higher than those of the UHPC-FPP-Titan-Tzwitt 1.9 and the UHPC-SPP-Halo-Tzwitt 2.7 columns.
229 In Fig. 2C, 2D, 1S C and D, the reduced van Deemter plots are reported. Reduced plate height
230 $h_r\left(=\frac{H}{d_p}\right)$ permits to properly evaluate the kinetic performance of columns packed with silica
231 particles of size. Extremely low h_r values were found with thiourea (1.49, 1.60 and 1.90 on the
232 UHPC-SPP-Halo-Tzwitt 2.7, the UHPC-SPP-Halo-Tzwitt 2.0 and the UHPC-FPP-Titan-Tzwitt 1.9
233 columns, respectively), proving the goodness of the packing process obtained for all columns.
234
235 **4.3 Kinetic performance limits**
236 The kinetic plot method was used to have a complete overview of the kinetic performance limits of
237 all columns. This method shows the highest efficiency achievable by a column in the shortest time,
238 working at the maximum pressure reachable by the instrument. Kinetic plots method is the best
239 choice to have a clear and proper comparison of kinetic performance of different columns (also with
240 different geometries) in various analytical conditions (HPLC, UHPLC or SFC). The t_0 *vs.* N kinetic
241 plot is the original one introduced by Giddings in 1965 [31]. This is the starting point for other
242 forms of kinetic plots, such as the so-called Poppe plot [32-33]. This plot permits to have a clearer
243 view on the C-term of van Deemter equation. In this work, for the preparation of kinetic plots, the
244 maximum operating pressure was set at 600 bar for columns packed with 2.7µm SPP CSP (silica

pressure limit) and 1000 bar (maximum operative pressure in most common UHPLC systems) for those packed with 2.0 µm SPP and 1.9 µm FPP CSPs. In Fig. 3, the kinetic plots for the three columns are reported. Fig. 3A (t_0 *vs* N) shows that the use of the UHPC-SPP-Halo-Tzwitt 2.0 column is worthwhile up to 190,000 plates. After this value, the best choice would be the UHPC-FPP-Titan-Tzwitt 1.9 column and beyond N = 226,000 the UHPC-SPP-Halo-Tzwitt 2.7 column gives the best kinetic performance, due to its very high permeability and low operating pressure. The same trend is observed in the Poppe plot (t_0/N *vs* N, Fig. 3B) and t_R*vs* N plot, on uracil, (Fig. 3C), where the column packed with the 2.0 µm SPP CSP overcomes the columns packed with the other two CSPs in the bottom left corner of the graph, which represents the maximum separation speed with realistic column length. In these two plots, where the data of uracil (k': 0.99-1.03) are reported, it is evident how the UHPC-Halo-Tzwitt 2.0 column exceeds, in terms of kinetic performance, the other two columns in every area of the plot thanks to its low C-term (1.8 and 2.1 times lower than that of the UHPC-FPP-Titan-Tzwitt 1.9 and the UHPC-SPP-Halo-Tzwitt 2.7 columns, respectively). L vs N plots (Fig. 3D) confirm the same trend. Indeed, by assuming an efficiency of 45,000 plates as the target value, one sees that a 18 cm long column packed with 2.0 µm SPPs can be used (at 1000 bar) against the 24 cm required by the UHPC-FPP-Titan-Tzwitt 1.9 column and the 28 cm of the UHPC-SPP-Halo-Tzwitt 2.7 one (but at 600 bar). Finally, the same kinetic behavior was observed, considering adenosine (k': 1.82-2.07) as the probe (Fig.2S). In this case, the C-term critically affects kinetic plots. Indeed the UHPC-SPP-Halo-Tzwitt 2.7 column seems to be the less efficient from the kinetic point of view. On the other hand, the UHPC-SPP-Halo-Tzwitt 2.0 column performs better than the other two columns across the entire range of these plots.

4.4 Chromatograms analysis of achiral samples

Chromatograms showing the separation of a mixture of achiral probes naphthalene (hold-up volume marker), thiourea, uracil and adenosine, on all the three columns at their optimal flow rate (on

thiourea) are shown in Fig. 4A. Since the optimal flow rate of the UHPC-SPP-Halo-Tzwitt 2.0 column is higher than those of the other columns, a reduction of almost 40% of analysis time could be achieved with this column. Fig. 4B and 4C show the same chromatographic separations at higher flow rates (2.0 and 2.5 mL/min). The column packed with 2.0 μm SPPs exhibits the lower drops in efficiency and resolution when compared with the other two columns as a confirmation of the flat C-branch of its van Deemter curve. Indeed, by considering thiourea, moving from the optimal flow-rate to 2.5 mL/min produced efficiency loss of 3%, 10% and 12% respectively on the UHPC-SPP-Halo-Tzwitt 2.0, the UHPC-FPP-Titan-Tzwitt 1.9 and UHPC-SPP-Halo-Tzwitt 2.7 columns. If one considers adenosine, the efficiency drop became even more evident, going from 23% to 27% and then to 42% for the three columns (considered in the same order as before). This efficiency loss is reflected in corresponding resolution values, which decrease in the same order (Table 2). In Fig. 4D all chromatograms are reported as a function of k'. As it can be noticed, analytes have almost the same retention factors on the two SPP CSPs, while they are 6-10% higher on the FPP one (see above).

4.5 van Deemter analysis on chiral compound

The kinetic profile of the different columns was completed by measuring van Deemter plots of the racemic chiral molecule 2-(4-chloro-phenoxy)-propionic acid (Fig. 5). The column packed with UHPC-SPP-Halo-Tzwitt 2.0 particles showed outstanding performance. Almost 300,000 N/m (H= 3.42μm, h_r= 1.71) were recorded for the first eluted enantiomer and roughly 280,000 plates/m for the second one, at optimal flow rates of 0.9 and 0.7 mL/min, respectively. The column packed with 1.9μm FPPs generated more than 260,000 N/m (H = 3.82μm, h_r= 2.01) at 0.7 mL/min for the first eluted enantiomer. As expected, the column packed with the UHPC-SPP-Halo-Tzwitt 2.7 particles was the less efficient, by producing 211,000 N/m for the first eluted enantiomer (H = 4.74μm, h_r = 1.76) at a flow rate of 0.5 mL/min. This gap in terms of efficiency is clearly pointed out in Fig. 5B

and 5C, where N/m is reported as a function of flow rates for both enantiomers for each column. An efficiency gain of about 11% and more than 40% on the second eluted enantiomer is achievable with the UHPC-SPP-Halo-Tzwitt 2.0 column if compared to the UHPC-FPP-Titan-Tzwitt 1.9 and UHPC-SPP-Halo-Tzwitt 2.7 ones (Fig. 5C), respectively. Moreover, the UHPC-SPP-Halo-Tzwitt 2.0 column still exhibits a flatter C-branch also for the separation of chiral samples. This is an essential advantage of this column for UHPLC enantioseparations, as it will be shown in the following.

4.6 Applications to chiral compounds

4.6.1 Kinetic performance

After evaluation of physical and kinetic properties, all columns were tested for practical applications. A broad range of chiral compounds (including N-derivatized amino acids, agrochemical and drugs or drug-like molecules) was employed under HILIC conditions. All kinetics data are summarized in Table 2S and Fig 3S. As expected, the most efficient column was the UHPC-SPP-Halo-Tzwitt 2.0, followed by the UHPC-FPP-Titan-Tzwitt 1.9 and the UHPC-SPP-Halo-Tzwitt 2.7. Indeed, looking at the bar plot in Fig. 3S, an average 35% loss of efficiency for the first eluted enantiomer was observed on the UHPC-SPP-Halo-Tzwitt 2.7 column in comparison to the UHPC-SPP-Halo-Tzwitt 2.0 one.

Chromatograms of four different chiral samples recorded at 1.0 mL/min on the three columns are shown in Fig. 6. Roughly 265,000 N/m were recorded on the UHPC-SPP-Halo-Tzwitt 2.0 column for the first eluted enantiomer of *Z-D,L*-Met (Fig. 6D). Smaller efficiencies were observed on the UHPC-FPP-Titan-Tzwitt 1.9 column (-10%) and UHPC-SPP-Halo-Tzwitt 2.7 one (-36%).

Moreover in Fig. 3S, N/m values for 15 pairs of racemic samples recorded at a flow rate of 1.0 mL/min are reported as a bar plot. In most cases, the column packed with 2.0 µm particles provides the highest efficiency for the first eluted enantiomer (see Fig. 3S A). On the opposite, the UHPC-FPP-Titan-Tzwitt 1.9 column has shown larger efficiency for the second eluted enantiomer (see Fig.

322 3S B). The effect is more pronounced for the N-derivatized amino acids with medium-high
323 retention factors. These findings seem to suggest the existence of different adsorption/desorption
324 kinetics on the two columns, which could be correlated to the different surface density of chiral
325 selector on particles (see above). However, further experimental and theoretical investigations are
326 necessary to explain this behavior and to deeply understand the adsorption mechanism occurring on
327 both SPP and FPP CSPs.
328
329 **4.6.2 Thermodynamic and resolution (R_s)**
330 Retention and enantioselectivity values for several chiral probes on the three CSPs are listed in
331 Table 3 and Fig. 4S. From these data it is evident that the two SPP CSPs have similar retention
332 behavior, while the UHPC-FPP-Titan-Tzwitt 1.9 column showed higher retention factors. Very
333 similar enantioselectivity was observed on the two SPP CSPs (average value about 1.5). They were
334 about 10% higher than those observed on the FPP CSP (Fig. 4S). This difference could be related to
335 the higher density of the teicoplanin selector on the two SPP silica in comparison to the fully porous
336 one (see above).
337 As far as resolution is concerned, the UHPC-SPP-Halo-Tzwitt 2.0 column showed the highest
338 resolution values for 12 out of 15 samples (Fig. 7A). For instance, the UHPC-SPP-Halo-Tzwitt 2.0
339 column exhibited a resolution value of about 4.0 for *D,L*-Proglumide (Fig. 6A), which is 40% and
340 37% higher than those observed on the UHPC-SPP-Halo-Tzwitt 2.7 and UHPC-FPP-Titan-Tzwitt
341 1.9 columns, respectively. In Fig. 7B, the ratio between resolution values and retention times of the
342 second eluted enantiomers is reported. This plot clearly shows the very high resolution power of the
343 UHPC-SPP-Halo-Tzwitt 2.0 column in comparison to both the UHPC-SPP-Halo-Tzwitt 2.7 and the
344 UHPC-FPP-Titan-Tzwitt 1.9 columns.
345
346 **4.7 Very-High speed and Ultra-High Performance columns.**

van Deemter analysis revealed the ability of these columns to work easily at flow rates higher than optimal ones. The behavior of these CSPs was thus investigated up to 6.0 mL/min flow rate (so-called very-high speed regime), by employing 50×4.6 mm in house packed columns and different chiral samples were tested (Fig. 5S). Fig. 8 shows the separation of a mixture of enantiomers of haloxyfop and ketorolac at flow rates ranging from 1.0 to 6.0 mL/min. The resolution drop was about 45% on both the UHPC-SPP-Halo-Tzwitt 2.0 and the UHPC-FPP-Titan-Tzwitt 1.9 columns and higher than 50% on the UHPC-SPP-Halo-Tzwitt 2.7 one (see Table 4). An average loss in efficiency of more than 60% was observed on both the UHPC-SPP-Halo-Tzwitt 2.0 and UHPC-FPP-Titan-Tzwitt 1.9 columns. On the other hand, a dramatic efficiency loss of about 80% was recorded on the UHPC-SPP-Halo-Tzwitt 2.7 column.

4.8 Ultra-High speed and Ultra-High Performance columns

To further assess the potential of the two most performant CSPs (UHPC-SPP-Tzwitt-Halo 2.0 and UHPC-FPP-Tzwitt-Titan 1.9) for Ultra-High speed and Ultra-High Performance enantioseparations, two additional 20×4.6 mm columns were in house packed with these phases. Various chiral samples were tested, at flow rates as high as 8.0 mL/min (Fig. 6S). The separation of a mixture of haloxyfop and ketorolac is reported in Fig. 9. Ultra-fast separations were obtained on both columns. 73 s and 61 s were necessary to achieve the complete separation at 1.0 mL/min on the UHPC-FPP-Tzwitt-Titan 1.9 column and on the UHPC-SPP-Tzwitt-Halo 2.0 one, respectively, with efficiencies higher than 200,000 N/m and resolutions close to 4.0. Increasing the flow rate at 8.0 mL/min, the separation of the mixture was completed in roughly 8 seconds. Remarkably, by considering only the enantiomers of haloxyfop they were resolved in only 4.0 and 3.4 seconds on the UHPC-FPP-Titan-Tzwitt 1.9 and UHPC-SPP-Halo-Tzwitt 2.0 columns, respectively. High efficiency values close to 60,000 N/m were obtained also at this flow rate on the UHPC-FPP-Titan-Tzwitt 1.9 column, whereas the UHPC-SPP-Halo-Tzwitt 2.0 one showed about 76,000 N/m (Table 4). It is important to

observe that the combination of high efficiency and high enantioselectivity allowed to maintain excellent R_S values also in ultrafast regimes. Indeed, the UHPC-FPP-Titan-Tzwitt 1.9 column showed R_S values of about 1.6 while the UHPC-SPP-Halo-Tzwitt 2.0 column exhibited R_S values higher than 2.0.

5. Conclusions

This work has demonstrated that the zwitterionic teicoplanin based CSP prepared on 2.0 μm SPPs is very promising towards the development of high efficient and ultrafast liquid chromatography. The columns packed with this CSP exhibited excellent performance (300,000 N/m), very close to that obtained in RP achiral chromatography. These columns were also characterized by extremely fast mass transfer (very flat C-branch of van Deemter equation). Therefore, they could be employed in ultrafast separation (up to 8 ml/min flow rate) without dramatically loosing performance. As a proof of concept example of the feasibility of ultrafast and high performance chiral separations on these columns, haloxyfop enantiomers were baseline resolved (resolution equal to 2) in only 3 seconds.

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Figure captions.

Figure 1. A) Backpressure ΔP_{col} vs. flow-rate plots on UHPC-SPP-Halo-Tzwitt 2.0 µm (Black square), UHPC-SPP-Halo-Tzwitt 2.7 µm (Red circle) and UHPC-FPP-Titan-Tzwitt 1.9 µm (Green triangle). B) Backpressure ΔP_{col}vs. μ_0 plots on the UHPC-SPP-Halo-Tzwitt 2.0 µm (Black square), UHPC-SPP-Halo-Tzwitt 2.7 µm (Red circle) and UHPC-FPP-Titan-Tzwitt 1.9 µm (Green triangle). Eluent: ACN/H_2O 85:15 + 20mM $HCOONH_4$, η: 0.41 × 10^{-3} Pa s, T: 35°C.

Figure 2. A) and B) van Deemter plots (H vs μ_{inter}) for thiourea (k' = 0.6) and uracil (k' = 1.0), respectively, on the UHPC-SPP-Halo-Tzwitt 2.0 µm (Black square), UHPC-SPP-Halo-Tzwitt 2.7 µm (Red circle) and UHPC-FPP-Titan-Tzwitt 1.9 µm (Green triangle). C) and D) Plots of the reduced plate height, h_r vs. μ_{inter} on thiourea and uracil, respectively. Eluent: ACN/H_2O 85:15 + 20mM $HCOONH_4$, T: 35°C.

Figure 3. Kinetic plots showing a comparison of the three different columns under HILIC conditions, mobile phase: ACN/H_2O 85:15 + 20mM $HCOONH_4$; η = 0.41 × 10^{-3} Pa s; T = 35°C. (A) t_0 vs N plot, (B) t_0/N vs N plot, (C) Uracil t_R vs N plot and (D) L vs N plot on uracil. ΔP_{max} = 1000 bar was set for the UHPC-SPP-Halo-Tzwitt 2.0 µm (Black square) and UHPC-FPP-Titan-Tzwitt 1.9 µm (Green triangle), but a ΔP_{max} = 600 bar was used for the UHPC-SPP-Halo-Tzwitt 2.7 µm (Red circle).

Figure 4. Separations of the achiral probes naphthalene (hold-up volume marker), thiourea, uracil and adenosine on the UHPC-SPP-Halo-Tzwitt 2.0 µm (Black line), UHPC-SPP-Halo-Tzwitt 2.7 µm (Red line) and UHPC-FPP-Titan-Tzwitt 1.9 µm (Green line) at their optimal flow-rates (A), at flow-

519 rate: 2.0 mL/min (B) and at 2.5mL/min (C). Graph (D) shows the same chromatograms as a
520 function of the retention factor (k'). Eluent: ACN/H_2O 85:15 + 20mM $HCOONH_4$, T: 35°C.
521
522 **Figure 5.** A) van Deemter plots of 1st (solid lines) and 2nd (dashed lines) eluted enantiomers of 2-(4-
523 chloro-phenoxy)-propionic acid on the UHPC-SPP-Halo-Tzwitt 2.0 µm (Black square), UHPC-
524 SPP-Halo-Tzwitt 2.7 µm (Red circle) and UHPC-FPP-Titan- Tzwitt 1.9 µm (Green triangle). B)
525 N/m of the 1st eluted enantiomer vs. flow-rate plots on the three columns. C) N/m of the 2nd eluted
526 enantiomer vs. flow-rate plots on the three columns Eluent: ACN/H_2O 85:15 + 20mM $HCOONH_4$,
527 T: 35°C.
528
529 **Figure 6.** Separations of the racemic analytes on the UHPC-SPP-Halo-Tzwitt 2.0 µm (Black line),
530 UHPC-SPP-Halo-Tzwitt 2.7 µm (Red line) and UHPC-FPP-Titan-Tzwitt 1.9 µm (Green line) at
531 flow-rate: 1.0 mL/min. (A) D,L-Proglumide, (B) Dansyl-D,L-Methionine, (C) Fmoc-D,L-
532 Glutamine, (D) Z-D,L-Methionine. Eluent: ACN/H_2O 85:15 + 20mM $HCOONH_4$, T: 35°C.
533
534 **Figure 7.** Bar plots of resolution (R_s) and $R_s/t_{R,2}$ obtained for different analytes on the UHPC-
535 SPP-Halo-Tzwitt 2.0 µm (Black), UHPC-SPP-Halo-Tzwitt 2.7 µm (Red) and UHPC-FPP-Titan-
536 Tzwitt 1.9 µm (Green) at flow-rate: 1.0mL/min. Eluent: ACN/H_2O 85:15 + 20mM $HCOONH_4$, T:
537 35°C.
538
539 **Figure 8.** Very-High speed / Ultra-High Performance separations of the enantiomers of Haloxyfop
540 and Ketorolac, at different flow-rates, on UHPC-SPP-Halo-Tzwitt 2.0 µm (Black line), UHPC-SPP-
541 Halo-Tzwitt 2.7 µm (Red line) and UHPC-FPP-Titan-Tzwitt 1.9 µm (Green line) packed in 50 × 4.6
542 mm columns. Eluent: ACN/H_2O 85:15 + 20mM $HCOONH_4$, T: 35°C.
543

Figure 9. Ultra-High speed / Ultra-High Performance separations of the enantiomers of Haloxyfop and Ketorolac, at different flow-rates, on UHPC-SPP-Halo-Tzwitt 2.0 µm (Black line) and UHPC-FPP-Titan-Tzwitt 1.9 µm (Green line) packed in 20 × 4.6 mm columns. Eluent: ACN/H_2O 85:15 + 20mM $HCOONH_4$, *T*: 35°C.

Table 1. Experimental van Deemter analysis data under Hilic conditions on UHPC-SPP-Halo-Tzwitt 2.0 µm, UHPC-SPP-Halo-Tzwitt 2.7 µm and UHPC-FPP-Titan-Tzwitt 1.9 µm. Eluent: ACN/H_2O 85:15 + 20mM $HCOONH_4$, T: 35°C.

UHPC-SPP-Halo-Tzwitt 2.0

Sample	k' (/)	H_{min} (µm)	h_{min} (/)	N/m	$\mu_{0,opt}$ (mm/s)	$\mu_{inter,opt}$ (mm/s)	Flow-rate$_{opt}$ (mL/min)
Thiourea	0.62	3.21	1.60_5	311 280	2.85	3.63	1.5
Uracil	0.99	3.22	1.61	310 660	2.28	2.91	1.2
Adenosine	1.82	3.71	1.85	269 750	2.28	2.91	1.2

UHPC-SPP-Halo-Tzwitt 2.7

Sample	k' (/)	H_{min} (µm)	h_{min} (/)	N/m	$\mu_{0,opt}$ (mm/s)	$\mu_{inter,opt}$ (mm/s)	Flow-rate$_{opt}$ (mL/min)
Thiourea	0.62	4.03	1.49	248 090	2.20	2.60	1.1
Uracil	0.99	4.19	1.55	238 920	1.59	1.89	0.8
Adenosine	1.93	4.50	1.67	222 040	1.00	1.18	0.5

UHPC-FPP-Titan-Tzwitt 1.9

Sample	k' (/)	H_{min} (µm)	h_{min} (/)	N/m	$\mu_{0,opt}$ (mm/s)	$\mu_{inter,opt}$ (mm/s)	Flow-rate$_{opt}$ (mL/min)
Thiourea	0.66	3.61	1.90	276 780	2.34	3.32	1.4
Uracil	1.06	3.77	1.98	265 220	1.68	2.37	1.0
Adenosine	2.10	4.20	2.21	238 300	1.34	1.90	0.8

581 **Table 2.** Percentage of efficiency and resolution loss moving from the optimal flow-rate of each
582 column up to flow-rate= 2.5 mL/min

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Column	Efficiency loss % [Φ_{opt} vs Φ 2.5mL/min]			Resolution loss % [Φ_{opt} vs Φ 2.5mL/min]	
	N/m Thiourea	N/m Uracil	N/m Adenosine	$Rs_{[Thiourea-Uracil]}$	$Rs_{[Uracil-Adenosine]}$
UHPC-SPP-Halo-Tzwitt 2.0	-3%	-14%	-23%	-7%	-11%
UHPC-SPP-Halo-Tzwitt 2.7	-12%	-30%	-42%	-13%	-21%
UHPC-SPP-Titan-Tzwitt 1.9	-10%	-18%	-27%	-9%	-13%

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Table 3. Chromatographic data for chiral separations obtained on UHPC-SPP-Halo-Tzwitt 2.0 (Col. 1), UHPC-SPP-Halo-Tzwitt 2.7 (Col. 2) and UHPC-FPP-Titan-Tzwitt 1.9 (Col. 3) at flow-rate: 1.0 mL/min. Columns geometry: 100x4.6 mm L.xI.D.. Eluent: ACN/H_2O 85:15 + 20mM $HCOONH_4$, T: 35°C.

Sample	k'₂ (/)			α (/)			Rs (/)		
Name	Col. 1	Col. 2	Col. 3	Col. 1	Col. 2	Col. 3	Col. 1	Col. 2	Col. 3
N-Fmoc-D,L-Ala	3.18	2.83	2.96	1.61	1.57	1.39	7.73	6.37	6.75
N-Fmoc-D,L-Phe	1.31	1.33	1.46	1.15	1.27	1.15	2.67	3.45	2.91
N-Fmoc-D,L-Glu	4.70	4.34	5.19	1.27	1.29	1.18	6.68	5.24	4.72
N-Fmoc-D,L-Leu	1.22	1.29	1.42	1.40	1.29	1.42	5.84	5.65	5.34
N-BOC-D,L-Met	1.99	1.89	2.14	1.42	1.45	1.30	8.50	7.13	6.21
Dansyl-D,L-Met	2.09	1.95	2.07	1.73	1.77	1.46	11.09	8.72	8.11
Dansyl-D,L-Phe	1.33	1.24	1.52	1.15	1.17	1.11	2.72	2.30	2.08
Z-D,L-Phe	1.91	1.88	2.21	1.19	1.30	1.20	4.24	4.88	4.46
Z-D,L-Met	2.91	2.77	3.05	1.77	1.83	1.57	13.90	11.53	11.50
Z-D,L-Ala	4.33	4.02	4.47	1.54	1.65	1.44	12.54	10.38	10.12
Mandelic Acid	11.44	9.65	11.90	2.97	2.73	2.38	27.82	21.96	24.99
D,L-4,5-Dihydro orotic Acid	25.53	20.59	25.80	1.42	1.34	1.29	11.78	8.71	8.87
D,L-Proglumide	1.65	1.47	2.01	1.20	1.16	1.11	3.99	2.41	2.48
Haloxyfop	1.46	1.20	1.42	1.78	1.67	1.39	11.58	7.50	6.72
Ketorolac	4.80	4.30	4.44	1.40	1.37	1.26	9.61	7.03	6.33

Table 4. Percentage of efficiency and resolution loss on 5-cm and 2-cm long columns (4.6 mm I.D.) moving from the flow-rate: 1.0 mL/min up to flow-rate: 6.0 (on the 50x4.6 mm) and 8.0 mL/min (on the 20x4.6 mm columns).

Flow-rate 1.0 mL/min vs. 6.0 mL/min (50x4.6 mm) / 8.0 mL/min (20x4.6 mm)							
LxI.D.	**CSP**	**Resolution drop (-%)**		**Efficiency drop (-%)**			
		Haloxyfop	**Ketorolac**	**1° enantiomer Haloxyfop**	**2° enantiomer Haloxyfop**	**1° enantiomer Ketorolac**	**2° enantiomer Ketorolac**
50 x 4.6 mm	**SPP-Halo 2.0**	42	45	62	66	65	66
	SPP-Halo 2.7	51	53	73	78	77	78
	FPP-Titan 1.9	46	47	67	70	69	70
20 x 4.6 mm	**SPP-Halo 2.0**	44	52	61	69	74	72
	FPP-Titan 1.9	50	52	69	72	73	66