Unmatched Kinetic Performance in Enantioselective Supercritical Fluid Chromatography by Combining Latest Generation Whelk-O1 Chiral Stationary Phases with a Low-Dispersion in-House Modified Equipment

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Abstract

This proof-of-concept work investigates the ultimate kinetic limits reachable in chiral supercritical fluid chromatography (SFC) with modern columns and advanced technological solutions. A commercial equipment (Waters Acquity UPC^2) has been in-house modified to minimize its overall extra-column variance through a series of technical adjustments including low-volume connecting tubings, reduced-volume flow cell, an in-house made external column oven, external low-dispersion injection system, and electronic temperature controller. Compared to the original (as-shipped) configuration, the extra-column variance on the low-dispersion equipment was reduced by more than 97%, from about 85 to slightly more than 2 μL^2 (measured at 2.0 mL/min). This was mainly achieved thanks to the occurrence of fully developed turbulent regime with a proper selection of capillary i.d. at significantly smaller flow rates (1.5–4 mL/min; CO2/methanol 80/20, v/v; 35 °C; back pressure regulator (BPR), 105 bar) than in entry-1 configuration. Ultrahigh efficiency columns of different geometries in-house packed with latest generation sub-2 μm UHPC-FPP-Whelk-O1 Chiral Stationary Phase (CSP) have been employed under sub- and supercritical fluid conditions. By carefully modulating the length and the internal diameter of connecting tubings in the function of column geometry, state of the art efficiencies (estimated in roughly 300 000 theoretical plates/m with reduced HETP of roughly 1.85) have been obtained on 4.6 mm i.d. chiral columns. Remarkably, for 3.0 mm × 100 mm (i.d. × length) columns, the efficiency gain on the fully modified SFC system (compared to an instrumental configuration where only the standard injector was replaced by the low-dispersion one) was greater than 90% for compounds with a retention factor of 1 and as large as 25% for retention factors of 2.5.

Introduction

Supercritical fluid chromatography (SFC) keeps attracting an increasingly growing number of users in different fields of research and industry. SFC allows for faster and "greener" separations than traditional reversed- or normal-phase (RP or NP) chromatography. It is much less demanding than RP-HPLC in terms of pressure drops (when using columns of

similar dimensions), inasmuch as the viscosity of SFC mobile phases is significantly lower than that of typical mixtures employed in RP-HPLC. As a matter of fact, SFC allows one to run 3−5 times faster separations than RP-HPLC on columns packed with particles that have comparable diameters. SFC has become one of the preferred techniques for chiral analysis and purification in the pharmaceutical industry as well. The innovation in materials and manufacturing of chiral particles has indeed allowed the preparation, in the past few years, of a new generation of sub-2 μm fully porous particles (1−6) and second-generation 2.7 and 2.0 μm superficially porous ones,(7−15) which have opened new scenarios in the field of ultrafast high-efficiency chiral separations. Extraordinary results in terms of efficiency and separation speed have been achieved by means of these chiral stationary phases (CSPs) in RP, NP, and hydrophilic interaction chromatography (HILIC).(12−17)

Despite latest generation CSPs seem to be extremely promising for application in SFC, only a few examples of their employment under these conditions have been reported.(1,19−24) Different reasons can be advocated to explain this, but the excessively large extra-column dispersion of commercial SFC equipment (generally roughly around 90 μL^2 vs 1–2 μL^2 of modern UHPLC chromatographs) is definitely one of the most important. Such an extra-column variance is incompatible with the dispersion generated by modern HPLC columns,(20) including the chiral ones.(21,25)

The most advanced and complete work aimed at exploring the potential of new generation chiral columns in SFC have been those by Berger(18,19) and Armstrong and co-workers.(22,26) In particular, Berger modified a commercial 1260 Infinity SFC from Agilent Technology, by replacing standard tubing (170 μm i.d.) and flow cell (13 μL internal volume) with 120 μm i.d. tubing (of shortest possible length, *L*) and a 2 μL internal volume cell. Extra column dispersion was reduced by about 1 order of magnitude compared to the plumbing of the original setup and diminished to about 6–9 μL^2.(19,21) Under these conditions, he was able to achieve reduced HETP of 1.93 (with an efficiency greater than 280 000 plates/m) by employing a prototype 50 mm × 4.6 mm (*L* × i.d.) column packed with 1.8 μm fully porous Whelk-O1 particles prepared by some of the authors of this work. By using a Jasco SFC equipment properly modified to reduce its extra-column volume, Barhate et al.(26) investigated in detail some unexpected effects due to the compressible nature of mobile phases employed in SFC by focusing on the complex relation of hold-up time, retention time, efficiency, and optimum velocity with tubing diameter and flow regimes. They used columns packed with CSPs prepared on 1.9 μm fully porous particles of narrow particle size distribution. In this remarkable contribution, they evaluated the effect of i.d. tubings on the kinetic performance of separation by using i.d. as small as 0.05 mm. Because of the interplay of different effects to band broadening, they observed the best performance with 0.25 mm i.d. tubing showing that, counterintuitively, diminishing extra-column volume is not always the right approach to increase efficiency. Patel et al.(22) explored the use of second generation chiral superficially porous particles to perform high efficiency, ultrafast chiral separations in SFC.

The purpose of this work is to contribute to the development of chiral SFC to fill the gap between this technique and UHPLC. A Waters Acquity UPC^2 system has been in-house modified through a series of technical solutions including not only the replacement of tubings, flow cell, and injection system (to minimize extra-column dispersion) but also the employment of an *ad hoc* designed external column oven. The 50 and 100 mm long columns (with i.d. either 3.0 or 4.6 mm) have been prepared by slurry packing fully porous 1.8 μm Whelk-O1

particles of the latest generation(1,7,27) and operated under sub- and supercritical fluid conditions.

It is worth noting that both particles and columns employed in this work are different from prototypes used by Berger in ref (19). Indeed, the new columns have been packed with Whelk-O1-based fully porous 1.8 μm particles obtained by using a monofunctional chlorosilane Whelk-O1 selector (Whelk-O1 selector-dimethyl-monochlorosilane) in place of the trifunctional silane reagent (mercaptopropyl trialkoxysilane) used for the preparation of columns used in ref (19). The new CSP has allowed to achieve a significantly higher selectivity (more than 20% based on our internal tests), owing to an higher selector loading and a better passivation of silica matrix. In addition, the packing procedure for the preparation of columns was different as well. As a matter of fact, the new columns provided better kinetic performance than those used by Berger as evidenced also by the fact that no fronting/tailing of peaks was observed contrary to what has been reported by Berger.(19)

Experimental Section

Materials and Chemicals

HPLC gradient grade solvents were filtered before use on 0.2 μm Omnipore filters (Merck Millipore, Darmstadt, Germany). Racemic *trans*-stilbene oxide (TSO) was purchased from Sigma-Aldrich. Kromasil silica (1.8 μm particle diameter, 100 Å pore size, 323 m^2/g specific surface area) was from Akzo-Nobel (Bohus, Sweden). Whelk-O1 chiral selector was generously donated by Regis Technologies Inc. (Morton Grove, IL). Stainless steel empty columns (50 and 100 mm long, i.d. either 3.0 or 4.6 mm) were from IsoBar Systems by Idex (Erlangen, Germany). Viper column inlet/outlet capillaries (250 and 350 mm long with 0.18, 0.13, and 0.10 mm i.d.) were from Thermo Fisher Dionex (Waltham, MA).

Apparatus

SFC

An Ultra Performance Convergence Chromatography UPC^2 Acquity Waters system (Milford, MA) was in-house modified through the replacement of (i) the original 8 μL flow cell by a 3 μL one, (ii) the standard 10 μL loop autosampler (model SM-C2S) by an external 200 nL fixed-loop injector from VICI (model number EHMA), and (iii) the existing injector-to-column and column-to-detector connecting tubings (600 mm long, 0.175 mm i.d.) by shorter Viper capillaries. In addition, the need of reducing as much as possible the extra-column volume required a major modification, i.e., (iv) the employment of an external in-house made column oven with a preheater (±0.1 °C by an electronic temperature controller) in place of the standard one (model CM-30, whose internal volume was estimated of about 40 μL) to heat the column oven and the mobile phase (before it enters the column) to the desired initial temperature. Column was insulated from the compartment walls in the stagnant air bath oven through rubber insulators (adiabatic conditions). The system allows a 4 mL/min maximum flow-rate with 415 bar maximum backpressure. Sampling rate was set at the maximum value of 80 Hz.(28) Depending on the modifications introduced, five different instrumental configurations were employed (in all of them the external 200 nL fixed-loop injector was used, see later on for explanation). For the sake of clarity, they are schematically reported

in Table 1 (entries 1–5). Some details and pictures of the modified equipment are reported in the Supporting Information (Figures S1 and S2).

External injector, 200 nL fixed-loop injector (see Experimental Section for details). Extra-column volume and variance were measured at 80/20 CO_2/methanol, v/v (back pressure regulator (BPR), 105 bar). Extra-column variances reported in this table refer to 2 mL/min flow rate (see text for details). They were measured through peak width at half height. CM-30 is the oven model with which the UPC^2 was originally shipped. 600 × 0.175 mm (*L* × i.d.) tubings are the original ones.

UHPLC

An Acquity Waters UPLC I-Class system (Milford, MA), equipped with a binary solvent manager (2.0 mL/min maximum flow-rate, 1200 bar maximum back-pressure), a 5 μL injection loop autosampler, a thermostated column compartment (operated in still air conditions(20)), and a diode array detector with a 500 nL flow cell (80 Hz acquisition rate) was also employed. Two Viper capillaries (respectively, 250 and 350 mm × 0.100 mm, *L* × i.d.) were used as inlet and outlet connectors.

CSP and Column Preparation

Chiral Whelk-O1 1.8 μm totally porous particles were prepared according to refs (1,7,and 27). Based on elemental analysis calculations, the loading of chiral selector was 395 μmol/g of silica (more information in the Supporting Information). The synthetic procedure (Regis Technologies Inc. proprietary bonding protocol) used to synthesize the Whelk-O1 CSP employed in this work is different from that used by some authors of this paper to prepare the chiral particles employed by Berger in ref (19). These modifications were introduced to achieve higher loading and selectivity (∼+20%). In addition, packing protocols were also different. Briefly, in this work, the slurry solution (functionalized particles/acetone, 10/90% w/v) was pushed into column by using hexane/2-propanol mixture 90/10% v/v as the pushing solvent by means of a pneumatically driven Haskel pump (1000 bar maximum back-pressure). Pressure was gently increased from 400 to 1000 bar during packing. Then, 100 mL of pushing solvent were pumped into column at 1000 bar constant pressure for bed consolidation. Decompression until atmospheric pressure was gradually performed. Under NP conditions (data not shown), it was found that the new “version” of the CSP allowed for better efficiency, enantioselectivity, and larger resolution than the former one.

Chromatographic Conditions

SFC separations were performed by using CO_2/methanol mixtures as the mobile phase. In UHPLC, the mobile phase was made of hexane/ethanol (90/10, v/v). Injected volumes were 200–500 nL. The hold-up time was estimated by injection of chloroform in SFC and in UHPLC conditions. All chromatographic measurements were performed at 35 °C. The extra-column volume was calculated by the slope of t_{extra} vs $1/F_v$ plot (Figure S3 of the Supporting Information), where t_{extra} represents the extra-column time (min) and F_v is the flow rate (mL/min). The extra-column variance, σ_{extra}^2, was calculated by using a zero-dead-volume (ZDV) connector in place of the chromatographic column. σ_{extra}^2 was calculated both through peak width at half height (as recommended by European Pharmacopoeia(29)) and by peak

moments.(30) Experimental peaks have been exported and fitted by means of an Exponential Modified Gaussian (EMG) function to calculate first and second central moments. The second central moment (corresponding to the peak variance) is defined as follows:

$$\mu_2' = \frac{\int_0^\infty (t-\mu_1)^2 C(t)\,dt}{\int_0^\infty C(t)\,dt} \quad (1)$$

where μ_1 is the first moment (corresponding to retention time):

$$\mu_1 = \frac{\int_0^\infty tC(t)\,dt}{\int_0^\infty C(t)\,dt} \quad (2)$$

Column efficiency through the height corresponding to a theoretical plate, *H*, was calculated by

$$H = \frac{\sigma_{peak}^2}{L} \quad (3)$$

where σ_{peak}^2 is the peak variance (and *L* the column length). In all measurements, an outlet pressure *P* of 105 bar was used. Retention factor *k* was traditionally calculated as

$$k = \frac{t_R - t_0}{t_0} \quad (4)$$

being t_R and t_0 the retention and hold-up times.

Results and Discussion

The characteristics of the five instrumental configurations investigated in this work are schematically summarized in Table 1 (entries 1–5). In all cases an external injector was used. Essentially, we observed that the as-shipped one did not allow to perform efficient injections (with peak splitting effects) when the column was removed and replaced with a ZDV connector (possibly, due to an insufficient back-pressure under these conditions). However, following Grand-Guillaume Perrenoud et al.,(25) an extra column variance of 85 μL^2 can be taken as the benchmark for the performance of the commercially available version of the Waters UPC^2. As it can be noticed by data reported in Table 1 (entry 1 vs 2), the replacement of the 8 μL cell by a 3 μL one allows for a significant relative improvement of system variance, which passes from 29 to 15 μL (−52%). However, if compared to σ_{extra}^2 of state-of-the-art UHPLC equipment (1–2 μL^2),(31) it is evident that this value is still dramatically large for high-efficiency applications. Thus, to further reduce the extra-column volume and variance, the next step was the substitution of original tubings (600 × 0.175, *L* × i.d.) connecting the injector to the column and the column to the detector with the shortest possible Viper capillaries (i.d. ranging between 0.18 and 0.10 mm, see Table 1). Simultaneously, the original oven was replaced by an in-house made one (see Figures S1 and S2 of the Supporting Information). After these modifications, σ_{extra}^2 (estimated through the half-height method(30)) significantly diminished from 15 μL^2 to either 3 or 2.2 μL^2, depending on 0.18 or 0.13 mm i.d. capillaries were, respectively, used. Contrary to our expectations, on the other hand, the employment of 0.10 mm i.d. capillaries did not allow to reduce the extra-column variance below previous limits. This is most likely due to the dominant contribution to variance by the 3 μL detector cell

volume (the smallest we could test). Therefore, unless a more efficient flow cell is available,(32) capillaries with i.d. smaller than 0.13 mm should not be used to avoid the unnecessary increase of system back-pressure (to push the mobile phase through the narrower capillaries) without getting any significant improve in dispersion. As it will be shown later on, depending on column geometry and experimental conditions, even 0.13 mm i.d. capillaries sometimes do not provide any advantage with respect other instrumental configuration mounting larger i.d. capillaries (see Table 1), which should be then preferred to avoid the previously mentioned drawbacks. To get also visually an idea of the dramatic effect on peak shape obtained by moving from the entry-1 configuration (see Table 1) to the others considered in this work (entries 2–5), readers can look at Figure S4 of the Supporting Information, where chromatograms recorded at F_v = 2 mL/min by injecting chloroform without a column are reported for each configuration. Incidentally it is important to stress that not only capillaries i.d. but also their physical properties (first, internal roughness and material) are extremely important factors affecting the onset of turbulence as deeply discussed by Broeckhoven et al. in ref (33).

Figure 1, on the other hand, shows how the system variance changes by changing the flow rate on the five different configurations with CO_2/methanol 80/20, v/v as the mobile phase. In this plot, σ_{extra}^2 was estimated through peak moment in order to maximize the accuracy of these data.(30)

The figure points out a major difference between systems equipped with the standard oven (entries 1 and 2, upper lines in the plot) and those where the in-house made oven was used (entries 3–5, bottom part of the plot). When the former are considered, indeed, one observes σ_{extra}^2 to decrease almost linearly with increasing the flow rate up to F_v about 3 mL/min. Then, for F_v > 3, σ_{extra}^2 reaches a sort of plateau. On the other hand, by looking at how σ_{extra}^2 changes with F_v for configurations where the external oven was used (entries 3–5), a quite different behavior can be evidenced. σ_{extra}^2 remains indeed essentially constant from F_v = 1 mL/min to 4 mL/min (maximum flow rate achievable with the equipment). Even though the understanding of these features is complex and definitely deserves more experimental investigation (see later on), a tentative explanation is based on the study of the flow regime inside capillaries as a function of experimental conditions and instrumental configuration. Reynolds number, *Re*, is the dimensionless quantity that is traditionally employed to predict flow pattern inside tubes or pipes. It is calculated as(34) $Re = \frac{u d_{tube}}{\eta}$ (5) where d_{tube} is the internal diameter of the tube (cm), u the linear velocity (cm/s) ($u = 4F_v/\pi d_{tube}^2$) and η the viscosity (cm^2 per second). Then, it is usually assumed that when *Re* < 2000–2300 the flow is laminar. On the other hand, if *Re* > 4000 turbulent flow occurs. A transient regime exists for *Re* values of 2000–4000. Equation 5 strictly holds for empty capillaries. Its application to systems considered in this work (entries 1–5 Table 1) is therefore an approximation since (i) two capillaries were connected through a ZDV union, (ii) in the flow pipeline to which Reynolds' analysis applies a detector (volume either 8 or 3 μL) is also present; and (iii) for entries 1–2 (equipped with the standard oven) also a flow path volume and geometry through the oven contributes to *Re*. However, this equation can still be useful to get an idea of the flow regime inside the system in its entirety. Application of eq 5 in SFC requires the additional knowledge of how viscosity (η) of CO_2/methanol mixtures varies with both temperature and pressure. By assuming constant temperature, the experimental determination of the pressure drop along tubings at different F_v values (see Figure S5 of the Supporting Information) allows for the calculation of η(*P*) and, with some degree of approximation, that of *Re* values for the different

configurations. Details of these calculations are reported in the Supporting Information (Figure S6). From them some conclusions can be drawn. First, the nonlinear dependence of system back pressure against flow rate (Figure S5 of the Supporting Information) clearly indicates the failure of Darcy's law, which predicts proportionality between flux and pressure drop per unit length. When turbulence is approaching, increasingly growing inertial effects become dominant and the relationship between P and F_v is not linear any longer. With reasonable approximation, Reynolds' analysis shows that for entries 1 and 2 in Figure 1, unless $F_v > 3$ mL/min, turbulent regime is not fully developed. In the region before ($F_v < 3$ mL/min), the transition regime prevails. On the other hand, for entries 3–5 (Figure 1) *Re* values show that turbulent regime occurs already at flow rates as small as 1–1.5 mL/min. The main consequence of turbulence is the increase in mass transfer. The erratic shifts in the entire flow stream (typical of turbulent conditions) indeed strongly enhances mass transfer (that under laminar conditions is largely controlled by molecular diffusion), in a mechanism that has been defined turbulent diffusion.(35,36) Where turbulence has well-developed, turbulent diffusivity increases roughly in proportion to mean flow velocity.(35,36) This means that, under these conditions, diffusivity can be replaced by a term proportional to u. Thus, this simple analysis could explain the quasi-constant behavior observed in Figure 1 between σ_{extra}^2 and F_v for both entries 1 and 2 when $F_v > 3$ mL/min (plateau zone) and for entries 3–5 (in the entire F_v range). On the other hand, the quasi linear σ_{extra}^2 decrease observed for entries 1–2 ($F_v < 3$ mL/min) makes sense with the change of regime and the following decrease in dispersion typical of the transition zone. From a more practical viewpoint, these data also show that the use of 0.13 mm i.d. capillaries vs 0.18 mm ones is advantageous in terms of σ_{extra}^2. On the other hand, the use of 0.10 mm i.d. capillaries (entry-5) is not convenient if compared to 0.13 mm i.d. ones. Indeed σ_{extra}^2 does not decrease at the cost of significant higher back-pressure (e.g., +30% at 2 mL/min by moving from 0.13 to 0.10 mm i.d. capillaries). However, the use of 0.10 mm i.d. capillaries could be advantageous if lower dispersion detector cells can be used. In order to get a deeper understanding of these phenomena, the knowledge of pressure, density, and viscosity effect on solute diffusion coefficients is required. Differently than in liquid chromatography, indeed, in SFC it cannot be assumed diffusion coefficients (and viscosity) not to be a significant function of pressure and/or density. This information however is still unknown and more fundamental work is needed in this area.(37) Another very important point that must be evidenced is that the net advantage of fully developed turbulent regime inside capillaries might be lost with the column in place. Presumably, indeed, at the maximum flow rates reachable on commercial SFC equipment(34) turbulence inside the packed column cannot be achieved. On the other hand, band broadening at these flow rates is controlled by a supposedly large C-term of the van Deemter equation. In this sense, the use of open-tubular chiral columns could be very promising in SFC. This is an old concept(34) that, thanks to the technological improvement of modern SFC instrumentation, seems mature enough to be reconsidered.

The comparative evaluation of systems (entries 1–5 Table 1) under working conditions is based on the study of the dependence of efficiency on F_v (traditional van Deemter analysis), by considering the enantiomers of TSO as probes (details under the Experimental Section). It starts by comparing the van Deemter curves of the two enantiomers on the entry-1 configuration of UPC^2 (see Table 1). Figure 2 reports the results of this investigation on both a 50 mm × 4.6 mm and 100 mm × 4.6 mm (L × i.d.) columns.

These findings demonstrate the detrimental effect of system variance on the efficiency in SFC. In confirmation of this, Figure 3 shows that the van Deemter curves of the two enantiomers switches if the same measurements are performed on entry-3 configuration (Table 1) with the 100 mm × 4.6 mm (*L* × i.d.) column or on entry-4 one with the 50 mm × 4.6 mm (*L* × i.d.) column, respectively.

Analogous studies performed on the 100 × 3.0 mm (*L* × i.d.) column have shown the same behavior, as it can be evinced from Figure 4 where van Deemter curves of the two enantiomers measured on the entry-1 configuration (Table 1) vs those measured on the entry-4 one are compared. If 3.0 mm i.d. columns of shorter length are considered, the effect of σ_{extra}^2 even in low-dispersion configuration can be very important (as it will be shown below).

To generalize these concepts, simple considerations based on the relationship between peak variance σ_{peak}^2, retention factor *k*, and column dimensions (*L* × i.d.) allows to get an idea of the impact of σ_{extra}^2 on column performance. This information can be taken as a rule of thumb to guide the selection of the most convenient instrumental configuration (which is always a compromise between σ_{extra}^2 and pressure drop) in function of the column that must be employed (or is available in the lab). Calculations are reported in detail in Table S1 of the Supporting Information for different columns geometries (column *L* between 20 and 100 mm, column i.d. either 3.0 or 4.6 mm) and retention factor *k* (0–3). These calculations are based on a theoretical reduced plate height, *h*, equal to 2 ($h = H/d_p$, being d_p the particle diameter)(7) and show that, for instance, by assuming *k* = 0 on the entry-1 configuration (σ_{extra}^2 = 29 μL^2), columns of dimensions smaller than 100 mm × 4.6 mm (*L* × i.d.) should be always avoided. In this case, indeed, σ_{peak}^2 is essentially the same as σ_{extra}^2. If *k* = 1.5, on the other hand, columns as short as 20 mm (with 4.6 mm i.d.) generate peak variance almost twice as large as σ_{extra}^2. If one considers 100 mm × 3.0 mm (*L* × i.d.) columns, the entry-1 configuration should be avoided unless *k* = 2 or larger. To conclude the study of σ_{extra}^2 effect on column performance, Figure 5 shows that the gain in efficiency on a 100 mm × 3.0 mm (*L* × i.d.) column, by moving from entry-1 configuration (bottom chromatogram) to entry-4 one (top chromatogram), is more than 85 and 20%, respectively, for *k* = 1.2 and *k* = 3.6 (see the figure caption for details).

Resolution ($R_s = \frac{2(t_2 - t_1)}{w_1 + w_2}$, being *t* and *w* retention time and peak width, respectively) was 26 (entry-4, red trace) and 21 (entry-1, black trace). Eluent, CO_2/methanol 80:20 v/v; flow-rate, 2.0 mL/min.

The effect of extra-column band broadening was also investigated as a function of the percentage of methanol in the mobile phase. For this purpose, methanol amount was varied from 5 to 40%, v/v. By increasing the amount of alcohol, not only density and viscosity of the mobile phase increase but retention decreases following a typical NP behavior. Even if, as mentioned before, one should know the dependence of diffusion coefficients on mobile phase composition at high pressures and when and where turbulence develops by changing methanol amount, this study serves as an empirical evaluation of the effect of mobile phase composition on the optimal flow-rate and maximum efficiency that can be achieved. The results are summarized in Table 2. Of remarkable importance are the very large efficiencies (*N*/m) obtained on entry-4 configuration (no matter the amount of methanol), with *h* as small as 1.88 (measured through half-height method(30)), while the effect of methanol amount on column performance on entry-1 configuration is dramatically evident (on the first eluted

enantiomer *N*/m decreases by more than 30% by increasing the fraction of methanol from 5 to 40%). As an example, Figure S7 of the Supporting Information shows the van Deemter curves obtained on entry-1 vs entry-4 configurations at different concentration of methanol in the mobile phase for the first eluted enantiomer (worse case).

Column: 50 mm × 4.6 mm (*L* × i.d.). See Table 1 for entry-1 and entry-3 configurations. First and the second values in each cell refer to first and second eluted enantiomers, respectively.

The last part of our study focuses on the comparative evaluation of kinetic performance of chiral SFC vs UHPLC. To this end, an I-Class equipment was used with σ_{extra}^2 of 1.0 μL^2 (at 1.0 mL/min, see the Experimental Section for details). The evaluation is essentially based on the comparison of efficiency (*N*/m) and analysis time achieved either on the 50 mm × 4.6 mm or the 100 mm × 3.0 mm (*L* × i.d.) columns operated under NP and SFC conditions. Figure 6 reports the chromatograms and the van Deemter curves recorded at the maximum efficiency achievable on the two systems (on the 50 mm long column) in function of mobile phase composition and flow rate.

This study reveals that not only the analysis time (as expected) is shorter in SFC than in UHPLC but that, remarkably, also the efficiency was (slightly) larger on the former case. In particular, in UHPLC, 290 000 *N*/m (at $F_{v,opt}$ = 1.4 mL/min) and 263 000 *N*/m ($F_{v,opt}$ = 1.2 mL/min) were obtained for the first (*k* = 0.7) and the second eluted enantiomer (*k* = 1.7), while in SFC (entry-4 configuration) the efficiency reached respectively 296 000 *N*/m (first eluted enantiomer, *k* = 0.9) and 287 000 *N*/m (second eluted enantiomer, *k* = 2.4) at $F_{v,opt}$ = 3.5 mL/min. Therefore, in terms of analysis time, this means a gain of more than 70% in SFC with the same (or slightly better) efficiency. The expression ultrahigh performance SFC (UHPSFC) can be properly used under these conditions. The comparison between UHPSFC and UHPLC can also be made under conditions where retention of enantiomers is similar. Thus, mobile phase composition in SFC was changed by 80/20 to 60/40 CO_2/methanol, v/v so that *k*s for first and second enantiomers moved from 0.9 and 2.4 to 0.7 and 1.7, respectively (see Figure S8 of the Supporting Information). The increase in methanol percentage causes the decrease of $F_{v,opt}$ at 2.7 mL/min. Due to the larger impact of σ_{extra}^2 in these conditions (see before), the efficiency of the first eluted enantiomer was found to be essentially the same in UHPSFC and UHPLC. On the opposite, for the second eluted enantiomer a +10% increase in efficiency was in any case observed. An analogous study on the optimal performance of UHPSFC vs UHPLC has been also performed by using the 100 mm × 3.0 mm (*L* × i.d.) column. Figure S9 of the Supporting Information shows the van Deemter curves measured in this case. Essentially, the conclusion is reached that when 3.0 mm i.d. columns are employed, a further step has to be performed to decrease σ_{extra}^2 in SFC in order to achieve comparable performance as in UHPLC (but at faster flow rates). This improvement most likely will require the reduction (or possibly a completely new design) of the detector cell.

Conclusions

The performance of a commercial UPC^2 equipment in SFC has been significantly enhanced by a series of technical modifications. Designed with the purpose of reducing the extra-column volume, these adjustments (in particular the substitution of the standard oven with one in-house made) have allowed one to modify the flow regime conditions inside capillaries. The most relevant consequence has been that turbulence in capillaries occurs much before (i.e., at significantly smaller back-pressure or flow rate) than on the original (as-shipped)

configuration. Turbulent transfer at high flow rate essentially restrains the increase of plate height to some constant value. This causes the σ_{extra}^2 to decrease dramatically if compared to the original configuration showing that turbulence in capillaries has an enormous potential when coupled to the latest generation high efficiency CSPs. In SFC chiral separations (by using the enantiomers of *trans*-stilbene oxide as probes), efficiency in the order of 300 000 *N*/m (*h* = 1.88) and 290 000 *N*/m (*h* = 1.91), respectively, for retention factors of 1 and 2.5 were obtained on 4.6 mm i.d. columns packed with the latest generation UHPC-FPP-Whelk-O1 1.8 µm fully porous particles. The comparison with UHPLC has shown not only that UHPSFC allows for much faster separations but also that the kinetic performance is better in UHPSFC than in UHPLC. On the other hand, if 3.0 mm i.d. columns are employed (unless their length is 100 mm or more), the too large σ_{extra}^2 even in low-dispersion SFC does not allow one to reach the same performance as in UHPLC. Definitely, some technical improvements are needed on commercial equipment to fill this gap. In particular, major improvements are expected through the design of low-dispersion ovens (ours was a prototype that can be definitely improved) and nanoliter flow cells. These findings could be the basis for a relaunch of chiral open tubular columns in SFC. The rationale for this is that most likely fully developed turbulent regimes could be maintained inside open tubular columns thus permitting to achieve unrivaled kinetic performance by operating them on low-dispersion SFC equipment.

Supporting Information

The Supporting Information is available free of charge: Elemental analysis, details of modified instrument, calculation of V_{extra}, chromatograms, Reynolds number calculation, van Deemter curves at different CO_2/MeOH mixtures, comparison between UHPLC and UHPSFC, and rule of thumb to decide the proper column length/i.d. combination as a function of retention to avoid extra-column effects.

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