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Recent achievements and future challenges in supercritical fluid chromatography for the enantioselective separation of chiral pharmaceuticals

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Abstract During the last years, Supercritical Fluid Chromatography (SFC) has attracted a continuously growing number of users. Thanks to the introduction of state-of-the-art equipments, this technique has allowed to run 3 to 5 times faster separations than in High Performance Liquid Chromatography (HPLC) on columns packed with particles of comparable dimension, at lower pressure drops and without loss of efficiency. Thanks to its high versatility, its high-throughput screenings capability and "green" character of the mobile phase, SFC has become particularly attracting for the separation of chiral drugs in pharmaceutical industries.

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1 Introduction

Most of the molecules that play a key role for living organisms (such as amino acids, nucleic acids, sugars, pharmaceuticals) are chiral [1, 2]. Since biological interactions are strictly stereospecific, the two enantiomers may exhibit a completely different biological activity. It has been demonstrated that, in many cases, while one enantiomer is effectively active as therapeutic, the other one could be totally inactive or even toxic for human body or the environment. As a consequence, identification of possible impurities and full characterization of chiral Active Pharmaceutical Ingredients (APIs) are crucial steps for the development of drug substances. For this reason, the availability of high performance analytical methods is fundamental at any stage of the production of pharmaceuticals or biomedical products, whose commercialization is strictly monitored by specific guidelines recommended by regulatory agencies [3–5].

Chromatography represents the most powerful technique nowadays in use for the separation of chiral compounds for both analytical and preparative purposes. Even if chiral High (or Ultra High) Performance Liquid Chromatography (HPLC/UHPLC) still remains the first choice for the separation of enantiomers, during the last years the attention of separation scientists has been moved towards alternative methods, in particular supercritical fluid chromatography (SFC). This technique is based on the same principles as those of LC and, as a matter of fact, it makes use of the same software, hardware and very similar instrumentation. The main difference is the replacement of common liquids used as mobile phases in LC with mixtures of high-pressurized carbon dioxide (CO_2) mixed with another solvent (most often methanol or other alcohols). The use of CO_2 above its critical point leads to several advantages from a chromatographic viewpoint. Thanks to a lower viscosity and higher diffusion coefficients, chromatographic separations in SFC can be carried out at high flow rates without remarkable loss of efficiency and with very limited pressure drop along the column.

The development of SFC as a separation method has been somehow slow and discontinuous. The first report on the use of supercritical fluids in chromatography traces back to 1960s [6] but, at that time, this approach did not attract much attention within the analytical community, having to compete with well established and traditional techniques as LC and GC. Twenty years later, neat supercritical fluid CO_2 found use especially with open tubular columns but also with packed ones [7–14]. Despite promising kinetic performance, the widespread use of SFC has been limited by different factors. Firstly, due to the poor eluting strength of CO_2 (comparable to that of pentane), this technique has been restricted mainly to the analysis of nonpolar compounds. Other issues come from instrumental limitations such as the lack of UV sensitivity as well as poor reproducibility and robustness of the system. During the 90s, SFC has started to be considered a real chromatographic method, especially for the purification of chiral compounds. Moreover, the introduction of a co-solvent opened the door to the analysis of polar molecules [15].

However, it is after 2010 that SFC has seriously started attracting a growing number of users not only for preparative applications but also for analytical purposes. In that period, latest generation SFC equipments have been commercialized with different technical improvements that enhanced reproducibility, sensitivity and reliability of the system. As a matter of fact, in state-of-the-art equipments, extra

column variance is still dramatically larger (70-100 μL^2) than in modern UHPLC equipment (1-2 μL^2). As it will be shown later on, this is a main issue when using very efficient columns of reduced internal diameter packed with fine particles [16–18].

In general, the use of supercritical fluid CO_2/co-solvent mixtures has gained popularity over the last years as a "green" alternative to normal phase or reversed phase (NP or RP) LC separations. Another advantage is that, differently from LC, polar and hydrophobic stationary phases can be operated in SFC with the same mobile phase, representing a powerful orthogonal method for different analytical applications, especially for chiral separations, as described in past detailed review papers. [14,15,19]

This review is not intended to be a comprehensive overview of SFC covering all the aspects of this technique from fundamentals of separation to instrumental aspects. Many detailed works [14,15,18–21] have been recently published to which the interested reader is referred to. Scope of this paper is to provide an overview of the most recent advances in chiral SFC. In particular, we will focus on the last applications reported in literature for rapid high-throughput separation of chiral pharmaceuticals and on the last achievements in ultrafast (sub-minute) chiral SFC separations.

2 SFC in a glance

In SFC, the mobile phase is a supercritical fluid. This is a particular state of matter reached when temperature and pressure are near or above the critical point. For pure CO_2, which is the most common supercritical fluid used in chromatography, these values are $T_c = 31^{\circ}C$ and $P_c = 74$ bar. Fluids exhibit particular properties in supercritical conditions that are intermediate between those of gases and liquids. In particular, density is similar to that of liquids, viscosity is comparable to that of gases and diffusivity is midway. For these reasons, SFC is considered an intermediate separation technique between gas chromatography (GC) and HPLC. As a marginal remark, the term SFC is also often extended to applications in which temperature is kept below than the critical one, since there is no phase transition when pressure is maintained above 74 bar.

Neat supercritical fluid CO_2 is a non polar solvent, comparable to pentane. However, it is rarely used as single eluent. Organic modifiers, such as methanol or other alcohols, are routinely used as co-solvents. Their introduction not only increases the polarity of the mobile phase, hence the solvating power, but it also affect density of the mobile phase. In addition, several different additives (e.g. phenols, polyhydroxy acids, trifluoroacetic acid, triethylamine, ammonium acetate) are often added to the mobile phase, enhancing solvating power or favouring ion-pairing with charged analytes. Also water is used sometimes as a ternary mixture, allowing for the elution of the most polar compounds, such as peptides and amphoteric molecules [21].

Density plays a key role on different chromatographic parameters in SFC. Firstly, molecular interactions, and hence retention, but also viscosity, diffusivity and mobile phase velocity are strongly influenced by changes in density. Density profile along the column is affected by any change in pressure and temperature. Significant variation in density could have a detrimental effect on column efficiency due

to the formation of radial temperature gradients when CO_2 is operated under high compressibility conditions [18]. For the reason above, modern SFC istruments are designed to ensure isothermal or adiabatic conditions of the column and a strict control of pressure.

3 Recent applications of SFC for the analysis of pharmaceuticals divided by the type of chiral stationary phase

3.1 Polysaccharide-based CSPs

The most employed class of CSPs used in SFC is that based on immobilized polysaccharide derivatives [14, 15, 22]. The widespread use of cellulose- and amylose-based CSPs can be ascribed especially to their large applicability and their high loadability. This latter characteristic has represented the main reason for the success of these CSPs for preparative purposes in the past [15, 23].
However, in the last few years, 3 or 5 μm fully porous particles (FPPs) polysaccharide based CSPs has been mainly used for analytical purposes. In the following, the main applications of polysaccharide CSPs in SFC will be revised.

3.1.1 High-throughput screenings

In a recent paper, different cellulose and amylose based 3 μm FPPs CSPs have been successfully employed for high-throughput screening of 20 pharmaceuticals (including ketoprofen, ibuprofen and epinephrine) in only 4 minutes, followed by 2 minutes of column equilibration [24]. The mobile phases used were CO_2/methanol or CO_2/2-propanol mixtures plus a combined additive of trifluoroacetic acid and diethylamine. The simultaneous presence of both the two organic modifiers was found to be beneficial for the enhancement of enantioselectivity.
Retention mechanism of thirteen pairs of enantiomers belonging to the same structural family (phenylthiohydantoin-amino acids) has been studied on two different polysaccharide stationary phases (Chiralpak AD-H, amylose based and Chiralpak OD-H, cellulose based) at five different temperatures ranging from 5 up to 40°C. [25] The mobile phase used was CO_2/methanol 90:10 %v/v. Some structural changes seem to affect both CSPs above 20-30°C. Below this limit it was found that retention is substantially unaffected by temperature changes on the cellulose based column. On the contrary, remarkable differences in retention factors have been observed on the Chiralpak AD-H by changing temperature. This result suggests the presence of more heterogeneous chiral sites on the amylose based CSP than on the cellulose one.
In order to enhance the possibility to carry out high-throughput analysis, Multiple Injections In a Single Experimental Run (MISER) chromatographic technique has been applied in SFC with a 10×4.0 mm (L×I.D.) Chiralpak AD-3 column packed with 3 μm FPPs. This high-throughput method is based on multiple injections within a single chromatographic run to produce a continuous trace of chromatograms. The separation of Tröger's base enantiomers in an entire 96 well microplate of samples has been performed in 33 min (Fig. 1). [26] However, this approach is currently limited by the speed of autosamplers. Faster instrument con-

trol softwares are required to extensively apply MISER SFC to rapid enantiopurity screenings of a large number of samples.

3.1.2 Determination of enantiopurity of APIs and their intermediates

One of the most challenging tasks for pharmaceutical industries is the reduction of the number of separation modes required for the analysis of an API and its intermediates. Due to its versatility, SFC appears one of the most appealing chromatographic method for this purpose.
Barhate et al. have investigated a large number of chromatographic CSPs by both RPLC and SFC for the determination of enantiomeric excess of verubecestat (employed in clinical trials for the treatment of Alzheimer's disease) and its intermediates [27]. The best results have been obtained in RPLC by using a teicoplanin based CSP made on 2.7 μm superficially porous particles (SPPs) but cellulose-based chiral columns produced very promising results in SFC for the determination of enantipurity of the entire verubecestat synthetic route.
Bu et al. have also recently applied SFC for the analysis of poor UV absorbing drugs and synthetic intermediates with charged aerosol detection (CAD) [28]. Enantiomeric excess has been determined under both gradient and isocratic elution conditions and compared with results obtained with UV detection. A strong correlation between UV and CAD responses under isocratic conditions was observed, while under gradient conditions higher absolute errors between the two measures were registered, due to the fact that high amount of organic modifier enhances CAD response of later eluting compounds. In the same work, the practical use of SFC-CAD has been investigated for high-throughput parallel screening of chemo- and bio-catalytic reactions for the quick identification of desired reaction conditions. 24 different hydrolase enzymes were screened in parallel on a well plate and the two isomeric monoacid products were separated on a Chiralpak AD-3 column in less than 2 hours.

3.1.3 Comparison between chiral SFC and HPLC

Different studies have been recently conducted in order to compare retention mechanism of enantiomers on polysaccharide based CSPs in SFC and different LC conditions.
West et al. have compared retention of 24 chiral sulfoxides on seven different polysaccharide CSPs with CO_2/methanol mixtures as mobile phase proving that chlorinated cellulose CSPs are better in terms of both retention and enantioselectivity towards molecules containig a chiral sulfur atom [29]. By means of molecular modelling measurements, the authors of this paper demonstrated that molecules that could adopt a folded U-shaped conformation were most efficiently discriminated compared to linear ones. Moreover, SFC was compared to polar organic mode (POM) HPLC. It was found that the chiral selector must adopt a different conformation in the two operating modes. However, SFC outperformed POM HPLC in terms of enantioresolution.
Recently, retention mechanisms of different pairs of enantiomers on polysaccharide CSPs have been explored in both SFC and NPLC conditions. It has been demonstrated that the transposability of methods from NPLC to SFC can be challenging in some cases, mainly due to different interactions (hydrogen bonding

and accessibility of chiral cavities) contributing to retention in the two chromatographic modes [30,31]. Separations in NPLC resulted in shorter retention times and higher enantioresolution for the separation of dihydropyridone derivatives, especially if bearing two chiral centers [32]. Indeed scope of this work was the investigation of chromatographic conditions transfer from NPLC to SFC (this explains reported larger retention times in SFC). Despite higher retention times, selectivity was not consistently better in SFC, meaning that the additional retention is due to non-specific interactions of enantiomers with the CSP.
Vera et al. have compared selectivity of a Lux Cellulose-1 towards retention of FMOC-protected amino acids in SFC, NPLC and RPLC conditions [33]. In terms of retention, SFC lies in the middle between RPLC and NPLC. Although RPLC gave comprehensively the best enantioresolution, the introduction of 2% formic acid as additive in CO_2/methanol mixture used in SFC provided comparable results in shorter run time, allowing for better resolution per unit of time.

3.1.4 Chiral SFC-MS methods

Even if RPLC-MS is still considered the gold standard for the analysis of serum, urine and plasma samples, in the last years SFC has been efficiently hyphenated with mass spectrometry giving very promising results.
A reliable SFC-MS/MS method for the separation of amphetamine enantiomers in biological samples has been developed and validated for the first time by Hegstad et al in ref. [34]. R- and S- amphetamine enantiomers were baseline resolved by using a Chiralpak AD-3 column and CO_2/2-propanol+0.2% cyclohexylamine as mobile phase. This method has been routinely used for the analysis of several human urine samples representing a reliable tool to discriminate between legal use of amphetamine as therapeutic (in most countries only the S-enantiomer is prescribed) and illegal use (as racemic mixture).
Jenkinson et al. have recently developed a new SFC-MS/MS method for the analysis of metabolites of vitamin D in human serum [35]. The separation has been achieved in 6 minute on a Lux Cellulose column by using CO_2/methanol+0.1% formic acid as eluent. Concentrations of metabolites measured on 41 routine human serum samples have been found to be in accordance with those measured by means of UHPLC-MS/MS. In addition, structurally similar metabolites, differing only for the position or direction of an hydroxyl group, have been resolved and quantified by means of the optimized SFC-MS/MS method. The only issue reported by the authors is that latest generation mass spectrometers are required for the quantitation of very low concentration of analytes (in the order of pg/mL) due to the low injection volumes used in SFC.
A 3 μm FPPs Chiralpak IA was efficiently used to separate panthenol enantiomers in cosmetic formulations (such as creams, body lotions and exfoliants) [36]. Since only the D-enantiomer of panthenol is active as therapeutic, reliable methods to assess enantiopurity of formulated cosmetics are required. The column was employed in SFC conditions (CO_2 with 11% methanol as mobile phase) with both UV and MS detection. The online coupling improved sensitivity (LOQ as low as 0.5 μg/mL) since underivatized panthenol has a poor signal in UV.

3.1.5 Multidimensional chromatography

Chiral SFC has also been applied as the second dimension in highly selective multidimensional chromatography approaches to assess purity of chiral APIs. A first (achiral) RPLC dimension is needed in order to assess the amount of impurities and related substances while the second (chiral) dimension is used to evaluate the possible presence of undesired enantiomers [37]. Such a system has been applied for the quantitative analysis of an API, its metabolites and their corresponding enantiomers in a mouse hepatocyte treated sample by using Chiralpak IB-3 and AD-3 columns (Fig. 2) [38].

3.1.6 Other applications

Polysaccharide based CSPs have been also successfully used for the separation of chiral compounds with biological activity such as pesticides containing sulfur or phosphorous atoms [39], fungicides (i.e. fenbuconazole) in foods [40] and herbicides (i.e napropramide) [41].

3.2 Pirkle-type CSPs

Pirkle-type (or brush-type) chiral selectors are among the most versatile, allowing for the separation of a broad range of compounds. They represent the first class of CSPs that has been prepared on sub-2μm format [42–44]. One of the main advantage of these CSPs is that they exist in both the enantiomeric versions. By using columns functionalized with the same chiral selector but with opposite configuration, it is possible to reverse the elution order of enantiomers (so-called "Inverted Chirality Columns Approach", ICCA). This method has been recently applied by Mazzoccanti et al. to determine the enantiomeric excess of phytocannabinoids in marijuana samples for therapeutic use [45]. Indeed, many problems can be faced when working with Cannabis plant extracts. Not only are they highly complex mixtures but also the minor enantiomer is not always available as reference sample. Moreover, in many cases it can partially coelute with the main enantiomer. Two complementary (S,S)- and (R,R)- Whelk-O1 CSPs made on sub-2μm FPPs have been employed in SFC conditions (CO_2/methanol, 98:2 %v/v) to determine the enantiomeric excess of (-)-Δ^9-THC in medicinal marijuana (Bedrocan®). Thanks to ICCA protocol, beside the major enantiomer ((-)-4 peak in Fig. 3), a not negligible concentration (0.13%) of the (+)-enantiomer ((+)-4 peak in Fig. 3) has been detected. The enantiomeric excess was estimated to be 99.73%.
The same sub-2μm FPPs (S,S)-Whelk-O1 CSP has been used for the high-throughput screening of a large library of pharmaceutical compounds with different chemico-physical properties including β-blockers, antidepressants, anticancers and benzodiazepines, to name but a few [46]. The overall screening was completed in 24 hours under fast gradient elution (9 minute total analysis time, including column reconditioning) by using a mixture of CO_2/methanol as mobile phase. Even basic racemic mixtures, whose separation is traditionally challenging on Whelk-O1 CSPs prepared on larger particles, have been resolved with the sub-2μm CSP used in this work.

3.3 Macrocyclic glycopeptide CSPs

Macrocyclic glycopeptides CSPs allow for the separation of underivatized amino-acids. This class of CSP has been rarely used in SFC to date.
Recently, however, a 5 μm teicoplanin CSP (Chirobiotic T2) has been efficiently employed for the separation of D,L- enantiomers of underivatized phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) amino acids. Baseline separations have been obtained in less than 7 minutes by using CO_2 and 40% of organic modifier made of a mixture methanol/water (90:10 %v/v) [47]. LOD in the range of 0.5-2.0 μg/mL allowed for the determination of D-enantiomers up to 0.2%. This method has been applied for the determination of enantiopurity of five commercial food supplements confirming the absence of impurities in all of them. The authors have also investigated the possibility to simultaneously determine D,L-Phe and D,L-Tyr by coupling a diol achiral column (first dimension) with the Chirobiotic T2 (second dimension). The separation was obtained in about 15 minutes.

3.4 Cyclofructan based CSPs

Recently developed cyclofructan based 2.7 μm SPP CSPs have been employed by Armstrong and coworkers to investigate the transposability of chromatographic methods from NPLC to SFC for the enantioseparation of 21 α-aryl ketones [48]. The mobile phase used in NPLC was a mixture of heptanol/ethanol with percentages ranging from 95:5 to 99:1 %v/v. The same compositions have been transposed to SFC by replacing heptanol with CO_2. 17 of the 21 compounds have been baseline separated in NP conditions, while 10 out to 21 compounds via SFC. Even if the latter allowed for lower analysis time, HPLC provided better resolutions due to greater enantioselectivity values.

3.5 Ion-exchange CSPs

The use of ion exchange CSPs in SFC is very recent. Lajkó et al. have firstly used Cinchona alkaloid based ZWIX(+) and ZWIX(-) CSPs for the enantioseparation of N_α-Fmoc proteinogenic amino acids [49]. The effect of methanol content in the mobile phase and different additives (water, acids and bases) have been investigated in order to optimize separation conditions. The addition of water led to the formation of carbonic acid, imparting acidic character to the mobile phase. It was also found that a reduction of temperature has a beneficial effect on enantioresolution, meaning that chiral recognition mechanism is enthalpically-controlled.
The chromatographic beavior of the ZWIX(+) column in both HPLC and SFC conditions was compared for the separation of acidic, basic and zwitterionic species [50]. In general, SFC provided better enantioresolution than HPLC but there is evidence that separation mechanism is completely different. By costantly increasing the amount of organic modifier (methanol) in SFC, it was found that the ion-exchange mechanism is strongly influenced by the formation of transient acidic species (carbonic acid mono methyl ester). Finally, it was proved that basic additives are not strictly necessary when using the zwitterionic column and they could have an effect only on basic analytes.

4 Towards high-efficiency ultrafast SFC enantioseparations

The recent achievements in particle manufacturing has allowed to prepare very efficient particle formats, such as SPPs or sub-2μm FPPs, functionalized with chiral selectors. The introduction of these new CSPs packed into columns of very short length has represented a real breakthrough in the field of UHPLC enantioseparations, not only in terms of efficiency (comparable to those of achiral RPLC) but also in terms of speed of separation (analysis time $<$ 1 sec) [27,43,46,51–62].
Since SFC allows to run chromatographic separations at higher flow rates than LC without remarkable loss of efficiency [63], the use of new generation CSPs under these conditions seems to be a promising approach to achieve even faster separations.
Some of the authors of this work have recently obtained very fast enantioseparations by using both Teicoplanin and Whelk-O1 CSPs under SFC conditions [46, 54].
In the first case, Teicoplanin was bonded to 1.9 μm FPPs and packed into a 20×4.6 mm (L×I.D) column operated at 4 mL/min. The enantiomers of Ketorolac have been resolved in less than 70 sec on a chiral selector which has been considered a "slow" one [54].
In the second case, by using a 50×4.6 mm (L×I.D) column packed with 1.8 μm FPPs functionalized with Whelk-O1, the separation of abscisic acid enantiomers has been obtained in less than 45 sec (flow rate 3.5 mL/min) with a resolution (R_s) of 2.2 (see Fig. 4a) [46].
Very remarkable results in terms of ultrafast enantioseparations have been reported by Armstrong and coworkers. Sub-minute separations of different pairs of enantiomers of pharmaceutical interest have been obtained on teicoplanin and teicoplanin aglycone CSPs made on 1.9 μm FPPs packed into 50×4.6 mm (L×ID) columns at a flow rate of 7 mL/min [64]. Moreover, by using a 30×4.6 (L×I.D) column packed with 2.7 μm SPPs functionalized with a quinine derivative, they were able to obtain the separation of different amino acids in 6-8 sec with a flow rate of 20 mL/min [65]. An example is shown in Fig 4b.
However, the use of new generation CSPs in SFC is often partially limited by some instrumental issues. The excessively large extra-column band broadening of current SFC instruments has a detrimental effect on the overall chromatographic performance.
Berger has recently modified a commercial 1260 Infinity SFC from Agilent Technology, by replacing standard tubing (170 μm ID) and flow cell (13 μL internal volume) with 120 μm ID tubing (of shortest possible length) and a 2 μL internal volume cell [66]. The extra column dispersion was reduced to about 6-9 μL^2.
With the new configuration, he was able to achieve more than 280,000 plates/m (reduced HETP of 1.93) by employing a prototype 50×4.6 mm (L×ID) column packed with 1.8 μm Whelk-O1 FPPs. In addition, he reported about the ultrafast separations of 5-methyl 5-phenyl hydrantoin enantiomers in roughly 10 sec (flow rate 5 mL/min).
By using the same instrumental setup, he has been the first to operate a sub-2μm immobilized polysaccharide CSP in SFC conditions [67]. By using a 50×3 mm (L×ID) column packed with an amylose-based CSPs made on 1.6 μm FPPs, he was able to obtain the ultrafast separation of warfarin enantiomers in less than 10 sec (flow rate 3.75 mL/min) with a resolution of 1.5 (see Fig. 4c).

Recently, some of the authors of this work have modified a commercial Waters Acquity UPC^2 SFC instrument by a series of technical adjustments including the replacement of (i) standard tubings with shorter and narrower capillaries; (ii) the 8 μL flow-cell with a 3 μL one; (iii) the injection system with a 200 nL fixed-loop external one and (iv), finally, by using an ad hoc designed external column oven [68]. The extra-column variance was reduced from about 85 μL^2 (original configuration) to slightly more than 2 μL^2 (optimized configuration) measured at 2.0 mL/min. Kinetic performance of a 50×4.6 mm (L×ID) column packed with 1.8 μm Whelk-O1 FPPs operated on the optimized SFC instrument have been compared with that obtained on a commercial UHPLC instrument (Waters Acquity I-Class) with extra-column variance of 1 μL^2. At the minimum of the van Deemter curve, SFC provided a gain of 10% on the efficiency of the second enantiomer (285,000 N/m *vs.* 260,000 N/m recorded in UHPLC) in roughly 50% shorter analysis time. The expression ultra-high performance SFC (UHPSFC) can be properly used under these conditions.

In addition, Barhate et al. have demonstrated that when running ultrafast SFC separations, some unexpected results could be observed [69]. These deviations, not detected in LC, are mostly due to the noise generated by back pressure regulators and the presence of low viscosity eluent inside connection tubings. The latter is responsible for the development of possible turbulent flow inside tubings which could change both retention time and peak shape.

5 Perspectives

Due to the unique properties of supercritical fluid CO_2, SFC can be considered not only a "greener" alternative to HPLC but also an orthogonal and, in some cases, more versatile method of separation. This is particularly important for high throughput screenings at the beginning of the production of new drugs, when the number of unknown impurities could be relevant.

One of the field in which SFC will be increasingly used is in multidimensional applications, in particular,

RPLC×SFC achiral-chiral separations. SFC is particularly attractive as second fast dimension in ^{2}D separations. However, particular attention has to be put on the interface between the first RPLC dimension and the SFC one, especially to avoid the injection of large volumes of water. Different approaches have been already proposed. Particularly interesting is the use of collection loops [70] or activate modulators [71] that seem to be able to solve some of the issues encountered in this coupling [72].

Thanks to the introduction of latest generation CSPs made on sub-3μm SPPs and sub-2μm FPPs packed into short columns (2-5 cm), the first examples of enantioseparations in less than one minute or even in the order of seconds have been obtained also in SFC. This is a very promising field in which SFC could be expected to emerge as a gold technique. However, as demonstrated in recent works [66–68], some technical optimizations aimed at the reduction of extra-column band broadening are needed on commercial equipments. This can be obtained not only by replacing standard tubings with small capillaries but also by using low-dispersion ovens and flow cells in the order of nanoliters. These improvements will increase competitiveness of UHPSFC towards UHPLC.

Fundamental studies on mass transfer phenomena in SFC are necessary in order to understand how diffusion coefficients possibly change with pressure and temperature, thus their effect on column efficiency. Moreover, due to the lower mobile phase viscosity, turbulent flow effects have been clearly demonstrated through capillaries connecting the injector system to the column and the column to the detector. From an experimental point of view, this is accompanied by a nonlinear dependence of system backpressure on the flow rate (contrary to what happens when the Darcy's law is applicable). When turbulence is developing, increasingly growing inertial effects become dominant and the relationship between flow and pressure is not linear any longer [73].
The main consequence of turbulence is the improvement in mass transfer [74]. On the other hand, experimental findings show that through a packed bed turbulence is much more difficult to develop (at least at the flow rates commonly employed in SFC). These findings could be the basis to renew the interest in chiral open tubular columns for SFC applications since through them maintaining of turbulent regime should be possible. These concepts were proposed more than 50 years ago in the fundamental work of J. C. Giddings [74] when, however, technology was not advanced enough to permit their practical realization. The use of open tubular chiral columns on low-dispersion SFC equipments could lead to unmatched kinetic performance in chromatography.

Compliance with Ethical Standards

Conflict of interest: the authors declare that they have no conflict of interest.

Ethical approval: this article does not contain any studies with human participants or animals performed by any of the authors.

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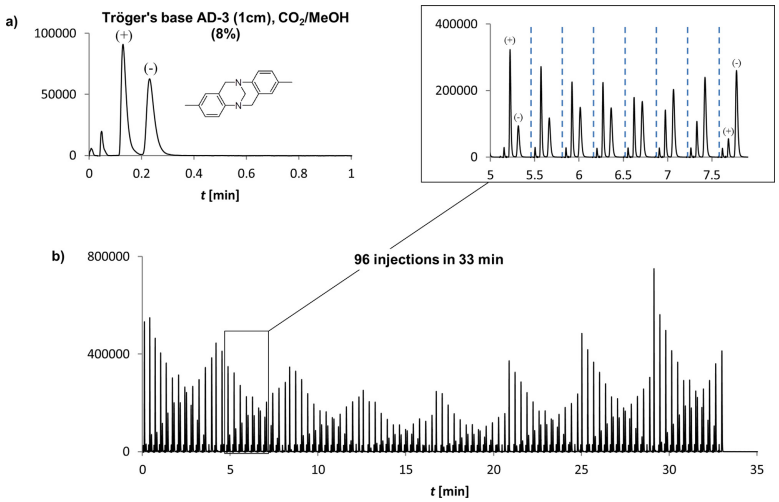

Fig. 1 a) Separation of Tröger's base enantiomers on a 10×4.0 mm (L×I.D.) Chiralpak AD-3 column packed with 3 μm FPPs functionalized with an amylose derivative. b) Injection of a 96 well plate with MISER approach on the same column. Tröger's base concentrations between 0.4-1 mg/mL. Reproduced with permission from Ref. [26].

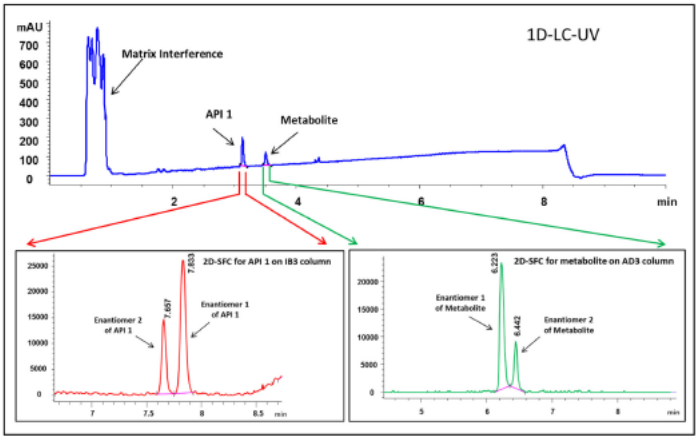

Fig. 2 2D LC-SFC analysis of an API and its metabolite. First dimension: achiral RPLC (top), second dimension: chiral SFC (bottom). Reproduced with permission from Ref. [38].

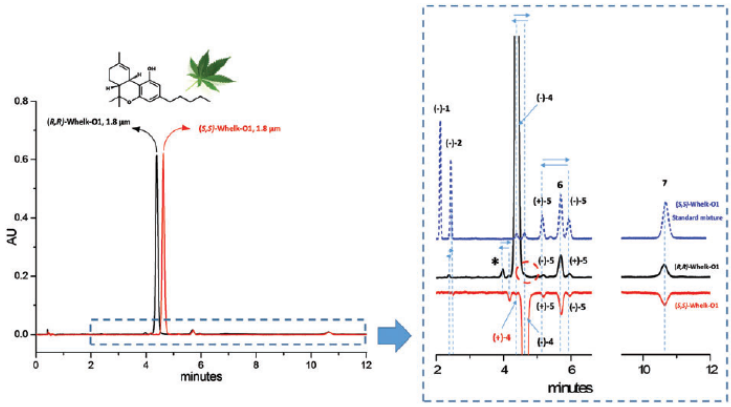

Fig. 3 Chromatograms of a Bedrocan® ethanol extract analyzed by applying the ICCA protocol. A zoom of the chromatogram between 2 and 6 minutes is shown in the inset together with the separation of a standard mixture of six-component cannabinoids (dotted chromatogram). The asterisk denotes a chiral unknown impurity. Reproduced from Ref. [45] with permission from The Royal Society of Chemistry.

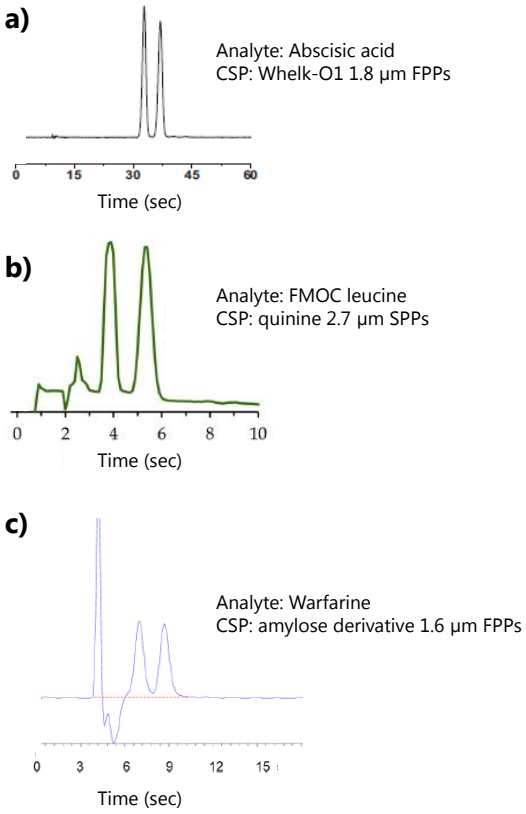

Fig. 4 Examples of ultrafast enantioseparations obtained in SFC. a) Abscisic acid enantiomers. Column: 50×4.6 mm (L×I.D), Whelk-O1 1.8 μm FPPs. Flow rate: 3.5 mL/min. Instrument: Waters Acquity UPC^2. Modified with permission from Ref. [46]. b) FMOC leucine enantiomers. Column: 30×4.6 (L×I.D), quinine-based 2.7 μm SPPs. Flow rate: 20 mL/min. Instrument: Jasco SFC-2000-7. Modified with permission from Ref. [65]. c) Warfarin enantiomers. Column: 50×3 mm (L×ID), amylose-based 1.6 μm FPPs. Flow rate: 3.75 mL/min. Instrument: Low dispersion modified Agilent 1260 Infinity SFC. Modified with permission from Ref. [67]

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Dear Editor,

following Your invitation to write a contribution for the special issue for the Birthday Jubilee of Chromatographia, I am pleased to submit the paper titled:

Recent achievements and future challenges in supercritical fluid chromatography for the enantioselective separation of chiral pharmaceuticals
*by: S. Felletti, O. H. Ismail, C. De Luca, V. Costa, F. Gasparrini, L. Pasti, N. Marchetti, A. Cavazzini, M. Catani**

This is a critical review on a topic which is receiving particular attention during the last years.

Supercritical Fluid Chromatography (SFC) is attracting a continuously growing number of users, especially for the analysis of chiral compounds. Due to the unique properties of the mobile phase employed (mixtures of supercritical fluid CO_2/co-solvent), SFC represents a faster and "greener" alternative to Reversed Phase (RP-) or Normal Phase (NP-) High Perfomance Liquid Chromatography (HPLC).

In this review we have considered the latest applications of SFC for the analysis of chiral compounds of pharmaceutical interest, covering main achievements of the last three years. We have also focused on some recent applications of SFC in ultrafast enantioseparations by using chiral columns of latest generation, such as those packed with sub-3μm Superficially Porous Particles (SPPs) or sub-2μm Fully Porous Particles (FPPs).
Finally, we have also discuss some technical improvements that are needed on commercial SFC equipment in order to reduce the still too large extra-column variance, that represents a technical limitation for high efficiency separations when using columns packed with very fine particles or with reduced internal diameter.

The paper is unpublished and has not been submitted for publication elsewhere.
Thank you in advance for your time and consideration.

With best regards,
Martina Catani and Alberto Cavazzini