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PRACTICAL APPLICATION OF SUPERCRITICAL FLUID CHROMATOGRAPHY FOR PHARMACEUTICAL RESEARCH AND DEVELOPMENT

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CHAPTER

2

Application space for SFC in pharmaceutical drug discovery and development

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Introduction

Drug discovery and development is an expensive and time consuming process, which takes approximately 12–15 years to produce a new drug and an estimated $2.6 billion to reach the market [1]. When searching for a new molecule that can modulate diseases during the initial phase of the drug discovery phase, it is important to keep in mind the breadth of chemical diversity among new chemical entities now considered by the pharmaceutical industry. Indeed, the number of small molecules with a molecular weight up to 500 Da, and containing only the most common atoms has been estimated at 10^{60} [2]. In addition, the number of possible peptides having 62 amino acids and composed of the 20 natural amino acids has been estimated at 10^{80} [3]. To date, only a small fraction of this available chemical space has been explored. It is also important to remember that information is rarely shared among companies about poorly behaved drug candidates (e.g., stability challenges, poor bioavailability, toxicity issues), making the drug discovery process incredibly difficult. Therefore, the procedures

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required in the pharmaceutical industry to find appropriate new compounds are remarkable. During the drug discovery process, it is necessary to screen millions of potential compounds (this number is small compared with the total number of possible small organic molecules) each year against a range of targets, and even then, success is not guaranteed. Before a drug molecule is synthesized and tested, and in an attempt to reduce attrition in drug discovery, the selection of compounds with suitable drug likeness is aided by rules developed using the physicochemical properties of the molecules. The most famous one is Lipinski's rule of five, which covers the number of hydrophilic groups (no more than 5 hydrogen bond donors and 10 hydrogen bond acceptors), molecular weight (less than 500 Da) and hydrophobicity (log P lower than 5) [4]. However, simple rules are not always accurate and may unnecessarily limit the chemical space of the search. Therefore, many companies also screen compounds that have properties outside these conditions, and many marketed drugs have features that cause them to score poorly on various drug likeness indices [5,6]. Among the exceptions, many of the common molecules originate from natural products. These molecules are recognized as contributing valuable chemical diversity in the design of molecular screening libraries [7,8], despite some difficulty in accessing renewable sources of plant-derived products [9]. To better understand the chemical space covered by synthetic molecules and natural products, Fig. 1 [10] highlights the obvious differences between three different groups of compounds, namely known natural products (NPs), readily obtainable natural products, and drugs [11], visualized through principal component analysis (PCA) plots.

Despite significant advances in technology, the drug discovery process remains expensive and time consuming. With the existence of such very large libraries of synthetic or natural compounds having diverse/heterogeneous chemical properties, there is a need for innovative analytical strategies, mostly based on chromatography. These are fast, automated and high-throughput screening methods adapted to be able to characterize the largest number

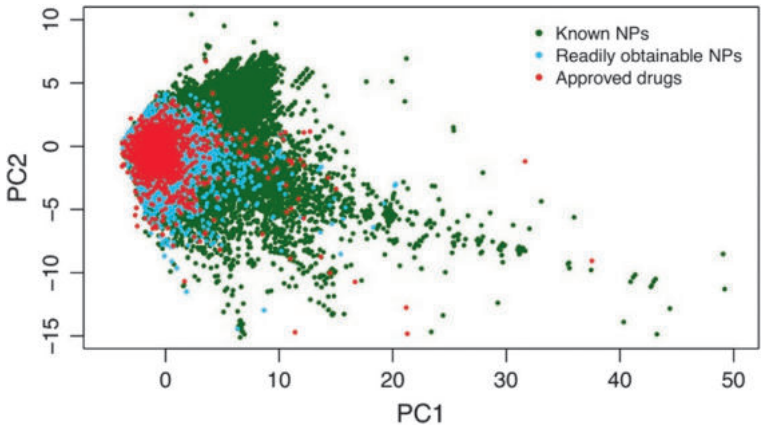

FIG. 1 Scatter plots of the second principal component (PC) against the first PC for the reference data sets based on 17 relevant physicochemical properties. The figure shows the data points of the known natural products (NPs), readily obtainable NPs and approved drugs. *Adapted with permission from Y. Chen, M. Garcia de Lomana, N.-O. Friedrich, J. Kirchmair, Characterization of the chemical space of known and readily obtainable natural products, J. Chem. Inf. Model. 58 (2018) 1518–1532. doi:10.1021/acs.jcim.8b00302.*

of compounds in the pharmaceutical research and development (R&D) environment. The main applications of chromatography in modern drug discovery can be summarized here [12]. First, reliable and generic (i.e., widely applicable) chromatographic methods have to be developed to check identity and amount of impurities in potential lead compounds from natural and/or synthetic sources. To meet these objectives, it is necessary to develop open-access, fully automated LC-UV and LC–MS systems that can be used by chemists [13]. In addition, there is a need to chromatographically purify sufficient amounts of compounds for pharmacokinetics/pharmacodynamics studies. This is particularly difficult when compounds contain a chiral center and the toxicity profile of each enantiomer has to be assessed.

From an analytical point of view, reversed phase liquid chromatography (RPLC) coupled with UV or mass spectrometric (MS) detection is considered as a gold standard analytical technology in the pharmaceutical industry. RPLC can indeed be applied to any compound having a log$P$ value (a measure of molecular hydrophobicity/lipophilicity) between -1 and 6 (this lipophilicity range fits very well with Lipinski's rule of five) as highlighted in Fig. 2. In RPLC, an apolar stationary phase is used (i.e., C4, C8, C18, phenyl), while the mobile phase is composed of a mixture of buffered water and polar organic solvents (i.e., acetonitrile, methanol, tetrahydrofuran). The retention mechanism of RPLC is mostly based on hydrophobic interactions, but other mechanisms can also take place depending on the mobile phase conditions and chemical nature of the stationary phase (i.e., ionic interactions, π–π interactions). The success of RPLC is mostly due to its versatility, simplicity, robustness, ease of hyphenation to MS and all contribute to its widespread use in the pharmaceutical industry.

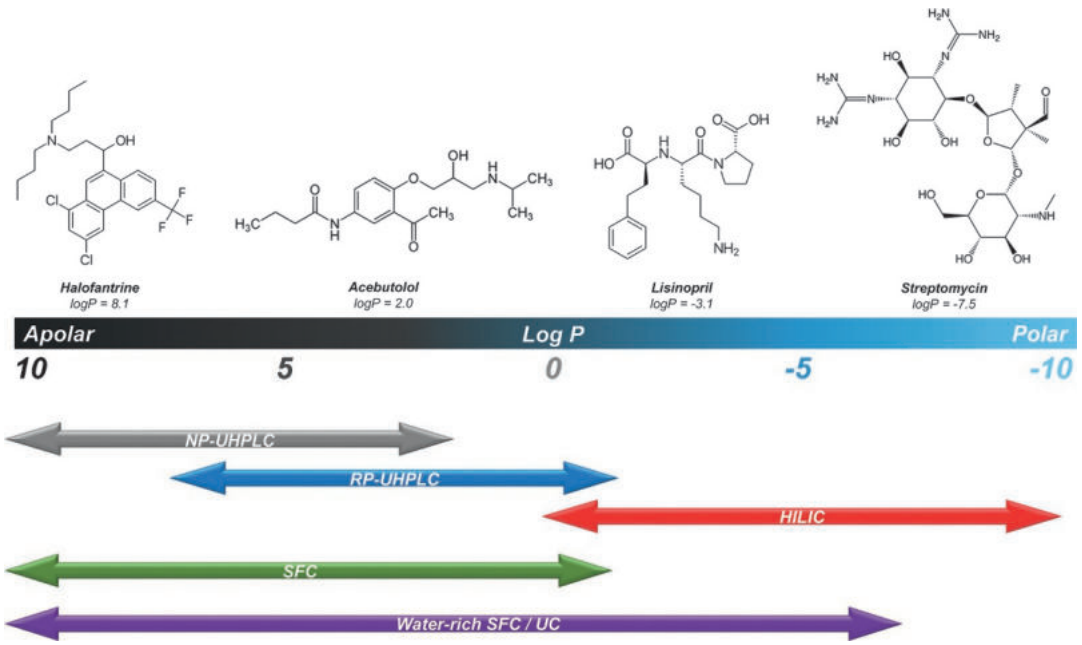

FIG. 2 Lipophilicity range covered by different chromatographic techniques (NPLC, RPLC, HILIC, SFC and water-rich SFC).

However, when considering compounds outside this lipophilicity range, alternative strategies are required. Among them, the use of normal phase liquid chromatography (NPLC) for the analysis of compounds having $\log P$ values ranging from 2 to >10 (e.g., halofantrine, cyclosporine A, propafenone, moclobemide, orlistat, amiodarone) which are all strongly retained under RPLC conditions. In NPLC, the stationary phase is polar (e.g., bare silica, amino, diol, cyano), while the mobile phase is mostly apolar and exclusively composed of organic solvents (e.g., hexane, ether, ethyl acetate, isopropanol). In NPLC, the retention mechanism is solely based on polar interactions (i.e., dipole–dipole, H-bonding). However, the use of NPLC has a significant number of drawbacks, including the use of highly toxic and expensive solvents, a poor applicability to ionizable compounds, a very limited compatibility with MS detection, and restricted robustness [14]. Conversely, when hydrophilic compounds ($\log P < 0$) require analysis (e.g., streptomycin, acarbose, lysinorile, ibandronic acid), retention is often insufficient in RPLC and alternative strategies have to be considered. Ion pairing reversed phase liquid chromatographic (IP-RPLC) was used in the 1990s and 2000s but has been progressively replaced by hydrophilic interaction chromatography (HILIC—Fig. 2). In HILIC, a polar protic stationary phase is used (e.g., bare silica, amide, diol, amino, zwitterion), while the mobile phase is composed of a small proportion of buffered water (5–40% v/v) mixed with an aprotic solvent (generally acetonitrile) [15]. The retention mechanism is complex, since it is a mix of hydrophilic partitioning (partition between a water enriched layer located at the surface of the stationary phase and the remaining mobile phase mostly composed of acetonitrile), and polar interactions (mostly ionic and H-bonding) [16]. HILIC provides suitable retention for hydrophilic compounds and excellent MS compatibility. However, it requires long equilibration times and is not as robust or versatile as RPLC.

As previously discussed, HILIC, RPLC and NPLC cover much of the chromatographic space for the wide breadth of molecules that may be found in drug discovery/drug development, whatever their physicochemical properties. However, HILIC and NPLC modes suffer from several limitations and are clearly not as robust in terms of resolution, repeatability, or selectivity and are not as compatible with as many compound classes as RPLC. For these reasons, supercritical fluid chromatography (SFC) has been suggested as a suitable alternative strategy to cover the widest possible range of molecules (Fig. 2) [17]. In SFC, any type of stationary phase can be considered (i.e., polar, apolar, aromatic, etc.). The mobile phase is mostly composed of supercritical CO_2 (CO_2 is used above its critical point, temperature >31 °C and pressure >72 bar) mixed with any type of organic solvent (from hexane to methanol) and additives (water, salts). The possibility to use almost any type of stationary phases and mobile phases makes SFC a highly versatile technique. However, in practice, a polar stationary phase is commonly used, and the mobile phase often consists of a mixture of CO_2, methanol and additives. Under such conditions, the retention mechanism is mostly based on polar interactions (dipole–dipole, ionic interactions and H-bonding), similar to what is found in NPLC. It is also important to note that SFC is considered to be a green technique (the mobile phase is mostly composed of supercritical CO_2), it offers excellent kinetic performance (low viscosity) and a strong potential for chiral separations and purifications. As demonstrated over 15 years ago [18], SFC is a highly valuable approach for high-throughput screening of large and diverse libraries of pharmaceutically relevant compounds, representative of what are commonly found in drug discovery.

Discussion

Historical overview of SFC applications

1980–2000: SFC for the analysis of apolar substances and chiral drugs

In its early days, SFC was identified as a suitable technique for the analysis of hydrophobic compounds [19,20]. The first applications involving SFC implemented pure supercritical CO_2 as the mobile phase. Although other molecules have been tested in their supercritical state, the choice fell quite rapidly on CO_2 due to the easiness in reaching its critical point. The polarity of supercritical CO_2 was estimated to be similar to that of apolar organic solvents, such as *n*-hexane or toluene according to the pressure applied. Thus, SFC was implemented for the analysis of apolar substances, such as lipids or hydrocarbons in petroleum samples [21,22]. The need to expand the application range of SFC became increasingly important, and therefore, the addition of polar liquid cosolvents, miscible with supercritical CO_2, was tested. Among the different solvents, small alcohols (e.g., methanol, ethanol, isopropanol, etc.) were often selected [23–25]. Their use provided a significant increase in the polarity of the mobile phase, thus allowing the analysis of compounds with limited hydrophobic character [23]. More importantly, SFC began to be considered as an attractive alternative to liquid chromatography (LC). Due to the similarity of the polarity to the mobile phase, SFC was tested for the analysis of compounds normally assessed using NPLC conditions. Subsequently, SFC was implemented mainly in the context of chiral analyses [26,27]. Chiral stationary phases, previously developed for LC also demonstrated good performance under SFC conditions. Additionally, a general reduction in the analysis time was obtained in SFC versus NPLC (Fig. 3), owing to the ability of SFC to reach higher flow-rates from the reduced backpressures generated under SFC conditions [26]. Moreover, the replacement of the organic solvents commonly employed in NPLC (i.e., *n*-hexane, *n*-heptane, dichloromethane, etc.) with a mobile phase rich in CO_2 lead to reduced environmental impact and lower costs. Furthermore, SFC showed its potential for purifying compounds as the supercritical CO_2 would simply evaporate when fractions were collected, leaving the purified sample in a minimal volume of the liquid cosolvent.

2000–2010: SFC as an alternative to RPLC for achiral applications

Starting from the early 2000s, more effort was invested to further develop SFC. The first stationary phases dedicated to SFC were developed and optimized, using polar selectors with some basic properties, such as 2-ethylpyridine (2-EP) or 4-ethylpyridine (4-EP), and LC columns were also used in SFC [28–30]. Innovative mobile phase conditions were also developed, in particular with the use of acidic and basic additives. The aim was to improve the peak shape (peak width and tailing) and subsequently the use of acidic or basic additives became more common [31]. Moreover, higher modifier percentages were being tested (up to 25–40% v/v). With a more abundant presence of the polar cosolvent, SFC started to increase its appeal for achiral applications involving the analysis of moderately polar compounds with a $\log P$ value as low as −1. This evolution made SFC compatible with impurity profiling in the pharmaceutical world, for the analysis of small synthetic API and their related impurities (e.g., beta-blockers, local anesthetics, benzodiazepines), as shown in Fig. 4 [32,33]. SFC demonstrated its orthogonality to RPLC by providing a different, and

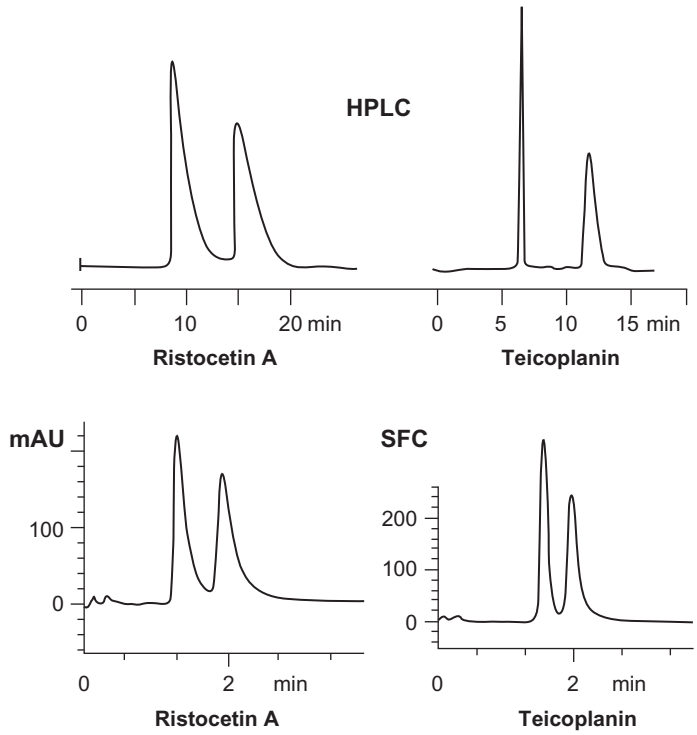

FIG. 3 Comparison of HPLC and SFC chiral separation of CBZ-norvaline enantiomers on Chirobiotic R (*left*) and Chirobiotic T stationary phases (*right*). *Reprinted, with permission, from Y. Liu, A. Berthod, C.R. Mitchell, T.L. Xiao, B. Zhang, D.W. Armstrong, Super/subcritical fluid chromatography chiral separations with macrocyclic glycopeptide stationary phases, J. Chromatogr. A 978 (2002) 185–204. doi:10.1016/S0021-9673(02)01356-0.*

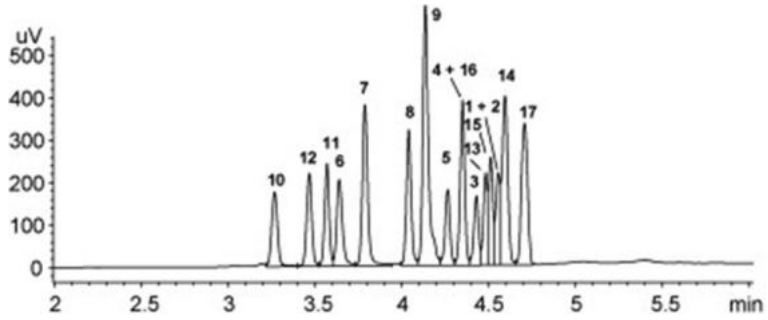

FIG. 4 Chromatograms of a complex pharmaceutical standard compound mixture analyzed on a cyanopropyl silica column under SFC conditions. *Reprinted, with permission, from C. Brunelli, Y. Zhao, M.-H. Brown, P. Sandra, Development of a supercritical fluid chromatography high-resolution separation method suitable for pharmaceuticals using cyanopropyl silica, J. Chromatogr. A 1185 (2008) 263–272. doi:10.1016/j.chroma.2008.01.050.*

in some cases better separation profile. A first example proposed a three-steps strategy in support of medicinal chemistry drug discovery: a preanalytical run enabling assessment of the separation conditions at preparative scale. A postanalytical step was used to verify compound purity [34]. Each run had a maximum time of 5 min with a purification rate above 90%. In a similar manner, another group proposed a generic SFC method using a 2-EP

stationary phase for high-throughput analysis and preparative steps [35]. Despite good results regarding method orthogonality and the purity level at the end of the process (above 95%), the method was not considered suitably generic for the whole range of compound functionalities observed within their discovery chemistry environment.

A similar approach was also proposed in the field of chiral compounds [36]. They successfully implemented a generic high-throughput chiral separation approach to speed up method development and provide fast and reliable results to the chemists. SFC provided faster separations and better resolution than their other assay methods (NPLC and RPLC). Nevertheless, several drawbacks were described for the implementation of this strategy in the laboratory: (i) the analysts were not familiar with SFC and training was not easy due to instrumentation complexity (in the beginning of the 2000s); (ii) the cost of the instrumentation was high in comparison with existing techniques; (iii) the disinterest of instrumentation manufacturers leading to nonoptimal design, poor robustness and low quality technical follow-up for existing systems.

Hence, during this 2000–2010 period, SFC struggled to find its space in the analytical portfolio of pharmaceutical laboratories, as it was not able to provide satisfactory robustness or quantitative performance. There are several reasons for the poor repeatability of SFC analytical methods: super/subcritical fluids are much more compressible than liquids, thus they are more exposed to the generation of density gradients inside the column when high backpressures are reached [37]. These density gradients are known to cause localized shifts in the elution strength of the mobile phase, especially in those regions where the supercritical CO_2 becomes denser. As the shifts in the mobile phase's elution power cannot be controlled easily, SFC methods have a high probability of experiencing limited retention times repeatability. Moreover, the sensitivity observed in SFC with a UV detector is often reduced compared to LC-UV [38]. The instrumentation available at the time did not always provide optimal mixing between the supercritical CO_2 and the liquid modifier, leading to significant refractive index effects. In addition, it also demonstrated the difficulties in keeping the mobile phase in its super/subcritical state, especially with increasing percentages of cosolvent. All these issues contributed to the generation of high UV background noise, making it very difficult for methods to reach the more stringent detection levels set by regulatory agencies. Finally, the introduction in the early 2000s of the first UHPLC systems, in combination with the development and use of columns with sub-2 μm silica particles contributed to make SFC a less attractive technique in analytical laboratories.

2010–2014: Transition from SFC to UHPSFC

The development of a new generation of SFC instruments in the early 2010s proved to be fundamental in establishing a future for this technique. These novel systems are mainly characterized by a much lower extra-column and gradient delay volumes, as well as an ability to reach and sustain backpressure values as high as 660 bar. Their release enabled SFC to accomplish a further evolution step, described by some as ultra-high-performance SFC (UHPSFC) [39]. UHPSFC was able to compete with UHPLC in terms of kinetic performance, regaining interest from different analytical laboratories. In parallel to the instrumental developments, column manufacturers made efforts to release a new and improved generation of SFC stationary phases, using sub-2 μm (including hybrid) silica particles, with a special focus on obtaining ligands providing complementary properties and, at the same time, improved peak

shapes for basic and acidic compounds [40]. However, one of the most important breakthroughs was the understanding of the role of different additives in tuning the elution strength and properties of the mobile phase. Not only did the use of MS-compatible salts such as ammonium formate (AmF) and ammonium acetate (AmAc) became more widespread, but it was also found that the addition of water, at low percentages in the liquid cosolvent, was vital in obtaining an impressive boost in the elution power of the SFC mobile phase for polar substances [41,42]. With the addition of water in the mobile phase, SFC experienced a further expansion of its application range, now focusing on more polar analytes (Fig. 5) [41,43,44]. This allowed SFC to be considered as an alternative not only to NPLC and RPLC, but also to HILIC. Furthermore, the analysis of substances (such as doping agents) in biological matrices (e.g., urine, plasma) became a reality with SFC [45]. The advantages of the new UHPSFC instrumentation provided a much-improved performance of SFC in routine laboratory use. Due to better design of the back-pressure regulator module, as well as a better technical understanding in the application of a super/subcritical fluid, SFC began providing more robust analyses [46]. Furthermore, since numerous SFC methods use a proportion of organic modifier up to 45%–50% (v/v), the mobile phase suffered less from compressibility effects, thus reducing the extent of the density gradient phenomenon. All this helped in establishing satisfactory reproducibility of retention times in UHPSFC, with this instrumentation also able to

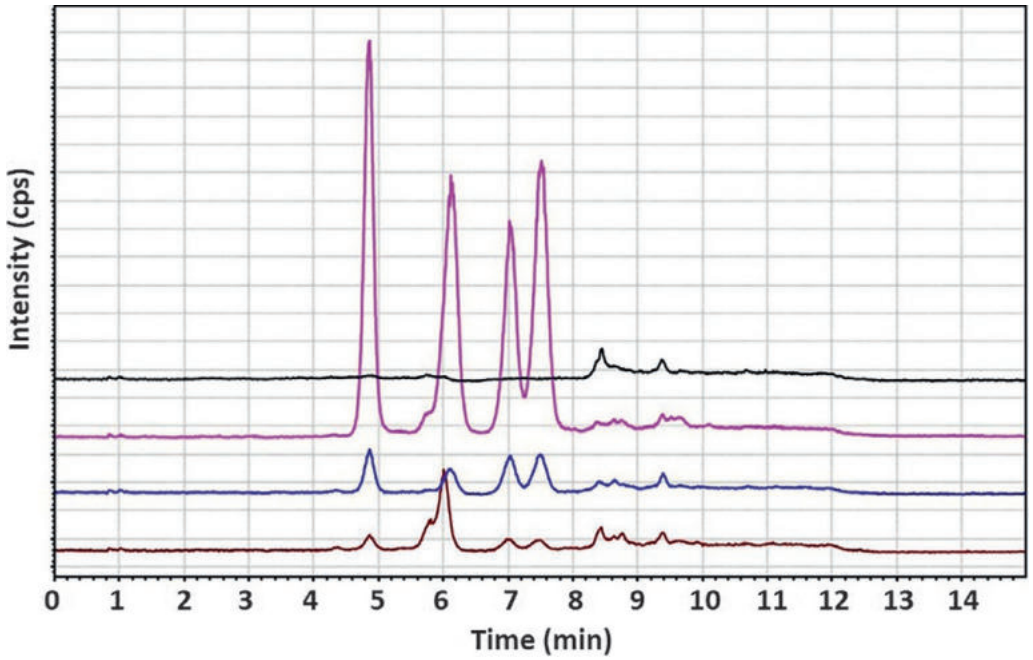

FIG. 5 SFC chromatograms of a real-life urine sample of a patient after administration of norketamine, before (black) and after 0–24 h (pink), 24–48 h (blue), and 48–72 h (brown). *Adapted, with permission from G.M. Fassauer, R. Hofstetter, M. Hasan, S. Oswald, C. Modeß, W. Siegmund, A. Link, Ketamine metabolites with antidepressant effects: fast, economical, and eco-friendly enantioselective separation based on supercritical-fluid chromatography (SFC) and single quadrupole MS detection. J. Pharm. Biomed. Anal. 146 (2017) 410–419. doi:10.1016/j.jpba.2017.09.007*

demonstrate good results during method validation [47]. Finally, the new instrumental design enabled SFC to move closer to LC in terms of UV sensitivity, with comparable performance.

2015–present day: SFC for highly polar compounds

Until now, our discussion has focused on the analysis of small molecules with a modest polar character, but SFC was also tested for more challenging molecules, such as peptides [48]. Preliminary experiments indicated the possibilities offered by SFC for analyzing highly complex compounds, as well as molecules possessing a high polarity. However, to verify if SFC would be able to provide good performance for these more complex molecules, the analytical conditions had to be adjusted. A clear example is given by the development of enhanced fluidity liquid chromatography (EFLC). In EFLC, CO_2 is no longer the main component of the mobile phase, it is rather employed to reduce the viscosity of the mobile phase that is mainly composed of a liquid [49]. The nature of the liquid itself is different: as the supercritical CO_2 is reduced, a higher percentage of water can be considered (up to 20–25% v/v) mixed with methanol. These extreme changes were successfully employed in the analysis of different biomolecules (e.g., polysaccharides, nucleobases, proteins), in an attempt to bridge liquid and supercritical fluid chromatography [50,51]. However, EFLC is poorly adapted to mildly polar or apolar analytes. Subsequently, some attempts were made to find conditions enabling SFC to successfully analyze apolar and polar substances simultaneously. Some laboratories developed an innovative SFC gradient approach called Unified Chromatography (UC) [52]. With UC, the analysis would begin with a high percentage of CO_2 (between 98% and 95%), as common with SFC applications. However, the gradient can reach percentages up to 100% solvent. The mobile phase would therefore completely pass from a supercritical to a liquid state within a single analytical run. The use of UC gradients were often combined with the addition of water in the mobile phase. Although it is not possible to reach the high percentages seen with EFLC gradients due to the predominant presence of CO_2 in UC-type gradients, percentages up to 5%–7% (v/v) of water have become more common in the works of different research groups. Moreover, the increased amount of water in the cosolvent allowed an increase in additives and salt concentrations, reaching in some cases values as high as 150 mM [53]. With such potential for optimized conditions, SFC started to be successfully utilized in areas of applications which were almost impossible to consider in the past: from metabolomics [54,55] to peptide and protein analysis [56]. Indeed, SFC is currently experiencing a growing interest and use in fields where HILIC or ion exchange chromatography were previously the only choices for chromatographers. In addition, UC-type gradients still offer the possibility to analyze mildly polar or apolar substances. As shown elsewhere (Fig. 6), UC-type gradients can still provide the necessary retention for apolar compounds such as lipids or fat-soluble vitamins, and simultaneously generate the required elution power for highly polar compounds that are strongly retained by the stationary phase [52,53]. Moreover, as most of the additives and salts chosen in SFC are fully MS-compatible, hyphenation with MS detection is still possible, with UC-type gradients. Such an approach was recently cited in the field of biopharmaceuticals drug discovery. A first paper reported the interest of SFC (almost UC) for the separation of a crude mixture of synthetic peptides of various sequence lengths [57]. Several stationary phases and additives (see Section "Considerations on SFC as an analytical tool in drug discovery and development") were tested to identify method(s) adapted to peptide length and conformation.

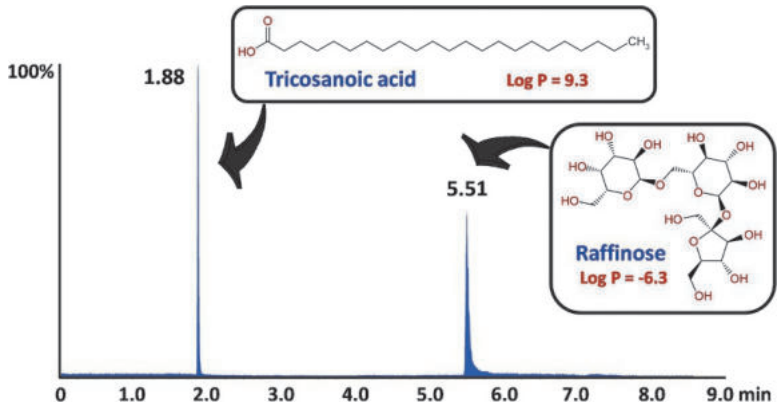

FIG. 6 Simultaneous analysis of triconasoic acid and raffinose under SFC/UC conditions. *Reprinted, with permission, from V. Desfontaine, G.L. Losacco, Y. Gagnebin, J. Pezzatti, W.P. Farrell, V. González-Ruiz, S. Rudaz, J.-L. Veuthey, D. Guillarme, Applicability of supercritical fluid chromatography–mass spectrometry to metabolomics. I–Optimization of separation conditions for the simultaneous analysis of hydrophilic and lipophilic substances. J. Chromatogr. A 1562 (2018) 96–107. doi:10.1016/j.chroma.2018.05.055.*

This study can be considered as a proof of concept. However, HPLC still outperformed SFC for peptides with long sequences. Another paper proposed SFC as a purification method for peptides [58]. Their results highlighted the utility of SFC for peptide purification at the lower preparative scale used in the field of drug discovery. These two examples highlighted the potential of SFC for larger analytes, which today represent a large proportion of new therapeutic molecules.

Considerations on SFC as an analytical tool in drug discovery and development

Mobile phase composition

As previously discussed, SFC underwent several evolutions in its history. Each time, one of the main factors which allowed it to overcome its perceived limit was the nature of the mobile phase. From the use of pure supercritical CO_2 in the 1980s and early 1990s to a ternary mixture of CO_2, methanol and water, with additives and salts at relatively high concentrations, the flexibility that SFC provides by simply modifying the composition of its mobile phase is a proposition that no LC technique can provide. This can be accomplished without changing the nature of the stationary phase, while in LC it is mandatory to change firstly the properties of the stationary phase (and, consequently, the mobile phase) to obtain good results with either apolar or polar analytes. Thus, it is simple to understand how SFC can provide scientists an alternative option to LC. This potential is provided by mixing, at different compositions, the four main components in the mobile phase (supercritical CO_2, organic modifier, water and additives/salts) [42,59]. If apolar, or water-unstable compounds are analyzed, the mobile phase should be predominantly composed of supercritical CO_2, with moderate percentages of organic cosolvent [60]. The increase in the mobile phase elution strength given by the addition of water or salts ensures the successful elution of highly polar substances. Additives

and, more specifically, water have become increasingly important in the context of method development in SFC, as they can provide suitable performance for polar and highly polar compounds. As recently demonstrated by an increasing number of papers, the use of water-rich mobile phases with additives of various nature in SFC has enabled the analysis of challenging samples such as nucleosides, amino acids, sugars and, even peptides and small proteins [51,56,61–63]. More details on the performance of SFC-based analytical methods for biomolecules and highly polar compounds can be found in Chapters 4 and 9.

Impact of the stationary phase

As previously mentioned, a significant amount of work has been undertaken to optimize SFC mobile phase conditions. Nonetheless, the performance of any chromatographic method, including SFC, remains linked to the chemical nature of the stationary phase. Unlike what was witnessed in RPLC with the proliferation of C18 stationary phases, the column screening phase is still an extremely relevant part of any method development process for SFC. Different chemistries have been developed specifically for this technique, for example selectors with a basic moiety, such as 2-ethylpyridine (2-EP) or diethylamine (DEA) and 2-picolylamine (2-PIC). In addition, LC stationary phases can be utilized as well, and diol-based, bare silica columns, aromatic stationary phases such as pentafluorophenyl (PFP), and even nonendcapped C18 columns have proven themselves useful in SFC. As they all demonstrate different chemical interactions, they guarantee different selectivity. However, a closer look highlights some common features among most of SFC columns. The majority of SFC columns possess some degree of polarity, as well as the ability to generate different interactions, such as H-bonding, π-π or dipole–dipole interactions. More details on the characteristics and main properties of SFC stationary phases can be found in Chapter 3. As explained above, the selection of a suitable SFC stationary phase remains an important step in method optimization. In this context, the selection of an orthogonal set of stationary phases can help method development [64]. In this study, API and degradation products of confidential development compounds were used to screen selected stationary phases before method optimization. This strategy helped selection of suitable conditions for the analytical separation before scaling-up to purification. It is important to note that the analytical SFC method was subsequently used to evaluate fraction purity.

In Section "2015–present day: SFC for highly polar compounds", it was highlighted how the choice of gradient profile has become an important factor today. There has been a rising trend to use unorthodox gradients in which either the supercritical CO_2 is not the main mobile phase component, as in the case of EFLC, or in performing the shift from supercritical to liquid conditions within a single analytical run, such as for UC-type gradients. These innovative gradients are being increasingly used in SFC for the analysis of biomolecules and compounds present in biological fluids, but there are some considerations that need to be highlighted. In both cases, EFLC and UC-type gradients utilize high percentages of modifier in the mobile phase, from 50% up to 80% to 100% (v/v). As the amount of cosolvent increases, column backpressure also drastically increases, which become more important when using columns packed with sub-2 μm particles. This can be problematic when using high flow rates, as the current generation of UHPSFC systems does not sustain backpressures higher than 400–660 bar. UC-type gradients tend to suffer more compared to EFLC, as high flow-rates are used in UC conditions, to ensure good kinetic performance, with the low percentages

of modifier employed at the beginning of the gradient. To address this issue, different strategies can be applied. One potential solution is to increase the column temperature to 50–70 °C to reduce mobile phase viscosity when high cosolvent percentages are reached, thus lowering the backpressures generated with sub-2 μm stationary phases. Moreover, higher temperatures have demonstrated improvement to the C-term of the van Deemter curve with a mobile phase predominant in the liquid modifier, following similar behavior to liquid chromatography [65]. A second strategy is to use a different morphology of silica particles, such as superficially porous particles (SPP) [66,67]. The introduction of sub-3 μm SPP was of great help to reduce the elevated backpressures generated by the sub-2 μm fully porous particles, without experiencing major loss in the kinetic performance. However, their use in SFC is still not widespread. A generic SFC gradient with a cosolvent percentage lower than 40–50% (v/v) does not generate elevated backpressures with a sub-2 μm stationary phase at relatively high flow-rates, thus there is currently limited interest for SPP in SFC. On the other hand, as EFLC and UC-type gradients become more common, the use of SPP phases will gain interest, as they can provide lower backpressures and almost identical kinetic performance.

Choice of injection solvent

The nature of the sample diluent can strongly affect peak shapes in SFC. Due to the peculiar retention and elution mechanism in SFC, it is important to choose an injection solvent which does not interact significantly with the stationary phase. The most common solvents used as sample diluent in the earliest applications were hexane, heptane, dichloromethane or methyl tert-butyl ether (MTBE). Those are aprotic, apolar solvents which provided good chromatographic performance, with minimized peak distortion [39]. Unfortunately, they also strongly limited the applicability range of SFC, since it was only possible to solubilize samples with limited polarity. Thus, several efforts were made in identifying alternative solvents, which would ensure sufficient solubility for more polar analytes, without compromising the peak shape and symmetry. Mixtures of the previously mentioned solvents with small alcohols such as isopropanol, ethanol and, more rarely, methanol became increasingly employed. In addition, acetonitrile (ACN) arose as a promising alternative. ACN is a good solvent not only for apolar compounds, but also for mildly polar substances such as synthetic drugs. It is perfectly miscible with methanol or water and, more importantly, it does not strongly interact with the stationary phase. It, therefore, guarantees good peak shapes and solubility of a wide range of molecules [68].

However, acetonitrile cannot always be successfully employed with more polar analytes or when biological matrices are considered. As SFC began its shift toward the use of increasingly higher percentages of cosolvent, it became possible to employ even more polar injection solvents. More importantly, the widespread use of EFLC and UC-type gradients seems to have enabled the use of methanol, or even water as a component of the sample diluent. In a few cases, pure water was used without any deterioration of the chromatographic parameters [56]. With compounds strongly retained by the column, which need high percentages of liquid modifier to elute, the impact of the sample diluent nature becomes less important. However, if analytes do not show a strong affinity for the stationary phase, they will inevitably suffer from peak distortion if protic solvents are used. This phenomenon is particularly important when compound mixtures with both poor and strong retention properties need to be analyzed simultaneously. In that case, it was demonstrated that a mixture of an aprotic

with a protic solvent could be an attractive strategy. In this example [53], a mixture of ACN and water with a 50:50 *v/v* ratio was fundamental in ensuring the best peak shape for analyzing simultaneously compounds with very different properties. More details on the strategy to choose the injection solvent for SFC analyses can be found in Chapters 7 and 8.

Hyphenation to mass spectrometer detectors

Similarly to LC, SFC can also be coupled to various detectors. Ultraviolet (UV) detection is the most widely used, but there has been a trend to use other detectors. Mass spectrometers (MS) are powerful analytical tools for molecule characterization and quantification and consequently, instrument providers have begun developing technical solutions to more easily hyphenate SFC with MS. Each hyphenation strategy brings its own advantages and disadvantages (more information can be found in Chapters 4 and 10). Indeed, due to the super/subcritical nature of the SFC mobile phase, system developers had to devise a solution to couple the MS and the back-pressure regulator module, as well as to handle the decompression of supercritical CO_2 in the connection tubing between the SFC and ionization source [69,70]. In this last segment, as the decompression phenomenon causes the CO_2 to pass in its gaseous state with a lowering of the temperature due to the endothermic nature of this process, there have been several precipitation issues reported as the mobile phase is unable to maintain solubility of the analytes. The addition of a make-up pump, delivering an additional solvent (generally MeOH), helped resolve this issue.

A make-up pump is essential for SFC-MS operation, more importantly when the SFC method does not use a high percentage of cosolvent (up to 20%–30%). The make-up pump's role might require a re-evaluation in the case of EFLC and UC-type gradients. As previously discussed, under these conditions, the amount of liquid modifier reaches elevated percentages (>70%–80% v/v), becoming the predominant element of the mobile phase. Quite logically, it can be expected that the current SFC-MS interfaces might not be best suited for these conditions. More specifically, the use of a make-up pump might become irrelevant when high modifier percentages are reached, thus avoiding the precipitation issues previously discussed. A possible solution would be to employ a flow-rate gradient for the make-up pump, to have the needed make-up solvent at the beginning of the analysis where the cosolvent levels are low and reduce this toward the end of the gradient profile where they are high. However, due to the limited research on this topic, it is hard to draw definite conclusions.

Quantitative performance of SFC

Analytical method validation is an important component of method development, especially in the pharmaceutical field. Method validation is required from (pre)-clinical studies onward (see Chapters 7 and 11). Method validation as described by ICH Q2 R1 [71] is not required in the drug discovery environment, but is advisable for pharmacokinetic studies. Furthermore, the assessment of SFC quantitative performance by means of method validation is required to confirm the reliability of this technique.

As mentioned above, SFC suffered for many years from a reputation for poor reliability and nonrobust chromatographic performance. Since the resurgence of SFC in the beginning of the 2010s and the launch of new instrumentations, several studies were performed to demonstrate the quantitative performance of this technique [72]. Firstly, the technical

improvements of new SFC instruments were clearly highlighted as beneficial to improve UV sensitivity, injection reproducibility and method robustness [73]. More recently, SFC-MS/MS was proposed as an alternative technique for nitrosamine analysis [74]. Indeed, the method was developed following the principles of analytical lifecycle management via a robust optimization strategy. This study highlighted that SFC is able to address complex analytical challenges and meet the associated regulatory requirements. Finally, besides the demonstration of SFC quantitative performance by means of method validation, the evaluation of SFC method reproducibility was also performed via an inter-laboratory study [75]. This study demonstrated the third level of method precision, i.e., reproducibility. The SFC method was successfully transferred to 18 laboratories with reproducibility RSD values better than those reported for the analogous LC impurities method. To summarize, these studies confirmed the quantitative performance and robustness of modern SFC, its reliability and its applicability to the field of drug development where its versatility and efficiency provide significant benefits.

On the specific topic of drug development, it is important to focus on degradation and impurity analysis. Indeed, after a first drug screening, drug candidates should be characterized before proceeding to pharmacological assay [76]. In this context, a paper reported a comparison between UHPLC and SFC for the impurity profiling of drug candidates [77]. At this stage of drug development, impurity profiling methods should be able to identify the API, evaluate the purity of the main component and identify all impurities at a level above a predefined threshold. In this context, the analytical method should be (i) selective for the API and impurities; (ii) precise and sensitive; and (iii) able to detect low levels of impurities. Based on previous discussion, SFC with UV and/or MS detection is proposed as an appropriate analytical technique to address these challenges.

Conclusions and perspectives

Today SFC can be considered a reliable analytical approach, which will be more widely adopted over the coming years for pharmaceutical drug discovery and development. While HPLC remains the chromatographic gold standard in the pharmaceutical industry, SFC offers a number of significant advantages, which should be considered. First of all, SFC has the potential to replace RPLC, NPLC and HILIC, for the analysis of highly hydrophilic to lipophilic substances, thanks to the use of adapted mobile phase and stationary phase conditions (i.e., EFLC, UC). It also offers orthogonal selectivity compared to RPLC, due to the very different retention mechanism (hydrophobic interactions in RPLC vs. polar interactions in SFC). In addition, modern SFC (also known as UHPSFC) offers excellent kinetic performance and ultrafast separations (important for HTS application), thanks to the use of columns packed with sub-2 μm particles and low mobile phase viscosity. SFC is also fully compatible with MS and various interfaces are now commercially available [69,70]. SFC should also be considered as a greener alternative to liquid chromatography, due to the fact the mobile phase is mostly composed of CO_2, which has a significant economic and ecological value. SFC is also regarded as one of the most powerful approaches for enantioseparations (a large majority of drugs are chiral) and is particularly beneficial at the preparative scale (CO_2 can be easily eliminated). Finally, it has been demonstrated that the robustness and quantitative performance

(method validation, inter-laboratory studies) obtained with modern SFC systems is comparable to that observed in RPLC, which is an important feature for pharmaceutical analysis.

However, it is also important to keep in mind that further development is required to expand its use in the pharmaceutical industry. First, more powerful SFC systems are required, to offer higher upper pressure limits (up to 1000 bar), and reduce system volumes (extra-column volume and dwell volume) [78]. Second, while fundamental studies have been published on SFC for more than 30 years, we still do not fully understand how additives (i.e., water, salts) and even methanol adsorb at the surface of the polar stationary phase, this still provides occasional unexpected results [79]. Method transfer (from SFC to UHPSFC, and from analytical to preparative SFC) is also more difficult in SFC than RPLC and requires further investigation [80].

In the last few years, there has been increasing interest toward the use of multidimensional LC (2D-LC) systems to further improve the overall performance in chromatography (i.e., higher peak capacity, enhanced selectivity, improved MS compatibility) [81]. In our opinion, the pioneering work of Venkatramani et al., highlighting the combination of achiral RPLC and chiral SFC is particularly interesting for pharmaceutical analysis [76], even if the experimental setup is complex and far from being able to be used routinely. A combination of apolar SFC column and polar SFC column could also be a valuable approach to improve analyte coverage (from hydrophilic to lipophilic analytes). So with a slow but steady evolution of SFC instrument design, the question is, "With recent advances in software, machine learning, automation and even miniaturization, what improvements will be designed into the next generation of SFC instrumentation?"

It is evident that instrument manufacturers continue to invest time and effort in improving SFC design. We see vendors increasing the targeted use of SFC from what was initially based around semipreparative/preparative instruments, to a more sensitive quantitative analytical tool. The analytical focus and shift to UHPSFC with sub-2 μm particle size columns provides even higher separation speeds, reduced solvent waste and improved peak resolution. Many labs from early discovery through to clinical development benefit from the ability to couple SFC-UV with important secondary detection modes like evaporative light scattering detector (ELSD) and MS. These additional detection methods work well with SFC and offer improved sensitivity for compounds not amenable to reversed-phase HPLC. Hence, the easy adaptation of SFC beyond the standard SFC-UV configuration has increased applications of SFC across the pharmaceutical industry to include areas such as drug excipient characterization (Chapter 8) and drug metabolism (Chapter 9). We anticipate developments in the future, which will further expand the application space of the technique.

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