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Modern analytical supercritical fluid chromatography using columns packed with sub-2 μm particles: A tutorial

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Highlights

► How to select a modern SFC system, compatible with sub-2 μm particles? ► How to choose a suitable stationary phase and mobile phase conditions in modern SFC? ► Which detection mode has to be employed for modern SFC operation? ► How to select an adapted dissolution solvent in SFC? ► How to analyze lipophilic, hydrophilic or ionizable compounds in SFC?

Graphical abstract

Lucie Nováková

Lucie Nováková is an Associate Professor at the Department of Analytical Chemistry, Charles University in Prague, Faculty of Pharmacy in Hradec Kralove, Czech Republic. Her research is oriented towards fast LC techniques, especially UHPLC, UHPSFC and UHPLC coupled to mass spectrometry. She is involved in a wide scope of research projects being focused on pharmaceutical analysis, plant analysis and bioanalytical applications. Therefore, an important part lays also in sample preparation step, where the focus is put on current trends

enabling facilitation, miniaturization and reduction of time and sample requirements. She has published over 45 scientific articles with about 900 citations.

Alexandre GRAND-GUILLAUME PERRENOUD

Alexandre Grand-Guillaume Perrenoud holds a master degree in Biochemistry from University of Fribourg, Switzerland and a Federal Diploma of Pharmacist from the University of Geneva, Switzerland. Since 2010, he is PhD student in pharmaceutical analytical chemistry group at the University of Geneva, under the supervision of Prof. Jean-Luc Veuthey and Dr. Davy Guillarme. His domain of expertise focuses on drug analysis using supercritical fluid chromatography and liquid chromatography approaches.

Isabelle FRANCOIS

Isabelle François conducted her PhD research in the lab of Prof. Pat Sandra at Ghent University in Belgium. During her research, she developed two-dimensional comprehensive fluid-based chromatography approaches (LCxLC and SFCxLC), focusing on the improvement of instrumental hardware. In 2009, she started working at ExxonMobil Chemical managing the chromatography lab in the Advanced Characterization Department. Since 2011, she works for Waters, currently as Product Specialist. Her areas of expertise are UHPLC, SFC, comprehensive and heartcutting 2D chromatography (LCxLC, SFCxLC) with optical detectors and MS. Isabelle has published over 10 papers in international peer viewed journals and is presenting at international conferences on a regular basis.

Caroline WEST

Caroline West completed a PhD in SFC in 2005, at the University of South of Paris, France. She then joined the University of Orleans, France and the Institute of Organic and Analytical Chemistry as an assistant professor in analytical chemistry. Her scientific interests lie in fundamentals of chromatographic selectivity, both in the achiral and chiral modes mainly in SFC, but also in HPLC. Her works are essentially devoted to improving the understanding of chromatographic separations in order to facilitate method development.

Eric LESELLIER

Eric Lesellier is Associate Professor at the University of Paris Sud (Orsay) for 1990, working for its research at the Centre National de la Recherche Scientifique CNRS ICOA (Institute of Organic and Analytical Chemistry; Orléans). Trying to better understand the behavior of compounds carry away by the sub/supercritical fluid through varied stationary phases, he mainly developed a chromatographic test for C18 bonded phases, and described the unification of several chromatographic areas by using CO₂/modifiers mobile phases.

Davy GUILLARME

Dr. Guillarme holds a PhD degree in analytical chemistry from the University of Lyon, France. Since 2004, he is senior lecturer in the laboratory of pharmaceutical analytical chemistry of the University of Geneva in Switzerland. Dr. Guillarme is co-author of more than 90 scientific papers related to pharmaceutical analysis. His expertise includes liquid chromatography, UHPLC, HILIC, LC-MS, supercritical fluid chromatography and analytical characterization of therapeutic proteins and mAbs. He is an editorial board member of several journals including LC-GC North America, Journal of separation science or American pharmaceutical review... Dr. Guillarme has also an extensive experience providing theoretical and practical training courses.

Abstract

This tutorial provides an overview of the possibilities, limitations and analytical conditions of modern analytical supercritical fluid chromatography (SFC) using columns packed with sub-2

μm particles. In particular, it gives a detailed overview of commercially available modern SFC instrumentation and the detectors that can be employed (UV, MS, ELSD, FID, etc.). Some advice on the choice of the stationary phase dimensions and chemistries, the nature of the mobile phase (choice of organic modifier and additives) and its flow rate as well as the backpressure and temperature are also provided. Finally, several groups of potentially problematic compounds, including lipophilic compounds, hydrophilic substances and basic drugs, are discussed in detail. All these families of analytes can be resolved with SFC but require specific analytical conditions.

KEYWORDS:

Supercritical fluid chromatography; ultra-high performance supercritical fluid chromatography; sub-2 μm particles; mobile phase selection; column selection; tutorial

1. Introduction**1.1. Properties of supercritical fluids**

Recently, supercritical fluid chromatography (SFC) has made a remarkable comeback within the separation sciences community. This technique, which has remained in the shadow of liquid chromatography (LC) for more than two decades and has been confined to only niche applications, is now benefitting from wider recognition and interest thanks to the implementation of new and important technological developments. Indeed, the explosion of LC separation costs related to the acetonitrile shortage in 2008 has forced both researchers and engineers to develop alternative strategies. In this context, SFC is considered a valuable option because its mobile phase requirements drastically differ from those of conventional chromatographic techniques. The SFC mobile phase consists of fluids that are heated and pressurised beyond their critical point. The nature of a supercritical mobile phase varies

markedly from that of liquid or gaseous mobile phases, and the properties are considered to be a hybrid of these two physical states. Supercritical fluids display diffusivity and viscosity features similar to those of a gas. Similarly to gas chromatography (GC), high solute diffusion coefficients (D_M) are encountered in SFC, leading to non-negligible chromatographic advantages [1-3]. On the other hand, the density and solvating power of supercritical fluids are more comparable to those of a liquid and allow for both relatively good solubility and good transportation of analytes. Density is often seen as a key feature for SFC, and special attention should be paid to influencing parameters, such as temperature and pressure.

Light hydrocarbons, N_2O , ammonia and chlorofluorocarbons have been quite successfully used as supercritical mobile phases [4, 5]. However, their success had to be balanced with serious safety issues, hardware damage, unsuitability for thermolabile compound analysis and environmental pollution. Safer and inert, carbon dioxide (CO_2) is by far the most commonly used supercritical mobile phase currently used. Its mild critical point (74 bar and 31 °C) constitutes a key advantage over other fluids, as these mild conditions can be easily reached on conventional chromatographic instrumentation (see Figure 1) [6, 7]. Despite this low critical point, most SFC separations are actually not performed under a rigorous supercritical state but rather in so-called subcritical conditions, primarily because a small proportion (2 to 40 %; v/v) of an organic modifier is generally added to enhance the limited polarity, solvating power and eluting strength of pure CO_2 [8].

Polar modifiers, such as methanol, ethanol and acetonitrile, are widely used to extend the flexibility of the supercritical/subcritical mobile phase [9]. Increases in the fluid solvent strength are mainly correlated to the modifier proportion rather than to the density modification associated with the modifier presence [10]. Meanwhile, the GC-like high diffusivity and low viscosity of the fluid remains poorly affected by the modifier [11]. Berger, who is considered the father of modern SFC, stated that a binary mobile phase displays the

benefits of both LC and GC, therefore placing SFC somewhere in between these two classical separation techniques [8].

Compared to these chromatographic approaches, SFC displays numerous practical and theoretical advantages due to its supercritical/subcritical mobile phase properties. Its low viscosity and elevated D_M allow for high speed (elevated linear velocity) and/or highly efficient (long column) separations with reasonable column pressure drop generation. Moreover, the addition of an organic modifier to the mobile phase allows the analysis of more polar compounds. Because only a small proportion of the modifier is generally required, organic solvent consumption is low compared to LC and allows decreases in both waste generation and costs related to its reprocessing [12]. This last point makes SFC a green separation technique, especially for large-scale or preparative applications, which involve large mobile phase volumes.

1.2. From capillary to packed column SFC

Despite the outstanding physicochemical properties of supercritical fluids, SFC has only been used episodically since its first description by Klesper *et al.* in 1962 [13]. SFC development was only anecdotic before the 80s because the chromatographic community mainly focused its interest on the already well-established method of GC and on the first developments of high performance liquid chromatography (HPLC). The first real breakthrough in SFC resulted from the pioneering work of Novotny *et al.* and Lee *et al.*, who have strongly contributed to the popularisation of SFC using pure supercritical fluids and long capillaries or open tubular stationary phases (cSFC) [14, 15]. In the early years, GC users showed great interest in the development of cSFC because they viewed it as an elegant way to extend the range of compounds that could be analysed [16]. The first instrumental developments in cSFC were also strongly inspired by the GC apparatus and included the use of (a) capillary columns, (b)

ovens, and (c) flame ionisation detectors (FID). Only slight modifications to GC hardware were required to properly function with a supercritical fluid. The critical pressure was maintained within the chromatographic instrument by a fixed terminal capillary restrictor possessing a small internal diameter. Capillary SFC systems, which were generally homemade, were often described as delicate and poorly reproducible. The technique itself rapidly declined in the early 90s because of strong limitations in terms of applications as the polarity of pure supercritical CO₂ was only suitable for analysing lipophilic compounds.

Surprisingly, the first commercial SFC system marketed in 1983 by Hewlett Packard was inspired by LC set-ups and was fully designed for the use of packed columns [17]. Packed column SFC (pSFC) was radically different from cSFC and aroused very limited enthusiasm among chromatographers. This instrument offered the possibility to directly modify the pure supercritical fluid with an organic co-solvent thanks to an innovative binary pump. The CO₂ pumping unit had to be cooled to pump the fluid in its liquid form from a pressurised tank, whereas the second pumping unit was dedicated to an organic co-solvent. GC and cSFC practitioners noted several drawbacks of pSFC, such as its incompatibility with FID and the smaller chromatographic efficiency achieved on short columns packed with small particles (5-10 µm) [18]. Conversely, pSFC was reported to offer shorter analysis times [19], the ability to analyse polar compounds, and improved selectivity [20, 21]. In addition, the first active backpressure regulator (BPR) was designed, facilitating the maintenance of a constant backpressure, even when an organic modifier gradient was used. Regarding detection, pSFC could use the same panel of detectors as LC, including a UV spectrophotometer, a mass spectrometer (MS) [22], an evaporative light scattering detector (ELSD) or a fluorescence detector [23]. Nevertheless, in the 90s and in the early 2000's, pSFC struggled to establish itself in other fields of applications beyond preparative scale and/or chiral separations. Its

repeatability and robustness, still below those achieved in LC, have somewhat hindered its implementations in analytical and QC laboratories.

The reluctance of analysts to use pSFC has faded since the recent introduction of a new generation of instruments dedicated to analytical purposes by several important manufacturers. These new systems benefit from a novel BPR design and are largely based on ultra-high performance liquid chromatography (UHPLC) technology, including higher upper pressure limits and reduced void volumes. Improved performance, reliability and full compatibility with the most modern stationary phases (sub-3 μm core-shell and fully porous sub-2 μm particles) have greatly broadened the application spectrum of this technique, making modern SFC competitive and complementary to UHPLC.

2. Selection of a modern SFC system

In order for SFC to become a mature and fully established technique, the system performance should be equivalent to that of modern GC and UHPLC. In addition to qualitative performance, which is influenced by column technology as well as system dispersion, the quantitative performance (precision, trueness, accuracy, robustness, etc.) is also of prime importance for an analytical strategy. Because previous generations of SFC equipment did not achieve these performance parameters, the implementation of SFC has remained very limited within the R&D environment. In SFC, the control of density is crucial. When the density is not properly managed, the solvent strength differs between analyses, leading to shifting retention times. The density is controlled by both pressure and temperature. In an SFC system, the BPR is responsible for the pressure control, so proper design is of crucial importance. Furthermore, changes in the temperature of the incoming CO_2 can also result in shifting retention times. Adequate and repeatable cooling of the incoming CO_2 is therefore mandatory. In addition to these issues, the injection volume flexibility was limited in older generations of

SFC instrumentation, reflecting imperfections in the design of the partial loop injector for SFC. From a detection perspective, the UV noise level caused by differences in the refractive indexes of the mobile phase constituents should also be minimised.

The revival of interest in SFC in recent years has been predominantly the result of the introduction of new state-of-the-art instrumentation. Instrument vendors have clearly identified the necessity to redesign SFC equipment in order to guarantee wider applicability of the technique. The following paragraphs describe the new systems that have recently been introduced.

2.1. Holistic system for SFC

The ACQUITY UltraPerformance Convergence Chromatography (UPC²) system was commercially introduced in 2012 by Waters (Milford, MA, USA). This instrument is dedicated for high performance analytical SFC and consists of a holistic design. To stress its difference from previous generations of SFC equipment, the term Convergence Chromatography, originating from a statement of Giddings [24], was introduced by Waters, indicating the broad applicability of the technology. The dispersion of the system has been significantly reduced in comparison to that of the legacy Berger and Thar SFC systems. The launch of the system was accompanied by the introduction of several SFC stationary phases available in the sub-2 μm particle scale. Important features include the efficient cooling of the CO₂ pump heads by Peltier and the design of a new dual stage BPR that is heated to avoid frost formation. The accurate control of these parts allows high repeatability even when mixing with low flow rate co-solvents (below 5%). The pump is able to aspirate both liquid as well as gaseous CO₂. The maximum flow rate and pressure of the instrument are 4 mL/min and 413 bar, respectively. However, the maximum pressure decreases linearly from 413 to 293 bar in the flow rate range from 3.25 to 4 mL/min. The instrument allows flexibility in

method development *via* the automated selection of up to four modifiers and up to six different stationary phases (only two columns can be selected in the “basic” single oven configuration, but the system does not need additional valves when up to four or six columns are desired). Partial loop volume injections are feasible due to the presence of an additional auxiliary valve, allowing compression of the sample plug prior to transfer towards the analytical column. The column is located in a still-air oven that can accommodate columns of up to 150 mm in length. An active preheater is included prior to the column to preheat the mobile phase at a suitable temperature before it enters the column. The column outlet is connected to a photo-diode array detector (PDA) including a high pressure UV cell (400 bar) with a volume of 8 μ L and a path length of 10 mm. Accurate pressure control is enabled by a dual-stage BPR consisting of a static and a dynamic part. The static part is located completely downstream and is responsible for the generation of a fixed back-pressure of 75-90 bar (differing among installations and instruments). The dynamic BPR generates the additional pressure (for example, 55-40 bar when a total BPR of 130 bar is set). This approach easily allows responding to changes in pressure originating from the application of gradient conditions. In addition to PDA detection, other available detectors are ELSD and MS. The coupling with MS consists of a double T with PEEK and PEEKsil tubing with fixed dimensions. The first T enables the addition of make-up solvent to the column effluent to enhance ionisation. In the second T, the flow is split towards the BPR and MS source. The instrument can be controlled by typical software packages, including Empower or MassLynx, provided by the manufacturer.

2.2. Hybrid system LC/SFC

The 1260 Infinity Hybrid SFC/UHPLC system from Agilent Technologies (Waldbronn, Germany) is a hybrid system allowing both UHPLC and SFC separations. Using switching

valves and two pumps (one for SFC and one for UHPLC), the system can be modified based upon the needs of the user. It is obvious that adequate rinsing when switching from one chromatographic mode to another is mandatory. This hybrid instrument was introduced in 2012, but the collaboration between Aurora and Agilent Technologies also resulted in the introduction of a dedicated analytical SFC system (1260 Infinity Analytical SFC system) in 2010. Aurora has officially been part of Agilent Technologies since 2011. All devices related to the SFC operation are located in the 1260 SFC Controller module (formerly known as the Aurora module), which is placed next to the 1260 Infinity system. This module is responsible for the compression of the incoming gaseous CO₂ with temperature control *via* a chilling liquid, while the BPR in this apparatus is a heated single-stage device. The maximum flow rates and pressures are 5 mL/min and 600 bar, respectively. The instrument allows flexibility in method development *via* column switching and modifier selection. The ‘basic’ system comes with only one modifier but can be extended with a switching valve to allow the selection of up to twelve solvents. A similar principle is applied in column switching, with a maximum of up to eight 50 mm length columns or up to four 250 mm length columns, but requires the purchase of additional modules. The system allows the injection of partial loop volumes. The detection possibilities are PDA, ELSD, MS and, since July 2013, also FID. The PDA is equipped with a micro flow cell with a volume of 1.7 µL and a 6 mm path length that is resistant to a pressure of 400 bar. The hyphenation with MS is performed based on a different approach than that used for the ACQUITY UPC² instrument. A make-up solvent is added to the column effluent prior to the BPR, and the total flow exiting the BPR is sent towards the MS source. Depending on the application, the effluent entering the MS can be heated using the Caloratherm heater (SandraSelerity Technologies, Kortrijk, Belgium). The 1260 Infinity Analytical SFC system is controlled *via* ChemStation or OpenLab.

3. The importance of the dissolution solvent

In RPLC, it is well established that the sample should be dissolved in a solvent as close as possible to or in a weaker eluent than the mobile phase in order to minimise band spreading. This is particularly critical for poorly retained analytes in the isocratic mode (“strong solvent effect”). A good laboratory practice is to dissolve the sample in a weaker diluent composed of the same constituents but in different proportions than the mobile phase into which the injection occurs. Because the mobile phase in SFC is made of a large proportion of supercritical CO₂, this solution cannot be envisaged, and it has been demonstrated that significant peak distortion caused by the sample diluents can also occur in SFC. Two recent studies dealing with the influence of the sample solvent composition in analytical SFC have been published [25,26].

In terms of the peak shape, non-polar solvents, such as hexane or heptane (as apolar as CO₂ itself), are considered the best sample diluents. However, such volatile solvents evaporate in the vial storage compartment and tend to continuously concentrate the samples in the vials. Storage compartment cooling and the use of sealed vials are therefore highly recommended, especially in the case of quantitative determination [25]. Another issue with using apolar dissolution solvents is their limited dissolving power, particularly for ionisable compounds. To achieve good solubility and acceptable peak shape, it is often recommended to blend non-polar solvents with more polar ones, taking into account possible miscibility issues within the diluent system.

In a first study [25], the peak profiles of butylparaben dissolved in eight solvent systems (*i.e.*, methanol, ethanol, isopropanol, tetrahydrofuran, 30:70 isopropanol:heptane, 50:50 tetrahydrofuran:heptane, 30:70 tetrahydrofuran:heptane and dimethylsulphoxide) were evaluated using columns of 150 x 2.1 mm, 5 µm and the Waters ACQUITY UPC² system. Because dimethylsulphoxide and methanol were the most polar solvents, distorted peaks were

systematically observed even when small injection volumes were used. The less retained compounds were always the most distorted. To limit peak distortion, the injection volume should be as small as possible, taking into account injection reproducibility and detection limits. In cases where a specific analyte cannot be dissolved in detectable quantities in a very weak solvent, a compromise must be made, and the best sample diluent was found to be a mixture of 30:70 tetrahydrofuran:heptane [25]. Figure 2 illustrates the chromatographic profiles of butylparaben achieved using methanol, isopropanol and 30:70 tetrahydrofuran:heptane as the sample diluent for several injected volumes ranging from 0.5 to 4 μL .

Another paper dealing with sample diluents in SFC was published in the meantime, but the experimental investigation was much more exhaustive [26]. Seven analytes of varying polarity were analysed on three different columns (bare silica, C18 and 2-EP columns of 150-250 x 4.6 mm, 5 μm) using 17 different sample diluents. Only pure solvents (no blending between polar and apolar solvents) were considered. The goal was to correlate the injection solvent properties with the chromatographic efficiency. Again, dimethylsulphoxide gave exceptionally broadened peaks and was found to be the worst sample diluent. This study also demonstrated that there was an interplay between the sample diluent, the stationary phase chemistry, the nature of the analyte and the mobile phase composition. As an example, the sample diluents may behave differently on a polar stationary phase (bare silica and 2-EP) and on an apolar phase (C18). For the two polar columns, the dielectric constant, dipole moment, hydrogen bonding and eluent strength of the sample diluents were highly correlated to poor efficiency, especially for polar analytes. This suggests that the injection solvent interacts with both the analyte and the stationary phase and thus minimises interactions with free silanols. On the contrary, analytes separated on a C18 column benefited from injection using solvents with higher dielectric constants and dipole moments. Finally, because the density and vapour

pressure affect the solubility of the injection solvent in the mobile phase, a low molar volume and high vapour pressure of the injection solvent positively affect the plate number on any type of column. Conversely, the viscosity and surface tension do not significantly influence the plate count.

Based on these two studies and on our own experience in modern SFC using columns packed with sub-2 μm particles, a compromise must be found between the sample solubility, the peak shape, the sample stability, the solvent volatility and the compound retention. The negative impacts of sample diluents on the peak profile can be dramatically mitigated by reducing the injected volume, but sensitivity could become an issue. When dealing with pharmaceutical compounds, we have observed that acetonitrile and tetrahydrofuran, both aprotic solvents with no H-bond donor capability, provided acceptable peak shape and solubility for a wide range of compounds. Another advantage of acetonitrile is that it is not retained and thus does not interfere with analysed compounds. It has been used in the past as a column dead time marker for columns packed with C18 or phenylhexyl groups [27]. Special care should be taken if the sample is dissolved with dimethylsulphoxide and dimethylformamide (commonly employed in the pharmaceutical industry). Indeed, these two solvents are UV active and are retained on polar columns. A strong interfering peak is therefore generally observed in the chromatogram. Last but not least, a rule of thumb for RPLC is to inject a volume equal to ~ 1 % of the column volume. In the case of SFC, the injected volume should be decreased to 0.1 - 0.5 % of the column volume.

4. Selection of the analytical column

4.1. Optimal column dimensions for modern SFC apparatus

4.1.1. Influence of extra-column band spreading

To determine which column dimensions are compatible with a given instrument, it is necessary to estimate the efficiency loss arising from instrumental contributions. For this purpose, the extra-column band spreading of a given system (σ_{ext}^2) has to be experimentally determined and compared with the column band spreading (σ_{col}^2). Normally, the σ_{ext}^2 should represent, at a maximum, 10% of the total band broadening ($\sigma_{\text{col}}^2 + \sigma_{\text{ext}}^2$) to maintain negligible contribution to band broadening from the instrument [28]. The two modern SFC devices, namely, the Waters ACQUITY UPC² and Agilent 1260 Infinity Analytical SFC systems, were experimentally characterised, and σ_{ext}^2 was equal to 85 μL^2 on both systems [29]. Compared to state-of-the-art UHPLC systems, which possess σ_{ext}^2 between 2 μL^2 (the best systems on the market) and $\sim 20 \mu\text{L}^2$, it is clear that there is still room for improvement in modern UHPSFC (ultra-high performance SFC) systems. The differences between UHPLC and UHPSFC instruments are mostly related to the tubing dimensions (length and internal diameter) and the UV detection cell volume. For a reference column widely employed in HPLC and SFC, namely, the 150 x 4.6 mm, 5 μm column, the σ_{col}^2 is estimated to be $\sim 20500 \mu\text{L}^2$ ($k = 8$). In this case, the ratio $\sigma_{\text{ext}}^2/\sigma_{\text{tot}}^2$ is equal to 0.4% for $\sigma_{\text{ext}}^2 = 85 \mu\text{L}^2$, showing that this column is perfectly compatible with modern UHPSFC instruments. However, the standard column dimension for UHPLC, namely, 50 x 2.1 mm, 1.7 μm , possesses a σ_{col}^2 of only $\sim 100 \mu\text{L}^2$, ($k = 8$). The latter is hardly compatible with modern instrumentation because the ratio $\sigma_{\text{ext}}^2/\sigma_{\text{tot}}^2$ is equal to 45%. This means that the intrinsic efficiency of the column will be reduced by 45% because of instrumental contributions. This simple calculation proves that current UHPSFC instruments are not ready to function properly with 2.1 mm I.D columns, except when the length exceeds 400 mm (see Table 1). Today, the best compromise for UHPSFC operation is to employ a column of 100 x 3 mm, 1.7 μm , possessing a σ_{col}^2 of $\sim 840 \mu\text{L}^2$ ($k = 8$), as the efficiency loss will be equal to only 9%. Calculations were also performed

for several other column dimensions and retention factors, and the corresponding data are reported in Table 1.

According to Table 1, it is clear that 4.6 mm columns are better than 3 mm I.D. columns. However, the upper flow rate limit of modern UHPSFC systems is limited, namely 4 and 5 mL/min on the Waters ACQUITY UPC² and Agilent 1260 Infinity Analytical SFC, respectively. Thus, the optimal linear velocity for 4.6 mm I.D. columns packed with sub-2 μm particles can barely be reached on these modern systems, and no further increase in the analysis speed can be envisioned. In addition, the solvent consumption (mobile phase composed of up to 30-40% MeOH in modern SFC) may be too high with 4.6 mm I.D. columns. For these reasons, we consider 3 mm I.D. columns to represent the best compromise between efficiency loss, solvent consumption and flow rate flexibility.

4.1.2. Influence of the system dwell volume

Another critical feature of SFC instruments is the dwell volume (or gradient delay volume), V_d , which corresponds to the total volume between the entrance of the mixing chamber and the column inlet in the high pressure binary system configuration [28]. This additional volume can be critical for high throughput experiments because it adds an unnecessary isocratic step at the beginning of the gradient. The two modern UHPSFC instruments possess V_d values of 440 μL (Waters ACQUITY UPC²) [30] and $\sim 700 \mu\text{L}$ (Agilent 1260 Infinity Analytical SFC). For comparison, the best UHPLC systems on the market possess V_d values of $< 100 \mu\text{L}$. The high V_d of UHPSFC systems is easily explained by the size of the mixing chamber. The larger mixing chamber is technically justified by the need to work at higher flow rates in UHPSFC vs. UHPLC and also by the need to mix liquid CO_2 with methanol without creating excessive background noise. Even if the V_d were 5 to 7 times larger in UHPSFC vs. UHPLC, the additional isocratic step duration that is created with columns of 100 x 3 mm and 50 x 2.1 mm

in UHPSFC and UHPLC, respectively, is similar on both types of instruments because of the higher flow rates employed in UHPSFC vs. UHPLC, which are related to both the higher u_{opt} value and the larger column I.D. This proves that the dwell volume is not a critical feature of modern UHPSFC systems.

4.2. Classification of stationary phase types

Due to the lack of water in the mobile phase, SFC is a unified separation method because it allows the use of both non polar and polar stationary phases with the same mobile phase [31,32]. Chromatographers should be aware that for achiral analyses, SFC can replace non aqueous reversed phase liquid chromatography (NARP-LC), RPLC, normal phase liquid chromatography (NPLC), and hydrophilic interaction chromatography (HILIC) if the studied compounds are soluble in the CO₂-rich mobile phase. This means that virtually all HPLC stationary phases (SP) can be used in SFC, from ODS-bonded silica to pure silica. Consequently, the choice of the best suited SP can be tricky, but the development possibilities are almost unlimited.

A classification system was developed with a solvation parameter model (or linear solvation energy relationship, LSER) that uses five descriptors (E, S, A, B, V) related to the interaction capabilities (charge transfer, dipole-dipole, hydrogen bonds acceptor and donor, dispersion) of the analytes with the chromatographic systems. All phase types were studied with the same mobile phase to better understand their differences and comprised phases based on alkyl bonded [33], aromatic [34-36], polar [37], or HILIC materials [38]. The difficulty is to provide a simple classification map allowing the comparison of all the columns on the basis of the five coefficient values (e, s, a, b, v) describing each SP [39-40]. For this purpose, a spidergram representation was proposed, which plots the five dimensions on a projected plan. Each point is placed at the extremity of the solvation vector described by the five LSER

coefficients, and the bubble size is related to the strengths of the interactions. More than 70 varied SPs were studied by this method, and the data are reported in Figure 3.

On this diagram, non-polar (*i.e.*, from C5 to C18, phenylhexyl) and polar SPs (*i.e.*, silica, ethyl-pyridine, cyano, diol, amino, etc.) are located in opposite areas, with the non-polar phases on the left and the polar phases on the right. In between, phases with mixed-mode behaviour can be found, such as the aromatic phases at the top (*i.e.*, phenyl, pentafluorophenyl and porous graphitic carbon) and the non-polar phases with polar embedded or end-capped groups at the bottom of the spidergram.

A recent study has shown that for five SPs packed with sub-2 μm particles and devoted to SFC (Waters HSS C18 SB, XSelect CSH Fluorophenyl, BEH (silica), BEH 2EP and BEH RP18 Shield), changing the particle size from 5 to 1.7 μm does not have a significant effect on the interactions [41]. These five chemistries can serve as a first screening because they are located in different areas of the spidergram, as illustrated in Figure 3 (underlined SP), confirming that they display varied selectivity properties. Among the available phases, it is important to mention ethyl-pyridine, which is often used in SFC column screening because of its basic moiety. Indeed, the latter offers good selectivity between acidic, neutral and basic compounds and often reduces the peak tailing for basic compounds [30, 42-45].

This diagram also displays the location of state-of-the-art columns packed with superficially porous particles (SPP) having a particle size of approximately 2.5 μm . This new generation of SP is particularly attractive due to its high efficiencies at reduced pressure relative to fully porous sub-2 μm particles [46]. As shown in [47], very high theoretical plate numbers were achieved by coupling several columns of this type for the analysis of complex mixtures. Another classification based on the use of the carotenoid test allowed the study of 10 different stationary phases packed with C18 SPP particles: Kinetex C18, Kinetex C18 XDB, Aeris WP, Aeris peptide, Accucore C18, Nucleoshell C18, Poroshell 120 C18, Halo C18, Halo peptide,

and Ascentis Express C18 [48]. This study shows that these phases can display varied behaviours in terms of polarity or shape recognition, which is particularly interesting for the discrimination of diastereoisomers.

Many other interesting stationary phases can be selected from the spidergram reported in Figure 3, such as Synergi polar RP (Phenomenex), Cosmosil HILIC and Cosmosil Pyrenyl (Nacalai Tesque), Acclaim Polar Advantage I (Thermo), Ace C18-PFP (ACT), Nucleodur Sphinx RP, (Macherey Nagel), and Luna HILIC (Phenomenex), but they are not yet available in sub-2 μm or SPP formats.

Finally, using this spidergram, it is possible to easily select an orthogonal set of SPs offering varied properties and selectivities [49].

5. Selection of the mobile phase

5.1. Selection of organic modifiers

It was previously mentioned that the low polarity of pure CO_2 makes it inconvenient as a mobile phase for the elution of polar and high molecular weight compounds. The polarity of CO_2 could be considered similar to that of hexane. To increase the solvent strength of such mobile phases and to favour compound solubility, the addition of a small amount of a more polar solvent (organic modifier) is needed. The additional effects of organic modifiers include the deactivation of the active sites on the surface of the column packing material and the change in mobile phase density. Furthermore, tuning of the selectivity is possible due to specific interactions, such as hydrogen bonding or dipole-dipole interactions [50, 51]. Depending on the choice of a modifier, several characteristics, including the dielectric constant, hydrogen-bonding capabilities, mass transfer characteristics and solvent viscosity, might be altered. The hydrogen bond acceptor capability of the surface silanol groups in the stationary phase might distort the peak shapes for hydrogen bond donor analytes. Organic

modifiers with hydrogen bond donor character (trifluoroethanol > methanol > ethanol > 2-propanol) were found to minimise these effects to a greater extent than non H-bond donor modifiers. To compensate for the hydrogen donating properties of silanol, competing acceptors, including dioxane, alcohols, THF or acetonitrile, are recommended. Based on the previous statements, alcohols were thus found to be the most universal modifiers, providing good overall efficiency, even though the selectivity was slightly compromised [52, 53].

Methanol is a very strong mobile phase modifier and is considered to be the first choice for the elution of polar compounds in SFC. It is completely miscible with CO₂ over a wide range of temperatures and pressures. Typically, a gradient program starting from 2-5% up to 30-40% of the modifier has to be initially performed as a scouting run. Other alcohols, such as ethanol or 2-propanol, are also quite commonly used, with increasing peak widths and longer analysis times generally reported [54, 55]. The most significant differences in selectivity, elution order and peak capacity were obtained when acetonitrile was used as an organic modifier. Significant increases in retention, decreases in peak capacity and poor peak symmetry are often observed [54, 55]. This has been attributed to the lack of hydrogen bond donation from acetonitrile and to the low coverage effect of silanols by acetonitrile [52, 55]. For this reason, acetonitrile alone is rarely used as an organic modifier in SFC. However, in some cases, a combination of polar alcohols with the addition of acetonitrile has been found advantageous for fine selectivity tuning [54, 56].

It is important to note that the addition of an organic modifier changes substantially the critical point of the mobile phase, depending on the nature and proportion of the modifier (Figure 4). However, in almost all practical circumstances, it is not significant whether the fluid is defined as a supercritical or subcritical. No significant changes in either the physical or chemical properties of the mobile phase have been observed within this state change.

5.2. Selection of mobile phase additives

The effect of an organic modifier alone is usually insufficient to overcome the chromatographically deleterious effects of residual silanol groups and/or to enable the elution of very polar or basic compounds [58]. The role of an additive includes providing coverage of the active sites and changing the stationary phase and mobile phase polarity. Stationary phases that require little or no additives represent a significant advantage in gradient SFC, as additives often contribute to increased baseline shift, especially when operating at low UV detector wavelengths. In SFC, this baseline shift cannot be compensated for by the addition of an equal amount of the same additive to the carbon dioxide [44].

The use of low concentrations of additives, usually acids or bases, in an organic modifier has a beneficial effect on the peak shape and selectivity in SFC [53]. Thus, the elution of organic acids can be improved by the addition of formic acid, trifluoroacetic acid (TFA) or citric acid, while the elution of bases can be improved by the addition of aliphatic amines, such as isopropylamine, diethylamine, ethyldimethylamine or triethylamine [58, 59]. The impacts of various additives, including TFA, formic acid, acetic acid, water, selected amines and phosphates, on their efficiency, selectivity and retention using LSER regression were evaluated on a cyano phase. Brønsted acids and bases were found to be effective at improving efficiency, while neutral additives demonstrated no effect under subcritical conditions. Any additive improved the efficiency under supercritical conditions, whereas the effect of an additive on the retention was highly dependent on the analyte structure [53].

In some cases, both acidic and basic additives were mixed into the mobile phase [60], or ionic additives, including various salts, such as lithium acetate, tetramethylammonium acetate or ammonium chloride, were tested [61]. Later, ammonia and volatile buffers, such as ammonium acetate [59, 61-635] or ammonium formate and carbonate [59], were successfully employed as additives and have been used for the analysis of basic compounds [64, 65]. The

main advantage of using ammonium salts or other volatile additives is their better compatibility with mass spectrometry detection. Another advantage might be easier post-purification treatment on the preparative scale, where the additive has to be removed.

An alternative solution consists of using water as an additive. Water has a very low solubility in supercritical CO₂ (~0.1% w/w) [66], but together with an organic modifier, it is miscible with CO₂. Water is more polar and possesses twice the hydrogen bonding capability of methanol. It becomes acidic in contact with CO₂ due to the formation and dissociation of carbonic acid [67]. So far, the use of water as an additive in SFC has not been widely reported, but the results obtained are promising due to several effects, including the enhancement of the mobile phase solvating power, the introduction of HILIC-like analyte partition, the simplification of preparative purifications and compatibility with mass spectrometry [68]. As an example, the addition of water at levels of up to 5% was found to improve substantially the peak shape of nucleobases with four different stationary phases. A comparison of water with other additives, including formic acid and ammonium acetate, is shown in Figure 5, with a pyridine stationary phase [69].

5.3. Choice of temperature and backpressure (tuning fluid properties)

In SFC, the analyte retention is influenced by the mobile phase density, which depends itself on the temperature, pressure and mobile phase composition. However, since the recognition of the composition of binary mixtures as the most important factor in controlling SFC separation [51], the programming pressure and/or density have become less important and are further used only for fine tuning [50].

Unlike in liquid chromatography, when the temperature is increased in SFC (at a constant pressure), the retention first increases due to the reduction in the mobile phase density. After reaching a maximum, the retention decreases at very high temperatures [55]. In SFC, the

temperature affects the vapour pressure of the solute and the density of the supercritical fluid, but it also influences the solubility parameters of both the solute and the supercritical fluid. It might also change the affinity of the compound for the stationary phase. Therefore, the resulting effect of temperature on retention in SFC is a combination of various mechanisms and can be quite complicated to explain depending on the experimental conditions, the properties of the solutes, and the nature of the supercritical fluid and the stationary phase [70]. To prevent peak shape degradation in SFC, it is important to ensure a single mobile phase state and beware of the formation of two phases. Because the critical temperature and pressure of binary mixtures methanol-CO₂ change with the fluid composition, they rapidly increase as the concentration of methanol increases (Figure 4). All binary mixtures of methanol-CO₂ have been reported to form a single phase at 40°C at a constant pressure of approximately 80 bar. Above this value, the density is a linear function of composition, regardless of whether the fluid is sub- or supercritical. On the other hand, at lower pressures, all mixtures of methanol in CO₂ separate into two phases. The higher temperatures used in SFC also require higher pressures if the formation of two phases is to be avoided [71].

Similarly, unlike in liquid chromatography, when the pressure increases in SFC, a small decrease in retention time is observed at a constant temperature. In a pure CO₂ mobile phase, this effect is relatively straightforward and simply means that higher pressures will lead to lower retention factors [70]. When performing a gradient of methanol in CO₂, this change is reported to be noticeable only for early eluting compounds (low concentration of organic modifier), while the elution of later eluting peaks is not affected by pressure modifications. Indeed, with higher percentages of organic modifier, the mobile phase compressibility is much lower, and subsequently variations in pressure have very little effect on the retention [54]. Changes in pressure also exert only minor effects on efficiency when the chromatographic conditions are well above the critical point of the mobile phase, *i.e.*, at

commonly used pressures > 150 bar [55]. Actually, column efficiency loss is typically observed with increasing axial density gradients. Tarafder *et al.* [72] have recently published detailed calculations of pressure and density decreases along the column under SFC conditions with CO_2/MeOH mobile phases using isopycnic plots overlaid on the P-T plane (Figure 6).

These plots enable the simultaneous identification of both the regions with the lowest density variations and those with reasonable compressibility, which can then be used as operating conditions for developing SFC methods. These regions did not correspond to the regions with the lowest pressure decreases. Conversely, lesser pressure drops resulted in higher density differentials. These drops were controlled by the compressibility of the mobile phase, which increased at lower pressures. Based on the results of these calculations, an example of which is given in Figure 6, a reasonable compromise appears to set-up SFC conditions at 150 bar and approximately 40 °C.

The addition of an organic modifier can be expected to increase density variations. However, because the addition of a modifier increases the viscosity and decreases the solvent compressibility simultaneously, leading to increased pressure, the resulting net change in density is ultimately small [72].

5.4. Selection of the mobile phase flow rate in modern SFC (van Deemter curves)

To evaluate the benefits of the latest generation of SFC instruments combined with columns packed with sub-2 μm particles, various configurations were compared using van Deemter curves and pressure plots. There have been numerous debates over the last few years about the validity of plotting van Deemter curves in SFC. Indeed, because the pressure inside the column changes with the flow rate, the mobile phase density also changes, leading to a reduction in the retention factor with increasing flow rate. Because the k value influences the

shape of the curves through changes in the B and C-terms, the reliability of van Deemter curves may be questionable. In our UHPSFC experiments at ~5% MeOH, we have observed that the retention factor varied by approximately 20% between the lowest and highest flow rate. This variation was of comparable magnitude to the change in retention factor observed in UHPLC over the entire range of applicable liquid mobile phase flow rates. The latter variation is known to be related to viscosity changes due to a frictional heating phenomenon that occurs in liquid conditions at very high pressure. In other words, the validity of van Deemter curves is comparable in UHPLC and UHPSFC in the presence of an organic modifier. However, using a pure, supercritical CO₂ mobile phase, the applicability of regular van Deemter curves for kinetic performance evaluation may be much more questionable because the higher compressibility of such a mobile phase will affect the k value to a greater extent. However, it should be noted that the use of such a particular mobile phase has become increasingly rare in modern SFC.

In Figure 7A, four different van Deemter curves, corresponding to HPLC, UHPLC, SFC and UHPSFC, are plotted. As expected, some significant differences were observed between these configurations because of the particle size differences and the different properties of the HPLC and SFC fluids. First, conventional HPLC was the least powerful strategy, as the optimal linear velocity was quite low, at approximately 0.7 mm/s, corresponding to a H_{\min} value of 11.6 μm . The benefits of SFC are illustrated in the corresponding van Deemter curve. Due to the significant reduction in viscosity of the supercritical fluid compared with the hydro-organic mobile phase employed in HPLC, the diffusion coefficients improved, leading to an increase in u_{opt} to up to ~ 3.5 mm/s for a H_{\min} value of 12.7 μm . Compared to conventional HPLC, the H_{\min} value achieved in UHPLC was drastically reduced (3.8 μm) because it is directly proportional to the particle size. In addition, the u_{opt} value increased proportionally to the reduction in particle size, up to 2.2 mm/s. Finally, the curve

corresponding to UHPSFC combines the advantages of both SFC and UHPLC because columns packed with sub-2 μm particles were employed in combination with a supercritical fluid. In this case, the u_{opt} value was approximately 10.2 mm/s ($F = 2.6$ mL/min for the 3 mm I.D. column), which corresponded to a 15-fold faster optimal linear velocity than for regular HPLC. The H_{min} value was slightly higher than that for the UHPLC conditions (~ 4.8 μm) but remained acceptable (*i.e.*, plate count of more than 21,000 plates for a 100 x 3 mm, 1.7 μm column at a flow rate 15-times higher than that used in conventional HPLC) [30].

It is important to mention that the exceptional gain in performance reported here between regular HPLC and UHPSFC may not always be so pronounced. The performance gain is highly dependent on the mobile phase viscosity. As an example, for UHPSFC experiments performed with a mobile phase containing between 10 and 20% MeOH (which represents common conditions), the gain in analysis time may range between 5- and 10-fold instead of 15-fold. Under such conditions, the kinetic performance of UHPSFC may be closer to that of UHPLC.

In addition to the plate count, it is also important to consider the generated pressure drops when selecting an adequate mobile phase flow rate. Figure 7B shows a plot of the column pressure drop as a function of the linear velocity. The highest pressure was systematically observed for the UHPLC configuration, even when a short column of only 50 mm was employed. Indeed, according to Darcy's law, the generated pressure drop is inversely proportional to the square of the particle size (when combining the u_{opt} from the van Deemter curves and Darcy's law, it even becomes proportional to d_p^3) and is also related to the viscosity of the fluid. In conventional HPLC with a 150 mm column, the pressure drop was equal to 54 bar at the optimal mobile phase linear velocity. In regular SFC with a 150 mm column, the backpressure was reduced to only 12 bar at u_{opt} because of the very low viscosity of the supercritical fluid. However, when using columns packed with sub-2 μm particles, the

pressure increased up to 322 and 185 bar in UHPLC and UHPSFC for 50 and 100 mm columns, respectively, at the optimal linear velocity [30].

Even if the pressures were reasonable in the SFC and UHPSFC modes, an additional backpressure of 120-150 bar must be applied downstream of the column to maintain the mobile phase in the subcritical/supercritical state. Thus, the upper limit of the Waters ACQUITY UPC² system (400 bar) is quickly attained, particularly when using a significant proportion of MeOH in the mobile phase. As an example, a maximum flow rate of only 2 mL/min could be applied with a column of 100 x 3 mm, 1.7 μ m when using a mobile phase containing 40/60 MeOH/CO₂. This issue was less noticeable on the Agilent 1260 Infinity Analytical SFC because the latter can withstand a maximum pressure of 600 bar. This upper pressure limit value is indeed required to perform UHPSFC with columns packed with sub-2 μ m particles.

5.5. Isocratic or gradient operation?

Because modern SFC looks more and more like UHPLC, the same rules can be applied for selecting the ideal elution mode, namely, isocratic or gradient. Thus, when analysing an unknown sample, it is advised to initially perform a generic scouting gradient from 2 to 40 % MeOH, including a short isocratic hold at 2 % MeOH and another one at 40 % MeOH. Even if it is a good strategy to perform a gradient with a proportion of MeOH higher than 40% for eluting the most polar compounds (particularly those possessing significant H-bonding capability) with a polar stationary phase, this is not recommended. Beyond this cut-off value, the interest for SFC from kinetic point of view (*i.e.*, low mobile phase viscosity and high diffusion coefficients) rapidly disappears, and the generated pressure becomes too critical when using columns packed with sub-2 μ m particles and a UHPSFC system with an upper pressure limit of 400 bar. Using this generic gradient procedure, it becomes possible to

determine the elution window of the analytes contained within the sample (first and last eluting peaks) and also to decide whether a gradient is required, taking into account the differences in elution composition between the first and last peaks.

Usually, the final gradients in UHPSFC (average gradient span of 20 % organic modifier) are narrower than those in UHPLC (average gradient span of 50 % organic modifier). In addition, because the linear velocity is higher in UHPSFC than in UHPLC, the column dead time is reduced. Because the gradient steepness depends on both the gradient span and the column dead time, the gradient time has to be reduced by a factor 2-3 in UHPSFC to achieve a similar gradient steepness (and thus comparable peak capacity) to UHPLC. Last but not least, similar to the well-known behaviour in HPLC/UHPLC, the resolving power and sensitivity achieved in UHPSFC gradient mode is often superior to that in the isocratic mode because the broadening of the most retained compounds is less pronounced.

6. Detection modes in SFC

In a GC-like SFC set-up, where only pure CO₂ is used as the mobile phase, flame ionisation detection (FID), electron capture detection (ECD), chemiluminescence, and various other detection approaches are possible [58]. Using FID for detection is only applicable when the mobile phase consists only of CO₂, and hence no modifier gradient can be programmed. The compounds are in this case eluted by applying a pressure gradient. The operation of a FID is based on the detection of ions formed during the combustion of organic compounds in a hydrogen flame. Therefore, the application of a solvent gradient results in a change in the baseline signal as the combustion of the modifier also takes place. This approach can only be applied to relatively apolar solutes and is mostly employed in the petrochemical industry for the group type separation of light oils and gasolines. Because FID is a universal detector, its most significant advantage is easy quantitation.

In an LC-like set-up, detection systems similar to those employed in HPLC are used [58,73]. While UV detection is the most popular choice, other systems, including ELSD [74-77], condensation nucleation light scattering detector (CNLSD) [78], charged aerosol detector (CAD) [75,79] and MS detection [80-83], have been used less commonly so far. Conversely, the hyphenation of SFC with MS is currently gaining in popularity.

With UV detection, it is necessary to control the backpressure in order to maintain sufficient mobile phase density; therefore, pressure resistant cells (up to 400 bar) are needed, and the back-pressure regulator is located after the detector. In theory, the low UV absorbance of CO₂ favours a good signal-to-noise ratio. However, there are several other factors contributing to increased noise in SFC-UV systems, leading to lower sensitivity compared to LC-UV. Pressure fluctuations (both due to the compression strokes of the pump or outlet pressure variations associated with the BPR control) are the major contributors to changes in density, together with refractive index fluctuations in the flow cell, creating additional background noise. Further optimisation of the SFC instrumental set-up is therefore needed to decrease these variations [84]. Recently, a report was published demonstrating the sufficient sensitivity of SFC-UV for impurity profiling in pharmaceutical analysis, which required a detection level of 0.05 % of the active principal ingredient [85].

ELSD and other detectors based on nebulisation have the potential to work better in SFC than in HPLC as a result of the easier evaporation of supercritical mobile phases. The addition of triethylamine or formic acid to the mobile phase has been reported to improve the ELSD response [76,77]. For ELSD and other nebulisation-based detectors (MS, CAD, CNLSD), an additional heating of the interfacing tube is often required to avoid the risk of dry ice formation, which is a result of the cooling effect when the supercritical fluid returns to atmospheric pressure after the BPR. For mobile phases containing only low concentrations of organic modifier, it is often recommended to add a make-up solvent after depressurisation to

avoid analyte precipitation or segmented flow due to the presence of separated phases in the transfer line [74,81,83]. To obtain a uniform response in gradient elution when using ELSD and CAD, it could also be advantageous to use gradient compensation, similar to what is sometimes employed in HPLC [74,79]. Finally, it has been recently reported that CAD can provide much better quantitative performance than can ELSD [75].

The coupling of SFC with MS should be more straightforward than LC-MS due to the high volatility of CO₂ and the extensive development of the API (atmospheric pressure ionisation) interfaces in LC-MS that could also be adopted in SFC-MS [80,81]. The commercial API sources used in LC-MS have been proven to be applicable in SFC-MS using ESI (electrospray ionisation) and APCI (atmospheric pressure chemical ionisation) [80-82]. Similar to LC-MS, the SFC mobile phase conditions can impact both the chromatographic performance and ionisation efficiency. Because in the majority of SFC-MS applications a make-up solvent is added post-column, the ionisation efficiency is enhanced independently from the chromatographic separation. The addition of make-up solvent is not only important for solute ionisation but also for generating a stable and homogeneous Taylor-cone in the MS source, especially for those compounds eluting at small modifier percentages. The detection sensitivity is more strongly dependent on the mobile phase flow-rate/pressure and the split-ratio for mass sensitive ionisation methods, such as APCI, than for concentration sensitive ionisation methods, such as ESI. Several interfacing approaches have been described, among which pre-BPR splitting is most likely the most promising approach [81].

7. Problematic compounds successfully analysed by modern SFC

7.1. Lipophilic compounds

Lipids display high complexity, and therefore the challenge for analytical methodologies is significant. In addition to GC [86-88] and (U)HPLC [88-92], SFC can be effectively

considered as a powerful tool for lipid analysis. For in-depth discussion on this topic, the readers can refer to a recent review by Bernal *et al.* [93]. Because UHPSFC is relatively new, the number of applications dealing with the analysis of lipids remains scarce, but is expected to expand in the future. As a general remark, C18 phases are most widely used for this type of application.

In 2002, Senoráns and Ibanez published a dedicated review of SFC separations of fatty acids (FAs) [94]. More recently, the analysis of free FAs has been described by Isaac *et al.* [95] for an oil sample. The separation was carried out on a C18 column packed with 1.8 μm particles using methanol and formic acid as a modifier. Recently, Taguchi *et al.* [96] described the separation and quantification of 25 bile acids present in rat serum by UHPSFC-MS/MS. Although SFC has always been regarded as a technology applicable to highly apolar compounds, the authors demonstrated that polar species can also be successfully analysed. In their paper, the authors stressed the advantage of simplified sample preparation, high throughput and excellent peak shapes.

The analysis of triacylglycerols (TAGs) by packed SFC has been performed using C18 [97-100], 2-ethylpyridine [101] or silver-ion exchange (SIC) [100, 102-104] columns. Due to the high complexity of these compounds and the lack of resolution, the use of multidimensional chromatography has been investigated for both esters of FAs as well as TAGs using SFC in one [105,106] or both dimensions [107,108]. The high orthogonality between LC and SFC is the main driver for these first configurations, and the current availability of UHPLC as well as UHPSFC opens new perspectives for these two-dimensional approaches.

The possibilities offered by UHPSFC for lipid analysis were recently illustrated by Jones *et al.* [109], who analysed inter-class separations of neutral and amphipathic lipids using BEH and HSS C18 columns packed with sub-2 μm particles.

In addition to lipids, SFC is also highly advantageous for the analysis of carotenoids and fat-soluble vitamins. Lesellier *et al.* [110,111] have thoroughly investigated the analysis of carotenoids by SFC, evaluating numerous stationary phases as well as modifiers. More recently, a number of application notes were published for such lipophilic compounds using UHPSFC conditions [112], showing the possibility to drastically reduce analysis times. Another notable benefit is that most of these compounds can be analysed using a single technology, and the sample preparation step is drastically simplified. Current LC or GC methodologies require extensive and different handling of the sample dedicated to the analysis and detection of only a limited number of compounds.

7.2. Hydrophilic compounds

A large variety of biologically active substances, including compounds of pharmaceutical interest, display high hydrophilicity and require challenging or problematic approaches to be properly analysed by LC. Indeed, the conventional C18 RPLC strategy struggles to achieve sufficient retention of highly hydrophilic compounds. Modified C18 materials displaying an embedded polar group (allowing for dipole–dipole and H-bond interactions) as well as analyte neutralisation using an appropriate mobile phase pH could be viable options but fail to be generic and generally require time-consuming, case-by-case optimisation [113]. NPLC is more suitable to achieving sufficient retention of hydrophilic substances but is environmentally unfriendly and suffers from solubility issues [114]. Ion-exchange chromatography and ion-pairing chromatography are elegant alternatives but are hardly compatible with MS detection [115]. Good retention, MS compatibility and straightforward method development have been recently reported by Periat *et al.* using hydrophilic interaction liquid chromatography (HILIC), which can be considered one of the best liquid approaches for hydrophilic compound analysis ($\log D_{\text{pH } 3}$ between -5 and 0) [116]. The same study also

underlines the potential of UHPSFC for the analysis of molecules of similar hydrophilicity by taking advantage of the good chromatographic selectivity and suitable retention provided by a normal phase-like separation mechanism (Figure 8).

In UHPSFC, the retention and selectivity are strongly dependent on H-bond interactions. A silica or hybrid silica stationary phase is therefore particularly advised as a first choice. Alternative chemistries, such as diol or 2-EP, could also be interesting for selectivity modification or ionisable polar compound analysis, respectively. When dealing with very polar substances, these compounds display high H-bond donor capability and tend to be extensively retained on polar materials. Thus, the stationary phase polarity must be adjusted. A C18 stationary phase could be a valuable option to decrease retention, but only non-end-capped C18 phases can be employed to maintain sufficient selectivity. Regarding the mobile phase conditions, a generic scouting gradient from 2 to 40 % MeOH is generally sufficient to elute the majority of compounds. A final isocratic step at 40 % MeOH could be used to elute the most hydrophilic compounds from the column. Peak shape issues and the elution of extremely retained compounds could be improved by enriching the supercritical mobile phase with 1 to 5 % water [117]. When using water as a polar additive, a HILIC-like retention mechanism involving analyte partition between the water-rich layer formed at the surface of the stationary phase and the bulk mobile phase has been recently postulated in supercritical conditions [118].

7.3. Basic drugs

Modern SFC is a competitive analytical tool for the analysis of a broad range of compounds displaying various physicochemical properties. However, many published SFC applications only address acidic, neutral or weakly basic analytes (pKa below 6). Indeed, the analysis of stronger basic compounds in modern SFC remains challenging for several reasons. First, the

presence of an alcoholic organic modifier confers acidic properties to the supercritical mobile phase (apparent pH of approximately 4-5) [119]. The formation of methylcarbonic acid by the reaction of MeOH and supercritical CO₂ is suspected to be the cause of this acidic pH [120]. Under such conditions, the base strength (pK_a value) is of particular importance [65]. While weakly basic compounds remain uncharged and can be easily analysed, stronger bases tend to become protonated and to establish secondary ionic interactions with the negatively charged residual silanols that are still present at the stationary phase surface. These ionic interactions significantly affect the kinetic performance and peak shape in chromatography. The use of end-capped stationary phases to limit silanol accessibility is not considered a viable option in UHPSFC because silanols are predominantly involved in both selectivity and retention. However, columns bearing a basic chemical moiety, such as 2-EP or diethylaminopropyl (DEAP), could be of interest. These basic moieties could also be protonated by the supercritical mobile phase. Their resulting positive charge further limits access to residual silanols by establishing both electrostatic repulsions with positively charged basic analytes and by quenching adjacent silanols *via* intra-stationary phase ionic or H-bond interactions. It is worth mentioning that important differences in terms of chromatographic behaviour and the resulting peak shape of basic analytes were observed between the different 2-EP manufacturers because of a variable amount of residual silanols, different silica pre-treatments and ligand density [65].

Another possible strategy to avoid peak degradation issues with basic analytes consists of using small proportions (< 1 %) of mobile phase additives. Strong basic additives, such as diethylamine, triethylamine and isopropylamine, are able to mask residual silanols or ion-pairing agents, such as trifluoroacetic acid, and have been successfully used in the past. The use of volatile salts, such as ammonium acetate, ammonium formate or ammonium hydroxide, (Figure 9) represents an elegant alternative [62, 64, 65, 121]. The main advantage of

ammonium salts lies in their compatibility with MS detection, while other acidic and basic additives should be avoided due to their massive contribution to ion suppression. In addition, typical ammonium salt concentrations of approximately 10 to 20 mM do not seem to affect the stationary phase nature or performance.

8. Conclusion

In conclusion, it is clear that UHPSFC using columns packed with sub-2 μm particles is a promising strategy for analytical scientists. However, although the approach appears to be similar to UHPLC, there are important differences that should be considered by users, particularly regarding the choice of stationary phase chemistry, mobile phase (organic modifier and additives), column dimensions, sample diluent and retention mechanism.

In terms of its application range, UHPSFC has the capacity to extend the possibilities of RP-UHPLC while also offering high throughput/high resolution separation. First, the compounds that are amenable to RPLC can also be analysed by modern SFC thanks to the non-negligible proportion of organic modifier in the mobile phase (on average 10-20 % and as high as 40 % MeOH). When analysing ionisable compounds, a suitable additive must be identified, and 20 mM ammonium formate appears as the best choice for the analysis of both acidic and basic substances. Due to obvious differences in retention mechanisms, the final separations achieved in UHPSFC and RP-UHPLC are fairly orthogonal. This opens the door to comprehensive SFCxSFC or LCxSFC experiments, allowing further improvements in resolving power [122]. In addition, because a polar stationary phase (*e.g.*, bare silica, 2-EP) is usually employed in UHPSFC, polar compounds can be more strongly retained than in RPLC without the need for ion-pairing reagents. Finally, it is also possible to use an apolar phase (C18 type) in UHPSFC without modifying the mobile phase components to analyse most apolar substances (*e.g.*, lipids, liposoluble vitamins etc.).

Last but not least, it is important for new SFC users to keep in mind that the mobile phase properties (*i.e.*, viscosity and especially density) can be strongly influenced by pressure and temperature. A generic temperature and backpressure of 40°C and 150 bar can be considered as a suitable first choice. However, changes in density as a function of pressure can be an issue when transferring methods from a column packed with sub-2 µm (analytical scale) to 5 µm (preparative scale) particles, and some solutions to limit this issue have recently been discussed [123].

Figure captions

Figure 1: Phase diagram of carbon dioxide.

Figure 2: Chromatograms of butylparaben dissolved in 30:70 tetrahydrofuran:heptane (top), isopropanol (middle), and methanol (bottom). The injection volumes represented are: 0.5 (black), 1.0 (red), 1.5 (blue), 2.0 (green), 2.5 (light blue), 3.0 (pink), and 4.0 μL (brown). Reprinted from [25], with permission.

Figure 3: Spidergram classification of various SPs commonly used in SFC. The existing columns packed with sub-2 μm particles dedicated for SFC operation, as well as a few SPs packed with sub-3 μm superficially porous particles, were also included in this representation.

Figure 4: Relationship between the calculated critical temperature, pressure and concentration of the organic modifier. Adapted from [57] with permission.

Figure 5: Separation of four nucleobases on a pyridine column using ethanol as an organic modifier and various additives, including 25 mM ammonium acetate, 5 mM ammonium acetate + 5% water, 5% water, 5 mM ammonium acetate and ethanol only. Reprinted from [69] with permission.

Figure 6: Variations in the density drop (in g/ml) in a column packed with 3 μm particles and eluted at 3 g/ml. The contour plots (solid curves) show how the density drop zones vary as a function of the outlet pressure and temperature. The dotted lines are the isopycnic lines of CO_2/MeOH mixtures of varying ratios: (A) CO_2/MeOH (95/5%, mol/mol), (B) CO_2/MeOH (90/10%, mol/mol), (C) CO_2/MeOH (85/15%, mol/mol), (D) CO_2/MeOH (80/20%, mol/mol). Reprinted from [72] with permission.

Figure 7: Kinetic performance and pressure drop for various LC and SFC configurations. A) van Deemter curves on 4 different chromatographic systems equipped with the most suitable column dimensions: HPLC column 150 x 4.6 mm, 5 μm (purple diamonds), SFC column 150 x 4.6 mm, 5 μm (green squares), UHPLC column 50 x 2.1 mm, 1.7 μm (red dots), UHPSFC

column 100 x 3.0 mm, 1.7 μm (blue triangles). B) Corresponding system pressure drops. Adapted from [30], with permission.

Figure 8: (B) Retention and selectivity achieved as a function of the percentage of organic solvent required for eluting individual hydrophilic compounds ($\log D_{\text{pH } 3}$ between -5 and 0) in the UHPSFC (A), HILIC (B) and RPLC (C) modes. The horizontal marks correspond to the average elution composition. Adapted from [116], with permission.

Figure 9: Chromatograms of basic analytes displaying a pK_a value below 5 (blue line 1, top chromatograms), between 6 and 7 (green line 2, middle chromatograms) and between 8 and 9.5 (red line 3, bottom chromatograms) obtained in UHPSFC on a BEH 2-EP phase without additives (chromatograms A) and on hybrid silica in the presence of 20 mM NH_4OH (chromatograms B). Adapted from [65], with permission.

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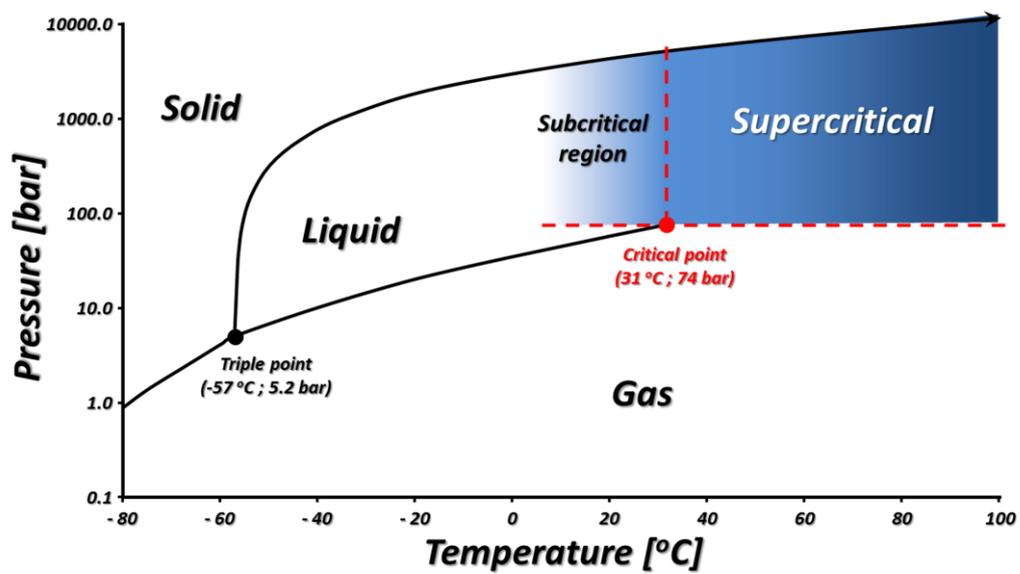


Figure 1

FIG 1 NEW .

Accepted

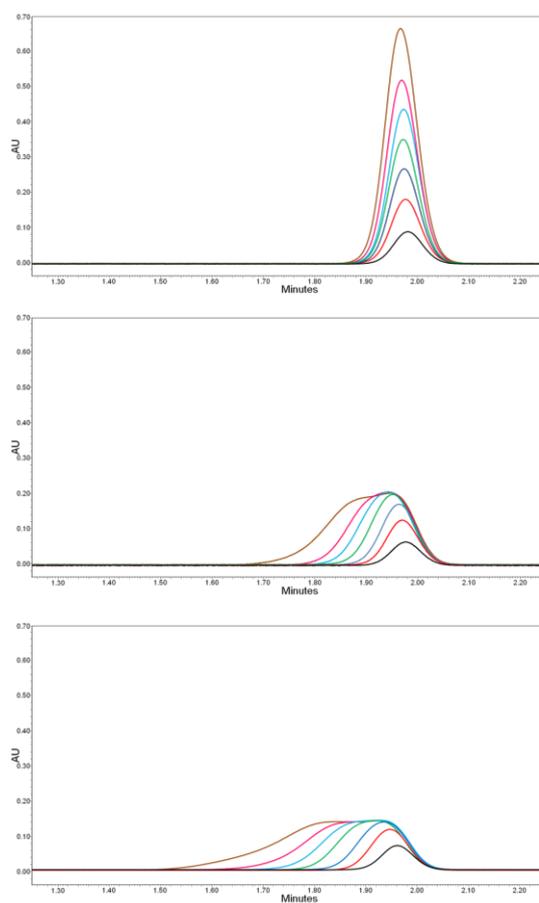


Figure 2

FIG 2 NEW .

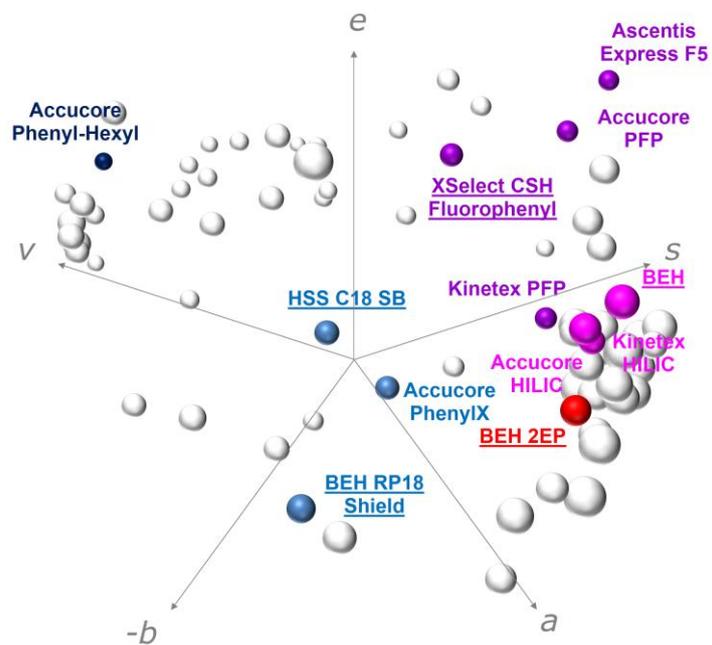


Figure 3

FIG 3 NEW .

Accepted

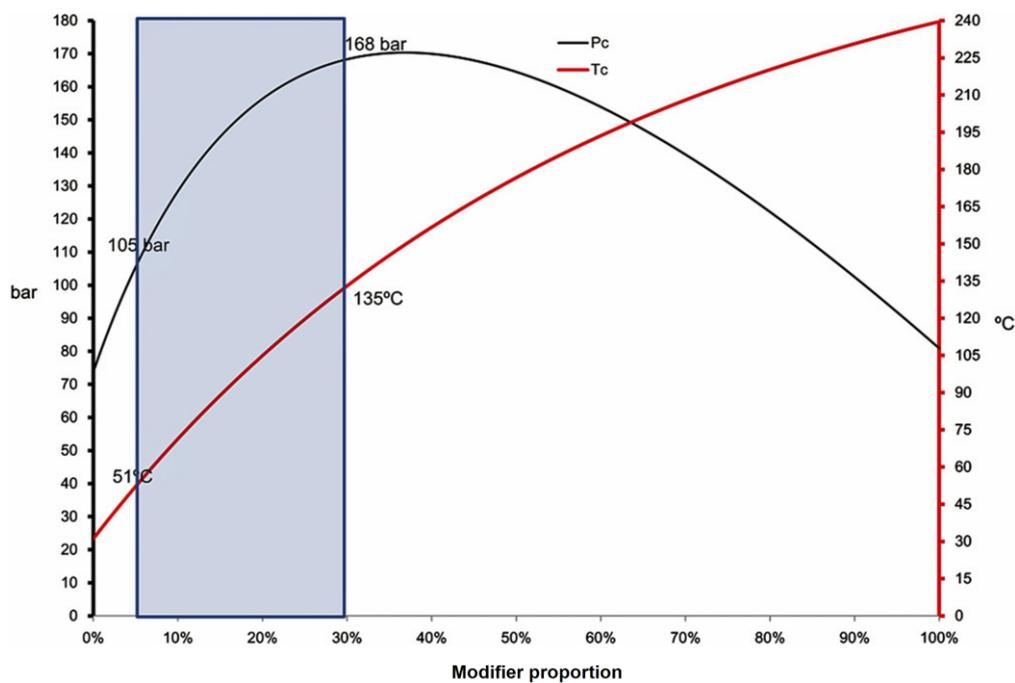


Figure 4

FIG 4 NEW .

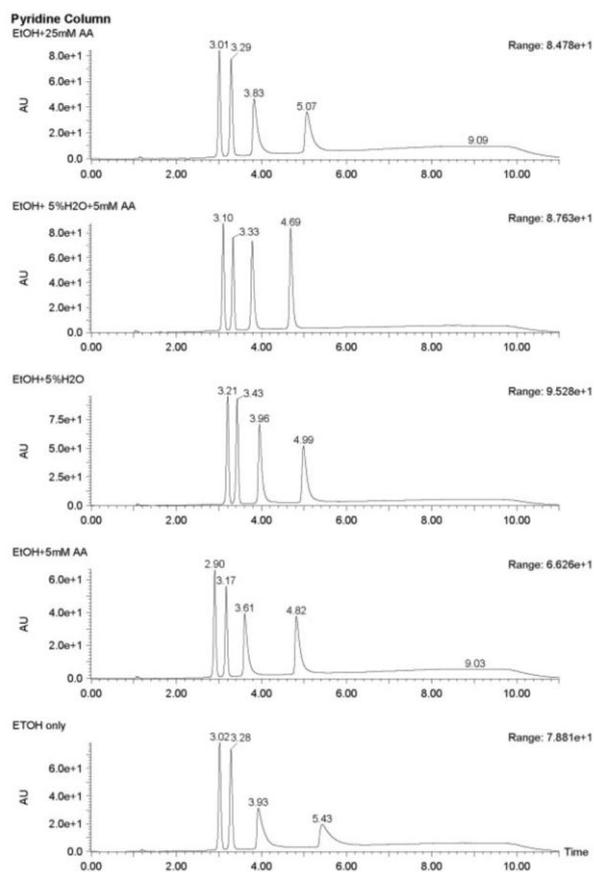


Figure 5

FIG 5 NEW .

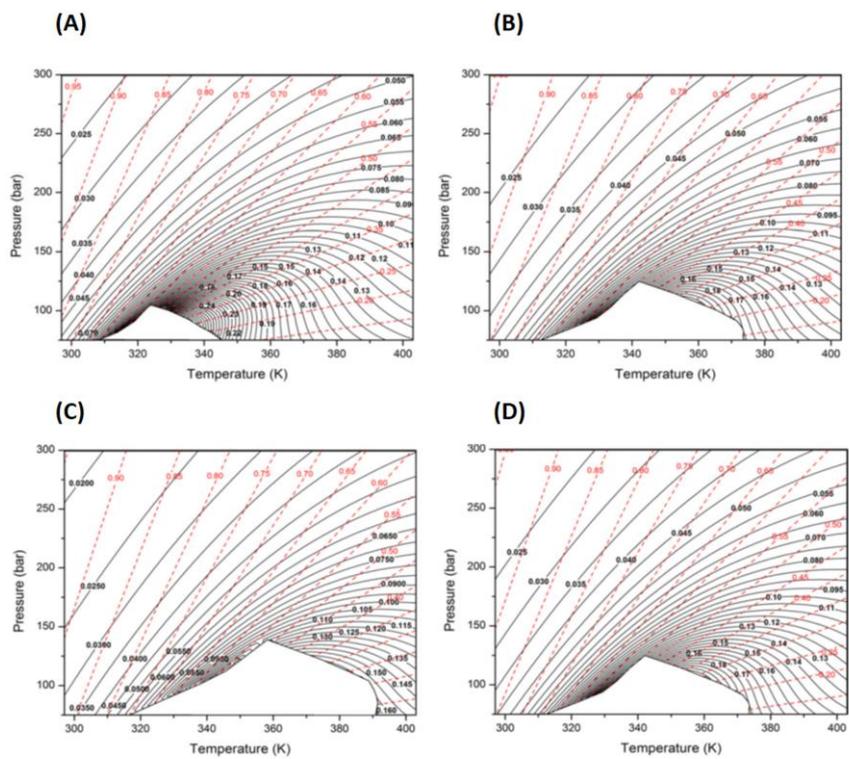


Figure 6

FIG 6 NEW .

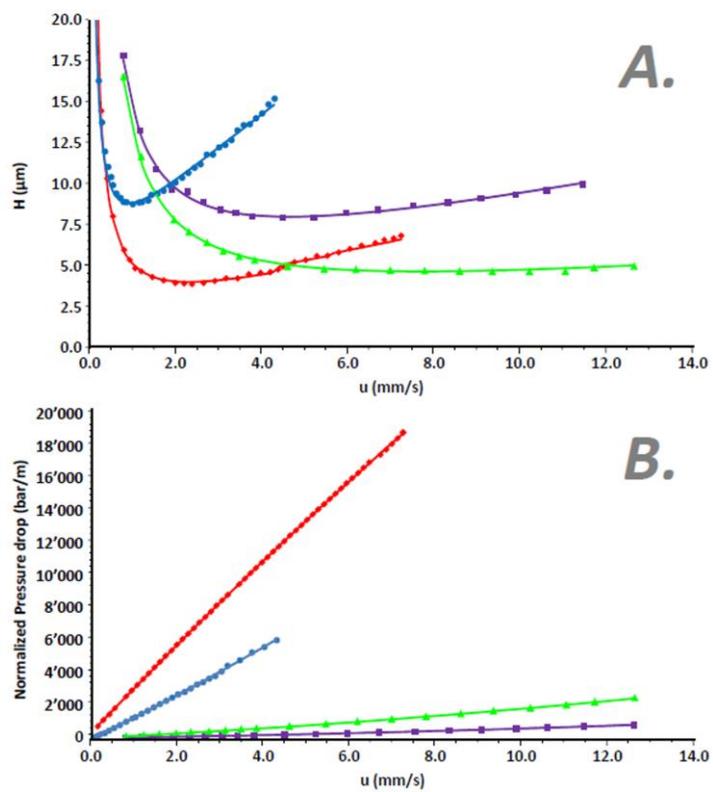


Figure 7

FIG 7 NEW .

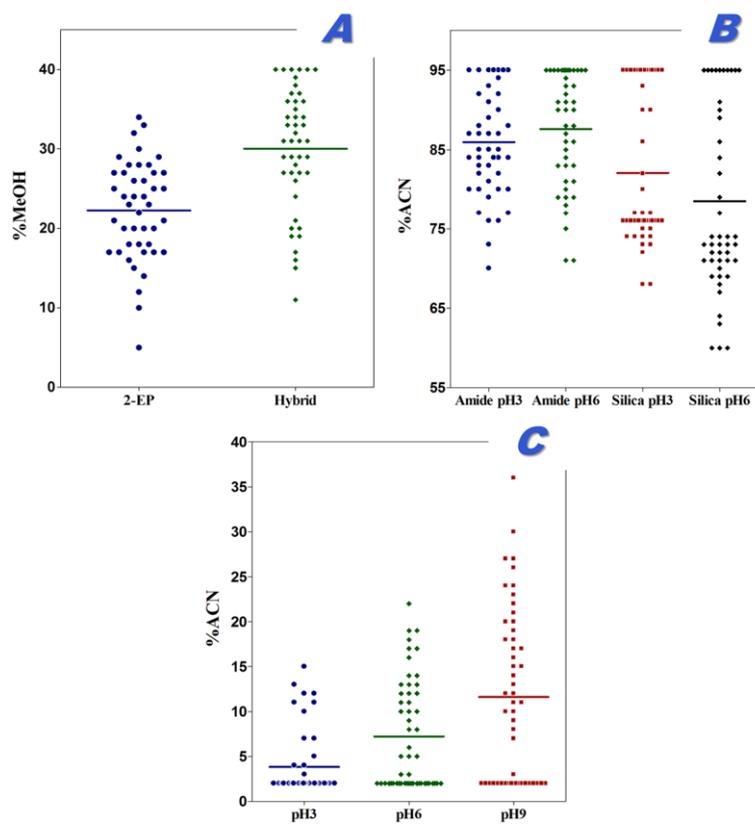


Figure 8

FIG 8 NEW .

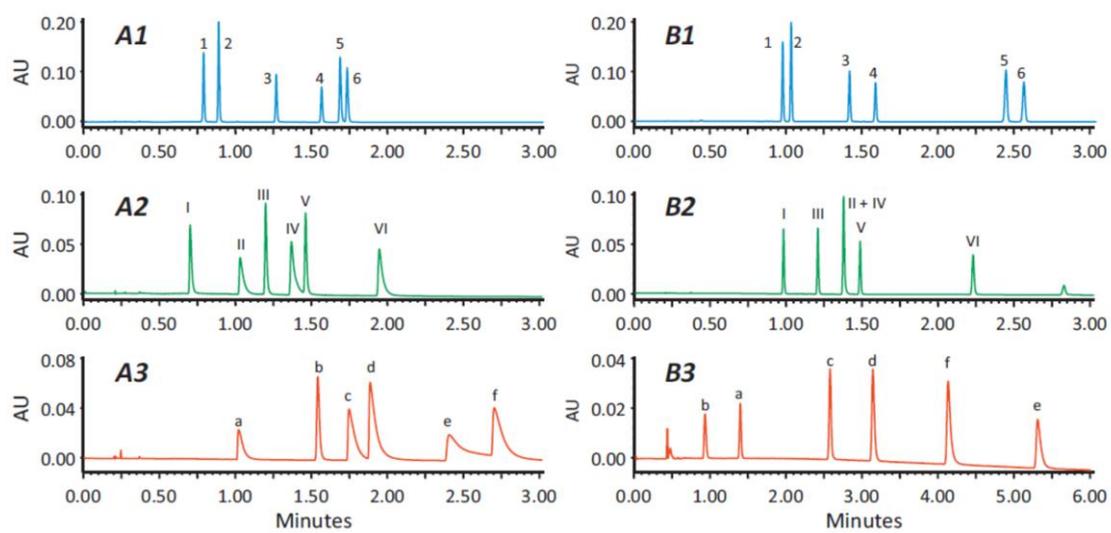


Figure 9

FIG 9 NEW .