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Evolution and Current Trends in Liquid and Supercritical Fluid Chromatography

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Abstract: The current trend in high performance liquid chromatography (HPLC) tends toward the achievement of higher separation efficiency and shorter analysis time. Indeed, better performance in LC has become increasingly important in recent years mainly driven by the challenges of either analyzing more complex samples or increasing the numbers of samples *per* time unit. In the recent development of particle technology, the use of fully porous sub-2 μm particles and sub-3 μm shell particles have received considerable attention. Beside packed columns, the new generation of silica-based monolithic columns also offers very high separation power. However, to take full advantage of these innovative phases, the chromatographic system has also to be drastically optimized in terms of upper pressure limit and system volume.

This revolution in column technology now spreads and covers several modes of liquid chromatography such as reversed-phase liquid chromatography (RPLC), hydrophilic interaction liquid chromatography (HILIC), or even supercritical fluid chromatography (SFC). The HILIC and SFC, which can be considered as alternative modes of chromatography, could also be useful to extend the applicability of chromatography towards the analysis of very hydrophilic and lipophilic compounds, respectively.

The present review gives an insight about the theory behind the success of current column technology and presents a summary of latest applications, using various modes of one-dimensional chromatography (RPLC, HILIC, SFC). This paper also shows that theoretically expected column efficiency could sometimes be compromised in practical work especially in the case of narrow bore columns.

Keywords: Core-shell, dwell volume, extra-column band broadening, fast analysis, high resolution separation, HILIC, instrumentation, monolith, SFC, UHPLC.

1. INTRODUCTION

During the last decade, there have been a lot of advances in the field of liquid chromatography, including new solid chromatographic supports and groundbreaking instruments, which have been summarized in (Fig. **1**). Thanks to these technical innovations, it is possible today to reach fast separation without compromising separation efficiency, very high resolution of complex samples at a reasonable throughput, significant changes in selectivity with alternative separation modes, or improved mass spectrometry (MS) sensitivity, thanks to chromatographic approaches involving higher proportion of organic modifier than reversed phase liquid chromatography (RPLC).

Since 2000, there has been a revolution in liquid chromatography with the objective to improve kinetic performance (plate numbers) of regular HPLC columns of 150 - 250 mm length packed with 5 μm particles. As reported in (Fig. **1**), various types of innovative material were commercially introduced or revisited, including silica-based monoliths in 2000 [1, 2], columns packed with fully porous sub-2 μm in 2004 [3, 4], and columns packed with core-shell sub-3 μm in 2007 [5, 6]. Using these approaches, it is indeed possible to strongly increase throughput and/or resolution in LC. It is also important to notice that the kinetic performance evaluation of state-of-the-art LC phases becomes a topic of high relevance for the scientific community in the last ten years [7-9]. Together with the evolution of chromatographic supports, the systems have also been drastically improved to be compatible with the most recent stationary phases [10-12]. Compared to old generation HPLC instruments, the current systems on the market possess an extended pressure range (up to 1000-1300 bar for most of them). In addition, the contribution of the system to band broadening was drastically reduced by using short and thin tubing, together with low injected volume and reasonable UV cell volume. Finally, in the case of gradient elution operation, the system dwell volume was also reduced on both high pressure and low pressure mixing systems, to meet the requirements of high throughput analysis.

Except kinetic performance, selectivity and retention remain much more important to resolve a mixture in liquid chromatography [13]. The first choice in LC is to employ a C18 or C8 phase, together with a mixture of acetonitrile and water in presence of buffer (in most cases 0.1% v/v formic acid, 0.1% v/v trifluoroacetic acid or phosphate buffer). However, depending on the complexity of the sample and

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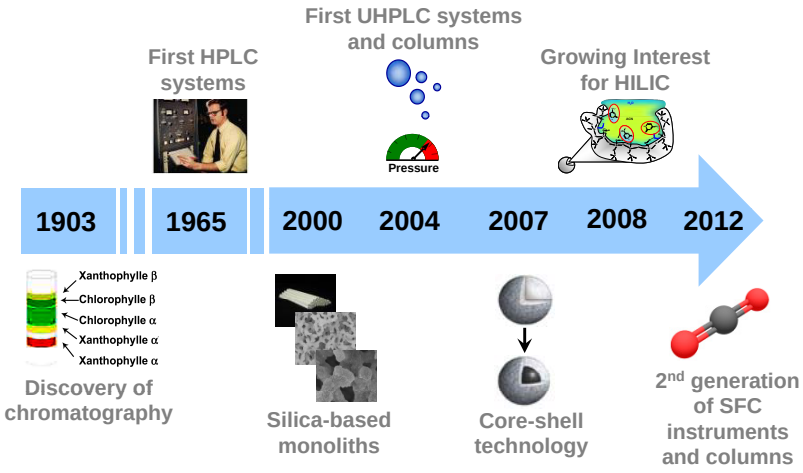

Fig. (1). Evolution of liquid chromatography from its discovery until now. As shown, there have been a significant number of advances during the last decade.

the physico-chemical properties of the analytes, it could be required to employ i) alternative stationary phases, ii) different mobile phase composition or even iii) orthogonal separation mode. Regarding RPLC stationary phase chemistry, pentafluorophenyl (PFP) bonding has strongly expanded in the last few years as it allows achieving orthogonal retention and selectivity for polar and ionisable compounds compared to a regular C18 material [14]. Numerous providers also propose now polar endcapped C18 material compatible with purely aqueous mobile phase [15]. With such columns, it is possible to slightly increase the retention of the most polar analytes and also to modify selectivity through additional interaction mechanisms. In terms of mobile phase, temperature up to 60-90°C is more commonly employed than in the past due to the improved resistance of silica-based stationary phases [16-18]. Indeed, elevated temperature allows an improvement of kinetic performance (thanks to lower mobile phase viscosity and increased diffusion) [19], a reduction of tailing with basic analytes and biomolecules (by increasing the rate of secondary ionic interactions kinetics) [20], and it also modifies retention and selectivity (due to a change in the system thermodynamic) [21]. Except temperature, pH remains a major parameter for tuning retention and selectivity of ionisable compound in RPLC [22]. Today, several providers offer hybrid silica-based columns compatible with pH up to 11 or 12 [23]. Finally, if separation cannot be resolved under RPLC conditions, there are two alternative separation modes which are highly promising, namely hydrophilic interaction liquid chromatography (HILIC) and supercritical fluid chromatography (SFC). The HILIC is particularly well adapted to the analysis of polar and ionisable compounds [24,25], while SFC is adapted to a large range of compounds, from relatively polar to very apolar ones [26, 27]. These two modes are not really new as the term HILIC has been coined in the 90's, while the first SFC experiments were performed during the 60's. However, with the recent introduction of innovative and more robust columns and systems, the interest for HILIC and SFC is growing in importance since 2008 and 2012, respectively (see Fig. **1**) [28].

Last but not least, MS becomes more and more important detection device coupled to LC and appears as the gold standard when sensitivity and/or selectivity of spectrophotometric detectors (UV, fluorescence) are inadequate [29]. Based on this observation, volatile mobile phase additives at low ionic strength (formic acid, acetate, formate or carbonate buffers), directly compatible with MS detection, are preferentially employed to develop new HPLC methods in many academic and industrial laboratories. It is worth mentioning that significant progress has been made in MS during the last decade, providing more sensitive, robust, user-friendly and faster systems [30]. The most important MS advances have been made on analyzer technology and on optics design for improved ion transmission and sensitivity. In the case where even higher sensitivity is required, it is also possible to play with the chromatographic conditions. For example, alternative chromatographic modes such as HILIC and SFC can offer lower limits of detection thanks to the use of large proportion of organic modifier, or due to the absence of water in the mobile phase, respectively [31-33].

Comprehensive separation of complex mixtures is a difficult challenge due to the presence of several components that vary from polar to non-polar and from very low to high concentrations, and that show diverse physico-chemical properties (acid-base properties, stability, solubility, detectability). The potential of conventional separation techniques such as liquid chromatography and detection approaches like UV or mass spectrometric detection is limited. In the last decade, comprehensive multidimensional separation techniques such as LC×LC have been gained in importance. These multidimensional techniques offer a huge separation power and are therefore ideally suited for the analysis of such mixtures. In contrast to off-line multidimensional separation techniques, where a particular fraction of the first-dimension separation is transferred and re-separated on a second-dimension column, the entire first dimension is analyzed in the second-dimension separation in LCxLC. Comprehensive LC×LC offers various advantages over both multidimensional off-line and conventional separation techniques, especially with respect to enhanced peak capacity, automation potential, reproducibility and shorter analysis time.

In the present review, all the technical solutions allowing faster separation, higher chromatographic resolution, im-

proved retention of critical compounds, alternative selectivity, and better sensitivity in one-dimensional liquid chromatography will be discussed in details. This contribution focuses especially on analytical scale separations. Based on this review, we hope that LC users will be able to make the good choice in their respective laboratories for columns, instruments and analytical conditions, considering the most recent materials and solutions available from providers.

2. CURRENT SOLUTIONS TO ACHIEVE FAST AND HIGH RESOLUTION SEPARATIONS IN LIQUID CHROMATOGRAPHY

In the last decade various analytical strategies have been developed to enhance separation speed and efficiency. Chromatographic supports based on monolithic material, small porous particles, and core-shell particles have been commercialized to improve throughput and separation efficiency.

2.1. Monolithic Supports

One possibility to enhance the separation speed is the reduction of the intrinsic flow resistance of the column. Increasing the external porosity and the flow-through pore size of the packing could lead to fast separations. The monolith approach, originally initiated by the works of Svec, Tennikova and Fréchet [34-36], Hjertén *et al.* [37], Horváth and co-workers [38], Tanaka and co-workers [39], already led to a number of well performing, commercially available polymeric and silica monolith columns [40, 41]. Monolithic columns have a unique chromatographic feature: high column efficiency and permeability [1, 42].

Two characteristics primarily differentiate the silica-based monoliths from the organic polymeric-based monoliths: pH stability and separation efficiency. While pH stability is definitely better for organic polymeric monoliths (pH 1-12), separation efficiency is undoubtedly improved with silica monoliths [1]. The organic monoliths are generally applied for the separations of large biomolecules, including oligonucleotides, peptides, and intact proteins [2, 43, 44]. On the other hand, silica-based monoliths are well adapted for the separation of small molecules, particularly since the introduction of the second generation of monoliths in 2011. The latter possesses macropores of 1.2 µm and mesopores of 15 nm. In the case of large molecules, widepore silica-based monoliths are however not yet commercially available but some promising data with prototype wide-pore silica monoliths were recently reported [45].

The first silica-based monolithic HPLC column (Chromolith, Merck Millipore) became commercially available in 2000 and attracted a lot of attention because of its novelty. This type of HPLC column consists of a porous silica rod that is encapsulated in a mechanically strong and solvent-resistant PEEK polymer and equipped with low-volume end-fittings [1]. The high porosity of silica-based monoliths is caused by macro- or through-pores that offer significant advantages compared to classical particle-packed columns, such as low column backpressure, operation at higher flow rates, fast analysis [1]. The mass-transfer kinetics of analytes is faster through monolithic columns compared to packed columns of comparable geometry and similar domain size. The kinetic efficiency of first generation silica monolith columns is comparable to columns packed with 3-4 µm totally porous particles.

With the advent of the second generation of commercial silica monolith columns, chromatographists now have an alternative to the sub-2 µm and core-shell packings for high-throughput, high-efficiency separations [1]. Compared to the first generation, this new generation possesses a more homogeneous porous silica network based on a well-designed silica skeleton in combination with a tailor-made bimodal pore structure (macro- and mesopores). The macropore size and corresponding domain size were systematically decreased. (Fig. **2**) shows scanning electron microscopic images of the first and second generation monoliths. At the same time, a much more homogeneous porous silica network has been obtained which causes a decrease in the eddy dispersion. The chromatographic performance of this new generation of monolithic columns demonstrates improved separation efficiency and peak symmetry, especially for basic compounds [1, 46].

Organic polymer monolithic stationary phases are well adapted to perform large molecules gradient separations, as the mass transfer is mainly driven by convection, rather than diffusion, due to the absence of mesopores [47, 48]. The fact that the solvent is forced to pass through the macropores of the polymer due to pressure leads to faster convective mass transfer, compared to the slow diffusion process into the stagnant pore liquid that is present in porous beads-packed columns. It was previously demonstrated that polymeric stationary phases led to superior performance over silica-based materials in the reversed-phase analysis of very large proteins (M_r >50 kDa) [49]. Porous polymer monoliths were recently employed at temperatures that exceeded 200°C for the separation of a range of simple solutes using pure water as the mobile phase [50]. These promising results suggested that polymer monoliths were suitable supports for the analysis of proteins at high temperatures (≥ 80°C), allowing i) the use of viscous organic modifiers such as 2-propanol, ethanol or methanol, ii) the use of extended column lengths and iii) the use of elevated linear velocities for fast separations. Finally, 5 cm-long poly(styrene-co-divinylbenzene) monolithic column of 1 mm I.D. were employed for the separation of intact proteins [51]. Using short capillary poly(styrene-co-divinylbenzene) monolith coupled to an LTQ Orbitrap XL mass spectrometer, a limit of detection in the low femtomol range was achieved after injecting a mixture of nine proteins with molecular weights ranging from 5.7 to 150 kDa [52]. It was shown that long (25 cm) monolithic columns with optimized morphologies could produce a peak capacity of 620 for the separation of intact proteins, by applying a 120 min-long gradient separation [53]. Monolithic capillary supports (200 µm I.D.) were also prepared for proteins and peptides separations by the polymerization of methylstyrene and the use of 1,2-bis(*p*-vinylphenyl)ethane (MS/BVPE) as a cross-linker in the presence of inert diluents [54].

Using short monolithic columns, or tube layers, instead of long columns, it is possible to achieve fast, high-resolution separations at low back-pressures [55-57]. Monolithic short columns can be used for rapid high-resolution compound screening, method screening, QC testing, as well as method and process development. Separations can be ac-

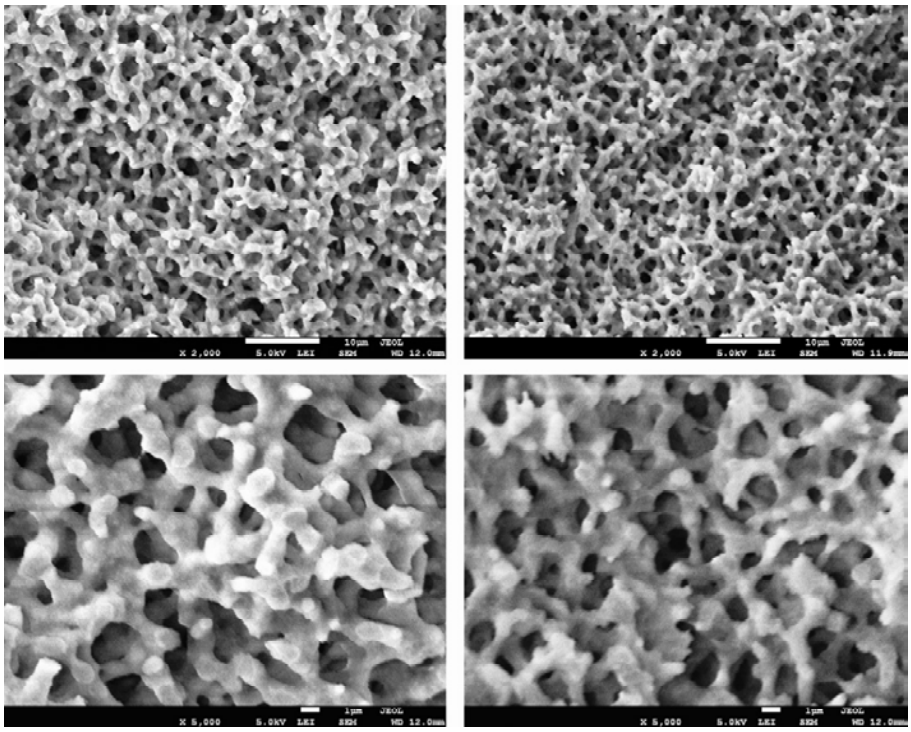

Fig. (2). SEM images of bare-silica rods (4.6 mm × 150 mm) from the 1st (left) and 2nd (right) Chromolith generation at 2000:1 (top) and 5000:1 (bottom) magnification. Reprinted from [2] with permission.

complished in seconds or minutes. This approach is well suited for process chromatography. Optimized methods are portable and can be transferred from research to production, given the consistent chromatographic performance of disk and tube monolithic columns. This technology can also be used for enzyme immobilization, bioconversions, or solid phase synthesis of peptides.

Protein analysis (using either specific protein quantitation by methods such as HPLC and immunoassays or structural analysis by methods such as LC-MS) usually requires significant sample preparation, including quantitative purification of the target protein from complex sample matrices and potentially enzymatic treatment or labeling. Now, several platforms for high-throughput microchromatography are available and capable of running around 100 small volume samples in parallel [58].

2.2. UHPLC

The technological approach of decreasing particle size in the packing material is a good way to enable faster separation, as the plate height (HETP) becomes very small and remains particularly low even under a higher flow-rate range, when small particles are employed.

On very fine particles (sub-2 μm), due to the narrow peaks, sensitivity and separation are improved at the cost of pressure. Knox and Saleem were the first to discuss the compromise between speed (pressure drop) and efficiency [59]. Halasz *et al.* demonstrated theoretically that the fastest HPLC separations could be obtained by employing the smallest particles [60]. It was also known that the minimum analysis time that could be achieved for a given separation was limited by the pressure limit of the HPLC system or the mechanical stability of the column bead. Later, Poppe confirmed that columns made with very fine particles are mainly useful for fast separations [61]. (Fig. **3**) presents some so-called Poppe plots of 1.4 - 7 μm particles to show the compromise between fast and high resolution separations. On the y axis, the plate-time (t_0/N that corresponds to the maximal achievable plate number in certain analysis time, or the minimal analysis time to achieve a given theoretical plate number) is plotted against the plate numbers (N) that are required for a given separation. Smaller particles outperform the larger ones (provide lower t_0/N) in the range of low plate numbers (left hand side of the plot) while larger particles offer faster separations in the high plate number region (right

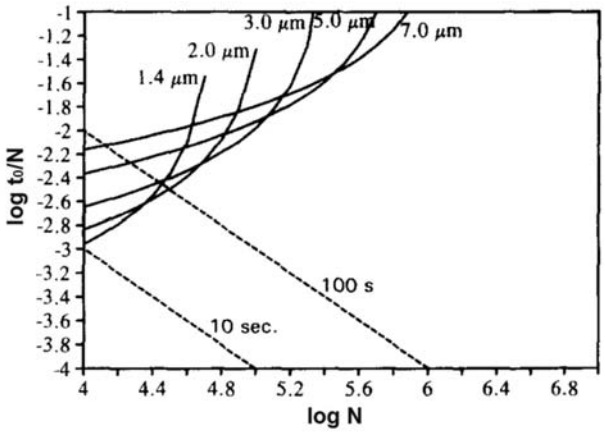

Fig. (3). Plot of plate time (t_0/N that corresponds to the maximal achievable plate number in certain analysis time or the minimal analysis time to achieve a given theoretical plate number) vs. required plate number in HPLC with various particle sizes. Reprinted from [61] with permission.

hand side of the plot). To overcome the pressure limitations of modern HPLC, the groups of Jorgenson [62, 63] and Lee [64] constructed dedicated instrumentation and columns to allow analysis at very high pressures. New nomenclatures have come with the term ultrahigh-pressure liquid chromatography, ultrahigh-performance liquid chromatography or very high-pressure liquid chromatography (UHPLC, UPLC, VHPLC or vHPLC). This term was employed to describe the higher backpressure requirement. The first ultra-high pressure system and commercial column packed with porous 1.7 μm hybrid silica particles were released in 2004. The new hardware was able to work up to 1000 bar (15 000 psi) and the system was called ultra performance liquid chromatography (UPLC™). Since then, several UHPLC systems have been commercialized that can work up to 1200-1300 bar (18 000 - 19 500 psi) and numerous sub-2 μm materials are available. With such innovative stationary phase technology, the throughput can be theoretically increased by 8-9 fold, while maintaining a similar separation efficiency compared to the conventional 5 μm packing [65]. However, the back-pressure generated by small particles could be prohibitive for conventional HPLC systems, as it is proportional to the square of reciprocal particle size, according to the Darcy's law [66]. Nowadays, a wide variety of columns packed with porous sub-2 μm particles (more than 100 different columns packed with 1.5 - 2 μm particles, from about 15 providers) as well as instruments (about 20 different systems with upper pressure limit comprised between 600 and 1,300 bar) are available on the market [3]. Commercially available columns packed with sub-2 μm fully porous particles were applied with great success in pharmaceutical, biomedical and environmental analysis in the past few years [67-74]. Several studies proved the excellent efficiency of sub-1 μm particles, however they are not so widespread because of the very high pressure generated and the difficulties to pack column [75-79]. It seems that currently the optimum particle size is somewhere between 1 and 2 μm. Particles smaller than 1 μm require a new generation of instrument and probably will be realized only in the future.

When working at very high pressure, a critical aspect is the effect of frictional heating, causing significant temperature gradients within the columns under very high pressures (ΔP > 400 bar). The radial temperature gradient, due to the heat dissipation at the column wall, can cause significant loss in plate count [80-82].

On the other hand, the smaller the particle diameter, the greater the difficulty in preparing a well-packed column bed is. Some reasons are the particle aggregation, frits blockage, or particle fracture, occurring when high pressure is required to pack sub-2 μm particles [83]. This is the main reason why the expected efficiency of columns packed with very fine particles is generally lower than the theory. The loss in efficiency with sub-2 μm particles has been a topic of interest, which has raised many possible explanations, including frictional heating [75, 84, 85], and radially inhomogeneous packing density [86]. The contribution from frictional heating does not explain the higher HETP value for 1.0 μm particles with pressures exceeding 6000 bar [86]. The packing is demonstrated to be inhomogeneous, but even accounting for this, still gives an excessively large value of the mass transfer resistance term [86]. Mobile phase compressibility has recently been shown to have an impact in increasing mass transfer resistance, in a study where pressure was increased from 2500 to 6300 bar [87], but it has yet not been explored at more reasonable pressures used in current commercial instruments. Beside the heat effects of high pressure, the mobile phase density, viscosity, diffusion coefficients, equilibrium constants, retention factors, efficiency parameters also mainly depend on the pressure. The compressibility of liquids can be considered as constant at constant temperature in the range of pressures used in conventional HPLC (below 400 bar). However, above 400 bar, the compressibility depends on the pressure. At 1000 bar the specific volume of common solvents decreases to approximately 90-98%, at 2500 bar to 84-93% than it is at atmospheric pressure [88]. The viscosity of liquids also increases with increasing pressure. At 1000 bar, the viscosity of organic solvents increases by a factor of 1.4 - 2 compared to atmospheric pressure [89, 89]. As a conclusion, the possible adverse effects of very high pressure and inhomogeneous packing density of narrow bore columns packed with very fine particles provide a compromised efficiency for the most recent columns. Summing up these effects, the overall efficiency of small narrow bore columns packed with various very fine particles (in the range of 1.5 - 2.1 μm) is nearly the same [74]. As example, it was showed that 1.7 μm Waters Acquity BEH C18, 1.9 μm Restek Pinnacle C18 and 2.1 μm Fortis C18 columns gave the same efficiency and separation speed when 5 cm long narrow bore columns were applied for the separation of steroids [74].

To conclude on UHPLC, by using current sub-2 μm fully porous materials and UHPLC instrumentations, the typical analysis time could be reduced to 1-5 minutes interval without loss of resolution and sensitivity. Previously, by using 15-25 cm long columns packed with 3 - 5 μm particles, the analysis time ranged generally between 10 and 60 minutes. In spite of the several complications of ultra-high pressure, UHPLC is currently the most widespread and popular technique in LC, especially in the pharmaceutical analysis field.

Nonporous and porous particles are the two major types of spherical packing materials that have been used for fast and ultrahigh-pressure separations [90]. The major difference between porous and nonporous particles is that porous particles have a resistance to mass transfer contribution from the stagnant mobile phase in the pores. Nonporous particles can provide lower mass transfer resistance and higher efficiency than porous particles but porous particles have greater surface areas and can provide much higher sample loading capacity. Seifar *et al.* estimated a 50-fold better sample capacity for porous particles compared to nonporous particles of the same size [91]. According to Wu *et al*. the loading capacity for 1.7 μm Acquity C18 porous particles is approximately 16.5 times larger than for Micra C18 nonporous 1.5 μm particles [92]. Another issue is the very low retention on nonporous particles, compared to totally porous ones. Probably these are the two main reasons why non-porous materials have not become so widespread until now.

2.3. Superficially Porous Particles

Superficially porous particles are made of a solid, nonporous core surrounded by a shell of a porous material that has properties similar to those of the fully porous

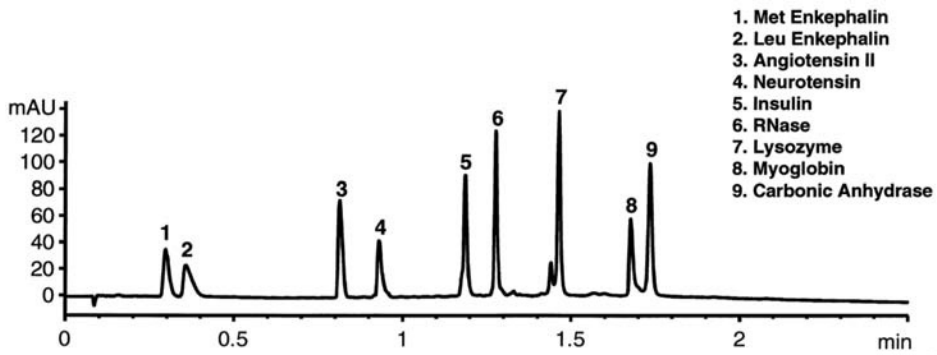

Fig. (4). Rapid separation of protein mixture on Poroshell 300 SB-C column: 75x2.1 mm, 5-µm, 0.25-µm porous shell. Reprinted from [96] with permission.

phases. This particle structure is also called shell, fused-core™, core-shell™, partially porous or pellicular.

The initial intend of applying superficially porous particles was to efficiently analyze large biomolecules. The rationale behind this concept was to improve the column efficiency by shortening the diffusion path that analyte molecules must travel and, in doing so, to improve their mass transfer kinetics [5, 93]. The concept of shell-type (pellicular) particles was imagined by Horváth and coworkers in the late 60s [94]. They prepared 50 µm pellicular particles with very thin active porous layer and applied this material for protein separation. Later on, Kirkland demonstrated that 30-40 µm diameter superficially porous packing provide much faster separations, compared with the large fully porous particles used earlier [95]. Several brands of superficially porous particles were developed and became popular in the 1970s. However, the major improvements in the manufacturing of high-quality, fully porous particles, that took place at this time, particularly by making them finer and more homogeneous, hampered the success of shell particles, which eventually disappeared. In 2000, the structure of those particles was reconsidered: the core diameter was reduced and the thickness of active layer was cut to 0.5 µm, while the whole particle diameter was only 5 µm [96]. (Fig. **4**) presents the fast and efficient separation of a protein mixture on 5 µm superficially porous particles. In 2007, a new revolution started with the commercialization of the first sub-3 µm superficially porous particle [97]. This material was made of 2.7 µm particles that consist of a 1.7 µm solid core surrounded by a 0.5 µm thick shell of porous silica. This particle structure/morphology seems to be a good compromise between fully porous and non-porous materials, as it manifests the advantages of porous and some benefit of nonporous particles. This design solved the problem of the low loading capacity of columns packed with the large, early pellicular particles because 75% of the volume of these particles is porous. Since then, several vendors commercialized different types of superficially porous particles. Now shell packing materials are commercially available in various diameters (5 µm, 4 µm, 3.6 µm, 2.7 µm, 2.6 µm 1.7 µm and 1.3 µm), with different shell thickness (0.6 µm, 0.5 µm, 0.35 µm, 0.25 µm, 0.23 µm, 0.20 µm and 0.15 µm).

With this current state-of-the-art column technology, very high efficiencies (comparable with sub-2 µm fully porous particles) can be attained at moderate back pressure. Therefore, this type of columns can provide faster separations for both small and macromolecules than the columns of same dimensions packed with fully porous particles. The new generation of superficially porous particles became very popular in pharmaceutical, biomedical, food and environmental analysis in the last few years and allows faster and more efficient separations [98, 99].

The separation power of shell particles increases with reducing shell thickness [100]. The smaller the diffusivity of the solutes, the larger the increase of the separation power is, compared to that of fully porous particles. On one hand, the theory suggests that the thickness of the porous layer should be decreased drastically to increase the separation efficiency of the columns for large molecular size compounds. On the other hand, there is a strict limitation to the decrease in the thickness of porous layer, since reducing the shell thickness decreases markedly the retention, the loadability of the column, making column overload easily, broadening the bands and decreasing the separation efficiency [100]. Therefore, the optimum shell thickness in reality is likely to be a compromise between efficiency, sample loading capacity and analyte retention, and is strongly sample dependent. Overload problems are likely to be more severe for both sub-2 µm porous as well as shell particles, due to the very high efficiencies produced by both types of columns [101].

The initial idea of preparing shell particles (pellicular) was to increase the column efficiency by reducing the mass transfer resistance across the particles. However, it appears from recent studies that transparticle mass transfer resistance is far from being the dominant contribution to band broadening in HPLC [5, 93].

The presence of a solid core inside the particles has a direct consequence on the longitudinal diffusion term (the B term in van Deemter equation), since it decreases this contribution to the plate height by about 20 %, when the ratio of the core to the particle diameter is ρ=0.63 (Halo, Ascentis Express…) and about 30 % when ρ=0.73 (Kinetex) [5, 84]. However, the reduced internal porosity of the superficially porous particles brings a limited improvement in their efficiency. Experimentally, it was implied that the solid core reduced the B term not more than 34 % in comparison with fully porous particle [97]. As a conclusion, it can be stated that recent superficially porous particles manifest a gain of approximately 20-30% in the longitudinal diffusion. This causes at best a gain of a ~10 % increase in the total column efficiency compared to that of columns packed with fully porous particles.

According to the theory, the intraparticle diffusivity (part of the C term in van Deemter equation) depends on the ratio of the diameter of the solid core to that of the particle in a superficially porous particle. As this ratio increases, the mass transfer kinetics becomes faster through the particles. Similarly, the external mass transfer also depends on the structure of the particles. According to some recent experimental measurements, the mass transfer kinetic is mostly accounted for by the external film mass transfer resistance across the thin layer of the mobile phase surrounding the external surface area of the particles [93]. This suggests that the initial idea of preparing shell or superficially porous particles with the purpose to increase the column efficiency by reducing the mass transfer resistance across the particles might provide only modest practical gains for the separation of low to medium molecular weight compounds [93].

According to several experimental results, the eddy dispersion term (A term in van Deemter equation) of the columns packed with superficially porous particles is significantly smaller (~30-40%) than that of the column packed with fully porous particles [5, 93]. This improvement was not predicted by theory since the eddy dispersion should not depend on particle structure. It is still unclear whether this significant improvement in efficiency is due to the particle size distribution (PSD) of superficially porous particles, which is significantly narrower than that of fully porous particles. Some recent studies, focusing on particles with a different design such as the superficially porous particles, have suggested that particles displaying a very narrow PSD can lead to unprecedented low minimal plate heights [5, 93]. It is however uncertain whether this finding can be purely related, because there are also other factors that might influence the packing quality. Superficially porous particles have a higher density and some of them are rougher than fully porous particles [5, 93]. This might also have an influence on the achieved packing quality, apart from the PSD.

To conclude on the efficiency of superficially porous particles, the success of these materials in the separation of small molecules is not primarily a result of the decrease in the C term, as it is often claimed in commercial brochures [93]. More importantly, the exceptional performance of columns packed with superficially porous particles is probably caused by the important reduction of the eddy dispersion term.

The use of shell particles has dramatically improved chromatographic peak efficiencies over fully porous particles in reversed phase chromatography [102-104] as well as in hydrophilic interaction (HILIC) separation mode, both in gradient and in isocratic elution mode. Shell particles are a relatively recent trend in chromatographic separation, but several pharmaceutical-bioanalytical [105-118], food analytical [119-129], environmental [130-134] and multidimensional [135-137] separations can be found in the literature. Very recently, new commercially available wide-pore stationary phases demonstrate exceptional efficiency for protein separations [138-140].

2.4. Future Perspectives for Column Technology

Future progress might be expected in the analysis of large biomolecules during the next decade [130]. Although faster chromatography of small molecules could remain possible with a generation of very fine (*e.g.* 1 μm particles), it also necessitates new instrument design with very high pressure capability (1500 - 3000 bar) and low dispersion (< 1 μL^2). The negative impact of frictional heating on column efficiency could be solved by applying small columns I.D. (*e.g.* 1.0 mm or less) or by replacing the conventional shell particles with a silica core in 2.1 mm I.D. narrow-bore columns with a particles made of a core having a larger thermal conductivity around 50 W/m/K, such as alumina or an equivalent material [141].

An alternative to packed and monolithic columns may be the so-called porous-layer open-tubular-type (PLOT) columns, which have been shown to provide very high column efficiency compared to conventional packed columns [142]. The kinetic performance of PLOT columns is very promising, but the use of the narrow PLOT columns initially led to several technical problems, such as very high pressures, detector coupling difficulties and extra-column band broadening [143, 144]. PLOT columns have increased in popularity after they were successfully coupled to nanospray-MS [145]. Recently, 10 μm inner diameter PLOT polystyrene-divinylbenzene (PS-DVB) columns have been designed and used for high-resolution LC-MS separations of peptides [146, 147].

Silica colloidal crystals also seem to be good alternatives in highly efficient separations [148]. Studies have shown that the eddy dispersion in columns increases as the particle size becomes smaller indicating that efficiency is limited by non uniform packing [149]. This arises from a radial distribution of packing densities in the cylindrical tube, giving a radial distribution of velocities that broadens peaks [150-153]. Recent experiments bear this out: nonporous 1.0 μm particles in capillaries of varying diameter show that the A term is primarily due to the radial heterogeneity of the packing. With silica colloidal crystals better packing is feasible because they form face centered cubic lattices [154]. Slabs of silica colloidal crystals have been used for separations of small molecules and for DNA and protein sieving [155, 156]. The plate heights were well above 1 μm yet these should achieve sub-micrometer plate heights by virtue of the crystalline packing [155, 156].

Recently, a new concept in chromatography columns, which is termed as "parallel segmented flow chromatography" (PSF) was introduced [157-159]. The concept behind these types of columns rests on management of the flow upon exit from the column. The flow from a PSF column elutes from two separated radial zones: The central flow region of the bed, which is separated from the peripheral or wall flow region. This is achieved by using an annular frit design, and a multi-channel end fitting. An impervious ring divides the outlet frit into two parts; an inner portion of frit channels flow from the central region of the bed out a central exit port on the outlet fitting, while an outer ring of frit channels solvent that migrates down the wall region out the peripheral ports on the outlet fitting. In essence, this design effectively establishes within the larger format column, a 'virtual'column having a narrower diameter, the dimensions of which are related to the volumetric ratio of flow exiting the column through the centre, relative to the flow exiting through the peripheral zones [160]. These PSF columns can

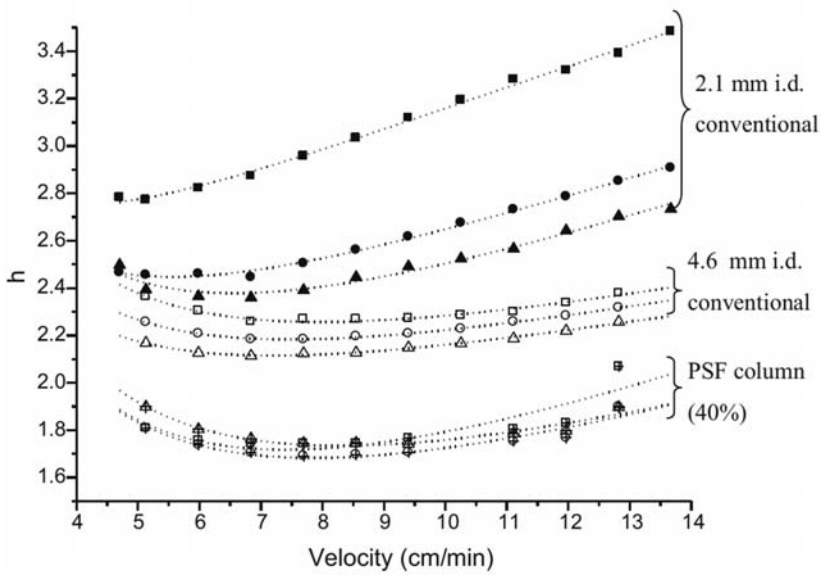

Fig. (5). Plots of reduced plate height (h) versus flow velocity obtained on the 4.6 and 2.1 mm i.d. conventional columns and a 4.6 mm i.d. parallel segmented flow column with 40% flow through column centre. Reprinted from [160] with permission.

provide significantly better efficiency (lower plate heights) than "conventional" columns. (Fig. **5**) shows the reduced plate heights versus flow rate for different columns including PSF column.

In the late 1990s, the group of Fred Regnier proposed the use of micro-fabricated pillar array columns as a novel support for liquid chromatography [161-163]. Pillar array columns have the advantage that the pillars in the bed can be arranged in a perfectly ordered conformation. The large possible gain (about a factor of 2-3) in efficiency that can be obtained when switching from a randomly packed column to a perfectly ordered array of pillars could be quantified using computational fluid dynamics computations [164]. This gain in efficiency can be attributed to the high degree of flow-path homogeneity that is introduced by using a perfectly ordered array of pillars as a support structure [165]. Apart from an improved efficiency, pillar array columns can also be designed so that the flow resistance can be drastically reduced compared to that in a packed bed of spheres [166]. Pillar array columns seem to be promising; several groups are currently working in the field.

3. THE IMPORTANCE OF INSTRUMENTATION IN FAST LIQUID CHROMATOGRAPHY

The success of highly efficient separations depends on both column efficiency and on preserving the efficiency by minimizing instrument-induced extra-column band spreading. Each improvement in column technology requires considerable progress in instrument design and manufacturing [11]. Extra-column band spreading affects the measured performance of columns, especially for columns with an internal diameter smaller than the standard 4.6 mm I.D. [12].

3.1. Extra-column Band Broadening

Extra-column band broadening effects are taking place in the "external" instrument (extra-column) volumes, including the injector system, the connector tubing, and the detector cell [10]. An additional contribution is related to time-based effects, namely on the time constant and sampling rate of the detector. Extra-column volume is a physical characteristic of the instrument and can be easily evaluated in terms of volume unit (*e.g.*, μL). On the other hand, the extra-column peak variance accounts for the sample dispersion before and after the column [10]. Extra-column dispersion (or variance) can be calculated in time square or volume square units. The extra-column peak variance is a function of the flow rate, the sample diffusion coefficient, the mobile phase viscosity, the temperature, and the injected amount. Theoretically, the different peak variances coming from the system and from the column itself are additive. The experimentally measured peak variance (σ^2_{total}) - that is sometimes called "apparent" peak variance - is the sum of the extra-column peak variance occurring before the column ($\sigma^2_{ec,b}$), the variance inside the column (σ^2_{col}), and the extra-column peak variance generated after the column ($\sigma^2_{ec,a}$). (Fig. **6**) shows a schematic view of the system components that contribute to band broadening.

Although the extra-column volume of modern UHPLC systems is significantly reduced, the column dimensions (*i.e.*, the column void volume) is also decreased, sometimes to a greater extent than the extra-column volume. Thus, the loss in apparent column efficiency can also be significant in UHPLC conditions [166]. The commercially available LC systems can be classified into three groups, (i) optimized systems for fast separation with very low dispersion (σ^2_{ec} <10 μL^2), (ii) hybrid LC systems recommended by the vendors for both fast and conventional separations (σ^2_{ec} =10-50 μL^2), and (iii) conventional LC systems with an extra column variance over 50 μL^2 [167]. The variance of Waters Acquity UPLC system was ca. 6 - 7 μL^2, the Agilent 1290 Infinity LC system performed ca. 4 - 8 μL^2, the variance of the standard Agilent 1200 Infinity and Shimadzu Nexera systems were measured at ca. 13 - 20 μL^2, the variance of the standard Perkin Elmer Flexar system was determined between 18 - 26 μL^2, the variance of the standard Agilent 1100 LC system was reported ca. 50 - 80 μL^2 while the conven-

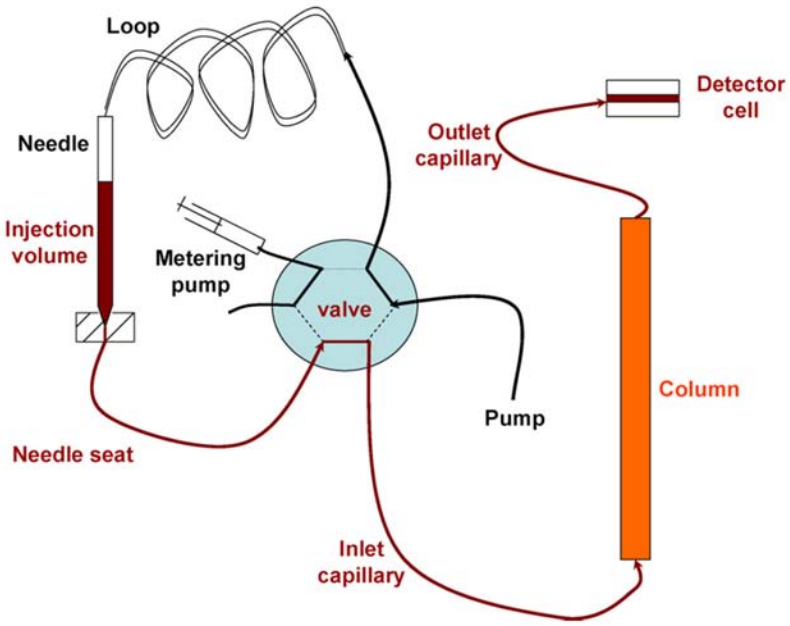

Fig. (6). Schematic view of LC system volumes. Reprinted from [11] with permission.

tional Merck LaChrom and Waters Alliance 2695 systems possess system variance around 100 - 200 μL^2 [167,168]. The recently launched Waters Acquity I-Class system was characterized in [169] and its system variance was measured between 0.5 - 4 μL^2. As expected, the difference between system variances of current instruments could be quite important.

Several attempts were made to minimize the system dispersion of existing instruments by changing the tubes, detector cell, or injection system. Wu and Bradley experimentally showed the impact of tubing diameter on the system variance [170]. The authors demonstrated that the extra-column system variance of an Acquity UPLC system can be decreased down to ca. 2 μL^2 by replacing the tubes with 0.0635 mm instead of 0.127 mm I.D. capillaries. Guiochon *et al.* demonstrated that the system variance of conventional HPLC instruments (*e.g.*, Agilent 1100 LC system) can be significantly decreased by changing the capillary tubes, needle seat, and detector cell [168]. After the optimization, an Agilent 1100 system performed σ^2_{ec} of ca. 5 - 10 μL^2. Similarly, Alexander *et al.* showed the optimization of a Waters Alliance 2695 system [171]. In this study, the extra-column system variance of the conventional system was reduced down to 15 μL^2 by changing the tubes and the injector system.

To further increase the observed column efficiency, Farkas *et al.* introduced a novel injection technique called Performance Optimizing Injection Sequence (POISe) or isocratic focusing [172]. The impact of the injection system on the observed chromatographic performance was eliminated. The POISe technique involves injecting a defined volume of weak solvent along with the sample to increase retention factors during sample loading. With this injection technique, 10-20% decrease in peak width was observed in isocratic mode for weakly retained solutes.

In conclusion, it is clear that further improvements in instrument design (smaller dispersion) are necessary to take the full advantage of the most recent very efficient small columns [167]. At the moment, it is not possible to fully use the potential of these small columns. The loss in column efficiency can reach 20 - 30% and even 30 - 80 % when using state-of-the-art 2.1 and 1.0 mm I.D. columns, respectively, with commercially available optimized UHPLC systems. Therefore, the chromatographers are suggested to optimize their system to keep the efficiency of their columns when doing highly efficient fast separations.

3.2. Gradient Delay Volume

Today, most LC separations performed in both industrial and academic laboratories are carried out in the gradient elution mode. Various theories of gradient elution were proposed [173-180] [1-3]. However, these theories are only valid as long as the system dwell volume (V_d), also known as gradient delay volume, is taken into account. The V_d of a system represents the volume from the mixing point of solvents to the head of the analytical column. Indeed, after the gradient has begun, a delay is observed until the selected proportion of solvent reaches the column inlet [181]. The sample is thus subjected to an undesired additional isocratic migration in the initial mobile phase composition. Two types of pumping systems are commercially available for HPLC operations, (i) high-pressure mixing systems, where the dwell volume comprises the mixing chamber, the connecting tubing and the autosampler loop; and (ii) low-pressure mixing systems, combining the solvents upstream from the pump, where additional tubing as well as volume of the pump head is added to the components of the high-pressure mixing system [182]. In the case of conventional HPLC systems, typical dwell volumes are in the range of 0.5 - 2 mL and 1 - 5 mL for high-pressure and low-pressure mixing systems, respectively. In comparison with conventional HPLC instruments UHPLC systems have dwell volumes of ca. 300 - 400 µL, with the best UHPLC systems having V_d of ca. 100 µL, and up to ca. 1 mL for some UHPLC instruments. Two

main concerns related to large system dwell volume are observed when performing fast separations in LC, including (i) unreliable gradient method transfer between columns of different geometries, and (ii) ultra-fast separations, which require more time than expected.

The problem observed during gradient method transfer has become a topic of particular interest since the introduction of phases containing fully porous sub-2 µm particles. Indeed, a significant number of scientific papers deal with the issue of method transfer from conventional HPLC to UHPLC. In the case of pharmaceutical analysis, analytical methods may be developed with UHPLC system in R&D laboratories since it allows for a significant decrease of method development timeframe. However, QC laboratories are often equipped with conventional LC instruments and the developed UHPLC methods have thus to be transferred to HPLC. The methodology for transferring a gradient method from one column geometry to another one is well-established. Basic rules have to be applied to scale the injected volume, mobile phase flow rate, gradient slope, and isocratic step duration [4-7, 181,183]. However, the system dwell volume also needs to be accounted during this transfer since it may differ between LC systems. Moreover, the extra isocratic step created at the starting of the chromatogram may also be different and could result in retention time variations, affecting the resolution during method transfer. To overcome this issue, the ratio of system dwell time on column dead time (t_d/t_0) must be ideally held constant, while changing column dimensions, particle size, or mobile phase flow rate [184]. Another issue related to large system dwell volume is the time spent for the additional isocratic hold produced at the beginning of the gradient which could be a severe issue when performing ultra-fast separations on micro or narrow-bore columns.

In conclusion, it is straightforward to determine which type of column geometry can be employed on which instrument, after system characterization. In theory, smaller V_d are highly recommended for fast and ultra-fast analysis, but some concerns have been reported with various UHPLC systems equipped with small mixer. Indeed, a problem of excessive blending noise has been described, caused by inadequate mixing of mobile phases from the binary pumps [185, 186]. This blending noise could be dependent on the pump design (*i.e.*, piston column, mixer volume, and presence of a damper). To avoid this issue, larger mixing volumes can be used, but at the expense of increasing dwell time. Many manufacturers thus offer large mixer with volumes comprised between 300 and 500 µL.

3.3. Upper Pressure Limit of Current Instrument

Nowadays, columns packed with fully porous sub-2 µm and core-shell sub-3 µm particles are very popular and are routinely applied in several pharmaceutical companies [187]. However, to obtain all the benefits of such columns, a chromatographic system possessing an extended upper pressure limit should be employed. The pressure generated in LC is proportional to the reciprocal of the particle size square. In this context, a large variety of chromatographic devices compatible with pressures in the range 600-1,300 bar have been developed in parallel to these new column technologies. Regarding the instrumentation, it remains challenging to consistently pump the mobile phase and introduce the sample in a reliable way under very high pressure (ca. 1,000 bar). Moreover, there are many design trade-offs that are required to balance system features (robustness, sensitivity, and safety) and performance characteristics.

Today, there is a large choice of column packed with fully porous sub-2 µm particles which are compatible with pressures up to 1,200 bar [3, 10, 11, 188]. These stationary phases consist of (i) hybrid silica material prepared from two monomers, *i.e.*, tetraethoxysilane and bis(triethoxysilyl) ethane which incorporates ethylene bridges (the high degree of cross-linking ensures strong mechanical and hydrolytic stability) [189], or (ii) classical silica simply packed under much higher pressure conditions. Because the commercial UHPLC systems and columns need to be routinely used, more extreme pressure conditions cannot be envisaged. Another reason for the upper pressure limit of current system is related to the frictional heating phenomenon, which is observed with columns packed with very fine particles operating at high mobile phase velocities, thus generating high pressure drop (as discussed in section 2.2.). Two solutions can be applied to limit this phenomenon, (i) reducing the column inner diameter, which remains difficult because of the strong contribution of extra-column variance to peak broadening, and (ii) reducing the backpressure inside the column [85]. Because current UHPLC instrumentations are hardly or even not compatible with columns of less than 1 mm I.D., the only solution to alleviate the frictional heating is to set the upper pressure limit of instruments at a reasonable value.

Based on several reported data, working with a chromatographic system possessing an elevated upper pressure limit is particularly relevant for high-resolution analysis (high plate count). Two recent papers have experimentally demonstrated this statement for the analysis of small molecules and peptides [190, 191]. Prototype columns packed with core-shell 2.6 µm particles were tested at a pressure up to 1,200 bar under gradient conditions. As expected, the analysis time was more than 2-fold reduced at 1,200 bar compared to 600 bar, using 300 mm column length, while the peak capacity remains identical between both conditions. Moreover, it was also possible to use a 450 mm column length at 1,200 bar to further increase peak capacity by ca. 30%.

4. ALTERNATIVE SELECTIVITY FOR POLAR AND IONISABLE COMPOUNDS WITH HILIC

4.1. Description of HILIC Mode

The term HILIC has been originally coined in 1990 by Alpert [192] to describe an innovative strategy allowing the retention of polar compounds. Indeed, these compounds are always problematic in RPLC, due to the absence of possible hydrophobic interactions. Alternatively, normal phase liquid chromatography (NPLC), ion-exchange chromatography (IEX) or ion-pairing chromatography can be employed with hydrophilic compounds but these approaches present some obvious limitations including a weak compatibility with mass spectrometry, low kinetic performance leading to broad peaks, the need to work with toxic solvents (NPLC) or the

on-column memory effects when using ion pairing reagents. For all these reasons, HILIC appears as a valuable alternative for the analysis of a large variety of biologically active substances such as pharmaceutical compounds and their impurities, amino acids, peptides, neurotransmitters, carbohydrates, oligosaccharides, nucleotides and nucleosides… Nevertheless, before 2003 the attention paid to HILIC was limited as the number of papers published with the keyword HILIC was less than 10 *per* year. From 2004 to 2007, this number increased to about 20 - 30 papers *per* year. The real breakthrough came in 2007 - 2008 and the number of papers in the period 2007-2012 growth up to 150-300 papers *per* year. There are two outstanding research papers published in 2006 and 2007 that explain the sudden interest for HILIC. In their review paper published in 2006 [25], Knut and Irgum explain in details the interaction mechanism in HILIC and provide detailed overview of available stationary phases and possible applications. In his research paper published in 2007, McCalley [193] demonstrated that HILIC was indeed interesting for the analysis of polar compounds but can also be considered as a viable alternative to RPLC for the separation of ionisable compounds. This extends significantly the possibilities offered by HILIC.

HILIC is characterized by a polar stationary phase such as bare silica, or silica derivatized with polar functional group including amide, diol, amino, cyano, sulfoalkylbetaine, cyclodextrin, polysuccinimide and a mixture of aqueous buffer and organic solvent as mobile phase. In terms of stationary phases, there is no versatile material such as the C18 in RPLC [24]. Among the polar chromatographic surfaces, the bare silica remains the most widely used (around 35% of the reported applications), followed by the zwitterionic sulfoalkylbetaine phase which has been used for 25% of the published works [194]. Amide and diol are also reference phases, each employed in about 15% of the cases. Finally, with a total of 4 columns it is possible to resolve a significant number of problems with HILIC. Some more exotic phases are also available for specific separations. Regarding mobile phase, it should ideally contain at least 60-70% v/v of an aprotic solvent such as acetonitrile or acetone. To summarize, the stationary phase employed in HILIC is similar to that used in NPLC, while the mobile phase is comparable to that commonly encountered in RPLC, but with a higher proportion of organic solvent.

The interaction mechanism has received a lot of attention during the last few years [195, 196]. This is obviously related to the diversity of stationary phase chemistries that can be employed in HILIC. It is now clearly established that a multimodal retention mechanism takes place, involving i) hydrophilic partitioning between a water enriched layer at the surface of the stationary phase and the less polar mobile phase, containing higher proportion of organic solvent, ii) ion exchange between a charged analyte and the charges available on the stationary phase (similarly to IEX), and iii) adsorption of analyte at the surface of the adsorbent via hydrogen bonds and dipole-dipole interactions (similarly to NPLC). The extend of these different mechanisms depends on the nature of the stationary phase, the analyzed compounds and the type of mobile phase (pH, nature of organic modifier, ionic strength).

4.2. Advantages and Limitations of HILIC

The main advantage of HILIC is related to its ability to retain the most polar compounds, difficult to analyze with other chromatographic modes such as RPLC, NPLC or IEX. In addition, HILIC does not necessitate the use of toxic solvents, or significant amounts of salt or ion pairing reagents. Besides hydrophilic compounds, HILIC can also be considered as a complementary alternative to RPLC for the analysis of ionisable analytes. Due to a very different retention mechanism involving strong ion exchange contribution, the separation appears to be quite orthogonal in HILIC vs. RPLC. Such behaviour has been reported for the analysis of small drugs and also for peptides [197, 198]. As example, (Fig. **7**) shows a separation of 9 peptides in RPLC and HILIC conditions. In this example, it is clear that the selectivity was completely different, as illustrated with peaks 5 and 6, eluted closely in RPLC and eluted as first and last peaks on the HILIC chromatogram. The same behaviour was also observed with peaks 8 and 9. Another major benefit of HILIC is the enhanced MS sensitivity with electrospray ionization mode [31, 199]. This is related to the more efficient desolvation and better spray stability in presence of large proportion of organic solvent in the mobile phase. The gain in sensitivity reported so far for HILIC-ESI-MS vs. RPLC-ESI/MS was comprised between 5 and 15, depending on the nature of the compounds, mobile phase composition and ionization source geometry. Thanks to the very low viscosity of HILIC mobile phase containing large proportion of organic solvent (in average 3-fold less than In RPLC), the generated backpressure remains systematically reasonable, allowing the use of longer columns, smaller particle sizes and higher mobile phase flow rates. A nice illustration has been published by McCalley who demonstrated the possibility to reach > 100'000 plates in less than 15 min under HILIC conditions, using the core-shell technology [200]. Lastly, HILIC can also be a valuable tool in bioanalysis due to the improvement of MS sensitivity but also to the better compatibility with SPE extracts. Indeed, SPE is usually performed with a large fraction of organic solvent that may be incompatible with the direct RPLC analysis. Then, a time-consuming evaporation/reconstitution step is added at the end of the extraction process. With HILIC separation, this high organic eluent can be directly injected into the column, thus increasing the throughput [201].

On the other hand, HILIC has also a number of limitations that need to be considered. HILIC is generally not as straightforward as RPLC, particularly for non-experienced users [202]. Even if it presents some obvious advantages over RPLC, there could still be reluctance in adopting this new technique. The main shortcoming of HILIC is related to the price and availability of acetonitrile, which could become a severe concern during shortage period. Some alternative solvents were investigated (*e.g.* acetone, isopropanol), but were found to be less appropriate from a separation or detection point of view. Another issue is the longer equilibration time required between successive gradient runs. This was attributed to the strong contribution of ion exchange mechanism, which is a slow process. In average, 2 to 3 times longer equilibrating time is required for HILIC vs. RPLC. Another challenge of HILIC arises from the accurate measurement

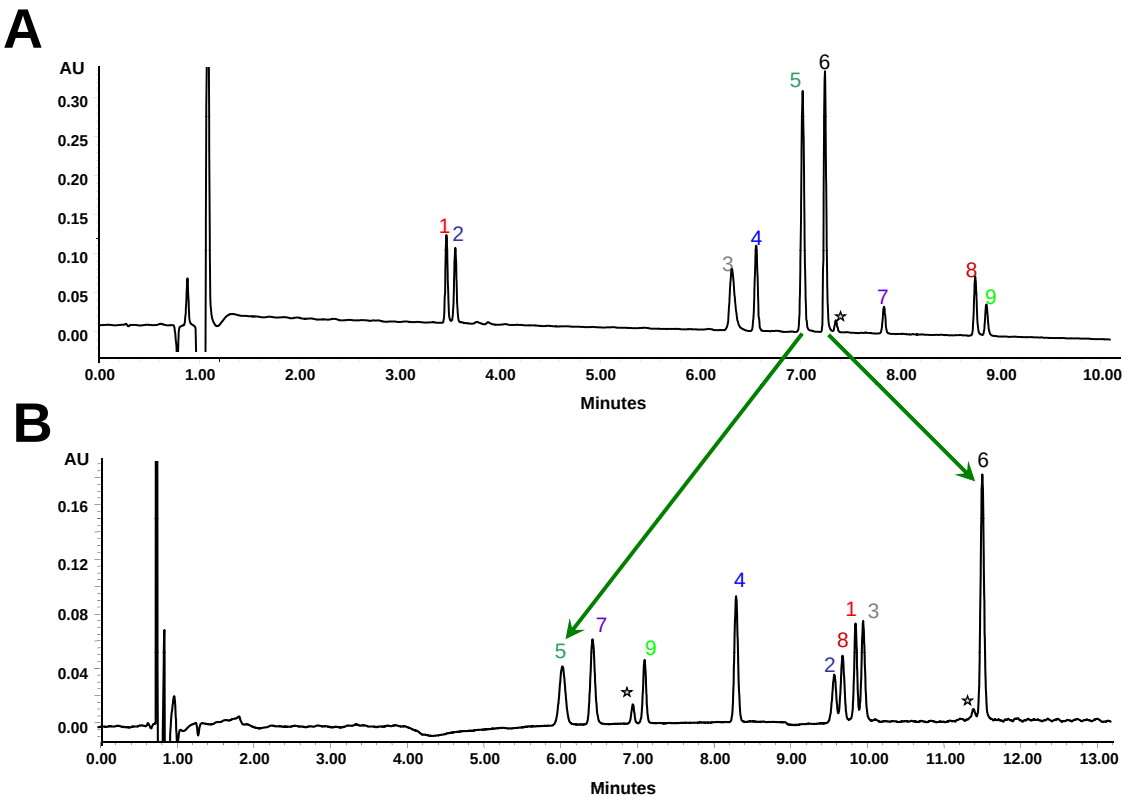

Fig. (7). Comparison of the chromatographic performance obtained by (A) RPLC *vs.* (B) HILIC for the analysis of a mixture of 9 model peptides with relative molecular masses comprised between 1 and 6 kDa. Conditions: (A) Column Acquity BEH C18 (2.1 mm id x 150 mm, 1.7 µm), flow rate of 400 µL/min, λ = 214 nm, volume injected = 5 µL, gradient profile: 10 to 90% ACN in 20 min with T = 30°C, (B) Column Acquity HILIC amide (2.1 mm id x 150 mm, 1.7 µm), flow rate of 500 µL/min, λ = 214 nm, volume injected = 5 µL, gradient profile: 90% ACN for 3 min, then 90 to 62% ACN in 9 min with T = 30°C. Reprinted from [198] with permission.

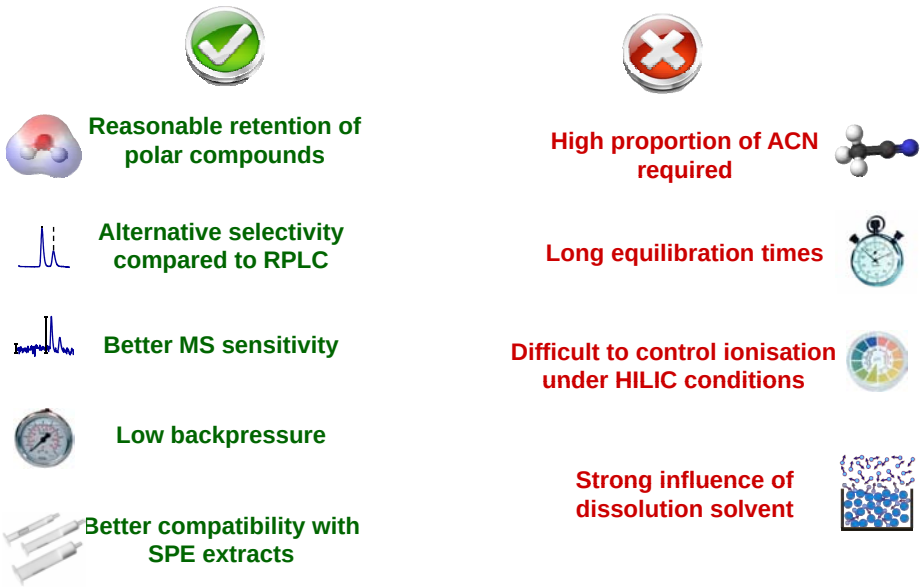

Fig. (8). Summary of advantages and limitations of HILIC *vs.* RPLC mode.

of mobile phase pH, analyte pKa and silanol pKa which cannot be easily determined in presence of large amounts of organic solvents (> 60% v/v ACN) [203]. In these conditions, it is thus difficult to know the ionisation state of the analyte. Then, the possible ionic interactions should be evaluated mostly in an empirical way instead of a rigorous

scientific way. Last but not least, peaks can appear more distorted (*i.e*. tailing, broadening, shouldering) in HILIC *vs.* RPLC. This behaviour can be explained mostly by the nature of the sample diluent which appears to have more impact on peak shape in HILIC *vs.* RPLC [198]. Fig. (**8**) summarized the main advantages and limitations of HILC compared to RPLC.

4.3. Current Trends in HILIC

Currently, there are two major trends in HILIC highlighted in the scientific literature.

First of all, the columns packed with sub-2 µm fully porous and core-shell sub-3 µm particles offer some very good performance in RPLC but are also available for HILIC operation, either under the form of bare silica or silica bonded with various polar groups. In RPLC, the main limitation of these two innovative approaches (particularly with sub-2 µm fully porous particles), is related to the important backpressure generated when percolating the mobile phase though a bed of very small particles [7]. In addition, the frictional heating effects occurring under high pressure may be detrimental for the separation as it could produce axial and longitudinal temperature gradients inside the column [82, 204]. As reported previously, the backpressure under HILIC conditions is always reasonable, due to the low viscosity of mobile phase containing between 60 and 95% v/v ACN. Consequently, columns packed with small particles are more compatible with old generation LC systems, provided that extra-column volume and dwell volume have been optimized for the column dimensions [10]. Moreover, the frictional heating is generally negligible in HILIC vs. RPLC. Based on these considerations, it is clear that columns packed with sub-2 µm fully porous and core-shell sub-3 µm particles have to be preferentially used in HILIC, due to obvious kinetic advantages [205]. The only restriction to their use is the limited choice of stationary phase chemistries. Currently, it is possible to find commercially available bare silica, hybrid silica, amide, amino, diol, zwitterionic and cyano bonding for sub-2 µm porous particles, while the core-shell sub-3 µm particles are only available under the form of bare silica and amide bonding. In a close future, the range of chemistries will probably be extended by providers.

Another topic of interest for HILIC is the development of screening methodology, similarly to what is commonly done in RPLC for method development. For this purpose, it is required to find out the most orthogonal conditions and the parameters which have the highest impact on retention and selectivity when using HILIC conditions. It has been demonstrated that the mobile phase pH and nature of the stationary phase were the most important variables in HILIC [206, 207]. Regarding pH, two initial conditions are sufficient for the screening procedure, namely pH 3 and 6. It has been reported that pH 9 could be of interest in some cases [207]. However, such pH conditions are only compatible with a restricted number of stationary phases such as hybrid silica, and cannot be easily employed on any HILIC phases. In terms of stationary phases, a lot of studies have been recently published to find out the most different HILIC columns [206, 208-212]. Based on multivariate data analysis and using large set of diverse compounds, it appears that a limited number of stationary phases, namely 4 to 5 different chemistries are sufficient for the initial screening procedure. Among them, the most promising ones are: (i) unmodified bare or hybrid silica, despite a possible strong adsorption of various types of polar compounds, (ii) amide bonded phase, because of its high versatility for high performance separation of polar drugs, (iii) amino phase thanks to the sufficient retention of negatively charged compounds such as acids, and (iv) zwitterionic sulfoalkylbetaine or poly(succinimide) bonded phases which also offer suitable retention for a wide range of analytes in spite of a lower kinetic performance compared to the other supports. As example, Periat *et al*. [206] proposed a screening strategy involving exclusively short columns (50 x 2.1 mm) packed with sub-2 µm particles, including unmodified bare silica, bare hybrid silica, silica bonded with zwitterionic group and hybrid silica bonded with amide. When testing these four columns at pH 3 and 6, and a generic gradient from 95 to 65% v/v ACN, all the screening procedure was performed in less than 1 hour, reequilibration included.

5. IS SFC A VIABLE ALTERNATIVE TO RPLC ?

5.1. Description of SFC

In 2008, the acetonitrile shortage forced academic and industrial analysts to find alternative strategies to LC. In this context supercritical fluid chromatography (SFC) has made a remarkable comeback. This chromatographic technique is well known for more than 50 years but has only been used sporadically since its discovery by Klesper *et al.* in 1962 [213]. SFC differs from other chromatographic techniques by the use of a mobile phase consisting of fluid that has been pressurized and heated beyond its critical point. In supercritical or near supercritical state, such fluid exhibits density and solvating power similar to a liquid and diffusivity and viscosity features close to a gas. Both properties allow good solubility and fast transport of analytes without generation of excessive pressure within the chromatographic system. Moreover, because the solutes diffusion coefficients (DM) were relatively high under SFC conditions, the inherent reduction of mass transfer resistance allows efficient analysis even at elevated linear velocity. Despite the outstanding physicochemical properties of supercritical fluids, SFC development was only anecdotic during its first two decades, mostly because of the significant interest of the chromatographic community for the already well-established gas chromatography (GC) and the first and promising developments of high performance liquid chromatography (HPLC). Real SFC breakthrough only occurs in the early 80's thanks to the original publications of Novotny *et al.* [214-216]. Their work, together with the successful studies led by Lee and co-workers have strongly contributed to the popularization of SFC using capillary or open tubular stationary phases [215, 217-219]. In the early years, capillary SFC (cSFC) was also strongly promoted by GC users and was mostly considered as an elegant extension of GC, allowing an expansion of the solubility domain of classical GC [220, 221]. Several fluids were successfully used as mobile phase such as light hydrocarbons [222, 223], N_2O and ammonia [224]. However, their success had to be balanced with serious safety issues and hardware damages. Safer and inert, carbon dioxide (CO_2) quickly established itself as the reference supercritical mobile phase amongst SFC users. Its modest supercritical point (31 °C and 74 bar) was also a key advantage

over other fluids, as these smooth conditions were easily achieved on classical chromatographic system. cSFC using CO_2 was successfully used for the analysis of thermolabile compounds [225, 226]. cSFC was also an extension of the GC in terms of instrumentation. Indeed the main GC features (a) capillary column, (b) oven, and (c) GC detectors mostly flame ionization detector (FID) were preserved in cSFC [227-229]. Only a few modifications of the GC hardware were required to work properly with supercritical fluid. Surprisingly, the first commercial SFC instrument marketed by Hewlett Packard in 1983, based on previous studies of Gere [230], was much more inspired by LC set-ups. This system was designed for the use of packed column to perform separation under supercritical conditions, and offered the possibility to modify the pure supercritical mobile phase with co-solvent thanks to an innovative binary pump [231]. The CO_2 dedicated pumping unit was cooled down to pump the fluid under its liquid form directly out of a pressurized tank, whereas the second pumping unit was dedicated to co-solvent (generally MeOH). At this time, packed column SFC (pSFC) aroused a very limited enthusiasm compared to the interest paid for cSFC. Indeed, being closer to LC, pSFC exhibited a radically opposed philosophy to cSFC. The smaller chromatographic efficiency inherent to the use of short columns packed with small particles of 3-10 µm and the incompatibility of FID with binary mobile phase were the most apparent drawbacks from the point of view of GC and cSFC users [232, 233]. On the other hand numerous advantages were pointed out (a) improved selectivity and enhanced retention [234-237], (b) short analysis time [238], (c) possible analysis of polar compounds [231, 239-241]. In spite of these advantages, pSFC using binary mobile phase took more than a decade to gain acceptance.

5.2. Advantages and Limitations of SFC

Capillary SFC rapidly declined in the early 90's because this technique was too limited in terms of applications and poorly reproducible. The lack of polarity and solvating power of pure supercritical CO_2 limited the technique to the analysis of non-polar compounds and to petrochemical applications. Conversely, greater mobile phase flexibility was offered in pSFC. In this context, Berger, who is now considered as the father of modern pSFC, has deeply contributed to the clarification of the incidence of organic modifier adjunction [242]. He stated that binary mobile phase has benefits of both LC and GC, placing therefore pSFC somewhere in between these two separative techniques [238]. Polar modifiers such as MeOH, EtOH and IpOH were found to drastically enhance both solvating power and eluent strength of a supercritical mobile phase in CO_2, even at low proportion [243]. This increase in the fluid solvent strength was reported to be correlated to the modifier proportion rather than to the modification of density associated to its adjunction [244] similarly to LC, while small proportions of modifier have a limited effect on the GC-like low viscosity and high diffusivity of the fluid [245].

Considering only the supercritical mobile phase properties, SFC displays numerous theoretical and practical advantages compared to LC. Low viscosity and increased molecular diffusion allow high speed (elevated linear velocity) and/or highly efficient (long column) separations with reasonable column pressure drop. Good diffusivity and high speed also reduce column equilibration time. In addition, organic solvent consumption is low and yields to decrease both waste generation and costs relative to its reprocessing [246]. This last point makes SFC a green separative technique, especially for large-scale or preparative applications, which have to deal with large mobile phase volumes. Preparative scale SFC is well-established in pharmaceutical industries and constituted an economical alternative for the production of large amount of compounds. Indeed and compared to preparative LC, the recovered fractions are highly concentrated and solvent evaporation is greatly facilitated by the absence of water. Preparative SFC constituted an alternative choice in the context of implementation of techniques with reduced environmental impact [247]. It has been relatively well documented through various successful industrial scale-up processes and is expected to grow in the upcoming years [248-250]. Nevertheless, it should be kept in mind that the sharp decrease in solvent consumption must be weighted by the large amount of energy required to pressurize the CO_2 and to regulate the temperature along the chromatographic path [251].

Orthogonal selectivity constitutes another key feature of SFC compared to reversed phase LC. Indeed the non-polar nature of CO_2 promotes the use of polar stationary phases, making SFC a normal phase like technique. In this context, chiral separations provide very good examples as most of enantiomeric separations under supercritical conditions are performed on polar polysaccharide chiral phases. In addition, chiral separations in SFC achieve similar or higher selectivity performance than NPLC but without the use of problematic normal phase solvents and with an improved throughput. Successful chiral separations under supercritical conditions have been extensively reviewed in the last few years [252-254]. Normal phase like mechanism also represents the vast majority of achiral SFC separations. Unlike reversed phase LC for which the C18 stationary phase is the gold standard and selectivity tuning is achieved by changing mobile phase conditions, method development in SFC is primarily performed by screening different stationary phases. To a lesser extent, the modification of organic modifier can also improve the separation. The compound polarity range that can be analysed with SFC is relatively wide from polar compounds that are generally difficult to retain in RPLC (log P down to -1/-2) to very apolar molecules such as petrochemical compounds or liposoluble vitamins. To have a broad range of possible applications, it is necessary to adapt the stationary phase to the polarity of the target compounds. A polar stationary phase (Silica, Diol, Cyano, 2-Ethypyridine) has to be selected for the analysis of polar or ionisable compounds involving normal phase mechanisms. Conversely, a non-polar phase (C18) will be preferred for the analysis of the most lipophilic molecules.

Regarding detection, pSFC uses the same panel of detectors as LC. Indeed, the addition of an organic co-solvent prevents its coupling with FID. The most common detector in pSFC is obviously the UV spectrophotometer [255], but the latter must be equipped with a special cell able to withstand the backpressure from the restrictor located downstream of the detector. The use of evaporative light scattering detector (ELSD) has also often been described in the literature [256-

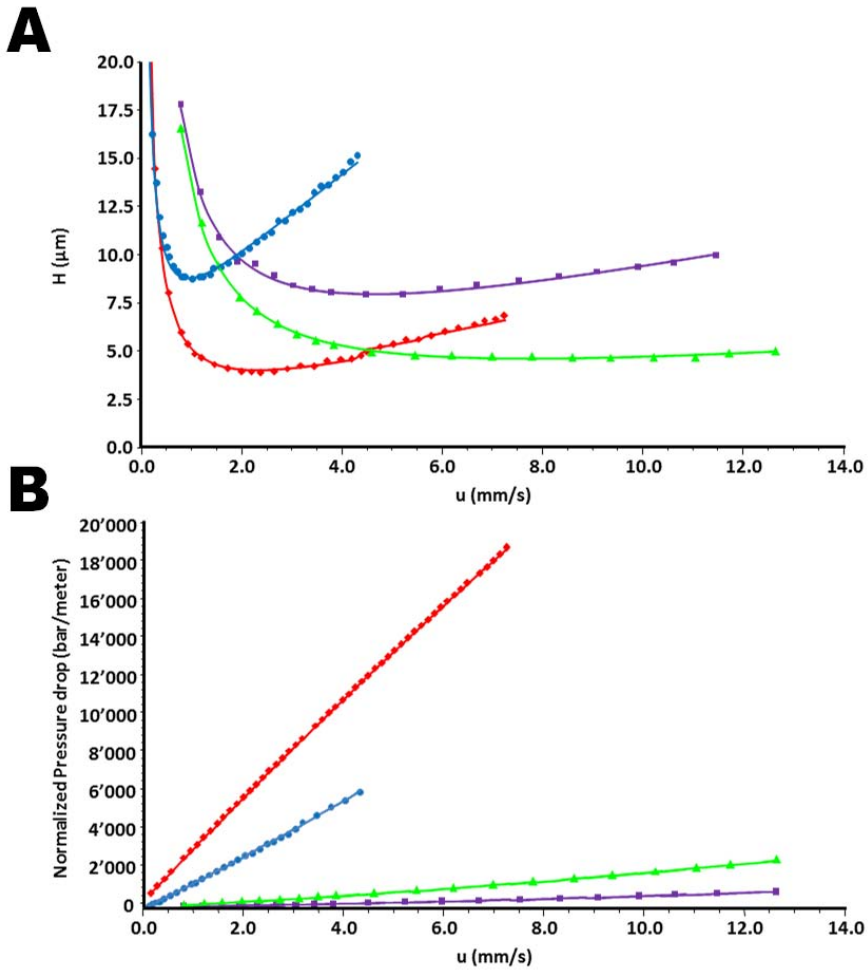

Fig. (9). (A) H-u plot representation of kinetic performance for 1.7 and 3.5 µm particles columns in UHPSFC (green triangles and purple squares, respectively) and in UHPLC (orange diamonds and blue dots, respectively). (B) Corresponding generated column pressure drop normalized to 1 m of column, to avoid influence of column geometry variations. Reprinted from [28] with permission. H corresponds to plate height expressed in µm and u is the linear velocity of the mobile phase in mm/s. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper).

258]. Finally, it is also possible to couple pSFC with MS and this hyphenation was already described over 20 years ago [259]. Although apparently more complicated at first glance that the LC-MS coupling due to the compressible nature of the supercritical mobile phase, the pSFC-MS hyphenation is relatively easy to implement with a few precautions and instrumental changes. pSFC-MS hyphenation and instrumentation have been extensively described by Pinkston who pointed out the advantages and drawbacks of different interface geometries [260]. Similarly to LC-MS, pSFC-MS can benefit from the robustness and versatility of the atmospheric pressure ionization source (API) such as APCI and ESI.

Finally, despite the obvious advantages of pSFC, this technique has struggled to establish itself in other fields of applications than preparative scale and/or chiral analysis. Albeit numerous system improvements have been described by Widmer [261-264], they were not able to overcome a serious lack of robustness and reproducibility. This has somewhat limited its use for analytical purpose as well as in the pharmaceutical industry where it has remained in the shadow of LC considered today as a reference technique.

5.3. Current Trends in SFC

Taking advantage of new technological advances in pump and back pressure regulator, several manufacturers have introduced a new generation of instrumentation, with improved performance, reliability and robustness. These new systems are largely based on recent developments of UHPLC instruments, including reduced void volumes and higher upper pressure limits. In addition, new instrumentation exhibits good compatibility with the most modern stationary phases (short, narrow-bore columns packed with sub-3 µm core-shell and fully porous sub-2 µm particles).

The use of short columns packed with fully porous sub-2 µm particles in SFC is relatively recent [265, 266]. Before the development of new systems, no benefit could be expected from such an association because the large system volume and limited upper pressure limit would compromise the separation. The high kinetic performance (>20,000 plates for 100 mm column length) obtained with sub-2 µm particles in supercritical conditions are comparable to those achievable under UHPLC on similar columns [28, 265], suggesting the introduction of the term ultra-high performance SFC

(UHPSFC) as a designation for the combination of SFC and sub-2 μm particles [267]. Similarly to LC, column dimensions have to be carefully chosen in UHPSFC, to limit the contribution of the system to band spreading, 3.0 x 100 mm columns were reported as the optimal dimensions for current UHPSFC instruments [268]. Compared to UHPLC, optimum linear velocity in UHPSFC is shifted towards higher values (by a factor of 3 to 5 fold), allowing ultra-fast and/or highly efficient analysis (Fig. **9A**) [28], at reasonable pressure drop (200-250 bar) (Fig. **9B**) [28]. However, evaluation of chromatographic performance in SFC in a proper way is not obvious [269]. Indeed compressibility of supercritical fluid varies with pressure drop and generates density, velocity and temperature gradients along the column [270] as well as reduction of retention factors. Because of these complex behaviours, the approaches used in LC to evaluate kinetic performance are not easily extrapolated to SFC conditions. In addition, these phenomena could impact more strongly columns packed with sub-2 μm than classic 5 μm particles, as the difference in pressure between the inlet and the outlet is higher. Guiochon and co-workers who have deeply investigated these parameters have recently proposed reliable models to explain kinetic performance in SFC [271-274]. The same group is also investigating the possibility to work at constant density (isopycnic) within the whole system by dynamically changing the outlet pressure (backpressure) to compensate the increase in column pressure drop to define suitable pressure and temperature working conditions for highest chromatographic efficiency [275-279]. The latter methodology has been successfully used to develop an accurate kinetic plot measurement [269]. Columns packed with core-shell 2.6 μm material were also evaluated in SFC and showed an impressive 50 % increase in efficiency when compared with 3 μm fully porous particles [280, 281].

Besides the column performance, the stationary phase nature plays a key role to achieve good separation under supercritical conditions. A great variety of silica based polar stationary phases have been recently developed [282]. Systematic classification of stationary phases was proposed by Lesellier and West who developed a tool based on solvatochromic model that groups the retention properties of a wide range of polar and non-polar phases. This tool is very useful for the column screening process, it allows the selection of sets of columns which exhibit the most orthogonal selectivities [283-285]. A derived approach was besides successfully implemented in the pharmaceutical industry [286].

The versatility of SFC in terms of analysed compounds opens up numerous application fields, including pharmaceutical analysis. Indeed, apart from its preponderant involvement in chiral and preparative separations already discussed, SFC penetrates gradually into the R&D and QC laboratories. The development of qualitative and quantitative methods orthogonal to LC for QC purposes has been recently described [287]. The range of compounds that can be analyzed with SFC is also expanded with the development of methodology for the analysis of highly polar or ionic compounds. As example, ion pairing SFC has been recently described for the analysis of peptides by Taylor and co-workers, who employed a ternary mobile phase consisting of CO_2, MeOH and 5% v/v of water containing an ion pairing agent [288]. Very good chromatographic behaviour was observed for polar molecules and HILIC-like retention mechanism has been postulated in supercritical conditions [289]. Complex matrixes have also been analyzed with SFC and applications on biological matrices such as blood, urine and tissues have been thoroughly reviewed [290]. Lipid metabolomics is also a challenging research field, in which SFC has already been highlighted as a very effective separation technique. However, due to the sample complexity, MS hyphenation is mandatory to extend detection throughput and obtain a detailed profile of individual components. Lipids analysis is currently the driving force of SFC-MS development [291]. Bamba and co-workers have showed that this technique was powerful to explore the large variety and polarity of important lipid classes such as carotenoids and phospholipids [292, 293]. Finally, SFC-MS is not restricted to this field and thanks to the versatility of atmospheric pressure ionisation source, is now gradually implemented in many new applications. Comparative studies between LC-MS and SFC-MS have demonstrated that the latter technique was highly competitive for drug analysis [294, 295]. In addition, several preliminary studies have recently described some favourable conditions for obtaining sharp peaks and good MS detection of basic pharmaceutical compounds [265, 296]. Fig. (**10**) shows an example of basic drugs analysis in supercritical conditions achieved on 2-ethylpyridine column and hybrid silica.

6. CONCLUSION

As shown in the present review, thanks to the most recent advances in LC illustrated in (Fig. **1**), it is possible to attain faster separations, higher chromatographic resolution, orthogonal selectivity, reasonable retention for problematic compounds (too hydrophilic or too lipophilic ones), and/or enhanced sensitivity, compared to conventional RPLC.

To increase the column performance, namely throughput and/or plate count, columns packed with fully porous sub-2 μm particles, introduced in 2004, appear as a suitable strategy but require the use of UHPLC instruments compatible with pressure up to 1000-1300 bar. To limit this issue, the core-shell sub-3 μm technology, was introduced in 2007 and presents some obvious benefits. On the market, there is today a huge competition between these two approaches, both possessing some advantages and drawbacks. On the other hand, despite recent improvement of kinetic performance brought to the silica-based monoliths (2^{nd} generation commercialized in 2012), the performance and above all the choice of column dimensions and chemistries remain too limited with monolithic columns. Finally, to take the full advantage of these innovative phases, it is mandatory to work with a suitable instrumentation, including an extended upper pressure limit, a limited contribution to band broadening and a reasonable gradient delay volume. If these new phases are employed on old-generation HPLC system without any optimization, theoretically expected column efficiency will be compromised in practical work, especially in the case of highly efficient, narrow bore columns.

Two alternative modes of chromatography, namely HILIC and SFC can be employed to further extend the pos-

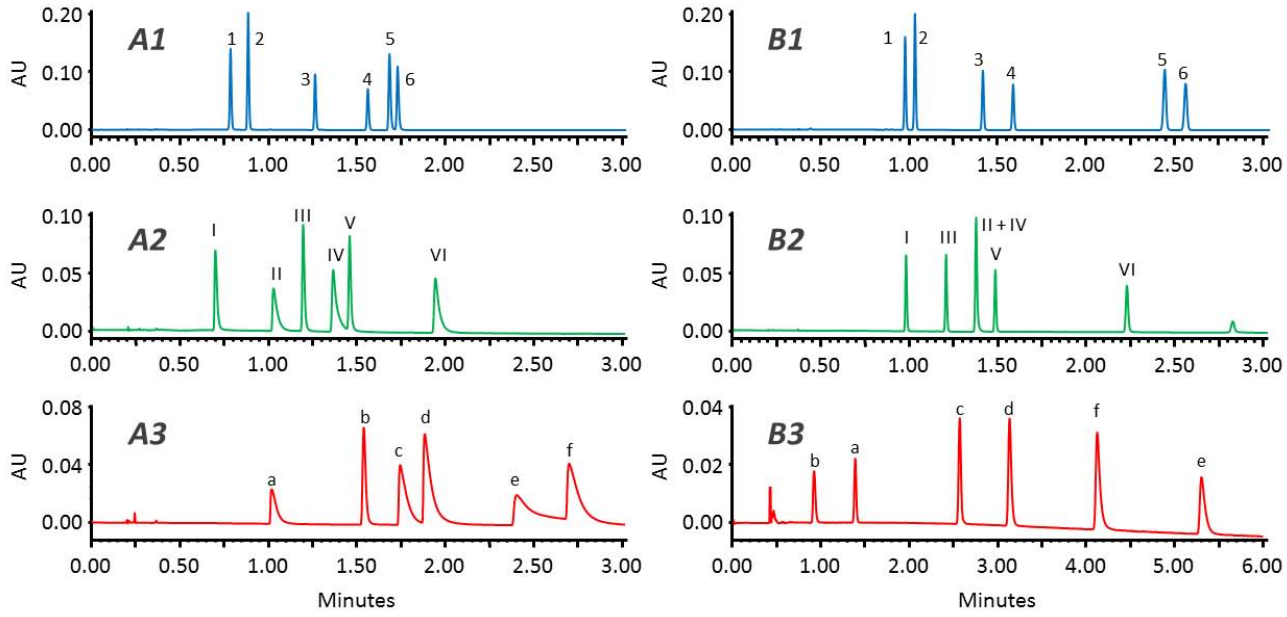

Fig. (10). Peak shape improvement strategies for basic drugs analysis in supercritical conditions. Separations of low pKa range compounds (blue line 1, top chromatograms), middle pKa range (green line 2, middle chromatograms) and high pKa range (red line 3, bottom chromatograms) achieved on 3.0 x 100 mm, 1.7 µm 2-Ethylpyridine column and 3.0 x 100 mm, 1.7 µm column hybrid silica in presence of 20 mM NH_4OH. Reprinted from [267] with permission. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper).

sibilities and applicability of RPLC to a wider range of compounds. It has been demonstrated that these modes were adapted to a large number of analytes traditionally analyzed by RPLC but offer a very different selectivity compared to RPLC. Due to the orthogonal interaction mechanism involved in HILIC and SFC, some other compounds hardly compatible with RPLC could also be successfully analyzed and retained. In addition, the kinetic performance achieved under HILIC and SFC modes was excellent, thanks to the low viscosity of the mobile phase and fast molecular diffusion. Finally, the sensitivity achieved with MS detection is expected to be higher under HILIC and SFC conditions, because of the use of large proportion of organic modifier, or due to the absence of water in the mobile phase, respectively.

According to these developments, HPLC appears as a mature, powerful technology, quite fast and also highly sensitive when coupled with MS detection. In the future, it is expected that the coupling of HPLC with MS will expand increasingly in laboratories, particularly with the introduction of more sensitive, robust, user-friendly and faster systems. Since few years, the importance of therapeutic proteins and monoclonal antibodies (glycoproteins of ~ 150 kDa) is growing in the pharmaceutical field and the expectations are high for this new class of compounds. However, the analytical characterization of biopharmaceuticals remains difficult and there is a need to improve the current analytical tools. Based on this consideration, it is likely that more and more specialized LC inert material possessing widepore particles will be commercialized, to improve the kinetic performance and reduce adsorption phenomena at the surface of the silica [139, 140].

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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ABBREVIATIONS

ACN	=	Acetonitrile
APCI	=	Atmospheric pressure chemical ionization
API	=	Atmospheric pressure ionization
BEH	=	Bridged ethylene hybrid particle
cSFC	=	Capillary supercritical fluid chromatography
DM	=	Molecular diffusion coefficient
ELSD	=	Evaporative light scattering detection
ESI	=	Electrospray ionization
EtOH	=	Ethanol
FID	=	Flame ionization detector
GC	=	Gas chromatography
HILIC	=	Hydrophilic interaction liquid chromatography
HPLC	=	High performance liquid chromatography
I.D.	=	Internal diameter
IEX	=	Ion exchange

IpOH = Isopropanol

LC = Liquid chromatography

LTQ = Linear trap quadrupole

MeOH = Methanol

M_r = Relative molecular mass

MS = Mass spectrometry

MS/BVPE = Methylstyrene and 1,2-bis(*p*-vinylphenyl)ethane copolymer

N = Plate number

NPLC = Normal phase liquid chromatography

PFP = Pentafluorophenyl

PLOT = Porous layer open tubular

POISe = Injection sequence for optimizing performance

PSD = Particle size distribution

PS-DVB = Polystyrene-divinylbenzene

PSF = Parallel segmented flow

pSFC = Packed column supercritical fluid chromatography

QC = Quality control

RPLC = Reversed phase liquid chromatography

SFC = Supercritical fluid chromatography

t_0 = Column dead time (elution time of a non-retained compound)

t_d = System dwell time

UHPLC = Ultra high-pressure liquid chromatography

UHPSFC = Ultra high-performance supercritical fluid chromatography

UPLC = Ultra performance liquid chromatography

UV = Ultraviolet detection

V_d = System dwell volume

VPLC = Very high-pressure liquid chromatography

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