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Sub 2 µm fully porous and partially porous (core-shell) stationary phases for reversed phase liquid chromatography

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Abstract

The need for increased throughput and/or superior performance has increased the demand for stationary phases with improved kinetic performance. Among them, increasing the sample throughput of the ever-growing number of necessary (routine) analyses has become a popular target to cut precious time. For the last thirty years, High-Performance Liquid Chromatography (HPLC) has been the leading technology when it comes to various analyses; however, its necessity of serial analyses taking typically 10-45 min has been a sample throughput-limiting barrier. Lately, the fundamentals of HPLC have been exploited to raise new technologies that can speed up analyses to ground breaking limits, without compromising separation efficiency. This paper reviews the most promising technologies, which are totally porous sub-2 µm (Ultra-Performance Liquid Chromatography or UPLC) and fused-core particle technology, these two have the potential to take LC to the next level. As each analytical method has its own demands, the advances of the above technologies are discussed for different applications where high throughput analysis can be meaningful. We discuss the perspectives of these technologies comparing them.

Keywords: Sub-2 µm fully porous stationary phases; core–shell stationary phases; wide pore stationary phases; increased kinetic performance stationary phases, hydrophilic interaction chromatography (HILIC); protein analyses.

1 Introduction.

After the introduction of high performance liquid chromatography (HPLC), and during the following decades, major efforts were made to develop more efficient stationary phases to improve the separations that can be achieved with this technique. During the first decades, the stationary phases used in HPLC are composed by particles with totally porous, irregular and relatively large particles. As technology advances, numerous new chemistries are developed and focus is given to reduce particle size and improve shape. Special interest is given to the development of smaller particles mainly because as particle size decreases there is an increase in the separation efficiency.

Several decades ago it was foreseen the trend to employ smaller particles for faster and more efficient separations. Not long ago, the goal was to achieve highly efficient packing with particles with a diameter between 5-10 µm.[1] Nowadays, this trend continues and current liquid chromatography stationary phases have even smaller particles, with diameters ranging between 1.3 and 3.5 µm. However, besides of smaller and more uniform particles with several different chemistries, currently there is also a need for stationary phases with increased kinetic performance to allow faster and more efficient separations. [2, 3] Scientists are particularly interested in using rapid and efficient procedures for qualitative and quantitative analysis to cope with a large number of samples and to reduce the time required for the delivery of results. In this aspect, reducing analysis time and ensuring the quality of a separation in HPLC, requires high kinetic efficiency.[4]

Higher efficiency and faster separations have always been of great interest in HPLC and have become increasingly important in recent years mainly driven by the challenges of more complex samples or by the increased the numbers of samples[4]. In areas such metabolomics and proteomics, where samples are very complex, it is

necessary to improve the separation efficiency of the stationary phase. This can be achieved by changing the chemistry of the stationary phase, by increasing the amount of stationary phase (column length) and/or reducing the particle diameter. To reduce analysis time, increased kinetics performance is needed. In this context, the ratio of the hold-up time to column efficiency can be used to assess the resolving power of a separation system, which is favoured with columns that possess high porosity and low plate heights while operating under high linear velocities, as summarized in Fig. 1.[5]

It is obvious that each application will require different conditions to achieve the proposed objective. Some applications can be achieved in short time because the resolution requirements are low, while some applications require high resolution and are not achievable in short times. This is largely dependent on the sample complexity, clearly suggesting the compromise between resolution and analysis time.[6, 7] For example, in order to analyse a relatively simple samples with only a few compounds in short times, the column length can be decreased and the linear velocity of the mobile phase may also be increased to reduce the retention of compounds by the stationary phase. In this case, short columns (10-50 mm) may be sufficient to afford a reasonable selectivity.

Combining high speed and high efficiency is the ideal scenario, but unfortunately it is difficult to achieve with complex samples, and as a result, it is necessary to sacrifice resolution for analysis time or vice versa. [6, 7] Thus, a balance between speed and high efficiency must be found. In this context, this review is dedicated to discuss the improved kinetic performance of totally porous sub 2 µm stationary phases and sub 3 µm partially porous (core-shell) stationary phases and their application for the analysis of macromolecules.

2 Sub 2 µm stationary phases.

In the past decade there has been a continuous drive to develop chromatographic stationary phases to perform fast LC separations, as sample throughput can be increased and therefore cost per sample reduced. One of the main strategies followed the ever need of producing smaller particles to increase efficiency of separations.

The effect of and particle size ($\boldsymbol{d}_p$), in efficiency can be explained by the van Deemter equation (Eq. **1**), which describes the relation between efficiency (expressed as the height equivalent to a theoretical plate, $\boldsymbol{H}$), linear velocity ($\boldsymbol{\mu}$), in which $\boldsymbol{\lambda}$ is a packing constant, $\boldsymbol{\gamma}$ an obstruction factor for diffusion in a packed bed, $\boldsymbol{D}_m$ the diffusion coefficient of analyte in the mobile phase and $\boldsymbol{f(k)}$ is a function of the retention factor ($\boldsymbol{k}$).

Eq. 1 $$H = 2\lambda d_p + \frac{2\gamma D_m}{\mu} + f(k)\frac{d_p^2}{D_m}\mu = A + \frac{B}{\mu} + C\mu$$

The dependence of $\boldsymbol{C}$-term, which is considered to mainly represent the resistance to mass transfer in the mobile phase, is direct proportional to the square of the particle size. Thus, decrease in particle diameter results into a large decrease in the plate height, especially at high linear velocities. [8]

The position of the minimum on the HETP curve, and the optimum linear velocity, can be determined by the use of differential calculus. The optimum linear velocity occurs when the slope of the $\boldsymbol{H}$ versus $\boldsymbol{\mu}$ curve is zero, i.e. when $dH/d\mu = 0$. This condition is satisfied in Eq. **2**.[8]

Eq. 2 $$\mu_{opt} = \sqrt{\frac{B}{C}}$$

Fig. 2 illustrates the he van Deemter equation (**Eq. 1**) for several stationary phases with particle size between 5-1.3 µm. It shows that stationary phases with reduced

particle size afford increased efficiencies. Furthermore, reduced particle sizes result in an increased μ_{opt}, as described in Eq. **2**.

On the other hand, reduction of particle size not only improves peak capacity, but also greatly increase the pressure generated by the column. For example, the pressure drop of the column, ΔP, is proportional to $1/d_p^2$ with the linear velocity (u) according with Kozeny-Carman equation[9]. (Eq. 3, where φ is the flow resistance factor, η is the viscosity of the solvent, L is the length of the packed bed, and d_p represents the particle diameter.[10-13]). Considering that reduced particle size results in an increased μ_{opt}, ΔP is proportional to $1/d_p^3$ at the optimal linear velocity (u_{opt}). For example, in Fig. 2, it was not possible to reach μ_{opt} using the stationary phases with 1.3 µm due the high back pressure generated by those very fine particles.

Eq. 3 $\Delta P = \frac{\varphi \eta L \mu}{d_p^2}$

Unfortunately, the increase of the pressure caused by smaller particles reaches the pressure limits of conventional HPLC systems (400 bar) with particles of approximately 3 µm. Smaller particles increase the pressure drop and will allow only a low mobile phase velocity, which will in turn provide low column efficiency (Fig. 2)[14].

In order to take advantage of packing materials with particles smaller than 2 µm (sub-2-µm particles) special systems are necessary. These systems have low dwell volumes and they are capable of withstanding the higher pressure caused by the sub-2-µm particles while maintaining a relative high linear velocity to provide high column efficiency and were termed ultrahigh-pressure liquid chromatography (UHPLC) systems. UHPLC has been defined as a type of liquid chromatography utilizing sub-2-µm particles. [15] Indeed, the main advantages of this technique are the high separation power (theoretical plate counts from 100,000 to 300,000) and reduced run times. As

mentioned, the main disadvantage of this technique is the high back pressure generated.[16-24,25, 26]

The first commercial UHPLC system (Acquity, Waters Corp.) and columns packed with porous 1.7 μm hybrid silica particles (Acquity BEH stationary phases) were introduced in 2004 and were able to withstand pressures up to 1000 bar. [23, 27, 28] Currently, there are more than 100 different columns packed with 1.5–2 μm particles, from about 15 providers, as well as, about 20 different UHPLC systems with pressure limits between 600 and 1300 bar that are available on the market.[28-30] For example, in Fig. 2, the optimal linear velocity and thus the lowest possible *H* value could not be reached before exceeding the upper pressure limit of the UHPLC instrument, Waters Acquity UPLC™ I-Class, used (1070 bar was reached at $u = 0.5$ cm/s at $T = 25$ °C). It means that with such column, the major contribution to band broadening is longitudinal diffusion (*B*-term dominated region).

Unfortunately, separation efficiency is not only influenced by the particle diameter and the mobile phase velocity, but also by several other parameters and characteristics of the system. Due to the higher efficiency achievable with smaller particles at high μ, column dimensions can be reduced to shorten analysis time and save solvent. Besides of shorter columns with reduced internal diameter, column void volume is also reduced in UHPLC columns to minimize diffusion of sample components and band broadening. Another characteristic of UHPLC systems when compared to conventional HPLC systems is the reduced extra-column volume.

Although there were great improvements in instrumentation in the last decades, the loss in apparent column efficiency can still be very significant, even in modern UHPLC equipment [28-30] and further improvements in instrument design (smaller

dispersion) are necessary to take full advantage of columns packed with sub 2 µm particles.[30] According to Fekete and Fekete,[30] it is not possible to fully utilize the potential of these small columns with current instrumentation. The loss in efficiency was estimated to reach 30–55% with commercially optimized UHPLC systems. This suggests that the performance of the stationary phases is being limited by equipment itself and not by the characteristics of the stationary phase.

Nowadays, there are several equipments that can reach backpressures between 600-1200 bar. However, fast chromatography has low retention volume and it needs equipments with low dead volume. For example, at 50-60 ◦C, HPLC equipments could work with a 50-100 mm long column packed with particles between 2.5-1.7 µm. In this case, the conventional HPLC equipment could generate a backpressure that is enough to run analyses using these columns, but band broadening from the equipment could destroy the separation.

Fig. 3 show several UHPLC equipments from different suppliers. This figure shown the maximum back pressure that those equipments can tolerate and their dead volume.[31]

In fact, the lower the retention volume the higher is the effect of extra column band broadening on efficiency loss. Thus, increased retention volume could reduce the efficiency loss due extra column band broadening. For example, in **Fig. 4**, theoretically, these columns should provide the same column efficiency. However, the column efficiency for the propiophenone peak decreases as column diameters are decreased.[32] The equipment used in this example is a Waters Acquity UPLC system (Milford, MA, USA). Looking to Fig. 3, we can observe that it is one of the UHPLC equipments with lower dead volume. Thus, columns with 3-4.6 mm diameter should be preferred instead of columns with 2.1 mm diameter.

When columns packed with particles of small diameter were used in a conventional LC instrument, the use of columns with diameter close to 4.6 mm could alleviate the efficiency loss due band broadening. For example, we run several analyses, in conventional HPLC equipment, using 50-100 mm long column packed with particles between 2.5-1.7 µm.

Another consideration in the upper pressure limit of current systems (1.100-1.300 bar) is related to the frictional heating phenomenon. Frictional heating is induced by movement of the mobile phase through the column bed at very high pressure. The generated heat dissipates along and across the chromatographic column, allowing for the formation of axial (longitudinal) and radial temperature gradients. [28-30] These thermal gradients may influence both the retention and the column efficiency. The efficiency loss due to these thermal gradients could be dramatic and perhaps the limit of increased performance via straightforward particle size reduction.[33] On the other hand, thermal conductivity is higher in core-shell columns due to the solid cores of the particles. The radial thermal gradients are lower than in columns of totally porous particles of the same particle size pumped with the same mobile phase at the same velocity. Better heat dissipation allows the further reduction of the core-shell particle size before encountering mobile-phase heating problems.[33]

It is also expected some alteration in retention due the high backpressure achieved with this technique. The pressure alone can have a significant effect on retention. However, the effects are much more pronounced with large and ionized analytes. An increase in the average column pressure of 500 bar can produce increases in the retention of ionized bases and acids by as much as 50% under typical operating conditions.[34-37] Large proteins, which have several ionizable functionalities and may undergo conformational changes under ultra-high pressure conditions, have shown a

pronounced increase in retention as the pressure is increased. For example, retention of myoglobin (MW ~17 kDa) increased more than 3.000% with the increase of pressure from 100 to 1.100 bar. [36]

In contrast, the use of elevated temperatures reduces the pressure of the system by affecting the viscosity of the mobile phase and reduces retention of analytes by increasing mass transfer rates.[38] Because the mobile phase viscosity can be dramatically reduced by an increase in temperature resulting in lower back pressures, separations carried out at high temperatures may take greater advantage of sub 2 µm particles.[39]

Extra-column variance leading to peak broadening and frictional heating are considered important factors limiting the performance of the separations with stationary phases with sub 2 µm particles. Another limiting factor is the difficulty of packing uniform beds with ever-smaller particles using current techniques. Due these drawn backs, the performance achieved in practice with sub 2 µm particles (totally porous or core shell) is smaller than the performance that can be theoretically achieved.[40-43]

To illustrate this aspect we can consider a recent study by Fekete et al.,[44] where several stationary phases with particle diameter ranging between 1.5 and3.0 µm were evaluated for the separation of pharmaceutical products, as shown in Fig. 5. They did this comparison using the Knox equation,[7] which use reduced plate heights ($h = H/d_p$) and linear velocities ($\nu = \mu d_p/D$). They observed that a similar efficiency can be achieved with columns packed with 1.9–2.1 µm particles and with smaller particles (1.5–1.8 µm). When the particle size was 2.5 µm or larger, the theoretically expected values and experimental data of plate heights were in good agreement, indicating that the full performance potential of the stationary phases were being used. It was suggested that the use of reduced particle size results in lower efficiencies than should be expected

due to high dead volume of equipments, frictional heating effects and due to the difficulties of packing uniform beds with smaller particles using current technologies. [44]

3 Partially porous (core-shell) stationary phases

Along with the reduction of particle size and improvement of the chromatographic system characteristics, huge efforts are also being made to improve the particles themselves. Until recently, most stationary phases were composed of totally porous particles. Stationary phases made of partially porous particles have several advantages over conventional particles and are being considered to be the next step in LC stationary phase technology. Fig. 6 illustrates the structure of a HALO peptide core-shell stationary.[45]

These partially porous particles (core-shell particles) consist of a solid inner core surrounded by a porous outer layer. In comparison with totally porous particles of similar diameters, the diffusion path is much shorter, because the inner core is solid fused silica, which is not accessible to the analytes interacting with the particle. The shorter diffusion path influences the resistance to mass transfer (the C term in the Van Deemter equation), which tends to limit the axial dispersion of solutes and minimize peak broadening, especially at elevated linear velocities. Additionally, this material has an exceptionally narrow particle size distribution and high packing density compared to porous particles (Fig. 7), leading to a smaller A term in the Van Deemter equation (i.e. eddy diffusion).[46]

Cabooter et al.,[47] have studied the particle size distribution (Fig. 8) and van Deemter curves of several sub 3 µm core-shell and 3-3.5 totally porous particles. They observed that core-shell particles have narrower distribution than totally porous particles as shown in Fig. 8. The core-shell also afforded higher efficiency than totally porous

particles. van Deemter plots have shown that core-shell stationary phases have lower A and B terms than totally porous particles, but not lower C term. In truth, for small molecules, such as the pharmaceuticals studied by Cabooter et al.,[47] core-shell stationary phases does not have lower C than totally porous stationary phases. It is due the roughness of core-shell stationary phases (see Fig. 6).

Core–shell particles have a much lower A-term contribution at high velocities compared to fully porous columns.[46] This implies that the superficially porous column can be operated at three to four times its optimum velocity and still have the same or better performance than the fully porous column. This is illustrated in Fig. 9, [48] where t example chromatograms are shown measured on the 250 mm 4.6 mm columns packed with the fully and superficially porous 5 µm particles. Fig. 9A and C shows the separation of the three alkylphenones at the optimum flow rate of 1mL/min for the Kinetex and Zorbax column respectively. Chromatograms measured on 5 µm core–shell and fully porous particle columns are compared at different flow rates. At the optimal flow rate u_{opt} the number of theoretical plates of the core–shell particle columns is circa 30% higher than the fully porous particle columns, which is of course directly related to the difference in (reduced) plate height. When going to a higher flow rate (e.g. almost three times u_{opt}) this difference even increases to more than 75% (see Eq. **2**), this does not happens because core-shell stationary phases have a lower C-term than totally porous stationary phases. it happens because core-shell stationary phases have lower A and B term than totally porous stationary phases. [48]

Indeed, due to its low A and B-term, sub 3 µm core-shell stationary phases can reach peak capacities comparable to sub 2µ totally porous particles.[49] For example, Fig. 10 show that sub 3µ core-shell stationary phases afford a peak capacity closely related to one of most popular sub 2µm totally porous stationary phase.[49]

When this review was written, core-shell stationary phases are available just as type B silica materials, while ZirChrom PDB is the only sub 2 µm non silica stationary phase available on the market (it is a polybutadiene-coated zirconia stationary phase).[50] The type C silica[51-56] is very promising material and it is possible to prepare sub 2 µm totally porous and core-shell stationary phases based on type C silica. However, there were not cores-shell and sub 2 µm stationary phases based in Type C silica, probably because type C silica has small market.

The core-shell technology is still evolving and the number of commercially available stationary is rapidly increasing and several new brands of core-shell stationary phases are introduced, Table 1 summarize some of the new brands, which are commercially evaluable.

The sub 3 µm core-shell and sub 2 µm totally porous stationary phases afford narrow peak shape. However, only half or one third pressure is required to operate with a column packed with sub 3 µm core-shell material, compared to a column packed with 2 µm totally porous stationary phase, in agreement with Darcy's law and the Karman-Kozeny equation. The relatively high specific permeability of columns packed with sub 3 µm core-shell particles ranges between $K_0 = 4.6 \times 10^{-11} cm^2$ and 6.4×10^{-11} cm^2, while the permeability of a column packed with 1.7 µm totally porous stationary phase is ~ 2.5×10^{-11} cm^2.[57-59]

A recent study evaluated the peak capacity of degradation products/impurities of ethinyl-estradiol using a 3 minute gradient time at a 0.8 mL/min flow rate with a sub 2 µm totally porous stationary phase (Acquity BEH C_{18}) and an 18 minute gradient time at 0.4 mL/mim flow rate using sub 3 µm core shell stationary phases (Kinetex C_{18}, Ascentis Express C_{18}, Poroshell C_{18}) with columns of 50 x 2.1 mm. Sub 3 µm core shell

stationary phases generated half of the back pressure in comparison to a sub 2 µm stationary phase while being able to maintain approximately the same peak capacity. [49]

On another study, the efficiency of core-shell stationary phases (Ascentis Express and Kinetex) and sub-2 µm totally porous stationary phases (Acquity BEH, Grace Vision HT and Hypersil Gold) was evaluated using estradiol and ivermectin as test probes. [41] Sub-3 µm core-shell and sub-2 µm totally porous materials provided very similar efficiency for both test compounds. However, one of the columns (Kinetex C_{18}) showed a smaller C term than the others (the Ascentis Express C18 and the sub-2 µm stationary phases). Additionally, the degradation products/impurities of ethinyl-estradiol were separated within the same time in sub-3 µm core-shell and sub-2 µm totally porous materials.

The enhanced performance of core-shell stationary phases is related to its higher permeability when compared to totally porous stationary phases, which is derived from the narrow particle size distribution. The sub 3 µm core-shell stationary phases have lower eddy diffusion and higher mass transfer resistance for small analytes than sub 2 µm totally porous stationary phases. In this aspect, Fekete et al., [46] compared a core-shell type stationary phase (Ascentis Express $C_{18;}$ 50 × 2.1 mm, 2.7 µm) with several sub-2 µm totally porous stationary phases [Acquity BEH C_{18} (50 × 2.1 mm, 1.7 µm), Grace Vision HT C_{18} column (50 × 2.0 mm, 1.5 µm) and Hypersil Gold C_{18} (50 × 2.1 mm, 1.9 µm)] using hormones as test probes. They observed that the plate heights generated by the 2.7 µm core-shell material were comparable to those produced by sub-2 µm particles. Surprisingly, the C term in the van Deemter formula for Ascentis Express C_{18} was higher than those observed with the sub-2 µm totally porous stationary phases, which means that the comparable efficiency of ~3 µm core-shell with sub-2 µm totally porous stationary phases are due to reduced eddy diffusion. The high C term

shown by core-shell stationary phases were attributed to its rough surface. Although it can be expected to observe lower efficiencies of the core shell column due to the higher value of the C term, because there is a reduced A term comparable efficiencies are observed for both core shell and sub 2 µm columns.

Fekete et al.,[46] observed that a sub 3 µm core-shell stationary phase [Ascentis express C18 (2.7 µm)] afford efficiencies comparable to sub 2 µm totally porous stationary phases [Grace Vision C18 (1.5 µm), Acquity BEH C18 (1.7 µm) and Hypersil Gold C18 1.9 µm] for the separation of a mixture of steroids. It was also observed that the core-shell stationary phase afforded a back pressure lower than sub-2 µm stationary phase due to its higher permeability. In addition, the core-shell stationary phases are able to separate a mixture of steroids in less than two minutes. [46]

In some cases, the pressure achieved with sub 3 µm stationary phases are in the 400 bar range, which is a pressure compatible with conventional LC equipment.[38, 60, 61] However, core-shell stationary phases or sub 2 µm totally porous stationary phases produce peaks with low retention volume, which means that the chromatographic performance might be severely compromised by extra volume from the equipment. The same discussion was done fin Fig. 4. When using sub 3 µm partially porous and sub 2 µm totally porous stationary phases, one of the primary considerations influencing the separation is the LC equipment. Even the most current UHPLC systems have limitations regarding how well they can reflect the true performance of a core–shell and sub 2 µm stationary phases.[8, 28]

The system dead volume influences the chromatographic performance of the separation and this factor increase its importance as the dimensions of the column gets smaller. However, judicious selection of the column dimensions can alleviate the

situation. Short columns are demanding since they generate peaks with very small volume and the effect of extra column volume will be less pronounced as the volume (both length and i.d.) of the column is increased.[62]

With the realization that column performance is being limited by the instrument characteristics and the wide presence of conventional HPLC in laboratories, researchers started to modify the configuration of their systems to improve the performance. Several modifications of conventional HPLC instrumentation are necessary to optimize system volume and achieve the full potential of core–shell stationary phases. It also has been suggested that separation conditions should be adjusted when using core–shell stationary phases to improve separations. [63] Conventional systems are not optimized for achieving fast and efficient separations when using small narrow-bore columns packed with sub 3 μm core–shell stationary phases. However, the use of columns with 4.6 mm I.D. may provide improved separations without much loss in column efficiency. [28, 64-66]

Bobály et al., [67] compared the chromatographic performance of Waters Cortecs 1.6 μm, Phenomenex Kinetex 1.3 μm and Phenomenex Kinetex 1.7 μm, which are sub 2μ core-shell stationary phases. In terms of kinetic performance, the Kinetex 1.3 μm particles provide exceptional performance (H_{min} of 1.95 μm), but suffers from a too low permeability. Thus, this column cannot be employed under optimal linear velocity conditions, even on the best UHPLC systems (ΔP_{max} of 1200 bar). Alternatively, the Kinetex 1.7 μm packing offers a twofold higher permeability, but the kinetic performance was lower (H_{min} of 3.17 μm). The best compromise seems to be the Cortecs 1.6 μm phase that possesses both a reasonable permeability (similar to that of Kinetex 1.7 μm) and excellent kinetic performance (H_{min} of 2.66 μm). This column outperforms the other two ones in the practically useful plate number and peak capacity ranges in terms of achievable analysis time. On the other hand, the was superior to the

other ones for ultra-fast analysis (e.g. $t_{grad} < 0.5$ min). Meanwhile, to attain the full benefits of Kinetex 1.3 μm, a system possessing $\sigma^2_{ec} \leq 1$ μL^2 is recommended.

The Bobály et al., [67] reinforce our opinion that, at the moment, there were not equipments which are able to work with sub 1.6 μm.

4 Silanophilic interactions of core-shell and sub 2 totally porous stationary phases.

Core-shell stationary phases can be successfully used to improve the separation and reduce analysis time of a wide range of analytes. In this sense, it is important to highlight the detrimental interactions of basic compounds with these stationary phases in low ionic strength mobile phases with organic and amino buffers.

Basic pharmaceutical analysis can also be successful using core-shell stationary phases, but one must take into account detrimental interactions of basic compounds with these stationary phases in low ionic strength mobile phases with organic and amino buffers. For example, Ruta et al., [68] used a sub 2 μm totally porous stationary phase [Acquity BEH C_{18}, (50 × 2.1 mm, dp 1.7 μm)] was compared with several sub 3 μm core-shell stationary phases [Poroshell 120 EC-C_{18} (50 × 2.1 mm, 2.7 μm), Kinetex C_{18}, (50 × 2.1 mm, 2.6 μm) and Halo C_{18} (50 × 2.1 mm, 2.7 μm)] for the analyses of 13 acidic and basic pharmaceuticals. Separation of the 13 pharmaceuticals was achieved in less than three minutes with all of the tested stationary phases. The separations were obtained under LC-MS compatible conditions (B = 0.1% formic, A = 0.1% formic acid in acetonitrile, gradient profile: 5% A for 1 min, then 5–95% A in 3 min.) and with phosphate buffer (A = phosphate buffer (20 mM, pH 6.85), B = ACN, gradient profile: 5% ACN for 1 min, then 5–95% ACN in 3 min). In addition, a similar loadability of the

stationary phases was observed for all stationary phases under LC-MS compatible conditions.

Another study compared the performance of totally porous (Acquity BEH-C_{18}) and partially porous (Kinetex-C_{18}) stationary phases with the same dimensions and particle size (50 x 2.1 mm, 1.7 µm) for the separation of small pharmaceuticals and larger charged molecules (peptides) under LC/MS compatible conditions (i.e. 0.1% formic acid, pH 2.8; 0.05% trifluoroacetic acid, pH 2.4; 10 mM ammonium acetate, pH 6.8; 0.1% ammonium hydroxide, pH 10.6; 10 mM ammonium formate, pH 2.8 and 10 mM ammonium formate pH 10.4). [68]

The partially porous column provided higher efficiency than the totally porous stationary phase for neutral solutes (ethylparaben and caffeine). However, both stationary phases provided lower efficiency for diphenhydramine than those obtained for caffeine. In both cases, the low efficiency values obtained were attributed to the poor peak shapes obtained. In this specific case, the partially porous column (Kinetex-C_{18}) was less efficient for small basic pharmaceuticals due detrimental interactions with the free silanols on the surface of this material. In contrast, the totally porous stationary pahse (Acquity BEH-C_{18}) is based on hybrid silica and it has lower amounts of free silanols than the partially porous column (Kinetex-C_{18}). [68]

5 HILIC with core-shell and sub 2 µm totally porous stationary phases.

Analysis of several hydrophilic molecules by reverse phase HPLC is hindered by the lack of retention of conventional stationary phases. Hydrophilic interaction liquid chromatography (HILIC) is a highly efficient alternative to effectively separate small

polar compounds on polar stationary phases.[69] Due to their characteristics, several pharmaceutical compounds can be separated in by HILIC.

The combination of the lower pressure caused by the lower viscosity of the solvents used in the mobile phase and partially porous columns can be explored to provide high resolution and short analysis times. For example, several basic (nortriptyline, diphenhydramine, benzylamine, procainamide) neutral (caffeine and phenol) and acid (2-naphthalenesulfonic acid and p-xylenesulfonic acid) pharmaceuticals were separated using a 45 cm long column packed with core-shell particles [three Halo silica columns (15 cm × 4.6 mm, 2.7 μm) coupled in series], with efficiencies higher than 100,000 plates per meter. Even though three columns were coupled in series, a relatively low system pressure was reported (250 bar) using acetonitrile-ammonium formate (85:15 v/v) as mobile phase, at a flow rate of 1 mL/min. [70].

However, an important aspect of superficially porous columns for HILIC separations is that column efficiency is not influenced at high flow rates, which means that shorter analysis times could be achieved at higher flows rate while generating acceptable pressure levels.

The use sub 3 μm core-shell and sub 2 μm totally porous stationary phases in the HILIC mode is a valuable tool for achieving fast and efficient separations of basic pharmaceuticals and to reduce frictional heating with sub 2 μm totally porous stationary phases. For example, Okusa et al.,[71] had shown that dextromethorphan is a very challenging test probe, even for modern RP stationary phases. In this context, separation midazolam, bupropion, dextromethorphan and their main metabolites (OH-midazolam, OH-bupropion, and dextrorphan), in the HILIC mode (mobile phase: 10mM formate

buffer (pH 3; 10 mM) and ACN. 30 ºC, Flow rate*:* 0.5 mL/min, gradient profile: 95% ACN for 1.20 min, then 95–80% MeCN in 3min, slope: 5%/min) using Acquity BEH HILIC (100 x 2.1 mm, 1.7 µm) and Ascentis HILIC (100 x 2.1 mm, 2.7 µm) columns was recently reported. The separation was achieved with symmetric peaks on both columns in 4.5 minutes. The observed backpressures were also reasonable (375 and 150 bar, for Acquity BEH HILIC and Ascentis HILIC, respectively). In this case, the high amount of organic modifier used was responsible for the low back pressure generated and for the good peak shape observed. [72]

High pressure drop and frictional heating obtained with sub 2 µm totally porous stationary phases are not relevant in HILIC, while the long equilibration times are observed with totally porous 5 µm stationary phases in the HILIC mode are reduced with sub 2 µm totally porous stationary phases. These observations were made by Periat et al.,[73] who provided a complete guide for method development using sub 2 µm totally porous stationary phases.

6 Partially porous and sub 2 µm totally porous stationary phases used for macromolecules analysis.

The importance of macromolecule analysis is increasing due to the development in several areas, specially the "omics" sciences. However, macromolecules are highly complex and their analysis is a challenging task, where several components need to be separated.

In this aspect, both partially porous and sub 2 µm totally porous stationary phases can provide several advantages. In fact, the core-shell materials were developed to limit diffusion of macromolecules into the pores of the stationary phase to improve their separation. [74]

Comparison of modern partially porous (Kinetex C_{18}) and sub 2 µm totally porous (Acquity BEH C_{18}) stationary phases (with same dimensions and particle size) for the separation of protein of different sizes revealed that for small molecules both stationary phases had similar efficiencies. In contrast, for large molecules the partially porous stationary phase showed higher efficiency than the totally porous. [75].

However, it is important to highlight that even partially porous stationary phases could result in poor resolution of macromolecules if its pore sizes are not large enough. For example, Gritti and Guiochon[76] observed that a partially porous stationary phase (Halo C_{18}) with 90 Å pore diameter provided lower efficiencies and higher C terms than totally porous stationary phases [Atlantis (dp 3µm and 101 Å pore diameter)] because the pore size was not large enough for the large proteins used as test probes in this study.

In large molecules analyses, core-shell stationary phases may afford higher efficiency than totally porous particles, when the pore sizes are large enough to allow penetration by macromolecules. This aspect is illustrated by a recent study, where a large pore core-shell stationary phase (Aeris WP C_{18} - 3.6 µm particle diameter), was compared with large pore totally porous stationary phases (Acquity BEH300 C_{18} and C_4; both with 3.6 µm particle diameter and 300 Å pore sizes), to analyze macromolecules (recombinant monoclonal antibodies). It was reported that the partially porous stationary phase provided higher efficiencies than totally porous stationary phases due the limited diffusion of macromolecules into the pores of the partially porous column. [77]

Another interesting example was reported by Ricker and co-workers [78]. They compared a wide pore (300 Å) 5 µm totally porous stationary phase (Zorbax 300SB-C_{18}

and Zorbax 300Extend-C_{18}) with (300 Å) 5 μm partially porous stationary phases (Poroshell 300SB-C_{18}, 300SB-C_8, 300SB-C_3, and 300Extend-C_{18}) for the separation of peptides and proteins with molecular weights from 1,673 to 950,000 Daltons. They observed that peptides and proteins show less peak broadening at high flow rates (linear velocity) on wide-pore, superficially porous particles as compared to that on totally porous particles.

Gritti et al.,[79] compared columns packed with the partially porous particles (2.7 μm) and with totally porous particles (3 μm), both stationary phases with 90 Å pore size for the separation of [naphthalene, insulin (5.8 kDa), lysozyme (14.3 kDa) and β-lactoglobulin (18.4 kDa) were used as test solutes]. They observed that shell structure does not seem to bring any advantage compared to a totally porous structure for low molecular weight compounds with respect to the mass transfer kinetics. It does lead to faster kinetics for high molecular weight compounds and allows markedly improved performance at high flow rates. For compounds with low diffusivities such as proteins or large peptides, the mass transfer kinetics are faster and the C term of the partially porous column is about one-half that of a column packed with totally porous silica particles.

In this context, in a recent review article it was suggested that the development of new stationary phases with wide-pore core–shell particles or fully porous sub-2 μm 300 Å particles make possible the fast and efficient separations of peptides and proteins in the RPLC mode. [77]. Recombinant monoclonal antibodies (mAbs) analysis is an interesting example of how development of wide pore sub 2 μm totally porous and wide pore totally porous particles have opened new horizons in macromolecule analysis. mAbs are glycoproteins that belong to the immunoglobulin (Ig)family. They have become particularly relevant for the treatment of autoimmune diseases or cancers. [80] In

2010, the global therapeutic mAbs market was a $48 billion. [80] Their analysis was mainly achieved by electrophoretic approaches,[80] but the introduction of wide pore sub 2 μm totally porous and wide pore totally porous particles boosted the development of very efficient analyses methods for mAbs in the RPLC mode. [80, 81] These methods [62, 63] were developed with the totally porous stationary phases (Acquity BEH-300 C_{18} and C_4 (1.7 μm particles with a pore size 300 Å) and partially porous columns (Aeris Widepore C_{18} and C_4, 3.6 μm particles and 300 Å pores).

There are several examples of applications of partially porous technology for the analysis of macromolecules. In one study, separation of monoclonal IgG2 disulfide isomers was achieved in 10 min using a partially porous stationary phase (Poroshell300SB-C_8 - 150 × 2.1□mm, 5□μm, 300□Å). [82]. A similar column was used [Poroshell 300SB-C_{18} (75 mm × 2.1 mm, 5 μm)] for a fast (7 minutes) and direct determination of Polysorbate 80 from an injection solution containing a four-helix bundle protein which belongs to the family of cytokines. [83]. Another study also used a a partially porous stationary phase (Halo Peptide-ES 160 Å; particle sizes ranging from 2.2 to 5 μm) for the analysis of several large molecules. [84]

Zirconia and titania have a large pore size (~300 Å). However, proteins are irreversible retained on this material.[85] This adsorption is the result of the high hydrophobicity of the polybutadiene coating and the strong Lewis acid sites on the zirconia surface causing strong interactions between proteins and the stationary phase. The combination leads to irreversible adsorption of proteins on polybutadiene-coated zirconia. However, while zirconia and titania are not useful as stationary phases in LC analysis of proteins,these materials are well-suited for solid phase extraction of phosphorylated peptides. [86] In addition, Rhinophase®-AB (ZirChrom, Anoka, USA) [prepared by refluxing particles of zirconia in a ethylenediamine-N,N'-

tetra(methylenephosphonic) acid (EDTPA) solution] can effectively purify a wide range of Mab subclasses, as well as polyclonal hIgG, IgA and IgM, as reported by Clausen et al.,[87]

Conclusions

Recent developments in chromatographic technology have resulted in core-shell and sub 2 µm stationary phases becoming more popular. Soon, it is likely that these particles will be dominant. However, at present, the 5 µm diameter is still the most used stationary phase support material.

Nowadays, in my working group, we just use sub 3 µm core shell stationary phases, in conventional LC equipment (Waters Alliance e2695), the columns are purchased in a 4.6 x 150 mm size to overcome band broadening. With these columns, we are able to achieve high efficient separations in short separation times. My working group had shared this experience with many separation groups in our University and all other groups had adopted core-shell stationary phases.

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Table 1: Examples of Commercial Core-Shell Stationary Phases

Supplier	Product name	Particle diameter (µm)	Shell thickness (µm)	Surface chemistries –
Macherey- Nagel (Düren, Germany)	Nucleoshell (90 Å)	2.7	0.5	RP-$_{18}$, Phenyl-Hexyl, Pentafluorophenyl and HILIC (Ammonium – sulfonic acid)
ChromaNik Technologies Inc (Osaka, Japan)	SunShell (90 Å)	2.6	0.5	C_{18}, C_8, Phenyl, PFP, 2-Ethylpyridine (2-EP), HILIC-Amide,
ChromaNik Technologies Inc (Osaka, Japan)	SunShell (160 Å)	2.6	0.5	WP-C_{18} and RP-AQUA
ChromaNik Technologies Inc (Osaka, Japan)	SunShell HFC18-16 (160 Å) and SunShell HFC18-30 (300 Å)	2.6	0.5	HFC_{18} stationary phase is an Hexa-Functional C18, it has six functional groups and it is prepared using a mixture of Hexamethydichlorotrisiloxane + Trimethylchlorosilane
Agilent (Palo Alto, CA, USA)	Poroshell 300 (300 Å)	5	0.25	SB-C_{18}, C_8, C_3, Extended
Agilent ((Palo Alto, CA, USA)	Poroshell 120 (120 Å)	2.7	0.50	EC-C_{18}, EC-C_8, EC-CN, SB-C_{18}, SB-C_{18}, SB-Aq, Bonus-RP, Phenyl-Hexil, HILIC (SB stationary phases are non-endcapped, EC stationary phases are endcapped)
Advanced Material Technology (Wilmington, Delaware, USA)	Halo (90 Å)	2.7 5.0	0.5 0.60	C_{18}, C_8, HILIC, RP-amide, phenylhexyl, pentafluorophenyl
Advanced Material Technology (Wilmington, Delaware, USA)	Halo Peptide-ES (160 Å)	2.7	0.50	C_{18}
Phenomenex (Torrance, California, USA)	Kinetex (100 Å)	5 2.6 1.7 1.3	 0.35 0.23	C_{18}, XB-C_{18}, C_8, HILIC and pentafluorophenyl
Sigma–Aldrich (Bellefonte, Pennsylvania, USA)	Ascentis Express (90 Å)	2.7 5.0	0.50	C_{18}, C_8, HILIC, RP-amide, phenylhexyl, pentafluorophenyl
Sigma–Aldrich (Bellefonte,	Ascentis Express Peptide-ES	2.7	0.50	C_{18}

Pennsylvania, USA)	160 Å			
Sigma–Aldrich (Bellefonte, Pennsylvania, USA)	BIOshell A160 Peptide (160 Å)	2.7 5		CN, C18 CN, C18
Sigma–Aldrich (Bellefonte, Pennsylvania, USA)	BIOshell A400 Protein (400 Å)	3.4		C4
Thermo Scientific (Faltam detalhes)	Accucore (80 Å)	2.6	0.50	C_{18}, aQ, RP-MS, HILIC, phenylhexyl, pentafluorophenylpropyl (PFP), Polar Premium,
Thermo Scientific	Accucore XL (80 Å)	4		C_8, C_{18} and Amide-HILIC
Thermo Scientific	Accucore nanoViper 150 (150 Å)	2.6	0.5	C_4 and C_{18}.
Thermo Scientific	Accucore (80 Å)	2.6	0.5	C_{18}, RP-MS, C_8, AQUA, Polar Premium, Phenyl-Hexyl, PFP, Phenyl-X, C_{30}, HILIC and Urea-HILIC
Phenomenex (Torrance, California, USA)	Aeris Widepore (200 A) Aeris Peptide (100 Å) Aeris Peptide (100 Å)	3.6 1.7 3.6	0.2 0.22 0.5	XB-C_{18}, XB-C_8, C_4
Wissenschaftliche Geratebau (Berlin, Germany)	BlueShell (80 Å)	2.6		C_{18}, C_{18} AQUA, HILIC
PerkinElmer (Waltham, MA, USA)	Brownlee (90 Å)	2.7	0.5	C_{18}, C_8, HILIC (bare silica), pentafluorophenylpropyl (PFP), phenylhexyl and RP-Amide
PerkinElmer (Waltham, MA, USA)	Brownlee Peptide ES-C18† (160 Å)	2.7	0.5	C_{18}
Shiseido (Japan)	CAPCELL CORE (90 Å)	2.7	0.5	polymer-coating
Waters (Milford, MA, USA)	CorTec (90 Å)	1.6	0.5	C_{18}, C_{18+} (CSH technology) HILIC
Protea Biosciences Group, Inc. (Morgantown, WV, USA)	Amplus (300 and 160 Å)	2.6		C_8, C_{18} and C_4
Waters (Milford, MA, USA)	CORTECS (90 Å)	1.6	0.5	C18, $C18_+$ (CSH technology) HILIC
Advanced Chromatography	ACE UltraCore (95)	2.5		C18, Phenyl-Hexyl

Technologies Limited. (Aberdeen, Scotland)		5		
Restek (Bellefonte, PA, USA)	Raptor (90 Å)	2.7		ARC-18 and Biphenyl
Nacalai (San Diego, CA, USA)	Cosmocore (90 Å)	2.6	1.6	C18

Figure Captions

Fig. 1: Main parameters affecting resolution and analysis time in HPLC.

Fig. 2: Experimental H – u plots of columns packed with 1.3, 1.7, 2.6 and 5 µm core–shell particles (peak widths were corrected for the extra-column band broadening). The test solution was eluted with water/acetonitrile 63/37 on the Kinetex 1.3 µm column. The mobile phase consists of water/ACN 62/38 (v/v) for the 1.7 µm and 2.6 µm Kinetex columns, and a mixture of water/ACN 60/40 (v/v) for the 5 µm column, to keep the same retention factors for the test solutes on the different columns. The column efficiency of butylparaben was considered. The mobile phase ensured a retention between k = 6–7.(Reproduced with permission from reference[14])

Fig. 3: Graphical representation of A. pressure tolerance and type of pumping system, and B. standard system dwell volume of all the UHPLC systems ($\Delta P > 600$ bar) commercially available. In A., light red expresses low pressure system volume while dark blue represents high pressure system volume. It is important to notice that the standard dwell volume reported in this figure can be modified on a few instruments either by bypassing the damper and mixer, or by changing the volume of the mixing chamber. (Figure reproduced from reference[31])

Fig. 4: Effect of columns diameter on efficiency. Chromatograms for columns with four different internal diameters. Conditions: Zorbax SB Extend C-18, 1.8 µm particles; 50 mm column length; flow rates for 4.6, 3.0, 2.1, 1.0 mm i.d. columns were 1.4, 0.60, 0.29, and 0.067 mL/min, respectively; the injection volumes 4.6, 3.0, 2.1, 1.0 mm i.d. columns were 4.8 µL, 2.0 µL, 1.0 µL, and 0.23 µL, respectively; the sample concentration was 0.1 mg/mL for each analyte; Peak identifications: from left to right (1) uracil; (2) benzylalcohol; (3) acetophenone; (4) propiophenone; and (5) benzophenone. (Figure reproduced from reference[32]).

Fig. 5: Knox curves of commercially available sub-3 µm and sub-2 µm packed columns obtained with ethinylestradiol. Experiments were conducted on 5 cm long narrow bore columns in 48/52 ACN/H_2O at 35 °C, $D_M = 1.15 \times 10^{-5}$ cm^2 (Reproduced with permission from reference[44])

Fig. 6: Cartoon graphic and SEM microphotograph of fused-core particle with 400 Å pores. (Reproduced with permission from reference[45])

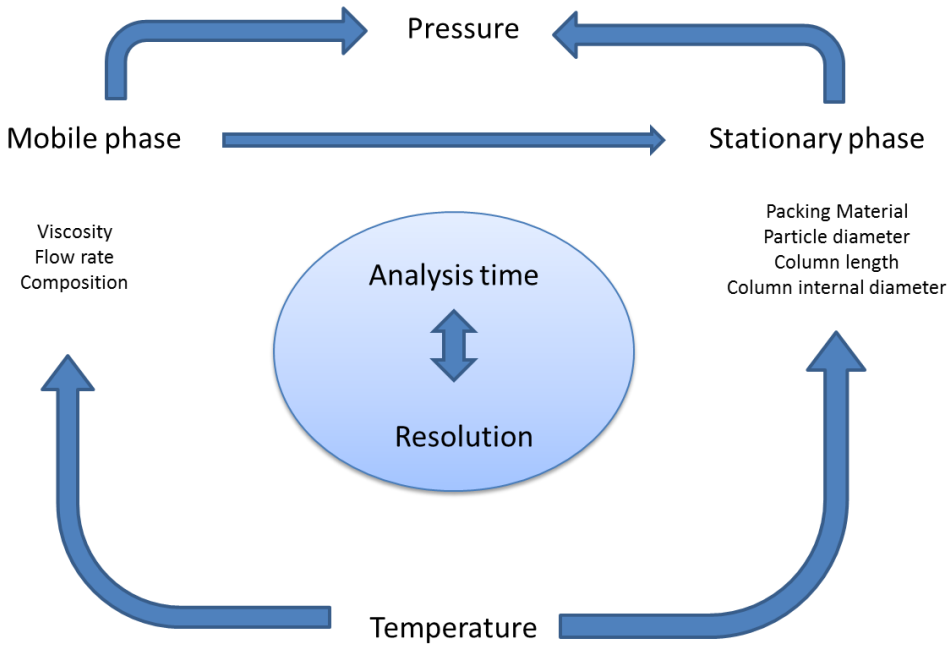
Fig. 7: Cumulative frequency (a) and particle size distribution (b) of Ascentis Express 2.7 µm shell particles and Waters UPLC BEH 1.7 µm porous particles. (Reproduced with permission from reference[46])

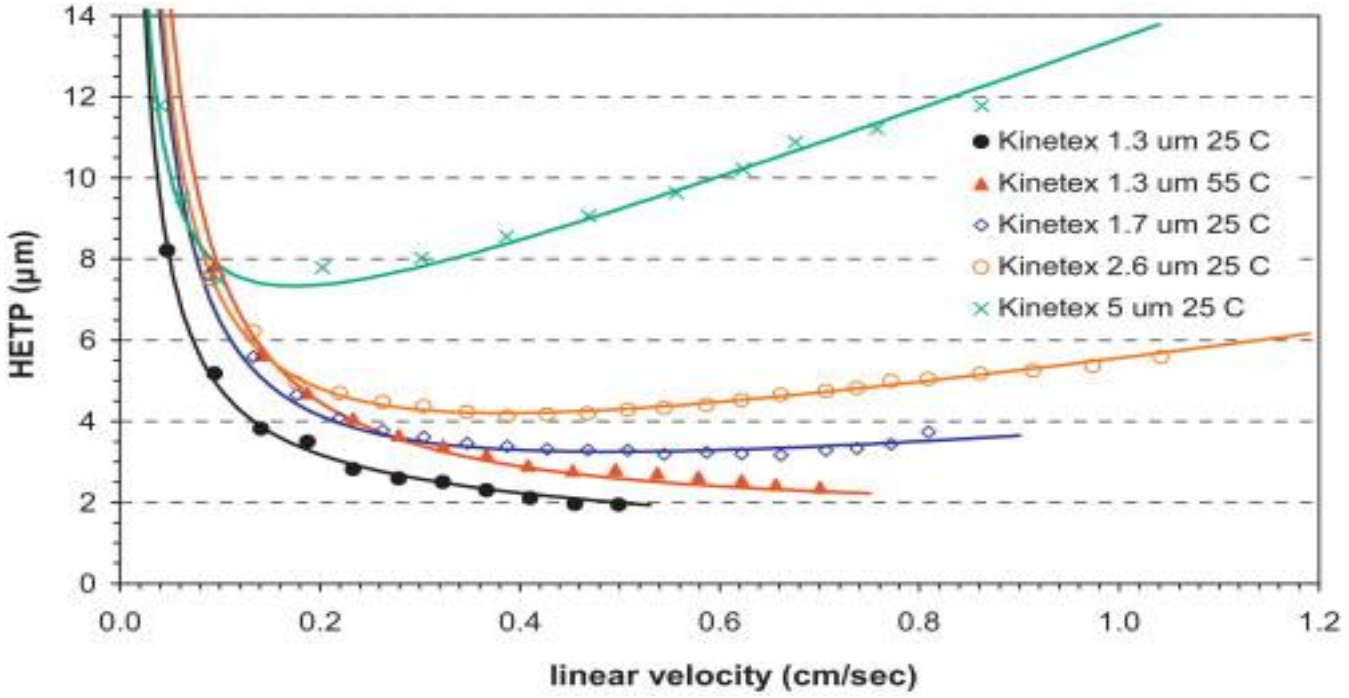
Fig. 8: Normalized particle size distributions of the different evaluated support types, determined from SEM pictures. XBridge C_{18}(d_p = 3.5 µm) (■), ACE3 C_{18} (d_p = 3.0 µm) (●), Gemini NX C_{18} (d_p = 3.0 µm) (◆), Hypersil Gold C_{18} (d_p = 3.0 µm) (▲), Kinetex

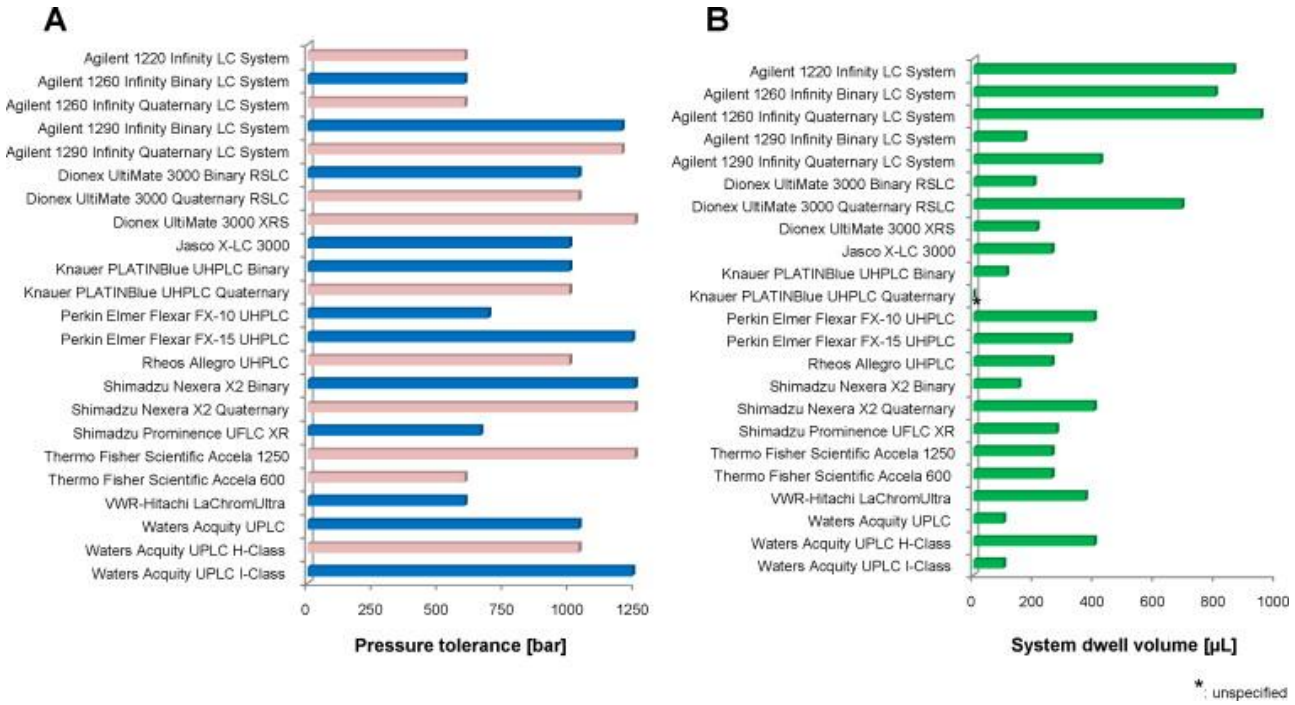
Fused Core C_{18} (d_p = 2.6 µm) (□), HALO Fused Core C_{18} (d_p = 2.7 µm) (▵) and Poroshell C_{18} (d_p = 2.7 µm) (○). (Reproduced with permission from reference[47])

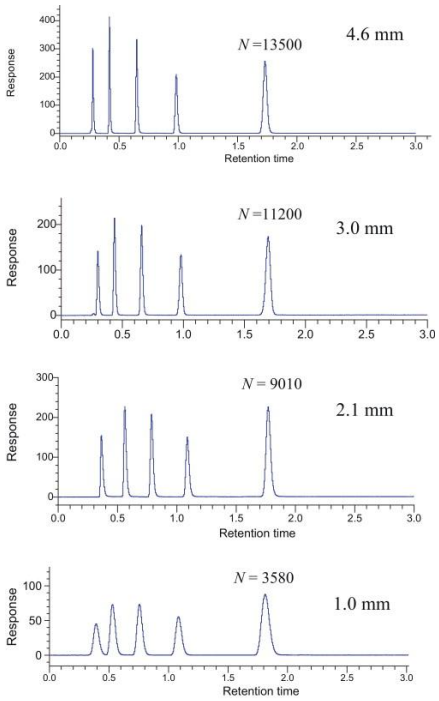
Fig. 9: Chromatograms and performance recorded on the Kinetex 250 mm × 4.6 mm 5 µm core–shell particle column (A and B) and the Zorbax 250 mm × 4.6 mm 5 µm fully porous particle column (C and D) at their optimal flow rate (A–C) and a flow rate almost three times higher (B–D). Teste solutes were uracil, butyrophenone, benzophenone and valerophenone . (Reproduced with permission from reference [48])

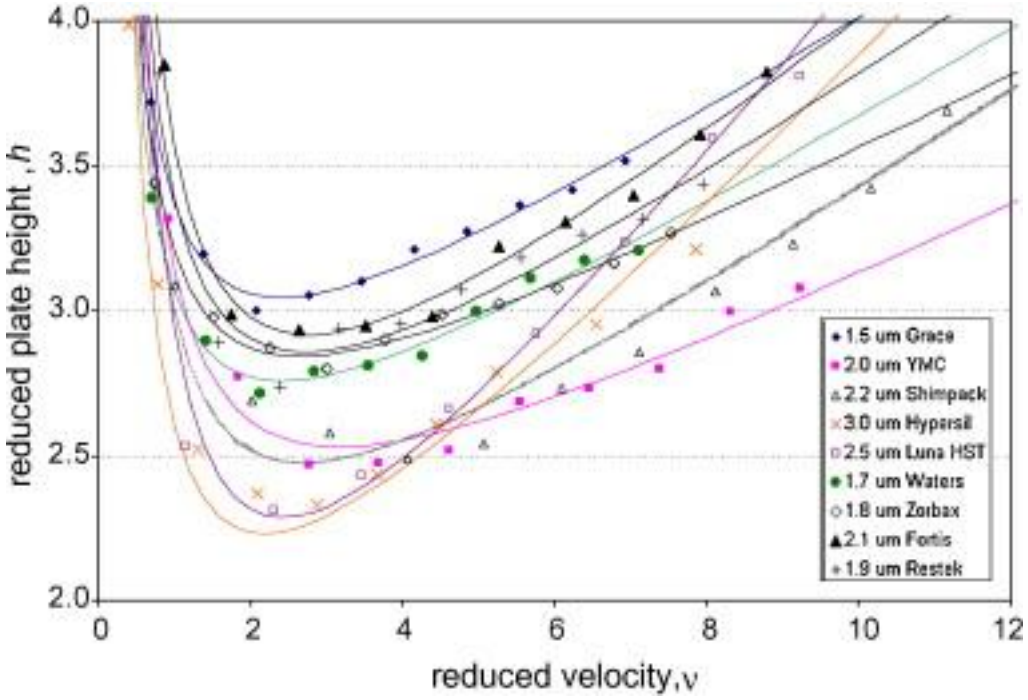
Fig. 10: Representative chromatogram of test samples. Conditions: the volume fractions of acetonitrile at the beginning and at the end of the gradient were set at 40 and 90%, the columns (5 cm × 2.1 mm) were thermo-stated at 30 °C, the injected volume was 0.5 µl. The gradient time was set as 3 min, at the flow-rate of 0.8 ml/min. Analytes: degradation products/impurities of ethinyl-estradiol (1,2,4,5,13), dienogest (3), ethinyl-estradiol (6), estradiol (7), finasteride (8), bicalutamide (9), gestodene (10), levonorgestrel (11), tibolone (12), and noretistherone-acetate (14). Peak 1,6,7,8,9,10,11,12,13 and 14 were considered for peak capacity calculations. (Reproduced with permission from reference[49])

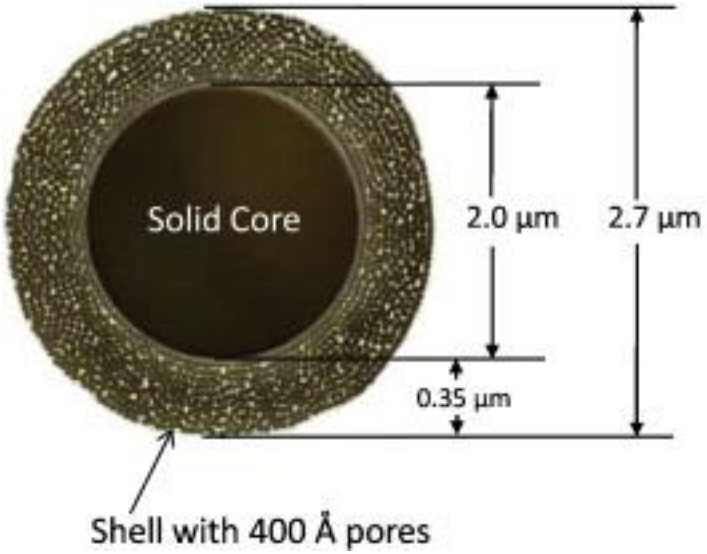


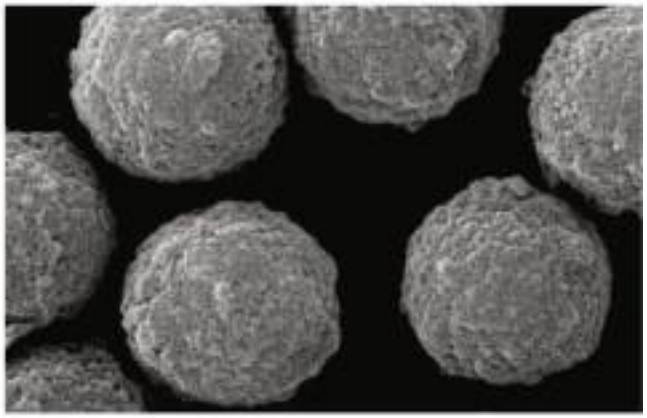


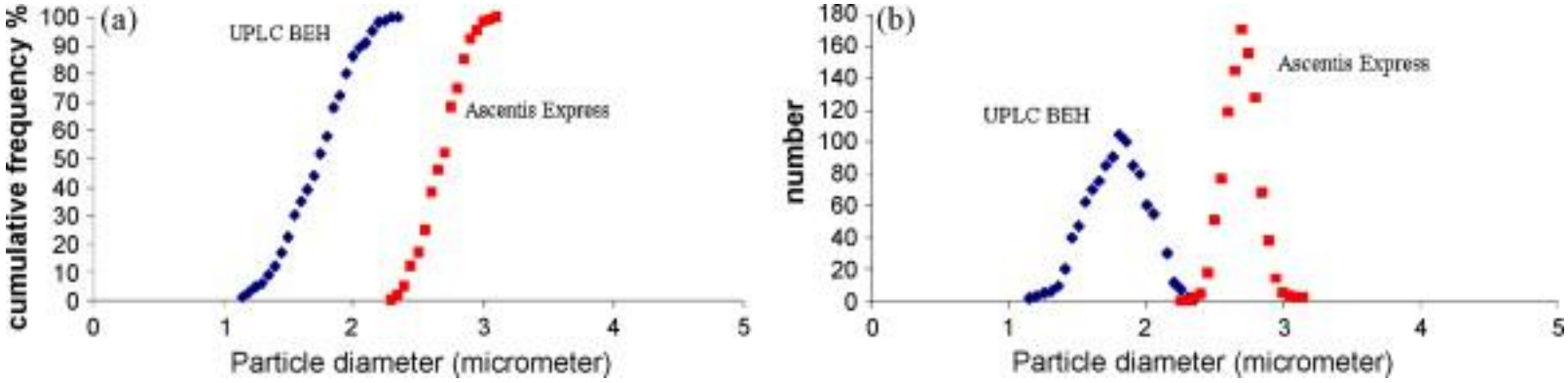












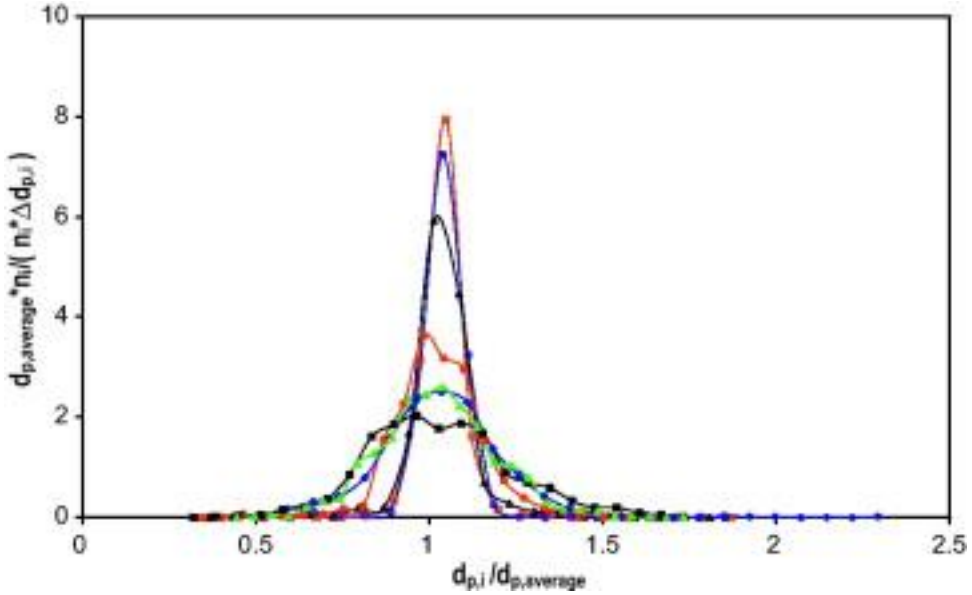


Fig. 2 Normalized particle size distributions of the different evaluated support types, determined from SEM pictures. XBridge C 18 (d p = 3.5 µm) (■), ACE3 C 18 (d p = 3.0 µm) (<ce:inline-figure baseline="0.0">

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Relationship between the particle size distribution of commercial fully porous and superficially porous high-performance liquid chromatography column packings and their chromatographic performance

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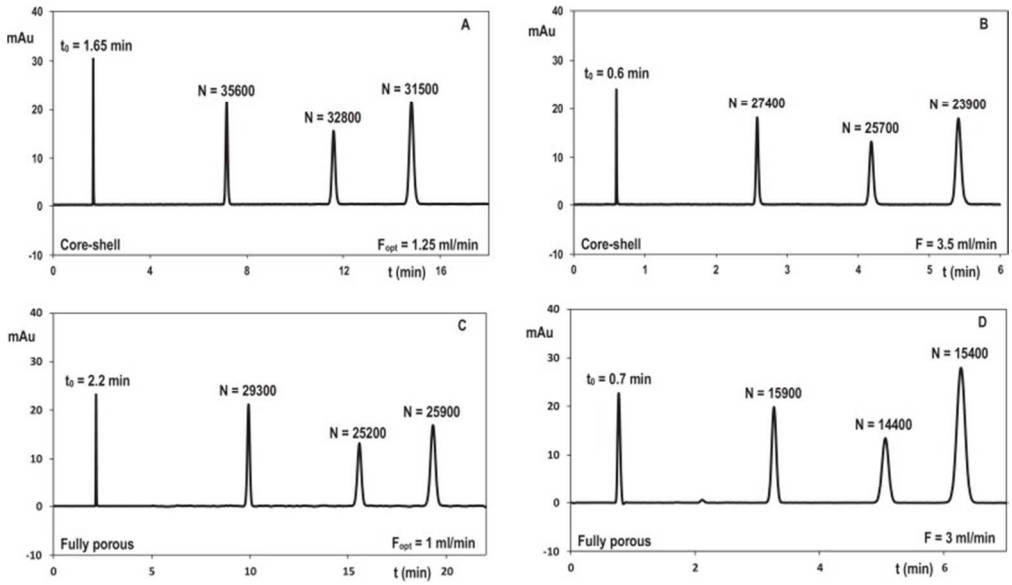

181x104mm (113 x 113 DPI)