



## Review

# Importance of instrumentation for fast liquid chromatography in pharmaceutical analysis

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## ABSTRACT

In the last decade, an important technical evolution has occurred in pharmaceutical analysis with numerous innovative supports and advanced instruments that have been proposed to achieve fast or ultra-fast separations in LC with an excellent sensitivity and ease of operation. Among the proposed strategies to increase the throughput, the use of short narrow-bore columns packed with sub-3 µm core-shell and porous sub-2 µm particles have emerged as the gold standards. Nevertheless, to take the full benefits of these modern supports, a suitable chromatographic system has to be employed. This review summarizes the instrumental needs and challenges in terms of extra-column variance, dwell volume, maximum system pressure, detector data acquisition rate, and injection cycle time. In addition, because of their reasonable pressure drop, the use of columns packed with sub-3 µm core-shell particles on a conventional LC instrument is discussed in detail. A methodology is proposed to check the compatibility between stationary phase and instrument, and some solutions are proposed to improve the performance of standard instruments.

Finally, because the column technology is evolving faster than instrumentation, it is nowadays possible to purchase short, narrow-bore columns packed with 1.3 µm core-shell particles. Micro columns (1 mm I.D.) packed with 1.7–1.9 µm porous particles are also available from several providers, which limit frictional heating effects and reduce solvent and sample consumption. However, it remains difficult to find instruments compatible with such column geometries and a severe loss of performance may be observed due to the system itself.

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## 1. Introduction: current solutions for fast analysis in LC

LC has been strongly evolving in the last few years with the emergence of numerous attractive supports that have been proposed to achieve rapid and efficient procedures for qualitative and quantitative analysis. Indeed, there is a growing demand for high-throughput separations in numerous fields (e.g., environmental,

food, or chemical analysis) where the time response delivery has to be reduced or the productivity enhanced, due to the large number of analyzed samples. The main driving force for faster separations remains the pharmaceutical field due to the need for enhanced productivity as well as reduced costs. In fact, a significant number of pharmaceutical applications, including purity assays, quality control, pharmacokinetic, and drug metabolism studies require high throughput separations.

Raising the mobile phase flow rate and reducing the column length while maintaining similar particle morphology appears to be the easiest way to decrease analysis time in LC. With this

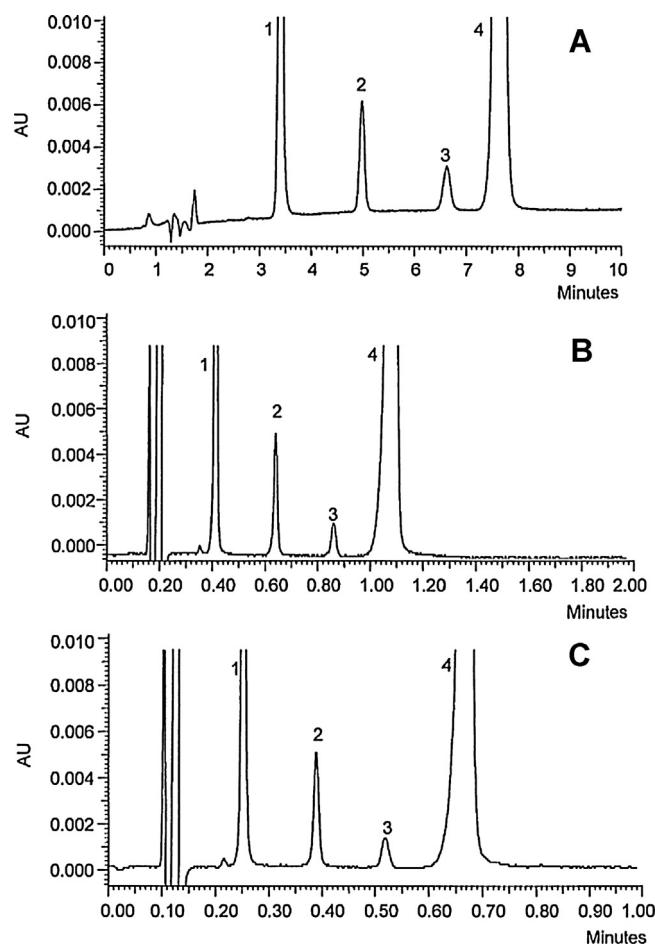
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approach, the kinetic performance is however strongly compromised, because the plate count is directly proportional to the column length and, according to the van Deemter curve, working beyond the optimal linear velocity generates an additional loss of efficiency. For this reason, this approach is often inappropriate, except for the analysis of simple mixtures of compounds [1].

An alternative approach to the traditional columns packed with spherical silica-based particles is the use of monolithic supports, which consist in a single rod of porous material possessing unique features related to its bimodal structure composed of macropores and mesopores. This material, originally developed in the 90's in academic laboratories by Hjerten, Frechet and Svec, and Tanaka and Nakanishi, became available in 2000 from Merck under the trademark Chromolith®. The commercial version of the silica-based monolith is composed of 2 µm macropores and 12 nm mesopores, providing a kinetic efficiency equivalent to the one obtained with a column packed with 3.5 µm particles and backpressure similar to a column packed with 11 µm particles [2]. In 2007, Guiochon stated that the recent invention and development of the monolithic columns was a major technological innovation in column technology. The design, the preparation, and the application of monolithic columns are probably the most vibrant area of research in LC [3]. However, despite the tremendous advantages of monoliths, less than 1% of liquid based separation applications are routinely using this column technology [4]. This can be explained by the lower performance compared to alternative approaches, such as UHPLC and core–shell technology. Nevertheless, a second generation of silica-based monoliths (macropores of 1.1 µm and mesopores of 13 nm) has been released in 2011. This new generation provides performance close to the one of sub-2 µm particles at a reduced pressure drop [5], due to an independent control of the macropore and mesopore sizes through the sol–gel synthesis process, allowing for tuning the permeability and the chromatographic efficiency. However, due to a limited number of available providers, chemistries, and dimensions, these phases also remain scarcely used.

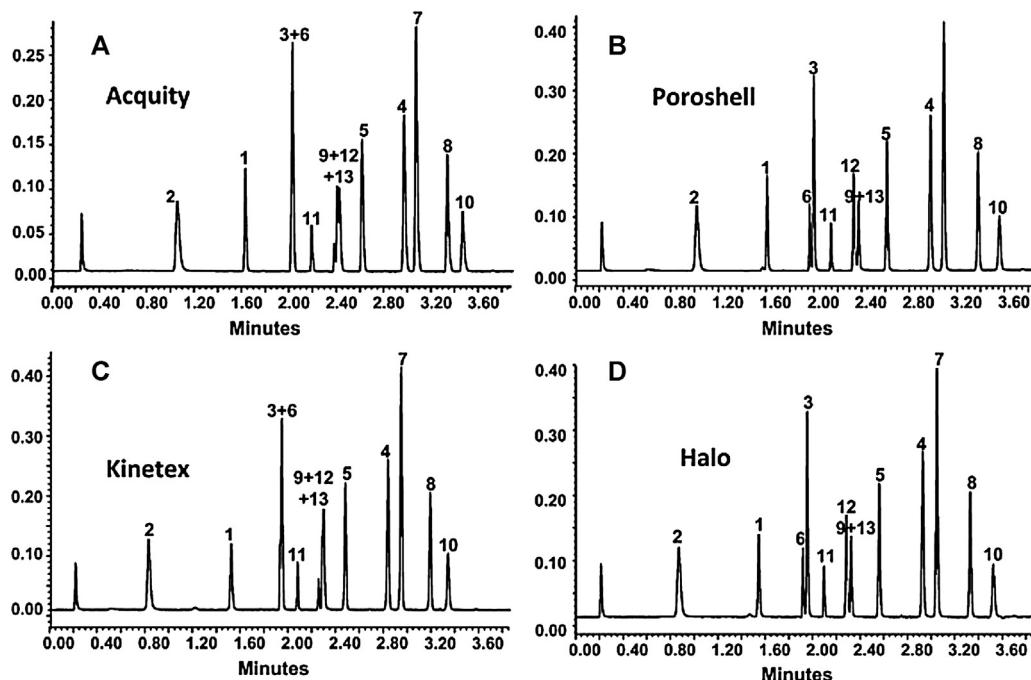
It has been very early reported by Knox and others that small particles lead to improved performance in LC [6–8]. However, the technical challenge to pack columns in a reliable way with very fine porous particles (below 2 µm) was only recently resolved. In addition, Knox explained in 1977 [7] that ultra-fast LC would require a new generation of instrumentation with reduced volumes, higher pressure capability, and improved data acquisition rate. In this context, 20–30 years passed before obtaining both appropriate columns and instruments for UHPLC operation. In 2004, the first commercial column packed with porous 1.7 µm hybrid silica particles, able to withstand pressure up to 1000 bar, was released on the market, combined with adequate UHPLC instrumentation [9,10]. With such innovative stationary phase technology, the throughput can be theoretically increased by 9-times, while maintaining a similar kinetic efficiency compared to the conventional 5-µm packing [11]. However, the backpressure generated by small particles could be prohibitive for conventional HPLC systems, as it is proportional to  $d_p^2$  according to the Darcy's law, and even to  $d_p^3$  when working under optimal linear velocity conditions [12]. Working with dedicated UHPLC systems compatible with pressure beyond 600 bar is therefore recommended. 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 1300 bar) are available on the market [13]. Fig. 1 illustrates an example of the possibilities offered by UHPLC in the pharmaceutical field. An isocratic method for the quality control of a pharmaceutical formulation of lidocaine was transferred from conventional HPLC (150 × 4.6 mm, 5 µm, Fig. 1A) to UHPLC (50 × 2.1 mm, 1.7 µm, Fig. 1B and C) [1]. Efficiency remained similar but the analysis time was ca. 8-fold



**Fig. 1.** Separation of a pharmaceutical formulation "Rapidocain" in isocratic mode. Experimental conditions: mobile phase containing ACN/phosphate buffer at pH 7.2 (40:60, v/v). A. Waters Xterra RP18, 150 × 4.6 mm, 5 µm,  $F = 1000 \mu\text{l}/\text{min}$ ,  $V_{\text{inj}} = 20 \mu\text{l}$ . B. Waters Acuity Shield RP18, 50 × 2.1 mm, 1.7 µm,  $F = 613 \mu\text{l}/\text{min}$ ,  $V_{\text{inj}} = 1.4 \mu\text{l}$ . C. Waters Acuity Shield RP18, 50 × 2.1 mm, 1.7 µm,  $F = 1000 \mu\text{l}/\text{min}$ ,  $V_{\text{inj}} = 1.4 \mu\text{l}$ . 1. methylparaben; 2, 2,6-dimethylalanilin; 3, propylparaben; 4, lidocaine. Reproduced by permission of The Royal Society of Chemistry (<http://www.rsc.org/shop/books/2012/9781849733885.asp>).

reduced at the optimal flow rate and even up to 12-fold at the maximal pressure of the UHPLC system.

High throughput analysis in LC can also be achieved with sub-3 µm superficially porous particles, also known as fused-core or core–shell technology. Core–shell columns are basically packed with 2.6 or 2.7 µm particles, including a 1.7 or 1.9 µm solid inner core surrounded by a thin 0.35 or 0.5 µm porous layer [14]. An efficiency of ca. 80% of the one achieved with fully porous sub-2 µm particles (UHPLC) has been reported with sub-3 µm core–shell particles but with a 2 to 3-times lower backpressure compared to UHPLC phases. This impressive kinetic performance has been extensively investigated by Guiochon and Gritti [15–17], Fekete *et al.* [18,19], Desmet *et al.* [20] or Guillarme *et al.* [21,22] which reported that this high plate count at moderate pressure was related to a reduced height equivalent to a theoretical plate ( $h_{\text{min}}$ ), comprised between 1.5 and 2 [21] and even 1–1.3 for standard columns of 4.6 mm I.D. [23], associated with a good permeability. This small  $h_{\text{min}}$  value was probably explained by the narrow particle size distribution and the high packing density, leading to a small A term (eddy dispersion) of the Van Deemter equation. Moreover, the reduction of the solutes diffusion path appeared to also positively impact the B (longitudinal diffusion) and C (mass transfer resistance) terms of the Van Deemter equation, especially in case of large



**Fig. 2.** Separation of 13 pharmaceutical compounds on A. Acuity BEH C18, 50 × 2.1 mm, 1.7 µm; B. Poroshell 120 EC-C18, 50 × 2.1 mm, 2.7 µm; C. Kinetex® C18, 50 × 2.1 mm, 2.6 µm, and D. Halo C18, 50 × 2.1 mm, 2.7 µm. See [21] for experimental conditions.

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solutes. Besides the kinetic performance, some studies demonstrated that the loading capacity, the retention, the selectivity, and the peak shape of small analytes [21] as well as large biomolecules [22] were very close to the ones obtained with fully porous sub-2 µm particles despite the lower amount of porous material. This behaviour is illustrated in Fig. 2 with a mixture of 13 pharmaceutical compounds. The same fast gradient analysis was performed on a fully porous sub-2 µm phase (Fig. 2A) and on three different core-shell sub-3 µm particles from various providers (Fig. 2B–D). The overall performance was comparable between the different conditions, but the backpressure was ca. 2-times reduced with the core-shell particles. Thanks to the commercial success of sub-3 µm core-shell particles, the range of available particle sizes has been recently extended to smaller (1.3–1.7 µm) [19] and larger (4–5 µm) [24] ones.

As previously discussed, a number of technical solutions for high throughput pharmaceutical analysis are nowadays available. However, to take the full advantage of the monolithic phases, the columns packed with fully porous sub-2 µm particles, and the stationary phases made of core-shell sub-3 µm particles, the instrumentation characteristics should be definitely considered. Indeed, fast or ultra-fast analyses present some constraints in terms of extra-column and dwell volumes, pumping pressure capability, injection time, and detector acquisition rate. This review aims to answer practical questions, i.e., (i) what are the instrumental requirements to properly work with the different column technology currently available, (ii) are the columns packed with sub-3 µm core-shell really compatible with any LC instruments, (iii) how is it possible to transform a relatively old-generation LC device to make it compatible with high throughput separations, and (iv) are the current chromatographic systems suitable for any existing column technology, focusing on the requirements and challenges.

## 2. Importance of extra-column volume and variance

The success of fast and efficient separations depends on the intrinsic column efficiency, but also on its preservation by

minimizing the instrument induced extra-column band spreading. Extra-column band broadening effects are much more critical when using small columns with smaller internal diameter than the standard ones, e.g., 4.6 or 3.0 mm. Several papers have discussed the extra-column effects as a major concern that negatively impacts the apparent column efficiency under fast and ultra-fast LC conditions [25–27].

Generally, the extra-column effects are taking place in the “external” instrument(extra-column) volumes, including the injector system, the connector tubing, and the detector cell. An additional contribution is related to time-based effects, namely on the time constant of the detector device. Obviously, the observed extra-column peak variance depends on the extra-column volume of the system. The latter is a physical characteristic of the used 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. 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 ( $\sigma_{\text{total}}^2$ ) is the sum of the extra-column peak variance occurring before the column ( $\sigma_{\text{ec,b}}^2$ ), the variance inside the column ( $\sigma_{\text{col}}^2$ ), and the extra-column peak variance generated after the column ( $\sigma_{\text{ec,a}}^2$ ) as expressed in Eq. (1):

$$\sigma_{\text{total}}^2 = \sigma_{\text{ec,b}}^2 + \sigma_{\text{col}}^2 + \sigma_{\text{ec,a}}^2 \quad (1)$$

The peak variance on the chromatographic column can be generally expressed by the Eq. (2):

$$\sigma_{\text{col}}^2 = \frac{V_r^2}{N_{\text{col}}} = \frac{V_0^2}{N_{\text{col}}} (1+k)^2 \quad (2)$$

where  $V_r$  is the retention volume,  $N_{\text{col}}$  is the column plate number and  $V_0$  is the column hold-up volume.

Then, the efficiency loss caused by the system can be estimated and the remaining column efficiency ( $E_r$ ) calculated with Eq. (3) [25]:

$$E_r = 100 \frac{\sigma_{\text{col}}^2}{\sigma_{\text{col}}^2 + \sigma_{\text{ec}}^2} \approx \frac{V_0}{V_{\text{ec}}} \quad (3)$$

where  $\sigma_{\text{ec}}^2$  is the sum of the extra-column peak variances ( $\sigma_{\text{ec},a}^2 + \sigma_{\text{ec},b}^2$ ).

$\sigma_{\text{col}}^2$  is a function of the column volume, while  $\sigma_{\text{ec}}^2$  depends on the extra-column volume ( $V_{\text{ec}}$ ). Then, the efficiency loss caused by the system is predominantly related to the ratio of the column/system volume [28,29]. 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 [29].

Extra-column variance depends on the injected volume ( $V_i$ ), tubing radius ( $r$ ) and length ( $l$ ), flow-cell volume ( $V_c$ ), detector time constant ( $\tau$ ), and flow rate ( $F$ ). It can be estimated according to Eq. (4) [1]:

$$\sigma_{V,\text{ext}}^2 = K_i \frac{V_i^2}{12} + K_c \frac{V_c^2}{12} + \tau^2 F^2 + \frac{r^4 l F^2}{7.6 D_M} \quad (4)$$

in which  $K_i$  and  $K_c$  are constants linked to the injection mode (i.e., speed and geometry) and the detector cell geometry, respectively. To minimize the extra-column dispersion, the injected volume, the detector cell volume, the tubing and the detector time constant have to be primarily optimized. Without going into details, the basic fundamentals of the different dispersion processes are briefly summarized hereafter.

The experimental investigation of injected solute spreading in different parts of chromatographic systems has been the subject of several studies [30–41]. The fundamentals of solute dispersion theory in tubes of circular cross-section were first implemented by Taylor for both laminar and turbulent flows [42,43]. Later, Aris extended Taylor's equation to the cases where the molecular diffusion in the axial direction could not be neglected [44]. The validity of the Taylor–Aris model was then demonstrated in several publications [45–47]. The most significant limitation of the Taylor–Aris equation is that it can only be correctly applied to long residence times (e.g., small flow rate or relatively large tube volumes). Alternatively, Atwood and Golay studied the dispersion in very short tubing lengths [47,48]. When the analyte diffusion was neglected, the dispersion could be estimated from the parabolic flow profile. Neude et al. used a random-walk computer model to find the transition between the classical Taylor–Aris dispersion in long tubes and the absence of diffusion described by Golay's proposal [27]. The Taylor–Aris, Golay and random-walk models are often used to evaluate the contribution of tubing to the band broadening. In all models, the contribution of tubing to the peak variance follows a function of the fourth power of its radius. Therefore, the smallest possible diameter tubes (compromised with pressure) have to be considered, particularly for fast chromatographic separations. The contribution of the detector volume ( $V_c$ ) and the injection plug ( $V_i$ ) to the solute dispersion is volumetric and has a direct effect on the observed peak variance as described in the literature [49,50]. In HPLC systems, detector cells of ca. 10  $\mu\text{l}$  are conventionally used which remains unacceptable for UHPLC separations. Currently, the use of 0.5–2  $\mu\text{l}$  cells with an average pathlength of 10 mm is quite common in state-of-the-art instrumentations. In addition to the volume-based contributions to band spreading, time-based contributions originating from the detector or in filters selected by the user, such as the time constant, need to be understood. The measurements of the influence of the time constant (often called as a

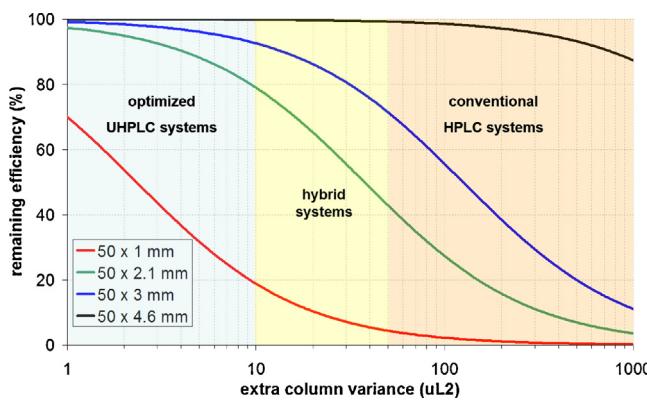
time-based filter) on the width of the apparent signal depend on the flow rate, in agreement with Eq. (4) [27]. At low flow rate, where the peaks are wide in time, the influence of the time-based filters is negligible, but they become important at higher flow rates.

The extra-column peak variances of a given system can be measured in different ways. Currently, there is no clear-cut methodology for the accurate and precise determination of extra-column variances. The most accepted ones are the half-height method and the moment method [51–53]. Both methods are based on a set of measurements without the chromatographic column. The latter is generally replaced by a zero dead volume union. The sample is injected at various flow rates and the band profile recorded.

The band variances can be calculated on the basis of the peak width measured at its half height or can be estimated with the first and the second central moments, which are calculated from the whole elution peak profiles. Since this approach necessitates the integration of the elution peak profile, the raw data have to be exported to such formats that are compatible with appropriate softwares (e.g., Excel, Origin). Calculations based on the exact value of second moment of the peaks may be more accurate than the values based on half-height method, but they are less precise due to the uncertainty of the estimations of the integration times' start and end. When peak profiles are asymmetrical, it has to be noted that the moment method is more adapted.

The extra-column volume can be accurately measured by the following procedure. Elution times have to be recorded at different flow rates with a zero dead volume union, as previously described. When plotting the elution times versus the reciprocal flow rate, the slope of the formed linear relationship directly gives the extra-column volume. In addition, the intercept provides the offset time of the system. The offset time refers to the difference between the moment when the zero time is recorded and the moment when the sample leaves the injection needle. This offset time is negligible in most cases (ca. 0.01 min).

The commercially available LC systems can be classified in three groups, (i) optimized systems for fast separation with very low dispersion ( $\sigma_{\text{ec}}^2 < 10 \mu\text{l}^2$ ), (ii) hybrid LC systems recommended by the vendors for both fast and conventional separations ( $\sigma_{\text{ec}}^2 = 10–50 \mu\text{l}^2$ ), and (iii) conventional LC systems with an extra column variance over 50  $\mu\text{l}^2$  [25]. The extra-column peak variances of several commercially available instruments were reported in the literature. The variance of Waters Acuity UPLC system was ca. 6–7  $\mu\text{l}^2$ , the Agilent 1290 Infinity LC system performed ca. 4–8  $\mu\text{l}^2$ , the variance of the standard Agilent 1200 Infinity and Shimadzu Nexera systems were measured at ca. 13–20  $\mu\text{l}^2$ , the variance of the standard Perkin Elmer Flexar system was determined between 18 and 26  $\mu\text{l}^2$ , the variance of the standard Agilent 1100 LC system was reported ca. 50–80  $\mu\text{l}^2$  while the conventional Merck LaChrom and Waters Alliance 2695 systems possess system variance around 100–200  $\mu\text{l}^2$  [25,26]. The recently launched Waters Acuity I-Class system was characterized in [54] and its system variance was measured between 0.5 and 4  $\mu\text{l}^2$ . As expected, the difference between system variances of current instruments could be quite important. Some model calculations were performed to illustrate its importance, which are based on experimental column efficiency data observed with UHPLC column packed with porous 1.7  $\mu\text{m}$  packing. Fig. 3 shows the effect of extra-column variance on the remaining column efficiency for 50 mm long columns of 1, 2.1, 3, and 4.6 mm I.D. (moderate retention of  $k=5$  is assumed). Clearly, when using the standard bore column (4.6 mm), no impact on the column efficiency is observed. This column dimension can be used with almost any conventional HPLC systems without loss in efficiency. When working with 3 mm I.D. columns, significant amount of the intrinsic column plate number can be lost when this column is operating in conventional systems (e.g., 50% loss at 120  $\mu\text{l}^2$  system variance).



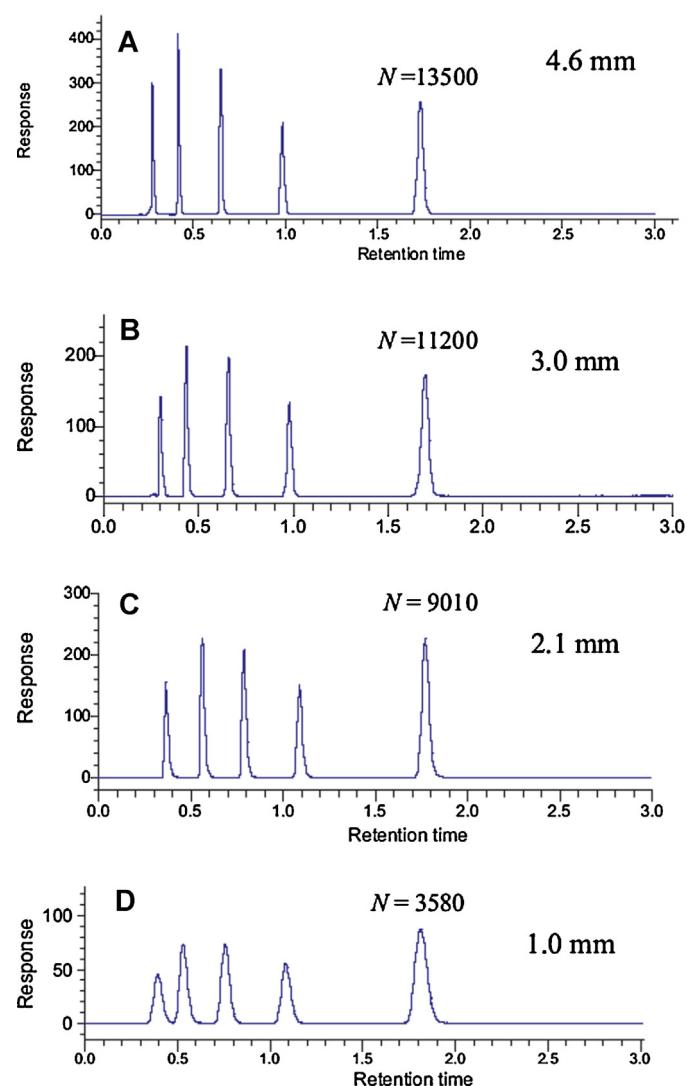
**Fig. 3.** Effect of the extra-column variance on the remaining column efficiency for 50-mm long columns of 1, 2.1, 3, and 4.6 mm I.D. A retention of  $k=5$  was assumed.

It becomes even more critical with narrow bore columns of 2.1 mm I.D. These small, very efficient columns can be only used on dedicated UHPLC systems. Only 80% of the intrinsic column efficiency can be achieved when working with a system having a  $10 \mu\text{L}^2$  variance. Finally, based on Fig. 3, the 1 mm ID columns are obviously incredibly critical in terms of loss in efficiency. Even by using the state-of-the-art instrumentation (e.g.,  $\sigma_{\text{ec}}^2$  of 1–3  $\mu\text{L}^2$ ), only 40–70% of the true column efficiency can be preserved.

Wu and Bradley recently reported experimental data on observed column efficiency of 50 mm long columns packed with  $1.8 \mu\text{m}$  particles and having different diameters (1, 2.1, 3, and 4.6 mm I.D.), with a Waters Acquity UPLC system [55]. The total extra-column volume of the system was measured at  $11.4 \mu\text{l}$ . Fig. 4 shows the change in observed column efficiency when varying the column diameter. These data confirm the calculations. Indeed, a significant loss of efficiency can be expected when using 2.1 or 1 mm I.D. columns with the most recent UHPLC systems. Lestremau and Szucs observed similar loss in performance in the gradient elution mode, when using 1 mm I.D. columns with the same UHPLC system [28].

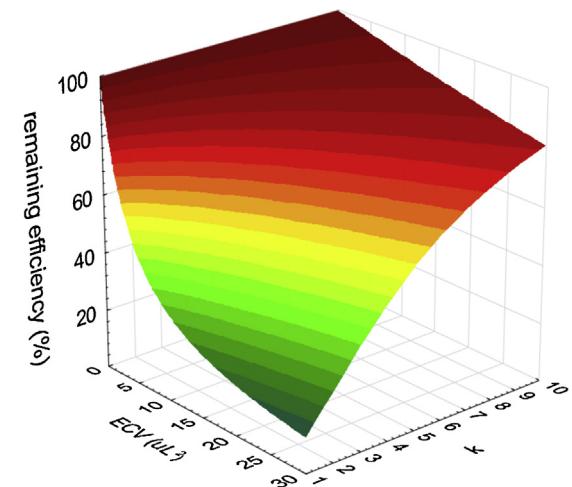
It is important to emphasize that the observed column efficiency also depends on the retention factor and not only on the system variance. Eq. (2) shows the evolution of peak variance with the solute retention. Fig. 5 illustrates the effect of solute retention factor and system variance on the remaining column efficiency. These model calculations are based on experimental peak variances observed by using a  $50 \times 2.1 \text{ mm}$  column packed with  $1.7 \mu\text{m}$  particles. Fig. 5 highlights that system variance is critical for weakly retained compounds, while it could be less important for highly retained compounds. When using narrow bore columns, the retention factor should be kept above  $k>3$ . On the other hand, when having  $k>8$ , this column can be used not only in UHPLC systems but in hybrid systems as well, keeping 75% of the intrinsic column efficiency.

Several attempts were made to minimize the system dispersion of existing instruments by changing the tubes, the detector cell, or the injection system. Wu and Bradley experimentally showed the impact of tubing diameter on the system variance [55]. The authors demonstrated that the extra-column system variance of an Acquity UPLC system can be decreased down to ca.  $2 \mu\text{L}^2$  by replacing the tubes with  $0.0635 \text{ mm}$  instead of  $0.127 \text{ mm}$  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, the needle seat, and the detector cell [26]. After the optimization, an Agilent 1100 system performed  $\sigma_{\text{ec}}^2$  of ca.  $5\text{--}10 \mu\text{L}^2$ . Similarly, Alexander *et al.* showed the optimization of a Waters Alliance 2695 system [56]. In this study, the extra-column system variance of the conventional



**Fig. 4.** Evolution of column efficiency on a 50-mm long column packed with  $1.8 \mu\text{m}$  particles with variation of column diameters. A. 4.6 mm I.D. B. 3.0 mm I.D. C. 2.1 mm I.D., and D. 1.0 mm I.D.

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**Fig. 5.** Effect of the solute retention factor  $k$  and the system variance (extra-column variance, ECV) on the remaining column efficiency (%). The model calculations are based on experimental peak variances observed by using a  $50 \times 2.1 \text{ mm}$  column packed with  $1.7 \mu\text{m}$  particles.

system was reduced down to  $15 \mu\text{l}^2$  by changing the tubes and the injector system. Surprisingly, the manual injection was found to provide significantly lower extra-column dispersion than the automatic system. Omamogho *et al.* optimized an Agilent 1200 LC system down to  $3\text{--}4 \mu\text{l}^2$  by changing the capillary tubing from 0.17 mm I.D. to 0.11 mm I.D., and the flow cell from 6 to 1.7  $\mu\text{l}$  [57].

In order to further increase the observed column efficiency, Farkas *et al.* introduced a novel injection technique called Performance Optimizing Injection Sequence (POISe) or isocratic focusing [58]. 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 [58].

In gradient elution mode, peak widths are continuously compressed during the elution, because the strong solvent concentration is slightly larger in the rear than in the front of the peaks. Therefore, the column efficiency is improved as a result of this thermodynamic compression effect. Consequently, the extra-column peak variance that is evolved before the column is mostly compensated by this compression effect. In most cases, the extra-column peak dispersion is practically negligible in gradient elution mode when using optimized UHPLC systems. However, it was shown that the true performance of the narrow-bore 2.1 mm I.D. BEH column (packed with  $1.7 \mu\text{m}$  particles) is deteriorated by the excessive contribution to overall band broadening of the parts of the instrument upstream the column (particularly for the less retained compounds) and by that of the parts placed downstream the column (for all compounds) [59]. Moreover, many analytes migrate along a significant fraction of the column length under isocratic conditions and they do not benefit from the compression of their band when the gradient flushes them out of the column. Therefore, the resolution power is lost when the compound isocratically migrates along the column [59]. This shows that the dwell volume of the instrument should be further minimized. Finally, there are also various additional contributions that could explain some unexpected band broadening in gradient mode. They include viscous fingering effects (related to a viscosity mismatch between the sample diluent and mobile phase [60]), solubility issues as well as heat effects.

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 [25]. 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 mm and 1.0 mm I.D. columns, with commercially available optimized UHPLC systems. Therefore, the chromatographers are suggested to optimize their system to keep the efficiency of their columns when doing efficient fast separations.

### 3. Importance of system dwell volume for fast analysis

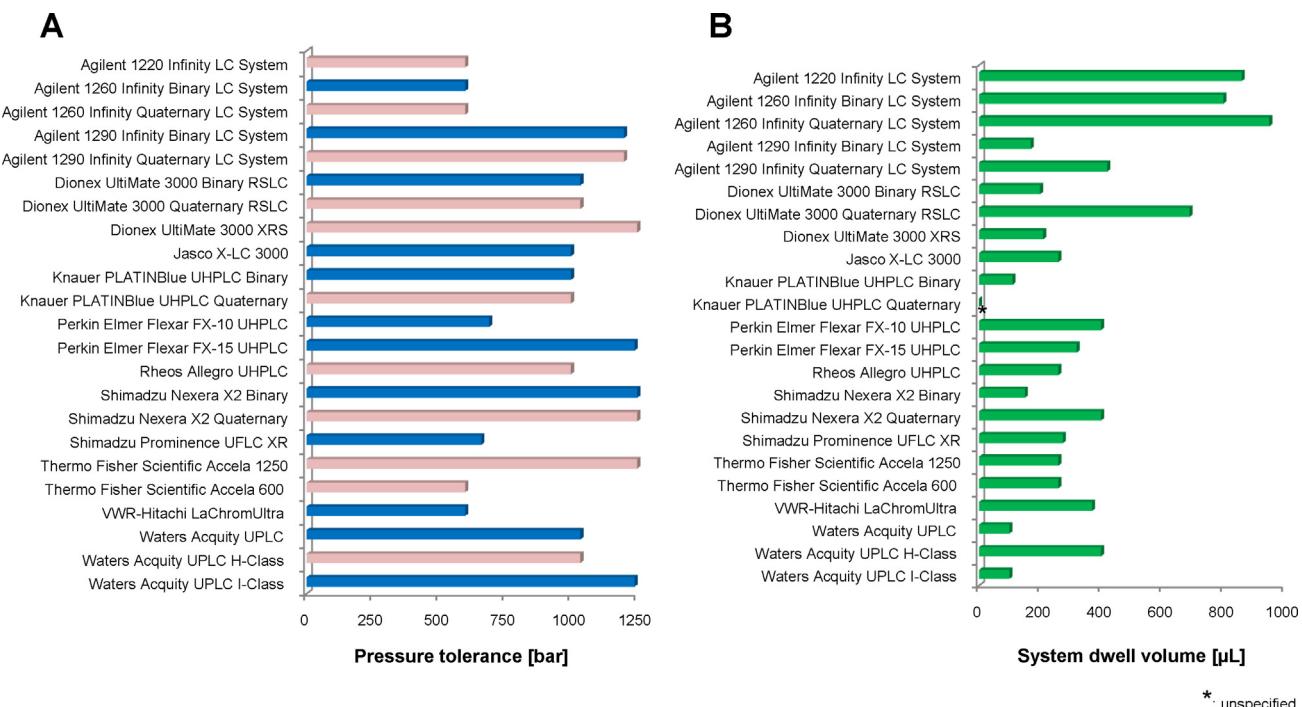
Nowadays, most LC experiments performed in industrial and academic laboratories are carried out in the gradient mode. This is particularly true in the pharmaceutical analysis, since complex separations of numerous compounds possessing a broad range of physico-chemical properties are often performed (e.g., impurity profiling, complex quality control, pharmacokinetics, and drug metabolism studies). The efficient use of gradient elution requires an understanding of the interplay between the achieved responses (retention, bandwidth, selectivity, and resolution) and the operating parameters (gradient profile, column geometry, and mobile phase flow rate). For this purpose, various theories of gradient

elution were proposed by Jandera and Churacek [61,62], Schoenmakers *et al.* [63–65], and Snyder, Dolan *et al.* [66–68]. 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 [69]. 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.e., (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 [70]. 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.

The dwell volume may differ from one instrument to another, but it can be easily measured according to several procedures described in the literature [70]. The system setup is rather simple and consists of using water for the A-solvent, and water spiked with 0.1% acetone for the B-solvent. The column is replaced by a zero-dead volume union, the detector is set to 265 nm, and the flow rate at a value adequately high to generate sufficient backpressure for reliable check valve operation. Finally, a 0–100% B gradient in ca. 20 min is programmed on the pumping system to obtain the final curve. Then, the dwell time can be measured by drawing a tangent to the main part of the gradient curve, and extrapolate the baseline to intersect this tangent. The time between the start of the gradient and this intersection represents the dwell time. The dwell volume is obtained by multiplying the dwell time by the flow rate. Alternatively, the delay volume can also be measured from a step gradient method with 10% gradient impulse steps from 0 to 100% B.

**Fig. 6** summarizes some instrument characteristics of all the UHPLC systems ( $\Delta P > 600$  bar) currently available on the market. The type of pumping system (low pressure vs. high pressure) is specified (Fig. 6A), as well as the system dwell volume (Fig. 6B). In comparison with conventional HPLC instruments which possess  $V_d$  between 0.5 and 5 ml, UHPLC systems have dwell volumes of ca. 300–400  $\mu\text{l}$ , with the best UHPLC systems having  $V_d$  of ca. 100  $\mu\text{l}$ , and up to ca. 1000  $\mu\text{l}$  for some UHPLC instruments. Two main concerns related to large system dwell volume when performing fast separations in LC are observed, 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  $\mu\text{m}$  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. Whatever the need, i.e., HPLC to UHPLC or UHPLC to HPLC, the methodology for transferring a gradient method from one column geometry to another one remains identical and some well-established rules have to be applied to scale the injected volume, the mobile phase flow rate, the gradient slope, and the isocratic step duration [1,11,69,71]. 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



**Fig. 6.** 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. Specifications were obtained by the providers and in [115]. 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

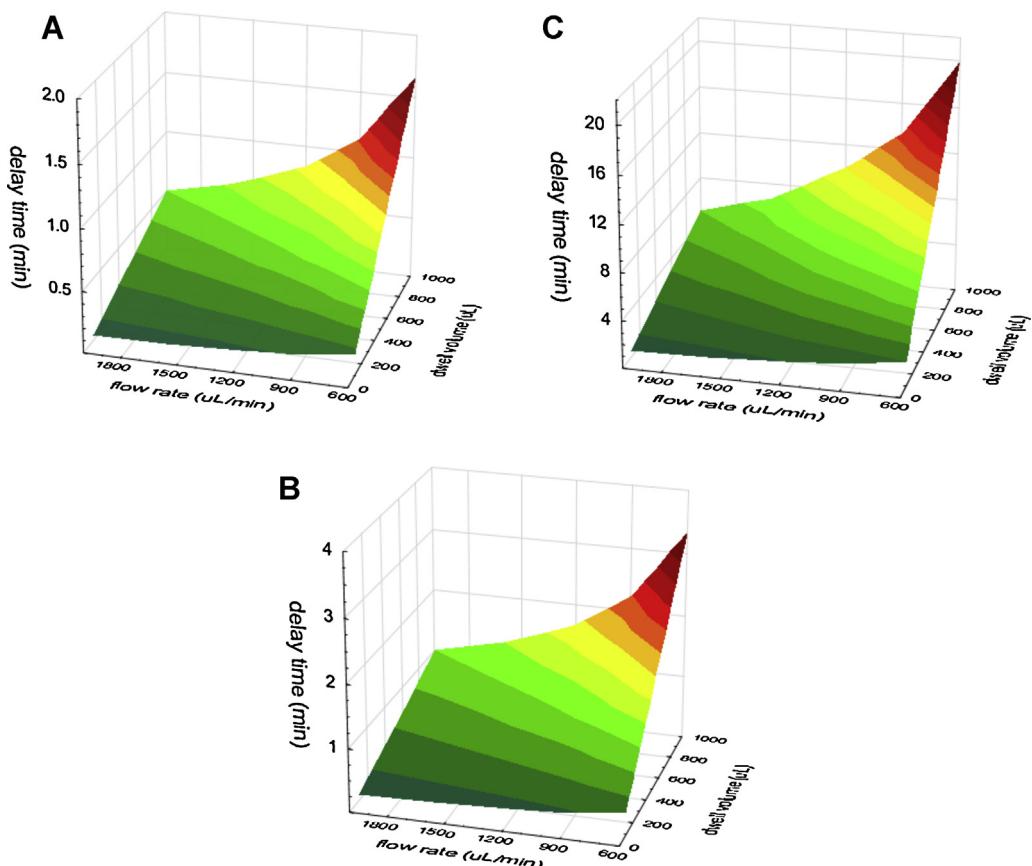
and could result in retention time variations, affecting the resolution during method transfer. To overcome this issue, the ratio of system dwell time on column dead time ( $t_d/t_0$ ) must be ideally held constant while changing column dimensions, particle size, or mobile phase flow rate [72]. Since the column dead time is severely reduced in fast-LC due to the use of short columns at high linear velocity compared to HPLC, the dwell time should also be significantly decreased, which leads to smaller dwell volumes of UHPLC instruments compared to conventional HPLC systems. In order to reach such low  $V_d$  values, some providers (i.e., Agilent Technologies and Shimadzu) proposed innovative devices based on micro-fluidic (e.g., Jet weaver technology) for an efficient mixing in a very low volume (less than 50  $\mu\text{L}$ ). To further reduce system dwell time, the mixing chamber as well as the damper (used to reduce pump ripples and associated UV noise) can be by-passed on few instruments, since the flow rates employed in UHPLC are rather low. Finally, due to the quite difficult method transfer on some instruments, it is also possible to (i) postpone the injection after the beginning of the gradient to simulate a higher dwell volume, or (ii) use a system simulation software to emulate another instrument possessing a higher dwell volume, to alleviate some of these differences [73].

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. This issue is illustrated in Fig. 7, with the representations of 3D-views of the isocratic hold duration for various column internal diameters and their corresponding mobile phase flow rates commonly employed with UHPLC and core-shell technologies. Several system dwell volumes varying between 100 and 1000  $\mu\text{L}$  were considered for this representation. As expected, a large dwell volume and a low mobile phase flow rate compromises ultra-fast separations. This is particularly significant when dealing with micro-bore columns of 1 mm I.D. (Fig. 7C) which are often employed in bio-analytical

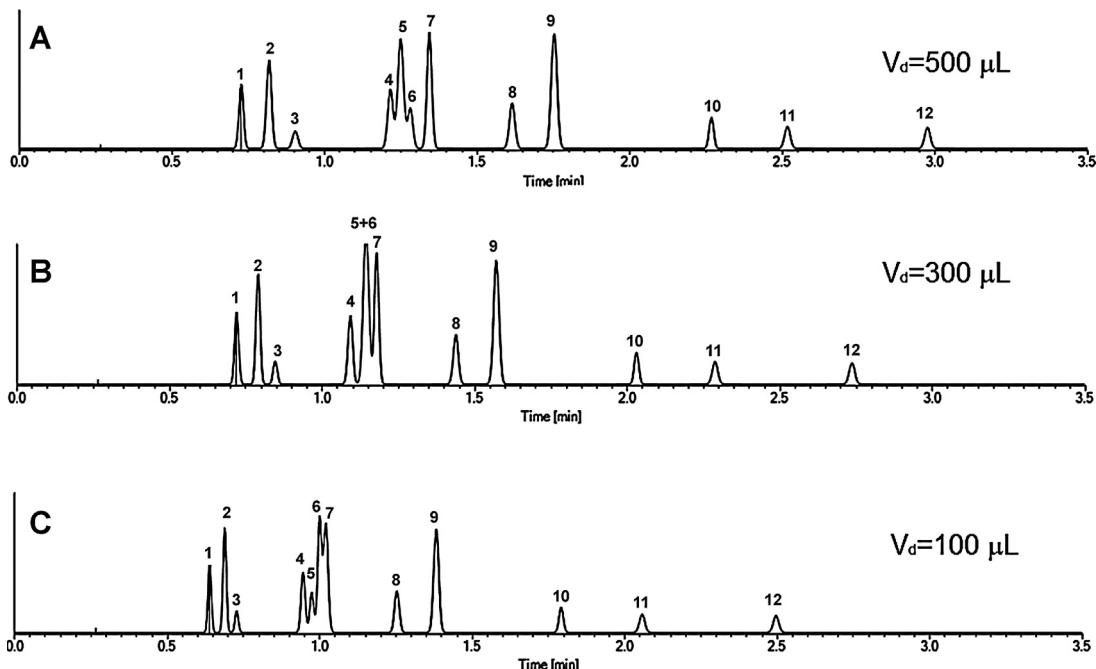
applications to improve MS sensitivity. In fact, the current UHPLC systems are not adapted to such column dimension in terms of gradient delay volume as the isocratic hold is on the minimum equal to 0.5 min (for  $V_d$  of 100  $\mu\text{L}$  and flow rate of 200  $\mu\text{L}/\text{min}$ , corresponding to a linear velocity of ca. 7 mm/s) and could be equal to 20 min in the worst conditions ( $V_d$  of 1000  $\mu\text{L}$  and flow rate of 50  $\mu\text{L}/\text{min}$ , corresponding to a linear velocity of ca. 1.7 mm/s). On the other hand, the 2.1 mm I.D. stationary phases (most widely used column I.D. in UHPLC and core-shell technologies, Fig. 7B) are more suitable with current instrumentation for high throughput separations, provided that the  $V_d$  remains reasonable ( $V_d < 300 \mu\text{L}$ ) and the flow rate is sufficiently high ( $F > 600 \mu\text{L}/\text{min}$ ). In such conditions, the initial isocratic step will be systematically lower than 0.5 min. Finally, the 3 mm I.D. (Fig. 7A) columns can accommodate larger  $V_d$ , up to 600  $\mu\text{L}$  at a flow rate of 1200  $\mu\text{L}/\text{min}$ , and even up to 1000  $\mu\text{L}$  at a flow rate of 2000  $\mu\text{L}/\text{min}$ . In both conditions, the initial isocratic hold will only length 0.5 min. This behaviour can be easily explained by the higher flow rates on larger I.D. columns, for a similar linear velocity, leading to reduced dwell times.

Fig. 8 shows the effect of transferring a pharmaceutical method between various UHPLC instruments possessing different dwell volumes (100, 300 and 500  $\mu\text{L}$ ). As expected, the retention, the selectivity and the overall resolution differ between these three chromatograms. This is particularly true for peaks number 4, 5, 6, and 7. In addition, the analysis time could be reduced from 3 min to 2.5 min, when reducing dwell volume from 500 to 100  $\mu\text{L}$ .

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 [74,75]. This blending noise could be dependent



**Fig. 7.** Representation of the isocratic hold duration for various mobile phase flow rates (600–2000  $\mu\text{L}/\text{min}$ ) and dwell volumes (100–1000  $\mu\text{L}$ ) with A. 3 mm I.D. column, B. 2.1 mm I.D. column, and C. 1 mm I.D. column.



**Fig. 8.** Method transfer between UHPLC instruments with various dwell volumes. The simulated chromatograms were obtained with Drylab® 2010 Plus modelling software (Molnar-Institute, Berlin, Germany). A. Dwell volume of 500  $\mu\text{L}$ , B. Dwell volume of 300  $\mu\text{L}$ , and C. Dwell volume of 100  $\mu\text{L}$ . 1, phthalic acid; 2, vanillic acid; 3, isovanillic acid; 4, anthmilic acid; 5, vanillin; 6, syringaldehyde; 7, ferulic acid; 8, ortho-vanillin; 9, benzoic acid; 10, trans-2,4-dimethoxycinnamic; 11, methyl benzoate; 12, ethyl benzoate. Conditions: Halo C18, 100  $\times$  2.1 mm, 2.7  $\mu\text{m}$ ; mobile phase containing water and ACN, gradient 15 to 90% ACN in 4 min,  $F=800 \mu\text{L}/\text{min}$ .

on the pump design (i.e., piston column, mixer volume, and presence of a damper). As a result, cyclical perturbations synchronized to the pump strokes are observed on the UV signal, leading to a decrease in sensitivity. The phenomenon is particularly relevant when the UV detection is carried out at relatively low wavelengths (200–230 nm) with volatile mobile phases (e.g., formate or acetate salts). To avoid this problem, 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  $\mu\text{l}$ , for a use at 214 nm with mobile phase containing trifluoroacetic acid (TFA).

#### 4. Importance of elevated system pressure for high throughput separations

For the last 20 years, columns packed with 3.5–5  $\mu\text{m}$  particles were the gold standards in HPLC. According to the Darcy's law (Eq. (5)) which takes into account the permeability of the stationary phase and the mobile phase viscosity, the backpressure generated by these phases usually ranges between 50 and 200 bar:

$$\Delta P = \phi \frac{\eta L u}{d_p^2} \quad (5)$$

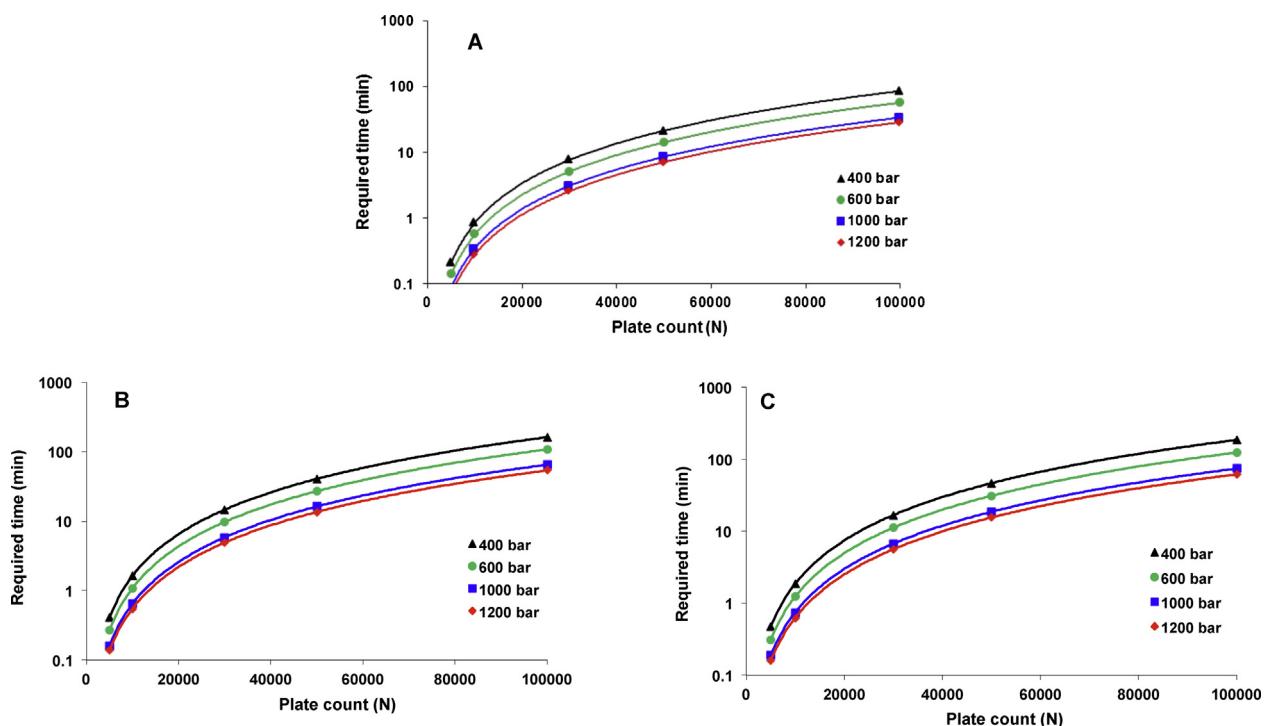
where  $L$  is the column length,  $u$  is the averaged mobile-phase velocity,  $\eta$  is the mobile phase viscosity,  $\Phi$  is the flow resistance, and  $d_p$  is the particle size. Until recently, conventional HPLC systems possessing an upper pressure limit of 400 bar were adapted for an appropriate work with columns packed with 3.5–5  $\mu\text{m}$  particles.

The interest of using smaller particle size in HPLC has been theoretically discussed in the 70's but has been experimentally tested in two academic state-of-the-art laboratories only at the end of the 90's. Jorgenson *et al.* were the first to evaluate the interest of using sub-2  $\mu\text{m}$  particles in HPLC and extensively published in this field [76–80]. The proof-of-concept was demonstrated in 1997, using a fused-silica capillary column of 52 cm  $\times$  30  $\mu\text{m}$  I.D. packed with 1.5  $\mu\text{m}$  non-porous particles and a UHPLC system able to work up to 4100 bar [76]. A plate count of 140,000–190,000 was attained for an analysis time of less than 10 min. Few years later, the upper pressure limit of the system and column was extended to 7000 bar and analysis time reduced as low as 4 min while plate numbers remained excellent (i.e., 190,000–300,000) using a 43 cm  $\times$  30  $\mu\text{m}$  I.D. column packed with 1.0  $\mu\text{m}$  non-porous particles [78]. Nearly at the same time, Lee *et al.* also investigated the possibilities of UHPLC using capillary columns of 12.5 cm  $\times$  29  $\mu\text{m}$  I.D. packed with 1.5  $\mu\text{m}$  non-porous particles, and a system able to work up to 3600 bar [81–85]. Using this strategy, a UHPLC separation of several benzodiazepines was carried out in less than 1 min [81]. The innovative works made in the groups of Jorgenson and Lee under extreme conditions prove that the system pressure capability has to be extended beyond the 400 bar limit to take the full advantage of columns packed with very fine particles.

Nowadays, columns packed with fully porous sub-2  $\mu\text{m}$  and core-shell sub-3  $\mu\text{m}$  particles are available from numerous providers and are routinely applied in several pharmaceutical companies [86]. However, in order to obtain all the benefits of such columns, a chromatographic system possessing an extended upper pressure limit should be used. In this context, a large variety of chromatographic devices compatible with pressures in the range 600–1300 bar have been developed in parallel to these new column technologies, as highlighted in Fig. 7.A [87]. Compared to the seminal works of Jorgenson and Lee ( $\Delta P_{\max}$  up to 7000 bar), the upper pressure limits of current UHPLC systems are more reasonable. The more restrictive pressure limitation can be explained by the construction, the robustness, and the safety of the UHPLC systems and columns, as well as the negative effects of very high pressure

on chromatographic efficiency, selectivity, retention, and repeatability. 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. 1000 bar). Moreover, there are many design trade-offs that are required to balance system features (robustness, sensitivity, and safety) and performance characteristics. These compromises often lead to specific technical characteristics of instruments from various providers, as presented in Fig. 7. Lee *et al.* devoted an interesting paper to the safety concerns in UHPLC [88]. Ruptures of columns and failures of the system components under ultra-high pressure can lead to dangerous high speed liquid jet and capillary projectiles. Column stability also needs to be considered for UHPLC operation. The works of Jorgenson and Lee up to 3000–7000 bar were exclusively performed on sub-2  $\mu\text{m}$  non-porous stationary phases, which are known to be more resistant than fully porous particles. However, this type of columns suffers from an unacceptable specific area and loading capacity, as well as a lack of appropriate retention. Today, there is a large choice of column packed with fully porous sub-2  $\mu\text{m}$  particles which are compatible with pressures up to 1200 bar [13,89]. 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) [90], 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 [91–95]. The frictional heating is induced by friction of the mobile phase percolating 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. These thermal gradients may influence both the retention and the column efficiency. Two solutions can be applied to limit this phenomenon, i.e., (i) reducing the column inner diameter, which remains difficult because of the strong contribution of extra-column variance to peak broadening, as described in section 2, and (ii) reducing the backpressure inside the column [96]. 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.

In order to evaluate the throughput and the resolution that can be obtained with commercially available UHPLC instruments using sub-3  $\mu\text{m}$  core-shell or sub-2  $\mu\text{m}$  fully porous particles, the kinetic plot methodology can be implemented. Indeed, this approach allows to better visualizing the performance limits of a given stationary phase technology on a particular instrument at an upper pressure limit. Kinetic plots were originally introduced by Giddings to discuss the advantages and limitations of LC and GC [97], and were also employed to compare performance of LC and CEC [8]. The construction of kinetic plot is based on the extrapolation of the van Deemter ( $H, u$ ) and permeability ( $K_{v,0}$ ) data obtained with a column of a certain length to shorter or longer columns, generating the maximal pressure drop that the chromatographic system could withstand. The final representation includes the extrapolated  $N$  and  $t_0$  values, calculated by simply rearranging the van Deemter and permeability data. Using the kinetic plot representation and by combining different column lengths and mobile phase flow rates for a given upper system pressure limit, it is possible to easily determine (i) the minimum time required to reach a particular efficiency, (ii) the maximal efficiency that can be achieved for a given analysis



**Fig. 9.** Calculation of the time required to attain a given plate number for A. column containing 2.6  $\mu\text{m}$  core–shell particles, B. column packed with 1.7  $\mu\text{m}$  core shell, and C. fully porous 1.7  $\mu\text{m}$  particles, assuming  $k = 10$ , at 400, 600, 1000 and 1200 bar.

time, and (iii) the maximal plate count that can be obtained without any time restriction. However, their construction remained tedious until the recent work of Desmet *et al.*, who proposed a simplified way to construct these curves using only two simple equations, i.e., Eq. (6) and Eq. (7) [98–102]:

$$N = \frac{\Delta P_{\max}}{\eta} \left( \frac{K_{v,0}}{u \times H} \right) \quad (6)$$

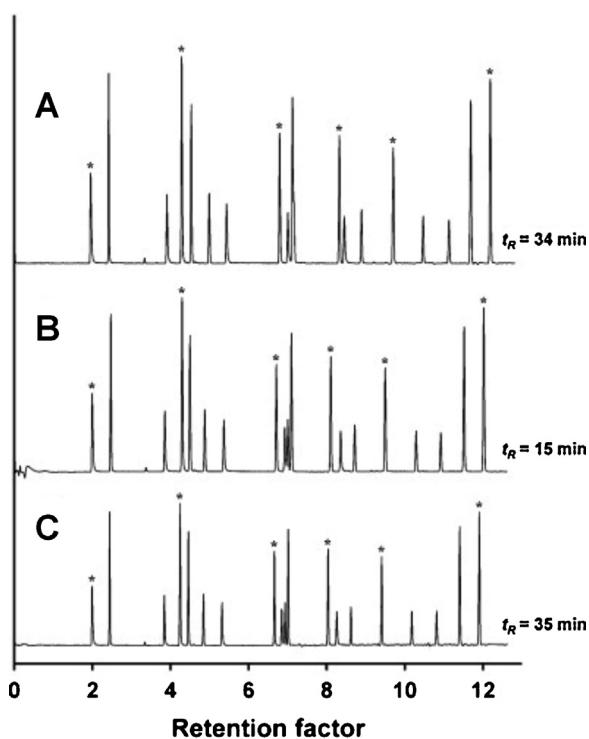
$$t_0 = \frac{\Delta P_{\max}}{\eta} \left( \frac{K_{v,0}}{u^2} \right) \quad (7)$$

where  $\Delta P_{\max}$  is the maximal system pressure (or column mechanical stability),  $K_{v,0}$  the chromatographic support permeability and  $H$  the height equivalent to a theoretical plate.  $H$ ,  $u$  and  $K_{v,0}$  can be easily determined experimentally, while  $\Delta P_{\max}$  and  $\eta$  are instrument and mobile phase dependent, respectively.

Using this approach, the minimum required time ( $k = 10$ ) to attain an efficiency of 5000, 10,000, 30,000, 50,000 and 100,000 plates using fully porous 1.7  $\mu\text{m}$ , core–shell 2.6  $\mu\text{m}$ , and core–shell 1.7  $\mu\text{m}$  were estimated. The corresponding values are reported in Fig. 9 for various system pressure limits equal to 400, 600, 1000 and 1200 bar. As shown in these figures, the performance of a column containing 2.6  $\mu\text{m}$  core–shell particles (Fig. 9A) systematically outperforms the one of a column packed with 1.7  $\mu\text{m}$  core shell (Fig. 9B) or fully porous 1.7  $\mu\text{m}$  particles (Fig. 9C) for a given pressure, within an efficiency range of 5000–100,000 plates. However, when considering the current pressure limits of these phases, i.e., 600 bar for the 2.6  $\mu\text{m}$  and 1000 bar for the 1.7  $\mu\text{m}$  particles, the performance are very close for the three column technologies (between 0.57 and 0.74 min for 10,000 plates and 56.8 – 74.4 min for 100,000 plates). Fig. 9 also clearly demonstrates the interest of having a chromatographic system possessing an elevated upper pressure limit to reduce the analysis time for a given plate number. Indeed, for a column packed with fully porous 1.7  $\mu\text{m}$  particles (UHPLC phase), an efficiency of 10,000 plates can be attained

in 1.86 min at a pressure of 400 bar, while it requires only 0.74 and 0.62 min at 1000 and 1200 bar, respectively. According to the kinetic plot methodology, the same reduction of analysis time can be expected for  $N = 100,000$  plates, for which analysis time was 186, 74 and 62 min for  $\Delta P_{\max} = 400, 1000$  and 1200 bar, respectively. Finally, the same characteristics could be observed for columns packed with core–shell of 2.6  $\mu\text{m}$  and 1.7  $\mu\text{m}$  particles. All these results demonstrate that the analysis time is always inversely proportional to the  $\Delta P_{\max}$  and that an elevated system pressure is always beneficial to maximize kinetic performance. Nevertheless, in the case of reasonable plate count (i.e., 10,000 plates), the 3-fold reduction in analysis time between a system possessing  $\Delta P_{\max}$  of 400 or 1200 bar remains marginal since in both cases the separation can be performed in less than 2 min. Considering the column reequilibration time and the injection cycle time, a system possessing high upper pressure limit is only interesting in the second dimension of a 2D-LC setup where ultra-fast separations (i.e., less than 30 s) are required. On the other hand, for high resolution separations (i.e., 100,000 plates), analysis time can also be reduced by 3-fold between systems possessing  $\Delta P_{\max}$  of 400 and 1200 bar. However, in this case, it corresponds to a drastic reduction of analysis time, from around 3 to only 1 h using columns packed with 1.7  $\mu\text{m}$  particles and from 85 to 28 min for column packed with 2.6  $\mu\text{m}$  core–shell particles.

Based on the data reported in Fig. 9, 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 [103,104]. Prototype columns packed with core–shell 2.6  $\mu\text{m}$  particles were tested at a pressure up to 1200 bar under gradient conditions. Fig. 10 presents the separation of a mixture of small molecules separated with core–shell 2.6  $\mu\text{m}$  particles at a pressure of 600 (Fig. 10A) and 1200 bar (Fig. 10B and C). As expected, the analysis time was more than 2-fold reduced at 1200 bar compared to 600 bar, using 300 mm



**Fig. 10.** Separation of a complex 19-compounds mixture on A. a 300 mm coupled column configuration at 600 bar ( $n_p = 222$  in 34 min), B. a 300 mm coupled column configuration at 1200 bar ( $n_p = 227$  in 15 min), and C. a 450 mm coupled column configuration at 1200 bar ( $n_p = 287$  in 35 min).

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column length, while the peak capacity remains identical between both conditions. Moreover, it was also possible to use a 450 mm column length at 1200 bar to further increase peak capacity by ca. 30% (Fig. 10C).

## 5. Injection cycle time and data acquisition rate

Injection cycle time could also represent an additional constraint in the case of fast or ultra-fast analyses. Indeed, in recent UHPLC systems, the time required for injecting sample is generally comprised between 30 s and more than 1 min for the slowest instruments. This additional time can strongly affect the throughput in case of ultra-fast separations where the analysis time itself can be reduced to less than 1 min. Various technical solutions were proposed to optimize the injection speed. Numerous providers, e.g., PerkinElmer, Shimadzu, and Dionex, demonstrated that the injection cycle time could be decreased with their instruments down to 8, 10 and 15 s, respectively. However, drawing the sample too rapidly can reduce the repeatability and increase the carryover. A significant contribution of the injection process is the time spent by the needle movements to and from the vial as well as into the flush port. These operations can be performed during the analytical separation, which is known as a so-called overlapped injection. Waters or Agilent Technologies have demonstrated the benefits of the overlapped injections on their instruments. Injection cycle times between 15 and 20 s can be achieved with this strategy, without affecting too much the autosampler performance.

Another technical issue encountered with high throughput separations is related to the detector acquisition rate. Indeed, the narrow peaks produced by state-of-the-art stationary phases, down to 1 s in some cases, could be critical. Indeed, for quantitative purpose, 10–15 acquisition points per peak are at least recommended to correctly define the chromatographic peak and

obtain suitable performance. This criterion is easy to achieve with spectroscopic detectors such as UV, UV-DAD, fluorescence (FD) or even with evaporative light scattering detectors (ELSD) and corona aerosol discharge (CAD), since detector speed up to 200 Hz has been reported with the last generation of instruments. However, obtaining high acquisition rate with MS devices is much more difficult as they must be capable of short dwell times and fast duty cycles [105,106]. Only the latest generation of instruments meets these requirements. Indeed, the most powerful triple quadrupole (QqQ) devices are able to perform SRM experiments in only 1 ms, while the best QqTOF/MS instruments on the market are able to scan  $m/z$  ratios from 100 to 1000 in only 10 ms with a resolution of ca. 40,000 with full width at half maximum (FWHM) [105].

## 6. Is core–shell technology compatible with conventional LC system?

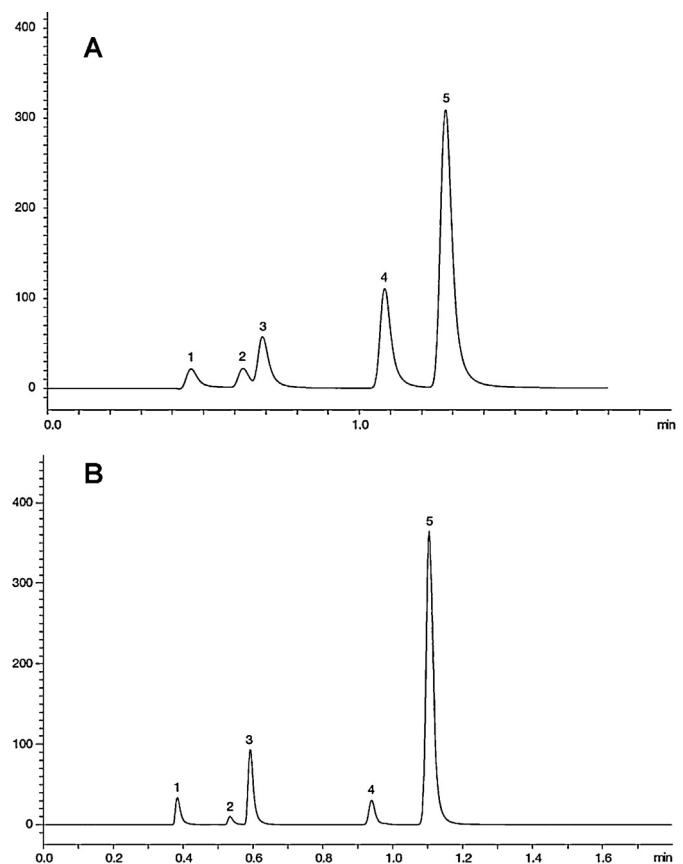
As expected from the theory, columns packed with sub-3  $\mu\text{m}$  core–shell particles, released in 2007, provide an efficiency comparable to the one obtained with columns packed with totally porous sub-2  $\mu\text{m}$  particles, but at reasonable operating pressure ( $P < 400$  bar). Gritti and Guiochon first reported reduced plate height minimum value of  $h_{\min} = 1.4$  for Halo<sup>TM</sup> column (Advanced Material Technology) with the injection of small molecular weight compounds [15]. These 2.7  $\mu\text{m}$  Halo<sup>TM</sup>, Ascentis Express<sup>TM</sup> (Sigma-Aldrich) and Poroshell 120 (Agilent Technologies) particles consist of a 1.7  $\mu\text{m}$  non-porous core and a 0.5  $\mu\text{m}$  porous silica layer [14]. A different structure in shell particles regarding the core and shell ratio was introduced by Phenomenex in 2009. This alternative technology offers particles, which consist of a 1.9  $\mu\text{m}$  or 1.24  $\mu\text{m}$  non-porous core and a 0.35  $\mu\text{m}$  or 0.23  $\mu\text{m}$  porous silica layer, respectively [19,23,107]. Several other vendors launched similar sub-3  $\mu\text{m}$  core shell packings in 2011 (Accucore, Nucleoshell<sup>®</sup>, SunShell<sup>®</sup>, and BrownLee<sup>TM</sup>). Because core–shell sub-3  $\mu\text{m}$  packings can operate at the half or at third pressure compared to fully porous sub-2  $\mu\text{m}$  particles, it is theoretically possible to operate such phases on a conventional HPLC instrument. In this context, the column manufacturers advertised several times that UHPLC efficiency can be achieved on HPLC systems, thanks to these innovative materials. Commercial statements promising that columns with sub-3  $\mu\text{m}$  core–shell allow for metamorphosing a HPLC as a UHPLC system are far to be valid and should be cautiously considered. Some considerations, examples and discussions are hereafter presented to determine if highly efficient and fast separations can be effectively achieved with these state-of-the-art core–shell columns on conventional HPLC systems.

In case of fast separations, the column length has to be decreased while the mobile phase linear velocity should be increased. However, maintaining good kinetic performance when using short column at high linear velocity requires the use of smaller particle size. The 50 mm long, narrow-bore (2.1 mm) columns recently became very popular, especially in the field of pharmaceutical analysis [108–110]. The pharmaceutical industry is 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.

Regarding the required system pressure, it is true that only the half or the third pressure is required to operate with a column packed with 2.6–2.7  $\mu\text{m}$  particles, compared to a column packed with 1.5–1.7  $\mu\text{m}$  particles, in agreement with the Darcy's law and Karman-Kozeny equation. The relatively high specific permeability of columns packed with 2.6–2.7  $\mu\text{m}$  core–shell particles ranges between  $K_0 = 4.6 \times 10^{-11} \text{ cm}^2$  and  $6.4 \times 10^{-11} \text{ cm}^2$ , while the permeability of a column packed with 1.7  $\mu\text{m}$  fully porous particles is of ca.  $2.5 \times 10^{-11} \text{ cm}^2$  [111–113]. Therefore, when using

the same column dimension, linear velocity, and mobile phase composition, the column made with 2.6–2.7  $\mu\text{m}$  particles generates significantly lower head pressure. In generic conditions, when using acetonitrile–water mobile phase, there is no need for more than 400 bar pressure with columns of 50  $\times$  2.1 mm packed with 2.6–2.7  $\mu\text{m}$  particles. However, higher pressure capability may be useful when using more viscous organic modifiers in the mobile phase such as methanol and isopropanol in order to tune the selectivity and/or for sustainable purposes. The maximum viscosity of acetonitrile–water mixture is around 1 cP at 25 °C, while the viscosity of methanol–water and isopropanol–water mixtures can reach 1.6 and 2.9 cP at 25 °C, respectively. The nominal mechanical stability of 2.6–2.7  $\mu\text{m}$  core–shell columns is 600 bar, while the pressure capability of conventional HPLC systems is 400 bar. To sum up, the pressure capability of conventional HPLC systems seems to be appropriate with columns of 2.6–2.7  $\mu\text{m}$  core–shell particles (50  $\times$  2.1 mm), but all the benefits of these phases cannot be attained. Indeed, operating these columns at a pressure higher than 400 bar can provide faster separations but also allows for the use of alternative solvents. Increasing the temperature in HPLC also offers a chance to reduce backpressure, as it drastically reduces the mobile phase viscosity. The suggested maximal operating temperature for Kinetex® columns is 60 °C, while the Halo™ columns can be operated up to 90–100 °C. When the conventional LC system can withstand 60–90 °C, some additional benefits from these 2.6–2.7  $\mu\text{m}$  core–shell columns can be obtained.

In terms of system volume and variance, the conventional HPLC systems are definitely not able to maintain the high efficiency of small columns packed with 2.6–2.7  $\mu\text{m}$  core–shell particles. Based on experimental results, some model calculations were performed to evaluate the peak variances that can be obtained with columns packed with 2.6  $\mu\text{m}$  core–shell particles of different geometries (i.e., 50  $\times$  2.1 mm, 50  $\times$  3 mm, and 50  $\times$  4.6 mm) [18]. Assuming retention factors of  $k=2$ –10, the expected peak variance on 50  $\times$  2.1 mm column ranges between  $\sigma_{\text{col}}^2 = 9$ –120  $\mu\text{l}^2$ , is expected around  $\sigma_{\text{col}}^2 = 25$ –330  $\mu\text{l}^2$  with the 50  $\times$  3 mm column, while the 50  $\times$  4.6 mm column performs peak variances of  $\sigma_{\text{col}}^2 = 130$ –1700  $\mu\text{l}^2$ . As discussed in Section 2, conventional systems contribute to the peak variance with  $\sigma_{\text{ec}}^2 = 50$ –150  $\mu\text{l}^2$ , which is clearly significant for the 50  $\times$  2.1 and 50  $\times$  3 mm columns. Considering  $\sigma_{\text{ec}}^2 = 50 \mu\text{l}^2$  extra-column system variance, the remaining column efficiency ranges between  $E_r = 15$ –70% for the 50  $\times$  2.1 mm column, while it is between  $E_r = 33$ –86% and 72–97% for the 50  $\times$  3 and 50  $\times$  4.6 mm columns, respectively. To benefit from the advantages of these very efficient small columns of 2.1 and 3 mm I.D., optimized systems are mandatory. A system having  $\sigma_{\text{ec}}^2 = 5 \mu\text{l}^2$  preserves 88 and 95% of the intrinsic column efficiency for the 2.1 and 3 mm I.D. column, respectively, assuming a retention factor of  $k = 5$ . Currently, Phenomenex offers a methodological guideline for the optimization of system volumes to use the potential of very efficient small columns [114]. This HPLC system optimization manual discusses in details the possible sources of non-desired peak dispersion, and makes suggestions to decrease the extra-column dispersion. It recommends replacing (i) the standard needle seat with a low volume needle seat (flow-through-needle injector models), (ii) the injector loop with a lower volume loop, (iii) the standard UV flow cell with a low volume flow cell (<2  $\mu\text{l}$ ), and (iv) the standard tubing with 0.005 in I.D. tubing. As additional suggestions, the column switching valves should be bypassed and the detector sampling rate and time constant should be optimized. Fig. 11 shows chromatograms obtained with a Kinetex® 100  $\times$  2.1 mm, 2.6  $\mu\text{m}$  column on conventional system without modification (Fig. 11A) and after optimization (Fig. 11B) [114]. In a systematic study, Gritti *et al.* showed that the combination of several changes in the system or the method allowed for the achievement of the full potential of



**Fig. 11.** Chromatograms obtained with a Kinetex® 100  $\times$  2.1 mm, 2.6  $\mu\text{m}$  column with A. a conventional HPLC system without modification, and B. after optimization. Reprinted from [114].

core–shell type phases, using a conventional LC system [26]. The first modification was the reduction of the extra-column volume of the instrument, without increasing too much its back pressure contribution, by changing the needle seat volume, the inner diameter and the length of the capillary connectors, as well as the detector cell volume of a standard instrument (Agilent 1100). The second adjustment consisted of injecting a volume of weak eluent (less than half the elution strength of the mobile phase) right after the sample. Experimental results showed that these changes could provide most of the efficiency expected from the true column performance. After applying these changes, the resolution of the 50  $\times$  2.1 mm, 50  $\times$  4.6 mm, and 100  $\times$  4.6 mm columns for compounds having retention factors close to 1 (worst case) were increased by about 180, 35, and 30%, respectively.

Finally, if gradient mode has to be employed, the dwell volume should also be considered. As explained in section 3, the system dwell volume could become critical when performing fast analysis by applying short narrow bore columns. Conventional HPLC systems possess 0.5–5 ml dwell volume that is clearly unacceptable for fast gradient separations. Similarly to extra-column band dispersion, the dwell volume of the system has to be also minimized. However, adjusting the dwell volume of a given low-pressure mixing system is not as simple as changing a detector cell or a tubing. When small dwell volume is required, high-pressure mixing systems are preferably applied. Several vendors (e.g., Dionex and Shimadzu) offer different volume mixers for their instrument to select the most appropriate mixing volume for the required flow rate (column dimension). As an example, Agilent offers its 1260 pump in both standard volume delay and low volume delay

configurations. Conventional HPLC systems with low pressure mixing systems are only acceptable for standard bore columns.

In conclusion, conventional systems are not suitable to achieve fast and efficient separations when using small narrow-bore columns packed with 2.6–2.7 µm core-shell particles. Without any modifications, only the 4.6 mm I.D. columns can be applied without too much loss in column efficiency. For 3 and 2.1 mm I.D. columns, the conventional LC systems must be optimized. The system volume and dwell volume have to be drastically reduced (e.g., tubing, detector cell and mixing chamber). After the appropriate modifications, most of the advantages of columns packed with 2.6–2.7 µm core-shell particles can be used without the need of very high pressure.

## 7. Conclusion

Historically, the importance of instrument has been neglected in LC because conventional HPLC columns of 150 or 250 × 4.6 mm, 5 µm, which offer analysis times between 20 and 60 min, are fully compatible with the vast majority of the conventional chromatographic systems. Recently, some new trends have emerged in the pharmaceutical industry to increase the throughput. In order to achieve fast (<5 min) or ultra-fast (<1 min) separations, short columns of 50 × 2.1 mm packed with fully porous sub-2 µm and core-shell sub-3 µm particles are increasingly used for real-life applications. However, a dedicated instrument is mandatory to fully benefit from these innovative stationary phases, which are able to produce high plate count per time unit values. The LC instrument should include a reasonably low dwell volume, extra-column variance, and injection cycle time, in combination with a sufficiently high upper pressure limit and data acquisition rate. In this context, since 2004 a new generation of instruments has been commercialized by numerous providers to meet these strict requirements. These recent instruments are not equivalent and their specific characteristics may differ. As example, dwell volume varies from ca. 100 to more than 800 µl, the extra-column variance is usually comprised from 1 to 25 µl<sup>2</sup>, while the upper pressure limit is included within the range 600–1300 bar. Moreover, some of the current UHPLC systems might be hardly compatible with state-of-the-art stationary phases. In addition, because the column technology evolves very rapidly, even the best LC instrument on the market could appear as insufficient in some cases. For instance, short narrow bore columns packed with 1.3 µm core-shell particles were recently released and appear as a good strategy for ultra-fast separations. However, the upper pressure limit and the extra-column variance of even the latest state-of-the-art commercially available UHPLC system are clearly insufficient to reach the full performance of such column technology. Similar limitations are observed when using a short column of 1 mm I.D. packed with sub-2 µm particles. Such column dimension is particularly useful to reduce the mobile phase and the sample consumption, but also to increase the compatibility with ESI-MS devices or reduce the frictional heating effects observed under very high pressure conditions. Once more, the dwell volume and the extra-column variance of current UHPLC systems are clearly too high to reach ultra-fast separations with high plate count on a 1 mm I.D. column [28]. Nevertheless, it is possible to bring some modifications to a commercial instrument to improve its performance but it is not recommended when working in a regulated environment, such as pharmaceutical industry.

It is important to notice here that nano-UHPLC systems, compatible with pressure up to 600 bar, have also been commercialized. They permit to work with columns of 50–100 µm I.D. However, such instruments are mostly dedicated to the proteomic field which requires high resolution and long analysis times. Such systems

would be incompatible with fast or ultra-fast separations, because of unsuitable dwell volumes or extra-column volumes, and are therefore out of the scope of the present review.

In conclusion, the column technology is nowadays evolving faster than the LC instrumentation. In this sense, there is a need to develop even better instrument than the current ones, including lower system dwell volume, reduced extra-column variance, faster injection cycle time, and higher upper pressure limit. However, all these improvements must not reduce the reliability, the sensitivity, the precision and the safety of existing systems, due to the high number of samples that have to be routinely analyzed in a strict regulated environment.

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