

Guidelines for the use of UHPLC Instruments

Requirements for UHPLC instruments, method development in UHPLC and method transfer from regular HPLC to UHPLC

Authors: Dr. Davy Guillarme, Prof. Jean-Luc Veuthey

Laboratory of Analytical Pharmaceutical Chemistry, School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Boulevard d'Yvoy 20, 1211 Geneva 4, Switzerland - Phone: +41 22 379 63 36 - Fax: +41 22 379 68 08 - E-mail: jean-luc.veuthey@unige.ch

1. INTRODUCTION

Over the past few years, there has been tremendous interest in approaches to speed up and/or increase the resolving power of the analytical separation process, particularly with the development of columns packed with porous sub-2 μm particles used in very high pressure conditions (namely UHPLC, for ultra high pressure liquid chromatography). Many laboratories want to transition some of their conventional HPLC methods to fast UHPLC methods, but their lack of experience sometimes acts as a deterrent to trying.

In this white paper, we share our experience in HPLC-to-UHPLC method transfer in the form of a tutorial introductory guide to help those who want to try this new and exciting UHPLC methodology to improve their productivity.

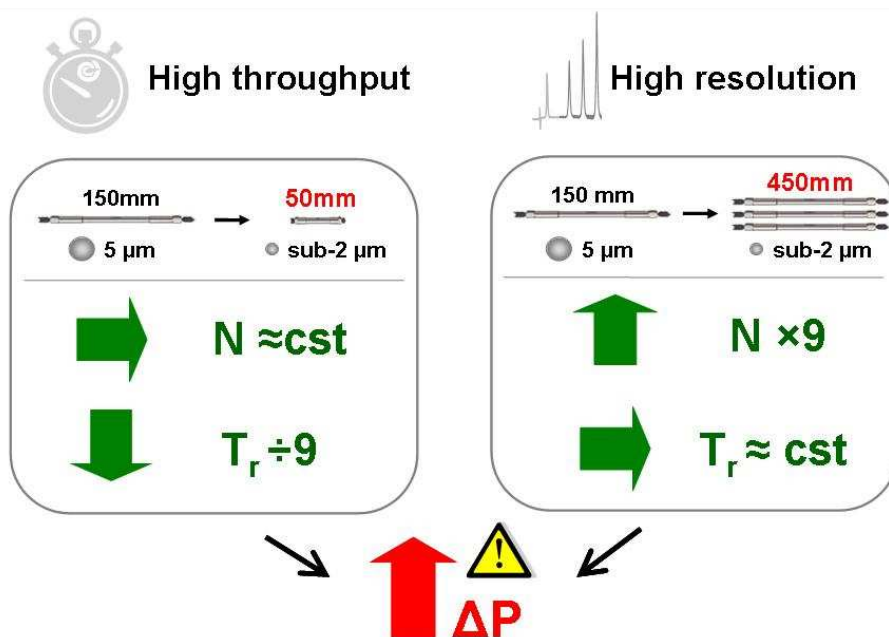


Figure 1: Columns packed with sub-2 μm particles: concept and interest

It is well known in liquid chromatography that the use of small particle size results in higher plate numbers, as well as faster separations. These effects are due to the fact that i) the chromatographic efficiency, N , is directly proportional to the ratio of column length and particle diameter, L/d_p and ii) the mobile phase linear velocity, u , is inversely proportional to the particle diameter, d_p . As illustrated in figure 1, for high throughput separations it is indeed possible to maintain an equivalent efficiency between a 150mm column packed with 5 μm particles and a 50mm column packed with sub-2 μm particles, while the analysis time is divided by 9-fold. It is also theoretically possible to maintain the analysis time equivalent between a conventional HPLC columns and a 450mm column packed with sub-2 μm , but with an efficiency enhancement by 9-fold with the latter.

However, the particle size reduction also generates a high backpressure (> 400 bar) not compatible with conventional instrumentation. Therefore, to benefit from the full potential of columns packed with small particles, it is recommended to work with a chromatographic system that withstands pressures up to 600 and even 1000 bar.

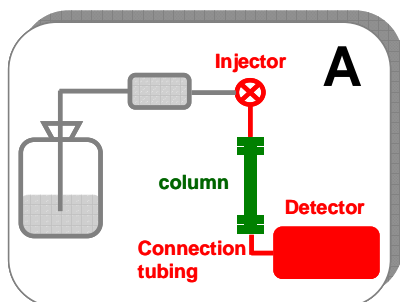
By comparing the intrinsic performance of such packing size with other existing techniques, such as monoliths, fused-core technology or high temperature liquid chromatography with conventional particle size, it is shown here that UHPLC, with a maximal pressure of 1000 bar is a very attractive strategy (i.e. approach that generates the lowest analysis time for a given efficiency) in the range 1,000 to 80,000 plates. Only the monolithic approach performs better than UHPLC for efficiencies higher than 80,000 plates (such efficiency is however often beyond the needs of a conventional LC analysis).

2. Requirements for UHPLC Experiments

2.1. UHPLC instrumentation

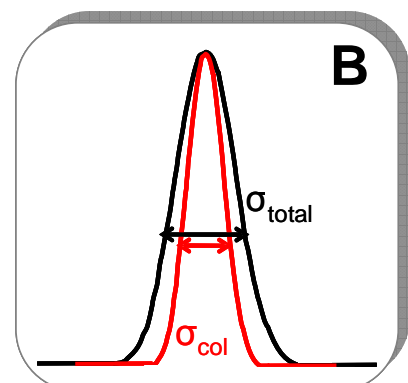
To work with columns packed with small particles, a specialized chromatographic system is needed. First, this instrument should be able to withstand the very high pressures generated by small particles and this is generally achieved by extending the pressure capabilities of the pumping system and by improving the sample introduction device to make it compatible with ultra-high pressures. Second, the chromatographic system should also be adapted to operate in fast and ultra-fast mode with reduced column volumes. Indeed, small diameter columns (1 and 2.1 mm I.D.) limit the frictional heating (generated under high pressure drops and high flow rates) and reduce the organic solvent consumption but require small extra-column volumes due to detection, tubing, and injection volume (figure 2). The following criteria have to be fulfilled to perform efficient separations:

The tubing volume should be reduced as much as possible. For this reason, the tubing length should be as short as possible and its diameter selected as a compromise between an acceptable generated pressure and a low volume. For this reason, **a system plumbed with 0.005" I.D. stainless steel tubing** and zero-dead volume fittings is generally preferred for UHPLC experiments.



$$\sigma_{tot}^2 = \sigma_{col}^2 + \sigma_{ext}^2$$

The injection volume should be selected in agreement with column geometry. A rule of thumb is to maintain the injected volume between 1 and 5% of the column dead volume. As most of the experiments carried out in UHPLC are performed with a 50x2.1 mm column ($V_0 = 120 \mu\text{L}$), the injected volume should be included **between 1 and 5 μL** , to limit band broadening. In addition, a fast injection cycle time is mandatory for analysis times lower than one or two minutes.



$$\sigma_{tot}^2 = \sigma_{inj}^2 + \sigma_{det}^2 + \sigma_{tubing}^2$$

Last but not least, the detector cell volume, time constant and acquisition rate should be carefully selected. The detector used in UHPLC should ideally possess a low cell volume (**2 μL or lower**) while the sensitivity shouldn't be lowered compared to that of a conventional HPLC instrument. Detector time constant has to be fast enough (**$\tau \leq 100 \text{ ms}$**) because peak widths are very small in UHPLC (only a few seconds). Finally, the detector sampling rate must be sufficiently high to acquire a suitable amount of data points across each peak (**> 20 Hz**).

It is noteworthy to note that to reduce the importance of extra-column volumes and avoid an unacceptable loss in efficiency, chromatographic conditions leading to high retention factors (**k at least equal to 3**) are often recommended in UHPLC (i.e. high σ_{col}^2 values inducing lower influence of σ_{ext}^2). However, due to the very small column dead time in UHPLC (e.g. $t_0 = 0.20 \text{ min}$ for conventional UHPLC conditions with a 50x2.1 mm, 1.9 μm columns operating at 600 $\mu\text{L}/\text{min}$), a high retention factor would not be detrimental for the analysis time. As an example, a retention factor of 5 in conventional HPLC leads to an analysis time of 10 minutes while it corresponds to only 1 minute in UHPLC.

Figure 2

The last critical parameter to perform ultra-fast separations in the gradient mode is the dwell volume of the system which corresponds to the time necessary for the gradient to reach the column inlet. To work with ultra-fast separations, a **small gradient delay volume** is required. With a large dwell volume, fast separations are compromised because an isocratic hold is generated at the beginning of the gradient, inducing potential changes in selectivity and longer analysis times.

To check the compatibility of an UHPLC instrumentation with a given column geometry, it is recommended to characterize the chromatographic system by determining the extra-column and dwell volumes. For optimal compatibility with ultra-fast separations, the former should be **lower than 20 μ L** while the latter should be no more than a few hundred μ L.

2.2. Quality of mobile phase and buffers

It is important to consider that the size of the frits and particles contained within UHPLC columns are much smaller than on regular HPLC packings. For example, the inlet frit pore size of an HPLC column is often equal to 2 μ m while it could be equal to only 0.2 μ m in UHPLC (this value strongly depends on the column provider). Therefore, small particles, potentially present within the mobile phase and which do not affect HPLC materials, can become critical in the UHPLC configuration. Therefore, it is important to check the absence of insoluble particles in the solvents and, for this purpose, several key rules have to be followed in UHPLC for the mobile phase preparation:

- Use only high grade organic solvents (ideally filtered through a 0.22 μ m membrane) – it is even possible to find acetonitrile of UHPLC grade from several suppliers (Biosolve[®], JT Baker[®], Fisher Scientific[®], etc...)
- Use high quality salts to prepare buffered mobile phases
- The water should be ultra pure and filtered through a 0.22 μ m membrane (Milli-Q[®] system or similar high quality water is recommended)
- Be careful with the microbiological growth (particularly when using phosphate buffer): always use freshly prepared mobile phases
- Be vigilant with the cleaning of glassware and do not top-off the bottles.

To limit the microbiological growth, the chromatographic system and columns should ideally be stored with pure organic solvents (methanol or acetonitrile).

2.3. UHPLC columns

One of the main criticisms made by early UHPLC users has been the reduced lifetime of columns packed with sub-2 μ m, compared to conventional columns. It is true that UHPLC columns are always exposed to very high pressures, but the packing pressure has been increased proportionally. In our laboratory, we have observed that lifetimes of UHPLC and regular HPLC columns are comparable. However, column lifetime also depends on the number of injections, number of column volumes or period of time used. With the latest generation of columns packed with sub-2 μ m, commercialized by different suppliers, it is possible to perform between 500 and 2,000 injections or even more on a single column. Such values correspond to about 5,000-20,000 column volumes, which are fully comparable with those obtained on standard HPLC columns. However, when considering the corresponding period of time, it is significantly reduced compared to conventional HPLC because of the higher throughput in UHPLC. For example, in a routine laboratory which performs a UHPLC analysis in 1 to 5 minutes, one thousand injections can be performed in a very short period of time. This time is significantly reduced compared to that using conventional instrumentation (10-fold longer) while a similar amount of work has been carried out. In addition, the re-equilibrating time needed during a UHPLC gradient should be drastically reduced compared to the regular HPLC, to limit the number of column volumes percolated.

Nevertheless, a real issue of UHPLC packing is linked to the very low volume of the column in conjunction with the high mobile phase linear velocity employed. Indeed, it is not recommended to let the system

continue pumping without performing any analysis because the column lifetime can be rapidly degraded (50 column volumes percolated through the column in only 10 minutes).

3. Method Development in UHPLC

The rules for developing a new method in UHPLC are slightly different from those of conventional HPLC because it is necessary to account for the backpressure constraint generated by the use of small particles.

3.1. Choice of column dimensions

Depending on the supplier, it is possible to find some columns dedicated to UHPLC with internal diameters of 1, 2.1 and 4.6 mm. As discussed previously, the 4.6 mm I.D. column is not of great interest because of the significant frictional heating generated by high mobile phase flow rates, generating a lack of repeatability of retention times and some potential difficulties in transferring methods from conventional HPLC. In addition, the consumption of organic solvent is critical as the flow rate should be in the range 3-5 mL/min. Concerning the 1mm I.D column, the effect of frictional heating should be neglected even at 1000 bar but the compatibility between this column geometry and any UHPLC instrument is extremely critical (particularly for tubing geometry). Due to these statements, **the 2.1 mm I.D. column should be considered as optimal for UHPLC operation.**

Regarding the column length, it should be selected according to the required efficiency (in isocratic mode) or peak capacity (in gradient mode). It has to be noted that there is no real limitation in UHPLC column length. Numerous suppliers propose some 150mm columns which can be coupled in series using low volume tubing if an experiment has to be performed with very long columns.

In the isocratic mode, it is well known from the basic equations of chromatography that efficiency is directly proportional to the column length. Therefore, a 50x2.1 mm, 1.9 μm column should generate around 10,000 plates, while the efficiency is increased to 20,000 and 30,000 plates with a 100 and 150x2.1 mm, 1.9 μm column respectively. Therefore, the column length should be chosen according to the requirement of the separation and the longest column always provides the highest efficiency. However, with 150 mm or longer columns, the mobile phase flow rate should be adapted to avoid over-pressurizing the analytical system.

In the gradient mode, the relationship between column length and chromatographic performance (expressed as peak capacity in gradient mode) is not trivial. In fact, the peak capacity depends both on efficiency and column dead time, but each to a different extent. Therefore, **the column length should be adapted according to the gradient time** and the longest column doesn't necessarily provide the highest peak capacity. It can be demonstrated that a 50x2.1 mm, 1.9 μm column has to be selected for gradient times lower than 5 minutes. The 100x2.1 mm, 1.9 μm column gives optimal performance for gradient times between 5 and 20 minutes and finally, the 150x2.1 mm, 1.9 μm column should only be used with gradients longer than 20 minutes.

3.2. Choice of mobile phase conditions

In UHPLC, the mobile phase flow rate has to be selected according to the Van Deemter curve (similarly to conventional HPLC) but also, according to the backpressure generated. For compounds with molecular weights in the range 100-400 $\text{g}\cdot\text{mol}^{-1}$, the optimal flow rate in isocratic mode for a 2.1 mm I.D. column packed with 1.9 μm particles is **around 400-600 $\mu\text{L}/\text{min}$** . Due to the low mass transfer resistance generated by small particles (because of the reduced diffusion path), it is even possible to work up to 1000 $\mu\text{L}/\text{min}$, with a limited loss in efficiency (around 20%). When dealing with larger molecules, the mobile phase flow rate should be reduced to 200-400 $\mu\text{L}/\text{min}$ due to a reduction of diffusion coefficients.

The rules in gradient mode are different, where the highest flow rate always provides the best peak capacity because it is dependent on the column dead time and, to a lesser extent, on efficiency. Therefore, the flow rate for gradient UHPLC experiments should be elevated but at the maximum equal to 80-90% of the maximum pressure withstood by the instrument. This solution is recommended to attain a sufficient level of robustness and to handle unexpected changes in column backpressure after hundreds of injections.

Regarding the mobile phase temperature, it could be valuable to work in UHPLC at a **mobile phase temperature of 40-50°C** instead of room temperature. With this strategy, the mobile phase viscosity is reduced and the backpressure diminishes by about 30% (at 50°C for an acetonitrile-water mobile phase) without affecting chromatographic performance.

Finally, it is well known that the viscosity of **acetonitrile-water hydro-organic mixtures** is on average 1.5 to 2-fold lower than that of methanol-water. Therefore, in the case of method development, the initial choice for mobile phase is acetonitrile as it generates significantly lower backpressure and more possibilities in UHPLC compared to methanol (particularly for the choice of column length).

3.3. Decision tree for method development

On the basis of the above discussion, it is possible to establish some generic conditions for the UHPLC method development: the column should be initially a C18 with geometry of 50x2.1 mm, 1.9 µm operating at a temperature of 40-50°C. The mobile phase should consist in a mixture of acetonitrile and buffer. It is generally better to begin the experiments in the gradient mode at a mobile phase flow rate close to the maximal pressure accepted by the UHPLC instrument. Regarding the choice of gradient time, it is extremely different in UHPLC compared to regular HPLC. Therefore, table 1 summarizes the optimal gradient time for various sets of UHPLC conditions.

Table 1: Calculated optimal gradient time according to column geometry.

Column geometry	Flow rate	Gradient profile	Optimal gradient time
150x4.6 mm, 5 µm	1 mL/min	5 - 95%	30 min
50x2.1 mm, 1.9 µm	600 µL/min	5 - 95%	3.5 min
50x2.1 mm, 1.9 µm	1 mL/min	5 - 95%	2.0 min
50x2.1 mm, 1.9 µm	600 µL/min	10- 60%	2.0 min
100x2.1 mm, 1.9 µm	300 µL/min	5 - 95%	14 min
100x2.1 mm, 1.9 µm	300 µL/min	10- 60%	8 min
150x2.1 mm, 1.9 µm	200 µL/min	5 - 95%	30 min
150x2.1 mm, 1.9 µm	200 µL/min	10- 60%	17 min

If the selectivity with the generic conditions previously described is not acceptable, it is possible to adapt various parameters (e.g. mobile phase pH, nature of the stationary phase and organic modifier). In UHPLC, the first parameter to change is the mobile phase pH, then the column chemistry and finally the organic modifier nature (because of the constraint in pressure with UHPLC).

4. Method Transfer in UHPLC

In various fields of application (i.e. pharmaceutical, environment, food,...), it is important to be able to transfer existing methods (performed in conventional HPLC conditions) to faster separations involving the use of columns packed with sub-2µm particles. As most of the providers now offer equivalent stationary phases packed with 5, 3 and sub-2µm particles, a geometrical transfer can be performed if the stationary phase chemistry remains identical between the original and final sets of conditions. For this purpose, some rules have to be applied in both isocratic and gradient modes.

4.1. Case of isocratic mode

In isocratic mode, two important parameters have to be modified, namely the injection volume and the mobile phase flow-rate.

To avoid a detrimental extra-column band broadening and maintain equivalent sensitivity, it is necessary to adapt the injection volume in line with the change of column dimensions. In liquid chromatography, the injected volume should represent only 1-5% of the column volume. The latter can be calculated simply from the column internal diameter (d_c) and length (L). Therefore, the injection volume is independent of the particle size and only proportional to the column volume. The new injected volume (V_{inj2}) can be determined simply by **maintaining the ratio of column dead volume and injected volume constant** between regular HPLC and UHPLC. Thus, the injected volume in UHPLC (V_{inj2}) can be calculated according to the following equation:

$$V_{inj2} = V_{inj1} \cdot \frac{d_{c2}^2}{d_{c1}^2} \cdot \frac{L_2}{L_1} \quad (1)$$

In this equation, subscripts 1 and 2 are related to HPLC and UHPLC column dimensions, respectively. For example, from a conventional 150x4.6 mm, 5 μm column to an UHPLC 50x2.1 mm, 1.9 μm column, the injected volume should be decreased by 14-fold. It can be noted that larger injection volumes than predicted can be used to maximize sensitivity. However, the sample should be dissolved in a solvent of weaker eluent strength than the initial mobile phase composition. This approach described as sample focusing (peak compression) allows the enrichment of the analytes on the top of the column.

Regarding mobile phase flow rate, it should be adapted to be close to the optimal value of the Van Deemter curve representation ($H=f(u)$). In liquid chromatography, it is well-known that the mobile phase linear velocity (u) is directly proportional to the square of column diameter and also depends on the particle size of the support. It is however, completely independent of the column length.

For a successful method transfer, it is mandatory to **maintain the product $u \cdot d_p$ constant**, to take into account simultaneous changes in column diameter and particle size of the support. Therefore, for a geometrical transfer, the UHPLC flow rate (F_2) can be calculated with the following equation:

$$F_2 = F_1 \cdot \frac{d_{c2}^2}{d_{c1}^2} \cdot \frac{d_{p1}}{d_{p2}} \quad (2)$$

As an example, from a regular 150x4.6 mm, 5 μm column to a UHPLC 50x2.1 mm, 1.9 μm column, the mobile phase flow rate should be decreased by 1.8-fold.

The expected analysis time of the transferred method (t_{ana2}) is directly **proportional to the change in column dead time** and can be estimated according to:

$$t_{ana2} = t_{ana1} \cdot \frac{F_1}{F_2} \cdot \frac{d_{c2}^2}{d_{c1}^2} \cdot \frac{L_2}{L_1} \quad (3)$$

The expected backpressure (ΔP_2) can be calculated from the Darcy's law which shows that ΔP is inversely proportional to d_p^3 and strictly related to the column length:

$$\Delta P_2 = \Delta P_1 \cdot \frac{L_2}{L_1} \cdot \frac{d_{p1}^3}{d_{p2}^3} \quad (4)$$

Finally, the expected solvent consumption of the transferred method (V_2) can be calculated by taking into account the change in column internal diameter, particle size and analysis time.

$$V_2 = V_1 \cdot \frac{d_{c_2}^2}{d_{c_1}^2} \cdot \frac{d_{p_1}}{d_{p_2}} \cdot \frac{t_{ana 2}}{t_{ana 1}} \quad (5)$$

Therefore, from a regular 150x4.6mm, 5 μ m column to an UHPLC 50x2.1 mm, 1.9 μ m column, the analysis time is reduced by **8-fold**. It has also to be noted that for the above-mentioned transfer, the efficiency would be **identical**, while the backpressure should be **6-fold** higher and the solvent consumption reduced by **14-fold**. This shows the benefits of the UHPLC strategy but also demonstrates the need to work with an instrument that withstands pressure higher than 400 bar.

Table 2: Optimal conditions to work with 6 column geometries that provide a similar theoretical efficiency of 10,000 plates. Changes in analysis time, backpressure and solvent consumption were also indicated.

Column geometry	Injected volume	Mobile phase flow rate	Change in analysis time	Change in backpressure	Change in solvent consumption
150x4.6 mm, 5 μ m	20 μ L	1.00 mL/min	-	-	-
100x4.6 mm, 3.5 μ m	13.3 μ L	1.43 mL/min	\div 2.1	x 1.9	\div 1.5
50x4.6 mm, 1.9 μ m	6.7 μ L	2.63 mL/min	\div 7.9	x 6.1	\div 3
150x2.1 mm, 5 μ m	4.2 μ L	0.21 mL/min	-	-	\div 4.8
100x2.1 mm, 3.5 μ m	2.8 μ L	0.30 mL/min	\div 2.1	x 1.9	\div 7.2
50x2.1 mm, 1.9 μ m	1.4 μ L	0.55 mL/min	\div 7.9	x 6.1	\div 14.4

Table 2 summarizes the injected volumes, mobile phase flow rates, analysis times, column backpressures and solvent consumption calculated with equations 1 to 5 for various typical column geometries.

It is possible to find in the literature a quantity of applications showing the possibility to transfer isocratic HPLC methods to columns packed with sub-2 μ m particles. For sake of clarity, only one example is reported but the approach can be applied to a wide variety of compounds and matrices. Figure 3 presents a method transfer from a conventional 150x4.6 mm, 5 μ m column to a 50x2.1 mm, 1.9 μ m column. Both columns provide an equivalent efficiency of around 10,000 plates (similar L/dp ratio). As shown on the chromatograms, efficiency, selectivity and resolution remain equivalent for the separation of seven common anxiolytic agents. After adjustment of mobile phase flow rate, the analysis time could be decreased by a factor of 7 (22 vs. 3.2 min), as expected from theory for a transfer from 5 to 1.9 μ m particles.

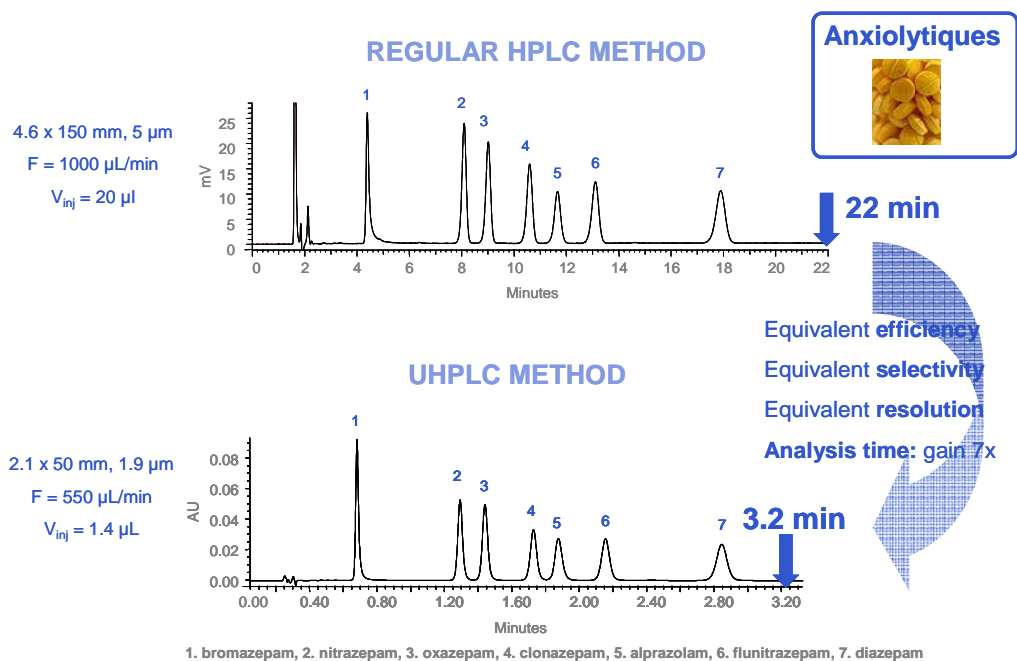


Figure 3: Isocratic method transfer from regular HPLC to UHPLC

4.2. Case of gradient mode

The rules for gradient method transfer are much more complex than isocratic ones but also based on basic principles of chromatography. First, the injection volume and mobile phase flow rate should be adapted in a similar way as the isocratic mode (see equations 1 and 2).

In linear or multi-linear gradient elution, the gradient profile can be decomposed as the combination of isocratic and gradient segments. The rules for efficient gradient transfer originally established by Snyder and Dolan (1) and recently updated by Carr *et al.* (2) should be strictly followed. For both parts, it is important to scale the gradient volume in proportion to the number of column volumes, to yield identical elution patterns, while the initial and final composition should be maintained constant. In fact, the number of column volumes percolated during the gradient in the regular HPLC system **should be equivalent** to that of the UHPLC set-up.

For any isocratic step within the gradient (i.e. initial isocratic step, isocratic step during a multi-linear gradient and also re-equilibrating time), the ratio between isocratic step time (t_{iso}) and column dead time (which depends on the mobile phase flow rate, column I.D. and length) should be maintained equivalent between conventional HPLC and UHPLC conditions. Therefore, the UHPLC isocratic step (t_{iso2}) can be determined with:

$$t_{iso2} = t_{iso1} \cdot \frac{F_1}{F_2} \cdot \frac{d_{c2}^2}{d_{c1}^2} \cdot \frac{L_2}{L_1} \quad (6)$$

As an example, from a regular 150x4.6 mm, 5 µm column to a UHPLC 50x2.1 mm, 1.9 µm column, the isocratic steps which occurred during the gradient process (including re-equilibrating time) should be reduced by 8-fold.

For slope segments, it is mandatory to keep the initial and final gradient composition (%B) constant. The new gradient time (t_{grad2}) can be expressed as:

$$t_{grad_2} = \frac{(\%B_{final_1} - \%B_{initial_1})}{slope_2} \quad (7)$$

The gradient slope ($slope_2$) should be calculated to maintain **the product of gradient slope and column dead time constant**. The new gradient slope ($slope_2$) can be expressed as:

$$slope_2 = slope_1 \cdot \frac{d_{c_1}^2}{d_{c_2}^2} \cdot \frac{L_1}{L_2} \cdot \frac{F_2}{F_1} \quad (8)$$

As an example, from a regular 150x4.6 mm, 5 μ m column to a UHPLC 50x2.1 mm, 1.9 μ m column, the gradient slope during the gradient process should be increased by 8-fold.

When transferring a gradient method from regular HPLC to UHPLC, some changes in selectivity could occur during the gradient run because of differences in dwell volume between the original and the UHPLC configuration. The system dwell volume (V_d) is also known as gradient delay volume. It refers to the volume between the mixing point of solvents and the head of the analytical column, as shown in figure 4. Low-pressure mixing systems possess generally larger dwell volumes than high-pressure mixing systems. After starting the gradient, it will take time until the selected proportion of solvent reaches the column. It means that the sample is subjected to an additional isocratic migration in the initial mobile phase condition. Since the gradient dwell volume may differ from one system to another, this extra isocratic step would be different and could result in retention time variations affecting resolution for early eluting peaks when transferring a method. To overcome this problem, **the ratio of system dwell time (t_d) and column dead time (t_0)** must be held constant while changing column dimensions, particle size or mobile phase flow rate.

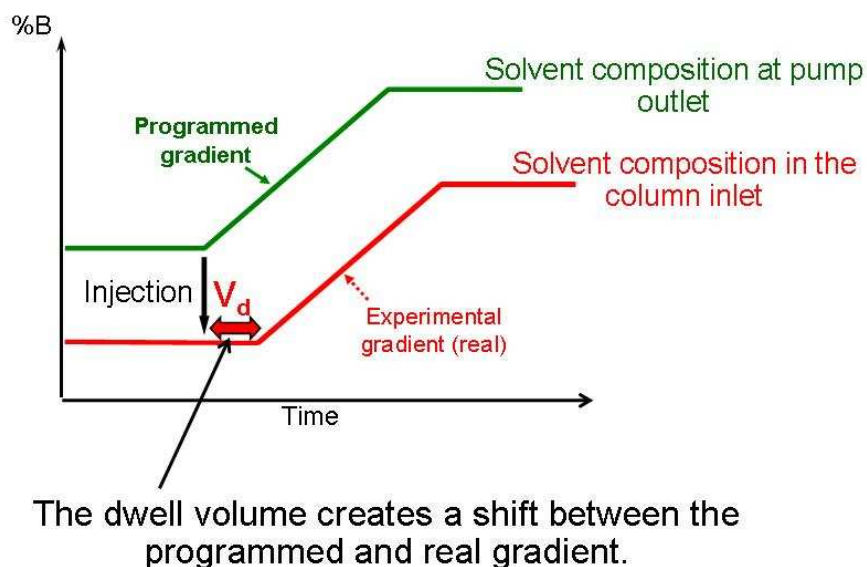


Figure 4: Importance of dwell volume in gradient method transfer

As the column dead time is reduced by around 8-fold between a regular 150x4.6 mm, 5 μ m column and a 50x2.1 mm, 1.9 μ m column, the system dwell time should be reduced by a similar factor. Therefore, it is mandatory to work in UHPLC with a system possessing a very low dwell volume (no more than a few hundred μ L) to limit the influence of V_d . When the difference between t_d/t_0 ratios remains too large, it is also possible to add an isocratic hold at the beginning of the UHPLC gradient.

Again, the applicability of the approach discussed can be found in the literature for method transfer between conventional and sub-2 μm packings. One example has been selected and is presented in figure 5. The separation of 12 pharmaceutical compounds was originally achieved using a 150x4.6 mm, 5 μm , C₁₈ column and further transferred to UHPLC with a 50x2.1 mm, 1.7 μm C₁₈ column possessing strictly similar chemistry. The original separation was performed in approximately 27 minutes and efficiently transferred to UHPLC in less than 3 min (reduction by a factor of 9). In addition, both separations were equivalent in terms of sensitivity, peak capacities and resolution, mainly because of an adequate reduction of system dwell volume (from 1.3 mL to 130 μL for HPLC and UHPLC, respectively).

Another relevant advantage of UHPLC is the re-equilibrating time reduction. In HPLC (150x4.6 mm column at 1 mL/min), re-equilibration took about 20 minutes, while using a short column packed with sub-2 μm particles (50x2.1 mm column at 600 $\mu\text{L}/\text{min}$), the re-equilibrating time decreases to only 2 minutes.

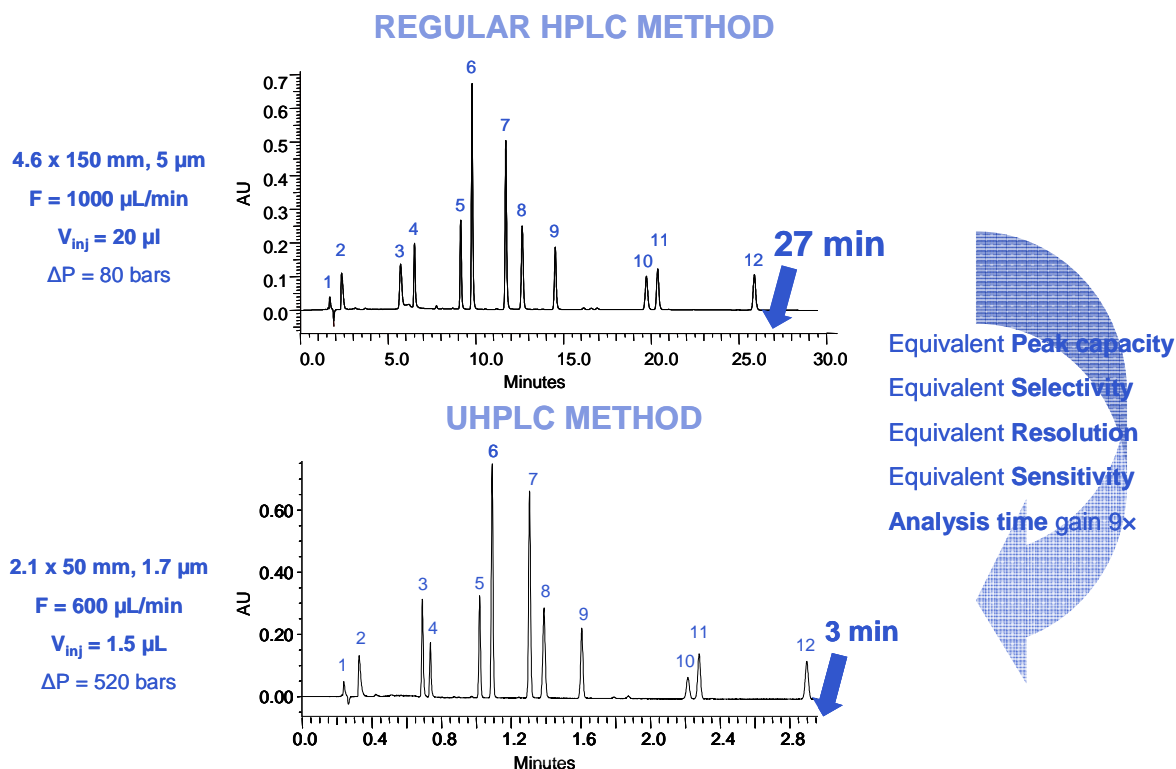


Figure 5: Gradient method transfer from regular HPLC to UHPLC

A last recommendation when working in UHPLC is to be careful when increasing mobile phase flow rate in the gradient mode. For an adequate transfer, it is necessary to adapt the gradient profile when increasing the flow rate, as illustrated by equations 6 and 8. Therefore, when changing mobile phase flow rate, the gradient slope and time of any isocratic step should be adapted in order to maintain equivalent selectivities.

Equations 1 to 8 can be used for determining the new parameters of a transferred isocratic or gradient method. However, at the University of Geneva, we developed a free program called "HPLC calculator" posted on our website (<http://www.unige.ch/sciences/pharm/fanal/lcap/divers/downloads.php>) which gives optimal conditions for method transfer in isocratic and gradient modes. In this calculator, the system dwell volume can also be considered for calculation in gradient mode.

5. Conclusion

The UHPLC technology (defined as columns packed with sub-2 μm particles used in very high pressure conditions) has proved to be a powerful approach to improving chromatographic analysis in terms of throughput and resolving power. By comparing its intrinsic performance with other existing techniques, such as monoliths or high temperature liquid chromatography, it is a very attractive strategy for improving chromatographic efficiencies in the range 1,000–80,000 plates.

An important factor in obtaining suitable performance with columns packed with sub-2 μ m particles is the choice of instrumentation. Indeed, it is mandatory to work with a chromatographic system that withstands pressure higher than 400 bar. The system should also possess very low extra-column volume, limited dwell volume and a sufficient detection acquisition rate.

The rules for method development in UHPLC are slightly different from that of regular HPLC because the high backpressure generated by the columns packed with very small particles has to be taken into account. For example, acetonitrile is the first choice for mobile phase organic modifier because of the lower viscosity compared to methanol. In addition, it is always beneficial to work at a temperature slightly higher than ambient (40-50°C).

One of the main advantages of UHPLC remains the possibility to transfer easily the existing HPLC methods to columns packed with sub-2 μ m particles, using basic equations of chromatography. In the isocratic mode, only the injected volume and mobile phase flow rate have to be adjusted. In the case of gradient elution, injected volume, flow rate, isocratic step duration and gradient slope must be adapted and dwell volume should be carefully considered.

6. References

1. J.W. Dolan, L.R. Snyder, Maintaining fixed band spacing when changing column dimensions in gradient elution, *J. Chromatogr. A* 799 (1998) 21-34
2. A.P. Schellinger, P.W. Carr, A practical approach to transferring linear gradient elution methods, *J. Chromatogr. A* 1077 (2005) 110-119

7. List of Abbreviations

Symbols:

d_c : column internal diameter

d_p : particle size of the support

F: mobile phase flow rate

k: retention factor of the compound

L: column length

N: chromatographic efficiency

t_0 : column dead time

t_{ana} : total analysis time

t_d : system dwell time

t_{iso} : initial isocratic hold

t_{grad} : gradient time

u: mobile phase linear velocity

V: Volume of solvent consumed

V_0 : column dead volume

V_{inj} : injection volume

Greek symbols:

ΔP : column backpressure

σ_{ext}^2 : extra-column dispersion

σ_{col}^2 : column dispersion

τ : time constant of the detector

%B_{initial}: initial percentage of organic modifier

%B_{final}: final percentage of organic modifier