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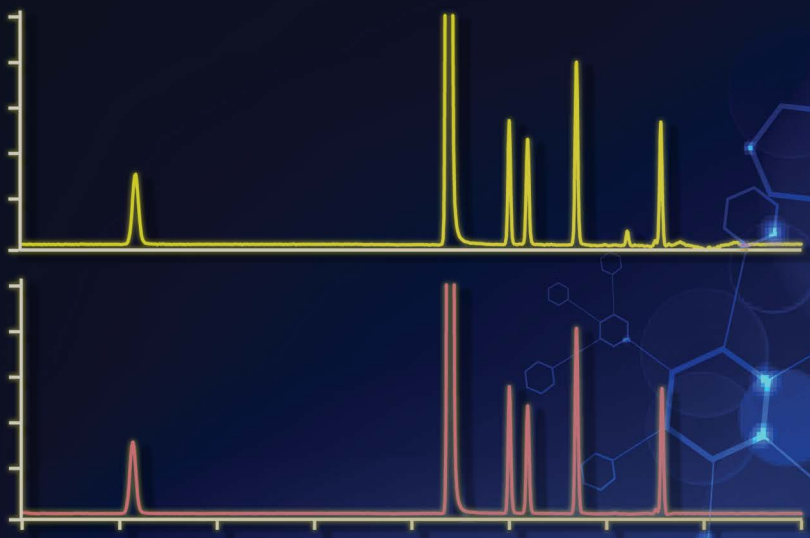

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An instrument parameter guide for successful (U)HPLC method transfer

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Goal

Explain in detail the instrumental parameters HPLC users need to consider during transfer of an analytical HPLC method between different instruments.

Introduction

The transfer of analytical procedures in liquid chromatography (LC) is a regular task in many laboratories. This challenge can be categorized into the following common scenarios:

A. Acceleration of methods, e.g. from HPLC to UHPLC methods
B. Method transfer to identical equipment, e.g. in another laboratory
C. Method transfer to a non-identical instrument, e.g. to a recently purchased system

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After the commercialization of ultra-high-performance liquid chromatography (UHPLC) instruments, and the simultaneous use of sub-2 µm column particles, scenario A became a common task in many laboratories. However, there are various publications available explaining the principles of method scaling.[1-3] Thus, scenario A is not further elaborated here, and the reader is referred to the existing literature.

For scenarios B and C, the aim of such a workflow is "simply" obtaining equivalent results between both systems to quickly have an operational method and to reduce revalidation efforts. For scenario B, the method robustness is the focus since the method is transferred between two identical systems. A discussion about criteria for method robustness/re-validation is not within the scope of this publication.

The challenge summarized under scenario C is often faced when transferring (validated) methods between different laboratories, e.g. from a developing laboratory to a QC laboratory or, similarly, from a sponsor laboratory to a contract laboratory. Here, the influence of instrument parameters on the chromatographic separation needs to be considered for successful transfer of an LC method from the originating to receiving laboratory.

This review explains instrumental parameters to be considered when transferring an LC method from one system to another. In addition, we will give recommendations on how to modify certain parameters to obtain equivalent results. These modifications are discussed with respect to USP General Chapter <621> Chromatography which describes the accepted limits of such modifications.[4] Finally, we give guidance on how to best characterize the root cause for common method transfer problems. This review focues solely on instrument parameters. Aspects such as correctly following an SOP, e.g. for buffer preparation, are not covered within this publication.

Categorization of (U)HPLC methods

The importance of instrument parameters for a successful method transfer became apparent over the last few years. The need to transfer methods gains importance due to the increasing involvement of external laboratories, such as contract research organizations, as well as the trend to transfer methods globally within a single company. In both cases, the chromatography instruments were often not identical, and difficulties occurred when reproducing results of the originating laboratory. In addition, the commercialization of UHPLC instruments with their significantly altered physical characteristics emphasized the influence of instrument parameters on a specific separation.

The extent to which a certain parameter influences the success rate of a method transfer process strongly depends on the actual application. Two important parameters are the column dimensions used (inner diameter and particle size) and the elution mode. Figure 1 shows the importance of the main instrument characteristics during the method transfer. For simplification purposes, the scenarios UHPLC (2.1 mm i.d. column, < 2 µm particles) versus HPLC (4.6 mm i.d. column, ≥ 3 µm particles) conditions, and isocratic versus gradient elution conditions are differentiated, as illustrated in Figure 1.

From these general considerations it becomes obvious that the gradient delay volume (GDV) is an important parameter for the transfer of a gradient elution method. Similarly, as the flow rates are generally lower for UHPLC separations, the importance of matching the GDV of the originating and receiving system is higher for UHPLC separations because small differences in GDV can affect retention times dramatically.

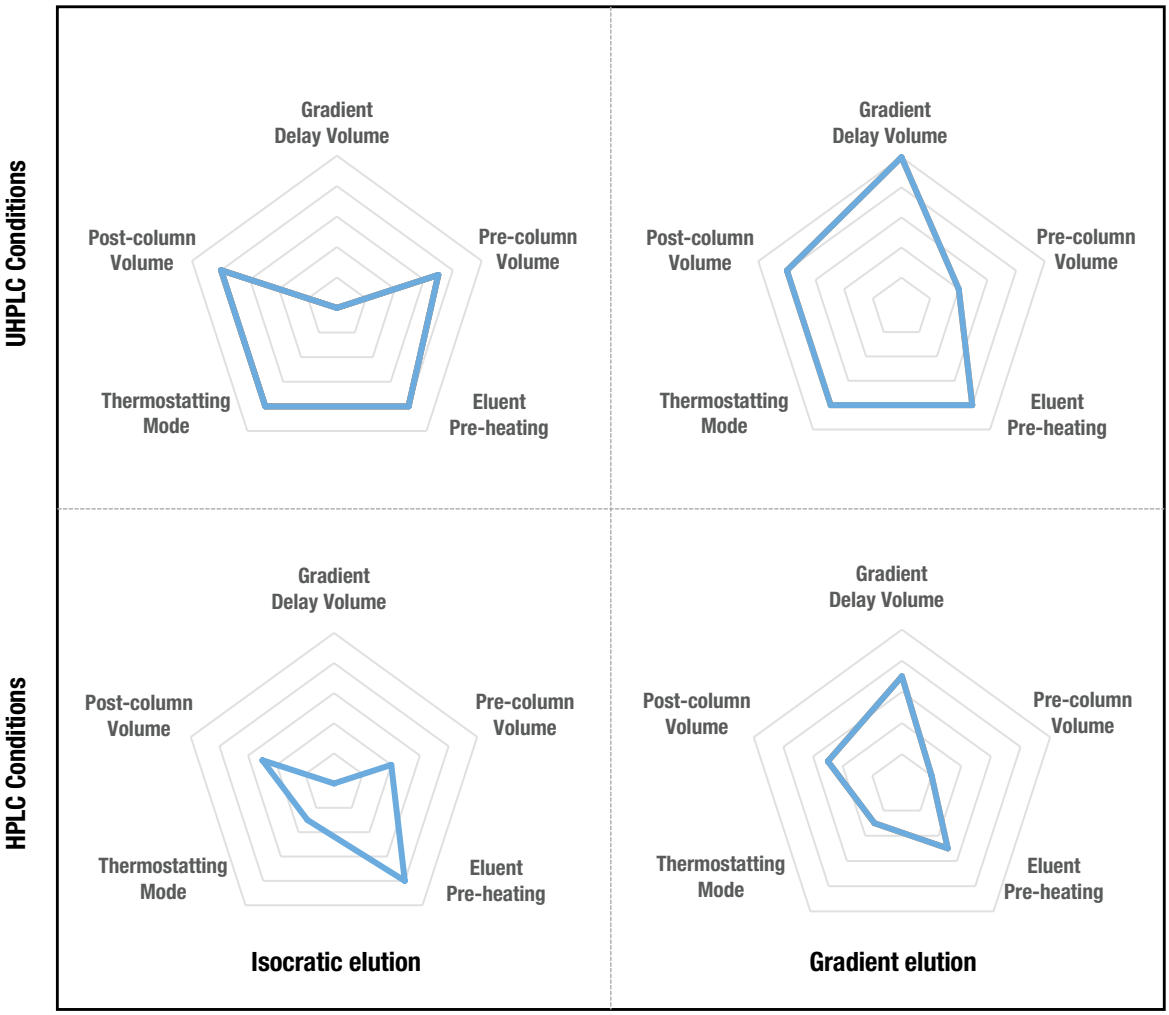


Figure 1. Instrument parameters and their importance for a successful method transfer. The further from the center of the graph, the more important the parameter. The importance value are estimates and dependent on additional method details such as separation temperature, flow rate, etc.

Furthermore, the thermostatting mode needs to be considered, which mainly describes how the instrument deals with frictional heating within the column. During standard HPLC separations, which regularly run below 400 bar (6000 psi), frictional heating is negligible. In contrast, under UHPLC conditions with pressures ranging up to 1500 bar (22,000 psi), significant frictional heating occurs. Thus, matching thermostatting modes is crucial when transferring UHPLC methods.

Gradient delay volume – What it is and how to measure it

The GDV is a physical characteristic of an HPLC system that describes the holding capacity of all interconnected components from the mixing point up to the entry of the column. Contributors to the GDV can include the pump, autosampler, and connecting capillaries. A consequence of the GDV is that a programmed elution gradient can enter the column with a delay, that can be calculated with the formula:

$$\textit{Delay time} = \frac{\textit{GDV}}{\textit{flow rate}}$$

As different HPLC instruments can have different GDVs, a particular solvent composition can arrive at different time points on the head of a column. Controlling the GDV can have a dramatic impact on reducing the amount of time required for a method transfer.

A common way to measure the GDV is to program a linear gradient from 0% to 100% B, with channel B containing a UV-absorbent compound. In this case, we used caffeine at a concentration of 12 mg/L (Figure 2).

The GDV is normally calculated by using the time when the UV trace reaches 50% of the maximal value (green arrow in Figure 2) according to the following formula:

$$GDV = (t_{50\%} - 0.5\ t_G) \times F$$

where $t_{50\%}$ is the time when the UV trace reaches 50% of the maximal value, t_G is the total gradient time, and F is the method flow rate.

An alternative approach is to use the time difference between the start of the gradient and the crossing of a linear extrapolation of the UV trace ramping up with the baseline (blue arrow in Figure 2). From our investigations, we found that using the method at 50% UV height (green arrow, Figure 2) is more reliable and thus we recommend this approach. In any case, care should be taken that no values are compared which originate from different evaluation methods.

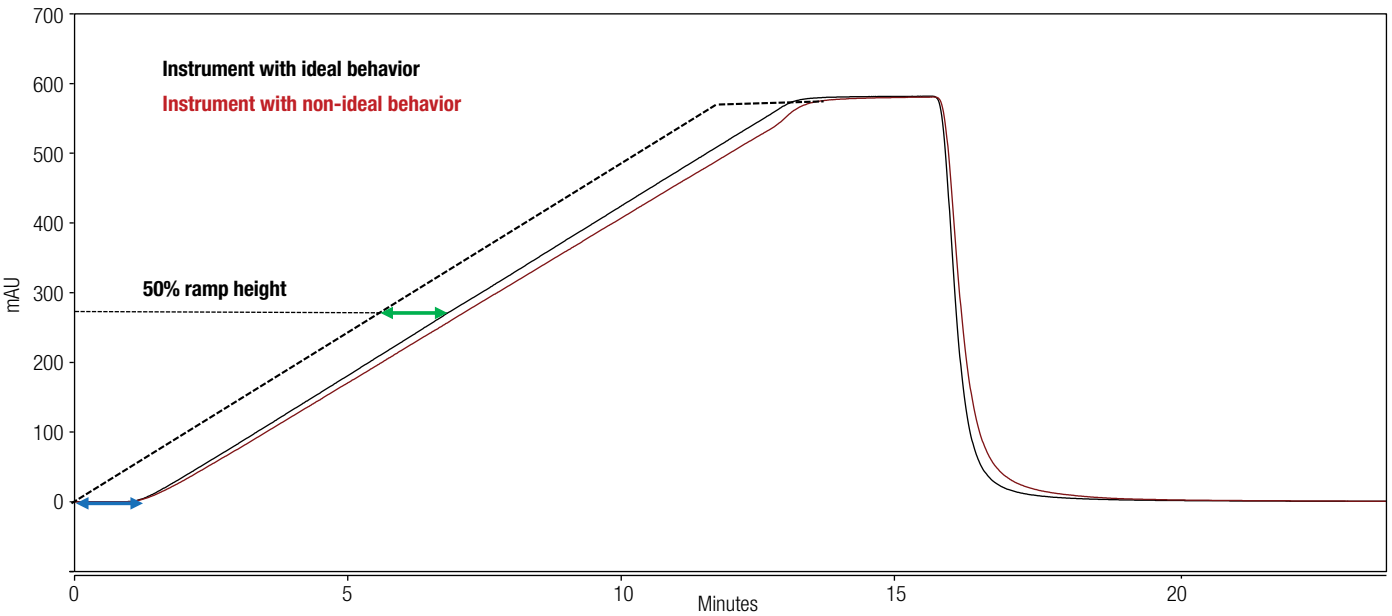


Figure 2. Method for determination of an instrument gradient delay volume. Two different instrument behaviors are shown as well as two commonly used data evaluation procedures (blue and green arrows).

In addition, the GDV is not a constant for specific HPLC or UHPLC instruments but depends on the flow rate and pressure applied. Figure 3 gives some examples for flow rate and pressure dependencies. Figure 3A shows the GDV of one system without a pulse damper and constant piston stroke volume at different flow rates while keeping the instrument backpressure constant. The differences between minimal and maximal GDV was up to 20%, with the lowest GDV observed at the highest tested flow rate of 3 mL/min. In contrast, Figure 3C shows the result of the same experiment using a system with a pulse damper and variable piston stroke volume. Here the GDV is more than 40% higher at the maximal flow rate of 3 mL/min compared to the lowest measured flow rate. This suggests that the GDV is not a fixed instrumental parameter but rather dependent on the applied method. For a successful method transfer, it will consequently be useful to determine the GDV under the original conditions.

Figure 3B shows the effect of the back pressure on the GDV. As expected, the GDV increases with increasing pressure by more than 40% when a pulse damper is used. However, in contrast to the flow rate, which is normally constant during one specific application, the pressure can change drastically during gradient elution. The result of this behavior is that retention times of compounds eluting during the gradient are affected by the dynamically changing GDVs and this needs to be considered for successful method transfer.

Table 1 gives an overview of commonly used HPLC systems equipped with a low pressure gradient type pump. As the measured GDV is flow rate dependent, a flow rate of 1 mL/min was used for all measurements to ensure best comparisons. Systems using a pulse damper have a high pressure dependency on their GDV. Even though it is not listed here, it should be noted that the GDV of high-pressure gradient type pumps is generally lower than for low-pressure gradient pumps, which makes the transfer between these instrument types more challenging.

Table 1. Summary of the GDV of several commonly used HPLC and UHPLC systems. Gradient tests were performed at a flow rate of 1 mL/min and a pressure of approximately 200 bar.

(U)HPLC System	GDV in µL
Thermo Scientific UltiMate 3000 SD Quaternary	1030
Thermo Scientific Vanquish Flex Quaternary	980
Agilent® 1100	1220
Agilent® 1260 Infinity® II Quaternary	1280
Waters® Alliance®	1150[5]
Shimadzu® LC-2010	1400
Shimadzu Nexera®-i	590 (40 µL mixer) 860 (300 µL mixer)

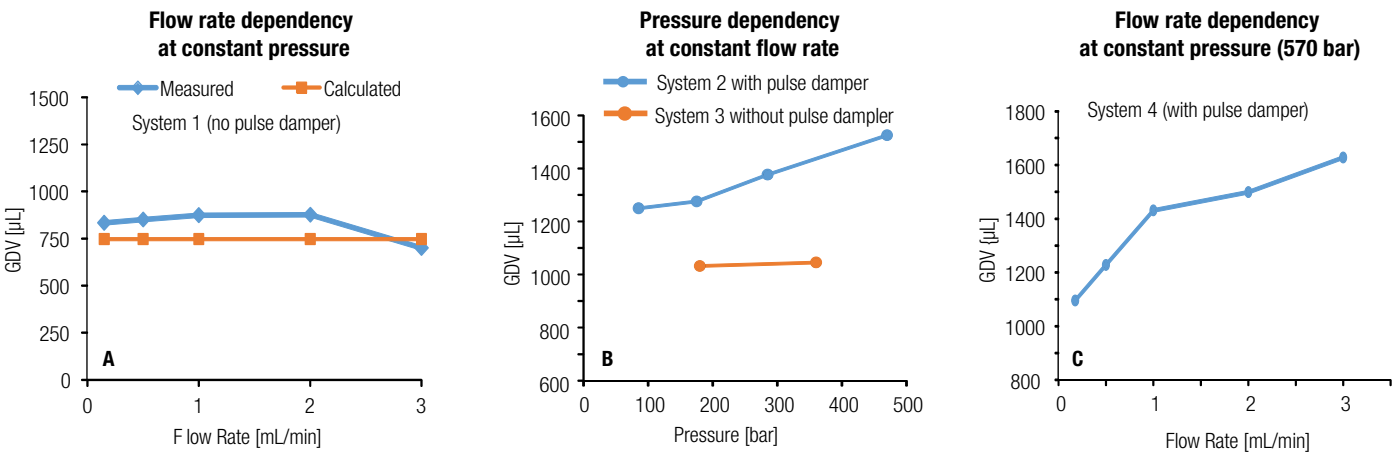


Figure 3. Dependency of gradient delay volume on flow rate and pressure for different types of instrumentation

Low-pressure vs. high-pressure mixing pumps

To form a gradient in liquid chromatography, two different gradient formation technologies exist—low-pressure gradient (LPG) and high-pressure gradient (HPG) proportioning. In the LPG, the convergence point of the solvents (normally up to 4) is before the pump head using a solenoid proportioning valve. LPG pumps generally have a higher GDV compared to HPG, since the pump heads contribute to the GDV.

Conversely, the HPG uses two independent pumps to deliver two solvents into the system. These two solvent streams converge after the pump on the high-pressure side of the HPLC. As the convergent point is after the pump heads on the high-pressure side, these pumps generally have a low GDV (Figure 4).

The difference in the gradient generation concept (e.g. solvent convergence either on the low- or on the high-pressure side of a pump) also has consequences on the flow and gradient accuracy as shown in Figure 5.

A simulated example is given for a programmed water/methanol gradient from 0% to 100% methanol at a flow rate of 1 mL/min (Figure 5A). For an HPG, both independent pumps deliver partial flow as determined by the desired gradient composition. For example, at a composition of 50% methanol, both pumps will deliver 500 µL/min. However, after converging both solvents on the high-pressure side of the pump, the resulting flow rate on the column will be less than 1 mL/min due to the volume contraction of both solvents. The contraction volume depends on the solvent and the mixture

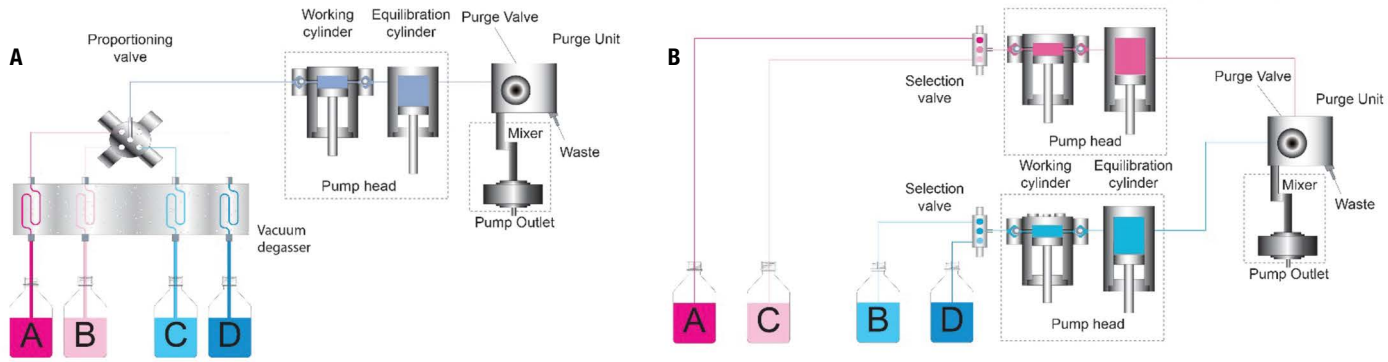


Figure 4. Schematic setup of a low-pressure gradient pump (A) and a high-pressure gradient pump (B). Note how the different solvent convergence points have effects on the gradient delay volume, which is defined as the volume between the convergent point of the solvents and the column head.

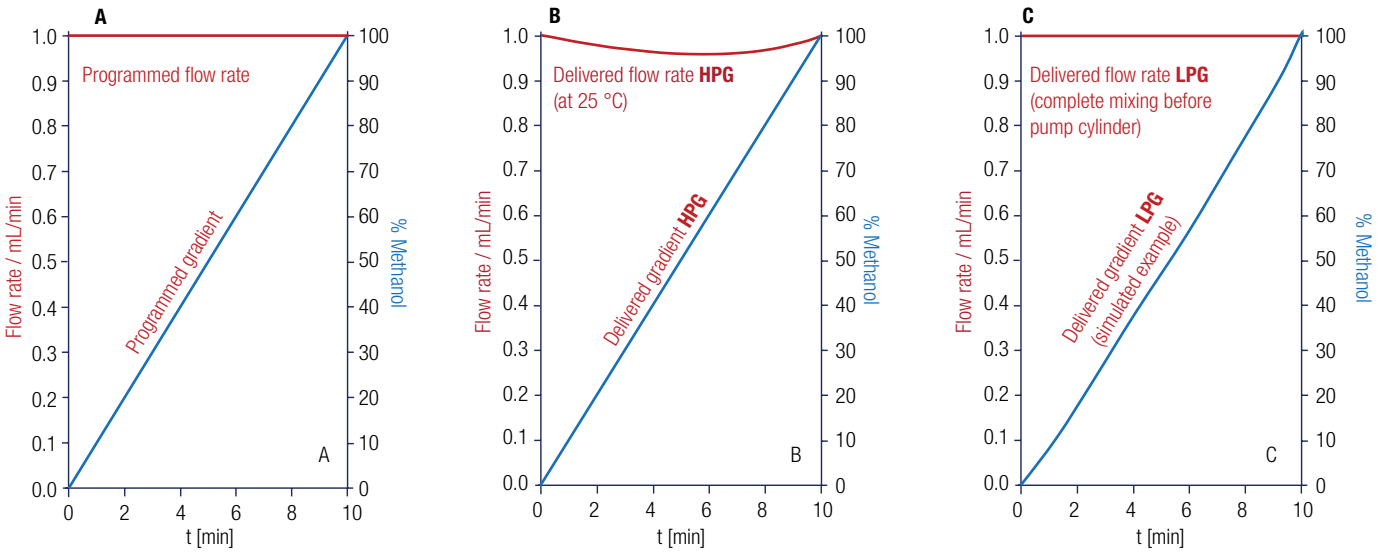


Figure 5. Flow rate and gradient accuracy of an HPG and LPG pump. Comparing A) programmed flow rate and gradient B) delivered flow rate and gradient of an HPG pump, and C) delivered flow rate and gradient of an LPG pump.

composition. For a methanol gradient, the error will be around 4% at a solvent composition of 55–60% methanol (Figure 5B). However, the gradient (solvent composition) delivered by an HPG pump is exactly as linear as the programmed gradient (Figure 5A). The LPG pump, in contrast, converges the solvents before the pump on the low-pressure side, and the delivered flow on the column will be 1 mL/min (Figure 5C). Furthermore, due to the volume contraction during the convergence of solvents at the proportioning valve, an LPG does not deliver the exact gradient composition as desired. Here the delivered gradient is not linear but rather bent.

As a consequence of this difference in the design of the pumps, it is generally recommended to consider the pump type (i.e., LPG or HPG) during a method transfer of the gradient. Preferably, methods should be transferred between the same pump type to avoid physical consequences of the design differences that may hamper method transfer results. Still, as described in the next chapter, care must be taken to reflect potential GDV differences that can appear even within one pump type.

Gradient delay volume adjustments

When a method is transferred, there are two general approaches used to adapt the different GDVs of the systems to facilitate the method transfer. Again, it should be considered that the transfer between HPG and LPG systems is normally accompanied with a significant difference in GDV and other differences that make method transfer more challenging. In addition to the two approaches explained in the next sections, the use of an isocratic hold at the beginning of a gradient program is a common practice in many HPLC laboratories. When such methods are transferred to a system with a larger GDV, the isocratic hold can simply be shortened. The change of the duration of the initial isocratic hold is allowed according to USP <621>.[4]

Adopting the GDV

An effective and straightforward way to compensate GDV differences between the originating and the receiving HPLC system is to physically change the GDV of the receiving system so that it matches the original system's GDV. An easy way to change the GDV is to adapt the mixer volume or sample loop volume of the instrument you are trying to transfer to. Such physical changes of the system are accepted and consistent with the USP guidelines.

Figure 6 gives an example of how compensation for the GDV differences was performed to transfer a method from an Agilent® 1260 Infinity® II system to a Thermo Scientific™ UltiMate™ 3000 Standard (SD) system. In this case, increasing the mixer volume from 400 µL to 800 µL on the UltiMate 3000 SD resulted in a good match of the gradient profile.

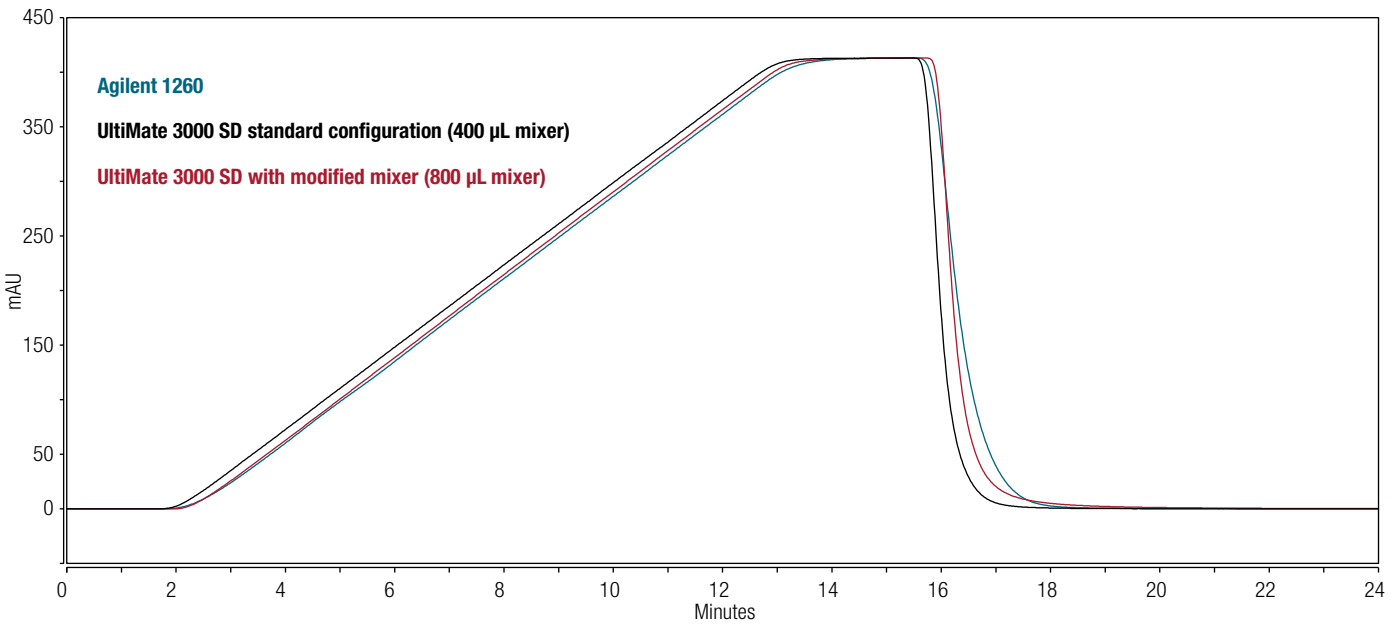


Figure 6. Overlaid gradient profiles of an Agilent 1260 LPG system, an UltiMate 3000 SD LPG system, and an UltiMate 3000 SD LPG system with increased mixer volume to compensate for GDV differences

Afterwards, the adopted instrumental setup was used to transfer the separation of 10 pesticides from the Agilent 1260 Infinity II system to the UltiMate 3000 SD system (Figure 7B). With this setup, the method could be transferred and a nearly identical separation was achieved. The same approach was also used to transfer a method for the separation of drugs used for the treatment of heart disease from an Agilent 1100 system to an UltiMate 3000 SD system. In this case, the installation of the 800 µL mixer kit also turned out to be successful (Figure 7A).

Besides changing the mixer of the pump (or the sample loop in the autosampler), the Thermo Scientific™ Vanquish™ UHPLC product line also allows the fine tuning of the GDV by adjusting the GDV via a metering device located in the autosampler which contributes to the system GDV. However, as this volume is adjustable with a simple software command, the user can gradually change the GDV for best method transfer. With this tool, it is possible to continuously vary the default GDV of any Vanquish system by a maximum of 100 µL. This feature is of help when already small differences in GDV hinder a successful method transfer (e.g. separation at flow rates around 400 µL/min or smaller or for the transfer between low GDV binary pumps of different vendors).

Changing the injection point relative to the gradient start

The second possibility to account for different GDVs between two HPLC systems is to move the injection time point relative to the gradient start. For instance, the originating system could have a GDV of 0.8 mL and the receiving system a GDV of 1.8 mL, resulting in a 1 mL difference. In this case, this difference can be compensated for by injecting the sample after the gradient start. For a flow rate of 1 mL/min, this would mean that the injection occurs one minute after the gradient program has started. In a practical sense, this would mean that the gradient starts at a time of -1 min relative to the injection, which always defines the zero point of a timetable. In this way, the slope and duration of the gradient would not be affected.

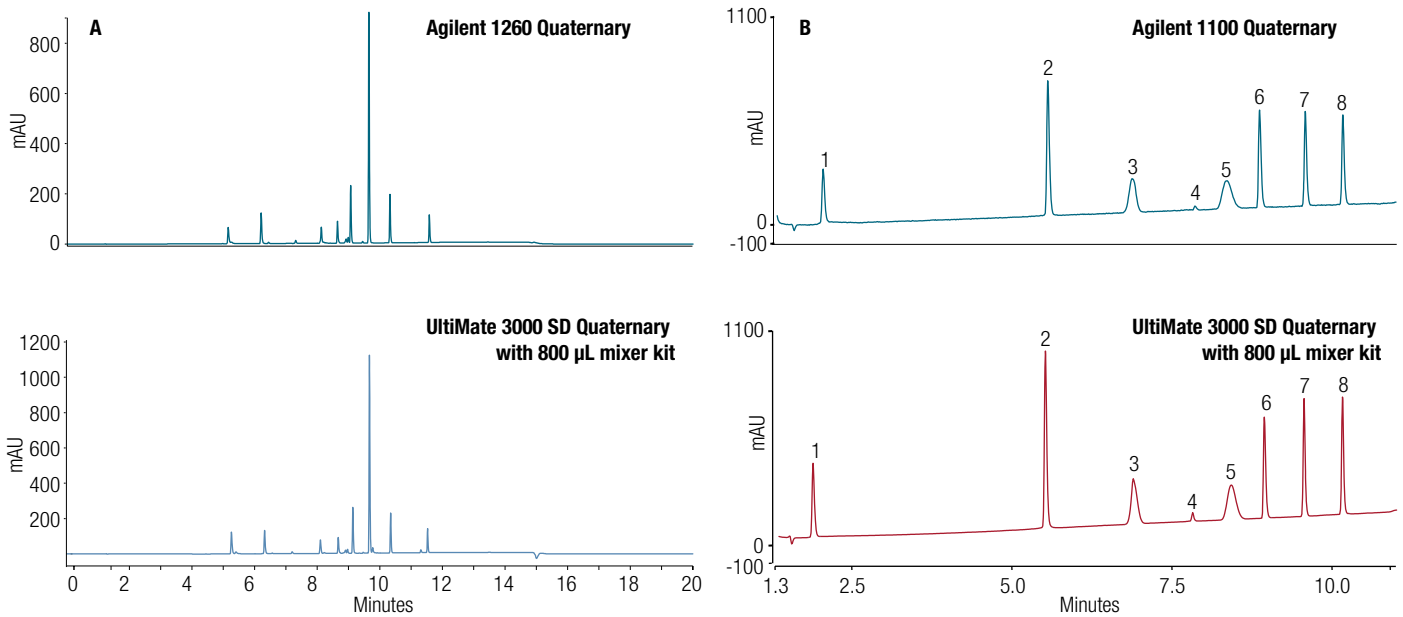


Figure 7. Transfer from an Agilent 1260 instrument to an UltiMate 3000 SD instrument (A) and transfer from an Agilent 1100 instrument to an UltiMate 3000 SD instrument (B). To match the gradient delay volume characteristics, the default mixer of an UltiMate 3000 SD system was exchanged to the 800 µL mixer kit.

In another example, Figure 8 shows the transfer of a method for acetaminophen and five impurities from an Agilent 1260 to an UltiMate 3000 SD instrument. The UltiMate 3000 SD system configuration has a lower default GDV. To compensate for this difference, an 800 µL mixer setup was installed. However, for this application that only runs at 120 bar, the additional mixer volume overcompensated the GDV difference (Figure 8, middle chromatogram). In such cases, a gradient pre-start can be programmed by the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software to start the gradient prior to the injection point. This resulted in a perfect overlay of both chromatograms (Figure 8, bottom) while smaller peak widths were observed for the UltiMate 3000 SD system.

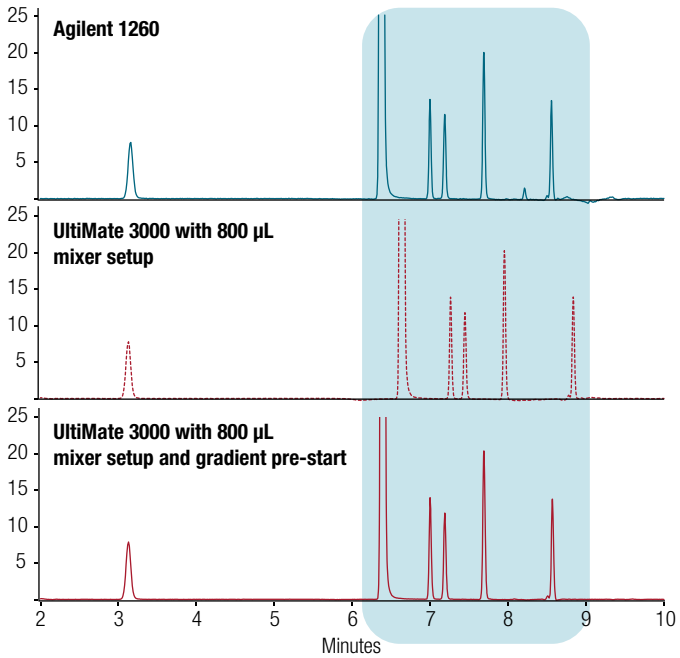


Figure 8. Transfer of a separation of acetaminophen and its impurities from an Agilent 1260 low pressure gradient to an UltiMate 3000 SD low pressure gradient system. For the UltiMate 3000 SD system the 800 µL mixer setup was used. To compensate for the higher gradient delay volume of the UltiMate 3000 SD system under these conditions, a gradient prestart was programmed.

Mobile phase pre-heating in front of the column

The temperature of a solvent entering a HPLC column may have an impact on both, resulting peak shapes and retention factors. Proper eluent temperature pre-conditioning is essential to achieve optimal column efficiencies, especially when working at column. temperatures above ambient. When the temperature of the incoming solvent is significantly lower than the column temperature, a radial temperature gradient between the center of the column and the column wall is formed, at least in the inlet part of the column. Such conditions are referred to as thermal mismatch effects and can have a strong impact on peak shape, resulting in peak broadening or peak distortion in the chromatogram. Thus, it is recommended to generally use the eluent pre-heating capability of an HPLC system.

For successful method transfer, care should be taken to also transfer the pre-heating capabilities of the originating system as accurately as possible. Beside the simple yes/no decision if a pre-heater needs to be included or not, the specific design, functional principle, and volume of the respective pre-heater must be considered.

Active and passive pre-heaters have two fundamentally different functional principles to distinguish. Passive pre-heaters (or temperature pre-conditioners) are more common and they work on the principle of a heat exchange device in mechanical contact to a temperature-controlled surface in the column compartment. From its surface, heat is transferred over the pre-heater into the incoming mobile phase along the temperature gradient. If this gradient has the opposite direction ($T_{Compartment} < T_{Eluent}$), heat flow occurs from the incoming eluent to the surface and the device acts as an eluent pre-cooler. This applies when the column compartment is cooled down below ambient conditions because the separation method requires low temperatures. Active pre-heaters are devices that are mostly independent from the temperature control of the column compartment. They use an internal heating element to regulate the temperature to actively control the resulting eluent temperature. The active eluent pre-heater of the Vanquish platform provides a unique opportunity to measure and control the temperature of the eluent streaming into the column, independent of the column compartment temperature. With this, it also allows the user to set the eluent temperature to a different value than the column compartment temperature, at least within certain limits. While column compartments mostly control the temperature by Peltier elements that can either heat or cool depending on the polarity of the applied voltage, the active eluent conditioners typically use a resistance heater, as this is a

much less bulky device to mount directly in front of the column. The consequence is that they can only heat and therefore cannot condition to sub-ambient temperatures. Table 2 provides an overview on the most important characteristics that distinguish active and passive pre-heaters.

Thanks to the flexible and independent temperature control of active pre-heaters, they provide clear advantages in method transfer scenarios. They can either mimic deviations from the expected outlet temperature of passive devices or compensate for deviations in the dissipation of frictional heat from the column. The advantage of these capabilities will be discussed in the section on column thermostatting.

In cases where a passive pre-heater is used, the volume should be considered, as this is normally the only readily available information. In general, a pre-heater with increased volume exhibits a more efficient pre-heating effect but also increases the extra column volume (Figure 9) and dispersion. That dispersion can be critical in method transfer especially for isocratic separations and UHPLC columns that generate very low peak volumes.

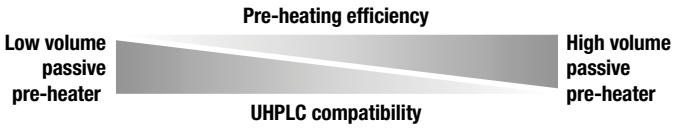


Figure 9. Passive pre-heater efficiency and UHPLC compatibility of different sized pre-heaters

It is thus important to match the pre-heater volume to the specific method requirements, keeping in mind the impact of the column design and flow rate. Elaboration of the experimental setup is required to study the effects of pre-heating since the temperature cannot be directly controlled with passive pre-heaters. The effects of pre-heating were investigated with an UltiMate 3000 forced air column thermostat using different passive pre-heaters and passing ambient temperature water through a column under different elevated temperature settings in the column compartment. The outlet temperature was recorded with a PT-1000 sensor in close contact to the outer surface of the 1/32" stainless steel capillary with thorough insulation using carved Styrodur™ foam.

Table 2. Comparison of passive temperature conditioners and active pre-heaters for features and benefits

	Passive eluent temperature conditioners	Active eluent pre-heaters
Cost	• Not significantly higher than connection capillaries with advanced fitting technique	• Significantly higher than capillaries with advanced fitting techniques by integrated temperature control device and temperature sensor
Temperature control	• Linked to compartment temperature, therefore can also cool down eluents • Lower heating performance for high temperatures and elevated flow rates • No control of heat/cool efficiency	• Temperature control independent of column compartment • Provides highest heating performance at relatively low volume • Heating efficiency can be monitored • Can only heat eluents
Mounting flexibility	• Requires solid contact to temperature-controlled surface in column compartment • Requires fix mounting position and typical size complicates very short connections to column	• Requires electrical contact, otherwise position is independent • Relatively small devices can be directly connected to column inlet
Availability	• Very common type that all manufacturers provide (often) with wide flexibility in volumes, contact materials and internal diameters	• Small selection of manufacturers, different volumes for different flow rates not required, flexibility in contact materials

Figure 10 shows the results for column compartment temperatures of 50 °C, 85 °C, and 105 °C under flow rates between 0.25 mL/min and 5 mL/min and pre-heater volumes of 2 µL, 7 µL, and 11 µL. At the lowest temperature, the 2 µL and the 7 µL pre-heaters were not different, therefore the results of the largest pre-heater are not shown. At low flow rates, the plots of all temperatures indicate that the temperature of the outgoing eluent is above the set-point of the column compartment. This removes the common misconception that passive pre-heaters can never heat to temperatures higher than the column compartment. The reason is that the compartment temperature is measured in the air surrounding the column and not at the plate where the pre-heater is mounted. This plate can be at higher temperature than the air in the center of the column compartment because of heat loss during thermostatting. Another observation is that the increasing slope of eluent temperature decreases with higher flow rate. These curves also show differentiation between the individual pre-heaters. As the pre-heater volume increases and is run at very high flow rates, the heating effect is greater due to the longer (but still considerably short) time the solvent spends in the device. Interestingly, the 2 µL and 7 µL curves cross at all temperature settings. To understand this effect, several pre-heater properties should be considered (Table 3).

Table 3 shows that all devices used in this study had different internal capillary diameters, resulting in substantially different surface-to-volume ratios. Smaller volume pre-heaters have higher surface-to-volume ratios, which improved the pre-heating effect at low flow rates when the time the solvent spends in the heat-exchanger is sufficiently long. Table 3 also shows the total volume of (including the connection capillary volume, which is substantially larger than the heated volume) and the internal diameter of the pre-heaters; both of which have a pronounced effect on the pre-column dispersion. Dispersion, which is expressed as resulting peak volume, decreases with the square of the tubing diameter (right column, Table 3). The trade-off between heating and dispersion will be discussed below. From the data in Figure 10 it can be concluded that the 2 µL pre-heater is effective for flow rates up to 2 mL/min for pure water, which has a markedly higher heat conductivity (factor 3 at 25 °C) than methanol and acetonitrile.[6]

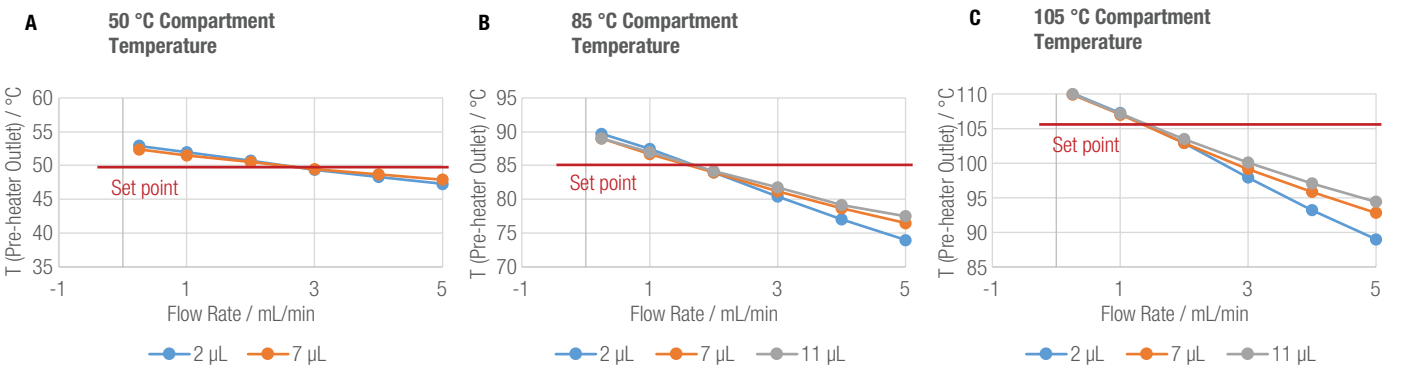


Figure 10. Passive eluent pre-heating effects on flow rate and pre-heater volume grouped by set compartment temperature

Table 3. Physical parameters of the different passive pre-conditioners studied

Nominal heated volume (µL)	Total volume with connectors (µL)	Internal capillary diameter (mm)	Surface to volume ratio (mm^2/mm^3)	Diameter induced dispersion effect (normalized to 1 µL pre-heater)
1	5	0.10	20	1.0
2	8	0.13	15	1.7
7	16	0.18	11	3.2
11	34	0.25	8	6.3

The combined effects of dispersion and eluent heating effectiveness of different passive pre-heaters can be seen from the chromatograms in Figure 11 and Figure 12. The black chromatograms on top show

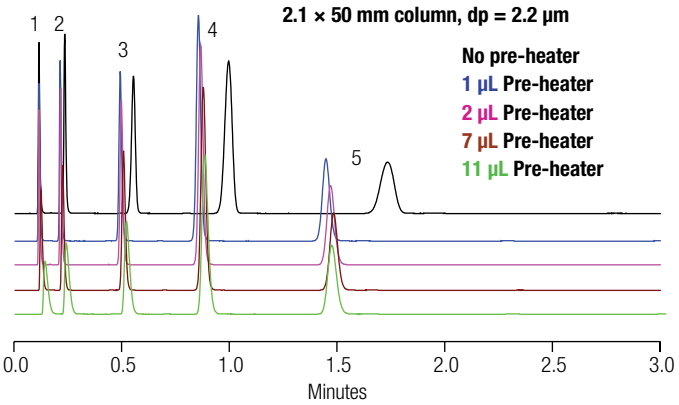


Figure 11. Standard isocratic column test on a column that produces small peak volumes show the effect of the pre-heater on peak shape and retention. Stationary phase: Thermo Scientific™ Acclaim™ RSLC 120 C18, eluent: water/acetonitrile 60/40 v/v, flow rate: 1.0 mL/min, column temperature: 70 °C. Peak assignment: 1: Uracil, 2: Nitroaniline, 3: Methylbenzoate, 4: Phenetole, 5: o-Xylene.

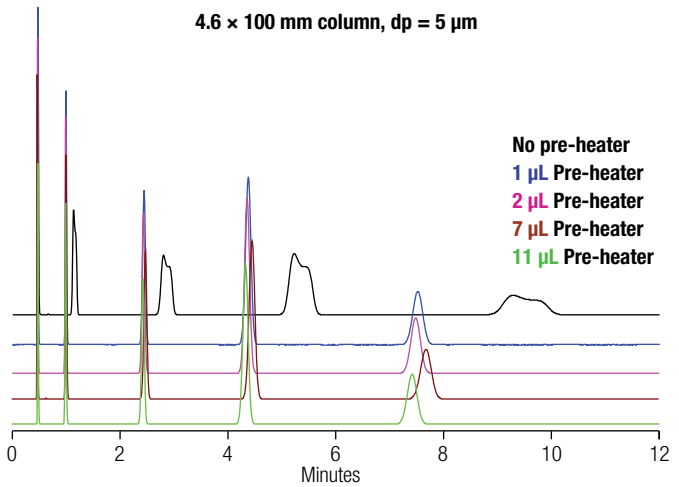


Figure 12. Standard isocratic column test on a wide bore column that produces relatively large peak volumes and is operated at elevated flow rate, show the effect of the pre-heater on peak shape and retention. Peak assignment, stationary phase, eluent, and column temperature as in Figure 11, flow rate: 2.0 mL/min.

the results without a pre-heater. The 2.1 mm column operated at 1 mL/min only shows broadened peaks (Figure 11), while the thermal mismatch in the 4.6 mm column leads to severe peak split or shoulder formation, which increases with the retention factor (Figure 12). This is caused by the less effective pre-heating in the connection capillary at high flow rates and the wider radial temperature gradient in a larger bore column.

As soon as a pre-heater is used, the peaks become much sharper and the retention factor is consistently reduced. These effects are more pronounced on the wide bore column and they result from the reduced thermal mismatch and higher average temperature inside the column when using a pre-heater. Also, the different pre-heater geometries have an effect on both retention and peak shapes that strongly varies with column dimension. While early eluting peaks become broad and asymmetric with the 2.1 mm column, there is no negative effect on peak shape with the conventional 4.6 mm column. It is also interesting to see how retention changes across the different pre-heaters. For both methods, the 7 µL pre-heater produces a lower internal temperature than the 2 µL pre-heater, which is in line with the data for 1 mL/min flow rate (Figure 9). When the 11 µL pre-heater is applied to the 4.6 mm column, it produces a separation with earlier elution of compounds than the 7 uL preheater. One might expect this with higher column temperatures, but it is due to the higher dwell time in a pre-heater with more similar surface-to-volume ratio. With the pre-heater outlet temperature measurement experiments applying pure water as mobile phase, this was at F = 2 mL/min only observed for T = 105 °C (Figure 9). Acetonitrile in the mobile phase of the chromatographic experiments conducts less heat, so the pre-heating conditions will be different relative to experiments with water.

The deepest insights into the effect of the pre-heater on peak shape can be obtained from plotting the determined plate number (N) of all peaks against their retention factor (k). Figure 13 compares the curves with and without pre-heaters in two different columns and methods. While the effect of thermal mismatch is expressed as a reduction in plate number with increasing retention, the effect of extra-column dispersion has the opposite characteristic. The N vs k plot can be used to characterize if the extent of extra-column dispersion of a system is appropriate for a certain column and method. Less extra-column dispersion can be tolerated with smaller peak volumes, in particular for early eluting peaks in isocratic methods. A basic rule of thumb demands 80% of the maximum efficiency that a column delivers in a given method should be achieved at a retention factor above 2. However, if plate numbers decrease in a method with increasing retention, a thermal mismatch effect is indicated. Although it is difficult to discriminate both effects occurring simultaneously, the N vs k plots can give valuable hints. The curves for small bore UHPLC columns are shown in Figure 13A. The operation without the pre-heater (blue) shows decreased efficiency with increasing retention, which clearly indicates thermal mismatch. The curve for the 1 µL pre-heater (orange) shows a normal characteristic of increasing plate number with the second peak at k=3.3 exhibiting 85% (6700) of the maximum plate number of 7900 which is acceptable. The curve for the 11 µL pre-heater (grey), starts with extremely low efficiency, while the second peak at k=2.7 only shows 37% (2200) of the maximum efficiency of 5900 plates, which is far below the 8000 plates that this column should provide in the respective method. Figure 13B shows the same scenarios for the conventional 4.6 mm column. The plate numbers without the pre-heater are included for completeness, but they are calculated from split peaks at high retention and are thus not meaningful. The curve for the 1 µL pre-heater (orange) shows a linear decrease in efficiency with increasing retention, thus pointing to a thermal mismatch effect. Looking at the curve from the 11 µL pre-heater (grey), one can see a normal behavior for an ideal column-to-system match. There is a slight effect of extra-column dispersion, which increases the plate number from 8400 to 9400 between the first and the second retained peak. After that, there is a slight decrease in plate number when going to very high retention. This effect is no thermal mismatch, but results from a stronger contribution of hindered mass transfer expressed as increasing C-term in the van Deemter or Knox equation with increased retention. This mass transfer effect is present in all scenarios and is more or less hidden by the thermal mismatch or extra-column dispersion effect. From the similarity of the orange and grey curve of the 4.6 mm column and from the generally good efficiencies with the 1 µL pre-heater, it can be deduced that the thermal mismatch with the small pre-heater and large column combination is not too severe, while the performance advantage of the 11 µL pre-heater is only minor. In other words, it would still be possible to use the 1 µL pre-heater for the conventional column, but the UHPLC column definitely requires a small volume pre-heater that keeps extra-column dispersion as low as possible.

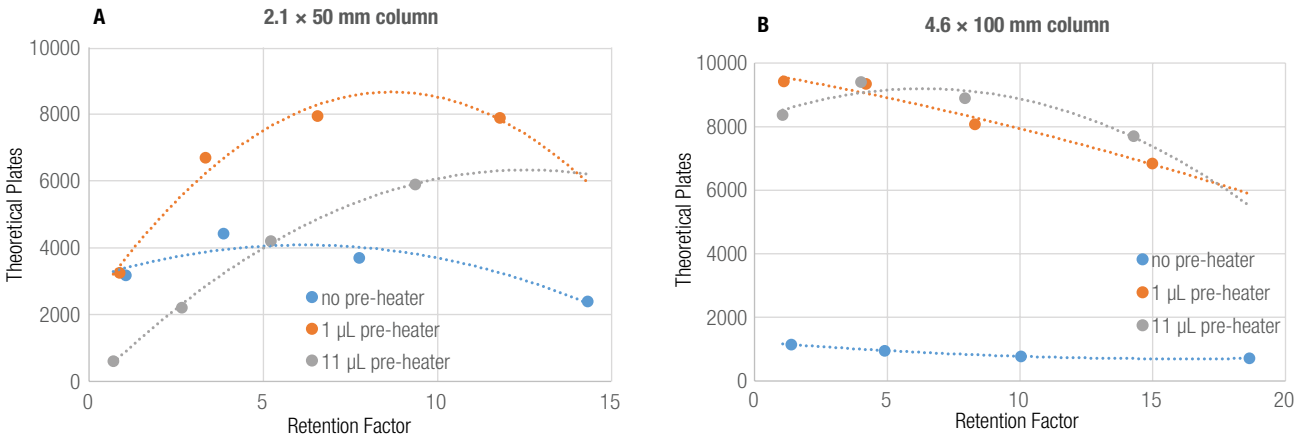


Figure 13. Plot of plate number against retention factor for both column type and experiment with no pre-heater, 1 µL pre-heater, and 11 µL pre-heater

The conclusion for the proper selection of a passive pre-heater in method transfer is not easy and straightforward. The simple rule to increase pre-heater volume with column volume could be demonstrated, but with highly heat-transfer effective small volume pre-heaters, the need for pre-heater volume increase is not always so strong, at least as long as flow rates do not exceed a certain limit. Predictions on the pre-heater volume that gives the best match to the behavior of the originating system will always be difficult, but it is advantageous to have a choice of devices to experimentally find the best one. In general, an appropriate pre-heater should always be used when the column temperature is 10 °C or more above ambient. If there is a choice, one should always start with the smallest available pre-heater. If the heating effect is not sufficient, this will be detected by poor efficiency of the peaks with higher retention and then the next larger pre-heater should be tested.

Column thermostatting and advantages of active pre-heaters

Effects of column thermostatting (even beyond the correct temperature control in the column compartment) are not typically considered in an HPLC or UHPLC method transfer scenario when it comes to root cause analysis of deviating chromatograms. For instance, if the retention times vary between the originating and the receiving system, differences in GDV or flush out behavior are often regarded as the only reason for the observed effect. Similarly, if differences in peak shapes are observed, an effect of the extra-column volume is regarded as the main problem. However, there are different column thermostatting modes applied for HPLC instruments that can have a significant effect on the chromatogram, especially when working at pressures above 400 bar (6000 psi).[7] For applications above 400 bar (6000 psi) the two thermostatting modes, forced and still air, will affect the produced frictional heating differently (Figure 14).

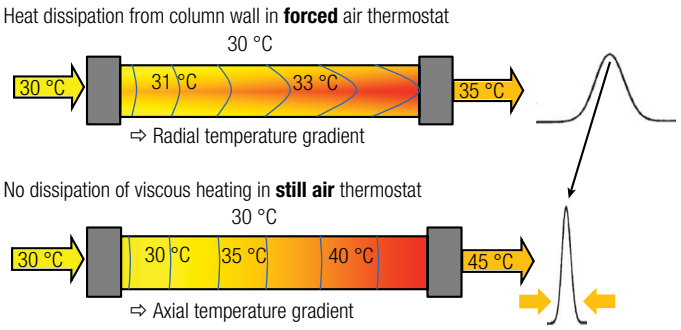


Figure 14. Schematic to show the differences in frictional heat dissipation for forced (top) and still air (bottom). For forced air, a radial temperature gradient occurs while for still air an axial temperature gradient occurs. The given temperatures are not real experimental data but simply serve to illustrate the effects.

In forced air, more frictional heat is removed, which causes a radial temperature gradient. Conversely, in still air thermostatting, the frictional heat is not removed, causing an overall higher separation temperature. The retention is dependent on the separation temperature as retention decreases with increasing temperature; the extent of this behavior is substance specific. In such a case, the effective column temperature also has an influence on the selectivity or distance of peaks.

This effect is illustrated with a separation of preservatives where the selectivity of the critical peak pair (dimethylphathalate/methylparabene) reacts strongly to the changes in column temperature. Moreover, the method produces relevant frictional heat at a pressure above 700 bar (10,000 psi), so a strong influence on the column thermostatting mode (or amount of heat dissipation) can be expected.

Figure 15 shows this effect in the context of transferring the respective isocratic method from an UltiMate 3000 BioRS system (top), which employs a forced air column thermostatting principle and passive eluent pre-heating, to a Vanquish Flex system operated in either forced air (bottom left) or still air thermostatting mode (bottom right) with an active pre-heater. In the forced air mode, the Vanquish Flex system allows method transfer with acceptable resolution of the critical peak pair. Still, the retention factors of peaks 2, 3, and 4 are somewhat reduced and so is the distance of peaks 2 and 3. These differences arise from the fact that the UltiMate 3000 TCC and the Vanquish TCC performance does not result in the exactly equivalent eluent pre-heating and temperature dissipation in their compartments. The still air mode, however, does not allow method transfer with sufficient separation of peaks 2 and 3 despite the overall better peak efficiency. The reason is that the overall higher temperature in the column, resulting from frictional heating, substantially reduces the selectivity between dimethylphathalate and ethylparabene. It would be desirable to take advantage of the still air thermostatting efficiency combined with the better selectivity from the lower column temperature with forced air thermostatting.

To influence the temperature in the column and thus the retention factors, one can take advantage of an independently controllable active pre-heater set at different temperatures. To test this, a series of separations starting from equal temperatures (40 °C) in the column compartment and active pre-heater was performed. The active pre-heater temperature was decreased gradually from 40 °C to 30 °C in 1 °C steps while keeping the column compartment temperature

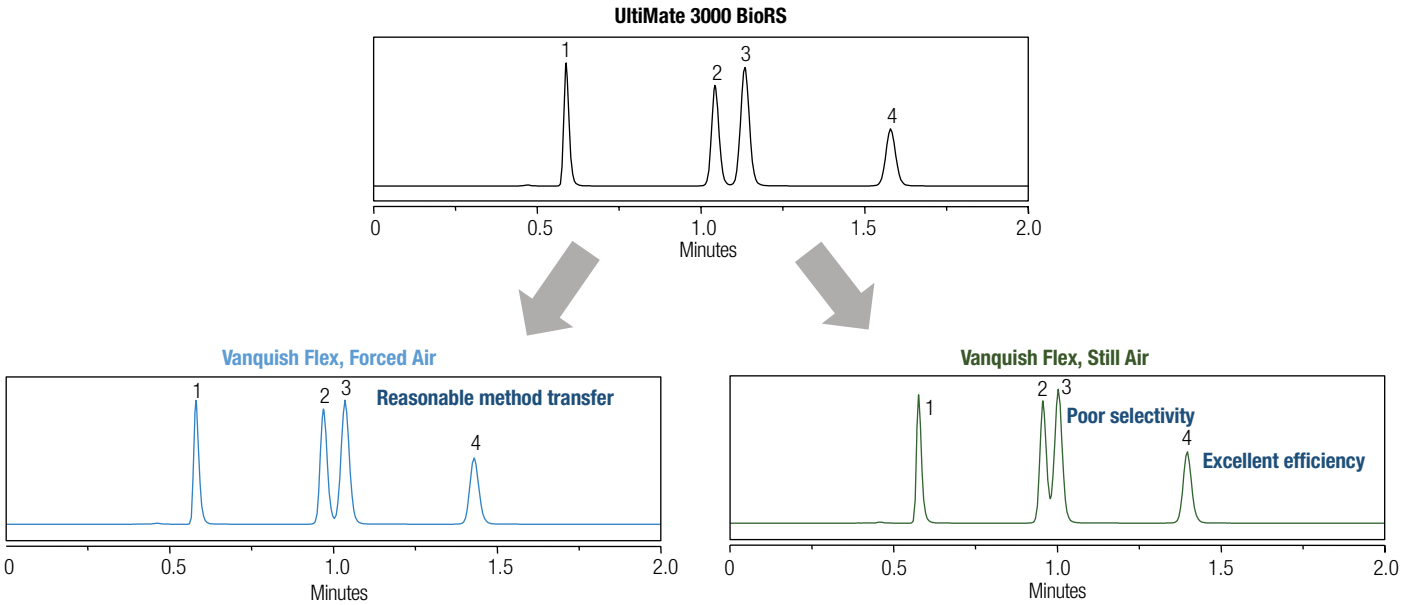


Figure 15. Influence of thermostatting mode on the transfer of a method

constant at 40 °C. To demonstrate the effect, the resulting retention factors were correlated with the temperature of the active pre-heater (Figure 16).

The retention factor of dimethylphthalate on the UltiMate 3000 BioRS system is shown as red dot in the chart at 40 °C with a value of 0.685 (Figure 16A). The retention factors on the Vanquish Flex system are represented as blue dots for the different active pre-heater temperatures. By plotting these two series in a chart, one can determine the intersection of the red and blue data on the y-axis to compare the retention factor on the Vanquish Flex system in still air mode with the retention factor on UltiMate 3000 BioRS system. The intersection can also indicate the corresponding temperature of the active pre-heater, on the x-axis, which in this case determines that an active pre-heater temperature of 30.5 °C leads to matching retention factors between the two systems for dimethylphthalate.

If applying this procedure to methylparaben and methylbenzoate accordingly (see other charts in Figure 16), one can find the active pre-heater temperature corresponding to matching retention factors for methylparabene at 34 °C and methylbenzoate at 32 °C. Since the compounds require three different incoming eluent temperatures to match the retention factor, one could take the average of 32 °C as a compromise to match all three retention factors as close as possible.

As stated above, one can benefit from the positive effects of still air mode under frictional heating at higher system pressures. Key criteria for this separation are the resolution of the critical pair and the overall peak efficiency translating into improved signal-to-noise ratio in the detector. To show the effects, the efficiency improvement of methyl benzoate in still air mode is plotted as a function of the set temperature in the active pre-heater. From Figure 17A, one can clearly see the

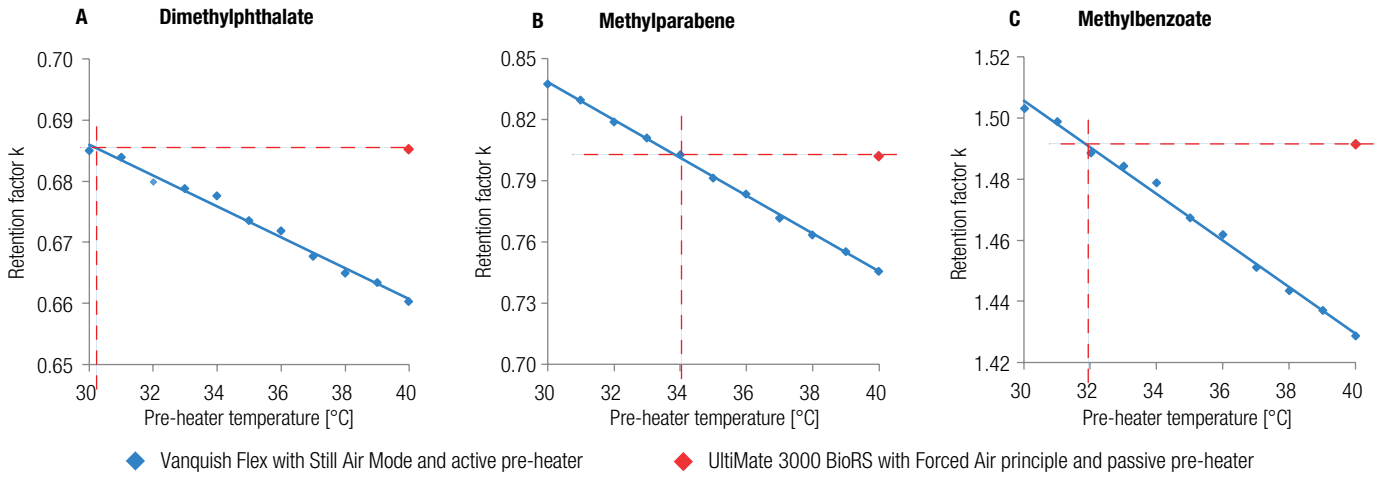


Figure 16. Influence of active pre-heater temperature on compound retention. Red for UltiMate 3000 RS system with passive pre-heater and blue for Vanquish Flex system with active pre-heater

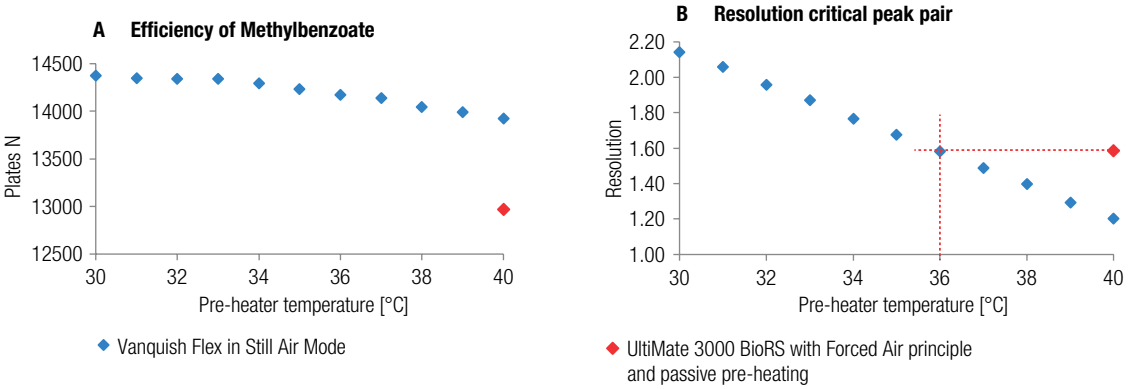


Figure 17. Influence of active pre-heater temperature on chromatographic efficiency and resolution

efficiency increase of 8% at 40 °C associated with still air thermostatting in the Vanquish Flex system. The red dot represents the result on the UltiMate 3000 BioRS system and the blue dots represent the result on the Vanquish Flex system in still air mode with varying pre-heater temperature. When reducing the active pre-heater temperature, it not only impacts the retention factors but also can increase the efficiency, in this case by 10%. The reason is a compensation of a minor radial temperature mismatch inside the column due to residual heat-flow (note that still air is not exactly adiabatic)—but this is only one part of the story. With this application, there is a critical peak pair that had a much worse resolution on the Vanquish Flex system in still air mode than on the UltiMate 3000 BioRS system. Because of influencing the retention factors by decreasing the active pre-heater temperature, the resolution of the critical peak pair changes. To demonstrate this, the resolution is plotted as a function of the active pre-heater temperature, and the intersection between the red dotted line and blue data points of the UltiMate 3000 BioRS system and the Vanquish Flex system, respectively, show the set point for the active pre-heater should be 36 °C. While the resolution is equivalent to the UltiMate 3000 BioRS system under these conditions, the retention factors do not match as shown before. When looking at the previously determined active pre-heater temperature of 32 °C (match of retention), the resolution of the critical peak pair on the Vanquish Flex system clearly exceeds the value observed on the UltiMate 3000 BioRS system.

Figure 18 compares the starting point on the UltiMate 3000 BioRS system at 40 °C and the optimized conditions for the run on the Vanquish Flex system, with the column compartment in still air mode at 40 °C and the active pre-heater set to 32 °C (setting values obtained from the previous evaluations).

By reducing the active pre-heater temperature to 32 °C while keeping the column compartment temperature at 40 °C, one can match the retention factors of the separated compounds of the UltiMate 3000 BioRS system with the Vanquish Flex system. These parameters on the Vanquish Flex system also exceed the resolution from the initial value of 1.58 to 1.93 and increased the efficiency by 11.5%. This example shows the positive effect of this unique property of active pre-heaters. Under frictional heating conditions, active pre-heaters can facilitate the transfer between different thermostatting modes, even without changing the controlled column compartment temperature, which is difficult in a regulated environment.

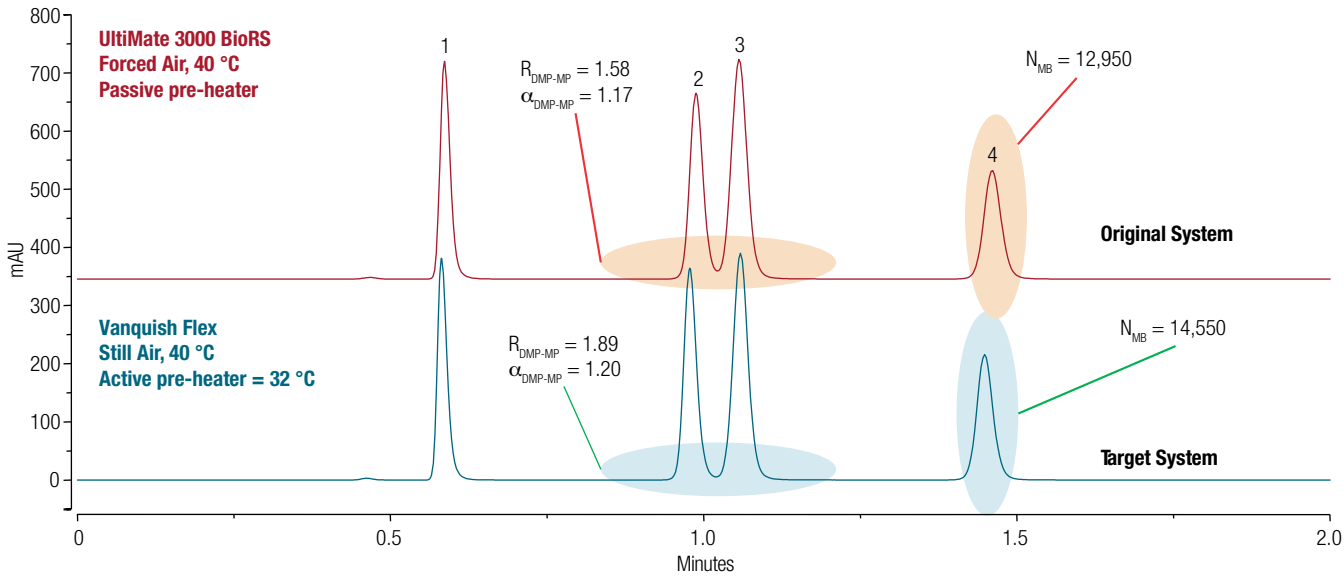


Figure 18. Match of retention times and improved peak shape and resolution with compensation of frictional heat by reduced inlet temperature of the column

Table 4 provides an overview on the column thermostatting modes of commonly used (U)HPLC systems.

Table 4. Thermostatting modes employed by various HPLC systems on the market

(U)HPLC System	Applied Thermostatting Mode
Thermo Scientific UltiMate 3000 Series	Forced Air
Thermo Scientific Vanquish Series	Still and Forced Air
Agilent 1100 and 1200 Series	Still Air
Waters Alliance Series	Forced Air
Waters® Acquity® Series	Still Air
Shimadzu series-i	Forced Air
Shimadzu LC-2010	Still Air

Effect of extra-column volume

The extra-column volume (ECV) is the volume from the injector to the detector excluding the volume in the column. The ECV can be further categorized into pre-column and post-column volume. The pre-column volume is determined mainly by instrument parts such as needle seat and connecting tubing, while the post-column volume also derives from the connecting tubing to the detector and capillaries within the detector, but mainly from the volume of the detector flow cell.

The impact of the ECV on the success rate of the method transfer strongly depends on the method itself. In general, the influence of the ECV becomes more prominent if the column volume decreases. This effect was reported for two column formats under isocratic elution conditions—adding an additional 15 µL ECV to a system with 4.6 × 150 mm column resulted in a small 1% loss in resolution for a low retaining compound (k=1) and no loss of resolution for a more retained compound (k=5). In contrast, for the more challenging column format of 2.1 × 150 mm, the loss in resolution was 19% and 3%, respectively, for the two compounds.[7] Thus, an instrument variation in ECV is of limited relevance when working with standard HPLC columns. If columns of 2.1 mm i.d. are used (UHPLC conditions) the effect of the ECV cannot be neglected.

Figure 19 shows the potential impact of additional ECV, generated by different tubing designs, on a chromatographic separation. Figure 19B gives a chromatographic example where, due to extended ECV, an impurity was not resolved from the main peak while with using Thermo Scientific™ Viper™ Fingertight capillaries and their minimized ECV, the impurity was distinguishable from the main compound. Such effects will be more pronounced for low diameter columns than for standard HPLC columns (4.6 mm i.d.). Thus, care should be taken on the fluidic connections when working with columns 2.1 mm i.d. or smaller.

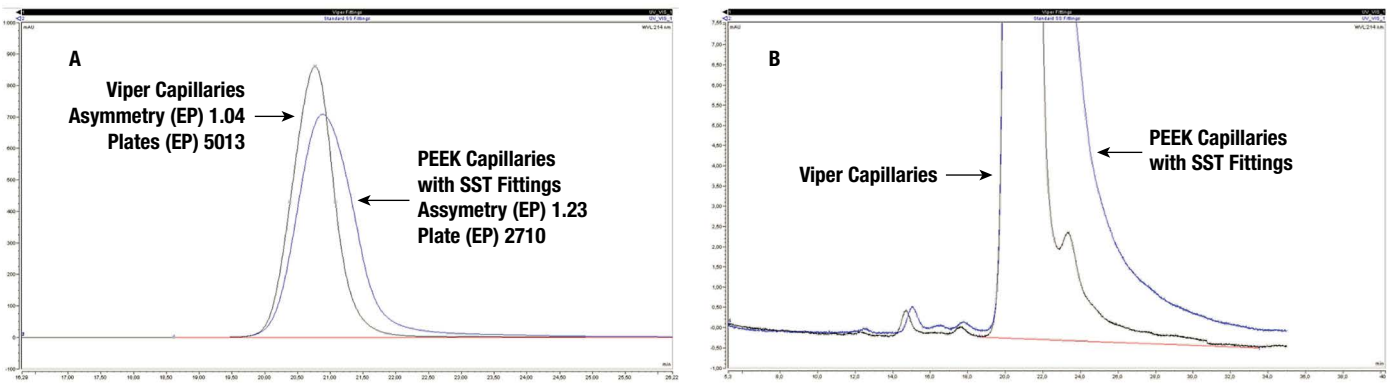


Figure 19. Comparison of Viper capillaries with ferrule-based fitting systems. (A) Asymmetry and plate counts of a single peak and (B) resolution of API and nearly eluting impurity

A significantly lower ECV in the receiving unit than in the originating unit has detrimental effects on the separation of early eluting substances when strong sample solvents are used.

To illustrate this behavior an isocratic separation was used under solvent mismatching conditions (sample in 100% methanol with 50:50 water/acetonitrile elution conditions). Figure 20A shows the plate counts for three different systems against the injection volume. The Vanquish Flex system clearly shows the highest chromatographic efficiency for the lowest injection volumes of 0.5 µL and 1 µL, whereas at 3 µL or higher no difference was observed. In addition, the sample mixing behavior was investigated by calculating a sample mixing factor (dividing the plate count at 3 µL injection volume by the plate count at 0.5 µL injection volume). In Figure 20B the mixing factor is plotted for the three instruments against the plate number at 0.5 µL injection volume and a correlation becomes obvious. Due to the lower general chromatographic efficiency, the Agilent 1260 system exhibits better pre-column sample mixing compared to the other systems. In this case it may make sense to artificially increase the pre-column volume, decrease the injection volume, or try to match the sample solvent with the eluent in order to transfer a method from a system with higher pre-column volume to a system with lower pre-column volume.

In Figure 21, the approach of reducing the injection volume to obtain a satisfactory peak shape is shown. The injection volume can be adjusted according to USP <621> if it fulfills the required precision and detection limits.[4]

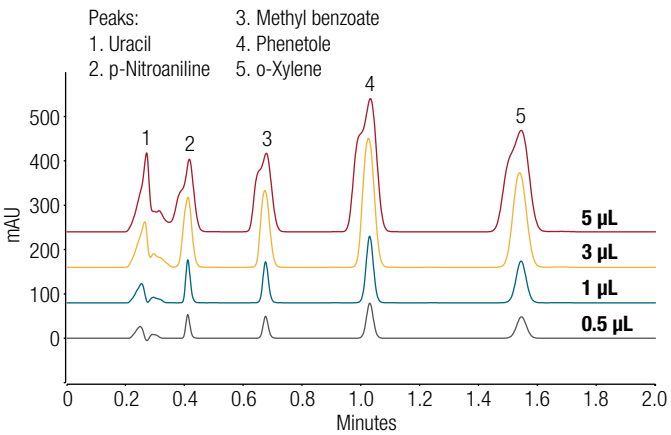


Figure 21. Effect of reducing the injection volume when the sample solvent (100% methanol) is stronger than the eluent (50:50 water/acetonitrile)

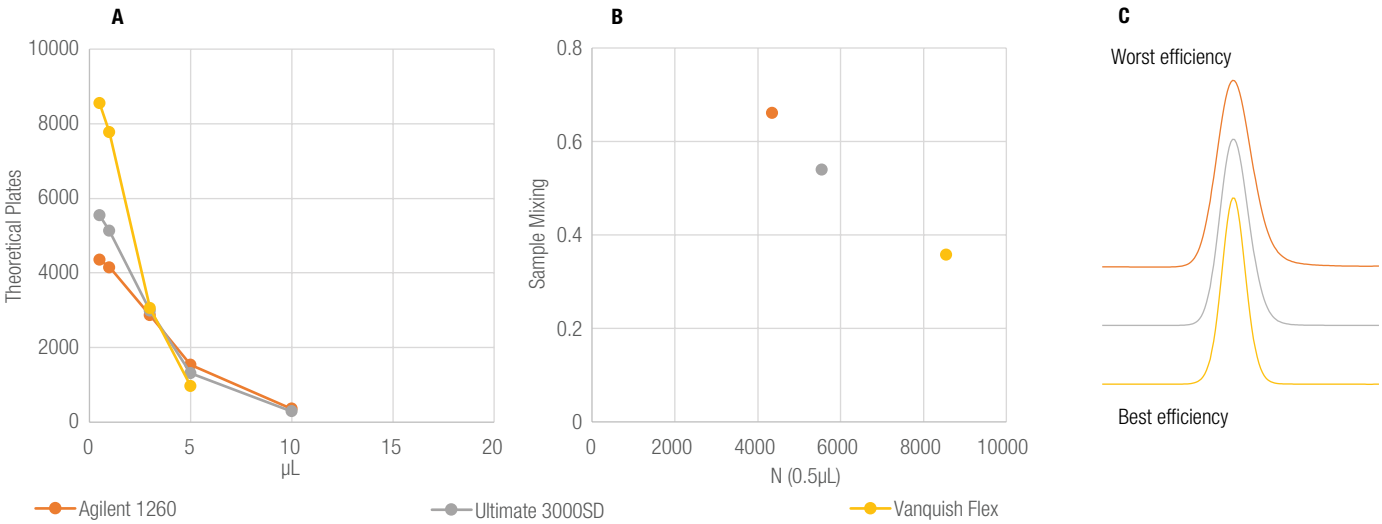


Figure 20. (A) Dependency of increasing injection volumes on system efficiency, (B) relationship between instrument sample mixing behavior and system efficiency with (C) respective peak shapes

For gradient separations the influence of the ECV is lower due to the peak re-focusing effect at the column head. Also, the post-column volume is more relevant than the pre-column volume, due to the on-column peak focusing in the gradient mode. Still, bad fluidic connections as well as inappropriate flow cell dimensions can result in different peak resolution between originating and receiving system when transferring a method (Figure 19).

Detector – flow cells and detector setting

The detector flow cell is critical to consider when transferring methods between different (U)HPLC systems. Care needs to be taken that the flow cell volume is in accordance with the peak volume and with the column diameter. As a rule of thumb, the flow cell volume should not be larger than 10% of the peak volume of the smallest peak. If the ratio between the peak volume and flow cell volume decreases, peak dispersion including a loss of efficiency and signal-to-noise will be the consequence.

The separations shown in Figure 22 were performed on a 1.0 × 100 mm, 2.1 × 100 mm, and 3.0 × 100 mm column, respectively.[8] For all separations a low dispersive UV monitor followed by a high sensitivity flow cell, with 13 µL illuminated flow cell volume and a light path of 60 mm, was used. In addition, the peak broadening factor was calculated by dividing the peak volume measured on the 13 µL flow cell by the peak volume measured with the UV monitor. From this data it becomes obvious that only marginal loss of resolution between the 45 nL and 13 µL flow cell is observed for the 3.0 × 100 mm column with peak volumes between 27 and 129 µL. For the last eluting peak in the 3.0 × 100 mm column, nearly no peak broadening is observed. Here the ratio of peak volume to flow cell volume is exactly 10. For the other column formats the high sensitivity 60 mm flow cell is not suitable. However, during a typical method transfer scenario it might be unrealistic that the column format is changed. Still, the same principle (flow cell volume 10% of peak volume) applies to method transfer scenarios where the column format is kept constant, but the flow cell volume is varied as different instruments are used.

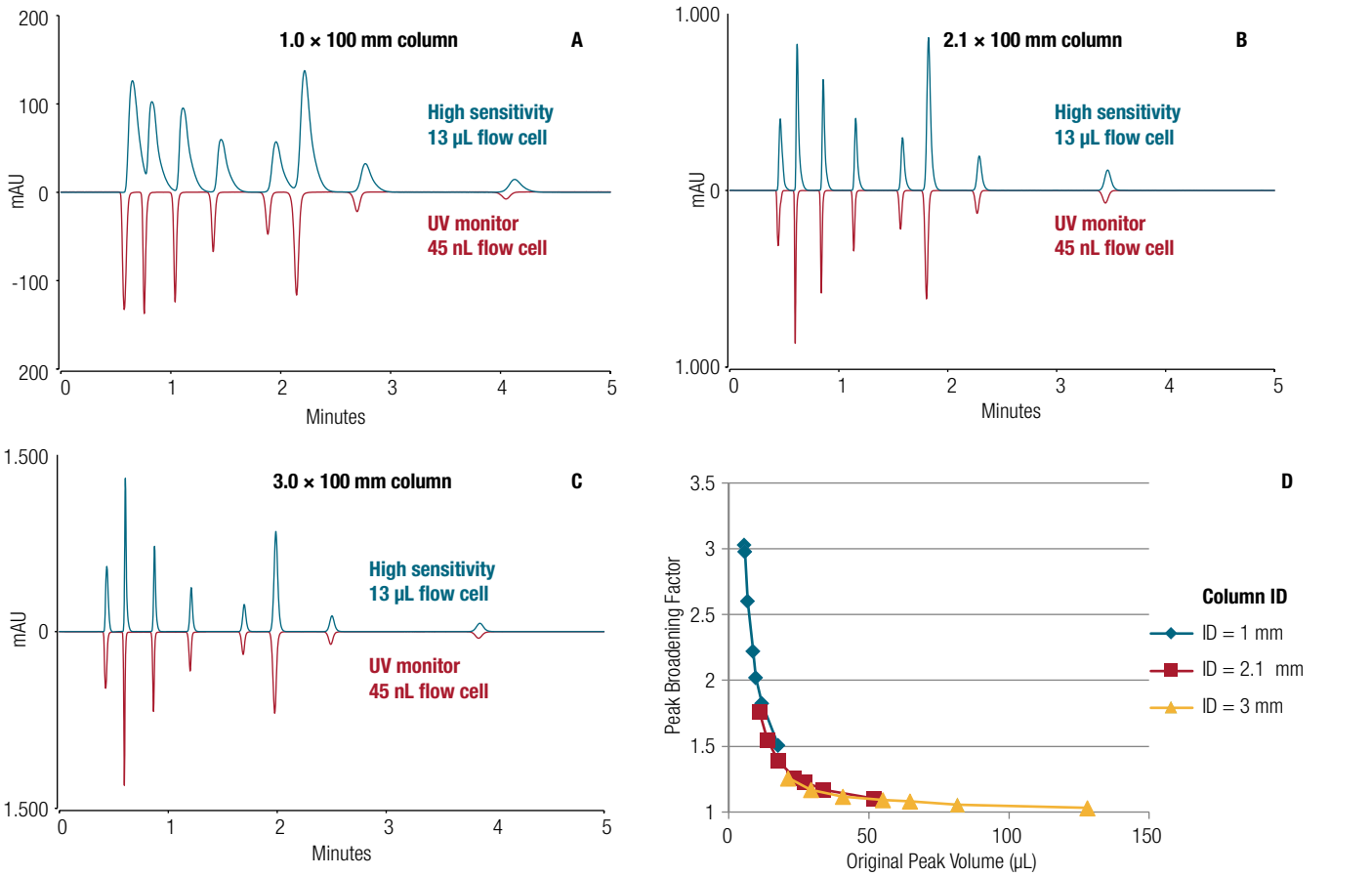


Figure 22. Isocratic separation of a standard mixture on a 1 mm column (A), 2.1 mm column (B), and 3.0 mm (C) using a high sensitivity 60 mm flow cell (blue trace) and a low dispersive flow cell (red trace). In addition, a peak broadening factor is given for all columns in dependence of the peak volume (D).

Besides the physical dimensions of the detector, or specifically the detector flow cell, the detector settings play a major role in obtaining similar results between different types of detectors or between different vendors. For successful method transfer, the setting for bandwidth, reference wavelength, and response time are of importance. The response time (also rise time or time constant) is in general a measure of how quickly the detector responds to a change in signal. An increasing response time reduces the signal noise but may simultaneously decrease the signal height and consequently influence the sensitivity. Furthermore, an increasing response time increases peak width and shifts the peak towards higher retention times.

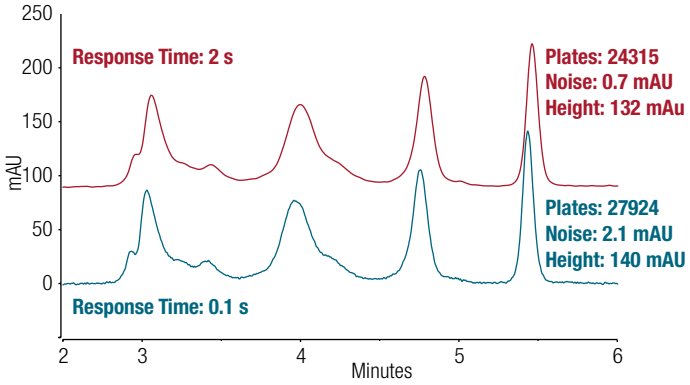


Figure 23. Comparison of isocratic size exclusion chromatography separations measured at different response times while all other parameters were kept constant

Figure 23 shows the effect on a practical example of a size exclusion chromatography (SEC) of a commercial standard. In this case, a decrease of the theoretical plates by nearly 13% was observed. This is especially critical for SEC as baseline separation between aggregates of biotherapeutics is often not easily achieved. In addition, the noise is dramatically decreased for the higher response time and improves overall signal-to-noise, so the user should find a compromise for best results. This compromise is normally provided by the CDS software, such as Chromeleon CDS software, which calculates optimal response times (and data collection rate) based on the obtained peak width.

A parameter influencing the relative quantitative results is the bandwidth of, for instance, a diode array detector. The bandwidth is the wavelength range that is used to record the chromatogram where the signal represents an averaged absorbance value for this wavelength range.

The effect of the bandwidth setting was investigated for an USP-based method analyzing acetaminophen with six different bandwidth settings. A first comparison of the spectra of acetaminophen and impurity B show very similar spectra for both compounds. Thus, the peak area ratio, which is often used for relative quantification purposes, is not affected (Figure 24, blue line). In contrast, the spectra of impurity C and 4-aminophenol have different spectra than the API, which is used for the calculation of the relative peak area. As a consequence, the relative quantification is affected by the bandwidth setting. For different analytes, this effect can even have different directions. While for aminophenol the relative response is decreasing with a broader bandwidth, the relative area of impurity C is increasing (Figure 24, green and purple line).

Thus, we recommend accurately considering corresponding detector settings during a method transfer. When the transfer is done on identical instruments this can be easily done. However, when instruments of different vendors are involved in the transfer, the standard instrument settings should be carefully evaluated.

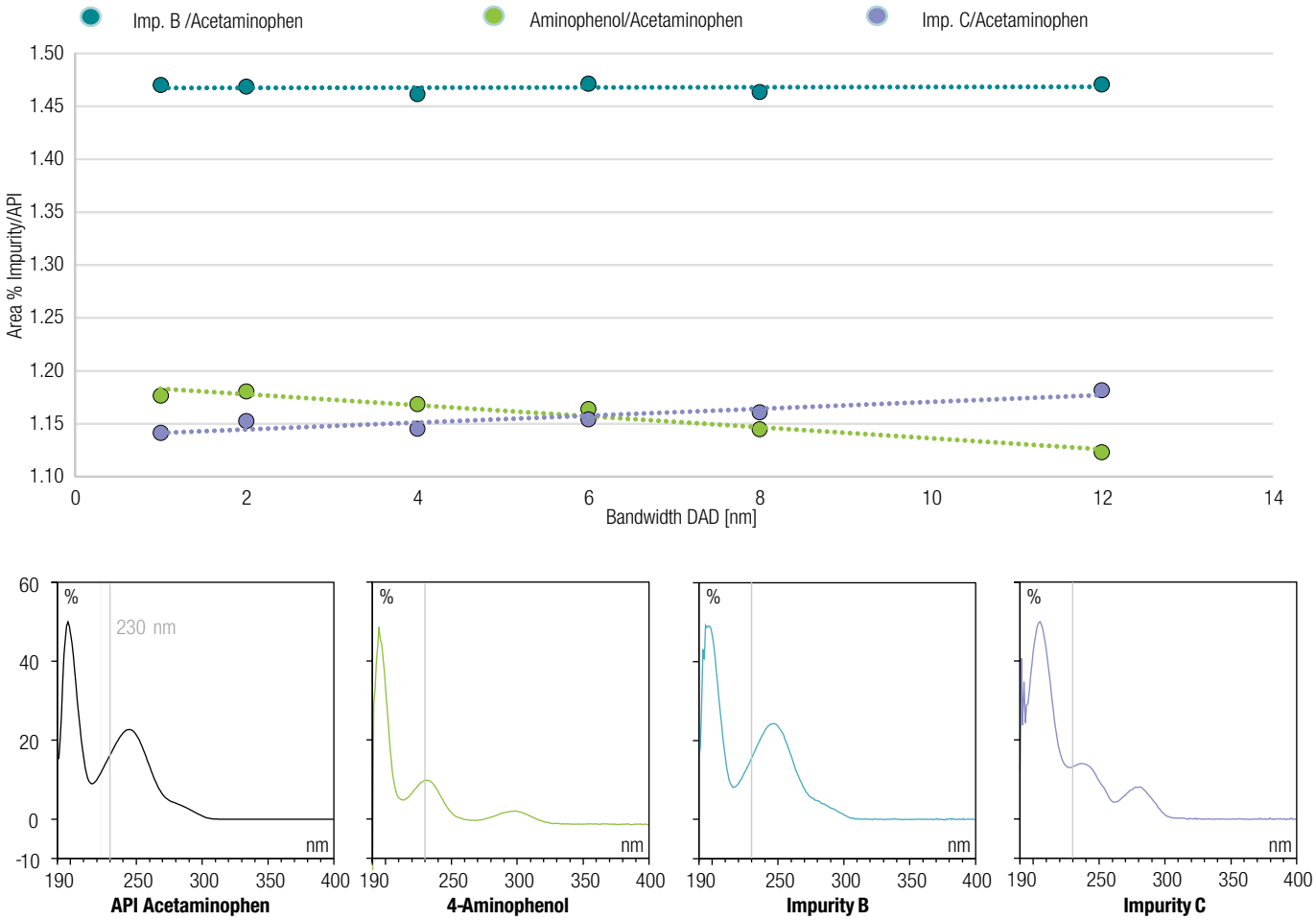


Figure 24. Relative peak areas of three impurities during the USP-based analysis of acetaminophen. Peak areas were recorded for six different bandwidth settings at 230 nm (indicated by gray vertical line) with the respective UV spectra of all involved compounds shown at the bottom.

Conclusions

Transferring HPLC methods depends on several different factors that often make this task very difficult for chromatographers. For instance, non-matching retention times can be caused by:

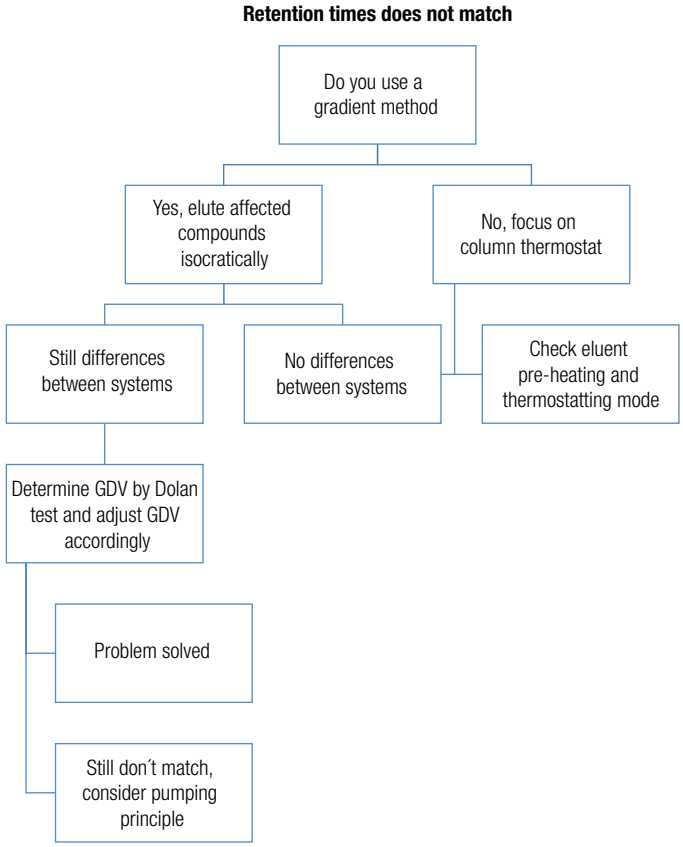
- Different pumping principles (LPG vs. HPG pumps)
- Different GDVs
- Different column thermostatting principles
- Different pre-heater usage

A loss of resolution also can be caused by multiple reasons such as:

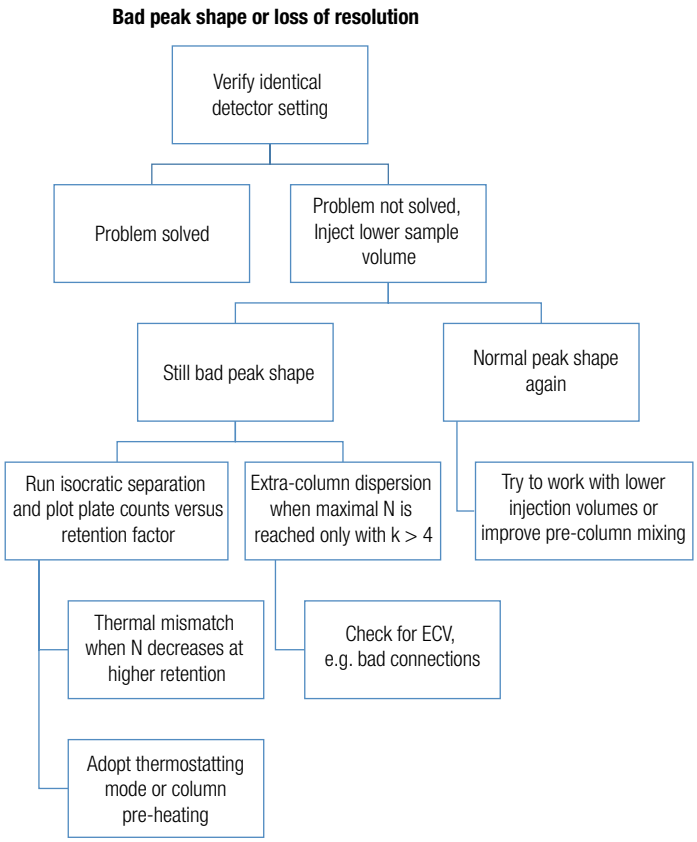
- Thermal mismatch due to pre-heating or column thermostatting
- Additional extra-column dispersion effects
- Sample solvent mismatch
- Detector settings

These two criteria illustrate how complex method transfer can be even when only the instrumental parameters are considered—aspects related to the column used, eluents, or other consumables are not even taken into account. The following flow schemes aim to provide guidance on how to transfer methods after certain observations. The guidance is primarily for the root cause analysis of deviation and not always the final fix of non-matching results, which was in depth discussed in all the sections above.

Retention time problem



Peak shape problem



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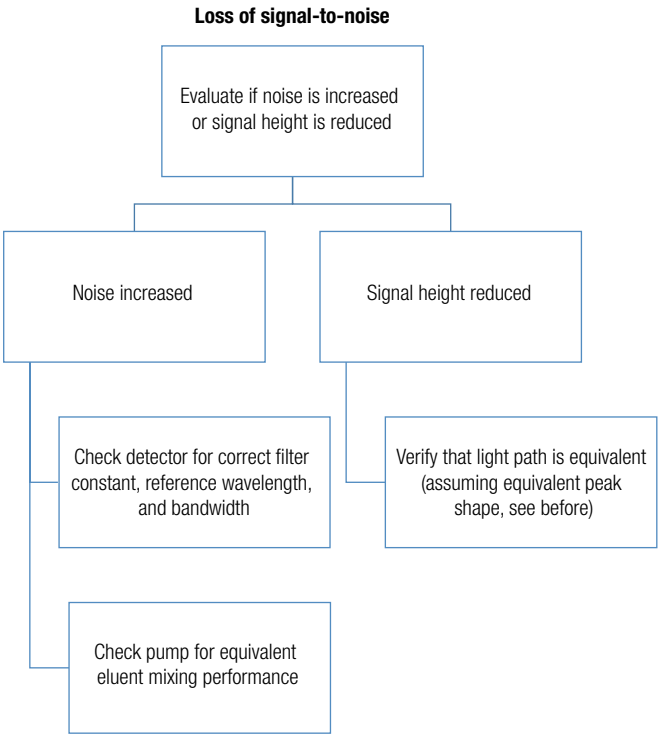
Signal-to-noise problem



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