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A successful liquid chromatography (LC) method transfer between different instruments is an integral step in highly regulated analytical laboratories. As LC methods are moved from the ‘transferring’ to the ‘receiving’ system, scientists are required to navigate multiple variables resulting from instrument settings or vendor configuration that directly impede method transferability.

In this compendium, we offer an all-in-one resource to help you tackle the common instrument challenges faced during method transfers to obtain consistent results and sustain regulatory compliance. As method transfers are inspected and audited, all processes need to comply with the standardized guidelines set by regulatory bodies such as the World Health Organization (WHO), the United States Pharmacopeia (USP) and, more recently, the EU GMP Guideline.

Technical challenges in method transfer

Instrument-to-instrument transfers, either between two departments such as R&D and quality control, or within the same laboratory between legacy instruments and their modern counterparts, often cause variability due to unique equipment characteristics. Our instrument parameters guide breaks down the root cause for instrument-related method transfer problems and shows you how to characterize them.

Of these, the factors that most commonly impact method transfers are **gradient delay volumes, column thermostatting, and sample dispersion effects**. Many modern LC systems, unfortunately, haven’t been designed to offer the flexibility to adjust these parameters, making it challenging to accurately transfer methods and risking non-compliance.

Optimized systems for seamless method transfer

The Thermo Scientific™ Vanquish™ Core HPLC system has been carefully configured to facilitate seamless method transfers and precise replication of methods, even across vendors. For instance, to address gradient delay volumes, one of the most critical parameters during method transfers, the Vanquish Core HPLC system has a built-in autosampler that can freely fine-tune the gradient delay volume to help make necessary adjustments without altering the gradient table.

Mismatched temperature control between two instruments can also directly influence the selectivity of analyte separation, resulting in poor peak shapes and altered selectivity. These thermal inconsistencies during a method transfer are easily addressed on the Vanquish Core HPLC system. Simply choose the most appropriate column thermostatting mode from the options in the settings to correctly mimic the column temperatures from the transferring instrument.

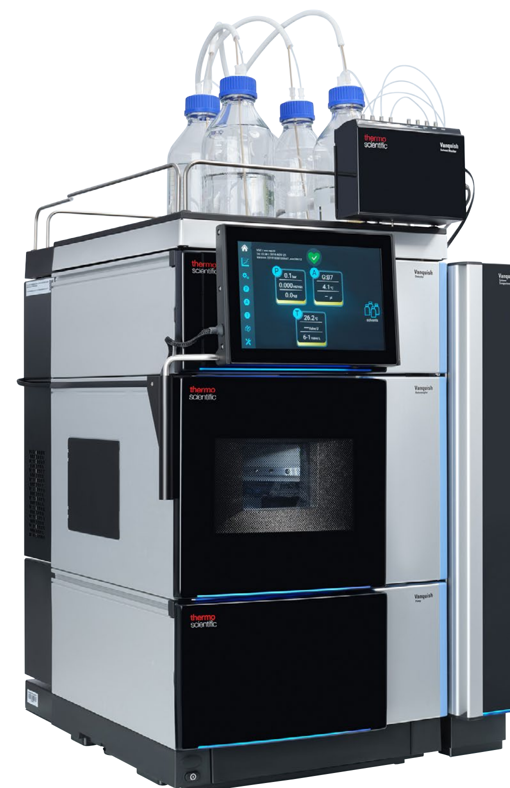
Often, insufficient sample mixing or dispersion effects caused by strong solvents can result in distorted peaks. Modifying parameters to address this issue, however, can alter the chromatographic conditions, which regulations do not permit. Having a flexible custom injection program, as included in the Vanquish Core HPLC system, can help you selectively adjust desired parameters without changing the experimental settings, thereby yielding improved peak shapes.

As individual parameters are modified to fully replicate the original protocol in a method transfer, it is important to ensure these changes are authorized within the USP 621 regulations to avoid any violation of rules. The Vanquish Core HPLC system provides flexibility to customize settings, while safeguarding regulatory guidelines.

Solidifying your method transfer processes

In addition to choosing appropriate LC systems, laboratories can maintain compliance across all workflows by using advanced software that integrates instruments across different locations. The Vanquish Core HPLC system, for example, can be operated by Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, which centrally controls all instruments within a workflow in a location-independent and vendor-neutral manner. By tracking user information and keeping audit trails, the CDS enables laboratories to maintain data integrity.

To address the different variables that influence a successful method transfer, we’ve curated a range of topics from specific case studies in intra-laboratory transfers to technical adjustments for vendor-related incompatibilities. The techniques provided here will enable you to confidently approach your next method transfer, perform necessary adjustments, and obtain reproducible results with continued regulatory compliance.



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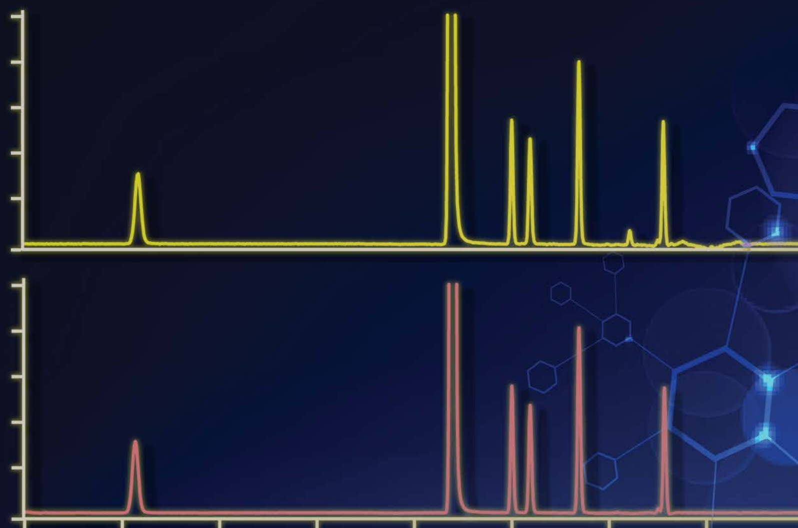

An instrument parameter guide for successful (U)HPLC method transfer

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Keywords
UHPLC, HPLC, USP <621>, gradient delay volume, column thermostatting, extra-column volume, detector settings

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.

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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.

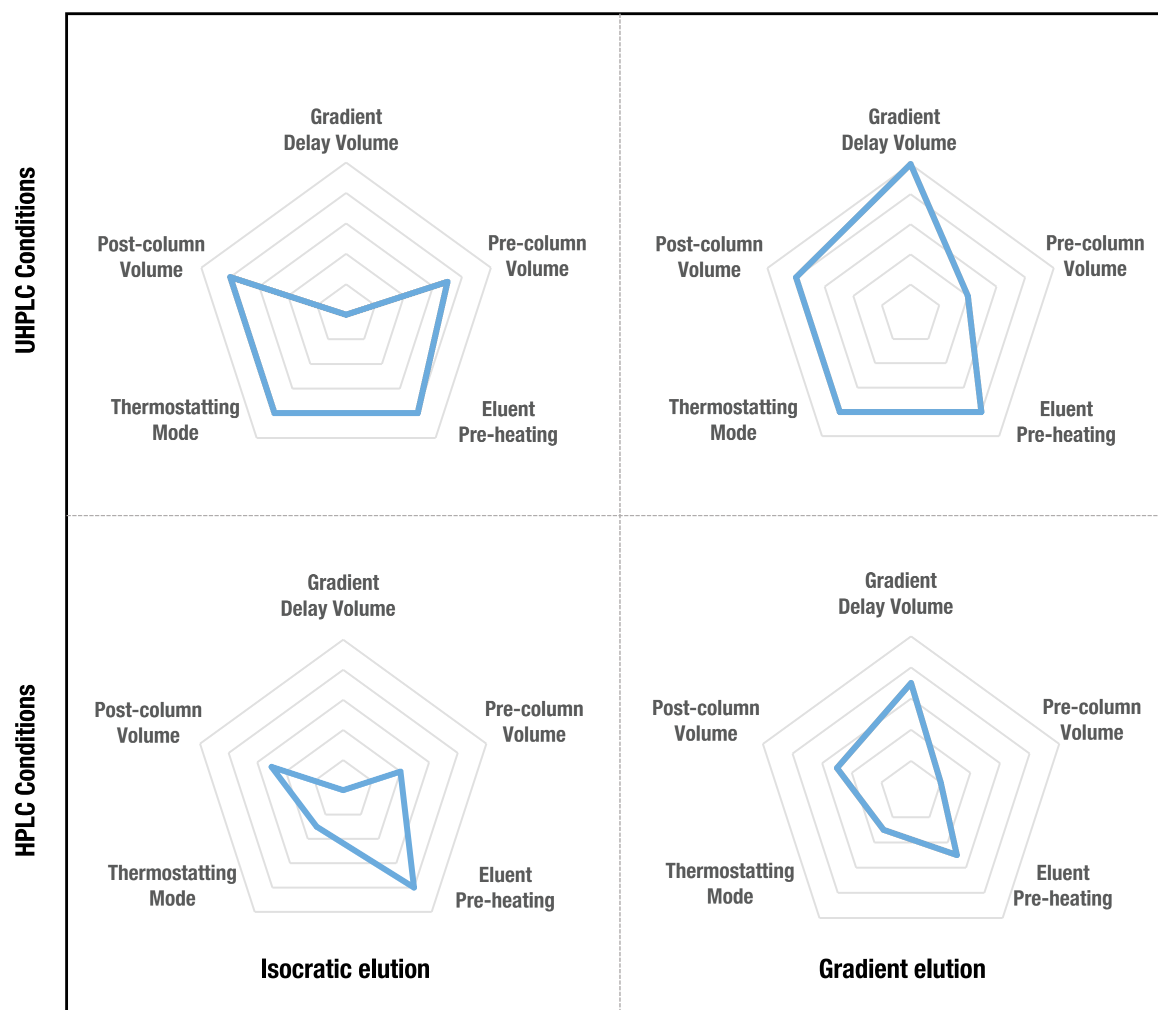

Figure 1. Instrument parameters and their importance for a successful method transfer. The further from the center of the graph, the more important the parameter. The importance value are estimates and dependent on additional method details such as separation temperature, flow rate, etc.

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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:

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

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.

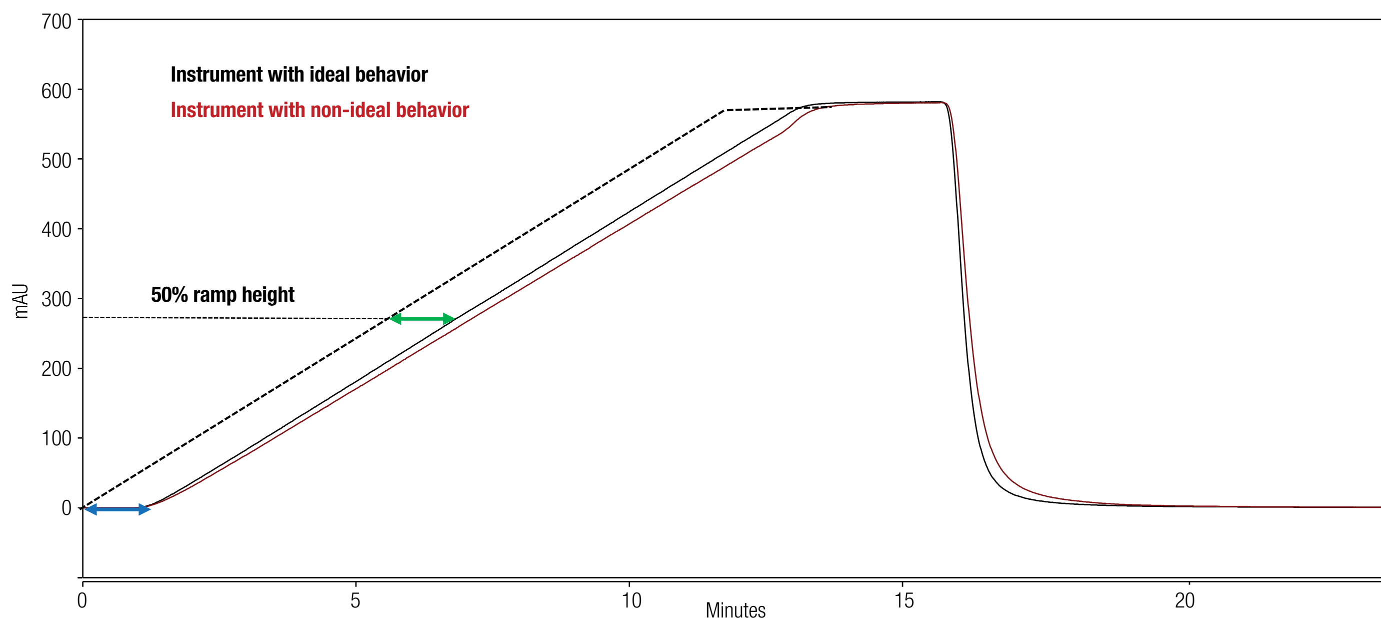

Figure 2. Method for determination of an instrument gradient delay volume. Two different instrument behaviors are shown as well as two commonly used data evaluation procedures (blue and green arrows).

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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)

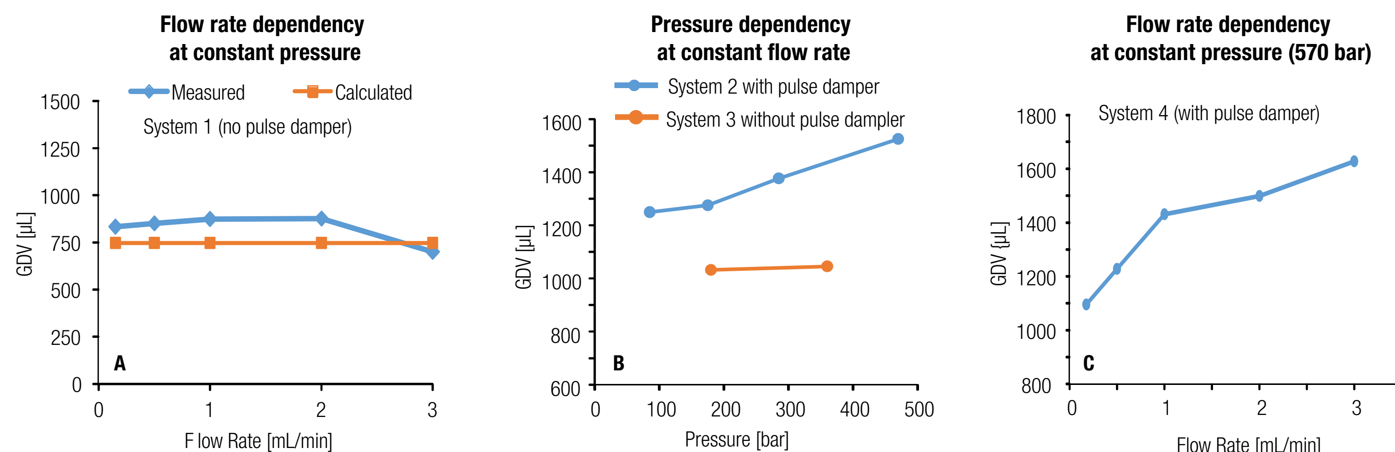

Figure 3. Dependency of gradient delay volume on flow rate and pressure for different types of instrumentation

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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

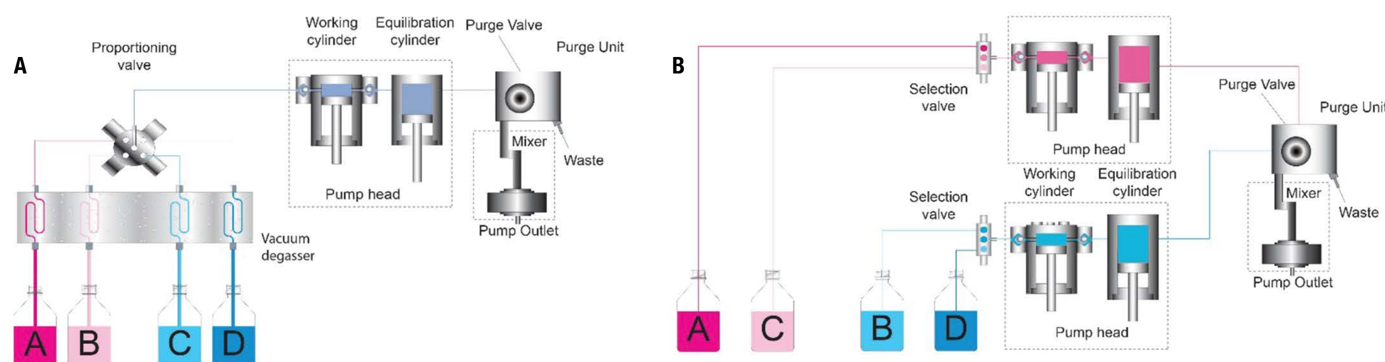

Figure 4. Schematic setup of a low-pressure gradient pump (A) and a high-pressure gradient pump (B). Note how the different solvent convergence points have effects on the gradient delay volume, which is defined as the volume between the convergent point of the solvents and the column head.

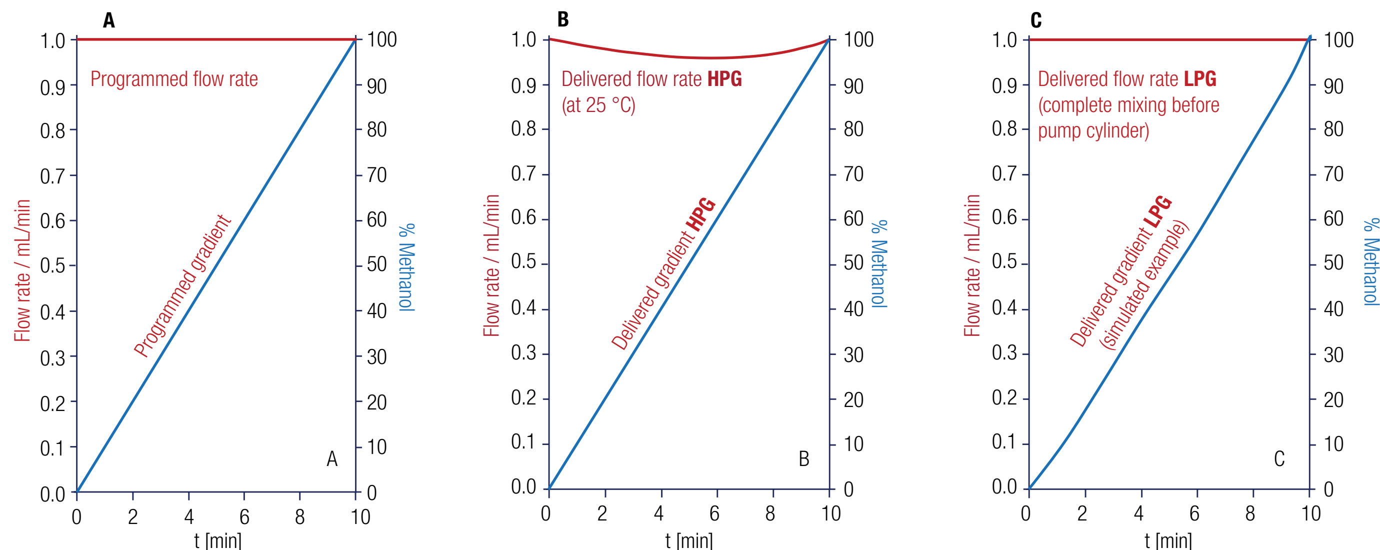

Figure 5. Flow rate and gradient accuracy of an HPG and LPG pump. Comparing A) programmed flow rate and gradient B) delivered flow rate and gradient of an HPG pump, and C) delivered flow rate and gradient of an LPG pump.

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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.

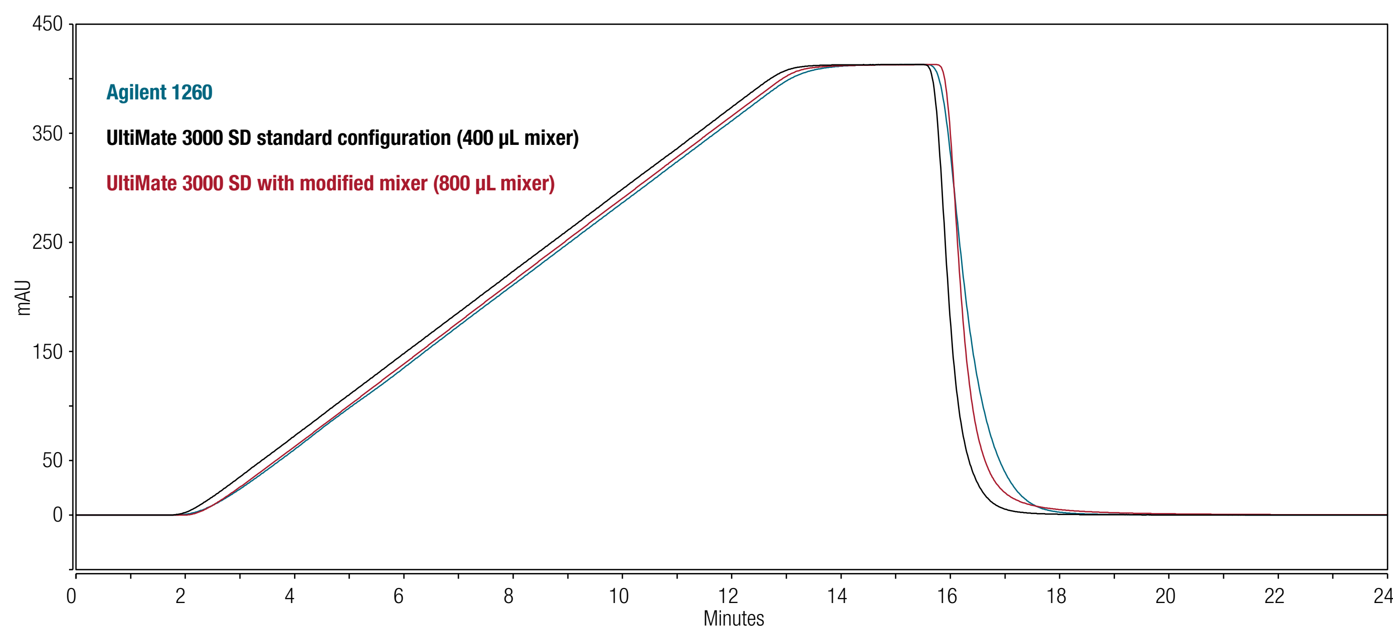

Figure 6. Overlaid gradient profiles of an Agilent 1260 LPG system, an UltiMate 3000 SD LPG system, and an UltiMate 3000 SD LPG system with increased mixer volume to compensate for GDV differences

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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.

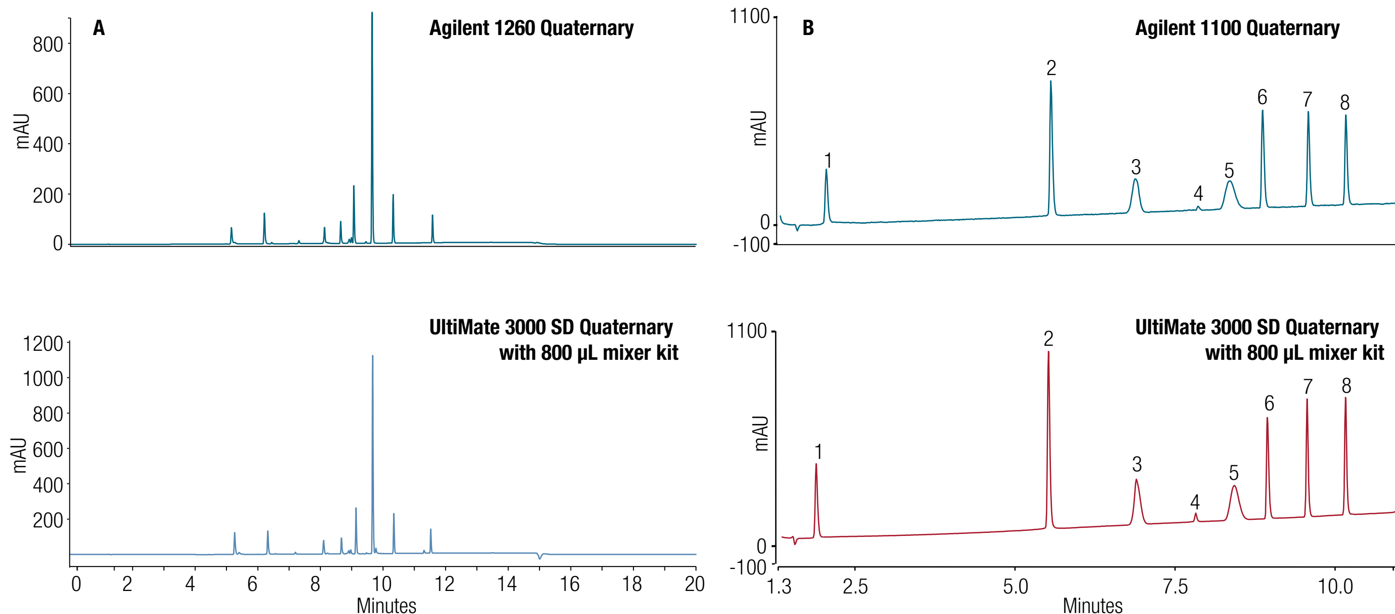

Figure 7. Transfer from an Agilent 1260 instrument to an UltiMate 3000 SD instrument (A) and transfer from an Agilent 1100 instrument to an UltiMate 3000 SD instrument (B). To match the gradient delay volume characteristics, the default mixer of an UltiMate 3000 SD system was exchanged to the 800 µL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

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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.

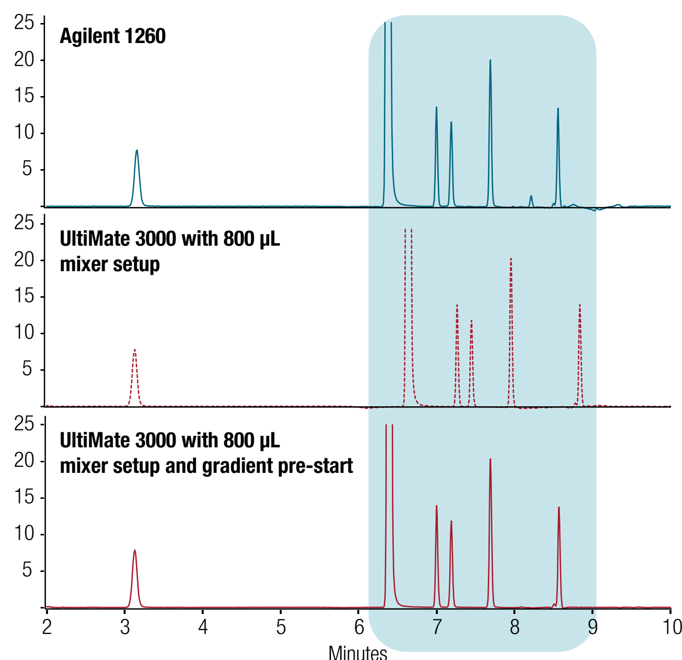

Figure 8. Transfer of a separation of acetaminophen and its impurities from an Agilent 1260 low pressure gradient to an UltiMate 3000 SD low pressure gradient system. For the UltiMate 3000 SD system the 800 µL mixer setup was used. To compensate for the higher gradient delay volume of the UltiMate 3000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

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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.

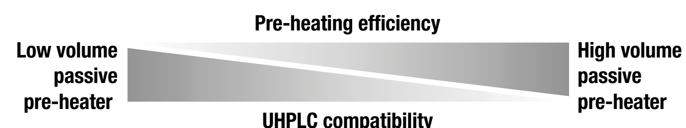

Figure 9. Passive pre-heater efficiency and UHPLC compatibility of different sized pre-heaters

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

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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]

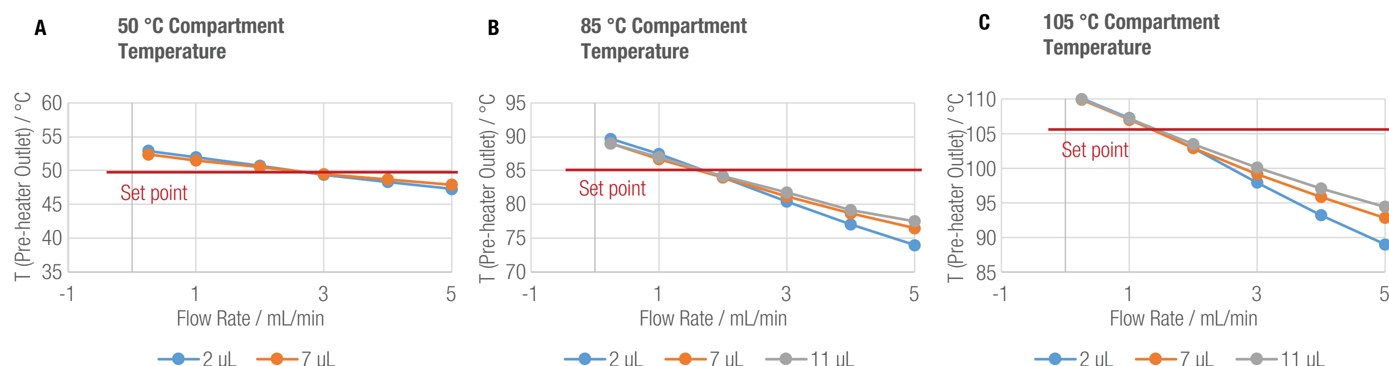

Figure 10. Passive eluent pre-heating effects on flow rate and pre-heater volume grouped by set compartment temperature

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

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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 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.

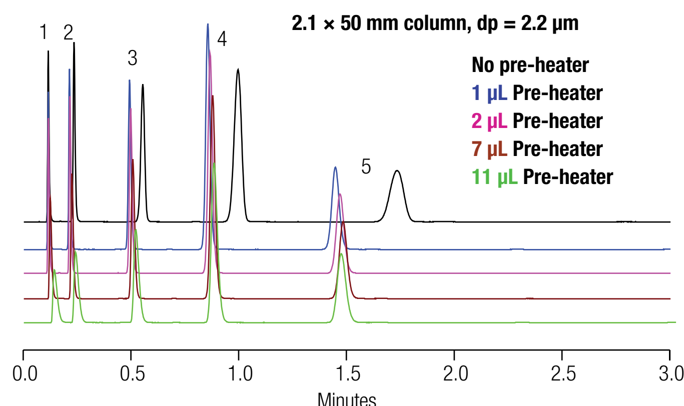

Figure 11. Standard isocratic column test on a column that produces small peak volumes show the effect of the pre-heater on peak shape and retention. Stationary phase: Thermo Scientific™ Acclaim™ RSLC 120 C18, eluent: water/acetonitrile 60/40 v/v, flow rate: 1.0 mL/min, column temperature: 70 °C. Peak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

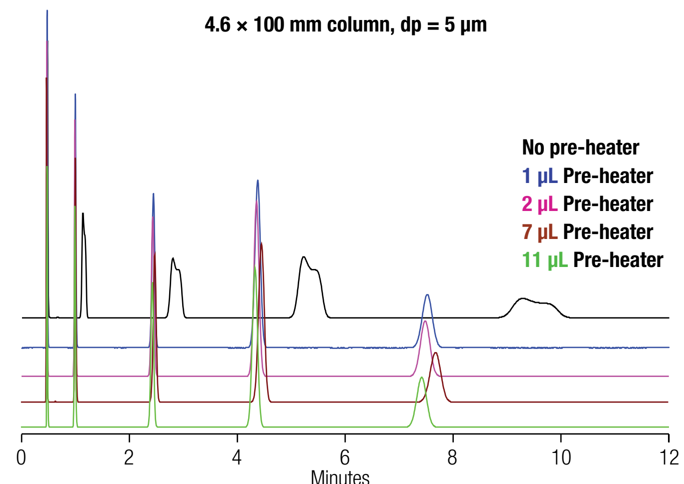

Figure 12. Standard isocratic column test on a wide bore column that produces relatively large peak volumes and is operated at elevated flow rate, show the effect of the pre-heater on peak shape and retention. Peak assignment, stationary phase, eluent, and column temperature as in Figure 11, flow rate: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

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.

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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.

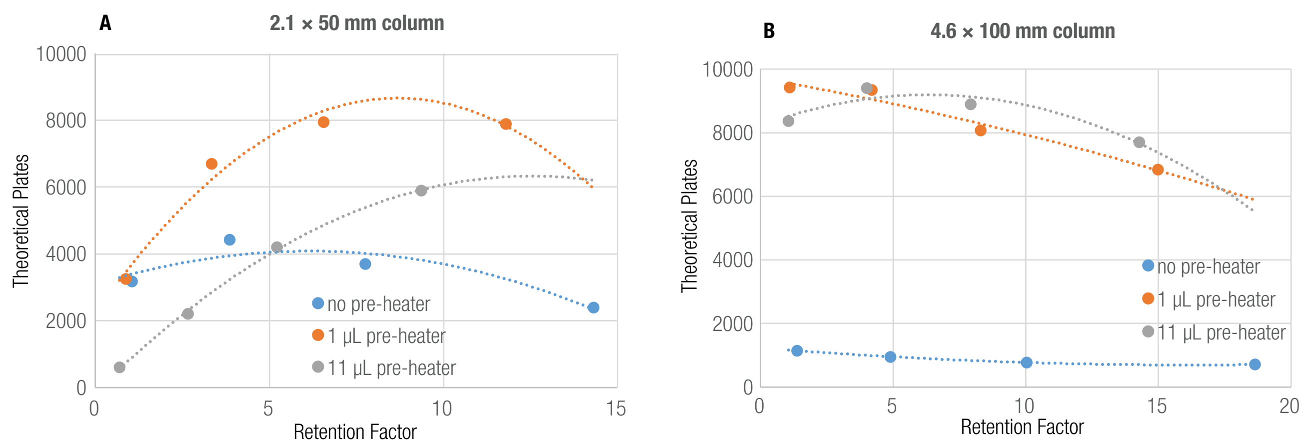

Figure 13. Plot of plate number against retention factor for both column type and experiment with no pre-heater, 1 µL pre-heater, and 11 µL pre-heater

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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).

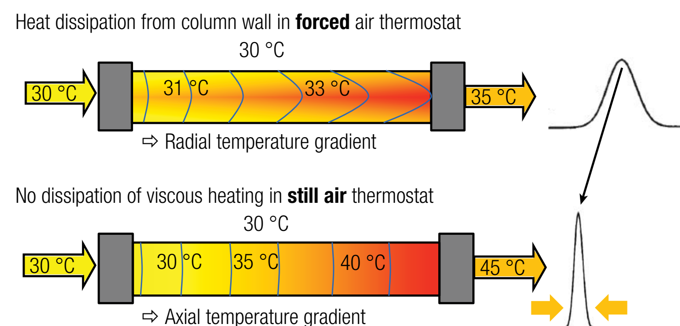

Figure 14. Schematic to show the differences in frictional heat dissipation for forced (top) and still air (bottom). For forced air, a radial temperature gradient occurs while for still air an axial temperature gradient occurs. The given temperatures are not real experimental data but simply serve to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

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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

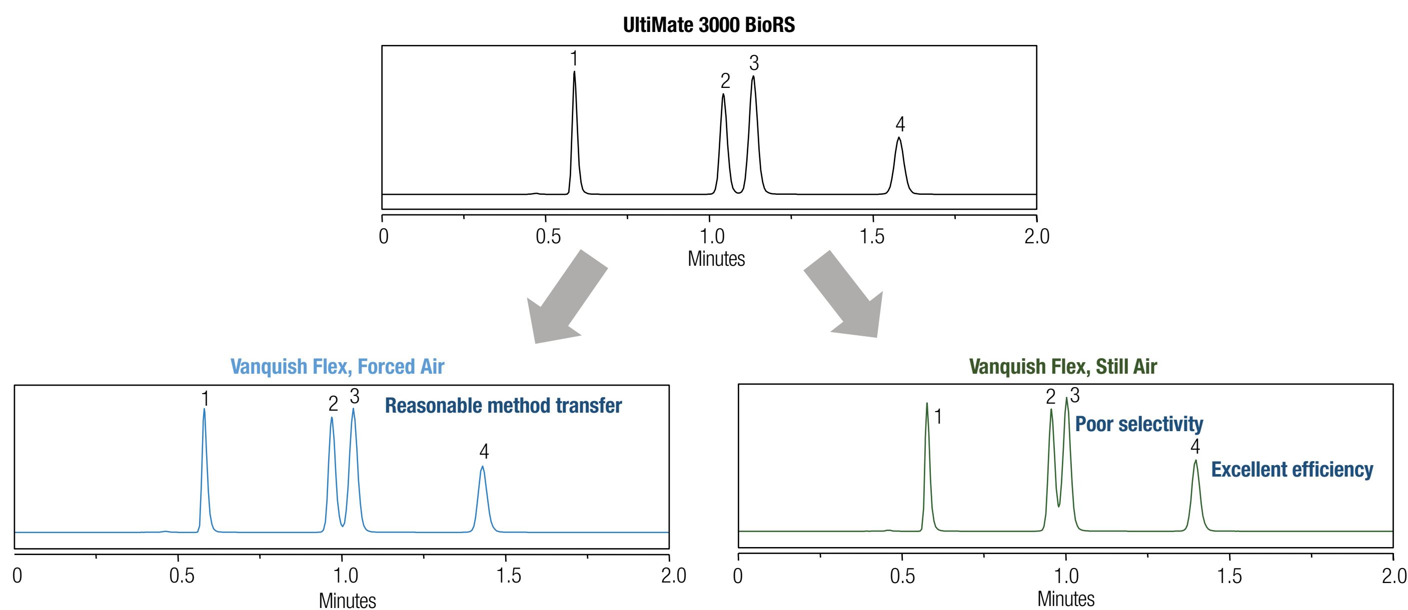

Figure 15. Influence of thermostatting mode on the transfer of a method

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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

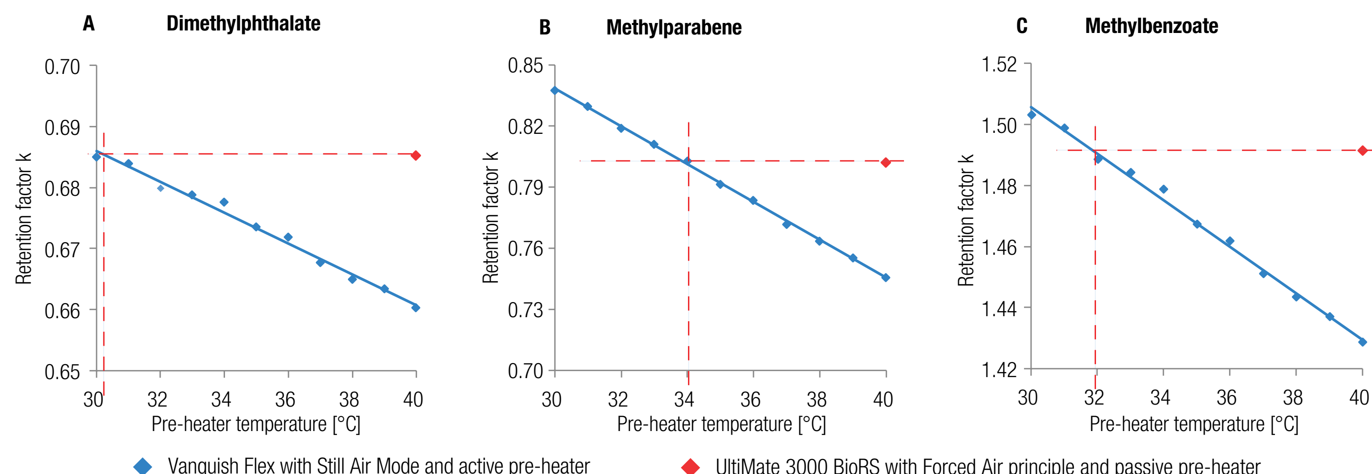

Figure 16. Influence of active pre-heater temperature on compound retention. Red for UltiMate 3000 RS system with passive pre-heater and blue for Vanquish Flex system with active pre-heater

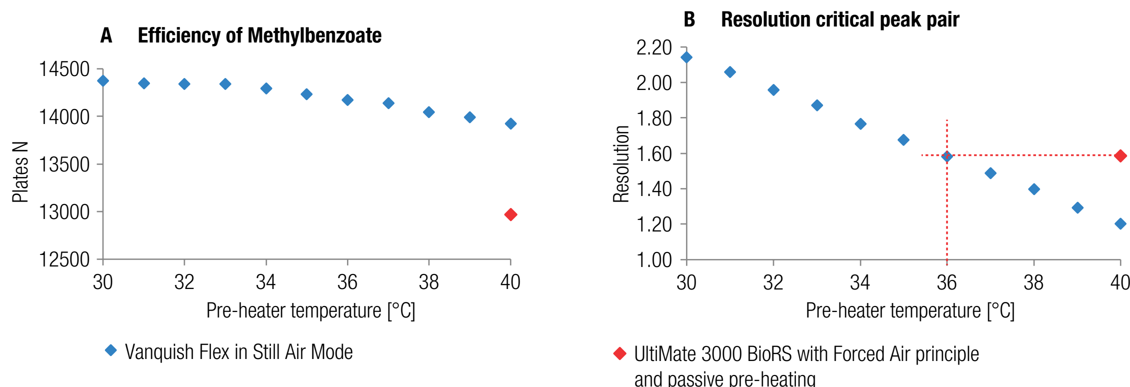

Figure 17. Influence of active pre-heater temperature on chromatographic efficiency and resolution

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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.

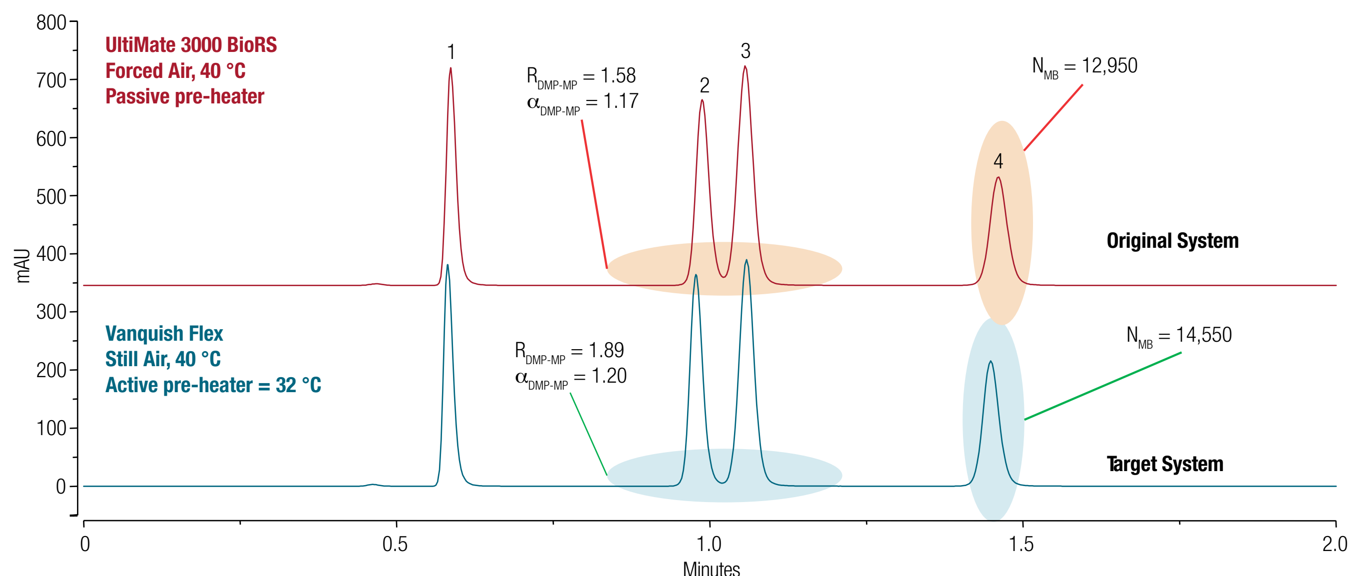

Figure 18. Match of retention times and improved peak shape and resolution with compensation of frictional heat by reduced inlet temperature of the column

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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.

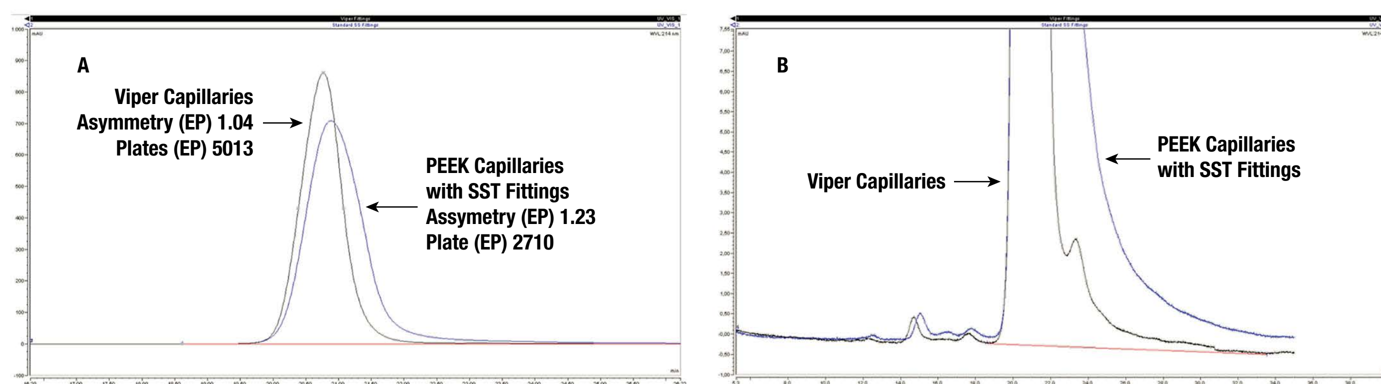

Figure 19. Comparison of Viper capillaries with ferrule-based fitting systems. (A) Asymmetry and plate counts of a single peak and (B) resolution of API and nearly eluting impurity

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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]

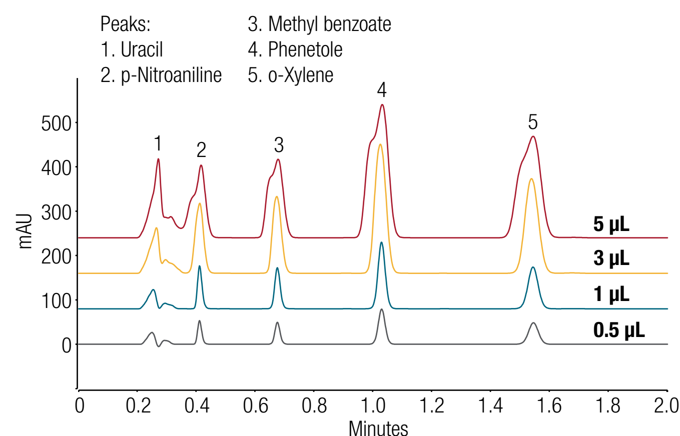

Figure 21. Effect of reducing the injection volume when the sample solvent (100% methanol) is stronger than the eluent (50:50 water/acetonitrile)

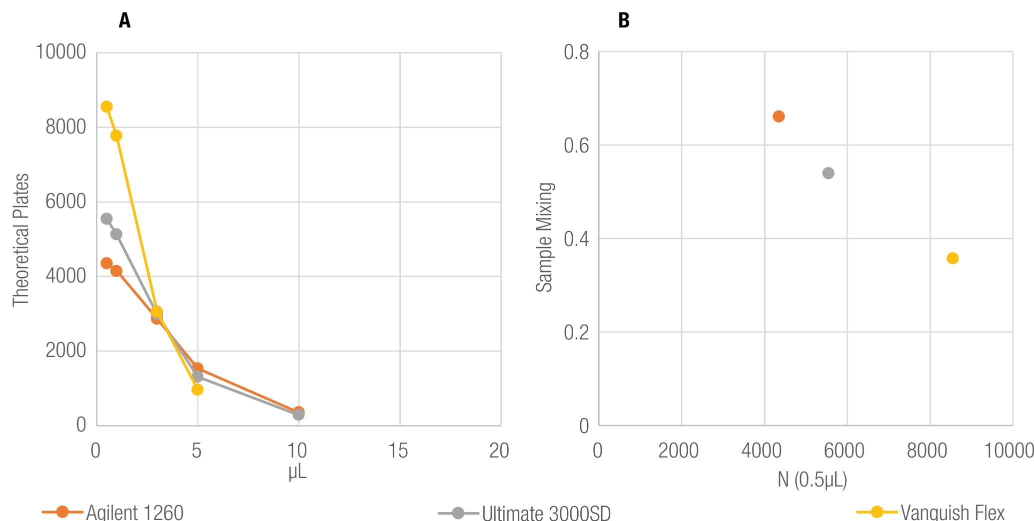

Figure 20. (A) Dependency of increasing injection volumes on system efficiency, (B) relationship between instrument sample mixing behavior and system efficiency with (C) respective peak shapes

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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.

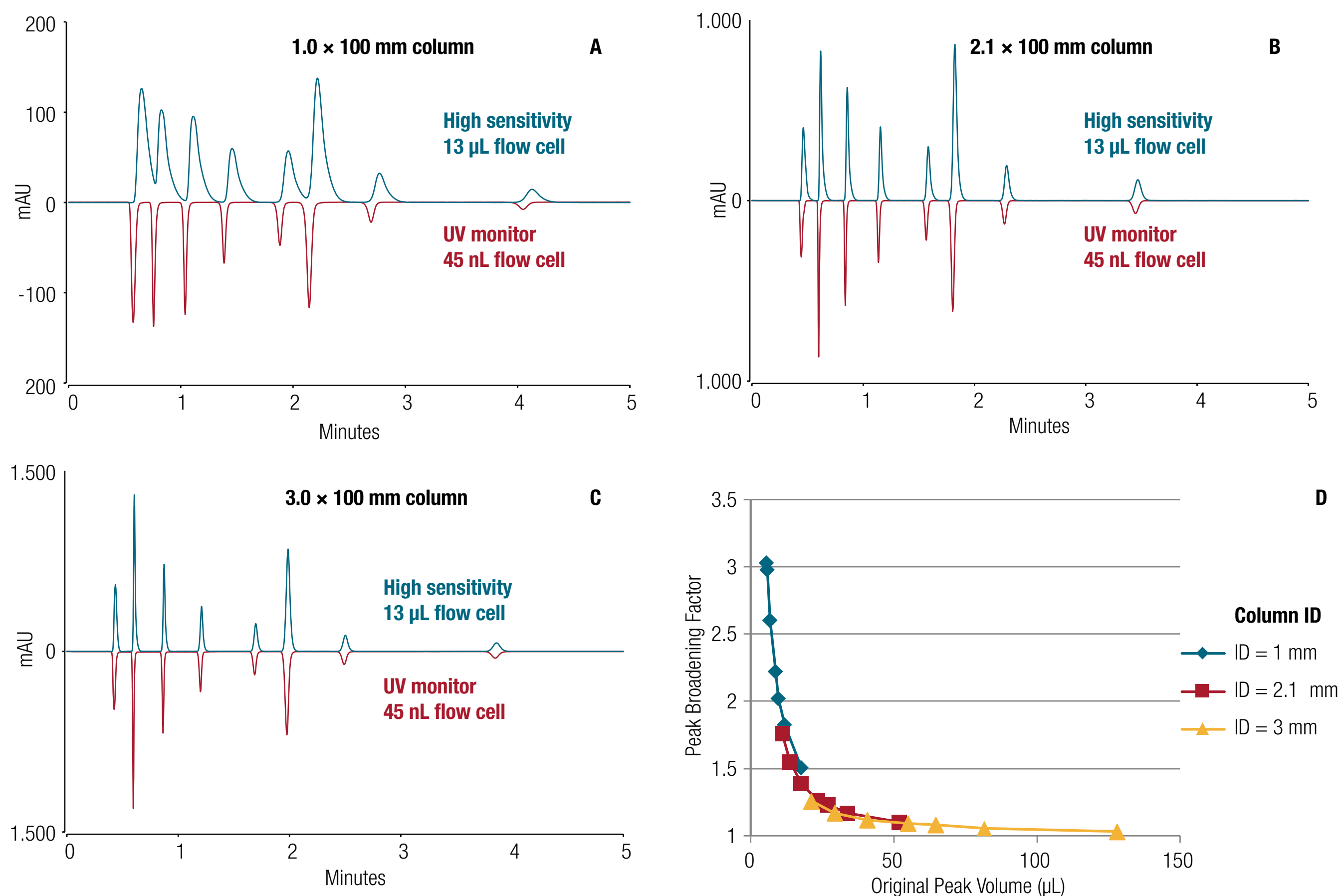

Figure 22. Isocratic separation of a standard mixture on a 1 mm column (A), 2.1 mm column (B), and 3.0 mm (C) using a high sensitivity 60 mm flow cell (blue trace) and a low dispersive flow cell (red trace). In addition, a peak broadening factor is given for all columns in dependence of the peak volume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

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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.

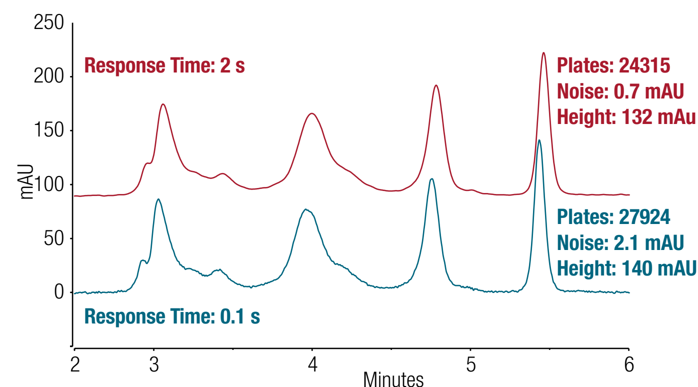

Figure 23. Comparison of isocratic size exclusion chromatography separations measured at different response times while all other parameters were kept constant

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.

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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.

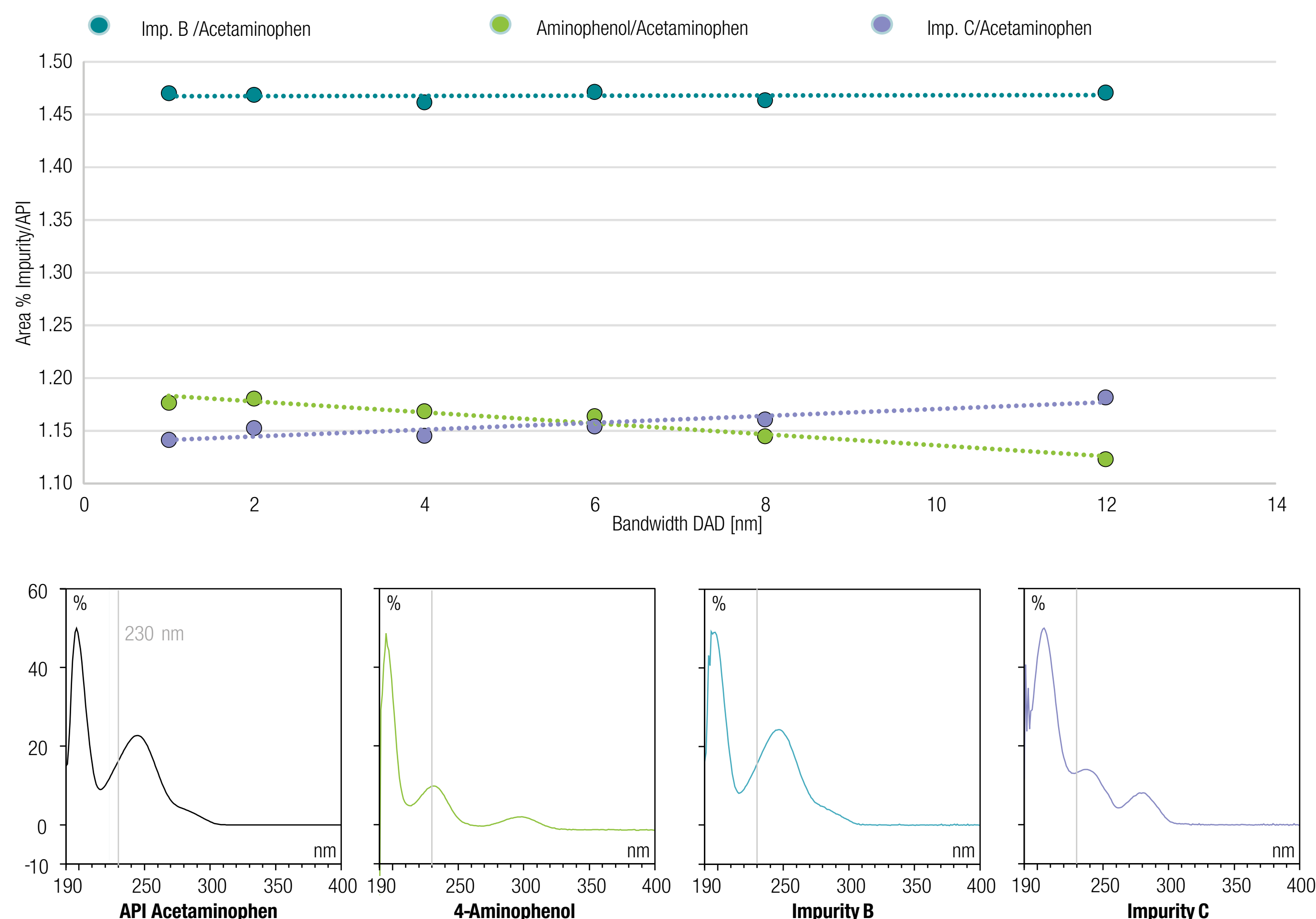

Figure 24. Relative peak areas of three impurities during the USP-based analysis of acetaminophen. Peak areas were recorded for six different bandwidth settings at 230 nm (indicated by gray vertical line) with the respective UV spectra of all involved compounds shown at the bottom.

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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:

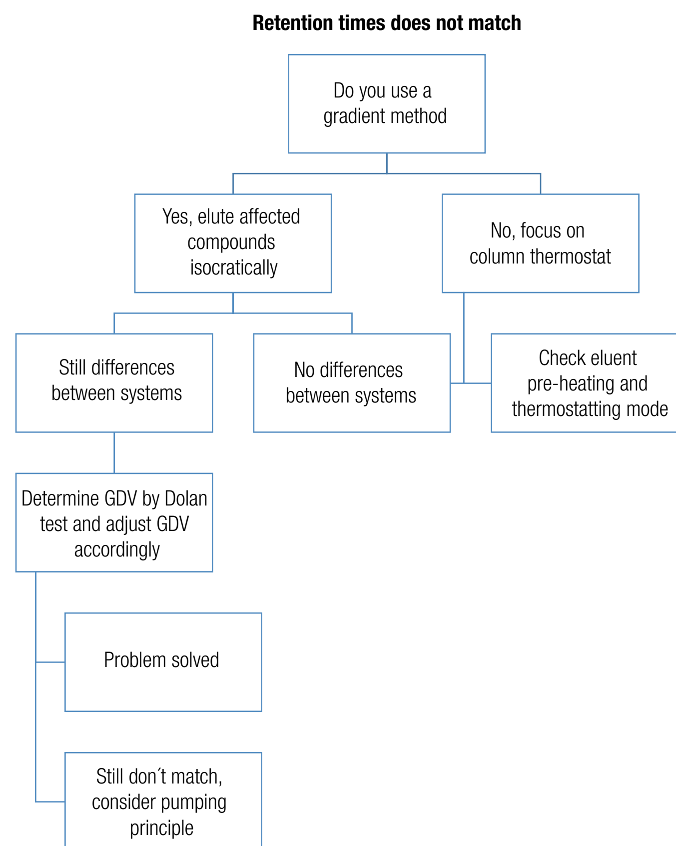
- Different pumping principles (LPG vs. HPG pumps)
- Different GDVs
- Different column thermostatting principles
- Different pre-heater usage

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

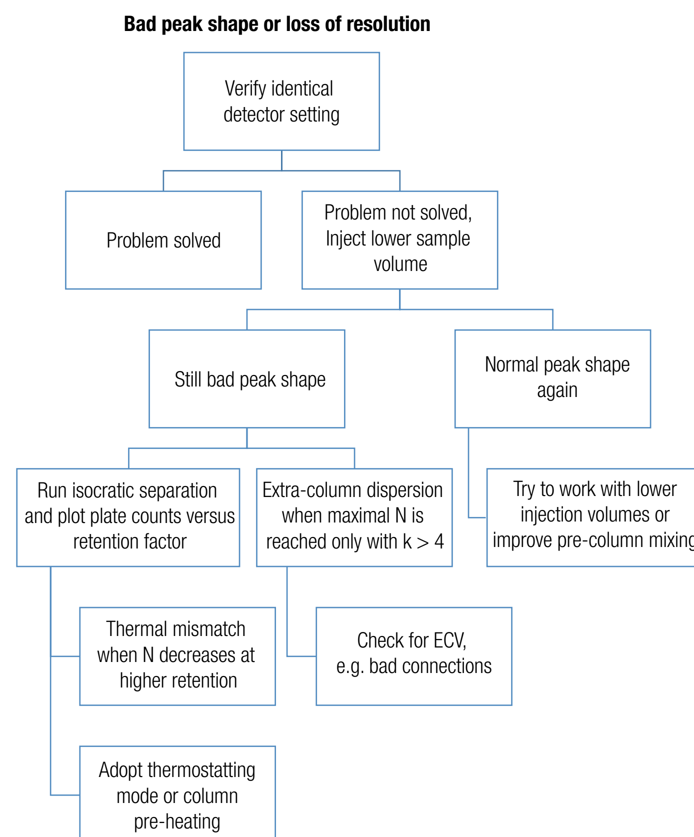
- Thermal mismatch due to pre-heating or column thermostatting
- Additional extra-column dispersion effects
- Sample solvent mismatch
- Detector settings

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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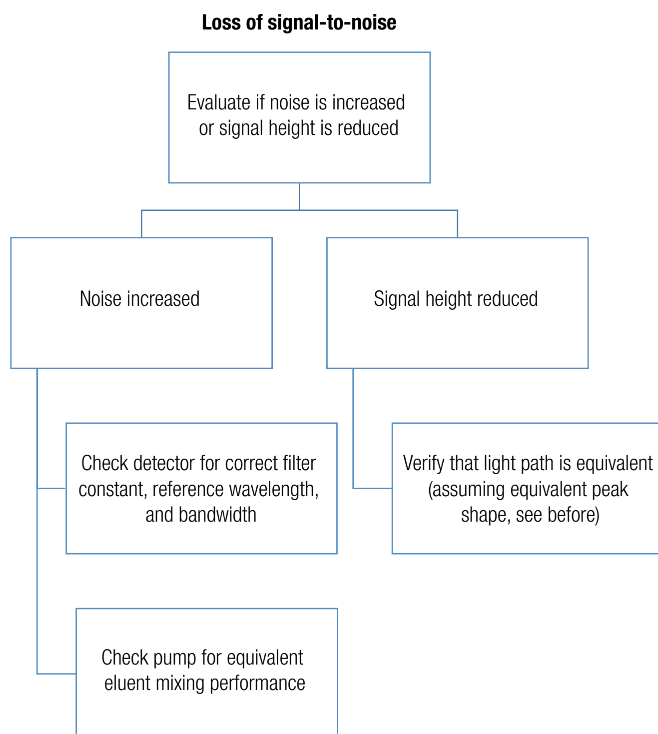
Signal-to-noise problem



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TECHNICAL NOTE 73371

Physical adjustment of gradient delay volume as a tool for successful transfer of HPLC methods

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Keywords: method transfer, gradient delay volume, adjustable GDV, metering device, idle volume, method transfer kit, regulatory compliance

Introduction

The transfer of chromatographic methods from an older HPLC instrument to a newer generation instrument or the transfer of methods between instruments of different vendors are challenging tasks for many HPLC practitioners. Differences in the hardware designs can affect chromatographic results, such as the thermostatting mode of the column compartment, the eluent pre-heating, and for gradient methods most importantly the instrument volumes as defined by the utilized pump technologies, autosampler designs and system plumbing[1].

The tools and possibilities for a user to mitigate the impact of instrument differences on validated chromatography methods are usually limited by regulatory bodies. For example, the United States Pharmacopeia (USP) Chapter <621> lists the permitted changes to a method without the need for revalidation and is often used as a reference for the degrees of freedom during HPLC method transfer[2]. Further changes which encompass specific system settings (e.g. forced air vs. still air thermostatting of a column oven) or mechanical changes to an instrument (e.g. changing a passive with an active column preheater) are usually considered acceptable as long as the instrument is qualified using a given configuration.

For gradient HPLC methods the instrument gradient delay volume (GDV) plays a crucial role for a successful method transfer. The GDV is defined as the volume from the point of mixing of the eluents to the column head[1]. Changes to the GDV or the length of an isocratic hold are explicitly permitted according to USP chapter <621>. Therefore, these changes are popular tools for successful method transfer. Changes to the isocratic hold are not universally applicable though, as they are limited to applications where an isocratic hold is prescribed[2]. Software-based compensation of differences in GDV, usually by a time-shift of the injection event or an alteration of the gradient profile can raise concerns of regulatory authorities.

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Gradient delay volume adjustment strategies

Manual changes to the gradient delay volume

Common strategies for changing the GDV of a system consist of adding large volume fluidic components into the flow path, i.e. placing mixers or large volume capillaries between the pump and the autosampler. While these changes usually help mimick a source instrument, the downsides are that mixers and capillaries have fixed volumes and thus do not allow for an exact setting of the GDV. Particularly in regulated environments, these hardware changes mandate a (re)qualification of the altered instrument and thus frequent changes to the system hardware is impractical. Consequently, this means that often a system configuration is locked to a fixed hardware configuration.

Switching of flow paths with different gradient delay volumes

Several commercial products try to circumvent this limitation by providing two separate flow paths; one providing a low GDV for short gradient responses required for applications using narrow-bore HPLC columns, and one large GDV flow path dedicated to providing compatibility with legacy methods/ HPLC instruments. Thus, with these products, the user can switch between full performance and legacy compatibility. Drawbacks of this approach are

1. striving for minimum GDV may lead to lower mixing performance,
2. both flow paths are static and cannot be further adjusted,
3. the instrument is optimized to transfer legacy methods from a single source system type only.

Freely tunable gradient delay volume

Thermo Scientific™ Vanquish™ Core HPLC systems use a metering device in the autosampler to aspirate the sample before the injection. The metering device is part of the flow path and contributes to the GDV of the HPLC system. The flushed through volume of the metering device can, however, be altered by the movement of a piston, thus changing the overall GDV of the HPLC system. This flushed through volume is called idle volume. The idle volume can be decreased by a software command to a low volume to mimic a system with a small GDV (Figure 1, left) or increased to a high volume to simulate a system with a higher GDV. The set idle volume and the injection volume are independent of each other, i.e. any combination of both is possible. The setting of the idle volume is tracked by the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software audit trail (version 7.2.10 MUa and higher) and therefore fully auditable. This functionality is also available with Thermo Scientific™ Standard Instrument Integration (SII) for Thermo Scientific™ Xcalibur™ software (version 1.6).

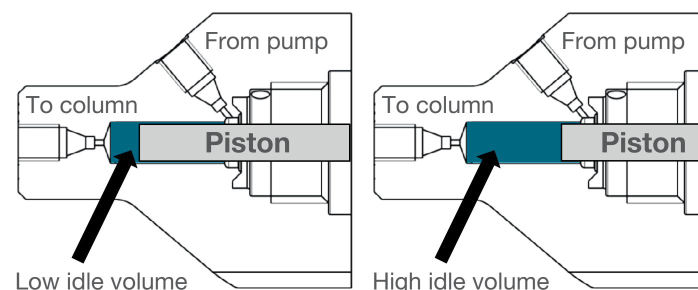

Figure 1. Effect of the metering device piston position on the gradient delay volume. The geometry is designed to ensure that, independent of the piston position, the entire idle volume is flushed, i.e. the geometry prevents stagnant zones from occurring, throughout all piston positions (idle volumes) and flow rates.

As the Vanquish Core system has a lower GDV than most routine HPLC systems, which typically have a GDV in the range of 1.1 to 1.4 mL (for low-pressure mixing pumps), the idle volume setting of up to 230 µL is usually sufficient to compensate GDV differences. A typical example is shown in Figure 2, where the idle volume setting was utilized to transfer a method for impurities in chlorhexidine from an Agilent 1260 Infinity LC system to a Vanquish Core HPLC system. By adaptation of the idle volume, the match of peak retention times could be greatly enhanced. For details of this method transfer example, refer to Application Note 73309: Straightforward transfer of an EP method for chlorhexidine impurity analysis from an Agilent 1260 Infinity LC system to a Vanquish Core HPLC system[3].

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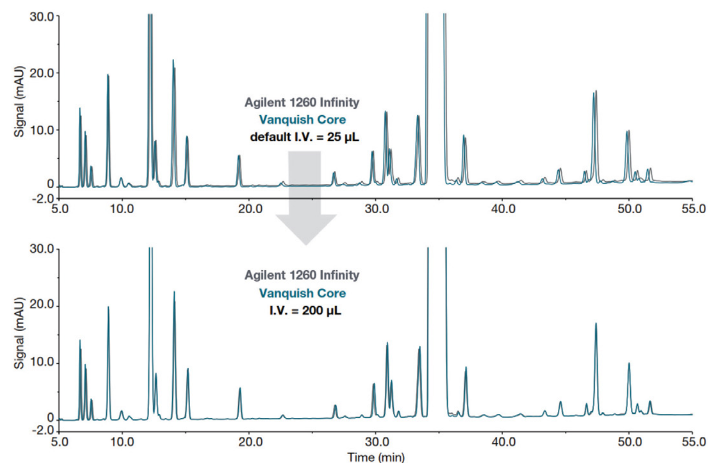

Figure 2. Example transfer from an Agilent 1260 Infinity Series HPLC system to a Vanquish Core HPLC system of a method determining impurities in chlorhexidine. Excellent overlap of the results could be achieved by increasing the system GDV by 175 µL. Reproduced from Application Note 73309: Straightforward transfer of an EP method for chlorhexidine impurity analysis from an Agilent 1260 Infinity LC system to a Vanquish Core HPLC system[3].

To further extend the GDV flexibility, a Method Transfer Kit (P/N 6036.2100) is available for Vanquish Core HPLC systems. It consists of a 6-port 2-position switching valve and a 200 µL loop which can be inserted into the flow path. The fluidic setup is depicted in Figure 3. Switching of the valve allows to increase the gradient delay volume by additional 200 µL. As this additional volume is placed before the point of injection, the system dispersion is not impacted.

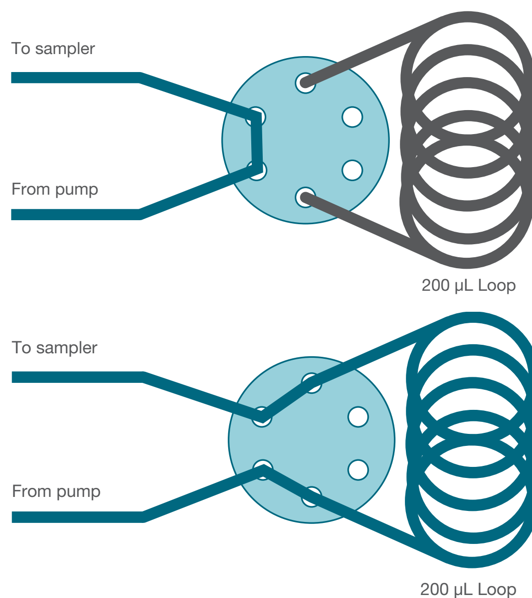

Figure 3. Switchable additional gradient delay volume. In bypass position, the flow from the pump is directly connected to the sampler, analogue to an instrument without installed method transfer kit (top). When the valve is switched, the loop is part of the flow path, adding the loop volume to the overall gradient delay volume of the system (bottom).

The GDV adjustment through the idle volume of the metering device of the Vanquish Core system is now combinable with the switching of the loop into the flow path. A combination of the two functions allows a 1 µL step tuning of the system GDV over a range of 430 µL decreasing it by 25 µL and increasing the system GDV by 405 µL compared to the default configuration. This is achieved by adjusting the idle volume of the metering device, the 200 µL loop, or a combination of both. This concept is also visualized in Figure 4.

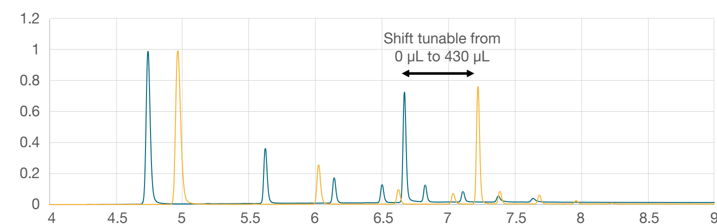

Figure 4. Impact of different GDV on a gradient separation using a Vanquish Core HPLC system. The lowest GDV can be achieved by an idle volume setting of 0 µL while switching the method transfer loop out of the flow path. The highest GDV can be implemented by an idle volume setting of 230 µL and simultaneously switching the loop into the flow path, the adjustment of the GDV is possible by 1 µL steps in a range of 430 µL.

Ease-of-use and software implementation

Whenever a Vanquish Method Transfer Kit (P/N 6038.2100) is installed, the system GDV can be influenced by using the loop, metering device, or both. To install the kit, it must be configured in the Chromeleon CDS (version 7.2.10 MUa or later) instrument configuration, as shown in Figure 5.

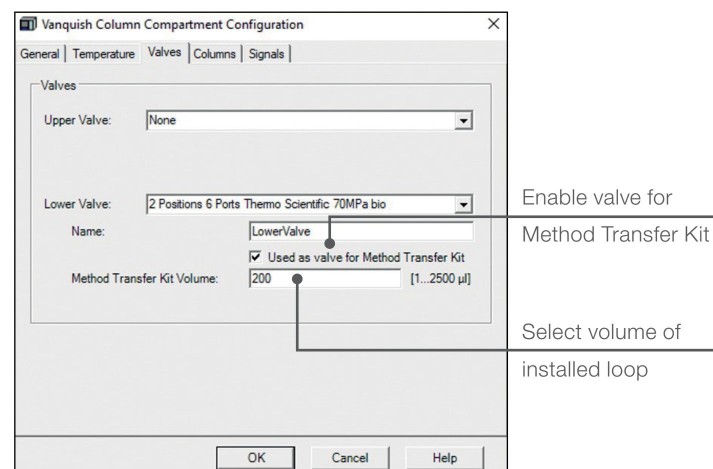

Figure 5. Instrument configuration screen of Chromeleon 7.3 CDS allowing the configuration of the method transfer kit and defining the loop volume thereof. 200 µL is the volume of the loop provided with the method transfer kit, other volumes for customized loops can also be entered.

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The volume of the loop shipped with the method transfer kit is 200 µL. In the Chromeleon software instrument configuration, it is also possible to define a different volume, for instance for a custom loop.

The Chromeleon Instrument Method Editor provides easy access to adjust the GDV as a regular method parameter, like for instance the sample temperature of the column compartment. This allows setting of the GDV within an instrument method and therefore individually for a sequence or even a specific sample. Figure 6 shows screen captures of the Chromeleon Instrument Method Editor (top) and Instrument Method Wizard (bottom) to illustrate this. The setting of the valve position and idle volume setting is fully tracked in Chromeleon CDS audit trails and can therefore be audited at any time.

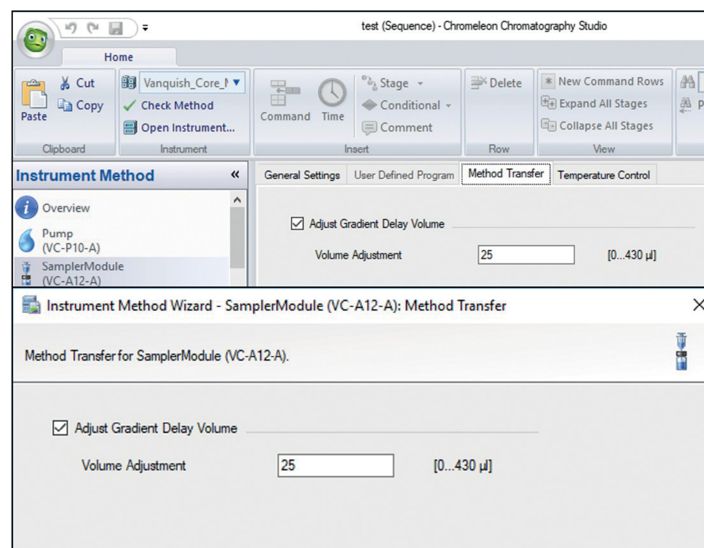

Figure 6. Chromeleon Instrument Method Editor (top) and Instrument Method Wizard (bottom), allowing to adjust the gradient delay volume of the system during method editing and method creation.

Limitations to using the gradient volume for method transfer

For a given gradient HPLC method, a change to the GDV or the length of the isocratic hold at the beginning of a method, can influence retention times across a chromatogram in an inconsistent way. Peaks eluting during the isocratic hold or close to it undergo a full or partial isocratic elution mechanism. Their retention times are usually not or minorly affected by GDV changes. Peaks eluted fully by the impact of the gradient typically show a deviation in accordance with the GDV difference. Both effects in a chromatogram can influence the chromatographic resolution.

To illustrate this effect, it is best to envision a non-retained and a strongly retained compound during a step gradient elution, as depicted in Figure 7. A non-retained compound will always elute at the same time, irrespective of a shift of the gradient step due to different gradient delay volumes. A strongly retained peak, however, will only elute once the gradient step reaches the analyte on the column, almost independent of the absolute length of the isocratic hold prior to the gradient step. In case of the latter, any increase of GDV directly translates into a shift of the retention time.

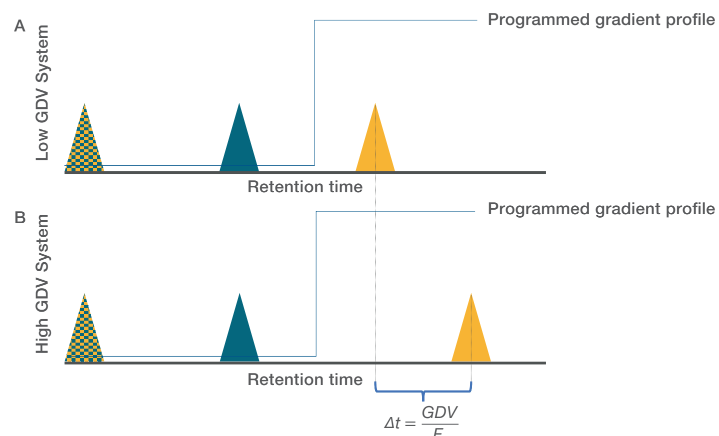

Figure 7. Impact of GDV on elution times of non-retained (blue) and strongly retained (orange) peaks during a step gradient elution (A) for a system with low GDV and (B) for a system with GDV. The retention time of the blue peak is not influenced by the GDV while the orange peak is directly shifted by the delay of the gradient (with GDV= gradient delay volume [mL], F=flow [mL/min] and Δt=retention time shift [min]).

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Guidelines for setting the GDV during method transfer

If no information on the GDV difference between source and target system is available, we recommend the following method transfer procedure:

i) Replicate the chromatogram on the Vanquish Core HPLC system with an idle volume setting of 0 µL and no loop connected, using the same gradient table and settings such as data collection rate and detector signal filter (e.g. time constant) as implemented at the source instrument.

ii) Compare results between source and target systems.

iii) In case of a retention time shift, identify whether a shift towards earlier retention times is present with the Vanquish Core HPLC system.

iv) Enter a volume shift into the Chromeleon Instrument Method Editor (Figure 6) to compensate the retention time shift (in case of a not fully consistent shift you might focus on late elution peaks) by:

a. either calculating the volume to compensate for the retention time shift with this simple equation:

$$\Delta V = (t_1 - t_2) \times F$$

With ΔV= delta gradient delay volume [µL], t_1=retention time of peak A on source system [min], t_2=retention time of peak A on target system [min], F=flow [µL/min]

b. or increase the volume in an iterative process until the best retention time overlap is achieved.[4]

These changes are compliant since all of the following are true:

- Compendial methods do not regulate system volumes.
- The fluidic setup of the HPLC system is not undergoing a manual change.
- Instrument parameter settings are fully trackable in the audit trail of the chromatography data system.

Compliance aspects

USP

In the method transfer for HPLC section of general chapter <621> in the USP41, modifications of the method are tolerated within limits. For the gradient delay volume (named dwell volume in the USP) it is stated that, "if adjustments are necessary, change in column packing (maintaining the same chemistry), the duration of an initial isocratic hold (when prescribed), and/or gradient delay volume adjustments are allowed."[2] This means that changes of the gradient delay volume as done by the method transfer kit are explicitly approved as a suitable tool for method transfer.

European Pharmacopeia

The European Pharmacopeia generally prefer a different design approach insofar that, "monographs preferably include an isocratic step before the start of the gradient program so that an adaptation can be made to the gradient time points to take account of differences in dwell volume between the system used for method development and that actually used. It is the user's responsibility to adapt the length of the isocratic step to the analytical equipment used."[5] Although the adaptation of the gradient delay volume is not explicitly mentioned as a tool, the effect, namely the length of the initial isocratic windows is actively encouraged as a changeable parameter. However, an approach that is based on altering the isocratic step length often fails to reflect the possible chromatographic differences due to different physical GDV, e.g. mixing effects. Therefore, if possible, physical adjustment of the GDV should be preferred.

Japanese Pharmacopeia

The Japanese Pharmacopeia can be considered liberal with respect to changing the operating conditions. A wide range of parameters "[…] may be modified within the ranges in which the liquid chromatographic system used conforms to the requirements of system suitability."[6] Adapting the GDV of a system can therefore be recommended as a suitable tool for simple method transfer.

Qualification of the Method Transfer Kit

Instrument qualification usually follows a holistic approach, testing whether an HPLC system works as intended. With Chromeleon CDS the required test sequences are generated automatically considering the used instrument configuration.

If the method transfer kit is installed and configured, Chromeleon CDS performs a duplicate of the pump gradient test. With that test, it is ensured that the flow path with and without additional loop is fully functional. Additional qualification tests besides the pump are not required as the method transfer kit does not influence autosampler, column compartment or detector performance. For more details, please refer to the *Thermo Scientific Chromeleon – Operational Qualification/ Performance Qualification for HPLC Instruments – Operating Instructions.*[7]

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Summary

- Gradient delay volume (GDV) is one of the most critical parameters during method transfer.
- In regulated environments, manual and not auditable changes to the HPLC system fluidics typically require a revalidation of the instrument.
- The Thermo Scientific Vanquish Core HPLC System provides a unique solution to support the method transfer from conventional HPLC instruments. The autosampler of the Vanquish Core HPLC system can freely tune the GDV of up to additional 230 µL. The optional Method Transfer Kit (P/N 6036.2100) allows to extend this range to up to 430 µL additional gradient delay volume to help transferring methods even from legacy design HPLC instruments with extensive GDV.
- Seamless integration into Chromeleon CDS and SII for Xcalibur offers fully compliant settings as the gradient delay volume is a method parameter logged in the audit trail.

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Find out more at **thermofisher.com/vanquishcore**

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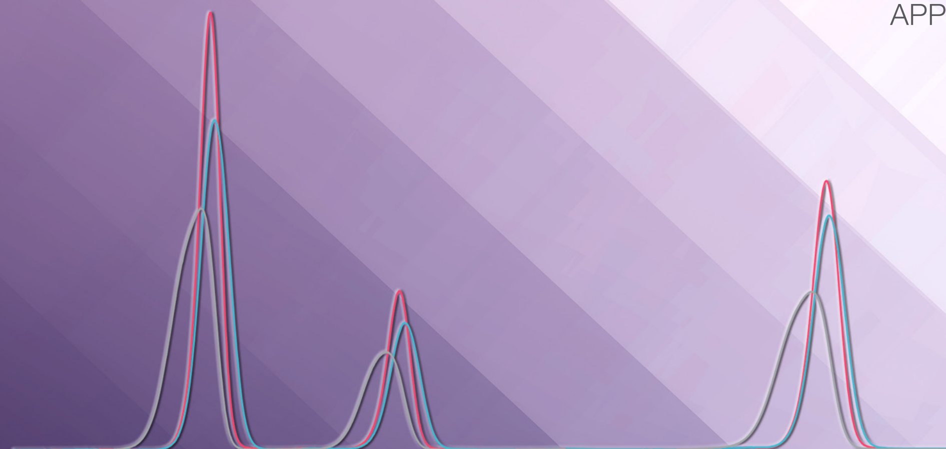

Improving peak results using a custom injection program to reduce solvent strength prior to sample injection

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Keywords
Mebendazole, system suitability, Vanquish, European Pharmacopoeia, custom injection program, sandwich injection, autosampler automation, automated liquid handling, peak results, resolution, asymmetry, peak width, efficiency

Application benefits

- Easy and flexible custom injection programs expand the possibilities of liquid handling in the Thermo Scientific™ Vanquish™ Split Sampler
- Custom injection provides the ability to improve certain system suitability parameters of an EP method without modifying chromatographic conditions

Goal

To demonstrate how custom injection programs affect chromatographic parameters such as asymmetry, resolution, and efficiency of a selected EP method.

Introduction

Pharmacopoeia monographs contain standardized analytical methods including acceptance criteria for the quality control of drug substances. Provided the regulations are strictly followed, the methods can be adopted without full validation.[1,2] To ensure adequate performance of the

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chromatographic system, a reference standard for the system suitability test (SST) needs to be injected and evaluated as indicated in the individual monograph. The general chapter of the European Pharmacopoeia (EP) (Chromatographic Separation Techniques 2.2.46) defines the permissible deviations from the EP method.[2] In particular, for gradient methods only a few adjustments are allowed, which can be critical to a method's success if poor chromatographic results are obtained with the original method.

Several monographs stipulate dissolution of the analytes in a solvent that is not the initial mobile phase composition of the method. In some cases, the recommended solvent has much higher elution strength. As a result, serious peak shape problems may occur. The reason for these anomalies lies in the fact that the analytes are transported to the column in a plug of strong solvent. Analyte molecules on the outside of the injection plug will mix with the mobile phase and will be retained. In contrast, molecules in the interior of the injection plug will not be retained and will migrate further into the column. These retention differences will cause peak distortion.[3]

With modern high-performance liquid chromatography (HPLC) instruments, system volumes are minimized so that a mismatch between sample solvent and initial gradient composition can result in insufficient mixing, which causes fronting or split peaks. A custom injection program that does not change the injection volume or solvent can be used to reduce the solvent strength of the solution to be injected. Thus, in this study, the EP method of mebendazole was not modified, except that a custom injection program was used to demonstrate the effect on system suitability criteria such as asymmetry, resolution, and efficiency.

Experimental

Chemicals

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Scientific™ Acetonitrile, Optima™ LC/MS grade (P/N A955-212)
- Fisher Scientific™ Ammonium acetate, LC/MS grade (P/N A114-50)
- Fisher Scientific™ N,N-Dimethylformamide, Acros Organics™, ACS reagent (P/N 10567942)
- EP reference standard: Mebendazole for system suitability CRS batch 1[4] (P/N Catalog code Y0000144)

Equipment

- Vials (amber, 2 mL), Fisher Scientific (P/N 11545884)
- Snap Cap with Septum (Silicone/PTFE), Fisher Scientific (P/N 10547445)

Preparation of standards

The mebendazole SST reference standard contains the API and seven impurities (impurity A–G). According to the EP monograph, 5 mg of mebendazole SST reference material was weighed into a 5 mL volumetric flask and filled to the line with dimethylformamide (DMF).

Instrumentation

A Thermo Scientific™ Vanquish™ Core Quaternary HPLC system equipped with the following was used for the analysis:

- Thermo Scientific™ Vanquish™ System Base Vanquish (VC-S01)
- Thermo Scientific™ Vanquish™ Quaternary Pump C (VC-P20)
- Thermo Scientific™ Vanquish™ Sampler CT (VC-A12)
- Thermo Scientific™ Vanquish™ Column Compartment C (VC-C10-A-03)
- Thermo Scientific™ Vanquish™ Diode Array Detector CG (VC-D11) with standard flow cell, 13 µL (P/N 6083.0510)

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Table 1. Chromatographic conditions

Column:	Thermo Scientific™ Hypersil GOLD™, 100 × 4.6 mm, 3 µm (P/N 25003-104630)	
Mobile phase:	A: 7.5 g/L ammonium acetate in water B: acetonitrile	
Flow rate:	1.2 mL/min	
Gradient:	*Time (min)*	*Mobile Phase B (%)*
	0	20
	15.0	30
	20.0	90
	25.0	90
	25.1	20
	30.0	20
Mixer volume:	350 µL + 50 µL	
Column temp.:	40°C with passive pre-heater	
Sampler temp.:	4 °C	
UV wavelength:	250 nm	
UV data collection rate:	10 Hz	
UV response time:	0.5 s	
Injection volume:	10 µL	

Custom injection program

In the present study, the custom injection program is used to reduce the solvent strength of the sample prior to injection. Various custom injection programs were tested, and two were selected as being the most effective. Details about the individual programs can be found in Tables 2 and 3.

If the custom injection mode is selected, predefined commands can be chosen from a drop-down menu when creating a new instrument method with the Thermo Scientific™ Chromeleon™ Chromatography Data System wizard. This allows quick and flexible programming of several pre-injection liquid handling steps. These commands are named as UDP (user-defined program) in Chromeleon software to distinguish from standard commands.

Data processing and software

The data acquisition and processing were done with Thermo Scientific™ Chromeleon™ 7.3 Chromatography Data System software.

Table 2. Custom injection A

Custom Injection A		
Command	**Parameter**	**Description**
UDP_PrepareLiquidHandling	Volume = 100 µL	Define the total liquid handling volume
UDP_Draw	Position = R:A2 Volume 10 µL Speed = 5 µL/s Needle Height = 2000	Draw 10 µL sample from the specified vial position (e.g., R:A2) with a draw speed of 5 µL/s, at needle height of 2000 µm
UDP_Draw	Position = R:A4 Volume = 90 µL Draw Speed = 20 µL/s Needle Height = 2000	Draw 90 µL initial mobile phase (20:80 acetonitrile:ammonium acetate buffer) from the specified vial position (e.g., R:A4) with a draw speed of 20 µL/s, at needle height of 2000 µm
UDP_Wait	10 s	Move needle to injection port and wait 10 s before injection
UDP_PrepareInject		End of liquid handling

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Table 3. Custom injection B

Custom Injection B		
Command	**Parameter**	**Description**
UDP_PrepareLiquidHandling	Volume = 100 µL	Define the total liquid handling volume
UDP_Draw	Position = R:A3 Volume = 45 µL Draw Speed = 5 µL/s Needle Height = 2000	Draw 45 µL mobile phase A (7.5 g/L ammonium acetate) from the specified vial position (e.g., R:A3) with a draw speed of 5 µL/s, at needle height of 2000 µm
UDP_Draw	Position = R:A2 Volume 10 µL Speed = 5 µL/s Needle Height = 2000	Draw 10 µL sample from the specified vial position (e.g., R:A2) with a draw speed of 5 µL/s, at needle height of 2000 µm
UDP_Draw	Position = R:A3 Volume = 45 µL, Draw Speed = 20 µL/s Needle Height = 2000	Draw 45 µL mobile phase A (7.5 g/L ammonium acetate) from the specified vial position (e.g., R:A3) with a draw speed of 20 µL/s, at needle height of 2000 µm
UDP_Wait	10 s	Move needle to injection port and wait 10 s before injection
UDP_PrepareInject		End of liquid handling

Results and discussion

System suitability (SST)

The mebendazole EP monograph requires that the reference standard for system suitability is dissolved and injected in DMF, probably to dissolve all seven impurities contained in the standard.[5] Literature data on the eluotropic strength of common HPLC solvents on stationary C18 phases suggest that DMF is a stronger solvent than acetonitrile.[6]

As can be seen in Figure 1, injection of the mebendazole SST standard into the initial mobile phase condition of the gradient method (20:80 acetonitrile:ammonium acetate buffer) causes peak distortion for the early eluting impurity peaks A–C. These peaks are detected with shoulders or show fronting, while the later eluting impurities D–G are less affected and elute as more symmetrical peaks. Peak distortion is typically observed if the injection solvent is stronger than the initial mobile phase composition, as is likely the case for impurities A–C. Another cause of peak fronting is overloading the column, as can be inferred for mebendazole, which is contained in the standard at a much higher concentration than the impurities.

In the following, impurities A–C are discussed in more detail to demonstrate the effect of using custom injection programs on various system suitability criteria.

In EP 9.2, chapter 2.2.46, section system suitability, the parameters usually employed for a system suitability check are listed:[1]

- Efficiency
- Retention factor
- Resolution
- Symmetry factor

A symmetry factor of 0.8–1.5 needs to be achieved. The asymmetry factors of the early eluting impurities A–C with normal injection are very close to the lower end of this requirement. Impurities A and C are even slightly outside at 0.76 and 0.77, respectively, as shown in Table 4.

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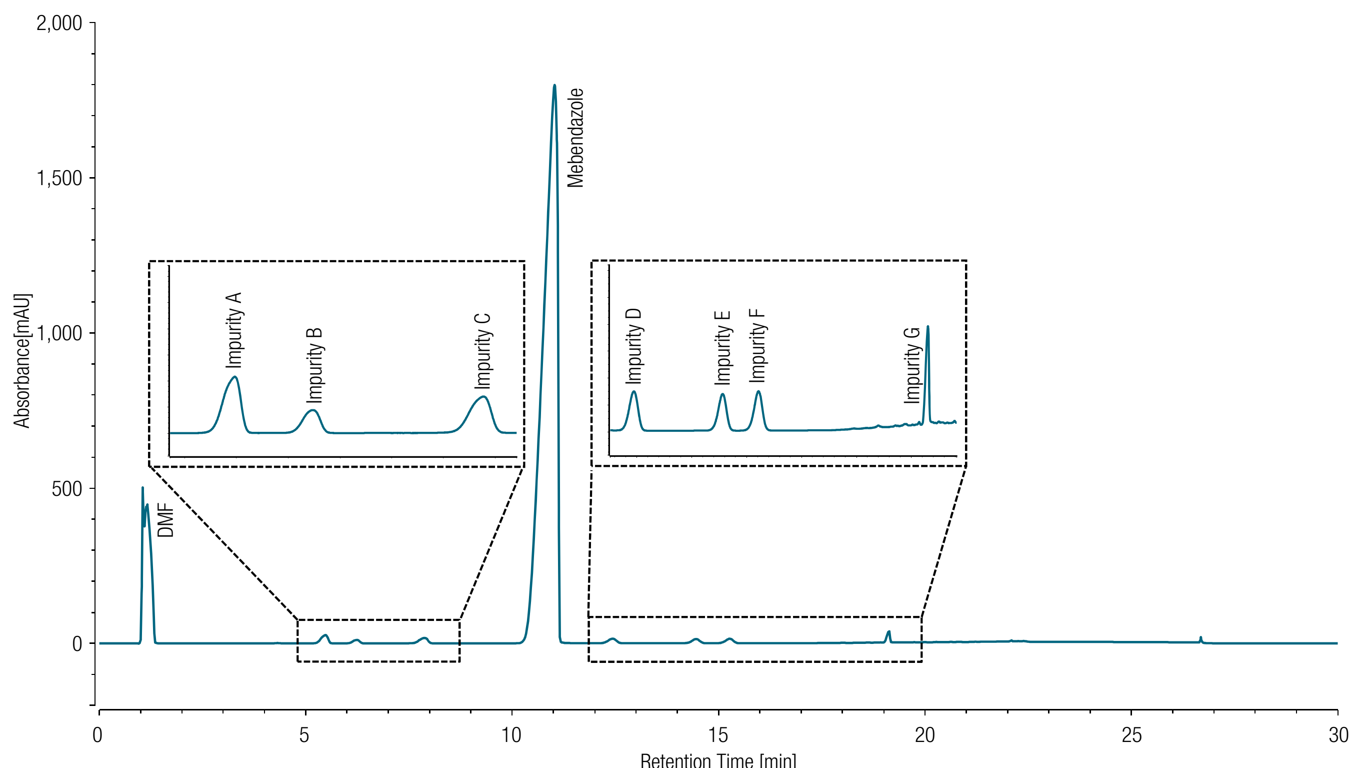

Figure 1. Chromatogram of a normal injection with enlargements of early eluting impurities A–C and later eluting impurities D–G. Sample: 1 mg/mL mebendazole SST in DMF; separation on a Hypersil GOLD column with 100 × 4.6 mm and 3 µm particle size; injection volume: 10 µL; for full details on the chromatographic method refer to Table 1.

Table 4. Asymmetry factors (EP) obtained for normal injection, custom injection A, and custom injection B. Averaged values of five consecutive injections; peak asymmetry improves with custom injections.

	Asymmetry (EP)		
Compound	Normal injection	Custom injection A	Custom injection B
Impurity A	0.76	0.98	0.89
Impurity B	0.83	0.95	0.87
Impurity C	0.77	0.93	0.89

Without adjusting the chromatographic conditions of the EP method, custom injection programs can be used to reduce the solvent strength in the sample prior to injection. Figure 2 illustrates the sample loop schematically. With the normal injection (I), after switching the autosampler valve to bypass, the loop is filled with initial mobile phase (20:80 acetonitrile:7.5 g/L ammonium acetate in water) from the column equilibration step and the sample is drawn. With the custom injection A program (II), first the sample is drawn and then the

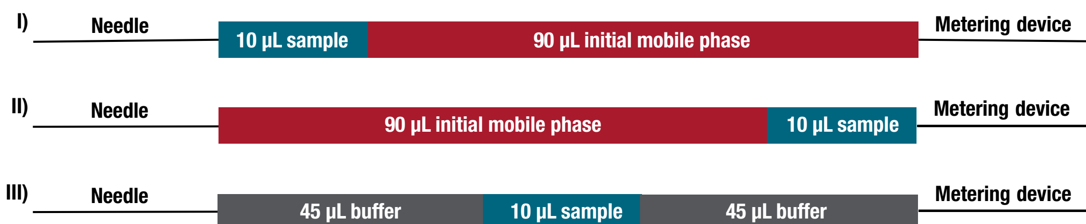

Figure 2. Schematic drawing of the sample loop on the three different injection methods. I = normal injection, II = custom injection A, III = custom injection B; sample = 1 mg/mL mebendazole SST standard in DMF; initial mobile phase = 20:80 acetonitrile:7.5 g/L ammonium acetate in water; buffer = 7.5 g/L ammonium acetate in water (refer to Tables 1–3 for more details)

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remaining loop volume is filled with the initial mobile phase. With the custom injection B program (III), the solvent strength is reduced even more by using a sandwich injection with the mobile phase A (7.5 g/L ammonium acetate in water). The sample is drawn between two aqueous buffer regions, which results in a lower solvent strength than the initial mobile phase and should result in a focusing effect of the analytes on the column head. Figure 3 demonstrates the effect of both customized injection programs on the peak shapes of impurities A–C compared to the normal injection. Both custom injection programs result in peaks with asymmetry values between 0.87 and 0.98 (Table 4). The closer the value is to 1, the more symmetric the peak is. Asymmetry values below 1 indicate a fronting peak shape, while values above 1 indicating peak tailing.

Overall, the peak area remains the same, while the peak height increases with an increase of the aqueous buffer content used in the custom injections (data not shown). This observation is in accordance with literature data published on the same approach but using different injection solvents instead of custom injection programs.[3]

The SST criteria of resolution and efficiency are not part of this particular EP monograph, but it can be demonstrated that custom injection programs improve both, compared to the normal injection (Figure 4). A better peak height, and thus narrower peaks, have a direct effect on resolution. The plate number increased up to 2 times with custom injection A and by a factor of 3–4 for custom injection B.

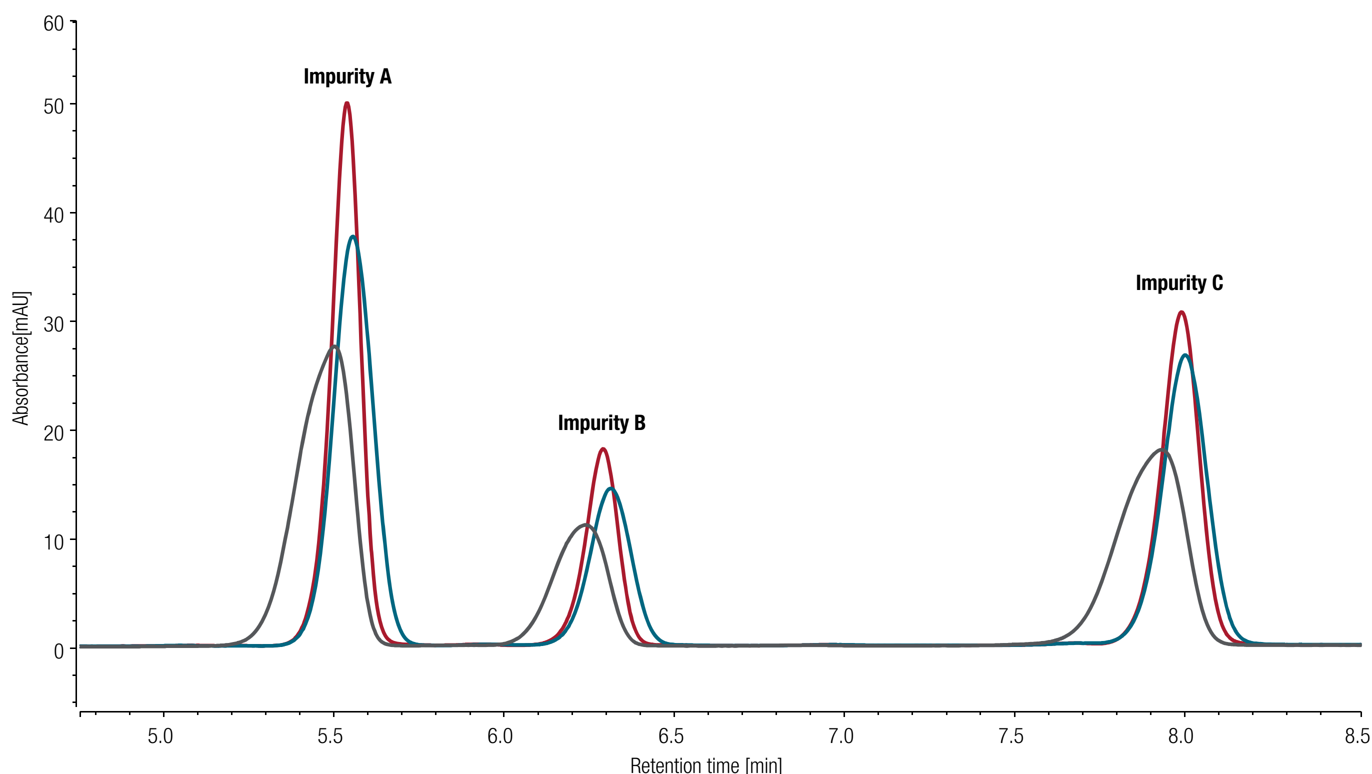

Figure 3. Overlaid chromatograms of normal injection (gray), custom injection A (blue), and custom injection B (red) of the impurities A–C. Sample: 1 mg/mL mebendazole SST in DMF; separation on a Hypersil GOLD column with 100 x 4.6 mm and 3 µm particle size; injection volume: 10 µL. For full details on the chromatographic method refer to Table 1; for full details on the custom injection programs refer to Table 2 and Table 3.

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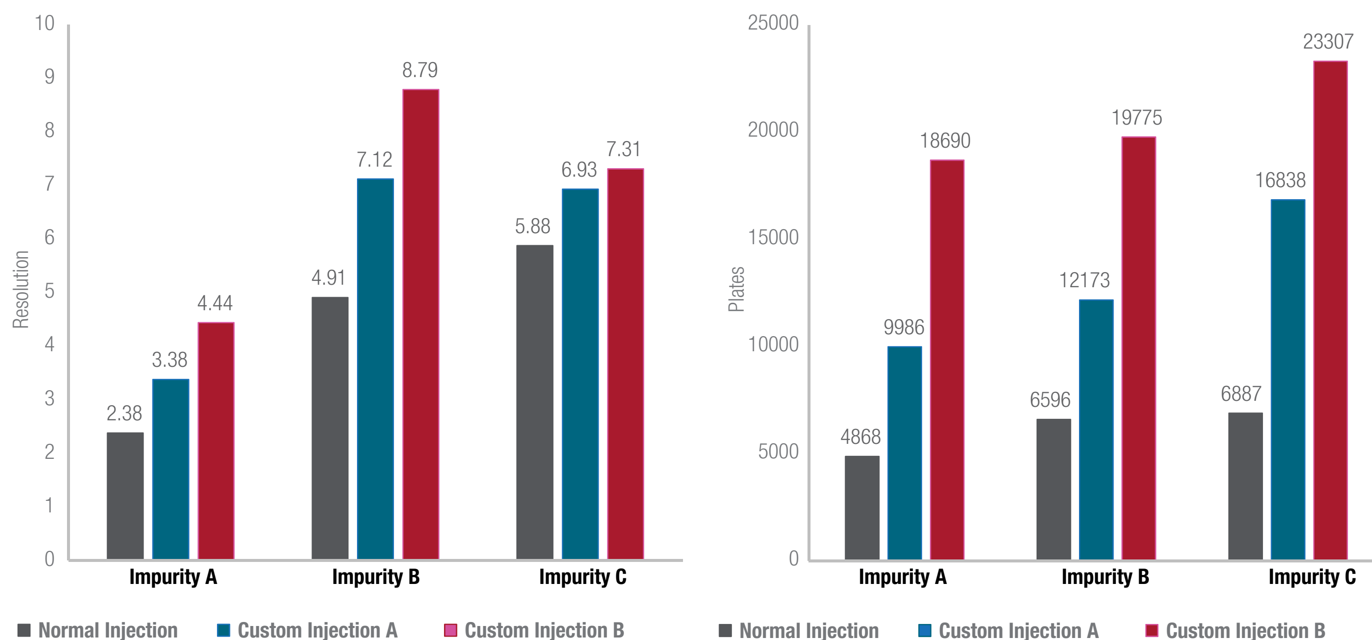

Figure 4. Peak resolution and plate number obtained for normal injection, custom injection A, and custom injection B. Resolution improved with custom injections; highest resolution was achieved for custom injection B; plate number increased with custom injections.

Instead of resolution, the EP monograph requires a peak-to-valley ratio for impurity D (relative to the API mebendazole) of at least 4 for system suitability.[5] This parameter is only used if two peak pairs are not fully baseline resolved. In the present study, baseline resolution was easily achieved with the Vanquish Core instrument and the Hypersil GOLD column. The peak-to-valley ratio is 137 for the normal injection mode, which is already far above the required limit.

Method performance

Method performance data on retention time and area precision were evaluated for five consecutive injections for each injection method (normal injection, custom injection A, custom injection B). The relative standard deviation (%RSD) of retention time (RT) and area are summarized in Table 5. The obtained %RSD RT values are extremely low (<0.1%) regardless of which method was applied.

In general, the %RSD area values are below 0.5%, or even lower (<0.2%) for the majority of impurities. Custom injection B shows slightly higher values in the range of 0.3%.

Table 5. Chromatographic results (%RSD RT and %RSD area) obtained for the different injection methods (n= 5). Excellent %RSD RT precision with <0.1% could be achieved; good area precision is obtained for impurities A–F; impurity G shows higher values up to 3.3%.

Compound	Normal injection		Custom injection A		Custom injection B	
	% RSD RT	%RSD area	%RSD RT	%RSD area	%RSD RT	%RSD area
Impurity A	0.06	0.06	0.09	0.06	0.08	0.26
Impurity B	0.03	0.10	0.08	0.09	0.06	0.27
Impurity C	0.06	0.14	0.08	0.06	0.05	0.23
Impurity D	0.02	0.17	0.04	0.10	0.02	0.34
Impurity E	0.02	0.47	0.04	0.12	0.02	0.23
Impurity F	0.01	0.03	0.04	0.09	0.02	0.25
Impurity G	0.02	3.31	0.01	1.90	0.01	3.03

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Only impurity G shows much higher %RSDs for peak area for all three injection methods up to 3.3%. Impurity G is structurally very different from the API and the other impurities and is therefore much more hydrophobic. This causes impurity G to elute during the steeper gradient region. As the substitution reaction product of impurity A and mebendazole (Figure 5), it is multiply positively charged with the mobile phase indicated for the EP method. Complex ionization behavior likely contributes to a worse peak shape and non-reproducible results for this basic molecule. To accurately measure impurity G, it may be necessary to adjust the pH of the mobile phase in order to reduce the degree of protonation.

Note, adjusting the pH is not permitted under 2.2.46 of the EP general chapter for gradient elution.

Custom injection programs are available for the entire Vanquish platform line (Horizon, Flex, and Core) and can be extremely useful in many applications. As demonstrated in this study, custom injections can be used to improve peak results to achieve system suitability criteria for critical applications. They can also be used to automate sample preparation steps, such as derivatization, generation of calibration standards for quantitative analysis, or addition of internal standards to the sample prior to injection.

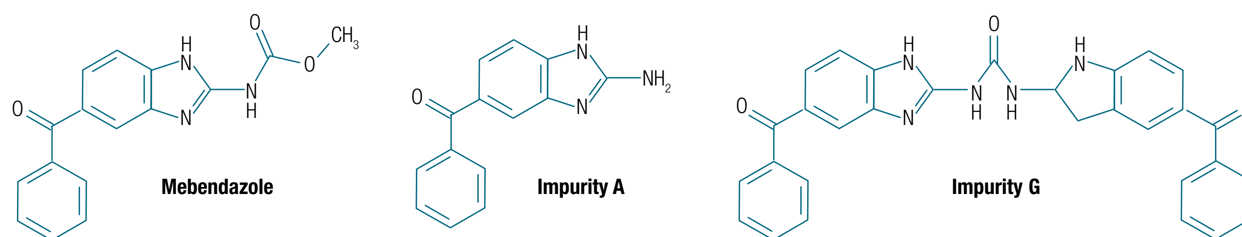

Figure 5. Chemical structures of mebendazole and its related impurities A and G

Conclusion

Custom injection programs provide the following benefits:

- Improved asymmetry factors, particularly for early eluting impurities A–C contained in the mebendazole system suitability standard, by reducing the solvent strength prior to injection.
- Increased column efficiency and resolution by producing narrower peaks.
- A practical and easy solution in cases where modifications of chromatographic conditions are not permitted.

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TECHNICAL NOTE 74138

Evaluation of custom injection programs and larger internal diameter capillary for strong solvent sample effects mitigation in liquid chromatography

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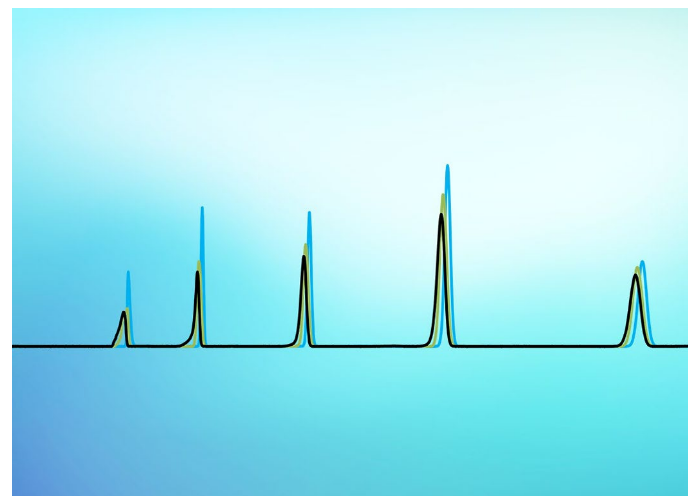
Themo Fisher Scientific, Germering, Germany

Keywords: Vanquish Core HPLC system, strong solvent samples, custom injection program, strong solvent loop, peak results, peak width, asymmetry, peak shape, dispersion



Goal

To determine the capability of custom injection programs and the strong solvent loop to reduce the strong solvent effects (SSE) caused by the sample on chromatographic performance for various diluents and column dimensions.

Introduction

Although in liquid chromatography (LC) analysis it is recommended to dissolve samples in weak eluting solvents,[1] occasionally samples must be injected in solvents stronger than initial mobile phase conditions (e.g., due to established analytical protocols, precipitation risks, the sample's solubility, detection limits, and many other factors). When the sample is dissolved in a solvent with a higher eluotropic strength/stronger elution power than the eluent, there is a risk of peak broadening, fronting, splitting or other peak distortions, which generally worsen with the diluent strength.[2] One of the common explanations for these peak distortions is the difference in eluotropic strength between the diluent and the mobile phase; another is a difference in viscosity causing hydrodynamic instability also known as "viscous fingering".[3] In most cases, it is difficult to distinguish between causes and therefore is referred to as the strong solvent effect (SSE), the sample diluent effect, or another similar term. In these instances, it is essential to find versatile strategies to deal with the possible consequences.

When the sample solvent is stronger than the eluent, the solvent plug must be thoroughly mixed with the mobile phase to obtain the expected retention of the analytes in the column. If the sample solvent is too strong and the mixing is not complete at the head of the column, some of the analytes or portions of them can (partially) break through the column with the strong diluent without

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interacting with the stationary phase. This results in a loss of resolution and/or peak distortion causing irreproducible chromatographic performance. The wider the column diameter and the smaller the injection volume, the more mixing will be promoted and therefore the separation performance less affected.

Even if no strong solvent is involved, similar negative consequences may result from injection volumes that are too large for the current column characteristics. Beyond a certain level, the high analyte concentration can saturate the packing material and partially prevent further sample interactions with the stationary phase. In addition, the high volume of sample diluent, which adds dispersion, can result in peak broadening, peak distortion, and change in retention time depending on each specific scenario (column overload).

The SSE is also noteworthy when LC methods are transferred from older to modern LC instruments. The larger capillary and needle seat internal diameters (ID) in legacy instruments comprise a larger volume between the injector and the column and allow better mixing of mobile phase and sample. In addition, the column diameters used with such instruments are typically larger than with modern systems, and are usually kept when a method is transferred. With modern high-pressure liquid chromatography (HPLC) instruments, the SSE is intensified due to narrower capillaries and columns. As a result, corresponding mitigation strategies need to be employed in cases such as a compendial method transfer, which does not permit much flexibility for changes in sample composition, injection volume, or method setup. (According to USP General Chapter <621>,[4] the injection volume can be adjusted as long as the results are within the established precision, linearity, and detection limits.)

Several strategies have already been proposed to mitigate the SSE in LC systems without modifying the composition of the injected sample including at-column dilution by a weak eluent from a second pump,[5] inline mixing of the sample and diluent,[6] installation of a mixer or a large ID capillary between the injector and the column,[7] and application of custom injection programs (CIPs).[8] Inevitably, each strategy comes with advantages and disadvantages, and its suitability may depend on the specific application.

This technical note describes two of the mentioned strategies to overcome or reduce the SSE, namely the Strong Solvent Loop (SSL) and CIPs. Both approaches can deliver satisfying chromatographic results with limited effort or system adaptation. The SSE is shown and discussed at five injection levels, and the mitigation of SSE by implementing SSL and CIPs is evaluated for two column dimensions and three different sample solvents.

Experimental

Chemicals

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Scientific™ Acetonitrile, Optima™ LC/MS grade (P/N A955-212)
- Uracil ≥99.0% (T), Sigma-Aldrich™ (P/N 94220)
- 4-Nitroaniline, 99%, ACROS Organics™ (P/N AC128371000)
- Methyl benzoate, 99%, ACROS Organics™ (P/N AC126340250)
- Phenetole, 98+%, ACROS Organics™ (P/N AC221491000)
- *o*-Xylene, 99%, pure, ACROS Organics™ (P/N AC140990010)

Sample handling

- Fisher Scientific™ Fisherbrand™ Mini Vortex Mixer (P/N 14-955-152)
- Thermo Scientific™ 11 mm Amber Glass Crimp/Snap Top Vials (P/N C4011-6W)
- Thermo Scientific™ 11 mm Autosampler Snap-It Caps (P/N C4011-54B)

Instrumentation

- Thermo Scientific™ Vanquish™ Core system consisting of:
  - System Base Vanquish Core (P/N VC-S01-A-02)
  - Vanquish Quaternary Pump C (P/N VC-P20-A)
  - Vanquish Split Sampler CT (P/N VC-A12-A)
  - Vanquish Thermostatted Column Compartment C (P/N VC-C10-A-03)
  - Vanquish Diode Array Detector CG (P/N VC-D11-A)
  - Standard flow cell, path length 10 mm (13 µL, SST, P/N 6083.0510)
  - Semi-micro flow cell, path length 7 mm (2.5 µL, SST P/N 6083.0530)
  - Strong solvent loop, V = 46.2 µL (P/N 6036.2200)

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Sample preparation

The stock sample preparation was performed by mixing uracil at 1.5 mg/100 mL, *p*-nitroaniline 8 mg/100 mL, methyl benzoate 40 µL/100 mL, phenetole 150 µL/100 mL, and *o*-xylene 250 µL/100 mL in 50/50 ACN/H_2O (v/v). The resulting mixture is used for reversed-phase (RP) column performance tests including the ones employed in these analyses. To create samples in strong diluents, the mix was diluted (1/10) in acetonitrile (ACN), methanol (MeOH), dimethyl sulfoxide (DMSO), and in mobile phase (MP) as a reference point without the SSE.

Chromatographic conditions

Parameter	Value
Columns	Thermo Scientific™ Hypersil GOLD™, 150 × 3 mm, 3 µm (P/N 25003-153030) Thermo Scientific™ Hypersil GOLD™, 250 × 4.6 mm, 5 µm (P/N 25005-254630)
Mobile phase	A: Water 50% B: Acetonitrile 50%
Flow rate	0.425 mL/min with the 3 mm ID column 1.000 mL/min with the 4.6 mm ID column
Column temperature	30 °C
Autosampler temperature	6 °C
Injection volume	1, 5, 10, 20, and 30 µL
Detector settings	UV wavelength: 254 nm Data collection rate: 20 Hz Response time: 0 s
Flow cell	2.5 µL with the 3 mm ID column 13 µL with the 4.6 mm ID column

Table 2. CIP settings. "X" is a variable volume that depends on the sample injection volume. The water volume and the sample volume must sum to 100 µL. Other parameters like position offset, draw speed, and needle height can be configured in addition to the vial position and volume.

No.	Command	Parameters	Description
1	UDP_Prepare Liquid Handling	Volume=100 µL	Sets the total handling volume
2	UDP_Draw	Position=water vial, Volume=45 µL	Draws the first water plug of 45 µL from the specified vial position
3	UDP_Draw	*(Parameters not specified in CIP, so injection table properties are used)*	Draws the sample from the specified vial position and volume (1, 5, 10, 20, and 30 µL) in the injection table
4	UDP_Draw	Position=water vial, Volume=X µL	Draws the second water plug, which depends on the sample volume X = 100 µL – 45 µL sample volume
5	UDP_Wait	10 s	Move needle to injection port and wait 10 s before injection
6	UDP_PrepareInject	-	End of liquid handling

Chromatography Data System

Thermo Scientific™ Chromeleon™ 7.3 Chromatography Data System (CDS) was used for data acquisition and analysis.

Results and discussion

The strong solvent effect

The SSE at two different column diameters over five injection volume levels is first evaluated in comparison to similar effects that may be caused by column overload. The extent of these effects is displayed for the samples dissolved in MP and in ACN.

Good practice in HPLC recommends that the maximum injectable volume is to be limited by the column length and cross-sectional area.[9] Nonetheless, the maximum injection volume still allowing suitable peak shapes may vary but also be dependent on the overall method conditions. If the injection volume is too high for the specific column, the peaks may be affected as a result of overload. The injection volume is also limited by the strength of the sample diluent in comparison to the MP: for stronger diluents the volume should be decreased, whereas for weaker diluents it can be increased.

Figure 1 illustrates the change of the resulting chromatograms for a 3 mm ID column as the injection volume is increased. When the sample is diluted in MP, the peaks slightly tail with increasing injection volume, likely due to column overload. However, when the sample is in strong diluent, the first (non-retained peak) is fronting and distorted at 5 µL and above because the mixing in front of the column is not sufficient, resulting in a solvent-mismatch effect. The retained peaks start fronting at 10 µL, more severely at 20 µL, and even result in shoulders with fronting at 30 µL. As the volume of injection is increased, less adequate mixing of the strong solvent with the MP takes place, carrying some of the analytes along without allowing sufficient interaction with the stationary phase.

For the 4.6 mm ID column (Figure 2), the sample diluted in MP results in optimal peak shapes. However, for the sample dissolved in strong diluent, the unretained peak shows fronting from 5 µL upwards and is less distorted. The other peaks start fronting only from injection levels of 20 µL and above, but to a much lesser extent than what has been observed with the 3 mm ID column.

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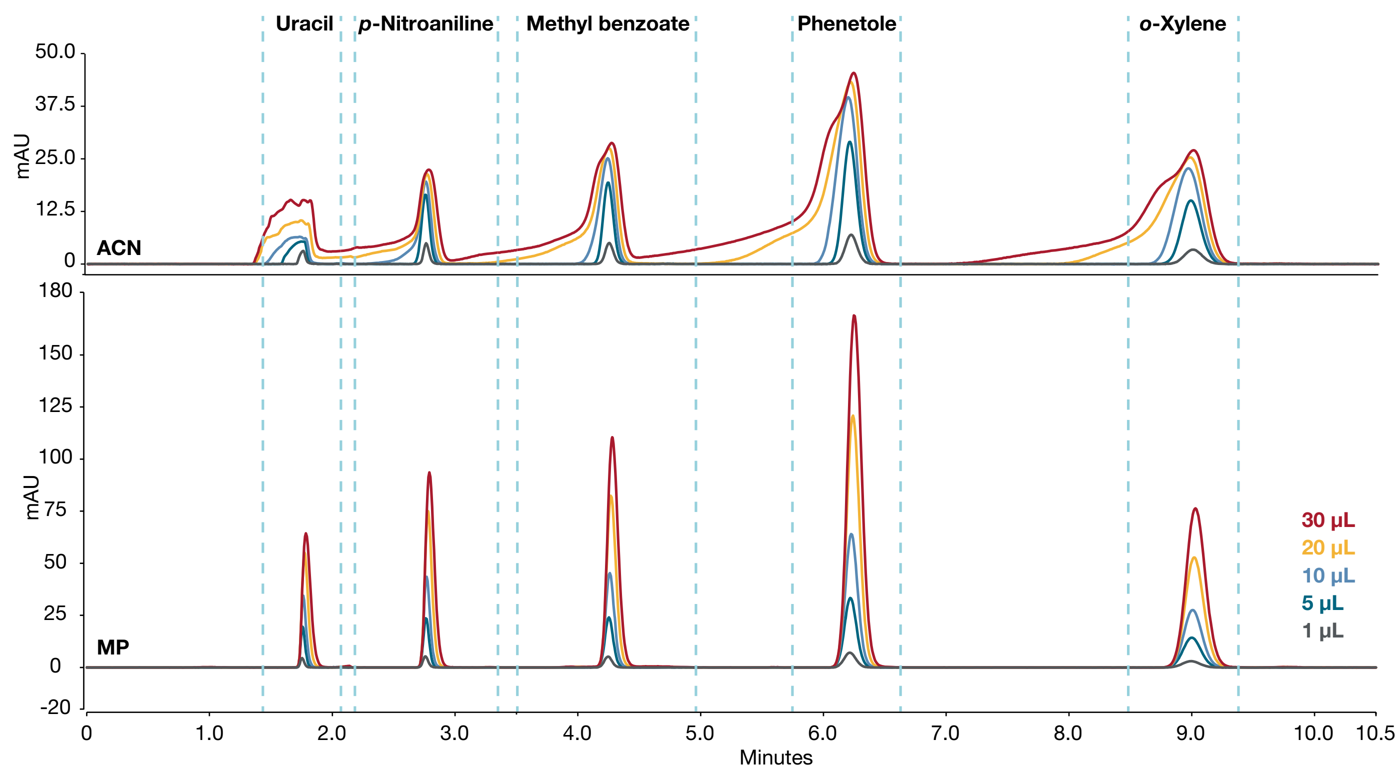

Figure 1. Overlay of chromatograms at different injection volumes (1, 5, 10, 20, and 30 µL) for the 3 mm ID column. Top: sample is dissolved in ACN; bottom: sample is dissolved in MP (50/50 ACN/water).

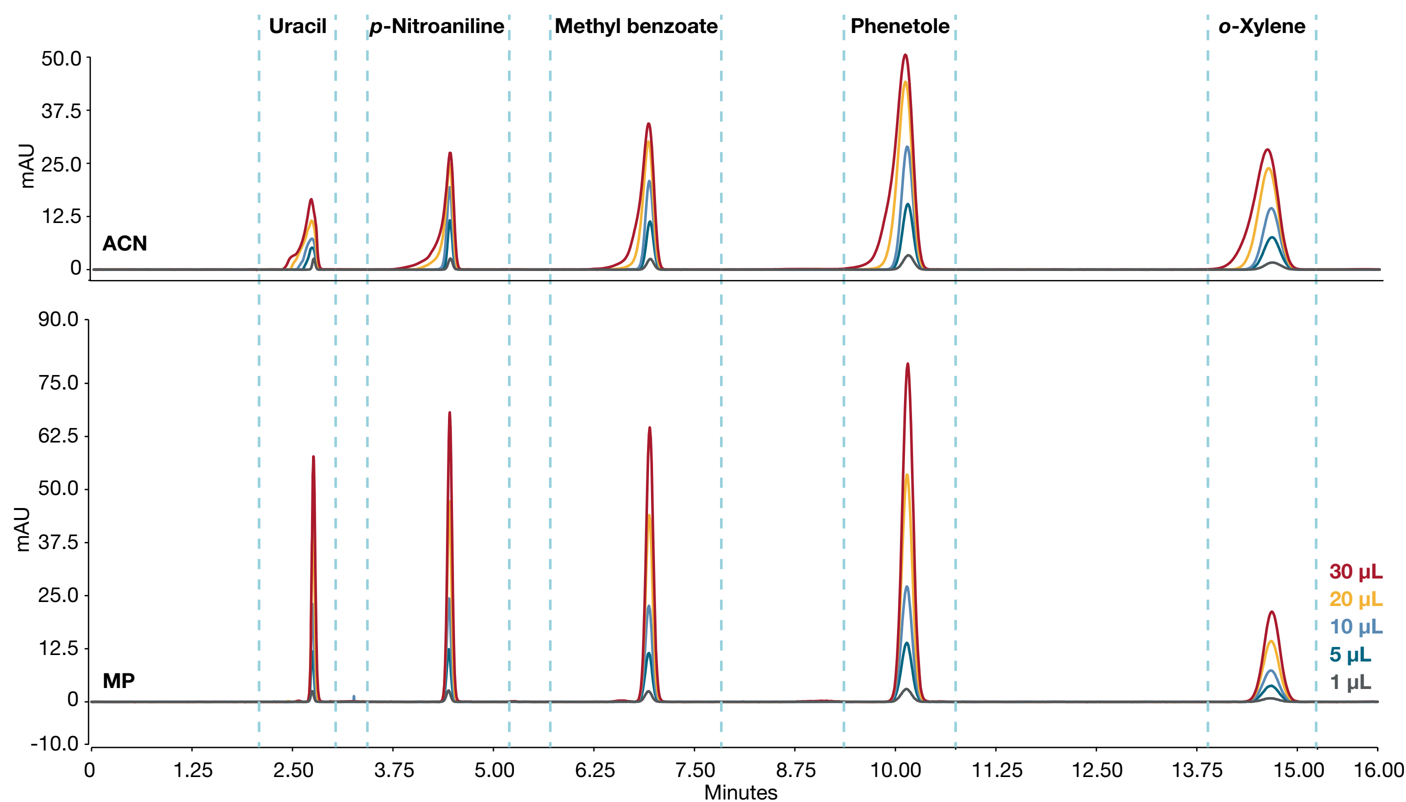

Figure 2. Overlay of chromatograms at different injection volumes (1, 5, 10, 20, and 30 µL) for the 4.6 mm I.D. column. Top: sample is dissolved in ACN; bottom: sample is dissolved in MP (50/50 ACN/water).

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In the previous examples, the effect of a strong solvent injection was shown to be less significant with increasing column dimensions. Moreover, the extent of peak distortion depends on the retention time (or retention factor) of the analyte, with a substantial difference between a non-retained and a later eluting analyte. The difference between a column overload effect and the SSE for the current conditions was also observed. The first resulted in tailing and the second in fronting, shouldering, and peak distortion. In short, for both columns the unretained peak was always affected from the 5 µL injection upwards and the rest of the peaks were affected to a different degree, which also depends on the column size.

The SSE induced by MeOH and DMSO results in similar distortion to ACN, but at different intensities (data not shown). ACN and MeOH have more similar and stronger effects than DMSO. Also, under the given conditions, DMSO results in a considerable artifact pattern that interferes with the first and sometimes the second peak in the chromatogram.

Mitigation strategies by SSL and CIPs

Custom injection programs based on simple commands called UDPs (user-defined programs), provide the user the ability to individually control the injection process. Here, it is implemented on the Vanquish Core HPLC system for the reduction of the sample solvent strength by diluting the sample with a weaker diluent in the injection loop prior to injection.[8] For this study, the program is described in Table 2. The total injection volume was 100 µL and the loop was filled as follows: 45 µL of water, followed by the sample volume (1, 5, 10, 20, or 30 µL), and the remaining volume of water to yield 100 µL total.

The second strategy involves the Strong Solvent Loop (Figure 3), which is a large ID capillary to improve the mixing of the sample plug and the mobile phase. It is installed between the sampler valve and the column inlet capillary and is also an easy way to mimic the dispersion of old systems. Both SSL and CIP were shown to significantly improve the performance of a compendial method.[10] However, the SSL may limit the applicability of UHPLC methods due to increased dispersion. The use of the SSL implies a change in the fluidic configuration, meaning that system re-qualification may be required to fulfill the guidelines of regulated laboratories.

Figure 3. Schematic of the strong solvent loop on the left and installed on the instrument on the right

Therefore, to align with regulatory guidelines, all the implications should be considered for each specific case. Either SSE mitigation strategy aims to improve mixing before the sample reaches the column. The CIP by mixing the sample with lower strength diluent in the sample loop and the SSL by facilitating mixing of the sample plug with the mobile phase by increased dispersion.

Evaluation of the mitigation extent

In Figure 4, the mitigation of SSE using CIP is shown for the 3 mm ID column. An injection volume of 10 µL was selected as representative for such a column format. SSL was also tested for this injection volume and column diameter, with modest improvements. The lower effectiveness of the SSL under these conditions is due to the extra-column dispersion contribution outweighing the beneficial effects of improved mixing (data not shown). Figure 13 shows decrease of efficiency under current conditions when the SSL was installed. In comparison with the no mitigation chromatograms, the CIP clearly provides better chromatographic results with narrower and higher peaks. Good peak shape was even obtained up to 20 µL injection volume and up to 30 µL for the last two peaks with MeOH and the last three with DMSO (data not shown). In the current chromatograms the unretained peak is distorted in all three solvents. The second peak is affected differently overall, presenting poor peak shape. Nonetheless, with the CIP the chromatogram is considerably improved, and very good peak shapes are obtained in each case except for the unretained peak.

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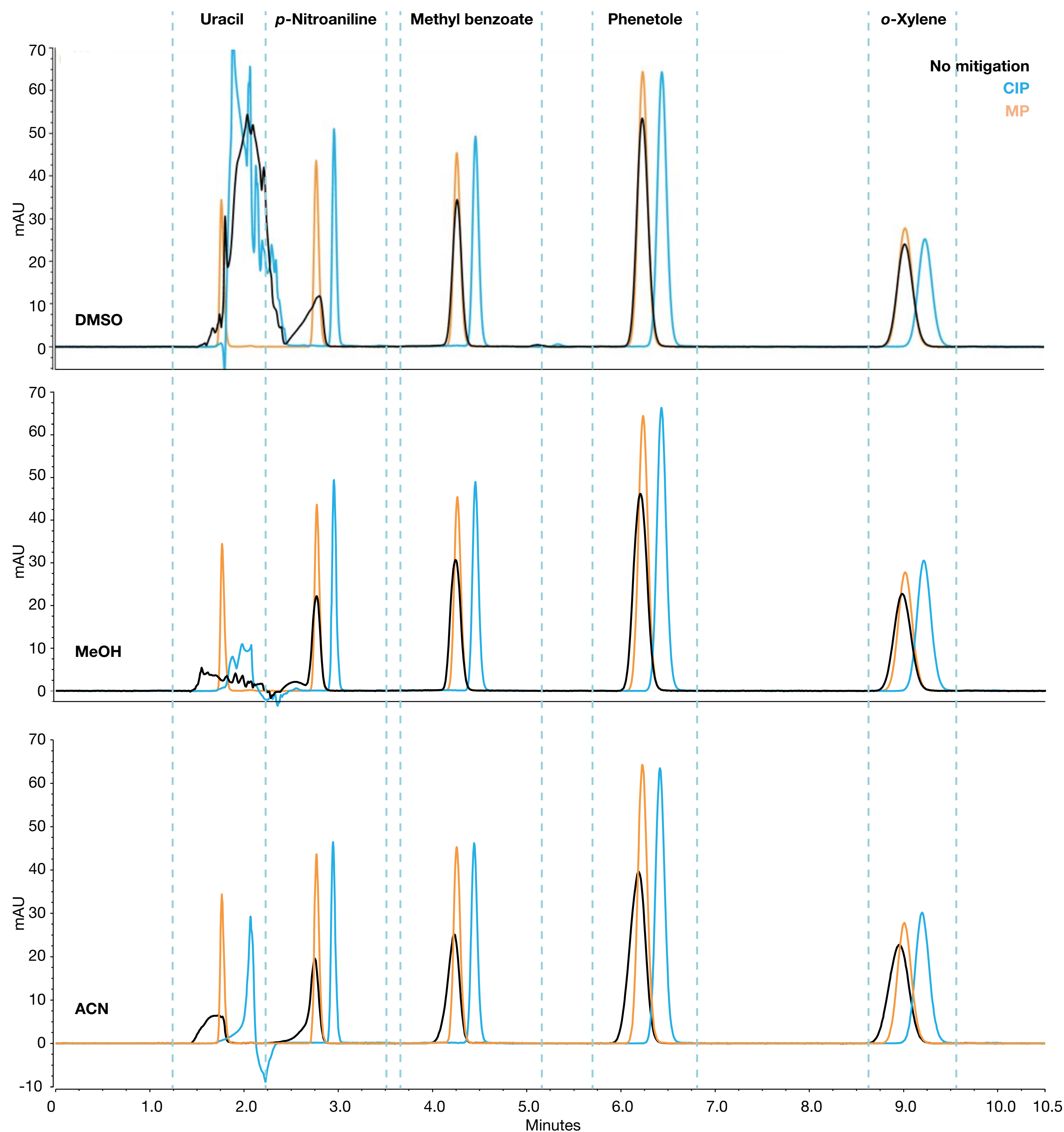

Figure 4. Overlay of chromatograms using the 3 mm ID column and 10 µL injection without any mitigation ("no mitigation" and sample dissolved in mobile phase (MP)) and with CIP for ACN, MeOH, and DMSO. The sample in MP is shown as a reference.

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An increase in the retention time (RT) when using CIP is observed compared to the results without mitigation. This outcome is due to the added water volume in the injection loop that is in front of the sample in the order of elution. For the current injection of 10 µL, another 45 µL of water was added before and after to sum to the total 100 µL as explained in Table 2. Therefore, the sample plug reaches the column head later than usual.

The peak width, asymmetry, and relative retention time (RRT) were compared across different conditions to assess the impact of the SSE. Uracil and *p*-nitroaniline were excluded from the comparison because they could not be integrated at any condition, due to poor peak shape.

The peak width results of the three late eluting peaks using the 3 mm ID column are displayed in Figure 5. The first thing to notice is that although the width varies considerably between the solvents, the CIP provides very similar peak widths in all conditions. This indicates that for this column and solvents CIP efficacy is not affected by the sample solvent. As a result, the peak width with CIP is comparable to the ideal situation where the sample is dissolved in the mobile phase. When no mitigation is applied, the severity of SSE is consistent with solvent strength. Peaks without mitigation are broader with ACN, followed by MeOH and DMSO.

In Figure 6, the asymmetry values with CIP are comparable to the values without mitigation and solvent dissolved in MP. Often, even under conditions where SSE is strong, peak symmetry is satisfactory even without CIP mitigation. Therefore, the beneficial effects of CIP are not always as obvious as for the peak width.

RRT values in Figure 7 are consistent across all conditions, meaning that there is no negative and disproportionate effect of the peak RT shift due to the CIP.

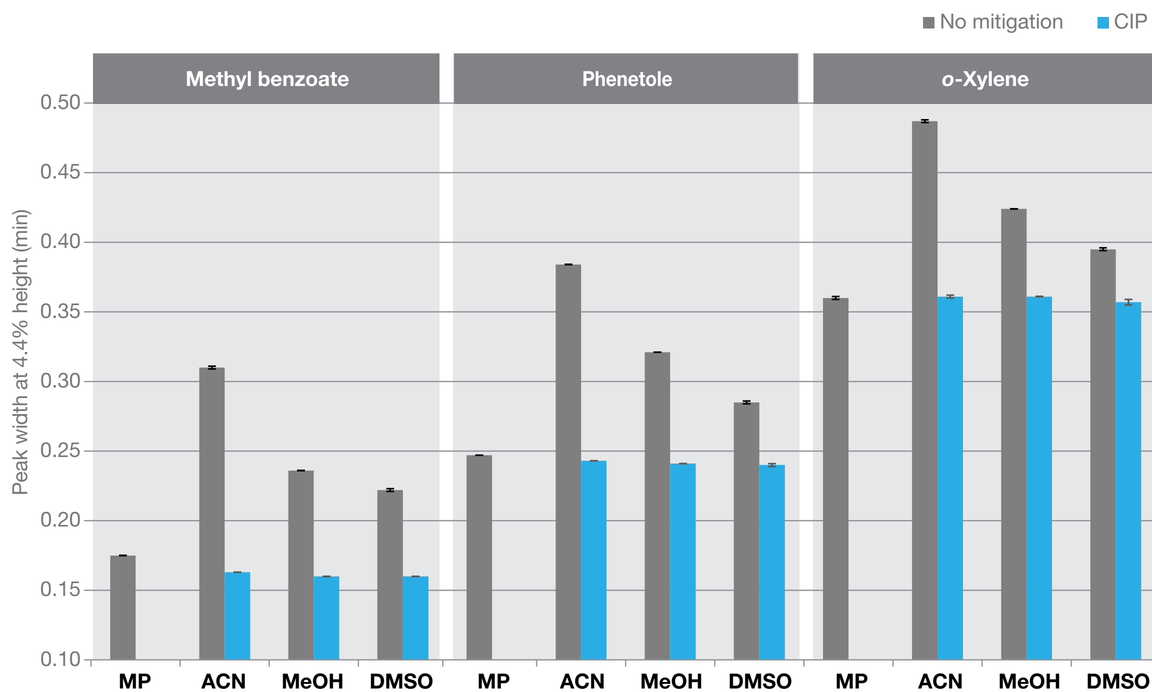

Figure 5. Peak width at 4.4% with 3 mm ID column and 10 µL injection volume with no mitigation and CIP for all sample solvents. Standard deviation is shown with the vertical error bars (N=3).

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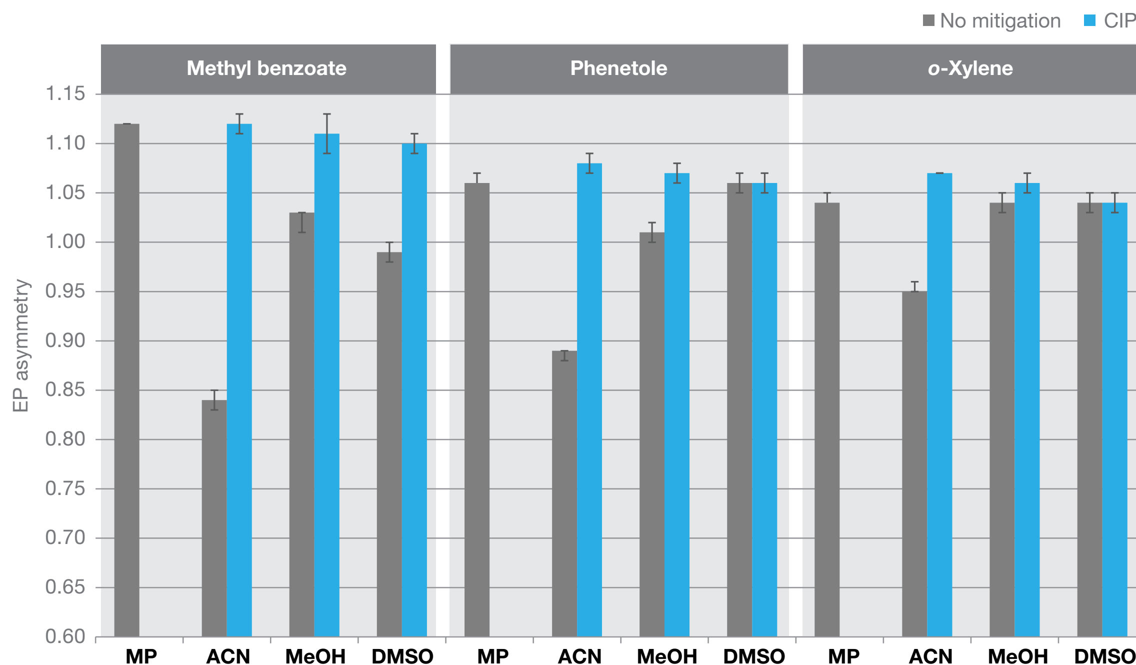

Figure 6. Peak asymmetry with 3 mm ID column and 10 µL injection volume with no mitigation and CIP for all sample solvents. Standard deviation is shown with the vertical error bars (N=3).

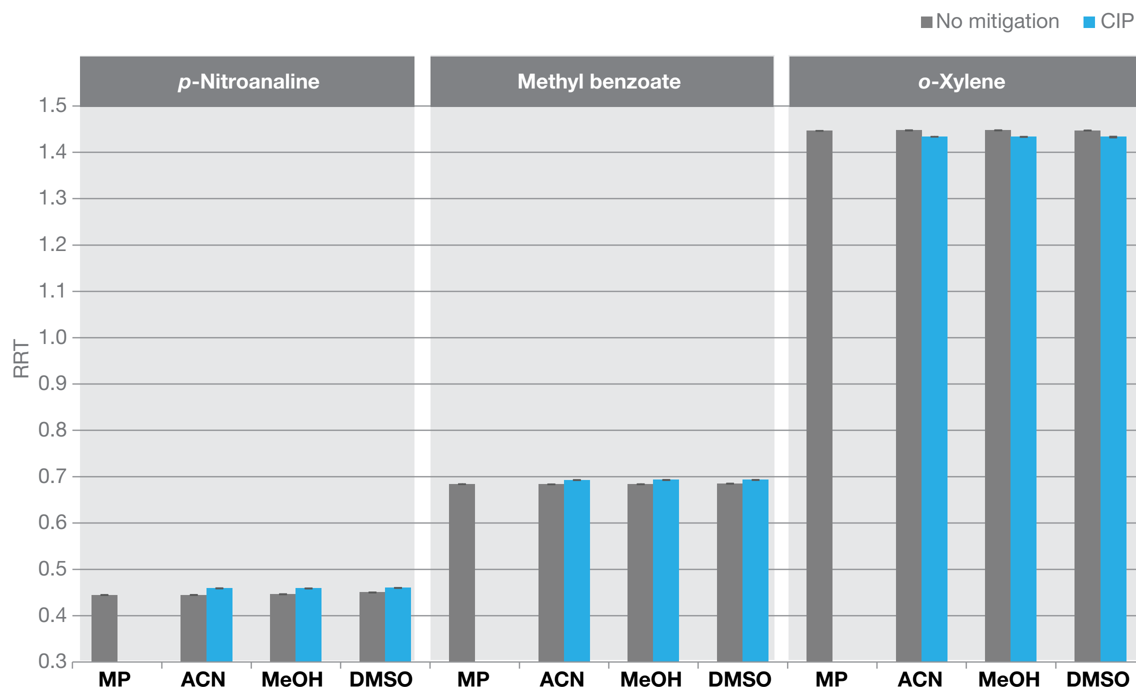

Figure 7. Retention time relative to phenetole (RRT) with 3 mm ID column and 10 µL injection volume with no mitigation and CIP for all sample solvents. Standard deviation is shown with the vertical error bars (N=3).

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The chromatograms obtained with the 4.6 mm column ID with both mitigation techniques (CIP and SSL) and without are displayed in Figure 8 for 20 µL injection volume, a typical injection volume for this column format. The unretained uracil is again not detected for the MeOH and DMSO due to the baseline artifacts, but is detected as a sharp peak for the sample dissolved in ACN when CIP is implemented. For the peak shape of *p*-nitroaniline, which is more strongly affected by SSE than the later eluting peaks, the CIP brings improvement for all conditions. Moreover, in the case that the strong solvent is ACN, the SSL is also effective and satisfactory peak shape is obtained.

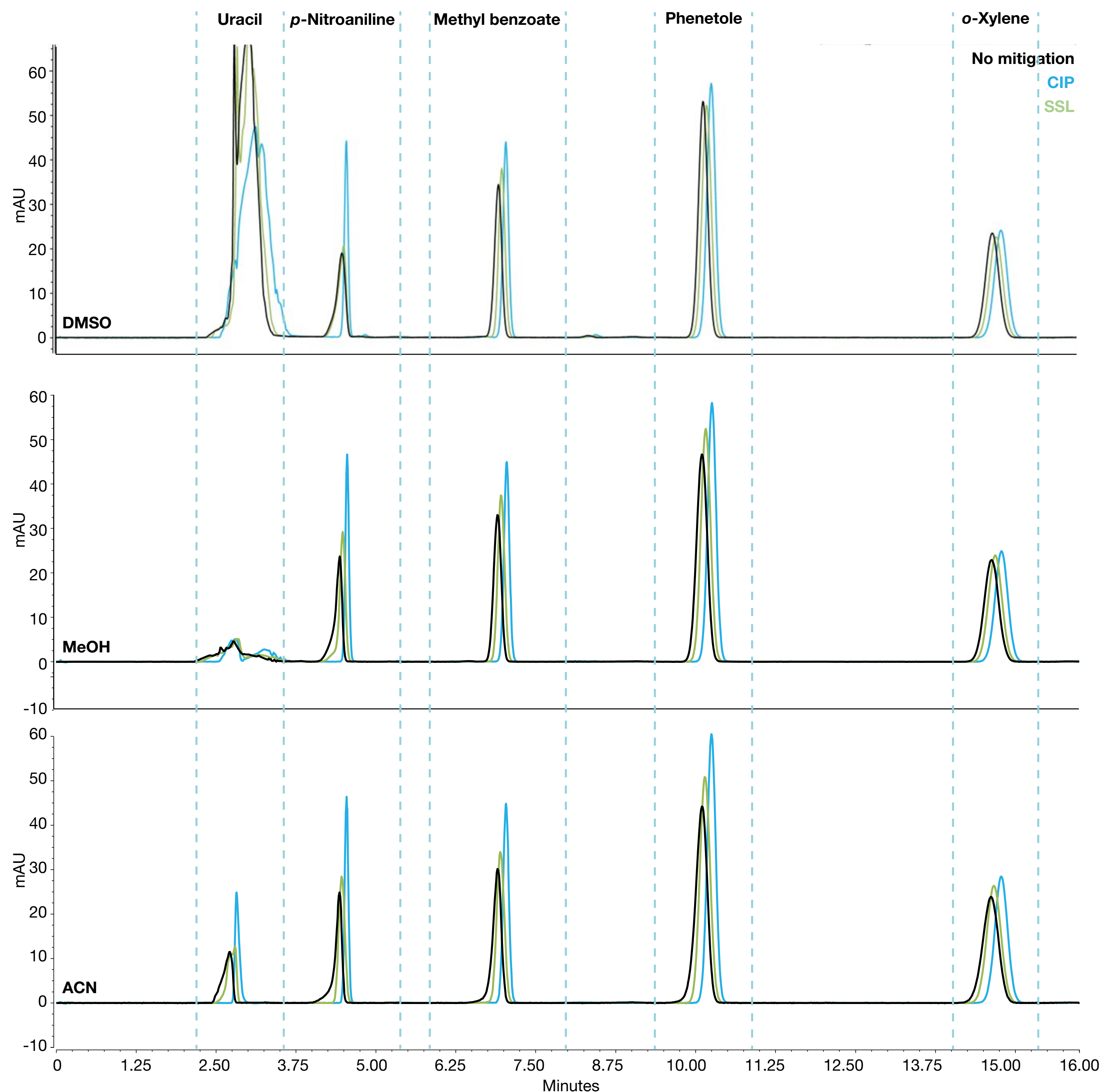

Figure 8. Overlay of chromatograms using the 4.6 mm ID column and 20 µL injection volume without any mitigation, with CIP and with SSL for all three sample solvents (ACN, MeOH, and DMSO)

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The same behavior of peak width as for the 3 mm ID column (both for no mitigation and CIP) is observed for the 4.6 mm ID column (Figure 9). However, the SSL reduces the peak width more for ACN, to a lesser extent for MeOH, and even less for DMSO. Therefore, the SSL mitigation in comparison to the CIP is less effective and more dependent on the sample solvent strength.

The CIP and "no mitigation" asymmetry patterns are similar for the 4.6 mm ID column and the 3 mm one, but even more symmetric for the 4.6 mm (Figure 10). The SSL usually performs very well and comparable to the CIP delivering good peak symmetry.

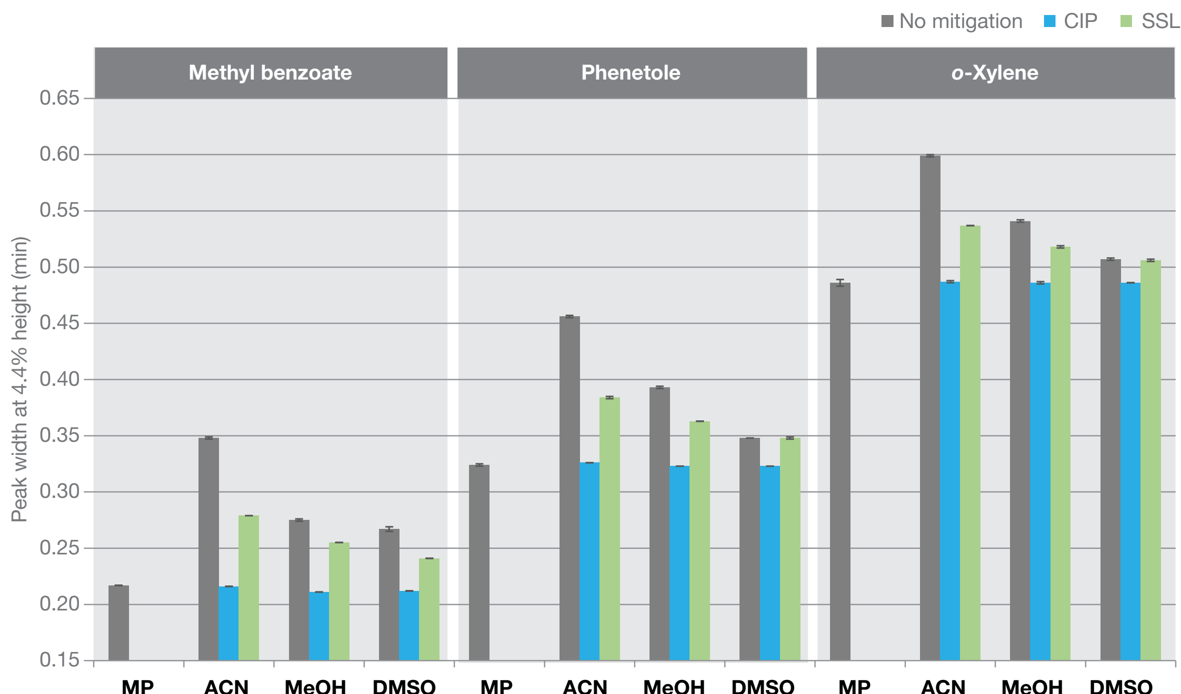

Figure 9. Peak width at 4.4% with the 4.6 mm ID column and 20 µL injection volume with no mitigation, CIP and SSL for all sample solvents. Standard deviation is shown with the vertical error bars (N=3).

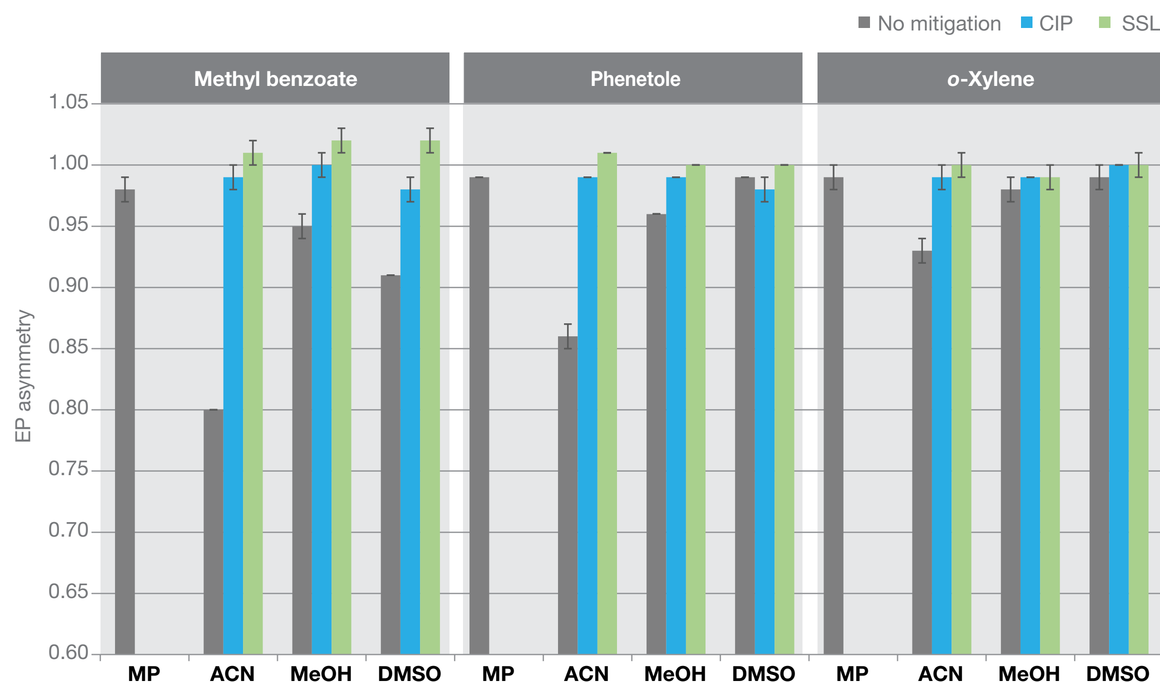

Figure 10. Peak asymmetry with the 4.6 mm ID column and 20 µL injection volume with no mitigation, CIP and SSL for all sample solvents. Standard deviation is shown with the vertical error bars (N=3).

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The relative retention time (relative to phenetole) shows again that although different absolute RT with the SSL and CIP are obtained compared to the method without mitigation, it is identical for all three solvents and methods (Figure 11).

There may be more specific or uncommon cases where one of the mitigation strategies may not be enough to obtain appropriate results. For the use of CIP at high sample volumes, when the portion of weak diluent in the injection loop is relatively low compared to the sample solvent, it may happen that the SSE is not adequately mitigated because of insufficient mixing between weak and strong solvent. In that case, employing CIP together with the SSL to enable a better dilution of the strong solvent could improve the peak shape. Figure 12 shows chromatograms for SSL, CIP, and a combination of both using a sample volume of 30 µL for the 3 mm ID column.

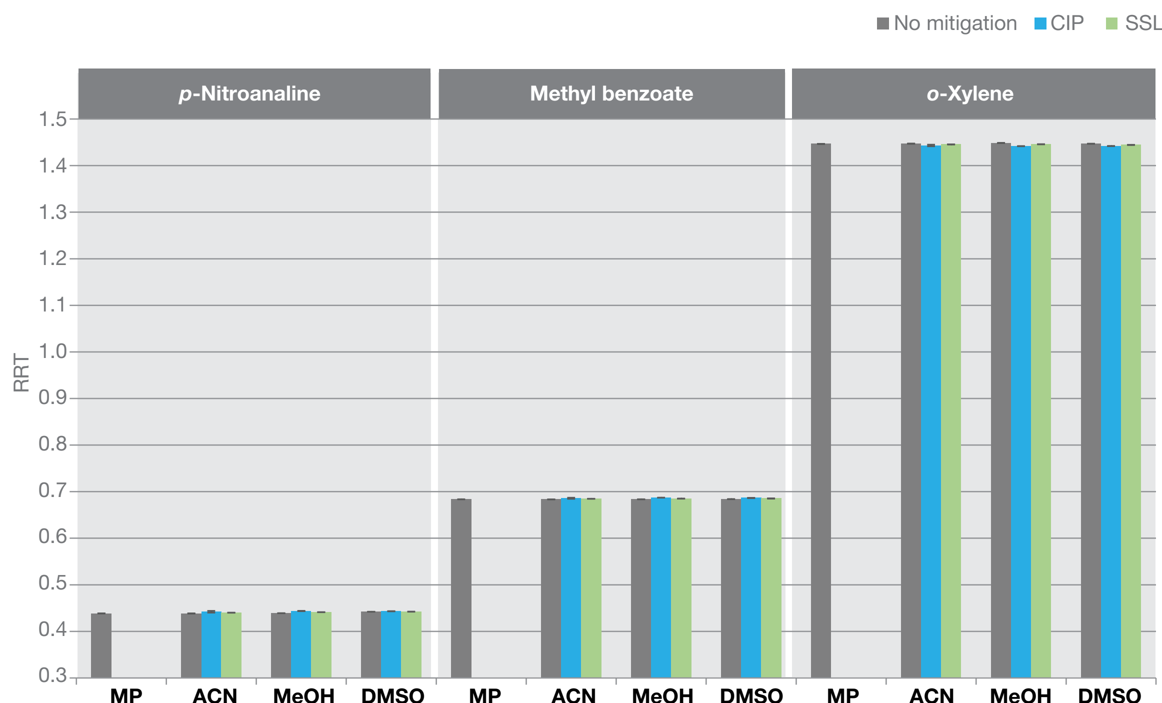

Figure 11. Retention time relative to phenetole (RRT) with the 4.6 mm ID column and 20 µL injection volume with no mitigation, CIP and SSL for all sample solvents. Standard deviation is shown with the vertical error bars (N=3).

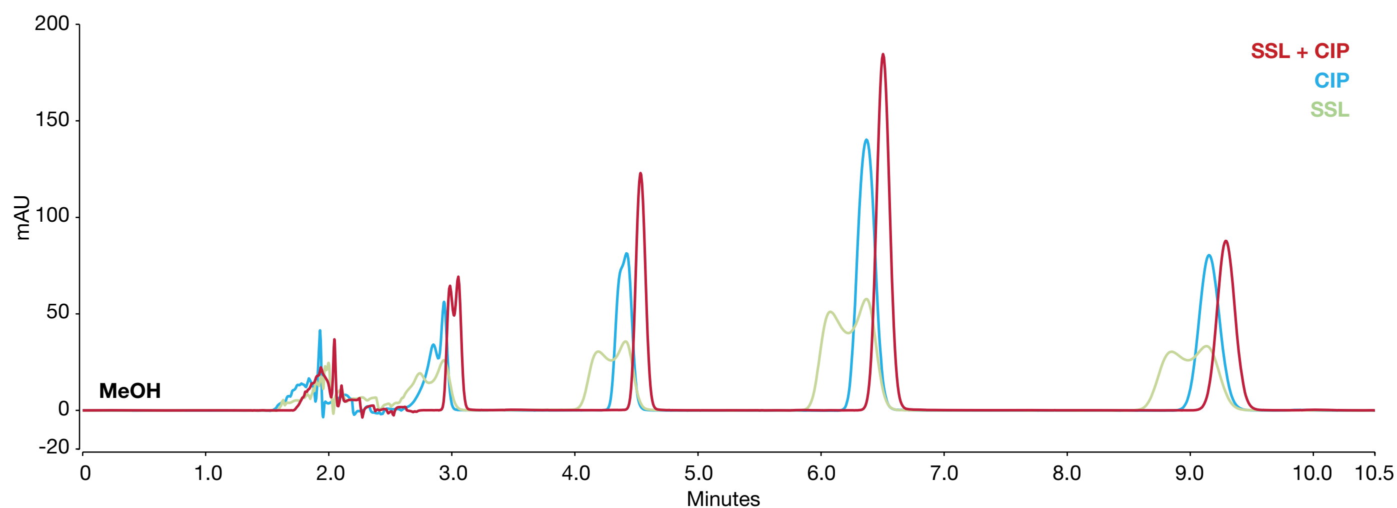

Figure 12. Overlay of chromatograms with SSL, CIP, and SSL+CIP using MeOH as a strong solvent for column 3 mm I.D and 30 µL injection

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With methanol as the sample diluent, the first peak cannot be detected. The second peak is improved but still split even if both strategies are applied in combination. The third peak has a shoulder with CIP, but with CIP and SSL it becomes narrower and higher and the shoulder disappears. The behavior of the fourth peak is the same as the third peak. In contrast, if the sample is already well diluted by CIP, and the SSL is employed in addition, the extra dispersion may negatively affect the peak width.

Finally, a comparison of the efficiency between "no mitigation" and SSL when the sample is in normal conditions (MP) and no SSE is present was carried out (Figure 13). This setup may be important to consider if another method with no SSE is run on the same instrument. Therefore, to avoid requalification of the system, some loss in the efficiency might be a worth trade off. Clearly, due to the added dispersion when using the SSL, the efficiency will decrease. As noted, it is more considerable as the column has smaller ID and less as the RT increases.

It should be taken into consideration that the SSE as well as its mitigation potential effectiveness may strongly vary from method to method. Therefore, the presented results need to be considered as a relevant, but still limited, subset of typical conditions in reversed-phase chromatography. The extent of the related effects depends on many factors, such as if the method is gradient or isocratic, the sample solvent (diluent) and its elution strength, the injection volume, the retention factor of the respective analyte, the column dimensions, and stationary phase characteristics. Refer to the already cited application notes[8,10] to evaluate their use in other methods.

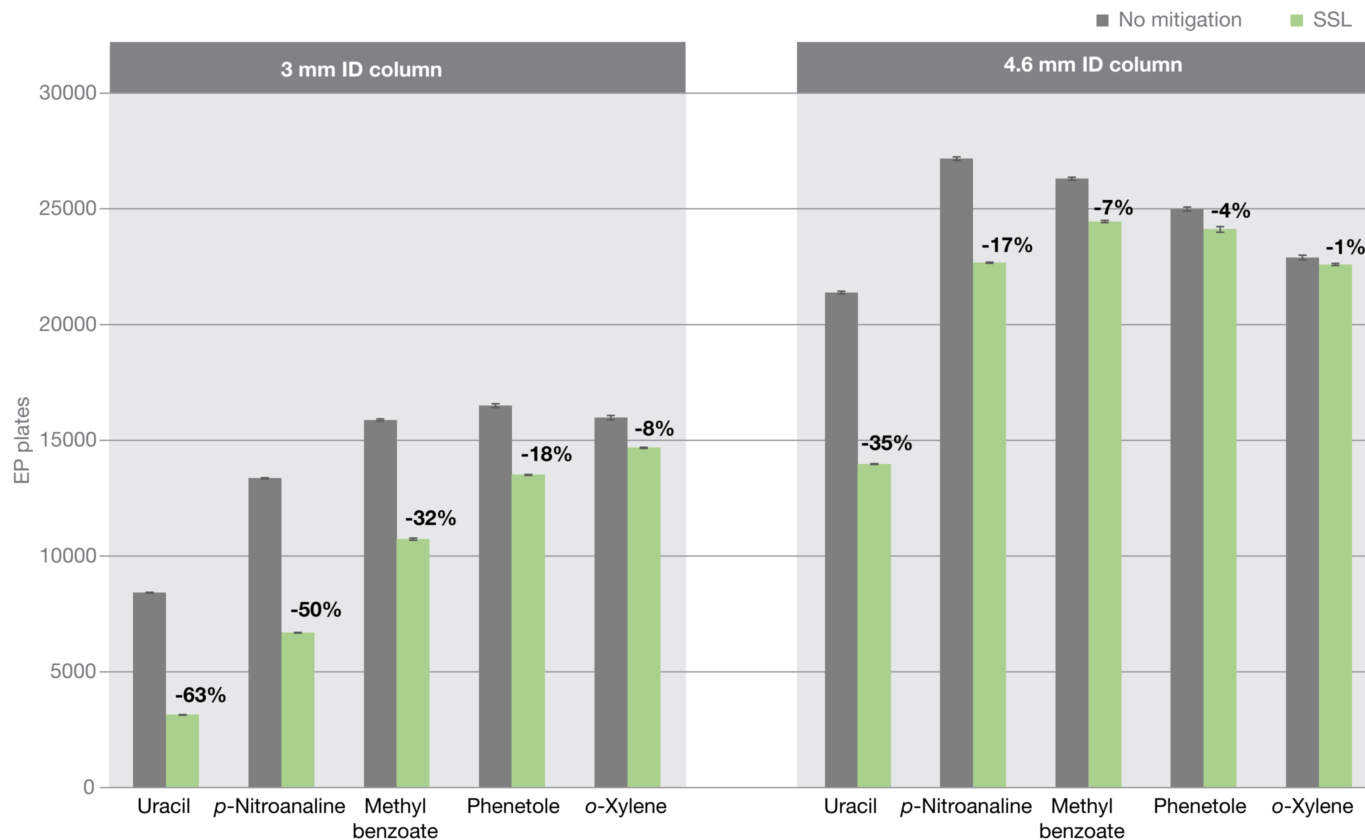

Figure 13. Comparison of the theoretical plates (N) of each peak between "no mitigation" and SSL when no SSE is present (sample in MP). For the 3 mm ID column, data of a 10 µL injection is shown and for the 4.6 mm ID column, of a 20 µL injection. The percentage refers to the decrease of plates from no mitigation to when SSL is installed. Standard deviation is shown with the vertical error bars (N=3).

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The SSE, causing peak fronting, broadening, splitting, and distortion, is more relevant and intense as the dimensions of the column decrease and the injection volume increases. There is also a difference between the solvents that are used for the sample in the present conditions, with ACN typically having a more intense effect upon the analyzed peaks, then MeOH, and finally DMSO. Due to the mismatch of the MP and diluent and/or the detectability of the impurities in the diluent, the unretained peak was covered by artifacts in most cases. It may have also been caused by the viscous fingering effect, which was beyond the scope of this study to investigate. This may be a problem depending on the method and the retention of the peak of interest because the CIP or SSL may not be able to avoid it. Usually, peaks of interest have higher retention factors and therefore this would not be an issue. Nevertheless, the CIP provided satisfactory results in every case for the second peak (less retained and closer to time zero) and even for the unretained one for the 4.6 mm ID column with ACN.

It can be concluded that the CIP is an excellent mitigation strategy of the SSE for all the considered scenarios. In comparison with the SSL it is expected to give better results because weak solvent is used, which in this case is more polar and enables focusing the analytes at the column inlet (reverse effect than the strong solvent), while the SSL only provides mixing with the mobile phase. Further optimization regarding the injection volume and diluent in the injection loop can be made depending on the scope and the conditions of the method. In addition, CIP mitigation strength was not solvent dependent in the considered peaks for the peak width and only slightly for the asymmetry. For asymmetry, more variability was found overall, presenting better results than no mitigation for the 4.6 mm ID column but not for the 3 mm where in some occasions the peaks with strong solvent were less asymmetric.

The SSL generally has lower mitigation power when compared to the CIP in the current conditions. In addition, its mitigation effect depends more on the type of sample solvent and its miscibility with the MP. For instance, it can be more clearly observed in the peak width and its capacity to fix the second peak. For the analyzed peaks, the asymmetry does not depend as much on the solvent because the values are in the same range. Nonetheless, it was shown to reduce the SSE, improving the peak width and asymmetry in various circumstances and even eliminated the fronting in the second peak for ACN, which has a small retention factor. This approach may work well when the SSE is not very strong because of the pre-requisite that the sample needs to be mixed with the MP. It also can improve the peak shape further when used in addition to CIP. Lastly, if other methods have to be run on the same instrument where no mitigation is required, the tradeoff between requalification (if needed)/loss of efficiency and SSE mitigation should be evaluated.

Conclusion

- The SSE intensifies as the dimensions of the column decrease and the injection volume increases, affecting more severely the unretained peak and distorting the rest of the peaks with shoulders, broadening, fronting, and splitting.
- The CIP is an excellent mitigation strategy of the SSE, which improves peak shape by narrowing it in the majority of the circumstances.
- The SSL improves the peaks in most of the scenarios although not as much as the CIP, and in some occasions, it can provide good peak shapes when the CIP is not sufficient by adding it in the configuration.
- The SSL is much more intuitive to use than programming a CIP. Nonetheless, the CIP is relatively easy to set up once the parameters are understood and permits many variations and setups.

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APPLICATION NOTE 73310

Transfer of a compendial LC method for impurity analysis of chlorhexidine from a Waters Alliance HPLC system to a Vanquish Core HPLC system

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Thermo Fisher Scientific, Germering, Germany

Keywords: HPLC method transfer, Vanquish Core HPLC System, Waters Alliance HPLC system, chlorhexidine, European Pharmacopoeia

Application benefits

- Seamless transfer of an EP monograph HPLC method from a Waters™ Alliance™ HPLC system to a Thermo Scientific™ Vanquish™ Core HPLC system is demonstrated.
- Equivalent chromatographic results are obtained with both systems, but improved resolution and system repeatability are provided by the Vanquish Core HPLC system.

Goal

To demonstrate the transfer of analytical HPLC methods from a Waters Alliance HPLC system to the Vanquish Core HPLC system.

Introduction

Instrument-to-instrument transfer of liquid chromatographic (LC) methods is a challenging task most analytical laboratories face frequently under several scenarios. For example, an established application needs to be run by several instruments within one lab to distribute major workload. On the other hand, inter-lab transfers are realized among method developing and method implementing

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laboratories, that is, from research and development (R&D) labs to quality control (QC) labs, or when specific tasks are outsourced, for example, to contract labs.[1] In both cases, the transferring and receiving laboratories' instruments can be either equivalent or different in vendor and configuration. A third scenario is the replacement of legacy instrumentation by modern technology. In either instance a transfer is only considered effective if equivalent results are obtained. The success and the required effort of such a transfer depend on multiple factors. The robustness of the method to be transferred as well as instrumentational deviations of the involved systems play an important role.[1] Some technical characteristics of a system, like its gradient delay volume (GDV), pump mixing mode, hydrodynamic behavior, column and eluent thermostatting options, may affect critical results like peak resolution or retention times.[2-4] The requirements of the chromatographer to the analytical outcome and the defined limits of acceptable deviations from the originating system determine the complexity of the transfer job. In addition, only very limited modifications of method parameters are usually accepted during a transfer to prevent the need of a time-consuming revalidation.

In the following, the HPLC method for impurity analysis of chlorhexidine digluconate given by the European Pharmacopoeia (EP) monograph[5] is transferred from a Waters Alliance HPLC system to a Thermo Scientific Vanquish Core HPLC system. Chlorhexidine is a common antiseptic and disinfectant, listed on the World Health Organization's (WHO) Model List of Essential Medicines.[6] It is available as an over-the-counter drug and is widely used in dental medicine and hygiene, for example, in mouthwashes and for skin disinfection purposes.

The selected Thermo Scientific™ Hypersil™ GOLD column complies well with the requirement for an end-capped C18 silica column of the monograph. Although we adhered to the EP monograph, the following discussions in general are also valid for the United States Pharmacopoeia (USP) method,[7] as the analytical method, i.e. column and gradient, are identical.

Experimental

Reagents and materials

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Scientific™ Optima™ Acetonitrile, LC/MS grade (P/N A955-212
- Thermo Scientific™ Pierce™ Trifluoracetic acid (TFA), LC-MS grade (P/N 85183)
- EP reference standard: Chlorhexidine for system suitability CRS batch 2, catalogue code Y0001545[8]

Sample preparation

According to the monograph, 5 mg of the reference standard, which contained chlorhexidine and various impurities, were dissolved in 1 mL of mobile phase A (see below).

Instrumentation and HPLC conditions

The instruments and the HPLC conditions used in this study are listed in Tables 1 and 2.

Table 1. Instruments

	Alliance Quaternary	Vanquish Core Quaternary
System base		System Base Vanquish Core (P/N VC-S01-A-02)
Solvent storage	Bottle Tray Kit	Solvent Rack (P/N 6036.1350)
Pump		Quaternary Pump C (P/N VC-P20-A-01)
Sampler	Separation Module 2695 (no mobile phase pre-heater)	Split Sampler CT (P/N VC-A12-A-02)
Column compartment		Column Compartment C (P/N VC-C10-A-03) (passive pre-heater P/N 6732.0170 included in System Base ship kit)
Detector	Photodiode Array Detector 2996	Diode Array Detector CG (P/N VC-D11-A-01)
Flow cell	Standard (10 mm)	Standard (10 mm, 13 µL, P/N 6083.0510)
System accessory		Method Transfer Kit Vanquish (P/N 6036.2100)

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Table 2. HPLC conditions

Parameter	Value
Column	Hypersil GOLD, 4.6 x 250 mm, 5 µm, 175 Å (P/N 25005-254630)
Mobile phase	A: 0.1% TFA in water/acetonitrile (80/20; v/v) B: 0.1% TFA in water/acetonitrile (10/90; v/v)
Flow rate	1 mL/min*
Gradient	0 min – 0% B, 2 min – 0% B, 32 min – 20% B, 37 min – 20% B, 47 min – 30% B, 54 min – 30% B, 55 min – 0% B, 62 min – 0% B
Column temperature	30 °C (forced air)
Autosampler temperature	8 °C
Detection	
Wavelength	254 nm
Bandwidth	6 nm
Data collection rate	5 Hz
Filter response/ response time	1 s
Injection volume	10 µL
Needle wash	Off

*Alliance: Stroke volume = 50 µL, as recommended for flow rates of 0.531–1.230 mL/min

Data processing and software

Thermo Scientific™ Chromeleon™ Chromatography Data System Software, version 7.3, was used for data acquisition and analysis.

Results and discussion

For best comparability, the following experiments were conducted with the same column, aliquots of the same sample, and the same mobile phase batch to exclude non-instrumental effects on the transfer. Seven consecutive injections were executed with each system. Figure 1 displays the comparison of both instruments under conditions outlined in the EP monograph. The chromatogram is populated over the complete run time with peaks of chlorhexidine, specified impurities, and unknowns not specified in the SST standard leaflet.[9] For reasons of clarity, the focus is on all peaks that exceeded a minimum peak area of 0.3 mAU·min in the following.

Similar chromatograms were generated by the Alliance and Vanquish Core HPLC instruments. For all peaks the absolute retention times differed less than 4% between the systems. A summary of relative retention times, experimentally obtained and provided by the EP monograph, is given in Table 3. Both instruments are in very good accordance with each other and well aligned with the EP objectives. Good agreement on relative peak areas related to the main peak is seen in Figure 2. However, the resolution values obtained with the Vanquish Core HPLC system are usually higher than those obtained with the Alliance system; this is mainly due to narrower peaks thanks to a lower system dispersion volume of the Vanquish Core HPLC system.

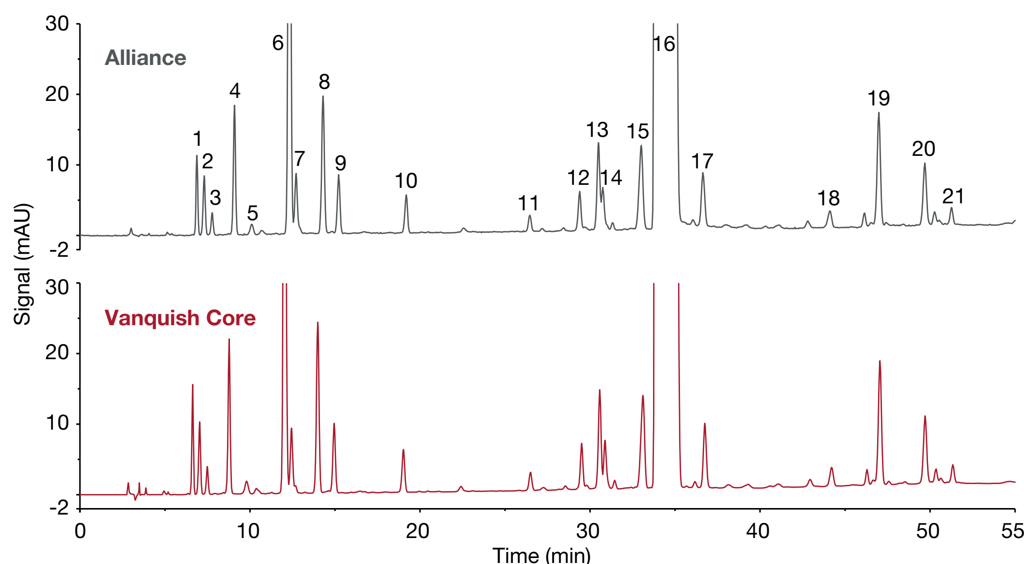

Figure 1. Transfer from Alliance system to Vanquish Core HPLC system according to the EP monograph for chlorhexidine gluconate; peak assignment according to impurity designation in EP monograph and standard leaflet[5, 9]

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Table 3. Relative retention times related to the main peak as stated in the EP monograph and averaged from Alliance and Vanquish Core chromatograms (Figure 1)

Peak #	Compound	EP monograph	Alliance	Vanquish Core
1	Unknown 1		0.20	0.20
2	Impurity L	0.23	0.22	0.21
3	Impurity Q	0.24	0.23	0.22
4	Impurity G	0.25	0.27	0.26
5	Unknown 2		0.30	0.29
6	Impurity N	0.35	0.36	0.36
7	Impurity B	0.36	0.38	0.37
8	Impurity F	0.50	0.42	0.41
9	Unknown 3		0.45	0.44
10	Impurity A	0.60	0.57	0.56
11	Unknown 4		0.78	0.78
12	Impurity H	0.85	0.87	0.87
13	Impurity O	0.90	0.90	0.90
14	Impurity I	0.91	0.91	0.91
15	Impurity J	0.96	0.97	0.98
16	Chlorhexidine	1.00	1.00	1.00
17	Unknown 5		1.08	1.08
18	Unknown 6		1.30	1.31
19	Impurity K	1.40	1.39	1.39
20	Unknown 7		1.47	1.47
21	Unknown 8		1.51	1.52

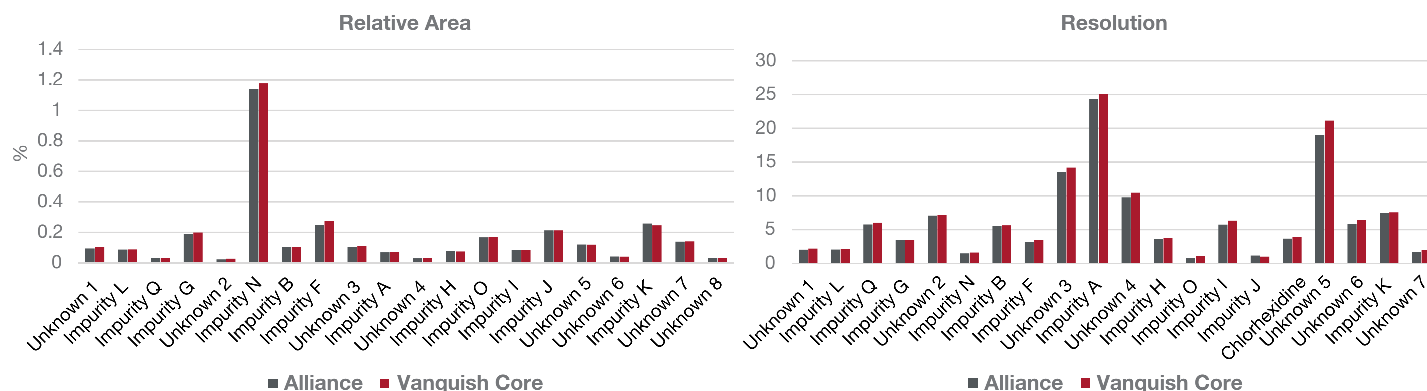

Figure 2. Chromatographic results with Alliance and Vanquish Core HPLC systems under conditions outlined in the EP monograph (Figure 1)

In addition, the repeatability of retention times and peak areas, expressed as relative standard deviations (RSD) over the seven injections, was massively improved by the Vanquish Core HPLC system as displayed in Figure 3. The RSDs of retention times for all peaks were 0.04% or lower with the Vanquish Core HPLC system, while they ranged up to 0.15% with the Alliance system. Area RSDs were 0.5% or lower for the Vanquish Core HPLC system and ranged between 0.6% and 2.8% for the Alliance. The effect is also visible in Figure 4.

The system suitability criteria given by the EP monograph, which requires a resolution of the impurity pair L and G of minimum 3 and a peak-to-valley ratio of impurity B of minimum 2, were easily met by either LC system with a resolution ~8 and a peak-to-valley ratio >5 (Alliance) and >7 (Vanquish Core). Thus, the EP method was successfully repeated with both systems without any method or hardware adaptions.

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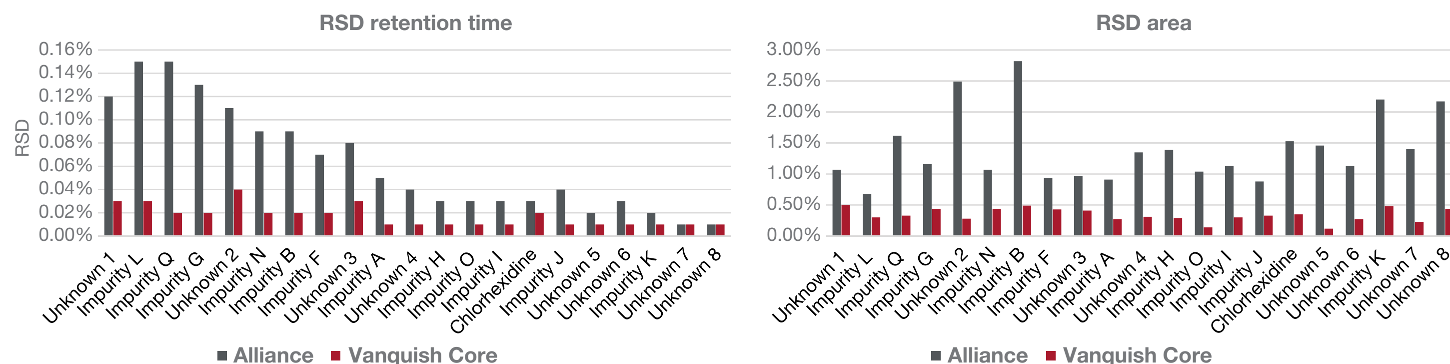

Figure 3. Relative standard deviations (RSD) of retention times and peak areas over seven injections obtained by the Alliance and Vanquish Core HPLC systems

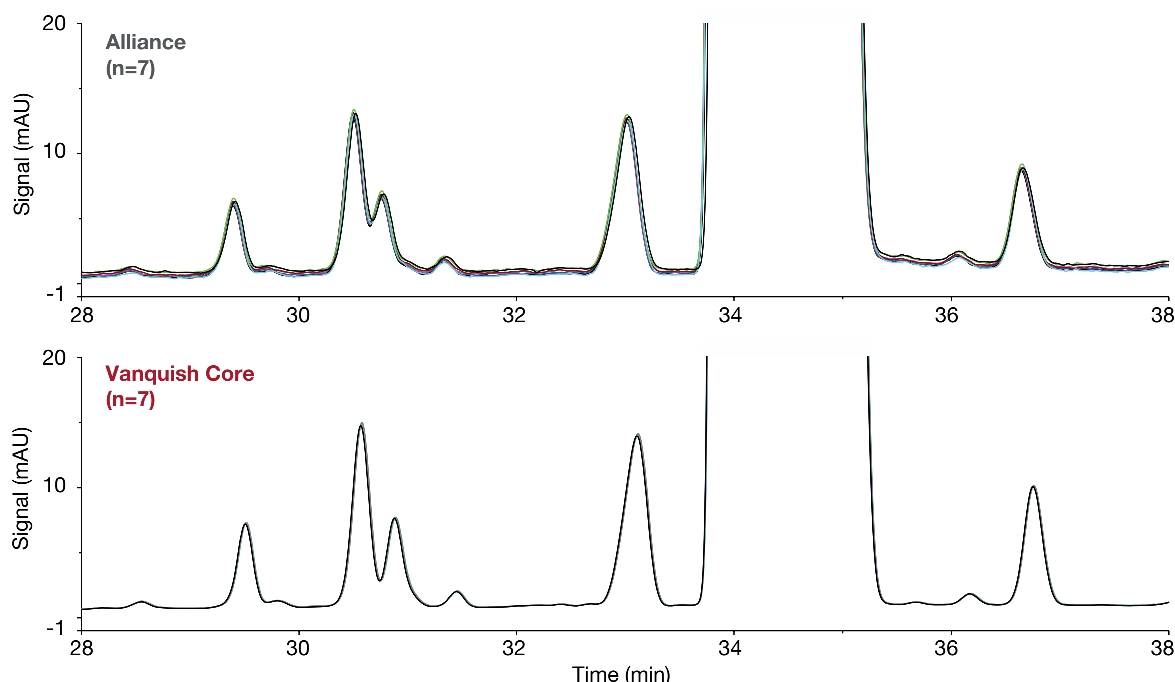

Figure 4. Overlay of seven injections by the Alliance and Vanquish Core HPLC systems, respectively, zoomed to a time range around the main peak, highlighting the improved analytical precision of the Vanquish Core HPLC system

Conclusion

- The straightforward transfer from a Waters Alliance HPLC system to a Thermo Scientific Vanquish Core HPLC system was demonstrated for the EP method for chlorhexidine impurity analysis.
- Equivalent chromatographic outcomes were provided by the two systems. However, improved peak resolution and system repeatability was provided by the Vanquish Core HPLC system.

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APPLICATION NOTE 73309

Straightforward transfer of an EP method for impurity analysis of chlorhexidine from an Agilent 1260 Infinity LC system to a Vanquish Core HPLC system

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Thermo Fisher Scientific, Germering, Germany

Keywords: HPLC method transfer, Vanquish Core HPLC System, Agilent 1260 Infinity LC system, chlorhexidine, European Pharmacopoeia

Application benefits

- Easy transfer of an EP monograph HPLC method from an Agilent® 1260 Infinity LC system to a Thermo Scientific™ Vanquish™ Core HPLC system is demonstrated.
- Enhanced hardware features of the Vanquish Core HPLC system enable flexible adjustments of the overall system gradient delay volume, which facilitates fine-tuning during the transfer.
- Equivalent chromatographic results are obtained with the originating and receiving instrument.

Goal

To showcase the transfer of analytical HPLC methods from an Agilent 1260 Infinity LC system to the Vanquish Core HPLC system and highlight the easy-to-use gradient delay volume (GDV) features of the Vanquish Core HPLC system.

Introduction

Instrument-to-instrument transfer of liquid chromatographic (LC) methods is a challenging task most analytical laboratories face frequently under several scenarios. For example, an established application needs to be run by several instruments within one lab to distribute the major workload. On the other hand, inter-lab transfers are realized among method developing and method implementing laboratories, that is, from research and development (R&D) labs to quality control (QC) labs, or when specific

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tasks are outsourced, for example, to contract labs.[1] In both cases, the transferring and receiving laboratories' instruments can be either equivalent or different in vendor and configuration. A third scenario is the replacement of legacy instrumentation by modern technology. In any instance a transfer is only considered effective if equivalent results are obtained. The success and the required effort of such a transfer depend on multiple factors. The robustness of the method to be transferred as well as instrumentational deviations of the involved systems play an important role.[1] Some technical characteristics of a system, like its gradient delay volume (GDV), pump mixing mode, hydrodynamic behavior, column and eluent thermostatting options, may affect critical results like peak resolution or retention times.[2-4] The requirements of the chromatographer to the analytical outcome and the defined limits of acceptable deviations from the originating system determine the complexity of the transfer job. In addition, only very limited modifications of method parameters are usually accepted during a transfer to prevent the need for a time-consuming revalidation. Thus, compliant hardware features are the preferred tools to assist in transferring LC methods, for example, the adaptable GDV options provided by the Vanquish Core HPLC system.

In the following, the HPLC method for impurity analysis of chlorhexidine digluconate given by the European Pharmacopoeia (EP) monograph[5] is transferred from an Agilent 1260 Infinity LC system (1260 Infinity) to a Thermo Scientific Vanquish Core HPLC system. Chlorhexidine is a common antiseptic and disinfectant, listed on the World Health Organization's (WHO) Model List of Essential Medicines.[6] It is available as an over-the-counter drug and is widely used in dental medicine and hygiene, for example, in mouthwashes and for skin disinfection purposes.

The selected Thermo Scientific™ Hypersil™ GOLD column complies well with the requirement for an end-capped C18 silica column of the monograph. Although we adhered to the EP monograph, the following discussions in general are also valid for the United States Pharmacopoeia (USP) method,[7] as the analytical method, i.e. column and gradient, are identical.

Experimental

Reagents and materials

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Scientific™ Optima™ Acetonitrile, LC/MS grade (P/N A955-212)
- Thermo Scientific™ Pierce™ Trifluoracetic acid (TFA), LC/MS grade (P/N 85183)
- EP reference standard: Chlorhexidine for system suitability CRS batch 2, catalogue code Y0001545[8]

Sample preparation

According to the monograph, 5 mg of the reference standard, which contained chlorhexidine and various impurities, were dissolved in 1 mL of mobile phase A (see below).

Instrumentation and HPLC conditions

The instruments and the HPLC conditions used in this study are listed in Tables 1 and 2.

Data processing and software

Thermo Scientific™ Chromeleon™ Chromatography Data System software, version 7.3, was used for data acquisition and analysis.

Table 1. Instruments

	Agilent 1260 Infinity Quaternary	Vanquish Core Quaternary
System base		System Base Vanquish Core (P/N VC-S01-A-02)
Solvent storage	Solvent Cabinet (5065-9981)	Solvent Rack (P/N 6036.1350)
Pump	Quaternary Pump (G1311B)	Quaternary Pump C (P/N VC-P20-A-01)
Sampler	High Performance Autosampler (G1367E) with Autosampler Thermostat (G1330B)	Split Sampler CT (P/N VC-A12-A-02)
Column compartment	Thermostatted Column Compartment with 6 µL heat exchanger (G1316A)	Column Compartment C (P/N VC-C10-A-03) (passive pre-heater P/N 6732.0170 included in System Base ship kit)
Detector	Variable Wavelength Detector (G1314F)	Variable Wavelength Detector C (P/N VC-D40-A-01)
Flow cell	Standard (10 mm, 14 µL (G1314-60186)	Standard (10 mm, 11 µL, P/N 6077.0250)
System accessory		Method Transfer Kit Vanquish (P/N 6036.2100)

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Table 2. HPLC conditions

Parameter	Value
Column	Hypersil GOLD, 4.6 x 250 mm, 5 µm, 175 Å (P/N 25005-254630)
Mobile phase	A: 0.1% TFA in water/acetonitrile (80/20; v/v) B: 0.1% TFA in water/acetonitrile (10/90; v/v)
Flow rate	1 mL/min
Gradient	0 min – 0% B, 2 min – 0% B, 32 min – 20% B, 37 min – 20% B, 47 min – 30% B, 54 min – 30% B, 55 min – 0% B, 62 min – 0% B
Column temperature	30 °C (still air)
Autosampler temp.	8 °C
Detection	254 nm, 5 Hz, response time 2 s (1260 Infinity) / 1 s (Vanquish Core)
Injection volume	10 µL
Needle wash	Off

Results and discussion

For best comparability, the following experiments were conducted with the same column, aliquots of the same sample, and the same mobile phase batch to exclude non-instrumental effects on the transfer. Seven consecutive injections were executed with each system. Figure 1 displays the comparison of both instruments under conditions outlined in the EP monograph. The chromatogram is populated over the complete run time with peaks of the main compound, specified impurities, and unknowns not specified in the SST standard leaflet.[9] For reasons of clarity, the focus is on all peaks that exceeded a minimum peak area of 0.3 mAU·min in the following.

Very similar chromatograms were generated by the 1260 Infinity and Vanquish Core instruments, implying a very similar chromatographic performance, as can also be seen in Figure 2 and Table 3. A summary of

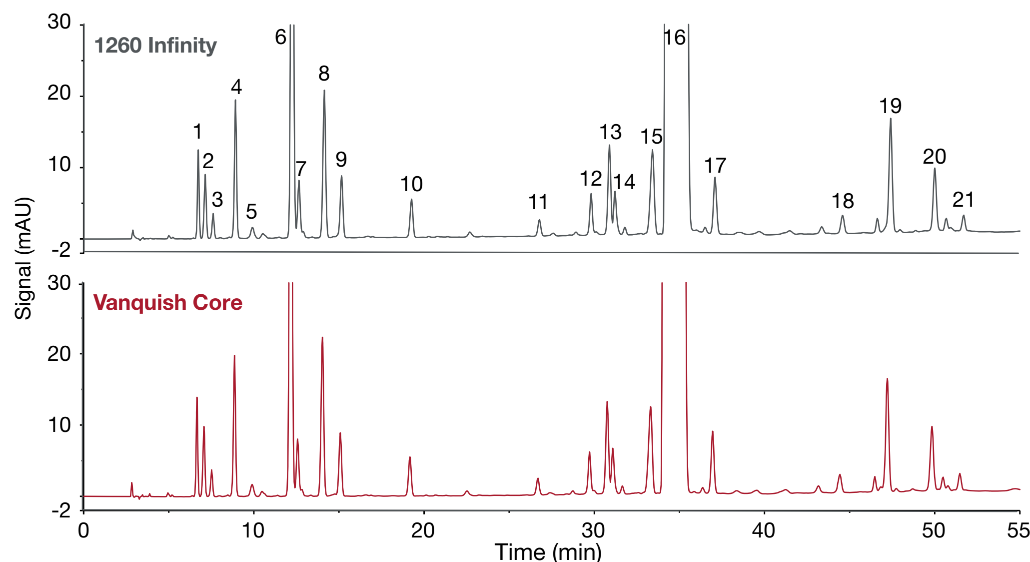

Figure 1. Transfer from 1260 Infinity system to Vanquish Core HPLC system according to the EP monograph for chlorhexidine gluconate; peak assignment according to impurity designation in EP monograph and standard leaflet[5, 9]

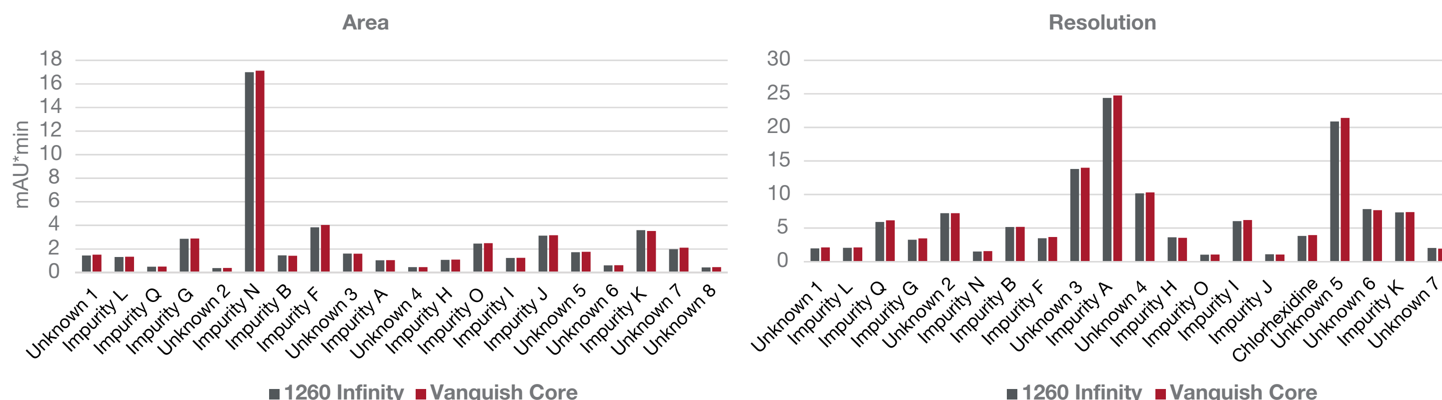

Figure 2. Chromatographic results with the 1260 Infinity and Vanquish Core HPLC systems under conditions outlined in the EP monograph (Figure 1)

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relative retention times, experimentally obtained and provided by the EP monograph, is given in Table 3. Both instruments are in excellent accordance with each other and well aligned with the EP objectives. In Figure 2 a full congruence in peak areas is displayed. Peak resolutions are in very good alignment as well, with slightly improved resolutions with the Vanquish Core HPLC system thanks to slightly narrower peaks. The retention time and peak area precisions obtained with the Vanquish Core HPLC system were excellent with a relative standard deviation of ≤0.02% for retention times and ≤0.6% for peak areas.

The system suitability criteria given by the EP monograph, claiming a resolution of the impurity pair L and G of minimum 3 and a peak-to-valley ratio of impurity B of minimum 2, are easily met by either LC system with a resolution ~8 and a peak-to-valley ratio ~6. Thus, the chlorhexidine impurity LC method was successfully repeated with both systems, giving equivalent results, and its transfer could be rated as straightforward and very successful.

However despite the perfect fit of *relative* retention times, in a direct overlay of both chromatograms, one can observe small deviations in the *absolute* retention times with all peaks eluting slightly earlier on the Vanquish Core HPLC system (Figure 3 top). These may be the results of a slightly smaller default GDV of the Vanquish Core HPLC system compared to the 1260 Infinity. The GDV of an LC system is defined as the volume between the point of mobile phase mixing in the pump and the column head. If a closer match of absolute retention times in gradient LC methods is required, for example, to meet prescribed acceptance limits, the deviations can be compensated by a tuning of the GDV of the Vanquish Core HPLC system by two different means.

Table 3. Averaged relative retention times related to the main peak as stated in the EP monograph and from 1260 Infinity and Vanquish Core chromatograms (Figure 1, default settings)

Peak #	Compound	EP monograph	1260 Infinity	Vanquish Core
1	Unknown 1		0.196	0.195
2	Impurity L	0.23	0.208	0.207
3	Impurity Q	0.24	0.222	0.220
4	Impurity G	0.25	0.260	0.260
5	Unknown 2		0.289	0.290
6	Impurity N	0.35	0.357	0.356
7	Impurity B	0.36	0.369	0.368
8	Impurity F	0.50	0.412	0.411
9	Unknown 3		0.442	0.442
10	Impurity A	0.60	0.562	0.562
11	Unknown 4		0.781	0.782
12	Impurity H	0.85	0.870	0.871
13	Impurity O	0.90	0.901	0.901
14	Impurity I	0.91	0.911	0.911
15	Impurity J	0.96	0.975	0.976
16	Chlorhexidine	1.00	1.000	1.000
17	Unknown 5		1.083	1.083
18	Unknown 6		1.301	1.302
19	Impurity K	1.40	1.383	1.383
20	Unknown 7		1.459	1.460
21	Unknown 8		1.508	1.508

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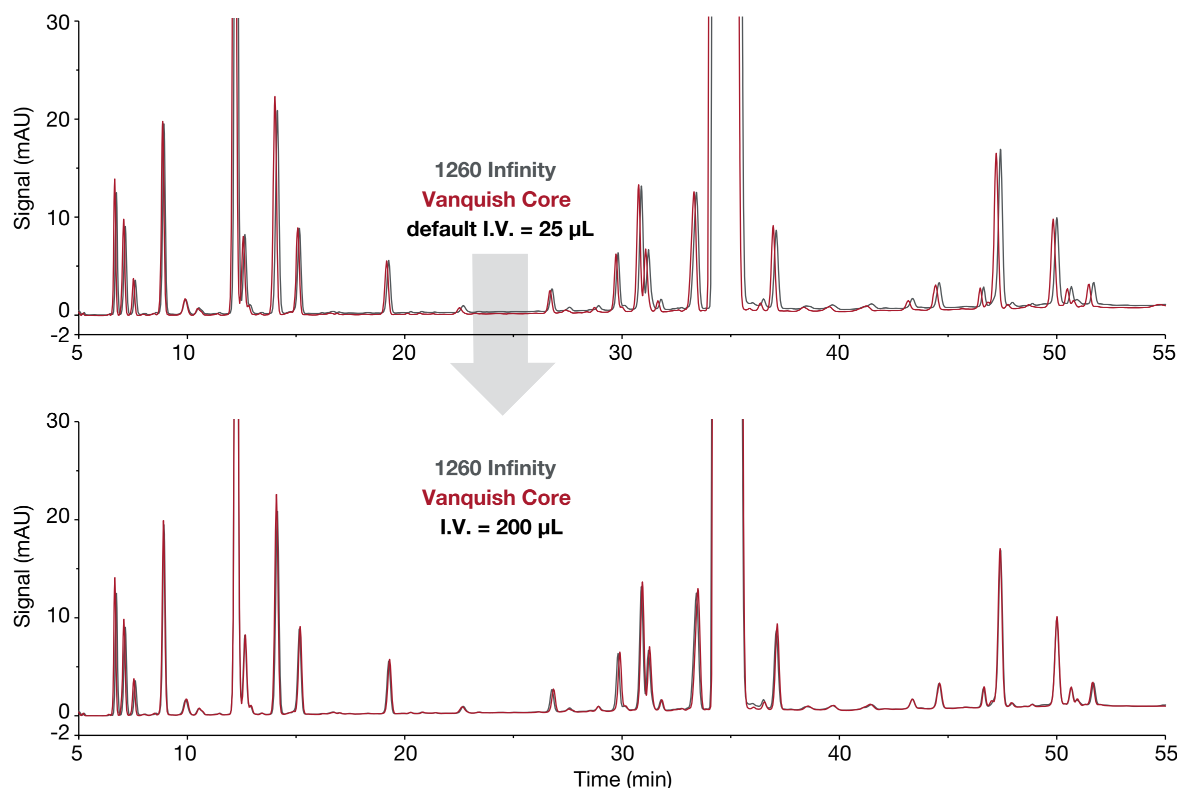

Figure 3. Retention time fine-tuning by idle volume (I.V.) adaption of the metering device in the Vanquish Core sampler

1. The idle volume setting of the autosamplers' metering device, which is the sample aspiration device, can be tuned in a range of 0–230 µL. The default setting is 25 µL.

2. An optional method transfer kit switches a 200 µL volume loop into the flow path between the pump and the autosampler.

Combining both approaches, the seamlessly tunable GDV portion of the Vanquish Core HPLC system is up to 430 µL. With this volume, retention times in gradient LC methods can be delayed in order to achieve a closer match with the originating system.

For the current application, the retention time deviations of the Vanquish Core HPLC system (default) compared to the 1260 Infinity system ranged from 0.02 to 0.22 min, increasing roughly over the run time (Figure 4). At a flow rate of 1 mL/min, these can be translated into volume differences of 20–220 µL with a mean of 115 µL. However, early eluting peaks in gradient methods often are affected by a mixture of isocratic and gradient elution and are less affected by the GDV. Thus, for GDV adaption one would rather take into account the mean deviation of later eluting peaks in the gradient. Due to that a first estimate to increase the idle volume from the default value (25 µL) to 200 µL markedly improved the retention time match of the Vanquish Core and the 1260 Infinity systems (Figure 3, bottom). In Figure 4 the improvement is outlined for each peak. As expected, early eluting peaks were hardly impacted by the GDV increase. For some later peaks, the GDV increase overcompensated the deviations, but in total the deviations were considerably decreased, demonstrating the benefit of adaptable system volumes for LC method transfers.

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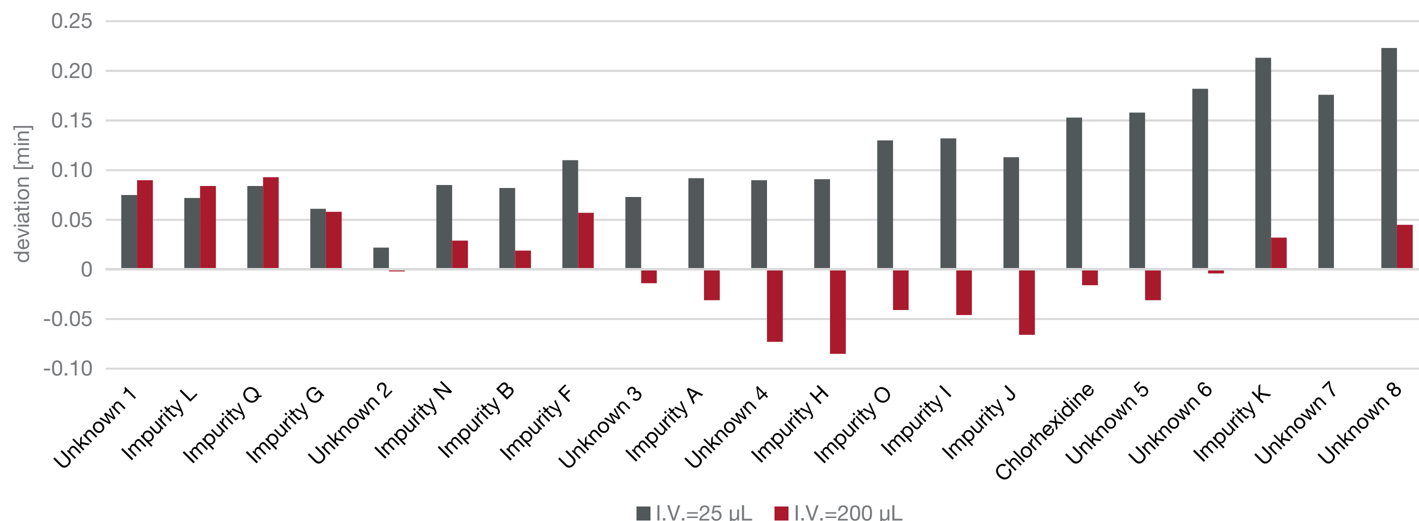

Figure 4. Retention time deviations of the 1260 Infinity system compared to the Vanquish Core HPLC system with an idle volume (I.V.) of 25 µL (default) and of 200 µL

In summary, for best retention time match, Vanquish Core HPLC system users should first replicate the chromatogram, compare with the chromatogram obtained with the source system, and then adjust the GDV of the system in an iterative process until the best retention time match is obtained.[10] The applied GDV changes are compliant since all of the following are true:

- Compendial methods do not regulate system volumes.
- The fluidic setup of the HPLC system is not undergoing a manual change.
- Instrument parameter settings are fully trackable in the audit trail of the chromatography data system.

Note that besides the GDV, other instrument-design differences may cause peak retention times to shift. One common example is thermal effects, for instance, induced by different eluent pre-heating efficiency or the absence or presence of a pre-heater.

Conclusion

- The straightforward transfer from an Agilent 1260 Infinity LC system to a Thermo Scientific Vanquish Core HPLC system was demonstrated for the EP method for chlorhexidine impurity analysis.
- Equivalent chromatographic outcomes were provided by the two systems.
- Small deviations of absolute retention times due to different system gradient delay volumes were easily decreased by adjustments of the idle volume of the Vanquish Core autosampler. For further GDV increase a Method Transfer Kit (P/N 6036.2100) is available. Either option is compliant and trackable.

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APPLICATION NOTE 73871

Setup and transfer of a gradient HILIC method for the impurity analysis of temozolomide

From an Agilent 1260 Infinity LC system to a Vanquish Core HPLC system

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Keywords: HPLC method transfer, Vanquish Core HPLC system, Agilent 1260 Infinity LC system, temozolomide, HILIC, Ph. Eur., USP

Application benefits

- A fast and reproducible gradient HILIC method was set up for the impurity analysis of the hydrophilic temozolomide.
- Straightforward transfer of the method from an Agilent™ 1260 Infinity™ LC system to a Thermo Scientific™ Vanquish™ Core HPLC system was demonstrated.
- Equivalent chromatographic results were obtained.

Goal

Develop an analytical HILIC method and transfer it from an Agilent 1260 Infinity LC system to the Vanquish Core HPLC system.

Introduction

The transfer of liquid chromatographic (LC) methods from one instrument to another is a recurring task in many analytical laboratories. It is required, for example, when a high workload has to be distributed over several instruments, when legacy instrumentation is replaced by modern technology, or when method development is realized in a lab different than the lab for the final method implementation, such as when outsourcing to contract labs. In each case, the transfer is only considered effective if equivalent results are obtained by the sending and receiving unit, but the challenge and effort depend on multiple factors. Usually a transfer is straightforward if equal instruments are involved, but deviations in technical characteristics of the systems like gradient delay volumes (GDV), pump mixing modes, column thermostatting and eluent pre-heating options, etc. may affect critical

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results like peak resolution or retention times.[1] In that, the complexity of the transfer job depends on the requirements of the chromatographer to the analytical outcome and the defined limits of acceptable deviations from the originating system. Usually very limited modifications of method parameters are acceptable during a transfer to prevent the need of a time-consuming revalidation. Hardware and software features of the Vanquish Core HPLC system are designed to assist in transferring LC methods while adhering to regulatory guidelines.[2,3]

Reversed-phase (RP) is the most common LC technique and it is widely used in pharmaceutical small molecule analyses. The majority of pharmacopeial LC methods rely on RP separations. However, hydrophilic interaction liquid chromatography (HILIC) is gaining more and more interest due to its polar retentivity. As very polar molecules are poorly retained by RP or only under special conditions like tedious chemical derivatization of analytes or ion-pairing reagents in the mobile phase, HILIC consolidates in its role as a powerful alternative technique. One example is the hydrophilic temozolomide: an alkylating cytostatic drug used in the chemotherapy of brain tumor patients. The chromatographic method for the impurity analysis of temozolomide provided by the monographs[4,5] of the European and the United States Pharmacopeia (Ph. Eur. and USP) specifies a C18 stationary phase and a highly aqueous mobile phase with the ion-pairing reagent hexanesulfonate to separate the active pharmaceutical ingredient (API) and four related impurities. In the current work, an alternative HILIC method was set up and was transferred from an Agilent 1260 Infinity LC (1260 Infinity) system to a Vanquish Core HPLC system.

Experimental

Instrumentation

Instruments listed below were used in the current study.

Reagents and materials

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Chemical™ Optima™ Acetonitrile LC/MS grade (P/N A955-212)
- Fisher Chemical™ Optima™ Ammonium acetate (NH_4Ac) LC/MS grade (P/N A114-50)
- Fisher Chemical™ Optima™ Acetic acid glacial (P/N A465-250)
- Fisher Chemical™ Hydrochloric acid 37% (P/N H/1200/PB08)
- Ph. Eur. reference standard: Temozolomide CRS batch 1, catalog code Y0001827[6]
- Ph. Eur. reference standard: Temozolomide for peak identification CRS batch 1, catalog code Y0001960[6]

Sample preparation

- Sample I: 5 mg of the reference standard temozolomide CRS were dissolved in 5 mL acetonitrile.
- Sample II: 3 mL of sample I were mixed with 3 mL of a 10.3 g/L aqueous hydrochloric acid solution. The mixture was heated in a boiling water bath for 1 hour in order to generate the impurities A, B, and E by forced degradation.
- Sample III: 1 mg of the reference standard temozolomide for peak identification CRS was dissolved in 1 mL acetonitrile. The standard contains the API temozolomide and low amount of impurity D.
- Sample IV: 450 µL of sample III were spiked with 15 µL of sample II to generate a sample of high API and low impurity concentration.

	Agilent 1260 Infinity Quaternary	Vanquish Core Quaternary
System base		System Base Vanquish Core (P/N VC-S01-A-02)
Pump	Quaternary pump (G1311B)	Quaternary Pump C (P/N VC-P20-A-01)
Sampler	High Performance Autosampler (G1367E) with thermostat module (G1330B)	Split Sampler CT (P/N VC-A12-A-02)
Column compartment	TCC with 6 µL heat exchanger (G1316A)	Column Compartment C (P/N VC-C10-A-03)
Detector	Variable Wavelength Detector (G1314F)	Variable Wavelength Detector C (P/N VC-D40-A-01)
Flow cell	Standard (10 mm, 14 µL, G1314-60186)	Standard (10 mm, 11 µL P/N 6077.0250)
System accessories		Method Transfer Kit Vanquish (P/N 6036.2100) Strong Solvent Loop (P/N 6036.2200)

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HPLC conditions

Parameter	Value
Column	Thermo Scientific™ Syncronis™ HILIC, 4.6 × 100 mm, 3 µm, 175 Å (P/N 97503-104630)
Mobile phases	A: 20 mM NH_4Ac pH 5.1 in water B: 20 mM NH_4Ac pH 5.1 in water/acetonitrile (10/90; v/v)
Flow rate	1 mL/min
Gradient	0.0 min – 100% B 5.0 min – 44% B 6.0 min – 44% B 6.1 min – 100% B 12 min – 100% B
Column temperature	30 °C (still air)
Autosampler temperature	10 °C
Detection	266 nm, 10 Hz, response time 1 s (1260 Infinity) / 0.5 s (Vanquish Core)
Injection volume	10 µL (5 µL for sample II)
Needle wash	Off

Experiments were generally verified by three consecutive injections, except for repeatability data, which was recorded with eight consecutive injections.

Data processing and software

Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) 7.3 was used for data acquisition and analysis.

Results and discussion

Method

The structures of the API temozolomide and the impurities A, B, D, and E, pursuant to the Ph. Eur. Nomenclature,[4] are summarized in Figure 1. The column selected for the separation was a Syncronis HILIC LC column with a proprietary zwitterionic stationary phase.

Figure 2 shows the chromatograms obtained by the described HILIC method with samples II, III, and IV with the 1260 Infinity LC system. In sample II, the majority of the API temozolomide was converted into the three impurities A, B, and E due to the forced degradation procedure in aqueous hydrochloric acid. Impurity D was detected as a small peak in sample III. Good separation of all peaks was ahieved, with a separation of impurities E and D to be expected from the chromatograms. To create a sample IV with all four impurities, sample III was spiked with a small amount of sample II. However, only three impurity peaks A, B, and D were detected with increased area of impurity D (compared to sample III) while no peak of impurity E was observed.

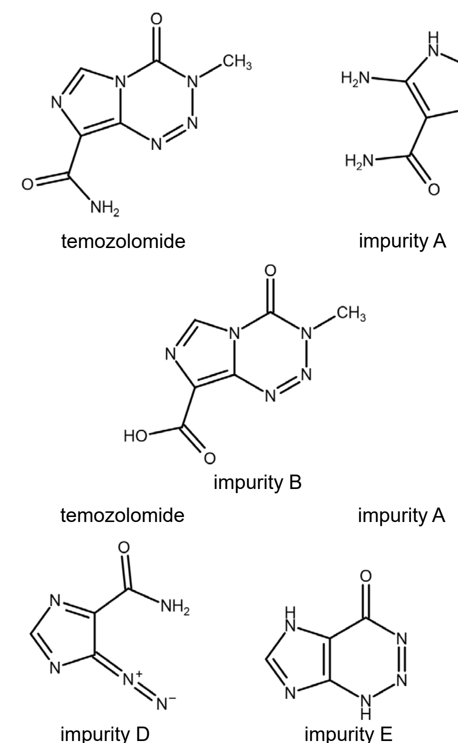

Figure 1. Structures of the API temozolomide and its impurities according to Ph. Eur.[4]

These and a few more experiments (data not shown) pointed to the assumption that impurity E transforms into impurity D by a ring opening under the much less acidic conditions in sample IV. No other sample preparation, mobile phase, etc. was found to create all four impurity peaks at the same time. Thus, injection of sample II and III is required for retention time comparison and impurity identification, which is in accordance with the Ph. Eur. procedure[4] for peak identification.

The gradient HILIC method was fast with a run time of 12 min and distinctly faster than the isocratic Ph. Eur. RP method, which can easily be around 30 min (specified as 3 times the retention time of temozolomide). As HILIC is commonly known to need long equilibration times, it is worth mentioning here that the equilibration time of 6 min was quite short, corresponding to just 5–6 column volumes. However, recent studies confirmed the validity of the “repeatable partial equilibration” concept in HILIC, proving that stable retention times can be achieved with not fully equilibrated columns.[7,8] Constant equilibration time was identified as a prerequisite, as selectivity changes may occur for different equilibration times. The effect is also shown in Figure 3 for the current method with additional equilibration time. As the resolution was impaired and no benefit was visible from longer equilibration times, the final method was kept as short as possible (6 min equilibration time).

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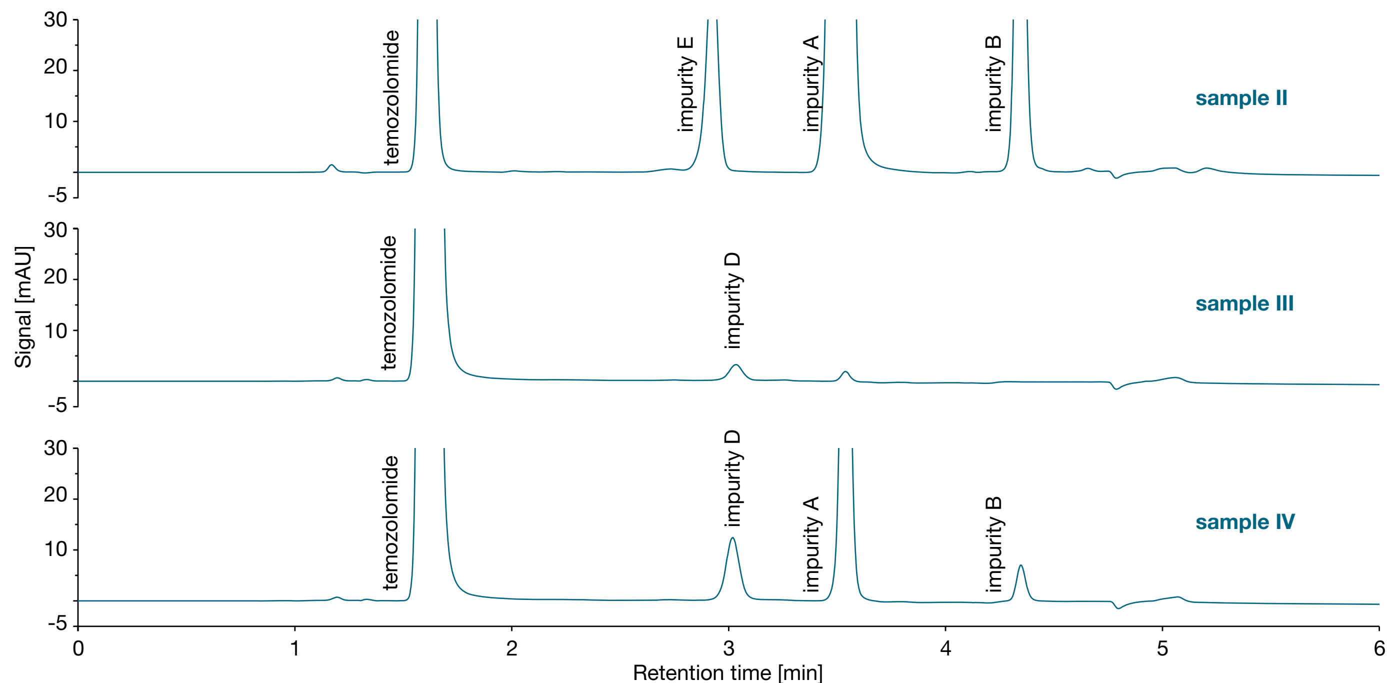

Figure 2. Chromatograms obtained by samples II, III, and IV with the 1260 Infinity system (6 min equilibration)

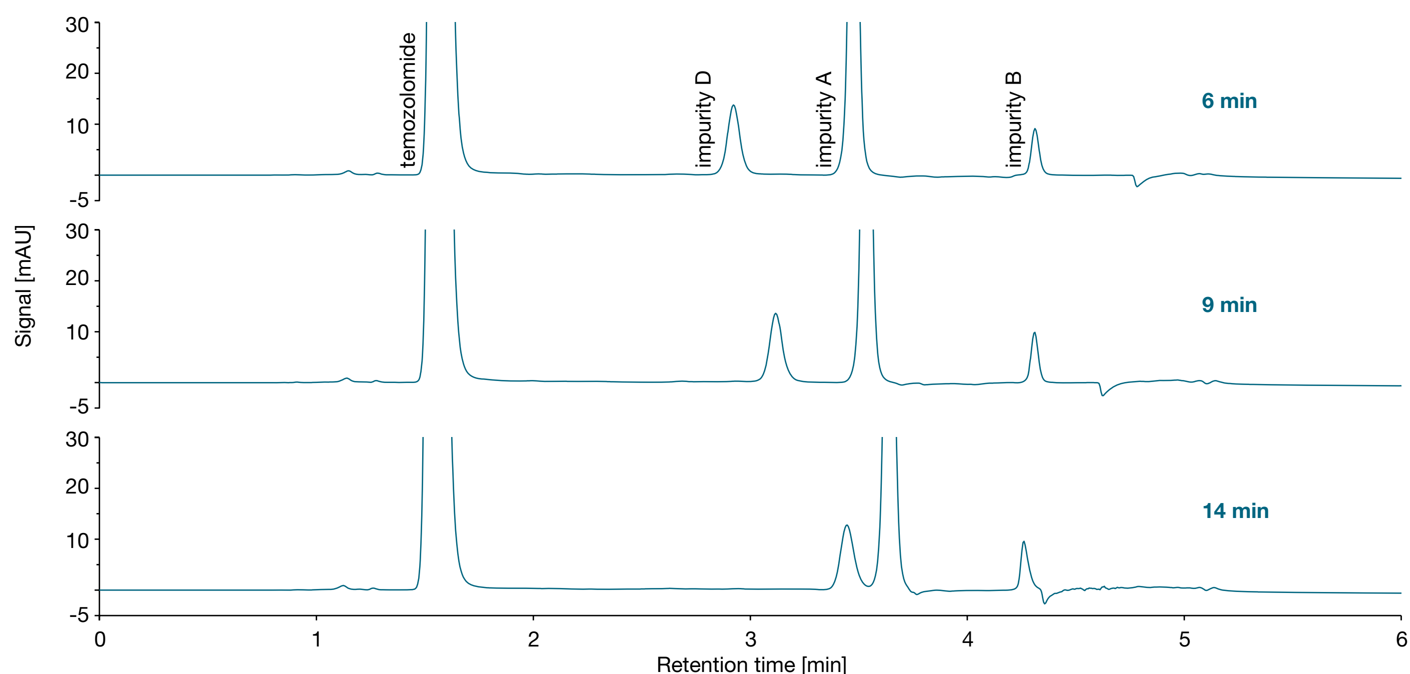

Figure 3. Selectivity changes of the HILIC method dependent on equilibration time (6, 9, and 14 min), shown for sample IV

Transfer

For best comparability, the transfer of the method from the 1260 Infinity system to the Vanquish Core HPLC system was conducted with the same column, aliquots of the same sample, and the same mobile phase batch to exclude non-instrumental effects on the transfer. Eight repeated injections of sample IV were executed with each system. Figure 4 displays the comparison of the chromatograms obtained with each system.

Very similar chromatograms were generated by the 1260 Infinity and Vanquish Core instruments, implying a very similar chromatographic performance. This can also be seen from Figure 5, which demonstrates a straightforward and successful method transfer. Retention times differed by less than 0.1 min and 3.5% from system to system. Both instruments provided repeatable retention times (t_R) and peak areas, expressed in low relative standard deviations (RSD). RSD in t_R is below 0.08% for each peak and system,

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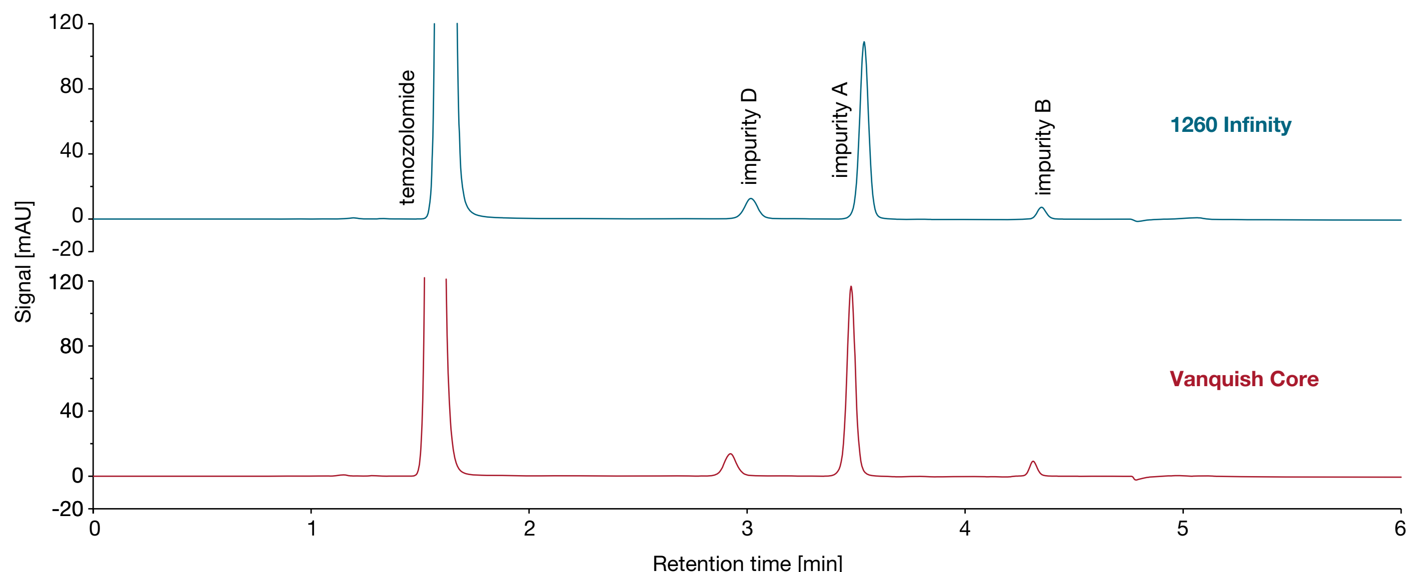

Figure 4. Transfer from 1260 Infinity system to Vanquish Core system

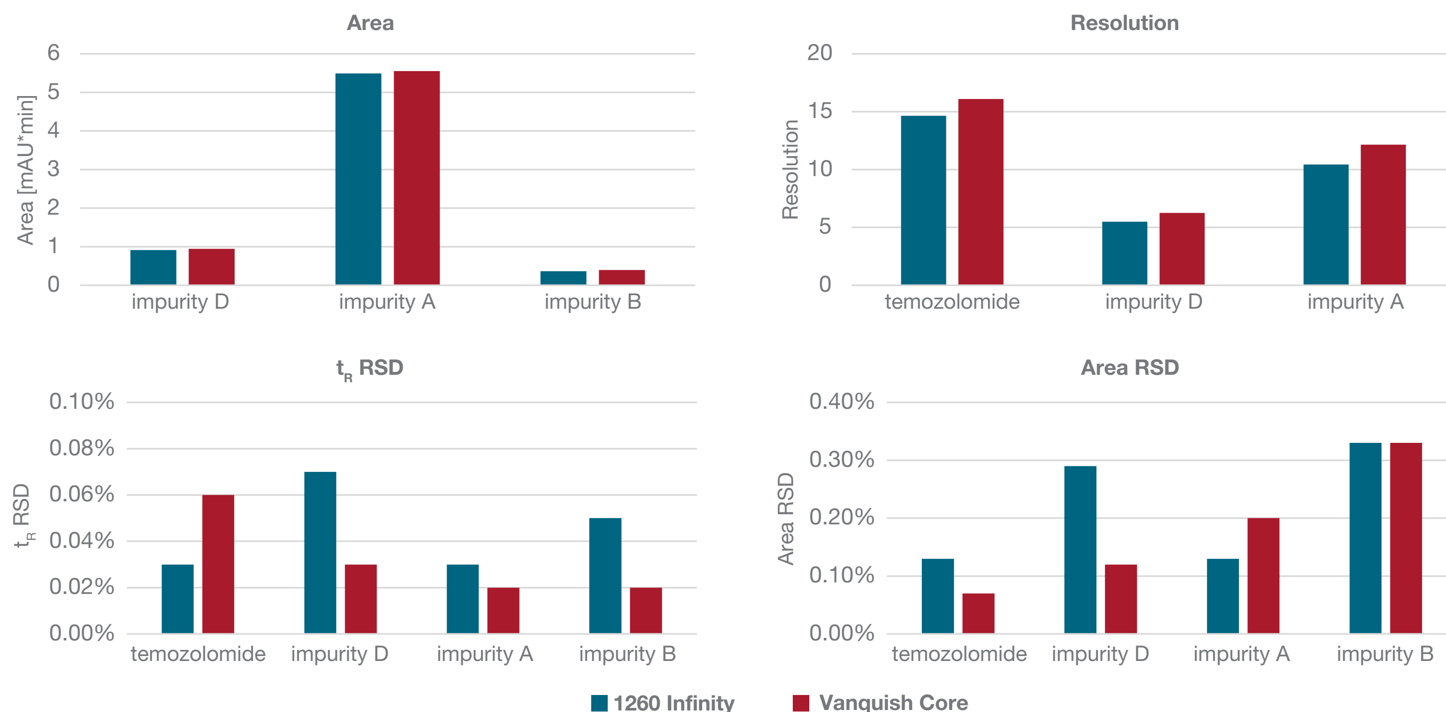

Figure 5. Chromatographic results and repeatability with 1260 Infinity and Vanquish Core systems

but overall better with the Vanquish Core system, while area RSD is below 0.4% and equivalent with both systems. The peak resolution is increased with the Vanquish Core system due to smaller peak widths (see appendix and Figure 8). The absolute peak areas of the impurities were in excellent accordance, for both systems. Areas of the API peaks differed distinctly, due to a difference in the detector response outside their linear range. The temozolomide peak considerably exceeded the specified linear range of both detectors (each 2500 mAU) as shown in Figure 6, which is common when APIs are overloaded to ensure the sensitive detection of low abundant impurities. Relative peak areas of impurities, referring to the API area, are affected by the mentioned response difference, and thus their use cannot be recommended when peaks overload detector capacities.

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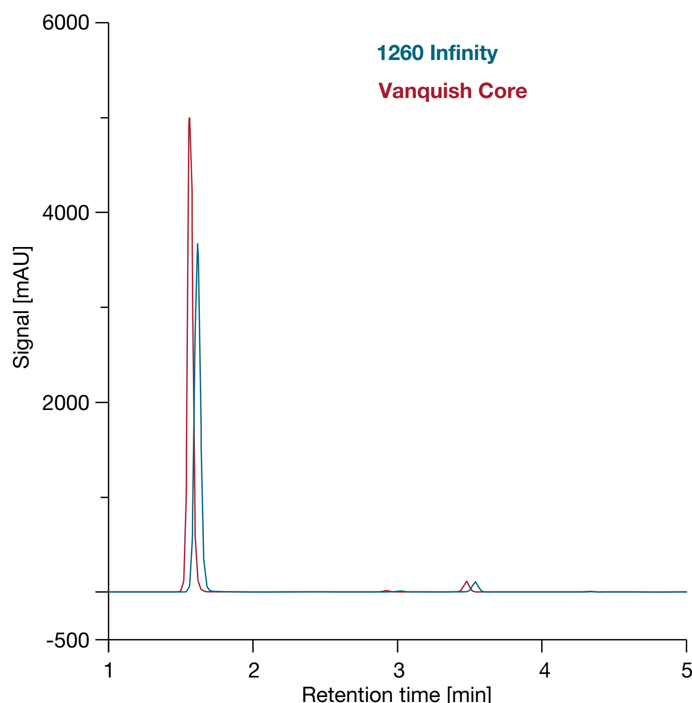

Figure 6. Unzoomed comparison of chromatograms obtained by 1260 Infinity and Vanquish Core instruments. While the temozolomide peak exceeds the linear range of both detectors, the dynamic response of the Vanquish Core VWD was distinctly higher than that of the 1260 Infinity VWD.

Conclusion

- A gradient HILIC method was set up for the impurity analysis of temozolomide with a run time of 12 min, giving reproducible results by applying the concept of repeatable partial equilibration.
- The transfer of the method from an Agilent 1260 Infinity LC system to a Thermo Scientific Vanquish Core HPLC system was a straightforward implementation, providing equivalent results with slightly more repeatable retention times and narrower peaks with the Vanquish Core system.
- Small offsets of absolute retention times could not be compensated by gradient delay volume adaption due to the effect on the partial equilibration of the column. Instead the root cause was found to be a difference in the extra column volumes of the systems (see Appendix).

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Appendix: Understanding residual retention time offsets

As mentioned above, the observed t_R deviations between the systems were small and did not compromise the success of the method transfer. A direct overlay is depicted in Figure 7A, showing slightly smaller t_Rs for each peak with the Vanquish Core system. Such t_R deviations may have several root causes like differences in the gradient delay volume (GDV) of the systems (volume between point of mobile phase mixing and column head), differences in the column thermostatting, differences in the extra-column volume (ECV, volume between points of injection and detection, excluding the column), or others. If a closer match of t_Rs is required, e.g., due to specified transfer limits, compensation of such instrumentational differences must be evaluated.

Usually, the first step is to vary the GDV, which can be easily implemented with the Vanquish Core instrument as it is equipped with a tunable idle volume of the autosamplers' metering device (0–230 µL and can be further increased by the Vanquish Method Transfer Kit to 430 µL).[2,3] However, in the current HILIC application, an increase of the GDV by moving the idle volume from default 25 µL to 100 µL did not result in a compensation of the t_R deviations but an unexpected reaction of t_Rs seen in Figure 7B with earlier elution of impurities D and A instead of later elution. This behavior can be explained by the phenomenon of partial equilibration of the HILIC column observed in the first section (Figure 3). With constant equilibration time but changing GDV, the column is in a slightly different equilibration state at the time of injection, which slightly affects the column selectivity. With much

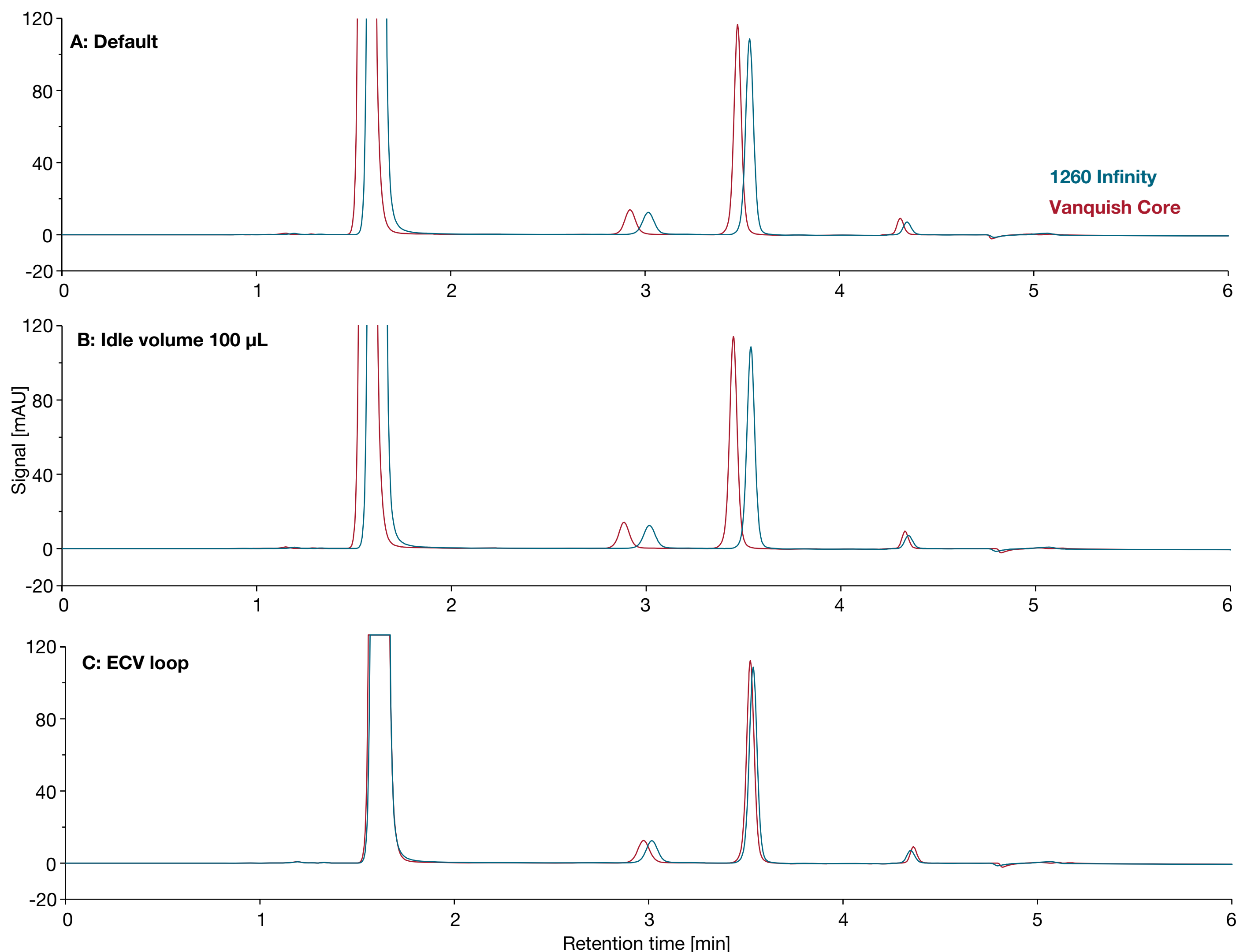

Figure 7. Overlay of chromatograms obtained by the 1260 Infinity instrument and the Vanquish Core instrument A) in default state (idle volume 25 µL); B) with idle volume set to 100 µL; C) with added ECV by 46 µL loop installed between injector and column. While the GDV adjustment could not compensate the t_R offset due to the partial column equilibration, the volume loop resulted in a closer match of ECVs.

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longer equilibration times, the t_Rs increased as expected with increasing the idle volume (data not shown). But as the method was chosen to be as short as possible, GDV adaption was not the proper tool to compensate t_R offsets in the current method transfer.

Instead, the fact that the deviations were relatively constant throughout the chromatogram and affected each peak, even the early eluting temozolomide, pointed to a difference in the ECV of the systems. The assumption was confirmed by two experiments: 1) a direct flow injection of a caffeine standard in a water flow of 1 mL/min in both systems without a column and 2) by the injection of a dead volume marker (anthracene) with the HILIC method applied. The calculated t_R differences between the systems were corresponding to an ECV difference of 45 µL for the caffeine injection (t_R 0.088 min vs. 0.043 min) and 47 µL for the anthracene injection (t_R 1.120 min vs. 1.167 min).

The ECV of an LC instrument is affected by contributions from tubing, connectors, fittings, and detector flow cell. In general, low ECVs are related to more efficient chromatography due to minimized dispersion of peak bands. The installation of an additional volume loop (P/N 6036.2200), corresponding to 46 µL, between the injector and the column in the Vanquish Core system resulted in a distinctly improved match of all t_Rs regarding the 1260 Infinity system with offsets not higher than 0.05 min as seen in Figure 7C and Figure 8. Although the loop technically also adds up to the GDV, its effect was different from changing the idle volume since the sample was also traveling through the added volume. The downside of that approach was an increase of peak widths compared to the default setup due to an increased peak dispersion by the loop (Figure 8). Thus, the installation of extra system volume is only recommended if other strong benefits apply, e.g., proper pre-column mixing of strong sample plugs and mobile phase.

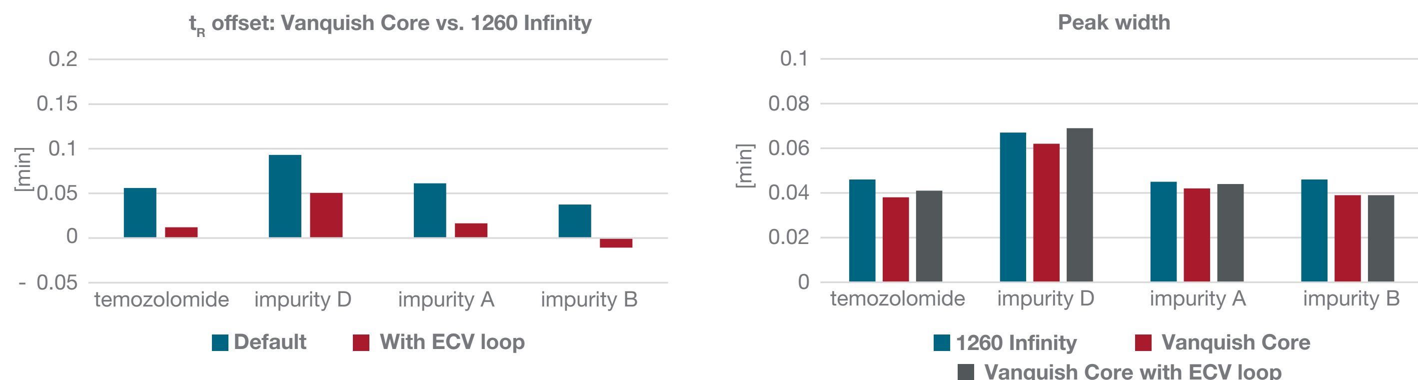

Figure 8. Effect of added ECV on the method transfer from the 1260 Infinity to the Vanquish Core system regarding t_R offset and peak widths (at half height)

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APPLICATION NOTE 73586

Easy transfer of an EP method for chlorhexidine impurity analysis from a Shimadzu Nexera-i system to a Vanquish Core HPLC system

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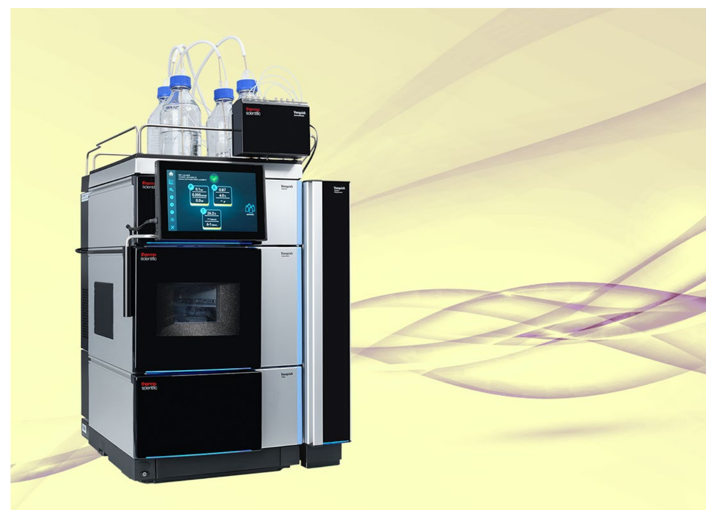
Keywords: HPLC method transfer, Vanquish Core HPLC system, Shimadzu Nexera-i, Chromeleon CDS, chlorhexidine, European Pharmacopoeia



Application benefits

- Straightforward transfer of an EP monograph HPLC method from a Shimadzu™ Nexera-i™ system to a Thermo Scientific™ Vanquish™ Core Quaternary HPLC system is demonstrated.
- Advanced hardware features of the Vanquish Core HPLC system enable flexible adjustments of the overall system gradient delay volume to facilitate compliant fine-tuning during the transfer.
- Equivalent chromatographic results are obtained with the originating and receiving instrument, but improved system precision is provided by the Vanquish Core HPLC system.

Goal

To showcase the transfer of analytical HPLC methods from a Shimadzu Nexera-i system to the Vanquish Core HPLC system and highlight the easy-to-use gradient delay volume (GDV) adjustment features of the Vanquish Core HPLC system.

Introduction

Instrument-to-instrument transfer of liquid chromatographic (LC) methods is a challenging task most analytical laboratories face frequently. For example, an established application needs to be distributed over several instruments within one lab to manage the workload. In another common situation, inter-lab transfers are realized among method developing and method implementing laboratories, that is, from research and development (R&D) labs to quality control (QC) labs, or when specific tasks are outsourced to contract labs.[1] In all cases, sending and receiving instruments may differ in vendor and configuration. A third scenario is the replacement of legacy instrumentation by modern technology. In any instance, a transfer is only

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considered effective if equivalent results are obtained. The success and the required effort of a transfer depends on the robustness of the method to be transferred as well as on instrumentational deviations of the involved systems.[1] Some technical characteristics of a system, like its gradient delay volume (GDV), pump mixing mode, hydrodynamic behavior, column and eluent thermostatting options, may affect critical results like peak resolution or retention times.[2-4] The complexity of the transfer job is determined by the requirements of the chromatographer to the analytical outcome and the defined limits of acceptable deviations from the originating system. In addition, only very limited modifications of method parameters are usually accepted during a transfer to prevent the need of a time-consuming revalidation. Thus, compliant hardware features, like the unique adaptable GDV options provided by the Vanquish Core HPLC system, are the preferred tools to assist in transferring LC methods.

In the following, the HPLC method for impurity analysis of chlorhexidine digluconate given by the European Pharmacopoeia (EP) monograph[5] is transferred from a Shimadzu Nexera-i system to a Vanquish Core Quaternary HPLC system. Chlorhexidine is a common antiseptic and disinfectant, listed on the World Health Organization's (WHO) Model List of Essential Medicines.[6] It is available as an over-the-counter drug and is widely used in dental medicine and hygiene, for example, in mouthwashes and for skin disinfection purposes.

The selected Thermo Scientific™ Hypersil GOLD™ column complies with the requirement for an end-capped C18 silica column of the monograph. Although we adhered to the EP monograph, the following discussions in general are also valid for the United States Pharmacopoeia (USP) method,[7] as the analytical method, i.e., column and gradient, are identical.

Experimental details

Reagents and materials

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Scientific™ Acetonitrile, Optima™ LC/MS grade (P/N A955-212)
- Thermo Scientific™ Pierce™ Trifluoracetic acid (TFA), LC-MS grade (P/N 85183)
- EP reference standard: Chlorhexidine for system suitability (SST) CRS batch 2 (catalog code Y0001545[8])

Instrumentation

See Table 1.

HPLC conditions

See Table 2.

Table 2. Chromatographic conditions

Parameter	Value
Column	Hypersil GOLD, 4.6 x 250 mm, 5 µm, 175 Å (P/N 25005-254630)
Mobile phase	A: 0.1% TFA in water/acetonitrile (80/20; v/v) B: 0.1% TFA in water/acetonitrile (10/90; v/v)
Flow rate	1 mL/min
Gradient	0 min – 0% B 2 min – 0% B 32 min – 20% B 37 min – 20% B 47 min – 30% B 54 min – 30% B 55 min – 0% B 62 min – 0% B
Column temp.	30 °C (forced air)
Autosampler temp.	8 °C
Detection	**Vanquish Core:** 254 nm, 5 Hz, response time 1 s **Nexera-i:** 254 nm, 4.1667 Hz, time constant 480 ms
Injection volume	7 µL
Needle wash	Off

Table 1. Instrumentation

	Shimadzu Nexera-i	Vanquish Core Quaternary
System base		System Base Vanquish Core (P/N VC-S01-A-02)
Pump	LC-2040C 3D MT; integrated system with quaternary solvent delivery, autosampler, column oven, photodiode array detector and two flow lines with UHPLC and HPLC delay volumes* *HPLC flow path used	Quaternary Pump C (P/N VC-P20-A-01)
Sampler		Split Sampler CT (P/N VC-A12-A-02)
Column compartment		Column Compartment C (P/N VC-C10-A-03)
Detector		Diode Array Detector (P/N VC-D11-A-01)
Flow cell	Fast flow cell i-series 3D (10 mm, 8 µL, 228-45618-54)	Standard (10 mm, 13 µL, P/N 6083.0510)
System accessory		Method Transfer Kit Vanquish (P/N 6036.2100)

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Sample preparation

According to the monograph, 5 mg of the reference standard, which contained the active pharmaceutical ingredient (API) chlorhexidine and various impurities, were dissolved in 1 mL of mobile phase A (see Table 2).

Data processing and software

Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS), version 7.3 was used for data acquisition and analysis. Shimadzu LC drivers for Chromeleon CDS were installed for the direct control of the Nexera-i instrument.

Results and discussion

For best comparability, the following experiments were conducted with the same column, aliquots of the same sample, and the same mobile phase batch to exclude non-instrumental effects on the transfer. In deviation from the EP monograph, the injection volume was 7 µL instead of 10 µL to avoid a saturation of the main peak signal with the Nexera-i detector due to exceedance of the linearity range. Seven consecutive injections were executed with each system. Figure 1 displays the comparison of both instruments under conditions outlined in the EP monograph. The chromatogram is populated over the complete run time with peaks of the main compound, specified impurities, and unknowns not specified in the SST standard leaflet.[9] For reasons of clarity, only the peaks that exceeded a minimum peak area of 0.25 mAU·min are considered in this work.

Very similar chromatograms were generated by the Nexera-i and Vanquish Core HPLC instruments, implying a very similar chromatographic performance as can also be seen in Table 3 and Figure 2. A summary of relative retention times, experimentally obtained and provided by the EP monograph, is given in Table 3. Both instruments are in excellent accordance with each other and well aligned with the EP objectives. In Figure 2, a very good accordance in relative peak areas and peak resolutions is displayed. The retention time and peak area precisions obtained with either system are shown in Figure 3. While the relative standard deviations (RSD) of retention times is not higher than 0.05% with the Vanquish Core HPLC system, they rank up to 0.09% with the Nexera-i system. The RSD of peak areas is below 0.5% for all peaks with the Vanquish Core HPLC system. The same is true for most of the peaks with the Nexera-i instrument with some exceptions exceeding this limit.

The system suitability criteria given by the EP monograph, requiring a resolution of the impurity pair L and G of minimum 3 and a peak-to-valley ratio of impurity B of minimum 2, are easily met by either LC system with a resolution > 8 and a peak-to-valley ratio > 6. Thus, the chlorhexidine impurity LC method was successfully repeated with both systems, giving equivalent results, and its transfer could be rated as straightforward and successful.

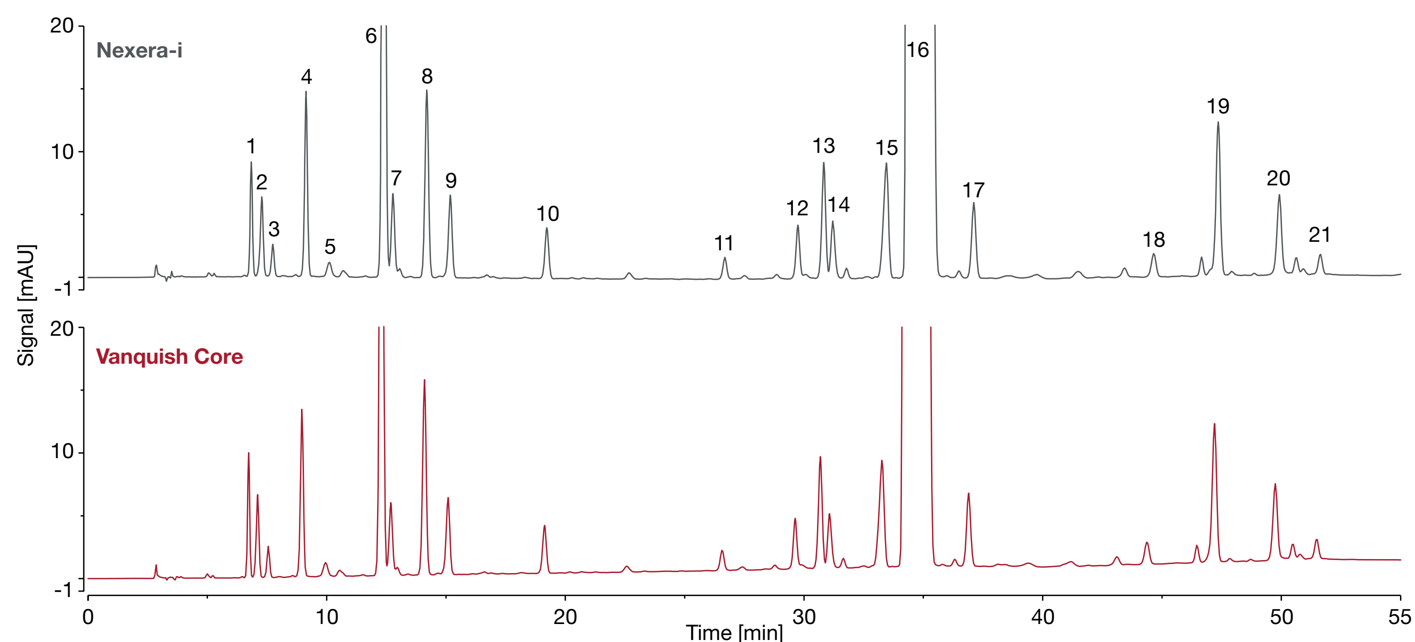

Figure 1. Transfer from Nexera-i system to Vanquish Core HPLC system according to the EP monograph for chlorhexidine gluconate; peak assignment according to impurity designation in EP monograph and standard leaflet[5, 9]

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Table 3. Averaged relative retention times related to the main peak as stated in the EP monograph and from Nexera-i and Vanquish Core chromatograms (Figure 1, default settings)

Peak #	Compound	EP monograph	Nexera-i	Vanquish Core
1	Unknown 1		0.199	0.197
2	Impurity L	0.23	0.212	0.208
3	Impurity Q	0.24	0.225	0.221
4	Impurity G	0.25	0.266	0.262
5	Unknown 2		0.294	0.291
6	Impurity N	0.35	0.360	0.359
7	Impurity B	0.36	0.372	0.371
8	Impurity F	0.50	0.413	0.412
9	Unknown 3		0.442	0.441
10	Impurity A	0.60	0.559	0.559
11	Unknown 4		0.776	0.776
12	Impurity H	0.85	0.865	0.866
13	Impurity O	0.90	0.897	0.896
14	Impurity I	0.91	0.907	0.907
15	Impurity J	0.96	0.973	0.972
16	Chlorhexidine	1.00	1.000	1.000
17	Unknown 5		1.079	1.078
18	Unknown 6		1.299	1.296
19	Impurity K	1.40	1.377	1.378
20	Unknown 7		1.452	1.453
21	Unknown 8		1.502	1.503

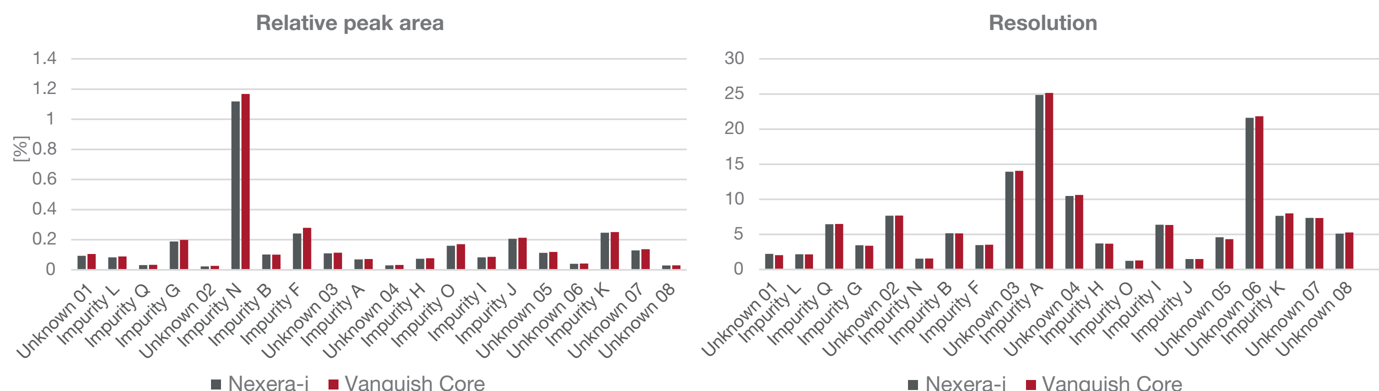

Figure 2. Chromatographic results with Nexera-i and Vanquish Core HPLC systems under conditions outlined in the EP monograph (Figure 1)

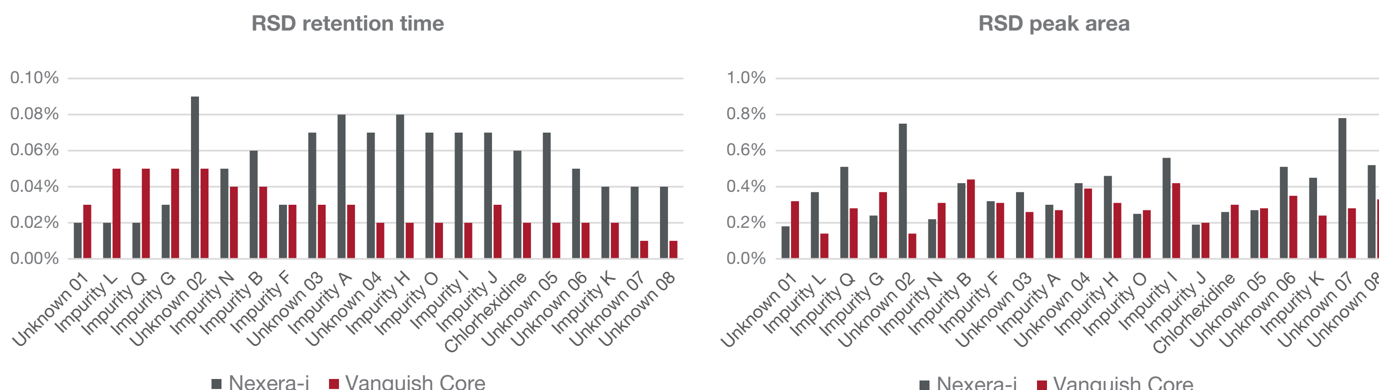

Figure 3. Relative standard deviations (RSD) of retention times and peak areas over seven injections obtained by the Nexera-i and Vanquish Core HPLC systems

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However, despite the excellent fit of *relative* retention times, in a direct overlay of both chromatograms, one can observe small deviations in the *absolute* retention times with all peaks eluting slightly earlier on the Vanquish Core HPLC system (Figure 4 top). These may be the results of a slightly smaller default GDV of the Vanquish Core HPLC system compared to the HPLC flow path of the Nexera-i system. The GDV of an LC system is defined as the volume between the point of mobile phase mixing and the column head. If a closer match of absolute retention times in gradient LC methods is required, for example, to meet prescribed acceptance limits, the deviations can be compensated by a tuning of the GDV of the Vanquish Core HPLC system by two different means.

1. The idle volume setting of the autosamplers' metering device, which is the sample aspiration device, can be tuned in a range of 0–230 µL. The default setting is 25 µL.
2. An optional method transfer kit switches a 200 µL volume loop into the flow path between the pump and the autosampler.

Combining both approaches, the seamlessly tunable GDV portion of the Vanquish Core HPLC system is up to 430 µL. With this volume, retention times in gradient LC methods can be delayed to achieve a closer match with the originating system.

For the current application, the retention time deviations of the Vanquish Core HPLC system (default) compared to the Nexera-i system ranged from 0.076 to 0.26 min (Figure 5). Increasing the idle volume from the default value (25 µL) to 125 µL markedly improved the retention time match of both systems (Figure 4, bottom). Early eluting peaks were less impacted by the GDV change, as can be expected from the mixed isocratic and gradient elution mechanisms affecting these peaks. For some other peaks, the GDV increase resulted in a slight overcompensation. In total, the retention time deviations were considerably decreased as outlined in Figure 5. After the idle volume adaption, the average of absolute retention time deviations decreased from 0.132 min with the default settings to 0.051 min, demonstrating the benefit of switchable system volumes for LC method transfers. If desired, further fine-tuning can be

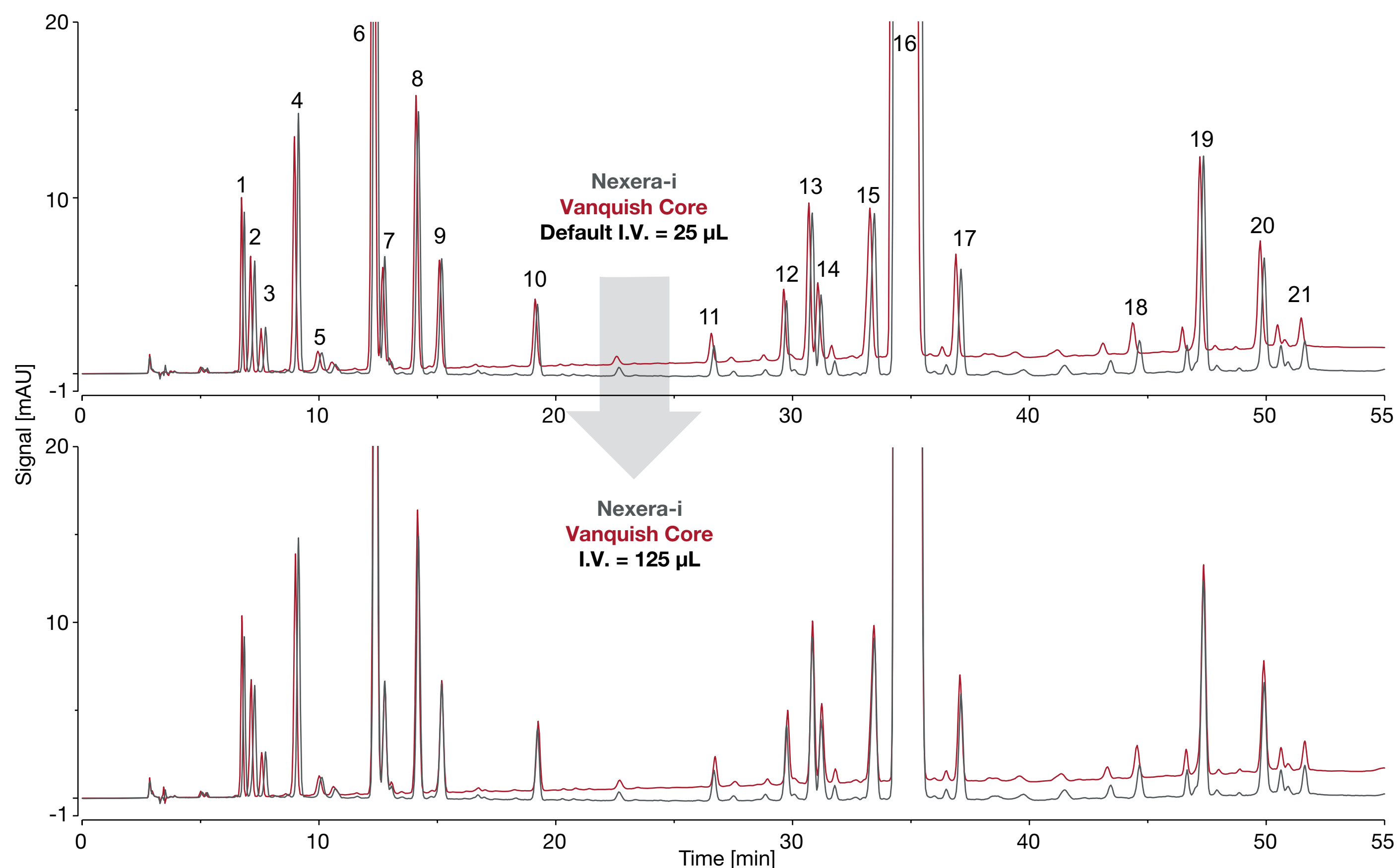

Figure 4. Retention time fine-tuning by idle volume (I.V.) adaption of the metering device in the Vanquish Core sampler

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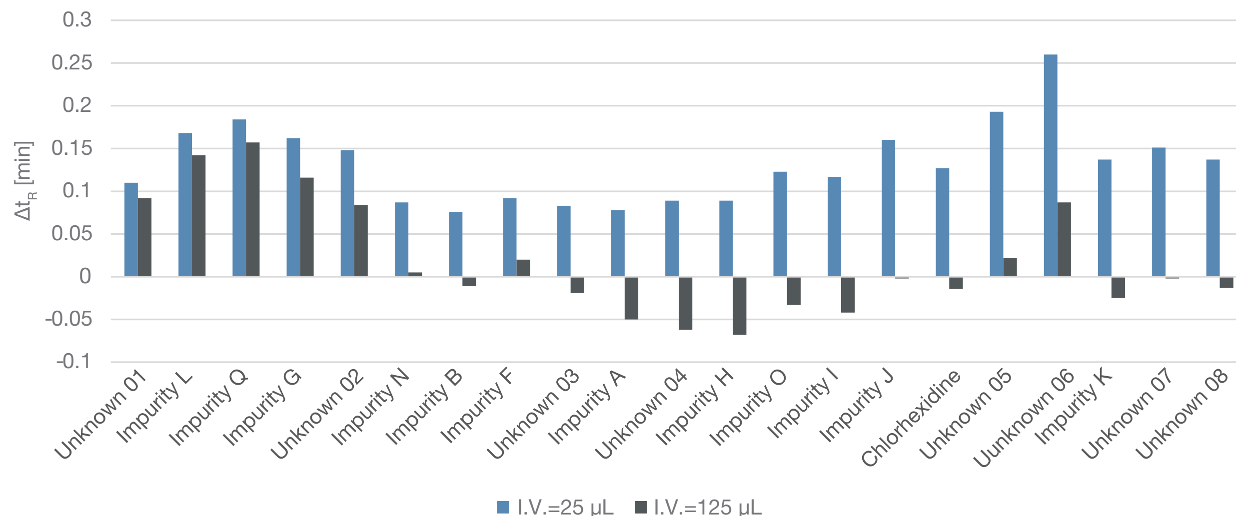

Figure 5. Retention time deviations of the Nexera-i system compared to the Vanquish Core HPLC system with an idle volume (I.V.) of 25 µL (default) and of 125 µL

done in an iterative way.[10] This flexibility is a major benefit in comparison to the method transfer concept provided by the Nexera-i system, which offers two static flow paths with two different pump mixers. In addition, the mobile phase mixing performance is not impacted by the flexible GDV tools of the Vanquish Core HPLC system.

The applied GDV changes are compliant because of the following.

- Compendial methods do not regulate system volumes.
- The fluidic setup of the HPLC system is not undergoing a manual change.
- Instrument parameter settings are fully trackable in the audit trail of the chromatography data system.

For interested readers, more details are outlined in Reference 10.

However, note that besides the GDV, other instrument-design differences may cause peak retention times to shift. Thermal effects are one common example, such as those induced by different eluent pre-heating efficiency or the absence or presence of a pre-heater.

Conclusion

- The straightforward transfer from a Shimadzu Nexera-i system to a Thermo Scientific Vanquish Core HPLC system was demonstrated for the EP method for chlorhexidine impurity analysis.
- Equivalent chromatographic outcomes were provided by the two systems with improved system precision of the Vanquish Core HPLC system.
- Small deviations of absolute retention times due to different system gradient delay volumes were easily decreased by an adjustment of the idle volume of the Vanquish Core autosampler. For further GDV increase, a Method Transfer Kit (P/N 6036.2100) is available. Either option is compliant and trackable.

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Seamless transfer of a compendial LC method for impurity analysis of chlorhexidine from an UltiMate 3000 Standard HPLC system to a Vanquish Core HPLC system

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Thermo Fisher Scientific, Germering, Germany

Keywords: HPLC method transfer, Vanquish Core HPLC System, UltiMate 3000 Standard HPLC system, chlorhexidine, European Pharmacopoeia

Application benefits

- Straightforward transfer of an EP monograph HPLC method from a Thermo Scientific™ UltiMate™ 3000 Standard HPLC system to a Thermo Scientific™ Vanquish™ Core HPLC system is demonstrated.
- Equivalent chromatographic results are obtained with both systems, but an improved resolution and retention time stability are provided by the Vanquish Core HPLC system.

Goal

To demonstrate the transfer of analytical HPLC methods from an UltiMate 3000 Standard HPLC system to a Vanquish Core HPLC system.

Introduction

Instrument-to-instrument transfer of liquid chromatographic (LC) methods is a challenging task most analytical laboratories face frequently under several scenarios. For example, an established application needs to be run by several instruments within one lab to distribute major workload. On the other hand, inter-lab transfers are realized among method developing and method implementing laboratories, that is, from research and development (R&D) labs to quality control (QC) labs, or when specific tasks are outsourced, for example, to contract labs.[1]

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In both cases, the transferring and receiving laboratories' instruments can be either equivalent or different in vendor and configuration. A third scenario is the replacement of legacy instrumentation by modern technology. In either instance a transfer is only considered effective if equivalent results are obtained. The success and the required effort of such a transfer depend on multiple factors. The robustness of the method to be transferred as well as instrumentational deviations of the involved systems play an important role.[1] Some technical characteristics of a system, like its gradient delay volume (GDV), pump mixing mode, hydrodynamic behavior, column, and eluent thermostatting options, may affect critical results like peak resolution or retention times.[2-4] The requirements of the chromatographer to the analytical outcome and the defined limits of acceptable deviations from the originating system determine the complexity of the transfer job. In addition, only very limited modifications of method parameters are usually accepted during a transfer to prevent the need of a time-consuming revalidation.

In the following, the HPLC method for impurity analysis of chlorhexidine digluconate given by the European Pharmacopoeia (EP) monograph[5] is transferred from an UltiMate 3000 Standard HPLC system (UltiMate 3000 SD) to a Vanquish Core HPLC system (Vanquish Core). Chlorhexidine is a common antiseptic and disinfectant, listed on the World Health Organization's (WHO) Model List of Essential Medicines.[6] It is available as an over-the-counter drug and is widely used in dental medicine and hygiene, for example, in mouthwashes and for skin disinfection purposes.

The selected Thermo Scientific™ Hypersil™ GOLD column complies well with the requirement for an end-capped C18 silica column of the monograph. Although we adhered to the EP monograph, the following discussions in general are also valid for the United States Pharmacopoeia (USP) method,[7] as the analytical method, i.e. column and gradient, are identical.

Experimental

Reagents and materials

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Scientific™ Optima™ Acetonitrile, LC/MS grade (P/N A955-212)
- Thermo Scientific™ Pierce™ Trifluoracetic acid (TFA), LC-MS grade (P/N 85183)
- EP reference standard: Chlorhexidine for system suitability (SST) CRS batch 2, catalogue code Y0001545[8]

Sample preparation

According to the monograph, 5 mg of the reference standard, which contained the chlorhexidine and various impurities, were solved in 1 mL of mobile phase A (see below).

Instrumentation and HPLC conditions

The instruments and the HPLC conditions used in this study are listed in Tables 1 and 2.

Table 1. Instruments

	UltiMate 3000 SD Quaternary	Vanquish Core Quaternary
System base		System Base Vanquish Core (P/N VC-S01-A-02)
Solvent storage	Solvent Rack SR-3000 (P/N 5035.9200)	Solvent Rack (P/N 6036.1350)
Pump	Quaternary Pump LPG-3400SD (P/N 5040.0031)	Quaternary Pump C (P/N VC-P20-A-01)
Sampler	Well Plate Autosampler WPS-3000TSL Analytical (P/N 5822.0020)	Split Sampler CT (P/N VC-A12-A-02)
Column compartment	Thermostatted Column Compartment TCC-3000SD (P/N 5730.0010) (passive pre-heater not included in default configuration)	Column Compartment C (P/N VC-C10-A-03) (passive pre-heater P/N 6732.0170 included in System Base ship kit)
Detector	Diode Array Detector DAD-3000 (P/N 5082.0010)	Diode Array Detector CG (P/N VC-D11-A-01)
Flow cell	Analytical (10 mm, 13 µL (P/N 6082.0100)	Standard (10 mm, 13 µL, P/N 6083.0510)
System accessory		Method Transfer Kit Vanquish (P/N 6036.2100)

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Table 2. HPLC conditions

Parameter	Value
Column	Hypersil GOLD, 4.6 x 250 mm, 5 µm, 175 Å (P/N 25005-254630)
Mobile phase	A: 0.1% TFA in water/acetonitrile (80/20; v/v) B: 0.1% TFA in water/acetonitrile (10/90; v/v)
Flow rate	1 mL/min
Gradient	0 min – 0% B, 2 min – 0% B, 32 min – 20% B, 37 min – 20% B, 47 min – 30% B, 54 min – 30% B, 55 min – 0% B, 62 min – 0% B
Column temperature	30 °C (forced air)
Autosampler temperature	8 °C
Detection	
Wavelength	254 nm
Bandwidth	4 nm
Data collection rate	5 Hz
Filter response/ response time	1 s
Injection volume	10 µL
Needle wash	Off

Data processing and software

Thermo Scientific™ Chromeleon™ Chromatography Data System Software, version 7.3, was used for data acquisition and analysis.

Results and discussion

For best comparability, the following experiments were conducted with the same column, aliquots of the same sample, and the same mobile phase batch to exclude non-instrumental effects on the transfer. Six consecutive injections were executed with each system. Figure 1 displays the comparison of both instruments in their default configurations under conditions as outlined in the EP monograph. The chromatogram is populated over the complete run time with peaks of the main compound chlorhexidine, specified impurities, and unknowns not specified in the SST standard leaflet.[9] For reasons of clarity, the focus is on all peaks that exceeded a minimum peak area of 0.3 mAU·min in the following.

The UltiMate 3000 SD and Vanquish Core HPLC systems differ in one major feature in their default configurations: the absence (UltiMate 3000 SD) and presence (Vanquish Core) of a passive eluent pre-heater. Nevertheless, very similar chromatograms were generated by both instruments, implying a very similar chromatographic performance. A summary of relative retention times, experimentally obtained and provided by the EP monograph, is given in Table 3. The instruments are in excellent accordance with each other and well aligned with the EP objectives. For all peaks, the absolute retention times differed less than 1.2% from system to system. In addition, full congruence in peak areas is seen in Figure 2, with less than 3% deviation between the systems for each peak.

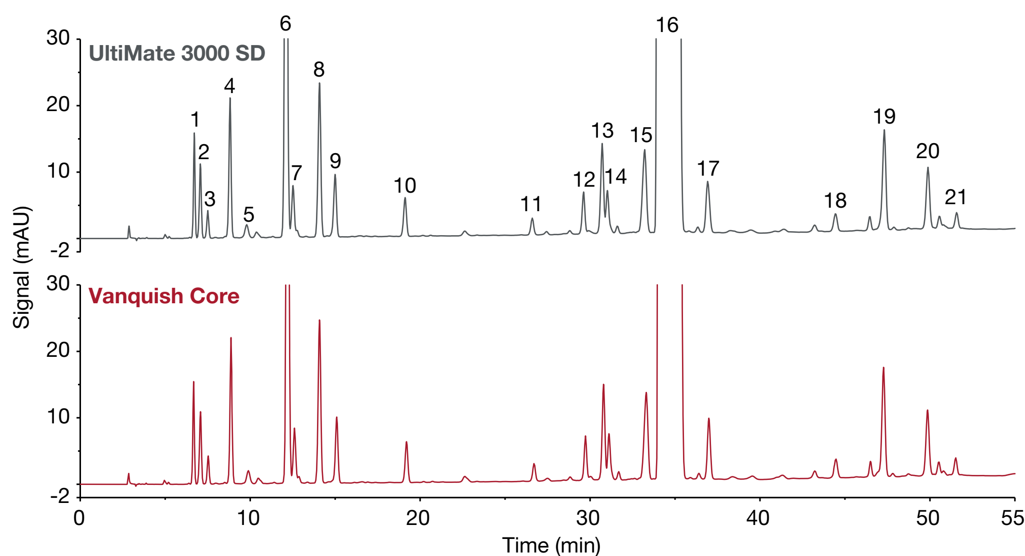

Figure 1. Transfer from UltiMate 3000 SD system to Vanquish Core HPLC system according to the EP monograph for chlorhexidine gluconate; peak assignment according to impurity designation in EP monograph and standard leaflet[5, 9]

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Table 3. Relative retention times related to the main peak as stated in the EP monograph and averaged from UltiMate 3000 SD and Vanquish Core chromatograms (Figure 1)

Peak #	Compound	EP monograph	UltiMate 3000 SD	Vanquish Core
1	Unknown 1		0.20	0.20
2	Impurity L	0.23	0.21	0.21
3	Impurity Q	0.24	0.22	0.22
4	Impurity G	0.25	0.26	0.26
5	Unknown 2		0.29	0.29
6	Impurity N	0.35	0.36	0.36
7	Impurity B	0.36	0.37	0.37
8	Impurity F	0.50	0.41	0.41
9	Unknown 3		0.44	0.44
10	Impurity A	0.60	0.56	0.56
11	Unknown 4		0.78	0.78
12	Impurity H	0.85	0.87	0.87
13	Impurity O	0.90	0.90	0.90
14	Impurity I	0.91	0.91	0.91
15	Impurity J	0.96	0.98	0.98
16	Chlorhexidine	1.00	1.00	1.00
17	Unknown 5		1.08	1.09
18	Unknown 6		1.31	1.30
19	Impurity K	1.40	1.39	1.39
20	Unknown 7		1.47	1.46
21	Unknown 8		1.52	1.51

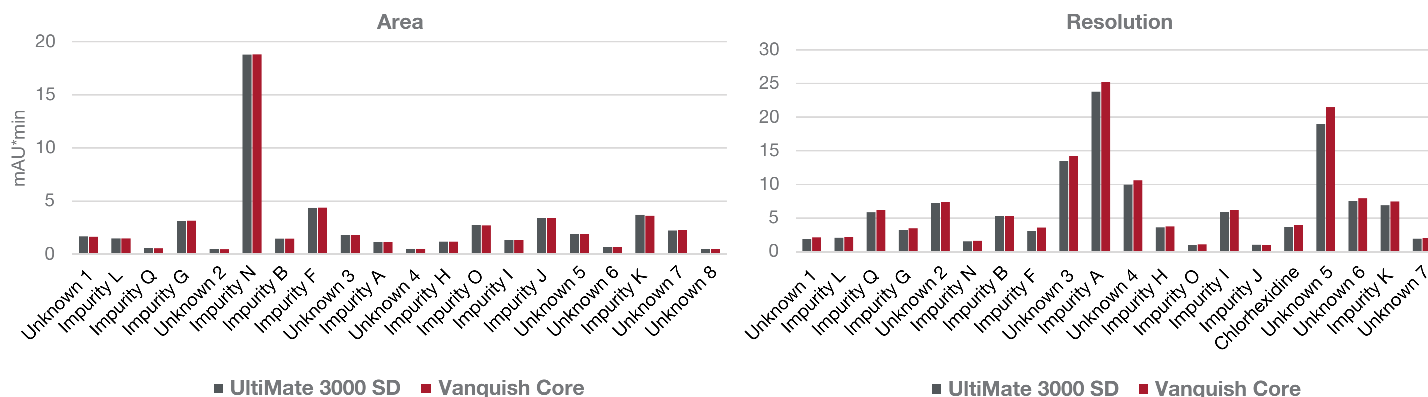

Figure 2. Chromatographic results with UltiMate 3000 SD and Vanquish Core HPLC systems under conditions outlined in the EP monograph (Figure 1)

However, the resolutions obtained by the Vanquish Core HPLC system are usually slightly higher than those obtained by the UltiMate 3000 SD system; this is mainly due to smaller peak widths caused by a lower dispersion volume of the Vanquish Core HPLC system. Both instruments provided equivalent repeatability of peak areas, expressed as relative standard deviations (RSD) over the six injections in Figure 3. Usually the RSD of peak areas was ≤0.5% with just one exception. The repeatability of retention times, however, was considerably improved by the Vanquish Core HPLC system as displayed in Figure 3. The RSDs of retention times for all peaks were lower than 0.03% with the Vanquish Core HPLC system, while they ranged up to 0.16% with the UltiMate 3000 SD system. The effect is also visualized in Figure 4.

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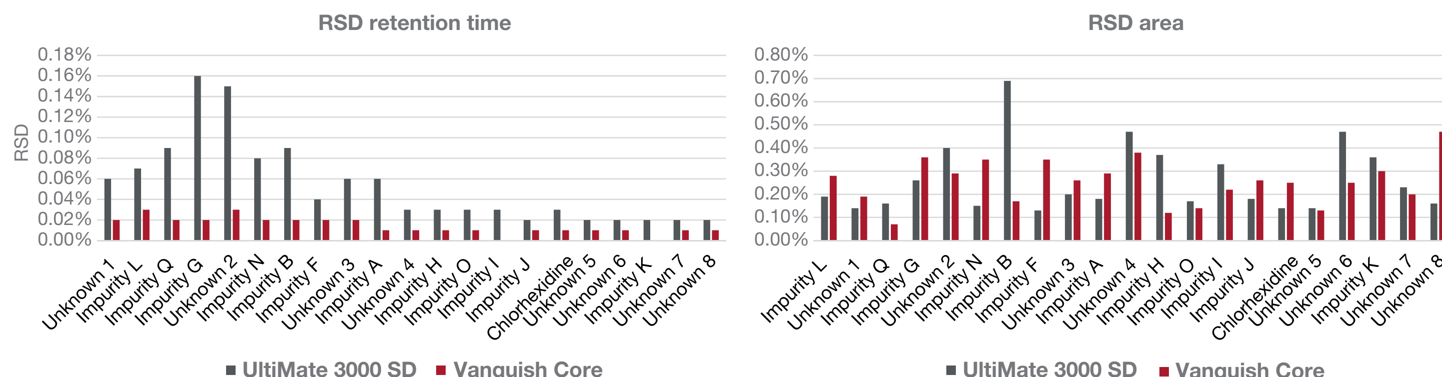

Figure 3. Relative standard deviations (RSD) of retention times and peak areas over seven injections obtained by the UltiMate 3000 SD and Vanquish Core HPLC systems

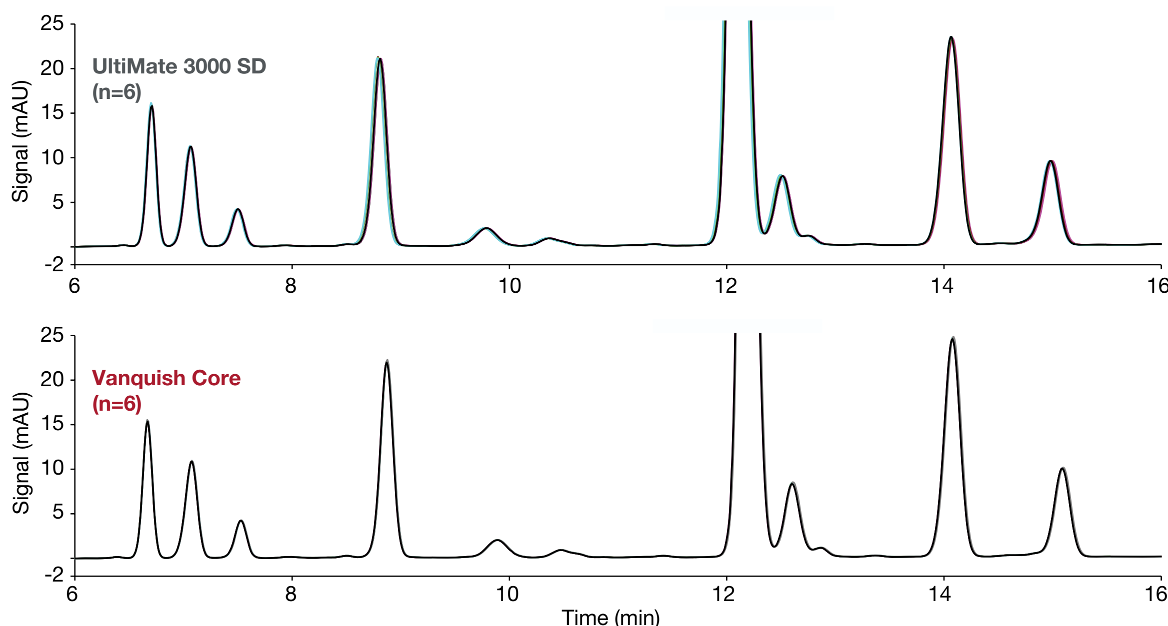

Figure 4. Overlay of six injections respectively by the UltiMate 3000 SD and Vanquish Core HPLC systems zoomed to a 10 min time segment, highlighting the improved analytical precision of the Vanquish Core HPLC system

The system suitability criteria given by the EP monograph, requiring a resolution of the impurity pair L and G of minimum 3 and a peak-to-valley ratio of impurity B of minimum 2, were easily met by either LC system with a resolution ~8 and a peak-to-valley ratio ~6 (UltiMate 3000 SD) and >7 (Vanquish Core). Thus, the EP method was successfully repeated with both systems and the transfer was successfully conducted without any method or hardware adaptions.

Conclusion

- The seamless transfer from a Thermo Scientific UltiMate 3000 Standard HPLC system to a Thermo Scientific Vanquish Core HPLC system was demonstrated for the EP method for chlorhexidine impurity analysis.
- Equivalent chromatographic outcomes were provided by the two systems. However, improved peak resolution and retention time repeatability was provided by the Vanquish Core HPLC system.

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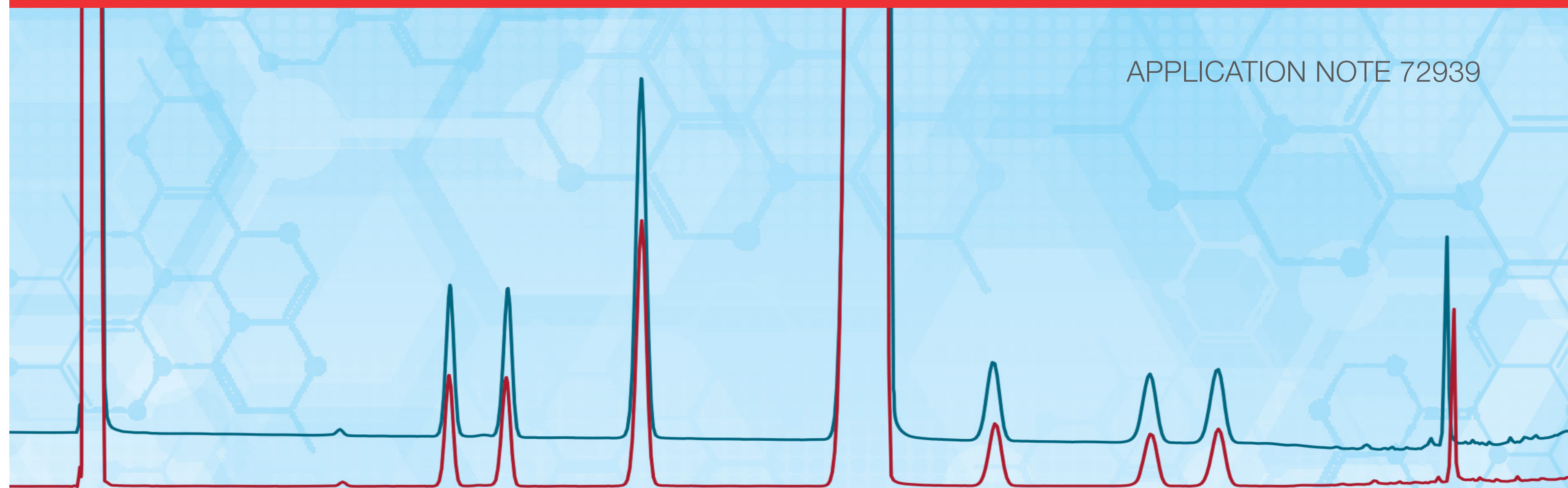

APPLICATION NOTE 72939

Transfer of an EP method for mebendazole from a Waters Acquity UPLC system to a Vanquish Horizon UHPLC system

Author
Maria Grübner

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Keywords
HPLC method transfer, Vanquish Horizon UHPLC system, Waters Acquity UPLC system, mebendazole, European Pharmacopoeia

Goal

To showcase the transfer of analytical HPLC methods from a Waters Acquity UPLC system to the Vanquish platform and highlight the impact of column thermostatting.

Application benefits

- Straightforward transfer of an EP monograph HPLC method from a Waters™ Acquity™ UPLC system to a Thermo Scientific™ Vanquish™ Horizon UHPLC system is demonstrated.
- During method transfer flexible thermostatting options provided by the Vanquish platform help to mimic the actual conditions at the original system.
- Substantial time and solvent savings are obtainable by speeding up conventional HPLC methods to UHPLC conditions without sacrificing chromatographic performance.

Introduction

Instrument-to-instrument transfer of liquid chromatographic (LC) methods is a challenging but frequently occurring task in most analytical laboratories. Within one lab, applications often need to be established at several instruments due to varying instrument availability and numbers of required analyses. Inter-lab transfers are commonly executed among method developing and method implementing laboratories.[1,2] In both cases, sending and receiving units can either equal or differ in configuration and vendor. Additionally, the replacement of legacy instruments by modern ones requires thorough method transfer, which is only effective if equivalent results are obtained.

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The success and required effort of a method transfer depend on multiple factors. The robustness of the transferred method plays an important role, especially when instrumentational variations affect the analysis.[1,2] It is well known that changing the pump type (low-pressure or high-pressure mixing) can have an impact on the separation, and also the gradient delay volume (GDV; hold-up volume from solvent mixing point at pump to column head) of a system is a commonly considered factor during method transfer.[3-5] However, other impacts, such as column thermostatting, are frequently underestimated but have a strong influence as will be shown in this application note. Furthermore, the claims of the chromatographer to the analytical outcome and the defined limits of acceptable deviations from the originating system add to the complexity of the transfer job.

In the following, an HPLC method for mebendazole impurity analysis according to the European Pharmacopoeia (EP) monograph[6] is transferred from a Waters Acquity UPLC system to a Thermo Scientific Vanquish Horizon UHPLC system. Mebendazole is a well-established anthelminthic drug for the treatment of various parasitic worm infestations. It is available as a generic drug and is listed on the World Health Organization's (WHO) Model List of Essential Medicines.[7]

The selected column is a Thermo Scientific™ Hypersil GOLD™ column that well complies with the requirement for a base-deactivated C18 column of the monograph.

Although we adhered to the EP monograph, the following discussions in general are also valid for the United States Pharmacopoeia (USP) method,[8] as the analytical method, i.e. column and gradient, are identical. The EP and USP monographs mainly differ in the preparation of sample solutions.

Experimental

Reagents and materials

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Scientific™ Acetonitrile, Optima™ LC/MS grade (P/N A955-212)
- Fisher Scientific N,N-Dimethylformamide, Acros Organics™, ACS reagent (P/N 10567942)
- Fisher Scientific Ammonium acetate, Optima LC/MS grade (P/N A115-50)
- EP reference standard: Mebendazole for system suitability CRS batch 1, catalogue code Y0000144[9]

Sample preparation

According to the monograph, 5 mg of the reference standard, which contained the active pharmaceutical ingredient (API) mebendazole and the impurities A, B, C, D, E, F, and G, were dissolved in 5 mL dimethylformamide (DMF).

Instrumentation

The instruments listed in Table 1 were used In the current study.

Table 1. Instrumentation standard configurations

	Acquity UPLC system	Vanquish Horizon UHPLC system
Pump	Binary Solvent Manager	Binary Pump H (P/N VH-P10-A)
Autosampler	Sample Manager	Split Sampler HT (P/N VH-A-10-A)
Sample Loop	10 µL	Default 25 µL (V=50 µL, P/N 6850.1911) or 10 µL (V=23 µL, P/N 6850.1915)
Column Compartment	High Temperature Column Heater	Column Compartment H (P/N VH-C10-A)
Detector	Tunable Ultraviolet Detector	Variable Wavelength Detector F (P/N VF-D40-A)
Flow Cell	Analytical (10 mm, 500 nL)	Semi-micro (7 mm, 2.5 µL, P/N 6077.0360)

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HPLC conditions

Column:	Hypersil GOLD, 4.6 × 100 mm, 3 µm, 175 Å (P/N 25003-104630)
Mobile Phase:	A: 7.5 g/L Ammonium acetate in water B: Acetonitrile
Flow Rate:	1.2 mL/min
Gradient:	0 min – 20% B 15 min – 30% B 20 min – 90% B 25 min – 90% B 25.1 min – 20% B 30 min – 20% B
Column Temp.:	40 °C (still air) with eluent pre-heating or as outlined elsewhere
Autosampler Temp.:	10 °C
Detection:	250 nm Vanquish Horizon: 10 Hz data collection rate, 0.5 s response time Acquity: 10 Hz data collection rate, normal filter time constant (0.2 s)
Injection Volume:	5 µL
Needle Wash:	Vanquish Horizon: Off Acquity: 200 µL Acetonitrile and 600 µL starting mobile phase

UHPLC conditions

Column:	Hypersil GOLD, 2.1 × 50 mm, 1.9 µm, 175 Å (P/N 25002-052130)
Mobile Phase:	A: 7.5 g/L Ammonium acetate in water B: Acetonitrile
Flow Rate:	0.8 mL/min
Gradient:	0 min – 20% B 2.35 min – 30% B 3.13 min – 90% B 3.91 min – 90% B 3.93 min – 20% B 4.7 min – 20% B
Column Temp.:	40 °C (still air) with eluent pre-heating or as outlined elsewhere
Autosampler Temp.:	10 °C
Detection:	250 nm Vanquish Horizon: 50 Hz data collection rate, 0.1 s response time Acquity: 40 Hz data collection rate, normal filter time constant (0.05 s)
Injection Volume:	1 µL
Needle Wash:	Vanquish Horizon: Off Acquity: 200 µL Acetonitrile and 600 µL starting mobile phase

Data processing and software

Thermo Scientific™ Chromeleon™ Software 7.2.9 Chromatography Data System (CDS) was used for data acquisition and analysis.

Results and discussion

Before a method transfer is started it is meaningful to take an in-depth review of the instrumentational differences of both systems and what kind of chromatographical differences could be expected from them. For example, in the current case the light paths of the detector flow cells differ by around 30%, so lower absolute peak heights and areas can be presumed for the Vanquish Horizon system. Additionally, instead of the passive mobile phase preheating that is accomplished by the column stabilizer assembly in the Acquity system, the Vanquish Horizon system has an active preheater available in the standard configuration, which may induce different thermal conditions at the column head. Furthermore, the Acquity system utilizes a pulled-loop Sample Manager with a 10 µL sample loop, while the Vanquish Horizon system autosampler is a split-loop design where sample loop with a default total loop volume of 50 µL and needle are part of the flow path. These sample loop differences as well as differences in pump mixing volumes translate into different GDVs, which may result in retention time shifts. Finally, differences in the pump design and flow control may cause minor deviations in the elution pattern.

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Transfer of EP method for mebendazole impurity analysis

For best comparability, all evaluations were conducted with the same column and sample and with five repeated injections. The chromatograms in Figure 1 display the comparison of both instruments under conditions as outlined in the EP monograph, and Table 2, Table 3, and Figure 2 summarize the chromatographic results. The relative retention times were well aligned with the EP monograph and in very good accordance with each other (see Table 3). In Figure 2 a full agreement on relative areas of impurity peaks is seen. The relative standard deviation (%RSD) of peak areas was not higher than 0.4% for the Vanquish Horizon system and equivalent or better than for the Acquity system. The signal-to-noise ratios of all impurity peaks were slightly higher for the Vanquish Horizon system despite the smaller light path length of the detector flow cell. Additionally, narrower peaks were produced by the Vanquish Horizon system and resolutions improved (Figure 2). The EP system suitability criterion of a peak-to-valley ratio of minimum 4 for the API and impurity D peaks was easily met by either system. Taking all of this together most chromatographers would rate this as a very successful method transfer without any special intervention and would conclude the method transfer evaluation.

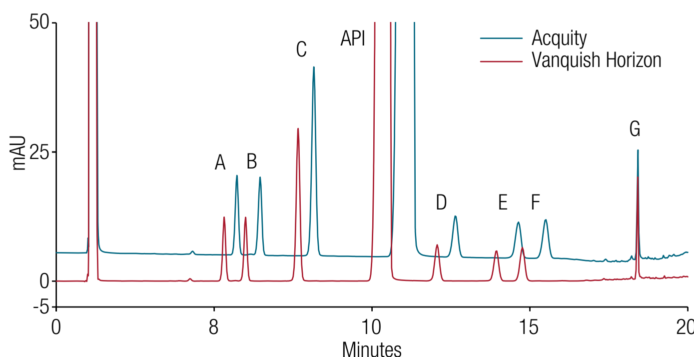

Figure 1. Transfer from Acquity system to Vanquish Horizon system according to EP monograph for mebendazole; peak assignment according to impurity designation in EP monograph

However, the deviations in absolute retention times (t_R) observed in Figure 1 and Table 2 might raise doubts or even pose an issue, if they exceed maximum acceptance limits defined in a certain lab. Thus, further elucidation is presented in a later section.

Table 2. Averaged absolute retention times in minutes over five injections for Acquity and Vanquish Horizon systems under conditions as outlined in EP monograph (Figure 1) and % deviation

Compound	Acquity	Vanquish Horizon
Impurity A	5.718	5.320 (Δ-7.0%)
Impurity B	6.454	5.996 (Δ-7.1%)
Impurity C	8.155	7.660 (Δ-6.1%)
Mebendazole (API)	11.225	10.526 (Δ-6.2%)
Impurity D	12.641	12.062 (Δ-4.6%)
Impurity E	14.635	13.938 (Δ-4.8%)
Impurity F	15.500	14.761 (Δ-4.8%)
Impurity G	18.425	18.417 (Δ-0.0%)

Table 3. Averaged relative retention times related to the API peak as stated in the EP monograph and from Acquity and Vanquish Horizon chromatograms (Figure 1)

Compound	EP monograph	Acquity	Vanquish Horizon
Impurity A	0.4	0.51	0.51
Impurity B	0.5	0.58	0.57
Impurity C	0.7	0.73	0.73
Mebendazole (API)	1.0	1.00	1.00
Impurity D	1.1	1.13	1.15
Impurity E	1.3	1.30	1.32
Impurity F	1.4	1.38	1.40
Impurity G	1.6	1.64	1.75

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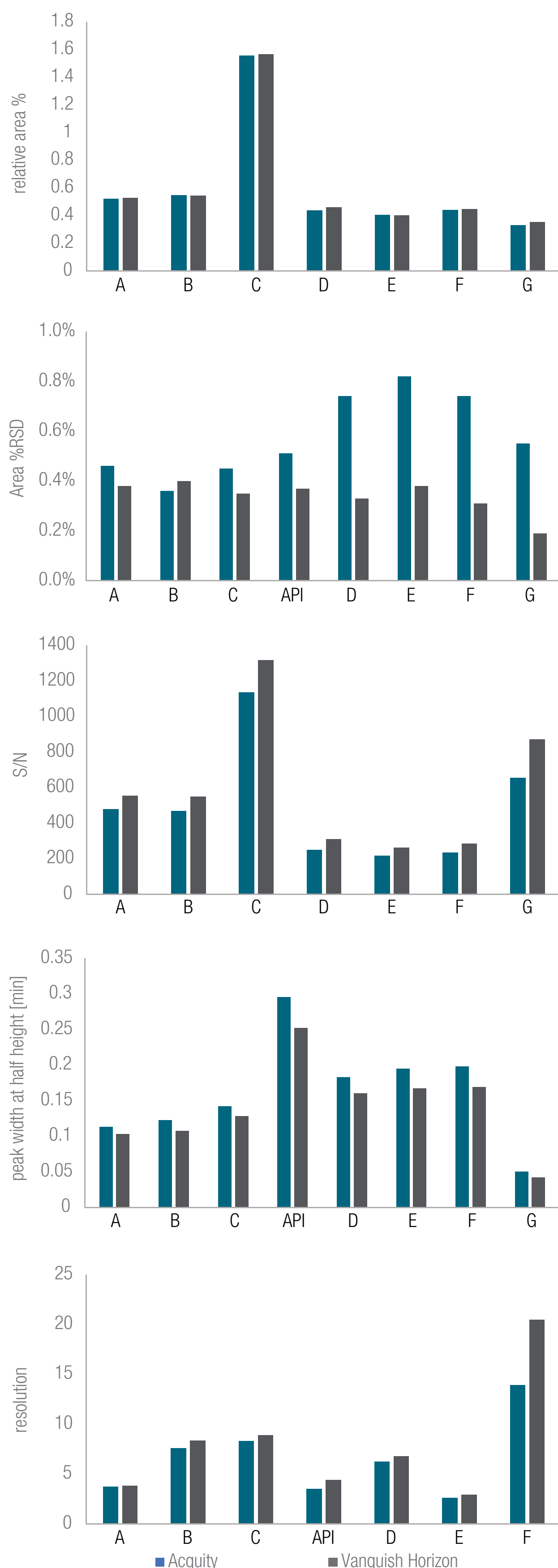

Figure 2. Chromatographic results with Acquity and Vanquish Horizon systems under conditions as outlined in the EP monograph (Figure 1); noise calculated from current chromatogram 2.0–3.0 min

Method scaling to UHPLC conditions

The fact that both systems utilized in the current study were designed to perform ultra-high-performance separations prompted the translation of the classical HPLC method for mebendazole analysis into a fast UHPLC method. Although currently EP and USP still refrain from permitting method scaling it is a worthwhile objective. A re-validation is required after such a translation but is usually justified by substantial savings in analysis time, solvent consumption, and costs. The speed-up method was easily calculated for the selected column dimension (2.1 × 50 mm, particle size 1.9 µm) by the new Thermo Fisher Scientific online tool.[10] Working with the Chromeleon CDS also offers the UHPLC speed-up calculator in the instrument method editing view. Savings of 90% eluent use and 84% run time resulted from the new method as depicted in Figure 3, which also shows the enormous gain in throughput. To reduce the GDV difference of both systems, the Vanquish Horizon system was now operated with a smaller sample loop (10 µL). The injection volume was set to 1 µL instead of the calculated 0.59 µL to deviate less from the Waters recommendation to use only injection volumes of 2–7.5 µL with the installed loop at the Acquity system.

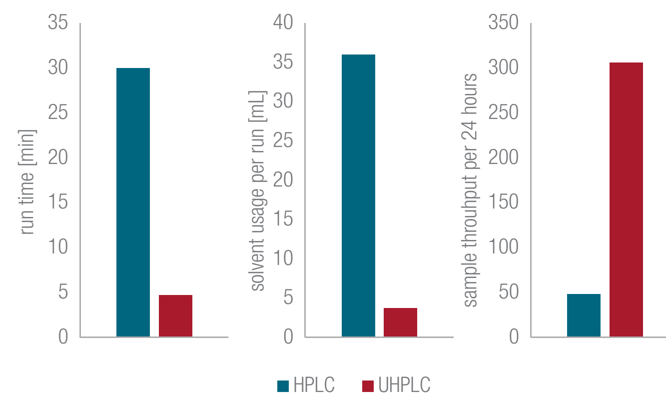

Figure 3. Comparison of EP HPLC method and speed-up UHPLC method with respect to analysis time, solvent consumption, and throughput

The obtained chromatograms are displayed in Figure 4. The relative retention times (related to the API peak) were in very good agreement with the original HPLC method; peak resolutions were only slightly decreased but never below 2.3, ensuring baseline separation of all peaks (Figure 5). The relative peak areas

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were well in line with the HPLC method results for the impurities A–F, which are structurally closely related to the API (Figure 5). In contrast, relative areas for impurity G were lower than under HPLC conditions as the dimer of the API impurity G structurally differs substantially from the other compounds. Hence a deviant UV response curve was expectable, resulting in different area ratios for different injection amounts.

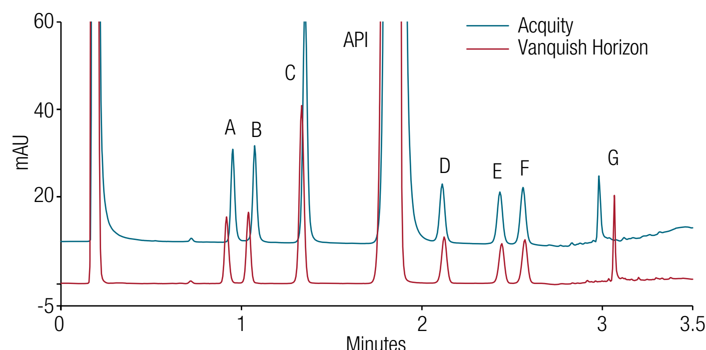

Figure 4. Chromatograms of down-scaled UHPLC method with Acquity and Vanquish Horizon systems; peak assignment according to impurity designation in EP monograph

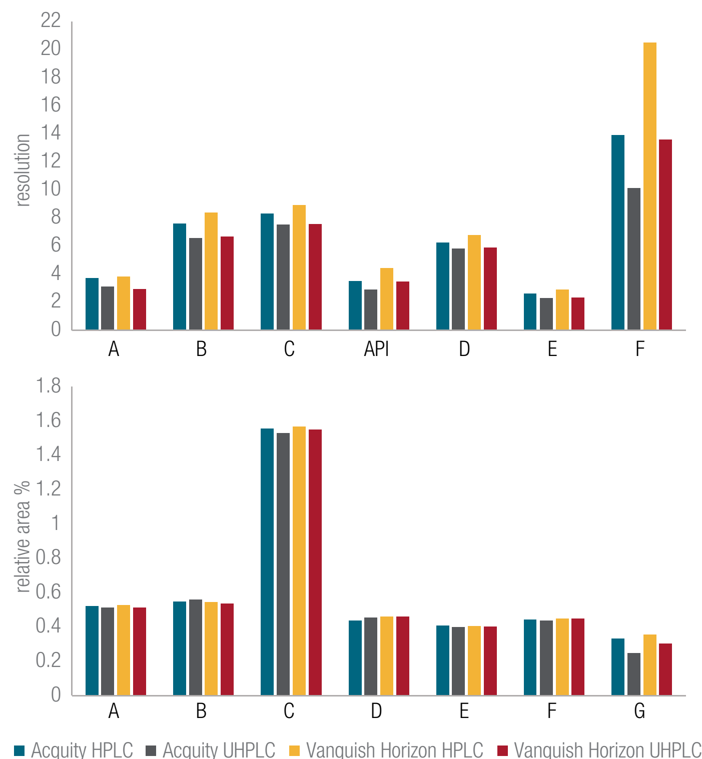

Figure 5. Peak resolution and relative peak areas for Acquity and Vanquish Horizon systems under HPLC conditions as outlined in the monograph and UHPLC conditions

The %RSD of peak areas was below 0.5% for the Vanquish Horizon system (Figure 6) and higher for the Acquity system; however, it should be noted that it was used outside its recommended injection volume range. Peak widths and S/N ratios were similar with both systems (Figure 6).

Figure 6. Chromatographic results with Acquity and Vanquish Horizon systems under UHPLC conditions (Figure 4); noise calculated from current chromatogram 0.45–0.60 min

Temperature effects on absolute retention times

As visible in Figure 1 and Figure 4, distinct deviations in absolute t_R were obtained with the Acquity and the Vanquish Horizon systems for the HPLC as well as the UHPLC methods. For the HPLC conditions, these are up to -7% for the Vanquish Horizon system (Table 1) and ranged from -3.9% to 2.9% for the UHPLC method.

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At first glance the early elution of the Vanquish Horizon system under HPLC conditions is surprising. On the one hand, because of the slightly larger GDV one would rather expect a later elution compared to the Acquity system. On the other hand, the deviations were too large to be explained just by GDV differences, as they would imply GDV differences of more than 500 µL for two systems that actually exhibit total GDVs of less than 200 µL. Thus, column thermostatting came into focus. To exclude such effects, the same methods as before were applied to both instruments but with column and column chambers equilibrated to ambient temperature (both instruments were located at the same air-conditioned lab, 2 m from each other). As shown in Figure 7A and D, the situation changed under the new conditions. Peaks eluted slightly later with the Vanquish Horizon system compared to the Acquity system as one could expect from a GDV perspective. These results gave evidence to deviating temperature conditions in the two column thermostats or eluent preheating devices when nominally set to 40 °C. The effective average temperature in the column appeared to be higher with the Vanquish system than with the Acquity system, causing earlier elution. The possible ways to go for a compensation and mimic the conditions of the Acquity instrument were 1) to adapt the column temperature setting, 2) to adapt the active preheater temperature setting, or 3) do both.

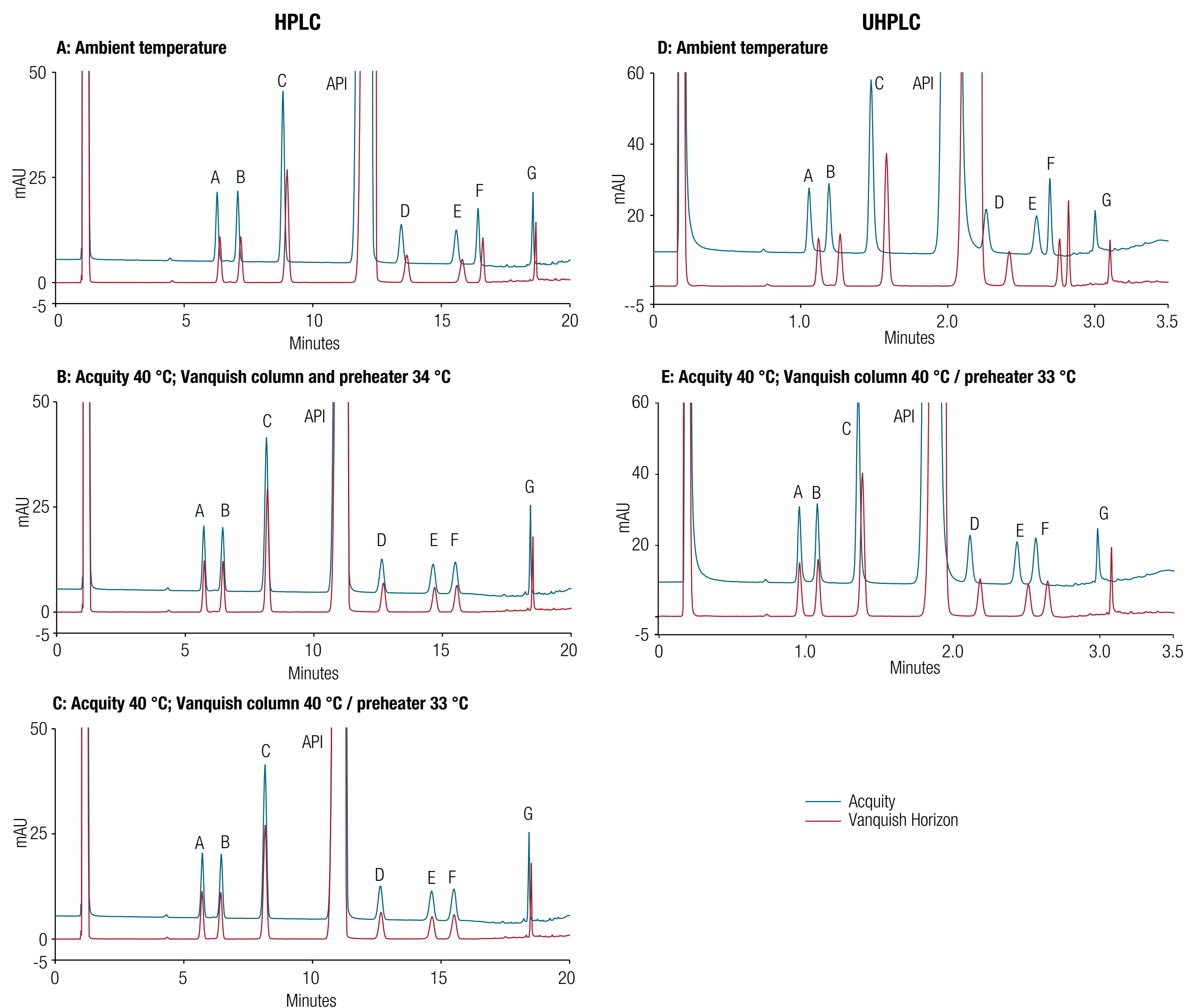

Figure 7. Temperature effects affecting the transfer from Acquity system to Vanquish Horizon system. (A–C) HPLC conditions; (D–E) UHPLC conditions; (A+D) ambient temperature; (B+C+E) Acquity chromatograms with column temperature set to 40 °C but Vanquish Horizon with adjusted temperature settings: (B) Vanquish Horizon column and preheater temperature set to 34 °C; (C) Vanquish Horizon column temperature set to 40 °C and preheater temperature set to 33 °C; (E) Vanquish Horizon column temperature set to 40 °C and preheater temperature set to 36 °C. Peak assignment is according to impurity designation in EP monograph.

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For the HPLC method a significant improvement of retention time overlay was achieved as depicted in Figure 7B and C and Figure 8. Reducing the column and preheater temperature at the Vanquish system to 34 °C resulted in t_R deviations of only 0.2% to 0.5% compared to the Acquity system at 40 °C (Figure 7B) and the deviations ranged from 0.3% to 0.5% when the column was kept at 40 °C but only the preheater was set to 33 °C (Figure 7C). In terms of pharmacopeial compliance either technique is applicable to a certain extent. Column temperature adjustments are permitted in a ±10 °C range in the USP guidelines, but only ±5 °C for gradient LC methods in the EP.[11,12] Thus, in the current application one should not go below 35 °C if EP compliance is required. Although the best t_R overlay was obtained at 34 °C, 35 °C will also generate a better t_R fit than keeping the Vanquish Horizon system at 40 °C. However, mobile phase preheating is not addressed in EP or USP monographs and hence the adaption of preheater temperatures is not regulated.

For the UHPLC method no temperature setting was found for the Vanquish Horizon system that generated an overlay of Acquity and Vanquish Horizon data as good as for the HPLC conditions. However, a smaller range of deviations (0.2–3.3%) could be attained by decreasing the active preheater temperature to 36 °C (Figure 7E and Figure 8). In UHPLC methods, as pressure, frictional heating, heat isolation, and heat dissipation play increasing roles, it is much more difficult to emulate the thermostatting of different systems.

Conclusion

- The successful transfer from a Waters Acquity UPLC system to a Thermo Scientific Vanquish Horizon UHPLC system was demonstrated for the EP method for mebendazole impurity analysis. The effort needed to obtain an adequate method transfer highly depends on the requirements of the user.
- Deviations of absolute retention times due to different effective temperatures in the column were compensated by adjustments of column oven temperature or mobile phase preheating temperature.
- Significant savings of 90% eluent consumption and 84% analysis time were obtained by method down-scaling to UHPLC conditions without compromising the chromatographical output.

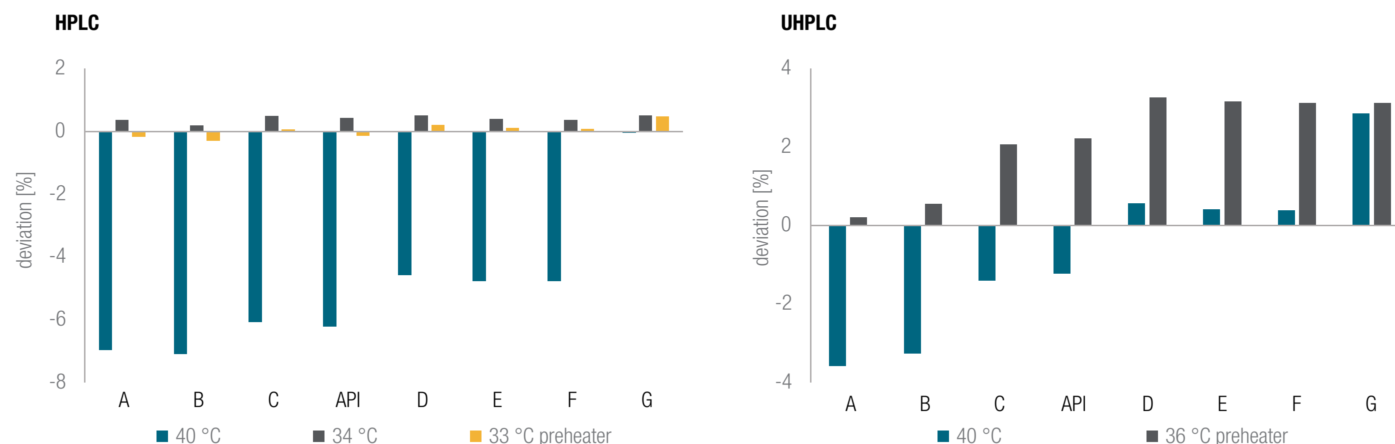

Figure 8. Summary of t_R deviations obtained with the Vanquish Horizon system at different column thermostatting settings with respect to the Acquity system set to 40 °C

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Transfer of a Heart Disease Treatment Analysis from an Agilent 1100 System to an UltiMate 3000 HPLC System

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Key Words
HPLC Method Transfer, Agilent 1100 System, UltiMate 3000 System, Gradient Application, Gradient Delay Volume Adjustment, Peak Dispersion, Peak Resolution

Goal

The goal of this Technical Note is to demonstrate a seamless transfer of a gradient HPLC method from an Agilent® 1100 HPLC system to a Thermo Scientific™ Dionex™ UltiMate™ 3000 HPLC system.

Introduction

Transfer of high performance liquid chromatography (HPLC) methods is common practice in analytical laboratories. Because an identical column format and chemistry are employed, users often expect the same chromatographic result; however, this is not always the case.[1] The transfer can involve different instruments, module generations,[2] laboratories, and companies, and the challenge related to it can therefore vary largely.

As instruments age and are no longer supported by vendors, like the 1100 Series from Agilent, which became obsolete at the end of May 2015, a situation can arise where an existing method needs to be transferred to a different instrument. Very often, one requirement for the method transfer is the best match to the previous chromatographic results. Many adverse effects encountered during the analytical method transfer can be traced to the instrument. One significant issue involves gradient separations that are in much more common use today than in the past.[3] Hence, this Technical Note focuses on comparative testing of a gradient HPLC application on a Quaternary Agilent 1100 system and a Quaternary UltiMate 3000 system.

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Experimental

Instrumentation

Agilent 1100 System	
Degasser:	G1322A Degasser
Pump:	G1311A QuatPump with standard mixer
Sampler:	G1367A WPALS
Sampler thermostat:	G1330B (in stack but not operated)
Column thermostat:	G1316A ColComp with 6 µL preheater
Detector:	G1315A DAD with analytical flow cell, 13 µL

Default capillaries were used for flow connections of the devices.

UltiMate 3000 SD System	
Degasser:	SRD-3400 (P/N 5035.9245)
Pump:	LPG-3400SD (P/N 5040.0031)
Sampler:	WPS-3000TSL (P/N 5822.0020)
Column thermostat:	TCC-3000SD (P/N 5730.0010) with 7 µL preheater
Detector:	DAD-3000 (P/N 5082.0010) with analytical flow cell, 13 µL
Mixer:	350 µL + 50 µL or 750 µL + 50 µL

Default Thermo Scientific™ Dionex™ Viper™ capillaries were used for flow connections of the devices.

Chromatographic Conditions and Settings	
Column:	Thermo Scientific™ Accucore™ XL column, C18, 4.6 × 150 mm, 4 µm, P/N 74104-154630
Mobile phase:	A: Water with 0.1% formic acid B: Methanol with 0.07% formic acid
Gradient:	

t [min]	%A	%B
0	90	10
10	20	80
11.5	20	80
12	90	10
17	90	10

Flow rate:	1.2 mL/min
Column temperature:	50 °C
Injection volume:	25 µL
UV detection wavelength:	214 nm
Data rate:	10 Hz
Response time:	0.5 s
Bandwidth:	4 nm
Slit width:	4 nm

Peak Identification and Concentration	
1. Hydrochlorothiazide	10 µg/mL
2. Chlorthalidone	20 µg/mL
3. Enalapril	60 µg/mL
4. Impurity	
5. Ramipril	60 µg/mL
6. Telmisartan	20 µg/mL
7. Azilsartan	20 µg/mL
8. Valsartan	20 µg/mL

Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software version 7.2

Results and Discussion

The same method parameters and the same column were used to separate the sample on the two instruments in the default configuration. Figure 1 shows a comparison of the obtained data. The red rectangles in the Agilent 1100 system data mark baseline artifacts resulting from the injection (left) and the gradient step at 12 min (right). These artifacts look very similar between the systems. More importantly, the chromatograms also look very similar, however peaks elute slightly earlier with the UltiMate 3000 system. This is a consequence of the optimized fluidics and the smaller gradient delay volume (GDV) of this system. Another consequence of the improved fluidics is that all peaks are higher and narrower.

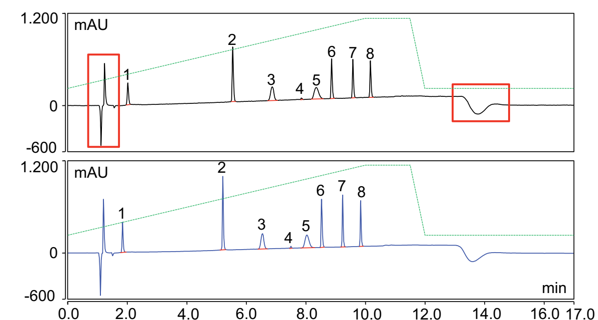

Figure 1. Gradient separation of heart disease treatment drugs performed on an Agilent 1100 system (black) and an UltiMate 3000 system (blue), both with default flow connections. The red rectangles indicate baseline artifacts caused by the injection and the final gradient step.

To increase the GDV and to shift the peaks closer toward the Agilent 1100 retention times, a larger mixer can be installed in the UltiMate 3000 pump. The UltiMate 3000 pump uses a flexible two-stage SpinFlow™ mixer with a radial and a longitudinal mixing part. Changing the mixing volume is both easy and fast. Different mixers covering a wide range of mixing volumes are available as shown in Table 1.

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Table 1. Available combinations of mixers and resulting mixing volume.

Description	SD Pumps* P/N	RS Pumps* P/N
Mixer for 35 µL mixing volume	6040.5000	6042.5000
Mixer for 100 µL mixing volume	6040.5100	6042.5100
Mixer for 200 µL mixing volume	6040.5110	
Mixer for 400 µL mixing volume	6040.5310	
Mixer for 800 µL mixing volume	6040.5750	
Mixer for 1550 µL mixing volume	6040.5450	

*except ISO-3100SD

We replaced the default 350 µL longitudinal mixer with a 750 µL mixer for a total mixing volume of 800 µL (P/N 6040.5750) to be more comparable with the Agilent 1100 system retention times. The overlay in Figure 2 shows how similar the peaks elute with this setup. Table 2 compares retention times of the peaks. Peaks 2-8 in Figure 2 have a maximum deviation of only 0.06 min; Peak 1 deviates by 0.19 min. The slightly pronounced retention time difference is likely to be caused by more efficient mobile phase pre-heating of the UltiMate 3000 system impacting the isocratic elution mechanism of the peak. If wanted, this difference could be reduced by a smaller volume pre-heater and by adding more extra column volume (ECV). However, this additional ECV would create more dispersion, reducing the improvements of the chromatography obtained with the UltiMate 3000 system (Table 3).

Table 2. Retention times obtained with Agilent 1100 and UltiMate 3000 systems (with 800 µL mixer).

Peak	Peak Name	Agilent 1100 System	UltiMate 3000 System	Retention Time Difference [min]
		Retention Time [min]	Retention Time [min]	
1	Hydrochlorothiazide	2.02	1.84	0.19
2	Chlortalidone	5.54	5.49	0.06
3	Enalapril	6.86	6.87	0.00
4	Impurity	7.85	7.78	0.07
5	Ramipril	8.35	8.41	-0.06
6	Telmisartan	8.86	8.92	-0.06
7	Azilsartan	9.58	9.55	0.03
8	Valsartan	10.17	10.15	-0.02

Table 3. Improvements on peak height, width, and resolution obtained with the UltiMate 3000 System (800 µL mixer) compared to the Agilent 1100 System.

Peak	Peak Name	Peak Height Improvement [%]	Peak Width Reduction (at 50% Peak Height) [%]	Resolution Improvement to Next Peak [%]
1	Hydrochlorothiazide	37%	12%	17%
2	Chlortalidone	31%	15%	23%
3	Enalapril	32%	19%	13%
4	Impurity	98%	19%	36%
5	Ramipril	22%	10%	11%
6	Telmisartan	3%	10%	-1%
7	Azilsartan	24%	19%	22%
8	Valsartan	30%	18%	n.a.

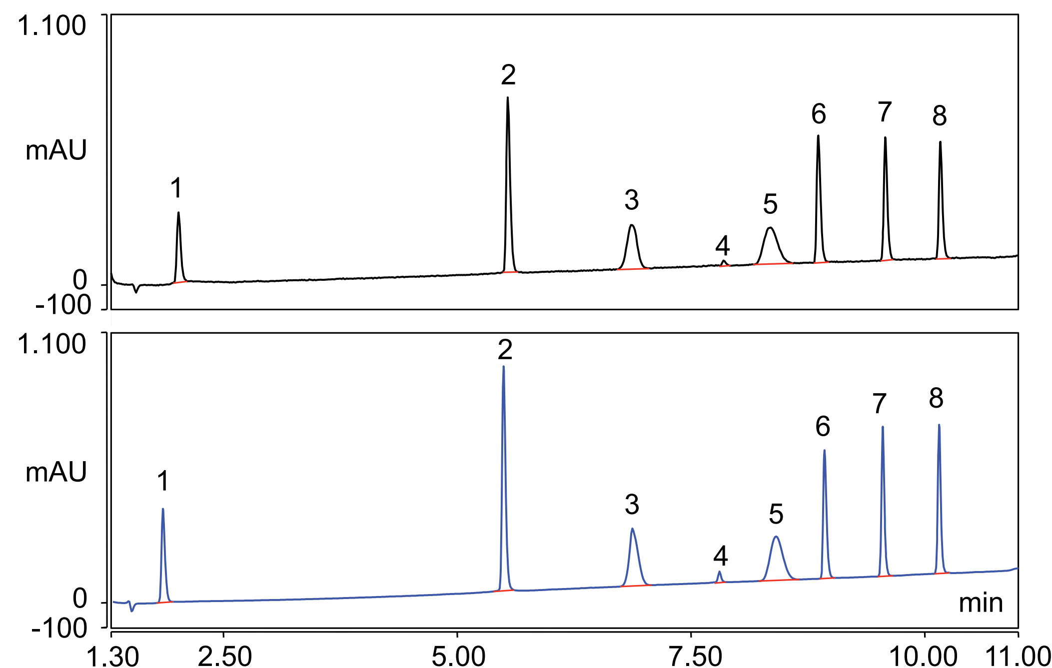

Figure 2. Gradient separation of heart disease treatment drugs performed on an Agilent 1100 system (black) and an UltiMate 3000 system (blue) with 800 µL mixer. The retention times obtained with both instruments match very well.

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Conclusion

The method transfer of a heart disease treatment gradient separation from an Agilent 1100 to an UltiMate 3000 system is exceptionally easy. After the installation of an 800 µL mixer, the peak retention times and the elution profiles are almost identical. At the same time, the UltiMate 3000 system creates less peak dispersion for higher and better resolved peaks.

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APPLICATION NOTE 72717

Method transfer of a USP derived acetaminophen assay from an Agilent 1260 Infinity system to an UltiMate 3000 SD system and a Vanquish Flex UHPLC system

Authors
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Keywords
HPLC method transfer, Vanquish Flex quaternary system, UltiMate 3000 Standard quaternary system, Agilent 1260 Infinity quaternary system, gradient delay volume, acetaminophen

Application benefits

- Flexible system volume adjustment in Thermo Scientific™ UltiMate™ 3000 systems and Thermo Scientific™ Vanquish™ UHPLC systems facilitate straightforward transfer of analytical HPLC methods.
- Fine tuning of retention times can be achieved by shifting the gradient start relative to injection time.
- If detection sensitivity is a critical issue, Thermo Scientific™ LightPipe™ technology provides an excellent remedy.

Goal

To demonstrate the straightforward transfer of analytical HPLC methods from an Agilent™ 1260 Infinity system to the UltiMate 3000 platform and the Vanquish platform.

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Introduction

The transfer of analytical liquid chromatographic (LC) methods from one instrument to another is a frequent but challenging task in most industries and is of particular importance in regulated environments.[1,2] Reasons for the need to transfer methods are manyfold, and procedures comprise application switching between the same or different types of instruments within the same laboratory, as well as transfers from legacy instruments to new ones due to replacement. Also, the transfer from developing laboratories to implementing laboratories of diverse location and equipment is very common. Proper transfer is only achieved if equivalent results are obtained with the sending and the receiving LC system.[1,2] The true complexity of this task highly depends on the robustness of the method to be transferred as well as on instrumentational differences of both systems.[1,2] To succeed in the challenge of maintaining retention times, resolutions, and other critical factors, specific technical characteristics of the systems like gradient delay volume (GDV), hydrodynamic behavior, or thermostatting mode must be taken into account. Additionally, as revalidation is time-consuming and expensive, modification of method parameters must be avoided as much as possible. Thus, hardware solutions become attractive features in method transfer.[3]

The current application note demonstrates the use of helpful features provided by the Thermo Scientific UltiMate 3000 and Vanquish platforms, like tunable GDVs and switchable thermostatting modes for the method transfer from another vendor's instrument (here the Agilent 1260 Infinity system). The selected application is derived from a USP assay for the analysis of the active pharmaceutical ingredient (API) acetaminophen, a common pain killer, and its impurities.[4] Analysis is performed with a Thermo Scientific™ Hypersil GOLD™ C8 stationary phase that matches the required USP level L7 and is well suited for analytes of medium hydrophobicity.

Experimental

Reagents and materials

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Scientific™ Optima™ Methanol, LC/MS grade (P/N 10767665)
- Fisher Scientific™ Sodium phosphate dibasic anhydrous (P/N 10182863)
- Fisher Scientific™ Potassium dihydrogen orthophosphate (P/N 10429570)
- Acetaminophen, 4-aminophenol, N-(4-hydroxyphenyl) propanamide (impurity B), 2-acetamidophenol (impurity C), acetanilide (impurity D), 4'-chloracetanilide (impurity J) were purchased from reputable vendors.

Sample preparation

Stock solutions of acetaminophen (20 mg/mL), 4-aminophenol, and the impurities B, C, D, and J (1 mg/mL each) were prepared in methanol. By dilution with methanol and mixing of stock solutions, a sample was prepared that contained 1 mg/mL acetaminophen and 10 µg/mL of each of the other compounds (corresponding to 1% of the API).

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Instrumentation

See Table 1 for the instruments used in this study.

Table 1. Instruments used in this study

Standard configurations			
	Agilent 1260 Infinity Quaternary	**UltiMate 3000 SD Quaternary**	**Vanquish Flex Quaternary**
			System Base (P/N VH-S01-A-02)
Pump	Quaternary Pump (G1311B)	Standard Quaternary Pump LPG-3400SD (P/N 5040.0031)	Quaternary Pump F (P/N VF-P20-A)
Sampler	High Performance Autosampler (G1367E) with thermostat module (G1330B)	Well Plate Autosampler WPS-3000TSL (P/N 5822.0020)	Split Sampler FT (P/N VF-A10-A)
Column Compartment	TCC with 6 µL heat exchanger (G1316A)	TCC-3000SD (P/N 5730.0010)	Column Compartment H (P/N VH-C10-A)
Detector	Diode Array Detector DAD VL (G1315D)	Diode Array Detector DAD-3000 (P/N 5082.0010)	Diode Array Detector FG (P/N VF-D11-A)
Flow Cell	Standard: 10 mm, 13 µL (G1315-60022)	Analytical: 10 mm, 13 µL (P/N 6082.0100)	Standard bio: 10 mm, 13 µL (P/N 6083.0540)
Hardware modifications applied for method transfer			
		• Add 7 µL eluent pre-heater (P/N 6722.0540) • Replace default static mixer 350 µL (P/N 6040.5310) by static mixer 750 µL (P/N 6040.5750)	• Replace default loop 25 µL (V=50 µL, P/N 6850.1911) by loop 100 µL (V=130 µL, P/N 6850.1913) • Modify idle volume from default 25 µL
Modifications applied for additional sensitivity enhancement			
			• Replace DAD FG by DAD HL (P/N VH-D10-A) with LightPipe standard flow cell (10 mm, P/N 6083.0100B) or LightPipe high sensitivity flow cell (60 mm, P/N 6083.0200)

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LC conditions

Column:	Hypersil GOLD C8, 4.6 × 100 mm, 3 µm, 175 Å (P/N 25203-104630)
Mobile Phase:	A: 1.7 g/L KH_2PO_4 and 1.8 g/L of Na_2HPO_4 in water B: Methanol
Flow Rate:	1 mL/min
Gradient:	0 min 1% B 3 min 1% B 7 min 81% B 7.1 min 1% B 12 min 1% B* (*when the UltiMate 3000 SD system was used with the 750 µL static mixer, equilibration was extended to 13 min)
Column Temp.:	35 °C (with eluent pre-heating)
Autosampler Temp.:	8 °C
Detection:	230 nm, 10 Hz data collection rate, 0.5 s response time
Inj. Volume:	1 µL
Needle Wash:	Off

Data processing and software

Thermo Scientific™ Chromeleon Software 7.2.8 Chromatography Data System was used for data acquisition and analysis.

Results and discussion

All method transfer experiments were conducted with the same column and sample, with consistent method parameters and seven repeated injections. The chromatograms in Figure 1 display the starting situation for the transfer from the Agilent 1260 Infinity system to the UltiMate 3000 SD system and to the Thermo Scientific™ Vanquish™ Flex system (all quaternary). The corresponding retention times are summarized in Table 2. In Figure 1a, Agilent 1260 Infinity system data are compared to data from the UltiMate 3000 Standard configuration system without an eluent pre-heater, and to data from the UltiMate 3000 system equipped with an optional 7 µL pre-heater. The distinct differences of both

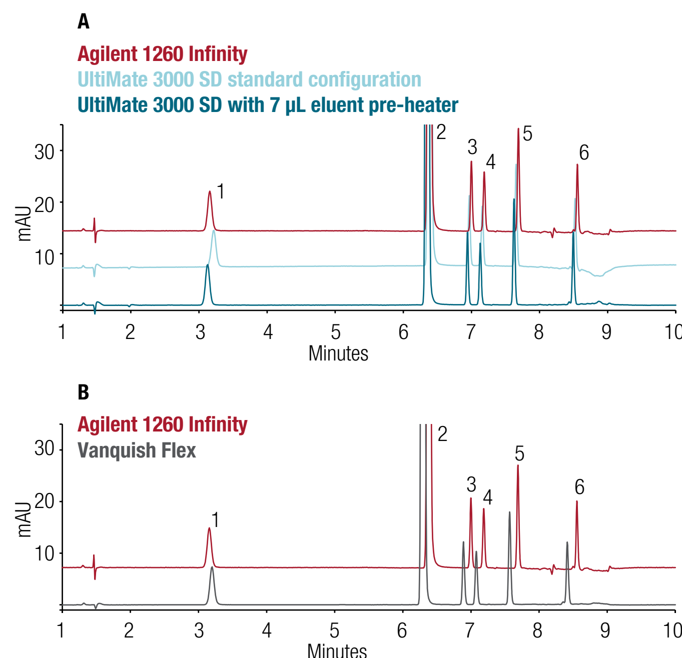

Figure 1. Starting situation of the method transfer. A) Chromatogram of Agilent 1260 Infinity system compared to UltiMate 3000 SD system in standard configuration and with optional eluent pre-heater; B) Chromatogram of Agilent 1260 Infinity system compared to Vanquish Flex system. For peak assignment see Table 2.

Table 2. Averaged retention times in minutes over seven injections for the systems and configurations stated in Figure 1 and % deviation for both pre-heated target systems from originating system

Peak No.	Compound	Agilent 1260 Infinity (originating system)	UltiMate 3000 SD system w/o pre-heating	UltiMate 3000 SD w/ pre-heating (target system)	Vanquish Flex (target system)
1	4-Aminophenol	3.16	3.21	3.13 (Δ 0.9%)	3.20 (Δ - 1.3%)
2	Acetaminophen (API)	6.38	6.39	6.34 (Δ 0.5%)	6.29 (Δ 1.3%)
3	Impurity B	7.00	6.97	6.94 (Δ 0.8%)	6.89 (Δ 1.6%)
4	Impurity C	7.19	7.16	7.13 (Δ 0.8%)	7.08 (Δ 1.5%)
5	Impurity D	7.69	7.66	7.63 (Δ 0.8%)	7.57 (Δ 1.6%)
6	Impurity J	8.56	8.52	8.50 (Δ 0.7%)	8.41 (Δ 1.7%)

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UltiMate 3000 system chromatograms clearly illustrate the noticeable impact of eluent thermostatting even at moderate separation temperatures. Thus, a successful method transfer should be conducted with adjusted thermostatting conditions and an installed pre-heater on the UltiMate 3000 system. This is especially emphasized by the behavior of the first peak (4-aminophenol), which elutes under isocratic conditions and is not affected by gradient effects. Without eluent pre-heating, it elutes later than on the Agilent 1260 Infinity system and approximates when pre-heating is applied. For the Vanquish Flex system an active pre-heater is included in the standard configuration and was activated for this method transfer, yielding similar retention of aminophenol (Figure 1b). In contrast, all peaks that elute during the gradient elute earlier on both Thermo Scientific instruments than on the Agilent 1260 Infinity system with enabled eluent pre-heating for the three systems. This is mainly due to a larger (and furthermore back-pressure dependent) GDV of the Agilent 1260 instrument. For that reason, a physical GDV adjustment by several features provided by the UltiMate 3000 and Vanquish portfolio is a promising way to minimize system differences for a successful method transfer.

Tables 3 and 4 give an overview of UltiMate 3000 SD and Vanquish system accessories available to stepwise modify system GDVs. For the transfer of the acetaminophen assay from the Agilent 1260 Infinity system to the UltiMate 3000 SD system, the default static mixer (350 µL) was replaced by the larger 750 µL mixer. As this volume difference overcompensated the GDV difference between

Table 3. Available UltiMate 3000 SD system consumables that can be used to modify the system GDV

Description	P/N
Mixer kit for pump 35 µL (25 µL capillary mixer +10 µL inline filter)	6040.5000
Mixer kit for pump 100 µL (25 µL capillary mixer + 75 µL static mixer)	6040.5100
Mixer kit for pump 200 µL (50 µL capillary mixer + 150 µL static mixer)	6040.5110
Mixer kit for pump 400 µL **(default configuration quaternary pump)** (50 µL capillary mixer + 350 µL static mixer)	6040.5310
Mixer kit for pump 800 µL (50 µL capillary mixer + 750 µL static mixer)	6040.5750
Mixer kit for pump 1550 µL (50 µL capillary mixer + 1500 µL static mixer)	6040.5450
Sample loop 25 µL (V=40 µL)	6820.2452
Sample loop 100 µL (V=130 µL) **(default configuration)**	6820.2451
Sample loop 250 µL (V=344 µL)	6820.2453
Sample loop 500 µL (V=667 µL)	6820.2454

Table 4. Available Vanquish system consumables that can be used to modify the system GDV

Description	P/N
Mixing system for pump 35 µL (25 µL capillary mixer + 10 µL inline filter)	6044.3870
Mixing system for pump 100 µL (25 µL capillary mixer + 75 µL static mixer)	6044.5100
Mixing system for pump 200 µL (50 µL capillary mixer + 150 µL static mixer)	6044.5110
Mixing system for pump 400 µL **(default configuration quaternary pump)** (50 µL capillary mixer + 350 µL static mixer)	6044.5310
Mixing system for pump 800 µL (50 µL capillary mixer + 750 µL static mixer)	6044.5750A
Mixing system for pump 1550 µL (50 µL capillary mixer + 1500 µL static mixer)	6044.5450A
Sample loop 10 µL (V=23 µL)	6850.1915
Sample loop 25 µL (V=50 µL) **(default configuration)**	6850.1911
Sample loop 100 µL (V=130 µL)	6850.1913

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sending and receiving instrument (see Figure 2a), a prestart of the gradient was then applied to match the retention times. With the prestart technique a smaller GDV can be emulated by shifting the point of injection relative to the method start. As the injection by definition is executed at 0.0 min, the method start is set to a negative time and all remaining steps of the method are shifted by the same value. Thus, no segment of the method is modified and the gradient table in total is not changed. For the current transfer, the extent of the time offset was -0.27 min and was derived from the average retention time difference of gradient-eluted peaks of the UltiMate 3000 system with the 750 µL static mixer and the Agilent 1260 Infinity system. Figure 2b illustrates the very good retention time match that was obtained by this technique, giving relative retention time deviations of <1% for aminophenol and <0.2% for peaks that elute in the gradient with respect to the originating method. While gradient-eluted peaks are shifted according to true or emulated GDV adjustments, peaks eluted under isocratic conditions are not affected. The minor difference in aminophenol retention thus might be the result of slightly different temperature conditions or proportioning of the isocratic conditions with 1 % of mobile phase B.

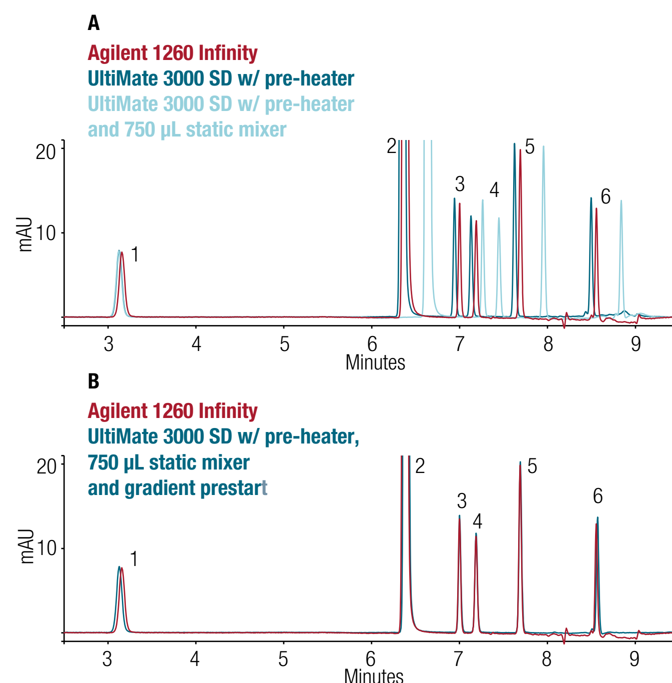

Figure 2. Method transfer from the Agilent 1260 Infinity system to the UltiMate 3000 SD system. A) Comparison of Agilent 1260 Infinity system and UltiMate 3000 SD system with eluent pre-heater and standard or larger static mixer; B) final transfer: comparison of Agilent 1260 Infinity system and UltiMate 3000 SD system with eluent pre-heater, larger static mixer, and gradient prestart. For peak assignment see Table 2.

In contrast, pre-starting the gradient was not necessary with the Vanquish Flex system to attain retention time congruence due to more flexible capabilities in GDV adjustment. At first the GDV difference of the Agilent 1260 Infinity and Vanquish Flex standard configured systems observed in Figure 1b was partially compensated by replacing the Vanquish standard sample loop by the 100 µL sample loop (actual GDV contribution 130 µL). The resulting retention times were closer to the originating instrument (see Figure 3a), and the remaining differences were in a range that could be offset by adjusting the idle volume of the autosampler metering device, the conducting unit of sample aspiration. This feature is unique to the Vanquish platform and can help in fine-tuning of the GDV as it is part of the sample loop flow path. The default idle volume setting of 25 µL was increased by 43 µL to a total of 68 µL, yielding the good alignment of retention times seen in Figure 3b with relative retention time deviations of 1.2 % for aminophenol and <0.4 % for peaks in the gradient.

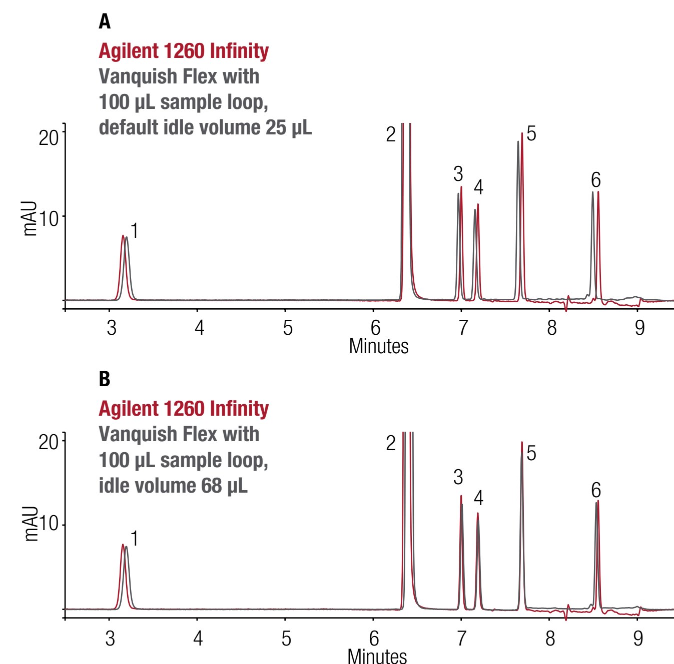

Figure 3. Method transfer from the Agilent 1260 Infinity system to the Vanquish Flex system. A) Comparison of Agilent 1260 Infinity system and Vanquish Flex system with 100 µL sample loop; B) final transfer: comparison of Agilent 1260 Infinity system and Vanquish Flex system with 100 µL sample loop and adapted idle volume to 68 µL. For peak assignment see Table 2.

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Another unique feature of Vanquish instruments is the switchable thermostatting mode of the column compartment, giving the choice of still or forced air column heating. The previous chromatograms were recorded in still air mode, as this reflects the thermostatting mode of the Agilent column compartment best. Figure 4 shows that for the current application the thermostatting mode has minor influence on retention times and is negligible here. However, in applications of higher pressure ranges (ultra-high-performance LC, UHPLC, > 600 bar) where frictional heating of the column becomes relevant, the column compartment mode is of certain importance.[3]

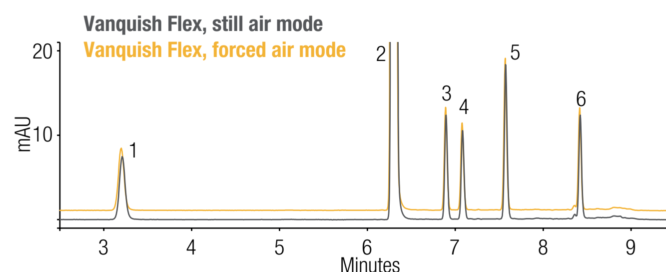

Figure 4. Negligible effect of column thermostatting mode for Vanquish Flex system in still and forced air mode. For peak assignment see Table 2.

In conclusion, retention times were successfully transferred from an Agilent 1260 Infinity instrument to an UltiMate 3000 SD instrument and a Vanquish Flex instrument by means of physical or simulated GDV adaption. This is in full agreement with the allowed adjustments according to the USP General Chapter <621>, which states: "If adjustments are necessary, a change in […] the duration of an initial isocratic hold (when prescribed), and/or the dwell volume are allowed."[5] Furthermore, critical chromatographic results were easily maintained during the transfer. The resolution of the critical pair of impurity B and C was 3.2 or better in all tested scenarios, and peak tailing factors ranged from 0.99 to 1.12. The relative standard deviation of peak heights was always far below 1% (Figure 5a). Thus, USP system suitability was accomplished by all three systems, both with and without GDV adaption. The relative areas of all impurity peaks were constant over the three instruments (Figure 5b).

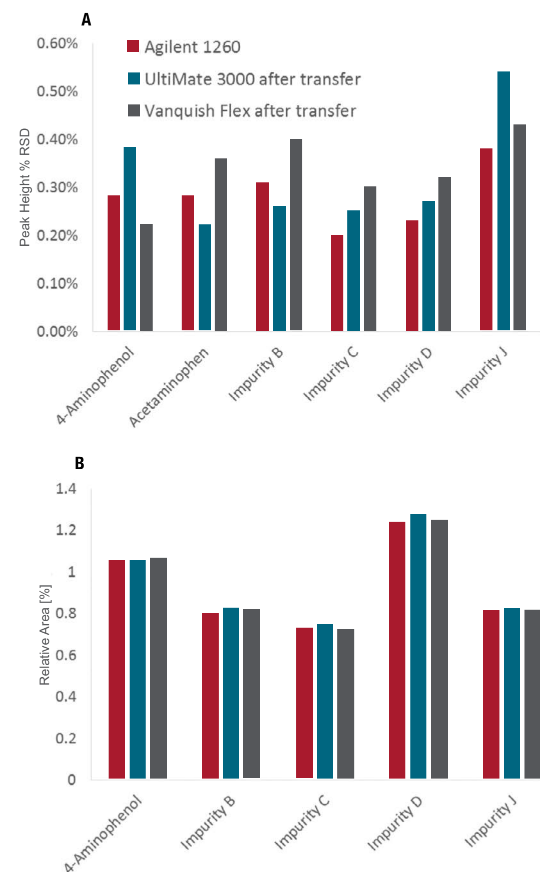

Figure 5. Averaged peak height precision (A) and relative areas of impurity peaks (B) for the originating system Agilent 1260 Infinity, UltiMate 3000 SD system after method transfer optimization (750 µL static mixer and gradient prestart), and Vanquish Flex system after method transfer optimization (100 µL sample loop and idle volume 68 µL)

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In Figure 6, the signal-to-noise (S/N) ratios of the transferred method are summarized, illustrating a distinct improvement of S/N performance from the originating system to the Ultimate 3000 SD system and the Vanquish Flex system in the present configuration. As an alternative to the Thermo Scientific™ Vanquish™ DAD FG, the Thermo Scientific™ Vanquish™ DAD HL provides an outstanding S/N performance driven by Thermo Scientific™ LightPipe™ technology, which is demonstrated by the additional bars in that graph. These results were obtained with the same Vanquish system as before but with a swapped detector; with both the standard flow cell with equal light path length as the three previous systems of 10 mm and the high sensitivity flow cell with 60 mm light path. While the S/N enhancement by the standard LightPipe flow cell is mainly caused by further noise reduction, the enormous gain with the high sensitivity flow cell is particularly generated by the sensitivity gain due to the long light path. This cell is especially suited for analyses with columns of 4.6 mm inner diameter.[6] Thus, the DAD HL is very suitable for the analysis of low-abundant impurities, and if S/N performance or sensitivity are of critical concern, the utilization of that dedicated DAD technology is highly recommended.

Conclusions

- During method transfer of an acetaminophen assay from an Agilent 1260 Infinity system to an UltiMate 3000 SD system as well as to a Vanquish Flex system (all quaternary), straightforward retention time matches were achieved by true and emulated GDV adjustments by diverse tools provided by the Thermo Scientific platforms, like exchangeable eluent pre-heaters, pump mixers, sample loops, and adjustable autosampler idle volume.
- Further critical chromatographic results like resolution of critical peak pair, peak asymmetries, peak height precision, and relative peak areas were easily maintained during transfer. Signal-to-noise ratios improved distinctly during the transfer.
- If detection sensitivity of the method is of particular concern, the utilization of DAD LightPipe technology is recommended for LC-UV applications.

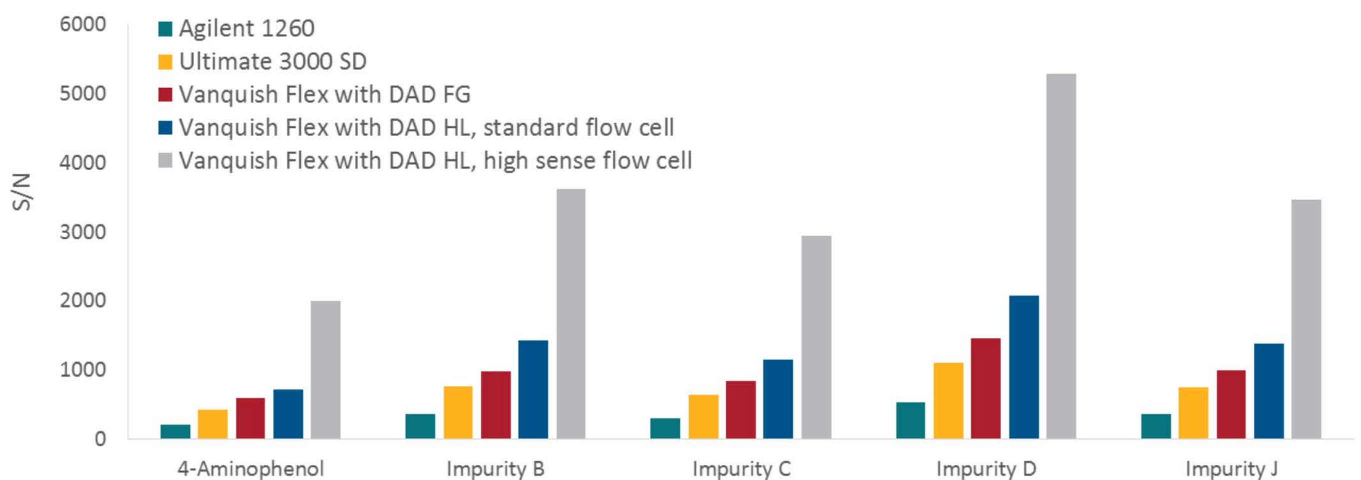

Figure 6. Signal-to-noise ratios (S/N) obtained with Agilent 1260 Infinity system, Ultimate 3000 SD system after method transfer optimization (750 µL static mixer and gradient prestart), Vanquish Flex system after method transfer optimization (100 µL sample loop and idle volume 68 µL) with DAD FG, DAD HL with 10 mm flow cell, and DAD HL with 60 mm high sense flow cell. Noise calculated from the current chromatogram 4.1–4.6 min.

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Transferring a Method for the Characterization of PTMs from the UltiMate 3000 BioRS System to the Vanquish Flex UHPLC System

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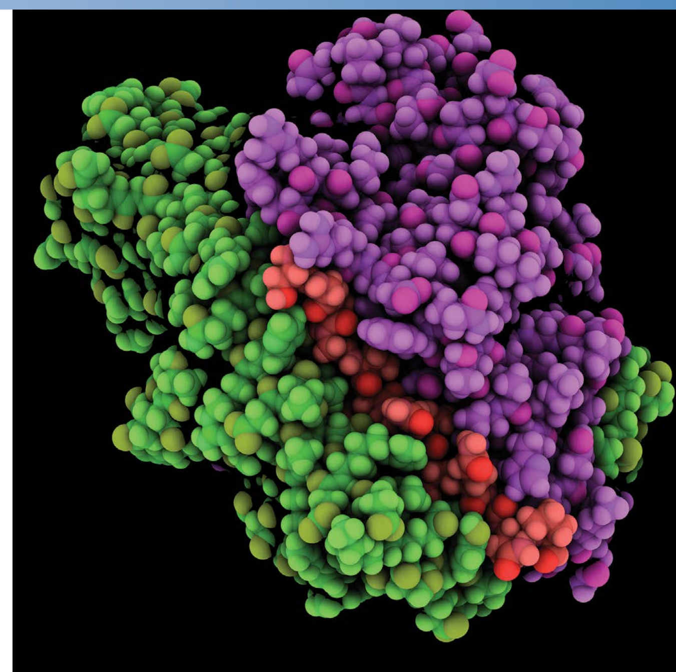
Key Words
Method Transfer, Thermo Scientific ProPac WCX-10, Biocompatible UHPLC, Protein Digest, Biotherapeutics Characterization, Biopharma, Deamidation

Goal
Demonstrating a successful transfer of an HPLC protein deamidation monitoring method from the Thermo Scientific™ UltiMate™ 3000 BioRS UHPLC to the Thermo Scientific™ Vanquish™ Flex UHPLC system.



Introduction
Therapeutic proteins play a key role in today's health care methods. Antibodies are especially suited for complex tasks in biological medication. The majority of biologics registered for therapeutic use in the last 15 years are antibodies. Antibodies have a molecular weight of about 150 kDa and are composed of approximately 500 amino acids. Due to their high molecular complexity, they can be affected by post-translational modifications (PTMs) like N- and O-glycosylation, deamidation, disulfide bonds, or oxidation. These can impair the biological functionality and reduce the efficacy. Deamidation is one of the most common PTMs resulting in a conversion of asparagine to aspartic acid. The change in the amino acid composition can cause allergy and affects the therapeutic protein in half-life, stability, and pharmacological activity.[1-3] According to regulatory guidelines, pharmaceutical companies have to ensure consistency and quality of their products. Therefore, checking for amino acid changes is mandatory.

Ion-exchange chromatography is a frequently used technique for the separation of deamidated therapeutic proteins and charge variants in general.[4] Further, enhanced loading capacity in comparison to reversed-phase chromatography favors the use of cation-exchange chromatography. As an additional benefit, the separation can be performed under native conditions for the evaluation of biological activity.

Analytical method transfer is one of the most discussed topics in regulated laboratories. After a developed method is validated and implemented in an industrial workflow, the method can be transferred considering specified criteria and regulatory guidelines to other systems.[5] Depending on the acceptance criteria, the receiving unit has to achieve the same validated results as in the original method.

A challenge in method transfer is to mimic different instrumental types with respect to gradient delay volume, hydrodynamic behavior, and thermostatting. The Vanquish Flex UHPLC system provides two different thermostatting modes as well as active and passive mobile phase pre-heating and an adjustable gradient delay volume. These instrumental features substantially support and facilitate method transfer, leading the best match of retention times, resolution, and peak area in compliance with validation criteria.

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In this application note we show an easy method transfer from the UltiMate 3000 BioRS UHPLC system to the Vanquish Flex UHPLC system. Both have entirely biocompatible fluidics providing high robustness and reliability, especially under harsh conditions like high salt concentrations. The Thermo Scientific™ ProPac™ WCX-10 column was used to provide high resolution, even for samples that differ only in one charged residue, and to minimize secondary (nonionic) interactions.

Experimental

Sample Preparation

The deamidation was forced by using a 1% ammonium bicarbonate solution. First, 15 mg of ribonuclease A (bovine pancreas) was dissolved in 1 mL starting conditions to get a protein concentration of 15 mg/mL. Then, 334 µL of the protein solution, 100 µL of 10% ammonium bicarbonate (w/v), and 566 µL of deionized water were combined in a 2 mL tube to create a final ribonuclease A concentration of 5 mg/mL. The tubes were placed in a thermo shaker for 10 minutes at 37 °C and aliquots were taken afterwards.

Instrumentation

Vanquish Flex UHPLC system consisting of:

- System Base (P/N VF-S01-A)
- Quaternary Pump F (P/N VF-P20-A)
- Split Sampler FT with 25 µL Sample Loop (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Active Pre-heater, 0.1 × 380 mm, VH-C1 (P/N 6732.0110)
- Diode Array Detector HL (P/N VH-D10-A) equipped with LightPipe™ Standard Flow Cell (P/N 6083.0100)

UltiMate BioRS 3000 system consisting of:

- LPG-3400RS Pump (P/N 5040.0036)
- WPS-3000TBRS Well Plate Autosampler with 25 µL Sample Loop (P/N 5841.0020)
- TCC-3000RS Thermostatted Column Compartment (P/N 5730.0000)
- Passive Pre-heater (P/N 6722.0540)
- DAD-3000RS Detector (P/N 5082.0020)
- Semi-micro Flow Cell for DAD, PEEK 2.5 µL volume, 7 mm pathlength (P/N 6082.0500)

Default Thermo Scientific™ Viper™ capillary fittings were used for flow connections of the devices.

Chromatographic Conditions	
Column	ProPac WCX-10 Analytical, 4 × 250 mm (P/N 054993)
Mobile Phase A	10 mM sodium phosphate in water, pH 6.0
Mobile Phase B	10 mM sodium phosphate, 1 M sodium chloride in water, pH 6.0
Gradient	0–30 min: 4–70% B; 30–40 min: 70–75% B; 40–42 min: 75–4% B; 42–55 min: 4% B
Flow Rate	1.00 mL/min
Temperature	30 °C (Forced air mode for Vanquish Flex system)
Maximal Pressure	152 bar (2204 psi)
Injection Volume	10 µL
Detection	280 nm
	Data Collection Rate: 10 Hz
	Response Time: 0.4 s

Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software, version 7.2 SR3, was used for data analysis.

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Results and Discussion

A salt-based gradient for a cation-exchange chromatography method was used to separate the deamidation products in a generic gradient program. The deamidation monitoring for both systems is shown in Figure 1. The chromatogram shows a match for all seven peaks. Comparing the two systems, the average relative deviation in retention time is 0.34% and there is an excellent standard deviation for the internal system reproducibility with three runs (Table 1). Moreover, the peak area ratio between the main variant of ribonuclease A and the two deamidation products (Peaks 2 and 3) match in this analytical method transfer with a deviation of 0.25% on average. The deamidation products as a percentage of the main peak area were 1.2% for Peak 2 and 3.3% for Peak 3 on the UltiMate 3000 BioRS system, and 1.4% and 3.6%, respectively, on the Vanquish Flex system. The resolution between the two deamidation products was 1.7 for both systems.

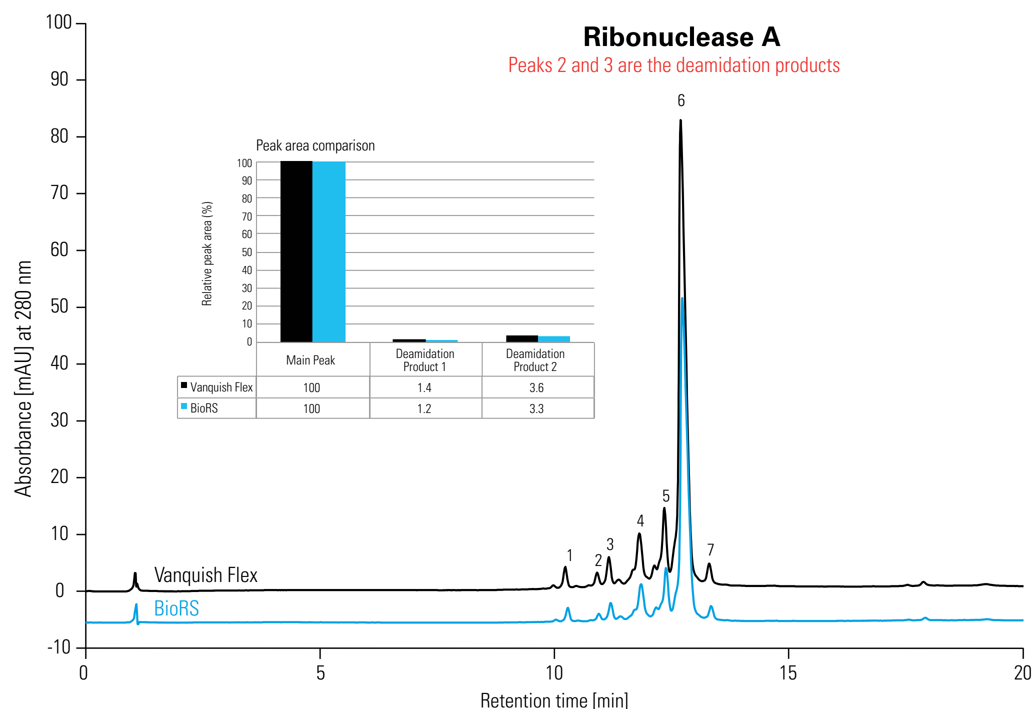

Figure 1. Overlay of a deamidation monitoring measured on the Vanquish Flex (black trace) and the UltiMate 3000 BioRs (blue trace) systems.

Table 1. Retention time data and deviations for both UHPLC systems. The relative deviation in retention time was calculated by dividing the delta in retention time by the average in retention time between both UHPLC systems (N=3).

Peak	Average Retention Time Vanquish Flex System [min]	Standard Deviation Vanquish Flex System [min]	Average Retention Time UltiMate 3000 BioRS System [min]	Standard Deviation UltiMate 3000 BioRS System[min]	Δ Average Retention Time [min]	Relative Deviation Retention Time Between Systems [%]
1	10.231	0.002	10.284	0.001	0.053	0.52
2	10.909	0.001	10.947	0.003	0.038	0.34
3	11.159	0.001	11.201	0.001	0.041	0.37
4	11.811	0.001	11.848	0.005	0.037	0.31
5	12.344	0.001	12.378	0.004	0.034	0.28
6	12.690	0.000	12.726	0.003	0.036	0.28
7	13.302	0.002	13.339	0.001	0.037	0.28

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Conclusion

Easy method transfer of a post-translational modification characterization from the UltiMate 3000 BioRS system to the Vanquish Flex system was shown. The average relative deviation in retention time between the methods on both UHPLC systems was 0.34%. Furthermore, the relative peak area ratios of the deamidation products deviate by only 0.25% between the sending unit (UltiMate 3000 BioRS system) and the receiving unit (Vanquish Flex system). Different thermostatting options and an adjustable gradient delay volume in the Vanquish Flex system provide the ability to physically recreate hardware conditions for successful method transfer.

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Get more from your liquid chromatography

No trade-offs in performance, robustness or ease of use

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Designed with innovative technology and attention to fine detail, Thermo Scientific™ Vanquish™ HPLC and UHPLC systems are the most advanced LC instruments available. The Vanquish systems improve performance and repeatability with no trade-offs in quality, robustness, or ease-of-use. Regardless of which Vanquish HPLC or UHPLC system is used, chromatographers have every tool they need to solve the toughest analytical challenges with confidence.

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- **Detect the lowest analyte quantities**—Higher detector sensitivity and lower baseline noise
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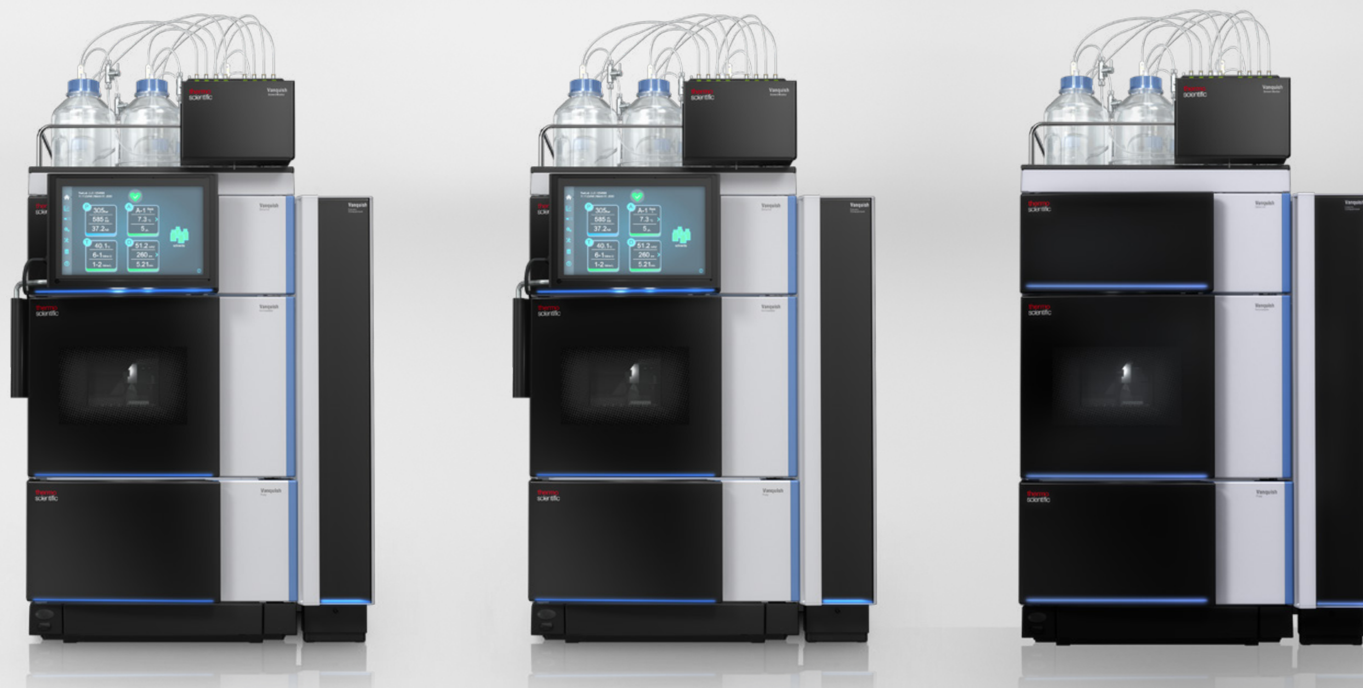
Run more samples, unattended

Designed for any application—whether HPLC or UHPLC, small or large molecules, simple or complex mixtures—the Vanquish platform was designed to handle it all. This innovative, simple-to-operate, and easy-to-maintain platform delivers confident separations and pushes chromatographic boundaries. Regardless of the configuration you choose, you get a highly integrated solution with optimized fluidics, providing unsurpassed retention time and peak area precision.



Thermo Scientific™ Vanquish™ Core HPLC system

Absolute dependability to enable worry-free applications

Thermo Scientific™ Vanquish™ Flex UHPLC systems

Complete flexibility for method development or fast and reliable UHPLC

Thermo Scientific™ Vanquish™ Horizon UHPLC system

Unrivaled performance and throughput for applications requiring high-end UHPLC

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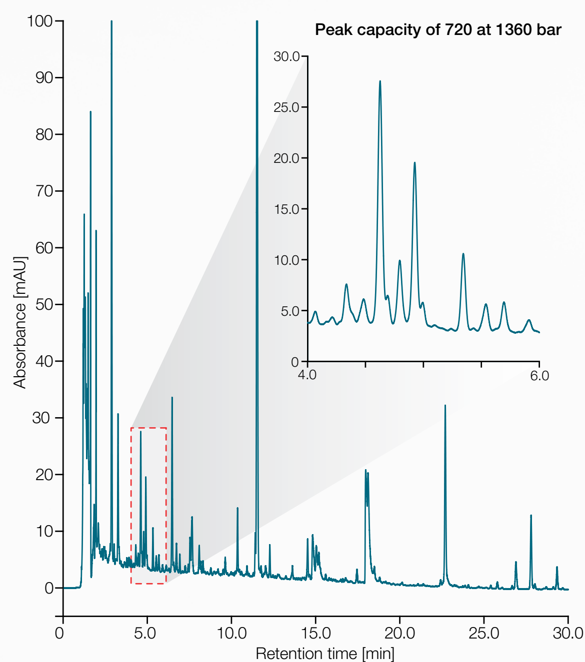
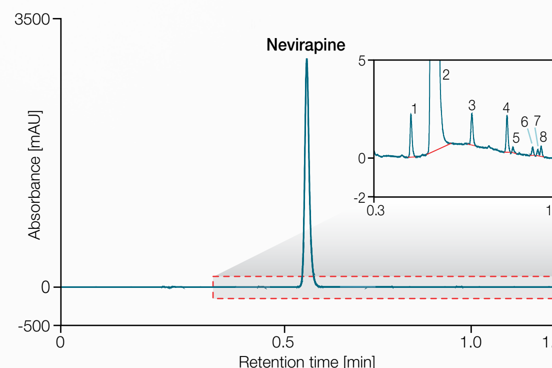
Deliver results without compromise

Vanquish Horizon UHPLC system

The Vanquish Horizon UHPLC system is an integrated, fully biocompatible, state-of-the-art binary UHPLC system with ultra-low gradient delay volume, designed to provide unrivaled performance for high resolution and high-throughput LC and LC-MS applications.



Ultra-high peak capacity for a complex herbal extract analysis.

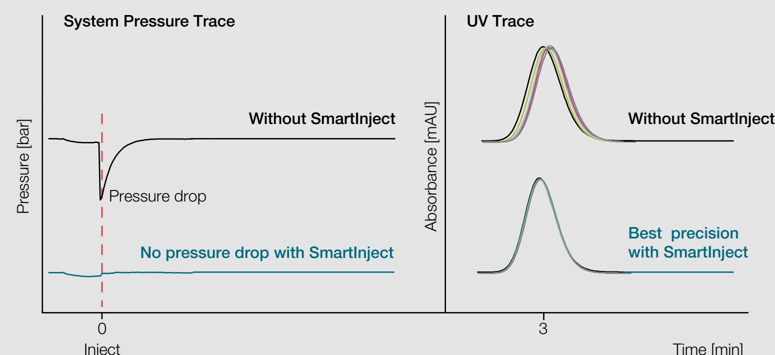


Impurity profiling of nevirapine (0.66 mg/mL) with UHPLC gradient and zoom into the baseline to show related impurities.

Why SmartInject Technology?

During injections, the sample loop at atmospheric pressure is placed in line with the high-pressure flow path, typically resulting in a pressure drop. With regular UHPLC systems, this adversely affects retention time precision and column lifetime.

Thermo Scientific™ SmartInject Technology automatically eliminates the pressure drop, improving data confidence and reduced cost of ownership.



Significant reduction of pressure drop after sample injection using SmartInject Technology resulting in improved retention time reproducibility (6 replicates) and enhanced column lifetime.

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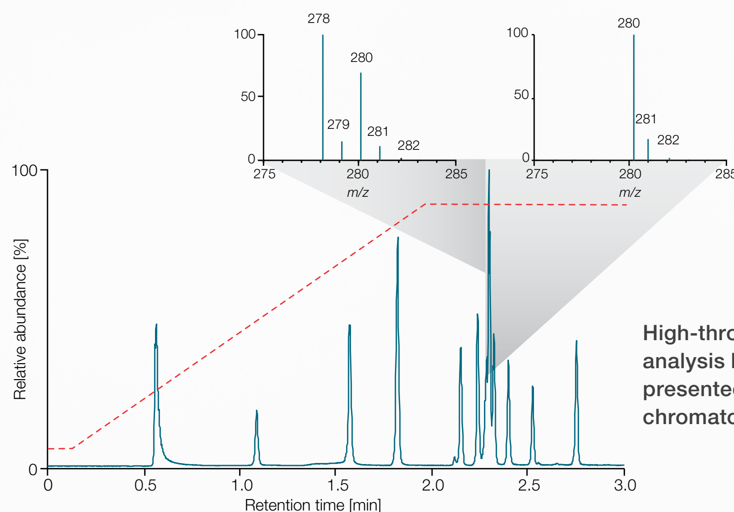
Easier method development and routine analysis

Vanquish Flex UHPLC systems

The Vanquish Flex systems offer binary and quaternary solvent blending. Binary solvent blending results in low gradient delay volumes ideal for routine UHPLC and LC-MS applications. Quaternary solvent blending allows multicomponent gradient formation for flexible UHPLC method development.



High-throughput pesticide analysis by full scan LC-MS presented as extracted ion chromatogram.

Vanquish Core HPLC system

Vanquish Core HPLC systems offer highly dependable HPLC analysis for binary, quaternary and isocratic solvent blending. A new level of user experience, the highest system productive time, and its ease to adopt within any given laboratory infrastructure make it ideal for all traditional HPLC analyses.

= System Suitability Test (SST) passed

Test sites of a Vanquish Core HPLC system round robin test using eight different instruments with individual samples, users, and columns. Each analysis passed the specified SST criteria highlighting the reliability of the instrument and the ease of use for each user resulting in a successful SST.

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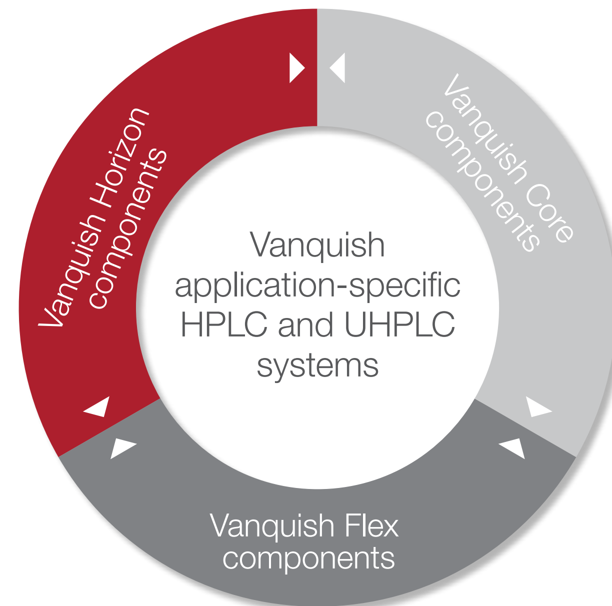
Customize your LC to your needs

While Vanquish HPLC and UHPLC systems are designed to address a broad range of analytical challenges, some applications require even more specialized solutions.

Vanquish application-specific systems leverage custom configurations of standard Vanquish components to address your most demanding applications. With this modular flexibility of the Vanquish platform, we offer systems tailored to boosting your productivity, increasing confidence in your analysis, and accelerating your HPLC and UHPLC method development.



Unique dual technology

The Vanquish platform contains two unique dual modules, which are utilized for application-specific systems. The Vanquish dual pumps merge two individual, low pressure mixing pumps in one housing enabling separate eluents and gradients for each pump while saving capital investment and bench space. The Vanquish dual split samplers possess two independently controlled injection units including separate injection valves and injection needles.

These two unique dual components are utilized across the various Thermo Scientific™ Vanquish™ Duo HPLC and UHPLC systems as well as the Thermo Scientific™ Vanquish™ Online 2D-LC systems to offer outstanding productivity for an improved return on investment and enhanced separation capabilities.

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Boost your productivity

Thermo Scientific Vanquish Duo HPLC and UHPLC systems

Increase your productivity with two completely independent flow paths for higher throughput and improved sample characterization.

Enhance your confidence

Thermo Scientific Vanquish Online 2D-LC systems

Enhance your confidence for the separation of complex samples and difficult-to-resolve analytes by utilizing a combination of two separation chemistries.

Accelerate your development

Thermo Scientific™ Vanquish™ Method Development HPLC and UHPLC systems

Accelerate your method development with automated column and solvent switching hardware and third party method development software compatibility.

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Two flow paths for maximized productivity

The Vanquish Duo UHPLC systems improve productivity and sample knowledge to deliver a faster return on investment. The Vanquish Duo UHPLC systems expand the benefits of our award-winning Vanquish platform with four workflows (Dual LC, Tandem LC or LC-MS, Dual LC-MS, and Inverse Gradient). Vanquish Duo UHPLC systems use a separate second flow path in one integrated system assembled from Vanquish Core, Vanquish Flex or Vanquish Horizon modules.

Vanquish Duo for Dual LC

Double your throughput, deepen your sample knowledge, and efficiently utilize your bench space with the Vanquish Duo for Dual LC workflow. Two completely independent flow paths for maximum productivity.

Vanquish Duo for Tandem LC or LC-MS

Increase your lab's throughput by 30% with the Vanquish Duo for Tandem LC or LC-MS workflow.[1] Maximize the utilization of your optical detector or mass spectrometer.

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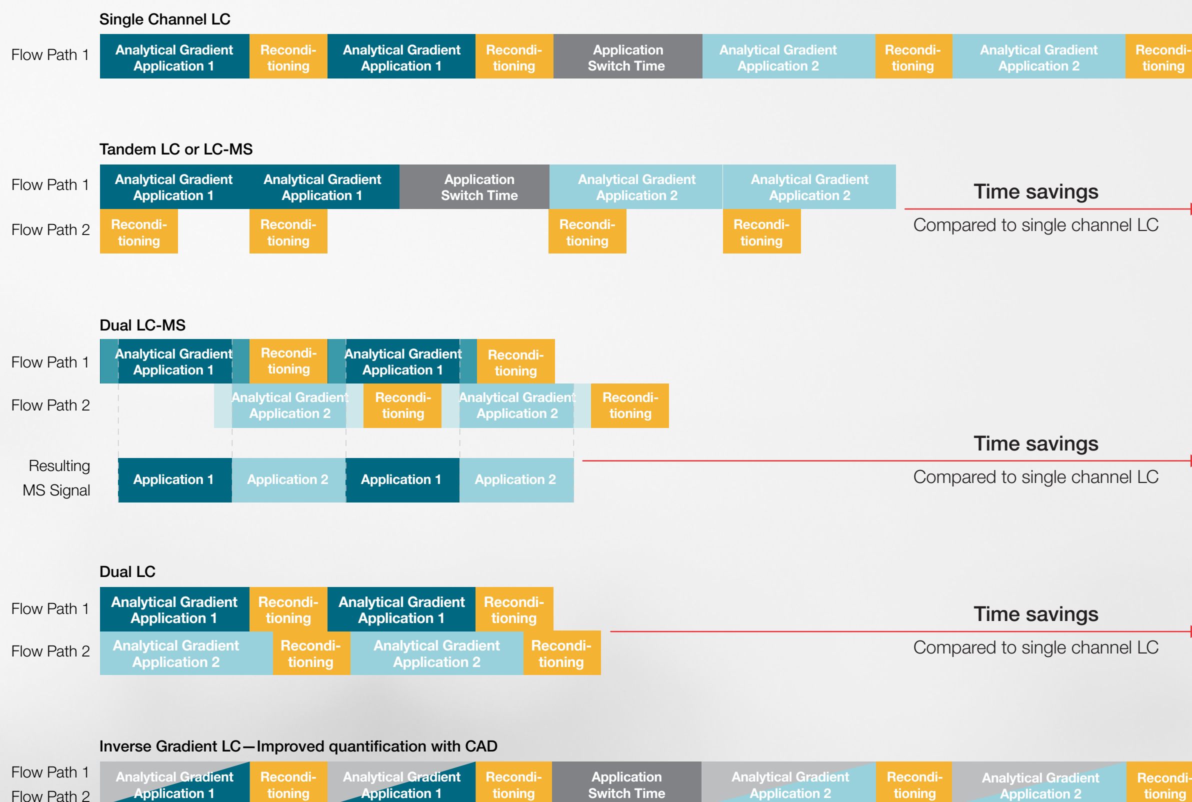

Vanquish Duo for Dual LC-MS

Double the throughput of your LC-MS application with the Vanquish Duo for Dual LC-MS. Run up to two applications simultaneously with a multi-channel LC while benefiting from the robustness, performance, and ease of use inherent of the Vanquish LC platform.

Vanquish Duo for Inverse Gradient LC

Improve your lab's quantification capabilities with the Vanquish Duo for Inverse Gradient, employing the unique and universal Charged Aerosol Detection, to quantify all non-volatile and semi-volatile compounds even when no individual reference standard is available.

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Increased confidence

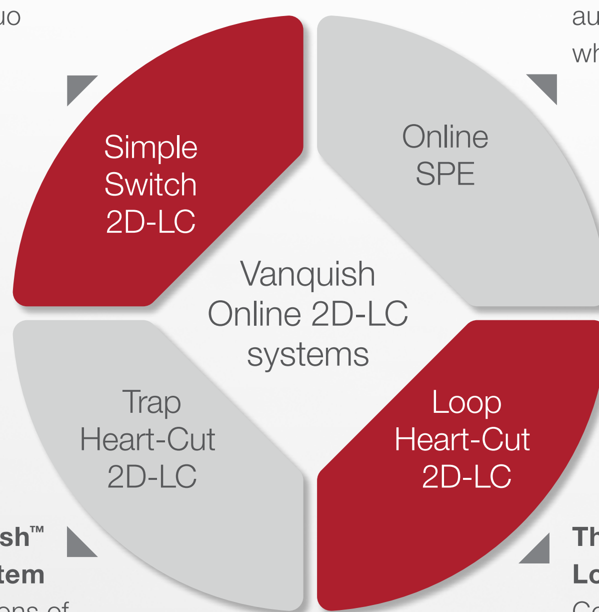
Complex samples with co-eluting peaks or interfering matrixes are not always successfully separated with conventional UHPLC utilizing a single column chemistry. In these cases, the analysis is improved by employing two-dimensional liquid chromatography to resolve co-eulting peaks and gain confidence in your separation.

Four different Vanquish 2D-LC systems can be configured from Vanquish Core, Vanquish Flex and Vanquish Horizon modules/components targeting different two-dimensional LC workflows:

Vanquish Online 2D-LC systems

Thermo Scientific™ Vanquish™ Simple Switch™ 2D-LC system
Achieve utmost application flexibility through seamless switching between a Vanquish Trap Heart-Cut 2D-LC instrument and a Vanquish Duo UHPLC system for Dual LC.

Thermo Scientific™ Vanquish™ Online SPE HPLC and UHPLC system
Ensure analyte pre-concentration and sample clean-up in an automated repeatable workflow while saving solvent and time.



Thermo Scientific™ Vanquish™ Trap Heart-Cut 2D-LC system
Transfer one or multiple fractions of the first dimension analytical column to a trap column before submitting to the second dimension for improved detection flexibility such as, making non-MS compatible separations MS compatible.

Thermo Scientific™ Vanquish™ Loop Heart-Cut 2D-LC system
Collect one or multiple fractions of the first dimension separation in a sample loop and transfer the cut-out fraction(s) to a complementary column chemistry for a second dimension separation for peak purity confirmation.

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Accelerate your development

Traditional method development requires weeks—months for skilled chromatographers to optimize a broad range of separation parameters. The Vanquish Method Development HPLC and UHPLC systems enable automated method development workflows including method scouting, optimization, sample profiling, robustness testing, and validation in a fraction of the time. A variety of hardware and software solutions means that the system can be customized to address your most demanding method development tasks from chiral to large biomolecule separations.

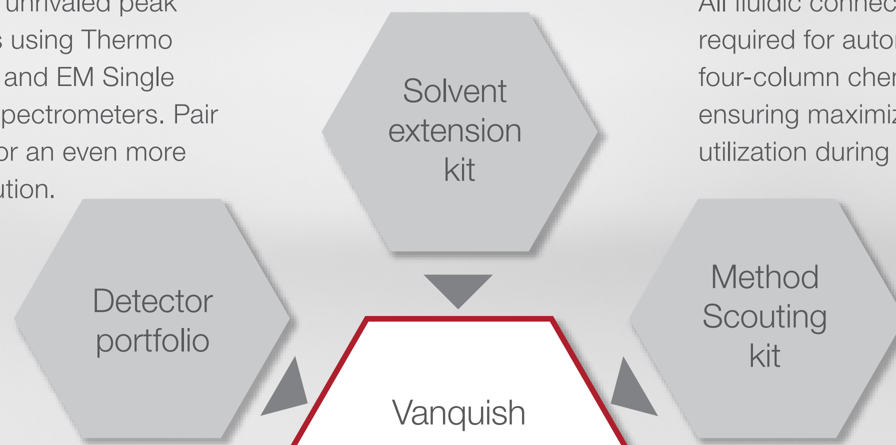
Vanquish Method Development HPLC and UHPLC systems

Hardware solutions

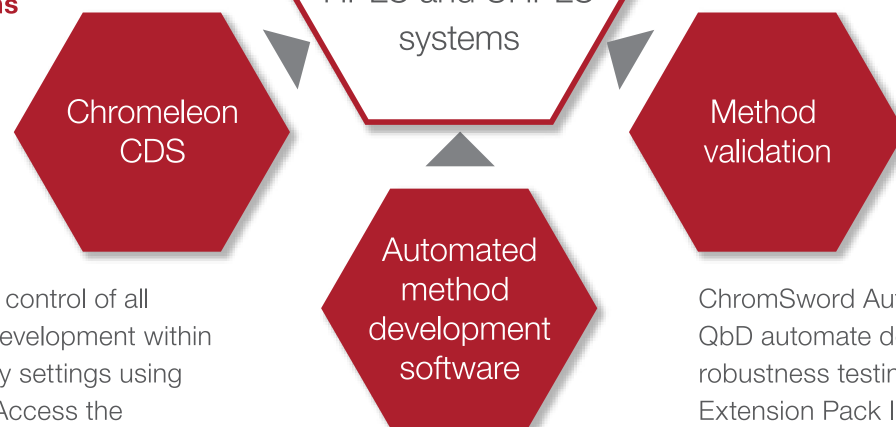
Enhanced confidence in method development with universal analyte detection using a Charged Aerosol Detector (CAD) and unrivaled peak tracking capabilities using Thermo Scientific™ ISQ™ EC and EM Single Quadrupole mass spectrometers. Pair multiple detectors for an even more comprehensive solution.

Accelerate method development through the use of up to nine additional solvents with automated column switching and purging.

All fluidic connections and capillaries required for automated scouting of four-column chemistries included, ensuring maximized instrument utilization during method development.



Software solutions



Intuitive and secure control of all stages of method development within compliant laboratory settings using Chromeleon CDS. Access the AppsLab Library of Analytical Applications for full, predefined eWorkflows™ procedures from sequence setup to result reporting.

Perform unattended method development in a fraction of the time with Vanquish-compatible third party method development software including ChromSwordAuto™ and Fusion QbD®.

ChromSword AutoRobust™ and Fusion QbD automate design and execution of robustness testing while Chromeleon Extension Pack ICH templates provide full workflows for method validation.

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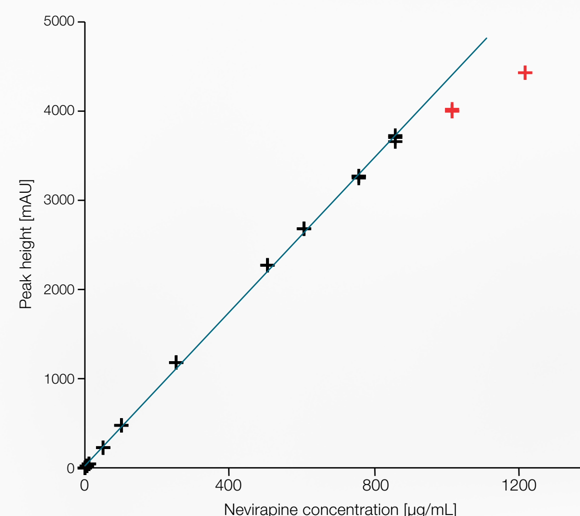
Detect the lowest sample quantities

Choosing the right detection technology is key to revealing all the components of interest in your sample. The Vanquish HPLC and UHPLC platforms offers a wide range of detection capabilities that can be easily integrated and combined to fit your methods.

Industry-leading diode array detection

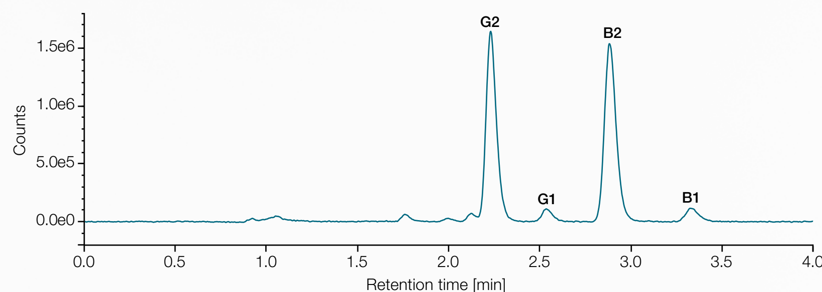
The Thermo Scientific Vanquish Diode Array Detector HL, with the Thermo Scientific™ LightPipe™ detection technology, delivers supreme analyte sensitivity through low baseline noise and longer effective light path by total internal reflection. The Thermo Scientific Vanquish Diode Array Detector FG offers cutting edge detector linearity, as well as a smooth and robust LC-MS integration. Based on diode array detection technology, the Multiple Wavelength Detector offers a cost-effective solution for simultaneously acquiring up to eight UV-Vis wavelength channels.



Concentration vs. peak height plot of nevirapine with data points that were considered for calibration (black) and data points that were eliminated from calibration due to curve decline (red). Linear calibration with permitted offset and no weighting.

Powerful fluorescence detection

Fluorescence detection enables high sensitivity detection of fluorescent compounds with a long-life lamp technology.



FLD chromatogram of the four aflatoxins: G2, G1, B2 and B1 at a concentration of 0.9 µg/kg for G2 and B2 and 2.9 µg/kg for G1 and B1.

Cost-effective, reliable UV-Vis

UV-Vis detection with excellent sensitivity and linearity is available to streamline in targeted compound analysis.

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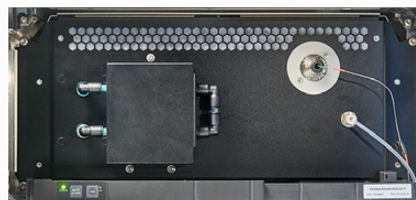
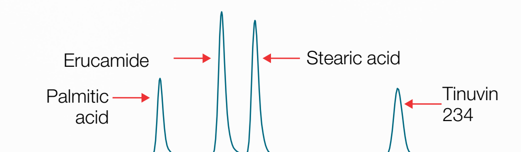
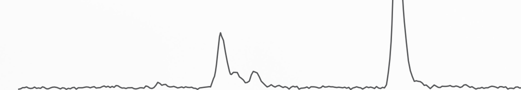
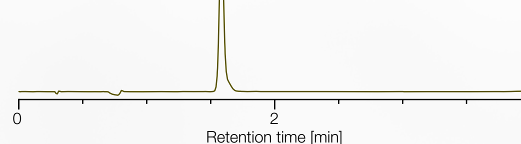


Uniquely universal charged aerosol detection

The Thermo Scientific™ Vanquish™ Charged Aerosol Detector is powerful and universal, able to detect virtually any large and small molecule that lacks a chromophore, or that poorly ionizes, with sub-nanogram-level sensitivity and near-uniform response. The flexibility and performance of charged aerosol detection is ideal for analytical R&D, while its simplicity and reproducibility benefit manufacturing QA/QC applications.



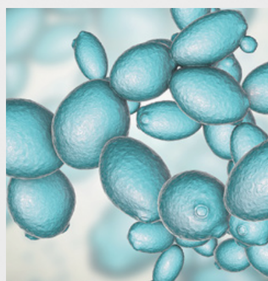




Unlike mass spectrometry (A) and UV (B), the CAD can measure all analytes in a sample. Mass spectrometry (MS) requires the analyte to form gas phase ions while response by a UV detector depends upon the nature of the chromophore.

Single quadrupole mass detection

Mass spectrometry gives access to valuable data no other technology can deliver. The easy to use ISQ EM single quadrupole mass spectrometer or the ISQ EC single quadrupole mass spectrometer integrates with LC systems for reliable, robust, and easy LC-MS routine analysis with an extended mass range for greater flexibility. Integrated software allows both novices and experts to quickly master MS to gain more insights from every sample.



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Vanquish HPLC and UHPLC Systems LC that takes your productivity to new heights

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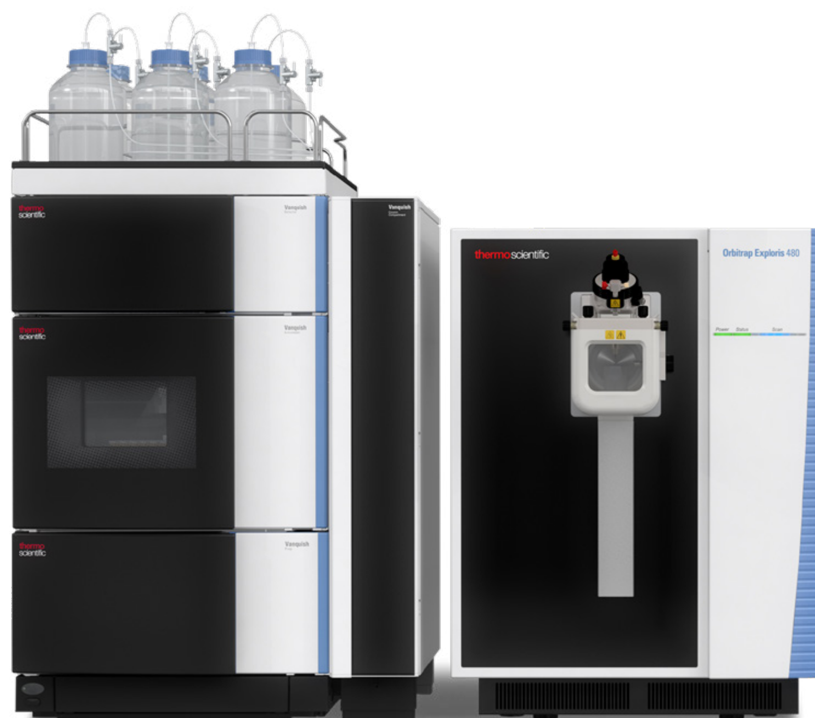
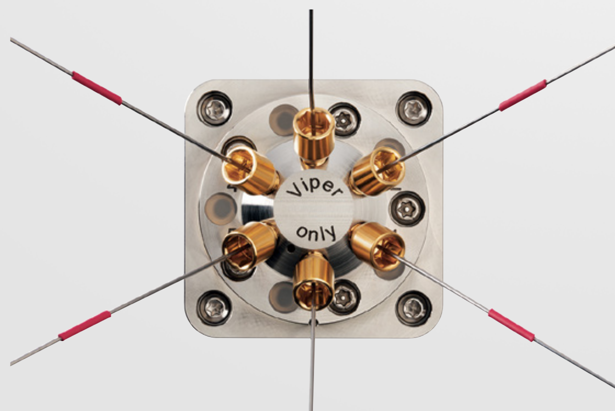
Seamlessly integrate MS

The Vanquish platform combines seamlessly with Thermo Scientific™ mass spectrometers, providing an extra level of information, confidence and productivity. Mass spectrometry provides sensitivity and selectivity for your analyses, giving you further insight into your samples and ability to resolve difficult separations, including co-eluting peaks, using differing mass-to-charge *(m/z)* ratios.



Thermo Scientific Vanquish system with the Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer.



Get better LC connections

Thermo Scientific™ Viper™ Fingertight Fittings revolutionize UHPLC connections. Viper fittings pave the way for easy setup of virtually dead-volume-free chromatography, even for your advanced column configurations. Now you can enjoy tool-free system fluidics setup and other connections.

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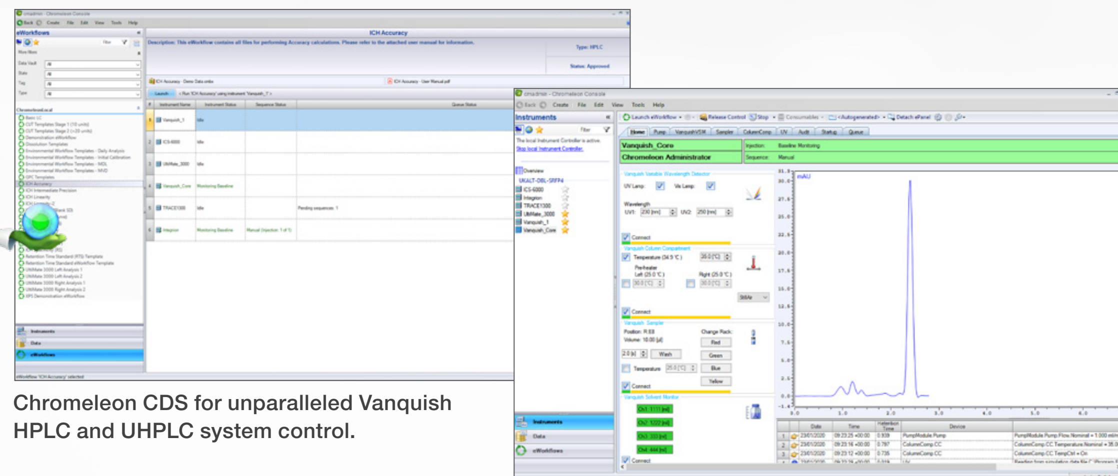
Maximize your results

One CDS to do it all

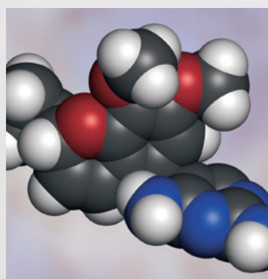
Benefit from the industry-leading, easy-to-use and compliant Chromeleon CDS with eWorkflow procedures for error-free sequence and method setup, dedicated ePanels for direct instrument control, walk-up Open Access software, and smart tools to streamline data processing and reporting. Chromeleon CDS delivers advanced system communication with single-point intelligent control and functionality. The control of selected Vanquish LC systems in Waters™ Empower™ and Agilent™ OpenLab™ CDS software is enabled by dedicated software plugins.

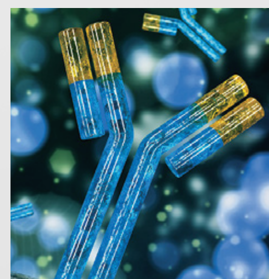


Chromeleon CDS for unparalleled Vanquish HPLC and UHPLC system control.

Optimize your separations with Thermo Scientific columns

The Vanquish HPLC and UHPLC systems are designed around the column, the core to every LC separation. Our family of Thermo Scientific™ Vanquish™ LC columns partner with the Vanquish platform to take advantage of its extended pressure capabilities and robustness, allowing you to optimize your separations faster and more easily. In addition, all Vanquish LC valves are biocompatible, have a long lifespan and are low maintenance, meeting the performance needs to make LC run robustly and routinely.





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Vanquish HPLC and UHPLC Systems
LC that takes your productivity to new heights

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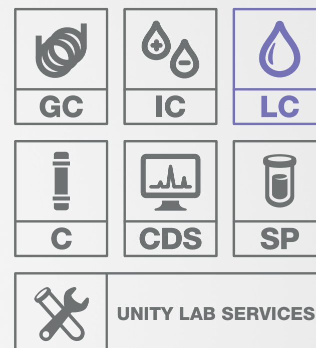
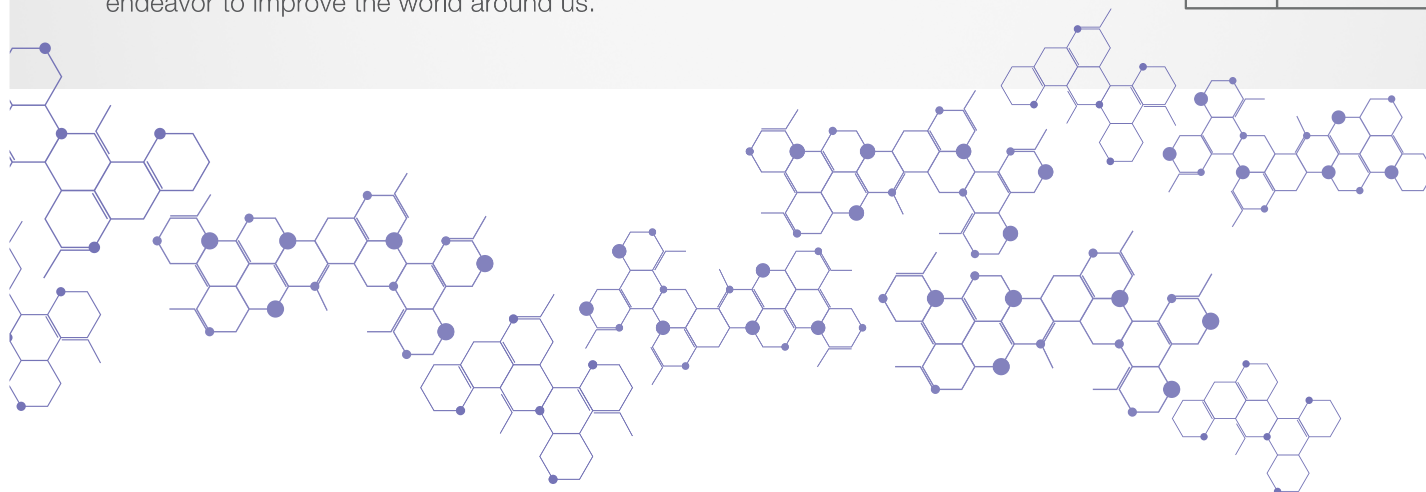
The collective power of chromatography

LC that takes your productivity to new heights

Laboratories are constantly asked to do more with less. Built from the ground up, Thermo Scientific HPLC and UHPLC instruments enable you to raise your productivity to the next level and give you confidence in your results. Time and cost associated with staff training are minimized allowing your laboratory to meet ever-increasing productivity demands, making it faster to bring products to market. With the largest portfolio of LC solutions, we remain a steadfast and committed partner in your endeavor to improve the world around us.





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Find out more at
thermofisher.com/liquidchromatography

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This compendium serves as a resource bundle to help plan and execute your next method transfer. It is packed with comprehensive information – application notes, handy guides – so you can prepare for, identify, and resolve instrument-related inconsistencies typically observed during a transfer.

Although a regular task for analytical scientists, method transfers pose unique problems as laboratories try to duplicate protocols and obtain consistent results between two instruments. In readjusting variables to mimic original conditions, scientists can spend valuable time in trial-and-error. Given the added responsibility of meeting regulatory requirements, routine method transfers can become rather tedious.

At Thermo Fisher Scientific, we're continually developing and updating our systems to address these pressing challenges. To this effect, we've designed our new Thermo Scientific™ Vanquish™ Core HPLC system to make method transfers easier than ever before. The features we've incorporated into this platform will equip your laboratory to obtain fast and reliable method transfers.

Using the Vanquish Core HPLC system you can:

- Match the gradient delay volume of your original system with our tunable gradient delay volume feature
- Resolve uneven pre-column volume and eliminate dispersion effects using the custom injection program or a dedicated strong solvent loop
- Replicate column temperatures by choosing between multiple column thermostatting modes as well as column pre-heating options

The customizable parameters offered in our Vanquish Core HPLC system give you complete control to adjust individual parameters and obtain identical settings, while providing you with the necessary guidance to remain compliant.

To learn more about the Thermo Scientific Vanquish Core HPLC system or request a demo, visit **www.thermofisher.com/vanquishcore**

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