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METHOD TRANSFER BETWEEN HPLC AND UHPLC INSTRUMENTS

EQUIPMENT-RELATED CHALLENGES AND SOLUTIONS

Today, ultra-high-performance liquid chromatography (UHPLC) has taken a firm foothold in the analytical laboratory. However, it is rare that 100% of instrumentation within a company is all from the same vendor and of the same model. Differences between equipment can lead to unexpected changes in the chromatogram when a method is transferred from one system to another. In this issue of *Separation Science* we examine these issues and how to address them...



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Equipment-Related Challenges in Method Transfer Between HPLC and UHPLC Instruments

Today, ultra-high-performance liquid chromatography (UHPLC) has taken a firm foothold in the analytical laboratory. However, it is rare that 100% of instrumentation within a company is all from the same vendor and of the same model. Differences between equipment can lead to unexpected changes in the chromatogram when a method is transferred from one system to another. Although this problem can be especially important for industries where regulatory rules require “equivalent” separation, such as pharmaceuticals, in reality, the same concerns exist for environmental, biochemical, petrochemical, and other industries, as well.

In the context of the present discussion, three abbreviations will be used to describe liquid chromatography (LC) systems. HPLC (high-performance liquid chromatography) systems are those systems designed to operate at pressures ≤ 400 bar (6000 psi), and represent most installed liquid chromatography systems worldwide. UHPLC (ultra-high-performance liquid chromatography) systems are designed to operate at pressures >400 bar (6000 psi), but will also work under HPLC conditions; UHPLC is replacing HPLC in many laboratories. LC is used in the present discussion to describe separations in general that may fit into either the HPLC or UHPLC categories.

In particular, the following challenges are common:

1. Conversion of “legacy” methods developed on older HPLC equipment to UHPLC conditions to reduce run time and/or to improve resolution and sensitivity.
2. Transfer of methods from one manufacturer’s equipment to another manufacturer’s.
3. “Legacy” methods must be run on newer HPLC or UHPLC equipment with the same results.
4. Transfer of methods developed on UHPLC equipment to conventional HPLC equipment while maintaining retention and resolution.

These challenges can exist within a

department, between laboratories in the same company, or between companies. For the pharmaceutical industry, regulations exist for transfer of methods (e.g., to shorter columns or smaller particle sizes), but sometimes these are hard to interpret or lag behind current technology. For example, the *United States Pharmacopoeia (USP)* Chapter <621>, “Chromatography,” contains guidelines about what adjustments can be made to chromatographic methods to meet system suitability. In addition, there is a proposal for the development of a new Chapter <1224>, “Transfer of Analytical Procedures,” but this does not exist as of this writing.

Much of the commercial and scientific



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literature concentrates on item 1 above, with a primary emphasis on scaling of the column and flow rate to take advantage of the smaller particles and higher pressures used in many UHPLC applications. Particular benefits are shorter run times for higher throughput and reduced solvent consumption. An example of this can be found in Agilent Application Note 5590-8428EN, “Scalability of Agilent Columns Across HPLC and UHPLC Instruments.” The current discussion does not consider this topic further.

Another aspect of method transfer that sometimes is encountered is the need to make small adjustments in the chemistry of the chromatographic system to “tweak” the separation so that acceptable results are obtained. For example, changes in flow rate, solvent composition, mobile-phase pH, or column temperature may be necessary to meet system suitability. In general, such changes involve an intimate understanding of the system chemistry and may require many trial-and-error experiments. Even then, the exact separation may not be obtained under the new conditions. *USP <621>* outlines the limits of such adjustments

for compendial methods. The safest way to allow for method adjustment is to validate the method in a manner that allows for small, defined changes in specific operating conditions.

Often, physical adjustments to instrument settings are necessary to transfer a method between different LC systems. This is the primary focus of the present discussion (topics 3-4 above), where the goal of the transfer process is to obtain the same results (retention times and resolution) when a method is transferred between two instruments.



Instrument-to-Instrument Method Transfer

Transfer of methods from one instrument to another is a common procedure over the lifetime of an LC method as it is moved from development and validation to routine application. With the increasing globalization of product development, methods not only have to be transferred between LC instruments within a single laboratory, but also between laboratories in different regions of the world. In particular, the pharmaceutical industry operates in a regulated environment that expects identical results regarding retention times and resolution wherever the method is applied.

Instrument-to-instrument method transfer generally is straight forward if the method is transferred between identical instruments. Even in these cases, however, different combinations of modules (autosamplers, pumps, etc.) and plumbing can still lead to differences. If methods are transferred between different instruments or between systems from different vendors, the process can be much more challenging. In the past, differences between HPLC equipment did not have a great impact on retention times and resolution because the differences of the instruments were relatively small considering that most methods were run on larger i.d. columns and had relatively long run times. Coincident with the introduction of UHPLC equipment came reductions in delay volume and improvements in mixer

performance. While these characteristics are beneficial for methods developed and run on the UHPLC equipment, they can add complexity to the method transfer between UHPLC and HPLC equipment. This is even more serious in contract research organizations (CROs) and other laboratories that must have a variety of equipment brands and models to meet client requirements.



Potential Instrument-Related Method Transfer Problems

When transferring methods between HPLC and UHPLC, several potential problems arise.

- Delay volume (dwell volume)
- On-line mixing characteristics
- Power range (pressure x flow rate)
- Injection volume
- Column oven performance
- Detector flow cell design
- Data acquisition rate
- Extra-column band-broadening (system dispersion)

The first two items, delay volume and mixing, often are the most important system characteristics that can cause method transfer problems. These are discussed in more detail in the next section. The remainder of this section will look briefly at each of the other variables.

Power range: The power range is a product of the system pressure and the flow-rate. When transferring a method from HPLC to UHPLC this rarely is an issue, because UHPLC systems are designed to operate at >400 bar, whereas HPLC systems have an upper

limit of 400 bar. On the other hand, a method developed on a UHPLC system, especially when <2- μ m particles are used, may exceed the pressure capabilities of an HPLC system. Although conventional HPLC systems may have a higher flow-rate capability than UHPLC, both types of systems typically are capable of flow rates of up to 5 mL/min – well above the normal flow rate for most methods, so this factor rarely is an issue. If it is known that a method developed on UHPLC will be transferred to HPLC, care can be taken to ensure that the operating pressure does not exceed 400 bar so the power range should not be a concern.

A possible approach could be to use superficially porous particle columns that show UHPLC performance but at a much lower back pressure due to larger particle size (2.7 μ m).

Injection volume: Most autosamplers, whether for conventional HPLC use or UHPLC, have similar injection volume capabilities. However, if a method is transferred between systems with

different autosampler types, i.e., fixed-loop and flow-through autosamplers, changes in delay volume can be observed. Also the operator must be aware of the functional differences of the different autosampler types (e.g., partial and complete loop fill for fixed-loop autosamplers.)

Column oven performance: Because isocratic retention changes by 1-2%/°C, it is ideal to use the same column oven for both HPLC and UHPLC methods or retention times will not be consistent since column temperature differences can affect not only retention times, but also peak spacing, as illustrated in Figure 1.

A temperature-related issue is the uniformity of temperature within the oven. A simple way to help ensure that the column temperature corresponds to the oven setting is to use the pre-heater tubing that is included with most column ovens. This is particularly important with Peltier-heated ovens, which rely on pre-heated solvent for best performance.

Figure 1

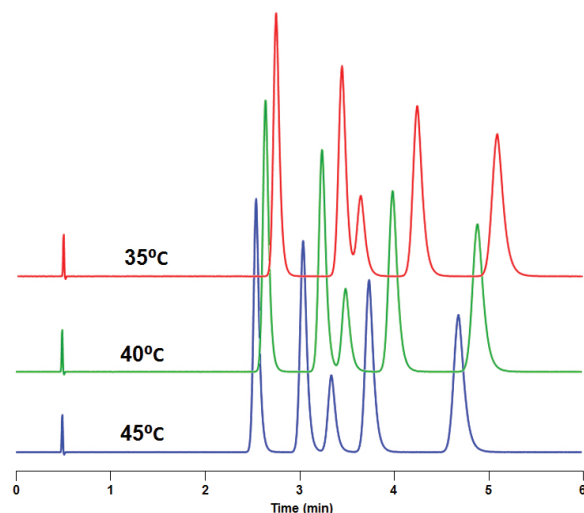
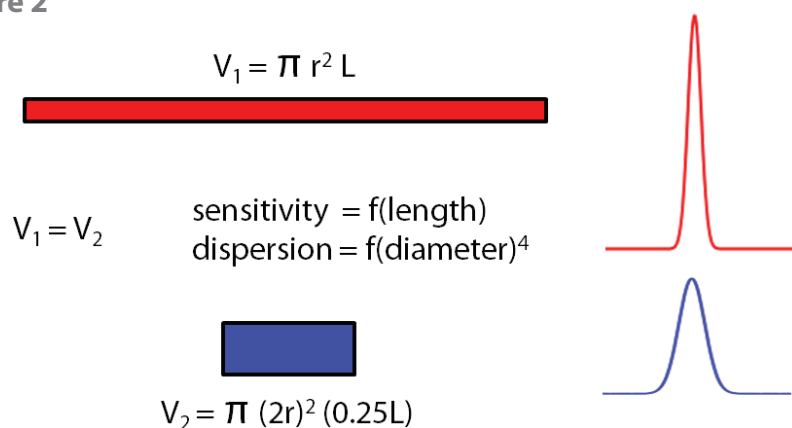


Figure 2



A final concern with column temperature is frictional heating (viscous heating) of the mobile phase as it travels through the column.

However, frictional heating is unlikely to be an issue with method transfer from HPLC to UHPLC equipment (same column and flow rate on a different instrument), or from UHPLC to HPLC, where lower pressures and larger particles will be the norm.

Detector cell design: Typical HPLC UV detectors have 10 mm long flow cells with volumes in the 8-15 μL range. The dispersion in these flow cells rarely causes problems of band broadening with conventional $\geq 3.5\text{-}\mu\text{m}$ packings and $\geq 2.1\text{-mm}$ i.d. columns. UHPLC systems generate much narrower peaks when short, narrow columns are used with sub- $2\text{-}\mu\text{m}$ particles. Narrower peaks require smaller, lower dispersion flow cells, with volumes of $<4\text{ }\mu\text{L}$, and often $\leq 1\text{ }\mu\text{L}$. Because dispersion is related to the fourth power of the cell diameter and sensitivity is related to the cell length, UHPLC detector cells can be constructed to lower dispersion and increase sensitivity by using a longer, narrower i.d. flow cell, as shown in

“Moving an HPLC method to a UHPLC system should have no issues with detector-related band broadening...”




Figure 2. Moving an HPLC method to a UHPLC system should have no issues with detector-related band broadening with such cells. The reverse transfer from UHPLC to HPLC, however, could be a problem for marginally separated peaks. The way around this problem is to ensure that sufficient separation exists in the UHPLC method that the somewhat larger HPLC detector cell will not compromise the separation.

Data acquisition rate: As a general rule, the data system should collect 10-20 data points across each peak to adequately capture the peak. Both HPLC and UHPLC data systems are capable of collecting data at a sufficiently high rate for methods on conventional columns (100-250 mm x 2.1-4.6 mm i.d., packed with $\geq 3.5\text{-}\mu\text{m}$ particles), so this should be of little concern in the present context.

Extra-column band-broadening: In addition to dispersion in the detector cell, other excess volume in the system can cause peaks to broaden, and thus lose sensitivity and perhaps resolution. However, this should be of little concern if good chromatographic practices are applied and short lengths of narrow-

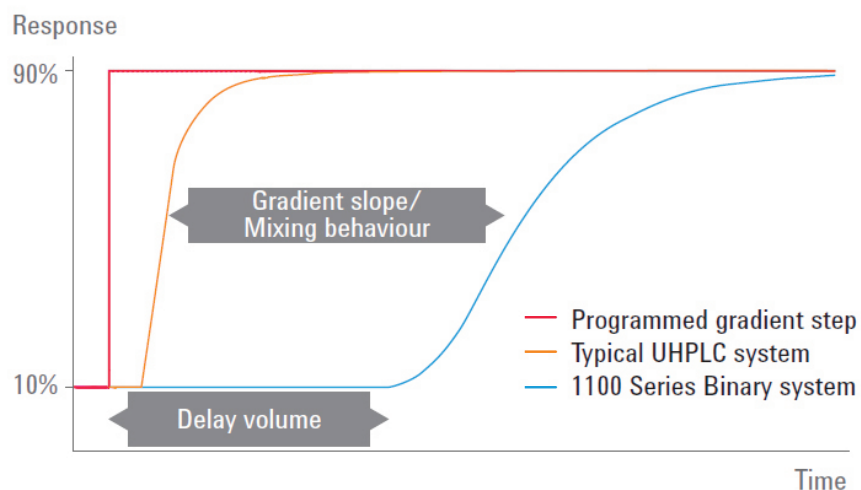
bore tubing are used to connect the various flow paths where sample travels. For example, the most popular tubing diameters for HPLC are 0.175 mm (0.007-in.) and 0.125 mm (0.005-in.) i.d.; these have volumes of $\approx 24\text{ }\mu\text{L/m}$ and $\approx 12\text{ }\mu\text{L/m}$, respectively. Short lengths of either type of tubing are unlikely to add significant dispersion to the system for routine methods. When tubing smaller than 0.175 mm is used, extra care needs to be taken, such as additional sample filtration, to avoid tubing blockage. With some UHPLC equipment, there is sufficient resistance to flow through all the narrow-bore tubing that pressures in excess of 70 bar may be observed with no column installed.

Gradient Delay and Mixing Characteristics

The remainder of this discussion will focus on the influence of gradient delay and mixing characteristics on the method transfer process. These aspects of the LC equipment are of little concern when isocratic separation is used, because no change in the mobile phase composition is required during a given chromatogram. However, gradients rely on reproducible changes in mobile phase composition to obtain reproducible separations, so the gradient delay and mixing characteristics can be very important.

Prior to the advent of UHPLC, gradient delay was the primary contributing factor when difficulty in transferring gradient HPLC methods was encountered. This is because, while different brands and models of HPLC equipment had different gradient delay values, the mixing properties were not much different. This is not the case with UHPLC equipment. The issues are illustrated in Figure 3.

Figure 3



In the example of Figure 3, a step gradient from 10 to 90% of the B-solvent is illustrated. The red line shows the programmed step, where there is a near-instantaneous change from 10% B to 90% B. The actual step generated by the LC equipment, however, deviates from the programmed step in two ways. First, there is a delay between the programmed time and the arrival of the gradient mixture at the column. (In the present example, the column was removed and replaced with a capillary tube, so the baseline rise represents the arrival of the gradient at the head of the column.) This is called the delay time or dwell time, and corresponds to the amount of time it takes the mobile

phase to mix and flow through the connecting tubing to the column. With the Agilent 1290 Infinity LC (yellow line), it can be seen that there is a short delay before the gradient reaches the column, then the trace rises sharply from the baseline. This short delay is due to the small delay volume (typically ≤ 150 μ L) for the 1290 Infinity LC. Compare this with an Agilent 1100 Series LC, an instrument designed to work with conventional columns. The step is delayed further than the 1290 Infinity LC step, because the delay volume is in the 1.5 mL range for the 1100 Series LC. An even greater delay may be seen with some older HPLC systems. Since the gradient delay is not the

only problem, a simple isocratic hold cannot compensate for the difference in delay time between instruments. The use of an isocratic hold to compensate for gradient delay differences was satisfactory in the past when HPLC systems of similar performance were standard.

The mixing characteristics of the system also can influence the behavior of gradients, as can be seen in Figure 3. Note that in addition to the delay of onset of the step, the shape of the step is quite different between the program, the 1290 Infinity LC, and the 1100 Series

LC. The onset of the gradient with the 1290 Infinity LC is quite sharp, but there is a noticeable slope to the plot and distinct rounding at the top. This is due to the wash-out characteristics of the mobile phase mixer. The change is even more dramatic with the 1100 Series LC trace. There is obvious rounding at both the onset and end of the step, as well as a considerably shallower slope. Also it can be seen that the delay between the 1290 Infinity LC (yellow) and 1100 Series LC (blue) curves increases as the %B increases. This added complexity makes it difficult, if not impossible, to compensate for mixing differences by a simple method adjustment.

It should be noted that the gradient delay and mixing characteristics of any given system, as illustrated in Figure 3, are not “bad” per se, but merely different. These differences can make transfer of gradient methods difficult between such instruments.

Next, we’ll look at different ways to compensate for the differences in gradient delay and mixing characteristics that were highlighted in Figure 3. These include the traditional addition of an isocratic hold and/or

delay tubing to the hardware as well as a unique feature of the 1290 Infinity LC system, Intelligent System Emulation Technology (ISET).

Two aspects of comparing instrument performance should be apparent, but need to be emphasized. First, it is the actual mobile phase composition at any point in the gradient that controls the retention of solutes, so comparison of mobile phase profiles is an appropriate way to illustrate instrument differences. Second, complex gradients, where more than one step or slope change occurs within a gradient, will be more challenging to transfer than simple, single-slope gradient. For these reasons, multiple-segment gradient profiles will be compared in the following discussion. To obtain the gradient profiles, such as in Figure 3, a UV-absorbing compound (e.g., uracil) is added to the B-solvent and the desired gradient is run; as the B-solvent is added, the baseline rises.

An example of the disparity between gradients on two different systems is shown in Figure 4. In this case, a multi-step gradient was developed on an 1100 Series LC and it was desired to

Figure 4

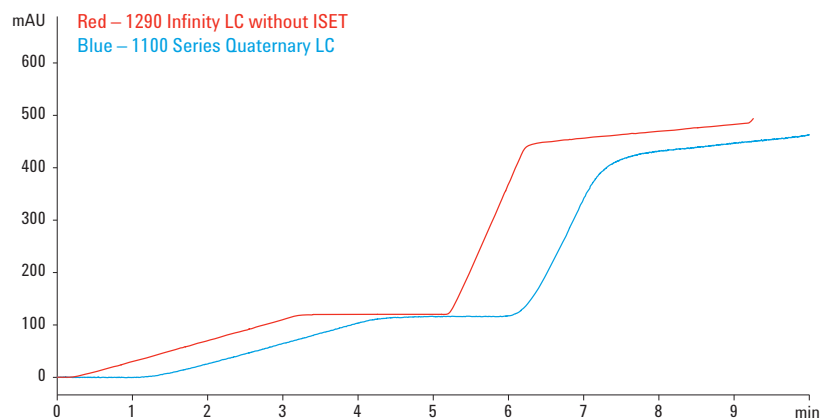
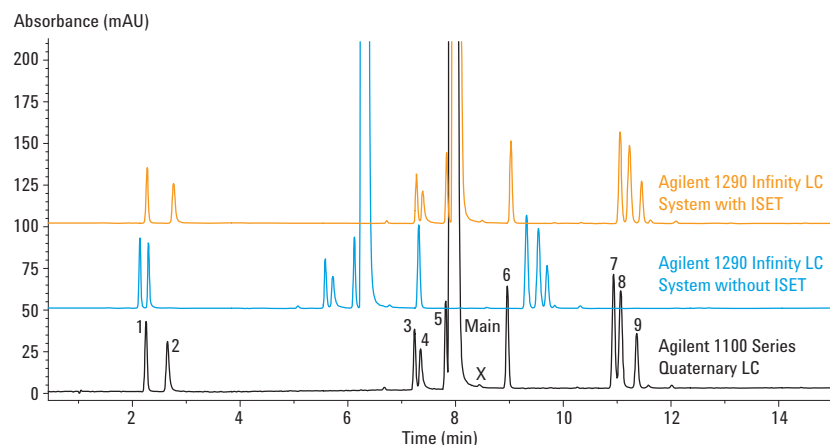


Table 1

Tracer (%)	Time (min) *	Additional 1 mL loop on an Agilent Infinity LC	Isocratic hold 1 min on an Agilent Infinity LC Time (min)	Isocratic hold 0.95 min on an Agilent Infinity LC Time (min)
5	0	0	0	0
5	n/a	n/a	1	0.95
20	3	3	4	3.95
20	5	5	6	5.95
60	6	6	7	5.95
65	9	9	10	9.95
95	10	10	11	10.95

* applied on 1100, 1290 Infinity LC with and without ISET.

Figure 5

move the method to an 1290 Infinity LC using the same mobile phase, column, and flow rate to obtain equivalent chromatographic results. The gradient program is listed in Table 1. The blue trace in Figure 4 shows the actual gradient produced by the 1100 Series LC when it reaches the column. It can be seen that there is effectively a 1.5-min isocratic hold at the beginning of the program. This is a result of ≈ 1.5 mL of delay volume in the 1100 Series LC being cleared out at a flow rate of 1 mL/min. The remainder of the tracing in Figure 4 follows the program of Table 1, except for the rounding of the gradient at the transition between each gradient step.

The same gradient program was entered in the Agilent 1290 controller and the program was run, resulting in the red trace in Figure 4. Two obvious differences exist between the two traces in Figure 4, just as they did in Figure 3. First, the isocratic hold at the beginning is shorter with the 1290 Infinity LC because the delay volume of the 1290 Infinity LC is much smaller. Second, the transitions between the gradient segments are much more angular with

the 1290 Infinity LC because of the more efficient wash-out characteristics of the system. However, even though the 1290 Infinity LC is a "better" system, it does not satisfactorily emulate the gradient profile of the 1100 Series LC, so the transfer of this gradient is not straight forward. The practical impact of these differences is shown in Figure 5 (with a different gradient program). The black chromatogram shows the separation developed and validated on an 1100 Series LC system. When transferred directly to the 1290 Infinity LC, the middle (blue) chromatogram was obtained. Retention times are shorter, as expected, because at any given time in the gradient, a stronger (higher %B) gradient has reached the column than with the original method. In addition to the retention-time shifts, there are changes in selectivity. Of particular interest are peaks 1 and 2 as well as 8 and 9, which are not as well separated on the 1290 Infinity LC. This simple method transfer does not produce equivalent chromatographic results.

Figure 6

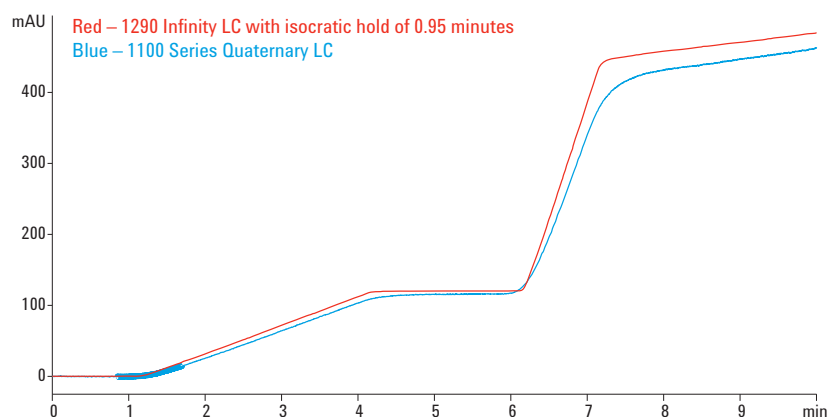
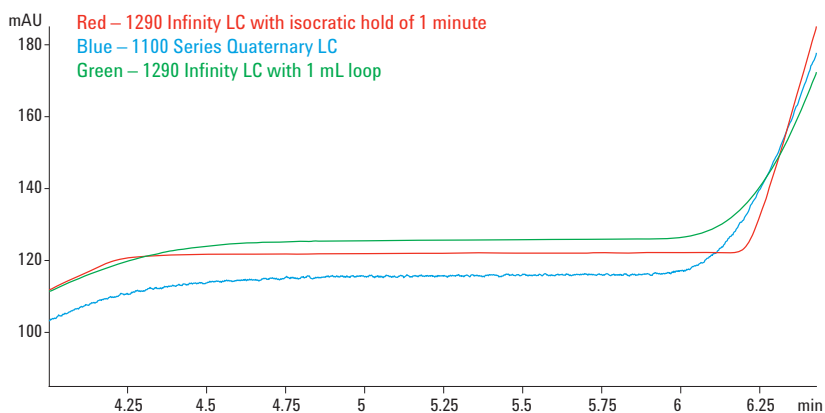


Figure 7



Isocratic hold: Careful examination of the two traces at the beginning of Figure 4 reveals that there is an offset of ≈ 1 min between the first rise in the baseline on the two instruments. One way to correct for this would be to insert a 1-min isocratic hold at the beginning of the program for the 1290 Infinity LC. This adjustment was tried, and improved the results, and a slight additional adjustment for a 0.95-min hold generated the data shown in Figure 6. Here it is seen that the start of the initial baseline rise aligns perfectly between the 1290 Infinity LC (red) and the 1100 Series LC (blue). This approach has been successful in the past for traditional HPLC systems where, although gradient delay-time differences might be significant, mixing characteristics were not dramatically different. This is no longer the case when transferring an HPLC method to a UHPLC system, as is seen by comparing the two traces at approximately 7-min in Figure 6. The 1290 Infinity LC does not replicate the rounding of the gradient trace in the 1100 Series LC. While the gradient profiles earlier in the run look to be equivalent, the differences in the end

of the run might result in unacceptable changes in retention and/or selectivity when method transfer is attempted.

Added delay volume: The differences in gradient performance, as in Figure 4, result from the larger delay time and different mixing characteristics of the 1100 Series LC and 1290 Infinity LC systems. The addition of an isocratic hold, as discussed above, compensates for the delay time, but not for the mixing characteristics. A modification of the flow path might downgrade the mixing performance of the 1290 Infinity LC so that it behaved like the 1100 Series LC. Such changes generally are not attempted, because they are tedious, require much fine-tuning, and may require re-qualification of the instrument. Furthermore, a manual changeover is required to switch the UHPLC instrument from “UHPLC” to “HPLC” mode, making it impossible to run both UHPLC and HPLC methods in the same sequence. However, to illustrate the effect of additional volume on the mixing characteristics, a 1-mL loop of tubing was added to the 1290 Infinity LC system. The results are shown

Figure 8

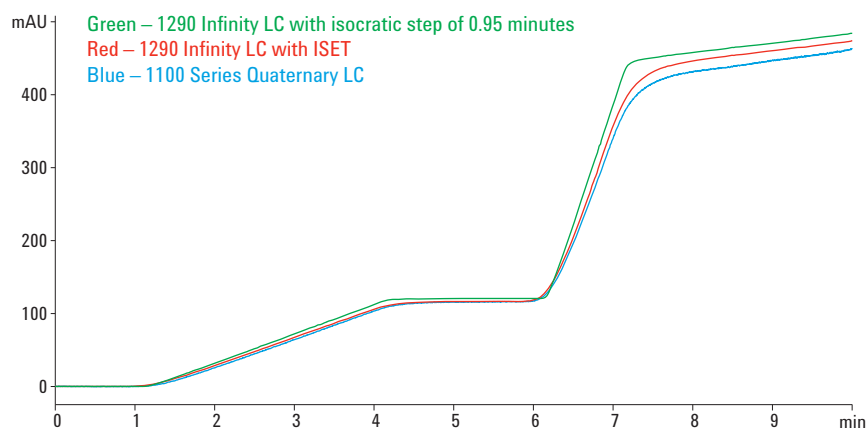
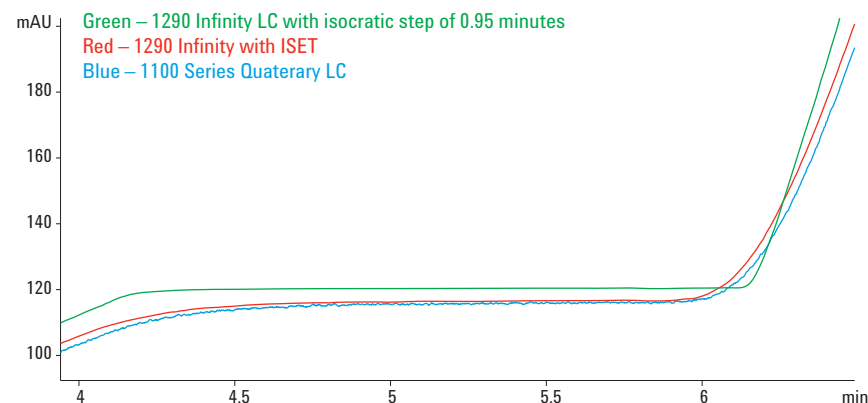


Figure 9



in Figure 7 for the first 6.5 min of the gradient program of Table 1. It can be seen that the 1290 Infinity LC with 1-mL of additional mixing (green trace) is much closer to the original 1100 Series LC program (blue) than is the 1290 Infinity LC with a 1-min delay (red).

Although adding a physical delay volume to the system, as in Figure 7, brings the results for the two system closer, they still don't match up perfectly. Part of the difficulty of trying to correct for instrument differences by adding delay time or delay volume (or both) is that there are design differences between the 1290 Infinity LC and 1100 Series LC that cannot be compensated by such changes.

Depending on the complexity of the HPLC method and the required level of reproducibility when transferred to a UHPLC system, the changes discussed above may be acceptable. However, it is possible to make even more accurate compensatory changes using a software feature available on the 1290 Infinity LC.

Intelligent System Emulation Technology: Near-perfect replication of gradient profiles can be achieved through a

combination of isocratic holds and subtle changes to the gradient shape to reflect mixing differences between equipment. Agilent's Intelligent System Emulation Technology (ISET) is a program that makes these changes automatically when a gradient program is moved to an 1290 Infinity LC system from another Agilent system with the current release of ISET but also from other non-Agilent systems with future releases. The delay volumes and mixing characteristics of all Agilent HPLC and UHPLC systems are pre-programmed into ISET, so all that needs to be done is to enter the model numbers of the pump and autosampler modules and ISET makes the adjustments automatically. This is illustrated in Figures 8 and 9 for the 1100 Series LC to 1290 Infinity LC transfer discussed above.

In Figure 8, the original 1100 Series LC program (blue) is compared to the best fit for the 1290 Infinity LC with a 0.95-min isocratic hold (green) and to the profile generated automatically using ISET with the 1290 Infinity LC (red). Although the programmed delay moves the gradient transitions to the right time points, it does not correct for gradient rounding.

ISET, however, does a good job at correcting for both the time offset and gradient rounding of the 1100 Series LC. The quality of the match generated by ISET is further illustrated in Figure 9 for the middle section of the gradient tracings of Figure 8. The real impact of ISET can be seen by examining the chromatograms of Figure 5 again. With ISET operational (red) the 1290 Infinity LC is able of accurately replicating the chromatograms obtained on the original 1100 Series LC system (black).

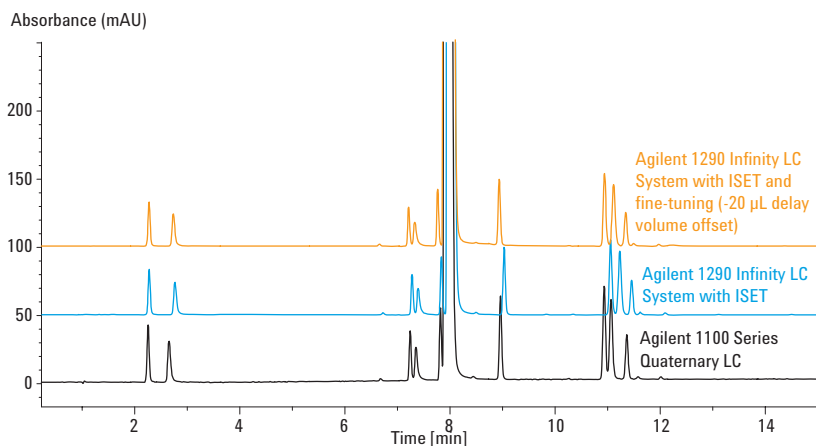
Further fine-tuning with ISET is

possible, if desired. Figure 10 repeats the chromatograms of Figure 5 for the 1100 Series LC (black), and 1290 Infinity LC with ISET (blue). It can be seen that, although the retention times of peaks through the first 8 min of the chromatogram are very close, the 1290 Infinity LC with ISET generates slightly longer retention times for the last 4 peaks in the chromatogram. ISET allows further manual fine-tuning through the entry of a pressure (120 bar in the present case) and a delay volume offset (20 μ L). These values can be fine-tuned to obtain results (red trace in Figure 10) that are extremely close to the originals. Adjustment of these values also can be used to emulate HPLC instruments with additional valves or capillaries leading to different delay volumes.

One major advantage of the 1290 Infinity LC with ISET is that it can be quickly and easily adjusted to replicate conventional HPLC methods without physical instrument modifications. Thus, the 1290 Infinity LC system can be used for method development and/or routine analysis of UHPLC methods as well as running legacy HPLC method developed on different LC platforms.

UHPLC to HPLC method transfer: The discussion above concentrates on how to make legacy HPLC methods run on UHPLC equipment. This might streamline laboratory operations when a legacy HPLC system was being maintained only to run a few legacy methods. By allowing these methods to be run on UHPLC equipment, the costs of instrument maintenance and operator training on the older equipment could be eliminated. One popular use of UHPLC systems in the pharmaceutical industry is to use the high-throughput capabilities of these systems to develop more robust methods more quickly. Then, because of the large install base of traditional HPLC equipment, operator skills, or other considerations, the UHPLC method is then converted to an HPLC method for routine analysis; Although such transfers usually are not too challenging, care must be taken that the new methods are not so demanding that they will not run adequately on HPLC systems. With ISET, it is easy to check this by running a method on an 1290 Infinity LC so that it emulates the results on conventional HPLC systems.

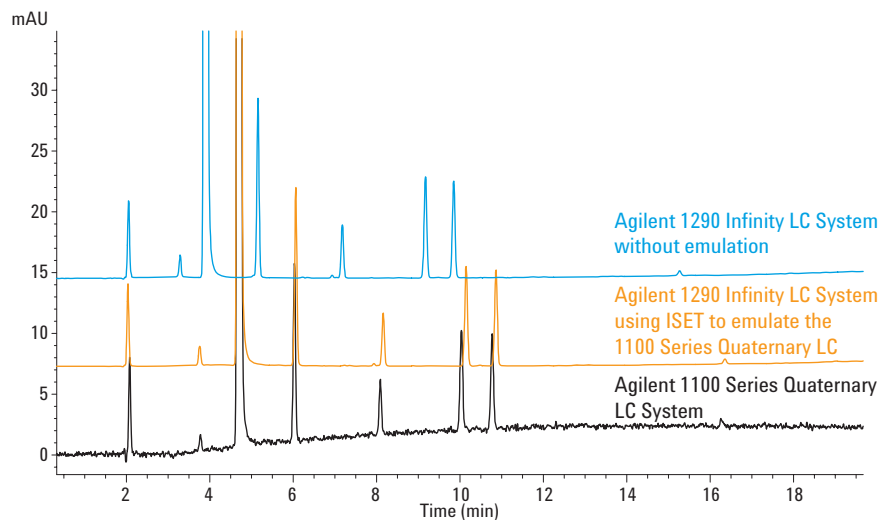
Figure 10



An example of this process is shown in Figure 11 for the gradient separation of an analgesic and several impurities. The initial method was developed on an 1290 Infinity LC, with the results shown as the blue chromatogram. Before validation, ISET is used to adjust the method so that it will appear as if it were run on an 1100 Series LC, with the chromatogram corresponding to the yellow trace. When the method is run

on an actual 1100 Series LC system, the results (black) are very close to those obtained with the 1290 Infinity LC with ISET. Retention time deviations for all peaks are <2% different between the two systems, acceptable agreement for most applications. With manual fine-tuning, as described above, it is expected that the match might be further improved.

Figure 11



Agilent 1290 Infinity LC with ISET



Conclusions

When liquid chromatographic methods are transferred between different systems, it is desirable to obtain the same retention times and separation of sample components. When such replication cannot be achieved, method modification may be required, with subsequent partial or full re-validation of the method, adding expense and delay to a project. One of the most challenging aspects of method transfer is related to the gradient generation capabilities of the instrument. Many times the addition of an isocratic hold and/or a physical delay loop may allow one system to emulate another. However, with the capabilities of the Agilent 1290 Infinity LC with ISET, such programming and physical changes are not necessary. Instead the ISET software accurately simulates the conditions created by other systems so that the same retention times and resolution can be obtained on the 1290 Infinity LC as for other LC equipment. By use of ISET, Quality by Design (QbD) principles can be followed, because the software allows testing instrument-to-instrument differences in advance of routine application. This will help to avoid regulatory problems that might otherwise be encountered when transferring LC methods between instruments.