Dwell Volume and Extra-Column Volume: What Are They and How Do They Impact Method Transfer?

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A chromatographic separation is impacted by numerous factors, including the LC system and its characteristics. The impact of the chromatographic system on the separation may not be obvious until the method is transferred to another LC system or scaled to a different column dimension. By characterizing the system and understanding these differences, strategies can be undertaken to increase the success of methods transfer. These approaches can use software or hardware tools – including the ACQUITY™ instrument control software – or follow the USP <621> Chromatography guidelines for scaling to translating methods.[1]

INTRODUCTION

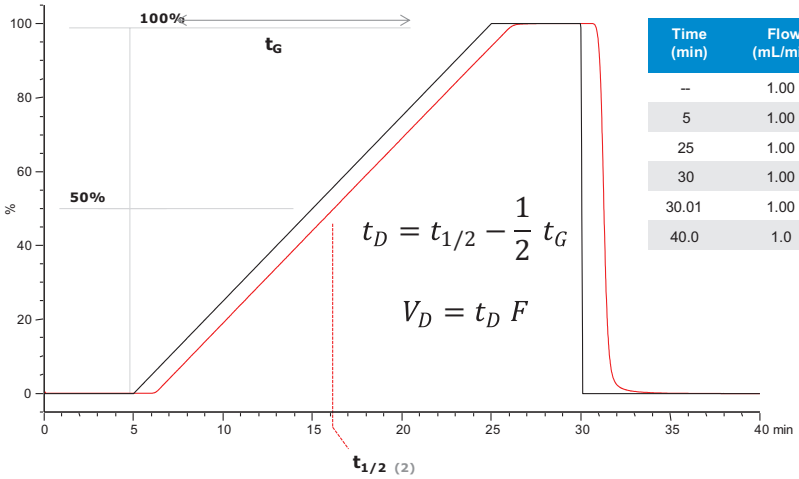
Method transfer or method scaling across different instrument platforms can be affected by both dwell volume and extra-column dispersion. Each characteristic will have a very different effect on the chromatographic separation. Dwell volume is the volume required for the change in a gradient to reach the column, or the volume difference between the point of mixing and the head of the column. Dwell volume impacts the retention times of a gradient separation, but can also affect selectivity, particularly for early eluting compounds. Extra-column dispersion is a measurement of the broadening of a peak that occurs between the injector and the detector and excludes the column. Extra-column dispersion or volume impacts peak width, resolution, and the overall efficiency of a separation. In this review, the differences of these attributes across a variety of instruments will be examined, specifically for their impact on methods transfer. We will also review strategies to minimize the impact on the method transfer. These recommendations will follow the USP <621> guidelines.

DWELL VOLUME

MEASURING DWELL VOLUME

For every chromatographic system, the dwell volume is a physical characteristic that is primarily a function of the pump. It represents the volume difference between the device that controls the delivery of the gradient and the head of the column. It is also commonly referred to as gradient delay volume. Dwell volume is impacted not only by the tubing (length and internal diameter (I.D.)), but also by any valves or mixers in the fluidic path up to the head of the column. To understand the variations among systems, we measured the volume difference of a programmed gradient and the delivery of said gradient to the detector (Figure 1). This was accomplished by using a gradient from 0–100% B where B contains a UV absorbent compound (in this case caffeine). Using a UV detector, we can then record the response of the UV absorber over time, which reflects the delay in the gradient delivery. For a more accurate assessment, the delay was calculated at 50% of the gradient. The analyses were conducted in triplicate with the average values recorded. This procedure has been extensively described in literature.[2,3]

The measured values (Figure 2), cover a range from <100 µL to over 1 mL for UPLC,™ UHPLC, and HPLC quaternary and binary systems. For each type of system, the pump characteristics determine the dwell volume. For quaternary pumps, changes in the gradient occur at the gradient proportioning valve (GPV). This valve, which is typically before the mixer, results in a greater dwell volume than in a binary system with the same mixer, tubing and valves, because the GPV is located *before* the solvent is pressurized by the pump. The total dwell volume not only includes the valve and the mixer, but also the internal volume of the pump heads themselves. In binary systems, the gradient change occurs at the mixer after the solvent has been pressurized by the pump; there is no gradient proportioning valve that affects the dwell volume. Rather, the gradient is formed by varying the flow rate of the A and B pumps to form the desired flow rate and composition.



Time (min)	Flow (mL/min)	%A	%B
--	1.00	100	0
5	1.00	100	0
25	1.00	0	100
30	1.00	0	100
30.01	1.00	100	0
40.0	1.0	100	0

Figure 1. Method for determining a system's dwell volume. A UV absorbent compound was placed in mobile phase B using a programmed gradient from 0–100% B. The chromatogram above shows the overlay of the programmed gradient (black) and the UV signal (red). The calculations were performed by determining the time at which the UV trace reached 50% of total absorbance ($t_{½}$) and then subtracting ½ of the programmed gradient (in time) to determine the dwell time (t_D). This value was converted to volume by multiplication with the flow rate (F).

Conditions: Mobile phase A: water; Mobile phase B: 10 mg/L caffeine in water; Wavelength: 273 nm. For all systems, a restrictor was used to ensure that the measurements were within a systems recommended operating pressures.

System	Dwell Volume (V_D) (mL)
Alliance HPLC (Quaternary) System	1.15
ACQUITY Arc™ (Quaternary) System	1.19 (Path 1) 0.770 (Path 2)
ACQUITY UPLC H-Class PLUS (Quaternary) System	0.375
ACQUITY UPLC I-Class PLUS (Binary) System	0.073

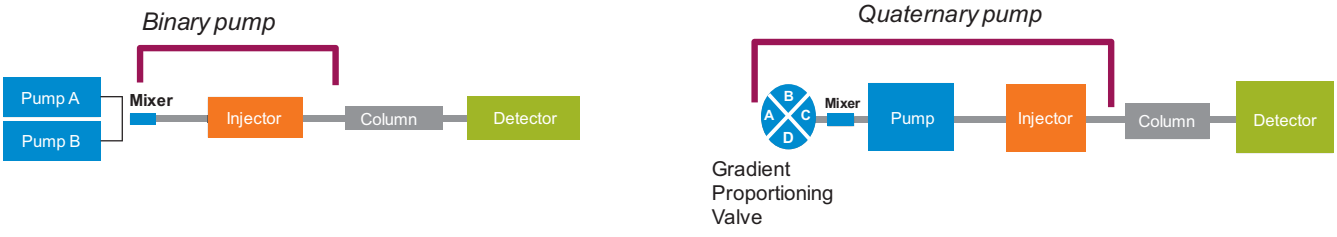


Figure 2. Measured dwell volumes for specific systems. All measurements were performed with the system in its default configuration. Values may vary from system to system based on column heater, tubing, and flow cell configuration. Red brackets indicate the parts of the system contributing to dwell volume.

COMPENSATING FOR DWELL VOLUME WHEN TRANSFERRING A METHOD

For each pump, differences in dwell volume and mixing behavior can impact retention times in gradient methods transfer. To illustrate these effects, the separation of flavonoids in orange extract was transferred from an ACQUITY UPLC H-Class PLUS (Quaternary) System to an Alliance™ HPLC (quaternary) System. When the method is transferred, the difference in retention time is apparent (Figure 3). In this separation, the retention time difference from UPLC (3A) to the HPLC (3B) is approximately 1 minute. The delay is throughout the gradient and also affects the time at which the gradient ends and the re-equilibration begins. Therefore, improper re-equilibration may occur, if the method is transferred to a system with larger dwell volume and no adjustments are made to the overall run time.

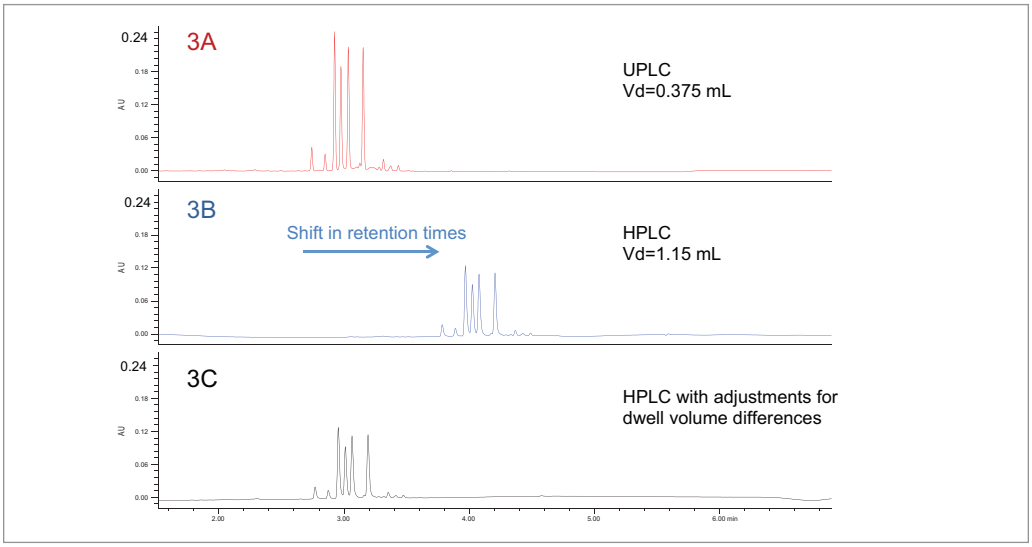


Figure 3. Effect of dwell volume on methods transfer. The separation of flavonoids in orange extract was performed on a UPLC and an HPLC system. The retention time offset is approximately 1.5 min between the two systems. This shift is expected given the dwell volume differences (0.775 mL) across the two systems. By adjusting the HPLC injection to occur after the gradient start, the dwell volume (and retention time) differences can be minimized.

Conditions: Column: CORTECS™ C_{18}+, 2.7 µm, 2.1 x 75 mm (p/n 186007396); Mobile phase A: 0.1% (v/v) HCOOH in H_2O; Mobile phase B: 0.1% (v/v) HCOOH in ACN; Flow rate: 0.6 mL/min; Gradient: 5–90% B in 4 min, 90% B for 1 min, 90–5% B in 1 min, 5% B for 5 min.

Note: The extra-column volume of the HPLC system (3B and 3C) negatively impacts the peak widths and resolution of the separation.

Adjustments for dwell volume differences are allowed per the USP <621>.[4] The typical approach to address dwell volume differences is to alter the gradient table, such as increasing the initial isocratic hold or shortening the gradient duration to emulate another system with different dwell volume. However, this approach requires making changes to the gradient table, which may result in the need to revalidate the method if operating in a regulated environment. As illustrated in the ACQUITY UPLC H-Class PLUS System and Alliance HPLC System method editor software (Figure 4), another approach towards addressing these differences is to adjust the gradient start time relative to the injection, through software. Using this feature, the previously described separation (Figure 3A) on an ACQUITY UPLC H-Class PLUS System was emulated on the Alliance HPLC System (3C). A pre-column volume of 775 µL (1.15–0.375 mL) was entered into the Alliance instrument method. The resulting chromatogram (3C) produces a shift in retention time on the Alliance HPLC System, with all the peaks eluting earlier (3B versus 3C). The new retention times are comparable to those observed on the UPLC PLUS System, illustrating an approach to minimize retention time difference across the two systems without changing the gradient.

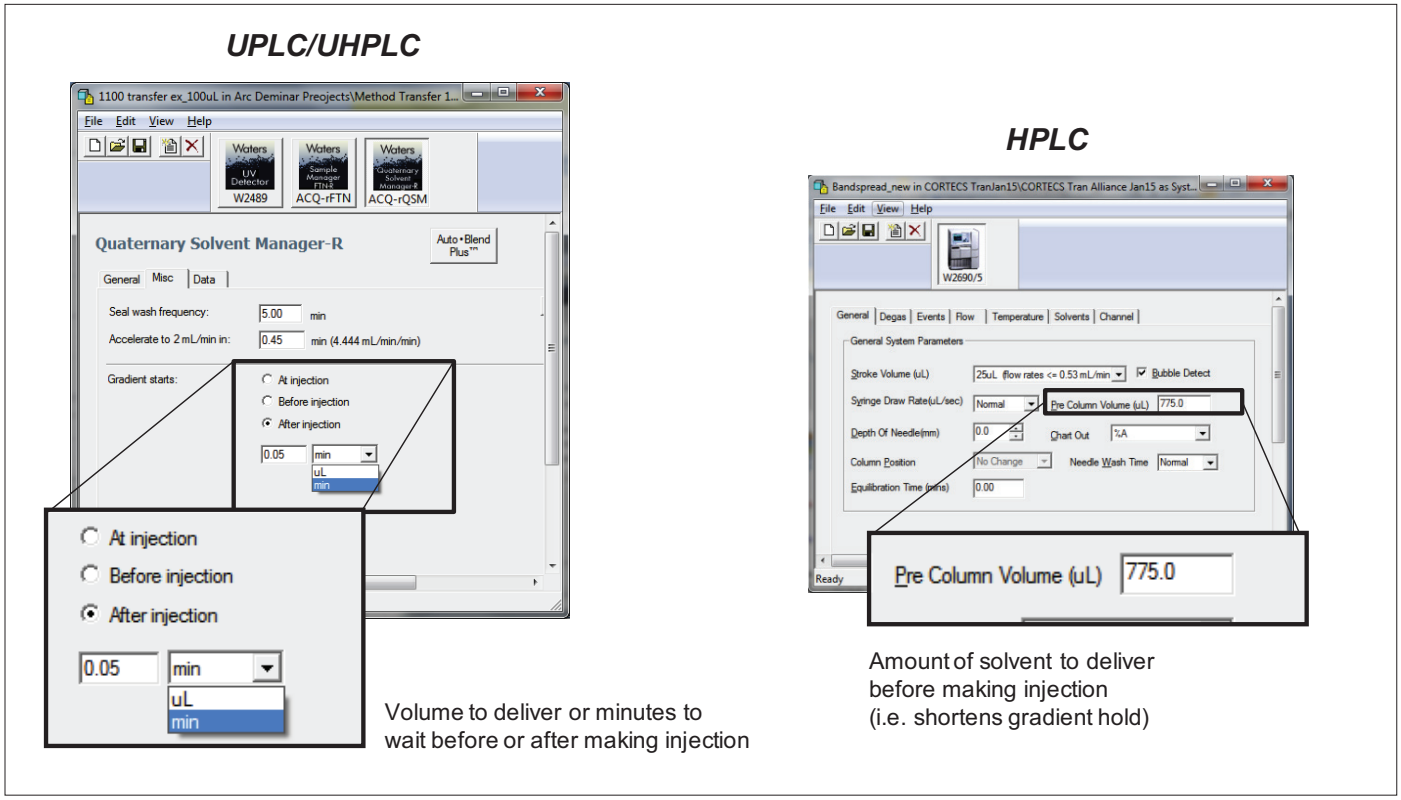


Figure 4. Tools for gradient adjustments between HPLC and UPLC. Instrument method editor options are available to adjust gradient start relative to the injection to compensate for dwell volume adjustments across systems.

EXTRA-COLUMN VOLUME

MEASURING EXTRA-COLUMN EFFECTS

Band broadening in HPLC is a function of both intra-column and extra-column effects. Extra-column effects are related to the volume from the injector to the detector. These effects are influenced by physical components of the system, including the needle seat, tubing connectors to the column, detector flow cells, preheaters, etc., as well as method conditions, such as injection volume and detector filtering.[5,6,7] Lastly, detector settings can also impact band variance.[8] The sum of all these factors can be represented by the band variance (σ) in the following equation.[5,6]

$$\sigma^2_{obs} = \sigma^2_{col} + \sigma^2_{extra\ -col}$$

where

$$\sigma^2_{extra\ -col} = \sigma^2_{needle\ \ seat} + \sigma^2_{injector\ \ volume} + \sigma^2_{injector\ \ valve} + \sigma^2_{tubing} + \sigma^2_{pre\,heater} + \sigma^2_{detector\ \ volume} + \sigma^2_{detector\ \ filter} + ..$$

To understand and characterize each LC's system, extra-column band broadening or dispersion was measured for HPLC, UHPLC, and UPLC instruments. Measurements were performed with each system in the standard configurations (tubing, flow cells, etc.) at a sampling rate of 40 Hz (enough to ensure adequate points across the peak). A zero dead volume union was used in place of a column (Figure 5). Measurements were calculated at 4σ (13.4% peak height) and 5σ (4.4% peak height) (Figure 6).

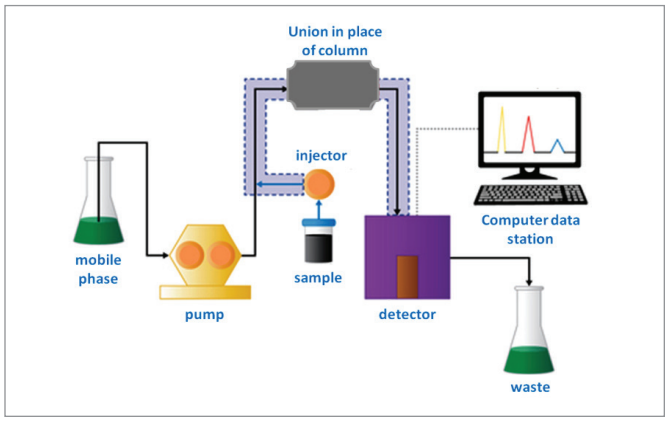


Figure 5. Typical system set up for measuring extra-column band broadening. Dotted blue lines represent potential areas of dispersion within the sample flow path. Each system was configured with standard tubing and flow cells, unless otherwise specified.

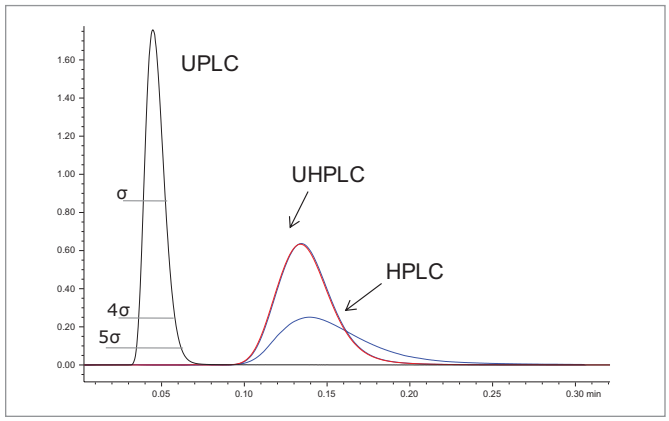


Figure 6. Extra-column effects. Using the Empower™ 3 Chromatography Data System, peak widths at 13.4% peak height (4σ) and 4.4% peak height (5σ) were recorded. The extra-column dispersion was determined by multiplying the peak width and the flow rate. The extra-column dispersion of the UHPLC (ACQUITY Arc System) is represented by the overlaid chromatograms of both Path 1 and Path 2.

Conditions: Flow rate: 0.3 mL/min; Mobile phase: 30:70 water:acetonitrile; Sample: 0.16 mg/mL caffeine in 1:9 water:acetonitrile (p/n 700002642), solution 7; Wavelength: 273 nm; Sampling rate: 40 pt/s (Hz); Injection volume: 1 µL; Low volume V-detail union (p/n 700002636).

The measured values vary significantly across HPLC to UPLC systems (Table 1). As the measurements show, column heaters and detectors can significantly impact extra-column effects of an LC system. Many LC systems have multiple column heater configurations with different I.D. and length of inlet and outlet tubing – all of which impact band broadening. System components, such as switching valves, preheaters, or flow cells, can also significantly affect these values. However, it should be noted that any volume before the injector, such as the Multi-flow path™ in the ACQUITY Arc System, do not impact extra-column dispersion. Given the influence of specific components, the injector, column heater, and detector configuration should be specified when reporting these values.

System	Extra-column band broadening @ 5 σ	Extra-column band broadening @ 4 σ
Alliance HPLC with 30 cm CH and HPLC PDA Detector	43–45	30–34
ACQUITY Arc System (Path 1 and 2) with 30 cm CHC and HPLC PDA Detector	25	19
ACQUITY UPLC H-Class PLUS with CH-A and ACQUITY PDA Detector	8	7
ACQUITY UPLC I-Class PLUS SM-FTN with CH-A and ACQUITY PDA Detector	7.5	5

Table 1. Extra-column band broadening of HPLC, UHPLC, and UPLC systems. Measurements were performed following the conditions previously specified. All systems were in the standard configuration unless otherwise specified. All detectors had standard flow cells.

**Ranges reflect differences in preheater and column compartment configurations.*

IMPACT OF EXTRA-COLUMN EFFECTS ON AN ISOCRATIC SEPARATION

The loss of efficiency for a separation due to extra-column effects varies with column dimensions and particle size.[5,9] Lower column volumes will be impacted more by extra-column band broadening then HPLC (4.6 mm I.D.) columns. This is because the ratio of on-column to extra-column band broadening is low, meaning extra-column effects are a significant portion of the overall band broadening.

To illustrate these effects, an isocratic separation (Figure 7) was performed on UPLC, UHPLC, and HPLC systems. Three different column configurations – scaled to keep L/d_p (length/particle size) constant – were analyzed on each system. On the UPLC System (Column C), extra-column band broadening was not significant (<10 µL), therefore, the efficiency and resolution of the separation was minimally impacted, regardless of the column dimensions.

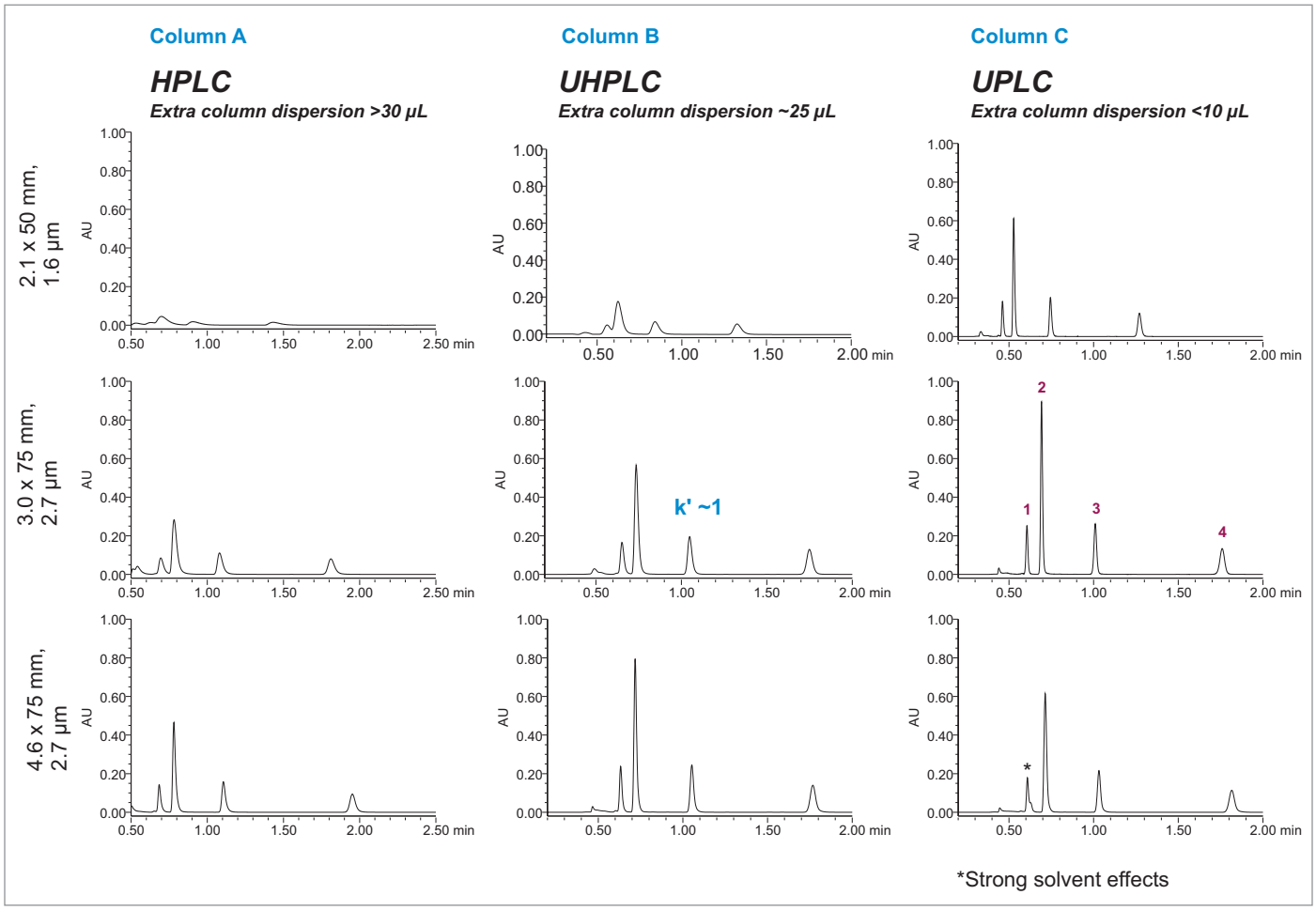


Figure 7. Isocratic separation and extra-column effects. An isocratic separation was evaluated on HPLC, UHPLC, and UPLC systems using 2.1, 3.0, and 4.6 mm I.D. columns with the appropriate method scaling.[1] In general, the HPLC system produced the broadest peaks and lowest sensitivity. However the differences varied with column dimensions. The greatest loss in efficiency was observed for the 2.1 mm column, while differences on the 4.6 mm were not as evident. In addition, some strong solvent effects were observed for the 4.6 x 75 mm column on the UPLC system due to sample diluent mismatch. These effects can be eliminated by matching the diluent to the mobile phase, adding pre-column volume or reducing injection volume.

Conditions: Sample: imipramine, chlorimipramine, 11-α-hydroxyprogesterone, and 17-α-hydroxyprogesterone at 0.1 mg/mL in 1:1 Water:methanol; Isocratic: 1:1 0.1% (v/v) TFA in water/0.1% (v/v) TFA in ACN; Wavelength: 254 nm; Column temperature: 35 °C; Method scaling: Column: CORTECS UPLC C_{18}, 1.6 µm, 2.1 x 50 mm (p/n 186007093), Flow rate: 0.3 mL/min, Injection volume: 1 µL; Column: CORTECS C_{18}, 2.7 µm, 3.0 x 75 mm (p/n 186007371), Flow rate: 0.612 mL/min, Injection volume: 3.1 µL; Column: CORTECS C_{18}, 2.7 µm, 4.6 x 75 mm (p/n 186007376), Flow rate: 1.44 mL/min, Injection volume: 7.2 µL.

On the HPLC and UHPLC system (Column A and B), the separation on the 2.1 x 50 mm column (top row) was noticeably impacted by the extra-column band broadening of each system. The resulting chromatography produced much lower efficiencies (USP plate count), and resolution than that observed on the UPLC system, as illustrated for peak 4 in Figure 8. On both the HPLC and UHPLC systems, a significant loss of efficiency (>50%) was observed as compared to the UPLC System.

Scaling the method to HPLC columns (middle and bottom rows) resulted in improved efficiencies on both the HPLC and UHPLC systems. In fact, the loss of efficiency for peak 4 on a 3.0 mm I.D. column was significantly less than observed with the 2.1 mm I.D. column (Figure 8). Scaling the separation to a 4.6 x 75 mm column resulted in comparable efficiencies on all systems. For the 4.6 mm I.D. column, the ratio of column volume to system dispersion is much more favorable, reducing the negative impact of the system dispersion on column efficiency.

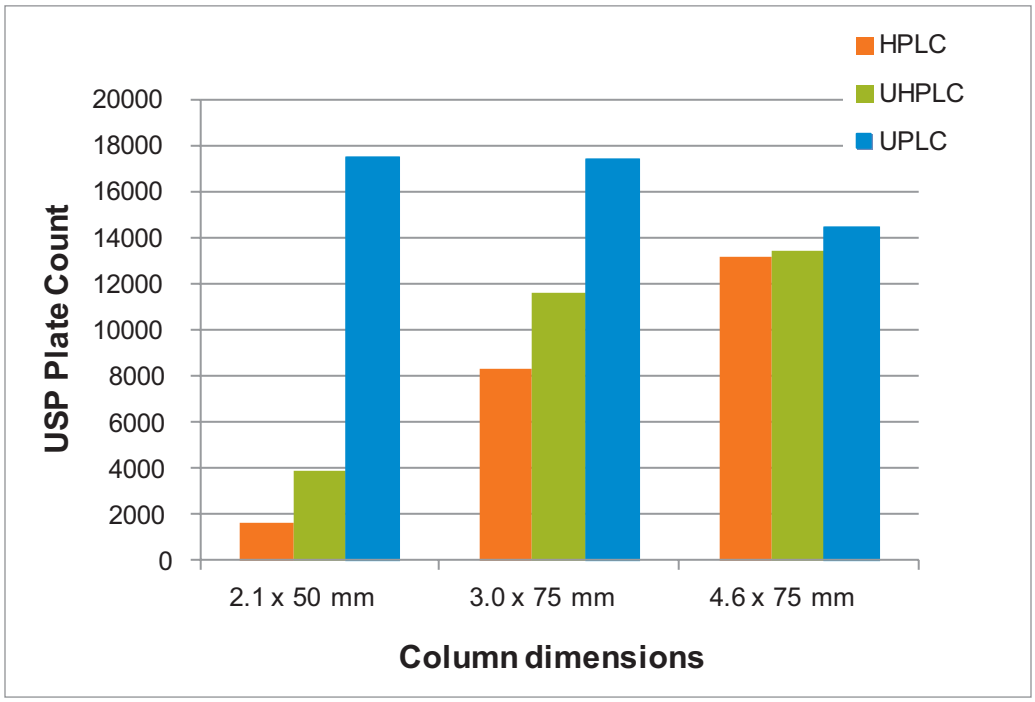


Figure 8. USP plate count for Peak 4. The loss of efficiency on the CORTECS UPLC C_{18}, 1.6 µm Column, 2.1 x 50 mm (p/n 186007093), is over 50% when comparing the UPLC to either the UHPLC or HPLC system. However, with the CORTECS C_{18}, 2.7 µm Column, 4.6 x 75 mm (p/n 186007376) the difference in USP plate count is much lower. The extra-column effects of the different systems have a much lower impact on performance as compared to the sub-2-µm 2.1 mm I.D. column.

IMPACT OF EXTRA-COLUMN DISPERSION ON A GRADIENT SEPARATION

While the effects of extra-column volume are particularly noticeable for isocratic separations, gradient separations are more forgiving of extra-column dispersion due to peak focusing at the head of the column. However, extra-column volume can affect resolution – particularly for critical pairs – since resolution is based on peak widths.

In this example, the separation on flavonoids in orange extract was performed on a 2.1 x 75 mm column on an HPLC system using a standard flow cell (8.4 µL, 10 mm path length) for the PDA detector (Figure 9A). The USP resolution for the flavonoids was between 1.1–1.8. To minimize extra-column band broadening and increase resolution, the same system was configured with a microbore flow cell (2.7 µL, 8 mm path length) (9B). Keeping all the inlet and outlet tubing the same and changing only the flow cell, resolution increased by almost 50%. The lower dispersion flow cell not only produced narrower peak widths, but similar sensitivity despite the shorter path length.

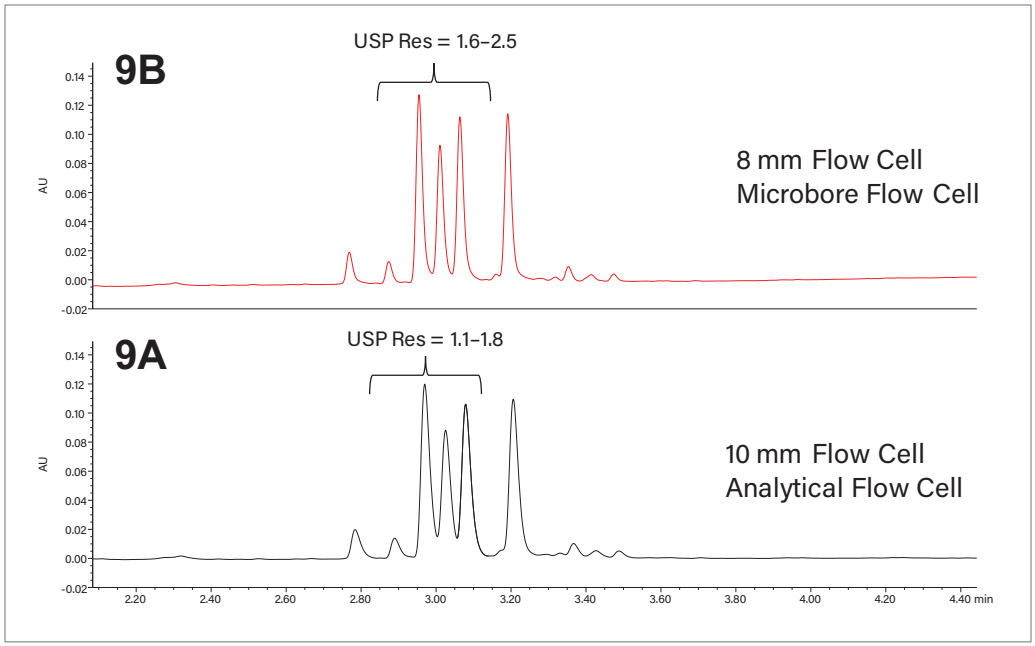


Figure 9. Effect of post column dispersion on a separation of orange extract. The same separation was performed on an HPLC system with two different flow cells. The lower dispersion (microbore) flow cell produced increased resolution of approximately 50%. Sensitivity was comparable despite the shorter path length due to the narrower peak widths observed in the lower dispersion flow cell.

Conditions: Alliance HPLC with 2998 Detector; Gradient: 5–90% B in 4 min; Column: CORTECS C_{18}+, 2.7 µm Column, 2.1 x 75 mm (p/n 186007396); Wavelength: 315 nm, Sampling rate: 20 pts/s (Hz), Filter time constant: Normal.

CONCLUSIONS

The success or failure of method transfer can be impacted by a wide range of system characteristics. Two of the more prominent attributes include dwell volume and extra-column volume. Each of these characteristics has a very different impact on the separation. While dwell volume can impact the time and slope of the delivery of the gradient, extra-column effects impact peak width, efficiency, and resolution of the separation. The impact of both of these characteristics can be minimized with proper adjustments. For dwell volume, adjusting the gradient start relative to the injection, whether through software or changes to the gradient table, helps minimize retention time differences. For dispersion effects, understanding the extra-column volume of each system and taking advantage of the instrument options, can improve the results of methods transfer. However, for smaller column dimensions and smaller particle size columns, the impact of extra-column volume may still affect the separation due to the unfavorable ratio of the column volume to extra-column effects of the system.

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