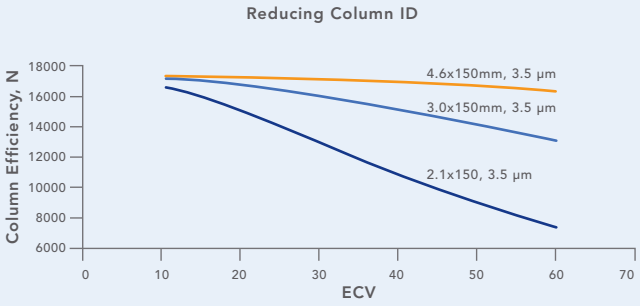
TECHNICAL REPORT

HOW TO MEASURE AND REDUCE HPLC EQUIPMENT EXTRA COLUMN VOLUME

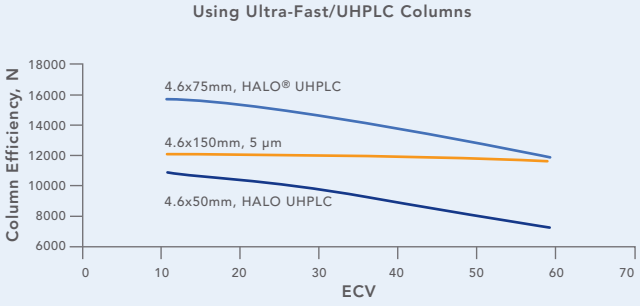
Because of the demand for higher productivity from analytical laboratories, the use of shorter columns packed with high efficiency particles, so-called ultra-fast or UHPLC columns, are becoming common place. Also, the desire to reduce solvent consumed by HPLC has many laboratories switching to columns with smaller internal diameters. Either of these actions results in chromatographic bands eluting from columns with much smaller peak volumes. If you are using newer UHPLC-type equipment, you should be able to use ultra-fast or smaller bore columns without any problems. However, if you are using conventional HPLC equipment, you are likely to experience a loss in resolution due to excessive extra column volume, ECV.

FIGURE 1: Effect of ECV on Column Performance

Note: Conventional HPLC equipment typically has ECV in the range of 30 to 50 µL. UHPLC equipment has ECV of ~10 µL.



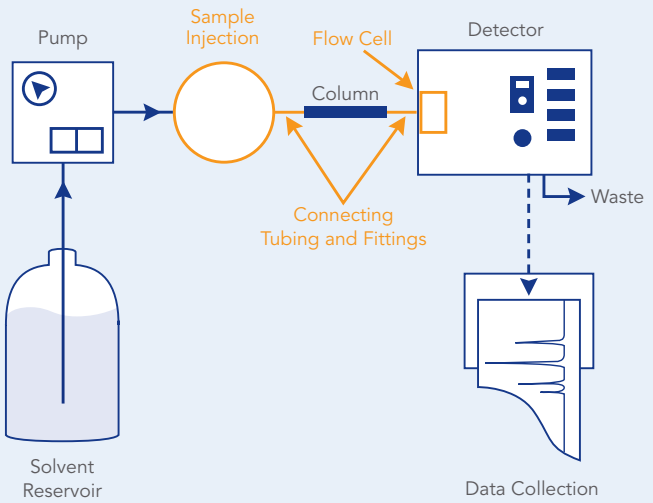
Reducing column ID is a great way to reduce solvent consumption, but the smaller the column ID the more susceptible the column is to ECV. All three of these columns have approximately the same efficiency, but the 2.1 mm ID column could have an "observed" efficiency over 30% less than that of the 4.6 mm ID column when used with typical conventional HPLC equipment with ECV greater than 30 µL.



Using shorter columns packed with higher efficiency packings, so called "ultra-fast" or "UHPLC" columns, can reduce separation times dramatically. However, to get the most resolving power from these columns when using conventional HPLC equipment, ECV must be reduced. Compared to the conventional 4.6 x 150 mm, 5 µm column, the 4.6 x 50 mm HALO UHPLC column can reduce separation time by over 80% while sacrificing less than 5% of the resolution. However, ECV must be minimized to achieve this performance.

ECV is the volume in an HPLC system external to the column that contributes to the total peak volume. ECV typically comes from the sample injector, including the volume of sample injected, the tubing connecting the sample injector (autosampler) to the column, the tubing connecting the column to the detector, the detector flow cell, plus any volume added by heat exchangers, fittings, connectors and inline filters. The schematic in Figure 2 identifies where ECV can be found in HPLC equipment.

FIGURE 2: Locating Extra Column Volume (ECV)

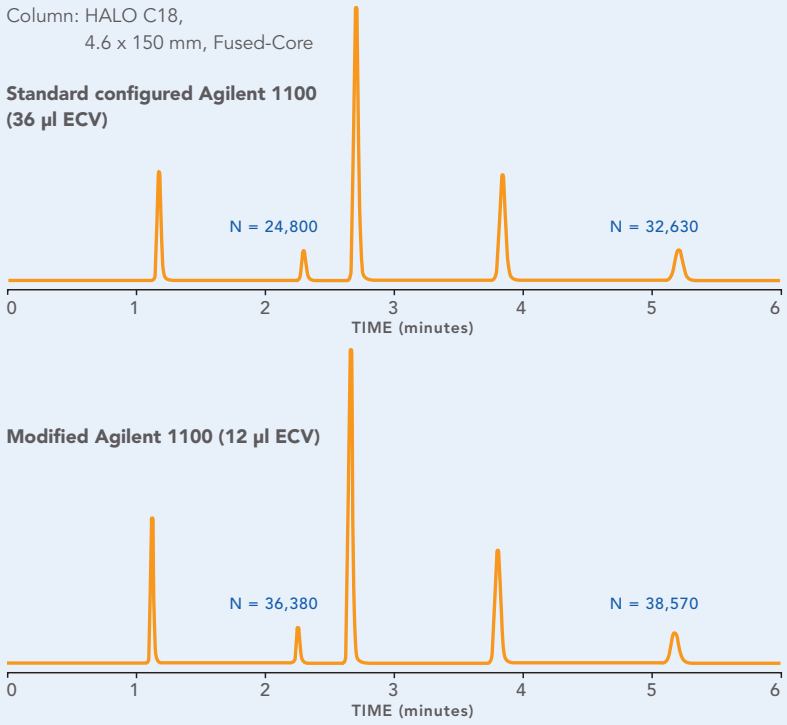


In this schematic of an HPLC system, the gold color indicates where extra column volume, ECV, is located.

The total peak volume, sometimes referred to as the "observed" peak volume, is the sum of the column peak volume plus ECV plus any volume contributed by dispersion in the system. In general, you should keep the ECV to less than half of the total peak volume in order to maintain more than 90% of the column's resolving power. Column peak volume will vary with both column length and column ID, but column ID will have the most effect on peak volume. Under isocratic conditions, peak retention (k values) will also affect peak volume. Early eluting peaks will have smaller peak volumes than late eluting peaks. In fact, one indication that your equipment ECV may be excessive for the column you are using is if the plate count (N) for an early eluting peak is significantly smaller than the plate count for a later eluting peak. See Figure 3 for an example.

Total peak volume = Column peak volume + ECV + apparent and/or real volume contributed by system dispersion

FIGURE 3: Indication of Excessive Equipment ECV for an Ultra-High Efficiency Column



An indication that you may have excessive ECV in your LC equipment for the column you are using is if the plate count for an early eluting peak is significantly lower than for a late eluting peak. Early eluting peaks have smaller volumes than late eluting peaks and are more vulnerable to ECV. In the standard configured Agilent 1100 the first eluting peak has 24,800 plates while the last eluting peak has over 32,000 plates. While this is good performance from the column, it does hint that much better performance could be achieved with this column by reducing the equipment's ECV. Indeed, the second chromatogram shows 36,380 plates for the first eluting peak and 38,570 plates for the last eluting peak using the same column, but after the Agilent 1100 had been modified to reduce the ECV.

When using gradient conditions, one can usually ignore the ECV that is before the column, unless the column is overloaded in terms of mass or volume, or your analytes have little or no retention in the starting gradient mobile phase. In most gradient separations, however, band compression occurs because the ever increasing stronger solvent at the back of the band accelerates the trailing portion of the band toward the front of the band. This effect essentially eliminates band broadening from pre-column ECV.

MEASURING ECV

There are three commonly used approaches to measuring the extra column volume in HPLC equipment. Simply adding the volumes from all contributors to equipment ECV is the easiest approach, although it depends on having accurate information from the equipment manufacturer as to detector flow cell volume, heat exchanger volume, etc. If this information is not available, or is suspect in its accuracy, then using one of the experimental methods may be preferable.

The experimental methods of measuring ECV actually measure extra column dispersion, rather than ECV. For the purpose of these calculations we will assume that extra column volume and extra column dispersion are approximately the same. A further discussion of extra column dispersion and how it relates to ECV is included later in this technical report.

Measuring Equipment Extra Column Volume

Method 1

Sum all the volume that the sample is exposed to, with the exception of the column volume.

ECV = Sample injection volume + volume of the capillary tubing from the sample injector/autosampler to the column compartment heat exchanger + column compartment heat exchanger volume + volume of the capillary tubing from the column compartment heat exchanger to the column + volume of the capillary tubing from the column to the detector flow cell + volume of detector flow cell + volume of pre-column filters and connectors, if any.

TABLE 1: Determining ECV in a standard configured Agilent 1100 by summing all the volume (non-column)

Description	Part No.	Volume (µL)
Needle seat capillary for standard autosampler 0.17 x 100 mm, Green	G1313-87101	2.5
Or, needle seat capillary for well-plate autosampler 0.17 x 100 mm, Green	G1367-87302	2.5
Stainless steel tubing from autosampler to column compartment heat exchanger 0.17 x 180 mm, Green	G1313-87305	4.5
Column compartment heat exchanger 3 uL		3.0
Stainless steel tubing from column compartment heat exchanger to column 0.17 x 90 mm, Green	G1316-87300	2.2
Stainless steel tubing from column to detector flow cell 0.17 x 380 mm, Green	G1315-87311	9.4
Standard flow cell for VWD 10 mm, 14 µL, 400 bar	G1314-60082	14.0
Standard flow cell for DAD/MWD 10 mm, 13 µL, 120 bar	G1315-60022	13.0
	Total ECV	**34.6/35.6**

TABLE 2: Estimating Capillary Tubing Volume

ID (inches)	ID (mm)	µL/cm
0.005	0.127	0.13
0.007	0.178	0.25
0.009	0.229	0.41
0.010	0.254	0.51

A considerable amount of connector tubing ECV can be reduced by using smaller ID tubing. For UHPLC applications, we recommend using 0.127 mm tubing.

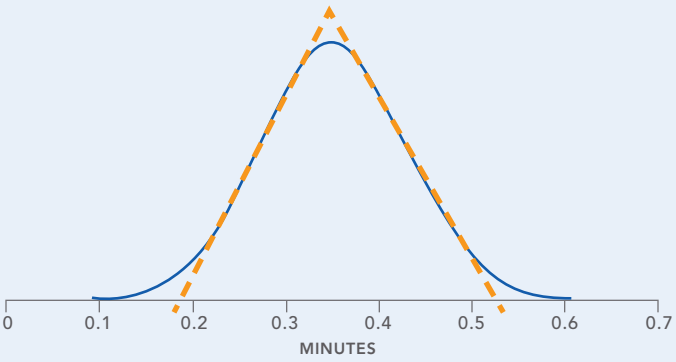
Method 2

Measure 4σ (peak volume) without contribution from the column.

1. Replace the column with a zero dead volume union.
2. Establish a flow rate of 100 µL/min with 50:50 organic modifier/water.
3. Set detector time constant to ≤ 0.1 sec and data rate to ≥ 10 Hz. (Detector response time equals time constant multiplied by 2.2.)
4. Allow flow rate to equilibrate for 10 minutes. Verify that the pressure and baseline are stable.
5. For best accuracy, measure the flow rate using a small (5 or 10 mL) volumetric flask.
6. Prepare an acetone solution with a concentration of 0.1% v/v in mobile phase.
7. Inject 0.5 µL of the acetone solution.
8. Print an expanded chromatogram so that the baseline width is > 50 mm. This will make the measurements more accurate.
9. Measure and calculate a conversion factor for axis distance in mm to time in min.
10. Draw tangents to the upslope and the downslope of the acetone peak.
11. Draw peak baseline from peak start to peak stop.
12. Measure distance, in mm, from the intersection of the upslope tangent line with the baseline to the intersection of the downslope tangent line with the baseline.
13. Calculate the peak tangent width (4σ) in minutes by multiplying the tangent width measured in step 12 (in mm) by the conversion factor calculated in step 9.
14. Calculate the system ECV by multiplying the flow rate (100 µL/min) by the peak tangent width calculated in step 13.

See Figure 4 for an example

FIGURE 4: Determining ECV by measuring peak volume (4σ) without a column



Example:
Flow rate = 100 µL/minute Tangent peak width = 0.35 minutes
ECV = 100 µL/minute x 0.35 minutes = 35 µL

Method 3

Use linear regression of peak variance to measure ECV.

1. Prepare standards of analytes that are homologues of each other. For example, uracil (void marker), benzyl alcohol, benzonitrile, nitrobenzene, 4-chloro-1-nitrobenzene, and toluene have been used successfully.
2. Set the LC pump for a flow velocity of 0.2 cm/sec. See Table 3 for a conversion of cm/sec to ml/min for various column IDs.
3. Set the mobile phase strength so that the retention of the standard analytes have k values between approximately 1 and 5.
4. Ensure that the flow rate and the baseline are stable.
5. Prepare a composite solution of the homologues, and inject a small volume, preferably less than 1 µL.
6. Get performance reports for the runs and obtain retention times and peak widths at half height. Measure both in seconds.
7. Calculate the σ (in seconds) via the following equation: σ = Peak width at half height ÷ 2.354.
8. Multiply σ by the flow rate to get σ in µL.
9. Multiply retention time (t_r) by the flow rate to get V_r in µL.
10. Plot σ^2 (µL) vs. V_r^2 (µL).
11. The y-intercept is equal to σ_{ec}^2.
12. To calculate ECV, multiply 4 times σ_{ec}

See Figure 5 for an example

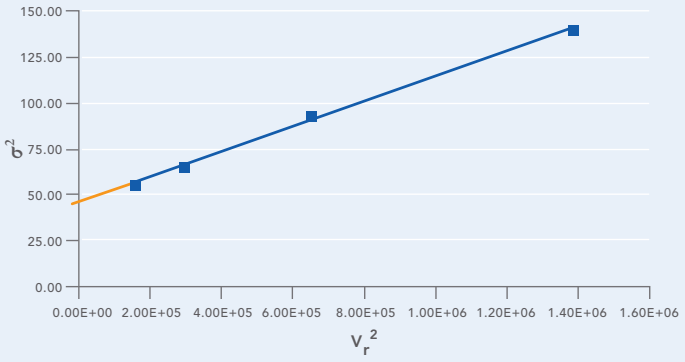
TABLE 3: Converting Mobile Phase Velocity (cm/sec) to Column Flow Rate (ml/min)

Mobile Phase Velocity	Column ID (mm) 1.0	2.1	3.0	4.6
0.1	0.030	0.13	0.27	0.63
0.2	0.059	0.26	0.53	1.3
0.3	0.089	0.39	0.80	1.9
0.4	0.12	0.52	1.1	2.5
0.5	0.15	0.65	1.3	3.1
0.6	0.18	0.79	1.6	3.8
0.7	0.21	0.92	1.9	4.4
0.8	0.24	1.0	2.1	5.0
0.9	0.27	1.2	2.4	5.7
1.0	0.30	1.3	2.7	6.3

FIGURE 5: Determining ECV by linear regression of peak variance

Example:
Flow rate = 0.95 ml/min or 15.83 µL/sec
σ = Peak width at half height (PW) divided by 2.354
t_r = analyte retention time
V_r = analyte retention volume

Compound	t_r (sec)	V_r (µL)	PW (sec)	σ (sec)	σ (µL)	σ^2 (µL)	V_r^2 (µL)
Uracil	25.2	399	1.11	0.472	7.466	55.7	1.59x105
Benzyl alcohol	34.2	541	1.20	0.510	8.071	65.1	2.93x105
Benzonitrile	51	807	1.43	0.609	9.645	93.0	6.52x105
Anisole	74.4	1178	1.76	0.747	11.82	140	1.39x106



Y-intercept = σ_{ec}^2 = 43 ECV = 4 x σ_{ec} = 4 x 6.6 µL = 26 µL

TABLE 4: Recommended equipment ECV, data rate and time constant for maintaining optimum resolving power of ultra-fast columns

Column I.D. (mm)	Column Length (mm)	Total Maximum ECV (µl)	Maximum Volume Contributed by Detector Flow Cell	Minimum Data Rate (Hz)	Maximum Time Constant (sec)
4.6	100	33	15 µl	5	0.22
4.6	75	28	5 µl	10	0.19
4.6	50	23	5 µl	10	0.16
4.6	30	18	5 µl	10	0.12
3.0	100	14	5 µl	5	0.22
3.0	75	12	2 µl	10	0.19
3.0	50	10	2 µl	10	0.16
3.0	30	8	2 µl	10	0.12
2.1	100	7	2 µl	5	<0.1
2.1	75	6	2 µl	10	<0.1
2.1	50	5	1 µl	10	<0.1
2.1	30	4	1 µl	10	<0.1

By keeping the LC equipment's ECV to less than the volumes listed above, close to optimum performance is achievable for the specific ultra-fast column dimensions indicated. These estimates are based on ultra-fast columns with average plate counts of 220,000 N/Meter, a peak retention factor (k) of 2 and maintaining over 90% of the resolving power of the ultra-fast columns. Even though gradient elution conditions may permit larger total ECV than what is recommended here, the maximum recommended volume for the detector flow cell should still be used.

ECV ≠ Extra Column Dispersion

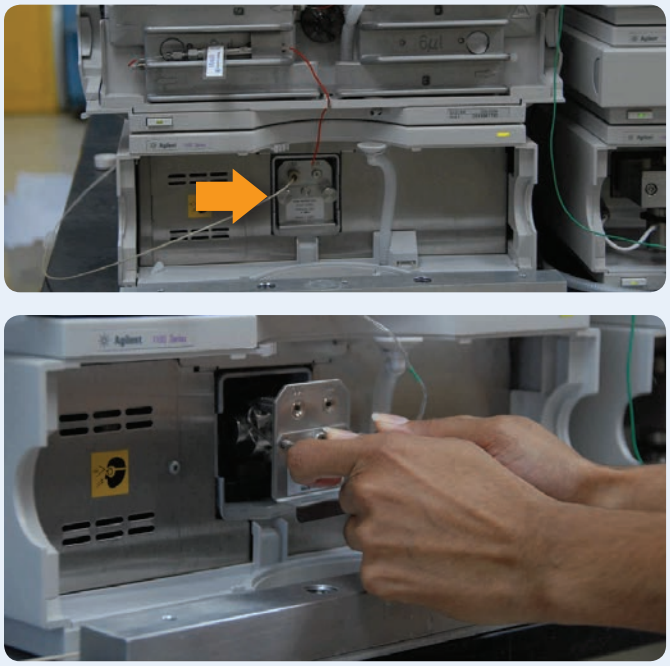
This report has been focused on extra column volume and its effect on band broadening. However, it is important to note that what we are really concerned about is extra column dispersion. Extra column volume is just one contributor to extra column dispersion. For convenience, we can often assume that other contributors to extra column dispersion are negligible compared to ECV. But sometimes an HPLC instrument system's extra column dispersion, and its negative impact on plates, is greater than what can be explained by ECV alone. The design of detector flow cells, for example, can have a significant effect on extra column dispersion, independent of their volume, as can poorly designed or poorly made capillary tubing connectors.

It is also important to recognize the contribution of the detector time constant and data collection rate to extra column dispersion. It is vitally important that these parameters be set appropriately for the column that you are using. The default values set by manufacturers of conventional HPLC systems are usually adequate for most conventional columns. However, when using ultra-fast columns that generate fast eluting peaks, faster detector time constants and data rates must be used, or poorer "observed" column performance will occur. Table 4 provides guidelines for setting detector time constants and data rates when using ultra-fast columns of different dimensions.

REDUCING ECV

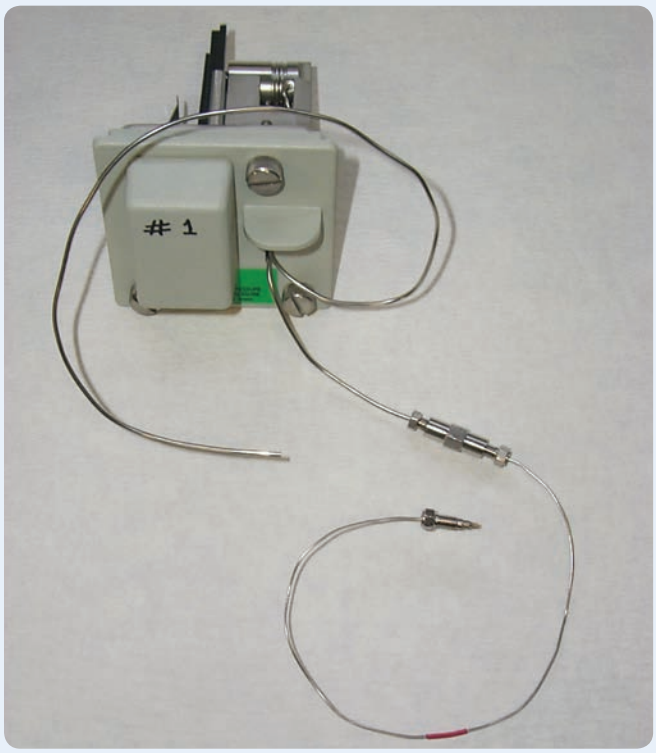
If you determine that your HPLC equipment's ECV is excessive for the column that you are using, then you will need to do some "plumbing" on your equipment to reduce the ECV to an acceptable volume. If you are using gradient conditions, you may only have to reduce the ECV that occurs after the column. Replacing the detector flow cell with one that has a smaller volume usually has the greatest effect on ECV, and is usually the easiest "plumbing" change to make. Unfortunately, it is also the most costly change, with detector flow cells typically costing between $1,500 and $2,500. Table 4 can help guide you in your choice of appropriate detector flow cells.

FIGURE 6: Replacing the detector flow cell in an Agilent 1100



Replacing the detector flow cell in an Agilent 1100 is simple. It is readily accessible and has just two thumb screws holding it in place.

FIGURE 7: Replacing the detector flow cell in a Waters Alliance



The Waters Alliance 2487 detector is shown sitting on top of the Waters Alliance system in the top photo. Removing the cover of the detector allows access to the detector flow cell assembly, shown in the bottom photo. Two screws hold the flow cell assembly in place, making it easy to install a smaller volume flow cell.

Recommended consecutive steps to take in "plumbing" HPLC equipment for reduced ECV:

1. **Replace the detector flow cell with a smaller volume flow cell.**
2. **Replace the capillary tubing connecting the column with the detector flow cell with smaller volume tubing of shorter lengths.**

If you are running gradient conditions, these first two steps may be sufficient in reducing your equipment's ECV to an acceptable level. However, if you plan to use isocratic conditions, these additional steps are recommended:

3. **Reduce sample injection volume to the minimum amount necessary to achieve acceptable peak response.**
4. **Keep the sample solvent weaker than the mobile phase.** If this is not possible, it will place more restrictions on the volume of sample that you can inject. If you are running gradient conditions, you will likely be able to inject considerably more sample volume than you would under isocratic conditions. However, it is still important to keep the sample solvent weaker than the initial strength of the mobile phase.
5. **If you are using a column compartment with a heat exchanger, replace it with a lower volume heat exchanger. Also, consider eliminating the heat exchanger, if you determine that it will not affect the quality of your chromatography.**
6. **Replace the capillary tubing connecting the sample injector/autosampler to the inlet of the column or to the column compartment heat exchanger.**

Although the detector time constant and the data rate, of course, have no effect on ECV, they still can have a significant effect on extra column dispersion and must be taken into consideration when trying to get the best performance from ultra-fast columns. Table 4 provides guidelines for selecting data rates and time constants for different ultra-fast column configurations.