(U)HPLC Method Parameter Quick Reference

When choosing a (U)HPLC column for an application the most important factor for high resolution is selectivity. This determining factor is mostly dependent on the analytes of interest but it can be altered, for example, by the choice of the stationary phase modification as well as organic solvents and additives of the mobile phase.

However other important parameters such as:

- **column inner diameter or radius (ID, r)**
- **column length (L)**
- **stationary phase particle diameter (d_p)**
- **mobile phase flow rate (F)**
- **injection volume (V_i)**
- **porosity of sample or mobile phase filters**

are all interconnected and need to be considered to achieve optimal resolution. Especially when transferring a method between different types of systems or detection methods, adjustments of these parameters are unavoidable.

This Expert Tip provides a quick reference of common combinations for column dimensions and suitable method parameters to help chromatographers in method transfer, scaling, optimisation and troubleshooting.

Adjusting flow rate to different stationary phase particle sizes and column ID

The optimal flow rate in isocratic separations is commonly described by the van Deemter equation. It is the linear velocity of the mobile phase (u) in cm*s^{-1} where various flow and kinetic parameters such as mass transfer and diffusion result in a minimum in the theoretical plate height (H) and therefore in the best separation efficiency.

$$H = A + \frac{B}{u} + (C_s + C_m) * u$$

The terms A (Eddy diffusion), B (longitudinal diffusion) and C (resistance to mass transfer; s = in stationary phase, m = in mobile phase) can be expanded further into several parameters and constants. In the scope of this quick reference however, it is important to note that linear increase of the stationary phase particle diameter will result in a proportionate increase of A and an exponential increase of C_s.

This means the optimal flow rate decreases when using bigger particle sizes and increases when using smaller ones.

To keep the linear mobile phase velocity and therefore the retention time and separation efficiency constant when changing the column inner radius (r), the flow rate (F) in mL*min^{-1} must be adjusted by a scaling factor (SF):

$$F_2 = F_1 * SF$$

Where SF is solely dependent on the inner radius of both columns:

$$SF = \left(\frac{r_2}{r_1}\right)^2$$

Because diffusion effects play a bigger part in SEC and HILIC separations, the optimal flow rate in these chromatography modes is usually about 50% of comparable RP applications.

Example

When scaling up an application from a column with dimensions 250 mm x 4.6 mm to a column with dimensions 250 mm x 10 mm the flow rate must be increased by a factor of 4.7.

$$SF = \left(\frac{5.0\ mm}{2.3\ mm}\right)^2 = \mathbf{4.7}$$

With an initial flow rate of 1 mL*min^{-1} the resulting flow rate is **4.7 mL*min^{-1}**.

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Table 1: Typical flow rates for reversed phase mobile phases (acetonitrile/water) depending on column ID and stationary phase particle size.

IDs [mm]	**typical flow rates in RP for particle size**			
	1.9 µm	**3 µm**	**5 µm**	**10 µm**
0.075	0.3 µL/min	0.15 µL/min	0.1 µL/min	–
0.1	0.6 µL/min	0.45 µL/min	0.3 µL/min	–
0.3	9 µL/min	4.5 µL/min	3 µL/min	–
0.5	30 µL/min	15 µL/min	10 µL/min	–
1.0	150 µL/min	75 µL/min	50 µL/min	–
2.1 / 2.0	0.6 mL/min	0.3 mL/min	0.21 mL/min	–
3.0	1.2 mL/min	0.6 mL/min	0.43 mL/min	–
4.0	–	1.0 mL/min	0.7 mL/min	–
4.6	–	1.5 mL/min	1.0 mL/min	0.7 mL/min
10	–	–	4.7 mL/min	7.7 mL/min
20	–	–	21 mL/min	18 mL/min
30	–	–	42 mL/min	38 mL/min

Since the optimal flow rate increases when stationary phase particle size decreases, both of these factors lead to an increased back pressure and adjustment of column length is a necessity to keep pressure below the maximum values for the chromatographic system, stationary phase and column hardware. Because of this some standard column dimensions have emerged when using specific stationary phase particle sizes or LC equipment.

Table 2: Typical column dimensions for different stationary phase particle sizes.

	stationary phase particle size			
	1.9 µm	**3 µm**	**5 µm**	**10 µm**
column length [mm]	≤ 100	≤ 150	≤ 250	≤ 250
IDs [mm]	≤ 3.0	≤ 4.6	≤ 10	≤ 30
typical example	***100 x 2.1 mm***	***150 x 3.0 mm***	***250 x 4.6 mm***	***250 x 20 mm***

Adjusting sample injection volume to total column volume and solvent strength

To obtain sharp and distinct peaks in an HPLC analysis the sample constituents need to be retained strongly at the top of the column. When the sample is dissolved in a weak solvent or an injection volume is small the resulting band of adsorbed compounds is narrow and peak efficiency is high, but when the sample solvent is strong or its volume is too high this band broadens and peak efficiency drops. Therefore, the maximum injection volume (V_i) is highly dependent on the specific analyte and the solvents in use. The injection volume should always be kept at a minimum determined by signal sensitivity and can be at maximum <3% of the total column volume (V_c) when using weak solvents and <0.1% when using strong solvents.

As a general guideline for injections in weak solvents it is recommended to use an injection volume of about 1% of the total column volume.

Table 3: Recommended injection volumes for weak sample solvents depending on column ID and length.

IDs [mm]	**Recommended injection volume per column length**						
	30 mm	50 mm	75 mm	100 mm	125 mm	150 mm	250 mm
0.075	1 nL	2 nL	3 nL	4 nL	5 nL	6 nL	–
0.1	2 nL	4 nL	6 nL	8 nL	10 nL	12 nL	–
0.3	20 nL	35 nL	50 nL	70 nL	85 nL	100 nL	–
0.5	60 nL	100 nL	150 nL	200 nL	250 nL	300 nL	–
1.0	240 nL	400 nL	600 nL	800 nL	1000 nL	1200 nL	–
2.1/2.0	1 µL	2 µL	2 µL	3 µL	4 µL	5 µL	–
3.0	–	3 µL	5 µL	7 µL	9 µL	11 µL	18 µL
4.0	–	6 µL	9 µL	13 µL	16 µL	19 µL	30 µL
4.6	–	8 µL	13 µL	17 µL	20 µL	25 µL	40 µL
10	–	40 µL	60 µL	80 µL	100 µL	120 µL	200 µL
20	–	150 µL	225 µL	300 µL	400 µL	500 µL	750 µL
30	–	350 µL	550 µL	700 µL	900 µL	1000 µL	1750 µL

Preserving column life time

Working at or near the pressure maximum of a HPLC column will in turn reduce its life time due to mechanical stress. Since adjusting flow rate, column length, inner diameter and stationary phase particle size will lead to changes in the observed backpressure this has to be considered when optimising these parameters. Working slightly below optimum is preferable for column life time when otherwise back pressure would be too close to its maximum.

Furthermore, when using particle sizes of 3 µm and smaller, the calculated interstitial space for the densest sphere packing between the packed stationary phase particles shrinks and becomes smaller than the usual porosity of column inlet frits (0.5–2 µm), mobile phase filters and some sample filters

Table 4: Stationary phase particle sizes with corresponding interstitial diameters and recommended sample filter porosity.

Particle size [µm]	**Interstitial diameter** [µm]	**Recommended filter porosity** [µm]
10	1.45	0.45
5	0.72	
3	0.43	0.2
1.9	0.28	

In turn particulate contamination smaller than the inlet frit porosity but larger than the interstitial volume of the stationary phase will be caught by the stationary phase itself and an increase in back pressure due to column fouling is inevitable.

Because of this, all mobile phase and sample filters should be chosen to have a pore size no larger than 0.2 µm when using small HPLC or UHPLC particle sizes and guard columns should be packed with the identical stationary phase in use.