Recommended Practices for Starting Off Successfully With a Brand-New Column

Background

Starting off with a brand-new column is not as simple as connecting both ends of the column to your HPLC or UHPLC instrument, and then flushing the column with your isocratic mobile phase or your starting gradient mobile phase composition. Instead, you need to be aware that during stationary phase manufacturing, column packing, and quality control (QC) testing, various proprietary solvents and surfactants may have been used to improve packing efficiency and column quality. Each column is carefully packed and then individually tested with an analyte mixture to demonstrate that the column performs at or above specifications for efficiency, retention, selectivity (alpha), and tailing factor. The QC testing is carried out, and the results (report and chromatogram) are included in the box with each new column. The QC-tested column is then stored and shipped in that QC mobile phase in most cases. For certain stationary phases, an additional step is used to flush the column with 100% organic modifier to ensure maximum stability of the stationary phase before use by the customer.

To ensure best performance from a new column, the following steps are recommended:

1. Flush the column, for 5–10 column volumes (refer to Table 1 below), with 100% organic modifier, using the solvent you will use for your method or analysis conditions. **Connect only the inlet end of the column to the LC system, and collect the effluent in a beaker or other appropriate container.**

2. Next, attach the column outlet to the LC system and continue to pump 100% organic modifier and monitor the baseline using the wavelength(s) for your method (and/or a low wavelength such as 210 or 230 nm, or a broadband signal from a diode-array detector such as 254 nm (bandwidth 80 or 100 nm)). For LC-MS systems, use the total ion current (TIC) to monitor the baseline. Allow the baseline to come to an equilibrium state using 10–25 mAUFS for UV detectors.

Table 1 Column Volume and Equilibration Time Estimator*

Particle Type	ID (mm)	Length (mm)	Porosity	V_M (mL)	Flow Rate (mL/min)	Time for 5 V_M (min)	Time for 10 V_M (min)
Totally porous	4.6	150	0.65	1.62	1.0	8.1	16.2
Totally porous	3.0	150	0.65	0.69	0.6	5.7	11.5
Totally porous	2.1	150	0.65	0.34	0.5	3.4	6.8
Superficially porous	4.6	150	0.5	1.25	1.0	6.2	12.5
Superficially porous	3.0	150	0.5	0.53	0.6	4.4	8.8
Superficially porous	2.1	150	0.5	0.26	0.5	2.6	5.2
Totally porous columns include Avantor ACE, Prontosil, etc.							
Superficially porous columns include Avantor ACE UltraCore, HALO, HALO Bioclass							

*Note that other times can be estimated for different flow rates and column lengths by ratioing the new flow rate or column length versus those in the table. For example, 10 column volumes for a 4.6 x 250 mm totally porous column at 1.5 mL/min would require [1.62 mL × 5 V_M × (10 V_M/5 V_M) × (250 mm/150 mm)] / 1.5 mL/min = 18.0 min.

Once a stable baseline has been established, it is prudent to inject a standard or sample two or more times sequentially to ensure that the column is equilibrated by comparing chromatograms and retention times between/among runs.

Gradient Separations

For gradient separations, a recommended equilibration volume consists of one or two gradient delay volumes and multiple column volumes. Literature research has shown that two gradient delay volumes, plus 5 to 10 column volumes for RPLC separations, allow the column to come to equilibrium ensuring repeatable retention times. As few as 3 column volumes and 1 gradient delay volume may provide reasonable equilibration for some methods and conditions, especially for injections as part of a sequence (Ref. 1). Some examples of gradient equilibration times are shown below in Table 2 below. The examples are shown for linear gradients from 5% to 95% organic using flow rates that are typical for linear velocities appropriate for the respective particle sizes and column IDs. Long equilibration times are necessary for instruments with larger delay volumes and for longer columns with larger IDs.

Before the column is used for a method that uses a mobile phase with a true buffer (acetate, formate, phosphate, citrate, etc.), it is wise to equilibrate the column with the desired isocratic or starting gradient composition without the buffered mobile phase first. For example, if the method uses a methanol/phosphate buffer at pH 2.5 and uses a gradient program that runs from 10% CH_3OH to 70% methanol, the column could be equilibrated with 5–10 column volumes of 70:30 CH_3OH/water, followed by 70:30 CH_3OH/buffer, followed by 10:90 CH_3OH/buffer, followed by 2 or 3 gradient runs from 10% to 70% CH_3OH/buffer before collecting meaningful results. Here is an additional caveat for working with buffered mobile phases: Test the upper limit for a gradient run for buffer solubility when mixed with organic modifier used for the method. For example, if a gradient method calls for a final gradient composition of 80:20 CH_3CN/20 mM buffer, prepare a premixed mobile phase on a small scale by mixing ~8 mL of CH_3CN with 2 mL of buffer. Mix the solution thoroughly by vortex mixing or hand shaking, and then allow the mixture to stand at room temperature for several hours. If any precipitate forms, you may have to reduce the buffer concentration or reduce the final percentage of CH_3CN in the gradient final composition. See reference 2 for more information.

Table 2 Gradient Equilibration Times for Different Scenarios for Different Column IDs, Lengths, and Particle Sizes

Parameter	2.1 mm ID	3.0 mm ID	4.6 mm ID
Delay Volume (V_D, mL)	0.125	0.47	1.1
ID (mm)	2.1	3	4.6
Length (mm)	50	100	250
Particle Size (µm)	1.7	2.7	5
Particle Morphology	TPP	SPP	TPP
Porosity	0.65	0.50	0.65
V_M (mL)	0.113	0.353	2.701
Flow Rate (mL/min)	0.60	0.85	1.2
t_0 (min)	0.188	0.416	2.250
Linear Velocity (mm/sec)	4.44	4.01	1.85
%B Initial	5	5	5
%B Final	95	95	95
Gradient Time (t_G, min)	3.0	12.0	40
k*	2.58	4.65	2.86
2 V_D (mL)	0.25	0.94	2.2
10 V_M (mL)	1.13	3.53	27.01
Total Equil. Volume (mL)	1.38	4.47	29.21
Total Equil. Time (min)	2.29	5.26	24.34

Reference

1. Adam P. Schellinger and Peter W. Carr, "Isocratic and gradient elution chromatography: A comparison in terms of speed, retention reproducibility and quantitation", Journal of Chromatography A, 1109 (2006) 253–266.
2. Adam P. Schellinger and Peter W. Carr, "Solubility of Buffers in Aqueous–Organic Eluents for Reversed-Phase Liquid Chromatography", LCGC NORTH AMERICA VOLUME 22 NUMBER 6 JUNE 2004, pp 544-548.