Column Equilibration in HILIC Mode

AKN0025

ACE Knowledge Note #0025

Introduction

In order to obtain stable and reproducible retention times, it is essential to fully equilibrate HILIC columns with mobile phase prior to analysis to ensure a stable adsorbed water layer exists at the stationary phase surface. Similarly, after performing a gradient run, the column requires re-equilibration to the gradient starting conditions and formation of a stable adsorbed water layer.

Equilibration

In order to obtain reproducible retention times in liquid chromatography, it is essential that the column is equilibrated with mobile phase to a steady state. Allowing insufficient time for equilibration in both isocratic and gradient modes is a common source of poorly reproducible separations.

Isocratic

In reversed-phase (RP) LC, at least 10 column volumes of mobile phase should be flushed through the column prior to analysis in order to sufficiently equilibrate the column. Table 1 shows approximate column volumes for popular LC column formats. If elevated temperatures are used, additional time to equilibrate the column temperature may also be necessary.

In HILIC mode, column equilibration time can be somewhat longer. This is because in order to obtain stable and reproducible retention times, a stable hydration layer has to be established and maintained around the silica surface.

The time required for HILIC equilibration can vary between different stationary phases, mobile phases and analytes and is often highly application dependant. Longer equilibration times when using buffered mobile phases have also been noted. To obtain robust HILIC methods it is therefore recommended that column equilibration times are examined and documented during method development to aid in future method transferability.

As a general rule, it is recommended that a newly purchased column is flushed with 60-80 column volumes to fully equilibrate with a new mobile phase (Figure 1). For example, a 100 x 4.6 mm column operated at 1.5 mL/min requires initial equilibration of 42-56 minutes. Once the run is completed, the column should be washed and stored according to the guidelines found on the reverse of the column QC test chromatogram supplied with the column. For subsequent runs, shorter equilibration times of 20 column volumes are sufficient (Figure 2).

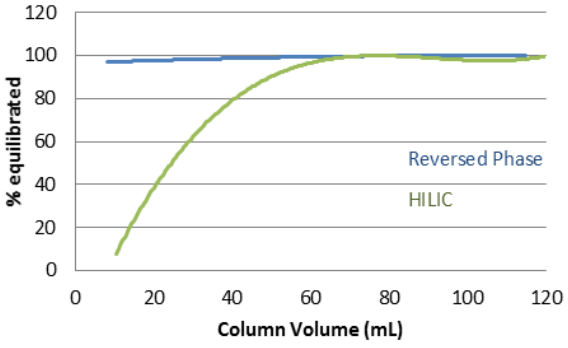

Figure 1: Plot of the number of column volumes required to achieve stable retention time in isocratic reversed-phase (blue) and HILIC (green) modes for brand new, unused columns

Column i.d. (mm) \ Column Length (mm)	50	75	100	125	150	250
1.0	0.025	0.037	0.049	0.062	0.074	0.124
2.1	0.109	0.164	0.218	0.273	0.327	0.546
3.0	0.223	0.334	0.445	0.557	0.668	1.113
4.6	0.523	0.785	1.047	1.309	1.570	2.617

Table 1: Approximate internal volume in millilitres of common LC column formats packed with fully porous particles.

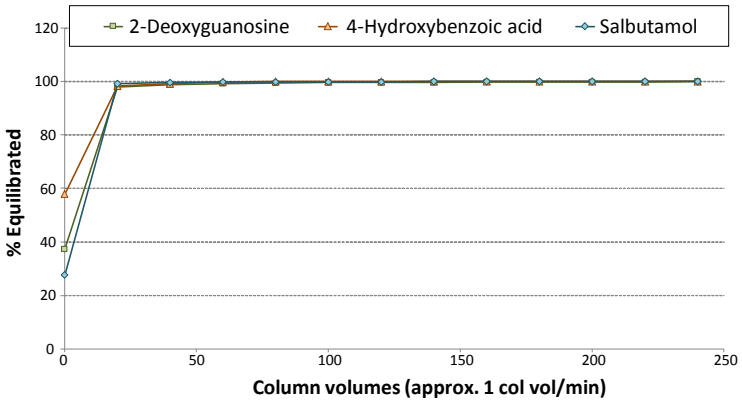

Figure 2: Plot of the number of column volumes required to achieve equilibration for second and subsequent analyses.
Column: ACE 5 HILIC-A, 100 x 3.0 mm, Mobile phase: 10 mM ammonium formate pH 4.7 in MeCN/H2O (90:10 v/v), Flow rate: 0.43 mL/min, Temperature: 25 °C, Detection: UV 214 nm, Sample: 2-deoxyguanosine, 4-hydroxybenzoic acid, salbutamol.

Gradients

Similar rules apply for gradient HILIC chromatography. A new HILIC column should be equilibrated for at least 60-80 column volumes before the first injection. After performing a gradient analysis, a re-equilibration time of 10 column volumes is usually sufficient to re-establish the gradient starting conditions and obtain robust retention times (Figure 3). Gradient re-equilibration times are typically more critical to reproducible retention times for HILIC gradients than for RP gradients.

It is recommended that the re-equilibration time is thoroughly assessed during method development and then recorded accurately within official method documentation to ensure reliable future use of the method.

When using buffered mobile phases in gradient HILIC mode, it is also advisable to maintain a constant buffer strength throughout the gradient. This is easily achieved by incorporating the same concentration of buffer in both solvent lines. Note that care should be taken to avoid buffer precipitation at high % organic.

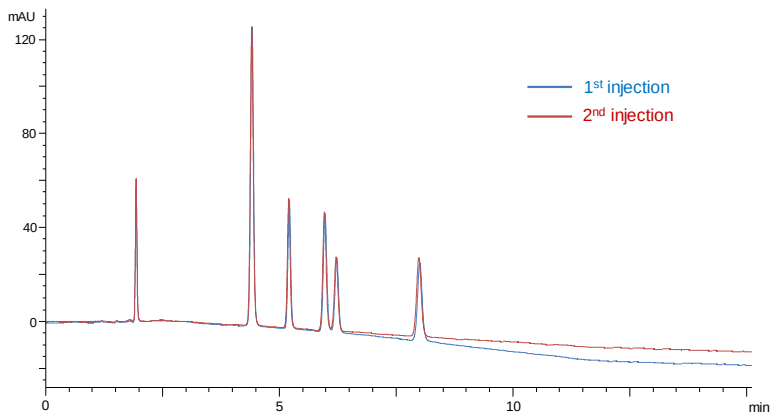

Figure 3: Repeat injections on a HILIC gradient method with re-equilibration time corresponding to 20 column volumes.
Column: ACE 5 HILIC-A, 150 x 4.6 mm, Mobile phase: 10 mM ammonium formate pH 3.0 in MeCN/H_2O, Gradient: 94 to 70% MeCN in 15 minutes, Flow rate: 1.5 mL/min, Temperature: 25 °C, Detection: 254 nm, Sample: theophylline, hypoxanthine, acebutolol, guanine, cytosine, cytidine.

Conclusion

HILIC methods typically require longer equilibration times than reversed-phase methods to obtain reproducible analyte retention. It is recommended that equilibration time is thoroughly assessed during method development. By following the guidelines outlined in this ACE Knowledge Note, reliable and reproducible HILIC methods can be developed using ACE HILIC columns.