Investigating the Effects of Chromatographic Parameters on Column Equilibration in Isocratic and Gradient HILIC Separations

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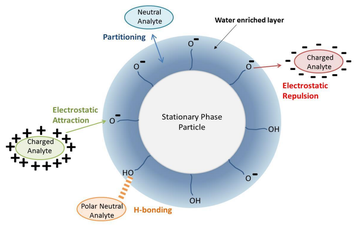
1. Introduction



- Hydrophilic Interaction Liquid Chromatography (HILIC) is a powerful technique for the separation of hydrophilic and polar compounds.
- HILIC utilises a polar stationary phase and high-organic containing mobile phase.
- The HILIC retention mechanism is complex and includes electrostatic, polar and partitioning interactions.
- The key to HILIC retention is formation of a water-enriched layer at the particle surface.
- Column equilibration to form a stable water-enriched layer is vitally important for reproducible chromatography.
- Long and variable equilibration times are often cited as a negative aspect of the HILIC technique.
- This work aims to assess key isocratic and gradient method parameters and how they affect column equilibration.

2. Experimental

- The three **ACE HILIC** phases (100 x 3.0 mm, 3 µm) were assessed in this study:
- **HILIC-A** (An acidic stationary phase showing high cation exchange capability)
- **HILIC-B** (A basic character phase with reasonable anion exchange capacity)
- **HILIC-N** (A neutral bonded phase with low anion and cation exchange capabilities)
- **Isocratic equilibration:** The following parameters were assessed:

Parameter	Values assessed
Fresh vs used column	Freshly packed column vs previously used column
Mobile phase ionic strength	5, 10 and 20 mM ammonium formate pH 3.0
Percentage water in the mobile phase	6, 10, 15 and 20 % water

- **Gradient equilibration:** The effect of post gradient re-equilibration time was investigated (re-equilibration times equivalent to 10, 20, 30 and 200 column volumes).

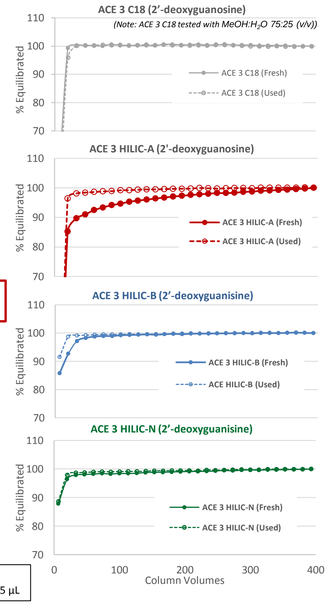
For all experiments, a test mix containing 4-hydroxybenzoic acid (acid), salbutamol (base) and 2'-deoxyguanosine (polar neutral) was injected.

3. Fresh vs Used Columns

- Freshly packed columns were equilibrated with mobile phase for 400 column volumes. Sample injected every 10 mins.
- The buffer was removed from the column (MeCN:H_2O 1:1) and stored for 48 h in IPA.
- Columns were then re-equilibrated with mobile phase for 400 column volumes and sample injected every 10 mins.

$$\%\ Equilibrated_{t=x} = \left(k_x / k_{final}\right) \times 100$$

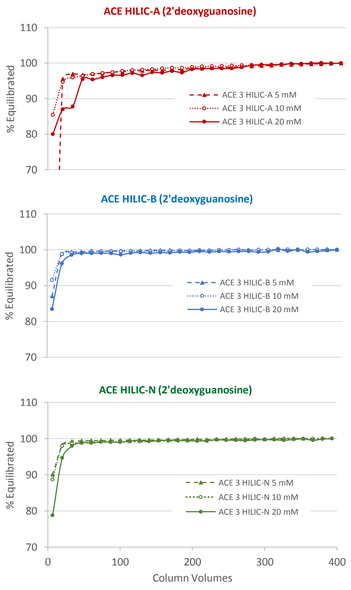
- Used columns were found to equilibrate faster than freshly packed columns, potentially due to the presence of residual non-aqueous packing solvents in pores of freshly packed columns.
- Equilibration times for used ACE HILIC columns were similar to the ACE C18.
- Under these conditions, fresh bonded phases appear to equilibrate faster than bare silica.



Mobile phase: 10 mM Ammonium formate pH 3.0 in MeCN/H_2O 90:10 (v/v)
Flow rate: 0.6 mL/min **Temp:** 35 °C, **Detector:** 214 nm **Injection volume:** 5 µL

4. Ionic Strength

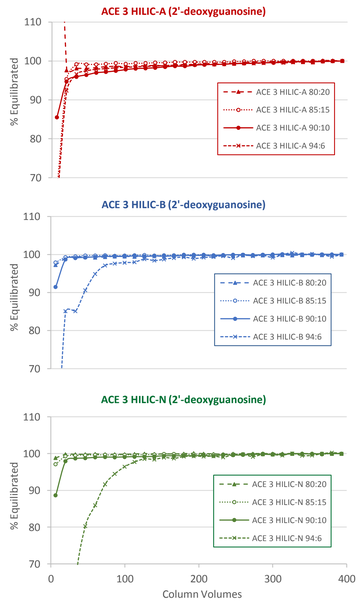
- All three phases were equilibrated with 5, 10 and 20 mM ammonium formate pH 3.0 in MeCN:H_2O 9:1 (v/v) for 400 column volumes. Sample injected every 10 mins.
- All three phases were >95% equilibrated after 20 column volumes with 5 and 10 mM buffer concentrations.
- The 20 mM ionic strength mobile phase required slightly longer to equilibrate (up to 50 column volumes on the HILIC-A).



Mobile phase: 5, 10 or 20 mM Ammonium formate pH 3.0 in MeCN/H_2O 90:10 (v/v)
Flow rate: 0.6 mL/min **Temp:** 35 °C
Detector: 214 nm **Injection volume:** 5 µL

5. Percentage Organic

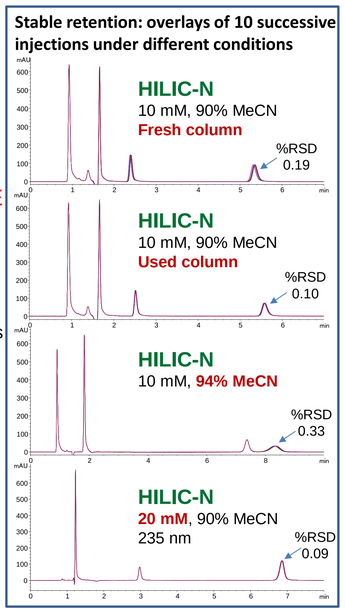
- All three ACE phases were equilibrated with 10 mM ammonium formate pH 3.0 in MeCN:H_2O at different volume fractions for 400 column volumes. Sample injected every 10 mins.
- 20 column volumes was sufficient to equilibrate (>95%) all ACE phases under most conditions.
- 94% MeCN took >100 column volumes to equilibrate on the ACE HILIC-B and HILIC-N.
- This implies that formation of a stable water layer takes longer when using low-aqueous containing mobile phases in HILIC mode for bonded phases, possibly due to the presence of a more substantial water-enriched region at the particle surface.



6. Isocratic: Observations

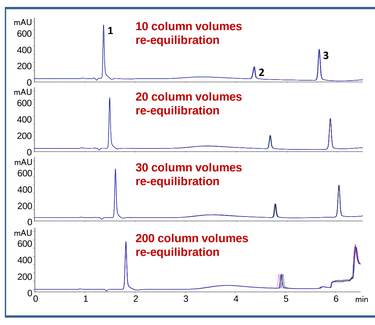
- Similar trends were observed for acidic (4-hydroybenzoic acid), neutral (2'-deoxyguanosine) and basic (salbutamol) analytes.
- Stable retention was achieved on all ACE phases once equilibrated.
- Fresh columns took longer to equilibrate than used columns
  - Minimum 20 column volumes for used columns
  - Minimum 60-80 column volumes for fresh columns
- Higher ionic strength mobile phases may require longer to equilibrate.
- Formation of a stable water-enriched layer may be substantially slower when using mobile phases containing ~5-6% water.

Stable retention: overlays of 10 successive injections under different conditions



7. Gradient Equilibration

- A HILIC gradient was run on each phase and the post-gradient equilibration time varied:
  - 10, 20, 30 and 200 column volumes
  - 1 column volume = 0.75 minutes (100 x 3.0 mm column, 0.6 mL/min)
  - 10 injections performed, 1st injection discarded
- The length of the re-equilibration stage was found to affect analyte retention.



Overlay of 9 consecutive injections for each re-equilibration time

Columns: ACE 3 HILIC-N, 100 x 3.0 mm
Mobile phase:
A: 10 mM ammonium formate pH3.0 in MeCN:H2O 94:6 (v/v)
B: 10 mM ammonium formate pH3.0 in MeCN:H2O 50:50 (v/v)

Gradient:	t	%B	
	0	100	
	10	0	
	15	0	
	16	100	
	XX	100	X = 23.5, 31.0, 38.5 or 166.0 (10, 20, 30, 200 column volumes)

Flow Rate: 0.6 mL/min Temperature: 35 °C
Injection Volume: 5 µL Detection: UV, 235 nm
Sample: 1. 4-hydroxybenzoic acid, 2. Salbutamol, 3. 2'-Deoxyguanosine

8. Gradient Equilibration

- Replicate injections (n=9) for each re-equilibration experiment showed excellent reproducibility (%RSD typically <0.3).
- This implies that re-equilibration times of 10-20 column volumes should be generally applicable to obtain reproducible chromatography.
- It is essential to accurately control gradient re-equilibration times in HILIC mode. Care should also be taken to account for differing system dwell volumes.
- Important to carry out a blank run before injecting or discard the first injection.

		Retention time (%RSD*)		
	Re-equilibration	4-Hydroxybenzoic acid	2'-Deoxyguanosine	Salbutamol
HILIC-A	10 column volumes	1.178 (0.09)	4.134 (0.22)	4.547 (0.25)
	20 column volumes	1.300 (0.06)	4.574 (0.18)	4.429 (0.12)
	30 column volumes	1.330 (0.11)	4.683 (0.21)	4.446 (0.13)
	200 column volumes	1.343 (0.61)	4.788 (0.61)	4.514 (0.46)
HILIC-B	10 column volumes	1.351 (0.18)	5.559 (0.09)	4.244 (0.14)
	20 column volumes	1.398 (0.30)	5.712 (0.07)	4.441 (0.13)
	30 column volumes	1.409 (0.31)	5.810 (0.09)	4.508 (0.15)
	200 column volumes	2.222 (0.14)	6.003 (0.41)	4.639 (0.61)
HILIC-N	10 column volumes	1.367 (0.08)	5.636 (0.05)	4.349 (0.12)
	20 column volumes	1.488 (0.08)	5.857 (0.04)	4.666 (0.08)
	30 column volumes	1.602 (0.08)	6.028 (0.05)	4.767 (0.13)
	200 column volumes	1.816 (0.11)	5.765 (0.76)	4.917 (0.43)

9. Conclusions

- It is essential to fully and consistently equilibrate HILIC columns to obtain reproducible chromatography.
- With adequate equilibration, ACE HILIC columns show similar reproducibility to reversed-phase columns.
- Fresh columns should be equilibrated for at least 60-80 column volumes before injecting.
- Equilibration times for used columns are shorter, 20 column volumes is usually sufficient.
- Under certain conditions, column equilibration may take longer than 20 column volumes.
  - High buffer concentrations
  - Low percentage of water in the mobile phase
- For HILIC gradients, varying the post-gradient re-equilibration time affects analyte retention and potentially selectivity. It is crucial to accurately control and record re-equilibration times.
- When transferring HILIC gradients, instrument dwell times may need compensating for to maintain consistent re-equilibration times.