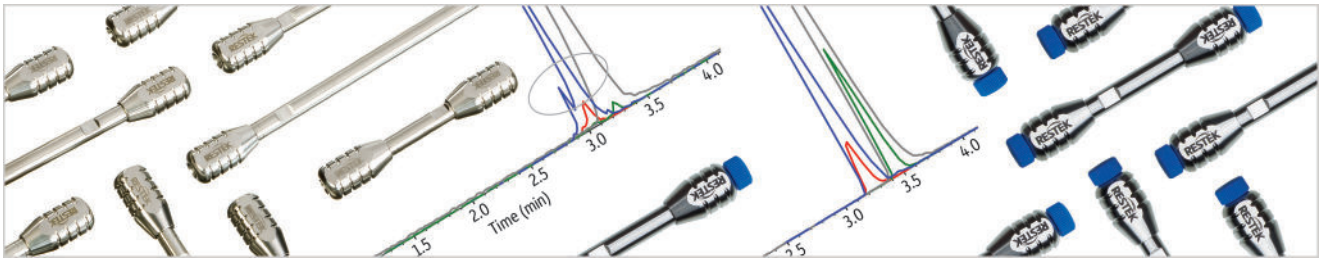


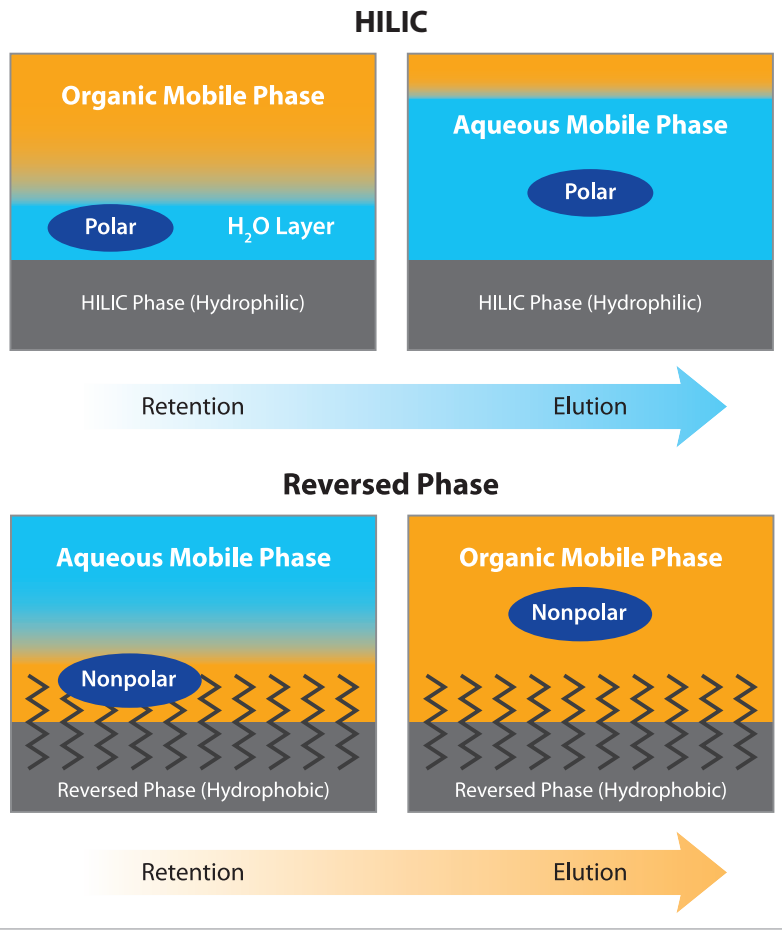
How to Avoid Common Problems with HILIC Methods

HILIC methodology is a powerful tool, especially for analyzing polar compounds, but it can be challenging to implement and there are several important considerations that you need to be aware of in order to avoid common pitfalls. This article will introduce the HILIC technique, discuss frequent problem areas, and help you successfully incorporate HILIC methods into your laboratory's repertoire.

"HILIC" stands for Hydrophilic Interaction Liquid Chromatography. It is a separation mode that uses a polar stationary phase, which can be bare silica, like the Raptor HILIC-Si column, or a polar bonded phase, like the Raptor FluoroPhenyl column. The solvents used in HILIC methods are similar to those employed in reversed-phase LC (e.g., water, methanol, and acetonitrile) and volatile buffers and modifiers may also be used. Because HILIC stationary phases are polar, the technique is well suited for the analysis of polar molecules that have limited or no retention on typical reversed phases like C8 and C18.

HILIC is sometimes called "reverse reversed-phase" because water is the "strong" or eluting solvent, which is the opposite of reversed-phase LC, where water is the "weak" solvent (Figure 1). In HILIC separations, the initial conditions are highly organic (typically 60% or more) and a water layer is adsorbed on the silica surface. The presence of this water layer creates what is sometimes described as a gradient within the mobile phase because it is increasingly water-rich as you get closer to the silica surface. Because they are soluble in the water layer, polar analytes can interact with the polar particle surface and are retained in multiple ways, including hydrogen bonding, dipole-dipole, and ion exchange. Raptor HILIC-Si columns, for example, offer a good combination of ion exchange and partitioning, which allows for alternate selectivity and retention compared to other HILIC-type phases.

Figure 1: Use HILIC when greater retention of polar analytes is needed. In HILIC mode, the aqueous mobile phase is the strong (or eluting) solvent versus the more familiar reversed-phase mode, where elution is the result of the organic solvent strength.



Column Conditioning and Re-Equilibration are Essential for Retention Time Reproducibility

The first step in ensuring success with HILIC methods is understanding that the analytical column must be properly conditioned before use and also sufficiently equilibrated between injections. Failure to condition and then equilibrate the column between runs can result in the water layer not being fully reestablished on the particle surface, which can lead to irreproducible retention times.

In order to condition your column prior to use, it must be flushed with the mobile phase that will be used during analysis. For isocratic HILIC methods, at least 50 column volumes should be used and, for gradient HILIC methods, at least 10 blank injections running the full-time program should be performed. Conditioning is also required when you change the mobile phase composition or the concentration of any additives. Use the same number of column volumes or blank injections as you did when conditioning the column for the first time.

In addition to the initial conditioning of the column with the mobile phase, it is critical that the column be re-equilibrated between injections. Because the separation mechanism in HILIC methods involves the adsorption of a water layer on the particle surface, it is very important to completely reestablish or "reset" this water layer in between injections to ensure retention time reproducibility. We recommend equilibration with a minimum of 10 column volumes starting when the gradient program returns to initial conditions, or in isocratic separations, at least 10 column volumes starting from the retention time of the last peak. The number of column volumes required for complete re-equilibration can be very analyte dependent, especially for isocratic separations, so make sure you investigate retention time reproducibility during HILIC method development.

Table I shows the column volume for each available dimension of the Raptor HILIC-Si column. When conditioning, multiply by the recommended 50 for isocratic methods to determine the total volume of conditioning solvent required, then divide by the flow rate to calculate the total conditioning time. For equilibration, multiply the value in the table corresponding to your column size by 10 for the equilibration volume required; then, divide by the flow rate to calculate the time interval for equilibration in your method program.

Table I: Column Volumes (mL) Based Upon Raptor HILIC-Si Column Dimensions

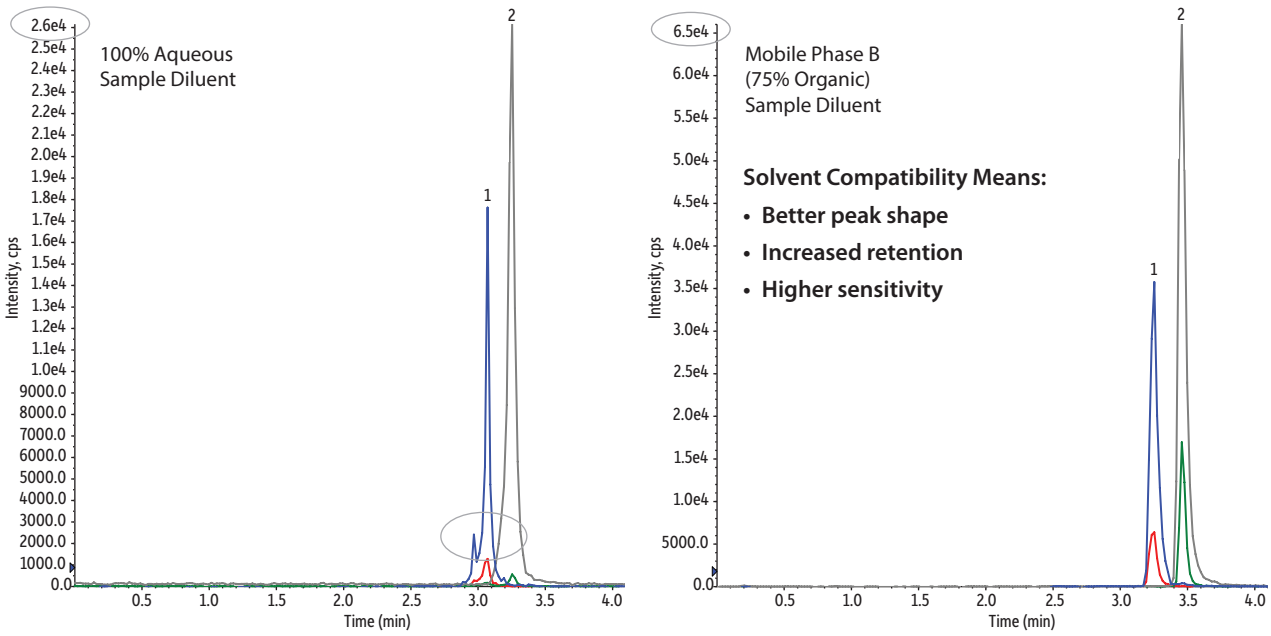
	Column Length			
Column ID	30 mm	50 mm	100 mm	150 mm
2.1 mm	0.05	0.1	0.2	0.3
3.0 mm	—	0.2	0.4	0.5
4.6 mm	—	0.4	0.8	1.2

For example, when using a 100 mm x 2.1 mm ID column, one column volume is 0.2 mL. If we are running an isocratic method with a flow rate of 0.30 mL/min, then the column should be conditioned with at least 10 mL (50 volumes x 0.2 mL volume). The total conditioning time would be 33 min (10 mL ÷ 0.30 min/mL). Similarly, the volume for re-equilibration between each injection would be 2 mL (10 volumes x 0.2 mL volume) and the re-equilibration time would be 7 min (2 mL ÷ 0.30 min/mL). These calculations can be used to establish a good starting point, but re-equilibration time is analyte dependent, so method development is required to ensure that re-equilibration is adequate and results in reproducible retention times.

Injection Solvent Matching is Critical for Good Peak Shape

If you have experience with reversed-phase separations, you know that the injection solvent makes a big difference in peak shape if it is very different from the solvent conditions that are on the column. The same is true in HILIC methods, so it's very important that the injection solvent be as close of a match as possible to the initial solvent conditions, which are high in organic content. The good news is that if you are using SPE for sample prep, you are eluting in a highly organic solvent, so the sample extract may already be ready for HILIC analysis. Figure 2 illustrates the impact of solvent matching on peak shape. If the sample is prepared in 100% aqueous diluent, peak shape is poor for diquat, both compounds elute early, and the signal is relatively low compared to the injection where the sample was dissolved in mobile phase B (75% acetonitrile). By matching the injection solvent to the initial mobile phase conditions, you get better peak shape, increased retention, and higher sensitivity.

Figure 2: Matching the injection solvent to the mobile phase results in better peak shape, retention, and sensitivity.



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Peaks	Precursor Ion 1	Product Ion 1	Precursor Ion 2	Product Ion 2
1. Diquat	183.3	157.2	183.3	130.1
2. Paraquat	185.3	170.3	171.3	77.2

Column	Raptor HILIC-Si (cat.# 9310A52)
Dimensions:	50 mm x 2.1 mm ID
Particle Size:	2.7 µm
Pore Size:	90 Å
Temp.:	45 °C
Sample	Paraquat & diquat calibration mix (cat.# 32437)
Conc.:	50 ng/mL (cat.# 32437 diluted to 50 ng/mL)
Inj. Vol.:	5 µL
Mobile Phase	
A:	Water, 50 mM ammonium formate, 0.5% formic acid
B:	25:75 Water:acetonitrile, 50 mM ammonium formate, 0.5% formic acid

Time (min)	Flow (mL/min)	%A	%B
0.00	0.6	0	100
4.00	0.6	35	65
4.01	0.6	0	100
7.00	0.6	0	100

Detector	MS/MS
Ion Mode:	ESI+
Mode:	MRM
Instrument	HPLC

Mobile Phase pH Effects Are Analyte Dependent

In addition to the importance of the mobile phase matching the injection solvent, the pH of the mobile phase can also affect chromatographic performance. In fact, method development and evaluation are most important in this area because the effect of pH on analyte charge state varies based on each compound's pKa. With HILIC methods, the high concentration of organic solvent in the mobile phase raises the pH, and the actual eluent pH can be 1–1.5 units higher than in the aqueous portion alone. The charge state of the column itself can also be affected. For example, in a Raptor HILIC-Si column, the bare silica has a pKa between 3.8 and 4.5, so the mobile phase pH changes the charge of the silica surface, making it neutral in very acidic conditions and ionized (negatively charged) as the pH begins to approach 3.8 and above. For this reason, if your analyte has one or more protonated amine or quaternary amine groups, it's a good candidate for analysis on a Raptor HILIC-Si column.

Buffer Choice Influences Both Chromatographic and Instrument Performance

Buffers are used to keep the eluent pH constant and can also assist in separating analytes of interest from interferences. Many HILIC separations use a mass spectrometer for the detector, so volatile buffers like ammonium formate and ammonium acetate are very common. The buffer concentration in HILIC separations is important to consider for two main reasons. The first is that the high organic content of the mobile phase can cause buffer salts to precipitate in the transfer tubing between the autosampler and the column, in the column frit, or on the column itself. All of these situations are likely to result in your instrument going offline for maintenance. The second consideration relates specifically to the retention mechanism happening in a Raptor HILIC-Si column. Recall that the silica surface has a negative charge under typical HILIC mobile phase conditions, so the positively charged buffer ions are competing with the target analytes for retention. If the buffer concentration is high, analyte retention can be reduced. Like with pH, method optimization is required, but 10 mM is a good starting point for buffer concentration. Both the A and B mobile phases should be buffered equally in order to keep the ionic strength constant during a gradient for the most consistent MS detector response. Check with your MS vendor for the maximum buffer concentration they recommend for your ESI source.

In Summary

As with any new technique, successful application depends in large part on understanding the differences between the new approach and more familiar procedures. HILIC methods provide a powerful new way to analyze polar compounds, but implementation can be challenging if analysts assume it is similar to reversed-phase LC. With HILIC methods, ensuring that the column is properly conditioned (both when new and when solvents and/or additives are changed) and also re-equilibrated between injections; matching the injection solvent to the initial mobile phase; and understanding the effects of mobile phase pH and buffer choice on your analysis are important steps toward getting good results. These guidelines provide a good starting point for establishing HILIC methods, but method development is essential to ensure optimum performance for your specific target analytes.

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