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PolyHYDROXYETHYL A™ Columns Hydrophilic Interaction Chromatography Mode (HILIC)

Principle of HILIC: If the stationary phase is more polar than the mobile phase, then solutes will be retained in proportion to their hydrophilicity. With PolyHYDROXYETHYL A, this is usually the case if the mobile phase contains > 60% organic solvent (usually acetonitrile [ACN] or propanol). The order of elution is generally the inverse of that with reversed-phase HPLC (RPC). Chaotropes decrease retention. These include TFA, urea, guanidinium hydrochloride, and unbuffered formic acid. Avoid their use unless the objective is to accelerate elution. Retention is influenced by the solvent used: ACN~PrOH>EtOH>MeOH.

Initial Use: Columns are shipped in methanol. Prepare the column for use in the following manner (200 x 4.6mm ID columns):

Flush with water for 20 minutes @ 1 mL/min
Flush with conditioning buffer for 1 hour @ 1 mL/min
Flush with water for 20 minutes @ 1 mL/min
Flush with mobile phase for 1 hour @ 1mL/min

A suitable conditioning buffer would be 100mM salt in the pH range 4.0-6.5, such as ammonium acetate or KH_2PO_4 .

It's a good idea to run at least one gradient cycle with a new column before injecting samples. Changes in the topography of the polymeric coating may lead to modest changes in the retention times during the first few runs following exposure to aqueous mobile phases.

New HPLC columns sometimes absorb small quantities of proteins or phosphorylated peptides in a nonspecific manner. The sintered metal frits have been implicated in this. Eluting the column for 20-24 hr. at a low flow rate with 40mM EDTA.2Na usually solves the problem. This passivates all metal surfaces in the HPLC system, as well as the column [CAUTION: This treatment can affect the integrity of the frits in some cases, and should probably be avoided with columns packed with 3- μm material. In some cases this has also caused the collapse of 5- μm , 200-Å column packings].

If a column is to be used in **mass spectroscopy**: Columns of all materials suitable for protein applications will leach coating components when new. This can result in an elevated background in mass spec. To accelerate the loss of leachable coating components, substitute 50 mM formic acid for the salt solution to condition a new column. Elute the column for 24 hours at a low flow rate. Then, flush out the formic acid with water and equilibrate with the HILIC mobile phases.

Routine use: Columns should be used at ambient temperatures. Filter mobile phases and samples before use. Failure to do so may cause the inlet frit to plug. If a salt gradient is being used, flush the column with 15 column volumes of the high-salt buffer before equilibrating with the low-salt buffer. At the end of the day, flush the column with 15 column volumes of water and plug the ends.

Loading Capacity: The loading capacity of a 4.6mm ID column is about 1 mg of polar solute, depending on the strength of the solute's binding to the support and the level of organic solvent in the sample solvent. High levels promote binding.

Storage: 1) Overnight: 100% mobile phase A. 2) Several days: Store in water. 3) Longer periods: Store in water in the refrigerator, with the ends plugged. **ACN can be added to the storage solvent (e.g., ACN:Water = 80:20) to retard microbial growth.**

Operation in the HILIC Mode: THE SAMPLE SOLVENT SHOULD MATCH THE STARTING MOBILE PHASE REASONABLY WELL. For example, if the mobile phase contains 80% ACN, then the sample should contain at least 70%. Otherwise, loading capacity is decreased and a pure solute may elute in multiple peaks or not be retained at all. The electrolytes should also be matched if possible. The mobile phase should contain at least 7-10 mM salt or electrolyte overall. The more highly charged the solute, the more salt is needed to yield symmetrical peaks. Use HPLC-grade salts if available.

HILIC of Peptides: Use a column with a pore diameter of 200 or 300 Å. If absorbance is to be monitored at low wavelengths (e.g., 220 nm), then include 10-15 mM triethylamine phosphate (TEAP) in the mobile phase; see separate instruction sheet for its preparation. Run a gradient from 85-5% ACN. If the mobile phase has to be volatile, then substitute ammonium formate or acetate for the TEAP. Retention will be higher at pH 3 than at pH values > 4. The following factors affect HILIC of peptides:

- 1) Retention is in proportion to how many hydrophilic residues the peptide contains. Basic residues are by far the most hydrophilic, followed by phosphorylated residues. Thereafter, retention follows the opposite trend seen with RPC; Asn- promotes retention the most, followed by Ser-, Gly-, etc., with Phe- and Leu- promoting retention least.
- 2) Juxtaposition of an acidic and a basic residue largely eliminates retention due to the basic residue.
- 3) At pH 2.7, only basic and phosphorylated or sulfated residues will be charged. At pH 5.0, carboxyl- groups will be charged as well. This factor can be used to manipulate selectivity.
- 4) As with cation-exchange, peptides will generally elute at pH 2.7 in order of increasing number of basic residues, since basic residues are so hydrophilic. However, unlike cation-exchange, a particularly hydrophilic peptide can be retained more strongly than a hydrophobic peptide with more basic residues. Thus, the selectivity of the two methods is complementary. Similarly, retention in HILIC isn't exactly the inverse of that in RPC, since a peptide with a lot of polar residues does not necessarily contain a corresponding number of nonpolar ones. Thus, a RPC-HILIC sequence can be used to fractionate complex mixtures of peptides.
- 5) If the peptide solution contains appreciable urea or another chaotrope, then dilute it to get the concentration of the chaotrope below 300 mM at least.

HILIC of Proteins: Use a pore diameter of 300 Å. Proteins contain more polar residues than do peptides (since they contain more residues of all kinds) and are better retained. One can frequently get good results with a gradient of 75%-5% organic solvent. There are two problems with HILIC of proteins that are usually not encountered with peptides:

- 1) Keeping the protein in solution in 75% organic solvent. Propanol is a better solvent for proteins than is ACN, while a blend of the two is frequently better than either solvent alone. Small amounts of chaotropes help in this, especially 50 mM hexafluoro-2-propanol (HFIP) and/or use of 50 mM formic acid as the electrolyte additive instead of a salt. Under these conditions, detergents and lipids will elute in the void volume, while proteins are retained. If solubility remains a problem at 75% PrOH, then start with 90% PrOH containing 100mM HFIP and 200mM formic acid.
- 2) Getting the proteins to elute. In contrast to the situation with peptides, it is frequently a good idea to replace ~ 15 mM salt with 50 mM formic acid in the mobile phases to be used for proteins. These conditions have been used successfully for online HILIC-MS of proteins up to 80 KDa.

HILIC of Metabolites and Small Polar Solutes: Use a pore diameter of 60 or 100 Å. Initially, try 85% ACN. Solutes of marginal polarity may require as much as 95% ACN for retention. Do include at least 7 mM salt in the mobile phases; TEAP for transparency at 220 nm or ammonium formate or acetate for volatility. Anomeric forms of reducing sugars are resolved; if this is a concern, contact PolyLC for ways to prevent this.

HILIC of Aminoglycoside Antibiotics: These represent an extreme in polarity. Success involves use of a column with pore diameter of 1000 Å (to decrease the surface area) and use of at least 125 mM ammonium acetate in the mobile phase to insure symmetrical peaks. Run a gradient from 85-5% ACN.

HILIC of Oligonucleotides and their Analogs: Try a shallow decreasing ACN gradient starting at 75%, with 100 mM triethylamine acetate, pH 6.0. C and G promote retention much more than do A and T.

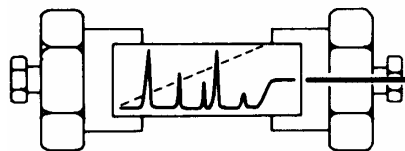
Preparation of TEAP Stock Solution

Example: 250 ml of 0.5 M TEAP, pH 3.0: Weigh 14.4 g. of 85% H₃PO₄ (HPLC grade) into a 250-ml beaker. Add a magnetic stirring bar. Slowly add ~ 150 ml water, with stirring. [DO THE NEXT PART IN THE HOOD]. Take a bottle of triethylamine out of the refrigerator and allow it to warm up to room temp. Place a pH electrode into the solution in the beaker. Using a pipet, add triethylamine to the stirred solution until the pH reaches 3.0 (CAUTION: When the pH reaches 2.8 or so, wait a few minutes till solution cools to room temp. before making the final pH adjustment). Pour the solution into a 250-ml volumetric flask (using a funnel). Rinse down the beaker with water and add the rinsing to the flask; repeat this once. Add water to the 250-ml mark and invert the flask 8-9 times. Filter & store in the refrigerator.

Preparation of HILIC Mobile Phase

Example: 1 L. of 80% acetonitrile (ACN) containing 15 mM TEAP: To a 1-L. vol. flask, add 30 ml of the 0.5 M TEAP stock solution. Add 170 ml water; swirl flask briefly. Now add ACN to several ml below the 1-L. vol. mark. Invert flask 8-9 times; contents will cool (due to the endothermic nature of mixing ACN and water). Let flask warm up to room temp. (this can be

accelerated by sonicating in a warm water bath) with occasional inversion of the flask to equilibrate the temperature of the contents. Now add ACN to the mark and invert 8-9x.



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PolyHYDROXYETHYL A™ Columns Size Exclusion Chromatography Mode (SEC)

Initial use: Flush new columns with 15 column volumes of water (*e.g.*, 30 ml for a 200x4.6-mm column), then condition with the mobile phase for a minimum of 4 hours. When using a denaturing mobile phase like 50 mM formic acid, one should condition overnight at a low flow rate in order to get reproducible retention times.

Routine use: Columns should be used at ambient temperatures. Filter mobile phases and samples before use. Failure to do so may cause the inlet frit to plug. At the end of the day, flush the column with 15 column volumes of water and plug the ends.

Mobile phases:

1) Nondenaturing mobile phase:

- a) Peptides: 0.2 M (Na)₂SO₄ + 5 mM KH₂PO₄, pH 3.0, containing 25% acetonitrile.
- b) Proteins: 50 mM KH₂PO₄ + 100 mM NaCl, pH 6.5, or some other solvent compatible with the proteins.

2) Denaturing mobile phase: 50 mM formic acid or any buffer between pH 3-7 containing 50 mM hexafluoro-2-propanol. The formic acid mobile phase is volatile but limits detection below 240 nm. For example, at 225 nm, a 50 mM formic acid solution has an absorbency of 1 AU. This high baseline can be subtracted, however, and absorbance monitored on top of it.

Loading Capacity: The loading capacity of a 4.6-mm ID column is roughly 0.4-0.8 mg peptide with no significant loss of resolution, but this number depends on the composition of the sample. The sample solvent should not differ greatly from the mobile phase in ionic strength or organic solvent content in order to prevent a significant difference in viscosity between the two. Otherwise, a sample might elute without encountering the stationary phase.

Storage: 1) Overnight: 100% mobile phase A. 2) Several days: Store in water. 3) Longer periods: Store in water in the refrigerator, with the ends plugged. **ACN can be added to the storage solvent (*e.g.*, ACN:Water = 80:20) to retard microbial growth.**

Fractionation range: These columns exhibit two alternative fractionation ranges, depending on the mobile phase used. For example, with a 200 Å pore column, a nondenaturing mobile phase will afford a range of approx. 200-40,000 Da. With a denaturing mobile phase, the range will be 20-1600 Da. The same column can be used interchangeably in either range simply by switching the mobile phase. Fractionation ranges also vary with pore diameter, per the following table:

Range (Da) in Different Mobile Phases

Pore Diameter	Denaturing	Nondenaturing
60Å	20-600	60-10,000
200Å	20-1,600	200-40,000
300Å	20-40,000	300-100,000
500Å	20-100,000	600-500,000
1000Å	20-500,000	1,000-1,000,000