
Hydrophilic Interaction Chromatography (HILIC): HILIC/SEC and HILIC/SCX

HILIC or RPC for Polar Molecules

To increase retention of hydrophilic molecules by RPC, there is a versatile, effective alternative to consider: hydrophilic interaction chromatography (HILIC). A rival technique to RPC for separating polar peptides, HILIC is easy to use and works well where RPC is ineffective--with polar solutes not retained well on RPC. HILIC has been used successfully with phosphopeptides, crude extracts, peptide digests, membrane proteins, carbohydrates, histones, oligonucleotides and their antisense analogs, polar lipids, and in preparative applications where changing the order of elution affects isolation yields.

How HILIC works

HILIC separates compounds by passing a hydrophobic or mostly organic mobile phase across a neutral hydrophilic stationary phase, causing solutes to elute in order of increasing hydrophilicity--the inverse of RPC. With neutral peptides one may use 15mm ammonium formate and reverse organic conditions. Highly charged molecules require low amounts (e.g., 10 mM) of salt for ion suppression and a slight perchlorate or sulfate gradient (in a high organic solvent concentration) to effect desorption.

PolySULFOETHYL or PolyHYDROXYETHYL Aspartamide

There are two column choices to perform HILIC separations, and both offer an alternative separation mechanism for added versatility. The PolyHYDROXYETHYL Aspartamide™ column (HEA) will retain solutes solely through hydrophilic interactions when using mobile phase concentrations in the range of 40-85% acetonitrile. Under non-HILIC conditions (mobile phase concentrations less than 40% acetonitrile) the column will perform small-molecule size exclusion separations¹.

The second column is the PolySULFOETHYL Aspartamide™ SCX column which performs either hydrophilic interaction separations superimposed upon electrostatic effects under HILIC conditions as above, or a cation exchange mixed-mode separation² where resolution is enhanced for peptides with the same net positive charge under non-HILIC conditions.

Operating Recommendations for HILIC Separations

Initial Use

When using either column to perform HILIC separations,

flush the new column with 25 ml water, then condition with at least 60 ml of a buffer solution with a salt concentration of 0.2 - 0.4 M and a pH in the range of 3 - 6 (exact figures are not important here). Flush again with another 20 ml water, then equilibrate with 30 ml of the mobile phase before injecting samples. (These volumes relate to 4.6 mm ID columns. For 9.4 mm ID columns, the volumes above should be multiplied by a factor of four). To prepare the HEA column for size exclusion chromatography or the SCX column for ion exchange chromatography, see the appropriate section below).

Routine Use

Be sure to filter samples and mobile phases before use. Flush and store HILIC columns in water when not in use. Operation at room temperature is recommended since elevated temperature shortens column lifetime.

General Mode of Operation

Salt is not required with solutes that are not electrolytes. In the case of electrolytes, use at least 5-10 mM buffer in the mobile phase. Gradient elution can be accomplished by a decreasing organic gradient (starting from 80-85% acetonitrile for peptides or 95% for phospholipids) or an increasing salt gradient (which typically gives flatter baselines). Solubility of salts can be a problem with mostly organic mobile phases, but sodium perchlorate works well and is transparent at low wavelengths (Fisher sells an HPLC grade). Buffer salts with reasonable solubility in 80% acetonitrile include triethylamine phosphate (TEAP) and sodium methylphosphonate (from methylphosphonic acid). Isocratic retention is typically several times greater with TEA salts than with the corresponding sodium or potassium salt. With 80% acetonitrile, concentrations of 75 mM (pH 5.0) or 100 mM (pH 2.8) TEAP are attainable. Gradient elution in HILIC generally requires one-fifth to one-tenth the concentration of salt required in ion-exchange chromatography.

A stock solution of TEAP is prepared by making a concentrated aqueous solution of phosphoric acid, adding TEA until the desired pH is attained, then diluting to give a known final concentration (e.g., 2 M in phosphate). Similar methods are used with phosphonate buffers. Prepare stock solutions fresh monthly and store in the refrigerator. For preparation of the mobile phase, add the appropriate amount of stock solution and water to a volumetric flask. Next, add the acetonitrile to a level several ml short of the mark. Mix, then put the flask in a sonication bath for 5 minutes. This degasses and warms the solution. Finally, add acetonitrile to the mark and mix.

Performing HILIC Separations (cont'd)

Organic solvents such as isopropanol can be used as alternatives to acetonitrile. But higher concentrations are usually required to attain the same degree of retention, and the resulting mobile phases are appreciably more viscous.

HILIC of Peptide and Proteins

Begin with a common mobile phase of 10 mM TEA, pH 2.8, containing 80% acetonitrile. Run gradients as described above. If retention is inadequate, try 85% acetonitrile.

The following factors affect retention of peptides in HILIC:

- 1. Retention is proportional to the hydrophilicity of a peptide:** Basic groups are the most hydrophilic, followed by phosphorylated residues. Thereafter, retention follows the opposite trend seen with reversed-phase HPLC: Asn promotes retention the most, followed by Ser-, Gly-, etc., with Phe- and Leu- promoting retention the least.
- 2. Juxtaposition of an acidic and basic residue:** An acidic next to a basic residue, or an acidic residue as the N-terminus, largely eliminates the normal retention effects of a basic residue.
- 3. Change in polarity with a change in pH:** At pH 2.8, only basic and phosphorylated groups will be charged. At pH 5.0, both acidic and basic residues will be charged. This factor can be used to manipulate selectivity.
- 4. Retention proportional to the number of basic residues:** In general, at pH 2.8 peptides will elute in order of increasing number of basic residues, as do cation-exchange separations. 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.

HILIC of Sugars and Oligosaccharides:

No salt is necessary unless the carbohydrate is charged. The mobile phase should contain 80-85% acetonitrile (with much lower levels used with amino- sugars). Anomeric forms of reducing sugars are resolved.

HILIC of Oligonucleotides

Try a salt gradient in 75% acetonitrile. C and G are retained much more than A and T, and may necessitate lower levels of acetonitrile.

HILIC of Phospholipids

Try a mobile phase of 15 mM ammonium formate pH 6.5 and 95% acetonitrile decreasing to 50%. Selectivity depends upon the pH and ionic strength.

HILIC of Drugs, Small Molecules and Miscellaneous Metabolites

Retention will be the opposite of that of reverse-phase HPLC. Initially, try mobile phases with 80% acetonitrile. Some experimentation with the salt level and pH will be necessary in each case.

Volatile Mobile Phases and Sequencing

The presence of 5-10 mM nonvolatile buffer salt does not interfere with many sequencing techniques for peptides. If a completely volatile mobile phase is needed, such as for mass spectroscopy, then ammonium formate can be used as the buffer salt, with a descending acetonitrile gradient. Unfortunately, formate absorbs and gives baseline artifacts in gradient elution at low wavelengths. No such problems are encountered at 254 or 280 NM.

NOTE: If the mobile phase contains over 80% organic solvent, then the sample should contain at least 70%. Otherwise, pure solutes may elute in multiple peaks.

References

1. Alpert, A.J., "Hydrophilic Interaction Chromatography (HILIC): A New Method for Separation of Peptides, Nucleic Acids, and Other Polar Solutes," J. Chromatogr. **499**, 177-196 (1990).
2. Zhu, B., Mant, C., Hodges, R., "Mixed-Mode Hydrophilic and Ionic Interaction Chromatography Rivals Reversed-Phase Chromatography for the Separation of Peptides," J. Chromatog., **594**, 75-86 (1992).
3. Boutin et al., HILIC of phosphorylated peptides and tyrosine kinase reaction mixtures, submitted to Anal. Biochem.
4. Fong et al., HILIC and SCX of Hydroxyproline-rich peptides from Douglas Fir cell wall proteins, submitted to Plant Physiol.
5. Kieliszewski et al., HILIC and SCX of Hydroxyproline-rich peptides from cell wall proteins of Zea maize (corn), submitted to Plant Physiol.
6. Przysiecki et al., HILIC, SEC and SCX of recombinant antistatin with a preproleader sequence, submitted to Arch. Biochem. Biophys.
7. Przysiecki et al., HILIC, SEC and SCX of recombinant antistatin with a preproleader sequence, submitted to Arch. Biochem. Biophys.
8. Przysiecki et al., Characterization of Recombinant Antistatin Secreted by *Saccharomyces cerevisiae*, "Protein Expression & Purification", **3**, 185-195 (1992).

Performing SEC Separations

Operating Recommendations for SEC Separations

Background on SEC Use

The PolyHYDROXYETHYL Aspartamide column was created specifically to perform HILIC and retain solutes solely through hydrophilic interactions. However, when used with a mobile phase which does not contain enough organic solvent to induce hydrophilic interaction, then it functions in the size exclusion chromatography mode. Swelling the coating with a suitable mobile phase causes the effective pore diameter to become the spacing between polymer chains of the coating (~15Å), allowing solutes as small as water to be separated by size.

The HEA column is available in three pore sizes, 200Å, 300Å and 1000Å. A column with 200Å pores has a fractionation range of 20-10,000 MW allowing resolution of the smallest of bioorganic molecules. The 300Å pore size accommodates a range of 20-80,000, and the 1000Å pore material can fractionate over a size range of 1,000 - 2,000,000, (over 5 orders of magnitude). The HEA columns may also be used with volatile mobile phases.

Start-up

Flush new columns (4.6 mm ID) with 25 ml water, then condition with at least 60 ml of a buffer solution with salt concentration of 0.2 M and a pH in the range of 3-6 (exact figures are not important here). Flush with another 20 ml water, then equilibrate for six hours (flow rate 0.5 ml/min.) using one of the mobile phases recommended below before injecting samples. (For 9.4 mm ID columns, the above volumes should be multiplied by a factor of 4, and the flow rate for equilibration is 2 ml/min.) It is not necessary to repeat this conditioning step thereafter unless the column is flushed with organic solvent for long-term storage or used under HILIC conditions.

HEA columns will exhibit two different fractionation ranges, depending on the mobile phase used. For a mobile phase of 0.2 M (Na)₂SO₄ + 5 mM K-PO₄, pH 3.0, containing 25% acetonitrile, the fractionation range will be approximately Mol. Wt. 400-10,000 for columns with 200Å pores (P1020 209). For HEA columns with 300Å pores (P1030 209, P1030 204), the fractionation range is approximately MW 1000-200,000.

For a mobile phase of 50 mM formic acid, the fractionation range will be approximately MW 20 - 1000 for columns with 200Å pores. For 300Å columns, MW 20 - 80,000.

The same column can be used for both fractionation ranges simply by switching between these two mobile phases. The formic acid mobile phase is volatile but precludes detection below 240 NM. The use of volatile mobile phases which are transparent at 215 NM (e.g. hexafluoro-isopropanol, HFIP) is experimental and hazardous, although it has the same effect on the fractionation range as formic acid.

Sample Composition

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 of the two. With high viscosity, solute molecules might not diffuse from the mobile phase to the stationary phase before the sample passes through the column. The loading capacity of a 4.6 mm I.D. 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.

Part Numbers and Prices

PolyHYDROXYETHYL Aspartamide

Description	Part #	Price
200Å, 2.1x200 mm 5µ	P202HY0502	\$545
200Å, 4.6x200 mm 5µ	P204HY0502	\$545
200Å, 9.4x200 mm 5µ	P209HY0502	\$1,080
300Å, 2.1x200 mm 5µ	P202HY0503	\$545
300Å, 4.6x200 mm 5µ	P204HY0503	\$545
300Å, 9.4x200 mm 5µ	P209HY0503	\$1,080
1000Å, 2.1x200 mm 5µ	P202HY0510	\$545
1000Å, 4.6x200 mm 5µ	P204HY0510	\$545
1000Å, 9.4x200 mm 5µ	P209HY0510	\$1,080
60Å, 2.1x200 mm 5µ	P202HY05006	\$545
60Å, 4.6x200 mm 5µ	P204HY05006	\$545
60Å, 9.4x200 mm 5µ	P209HY05006	\$1,080
60Å, 1.0x50 mm 5µ	P051HY05006	\$445
60Å, 2.1x35 mm 5µ	P03.52HY05006	\$370
60Å, 4.6x35 mm 5µ	P03.54HY05006	\$370

PolySULFOETHYL Aspartamide SCX

200Å, 2.1x50 mm 5µ	P052SE0502	\$445
200Å, 2.1x100 mm 5µ	P102SE0502	\$475
200Å, 4.6x50 mm 5µ	P054SE0502	\$395
200Å, 4.6x100 mm 5µ	P104SE0502	\$475
300Å, 2.1x50 mm 5µ	P052SE0503	\$445
300Å, 2.1x100 mm 5µ	P102SE0503	\$475
300Å, 2.1x200 mm 5µ	P202SE0503	\$545
300Å, 4.6x50 mm 5µ	P054SE0503	\$395
300Å, 4.6x100 mm 5µ	P104SE0503	\$475
300Å, 4.6x200 mm 5µ	P204SE0503	\$545
300Å, 9.4x200 mm 5µ	P209SE0503	\$1,080

Performing SCX Separations

Operating Recommendations for SCX Separations

The PolySULFOETHYL Aspartamide SCX column in the ion exchange mode is useful for n-terminal variant analysis, neuropeptides, growth factors, CNBr peptide fragments, and synthetic peptides as a complement to RPC.

Initial Use

Flush the methanol storage solvent from the column with at least 40 ml water before elution with salt solution to prevent salt precipitation. Then elute the column with a strong buffer for at least one hour prior to its initial use. A convenient solution to use is 0.2 M monosodium phosphate + 0.3 M sodium acetate.

Routine Use

Filter samples and mobile phases before use. Flush and store the column in water when not in use. Operation at room temperature is recommended since elevated temperature shortens column lifetime. Use of 0.1% TFA or high concentrations of formic acid in the mobile phase is not recommended. The conditioning process is reversed by exposing the column to pure organic solvents. Accordingly, to minimize the time to start the column for a 1-2 day storage, the column should be flushed with at least 40 ml of deionized water (not methanol), and the ends should be plugged. For extended storage, it is recommended that at least 25% methanol be used to prevent bacterial growth and contamination. Exercise care when using organic solvents to prevent precipitation of salts.

General Mode of Operation

Selectivity can be significantly enhanced by varying the pH, ionic strength, or organic solvent concentration in the mobile phase. Mobile phase modifiers help to improve peptide solubility or to mediate the interaction between peptide and stationary phase. For more strongly hydrophobic peptides, a non-ionic surfactant (at a concentration below its CMC) and/or acetonitrile or n-propanol as mobile phase modifiers, can substantially improve resolution and recovery over conventional reverse phase methods. You may obtain additional selectivity by simply changing the slope of the KCl or (NH₄)₂SO₄ gradient.

Using this column at pH 3 is better for retention of neutral to slightly acidic peptides. Use of a higher pH may be considered for basic hydrophobic peptides.

Adding MeCN or propanol to the A&B solvents changes the separation mechanism and results in a separation based

not only on positive charge, but also on hydrophobicity. Experimentation with organic content is encouraged.

One set of operating conditions for these applications for an analytical column would be:

Buffer A: 5 mM K-PO₄ + 25% MeCN;

Buffer B: 5 mM K-PO₄ + 25% MeCN

+ 300-500 mM KCl;

Linear gradient, 30 min. at 1 ml/min.

Peptides

Peptides are retained by the positive charge of at least the n-terminal amine and eluted by a combination of total charge, charge distribution, and hydrophobicity. If the peptide does not stick to the column, be sure it is in a small amount of buffer, or decrease the concentration of organic in the A&B solvents to 10% or 5%. (Organic solvent concentration is empirically determined).

Since total binding capacity is approximately 100 mg/gm of packing (for nonresolved materials), there will be a considerable Donan effect present. To prevent exclusion from the column, put your sample in 5-15 mM of salt or buffer. Additionally, the gradient at the outlet of the column will be much more concave than that observed on the chart paper. Consequently, if you have had no prior experience using this column, we recommend following a standard methods development protocol to be sure that your peptide is eluting properly. The Nest Group recommends an upper load limit of 1 mg of protein digest for a 4.6 mmID x 50 mm 200Å column, although up to 40 mg of a soluble synthetic, +3 peptide has been separated on a 4.6 mm x 200 mm column.

When using a guard column as a methods development column, we recommend a load limit of one-tenth of a milligram with gradient times shortened to 8-10 min. at the same flow rate since the void volume is only 0.3 ml.

General Cleaning

To remove ionic species, first flush column with 3 column volumes of water, then 1.0M salt for 5 column volumes. Be sure to remove all organic solvent before beginning this procedure. Then wash again with water and flush with 100% MeCN to remove non-polar species.

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