Hydrophilic-Interaction-Chromatography (HILIC) as a Go-To Alternative for Synthetic Peptide Analysis and Purification

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Abstract

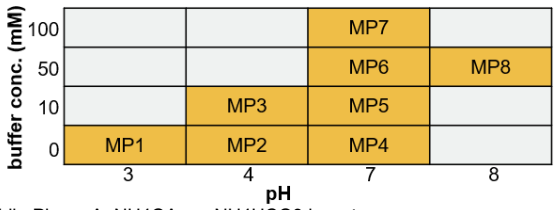
We conducted a multifactor study encompassing: (a) HILIC stationary phases with distinct surface chemistries, (b) TFA-free mobile phases varying in buffer strength and pH, (c) a representative panel of peptides spanning a wide range of length, charge, hydrophobicity, and sequence composition. Compared with C18 reversed-phase liquid chromatography (RP-LC), HILIC covers a comparable chemical space while providing different retention, selectivity, and resolution. HILIC also enhanced separation of structurally subtle and process-relevant impurities. Moreover, we established one-step HILIC purifications of peptide crudes, beneficial for process sustainability and sample in vivo readiness.

Methods

Initial Screening

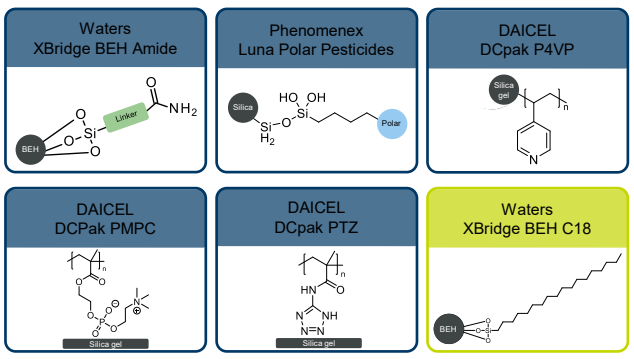
8 mobile phase pairs × (5 HILIC columns + 1 RP C18 column)

Mobile Phase Conditions



Mobile Phase A: NH4OAc or NH4HCO3 in water
Mobile Phase B: +/- 0.5% AcOH in acetonitrile/water 95/5

Column Chemistry



2.1 × 100 mm, 2.5 µm (C18, Amide) or 3.0 µm (Luna, P4VP, PMPC, PTZ)

Table 1 The peptide test-set for screening

Peptide Name	Molecular Weight (g/mol)	pI	Structure Class
Leucine Enkephaline	555.6	6.0	linear
Angiotensin II	1046.2	7.7	linear
Carbetocin	988.2	N.A.	cyclic, thioether
Desmopressin	1069.2	8.7	cyclic, disulfide
Leuprolide	1209.4	9.7	linear
Triptorelin	1311.4	7.7	linear
Somatostatin	1637.9	8.9	cyclic, disulfide
Liraglutide analogue*	3512.8	4.6	linear
ACTH (Porcine)	4541.1	9.4	linear

(*) Liraglutide analogue refers to the parental peptide minus the lipid moiety
The peptides were mixed and dissolved in ACN/water (1/1, v/v) to a final concentration of 0.5 mg/mL each.

Real sample testing

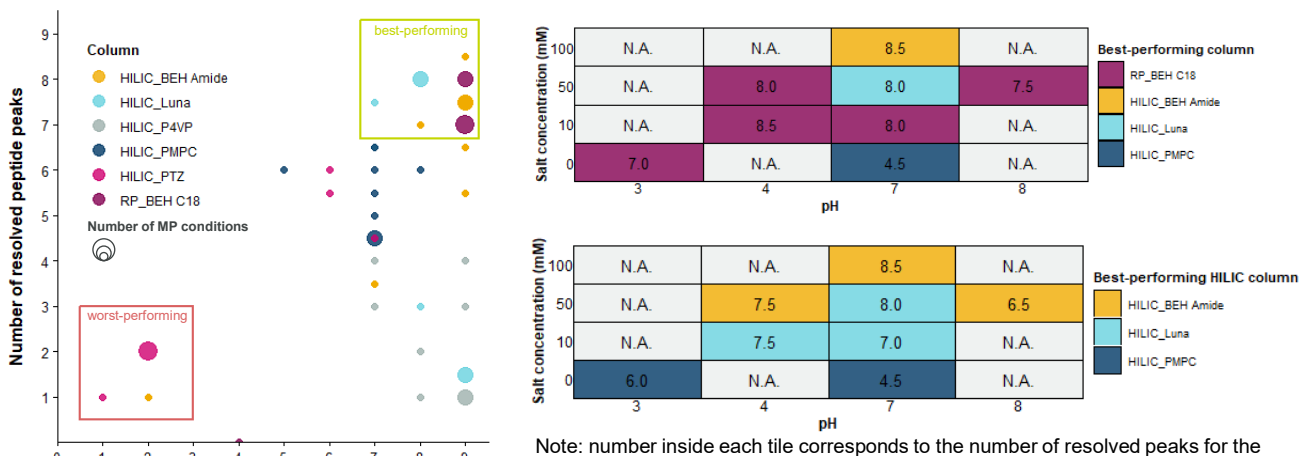
The peptide crudes were analyzed on Waters XBridge BEH C18 and XBridge BEH Amide for comparison.

Table 2 The peptide crudes with challenging impurities

Peptide	Molecular Weight (g/mol)	Structure Class
P1	4310.8	linear
P2	1993.1	maleimide-bridged monocyclic
P3	1526.8	bicyclic
P4	2645.0	peptide-small molecule conjugate

Results

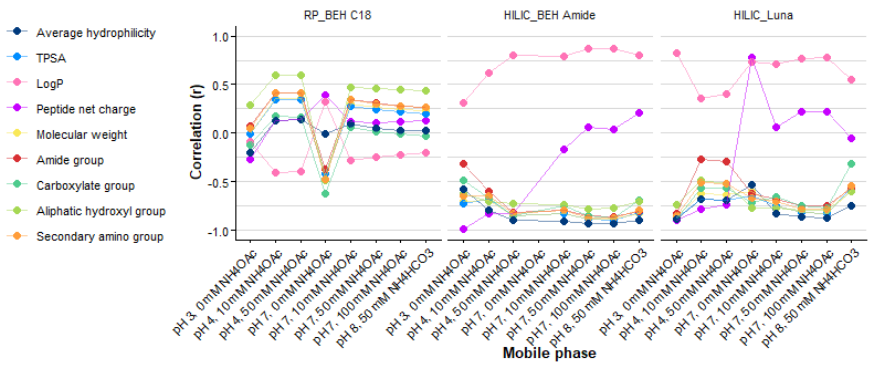
HILIC Column Screening for Generic Peptide Separation Method



Note: number inside each tile corresponds to the number of resolved peaks for the best-performing column at a specific mobile phase composition.

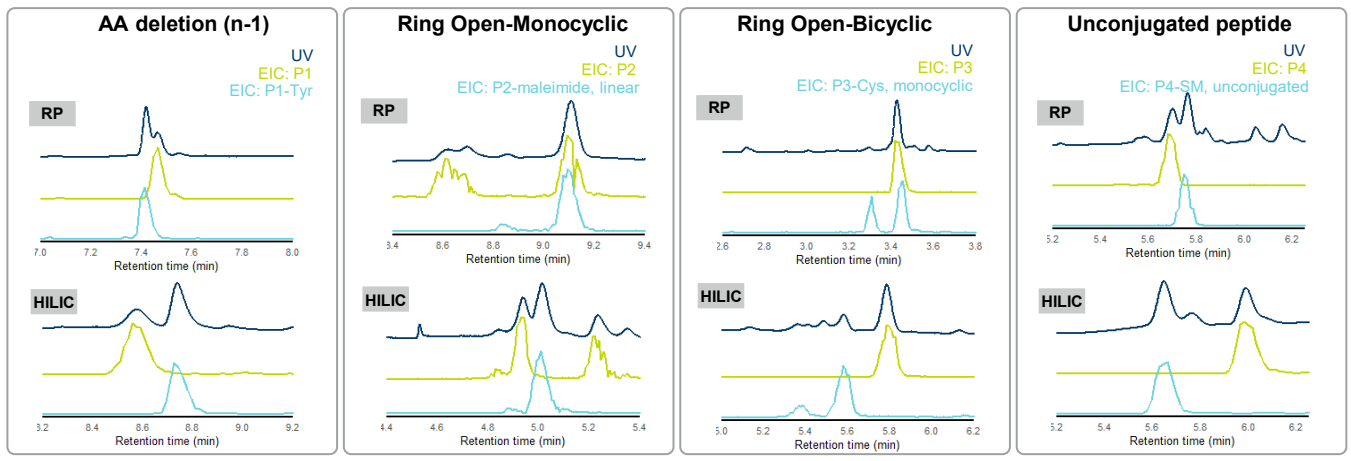
- **Selection criteria for initial screening:** Peptide coverage and chromatographic resolving power
- **Benchmarked by RP**: HILIC can provide comparable performance per selection criteria
- **Best-performing HILIC columns:** XBridge BEH Amide and Luna Polar Pesticides
- **Salt concentration impact:**
  - **DCPak PMPC:** Best under low- or no-salt conditions
  - **Luna Polar Pesticides:** Optimal at low-to-medium salt concentrations
  - **XBridge BEH Amide:** Optimal at medium-to-high salt concentrations
- **pH compatibility:** XBridge BEH Amide provided the broadest pH operating range

Correlation of peptide properties with retention on HILIC columns



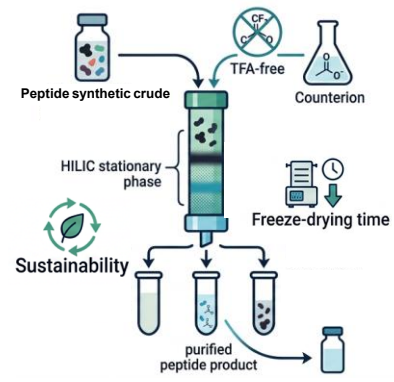
- **Charged HILIC columns**: No consistent relationship between peptide properties and retention (data not shown)
- **Neutral HILIC columns** (BEH Amide, Luna):
  - **LogP**: Strong positive correlation with retention
  - **Average hydrophilicity**: Inverse correlation with retention

New chance for resolving challenging synthetic peptide impurities



Mobile phase A: 50 mM NH4OAc + 0.5% AcOH in water; mobile phase B: 0.5% AcOH in ACN/water 95/5.
RP gradient: 10-50 % B in 10 min; HILIC gradient: 100-60% B in 10 min

One-step TFA-free purification on HILIC



- **One-step purification**: HILIC enables single-step peptide processing without compromising purity
- **Workflow flexibility**: Applicable as a standalone method or complementary dimension in RP-based 2D-LC workflows
- **Hydrophobic peptide compatibility**: High-organic mobile phase supports dissolution and purification of highly hydrophobic peptides
- **In vivo readiness**: Acetate counterion in purified peptide products
- **Sustainability**: TFA-free mobile phase
- **Process efficiency**: Reduced freeze-drying time for HILIC fractions

Conclusions

We have evaluated HILIC as a versatile, TFA-free platform for efficient peptide analysis and purification. HILIC can be particularly advantageous for hydrophobic therapeutic peptides that require high organic content for dissolution, as well as for method development focused on challenging impurity profiles.

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