Why Aqueous Normal Phase and HILIC are Different

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The Essential Feature of the Aqueous Normal Phase Mode is the Presence of the Hydride Surface
WHAT IS HILIC RETENTION?

HILIC Retention

% Organic in Mobile Phase

Retention Time (min)

- - Hydrophilic Compound
- - Hydrophobic Compound

% Organic in Mobile Phase
WHAT IS AQUEOUS NORMAL PHASE RETENTION?

A continuum of retention that provides a transition from the reversed-phase to the normal phase modes with water as a constituent in the mobile phase

Three distinct retention patterns are possible:

1. No overlap of reversed phase and normal phase retention for two or more compounds.

2. Overlap of reversed phase and normal phase retention for two or more compounds.

3. Individual compounds that can be retained by both reversed phase and normal phase modes.
No overlap of reversed phase and normal phase retention for two or more compounds.

ANP 1

Retention Time (min)

% Organic in Mobile Phase

ANP Compound
Reversed-Phase Compound
Overlap of reversed phase and normal phase retention for two or more compounds.

Aqueous normal phase (1) and reversed-phase (2) compounds at three mobile phase compositions: A, 50:50 acetonitrile, DI water; B, 80:20 acetonitrile, DI water; and C, 85:15 acetonitrile, DI water.
Individual compounds that can be retained by both reversed phase and normal phase modes

ANP 3
DIFFERENCES BETWEEN AQUEOUS NORMAL PHASE AND HILIC

Aqueous Normal Phase
Silica Hydride-Based Column

• Retains nonpolar compounds by reversed phase mechanism
• Retains polar compounds by normal phase mechanism
• Both reversed phase and normal phase mechanisms can operate simultaneously
• Can separate samples with both polar and nonpolar compounds

Hydrophilic Interaction Chromatography (HILIC) uses ordinary Silica-Based Column or zwitterionic polymers

• Retains polar compounds by a normal phase mechanism
• Does not retain nonpolar compounds
• Cannot usually separate samples having both polar and nonpolar compounds
USE OF VARIOUS MOBILE PHASE SOLVENTS IN ANP

Acetonitrile is the most common organic mobile phase component for ANP, but others are possible.

Methanol is usually only possible with strongly basic compounds.

Acetone is possible when using MS detection. ANP behavior similar to acetonitrile.
EFFECT OF TEMPERATURE IN ANP
OPPOSITE TO REVERSED-PHASE FOR MANY SOLUTES
EFFECT OF STATIONARY PHASE ON ANP RETENTION

Hydride Based Cholesterol: Choline/Acetylcholine

- Acetylcholine
- Choline

Retention time (min.)

% Acetonitrile in DI water + 0.5% FA

0 5 10 15 20 25 30

Hydride Based BD C18: Choline/Acetylcholine

- Choline
- Acetylcholine

Retention time (min.)

% Acetonitrile in DI water + 0.5% FA

0 5 10 15 20 25
The table shows retention time reproducibility for nine amino acids at two temperatures. Four replicates were performed at each temperature. The reproducibility was 0.28% or better for the amino acids. This is a significant improvement over what is usually observed for most HILIC analyses, especially considering this is gradient data with only a 5 minute re-equilibration time between runs.
ANP RESULTS IN IMPROVED SENSITIVITY WHEN USING MS FOR DETECTION

SIGNAL INTENSITY AS A FUNCTION OF MOBILE PHASE COMPOSITION

GLUCOSE SIGNAL INCREASES AS % ACETONITRILE INCREASES UP TO 95% IN THE MOBILE PHASE
COLUMN EFFICIENCY OF ANP STATIONARY PHASES

Plots of efficiency (HETP) vs. flow rate

(A) glucose and sorbitol on DH column (2.1 x 150 mm, particle size 4 µm) in a 80:20 ACN/DI water + 0.1% formic acid mobile phase.

(B) Comparison of commercial HILIC (4.6 x 150 mm, particle size 3.5 µm) and DH (4.6 x 150 mm, particle size 4.0 µm) columns for ANP retention of uracil.
EXAMPLES OF HYDROPHILIC COMPOUNDS RETAINED BY ANP

Glucosamine on Bidentate C18

Retention Time (min.)

% Acetonitrile in DI Water + 0.5% FA

- Glucosamine
Extracted Ion Chromatogram Of Nineteen Amino Acid Separation

All of the critical amino acid pairs (those that are isobaric or have masses within one mass unit) are separated under these conditions except for the Leucine / Isoleucine pair.
EXTRACTED ION CHROMATOGRAMS OF ORGANIC ACIDS

maleic acid (1)
aconitic acid, trans (2)
aconitic acid, cis (3)
aconitic acid, impurity (4)
fumaric acid (5)
citric acid (6)
oxaloacetic acid (7)
NUCLEOTIDES ON UNDECENOIC ACID COLUMN

1 = adenosine-3’,5’-cyclic monophosphate;
2 = adenosine-5’-monophosphate;
3 = adenosine-5’-triphosphate;
4 = thymidine-5’-triphosphate;
5 = uridine-5’-triphosphate;
6 = cytosine-5’-triphosphate;
7 = guanosine-5’-triphosphate.

85:15 90% acetonitrile/10% DI water + 0.1% ammonium formate/ DI water + 0.1% ammonium formate.
Highly polar metabolites: Uridine 5′-diphosphate (UDP) and phosphorylated sugars analyzed using Diamond Hydride column.

1 - the monitored MRM transitions were m/z 535 to m/z 323
2 - the monitored MRM transitions were m/z 564 to m/z 322
3 - UDP hexanolamine (internal standard) - the monitored MRM transitions were m/z 502 to m/z 258
ANP RETENTION CAN BE OBSERVED FOR HYDROPHILIC PEPTIDES

GENERAL PEPTIDE STRUCTURE: Ac-AXEXAHKAY-NH₂
### SEPARATION OF A MIXTURE OF POLAR AND NONPOLAR COMPOUNDS

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<td>1</td>
<td>Cytidine-R1</td>
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<td>12.12</td>
<td>3.73</td>
<td>1.54</td>
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<td>454</td>
<td>12.07</td>
<td>3.73</td>
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<td>12.09</td>
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<td>536</td>
<td>8.65, 8.68</td>
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<td>3.46</td>
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<td>7.91, 10.98</td>
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<td>Benzopyran</td>
<td>396</td>
<td>-</td>
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Mobile Phase: 60:40 acetonitrile/water

![Chromatogram](image.png)
FOR MORE INFORMATION ON ALL TYPE C SILICA HYDRIDE PHASES

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