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HYDROPHILIC INTERACTION CHROMATOGRAPHY (HILIC) FOR SMALL MOLECULES: RETAINING VERY POLAR ANALYTES

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OVERVIEW-

Hydrophilic interaction chromatography (HILIC) has been described as an alternative to traditional reversed-phase chromatography for the retention of very polar analytes. It is a *variation* of normal-phase chromatography without the disadvantages of using solvents that are not miscible in water.
In this mode, a high organic-low aqueous mobile phase is used with a polar stationary phase to achieve retention of very polar analytes. This highly organic (> 80%) mobile phase is ideal for enhancing sensitivity in mass spectrometry.

INTRODUCTION-

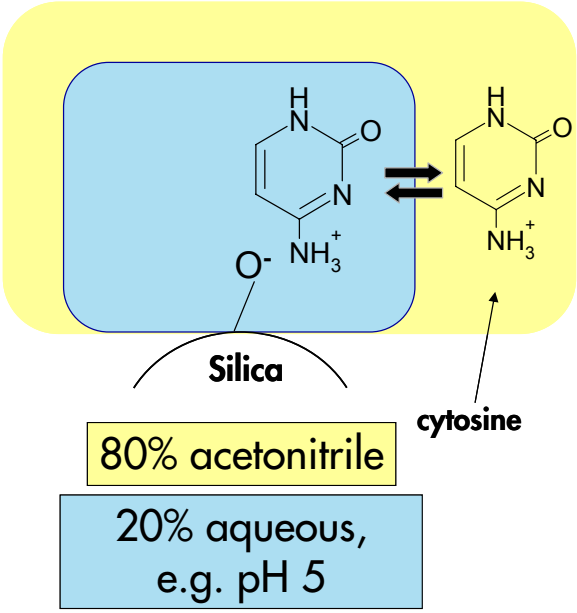
We have developed and optimized a stationary phase for HILIC that provides good retention of very polar basic analytes, including several active pharmaceutical ingredients and their metabolites and/or impurities. We have examined the effects of sample diluents on peak shape and sensitivity and will make recommendations based on this data. We have also compared the differences in LC/MS sensitivity between HILIC conditions (i.e. > 80% organic) on the HILIC column and 100% aqueous conditions on a reversed-phase column designed to retain polar analytes. Because of the high organic mobile phase starting conditions, plasma samples cleaned-up by protein precipitation or mixed-mode SPE can be directly analyzed by LC/MS without solvent evaporation and reconstitution.

Atlantis™ Columns

ATLANTIS™ HILIC SILICA –

- First HILIC column in a family of columns optimized for the retention of highly polar analytes.
- Silica column that has been optimized for HILIC.
- Atlantis™ HILIC Silica is ideal for the retention of very **polar bases**

RETENTION MECHANISM ON SILICA-



- Polar analyte **partitions** into and out of adsorbed water layer
- Charged polar analyte can undergo **cation exchange** with charged silanol groups
- **Combination** of these mechanisms results in **enhanced** polar retention

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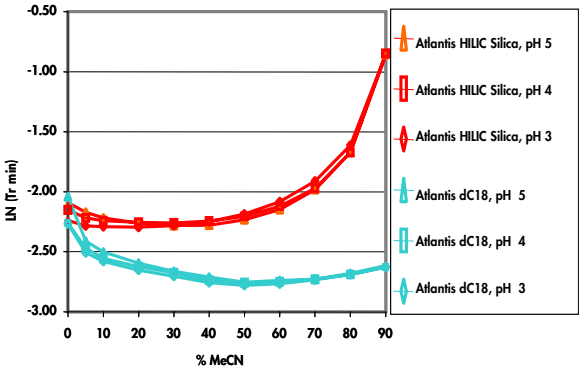
HILIC VS. REVERSED-PHASE RETENTION CHARACTERISTICS-

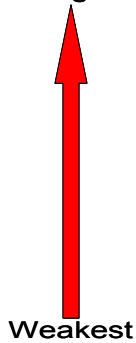


- Retention characteristics of cytosine were monitored under HILIC and reversed-phase conditions at pH 3, 4, and 5 with 10% incremental values of acetonitrile from 0 to 90%.
- The optimum operating range to achieve retention is above 70% organic for HILIC and below 20% organic for reversed-phase chromatography
- HILIC offers significantly more retention than reversed-phase chromatography for polar basic analytes in their respective optimum operating ranges for retention.

SOLVENT STRENGTH: HILIC-

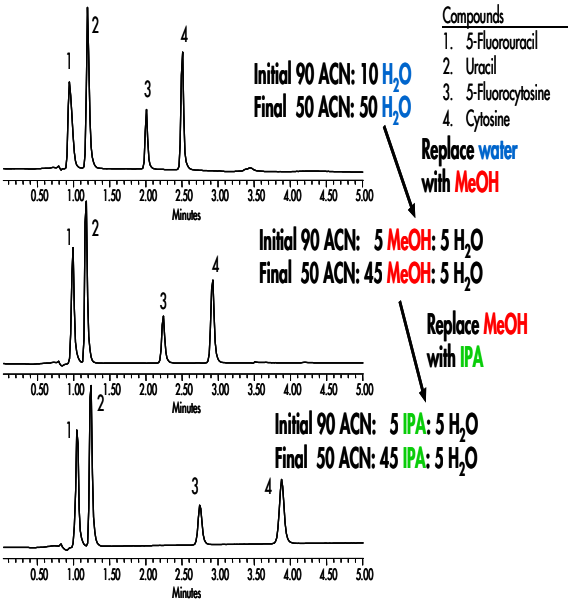
Solvent
Water
Methanol
Ethanol
Isopropanol
Acetonitrile
Acetone
Tetrahydrofuran

Strongest



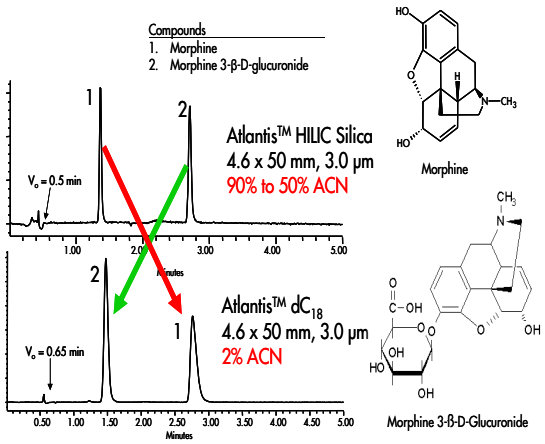
Weakest

In HILIC, utilizing a ***less*** polar solvent can ***increase*** the retention of polar analytes



Retention of later eluting peaks increases by substituting a portion of the mobile phase with a less polar solvent

COMPLEMENTARY SELECTIVITY TO REVERSED-PHASE CHROMATOGRAPHY-



Both techniques offer good retention and peak shape of the analytes. However, the ***polar*** metabolite elutes ***after*** the compound of interest on the Atlantis™ HILIC Silica column.

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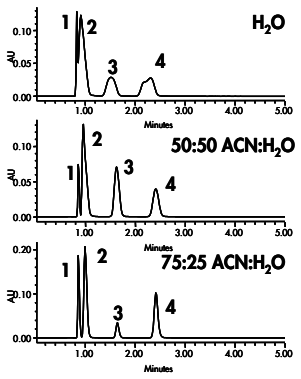
IMPORTANCE OF SAMPLE DILUENT-

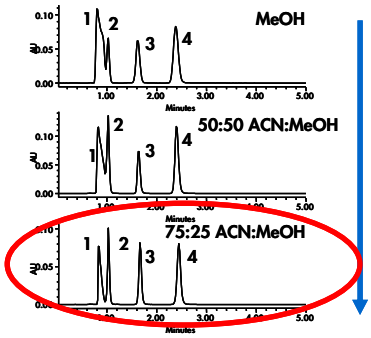


Atlantis™ HILIC Silica
4.6 x 50 mm, 3 µm
10.0 µL Injection volume

Compounds	Sample Conc.
1. 5-Fluorouracil	25 µg/mL
2. Uracil	25 µg/mL
3. 5-Fluorocytosine	25 µg/mL
4. Cytosine	25 µg/mL

Peak shape improves as % organic in the diluent increases.

Peak shape can be further improved by removing the aqueous portion of the diluent.



Atlantis™ HILIC Silica
4.6 x 50 mm, 3 µm
10.0 µL Injection volume

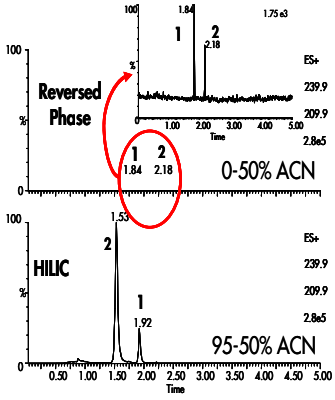
Compounds	Sample Conc.
1. 5-Fluorouracil	25 µg/mL
2. Uracil	25 µg/mL
3. 5-Fluorocytosine	25 µg/mL
4. Cytosine	25 µg/mL

Peak shape improves as % acetonitrile in the diluent increases.

Peak shape is improved by increasing the non-polar portion of the diluent.

- In HILIC, it is important for the sample diluent to be 100% organic – remember, the organic mobile phase is the weak solvent.
- The sample diluent can significantly influence peak shape and peak area of your analytes of interest
- However, polar analytes often have low solubilities in organic solvents
- We ran an extensive series of experiments to determine a generic diluent
- **75:25 acetonitrile:methanol** is a useful generic diluent for most polar analytes
- This diluent is a compromise between solubility and peak shape

ENHANCED MS SENSITIVITY-



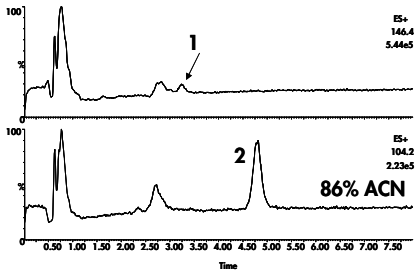
Atlantis™ dC18	Peak Area
2.1 x 50 mm, 3 µm	
1. Albuterol 100 pg/µL	21
2. Bamethan 50 pg/µL	14

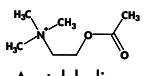
Atlantis™ HILIC Silica	Peak Area
2.1 x 50 mm, 3 µm	
2. Bamethan 50 pg/µL	110085
1. Albuterol 100 pg/µL	19567

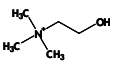
The retention of polar analytes by reversed-phase chromatography often requires non-volatile highly aqueous mobile phases which is not ideal for compound ionization by ESI-MS. Since HILICrequires a highly volatile high organic mobile phase, a dramatic increase in sensitivity can be observed.
The example above shows an increase in sensitivity by 3 to 4 orders of magnitude on the Atlantis™ HILIC Silica for these analytes. A lower limit of detection can be achievable because of the sensitivity that these highly volatile mobile phases promote.

LOW LIMITS OF DETECTION-

Atlantis™ HILIC Silica	Concentration
2.1 x 50 mm, 3 µm	
1. Acetylcholine	**100 fg/µL**
2. Choline	**100 fg/µL**




Acetylcholine


Choline

Low limits of detection are possible by HILIC due to the higher sensitivity achieved from utilizing highly volatile mobile phases. A limit of detection of **100 fg/µL** of Acetylcholine and Choline was achieved using an Atlantis™ HILIC Silica column on a Waters® ZQ™ single quadrupole mass spectrometer

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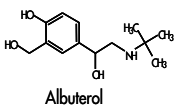
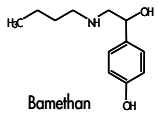
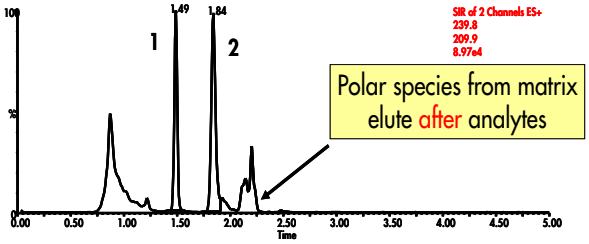
DIRECT INJECTION OF SPE ELUENT ONTO HILIC COLUMN-





Atlantis™ HILIC Silica
2.1 x 50 mm, 3 µm
1. Bamethan 10 pg/µL
2. Albuterol 50 pg/µL



- Evaporation and reconstitution are often the most lengthy steps of solid phase extraction. The elimination of these steps would prove useful by reducing sample preparation time and increasing throughput and productivity.
- The high organic SPE eluent is compatible with the high organic mobile phase conditions that HILIC requires, thus eliminating the need to evaporate and reconstitute the SPE eluent.
- If the SPE method is not optimized and there is residual matrix post SPE, the polar species from the matrix elute after the analytes of interest thus reducing potential suppression from matrix interferences.

CONCLUSIONS –

Atlantis™ HILIC Silica columns offer:

- Complementary selectivity to reversed-phase chromatography
- Retention of highly polar basic analytes not retained by reversed-phase chromatography
- Enhanced sensitivity in ESI-MS due to highly volatile mobile phases (> 80% organic)
- Lower limits of detection
- Shorter sample preparation procedures due to the elimination of the evaporation and reconstitution steps and directly injecting the eluent

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