

Interplay of Chromatographic Parameters and Analyte Physical Properties on Retention and Selectivity in Hydrophilic Interaction Liquid Chromatography

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Introduction

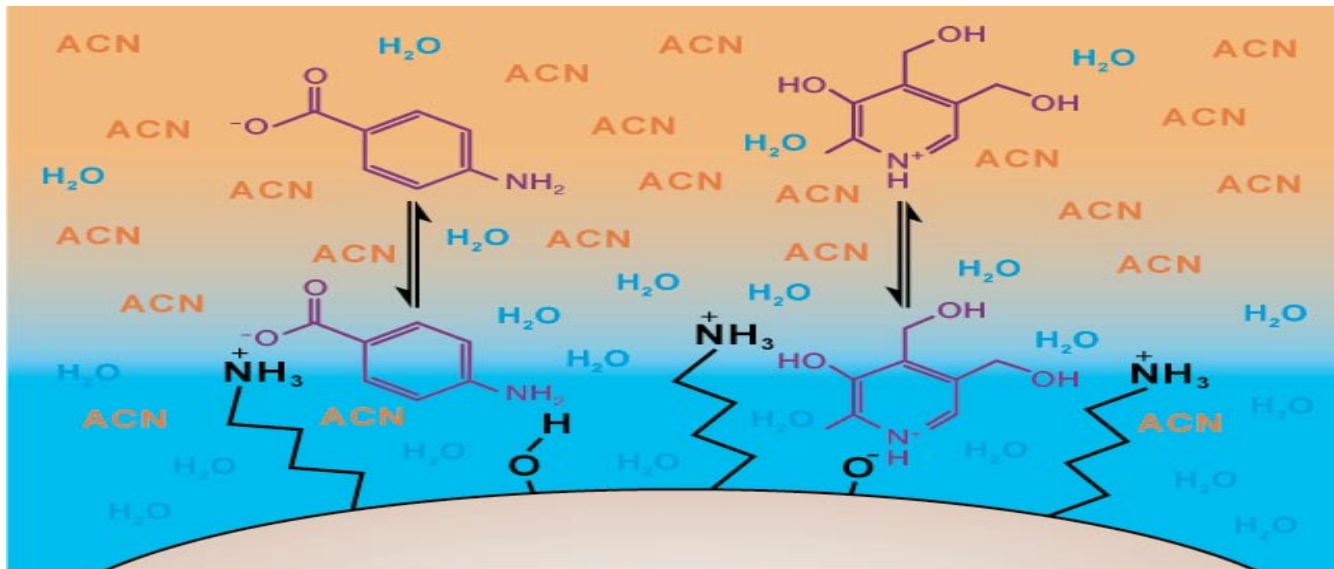
The analysis of polar compounds using mass spectrometric compatible conditions is becoming increasingly important as drug compounds continue trending toward more polar molecules. Hydrophilic interaction liquid chromatography (HILIC) is a unique separation mode that addresses this growing need; however, further research characterizing this separation mode is needed to reduce it to practice. The chromatographic conditions employed in HILIC mode separations have a dramatic effect on both analyte and chromatographic sorbent physical properties. To date, little research has been devoted to

rationalizing the effect of operating parameters on analyte-sorbent interactions in HILIC mode. In this presentation, changes in analyte and sorbent physical properties in the high organic environment of HILIC are used to explain the observed effects on retention and selectivity with several model compounds and chromatographic sorbents.

Hydrophilic Interaction Chromatography (HILIC)

- **HILIC retention mechanism**

- Primarily partitioning of analytes between water enriched layer of solvent near sorbent surface and the relatively more hydrophobic bulk eluent
- Partitioning based on relative solubility in each layer
- H-bonding and Ion-exchange with the sorbent also important



Primary HILIC Chromatographic Parameters

- **Column Chemistry**

- Silica, amino, cyano, PFP, diol, zwitterion, ion-exchange, “specialty”

- **Type and % Organic**

- Acetonitrile primary weak eluent

- Isocratic: 95-70 % Acetonitrile typical

- Gradient: 90-50 % Acetonitrile

- Modifiers – IPA, methanol, ethyl acetate, others

- **Buffer pH**

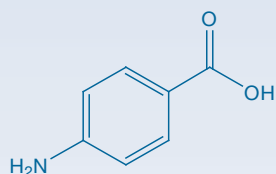
- pH 2 – 7

- **Ionic Strength (IS)**

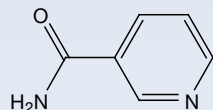
- 5-10 mM buffer concentration

Probe Compound Physical Properties

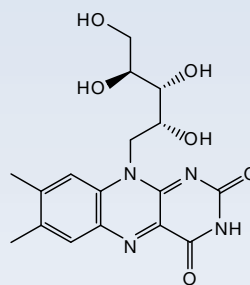
● p-Aminobenzoic Acid (1)
 pK_a 4.7, H^+ pK_a 2.7
 $\log P$ 0.83



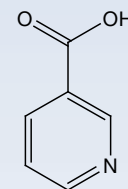
○ Nicotinamide (2)
 H^+ pK_a 3.35
 $\log P$ - 0.37



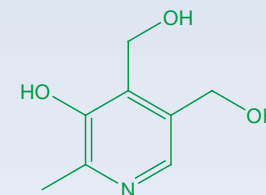
○ Riboflavin (3)
 pK_a 10.2
 $\log P$ - 1.46



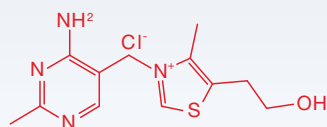
○ Nicotinic Acid (4)
 pK_a 4.7, H^+ pK_a 3.0
 $\log P$ 0.36



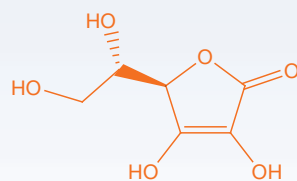
● Pyridoxine (5)
 H^+ pK_a 5.6, pK_a 8.6
 $\log P$ - 0.77



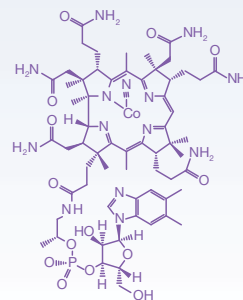
● Thiamine (6)
 H^+ pK_a 5.5
 $\log P$ - 4.6



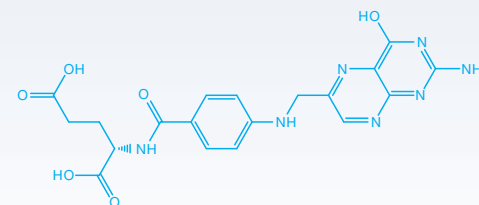
● Ascorbic Acid (7)
 pK_a 4.1, 11.2
 $\log P$ - 1.85



● Vitamin B12 (8)
 pK_a 1.59
 $\log P$ - 0.90

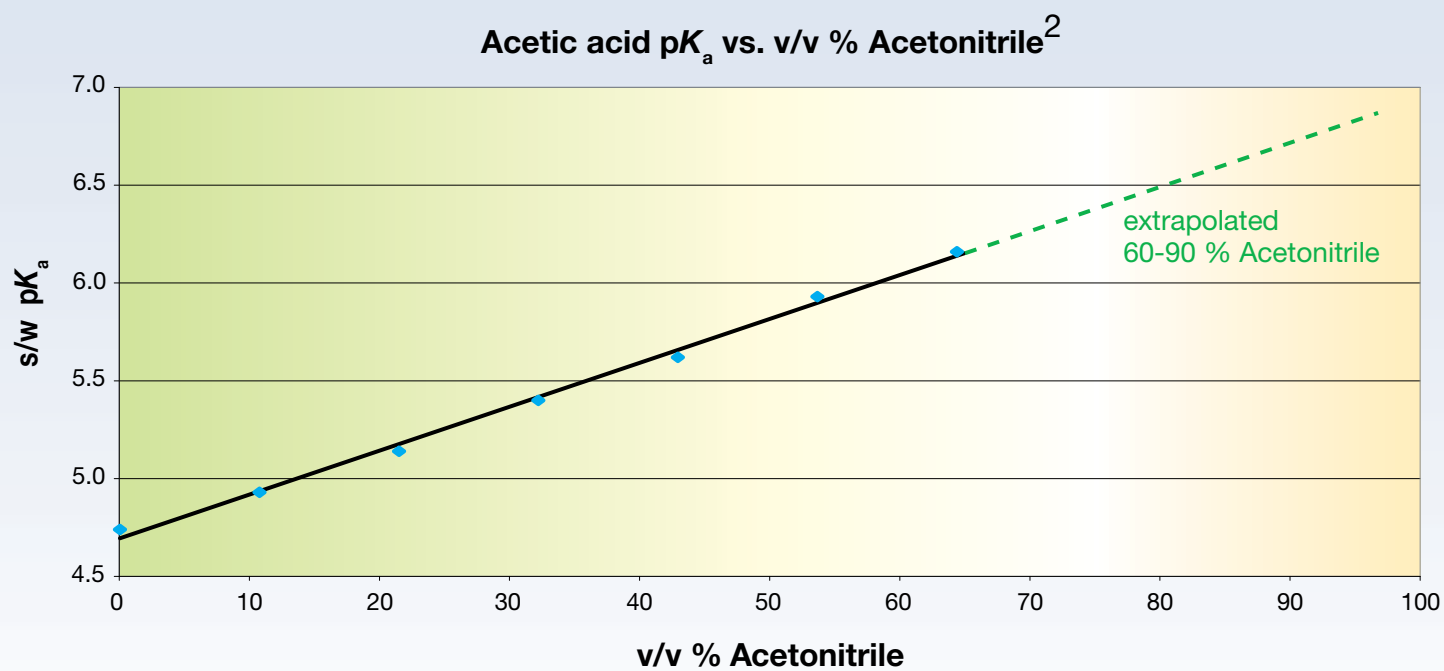


● Folic Acid (9)
 pK_a 2.7, 4.1, 8.9
 $\log P$ - 0.02



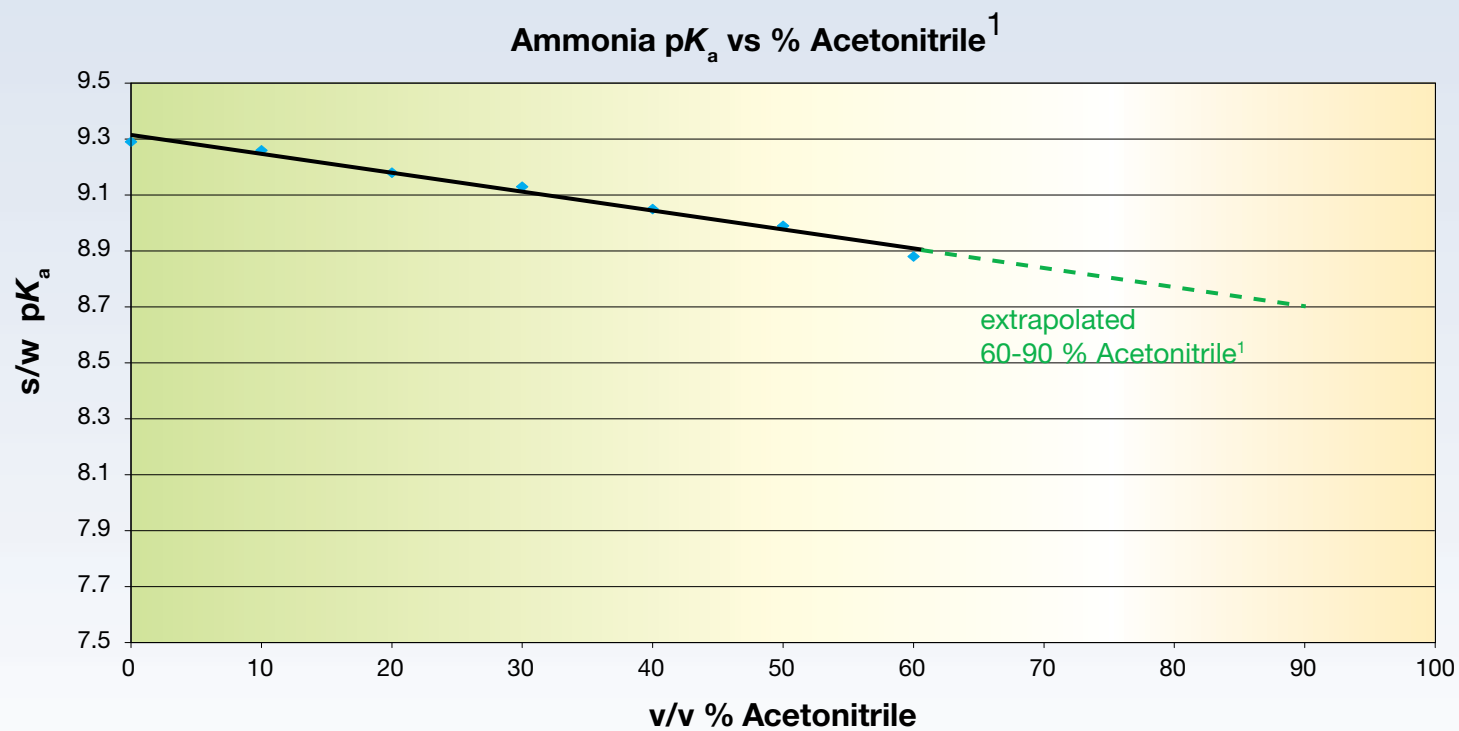
Weak Acid pK_a vs. % Organic

- pK_a of weak acids *increase* with increasing organic solvent concentration
- Sorbent silanols expected to follow similar trend



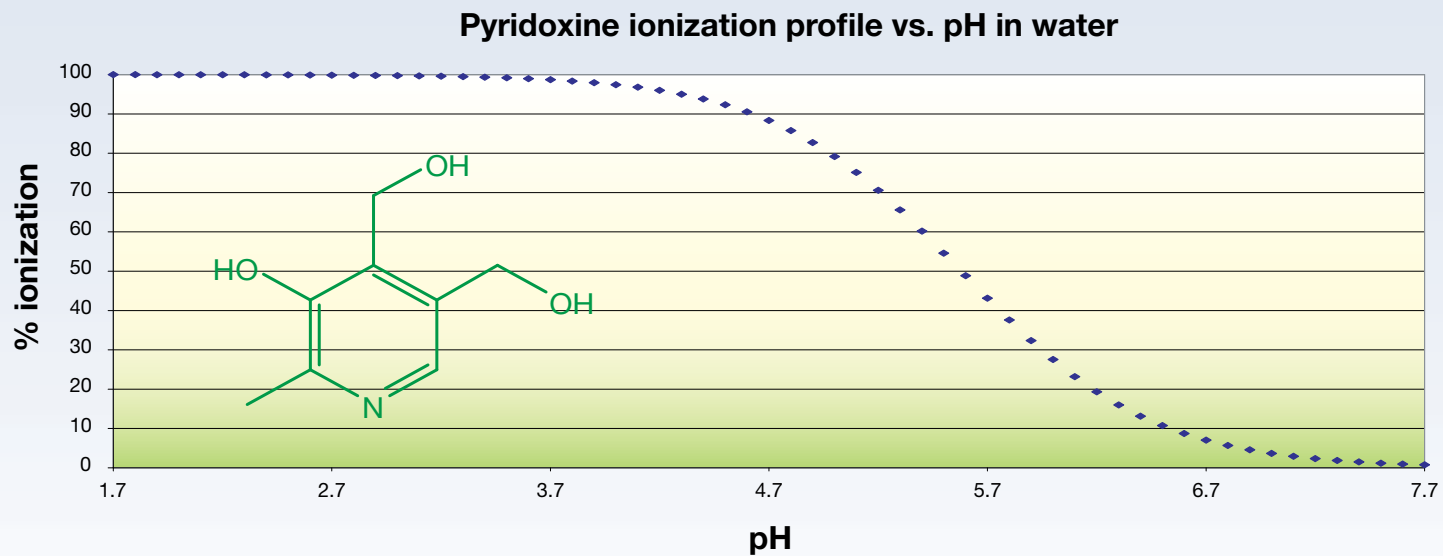
Weak Base pK_a vs. % Organic

- pK_a 's of weak bases decrease with increasing organic solvent concentration
 - Smaller changes than acids



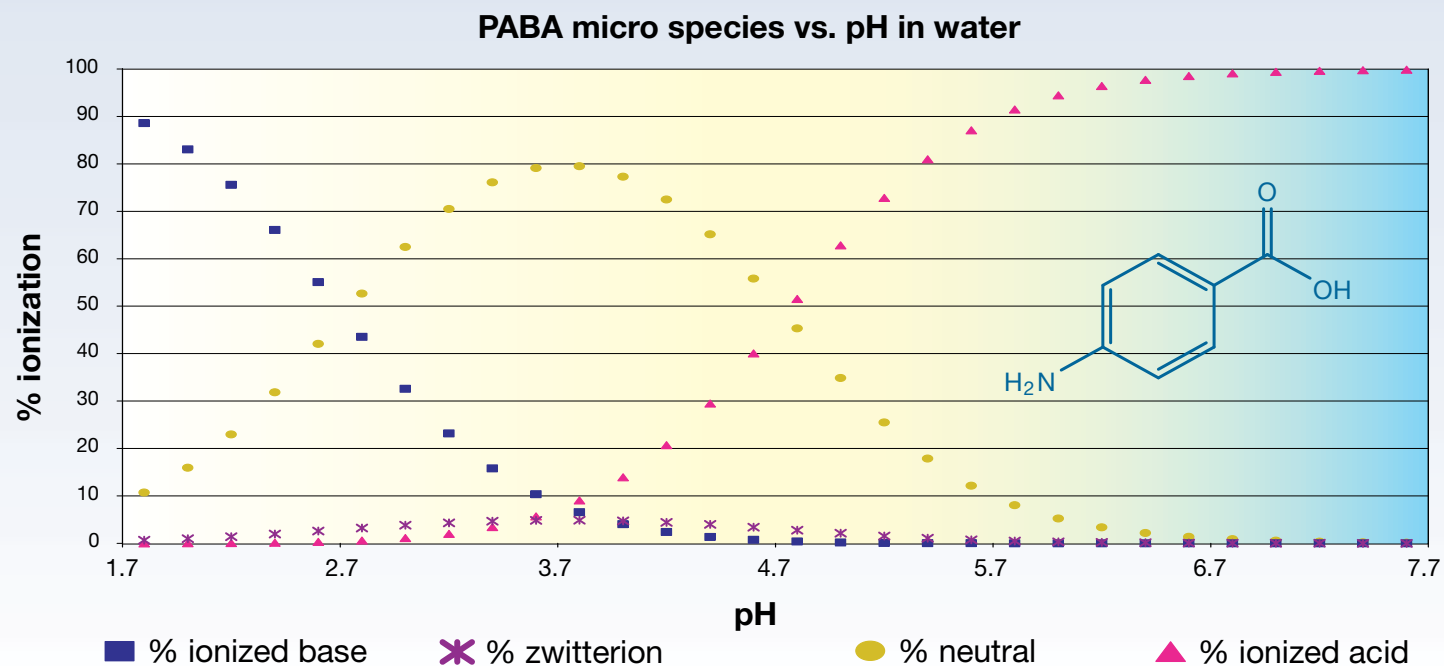
Basic Analyte % Ionization

- As in RPLC, ionization of analytes greatly effects retention in HILIC
- Retention in HILIC generally follows ionization profile
 - In HILIC retention increases with increased ionization



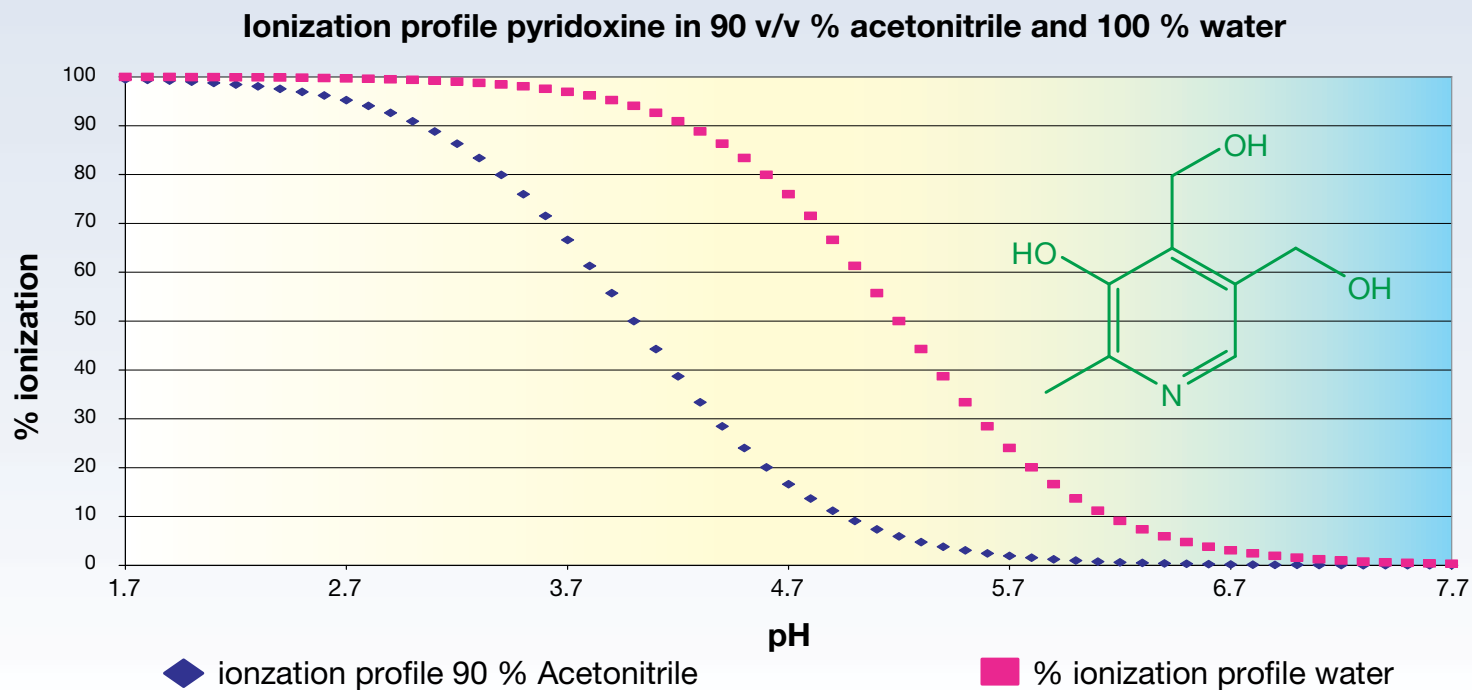
Zwitterionic Analyte % Ionization

- For zwitterionic analytes, all ionizable groups must be considered to understand retention changes with pH
- p-amino benzoic acid (PABA) micro species vs. pH in water
 - 4 species: neutral, ionized acid, ionized base, zwitterion



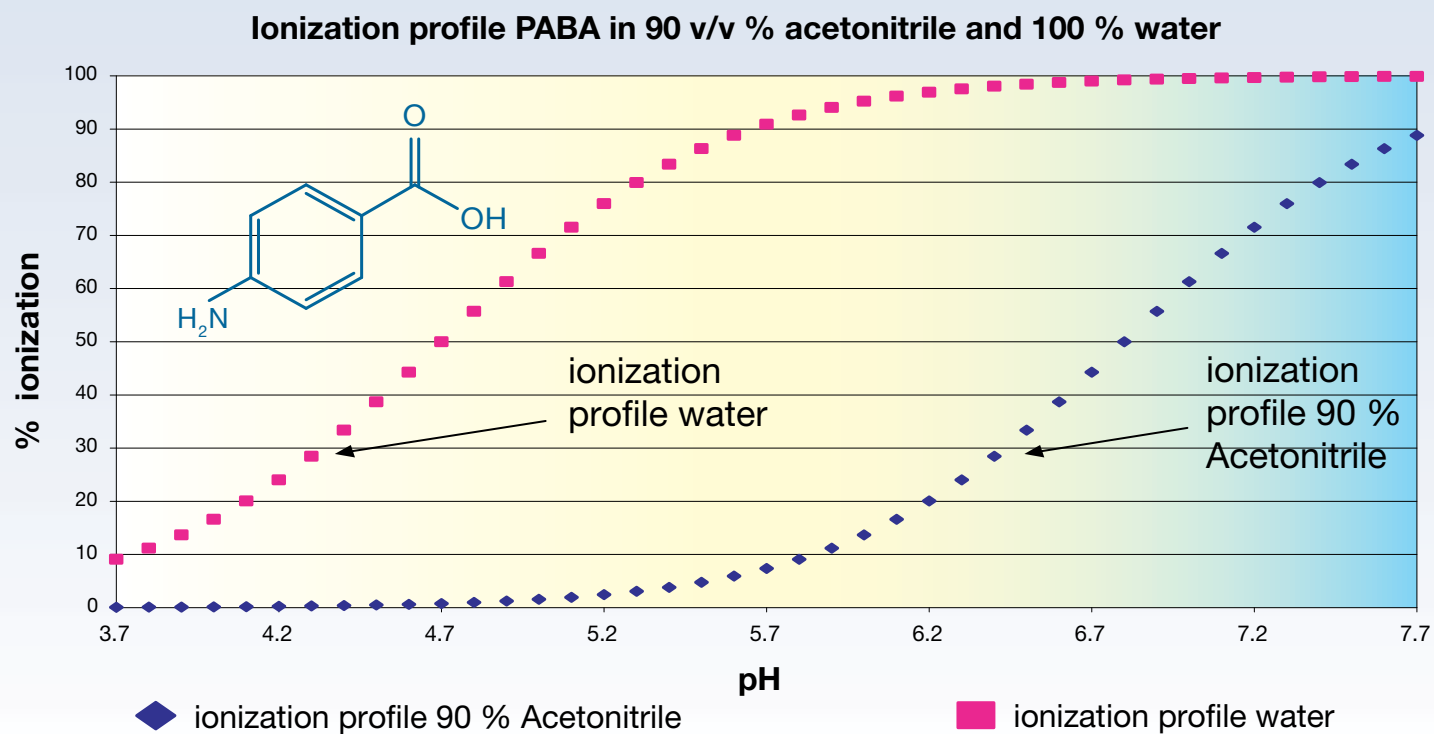
High Organic Concentration Effect on Analyte pK_a and % Ionization

- High organic concentration shifts pK_a 's and changes ionization profile
- Decreased ionization due to organic solvent gives decreased retention



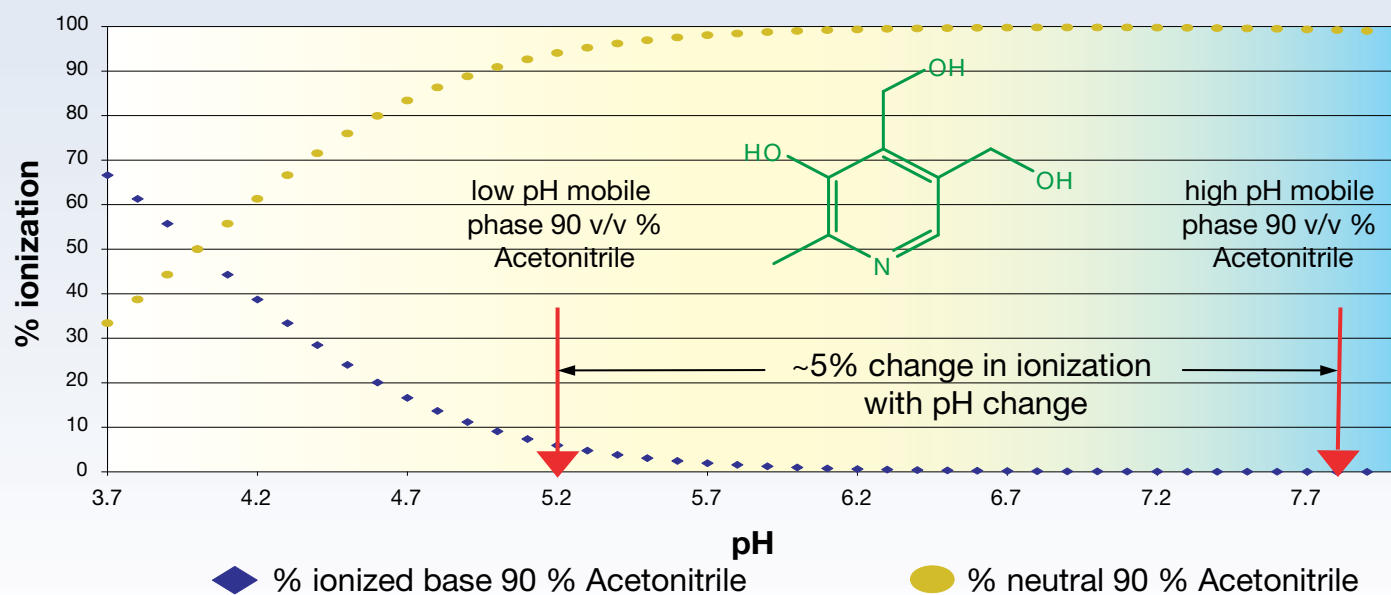
Shifting pK_a and % Ionization PABA

- With high organic solvent concentration the difference between acidic and basic pK_a 's increase which reduces the number of species present in solution



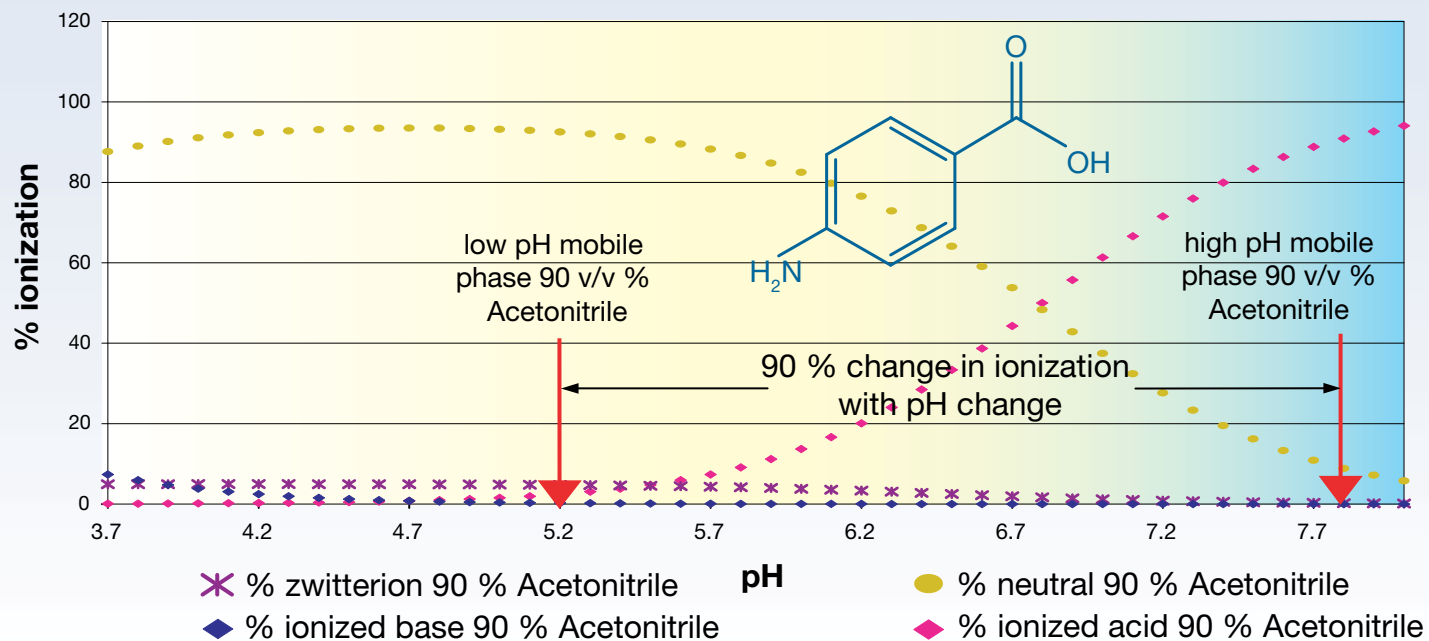
Analyte Retention vs. % Ionization in 90% Acetonitrile

- In HILIC, retention increases with increasing polarity
- Increasing ionization increases polarity and thus increases retention
- For pyridoxine, ionization changes only slightly at the two mobile phase pHs used
 - Retention is not expected to be significantly affected



Zwitterionic Analyte Retention vs. % Ionization in 90 % Acetonitrile

- Due to pK_a shifts, only the acidic group of PABA ionizes
 - Basic pK_a decreased while acidic pK_a increased
- For PABA, ionization changes significantly at the two mobile phase pHs used
 - Retention is expected to change significantly

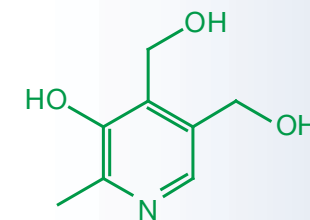
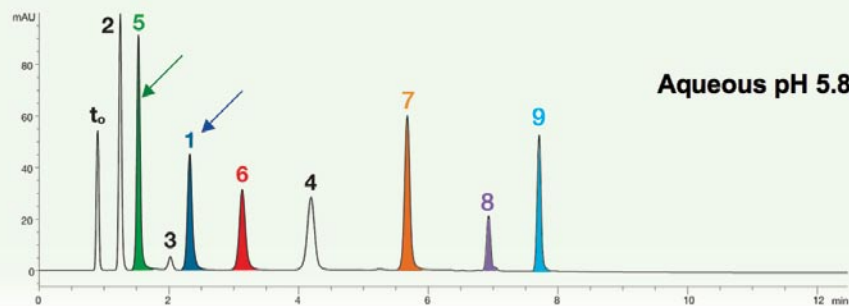
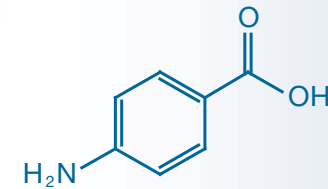
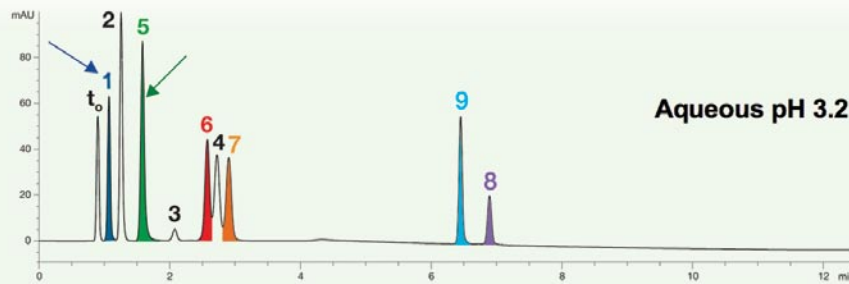


Pyridoxine & PABA Retention Aqueous pH 3.2 & 5.8

- Significant retention and selectivity changes with mobile phase pH change
 - 5 μm LUNA HILIC
 - For pyridoxine, minor change in ionization between pHs, no change in retention
 - For PABA, major change in ionization which results in major change in retention

Conditions	
Column:	150 x 4.6 mm
Flow Rate:	2 mL/min
Temperature:	Ambient

Gradient Program:			
Time	% Acetonitrile	H ₂ O	Buffer
0	90	5	5
2.5	90	5	5
10.0	50	45	5
12.5	50	45	5

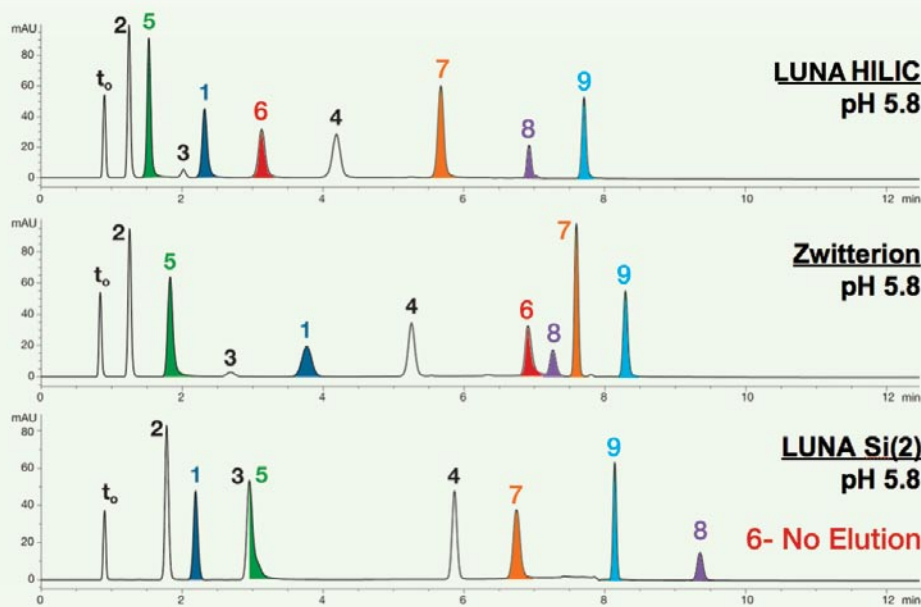


Effect of Column Chemistry H-bonding & Ion-Exchange

- Significant retention and selectivity differences with changes in column chemistry
 - Likely due to differences in H-bonding and ion-exchange

Conditions	
Column:	150 x 4.6 mm
Flow Rate:	2 mL/min
Temperature:	Ambient

Gradient Program:			
Time	% Acetonitrile	H ₂ O	Buffer
0	90	5	5
2.5	90	5	5
10.0	50	45	5
12.5	50	45	5

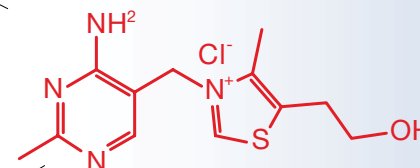
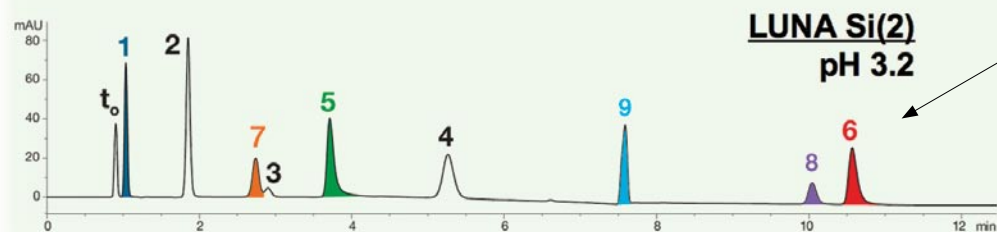
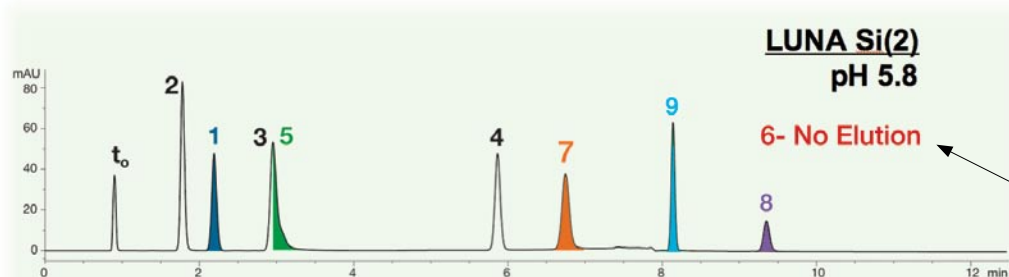


pH Effect on Column Properties

- Thiamine (quaternary amine) adsorbed on silica at pH 5.8, elutes at pH 3.2
 - Surface silanols ionized at pH 5.8

Conditions	
Column:	150 x 4.6 mm
Flow Rate:	2 mL/min
Temperature:	Ambient

Gradient Program:			
Time	% Acetonitrile	H ₂ O	Buffer
0	90	5	5
2.5	90	5	5
10.0	50	45	5
12.5	50	45	5



Conclusions

- Retention and selectivity changes in HILIC can be understood by the effect of chromatographic conditions, such as pH and percent organic, on analyte and sorbent physical properties
- The high organic concentration used in HILIC significantly alters analyte, sorbent and buffer pK_a 's
 - pK_a 's of acids increase with increasing organic concentration
 - pK_a 's of bases decrease with increasing organic concentration
 - Aqueous buffer pH and thus mobile phase pH changes with addition of organic
 - Analyte pK_a and mobile phase pH change with increasing organic solvent
 - Both of these affect ionization and thus retention
- In HILIC analyte retention increases with increased ionization
- Sorbent surface chemistry significantly affects selectivity and retention thru differences in H-bonding and ion-exchange
- H-bonding and ion-exchange character of analytes and sorbents affected by changes in ionization

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