WA20262

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# Efficient Method Development:

## *Exploring pH, Solvent and Stationary Phase*

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*HPLC 2002 – Montreal*

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# Abstract

We outline an efficient starting point for the development of an HPLC method based on our evaluation of the major selectivity differences between the three major variables of pH, organic solvent and stationary phase. We found that the largest selectivity differences for ionizable compounds were caused by pH changes. The organic modifier, methanol or acetonitrile produced the second largest selectivity changes. The third group of selectivity changes was due to the stationary phase itself. However, only very small changes were found when stationary phases of different chain lengths were compared. Larger selectivity effects were found when a classical stationary phase was compared to one with an incorporated polar group. In our general method development set-up, we combined both phases with a phenyl ligand to maximize the selectivity differences between different stationary phases. We show an example of how to quickly generate data to assess the selectivity differences and then use those data to develop the method.

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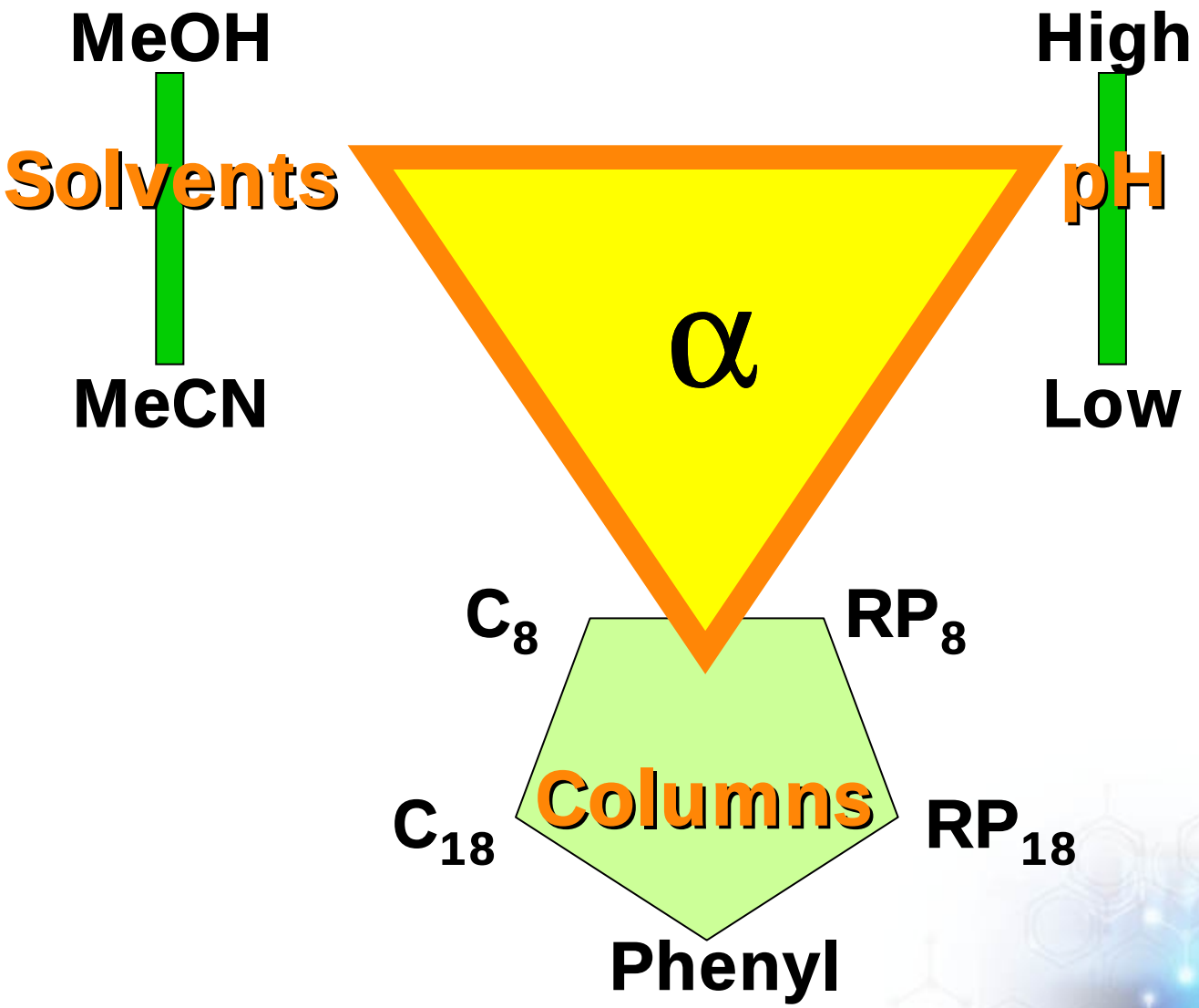
# Selectivity Studies

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# Example of Selectivity Study

**Chromatographic Conditions:**

Columns and mobile phases:
as indicated
Column dimensions:
4.6 mm x 50 mm, 3.5 µm
Gradient elution:
from 0% to 80%, $t_g$=15 min
MeOH or MeCN
Low and High pH
Flow rate: 2.0 mL/min
Wavelength: 254 nm
Temperature:30°C

**Compounds: Diuretics**

1) Amiloride
2) Furosemide
3) Althiazide
4) Chlorthalidone
5) Benzthiazide
6) Probenecid
7) Triamterene
8) Bumetanide
9) Bendroflumethiazide
10) Ethacrynic acid
11) Canrenoic acid

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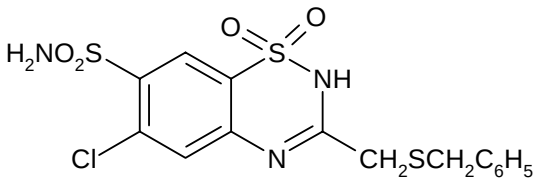
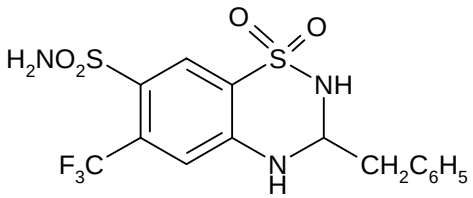
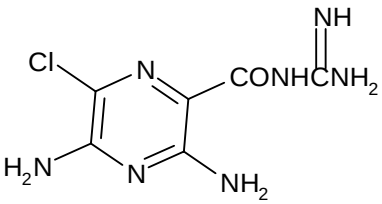
# Test Compounds



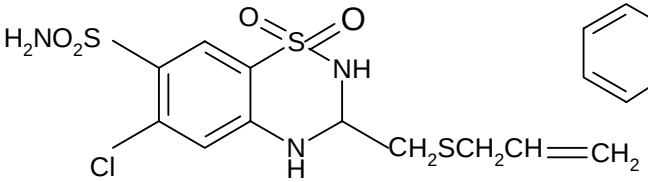

**Benzthiazide (WA)**



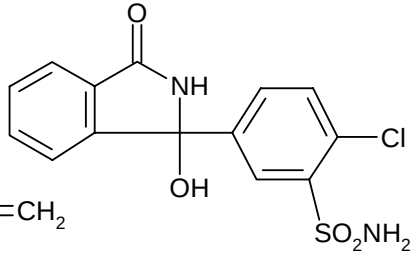

**Bendroflumethiazide (WA)**



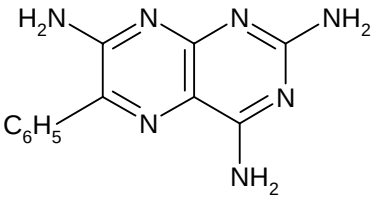

**Amiloride (B)**



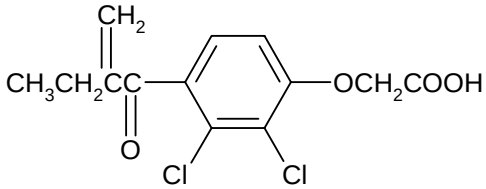

**Althiazide (WA)**



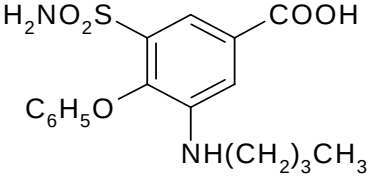

**Chlorthalidone (WA)**



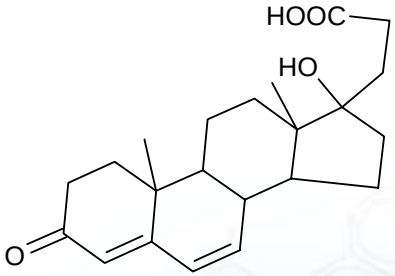

**Triamterene (B)**



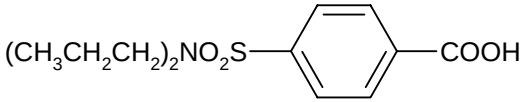

**Ethacrynic acid (A)**



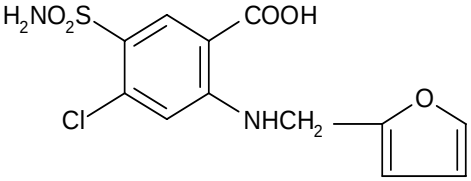

**Bumetanide (Z)**




**Canrenoic acid (A)**




**Probenecid (A)**




**Furosemide (Z)**

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# Measuring and Ranking Selectivity

- Can be done simply by visual inspection – or –
- Quantitative Alternative:
  Measure correlation coefficient { $r^2$ } of the retention times, when comparing different columns or chromatographic conditions !
- Measure of the **Selectivity Difference:**

$$\text{Selectivity Difference} = s = 100 \cdot \sqrt{\left(1 - r^2\right)}$$

- Simple tool can be used by anyone

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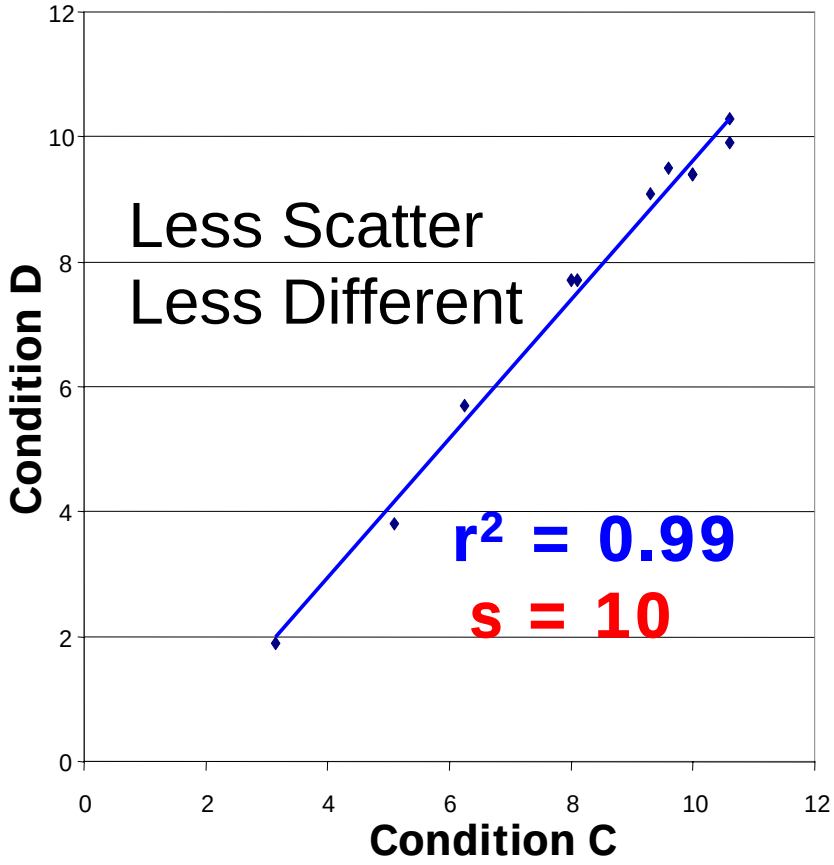
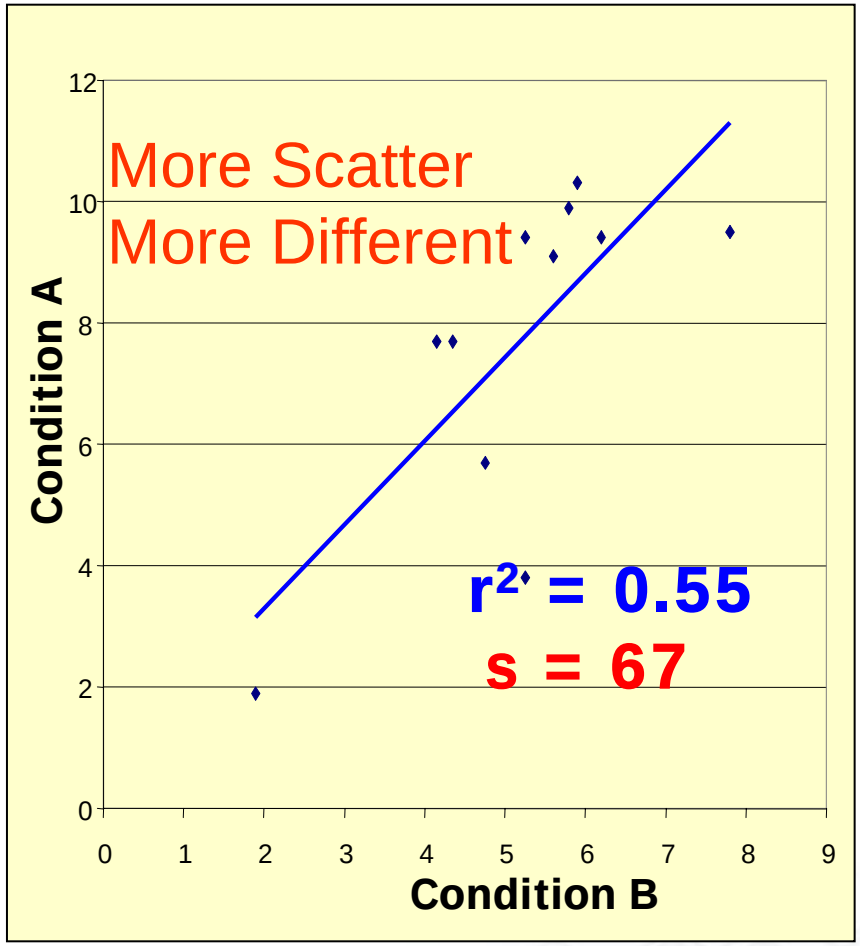
# Selectivity Measurement
## (Plot of Retention Times under Different Conditions)




**Condition A is very different than Condition B (s = 67)**

**Condition D is similar to Condition C (s = 10)**

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# Solvent Selectivity

- **Common solvents:**
  - **Methanol (MeOH)**
  - **Acetonitrile (MeCN)**
- **Less common:**
  - **Isopropanol (IPA)**
  - **Ethanol (EtOH)**
  - **THF (Tetrahydrofuran)**

**The use of different solvents provides changes in selectivity as well as elution strength**

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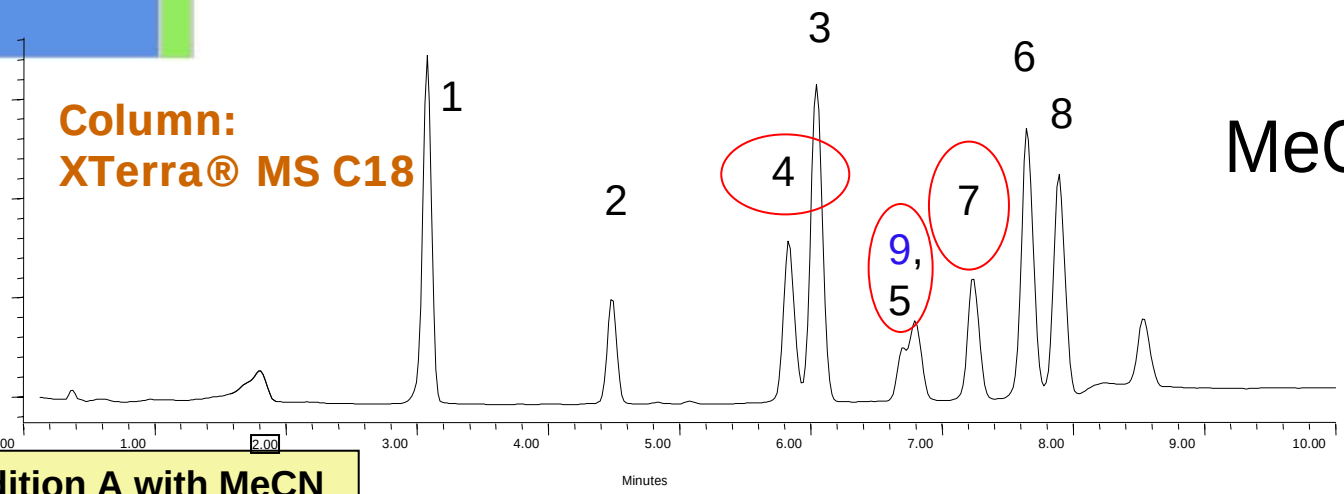
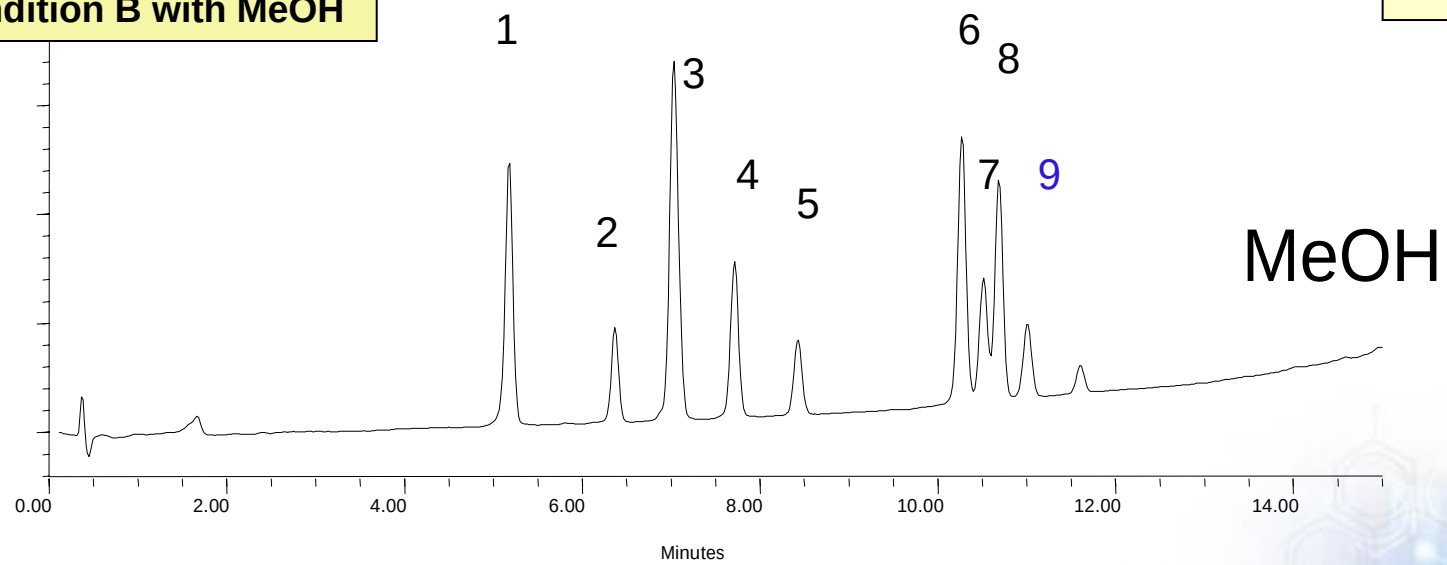
# Comparison of Solvent Selectivity

**Column:**
**XTerra® MS C18**




**Condition A with MeCN**
**Condition B with MeOH**

s = 43

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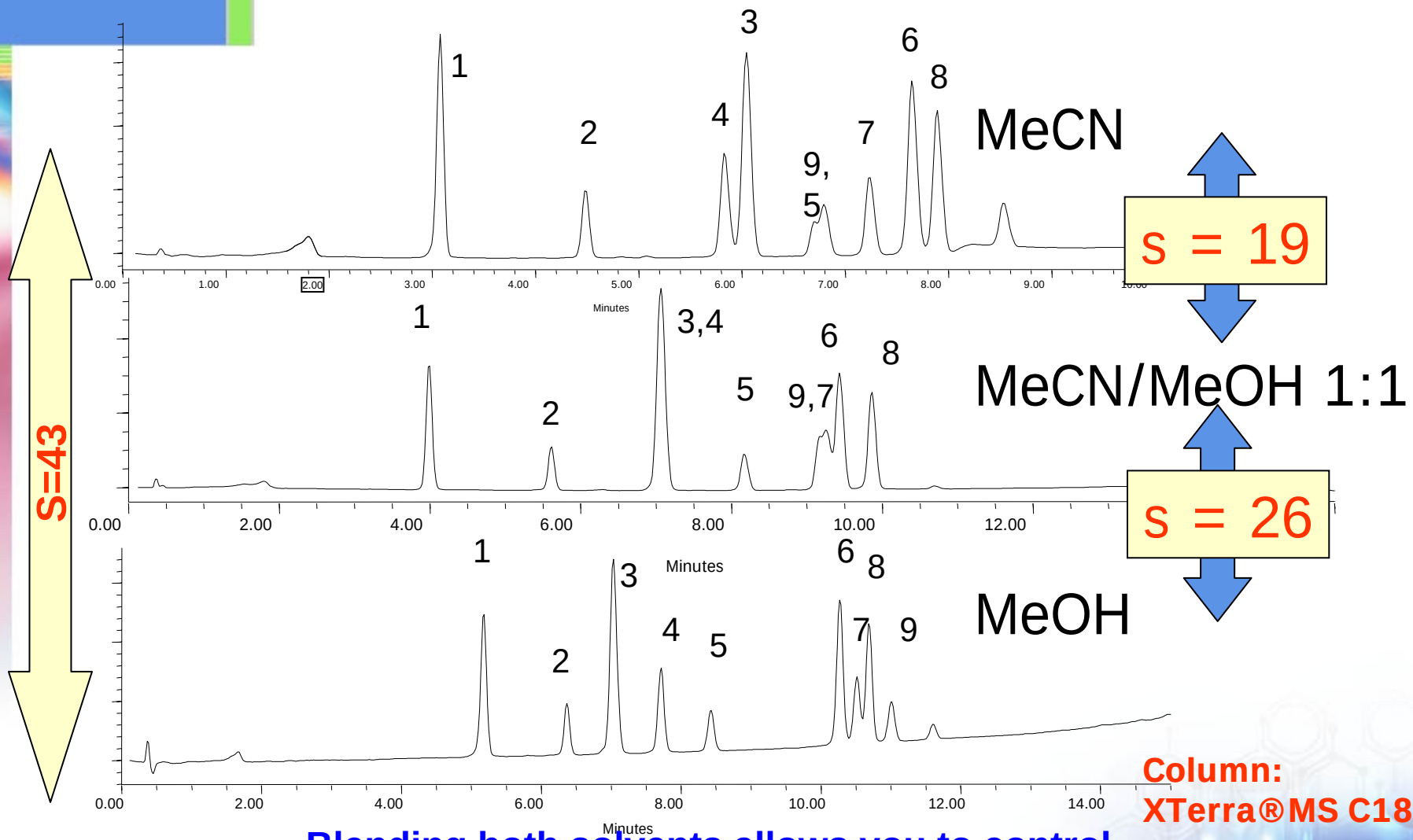
# Solvent Selectivity is Continuous




**Column: XTerra®MS C18**

**Blending both solvents allows you to control the degree of selectivity difference**

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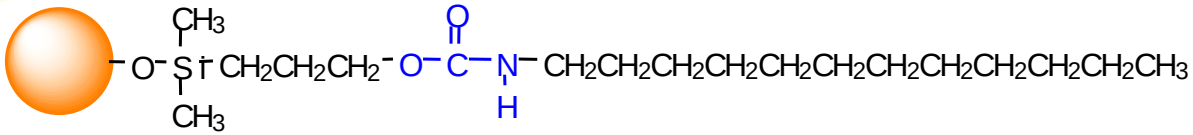
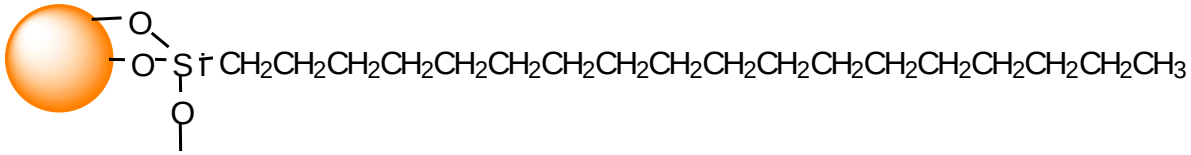
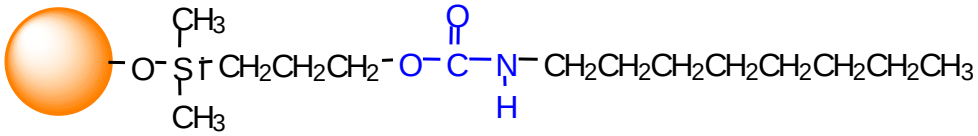
# Column Selectivity: XTerra® Columns



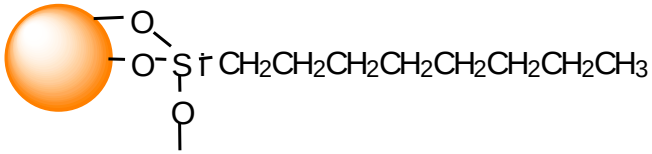
**$RP_{18}$**



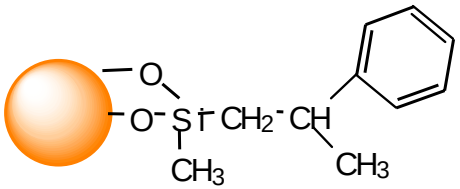
**MS $C_{18}$**



**$RP_8$**



**MS $C_8$**



**Ph**

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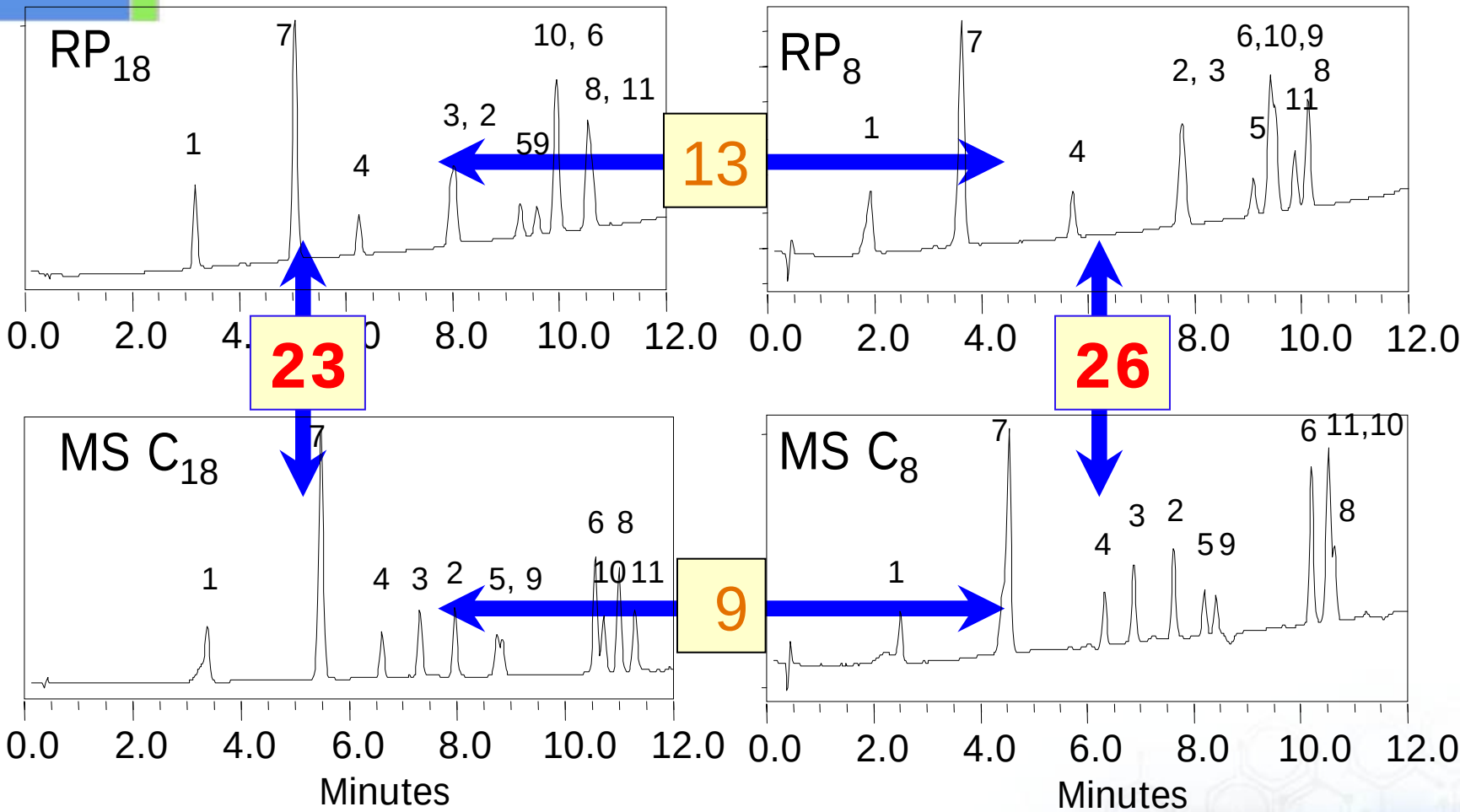
# Selectivity Comparison at a Single pH Value (low)




**Selectivity shift from ligand type (23, 26) is larger than selectivity shift from chain length (13, 9)**

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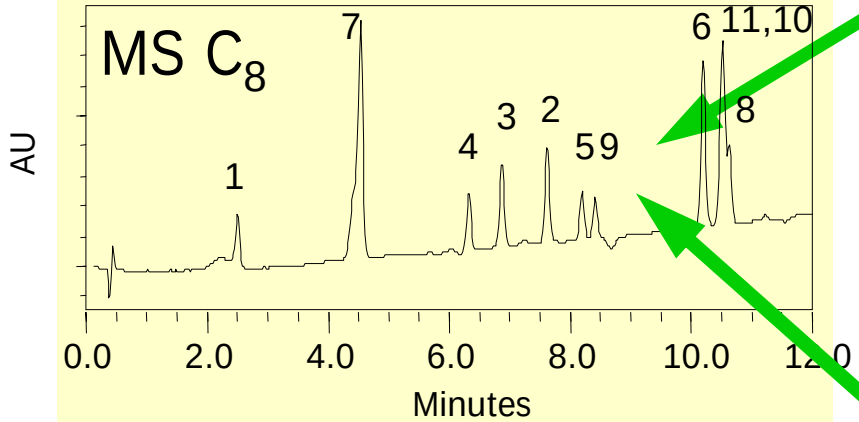
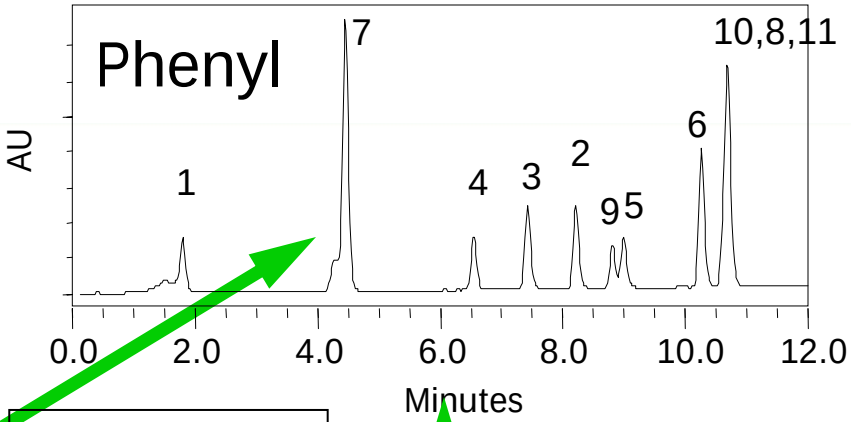
# Selectivity Comparison
## (Short chains only)

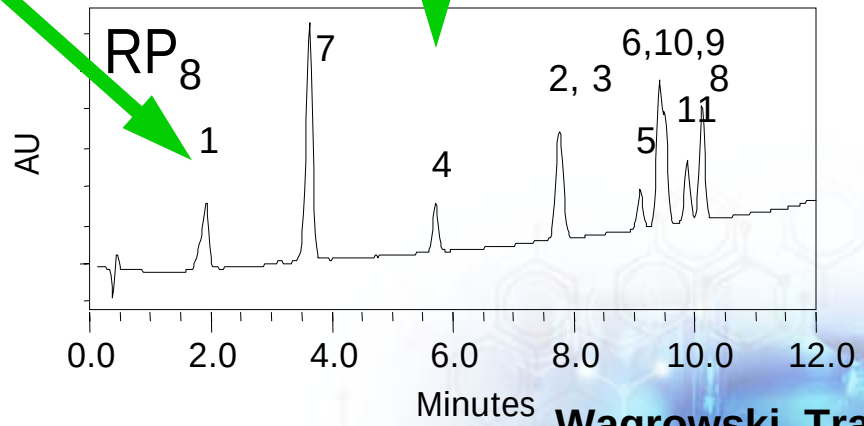



s = 13

s = 22

s = 26




Most drastic selectivity shift between classical phase and phase with embedded polar group

**s = 26**

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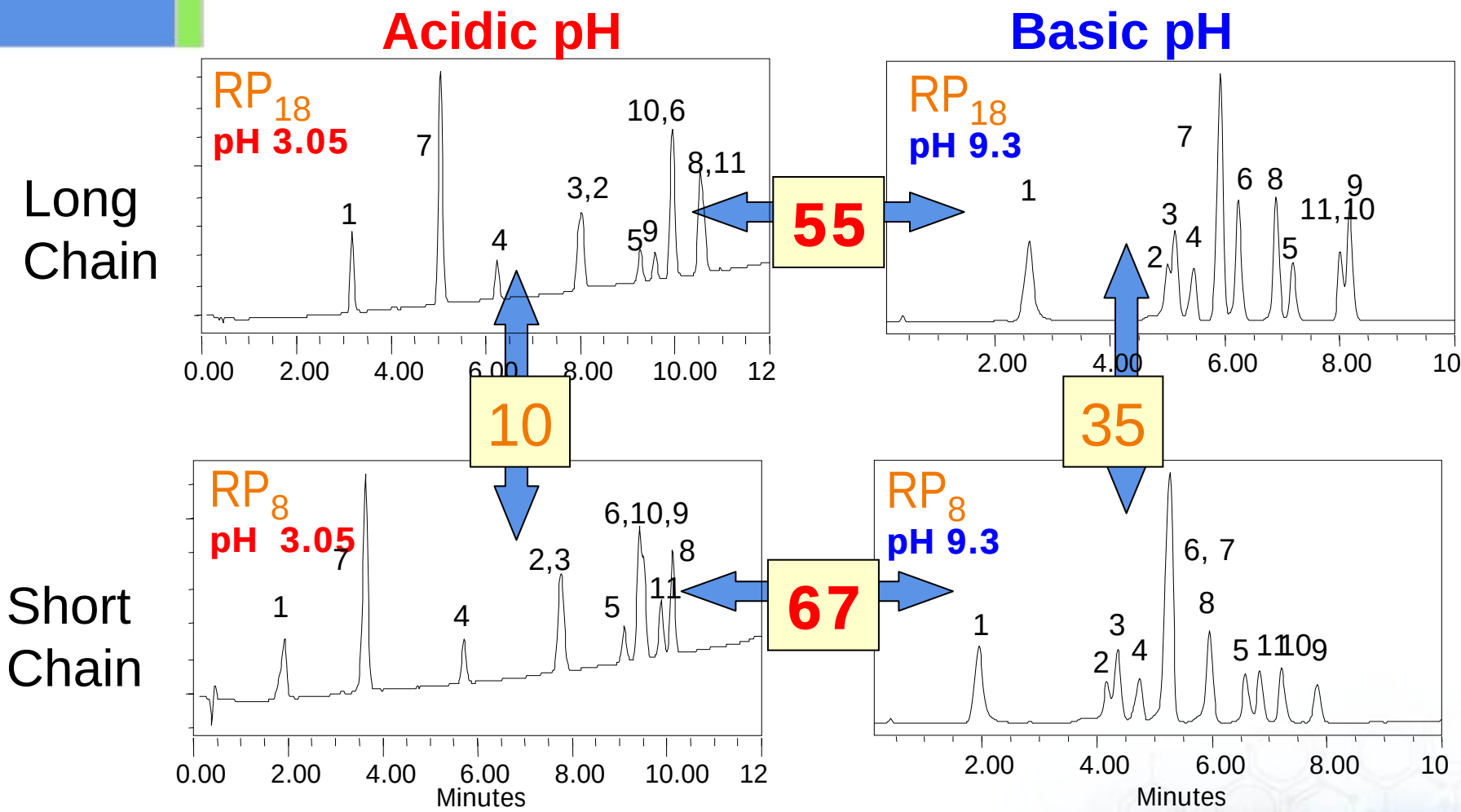
# pH and Chain Length




**pH provides more selectivity difference than does chain length**

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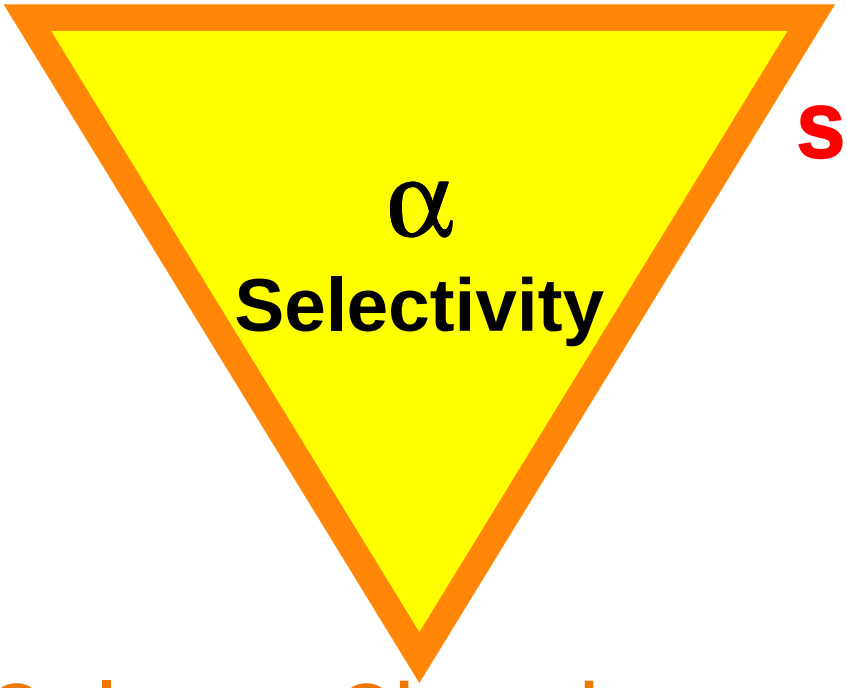
# Rating of Selectivity Factors

Solvent

**s = 45**

**pH**

**s = 50-67**




Column Chemistry **s < 50**

$C_{18}$-$RP_{18}$ ~ 20-25

$C_8$-$C_{18}$ ~ 10

$RP_8$-Phenyl ~ 20-25

$C_{18}$-$RP_{18}$ for Phenols = 45

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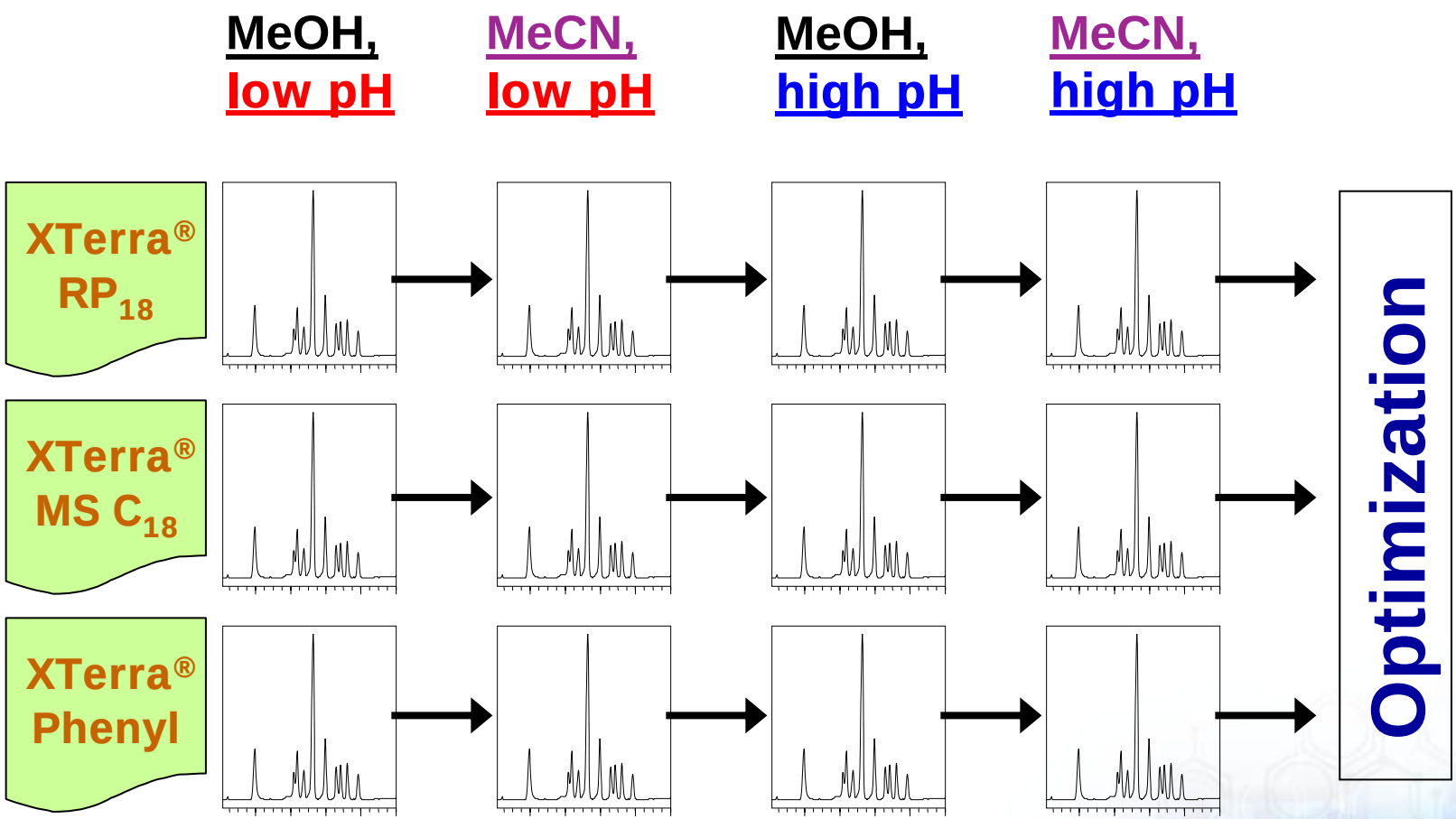
# Exploratory Method Development

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# Chromatographic Conditions

- Column Dimensions
  - 4.6 mm x 50 mm, 3.5 μm
- Gradient elution
  - 0% to 80%, $t_g$ = 15 min
  - MeOH and MeCN
  - pH 3 (Ammonium formate buffer) and pH 10 (Ammonium bicarbonate buffer)
- Flow rate = 2 mL/min
- Temperature = 30 $^{o}$C
- Alliance™ LC Systems

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# Example: Parent Drug and Metabolites in Plasma

- **Problem: a small number of compounds of interest in an ocean of interferences**
- **Goals:**
  - **Separation of compounds of interest from each other**
  - **Elimination of background interferences**
- **Analytes: verapamil, methoxyverapamil (ISTD) and norverapamil in rat plasma**
- **Sample Preparation:**
  - **Oasis® cartridges, conditioned with methanol and water**
  - **Load acidified plasma sample (2% $H_3PO_4$)**
  - **Wash with 5% methanol in water**
  - **Elute with methanol**

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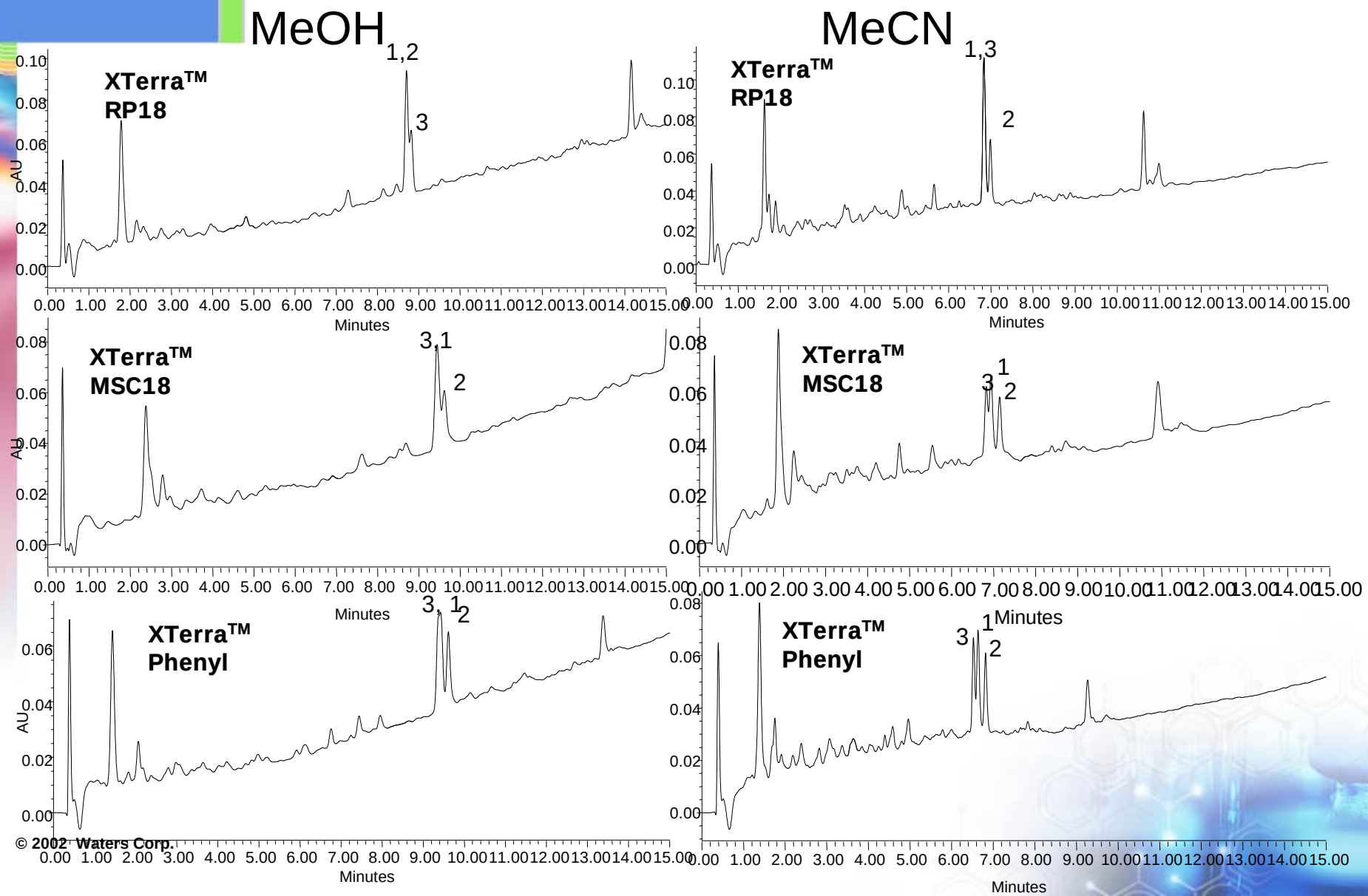
# Initial Gradient Study: pH 3.64

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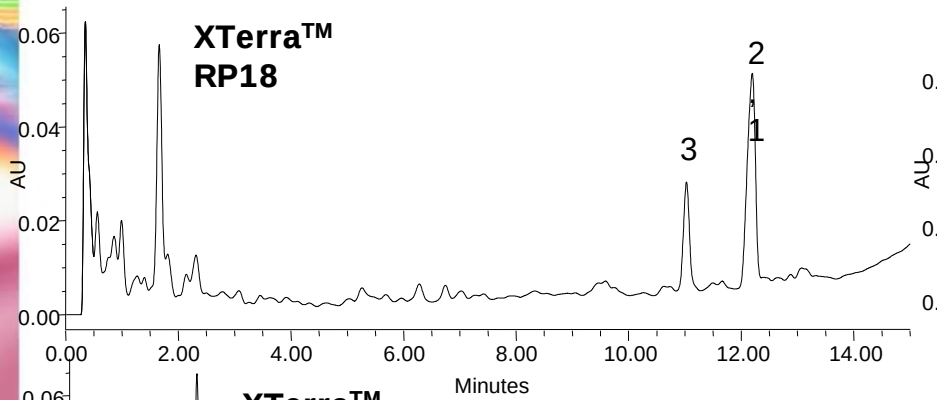
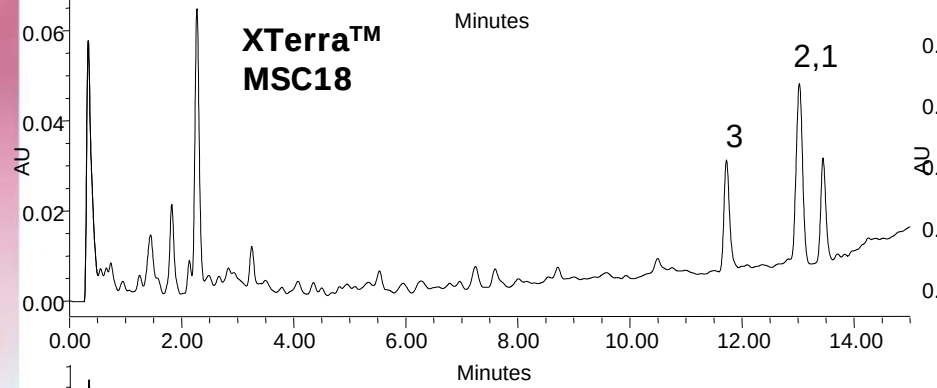
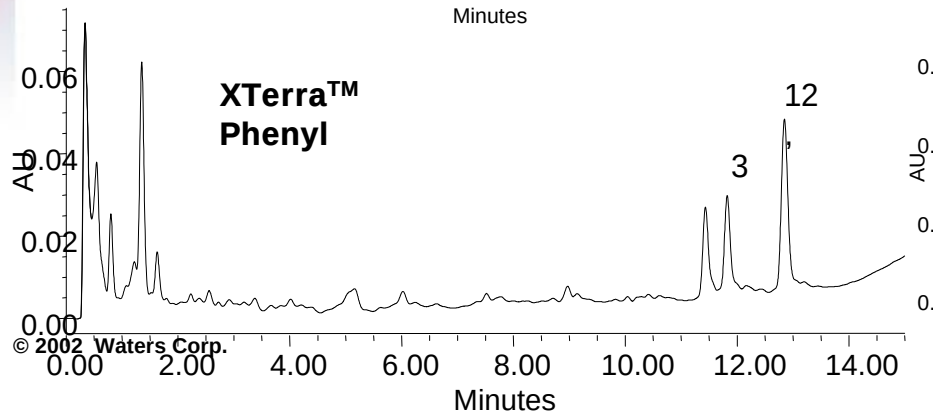
# Initial Gradient Study: pH 9.0

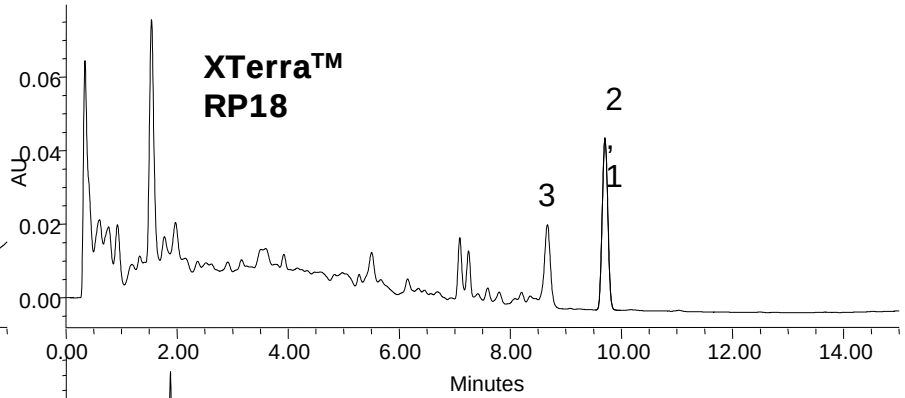
MeOH

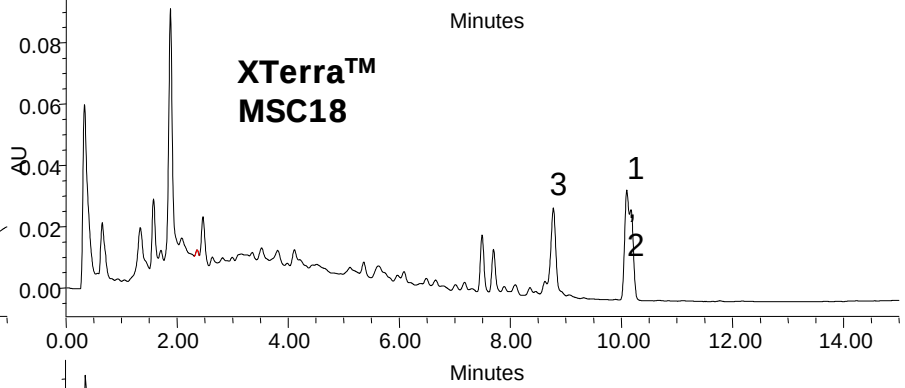


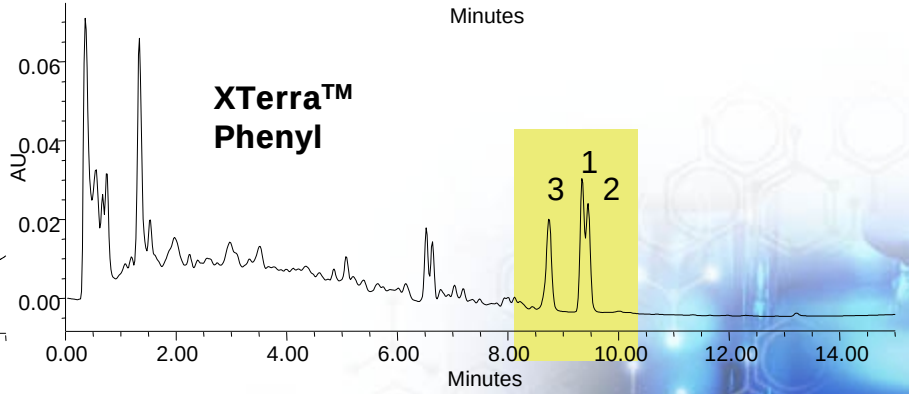




MeCN

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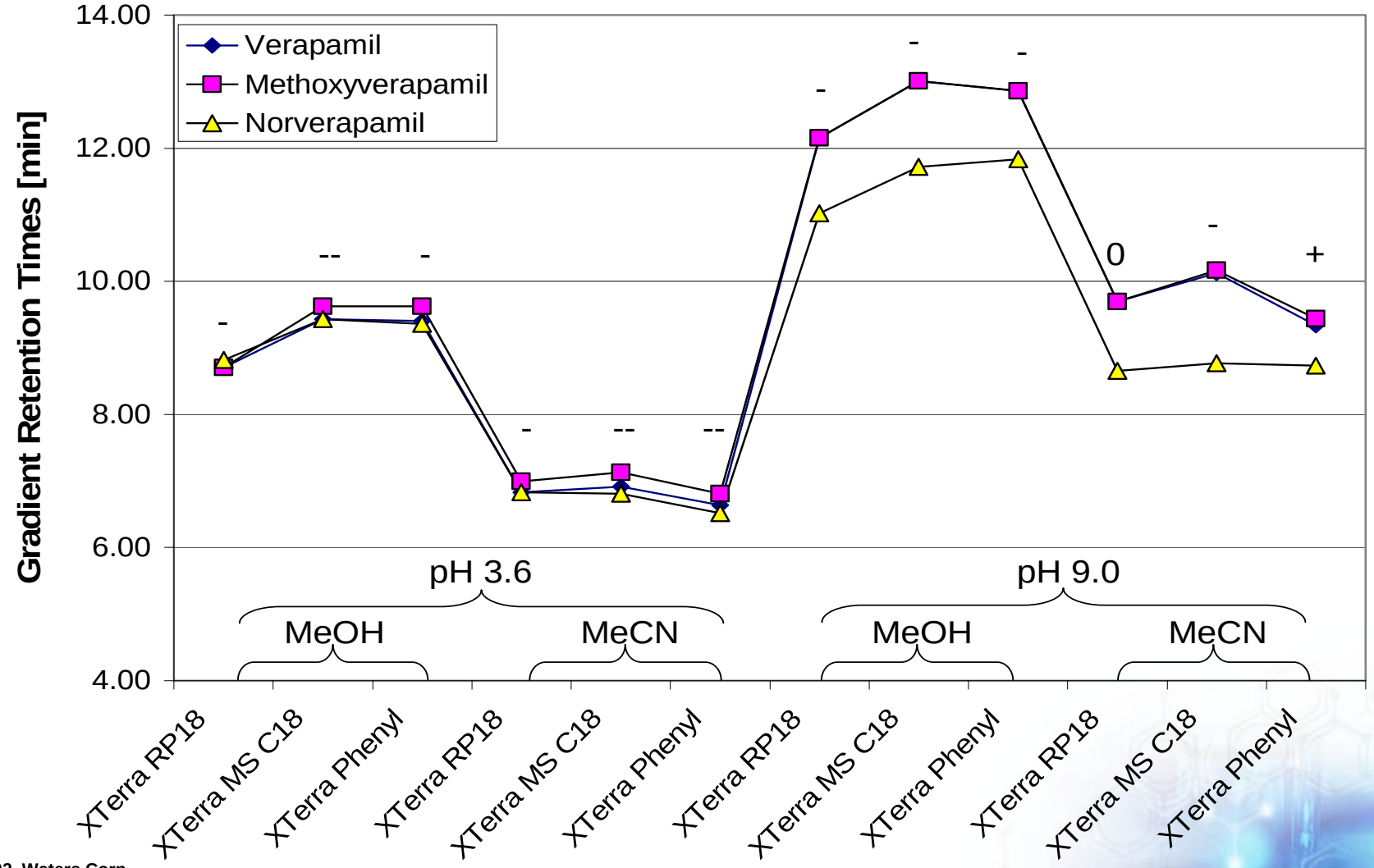
# Summary of Initial Gradient Studies

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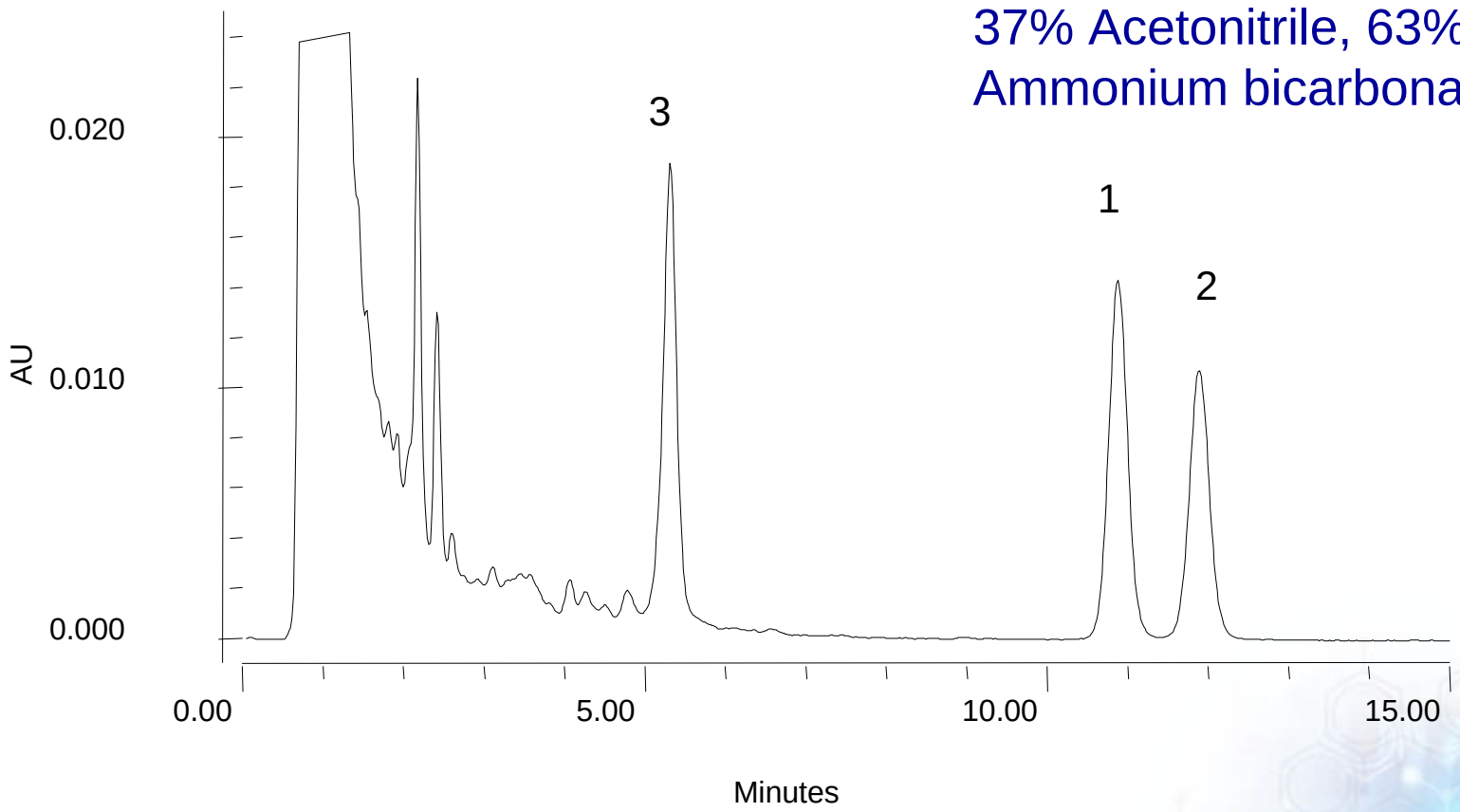
# Final Isocratic Method

**XTerra® Phenyl**
4.6 mm x 100 mm, 3.5 µm
37% Acetonitrile, 63% 10 mM
Ammonium bicarbonate, pH 9

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# Conclusions

- Exploration of selectivity tools
  - pH is the most powerful tool in the manipulation of the separation of ionizable analytes
  - Solvent and type of bonded phase (classical phase vs. phase with incorporated polar group) are the second choices

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# Conclusions (cont.)

- Rapid method development possible by using
  - High pH and low pH
  - Acetonitrile and methanol
  - XTerra® MS $C_{18}$, XTerra® $RP_{18}$, and XTerra® Phenyl stationary phases

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# Literature

U. D. Neue, C. H. Phoebe, K. Tran, Y.-F. Cheng, Z. Lu, “Dependence of Reversed-Phase Retention of Ionizable Analytes on pH, Concentration of Organic Solvent and Silanol Activity”, J. Chromatogr. A 925 (2001), 49-67

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