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Session F6

A Systematic Approach Towards UPLC® Method Development

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Challenges of Method Development

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- Chromatographic methods are developed for different applications constantly, like throughout the drug development process
  - Samples vary in complexity
  - Redundancy exists across an organization
- Method development is costly and time consuming
  - Desire to streamline processes to bring products to market faster
  - Faster chromatographic methods will improve profitability

Critical Components of Method Development

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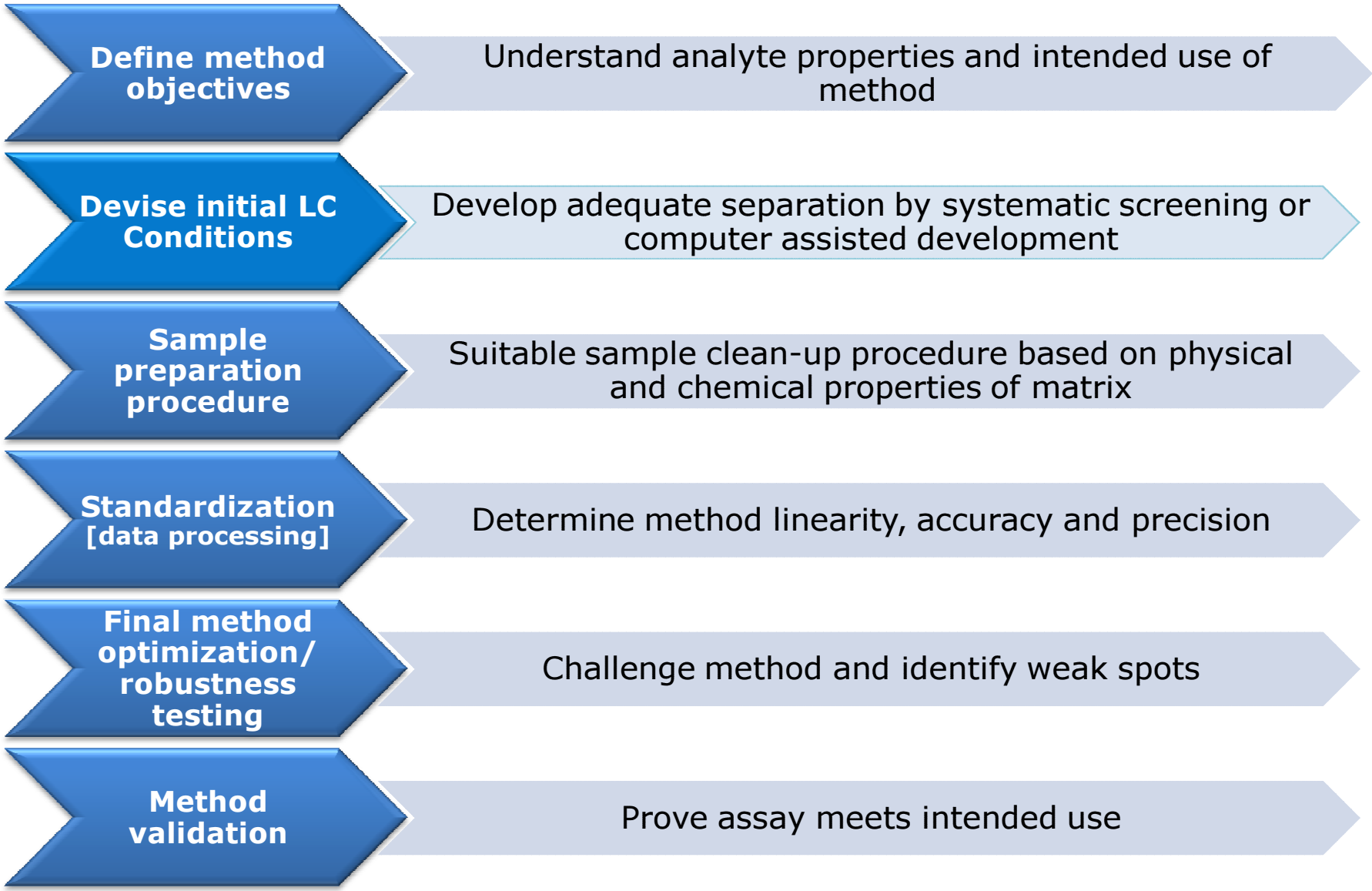

Outline

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- **Introduction**
  - Approaches Toward Method Development
  - UPLC Technology
  - Success Criteria
- **Controlling Selectivity and Retention**
  - Stationary Phase and Particle Substrate Design
  - Organic Modifier
  - Mobile Phase pH
- **Method Development Strategy**
  - Systematic Screening Protocol
  - Quality by Design [Q_bD] Approach
- **Implementing the Approach**
  - Case Study
- **Conclusion**

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Approaches Toward Method Development:
Deriving Initial LC Conditions

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- Match LC conditions to the chemical properties of the analyte[s]
  - Educated guess based on past experience [speculation]
  - Usually supplemented with a literature search
  - Ask a colleague

- Stepwise incremental approach
  - Next step experimental design based on results from previous experiment

★Systematic screening protocol
  - Evaluate combinations of mobile phase pH, organic modifier and stationary phase
    - Select best combination of these parameters
  - Method optimization
    - Gradient slope/Temperature

UPLC Technology Can Streamline Method Development

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UPLC Technology enables faster method development

ACQUITY UPLC H-Class

Develop methods in a single work day!

- Systematic screening protocol involving pH, organic modifier and column chemistry
- High resolution sub 2 µm column technology creates high resolution separations, faster
- Automated column and mobile phase selection
- Quaternary solvent mixing [ACQUITY UPLC H-Class]

Before You Start:
Information Gathering

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- Chemical properties [functional groups]
  - Ionizable species, polarity, pKa, molecular weight
- Sample solubility
- Number of compounds present
  - How many components are you trying to separate?
- Sample matrix
- Detection technique [UV, ELS, RI, FL, MS etc.]
  - Based on available equipment or sensitivity requirements of assay
- Criteria for success
  - Concentration range and quantitative requirements
  - System suitability

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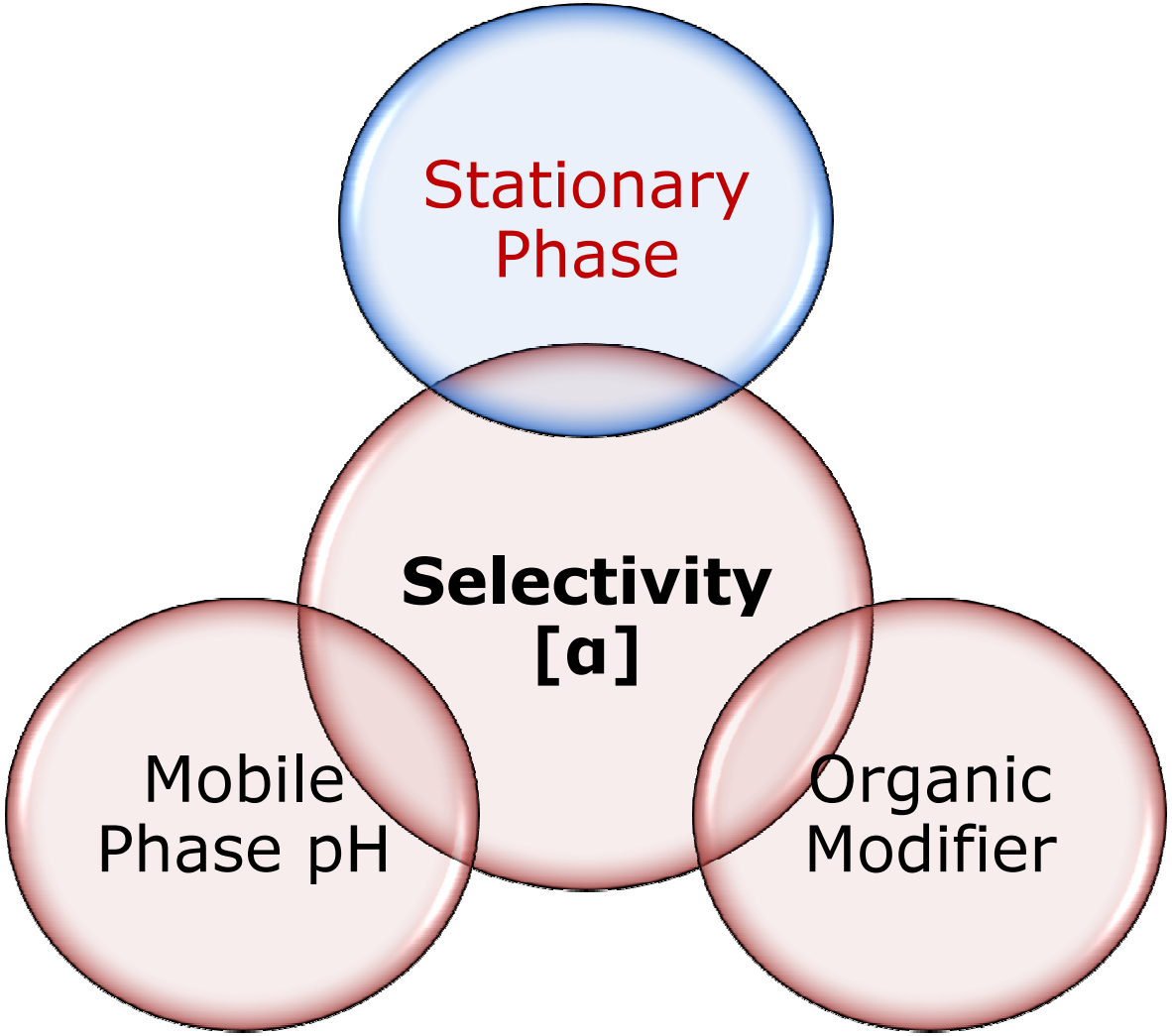
Chemical Factors that Impact Selectivity



Improving Resolution with Complementary Selectivity

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$$Rs = \frac{\sqrt{N}}{4} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k}{k+1}$$

Physical

Maximized in UPLC Separations by:

- Ultra-low dispersion system
- Small [< 2 µm] particles
- Higher pressure capability
- Well-designed columns

Chemical

Maximized in UPLC Separations by:

- Range of column chemistries
- Multiple particle substrates
- Wide usable pH range [BEH]
- High retentivity [HSS]
- Wide range in selectivity [CSH]

Impact on Resolution % Improvement

Double N	20 – 40%
Double k	15 – 20%
Double α	> 400%

Stationary Phase Selectivity:
Bonded-Phase [Ligand] and Particle Substrate

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- Silanol activity and surface charge
  - Influences secondary interactions [ion-exchange], peak shape and sample loadability
- Hydrophobicity
  - Longer alkyl chain lengths will provide increased retention
  - Shorter, ionizable ligands will increase polarity
- Hydrolytic stability
  - Column lifetime will be impacted by the number of attachment points to the particle surface
- Ligand density
  - Influences retention and sample loadability

The Widest UPLC Column Offering

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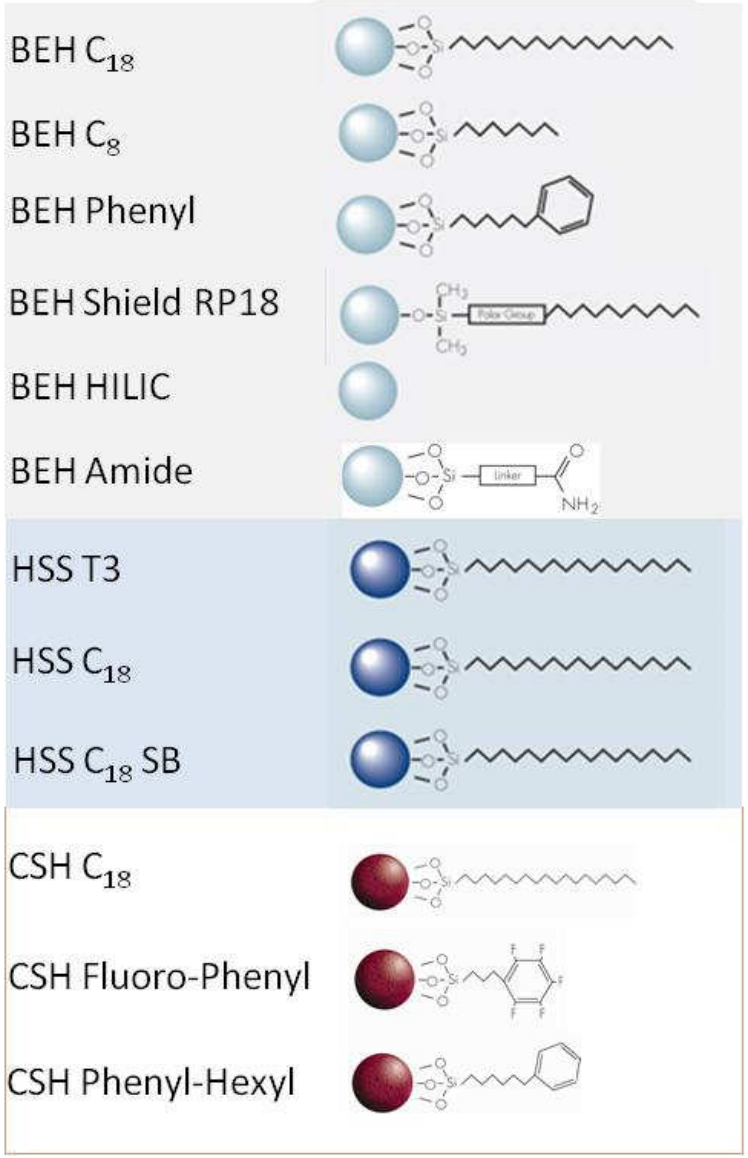
- **Five particle substrates**
  - 130Å, 200Å and 300Å BEH [Ethylene Bridged Hybrid], HSS [High Strength Silica] and **CSH [Charged Surface Hybrid]**
  - All are available in HPLC and UPLC particle sizes
- **Wide and growing selection of column chemistries**
  - BEH 130Å C_{18}, C_8, Shield RP_{18}, Phenyl, HILIC and Amide
  - BEH 300Å C_{18} and C_4
  - BEH 200Å SEC
  - HSS C_{18}, T3, C_{18} SB (and soon Cyano and PFP)
  - **CSH C_{18}, Fluoro-Phenyl and Phenyl-Hexyl**
- **Proven application-based solutions**
  - AAA, OST, PST, PrST and Glycan
- **Transferability between HPLC and UPLC**
  - XBridge HPLC and ACQUITY UPLC BEH columns
  - HSS HPLC and ACQUITY UPLC HSS columns
  - **XSelect HPLC and ACQUITY CSH columns**
- **VanGuard Pre-columns**
- **eCord Technology**



Industry Trends:
Current State of Reversed-Phase Separations

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- Advances in stationary phase design
  - Hybrid particle technology
    - Extended usable pH range [1-12]
    - Exceptional peak shape and efficiency
    - Rugged and reliable column life
  - Sub 2 µm particle technology
    - Improvements in resolution, sensitivity and speed of analysis
  - Pellicular [core-shell] particles

- Instrument platform of choice
  - UltraPerformance LC with UV and mass spectrometry [UPLC/MS/[MS]]
    - Requires volatile mobile phases
      - Excludes typical UV-based buffers [i.e., phosphate buffers]
    - Preference towards low ionic strength additives [i.e., formic acid, acetic acid, ammonium hydroxide]
      - Avoid preparation of buffers if possible

Defining the Problem:
Low Ionic Strength Mobile Phases

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- Poor mass loading of charged cationic [basic] solutes in low pH mobile phases due to limited sample capacity
  - High tailing factors
  - Poor signal intensity
- Slow equilibration at low pH
  - Drifting retention times with repeat injections
- Elution [retention] time shift after exposure to a higher pH mobile phase*[1]
  - Irreproducible assay performance when performing method screening
    - Low/high pH switching with un-buffered mobile phases

*1 Marchand, D.H., et al., J. Chromatogr. A **2003**, 1015, 53-64

Explanation of CSH Technology

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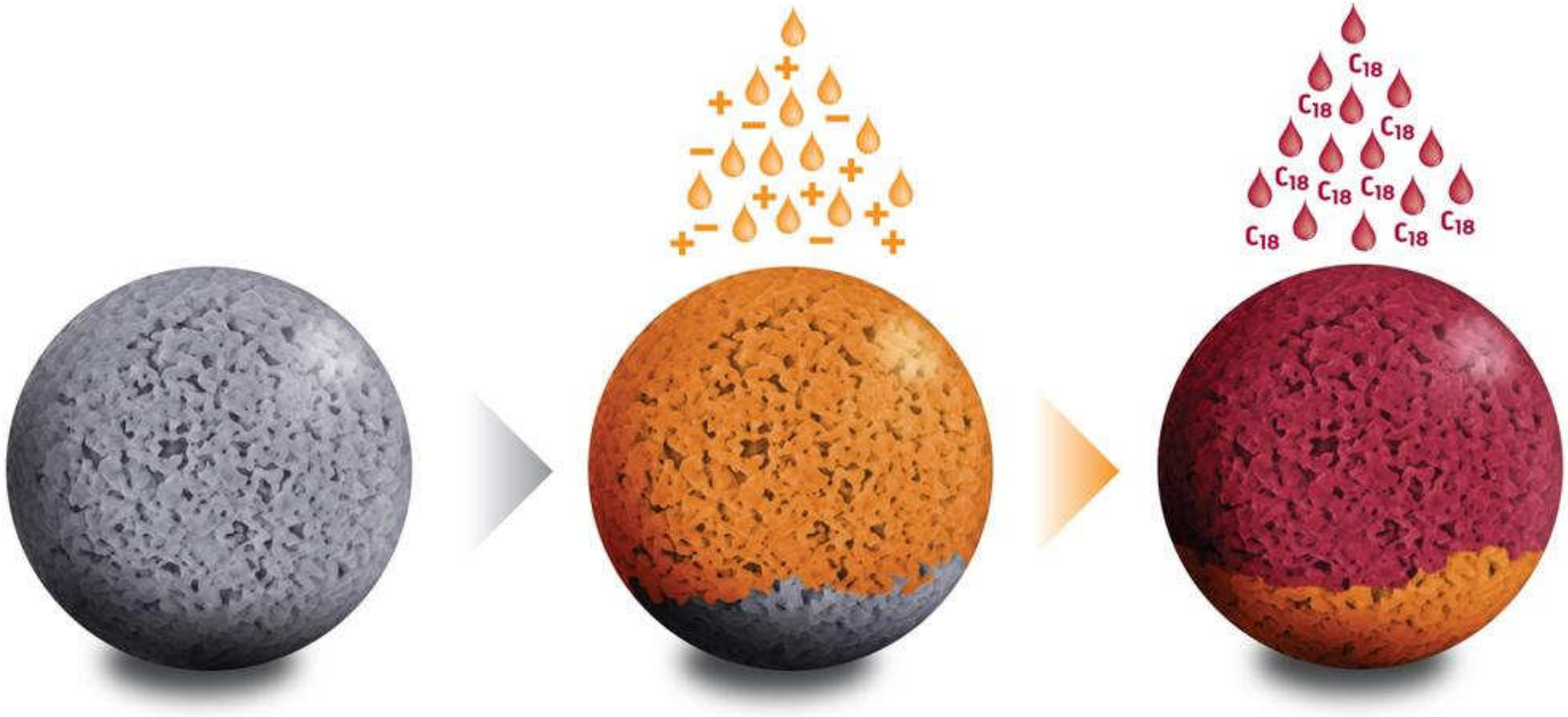
Step 1

Step 2

Step 3



Unbonded BEH Particle

Start with the rugged, ultra-efficient, ethylene bridged hybrid (BEH) particle

Apply Controlled Surface Charge

Add reproducible low-level charge to particle surface

Bond and End Cap

Functionalize with appropriate bonded phase chemistry

CSH Technology:
Controlled Surface Charge Yields High Performance

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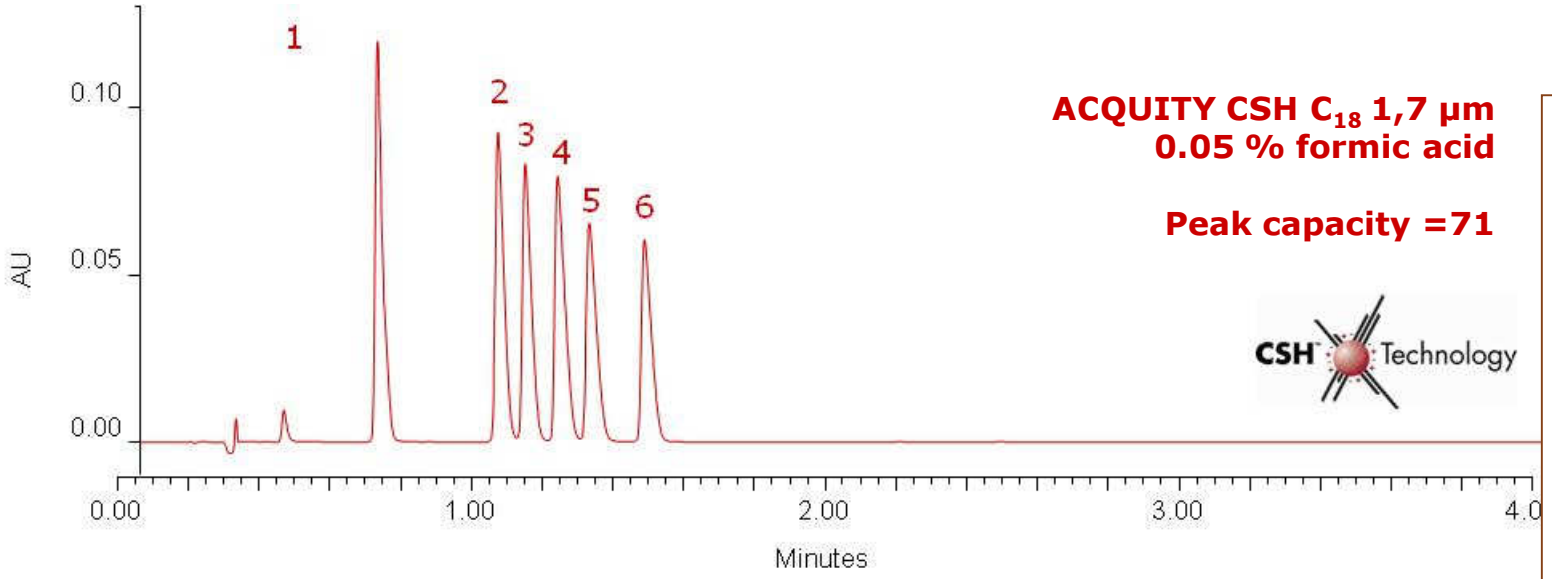
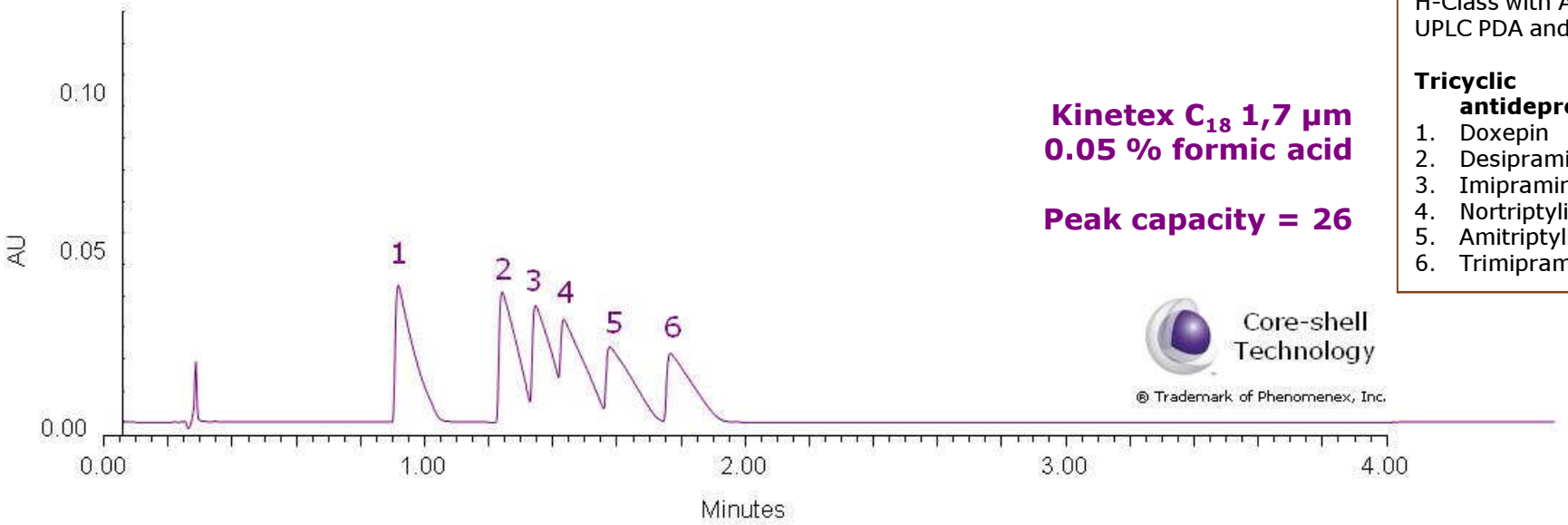


ACQUITY CSH C_{18} 1,7 µm
0.05 % formic acid

Peak capacity =71

CSH Technology



Kinetex C_{18} 1,7 µm
0.05 % formic acid

Peak capacity = 26

Core-shell Technology
® Trademark of Phenomenex, Inc.

Columns: 2,1 x 50 mm
MP A: water
MP B: acetonitrile
MP C: 2% formic acid
Gradient: 25-35%B in 2 min, 35 – 95%B from 2 - 3 min; [2.5% C held constant]
Inj. Vol.: 5 µl
Sample Conc.: 10 µg/ml
Detection UV @ 254 nm
Sampling rate: 20 Hz
Filter: 0.1 sec
System: ACQUITY UPLC H-Class with ACQUITY UPLC PDA and SQD

Tricyclic antidepressants:

1. Doxepin
2. Desipramine
3. Imipramine
4. Nortriptyline
5. Amitriptyline
6. Trimipramine

CSH Technology:
Influence of Sample Loading on Trace Impurity Detection

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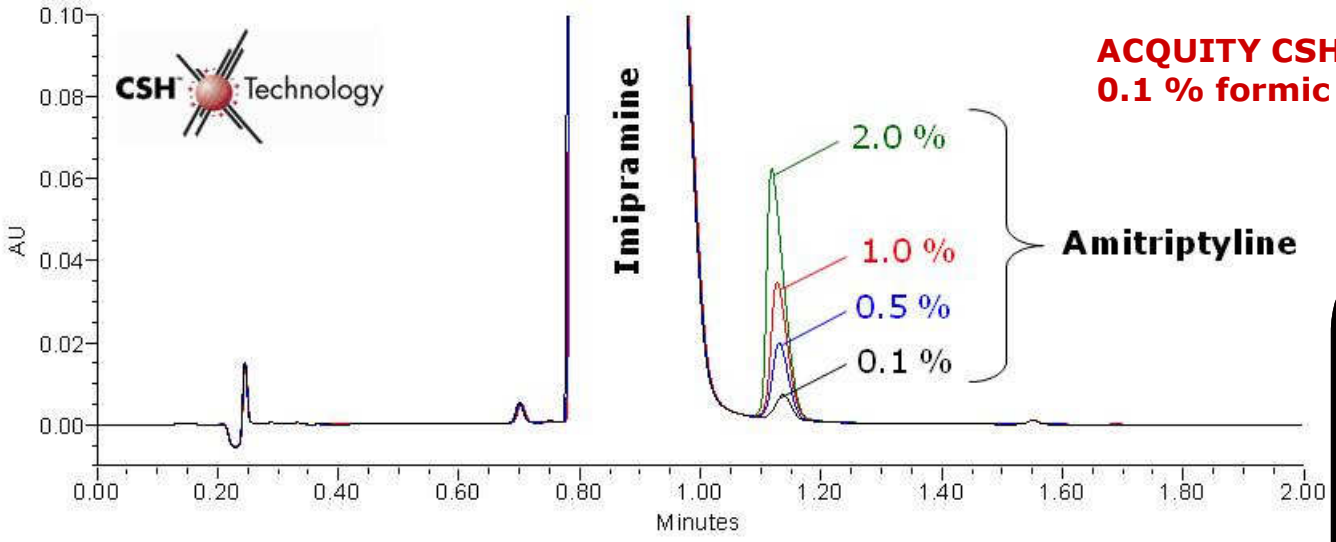
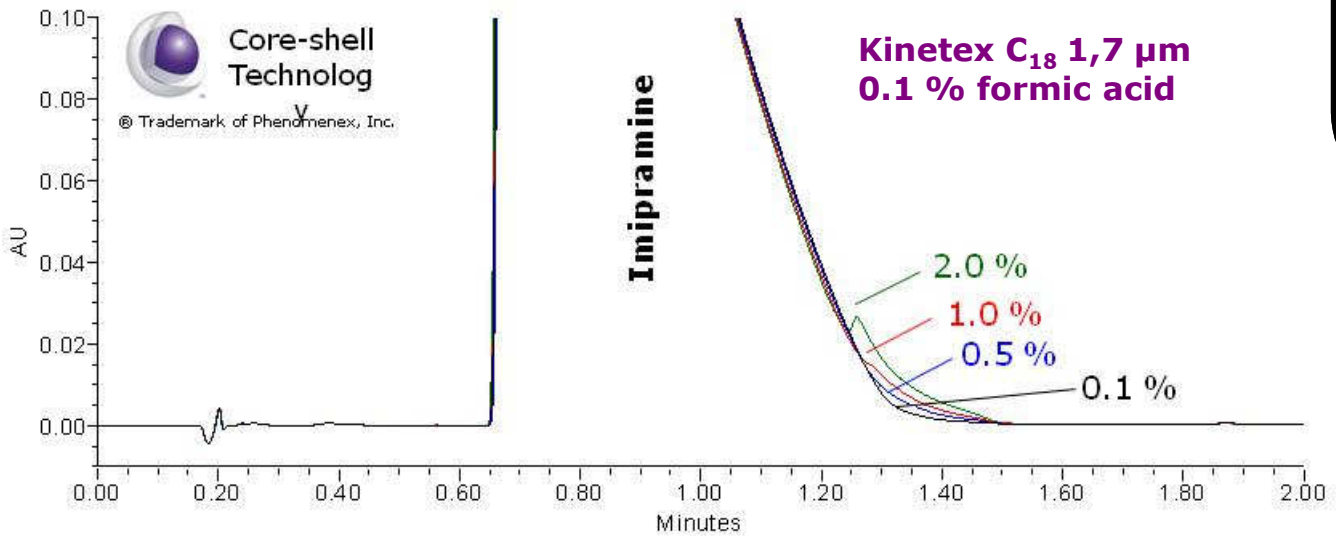


ACQUITY CSH C_{18} 1,7 µm
0.1 % formic acid



Kinetex C_{18} 1,7 µm
0.1 % formic acid

Observations

CSH Technology enables superior peak shape and efficiency in low ionic strength mobile phases

Improved sensitivity for trace level impurity analysis

Imipramine concentration held constant at 0.5 mg/ml;

0.1% formic acid mobile phase

CSH [Charged Surface Hybrid] Chemistries of UPLC Technology

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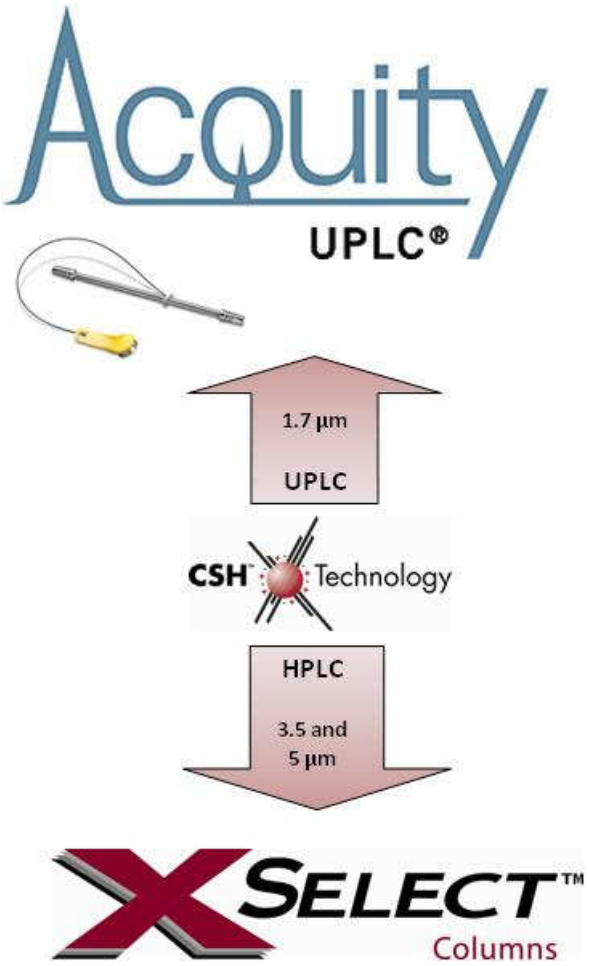
- **CSH C_{18}**
  - Trifunctionally bonded C_{18}
  - Wide pH range for maximum selectivity [pH 1 - 11]
  - Superior peak shape and efficiency in buffered and low ionic strength mobile phases
- **CSH Phenyl-Hexyl**
  - Trifunctionally bonded C_6-Phenyl
  - Wide pH range [1 - 11]
  - Complementary selectivity for aromatic species
- **CSH Fluoro-Phenyl**
  - Trifunctionally bonded, non-endcapped, pentafluorophenyl [pH 1 – 8]
  - Unique selectivity compared to alkyl columns
  - Stable and reproducible manufacturing process



ACQUITY UPLC Column Selection:
Systematic Screening

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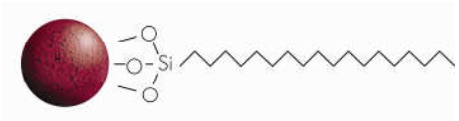
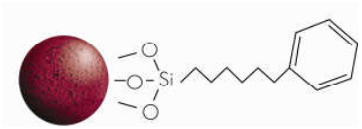


- **CSH C_{18}**
  - Wide pH range for maximum selectivity [pH 1 - 11]
  - Superior peak shape and efficiency in buffered and low ionic strength mobile phases



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  - Trifunctionally bonded C_6-Phenyl [pH 1 - 11]
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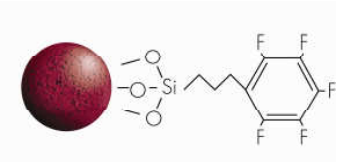

- **CSH Fluoro-Phenyl**
  - Trifunctionally bonded pentafluorophenyl, non-endcapped [pH 1 – 8]
  - Unique selectivity compared to alkyl columns

- **HSS C_{18} SB [S**electivity for **B**ases**]**
  - Low ligand density, trifunctionally bonded C_{18} [pH 2 – 8]
  - Non-endcapped C_{18} designed for silanophilic interactions and alternate selectivity with exceptional peak shape for bases

Systematic Screening Protocol

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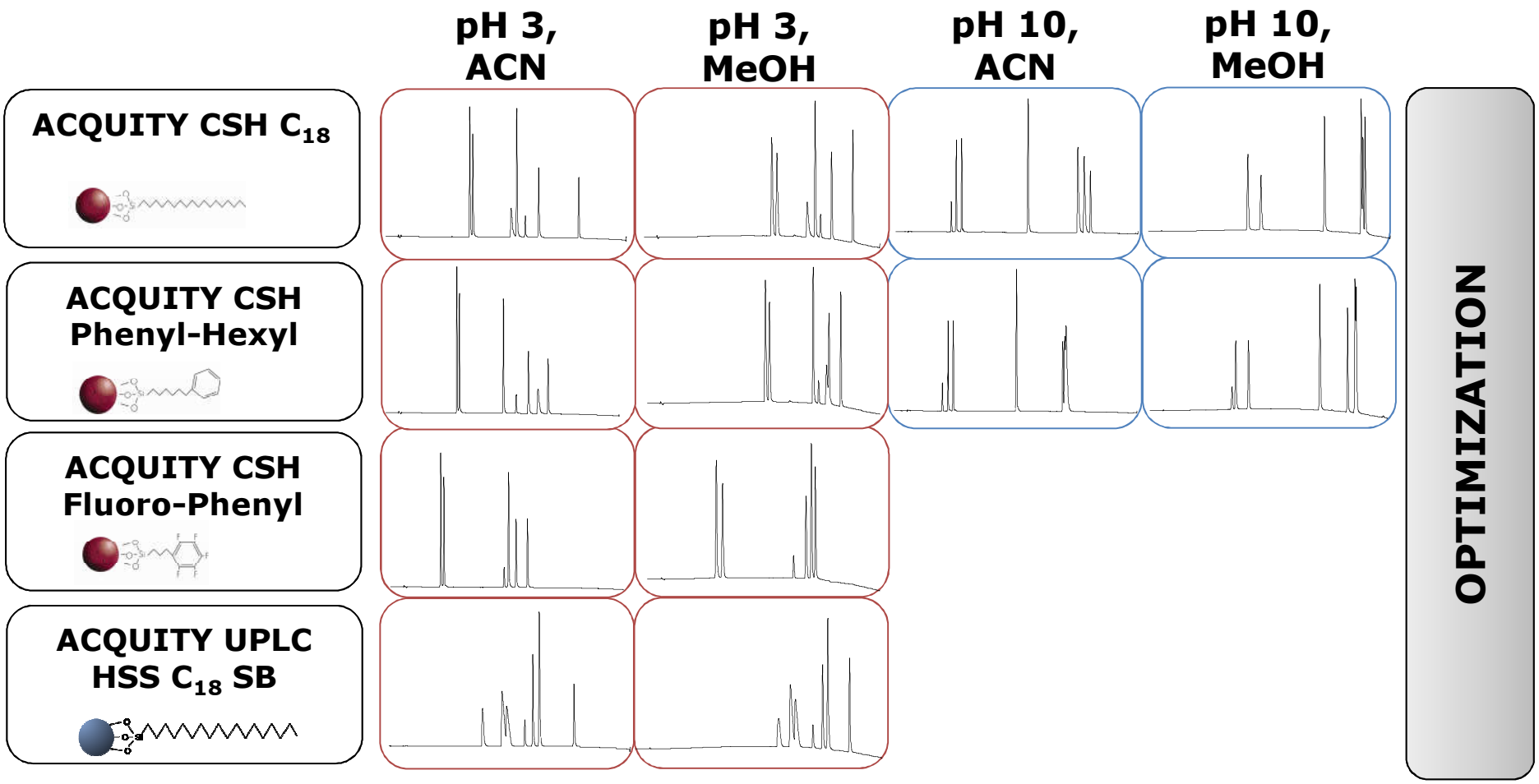
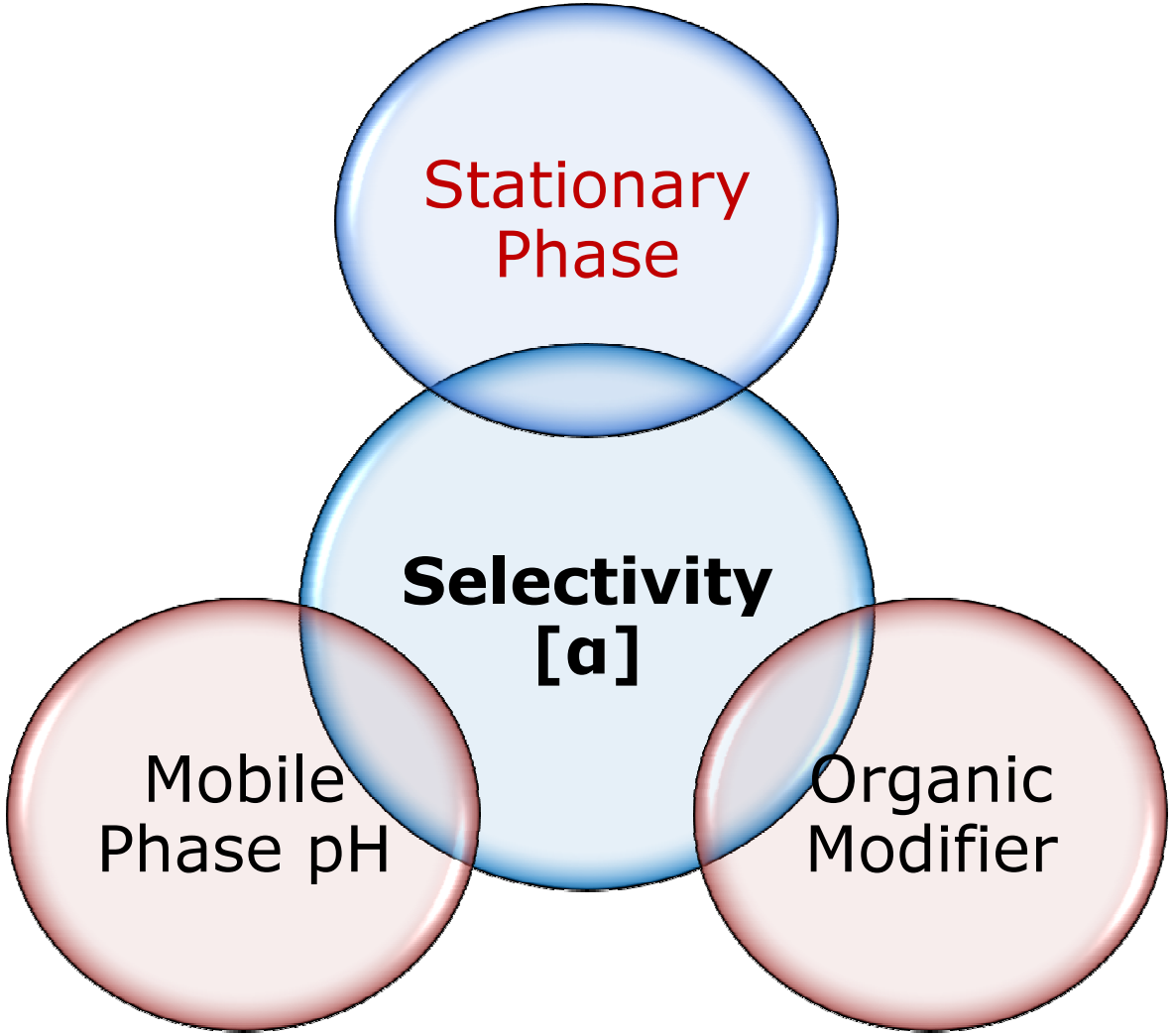


Chemical Factors that Impact Selectivity



Demonstrating Selectivity:
Chemical Structures

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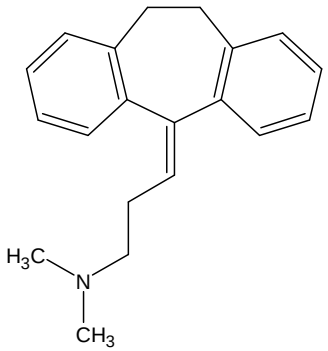
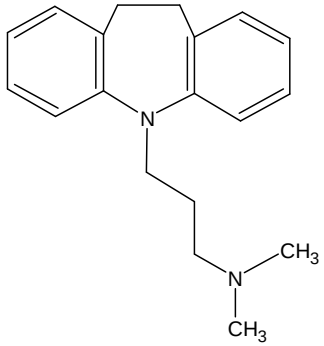
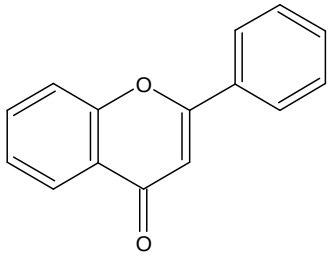


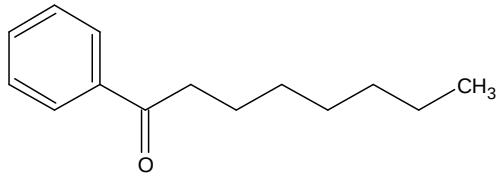
Amitriptyline [B]
m.w. 277.40



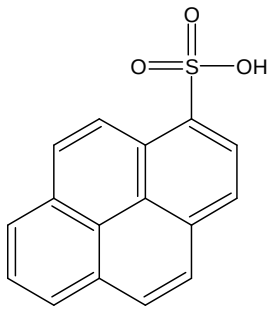
Imipramine [B]
m.w. 280.40



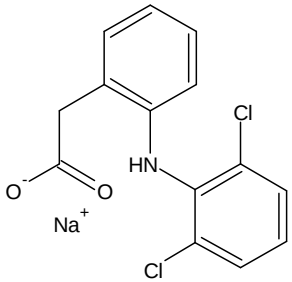
Flavone [N]
m.w. 222.24



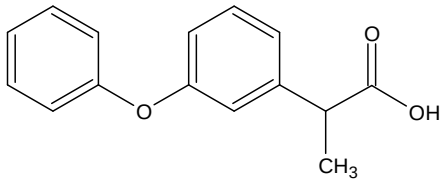
Octanophenone [N]
m.w. 204.31



1-pyrenesulfonic acid [A]
m.w. 304.3



Diclofenac [A]
m.w. 318.13



Fenoprofen [A]
m.w. 242.27

Stationary Phase Selectivity:
Basic and Neutral Compounds

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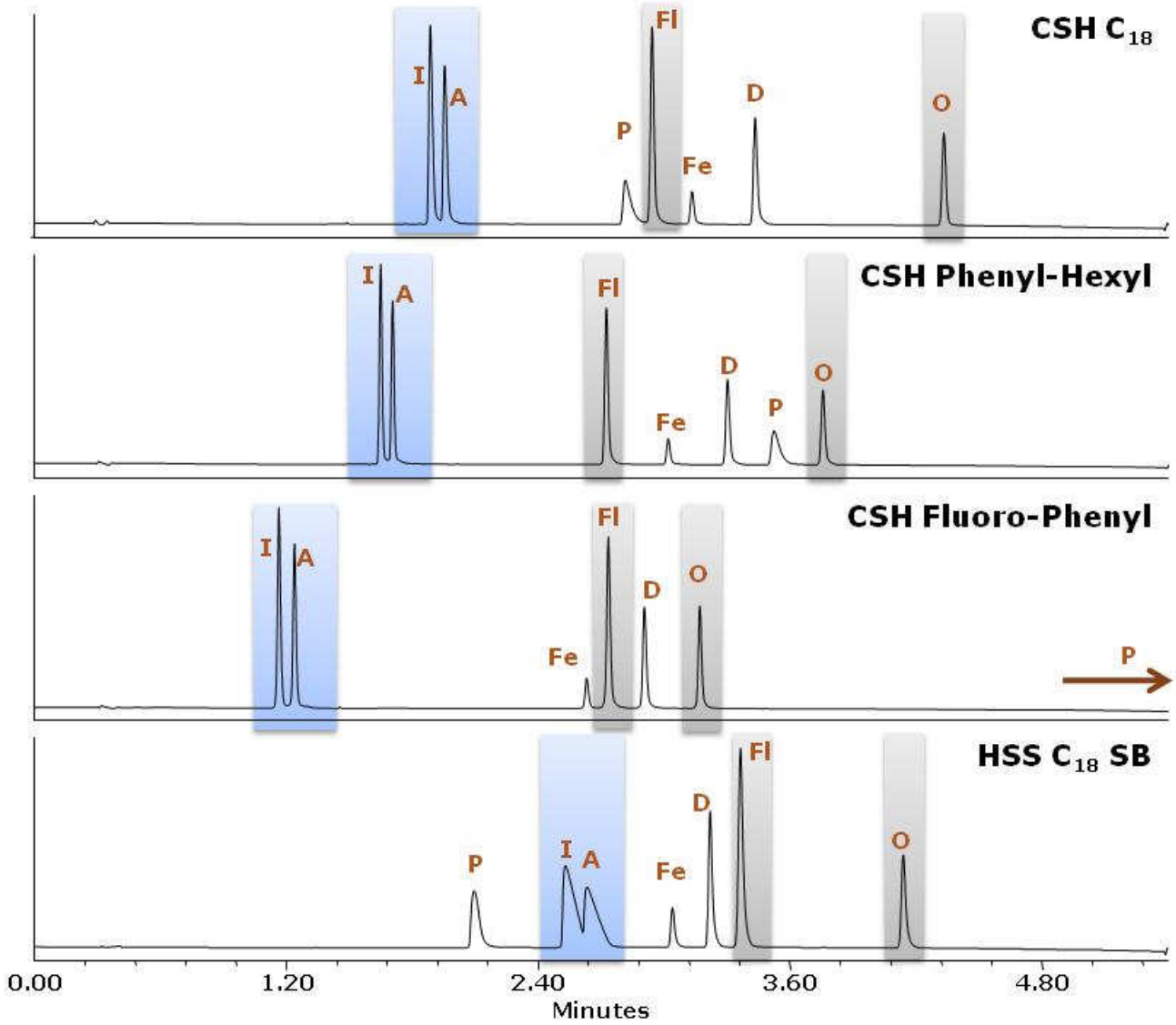

Acetonitrile, pH 3.0

Test Probes:
I: Imipramine [B]
A: Amitriptyline [B]
Fl: Flavone [N]
O: Octanophenone [N]

Observations

Similar selectivity α between the bases

Large differences in stationary phase selectivity for overall mixture

Stationary Phase Selectivity:
Acidic Compounds

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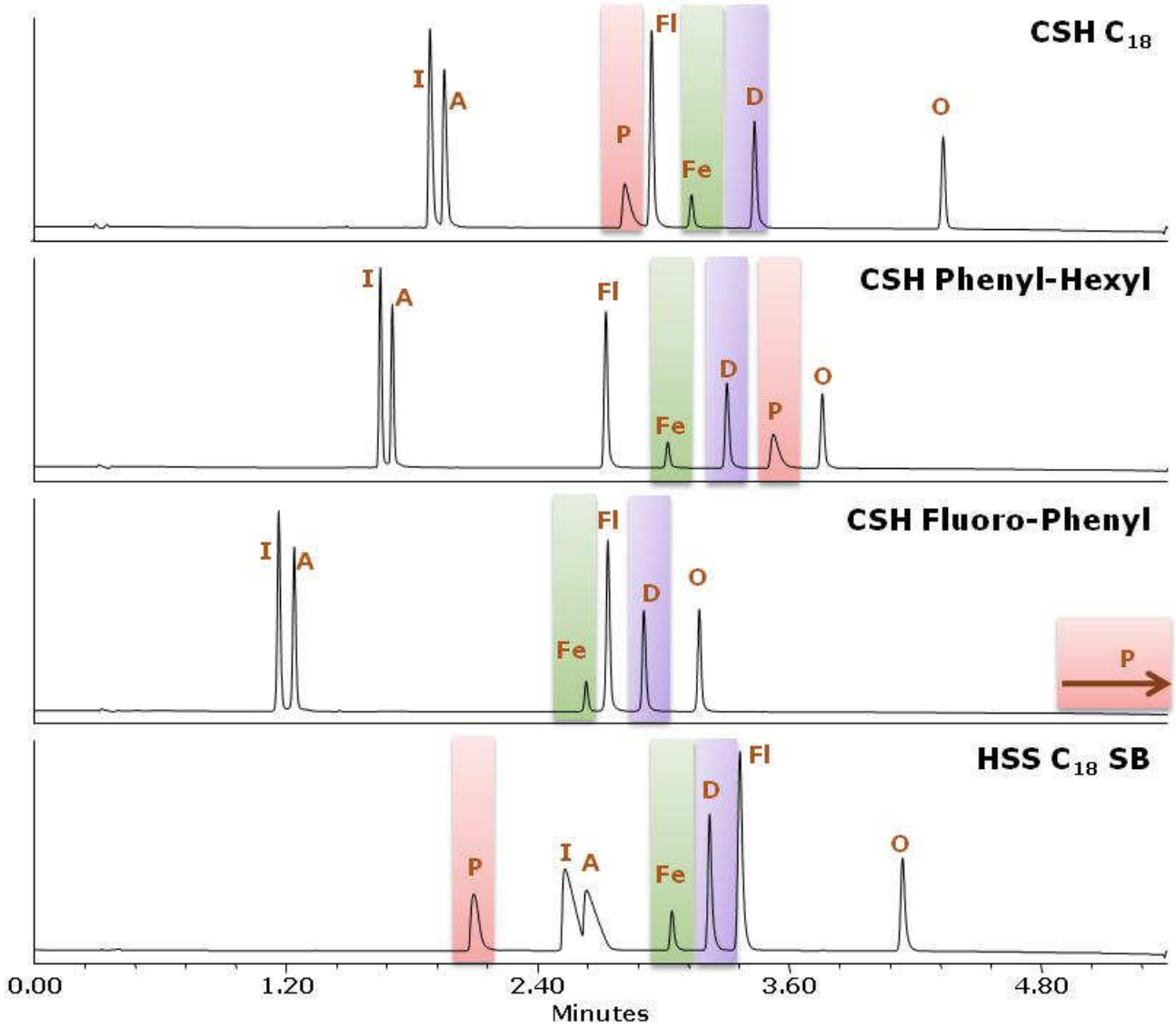


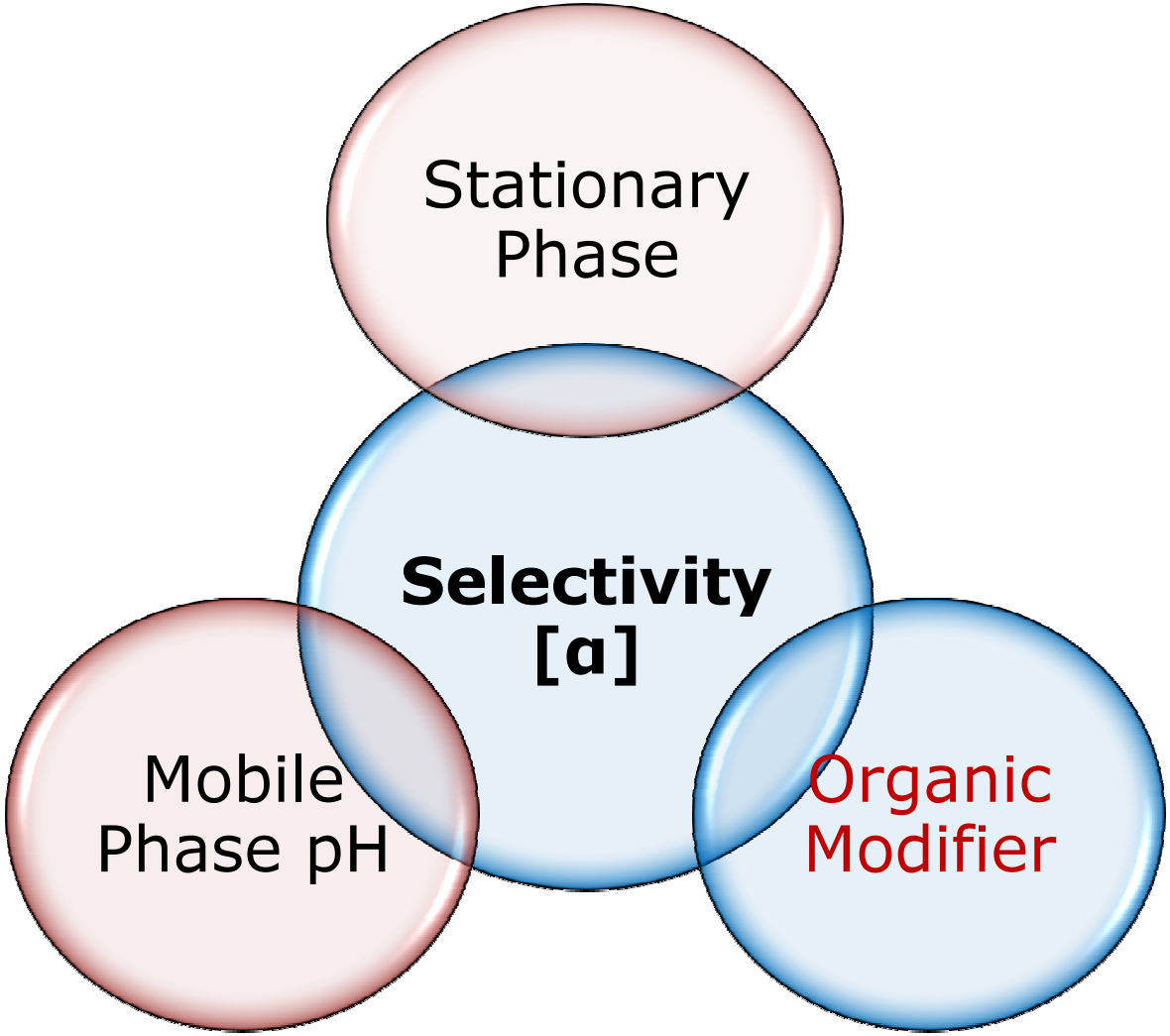
Acetonitrile,
pH 3.0

Test Probes:
P: 1-pyrenesulfonic acid [A]
Fe: fenoprofen [A]
D: diclofenac [A]

Observations

Large differences in stationary phase selectivity for acids

Chemical Factors that Impact Selectivity



Organic Solvent Properties

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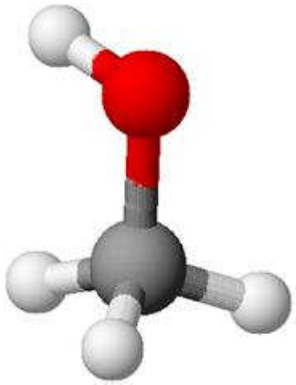
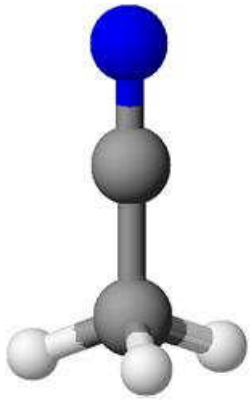
- Methanol
  - Protic solvent [hydrogen bond donor]
  - Weak elution solvent [compared to acetonitrile]
  - Higher viscosity than acetonitrile



- Acetonitrile
  - Aprotic solvent [hydrogen bond acceptor]
  - Strong elution solvent [compared to methanol]
  - Low viscosity



Solvent Selectivity:
Basic and Neutral Compounds

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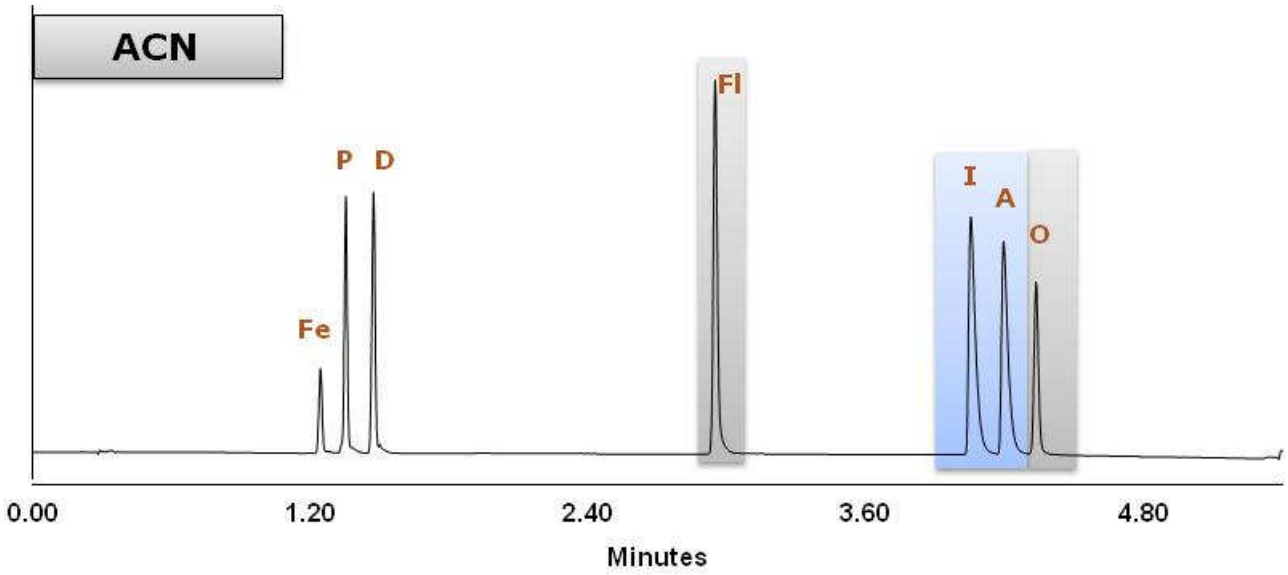

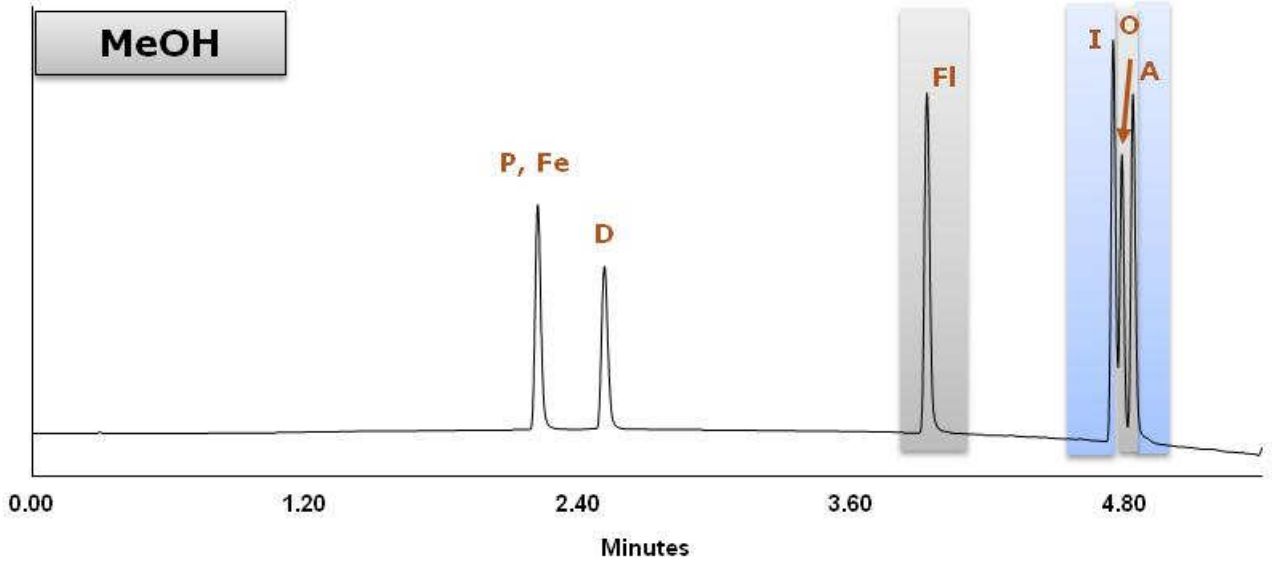

ACQUITY CSH C_{18}, pH 10

Test Probes:
I: Imipramine [B]
A: Amitriptyline [B]
Fl: Flavone [N]
O: Octanophenone [N]

Observations

Methanol is a weaker elution solvent than acetonitrile resulting in greater retention for all analytes

Selectivity difference between bases relative to neutral test probes

Solvent Selectivity:
Acidic Compounds

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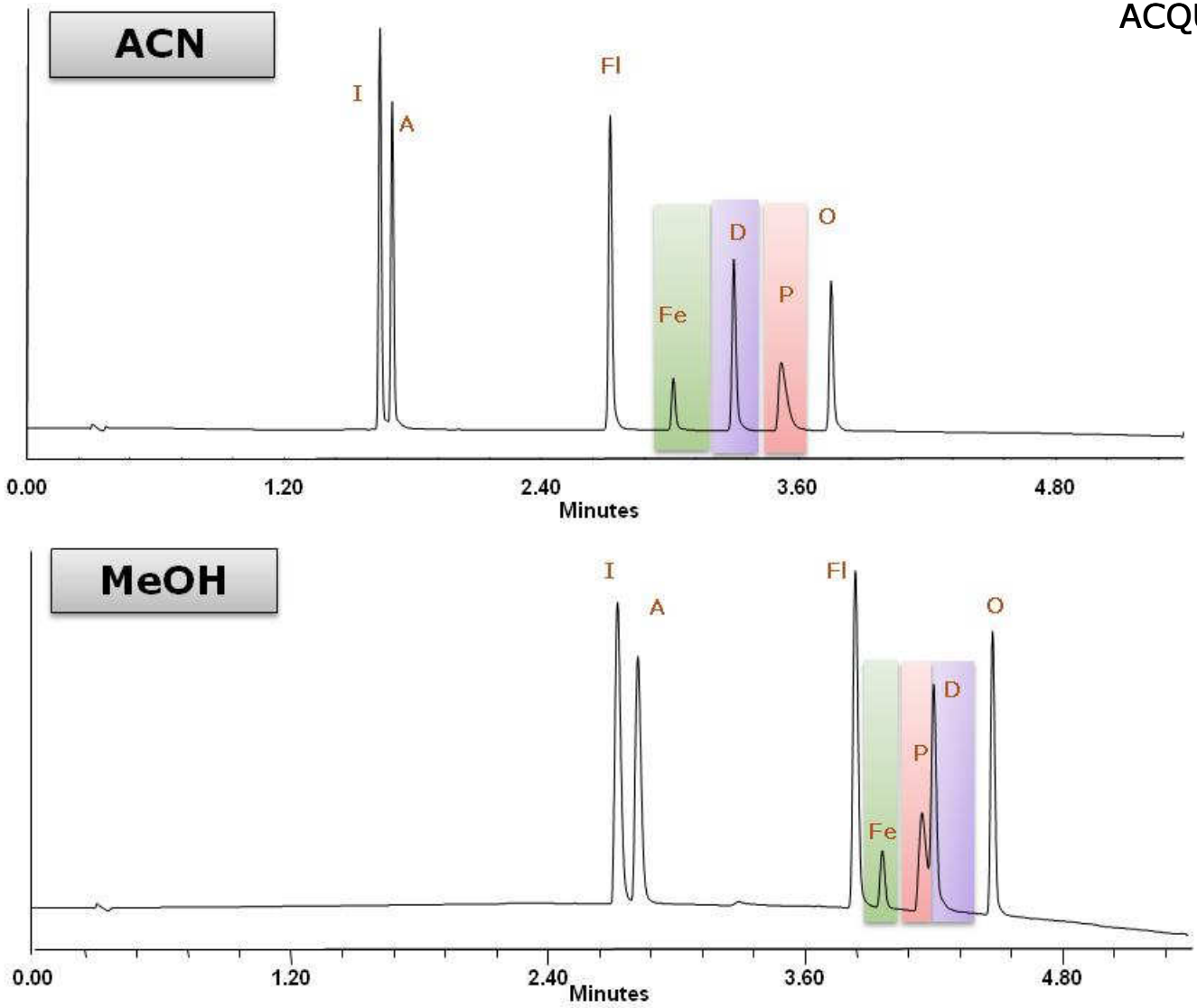
ACQUITY CSH Phenyl-Hexyl, pH 3



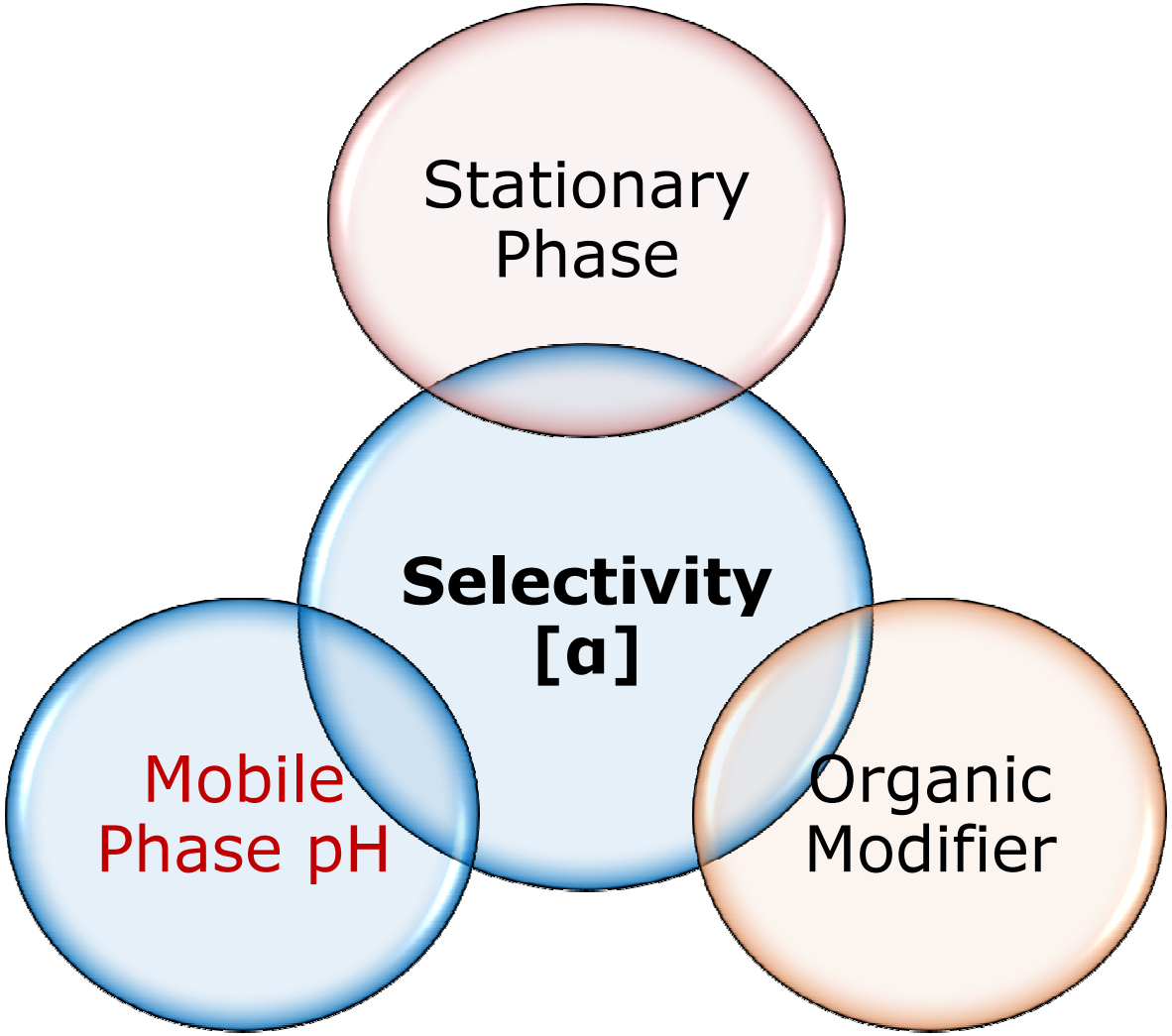
Test Probes:
P: 1-pyrenesulfonic acid [A]
Fe: fenoprofen [A]
D: diclofenac [A]

Observations

Methanol is a weaker elution solvent than acetonitrile resulting in greater retention for all analytes

Elution order change for acidic test probes

Chemical Factors that Impact Selectivity



Impact of Mobile Phase pH on Retention and Selectivity

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- Impacts analytes with ionizable functional groups
  - Amines
  - Carboxylic acids
  - Phenols
- Some compounds contain more than one ionizable group
- Strong selectivity changes can be observed with changes in mobile phase pH

Reversed-Phase Retention Map:
The Impact of pH on Ionizable Compounds

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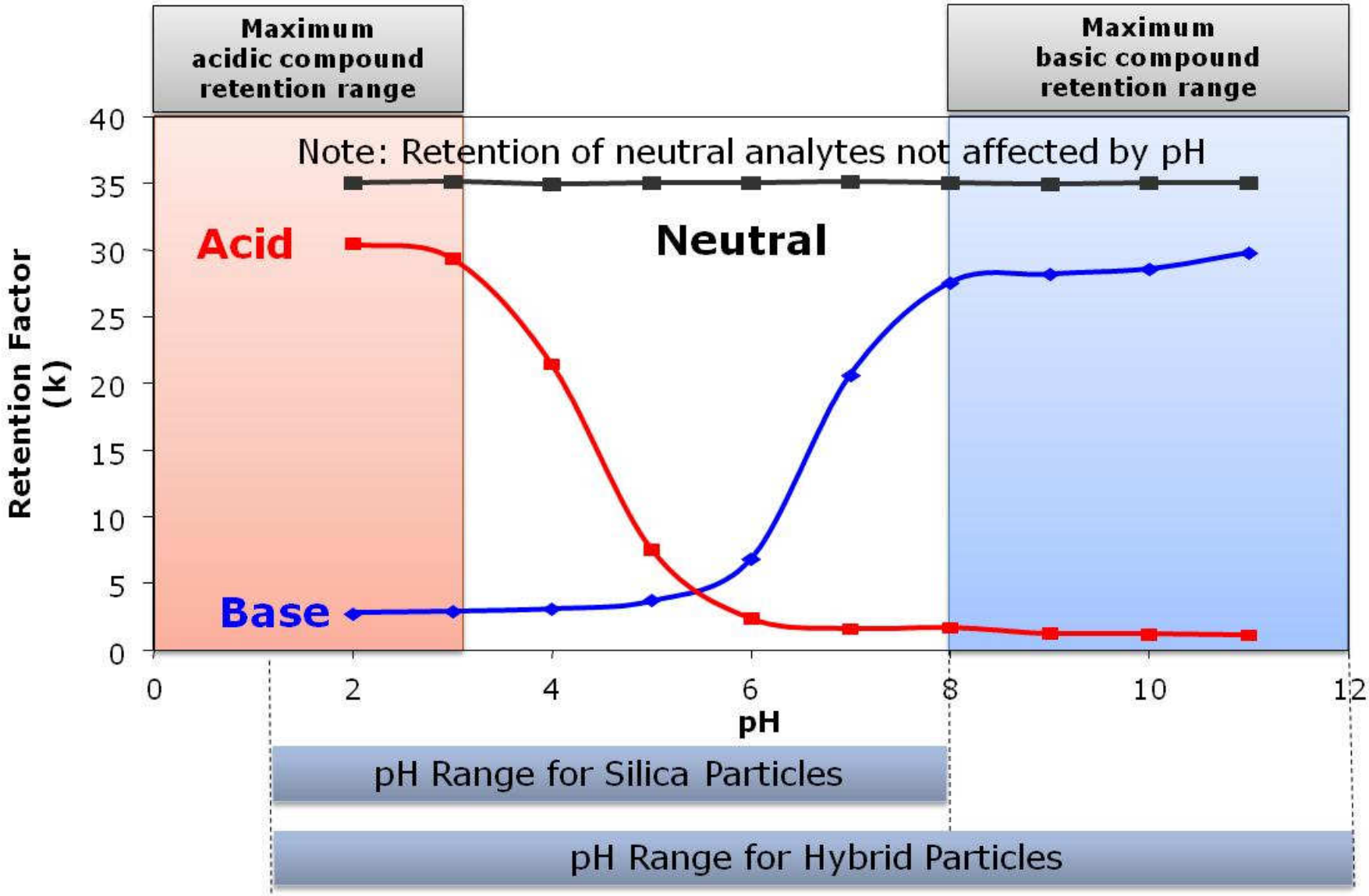

Mobile Phase pH Selectivity:
Basic and Neutral Compounds

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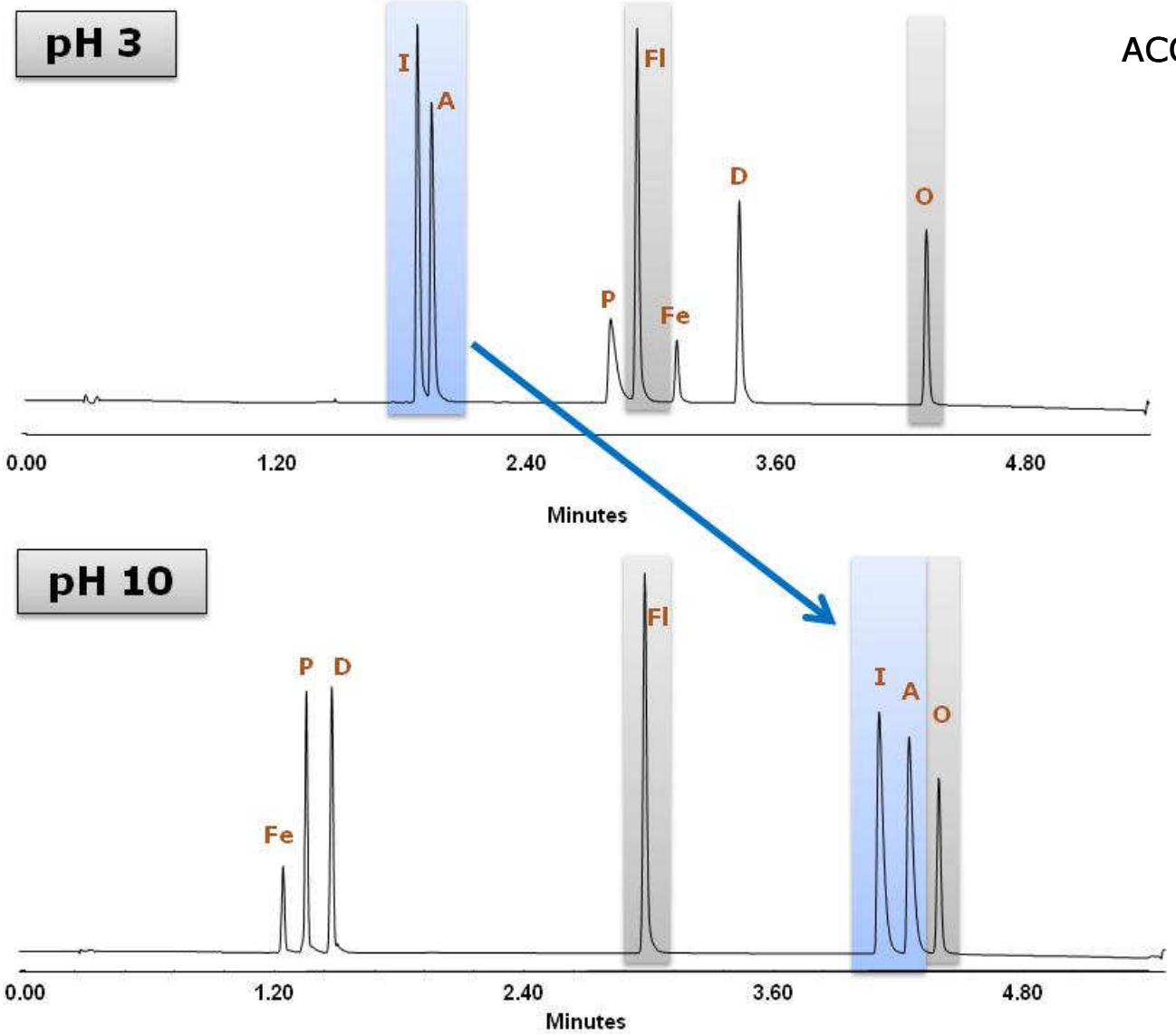

ACQUITY CSH C_{18}, acetonitrile

Test Probes:
I: Imipramine [B]
A: Amitriptyline [B]
Fl: Flavone [N]
O: Octanophenone [N]

Observations

At high pH, bases are in their neutral [unionized] form, resulting in greater retention

Neutral compounds are unaffected by pH

Mobile Phase pH Selectivity:
Acidic and Neutral Compounds

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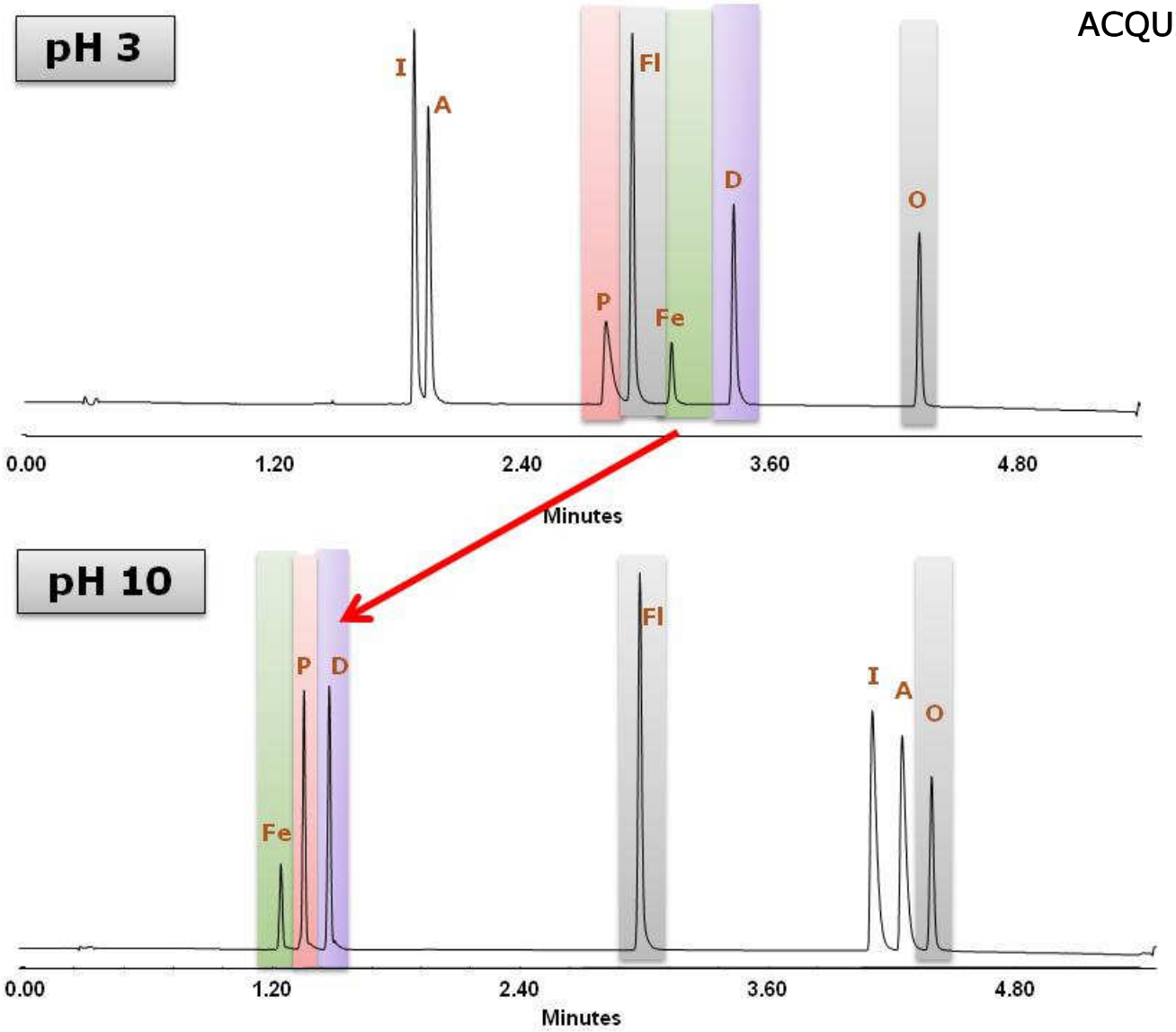

ACQUITY CSH C_{18}, Acetonitrile

Test Probes:
- P: 1-pyrenesulfonic acid [A]
- Fe: fenoprofen [A]
- D: diclofenac [A]
- Fl: Flavone [N]
- O: Octanophenone [N]

Observations

At low pH, acids are in their neutral [unionized] form, resulting in greater retention

Elution order change for acidic compounds

Neutral compounds are unaffected by pH

Implementing Mobile Phase pH Switching:
Monitoring Column Performance

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- Our systematic screening protocol evaluates high and low pH mobile phases.
  - Screen multiple columns and organic modifiers at pH 3 and pH 10
  - Stationary phase must be re-equilibrated when exposed to a new set of conditions

- With low ionic strength mobile phases [i.e., formic acid, ammonium hydroxide], column performance [retention and selectivity] can change *[1]
  - Slow surface equilibration at low pH
  - Inconsistent selectivity can impact open access systems and method transfer

*1 Marchand, D.H., et al., J. Chromatogr. A **2003**, 1015, 53-64

Implementing Mobile Phase pH Switching: Monitoring Column Performance

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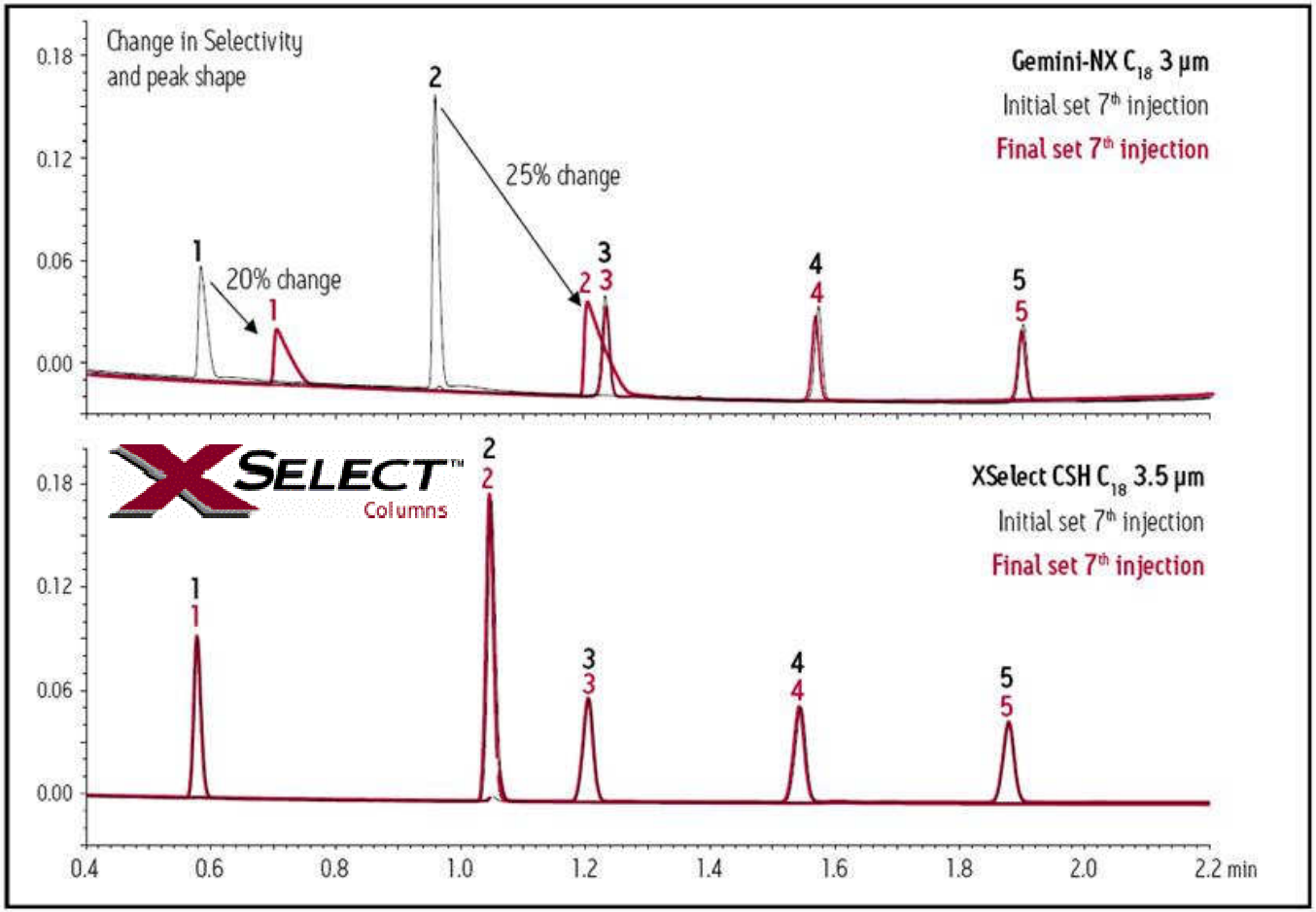


Separations on Gemini-NX C18 (top) and XSelect CSH C18 (bottom) columns (both 2,1 x 50 mm) before and after exposure to a pH 10 mobile phase. Gradient: A: 0.1% formic acid in water; B: acetonitrile; 5 to 95% B linear in 2.5 minutes. Temperature: 30 °C. Injection volume: 2 µL. Detection: 260 nm. Flow rate: 0.8 ml/min. Analytes: (1) metoprolol; (2) amitriptyline; (3) dimethylphthalate; (4) diethylphthalate; (5) dipropylphthalate. System: ACQUITY UPLC.

Observations

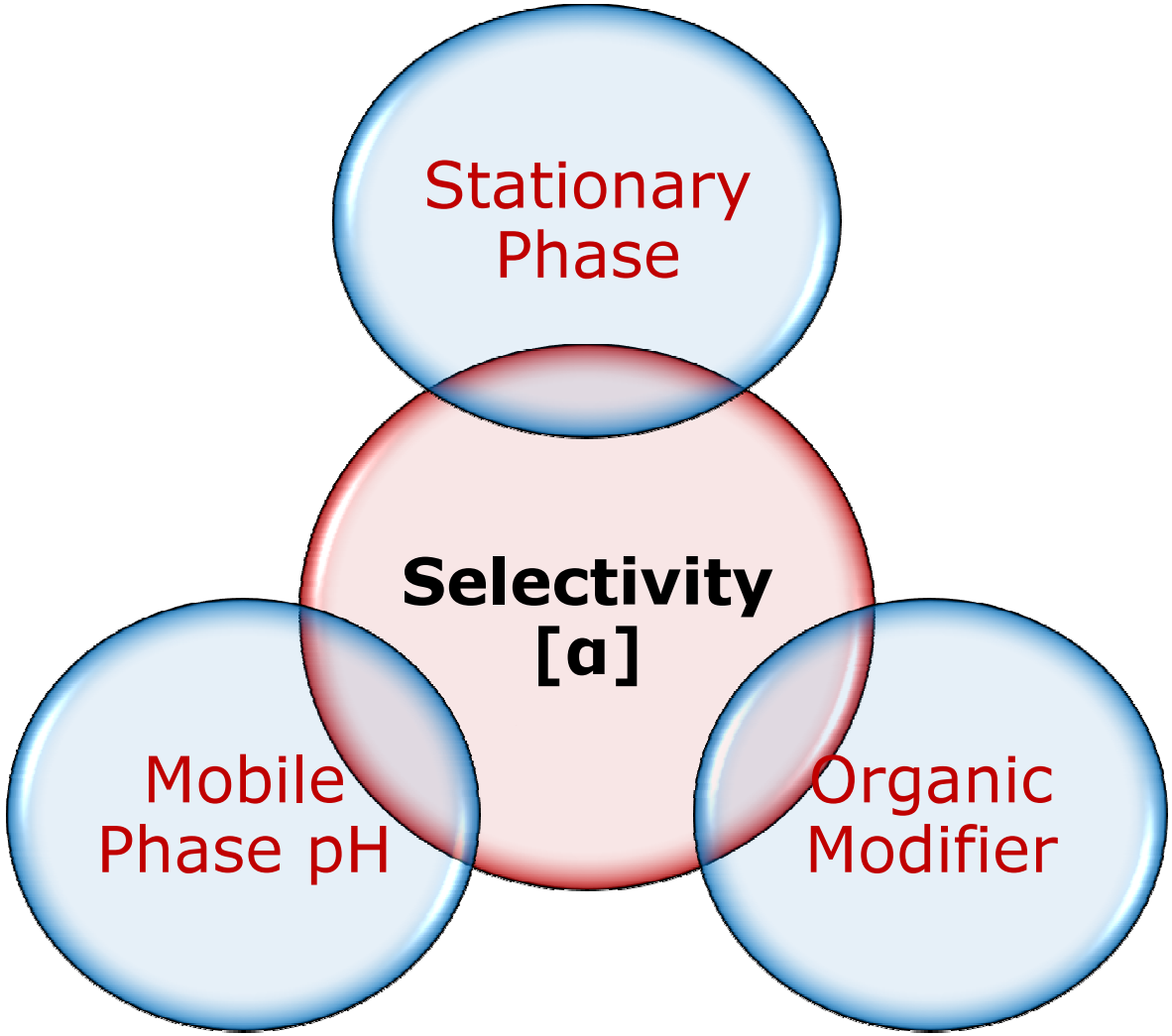
Gemini-NX C_{18} shows a 20 – 25 % change in retention at low pH, after exposure to high pH mobile phases.

No significant retention or selectivity shift was observed on the XSelect column.

CSH™ Technology

Gemini is a trademark of Phenomenex, Inc.

Chemical Factors that Impact Selectivity



Maximizing Selectivity Differences:
Combining Stationary Phase, Organic Modifier and Mobile Phase pH

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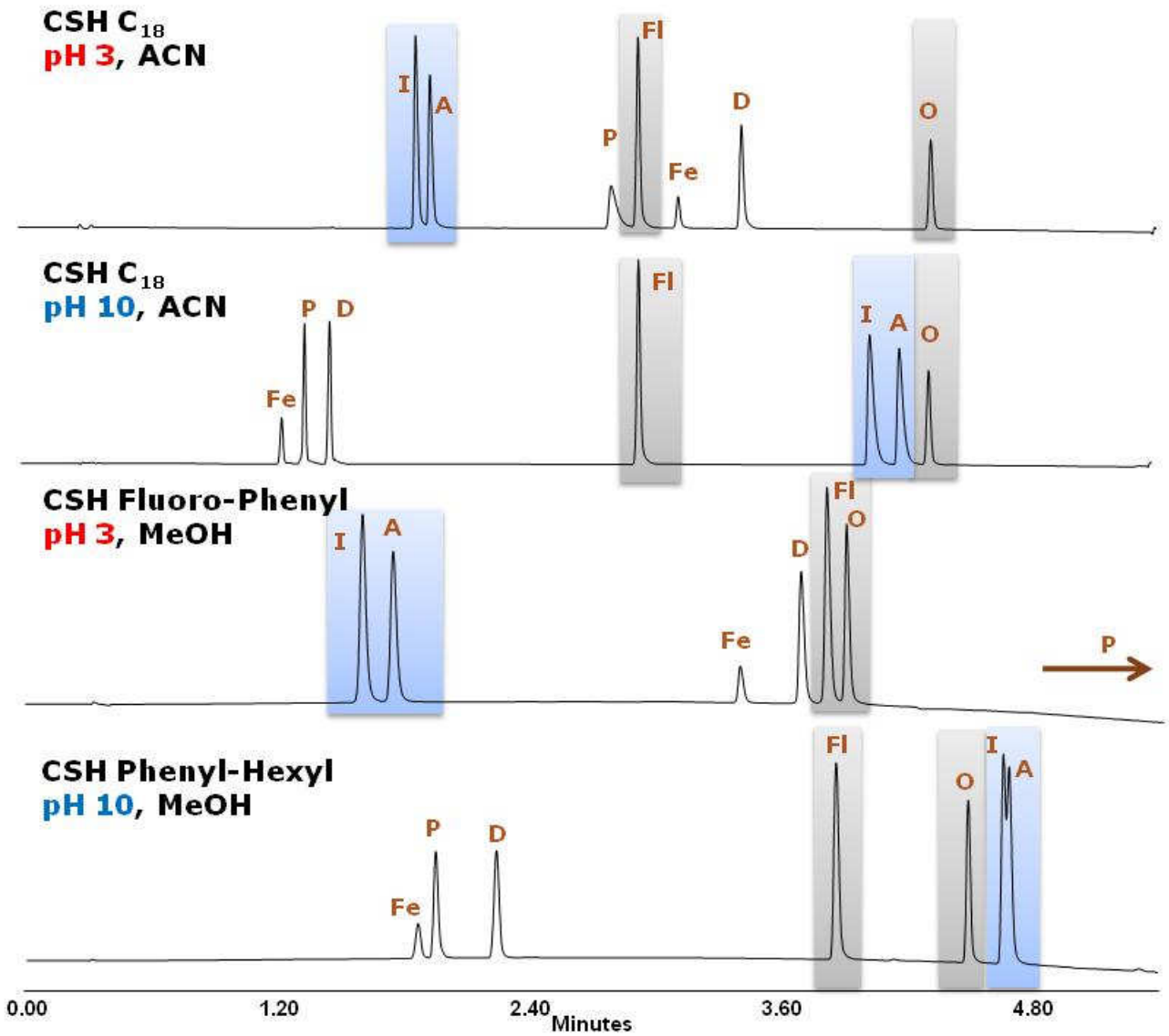

BASIC AND NEUTRAL TEST PROBES

Test Probes:
- I: Imipramine [B]
- A: Amitriptyline [B]
- Fl: Flavone [N]
- O: Octanophenone [N]

Observations

Large differences in selectivity are observed when evaluating combinations of stationary phase, organic modifier and mobile phase pH

Maximizing Selectivity Differences:
Combining Stationary Phase, Organic Modifier and Mobile Phase pH

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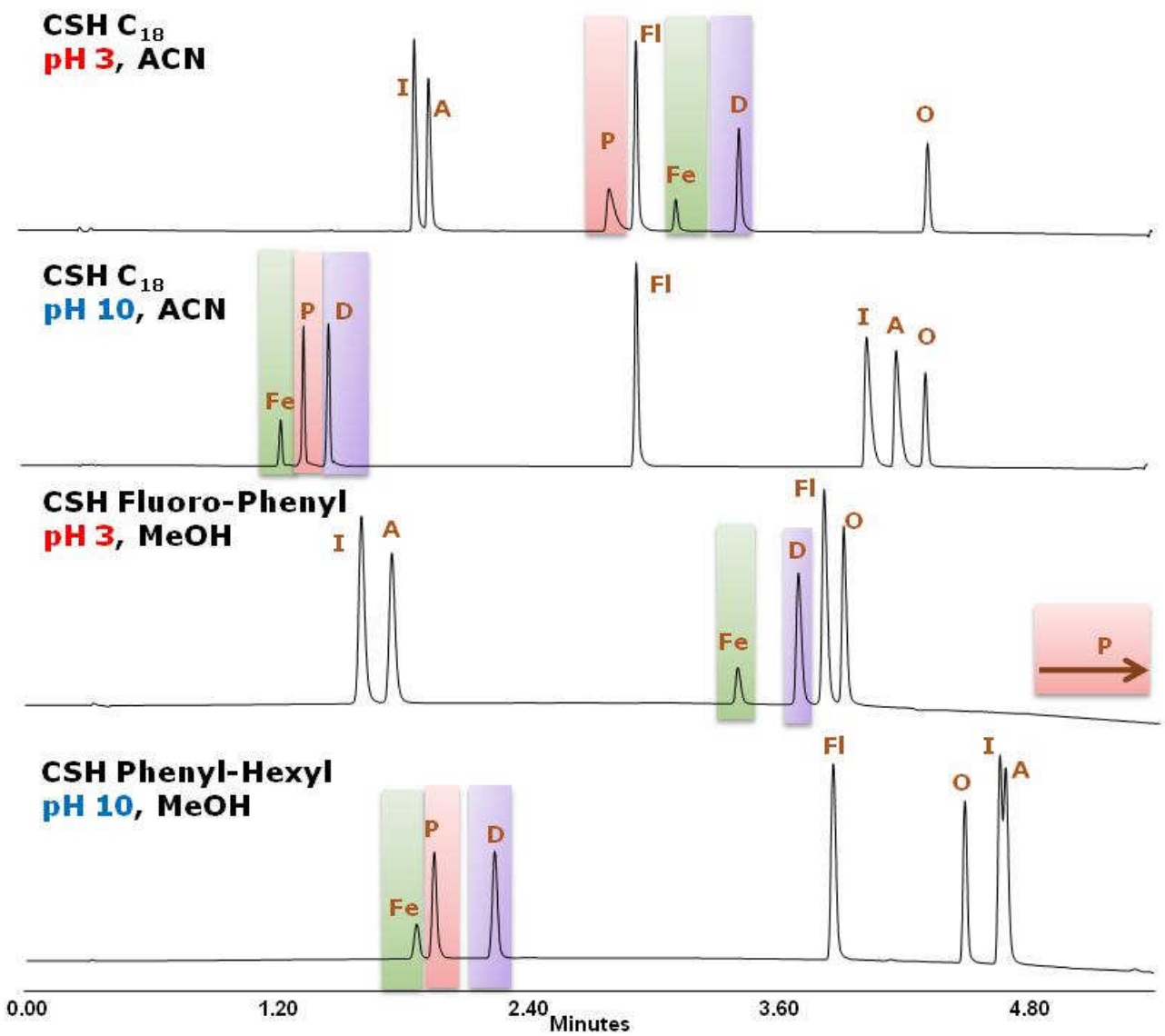

ACIDIC TEST PROBES

Test Probes:
P: 1-pyrenesulfonic acid [A]
Fe: fenoprofen [A]
D: diclofenac [A]

Observations

Large differences in selectivity are observed when evaluating combinations of stationary phase, organic modifier and mobile phase pH

Selectivity Observations

- Analytes in their un-ionized [neutral] form yield greater retention
- Methanol is a weaker elution solvent than acetonitrile, and therefore exhibits increased retention of all components, as well as selectivity differences, compared to acetonitrile
- Large differences in selectivity are observed when a change in mobile phase pH alters the charge state of the analyte
- Large selectivity differences are observed between the stationary phases at any given condition
- The most significant selectivity differences occur when comparing combinations of stationary phase, organic modifier and mobile phase pH

Selectivity Summary

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- Manipulation of parameters for method development [as described previously] is applicable to both HPLC and UPLC separations
  - Column selectivity [ACQUITY CSH or XSelect CSH HPLC columns]
  - Acetonitrile and methanol
  - pH 3 and pH 10 mobile phases
- Hybrid particle technology enables the exploration of pH extremes in method development
  - Stability from pH 1 – 11
- CSH Technology columns facilitate:
  - Selectivity differences independent of the mobile phase conditions employed
  - The use of low ionic strength mobile phases with high sample capacity
  - Reliable performance when switching between mobile phase pH's
- Evaluation of data from the complete systematic screening protocol is essential to fully understand the analytes chromatographic behavior

Why UPLC Technology for method development?

Develop Methods Faster with UPLC Technology: Maintaining Separation Power

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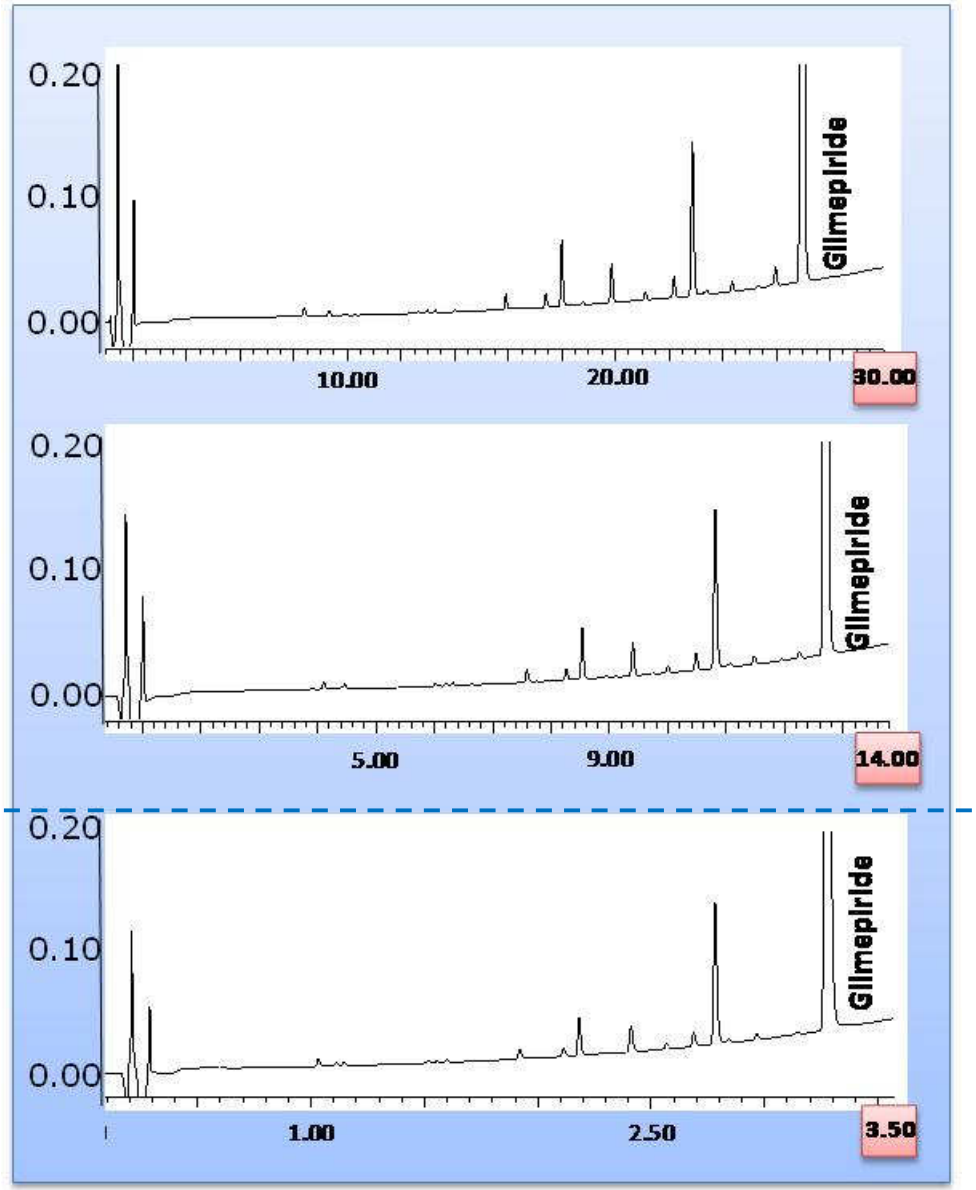
HSS
HIGH STRENGTH SILICA

HPLC
3,5 and 5 µm

1,8 µm
UPLC

Acquity
UPLC®



HPLC

5 µm – 150 mm
L/d_p = 30 000

3,5 µm – 100 mm
L/d_p = 28 571

UPLC

1,8 µm – 50 mm
L/dp = 27 778

Develop Methods Faster with UPLC Technology:
Time Savings

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UPLC Gradient Conditions:

Column: 2,1 x 50 mm, 1,7/1,8 µm
Flow Rate: 0,5 ml/min
Gradient:

Time [min]	Profile %A	%B	%C
0.0	90	5	5
5.0	5	90	5
5.1	90	5	5
5.5	90	5	5

Peak Capacity [P_c] = 150

HPLC Gradient Conditions:

Column: 4,6 x 100 mm, 3,5 µm
Flow Rate: 1,17 ml/min
Gradient:

Time [min]	Profile %A	%B	%C
0.0	90	5	5
20.6	5	90	5
21.0	90	5	5
22.6	90	5	5

Peak Capacity [P_c] = 150

$$P_c = 1 + \frac{t_g}{w}$$

UPLC can achieve the same separation 4X faster than 3.5 µm HPLC

Develop Methods Faster with UPLC Technology: Time Savings

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UPLC Method Development Protocol:
2.1 x 50 mm, 1.7/1.8 µm, 0.5 mL/min

pH 3/ acetonitrile	Time
Flow ramp	3 min
Column conditioning [2 blank gradients]	11 min
Sample injection [2 replicates]	11 min
pH 3/ methanol	**Time**
Flow ramp	3 min
Column conditioning [2 blank gradients]	11 min
Sample injection [2 replicates]	11 min
Column purge	10 min
pH 10/ acetonitrile	**Time**
Flow ramp	3 min
Column conditioning [2 blank gradients]	11 min
Sample injection [2 replicates]	11 min
pH 10/ methanol	**Time**
Flow ramp	3 min
Column conditioning [2 blank gradients]	11 min
Sample injection [2 replicates]	11 min
Column purge	10 min
SCREENING TIME	120 min

2 hours/ column [low/high pH switching]
x 2 column
1 hour/ column [low pH only]
x 2 column

TOTAL SCREENING TIME: 6 HOURS

HPLC Method Development Protocol:
4.6 x 100 mm, 3.5 µm, 1.17 mL/min

pH 3/ acetonitrile	Time
Flow ramp	3 min
Column conditioning [2 blank gradients]	45.6 min
Sample injection [2 replicates]	45.6 min
pH 3/ methanol	**Time**
Flow ramp	3 min
Column conditioning [2 blank gradients]	45.6 min
Sample injection [2 replicates]	45.6 min
Column purge	41min
pH 10/ acetonitrile	**Time**
Flow ramp	3 min
Column conditioning [2 blank gradients]	45.6 min
Sample injection [2 replicates]	45.6 min
pH 10/ methanol	**Time**
Flow ramp	3 min
Column conditioning [2 blank gradients]	45.6 min
Sample injection [2 replicates]	45.6 min
Column purge	41 min
SCREENING TIME	459 min

7.65 hours/ column [low/high pH switching]
x 2 column
3.82 hour/ column [low pH only]
x 2 column

TOTAL SCREENING TIME: 23 HOURS

Develop methods 4X faster with UPLC

Outline

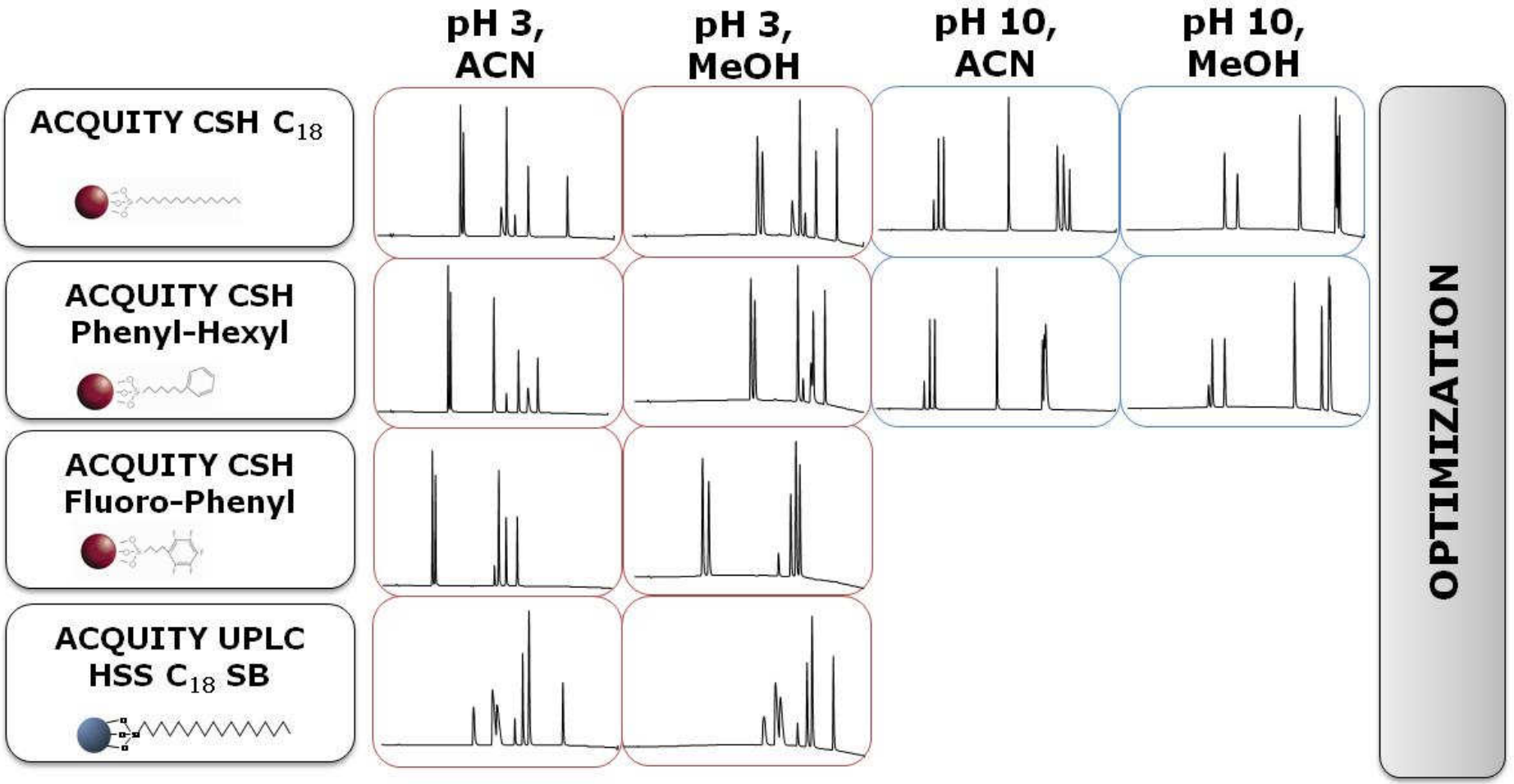
- **Introduction**
  - Approaches Toward Method Development
  - UPLC Technology
  - Success Criteria
- **Controlling Selectivity and Retention**
  - Stationary Phase and Particle Substrate Design
  - Organic Modifier
  - Mobile Phase pH
- **Method Development Strategy**
  - Systematic Screening Protocol
  - Quality by Design [Q_bD] Approach
- **Implementing the Approach**
  - Case Study
- **Conclusion**

Automated Method Development

Systematic Screening Protocol

- ACQUITY UPLC H-Class Quaternary Solvent manager [QSM] with solvent select valve
  - Mix up to 4 solvents
  - Optional solvent select valve enables an additional 5 solvent lines
- ACQUITY UPLC H-Class Column Manager
  - Flexible modules to select between 2 and 6 columns
  - Utilize 2,1 x 50 mm, 1,7/1,8 µm columns
- Fast, 5 minute gradient from 5 – 90 % organic at 0,5 ml/min

Systematic Screening Protocol



Method Development:
Quality by Design [Q_bD] Approach

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- Systematic Screening Protocol
  - Good first pass, rapid method development
  - Choice of best combination of parameters [i.e., stationary phase, organic modifier, mobile phase pH] is subjective
  - Optimum separation conditions may be outside of screening approach [i.e., pH 5 with a mixture of acetonitrile and methanol]

- Quality by Design with Design of Experiments [DOE] Approach
  - Start with systematic screening protocol
  - Define separation objectives
  - Gain knowledge about the product or process
  - Create sufficient scientific understanding to establish a design space, specifications and controls
  - Defines robust operating space

Method Development:
Quality by Design [Q_bD] Approach

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Design of Experiments [DOE] Approach

- Fusion AE Method Development Software
  - Aligned with FDA and EMA Q_bD initiatives
  - Applies DOE approach to method development using simple templates
  - Facilitates data interpretation
  - Incorporates robustness modeling into the chromatographic development process
  - Automates sample and method set creation in Empower

Empower 2 software

Fusion AE™

[1] Define Experimental Region

Select study variables | Define study ranges

[2] Develop Knowledge Space

Conduct experimental design | Analyze results and study variable effects

[3] Establish Design Space

Define optimum conditions | Define robust operating space

Outline

- **Introduction**
  - Approaches Toward Method Development
  - UPLC Technology
  - Success Criteria
- **Controlling Selectivity and Retention**
  - Stationary Phase and Particle Substrate Design
  - Organic Modifier
  - Mobile Phase pH
- **Method Development Strategy**
  - Systematic Screening Protocol
  - Quality by Design [Q_bD] Approach
- **Implementing the Approach**
  - Case Study
- **Conclusion**

Implementing the Approach:
Mirtazapine and Impurities

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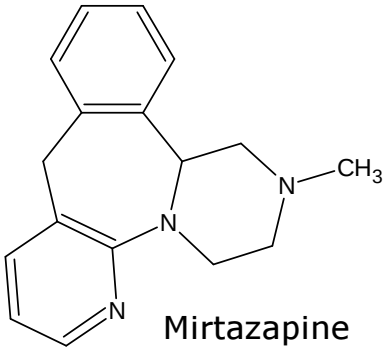
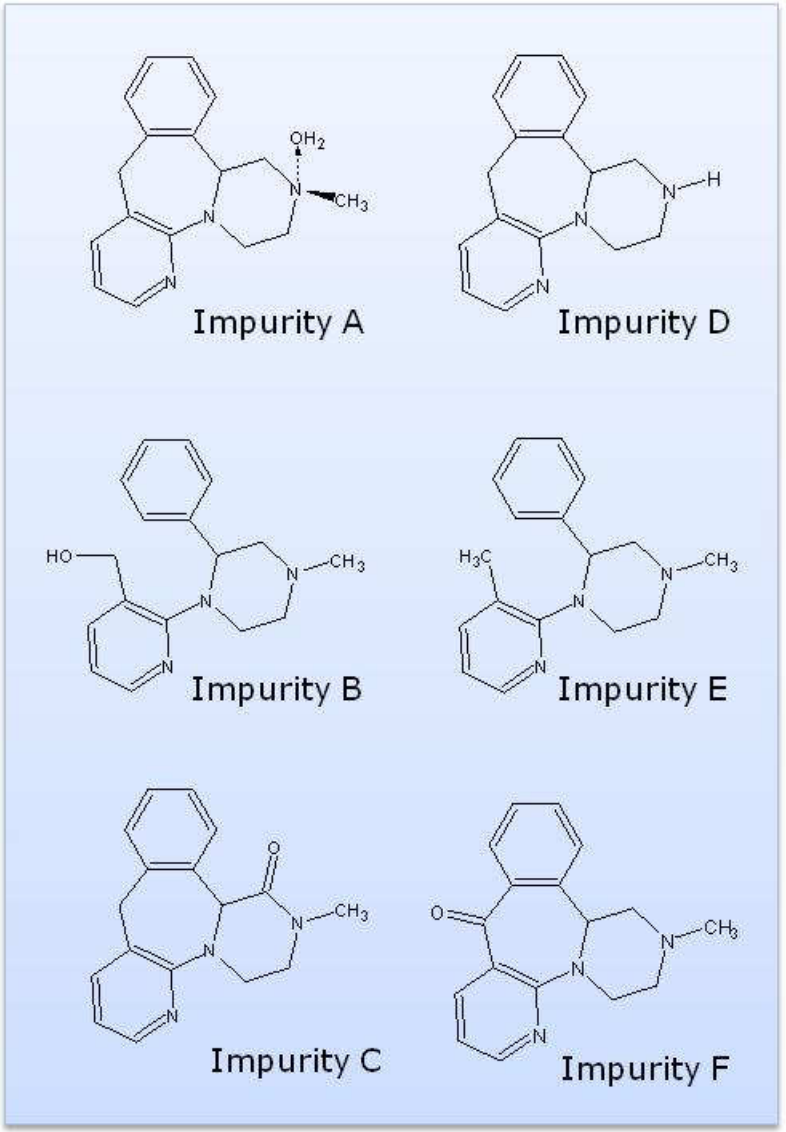


Mirtazapine

Mirtazapine [m.w. 265.35]

Used primarily for the treatment of clinical depression

USP mirtazapine resolution mix RS 1.0 mg/ml in 50:50 ACN:H_2O



Method development and optimization

- Empower 2 CDS with Fusion AE method development software
  - Uses statistically significant combination of different parameters [*software will not run every combination of every parameter*]
    - 4 column chemistries
    - 2 organic modifiers
    - 2 mobile phase pH's
    - Gradient times: 2, 3.1, 4.3, 5.4, 6.5 min

Fusion AE Experimental Design

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Replicates built into design:

1.a.1.a, 12.a.1.a; 2.a.1.a, 13.a.1.a
3.a.1.a, 14.a.1.a; 4.a.1.a, 15.a.1.a
18.a.1.a, 19.a.1.a; 22.a.1.a, 30a.1.a
23.a.1.a, 28.a.1.a; 24.a.1.a, 32.a.1.a
26.a.1.a, 33.a.1.a; 34.a.1.a, 37.a.1.a

Run No.	Gradient Time (min)	Organic Solvent Type (*)	pH (*)	Column Type (*)
Wash - 1	0.1	Acetonitrile	2.6	CSH HL C18
Wash - 2	0.1	Acetonitrile	2.6	CSH Phenyl-Hexyl
Wash - 3	0.1	Acetonitrile	2.6	CSH Fluoro-Phenyl
Wash - 4	0.1	Acetonitrile	2.6	HSS C18 SB
1.a.1.a	4.3	Acetonitrile	2.6	CSH HL C18
2.a.1.a	4.3	Ac		
3.a.1.a	4.3	A		
4.a.1.a	4.3	A		
5.a.1.a	5.4	A		
6.a.1.a	6.5	A		
7.a.1.a	2	A		
8.a.1.a	2	A		
9.a.1.a	6.5	A		
10.a.1.a	6.5	A		
11.a.1.a	2	A		
12.a.1.a	4.3	A		
13.a.1.a	4.3	A		
14.a.1.a	4.3	A		
15.a.1.a	4.3	A		
Wash - 5	0.1	Ac		
Wash - 6	0.1	Acet		
16.a.1.a	2	Acetonitrile	10.6	CSH Phenyl-Hexyl
17.a.1.a	4.3	Acetonitrile	10.6	CSH HL C18
18.a.1.a	6.5	Acetonitrile	10.6	CSH Phenyl-Hexyl
19.a.1.a	6.5	Acetonitrile	10.6	CSH Phenyl-Hexyl
Wash - 7	0.1	Methanol	2.6	CSH HL C18
Wash - 8	0.1	Methanol	2.6	CSH Phenyl-Hexyl
Wash - 9	0.1	Methanol	2.6	CSH Fluoro-Phenyl
Wash - 10	0.1	Methanol	2.6	HSS C18 SB

Fusion AE software automatically constructs a set of experiments by selecting the most efficient statistical experimental design

Instrument methods, method sets and sample sets are automatically created in Empower 2 to carry out the experiment

20.a.1.a	3.1	Methanol	2.6	CSH Phenyl-Hexyl
				HSS C18 SB
				CSH Fluoro-Phenyl
				CSH Phenyl-Hexyl
				HSS C18 SB
				CSH Phenyl-Hexyl
				CSH HL C18
				CSH HL C18
				CSH Phenyl-Hexyl
				CSH Fluoro-Phenyl
				CSH Fluoro-Phenyl
				CSH Fluoro-Phenyl
				HSS C18 SB
				CSH HL C18
				CSH HL C18
				CSH Phenyl-Hexyl
				CSH HL C18
35.a.1.a	2	Methanol	10.6	CSH Phenyl-Hexyl
36.a.1.a	2	Methanol	10.6	CSH HL C18
37.a.1.a	6.5	Methanol	10.6	CSH HL C18
38.a.1.a	5.4	Methanol	10.6	CSH Phenyl-Hexyl
Wash - 13	0.1	Methanol	10.6	CSH HL C18
Wash - 14	0.1	Methanol	10.6	CSH Phenyl-Hexyl
Wash - 15	0.1	Methanol	2.6	CSH Fluoro-Phenyl
Wash - 16	0.1	Methanol	2.6	HSS C18 SB

Stationary Phase Selectivity:
Mirtazapine and Impurities

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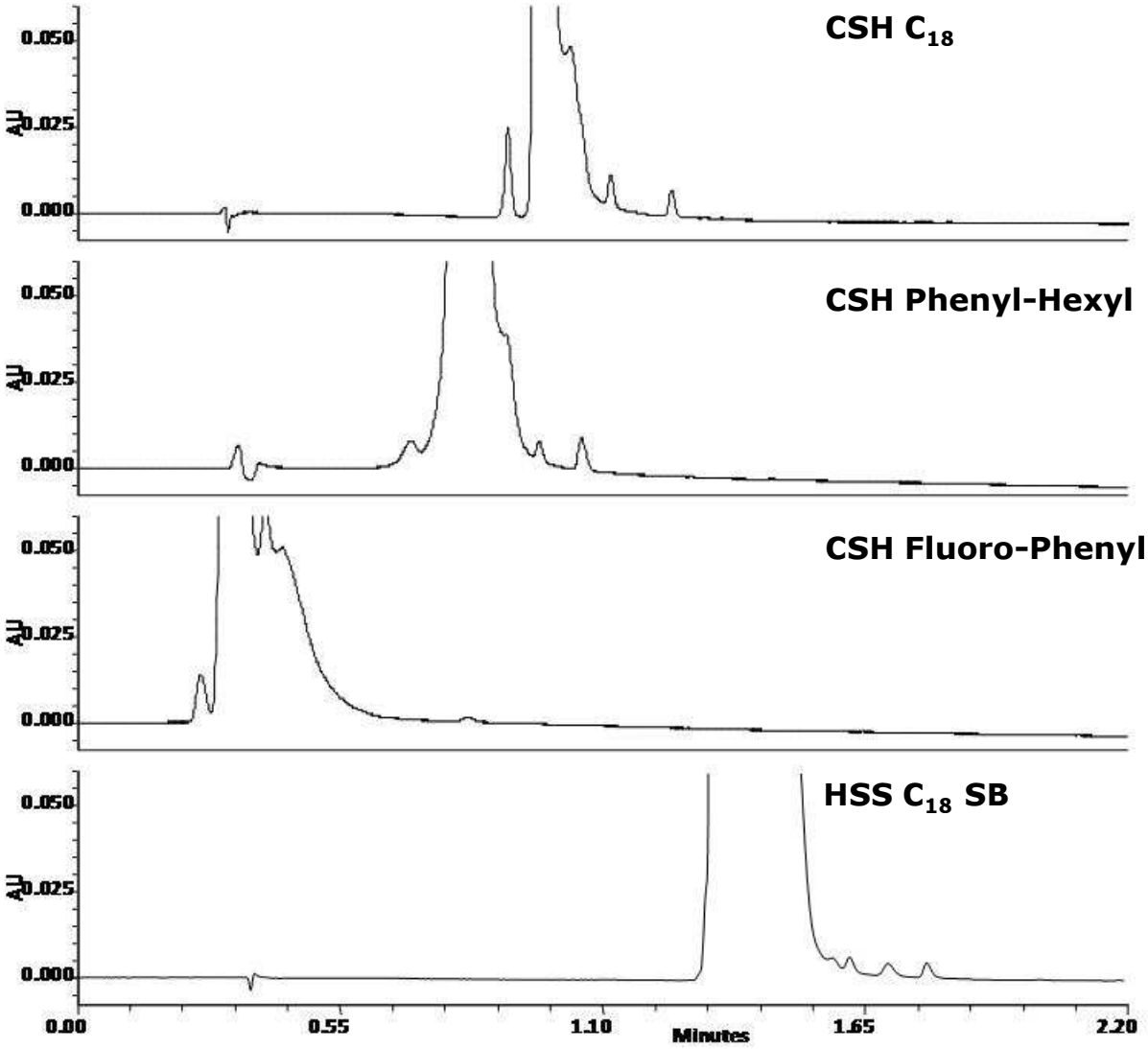

Acetonitrile, Low pH
T_g = 4.3 min 5-95% B

Observations:

Selectivity differences observed between the different columns

Inadequate retention on Fluoro-Phenyl

Poor resolution of mirtazapine and 6 impurities on all columns

Action:

Investigate mobile phase pH

Mobile Phase pH Selectivity:
Mirtazapine and Impurities

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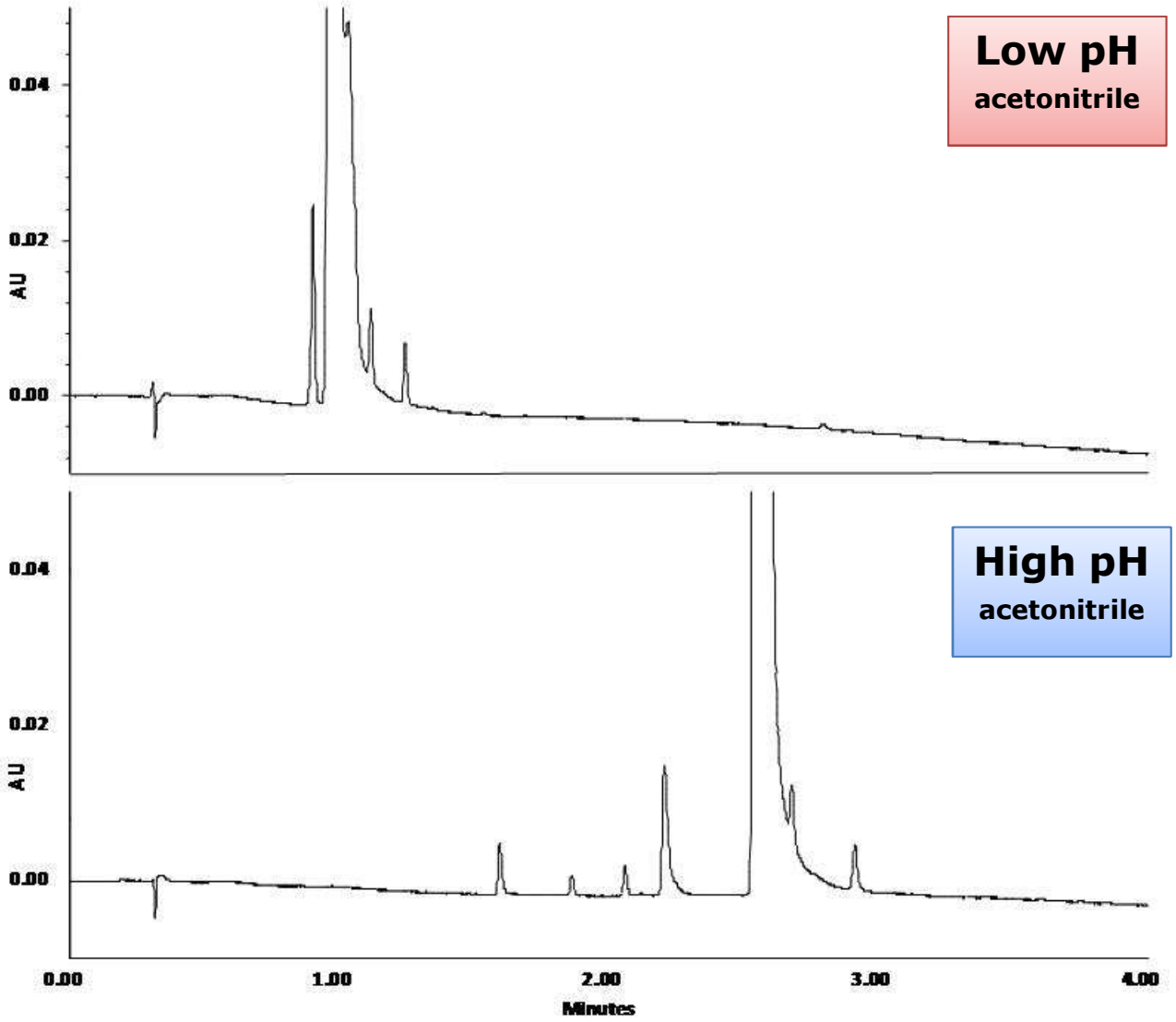

Low pH
acetonitrile

High pH
acetonitrile

ACQUITY CSH C_{18}
T_g = 4.3 min 5-95% B

Observations:

Better retention at high pH due to the neutral charge state of the analytes

Improved resolution of impurities from mirtazapine at high pH

Action:

Compare stationary phase selectivity at high pH

Stationary Phase Selectivity, High pH:
Mirtazapine and Impurities

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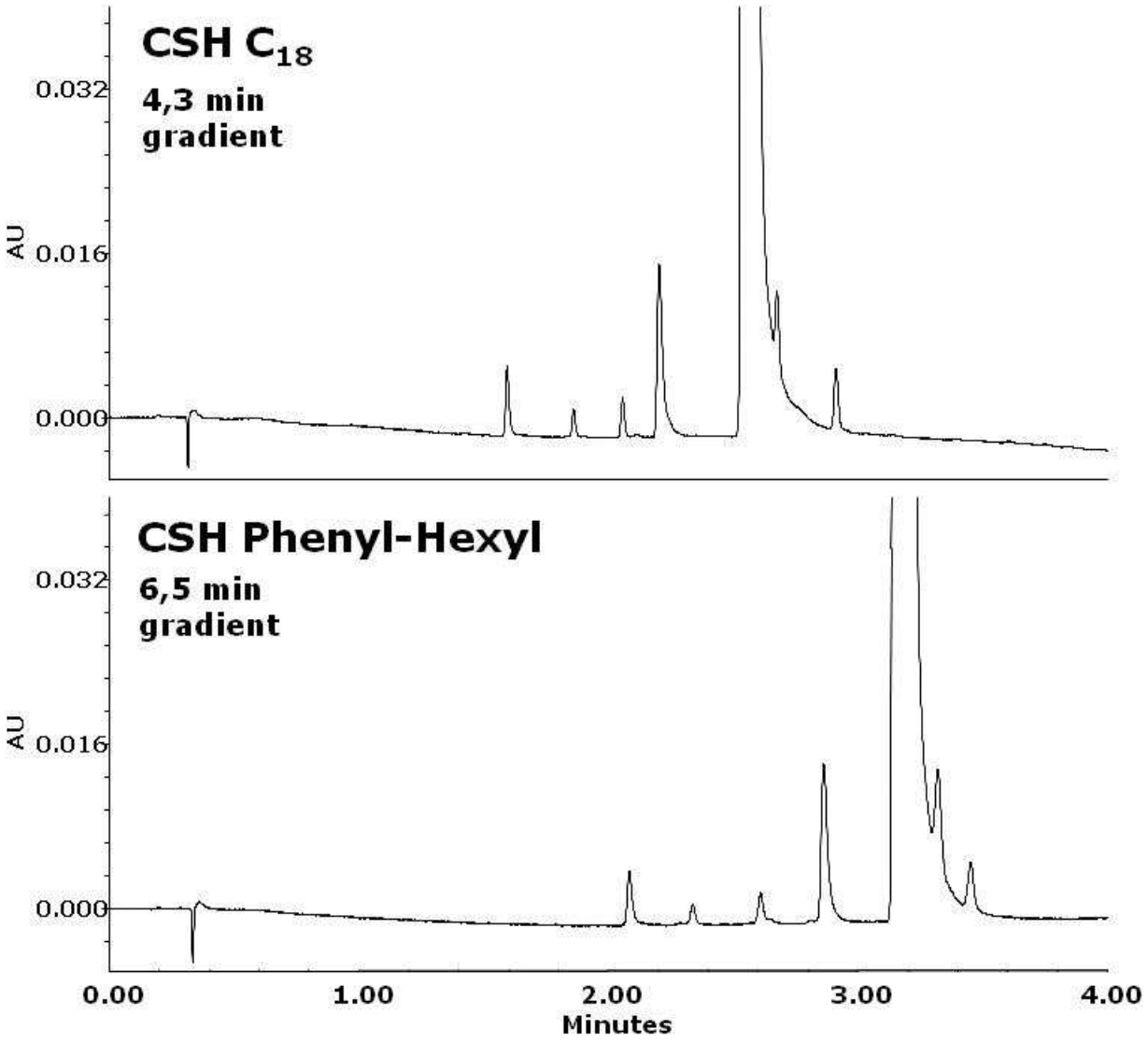

Acetonitrile, High pH
5-95% B

Observations:

Similar selectivity and resolution on both columns at high pH

Action:

Investigate organic modifier

Solvent Selectivity, High pH:
Mirtazapine and Impurities

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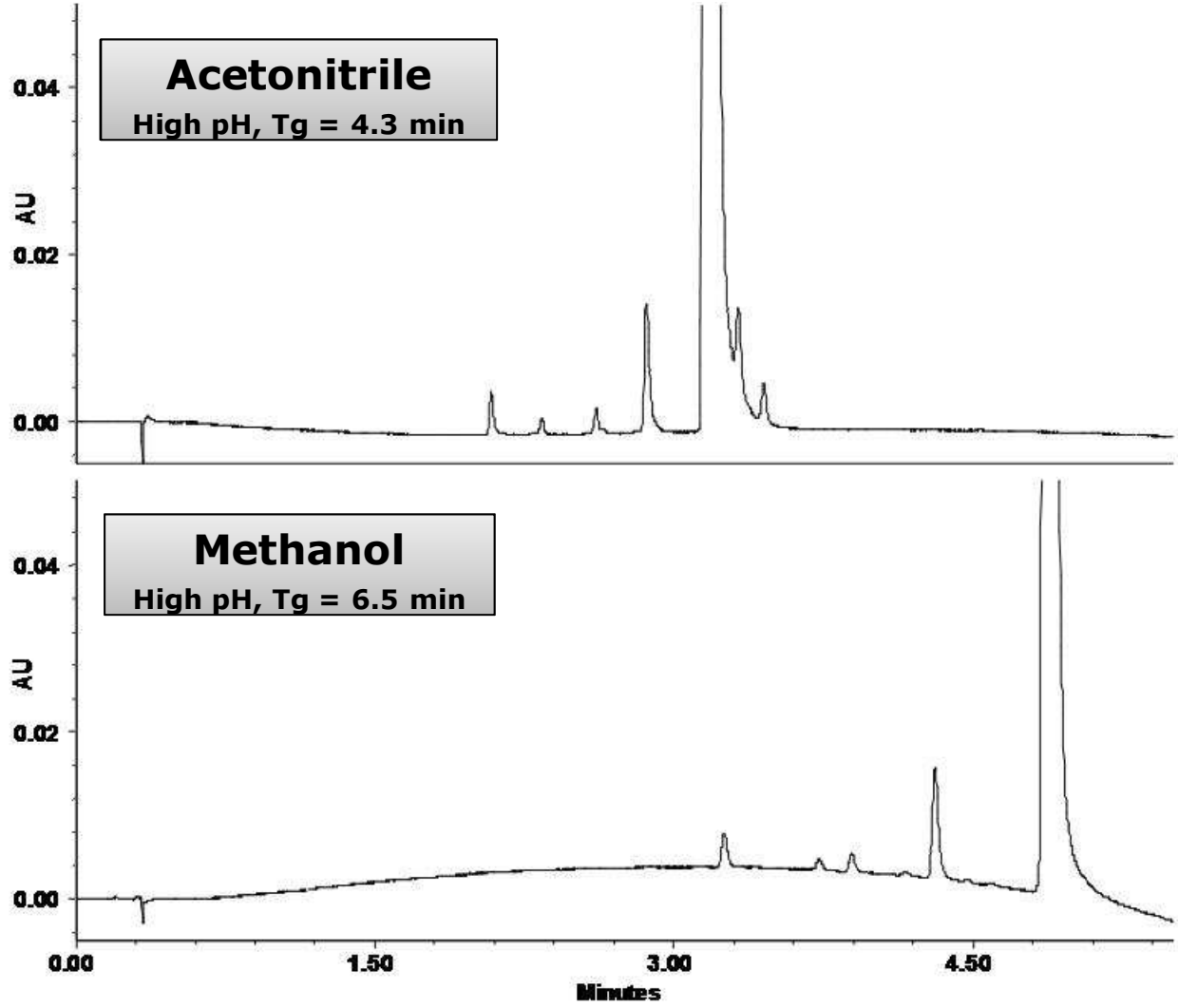

ACQUITY CSH C18
5-95% B

Observations:

Methanol is a weaker elution solvent resulting in greater retention

Better resolution of impurities from mirtazapine with acetonitrile

Action:

Fusion AE software Automated Method Optimizer

Data Analysis from Screening Protocol:
Automated Method Optimizer

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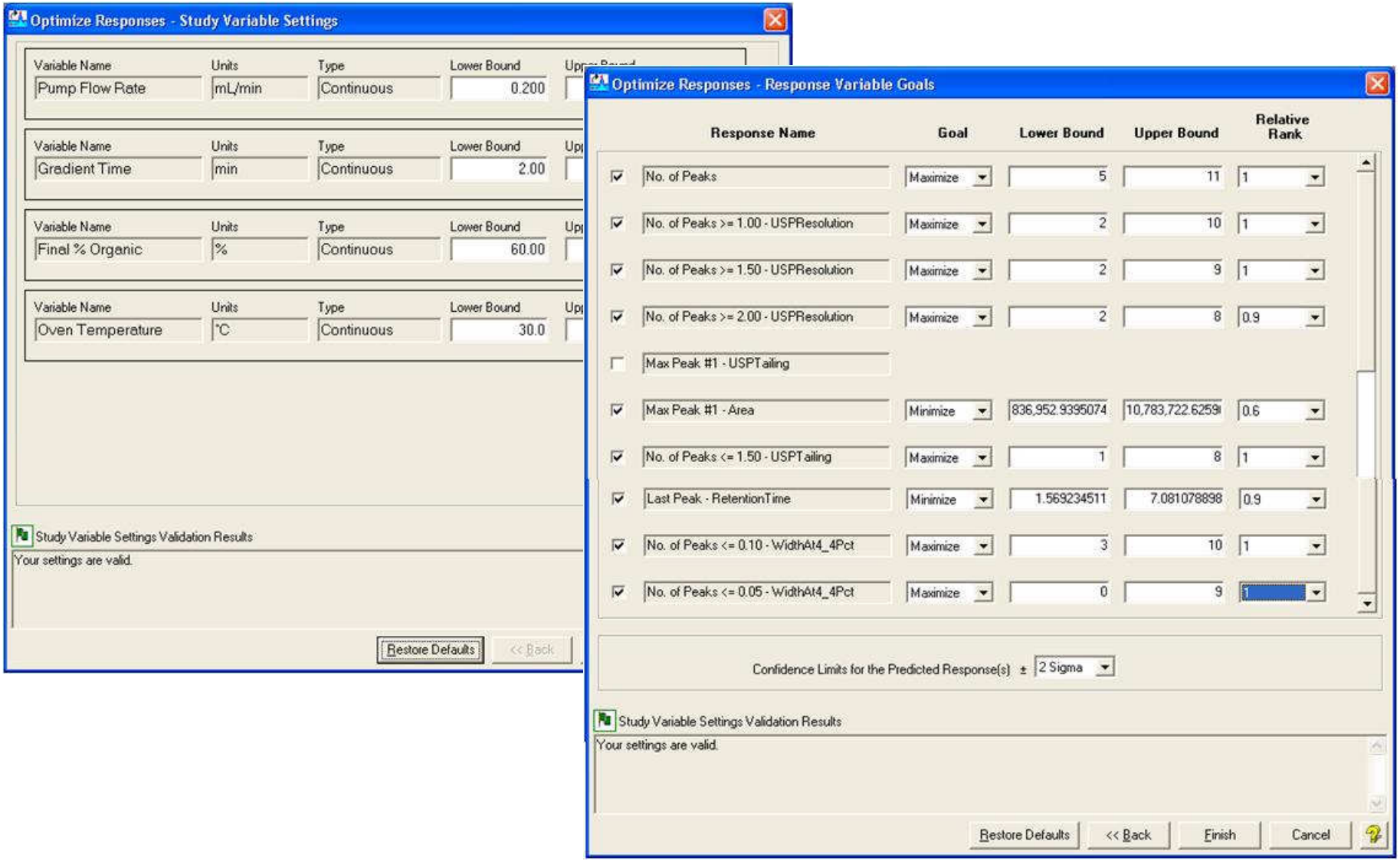

Automized Method Optimization:
Overlay Plot

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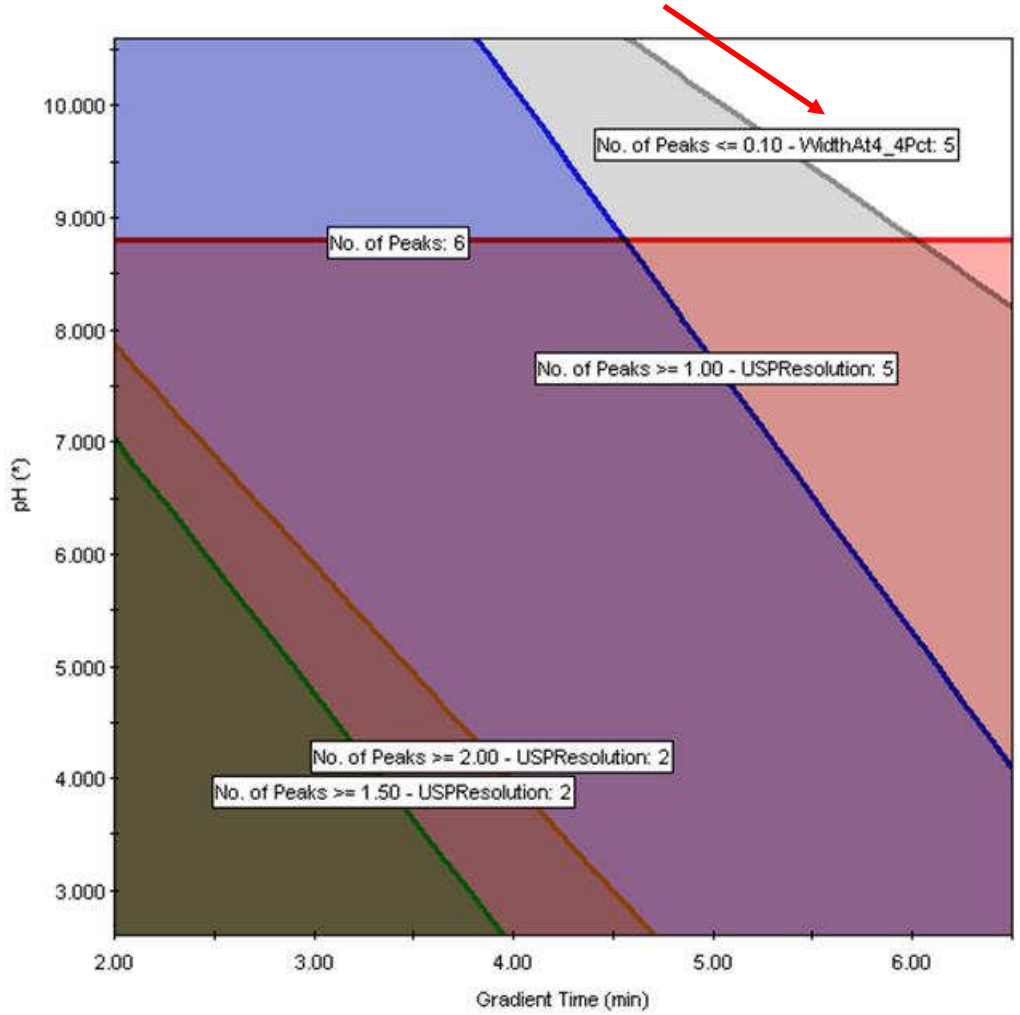
White region represents operating region that meets specified success criteria



Optimized Results From Screening Protocol:

ACQUITY CSH C18
Acetonitrile, pH 10.6, T_g = 5.13 min

All 28 conditions included in the data analysis returned the same answer

Optimizer Answer #1: 28 of 28

Study Variable Data

Study Variable Name	Optimizer Answer Level Setting
Gradient Time	5.13
Organic Solvent Type	Acetonitrile
pH	10.600
Column Type	CSH HL C18

Optimization Parameters

- Flow rate
  - Set window: 0,2 – 0,7 ml/min
- Gradient end point
  - Set window: 60 – 95 % acetonitrile
- Gradient time
  - Set window: 2 – 6,5 minutes
- Column temperature
  - Set window: 30 – 45 ^{o}C

Action:

Using column and mobile phase selections determined from screening protocol, Fusion AE determines an experimental design to optimize secondary effectors of selectivity

Determines interactions between variables including:

- Linear additive effects
- Simple interactions
- Complex interactions

Method Optimization Experimental Design

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Run No.	Sample Set No.	Pump Flow Rate (mL/min)	Gradient Time (min)	Final % Organic (%)	Oven Temperature (°C)
Wash - 1	1	0.45	0.1	95	30
1.a.1.a	1	0.7	6.5	95	30
2.a.1.a	1	0.2	6.5	95	30
3.a.1.a	1	0.7	2	95	30
4.a.1.a	1	0.2	2	95	30
5.a.1.a	1	0.7	6.5	60	30
6.a.1.a	1				
7.a.1.a	1				
8.a.1.a	1				
9.a.1.a	1				
10.a.1.a	1				
11.a.1.a	1				
12.a.1.a	1				
13.a.1.a	1				
14.a.1.a	1				
15.a.1.a	1				
16.a.1.a	1				
17.a.1.a	1				
18.a.1.a	1				
19.a.1.a	1				
20.a.1.a	1				
21.a.1.a	1				
22.a.1.a	1				
23.a.1.a	1	0.575	5.4	69	37.5
24.a.1.a	1	0.325	5.4	69	37.5
25.a.1.a	1	0.575	3.1	69	37.5
26.a.1.a	1	0.575	5.4	86	37.5
27.a.1.a	1	0.325	3.1	86	37.5
28.a.1.a	1	0.7	6.5	60	45
29.a.1.a	1	0.2	6.5	60	45
30.a.1.a	1	0.7	2	60	45
31.a.1.a	1	0.2	2	60	45
32.a.1.a	1	0.45	4.3	78	45
33.a.1.a	1	0.7	6.5	95	45
34.a.1.a	1	0.2	6.5	95	45
35.a.1.a	1	0.2	4.3	78	45
36.a.1.a	1	0.45	4.3	78	45
				95	45
				95	45
				86	52.5
				69	52.5
				86	52.5
				60	60
				60	60
				60	60
				60	60
				95	60
				95	60
				78	60
				95	60
				95	60
				78	60
				95	60
				95	60
54.a.1.a	1	0.7	2	78	60
55.a.1.a	1	0.7	2	95	60
56.a.1.a	1	0.2	2	78	60
57.a.1.a	1	0.2	2	95	60
58.a.1.a	1	0.45	6.5	60	60
59.a.1.a	1	0.45	4.3	78	60
60.a.1.a	1	0.7	4.3	60	60
61.a.1.a	1	0.7	6.5	60	60
62.a.1.a	1	0.2	4.3	60	60
Wash - 2	1	0.45	0.1	60	60

Fusion AE software automatically constructs a set of experiments by selecting the most efficient statistical experimental design

Instrument methods, method sets and sample sets are automatically created in Empower 2 to carry out the experiment

Sample set run time: < 14 hours
Replicates highlighted

Automized Method Optimization:
Overlay Plot

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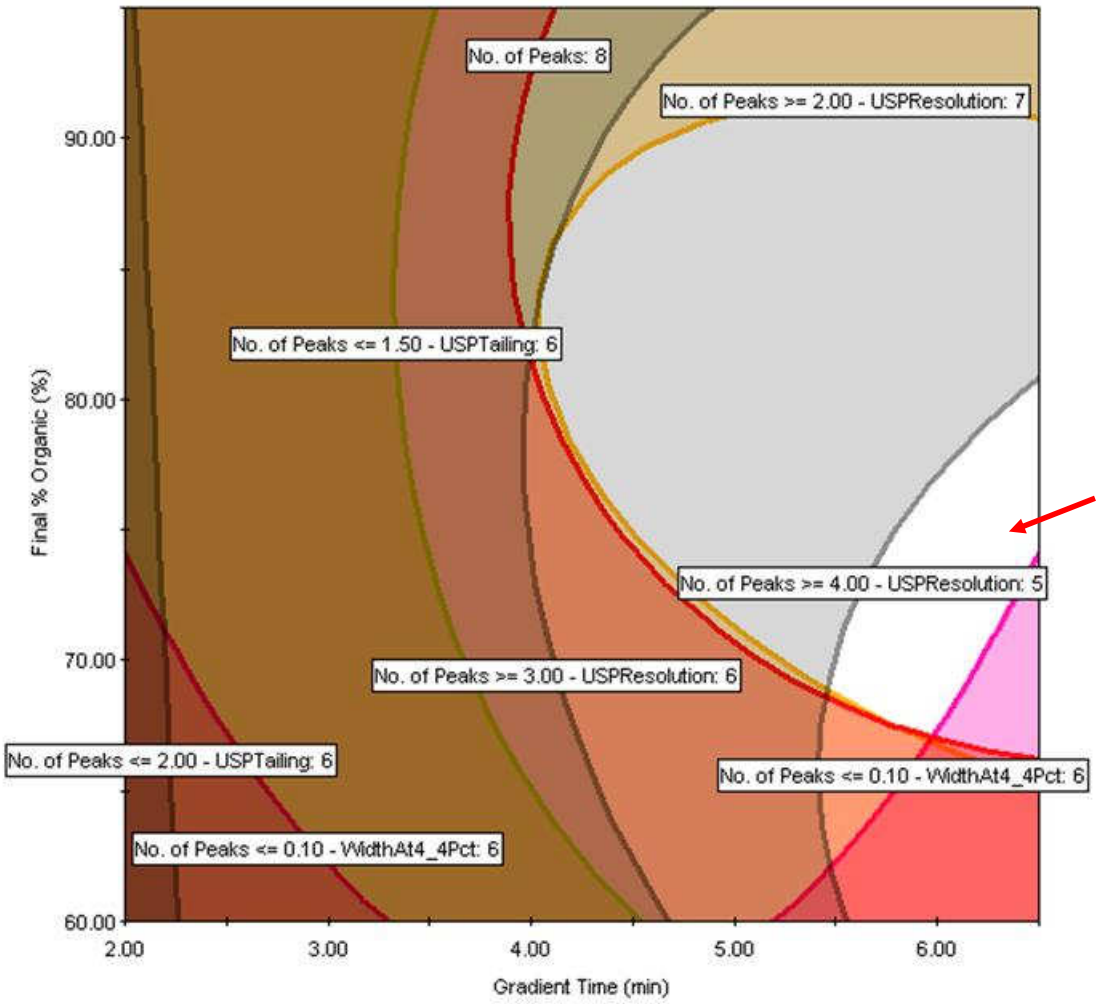
Control space



The Final Result:

ACQUITY CSH C_{18}, 2,1 x 50 mm, 1,7 µm
pH 10.6, T_g = 6.5 min, 5 – 78% acetonitrile Flow rate = 0.7 ml/min, T = 45 °C

Optimizer Answer #4: 5 of 35

Study Variable Data

Study Variable Name	Optimizer Answer Level Setting
Pump Flow Rate	0.700
Gradient Time	6.50
Final % Organic	77.98
Oven Temperature	45.0

Final Optimized Result:
Mirtazapine and Impurities

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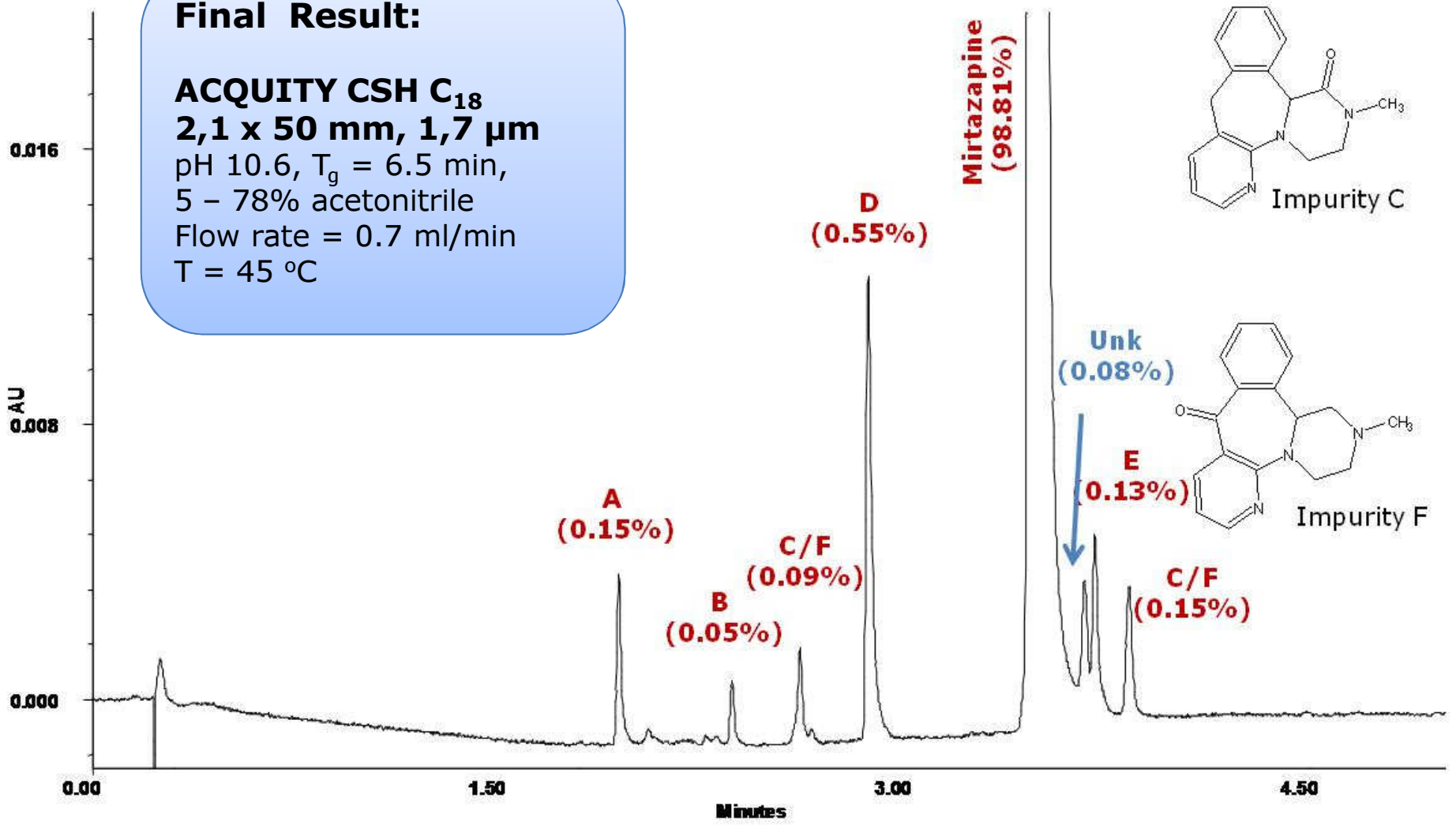
Final Result:

ACQUITY CSH C_{18}
2,1 x 50 mm, 1,7 µm
pH 10.6, T_g = 6.5 min,
5 – 78% acetonitrile
Flow rate = 0.7 ml/min
T = 45 °C



Outline

- **Introduction**
  - Approaches Toward Method Development
  - UPLC Technology
  - Success Criteria
- **Controlling Selectivity and Retention**
  - Stationary Phase and Particle Substrate Design
  - Organic Modifier
  - Mobile Phase pH
- **Method Development Strategy**
  - Systematic Screening Protocol
  - Quality by Design [Q_bD] Approach
- **Implementing the Approach**
  - Case Study
- **Conclusion**

Concluding Remarks

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- UPLC Technology facilitates rapid development of robust methods
  - Systematic screening protocol involving pH, organic modifier and column chemistry
  - High resolution sub 2 µm column technology creates high resolution separations, faster
  - Automated column and mobile phase selection
  - Quaternary solvent mixing [ACQUITY UPLC H-Class]
- The principles of method development described here can implemented for both HPLC and UPLC
  - XSelect HPLC columns and ACQUITY CSH UPLC columns provide a broad range of selectivity [C18, Phenyl-Hexyl and Fluoro-Phenyl] to efficiently develop robust methods
- Combining Fusion AE method development software with UPLC Technology enables a rapid yet comprehensive approach to Q_bD method development
  - Develop robust methods in a matter of days
  - Incorporates robustness modeling into the method development process
  - Aligned with FDA and EMA Q_bD initiatives

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Questions? Comments?