Multi-Dimensional LC/MS Using Orthogonal Reversed-Phase Stationary Phases

David S. Bell and Shawn R. Wyatt

Supelco

595 North Harrison Road, Bellefonte, PA 16823

Outline

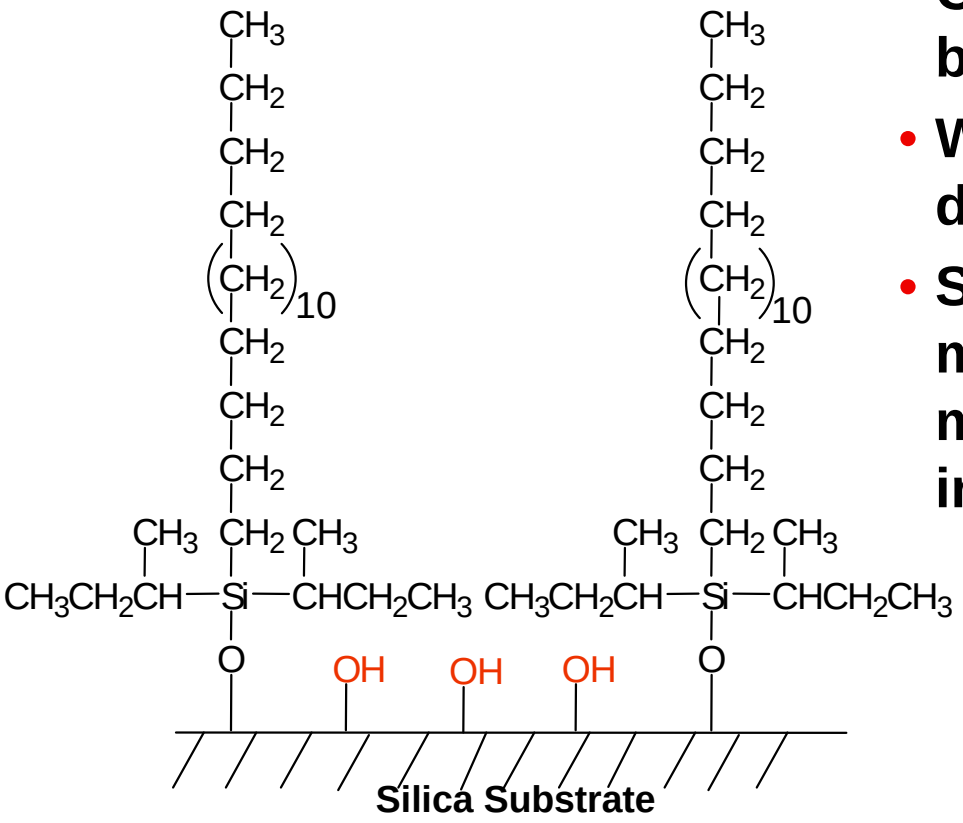
- **Introduction**
  - What is multi-dimensional chromatography?
  - Molecular interactions
- **Methods using multi-dimensional chromatography**
  - Ion-pair chromatography
  - Coupling phases of distinctly different chemistries
  - Coupling phases of complementary chemistries
  - Multi-dimensional chromatography on a single phase
- **Summary/Conclusions**
- **Q&A**

Introduction

- **What is meant by multi-dimensional chromatography?**
  - The use of multiple molecular interactions to impart separation of target analytes
- **Types of molecular interactions:**
  - Dispersive (hydrophobic, partition, transient interactions)
  - Polar (dipole-dipole, hydrogen bonding)
  - Ionic (ion-exchange)

  - It is a combination of these molecular interactions that result in retention and resolution

Introduction



- **C18 stationary phases are by far the most utilized**
- **Work primarily on dispersive interactions**
- **Strong efforts have been made by manufacturers to minimize other interactions**

Introduction

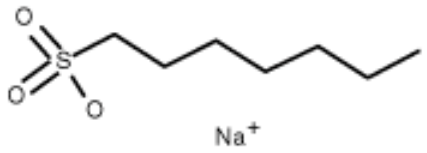
- **What if there is no retention on C18 for a particular target analyte?**
- **What if dispersive interactions alone do not result in resolution?**
- **There is often the need to invoke additional interactions for a given analysis (multi-dimensional chromatography)**

Introduction

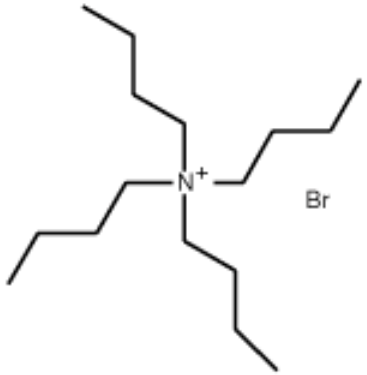
- **Methods for adding additional interactions:**
  - Addition of mobile phase modifiers to the existing C18 system (ion-pair reagents, for example)
  - Coupling columns of differing stationary phase chemistries
    - Distinctly different
    - Complementarily different
  - Use a single stationary phase that permits dispersive, polar and ionic interactions

Addition of Mobile Phase Modifiers: Ion-Pair Chromatography

- **The addition of ion-pairing agents imparts an ionic interaction on top of the dispersive interactions available on C18 phases**



Heptanesulfonic Acid



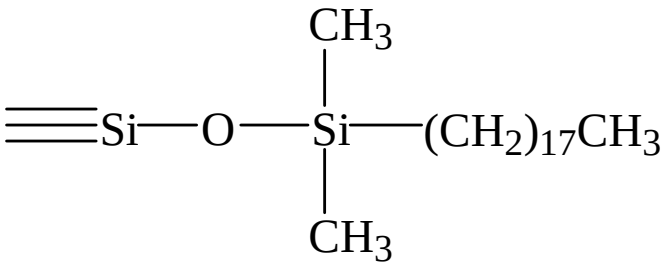
Tetrabutylammonium bromide

- **Tend to be non-volatile and can suppress ionization in LC/MS systems**
- **Due to the dynamic situation, reproducibility often suffers**
- **Gradients?**

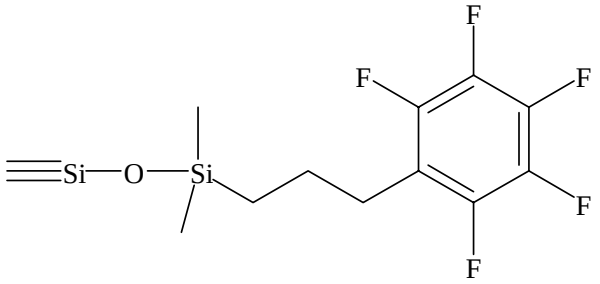
Coupling Columns of Differing Stationary Phase Chemistries – 2D Separations

- **Different stationary phase chemistries may impart supplemental interactions to a separation system**
  - A more polar phase may induce retention of a more polar target analyte, while a C18 may best retain a neutral component
  - An ion-exchange column may be used to fractionate a complex mixture prior to separation via dispersive interactions on a C18
- **Considerations**
  - Can the mobile phase be altered such that only one of the phases is affected?
  - What phases provide alternative interactions to C18?
  - Can a single mobile phase condition be used to utilize both phases?
  - When is this a viable alternative?
  - What are the consequences?

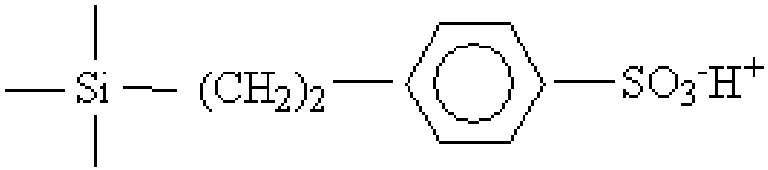
Types of Stationary Phases



Alkyl (C18, C8) primarily dispersive interactions



Polar Reversed-Phase (PFPP), dispersive, polar and ionic interactions



Ion-exchange (SCX,SAX), primarily ionic interactions

Coupling Distinctly Different Phases

- **Most common multidimensional chromatography is performed using distinctly different phases**
  - IEX and RP
- **The intention is typically to reduce the complexity of a sample**
  - Protein digests

β-Galactosidase

From E. Coli

Protein MW : 116484Da

Trypsin digestion of Beta-Gal gives around 100 to 150 peptides.

```
    1          11         21         31         41         51
    |          |          |          |          |          |
   1 TMITDSLAVV LQRRDWENPG VTQLNRLAAH PPFASWRNSE EARTDRPSQQ LRSLNGEWRF    60
  61 AWFPAPEAVP ESWLECDLPE ADTVVVPSNW QMHGYDAPIY TNVTYPITVN PPFVPTENPT   120
 121 GCYSLTFNVD ESWLQEGQTR IIFDGVNSAF HLWCNGRWVG YGQDSRLPSE FDLSAFLRAG   180
 181 ENRLAVMVLR WSDGSYLEDQ DMWRMSGIFR DVSLLHKPTT QISDFHVATR FNDDFSRAVL   240
 241 EAEVQMCGEL RDYLRVTVSL WQGETQVASG TAPFGGEIID ERGGYADRVT IRLNVENPKL   300
 301 WSAEIPNLYR AVVELHTADG TLIEAEACDV GFREVRIENG LLLLNGKPLL IRGVNRHEHH   360
 361 PLHGQVMDEQ TMVQDILLMK QNNFNAVRCS HYPNHPLWYT LCDRYGLYVV DEANIETHGM   420
 421 VPMNRLTDDP RWLPAMSERV TRMVQRDRNH PSVIIWSLGN ESGHGANHDA LYRWIKSVDP   480
 481 SRPVQYEGGG ADTTATDIIC PMYARVDEDQ PFPAVPKWSI KKWLSLPGET RPLILCEYAH   540
 541 AMGNSLGGFA KYWQAFRQYP RLQGGFVWDW VDQSLIKYDE NGNPWSAYGG DFGDTPNDRQ   600
 601 FCMNGLVFAD RTPHPALTEA KHQQQFFQFR LSGQTIEVTS EYLFRHSDNE LLHWMVALDG   660
 661 KPLASGEVPL DVAPQGKQLI ELPELPQPES AGQLWLTVRV VQPNATAWSE AGHISAWQQW   720
 721 RLAENLSVTL PAASHAIPHL TTSEMDFCIE LGNKRWQFNR QSGFLSQMWI GDKKQLLTPL   780
 781 RDQFTRAPLD NDIGVSEATR IDPNAWVERW KAAGHYQAEA ALLQCTADTL ADAVLITTAH   840
 841 AWQHQGKTLF ISRKTYRIDG SGQMAITVDV EVASDTPHPA RIGLNCQLAQ VAERVNWLGL   900
 901 GPQENYPDRL TAACFDRWDL PLSDMYTPYV FPSENGLRCG TRELNYGPHQ WRGDFQFNIS   960
 961 RYSQQQLMET SHRHLLHAEE GTWLNIDGFH MGIGGDDSWS PSVSAEFQLS AGRYHYQLVW  1020
1021 CQK
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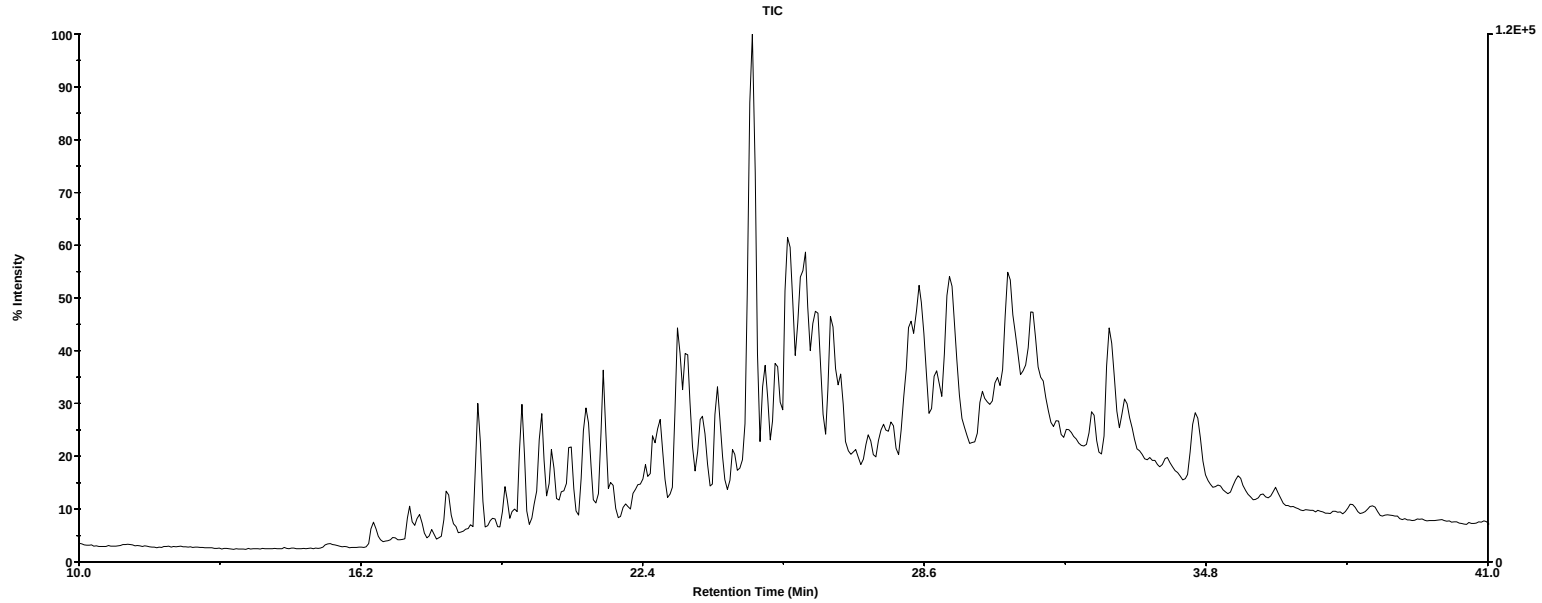
Compliments of Qian Chen, The Pennsylvania State University

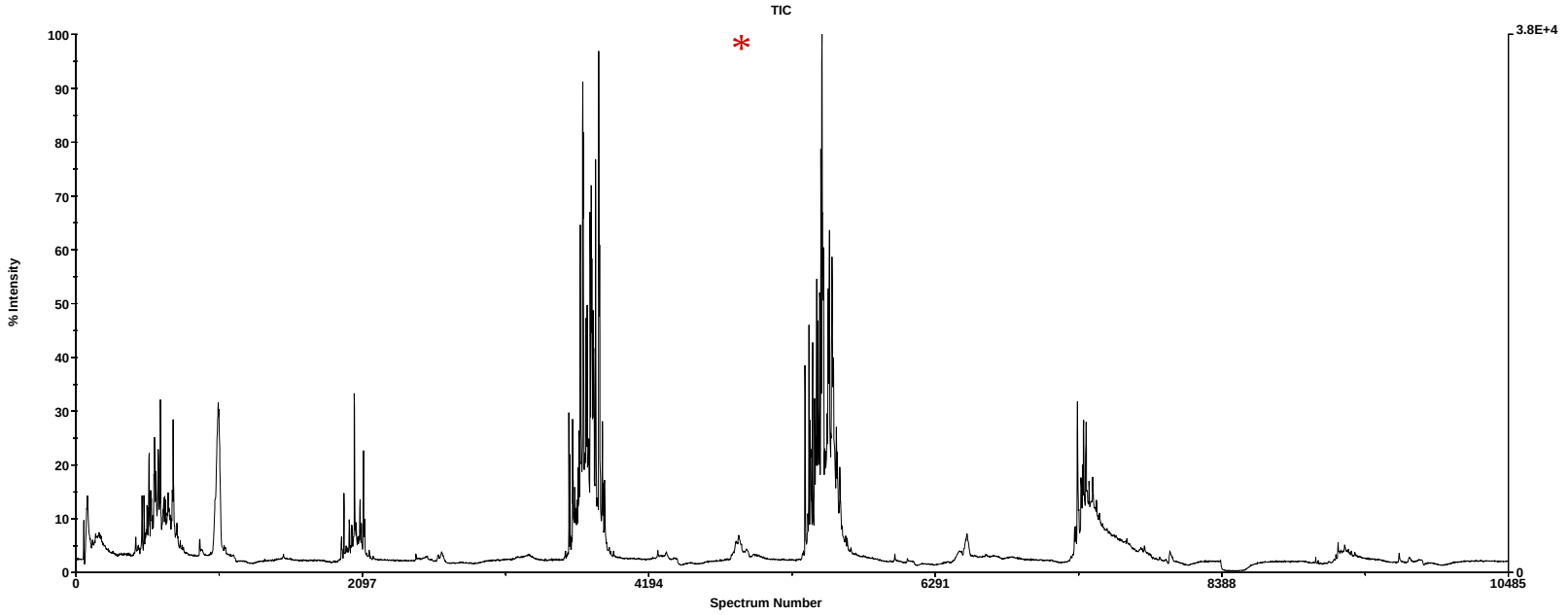
Results of 1-D RPLC/ESI-MS System of Tryptic Digest of β-Galactosidase



TIC of 1-D RPLC/MS of digest

Compliments of Qian Chen, The Pennsylvania State University

Results of 2-D LC/LC/ESI-MS System of Tryptic Digest of β-Galactosidase



TIC of 2-D LC/LC/MS results.

Compliments of Qian Chen, The Pennsylvania State University

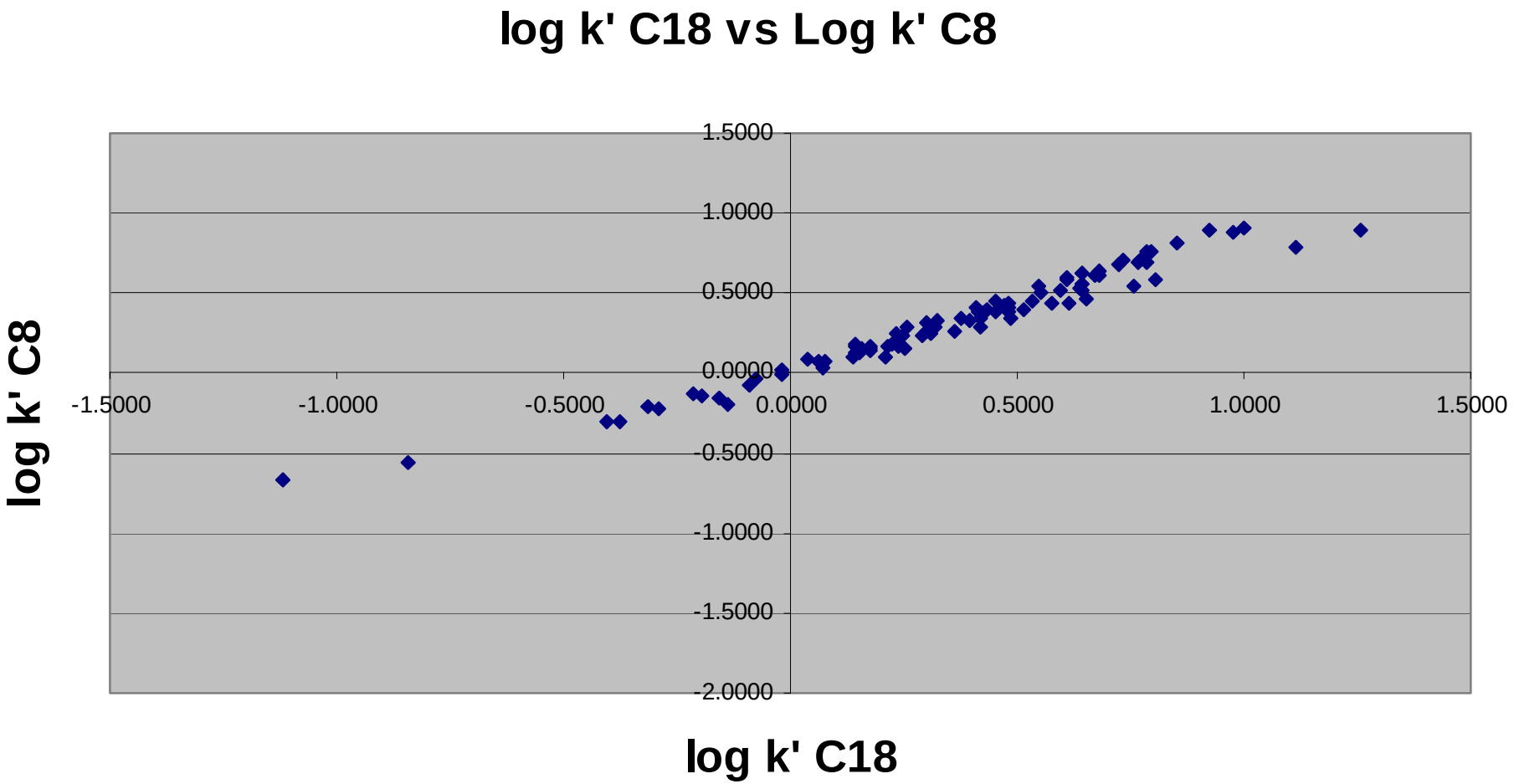
Coupling Distinctly Different Phases

- **Unique in that a mobile phase change effects one phase but not the other (in theory!)**
  - Increasing the ionic strength in the IEX dimension minimally affects the elution from the RP phase
  - Increasing the organic composition of the MP in the second step, minimally affects the IEX
  - Reasonably easy to control

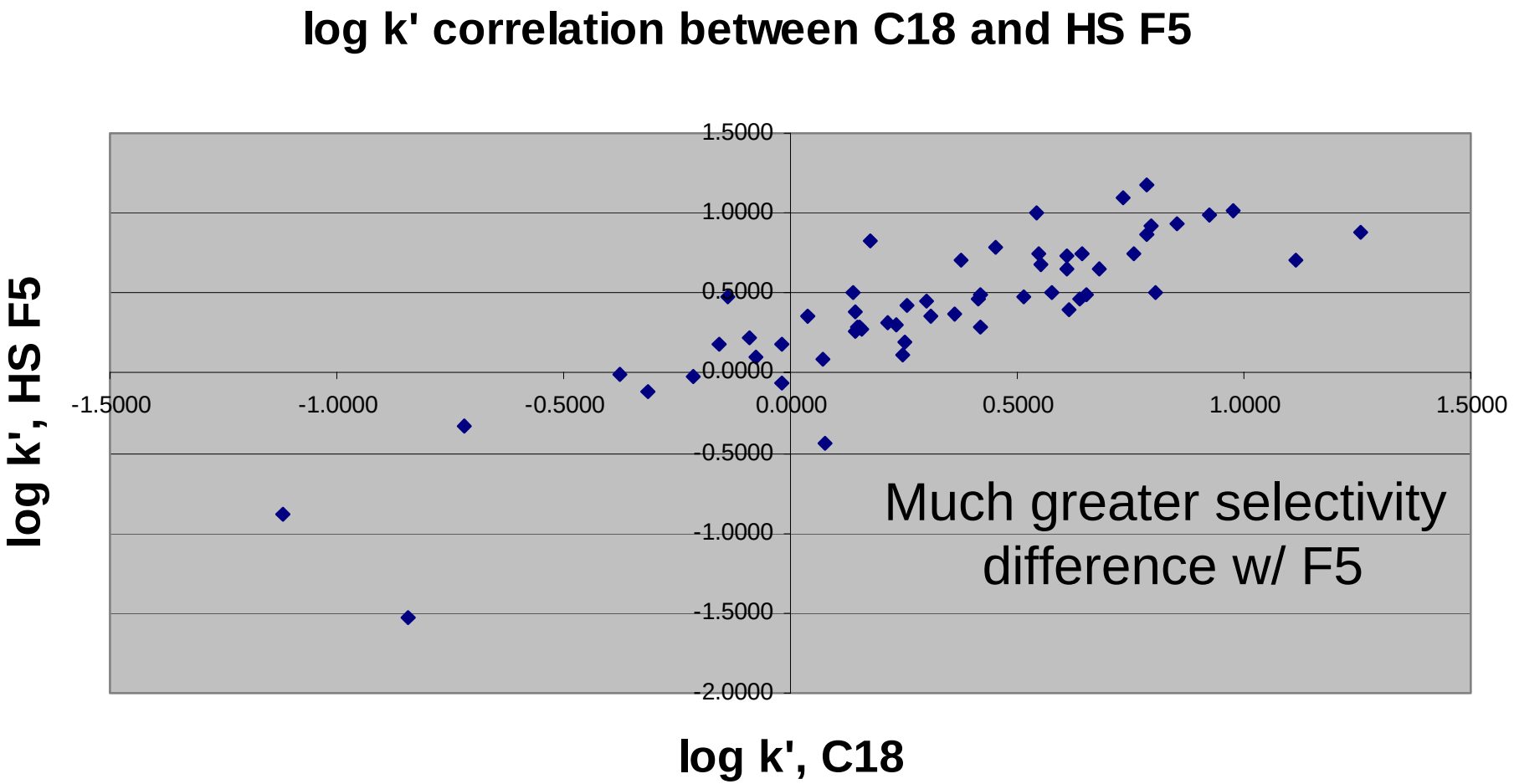
Coupling Complementary Stationary Phases

- **Coupling two different RP stationary phases with complementary interactions is different**
  - A change in mobile phase will usually affect both columns as the interactions are not as distinct
  - Simultaneous separation rather than fractionation/separation

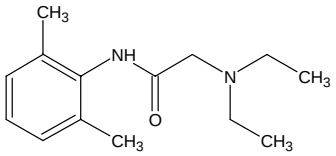
Coupling Complementary Stationary Phases



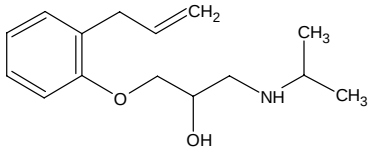
Coupling Complementary Stationary Phases



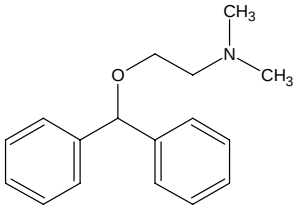
Coupling Complementary Stationary Phases



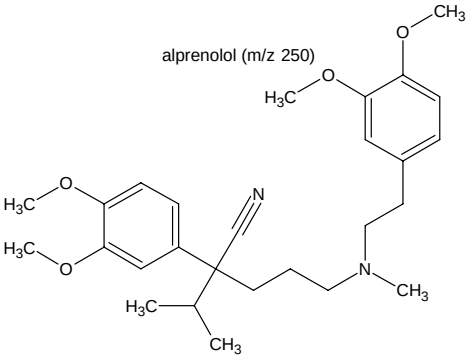
lidocaine (m/z 235)



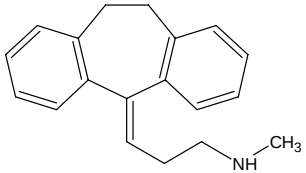
alprenolol (m/z 250)



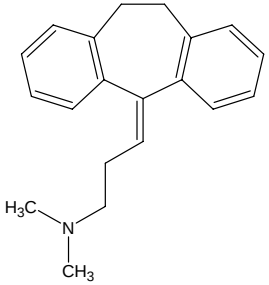
diphenhydramine (m/z 256)



verapamil (m/z 455)



nortriptyline (m/z 264)



amitriptyline (m/z 278)

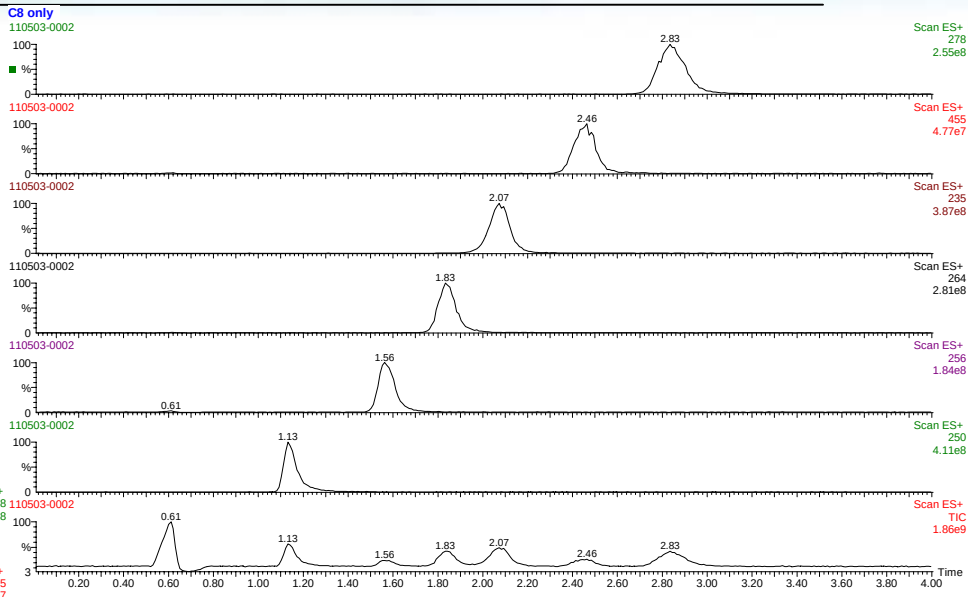
Run on C8 and C18 under identical conditions and compare selectivities

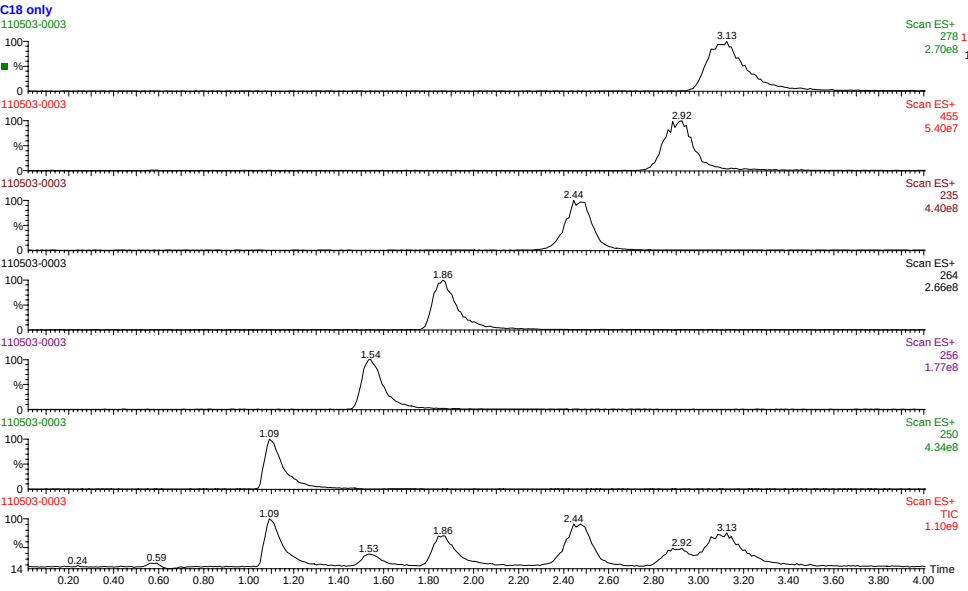
Run on C18 and F5 under identical conditions and compare selectivities

Coupling Complementary Stationary Phases

Discovery C8 →

Similar retention and selectivity:
Coupling provides no advantages





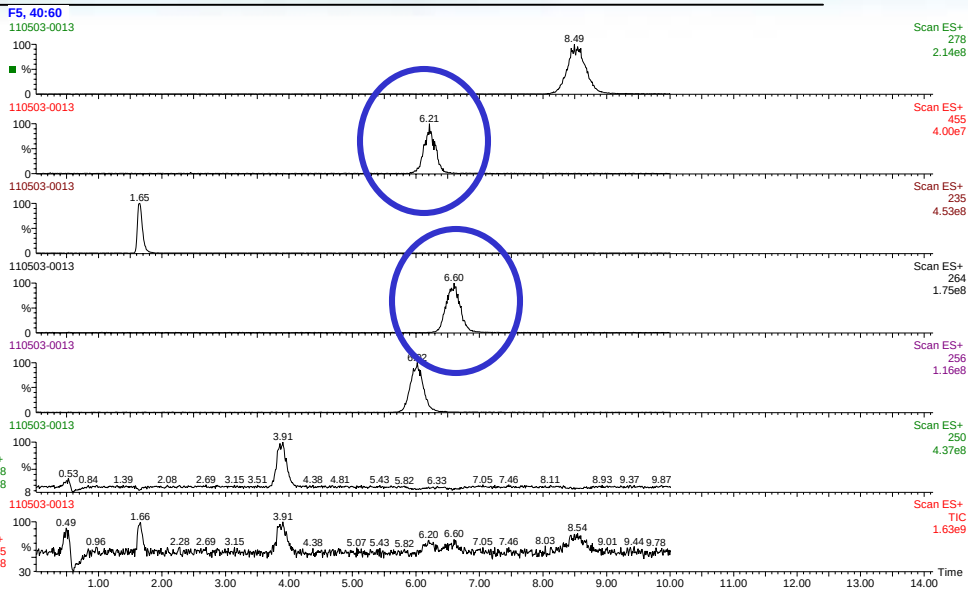
← Discovery C18

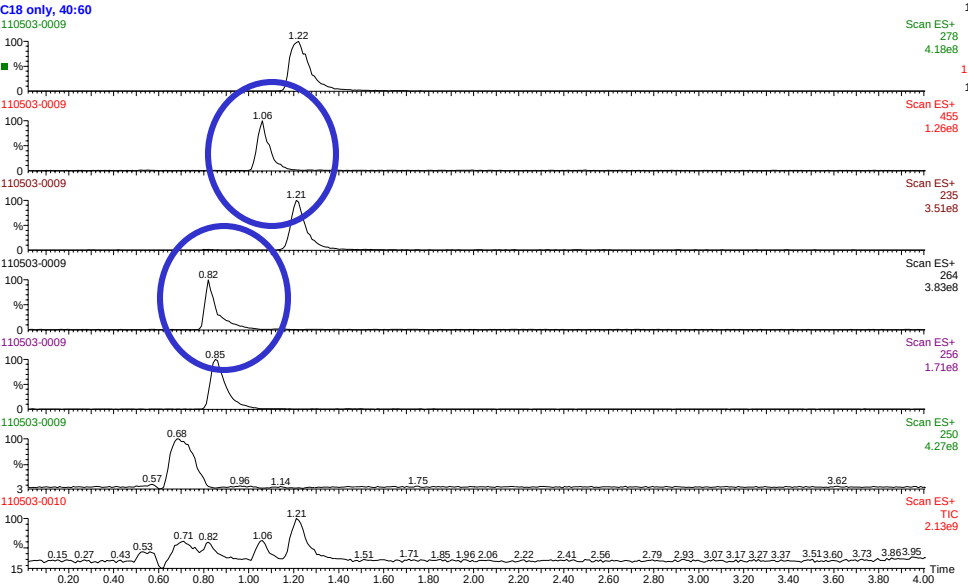
Mobile Phase:	20mM ammonium acetate, pH 6.7:Acetonitrile (60:40, v/v)
Flow Rate:	1mL/min
Detection:	ESI (+)
Temperature:	35ºC
Sample Volume:	10µL

Coupling Complementary Stationary Phases

Discovery HS F5 →

Different retention and selectivity:
Coupling may offer unique separation

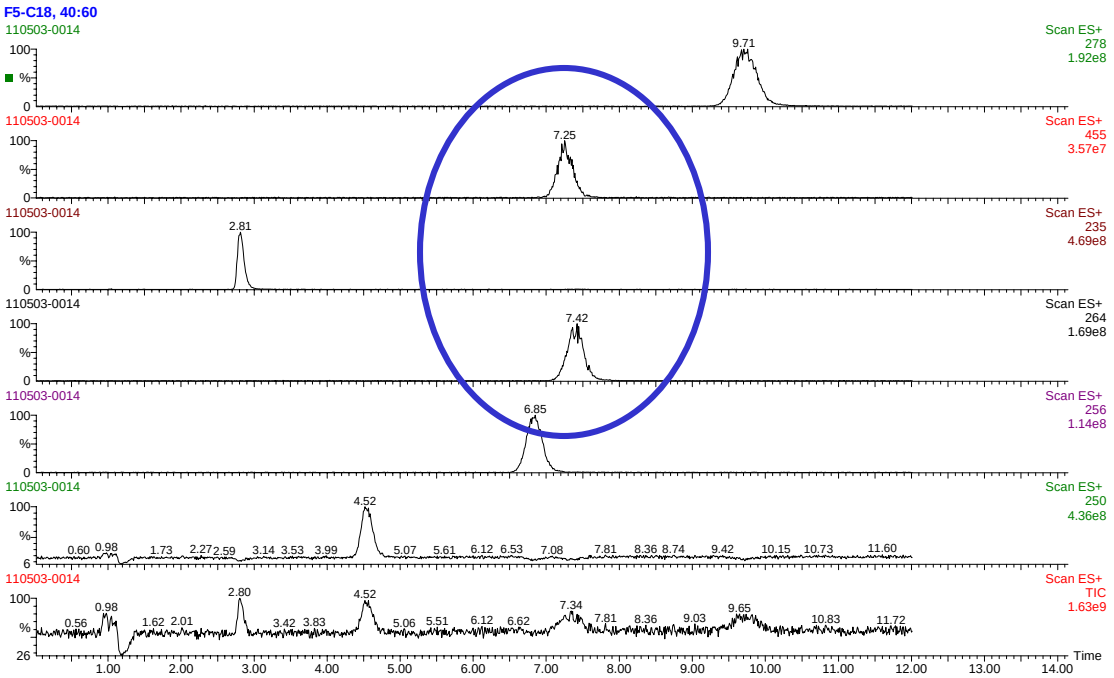




← Discovery C18

Mobile Phase:	20mM ammonium acetate, pH 6.7:Acetonitrile (40:60, v/v)
Flow Rate:	1mL/min
Detection:	ESI (+)
Temperature:	35ºC
Sample Volume:	10µL

Coupling Complementary Stationary Phases



Coupled C18 and HS F5

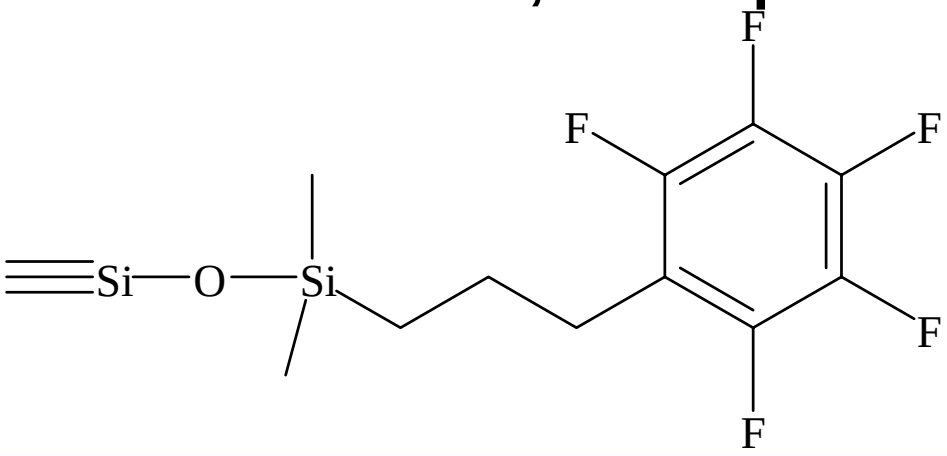
- Common issue when coupling: The mobile phase conditions are not optimal for each column chemistry
- Presents difficulties in optimizing the interactions on BOTH phases

Coupling Complementary Stationary Phases

- **Situations arise where coupling of complementary phases works**
  - Late stage method development where a “new” impurity is discovered
- **In most cases properly choosing a single stationary phase with the needed interactions will suffice**

Multi-Dimensional Separations on a Single Stationary Phase

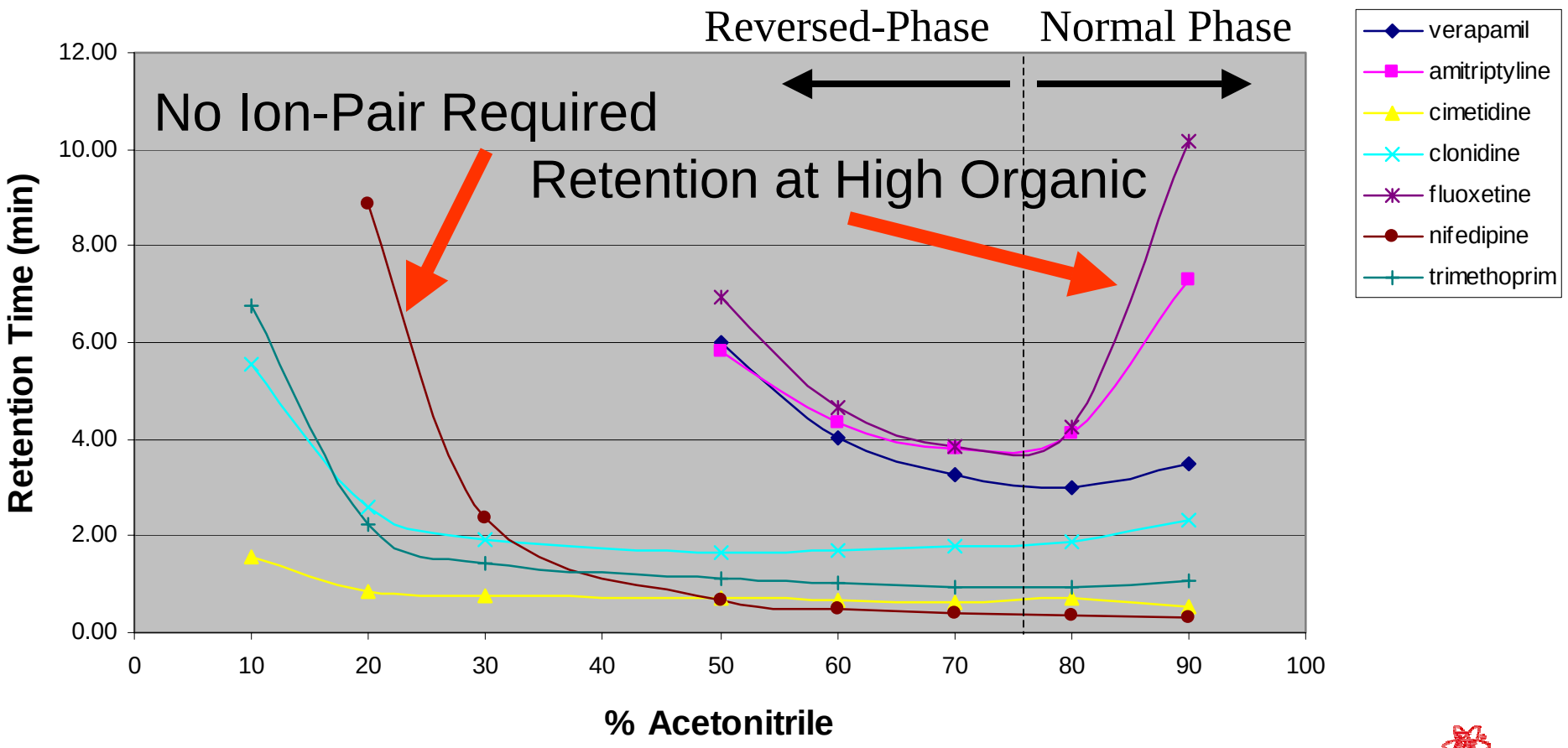
- **Alkyl phases do not complement polar/ionic interactions**
- **Polar interactions are of a secondary nature**
  - Difficult to control
  - Highly variable
- **Polar-RP phases, by design, provide polar/ionic interactions, which provide alternative selectivity**



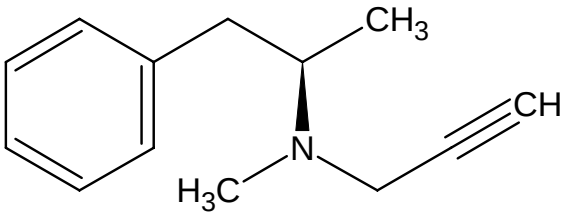
- Dipole interactions
- IEX interactions
- Dispersive interactions

Multi-Dimensional Separations on a Single Stationary Phase

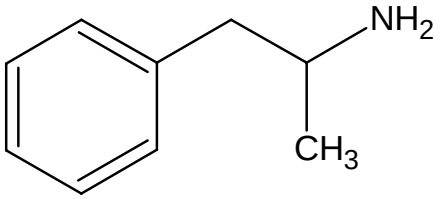
"U-Shape" Retention on Discovery HS F5



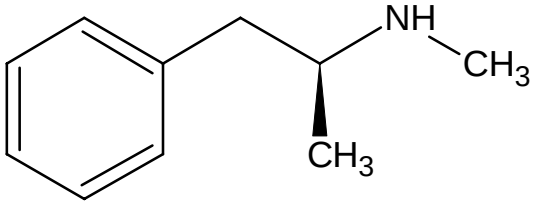
Multi-Dimensional Separations on a Single Stationary Phase



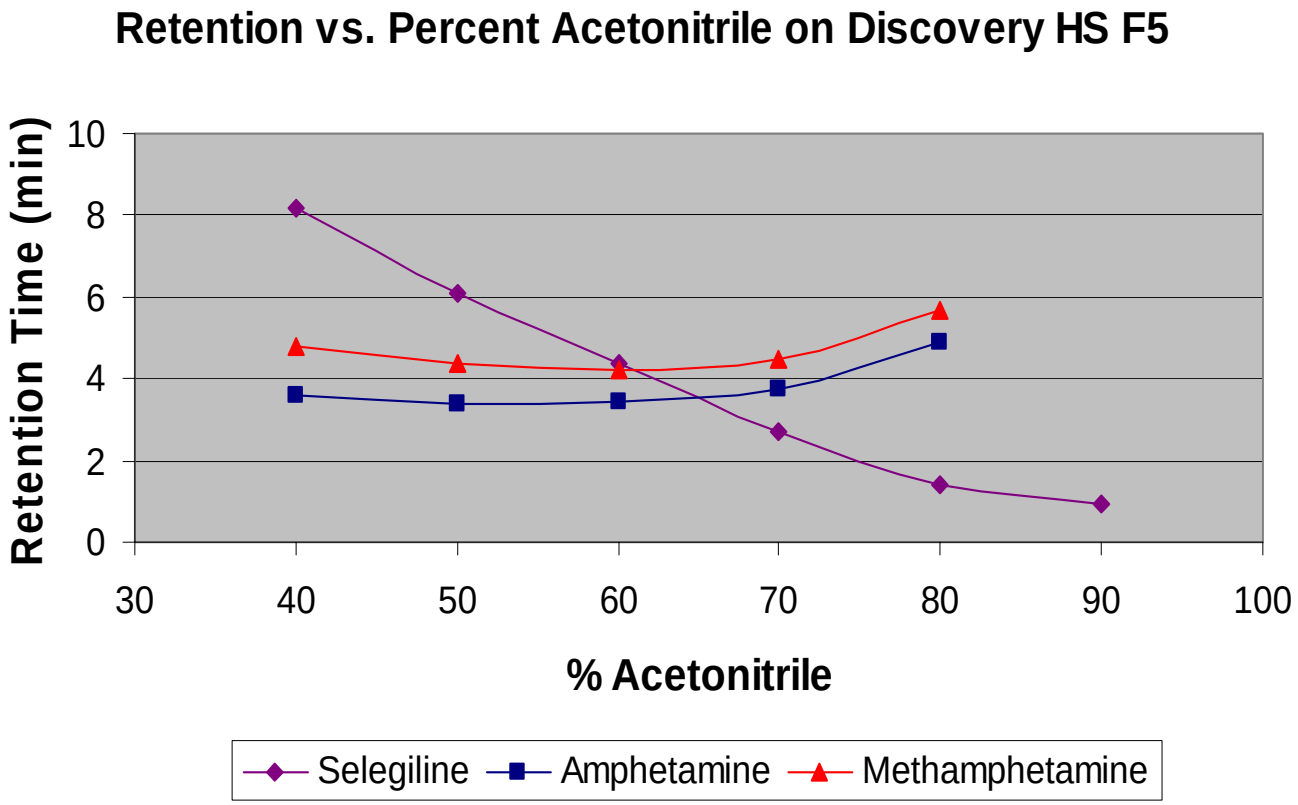
Selegiline



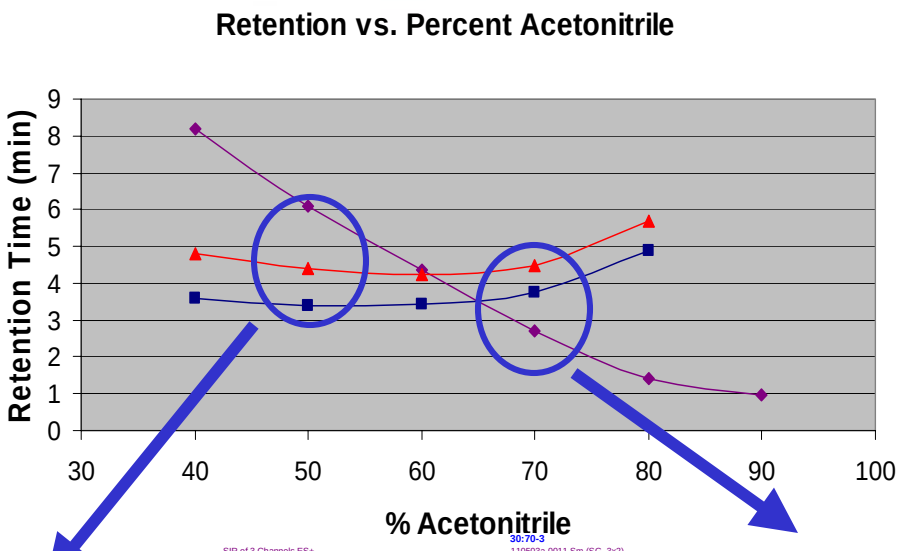
Amphetamine

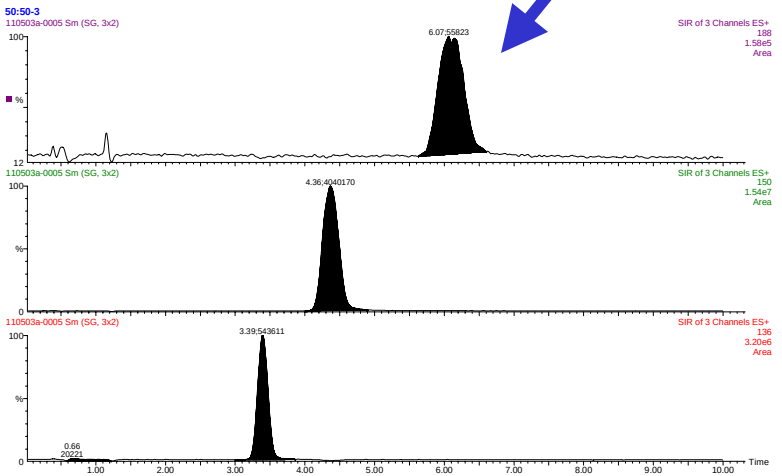


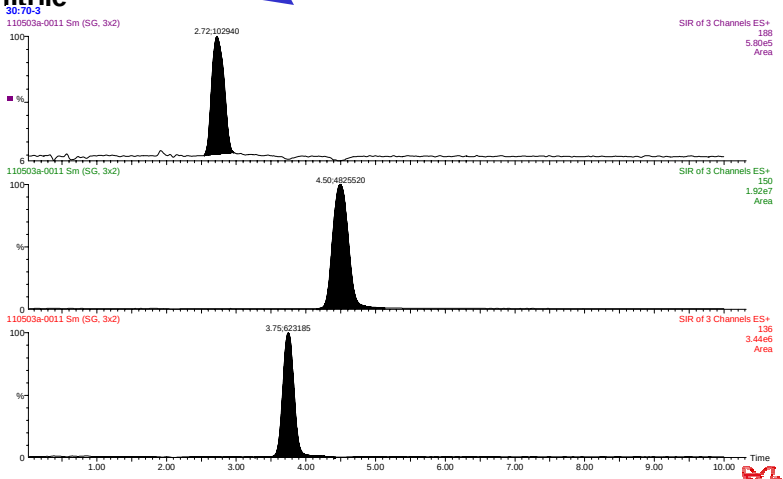
Methamphetamine



Multi-Dimensional Separations on a Single Stationary Phase







Multi-Dimensional Separations on a Single Stationary Phase

- **Retention and separation are a manifestation of various interactions**
  - Dispersive, polar, and ionic
- **Traditional alkyl phases primarily complement dispersive**
  - Polar interactions are secondary
- **Polar RP phases are designed to provide polar and ionic interactions**
  - Improved control
  - Increased ruggedness
  - Alternative selectivity

Summary

- **Multi-dimensional chromatography involves the use of multiple interactions to impart a given separation**
  - Mobile phase additives
    - Generally impairs LC/MS detection
  - Coupling columns packed with distinctly different phases
    - Fractionate/separate
    - Mobile phase adjustments, in theory, affect one phase but not the other
    - Generally easy to control
  - Coupling columns packed with complementary chemistries
    - Separate/separate
    - Mobile phase adjustments tend to effect both phases
    - Often difficult to maximize the benefits of each phase with a single set of mobile phase conditions

Summary

- Multi-dimensional chromatography on a single phase
  - Many stationary phases available that permit multiple interactions
  - Understanding these interactions permits one to maximize their utility for a given separation
  - Often the easiest mode of multi-dimensional chromatography when the appropriate phase is chosen

Acknowledgements

- Qian Chen, The Pennsylvania State University
- Shawn R. Wyatt, Supelco
- Richard Henry, Consultant