# Strategies for Chiral HPLC Method Development

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T409133

# Agenda:

## Introduction and Background

- Overview of stationary phases
  - Cyclodextrins (CYCLOBOND)
  - Macrocyclic glycopeptides (CHIROBIOTIC)
- Modes of chromatography

## Analytical Chiral HPLC

- LC/MS Screening Background
- Study of Impact of variables on retention and selectivity in polar ionic mode
  - Buffer Type
  - Buffer Concentration
  - Acid/base Ratio
  - Column phase chemistry

## Preparative Chiral HPLC

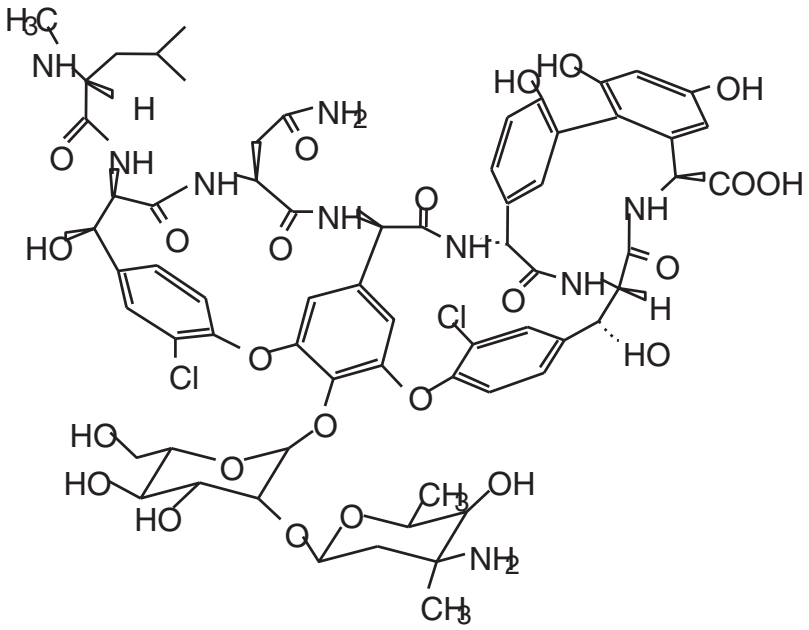
- Reversed Phase
- Trouble Shooting

# Introduction

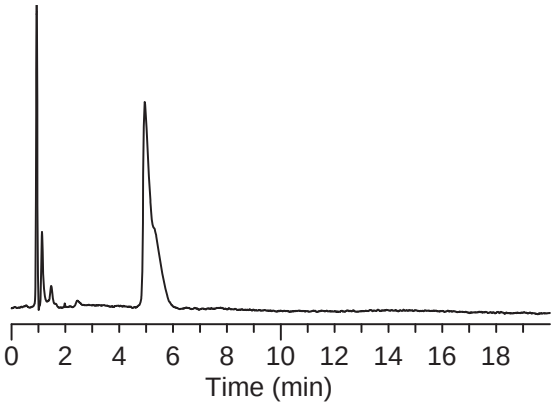
- Retention mechanisms in chiral separations are highly complex and analyte specific, therefore many columns and conditions must be screened to ensure that optimum conditions are found.

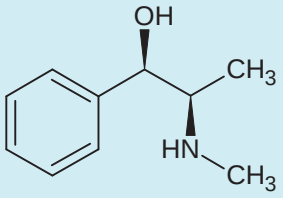
- The approach of using ‘experience’ is not as effective as it is with typical reversed-phase method development.

- Why Not?

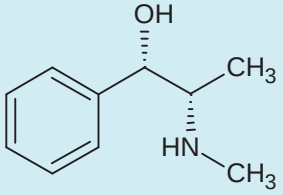


# Ephedrine/Pseudoephedrine

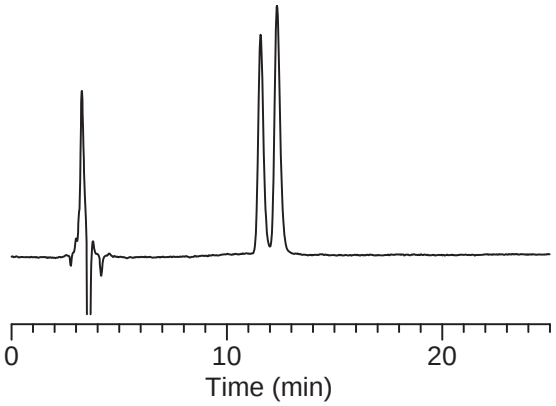


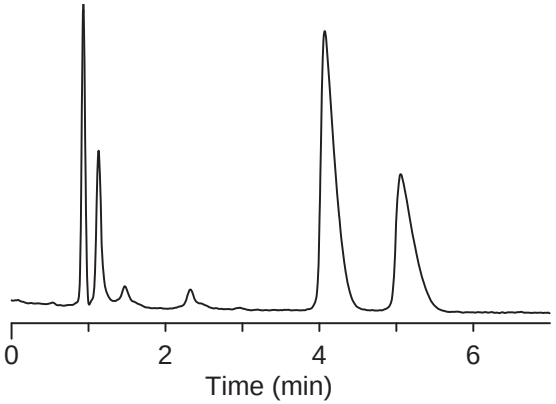




(1R, 2R)-(-)-Pseudoephedrine

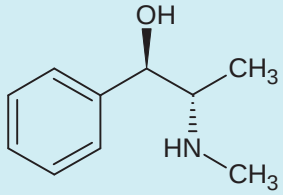



(1S, 2S)-(+)-Pseudoephedrine

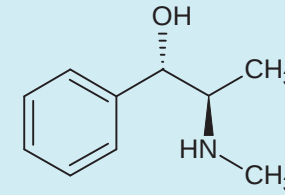


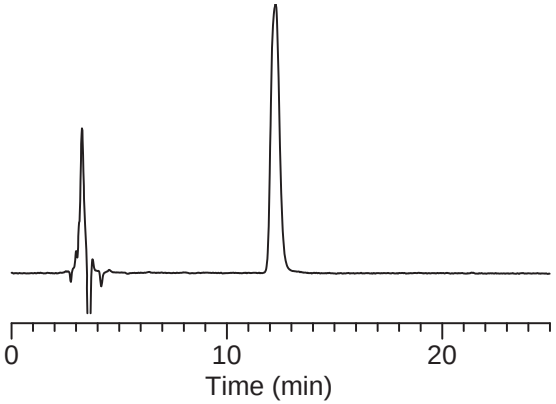

Chiral AGP Column
10 mM ammonium phosphate/
1 mM octanoic acid




(1R, 2S)-(-)-Ephedrine

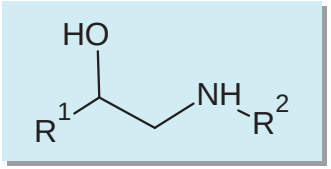



(1S, 2R)-(+)-Ephedrine

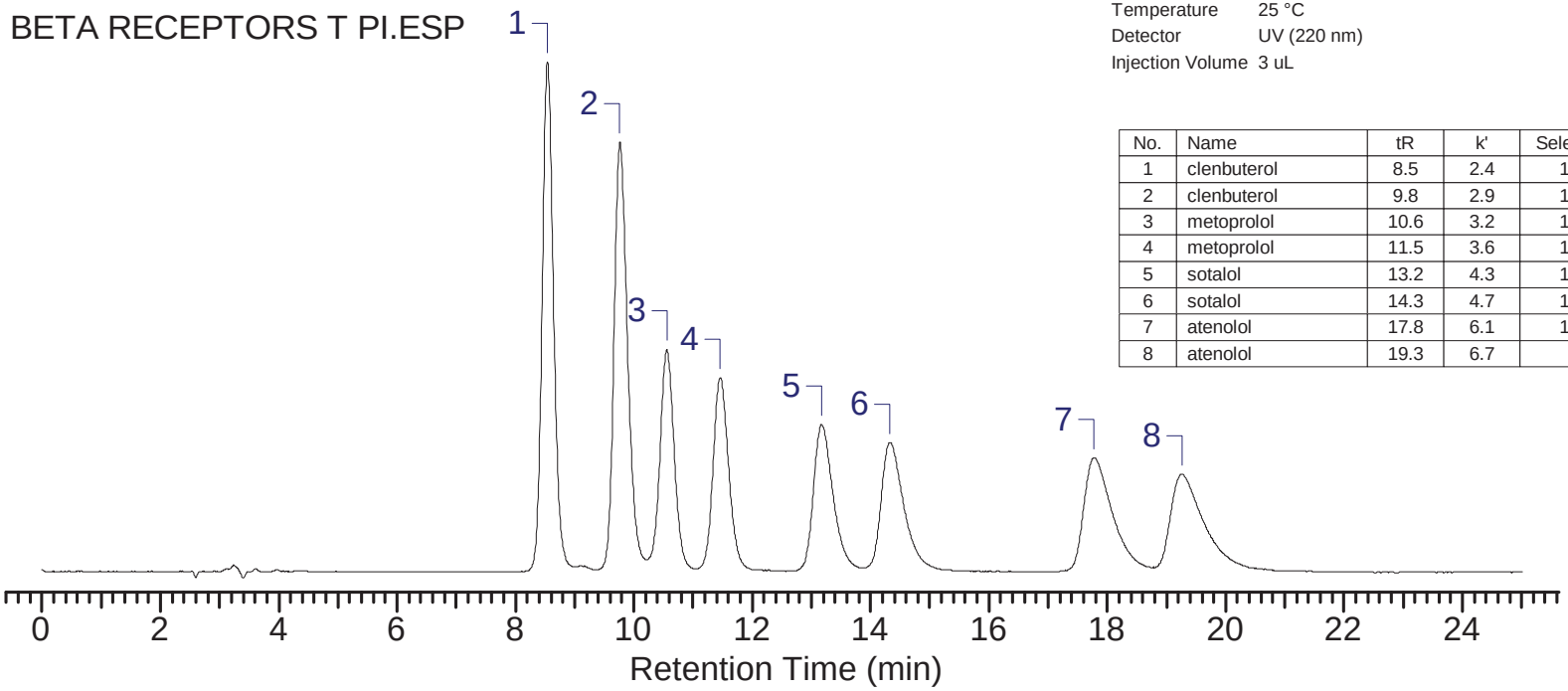



Chirobiotic T
Methanol:Acetic Acid:TEA

# β-Blocker Separation on Chirobiotic T




| | |
|---|---|
| Column Name | CHIROBIOTIC T |
| Length | 25 cm |
| Diameter | 0.46 cm |
| Particle Size | 5 um |
| Mobile Phase | methanol; 15mM ammonium formate |
| Flow Rate | 1 ml/min |
| Temperature | 25 °C |
| Detector | UV (220 nm) |
| Injection Volume | 3 uL |




| No. | Name | tR | k' | Selectivity |
|---|---|---|---|---|
| 1 | clenbuterol | 8.5 | 2.4 | 1.20 |
| 2 | clenbuterol | 9.8 | 2.9 | 1.11 |
| 3 | metoprolol | 10.6 | 3.2 | 1.11 |
| 4 | metoprolol | 11.5 | 3.6 | 1.19 |
| 5 | sotalol | 13.2 | 4.3 | 1.11 |
| 6 | sotalol | 14.3 | 4.7 | 1.29 |
| 7 | atenolol | 17.8 | 6.1 | 1.10 |
| 8 | atenolol | 19.3 | 6.7 | - |

# Cyclodextrin (CYCLOBOND™) and Macrocyclic glycopeptide (CHIROBIOTIC™) Chiral Stationary Phases

- CHIROBIOTIC & CYCLOBOND HPLC columns provide method development complementary to polysaccharide columns
- Multiple bonded chiral selectors make them extremely robust and compatible with all commonly used HPLC solvents – including chlorinated ones
- Both stationary phase types are MS compatible
- CHIROBIOTIC CSPs are especially useful for the chiral separation of polar compounds

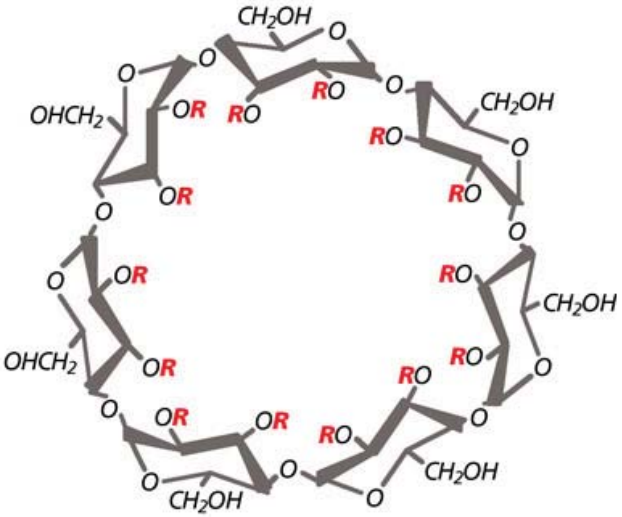
# CYCLOBOND Chiral Stationary Phases

Consist of covalently bonded β-cyclodextrin or modified (derivatized) β-cyclodextrin to porous silica

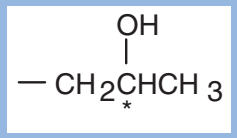
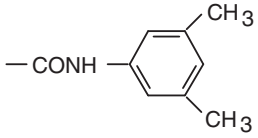
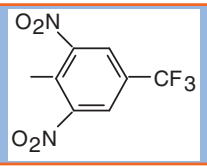
- Chiral, apolar interior of the cyclodextrin “basket”
- Chemical modifications to the secondary hydroxyls (50%-100%) of the cyclodextrin to add additional interactions

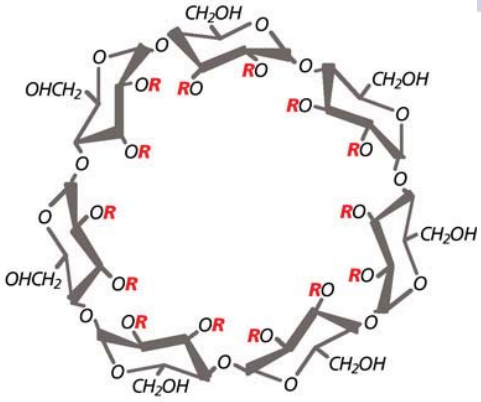
Types of interactions:

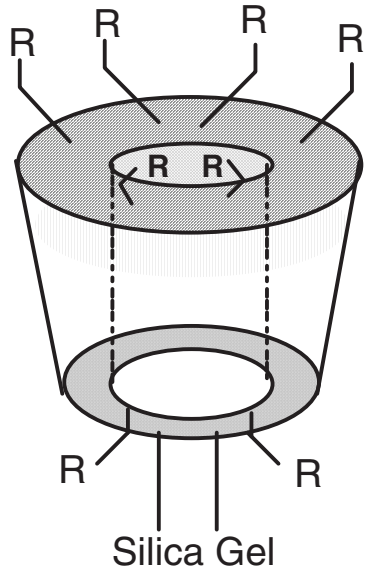
- Hydrophobic inclusion
- Hydrogen bonding interactions
- Steric interactions
- Dipole-dipole interactions
- π-π interactions



# CYCLOBOND: Bonded Cyclodextrins for HPLC

| R = | Designation | CD Type |
|---|---|---|
| none | **CYCLOBOND I 2000**<br>**CYCLOBOND II 2000** | β CD<br>γ CD |
| - $OCH_3$ | **CYCLOBOND I 2000 DM**<br>(methylated) | β CD |
| - $COCH_3$ | **CYCLOBOND I 2000 AC**<br>**CYCLOBOND II 2000 AC**<br>(acetylated) | β CD<br>γ CD |
|  | **CYCLOBOND I 2000 RSP or HP-RSP or SP**<br>(Racemic or S-hydroxypropyl ether,<br>HP = high performance) | β CD |
|  | **CYCLOBOND I 2000 DMP**<br>(3,5-dimethylphenyl carbamate) | β CD |
|  | **New: CYCLOBOND I 2000 DNP**<br>(2,6-dinitro-4-trifluoromethyl phenyl ether) | β CD |





# CHIROBIOTIC Chiral Stationary Phases

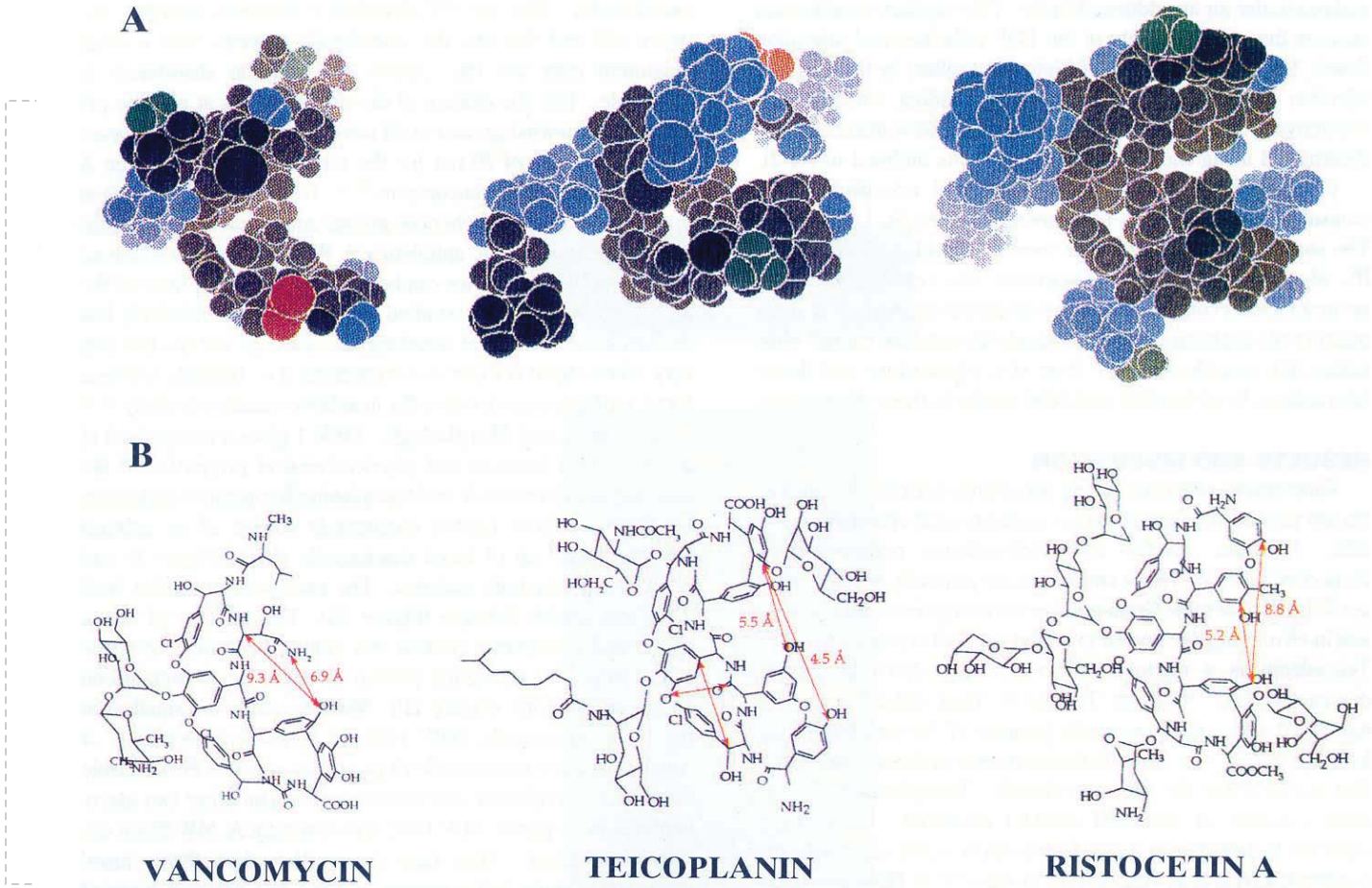
- **Macrocyclic glycopeptides** provide a multi-modal chiral surface capable of a wide variety of different interactions
- To date, there are 6 types of CSPs commercially available
- Subtle differences between them help to reveal the dominant mechanisms that lead to enantiomeric recognition
- Among these mechanisms, **ionic interactions** dominate for ionizable molecules
- *Macrocyclic glycopeptides CSPs provide a valuable source of separations for polar molecules*

# CHIROBIOTIC Chiral Stationary Phases

| | |
|---|---|
| CHIROBIOTIC V, V2 | Vancomycin |
| CHIROBIOTIC T, T2 | Teicoplanin |
| CHIROBIOTIC R | Ristocetin |
| CHIROBIOTIC TAG | Teicoplanin Aglycone |
| NOTE: V2, T2 differ from V, T in the chemistry used to bond the glycopeptide to the silica.<br>The V2 and T2 often give higher selectivity for many applications and both have higher capacity for prep. | |

- **Broad based** chiral stationary phases for basic, acidic *and* neutral molecules
- **Chemically** bonded to pure silica (>4 linkages), so very robust
- **Stable** to high flow rates and pressures

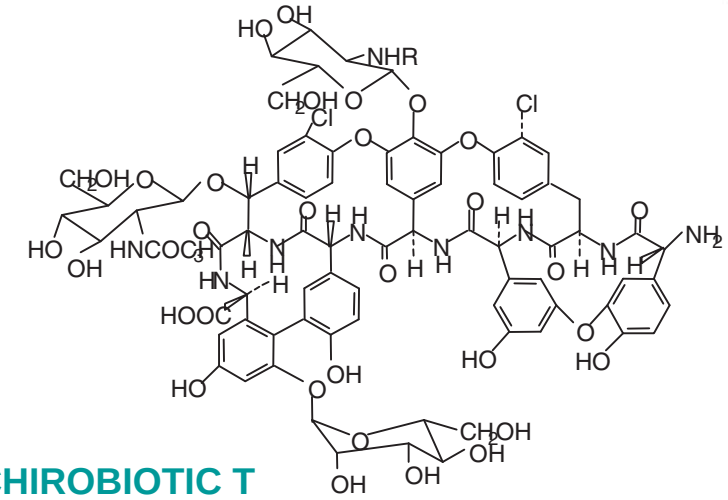
# Macrocyclic glycopeptides (CHIROBIOTICs)



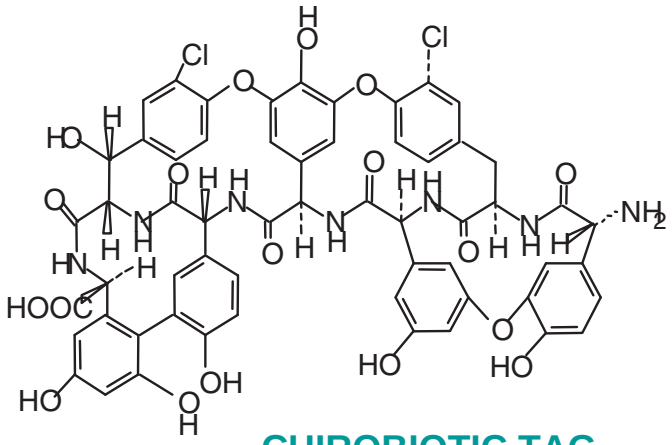

A. Profile view of the aglycone basket of space-filling molecular models
B. Stick figures

# Mobile Phase Types for CHIROBIOTIC CSPs

- **Polar Ionic Mode** – a ***non-aqueous*** mobile phase. Unique to CHIROBIOTICS: fast, perfect for prep, **MS** detection
  - for ***ionizable*** molecules – any acid or base
- **Reversed Phase** – **MS** compatible, ideal for manufacturing QC, bioanalysis
  - for ***all types*** of molecules
- **Normal Phase** –
  - about 15% of all applications



**CHIROBIOTIC T**



**CHIROBIOTIC TAG**

# Advantages in the Polar Ionic Mode (PIM)

- Good sample solubility
- MS compatible
- Broad selectivity, high efficiency
- Easy to manipulate
- Low pressure, long column life
- Low volatility/high solubility solvent, excellent for prep

# CHIROBIOTIC CSPs and LC-MS to Study Retention and Selectivity

- CHIROBIOTIC stationary phases
  - Especially good for the separation of polar compounds
  - Operate well in both reversed-phase and polar ionic modes
  - Highly amenable to the use of LC-MS systems

- LC-MS has the ability to separate in the mass/charge dimension
  - Run a composite set of probes to assess the impact of operational parameters on enantiomeric selectivity for many analytes simultaneously.

# Purpose of Study

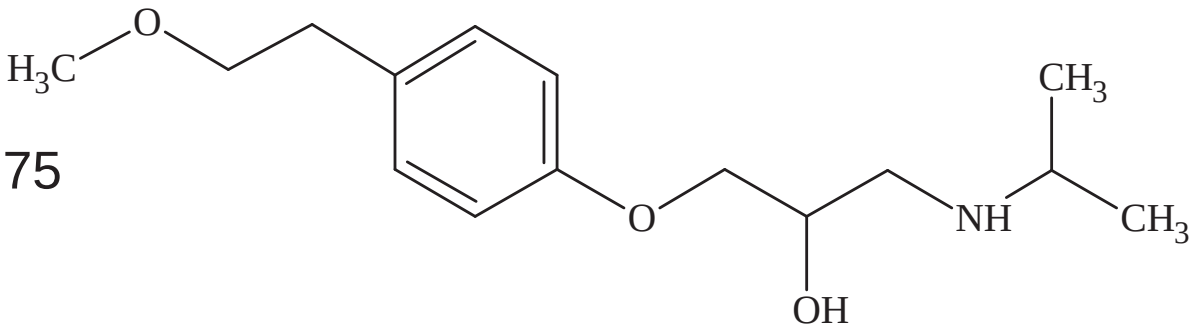
- Utilize the LC-MS approach for a set of basic probes differing in $pK_a$ values, hydrophobicity and molecular weight to determine the impact of variables on retention and selectivity.
  - buffer (salt) type,
  - buffer concentration
  - acid/base ratio
  - Chirobiotic stationary phases
- Move from traditional UV screening to LC-MS-based screening protocol
  - Increase throughput – multiple samples/run
  - Decrease false-positives
- Resolve issues with traditionally-used modifiers
  - TFA
  - TEA
  - Non-volatile salts
- Overcome additional obstacles

# Experimental

| | |
|---|---|
| Instrument: | Waters/Micromass ZQ, Single Quadrupole, Waters Alliance 2690 |
| Column: | Chirobiotic T, 150 cm x 4.6 mm, 5 µm |
| Temperature: | 35° C |
| Flow Rate: | 1 mL/min |
| Mobile Phase: | 0.1%, w/v ammonium acetate in methanol (Polar Ionic Mode) |
| Detection: | ESI, Positive Ion Mode, scan range m/z 150–500 |
| Inj. Vol.: | 5 µL |

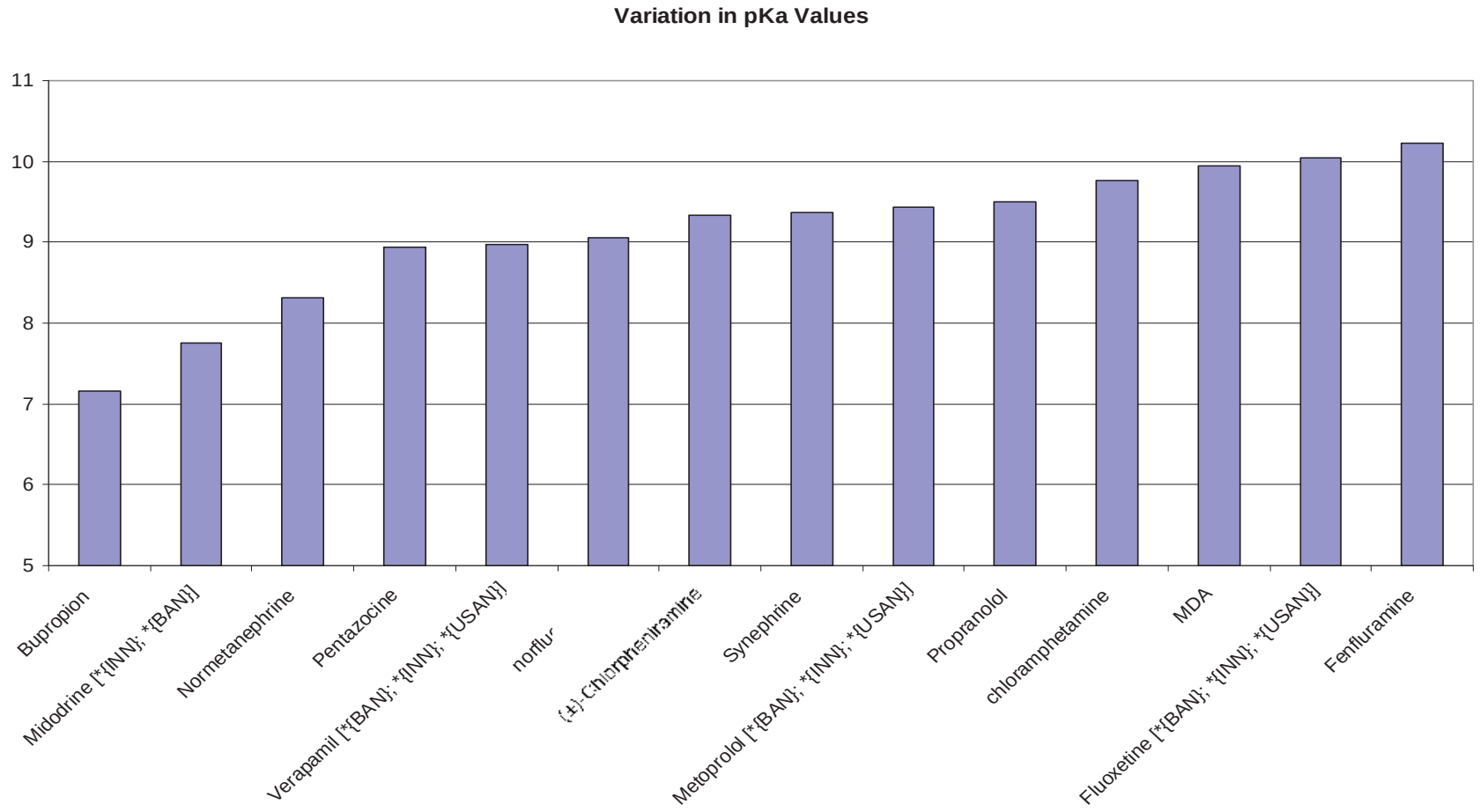
# Composite Test Probe- Basic Analytes

synephrine, m/z 168
chloramphetamine, m/z 170
methylenedioxyamphetamine (MDA) m/z 180
normetanphrine, m/z 184 (base peak m/z 166 $-H_2O$)
fenfluramine, m/z 232
bupropion, m/z 240
midodrine, m/z 255
propranolol, m/z 260
metoprolol, m/z 268
chlorpheniramine, m/z 275
pentazocine, m/z 286
norfluoxetine, m/z 296
fluoxetine, m/z 310
verapamil, m/z 455

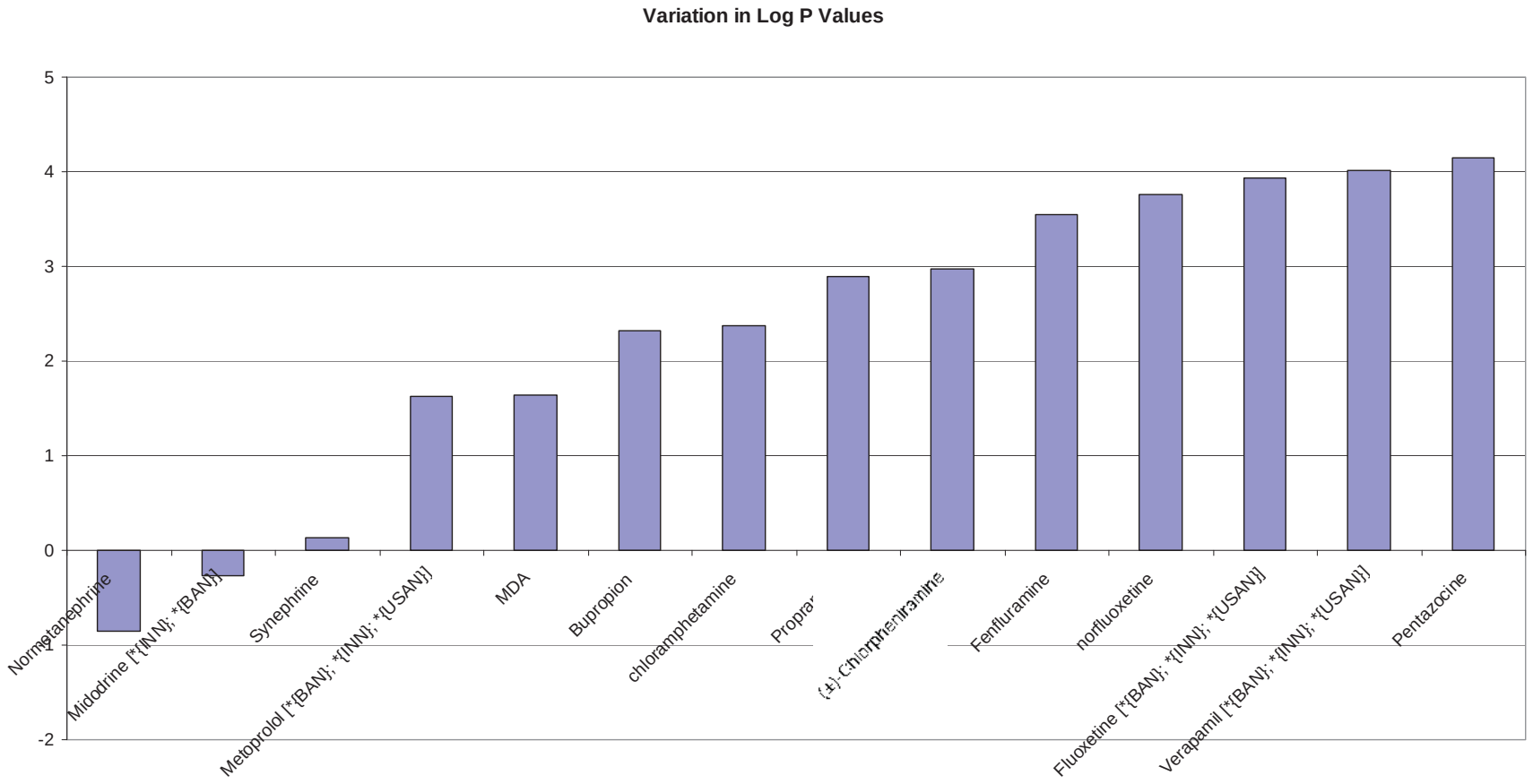



Metoprolol

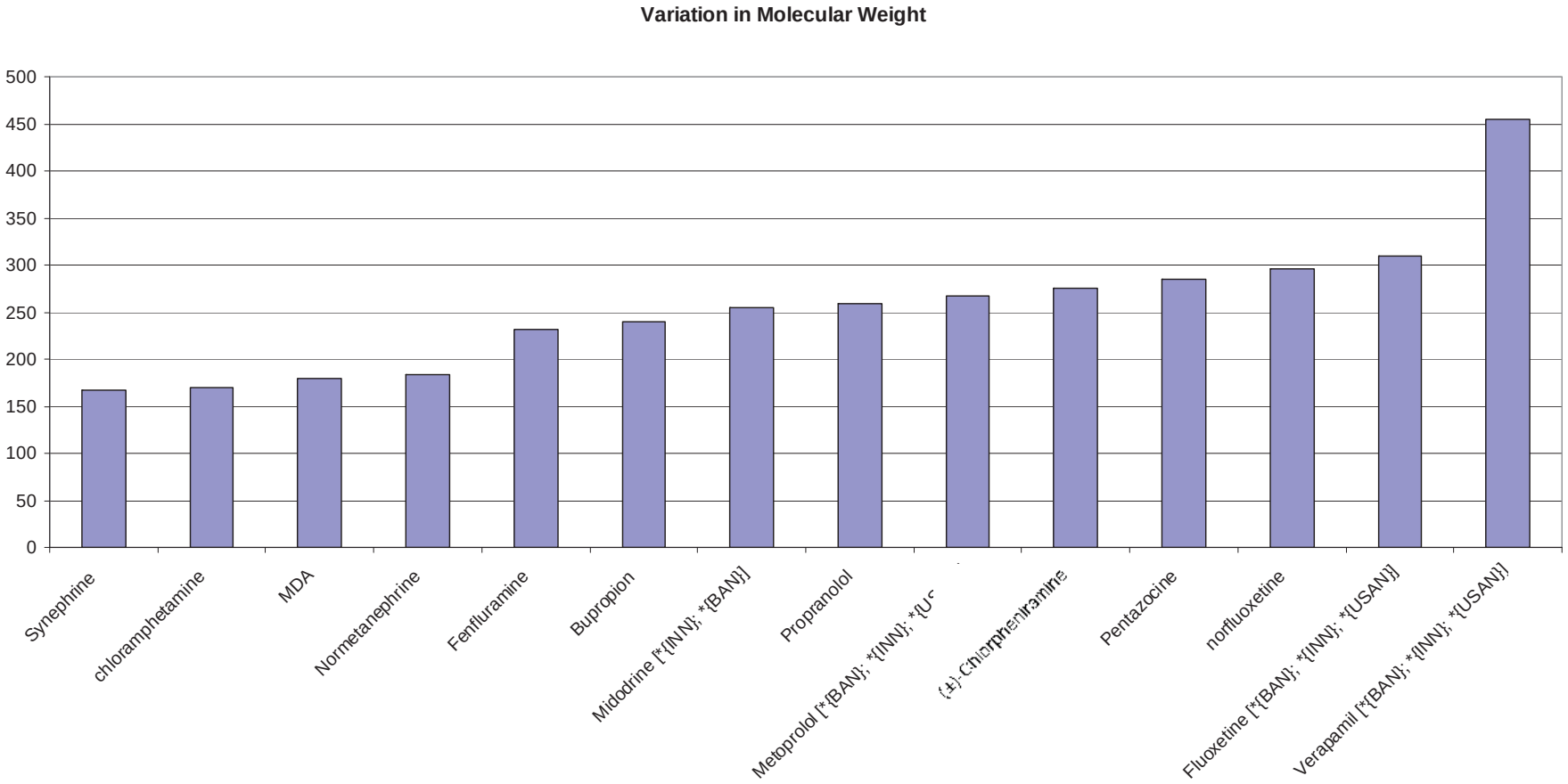
# $pK_a$ Values for Probe Analytes



# Log P Values for Probe Analytes



# Molecular Weights for Probe Analytes



# Validation of LC-MS Approach

- Chirobiotic T has been shown to be effective for the separation of β-blocker enantiomers (1-2).

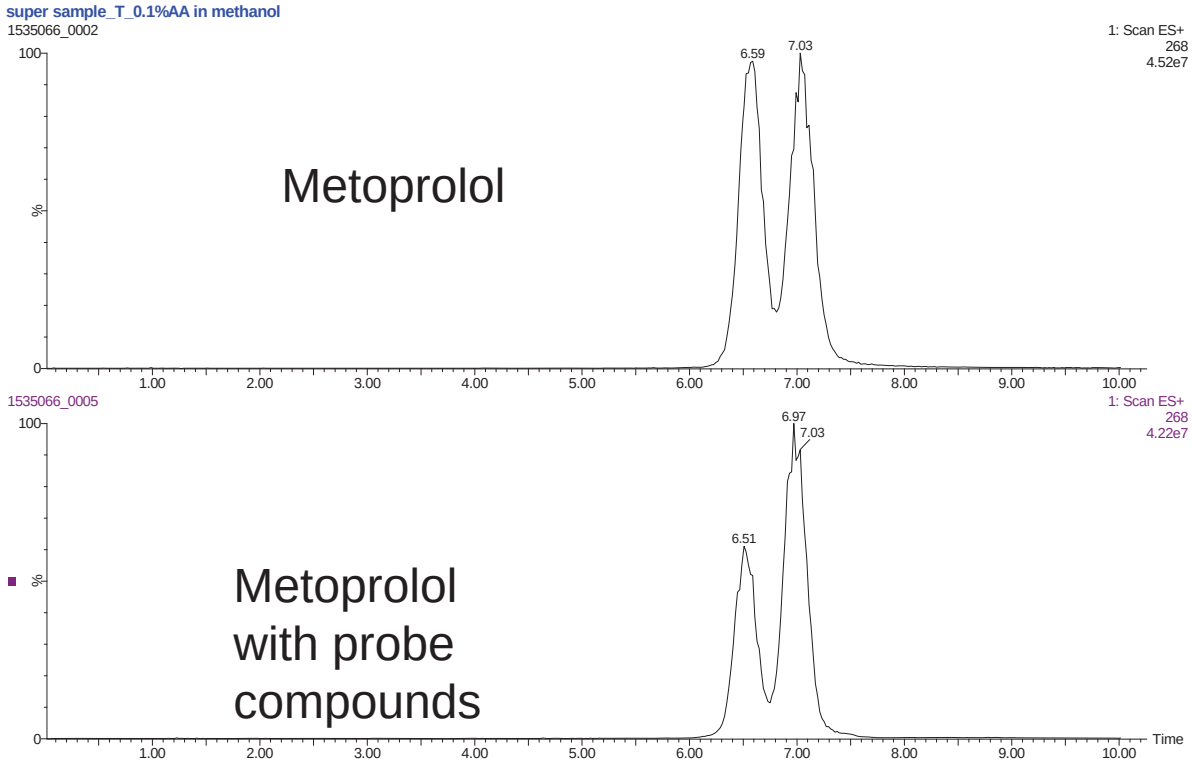
- In this study, the β-blocker metoprolol was first injected alone and then in the presence of the 13 additional compounds; significant retention time overlap was observed.

- The success of the approach was confirmed and then utilized to investigate the impact of buffer type, concentration and acid/base ratio on retention and selectivity in Polar Ionic Mode.


1. Jensen, B. P., C. F. Sharp, et al. (2008). "Development and validation of a stereoselective liquid chromatography-tandem mass spectrometry assay for quantification of S- and R-metoprolol in human plasma." Journal of Chromatography B 865(1-2): 48-54.

2. Bell, D., C. Aurand, J. Claus, D. Schollenberger and J. Jones, Chiral LC-MS Analysis of Drug Substances (Beta-Blockers) from Plasma Using Macrocyclic Glycopeptide Chiral Stationary Phases, Pittcon 2009, Poster Tuesday PM.

# Comparison of Metoprolol Alone and in Probe Mix



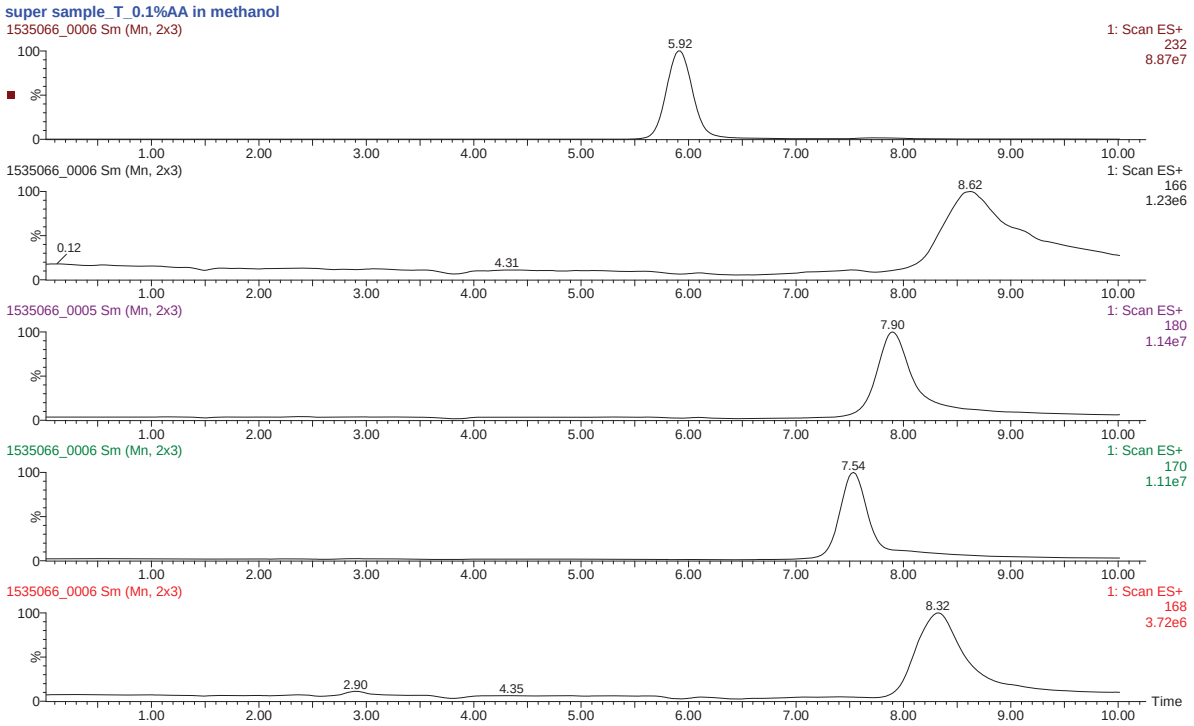

Note a slight variation in enantiomer response due to ion-suppression by coeluting peaks; however, retention and selectivity is not compromised

Chirobiotic T, 0.1% $NH_4Ac$ in Methanol (Polar Ionic Mode), ESI+ (Extracted Ion Current).

# Probe Mix using 0.1% Ammonium Acetate in Methanol

Set 1:

**Extracted Ion Current (XIC)**




Fenfluramine

Normetanphrine

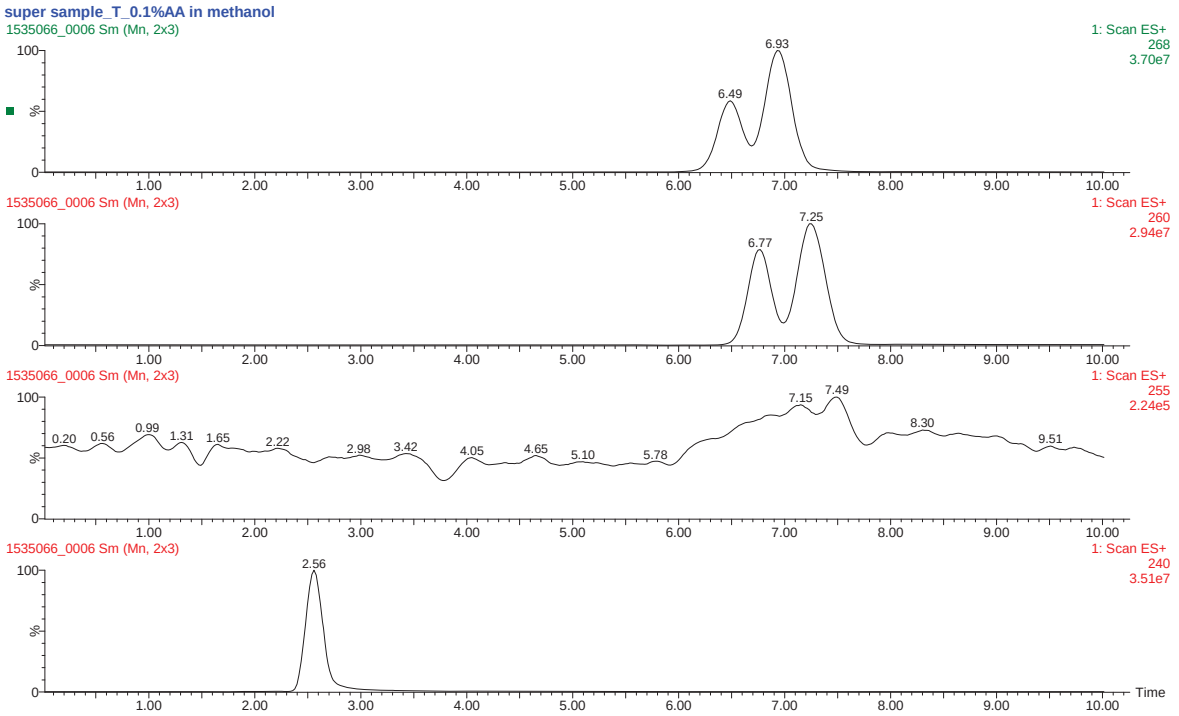
MDA

Chloramphetamine

Synephrine

# Probe Mix using 0.1% Ammonium Acetate in Methanol

Set 2:




Metoprolol

Propranolol

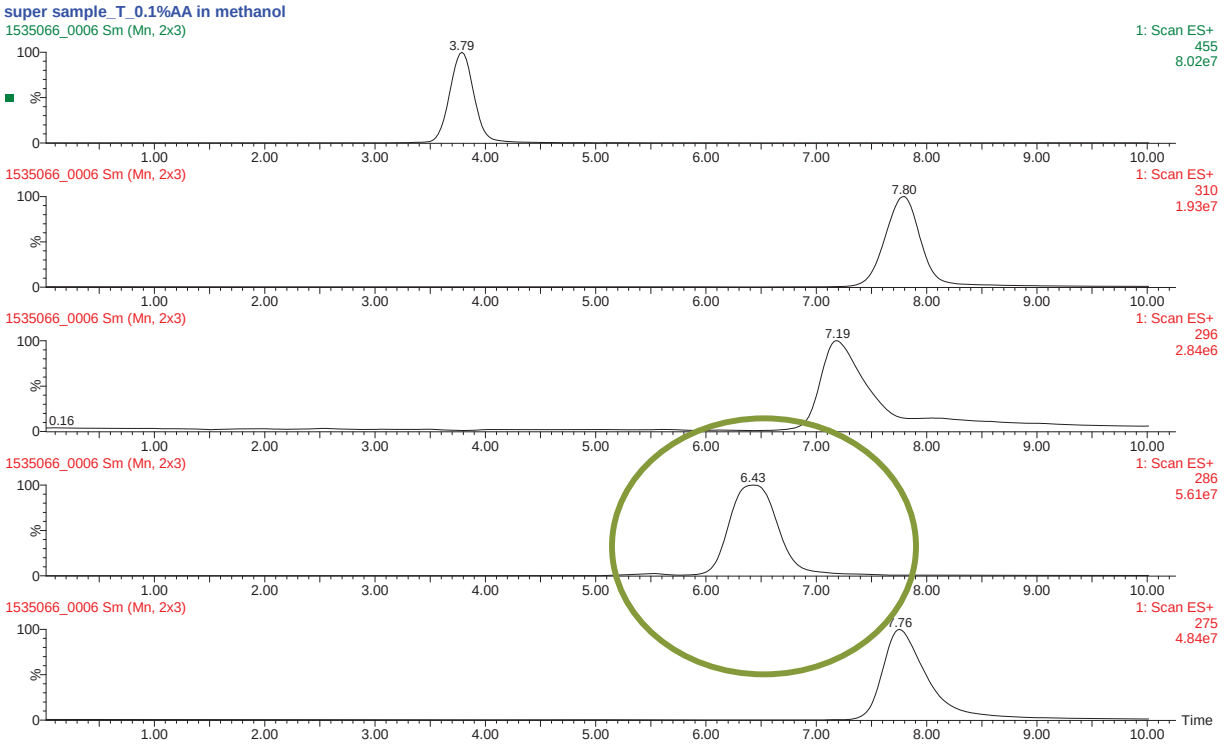
Midodrine[1]

Bupropion

1. Midodrine was not observed under these conditions.

# Probe Mix using 0.1% Ammonium Acetate in Methanol

## Set 3:




Verapamil

Fluoxetine

Norfluoxetine

Pentazocine

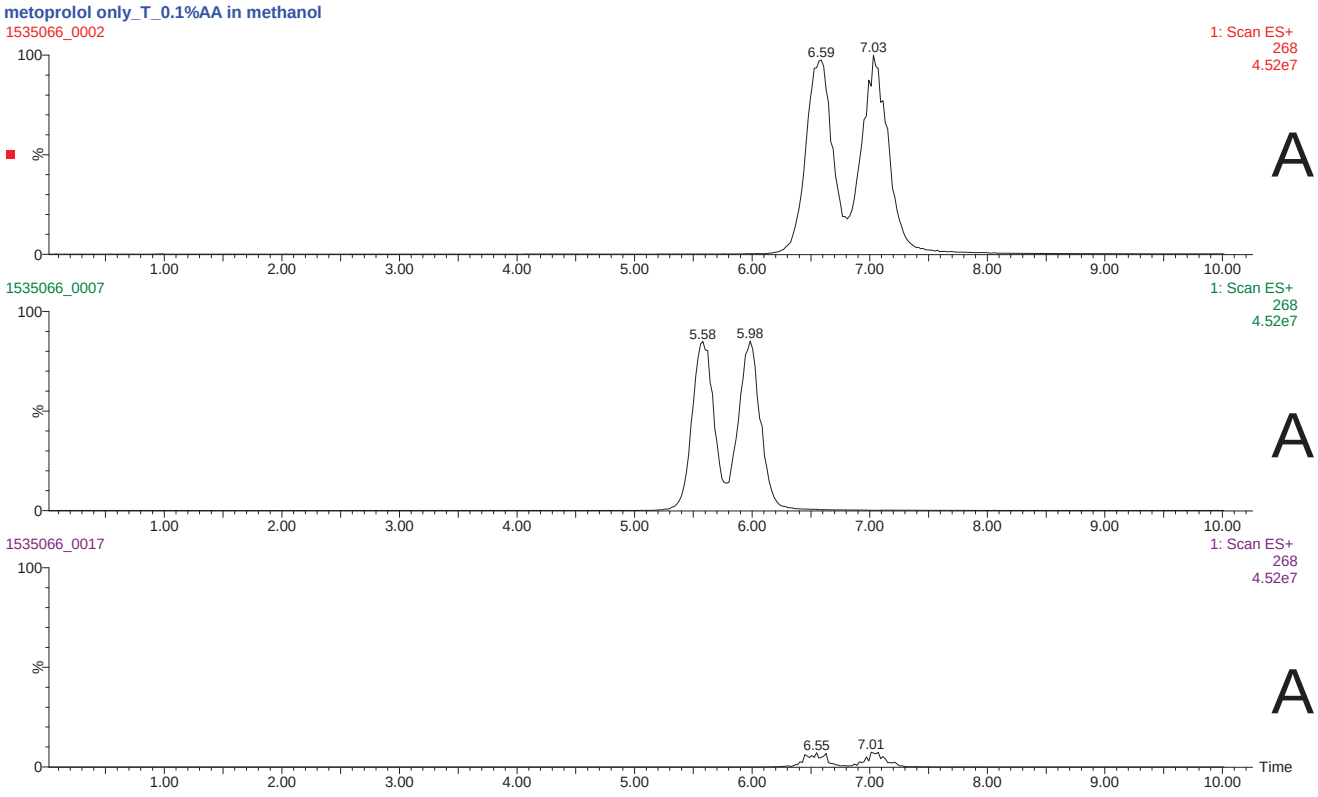
Chlorpheniramine

# Impact of Buffer Type on Retention and Enantiomeric Selectivity

- 0.1% wt/v ammonium acetate, ammonium formate and ammonium trifluoroacetate were prepared in methanol.
- Retention and selectivity were monitored as a function of the buffer type.
- The complex probe mixture was run using multiple injections to confirm system equilibration.

# Comparison of Buffers – Metoprolol in Probe Mix

Anion has a major effect on response, a slight effect on retention, and no significant effect on selectivity.




Ammonium Acetate

Ammonium Formate

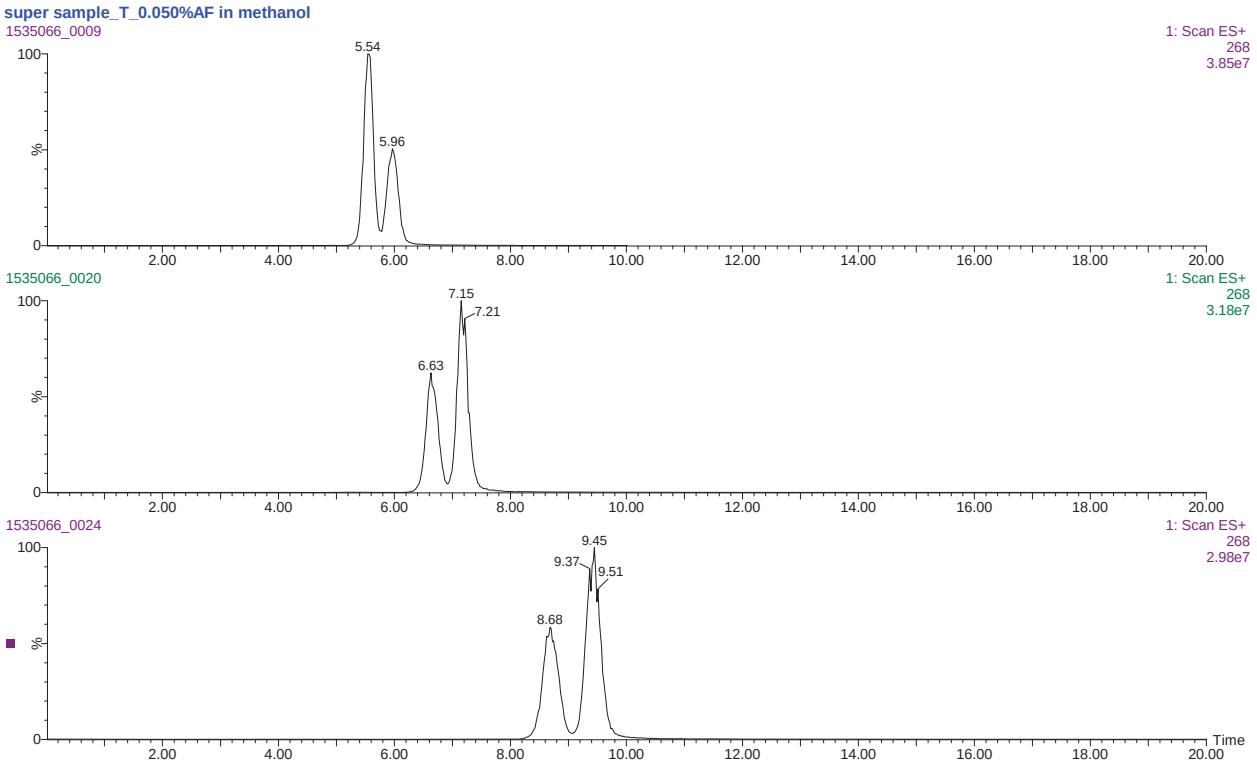
Ammonium TFA

# Impact of Buffer Concentration on Retention and Enantiomeric Selectivity

- The complex probe mixture was run using 0.1%, 0.075% and 0.05% ammonium formate (AF) in methanol.
- Retention and selectivity were monitored as a function of the buffer concentration.

# Impact of Buffer Concentration on Metoprolol Retention and Selectivity

Concentration has an effect on retention, but no major effect on selectivity or response.



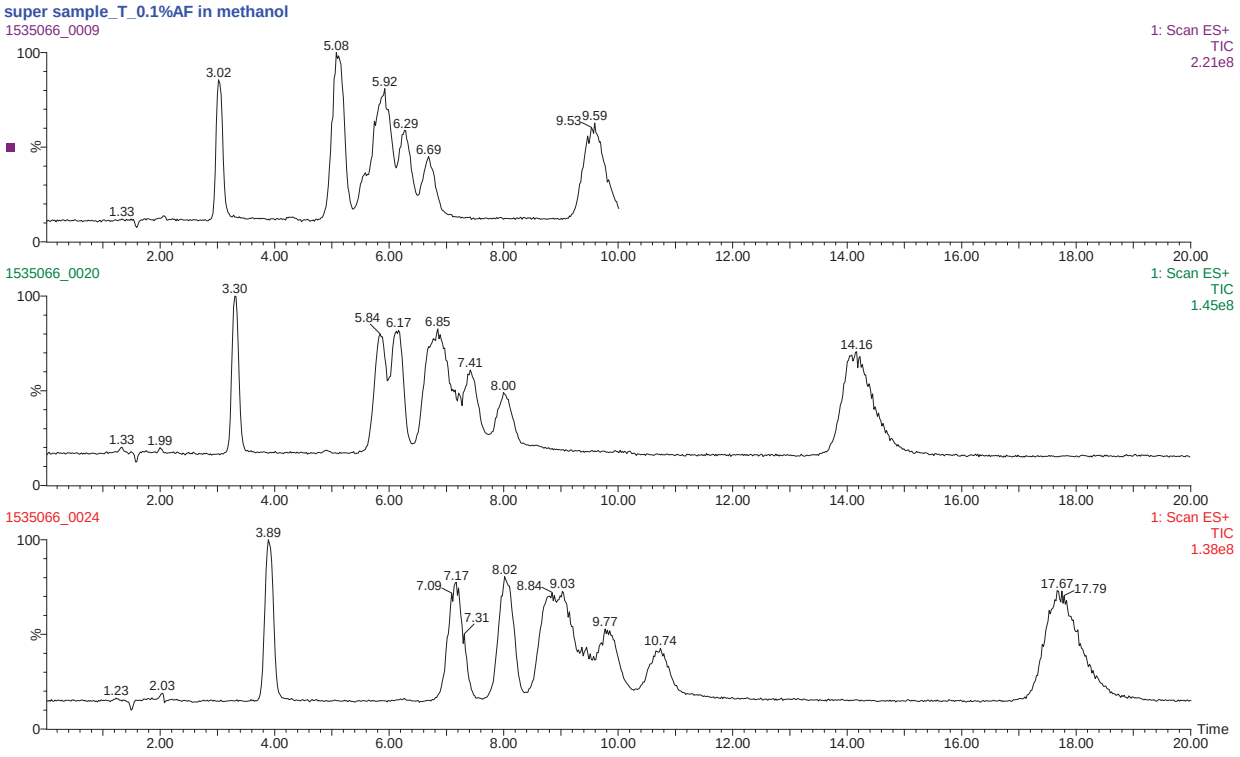

0.1% AF

0.075% AF

0.05% AF

# Impact of Buffer Concentration on Retention and Selectivity for Complex Probe Mix

Concentration of ammonium formate has an effect on retention, but no major effect on selectivity or response.




0.1% AF

0.075% AF

0.05% AF

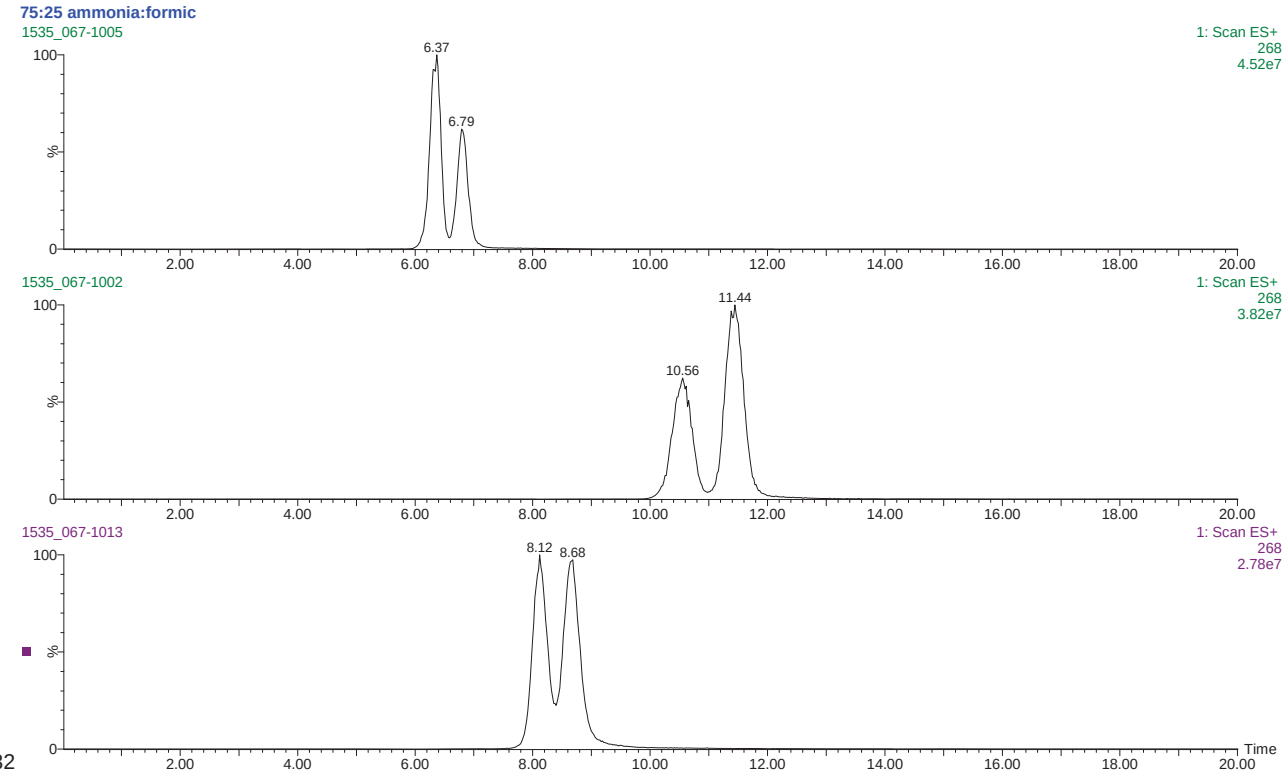
# Impact of Buffer Component Ratio on Retention and Selectivity

- 13 mM ammonium hydroxide and 13 mM formic acid were independently prepared in methanol
- The complex sample was run using acid:base ratios of 3:1, 1:1 and 1:3
- Retention and enantiomeric selectivity were monitored
- Runs were repeated to ensure equilibration

# Impact of Buffer Component Ratio on Metoprolol Retention and Selectivity

Ratio creates a significant change in retention plus some change in selectivity.

Ratio is base:acid




1:3

1:1

3:1

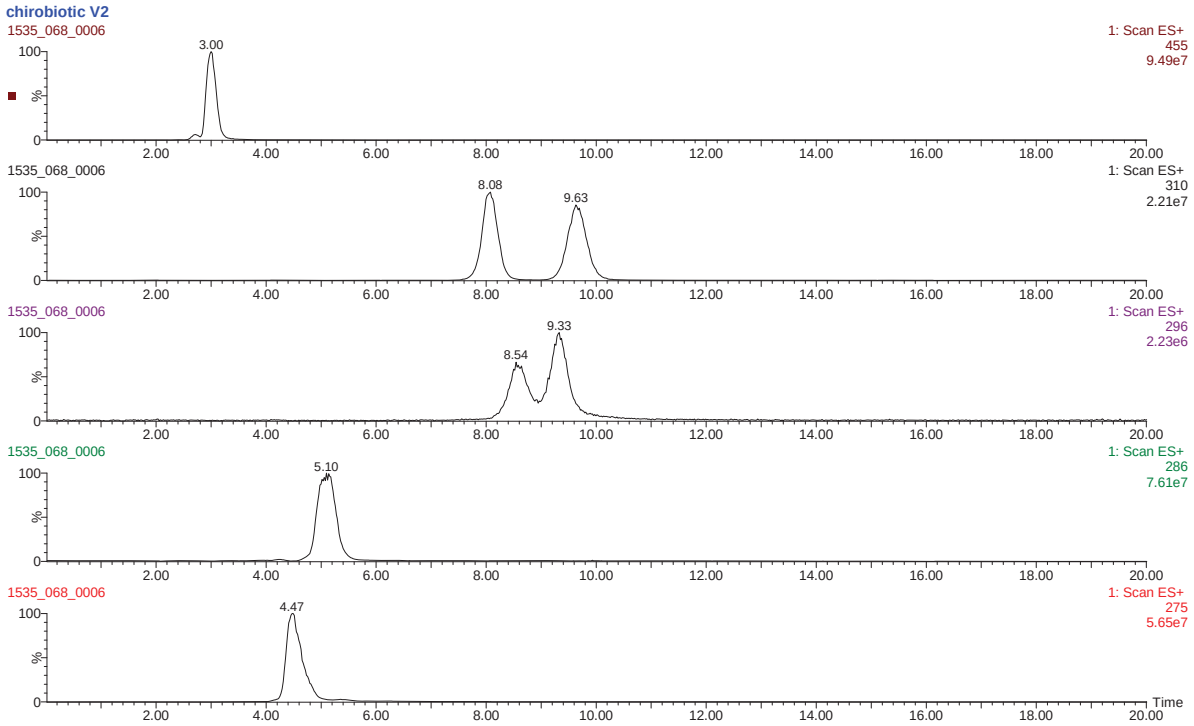
# Impact of Stationary Phase Chemistry for Three CSPs on Retention and Selectivity

Chirobiotic V2, TAG and R were run to assess the impact of stationary phase on the set of basic analytes

| | |
|---|---|
| Instrument: | Waters/Micromass ZQ, Single Quadrupole, Waters Alliance 2690 |
| Column: | Chirobiotic V2, TAG and R, 150 x 4.6 mm |
| Temperature: | 35° C |
| Flow Rate: | 1 mL/min |
| Mobile Phase: | Ammonium formate in methanol (13 mM) |
| Detection: | ESI, Positive Ion Mode, scan range m/z 150–500 |
| Inj. Vol.: | 5 µL |

# Chirobiotic V2 Shows Selectivity Towards Fluoxetine and Norfluoxetine

Unique selectivity between V2 phase and certain solutes shows up in complex probe mix.




Verapamil

Fluoxetine

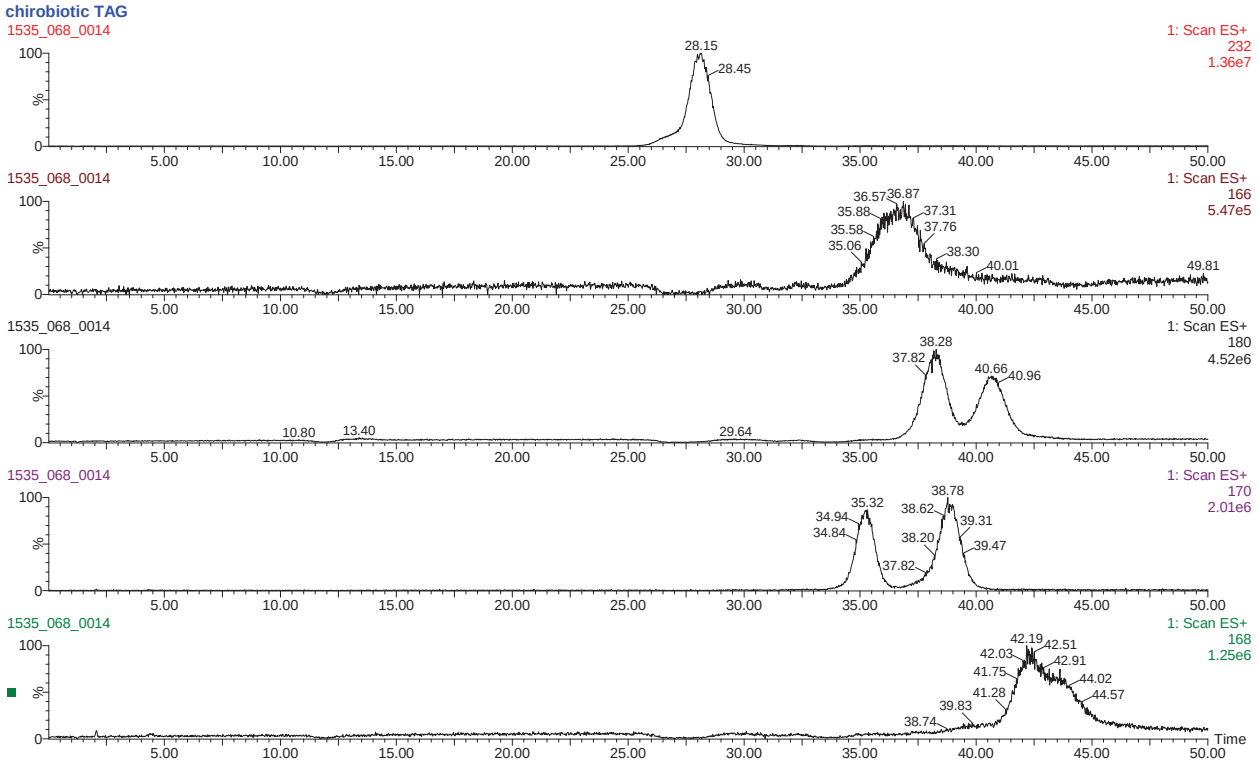
Norfluoxetine

Pentazocine

Chlorpheniramine

# Chirobiotic TAG Shows Selectivity Towards the Amphetamines

Unique selectivity between TAG phase and certain solutes shows up in complex probe mix.




Fenfluramine

Normetanphrine

MDA

Chloramphetamine

Synephrine

# Column Screening

- Changing the CSP is still the most useful means of altering enantiomeric selectivity.
- Each of the Chirobiotic phases showed selectivity toward different analytes (except R, which is generally more applicable to acidic compounds).
- Other than very general trends, selectivity remains unpredictable, necessitating a column screening approach to method development.
- When CSPs are compatible, LC-MS can make this easier by allowing mixtures of different enantiomers to be screened simultaneously.

# Published Statistics – 53 Chiral Compounds

| % Positive | CSP | Mobile Phases | No. Operating Parameters |
|---|---|---|---|
| 87% | AS, AD, OD, OJ | 5 | 20 |
| 65%* | V, T, R | 2 | 6 |
| 96% | Combined | 7 | 26 |

*If HP-RSP had been added, % would have gone to 74% due to antifungal agents.
*If DNP had been added in the POM, % would have gone to 89%.

Ref: Evaluation of Generic Liquid Chromatography Screens for Pharmaceutical Analysis, Andersson, M.E., Aslan, D., Clarke, A., Roeraade, J. Hagman, G., Journal of Chromatography A, 1005 (2003) 83-101.

# HPLC CHIROBIOTIC and CYCLOBOND Column Screening

Established protocol includes a 3 mobile phase set of conditions run using 6 different stationary phase chemistries as a front line screening. This front line is generally providing 80% of our screening successes.

**Columns:**

- **CHIROBIOTIC™**
  - V2
  - T
  - TAG
- **CYCLOBOND™**
  - β-CD
  - DMP
  - HP-RSP

**Mobile Phases:**

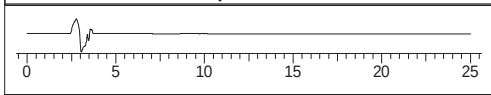
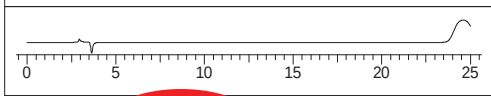
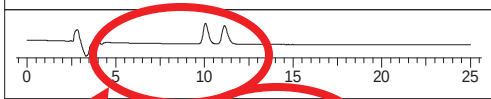
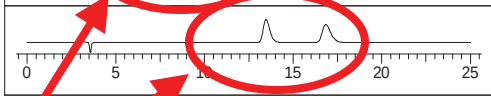
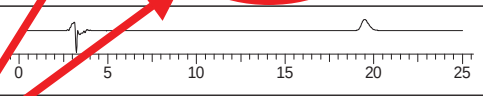
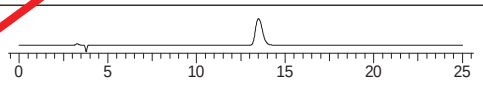
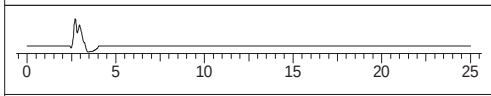
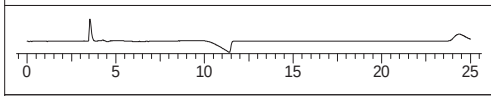
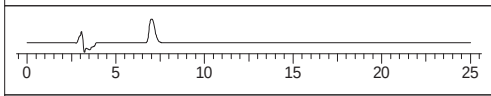
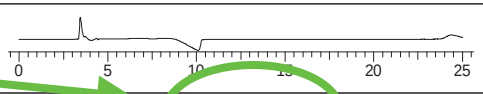
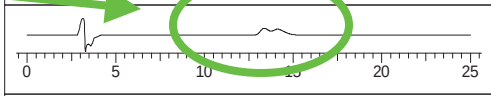
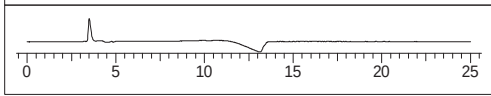
- **Polar-Ionic Mode (PIM)**
  - 100:0.1:0.1, methanol:acetic acid:triethylamine
- **Reversed-Phase (RP)**
  - 70:30, 20 mM ammonium acetate (pH 4.0):acetonitrile
- **Polar-Organic Mode (POM)**
  - 95:5:0.3:0.2, acetonitrile:methanol: acetic acid:triethylamine.

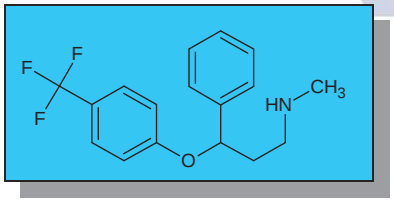
*Variations made based on customer-specific information. LC-MS specific protocol also available.

# Results of Fluoxetine Column Screen

(+/-)-Fluoxetine underwent the primary screening protocol and yielded positive results.

**CHIROBIOTIC V2 in both RP and PIM provided excellent selectivity while CYCLOBOND I 2000 DNP showed some selectivity in RP**

| Spectrum | Column | mode | elution |
|---|---|---|---|
|  | CHIROBIOTIC TAG | RP | No Elution |
|  | CHIROBIOTIC TAG | PIM | No Separation |
|  | CHIROBIOTIC V2 | RP | Separation |
|  | CHIROBIOTIC V2 | PIM | Separation |
|  | CHIROBIOTIC T | RP | No separation |
|  | CHIROBIOTIC T | PIM | No Separation |
|  | Cyclobond I 2000 | RP | No Retention |
|  | Cyclobond I 2000 | POM | No Separation |
|  | Cyclobond 2000 HP-RSP | RP | No Separation |
|  | Cyclobond 2000 HP-RSP | POM | No Separation |
|  | Cyclobond 2000 DNP | RP | Separation |
|  | Cyclobond 2000 DNP | POM | Unknown |




•Screening results are viewed in a tabular form for easy review and comparison.





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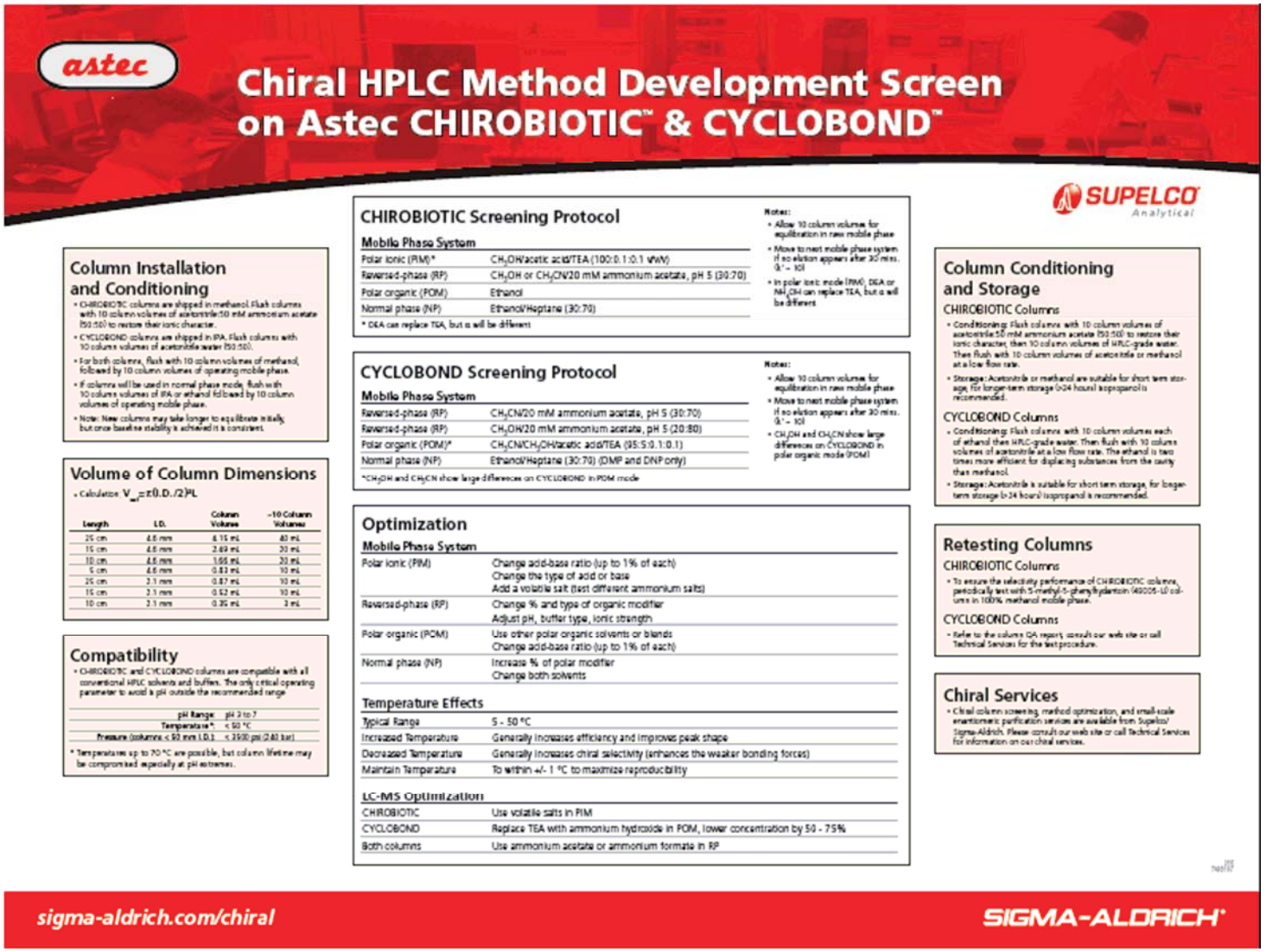
# Chiral Wall Chart



astec

## Chiral HPLC Method Development Screen on Astec CHIROBIOTIC™ & CYCLOBOND™

SUPELCO Analytical

### Column Installation and Conditioning

- CHIROBIOTIC columns are shipped in methanol. Flush columns with 10 column volumes of acetonitrile:50 mM ammonium acetate (50:50) to restore their ionic character.
- CYCLOBOND columns are shipped in IPA. Flush columns with 10 column volumes of acetonitrile:water (50:50).
- For both columns, flush with 10 column volumes of methanol, followed by 10 column volumes of operating mobile phase.
- If columns will be used in normal phase mode, flush with 10 column volumes of IPA or ethanol followed by 10 column volumes of operating mobile phase.
- Note: New columns may take longer to equilibrate initially, but once baseline stability is achieved it is consistent.

### Volume of Column Dimensions

- Calculation: $V_m = \pi (I.D./2)^2 L$

| Length | I.D. | Column Volume | ~10 Column Volumes |
|---|---|---|---|
| 25 cm | 4.6 mm | 4.15 mL | 40 mL |
| 15 cm | 4.6 mm | 2.69 mL | 30 mL |
| 10 cm | 4.6 mm | 1.66 mL | 20 mL |
| 5 cm | 4.6 mm | 0.83 mL | 10 mL |
| 25 cm | 2.1 mm | 0.87 mL | 10 mL |
| 15 cm | 2.1 mm | 0.52 mL | 10 mL |
| 10 cm | 2.1 mm | 0.35 mL | 3 mL |

### Compatibility

- CHIROBIOTIC and CYCLOBOND columns are compatible with all conventional HPLC solvents and buffers. The only critical operating parameter to avoid is pH outside the recommended range

| | |
|---|---|
| pH Range: | pH 2 to 7 |
| Temperature*: | < 50 °C |
| Pressure (columns < 50 mm I.D.): | < 3500 psi (240 bar) |

* Temperatures up to 70 °C are possible, but column lifetime may be compromised especially at pH extremes.

### CHIROBIOTIC Screening Protocol

**Mobile Phase System**

| | |
|---|---|
| Polar Ionic (PIM)* | $CH_3OH$/acetic acid/TEA (100:0.1:0.1 v/v/v) |
| Reversed-phase (RP) | $CH_3OH$ or $CH_3CN$/20 mM ammonium acetate, pH 5 (30:70) |
| Polar organic (POM) | Ethanol |
| Normal phase (NP) | Ethanol/Heptane (30:70) |

* DEA can replace TEA, but α will be different

Notes:
- Allow 10 column volumes for equilibration in new mobile phase
- Move to next mobile phase system if no elution appears after 30 mins. (k' ~ 10)
- In polar ionic mode (PIM), DEA or $NH_4OH$ can replace TEA, but α will be different

### CYCLOBOND Screening Protocol

**Mobile Phase System**

| | |
|---|---|
| Reversed-phase (RP) | $CH_3CN$/20 mM ammonium acetate, pH 5 (30:70) |
| Reversed-phase (RP) | $CH_3OH$/20 mM ammonium acetate, pH 5 (20:80) |
| Polar organic (POM)* | $CH_3CN$/$CH_3OH$/acetic acid/TEA (95:5:0.1:0.1) |
| Normal phase (NP) | Ethanol/Heptane (30:70) (DNP and DNP only) |

*$CH_3OH$ and $CH_3CN$ show large differences on CYCLOBOND in POM mode

Notes:
- Allow 10 column volumes for equilibration in new mobile phase
- Move to next mobile phase system if no elution appears after 30 mins. (k' ~ 10)
- $CH_3OH$ and $CH_3CN$ show large differences on CYCLOBOND in polar organic mode (POM)

### Optimization

**Mobile Phase System**

| | |
|---|---|
| Polar Ionic (PIM) | Change acid-base ratio (up to 1% of each)<br>Change the type of acid or base<br>Add a volatile salt (test different ammonium salts) |
| Reversed-phase (RP) | Change % and type of organic modifier<br>Adjust pH, buffer type, ionic strength |
| Polar organic (POM) | Use other polar organic solvents or blends<br>Change acid-base ratio (up to 1% of each) |
| Normal phase (NP) | Increase % of polar modifier<br>Change both solvents |

**Temperature Effects**

| | |
|---|---|
| Typical Range | 5 - 50 °C |
| Increased Temperature | Generally increases efficiency and improves peak shape |
| Decreased Temperature | Generally increases chiral selectivity (enhances the weaker bonding forces) |
| Maintain Temperature | To within +/- 1 °C to maximize reproducibility |

**LC-MS Optimization**

| | |
|---|---|
| CHIROBIOTIC | Use volatile salts in PIM |
| CYCLOBOND | Replace TEA with ammonium hydroxide in POM, lower concentration by 50 - 75% |
| Both columns | Use ammonium acetate or ammonium formate in RP |

### Column Conditioning and Storage

**CHIROBIOTIC Columns**

- Conditioning: Flush columns with 10 column volumes of acetonitrile:50 mM ammonium acetate (50:50) to restore their ionic character, then 10 column volumes of HPLC-grade water. Then flush with 10 column volumes of acetonitrile or methanol at a low flow rate.
- Storage: Acetonitrile or methanol are suitable for short term storage, for longer-term storage (>24 hours) isopropanol is recommended.

**CYCLOBOND Columns**

- Conditioning: Flush columns with 10 column volumes each of ethanol then HPLC-grade water. Then flush with 10 column volumes of acetonitrile at a low flow rate. The ethanol is two times more efficient for displacing substances from the cavity than methanol.
- Storage: Acetonitrile is suitable for short term storage, for longer-term storage (>24 hours) isopropanol is recommended.

### Retesting Columns

**CHIROBIOTIC Columns**

- To ensure the selectivity performance of CHIROBIOTIC columns, periodically test with 5-methyl-5-phenylhydantoin (40005-LC) columns in 100% methanol mobile phase.

**CYCLOBOND Columns**

- Refer to the column QA report, consult our web site or call Technical Services for the test procedure.

### Chiral Services

- Chiral column screening, method optimization, and small-scale enantiomeric purification services are available from Supelco/Sigma-Aldrich. Please consult our web site or call Technical Services for information on our chiral services.

sigma-aldrich.com/chiral

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# Conclusions, LC-MS screening study

- The utility of LC-MS to study the impact of variables on retention and selectivity simultaneously for a large sample set in chiral separations has been demonstrated.

- Variables such as buffer type, buffer concentration, acid/base ratio and column phase chemistry were investigated in polar ionic mode.

- Selectivity was impacted the greatest by changing stationary phase, confirming the need for column screening as a first step in method development.

- Once selectivity has been observed on a CSP, the adjustment of buffer component ratios appears to have the greatest impact on enantiomeric resolution.

# Conclusions, Contd.

- Both the type of buffer salt and the concentration can be used to manipulate peak shape and retention, but have limited impact on selectivity.

- Batch screening shows excellent potential for speeding up the column selection process.

- Further work is planned to rapidly investigate variables on Cyclodextrin and other CSPs using this batch LC-MS screening technique.

Poster:

Bell, D., J. Claus, J. Jones, Significant Improvements in Chiral Method Development using an LC-MS-Based Screening Approach, Chirality 2009, Poster Monday and Tuesday PM.

# Preparative Chiral HPLC

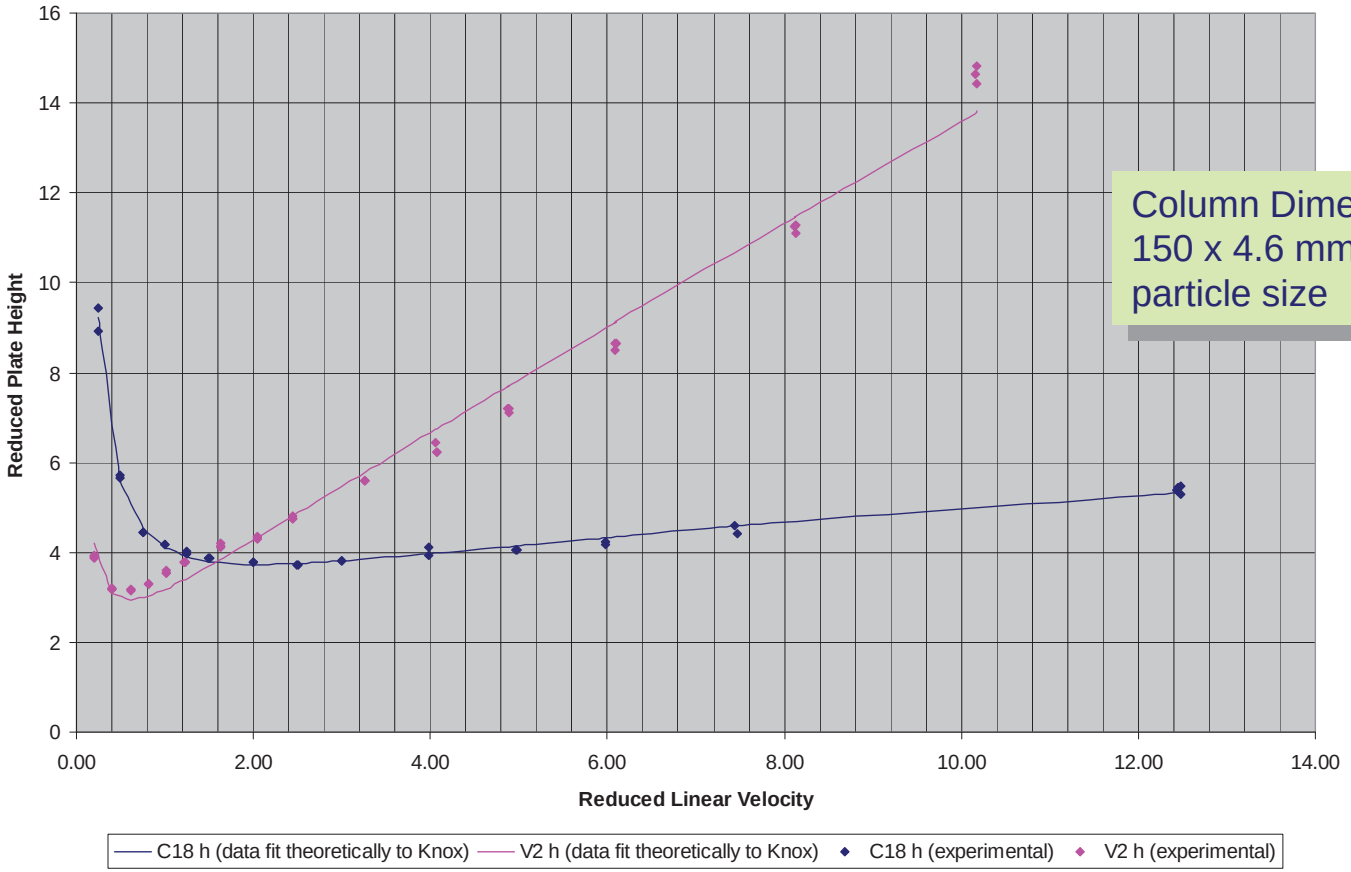
# Benefits of Preparative Separations in Reversed-Phase

- Improved Sample Solubility of Polar Analytes
  - Preparative work cannot be completed if the sample cannot be dissolved in the solvent
- Less Toxic than Normal-Phase
  - Reversed Phase: mobile phase is mostly aqueous
  - Normal Phase: 100% organic solvents and usually between 70% and 100% hexane or heptane
    - Hexane has been known to produce neurotoxic effects
- Fast Sample Recovery
  - Aqueous fractions containing analyte can be run through a C18 flash cartridge.
  - Analyte adheres to cartridge and can be washed with water to remove mobile phase additives
  - Analyte eluted from flash cartridge using minimal amount of MeOH, that can be removed quickly *in vacuo*.

# Low Flow on CHIROBIOTICS

- Greater Efficiency at Low Flow on the CHIROBIOTIC Phases.
  - Optimal linear velocity on the CHIROBIOTIC V2 is significantly lower than that observed on the Ascentis C18. Therefore, the CHIROBIOTIC columns produce optimal efficiency at flow rates significantly lower than that observed with traditional non-chiral columns, such as the Ascentis C18.

# The Knox plot of Fluoxetine on the CHIROBIOTIC V2 vs. the Ascentis C18




Column Dimensions: 150 x 4.6 mm, 5 um particle size

The optimal reduced linear velocity on the V2 is 0.61, which translates into a flow rate of 0.15 mL/min on the analytical column used in this study.





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# Flow Rate Effects: Three Examples

| **Mobile Phase:** | 100%MeOH | 100/0.1w%, MeOH/NH4formate | |
|---|---|---|---|
| **Sample:** | 5,5 hydantoin | terbutaline | clenbuterol |
| **Column:** | **CHIROBIOTIC T, 5u, 25cmx4.6mm** | | |
| **Flow Rate (mL/min)** | Plate Counts (peak 1) | | |
| 1.5 | 10748 | 7922 | 8932 |
| 1 | 13666 | 10441 | 11133 |
| 0.5 | 17849 | 14978 | 15270 |
| 0.3 | 20784 | 18286 | 19054 |
| 0.25 | 20935 | 19452 | 19150 |
| 0.2 | 21543 | 20075 | 19883 |
| 0.15 | 20475 | 20469 | 19754 |
| 0.1 | | 19377 | |
| % Gain, Y/G | 58% | 96% | 79% |

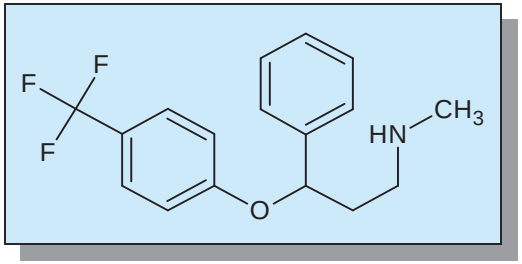
# The Analytical Separation of Fluoxetine in Reversed-Phase

**Conditions:**

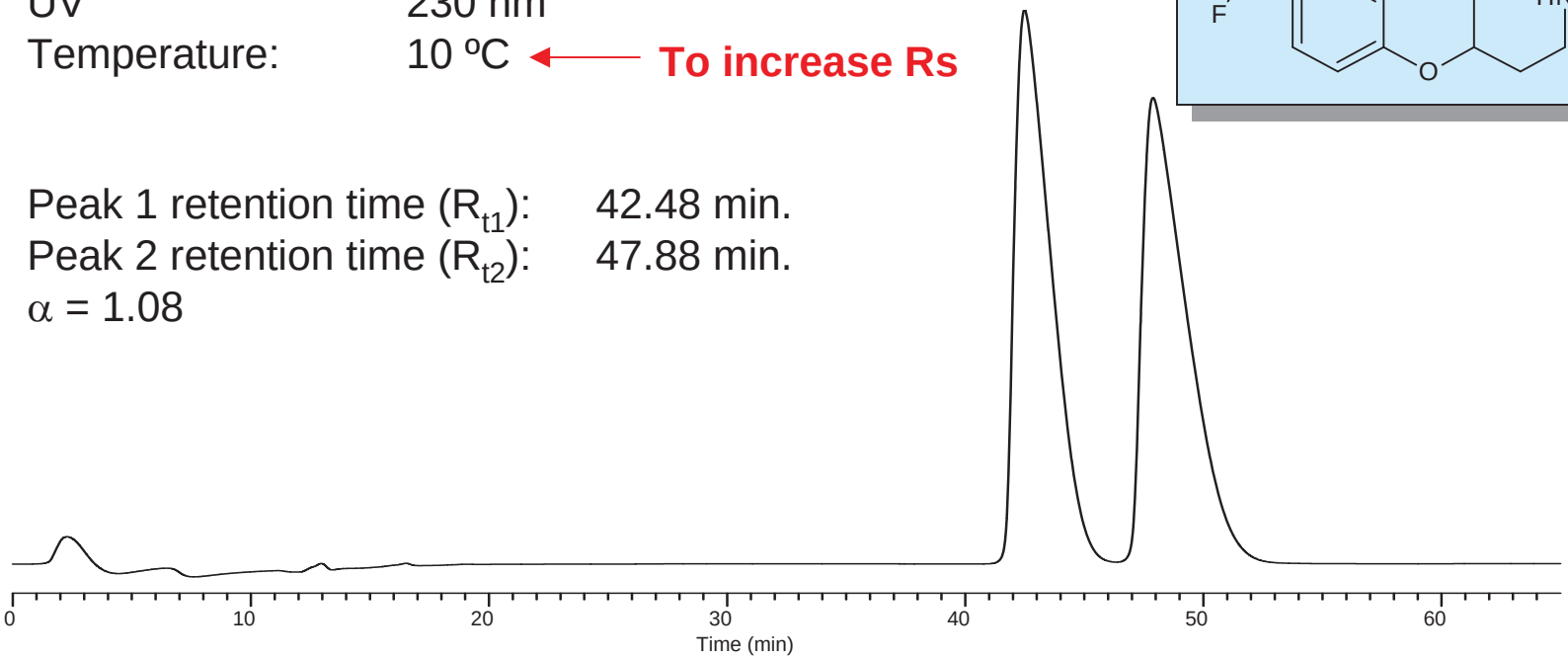
| | |
|---|---|
| Column: | CHIROBIOTIC V2, 150 x 4.6 mm, 5 µm particles |
| Mobile Phase: | 70:30, 20 mM $NH_4OAc$ (pH 4): ACN (Reversed-Phase Mode): |
| Sample: | 12.5 mg/mL in mobile phase (racemic) |
| Injection Volume: | 2 µL |
| Flow Rate: | 0.15 mL/min |
| UV | 230 nm |
| Temperature: | 10 ºC |

**To optimize efficiency**

**To increase Rs**




Peak 1 retention time ($R_{t1}$): 42.48 min.
Peak 2 retention time ($R_{t2}$): 47.88 min.
$\alpha$ = 1.08





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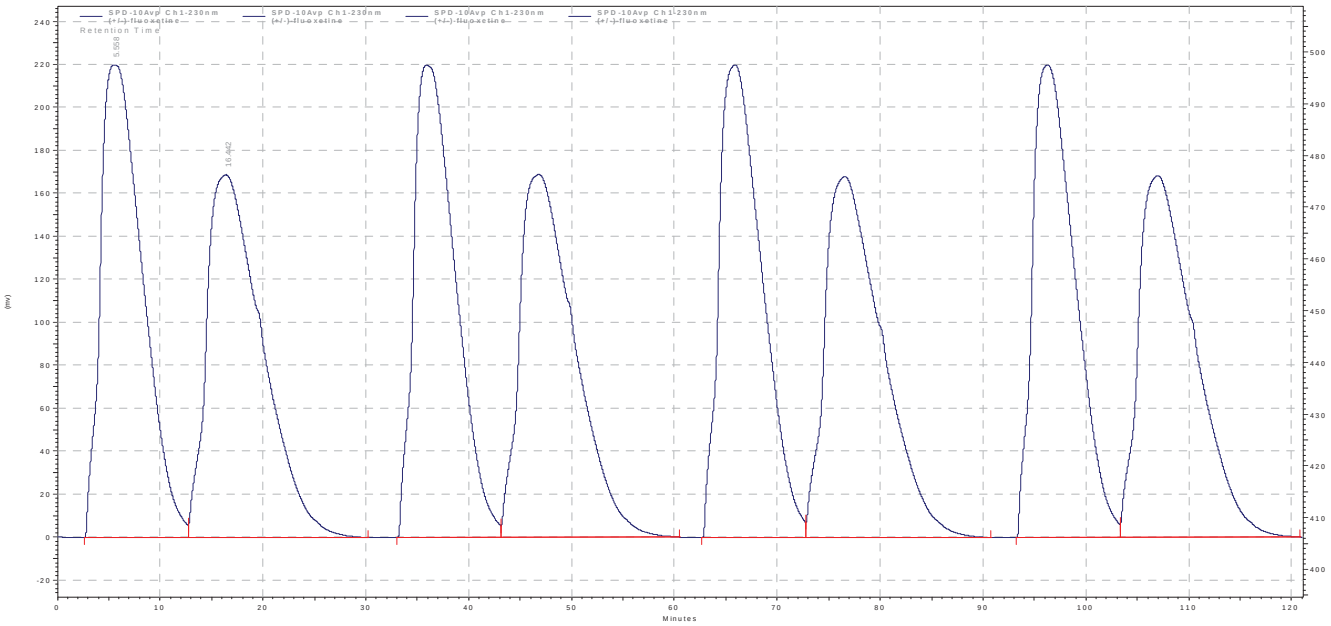
# Fluoxetine Prep Separation with Stacked Injections

**Conditions:**

| | |
|---|---|
| Column: | **CHIROBIOTIC V2, 250 x 21.2 mm, 5 µm particles** |
| Mobile Phase: | 70:30, 20 mM $NH_4OAc$ (pH 4): ACN (Reversed-Phase Mode): |
| Sample: | **50 mg/mL in mobile phase (racemic)** |
| Injection Volume: | **88 µL** |
| Flow Rate: | **3.2 mL/min** |
| UV | 230 nm |
| Temperature: | 10 ºC |



# General Sample Recovery from RP and PIM

## Reversed-Phase:

- Because the reversed-phase mobile phase is composed mostly of water, traditional evaporation would be time consuming. Also, the resultant dried product would be contaminated with buffer salts.
- To eliminate these obstacles, aqueous fractions containing analyte can be run through a C18 flash cartridge.
  - The analyte adheres to cartridge and can be washed with water to remove mobile phase additives.
  - The analyte is then eluted from flash cartridge using a minimal amount of MeOH, that can be removed quickly *in vacuo*.

## Polar-Ionic Mode:

- To remove the mobile phase additives resulting from prep work done in PIM, the mixture is concentrated to a residue, dissolved in diethyl ether, loaded onto a silica flash cartridge, and washed with diethyl ether. The final product is eluted with a minimal amount of methanol.

# Sample Recovery

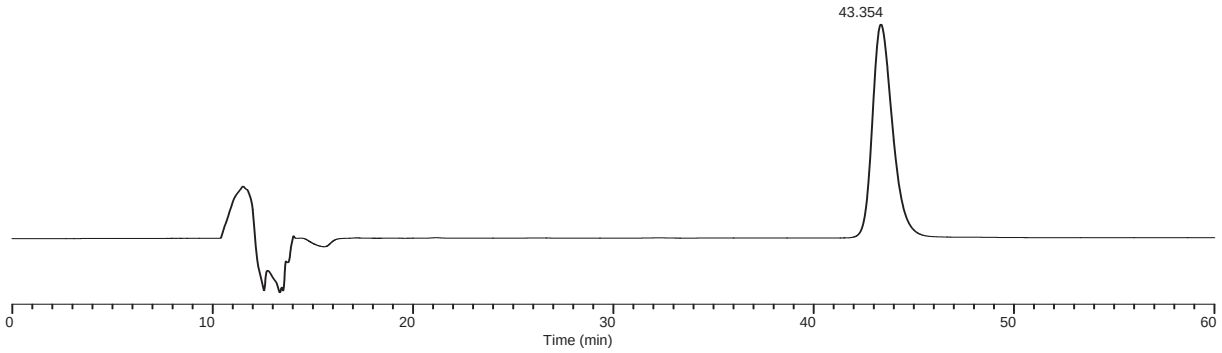
## For Peak 1:

- Connected VersaPak™ C18 flash cartridge (40 x 150 mm) to detector.
- Equilibrated with mobile phase, 10 mL/min for 30 min.
- Combined fractions for peak one (total 1600 mL). Diluted to 3200 mL with water.
- Loaded all of peak 1 onto cartridge at 10 mL/min.
- Washed cartridge containing peak with water (1000 mL).
- Eluted peak 1 from cartridge with 900 mL MeOH. Collected 300 mL of peak 1.
- Concentrated *in vacuo* at 40 ºC.
- Transferred to a tared vial, and concentrated under nitrogen.

# Purity of Peaks

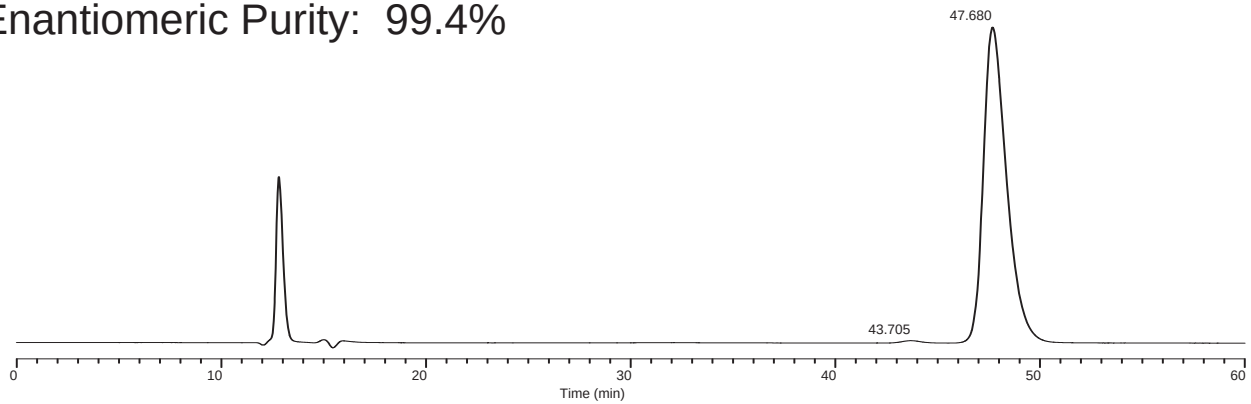
Peak 1:

- Enantiomeric Purity:  99.9%




Peak 2:

- Enantiomeric Purity:  99.4%

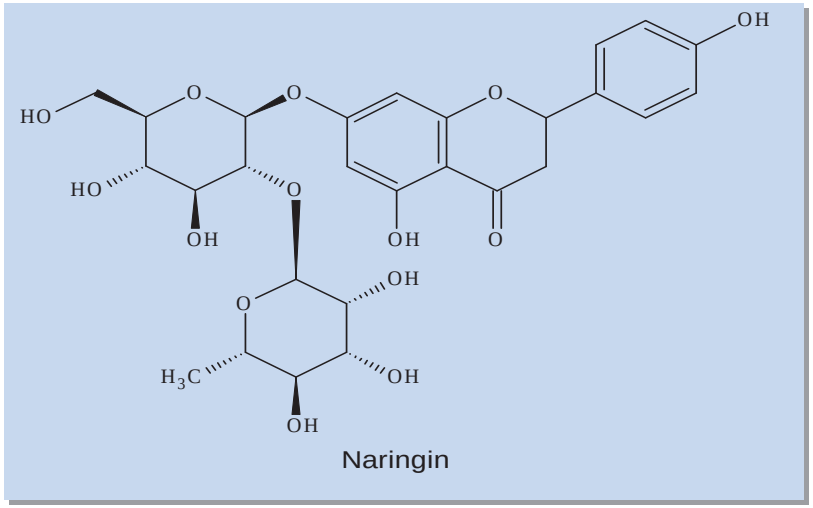


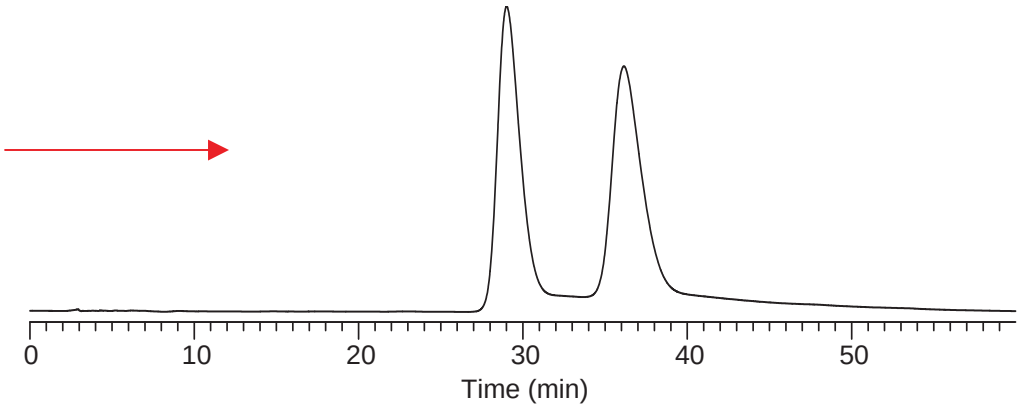
# Conclusions, CHIROBIOTIC prep

- A reversed-phase low flow approach was applied to the preparative separation of (+/-)-Fluoxetine on the CHIROBIOTIC V2.
- A total of 257.2 mg of racemic material was processed to yield 102.6 mg of 99.9% enantiomerically pure peak 1 (89% recovery). Peak 2 had an enantiomeric purity of 99.4%.
- Solvent consumption for the preparative process totaled less than 6 L. Of this total, more than 4 L was aqueous based. Less than 2 L of organic solvent waste was produced. Therefore, prep separations done in reversed-phase mode produce minimal organic waste.
- Decreasing the flow gave optimal efficiency, allowing for larger injections to be made, and therefore, saved time and solvent.
- A benefit of using reversed-phase and polar ionic/polar organic modes over normal phase modes for prep separation includes possible improvements in sample solubility, which can lead to increased capacity, throughput, and yields.
- In summary, the use of reversed-phase and polar ionic/polar organic modes for prep separation at low flow rates are safe and efficient ways to execute successful chiral preparatory separations.

# Preparative Chiral Separation of Naringin

- Responsible for bitter taste in grapefruit.
- Causes some drug-drug interactions in vivo, and interferes with absorption and metabolism of other drugs.
- Chiral screen showed enantiomeric selectivity in RP mode on the CYCLOBOND HP-RSP column.
- Optimization with prep in mind:
  - RSP instead of HP-RSP
  - Methanol instead of acetonitrile
  - Removed mobile phase additives
  - Decreased temperature



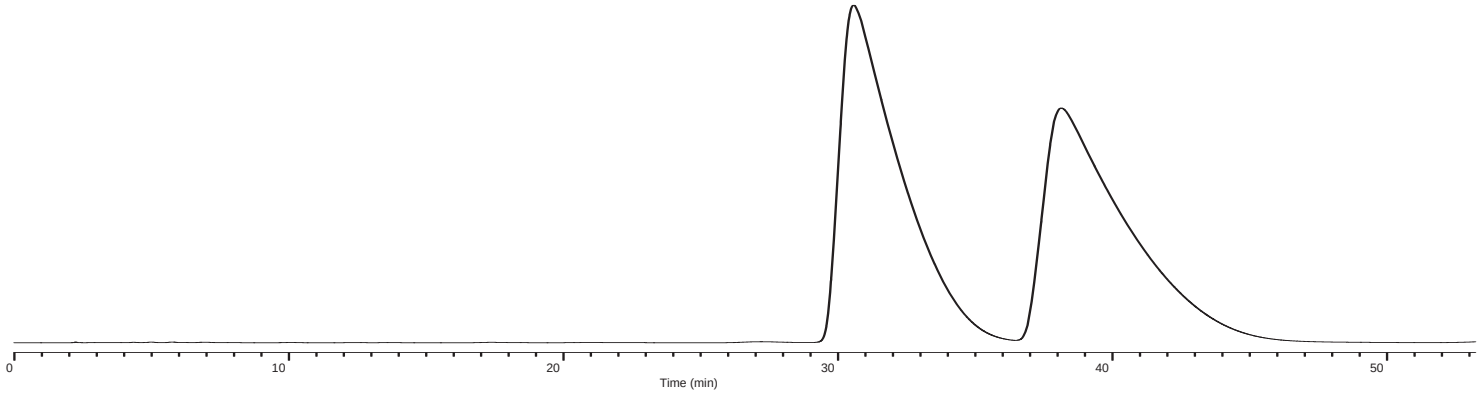
# Analytical Conditions Optimized for Prep

## Analytical Conditions:

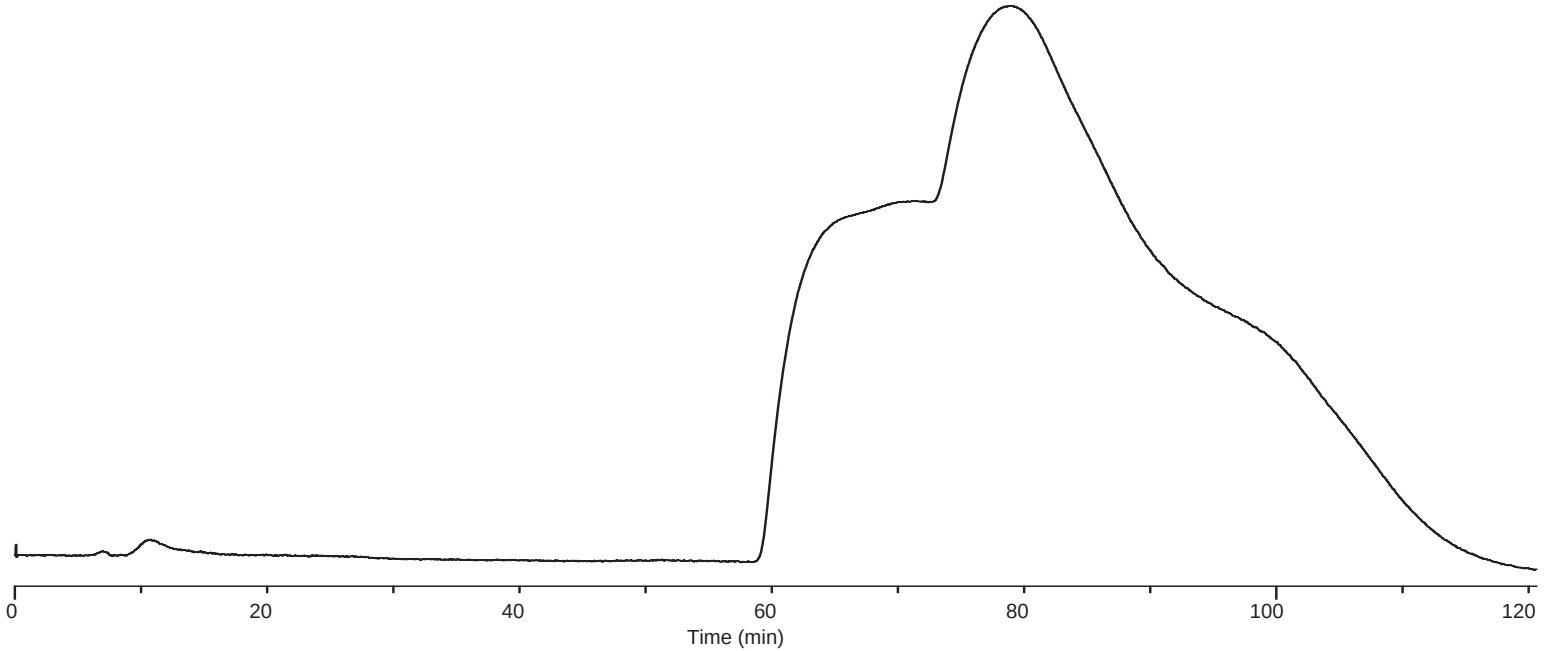
| | |
|---|---|
| **Column:** | CYCLOBOND RSP |
| **Dimensions:** | 25 cm x 4.6 mm I.D. |
| **Mobile Phase:** | 80:20, water:methanol |
| **Temperature:** | 10 °C |
| **Flow Rate:** | 1 mL/min |
| **Detection:** | UV at 220 nm |
| **Inj. Volume:** | 80 µL |
| **Sample:** | 10 mg/mL in mobile phase |

## Prep Conditions:

| | |
|---|---|
| **Column:** | CYCLOBOND RSP |
| **Dimensions:** | **25 cm x 21.2 mm I.D.** |
| **Mobile Phase:** | 80:20, water:methanol |
| **Temperature:** | 10 °C |
| **Flow Rate:** | **10 mL/min** |
| **Detection:** | UV at 220 nm |
| **Inj. Volume:** | **3000 µL** |
| **Sample:** | **5 mg/mL in mobile phase** |



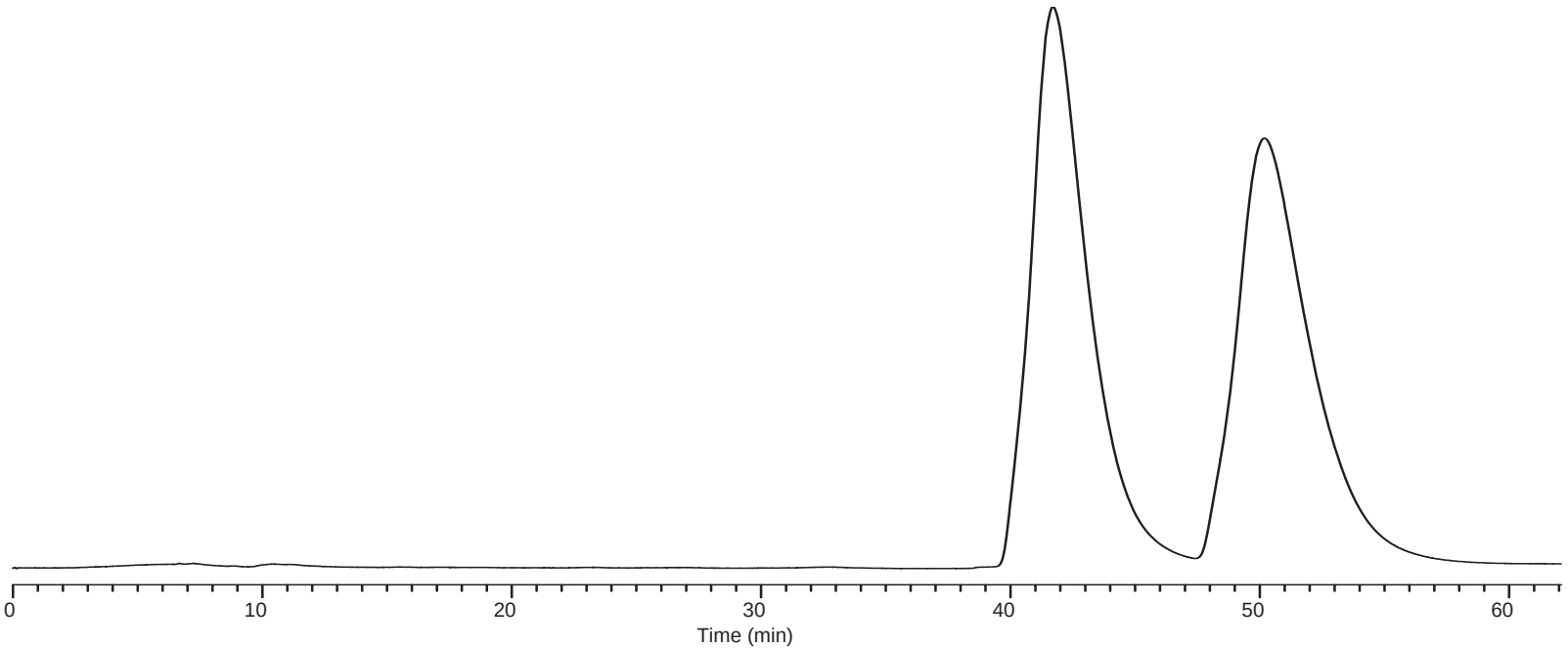
# First Prep Injection



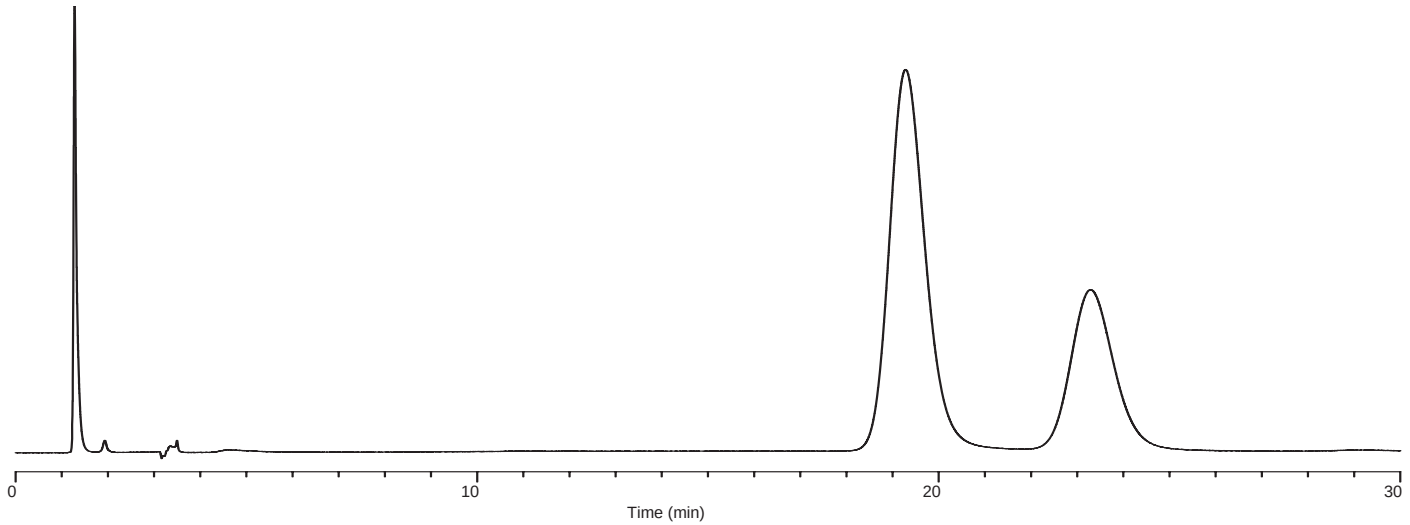

- Due to insolubility of the analyte in the mobile phase, a number of problems arose when trying to scale to prep.
- 10 °C temperature caused the analyte to crash out of solution, causing the column to become blocked and the system to overpressure.

# Temperature and Sample Injection Adjustments

| | |
|---|---|
| Temperature: | 25° C |
| Inj. Vol.: | 3000 µL |
| Sample Conc.: | 0.5 mg/mL in Mobile Phase |



# Racemization of the First Eluting Enantiomer Observed After Recovery




- Peak 1 only 68% enantiomerically pure.

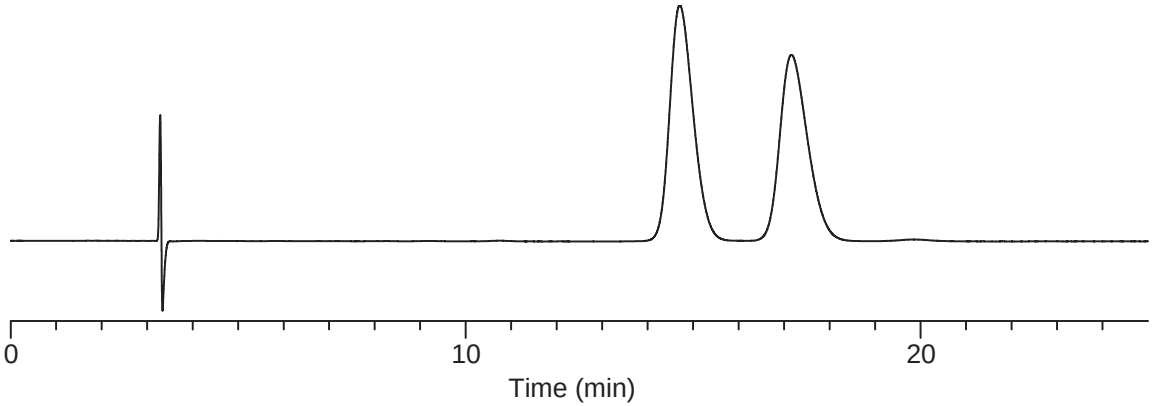
# Literature Supporting Naringin Racemization

- Caccamese, S.; Bianca, S.; Santo, D. Racemization at C-2 of naringin in sour oranges with increasing maturity determined by chiral high-performance liquid chromatography. *J. Agric. Food Chem.* **2007**, *55*, 3816-3822.
  - "…in the HPLC ethanolic solution of some samples a spontaneous racemization at C-2 was observed during standing at room temperature."
  - "…(2*S*)-naringin, the flavanoid glycoside present in immature grapefruit, undergoes nonenzymatic racemization at the C-2 position via ring opening, leading to nearly equal amounts of (2*S*)- and (2*R*)-naringin…"
  - Also states that "less acidic" samples tended to racemize more readily.
- Krause, M.; Galensa, R. High-performance liquid chromatography of diastereomeric flavanone glycosides in Citrus on a β-cyclodextrin-bonded stationary phase (cyclobond I). *J. Chromatogr.* **1991**, *588*, 41-45.
  - "The aglycone in naringin was easily racemized by heating (2*S*)-naringin at 70° C in aqueous methanolic solution."

# Naringin Prep, Plan B

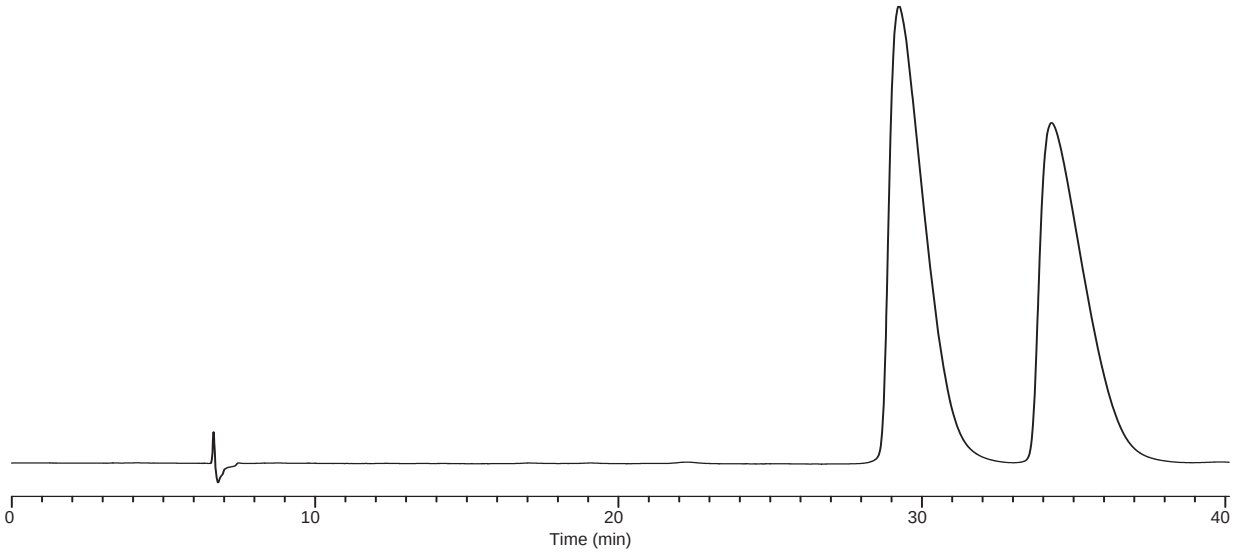
- New mobile phase
  - No methanol, ethanol, or other protic organic solvent
  - Keep acidic
- No heating

| | |
|---|---|
| Column: | CYCLOBOND RSP, 25 cm x 4.6 mm I.D., 5 µm particles |
| Temperature: | 25° C |
| Flow Rate: | 1 mL/min |
| Mobile Phase: | 90:10, water:acetonitrile with 0.1% formic acid |
| Detection: | UV at 220 nm |
| Inj. Vol.: | 10 µL |
| Sample Conc.: | 1 mg/mL in 50:50, water:acetonitrile with 0.1% formic acid |



# First Prep Injection Under New Conditions

| | |
|---|---|
| Column: | CYCLOBOND RSP, 25 cm x 21.2 mm I.D., 5 µm particles |
| Temperature: | 25° C |
| Flow Rate: | 10 mL/min |
| Mobile Phase: | 90:10, water:acetonitrile with 0.1% formic acid |
| Detection: | UV at 220 nm |
| Inj. Vol.: | 3000 µL |
| Sample Conc.: | 0.5 mg/mL in mobile phase |



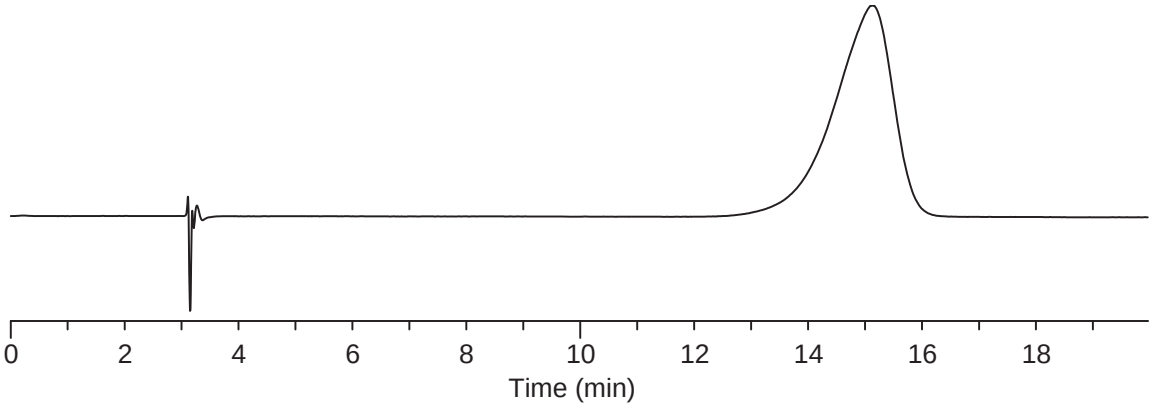
# Sample Recovery for Naringin

- Fractions were loaded onto a C18 preparative column, washed with water to remove the formic acid, and eluted in **acetonitrile**.
- The acetonitrile eluent for each peak was injected on the analytical system to ensure that no racemization had occurred.
- Eluent was concentrated to dryness under vacuum, followed by drying under a nitrogen stream with no heat. The concentrated material was then sealed and placed in the freezer.

# Enantiomeric Purity

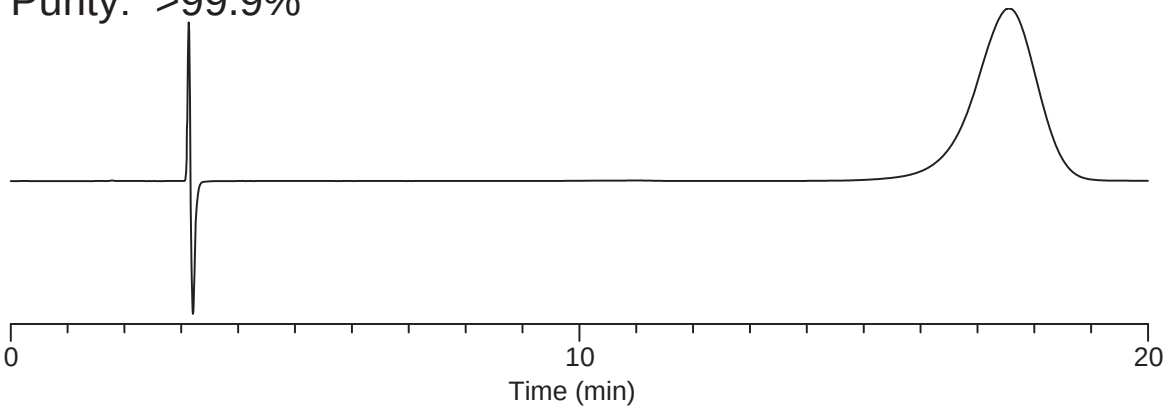
Peak 1:

- Enantiomeric Purity: >99.9%




Peak 2:

- Enantiomeric Purity: >99.9%






*Note: peak shape due to dissolution of the analyte in acetonitrile instead of mobile phase*

# Conclusions

- Although the use of analytical HPLC is valuable in the determination of a chiral preparative HPLC method, as seen with naringin, scaling from analytical HPLC to preparative HPLC is not always as straight forward as it may seem.

- Every chiral analyte is unique, and its properties must be taken into consideration upon performing chiral preparative work.

- The use of CHIROBIOTIC and CYCLOBOND allows solvent flexibility and choices in mobile phase techniques.

- Although initial efforts at separating the enantiomers of naringin produced racemization, literature searches provided information that enabled the racemization problem to be solved with the use of an acidic non-methanolic mobile phase.

- Of the 120 mg of racemic naringin processed, a total of 46.6 mg of enantiomer 1 was recovered and a total of 39.6 mg enantiomer 2 was recovered. Both enantiomers were in the form of off-white solids.

- Purity analysis indicates that the final solid material of enantiomer 1 has an enantiomeric purity of 99.9% and the final solid material of enantiomer 2 also has an enantiomeric purity of 99.9%.

# Summary

- CHIROBIOTIC CSPs are MS compatible
- PIM and RP mobile phases are MS compatible
- CHIROBIOTICS useful for polar compounds
- Improved sample solubility in PIM and RP
- LC-MS separates in the mass/charge dimension

→ LC-MS was successfully used to study the impact of variables on retention and selectivity simultaneously for a large sample set of chiral analytes.

- Changing the CSP is the most useful means of altering enantiomeric selectivity.
- Each CSP selective toward different analytes
- Enantiomeric selectivity remains unpredictable

→ Column screening is highly beneficial and recommended

- Improved sample solubility of polar analytes in RP
- Fast sample recovery from prep in RP
- RP less toxic than NP
- Low flow improves efficiency on CHIROBIOTICS

→ Reversed-phase mode at low flow is useful for the safe and efficient execution of chiral preparative separations.

# Acknowledgements

Supelco/Sigma-Aldrich

- Dave Bell
- Jay Jones
- Hugh Cramer
- J.T. Lee
- Craig Aurand
- Dan Shollenberger
- Dick Henry
- Tracy Ascah

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8. M. Krause et. al. High-performance liquid chromatography of diastereomeric flavanone glycosides in citrus on a β-cyclodextrin-bonded stationary phase (Cyclobond I). *J. Chromatogr.* **1991**, *588*, 41-45.