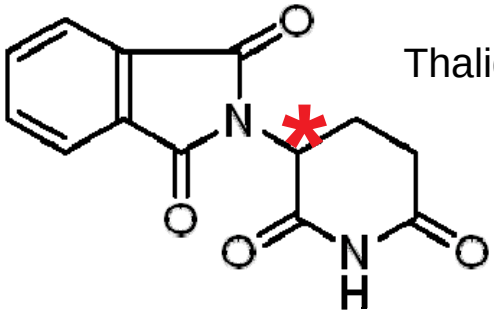
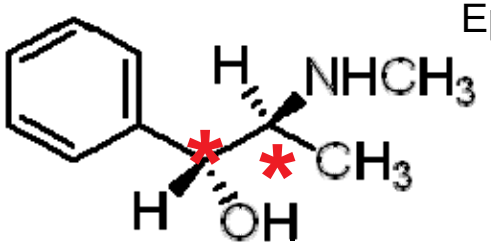
Practicing Chiral Chromatography in Your Mobile Phase Comfort Zone

Enantiomers

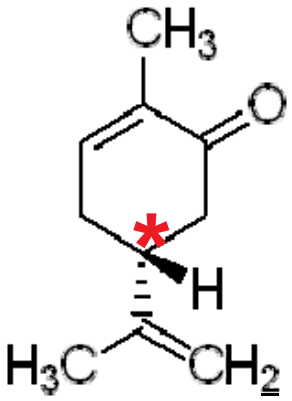
- Stereoisomers that differ in the direction they rotate a plane of polarized light are called optically active, or chiral, and their isomers are called **enantiomers**
- They are non-superimposable mirror images
- They are Important because **biological systems recognize chirality**



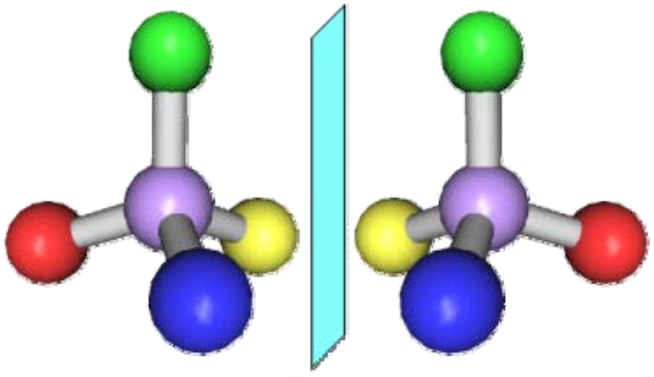
Thalidomide



Ephedrine



Carvone



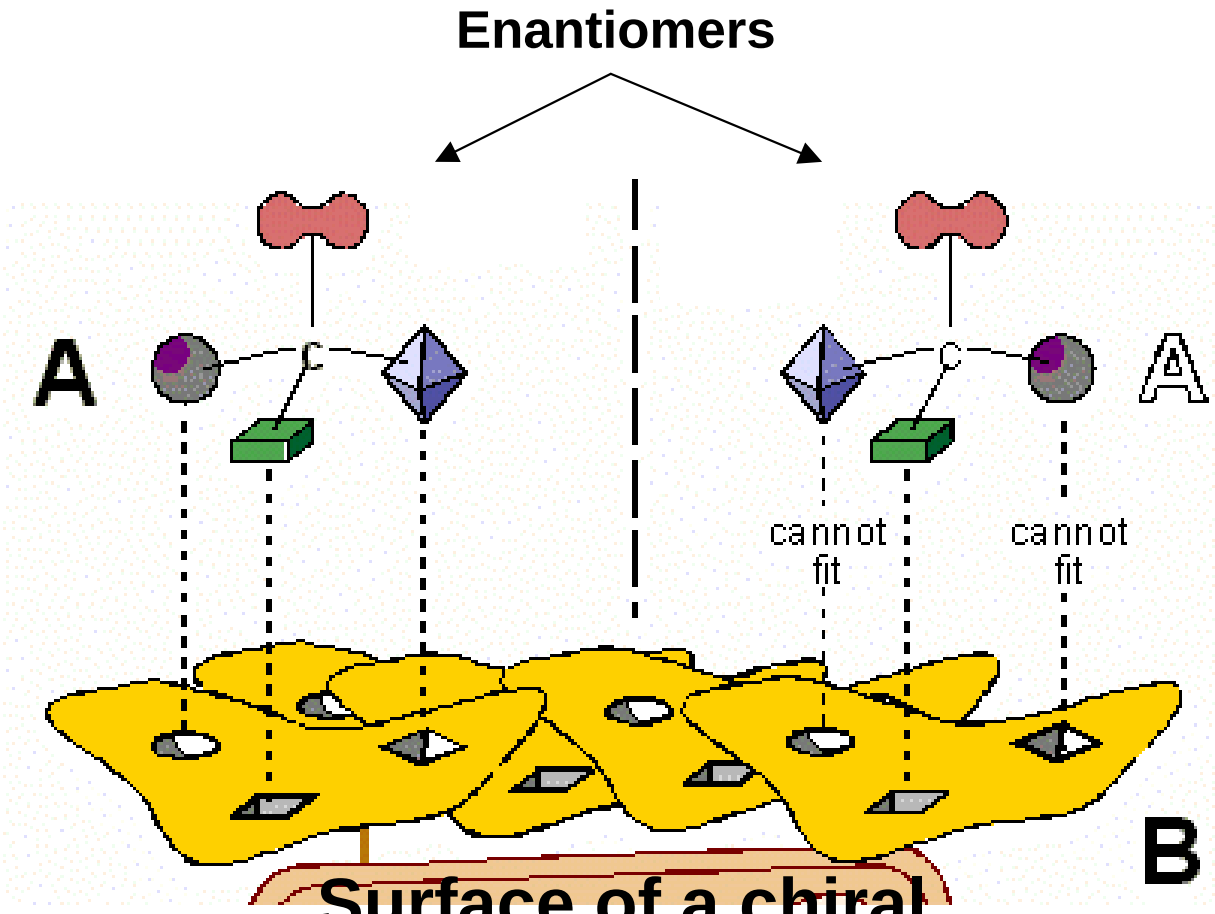
Two chiral molecules (enantiomers). The asymmetric carbon is the purple atom in the middle.

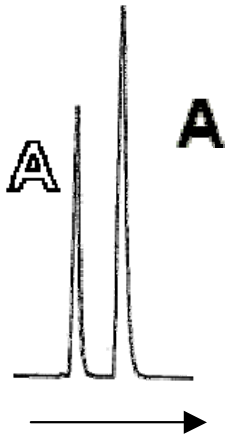
Distinguishing Between Enantiomers

Enantiomers differ in two ways:

1. The direction they rotate a plane of polarized light
2. Their interaction with other chiral molecules:
   - → Biological molecules (e.g. proteins)
   - → **Chiral HPLC & GC stationary phases**
3. We can leverage these differences to separate enantiomers

Schematic of How Chiral Chromatography Works: 3-Point Interaction



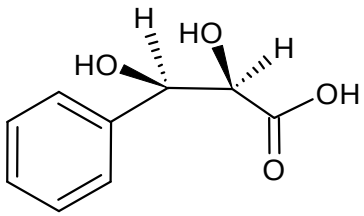


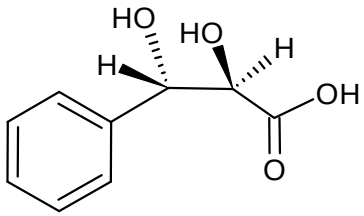
http://www.ipc.uni-tuebingen.de/weimar/pictures/chiral_recognition.gif

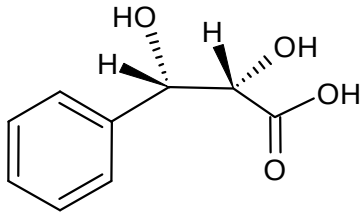
The Power of Chiral Chromatography

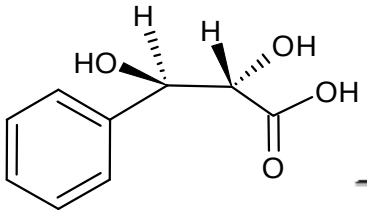
These four compounds only differ in the orientation of the –OH and –H groups, and demonstrate the ability of chiral HPLC to separate enantiomers and diastereomers

2,3-Dihydroxy-3-phenyl-propionic Acid Isomers:



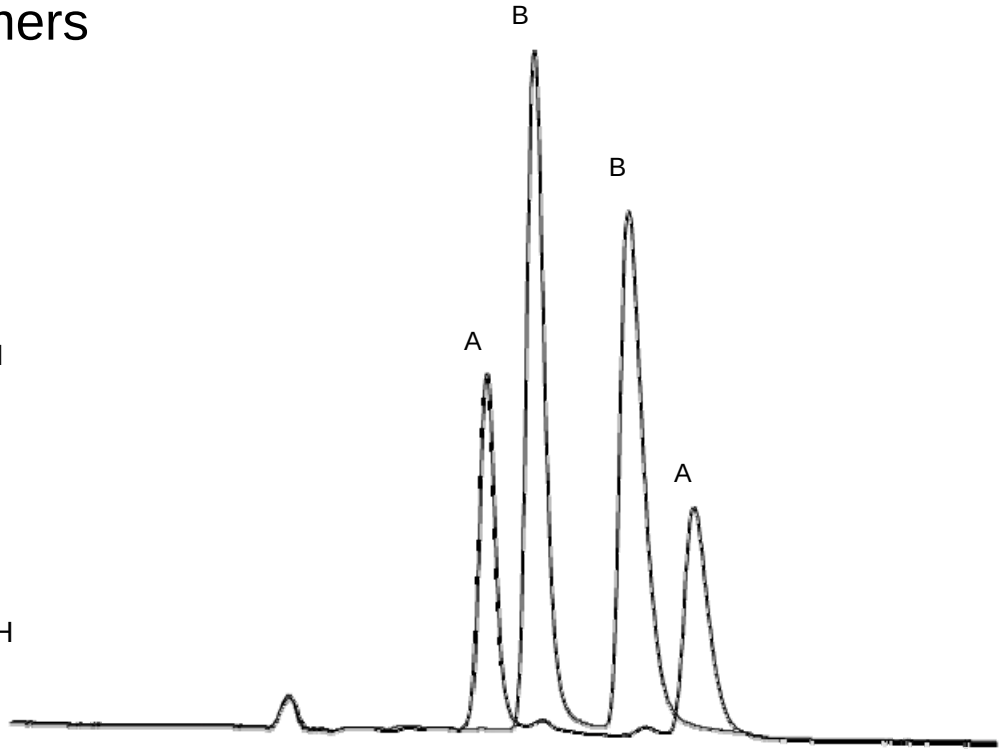






racemate A
-OH groups on the same side

racemate B
-OH groups on the opposite side



column:	CHIROBIOTIC R, 25 cm x 4.6 mm I.D., 5 µm particles (13024AST)
mobile phase A:	0.1% ammonia, pH 4.1 with formic acid
mobile phase B:	methanol
mobile phase ratio:	50:50
flow rate:	1 mL/min.
temp.:	ambient
det.:	UV at 258 nm
injection:	10 µL

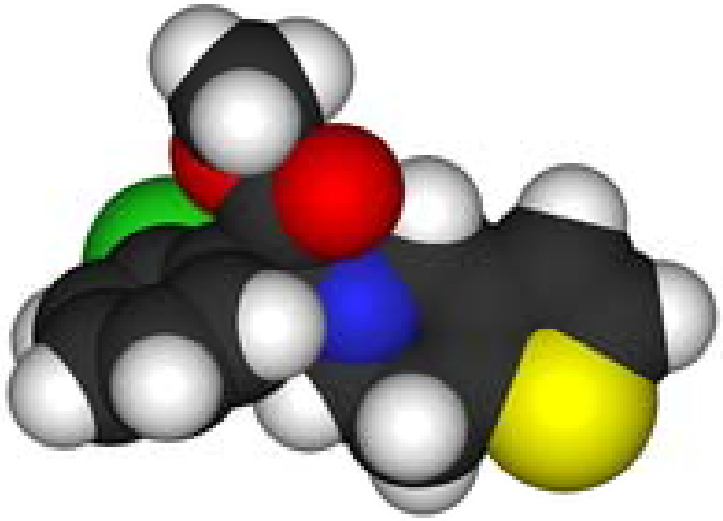
Chiral HPLC

Chiral stationary phases for HPLC

Familiar HPLC and LC-MS mobile phases

Chiral screening and LC-MS

Preparative considerations



Types of Chiral Stationary Phases (CSP)

Chiral molecules

- Cellulose/amylose
- Macrocyclic glycopeptides
- Cyclodextrins
- Synthetic polymers
- Proteins
- Amino acids
- Other small, chiral molecules

The more different types of functional groups in the CSP, the more types of interactions it can undergo with the analytes (more potential for success).

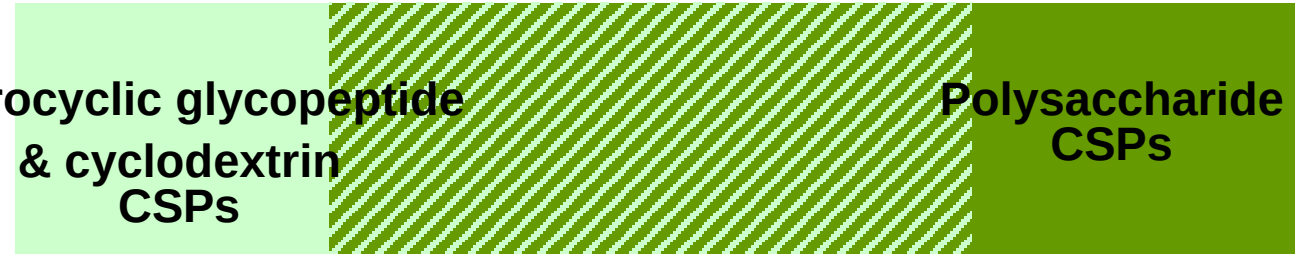
Cyclodextrin (CYCLOBOND) and Macrocyclic glycopeptide (CHIROBIOTIC)

- Innovative CSPs invented by Prof. Daniel Armstrong
- Naturally-occurring compounds
- Multiple chiral selectors tightly bonded to pure silica
- Versatile and extremely robust
- Use familiar mobile phases, like reversed-phase
- Useful for polar and ionic compounds
- MS-compatible; ionic mobile phases promote sensitive MS response
- Complement cellulose and amylose polysaccharide type columns
- “Greener” than normal phase

Success of Co-screening with Polysaccharide CSPs

The cyclodextrin and macrocyclic glycopeptide CSPs are complementary to the more common polysaccharide CSPs. Generally:

- 25% chance that polysaccharide gives best separation
- 25% chance that the cyclodextrin and macrocyclic glycopeptide CSPs give best separation
- 50% overlap (both work)



(polar molecules dominate) (nonpolar molecules dominate)

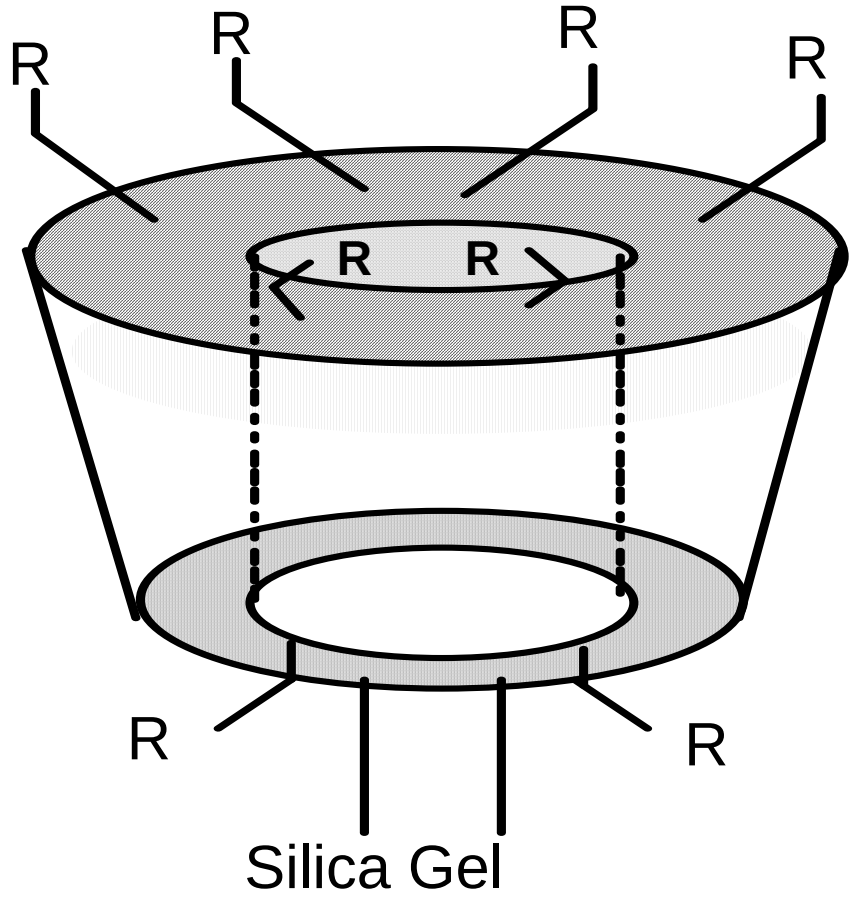
Cyclodextrin (Astec CYCLOBOND)

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

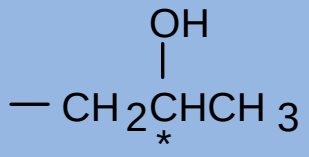
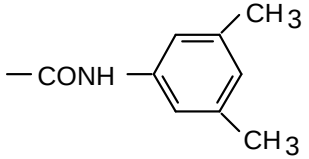
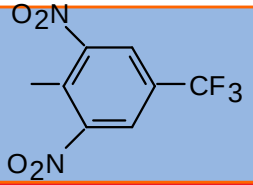
- 35 chiral centers
- Chiral, non-polar interior “basket”
- Chemical selectors on the outside surface

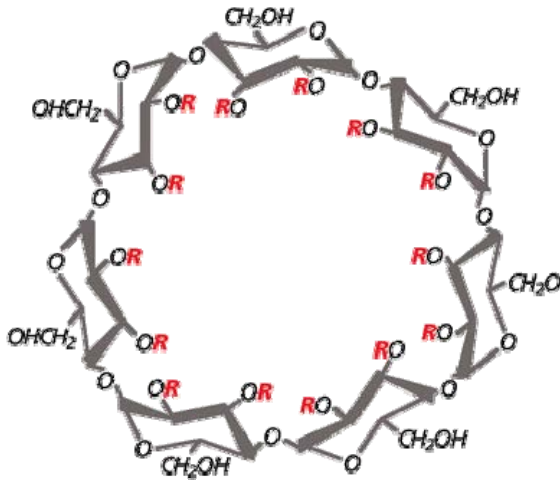
Types of possible interactions:

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



Astec CYCLOBOND Derivatives

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



Most successful selectors:

- CB I 2000
- HP-RSP
- DNP (Pi Acid)
- DMP (Pi Base)

Astec CYCLOBOND Separation

Column: Astec CYCLOBOND I 2000,
25 cm x 4. 6mm I.D., 5µm particles

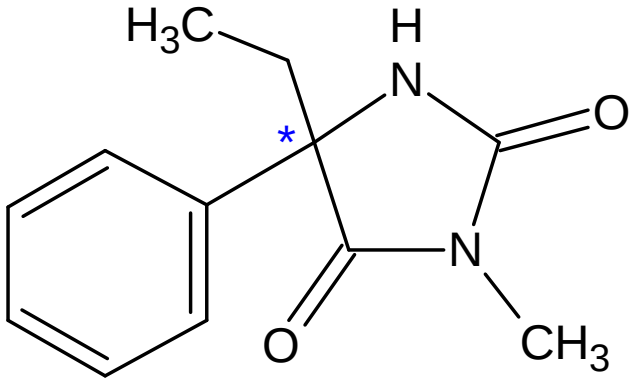
Mobile Phase: 25/75, ACN/20mM NH4OAc,
pH 4.0

Flow Rate: 0.6 mL/min

UV: 230 nm

Temperature: 25°C

Analyte: Mephenytoin



Mephenytoin (an anticonvulsant)

Astec CHIROBIOTIC

Macrocyclic glycopeptides

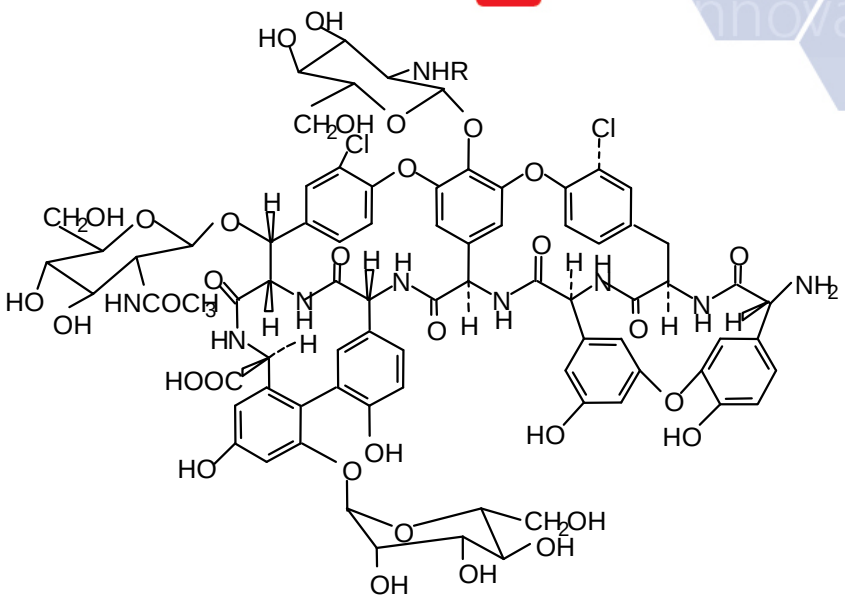
Multi-modal chiral surface capable of a wide variety of different interactions

Unique ionic interactions – very useful for polar and ionic compounds and mobile phases

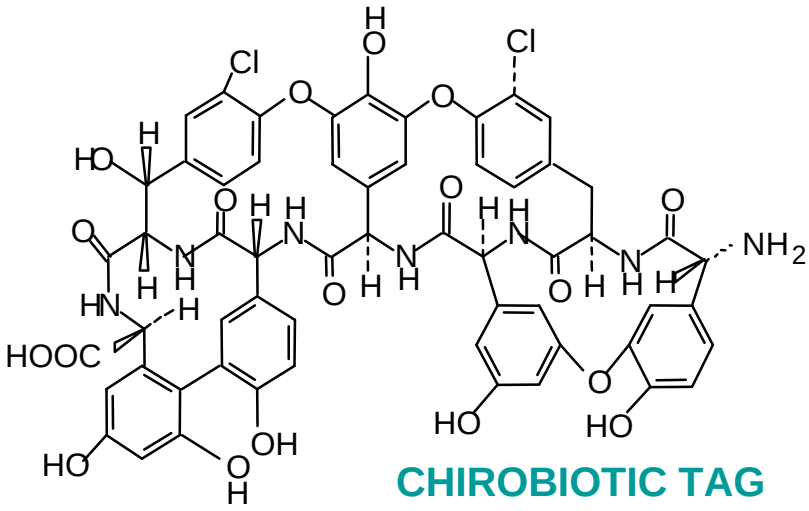
Very robust chemistry:

- Chemically bonded to pure silica (>4 linkages)
- Stable to high flows, solvents, temperature (50°C) and pressures (3500 psi)

Choices in enantioselectivity among 6 phases



CHIROBIOTIC T (Teicoplanin)

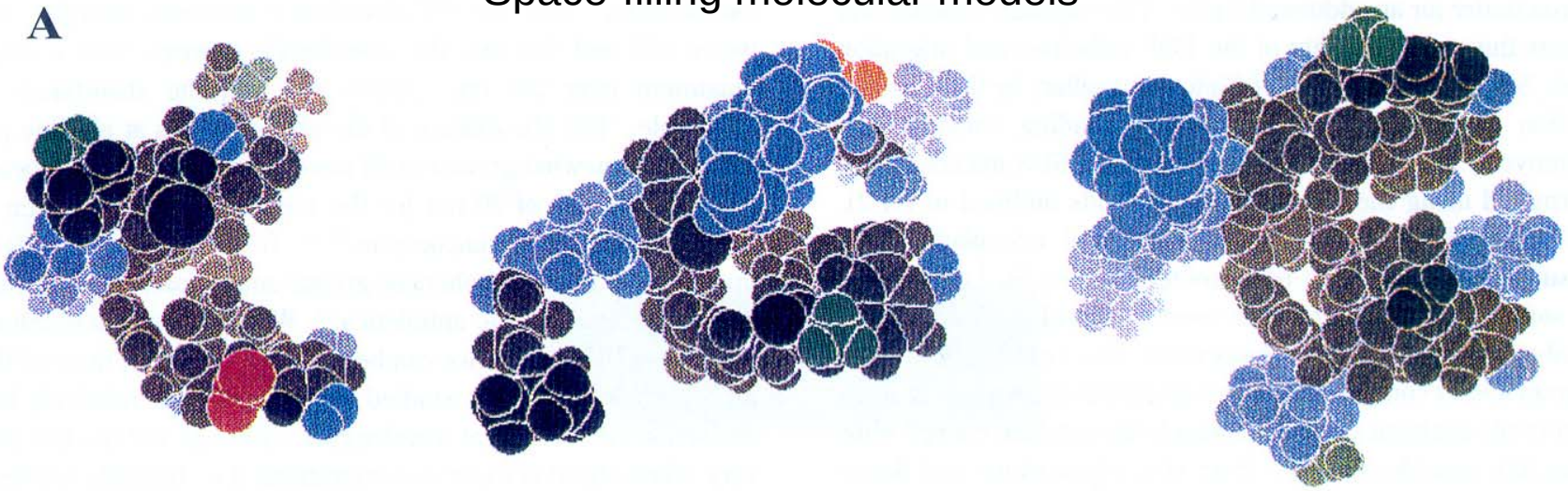


CHIROBIOTIC TAG

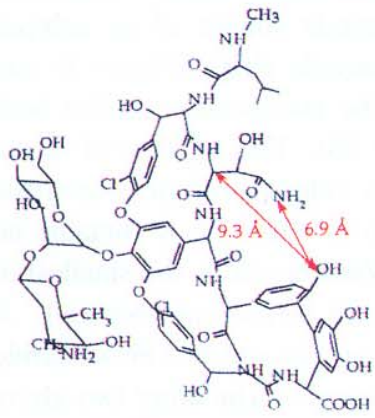
Astec CHIROBIOTIC Structures

Space-filling molecular models

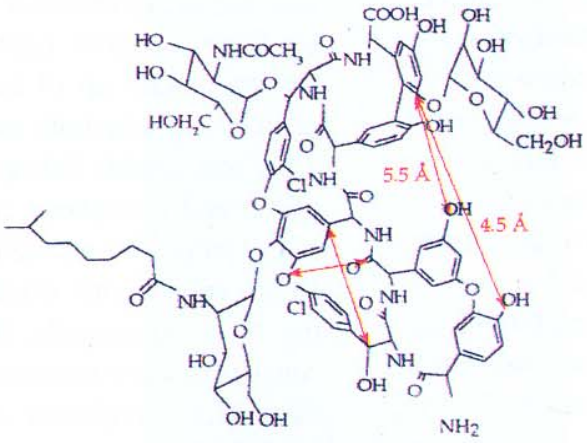
A



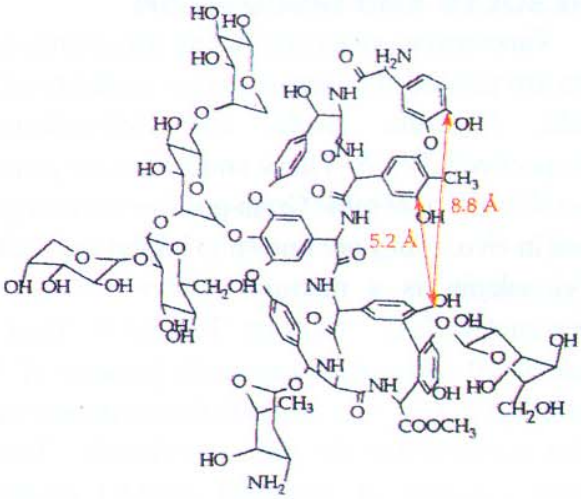
B



VANCOMYCIN



TEICOPLANIN



RISTOCETIN A

Astec CHIROBIOTIC Phases

Macrocyclic Glycopeptide	Designation
Vancomycin	**Astec CHIROBIOTIC V and V2**
Teicoplanin	**Astec CHIROBIOTIC T and T2**
Ristocetin	**Astec CHIROBIOTIC R**
Teicoplanin Aglycone	**Astec CHIROBIOTIC TAG**

CHIROBIOTIC Separation

Column: Astec CHIROBIOTIC V,
25 cm x 4. 6mm I.D., 5µm particles

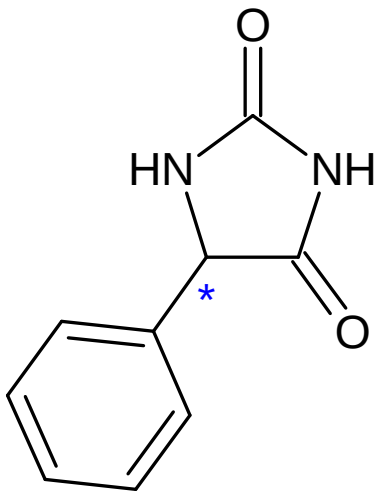
Mobile Phase: 10/90, ACN/20mM NH4OAc,
pH 5.5

Flow Rate: 1 mL/min

UV: 210 nm

Temperature: 25°C

Analyte: 5-Methyl-5-phenylhydantoin



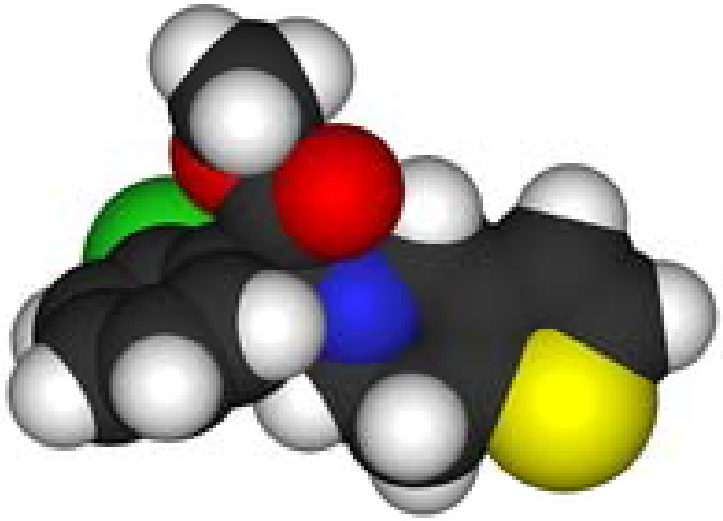
Chiral HPLC

Chiral stationary phases for HPLC

Familiar HPLC and LC-MS mobile phases

Chiral screening and LC-MS

Preparative considerations



3-Point Interactions on Multi-modal CHIROBIOTIC and CYCLOBOND CSPs

The most likely interactions between analyte and CSP in descending order of strength under different mobile phase conditions:

Reversed Phase Conditions (all compounds)

- Ionic
- Hydrogen Bonding
- Steric/Inclusion/Hydrophobic

Polar Ionic Conditions (ionizable compounds only)

- Ionic
- Hydrogen Bonding
- Steric/π-π

Polar Organic & Normal Phase Conditions (neutral compounds)

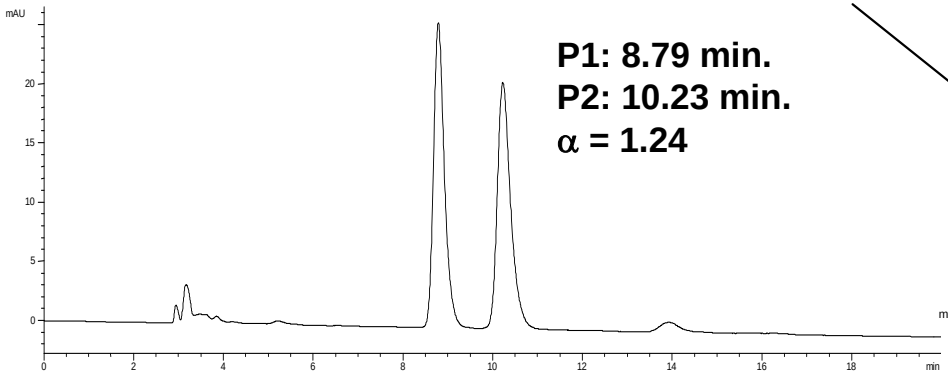
- Hydrogen Bonding
- π-π
- Steric/Dipole

Chiral Reversed-Phase

- Comprises water (buffers) with CH_3OH or CH_3CN
- In most HPLC analysts' "comfort zone"
- Highly compatible with mass spectrometry detection
- Especially valuable for the separation of polar and ionic chiral analytes
- Distinct and valuable benefits for preparative separations
- "Greener" – more water, less hazardous solvents
- Many parameters to vary to optimize:
  - pH
  - Buffer type and concentration
  - Organic modifier type and concentration
  - Temperature
  - Flow rate
- Permitted on CHIROBIOTIC and CYCLOBOND columns

Bupivacaine in Reversed-Phase Mode on CHIROBIOTIC V2

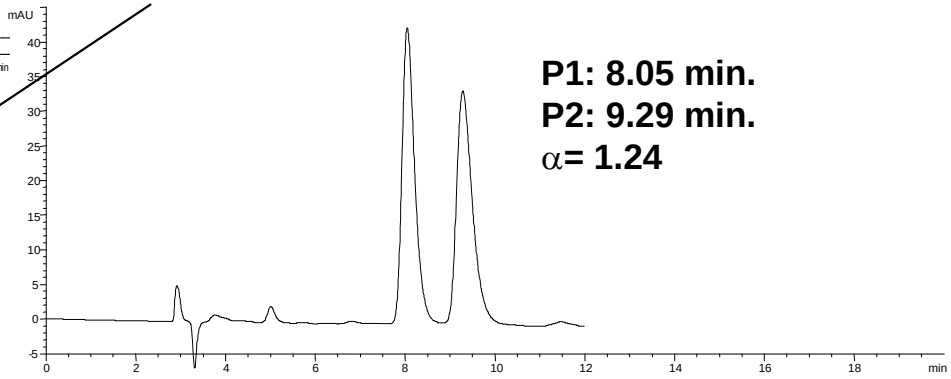
80/20: MeOH/10mM NH_4OAc, pH 4.1



P1: 8.79 min.
P2: 10.23 min.
α = 1.24

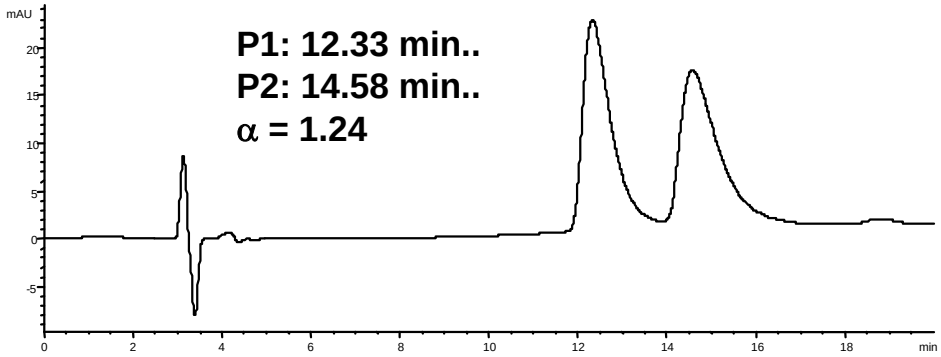
Large changes in % water may not change chiral retention and selectivity

50/50: MeOH/10mM NH_4OAc, pH 4.1



P1: 8.05 min.
P2: 9.29 min.
α= 1.24

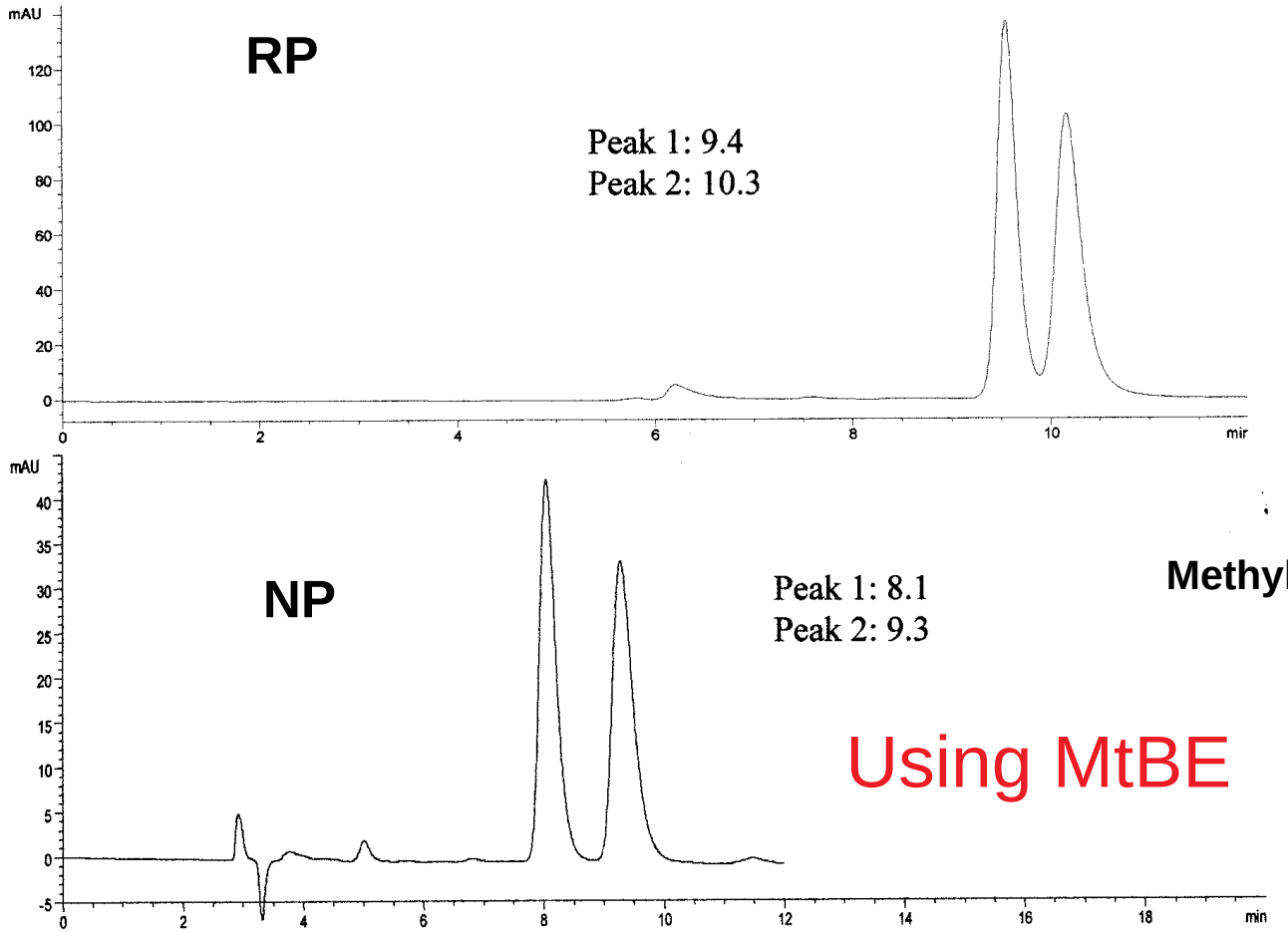
25/75: MeOH/10mM NH_4OAc, pH 4.1

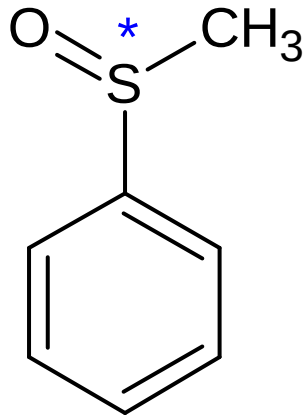


P1: 12.33 min..
P2: 14.58 min..
α = 1.24

Although the polar ionic mode tends to give sharper peaks with less retention, CHIROBIOTIC phases can also provide the flexibility of using reversed-phase mode to control the retention (U-shape) and to increase sensitivity in LC-MS.

Conversion from Reversed Phase to Normal Phase Easily Accomplished





Methyl phenyl sulfoxide

Using MtBE

Methyl phenyl sulfoxide separation by CHIROBIOTIC V (250x4.6mm).
Top: 20/80, THF/20 mM NH_4NO_3. Bottom: 97/2/1/, MtBE/ACN/MeOH.

Unique Polar Ionic Mode for Astec CHIROBIOTIC

Unique to CHIROBIOTIC CSPs

Comprises methanol or acetonitrile with organic salt, acid or base

- e.g. 10 mM ammonium formate in methanol

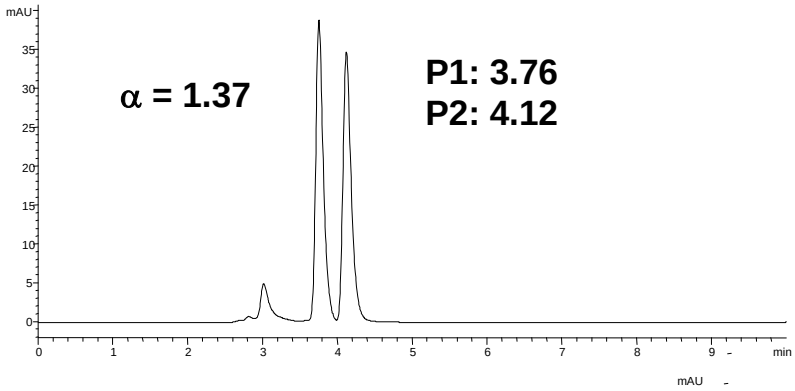
Ideal for LC-MS (volatile salts)

Ideal for prep (volatile)

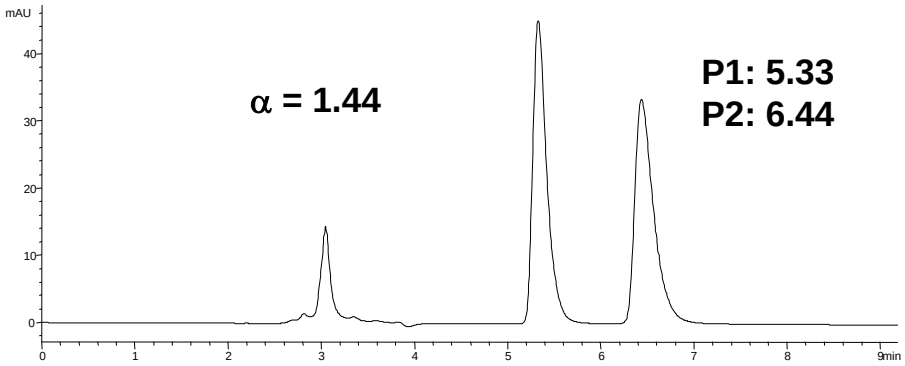
Analytes are ionizable molecules – any acid or base

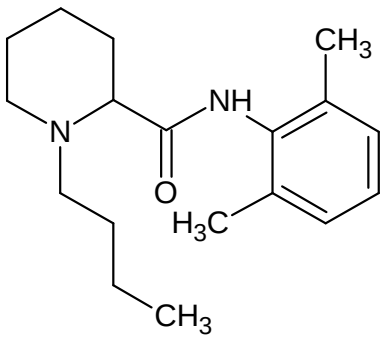
Bupivacaine in Polar Ionic and Reversed-Phase Modes on CHIROBIOTIC V2

Mobile Phase:
100/0.1w%: MeOH/NH_4Formate

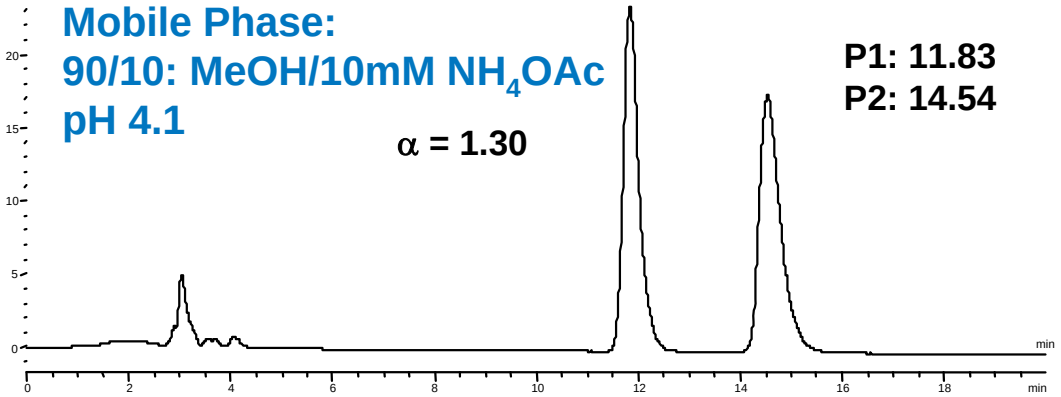


Mobile Phase:
100/0.1w%: MeOH/NH_4TFA





Mobile Phase:
90/10: MeOH/10mM NH_4OAc
pH 4.1



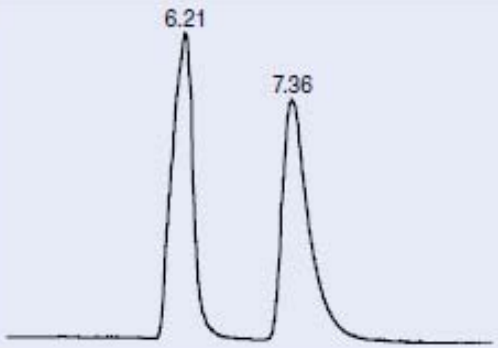
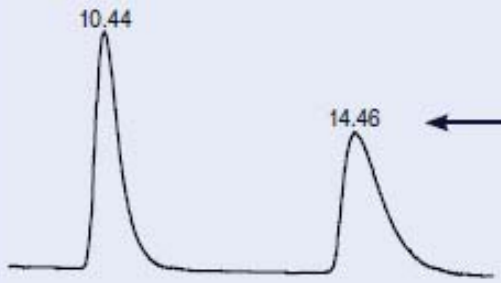
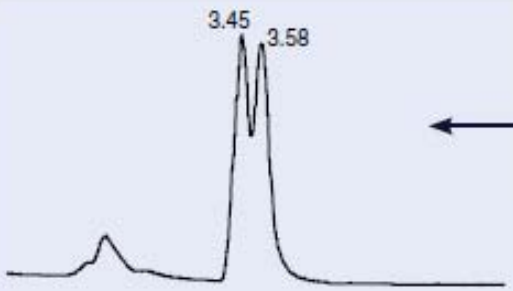
CHIROBIOTIC V2, 250x4.6mm

Buffer Component Ratio in Polar Ionic Mode

Acid:Base = **1:1**

Acid:Base = **3:1**

Acid:Base = **1:3**

Column	CHIROBIOTIC V
Mobile Phase	CH_3OH/Acetic Acid/Triethylamine
100/0.1/0.1 Acid:base ratio=1:1	
100/0.15/0.05 Acid:base ratio=3:1	
100/0.05/0.15 Acid:base ratio=1:3	

Mianserin

High acid: Nitrogen on mianserin is positively charged, while -COOH group on vancomycin is negatively charged: **strong ionic interaction**

High base: Nitrogen on mianserin group is free amine, but -COOH group on vancomycin is fully charged: **weak ionic interaction**

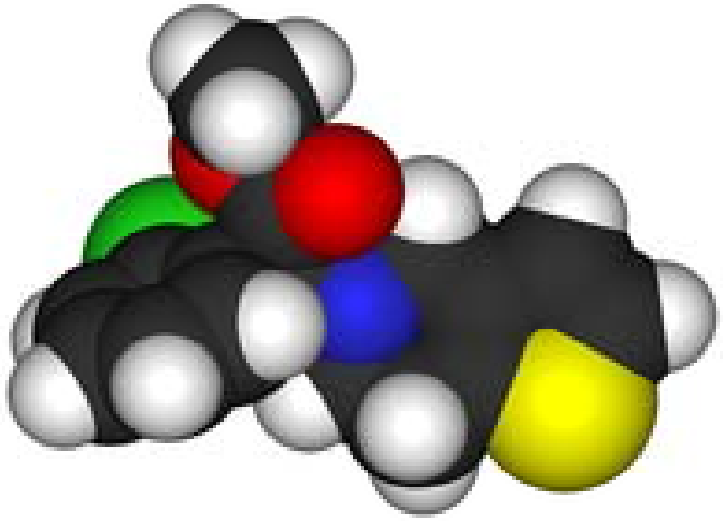
Chiral HPLC

Chiral stationary phases for HPLC

Familiar HPLC and LC-MS mobile phases

Chiral screening and LC-MS

Preparative considerations



LC-MS Chiral Column Screening Strategy

- Chiral method development = Screening several columns!
  - Impossible to predict which CSP will work
- LC-MS can make this easier by allowing mixtures of different enantiomers to be screened simultaneously
  - CHIROBIOTIC and CYCLOBOND are compatible with LC-MS

Published Data – 53 Chiral Compounds

(Three CHIROBIOTIC and four CHIRALPAK columns)

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

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 Recommended Screening Columns

Protocol includes three mobile phase conditions run with six different stationary phase chemistries as a front line screening*. This front line has generally provided ca. 80% screening success.

Columns:

- **CHIROBIOTIC™**
  - V2
  - T
  - TAG
- **CYCLOBOND™**
  - β-CD
  - DNP (or 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.

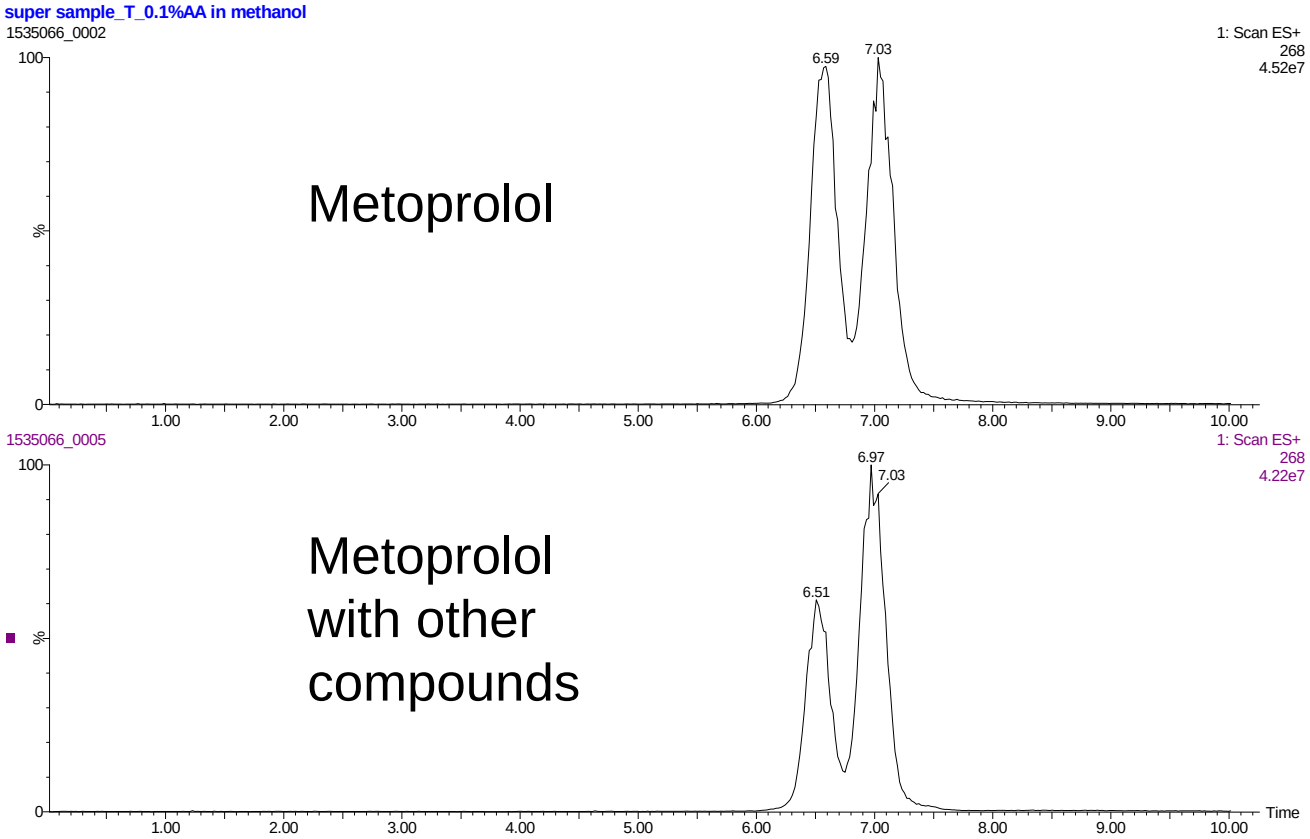
* Protocol may be adjusted for LC-MS or for certain sample types.

Chiral LC-MS Column Screening Protocol

LC-MS analysis

- Has ability to separate in the mass/charge dimension, making it possible to evaluate many analytes in the same injection.
- Operates well in both reversed-phase and polar ionic/polar organic modes; ammonium salts usually preferred over TEA in mobile phases.
- Compatible with both the CHIROBIOTIC and CYCLOBOND CSPs.
- May be used as a valuable tool for rapid chiral screening of multiple chiral samples using simultaneous injection and selective ion detection.

Comparison of Metoprolol Alone and in 13-Component Chiral Mixture by LC-MS



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).

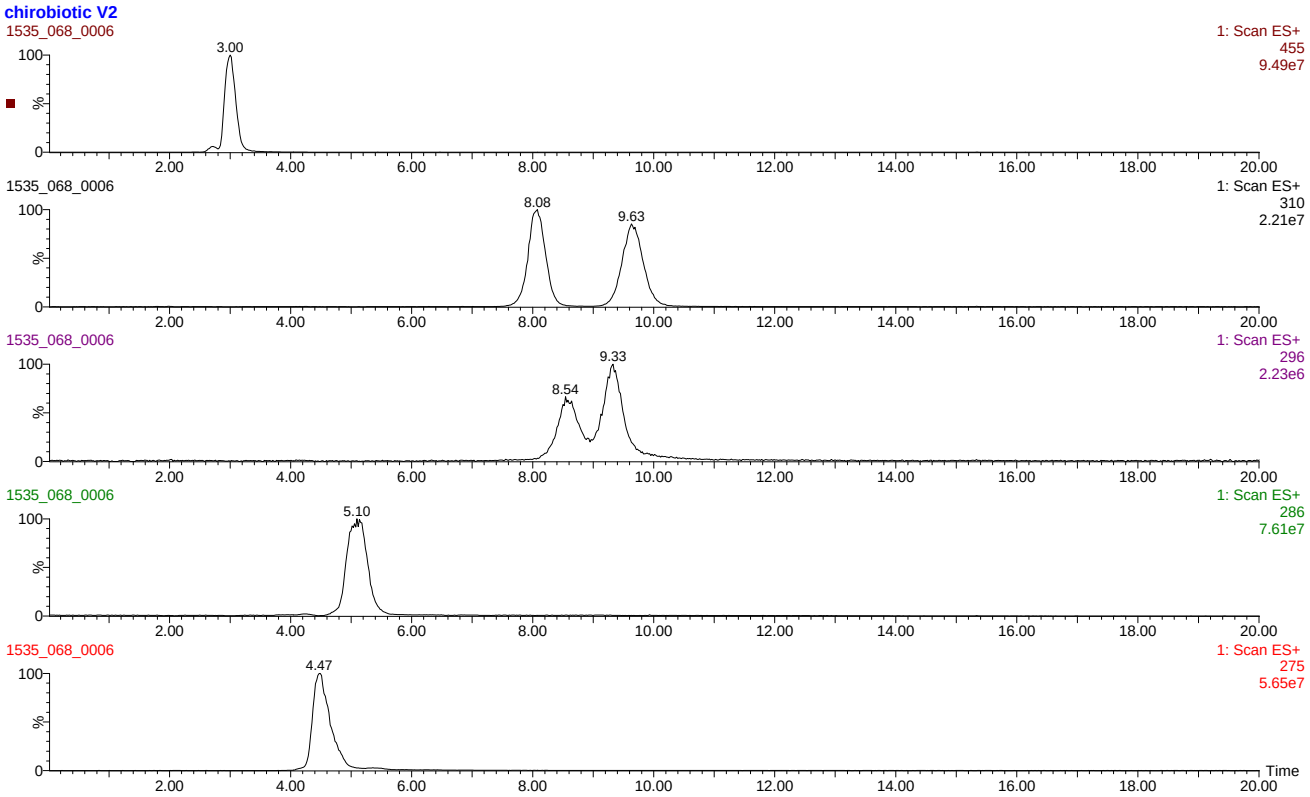
Changing Chiral Stationary Phase Provides Greatest Impact on Selectivity

CHIROBIOTIC V2, TAG and R were screened to assess the impact of stationary phase on a 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 for Fluoxetine and Norfluoxetine

Unique selectivity between V2 phase and certain solutes shows up in LC-MS screen.



Verapamil

Fluoxetine

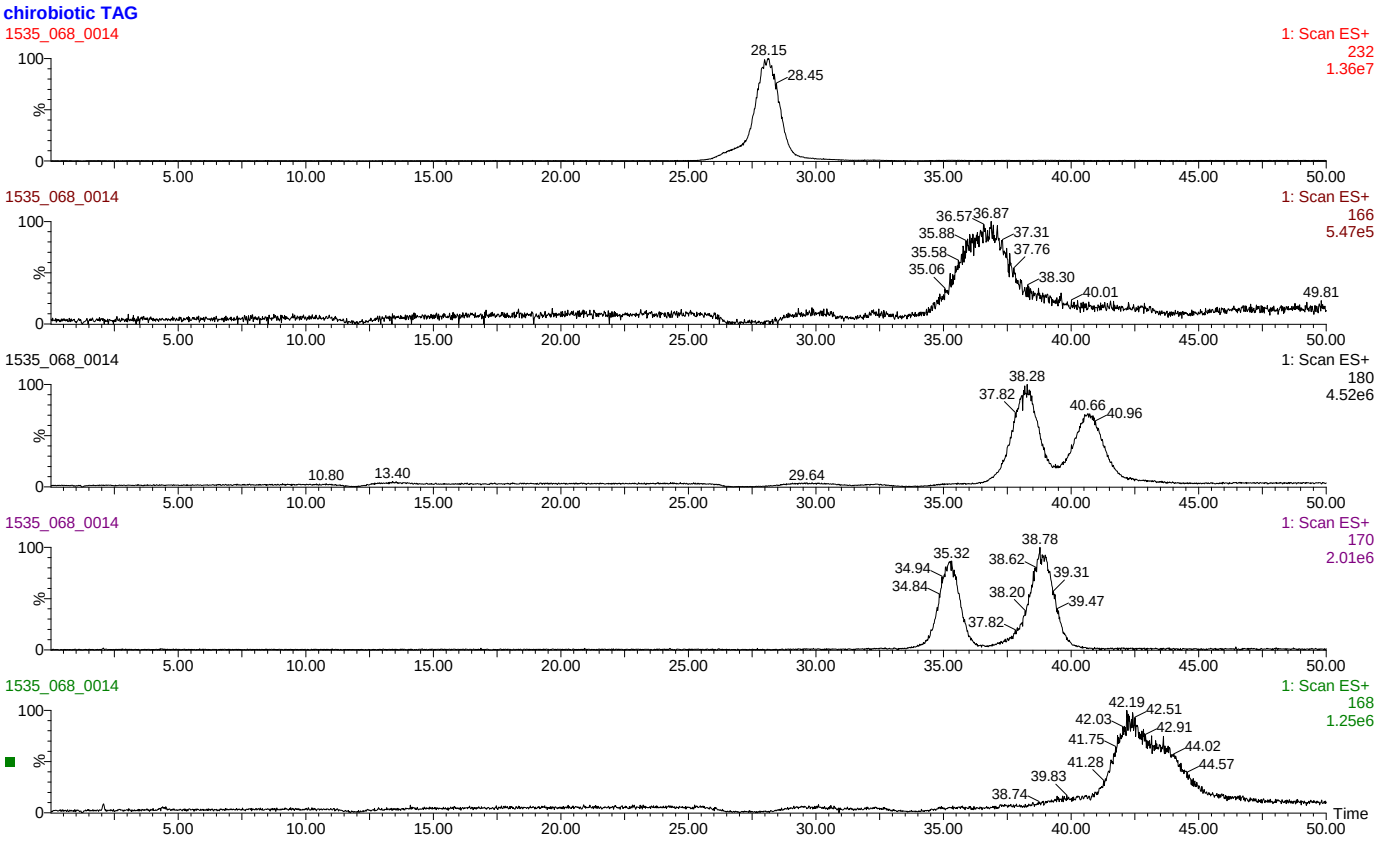
Norfluoxetine

Pentazocine

Chlorpheniramine

CHIROBIOTIC TAG Shows Selectivity for Amphetamines

Unique selectivity between TAG phase and certain solutes shows up in LC-MS screen.



Fenfluramine

Normetanephrine

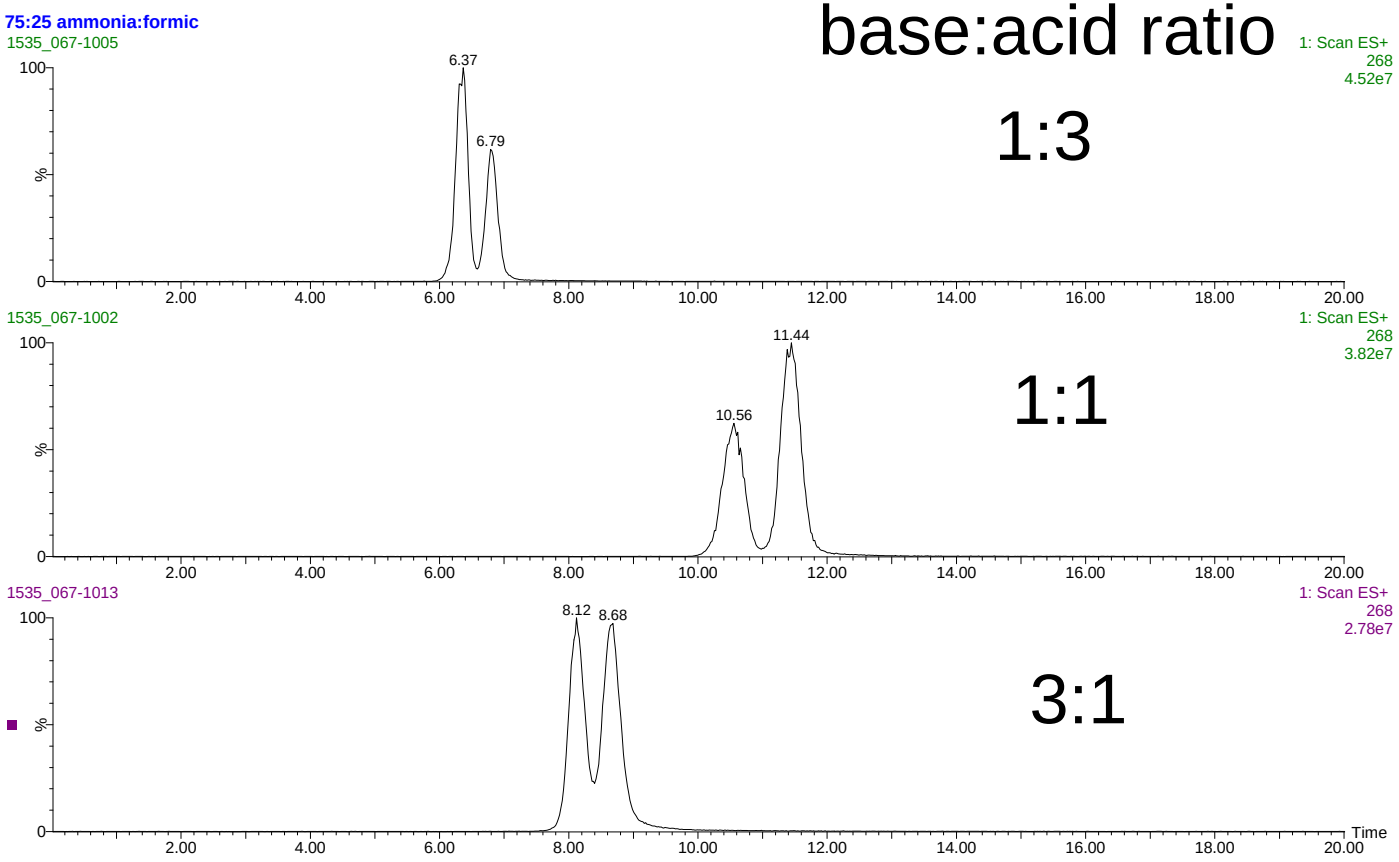
MDA

Chloramphetamine

Synephrine

Impact of Buffer Component Ratio on Metoprolol Retention and Selectivity

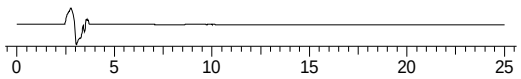
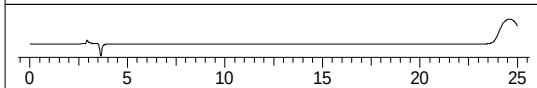
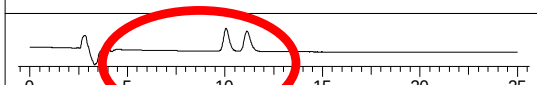
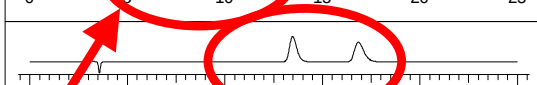
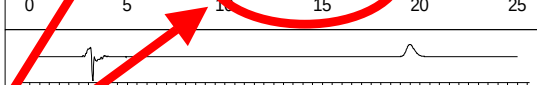
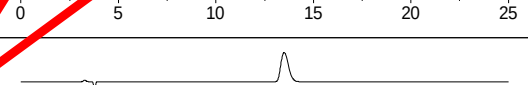
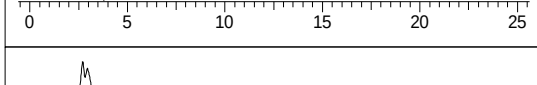
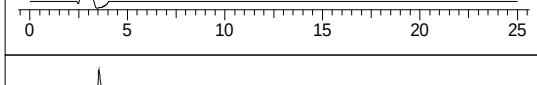
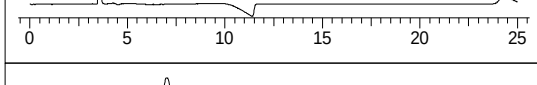
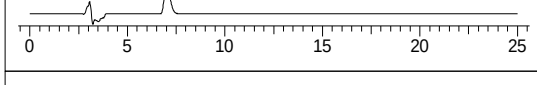
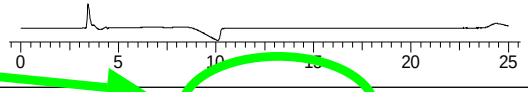
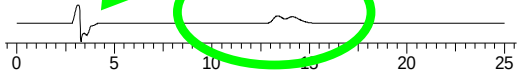
13 mM ammonium hydroxide and 13 mM formic acid were independently prepared in methanol.

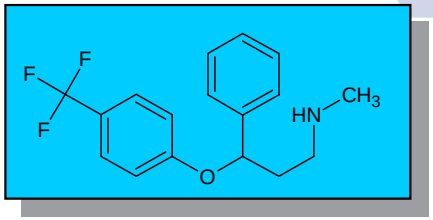


Results of Fluoxetine Column Screen

(+/-)-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.

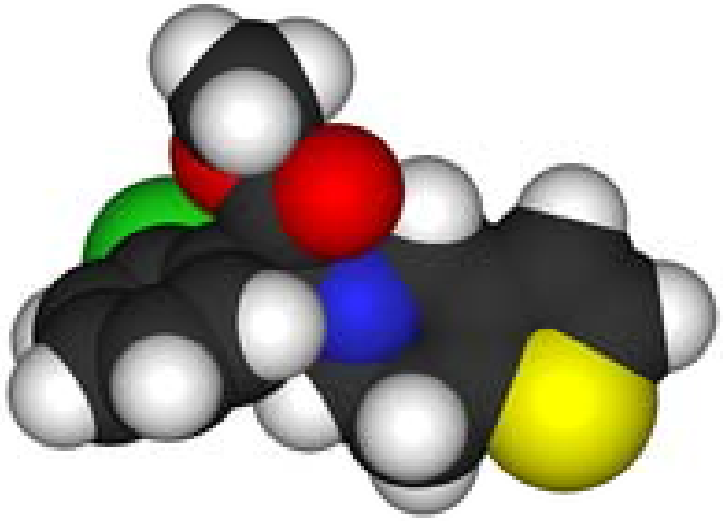
Chiral HPLC

Chiral stationary phases for HPLC

Familiar HPLC and LC-MS mobile phases

Chiral screening and LC-MS

Preparative considerations

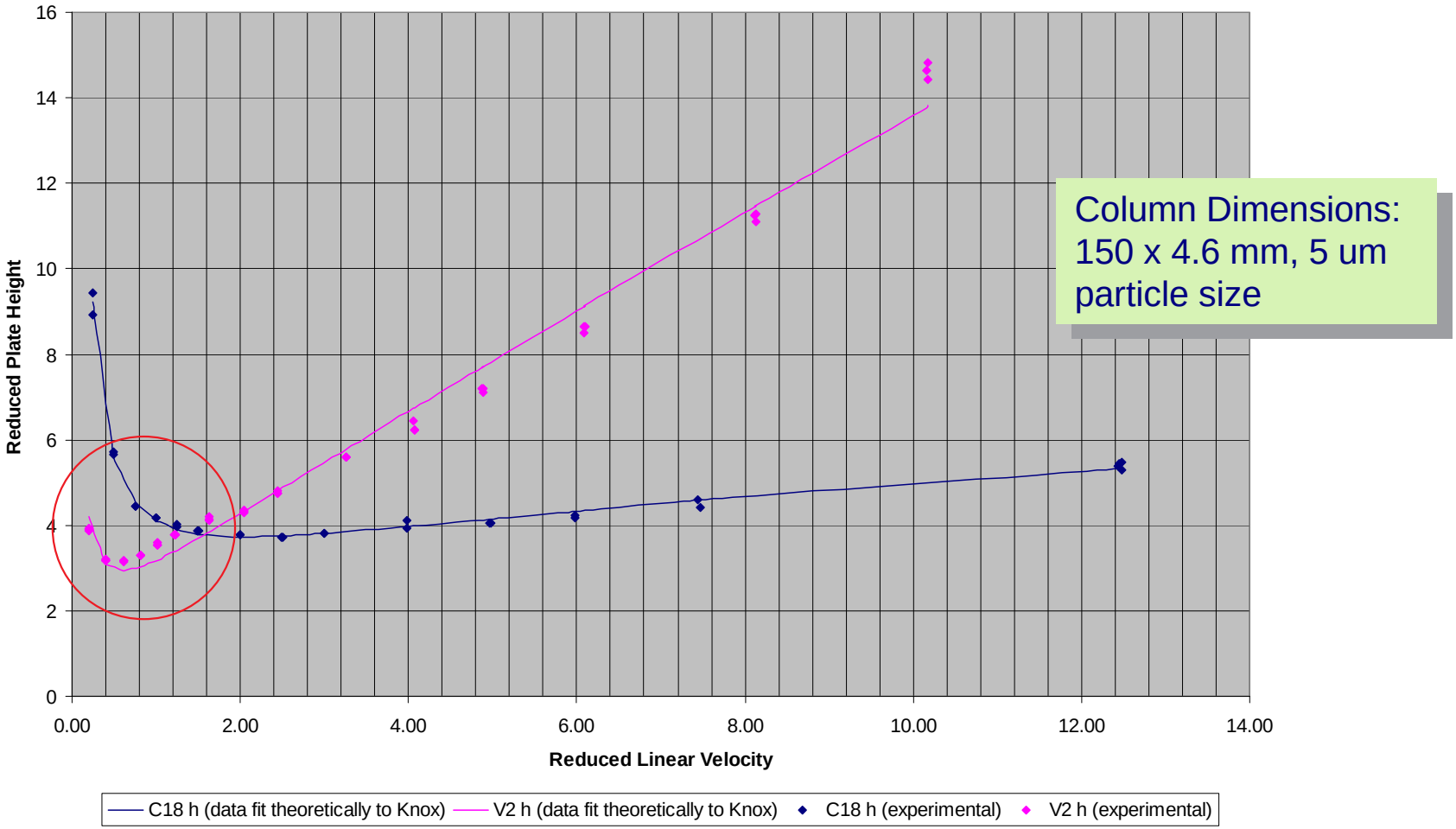


Prep Benefit: Low Flow Rate on CHIROBIOTIC

Optimal linear velocity on CHIROBIOTIC is significantly lower than that observed on achiral (C18) columns.

Low flows increase analysis time but greater efficiency and resolution increase sample capacity in prep separations.

Benefits of Low Flow for Fluoxetine on CHIROBIOTIC V2 *vs*. Ascentis C18



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.

Low Flow Advantage: 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%

Prep Benefit: Reversed-Phase Mode

Improved **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

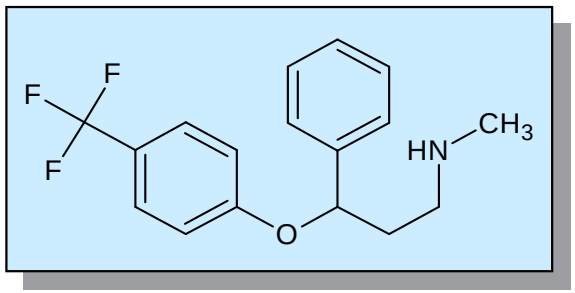
Optimizing Separation of Fluoxetine for Preparative HPLC

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

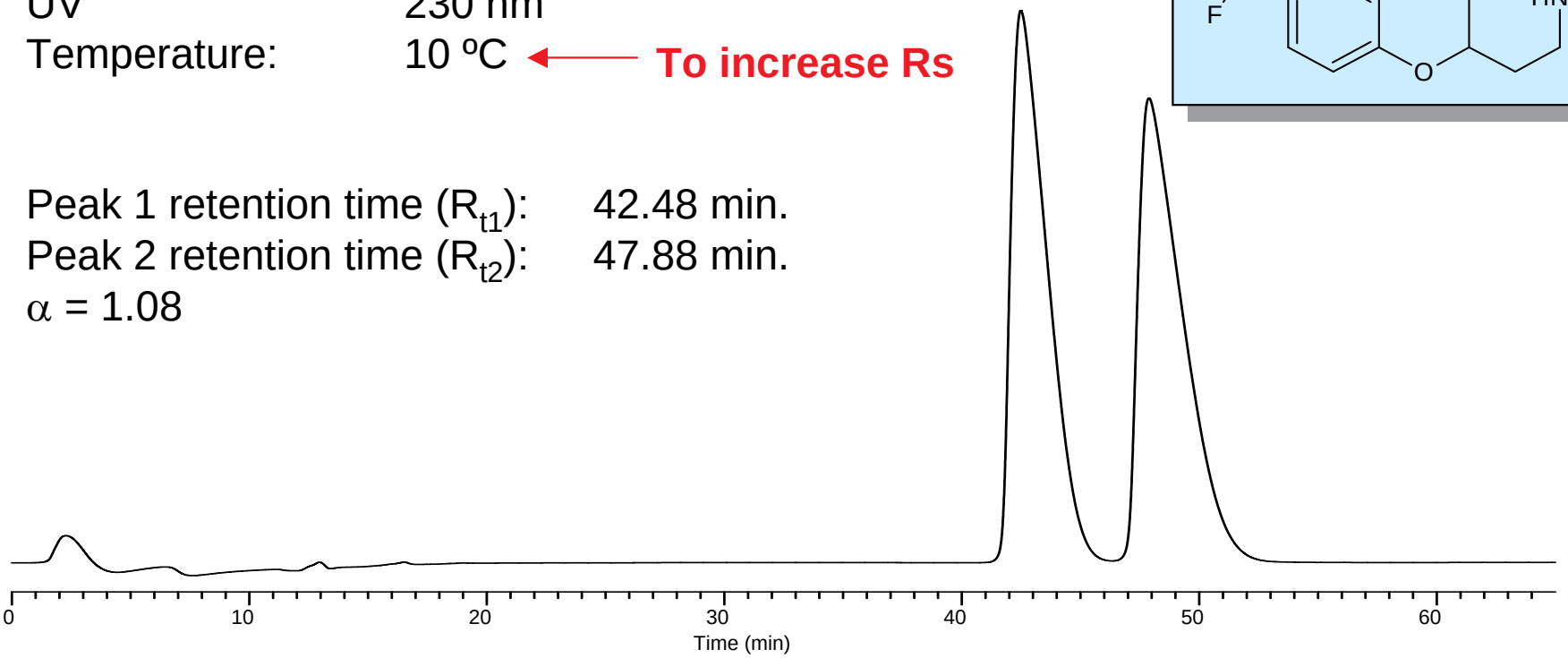
To increase Rs



Peak 1 retention time (R_{t1}): 42.48 min.

Peak 2 retention time (R_{t2}): 47.88 min.

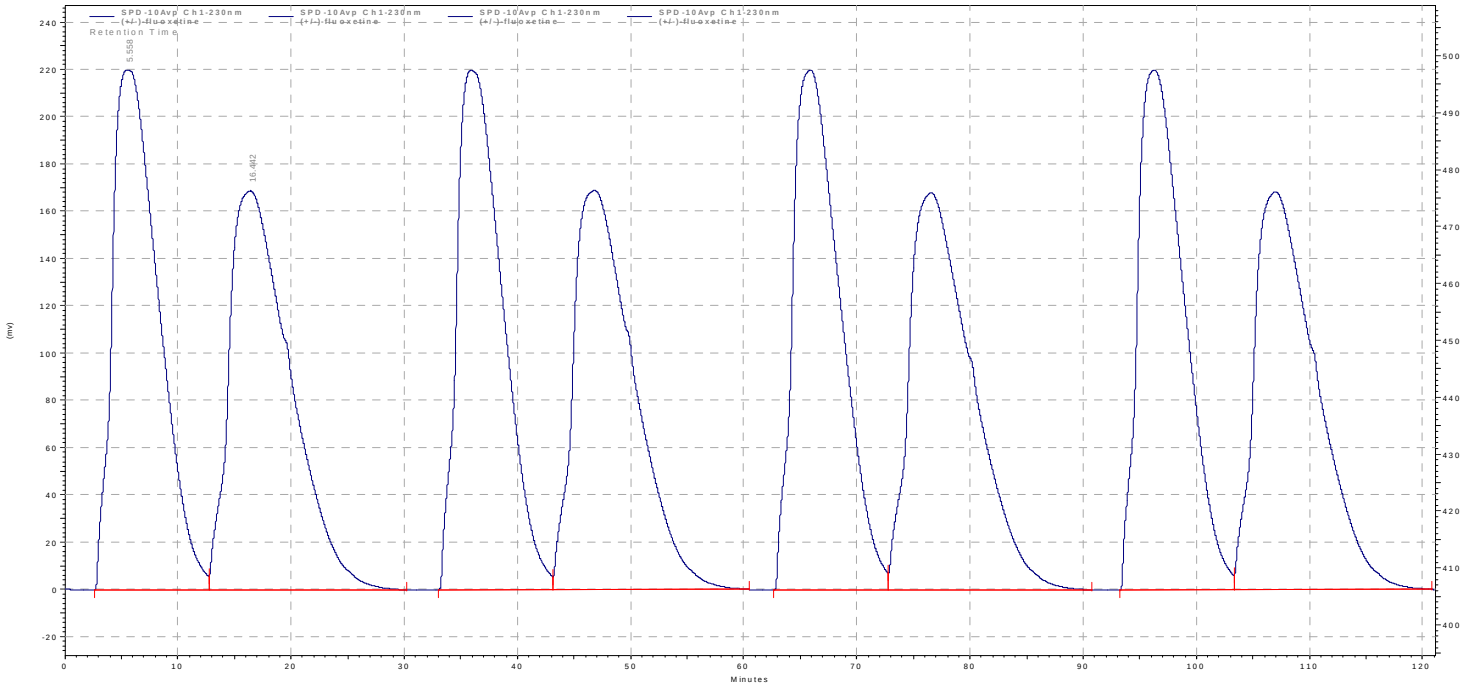
α = 1.08



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



Sample Recovery in RP and PIM Modes

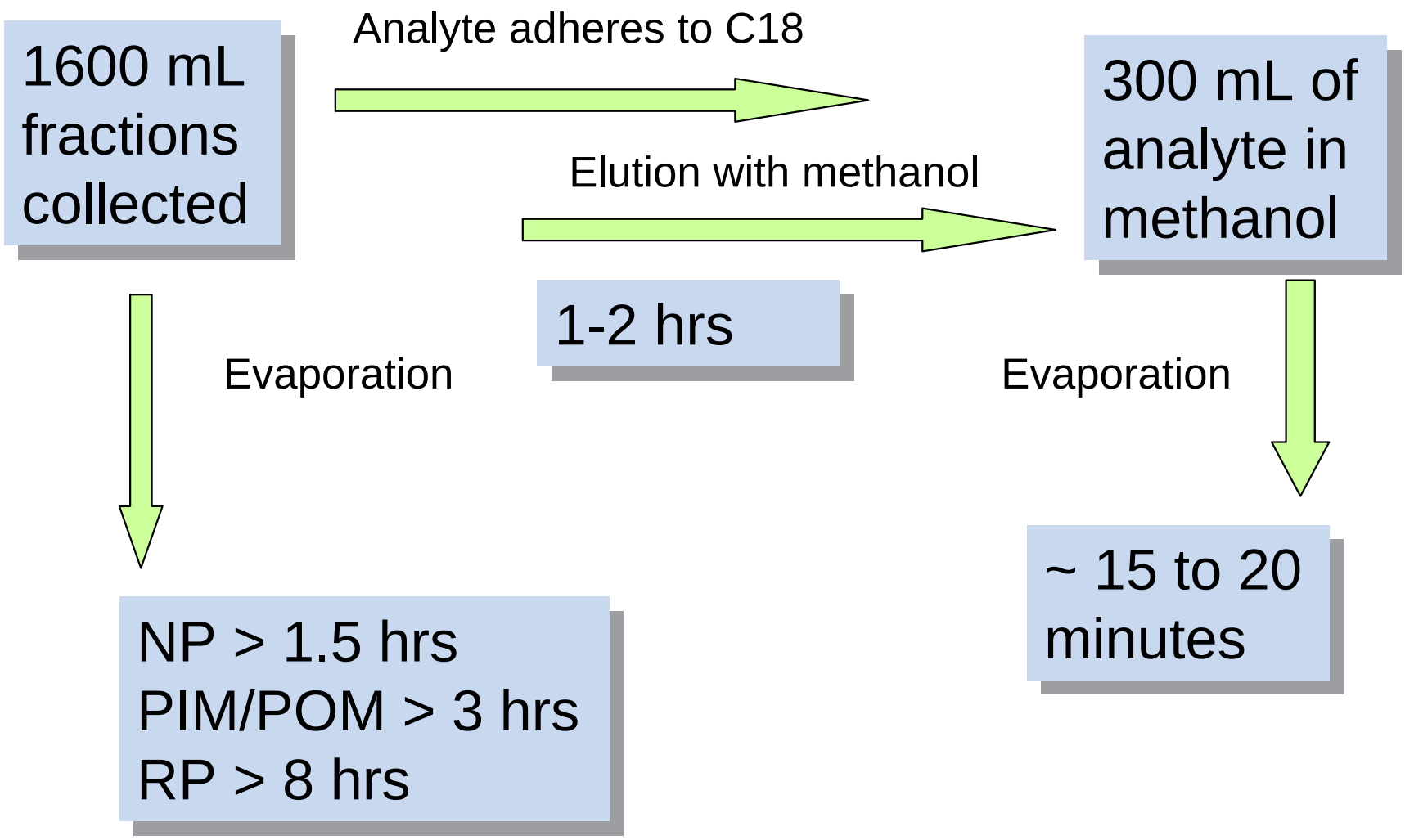
Reversed-Phase:

- Problems:
  - Lengthy time to remove water by evaporation
  - Buffer sales
- Solution:
  - Trap eluant containing analyte on C18 flash cartridge, wash
  - Elute in a minimal amount of MeOH

Polar Ionic Mode:

- Problems:
  - Mobile phase additives (salts)
- Solutions:
  - Concentrate, dissolve in diethyl ether
  - Load onto a silica flash cartridge, wash
  - Elute with a minimal amount of MeOH

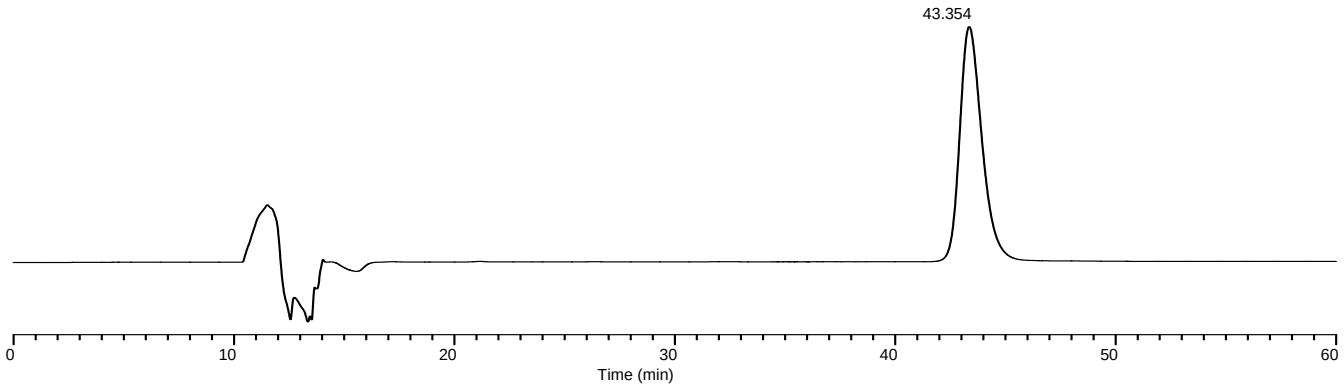
Sample Recovery Using Reversed-Phase C18 Flash Column Method



Purity of Prep Fractions

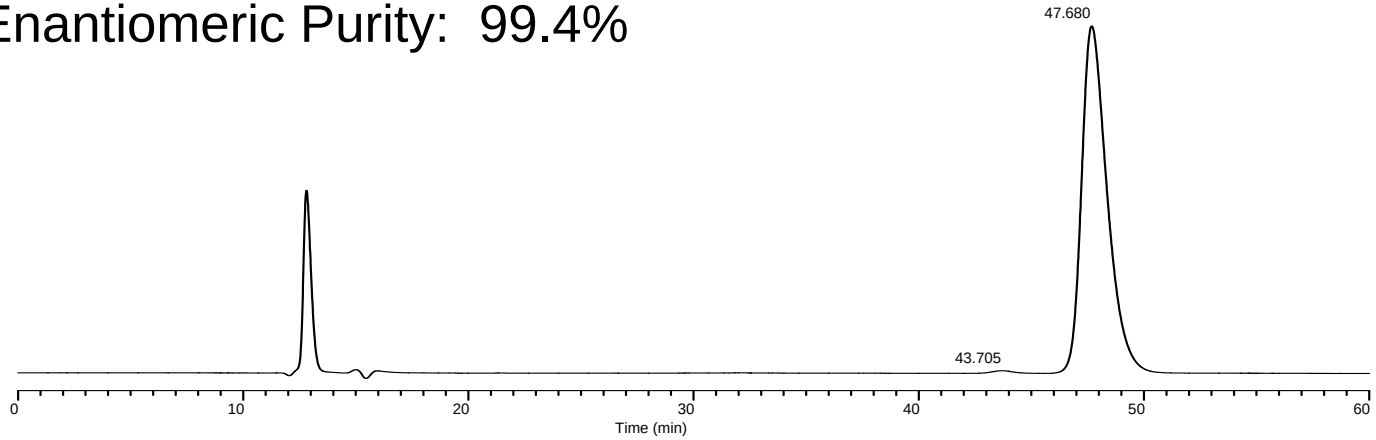
Peak 1:

- Enantiomeric Purity: 99.9%



Peak 2:

- Enantiomeric Purity: 99.4%



Important Points in Reversed-Phase Chiral Preparative Separations

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.

- Fast sample recovery from prep in RP using C18 recovery method
- RP less toxic than NP
- Low flow improves efficiency

Overall Chiral Summary

Chiral stationary phases for HPLC

- CYCLOBOND CSPs: Covalently bonded β-cyclodextrin or derivatized β-cyclodextrin
  - Reversed-phase, polar organic mode, normal phase modes
- CHIROBIOTIC CSPs: Macrocyclic glycopeptides
  - Especially useful for separation of polar analytes
  - Reversed-phase, polar-ionic, or normal phase modes
- Robust and compatible with all HPLC solvents
- MS detection and prep compatible

Familiar HPLC and LC-MS mobile phases

- Solvents and conditions familiar to most analytical chemists
- MS compatible
- Ideal for separation of polar and ionic compounds

CHIROBIOTIC HPLC Columns

LC-MS compatible

RP mobile phases

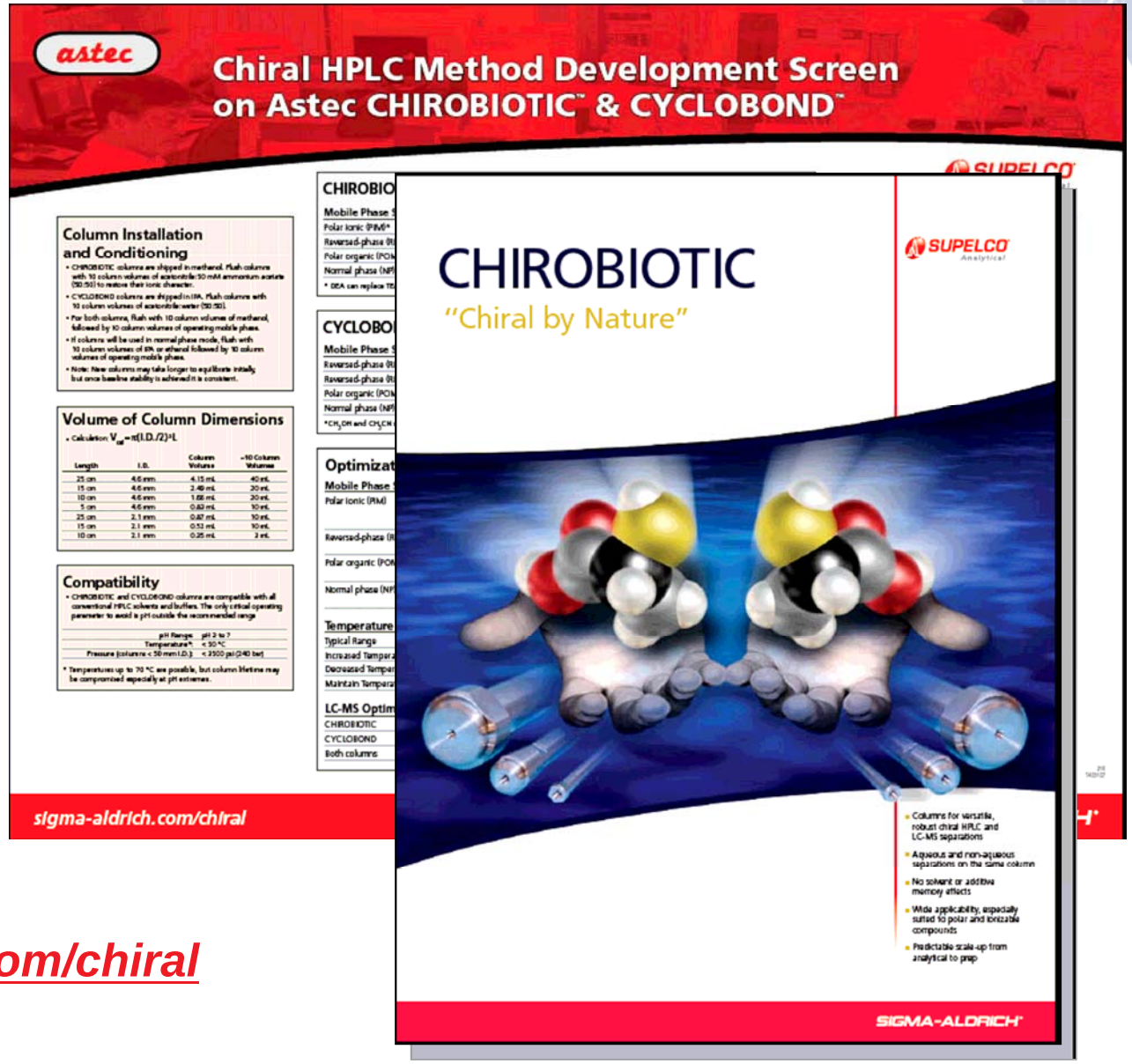
Analytical to prep

Phases:

- CHIROBIOTIC T and T2
- CHIROBIOTIC V and V2
- CHIROBIOTIC TAG
- CHIROBIOTIC R

Complements other Astec phases, CYCLOBOND, P-CAP, and chiral GC

Analytical Services



http://www.sigma-aldrich.com/chiral

Supelco Chiral Services

Chiral column screening (HPLC & GC)

Method optimization

Small-scale purification (<10 grams of each enantiomer)

Larger scale through our SAFC partners

http://www.sigma-aldrich.com/chiral

Acknowledgements/Collaborators

Prof. Daniel Armstrong, U. Texas Arlington

Dick Henry, Consultant

Supelco and Fluka R&D Teams

For more information on the subjects presented here, please contact techservice@sial.com or your regional sales team.