

# Basics of Chiral HPLC

Definitions

Principles

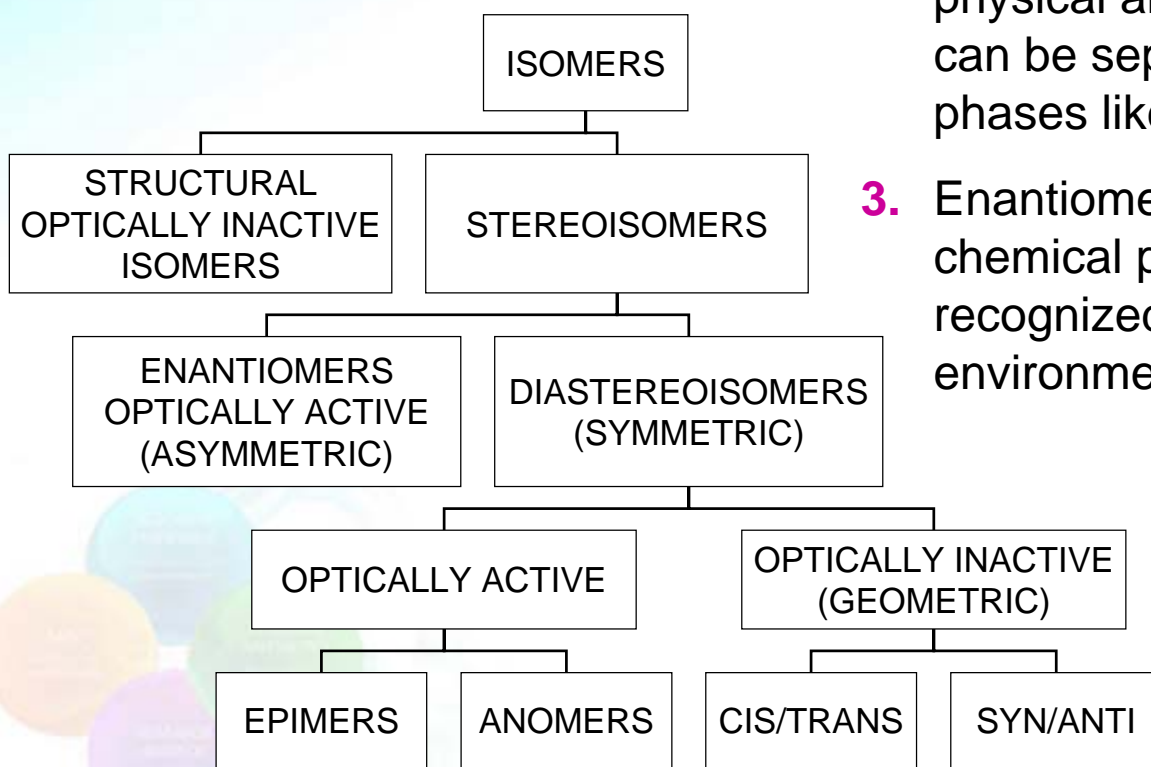
Available CSPs

Mobile phase types

T408109

# The Field of Stereochemistry

1. All isomers have same chemical formula but differ in the arrangement of certain chemical groups in space.
2. Many types of isomers have different physical and chemical properties and can be separated by conventional phases like C<sub>18</sub>.
3. Enantiomers have same physical and chemical properties and can be recognized or separated only in chiral environment.



# The Inclusion Complex

The basis for many chiral separations, especially in the reversed phase mode is a phenomena called inclusion complexing. First described for the polyglucose structures, cyclodextrins, it has been identified as a mechanism for the macrocyclic glycopeptides as well as the cellulose and amylose CSPs. An understanding of this phenomena is then imperative to an understanding of how these phases separate.

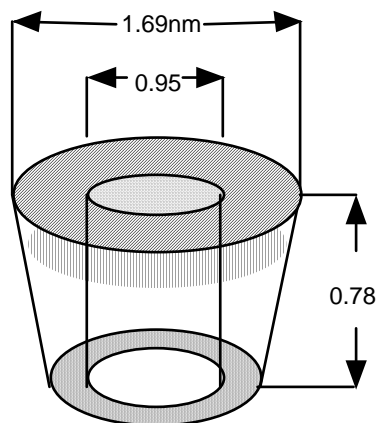
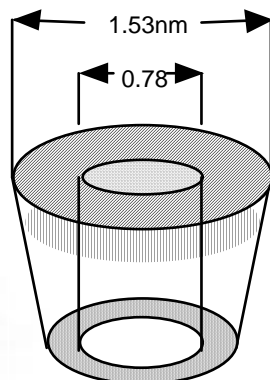
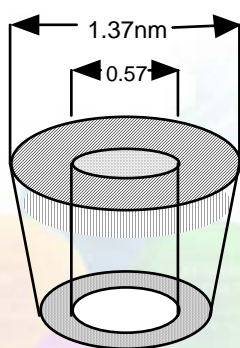
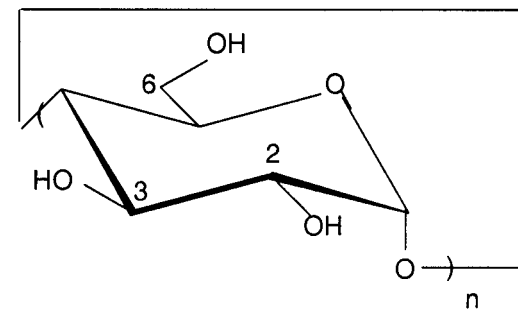
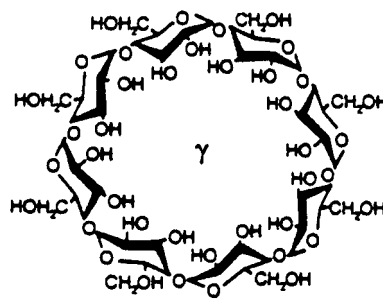
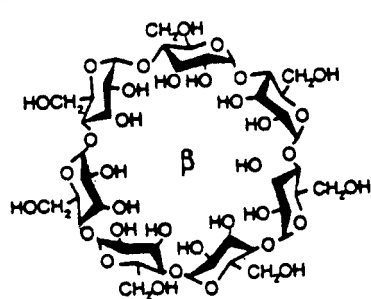
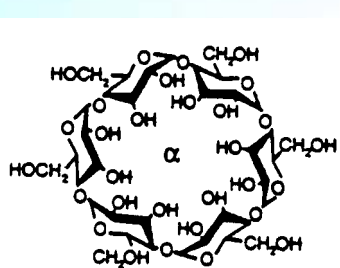
Structure and dimensions of the most common cyclodextrin molecules.

**$\alpha$ -cyclodextrin**

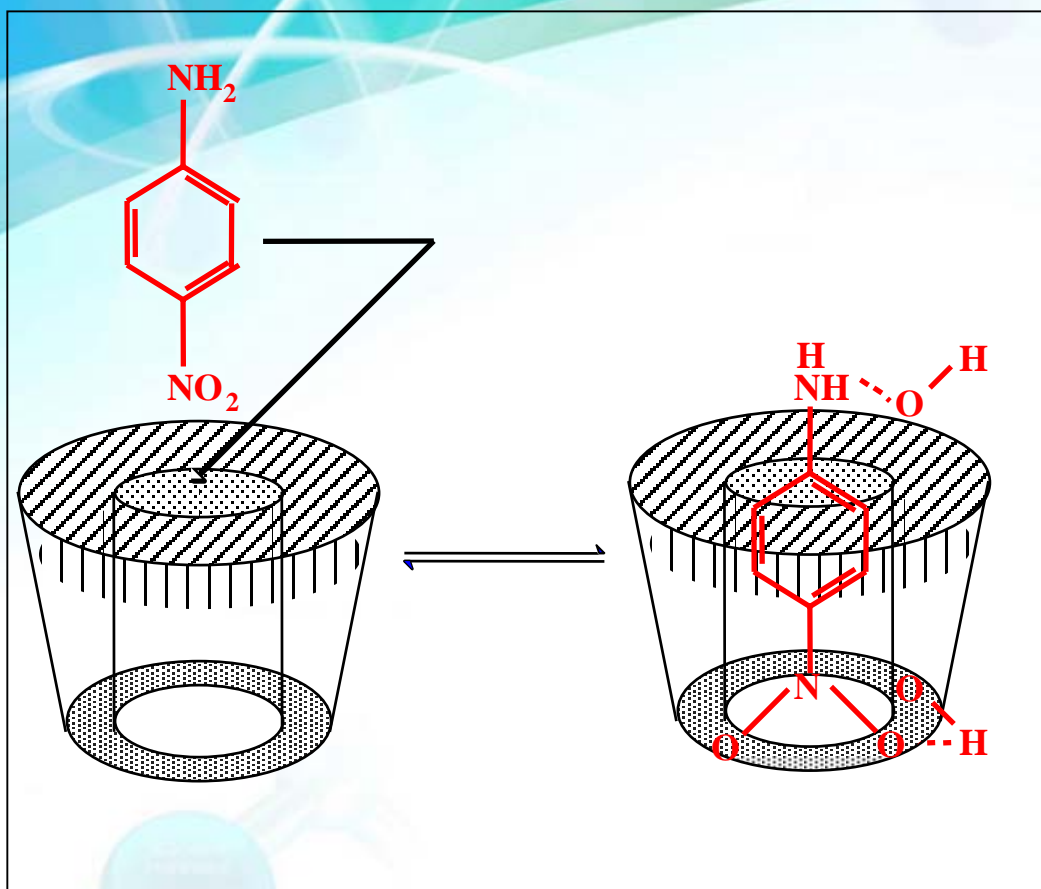
**$\beta$ -cyclodextrin**

**$\gamma$ -cyclodextrin**

**Schematic of one glucose unit in a cyclodextrin molecule**



# Inclusion Complex Schematic



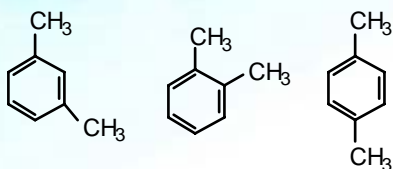
Inclusion complexing is accomplished in reversed phase systems. Its effectiveness is dependent upon the position and strength of substituents on benzene or fused ring analytes. The elution order is typically meta<ortho<para. The bulky character of the meta and ortho limits full inclusion. The linear nature of the para structure results in full inclusion. Selectivity for this isomer is generally highest when strong hydrogen bonding groups are present.

Acetonitrile accelerates release from the cavity. Methanol reduces hydrogen bonding effects to the cyclodextrin hydroxyl groups. Combinations of acetonitrile and methanol in water are useful in optimizing a separation.

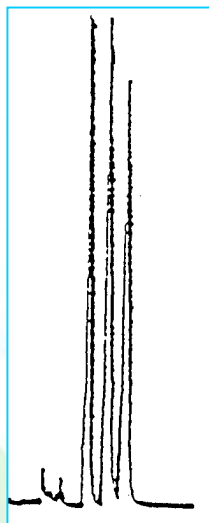
# Examples of Inclusion Phenomena

## Positional Isomers

### Xylenes

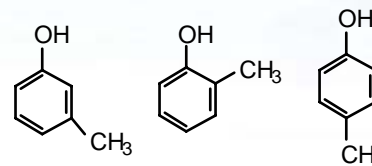


Peak 1 - 5.74 min. (ortho)  
Peak 2 - 6.38 min. (meta)  
Peak 3 - 6.83 min. (para)

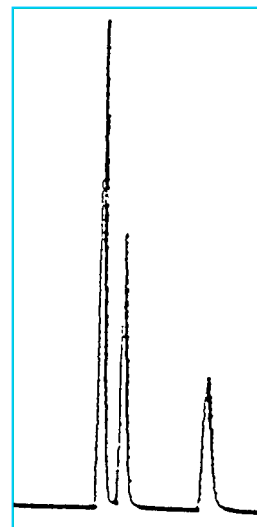


CYCLOBOND I 2000  
Mobile Phase: 30/70: CH<sub>3</sub>CN/H<sub>2</sub>O

### Cresols

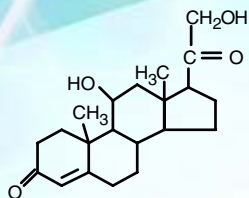


Peak 1 - 6.62 min. (ortho)  
Peak 2 - 7.31 min. (meta)  
Peak 3 - 9.82 min. (para)

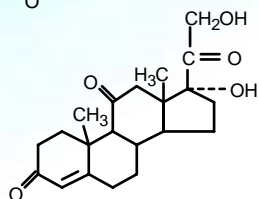


CYCLOBOND I 2000  
Mobile Phase: 40/60: CH<sub>3</sub>OH/H<sub>2</sub>O

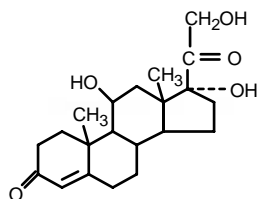
# Structural Isomers



**Corticosterone**



**Cortisone**

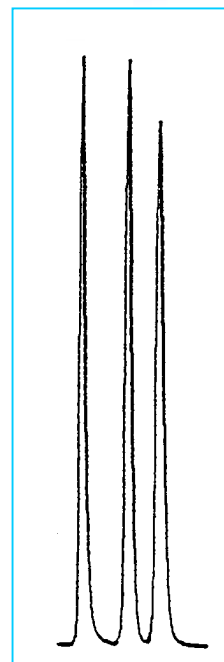


**Hydrocortisone**

Peak 1 - 6.29 min.

Peak 2 - 8.02 min.

Peak 3 - 9.18 min.

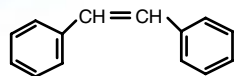


CYCLOBOND I 2000

Mobile Phase:40/60: CH<sub>3</sub>CN/H<sub>2</sub>O

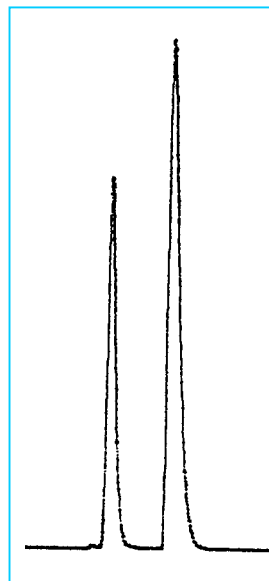
# Geometric Isomers

## cis/trans Stilbene



Peak 1 - 5.60 min.

Peak 2 - 7.14 min.

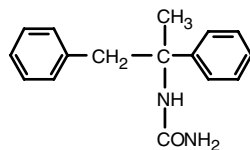


CYCLOBOND I 2000

Mobile Phase: 70/30: MeOH/H<sub>2</sub>O

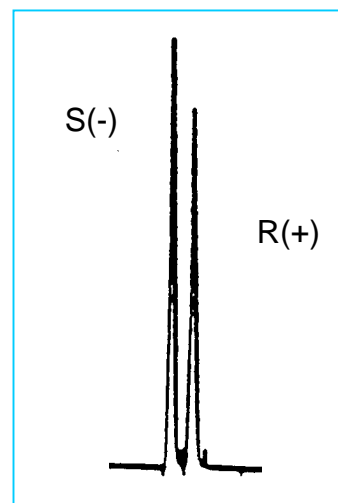
# Enantiomers

## Remacemide



Peak 1 - 5.74 min.

Peak 2 - 6.79 min.



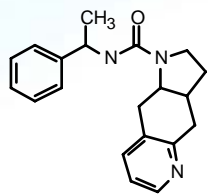
CYCLOBOND I 2000

Mobile Phase:

3.5/96.5: CH<sub>3</sub>CN/PO<sub>4</sub>(0.1M) pH 3.5

# Diastereoisomers

## 2,2,3a,4,9,9a-Hexahydro-14-Pyrrolo[2,3g]quinoline-1-carboxylic (1-phenylethyl) amide



Peak 1 - 8.01 min.

Peak 2 - 11.66 min.



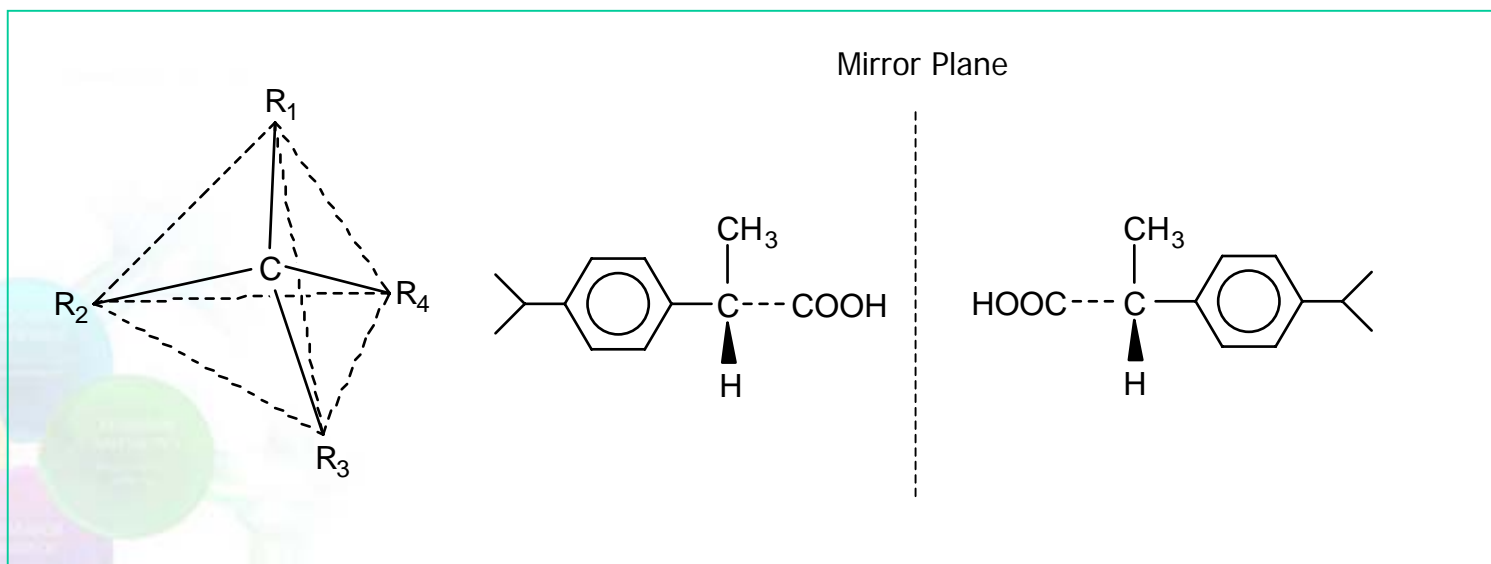
CYCLOBOND I 2000

Mobile Phase:

40/60: MeOH/0.1% TEAA, pH 6.0

# Terminology and Definitions

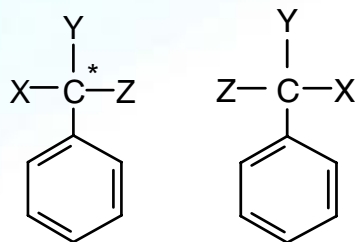
**CHIRALITY** deals with stereoisomers which are compounds with the same molecular formula and structure but different spatial orientations around a stereogenic center or axis commonly defined as a plane of symmetry. The molecular dissymmetry is based on the geometric nature of atoms like carbon, sulfur, phosphorous and nitrogen.



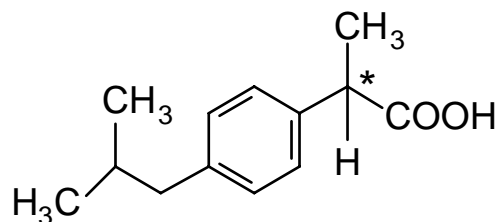
# Models for Identification of Potential Racemates

## A. Enantiomers based on **CARBON**

### Type 1: **Stereogenic Center**

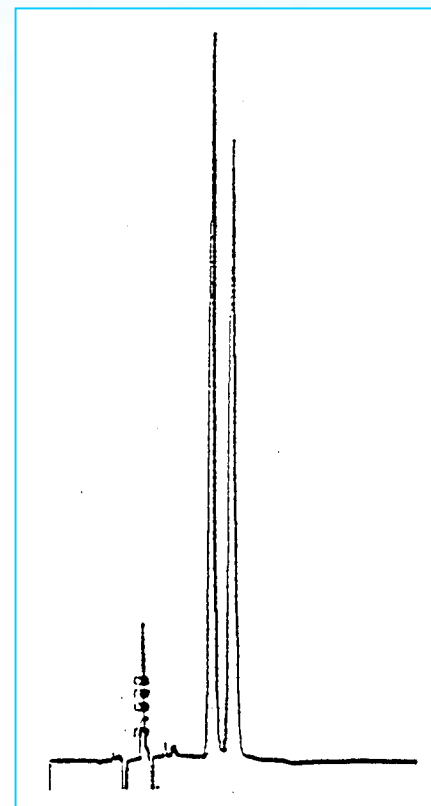


## Ibuprofen



Peak 1 - 5.77 min.

Peak 2 - 6.47 min.

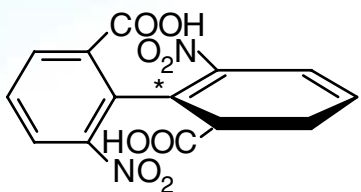


CHIROBIOTIC V

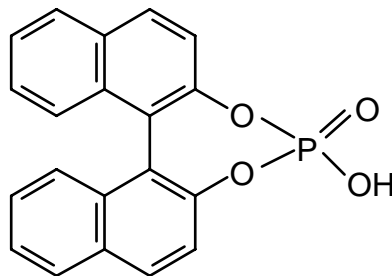
10/90: THF/20mM Na Citrate, pH 6.3

# Models for Identification of Potential Racemates

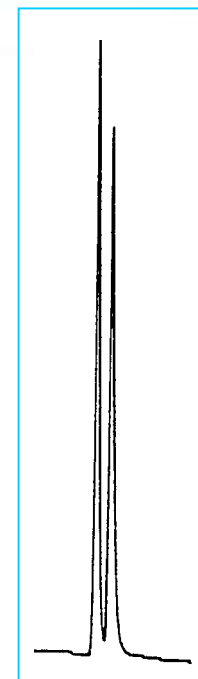
## Type 2: Axis of Symmetry



## 1,1'-Binaphthyl-2,2'-diylhydrogenphosphate



Peak 1 - 9.12 min.  
Peak 2 - 10.03 min.



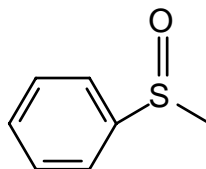
CHIROBIOTIC V  
30/70: ACN/0.1% TEAA, pH 4.1

# Models for Identification of Potential Racemates

## B. Enantiomers based on **SULFUR**

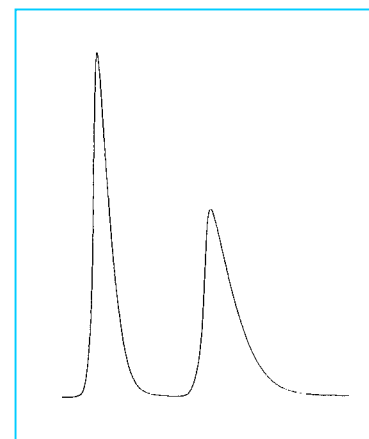
Possibilities include sulfoxide, sulfoximide, sulfinic acid and sulfonium ion.

### Methyl phenyl sulfoxide



Peak 1 - 10.86 min.

Peak 2 - 14.49 min.



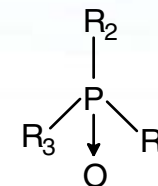
CHIROBIOTIC TAG  
40/60: EtOH/Hex

# Models for Identification of Potential Racemates

## C. Enantiomers based on **PHOSPHORUS**

Possibilities include phosphine, phosphine oxide, phosphinate and phosphonium ion.

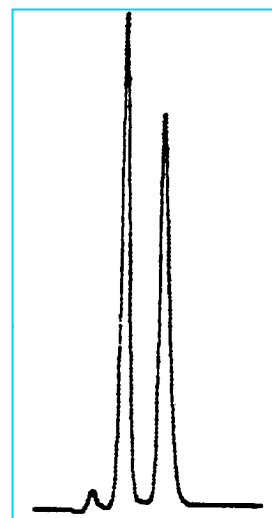
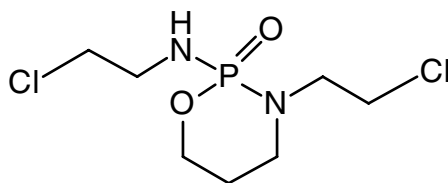
### Phosphine Oxide



Peak 1 - 7.53 min.

Peak 2 - 8.48 min.

### Ifosfamide

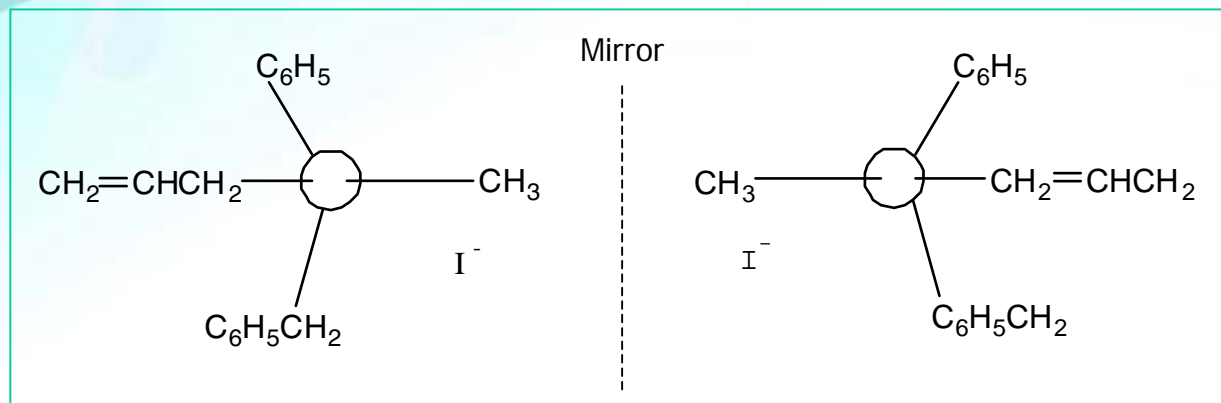


CHIROBIOTIC T  
10/90: THF/H<sub>2</sub>O

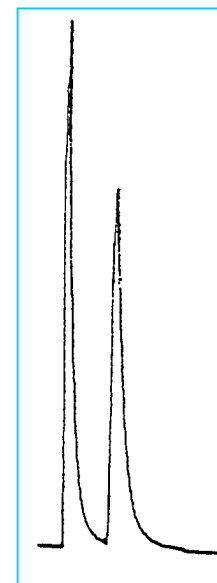
# Models for Identification of Potential Racemates

## D. Enantiomers based on **NITROGEN**

Possibilities include amine oxide and ammonium ion.



Peak 1 - 8.31 min.  
Peak 2 - 11.40 min.



**(+) and (-)-Methylallylphenylbenzyl  
ammonium iodide**

CHIROBIOTIC V  
100/0.02/0.01: MeOH/HOAc/TEA

# Definitions

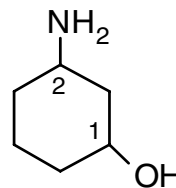
## R,S-CONFIGURATIONS:

Term used to describe the absolute conformation of a chiral compound (by Cahn, Ingold and Prelog) according to their sequence rules. “R” is the abbreviation for rectus (Latin, meaning right or clockwise), while “S” is the abbreviation for sinister (Latin, meaning left or counterclockwise).

Ref: J. Chem. Soc., 612 (1952); Experientia, 12, 81 (1956); Angew. Chem. Int. Ed., 5, 385 (1966).

## THE 2<sup>n</sup> RULE:

The maximum number of stereoisomers that can exist for a compound containing more than one chiral center is  $2^n$ , where n is the number of chiral centers.



$$N=2$$

$$\text{Number isomers} \therefore (2^2)=4$$

# ENANTIOMERIC EXCESS:

Used to describe the percent purity of an enantiomer. It can be calculated from the chromatographic data as follows:

$$\% ee = \left[ \frac{\text{Area A} - \text{Area B}}{\text{Area A} + \text{Area B}} \right] \times 100$$

## Example:

If the specific rotation of enantiomer A was  $+60^\circ$  and a partially racemized mixture was  $+30^\circ$ . What was the % optical purity or the enantiomeric excess? What are the relative amounts of (+) and (-) enantiomers in the mixture?

$$1) \% \text{ optical purity} = \frac{+30^\circ}{+60^\circ} \times 100 = 50\%$$

2) Amount (+) and (-) enantiomers

Let  $x$  = % of (+) enantiomer

Let  $y$  = % of (-) enantiomer

$$x + y = 100\%$$

$$x - y = 50\%$$

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$$2x = 150\%$$

$$x = 75\% (+)$$

$$75 + y = 100$$

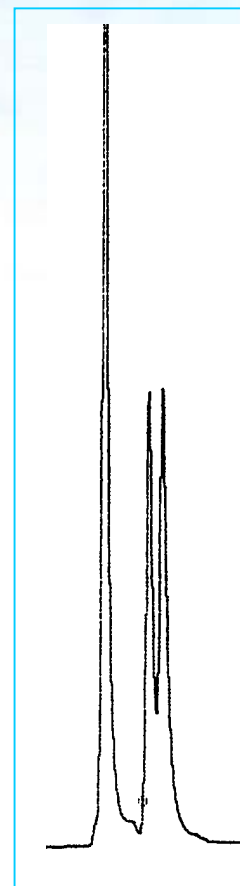
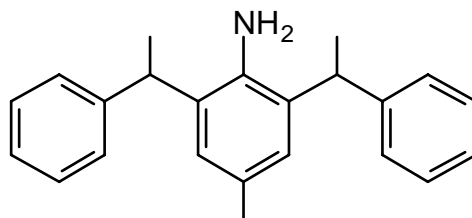
$$y = 100 - 75$$

$$y = 25\% (-)$$

## MESO COMPOUND:

A compound whose functional groups are superimposable on their mirror images even though they contain chiral centers. It is optically inactive.

### 2,6-bis-(1-phenyl-ethyl)-4-methylaniline

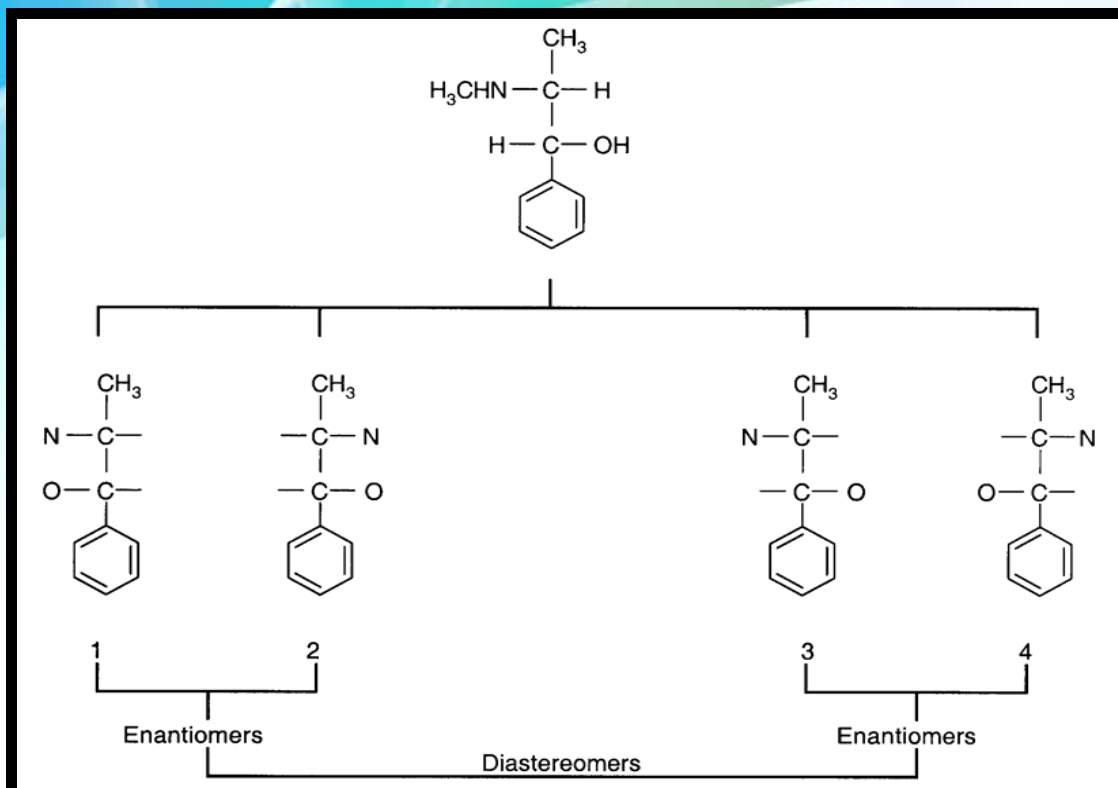


Peak 1 – 6.19 (meso)  
Peak 2 – 7.96  
Peak 3 – 8.47

CYCLOBOND I 2000 RSP  
250x4.6mm

30/70: CH<sub>3</sub>CN/0.1% TEAA, pH 6.5  
1.0 mL/min.

# DIASTEREOMERIC/ENANTIOMERIC STRUCTURES:

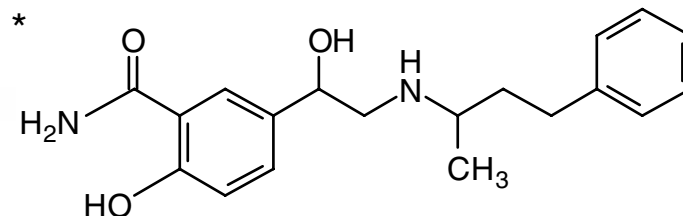


**Note:** Conventional reversed phase would separate this analyte into 2 peaks (diastereomers). Only a chiral stationary phase would resolve all 4 enantiomers.

Peak 1 – 11.54 min.  
Peak 2 – 12.29 min.  
Peak 3 – 16.57 min.  
Peak 4 – 19.35 min.



## Labetalol



CHIROBIOTIC V  
100%: MeOH/0.1% ATFA, v/w

## RACEMATES OR RACEMIC MIXTURE:

A 50:50 mixture of enantiomers. This mixture is optically inactive due to the rotation of one molecule exactly cancelling the opposite rotation of its enantiomer.

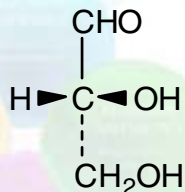
## OPTICAL ACTIVITY:

Rotation of the plane of polarized light caused by the presence of a stereogenic center or axis in a compound.

## NOMENCLATURE:

Amino acids, sugars and related compounds still refer to the D,L designation. This system, invented by Emil Fisher, refers to the configuration of the glyceraldehyde. He arbitrarily assigned the + isomer of glyceraldehyde as the D isomer.

### D-(+)-Glyceraldehyde



The d and l designation refers to the rotation of plane polarized light (Sodium d-line). If this light is rotated to the right, the designation is “d” or (+). This assignment has problems as D-glutamic acid is actually l or (-) for the rotation of polarized light. Care must be exercised in using D,L or d,l.

# Principles Governing Chiral Separation

**Concept:** formation of a **diastereomeric complex** in a chromatographic equilibrium such that the nonchiral interactions are at minimum strength and the differential chiral interaction is at maximum strength. Identifying those points of interaction between the stationary phase and the racemate guides you in the choice of CSPs and the best conditions under which to operate.

Non-chiral interactions generally anchor a molecule and, therefore, assist in the formation of the diastereometric complex. Both  $\pi$ - $\pi$  interactions driven in normal phase phase solvents and inclusion complexation driven in reversed phase modes are the first significant areas to address the potential of an appropriate chiral stationary phase.

# Types of Interaction

All forces in the chiral interaction process **do not** have to be attractive, they can be attractive as well as repulsive. Commonly described forces for chiral recognition are listed as follows:

- $\pi-\pi$
- Hydrogen bonding
  - a. Hydrogen donor site
  - b. Hydrogen acceptor site
- Inclusion complexation
- Steric hindrance
- Dipole-dipole
- Ionic interaction

# Modern Chiral Stationary Phases

Polymeric

Synthetic

- Methacrylate
- Polycyclic amine-3

Focus – normal phase,  
low cost,  
reproducible, high  
capacity

Astec-Supelco  
Phases

Natural

- Cellulose
- Amylose
- Proteins

Small  
molecule  
ligands

- Copper complex-2
- $\pi$ -complex
- Crown ether
- Cyclodextrin-12
- Macrocyclic glycopeptides-6

These 3 cover applications that  
are possible on 140 CSPs that  
have been created worldwide in  
this area.

# Astec-Supelco Chiral Phases

## Polycyclic Amine Phases

P-CAP, P-CAP-DP, P-CAP-EV

## Copper Complex Phases

CLC-D, CLC-L

## Macrocyclic Glycopeptide Phases

CHIROBIOTIC V, CHIROBIOTIC V2,  
CHIROBIOTIC T, CHIROBIOTIC T2,  
CHIROBIOTIC TAG, CHIROBIOTIC R

# Astec-Supelco Chiral Phases con't

## Cyclodextrin Phases

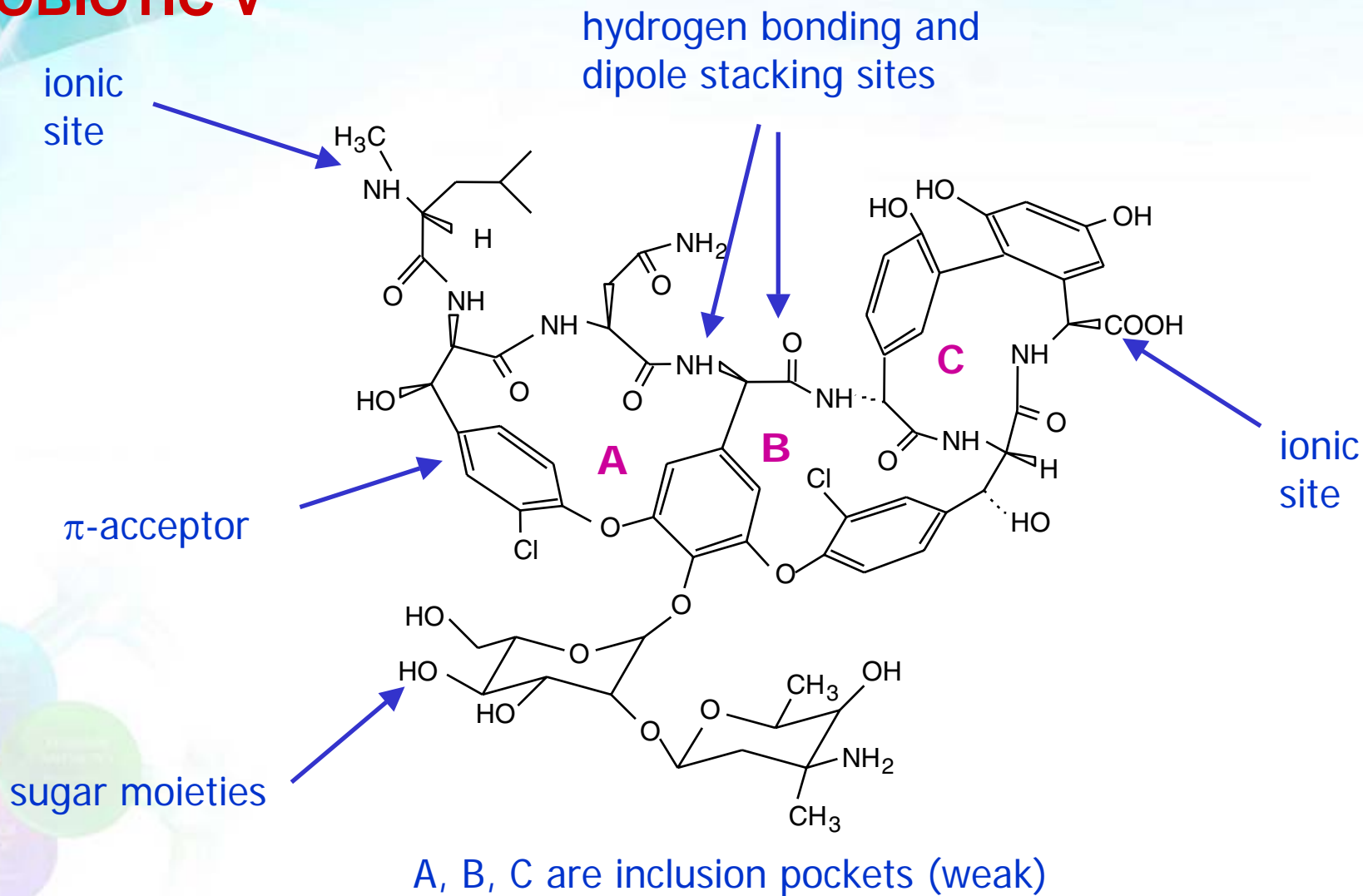
CYCLOBOND I 2000

CYCLOBOND I 2000 AC, CYCLOBOND I 2000 SP,  
CYCLOBOND I 2000 RSP, CYCLOBOND I 2000 HP-RSP,  
CYCLOBOND I 2000 SN, CYCLOBOND I 2000 RN,  
CYCLOBOND I 2000 DM, CYCLOBOND I 2000 DMP,  
CYCLOBOND I 2000 DNP,  
CYCLOBOND II, CYCLOBOND II AC

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# Structure of Vancomycin CSP

## CHIROBIOTIC V

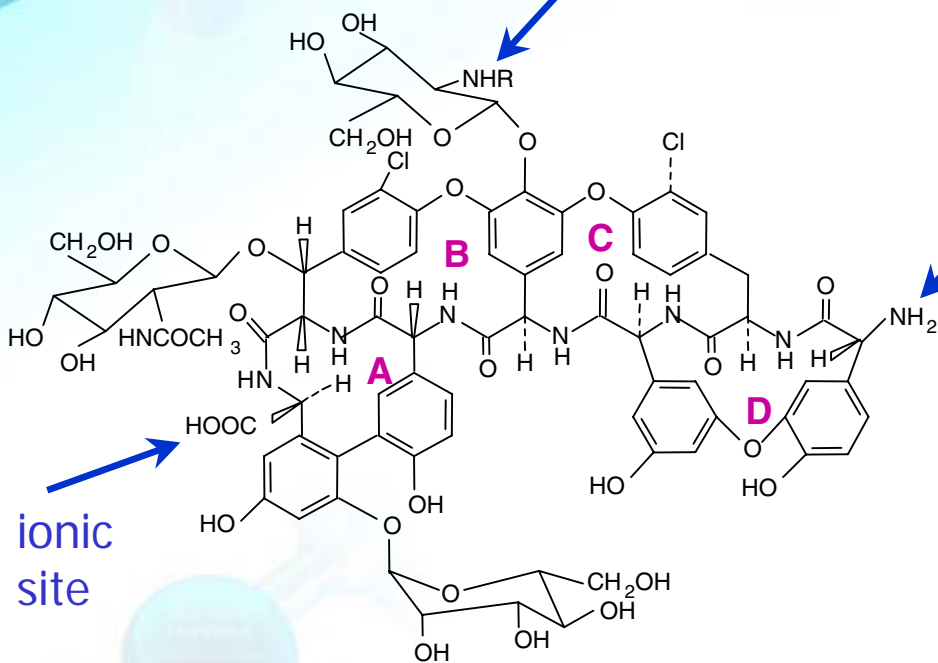


# Structure of Teicoplanin CSP

**Teicoplanin,  
CHIROBIOTIC T**

→ Key sites

sugar and alkyl chain

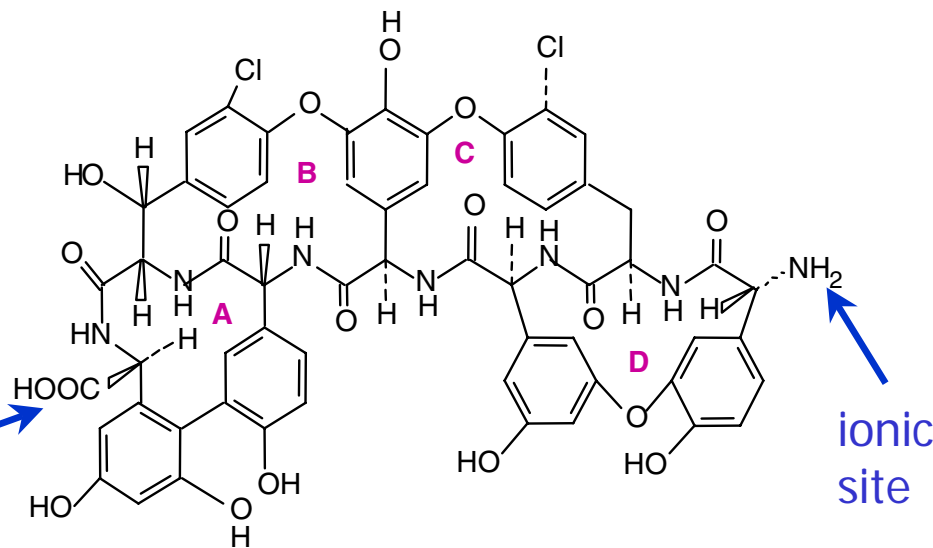


ionic site

**Teicoplanin Aglycone,  
CHIROBIOTIC TAG**

ionic site

ionic site



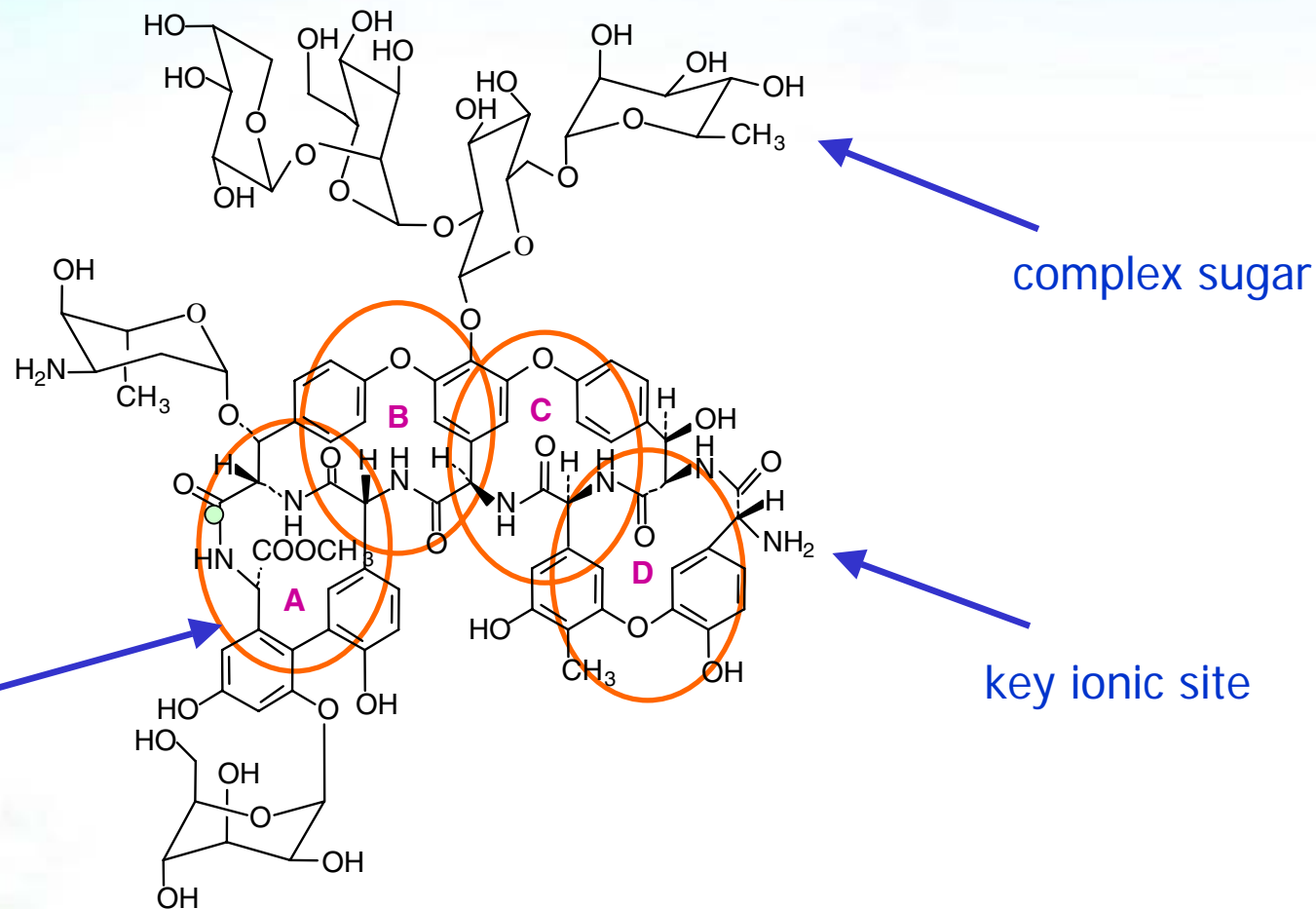
ionic site

# CHIROBIOTIC V2 and CHIROBIOTIC T2

- Extensions of the CHIROBIOTIC V2 and T2 created by changing the position of several linkages and the chain length used to anchor the ligand.
- These changes can **enhance** the **selectivity** and **capacity** of these phases mainly in the polar ionic and polar organic modes, and sometimes in reversed phase.

# Proposed Structure of Ristocetin A CSP

## CHIROBIOTIC R

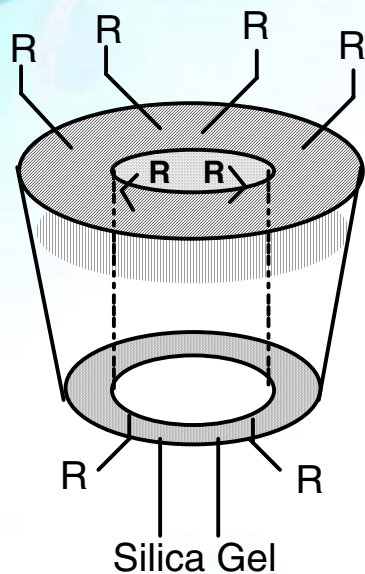


inclusion cavities

complex sugar

key ionic site

# Bonded Derivatized Cyclodextrins



\* Signifies stereogenic center

R =	Suffix	CD Type
- OCH <sub>3</sub>	DM (methylated)	β-CD
- COCH <sub>3</sub>	AC (acetylated)	β-CD γ-CD
$\begin{array}{c} \text{OH} \\   \\ -\text{CH}_2\text{CH}^*\text{CH}_3 \end{array}$	SP or RSP/HP-RSP (hydroxypropyl ether)	β-CD
$\begin{array}{c} \text{CH}_3 \\   \\ -\text{CONHCH}^* \end{array}$	RN or SN (naphthylethyl carbamate)	β-CD
$\begin{array}{c} \text{CH}_3 \\   \\ -\text{CONH} \end{array}$	DMP (3,5-dimethylphenyl carbamate)	β-CD
	DNP (2,6-dinitro-4-trifluoromethyl phenyl ether)	β-CD

# Most Productive CYCLOBOND Phases

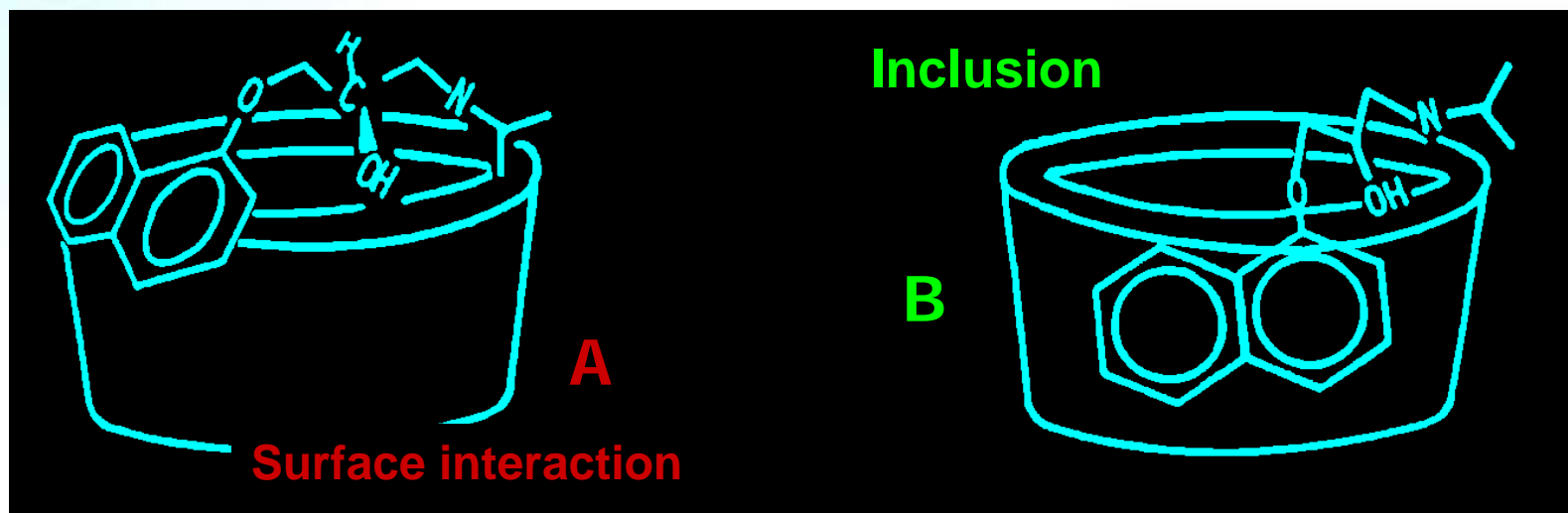
- **CYCLOBOND I 2000**
- **CYCLOBOND I 2000 HP-RSP (highest hit rate)**

Primarily basic chiral compounds have been resolved on the RSP and to a lesser extent both neutrals and acidics. All successful separations have been in the reversed phase mode with the organic component in the range of 5-40%.
- **CYCLOBOND I 2000 DMP (second most productive CSP in this line)** This is a  $\pi$ -basic phase.
- **CYCLOBOND I 2000 DNP (new addition)**

This is a  $\pi$ - acidic phase that has demonstrated separations not previously possible on any cyclodextrin phase.

# CYCLOBOND

two different enantioselective retention mechanisms



**A: Polar organic mode (surface)**

**B: Reversed phase mode (inclusion)**

# Polar Organic: Structural/Functional Group Requirements

- Structural Requirements:

2 functional groups capable of interacting with the stationary phase

- Types of Functional Groups:

Halogen: I > Br > Cl > F

Amine: 3° ~ 2° > 1°

Carbonyl: -COOH, -CHO, -C=O, -COOR

Sulfo-, phospho- and hydroxyl groups

One of these functional groups must be on or alpha to the stereogenic center

# Mobile phases types and mechanisms

Normal Phase

Reversed Phase

Polar Organic

Polar Ionic

# Normal phase and types of interaction

## Composition:

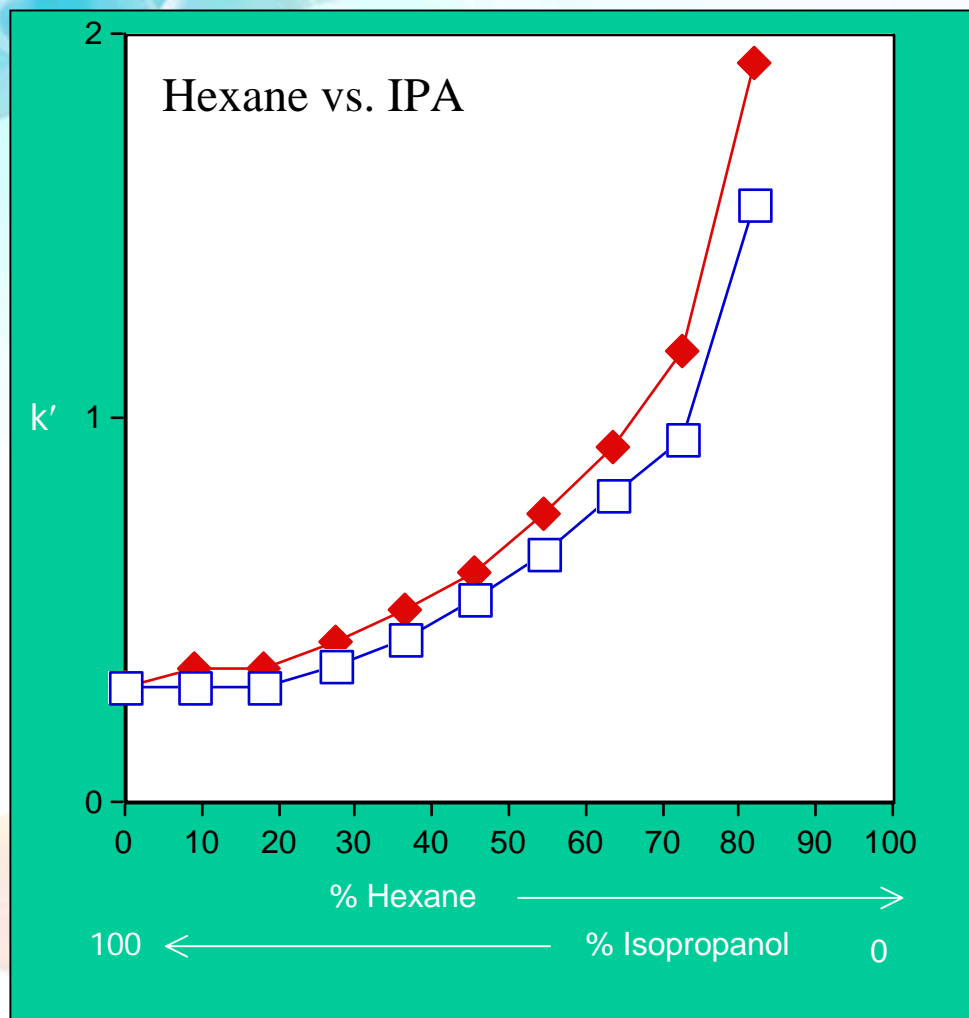
Hydrocarbon solvent: hexane or heptane + polar alcohol: IPA or EtOH

Dominant interactions:  $\pi$ - $\pi$  interaction, hydrogen bonding

## Type of CSPs:

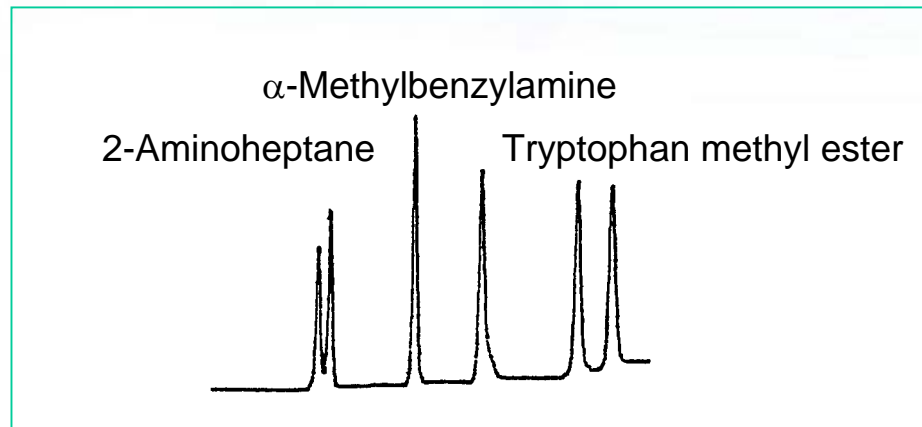
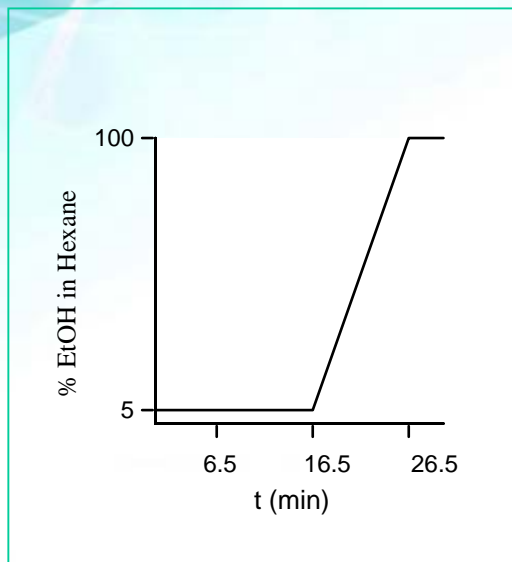
CYCLOBOND I 2000 DMP, SN, RN,  
CHIROBIOTIC V, T, R and TAG

# Normal Phase Solvents Mephenytoin



Ref: Anal. Chem., Vol. 66 (9),  
p 1473-184, May 1994.  
Armstrong, Tang, S. Chen, Zhou,  
Bagwill and J.R. Chen.

# Gradient Separation in the Normal Phase



Derivative: 3,4-Dinitrobenzoyl  
CYCLOBOND I 2000 SN  
Hexane/EtOH Gradient

## Reversed Phase

### Composition:

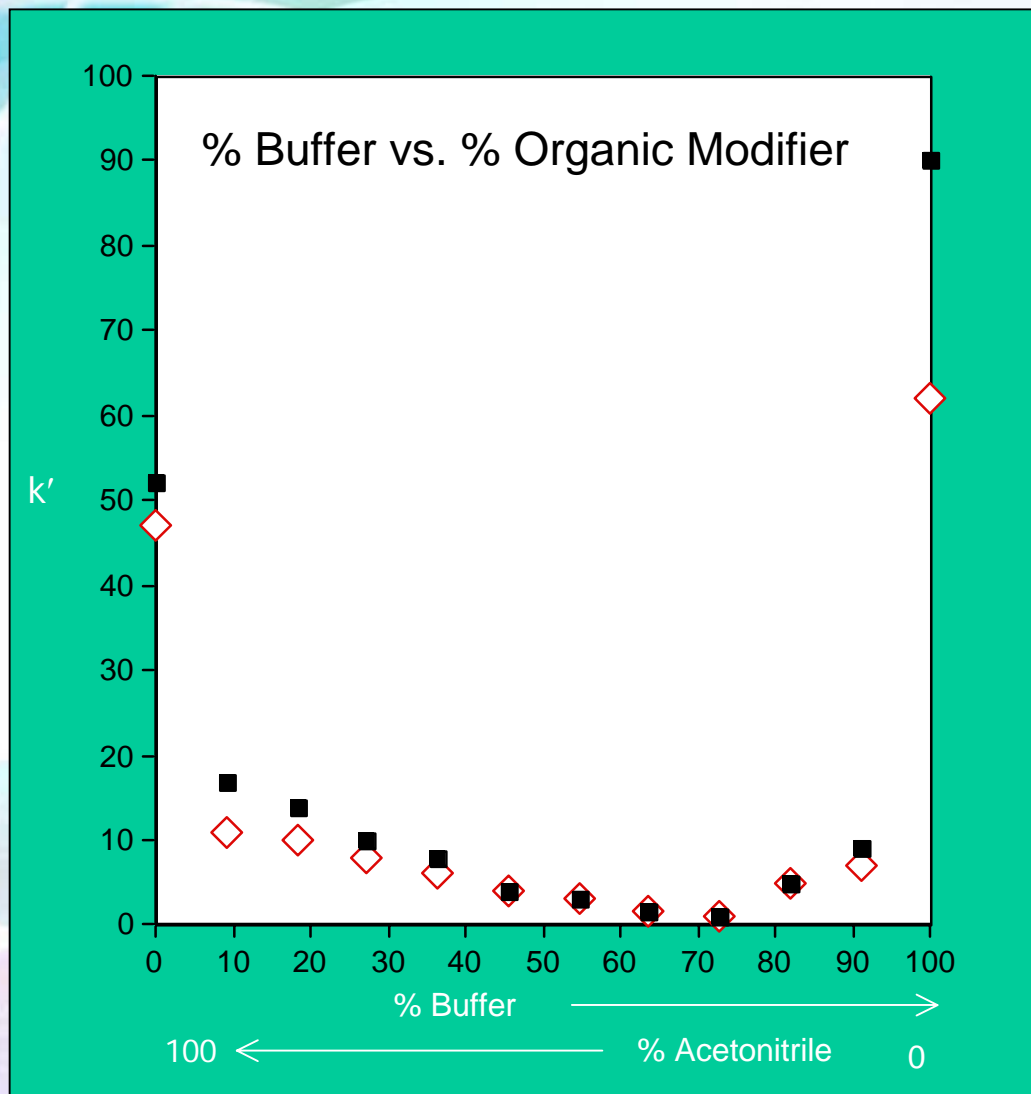
Organic solvent: ACN, MeOH or THF + aqueous buffer: TEAA, NH<sub>4</sub>OAc

Dominant interactions: inclusion, hydrogen bonding

### Type of CSPs:

CYCLOBOND I 2000, RSP,AC, DMP,SN, RN, II, III, CHIROBIOTIC V, T, R and TAG

# Reversed Phase Separation 5-Methyl-5-phenyl hydantoin



Ref: Anal. Chem.,  
Vol.66(9), p. 1473-  
1484, May 1994.

# Reversed Phase Solvents

## Factors Influencing a Separation

- pH
- Organic modifier
- Buffer type and concentration
- Flow rate
- Temperature

## Polar Organic Phase

### Composition:

ACN + MeOH + HOAc + TEA

### Dominant interactions:

Hydrogen bonding, dipole-dipole

### Type of CSPs:

**Cyclodextrins only –**  
CYCLOBOND I 2000, RSP, AC, DMP, SN, RN

# Polar Organic Solvents

## Factors Influencing Retention and Selectivity

### *CYCLOBOND Phases Only*

There are four components to the original polar organic mode (v/v/v/v):

Acetonitrile	50-100 parts
Methanol	0-50 parts
Anhydrous, Glacial Acetic Acid	0.1 to 1.0 parts
Anhydrous Triethylamine	0.1 to 1.0 parts

This composition is primarily used for cyclodextrins and cyclodextrin derivatives.

- To increase selectivity alter ratio of acid to base
- To decrease retention without affecting selectivity:
  - Increase acid/base concentration at same ratio
  - Increase methanol concentration
- To increase retention:
  - Reduce or eliminate methanol
  - Decrease acid/base concentration at same ratio

# New Polar Ionic Mode

**Composition:**

MeOH + HOAc + TEA

**Dominant interactions:**

Ionic interaction, hydrogen bonding

**Type of CSPs:**

Macrocyclic glycopeptides only -  
CHIROBIOTIC V, T, R and TAG

# Polar Ionic Mode: *CHIROBIOTIC Phases Only*

## BENEFITS:

- **Faster, more efficient separations, low pressure, long column life**
- **Faster method development, simple optimization, broad selectivity**
- **Best use ammonia and formic acid or acetic acid modifiers (0.01-1.0 parts) in 100 parts methanol for LC/MS/MS compatibility.**
- **Analyte salts easily disassociated**
- **Complimentary to normal phase separations on polysaccharide CSPs**
- **Very useful in preparative purifications to replace hexane/ethanol.**
  - **Lower boiling point than heptane or hexane, higher evaporation rate**
  - **Less toxic**
  - **Higher evaporation rate**

# POLAR IONIC MODE

## Mobile Phase Components

### ▪ Methanol/Acid/Base Components:

- Methanol, anhydrous
- Acid: anhydrous TFA, acetic acid, formic acid
- Base: TEA, DEA, NH<sub>3</sub>
- Alternatives: ammonium formate or acetate

### ▪ Ratio of Acid to Base

- Controls only selectivity
- Rate: 4:1 to 1:4, most typical 2:1

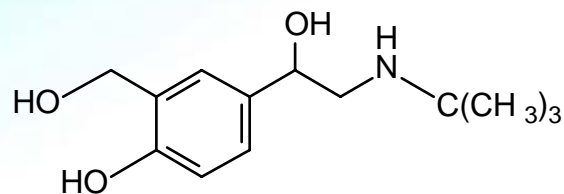
### ▪ Concentration of Acid + Base

- Controls retention and selectivity
- Concentration range: 0.001 to 1.0 part per 100, most typical 0.10.

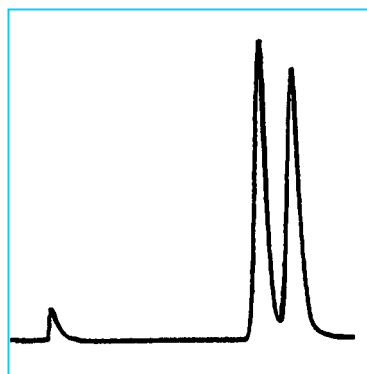
# Conversion from Old Polar Organic Mode to New Polar IONIC Mode

*For CHIROBIOTIC Phases Only*

## Albuterol

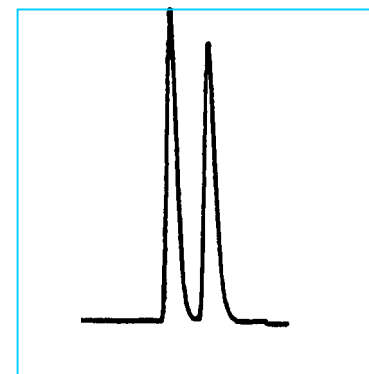


Peak 1 - 17.04 min.  
Peak 2 - 18.37 min.



70/30/0.5/0.2:  
**CH<sub>3</sub>CN/CH<sub>3</sub>OH/HOAc/TEA**

Peak 1 - 11.28 min.  
Peak 2 - 12.45 min.

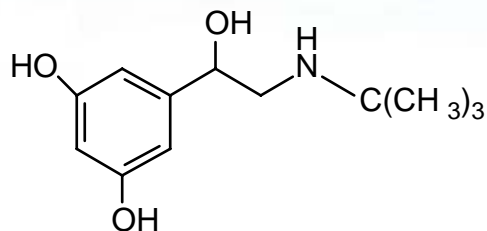


100/0.01/0.01:  
**CH<sub>3</sub>OH/TFA/NH<sub>4</sub>OH**

# Acid-Base Effects

With the new polar organic mode different acids and bases could be used, unlike the situation with the cyclodextrin, which demonstrates selectivity only employing acetic acid and triethylamine.

## Terbutaline

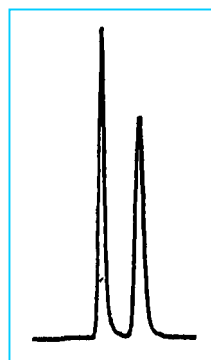


$\alpha = 1.38$



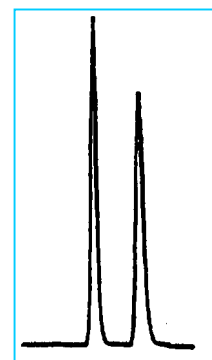
CH<sub>3</sub>OH/HOAc/TEA  
100/0.3/0.2

$\alpha = 1.30$



CH<sub>3</sub>OH/20mM  
NH<sub>4</sub>Ac

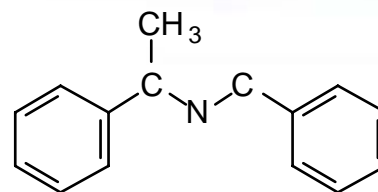
$\alpha = 1.30$



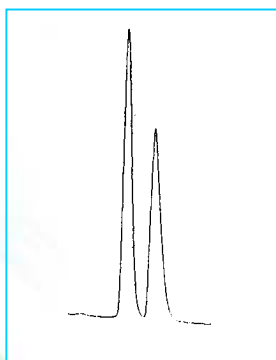
CH<sub>3</sub>OH/TFA/NH<sub>4</sub>OH  
100/0.05/0.05

# Comparison Using HOAc/TEA vs ATFA in the Polar IONIC Mode

N-Benzyl-a-methylbenzylamine  
CHIROBIOTIC V, 250x4.6mm

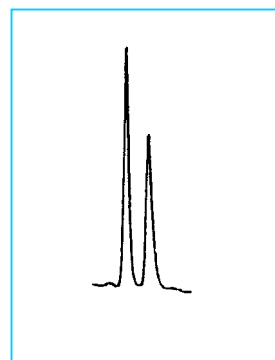


Peak 1 – 17.09 min.  
Peak 2 – 18.82 min.



HOAc/TEA in Mobile Phase  
100/0.02/0.01: MeOH/HOAc/TEA

Peak 1 – 15.21 min.  
Peak 2 – 16.66 min.



ATFA\* in Mobile Phase  
100/0.02 v/w:  
MeOH/NH<sub>4</sub>OOCF<sub>3</sub>

# Comparison of Four Basic Mobile Phase Types for CHIROBIOTIC CSPs

Compound is:

## Polar IONIC mode composition:

Methanol + Acid + Base (100+0.1+0.1, v/v/v)  
or Methanol + Volatile Ammonium Salt (100+0.1% v/w)

Ionizable

Neutral

## Normal Phase mode composition:

Polar + Nonpolar (EtOH+Heptane)

Neutral/Polar

## Polar organic mode composition:

Polar/Nonpolar (MeOH or EtOH or ACN or combinations,  
eg MeOH/ACN)

All types

## Reversed Phase composition:

Organic + Aqueous Buffer (ACN+TEAA; ACN & NH<sub>4</sub>OAc)

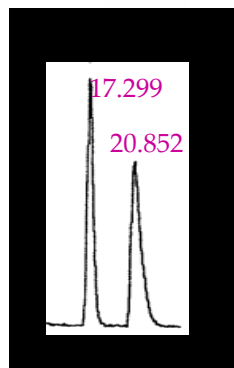
# Temperature Effects

- Shorter analysis time
- Higher efficiency

## Arginine

CHIROBIOTIC T,  
25°C

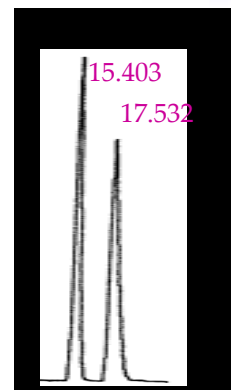
30/70 MeOH/10 mM  
NH<sub>4</sub>OAc (pH4.0)  
1.0 mL/min, ELSD



## Arginine

CHIROBIOTIC T,  
45°C

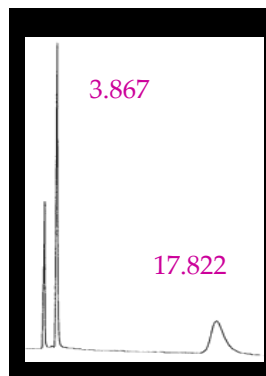
30/70 MeOH/10 mM  
NH<sub>4</sub>OAc (pH4.0)  
1.0 mL/min, ELSD



## Diacetyl-cysteine (N<sup>15</sup>)

CHIROBIOTIC T,  
25°C

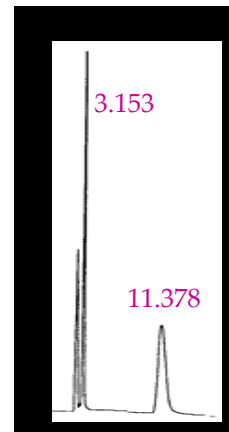
100/0.1% (v/w)  
MeOH/NH<sub>4</sub>OAc  
1.0 mL/min, ELSD



## Diacetyl-cysteine (N<sup>15</sup>)

CHIROBIOTIC T,  
55°C

100/0.1% (v/w)  
MeOH/NH<sub>4</sub>OAc  
1.0 mL/min, ELSD



# Application of Temperature Effect in Loading Study of Diacetyl-Cysteine (N<sup>15</sup>)

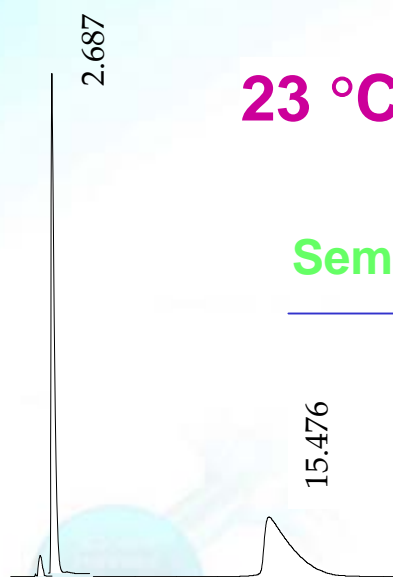
CHIROBIOTIC T, 250x4.6mm

Mobile Phase: MeOH/NH<sub>4</sub>OAc (0.1% v/w)

Detection: UV@230nm

Less Time!

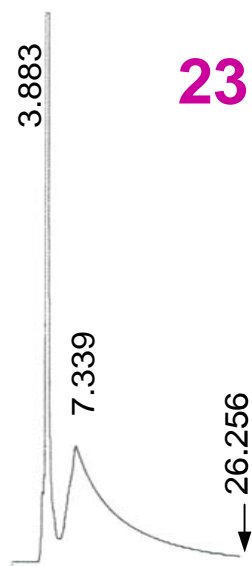
Less mobile phase additive!



23 °C

Semi-Prep

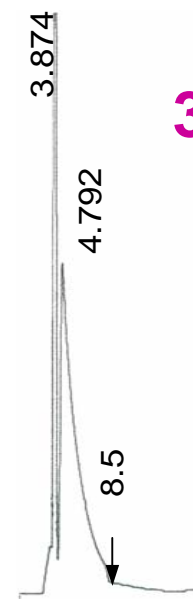
Flow Rate: 2.0 mL/min



23 °C

Higher Temp

Loading: 15 mg  
Flow Rate: 1.0 mL/min



35 °C

Loading: 15 mg  
Flow Rate: 1.0 mL/min



# Practical Guides for CSP Screening

# Historical Chiral Screening Approaches

- Emphasized molecular structure and analysis of analyte-CSP surface interaction as a predictive tool
- Utilized databases and literature searches for similar molecules [but minor structural differences often resulted in a loss of selectivity for some CSPs]
- Screen all CSPs!!

# Aim Method Development Process

- To quickly identify a suitable column for selectivity, in the minimum number of experiments

# Method Development Approaches

- Single column with multi-mobile phases (mainly CHIROBIOTIC phases)
- Multi-column switching with minimal number of simple mobile phases

# Screening Strategy

Choose a small set of CSPs that:

- are broad-based to increase chances of success for a wide range of molecular types
- offer selectivity in a wide range of mobile phases for increased selectivity possibilities and sample solubility
- are complementary to each other, minimum overlap in selectivity

# Complementary

## Defined as:

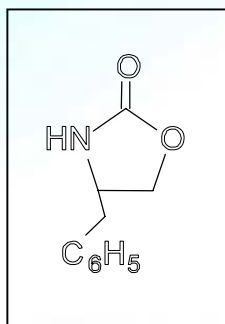
Offering separation potential in areas not possible with a given CSP, i.e., CHIROBIOTIC phases handle more polar molecules better than amylose or cellulose.

Offer increased selectivity in same mobile phases conditions, i.e., substituting one CHIROBIOTIC phase for another.

# Complementary Separations

## CHIROBIOTIC R versus CHIROBIOTIC V

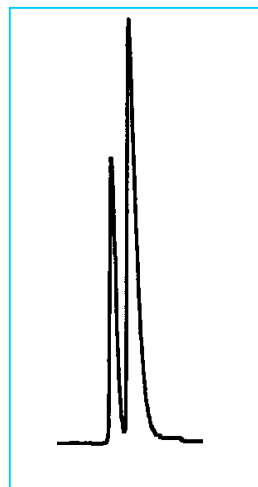
### 4-Benzyl-2-oxazolidinone



Peak 1 (S) 6.01  
Peak 2 (R) 6.91



Peak 1 (R) 6.54  
Peak 2 (S) 7.15



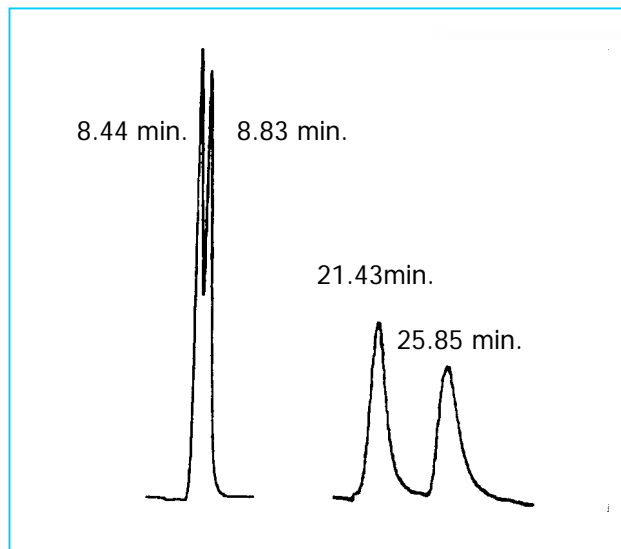
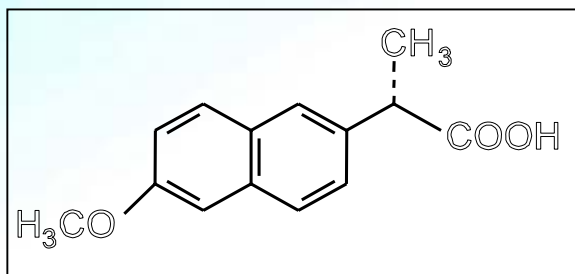
**CHIROBIOTIC R**    **CHIROBIOTIC V**

Mobile Phase: 50/50: Hex/EtOH

# Complementary Separations

## CHIROBIOTIC T versus CHIROBIOTIC R

### Naproxen



T

R

Mobile Phase: 30/70: MeOH/0.1% TEAA, pH 4.1

# Screening Strategy

Current industry trend is for generic screening methods with:

- a simple set of columns combining CHIROBIOTIC and Chiralcel/pak\* CSP's
- a minimum number of solvents covering all five mobile phase types

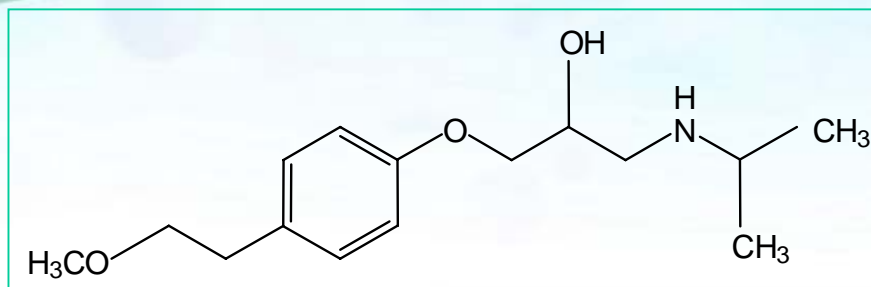
\*Chiracel and Chiralpak are the trademarks of Daicel.

# Distinct Mobile Phase Choices

- Normal phase
- Polar mobile phases
  - polar ionic mode: CHIROBIOTIC phases only
  - polar organic mode: CYCLOBOND phases only
  - Polar mode: Chiralcel/pak and CHIROBIOTIC phases
- Reversed phase

# Normal Phase vs Polar Ionic Mode<sup>©</sup>

## Metoprolol

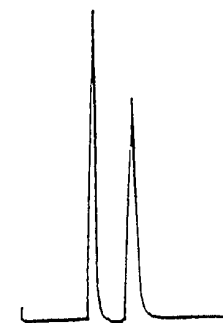


### CHIRACEL OD<sup>®</sup>

Peak 1 – 11.9 min.  
Peak 2 – 18.2 min.

*Normal Phase*

20/80/0.1: IPA/Hex/DEA

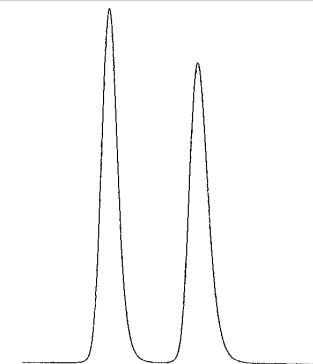


### CHIROBIOTIC T<sup>®</sup>

Peak 1 – 15.36 min  
Peak 2 – 17.11 min

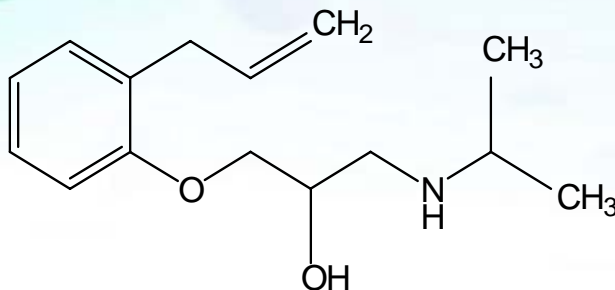
*Polar Ionic Mode*

MeOH/0.1% ATFA



# Normal Phase vs Polar Ionic Mode<sup>©</sup>

## Alprenolol

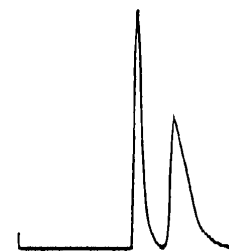


### CHIRACEL OD

Peak 1 - 12.4 min.  
Peak 2 - 16.4 min.

### Normal Phase

20/80/0.1: IPA/Hex/TFA

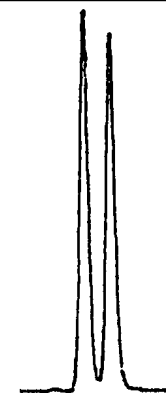


### CHIROBIOTIC V

Peak 1 - 7.69 min.  
Peak 2 - 8.33 min.

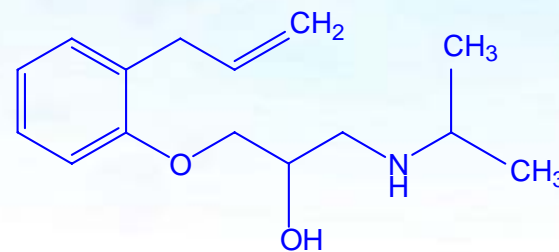
### Polar Ionic Mode

100/0.01/0.01: MeOH/HOAc/TEA



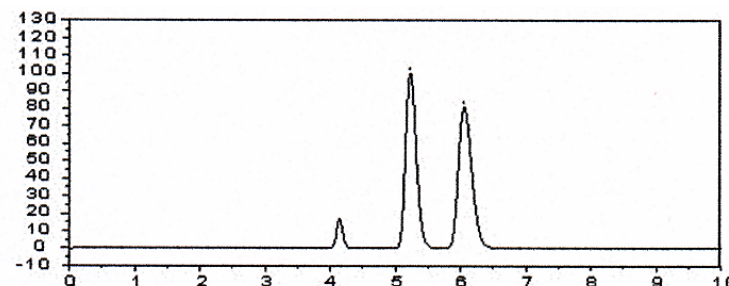
# Cellulosic vs CHIROBIOTIC CSP's : RP vs PIM

Tolperisone



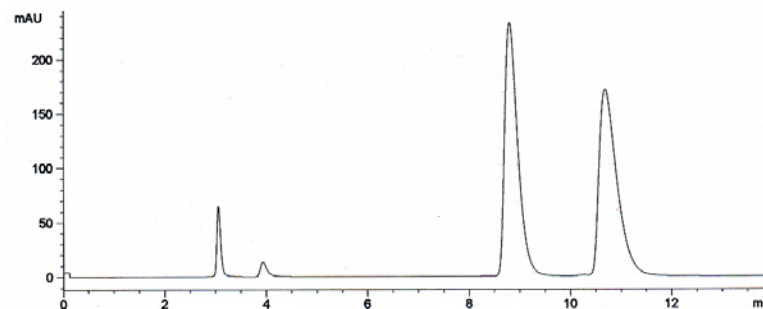
**CHIRALPAK® AD-RH** Peak 1 – 5.23 min  
Peak 2 – 6.06 min

**Reversed Phase** 60/40: ACN/20mM Borate



**CHIROBIOTIC V2** Peak 1 – 8.80 min  
Peak 2 – 10.69 min

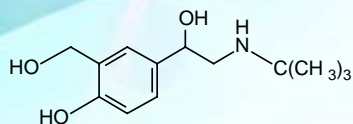
**Polar Ionic Mode** 100/0.1%: MeOH/ATFA



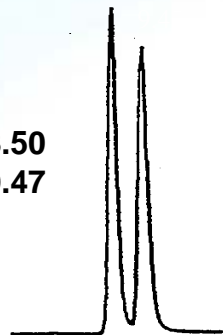
CHIRALPAK® is the registered trademark of Daicel Chemical Industries, Ltd

# Broad Selectivity Based on the Same Stereogenic Center with CHIROBIOTIC T Example: Amino Alcohols

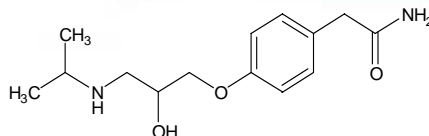
**Albuterol**



1. 8.50
2. 9.47



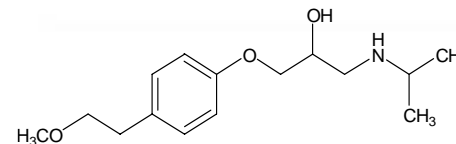
**Atenolol**



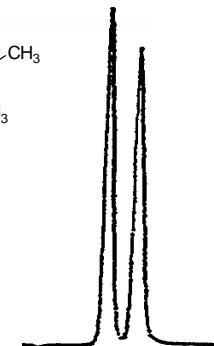
1. 12.64
2. 13.84



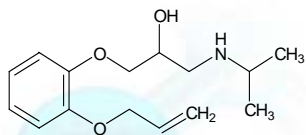
**Metoprolol**



1. 6.81
2. 7.48



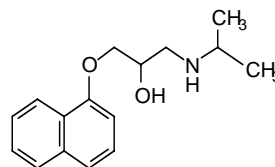
**Oxprenolol**



1. 11.82
2. 12.55



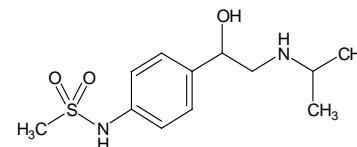
**Propranolol**



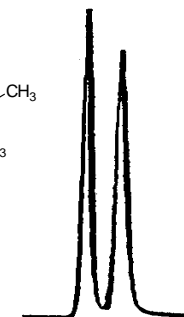
1. 7.08
2. 7.83



**Sotalol**



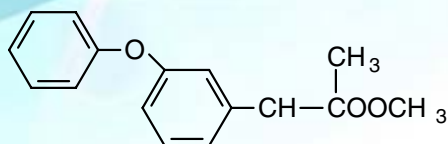
1. 8.71
2. 9.66



Mobile Phase: 100/0.1/0.1:MeOH/HOAc/TEA @ 2.0 mL/minute

# Broad Selectivity Based on the Same Stereogenic Center with CHIROBIOTIC V: Example: Profens

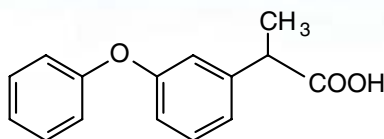
## Fenoprofen Methyl Ester



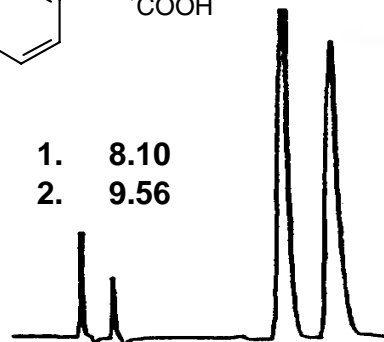
1. 8.31
2. 9.72



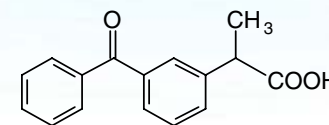
## Fenoprofen



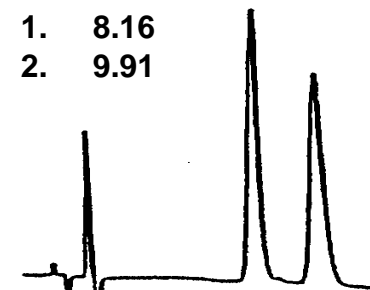
1. 8.10
2. 9.56



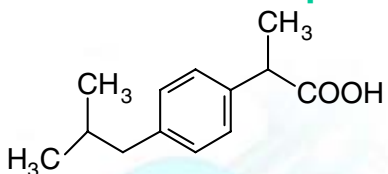
## Ketoprofen



1. 8.16
2. 9.91



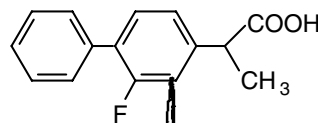
## Ibuprofen



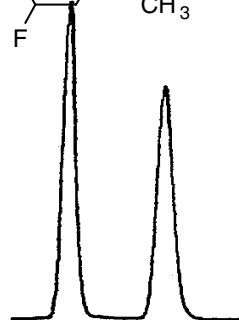
1. 5.77
2. 6.47



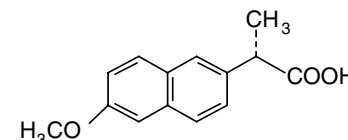
## Flurbiprofen



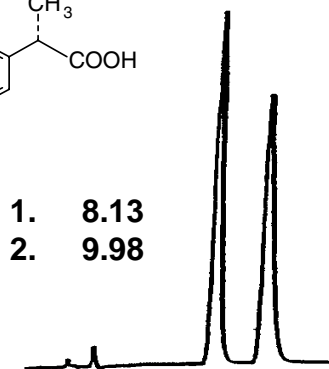
1. 8.53
2. 11.19



## Naproxen



1. 8.13
2. 9.98



Mobile Phase: 10/90: THF/10mM Na Citrate, pH 6.3 @ 1.0 mL/min.

# Purpose of Assay

## Generic screening develops options for a variety of applications

- Quick analytical method (possibly suitable for later optimization and validation)
- Suitable for possible small scale prep
- Trace analysis of unwanted isomer
- Impurity profiling
- LC-MS method

# Complementary Method Development

<b>Cellulose &amp; Amylose (CHIRALCEL/CHIRALPAK)</b>	<b>Macrocyclic Glycopeptides (CHIROBIOTIC)</b>
Broad applicability over wide range of compound types	Broad applicability over wide range of compound types
Traditionally NP, but now also RP, POM (different columns)	Designed to provide selectivity in PIM, RP, NP (same column)
Chiral interaction sites via different chemistries & helical structure	Large number of chiral interaction sites
Most useful are AD, OD, AS, OJ	Most useful are V2, T, R, TAG
Coated phases	Chemically bonded

# Complementary Method Development

## CHIRALCEL/PAK:

- Compound must be in **neutral form** - interaction always non-ionic
- Separate samples into acids, bases and neutrals (neutrals can be screened with either acids or bases)

## CHIROBIOTIC:

- Compound can be **ionised**, or can be a salt – ionic interactions are a key mechanism
- Same mobile phase screens are used for all samples, but can choose selective screening for acid, bases or neutrals

***Note: Functional group on or near stereogenic center dictates whether analyte is acid or base***

## 1. COLUMN INSTALLATION

CHIROBIOTIC™ columns are shipped in methanol. Before starting to use a new column, wash with 20 mL HPLC grade methanol at 1 mL/min. The column test standard, 5-methyl-5-phenylhydantoin, can be injected at this stage.

CYCLOBOND™ columns are shipped in IPA and should be washed with 30 mL HPLC grade water at 0.8 mL/min before starting the method development screen.

## 2. MOBILE PHASE CHOICE

No.	Mobile Phase	Composition (% v)
<b>REVERSED PHASE MODE:</b>		
1	MeOH/20mM NH <sub>4</sub> OAc, pH 4.0	20/80
2	MeOH/20mM NH <sub>4</sub> OAc, pH 6.0	20/80
3	ACN/20mM NH <sub>4</sub> OAc, pH 4.0	30/70
4	ACN/20mM NH <sub>4</sub> OAc, pH 6.0	30/70
<b>POLAR IONIC MODE®:</b>		
5	MeOH/HOAc/TEA*	100/0.1/0.1
<b>POLAR ORGANIC MODE:</b>		
6	ACN/MeOH/HOAc/TEA	95/5/0.3/0.2
<small>CHIROBIOTIC PHASES ONLY</small> If not progressing to normal phase, wash with MeOH at this stage to test and store the column.		
<b>NORMAL PHASE MODE:</b>		
7	EtOH/Hexane (or heptane, isohexane)	30/70
8	Washing cycle	100% EtOH
9	Column storage: CHIROBIOTIC	100% MeOH
	CYCLOBOND	100% IPA

\* Use salts (NH<sub>4</sub>O<sub>2</sub>CCF<sub>3</sub> for bases, NH<sub>4</sub>OAc for acids) when developing methods for prep.

## 3. COLUMN CHOICE AND RUN TABLE

Select your choice of columns from the list below. For a 6-column switching system, we recommend CHIROBIOTIC V2, T, R and CYCLOBOND I 2000, DNP and HP-RSP.

No.	Column Type (250x4.6mm)	1	2	3	4	5	6	7	8
I	CHIROBIOTIC V2	y		y		y		y	y
II	CHIROBIOTIC T	y	y		y	y		y	y
III	CHIROBIOTIC R		y		y	y		y	y
IV	CHIROBIOTIC TAG	y	y		y	y		y	y
V	CYCLOBOND I 2000	y		y			y		y
VI	CYCLOBOND I 2000 DNP	y		y			y	y	y
VII	CYCLOBOND I 2000 DMP	y		y			y	y	y
VIII	CYCLOBOND I 2000 HP-RSP	y		y	y		y		y

## 4. RUN CONDITIONS

Flow Rate: 1.0 mL/min.  
 Equilibration Time: 25 minutes  
 Run Time: 25 minutes  
 Temperature: Ambient  
 Detector: UV - 230nm  
 Sample: 1 mg/mL in MeOH

## Notes

- The recommended protocol assumes the use of 250 x 4.6mm columns. For 100 x 4.6mm columns, use the same conditions at 0.5 mL/min.
- It is permissible to run straight from the reversed phase to the polar ionic mode®, and from the polar ionic mode to normal phase without an intermediate solvent wash.
- If any screening run results in a retention time less than 5 minutes, reduce the strength of the mobile phase and re-run. Aim for retention times from 5 to 20 minutes. In reversed phase mode reduce organic component, in polar ionic mode® or polar organic mode reduce acid/base concentration. Retention times can be later reduced in the optimization process.
- If a separation occurs in the polar ionic mode®, for a neutral molecule, change to 100% organic solvent (i.e. MeOH, EtOH or ACN).
- If the compound does not elute in reversed phase, increase the organic content to 40%. In the polar ionic mode®, increase the acid/base concentration up to 1.0/1.0. In the polar organic mode for the CYCLOBOND columns, increase the MeOH concentration up to 10%.

## 5. OPTIMIZATION PROCEDURES

Polar ionic mode® (CHIROBIOTIC phases only)	<ul style="list-style-type: none"> <li>● Test alternative acid/base ratios (generally higher acid for basic molecules, higher base for acidic molecules)</li> <li>● To change acid/base to a volatile salt, use ammonium trifluoroacetate for basic compounds and ammonium acetate for acidic compounds at a concentration of 0.1wt%, adjust accordingly. Ammonium formate may be used as a compromise for both acidic and basic compounds.</li> </ul>
Polar organic mode	<ul style="list-style-type: none"> <li>● Eliminate MeOH</li> <li>● Test alternative acid/base ratios</li> </ul>
Reversed phase mode	<ul style="list-style-type: none"> <li>● Test smaller pH changes</li> <li>● Change organic to THF, ACN, MeOH</li> <li>● Change buffer type and buffer concentration</li> <li>● Change temperature</li> </ul>
Normal phase mode	<ul style="list-style-type: none"> <li>● Change EtOH concentration</li> </ul>

## 6. OPTIMIZING FOR MS DETECTION

**CHIROBIOTIC:** Use salts, as in Step 5, when using the polar ionic or polar organic modes.

**CYCLOBOND:** Use NH<sub>4</sub>OH to replace TEA in polar organic mode, lower concentration by 50 to 75%.  
**Both Phases:** Use ammonium acetate or ammonium formate when using in reversed phase.

## 7. RETESTING YOUR METHOD DEVELOPMENT COLUMNS

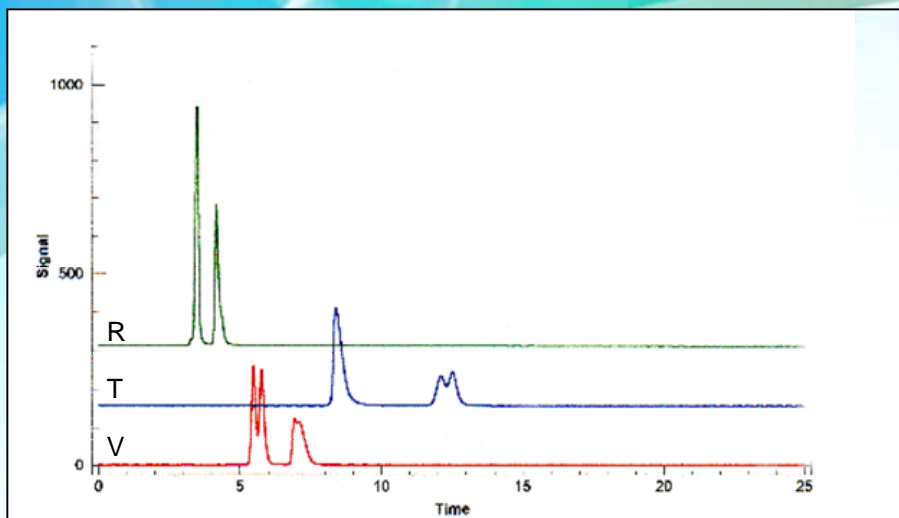
To ensure the selectivity performance of CHIROBIOTIC columns, periodically test with 5-methyl-5-phenylhydantoin in 100% MeOH. For testing CYCLOBOND columns, please refer to your CYCLOBOND Handbook.

ADVANCED SEPARATION TECHNOLOGIES

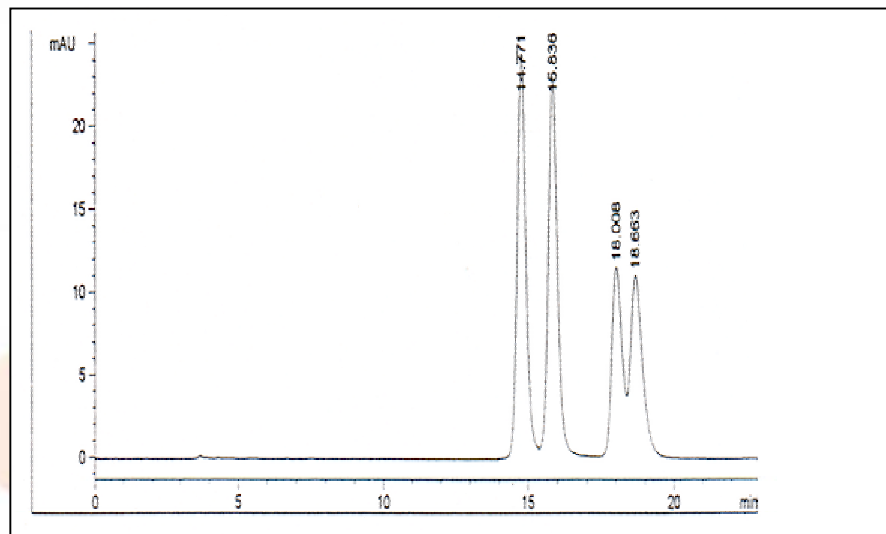
World Headquarters: 37 Leslie Court, Post Office Box 297, Whippany, NJ 07981 USA Tel: (973) 428-9080 Fax: (973) 428-0152  
 E-mail: info@astecusa.com www.astecusa.com

UK and Ireland Sales Office: 1 Blake Street, Congleton, Cheshire CW12 4DS UK Tel: +44 (0) 1260 276276 Fax: +44 (0) 1260 290067  
 E-mail: info@asteceuro.com www.asteceuro.com

# Typical Screening Results

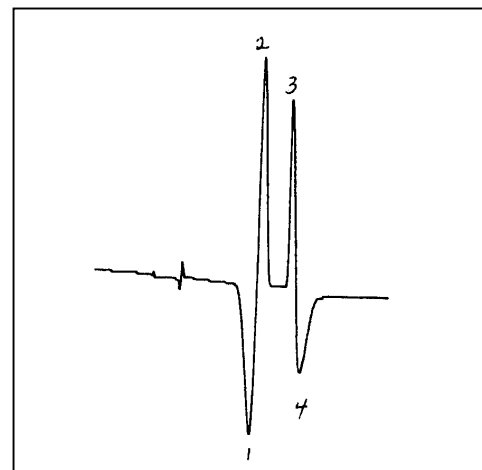


Optimized CHIROBIOTIC V



MeOH/NH<sub>4</sub>TFA; 100/0.02 w%

Chiralizer® Optical Rotation



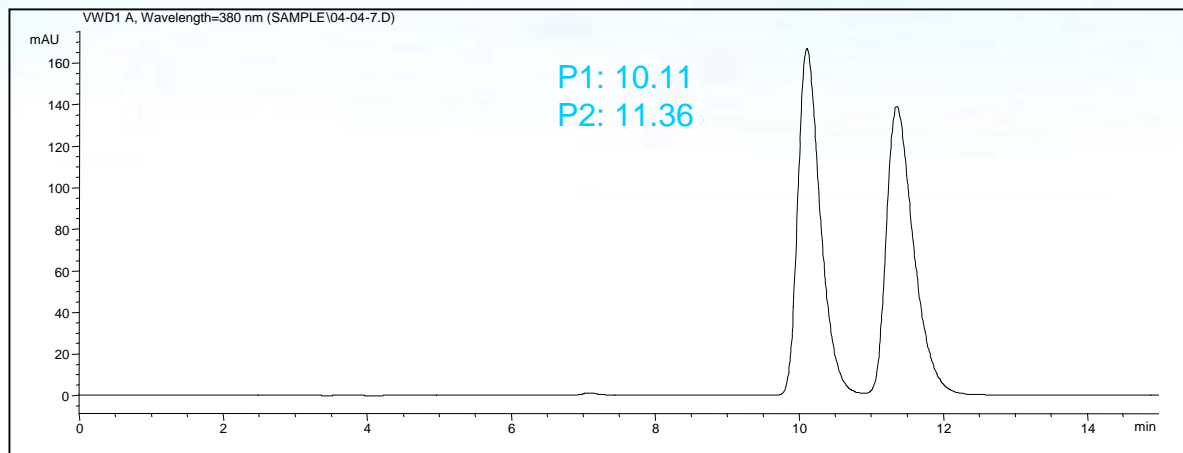
# Best Positive Screening Results

Column: **CHIROBIOTIC V2, 250x4.6mm**

Mobile Phase: 40/60, ACN/10 mM NH<sub>4</sub>OAc, pH 3.8

Flow Rate: 0.8mL/min

UV: 380 nm



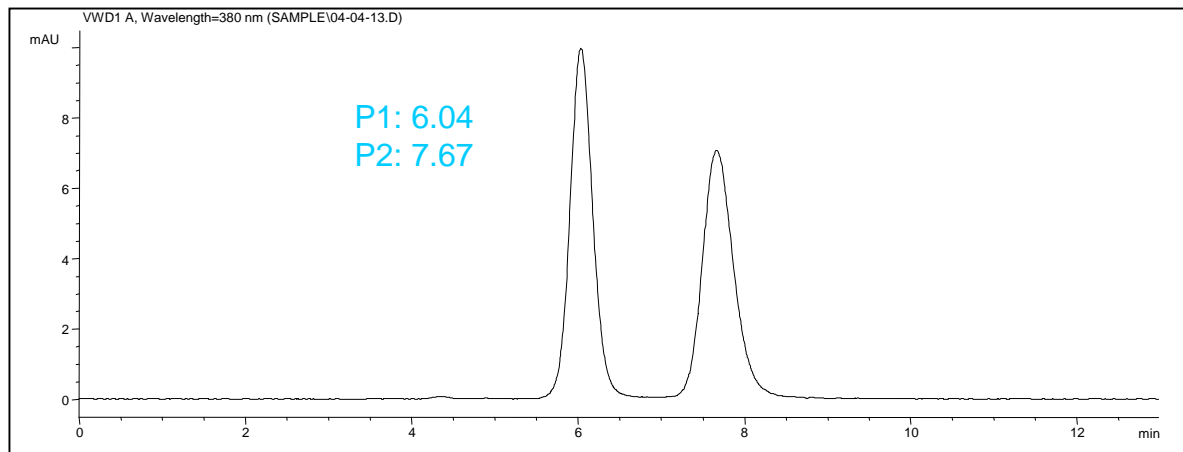
Column: **CHIROBIOTIC V2, 150x4.6mm**

Mobile Phase: 50/50, MeOH/5 mM NH<sub>4</sub>OAc, pH 3.5

Flow Rate: 0.8mL/min

UV: 380 nm

**Optimized Method for LC/MS application**

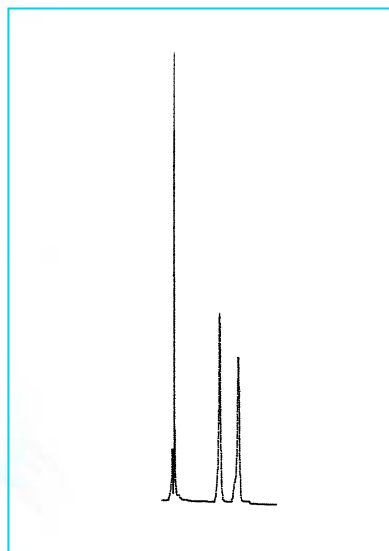


# Optimization Step: Polar Ionic Mode

## Enhanced Selectivity T → T2

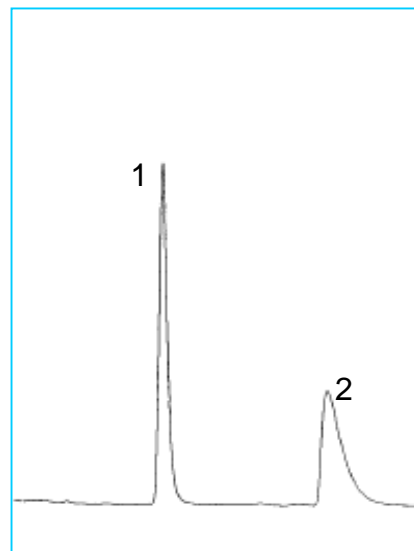
### Terbutaline

Peak 1: 9.72  
Peak 2: 10.92



CHIROBIOTIC T,  
100/0.2/0.1; MeOH/AcOH/TEA

Peak 1: 9.70  
Peak 2: 16.06



CHIROBIOTIC T2,  
100/0.1w%; MeOH/NH<sub>4</sub>TFA

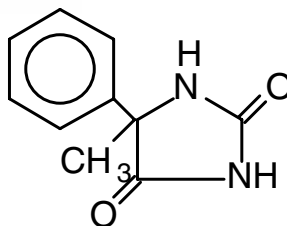
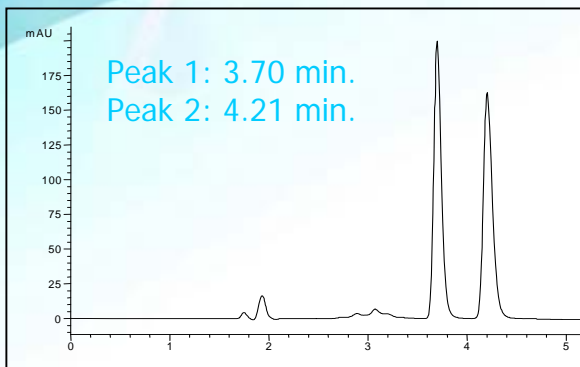
# Method Development Screen

- Monitor column performance *regularly*
- Store columns in correct solvents (free of additives):
  - CHIROBIOTIC: 100% MeOH
  - CYCLOBOND: 100% 2-PrOH
  - CHIRALCEL/PAK: Hexane/2-PrOH, 90/10

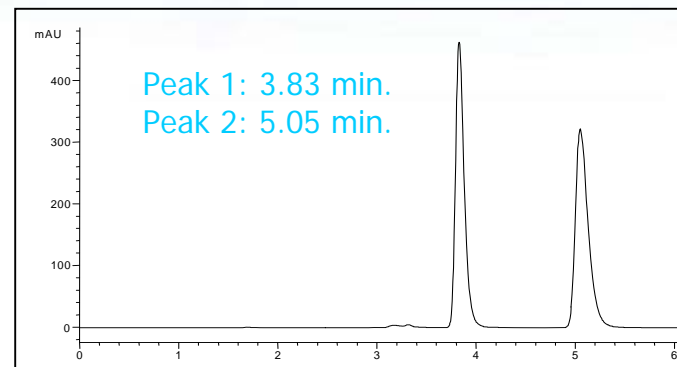
# Performance Tests for CHIROBIOTIC Phases

To ensure the selectivity and performance of all CHIROBIOTIC LC columns, periodically test your columns. This can now be accomplished with a single compound for all the CHIROBIOTIC phases in a very simple mobile phase of 100% MeOH

## CHIROBIOTIC V



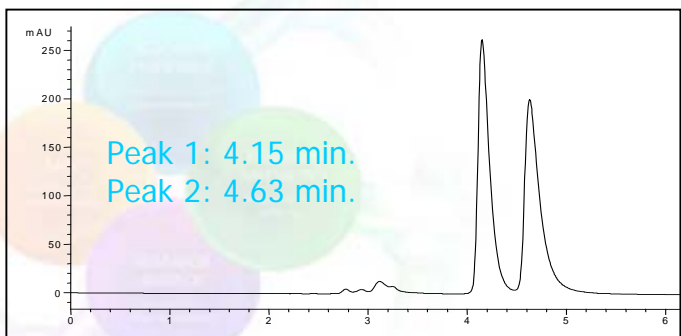
## CHIROBIOTIC T



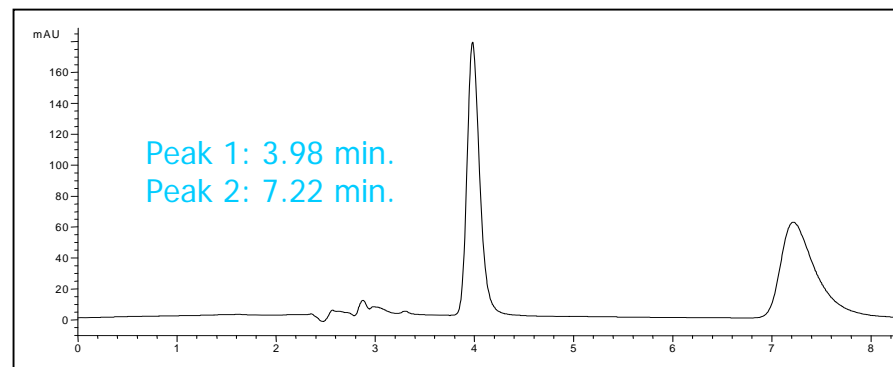
Conditions for all columns:

Sample: 5-Methyl-5-Phenylhydantoin (Aldrich 18,082-3)  
Column size: 250x4.6mm  
Mobile phase: 100% MeOH  
Flow rate: 1 ml/min.  
UV: 220nm

## CHIROBIOTIC R



## CHIROBIOTIC TAG

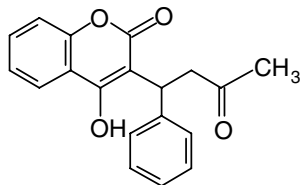


# Performance Tests for CYCLOBOND

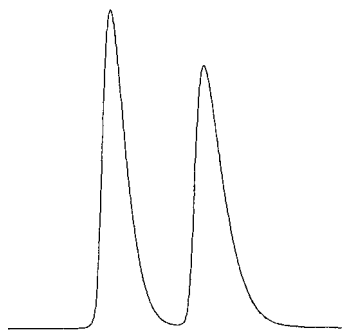
## CYCLOBOND I 2000



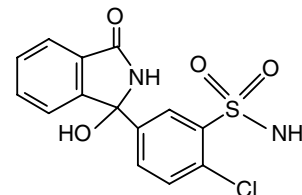
Warfarin  
(Sigma # A2250)  
Peak 1: 6.89 min.  
Peak 2: 7.90 min.



## CYCLOBOND I 2000 RSP



Chlorthalidone  
(Sigma # C2775)  
Peak 1: 12.04 min.  
Peak 2: 13.73 min.



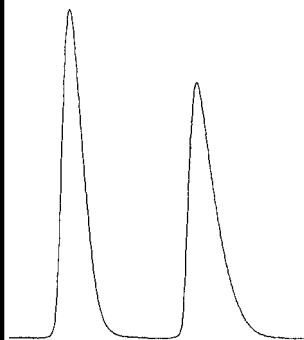
Mobile Phase:  
Flow Rate:  
Injection Vol.:  
Sample Conc:  
Detection:

100/0.3/0.2: ACN/HOAc/TEA  
1.0 mL/min.  
5 µL  
5 mg/mL  
254 nm

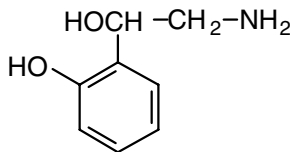
Mobile Phase:  
Flow Rate:  
Injection Vol.:  
Sample Conc:  
Detection:

10/90: ACN/0.1% TEAA, pH 4.1  
1.5 mL/min.  
3 µL  
5 mg/mL  
230 nm

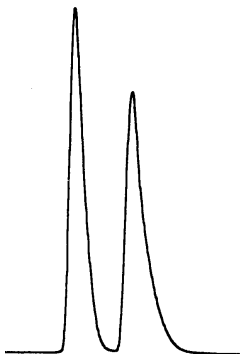
## CYCLOBOND I 2000 AC



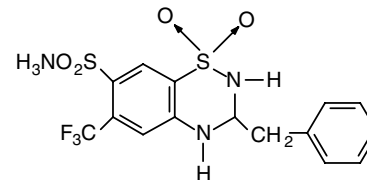
Norphenylephrine  
(Aldrich #11,372-7)  
Peak 1: 7.50 min.  
Peak 2: 8.77 min.



## CYCLOBOND I 2000 SN



Bendroflumethiazide  
(Sigma # B5775)  
Peak 1: 6.80 min.  
Peak 2: 7.40 min.



Mobile Phase:  
Flow Rate:  
Injection Vol.:  
Sample Conc:  
Detection:

10/90: MeOH/0.1% NaAc, pH 5.5  
1.0 mL/min.  
3 µL  
5 mg/mL  
230 nm

Mobile Phase:  
Flow Rate:  
Injection Vol.:  
Sample Conc:  
Detection:

50/50:ACN/0.1% TEAA, pH 4.1  
1.0 mL/min.  
5 µL  
5 mg/mL  
254 nm

# Isocratic or Gradient?

- Gradients suitable for NP, unsuitable for RP
- Recent papers suggest that gradient method development not much faster and has no greater success rate
- In PIM, ammonium formate gradient possible from 0.01% to 0.05% in, methanol to replace HOAc which has a very broad window

# Results of Complementary Generic Screening (1)

## Two recent studies showed:

- Screening\* four polysaccharides over 5 (NP, POM) mobile phases provided selectivity for 87% of a set of 53 compounds: the same study tested three CHIROBIOTIC phases over just 2 (RP, PIM) mobile phases with selectivities of 65%.
- It was noted that, together, they provided a 96% success rate, with increased selectivity for certain families of compounds on the CHIROBIOTIC CSPs confirming the complementary effect.

\* M E Andersson et al, J chrom A, 1005 (2003) 83

# Sales Tools Available

- Product Guide
- Handbooks (4)
- New Product Bulletins
- New Applications will be available soon on web
- Seminar Program (CD)
- Method Development program
- Website: [www.astecusa.com](http://www.astecusa.com)
- E newsletter

# CONCLUSIONS

- Chirobiotic phases are complimentary to cellulose and amylose CSPs
- Polar ionic mode is best mobile phase for ionic racemates
- Reversed phase can be run on all columns for any type of analyte
- Cyclobond phases have solve chiral separation problems not possible on any other CSP especially the Cyclobond HPRSP.
- Generic screening has proven to be the most efficient methodology for chiral selectivity

# The Supelco Chiralyser

Optical rotation is a very useful tool when dealing with chiral entities. Up to now the major problem has been the sensitivity and ease of use of the commercially available devices. The Astec Chiralyser has come a long way in solving those problems. Two available scientific tools have made this a reality.

1. The availability of single wavelength LED's which allowed the choice of the best, most sensitive wavelength (430 nm) as a light source. In addition, from the LED technology you get long life > 100,000 hours.
2. Use of the Faraday effect that allows nulling of the parallel magnetic field electronically so that the entire sample in the cell can be read instantaneously.

# What are the benefits of a Chiralyser in the area of chiral separations?

1. Validates peaks as (-) and (+). Eliminates peaks that are achiral contaminants.
2. Identifies a reversal of elution order. A common occurrence between CSP's and utilizing different mobile phase conditions.
3. Identifies enantiomeric pairs. A useful determination as the introduction of an achiral guard column can easily complete resolution if diastereoisomers are overlapping.
4. Detects analytes with no UV unlike circular dichroism.
5. System is easy to set up and economical to use, no complicated software.

Below is a list of compounds that have been run in our lab. It demonstrates the reversal of elution order problem and the variety of chiral molecules that have been assayed.

Compound	Column	Mobile Phase	First peak
5,5 Hydantoin	All Chirobiotics	MeOH	(-)
Oxazepam	V/T/R	MeOH	(+)
*N-Amine	V/V2	100/0.1w%,MeOH/ATFA	R(+)
*N-Amine	V	30/70,MeOH/TEAA,4.1	S(-)
Methodone	V2	100/0.1w% MeOH/ATFA	R(-)
Methodone	HP-RSP	20/80, ACN/NH <sub>4</sub> OAc,3.6	R(-)
*Propranolol	T/T2	100/0.1w% MeOH/ATFA	S(-)
*Propranolol	TAG	100/0.1w% MeOH/ATFA	R(+)
Terbutaline	T/T2/TAG/V	100/0.1w% MeOH/ATFA	(-)
Metoprolol	T	100/0.1w%,MeOH/ATFA	R(+)
*Bendroflumethiazide	V	10/90,THF/NH <sub>4</sub> NO <sub>3</sub>	(+)
*Bendroflumethiazide	T	30/70, MeOH/H <sub>2</sub> O	(-)
Naproxen	V	10/90, THF/NaCitrate	S(+)
Naproxen	R	20/80, MeOH/TEAA,5.5	S(+)
Ibuprofen	V	10/90, THF/NaCitrate	R(-)
Albuterol	V/T	100/0.1w%,MeOH/ATFA	(-)

\* Reversed elution order

*Mianserin	V	100/0.1w%,MeOH/ATFA	(-)
*Mianserin	T	100/0.1w%,MeOH/ATFA	(+)
Mandelic acid	T/R	30/70, MeOH/pH 4.5	S(+)
Mandelic acid	T/R	100/0.1w%,MeOH/ATFA	S(+)
Bupvacaine	V/V2	100/0.1w%,MeOH/ATFA	S(-)
Nicardipine	V/V2/T	100/0.1w%,MeOH/ATFA	(-)
*Ritalin	V/V2	100/0.1w%,MeOH/ATFA	(-)
*Ritalin	T2	100/0.1w%,MeOH/ATFA	(+)
Omeprazole	R	40/60, EtOH/Heptane	R(+)
Omeprazole	R	30/70, MeOH/HOAc,4.1	R(+)
Amphetamine	V2	100/0.05w%,MeOH/ATFA	S(+)
Methamphetamine	V2	100/0.05w%,MeOH/ATFA	S(+)
Dextro/Levorphanol	V/V2	100/0.1w%,MeOH/ATFA	D(+)
Dextro/Levo methorphan	V/V2	100/0.1w%,MeOH/ATFA	D(+)
Butorphanol	T/T2	100/0.1w%,MeOH/ATFA	D(+)
Ketoprofen	R	100/0.02w%,MeOH/HOAc	S(+)
*a-Me a-Ph succinimide	R	20/80,EtOH/Hex	(-)
*a-Me a-Ph succinimide	TAG/T/V	20/80,EtOH/Hex	(+)
*Warfarin	B-CD	100/0.3/0.2, ACN/HOAc/TEA	S(-)
*Warfarin	V	30/70, ACN/NH <sub>4</sub> OAc,4.1	R(+)

\* Reversed elution order