Amino Acid and Peptide Chiral Separations

1.0 Introduction

Over the past decade, chiral analysis in the separation sciences has become increasingly important. The diverse functions and properties of enantiomeric amino acids and peptides make their separation necessary as research progresses in many applicable fields of science and technology. Basic enantiomeric purity of the building blocks for peptides and proteins is essential for the final quality of the products of the future – whether for diagnosis or treatment.

Astec has been on the forefront of research into bonded chiral stationary phases (CSPs) for over 22 years. Many of our products have shown excellent results in the analysis of all types of natural and synthetic amino acids, including α-, β-, γ-, cyclic, and various N-blocked amino acids as well as peptides up to 14 amino acids to date. The types of CSPs used for the HPLC separations of these molecules include CHIROBIOTIC, CYCLOBOND, P-CAP, and Astec CLC. In capillary GC, a number of the CHIRALDEX phases have been employed. The goal of Astec is not only to provide a comprehensive range of products, such as those listed above, but also to pass along the knowledge of using our products and of optimizing the separations being attempted, whether analytical or preparative.

The purpose of this handbook is to share work done by Astec scientists, co-workers, and authors as well as that published by leading researchers in the field, to enable anyone doing a chiral separation in this area to achieve success with the guidance provided. This Handbook will define the specific CSPs and mobile phases (and their classification) to give the best selectivity for chiral separation. Certainly, all the questions and answers are not always found in such a review, but additional help from Astec is only a phone call or e-mail away.

The table of contents on the previous page will lead you to the various sections included in the Handbook. Some of you may already be familiar with Astec chiral columns and their use, thus you may want to skip to a section further on. However, brief descriptions of the CSPs have also been included here to make this a complete a Handbook as possible. Most of the information needed for mobile phase design and optimization of amino acid or peptide separations has been included in this Handbook. Should further information be needed the companion CHIROBIOTIC Handbook and CYCLOBOND Handbook can be consulted. Astec would be happy to send you a copy of these handbooks with a simple call or e-mail to us. Rather than repeat all of the information in these Handbooks essential pieces only were copied here. Mechanistically, the chiral phase selectivity and optimization is identical no matter what the compound type may be.

To begin working with enantiomeric separation of amino acids, N-blocked amino acids, or peptides, it is first suggested reading through this brochure in its entirety to see what information has been presented that might pertain to your research. Matching your separation problem to what is presented here can save you large blocks of time on the learning curve.

You should then begin by consulting the LC Decision Tree on page 14 for a quick guide to resolving natural free and N-blocked amino acids. This decision tree will lead you to the type of Astec chiral HPLC column that has been shown to work well for a particular type of natural amino acid or derivative. Likewise, each type of column used for a separation has a mobile phase type (POM, PIM, NP, RP) associated with it to give the best results. A brief discussion of each of these is given in the section on mobile phase design. Suggestions for the optimization are given in the order of what parameter often results in the greatest potential for success in resolving the enantiomeric pair.

Various tables are given with the results of the more common naturally occurring amino acids and their derivatives. The tabular information shown in the following pages was extracted from published papers, and/or was revalidated in the Astec laboratories. If other synthetic, modified or amino acid analogues are to be separated and they are not to be found in the tables, consult the short abstracts to find a closer match.

Lastly, for complex matrices, e.g. food products, where detection is a key issue, Astec CLC columns with the copper coordination

complexation first described by Davenkov[1] can be used. Details on its use and optimization are found in this Handbook.

It is also recommended that the original paper be consulted to get further details that cannot be condensed into a simple abstract or into other tables in this Handbook. Comments in these original papers might also remind you of alternative approaches to the separations you will be attempting, or comments to allow you to reach your goals more quickly. These published papers are easily found in most university libraries. For others, the e-mail address of the principle author is given for your convenience.

Also included in this Handbook are comments on scaling up your chiral separation should you need to isolate larger quantities than can be easily obtained by multiple injections on an analytical size column (Section 8, page 30). In addition, a few references on the use of MS or tandem MS in conjunction with an Astec chiral HPLC column are given but this is a field unto its own and rapidly developing. To use MS detection, it is still necessary to get good separations by choosing the right chiral column and optimizing the mobile phase, and then making the mobile phase components compatible with the type of MS ionization being used.

2.0 Description of Applied Chiral Stationary Phases (CSPs)

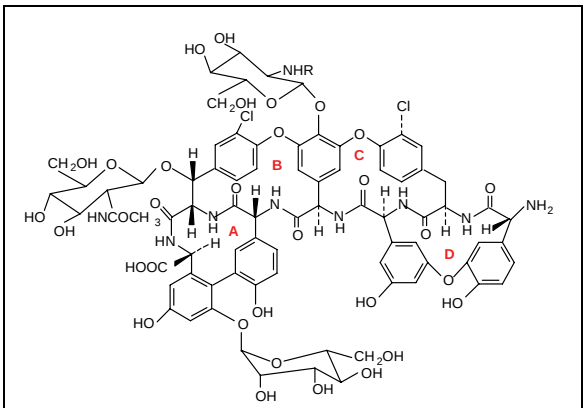
2.1 CHIROBIOTIC™ Bonded Macrocyclic Glycopeptide Phases

CHIROBIOTIC phases are manufactured by chemically linking macrocyclic glycopeptides through five covalent bonds to a silica surface. The appropriate CHIROBIOTIC phases used in this study include teicoplanin (CHIROBIOTIC T), teicoplanin aglycone (CHIROBIOTIC TAG) and ristocetin A (CHIROBIOTIC R). Since their general introduction by Dr. Daniel Armstrong as chiral stationary phases for HPLC in 1994, these products have established themselves as valuable tools for the separation of a wide variety of chiral molecules. In addition to their broad selectivity, these phases have demonstrated the ability to differentiate small changes in molecular structure and, therefore, have been ideal for drug stability and metabolism studies, monitoring biocatalysis reactions and following the course of a compound throughout its development. The stability of these phases has made them most useful for the above applications since the type of solvent, be it aqueous buffer (bioreactors) or halogenated solvent, has no material effect on the performance or stability of the stationary phase. The mobile phases used are compatible with the requirements for mass spectrometry which has further contributed to their success. Over 21 LC-MS publications have been published in the last two years.

In 2003, two new versions were added, the CHIROBIOTIC V2 and T2. Development came about as a result of an intense study on the effect of the type of linkage used to bond these unique chiral structures to silica. The position of those linkages and the length of the spacer was investigated. It was noted that the response to these factors studied was also a function of the type of mobile phase used to evaluate them. Since the polar organic (POM) and polar ionic (PIM) modes are the most desirable mobile phases from the standpoint of their utility in preparative and LC-MS applications, the study focused on increasing the performance of these two phases in those particular modes. Additionally, we found a significant increase in capacity for preparative applications by factors of 20-30 times. For amino acids and peptides the CHIROBIOTIC T2 is valuable when used in the optimization process in the polar organic and polar ionic modes primarily.

2.1.1 CHIROBIOTIC T and T2 (Teicoplanin)

Proposed Structure of the Macrocyclic Glycopeptide Teicoplanin



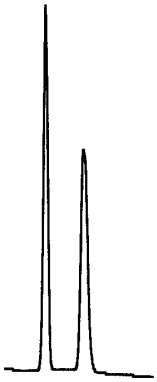
[1] V.A. Davenkov and S.P.V. Rogozhin, J. Chromatogr., 60, 280 (1971).

General Physical Data:
Molecular Weight ~ 1885
Chiral Centers – 23
Sugar Moieties – 3
Inclusion Cavities – 4 (A,B,C,D)
R- (8-Methylnonanoic Acid)

Types of Chiral Analytes Separated on CHIROBIOTIC T and T2

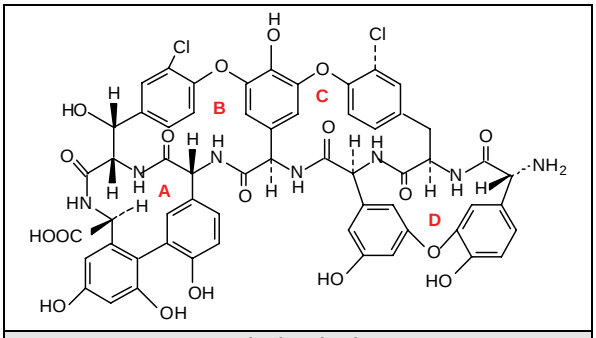
The CHIROBIOTIC T and T2 have unique selectivity for a number of classes of molecules, specifically underivatized α-, β-, γ- or cyclic amino acids, N-derivatized amino acids, i.e., FMOC, CBZ, t-BOC and alpha-hydroxycarboxylic acids, acidic compounds including carboxylic acids and phenols, small peptides, neutral aromatic analytes and cyclic aromatic and aliphatic amines. Separations normally obtained on a chiral crown ether or ligand exchange type phase are possible on CHIROBIOTIC T or when the polar organic or polar ionic modes are used on the CHIROBIOTIC T2. Determining single amino acid differences in a peptide chain or the chirality of a single amino acid in the chain has been demonstrated on the CHIROBIOTIC T2. The CHIROBIOTIC T, T2, TAG and R are complementary to each other.

Some Examples of Amino Acid Separations on CHIROBIOTIC T

Citrulline	Aspartic Acid
Peak 1 – 4.49 min. Peak 2 – 5.74 min.	Peak 1 – 5.94 min. Peak 2 – 7.35 min. 
CHIROBIOTIC T 50/50: MeOH/H_2O 1 mL/min. 25°C ELSD Detector	CHIROBIOTIC T 80/20/0.02: MeOH/H_2O/H_2CO_2 0.8 mL/min. Ambient ELSD Detector

2.1.2 CHIROBIOTIC TAG (Teicoplanin Aglycone)

Proposed Structure of the Macrocyclic Glycopeptide Teicoplanin Aglycone

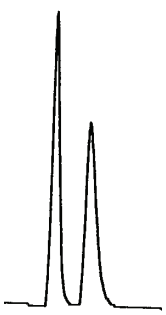
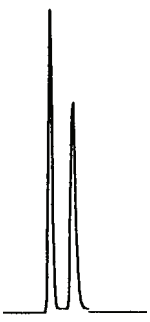


General Physical Data:
Molecular Weight ~ 1197
Chiral Centers – 8
Inclusion Cavities – 4 (A,B,C,D)

Types of Chiral Analytes Separated on CHIROBIOTIC TAG

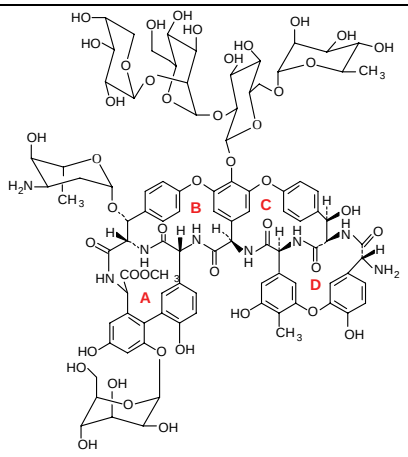
The CHIROBIOTIC TAG has shown excellent complementary selectivity to the CHIROBIOTIC T and T2. The removal of the three carbohydrates has enhanced resolution for many of the amino acids, α-, β-, γ- and cyclic. It has shown remarkable selectivity especially towards sulfur containing molecules (sulfoxides) including amino acids methionine, histidine and cysteine. Resolution of classes of molecules like amino alcohols which were excellent on the CHIROBIOTIC T have shown reduced resolution on the TAG with the exception of propranolol. A number of neutral molecules like the oxazolidinones, hydantoins and diazepines have shown enhanced resolution and, more remarkably, in single solvents like methanol, ethanol or acetonitrile. Some acidic molecules have also shown increased selectivity.

Some Examples of Amino Acid Separations on CHIROBIOTIC TAG

Carnitine	Serine
Peak 1 – 12.19 min. Peak 2 - 14.89 min. 	Peak 1 – 3.74 min. Peak 2 – 4.46 min. 
CHIROBIOTIC TAG	CHIROBIOTIC TAG

85/15: MeOH/25mM NH_4OAc, pH 6.0 1 mL/min. ELSD Detector	30/70: MeOH/H_2O 1 mL/min. ELSD Detector

Proposed Structure of the Macrocyclic Glycopeptide Ristocetin A



General Physical Data:
Molecular weight ~ 2066
Chiral centers – 38
Sugar moieties - 6
Inclusion cavities – A,B,C,D

Types of Chiral Analytes Separated on CHIROBIOTIC R

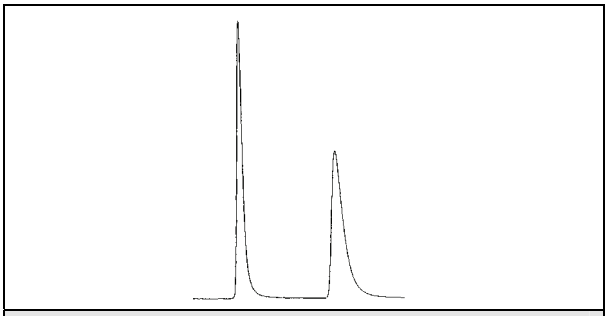
CHIROBIOTIC R has demonstrated unique selectivity for racemates with an acidic chiral center like α-hydroxy/halogenated acids, profens, N-blocked amino acids, imides, hydantoins and substituted aliphatic acids. Many FMOC and t-BOC derivatized amino acids can be resolved on this phase. The CHIROBIOTIC R is complementary to the CHIROBIOTIC T, T2 and TAG.

Examples of FMOC Amino Acid Separations on CHIROBIOTIC R in the Polar Ionic Mode

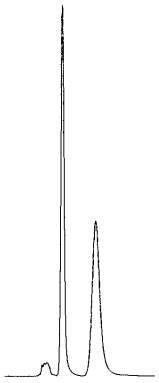
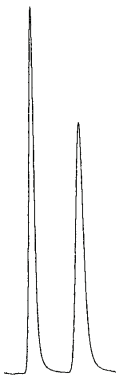
FMOC-Glutamine
Peak 1 – 8.11 min. Peak 2 – 13.66 min.

The preparation and applications of the aglycone form of CHIROBIOTIC T was a concept conceived and published by Dr. Francesco Gasparrini, Universita Degli Studi Di Roma, Italy. It was under his guidance that we have produced this product.

2.1.3 CHIROBIOTIC R (Ristocetin A)



100/0.1%: MeOH/NH_4TFA
(LC-MS Compatible)

FMOC-Alanine	FMOC-Isoleucine
Peak 1 – 2.95 min. Peak 2 – 4.40 min.	Peak 1 - 4:05 min. Peak 2 – 5.16 min.
	
100/0.1%: MeOH/NH_4TFA (LC-MS Compatible)	100/0.1%: MeOH/NH_4TFA (LC-MS Compatible)

2.1.4 CHIROBIOTIC Phases - Complementary Separations for Amino Acids and/or Diastereomers

Complementary Separations

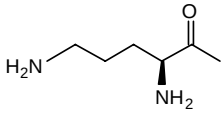
Each of the CHIROBIOTIC stationary phases has unique selectivity characteristics and, in addition, have been found to offer complementary separations. The use of the term 'complementary' describes the condition where an increase in selectivity is obtained in the exact mobile phase conditions on a different CHIROBIOTIC phase. This complementary nature allows for improved resolution by simply

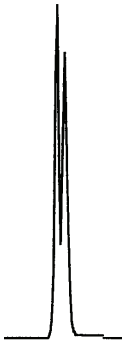
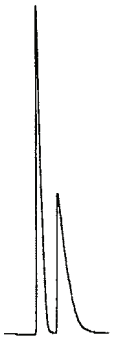
substituting the CHIROBIOTIC T with the CHIROBIOTIC R or the CHIROBIOTIC TAG with the CHIROBIOTIC T or CHIROBIOTIC T2. The reasons for this phenomena have to do with the subtle differences in diastereomeric binding sites between the four different CHIROBIOTIC phases. The two mobile phase conditions that have demonstrated this phenomena include the polar ionic and reversed phase modes.

The CHIROBIOTIC T has been the column of choice for the majority of this work but if attempts to optimize a separation fail to obtain complete resolution, the CHIROBIOTIC T2, CHIROBIOTIC R and/or CHIROBIOTIC TAG is switched into the exact same mobile phase. The choice among these alternatives can only come from a review of the applications.

CHIROBOTIC T vs. CHIROBIOTIC TAG

Ornithine

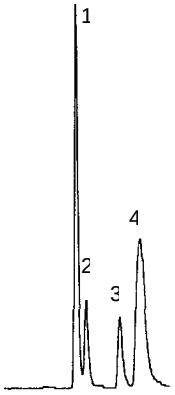
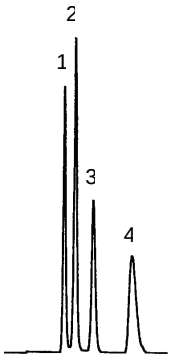


CHIROBIOTIC T	CHIROBIOTIC TAG
Peak 1 – 7.87 min. Peak 2 – 8.15 min.	Peak 1 – 15.58 min. Peak 2 – 18.29 min.
	

20/80: MeOH/20mM NH_4OAc, pH 4.5
(LC-MS Compatible)
1.0 mL/min
ELSD Detector

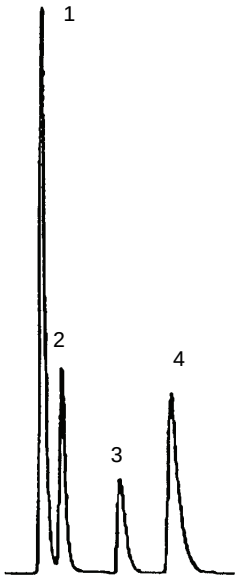
Amino Acid Diastereomers

A number of amino acids like isoleucine, threonine and valine have isomeric forms. The CHIROBIOTIC R, T and TAG have proven useful for the separation of these pairs of enantiomers in simple alcohol/water mixtures.

Isoleucine / Allo Isoleucine	Threonine / Allo Threonine
Peak 1 – 6.13 min. L-Ileu Peak 4 – 8.42 min. D-Ileu Peak 2 – 6.59 min. L-AIleu Peak 3 – 7.82 min. D-AIleu	Peak 1 – 3.4 min. L-Thr Peak 3 – 4.78 min. D-Thr Peak 2 – 3.94 min. L-AThr Peak 4 – 6.58 min. D-AThr
	
CHIROBIOTIC R 250x4.6mm 60/40: MeOH/H_2O 0.6 mL/min. ELSD Detector	CHIROBIOTIC TAG 250x4.6mm 60/40: MeOH/H_2O 1.0 mL/min. ELSD Detector

Valine / Isovaline

Peak 1 – 7.60 min. L-Val
Peak 4 – 13.37 min. D-Val
Peak 2 – 8.58 min. L-IVal
Peak 3 – 11.10 min. D-IVal



CHIROBIOTIC T
250x4.6mm
90/10: MeOH/H_2O
0.8 mL/min.
ELSD Detector

2.2 CYCLOBOND Bonded Cyclodextrin Phases

CYCLOBOND phases are manufactured by bonding to a silica media through an appropriate

spacer to one or two of the hydroxyl groups of crystalline beta-cyclodextrin. This linkage is a non-nitrogen covalent bond that gives the linked cyclodextrin excellent stability in a wide variety of mobile phase conditions. This cyclodextrin is composed of 6 glucose units arranged in the form of a toroidal basket. The 2, 3 and 6 position hydroxyl groups are arranged on the outside of the basket giving a chiral hydrogen bonding surface with 35 stereogenic centers for beta and 42 for the gamma form. The cavity is composed of the glucoside oxygens and methylene hydrogens giving it an apolar character. As a consequence of this structure, CYCLOBOND can include other apolar molecules of appropriate dimensions and bind them through dipole-dipole interactions, hydrogen bonding or London dispersion forces. In addition, the surface can interact to form diastereomeric complexes through both hydrogen donor and hydrogen acceptor exchange for molecules with counter hydrogen interacting groups.

2.2.1 CYCLOBOND I *2000* and CYCLBOND II Beta-cyclodextrin and Gamma-cyclodextrin

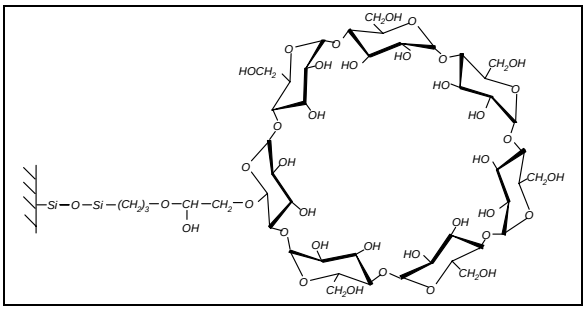
The bonded beta-cyclodextrin is designated as CYCLOBOND I *2000* and the bonded gamma is CYCLOBOND II. Both of these forms have been useful in amino acid analysis.

Types of Chiral Analytes Separated on CYCLOBOND I *2000* and CYCLOBOND II

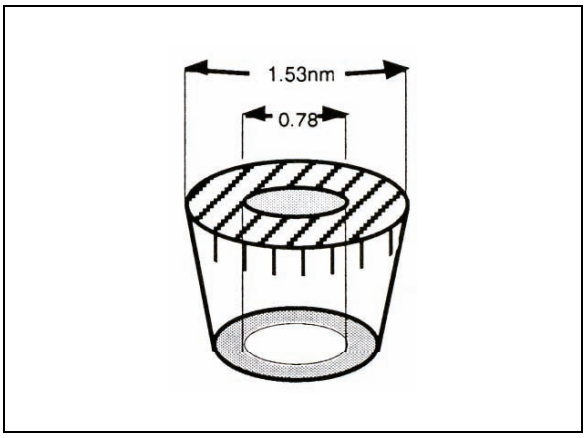
CYCLOBOND I *2000* has demonstrated by far the broadest suitability for analytes of general interest in the pharmaceutical, chemical and environmental areas. As a result of this diversity, CYCLOBOND I *2000* has been used as the basis for several derivatives to further extend the ability to form more selective chiral interactions.

CYCLOBOND II demonstrates increased selectivity for ring sizes greater than naphthalene. The fluorene structure of the N-TAG, FMOC is a perfect example.

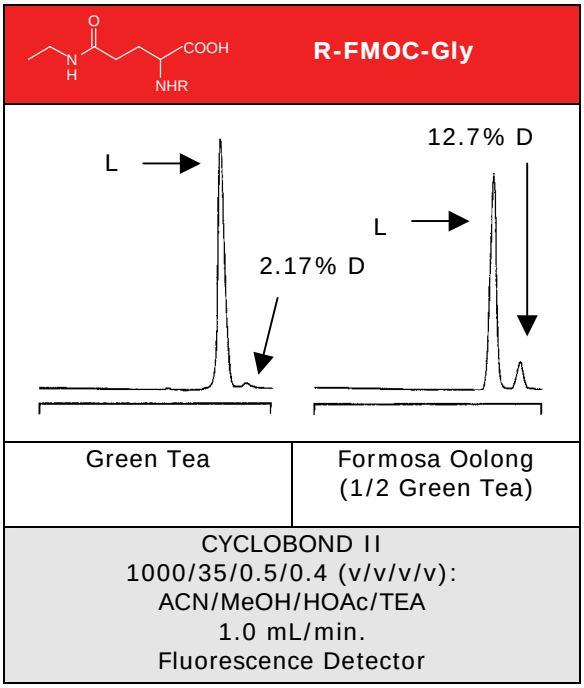
Beta-cyclodextrin Bonded to Silica



Schematic of Beta-cyclodextrin Dimensions



Determination of Theonine in Tea (CB-152)



2.2.2 CYCLOBOND I *2000* RSP Beta-cyclodextrin, R,S-Hydroxypropyl ether bonded

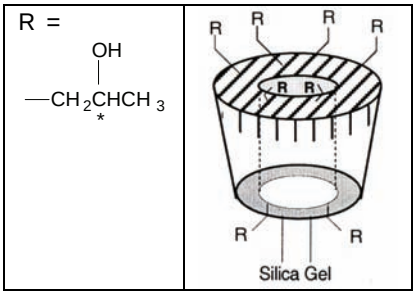
The sterically fixed hydroxyl groups of the beta-cyclodextrin are reacted with racemic propylene oxide for this derivative. These appendant hydroxyl groups increase hydrogen bonding flexibility and provide additional sites for hydrogen bonding thereby assisting in immobilization of the solute and steric interactions especially for enantiomers which have bulky substituents beta to the stereogenic center. Molecular structures that fit this derivative have hydrogen bonding groups or acceptor sites that are within 2-4 carbons of an aromatic or heterocyclic ring. This chiral stationary phase functions in both reversed phase and polar organic phase modes.

A complementary version, CYCLOBOND I *2000* HP-RSP based on changing linkage and substitution level of the hydroxypropyl has resulted in increased performance for certain classes of compounds. Conazoles in particular have benefited from this new version but not t-BOC amino acids.

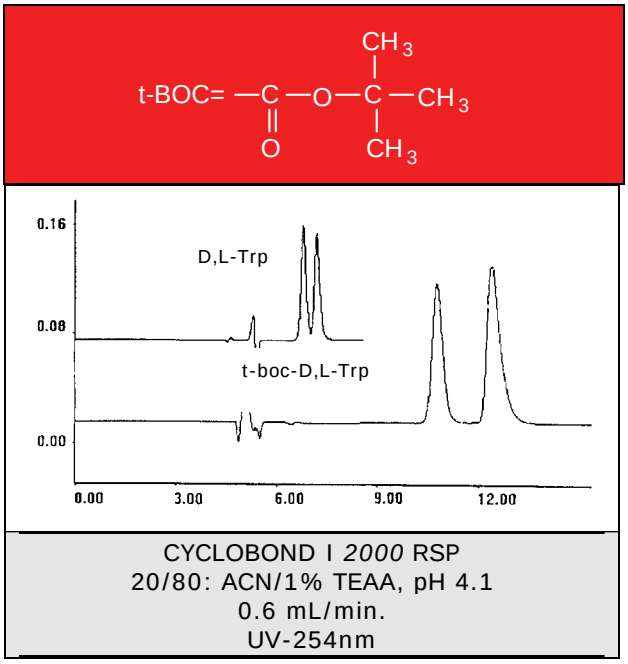
Types of Chiral Analytes Separated on CYCLOBOND I *2000* RSP

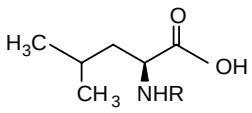
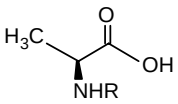
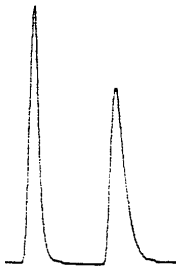
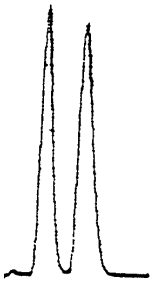
Certain N-blocked amino acids, like AQC, t-BOC, acids, alcohols and amines.

Schematic of Hydropropyl Substitution on β-cyclodextrin



Enantioseparation of D,L-Tryptophan and N-t-BOC Tryptophan (CB-113)



Separation of t-BOC Amino Acids	
R = t-BOC	
Leucine	Alanine
	
Peak 1 – 8.21 min. Peak 2 – 11.34 min.	Peak 1 – 10.49 min. Peak 2 – 11.62 min.
	
CYCLOBOND I *2000* RSP 7/93: CH_3CN/1% TEAA, pH 6.9 0.8 mL/min. UV – 214nm	

2.2.3 CYCLOBOND I *2000* RN and SN Beta-cyclodextrin, S-naphthylethyl and R-naphthylethyl bonded

The carbamate coupling of the π base, 1-naphthylethyl to a bonded cyclodextrin creates a complex chiral environment that has demonstrated diverse chiral separations. It has been labeled a multi-model chiral stationary phase because it has been operated successfully in three distinctly different mobile phases that function by three distinctly different mechanisms. It has, therefore, produced a range of separations not previously possible and has also enhanced certain separations due to more favorable diastereomeric complexation.

The three possible mobile phase modes are normal phase, polar organic phase and reversed phase.

The best starting point for choosing the proper mobile phase is dictated by the analyte structure, solubility and stability. For example, if the analyte is π acidic, an immediate test in a normal phase solvent is essential. If the analyte is not π acidic but contains 2 hydrogen bonding groups, one on or near the stereogenic center, then a first test should be the polar organic mode. Reversed phase offers the broadest possibilities for success.

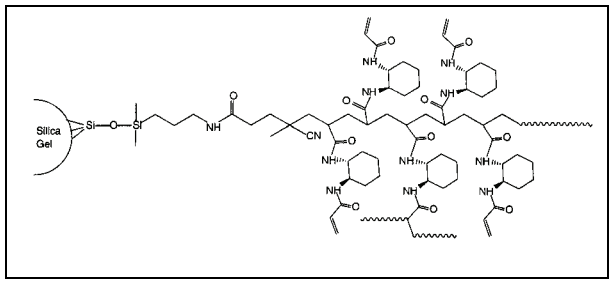
Of the carbamates available, the S-naphthylethyl carbamate (SN) has shown statistically, so far, the greatest selectivity and versatility especially in the polar organic mode. However, since the naphthylethyl carbamate configuration does play a role in enantioselectivity, the R form can be useful if separation does not occur on the S form.

Types of Chiral Analytes Separated on CYCLOBOND I *2000* RN and SN

AQC amino acids, proline, 3,5-DNB amino acids, π-acidic and other primary and secondary amines.

2.3 P-CAP™ S,S and R,R HPLC Columns

Reproducible, Repeating Polymer Composed of a Bi-functional Chiral Ligand (P-2)



P-CAP is new polymer technology for the separation of racemic compounds in any mobile phase system. The CSP technology is based on the reproducible polymerization of a cyclic diamine to the silica surface. The method offers maximum protection of the silica and excellent availability of the short chain polymeric ligand that ensures high capacity. The resulting thin, ordered layer of polymer does not alter the porous structure of the silica. (R,R) and (S,S) P-CAP have opposite configurations of the cyclic diamine allowing for easy reversal of elution order by substitution of the column in the existing mobile phase.

The repeating chiral moiety offers both structural conformation and hydrogen bonding interactions as the driving mechanism for chiral recognition. Preparative separations can be run in a variety of solvents without any large impact on the selectivity to meet solvent requirements (i.e, solubility, recovery, subsequent character-ization). As a result of the juxtaposition of the binding sites, molecules with two or more functional groups near the stereogenic center

demonstrate the highest selectivity. Separations have been run in pure acetone, ethyl acetate, heptane/ethanol, dichloromethane/methanol, acetonitrile/water or acetonitrile/NH_4OAc. Mobile phases are easily adapted to ideal MS compatible systems.

These CSPs have no memory effect and selectivity can be obtained in a variety of solvent choices with different efficiencies. Salt and/or acetic acid can be added to improve efficiency or enhance detection in mass spectrometry.

Types of Chiral Analytes Separated on P-CAP

P-CAP phases show no enantioselectivity towards underivatized amino acids and peptides or their esters. All N-derivatized α-amino acids can be well resolved in both the polar organic and normal phase modes. In general, the polar organic mode gives higher efficiency and shorter retention while the normal phase mode provides higher selectivity as shown in Figure 1A and 1C on page 9. Figure 1B shows the lack of selectivity with the choice of a wrong additive. This phase offers excellent mobile phase conditions for LC-MS and easy reversal of elution order for minimum levels of detection.

Other separations include benzenesulfonamides, binaphthols, benzodiazepins, phosphonic acids, bis-sulfones and chromemones.

Figure 2A versus 2B on page 9, demonstrates the ease of elution order reversal with the substitution of the (S,S) P-CAP column (B) for the (R,R) P-CAP configuration (A).

The Astec Chiralyser optical rotation detector easily identifies the optical rotation order from the R,R versus S,S configurations as can be seen in Figure 3B on page 10.

P-CAP is manufactured under license from La Sapienza, Università degli Studi di Roma. Patent Pending.

Figure 1. Effect of additives and mobile phase type on enantiomeric separations of FMOC-phenylalanine using (R,R) P-CAP.

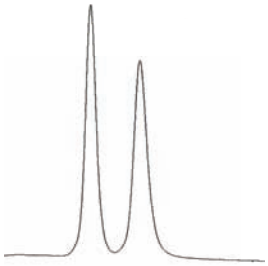
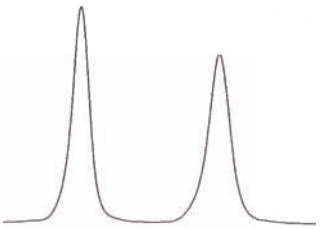
A) Polar Organic Mode: 70/30/20mM (v/v/c): ACN/MeOH/NH_4OAc 1 mL/min, 25°C	**B) Polar Organic Mode: 70/30/0.1 (v/v/v): ACN/MeOH/TFA 1 ml/min, 25°C**	**C) Normal Phase Mode: 50/50/0.1 (v/v/v) : EtOH/Heptane/TFA 1 mL/min, 25°C**
Peak 1 – 7.04 min. Peak 2 – 7.87 min. 	Peak 1 – 5.36 min. 	Peak 1 – 9.13 min. Peak 2 – 11.31 min. 

Figure 2. Reversal of the elution order of t-BOC-Glutamine
Conditions: 70/30/20mM (v/v/c) ACN/MeOH/NH_4OAc, 1 mL/min, 25ºC

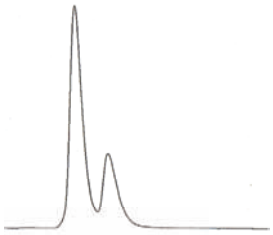
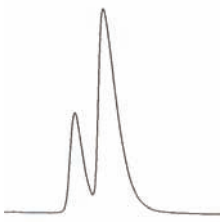
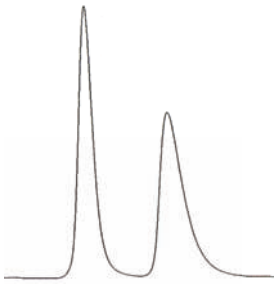
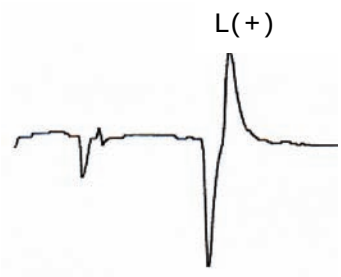
A) (R,R) P-CAP	**B) (S,S) P-CAP**
Peak 1 – 8.18 min. (L) Peak 2 – 8.84 min. (D) 	Peak 1 – 8.01 min. (D) Peak 2 – 8.50 min. (L) 

Figure 3. Reversal of the elution order of N-acetyl-DL-methionine with UV detection (A) and optical rotation detection (B) (Chiralyser from Astec)

A) UV detection: (R,R) P-CAP, 70/30/20mM (v/v/c) ACN/MeOH/NH_4OAc, 1mL/min, 25°C	**B) Optical rotation detection using the Chiralyser**	
Peak 1 – 8.44 min. Peak 2 – 9.83 min. 	(R,R) P-CAP L(+) D(-)	(S,S) P-CAP L(+)  D(-)

2.4 Astec CLC Ligand Exchange Phases

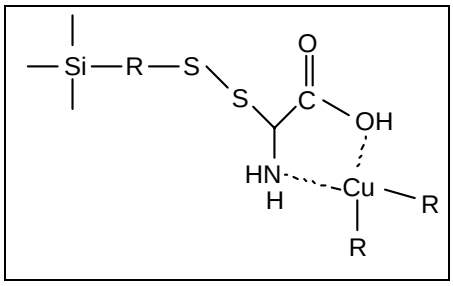
The ligand exchange concept was the first practical approach to the separation of underivatized amino acids, amino alcohols and hydroxy carboxylic acids from the original work of Davankov and co-workers[2]. This requires a transition metal with a +2 valence, copper being the most effective. The copper complex formed with the free amino acid in solution can be visualized at 254nm UV. In addition to alpha amino acids, alpha hydroxy acids and amino alcohols have been resolved on these types of CSPs.

Various alcohols up to 30% can be used to effect retention and resolution. Elution order can be reversed by using opposite enantiomeric form of the bidentate ligand.

Types of Chiral Analytes Separated on Astec CLC

For the separation of amino acids, amino alcohols, hydroxy carboxylic acids and other α-bifunctional compounds.

Astec CLC



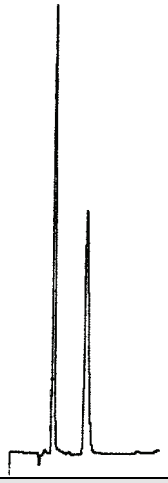
For the CLC-D column, analytes generally elute as the L enantiomer before the D with the exception of tartaric acid where the D elutes first. The CLC-L column has the opposite elution order, the D enantiomer eluting before L.

For low levels of detection, proline, which is typically run in pure water on the CHIROBIOTIC T and aspartic acid which requires a mobile phase with a pH of 3.6 on the CHIROBIOTIC T, can both be resolved on the CLC-D or CLC-L in 5mM $CuSO_4$ with the usual reversal of elution order from the CLC-D and CLC-L and enabling detection at 254nm UV.

[2] V.A. Davankov and S. V. Rogoshin, J. Chromatogr. 60, 280 (1971).

(D,L)-Proline

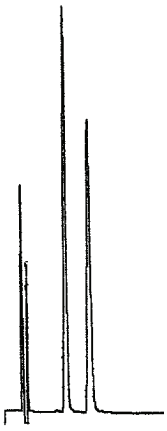
t_o – 1.75
Peak 1 (L) – 4.28 min.
Peak 2 (D) – 7.49 min.



Astec CLC-D, 150x4.6mm
5mM $CuSO_4$ (pH 4.0) buffered with 0.05M HOAc
1.0 mL/min.
UV - 254nm
5µL Inj. Vol.
Sample Concentration – 5mg/mL Proline in water

(D,L)-Leucine

t_o – 1.75
Peak 1 (L) – 6.31 min.
Peak 2 (D) – 8.77 min.



Astec CLC-D, 150x4.6mm
5mM $CuSO_4$ + 5% IPA
1 mL/min.
UV - 254nm
5µL Inj. Vol.
Sample Concentration – 5mg/mL Leucine in water

3.0 Mobile Phase Design and Optimization Protocols

For amino acids and their various derivatives, analysis can be accomplished through a variety of different mobile phase conditions. Solubility is the primary issue that makes the CHIROBIOTIC phases a good choice because they have the built in mechanisms that provide chiral interaction in a variety of mobile phase conditions. Reversed phase is the most general mobile phase but the polar organic (using CYCLOBOND and P-CAP phases) and the polar ionic modes (CHIROBIOTIC phases) offer unique opportunities for amino acids and peptides with good solubilities in high concentrations of methanol or acetonitrile/methanol. The polar organic and polar ionic mode mobile phases have perfect compatibility for LC-MS platforms and for preparative applications. The following chiral stationary phase (CSP) and mobile phase relationships have been found to exist.

3.1 Reversed Phase (RP)

Reversed phase is the most useful mobile phase for the separation of free amino acids and peptides of all types and for a number of N-blocked derivatives. As is typical in this mobile phase there are two components, organic and aqueous. Both methanol and acetonitrile are useful organic components. The aqueous portion plays a significant role in selectivity. For simple amino acids a good grade of water is sufficient. For bi-functional amino acids and peptides the aqueous portion must be buffered and both ammonium acetate and formic acid have produced excellent results. For LC-MS, the use of ammonium trifluoroacetate plus formic has been most effective.

Components: MeOH or ACN/H_2O, for multi-functional + NH_4OAc or ATFA/H_2CO_2

Dominant Interactions: Ionic, hydrogen bonding

Applicable CSPs: CHIROBIOTIC T, T2, TAG, R, CYCLOBOND I *2000*, RSP, CYCLOBOND II

Or Astec CLC ligand exchange using MeOH/1-5mM $CuSO_4$

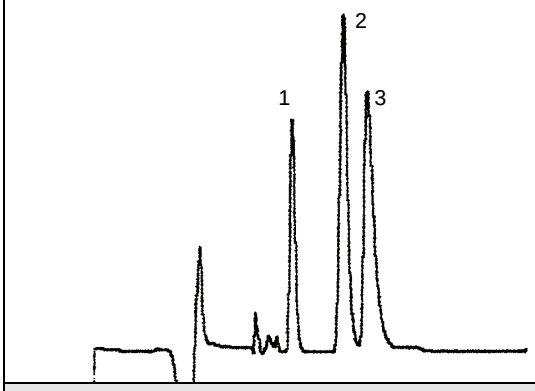
A. CHIROBIOTIC Mobile Phases (RP)

As in most reversed phase systems retention is controlled by the concentration of the organic modifier. The major difference for these CSPs is that the choice of organic modifier can have a larger impact than with conventional chromatography. Two organic modifiers have worked well for this class of racemates; methanol and acetonitrile, the latter working better for the more lipophillic compounds. Several other factors differ from conventional chromatography:

1. For the CHIROBIOTIC phases the carboxyl group on the amino acid must be free to obtain selectivity but any N-blocked group, while affecting retention, does not have as significant an impact on selectivity.

Ester versus Free Acid Selectivity

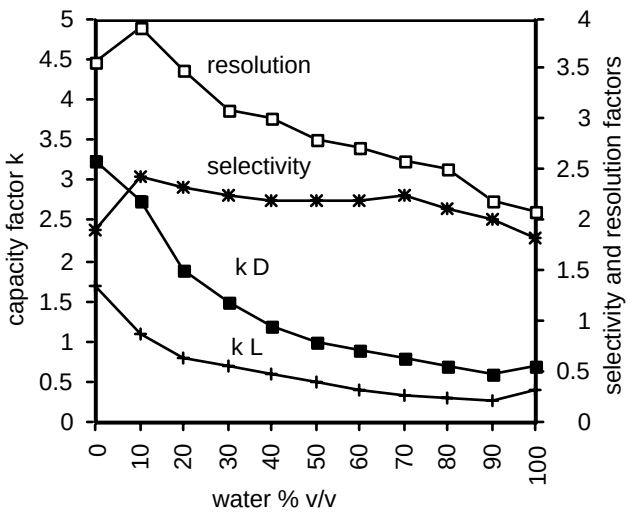
Peak 1 – 7.65 min. D,L-Phenylalanine Methyl Ester
Peak 2 – 9.95 min. L-Phenylalanine
Peak 3 – 11.22 min. D-Phenylalanine



CHIROBIOTIC T
20/80: EtOH/H_2O
0.8 mL/min.
UV-254nm

2. Increased resolution has been observed by increasing the organic modifier for neutral amino acids. For peptides and other amino acids, the reverse is true.

Influence of % H_2O in Mobile Phase on Separation Factors - Methionine



CHIROBIOTIC Phases Optimization in the Reversed Phase Mode (RP)

For efficient optimization, the following steps should be observed in order:

1. Evaluate the organic modifier: MeOH>ACN. Acetonitrile works best for more lipophillic amino acids and peptides.

2. Evaluate the concentration of organic modifier. For underivatized amino acids higher concentrations of organic modifier increases retention while for derivatized amino acids, lower concentrations increase retention. Both scenarios generally lead to higher selectivity.

3. Evaluate the effect of pH.

   a) For neutral amino acids, buffer is generally not required, while for the acidic and basic amino acids it is essential for proper retention and selectivity. While higher pH may increase alpha values, the best resolution is generally obtained at lower pH.

   b) For derivatized amino acids, higher pH reduces retention and increases alpha values.

4. Evaluate the best buffer: NH_4TFA, NH_4OAc, NH_4HCO_2.

5. Evaluate the concentration of buffer. The typical range is 0.1 – 1.0%. Lower concentrations can increase selectivity while higher concentrations lead to better peak shape, especially for the basic amino acids.

6. Evaluate flow rate. Lower flow rates can increase resolution. Typical range is 0.4 to 1.0 mL/min. for 250x4.6mm columns.

7. Evaluate temperature. Lower temperature can increase resolution, higher temperatures can increase efficiencies by reducing or eliminating tailing. Higher temperatures can also increase solubility for higher loading capacities during a scale-up separation.

B. CYCLOBOND I *2000*, CYCLOBOND I *2000* RSP, CYCLOBOND I *2000* RN and SN and CYCLOBOND II Mobile Phases (RP)

The majority of work in the area of amino acids and N-blocked amino acids has been done in the polar organic mode on CYCLOBOND phases. The major area of application for reversed phase has been the t-BOC amino acid derivatives which has been developed on the CYCLOBOND I *2000* RSP phase. For those cases requiring the reversed phase mode, the best starting mobile phase composition has been 5/95: ACN/1% TEAA, pH 7 or pH 4.

CYCLOBOND Phases Optimization in the Reversed Phase Mode (RP)

Increasing or decreasing the acetonitrile concentration has been the most effective step for controlling retention and resolution. Substitution of ammonium acetate for the triethylamine acetate (TEAA) has been successful in a limited number of cases, the concentration varying widely.

C. CLC-D and CLC-L Copper Sulfate Mobile Phases (RP)

The CLC columns are based on the use of a metal ligand to complete the coordination complex between the two functional groups of the bonded ligand and the two functional groups of the amino acid. The best metal ion to accomplish this task is copper in its sulfate form.

CLC Phases Optimization in the Reversed Phase Mode (RP)

The concentration of copper sulfate should range between 1-5mM. Lower concentrations of copper salt increase retention while higher

concentrations alone or in combination with an alcohol can decrease retention.

The CLC columns will tolerate up to 30% methanol, 20% ethanol and 15% isopropanol in the presence of the copper sulfate. The best resolution results are obtained as a result of minimization of the retention factor.

3.2 Polar Ionic Mode (PIM)

This mobile phase was designed specifically for the CHIROBIOTIC stationary phases. It is an ideal mobile phase for enhancing detection when using LC-MS platforms and in preparative processing. It has been widely used for many derivatized and novel synthetic amino acids.

Components: MeOH + HOAc + TEA. Note that NH_4OAc salt can be substituted for HOAc/TEA

Dominant Intertactions: Ionic, hydrogen bonding

Applicable CSPs: CHIROBIOTIC T, T2, TAG and R

The components are volatile and the volatile salts that can be substituted are of low concentration, typically 0.01-0.2% of ammonium acetate or ammonium formate. The window for selectivity is, however, widest for a single composition of HOAc/TEA which is why it is used for screening. Once selectivity is observed the right type and concentration of salt can be quickly found.

CHIROBIOTIC Phases Optimization in the Polar Ionic Mode (PIM)

1. Evaluate the acid/base ratio. The proper ratio has a dramatic effect on selectivity. Once the proper ratio is determined then the concentration at that ratio can be increased to reduce retention.

2. Evaluate flow rate. Lower flow rates generally lead to higher resolution.

3. Evaluate temperature. Lower temperature often results in higher selectivity, while increased temperature can reduce tailing, increase solubility and reduce retention.

4. Evaluate complementary effect. If the method was developed on the CHIROBIOTIC T then substituting the CHIROBIOTIC T2 and/or TAG in the same mobile phase composition could lead to higher selectivity.

3.3 Polar Organic Mode (POM)

This mobile phase was designed specifically for the CYCLOBOND and P-CAP phases. Two solvent modifiers, ACN and MeOH control retention. As the methanol concentration is increased, retention decreases. There are many cases where the methanol has been completely eliminated. The second two components, HOAc/TEA, primarily control selectivity. Altering the ratio can have a dramatic effect on selectivity. For amino acids the acid is typically favored. There are numerous publications where this mode has been used for derivatized amino acids and small peptides, especially when derivatized with FMOC-Gly and Accu-TAG* reagents. P-CAP phases only require ratios of ACN and MeOH.

Components: ACN + MeOH + HOAc + TEA

Dominant Interactions: Hydrogen bonding, dipole-dipole, steric interaction

Applicable CSPs: CYCLOBOND I *2000*, CYCLOBOND I *2000* RSP, CYCLOBOND I *2000* RN or SN and P-CAP

CYCLOBOND Phases Optimization in Polar Organic Mode (POM)

1. Evaluate the methanol concentration. Decreased methanol increases retention.

2. Evaluate acid/base ratio. The ratio typically favors the acid. Most typical is 2:1 but ratios as high as 5:1 have been observed.

3. Evaluate flow rate. Decreased flow rate increases resolution but the effect is not as dramatic as observed in the reversed phase mode.

4. Evaluate temperature. Decreased temperature increases resolution but for amino acids increased temperature has a number of beneficial effects including reduced tailing, decreased retention and increased solubility. The latter benefit helps both in lower detection limits and in preparative processing by increasing throughput.

*Accu-TAG is a trademark of Waters Corporation.

3.4 Normal Phase Mode (NP)

Normal phase applications on CYCLOBOND have been primarily for the DNP derivatives of amino acids. The typical composition is Hexane/EtOH and in some cases Hexane/IPA. In a number of cases pure EtOH or IPA have been used. For the P-CAP phases ACN/MeOH has been used.

Components: Hexane or heptane + polar alcohol, IPA or EtOH; ACN/MeOH, and there are cases where pure ethanol has been used.

Dominant Interactions: π-π, hydrogen bonding

Applicable CSPs: CHIROBIOTIC T, T2, TAG, R and P-CAP

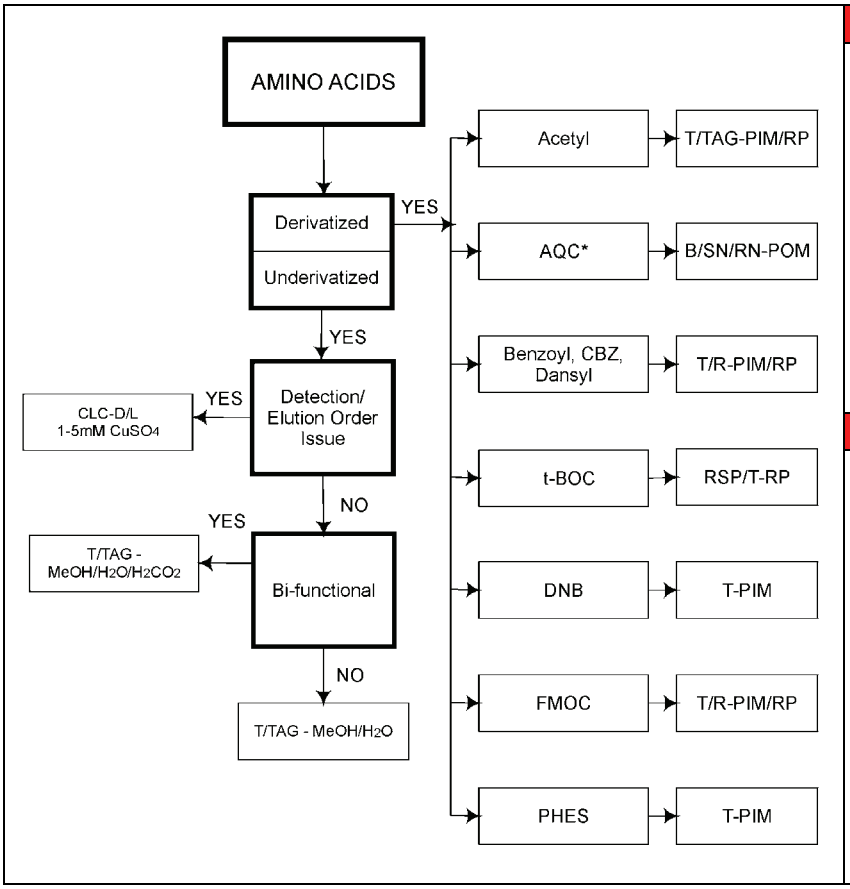
CHIROBIOTIC Phases Optimization in Normal Phase (NP)

1. Evaluate the ratio of alcohol to hydrocarbon. Heptane, isoheptane and isohexane have been used as good substitutes for the neurotoxic hexane and may give better selectivity.

2. Evaluate the effect of adding acid or base. Small amounts of acid or base can be added to suppress unwanted interactions from acid or base functionalities in the compound to be analyzed.

For optimization methods on P-CAP phases please refer to pages 8 and 9.

4.0 LC Decision Tree for the Separation of Natural D,L-Amino Acids and their N-blocked Derivatives



LC Column Designations	
CLC-D	CLC, D configuration
CLC-L	CLC, L configuration
B	CYCLOBOND I *2000*
RSP	CYCLOBOND I *2000* RSP
RN	CYCLOBOND I *2000* RN
SN	CYCLOBOND I *2000* SN
T	CHIROBIOTIC T
TAG	CHIROBIOTIC TAG
R	CHIROBIOTIC R

LC Mobile Phase Designations	
POM	Polar Organic Mode
PIM	Polar Ionic Mode
RP	Reversed Phase
NP	Normal Phase

**AQC is a trademark of Waters Corporation*

4.1 Best Starting Mobile Phase Compositions for Natural Amino Acids

Type	Column	Mobile Phase
Underivatized Amino Acids		
Neutral	CHIROBIOTIC T, T2, TAG, R	50/50: ACN/H_2O and MeOH/H_2O
	CLC	10/90: MeOH/5mM $CuSO_4$
Acidic	CHIROBIOTIC T, T2, TAG, R	50/50/0.02 (v/v/v): MeOH/H_2O/H_2CO_2
	CLC	10/90: MeOH/5mM $CuSO_4$
Basic	CHIROBIOTIC T, T2, TAG, R	50/50: MeOH/20mM NH_4OAc, pH 4.1
	CLC	10/90: MeOH/5mM $CuSO_4$
N-blocked Amino Acids		
	CHIROBIOTIC T, T2, TAG, R	100/0.1w%: MeOH/NH_4OAc or 50/50: MeOH/20mM NH_4OAc
	P-CAP	70/30/20mM (v/v/c): ACN/MeOH/NH_4OAc
N-t-BOC Amino Acids Only		
	CHIROBIOTIC T, T2, TAG, R	20/80: MeOH/20mM NH_4OAc, pH 4.1
	P-CAP	85/15: ACN/MeOH/20mM NH_4OAc, v/v/c
	CYCLOBOND I *2000* RSP	10/90: ACN/20mM NH_4OAc

4.2 Enantioresolution of Underivatized Natural α-Amino Acids**

R—CH(NH$_2$)—COOH		CHIROBIOTIC T[1]		CHIROBIOTIC TAG[2]		CHIROBIOTIC R[3]	
α-Amino Acid	R-Moiety	k′*	Rs	k′	Rs	k′	Rs
Alanine	-CH_3	0.56	2.9	0.16	4.0	0.30	1.7
Arginine	-$(CH_2)_3$-NH-CNH-NH_2	1.17	2.1	2.17	3.0	N/A	N/A
Aspartic	-CH_2-COOH	1.49	1.9	0.95	2.0	N/A	N/A
Asparagine	-CH_2-CO-NH_2	0.58	2.1	0.29	3.7	1.45	1.56
Cysteine	-CH_2-SH	0.45	1.6	0.20	1.8	1.78	1.50
Glutamic	-CH_2-CH_2-COOH	1.15	2.2	0.64	2.5	N/A	N/A
Glutamine	-$(CH_2)_2$-$CONH_2$	1.13	1.6	0.82	3.5	N/A	N/A
Histidine		3.10	1.5	3.96	1.5	1.13	1.45
Isoleucine	-CH(CH_3)-CH_2-CH_3	0.40	2.5	0.18	3.0	1.03	2.9
Leucine	-CH_2-CH-$(CH_3)_2$	0.47	3.5	0.60	5.5	0.27	2.2
Lysine	-$(CH_2)_4$-NH_2	0.81	2.2	1.21	2.5	1.27	1.97
Methionine	-CH_2-CH_2-S-CH_3	0.55	3.3	0.47	3.5	1.23	2.5
Phenylalanine		0.87	2.0	0.98	7.2	0.64	2.5
Proline		0.58	2.5	0.43	6.2	2.00	3.24
Serine	-CH_2OH	0.69	1.5	0.11	1.9	1.13	0.8
Threonine	-CHOH-CH_3	0.75	1.4	0.46	4.0	0.19	1.0
Tyrosine		0.60	1.9	0.76	2.9	0.52	1.0
Tryptophan		1.01	2.0	2.05	3.5	1.12	2.0
Valine	-CH(CH_3)$_2$	0.56	1.9	2.48	4.5	1.22	2.0

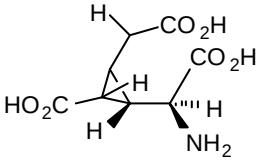
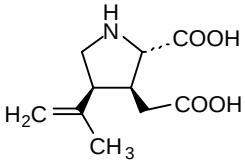
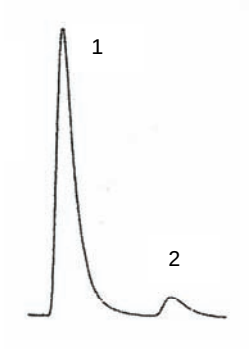
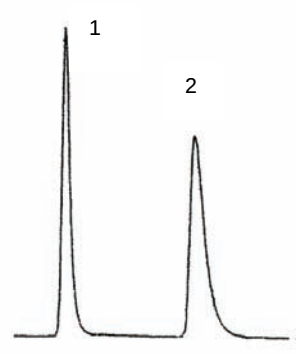
N/A - not available.

*L-form eluted first for all cases.
**Data in this table validated in Astec laboratories.
Note: For LC-MS use ATFA/H_2CO_2. See LC-MS section. Ammonium acetate can be used but peaks are more efficient with either NaH_2PO_4 or ATFA/H_2CO_2.

For more information, please consult the following articles:
1. (C-4) Berthod, Liu, Bagwell and Armstrong, J. Chromatog. A., 731, 123-137 (1996).
2. (C-23) Berthod, Gasparrini and Carotti, Anal. Chem. Vol. 72, 1767-1780 (2000).

3. (C-8) Ekborg-Ott, Liu and Armstrong, Chirality 10, 434-483 (1998).

4.3 Underivatized Synthetic Amino Acids

(2S,2'R,3'R)-2-(1'-[H],2',3'-dicarboxycyclopropylglycine	Kainic Acid
	
Peak 1 – 12.03 min. (S) Peak 2 – 15.74 min. (R) Impure Mixture 	Peak 1 – 5.33 min. (L) Peak 2 – 8.07 min. (D) 
CHIROBIOTIC T 90/10: MeOH/HOAc, pH 3.8 1.0 mL/min. UV-254mn	CHIROBIOTIC T 80/20: MeOH/50mM HOAc 1.0 mL/min. UV-210nm

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4.4 Best Starting Mobile Phase Composition for Peptides
Columns: CHIROBIOTIC T, T2 and TAG, 250x4.6mm, 5µm

Di and Tri-peptides	
Starting mobile phase	50/50: ACN/5mM NH_4OAc, pH 4.1
Variables for optimization	% ACN or combination of ACN and MeOH pH (2.8–7.0) Concentration of buffer (2-50mM) Temperature (5-45˚C) Complementary effects (CHIROBIOTIC T, T2 and TAG)
Bi-functional Peptides	
Starting mobile phase	50/50: ACN/0.1% H_2CO_2
Variables for optimization	% ACN (U-shaped effects) Concentration of HCOOH (0.01-0.5%) Temperature (5-45°C) Complementary effects (CHIROBIOTIC T, T2 and TAG)
Neutral Peptides	
Staring mobile phase	50/50: ACN/5mM NH_4HCO_2
Variables for optimization	% ACN (U-shaped effects) pH (2.8-7.0) Concentration of buffer (2-50mM)

	Temperature (5-45°C) Complementary effects (CHIROBIOTIC T, T2 and TAG)

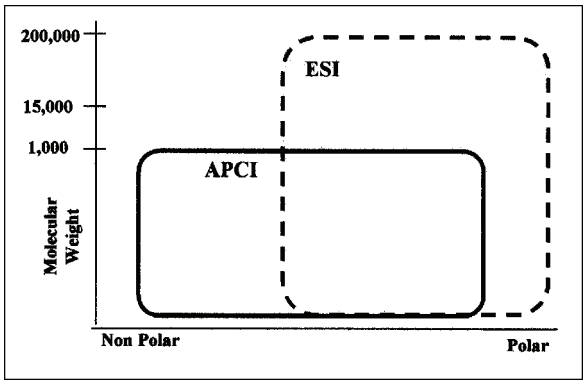
5.0 LC-MS Methods for Amino Acids and Peptides

Since 1996, the LC-MS platform has gained increasing status as an analytical and developmental tool especially within the pharmaceutical industry. To date, 27 of all the publications relating to chiral separations utilizing this technique, one applied our CYCLOBOND and 21 cited our CHIROBIOTIC phases. Please refer to CHIROBIOTIC bibliography references C-30 and C-57 for published method development techniques for LC-MS.

CHIROBIOTIC phases have gained much interest with the success of the polar ionic mode as a simple, effective mobile phase resulting in easy to validate methods. CHIROBIOTIC phases avoid inorganic buffers and rarely require normal phase solvents like hexane. Instead, a typical mobile phase would be methanol with low concentrations (0.1–0.001%, v/wt) of volatile salts like ammonium trifluoroacetate, ammonium formate or ammonium acetate, enhancing MS detection. Speed of analysis is another very favorable factor, especially when using the polar ionic mode. Further, CHIROBIOTIC technology allows for use of any solvent compatible with LC-MS even halogenated solvents, which is not possible with cellulose, amylose and other alternative CSPs.

Since CHIROBIOTIC phases operate in both the polar ionic and reversed phase modes, they are useful in APCI and ESI detection methods. Their application area can be seen summarized in the figure following.

Analyte Compatibility



Note: APCI is a good method for amino acid and peptides since these molecules are temperature stable.

5.1 Adapting LC Methods to LC-APCI-MS

SOLVENTS

All known solvents compatible with MS are compatible with the CHIROBIOTIC phases including methanol, ethanol, acetonitrile, water, isopropyl alcohol, dichloromethane, chloroform, hexane and tetrahydrofuran. The P-CAP phases described on page 8 also have universal solvent compatibility. Depending on the ion source, certain additives will aid or inhibit ion formation and hence detectability.

ADDITIVES

Acids

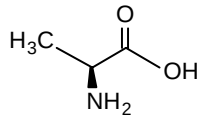
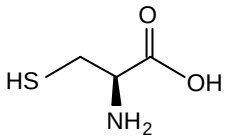
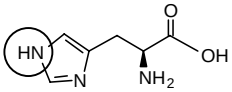
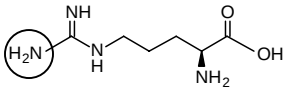
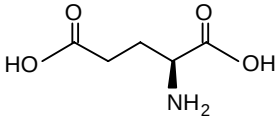
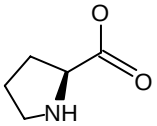
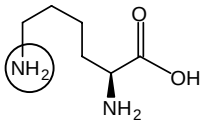
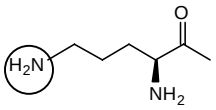
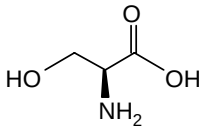
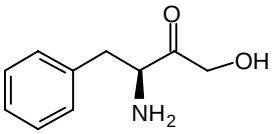
Formic and acetic acid are recommended. Trifluoroacetic acid sometimes causes ion suppression in both positive and negative modes and is, therefore, not recommended.

Bases

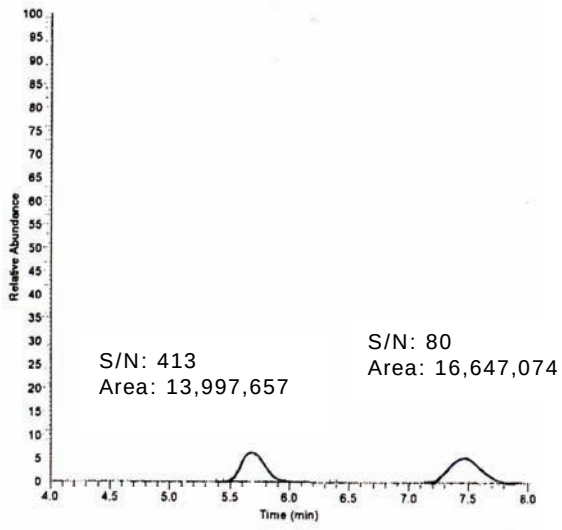
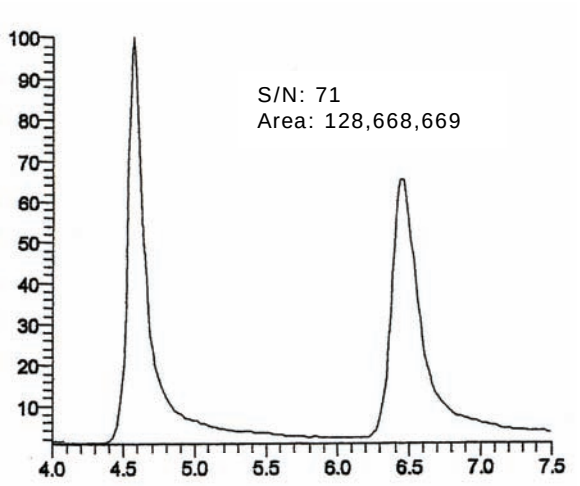
Ammonium hydroxide and ammonia solutions are recommended. Triethylamine and trimethylamine may depress ionization for positive ion formation of basic analytes.

Buffers/Salts

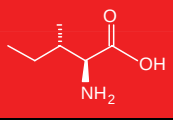
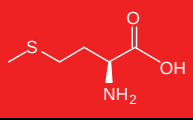
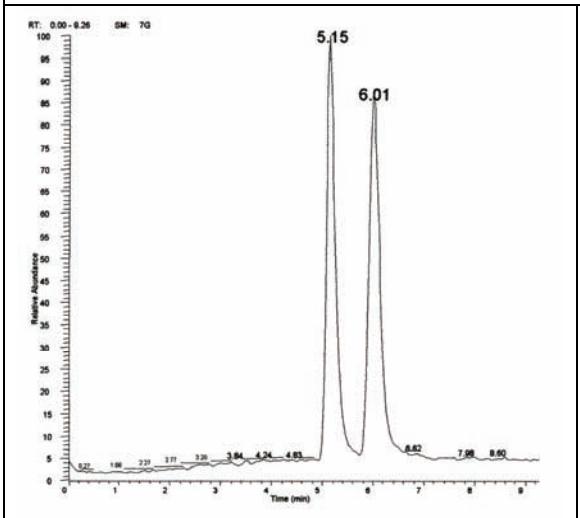
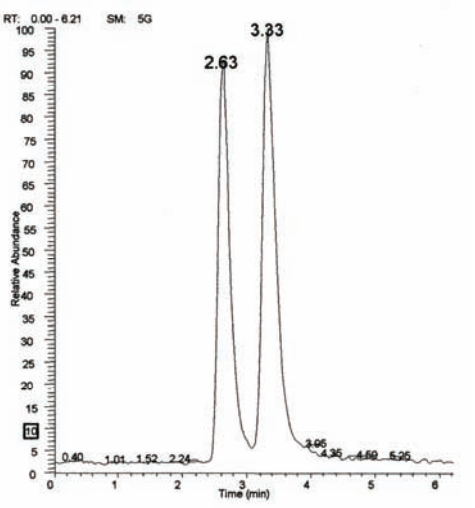
Ammonium formate, low concentrations (<0.1%w) of ammonium trifluoroacetate and ammonium acetate have been successfully used.

Amino acids separated and detected without additives on CHIROBIOTIC T		Amino acids not eluted from CHIROBIOTIC T without additives	
 Alanine MW - 89	 Cysteine MW - 121	 Histidine MW - 155	 Arginine MW - 174
 Glutamic Acid MW - 147	 Proline MW - 115	 Lysine MW - 146	 Ornithine MW - 132
 Serine MW - 105	 Phenylalanine MW - 165	Ammonium trifluoroacetate (1%) in methanol combined with 0.1% formic acid in water results in the elution of basic amino acids from CHIROBIOTIC T. The combination of ATFA/H_2CO_2 lowers pH appropriately and is especially good for APCI detection as can be seen below.	

Additives Enhance Ionization

D,L-leucine Without Additives	D,L-leucine With Additives ATFA / H_2CO_2
100 ng	
	

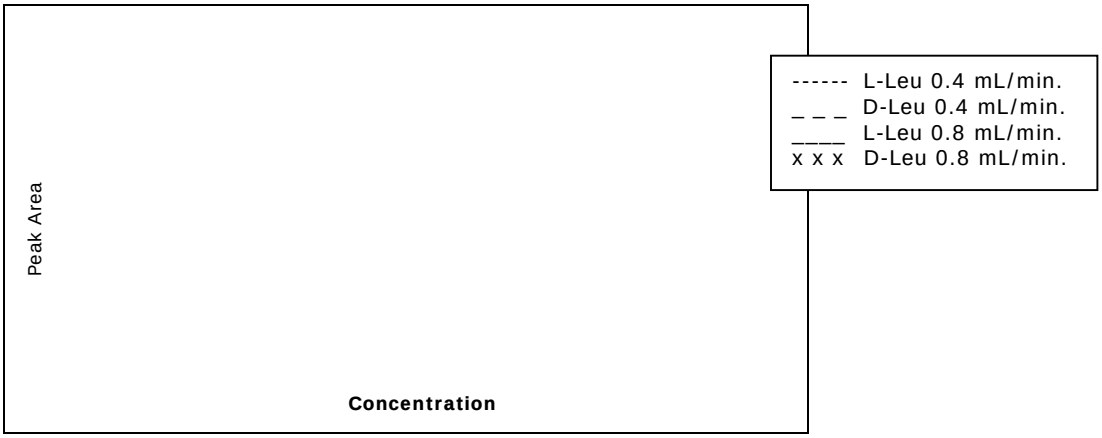
Sensitivity of LC-MS Without Derivatization

Isoleucine **MW – 131** 	**Methionine** **MW – 149** 
500 picograms	250 picograms
APCI Probe Temp: 300°C	
	
CHIROBIOTIC T 50/50: MeOH/H_2O 0.4 mL/min.	CHIROBIOTIC T 25/75: MeOH/H_2O 0.4 mL/min.

5.2 Flow Rate Effects

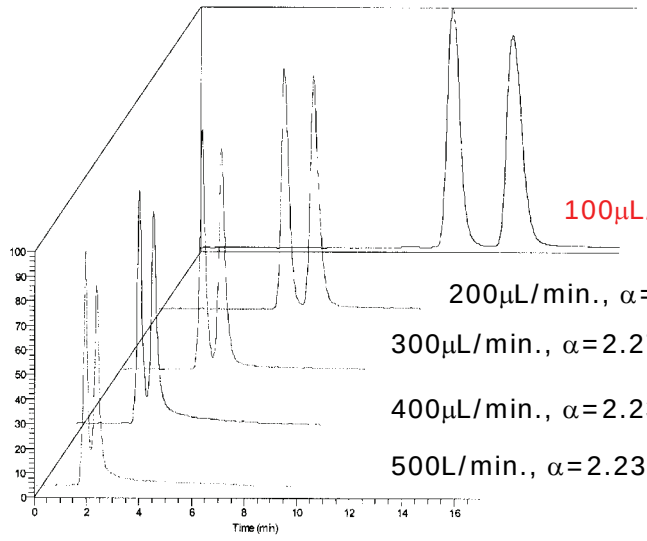
Flow rate is an important parameter in the optimization of any chiral or non-chiral LC/ESI-MS method. The best sensitivity is often achieved with moderate to low flow rates.

Flow Rate Dependence on Senstivity for ESI



Flow Rate Effect on Resolution in ESI Mode

Conditions:
CHIROBIOTIC T, 250x2.1mm
80/20: MeOH(1% AFTA)/H_2O(0.1% H_2CO_2)
Analyte: D,L-Phenylalanine



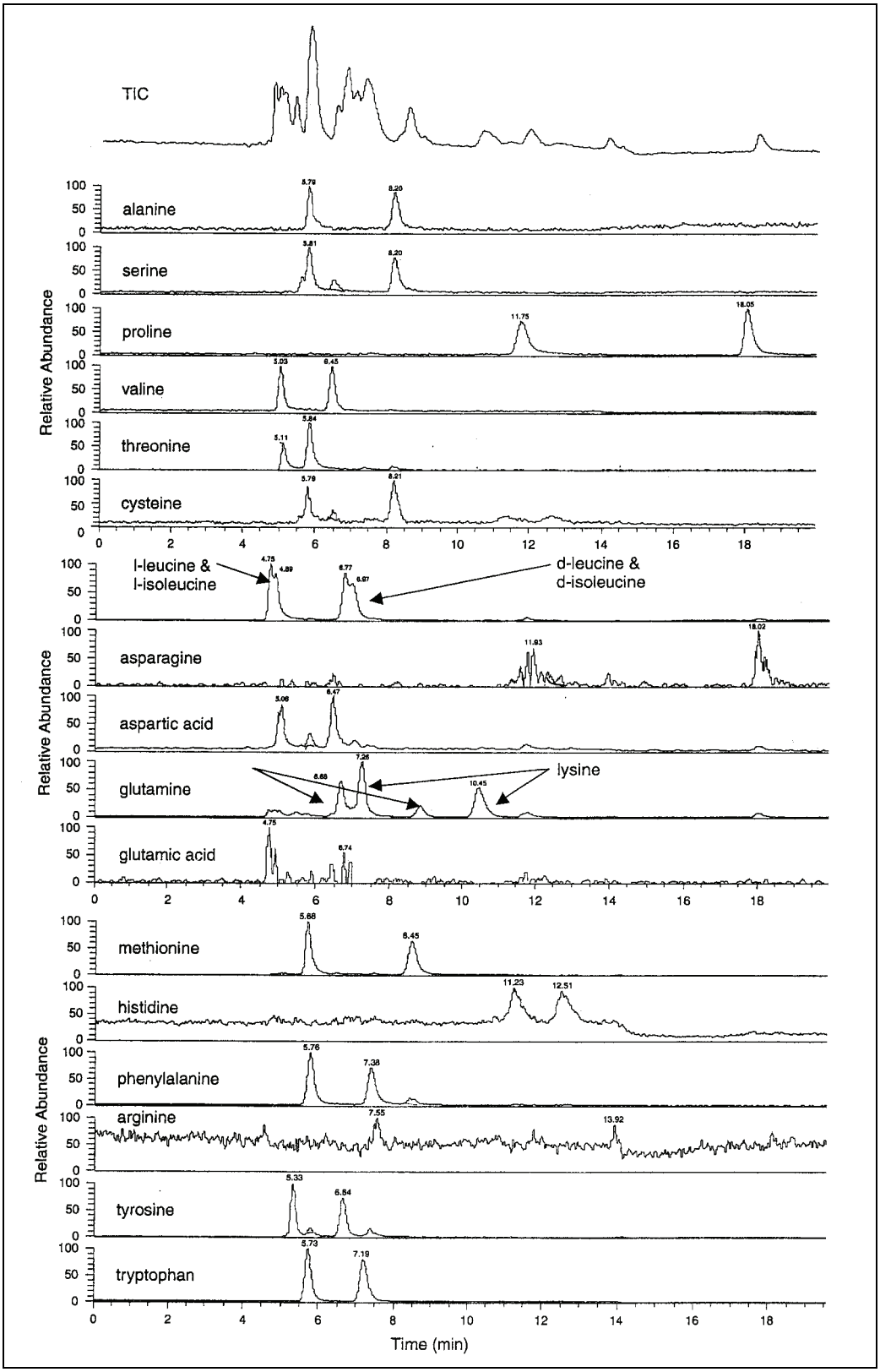
5.3 Detection Sensitivity

Detection Limits: UV/Vis vs. LC-MS

Compound	Class	UV/Vis @ 210nm	APCI-MS SIM @ [M+H]	Separation Conditions
Leucine	Neutral	10 µg	500 pg	50/50: MeOH/H_2O
Proline	Neutral	5 µg	1 ng	100% H_2O
Phenylalanine	Neutral	100 ng	1 ng	80/20: A:B*
Methionine	Neutral	5 µg	250 pg	80/20: A:B*
Glutamine	Neutral	10 µg	10 ng	80/20: A:B*
Glutamic Acid	Acidic	1 µg	10 ng	80/20: A:B*
Lysine	Basic	10 µg	10 ng	80/20: A:B*

*A: 1.0% NH_4TFA in MeOH; B: 0.1% formic acid in H_2O

Enantiomeric Separation of all 19 Native Amino Acids in a Single Run



Step gradient: 80:20 A:B – 7 min. then 20:80 A:B 7-20 min.
A: 1.0% NH_4TFA in MeOH B: 0.1% formic acid in H_2O
CHIROBIOTIC T LC-APCI-MS Full Scan Mode m/z range: 87.0-207.0

5.4 Abstracts of LC-MS Referenced Papers

As mass spectrometry has become the detector of choice, we have dedicated a special heading to cover this topic. The mobile phase requirements for enhanced detection is a critical factor as is the flow rate which affects both small diameter column performance but is also influenced by MS operating parameters. Additionally, depending on the ion source, certain additives will aid or inhibit ion formation and hence selectivity and sensitivity. The following abstracts are meant to give the reader a brief summary of the content of the paper and specific operational details. Further details can be found in the original papers which can be obtained from the key author whose e-mail address has been given. It should also be noted that a greater range of applications (21 to date) have been summarized for chiral LC-MS which is available from Astec as Lab Notes #LC005.

5.4.1. Method Development Techniques

Amino Acids – Underivatized – LC-MS (Reference C-40)

Simultaneous analysis of underivatized chiral amino acids by liquid chromatography-ionspray tandem mass spectrometry using a teicoplanin chiral stationary phase, K.Petritis, A.Valleix, C.Elfakir, and M.Dreux, J.Chromatogr.A, 913, 331-340 (2001); email: konstantinos.petritis@pnl.gov

Abstract: A total of 15 underivatized chiral amino acids from proteinogenic and non-proteinogenic sources were analyzed. The column used was a CHIROBIOTIC T column (250 x 4.6mm, 5µm). A number of methanol/water and acetonitrile/water combinations were examined to determine the best ratios for various pairs of amino acids such as L,D-isoleucine/leucine and L,D-isovaline/valine. These studies show that the teicoplanin column not only has the ability to separate the L and D enantiomers of the amino acids, but also the enantiomers of the positional isomers of amino acids (with the methyl group on the α-, β-, or γ-carbon atoms). All L-amino acids having the methyl group on the α carbon were eluted before the L-amino acids having a β-methyl group, while all the L-amino acids having the methyl group on the β carbon were eluted before the L-amino acids having a γ methyl group.

Summary:

Analytes:	Underivatized 15 protein amino acids
Sample Prep:	Standards
Column:	CHIROBIOTIC T (5µm), 250 x 4.6 mm
Mobile Phase:	75/25: ACN/H_2O
Flow Rate:	0.8 mL/min
Analysis Time:	Under 25 minutes
Detection limits:	0.25 to 5.0 µg/L depending on the amino acid

Amino Acids and Peptides – Underivatized – LC-MS (Reference C102)

Analysis of native amino acid and peptide enantiomers by high-performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry, M.J.Desai and D.W.Armstrong, J.MassSpectrom. 39, 177-187 (2004); email: sec4dwa@iastate.edu

Abstract: Aside from the importance of the enantiomeric separation of amino acids and peptides, the ability to use mass spectrometry for their characterization has become just as important. These researchers have developed a number of mobile phases compatible with atmospheric pressure chemical ionization (APCI) mass spectrometry, which can give an order of magnitude better sensitivity over electrospray ionization (ESI). The columns used are CHIROBIOTIC T and CHIROBIOTIC TAG (250 x 4.6mm or 250 x 2mm, 5µm). Non-chiral peptide studies were done on an Astec C_{18} column. Amino acid detection limits are compared for this LC-MS work to UV at 210nm. Refer to the paper for their discussion on the separation and MS of di- and tri-peptides. A table of the separations and mobile phases based on information in this paper is given in this Handbook in Section 5.5.

Summary:

Analyte:	Underivatized amino acids (Section 5.5)
Sample prep:	Standards
Column:	CHIROBIOTIC T/TAG, (5µm) 250 X 4.6mm or 250 x 2.0mm
Mobile Phase:	Various, see Section 5.5
Flow rate:	0.4 or 0.8 mL/min
Analysis time:	20 min or less
Detection limits:	250pg - 10ng/ml

5.4.2. Nutraceuticals

Selenomethionine Enantiomers (Reference 36)

Chiral speciation and determination of selenomethionine enantiomers in selenized yeast by HPLC-ICP-MS using a teicoplanin-based chiral stationary phase. Mendez, S.P., Gonzalez, E.B., Medel, A.S., J. Anal. At. Spectrom. 15, 1109-1114 (2000).

Abstract: Underivatized selenoamino acids could be resolved with very good selectivity and excellent detection limits, 1.9 µg/L for each selenomethionine enantiomer on a 250x4.6mm analytical CHIROBIOTIC T column in under 8 minutes. This publication evaluated the methanol content, pH, ionic strength of the mobile phase on retention and resolution for the selenoaminoacid enantiomers. It was determined that the method was very robust and not sensitive to changes in buffer. The HPLC-ICP-MS method was successfully applied to the chiral speciation and determination of D- and L-selenomethionine enantiomers in real samples of selenized yeast.

Summary:

Analytes:	Selenomethionine and selenthionine
Sample Prep:	Enzymatic hydrolysis/aqueous extraction
Column:	CHIROBIOTIC T, 250 x 4.6mm
Mobile Phase:	98/2: Methanol/water
Flow Rate:	1.0 mL/min.
Analysis Time:	Under 9 minutes
Detection:	0.8 µg/mL as selenium, Sensitivity 26x > crown ether

Selenomethionine Enantiomers (Reference C-79)

Hybridation of different chiral separation techniques with ICP-MS detection for the separation and determination of selenomethionine enantiomers: chiral speciation of selenized yeast, S.P. Mendez, E.B.Gonzales, and A. Sanz-Medel, Biomed.Chrom., 15, 181-188 (2001): email: ASM@sauron.quimica.uniovi.es

Abstract: Selenoproteins are found in the human body and are an essential element for our health. Selenomethionine is the amino acid that can be absorbed and serves for selenium storage in proteins. Dietary supplements containing selenium enriched yeast are made for human consumption. This paper analyzes the enantiomeric content of these. To increase the sensitivity of the detection of these species, the inductively coupled plasma mass spectrometer (ICP-MS) was used. After extraction of the sample, direct separation was achieved using a CHIROBIOTIC T column (250 x 4.6mm, 10µm) with a mobile phase of 2% methanol in water, at a flow rate of 1 ml/min. The L enantiomer elutes at 4.9 min., the D enantiomer elutes at 6.0 min.

Summary:

Analyte:	D and L selenomethionine
Sample prep:	Enzymatic hydrolysis, filtration
Column:	CHIROBIOTIC T, (10µm) 250 X 4.6mm
Mobile Phase:	2% Methanol in water v,v
Flow rate:	1.0 mL/min.
Analysis time:	7 min.
Detection limits:	0.8 µg/L (as Se)

Selenomethionine Enantiomers (Reference C-107)

Comparative study of the instrumental coupling of high performance liquid chromatography with microwave-assisted digestion hydride generation atomic fluorescence spectroscopy and inductively coupled plasma mass spectrometry for chiral speciation of selenomethionine in breast and formula milk, J.L.Gomez-Ariza, V.Bernam-Daza, and J.M.Villegas-Portero, Analy.Chem.Acta 520, 229-235 (2004); email: ariza@uhu.es

Abstract: This is a more recent paper dealing with the determination of the enantiomers of selenomethionine. Coupling various detection devices has become more common to eliminate intermediate steps and losses, and leads to greater reproducibility and sample throughput. The chiral separation is accomplished on a CHIROBIOTIC T column (250 x 4.6mm, 10µm) – the mobile phase was pure water at 1 ml/min. The limits of detection were 3.1 ng/mL for L-selenomethionine and 3.5 ng/mL for the D-enantiomer. See the paper for the other details of instrument coupling.

Summary:

Analyte:	D and L selenomethionine
Sample prep:	Precipitation, conc. SCX
Column:	CHIROBIOTIC T,(10µm) 250 X 4.6mm
Mobile Phase:	Milli-Q water*
Flow rate:	1.0 mL/min
Analysis time:	9 min.
Detection limits:	3.1 (L), 3.5(D) ng/mL

*Trademark of Millipore Corporation.

5.4.3. Clinical Applications

2-Hydroxyglutaric Acid Enantiomers – LC-MS (Reference C-38)

Chiral liquid chromatography tandem mass spectrometry in the determination of the configuration of 2-hydroxyglutaric acid in urine, M.S.Rashed, M.AlAmoudi, and H.Y.Aboul-Enein, Biomed. Chromatogr., 14, 317-320 (2000); email: rashed@kfshrc.edu.sa

Abstract: Both the D- and L- forms of 2-hydroxyglutaric acid lead to distinct inherited metabolic diseases which have been found in children. The possibility of now being able to distinguish between these can lead to improved treatment of each. The detection used was negative-ion electrospray ionization (ESI) on a LC-MS/MS system. After sample preparation, direct separation was achieved using a CHIROBIOTIC R column (250 x 4.6mm, 5µm) with a mobile phase of a) 5mM triethylamine adjusted to pH 7.0 with acetic acid and b) methanol in a ratio of 9:1. The flow rate was 0.5 mL/min. The stream was split to allow only 60 µL/min of the effluent into the ion source. The L enantiomer elutes at 4.85 min, the D enantiomer elutes at 5.5 min.

Summary:

Analytes:	D,L-2-hydroxyglutaric acid
Sample Prep:	Urine dilution (100 µL) with mobile phase (900 µL), filtration
Column:	CHIROBIOTIC R, (5µm) 250 x 4.6 mm
Mobile Phase:	5mM TEAA, pH-7.0/MeOH: 9/1, v/v
Flow Rate:	0.5 mL/min; 60µl/min to ion source
Analysis Time:	Under 6 minutes
Detection Limits:	Not specified

Pipecolic Acid Enantiomers (Reference 53)

Determination of L-pipecolic acid in plasma using chiral liquid chromatography-electrospray tandem mass spectrometry, Rashed, M.S., Al-Ahaidib, L.Y., Aboul-Enein, H.Y., Al-Amoudi, M., Jacob, M., Clinical Chemistry 47:12, 2124-2130 (2001).

Abstract: L-pipecolic acid is an important biochemical marker for the diagnosis of peroxisomal disorders. This LC-MS-MS method uses CHIROBIOTIC T to clearly establish healthy individuals from peroxisomal disease patients. From a 50 µL plasma sample after deproteinization, evaporation and injection mean recoveries of pipecolic acid spike samples were 95% and 102% at concentrations of 1-50 µmol/L. The method is simple, rapid and stereoselective. An internal standard of phenylalanine-d_5 was used. Turnaround time for the complete assay was 20 minutes.

Analytes:	Pipecolic acid in plasma, urine and cerebrospinal fluid
Sample Prep:	Acidified acetonitrile/evaporation/ MeOH/water
Column:	CHIROBIOTIC T, 250 x 2.0 mm
Mobile Phase:	MeOH/H2O: 60/40, v/v
Flow Rate:	200 µL/min
Analysis Time:	Under 12 minutes
Detection Limits:	0.5 to 80 µmol/L

5.4.4. Food and Beverage Analysis

Theanine enantiomers (from tea) – LC-MS (Reference C-103)

Analysis of derivatized and underivatized theanine enantiomers by high-performance liquid chromatography/atmospheric pressure ionization- mass spectrometry, M.J.Desai and D.W.Armstrong, Rapid Comm. Mass Spectrom., 18, 251-256 (2004); email: sec4dwa@iastate.edu

Abstract: Theanine is a naturally occurring non-proteinic amino acid found in tea leaves. It has shown many positive physiological activities including reduced blood pressure and enhancing the anti-tumor effect of chemotherapy drugs. As with many compounds exhibiting chirality, interest in which enantiomer is responsible for what physiological activity is very important. Both the underivatized and the derivatized (FMOC, AQC, Dansyl) theanine were able to be separated on a CHIROBIOTIC T column (250 x 4.6mm, 5µm) with ratios of a) 80/20: MeOH(1% ATFA)/H_2O(0.1% H_2CO_2, b) 30/70: MeOH(1% ATFA)/MeOH, c) 30/70: or 35/65: MeOH(1% ATFA/H_2O. Flow rates were either 0.4 or 0.8 mL/min. MS work with APCI (atmospheric pressure chemical ionization) for the underivatized enantiomers and ESI (electrospray ionization) for the underivatized and derivatized enantiomers is shown. The limit of detection for the theanine is 10 ng/mL.

Summary:

Analyte:	Underivatized/derivatized theanine
Sample prep:	Standards and samples, without or with derivatization
Column:	CHIROBIOTIC T, (5µm) 250 X 4.6mm
Mobile Phase:	See abstract above
Flow rate:	0.4 or 0.8 ml/min.
Analysis time:	Underivatized, 10 min; Derivatized, up to 40 min.
Detection limits:	10 ng/mL

5.5 Enantioresolution of Underivatized Amino Acids with Mass Spectometry Detection APCI (Ref. C-102)

R—CH(NH$_2$)—COOH					Results	
α-Amino Acid	**R-Moiety**	**Molecular Mass**	**CHIROBIOTIC T/TAG**	**Mobile Phase Composition and Flow Rate**	**Rs**	**α**
Alanine	-CH_3	89	T	50/50: EtOH/H_2O @ 0.4 mL/min.	1.72	1.46
			T	80/20: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH) @ 0.8 mL/min.	2.4	1.6
Serine	-CH_2OH	105	T	85/15: EtOH/H_2O @ 0.4 mL/min.	1.55	1.63
			T	80/20: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH) @ 0.4 mL/min.	1.75	1.19
Proline	-CH_2-CH_2-CH_2-	115	T	100% H_2O, 0.4 mL/min.	1.52	1.30
Valine	-$CH(CH_3)_2$	117	T	50/50: EtOH/ H_2O @ 0.4 mL/min.	1.52	1.30
			T	80/20: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH) @ 0.8 mL/min.	2.52	1.37
Norvaline	-CH_2-CH_2-CH_3	117	T	50/50: EtOH/H_2O @ 0.2 mL/min.	3.40	2.21
			T	80/20: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH) @ 0.8 mL/min.	5.29	2.41
Threonine	-CHOH-CH_3	119	T	70/30: EtOH/ H_2O @ 0.8 mL/min.	1.94	1.22
			T	80/20: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH) @ 0.8 mL/min.	1.94	1.22
Cysteine	-CH_2-SH	121	T	50/50: EtOH/H_2O @ 0.8 mL/min.	2.60	1.19
			T	80/20: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH) @ 0.8 mL/min.	2.60	1.19
Isoleucine	-CH(CH_3)-CH_2-CH_3	131	T	50/50: EtOH/H_2O @ 0.4 mL/min.	1.67	1.21
			T	80/20: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH) @ 0.8 mL/min.	4.11	1.64
Leucine	-CH_2-CH(CH_3) $_2$	131	T	50/50: EtOH/ H_2O @ 0.4 mL/min.	2.26	1.64
			T	80/20: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH) @ 0.8 mL/min.	3.90	1.60
Norleuceine	-CH_2-CH_2-CH_2-CH_3	131	T	80/20: EtOH/H_2O @ 0.2 mL/min.	3.87	2.38
			T	80/20: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH) @ 0.8 mL/min.	4.72	1.84
Asparagine	-CH_2-CO-NH_2	132	T	50/50: MeOH/ H_2O @ 0.8 mL/min.	3.50	1.22
			T	80/20: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH) @ 0.8 mL/min.	5.31	1.65
Aspartic acid	-CH_2-COOH	133	T	100% MeOH @ 1.0 mL/min.	3.0	1.58
			T	80/20: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH) @ 0.8 mL/min.	2.67	1.28
Glutamine	-(CH_2) $_2$-$CONH_2$	146	T	50/50; MeOH/ H_2O @ 0.8 mL/min.	2.55	1.18
			T	80/20: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH) @ 0.8 mL/min.	5.44	1.43
Lysine	-$(CH_2)_4$-NH_2	146	T	80/20: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH) @ 0.8 mL/min.	6.08	1.50
Glutamic	-CH_2-CH_2-$CONH_2$	147	TAG	80/20; MeOH/ H2O 1.0 mL/min.	1.54	1.77
			T	80/20: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH) @ 0.8 mL/min.	4.41	1.39
Methionine	-CH_2-CH_2-S-CH_3	149	T	25/75 EtOH/H_2O @ 0.4 mL/min.	1.50	1.37
			T	80/20: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH) @ 0.8 mL/min.	5.75	1.70
Histidine		155	TAG	60/40: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH) @ 0.2 mL/min.	2.23	1.24
			T	80/20: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH) @ 0.8 mL/min.	1.53	1.13
Phenylalanine		165	T	50/50: EtOH/ H_2O @ 0.4 mL/min.	1.53	1.40
			T	80/20; MeOH(1.0% NH4TFA)/H_2O (0.1%HCOOH), 0.8 mL/min.	3.44	1.39
Arginine	-$(CH_2)_3$-NH-CNH-NH_2	174	T	80/20: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH) @ 0.8 mL/min.	4.96	1.64
Tyrosine		181	T	55/45: EtOH/ H_2O @ 0.4 mL/min.	1.58	1.38
			T	80/20: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH) @ 0.8 mL/min.	3.24	1.34
Tryptophan		204	T	20/80: EtOH/ H_2O @ 0.4 mL/min.	1.3	1.26
			T	80/20: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH) @ 0.8 mL/min.	3.33	1.38
Other Amino Acids						
2-Pyrrolidone-5-carboxylic acid		129	TAG	50/50: EtOH/ H_2O @ 0.4 mL/min.	1.33	1.19
			TAG	80/20: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH) @ 0.8 mL/min.	1.43	1.13

α-Amino Acid	R-Moiety	Molecular Mass	CHIROBIOTIC T/TAG	Mobile Phase Composition and Flow Rate	Rs	α
Ornitine		133	TAG	50/50: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH), 0.4 mL/min.	1.69	1.38
			TAG	80/20: MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH) @ 0.8 mL/min.	3.33	1.38
cis-2-Amino-cyclohexane carboxylic acid		143	T	90/10;EtOH/ H_2O) 0.2 mL/min.	1.27	1.22
Carnitine		161	TAG	70/30; MeOH/ H_2O) 0.2 mL/min.	1.85	1.66
DOPA		197	TAG	50/50; EtOH/ H_2O) 0.2 mL/min.	6.44	8.25
			T	80/20; MeOH(1.0% NH_4TFA)/H_2O (0.1%HCOOH), 0.8 mL/min.	5.86	1.63
Acetylcarnitine		203	T	62.5/37.5; MeOH/ H_2O) 0.4 mL/min.	1.71	1.15
Ref: Analysis of native amino acid and peptide enantiomers by high-performance liquid chromatography/atmospheric pressure chemical Ionization mass spectrometry, M.J. Desai & D.W. Armstrong, J. Mass Spectrom., 39, 177-187 (2004) – (Ref: C-102)						

Notes on LC-MS and CHIROBIOTIC Phases

1. Polar ionic and polar organic modes are ideal for ESI.
2. APCI is usually best for reversed phase separations but requires more optimization.
3. Additives effect both separation selectivity and detection sensitivity.
4. Native amino acids work best with APCI and alcohol/water mixtures.
5. Lower flow rates tend to enhance detection sensitivity and overall performance.

6.0 Trace Analysis

The development of pure enantiomers has been a major effort since the first discovery of the ill effects of a drug racemate. It has accelerated dramatically as more cost effective means were sought for their production. Traditionally, molecules from natural sources have been used in chiral synthesis since they were often available at reasonable cost. More recently an increasing number and variety of new chiral agents have become available. Many of these are not from natural sources and need to be analyzed for their chiral impurities in order for the final product to meet the industry requirements of >99% purity.

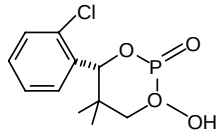
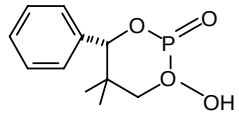
In the work of D. W. Armstrong and J. T. Lee, enantiomeric impurities in chiral starting materials were found to range from a low of 0.01% to a high of 10%. Chiral contaminants of 1-10% were not uncommon. The surprising fact is that suppliers and consumers alike were not able to verify the chiral purity of their starting materials. Only the non-chiral impurities were evaluated.

In the first of two published works of Armstrong and Lee (Reference CB-156), 83 chiral reagents were studied. For these analyses, 4 chiral GC phases (42.9% on the CHIRALDEX G-TA), 5 chiral HPLC phases (33.3% on the CYCLOBOND I *2000* RSP) and 3 types of CE chiral additives were used. In all 39 different sets of conditions were employed to evaluate the 83 chiral reagents. In this publication two chiral resolving agents used for amino acids were tested for enantiomeric purity. The results follow.

In the second study (Reference CB-157), 109 chiral reagents were evaluated. This was accomplished with 5 chiral GC phases (39.3% on the CHIRALDEX G-TA, 33.7% on the CHIRALDEX B-DM) and 6 chiral HPLC phases, the largest percentage being on the CHIROBIOTIC V (23.5%).

Approximately 64% of these latter reagents had moderate to high levels of enantiomeric impurities. The range was from >0.1% to <16%.

Chiral Resolving Agents (Reference CB-156)
Resolution of amines, amino acids and amino alcohols

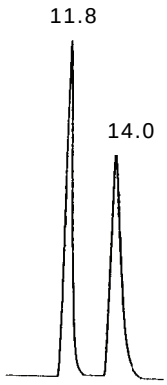
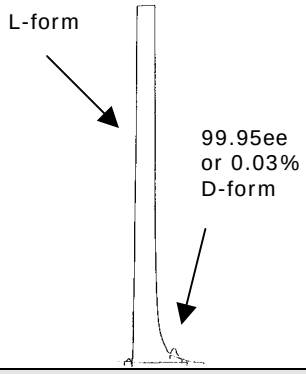
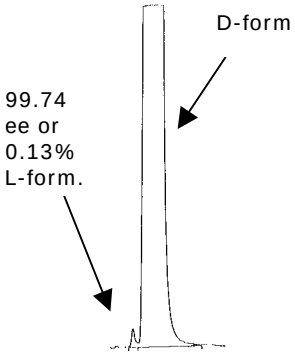
Chiral Resolving Agent	Purity (ee)		Column
4-(2-Chlorophenyl)-2-hyroxy-5,5-dimethyl-1,3,2-dioxaphosphorinane 2-oxide 	(S) 0.60[a] (R) 3.39[a]	(R) 98.80[a] (S) 93.22[a]	CYCLOBOND I *2000*[1] CYCLOBOND I *2000* RSP[2]
2-Hydroxy-5,5-dimethyl-4-phenyl-1,3,2-dioxaphosphorinane 2-oxide 	(S) 0.03 (R) 0.41	(R) 99.94 (S) 99.18	CYCLOBOND I *2000* RSP[3]

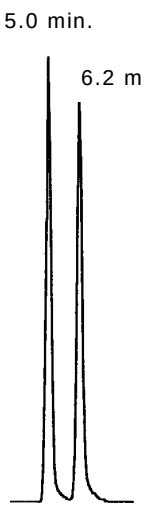
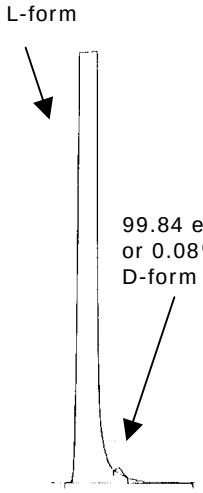
[a]CYCLOBOND I *2000* and CYCLOBOND I *2000* RSP have opposite elution orders for this compound.

[1]CYCLOBOND I *2000*, 95/5/0.3/0.2: ACN/MeOH/HOAc/TEA
[2]CYCLOBOND I *2000* RSP, 30/70: MeOH/1% TEAA, pH 4.1.
[3]CYCLOBOND I *2000* RSP, 20/80: MeOH/1% TEAA, pH 4.1

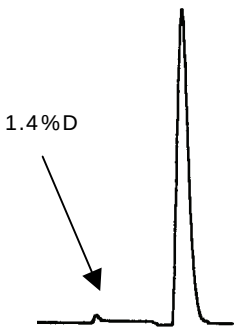
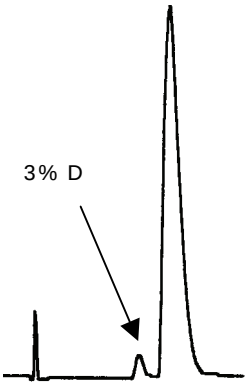
Enantiomeric Impurity Analysis of Amino Acids

Evaporative Light Scattering Detector (ELSD) in general has a more stable baseline and better sensitivity for all amino acids. ELSD requires volatile salts when buffers are used.

Phenylalanine		
Racemate	Evaluation Pure L	Evaluation Pure D
		
CHIROBIOTIC T 80/20: ACN/H_2O 1.0mL/min. UV-254nm		

Aspartic Acid	
Racemate	**Evaluation Pure L**
	
CHIROBIOTIC T 80/20: MeOH/10mM NH_4OAc, pH 3.8 0.8mL/min. 25°C ELSD Detector	

Trace Analysis on Astec CLC Columns

Proline	Aspartic Acid
Peak 1 – 6.29 min. (D) Peak 2 – 11.87 min. (L)	Peak 1 – 9.17 min. (D) Peak 2 – 11.14 min. (L)
	
Astec CLC-L 5mM $CuSO_4$ UV-254nm	

7.0 Enantiomeric Separations by Supercritical Fluid Chromatography (SFC)

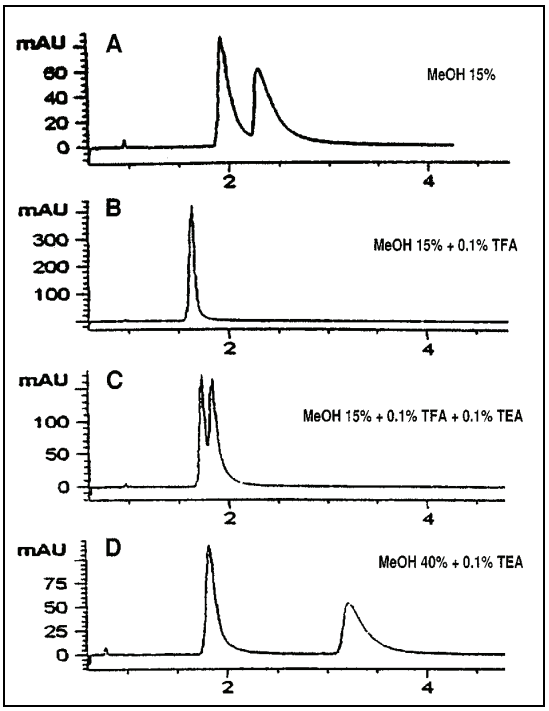
As a technique, SFC works best when separations from LC were based on normal phase or polar organic type mobile phases. If additives had been used in LC they will typically be required in SFC. Additive concentrations are generally higher with acids and bases on the CHIROBIOTIC phases due to ionic interactions and lower or absent for P-CAP phases. Neutral molecules are, therefore, more ideally suited to SFC methodologies.

7.1 SFC on CHIROBIOTIC Phases

All packed CHIROBIOTIC phases are stable to subcritical and supercritical fluid methodologies and have been tested under a variety of operating conditions. Several recent publications attest to the effectiveness of these stationary phases in this methodology. Because of the polarity of these phases and the presence of ionic functionality, additives such as methanol in the range of 10-25% had to be added to the carbon dioxide. For some ionizable compounds additional additives like TFA and TEA have to be added in the range of 0.05-0.5% v/v.

Effect of Additives in SFC

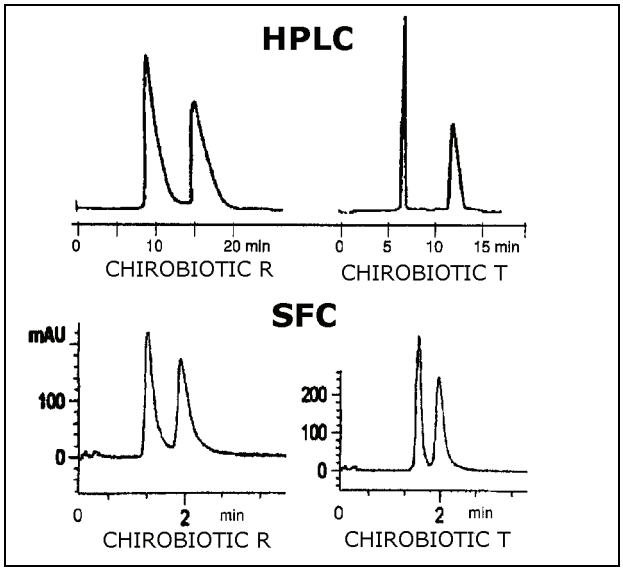
DNPyr-leucine
CHIROBIOTIC T, 250x4.6mm
SFC Mobile Phase @ 4 mL/min.



Armstrong has published the first super critical separation of amino acids (Reference C-70).

Comparison HPLC/SFC

CBZ-Norvaline
HPLC: 20/80: MeOH/TEAA, pH 4
SFC: 60/39.96/0.4:CO_2/MeOH/TEA

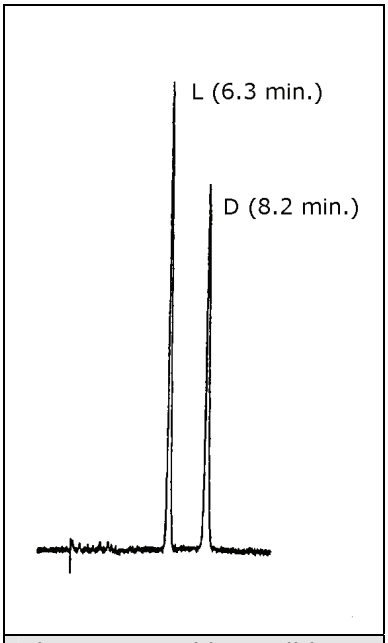


7.2 SFC on CYCLOBOND Phases

Comparisons between LC and SFC demonstrate that, although selectivity was sometimes lower in SFC than in LC, the improved efficiency in SFC resulted in higher resolution. Identification of optimum chromatographic parameters is facilitated in SFC, and most of the compounds investigated were resolved with a carbon dioxide/methanol eluent. However, the alcohol modifier plays an important role in enantioselectivity in SFC, and the nature of this role is not the same for all analytes. Only when run in the polar organic mode is a substantial savings in time noted for LC. Resolution is also better for LC in this mode.

Ref: Williams, K.L., Sander, L.C., Wise, S.A., Comparison of Liquid and Supercritical Fluid Chromatography Using Naphthylethyl-carbamoylated-β-cyclodextrin Chiral Stationary Phases. See CYCLOBOND bibliography reference CB-145.

Separation of N-(3,5-dinitrobenzoyl)-DL-valine methyl ester by SFC on CYCLOBOND I 2000 RN



Chromatographic conditions: 90/10: Carbon Dioxide/Methanol, 2.0 mL/min. 30°C, 15 MPa.

Note: P-CAP phases have demonstrated excellent performance in SFC. However, no work on N-blocked amino acids or peptides has yet been published.

8.0 Preparative Separations

CHIROBIOTIC phases offer unique opportunities for preparative purifications. Some key factors for consideration:

1. No solvent limitations. Halogenated solvents as well as very polar solvents are well tolerated on these CSPs. This solvent tolerance is especially useful when optimizing for sample solubility.

2. Can be run in four distinctly different mobile phase types. Use of acid/base on any one of these CSPs does not preclude their use in other mobile phases. Mobile phases listed here in the order of success:

   - Polar ionic mode: MeOH/Acid/Base or MeOH/NH_4 Salt
   - Reversed phase mode* ACN/Buffer
   - Polar organic mode: MeOH/EtOH, ACN or combination
   - Normal phase mode: Heptane/EtOH

*Please note efficient workup/isolation procedures available for the reversed phase mode. Method outlined for analytical C_{18} column. See workup on page 33.

3. Very long term stability with these CSPs due, in part, to the multiple linkages used in anchoring the CSP and, secondly, to the mild conditions typically required.

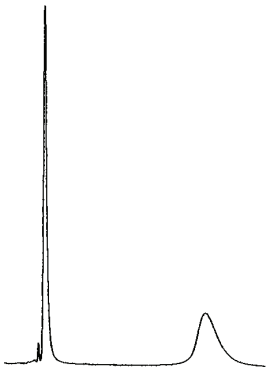
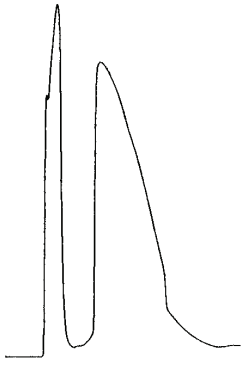
4. Range of capacities is compound dependent. CHIROBIOTIC phases significantly overlap cellulose and amylose phases based on throughput primarily because separations on these CSPs are usually very fast. Capacities on CHIROBIOTIC V2 and T2 phases have been 2.5 mg/gm with an alpha of 1.5. Maximum capacity achieved was 300 mg “on column” using a 250x21.2mm column with an alpha value of 2.0.

5. Excellent economics are possible especially with the polar organic and polar ionic modes. Ionic interactions play a significant role in the chiral recognition mechanism on these phases. Solvents here are anhydrous, more

volatile and less toxic than the typical normal phase solvents.

6. N-Acetylated amino acids generally have higher solubility and substantially higher selectivity on CHIROBIOTIC phases. Acetylated amino acids are easily cleaved chemically or enzymatically after separation and recovery.

Derivatization Study: N-Acetyl Tryptophan

ANALYTICAL	PREPARATIVE
CHIROBIOTIC TAG 250x4.6mm, 5µm Detection: 254nm UV Flow Rate: 1.0 mL/min.	CHIROBIOTIC TAG 250x21.2mm, 5µm Detection: 300nm UV Flow Rate: 35.0 mL/min. Load: 200mg in 6mL
Mobile Phase – Polar Ionic Mode 100/0.1w%: MeOH/NH_4OAc	
Peak 1 – 3.80 min. Peak 2 – 15.59 min. 	Peak 1 – 2.86 min. Peak 2 – 4.53 min. 
k_1 = 0.36, α = 12.7	Throughput – 20 mg/g CSP/hr.

8.1 Preparative Method Development Procedure

1. Test sample solubility in various solvent systems (see Mobile Phase Design Section 3.0).

2. Screen multiple columns in the best mobile phase for maximum analyte solubility and maximum capacity.

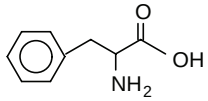
3. Detune detector sensitivity.

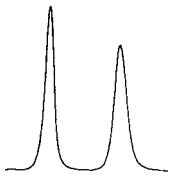
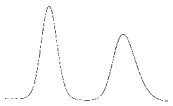
4. Optimize conditions on the best CSP (see optimization steps under mobile phase design).

5. Calculate need for preparative purification from analytical (5µm) to preparative (10µm or 16µm) packing materials.

Comparison of 5µm, 10µm and 16µm CHIROBIOTIC T

Phenylalanine



5µm	10µm	16µm
Peak 1 – 5.75 Peak 2 – 7.17	Peak 1 – 5.86 Peak 2 – 7.13	Peak 1 – 5.84 Peak 2 – 7.57
		

CHIROBIOTIC T, 250x4.6mm
50/50: EtOH/H_2O
0.9 mL/min.

6. Overload an analytical column packed with 10 or 16µm material. Target ~85% resolution. Calculate column dimensions required.

Because the prep method has been developed on an analytical column that contains the identical packing that is in the prep column, scale the flow rate and sample load by the proportional cross sectional area between the analytical and prep column. Continue your loading study in order to get the best throughput.

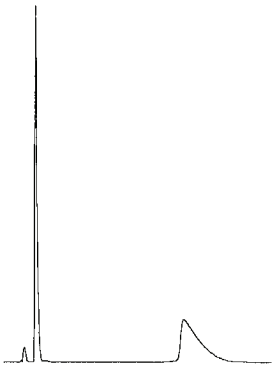
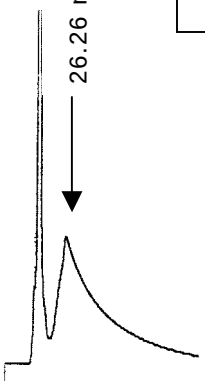
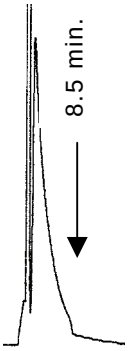
Scale-Up Guide

This guide was developed from a range of racemates. As a general rule, sample capacity of a column can be squared when the column diameter is doubled, i.e., 2 x ID = $(Cp)^2$ @ 4 x mL/min.

Column Diameter	Av. Sample Capacity	Typical Flow Rates	Injection Volume
10.0mm ID	2-20mg	4-10 mL/min.	0.2-2.0 mL
21.2mm ID	20-200mg	15-50 mL/min.	1.0-10.0 mL
50.0mm ID	200mg-5g	60-250 mL/min.	5.0-50.0 mL

7. Study temperature effects.

Temperature Effect in Loading Study of Diacetyl-Cysteine (N^{15})

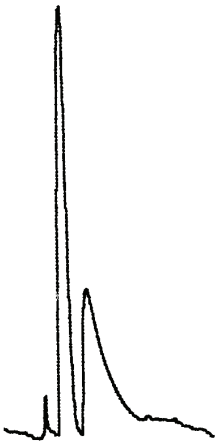
23°C	23°C	35°C
Peak 1 – 2.69 min. Peak 2 – 15.48 min.	Higher Temperature 23°C → 35°C 26.26 min.	8.5 min.
		
Loading - 20μg	Loading – 15mg	Loading- 15mg
CHIROBIOTIC T, 250x4.6mm 100/0.1% (v/w): MeOH/NH_4OAc 2.0 mL/min. 230nm UV		

8. Determine purity and yield from an analytical run.

9. Determine options for any interfering impurities.

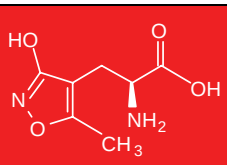
10. Evaluate systems and features available as maximum flow rate versus operating pressure, recycling and shaving, variable wavelength and minimized data point collection, all to determine final column dimensions.

Preparative Purification of Isoleucine on CHIROBIOTIC TAG

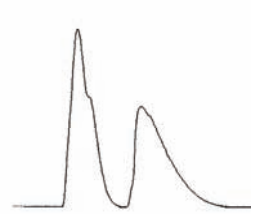


CHIROBIOTIC TAG
250x21.2mm
30/70: MeOH/H_2O
20mL/min.
Inj. 170mg in 10mL

Prep Purification of the Synthetic Amino Acid – AMPA



Peak 1 – 5.13 min.
Peak 2 – 6.38 min.

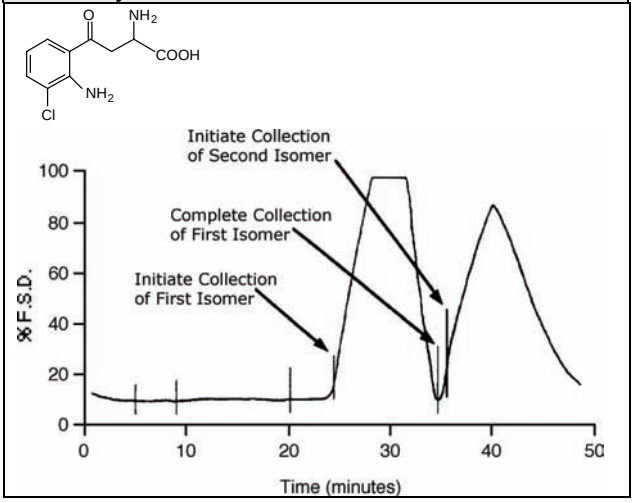


CHIROBIOTIC T
250x21.2mm
80/20: MeOH/10mM Ammonium Bicarbonate, pH 4
20 mL/min.
Inj. 50mg in 1mL

Printed with the kind permission of Tocris Biosciences.

Preparative Separation of the Enantiomers of Chlorokynurenine on CHIROBIOTIC T. An Example of Displacement Chromatography

Column:	CHIROBIOTIC T (10µm), 5x50cm
Mobile Phase:	1/1:EtOH(95%)/H_2O
Temperature:	40°C
Inj. Volume:	400mL
Concentration:	800mg
Purity:	Peak 1 – 99%, Peak 2 – 98%
Recovery:	95%



Courtesy Drs. Dolak and Seest, Pharmacia–Upjohn.

8.2 Work-up/Isolation Procedure for Aqueous Mobile Phase Systems

This technique utilizes the ability of C_{18} phases to remove organics from high aqueous systems. The degree of hydrophobicity of the analyte is the main criterion in applying this technique. The following procedure can be used to ascertain the capacity of the C_{18} column and the size column required for recovery of separated analytes from a full scale prep run.

1. Equilibrate an analytical C_{18} column with HPLC grade water.

2. Pump the recovered chiral column eluant containing the enantiomer through the column until the compound breaks through. Adjust pH of mobile phase to increase hydrophobicity and convert all cations and anions to neutral salt. The principle is to suppress the ionization of the analyte.

3. Wash column with water to remove any buffer present and elute with an appropriate organic solvent. Methanol is often the best choice but ethanol or acetonitrile can be used. Measure the volume of collected solvent and assay for recovered analyte.

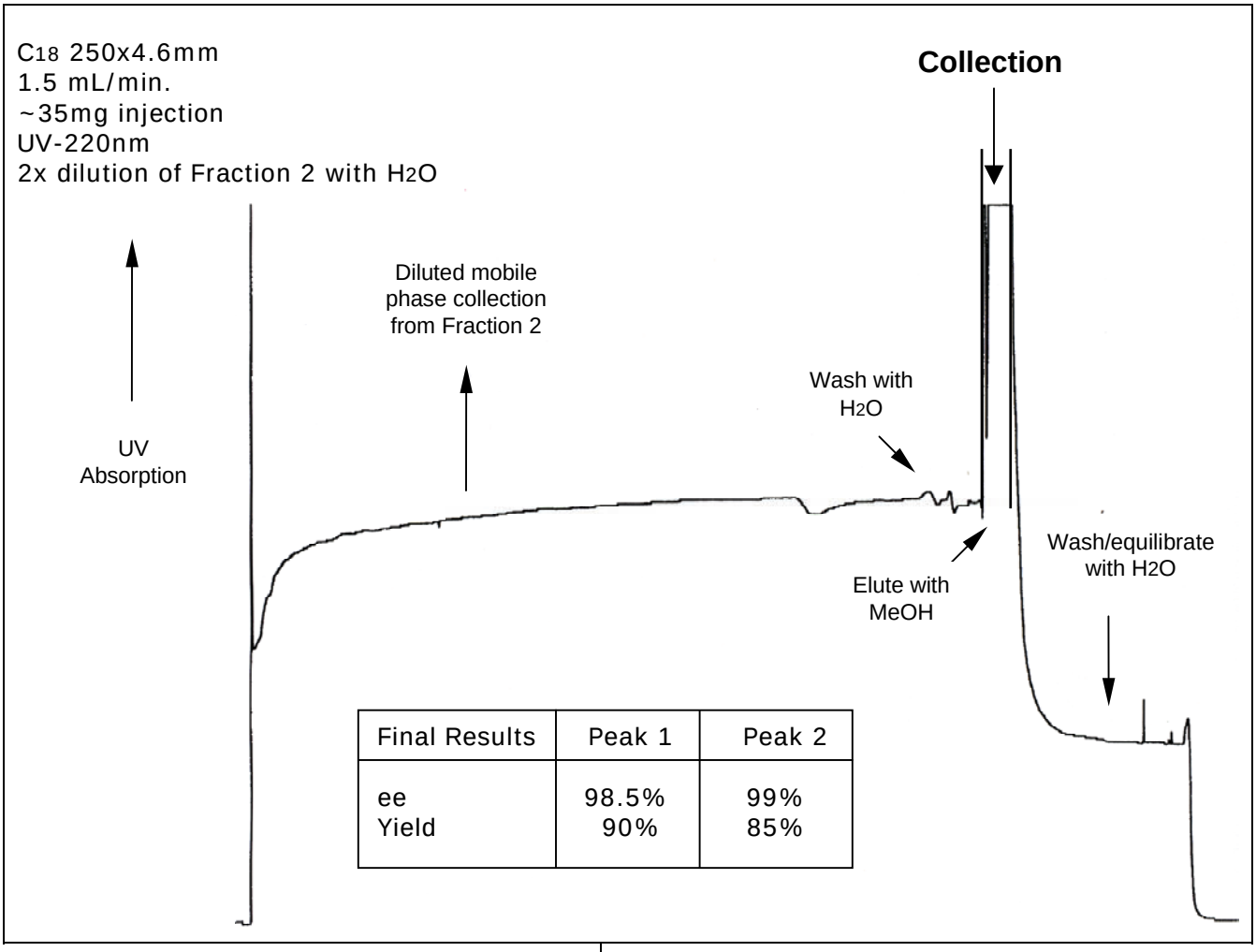
4. If recovered amount falls below anticipated capacity it is always possible to further dilute the eluant that is being charged with water. In addition, larger size C_{18} columns can be used.

5. After elution of the compound of interest the column is equilibrated with water for the next addition.

6. From analytical runs it is possible to calculate the size of the column required for a larger scale run. Typically, doubling the column diameter is equivalent to four times the volume.

Product Recovery: Case Study Warfarin in the Reversed Phase Mode

This C_{18} recovery method makes for efficient use of the reversed phase mode with CHIROBIOTIC phases. Also note speed of recovery of the separated enantiomers from a C_{18} is faster than evaporation of an equivalent volume of heptane.



Final Results	Peak 1	Peak 2
ee	98.5%	99%
Yield	90%	85%

8.3 Optimization Studies

1. Prime consideration in dealing with preparative applications is the balance between selectivity and sample solubility.

2. Check the CHIROBIOTIC T2 if the polar organic or polar ionic modes have been chosen. This CSP may offer increased resolution and increased capacity primarily in these modes. For neutral molecules, the polar organic mode may work best eliminating the need for acid/base or volatile salt.

3. When operating in the polar ionic mode check carefully the choice of volatile salt as it may effect selectivity dramatically. Ammonium trifluoroacetate favors basic compounds while ammonium acetate favors acidic compounds.

Specific Examples of CSP Capacity

Compound	Loading Capacity *	Column
Dehydroproline	10.0	CHIROBIOTIC T
Phenylalanine	10.0	CHIROBIOTIC T
Isoleucine	24.0	CHIROBIOTIC TAG

**mg racemate per gram CSP/hour*

The preceding examples were chosen as simple demonstrations of some of the points covered. They do not represent the best or the worst examples of what is currently a broad base of experience.

8.4 Conclusions for Preparative LC

1. The 10µm and 16µm CHIROBIOTIC phases show excellent scalability directly from analytical to 2" (5cm) ID column.

2. The retention times and resolution data were nearly identical to published application notes.

3. The CHIROBIOTIC phases can be operated sequentially in normal, reversed phase, polar ionic and polar organic modes with no deleterious effects on the stationary phase.

4. CHIROBIOTIC columns up to 2″ ID have stable beds at linear velocities up to 400 mL/min.

5. CHIROBIOTIC phases have demonstrated no solvent limitations.

6. CHIROBIOTIC phases have demonstrated capacities from 2 to 24 grams/hour/kilo CSP.

7. LC-MS methods are available to identify ligand bleed. Current robustness testing shows no bleed >0.5 ng/mL.

9.0 Peptide Separations

Selective separations of peptides with sequence deletions, single chiral or achiral amino acid differences and/or epimeric centers is possible using CHIROBIOTIC LC stationary phases. Peptide separations are important in many areas such as protein sequencing, synthesis of new peptide drugs and pharmacological studies.

Very similar peptides (differing by only one or two single amino acids or the chirality of an amino acid) can be challenging for traditional LC modes such as ion-exchange, ion pairing and reversed phase. In addition, mobile phases used in traditional modes may not be ESI-MS compatible.

The CHIROBIOTIC phases have demonstrated the ability to separate closely related peptides with ESI-MS compatible mobile phases.

Some Example Peptides Separated

Bradykinins	Neurotensins	Leucokinins
PPGFSFR	pELYENKKPRRP-***dW***-IL	DP***A***F***S***SWG-NH_2
PPGFSPFR	pELYENKPRRP-***dY***-IL	DP***G***F***S***SWG-NH_2
RPGFSPFR	pELYENKPRRP***Y***IL	DP***A***F***N***SWG-NH_2
RPRGFSPFR	pELYENKPRRP***F***IL	***β-Casomorphins***
Lutenizing Hormone Releasing Factor	pELY***Q***NKPRRP***Y***IL	Y-dA-F-***dA***-Y-NH_2
PE-***dF***-WSY-***dA***-LRPG-NH_2	pELYENK***S***RRP***Y***IL	Y-dA-F-***dP***-Y-NH_2
pEHWSY-***dA***-LRPG-NH_2	***Vasopressins***	Y-dA-F-***Hyp***-Y-NH_2
pEHWSY-***dK***-LRPG-NH_2	CYFQNCP***K***G-NH_2	
HWSY***G***LRPG-NH_2	CYPQACP***R***G-NH_2	

See reference C-14 for decoding into 3 letter amino acid designation.

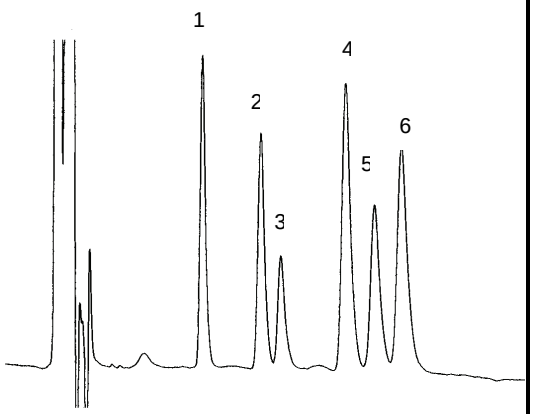
9.1 Single Amino Acid Differences, Chiral or Achiral in Peptide Sequences

9.1.1 Enkephalins

Peak 1: 17.06 min. Tyr-D-Ala-Gly-Phe-D-leu
Peak 2: 21.96 min. Tyr-D-Ala-Gly-Phe-Met
Peak 3: 23.59 min. Tyr-D-Ala-Gly-Phe-Leu
Peak 4: 29.10 min. Tyr-Gly-Gly-Phe-Met
Peak 5: 31.47 min. Tyr-Gly-Gly-Phe-Leu
Peak 6: 33.74 min. Tyr-Ala-Gly-Phe-Leu

Single Amino Acid Analogues:
Peak 2 and 3
Peak 4 and 5
Peak 5 and 6

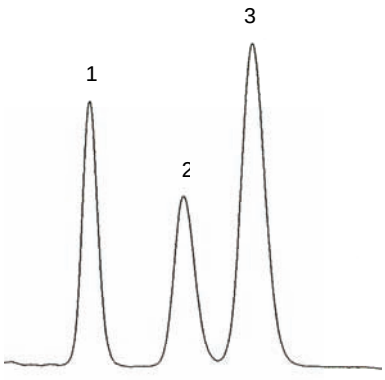
Chiral Amino Acid Analogues:
Peak 1 and 3
Peak 3 and 6



CHIROBIOTIC T2, 250x4.6mm
75/25: ACN/5mM NH_4HCO_2, pH 3.3
0.5 mL/min.
25°C
UV-220nm

9.1.2 Neurotensin

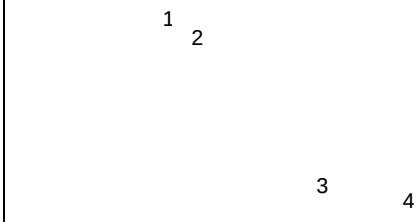
Peak 1: 20.63 min. pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-**Tyr**-Ile-Leu
Peak 2: 26.86 min. pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-**Phe**-Ile-Leu
Peak 3: 31.41 min. pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-**D-Trp**-Ile-Leu



CHIROBIOTIC T2, 250x4.6mm
25/75: ACN/0.1% H_2CO_2
1.0 mL/min.
35°C
220nm UV

9.1.3 Luteinising hormone-releasing hormone, LHRH

Peak 1: 4.29 min. pGlu-**D-Phe**-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-Gly-NH2 Peak 2: 5.38 min. pGlu-**His**-Trp-Ser-Tyr-**D-Ala**-Leu-Arg-Pro-Gly-NH2 Peak 3: 9.60 min. pGlu-His-Trp-Ser-Tyr-**D-Lys**-Leu-Arg-Pro-Gly-NH2 Peak 4: 12.36 min. His-Trp-Ser-Tyr-**Gly**-Leu-Arg-Pro-Gly-NH2

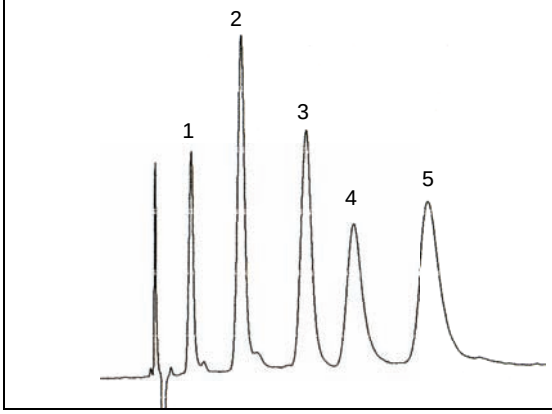


CONDITIONS:

Column:	CHIROBIOTIC T2, 250x4.6mm
Mobile Phase:	40/60: ACN/0.1% H_2CO_2
Flow Rate:	1.0 mL/min.
Temperature:	35°C
Detection:	220nm UV

9.1.4 Angiotensin II

Peak 1: 5.53 min. Glu-Gly-Val-Tyr-Val-His-Pro-Val Peak 2: 8.00 min. Asp-Arg-Val-Tyr-Val-His-Pro-Phe Peak 3: 11.24 min. Sar-Arg-Val-Tyr-Ile-His-Pro-Thr Peak 4: 13.63 min. Asp-Arg-Val-Tyr-Ile-His-Pro-Phe Peak 5: 17.35 min. Sar-Arg-Val-Tyr-Ile-His-Pro-Phe



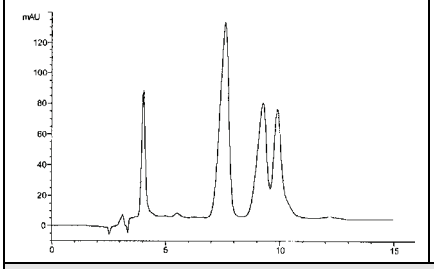
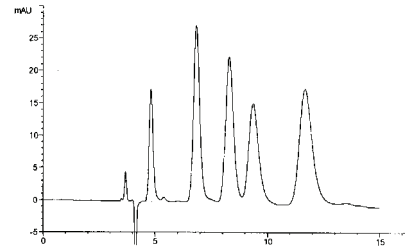
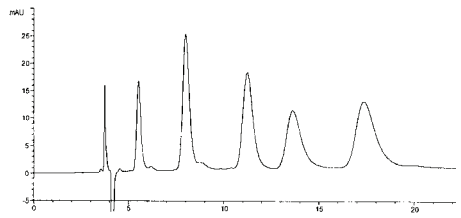
CONDITIONS:

Column:	CHIROBIOTIC T2, 250x4.6mm
Mobile Phase:	20/80: ACN/0.1% H_2CO_2
Flow Rate:	0.8 mL/min.
Temperature:	35°C
Detection:	220nm UV

9.2 Method Development Protocol for Peptides

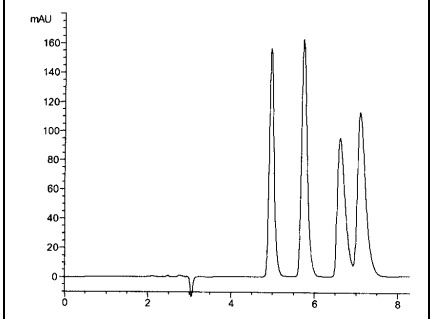
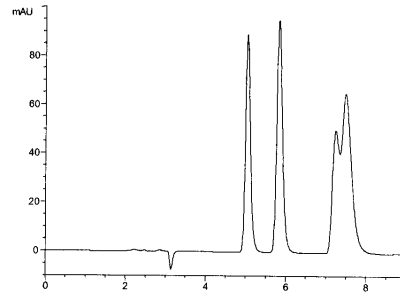
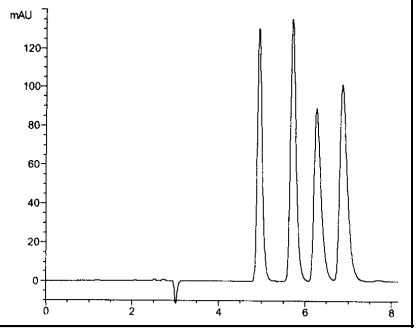
Peptide Type	Di/Tri-Peptides	Bi-Functional	Neutral
Starting mobile phase: 50% ACN with:	5mM NH_4OAc, pH 4.1	0.1% H_2CO_2	5mM NH_4 Formate
Optimization: Organic	% ACN or combination of ACN and MeOH	% ACN (U-shape effect)	% ACN (U-shape effect)
pH	2.8-6.8		2.8-6.2
Buffer	2-50mM	0.01-0.5%	2-50mM
Temperature	4-45°C	4-45°C	4-45°C

9.3 Solvent Effects

50/50	25/75	Best Result - 20/80
		

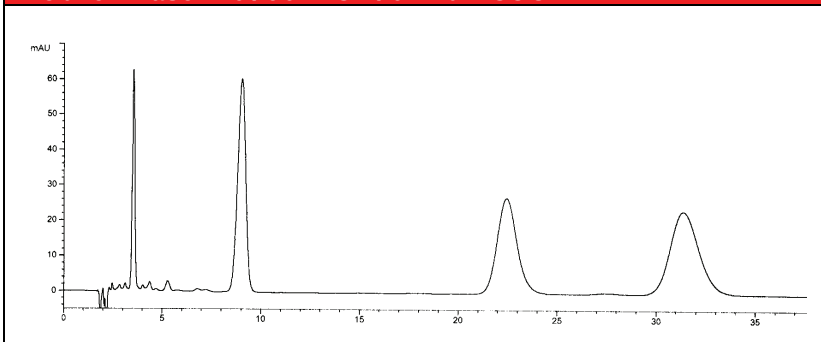
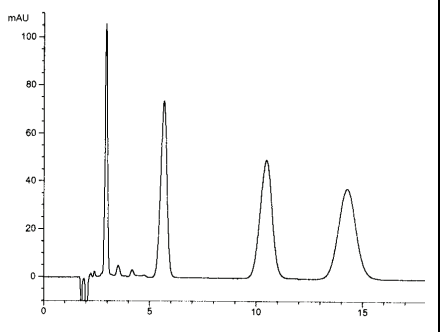
Conditions: CHIROBIOTIC V2, 250x4.6mm, Mobile phase – ACN/0.1% H_2CO_2 @ 0.8 mL/min. Temperature: 35°C, Detection – UV 220nm. Sample is Angiotensin II.

9.4 Temperature Effects

25°C	15°C	Best Result 35°C
		

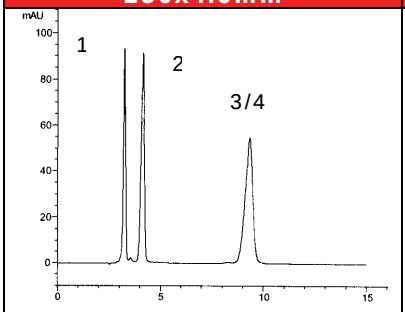
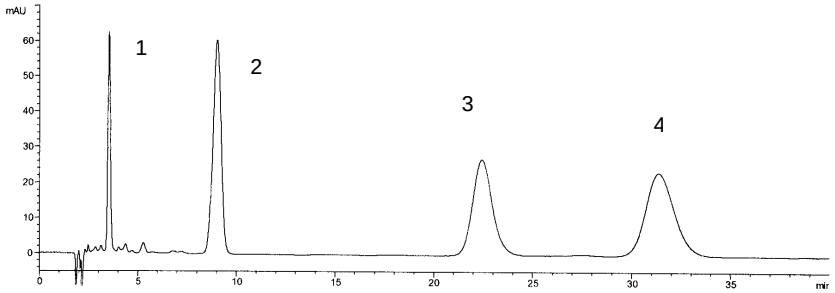
Conditions: CHIROBIOTIC T, 250x4.6mm, Mobile phase – 50/50: ACN/10mM NH_4OAc, pH 4.1 @ 1.0 mL/min. Detection – UV 220nm. Sample is DL-Ala-DL-Val.

9.5 Buffer Concentration Effects

Mobile Phase: 40/60: ACN/0.1% HCOOH	40/60: ACN/0.2% HCOOH
	

Conditions: CHIROBIOTIC TAG, 250x4.6mm, Flow Rate – 1.5 mL/min. Temperature @ 35°C, Detection – UV 220nm. Sample is Bradykinin.

9.6 Complementary Effects T2 vs TAG

CHIROBIOTIC T2 250x4.6mm	CHIROBIOTIC TAG 250x4.6mm
	

Conditions: Mobile phase – 40/60: ACN/0.1% H_2CO_2 @ 1.5 mL/min., Temperature @ 35°C, Detection UV-220nm. Sample – Bradykinin.

9.7 Summary of observations on the separation of peptides on CHIROBIOTIC Phases

1. Using ACN/buffer as the mobile phase, the retention of peptides were usually found to be the shortest when the % ACN was in the range of 40-60%.

2. Higher buffer concentrations were found to correlate with lower retentions.

3. Some elution order changes were observed between CHIROBIOTIC T and T2.

4. Mobile phases are mostly LC/MS compatible in either APCI or ESI mode.

5. The methods reported allow for conformational validation by altering temperature, buffer concentration and the stationary phase in the same mobile phase (complementary effect) to alter elution order. Therefore, an orthogonal platform can be developed, if necessary.

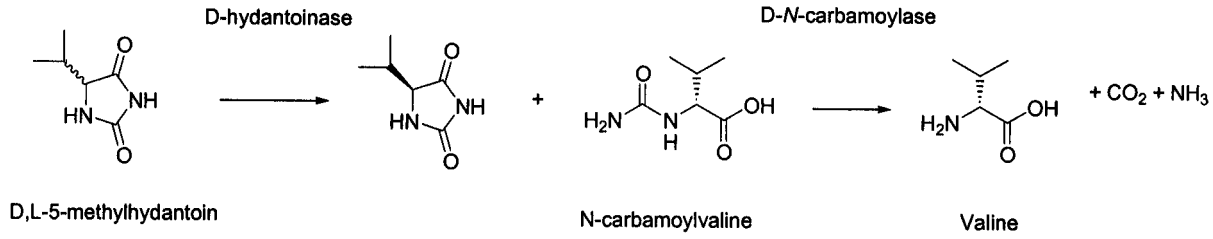
6. Peptide families with up to 13 amino acid units so far have been successfully separated for sequence variations/deletions as well as chiral analogues.

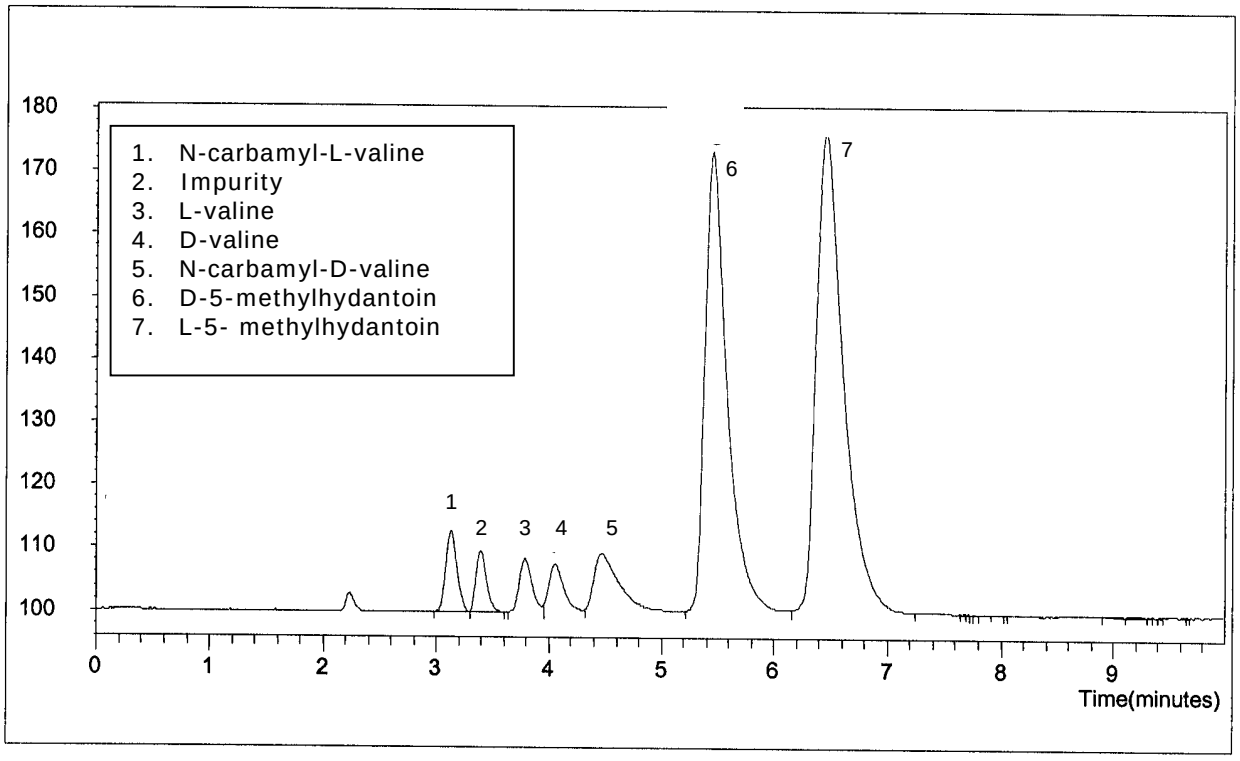
7. The peptide containing the D-amino acid elutes before the L-amino acid peptide.

10.0 Biocatalysis

CHIROBIOTIC chiral stationary phases are very useful for monitoring biocatalytic conversions because no sample preparation is necessary. Aqueous reactions can be injected directly and CHIROBIOTIC T is very sensitive to structural differences allowing for monitoring of all steps in the reaction process.

Example
Monitoring a Multi-stage Biocatalytic Process by Direct Injection and Separation of All Intermediates and Final Product





Conditions: Column - CHIROBIOTIC T, 250x4.6mm, 5µm Mobile Phase - 80/20: 15mM NH_4OAc, pH 4.1/MeOH @ 1.0 mL/min.

Courtesy of Dr. Lucien Duchateau, DSM Fine Chemicals, Galeen, The Netherlands.

11.0 Tables of Published Separation Data for Amino Acids

11.1 Underivatized Aromatic Amino Acids – Reference C-51 (CHIROBIOTIC Bibliography)

R—CH(NH$_2$)—COOH	CHIROBIOTIC T			
α-Amino Acid	**K$_1$ (L)**	**K$_2$ (L)**	**Rs**	**Mobile Phase**
Phenylalanine	2.23	4.42	4.40	A
	1.48	3.07	5.60	B
	1.12	2.90	4.70	C
α-Methylphenylalanine	1.62	1.94	1.30	A
	1.33	1.51	0.8	B
	0.93	1.03	0.60	C
Tyrosine	2.01	3.99	4.20	A
	1.29	2.68	5.10	B
	0.97	1.84	4.20	C
α-Methyltyrosine	1.48	1.65	0.60	A
	1.18	1.30	0.60	B
	0.85	0.85	-	C

A) 100/0.005/0.005 (v/v/v): Methanol/HOAc/TEA
B) 80/20 (v/v): Ethanol/water
C) 90/10 (v/v): Methanol/water

11.2 Derivatized Amino Acids – Reference C-108 (CHIROBIOTIC Bibliography)

R—CH(NH$_2$)—COOH		CHIROBIOTIC T (250x4.6mm, 5μm)				
α-Amino Acid	**R-Moiety**	**Derivative**	**K$_1$**	**α**	**Rs**	**Mobile Phase**
Alanine	-CH$_3$	3,5-DNB	3.11	1.77	3.53	B
		3NB	6.49	1.46	2.34	A
		4NB	6.57	1.14	1.13	A
		4BrB	4.29	1.11	0.95	C
		3ClB	4.41	1.25	2.09	A
		4ClB	7.40	1.09	0.89	A
		4FB	3.64	1.15	0.93	C
Leucine	-CH$_2$-CH(CH$_3$)$_2$	3,5-DNB	2.48	1.96	2.83	D
		3,5-DNB	1.82	2.15	3.05	C
		3NB	4.39	1.43	2.90	A
		4NB	5.13	1.20	2.51	A
		4BrB	4.74	1.24	1.74	A
		3ClB	4.41	1.25	2.09	A
Norleucine	-CH$_2$-CH$_2$-CH$_2$-CH$_3$	3,5-DNB	1.86	2.15	3.11	C
		3NB	4.00	1.45	2.23	A
		4NB	3.87	1.24	1.63	A
		3ClB	4.04	1.24	1.76	A
tert-Leucine	-C-(CH$_3$)$_3$	3,5-DNB	0.99	2.64	1.90	C
		3NB	2.83	1.17	1.09	A

		4NB	2.79	1.11	0.91	A

α-Amino Acid	R-Moiety	Derivative	K_1	α	Rs	Mobile Phase
Methionine	$-CH_2-CH_2-S-CH_3$	3,5-DNB	2.35	2.27	3.17	C
		3,5-DNB	1.77	2.31	2.52	D
		3NB	4.47	1.42	2.69	A
		4NB	4.17	1.21	1.49	A
		4BrB	4.64	1.20	1.19	A
		3CIB	4.21	1.21	1.73	A
Phenylalanine		3,5-DNB	2.44	1.66	2.64	B
		3NB	4.33	1.28	1.92	A
		4NB	4.09	1.18	1.75	A
		3CIB	4.37	1.16	1.14	A
		4BrB	4.69	1.16	1.06	A
		4FB	2.85	1.11	0.90	C
Serine	$-CH_2OH$	3,5-DNB	3.05	1.42	1.70	C
		3NB	4.89	1.27	1.64	C
		4NB	9.03	1.08	0.68	A
		4NB	4.87	1.27	1.64	C
		3CIB	8.40	1.15	0.90	A
		4FB	4.22	1.12	0.82	A
Homoserine	$-CH_2CH_2OH$	3,5-DNB	4.03	1.60	2.29	C
		3NB	5.56	1.31	1.91	C
		4NB	5.56	1.17	1.25	C
Threonine	$-CHOH-CH_3$	3,5-DNB	2.17	1.76	2.49	C
		3NB	5.30	1.22	1.28	A
		3NB	2.90	1.24	1.26	C
		4NB	4.59	1.13	0.91	A
		4BrB	5.49	1.08	0.65	A
Tyrosine		3,5-DNB	5.15	1.44	1.55	C
		3NB	6.76	1.20	1.28	C
		4NB	6.71	1.17	1.43	C
m-Tyrosine		3,5-DNB	4.45	1.32	1.43	C
		3NB	2.21	1.34	2.00	C
		4NB	6.21	1.24	1.42	C
Tyrptophan		3,5-DNB	3.33	1.36	1.67	C
		3NB	6.76	1.20	1.28	C
		4NB	4.88	1.14	1.03	C
		3CIB	8.84	1.12	1.15	A
5-Methyltryptophan		3,5-DNB	1.41	1.3	2.7	C
		3NB	4.84	1.12	1.07	C
		4NB	4.69	1.11	1.12	C
		3CIB	8.82	1.08	0.88	A
		4CIB	9.34	1.07	0.61	A
		4BrB	9.71	1.06	0.65	A
		4FB	4.62	1.06	0.75	C
Valine	$-CH(CH_3)_2$	3,5-DNB	1.65	1.9	2.24	C
		3NB	4.05	1.22	1.75	A
		4NB	3.89	1.13	1.13	A
		4BrB	4.54	1.08	0.79	A
		3CIB	4.03	1.10	1.07	A
Norvaline	$-(CH_2)_2CH_3$	3,5-DNB	1.98	2.32	2.92	C
		3NB	4.36	1.50	2.63	A
		4NB	2.45	1.32	1.77	C
		3CIB	4.46	1.26	2.20	A
Derivatives:						
3,5-DNB = 3,5-dinitrobenzoyl						
3NB = 3-nitrobenzoyl						
4NB – 4-nitrobenzoyl						
3CIB = 3-chlorobenzoyl						
4CIB = 4-chlorobenzoyl						
4BrB = 4-bromobenzoyl						
4FB = 4-fluorobenzoyl						
Mobile Phases						
A) acetonitrile/methanol/gl acetic acid/triethylamine (490:10:1:1, v/v/v/v) 1mL/min						

Other compounds not included in this table (see original paper)

B) acetonitrile/methanol/gl acetic acid/triethylamine (490:10:1:2, v/v/v/v), 1mL/min
C) acetonitrile/methanol/gl acetic acid/triethylamine (480:20:1:2, v/v/v/v) 1mL/min
D) acetonitrile/methanol/gl acetic acid/triethylamine(480:20:1:3, v/v/v/v), 1mL/min
E) acetonitrile/methanol/gl acetic acid/triethylamine (490:10:1:1.5, v/v/v/v), 1mL/min

11.3 Derivatized Amino Acids – Reference P-1, P-2 (P-CAP Bibliography)

R—CH(NH$_2$)—COOH		P-CAP (250x4.6mm, 5µm)					
α-Amino Acid	R-Moiety	Derivative (From Ref 1 unless stated otherwise)	K1	α	Rs	Mobile Phase	Detection
Alanine	-CH$_3$	N-BOC	1.34	1.14	1.46	H	210nm
		N-Z	1.79	1.21	1.64	H	254nm
		N-FMOC	1.06	1.23	2.02	F	254nm
		N-DNB	0.97	1.18	1.25	F	254nm
Leucine	-CH$_2$-CH(CH$_3$)$_2$	N-BOC	1.16	1.33	2.82	H	210nm
		N-Acetyl	0.97	1.28	1.85	F	210nm
Isoleucine	-CH(CH$_3$)-CH$_2$-CH$_3$	N-DNB	0.76	1.40	2.30	F	254nm
Methionine	-CH$_2$-CH$_2$-S-CH$_3$	N-BOC	1.27	1.36	3.09	H	210nm
		N-DNB	0.94	1.30	1.64	F	254nm
Phenylalanine		N-BOC	1.29	1.24	2.53	H	254nm
		N-Z	1.67	1.26	2.43	H	254nm
		N-FMOC	1.04	1.31	2.72	F	254nm
		N-FMOC (Ref 2)	1.85	1.37	3.5	M	254nm
		N-Acetyl	1.02	1.16	1.40	F	254nm
Cysteine	-CH$_2$-SH	Diacetyl (Ref 2)	2.61	1.30	1.5	N	na
Phenylglycine	-C$_6$H$_5$	N-Acetyl	1.59	1.14	0.91	F	254nm
N-BOC = N-tert-butoxycarbonyl							
N-Z = Benzyloxycarbinyl							
N-FMOC = N-Fluorenoxymethylcarbonyl							
N-DNB = N-Dinitrobenzoyl							
Mobile Phases:							
F) acetonitrile/methanol (70:30, v/v) with 20mM ammonium acetate							
H) acetonitrile/methanol (85:15, v/v) with 20mM ammonium acetate							
M) acetonitrile/methanol/trifluroracetic acid (95:5:0.1, v/v/v)							
N) acetonitrile/methanol/ammonium acetate (80:20:0.2, v/v/w)							
Flow rate: 1mL/min.							

11.4 – N-tert-Butoxycarbonyl (t-Boc) Amino Acids – References C-8, C-16, CB-113

The cyclodextrin phase CYCLOBOND I *2000* RSP has been the most widely used stationary phase for the separation of this class of derivatized amino acids. It has been found, however, that the P-CAP, CHIROBIOTIC T and CHIROBIOTIC R complement this phase quite well. The chart below can be used as a guide for the separation of this class of analytes.

Chiral Separation of N-t-BOC Amino Acids

Compound	Mobile Phase	Column	k1[2]	α	Rs
Alanine	20/80: MeOH/0.1%TEAA, pH 4.1 10/90: MeOH/0.1%TEAA, pH 4.1 5/95: ACN/1% TEAA, pH 4.1 70/30/ 20mM ACN/MeOH/NH$_4$OAc	R T RSP (R,R)P-CAP[1]	1.08 0.45 1.30 1.38	1.77 1.55 - 1.06	3.3 2.4 1.8 0.8
Arginine	20/80: ACN/1% TEAA, pH 4.1	T	1.6	-	2
Asparagine	20/80: MeOH/0.1%TEAA, pH 4.1 70/30/20 mM ACN/MeOH/NH$_4$OAc	T (R,R)P-CAP	1.30 2.29	1.36 1.06	2.0 0.8
Glutamine	20/80: MeOH/0.1%TEAA, pH 4.1 10/90: MeOH/0.1%TEAA, pH 4.1 70/30/20 mM ACN/MeOH/NH$_4$OAc	R T (R,R)P-CAP	1.10 0.41 1.78	1.38 1.37 1.13	2.0 1.4 1.2
Histidine	20/80: MeOH/0.1%TEAA, pH 6.0 20/80: MeOH/0.1%TEAA, pH 4.1	R T	0.81 1.53	1.37 1.66	1.8 2.0
Isoleucine	20/80: MeOH/0.1%TEAA, pH 4.1 10/90: ACN/20mM NH4OAc	R RSP	1.67 2.00	1.25 1.54	1.6 1.6

	70/30/20 mM ACN/MeOH/NH_4OAc	(R,R)P-CAP	0.99	1.13	1.2
Leucine	5/95: ACN/1% TEAA, pH 4.1	RSP	1.5	-	4.6

Compound	Mobile Phase	Column	k_1[2]	α	Rs
Methionine	20/80: MeOH/0.1%TEAA, pH 6.0	R	0.34	20.3	12.0
	20/80: MeOH/0.1%TEAA, pH 4.1	T	0.48	3.90	5.5
	10/90: ACN/20mM NH_4OAc	RSP	1.20	4.09	10.0
	70/30/20 mM ACN/MeOH/NH_4OAc	(R,R)P-CAP	1.18	1.63	5.6
Phenylalanine	20/80: MeOH/0.1%TEAA, pH 6.0	R	1.02	3.30	4.8
	10/90: MeOH/0.1%TEAA, pH 6.0	T[3]	0.44	1.55	1.6
	7/93: ACN/1% TEAA, pH 4.1	RSP	3.0	-	2.7
	70/30/20 mM ACN/MeOH/NH_4OAc	(R,R)P-CAP	1.24	1.10	1.1
Phenylglycine	20/80: MeOH/0.1%TEAA, pH 6.0	R	0.15	9.24	5.0
	20/80: MeOH/0.1%TEAA, pH 4.1	T	0.52	4.65	3.5
	10/90: ACN/20mM NH4OAc	RSP	3.59	1.29	1.5
	70/30/20 mM ACN/MeOH/NH_4OAc	(R,R)P-CAP	1.52	1.08	1.0
Serine	20/80: MeOH/0.1%TEAA, pH 4.1	R	0.88	1.30	2.4
	70/30/20 mM ACN/MeOH/NH_4OAc	(R,R)P-CAP	1.95	1.09	1.0
Tryptophan	20/80: MeOH/0.1%TEAA, pH 6.0	R	0.61 (D)	3.89	5.4
	20/80: MeOH/0.1%TEAA, pH 4.1	T	0.73 (D)	2.17	2.2
	10/90: ACN/20mM NH4OAc	RSP	1.46 (D)	2.96	5.7
	70/30/20 mM ACN/MeOH/NH_4OAc	(R,R)P-CAP	1.33 (D)	1.50	2.1
p-Tyrosine	20/80: MeOH/0.1%TEAA, pH 6.0	R	0.79	4.31	5.5
	10/90: MeOH/0.1%TEAA, pH 6.0	T[3]	0.24	1.77	1.4
	7/93: ACN/1% TEAA, pH 4.1	RSP	2.1	-	3.0
	70/30/20 mM ACN/MeOH/NH_4OAc	(R,R)P-CAP	1.69	1.09	1.0
Valine	20/80: MeOH/0.1%TEAA, pH 4.1	R	1.44	1.26	2.0
	10/90: ACN/20mM NH4OAc	RSP	1.64	1.45	1.6
	70/30/20 mM ACN/MeOH/NH_4OAc	(R,R)P-CAP	1.03	1.11	1.1

Legend: TEAA = Triethylammonium acetate;
NH_4OAc = Ammonium acetate
T = CHIROBIOTIC T
R = CHIROBIOTIC R
RSP = CYCLOBOND I *2000* RSP

[1]P-CAP separations were run at Astec laboratories, non-optimized with the screening mobile phase. All elution orders on R,R-PCAP can be reversed by substituting the S,S-PCAP column.

[2]For all compounds tested, L-form eluted first except for tryptophan.

[3]Flow rate = 1mL/min; *0.5mL/min

UV = 220 nm

Other t-Boc Amino Acids on CHIROBIOTIC R, 250x4.6 mm

Compound	k_1	Rs	Mobile Phase
N-(*tert*-Butoxycarbonyl)-O-benzyl-serine	1.26(L)	0.8	20/80: MeOH/0.1% TEAA, pH 7.0
N-(*tert*-Butoxycarbonyl)-4-chloro-phenylalanine	9.28(L)	3.89	20/80: MeOH/0.1% TEAA, pH 4.1
N-(*tert*-Butoxycarbonyl)-3-(2-naphthy)alanine	11.79(L)	3.05	10/80: MeOH/0.1% TEAA, pH 4.1
N-(*tert*-Butoxycarbonyl)-4-nitro-phenylalanine	2.94(L) 9.17(L)	1.68 1.79	20/80: MeOH/0.1% TEAA, pH 7.0 10/80: MeOH/0.1% TEAA, pH 4.1
N-(*tert*-Butoxycarbonyl)-Nω-p-tosyl-arginine	2.33 7.23	0.8 0.9	20/80: MeOH/0.1% TEAA, pH 4.1 10/80: MeOH/0.1% TEAA, pH 7.0

11.5 Chiral Separation of N-FMOC Amino Acids, References – Validated at Astec Laboratories

Compound	Mobile Phase	Column	k1	α	Rs
Alanine	50/50, MeOH/20mM NH_4OAc	R	0.38	3.89	3.5
	40/60, MeOH/1% TEAA, pH 4.1	T	1.26	2.27	5.5
	100/0.02w%, MeOH/NH_4OAc	R	0.57	2.37	2.2
Arginine	20/80, MeOH/0.1%TEAA, pH 6.8	R	3.28	1.46	1.6
	100/0.1w%, MeOH/NH_4TFA	R	1.69	2.95	4.6
Asparagine	100/1/1, MeOH/HOAc/TEA	T			1.7
	40/60, MeOH/1% TEAA, pH 4.1	T	0.63	1.81	3.0
	100/0.1w%, MeOH/NH_4TFA	R	4.41	1.22	1.3
	30/70, MeOH/20mM NH_4OAc	R	1.55	1.49	1.8
Aspartic acid	20/80, MeOH//0.1%TEAA, pH 6.8	R			2.0
	40/60, MeOH/0.1% TEAA, pH 4.1	T	0.46	1.68	1.8
	100/0.1w%, MeOH/NH_4TFA	R	2.59	1.23	1.3
Citrulline	40/60,MeOH/0.1% TEAA, pH 4.1	T	1.07	2.50	4.0
	100/1/1, MeOH/HOAc/TEA	T			3.0
	30/70, MeOH/20mM NH_4OAc	R	1.34	2.05	2.6
Cysteine	65/35, EtOH/Hexane	R			1.7
Glutamic acid	20/80, MeOH/0.1%TEAA, pH 6.8	R			1.6
	100/1/1, MeOH/HOAc/TEA	T			1.3
	40/60, MeOH/0.1% TEAA, pH 4.1	T	1.07	1.60	3.8
Glutamine	40/60, MeOH/0.1% TEAA, pH 4.1	T	0.61	2.85	5.0
	100/0.1w%, MeOH/NH_4OAc	R	1.90	2.04	3.8
	30/70, MeOH/20mM NH_4OAc	R	0.93	2.46	3.6
Histidine	20/80, MeOH/0.1%TEAA, pH 4.1	R			1.0
Isoleucine	40/60, MeOH/0.1% TEAA, pH 4.1	T	1.08	1.78	2.2
	100/0.1w%, MeOH/NH_4OAc	R	0.45	1.87	2.3
	30/70, MeOH/20mM NH_4OAc	R	2.32	1.85	1.6
Isoleucine,allo	100/0.1w%, MeOH/NH_4OAc	R	0.53	1.57	2.0
Isoserine	65/35, EtOH/Hexane	R			1.5
	50/50, MeOH/1% TEAA, pH 5.5	T			4.5
Leucine	40/60, MeOH/0.1% TEAA, pH 4.1	T	1.03	2.45	5.0
	100/0.1w%, MeOH/NH_4TFA	R	0.46	2.41	3.5
Lysine	50/50, MeOH/1% TEAA, pH 5.5	T			1.4
	100/0.1w%, MeOH/NH_4TFA	R	0.79	2.12	3.4
Methionine	40/60, MeOH/0.1% TEAA, pH 4.1	T	0.96	3.43	6.0
	100/1/1, MeOH/HOAc/TEA	T			3.0
	100/0.1w%, MeOH/NH_4TFA	R	0.94	2.70	4.5
	50/50, MeOH/20mM NH_4OAc	R	0.27	5.77	5.4
Norleucine	40/60, MeOH/0.1%TEAA, pH 4.1	T	1.20	2.87	6.5
	100/0.1w%,MeOH/NH_4TFA	R	0.50	2.0	3.0
	30/70, MeOH,20mM NH_4OAc	R	2.92	2.15	3.0
Norvaline	100/0.1w%, MeOH/NH_4TFA	R	0.61	2.56	3.5
	30/70, MeOH/20mM NH_4OAc	R	2.12	3.56	6.5
	40/60, MeOH/0.1% TEAA, pH 4.1	T	0.99	3.19	5.5
Ornithine	50/50, MeOH/1% TEAA, pH 5.5	T			1.4
	100/0.1w%, MeOH/NH_4TFA	R	1.22	1.72	3.0
Phenylalanine	100/1/1, MeOH/HOAc/TEA	T			3.0
	40/60, MeOH/0.1% TEAA, pH 4.1	T	1.54	2.82	6.0
	100/0.02w%, MeOH/NH_4OAc	R	0.45	6.65	5.0
	50/50, MeOH/20mM NH_4OAc	R	0.12	8.53	6.0
Proline	100/0.02/0.01, MeOH/HOAc/TEA	R			1.0
	95/5/0.3/0.2, ACN/MeOH/AA/TEA	CBII			1.4
	100/0.4/0.6, ACN/HOAc/TEA	RN			4.1

Compound	Mobile Phase	Column	k_1	α	Rs
Serine	20/80, MeOH/0.1%TEAA, pH 6.8	R			1.6
	100/1/1.5, MeOH/HOAc/TEA	T			1.4
	20/80, MeOH/0.1% TEAA, pH 4.1	T	2.28	1.61	3.3
	30/70, MeOH/20mM NH_4OAc	R	1.07	1.80	2.5
Threonine	30/70, MeOH/0.1%TEAA, pH 4.1	T	1.56	1.35	1.7
	30/70, MeOH/20mM NH_4OAc	R	0.93	1.44	1.8
Tryptophan	65/35, EtOH/Hexane	R			1.1
	40/60, MeOH/0.1% TEAA, pH 4.1	T	2.04	1.88	3.7
	100/0.1w%, MeOH/NH_4TFA	R	1.21	6.16	5.8
	100/0.02w%,MeOH/NH_4OAc	R	0.34	9.30	5.3
p-Tyrosine	100/0.02w%,MeOH/NH_4OAc	R	0.44	6.76	5.0
Valine	40/60, MeOH/0.1% TEAA, pH 4.1	T	0.90	1.90	4.0
	30/70, MeOH/20mM NH_4Oac	R	2.01	2.68	4.0
	100/0.1w%, MeOH/NH_4TFA	R	0.70	1.76	2.5

Legend: TEAA = Triethylammonium acetate
T = CHIROBIOTIC T
R = CHIROBIOTIC R
RN = CYCLOBOND I *2000* RN (R-naphtylethyl carbamate) (See CYCLOBOND HANDBOOK).
CBII = CYCLOBOND II (gamma-cyclodextrin) (See CYCLOBOND HANDBOOK).

12.0 Bibliography

12.1 Relevant References for Amino Acid and Peptide Separations on CHIROBIOTIC Phases (C-)

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CHIROBIOTIC HPLC COLUMNS

Microbore CHIROBIOTIC Columns – 5 µm

Phase Type	Microbore Guard Column 2cmx1.0mm*	100x2.1mm	150x2.1mm	250x2.1mm
CHIROBIOTIC T	12101	12018	12019	12020
CHIROBIOTIC T2	16101	16018	16019	16020
CHIROBIOTIC TAG	14101	14018	14019	14020
CHIROBIOTIC R	13101	13018	13019	13020

*No guard column holder required for microbore guard columns.

Analytical CHIROBIOTIC Columns – 5 µm

Phase Type	Guard Cartridge* 2cmx4.0mm	50x4.6mm	100x4.6mm	150x4.6mm	250x4.6mm	500x4.6mm
CHIROBIOTIC T	12100	12021	12022	12023	12024	12026
CHIROBIOTIC T2	16100	16021	16022	16023	16024	16026
CHIROBIOTIC TAG	14100	14021	14022	14023	14024	14026
CHIROBIOTIC R	13100	13021	13022	13023	13024	13026
Guard Column Holder	21150					

Semi-preparative CHIROBIOTIC Columns - 5µm

Phase Type	250x10mm	500x10mm	250x21.2mm	500x21.2mm	250x30.0mm
CHIROBIOTIC T	12034	12036	12044	12046	12054
CHIROBIOTIC T2	16034	16036	16044	16046	16054
CHIROBIOTIC TAG	14034	14036	14044	14046	14054
CHIROBIOTIC R	13034	13036	13044	13046	13054

Availability - CHIROBIOTIC T, T2, TAG and R™ HPLC Columns - 10µm "Scout Columns" for Loading Studies for Preparative Separations

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Catalog No. 10200
The kit contains:
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1 - CHIROBIOTIC T HPLC Column, 100x4.6mm
1 - CHIROBIOTIC TAG HPLC Column, 100x4.6mm
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The CHIROBIOTIC T, T2, TAG and R are also available in 2" and 4" prepacked columns and bulk 10µm and 16µm media for preparative separations. We also offer contract services for the preparation of purified enantiomers in quantities up to 1 kilogram. Please contact our Sales Department for specific information.

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Microbore CYCLOBOND I *2000* Columns - 5µm

Phase Type	Microbore Guard Column 2cmx1.0mm	100x2.1mm	150x2.1mm	250x2.1mm
CYCLOBOND I *2000*	21010	20018	20019	20020
CYCLOBOND I *2000* RSP	21013	20318	20319	20320
CYCLOBOND I *2000* HP-RSP	24101	24018	24019	24020
CYCLOBOND I *2000* SN	21015	20518	20519	20520
CYCLOBOND I *2000* RN	21016	20618	20619	20620

Note: Microbore guard columns do not require a holder.

Analytical CYCLOBOND Columns – 5 µm

Phase Type	Guard Cartridge* 2cmx4.0mm	50x4.6mm	100x4.6mm	150x4.6mm	250x4.6mm	500x4.6mm
CYCLOBOND I *2000*	21100	20021	20022	20023	20024	20026
CYCLOBOND I *2000* RSP	21103	20321	20322	20323	20324	20326
CYCLOBOND I *2000* HP-RSP	24100	24021	24022	24023	24024	24026
CYCLOBOND I *2000* SN	21105	20521	20522	20523	20524	20526
CYCLOBOND I *2000* RN	21106	20621	20622	20623	20624	20626
Guard Column Holder	21150					

Semi-preparative CYCLOBOND I *2000* Columns - 5µm

Phase Type	250x10mm	500x10mm	250x21.2mm	500x21.2mm	250x30.0mm
CYCLOBOND I *2000*	20034	20036	20044	20046	20054
CYCLOBOND I *2000* RSP	20334	20336	20344	20346	20354
CYCLOBOND I *2000* HP-RSP	24034	24036	24044	24046	24054
CYCLOBOND I *2000* SN	20534	20536	20544	20546	20554
CYCLOBOND I *2000* RN	20634	20636	20644	20646	20654

CYCLOBOND I *2000* HPLC COLUMNS - 10µm "Scout Columns" for Loading Studies for Preparative Separations

Phase Type	250x4.6mm
CYCLOBOND I *2000*	22024
CYCLOBOND I *2000* RSP	22324
CYCLOBOND I *2000* HP-RSP	24124
CYCLOBOND I *2000* SN	22524
CYCLOBOND I *2000* RN	22624

Microbore CYCLOBOND II Columns - 5µm

Phase Type	Microbore Guard Column 2cmx1.0mm	100x2.1mm	150x2.1mm	250x2.1mm
CYCLOBOND II	41001	46018	46019	41021

Analytical CYCLOBOND II Columns- 5µm

Phase Type	Guard Cartridge* 2cmx4.0mm	50x4.6mm	100x4.6mm	150x4.6mm	250x4.6mm	500x4.6mm
CYCLOBOND II	42120	46021	40020	46023	41020	40023
Guard Column Holder	21150					

Semi-preparative CYCLOBOND II Columns - 5µm

Phase Type	250x10mm	500x10mm	250x21.2mm	500x21.2mm
CYCLOBOND II	40025	40026	40028	41420

CYCLOBOND II - 10µm "Scout Columns" for Loading Studies for Preparative Separations

Phase Type	250x4.6mm
CYCLOBOND II (10µm)	44024

CYCLOBOND Preparative Columns and Media

CYCLOBOND phases are also available in 2" and 4" prepacked columns and bulk 10µm and 16µm media for preparative separations. We also offer contract services for the preparation of purified enantiomers in quantities up to 1 kilogram. Please contact our Sales Department for specific information.

P-CAP HPLC COLUMNS

Microbore P-CAP Columns – 5 µm

Phase Type	Particle Size	Microbore Guard Column 2cmx1.0mm	150x2.1mm	250x2.1mm
(R,R) P-CAP	5µm	31101	31019	31020
(S,S) P-CAP	5µm	33101	33019	33020

Analytical P-CAP Columns – 3µm and 5µm

Phase Type	Particle Size	Guard Cartridge* 2cmx4.0mm	150x4.6mm	250x4.6mm
(R,R) P-CAP	3µm	31100	30023	
(R,R) P-CAP	5µm	31100	31023	31024
(S,S) P-CAP	3µm	33100	32023	
(S,S) P-CAP	5µm	33100	33023	33024
Guard Column Holder		21150		

Semi-preparative P-CAP Columns - 5µm

Phase Type	Particle Size	250x10mm	250x21.2mm
(R,R) P-CAP	5µm	31034	31044
(S,S) P-CAP	5µm	33034	33044

Availability – P-CAP - 10µm "Scout Columns" for Loading Studies for Preparative Separations

Phase Type	Particle Size	250x4.6mm
(R,R) P-CAP	10µm	31124
(S,S) P-CAP	10µm	33124

P-CAP Preparative Columns and Media

P-CAP phases are also available in 2" and 4" prepacked columns and bulk 10µm and 16µm media for preparative separations. We also offer contract services for the preparation of purified enantiomers in quantities up to 1 kilogram. Please contact our Sales Department for specific information.

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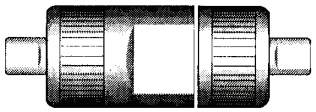
Guard Cartridge Holder



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