Advances in Chiral Resolution of Amino Acids by LC and LC/MS

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Abstract

Proteomics and the analysis of amino acids for human health coupled with the utility of amino acids in biotechnology and other pharmaceuticals has made the chiral analysis of amino acids imperative. There is a long history in chiral separation of amino acids from the development of the first LC methods for separating various alpha, primary amines by the chiral crown ether design of Cram, to the work of Davenkov and ligand exchange chromatography. The application of macrocyclic glycopeptides since 1995 however, has opened the doors to an array of applications for all forms of amino acids including primary, secondary as well as beta and gamma amino acids. This technique has also allowed for the resolution of a wide variety of N-blocked amino acids. This presentation will cover the history of chiral stationary phase development in this area and focus primarily on developments with macrocyclic glycopeptides. Very large increases in selectivity have been obtained with the aglycone forms of at least one macrocyclic in particular, teicoplanin. Comparison of amino acid separations on different versions of these various CSP’s will be presented and the utility of some for preparative application will be outlined. The use of these novel stationary phases has not only expanded the applications possible in this area but has tied the technology to a wide variety of methodologies like SFC and LC/MS.
Amino Acids - the Building Blocks of Peptides, Proteins and Pharmaceutical Targets

Chiral Purity is Essential

Presentation contents:

* Historical Development of CSP’s for Amino Acids Chiral Recognition

* Applications:
  Free alpha, beta and gamma amino acids
  Cyclic and unusual amino acids
  N-blocked amino acids
  LC/MS of amino acids
  Clinical applications for genetic diseases, (LC/MS)
Ligand Exchange

Schematic of the three dimensional complex formed between the bonded amino acid proline, copper (II) and an amino acid.

Key Points for Ligand Exchange

1. First practical approach to the separation of underivatized amino acids.
2. Requires a bidentate ligand. Examples of the type of chiral ligands employed include but are not limited to proline, hydroxyproline, histidine, penicillamine and phenylalanine amide.
3. Requires a transition metal with a +2 valence, copper being the most effective.
4. Copper complex formed with the free amino acid in solution can be visualized at 254 nm UV.
5. In addition to alpha amino acids, alpha hydroxy acids and amino alcohols have been resolved on these types of CSP’s.
6. Various alcohols and pHs can be used to effect retention and resolution.
7. Elution order can be reversed by using different enantiomeric form of the bidentate ligand.
Separation of Alpha Hydroxy Acids

**Lactic Acid**

Astec CLC-L
Peak 1: 9.24 min.
Peak 2: 12.58 min.

Astec CLC-D
Peak 1: 9.27 min.
Peak 2: 12.18 min.

Mobile Phase: 5mM CuSO₄
Separation D, L Proline
Astec Ligand CLC-D

Peak 1: 4.25 min.
Peak 2: 7.41 min.

Mobile Phase: 3 mM CuSO₄
Flow Rate: 1.5 mL/min
UV: $\lambda$=254 nm
Inj: 2 $\mu$L (10 mg/mL)
Trace Analyses on Astec CLC Columns

**Proline**

Astec CLC-L

Peak 1: 6.29 min. (D)
Peak 2: 11.87 min. (L)

**Lactic Acid**

Astec CLC-L

Peak 1: 9.24 min. (D)
Peak 2: 12.58 min. (L)

**Aspartic Acid**

Astec CLC-L

Peak 1: 9.17 min. (D)
Peak 2: 11.14 min. (L)
Chiral Crown Ethers

References: Cram, et al.,
Key Points for Chiral Crown Ethers

1. Expands the reach of chiral separations to a greater variety of primary amine containing analytes.

2. Crown-6-polyethers chirality is obtained with the attachment of steric bulk in the form of chiral planes that can be made (+) or (-).

3. Amine must be in the form of an ammonium ion to form the required inclusion complex.

4. Uses only perchloric acid as the mobile phase to promote and maintain the ammonium ion formation.

5. Secondary amines do not separate.

6. Temperature can be used as an operating parameter to effect retention and selectivity.
Crown Ether Separations

Glutamine
- Load: 5 mM, 2 μL
- Eluent: Aq. HClO₄ pH 2.0
- Flow rate: 0.4 mL/min
- Temperature: 25°C
- Detection: UV 200 nm

Methionine
- Load: 5 mM, 2 μL
- Eluent: Aq. HClO₄ pH 2.0
- Flow rate: 0.8 mL/min
- Temperature: 25°C
- Detection: UV 200 nm

Isoleucine
- Load: 5 mM, 10 μL
- Eluent: Aq. HClO₄ pH 2.0
- Flow rate: 0.4 mL/min
- Temperature: 0°C
- Detection: UV 200 nm
Proposed Structures of Glycopeptide CSP’s

Vancomycin

Ristocetin A
Proposed Structures of Glycopeptide CSP’s

Teicoplanin

Teicoplanin Aglycone
Possible Mechanism for the Separation of Amino Acids on CHIROBIOTIC CSP’s

Work of Berthod and Armstrong:


Indicated that the primary amine on the CHIROBIOTIC T was largely responsible for the separation of racemic amino acids but that several other mechanisms were also at work.

Work of Jandera:


Indicated that at least two selective sites and one non-selective site take part in chiral recognition on the CHIROBIOTIC T.
Key Functional Group for CSP Interaction

Column: CHIROBIOTIC T (250x4.6mm)
Mobile Phase: 50/50, EtOH/H2O
Flow rate: 0.8mL/min.
UV: 254nm

Phenylalanine

Phenylalanine methyl ester
Underivatized D, L-Amino Acids

Effect of Alcohol Modifier on Retention, Selectivity and Resolution

Sample: Methionine

Underivatized D, L-amino acids can be separated in simple ethanol or methanol and water mobile phases. This type of mobile phase allows for low UV detection or the use of light scattering detectors. Note in the following chart the effect of organic composition on resolution. An increase in the alcohol content of the mobile phase typically results in a dramatic increase in resolution while selectivity is largely unaffected.
Decision Tree for the Separation of D, L-amino acids and N-blocked Amino Acids

Amino Acids

CLC-D
CLC-L

Yes

Detection/Elution Order Issue

Yes

No

No

Bi-functional

No

T - EtOH/Buffer
TAG - MeOH/Buffer

TM - EtOH/H2O
TAG - MeOH/H2O

Acetyl

T/TAG - RP
T/TAG - POM

AQC

RN/B - POM
DMP/RSP-RP

Benzoyl
CBZ
Dansyl

T/R - RP
T/R - POM

t-BOC

R/T/RSP - RP

DNB

RN/SN - NP

FMOC

T/R - RP
T/R/RN - POM

*Trademark Waters Corporation
# Enantioresolution of Underivatized $\alpha$-Amino Acids

<table>
<thead>
<tr>
<th>$\alpha$-Amino Acid</th>
<th>R-Moiety</th>
<th>CHIROBIOTIC T$^{(1)}$</th>
<th>CHIROBIOTIC TAG$^{(2)}$</th>
<th>CHIROBIOTIC R$^{(3)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>-CH$_3$</td>
<td>0.56 2.9</td>
<td>0.09 4.0</td>
<td>1.35(L) 1.45</td>
</tr>
<tr>
<td>Arginine</td>
<td>-(CH$_2$)$_3$-NH-C(NH$_2$)$_2$</td>
<td>1.17 2.1</td>
<td>2.17 3.0</td>
<td>N/A N/A</td>
</tr>
<tr>
<td>Aspartic</td>
<td>-CH$_2$-COOH</td>
<td>1.49 1.9</td>
<td>0.34 3.4</td>
<td>N/A N/A</td>
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<tr>
<td>Asparagine</td>
<td>-CH$_2$-CO-NH$_2$</td>
<td>0.58 2.1</td>
<td>0.21 3.7</td>
<td>1.45(L) 1.56</td>
</tr>
<tr>
<td>Cysteine</td>
<td>-CH$_2$-SH</td>
<td>0.45 1.6</td>
<td>0.20 1.8</td>
<td>1.78(L) 1.50</td>
</tr>
<tr>
<td>Glutamic</td>
<td>-CH$_2$-CH$_2$-COOH</td>
<td>1.15 2.2</td>
<td>0.64 2.5</td>
<td>N/A N/A</td>
</tr>
<tr>
<td>Glutamine</td>
<td>-(CH$_2$)$_2$-CO-NH$_2$</td>
<td>1.13 1.6</td>
<td>0.82 3.5</td>
<td>N/A N/A</td>
</tr>
<tr>
<td>Histidine</td>
<td></td>
<td>3.10 0.8</td>
<td>8.45 2.3</td>
<td>1.13(L) 1.45</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>-(CH$_3$)$_2$-CH-CH$_3$</td>
<td>0.40 2.5</td>
<td>0.18 3.0</td>
<td>1.25(L) 1.54</td>
</tr>
<tr>
<td>Leucine</td>
<td>-CH$_2$-CH-(CH$_3$)-CH$_3$</td>
<td>0.47 3.5</td>
<td>0.60 6.5</td>
<td>1.48(L) 1.45</td>
</tr>
<tr>
<td>Lysine</td>
<td>-(CH$_2$)$_4$-NH$_3$</td>
<td>0.81 2.2</td>
<td>1.21 2.5</td>
<td>1.27 1.97</td>
</tr>
<tr>
<td>Methionine</td>
<td>-CH$_2$-CH$_2$-S-CH$_3$</td>
<td>0.55 3.3</td>
<td>0.47 3.5</td>
<td>1.52(L) 1.52</td>
</tr>
</tbody>
</table>
Enantioresolution of Underivatized $\alpha$-Amino Acids (continued)

<table>
<thead>
<tr>
<th>Amine</th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>$R_3$</th>
<th>$R_4$</th>
<th>$R_5$</th>
<th>$R_6$</th>
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</thead>
<tbody>
<tr>
<td>Phenylalanine</td>
<td>0.87</td>
<td>2.0</td>
<td>0.98</td>
<td>5.2</td>
<td>2.04(L)</td>
<td>1.63</td>
</tr>
<tr>
<td>Proline</td>
<td>-CH$_2$-CH$_2$</td>
<td>2.4</td>
<td>2.5</td>
<td>0.43</td>
<td>6.2</td>
<td>2.00(L)</td>
</tr>
<tr>
<td>Serine</td>
<td>-CH$_3$OH</td>
<td>0.69</td>
<td>1.5</td>
<td>0.04</td>
<td>1.9</td>
<td>1.13(L)</td>
</tr>
<tr>
<td>Threonine</td>
<td>-CHOH-CH$_3$</td>
<td>0.75</td>
<td>1.4</td>
<td>0.46</td>
<td>4.0</td>
<td>N/A</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.60</td>
<td>1.9</td>
<td>0.76</td>
<td>2.9</td>
<td>1.73(L)</td>
<td>1.52</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.01</td>
<td>2.0</td>
<td>2.05</td>
<td>6.5</td>
<td>2.36(L)</td>
<td>1.55</td>
</tr>
<tr>
<td>Valine</td>
<td>-CH$_2$(CH$_3$)-CH$_3$</td>
<td>0.56</td>
<td>1.9</td>
<td>0.48</td>
<td>5.5</td>
<td>1.42(L)</td>
</tr>
</tbody>
</table>

N/A - not available.

Typical mobile phases:  
T = 60/40: EtOH/H$_2$O and EtOH/H$_2$O @ pH 3.8  
TAG = 20/80: MeOH/H$_2$O/pH 3.8 and/or 0.1% TEAA, pH 4.1  
R = MeOH/H$_2$O: 50/50 and MeOH/0.1% TEAA, pH 4 and pH 7

Selectivity Comparison T vs TAG for Aliphatic $\alpha$-Amino Acids

CHIROBIOTIC T

- Alanine
- 4.36 min.
- 5.61 min.

50/50: EtOH/H20
1.0 mL/min.
$\alpha$=1.80

CHIROBIOTIC TAG

- 3.49 min.
- 5.49 min.

30/70: MeOH/H20
1.0 mL/min.
$\alpha$=5.19
Selectivity Comparison T vs TAG for Aromatic α-Amino Acids

CHIROBIOTIC T

5.63 min.

6.80 min.

30/70: EtOH/H2O
1.0 mL/min.
\( \alpha = 1.42 \)

CHIROBIOTIC TAG

7.11 min.

10.46 min.

60/40: MeOH/H2O
1.0 mL/min.
\( \alpha = 1.82 \)
Selectivity Comparison T vs TAG for Basic $\alpha$-Amino Acids

CHIROBIOTIC T

- Lysine: 4.81 min.
- H$_2$N\text{-}\text{CH}$_2$\text{-}\text{CH}$_2$\text{-}\text{CH}$_2$\text{-}\text{COOH}
- 20/80: EtOH/100mM NaH$_2$PO$_4$
- 1.0 mL/min.
- $\alpha = 1.32$

CHIROBIOTIC TAG

- Lysine: 7.07 min.
- H$_2$N\text{-}\text{CH}$_2$\text{-}\text{CH}$_2$\text{-}\text{CH}$_2$\text{-}\text{COOH}
- 20/80: MeOH/100mM NaH$_2$PO$_4$
- 1.0 mL/min.
- $\alpha = 1.80$
Selectivity Comparison T vs TAG for Sulfur Containing $\alpha$-Amino Acids

**CHIROBIOTIC T**

4.34 min. 5.39 min.

20/80: EtOH/H2O
1.0 mL/min.
$\alpha = 1.32$

**Methionine**

\[
\text{H}_3\text{C}\text{S}\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH}
\]

**CHIROBIOTIC TAG**

4.69 min. 8.01 min.

30/70: MeOH/H2O
1.0 mL/min.
$\alpha = 2.98$
Selectivity of Bonded Teicoplanin Aglycone for Sulfur Containing Racemates

Methyl-phenyl sulfoxide

![Methyl-phenyl sulfoxide structure]

- 10.86 min.
- 14.49 min.

40/60: EtOH/Hexane
1.5 mL/min.

Cysteine

![Cysteine structure]

- 3.60 min.
- 5.20 min.

60/40: MeOH/H2O
1.0 mL/min.
Acidic Amino Acids (D, L)-Asp and (D, L)-N-Me-Asp

Column: CHIROBIOTIC TAG (250x4.6 mm I.D.)
Eluent: 85/15, MeOH/10 mM NH4OAc, pH = 3.8
Flow rate: 1 mL/min,
Detector: ELSD (T = 80 C, P = 3.1 bar)
Separation of β-Amino Acids

(1) 3-Amino-butanoic acid
(2) 3-aminopentanoic acid
(3) 3-amino-4-methylpentanoic acid
(4) 3-amino-4, 4 dimethylpentanoic acid
(5) 3-amino-4-ethylhexanoic acid
(6) 3-amino-3-cyclohexyl-propanoic acid
(7) 3-amino-3-(3-cyclohexene-1-yl) propanoic acid
(8) and 3-amino-3-phenylpropanoic acid


CHIROBIOTIC T – Separates 2, 3, 4, 5, 6 & 8
CHIROBIOTIC R – Separates 1, 2, 4 & 8
Reversed Phase Separations
CHIROBIOTIC TAG

No significant change has been observed for use of this phase in the reversed phase mode.

All of the points made under the CHIROBIOTIC T are applicable here, including the selectivity of methanol as the primary organic component.

Carnitine

(CH₃)₃NCH₂CH(OH)CH₂COOH

Peak 1: 12.19 min.
Peak 2: 14.89 min.

85/15:MeOH/25mM NH₄OAc, pH 6.0
1.0 mL/min.
Detector: ELSD, T=80°C
Nitrogen Flow: 1.0 SLPM
Amino Acid Diastereomers

A number of amino acids i.e., isoleucine and threonine, have *allo-forms*.

The CHIROBIOTIC R and CHIROBIOTIC TAG have proven useful for the separation of these pairs of enantiomers in simple alcohol/water mixtures.

**Isoleucine**

- Peak 1/4: L/D-Isoleucine
- Peak 2/3: L/D-Allo-Isoleucine

CHIROBIOTIC R, 250x4.6mm
80/20: MeOH/H2O @ 0.6 mL/min.
Detector: ELSD, T=80°C
Nitrogen Flow: 0.8 SLPM

**Threonine**

- Peak 1/3: L/D-Threonine
- Peak 2/4: L/D-Allo-Threonine

CHIROBIOTIC TAG, 250x4.6mm
60/40: MeOH/H2O @ 1.0 mL/min.
Detector: ELSD, T=85°C
Nitrogen Flow: 0.9 SLPM
Preparative Purification of Isoleucine on Teicoplanin Aglycone

Column: CHIROBIOTIC TAG
Size: 250 x 22.1 mm
Mobile Phase: 30/70: MeOH/H2O
Flow Rate: 20 mL/min
Injection: 170 mg in 10 mL
Chiral Separation on Unusual Amino Acids – (1)

A. Tyrosine analogs

B. Phenylalanine analogs
Chiral Separation on Unusual Amino Acids – (2)

C. Tetrahydroisoquinoline analogs

D. Amino tetralin analogs

E. Tryptophan analogs

2. G. Torok et al., Chirality 13 (2001) 648 - 656
1. N-FMOC (9-Fluorenyl methyl chloroformate) amino acids
# Resolution of N-FMOC D, L-Amino Acids

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mobile Phase</th>
<th>Column</th>
<th>k1</th>
<th>α</th>
<th>Rs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>50/50, MeOH/20mM NH4OAc</td>
<td>R</td>
<td>0.38</td>
<td>3.89</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>40/60, MeOH/1% TEAA, pH 4.1</td>
<td>T</td>
<td>1.26</td>
<td>2.27</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>100/0.02w%, MeOH/NH4OAc</td>
<td>R</td>
<td>0.57</td>
<td>2.37</td>
<td>2.2</td>
</tr>
<tr>
<td>Arginine</td>
<td>20/80, MeOH/0.1% TEAA, pH 6.8</td>
<td>R</td>
<td>3.28</td>
<td>1.46</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>100/0.1w%, MeOH/NH4TFA</td>
<td>R</td>
<td>1.69</td>
<td>2.95</td>
<td>4.6</td>
</tr>
<tr>
<td>Asparagine</td>
<td>100/1/1, MeOH/HOAc/TEA</td>
<td>T</td>
<td>0.63</td>
<td>1.81</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>40/60, MeOH/1% TEAA, pH 4.1</td>
<td>T</td>
<td>4.41</td>
<td>1.22</td>
<td>3.0</td>
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<tr>
<td></td>
<td>100/0.1w%, MeOH/NH4TFA</td>
<td>R</td>
<td>1.55</td>
<td>1.49</td>
<td>1.3</td>
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<tr>
<td></td>
<td>30/70, MeOH/20mM NH4OAc</td>
<td>R</td>
<td>0.55</td>
<td>0.57</td>
<td>1.8</td>
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<tr>
<td>Aspartic acid</td>
<td>20/80, MeOH/0.1% TEAA, pH 6.8</td>
<td>R</td>
<td>0.46</td>
<td>1.68</td>
<td>2.0</td>
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<tr>
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<td>2.59</td>
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<td>1.8</td>
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<td>R</td>
<td>1.69</td>
<td>2.95</td>
<td>4.6</td>
</tr>
<tr>
<td>Citrulline</td>
<td>40/60, MeOH/0.1% TEA, pH 4.1</td>
<td>T</td>
<td>1.07</td>
<td>2.50</td>
<td>4.0</td>
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<tr>
<td></td>
<td>100/1/1, MeOH/HOAc/TEA</td>
<td>T</td>
<td>1.34</td>
<td>2.05</td>
<td>3.0</td>
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<td>30/70, MeOH/20mM NH4OAc</td>
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<td>1.34</td>
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<td>2.6</td>
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<tr>
<td>Cysteine</td>
<td>65/35, EtOH/Hexane</td>
<td>R</td>
<td>1.7</td>
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<td>Glutamic acid</td>
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<td>R</td>
<td>1.07</td>
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<td>1.60</td>
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<td>40/60, MeOH/0.1% TEAA, pH 4.1</td>
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<td>1.07</td>
<td>1.60</td>
<td>3.8</td>
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<td>Glutamine</td>
<td>20/80, MeOH/0.1% TEAA, pH 6.8</td>
<td>R</td>
<td>0.61</td>
<td>2.85</td>
<td>1.6</td>
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<tr>
<td></td>
<td>65/35, EtOH/Hexane</td>
<td>R</td>
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<td>2.04</td>
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<td>40/60, MeOH/0.1% TEAA, pH 4.1</td>
<td>T</td>
<td>0.93</td>
<td>2.46</td>
<td>5.0</td>
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<td>100/0.1w%, MeOH/NH4OAc</td>
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<td>R</td>
<td>0.93</td>
<td>2.46</td>
<td>3.6</td>
</tr>
<tr>
<td>Histidine</td>
<td>20/80, MeOH/0.1% TEAA, pH 4.1</td>
<td>R</td>
<td>1.0</td>
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</table>
Resolution of N-FMOC D, L-Amino Acids (continued)

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Mobile Phase Conditions</th>
<th>T</th>
<th>R</th>
<th>2.2</th>
<th>2.3</th>
<th>1.6</th>
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<tbody>
<tr>
<td>Isoleucine</td>
<td>40/60, MeOH/0.1% TEAA, pH 4.1</td>
<td>1.08</td>
<td>1.78</td>
<td>2.2</td>
<td>2.3</td>
<td>1.6</td>
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<td></td>
<td>100/0.1%, MeOH/NH4OAc</td>
<td>0.45</td>
<td>1.87</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>30/70, MeOH/20mM NH4OAc</td>
<td>2.32</td>
<td>1.85</td>
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<tr>
<td>Isoleucine, allo</td>
<td>100/0.1%, MeOH/NH4OAc</td>
<td>0.53</td>
<td>1.57</td>
<td>2.0</td>
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</tr>
<tr>
<td>Iserine</td>
<td>65/35, EtOH/Hexane</td>
<td></td>
<td></td>
<td>1.5</td>
<td>4.5</td>
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<tr>
<td></td>
<td>50/50, MeOH/1% TEAA, pH 5.5</td>
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<tr>
<td>Leucine</td>
<td>40/60, MeOH/0.1% TEAA, pH 4.1</td>
<td>1.03</td>
<td>2.45</td>
<td>5.0</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100/0.1%, MeOH/NH4TFA</td>
<td>0.46</td>
<td>2.41</td>
<td>5.0</td>
<td>3.5</td>
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<td>Lysine</td>
<td>50/50, MeOH/1% TEAA, pH 5.5</td>
<td>0.79</td>
<td>2.12</td>
<td>1.4</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100/0.1%, MeOH/NH4TFA</td>
<td></td>
<td></td>
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<tr>
<td>Methionine</td>
<td>40/60, MeOH/0.1% TEAA, pH 4.1</td>
<td>0.96</td>
<td>3.43</td>
<td>6.0</td>
<td>3.0</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>100/1/1, MeOH/HOAc/TEA</td>
<td>0.94</td>
<td>2.70</td>
<td>6.0</td>
<td>3.0</td>
<td>4.5</td>
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<tr>
<td></td>
<td>100/0.1%, MeOH/NH4TFA</td>
<td>0.27</td>
<td>5.77</td>
<td>4.5</td>
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</tr>
<tr>
<td></td>
<td>50/50, MeOH/20mM NH4OAc</td>
<td></td>
<td></td>
<td>5.4</td>
<td></td>
<td></td>
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<tr>
<td>Norleucine</td>
<td>40/60, MeOH/0.1% TEAA, pH 4.1</td>
<td>1.20</td>
<td>2.87</td>
<td>6.5</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>100/0.1%, MeOH/NH4TFA</td>
<td>0.50</td>
<td>2.0</td>
<td>3.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30/70, MeOH/20mM NH4OAc</td>
<td>2.92</td>
<td>2.15</td>
<td></td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>Norvaline</td>
<td>100/0.1%, MeOH/NH4TFA</td>
<td>0.61</td>
<td>2.56</td>
<td>3.5</td>
<td>6.5</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>30/70, MeOH/20mM NH4OAc</td>
<td>2.12</td>
<td>3.56</td>
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<tr>
<td></td>
<td>40/60, MeOH/0.1% TEAA, pH 4.1</td>
<td>0.99</td>
<td>3.19</td>
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<td>Ornithine</td>
<td>50/50, MeOH/1% TEAA, pH 5.5</td>
<td>1.22</td>
<td>1.72</td>
<td>1.4</td>
<td>3.0</td>
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</tr>
<tr>
<td></td>
<td>100/0.1%, MeOH/NH4TFA</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>100/1/1, MeOH/HOAc/TEA</td>
<td>1.54</td>
<td>2.82</td>
<td>3.0</td>
<td>6.0</td>
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<tr>
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<td>40/60, MeOH/0.1% TEAA, pH 4.1</td>
<td>0.45</td>
<td>6.65</td>
<td>6.0</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>100/0.2%, MeOH/NH4OAc</td>
<td>0.12</td>
<td>8.53</td>
<td></td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50/50, MeOH/20mM NH4OAc</td>
<td></td>
<td>6.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CHIROBIOTIC T (Reversed Phase Mode)

LC→LC/MS Compatible Mobile Phase

Example: FMOC Leucine

Mobile Phase: MeOH/ Buffer*, 40/60

*Buffer: 0.1% TEAA, pH=4.1
*Buffer: 20mM NH4OAc, pH=4.1
N-FMOC (9-Fluorenylmethyl Chloroformate) Amino Acids

Column: CHIROBIOTIC T (Reversed Phase Mode)

Peak 1: 5.55 min.  
Peak 2: 6.73 min.

Peak 1: 7.87 min.  
Peak 2: 10.30 min.

Mobile Phase: 40/60: MeOH/0.1% TEAA, pH=4.1
N-FMOC (9-Fluorenethylmethyl Chloroformate) Amino Acids

Column: CHIROBIOTIC R (Polar Organic Mode)

Norleucine
Peak 1: 4.20
Peak 2: 5.61

Norvaline
Peak 1: 4.51
Peak 2: 7.17

Methionine
Peak 1: 5.4
Peak 2: 9.90

Mobile Phase: 100/0.1w%: MeOH/NH4TFA (LC/MS Compatible)
**N-FMOC (9-Fluorenylmethyl Chloroformate) Amino Acids**

**Column:** CHIROBIOTIC R (Reversed Phase Mode)

- **Asparagine**
  - Peak 1: 7.66
  - Peak 2: 9.95

- **Glutamine**
  - Peak 1: 6.22
  - Peak 2: 8.78

- **Serine**
  - Peak 1: 5.79
  - Peak 2: 9.87

**Mobile Phase:** 30/70: MeOH/20mM NH₄OAc (LC/MS Compatible)
2. N-tert-Butoxycarbonyl (t-BOC) Amino Acids

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 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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mobile Phase</th>
<th>Column</th>
<th>k1</th>
<th>α</th>
<th>Rs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>20/80, MeOH/0.1%TEAA, pH 4.1</td>
<td>R</td>
<td>1.08</td>
<td>1.77</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>10/90, MeOH/0.1%TEAA, pH 4.1</td>
<td>T</td>
<td>0.45</td>
<td>1.55</td>
<td>2.4</td>
</tr>
<tr>
<td>Asparagine</td>
<td>20/80, MeOH/0.1%TEAA, pH 4.1</td>
<td>T</td>
<td>1.30</td>
<td>1.36</td>
<td>2.0</td>
</tr>
<tr>
<td>Glutamine</td>
<td>20/80, MeOH/0.1%TEAA, pH 4.1</td>
<td>R</td>
<td>1.10</td>
<td>1.38</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>10/90, MeOH/0.1%TEAA, pH 4.1</td>
<td>T</td>
<td>0.41</td>
<td>1.37</td>
<td>1.4</td>
</tr>
<tr>
<td>Histidine</td>
<td>20/80, MeOH/0.1%TEAA, pH 6.0</td>
<td>R</td>
<td>0.81</td>
<td>1.37</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>20/80, MeOH/0.1%TEAA, pH 4.1</td>
<td>T</td>
<td>1.53</td>
<td>1.66</td>
<td>2.0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>20/80, MeOH/0.1%TEAA, pH .1</td>
<td>R</td>
<td>1.67</td>
<td>1.25</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>10/90, ACN/20mM NH4OAc</td>
<td>RSP</td>
<td>2.00</td>
<td>1.54</td>
<td>1.6</td>
</tr>
</tbody>
</table>
## 2. N-tert-Butoxycarbonyl (t-BOC) Amino Acids

<table>
<thead>
<tr>
<th></th>
<th>20/80, MeOH/0.1%TEAA, pH 6.0</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Methionine</td>
<td>20/80, MeOH/0.1%TEAA, pH 4.1</td>
<td>10/90, ACN/20mM NH4OAc</td>
<td>R</td>
<td>T</td>
<td>0.34</td>
<td>20.3</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.48</td>
<td>3.90</td>
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<td></td>
<td></td>
<td></td>
<td>1.20</td>
<td>4.09</td>
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<tr>
<td>Phenylalanine</td>
<td>20/80, MeOH/0.1%TEAA, pH 6.0</td>
<td>10/90, MeOH/0.1%TEAA, pH 6.0</td>
<td>10/90, ACN/20mM NH4OAc</td>
<td>R</td>
<td>T*</td>
<td>1.02</td>
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<td></td>
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<td></td>
<td></td>
<td>0.44</td>
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<td></td>
<td></td>
<td></td>
<td>1.90</td>
<td>1.16</td>
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<tr>
<td>Phenylglycine</td>
<td>20/80, MeOH/0.1%TEAA, pH 6.0</td>
<td>20/80, MeOH/0.1%TEAA, pH 4.1</td>
<td>10/90, ACN/20mM NH4OAc</td>
<td>R</td>
<td>T</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>0.52</td>
<td>4.65</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>3.59</td>
<td>1.29</td>
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<tr>
<td>Serine</td>
<td>20/80, MeOH/0.1%TEAA, pH 4.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.88</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>20/80, MeOH/0.1%TEAA, pH 6.0</td>
<td>20/80, MeOH/0.1%TEAA, pH 4.1</td>
<td>10/90, ACN/20mM NH4OAc</td>
<td>R</td>
<td>T</td>
<td>0.61 (D)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.73 (D)</td>
<td>2.17</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>1.46 (D)</td>
<td>2.96</td>
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<tr>
<td>p-Tyrosine</td>
<td>20/80, MeOH/0.1%TEAA, pH 6.0</td>
<td>10/90, MeOH/0.1%TEAA, pH 6.0</td>
<td>10/90, ACN/20mM NH4OAc</td>
<td>R</td>
<td>T*</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.24</td>
<td>1.77</td>
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<td></td>
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<td>1.05</td>
<td>1.16</td>
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<td>Valine</td>
<td>20/80, MeOH/0.1%TEAA, pH 4.1</td>
<td>10/90, ACN/20mM NH4OAc</td>
<td>R</td>
<td>RSP</td>
<td>1.44</td>
<td>1.26</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>1.64</td>
<td>1.45</td>
</tr>
</tbody>
</table>

Legend: TEAA = Triethylammonium acetate
T = CHIROBIOTIC T
R = CHIROBIOTIC R
Flow rate = 1mL/min; *0.5mL/min.
UV = 220 nm
For all compounds tested, L-form eluted first except for truptophan
Enantioseparation of D, L-Try and N-t-BOC-Tryptophan on CHIROBIOTIC T

Mobile Phase: 20/80, Acetonitrile/1% Triethylamine Acetate, pH 4.1
N-t-BOC Amino Acids

Column: CHIROBIOTIC R

Peak 1: 5.43  
Peak 2: 6.32

Peak 1: 6.06  
Peak 2: 13.24

Peak 1: 4.84  
Peak 2: 10.12

Histidine  
Phenylalanine  
Tryptophan

Mobile Phase: 20/80: MeOH/0.1% TEAA, pH=6.0
3. Enantioseparation of N-acetyl Serine

30/70: Methanol/0.1% Triethylamine Acetate, pH 4.1 @ 1.0 mL/min.
Enantioseparation of N-acetyl Amino Acids

<table>
<thead>
<tr>
<th>N-Acetyl Amino Acid</th>
<th>Column</th>
<th>$k_1$</th>
<th>Rs</th>
<th>Mobile Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryp</td>
<td>CHIROBIOTIC T</td>
<td>0.19</td>
<td>2.5</td>
<td>30/70: MeOH/0.1% TEAA, pH 4.1</td>
</tr>
<tr>
<td>Tryp</td>
<td>CHIROBIOTIC TAG</td>
<td>0.51</td>
<td>5.0</td>
<td>100/0.1% MeOH, MeOH/NH4OAc</td>
</tr>
<tr>
<td>Tryp</td>
<td>CHIROBIOTIC TAG</td>
<td>1.23</td>
<td>3.0</td>
<td>30/70: MeOH/20 mM NH4OAc, pH 6.0</td>
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<tr>
<td>Val</td>
<td>CHIROBIOTIC R</td>
<td>0.42</td>
<td>1.5</td>
<td>50/50: ACN/H2O</td>
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<tr>
<td>2-fluoro-Phe</td>
<td>CHIROBIOTIC R</td>
<td>0.52</td>
<td>1.5</td>
<td>50/50: ACN/H2O</td>
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<tr>
<td>3-fluoro-Phe</td>
<td>CHIROBIOTIC T</td>
<td>0.55</td>
<td>2.5</td>
<td>50/50/0.2/0.2: ACN/MeOH/HOAc/TEA</td>
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<tr>
<td>4-fluoro-Phe</td>
<td>CHIROBIOTIC T</td>
<td>1.43</td>
<td>5.6</td>
<td>20/80: MeOH/1% TEAA, pH 4.1</td>
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</tbody>
</table>
Simultaneous Isocratic Analysis of 15 Underivatized Amino Acids by LC-ISP-MS-MS on CHIROBIOTIC T 75/25: ACN/H₂O

Determination of Pipecolic Acid in Plasma by LC/MS/MS

**Peroxisomal Disorders:**
Indicated by higher level of L-PA

Higher concentration of D-PA indicated liver dysfunction.

LC/MS/MS methodology utilizes 50 μL of plasma, no derivatizing agents and no interfering substances utilizing the CHIROBIOITIC T, clearly distinguishing between healthy individuals and peroxisomal disease patients.

Linear calibration curves were obtained in the range of 0.5 – 80 μmol./L.

Transition: \( m/z 130 \rightarrow m/z 84 \)

(A), mixture of D- and L-PA calibrators; (B) L-PA calibrator.
Determination of D and L 2-Hydroxyglutaric Acid

Two distinct inborn metabolic disorders:

LC/MS/MS methodology utilizes 100 μL urine + 900 μL mobile phase and filtered through a 0.45 μM membrane filter.

LC-MS/MS negative ion chromatograms obtained in the MRM mode of analysis using CHIROBIOTIC R column.

(A) A mixture of D- and L-2-HG
(B) and (C) urine samples from patients 1/2
(D) urine sample from patient 2 spiked with standard D-2-HG.

Conclusions

- Macrocyclic glycopeptide CSPs offer the best solution for all kinds of amino acids chiral separation needs in reversed phase and polar organic mode.

- Mobile phases are LC/MS compatible for simultaneous detection of amino acids mixtures and for biological samples.

- The loading capacity for amino acids is fairly high and the eluent is easily removed for sample recovery.