Facile HPLC Separation of Peptides: Resolution of Chiral/Achiral Single Amino Acid Analogues using Teicoplanin-based Chiral Stationary Phases

J.T. Lee and Thomas E. Beesley
Advanced Separation Technologies Inc. (astec),
37 Leslie court, P.O. Box 297, Whippany, NJ 07981 USA
Website: www.astecusa.com; Email: info@asteusa.com

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Abstract

- Recently, a new wave of peptide therapeutics are coming through pharmaceutical and biotech companies. Compared to small molecules, peptides are more potent with higher specificity, and most importantly, peptides clear from the body faster and have fewer toxicology problems.
- The separation of closely-related peptides that are different from one another by a particular amino acid, or by the chirality of an amino acid, often involves HPLC methods that are either complex or not ESI-MS compatible. This study describes the HPLC method development for resolution of small chiral peptides and single amino acid chiral/achiral analogues in peptide sequences utilizing teicoplanin-based chiral stationary phases. The mobile phases used are generally acetonitrile-buffer systems that are LC-MS compatible, enabling higher sensitivity than UV detection. More importantly, these phases provide different selectivity and higher resolution when compared to the conventional C_{18}, ion pairing and ion exchange methods.
- This presentation is aimed to give analytical chemists a tool on LC-MS platforms to test the quality and quantity of peptide products. Amino acid variation for peptides with up to 13 amino acid residues have been separated so far, but it is not known what the limit in peptide length will be. Contrary to amino acids, the elution order for peptides with chiral analogue has shown to be D before L. Examples of separation on different peptide families and several racemic di- and tri-peptides will be given. Detailed information on the method development and optimization procedures will be outlined. Finally, the complementary effect on the CHIROBIOTIC T/T2/TAG columns will also be presented.

Separation of Peptide Analogues

These are closely related peptides that differ from each other because of a particular amino acid or chirality of an amino acid either within the peptide or at its terminus

- Current LC Methods:
  - Reversed phase (C_{18}) – often needs ion pairing reagents that modify the column and are not MS compatible
  - Ion exchange – uses buffers that are generally not MS compatible

- CHIROBIOTIC Phases
  - Use ACN/ buffers that are LC-MS compatible
  - Provide different selectivity from C_{18} and ion exchange methods

REFERENCE:
B Zhang, R Soukup & D W Armstrong; J Chrom A, 1053 (2004) 89-99

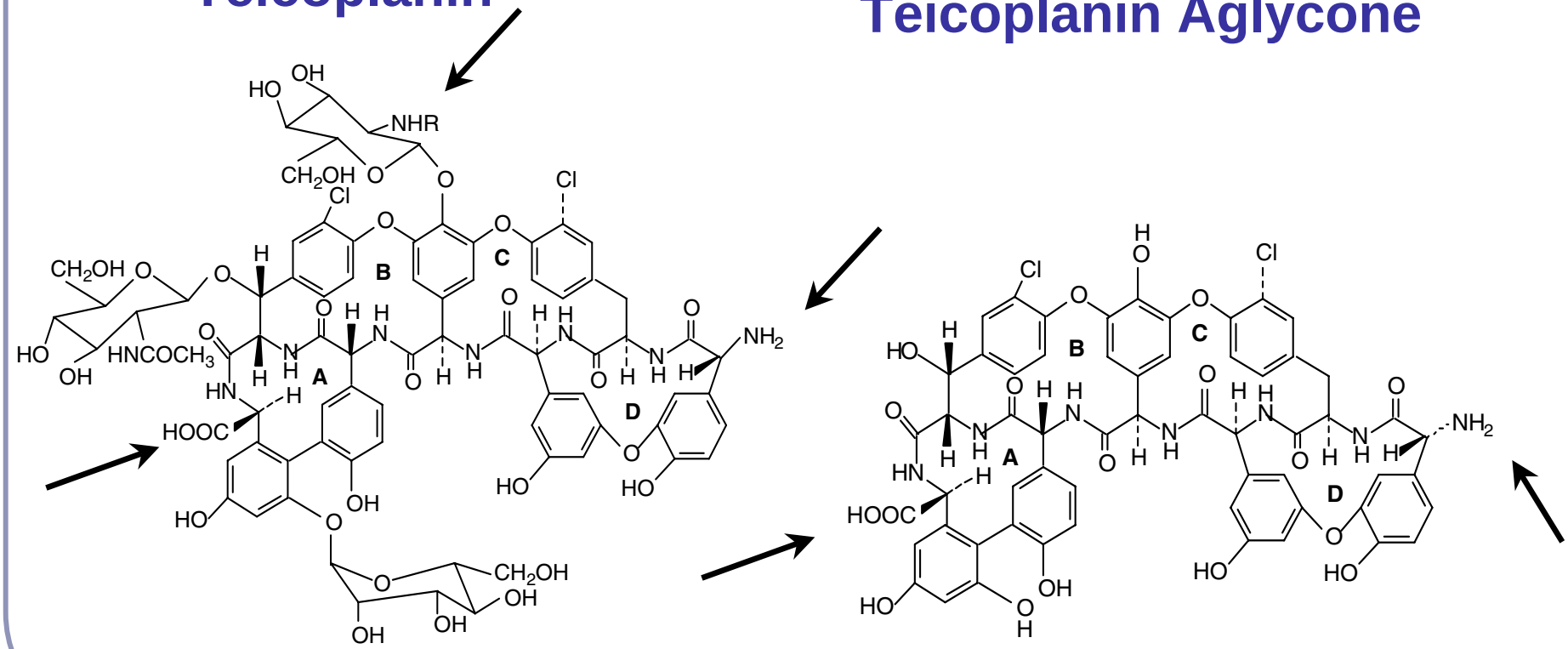
Separation of Peptide Analogues

- Astec CHIROBIOTIC phases have demonstrated large selectivity factors for:
  - Single amino acid differences within peptides of up to 13 amino acid residues
  - Single chiral amino acid differences where one amino acid in the peptide is present as either the D or L form
- Maximum peptide chain length that can be separated is as yet unknown.
- D elutes before L when it is a chiral analogue.

Proposed Structures of CSPs
Macrocyclic Glycopeptides

Teicoplanin

Teicoplanin Aglycone



→ Key sites

Methods Development

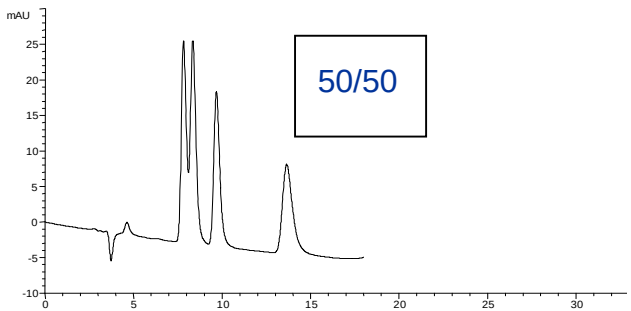
Peptide type	Di/Tri-peptides	Bi-functional	Neutral
Starting mobile phase: **50/50: ACN/Buffer**	5mM NH_4OAc, pH 4.1	0.1% HCOOH	5mM NH_4 formate
Optimization: **Organic**	% ACN or combination of ACN and MeOH	% ACN (U-shape effect)	% ACN (U-shape effect)
pH	2.6 – 6.8		2.6 – 6.2
Buffer	2-50mM	0.01 – 0.5%	2-50mM
Temperature	5-45^oC	5-45^oC	5-45^oC

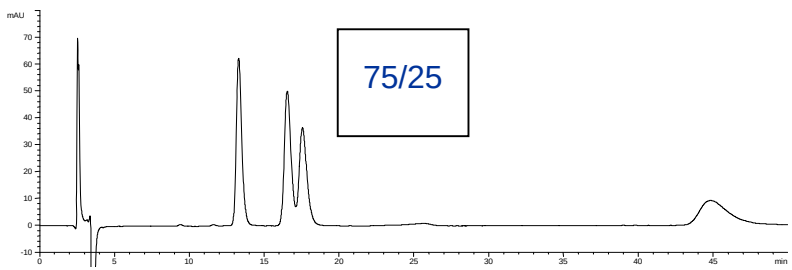
All peptide types showed complementary effects between CHIROBIOTIC T, T2 AND TAG CSPs

Optimization: [ACN] Effects Tri-peptide

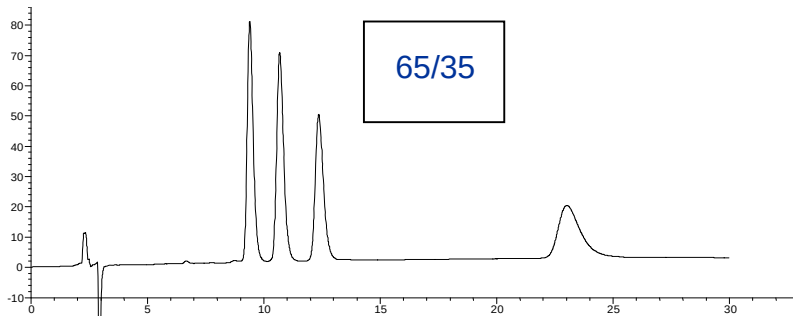
Sample: Gly-DL-Leu-DL-Ala

Column:	CHIROBIOTIC TAG, 250x4.6mm
Mobile phase:	ACN/5mM NH_4OAc, pH 4.1
Temperature:	25°C
Flow rate:	1 mL/min
UV:	220 nm

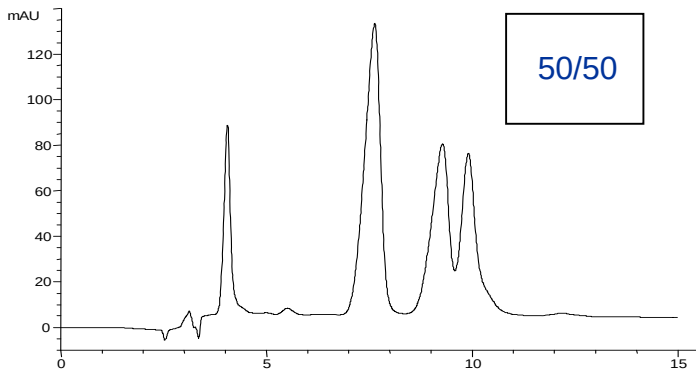


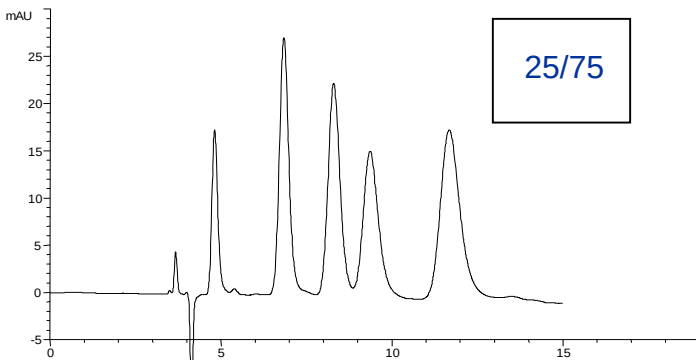


Best result



Optimization: [ACN] Effects – 5 Angiotensins II

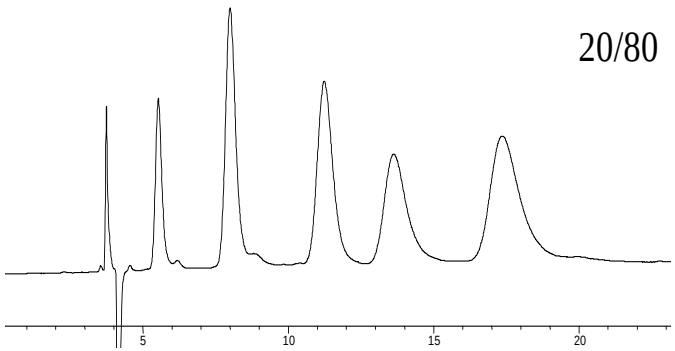




Column:	CHIROBIOTIC T2, 250x4.6mm
Mobile phase:	ACN/0.1% HCOOH
Temperature:	35˚C
Flow rate:	0.8 mL/min
UV:	220 nm

Optimization: [ACN] Effects
5 Angiotensins II

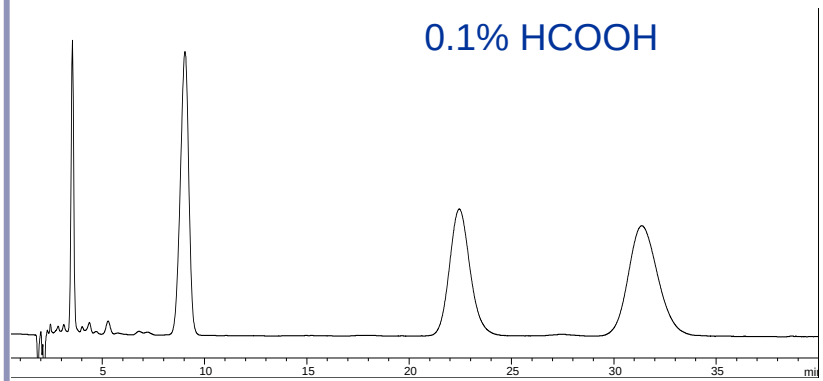
Best result



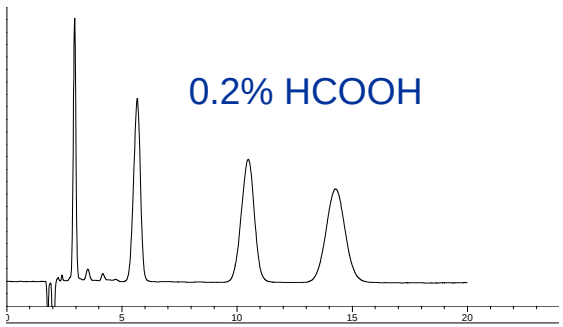
Column:	CHIROBIOTIC T2, 250x4.6mm
Mobile phase:	20/80: ACN/0.1% HCOOH
Flow rate:	0.8 mL/min
Temperature:	35˚C
UV:	220 nm

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: 17.36 min Sar-Arg-Val-Tyr-Ile-His-Pro-Phe

Optimization: [Buffer] Effects
4 Bradykinins



Column:	CHIROBIOTIC TAG, 250x4.6mm
Mobile phase:	50/50: ACN/HCOOH
Flow rate:	1.5 mL/min
Temperature:	5˚C
UV:	220 nm



Peak 1: 2.95min,
Pro-Pro-Gly-Phe-Ser-Pro

Peak 2: 5.67 min,
Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

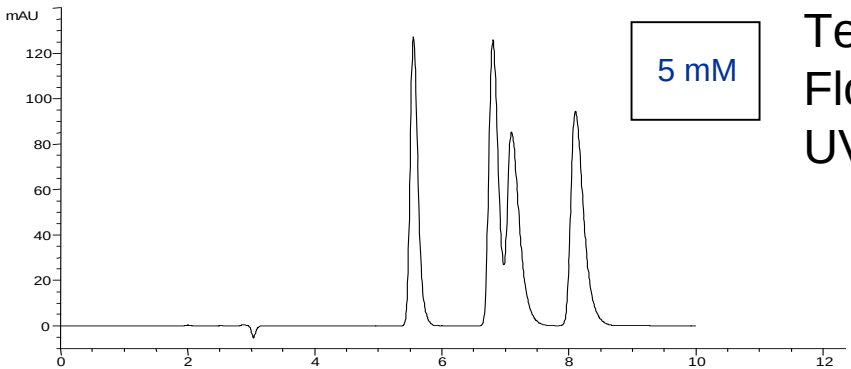
Peak 3: 10.48 min,
Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg

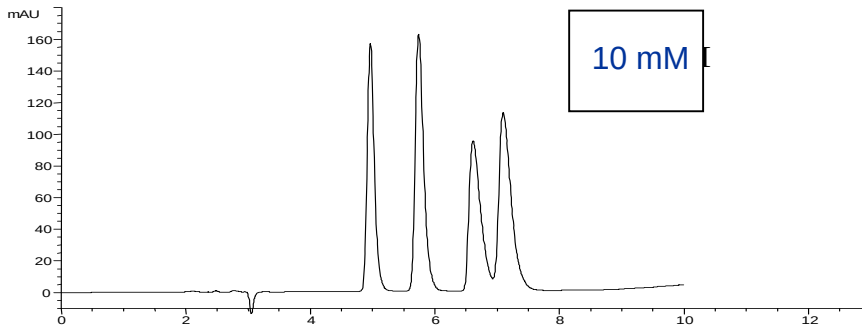
Peak 4: 14.28 min,
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

Optimization: [Buffer] Effects Di-peptide

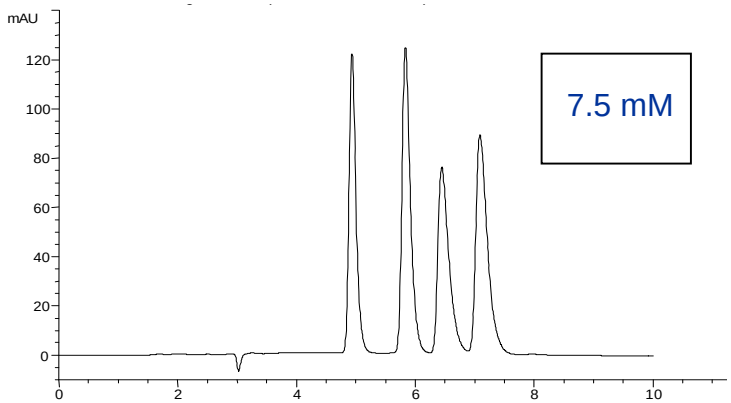
Sample: DL-Ala-DL-Val

Column:	CHIROBIOTIC T, 250x4.6mm
Mobile phase:	50/50: ACN/NH_4OAc, pH 4.1
Temperature:	25˚C
Flow rate:	1mL/min
UV:	220 nm





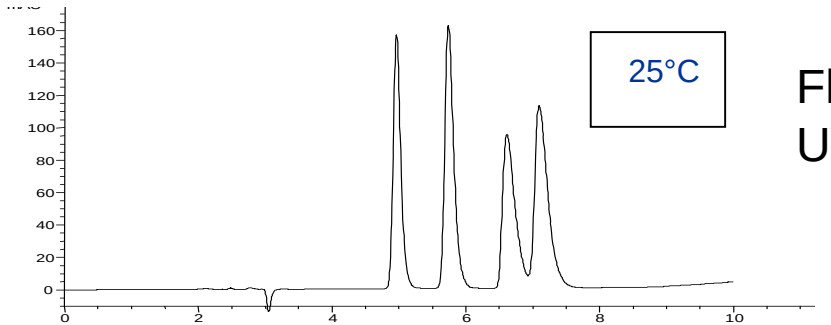
Best result

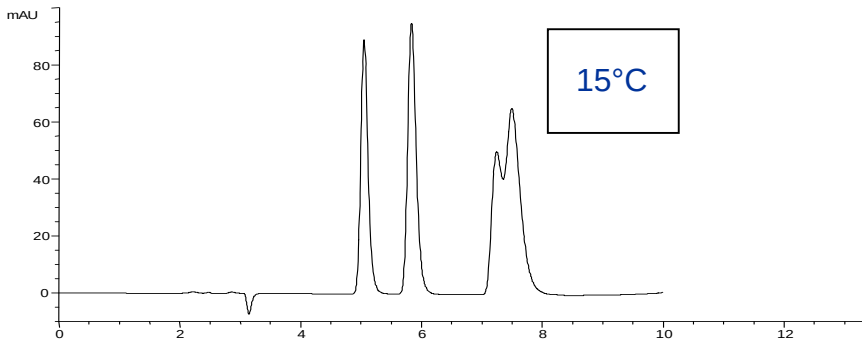


Optimization: Temperature Effects Di-peptide

Sample: DL-Ala-DL-Val

Column: CHIROBIOTIC T, 250x4.6mm
Mobile phase: 50/50: ACN/10mM NH_4OAc, pH 4.1
Flow rate: 1mL/min
UV: 220 nm





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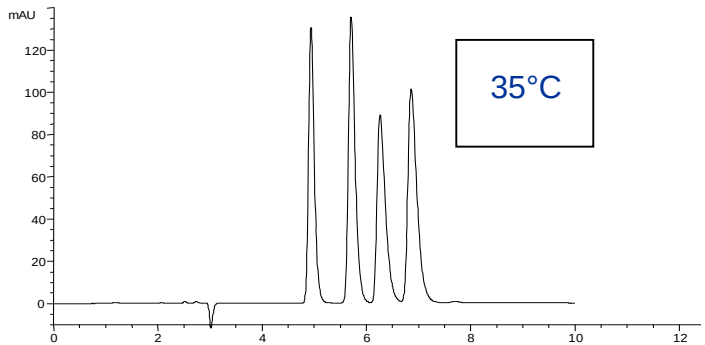
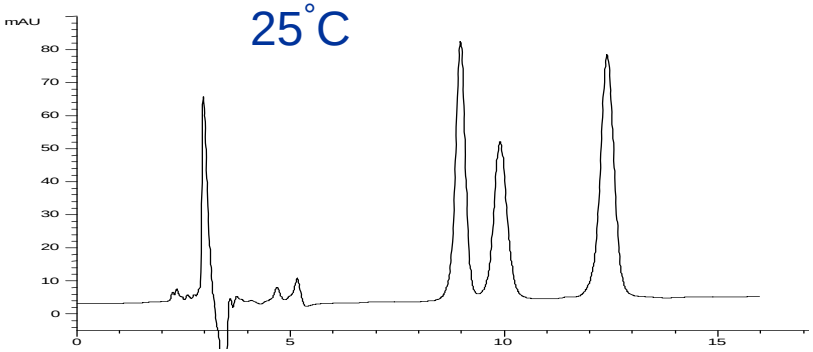
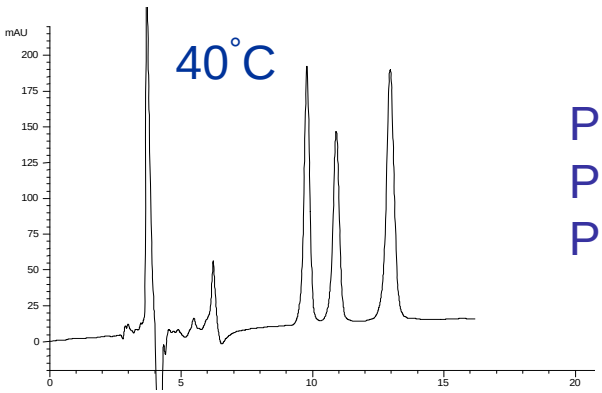


Optimization: Temperature Effects 3 β-Casomorphins



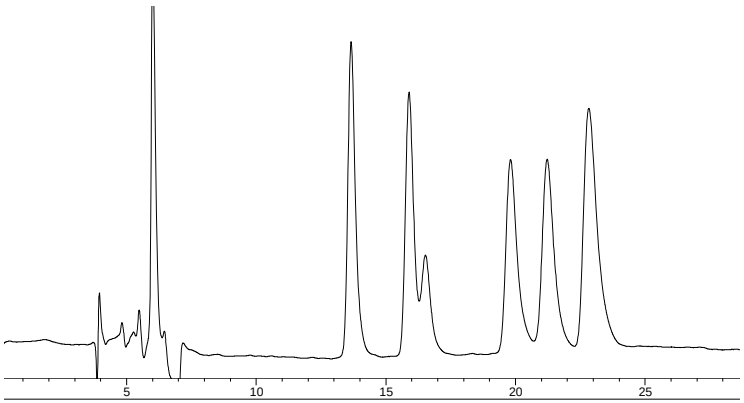
Column: CHIROBIOTIC T, 250x4.6mm
Mobile phase: 75/25: ACN/20mM NH_4Formate
Flow rate: 1 mL/min
UV: 230 nm



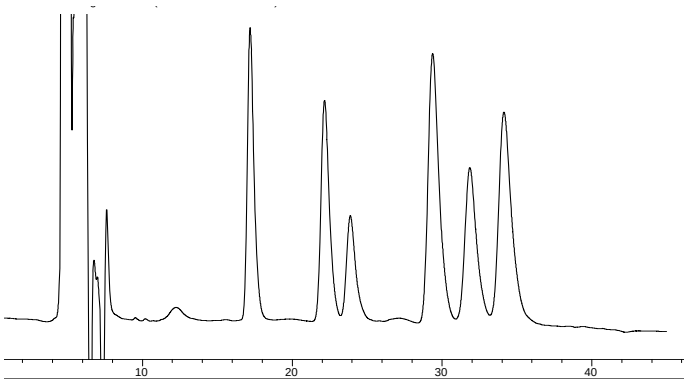
Peak 1: 8.98 min Tyr-D-Ala-Phe-**D-Ala**-Tyr-NH2
Peak 2: 9.91 min Tyr-D-Ala-Phe-**D-Pro**-Tyr-NH2
Peak 3: 12.41 min Tyr-D-Ala-Phe-**Hyp**-Tyr-NH2

Complementary Effect: T/T2
6 Enkephalins

CHIROBIOTIC T, 250x4.6mm



CHIROBIOTIC T2, 250x4.6mm



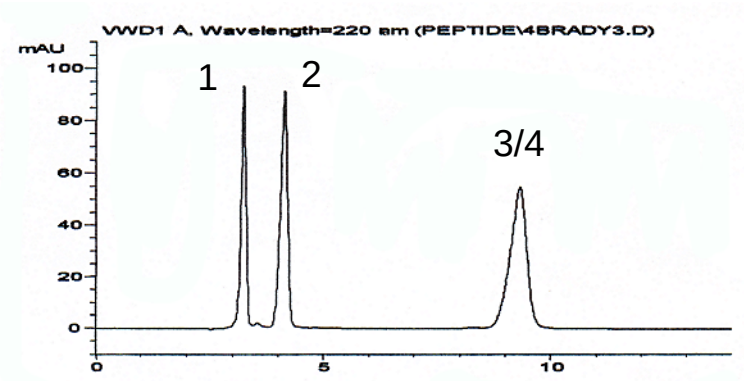
Mobile phase:	75/25: ACN/ 5mM NH_4Formate, pH 3.3
Temperature:	25˚C
Flow rate:	0.5mL/min
UV:	220nm

Best result

Column:
CHIROBIOTIC T2, 250x4.6mm

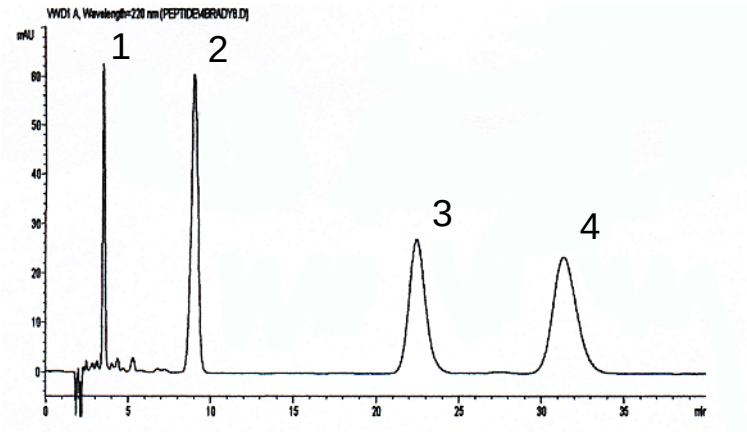
Complementary Effect: T2/TAG
4 Bradykinins

CHIROBIOTIC T2, 250x4.6mm



Mobile phase: 40/60: ACN/0.1% HCOOH
Flow rate: 1.5 mL/min
Temperature: 35˚C
UV: 220 nm

CHIROBIOTIC TAG, 250x4.6mm

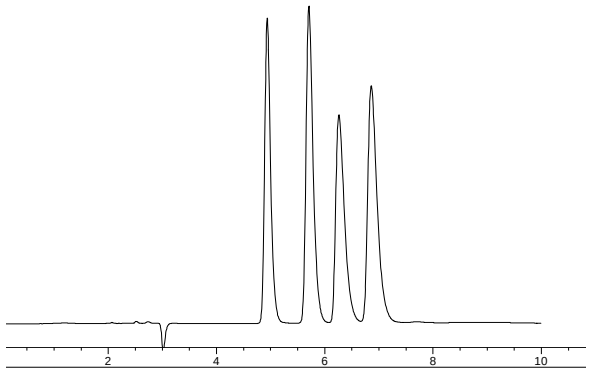


Best result

Column:
CHIROBIOTIC TAG, 250x4.6mm

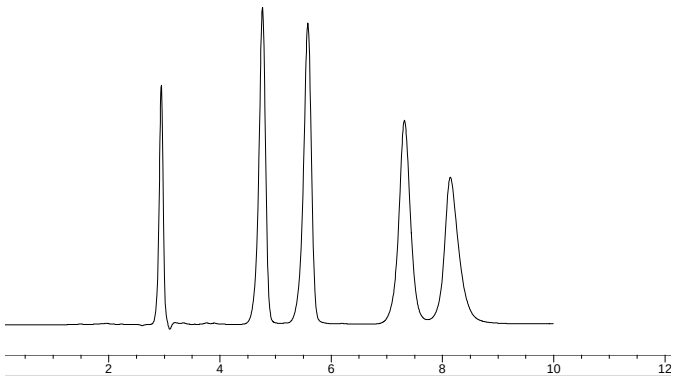
Best Conditions: Di-peptides

Sample: DL-Ala-DL-Val



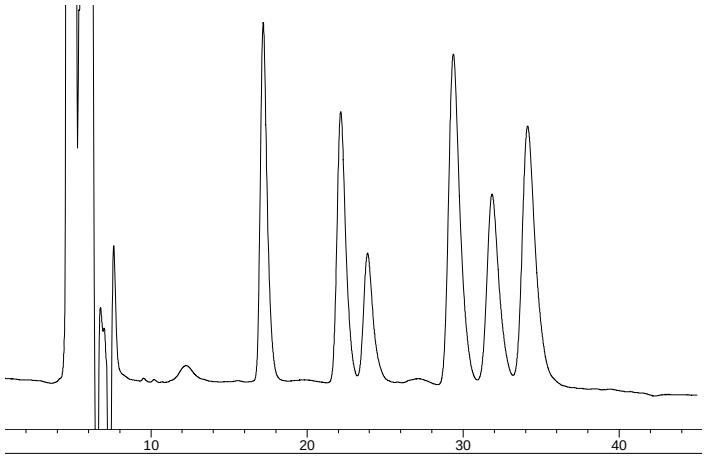
Column:	CHIROBIOTIC T, 250x4.6mm
Mobile phase:	50/50: ACN/10mM NH_4OAc, pH 4.1
Temperature:	35˚C
Flow rate:	1 mL/min
UV:	220 nm

Sample: DL-Ala-DL-Phe



Column:	CHIROBIOTIC T, 250x4.6mm
Mobile phase:	50/50: ACN/5mM NH_4OAc, pH 4.1
Temperature:	25˚C
Flow rate:	1 mL/min
UV:	220 nm

Application: 6 Enkephalins



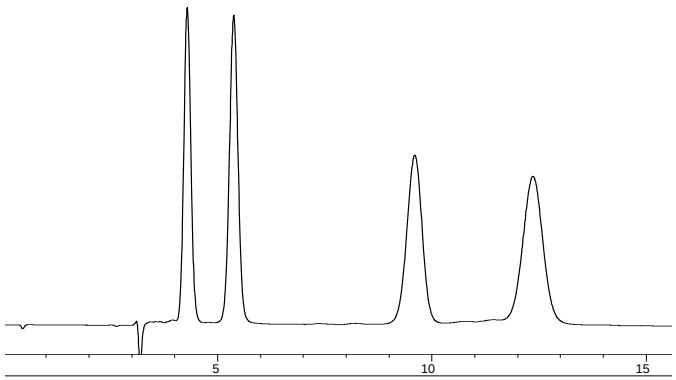
Column:	CHIROBIOTIC T2, 250x4.6mm
Mobile phase:	75/25: ACN/5mM NH_4Formate, pH 3.3
Temperature:	25˚C,
Flow rate:	0.5 mL/min
UV:	220 nm

- 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

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

Application: Luteinising Hormone-Releasing Hormone

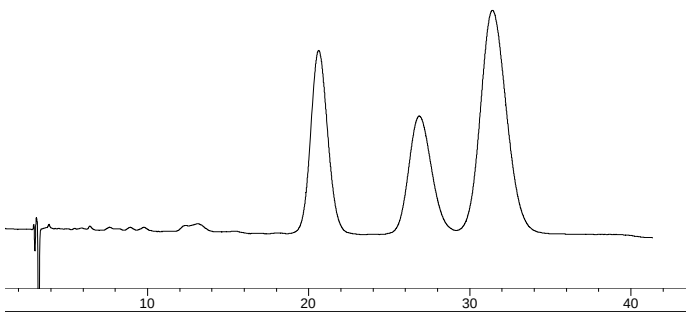
Sample: LHRH



Column:	CHIROBIOTIC T2, 250x4.6mm
Mobile phase:	40/60: ACN/0.1% HCOOH
Flow rate:	1.0 mL/min
Temperature:	35˚C
UV:	220 nm

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

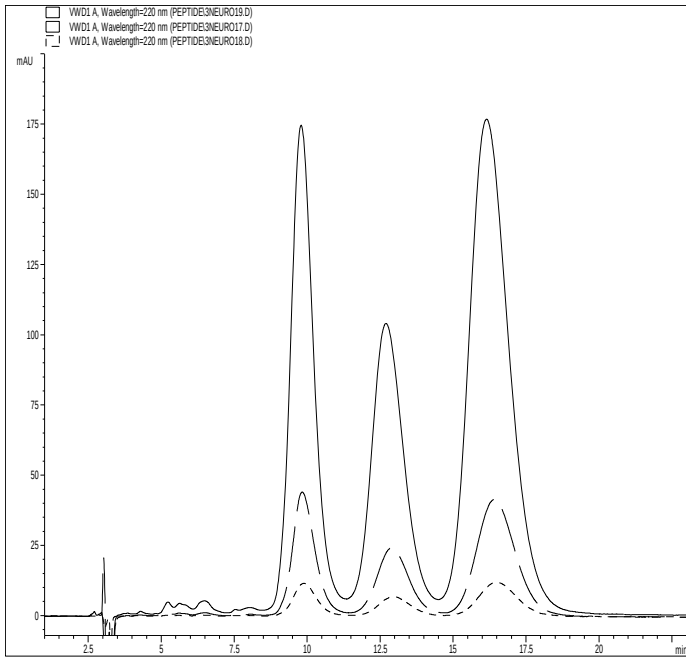
Application:
3 Neurotensins (13 AA residues)



Column:	CHIROBIOTIC T2, 250x4.6mm
Mobile phase:	25/75: ACN/0.1% HCOOH
Flow rate:	1.0 mL/min
Temperature:	35˚C
UV:	220 nm

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

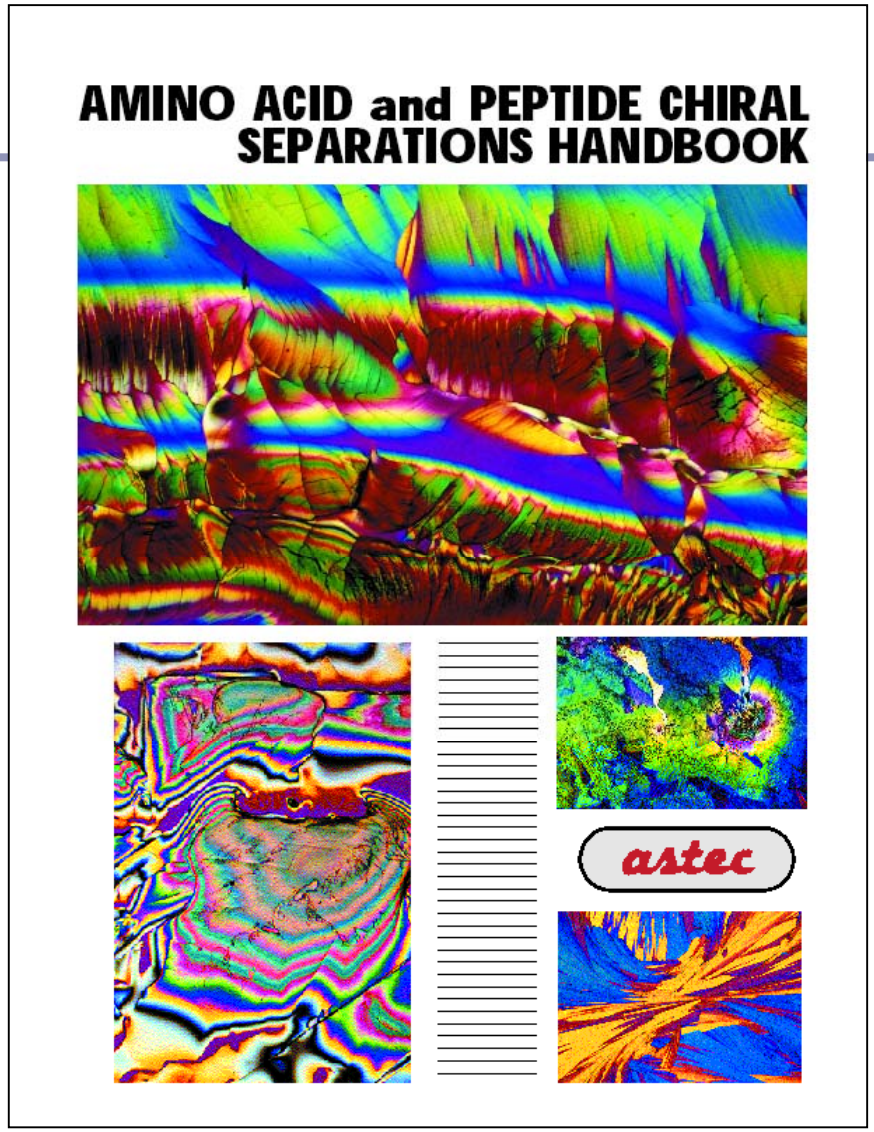
No Overloading Issue
ex: 3 Neurotensins



Column:	CHIROBIOTIC T2, 250x4.6mm
Mobile phase:	20/80: ACN/0.2% HCOOH
Temperature:	35˚C
Flow rate:	1mL/min
UV:	220nm
Load:	Each peptide contains:
_____	2µg
----------	0.5µg
……	0.14µg

CONCLUSIONS

- Using ACN/buffer as the mobile phase, the retention of peptides was usually found to be the shortest when the % ACN was in the 40-60% range.
- Higher buffer concentrations were found to correlate with lower retentions.
- Some elution order changes were observed between CHIROBIOTIC T and T2.
- Mobile phase compositions are LC-MS compatible utilizing APCI or ESI mode.
- The methods reported are flexable in composition and operating conditions allowing an orthogonal platform to be developed, if necessary.
- Sample capacity is higher then traditional C_{18} phases.
- Peptide families with up to 13 amino acid units have been successfully separated for sequence variations/deletions, even chiral analogues.
- Complementary effects among CHIROBIOTIC T/T2/TAG.



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