



# Ion Pair Supercritical Fluid Chromatography of Isomeric Protect and Unprotected Polypeptide Pairs

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# Introduction

The characterization and determination of peptides is of great importance in the pharmaceutical industry as is the ability to rapidly perform targeted determinations of bioactive peptides in complex matrices. Traditionally, peptides are separated using reversed phase HPLC[1], however the separation are often difficult or impossible due to the complex nature of peptides mixtures. Supercritical fluid chromatography (SFC) can provide advantages in many of these areas where traditional HPLC of peptides has encountered challenges. We have previously reported the separation of protected peptides by SFC[2], we have extended this study to a pair of unprotected peptides pairs of the same mass but different amino acid sequence. This poster will discuss the results we have obtained.



# Experimental

The uncapped peptides used for this research were synthesized by CPC Scientific Inc. (San Jose, CA) and were supplied as salts of trifluoroacetate (Table 1). Columns of different stationary phases, particle size and pore size were evaluated. All columns used for this study were 4.6x250mm. Each column was pre-equilibrated with 95:5 CO<sub>2</sub>: modifier for 180sec. The flow rate was 2mL/min and the back pressure regulator was set at 100 bar. The oven temperature was set to 40°C. The concentration of each peptide in methanol was ~ 1.0mg/mL and 0.5 mg/mL per peptide for the two component mixture. The modifier was 100% methanol or 90:10 Methanol:Water and the additives were 0.2% (v/v) trifluoroacetic acid (TFA), 0.1% (v/v) iso-propylamine (IPAm) + 0.1% TFA. The linear gradient schedule used throughout this study is shown in Table 2. A Thar/Waters analytical SFC and ZQ mass spectrometer with an electrospray ion mode with EMPOWER control was used for the experiments.



# Table 1: Summary of Unprotected Peptide Samples

Product #	Lot #	Sequence	Molecular Wt
831057	CI-09-00444	GLY- <u>VAL</u> -LEU-GLY-LEU-ALA-LEU-GLY- <u>GLY</u> -LEU-LYS-LYS	1125.4
831058	CI-09-00457	GLY- <u>GLY</u> -LEU-GLY-LEU-ALA-LEU-GLY- <u>VAL</u> -LEU-LYS-LYS	1125.4
831055	CI-09-00447	GLY- <u>PHE</u> -LEU-GLY-LEU-ALA-LEU-GLY- <u>GLY</u> -LEU-LYS-LYS	1173.5
831056	CI-09-00448	GLY- <u>GLY</u> -LEU-GLY-LEU-ALA-LEU-GLY- <u>PHE</u> -LEU-LYS-LYS	1173.5

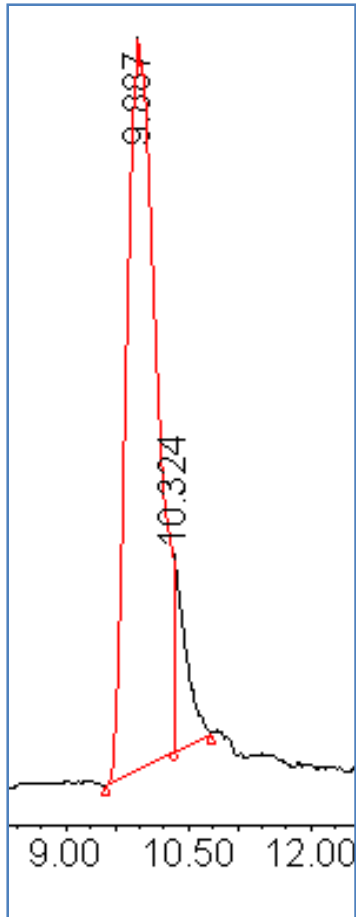
## Table 2: Generic Gradient Table

Time (min)	% CO <sub>2</sub>	% Modifier
0	95	5
1.0	95	5
10.0	50	50
15.0	50	50
17.0	95	5

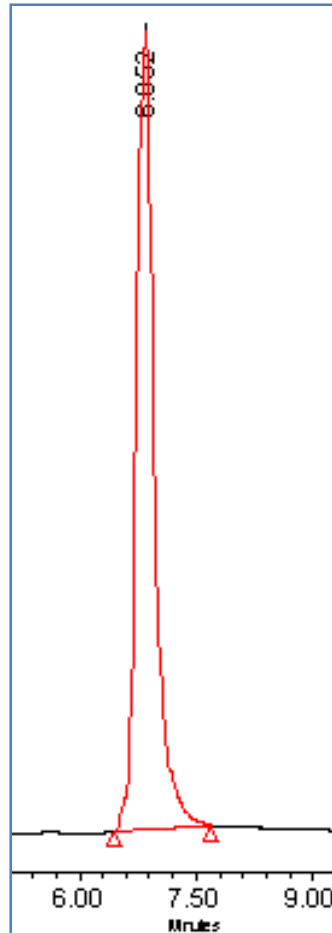




## Figure 2: Unprotected Peptides on 2-Ethyl Pyrdine Column



0.2% TFA-MeOH



90:10 MeOH:H<sub>2</sub>O  
With 0.2% TFA



90:10 MeOH:H<sub>2</sub>O  
With 0.1% TFA + 0.1% IPAm

# Figure 3: Unprotected Peptides on C8 Column



0.2% TFA-MeOH



90:10 MeOH:H<sub>2</sub>O  
With 0.2% TFA



90:10 MeOH:H<sub>2</sub>O  
With 0.1% TFA + 0.1% IPAm



# Discussion

The goal of this research was to demonstrate the feasibility of packed column SFC for chromatographically isolating individual unprotected peptides of identical mass, composition, and charge that differ only in amino acid sequence. The experiments in this study showed that:

- Chromatographic resolution of the unprotected peptide pairs was achieved on a Silica column when 5% water was added to 0.2% TFA-Methanol modifier (Figure 1). Addition of 5% water to 0.2% TFA-Methanol modifier made no difference in resolution on the 2-EP column. The Diol column exhibited similar resolution behaviour as the Silica column but complete resolution was not achieved.
- Unlike the protected peptides, 0.2% TFA-MeOH was unsuccessful for the resolution of the unprotected peptide pairs on the EP or Amino columns (Figure 2). Addition of Water and Isopropyl amine to the modifier made no significant difference on the resolution of the critical pair.



# Discussion continued/Conclusions

- The unprotected peptides had better elution on the non-polar columns such as C8, C4 and C18 when water was added to the modifier. Addition of Isopropyl amine to the modifier also proved to improve peak shape when compared to TFA-MeOH only (Figure 3)



# Summary/future work

- Two pairs of water soluble protected peptides with molecular mass approximately 1200Da that differ only in amino acid sequence have been separated by SFC.
- Detection of the peptides was done both by a mass spectrometer and an evaporative light scattering detector
- Water appears to aid the elution of peptides from many non-polar and polar stationary phases
- 2-EP and 4-EP failed to yield a good separation of isomers of unprotected peptides; whereas they were successful with the protected peptides
- Bare silica appears to be the only phase that yields isomer resolution

## Future work:

- Investigate the effect of water on silica column
- Evaluate the use of water on silica column for the protected peptides



# References

1. J. W. Eschelbach, J. W. Jorgenson, "Improved Protein Recovery in Reversed Phase Liquid Chromatography by the Use of Ultrahigh Pressures", *Anal. Chem.*, 78, 2006, 1697-1706.
2. M.A. Patel, F. Riley, J. Wang, M. Lovdahl, L.T. Taylor, "Packed column supercritical fluid chromatography of isomeric polypeptide Pairs", *Journal of Chromatography A*, Volume 1218, Issue 18, 6 May 2011, Pages 2593-2597