



# Packed Column Supercritical Fluid Chromatography of Isomeric Protected 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 peptide mixtures. Supercritical fluid chromatography (SFC) can provide advantages in many of these areas where traditional HPLC of peptides has encountered challenges. In this regard, we wish to report a study concerned with packed column SFC separation of two pairs of specially synthesized, capped polypeptides wherein each pair has the same molecular weight and amino acid content, but the sequence within each pair has two amino acid fragments interchanged.

## EXPERIMENTAL

The capped peptides used for this research were synthesized by CPC Scientific Inc. (San Jose, CA) and were supplied as salts of trifluoroacetate (Table 1). Various 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 ~ 0.4mg/mL and 0.2 mg/mL per peptide for the two component mixture. The primary modifier was 100% methanol and the additives were 0.2% (v/v) trifluoroacetic acid (TFA) in methanol, 0.2% (v/v) iso-propylamine (IPAm) in methanol, and 10mM NH<sub>4</sub>OAc (AA) in methanol. 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 Peptide samples

Lot Number	Sequence	purity	Mass
CI-05-00551	Ac-GGLGLALGV LKK-NH <sub>2</sub>	97.9%	1166.4
CI-05-00547	Ac-GVLGLALGGLKK-NH <sub>2</sub>	99.0%	1166.4
CI-05-00541	Ac-GFLGLALGGLKK-NH <sub>2</sub>	96.1%	1214.5
CI-05-00544	Ac-GGLGLALGFLKK-NH <sub>2</sub>	96.0%	1214.5

Table 2: Generic Gradient table

Time (sec)	% CO <sub>2</sub>	% Modifier
0	95	5
60	95	5
540	50	50
300	50	50
120	95	5

## RESULTS

Figure 1: HA-Pyridine with 0.2% TFA-MeOH

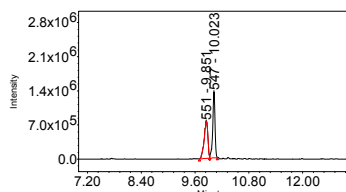


Figure 2: Aminopropyl with 0.2% TFA-MeOH

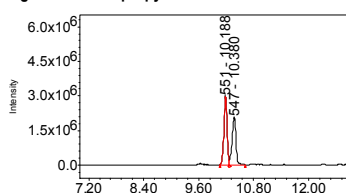


Figure 3: 2-ethyl pyridine with 0.2% TFA-MeOH

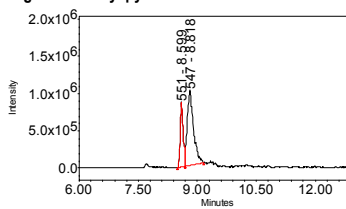


Figure 4: HA-Pyridine with 0.2% IPAm-MeOH

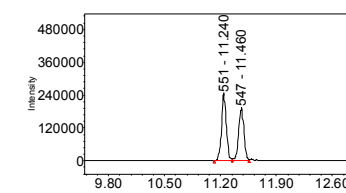


Figure 5: 4-Ethylpyridine with 0.2% IPAm-MeOH

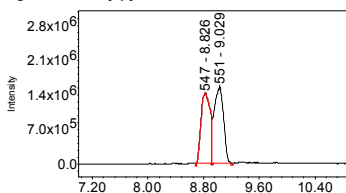
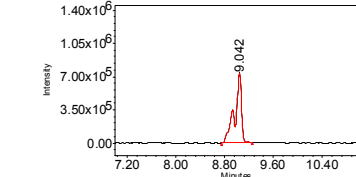
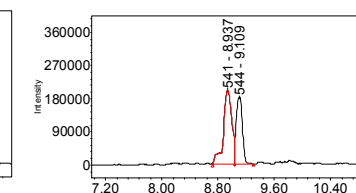
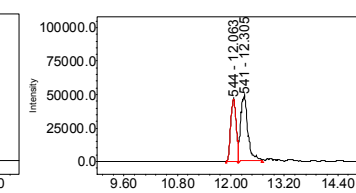
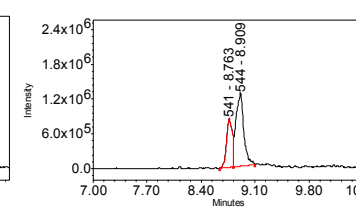
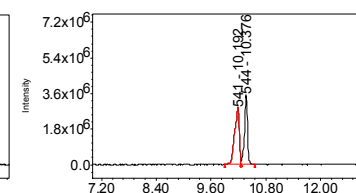
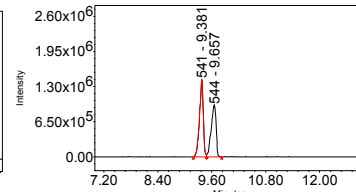
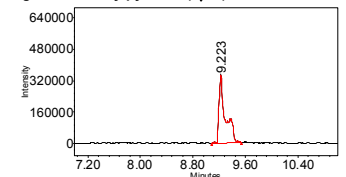


Figure 6: 2-Ethylpyridine (3µm) with 0.2% IPAm-MeOH



## DISCUSSION

The goal of this research was to demonstrate the feasibility of packed column SFC for chromatographically isolating individual peptides of identical mass, composition, and charge that differ only in amino acid sequence. All the chromatograms presented in this poster are extracted ion chromatograms for the mass of each specific peptide. The experiments in this study showed that:

- 0.2% TFA-MeOH was most successful modifier for elution of the four peptides
- Ammonium acetate and 2-propylamine showed poor results on all columns except the HA-Pyridine column
- Injection of individual peptides yielded a single highly sharp peak on all columns except on the phenyl hexyl column.
- Partial to nearly baseline separation of the isomeric pairs was obtained on the HA-Pyridine, Aminopropyl and the 2-ethyl pyridine (5 µm) columns when 0.2% TFA-MeOH was used (Figure 1 – 3).
- HA-Pyridine also showed partial separation of the isomeric peptide pairs when 0.2% 2-propylamine was used (Figure 4)
- HA-Pyridine phase was slightly favored over the amino propyl phase and the ethyl pyridine phase.
- Phenyl-Hexyl, Hilic Diol and Silica columns did not exhibit the same degree of resolution for the isomeric peptide pairs

Additional experiments were performed to determine if chromatographic resolution of the peptide pairs could be enhanced by making small modifications such as changing particle size and phase chemistry. Experiments show that:

- Small modifications to the stationary phase (i.e. 4-ethyl substituted for 2-ethyl) with identical particle and pore size actually resulted in loss of resolution (Figure 5).
- Using the smaller particle size (3µm) 2-Ethylpyridine column did not afford better resolution, in fact, the resolution was further diminished (Figure 6).

In summary, two pairs of water soluble protected peptides with molecular mass approximately 1200Da that differ only in amino acid sequence have been separated by SFC with mass spectrometric detection. Work is underway to determine if comparable resolution can be achieved with unprotected peptides that differ solely in amino acid sequence

## 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.

## ACKNOWLEDGMENTS

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