Separation of Peptides by Hydrophilic Interaction Chromatography Using TSKgel® Amide-80

TSKgel
APPLICATION NOTE

Abstract

The TSKgel® Amide-80 column provides excellent selectivity for separating peptides in hydrophilic interaction chromatography due to polar carbamoyl functionalities bonded to a silica base material.

Introduction

The separation of peptides with many acidic and basic residues has always proved to be problematic with RPLC gradient elution runs common in LC/MS and/or high throughput methodologies. Several options are available to increase retention of polar peptides including mobile phase adjustment[1] or switching to a polar adsorptive mechanism used in both normal phase and hydrophilic interaction chromatography (HILIC)[2,3]. HILIC methods are often advantageous because aqueous and polar organic mobile phase systems, common to RPLC, are acceptable. The data presented within will highlight the rational, recovery and reproducibility associated with a HILIC method developed within Tosoh laboratories for the separation of peptides.

Experimental Conditions

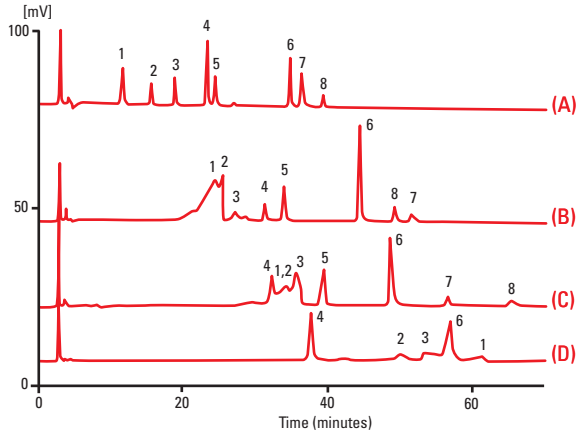
A 5µm TSKgel Amide-80 column (4.6mm ID x 25cm) was used in conjunction with a Tosoh HPLC system. The peptides were purchased from the Peptide Institute (Osaka, Japan) and Sigma (St. Louis, MO). Peptide products from the Peptide Institute (http://www.peptide.co.jp/en/) are available as 500µg per vial. After weighing out 250µg of peptide, a 10µL solution of acetonitrile:water:formic acid=5:50:50 or water:formic acid=50:50 was added to the peptide. If needed to aid peptide dissolution, 10µL of formic acid was added. An additional volume of 90µL acetonitrile was added to the peptide solution to obtain the final peptide samples with concentrations of 250µg/100µL or 250µg/110µL. Of each peptide sample, we injected 10 to 50µL into the HPLC system.

Final mobile phase conditions: Eluent A (initial eluent) contained 0.1% trifluoroacetic acid (TFA) in acetonitrile (ACN):water (97:3) and Eluent B contained 0.1% TFA in ACN:water (55:45). The peptides were dissolved in 10µL of ACN:water:formic acid (5:45:50) and subsequently diluted to a final volume of 50µL with Eluent A. A linear gradient from A to B was administered at 1.0mL/min over 70 minutes at 0.6% water/min. UV Detection was monitored at 265nm.

Results

In the study to find suitable method conditions for the separation of peptides by HILIC, the effect of acid and ACN concentration were examined. It is commonly understood that residual silanols, present with functionalized silica based materials, can interact with the ionic residues of peptides. This interaction affects recovery and/or causes peak tailing. For this reason, TFA, acetic acid, formic acid and no acid were investigated to determine the appropriate acid strength for eliminating IEX interactions. As shown in *Figure 1*, elution order and peak shape were affected differently depending the acid investigated. Without the addition of acid, three of the peptides failed to elute and tailing was evident on the remaining peptides. The addition of weak acids (acetic or formic) allowed the elution of all components but did not eliminate tailing. TFA at 0.1% was strong enough to eliminate tailing and sequester the apparent IEX interactions. Subsequent work (not shown) indicates little improvement with the addition of equal molar amounts of TFA and TEA at 0.1% or 0.2% over what is possible with TFA alone[4].

Figure 1. Effect of Various Acids on Separation of Peptides with TSKgel Amide-80.



Effect of various acids on separation of peptides with TSKgel Amide-80. The conditions are as mentioned in experimental conditions with the exception of the elimination of replacement of the acid for experiments B-D. (A) 0.1% TFA (B) 0.1% formic acid (C) 0.1% acetic acid (D) no acid. The corresponding eight sample peaks to peptide sequence is provided in *Table 1*.

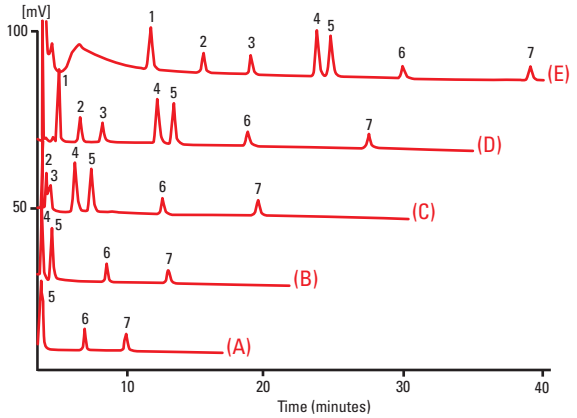
Table 1. Recovery of Peptides from TSKgel Amide-80 with a Sample Injection of approx 1µg.

Peak/Peptide Number	Sequence	Recovery from TSKgel Amide-80	(%) Recovery from TSKgel ODS-80T$_S$*
1	FY	96	96
2	FGGF	101	89
3	FLEEI	98	93
4	DYMGWMDP-NH2	90	74
5	NFTYGGF	90	95
6	AGSQ	96	65
7	WAGGDASGE	85	96
8	YGGFMTSQKSQTPLVT	92	96
9	ASTTTNYT	94	89
10	VLSEGEWQLVLHVW AKVEADVAGHGQDI LIRLFKSHPETLEKFD RFKHLKTEAEM	80	62

*TSKgel ODS-80T$_S$ run was at 83.3 min. linear gradient of ACN from 5 to 55% in 0.1% TFA

The advantage of using TFA to eliminate IEX interactions is the need for desalting is eliminated when a volatile mobile phase is used. PolyHydroxyethyl A, {poly (2-hydroxyethyl aspartamide) silica} a commonly used HILIC column for peptides, has been reported to exhibits IEX interactions[5,6]. Thus, salts or non-volatile acids such as orthophosphoric acid are recommended in the mobile phase to eliminate such effects. Peptide isolation subsequently becomes more tedious with the use of a PolyHydroxyethyl A type column.

Figure 2. Effect of ACN Concentration in Initial Mobile Phase with TSKgel Amide-80.

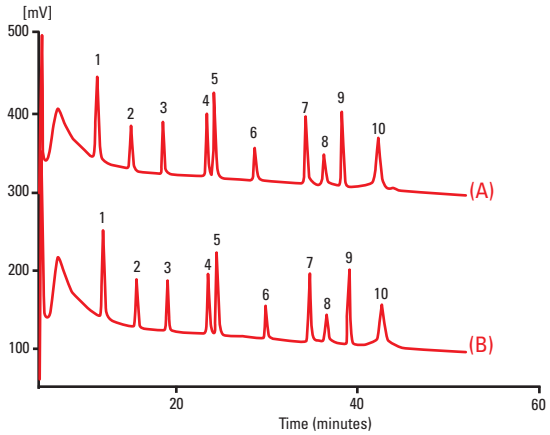


Effect of ACN concentration in initial mobile phase with TSKgel Amide-80. The conditions are as mentioned in experimental conditions with the exception of eluent A ACN:water ratio for experiments A thru D. (A) 75:25 (B) 80:20 (C) 85:15 (D) 90:10 (E) 97:3 Corresponding eight sample peaks to peptide sequence is provided in *Table 1*.

The effect of ACN concentration on peptide retention was also examined. Opposite of RPLC, increasing the percent organic increases retention of polar compounds with HILIC stationary phases. As evident in *Figure 2*, the higher the initial concentration of ACN, the better the resolution. Resolution of all peptides is possible above an 85% initial ACN concentration. Furthermore, recovery of the various peptides averaged 92% as shown in *Table 1*. Recovery with the TSKgel Amide-80 column was higher when compared to a column packed with an 80Å pore size, end capped ODS material[3].

Reproducibility and repeatability were determined by examining both the variability between injections and the variability between columns. Ten replicates of a peptide mixture were injected on a new TSKgel Amide-80 column and a TSKgel Amide-80 column that had seen over 500 injections. As expected, the coefficient of variation for the retention time of the various peptides between injections averaged below 0.4% for both columns.

Figure 3. Comparison of Peptide Separation Chromatograms.



Comparison of peptide separation chromatograms on (A) used TSKgel Amide-80 column and (B) new TSKgel Amide-80 column. Corresponding ten sample peaks to peptide sequence is provided in *Table 1*.

The coefficient of variation for peak heights, which relates directly to efficiency, averaged 1.2% and 2.4% for the new and used columns respectively. *Figure 3* provides a visual comparison of traces from a new and used column indicating that good reproducibility and long lifetimes can be achieved with this method and column type.

Conclusions

The separation of polar compounds has long been problematic for reasons related to retention, peak shape and reproducibility. The data shared within indicates the superior performance of both the TSKgel Amide-80 column and HILIC method for the separation of peptides. The carbamoyl stationary phase of TSKgel Amide-80 offers a unique selectivity and long lifetime for scientists searching for alternatives to separating polar compounds.

References

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