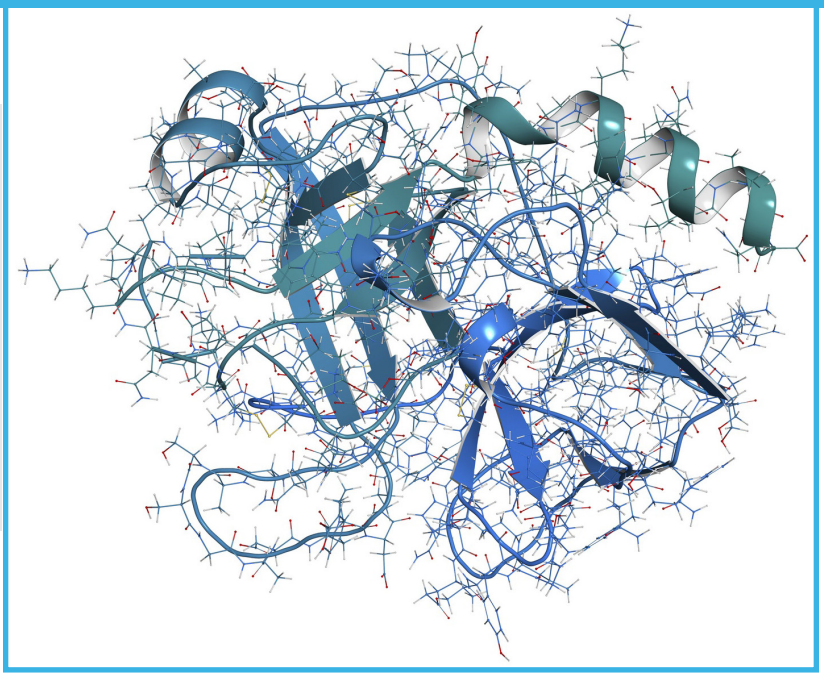
HALO®

TECHNICAL REPORT: AMT_TR_BIO_24

TITLE: **IMPROVED BIOSEPARATIONS WITH HALO® PCS C18 PEPTIDE**

MARKET SEGMENT: BIOCLASS



AUTHOR:

Stephanie Schuster, Ph.D.
Senior Technical Support Scientist

ABSTRACT

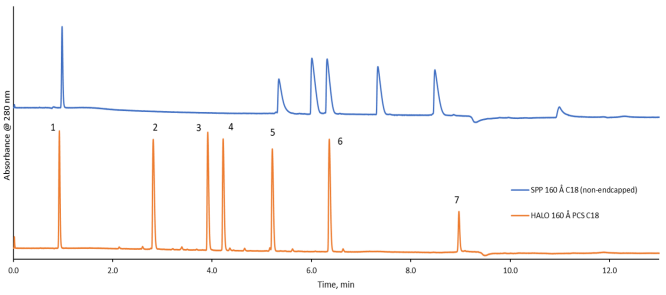
A new stationary phase with a positively charged surface (HALO 160 Å PCS C18) is available for analyses of peptides using low ionic strength mobile phase. The advantages of such a phase are highlighted in comparisons to a standard peptide phase.

INTRODUCTION

HALO® PCS C18 Peptide is a reversed-phase stationary phase designed for LC and LC-MS separations of peptides, tryptic digests, and post-translational modifications using low ionic strength mobile phase conditions. The stationary phase is composed of both positively charged ligands and C18 ligands to give improved peak shape, improved sample loading, and better impurity characterizations when using low ionic strength mobile phases, such as formic acid. HALO® PCS C18 Peptide is built on a 2.7 µm Fused-Core® particle with 160 Å pores in the 0.5 µm thick silica shell.

When acidic pH mobile phase conditions are used, basic compounds become protonated. For peptides, basic amino acids in the sequence become protonated. This can lead to peak tailing. There are a few reasons why the tailing occurs, but none have been absolutely confirmed. One of the leading possibilities is that repulsion between the positively charged compounds prevents additional positively charged compounds from being able to access the available surface area.[1] A stationary phase with a positive charged surface (PCS) uses chemistry which is positively charged at pH conditions less than 5.5. With a positive charge surface stationary phase, the repulsion is reduced among the analytes and leads to improved peak shapes (reduced tailing). This type of stationary phase shows advantages for LC and LC-MS of peptides.[2]

For example, Figure 1 shows the same separation run using a traditional C18 column (blue trace) compared to a HALO® PCS C18 Peptide column (orange trace). The peak shapes are much narrower with reduced tailing on the HALO® PCS C18 Peptide column compared to the traditional C18 column. The retention is decreased with the HALO® PCS C18 Peptide column since there is repulsion between the positively charged phase and the positively charged amino acids present in the peptide sequences.



Test Conditions:
HALO 160 Å PCS C18,2.7µm, 2.1x100 mm; Comparison:SPP 160 Å C18, 2.7 µm, 2.1 x 100 mm; Mobile Phase A: Water/ 0.1% Formic Acid; Mobile Phase B: Acetonitrile/ 0.1% Formic Acid; Gradient: 2-35%B in 10min; 30°C; 0.3 mL/min; 280nm;1 µL

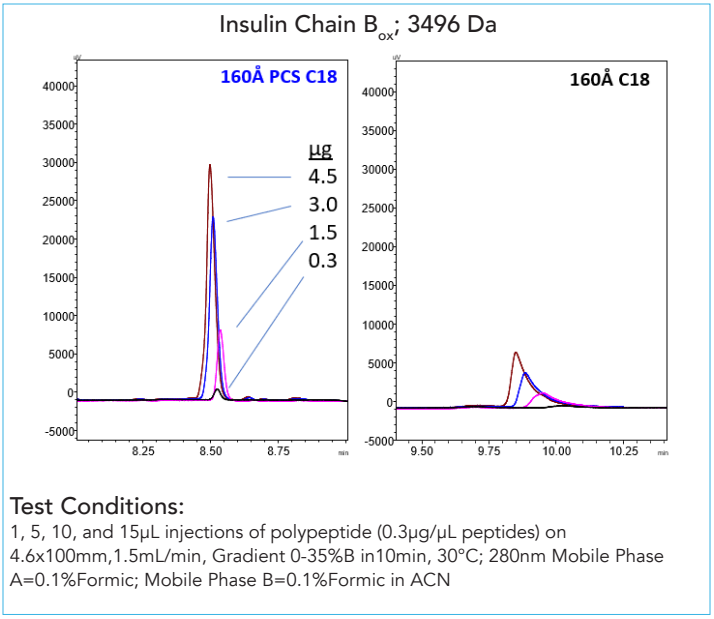
Peaks:
1.Uracil
2. S1Y Sequence: RGAGGLYLGK-NH2
3. S2Y Sequence: Ac-RGGGGLYLGK-NH2
4. S3Y Sequence: Ac-RGAGGLYLGK-NH2
5. S4Y2 Sequence: Ac-RGVGYLGLGK-NH2
6. S5Y Sequence: Ac-RGVVGLYLGK-NH2
7. Insulin Chain B Oxidized

Figure 1. Improved peak shape with the HALO® PCS C18 Peptide column compared to a traditional C18 column using a gradient with water/ ACN/0.1% formic acid for a separation of 5 peptides and insulin B_{ox}.

KEY WORDS:

Positively charged phase, peptides, tryptic digests, antibody-drug conjugates

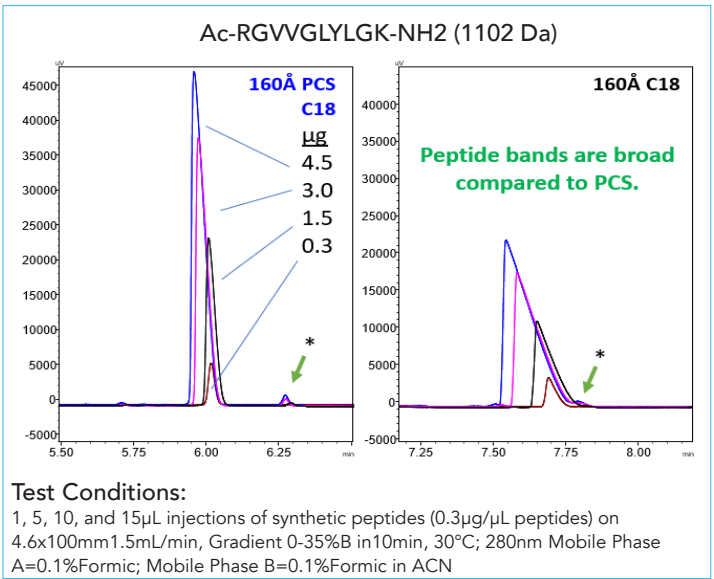
The HALO® PCS C18 Peptide column also demonstrates improved sample loading compared to a standard Peptide C18 column. The results in Figure 2 show sharp, narrow peaks for insulin B_{ox} from sample loads of 0.3 to 4.5 µg on column. This is contrasted to the broad, tailed peaks that are observed on the standard Peptide C18 column.



Test Conditions:
1, 5, 10, and 15µL injections of polypeptide (0.3µg/µL peptides) on 4.6x100mm,1.5mL/min, Gradient 0-35%B in10min, 30°C; 280nm Mobile Phase A=0.1%Formic; Mobile Phase B=0.1%Formic in ACN

Figure 2. Sample loading using insulin B_{ox} chain. The results using the HALO® PCS C18 Peptide column maintain their sharpness and do not exhibit tailing in contrast to the results observed on the standard Peptide C18 column.

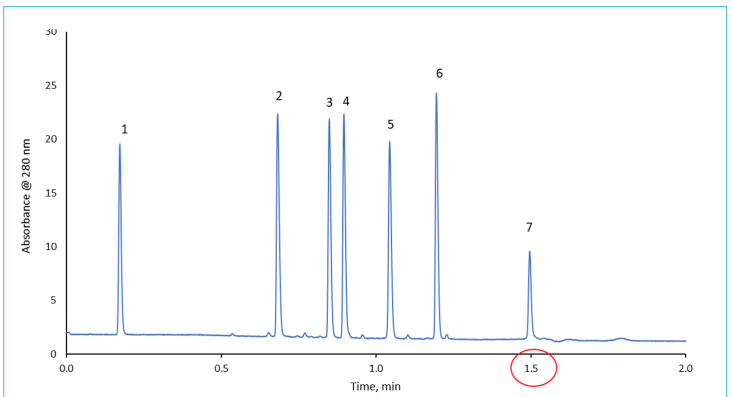
A similar loading study was conducted using a synthetic peptide which was 1102 Da and contained a small impurity. In this experiment, the resolution of the impurity peak was maintained across the range of 0.3 to 4.5 µg loaded on column for the HALO® PCS C18 Peptide column. However, resolution of the impurity peak was lost when the standard Peptide C18 column was used since the main peak showed increased tailing which then obscured the impurity peak. The results for this experiment can be found in Figure 3.



Test Conditions:
1, 5, 10, and 15µL injections of synthetic peptides (0.3µg/µL peptides) on 4.6x100mm1.5mL/min, Gradient 0-35%B in10min, 30°C; 280nm Mobile Phase A=0.1%Formic; Mobile Phase B=0.1%Formic in ACN

Figure 3. Sample loading experiment highlighting the presence of an impurity indicated by an asterisk, which is clearly visible on the HALO® PCS C18 Peptide column, but obscured on the standard Peptide C18 column.

The HALO® PCS C18 Peptide column is also capable of rapid separations due to the Fused-Core® or superficially porous design of the particle with a solid silica core and a porous silica shell. With a reduced path length for analytes to diffuse into and out of, the flow rate for HALO® columns may be increased to more than double the flow rate that is typically used for fully porous particles. This is made possible by the relatively flat van Deemter curves exhibited by HALO® columns.[3] To illustrate this see Figure 4 where a mix of peptides is separated with a 2.1 x 50 mm column using a flow rate of 1 mL/min in 1.5 minutes. For reference, the typical flow rate for a 2.1 mm ID FPP column would be 0.3 mL/min.

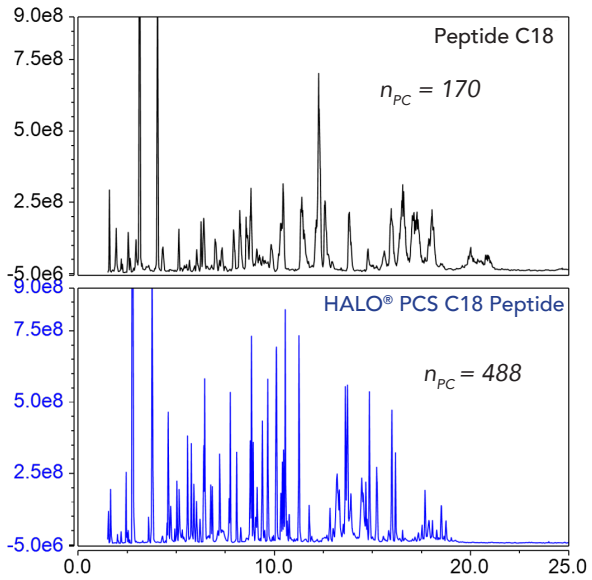


Peak Indentities:
1. Uracil
2. S1Y Sequence: RGAGGLYLGK-NH2
3. S2Y Sequence: Ac-RGGGGLYLGK-NH2
4. S3Y Sequence: Ac-RGAGGLYLGK-NH2
5. S4Y2 Sequence: Ac-RGVGYLGLGK-NH2
6. S5Y Sequence: Ac-RGVVGLYLGK-NH2
7. Insulin Chain B Oxidized

Test Conditions:
1µL of Synthetic Peptide Standard (0.3µg/µL) PCS C18 Peptide 2.1x50mm 2.7µm ,1mL/min 30°C (360bar), 0-35%B in 1.5min; Mobile Phase A=0.1% Formic Acid; Mobile Phase B=0.1% Formic Acid in ACN

Figure 4. Rapid separation completed in 1.5 minutes on a HALO® PCS C18 Peptide column.

A tryptic digest of trastuzumab was analyzed using a HALO® PCS C18 Peptide column and a standard Peptide C18 column. The results are shown in Figure 5. Peak capacities for both separations were calculated based on ID peptides with the HALO® PCS C18 Peptide column yielding a peak capacity of 488 compared to only 170 for the standard Peptide C18 column. The large increase in peak capacity is attributed to the much narrower peaks that are observed with the HALO® PCS C18 Peptide column.

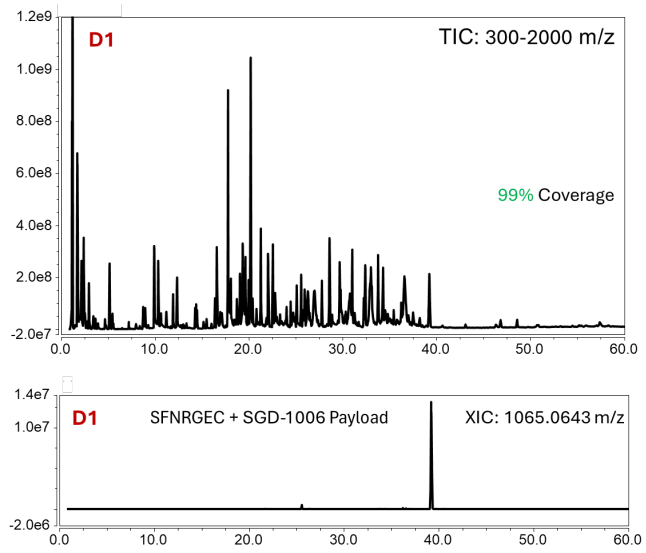


Test Conditions:
2.7 µm, 2.1 x 150 mm; 0.4mL/min; 60°C; 3-50% in 30 min; 2 µg tryptic digest; Mobile Phase A=0.1% Formic Acid in H_2O; Mobile Phase B=0.1% Formic Acid in ACN; Shimadzu NexeraX2 -> diverter valve -> QExactive HF (res=240,000)
MarvelXACT Post-Column Plumbing:
50 µm x 350 mm from column to diverter valve
50 µm x 350 mm from diverter valve to union
50 µm x 150 mm from grounding union to HESI II

n_{PC} based on ID peptides

Figure 5. The HALO® PCS C18 Peptide column shows more than double the peak capacity of the standard Peptide C18 column.

HALO® PCS C18 Peptide is available in 1.5 mm ID column dimension for increased signal and reduced solvent consumption. A HIC isolated sample of enfortumab vedotin-ejfv, which is an antibody-drug conjugate (ADC) used to treat bladder cancer, was reduced and alkylated. Then the sample was digested using trypsin. The ADC tryptic digest was run on a 1.5 x 150 mm HALO® PCS C18 Peptide column using MS detection. See Figure 6.



Test Conditions:
PCS C18 160 Å 2.7µm 1.5 x 150 mm: 0.25mL /min; 2-50 %B in 60 min; 60°C; Mobile Phase A = 0.1% Formic Acid in H_2O; Mobile Phase B = 0.1% Formic Acid in ACN

Figure 6. LC-MS of an ADC tryptic digest using a 1.5 x 150 mm HALO® PCS C18 Peptide column with XIC of the payload attached to tryptic peptide in the lower panel.

Using MS/MS to verify the result, a single L-chain cystine site was occupied by the payload. The selectivity of the column allows for the ability to obtain a clear spectrum that can be further fragmented and analyzed for features of the payload and the peptide sequence. Furthermore, 99% coverage of the protein sequence was achieved demonstrating the capabilities of the 1.5 x 150 mm HALO® PCS C18 Peptide column.

CONCLUSION:

The new positively charged HALO® PCS C18 Peptide column for peptides, tryptic digests, and post-translational modifications has the advantages of improved peak shape, sample loading, and better impurity characterizations compared to standard Peptide columns. It is ideal for use with low ionic strength mobile phases for LC and LC-MS to help solve challenging bioseparations.

REFERENCES:

1. *D. V. McCalley, Understanding and managing peak shape for basic solutes in reversed-phase high performance liquid chromatography. Chemical Communications, 2023, 59, 7887-7899.*
2. *J. Lenco, S. Jadeja, D. K. Naplekov, O. V. Krokhin, M. A. Khalikova, P. Chocholous, J. Urban, K. Broeckhoven, L. Nováková, and F. Svec, Reversed-Phase Liquid Chromatography of Peptides for Bottom-Up Proteomics: A Tutorial. Journal of Proteome Research, 2022, 21, 2846-2892.*
3. *J.J. DeStefano, T.J. Langlois, and J.J. Kirkland, Characteristics of Superficially-Porous Silica Particles for Fast HPLC: Some Performance Comparisons with Sub-2-µm Particles. Journal of Chromatographic Science, 2008, 46, 254-260.*