Charged-Surface Stationary Phase Improves Pepide Separations in Low Ionic Strength Mobile Phases

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Introduction

Superficially porous particles (SPPs) enable rapid, high-resolution separations with favorable compromise between permeability of packed beds (pressure) and excellent chromatographic efficiency. We have previously demonstrated that 160 Å pore size 2.7µm diameter particles are favored for highly efficient separations of peptides e.g. from proteolytic digests, synthetic peptides, and therapeutic biopolymers. The use of formic acid (FA) modified mobile phases for LC/MS separations of peptides in reversed-phase HPLC (RPC) can have limitations. Peptides are variably charged in weakly acidic mobile phases, but cationic species often show load dependent tailing in weak acids. The use of perfluorinated acid modifiers, such as trifluoracetic acid (TFA), yields superior peak shape and load tolerance in RPC, but poor LC/MS detection resulting from tight ion pair formation, leading to poor ionization efficiency. Our previous results have illustrated means to gain improvements in RPC, via mobile phase selection, while reducing ionization suppression for peptide and proteomic analyses (shown in Fig. 1; Johnson, et al., 2013 J. Biomol.Tech., 24, 187-197). Stationary phase manipulation presents another tool for method development. A novel 160Å 2.7µm SPP possessing a positive charge surface (PCS), with RPC retention afforded by the C18 stationary phase is shown for a variety of peptide and protein digest analyses..

Fig.1 Synthetic Peptide Mixture LC/MS in Several Acidic Modifiers

Peptide	Abbrev.	MW
[Leu5]-enkephalin	L	555.6
angiotensin I, human acetate hydrate	A	1297
substance P acetate salt hydrate	S	1348
Melittin, honey bee venom	M	2847
beta-endorphin, human	β	3465

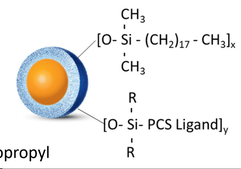
New PCS Surface Modification for Silica Surfaces

The novel reversed-phase positively charged surface chemistry modification has recently been developed by AMT, for modification of 90 Å and 160 Å pore size Spp silica particles. The controlled surface charge density suppled by this ligand is paired with suitable organosilane modified reversed-phase surfaces (in this case dimethyl-C18).

Reproducible RPC separations require total and relative density control of charged and hydrophobic surface ligands. A steric-protected positively charged ligand (diisopropyl silane) provides superior stability and performance with elevated temperature and acidic mobile phase use conditions common for peptide separations.

HALO 90 Å and 160 Å pore size, 2.7 µm PCS C18

- Excellent peak shape and increased loading capacity for basic compounds
- Alternate L1 selectivity (PCS C18)
- Alternate L11 selectivity (PCS Phenyl-Hexyl)
- Built upon Fused-Core® technology for fast,
- efficient and reliable separations



Peptide Separations Comparing Traditional and PCS Modified Surfaces

Fig.2 Peptide mixture resolution and peak shape in Formic Acid Mobile Phase

- Gradient separation of a QA mixture including 5 variant synthetic peptides + insulin B_{ox}
- Reduced retention time for HALO® PCS C18 Peptide compared to traditional Peptide ES-C18
- Improved peak widths and reduced tailing in formic acid

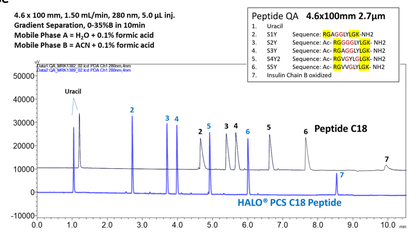


Fig.3 Peptide peak shape in Formic Acid Mobile Phase at increased load

- Varying load of a 10 residue synthetic peptide and insulin B_{ox} on a traditional and charged surface RPC material
- Notable improvement in load tolerance (peak width and peak tailing) on PCS as load increased from 0.3 to 4.5 µg on column

4.6x100mm; 1.5mL/min, 0-35%B in 10min, 30°C; 280nm
A=0.1%Formic; B=0.1%Formic in AcN, 0.3 µg/µL peptide

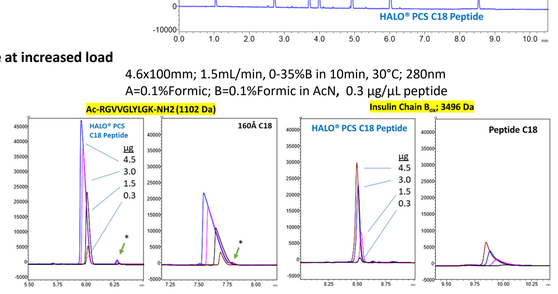
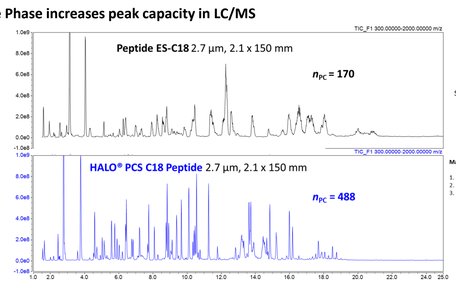


Fig.4 Tryptic digest analysis in Formic Acid Mobile Phase increases peak capacity in LC/MS

- Peak capacities (n_{PC}) measured with modest load (2 µg) of trastuzumab tryptic digest on a 2.1 mm ID column
- n_{PC} based on 12 ID peptides measured using extracted ions (XICs) $PW_{1/2}$, t_R and Δt_G for this specific sample set
- Decreased peak widths effect notable increase in peak capacity

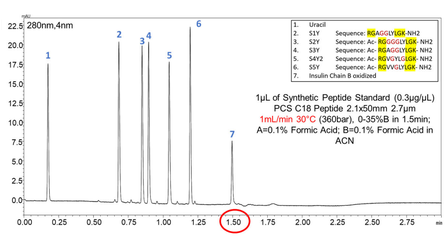


0.4mL/min; 60°C; 3-50% in 30 min; 2 µg tryptic digest
A=0.1% Formic Acid in H2O
B=0.1% Formic Acid in ACN
Shimadzu Nexera X2 -> diverter valve -> QExactive HF (res=240,000)

MarvelXACT Post-Column Plumbing:
1. 50 µm x 350 mm from column to diverter valve
2. 50 µm x 350 mm from diverter valve to union
3. 50 µm x 150 mm from grounding union to HESI II

Fig.5 Very high-speed analysis of a synthetic peptide standard on the 50 mm PCS C18 Peptide Column

- The highly efficient 160 Å pore superficially porous particle permits very high throughput analysis
- The example shows separation conducted in less than 2 minutes, with modest backpressure, even at moderate temperature.



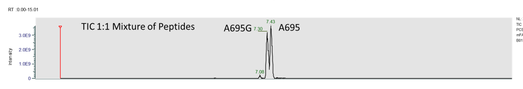
Application of PCS-C18 Peptide to Structure Analysis by LC/MS

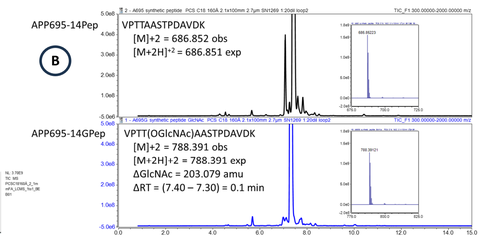
Fig.6 Peptide/Glycopeptide Pair Impurity Analysis

	Structure	Mass (mi.)	$[M+2H]^{+2}$
APP695-14Pep	$VPT^{291}T^{292}AASTPDAVDK$	1371.6881	686.8513
APP695-14GPep	VPTT(GlcNAc)AASTPDAVDK	1574.7675	788.3910

Synthetic peptide of 14 aa, glycosylated at position 4 (Thr292), purity by LC/UV = 98+%
What Impurities are present? How much Parental peptide is in the Glycosylated Peptide sample?

HALO® PCS C18 Peptide, 2.1 x 100 mm
0.5 mL/min, 2-12%B in 15 min, 30°C; A=0.1%Formic; B=0.1%Formic in AcN
QExactive HF, 200 ng each injected (0.4 µL)





- Resolution of the glycosylated (A695G) and unmodified (A695) peptide in RP (panel A)
- A695G shows minor impurities, including potential A695 impurity (panel B)
- XIC for A695 shows minor signal for in-source glycan loss A695G (*), and authentic small impurity (c. 0.1%) of A695 in the glycopeptide, based on response from spike sample signal for 1/500 level of A695 spike into A695G (panel C)

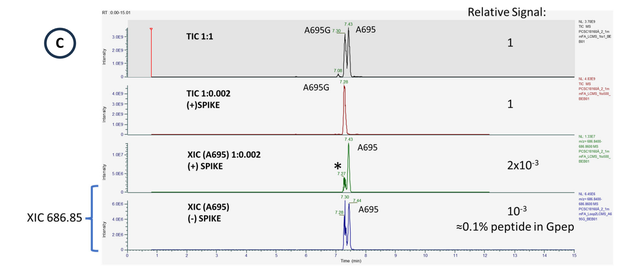
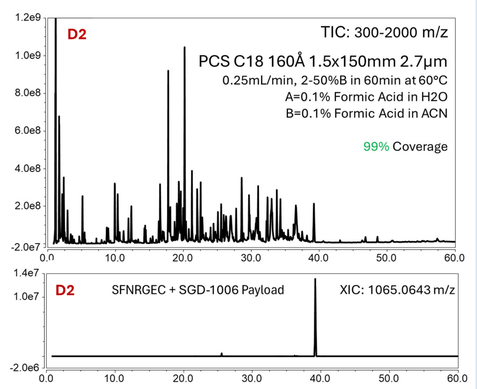


Fig.7 Tryptic digest LC/MS analyses to identify payload site for isolated DAR2 ADC

- HIC isolated mAb with 2 vedotin-ejfv payload conjugated to enfortumab was digested with trypsin. A single L-chain cystine site was occupied by the payload, with retention identified in the XIC, verified by MS/MS.



Conclusions

HALO® 160 Å PCS C18 exhibits favorable peak shape for peptides in weakly acidic or ion pairing mobile phases – expanding choice of mobile phased for effctive LC/MS.

HALO® PCS phases improve load tolerance in FA vs. traditional stationary phases – useful for analysis of less abundant variants/impurities.

All HALO® PCS phases exhibit the speed and resolution advantages of Fused-Core® superficially porous particles.

New material shows high resolution for complex mixtures, as obtained from fragments of mAbs and ADCs.

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