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Application Note

Maximizing Phosphopeptide Recovery in LC-MS Studies with MaxPeak High Performance Surfaces Technology

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Abstract

Interactions between transition metals in chromatography systems and analytes containing phosphate groups can lead to poor chromatographic performance or analyte loss. Waters has developed a class of new technologies, known as MaxPeak High Performance Surfaces (HPS), and these have been applied in the development of a new liquid chromatographic system called the ACQUITY Premier. When utilized in the components of an LC system and chromatographic column, MaxPeak HPS provides a highly effective surface barrier that mitigates undesired interactions with metal surfaces and can help in the analysis of certain compounds–phosphopeptides being one such class of analyte. The new technology, when applied to both the chromatographic system wetted components and column housing, can greatly improve the recovery of phosphopeptides. Some peptides analyzed in this study are found to go from not being detected with a standard system to very strong signals with an HPS fluidic path.

The data described in this note compares the performance between a conventional system and HPS treated

system to investigate the phosphopeptide response. Tryptic digests of Alpha and Beta casein, along with synthetic PhosphoMix standards, were analyzed and showed that recovery rates varied based upon the number of phosphorylation sites and number of amino acid residues. In extreme cases, it is found that some peptides go from not being observed at all with the conventional configuration to being detected with large ion counts, when using the HPS treated system.

Benefits

- Enhanced phosphopeptide detection
- Multiple site modified peptides observed
- Chromatographic peak shape improvement for modified peptides

Introduction

Despite the continued advances of mass spectrometry as an analytical technique, detection of phosphopeptides by liquid chromatography coupled with mass spectrometry (LC-MS) still remains a very challenging application in the field of proteomics. One major problem is that phosphopeptides do not protonate efficiently due to the presence of one or more acidic phosphate groups, making their detection difficult.[1] However, there are other significant mechanisms which contribute to the difficulties in phosphopeptide analysis by LC-MS.

Incomplete recovery from the wetted components of an LC system is another major cause of compromised phosphopeptide detection efficiency. Metal ions interact with the phosphate group via a lewis acid/lewis base interaction resulting in partial or even complete retention and poor peak shape. It tends to follow that the more phosphate groups a peptide contains, the worse their recovery and chromatographic peak shape. Current strategies to improve phosphopeptide recovery in LC-MS analyses include the addition of EDTA or citrate to samples, with both acting as metal chelators, or multiple injections of these solutions to attempt passivation of the fluidic path prior to sample analyses.[2] However, these strategies do present drawbacks as they can cause issues with chromatographic performance and sensitivity of the MS measurement.

In this application note, we have investigated the benefits of MaxPeak HPS Technology in the analysis of phosphopeptides. MaxPeak HPS hardware takes advantage of a hybrid inorganic/organic silica surface layer

which is chemically similar to bridged-ethyl hybrid silica. When applied to metallic surfaces wetted during an LC separation, such as the column and LC fluidic path, MaxPeak HPS imparts a resilient barrier but does not participate in separation of sample constituents. Experiments were performed in which the recovery response of numerous phosphopeptides were measured using both conventional and HPS modified systems.

Experimental

Samples

In solution tryptic digests of both Alpha Casein and Beta Casein were performed and the presence of most of the expected phosphopeptides in the resulting sample was confirmed by infusing, without use of the LC system, into the ESI source at 5 µL/min. The largest multiply phosphorylated peptides from the digests, QMEAESISSSEEIVPNSVEQK and RELEELNVPGEIVESLSSSEESITR, were found to be present by infusion but at a very low level. Further phosphopeptide species were obtained from standard synthetic phosphopeptide mixes 1 and 3 (Sigma-Aldrich), which consist of mono-, di-, tri-, and tetra-phosphorylated synthetic peptides. Samples were dissolved in aqueous 0.1% formic acid to a concentration of 1 pmol/µL of each peptide. For each analysis, 1 pmol was injected onto the column.

LC Conditions

LC system(s):	ACQUITY UPLC I-Class (Conventional) and ACQUITY Premier (HPS)
Column(s):	CSH 2.1 mm x 100 mm (Conventional and HPS)
Column temp.:	55 °C
Sample temp.:	8 °C
Flow rate:	150 µL/min (2.1 mm)

Mobile phase A:	Water + 0.1% formic acid
Mobile phase B:	Acetonitrile + 0.1% formic acid
Gradient:	1 to 40% B in 15 mins

MS Conditions

MS system:	SYNAPT XS
Ionization mode:	Electrospray Positive Ion
Acquisition mode:	ToF MS^E
Acquisition range:	50-2000 Da
Collision energy:	Trap CE ramp (14-40 eV)
Capillary voltage:	2.2 kV
Cone voltage:	30 V
Lock mass solution:	Glu Fibrinopeptide B (2+, *m/z* 785.8426)

Data Management

Chromatography software:	MassLynx v4.2
MS software:	MassLynx v4.2
Informatics:	PLGS 3.0.3, SkyLine (University of Washington)

Results and Discussion

In general, use of the HPS column greatly improves recovery for most peptides. The data presented in Table 1 highlights the results obtained from the expected phosphopeptides from all the samples analyzed. The two far right colored columns describe the recovery of the peptide based upon signal and peak shape, using reconstructed ion chromatograms of raw data in MassLynx and the data extraction in Skyline software. An example of phosphopeptide recovery improvement achieved using the HPS column is shown in Figure 1. The raw data chromatograms based on 1 pmol Alpha Casein injections are shown, with the data from the HPS system provided in the top trace. It is apparent that the change to HPS has very little effect in the response of the non-modified peptides present in the sample, however, by expanding a small retention time range from approximately 8.5 to 10.5 minutes, two peaks are shown to go from virtually non-existent response with the conventional system to large signal response with the HPS. Further examination of the data when extracted using Skyline, Figure 2, reveals another singly phosphorylated peptide, VPQLEIVPN<u>S</u>AEER, changing from no observed signal with the conventional system to large signal and excellent peak shape with the HPS system-column. The extracted ions are the precursor 2+ and 3+. An example peptide with more than one phosphorylation site, ADEP<u>SS</u>EE<u>S</u>DLEIDK, is shown in Figure 3. Generally, multiply phosphorylated peptides such as this exhibit extremely poor recovery as illustrated with the conventional configuration. In comparison, the HPS system shows significant increases in recovery for these multiply phosphorylated species. Figure 4 shows ProteinLynx Global Server (PLGS) search reports for a Beta Casein digest analyzed with conventional and HPS treated systems. The protein sequence coverage maps highlight regions of the protein sequence where the modified peptide resides. Observed is the absence of FQ<u>S</u>EEQQQTEDELQDK when analyzed with the conventional configuration and clear presence indicated with the HPS treated setup. The PLGS matched amino acid sequence for the peptide is also shown.

Protein/Sigma mix	Peptide (mod)	*m/z* (2+, 3+)	Conventional system	High perf surface
Alpha	VPQLEIVPN**S**AEER	830.90, 554.27		
Alpha	DIG**S**E**S**TEDQAMEDIK	964.35, 643.24		
Alpha	QMEAE**S**I**SSS**EEIVPN**S**VEQK	1360.96, 907.64		
Alpha	YKVPQLEIVPN**S**AEER	976.48, 651.32		
Alpha S2	TVDME**S**TEVFTK	733.81, 489.54		
Beta	FQ**S**EEQQQTEDELQDK	1031.42, 687.95		
Beta	RELEELNVPGEIVE**S**L**SSS**EESITR	1561.64, 1041.43		
1_1	VLHSG**S**R	418.19		
1_2	RS**Y**S**S**RSR	536.21		
1_3	RDSLG**T**YSSR	611.27, 407.85		
1_4	**T**KLI**T**QLRDAK	723.86, 482.91		
1_5	EVQAEQPSS**S**SPR	741.32, 494.55		
1_6	ADEP**S**SEESDLEIDK	872.35, 581.90		
1_7	ADEPS**S**EE**S**DLEIDK	912.33, 608.56		
1_8	FEDEGAGFEES**S**ETGDYEEK	1167.93, 778.95		
1_9	ELSN**S**PLRENSFG**S**PLEFR	1170.01, 780.34		
1_10	SPTEYHEPV**Y**ANPFYRPT**T**PQR	1405.60, 937.41		
3_1	SL**S**Y**S**PVER	639.22, 426.78		
3_2	LQG**S**GV**S**LA**S**K	643.75, 429.50		
3_3	PP**Y**S**S**RVI**T**QR	728.79, 486.20		
3_4	**S**RSR**S**Y**T**PE**Y**R	861.28, 574.52		
3_5	ADEP**S**S**S**EE**S**DLEIDK	952.31, 635.21		

Observed with good signal and peak shape

Observed but with compromised signal and/or peak shape

Not observed

Table 1. Phosphopeptide sequences, m/z and recovery description from all the samples analysed. The colored key describes the response based upon signal and peak shape observed.

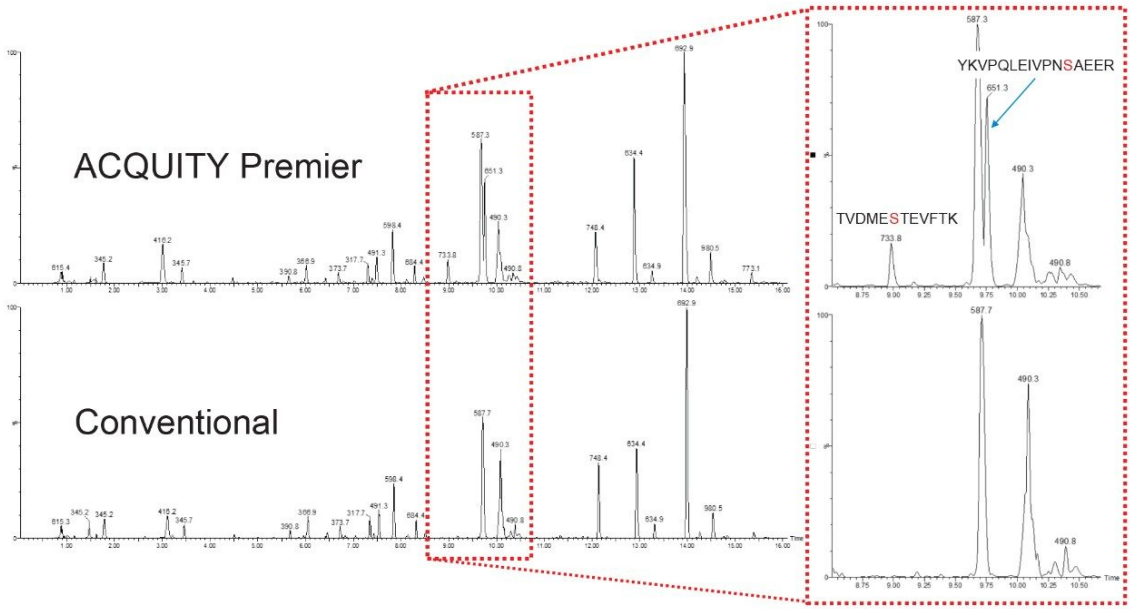

Figure 1. Chromatograms from injection of 1 pmol Alpha Casein, with a zoomed in region from retention time 8.5 to 10.5 min highlighted on the right hand side. Observed is a clear recovery of phosphopeptides whilst the responses of non-modified peptides do not significantly alter.

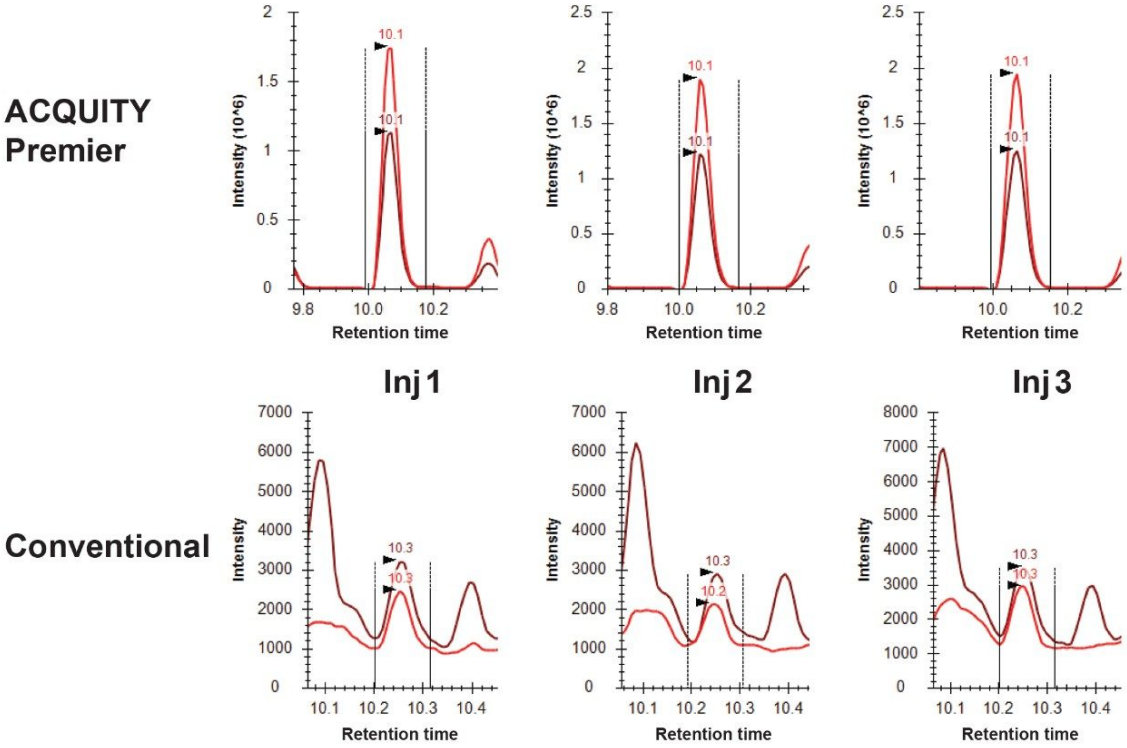

Figure 2. Peak area improvement for phosphopeptide VPQLEIVPNSAEER from Alpha Casein tryptic digest. The extracted ions are m/z for the precursor 2+ and 3+ ions.

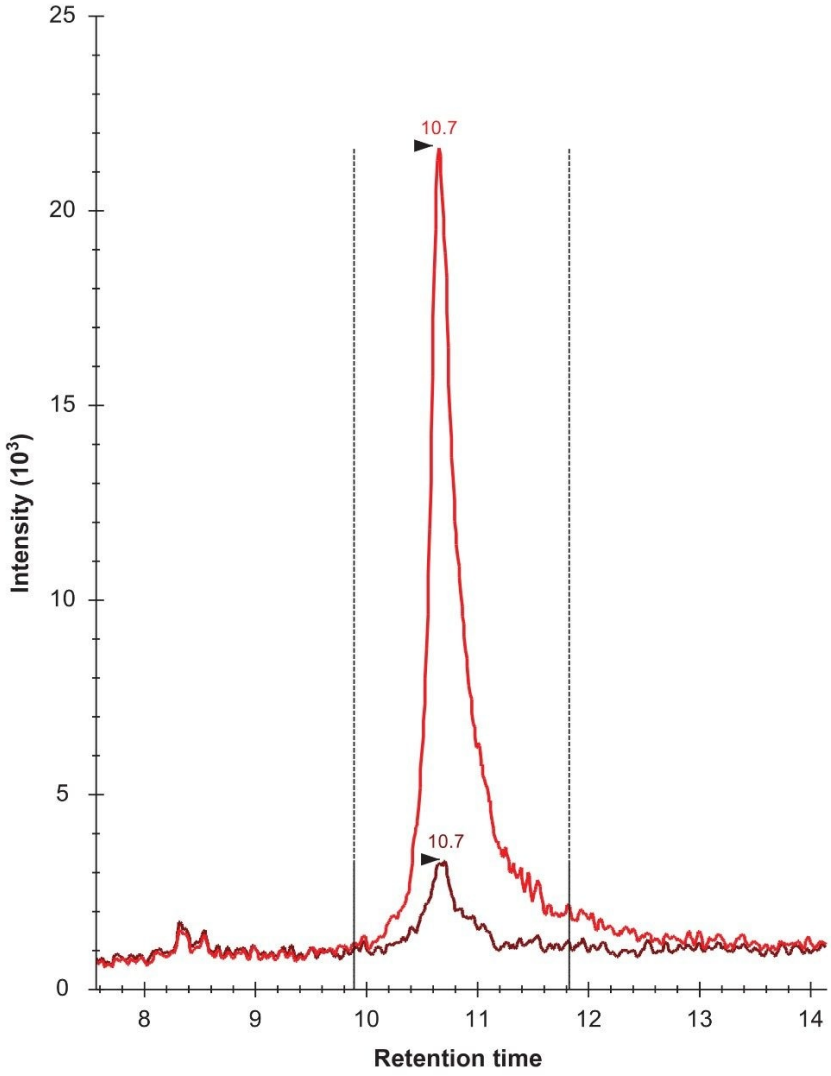

Figure 3. Peak shape of tri phosphorylated peptide ADEPSSEESDLEIDK from the Sigma Mixture 3. With the conventional configuration, this peptide was not observed but with the HPS configuration a significant increase in recovery is observed.

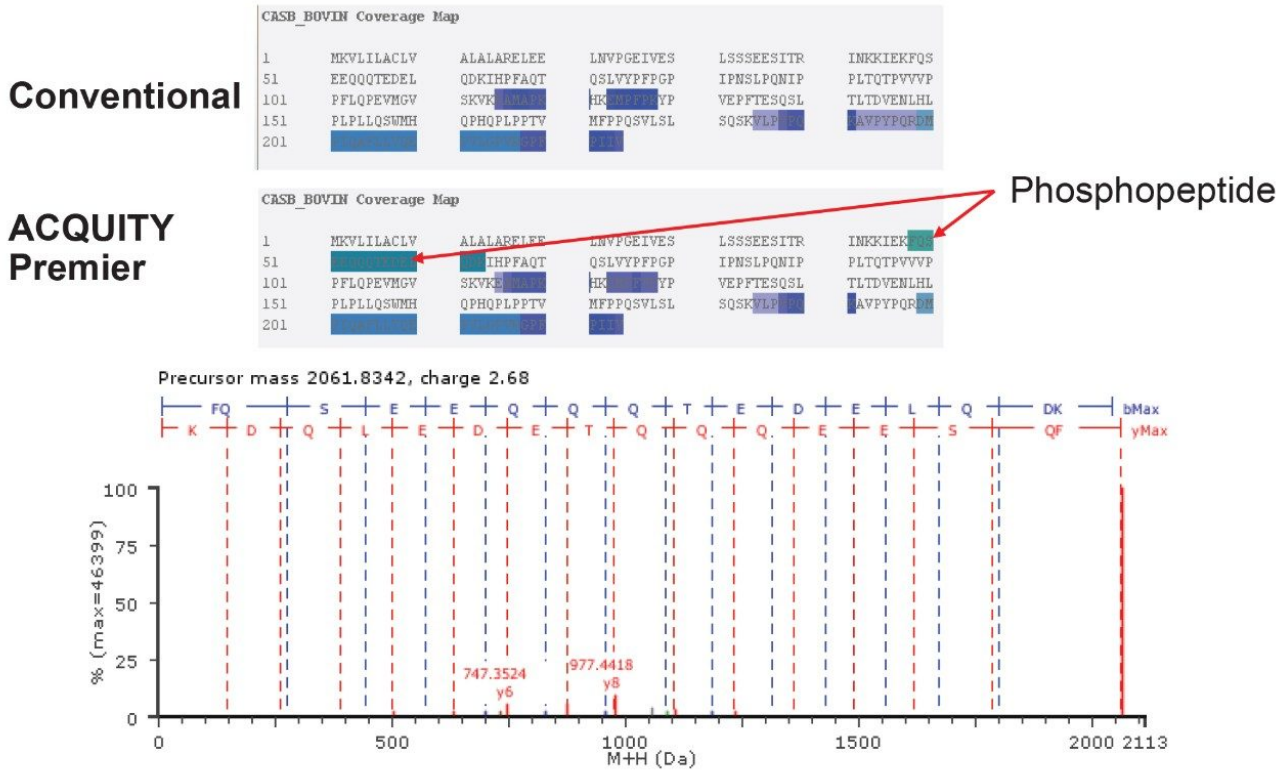

Figure 4. PLGS search report for Beta Casein digest. Coverage map shows the absence of FQS EEQQQTEDELQDK from the sequence coverage analysed with the conventional column and presence with the HPS treated. Also shown is matched amino acid sequence ions for this peptide.

Conclusion

Treatment of the LC fluidic path with a hybrid organic/inorganic barrier based on an ethylene-bridged siloxane structure have been shown to dramatically improve, in terms of mass spectrometry response and chromatographic peak shape, the recovery response of phosphopeptides in LC-MS studies. This treated surface, known as MaxPeak High Performance Surfaces (HPS), limits the metal ion interaction with the phosphate group present in the peptide which is the cause of problems seen with phosphoproteomics analyses utilizing a normal LC system. Various peptide containing samples have been studied, with varying degrees of improvement. In the most extreme cases, we observe that certain peptides analyzed in this study go from not being detected with a

standard system to very strong signals with an HPS fluidic path.

References

1. Liu *et al*, *Rapid Commun Mass Spectrom*. 2005;19(19):2747–56.

2. Winter *et al, J Proteome Res*. 2009 Jan;8(1):418–24.

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