

# Increased Sensitivity for LC-MS Analysis of Phosphopeptides on an ACQUITY QDa Detector Using XSelect Premier Columns

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## Abstract

Absorptive losses have been known to occur with phosphorylated peptides using metal surfaces such as stainless-steel. While inert hardware such as PEEK-lined columns have been employed to minimize the interaction between acidic moieties and metal surfaces, a more pressure stable and widely applicable technology exists. MaxPeak Premier Columns employ a novel surface technology to greatly reduce, if not eliminate, binding of analytes to metal surfaces. The work shown here demonstrates the improved MS sensitivity of phosphopeptides using a single quadrupole MS detector that can be achieved by using MaxPeak Premier Columns.

## Benefits

- Increased MS sensitivity for phosphopeptides
- Improved reproducibility and analyte recovery
- Out of the box performance with minimal column conditioning

## Introduction

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Metal surfaces interact with acidic compounds through Lewis acid-base interactions that may lead to adsorption of the compounds onto the metal surfaces. This can prove detrimental to the analysis of these acidic compounds by LC and LC-MS as most systems and packed columns consist of metal hardware. Detrimental effects because of this non-specific binding include peak tailing and low analyte recoveries. While strategies exist to mitigate these interactions, like using a passivation agent or inert hardware such as PEEK, these methods pose their own issues, including decreased operating pressures or MS interferences.

MaxPeak High Performance Surfaces (HPS) Technology was introduced specifically to address the concerns of metal-analyte interactions without sacrificing operating pressure. MaxPeak Premier Columns include an organic/inorganic barrier that mitigates interactions between acidic analytes, such as phosphorylated probes, and chromatographic metal surfaces. This benefit has been documented for small molecule, oligo, and peptide separations.<sup>1</sup> In addition, MaxPeak High Performance Surfaces (HPS) hardware does not require any special passivation solvents and is compatible with a wide range of mobile phases to allow for method development and optimization activities.

To demonstrate the benefits of MaxPeak Premier Columns for LC-MS analysis, the MassPREP Phosphopeptide Standard Enolase was analyzed on three columns using an ACQUITY UPLC H-Class System coupled to an ACQUITY QDa Mass Detector for MS detection. The XSelect Peptide CSH C<sub>18</sub> Column and a competitor peptide column was compared against an XSelect Premier Peptide CSH C<sub>18</sub> Column with MaxPeak HPS Technology. A phosphopeptide standard was chosen as they are generally less abundant than non-phosphorylated peptides and are known to adsorb to metals. Additionally, the accurate analysis of these peptides is critical for many workflows. Comparisons between all three columns were made regarding analyte recovery through peak areas and peak heights. Here, the XSelect Premier Column provided the highest sensitivity and recovery for all phosphorylated peptides, leading to increased reproducibility from injection to injection.

## Experimental

### Sample Description

The MassPREP Phosphopeptide-Enolase standard (PN: [186003285 <https://www.waters.com/nextgen/us/en/shop/standards--reagents/186003285-massprep-phosphopeptide-standard-enolase.html>](https://www.waters.com/nextgen/us/en/shop/standards--reagents/186003285-massprep-phosphopeptide-standard-enolase.html)) contains 1 nmol each of four synthetic enolase phosphopeptides: T18 (1P, tyrosine), T19 (1P, serine), T43 (1P, threonine), and T43 (2P, serine). The vial contents were reconstituted in 50 µL Milli-Q water to give a 20 pmol/µL solution and vortexed for 10 seconds prior to injection.

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## Method Conditions

### LC Conditions

LC system:	ACQUITY UPLC H-Class System
Detection:	ESI+ Full Scan
Vials:	TruView LCMS Certified Clear Glass Vial Total Recovery (PN: 186005669CV)
Column(s):	XSelect Peptide CSH C <sub>18</sub> XP Column, 2.1 x 50 mm, 2.5 µm (PN: 186006941)  XSelect Premier Peptide CSH C <sub>18</sub> Column, 2.1 x 50 mm, 2.5 µm (PN: 186009904)  Competitor Peptide Column, 2.1 x 50 mm, 2.7 µm
Column temp.:	60 °C
Sample temp.:	Ambient
Injection volume:	1 µL
Flow rate:	0.45 mL/min
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile
Gradient:	See Table

## Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
0.00	0.45	99	1	6
7.35	0.45	75	25	6
8.09	0.45	75	25	6
8.10	0.45	99	1	6
9.00	0.45	99	1	6

## Data Management

Chromatography software:

Empower 3 Feature Release 4

MS software:

Empower 3 Feature Release 4

Informatics:

Empower 3 Feature Release 4

## Results and Discussion

To demonstrate the benefits of MaxPeak Premier LC Columns, we evaluated the separation and recovery of a phosphopeptide standard using a conventional XSelect Peptide CSH C<sub>18</sub> Column, a competitive peptide column, and an XSelect Premier Peptide CSH C<sub>18</sub> Column. Figure 1 shows the extracted ion chromatograms of the first injection for each column. MS detection was performed using an ACQUITY QDa Mass Detector at the (M+2H)<sup>+2</sup> mass indicated in Table 1.

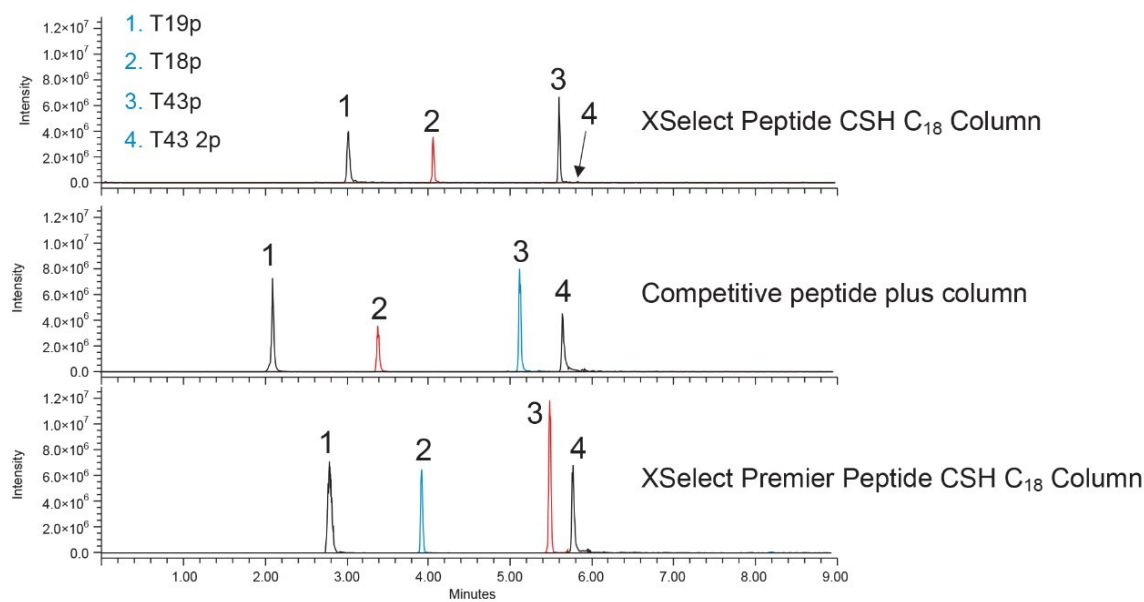


Figure 1. Separation of four phosphopeptides on three columns. Extracted ion chromatograms of the first injection of five for the  $[M+2H]^{+2}$  ion taken from a full scan ESI+ spectrum.

Peptide	Sequence	Average MW (g/mol)	Isotopic mass, (M+H) 1+	Isotopic mass, (H+2H) 2+
T18 1P or T18p	NVPLpYK	812.858	813.3912	407.1995
T19 1P or T19p	HLADLpSK	862.875	863.4028	432.2053
T43 1P or T43p	VNQIGpTLSESIK	1368.444	1368.6776	684.8428
T43 2P or T43pp	VNQIGTLpSEpSIK	1448.424	1448.6439	724.8259

Table 1. Synthetic enolase phosphopeptides in the MassPREP Phosphopeptide standard and mass to charge ( $m/z$ ) ratios for the various charge states.<sup>2</sup>

The XSelect Peptide CSH C<sub>18</sub> Column produces the lowest signal intensity for all peptides, with the T43 2p peak almost undetectable from background noise and considerably lower in intensity compared to the singly phosphorylated peptides. The doubly phosphorylated peptide is more difficult to recover due to its extra acidic phosphate group and shows increased signal intensity using the competitive peptide column. However, the highest signal intensity achieved for T43 2p was with the XSelect Premier Peptide CSH C<sub>18</sub> Column. In fact, all probes have higher signal intensities using XSelect Premier Peptide CSH C<sub>18</sub> Column compared to the other columns tested. The singly phosphorylated peptides, though less acidic than the doubly phosphorylated T43 2p, still show adsorptive losses as indicated with lower signal intensities and peak areas for the conventional XSelect Peptide CSH C<sub>18</sub> and competitor peptide columns (Table 2). Apart from the differences in signal intensity, the XSelect Premier Peptide CSH C<sub>18</sub> Column achieves similar selectivity compared to the XSelect Peptide CSH C<sub>18</sub> Column, especially for the T43p and T43 2p peaks. The competitive peptide plus column shows a better

separation of those two probes but is overall less retentive than the XSelect CSH C<sub>18</sub> chemistries, with or without the MaxPeak HPS hardware. A slight difference in retention is seen between the XSelect Premier Peptide CSH C<sub>18</sub> Column and the XSelect Peptide CSH C<sub>18</sub> Column with the standard hardware column showing slightly higher retentions, likely due to the ionic interactions between the probes and the metal surfaces. Being able to switch from a standard column to a MaxPeak Premier Column without major selectivity differences allows for faster adoption of MaxPeak HPS Technology in critical assays.

Compound	Xselect Peptide CSH C <sub>18</sub> Column	XSelect Premier Peptide CSH C <sub>18</sub> Column	Competitor peptide column
T19p	10409467	28055540	14101364
T18p	631465	14236846	7405508
T43p	9679489	26468286	16095331
T43 2p	305512	24497628	11621357

Table 2. Peak areas for the four compounds on all three stationary phases.

The most striking difference when considering peak area is again with the T43 2p peptide (Table 2) versus the MaxPeak Premier Column, upon first injection, peak areas for the T43 2p peptide were ~50% lower on the competitor peptide column and ~99% lower on the XSelect Peptide CSH C<sub>18</sub> Column. However, when running successive injections, the peak areas for all phosphopeptides using both the conventional XSelect Peptide CSH C<sub>18</sub> and competitor peptide columns begin to increase, as shown in Figure 2.

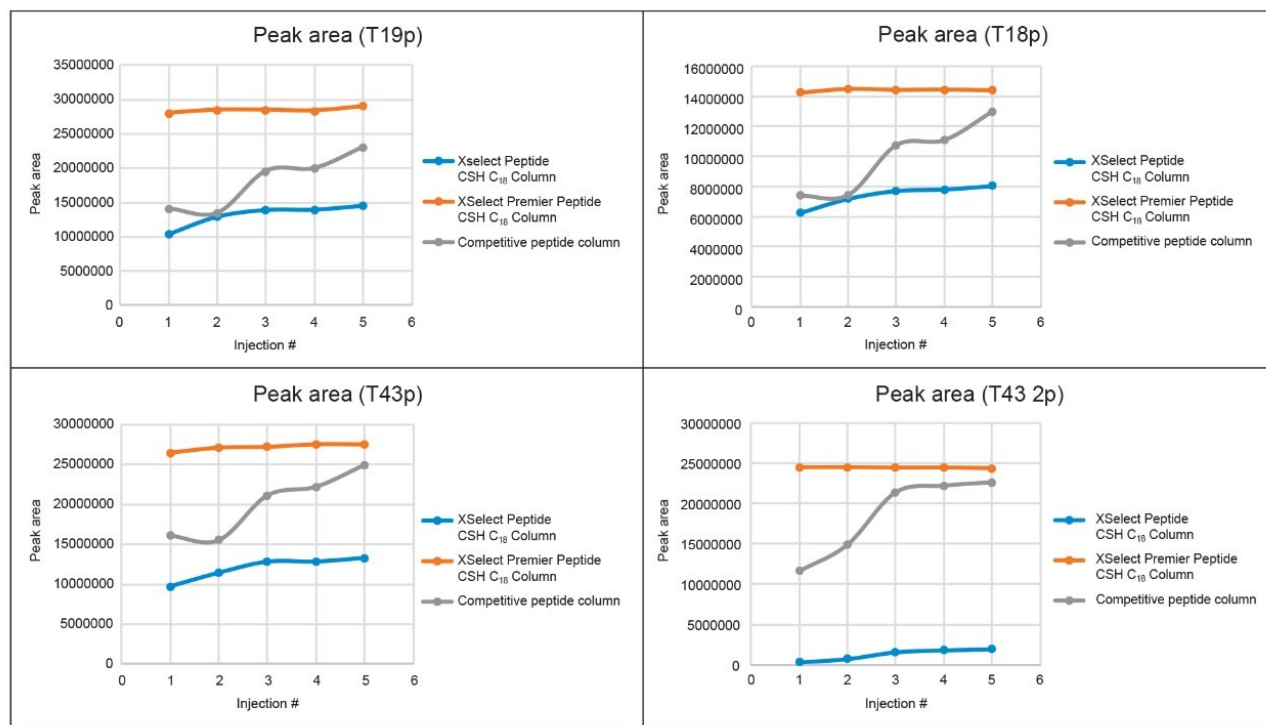


Figure 2. Peak area trends of the four phosphopeptides over five injections.

In Figure 2, the XSelect Premier Peptide CSH C<sub>18</sub> Column yields reproducible peak areas from the first injection, in contrast to the other two columns. The XSelect Peptide CSH C<sub>18</sub> Column demonstrates a very slow increase in peak area over five injections while the competitive peptide column improves more dramatically, but without a linear increase over time. In both cases, neither column reaches the performance of the MaxPeak Premier Column for any of the phosphopeptides within five injections, suggesting that either further column conditioning is needed or that the level of performance achieved with MaxPeak Premier Columns cannot be attained. The enhanced recovery of MaxPeak Premier Columns demonstrates the importance and utility of inert chromatographic surfaces for phosphopeptide analysis.

## Conclusion

With MaxPeak Premier Columns, metal interactions between acidic analytes such as phosphopeptides and chromatographic surfaces have been drastically reduced. As demonstrated in this application note, using MaxPeak Premier LC Columns can improve the recovery of phosphopeptides for detection using single quadrupole mass spectrometers without significant changes in selectivity or analyte retention. The increased sensitivity using the XSelect Premier CSH C<sub>18</sub> Column can allow for lower levels of peptides to be detected,

granting better characterization of biological systems or protein digestions via peptide mapping. MaxPeak Premier Columns could thus be very useful when analyzing low concentrations, especially for low abundant phosphorylated peptides that often need enrichment prior to analysis.

## References

1. DeLano M, Walter TH, Lauber M, Gilar M, Jung MC, Nguyen JM, Boissel C, Patel AV, Bates-Harrison A, Wyndham K. Using Hybrid Organic-Inorganic Surface Technology to Mitigate Analyte Interactions with Metal Surfaces in UHPLC. *Analytical Chemistry* (93) 2021. 5773–5781.
2. MassPrep Phosphopeptide Standard-Enolase Care and Use Manual. [715001599 <https://www.waters.com/webassets/cms/support/docs/715001599.pdf>](https://www.waters.com/webassets/cms/support/docs/715001599.pdf).

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