

## Increasing Chromatographic Performance of Acidic Peptides in RPLC-MS-based Assays with ACQUITY Premier featuring MaxPeak HPS Technology

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### Abstract

Metal-ion mediated adsorption of analytes as a contributing factor in poor peak shape, tailing, and diminished recovery of compounds in LC-based techniques can negatively impact data quality and assay robustness. Analytes exhibiting phosphate groups, uncharged amines, and deprotonated carboxylic acids are particularly susceptible to these phenomena and are commonly encountered in the development and manufacturing of protein-based therapeutics. Current methods for addressing analyte/surface adsorption include ion-pairing additives, hardware passivation, and high-ionic strength mobile phase. These strategies, while proven effective, can be challenging to deploy in terms of instrument/technique compatibility in the case of MS-based methods as well ensuring they can be implemented in a safe and efficient manner with respect to lengthy passivation procedures that can involve corrosive reagents. The newly introduced ACQUITY Premier brand columns with MaxPeak HPS Technology is Waters solution to these challenges. Waters ACQUITY Premier Columns are designed to deliver exceptional chromatographic performance while minimizing analyte/surface interactions of

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sensitive compounds. In this study, the performance gain of ACQUITY Premier Columns with MaxPeak HPS Technology is demonstrated with increased recovery, reproducibility, and robustness of RPLC-MS-based peptide mapping assays using the Waters NIST mAb tryptic digest standard. Collectively, this study establishes MaxPeak HPS Technology can be broadly applied in the development and manufacturing of therapeutic drug products to deliver the chromatographic performance expected from Waters technologies while increasing reproducibility, peak shape, and recovery of analytes prone to surface interactions.

## Benefits

- Increased sensitivity through improved recovery and lower peak tailing
- Compatibility with legacy methods without the use of additional additives
- Improved method robustness through increased assay reproducibility
- Increased productivity through reduced method development time

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## Introduction

Analyte/surface adsorption in liquid chromatography (LC) has been established as a contributing factor in poor peak shape, tailing, and diminished recovery of compounds in LC-based techniques.<sup>1-3</sup> Recently, metal-ion mediated adsorption has been identified as a specific adsorption mechanism for analytes that exhibit Lewis acid/base characteristics.<sup>4</sup> The hypothesis being analytes bearing electron rich moieties such as phosphate groups, uncharged amines, and deprotonated carboxylic acids act as Lewis Bases which can adsorb in a non-covalent manner to electron deficient sites on the metal surface which act as a Lewis Acid. Conventional strategies to suppress metal-ion mediated adsorption include ion-pairing, hardware passivation, and high-ionic strength mobile phases. While largely successful, challenges still exist in certain instances where analytes exhibit inordinately strong interactions with metal surfaces (e.g. bearing multiple electron-rich moieties) and/or assays are performed with non-optimal conditions (e.g. weak vs. strong ion-pairing). This is particularly evident in RPLC-MS-based peptide analyses wherein peptide fragments containing aspartic acid (D) or glutamic acid (E) residues can interact with metal surfaces which can exacerbate adsorption characteristics resulting in increased tailing and reduced sensitivity of analytes prone to metal-ion mediated adsorption as shown in Figure 1. Recently, it was shown that metal chelators can be used as mobile phase additives to mitigate adsorption artifacts with notable

success.<sup>5</sup> However, incorporation of such additives is not always ideal as they can introduce new chromatographic artifacts as well as suppress ionization in MS-based analyses. These challenges highlight the need for novel columns and instrumentation that can mitigate metal-ion mediated adsorption without the need for additional additives or lengthy passivation processes.

The newly introduced ACQUITY Premier brand columns with MaxPeak HPS Technology is Waters solution to these challenges. The ACQUITY Premier Columns are designed to deliver exceptional chromatographic performance while minimizing analyte/surface interactions of sensitive compounds. The objective of this application note is to demonstrate how ACQUITY Premier with MaxPeak HPS Technology can increase productivity in the lab and mitigate risk through increased reproducibility, recovery, and robustness of assays performed in the development and manufacturing of biopharmaceutical drug products.

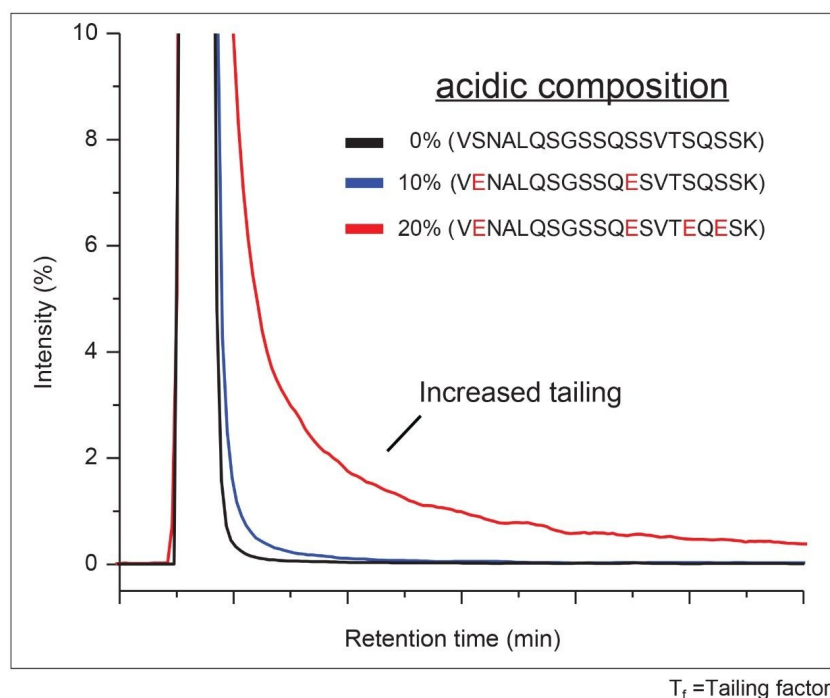


Figure 1. Synthetic acidic peptide ladder. Tailing was evaluated for 3 synthetic peptides manufactured with 0, 2, and 4 glutamic acid (E) residues representing 0%, 10%, and 20% acidic content by composition. Under isocratic conditions (MP A: 89%, MP B: 11%), tailing was observed to increase significantly for peptides containing multiple acidic residues.

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## Experimental

Columns were conditioned as outlined by the column care and use manual. Waters mAb Tryptic Digestion Standard (p/n: [186009126](#)) was reconstituted in MS-grade water with 0.1% FA at a concentration of 0.2 mg/mL (SYNAPT XS) and 0.5 mg/mL (ACQUITY QDa), aliquoted and stored at -80 °C prior to use.

## LC Conditions

LC system:	ACQUITY H-Class Binary Bio PLUS
Detection:	TUV, 10 mm Analytical FC, $\lambda=214$ nm
Vials:	QuanRecovery with MaxPeak HPS (300 $\mu$ L/25pk, p/n:186009242)
Column(s):	CSH 130 Å C <sub>18</sub> Column (2.1 x 100 mm, 1.7mm p/n: 186005297)  ACQUITY Premier Peptide CSH 130 Å C <sub>18</sub> Column (2.1 x 100 mm, 1.7mm p/n: 186009461)
Column temp.:	60 °C
Sample temp.:	6 °C
Injection volume:	QDa = 10 $\mu$ L, Synapt XS = 1.0 $\mu$ L
Flow rate:	0.200 mL/min
Mobile phase A:	H <sub>2</sub> O, 0.1 % Formic acid

Mobile phase B:

Acetonitrile, 0.1 % Formic acid

### Gradient Table (Figure 2-7)

Time	Flow (mL/min)	% A	% B	Curve
Initial	0.200	99	1	6
2.00	0.200	99	1	6
52.00	0.200	65	35	6
57.00	0.200	15	85	6
62.00	0.200	15	85	6
67.00	0.200	99	1	6
80.00	0.200	99	1	6

### MS Conditions (Figure 1 and 6)

MS system: ACQUITY QDa

Ionization mode: ESI+

Acquisition range: 250–1250 *m/z*

Capillary voltage: 1.5 kV

Cone voltage: 10 V

Probe temp.: 600 °C

## MS Conditions (Figure 2-5)

MS system: SYNAPT XS

Ionization mode: ESI+

Acquisition range: 50–2000 *m/z*

Capillary voltage: 2.2 kV

Cone voltage: 20 V

Source temp.: 120 °C

Desolvation temp.: 350 °C

Cone gas: 35 L/hr

Desolvation gas: 500 L/hr

Lockmass: Glu fibrinopeptide B at 100 fmol/mL in  
75/25 acetonitrile/water, 0.1% formic acid

## Data Management

Chromatography software: Empower 3 FR4

MS software: MassLynx 4.2

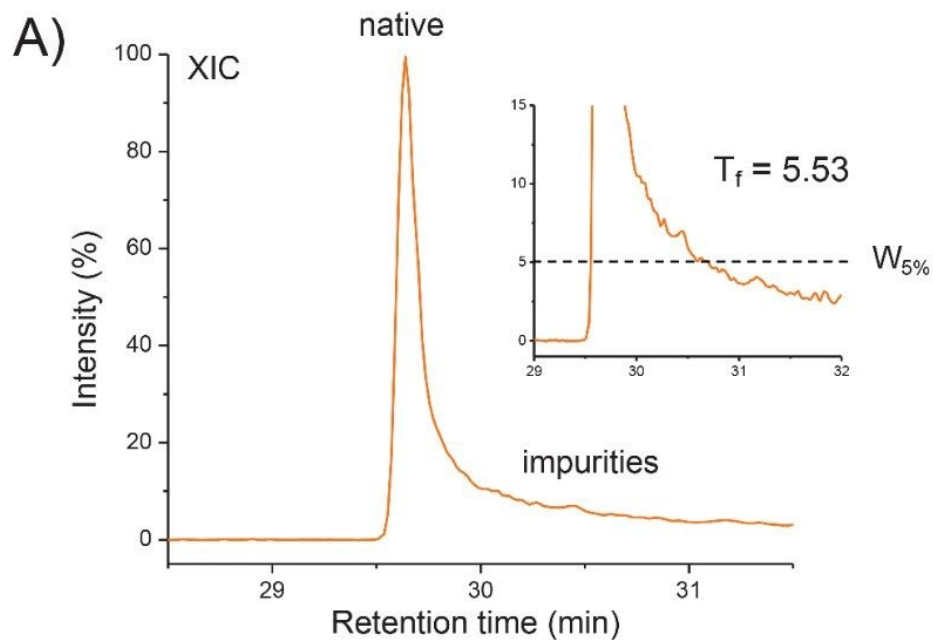
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## Results and Discussion

### Industry Relevance:

Peptide based analyses have proven to be an invaluable tool in the characterization and quality control of protein-based therapeutics as part of a quality lifecycle management process. As part of this process, LC-MS-based data plays a critical role in providing information used to determine primary sequence protein modifications and their related impurities. The value of mass information in these analyses has contributed in part to renewed interest in expanding the role of MS-based methods to improve productivity and data quality in the development and manufacturing of drug products. However, peptide assays with MS detection are often deployed with weaker mobile phase additives such as formic acid in favor of sensitivity over chromatographic performance. This can be problematic for trace impurities that are prone to metal-ion mediated adsorption (e.g. "acidic" peptides) as assay reproducibility and accuracy of results can vary based on the severity of the analyte/surface interaction. An example of this is shown in Figure 2A in the case of deamidation of asparagine.

## Conventional technology



## ACQUITY Premier with MaxPeak HPS Technology

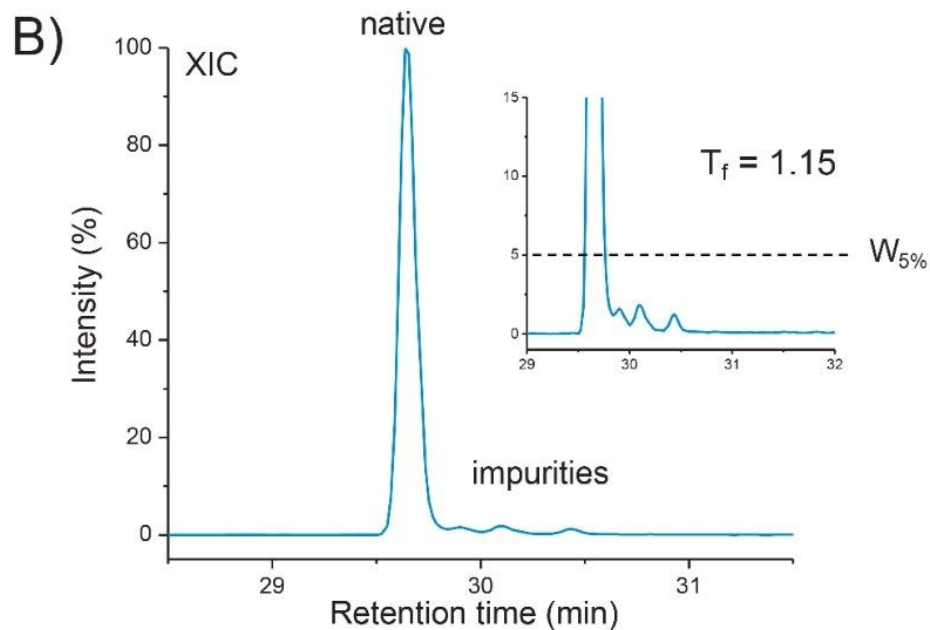


Figure 2. Critical quality attribute related T37 PENNYK peptide. A) Tailing factor was calculated at 5.53 for the



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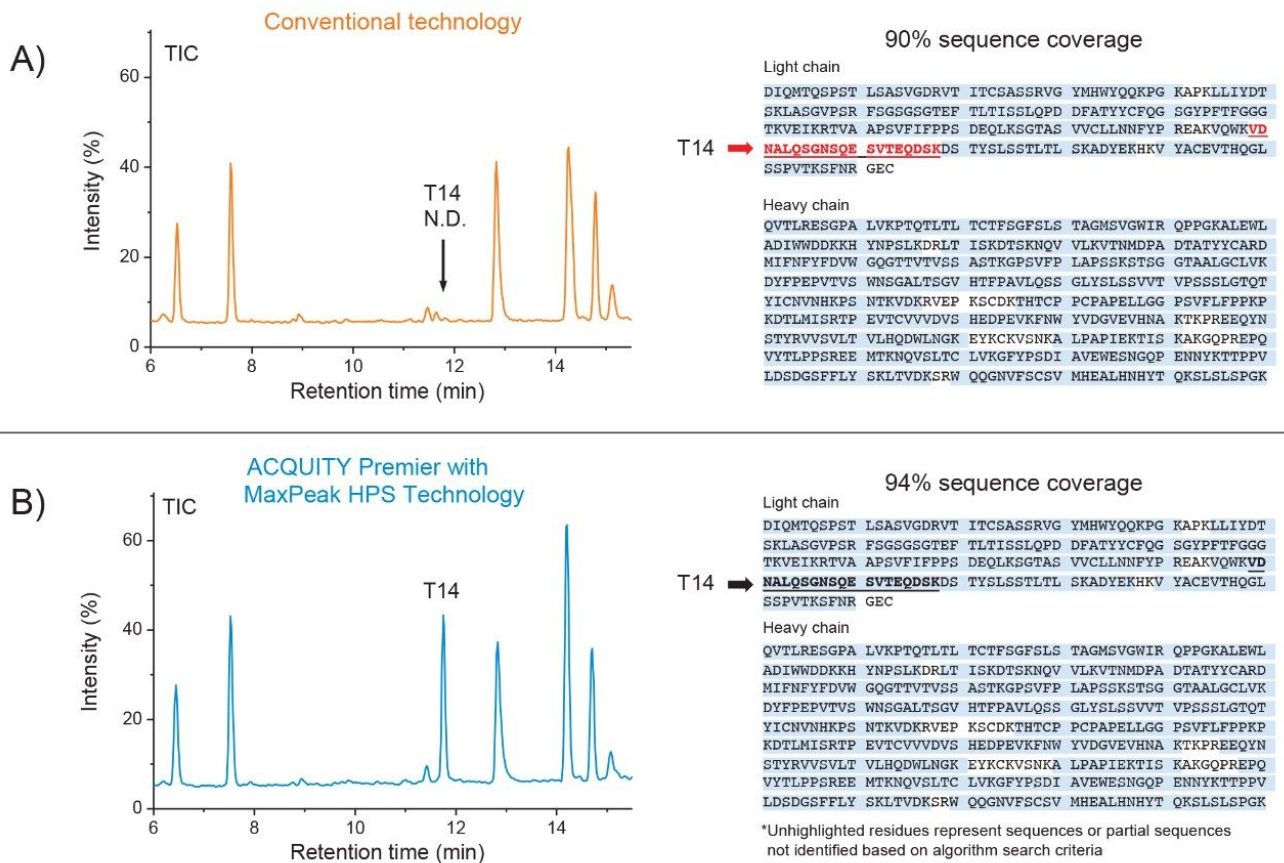
*T37 native peak using a conventional stainless-steel column which resulted in the inability to detect closely eluting deamidated related impurities. B) Tailing was reduced by 79% to a value of 1.15 for the native peak when using a ACQUITY Premier Column with MaxPeak HPS Technology allowing for detection of CQA related deamidated impurities.*

Deamidation of asparagine to aspartic acid and iso-aspartic acid is a common post-translational modification of monoclonal antibodies (mAbs) that has been correlated to drug efficacy. As a result, biopharmaceutical companies invest a significant amount of resources in the control and monitoring of critical quality attributes (CQAs) such as deamidation. Of the deamidated species monitored, the "PENNYK" T:37 peptide (sequence: GFYPSDIAVEWESNGQPENNYK) is of notable interest in that it is a routinely monitored Fc domain peptide which is known to be susceptible to post translation modifications such as deamidation and contains 4 "acidic" residues (3 glutamic acid, 1 aspartic acid). As shown in Figure 2A, the impurities associated with the PENNYK peptide elute closely to the native peptide due to similar physicochemical properties making them susceptible to missidentification/integration errors due to tailing artifacts. In this example a tailing factor of 5.53 was observed for the native PENNYK peptide when performing a formic acid-based RPLC peptide map on a conventional LC system using the Waters ACQUITY UPLC CSH 130 Å C<sub>18</sub> Column. This resulted in the inability to detect closely eluting impurities due to excessive tailing of the native peak. In contrast, when the same separation was performed using the Waters ACQUITY Premier Peptide CSH 130 Å C<sub>18</sub> Column, tailing was reduced by 79% with a tailing factor of 1.15 observed for the native peptide. The observed performance gain allowed for the chromatographic separation of both deamidated impurities which were approximately baseline resolved from the native peak. These results demonstrate the value ACQUITY Premier with MaxPeak HPS Technology can bring to the lab to improve chromatographic performance for the development and manufacture of safe and efficacious drug products.

## Increased Recovery:

The performance gains observed when using ACQUITY Premier with MaxPeak HPS Technology find merit for its application in both upstream and downstream activities. The reoccurring frequency of glutamic acid and aspartic acid residues in peptide fragments suggests all acidic residue containing peptides stand to benefit from MaxPeak HPS Technology in terms of reproducibility, peak shape, and recovery by minimizing analyte/surface interactions. As shown in Figure 3, the total ion chromatogram (TIC) of the NIST mAb tryptic digest was evaluated for the recovery of the T:14 peptide fragment (sequence = VDNALQSGNSQESVTEQDSK), which

contains 4 acidic residues (20%), using a Waters UPLC CSH 130 Å C<sub>18</sub> Column (Figure 3A) as well as a Waters ACQUITY Premier Peptide CSH 130 Å C<sub>18</sub> Column featuring MaxPeak HPS Technology (Figure 3B) with detection being performed with a Waters SYNAPT XS Mass Spectrometer. As shown in Figure 3A, the T:14 peptide fragment was not detected at a mass load of 0.2 µg resulting in a reduced sequence coverage of 90% when using the ACQUITY UPLC CSH 130 Å C<sub>18</sub> Column. In contrast, the T:14 peptide was observed to have a significant increase in recovery when the same sample was separated using the same method with the ACQUITY Premier Peptide CSH 130 Å C<sub>18</sub> Column (Figure 3B) resulting in 94% sequence coverage for the peptide mapping assay. The benefits afforded by ACQUITY Premier with MaxPeak HPS Technology extend beyond increased recovery alone.



**Figure 3. Increased Recovery.** A) The T14 peptide fragment of the Waters NIST mAb digest standard was not detected in a LC-MS-based peptide mapping assay at a 0.2 µg mass load when using a conventional stainless-steel column, resulting in 90% sequence coverage. B) Recovery of the T14 peptide fragment when using an ACQUITY Premier Column with MaxPeak HPS Technology enabled detection and identification of the T14 peptide fragment resulting in increased sequence coverage (94%).

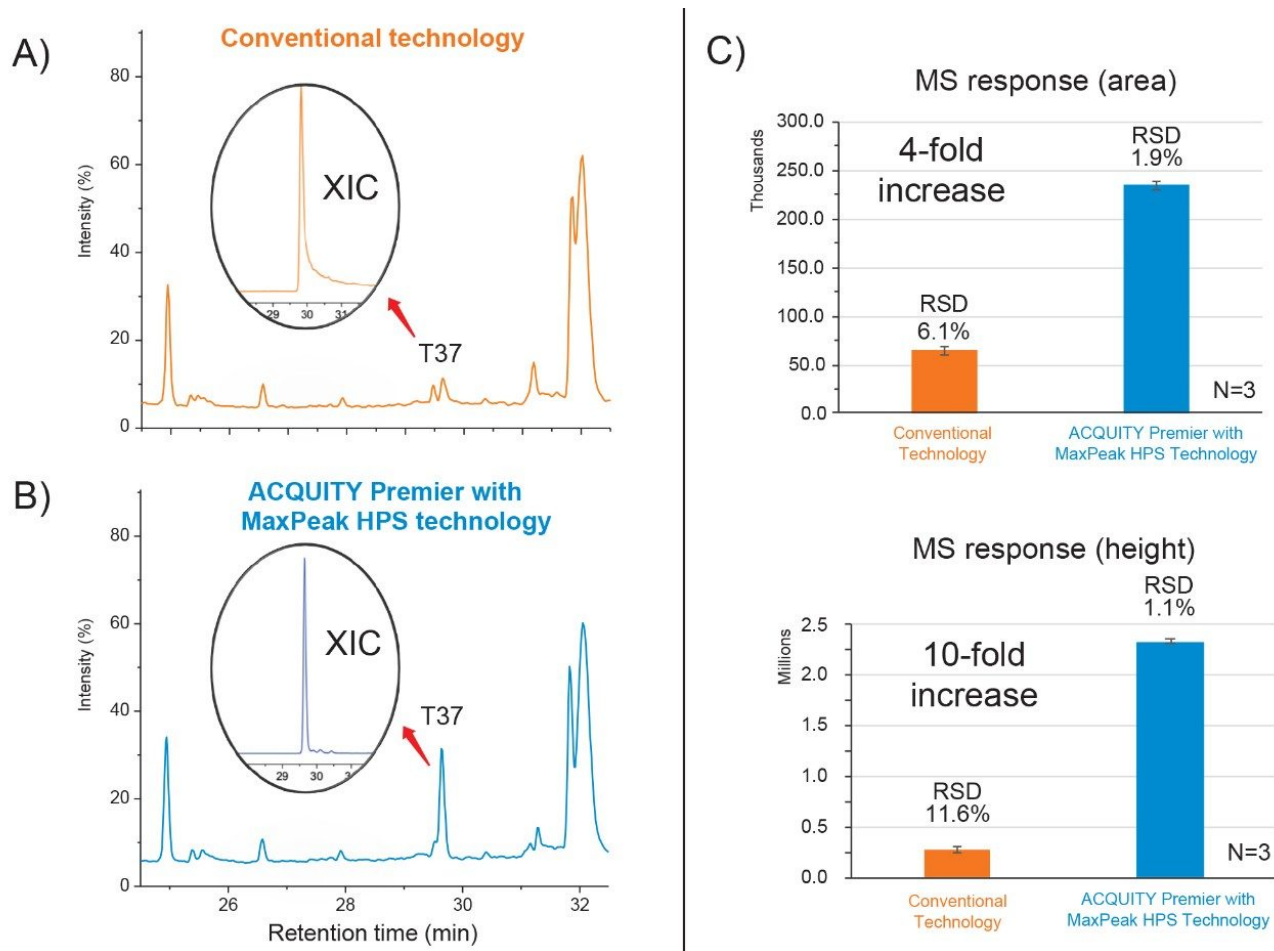


Figure 4. Increased response. Recovery of the T37 peptide fragment from a tryptic digest of the NIST reference mAb standard was evaluated for a peptide map performed on A) a conventional column (stainless-steel) as well as a B) ACQUITY Premier Column with MaxPeak HPS Technology. C) A 10-fold increase in peak height was observed due to the reduced tailing using MaxPeak HPS Technology resulting in a 4-fold increase in peak area. The observed improvement in recovery resulted in a ~90% reduction in MS-response variability (height %RSD 11.6% vs. 1.1%) across 3 replicate injections when using the ACQUITY Premier Column with MaxPeak HPS Technology.

### Increased Response:

As shown in Figure 4, the T:37 peptide was evaluated concurrently with the T:14 peptide fragment using the same sample and system configuration. As shown in Figure 4A, the XIC (848.7174  $m/z$ ) of the T:37 peptide fragment at a 0.2  $\mu\text{g}$  mass load exhibited significant tailing which impeded detection of related impurities that

closely elute with the native peak as well as introducing increased variability in instrument response (Figure 4C). In contrast, the same peptide showed a 10-fold increase in peak height and a 4-fold increase in peak area as a result of reduced tailing and increased recovery when the same sample was separated using the same method on an ACQUITY Premier Column with MaxPeak HPS Technology (Figure 4B). The increased recovery and improved peak shape afforded by MaxPeak HPS Technology resulted in increased assay reproducibility with a 90% reduction in MS-response (height) variability calculated at an R.S.D. of 1.1 % for 3 replicate injections. These results demonstrate ACQUITY Premier with MaxPeak HPS Technology can be broadly applied to improve chromatographic performance of acidic residue containing peptides for increased assay robustness and reproducibility.

### Improved Peptide Characterization Data Quality:

The recovery and chromatographic performance gains observed with ACQUITY Premier and MaxPeak HPS Technology has the added benefit of improving the quality and interpretation of data. As part of upstream activities, characterization of protein-based therapeutics relies heavily on peptide mapping to determine amino acid sequence and related impurities of prospective drug candidates. In this respect, the ability of ACQUITY Premier with MaxPeak HPS Technology to reduce tailing and resolve critical species of sensitive peptides enables analysts to sequence drug candidates with increased confidence. This is demonstrated with the fragmentation profile of the T:37 peptide fragment as shown in Figure 5. Using MS<sup>E</sup> acquisition mode (Data Independent Acquisition) on the Waters SYNAPT XS Mass Spectrometer, spectra for each of the 4 peaks (inset) related to T:37 (GFYPSDIAVEWESNGQPENNYK) species are shown in Figure 5. In this instance, critical y series fragmentation ions were observed and used to assign the deamidation sites for unmodified (blue) as well as deamidated or succinimide forms (red). Beginning with the y3 ion, which corresponds to N393, is unmodified in all peaks. Next, the y4 ion is unmodified in all but Peak 3, which shows a +0.9801 Da shift, pointing to deamidation on N392. The y series for Peak 3 continue to show the mass shift for deamidation, as expected. It is not until y9 ion that any mass shifts are observed for Peaks 2 and 4. The y9 ion, corresponding to N387, is observed with +0.9870 Da for Peak 2 (deamidation) and -17.0052 Da for Peak 4 (succinimide intermediate). Therefore, we can unambiguously assign these chromatographically resolved T:37 species as follows: Peak 1- unmodified, Peak 2- N387 deamidation, Peak 3- N392 deamidation, Peak 4- N387 succinimide intermediate. These results demonstrate ACQUITY Premier with MaxPeak HPS Technology not only can improve recovery and reproducibility of assays but also increase confidence in data interpretation and peptide identification through reduced tailing to resolve co-eluting peaks and increased detector response to support both upstream and downstream activities associated in the development and manufacture of therapeutic drugs.

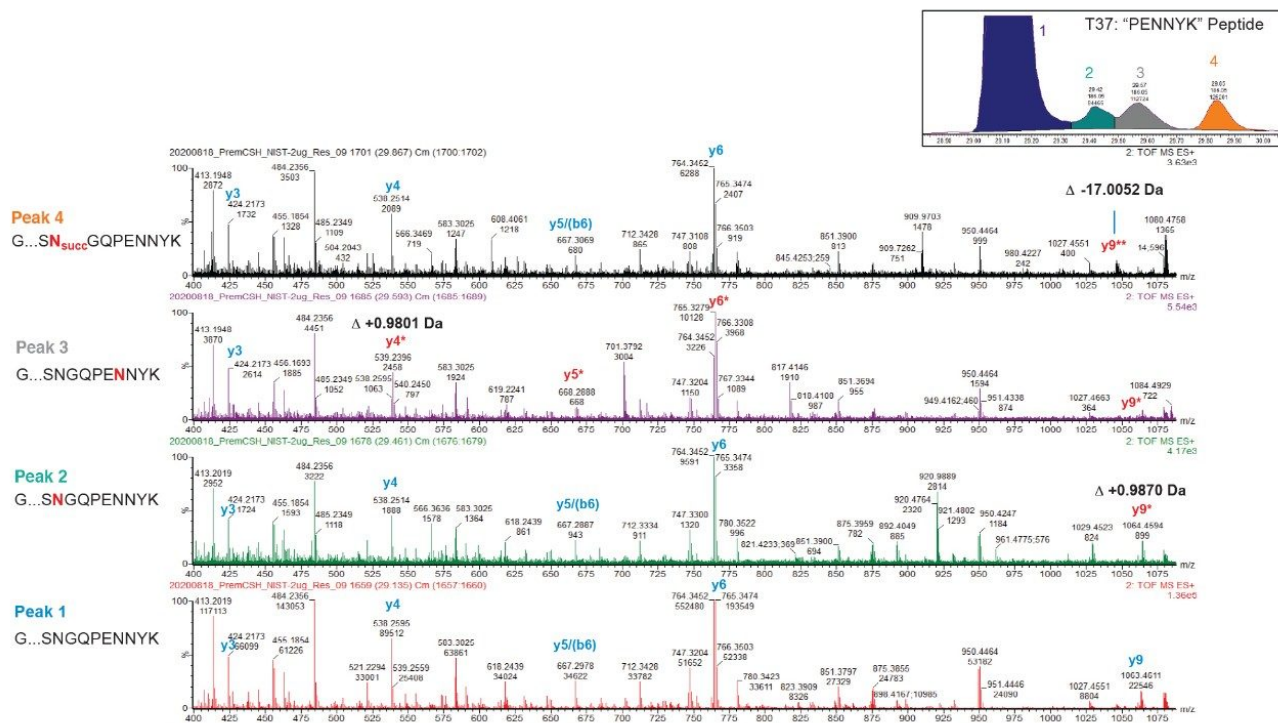


Figure 5. Enhanced data quality.  $MS^E$  high energy fragment spectra ( $m/z$  400-1150) with labeled critical  $y$  series fragmentation ions for GFYPSDIAVEWESNGQPENNYK peptide and its respective deamidation species (shown in the XIC in the top right). The bottom panel corresponds to  $MS^E$  spectra of Peak 1 (unmodified), the center panels correspond to Peaks 2 and 3 (deamidated species), and the top panel corresponds to Peak 4 (a succinimide intermediate). Unmodified  $y$  ions are labeled in blue, while deamidated (\*) and succinimide (\*\*) species are labeled in red.

## Reproducible Chromatographic Performance:

As methods are transferred from development to manufacturing environments, reproducibility as an assay criterion is critical to ensure consistent results can be obtained in an efficient manner and accurately reflect CQAs in a drug product (a lack of which can result in costly delays and in some instances investigations when results are out of specification as in the case of regulated environments). To this end, chromatographers often incorporate passivation procedures as part of a standard operating procedure to aid in stabilizing chromatographic performance. These passivation procedures range from relatively benign practices such as repeated injections of a sample matrix to more aggressive techniques that can include the use of corrosive

solvents such as nitric and/or phosphoric acid to passivate metal surfaces. While these practices are not without merit, they do take time to execute and prevent the full utilization of lab and instrument resources. ACQUITY Premier with MaxPeak HPS Technology offers users the ability to bypass these lengthy and sometimes hazardous procedures with consistent and reliable performance out-of-the box.

An example of this is shown in Figure 6 for the CQA associated PENNYK peptide. Using a newly cleaned ACQUITY UPLC System (phosphoric acid wash), tailing factor for the PENNYK peptide was monitored using a single quadrupole mass detector (ACQUITY QDa) with selected ion recording (SIR) set at a value of 849.20  $m/z$  in a peptide map separation with and without ACQUITY Premier MaxPeak HPS Technology. As shown in Figure 6A, peak tailing increased in the separation without MaxPeak HPS Technology (conventional technology) with a %RSD of 8.87% over a 48-hr time period as the system approached a steady-state. In contrast to this, chromatographic performance was stable and consistent when the assay was performed on a newly cleaned system using ACQUITY Premier with MaxPeak HPS Technology with a %RSD of 1.01 % for peak tailing over the same time period. The reproducibility of MaxPeak HPS Technology in this instance translates to stable and consistent chromatographic performance. As shown in Figure 6B, when using MaxPeak HPS Technology, chromatographic performance for the PENNYK peptide was consistent and highly reproducible over the 48-hr injection series enabling accurate determination of relative abundance with %RSDs below 2.5%. These results demonstrate ACQUITY Premier with MaxPeak HPS Technology can deliver consistent and reliable results for increased productivity in the lab by reducing instrument down time and variability of results, assay qualities which are critical in ensuring safe and efficacious drug products can be brought to market in an efficient manner.

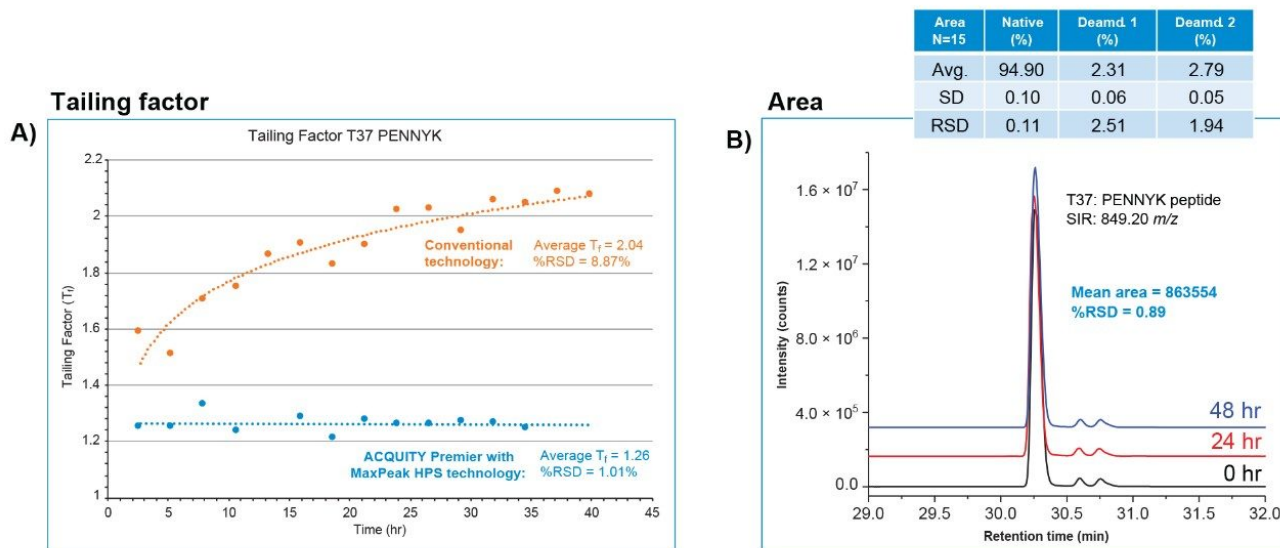


Figure 6. Reproducibility of ACQUITY Premier with MaxPeak HPS Technology. A) Tailing factor for the PENNYK peptide was monitored over a 48-hr period in a newly cleaned system with (blue data trace) and without (orange data trace) MaxPeak HPS Technology. Peak tailing % RSD was calculated at 8.87% for the separation performed without MaxPeak HPS Technology compared to 1.01% when using the ACQUITY Premier with MaxPeak HPS Technology. B) Chromatographic performance for the PENNYK peptide and associated impurities was consistent over the 48-hr injection series with %RSDs for relative abundance below 2.5% (table inset).

## Legacy Compatibility:

Evaluation of new technology to improve the lifecycle of drug products is recommended as part of a pharmaceutical quality system. As part of the evaluation pharmaceutical companies will often perform comparability studies to determine what impact the new technology may have on existing methods deployed in the development and manufacturing of therapeutic drugs. Using these principals, retention time and selectivity was compared for a NIST mAb digest peptide map performed with and without Maxpeak Premier Technology. To ensure comparability, both data sets were generated in an identical fashion with respect to system preparation (acid washed prior to data acquisition) and column conditioning (15 injections of a mAb tryptic digest) prior to data acquisition. As shown in Figure 7A and 7B, chromatograms were analyzed between both separations to assess differences. To facilitate comparison, UV chromatograms were time aligned to adjust for dwell volume differences and plotted on the same scale. Qualitatively, the separation performed with MaxPeak HPS



Technology showed improvement in peak tailing and recovery for peptides T:14 and T:37 (insets) that were consistent with previous findings. More notably, the peptide profiles were highly consistent with each other with respect to profile and abundance indicating MaxPeak HPS Technology did not negatively impact the separation. Using a more rigorous approach peaks with a  $S/N \geq 3$  were integrated and plotted against each other as a function of relative retention time using the last eluting peak as the reference peak. As shown in Figure 7C, the orthogonal comparison indicated good retention time agreement (slope = 1.00) and a negligible time offset (y-intercept = -0.005) between the separations indicating selectivity was predominantly conserved when using MaxPeak HPS Technology. These results demonstrate ACQUITY Premier with MaxPeak HPS Technology can be applied to legacy RPLC-based methods with negligible impact to chromatographic selectivity while increasing reproducibility, peak shape, and recovery of sensitive analytes by minimizing analyte/surface interactions.

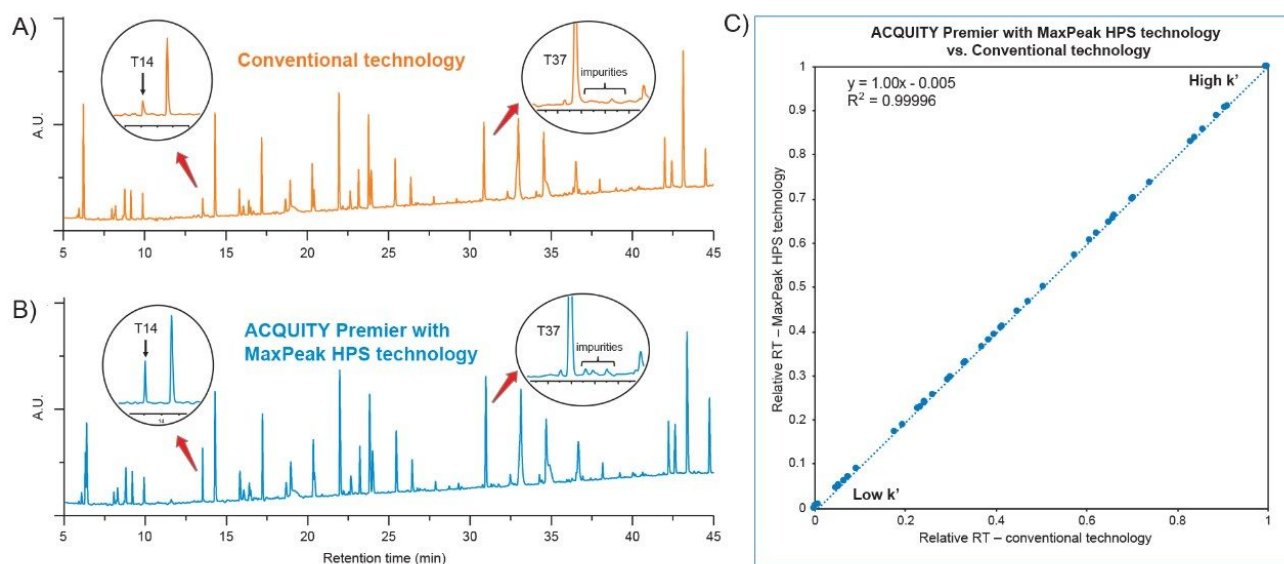


Figure 7. Comparability. Peptide profiles were highly comparable with respect to profile and abundance for a NIST mAb digest peptide map performed using A) conventional LC technology versus B) ACQUITY Premier with MaxPeak HPS Technology. C) An orthogonal comparison of relative retention time of peaks with  $S/N \geq 3$  indicated good retention time agreement (slope = 1.00) and a negligible time offset (y-intercept = -0.005) indicating selectivity was predominantly conserved when using ACQUITY Premier with MaxPeak HPS Technology.

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## Conclusion

Analyte/surface adsorption in liquid chromatography as a contributing factor to poor peak shape, tailing, and diminished recovery can lead to increased assay variability, reduced assay sensitivity, and missinterpretation of results for analytes susceptible to surface interactions. ACQUITY Premier with MaxPeak HPS Technology is Waters, solution to these challenges. Waters, ACQUITY Premier Columns are designed to deliver exceptional chromatographic performance while minimizing analyte/surface interactions of sensitive compounds. Collectively, this study demonstrates MaxPeak HPS Technology can be broadly applied in the development and manufacturing of new or existing therapeutic drug products to deliver the chromatographic performance expected from Waters technologies while increasing reproducibility, peak shape, and recovery of analytes prone to surface interactions.

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