

## The BioAccord System With ACQUITY Premier for Improved Peptide CQA Monitoring

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### Abstract

Peptide MAM is an LC-MS based assay for direct biotherapeutic product attribute analysis that is increasingly used in protein biotherapeutic quality assessment. As part of lifecycle management, assays need to remain accurate and consistent to ensure continued drug product quality and safety. In this respect, robust system performance plays a critical role in MAM data quality. Non-specific adsorption of acidic peptides to metal surfaces is a well-known phenomenon affecting LC-MS analyses, causing asymmetric peaks, loss of peptides, and increased variability in detector response for quantitative measurements. This work demonstrates the performance gains of the BioAccord configured with the inert ACQUITY Premier resulting in increased peptide recovery and robust MS response, with more reproducible results for attributes spanning 3-orders of magnitude in dynamic range. The improved performance from MaxPeak High Performance Surfaces (HPS) Technology demonstrates that the BioAccord System with ACQUITY Premier controlled by the compliant-ready waters\_connect informatics is optimally suited as a LC-MS platform for MAM-based assays.

### Benefits

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- Improved acidic peptide recovery from MaxPeak HPS System and column surfaces
- Peptide quality attribute monitoring at low loading levels
- Stable MS signal at high and low measurement levels
- Lowered %RSD levels for monitored attributes

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## Introduction

LC-MS based multi-attribute method (MAM) assays continue to gain popularity within the biopharmaceutical industry due to their ability to directly measure multiple attributes such as product variation and degradation. These assays complement, and can potentially replace, multiple conventional optical based assays due to their increased specificity and sensitivity in a single analysis.<sup>1</sup> To this end, efforts have been made to develop and validate MAM-based assays using critical quality attributes (CQAs) of the drug product as part of lifecycle management.<sup>1,2</sup> Assays need to be robust, accurate, and consistent to ensure methods can be readily deployed across an organization to support product development, manufacturing, and release activities. It has become clear that everything from sample preparation, chromatographic robustness, detection consistency, and informatic processing can affect overall assay robustness, with abundance (trace-level) of targeted attributes having the greatest challenges for assay performance and reproducibility.

Recently, it was reported that chromatographic performance for peptides containing multiple acidic residues (glutamic/aspartic acid or residues with acidic modifications) can be compromised with generic RPLC-MS methods when performed on conventional stainless-steel LC systems.<sup>3</sup> Peptides bearing electron-rich moieties such as carboxylic acids interact with or adsorb to the metal oxide surfaces in the instrument and column resulting in reduced recovery and increased peak tailing for these metal sensitive analytes. Adsorption phenomena such as these can be particularly challenging for peptide MAM analyses, as suboptimum performance brought on by metal/surface interaction of analytes can reduce the quantitative accuracy and reproducibility of the assay in the assessment of product attributes. Substantial efforts have been made to reduce these metal-peptide interactions in RPLC assays using alternate ion-pairing reagents, passivation, and high ionic strength solvents that may require additional method development efforts that are not always MS-compatible.<sup>4,5</sup> While these mitigation strategies have been shown to improve chromatographic performance, careful consideration must be given during method optimization as introduction of additional method complexity

increases the risk associated with method robustness. The ACQUITY Premier UPLC System is designed to improve the chromatographic performance for acidic compounds without the need for additional method development or extensive optimization efforts. This is made possible through the incorporation of MaxPeak High Performance Surfaces (HPS) Technology in the high surface area components of the LC system and column hardware which introduces a barrier layer to minimize analyte/surface interaction of metal sensitive analytes.<sup>6</sup> The purpose of this study is to demonstrate the impacts of MaxPeak HPS Technology to MAM-based assays through its ability to improve recovery<sup>7</sup> and peak shape of metal sensitive analytes, resulting in improved assay sensitivity and robustness without changing the RPLC-MS method or conditions.

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## Experimental

### Sample Description

mAb Tryptic digestion standard (p/n: [186009126 < https://www.waters.com/nextgen/us/en/shop/standards--reagents/186009126-mab-tryptic-digestion-standard.html>](https://www.waters.com/nextgen/us/en/shop/standards--reagents/186009126-mab-tryptic-digestion-standard.html) ) was dissolved in 200  $\mu\text{L}$  of 0.1% formic acid to yield a final concentration of 0.2  $\mu\text{g}/\mu\text{L}$ . The injection volume of the sample was 5.0  $\mu\text{L}$  (1.0  $\mu\text{g}$ ).

### Method Conditions

Data was acquired on both a (1) BioAccord System with ACQUITY UPLC I-Class PLUS (Stainless-Steel) and a (2) BioAccord System with ACQUITY Premier (Inert-MaxPeak HPS) for direct comparison. Column technology consistent with each system architecture were used with a common stationary phase packing.

### LC Conditions

Detection:	TUV, MS
Vials:	QuanRecovery with MaxPeak HPS Vials (p/n: 186009186)

Column(s): (1) ACQUITY UPLC Peptide CSH C<sub>18</sub> Column (p/n: 186006938)  
(2) ACQUITY Premier Peptide CSH C<sub>18</sub> Column (p/n: 186009489)

Column temp.: 60 °C

Sample temp.: 6 °C

Injection volume: Blank 10 µL, sample 2–10 µL

Flow rate: 0.2 mL/min

Mobile phase A: 0.1% Formic acid in water

Mobile phase B: 0.1% Formic acid in acetonitrile

## Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
0.00	0.2	99	1	initial
3.00	0.2	99	1	6
78.00	0.2	65	35	6
85.70	0.2	15	85	6
93.00	0.2	15	85	6
100.70	0.2	99	1	6
120.00	0.2	99	1	6

## MS Conditions

MS system: ACQUITY RDa Detector

Ionization mode: ESI+

Acquisition range: m/z 50–2000

Capillary voltage: 1.2 kV

Collision energy: 60–120 V

Cone voltage: 20 V

## Data Management

Informatics: waters\_connect with Peptide MAM App,  
LC-MS Tool Kit

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## Results and Discussion

In this study, we conducted a side-by-side comparison of a conventional stainless-steel flow path BioAccord System vs. the BioAccord System configured with an inert MaxPeak HPS Surfaces ACQUITY Premier LC System and Column (Figure 1). In this comparative evaluation, both BioAccord Platforms included the ACQUITY RDa Mass Detector and used the waters\_connect Peptide MAM application workflow to generate results. Analyte recovery, assay sensitivity, and reproducibility of selected CQAs from a digest of the NIST mAb reference material were used to assess system performance and the suitability of the BioAccord System with ACQUITY Premier to support MAM-based assays.



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*Figure 1. The BioAccord System with ACQUITY Premier. The ACQUITY Premier Technology contains flow paths, frits, and columns with MaxPeak High Performance Surfaces (HPS) to reduced metal surface adsorption of analytes.*

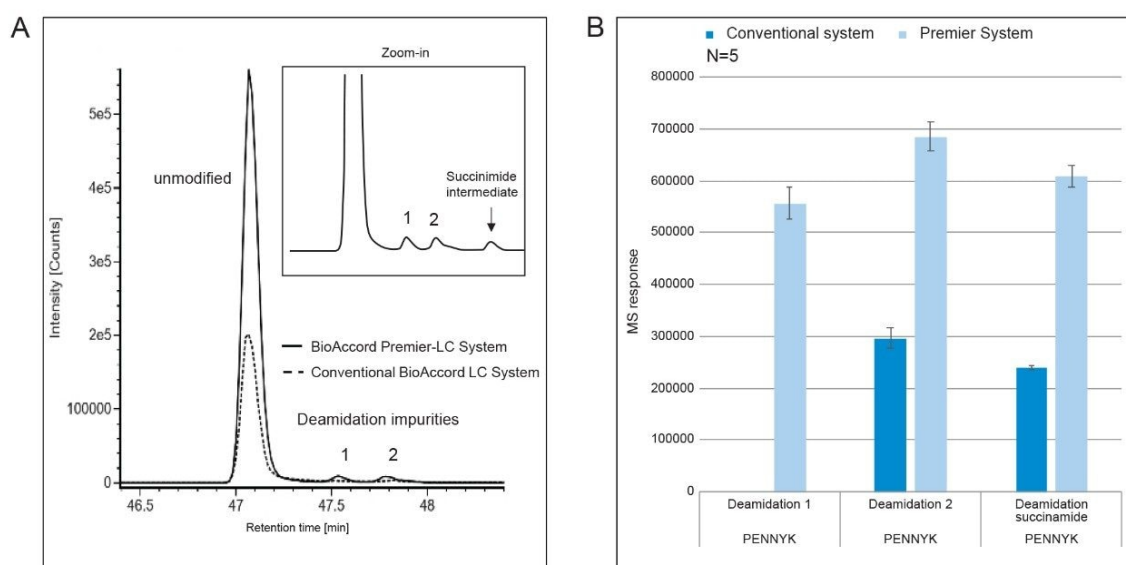
## Increased Recovery

Deamidation of asparagine to aspartic acid and iso-aspartic acid is a common PTM of monoclonal antibodies (mAbs) that has been shown to impact their efficacy and potency.<sup>8</sup> As part of the development process of mAb-based drug products, sequences susceptible to deamidations are frequently characterized and monitored to maintain the consistent quality of the biotherapeutic. The “PENNY” peptide contains a known sequence with multiple likely deamidation site(s) that is reported to impact antigen binding.<sup>8</sup> As a sequence located in the constant domain (Fc) of the heavy chain, it is a peptide routinely monitored during development and manufacturing of mAb-based therapeutics to monitor process consistency and product quality. For the NISTmAb reference antibody, after enzymatic treatment with trypsin, the HC:T37 “PENNY” peptide already contains four acidic residues (sequence: GFYPSDIAVEWESNGQPENNYK) making it highly susceptible to adsorption to metal surfaces in the LC flow path and column hardware, and making it an ideal candidate analyte to evaluate the MaxPeak Premier HPS Technology.

In this comparison study, two deamidation sites which are commonly monitored for the PENNY peptide were

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observed with both systems (Figure 2A, peak 1 & 2), as well as a peak corresponding to the succinimide intermediate (Figure 2A inset). While present in both data sets, the BioAccord System with ACQUITY Premier exhibited improved recovery overall with a 2-fold or greater increase in MS signal intensity for the "PENNY" peptide and its related variants as shown in Figure 2B. Interestingly, while visible, deamidation peak 1 was below the detection limit for the processing method in the conventional data set due to its low recovery and poor peak shape. In this context, the data illustrates how MaxPeak HPS Technology can improve data analysis, particularly in automated workflows that use the same processing method.



*Figure 2. Recovery Comparison. A) Extracted ion chromatograms (XIC) for the HC:T37 peptide fragment (sequence: GFYPSDIAVEWESNGQPENNYK) and associated impurities from a tryptic digest of the NISTmAb reference material separated on a conventional BioAccord System (dashed line) and a BioAccord System with ACQUITY Premier featuring MaxPeak HPS Technology (solid line). B) Total normalized peak area for the HC:T37 impurities were calculated for a set of 5-injections on both systems.*

Furthermore, the performance gain exhibited by MaxPeak Premier HPS Technology was observed to directly impact the quality of MS data. As shown in Figure 3, the increased recovery of the PENNY peptide and its associated variants resulted in an increase in the number and intensity of b/y fragment ions (3 vs. 7) for the PENNY peptide. This data demonstrates that ACQUITY Premier Technology can be used to increase recovery of



product quality attribute related peptides for improved confidence in peak assignment in MAM-based assays.

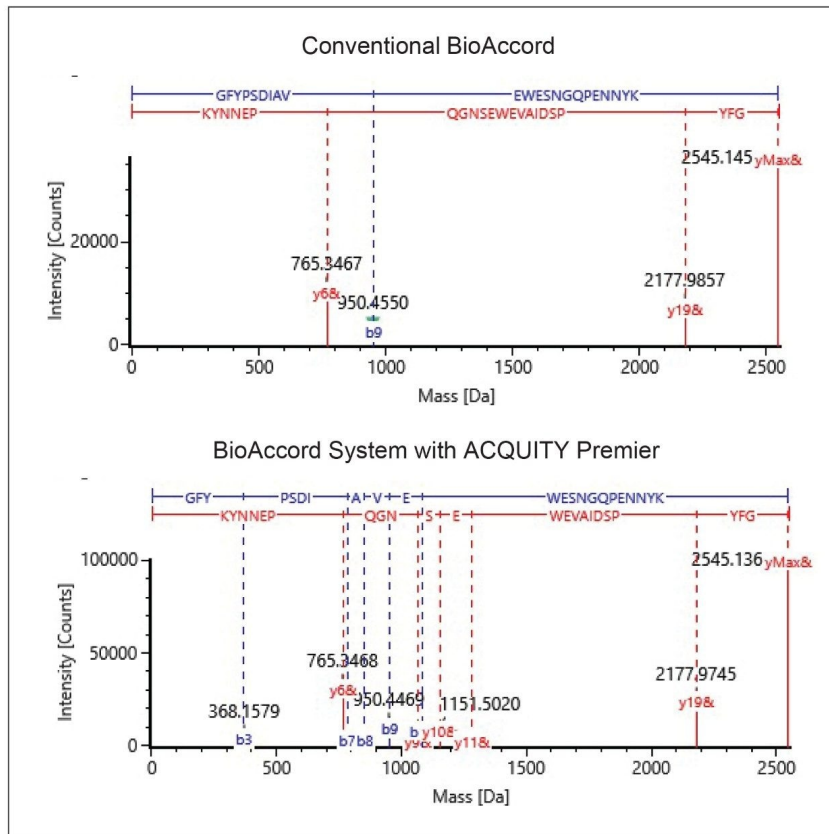


Figure 3. Improving MS data quality. The b/y fragmentation spectra acquired for PENNY deamidation-2 on conventional and ACQUITY Premier Systems. Evaluation of fragmentation data for the HC:T37 deamidated impurity (peak 2) using MS with fragmentation mode (data independent acquisition) exhibited improved intensity for fragment ions on the BioAccord System with ACQUITY Premier (bottom panel) when compared to the conventional BioAccord System (top panel).

## Robust Technology

Developing robust methods that can be readily scaled and deployed across an organization is critical to maintain productivity and facilitate easier method transfer between labs. This can be particularly challenging for LC-MS

methods when working with samples across labs that may vary in complexity and concentration. As shown in the previous data, ACQUITY Premier with MaxPeak HPS Technology is able to improve the recovery and MS response of metal sensitive peptides.

To further evaluate the robustness of the BioAccord System with ACQUITY Premier in its ability to support development and manufacturing activity, a panel of product quality associated attributes (Table 1) was monitored over a broad range of concentrations (0.1 µg–2.0 µg). As shown in Figure 4, the BioAccord System with MaxPeak Premier Technology is able to accurately and consistently report area % of attributes over the measured range with %RSD of individual attributes not exceeding 20% for the majority of attributes. An example of this performance stability can be seen with the DTLMISR oxidation modification which consistently responded over the increasing mass load with a calculated %RSD of 6.3%. Higher variability was observed with lower abundant species at lower mass loads, particularly glycopeptides, however this is not entirely unexpected as glycopeptides are poorly ionizing and only present at 1.03% MS response relative to the base peak of the peptide digest. This data illustrates the BioAccord System when coupled to the ACQUITY Premier is a robust LC-MS platform that can be broadly applied in labs to support development and manufacturing activity and is well suited for MAM-based assays which inherently require instruments that can detect multiple analytes across varying abundance levels.

Peptide sequence	Modification	Mean % mod
VVSVLTVLHQDWLNGK	(Base peak)	95.52%
DIQMTQSPSTLSASVGDR	oxidation	0.94%
DMIFNFYFDVWGQGTTVTVSSASTK	oxidation	1.02%
DTLMISR	oxidation	1.60%
GFYPSDIAVEWESNGQPENNYK	Deamidation 1	2.06%
GFYPSDIAVEWESNGQPENNYK	Deamidation 2	1.72%
GFYPSDIAVEWESNGQPENNYK	Deamidation succinamide	1.88%
VTNMDPADTATYYCAR	oxidation	0.74%
VVSVLTVLHQDWLNGK	Deamidation 1	1.01%
VVSVLTVLHQDWLNGK	Deamidation succinamide	2.92%
<b>Glycopeptide</b>		
EEQYNSTYR	(base peak)	1.03%
EEQYNSTYR	G0F	43.30%
EEQYNSTYR	G1F	40.47%
EEQYNSTYR	G2F	9.06%
EEQYNSTYR	G0F-GlcNAc	1.82%
EEQYNSTYR	G1F-GlcNAc	2.82%
EEQYNSTYR	Man5	1.40%

*Table 1. A selected list of NIST mAb critical quality attributes measured across different mass loads (0.1 µg–2.0 µg) of the digest on a BioAccord Premier.*

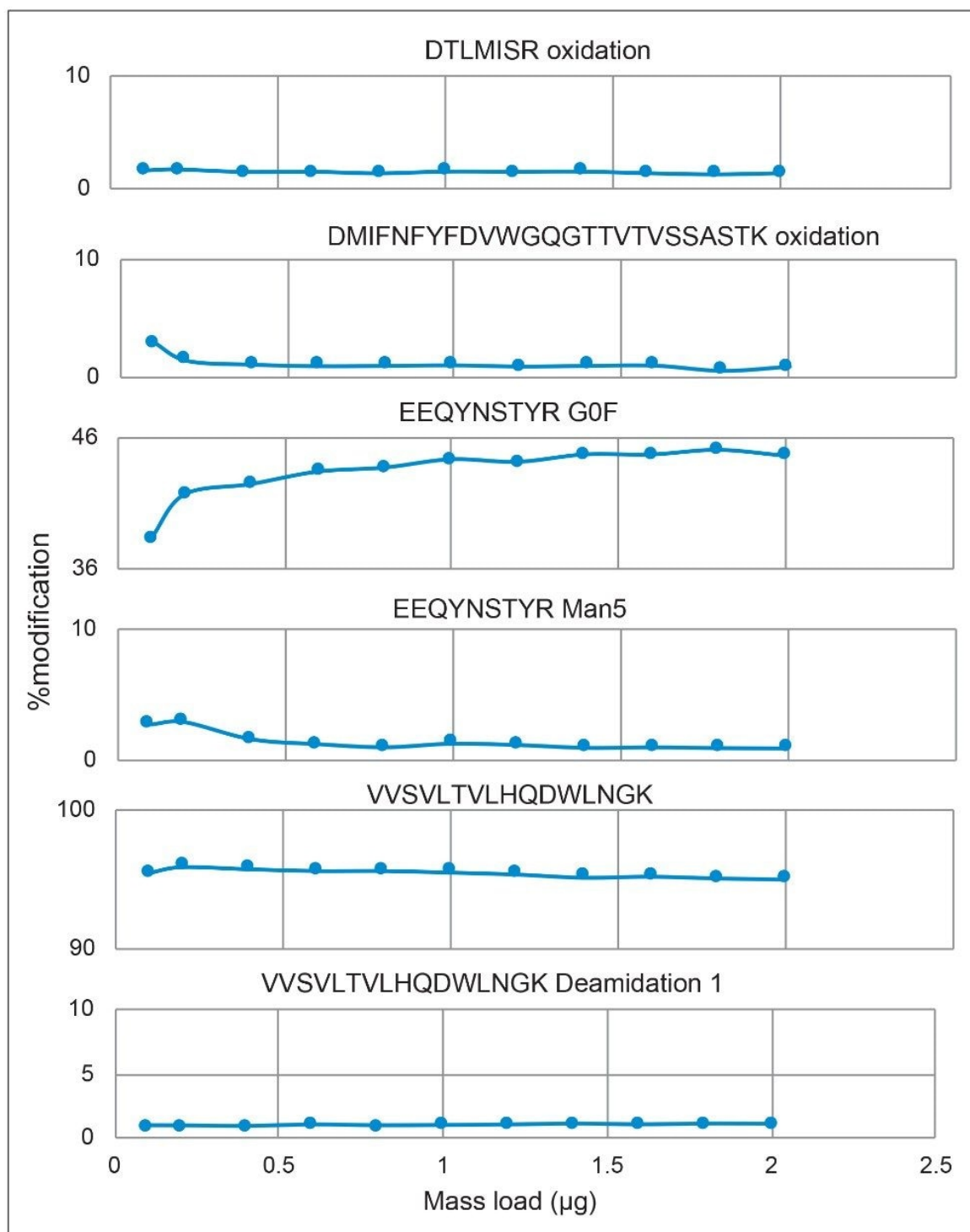


Figure 4. Consistence Performance. Calculated % modification levels for CQAs of the NISTmAb RM 8671 based

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*on % normalized MS response for increasing mass load.*

## Reproducible and Broad Applicability

As previously shown, the BioAccord System with ACQUITY Premier as an LC-MS platform can deliver consistent and accurate results and is able to support the analytical needs across an organization. However, MAM-based assays contain a particular challenge with respect to linear dynamic range. At a fundamental level, the concept of determining the dynamic range of a simplex assay relies on the accuracy and precision of an assay in response to a reference standard which is used to set the acceptance criteria. This can be more challenging for MAM assays as the dynamic range represents a spectrum of multiple analytes with varying levels of baseline peptide ionization efficiency and relative abundance to their unmodified form. To ensure reproducible and accurate measurement of CQAs in MAM-assays the LC-MS platform must demonstrate a linear response for a given analyte and a broad spectral dynamic range to address complex samples.

To evaluate this practical dynamic range issue, normalized response for the T26 peptide fragment base peak (sequence: VVSVLTVLHQDWLNGK) was plotted against increasing mass load. As shown in Figure 5A, MS source saturation was observed above loadings of 1.2  $\mu\text{g}$  with the linear range spanning 0.1  $\mu\text{g}$ –1.0  $\mu\text{g}$  with an  $R^2 = 0.99$ . Using this information, it was determined a mass load of 1  $\mu\text{g}$  would allow for an acceptable mass load with no peaks exceeding upper response limits and maximum sensitivity towards trace-level CQAs. This is demonstrated in Figure 5B where the BioAccord Premier LC was able to detect the Man 5 glycosylation species (0.08% of base peak) of the T25 peptide fragment (sequence: EEQYNSTYR) with a high degree of reproducibility (Response %RSD = 2.78%). More notable is the fact that the in-spectrum detector response represents a spectral dynamic range that spans 3-orders of magnitude. Thus, the BioAccord System with ACQUITY Premier is able to acquire accurate and reproducible data for a diverse set of analyte peptides with a sensitivity that is well suited for routine use MAM-based assays. This is further demonstrated in Table 2 where %RSD for the monitored attributes were observed to be below 4% when performing the MAM assay on the BioAccord System with ACQUITY Premier which was 1.5–2.5-fold lower than the conventional BioAccord System. As expected, the largest differences observed were with acidic residue containing peptides demonstrating the value MaxPeak HPS Technology brings to MAM-based assays.

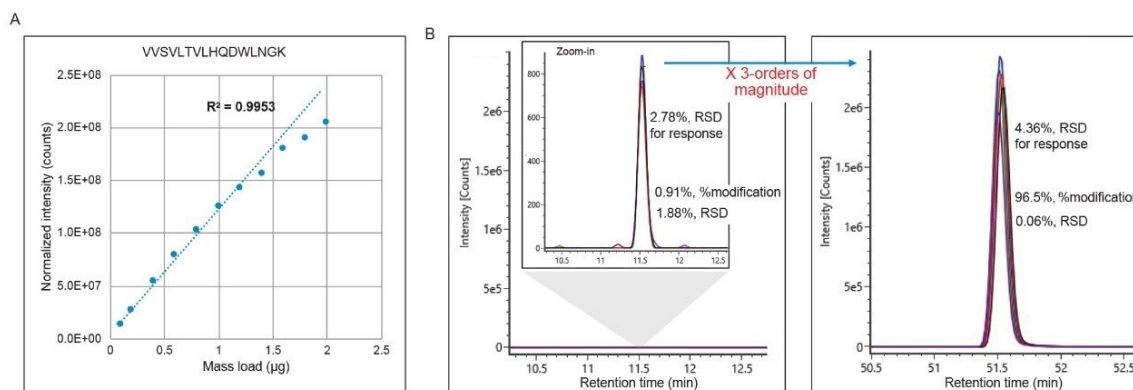


Figure 5. Dynamic range, A) Evaluating the linearity of normalized response across different mass loads. The selected peptide T26:VVSVLTVLHQQDWLNGK is the base peak peptide of the MS spectrum. The abundance of lowest measured attribute T25:EEQYNSTYR Glycosylation Man 5 relative to the given T26 peak is 0.08%. B) chromatograms of and VVSVLTVLHQQDWLNGK measured at 1 µg optimum loading levels. The data delivered stable MS responses and %modification levels across 5-consecutive injections.

Peptide sequence	Modification	%Modification conventional system	%Modification ACQUITY Premier System	%RSD conventional system	%RSD ACQUITY Premier System
VVSVLTVLHQDWLNGK	(base peak)	95.88	96.55	0.04	0.06
DIQMTQSPSTLSASVGDR	oxidation	0.86	0.86	7	4
DMIFNFYFDVWGQGTTVTVSSASTK	oxidation	1.69	1.06	7.3	1.18
DTLMISR	oxidation	1.33	1.66	3.05	3.45
GFYPSDIAVEWESNGQPENNYK	Deamidation 1	2.1	1.71	7.4	2.81
GFYPSDIAVEWESNGQPENNYK	Deamidation 2	-	2.1	-	1.33
GFYPSDIAVEWESNGQPENNYK	Deamidation succinamide	1.99	1.87	2.68	0.89
VTNMDPADTATYYCAR	oxidation	0.46	0.67	7.45	2.4
VVSVLTVLHQDWLNGK	Deamidation 1	0.92	0.92	3.44	1.48
VVSVLTVLHQDWLNGK	Deamidation succinamide	2.74	2.53	2.15	1.94
EEQYNSTYR	(base peak)	0.68	0.56	4.81	2.41
EEQYNSTYR	G0F	43.81	43.81	0.27	0.46
EEQYNSTYR	G1F	41.51	41.65	0.44	0.31
EEQYNSTYR	G2F	8.17	7.65	0.57	0.93
EEQYNSTYR	G0F-GlcNAc	2.36	2.47	2.68	1.41
EEQYNSTYR	G1F-GlcNAc	2.56	2.85	1.25	1.64
EEQYNSTYR	Man5	0.91	1	3.06	1.88

Table 2. A selected list of NISTmAb critical quality attributes measured at 1 µg mass load with conventional system and a BioAccord System with ACQUITY Premier. The % modification levels determined using waters connect, Peptide MAM application are reported here with respective %RSD levels measured across 5-injections.

## Conclusion

To develop a robust peptide MAM assay, an LC system that delivers and unbiased and consistent protein digest separation is a necessity. In this study we evaluated the BioAccord System with ACQUITY Premier featuring MaxPeak HPS Technology against a conventional stainless-steel LC-MS platform for its ability to obtain optimal chromatographic performance for peptides representing product quality attributes in a RPLC-MS based peptide MAM assay. In this comparison, it was demonstrated that the MaxPeak HPS Technology minimizes adsorption of metal sensitive analytes, enabling robust method execution with improved recovery, assay sensitivity, and method reproducibility. In summary, the BioAccord System with ACQUITY Premier represents a robust and flexible LC-MS platform that is ideal for deployment across development, manufacturing, and quality organizations.

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